



Episulfonium chemistry for versatile protein modifications and crosslinking

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Abstract

Proteins mediate intracellular processes through sequence-encoded three-dimensional structures and are dynamically regulated by post-translational modifications. Facile access to well-defined protein conjugates through chemical protein modification for investigation of protein functions is challenging because chemical reactions on biomacromolecules must proceed rapidly in aqueous buffer under biocompatible conditions and provide high site selectivity. Cysteine-selective protein modification strategies offer an attractive solution due to low native cysteine abundance and high thiol nucleophilicity, but many established approaches require synthesis of prefunctionalized electrophiles, multi-step procedures with isolation of intermediates, as well as incorporation of long or unstable linkers. A recently developed vinylthianthrenium-mediated bioconjugation enables fast cysteine-selective protein modification and incorporation of different payloads via a short and stable ethylene linkage.

The first part of this work describes the exploration of the reaction scope for versatile protein and peptide bioconjugation with vinylthianthrenium reagents, as well as mechanistic studies on the bioconjugation. The proposed mechanism involves umpolung of nucleophilic cysteinyl thiols via formation of electrophilic episulfonium intermediates, followed by rapid trapping by neighboring nucleophiles, thereby suggesting a fundamental difference from previously established multi-step protocols.

The rest of the work is dedicated to the investigation of the episulfonium chemistry for protein labeling and crosslinking *in cellulo*. Introduction of covalent crosslinks between native amino acids in proteins enables intracellular protein structure probing and characterization of protein–protein interactions based on the identity of the crosslinked residues. However, crosslinking with commonly used bifunctional reagents occurs in two separate steps and can thereby potentially capture unfavorable conformations, whereas crosslinking via irradiation of photoreactive amino acids relies on unnatural amino acid incorporation and activation with UV light.

Single-step crosslinking via episulfonium formation provides an alternative to both methods and enables incorporation of short and stable crosslinks. In this work, formation of episulfonium intermediates on protein surfaces for intracellular protein labeling and crosslinking in different cell types was established. The elucidated reactivity profile of vinylthianthrenium-mediated crosslinking suggests versatile reactivity of episulfonium intermediates with all nucleophilic amino acids and formation of short-distance restraints for reliable protein structure probing. Finally, a method for inter-protein crosslinking using a new thianthrenium reagent that enables enrichment of modified products and facilitates identification of protein–protein interactions in cells is presented in the last part of the work. The combined data demonstrate broad applicability of the site-selective episulfonium generation strategy for synthesis of well-defined protein conjugates, covalent disulfide rebridging, and versatile protein crosslinking.

Zusammenfassung

Proteine vermitteln intrazelluläre Prozesse über sequenzkodierte dreidimensionale Strukturen und werden dynamisch durch posttranslationale Modifikationen reguliert. Für die Untersuchung von Proteinfunktionen ist der Zugang zu wohldefinierten Proteinkonjugaten über chemische Proteinmodifikation zentral, jedoch anspruchsvoll. Reaktionen an Biomakromolekülen müssen in wässrigen Puffern unter biokompatiblen Bedingungen rasch ablaufen und zugleich eine hohe Selektivität für eine bestimmte Aminosäure gewährleisten. Cystein-selektive Modifikationsstrategien sind dafür besonders attraktiv, da die native Cystein-Abundanz gering ist und Thiole eine hohe Nukleophilie aufweisen. Viele etablierte Verfahren sind dennoch aufwendig, weil sie die Synthese vorfunktionalisierter Elektrophile erfordern, mehrstufige Abläufe mit Isolierung von Intermediaten beinhalten und häufig zur Einführung langer oder instabiler Linker führen. Eine kürzlich entwickelte, vinylthianthrenium-vermittelte Biokonjugation adressiert diese Einschränkungen, indem eine schnelle Cystein-selektive Proteinmodifikation und die Einführung verschiedener Payloads über einen kurzen und stabilen Ethylen-Linker ermöglicht werden.

Der erste Teil dieser Arbeit untersucht die Anwendungsbreite der Reaktion für eine vielseitige Protein- und Peptidbiokonjugation mit Vinylthianthrenium-Reagenzien. Außerdem werden mechanistische Aspekte dieser Biokonjugation analysiert. Ein vorgeschlagener Mechanismus beruht auf der Umpolung nukleophiler Cystein-Thiolgruppen über die Bildung elektrophiler Episulfonium-Intermediate, die anschließend rasch durch benachbarte Nukleophile abgefangen werden. Damit unterscheidet sich der zugrunde liegende Reaktionsablauf grundlegend von bislang verbreiteten mehrstufigen Vorgehensweisen.

Der verbleibende Teil der Arbeit ist der Untersuchung der Episulfonium-Chemie für Protein-Labeling und Crosslinking *in cellulo* gewidmet. Die Einführung kovalenter Crosslinks zwischen nativen Aminosäuren in Proteinen erlaubt die Untersuchung von Proteinstrukturen in ihrem zellulären Kontext und ermöglicht die Charakterisierung von Protein–Protein-Interaktionen anhand der Identität der crosslinkten Aminosäurereste. Allerdings erfolgt Crosslinking mit häufig verwendeten bifunktionellen Reagenzien in zwei getrennten Schritten und kann dadurch potenziell auch ungünstige Konformationen erfassen, während Crosslinking über die Bestrahlung photoreaktiver Aminosäuren auf dem Einbau unnatürlicher Aminosäuren und der Aktivierung mit UV-Licht beruht.

Ein einstufiges Crosslinking über die Bildung von Episulfonium-Intermediaten stellt eine Alternative zu beiden Ansätzen dar und ermöglicht die Einführung kurzer und stabiler Crosslinks. In dieser Arbeit wird die Erzeugung von Episulfonium-Intermediaten auf Proteinoberflächen für intrazelluläres Protein-Labeling und Crosslinking in verschiedenen Zelltypen etabliert. Das aufgeklärte Reaktivitätsprofil des Crosslinkings mit Vinylthianthrenium-Reagenzien zeigt eine breite Reaktivität der Episulfonium-Intermediate gegenüber nukleophilen Aminosäuren und führt zu kurzreichweitigen Abstandsbeschränkungen, die eine zuverlässige Aufklärung von Proteinstrukturen unterstützen. Abschließend wird eine Methode für inter-protein Crosslinking

vorge stellt, die auf einem neuen Thianthrenium-Reagenz basiert. Dieses Reagenz ermöglicht die Anreicherung der modifizierten Produkte und erleichtert die Identifizierung von Protein–Protein-Interaktionen in Zellen. Die Gesamtheit der Daten belegt die breite Anwendbarkeit einer selektiven Erzeugung von Episulfonium-Intermediaten für die Synthese wohldefinierter Proteinkonjugate, kovalentes Disulfid-Rebridging und vielseitiges Protein-Crosslinking.

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Note

Portions of this thesis have been taken, with permission, from the following publications:

- Hartmann, P.; **Bohdan, K.**; Hommrich, M.; Juliá, F.; Vogelsang, L.; Eirich, J.; Zangl, R.; Farès, C.; Jacobs, J. B.; Mukhopadhyay, D.; Mengeler, J. M.; Vetere, A.; Sterling, M. S.; Hinrichs, H.; Becker, S.; Morgner, N.; Schrader, W.; Finkemeier, I.; Dietz, K.-J.; Griesinger, C.; Ritter, T., Chemoselective Umpolung of Thiols to Episulfoniums for Cysteine Bioconjugation. *Nature Chemistry* **2024**, *16* (3), 380-388.
- Hartmann, P.*; **Bohdan, K.***; Vogelsang, L.; Marchionni, D.; Preisinger, C.; Vetere, A.; Dietz, K.-J.; Ritter, T., *In Cellulo* Cysteine Umpolung for Protein Structure Probing. *Journal of the American Chemical Society* **2025**, *147* (39), 35587-35594.
- **Bohdan, K.***; Hartmann, P.*; Müller, S.; Marchionni, D.; Preisinger, C.; Jacobs, J. B.; Vogelsang, L.; Sterling, M. S.; Dietz, K.-J.; Ritter, T., Thianthrenium Chemistry for Identification of Protein–Protein Interactions in Cells. *Journal of the American Chemical Society* **2025**, *147* (49), 45576-45584.

*These authors contributed equally.

List of abbreviations

6-FAM	6-Carboxyfluorescein
AA	Amino acid
BCA	Bicinchoninic acid
BIS-TRIS	2-[Bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol
BSA	Bovine serum albumin
CuAAC	Copper-catalyzed azide-alkyne cycloaddition
Da	Dalton (unit)
Dha	Dehydroalanine
DMEM	Dulbecco's Modified Eagle Medium
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate-buffered saline
DSSO	Disuccinimidyl sulfoxide
DTT	Dithiothreitol
Et	Ethyl
FCS	Fetal calf serum
FDR	False discovery rate
FWHM	Full width at half maximum
GO	Gene ontology
GSH	Glutathione
h	Hour (unit)
HCD	Higher-energy C-trap dissociation
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High-performance liquid chromatography
HRMS	High-resolution mass spectrometry
Hz	Hertz (unit)
IAA	Iodoacetamide
IR	Infrared (spectroscopy)
KIE	Kinetic isotope effect
L	Liter (unit)
LB	Luria–Bertani (medium)
LC–MS	Liquid chromatography–mass spectrometry
LC–MS/MS	Liquid chromatography–tandem mass spectrometry
Me	Methyl
MeCN	Acetonitrile
min	Minute (unit)
MQ	Milli-Q®
MS	Mass spectrometry

NEM	<i>N</i> -Ethylmaleimide
NHS	<i>N</i> -Hydroxysuccinimide
NMR	Nuclear magnetic resonance
NP-40	Nonidet P-40
Nu	Nucleophile
OD ₆₀₀	Optical density at 600 nm
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PDB	Protein data bank
PEG	Polyethylene glycol
P _i	Inorganic phosphate
pLDDT	Predicted Local Distance Difference Test
PMSF	Phenylmethylsulfonyl fluoride
POI	Protein of interest
PPI	Protein–protein interaction
ppm	Parts per million
PTM	Post-translational modification
RIPA	Radioimmunoprecipitation assay buffer
RPKA	Reaction Progress Kinetic Analysis
rpm	Revolutions per minute (unit)
RPMI	Roswell Park Memorial Institute (medium)
s	Second (unit)
SDS	Sodium dodecyl sulfate
SP3	Single-pot, solid-phase-enhanced sample preparation
SPAAC	Strain-promoted azide–alkyne cycloaddition
TCEP	Tris(2-carboxyethyl)phosphine
TFA	Trifluoroacetic acid
THPTA	Tris(3-hydroxypropyltriazolylmethyl)amine
TPSA	Topological polar surface area
TSP	3-(Trimethylsilyl)propionic-2,2,3,3-d ₄ acid sodium salt
TT	Thianthrene
TTO	Thianthrene- <i>S</i> -oxide
UV-vis	Ultraviolet–visible (spectroscopy)
VTFT	Tetrafluorovinylthianthrenium tetrafluoroborate
VTNA	Variable time normalization analysis
VTT	Vinylthianthrenium tetrafluoroborate
XL	Crosslinking
YPD	Yeast extract peptone dextrose (medium)

Chapter 1: Site-selective protein modification via cysteine umpolung

Introduction: Bioconjugation approaches

Chemical modifications of proteins

Basic intracellular processes are controlled by proteins, macromolecules composed of a set of 20 canonical amino acid building blocks that form polymers consisting from dozens to thousands of amino acids¹. Protein folding in an aqueous medium depends on the primary amino acid sequence and leads to macromolecular structures of various shapes, adapted to carrying out a specific set of functions over years of evolution². Apart from protein size and shape, another important factor controlling protein functions is the presence of covalent modifications, so-called post-translational modifications (PTMs), which are introduced into the proteins after ribosomal synthesis. The introduced PTMs can consist of several atoms (e.g., a phosphoryl group in phosphorylation) or complex molecules (e.g., the 8.6 kDa protein ubiquitin in ubiquitinylation), and enable control of intracellular processes by modulating a protein's properties in a precise manner³. Because PTMs are vital for cell biology, their misregulation leads to diseases, which makes the study of PTMs important for understanding protein functions and for the development of methods for disease treatment.

Scientists have developed various methods to introduce PTMs into protein structures⁴. Installation of PTMs enables isolation of proteins with altered properties for investigation of protein functions⁵, whereas bioconjugation via attachment of fluorescent molecules or cytotoxic payloads enables intracellular protein tracking and synthesis of therapeutic macromolecules such as antibody–drug conjugates^{6,7}. In order to obtain a well-defined protein conjugate, installation of a payload of interest must occur at a specific position and proceed without detrimental effects on the protein structure.

Development of genetic code expansion enabled incorporation of unnatural amino acids that carry a PTM or a bioorthogonal handle during the protein synthesis step, and thereby enables direct isolation of proteins precisely modified at a specific position as well as incorporation of linchpins for follow-up transformations⁸. Although useful in the context of PTM installation in living cells, an inherent disadvantage of the method is the need for design of orthogonal AA-tRNA synthetase–tRNA pairs for each particular noncanonical amino acid, as well as optimization for a specific cell type. Incorporation of noncanonical amino acids carrying bulky substituents or incorporation of several noncanonical amino acids into the same protein remains challenging, which limits the applicability of the method⁴.

Chemical protein modification represents an alternative strategy for incorporation of a modification after protein synthesis⁹. A wide variety of available reactive functional groups can be used to attach different handles to a protein of interest (POI) and thereby presents an opportunity for

synthesis of different protein conjugates based on the same starting material (Figure 1.1). To prevent protein degradation or aggregation, chemical protein modifications must proceed on a timescale of minutes to hours in aqueous buffered solutions at neutral pH (6.0–8.0) and temperatures of up to 37 °C. Moreover, in order to prevent formation of product mixtures, developed methods must be selective for a particular residue to enable installation of a payload at a specific position of the POI¹⁰. In order to achieve high selectivity of protein modification, low-abundance amino acids such as cysteine (Cys)^{11,12} and methionine (Met)¹³, as well as the N-terminus¹⁴, are usually targeted. Apart from low native abundance, site-selective protein modification relies on distinct inherent reactivity modes of the targeted amino acids. For example, Met-selective bioconjugation approaches rely on selective oxidation of thioethers in the presence of other amino acids, Cys residues can be selectively modified using electrophilic reagents due to their inherent nucleophilicity, and selective N-terminal modification is achieved by pH control and utilization of amine-selective reaction chemistry.

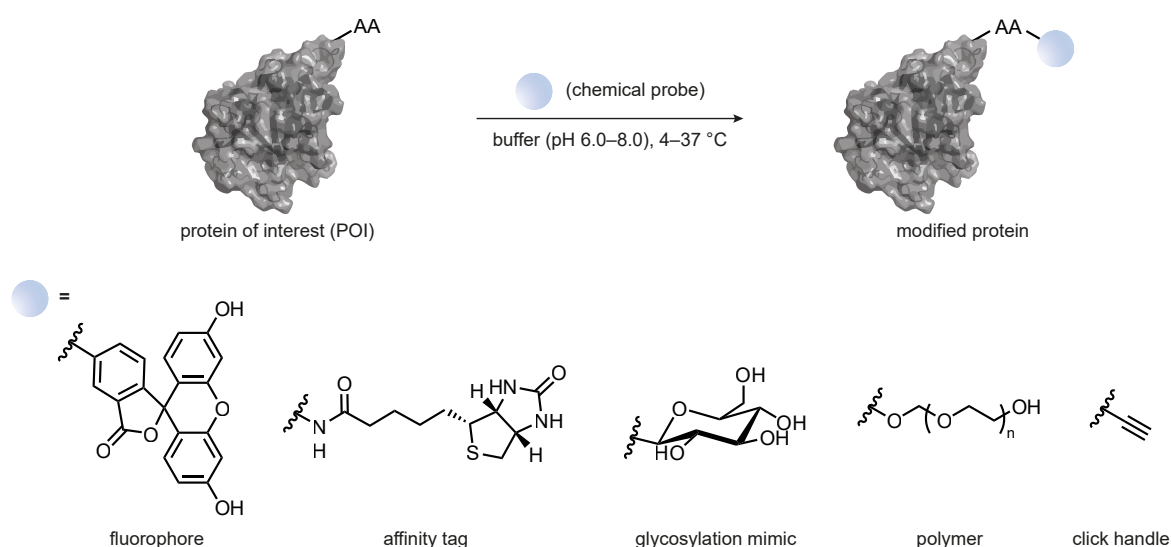


Figure 1.1: General strategy for protein modification using chemical probes. AA, amino acid.

Cysteine-based protein modification

Cys-selective protein modification has evolved as a privileged method for site-selective protein functionalization because most proteins do not contain reduced Cys, a potent nucleophilic amino acid at neutral pH. Therefore, incorporation of Cys at a specific position of a POI, followed by reaction with an electrophilic reagent, provides an efficient strategy for site-selective protein modification¹⁵. Moreover, the reactivity of the targeted Cys depends on the local microenvironment, which has been used to develop methods that enable site-specific functionalization of one Cys residue in the presence of another Cys in the same protein and thereby enable protein modification with several payloads^{16–18}. The practicality of this approach makes Cys-selective bioconjugation a valuable alternative to protein functionalization methods that rely on genetic code expansion.

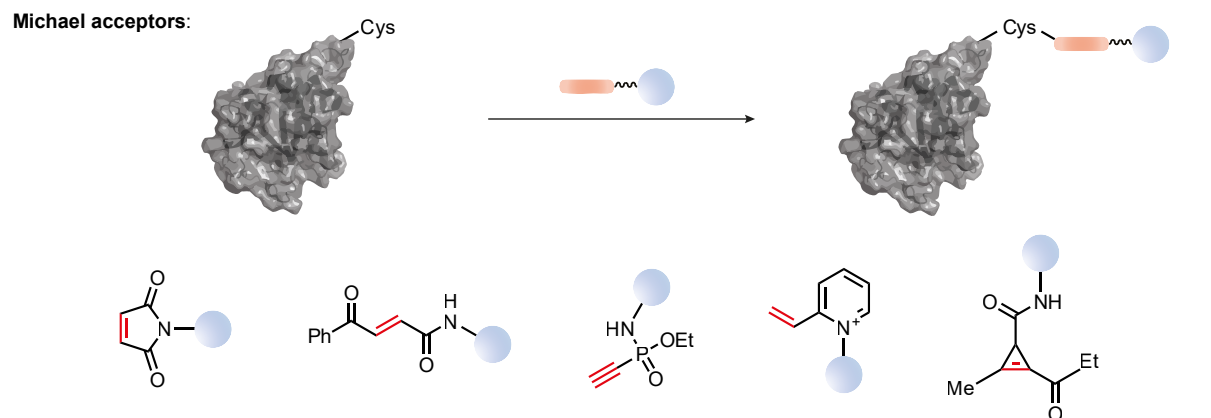
Dozens of methods have been developed to enable fast incorporation of different payloads into a POI via Cys-selective functionalization¹⁹. Various electrophilic reagents that carry frequently used payloads such as fluorophores or PEG chains have been commercialized and can readily be used by non-experts. However, the search for novel methods for Cys-selective protein modification continues, as robust methods that enable fast incorporation of novel payloads remain

desirable. All existing methods for Cys-selective modification can be divided into two broad categories, and understanding the differences between these approaches is important for identifying the strengths and weaknesses of existing methods and for comparison with novel methods.

Utilization of prefunctionalized payloads

Historically, most Cys-selective bioconjugation methods have utilized electrophilic reagents that consist of a reactive electrophilic moiety, a payload of interest, and a linker connecting them (Figure 1.2, top). Maleimide-based reagents have been extensively utilized for the synthesis of protein conjugates due to their high Cys selectivity and reactivity²⁰. However, instability of the formed conjugates in the presence of thiols created a need for novel approaches that enable formation of conjugates with stable linkers and that can proceed rapidly under biocompatible conditions (aqueous buffered mixtures, temperatures of up to 37 °C). Alternative Michael acceptors²¹ and activated alkyne electrophiles such as phosphonamidates²² were developed (Figure 1.2, top), along with hypervalent sulfur²³ and iodine²⁴ reagents, perfluoro- and heteroarenes²⁵, and electrophilic organometallic compounds^{26,27} (Figure 1.2, bottom). Stable and well-defined conjugates can be accessed rapidly using many of the available methods. Although practical when a prefunctionalized reagent carrying the payload of interest is readily available, incorporation of new payloads requires synthesis of new reagents, which reduces the applicability and versatility of the approach.

Michael acceptors:



Other electrophiles:

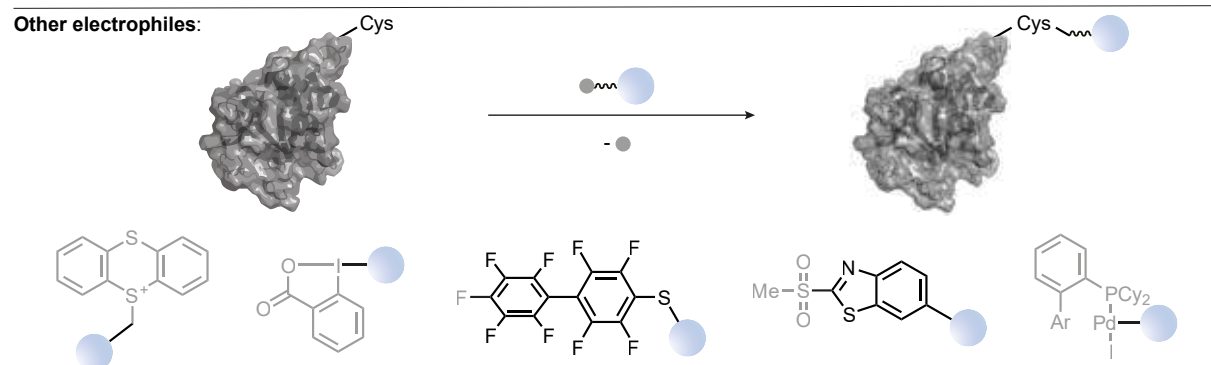


Figure 1.2: Common payload-based approaches for Cys-selective protein modification. The wavy line and the blue ball represent the incorporated linker and payload, respectively. The reactive electrophilic centers of Michael acceptors are highlighted in orange (top), and the leaving groups are depicted in gray (bottom).

Generation of reactive linchpins

An alternative approach for Cys-selective functionalization involves transformation of Cys into an electrophilic linchpin for installation of payloads of interest in a subsequent step²⁸. To achieve this, reagents with two electrophilic centers have been used, such as maleimide-based^{29–31}, organometallic³², or sulfonium-based³³ biselectrophilic reagents (Figure 1.3). The overall modification is achieved in two sequential steps via substitution with two nucleophiles, one of which is the Cys thiol on the protein and the second of which is a thiol-containing molecule carrying a linchpin of interest.

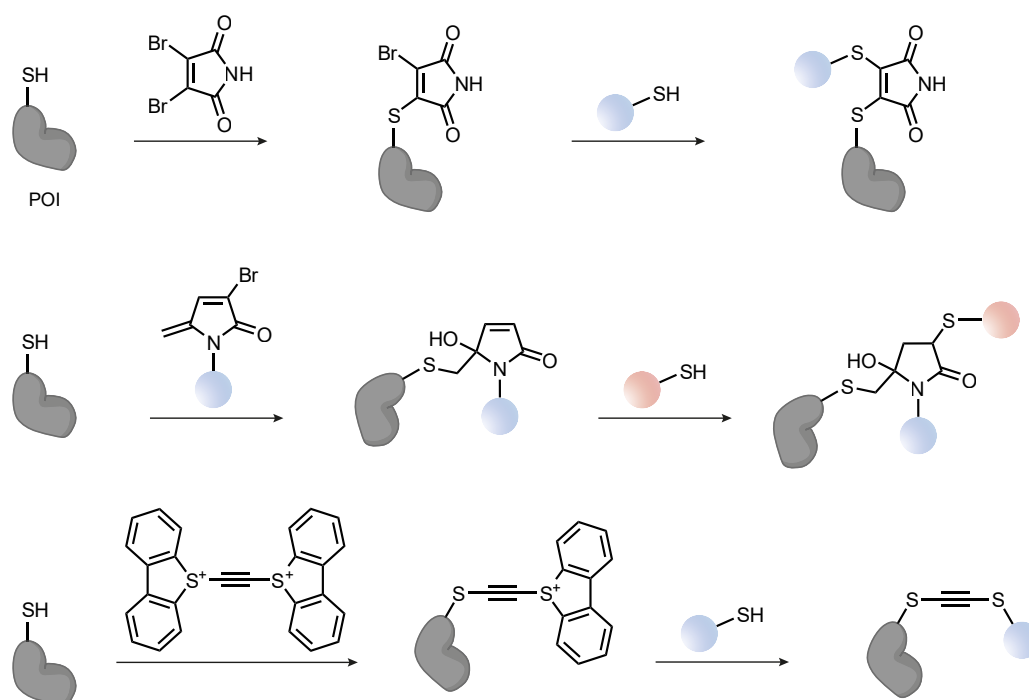


Figure 1.3: Overview of biselectrophilic reagents used for two-step Cys functionalizations. The balls represent incorporated payloads.

Alternatively, Cys itself can be turned into an electrophilic linchpin via formation of sulfonium cations that generate an electrophilic dehydroalanine (Dha) linchpin upon elimination in a basic buffer³⁴. The obtained product reacts with different thiol- and nitrogen-based nucleophiles in a separate step, providing a broad scope of functionalizations that can be achieved using the same starting material (Figure 1.4).

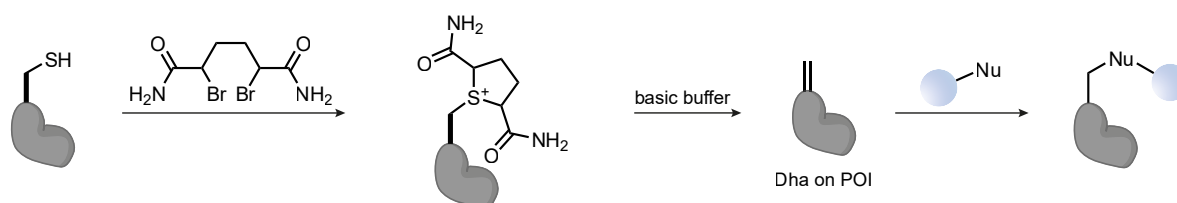


Figure 1.4: Formation of dehydroalanine (Dha) on protein of interest (POI) surface for follow-up modifications with nucleophiles (Nu).

Although versatile conjugates can be accessed, both utilization of biselectrophilic reagents and protein modification via Dha formation rely on two-step procedures that impose inherent limitations on the versatility of the methods. To avoid cross-reactivity between nucleophiles and the electrophilic reagents used for linchpin installation, electrophilic products are isolated prior to

addition of the nucleophile that carries a payload of choice. The formed electrophilic intermediates must therefore be stable enough to enable quantitative linchpin formation and handling of the product prior to the second step. However, a downside of high linchpin stability is low reactivity in the second transformation, which is why thiols are used as nucleophiles in most cases.

To address this limitation, alternative reactivity modes have been explored, and a substantial expansion of the substrate scope for Dha-based modifications has been achieved via generation of carbon-centered radicals that add to Dha^{35,36}. Nevertheless, the reactions proceed in a denaturing buffer and require handling under an oxygen-free atmosphere, which limits applicability of the method. Therefore, development of novel approaches for versatile linchpin-based Cys functionalization under biocompatible reaction conditions is desirable.

Vinylthianthrenium-based bioconjugation

The Ritter group has previously reported synthesis of vinylthianthrenium tetrafluoroborate (**VTT**), a bench-stable compound that can be obtained from ethylene gas in one step on a multigram scale³⁷ (Figure 1.5). In contrast to previously reported vinylating electrophilic reagents such as vinyl bromide and vinyl diphenylsulfonium salts, **VTT** is a non-hygroscopic, easy-to-handle solid reagent that can be used for annulation reactions with bis-nucleophiles and for cross-coupling reactions.

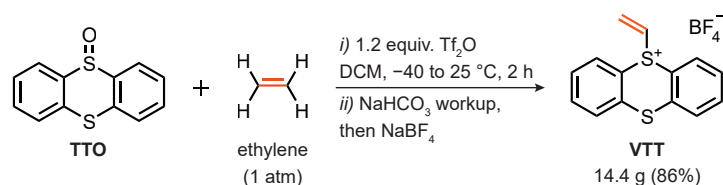


Figure 1.5: Synthesis of **VTT** from ethylene gas. DCM, dichloromethane; Tf₂O, trifluoromethanesulfonic anhydride; **TTO**, thianthrene-S-oxide.

Dr. Philipp Hartmann and Prof. Dr. Fabio Juliá hypothesized that, apart from intramolecular cyclization reactions, **VTT** might also be used to modify proteins and peptides and to introduce payloads via a short ethylene linkage. Indeed, treatment of the Cys-containing tripeptide glutathione (**GSH**) with **VTT** in the presence of excess sodium azide in an aqueous buffer led to formation of the azidated conjugate in high yield (Figure 1.6) and demonstrated that **VTT** can be used to introduce an azide click handle into Cys-containing biomacromolecules without the need for synthesis of an azide-containing electrophilic reagent.

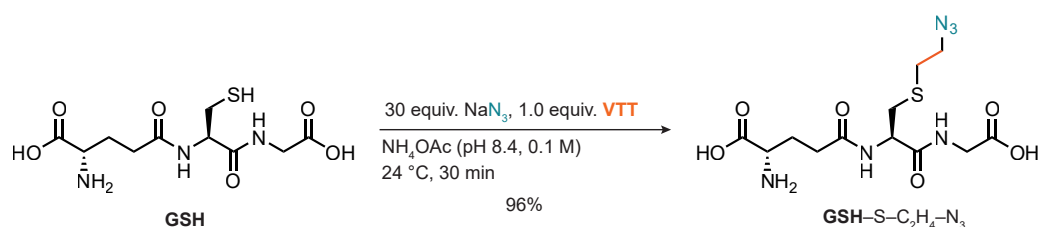


Figure 1.6: **VTT** enables efficient azide incorporation into Cys-containing peptide **GSH**.

In contrast to previously reported methods for Cys functionalization via linchpin formation, the transformation affords the product in a single step without the need for isolation of the reactive intermediate. This observation indicated that the procedure might be extended to different

nucleophiles by varying the nature of exogenous nucleophiles added to the mixture to provide a method for versatile peptide and protein bioconjugation. Moreover, additional studies were required for elucidation of the reaction mechanism and identification of the putative reactive intermediate.

Elucidation of reaction mechanisms

General considerations for mechanistic studies

Elucidation of a reaction mechanism starts with the characterization of the reactants, products, and by-products that are involved in or formed during the reaction³⁸. Once a reaction is optimized to ensure formation of the desired product in high yield, initial control experiments enable identification of crucial components and the influence of reaction conditions on the reaction outcome. Based on these observations, a set of chemically plausible mechanisms is proposed, each consistent with the previously obtained experimental data. In the next step, experiments are designed to obtain data that enable distinction between the proposed mechanisms and disproval of those that do not align with the observations. The proposed mechanisms may be refined at each step based on insights from the obtained data. Ultimately, the goal of a mechanistic study is to design experiments that narrow the set of viable mechanisms as far as possible. A mechanism that is consistent with all obtained data can then be considered a plausible description of the reaction pathway, but the proposed mechanism cannot be regarded as definitively proven, since the existence of alternative, unconsidered mechanisms can never be completely excluded³⁸.

Kinetic analysis represents one of the most fundamental tools for mechanistic elucidation, since quantitative information on the dependence of the reaction rate on reactant concentrations can be used to distinguish between different mechanistic proposals. Studies of linear free energy relationships and kinetic isotope effects provide further insight into the nature of the transition state and thus enable additional differentiation between proposed mechanisms. Additional control experiments and computational studies can further contribute to mechanistic understanding by probing the feasibility of specific intermediates and elementary steps³⁸.

Visual kinetic analysis methods for kinetic studies

Determination of the reaction order for each reaction component provides valuable data for mechanistic analysis, as it allows distinction between different proposed mechanisms³⁹. In the standard kinetic analysis approach, the influence of reagents on the reaction rate is studied under pseudo-first-order conditions, where the concentration of one substrate is taken in a large (>10-fold) excess and assumed to be constant during the reaction progress³⁸. The resulting rate law equation of the studied reaction is simplified, and the order in each component can be determined from the relationship between the observed reaction rate and the substrate concentration. For example, for a bimolecular reaction, a linear relationship obtained under pseudo-first-order conditions indicates that the reaction is first order in the substrate whose concentration was varied³⁸. Although the reaction rate constant can also be calculated based on the obtained data, this approach has several drawbacks. First, pseudo-first-order conditions often do not reflect the actual reaction conditions used in synthesis, and the required reagent excess may lead to changes in the reaction's kinetic profile⁴⁰. Second, determination of the reaction order in a single component requires measurement of the observed rate constant under several dif-

ferent reaction conditions. To ensure that the obtained values are not affected by experimental error, these measurements must be repeated multiple times, which increases the total number of experiments needed to obtain a single data point.

Reaction Progress Kinetic Analysis (RPKA) is an alternative method for studying reaction kinetics that allows determination of rate laws without the use of pseudo-first-order conditions⁴¹. Reaction profiles are obtained by plotting the reaction rate against the concentration of a reagent of interest, **R**, yielding a so-called graphical rate equation. A second graphical rate equation is obtained by performing the reaction with a different initial concentration of **R**, while keeping the concentrations of the other reagents constant. The reaction order in **R** is then obtained by plotting the reaction profiles as $\text{rate}/[\mathbf{R}]^r$ against the concentration of the reagent that was held constant. The order corresponds to the value of r at which the curves overlap. Therefore, determination of the reaction order can be achieved based on analysis of the full reaction profile using only two experiments under conditions that closely resemble synthetically relevant reaction conditions.

To plot the reaction profiles, RPKA requires rate values that can be obtained using either direct measurements or derivation from reagent concentration values obtained at various time points. However, only a few experimental techniques enable direct determination of instantaneous rates, whereas obtaining rates from concentration changes requires initial fitting of the data to a polynomial function, which might introduce artifacts before the data are used for RPKA⁴⁰. Variable Time Normalization Analysis (VTNA) was developed to enable direct graphical analysis of reaction profiles from concentration profiles⁴². Similar to RPKA, the order in a reagent **R** can be obtained from two experiments in which the concentration of **R** is the only varied parameter, by plotting the concentration change of the product against $\sum [\mathbf{R}]^r \Delta t$, or $[\mathbf{R}]^r t$ if the concentration of **R** does not change significantly during the course of the reaction^{40,43}. Notably, the analysis can be performed even if the obtained data exhibit substantial experimental noise⁴⁰.

Project goals

The main goal of the project was elucidation of the **VTT**-mediated bioconjugation reaction mechanism using modification of glutathione with **VTT** in the presence of sodium azide as a model reaction. Exploration of the reaction scope and applicability of the approach for peptide and protein bioconjugation was the second aim. Together, the combined data should provide a comprehensive characterization of the bioconjugation reaction for comparison with state-of-the-art methods for Cys-selective functionalization.

VTT-mediated bioconjugation: Results and discussion

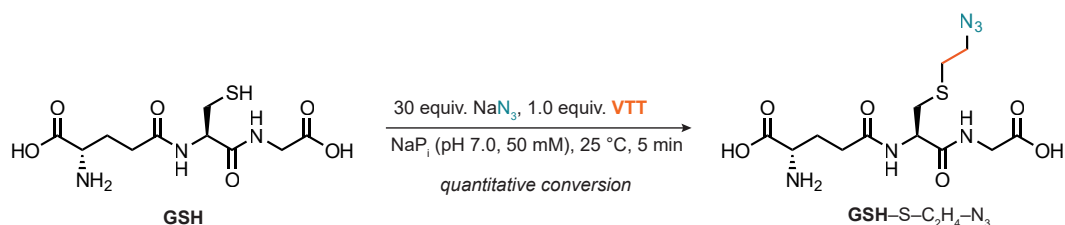
Contributions to the project: Prof. Dr. Fabio Juliá and Dr. Philipp Hartmann developed the bioconjugation reaction. The author performed the kinetic studies and synthesis of disulfide-rebridged peptides. Marie Sophie Sterling and Heike Hinrichs performed the LC–MS analysis. Pia Münstermann, Nadine Sauerborn, and Corinna Heidgen performed HPLC purifications. Julia Beatrice Jacobs acquired and analyzed the NMR data for isolated peptides.

Dr. Lara Vogelsang synthesized the proteins used in the studies. The author, together with Dr. Philipp Hartmann and Moritz Hommrich performed reactions with proteins. Dr. Rene Zangl, Dr. Alessandro Vetere, Marie Sophie Sterling, and Heike Hinrichs acquired the MS data on proteins (not shown; see ref.⁴⁴), which were interpreted by Dr. Philipp Hartmann, Dr. Rene Zangl, Dr. Alessandro Vetere, Marie Sophie Sterling, and Heike Hinrichs.

Kinetic studies of the bioconjugation reaction

Initial data on the VTT-mediated bioconjugation

VTT reacts with Cys-containing peptide **GSH** in the presence of excess sodium azide in aqueous buffers to afford the conjugated azide-containing product:



Because both **GSH** and sodium azide are water-soluble reagents and quantitative bioconjugation product formation can be achieved, the reaction was selected for the elucidation of the VTT-mediated bioconjugation mechanism under biocompatible conditions. The initial control experiments revealed that **GSH** reacts with VTT at 5–10 mM scale in buffered mixtures to yield the azidated product in less than five minutes, whereas no reaction with sodium azide is observed within the same time frame. The observations were consistent with a mechanism that involves addition of Cys to VTT in the first step, followed by reaction with azide. However, a more detailed mechanistic study was required to identify the nature of the reactive electrophilic intermediate and to obtain the rate law of the reaction.

Deuterium incorporation studies

The first part of the mechanistic studies involved identification of the putative intermediate that reacts with azide to form the final product. Initial Michael addition of deprotonated cysteinyl thiol to VTT should lead to a sulfur ylide intermediate, which would form a primary alkylthianthrenium intermediate after fast proton transfer. Alkylthianthrenium species are potent electrophiles and could undergo a nucleophilic substitution reaction with azide⁴⁵. Alternatively, the lone electron pair of sulfur in the thioether can act as a nucleophile to displace thianthrene and form a cyclic episulfonium intermediate, similar to the known reactivity of sulfur mustard⁴⁶ (Figure 1.7).

In order to distinguish between the two possible mechanisms, a deuterium incorporation experiment was performed. In deuterated buffer, the sulfonium ylide becomes deuterated to yield the alkylthianthrenium intermediate with an incorporated deuterium atom. If product formation oc-

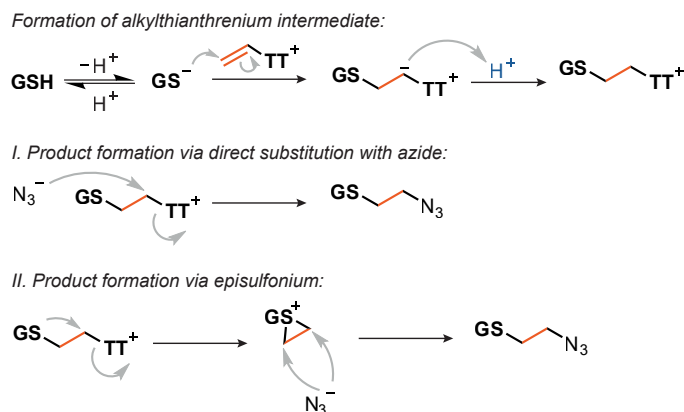


Figure 1.7: Proposed mechanisms for the product formation from alkylthianthrenium intermediate.

curs via reaction of azide with the alkylthianthrenium intermediate, the obtained product should be monodeuterated at the carbon bearing the azide substituent of the ethylene linker. If, however, an episulfonium intermediate is formed instead, azide will react with a cyclic intermediate, which should lead to the equal deuterium incorporation grade at both positions of the ethylene bridge in the product. NMR analysis of the product mixture revealed equal deuterium incorporation at the two positions of the ethylene linkage (Figure 1.8). This observation is consistent with a mechanistic pathway that involves reaction between azide and an episulfonium intermediate and excludes product formation via a direct azide attack on the alkylthianthrenium intermediate.

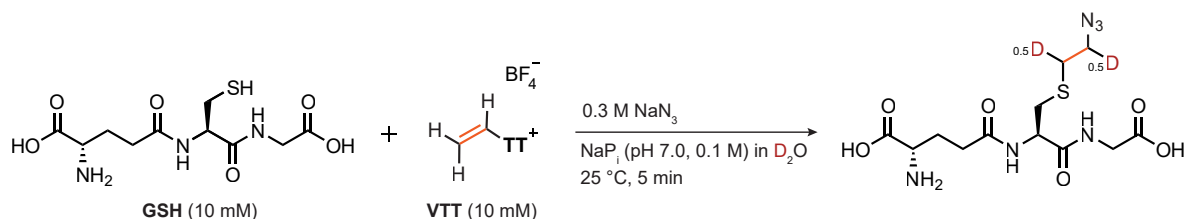


Figure 1.8: Deuterium incorporation experiment with VTT.

The deuterium incorporation experiment provided data that are consistent with product formation via a cyclic intermediate. Kinetic studies were then performed to identify the rate-determining step of the overall transformation and to determine the influence of each substrate on the reaction rate.

VTNA method for kinetic studies

VTNA was used in this work to identify the rate law of the VTT-mediated bioconjugation reaction. Because reaction between VTT and GSH leads to formation of an equimolar amount of thianthrene (TT), reaction rate profiles can be obtained by monitoring reaction progress by UV-vis spectroscopy and deriving concentrations of the reagents from the measured concentration of TT at each time point. The UV-absorbing TT, rather than VTT, was selected to monitor reaction progress because TT possesses a higher molar absorption coefficient at its absorption maximum and thereby enables more precise measurements of concentration changes, as confirmed by monitoring absorption profile changes during the course of the reaction between GSH and VTT in the presence of excess sodium azide (Figure 1.9). Acetonitrile (30%, v/v) was used as a co-solvent to monitor the reaction progress with UV-vis in order to ensure the homogeneity of the mixture upon formation of water-insoluble TT.

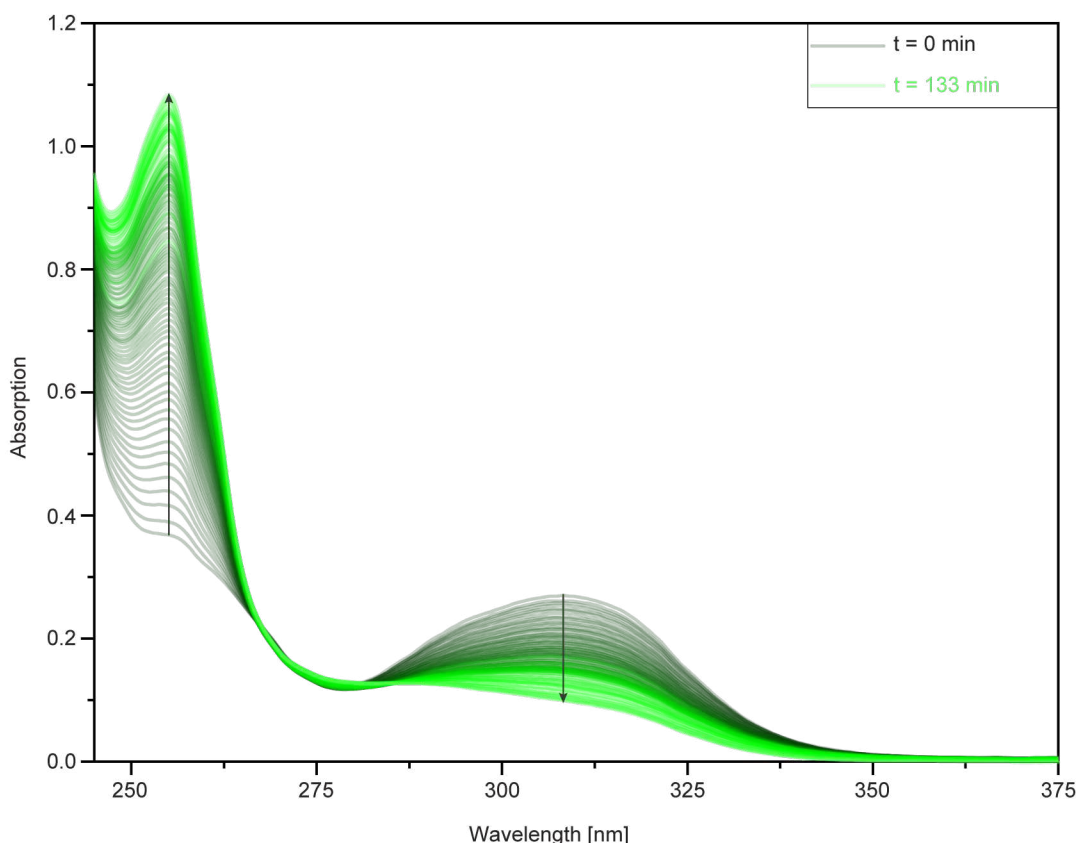


Figure 1.9: Absorption change for the reaction of 40 μM **GSH** with 50 μM **VTT** and 1.5 mM NaN_3 in sodium phosphate buffer (pH 7.4, 50 mM) containing 30% MeCN. The spectra were obtained with a NanoDrop™ One^C at $(37 \pm 0.1)^\circ\text{C}$. The increase of the absorbance intensity at 255 nm corresponds to **TT** formation, whereas the absorbance decrease at 307 nm corresponds to consumption of **VTT** in the course of the reaction.

VTNA revealed the linear dependence of the reaction rate on the concentration of both **VTT** and **GSH** and no influence of sodium azide concentration on the reaction rate (Figure E.33–Figure E.44). Based on the obtained values, the rate law of the reaction is:

$$\text{rate} = k[\text{GSH}]^1[\text{VTT}]^1[\text{NaN}_3]^0 \quad (1.1)$$

Elucidation of the rate-determining step

The observation that sodium azide concentration does not influence the reaction rate is consistent with a rapid reaction between the *in situ*-generated episulfonium intermediate and azide that occurs after the rate-determining step. However, the obtained rate law does not allow distinction between rate-limiting Michael addition of **GSH** to **VTT** and rate-limiting formation of the episulfonium intermediate via intramolecular cyclization and thianthrene displacement, both occurring prior to the reaction with azide. It was hypothesized that the hybridization change at the reactive atoms that occurs in each of the two steps can be used to determine the rate-limiting step of the reaction. Michael addition at an sp^2 -hybridized carbon atom yields a primary alkylthianthrenium intermediate with an sp^3 -hybridized carbon atom, whereas the subsequent thianthrene displacement leads to formation of a cyclic episulfonium intermediate. In strained molecules such as episulfonium ions, the atoms that form the cycle undergo partial rehybridiza-

tion to accommodate small bond angles by increasing p-character in the bonds within the cycle. Increased p-character in the episulfonium ring bonds is expected to be accompanied by increased s-character in the exocyclic C–H bonds at the ring carbon⁴⁷. Therefore, whereas Michael addition causes a hybridization change from sp^2 to sp^3 at the reacting carbon, episulfonium formation should shift the exocyclic C–H hybridization from sp^3 toward greater s-character (sp^3 to sp^{3-x} ; Figure 1.10).

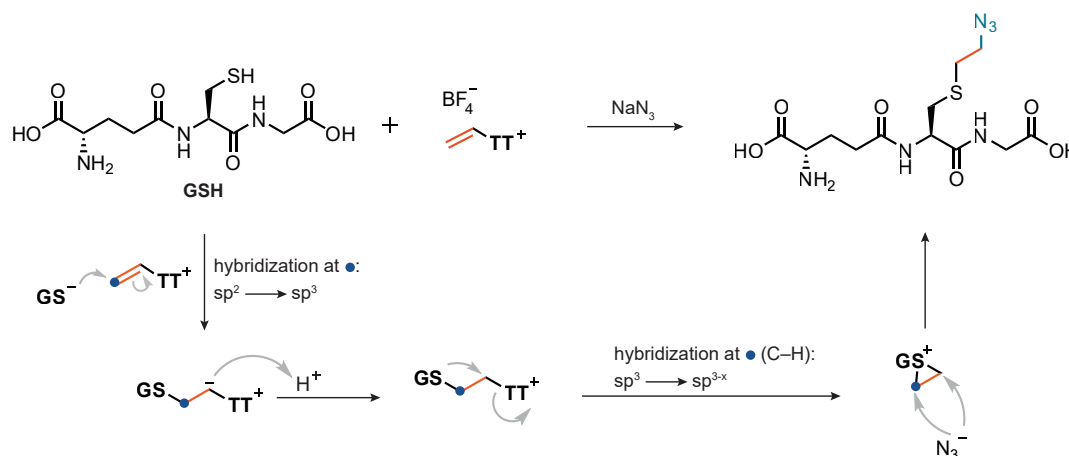


Figure 1.10: Expected hybridization changes at the reactive center in the first reaction steps.

If a hybridization change appears in the rate-determining step, the reaction rate should change when comparing isotopologues with different isotopes at the C–H bond involved in the rate-determining step because of the kinetic isotope effect (KIE)⁴⁸. For the reaction between **GSH** and **VTT**, a rate-determining hybridization change from sp^2 to sp^3 should produce an inverse secondary isotope effect, whereas a rate-limiting episulfonium formation is expected to produce either no or a very small secondary KIE. To test this hypothesis, an intermolecular competition experiment between **VTT** and $^2\text{H}_3\text{-VTT}$ was performed by incubating **VTT** and $^2\text{H}_3\text{-VTT}$ in equimolar amounts, each in excess relative to **GSH**, in the same reaction mixture and quantifying the ratio of the corresponding products. The obtained ratio between products formed from reaction with **VTT** and $^2\text{H}_3\text{-VTT}$, respectively, was 0.82 (Figure 1.11). This value is in line with the KIE obtained by determining the second-order rate constants of the two reactions ($k_H/k_D = 0.79$) and is in agreement with the value expected for an inverse secondary KIE caused by the hybridization change from sp^2 to sp^3 in the rate-determining step⁴⁸. Therefore, the KIE experiments provided data consistent with a rate-determining Michael addition of the cysteinyl thiol to **VTT** and exclude rate-limiting thianthrene displacement.

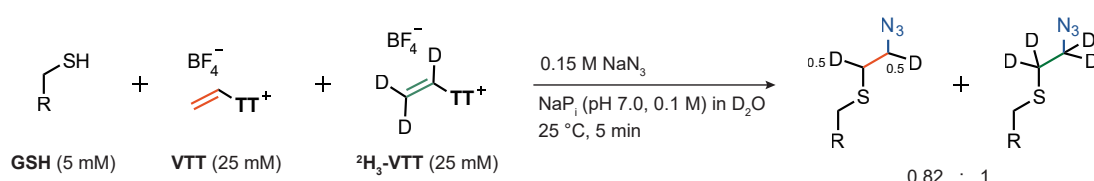


Figure 1.11: Observed KIE from the competition experiment between **VTT** and $^2\text{H}_3\text{-VTT}$.

Summary of the mechanistic studies

The combined data are in line with a mechanism that involves rate-determining Michael addition of Cys to **VTT**, followed by fast generation of a cyclic episulfonium intermediate that undergoes rapid reaction with azide present in the mixture (Figure 1.12). The described reactivity mode

has important practical implications: because **VTT** is a potent Michael acceptor, it undergoes reaction with Cys even in the presence of excess sodium azide in the mixture, thereby providing a method for Cys-selective modification in complex mixtures.

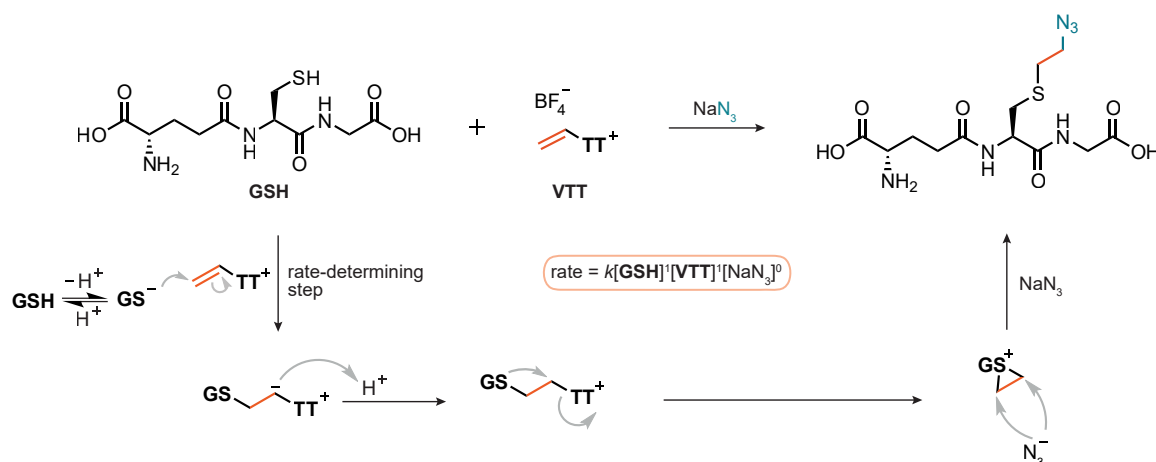


Figure 1.12: Overview of the proposed mechanism for the bioconjugation reaction.

Unlike other Cys-reactive Michael acceptors, Michael addition to **VTT** leads to umpolung of nucleophilic Cys into a fleeting electrophilic intermediate that is more reactive than **VTT** itself, thereby enabling single-step product formation with exogenous nucleophiles. Bioorthogonal nucleophiles present in the mixture readily react with the *in situ*-generated episulfonium intermediate to form conjugates with a stable ethylene linkage that, unlike commonly used maleimide-based reagents, does not undergo retro-Michael reaction⁴⁹. Because an inorganic azide source is introduced into the reaction as part of the buffer mixture, no synthesis or handling of explosive organic azides is required to incorporate the widely used bioorthogonal azide tag^{50,51}. Moreover, the azide moiety in the formed conjugate is located close to the protein surface and does not perturb the protein tertiary structure⁴⁴. Nevertheless, subsequent derivatization of the introduced azide linchpin in a follow-up step using Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC) or strain-promoted azide–alkyne cycloaddition (SPAAC) reactions can be readily achieved to introduce a payload of interest⁴⁴.

Determination of second-order rate constants

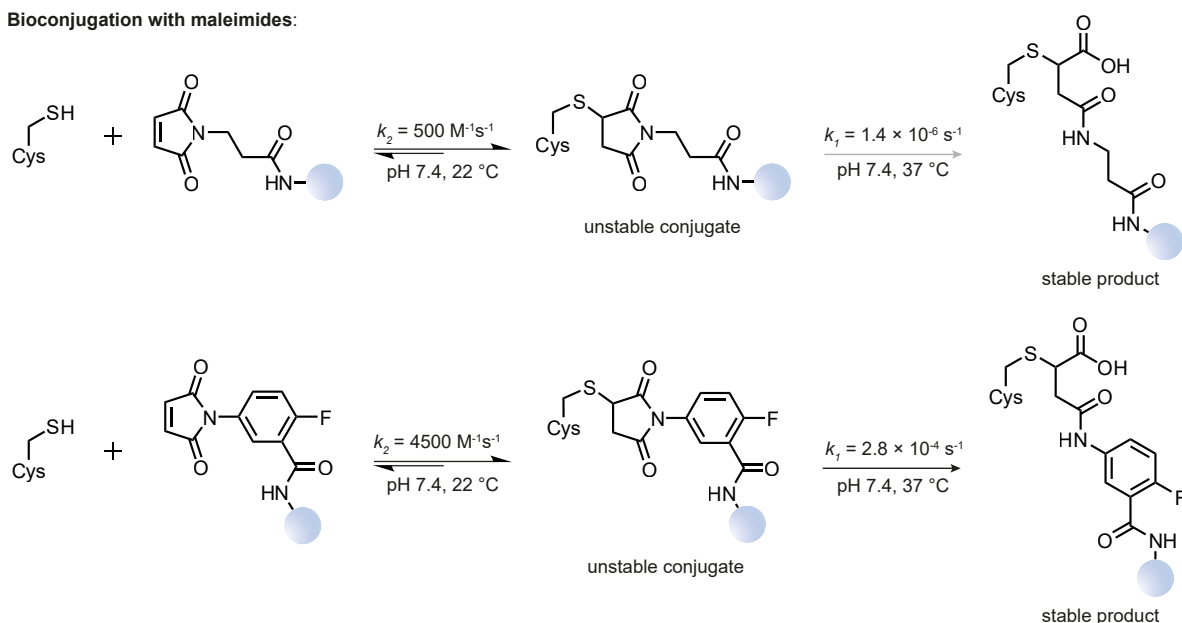
Efficient bioconjugation reactions rely on fast transformations because proteins of interest are used at low concentrations, usually in the micromolar range. Fast bioconjugation ensures that the reaction reaches completion within a timeframe of minutes to hours before protein degradation takes place⁵². In the case of *in vivo* labeling experiments, high reaction rates are essential to ensure that the readout can be obtained on a timescale comparable to that of the studied intracellular processes. Moreover, a high reaction rate ensures that the reagent does not need to be used in large excess and therefore prevents off-target reactions and enhanced cytotoxicity¹⁹.

Having established that the reaction rate is first order in both **GSH** and **VTT**, the reactivity of **VTT** toward **GSH** under the studied conditions can be quantified by determining the second-order rate constant. The rate constant for the reaction between **GSH** and **VTT** in sodium phosphate buffer at pH 7.4 is $38.1 \pm 0.1 \text{ M}^{-1}\text{s}^{-1}$. As a reactive Michael acceptor, **VTT** reacts with Cys 40 times faster than iodoacetamide ($0.9 \pm 0.05 \text{ M}^{-1}\text{s}^{-1}$)⁵³, a reagent that has been widely used in Cys-based intracellular chemoproteomics⁵⁴. Bioconjugation with **VTT** also occurs faster than copper-catalyzed or strain-promoted azide–alkyne cycloaddition reactions ($3.4 \text{ M}^{-1}\text{s}^{-1}$ and $0.9 \text{ M}^{-1}\text{s}^{-1}$, respectively⁵⁵) that are commonly used for chemoselective ligation. Cys modification with alkylmaleimide reagents occurs faster ($500 \text{ M}^{-1}\text{s}^{-1}$) but leads to formation of unstable conjugates²⁰.

Arylmaleimides have been developed to achieve faster Cys bioconjugation and to ensure that the retro-Michael decomposition pathway is suppressed by rapid succinimide hydrolysis. Indeed, second-order rate constants of $4500 \text{ M}^{-1}\text{s}^{-1}$ can be achieved with specifically designed aryl substituents²⁰ (Figure 1.13, top). However, introduction of the aryl groups that is necessary for formation of more stable conjugates leads to bulkier, less water-soluble reagents⁵⁶. In contrast to reagents such as maleimides, for which modification of the reagent scaffold ultimately leads to changes in linker properties, fine-tuning of **VTT** reactivity can be achieved without introducing changes in the linker that connects the biomolecule and the introduced payload. Because thianthrene displacement occurs upon episulfonium generation and prior to the product formation, a modified thianthrenium moiety influences the reactivity of the reagent in the first, rate-limiting Michael addition step but has no effect on the episulfonium reactivity or the structure of the final product. For example, **VTFT**, a tetrafluoro derivative of **VTT**, can be synthesized from commercially available ethylene gas and tetrafluorothianthrene-S-oxide in one step, and was isolated to obtain a more reactive derivative of **VTT**⁴⁴.

Mechanistic studies revealed identical kinetic profiles for the reactions between **GSH** and **VTT** or **VTFT** in the presence of azide, consistent with a common reaction mechanism that leads to formation of the same ethylene-bridged conjugate. The determined second-order rate constant for the reaction between **GSH** and **VTFT** in the presence of NaN_3 is $310 \pm 8 \text{ M}^{-1}\text{s}^{-1}$, an order of magnitude higher than that for **VTT** and of the same order of magnitude as Cys functionalization with *N*-alkylmaleimides under similar conditions ($500 \text{ M}^{-1}\text{s}^{-1}$)^{20,55} (Figure 1.13, bottom). The high reaction rate is not limited to Cys-containing peptides such as **GSH**, and the potent reactivity of **VTFT** enables modification of proteins within minutes. For example, functionalization of a Cys-containing superfolder green fluorescent protein (sfGFP) mutant S147C proceeds with full conversion in just two minutes, and leads to formation of the same ethylene-linked conjugate as the single identified product, consistent with data obtained for **VTT**⁴⁴.

Bioconjugation with maleimides:



VTT-mediated bioconjugation:

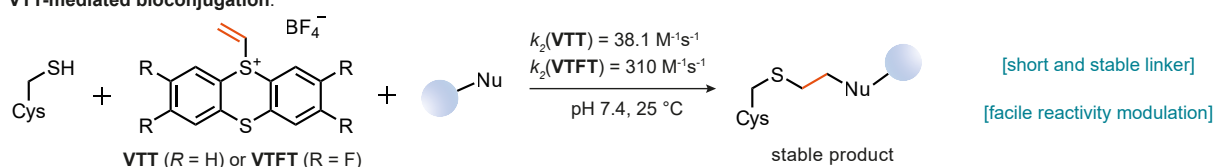


Figure 1.13: Comparison of maleimide-mediated (top) and vinylthianthrenium-mediated (bottom) bioconjugation strategies. Introduction of an aryl group at the maleimide nitrogen increases the bioconjugation rate and enables fast formation of stable products via enhanced hydrolysis of the succinimide ring. However, the introduced bulky aryl group remains as a part of the linker in the obtained conjugate. **VTT**-mediated bioconjugation does not require payload incorporation into the reagent and enables control of the reaction rate via modification of the thianthrenium moiety, which does not influence the linker structure.

The described experiments revealed that bioconjugation with **VTT** and its more reactive derivative **VTFT** occurs via the same mechanism that involves rate-determining Michael addition to vinylthianthrenium, followed by Cys umpolung via formation of a reactive episulfonium intermediate that engages in reaction with exogenous azide present in the mixture to yield the conjugation product. The determined rate constants for bioconjugation with both **VTT** and **VTFT** compare favorably with commonly used bioconjugation methods and allow protein modification in minutes, thereby enabling rapid introduction of the bioorthogonal azide moiety through a short ethylene linker. The next section explores the applicability of the approach for synthesis of different protein and peptide bioconjugates.

Protein bioconjugation and peptide stapling

Scope of protein bioconjugation

VTT and **VTFT** are reactive Michael acceptors capable of Cys-selective protein modification in complex lysate mixtures⁴⁴. Quantitative conversion of Cys-containing proteins of various sizes and formation of azide-containing products can be achieved in under five minutes at 25 °C with **VTFT** (Figure 1.14). The high reaction rate enables synthesis of azidated conjugates from native or recombinant proteins by utilizing **VTFT** in an excess of up to 10 equivalents relative to TCEP (tris(2-carboxyethyl)phosphine), which was initially added to the mixture to ensure formation of reduced cysteinyl thiols on the protein surfaces prior to addition of **VTFT**.

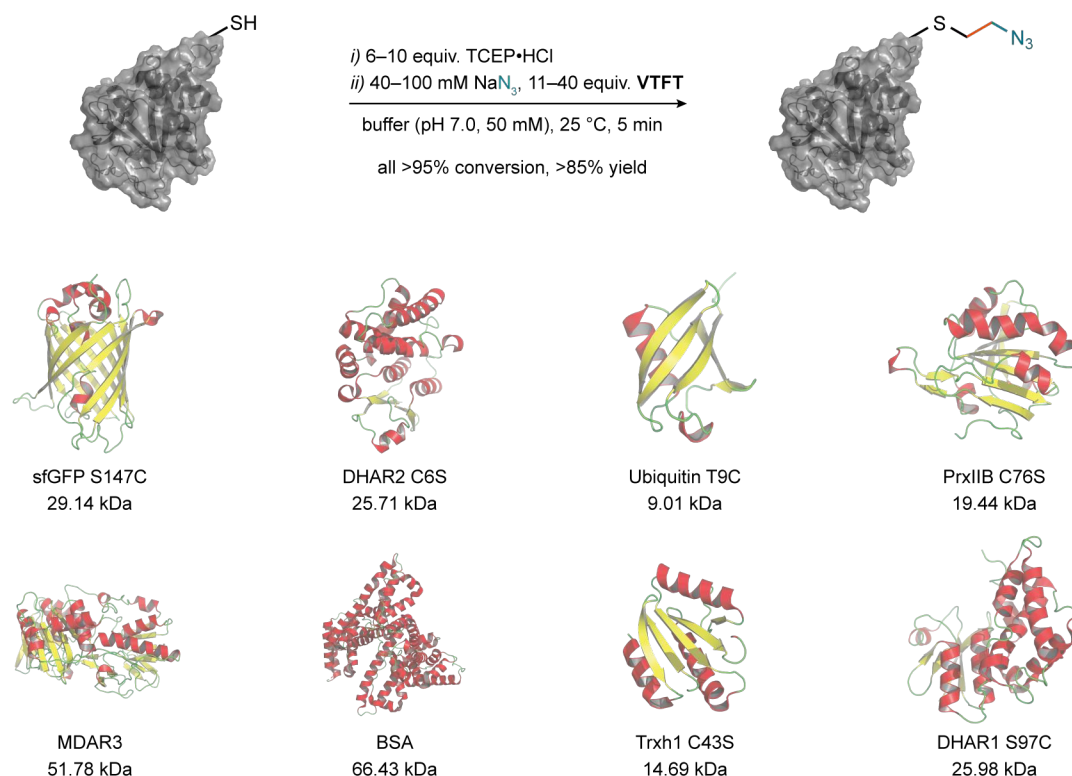


Figure 1.14: Scope of proteins used for bioconjugation reactions with **VTFT** and NaN₃. NaP_i and HEPES were used as buffers.

Episulfonium is a reactive intermediate that, when generated in aqueous solutions, undergoes rapid hydrolysis unless there is a nucleophile in close proximity⁵⁷. Therefore, formation of versatile bioconjugates via *in situ*-generated episulfonium should be feasible if the nucleophile of interest is added to the mixture in large excess to outcompete reaction with water. Quantitative formation of the azidated conjugates can be achieved when **VTT** is added to the protein mixtures containing sodium azide at concentrations of 40 mM and higher. The optimized reaction conditions for bioconjugation with azide can also be extended to incorporation of anilines to introduce NMR-active tags or a bioorthogonal linchpin (Figure 1.15). This result is notable because anilines are weak nucleophiles: the Mayr nucleophilicity parameters^{58,59} of aniline in water are $N = 12.99$, $s_N = 0.73$ ⁶⁰, substantially lower than those of the reactive thiolate of glutathione (**GSH**, $N = 20.97$, $s_N = 0.56$)⁶¹, indicating that episulfonium formation enables protein modification with nucleophiles that are orders of magnitude less reactive than the thiol-based nucleophiles that are commonly used for protein functionalizations via formation of electrophilic linchpins.

Episulfonium is also able to trap other nucleophiles, including thiocyanate, thiosulfate, and thio-

phosphate, enabling single-step incorporation of an IR label or PTM mimics. These results demonstrate the broad practical applicability of **VTT**-mediated bioconjugation for versatile modifications enabled by episulfonium formation. In contrast to methods that rely on transformation of Cys into an electrophilic linchpin for follow-up modification that require isolation of the protein bearing the introduced linchpin, episulfonium is generated *in situ* and reacts with nucleophiles that cannot be used in standard linchpin approaches to yield the desired products in a single step (Figure 1.15).

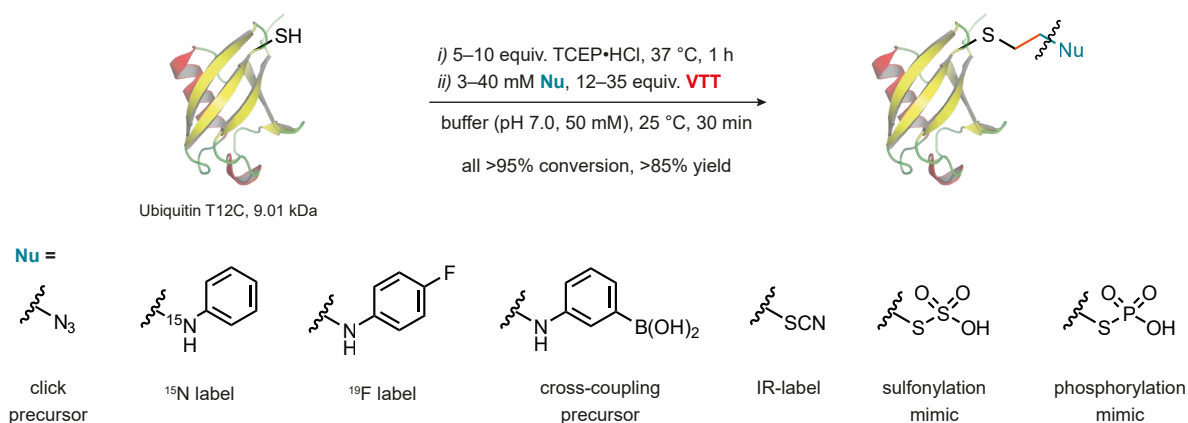


Figure 1.15: Scope of nucleophiles for protein modification introduced with **VTT**. NaP_i and HEPES were used as buffers.

Thiol-based nucleophiles cannot be used directly in **VTT**-mediated bioconjugation because of the competing reactivity with cysteinyl thiols. However, formation of primary alkyl iodide species in the reaction between the episulfonium intermediate and iodide can be used to achieve umpolung in a separate step prior to addition of reactive thiol nucleophiles and thereby expand the scope of nucleophiles that can be used in **VTT**-mediated bioconjugation (Figure 1.16).

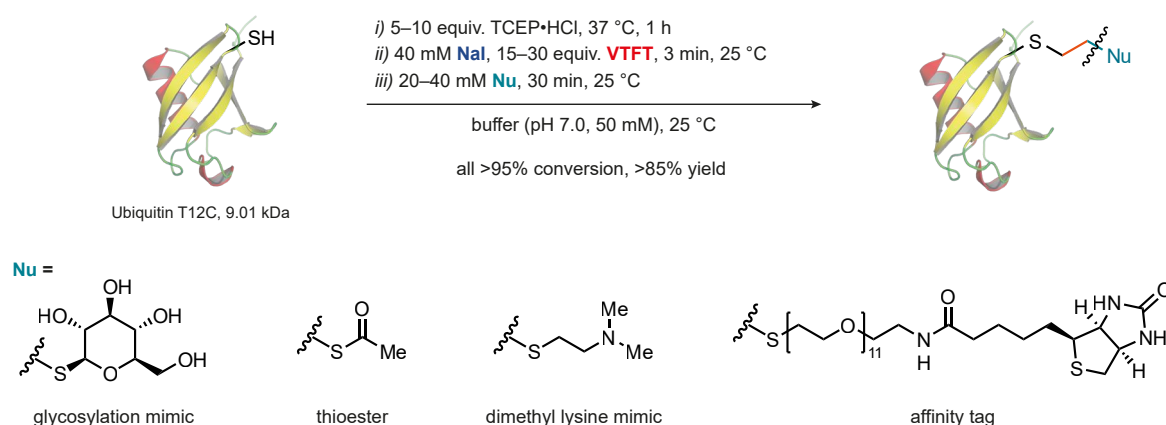


Figure 1.16: Scope of nucleophiles introduced via formation of alkyl iodide intermediate. NaP_i , BIS-TRIS, and HEPES were used as buffers.

Because alkyl iodide intermediates undergo hydrolysis in buffered mixtures, **VTF** was used for synthesis of bioconjugates via iodide trapping to ensure fast formation of the alkyl iodide intermediate prior to the addition of the second nucleophile, and thereby suppress irreversible hydrolysis of the primary alkyl intermediate. The method enabled fast formation of products with thioglucose, thioacetate, and dimethylaminoethanethiol as a glycosylation mimic, a thiolate protecting group, and a dimethyllysine mimic, respectively. Reaction with a biotin–PEG–thiol

reagent allowed direct incorporation of an affinity tag. The combined data demonstrate that **VTT**-mediated Cys umpolung enables access to products from nucleophiles that cannot be utilized via established linchpin methods due to high stability and low reactivity of the introduced linchpins, while not being limited to nucleophiles that possess low reactivity toward **VTT** in buffer.

Peptide stapling via disulfide rebridging

The applicability of episulfonium chemistry is not limited to protein and peptide functionalizations with exogenous nucleophiles. If a nucleophilic amino acid is located in close proximity to the *in situ*-generated episulfonium, the nucleophilic group of the neighbouring amino acid attacks the episulfonium intermediate to form ethylene-bridged crosslinked peptides⁴⁴.

Moreover, vinylthianthrenium chemistry can be used to stabilize labile disulfide bonds in cyclic peptides via introduction of an ethylene linkage. To obtain reactive precursors for peptide crosslinking, commercially available disulfide-containing peptide therapeutics oxytocin, lypressin, and octreotide were reduced in the presence of TCEP in sodium phosphate buffer at neutral pH. Formation of stapled products was achieved by using an equimolar amount of **VTFT**, which enabled isolation of disulfide-rebridged peptides in 61–74% yield (Figure 1.17), and identity of the crosslinked residues was confirmed by NMR and MS/MS analysis⁴⁴.

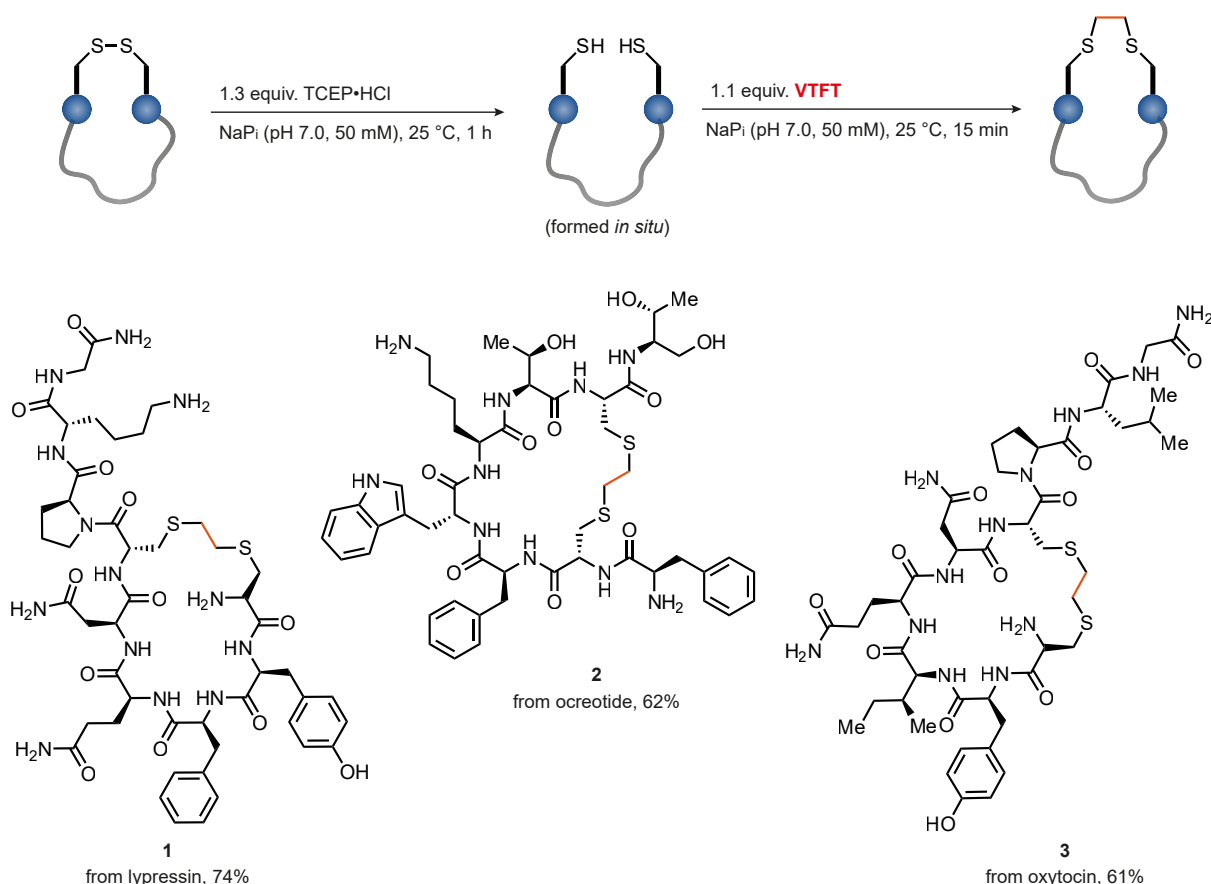


Figure 1.17: Scope of disulfide-rebridged peptides.

In contrast to commonly used olefin metathesis or cycloaddition reactions for peptide stapling^{62,63}, no unnatural amino acid incorporation is required during peptide synthesis, and the reactive precursor for the stapling reaction can be obtained by incubation with a mild reductant such as TCEP. Disulfide-rebridged products with a short methylene linkage have been previously synthesized⁶⁴, but the method relies on use of light-sensitive diodomethane⁶⁵ and is limited to Cys–Cys crosslinking. In contrast, both **VTT** and **VTFT** are bench-stable compounds that generate a common reactive intermediate capable of engaging amino acids such as lysine and glutamic acid in peptide stapling for synthesis of different types of crosslinked peptides⁴⁴.

VTT-mediated bioconjugation: Conclusions

The kinetic studies described in this part provided a basis for elucidation of the bioconjugation reaction mechanism and determination of the bioconjugation rate constants. The same mechanism for **VTT**- and **VTFT**-mediated bioconjugation was proposed and involves rate-limiting addition of Cys to the reagents, followed by formation of a common episulfonium intermediate that readily engages various nucleophiles in a reaction to form conjugates with a short and stable ethylene linkage in one step. The reaction occurs with rates comparable to or higher than those of commonly used protein ligation methods. Versatility of the method was demonstrated by synthesis of different protein conjugates and showed wide applicability of the episulfonium-based approach for protein bioconjugation based on a single reagent. Moreover, **VTT** chemistry enables stabilization of labile disulfide bonds in peptides via introduction of an ethylene bridge. No previously available approach provides such broad applicability for both protein conjugation and peptide stapling, since **VTT**-mediated episulfonium formation enables *in situ* umpolung of reactive and nucleophilic Cys into an electrophilic episulfonium intermediate in the presence of various other nucleophiles that do not compete with Cys in the initial reaction step. The obtained data indicate that formation of reactive linchpins should be a more promising approach for versatile bioconjugation in comparison to synthesis of reagents that carry different payloads. Therefore, development of new linchpin-based methods for versatile bioconjugation that selectively tag amino acids other than Cys would provide valuable enrichment to currently available and traditionally used bioconjugation methods.

Chapter 2: *In cellulo* crosslinking for protein structure probing

Introduction: Protein crosslinking

MS-based proteomics

Proteins often occur as multiple proteoforms, distinct molecular variants of the same protein defined by specific PTM patterns that can exhibit different activities and interaction profiles. Therefore, characterizing the entirety of proteoforms is essential for understanding differences in the proteome between healthy and disease states⁶⁶. Bottom-up mass spectrometry (MS) proteomics is a well-established and sensitive method for studying protein expression levels, characterizing proteoforms and protein conformations in solution, as well as identifying protein–ligand and protein–protein interactions in cells⁶⁷. In contrast to top-down proteomics, which relies on MS characterization of intact proteins and typically provides lower proteome coverage depth, data on protein states in bottom-up proteomics are obtained via analysis of peptides generated after enzymatic proteolysis, which undergo chromatographic separation followed by MS identification⁶⁸. Deep proteome coverage can therefore be achieved, since proteins of all sizes undergo proteolytic digestion to yield a mixture of peptides that can be easily ionized, detected, and characterized using sensitive MS setups.

The general workflow consists of the following steps: proteins are extracted from biological samples of interest, such as cells, tissues, or biological fluids, and protein concentration is determined using colorimetric or fluorimetric methods⁶⁹. Before digestion, proteins are solubilized in denaturing buffers, disulfide bonds are reduced, and free Cys residues are alkylated to decrease complexity of the analyzed sample in downstream analysis. In the next, most crucial step of the sample preparation, a protease is added to initiate hydrolysis of peptide bonds⁶⁷ (Figure 2.1). Various digestion enzymes with different specificities, or combinations of proteases, can be used to control digestion depth and length of the obtained peptides. Depending on conditions used for the proteolysis step, obtained samples may need to be purified to remove interfering compounds introduced during previous steps prior to the analysis⁶⁷.

In the next step, the peptide mixture is resolved using high-performance liquid chromatography. The separated peptides are analyzed by tandem MS, in which the mass of peptide ions is measured, after which selected peptide ions (precursor ions) undergo fragmentation, providing precursor m/z values and intensities as well as fragment-ion spectra that are used for elucidation of peptide sequence and PTM localization⁶⁷. During follow-up data analysis, detected precursor masses and fragmentation patterns for each peptide are compared to theoretically predicted m/z values of peptides expected to be present in the mixture, given the protein sequences and the specificity of the utilized protease. Various software packages are used for automatic processing of raw LC–MS/MS data and provide peptide identities and observed intensities of the detected peptide ions, thereby enabling quantification of relative protein abundances and infer-

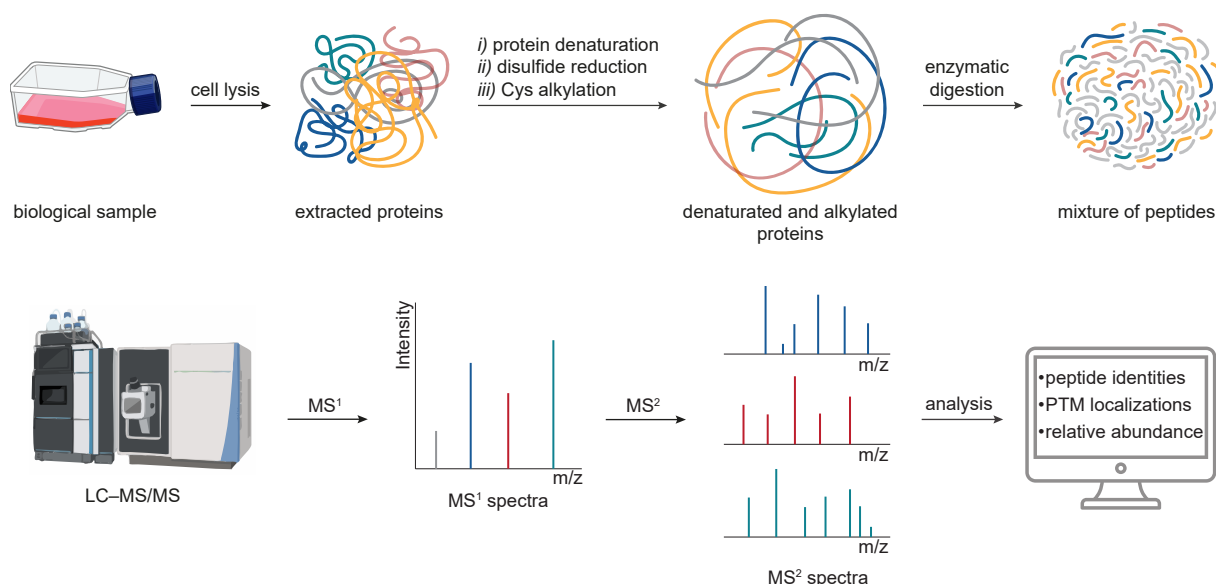


Figure 2.1: General scheme for sample preparation and analysis in proteomics studies. Analysis of LC-separated peptides consists of detection of peptide ions, followed by fragmentation of selected ions. The obtained precursor masses and fragmentation patterns provide information on peptide identity and sequence, as well as localization of PTMs.

ence of PTM localizations in the proteins, both essential for characterization of proteoforms⁷⁰.

Chemoproteomic studies

Chemoproteomic studies utilize reactive chemical probes to detect protein sites that exhibit distinct reactivity profiles, which can be used to identify novel drug targets⁷¹. Due to its high nucleophilicity, Cys is a reactive amino acid that undergoes various PTMs that regulate catalytic enzyme activity and protein localization⁷². Therefore, development of Cys-selective, cell-permeable probes enables characterization of Cys oxidation states, whereas PTM-specific reporters are used for selective targeting and identification of protein sites that undergo a PTM of interest. Moreover, chemical probes equipped with Cys-reactive warheads have been used to identify novel targets for development of covalent inhibitors⁵⁴. Development of light- and heavy-labeled probes for fast Cys-selective protein modification is particularly valuable for reliable quantitative LC-MS/MS analysis⁷³. In this case, cell samples from a control experiment and cells subjected to a stimulus of interest can be treated with different isotopologues of the same reagent under the same conditions. Next, protein mixtures are extracted via cell lysis, followed by normalization of protein concentrations, mixing of the two samples, protein digestion, and LC-MS/MS analysis. Quantification of the identified labeled peptides by comparing the paired MS¹ signal intensities of the isotopically distinct labels enables quantitative characterization of stimulus-induced changes in relative protein abundances⁷⁴ (Figure 2.2). Development of novel reagents that possess high cell permeability and enable fast intracellular protein labeling while preserving intracellular homeostasis is desirable to ensure that the obtained readout of quantified protein abundances closely represents native conditions⁷⁵.

Crosslinking MS

Crosslinking MS for protein structure probing

Elucidation of protein structure is important for understanding protein function and identification of binding sites in development of new drugs⁷⁶. Although nearly 250,000 protein structures

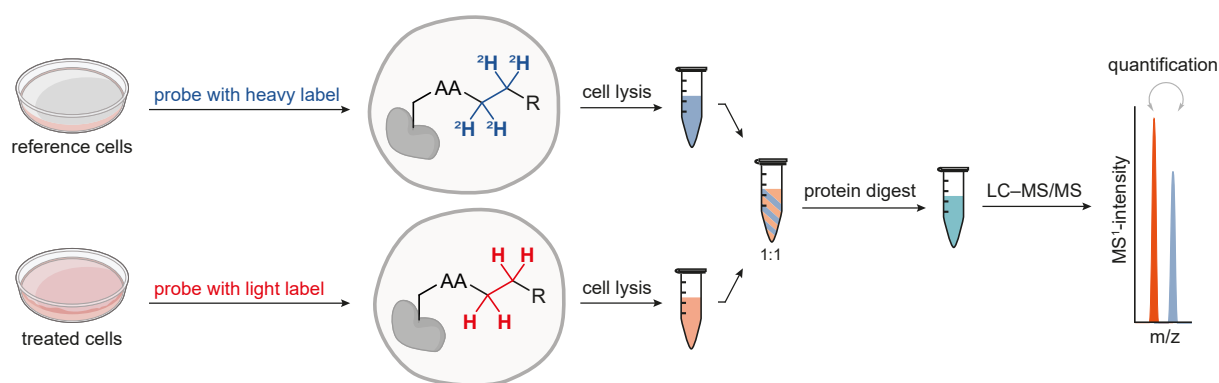


Figure 2.2: A general workflow for quantitative chemoproteomic studies. Quantification of ion intensities corresponding to peptides modified with different isotopologues enables inference of changes in protein abundance in samples treated with a stimulus of interest in comparison to reference samples. AA, amino acid.

are currently available based on X-ray crystallography data⁷⁷, formation of crystals suitable for analysis is very challenging for some proteins⁷⁸. Moreover, X-ray structures might not represent native protein structures in solution. Protein NMR enables elucidation of the protein structure in solution and even in cells⁷⁹, but is limited to small proteins. Structures of large proteins can be resolved using cryo-electron microscopy, but the method is currently not suitable for small (<50–100 kDa) proteins and also relies on protein structure elucidation in an artificial environment⁸⁰.

Crosslinking MS has emerged as a useful alternative method for protein structure probing in solution that can be applied to isolated proteins as well as complex protein mixtures in lysates or cells⁸¹. In a typical experiment, a sample of interest is incubated with a bifunctional reagent that contains two electrophilic warheads separated by a linker of a particular length. The reagent reacts with solvent-accessible residues on protein surfaces and thereby introduces covalent linkages between amino acids that are in close proximity⁸². The treated protein sample is then processed and digested according to standard proteomics sample preparation protocols. The LC–MS/MS measurement is then performed to identify the crosslinked peptides. Because crosslinked peptides are typically larger and contain more protonated sites than linear peptides, they often ionize to higher precursor charge states. Therefore, partial enrichment of crosslinked peptides can be achieved during LC–MS/MS acquisition by restricting precursor selection to higher charge states (e.g., $z \geq 3+$)⁸³.

In the next step, the obtained data are processed to elucidate the identity of the crosslinked residues and to map identified peptides to specific protein sequences expected to be present in solution (Figure 2.3). Because assignment of two peptides is required to determine a crosslink identity, analysis of crosslinking MS data is more complex than a modification search on linear peptides, as it requires considering all pairwise peptide combinations and deconvoluting MS/MS spectra comprising fragments from two linked peptides.

Based on the identity of the crosslinked residues and linker length of the utilized crosslinking reagent, the obtained crosslinking data can be used to quantify conformational changes of proteins in solution as well as to provide additional support for protein structure prediction^{84,85}. Although machine-learning methods such as AlphaFold2 enable prediction of structures for hundreds of millions of protein sequences⁸⁶, accurate prediction remains challenging for intrinsically

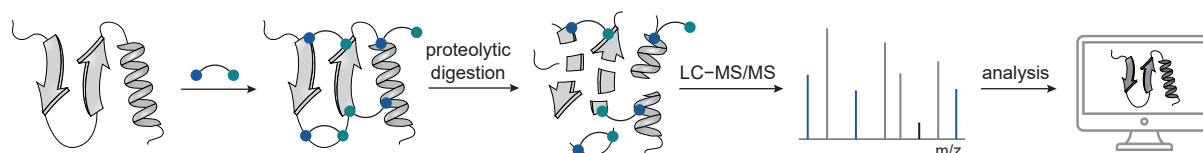


Figure 2.3: Protein structure probing using crosslinking MS.

disordered regions and proteins for which only few homologous sequences are known. Only 36% of residues in the human proteome are predicted with very high confidence (Predicted Local Distance Difference Test (pLDDT) score > 90)⁸⁷. Crosslinking data can be used to guide protein structure prediction using identified distance restraints between crosslinked residues and lead to increased quality of the predictions when compared to the results obtained with tools like AlphaFold⁸⁸. A recently developed algorithm, AlphaLink2, predicts protein structures from an amino acid sequence while incorporating distance restraints, and can therefore be used for crosslinking-guided protein structure probing by users without specialized modeling expertise⁸⁹.

Common crosslinking approaches

Most commonly used crosslinking protocols are based on the use of biselectrophilic Lys-selective reagents due to high abundance of Lys residues on protein surfaces and potent reactivity of Lys with *N*-hydroxysuccinimide (NHS) esters⁹⁰ (Figure 2.4). A wide variety of Lys-selective crosslinking reagents is commercially available and includes reagents with cleavable and non-cleavable linkers, different linker lengths, enrichment handles, and isotopologues for quantitative crosslinking studies⁹¹. MS-cleavable crosslinking reagents with an incorporated labile group in the linker structure can be utilized to facilitate identification of crosslinked peptides by generating peptide-specific fragments upon MS/MS cleavage, yielding diagnostic peptide fragments that allow independent assignment of each peptide⁹². Nevertheless, development of new database search algorithms allows reduction of the total search space and analysis of crosslinking data obtained even from complex protein mixtures^{83,93}. Non-cleavable crosslinking reagents are therefore widely applicable and frequently used⁹⁰. Although practical for identification of a large number of crosslinks due to targeting of abundant Lys residues, the two-step crosslinking process is characterized by relatively long (minutes to hours) lifetimes of the formed intermediates, which makes trapping of unfavorable protein conformations feasible⁹⁴. Combined with relatively high flexibility of Lys side chains and linker lengths of 8–12 Å, long-distance restraints of more than 30 Å are obtained, which further complicates precise protein structure mapping.

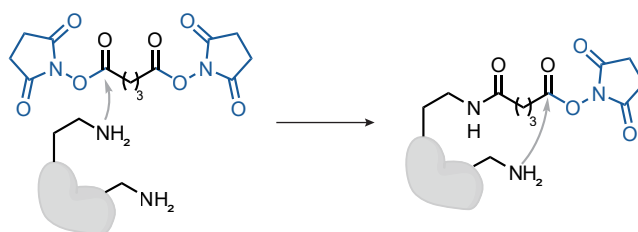


Figure 2.4: Crosslinking with homobifunctional crosslinking reagents.

Heterobifunctional Lys-selective crosslinking reagents containing photoreactive groups such as diazirines have emerged as an alternative to homobifunctional reagents for more reliable structure mapping⁹⁴. Reaction with Lys residues leads to incorporation of diazirines into protein

surfaces. Upon irradiation with UV light, short-lived carbene intermediates are formed and undergo insertion reactions with neighboring amino acids (Figure 2.5). Fast reaction rates of crosslink formation, shorter length of the incorporated linker, and high crosslink mapping density due to formation of versatile crosslinking products enable protein structure probing with higher resolution⁹⁵. However, the overall crosslinking process also proceeds in two separate steps and requires exogenous activation for the formation of the reactive intermediates. Isotopically coded heterobifunctional crosslinking reagents have been synthesized⁹⁶ but have not yet found wide applicability in quantitative crosslinking studies, possibly because of broad carbene reactivity that leads to formation of various coeluting products with low peak intensities⁹⁷.

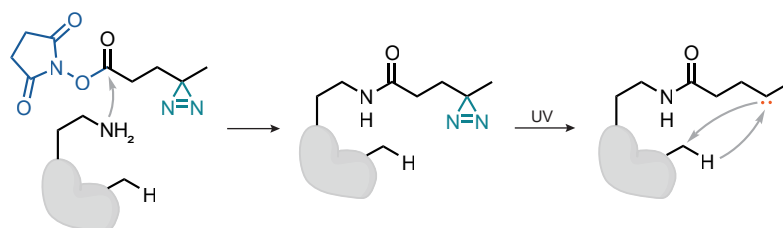


Figure 2.5: Crosslinking with heterobifunctional crosslinking reagents.

Fast crosslink formation can also be achieved if the photoreactive group is introduced into the protein structure via an unnatural amino acid during protein synthesis in cells. Protein-wide incorporation of photo-leucine and photo-methionine is an established method that leads to introduction of diazirines in close proximity to protein surfaces and does not lead to protein structure perturbation⁹⁸. Reactive carbene intermediates for fast crosslinking are then generated upon UV irradiation. Fast formation of short crosslinks enables reliable protein structure probing based on short-distance restraints obtained in the experiment (Figure 2.6).

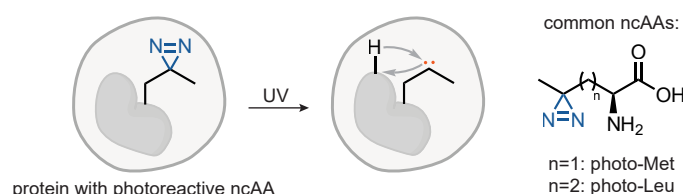


Figure 2.6: Crosslinking with unnatural photoreactive amino acids (ncAA, non-canonical amino acid).

Nevertheless, incorporation of noncanonical amino acids requires optimization for a specific cell type⁹⁹, and the crosslinking procedure relies on activation with UV light, which requires careful control of the exposure dose to minimize DNA damage¹⁰⁰. Development of cell-permeable chemical probes that enable fast and versatile crosslinking of natural amino acids can therefore provide an alternative to existing methods for reliable protein structure probing studies in different cell types.

Project goals

The previously described Cys-selective reactivity of **VTT**, as well as the versatile chemistry of episulfonium intermediates formed upon Cys umpolung with **VTT**, indicated that **VTT** can be used both for fast proteome-wide Cys labeling and as a versatile crosslinking reagent. The goals of the project were investigation of cell permeability of **VTT** and quantification of its intracellular protein labeling efficiency in different cell types, as well as establishment of episulfonium-mediated intracellular crosslinking.

***In cellulo* protein crosslinking: Results and discussion**

***In cellulo* protein labeling with VTT**

Contributions to the project: Dr. Philipp Hartmann initiated the project and discovered cell permeability of **VTT** together with Dr. Lara Vogelsang. Dr. Lara Vogelsang performed experiments with bacterial, plant, and yeast cells, as well as protein overexpression in *E. coli* cells. The author, together with Dr. Philipp Hartmann, Dr. Dario Marchionni, and Dr. Christian Preisinger performed experiments with mammalian cells. The author and Dr. Philipp Hartmann performed cytotoxicity assays and cell viability experiments. The author and Dr. Philipp Hartmann performed *in cellulo* competition experiments, and Dr. Dario Marchionni performed *in vitro* competition experiments between **VTT** and maleimide. The author and Dr. Philipp Hartmann prepared samples for LC–MS/MS analysis. Dr. Christian Preisinger and Dr. Alessandro Vetere acquired LC–MS/MS data. The author performed LC–MS/MS data processing, database searches for modified and crosslinked peptides, and data analysis.

VTT is a membrane-permeable reagent that enables Cys umpolung *in cellulo*, as discovered by Dr. Philipp Hartmann and Dr. Lara Vogelsang¹⁰¹. Incubation of *E. coli* cells with sodium azide, followed by addition of **VTT**, led to proteome-wide incorporation of the azide tag into proteins, as revealed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of mixtures obtained after cell lysis and a CuAAC reaction of proteins in the lysates with a fluorescein tag (Figure 2.7). Incorporation of azide into the extracted proteins was additionally confirmed via LC–MS/MS, consistent with episulfonium formation in cells that enables incorporation of a bioorthogonal tag for follow-up modifications. The qualitative data demonstrated feasibility of fast intracellular protein modification with **VTT** and indicated feasibility of **VTT**-mediated crosslinking in different cell types.

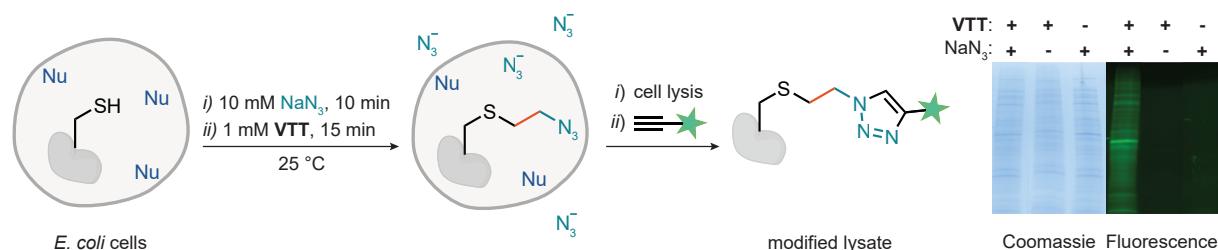


Figure 2.7: *In cellulo* incorporation of azide tag in *E. coli* cells.

Cytotoxicity of VTT

Mammalian cells do not contain rigid cell walls and are typically easier to lyse by mechanical, osmotic, chemical, and electrical methods than intact bacterial or plant cells¹⁰². Two model mammalian cell cultures, K562 and HEK293, were used to investigate cytotoxicity of **VTT**. K562 is a leukemia cell line that grows in suspension and is commonly used as a blood cancer model¹⁰³, whereas HEK293 is an adherent cell line that is widely used in recombinant protein production¹⁰⁴. To establish the concentration range at which cells can be treated with **VTT** without inducing membrane damage, a cytotoxicity assay was performed with both cell types. When mammalian cells undergo lysis, DNA is released into the mixture and can be detected to quantify the number of lysed cells. The commercially available CellTox™ dye does not penetrate intact cells and forms fluorescent products upon binding to DNA, thereby enabling fast detection of cells with damaged cell membranes.

No cell lysis was observed when K562 cells were treated with **VTT** at concentrations up to 1 mM for one hour at 37 °C (Figure 2.8). Moreover, no membrane damage was observed when measurements were performed three hours after addition of **VTT** to the cells. Increased fluorescence intensity was observed only at 2 mM **VTT** and indicated membrane degradation at **VTT** concentrations higher than 1 mM. Experiments with HEK cells also yielded data consistent with absence of membrane degradation for cells treated with up to 1 mM **VTT** for one hour at 37 °C. Longer incubation times led to membrane damage at **VTT** concentrations higher than 0.5 mM (Figure E.80). Higher fragility of the HEK cell membrane most likely arises because treatment of adherent HEK cells in PBS buffer led to detachment from the surface, followed by anoikis, the detachment-induced cell death¹⁰⁵. Nevertheless, the combined experimental evidence indicated that intracellular labeling without cell membrane disruption can be achieved with **VTT** at concentrations as high as 1 mM.

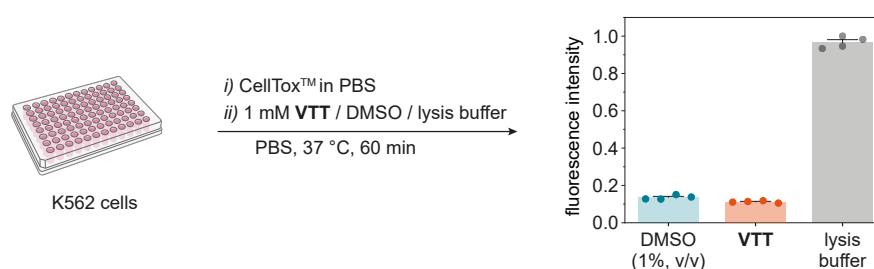


Figure 2.8: Normalized fluorescence intensity values from cytotoxicity (membrane integrity) assay performed with K562 cells after incubation with **VTT**, DMSO (1%, v/v), or lysis buffer (LB). Data are means $\pm$ SE of n=4 independent experiments.

Although the cytotoxicity assay provides valuable data on membrane integrity of cells exposed to **VTT**, it does not allow estimation of the direct influence of intracellular protein labeling on metabolic activity in cells. Given that intracellular alkylation of catalytic Cys residues likely perturbs redox signaling¹⁰⁶, a metabolic activity assay based on quantification of cellular reducing power can provide a readout of a relevant metabolic response to intracellular Cys alkylation with **VTT**. Resazurin is a nonfluorescent, cell-permeable, and nontoxic dye that is reduced to fluorescent resorufin by active reductases in viable cells and has been widely used for cell viability studies^{107,108}. No decrease in the redox activity was observed when K562 cells were treated with up to 63 μ M **VTT** for 25 minutes at 37 °C (Figure 2.9). Even after incubation in the presence of 1 mM **VTT**, cells were still able to produce $52 \pm 2\%$ of the resorufin amount observed in the nontreated samples within the same timeframe.

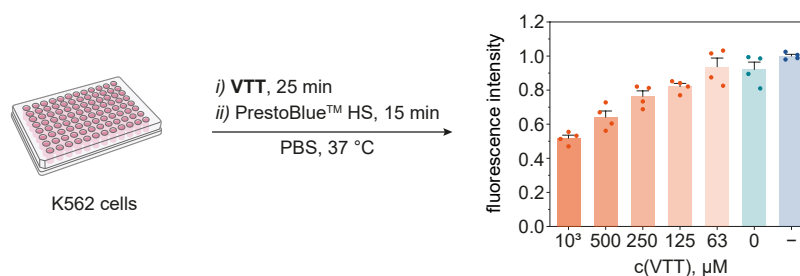


Figure 2.9: Resazurin-based cell viability assay performed with K562 cells after incubation with **VTT** at various concentrations, DMSO (1%, v/v), or PBS (-) for 25 minutes. Data are means $\pm$ SE of n=4 independent experiments. PrestoBlue™ HS, the resazurin reagent used for the assay.

The same assay was also performed with *N*-hex-5-ynyl-2-iodoacetamide (**IAA** alkyne) and *N*-

ethylmaleimide (**NEM**) to compare **VTT** with reagents that are commonly used for Cys-selective protein labeling. **IAA** alkyne has been widely used for reactive Cys profiling in cells⁵⁴, whereas **NEM** has served as a model compound that enables fast Cys alkylation¹⁰⁹. The experiments revealed that incubation with **IAA** alkyne perturbs intracellular redox metabolism to an extent that is not significantly different from that observed for **VTT**. For example, treatment of K562 cells with 0.25 mM reagent led to a moderate decline in measured metabolic activity for both **VTT** ($76 \pm 3\%$) and **IAA** alkyne ($72 \pm 1\%$). In contrast, incubation with 0.25 mM **NEM** led to significantly lower activity ($47.7 \pm 0.3\%$), similar to the value obtained for a higher (1 mM) **VTT** concentration.

After the initial measurement, cells were incubated for another four hours at 37 °C to determine whether cells treated with alkylating reagents were still able to convert resazurin into resorufin. Notably, fluorescence intensity in samples treated with **NEM** increased 25% more slowly in comparison to both **VTT** and **IAA** alkyne (Figure 2.10). The combined data are consistent with significantly higher cellular toxicity of **NEM** compared to both **VTT** and **IAA** alkyne.

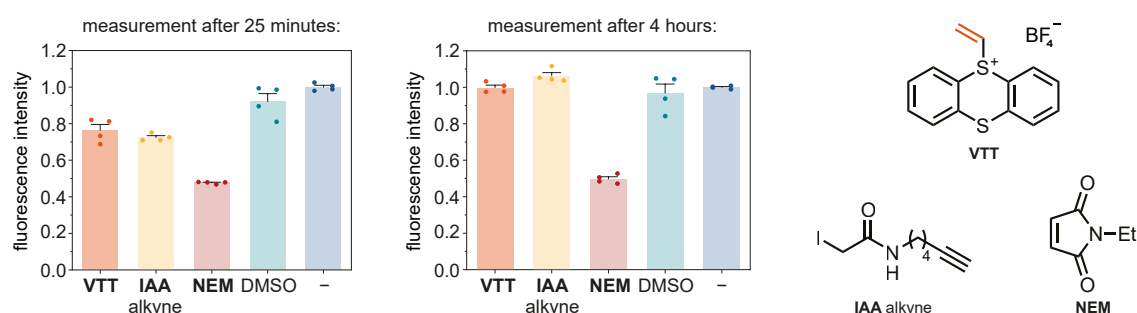


Figure 2.10: Resazurin-based cell viability assay performed with K562 cells after incubation with 250 μ M **VTT**, **NEM**, **IAA** alkyne, DMSO (1%, v/v), or PBS (-) for 25 minutes (left) or four hours (right). Data are means $\pm$ SE of $n=4$ independent experiments.

Intracellular labeling efficiency

VTT can be used for Cys-selective labeling in lysates with high Cys coverage⁴⁴. To evaluate how the cellular membrane influences labeling efficiency *in cellulo*, K562 cells and lysates were treated with 0.25 mM **VTT** at 37 °C for 30 minutes and subsequently incubated with dithiothreitol (DTT) to ensure that proteins do not get alkylated with unreacted **VTT** upon cell lysis. The cells were lysed, and the trypsin-digested peptide mixtures were analyzed by LC-MS/MS. Database searches for modified Cys-containing peptides (Cys-S-C₂H₄OH) revealed that the numbers of modified peptides detected under *in vitro* and *in cellulo* conditions were of the same order of magnitude, indicating that efficient intracellular labeling with **VTT** can also be achieved (Figure 2.11).

In addition to the cell membrane, which poses a challenge for efficient protein modification, compartmentalization of eukaryotic cells provides an additional obstacle to obtaining a comprehensive proteome-wide readout using chemoproteomic methods¹¹⁰. Isolation of organelles and utilization of specifically designed reagents are often required to achieve efficient protein labeling in organelles such as mitochondria^{111,112}. To evaluate whether **VTT** can label proteins in different cellular compartments, a GO (gene ontology) annotation of proteins identified as modified in at least two biological replicates was performed for datasets obtained in both *in cellulo* and *in vitro* setups (Figure 2.12). The analysis revealed that **VTT** modified proteins localized in

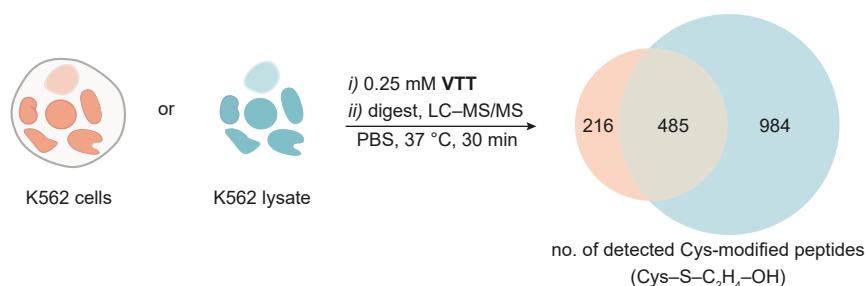


Figure 2.11: Modification of Cys-containing proteins in K562 cells and lysate. The left and right circles represent the numbers of unique modified peptides identified in at least one biological replicate from *in cellulo* or *in vitro* experiments, respectively.

all major cellular compartments. Moreover, comparison with annotation of proteins modified in lysate samples demonstrated a similar distribution of protein localization and provided additional evidence for efficient intracellular modification achievable with **VTT**.

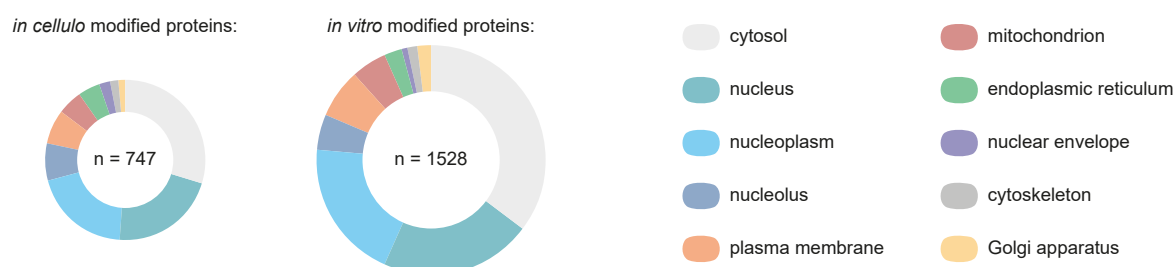


Figure 2.12: Gene ontology annotation (top 10) of proteins that were labeled in K562 cells and lysate.

To evaluate the kinetic profile of intracellular protein labeling, efficiency of protein labeling with **VTT** was benchmarked in a competition experiment against **NEM**, a Cys-alkylating reagent that is more reactive than **VTT** under *in vitro* conditions. This comparison was used to assess how cellular membrane permeability affects the relative labeling efficiencies of the two reagents. To obtain a quantitative readout, the ²H₃-**VTT** isotopologue of **VTT** was used to enable MS-based quantification of products arising from protein modification with either light **VTT** or heavy **VTT**⁴⁴. In a reference experiment performed to obtain the maximum labeling efficiency achievable by **VTT** in the absence of **NEM**, K562 cells were treated with the ²H₃-**VTT** isotopologue. In the competition experiment, cells were treated with an equimolar mixture of **VTT** and **NEM**. In both cases, cells were pre-incubated with NaN₃ to enable downstream enrichment of peptides modified with **VTT** or ²H₃-**VTT**. Incubation with reagents was performed for five minutes and was followed by addition of DTT to enable comparison of labeling efficiency over a short time frame. The cells were lysed, the protein concentration was normalized, and lysate mixtures from the control experiment with ²H₃-**VTT** were mixed 1:1 with lysate mixtures obtained from the competition experiment. A CuAAC reaction with a biotin alkyne was performed to incorporate an affinity tag into Cys residues modified with **VTT** isotopologues and NaN₃. Proteins were digested, and peptides carrying the incorporated biotin moiety were enriched by capture on NeutrAvidin beads, a solid support coated with a deglycosylated avidin variant that forms a high-affinity interaction with biotin.

LC-MS/MS analysis of the biotin-enriched samples revealed a heavy-to-light ratio of 2.0 ± 0.1 for products originating from the reference experiment with heavy-labeled ²H₃-**VTT** relative to

products obtained in the competition experiment with **NEM** (Figure 2.13). Because presence of equimolar **NEM** led to a twofold decrease in labeling efficiency with **VTT**, the obtained ratio indicates similar rate constants for **VTT** and **NEM** in the rate-determining step of the labeling process. Since **NEM** is a more reactive electrophile than **VTT** when reaction with Cys is performed *in vitro*, the obtained product ratio after intracellular labeling suggests that cell permeability, and not the reaction between Cys and alkylating reagents, was the rate-limiting step of the overall process. Moreover, the experiment demonstrates that formation of reactive episulfonium intermediates on protein surfaces is achieved within minutes, further supporting efficient intracellular labeling with **VTT**.

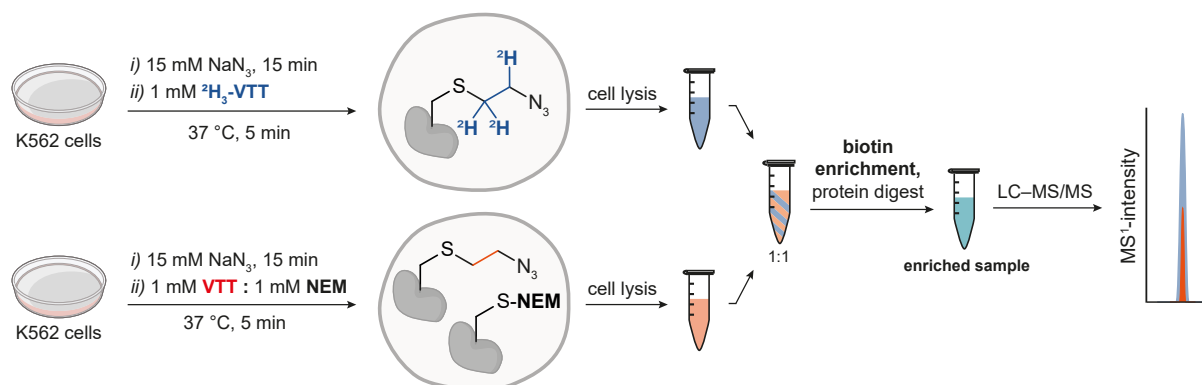


Figure 2.13: Affinity enrichment of labeled polypeptides for LC–MS/MS analysis after competition experiments between **VTT** and *N*-ethylmaleimide (**NEM**). Only one of the two possible constitutional isomers for the $^2\text{H}_3$ -**VTT**-labeled product is depicted. The obtained ratio of 2.0 ± 0.1 is mean $\pm$ SE of $n=3$ independent experiments.

It is noteworthy that intracellular protein modification with **VTT** proceeds on the same timescale as with **NEM**, while having a significantly less pronounced negative effect on intracellular metabolism, potentially due to the differences in the off-target reactivity and modification specificity⁴⁴. Having established that **VTT** enables fast and efficient intracellular labeling in K562 cells, labeling experiments were performed with different cell types to evaluate whether similar labeling efficiency can be achieved in all cases, including cells that contain more rigid cell walls (Figure 2.14).

Mammalian HEK cells, bacterial *E. coli* and *B. subtilis* cells, plant cells of *A. thaliana*, *B. vulgaris*, and *N. tabacum*, as well as yeast cells were treated with **VTT**, lysed, and trypsin-digested mixtures were analyzed by LC–MS/MS. The number of peptides carrying the Cys–S–C₂H₄OH modification was normalized to the total number of the identified Cys-containing peptides to enable comparison between organisms that differ in the proteome size. Labeling efficiencies in the range of 19–28%, with an average of 24%, were achieved for all cell types used in the study, and demonstrate wide applicability of **VTT** for efficient intracellular episulfonium generation across diverse cell types.

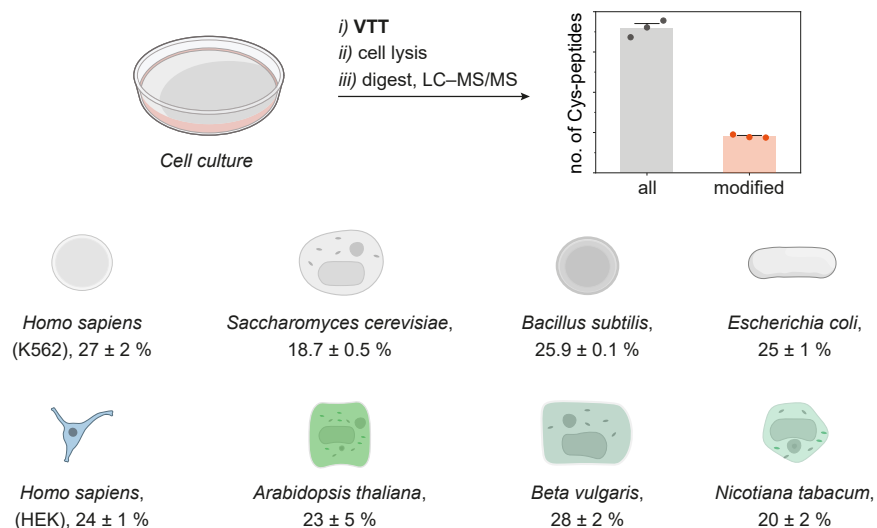


Figure 2.14: Evaluation of the labeling efficiency after *in cellulo* protein modification with VTT in various cell types. The values are means $\pm$ SE of n = 3 independent experiments.

***In cellulo* protein crosslinking for protein structure probing**

The combined experimental data presented so far demonstrate that **VTT** enables fast Cys umpolung into episulfonium intermediates on protein surfaces in different cells. Given that episulfonium formation in peptides in close proximity to nucleophilic amino acids such as Cys, Lys, and Glu was used for synthesis of crosslinked peptides with short linkages⁴⁴, generation of episulfonium *in cellulo* should provide a method for intracellular single-step protein crosslinking. In contrast to crosslinking with commonly used bifunctional reagents, which proceeds in two steps and can lead to identification of crosslinked products formed after protein conformational changes, the generated episulfonium intermediate undergoes rapid hydrolysis unless a crosslinking event with a neighbouring amino acid occurs, and should allow reliable information on the local protein microenvironment to be extracted from the obtained crosslink identities.

The following section describes utilization of **VTT** for intraprotein crosslinking studies. The aim of the work was to characterize versatility of episulfonium-mediated crosslinking chemistry *in cellulo*, determine the range of distance restraints for protein structure modeling provided by **VTT**-mediated crosslinking, and establish applicability of **VTT** for structure probing of proteins in various cell types.

Episulfonium crosslinking reactivity profile

Although episulfonium reactivity for protein crosslinking has not been systematically studied prior to this work, precedents for electrophilic linchpin formation enabling versatile crosslinking exist and suggested that episulfonium-mediated crosslinking may not be limited to highly nucleophilic residues. For example, Zhang et al. reported epoxide-containing unnatural amino acids that can be incorporated into protein structures for versatile protein crosslinking¹¹³. As expected for the high reactivity of the employed epoxide linchpin, crosslink formation with all nucleophilic amino acids was observed. When incubated in DMEM or LB, the cell culture media used for mammalian and bacterial cells, respectively, only partial (up to 23%) decomposition of the epoxide probe was observed after 24 hours, indicating suitability of the probe for efficient introduction of an electrophilic epoxide linchpin into proteins. Given the high reactivity of episulfonium ions, which undergo rapid hydrolysis in aqueous solutions upon formation⁵⁷, **VTT**-mediated intraprotein crosslinking between Cys and nucleophilic amino acids should be feasible and provide an alternative to approaches that rely on generation of reactive carbenes on protein surfaces for fast and versatile crosslinking without the need for unnatural amino acid incorporation.

To characterize the reactivity profile of episulfonium intermediates formed *in cellulo*, K562 cells treated with 0.25 mM **VTT** were lysed, and proteins were trypsin-digested. Because crosslinked peptides are larger in size and possess higher charge states than linear peptides, partial enrichment of crosslinked peptides during LC–MS/MS data acquisition can be achieved by excluding singly and doubly charged ion precursors from fragmentation⁸³. The digested K562 samples were analyzed by LC–MS/MS using settings commonly applied for identification of crosslinked peptides. Database searches were performed to identify crosslinked peptides in which an ethylene linkage was incorporated between Cys and one of the nucleophilic amino acids (Arg, Asp, Cys, Glu, His, Lys, Ser, Thr, and Tyr). Indeed, crosslinks involving all corresponding residue pairs were identified, consistent with the expected high electrophilicity of episulfonium ions (Figure 2.15).

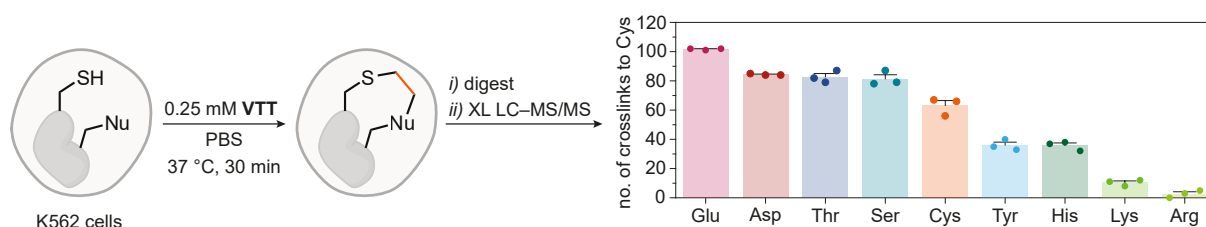


Figure 2.15: The number of the detected crosslinks from Cys to each of the searched amino acids detected after incubation of K562 cells with **VTT**. Data are means $\pm$ SE of $n = 3$ independent experiments.

To gain a deeper understanding of the episulfonium reactivity profile, the obtained crosslink frequencies were normalized by relative frequencies of each crosslinked amino acid in the proteome. The analysis revealed that crosslinking with Cys occurs more frequently than expected based on relatively low Cys abundance in the proteome (Figure 2.16). This result is consistent with the high nucleophilicity of Cys residues¹¹⁴. Crosslinked products with Lys and Arg were identified less frequently than expected, consistent with lower reactivity of these residues in the protonated state at physiological pH¹¹⁵. However, since the lysate mixtures were digested with trypsin, a protease that cleaves peptide bonds on the C-terminal side of Lys and Arg residues, lower identification frequency for these residues due to the utilized digestion protocol cannot be excluded¹¹⁶. The results demonstrate that episulfonium intermediates formed on protein surfaces *in cellulo* possess high reactivity and readily react with nucleophilic amino acids, thereby yielding versatile crosslinks in a single step.

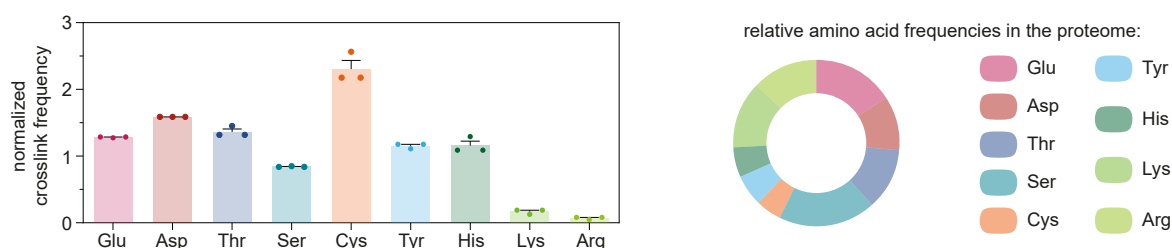


Figure 2.16: Normalized frequency of the identified crosslinks from Cys to each of the searched amino acids. Data are means $\pm$ SE of $n=3$ independent experiments.

Crosslinking with **VTT** leads to incorporation of ethylene linkages between crosslinked residues. Because the reactive episulfonium intermediate undergoes fast hydrolysis unless a crosslinking event takes place, crosslink formation is expected to occur for amino acids that are located in close proximity to the generated episulfonium. Therefore, identification of **VTT**-crosslinked peptides should provide experimental evidence on the local environment of the reacted Cys residue. Identity of the crosslinked residues can provide important data for protein structure modeling, given that the expected range of distances between crosslinked residues is known for the utilized crosslinking reagent. To determine the distance restraints that **VTT**-mediated crosslinking provides, crosslinks identified in two or more biological replicates in K562 samples were mapped to reported high-resolution protein X-ray structures or, if no structure was available, to high-confidence AlphaFold¹¹⁷ predictions. To ensure unambiguous assignment of intraprotein crosslinked distances, proteins known to form dimers were excluded from the analysis. Distances between the α -carbon atoms ($C\alpha-C\alpha$) of crosslinked residues were calculated and revealed a range of 4–32 Å with a median distance of 10–14 Å for all amino acids (Figure 2.17), in line with high reactivity of the generated episulfonium intermediate that does not

discriminate strongly between amino acids of different nucleophilicity.

X-ray crystal structures of disordered coil and loop protein regions may correspond to a conformation stabilized by lattice contacts and thus not necessarily to the lowest free-energy conformation in solution¹¹⁸. Given that predictive models such as AlphaFold are trained on reported crystal structures, these models perform poorly in predicting conformations of disordered regions¹¹⁹. Therefore, when the crosslinked residues are mapped to experimentally obtained or predicted disordered protein structural motifs, the obtained distances may provide unreliable estimates of distance restraints between the crosslinked residues in solution¹²⁰. To obtain a more accurate estimation of **VTT**-generated distance restraints, mapped distances between residues involved in coils and loops were excluded from the analysis, leaving only crosslinks between well-defined structural elements. The resulting range of 4–23 Å provides a more reliable estimation of **VTT**-generated distances and revealed that crosslinking occurred primarily between residues located 8–16 Å apart. Exclusion of crosslinks in loop and coil regions decreased the median calculated C α –C α distance from 13.5 Å (calculated for all crosslinks) to 11.1 Å. Both values are similar to the median C α –C α distance reported for photo-leucine crosslinks⁸⁸ and indicate that **VTT**-mediated crosslinking produces short-distance restraints comparable to state-of-the-art methods that rely on carbene generation via unnatural amino acid incorporation.

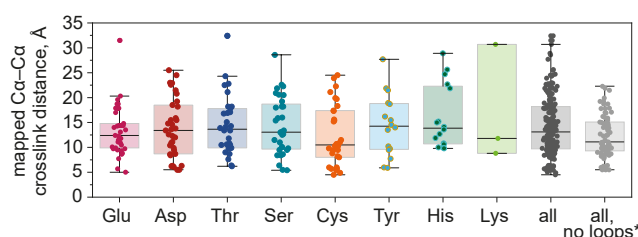


Figure 2.17: Distribution of C α –C α distances for the mapped crosslinks that were identified in at least two biological replicates in K562 cells. Each box spans from the first quartile (the 25th percentile) to the third quartile (the 75th percentile). Horizontal line indicates the median, whiskers extend to $1.5 \times$ interquartile range from box boundaries. *data were obtained after excluding mapped crosslinks in non-ordered protein structural motifs.

***In cellulo* protein crosslinking with VTT**

VTT-mediated episulfonium generation *in cellulo* enables fast single-step crosslinking for protein structure probing. **VTT** achieves efficient intracellular labeling in various cell types, providing a general approach for formation of crosslinked proteins in the native cellular environment. Having established the episulfonium reactivity profile in mammalian K562 cells, digested samples obtained after treatment of different cell types with **VTT** were analyzed using crosslinking LC–MS/MS. Crosslinked peptides identified in at least two biological replicates were used for protein structure prediction (Figure 2.18). Because crosslinks were formed in regions with well-defined folds for which high-confidence predictions with AlphaFold are available, structure prediction using experimentally obtained distance restraints did not lead to an increase in prediction confidence scores (pLDDT) for the depicted proteins. Nevertheless, the obtained results demonstrate that **VTT** can be used for protein structure probing in fungal, mammalian, plant, and bacterial cells.

The protein crosslinking data discussed so far were obtained from measurements of full lysate samples using standard liquid chromatography separation protocols. Additional enrichment of

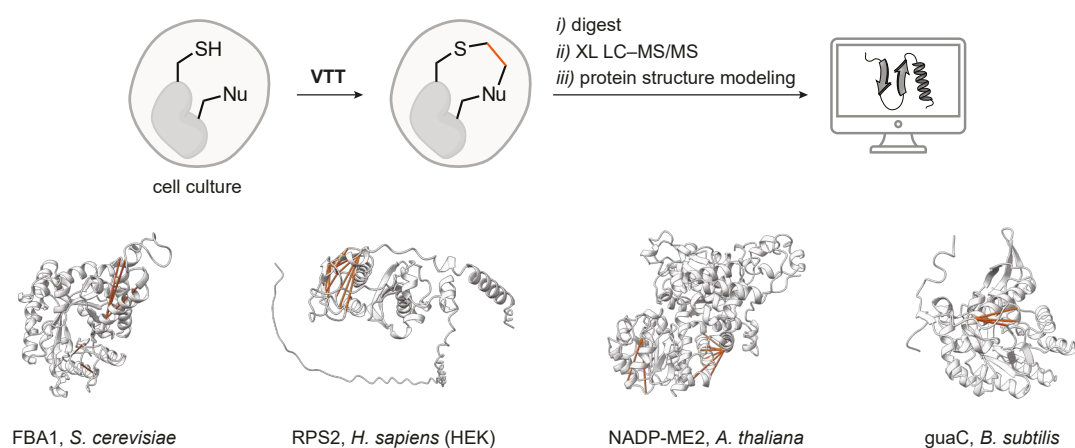


Figure 2.18: Representative proteins and mapped detected crosslinks from different organisms. The protein structures were predicted using AlphaLink2⁸⁸ and visualized with ChimeraX¹²¹.

low-abundance crosslinked peptides could be achieved via fractionation of digested samples¹²², but this would lead to an increase in data acquisition and processing time for analysis of a single digested sample. Moreover, if a protein of interest possesses relatively low abundance in the proteome, corresponding crosslinked peptides might remain undetected even after extensive sample enrichment by chromatographic separation, since the product mixture will predominantly contain peptides arising from highly abundant proteins¹²³. To address this limitation, crosslinking of an isolated POI can be performed *in vitro*, but *in cellulo* crosslinking studies are more desirable to obtain information on protein conformation in its native cellular environment¹²⁴. An alternative solution for enrichment of low-abundance peptides consists of affinity purification of a POI to separate the low-abundance protein from other proteins in the lysate prior to sample digestion¹²⁵. In this case, protein crosslinking can take place in the intracellular environment, while the crosslinked peptides can still be identified even for naturally low-abundance proteins.

GrxC5 is a protein of moderate abundance in the *Arabidopsis thaliana* proteome (14.3 ppm; ranked among the top 25% of proteins by abundance in PaxDB¹²⁶). The Cys-containing oxidoreductase is expected to react with **VTT** and form crosslink pairs. No GrxC5-characteristic peptides were identified in samples obtained after treatment of *A. thaliana* cells with **VTT**. To demonstrate feasibility of **VTT**-mediated crosslinking in cases where no information can be extracted from full lysate samples, the model protein GrxC5 with an incorporated His tag was overexpressed and crosslinked with **VTT** in *E. coli* cells. The cells were washed, and affinity purification of GrxC5 on a Ni column, followed by isolation of the protein monomer by SDS-PAGE, enabled enrichment of putative crosslinked protein species. The obtained product was digested with trypsin, and peptide mixtures were analyzed by crosslinking LC-MS/MS. Four crosslinks were identified in two out of three biological replicates (Figure 2.19). These results demonstrate that **VTT**-mediated intracellular protein structure probing can be performed even for naturally low- and medium-abundance proteins via incorporation of an affinity tag into the protein of interest and subsequent protein separation using standard biological methods.

Comparison of VTT-mediated crosslinking to other methods

VTT is a commercially available reagent that is able to incorporate short crosslinks on protein surfaces via efficient intracellular episulfonium generation. Fast cell permeability of **VTT** can be rationalized by its relatively small calculated topological polar surface area (TPSA, 50.6

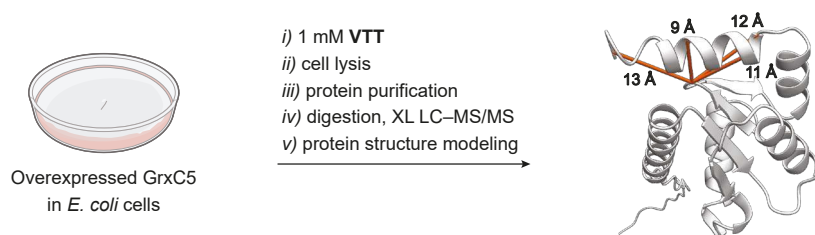


Figure 2.19: *In cellulo* crosslinking of overexpressed GrxC5 and detected crosslinks. The protein structure was predicted using AlphaLink2⁸⁸ and visualized with ChimeraX¹²¹.

Å²) and high lipophilicity index ($\log P = 4.6$), since low TPSA and high $\log P$ correlate with fast passive membrane permeability¹²⁷. Comparison with other Cys-selective crosslinking reagents demonstrates that **VTT** is more compact and lipophilic than most other commercially available crosslinking reagents (Figure 2.20) and that **VTT** belongs to the class of reagents that incorporate short linkages.

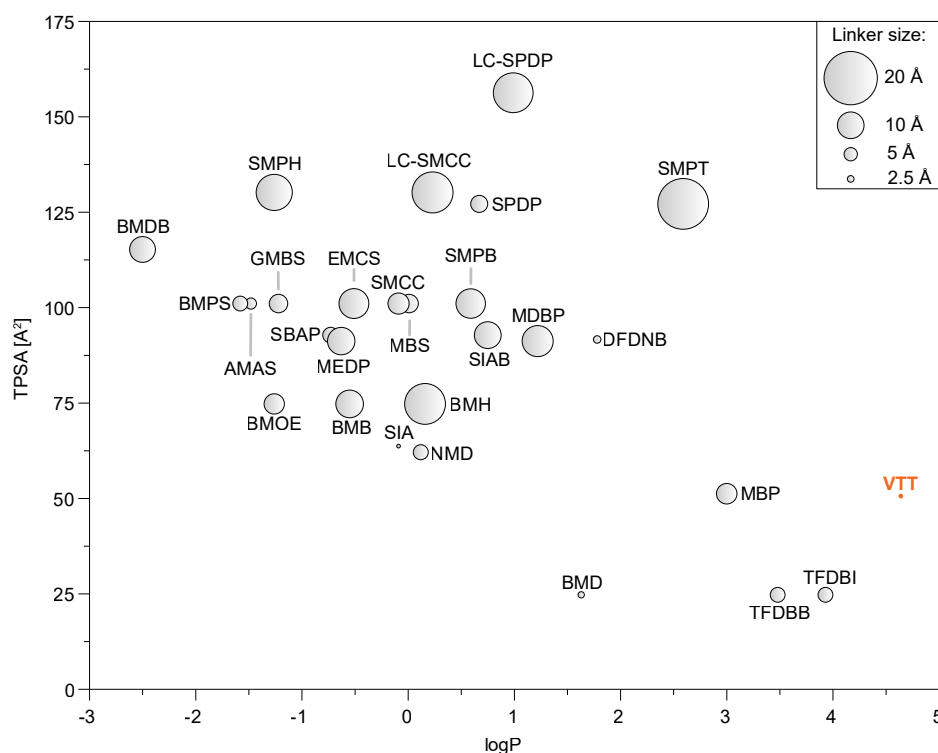


Figure 2.20: Comparison of **VTT** with other crosslinking reagents based on the calculated topological polar surface area (TPSA), the octanol-water partition coefficient (P), and linker size.

As seen in Figure E.115, the majority of commercially available Cys-reactive crosslinking reagents contain maleimides or disulfides that form labile products upon conjugation with Cys^{49,128}. Cys crosslinking with bromo- or iodoacetamides enables incorporation of stable linkers but occurs orders of magnitude slower than reactions with maleimides^{106,129}.

Comparison with other commonly utilized chemical crosslinking methods demonstrates that **VTT** enables formation of shorter linkers in a single step and, unlike diazirine-based approaches, does not rely on exogenous activation with UV light for generation of reactive intermediates (Figure 2.21). Isotopologues of lysine-selective bifunctional crosslinking reagents are commercially available and have been widely used in quantitative crosslinking studies¹³⁰. However, the

relatively high stability of NHS esters formed after the initial reaction with lysine may lead to trapping of non-native protein conformations and provide unreliable distance restraints¹³¹. **VTT** isotopologues are readily accessible from labeled ethylene gas and can be used for reliable quantitative crosslinking studies.

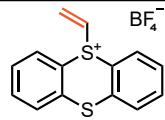
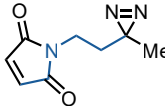
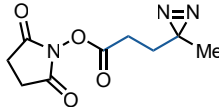
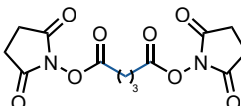
crosslinker	structure	stable linker	isotopologues for quant. MS	single-step crosslinking	linker size	selectivity	crosslinked amino acids
VTT		+	+	+	1.5 Å	Cys	Glu / Asp / Thr / Ser / Cys / Tyr / His / Lys / Arg
NMD		—	—	—	6.0 Å	Cys	all amino acids
SDA		+	—	—	3.9 Å	Lys (Ser, Thr, Tyr)	all amino acids
DSG		+	+	—	7.7 Å	Lys (Ser, Thr, Tyr)	Lys (Ser, Thr, Tyr)

Figure 2.21: Comparison of **VTT**-mediated crosslinking to other approaches. **NMD**, *N*-maleimido-diazirine¹³²; **SDA**, succinimidyl azipentanoate¹³³; **DSG**, disuccinimidyl glutarate¹³⁴.

Conclusions: *In cellulo* protein crosslinking

VTT enables efficient labeling of Cys-containing proteins in various cell types. Competition experiments demonstrated that **VTT** achieves fast intracellular labeling with rates comparable to those of highly reactive maleimide-based reagents. At the same time, Cys alkylation with **VTT** leads to only a moderate decrease in cellular metabolic activity, whereas treatment of mammalian cells at concentrations as high as 1 mM does not lead to membrane degradation. The combined data demonstrate that **VTT** can be used as an alternative to commonly used Cys-alkylating reagents when fast intracellular alkylation is desired. Moreover, the results indicate that development of positively charged probes for intracellular protein labeling may represent a promising strategy to enhance cell permeability of hydrophobic organic molecules.

In contrast to commonly used Cys-alkylating reagents, **VTT**-mediated protein labeling leads to generation of reactive episulfonium intermediates that are able to trap nucleophilic amino acids to form stable crosslinked products in a single step. High episulfonium reactivity and incorporation of short ethylene linkers in the products enable generation of short-distance restraints for protein structure modeling, thereby providing a method for studying protein conformations in native cellular environments. **VTT** is a commercially available reagent, and its isotopologues can be accessed from labeled ethylene gas in one step. Applicability of the described crosslinking method can be further expanded by utilizing **VTT** isotopologues in quantitative crosslinking studies and should thereby enable investigation of protein conformation changes in cells. A protein of interest can be equipped with Cys and crosslinked with **VTT** in cells to study protein conformation or interactions with other proteins, providing a more general approach to intracellular protein crosslinking compared with frequently used approaches based on unnatural amino acid incorporation.

Chapter 3: *In cellulo* identification of protein–protein interactions

Introduction: Identification of protein–protein interactions

Protein–protein interactions and their characterization

Most cellular functions are regulated via interactions between proteins that form transient and permanent complexes¹³⁵, and estimates suggest that more than 80% of proteins in humans act in complexes to form approximately 650,000 interactions^{136,137}. Apart from understanding basic intracellular mechanisms, identification of novel protein–protein interactions (PPIs) is important for development of therapeutics, since abnormal PPIs cause diseases¹³⁸. Therefore, in addition to detection of previously unknown PPIs, characterization of PPI interfaces as well as protein conformational changes upon PPI formation is essential for development of suitable inhibitors¹³⁹. Although experimental structures of approximately 10,000 human PPIs are available and a comparable number of modeled PPIs exists, more than 85% of known human PPIs lack structural data¹⁴⁰.

Various methods are available for identification of PPIs *in vitro* and *in cellulo* on both large and small scales^{141,142}. Crosslinking mass spectrometry is a sensitive method for PPI detection that provides high-resolution structural information on interaction sites and therefore can be used to obtain data for modeling of PPI surfaces^{143,144}. Similar to crosslinking methods for protein structure probing, identification of PPIs relies on incorporation of covalent crosslinks between residues of interacting proteins, followed by LC–MS/MS analysis of the digested samples (Figure 3.1). Identification of crosslinked residues reveals information on protein sites that are located in close proximity within a PPI and enables detection of both transient and stable interactions *in vitro* and *in cellulo*¹⁴⁵. The obtained distance restraints can be integrated into PPI structure modeling, which has been shown to provide more accurate predictions than standard *in silico* approaches that infer inter-protein contacts from sequence coevolution data and use these contacts as distance restraints or scoring terms to guide and rank docking models⁸⁹.

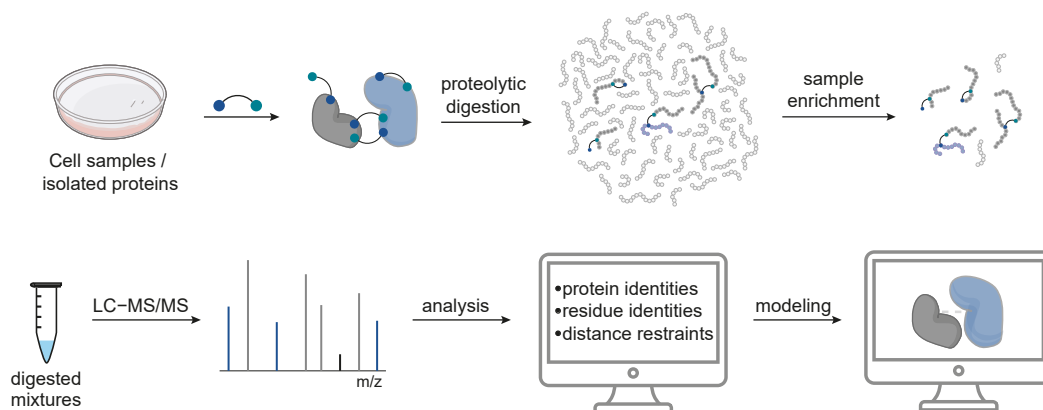


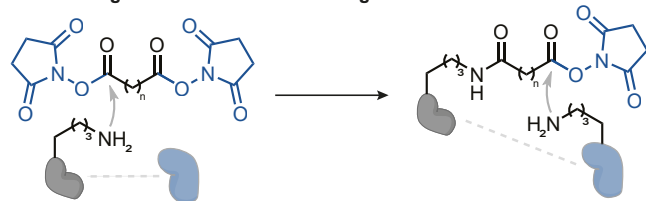
Figure 3.1: Crosslinking MS for PPI identification and PPI interface modeling.

Common crosslinking approaches for PPI identification

The crosslinking approaches utilized for identification of PPIs are analogous to the previously described crosslinking approaches for intra-protein crosslinking. Identification of inter-protein crosslinks is generally more challenging due to relatively low abundance of heteromeric crosslinks in complex peptide mixtures obtained after sample digestion. Purification techniques such as chromatographic enrichment of crosslinked peptides are therefore often applied to increase the sensitivity of the method¹⁴⁶. Alternatively, enrichment of crosslinked peptides prior to LC–MS/MS analysis can be achieved using affinity tags such as biotin, which gets incorporated into the modified peptides⁹⁴. The affinity tag can be directly incorporated into the reagent structure or introduced in a step following the crosslinking via azide–alkyne cycloaddition reaction with an incorporated azide or alkyne that serves as an enrichment handle. The latter approach requires an additional step but is advantageous when maintaining the crosslinker size and cell permeability is critical, as the bulky affinity tag is introduced only after lysis of the cells treated with the crosslinker reagent¹⁴⁷.

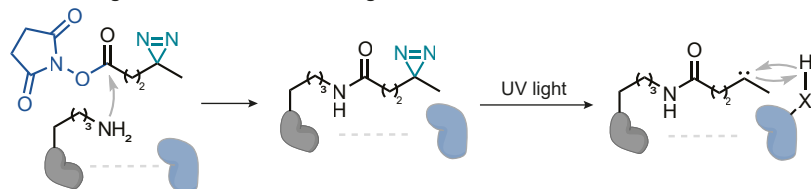
As in the case of protein structure probing, utilization of homobifunctional crosslinking reagents for PPI identification suffers from identification of false-positive PPIs due to low reactivity of intermediates formed on protein surfaces⁹⁴ (Figure 3.2).

Crosslinking with homobifunctional reagents:



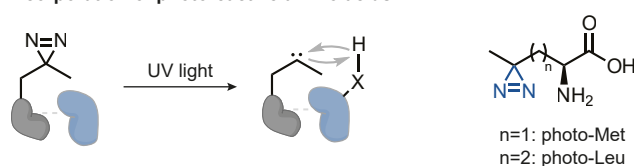
long linkers ($n > 2$)
long intermediate half-life
affinity tag incorporation

Crosslinking with heterobifunctional reagents:

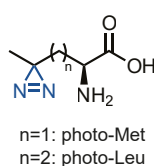


two-step process
short intermediate half-life
affinity tag incorporation

Incorporation of photoreactive amino acids:



common ncAAs:



short linker
short intermediate half-life
no affinity tag incorporation

Figure 3.2: Crosslinking approaches for PPI identification and main advantages (green) and disadvantages (red) of each approach.

Crosslinking based on reactive carbene generation proceeds in two steps via initial protein modification with heterobifunctional reagents or requires incorporation of unnatural amino acids. Although more reliable distance restraints are obtained, enrichment of the crosslinked peptides in both cases relies on chromatographic separation that provides only partial removal of linear peptides and typically requires extensive fractionation, thereby increasing the sample preparation, data acquisition, and data processing time^{122,146}. Metabolic proteome-wide incorporation of unnatural amino acids bearing enrichment tags is currently not achievable due to limited trans-

lational compatibility of bulky unnatural amino acids, whereas incorporation of an enrichment tag into heterobifunctional reagents leads to extension of the linker size¹⁴⁸. Therefore, novel approaches for identification of PPIs based on fast crosslinking between native residues and incorporation of short linkages without the need for activation with UV light can serve as valuable alternatives to the existing methods.

Project goals

Having established that fast episulfonium reactivity enables rapid crosslinking for protein structure probing in cells, the results indicated that episulfonium-mediated crosslinking can be used for identification of PPIs *in cellulo*. The goals of the project were establishment of a protocol for reliable PPI identification via intracellular episulfonium generation and comparison of the method with commonly used crosslinking approaches for PPI detection.

***In cellulo* PPI identification: Results and discussion**

Contributions to the project: *The author designed and synthesized the crosslinking reagent and explored its reactivity in aqueous buffer, with cysteine, and with proteins. Dr. Lara Vogelsang synthesized the ubiquitin K63C mutant. Marie Sophie Sterling acquired and analyzed protein LC–MS data. Heike Hinrichs performed LC–MS analysis of the cysteine conjugate. Pia Münstermann performed HPLC purification of the cysteine conjugate. Julia Beatrice Jacobs acquired and analyzed the NMR data. The author, together with Dr. Philipp Hartmann, Dr. Dario Marchionni, and Dr. Christian Preisinger performed experiments with mammalian cells. The author performed experiments with E. coli cells, cytotoxicity assays, and cell viability experiments. The author and Dr. Philipp Hartmann optimized the enrichment protocol. The author and Sven Müller performed enrichment of the samples used for analysis. Dr. Christian Preisinger and Dr. Alessandro Vetere acquired LC–MS/MS data. The author performed LC–MS/MS data processing, open and closed database searches, and crosslinking data analysis. The author performed docking studies. The author and Dr. Philipp Hartmann analyzed the obtained docking structures.*

VTT-mediated inter-protein crosslinking

Intracellular protein labeling with **VTT** enables formation of reactive episulfonium intermediates on protein surfaces that undergo fast crosslinking with neighboring nucleophilic amino acids. Because episulfonium intermediates possess low stability in aqueous solution, **VTT**-mediated Cys umpolung should serve as a reliable method for identification of PPIs in cells based on detection of **VTT**-crosslinked products. Formation of inter-protein crosslinks with **VTT** should occur only if two proteins are located in close proximity, since the generated episulfonium intermediate will undergo fast hydrolysis or intra-protein crosslinking unless reactive residues of an interacting protein are located close to the generated episulfonium. Moreover, because episulfonium readily reacts with various nucleophilic amino acids, formation of several crosslinked products that support identification of a single PPI is expected for **VTT**-mediated inter-protein crosslinking, thereby providing additional confidence in the PPI identification.

Initial experiments suggested the feasibility of **VTT**-mediated crosslinking for PPI identification. HEK cells were treated with **VTT**, washed three times with PBS to ensure that the identified crosslinked products were formed *in cellulo*, lysed, and the protein mixtures were digested with trypsin. XL LC–MS/MS analysis of the mixtures and database searches for crosslinked peptides revealed 23 inter-protein crosslinks, in which the two linked peptides were assigned to different proteins, and were mapped to seven PPIs (Figure 3.3). These results demonstrated that intracellular PPI identification with **VTT** is feasible but indicated low detection sensitivity as a limitation of the method: only three of the seven identified PPIs were supported by three or more crosslinked products and can therefore be considered as high-confidence PPIs. In addition, only one of the three high-confidence PPIs has been previously reported, which limits benchmarking and meaningful comparison of the method with previously reported approaches for PPI detection.

Although sensitivity of the method could be enhanced via sample fractionation prior to measurements to achieve partial enrichment of crosslinked species, design of a reagent that enables enrichment of the crosslinked products without the need for extensive chromatographic purification was foreseen as a more promising strategy for the development of a practical method.

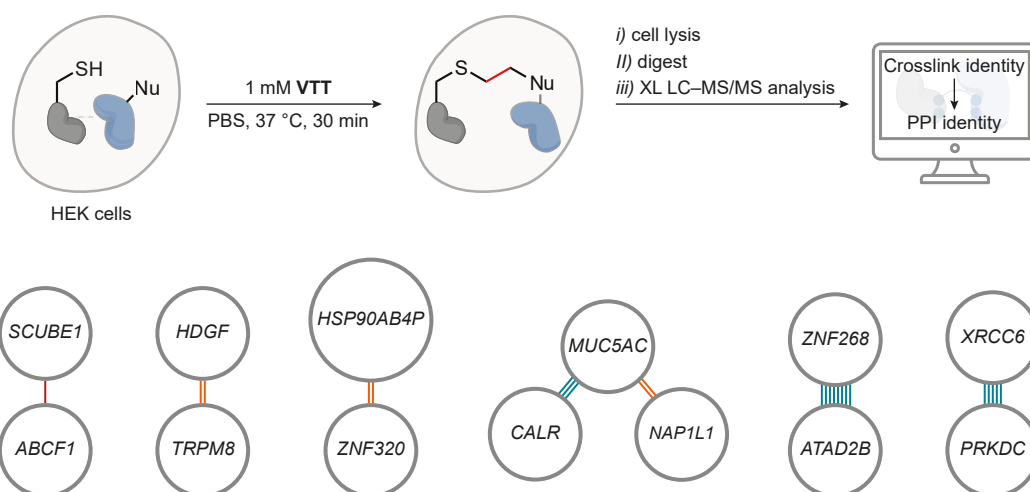


Figure 3.3: Identification of PPIs in HEK cells with VTT. The number of lines corresponds to the number of the detected inter-protein crosslinks that supported the identification of the interaction. High-confidence PPIs were identified via three or more crosslinks (green lines), low- and medium-confidence PPIs were identified with one or two crosslinks (red and orange lines, respectively). SCUBE1, Signal peptide, CUB and EGF-like domain-containing protein 1; ABCF1, ATP-binding cassette sub-family F member 1; HDGF, Hepatoma-derived growth factor; TRPM8, Transient receptor potential cation channel subfamily M member 8; HSP90AB4P, Putative heat shock protein HSP 90-beta 4; ZNF320, Zinc finger protein 320; MUC5AC, Mucin-5AC; CALR, Calreticulin; NAP1L1, Nucleosome assembly protein 1-like 1; ZNF268, Zinc finger protein 268; ATAD2B, ATPase family AAA domain-containing protein 2B; XRCC6, X-ray repair cross-complementing protein 6; PRKDC, DNA-dependent protein kinase catalytic subunit.

Design, synthesis, and properties of the crosslinking reagent

The goal of the project was to design a reagent that, like **VTT**, enables intracellular episulfonium formation for inter-protein crosslinking while allowing enrichment of the crosslinked peptides via an incorporated bioorthogonal tag that does not negatively influence the reagent cell permeability. The hypothesis was that incorporation of a terminal alkyne group into the **VTT** structure using a short linker would preserve high Cys reactivity and cell permeability of the compound while equipping modified products with a bioorthogonal handle. To compensate for introduction of a nonpolar alkyl chain and the alkyne group, a short linker containing a polar ether group was designed (Figure 3.4).

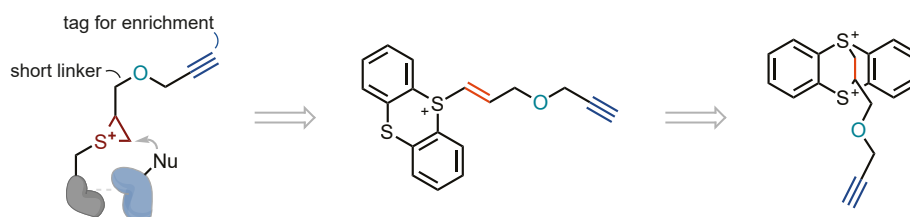


Figure 3.4: Design of the crosslinking reagent with an alkyne tag for PPI identification via episulfonium formation.

The substituted episulfonium ion can be obtained via reaction between Cys and an alkenylthianthrenium salt. The alkenylthianthrenium salt can be formed by deprotonation of a dicationic thianthrenium reagent obtained via cycloaddition between a substituted alkene and an activated thianthrene-*S*-oxide. Indeed, thianthrenation of commercially available 3-allyloxy-1-propyne yielded a bench-stable, water-soluble ($c = 68$ mM) thianthrenium dication **TTD** in 82% yield on a gram scale (Figure 3.5).

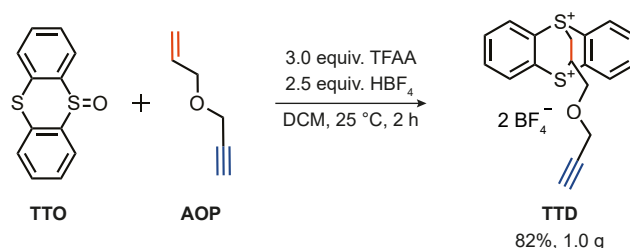


Figure 3.5: Single-step synthesis of alkyne-containing thianthrenium reagent **TTD**. TTO, thianthrene-*S*-oxide; AOP, 3-allyloxy-1-propyne; TFAA, trifluoroacetic anhydride; DCM, dichloromethane.

TTD undergoes fast ($t_{1/2} = 3$ s) deprotonation in sodium phosphate buffer at pH 7.4 to yield a mixture of isomeric alkenylthianthrenium salts. Reaction of **TTD** with 2.5 equiv. of cysteine leads to formation of a single constitutional isomer of the crosslinked conjugate and proceeds with deuterium incorporation when the reaction is performed in deuterated buffer. These data are consistent with a mechanism that involves fast *in situ* generation of alkenylthianthrenium salts that all react with Cys via formation of sulfur ylides, proton transfer, followed by generation of a common episulfonium intermediate (Figure 3.6), and exclude a mechanism involving direct substitution of thianthrenium in **TTD** with Cys. Because fast *in situ* deprotonation of **TTD** leads to formation of alkenylthianthrenium salts that share the same reactivity toward Cys, the overall transformation proceeds in a single step and indicates that **TTD** can be used directly for crosslinking studies without the need for isolation of intermediate alkenylthianthrenium salts.

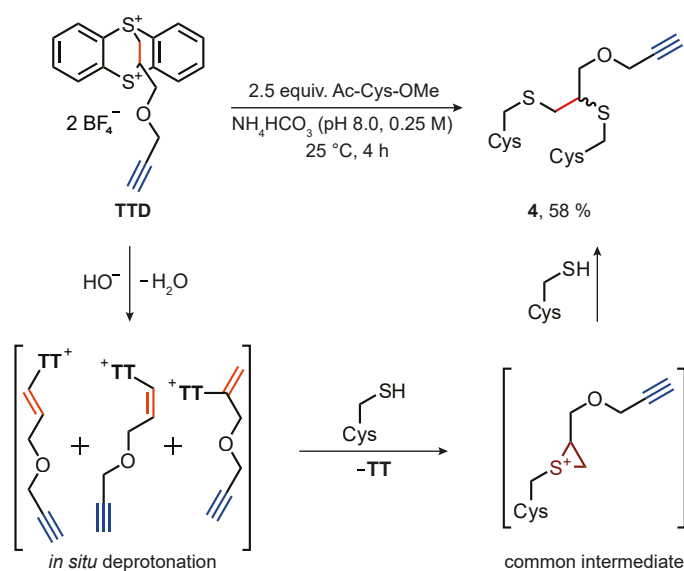


Figure 3.6: Proposed mechanism for reaction of **TTD** with *N*-acetyl-L-cysteine-methylester (Ac-Cys-OMe) in ammonium bicarbonate buffer.

Comparison between TTD and VTT

In vitro experiments

To verify whether **TTD** can be used directly for protein crosslinking and to compare its reactivity with **VTT** *in vitro*, a Cys-containing ubiquitin mutant K63C was treated with **TTD** or **VTT**. The mixtures were analyzed by LC–MS and revealed formation of species with masses corresponding to products containing an incorporated crosslink as the main products, along with products with masses corresponding to episulfonium hydrolysis products in both cases (Figure E.11–Figure E.22). The same product distribution was obtained when either **VTT** or its dicationic precursor was used in the reaction (Figure E.16, Figure E.23), further demonstrating that alkenylthianthrenium salts can be generated *in situ* from bench-stable thianthrenium dications and suggesting that the dication deprotonation as a separate synthetic step is redundant. A control experiment with native ubiquitin revealed no product formation with **TTD** (Figure E.29–Figure E.31). The combined data suggest that **TTD** enables fast and Cys-selective protein crosslinking with simultaneous introduction of an alkyne tag for downstream derivatization of modified products.

In cellulo competition experiments

Having established the reactivity profile of **TTD** *in vitro*, the next goal was to verify whether **TTD** can enable fast episulfonium generation *in cellulo*. The alkenylthianthrenium salts formed after **TTD** deprotonation in buffer have polarity and size similar to those of **VTT**: the calculated lipophilicity index (logP) values of 4.28–4.42 are slightly lower than that of **VTT** (4.6), whereas the topological polar surface area (TPSA) of each compound ($59.8 \text{ \AA}^2$) is only 18% higher than the TPSA calculated for **VTT** ($50.6 \text{ \AA}^2$). Therefore, the calculated logP and TPSA values suggest that introduction of the alkyne-containing linker should not lead to a pronounced negative effect on the cell permeability of alkenylthianthrenium salts generated *in situ* from **TTD** when compared to **VTT**¹⁴⁹.

A competition experiment in *E. coli* cells was performed to compare intracellular labeling efficiency of **VTT** and **TTD**. Isotopologues of **VTT** were used to obtain a quantitative readout of how an equimolar amount of **TTD** influences the labeling efficiency of **VTT**. In the control experiment, *E. coli* cells were treated with 1 mM heavy-labeled $^2\text{H}_3$ -**VTT** in LB medium at 37 °C. In the competition experiment, the cells were treated with a stock solution containing 1 mM **VTT** and 1 mM **TTD**. Treatment with reagents was performed for either 5 minutes or 1 hour to compare the labeling rates at early and late stages of the intracellular labeling process. The treated cells were washed and lysed, protein concentrations were determined, and samples from the control experiment were mixed 1:1 with samples from the competition experiment. The mixtures were then digested with trypsin and analyzed by LC–MS/MS. A median heavy-to-light (H/L) ratio of 1.18 ± 0.05 was obtained for the competition experiment performed for 5 minutes. In the experiment performed for 1 hour, the H/L ratio was slightly higher (1.313 ± 0.003) (Figure 3.7), both in line with higher labeling rates for **VTT**. It is not surprising that **VTT** outcompetes **TTD** in protein labeling, since presence of a substituent in the β position of alkenylthianthrenium salts formed upon deprotonation of **TTD** is expected to lower the reactivity when compared with the unsubstituted **VTT**¹⁵⁰. However, the observed influence of **TTD** on labeling efficiency of **VTT**, although small, indicates that overall **TTD**-mediated intracellular labeling occurs with rates comparable to those of **VTT**, and faster than those with **IAA** alkyne.

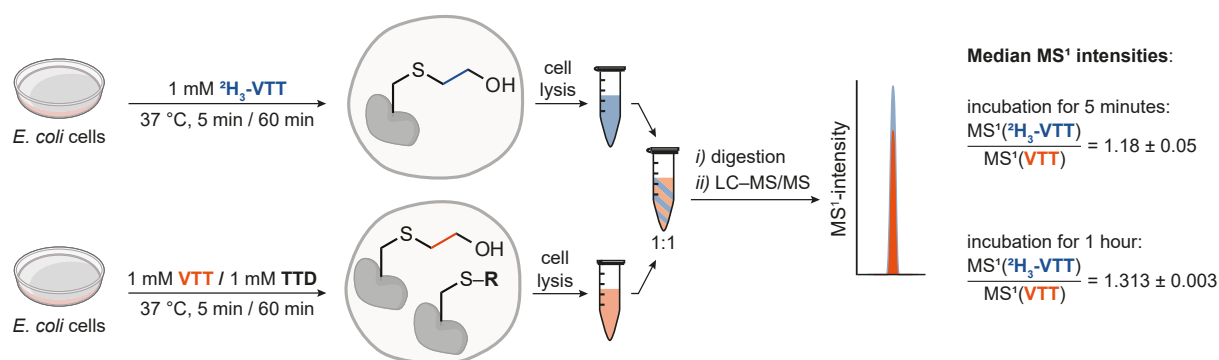


Figure 3.7: Competition experiment between **VTT** and **TTD**. The values are means $\pm$ SE based on n=3 experiments. **R** represents the moiety introduced via alkylation with **TTD**.

Comparison of cytotoxicity

Cytotoxicity studies were performed to identify the concentration range at which treatment of mammalian HEK cells with **TTD** does not lead to membrane damage and to evaluate the influence of **TTD** treatment on the cellular reducing capacity. To evaluate the membrane integrity of cells treated with **TTD**, the previously described cytotoxicity assay was used to quantify DNA released by the lysed cells. Incubation of cells with **TTD** at concentrations as high as 1 mM for 1.5 hours at 37 °C did not lead to any detectable cell lysis (Figure 3.8).

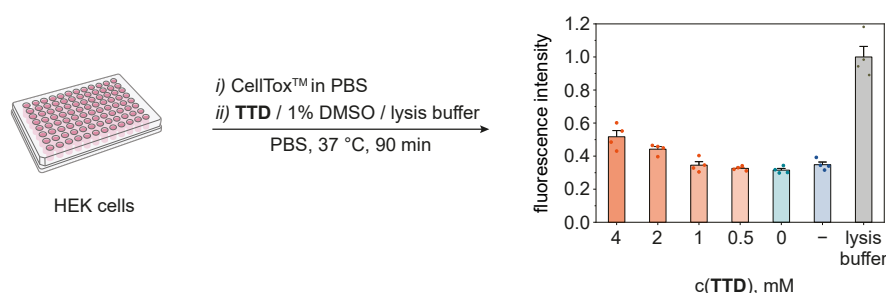


Figure 3.8: Membrane integrity assay with **TTD**. The values are means $\pm$ SE based on n=4 experiments.

When the cells were treated with resazurin reagent, followed by addition of 1 mM **VTT** or **TTD** and incubation for 1 hour at 37 °C, fluorescence measurements revealed that cells treated with **TTD** reduced approximately twice the amount of nonfluorescent resazurin to fluorescent resorufin compared with **VTT** (Figure 3.9), consistent with faster intracellular alkylation by **VTT** that induces more pronounced perturbation of the redox metabolism. The combined data suggested feasibility of the performing intracellular crosslinking with **TTD** in intact cells.

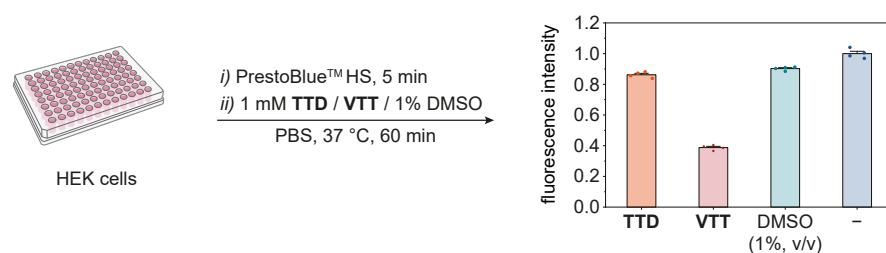


Figure 3.9: Resazurin-based viability assay with **TTD** and **VTT**. The values are means $\pm$ SE based on n=4 experiments.

TTD-mediated alkyne incorporation for downstream analysis

Competition experiments with **VTT** indicated that **TTD** competes with **VTT** in intracellular protein labeling. To verify that **TTD** can be used for efficient intracellular protein labeling with mammalian cells and alkyne incorporation for downstream enrichment of the modified products, HEK cells were treated with 1 mM **TTD** for 1 hour, washed with PBS, lysed, and the obtained mixtures were subjected to a CuAAC reaction with an azide-containing fluorescein probe to visualize proteins modified with **TTD** *in cellulo*. SDS-PAGE and in-gel fluorescence analysis revealed that treatment with **TTD** led to proteome-wide incorporation of the alkyne tag (Figure 3.10).

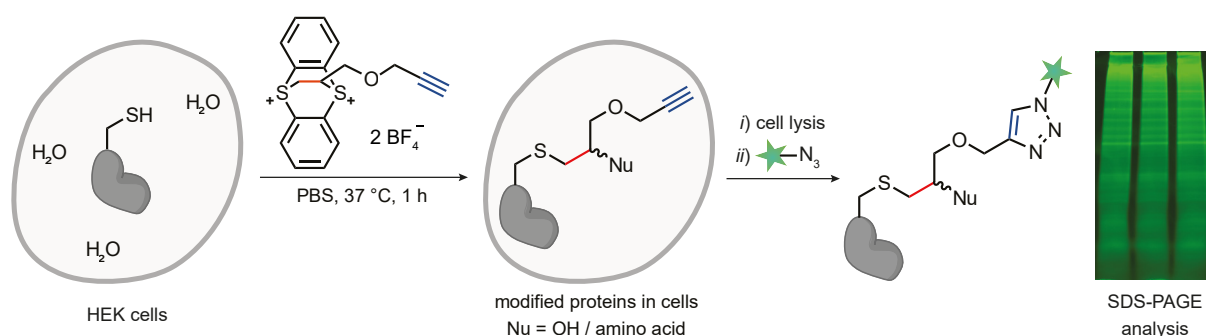


Figure 3.10: Proteome-wide alkyne incorporation and fluorescence SDS-PAGE analysis of the isolated lysate after CuAAC reaction with 6-FAM (6-carboxyfluorescein) azide. PBS, phosphate-buffered saline; Nu, nucleophile; AA, amino acid. Only one of the two possible constitutional isomers of each product is depicted.

The experiment showed that products obtained after intracellular protein modification with **TTD** can be further derivatized using standard click reaction protocols and therefore provide a suitable starting material for enrichment of modified products. A CuAAC reaction with an azide-containing biotin probe was set up to introduce an affinity tag for a follow-up enrichment step. The protein mixture was trypsin-digested, and the obtained modified peptides were enriched using NeutrAvidin beads¹⁵¹ (Figure 3.11).

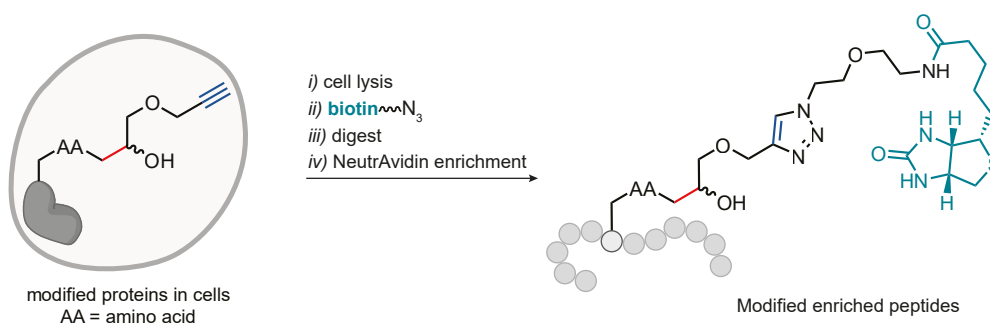


Figure 3.11: Biotin-based enrichment of modified peptides. AA, amino acid.

The samples were analyzed by LC–MS/MS, and a database search for episulfonium hydrolysis products on all nucleophilic amino acids was performed to verify Cys selectivity of **TTD** in intracellular protein labeling. The search revealed that protein labeling predominantly occurred on Cys, with 86% of the identified modified peptides carrying the biotin modification on Cys (Figure 3.12).

Efficient enrichment of modified peptides is crucial for enhancing sensitivity of PPI identification with the developed reagent. To evaluate the enrichment efficiency of the protocol, the lysate

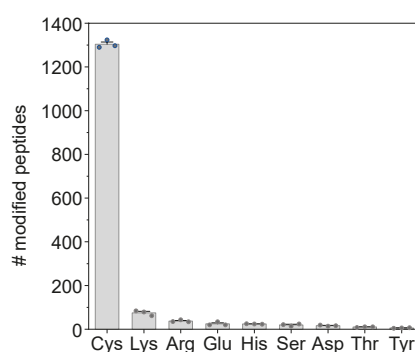


Figure 3.12: The selectivity profile of **TTD**-modified peptides detected by LC–MS/MS. The values are means $\pm$ SE of $n=3$ independent experiments.

samples obtained after lysis of **TTD**-treated cells were trypsin-digested and analyzed by LC–MS/MS in the same way as biotin-enriched samples to enable direct comparison between the enriched and non-enriched samples. A database search for Cys-modified peptides carrying the alkyne tag in the non-enriched samples and the biotin tag in the enriched samples was performed and revealed that the enrichment step increased the identification sensitivity of modified peptides by a factor of 14, with an overlap of 94% for the modified peptides (Figure 3.13).

Comparison of the number of modified peptides in both sample groups revealed that only $1.51 \pm 0.01\%$ of peptides identified in the non-enriched samples were modified, whereas the enriched samples predominantly consisted of peptides carrying the biotin modification ($92 \pm 1\%$). Because the samples also contained crosslinked peptides that were not considered in this calculation, the actual enrichment specificity should be even higher. The large overlap between the two sample groups and the observed levels of the enrichment specificity indicated that efficient enrichment of modified peptides was achieved and suggested that LC–MS/MS data obtained from the enriched samples were suitable for identification of low-abundance inter-protein crosslinks.

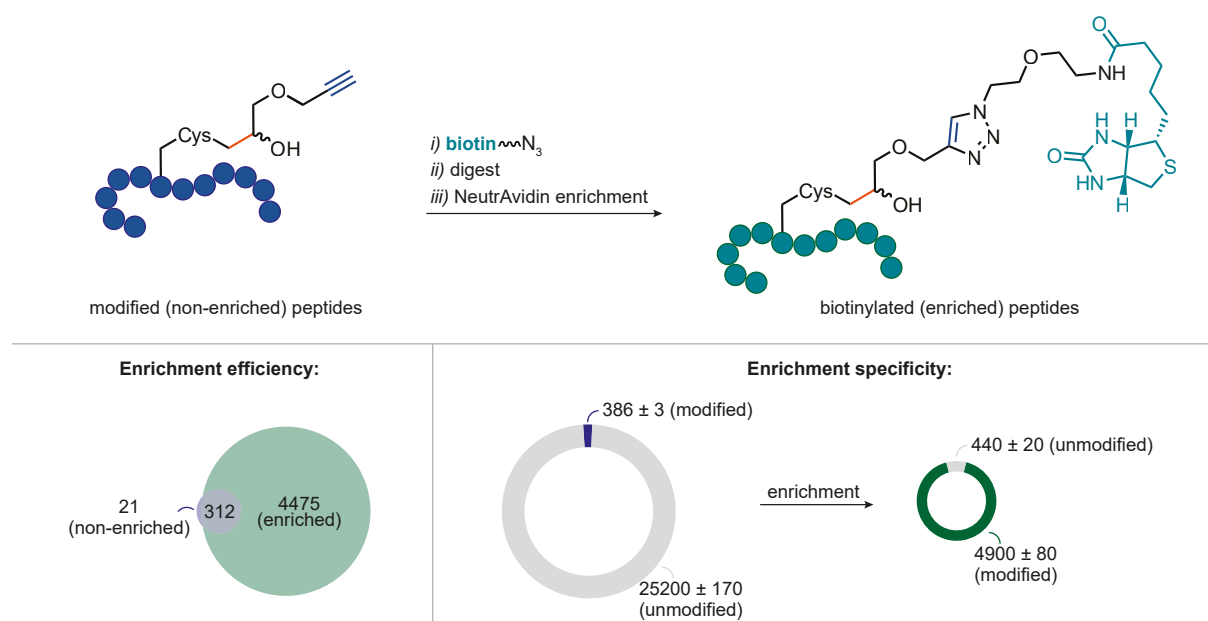


Figure 3.13: Quantification of enrichment efficiency and specificity for the utilized protocol.

Analysis of the crosslinking data

Having established that **TTD**-mediated proteome-wide incorporation of the alkyne tag occurred predominantly on Cys, a search for crosslinked peptides was performed to identify peptides containing an introduced covalent linkage between Cys and one of the nucleophilic amino acids (Asp, Glu, Cys, His, Tyr, Ser, Thr, Lys, Arg), as well as protein C- and N-termini. The resulting list of matches was filtered to include only peptides with at least three detected fragment peaks to ensure that only high-quality MS fragmentation spectra were used for assignment of the crosslinked species. Subsequently, the results were filtered to obtain a list of the crosslinked peptides that passed a threshold of 1% calculated false discovery rate (FDR). The FDR provides an estimate of the proportion of obtained matches that are false positives¹⁵². In total, 1534 intra-protein and 100 inter-protein crosslinks were identified in the enriched samples. Given the applied 1% FDR threshold at the residue-pair level, the obtained dataset is expected to contain approximately 15 false-positive intra-protein crosslinks, whereas only one of the identified inter-protein crosslinks is expected to be a false positive^{153–155}. The low fraction (6%) of inter-protein crosslinks in the dataset is consistent with the reactivity mode of episulfonium intermediates and the incorporation of short two-carbon crosslinks¹⁵⁶: formation of an inter-protein crosslink can occur only when two proteins are in close proximity and the crosslinking reaction takes place before the episulfonium reacts with water or undergoes intramolecular crosslinking.

When the same analysis was applied to data obtained from the non-enriched samples, only 12% of the intra-protein crosslinks observed in the enriched dataset were identified (Figure 3.14).

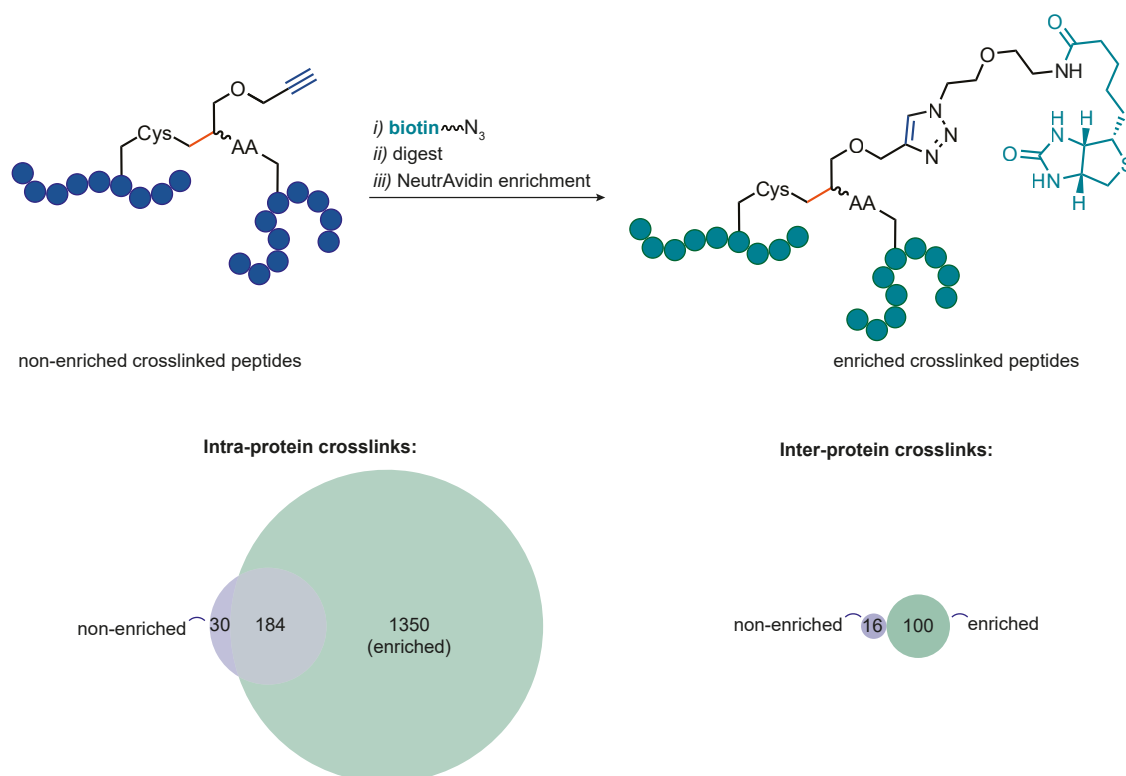


Figure 3.14: Overlap between the lists of crosslinked peptides identified in the non-enriched and enriched samples. AA, amino acid.

Moreover, the 1% FDR threshold could not be applied to inter-protein crosslinks detected in the non-enriched dataset in a meaningful way because only 16 matches were obtained. Therefore,

at this low number of matches, FDR estimation is not statistically meaningful below 1/16, such that the smallest non-zero FDR value that can be supported is 6.25%. As in the case of **VTT**-derived inter-protein crosslinks, analysis of the non-enriched **TTD** samples is not suitable for a reliable benchmarking of the method for PPI detection: only four of the identified PPIs were supported by at least three crosslinks, and only one of these PPIs has been previously reported. These results further support the importance of the enrichment step for reliable identification of low-abundance episulfonium-derived inter-protein crosslinks.

Gene ontology annotation of identified crosslinked proteins in the enriched samples showed that both intra-protein and inter-protein crosslink formation occurred in the major cellular compartments, further supporting efficient cell permeability and intracellular labeling efficiency of the reagent (Figure 3.15).

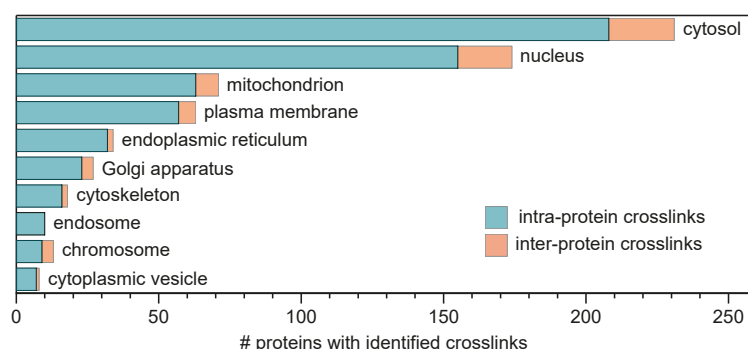


Figure 3.15: Gene ontology-annotated intracellular localization of proteins for which at least two crosslinks were detected in the enriched dataset.

Crosslinks to all nucleophilic amino acids were identified in the obtained dataset, demonstrating high reactivity of the **TTD**-derived episulfonium. Comparison of amino acid distributions in the identified intra- and inter-protein crosslinks with the relative abundances of nucleophilic residues in the proteome revealed no observable selectivity of the episulfonium toward most amino acids included in the analysis (Figure 3.16).

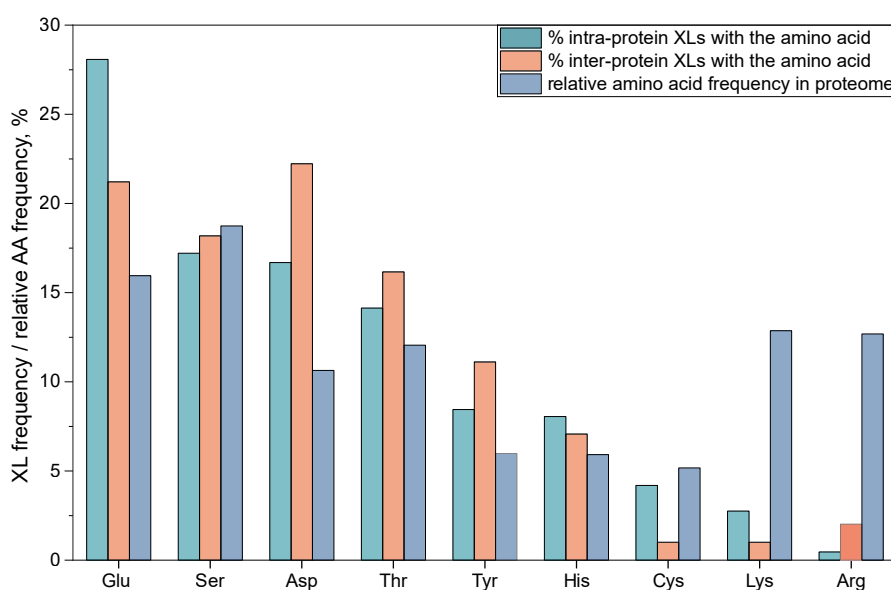


Figure 3.16: Distribution of amino acids in the detected crosslinks.

Basic amino acids Arg and Lys, which are protonated at neutral pH, represented the only exception, in line with the previously observed reactivity trend for **VTT**-mediated crosslinking.

Because the **TTD**-derived episulfonium intermediate enables versatile crosslinking with various amino acids, generation of the episulfonium intermediate in close proximity to another protein should enable formation of several crosslinked products that support identification of a single PPI. Indeed, the 100 detected heteromeric crosslinks were mapped to 22 PPIs involving 39 proteins. In contrast to the inter-protein crosslinking data obtained from the non-enriched samples, distribution of crosslinks among PPIs identified in the enriched samples revealed that 20 of the 22 PPIs were supported by at least three crosslinks (Figure 3.17), thereby providing a dataset that predominantly consisted of high-confidence PPIs. The two PPIs identified by fewer than three crosslinks were not considered in the follow-up analysis to ensure that method benchmarking was performed using a high-confidence dataset.

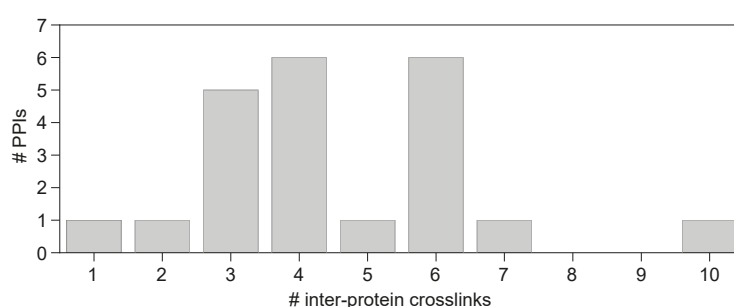


Figure 3.17: Number of heteromeric crosslinks per identified PPI.

Among the 20 PPIs included in the analysis, 15 have been previously reported and are annotated in the STRING database¹⁵⁷, which provides a measure of PPI validity¹⁵⁸ (Figure 3.18). Only two interactions exhibited STRING scores below the high-confidence threshold of 0.9, while being above the medium-confidence cutoff of 0.5. Given that all considered PPIs were supported by at least three high-quality crosslink identifications passing stringent FDR control, these results support the suitability of **TTD**-mediated crosslinking for reliable PPI identification.

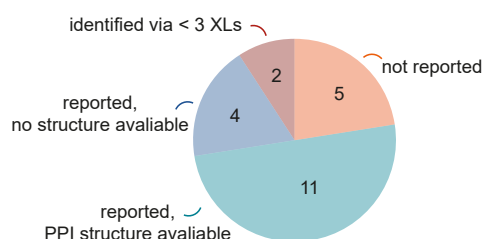


Figure 3.18: Overview of the PPIs that were identified in the enriched dataset.

Validation of the inter-protein distance restraints

Beyond accurate identification of PPIs, a useful crosslinking method should provide rigid distance restraints for reliable modeling of protein interaction surfaces. Fast episulfonium hydrolysis and intra-protein crosslinking compete with the formation of inter-protein crosslinks and should prevent identification of PPIs if two proteins are not in close proximity. Moreover, only a two-carbon linkage is inserted between the reacted residues, which suggests that **TTD**-mediated inter-protein crosslinking should yield short distance restraints comparable to those produced by **VTT**.

To evaluate this hypothesis, the crosslinked residue pairs from an identified PPI were mapped onto a reported high-resolution protein complex structure. The interaction between CCT5 and CCT7 within the TCP-1 ring complex was selected, as a high-quality PPI structure is available for the interaction complex, and mapping of all identified crosslinks could be performed. The resulting $C\alpha$ – $C\alpha$ distances are in the range from 11 to 17 Å (Figure 3.19), consistent with the 4–23 Å distance range determined for **VTT**-mediated crosslinking (Figure 2.17).

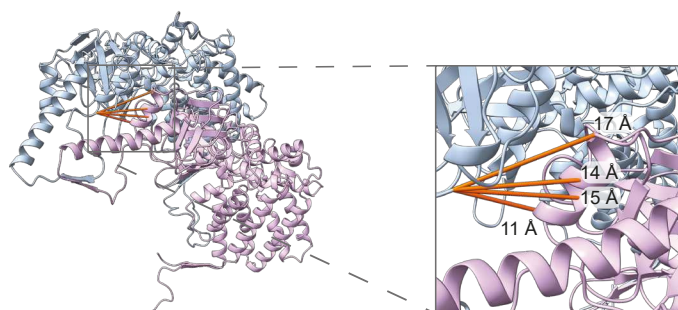


Figure 3.19: Reported structure of interacting proteins in the chaperonin TCP-1 ring complex with the mapped detected crosslinks. The structure was visualized using Chimera X 1.10¹²¹.

The obtained mapped distance values and the observed reactivity profile of the **TTD**-derived episulfonium intermediate indicated that distance restraints of up to 23 Å, as obtained for **VTT**-mediated crosslinking, can be used to achieve reliable prediction of inter-protein interaction surfaces based on the identity of the crosslinked residues. To test this hypothesis, a docking study was performed using the geometries of CCT5 and CCT7 together with the list of the crosslinked residue pairs and distance restraints of up to 23 Å between the crosslinked residues.

The degree of structural agreement between the predicted and reported structures was evaluated using DockQ¹⁵⁹, which estimates model quality by quantifying the number of preserved native contacts at the interaction interface with spatial overlap between the predicted and native structures. A DockQ score of 0.95 was obtained, indicating an almost perfect match between the modeled PPI complex and the native structure (Figure 3.20). These results further demonstrate that **TTD**-mediated crosslinking enables identification of high-confidence PPIs and that the crosslinked residue identities can be used to model inter-protein interaction surfaces using short distance restraints, yielding reliable PPI structure predictions.

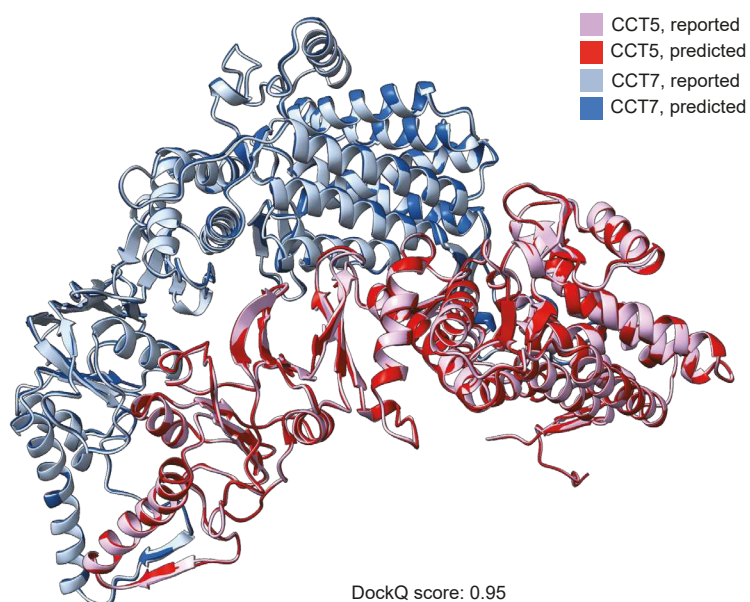


Figure 3.20: Evaluation of the crosslinking approach for structure prediction of interacting proteins CCT5 and CCT7. Docking was performed with HADDOCK 2.4¹⁶⁰ and the structure was aligned with the reported PDB structure (7NVL) and visualized using Chimera X 1.10¹²¹.

Prediction of PPI structures for previously reported PPIs

The accurate structure prediction of the CCT5–CCT7 complex provided initial evidence that reliable PPI identification can be achieved using the obtained dataset. To further assess the reliability of the predictions, PPIs that have been previously reported but for which no interaction-surface structures are available were analyzed. Although these interactions lack reference structures for a direct benchmarking, the plausibility of the predicted interaction surfaces was evaluated based on the reported biological functions and known properties of the interacting proteins. Three PPIs involved proteins for which either an experimentally determined crystal structure or a high-confidence AlphaFold-predicted structure was available and were therefore suitable for docking studies.

A docking study of the interaction between Cullin-4A (CUL4A) and Cullin-associated NEDD8-dissociated protein 1 (CAND1) did not yield a single dominant cluster of predicted structures with scores clearly exceeding those of alternative models. Although the applied distance restraints were satisfied in all five top-scoring clusters, indicating that the crosslinking data effectively constrained the interaction space, no model could be unambiguously selected as the most plausible interaction geometry. Consequently, this PPI was not considered further in the docking analysis. The interaction surfaces of the remaining PPIs could be successfully modeled and were analyzed in greater detail.

RACK1–vigilin PPI structure

Receptor for activated C kinase 1 (RACK1) is a conserved scaffold protein and an integral part of the ribosomal 40S subunit¹⁶¹. RACK1 associates with vigilin, the high-density lipoprotein binding protein (HDLBP), a conserved protein that can bind RNA and thereby regulate translation¹⁶². Moreover, vigilin can act as a host protein in the replication of various viral RNAs¹⁶². Recent studies have examined the role of the RACK1–vigilin interaction in the replication of DENV (dengue virus) and revealed that viral replication depends on vigilin's interaction with RACK1, and not on the ability of vigilin to bind viral RNA^{163,164}. Vigilin consists of 14 RNA-binding domains, and mutational studies in different cell types revealed that its C-terminal KH domains 13 and 14 are essential for interaction with RACK1¹⁶³. Although the existing data provide insight into how the two proteins interact, no structure of the interaction surface is currently available, and the interaction site on RACK1 has not been identified.

Seven crosslinks between vigilin and RACK1 were identified after **TTD** crosslinking and used to guide docking of the two proteins. The top-scoring model placed RACK1 in close proximity to the KH14 domain of vigilin, consistent with the previously reported data, and indicated that the WD-1 and WD-2 domains of RACK1 are responsible for the interaction ([Figure 3.21](#)). The obtained structure provides the first predicted interaction surface between RACK1 and vigilin and is consistent with prior studies, further supporting the reliability of PPI structure predictions based on **TTD**-derived crosslinking data.

VDAC2–VDAC3 PPI structure

VDAC2 and VDAC3 are voltage-dependent anion channel protein isoforms located in the outer mitochondrial membrane of human cells, where they control the transfer of ions and metabolites between the mitochondria and the cytosol¹⁶⁵. Although the biological functions of VDAC3 have not been elucidated to the same extent as those of the other VDAC proteins, interaction between

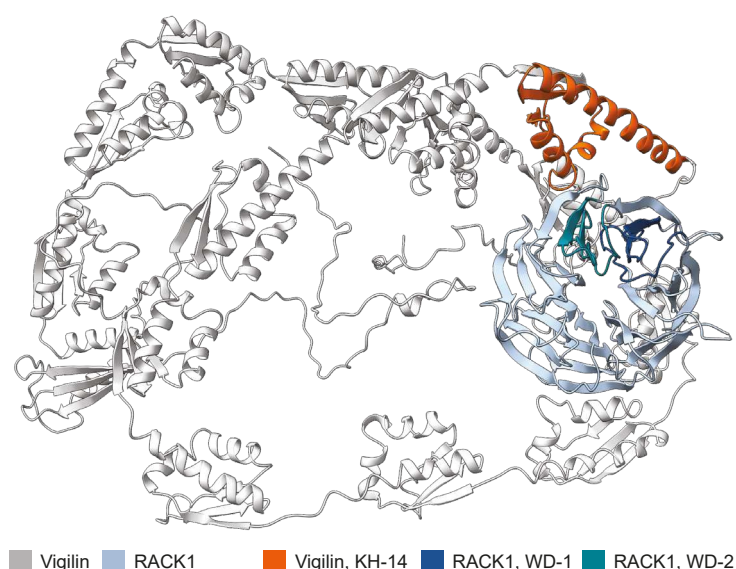


Figure 3.21: Predicted structure of interaction between RACK1 and vigilin based on the obtained crosslinking restraints. Docking was performed with HADDOCK 2.4¹⁶⁰ and the structures were visualized using Chimera X 1.10¹²¹.

VDAC2 and VDAC3 has been identified in several studies in mammalian cells^{166,167}, suggesting cooperative action of the two proteins. For example, studies of ferroptosis, an iron-dependent oxidative cell death, revealed that the antitumor drug erastin acts by binding to both VDAC2 and VDAC3¹⁶⁸, linking their interaction to erastin-induced oxidative cell death^{169,170}.

Although important for understanding the mechanism of ferroptosis activation, no structural data on the VDAC2–VDAC3 interaction surface exist, which is unsurprising given that VDAC3 is not yet a well-studied protein and its crystal structure has not been obtained¹⁶⁵. Although a high-confidence prediction of the VDAC3 structure exists (pLDDT = 95.4), modeling of the PPI structure with AlphaFold-Multimer did not produce reliable predictions¹⁷¹, highlighting the limitations of theoretical approaches for PPI structure modeling when not guided by experimental data. **TTD**-mediated crosslinking enabled identification of four crosslinks between VDAC2 and VDAC3 in the obtained dataset, and the identity of the crosslinked residues was used as input for a docking study. Additional docking was performed based on the identity of crosslinked residues reported for the VDAC2–VDAC3 interaction in a previous crosslinking study using the crosslinker **DSSO** (disuccinimidyl sulfoxide)¹⁷². **DSSO** is a bifunctional, lysine-selective crosslinking reagent that connects two lysine residues via a linker that is approximately 8 Å longer than the two-carbon crosslink introduced by **TTD** (Figure 3.22). Therefore, whereas distance restraints of 23 Å were used for PPI modeling with the **TTD**-derived crosslinking data, the commonly used^{173,174} restraints of 35 Å were applied for modeling based on the **DSSO** crosslinking data.

Previously reported data on the structure of VDAC channels^{175–177} indicate a parallel orientation of the β -barrels embedded in the mitochondrial membrane, suggesting that a parallel arrangement of interacting VDAC2 and VDAC3 proteins should be expected. Indeed, docking studies using the **TTD**-derived crosslinking data yielded a model in which the two proteins were oriented parallel to each other, consistent with the biological function of these pore-forming proteins (Figure 3.23). When the PPI structure was modeled using a distance restraint of 35 Å between

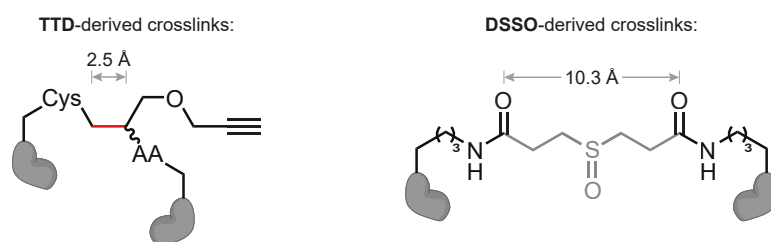


Figure 3.22: Comparison of structures of crosslinked products obtained via crosslinking with **DSSO** and **TTD**. AA, amino acid.

the crosslinked residues identified in the previously reported **DSSO**-based crosslinking study, the top-scoring prediction placed the two proteins in an orthogonal orientation. This arrangement does not align with the expected parallel orientation of the pore-forming proteins in the membrane and can therefore be considered a false prediction. These results demonstrate the superiority of the episulfonium-based crosslinking approach for reliable PPI structure modeling using short distance restraints, compared with traditional approaches that rely on homobifunctional reagents with long linker lengths, especially in cases when no reference structure of a similar PPI or additional data on the interaction surface are available.

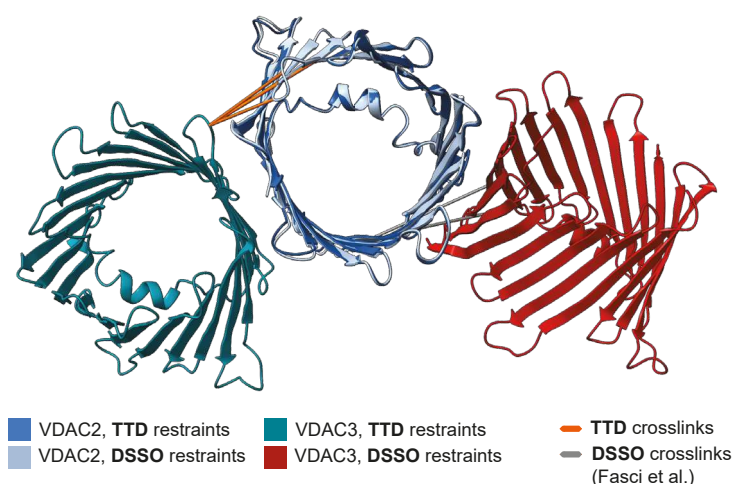


Figure 3.23: Predicted structures of interaction between VDAC2 and VDAC3 based on crosslinking restraints obtained in this study or reported by Fasci et al.¹⁷², and mapped identified crosslinks. Docking was performed with HADDOCK 2.4¹⁶⁰ and the structures were visualized using Chimera X 1.10¹²¹.

The crosslinking data on the RACK1–vigilin and VDAC2–VDAC3 interactions obtained with **TTD** provided additional evidence for the reliability of the **TTD**-based crosslinking approach. First, the obtained models align well with what has previously been known about the protein sites that participate in the interaction (RACK1–vigilin) or the orientation of analogous proteins in the complex (VDAC2–VDAC3). Second, the importance of short distance restraints for reliable PPI modeling was demonstrated through comparison with crosslinking data obtained using a frequently used homobifunctional crosslinker. The combined data further demonstrate that the fast and versatile reactivity of the episulfonium intermediate, the short linkages introduced in the crosslinked products, and the identification of a single PPI supported by several crosslinks enable reliable PPI identification, whereas the obtained distance restraints can be used to obtain additional insight into the PPI surface when no experimental PPI structure is available.

Identification of previously undocumented PPIs

The analysis of the data on previously reported PPIs suggested that the episulfonium-mediated crosslinking should also enable reliable identification of previously undocumented PPIs, given that the interaction is supported by several heteromeric crosslinks that pass stringent FDR filtering. The set of the identified PPIs contained five interactions that had not been previously reported, all supported by at least three detected inter-protein crosslinks and therefore representing high-confidence hits. To further verify the plausibility of these identifications, the cellular localization of the proteins involved in the detected interactions was assessed using an automatic gene ontology annotation. The analysis revealed that proteins in all PPIs are known to be localized either in the same cellular compartment or in compartments for which cross-compartment PPI formation has been reported, such as the centrosome and cytosol or the endoplasmic reticulum and mitochondrion^{178–180}. It is important to note that the annotation was performed based on entries with existing experimental evidence and did not include annotations based on sequence similarity. Therefore, co-localization of all protein pairs involved in the identified PPIs within the same cellular compartments cannot be excluded.

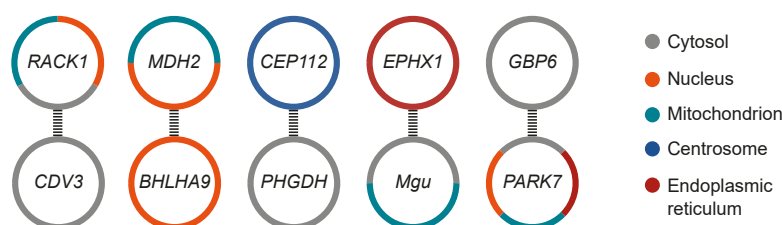


Figure 3.24: Annotation of cellular localization for proteins in previously undocumented PPIs that were identified in this work. Dashed lines indicate detected crosslinks between the proteins. RACK1, receptor of activated protein C kinase 1; CDV3, protein CDV3 (carnitine deficiency-associated gene expressed in ventricle 3) homolog; MDH2, mitochondrial malate dehydrogenase; BHLHA9, class A basic helix-loop-helix protein 9; CEP112, centrosomal protein 112; PHGDH, D-3-phosphoglycerate dehydrogenase; EPHX1, epoxide hydrolase 1; Mgu, uroporphyrinogen-III synthase; GBP6, guanylate-binding protein 6; PARK7, Parkinson disease protein 7.

The set of the obtained PPIs was analyzed to identify candidates for docking studies based on the detected crosslinked residues. One of the identified interactions was between RACK1 and CDV3, a putative protein that has been linked to cancer in different tissues¹⁸¹. Although plausible given the identification of the previously reported interaction between RACK1 and vigilin, no meaningful prediction of the interaction surface can be obtained for the PPI between RACK1 and CDV3 because no structural data or reliable prediction of the protein structure is available for CDV3. The interaction between mitochondrial malate dehydrogenase (MDH2) and the transcription factor BHLHA9 was also not considered due to the unavailability of a reported protein structure or a high-confidence prediction for the structure of BHLHA9.

The existence of all identified PPIs is plausible based on the number of detected inter-protein crosslinks and the cellular localization data, but the crosslinking data alone do not provide a strong indication of whether a detected PPI corresponds to a stable complex or a transient interaction. However, heteromeric crosslinks between the centrosomal protein 112 (CEP112) and D-3-phosphoglycerate dehydrogenase (PHGDH) were identified in two out of three biological replicates and therefore suggested that the detected PPI may correspond to a stable complex. This interaction was therefore selected for a docking study and yielded a top-scoring prediction

that places PHGDH in close proximity to the N-terminal region of CEP112 (Figure 3.25). Analysis of contact residue pairs^{182,183} suggested that the interaction in the modeled PPI is mostly governed by electrostatic forces, and the predicted dissociation constant value of 3×10^{-7} ($pK_d \approx 6.5$, Figure 3.25) places the PPI in the range of medium-affinity complexes¹⁸⁴, consistent with the identification of crosslinks between the two proteins in two biological replicates. The previously reported involvement of both PHGDH¹⁸⁵ and CEP112¹⁸⁶ in translational regulation, as well as the reported association of centrosomal proteins with metabolic enzymes¹⁸⁵ further suggests that formation of a complex between the two proteins is feasible, although additional studies are required to confirm the presence of the interaction in viable cells and to dissect its biological role.

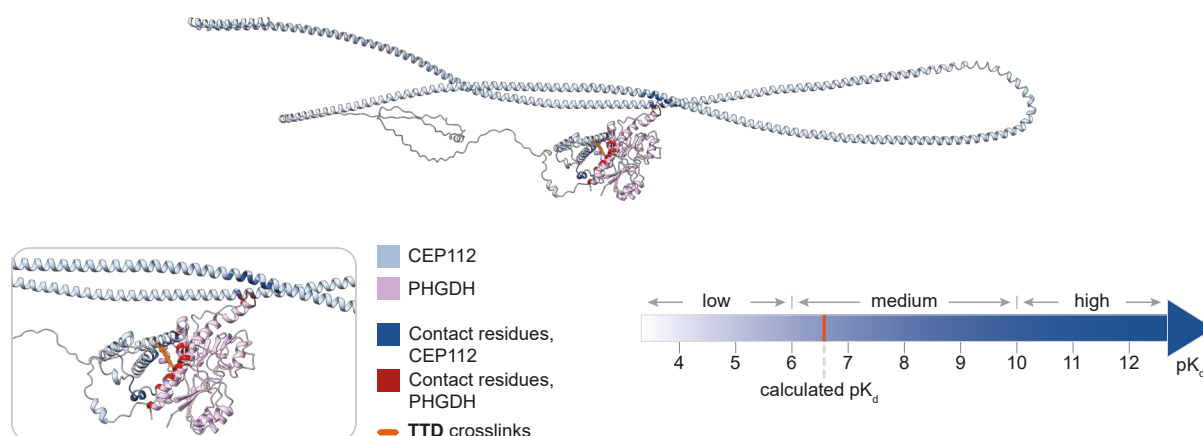


Figure 3.25: Predicted structure of interaction between PHGDH and CEP112, zoom on the identified contact residues and mapped **TTD**-derived crosslinks, and the predicted dissociation constant (K_d). Docking was performed with HADDOCK 2.4¹⁶⁰, contact residues were identified using PRODIGY^{182,183}, and the structures were visualized using Chimera X 1.10¹²¹.

Overall, the obtained evidence shows that **TTD**-mediated crosslinking enables identification of previously undocumented PPIs in the cellular environment and that the resulting data on the identity of the crosslinked products enable modeling of interaction surfaces using short distance restraints. Although low-abundance Cys residues are targeted with the described method, generation of a reactive episulfonium intermediate enables formation of versatile crosslinked products that support identification of a single PPI. Therefore, the method provides a means of achieving single-step crosslinking for reliable PPI identification based on previously unappreciated Cys-based inter-protein crosslinking.

Intracellular episulfonium chemistry beyond protein crosslinking

The intracellular environment contains various nucleophilic species that could react with the generated episulfonium intermediates and thereby expand the applicability of the episulfonium-mediated crosslinking elucidated in this work. Because episulfonium intermediates formed on protein surfaces predominantly undergo hydrolysis or intra-protein crosslinking, identification of other low-abundance products is challenging if the samples are not enriched in modified peptides. The observed high enrichment efficiency after **TTD**-mediated intracellular episulfonium generation, as well as identification of low-abundance inter-protein crosslinks, suggested that identification of other low-abundance products in the enriched samples should be feasible.

To identify potential candidates, an open database search of the obtained LC–MS/MS data was performed. In contrast to so-called closed modification searches, in which the algorithm matches experimentally obtained mass spectra to theoretical spectra of peptides expected to carry a specific, pre-defined modification within a narrow mass deviation window, open modification search algorithms allow a wide mass difference between the theoretical and experimentally observed peptide masses and yield a list of the most frequently observed mass shifts within a specified mass range¹⁸⁷. Although less sensitive and more computationally demanding than conventional modification searches, open modification search is useful for discovery of novel post-translational modifications¹⁸⁸ or unexpected transformations of modified peptides that occurred during sample preparation¹⁸⁹.

An open database search of the enriched data obtained in the crosslinking experiments with **TTD** revealed an unannotated mass shift of 757.2884 Da that was identified approximately five times less frequently than the episulfonium hydrolysis product, indicating that detection of other episulfonium-derived products beyond the episulfonium hydrolysis product should be feasible. Due to the relatively high (up to 4.5 mM) concentration of the Cys-containing tripeptide glutathione in HEK cells¹⁹⁰, as well as the high Cys-reactivity of glutathione, the first hypothesis was that the observed unassigned mass shift corresponded to a glutathione conjugate. Indeed, the expected modification mass of 757.2887 Da matches well with the observed mass shift, and a closed search using the corresponding modification formula enabled identification of 100 ± 10 glutathione-modified peptides across three biological replicates. Moreover, conjugates with less abundant¹⁹¹ free cysteine were also identified, supported by high-confidence fragment ion matches of peptides that were detected in all three biological replicates (Figure 3.26), further demonstrating the wide reactivity scope of the episulfonium intermediate.

Because intracellular protein modification with **TTD** predominantly occurs on Cys residues (Figure 3.12), the crosslinked products as well as episulfonium hydrolysis products should originate from the initial reaction of *in situ*-generated alkenylthianthrenium salts with Cys, followed by formation of an episulfonium intermediate and its reaction with nucleophilic residues or water. However, the Cys-containing glutathione should also readily react with alkenylthianthrenium salts, which means that some of the identified protein–glutathione conjugates could arise from the initial episulfonium generation on glutathione, followed by its reaction with a nucleophilic residue on a protein, thereby representing an alternative reactivity mode (Figure 3.27).

To elucidate the distribution of modified protein residues in the detected glutathione conjugates, a modification search was performed and the possible modification localization site was re-

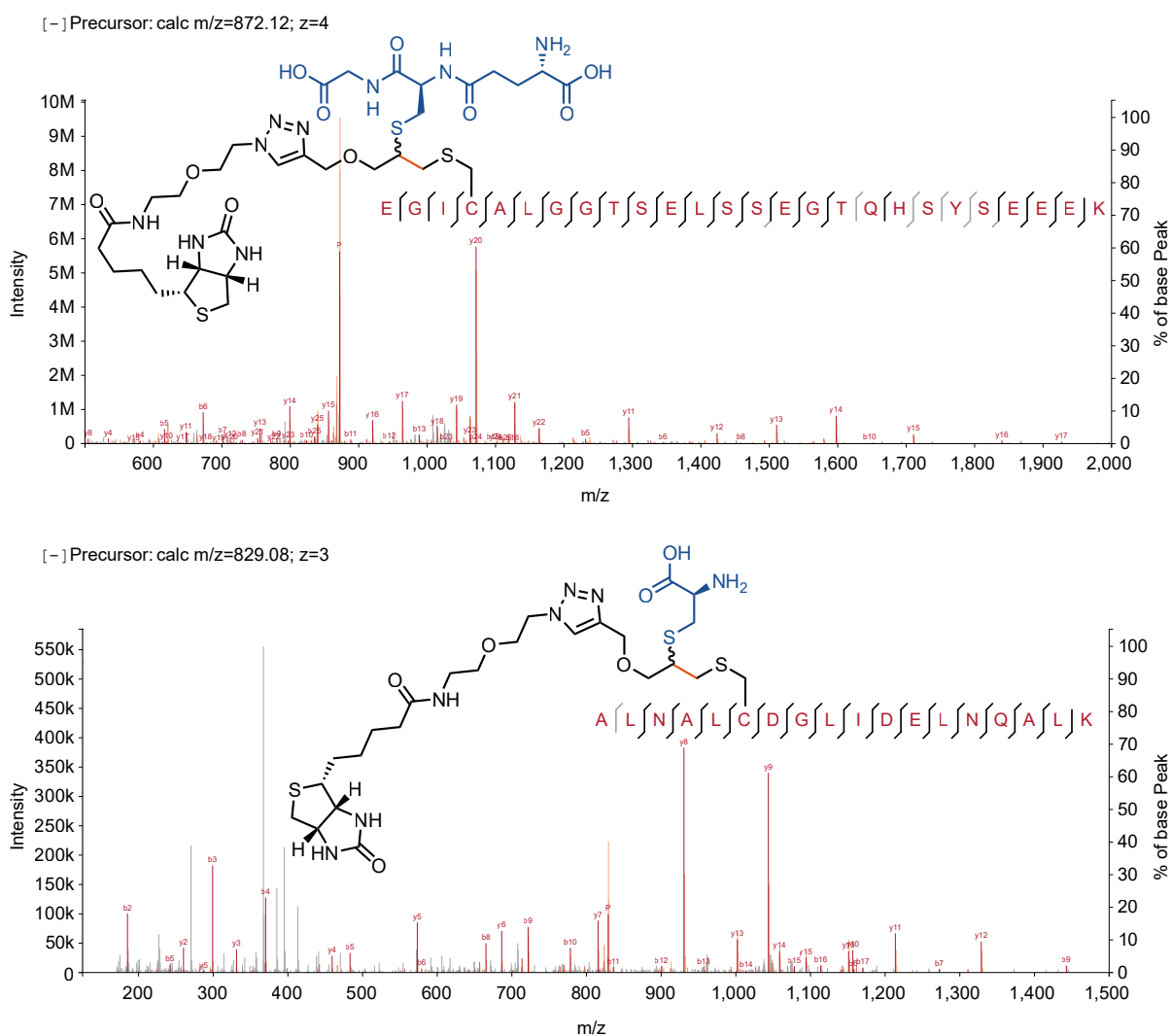


Figure 3.26: Exemplary MS² spectra of identified conjugates between Cys-containing peptides and **GSH** (top) or free Cys (bottom), each identified in three biological replicates. The spectra were visualized using xiSPEC¹⁹².

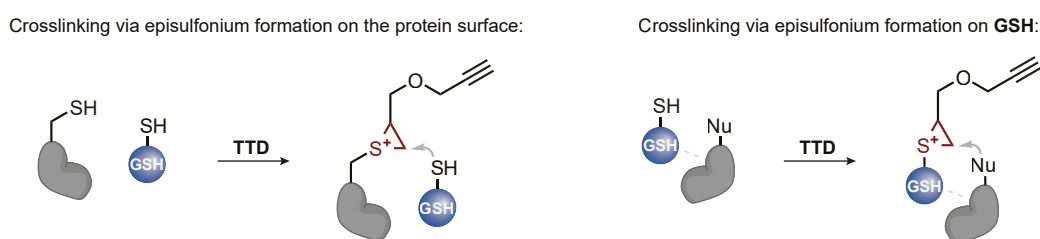


Figure 3.27: The two possible episulfonium generation and reactivity pathways that lead to **TTD**-mediated formation of protein conjugates with **GSH**.

stricted to nucleophilic amino acids (Asp, Glu, Cys, His, Tyr, Ser, Thr, Lys, Arg). The resulting matches were filtered to obtain a list of modified peptides for which the localization probability was at least 95%. Comparison of the relative modification frequencies to the previously obtained data on the selectivity of **TTD**-mediated protein modification revealed a decrease in Cys selectivity from 86% to 51% and a significantly more frequent identification of conjugated products on Glu and His, consistent with a mechanism that involves initial episulfonium formation on glutathione (Figure 3.28).

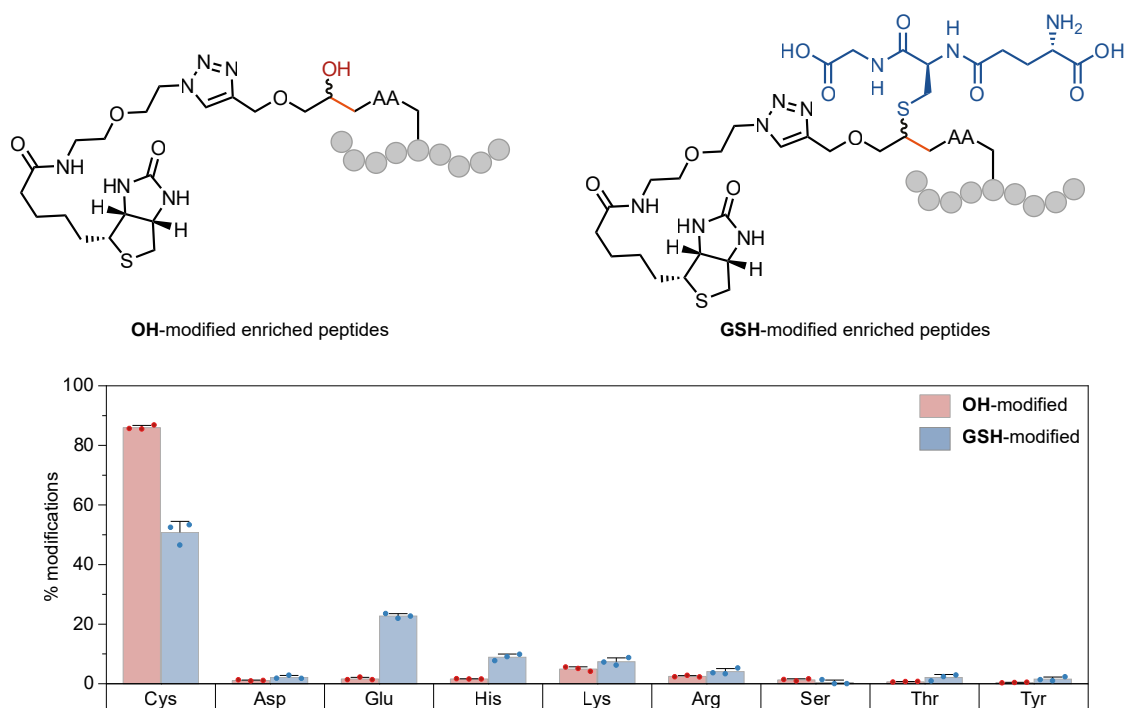


Figure 3.28: Selectivity profiles of modified peptides that correspond to episulfonium hydrolysis product or conjugation with **GSH**. The values are means $\pm$ SE based on $n=3$ experiments. AA, amino acid.

Regardless of whether the detected conjugate was formed via initial episulfonium formation on the protein surface or on glutathione, identification of the corresponding conjugate product suggests close proximity between glutathione and the reacted protein residue. In the case of proteins for which conjugate formation was observed on Cys, the data might provide initial hits for discovery of novel glutathionylation sites based on the identity of the reacted Cys residues. This strategy would enable identification of transient *S*-glutathionylation sites¹⁹³ and, in contrast to other methods, would not require synthesis and introduction of exogenous glutathione probes^{194,195} or enzymatic glutathione labeling¹⁹⁶. Further experiments are needed to determine whether formation of the detected **GSH**-containing conjugates reflects preferential trapping at sites that undergo *S*-glutathionylation, rather than nonspecific capture driven by the high cytosolic abundance of **GSH**. Nevertheless, the obtained dataset shows an overlap between reported *S*-glutathionylation sites and protein residues that underwent **TTD**-mediated conjugate formation with **GSH**. More than half of the modified cysteine residues have not been annotated as known *S*-glutathionylation sites¹⁹⁷, and no data on Cys glutathionylation are available for 50 proteins identified using **TTD**-mediated crosslinking (Figure 3.29), thereby providing new potential targets for studying glutathionylation of native Cys residues that can yield additional insight into cellular redox homeostasis and signaling^{198,199}.

Apart from protein glutathionylation, glutathione can also act as a ligand to mediate protein functions via non-covalent interactions^{200–202}. To identify and study such interactions, glutathione-based photoaffinity probes can be used to induce covalent crosslinking and enable identification of the interaction sites²⁰³. However, this approach relies on activation with UV light and requires modification of the glutathione core for the synthesis of the probes. **TTD**-mediated crosslinking enables single-step formation of crosslinks between native glutathione and protein sites that are located in close proximity to native glutathione and could therefore serve as an alterna-

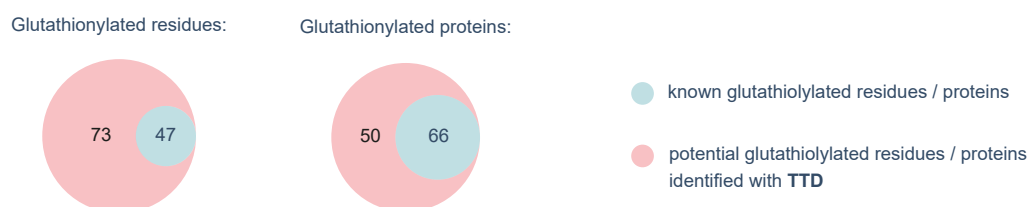


Figure 3.29: Overlap of identified Cys residues that underwent **TTD**-mediated conjugation with glutathione **GSH** and the Cys residues known to undergo glutathionylation (left) and the overlap of the corresponding proteins (right). Only modified peptides identified in at least two biological replicates, with a Cys-localization probability of at least 95% were considered. The reference list of the known glutathionylation sites was obtained from the iCysMod database¹⁹⁷.

tive strategy for studying protein–glutathione interactions in cells. Additional benchmarking will be required to define to what extent the obtained data represent protein–**GSH** interaction sites. Crosslinking studies using well-characterized **GSH**-binding proteins and corresponding mutants with reduced **GSH** affinity could be performed to evaluate the sensitivity of the method and determine whether the conjugate product formation takes place due to the high intracellular **GSH** concentration rather than protein–**GSH** complex formation.

The approach could be extended to crosslinking studies with exogenous probes equipped with Cys, thereby providing an alternative to photoaffinity labeling that requires exogenous activation and incorporation of both photoreactive groups and enrichment tags into the probes²⁰⁴. Overall, the episulfonium-mediated approach offers wide flexibility for affinity crosslinking studies: both incorporation of Cys into proteins of interest and utilization of thiol-containing probes for single-step intracellular crosslinking appear to be feasible.

***In cellulo* PPI identification: Conclusions**

In this part of the work, episulfonium-mediated crosslinking was successfully applied to inter-protein crosslinking studies, enabled by identification of low-abundance crosslinked species. The developed synthetically accessible thianthrenium reagent enables both fast intracellular episulfonium generation on protein surfaces for versatile single-step crosslinking, as well as incorporation of an alkyne tag into the modified products. Efficient enrichment of the crosslinked products enabled detection of diverse inter-protein crosslinks and identification of PPIs in different cellular compartments in mammalian cells. Because episulfonium intermediates readily react with various nucleophilic residues, almost all identified PPIs were supported by detection of at least three crosslinked products, thereby providing a reliable method for PPI detection. Short and stable crosslinks are formed, allowing PPI interaction surfaces to be modeled with higher precision compared to commonly used methods that rely on two-step crosslinking procedures and utilization of crosslinking reagents with long linkers. Moreover, the Cys-targeting crosslinking strategy enabled identification of previously unreported PPIs, further indicating the importance of expanding the scope of available chemical toolkits for protein crosslinking studies.

Although the focus of this work was primarily on the identification of inter-protein crosslinked products, the results described in the last section demonstrate that efficient enrichment of modified products arising from episulfonium reactions with nucleophilic species further expands the applicability of the approach to identification of potential glutathionylation sites, based on detection of protein–glutathione conjugates without the need for specifically engineered glutathione-based probes. The combined data indicate the possibility of studying protein–protein as well as protein–ligand interactions in the cellular environment via either introduction of Cys residues into a protein of interest or utilization of thiol-containing probes. Overall, the versatile episulfonium reactivity, combined with strategies for efficient enrichment of the modified products, demonstrates that intracellular episulfonium chemistry enables a wide variety of applications beyond Cys-selective alkylation.

Final conclusions

This thesis demonstrates *in situ* episulfonium generation as a general strategy for fast, site-selective protein modification and crosslinking under biocompatible conditions. The versatile reactivity of episulfonium ions was studied *in vitro* and *in cellulo*, and a broad variety of applications for site-selective episulfonium formation on protein surfaces has been established. Across all the described applications, a common reactivity principle provides the distinguished properties of the episulfonium-based approach for protein modification and crosslinking: upon rate-limiting reaction of an alkenylthianthrenium Michael acceptor with cysteinyl thiols, transient electrophilic episulfonium intermediates are formed and get rapidly trapped by diverse nucleophiles. The reactivity mode therefore enables formation and isolation of different products based on the nature of the reacted nucleophiles with a single reagent.

Apart from the elucidation of the bioconjugation reaction mechanism, distinct characteristics of the episulfonium-mediated approach for protein structure probing via single-step **VTT**-mediated crosslinking were established in this work. The *in situ* formation of highly reactive episulfonium intermediates on protein surfaces proceeds without exogenous activation and enables the formation of versatile crosslinking products with short covalent linkages, thereby providing data for reliable protein structure probing. Combined with the fast cell permeability and efficient protein labeling by vinylthianthrenium in different cell types, as well as moderate cell toxicity, the described approach provides an efficient tool for probing protein structures in native cellular environments. Moreover, the facile access to isotope-labeled derivatives of vinylthianthrenium further suggests the potential for quantitative studies of protein conformational changes and interaction dynamics *in cellulo*.

The design of a synthetically accessible thianthrenium reagent with an incorporated tag for enrichment further extended the applicability of the episulfonium approach to inter-protein crosslinking for identification of protein–protein interactions in cells. The new reagent preserved fast intracellular protein labeling while enabling efficient affinity enrichment of the modified products. Crosslinking in mammalian cells followed by biotin-based enrichment enabled the identification of a set of high-confidence PPIs, each supported by multiple independent crosslinked products. The benchmarking of the method revealed that short crosslinks are incorporated, and that short distance restraints can be used for reliable structure modeling of PPI surfaces. Moreover, the efficient enrichment enabled identification of previously undocumented PPIs in cells, further demonstrating the applicability of crosslinking via Cys umpolung. In addition, identification of the conjugation products with free cysteine and glutathione in cells further indicates the potential applicability of the method for probing protein–ligand interactions *in cellulo*.

Taken together, the results demonstrate that episulfonium-mediated Cys umpolung provides a broadly applicable approach for fast bioconjugation, intracellular structure probing, and PPI identification through a shared reaction mechanism. The work shows that the design of protein conjugation reactions around *in situ* generated reactive linchpins, rather than the utilization of prefunctionalized reagents for different purposes, offers a route to versatile protein functionalization and crosslinking in complex biological systems and motivates the development of analogous approaches for selective targeting of residues beyond Cys.

Experimental section

NOTE: For each experimental section, a dedicated statement at the beginning of the section describes the author's contributions in detail.

Materials and methods

Starting materials

Reagents

N-ethylmaleimide (**NEM**) was purchased from ThermoFischer (#156100050). *N*-hex-5-ynyl-2-iodoacetamide (**IAA** alkyne) was purchased from BLD Pharmatech (#BD324491). 6-FAM alkyne was purchased from Lumiprobe (#C51B0). 6-FAM azide was purchased from Carl Roth (#7806.2). Biotin-PEG3-alkyne was purchased from BroadPharm (#BP-40761). Biotin-PEG1-azide was purchased from BroadPharm (#BP-40775). THPTA was purchased from TCI Chemicals (#T3171). TCEP•HCl was purchased from Carl Roth (#HN95.1). DTT was purchased from Chempur (#GC3928). 3-(Allyloxy)-1-propyne was purchased from Sigma Aldrich (#763659).

Solvents

Water used to prepare buffers and as solvent was of ultra-high quality (UHQ) grade (18.2 MΩ·cm⁻¹). Methanol (>99%) was purchased from Sigma-Aldrich, dichloromethane (>99%), dimethylsulfoxide (>99%), dimethylformamide (>99%), and acetonitrile (>99%) were purchased from Fisher Scientific. Anhydrous DCM was obtained from Phoenix Solvent Drying Systems.

Mass spectrometry

High resolution Mass Spectrometry (HRMS) experiments for small molecules and peptides were performed on a Thermo Scientific™ Q Exactive Plus or a Thermo Scientific™ Q Exactive GC Orbitrap device.

NMR Spectroscopy

Chemical shifts are reported in ppm (δ) relative to tetramethylsilane (TMS) with the solvent (residual) peak as the internal standard. For ¹H NMR: HDO, δ 4.79; (CHD₂)(CD₃)NC(O)D, δ 2.75; (CHD₂)(CD₃)SO, δ 2.50; CHD₂CN, δ 1.94. For ¹³C NMR: (CD₃)₂NC(O)D, δ 29.76; (CD₃)₂SO, δ 39.52; CD₃CN, δ 1.323. ¹⁹F and ¹⁵N NMR spectra were referenced relative to ¹H using a unified chemical shift scale. Data are reported as follows: s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, m = multiplet, br = broad; coupling constants in Hz.

NMR spectra of small molecules and peptides were recorded on following instruments:

- Bruker Avance III 500 spectrometer equipped with a BBFO probe head, operating at 500 MHz, 471 MHz, and 126 MHz, for ¹H, ¹⁹F, and ¹³C acquisitions, respectively.
- Bruker Avance III 500 spectrometer equipped with a BBFO probe head, operating at 470 MHz for ¹⁹F acquisitions.
- AVANCE III 600 spectrometer equipped with a triple-channel "TCI" cryogenic probehead

(Bruker GmbH, Rheinstetten) operating at 600 MHz, 61 MHz, and 151 MHz, for ^1H , ^{15}N , and ^{13}C acquisitions, respectively. All experiments used standard Bruker pulse sequences with standard parameter sets found in libraries of Topspin 3.6.

LC–MS

Analytical LC–MS measurements of small molecules and peptides

Analytical LC–MS measurements of small molecules and peptides were performed on following instruments:

- Agilent 1260 Infinity Automated LC System
- Agilent 1290 infinity II
- Shimadzu LCMS-2020

The utilized methods are described in detail for each performed analysis.

Analytical LC–MS measurements of proteins

Measurements were performed on Shimadzu LCMS-9030 with SCL-40, 2x LC-40D XS, SIL-40C XS, CTO-40C, SPD-M30A, equipped with a YMC mm Accura Triart Bio C4 column, 100 mm × 2.1 mm, 3 μm , flow rate 0.2 mL / min, 323 K.

Eluent A: 0.1% formic acid in UHQ- H_2O ; Eluent B: acetonitrile + 0.1% formic acid.

The samples were separated using a linear gradient method. Initially, the gradient began with an isocratic condition of 10% B for 2.5 minutes, transitioning to 80% B over a period of 12.5 minutes. This was followed by a 3-minute isocratic run at 80% B. Subsequently, a linear gradient was applied to return to 10% B over 18 seconds. Online desalting of the samples was performed using an Agilent AdvanceBio Desalting-RP column (dimensions: 12.5 x 2.1 mm) with a mobile phase of 10% B from 0.32 to 2.5 minutes, at a flow rate of 0.4 mL/min. Sample injection occurred at 0.1 minutes. Absorption spectra were recorded at a wavelength of 214 nm using a diode array detector (DAD).

LabSolutions 5.128 and LabSolutions Insight Explore 4.2 software was used for analysis and deconvolution.

Preparative HPLC separations

Preparative HPLC separations of small molecules and peptides were performed on a Shimadzu system using 2 x LC-20AP, SIL-20A HT with 2 mL sample coil, CTO-20AC, SPD-20A variable cell 0.5 mm, CBM-20A, and FRC-10A modules.

LC–MS/MS

LC–MS/MS method A

Acquisition of mass spectra was performed on an Orbitrap Eclipse Tribrid Mass Spectrometer (Thermo Fisher Scientific) connected to a Vanquish Neo UHPLC system (Thermo Fisher Scientific). The samples were dissolved in 0.1% (v/v) aqueous formic acid, loaded onto a trapping column (Acclaim PepMap100, C18, 5 μm , 100 Å, 300 μm i.d. × 5 mm, Thermo Scientific) using a loading volume of 5 μL , and eluted onto the analytical column in reverse direction. Separation was achieved on a PepMap Neo capillary column (C-18, 50 cm, 75 μm internal diameter, 2 μm particle size, 100 Å pore size, Thermo Fisher Scientific). The peptide separation was performed with a binary mobile phase gradient using 0.1% (v/v) aqueous formic acid as mobile phase A

and 0.1% (v/v) formic acid in acetonitrile as mobile phase B at a flow rate of 300 nL min⁻¹: 0–103 min, 1.5→24% B; 103–128 min, 24→32% B; 128–135 min, 32→80% B; and 135–139 min, 80→90% B. Electrospray ionization was achieved with a Nanospray Flex ESI source equipped with a stainless steel emitter.

The MS data were acquired in data-dependent mode with varying cycle settings. In each case, the full-scan mass spectrum was recorded in the Orbitrap at a resolution of R = 60,000 (FWHM at m/z 200) in the range of m/z 375 to 1500. Ions with a precursor charge state between +2 and +5 were isolated using a 2 Da isolation window and fragmented using Higher-energy collisional dissociation at a normalized energy level of 30%. Dynamic exclusion was enabled after two repeats with 30 s exclusion duration. Fragment spectra of intensity ordered parent ions (charge state +2 to +5) were recorded in the linear ion trap over a cycle time of 2 s using rapid resolution setting. A Real-Time-Search was employed, searching for modified target peptides against the corresponding FASTA database for the respective organism. Identified hits were analyzed using an additional MS² scan in the Orbitrap at a resolution of R = 15,000 (FWHM at m/z 200).

LC–MS/MS method B

Acquisition of mass spectra was performed on an Orbitrap Eclipse Tribrid Mass Spectrometer (Thermo Fisher Scientific) connected to a Vanquish Neo UHPLC system (Thermo Fisher Scientific). The samples were dissolved in 0.1% (v/v) aqueous formic acid, loaded onto a trapping column (Acclaim PepMap100, C18, 5 µm, 100 Å, 300 µm i.d. × 5 mm, Thermo Scientific) using a loading volume of 5 µL, and eluted onto the analytical column in reverse direction. Separation was achieved on a PepMap Neo capillary column (C-18, 50 cm, 75 µm internal diameter, 2 µm particle size, 100 Å pore size, Thermo Fisher Scientific). The peptide separation was performed with a binary mobile phase gradient using 0.1% (v/v) aqueous formic acid as mobile phase A and 0.1% (v/v) formic acid in acetonitrile as mobile phase B at a flow rate of 300 nL min⁻¹: 0–103 min, 1.5→24% B; 103–128 min, 24→32% B; 128–135 min, 32→80% B; and 135–139 min, 80→90% B. Electrospray ionization was achieved with a Nanospray Flex ESI source equipped with a stainless steel emitter.

The MS data were acquired in data-dependent mode with varying cycle settings. In each case, the full-scan mass spectrum was recorded in the Orbitrap at a resolution of R = 60,000 (FWHM at m/z 200) in the range of m/z 375 to 1500. Ions with a precursor charge state between +2 and +5 were isolated using a 2 Da isolation window and fragmented using Higher-energy collisional dissociation at a normalized energy level of 30%. Dynamic exclusion was enabled after two repeats with 30 s exclusion duration. Fragment spectra of the 20 most intense parent ions (charge state +2 to +5) from the full scan spectrum were recorded in the Orbitrap at a resolution of R = 15,000 (FWHM at m/z 200).

LC–MS/MS method C

Acquisition of mass spectra was performed on an Orbitrap Eclipse Tribrid Mass Spectrometer (Thermo Fisher Scientific) connected to a Vanquish Neo UHPLC system (Thermo Fisher Scientific). The samples were dissolved in 0.1% (v/v) aqueous formic acid, loaded onto a trapping column (Acclaim PepMap100, C18, 5 µm, 100 Å, 300 µm i.d. × 5 mm, Thermo Scientific) using a loading volume of 5 µL, and eluted onto the analytical column in reverse direction. Separation was achieved on a PepMap Neo capillary column (C-18, 50 cm, 75 µm internal diameter, 2 µm

particle size, 100 Å pore size, Thermo Fisher Scientific). The peptide separation was performed with a binary mobile phase gradient using 0.1% (v/v) aqueous formic acid as mobile phase A and 0.1% (v/v) formic acid in acetonitrile as mobile phase B at a flow rate of 300 nL min⁻¹: 0–103 min, 1.5→24% B; 103–128 min, 24→32% B; 128–135 min, 32→80% B; and 135–139 min, 80→90% B. Electrospray ionization was achieved with a Nanospray Flex ESI source equipped with a stainless steel emitter.

The MS data were acquired in data-dependent mode with varying cycle settings. In each case, the full-scan mass spectrum was recorded in the Orbitrap at a resolution of R = 60,000 (FWHM at m/z 200) in the range of m/z 375 to 1500. Ions with a precursor charge state between +3 and +8 were isolated using a 2 Da isolation window and fragmented using higher-energy collisional dissociation at a normalized energy level of 30%. Dynamic exclusion was enabled after two repeats with 30 s exclusion duration. Fragment spectra of the 10 most intense parent ions (charge state +3 to +8) from the full scan spectrum were recorded in the Orbitrap at a resolution of R = 15,000 (FWHM at m/z 200).

LC–MS/MS method D

Acquisition of mass spectra was performed on an Exploris 480 mass spectrometer (Thermo Scientific) coupled to an RSLCnano UHPLC system (Thermo Scientific). The samples were dissolved in 3% (v/v) formic acid / 1% (v/v) acetonitrile, loaded onto a trapping column (Acclaim PepMap100, C18, 5 µm, 100 Å, 300 µm i.d. × 5 mm, Thermo Scientific) over 10 min, and then eluted onto the analytical column. Separation was achieved on an Aurora Ultimate column (C18, 25 cm, 75 µm internal diameter, IonOpticks) maintained at 45 °C. The peptide separation was performed with a binary mobile phase gradient using 0.1% (v/v) aqueous formic acid as mobile phase A and 80% (v/v) acetonitrile / 0.1% (v/v) formic acid as mobile phase B with a flow rate of 300 nL min⁻¹: 0–2 min, 2% B; 2–105 min, 2→30% B; 105–130 min, 30→40% B; 130–137 min, 40→99% B; 137–142 min, 99% B; 142–145 min, 95→2% B; and 145–160 min, 2% B.

Electrospray ionization was achieved with a 2 kV spray voltage and a capillary temperature of 275 °C. MS data were acquired in data-dependent mode based on the previously reported settings²⁰⁵. The MS1 spectra were recorded over m/z 375–1500 at a resolution of 60,000 with an automated gain control (AGC) of 300% and a minimum intensity threshold of 5 × 10³. Precursors with charge states from +3 to +8 were selected for fragmentation with dynamic exclusion set to automatic. Fragment spectra of the top 10 precursors were acquired at a resolution of 30,000, using a 1 Da isolation window, normalized stepped HCD collision energies of 21%, 27%, and 33%, normalized AGC of 100%, and a maximum injection time of 64 ms.

LC–MS/MS method E

Acquisition of mass spectra was performed on an Orbitrap Eclipse Tribrid Mass Spectrometer (Thermo Fisher Scientific) connected to a Vanquish Neo UHPLC system (Thermo Fisher Scientific). The samples were dissolved in 0.1% (v/v) aqueous formic acid, loaded onto a trapping column (Acclaim PepMap100, C18, 5 µm, 100 Å, 300 µm i.d. × 5 mm, Thermo Scientific) using a loading volume of 5 µL, and eluted onto the analytical column in reverse direction. Separation was achieved on a PepMap Neo capillary column (C-18, 50 cm, 75 µm internal diameter, 2 µm particle size, 100 Å pore size, Thermo Fisher Scientific). The peptide separation was performed with a binary mobile phase gradient using 0.1% (v/v) aqueous formic acid as mobile phase A

and 0.1% (v/v) formic acid in acetonitrile as mobile phase B at a flow rate of 300 nL min⁻¹: 0–120 min, 4.8→24.8% B; 120–125 min, 24.8→40% B; 125–130 min, 40→72% B; and 130–140 min, 90% B. Electrospray ionization was achieved with a Nanospray Flex ESI source equipped with a stainless steel emitter.

The MS data were acquired in data-dependent mode with varying cycle settings. In each case, the full-scan mass spectrum was recorded in the Orbitrap at a resolution of R = 60,000 (FWHM at m/z 200) in the range of m/z 375 to 1500. Ions with a precursor charge state between +2 and +6 were isolated using a 0.7 Da isolation window and fragmented using Higher-energy collisional dissociation at a normalized energy level of 30%. Dynamic exclusion was enabled after one repeat with 60 s exclusion duration. Fragment spectra of intensity ordered parent ions (charge state +2 to +6) were recorded in the linear Orbitrap over a cycle time of 3 s at a resolution of R = 15,000 (FWHM at m/z 200).

SDS-PAGE

SDS-PAGE sample preparation and analysis was carried out as instructed in the manual of Bio-Rad. Fluorescence measurements were carried out as instructed in the manual of Bio-Rad utilizing the “Fluorescein” analysis of the Bio-Rad Laboratories ChemiDocMP system. Coomassie Blue staining of the gels was performed as instructed by the manufacturer. All proteins were analyzed with Tris/Glycine/SDS gel electrophoresis. Image Lab Version 6.1.0 build 7 software from Bio-Rad Laboratories Inc. was used for analysis and processing of SDS-PAGE data. Reagents used in the SDS-PAGE workflow:

- Bio-Rad Laboratories Any kD MP TGX Stain-Free 10W 30 µL
- Bio-Rad Laboratories 10x Tris/Glycine/SDS buffer
- Bio-Rad Laboratories 4x Laemmli sample buffer
- Bio-Rad Laboratories QC Colloidal Coomassie stain
- Bio-Rad Laboratories Precision Plus Protein™ Dual Xtra Prestained Protein Standards

Devices used in the SDS-PAGE workflow:

- Bio-Rad Laboratories ChemiDocMP
- Bio-Rad Laboratories Mini-PROTEAN Tetra Cell
- Bio-Rad Laboratories PowerPac Basic

Miscellaneous

Lyophilization of purified peptides was performed using a BÜCHI Lyovapor™ L-200. Centrifugation was performed using a Thermo Scientific™ MegaFuge™ 8R. Pipetting of small volumes was performed with an Eppendorf Research® plus – Mechanical Pipette, 1-channel, 0.5–10 µL, an Eppendorf Eppendorf Research® plus - Mechanical Pipette, 1-channel, 10–100 µL, or an Eppendorf Eppendorf Research® plus – Mechanical Pipette, 1-channel, 100–1,000 µL. Protein reactions were conducted in an Eppendorf ThermoMixer® C equipped with an Eppendorf SmartBlock™ 1.5 mL. Sample preparation for LC–MS/MS analysis was performed in Eppendorf Protein LoBind® Tubes.

Protein desalting and rebuffing was performed with AMICON[®] filter units from Sigma Aldrich. Protein concentrations were determined by A280 absorption or BCA assay (ThermoFisher, #23225) using a Thermo Scientific[™] NanoDrop[™] One^C. Extinction coefficients of the proteins used in this work were calculated based on the FASTA sequences with the assumption that all Cys residues are reduced. Lysate concentrations were determined via BCA assay. Fluorescence measurements were performed on Plate reader SpectraMax iD3 (Molecular Devices) and the results were analyzed with Soft Max Pro[®] 7 software. The Countess 3 from Invitrogen was used for determination of the number of mammalian cells. Cell lysis via sonication was performed with BANDELIN Sonoplus HD 2070 with Sonotrode MS 73. Precellys Evolution system from Bertin Technologies was used for cell homogenization. Unless stated otherwise, Milli-Q[®] water (MQ-H₂O) was used in all experiments.

Synthesis of compounds

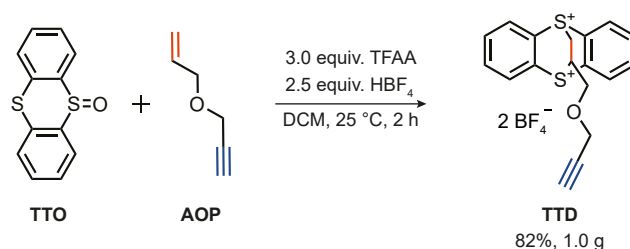
Thianthrenium salts

Vinylthianthrenium reagents

Synthesis of **VTT**, the dicationic precursor for the synthesis of **VTT**, **²H₃-VTT**, **²H₃-VTFT**, and **VTFT** was performed according to the reported methods^{37,44}.

TTD

TTD was synthesized and characterized by the author.



Under ambient atmosphere, a 25 mL round-bottom flask was equipped with a Teflon-coated magnetic stirring bar, thianthrene-S-oxide (580 mg, 2.50 mmol, 1.00 equiv.), allyloxy-1-propyne (260 mg, 2.65 mmol, 1.05 equiv.), and 10.0 mL of anhydrous DCM (c = 0.25 M). The flask was capped with a rubber septum, a balloon filled with argon was connected, and the mixture was cooled down to -40 °C in an acetonitrile dry ice bath. Trifluoroacetic anhydride (1.0 mL, 1.6 g, 7.5 mmol, 3.0 equiv.) was added to the stirring (500 rpm) mixture, followed by dropwise (2 drops / second) addition of tetrafluoroboric acid-diethyl ether complex (0.85 mL, 1.0 g, 6.2 mmol, 2.5 equiv.). The mixture was stirred for 30 minutes at -40 °C. Afterwards, the cooling bath was removed and the mixture was stirred for 3.5 hours at 25 °C. The balloon and the rubber septum were removed, the precipitate was collected by filtration, washed with DCM (20 mL), Et₂O (20 mL), and dried under vacuum to give the title compound as a colorless powder (1.00 g, 2.06 mmol, 82.4% yield).

NMR Spectroscopy:

¹H NMR (600 MHz, CD₃CN, 298 K, δ): 8.58 – 8.50 (m, 4H), 8.15 – 8.07 (m, 4H), 4.82 (dtd, J = 8.9, 5.9, 4.0 Hz, 1H), 4.42 (dd, J = 13.7, 8.9 Hz, 1H), 4.28 (dd, J = 11.4, 4.0 Hz, 1H), 4.14 – 4.04 (m, 2H), 3.83 (dd, J = 13.7, 5.8 Hz, 1H), 3.53 (dd, J = 11.4, 6.0 Hz, 1H), 2.81 (t, J = 2.4 Hz, 1H).

¹³C NMR (151 MHz, CD₃CN, 298 K, δ): 137.5, 137.1, 137.0, 137.0, 136.9, 136.8, 136.6, 136.3, 126.8, 126.3, 125.4, 124.9, 78.8, 77.9, 66.7, 59.5, 56.2, 40.1.

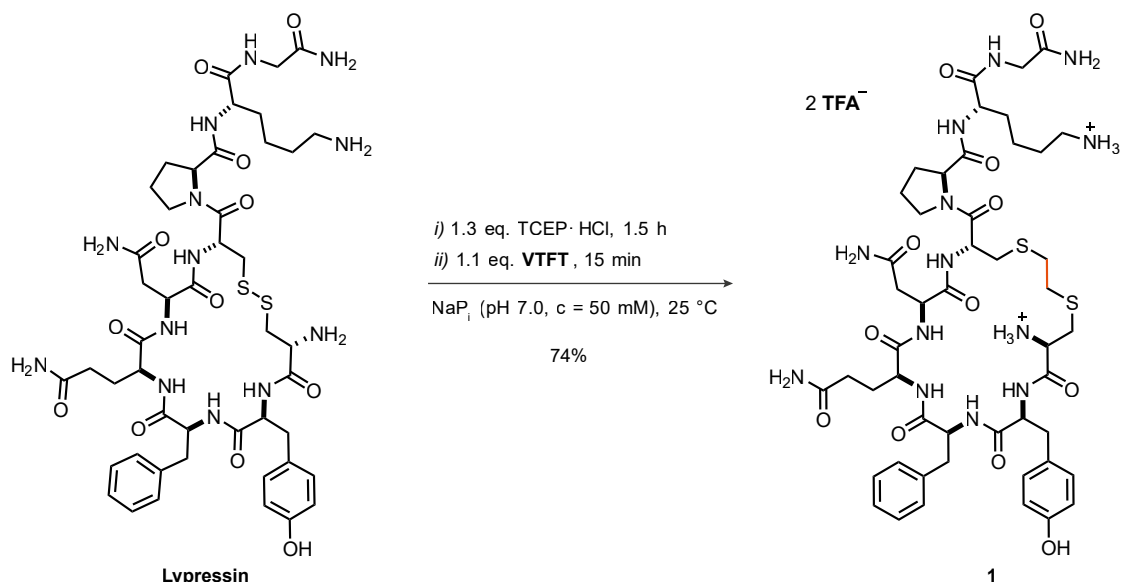
¹⁹F NMR (471 MHz, CD₃CN, 298 K, δ): -151.0.

HRMS-ESI (m/z) calc'd for C₁₈H₁₆OS₂ [M-2BF₄]²⁺, 156.0316; found, 156.0316; deviation: 0.0 ppm.

Ethylene-bridged peptides

The author performed synthesis and isolation of the ethylene-bridged peptides. Marie Sophie Sterling and Heike Hinrichs performed the LC–MS analysis. Pia Münstermann, Nadine Sauerborn, and Corinna Heidgen performed HPLC purifications. Julia Beatrice Jacobs acquired and analyzed the NMR data for the isolated peptides, and Frank Kohler performed the MS and MS/MS analysis of the obtained products.

Ethylene-bridged lypressin (1)



At 20–25 °C, a round-bottom flask (25 mL) was charged with lypressin (10.5 mg, 9.94 μmol , 1.00 equiv.) and a Teflon-coated magnetic stirring bar. Then, 5.0 mL of sodium phosphate buffer (pH 7.0, $c = 100 \text{ mM}$) and 4.1 mL of H_2O were added to the flask and the starting material was dissolved while stirring at 300 rpm. Next, a freshly prepared 20 mM solution of TCEP·HCl in H_2O (0.65 mL, 13 μmol , 1.3 equiv.) was added to the flask and the mixture was stirred at 300 rpm for 1.5 hours at 25 °C. A 50 mM solution of **VTFT** in DMF (0.21 mL, 10 μmol , 1.1 equiv.) was added dropwise over 1 minute and the mixture was stirred for 15 minutes. The reaction mixture was filtered, the filtrate was collected, the filter cake was washed with H_2O (6 x 5 mL), and the combined filtrate was lyophilized. The obtained residue was purified by HPLC on a YMC Triart C18 column ((150×30mm, 5 μm) with an YMC Triart C18 precolumn (50×30mm, 5 μm), flow rate = 42.5 mL/min, 35°C) with a linear gradient from 10:90 v/v (MeCN : 0.1% v/v TFA in H_2O) to 22:78 v/v (MeCN : 0.1% v/v TFA in H_2O) over 18.0 minutes. The fractions containing the product ($t \approx 16.9$ minutes) were collected and lyophilized to afford the title compound as a colorless powder (9.7 mg, 7.4 μmol , 74% yield).

NMR Spectroscopy:

^1H NMR (600 MHz, DMF-d_7 , 298 K, δ): 9.56 (s, 1H), 9.22 (d, $J = 8.4 \text{ Hz}$, 1H), 8.85 (d, $J = 5.1 \text{ Hz}$, 1H), 8.85 (d, $J = 5.0 \text{ Hz}$, 1H), 8.24 (d, $J = 8.0 \text{ Hz}$, 1H), 8.03 (d, $J = 8.5 \text{ Hz}$, 1H), 8.02 (t, $J = 6.0 \text{ Hz}$, 1H), 7.77 (d, $J = 8.0 \text{ Hz}$, 1H), 7.73 – 7.71 (m, 1H), 7.53 – 7.51 (m, 1H), 7.36 (s, 1H), 7.35 – 7.29 (m, 4H), 7.26 – 7.23 (m, 1H), 7.14 (d, $J = 1.6 \text{ Hz}$, 1H), 7.11 (s, 1H), 7.08 – 7.04 (m, 2H), 6.92 (s, 1H), 6.74 – 6.69 (m, 2H), 4.85 (q, $J = 5.6 \text{ Hz}$, 2H), 4.83 (td, $J = 8.5, 5.3 \text{ Hz}$, 1H), 4.63 (ddd, $J = 10.0, 8.3, 5.3 \text{ Hz}$, 1H), 4.51 (dt, $J = 9.6, 5.3 \text{ Hz}$,

1H), 4.42 (t, *J* = 5.8 Hz, 1H), 4.39 (dd, *J* = 8.5, 4.3 Hz, 1H), 4.33 (td, *J* = 10.0, 4.5 Hz, 1H), 4.11 (dt, *J* = 8.5, 5.0 Hz, 1H), 3.84 (dd, *J* = 16.9, 6.1 Hz, 1H), 3.76 (dd, *J* = 16.9, 5.8 Hz, 1H), 3.75 (t, *J* = 6.8 Hz, 2H), 3.32 (dd, *J* = 14.6, 5.1 Hz, 1H), 3.28 (dd, *J* = 14.2, 5.3 Hz, 1H), 3.17 (dd, *J* = 14.6, 6.5 Hz, 1H), 3.12 (dd, *J* = 14.1, 9.6 Hz, 1H), 3.11 (dd, *J* = 13.9, 5.3 Hz, 1H), 3.06 (dd, *J* = 14.4, 5.6 Hz, 1H), 3.04 (t, *J* = 7.6 Hz, 2H), 2.94 (dd, *J* = 14.4, 7.6 Hz, 1H), 2.88 (dd, *J* = 15.8, 8.0 Hz, 1H), 2.86 – 2.84 (m, 1H), 2.81 (dd, *J* = 14.0, 10.0 Hz, 1H), 2.80 (dd, *J* = 15.8, 5.1 Hz, 2H), 2.79 – 2.78 (m, 2H), 2.40 – 2.34 (m, 2H), 2.17 – 2.11 (m, 1H), 2.09 (dtd, *J* = 14.1, 7.4, 5.0 Hz, 1H), 2.04 – 1.90 (m, 5H), 1.78 – 1.66 (m, 3H), 1.58 – 1.45 (m, 2H).

¹³C NMR (151 MHz, DMF-d₇, 298 K, δ): 175.2, 173.3, 172.9, 172.6, 172.3, 171.9, 171.7, 169.9, 168.3, 159.6, 138.0, 130.4, 129.6, 128.6, 128.2, 126.8, 117.15 (q, *J* = 300.0 Hz), 115.3, 61.3, 57.1, 56.0, 55.3, 53.3, 52.6, 52.3, 50.4, 47.6, 42.5, 39.9, 37.3, 37.0, 36.6, 33.6, 33.2, 32.8, 32.7, 31.8, 30.8, 26.9, 25.1, 22.8.

¹⁹F NMR (471 MHz, DMF-d₇, 298 K, δ): –74.9.

HRMS-ESI (*m/z*) calc'd for C₄₈H₇₁N₁₃O₁₂S₂ [M–2TFA]²⁺, 542.7388; found, 542.7391; deviation: +0.6 ppm. The full NMR-characterization and MS/MS spectra of the product are reported elsewhere⁴⁴.

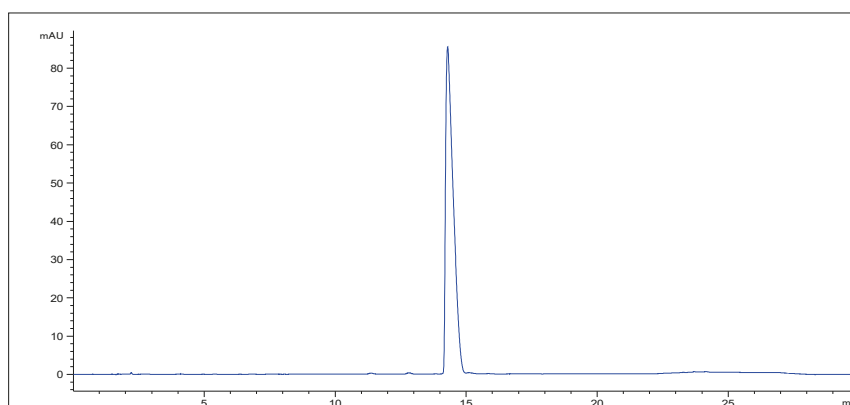
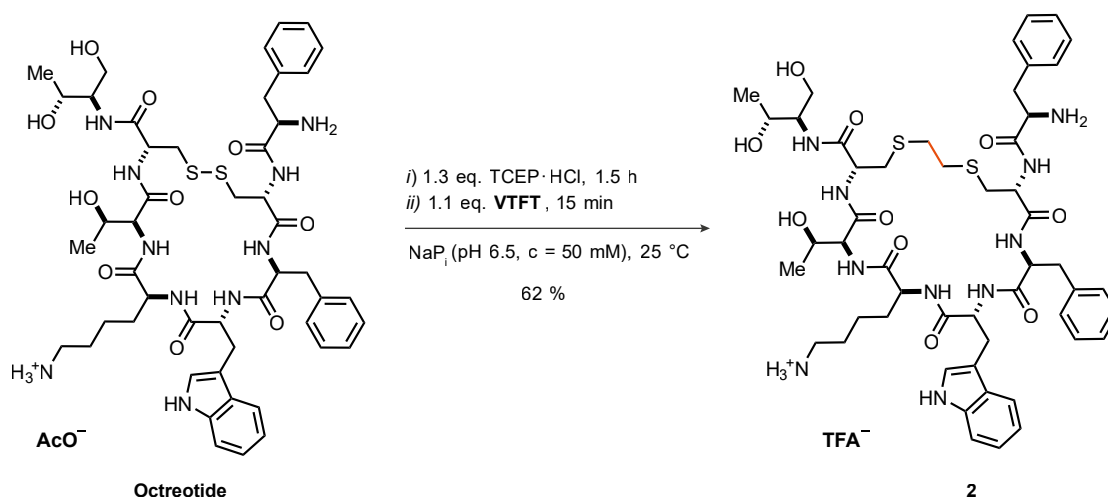


Figure E.1: Analytical HPLC trace of **1** (YMC-Triart C18 column, 150 x 4.6 mm, 5 μ m, flow rate = 1.0 mL $\cdot$ min^{–1}) with a linear gradient from 10:90 v/v (MeCN : 0.1% v/v TFA in H₂O) to 22:78 v/v (MeCN : 0.1% v/v TFA in H₂O) over 18.0 minutes.

Ethylene-bridged octreotide (**2**)



At 20–25 °C, a round-bottom flask (25 mL) was charged with octreotide acetate (11.3 mg, 10.5 μ mol, 1.00 equiv.) and a Teflon-coated magnetic stirring bar. Then, 5.2 mL of sodium phosphate buffer (pH 6.5, c = 100 mM) and 4.3 mL of H₂O were added to the flask and the starting material was dissolved while stirring at 300 rpm. Next, a freshly prepared 20 mM solution of TCEP·HCl in H₂O (0.68 mL, 14 μ mol, 1.3 equiv.) was added to the flask and the mixture was stirred at 300 rpm for 1.5 hours at 25 °C. A 50 mM solution of **VTFT** in DMF (0.22 mL, 11 μ mol, 1.1 equiv.) was added dropwise over 1 minute and the mixture was stirred for 15 minutes. The reaction mixture was filtered, the filtrate was collected, the filter cake was washed with H₂O (6 x 5 mL), and the combined filtrate was lyophilized. The obtained residue was purified by HPLC on a YMC Triart C18 column ((150×30mm, 5 μ m) with a YMC Triart C18 precolumn (50×30mm, 5 μ m), flow rate = 42.5 mL/min, 35°C) with a linear gradient from 23:77 v/v (MeCN : 0.1% v/v TFA in H₂O) to 27:73 v/v (MeCN : 0.1% v/v TFA in H₂O) over 33.5 minutes. The fractions containing the product (t $\approx$ 24.8 min) were collected and lyophilized to afford the title compound as a colorless powder (7.6 mg, 6.5 μ mol, 62% yield).

NMR Spectroscopy:

¹H NMR (600 MHz, DMF-d₇, 298 K, δ): 10.99 (s, 1H), 9.36 (d, J = 9.2 Hz, 1H), 9.06 (d, J = 4.9 Hz, 1H), 8.86 (d, J = 8.9 Hz, 1H), 8.56 (d, J = 7.6 Hz, 1H), 8.49 (d, J = 9.0 Hz, 1H), 8.11 (d, J = 9.3 Hz, 1H), 7.93 (d, J = 9.0 Hz, 1H), 7.50 (m, 2H), 7.49 (d, J = 8.0 Hz, 1H), 7.44 (d, J = 8.1 Hz, 1H), 7.37 (m, 2H), 7.33 (m, 1H), 7.30 (m, 2H), 7.25 (m, 1H), 7.19 (m, 2H), 7.11 (ddd, J = 8.2, 7.0, 1.2 Hz, 1H), 7.08 (s, 1H), 7.03 (ddd, J = 7.9, 6.9, 1.0 Hz, 1H), 5.19 (dt, J = 9.2, 6.5 Hz, 1H), 5.18 (dt, J = 8.9, 7.5 Hz, 1H), 5.00 (s, 1H), 4.75 (ddd, J = 8.3, 7.6, 6.4 Hz, 1H), 4.71 (dd, J = 9.1, 6.0 Hz, 1H), 4.51 (s, 1H), 4.38 (ddd, J = 9.2, 6.3, 5.2 Hz, 1H), 3.88 (dddd, J = 8.9, 7.8, 5.3, 2.3 Hz, 1H), 3.71 (dd, J = 10.7, 7.8 Hz, 1H), 3.62 (dd, J = 10.8, 5.3 Hz, 1H), 3.17 (dd, J = 14.3, 9.2 Hz, 1H), 1.90 (dddd, J = 13.8, 10.0, 6.7, 3.2 Hz, 1H), 1.59 (m, 1H), 1.52 (m, 1H), 1.37 (dtd, J = 13.8, 10.5, 4.5 Hz, 1H), 1.22 (d, J = 6.4 Hz, 3H), 1.15 (d, J = 6.4 Hz, 3H), 0.96 (m, 1H), 0.89 (m, 1H).

¹³C NMR (151 MHz, DMF-d₇, 298 K, δ): 172.3, 171.9, 171.8, 171.7, 170.6, 170.2, 169.8, 159.5 (br m), 137.6, 137.0, 135.9, 130.1, 129.4, 128.9, 128.6, 127.7, 127.5, 126.9, 124.1, 121.4, 118.7, 118.5, 118.1 (q, J = 296.0 Hz), 111.8, 109.7, 68.3, 65.2, 61.5, 59.4, 56.5, 56.2, 55.3, 55.1, 53.0, 51.8, 51.4, 39.7, 38.8, 38.6, 34.1, 33.6, 30.5, 30.3, 30.0, 27.2, 26.4, 22.7, 19.9, 19.7.

¹⁹F NMR (471 MHz, DMF-d₇, 298 K, δ): –74.8.

HRMS-ESI (m/z) calc'd for C₅₁H₇₁N₁₀O₁₀S₂ [M–TFA]⁺, 1047.479; found, 1047.479; deviation: 0.0 ppm. The full NMR-characterization and MS/MS spectra of the product are reported elsewhere⁴⁴.

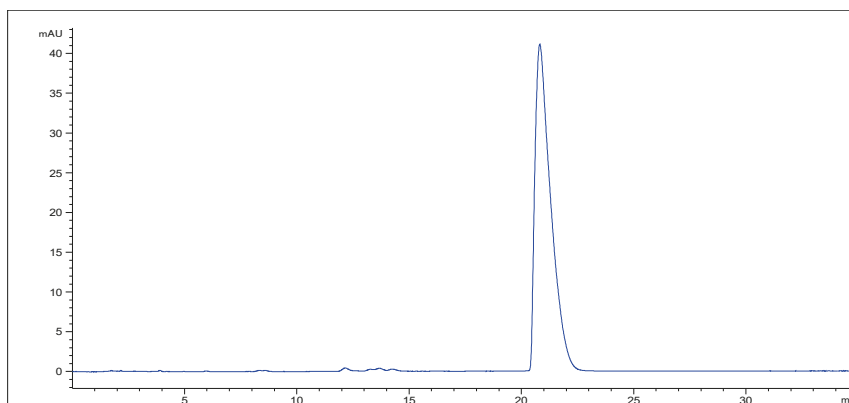
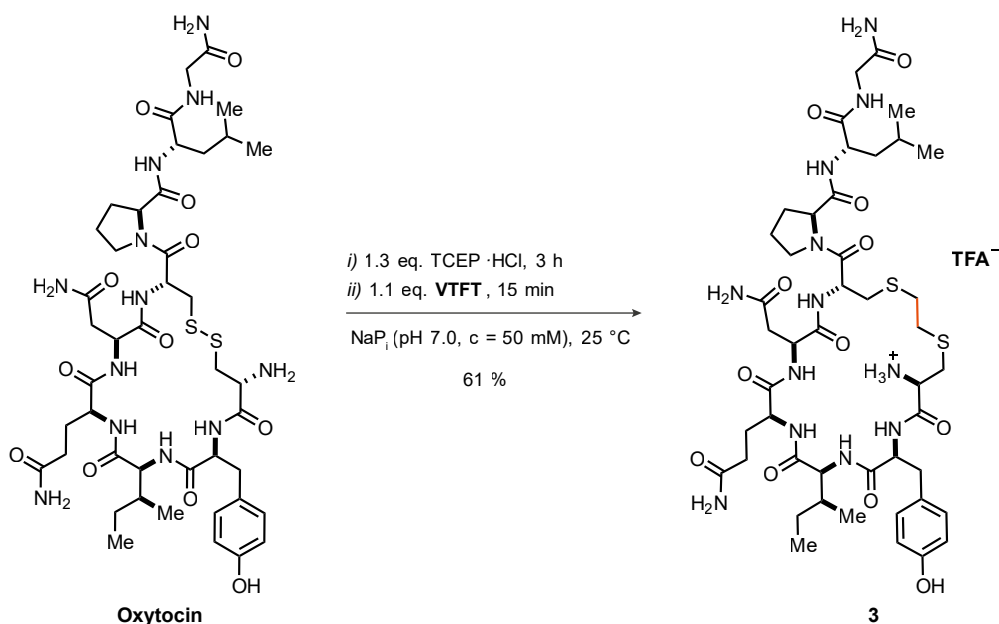


Figure E.2: Analytical HPLC trace of **2** (YMC-Triart C18 column, 150 x 4.6 mm, 5 μ m, flow rate = 1.0 mL $\cdot$ min⁻¹) with a linear gradient from 23:77 v/v (MeCN : 0.1% v/v TFA in H₂O) to 26:74 v/v (MeCN : 0.1% v/v TFA in H₂O) over 25 minutes.

Ethylene-bridged oxytocin (**3**)



At 20–25 °C, a round-bottom flask (25 mL) was charged with oxytocin (10.0 mg, 9.97 μ mol, 1.00 equiv.) and a Teflon-coated magnetic stirring bar. Then, 5.0 mL of sodium phosphate buffer (pH 7.0, c = 100 mM) and 4.1 mL of H₂O were added to the flask and the starting material was dissolved while stirring at 300 rpm. Next, a freshly prepared 20 mM solution of TCEP $\cdot$ HCl in H₂O (0.65 mL, 13 μ mol, 1.3 equiv.) was added to the flask and the mixture was stirred at 300 rpm for 3 hours at 25 °C. A 50 mM solution of VTFT in DMF (0.21 mL, 10 μ mol, 1.1 equiv.) was added dropwise over 1 minute and the mixture was stirred for 15 minutes. The reaction mixture was filtered, the filtrate was collected, the filter cake was washed with H₂O (6 x 5 mL), and the combined filtrate was lyophilized. The obtained residue was purified by HPLC on a YMC Triart C18 column ((150 x 30 mm, 5 μ m) with a YMC Triart C18 precolumn (50 x 30 mm, 5 μ m), flow rate = 42.5 mL/min, 35 °C) with a linear gradient from 20:80 v/v (MeCN : 0.1% v/v TFA in H₂O) to 27:73 v/v (MeCN : 0.1% v/v TFA in H₂O) over 10.5 minutes, followed by linear gradient to 60:40 v/v (MeCN : 0.1% v/v TFA in H₂O) over 1.5 minutes. The fractions containing the product (t $\approx$ 10.4 min) were collected and lyophilized to afford the title compound as a colorless powder (7.0

mg, 6.1 μ mol, 61% yield).

NMR Spectroscopy:

^1H NMR (600 MHz, DMF-d_7 , 298 K, δ): 9.43 (d, J = 8.1 Hz, 1H), 8.92 (s, 1H), 8.88 (d, J = 4.5 Hz, 1H), 8.70 (s, 3H), 8.20 (d, J = 8.0 Hz, 1H), 7.98 (t, J = 6.5 Hz, 1H), 7.96 (s, 1H), 7.86 (d, J = 8.7 Hz, 1H), 7.72 (d, J = 8.5 Hz, 1H), 7.62 (d, J = 1.7 Hz, 1H), 7.29 (d, J = 1.5 Hz, 1H), 7.28 – 7.23 (m, 2H), 7.09 (d, J = 1.5 Hz, 1H), 7.07 (d, J = 1.7 Hz, 1H), 6.94 (d, J = 1.7 Hz, 1H), 6.79 – 6.68 (m, 2H), 4.92 (dt, J = 8.5, 6.5 Hz, 1H), 4.91 (td, J = 9.5, 4.5 Hz, 1H), 4.79 (ddd, J = 11.3, 8.1, 3.4 Hz, 1H), 4.52 (t, J = 5.8 Hz, 1H), 4.42 – 4.37 (m, 1H), 4.35 (q, J = 7.8 Hz, 1H), 3.87 (dd, J = 16.8, 6.5 Hz, 1H), 3.76 – 3.69 (m, 2H), 3.67 (dd, J = 16.8, 5.7 Hz, 1H), 3.37 (dd, J = 14.2, 3.3 Hz, 1H), 3.32 (dd, J = 14.7, 5.1 Hz, 1H), 3.26 (dd, J = 14.6, 5.8 Hz, 1H), 3.04 – 2.99 (m, 1H), 3.00 (t, J = 14.2 Hz, 2H), 2.99 (dd, J = 15.6, 9.5 Hz, 1H), 2.87 (dd, J = 15.7, 4.4 Hz, 1H), 2.84 – 2.78 (m, 3H), 2.42 (dt, J = 16.0, 6.7 Hz, 1H), 2.37 (dt, J = 15.9, 6.7 Hz, 1H), 2.20 – 2.13 (m, 1H), 2.06 – 1.90 (m, 6H), 1.69 – 1.65 (m, 3H), 1.59 (ddd, J = 13.7, 7.5, 4.8 Hz, 1H), 1.29 (ddd, J = 13.6, 8.6, 7.3 Hz, 1H), 0.99 (d, J = 6.9 Hz, 3H), 0.91 (t, J = 7.4 Hz, 3H), 0.89 (d, J = 6.3 Hz, 3H), 0.86 (d, J = 6.2 Hz, 3H).

^{13}C NMR (151 MHz, DMF-d_7 , 298 K, δ): 175.7, 174.4, 173.0, 172.8, 172.8, 172.3, 171.8, 171.8, 171.8, 170.2, 168.3, 159.3 (q, J = 35.0 Hz), 156.7, 130.6, 128.7, 117.0 (q, J = 293.0 Hz), 115.2, 61.8, 60.9, 55.9, 55.7, 52.4, 52.3, 52.1, 50.7, 47.6, 42.7, 39.9, 37.5, 36.3, 36.1, 33.9, 33.2, 33.0, 32.5, 31.8, 29.4, 26.3, 26.0, 25.1, 25.0, 23.1, 21.0, 15.6, 11.4.

^{19}F NMR (471 MHz, DMF-d_7 , 298 K, δ): –74.7.

HRMS-ESI (m/z) calc'd for $\text{C}_{45}\text{H}_{71}\text{N}_{12}\text{O}_{12}\text{S}_2$ $[\text{M-TFA}]^+$, 1035.475; found, 1035.475; deviation: 0.0 ppm. The full NMR-characterization and MS/MS spectra of the product are reported elsewhere⁴⁴.

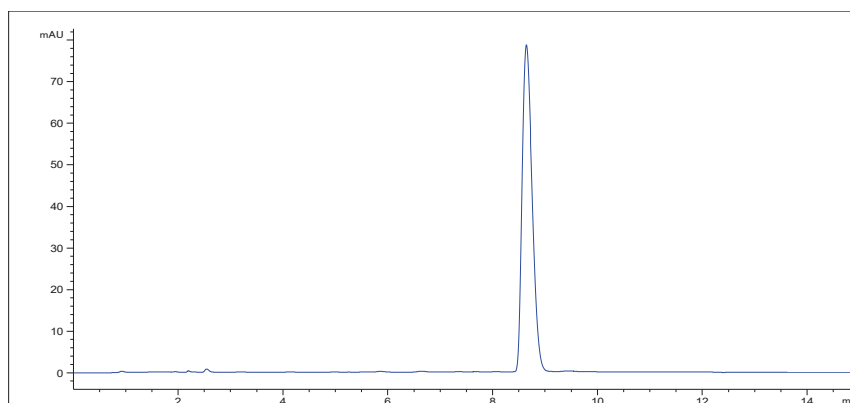
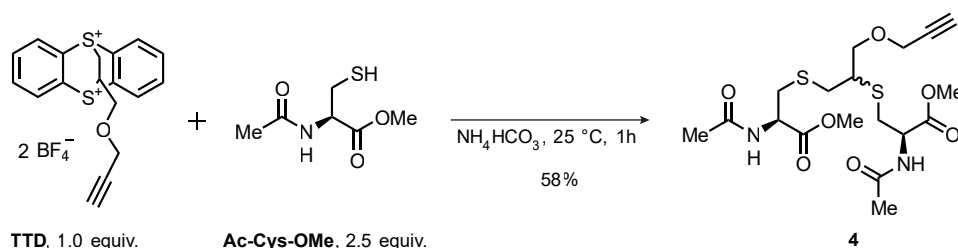


Figure E.3: Analytical HPLC trace of **3** (YMC-Triart C18 column, 150 x 4.6 mm, 5 μ m, flow rate = 1.0 mL $\cdot$ min⁻¹) with a linear gradient from 20:80 v/v (MeCN : 0.1% v/v TFA in H_2O) to 27:73 v/v (MeCN : 0.1% v/v TFA in H_2O) over 10.0 minutes.

TTD-crosslinked Cys (4)



Under ambient atmosphere, a 100 mL round-bottom flask was equipped with a Teflon-coated magnetic stirring bar and *N*-acetyl-L-Cysteine methyl ester (177 mg, 1.00 mmol, 2.50 equiv.). Then, 40 mL of a freshly prepared ammonium bicarbonate solution (0.25 M) was added, and the mixture was stirred for 2 minutes at 500 rpm at 25 °C. Next, solid **TTD** (194 mg, 400 μ mol, 1.00 equiv.) was added to the flask over 1 minute and the mixture was stirred at 500 rpm at 25 °C for 1 hour. The reaction mixture was filtered, the filtrate was collected, and the filter cake was washed with H₂O (6 x 5 mL). The combined filtrate was lyophilized and the obtained residue was purified by HPLC on a YMC Triart C18 column ((150×30 mm, 5 μ m) with a YMC Triart C18 precolumn (50×30mm, 5 μ m), flow rate = 42.5 mL/min, 35 °C) with a linear gradient from 20:80 v/v (MeCN : 0.1% v/v TFA in H₂O) to 30:70 v/v (MeCN : 0.1% v/v TFA in H₂O) over 20 minutes. The fractions containing the product ($t \approx 17$ min) were collected and lyophilized to afford the title compound as a colorless solid (103 mg, 230 μ mol, 57.5% yield).

NMR Spectroscopy:

¹H NMR (600 MHz, DMSO-*d*₆, 298 K, δ): 8.40 – 8.35 (m, 2H), 4.47 – 4.40 (m, 2H), 4.15 (t, *J* = 2.5 Hz, 2H), 3.68 – 3.62 (m, 7H), 3.60 – 3.55 (m, 1H), 3.45 – 3.42 (m, 1H), 3.08 – 3.01 (m, 1H), 3.00 – 2.89 (m, 2H), 2.86 – 2.72 (m, 4H), 1.86 (s, 6H).

¹³C NMR (151 MHz, DMSO-*d*₆, 298 K, δ): 171.3, 171.2, 171.2, 169.5, 169.5, 169.4, 80.0, 80.0, 77.5, 77.4, 70.7, 70.6, 57.7, 57.7, 52.4, 52.4, 52.2, 52.1, 45.3, 45.2, 40.1, 39.9, 39.8, 39.7, 39.4, 39.2, 39.1, 34.5, 34.3, 33.7, 33.6, 32.2, 22.3.

HRMS-ESI (*m/z*) calc'd for C₁₈H₂₈N₂O₇S₂Na [M+Na]⁺, 471.1230; found, 471.1231; deviation: 0.2 ppm. The full NMR-characterization of the product is reported elsewhere.

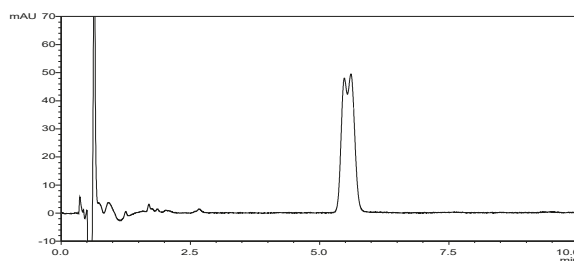
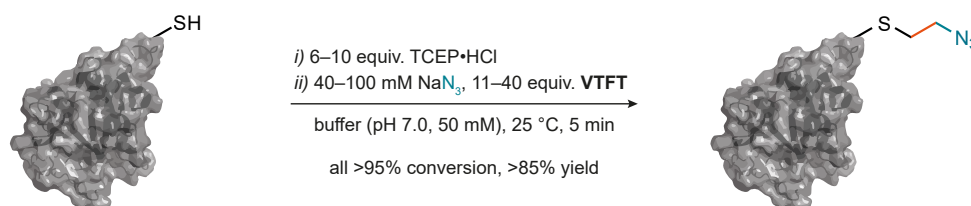


Figure E.4: Analytical (UV-Vis) HPLC trace of **4** (YMC-Triart C18 column, 50 x 3.0 mm, 1.9 μ m, flow rate = 0.5 mL/min), eluent: 20:80 v/v MeCN : 0.1% v/v TFA in H₂O.

Reactions with proteins

Dr. Lara Vogelsang synthesized the proteins used in the studies. The author, together with Dr. Philipp Hartmann and Moritz Hommrich performed protein modification reactions with **VTT** and **VTFT**. Dr. Rene Zangl, Dr. Alessandro Vetere, Marie Sophie Sterling, and Heike Hinrichs acquired the LC–MS and MS data on the modified proteins, which were interpreted by Dr. Philipp Hartmann, Dr. Rene Zangl, Dr. Alessandro Vetere, Marie Sophie Sterling, and Heike Hinrichs. Only summaries of the optimized protocols are presented below. The detailed protocols for the synthesis of each product as well as characterization of the products are reported elsewhere⁴⁴.

Protein azidation protocol with VTFT

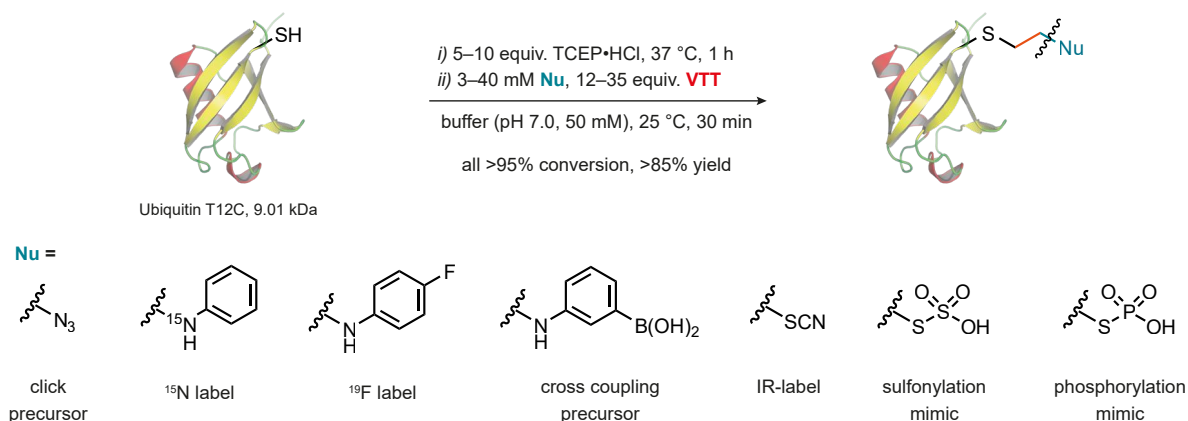


At 20–25 °C, the protein starting material in NaP_i or HEPES buffer (pH 7.0, 50 mM) was added to a 1.5 ml Eppendorf tube. Next, the protein solution was diluted with buffer, and TCEP (6–10 equiv.) in H₂O was added. The mixture was vortexed for 1 s, transferred into a Thermocycler preheated at 37 °C, and incubated at 37 °C for 1 h at 400 rpm. Next, a stock solution of sodium azide in the respective buffer (pH 7.0, 50 mM) was added to the mixture at 20–25 °C to obtain a nucleophile concentration of 40–100 mM and a protein concentration of 10–15 µM (see [Table E.1](#)). Next, **VTFT** (11–40 equiv.) was added as a stock solution in DMF. The amount of DMF did not exceed 1% v/v of the final mixture volume. The reaction mixture was vortexed for 1 s, transferred into a Thermocycler preheated at 25 °C, and incubated at 25 °C at 400 rpm for 5 min. The obtained mixture was analyzed by LC–MS and the conversion and yield were identified via integration of deconvoluted protein MS peaks.

Table E.1: Conditions used for protein modification with **VTFT**

Protein	Buffer	c _{final} (NaN ₃), mM	eq. VTFT	Conversion/Yield,%
sfGFP S147C	NaP _i	40	11	>95 / 85
PrxIIB C76S	NaP _i	100	30	>95 / 88
Trxh1 C43S	NaP _i	40	20	>95 / 87
Ubiquitin T9C	HEPES	40	20	>95 / 86
DHAR1 S97C	HEPES	40	20	>95 / 92
DHAR2 C6S	NaP _i	40	20	>95 / n.d.
MDAR3	HEPES	100	40	>95 / n.d.
BSA	NaP _i	40	20	>95 / n.d.

Protein modifications with VTT: scope of nucleophiles

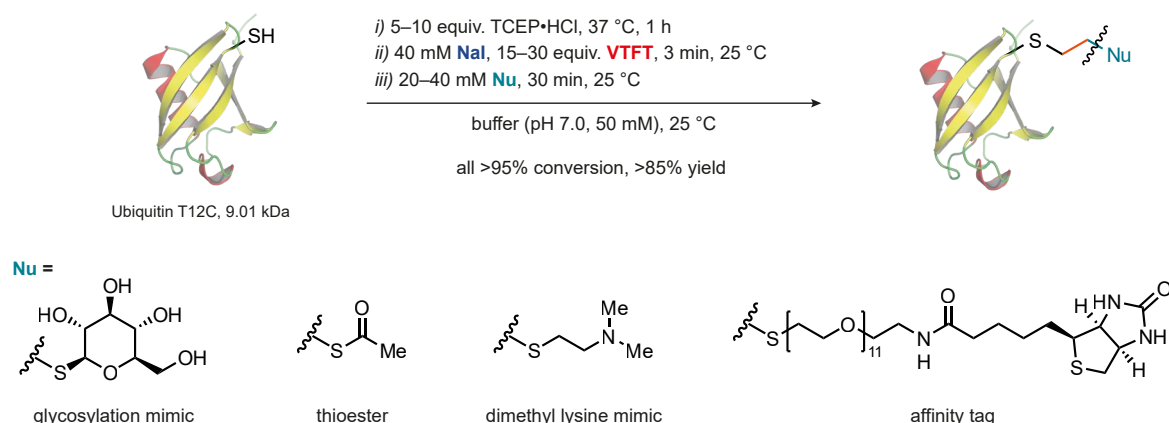


At 20–25 °C, a stock solution of ubiquitin T12C in NaP_i or HEPES (pH 7.0, 50 mM) with 125 μM DTT was added to a 1.5 ml Eppendorf tube. Next, the protein solution was diluted with buffer, and TCEP (5–10 equiv.) in H₂O was added. The mixture was vortexed for 1 s, transferred into a Thermocycler preheated at 37 °C, and incubated at 37 °C for 1 h at 400 rpm. Next, a nucleophile stock solution in the respective buffer (pH 7.0, 50 mM) was added to the mixture at 20–25 °C to obtain a nucleophile concentration of 3–40 mM and a protein concentration of 15–30 μM. The nucleophile concentration varied depending on the chosen nucleophile (see Table E.2). Next, a vinyl thianthrenium salt (12–35 equiv.) was added as a stock solution in DMF. The amount of DMF did not exceed 1% v/v of the final mixture volume. The reaction mixture was vortexed for 1 s, transferred into a Thermocycler preheated at 25 °C, and incubated at 25 °C at 400 rpm for either 5 min (**VTFT**) or 30 min (**VTT**). The obtained mixture was analyzed by LC–MS and the conversion and yield were identified via integration of deconvoluted protein MS peaks.

Table E.2: Conditions used for protein modification with **VTT** and **VTFT**

Nucleophile	c _{final} (Nu), mM	Buffer	Reagent	Conversion/Yield,%
sodium azide	40	HEPES	15 eq. VTT	>95 / 100
sodium azide	40	HEPES	20 eq. VTFT	>95 / 100
¹⁵ N-aniline	40	HEPES	15 eq. VTT	>95 / 88
4-fluoroaniline	40	HEPES	12 eq. VTT	>95 / 87
3-aminophenylboronic acid	40	HEPES	15 eq. VTT	>95 / 87
sodium thiocyanate	40	NaP _i	25 eq. VTT	>95 / 100
sodium thiosulfate	30	NaP _i	25 eq. VTT	>95 / 100
sodium thiophosphate	3	NaP _i	35 eq. VTT	>95 / 100

Protein modifications with VTT: scope of thiol-containing nucleophiles



At 20–25 °C, a ubiquitin mutant in NaP_i, HEPES or Bis-Tris buffer (pH 7.0, 50 mM) was added to a 1.5 ml Eppendorf tube. Next, the protein solution was diluted with buffer, and TCEP (5–10 equiv.) in H₂O was added. The mixture was vortexed for 1 s, transferred into a Thermocycler preheated at 37 °C and incubated at 37 °C for 1 h at 400 rpm. Next, a sodium iodide stock solution in the respective buffer (pH 7.0, 50 mM) was added to the mixture at 20–25 °C to obtain an iodide concentration of 40 mM and a protein concentration of 15–30 μM. Next, 15–30 equiv. of **VTFT** was added as a stock solution in DMF. The amount of DMF did not exceed 1% v/v of the final mixture volume. The reaction mixture was vortexed for 1 s, transferred into a Thermocycler preheated at 25 °C, and incubated at 25 °C at 400 rpm for exactly 3 min. Immediately afterwards, a thiol stock solution in the respective buffer (pH 7.0 or 8.0, 50 mM) was added to the mixture to obtain a nucleophile concentration of 20–40 mM. The concentration varied depending on the chosen thiol (see Table E.3). The mixture was incubated at 25 °C at 400 rpm for 30 min. The obtained mixture was analyzed by LC–MS and the conversion and yield were identified via integration of deconvoluted protein MS peaks.

Table E.3: Conditions used for protein modifications with thiol nucleophiles

Nucleophile	c _{final} (Nu), mM	Buffer	eq. VTFT	Conversion/Yield,%
thioglucose	40	NaP _i	25	>95 / 100
potassium thioacetate	40	Bis-Tris	15	>95 / 100
2-dimethylamino-ethanthiol	40	NaP _i	25	>95 / 100
biotin-PEG11-thiol	20	HEPES	30	>95 / 74

Protein crosslinking with VTT and TTD

Dr. Lara Vogelsang synthesized ubiquitin K63C. The author performed reactions with proteins. Marie Sophie Sterling acquired the LC–MS data, which were interpreted by the author and Marie Sophie Sterling.

Characterization of protein starting materials

Ubiquitin was purchased from Sigma Aldrich (#93950). Ubiquitin K63C was recombinantly expressed and purified as described previously⁴⁴, using a construct generated by subcloning ubiquitin K63C from a plasmid obtained from Addgene (pMAL-c5X, Plasmid #86589). The gene was amplified using primers 5'-AAAAGCTAGCCAGATCTTCGTCAAGACGTTAAC-3' and 5'-TTTTAAGCTTTTAACCACCTCTTAGTCTTAAGACAAG-3' and inserted into pET28a via NheI and HindIII restriction sites. Protein expression in *E. coli* NiCo21 (DE3), Ni-NTA purification, dialysis and thrombin cleavage for removal of the His-tag followed the protocol described previously⁴⁴. The protein mutant sequence: GSHMASQIFVKLTGKTITLEVEPSDTIENVKAK-IQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQCESTLHLVLRRLRGG*

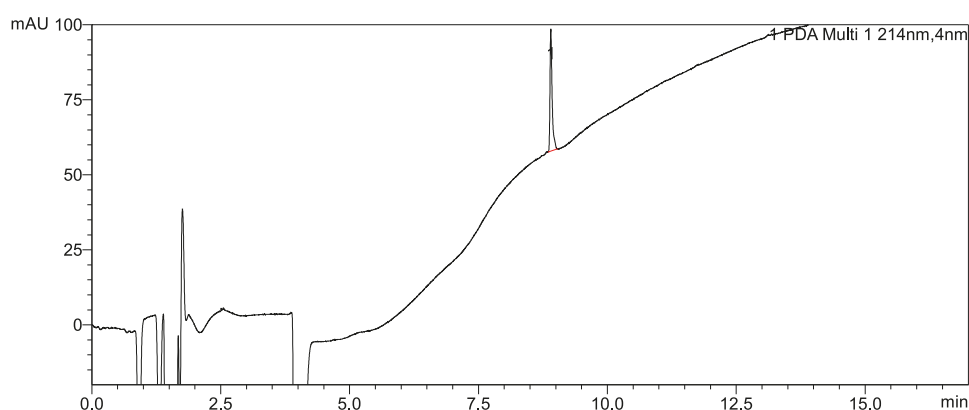


Figure E.5: LC–MS analysis of ubiquitin pre-reduced with 20 equiv. TCEP: UV trace.

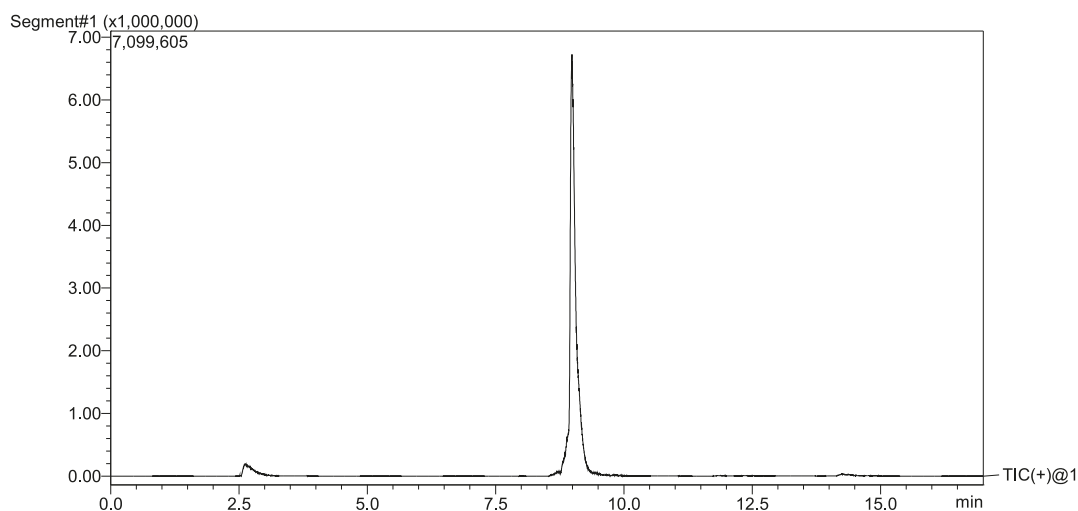


Figure E.6: LC–MS analysis of ubiquitin pre-reduced with 20 equiv. TCEP: total ion chromatogram.

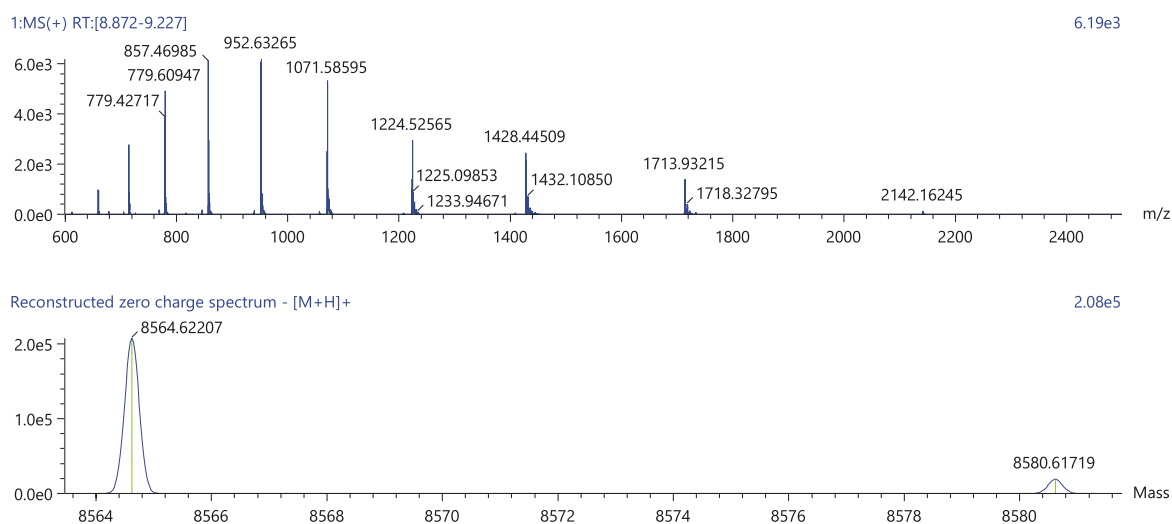


Figure E.7: LC-MS analysis of ubiquitin pre-reduced with 20 equiv. TCEP: ion series and deconvoluted spectrum of the protein peak; calculated mass: Ubiquitin, 8564.751 Da. Observed masses: 8564.622 Da, 8580.617 Da.

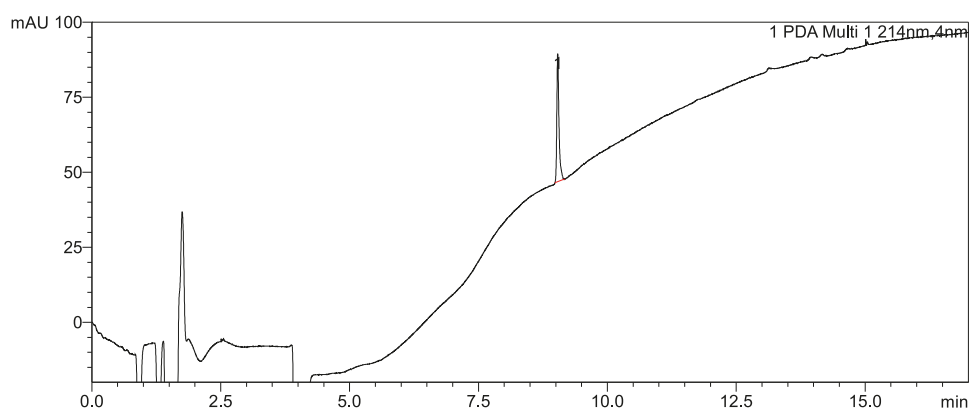


Figure E.8: LC-MS analysis of ubiquitin K63C pre-reduced with 20 equiv. TCEP: UV trace

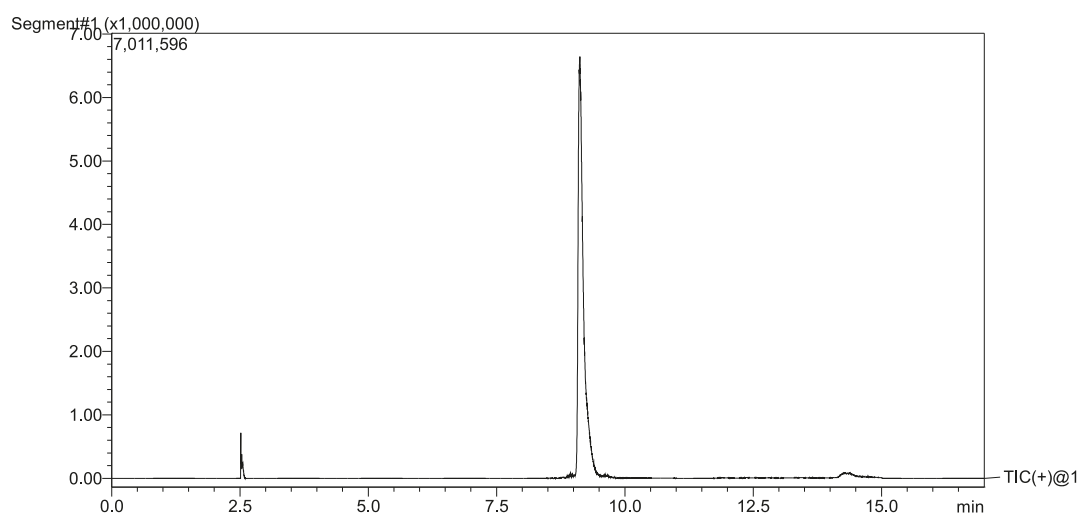


Figure E.9: LC-MS analysis of ubiquitin K63C pre-reduced with 20 equiv. TCEP: total ion chromatogram.

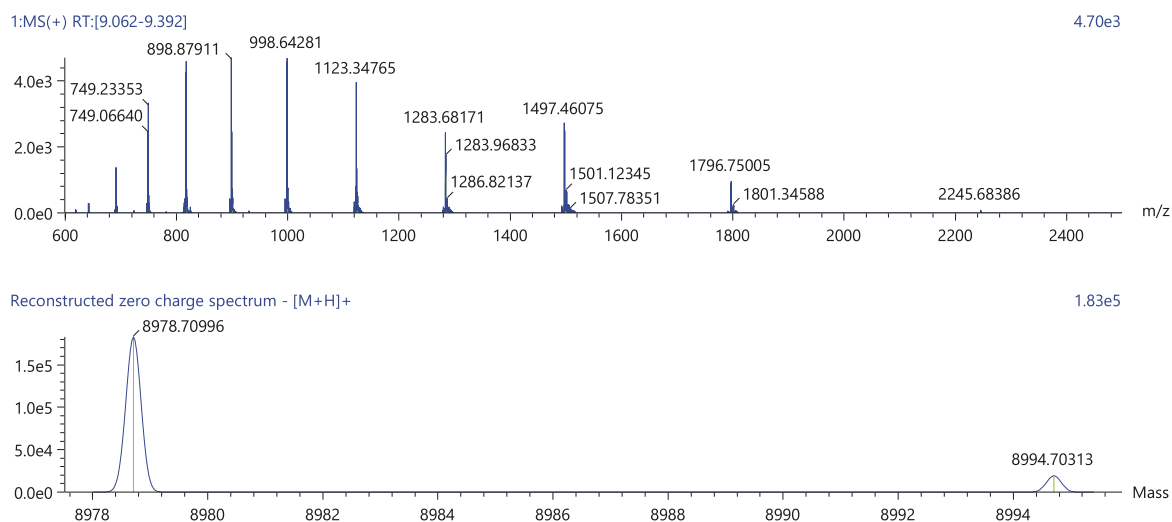
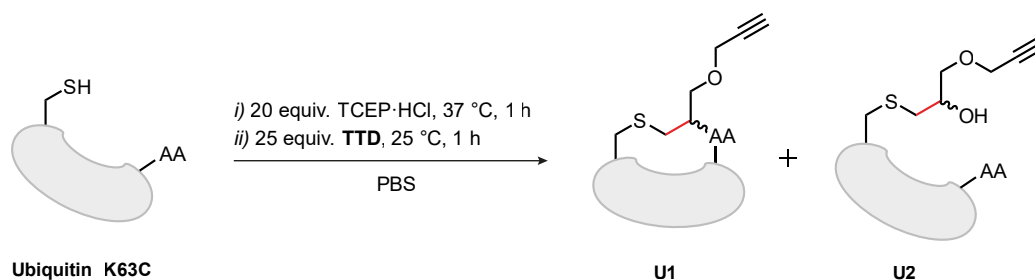


Figure E.10: LC–MS analysis of ubiquitin K63C pre-reduced with 20 equiv. TCEP: ion series and deconvoluted spectrum of the protein peak; calculated mass: Ubiquitin K63C, 8979.1454 Da. Observed masses: 8978.710 Da, 8994.703 Da.

Reaction between ubiquitin K63C and TTD



At 20–25 °C, 6.0 μ L of ubiquitin K63C (2.99 mg/mL, 1.0 equiv.) in NaP_i buffer (pH 7.0, c = 50 mM) was added to a 1.5 mL Eppendorf tube. Next, 92 μ L phosphate-buffered saline (PBS) was added. Then, 1.0 μ L of a TCEP stock solution (40 mM, 20 equiv.) in H₂O was added. The mixture was vortexed for 5 seconds, transferred into a Thermocycler pre-heated at 37 °C, and incubated at 37 °C for 1 hour at 600 rpm. Next, 1.0 μ L of a freshly prepared stock solution of **TTD** in DMSO (50 mM, 25 equiv.) was added to the mixture. The reaction mixture was vortexed for 5 seconds, transferred into a Thermocycler pre-heated at 25 °C, and incubated at 25 °C at 600 rpm for 1 hour. A stock solution of β -mercaptoethanol (1.0 μ L, 50 mM, 25 equiv.) in H₂O was added, the mixture was incubated for 15 minutes at 25 °C, centrifuged for 3 minutes at 17,000 $\times$ g, and the supernatant was analyzed using LC–MS.

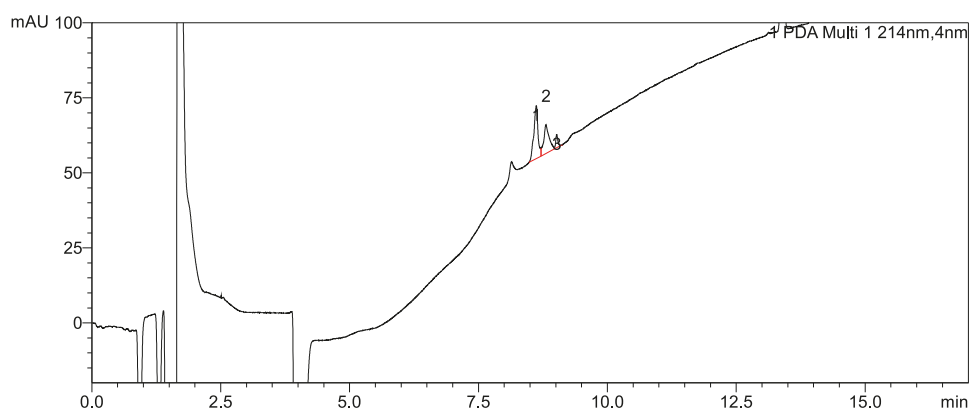


Figure E.11: LC–MS analysis of the reaction mixture with ubiquitin K63C: UV trace. Relative calculated areas: peak 1: 55%, peak 2: 38%, peak 3: 7%.

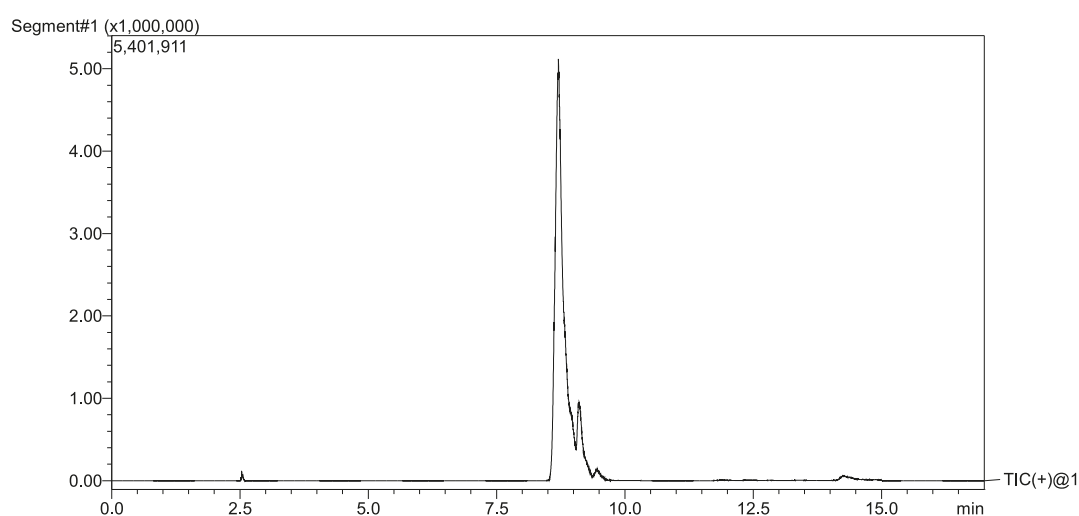


Figure E.12: LC–MS analysis of the reaction mixture with ubiquitin K63C: total ion chromatogram.

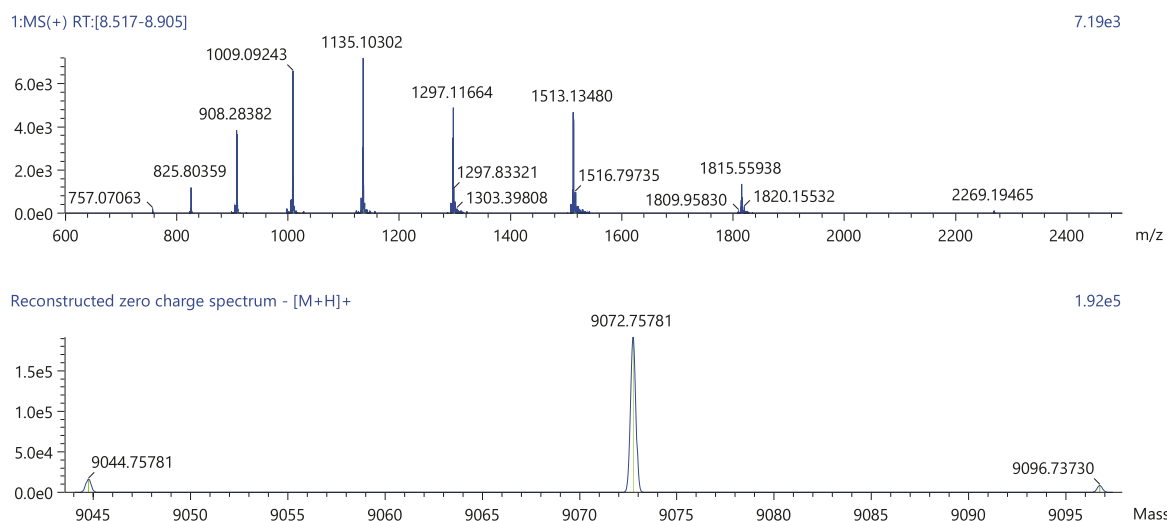


Figure E.13: LC–MS analysis of the reaction mixture with ubiquitin K63C: ion series and deconvoluted spectrum of protein peak 1; calculated mass: **U1**, 9073.187 Da. Observed masses: 9072.758 Da, 9044.758 Da, 9096.737 Da.

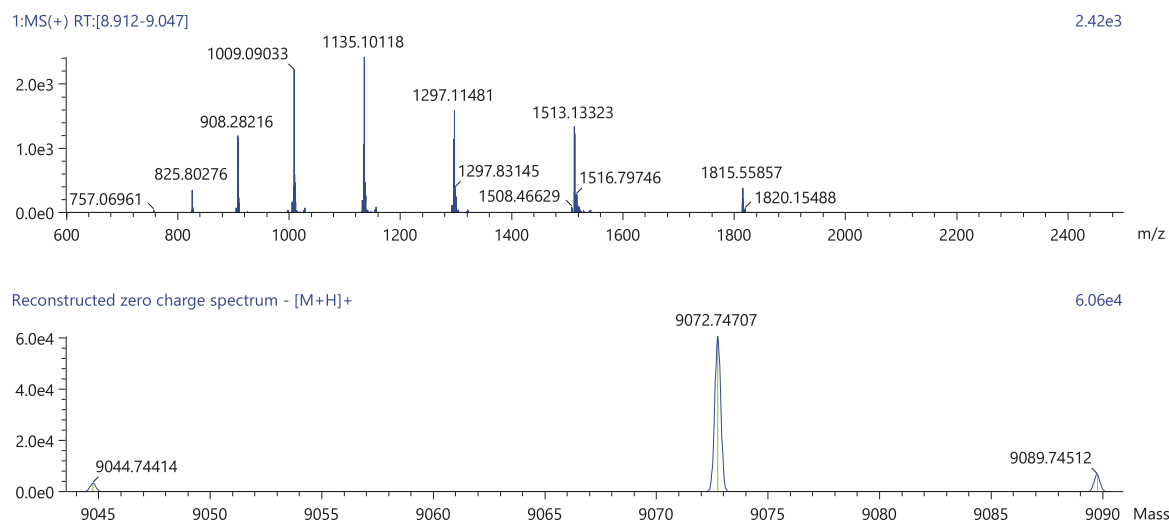


Figure E.14: LC–MS analysis of the reaction mixture with ubiquitin K63C: ion series and deconvoluted spectrum of protein peak 2; calculated mass: **U1**, 9073.187 Da. Observed masses: 9072.747 Da, 9044.744 Da, 9089.745 Da.

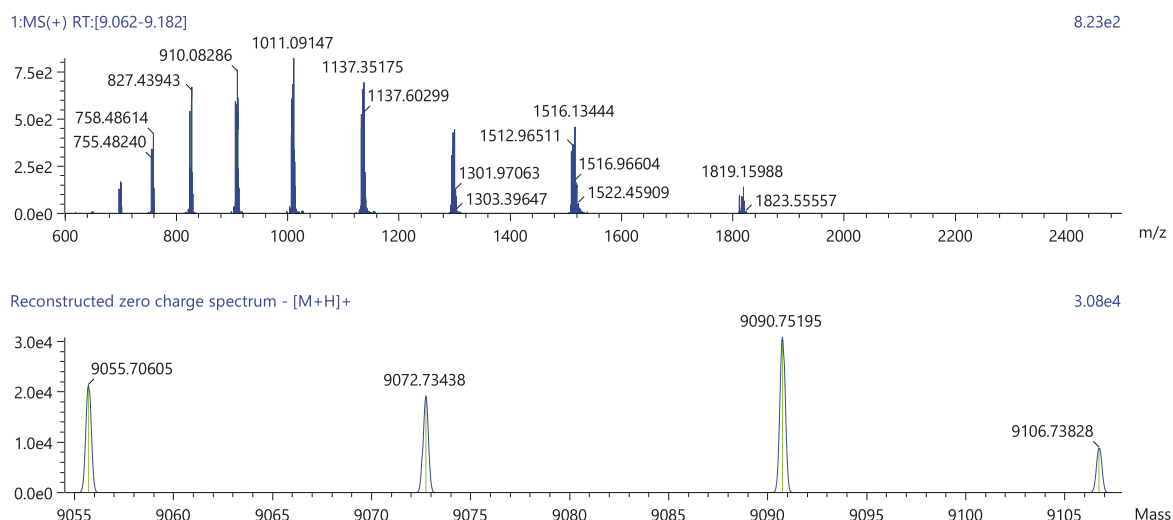
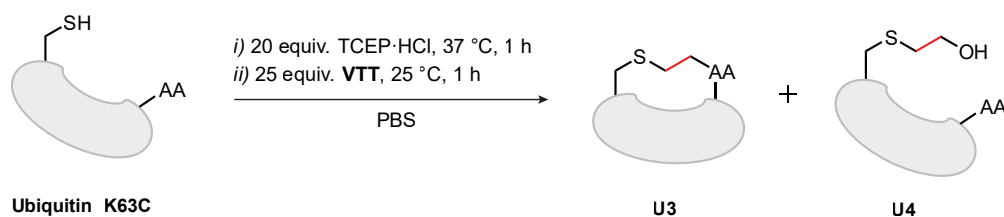


Figure E.15: LC–MS analysis of the reaction mixture with ubiquitin K63C: ion series and deconvoluted spectrum of protein peak 3; calculated mass: **U1**, 9073.187 Da; **U2**, 9091.198 Da. Observed masses: 9055.706 Da, 9072.734 Da, 9090.752 Da, 9106.738 Da.

Reaction between ubiquitin K63C and VTT



At 20–25 °C, 6.0 μL of ubiquitin K63C (2.99 mg/mL, 1.0 equiv.) in NaP_i buffer (pH 7.0, $c = 50\text{ mM}$) was added to a 1.5 mL Eppendorf tube. Next, 92 μL phosphate-buffered saline (PBS) was added. Then, 1.0 μL of a TCEP stock solution (40 mM, 20 equiv.) in H_2O was added. The

mixture was vortexed for 5 seconds, transferred into a Thermocycler pre-heated at 37 °C, and incubated at 37 °C for 1 hour at 600 rpm. Next, 1.0 μ L of a freshly prepared stock solution of **VTT** in DMSO (50 mM, 25 equiv.) was added to the mixture. The reaction mixture was vortexed for 5 seconds, transferred into a Thermocycler pre-heated at 25 °C, and incubated at 25 °C at 600 rpm for 1 hour. A stock solution of β -mercaptoethanol (1.0 μ L, 50 mM, 25 equiv.) in H₂O was added, the mixture was incubated for 15 minutes at 25 °C, centrifuged for 3 minutes at 17,000 xg, and the supernatant was analyzed using LC–MS.

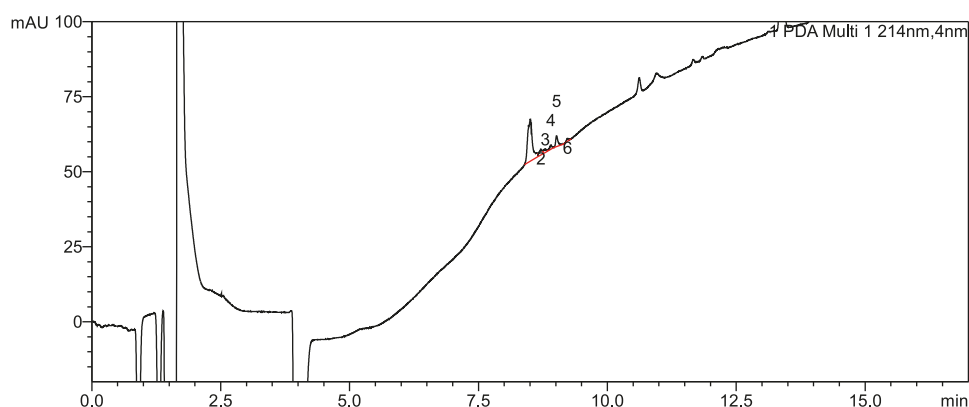


Figure E.16: LC–MS analysis of the reaction mixture with ubiquitin K63C: UV trace. Relative calculated areas: peak 1: 74%, peak 2: 7%, peak 3: 4%, peak 4: 3%, peak 5: 10%, peak 6: 2%.

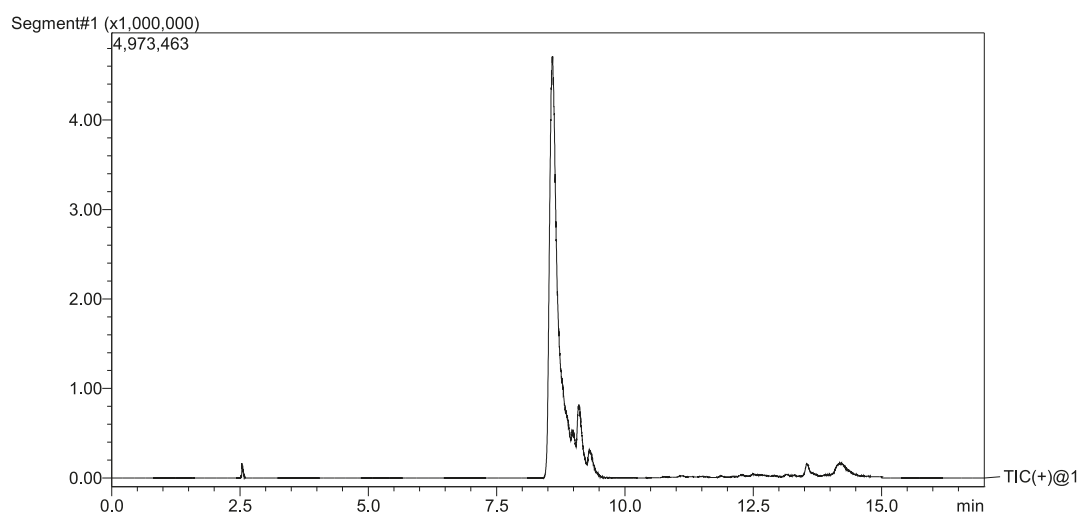


Figure E.17: LC–MS analysis of the reaction mixture with ubiquitin K63C: total ion chromatogram.

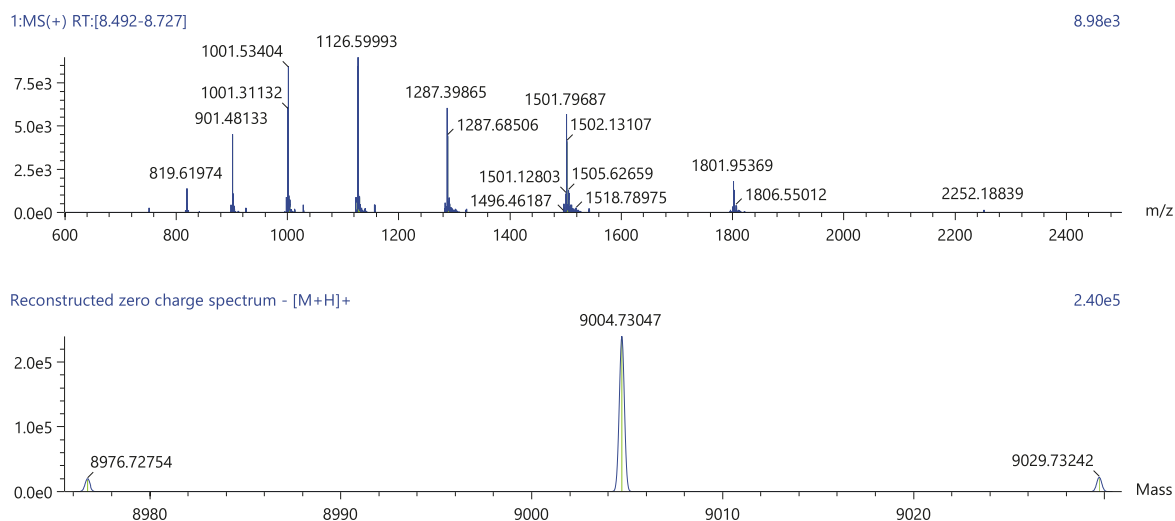


Figure E.18: LC–MS analysis of the reaction mixture with ubiquitin K63C: ion series and deconvoluted spectrum of protein peak 1; calculated mass: **U3**, 9005.161 Da. Observed masses: 9004.730 Da, 8976.727 Da, 9029.732 Da.

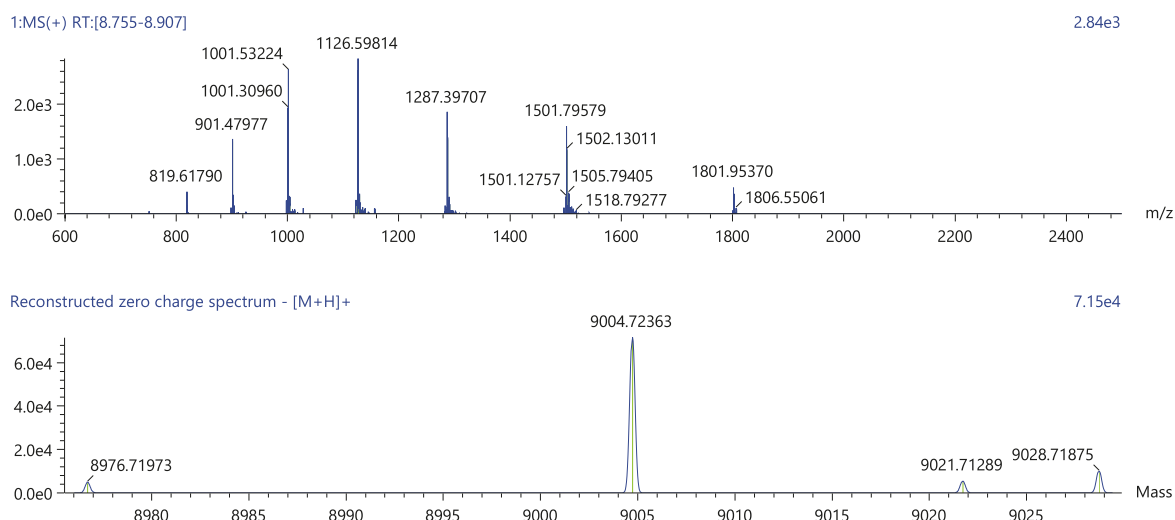


Figure E.19: LC–MS analysis of the reaction mixture with ubiquitin K63C: ion series and deconvoluted spectrum of protein peaks 2+3; calculated mass: **U3**, 9005.161 Da; **U4**, 9023.172 Da. Observed masses: 9004.724 Da, 8976.720 Da, 9021.713 Da, 9028.719 Da.

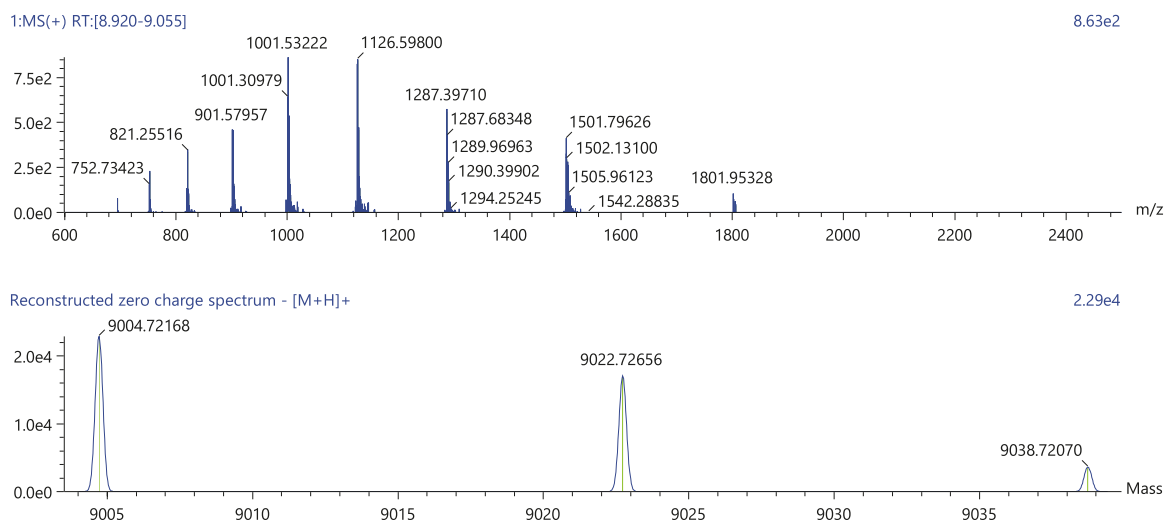


Figure E.20: LC–MS analysis of the reaction mixture with ubiquitin K63C: ion series and deconvoluted spectrum of protein peak 4; calculated mass: **U3**, 9005.161 Da; **U4**, 9023.172 Da. Observed masses: 9004.722 Da, 9022.727 Da, 9038.721 Da.

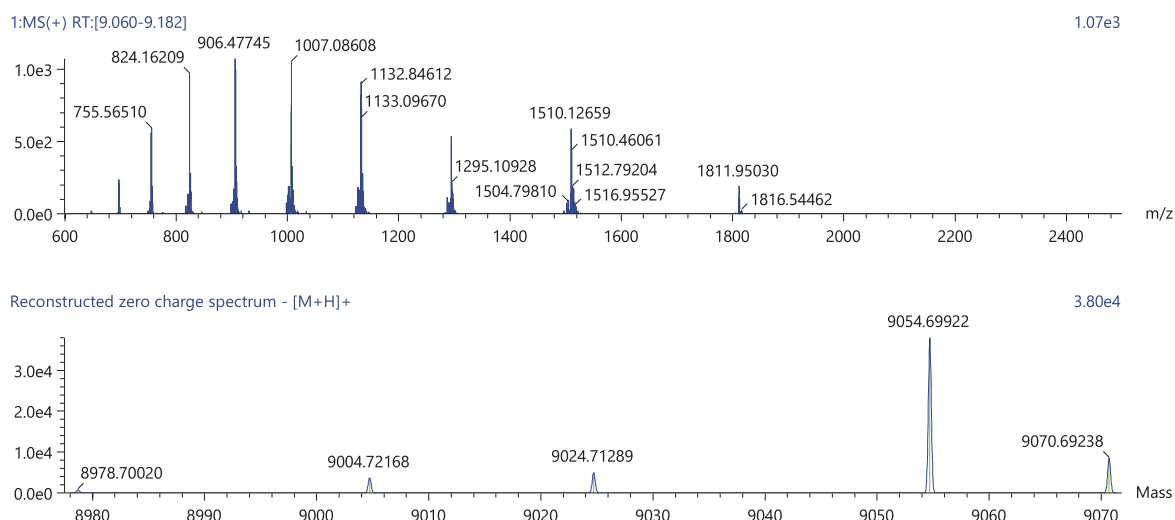


Figure E.21: LC–MS analysis of the reaction mixture with ubiquitin K63C: ion series and deconvoluted spectrum of protein peak 5; calculated mass: **U3**, 9005.161 Da; **U4**, 9023.172 Da. Observed masses: 9054.699 Da, 8978.700 Da, 9004.722 Da, 9024.713 Da, 9070.692 Da.

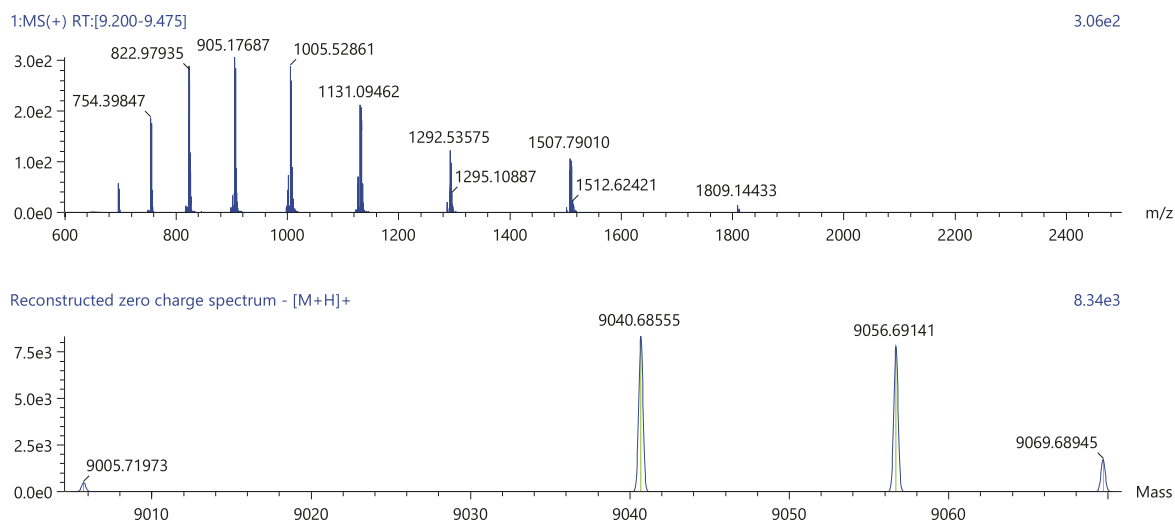
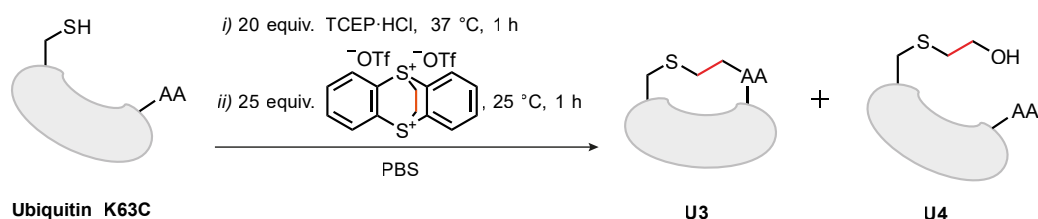


Figure E.22: LC–MS analysis of the reaction mixture with ubiquitin K63C: ion series and deconvoluted spectrum of protein peak 6; calculated mass: **U3**, 9005.161 Da. Observed masses: 9040.686 Da, 9056.691 Da, 9005.720 Da, 9069.689 Da.

Reaction between ubiquitin K63C and dicationic VTT precursor



At 20–25 °C, 6.0 μL of ubiquitin K63C (2.99 mg/mL, 1.0 equiv.) in NaP_i buffer (pH 7.0, $c = 50$ mM) was added to a 1.5 mL Eppendorf tube. Next, 92 μL phosphate-buffered saline (PBS) was added. Then, 1.0 μL of a TCEP stock solution (40 mM, 20 equiv.) in H_2O was added. The mixture was vortexed for 5 seconds, transferred into a Thermocycler pre-heated at 37 °C, and incubated at 37 °C for 1 hour at 600 rpm. Next, 1.0 μL of a freshly prepared stock solution of the cyclic **VTT** precursor in DMSO (50 mM, 25 equiv.) was added to the mixture. The reaction mixture was vortexed for 5 seconds, transferred into a Thermocycler pre-heated at 25 °C, and incubated at 25 °C at 600 rpm for 1 hour. A stock solution of β -mercaptoethanol (1.0 μL , 50 mM, 25 equiv.) in H_2O was added, the mixture was incubated for 15 minutes at 25 °C, centrifuged for 3 minutes at 17,000 xg , and the supernatant was analyzed using LC–MS.

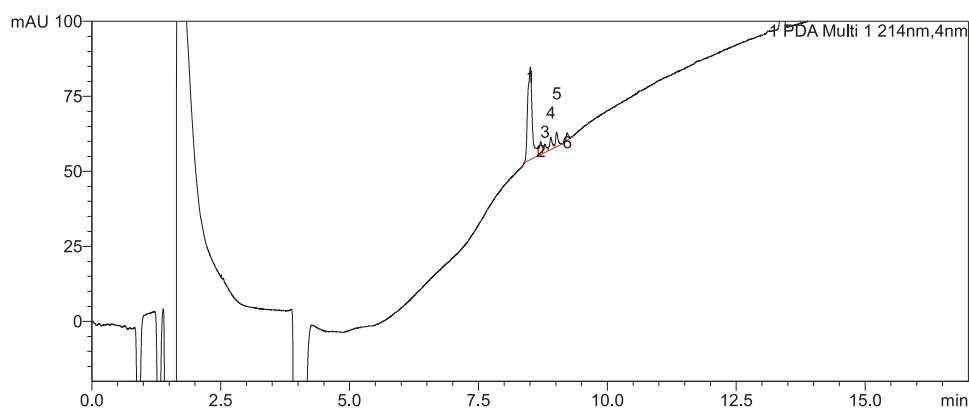


Figure E.23: LC–MS analysis of the reaction mixture with ubiquitin K63C: UV trace. Relative calculated areas: peak 1: 74%, peak 2: 6%, peak 3: 5%, peak 4: 6%, peak 5: 6%, peak 6: 3%.

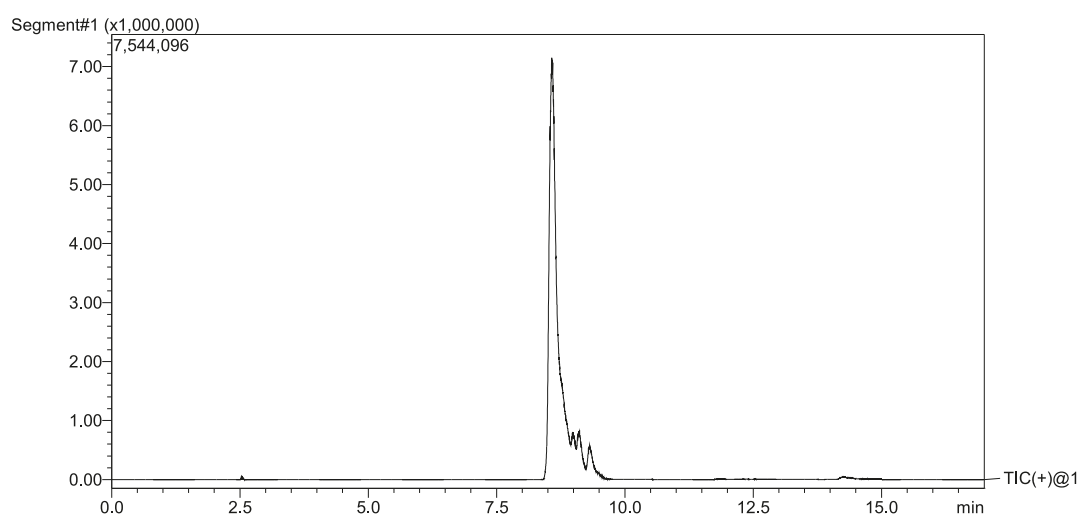


Figure E.24: LC–MS analysis of the reaction mixture with ubiquitin K63C: total ion chromatogram.

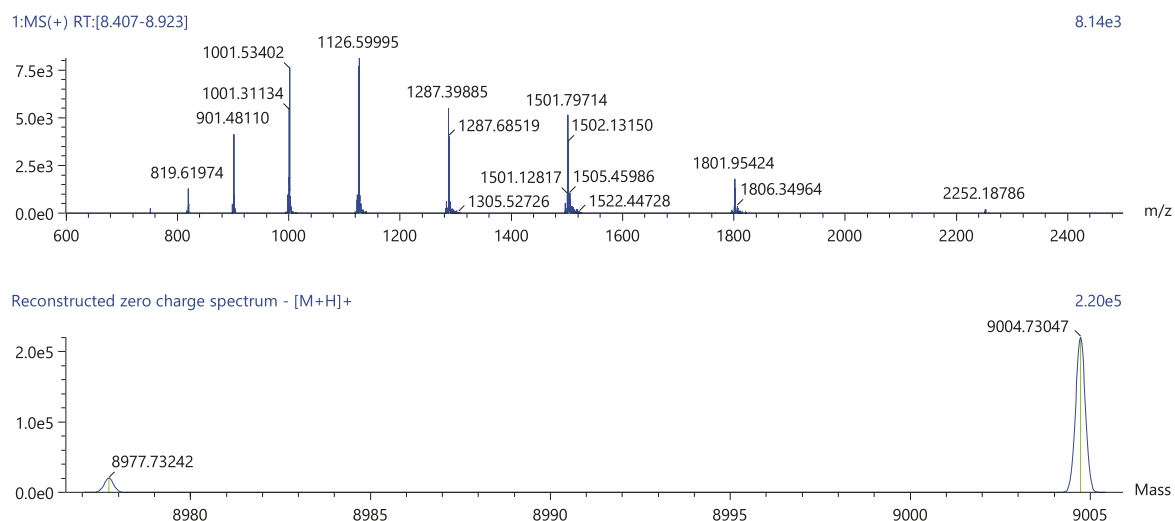


Figure E.25: LC–MS analysis of the reaction mixture with ubiquitin K63C: ion series and deconvoluted spectrum of protein peaks 1,2,3; calculated mass: **U3**, 9005.161 Da. Observed masses: 9004.730 Da, 8977.732 Da.

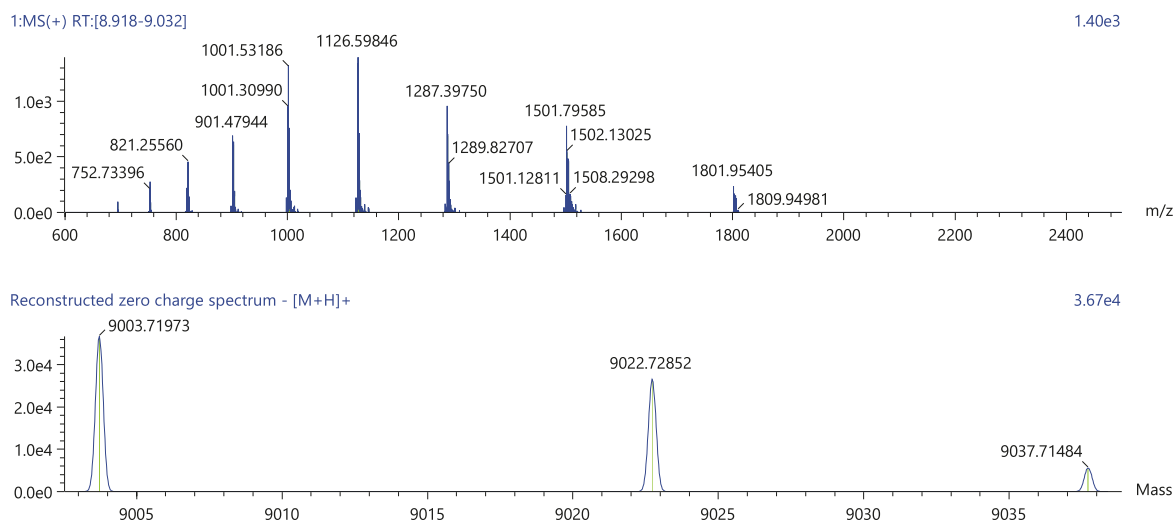


Figure E.26: LC–MS analysis of the reaction mixture with ubiquitin K63C: ion series and deconvoluted spectrum of protein peak 4; calculated mass: **U3**, 9005.161 Da; **U4**, 9023.172 Da. Observed masses: 9003.720 Da, 9022.729 Da, 9037.715 Da.

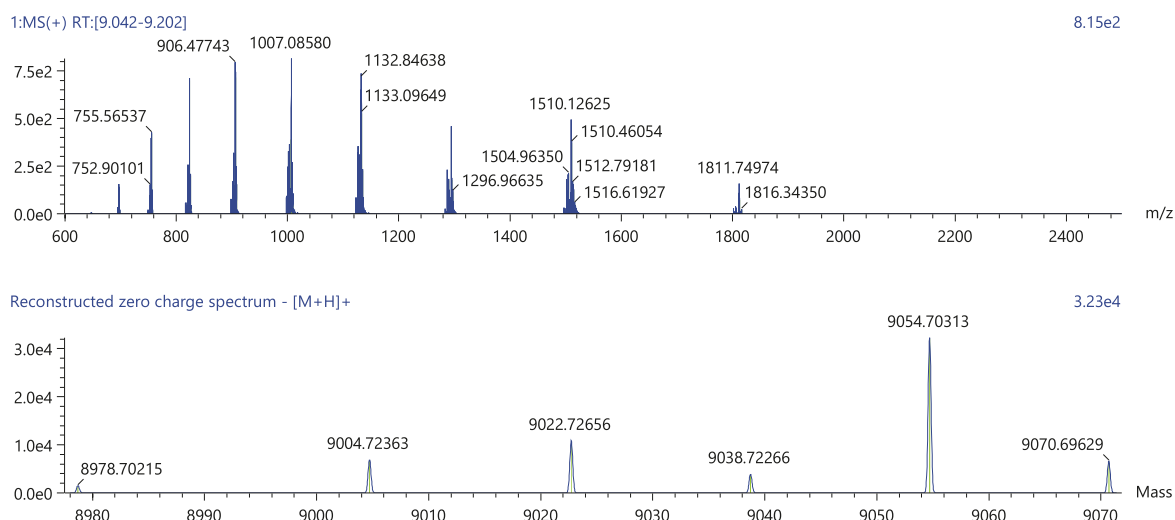


Figure E.27: LC–MS analysis of the reaction mixture with ubiquitin K63C: ion series and deconvoluted spectrum of protein peak 5; calculated mass: **U3**, 9005.161 Da; **U4**, 9023.172 Da. Observed masses: 9054.703 Da, 8978.702 Da, 9004.724 Da, 9022.727 Da, 9038.723 Da, 9070.696 Da.

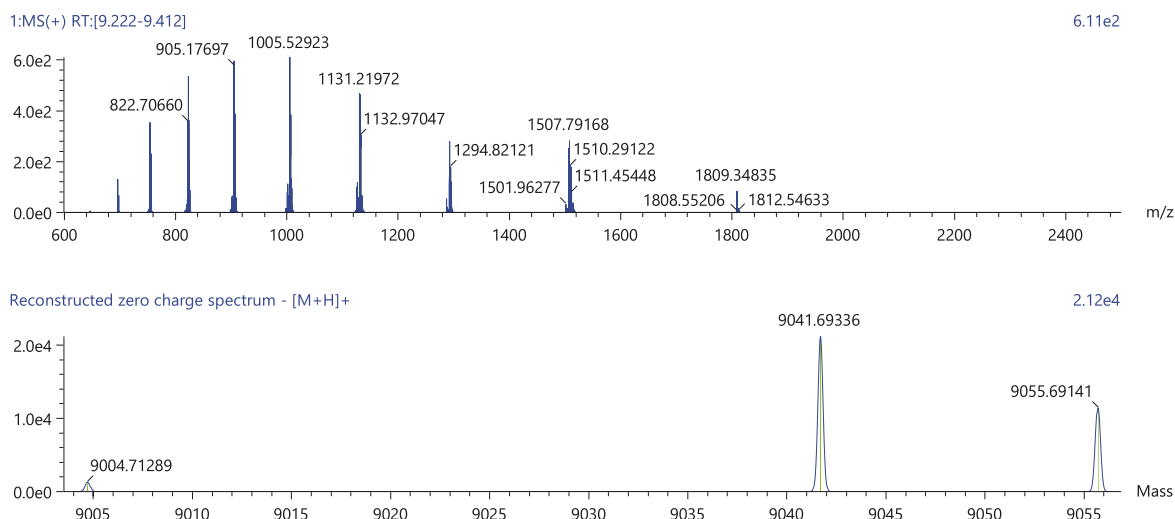
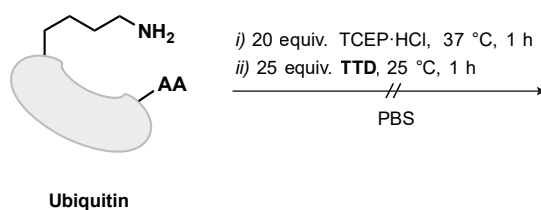


Figure E.28: LC–MS analysis of the reaction mixture with ubiquitin K63C: ion series and deconvoluted spectrum of protein peak 5; calculated mass: **U3**, 9005.161 Da. Observed masses: 9041.693 Da, 9056.691 Da, 9004.713 Da, 9055.691 Da.

Reaction between ubiquitin and TTD



At 20–25 °C, 69 μ L of ubiquitin (0.25 mg/mL, 1.0 equiv.) in NaP_i buffer (pH 7.4, $c = 50$ mM) was added to a 1.5 mL Eppendorf tube. Next, 29 μ L PBS was added. Then, 1.0 μ L of a TCEP stock solution (40 mM, 20 equiv.) in H_2O was added. The mixture was vortexed for 5 seconds, transferred into a Thermocycler pre-heated at 37 °C, and incubated at 37 °C for 1 hour at 600 rpm. Next, 1.0 μ L of a freshly prepared stock solution of **TTD** in DMSO (50 mM, 25 equiv.) was added to the mixture. The reaction mixture was vortexed for 5 seconds, transferred into a Thermocycler pre-heated at 25 °C, and incubated at 25 °C at 600 rpm for 1 hour. A stock solution of β -mercaptoethanol (1.0 μ L, 50 mM, 25 equiv.) in H_2O was added, the mixture was incubated for 15 minutes at 25 °C, centrifuged for 3 minutes at 17,000 $\times g$, and the supernatant was analyzed using LC–MS.

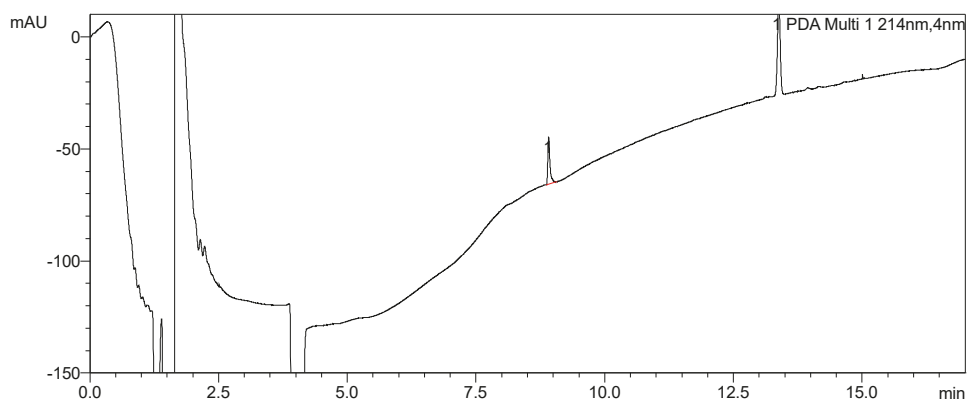


Figure E.29: LC–MS analysis of the reaction mixture with ubiquitin: UV trace.

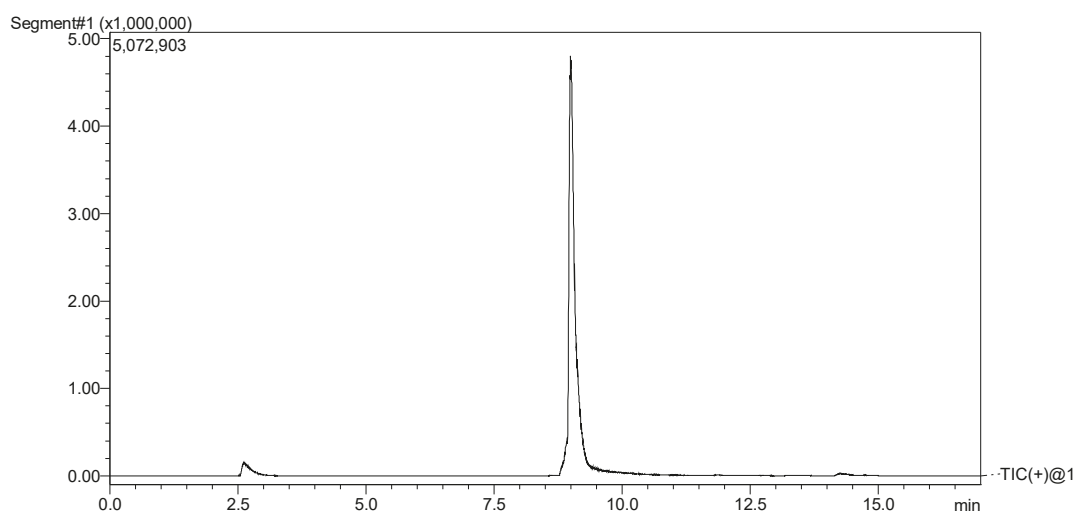


Figure E.30: LC–MS analysis of the reaction mixture with ubiquitin: total ion chromatogram.

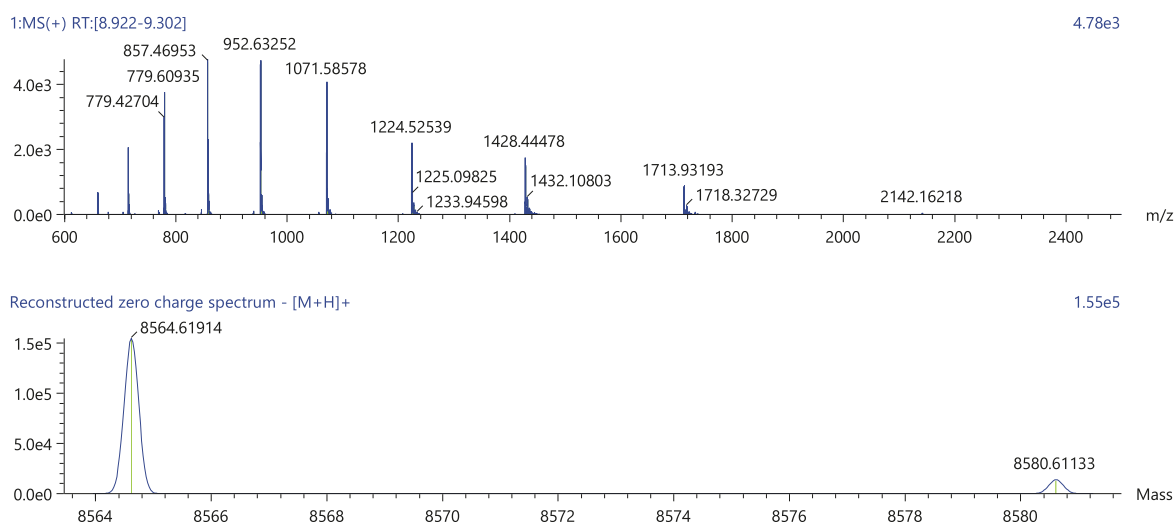


Figure E.31: LC–MS analysis of the reaction mixture with ubiquitin: ion series and deconvoluted spectrum of the protein peak; calculated mass: ubiquitin, 8564.751 Da. Observed masses: 8564.619 Da, 8580.611 Da.

Kinetic studies

The UV-vis data were acquired and analyzed by the author. Julia Beatrice Jacobs acquired the NMR data, which were analyzed by the author and Julia Beatrice Jacobs.

Kinetic reaction profile of bioconjugation with VTT

General considerations

Due to the high reaction rates (see Figure E.69, Figure E.71), the reactions between **GSH** and **VTT** or **VTFT** at 5 mM scale achieve full conversion in less than 5 minutes. Therefore, NMR studies were only used to analyze the product distribution in the reaction mixtures, whereas UV-vis spectroscopy was used to obtain the kinetic profile of the reaction and the rate constants. All UV-vis measurements were recorded on a Shimadzu UV-vis Spectrophotometer UV-2600 with temperature controller Shimadzu S-1700 at $(25 \pm 0.1)^\circ\text{C}$ unless stated otherwise.

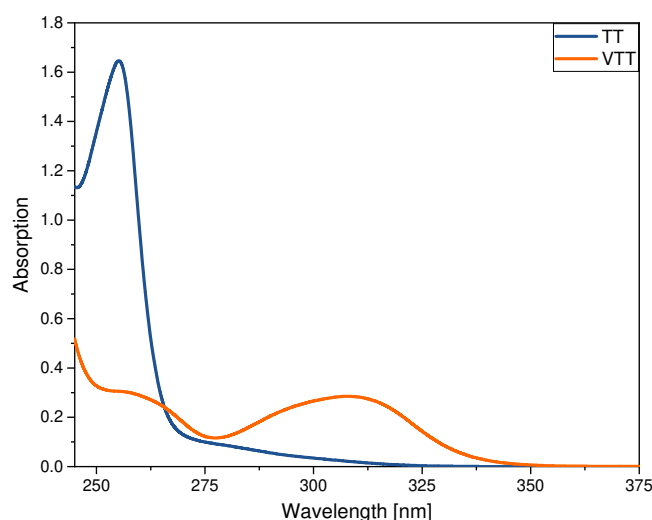


Figure E.32: Absorbance spectra of 50 μM **TT** and **VTT** aqueous solutions containing 30% MeCN.

Kinetic studies with the Variable Time Normalization Analysis

General procedure:

Stock solutions of **GSH** (1.85 mM) and NaN_3 (17.8 mM) were prepared in NaPi (pH 7.4, $c = 71$ mM). NaPi (pH 7.4, $c = 71$ mM), MeCN, NaN_3 , and **GSH** stock solutions were pipetted into a quartz cuvette charged with a Teflon-coated magnetic stirring bar. The cuvette was thermostated for 5 minutes at $(25 \pm 0.1)^\circ\text{C}$ in the cell holder. After performing the background measurement, 10 μL of a **VTT** stock solution in DMF was added to the cuvette. The obtained buffered mixture ($V = 2.0$ mL; 50 mM NaPi ; 30% v/v MeCN) contained 10–40 μM of **GSH**, 12.5–50 μM of **VTT**, and 0.5–4.5 mM of NaN_3 . The reaction mixture was homogenized by pipetting the mixture up and down. Then, the cuvette was capped and the release of **TT** was monitored by measuring absorbance at 255 nm while stirring.

Reaction order in GSH

To determine the reaction order in **GSH**, $c(\text{GSH})$ was set to 10 μM , 20 μM , and 40 μM , keeping the concentrations of the other reagents constant: $c(\text{VTT}) = 50$ μM and $c(\text{NaN}_3) = 1.5$ mM. The concentration of **TT** was plotted against $\sum [\text{GSH}]^\beta \cdot \Delta t$, generating graphs for different values of β (0, 0.5, 1.0, and 2.0).

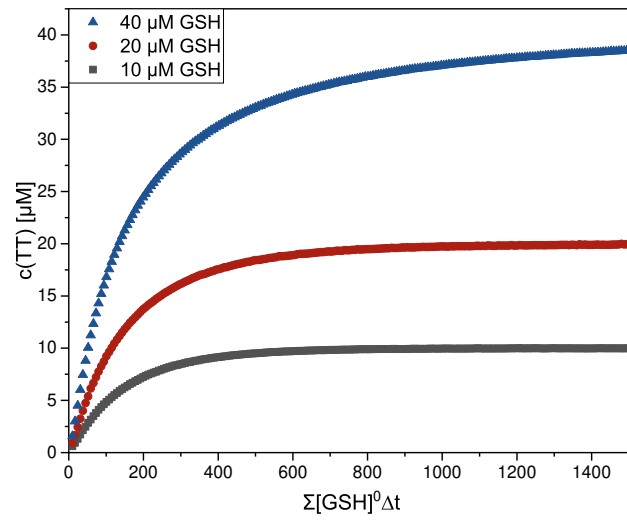


Figure E.33: VTNA analysis for $\beta = 0$.

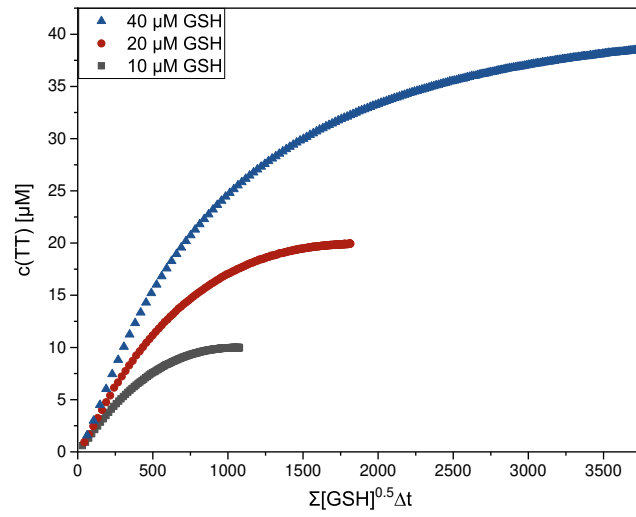


Figure E.34: VTNA analysis for $\beta = 0.5$.

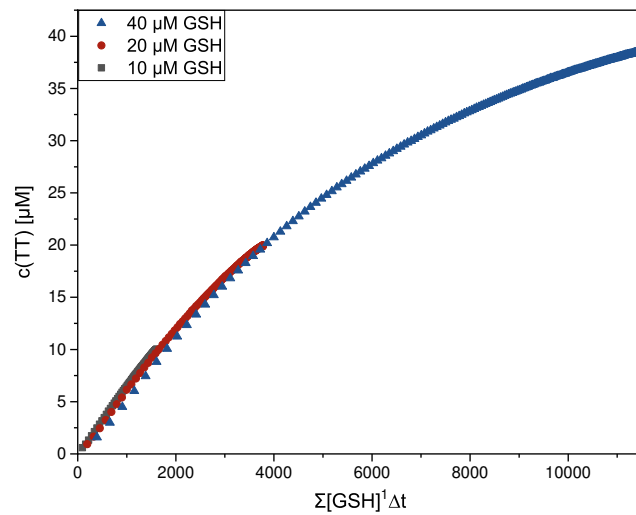


Figure E.35: VTNA analysis for $\beta = 1.0$.

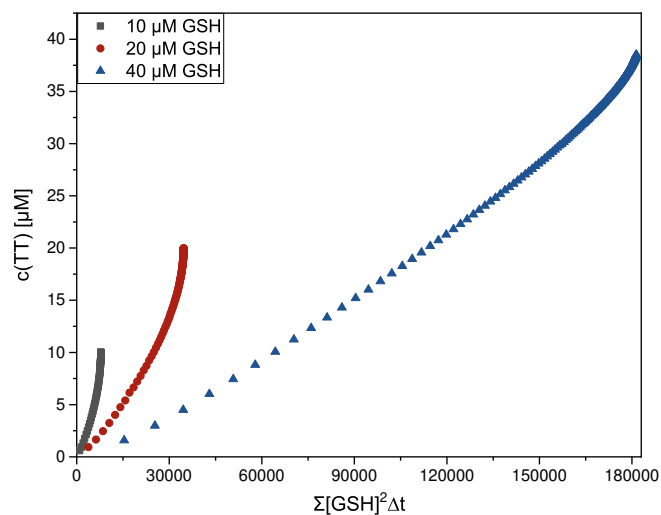


Figure E.36: VTNA analysis for $\beta = 2.0$.

Reaction order in VTT

To determine the reaction order in **VTT**, $c(\text{VTT})$ was set to 12.5 μM , 25 μM , and 50 μM , keeping the concentrations of the other reagents constant: $c(\text{GSH}) = 10 \mu\text{M}$ and $c(\text{NaN}_3) = 1.5 \text{ mM}$. The concentration of **TT** was plotted against $\sum[\text{VTT}]^\beta \cdot \Delta t$, generating graphs for different values of β (0, 0.5, 1.0, and 2.0).

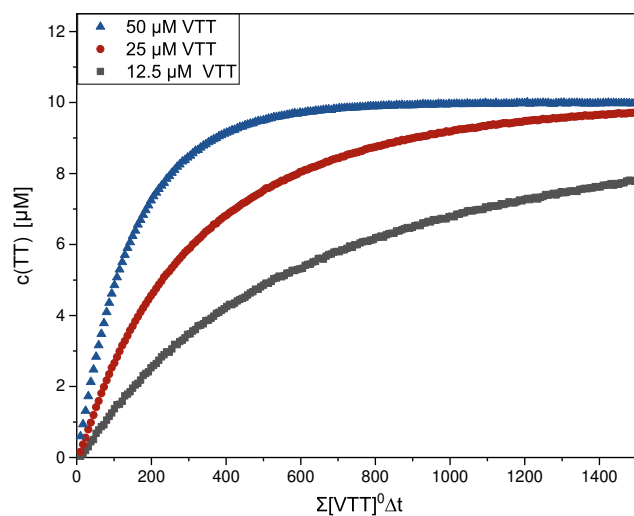


Figure E.37: VTNA analysis for $\beta = 0$.

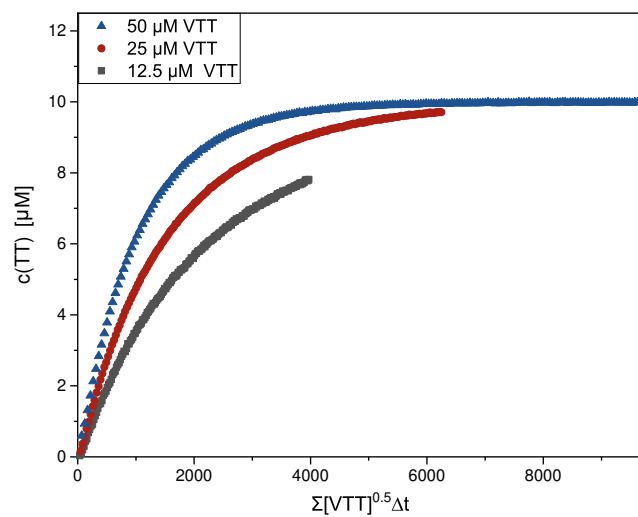


Figure E.38: VTNA analysis for $\beta = 0.5$.

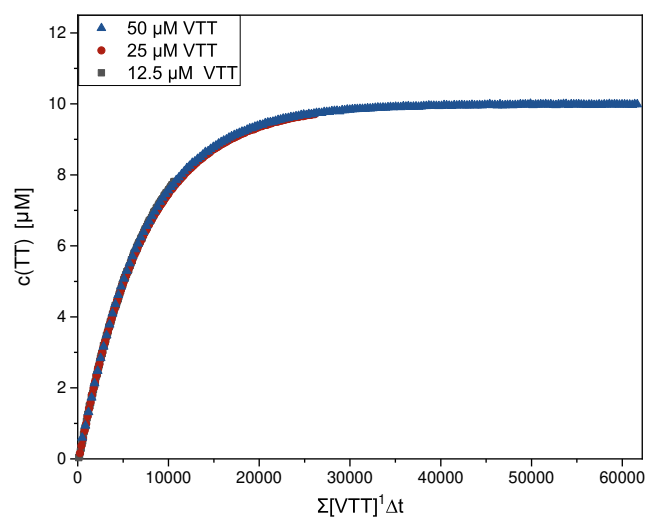


Figure E.39: VTNA analysis for $\beta = 1.0$.

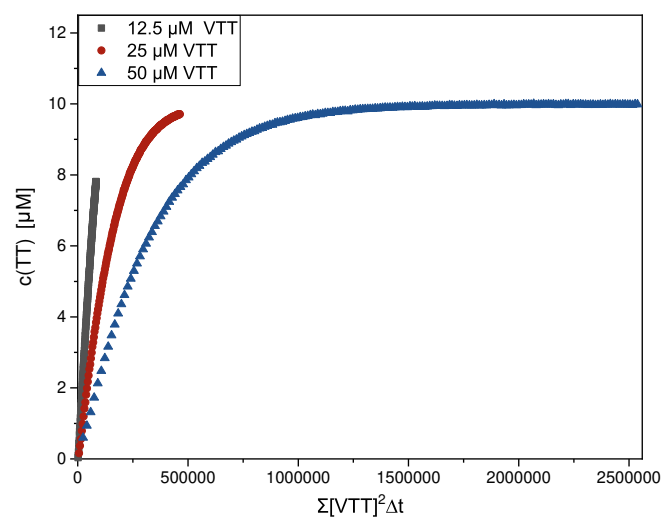


Figure E.40: VTNA analysis for $\beta = 2.0$.

Reaction order in NaN_3

To determine the reaction order in NaN_3 , $c(\text{NaN}_3)$ was set to 0.5 mM, 1.5 mM, and 4.5 mM, keeping the concentrations of other reagents constant: $c(\text{GSH}) = 10 \mu\text{M}$ and $c(\text{VTT}) = 50 \mu\text{M}$. The concentration of **TT** was plotted against $t \cdot [\text{NaN}_3]^\beta$, generating graphs for different values of β (0, 0.5, 1.0, and 2.0).

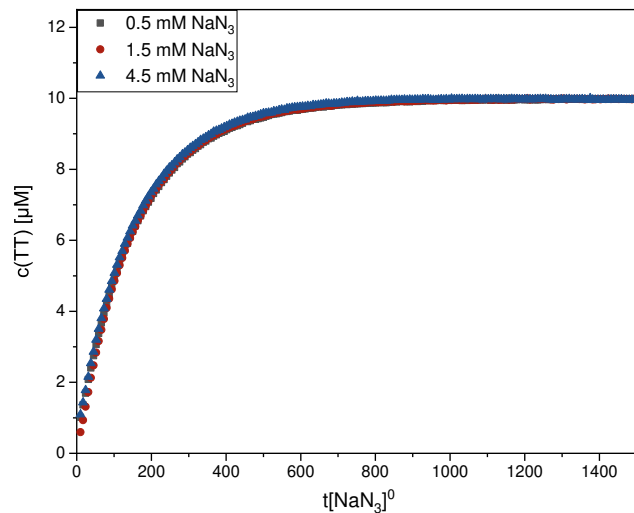


Figure E.41: VTNA analysis for $\beta = 0$.

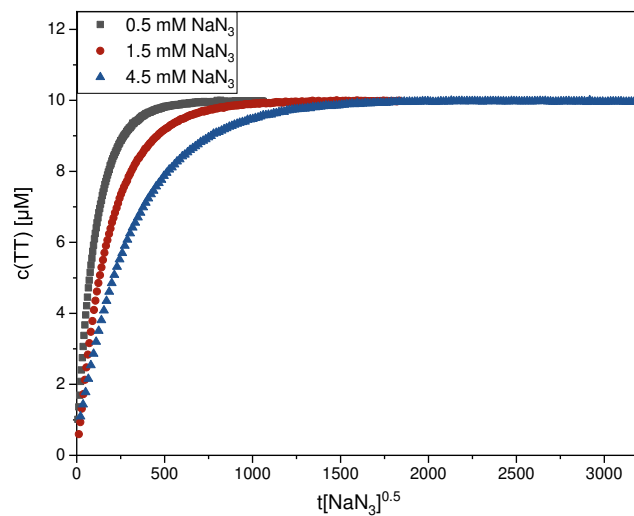


Figure E.42: VTNA analysis for $\beta = 0.5$.

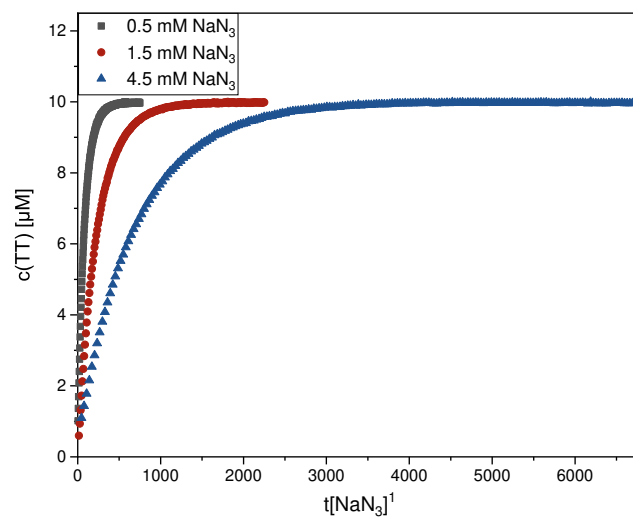


Figure E.43: VTNA analysis for $\beta = 1.0$.

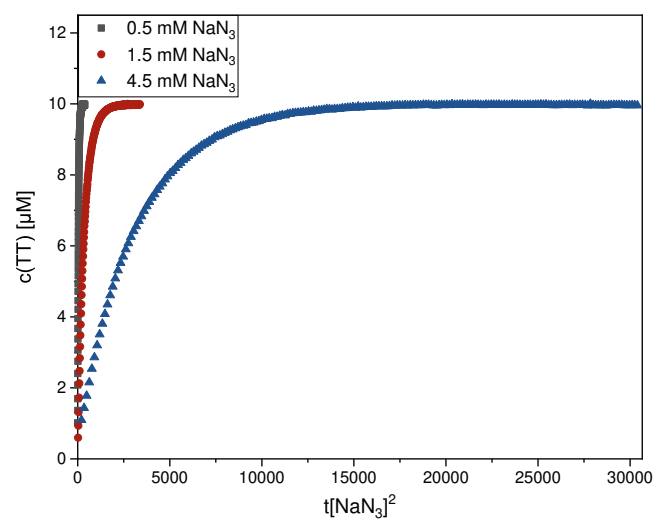


Figure E.44: VTNA analysis for $\beta = 2.0$.

Kinetic reaction profile of bioconjugation with VTFT

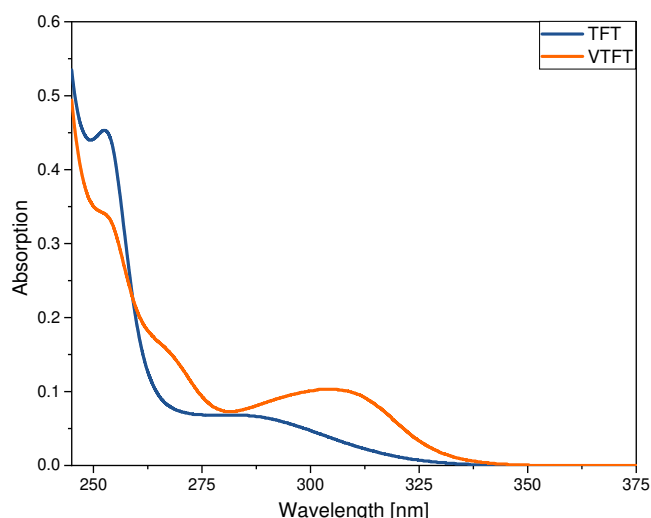


Figure E.45: Absorbance spectra of 50 μM **TFT** and **VTFT** aqueous solutions containing 30% MeCN.

Kinetic studies with the Variable Time Normalization Analysis

General procedure:

Stock solutions of **GSH** (1.87 mM) and NaN_3 (17.6 mM) were prepared in sodium phosphate buffer (pH 7.4, 71 mM). NaP_i (pH 7.4, 71 mM), MeCN, NaN_3 , and **GSH** stock solutions were pipetted into a quartz cuvette charged with a Teflon-coated magnetic stirring bar. The cuvette was thermostated for 5 minutes at $(25 \pm 0.1)^\circ\text{C}$ in the cell holder. After performing the background measurement, 10 μL of a **VTFT** stock solution in DMF was added to the cuvette. The obtained buffered mixture ($V = 2.0\text{ mL}$; 50 mM NaP_i ; 30% v/v MeCN) contained 10–30 μM of **GSH**, 12.5–50 μM of **VTFT**, and 0.5–4.5 mM of NaN_3 . The reaction mixture was homogenized by pipetting the mixture up and down. Then, the cuvette was capped and the release of **TFT** was monitored by measuring absorbance at 253 nm while stirring.

Reaction order in GSH

To determine the reaction order in **GSH**, $c(\text{GSH})$ was set to 10 μM , 20 μM , and 30 μM , keeping the concentrations of the other reagents constant: $c(\text{VTFT}) = 50\text{ }\mu\text{M}$ and $c(\text{NaN}_3) = 1.5\text{ mM}$. The concentration of **TFT** was plotted against $\sum [\text{GSH}]^\beta \cdot \Delta t$, generating graphs for different values of β (0, 0.5, 1.0, and 2.0).

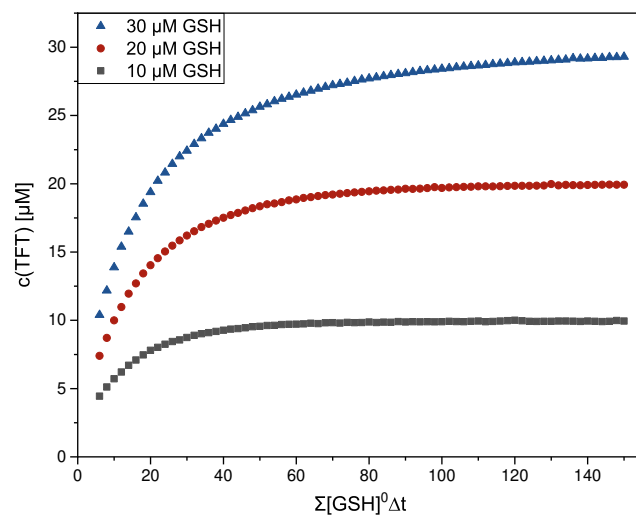


Figure E.46: VTNA analysis for $\beta = 0$.

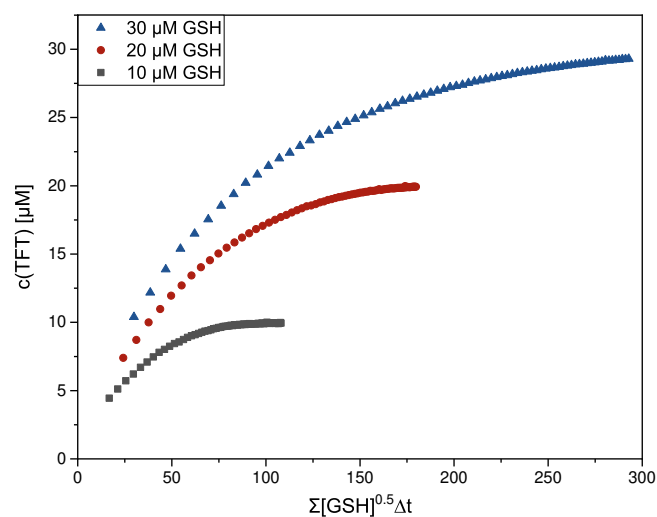


Figure E.47: VTNA analysis for $\beta = 0.5$.

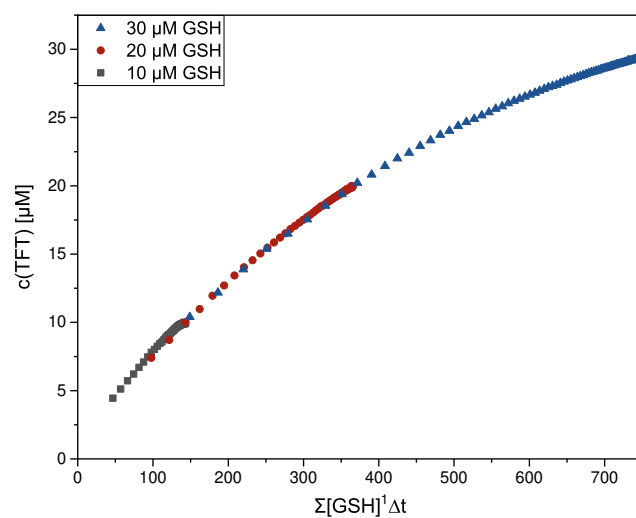


Figure E.48: VTNA analysis for $\beta = 1.0$.

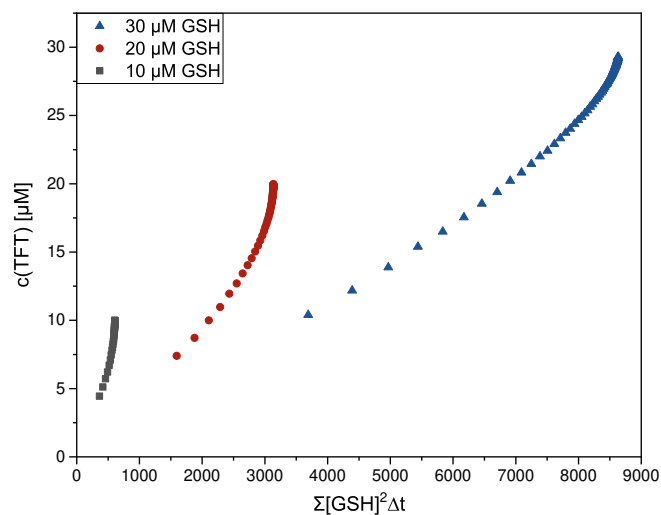


Figure E.49: VTNA analysis for $\beta = 2.0$.

Reaction order in VTFT

To determine the reaction order in **VTFT**, $c(\text{VTFT})$ was set to 12.5 μM , 25 μM , and 50 μM , keeping the concentrations of the other reagents constant: $c(\text{GSH}) = 10 \mu\text{M}$ and $c(\text{NaN}_3) = 1.5 \text{ mM}$. The concentration of **TFT** was plotted against $\sum[\text{VTFT}]^\beta \cdot \Delta t$, generating graphs for different values of β (0, 0.5, 1.0, and 2.0).

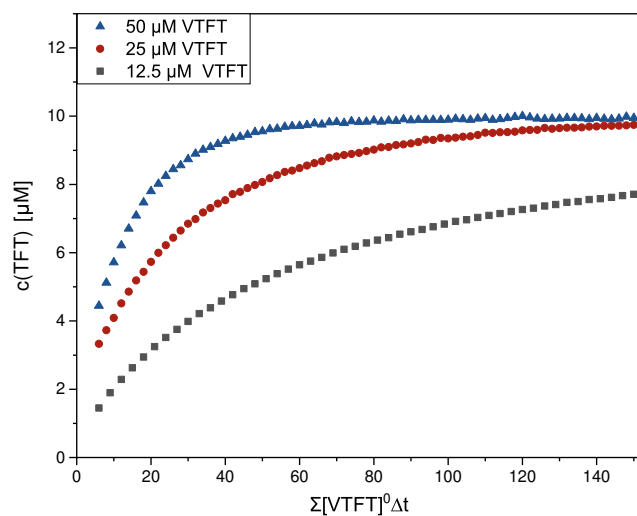


Figure E.50: VTNA analysis for $\beta = 0$.

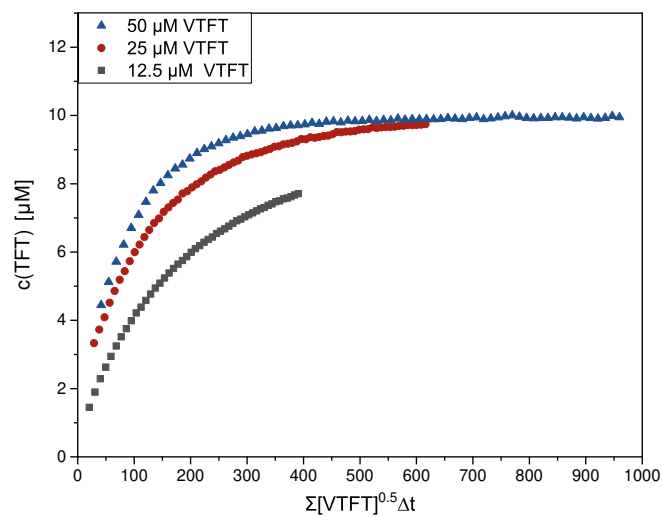


Figure E.51: VTNA analysis for $\beta = 0.5$.

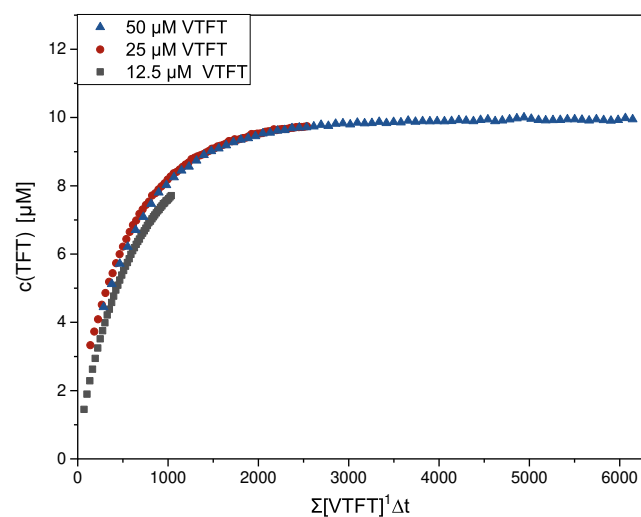


Figure E.52: VTNA analysis for $\beta = 1.0$.

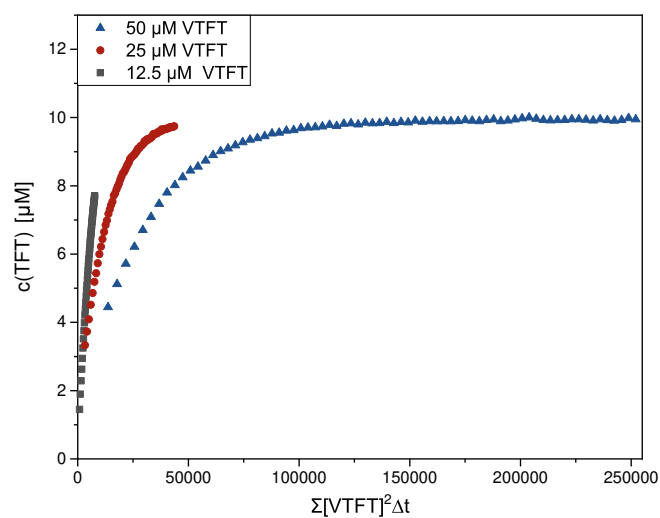


Figure E.53: VTNA analysis for $\beta = 2.0$.

Reaction order in NaN_3

To determine the reaction order in NaN_3 , $c(\text{NaN}_3)$ was set to 0.5 mM, 1.5 mM, and 4.5 mM, keeping the concentrations of other reagents constant: $c(\text{GSH}) = 10 \mu\text{M}$ and $c(\text{VTFT}) = 50 \mu\text{M}$. The concentration of **TFT** was plotted against $t \cdot [\text{NaN}_3]^\beta$, generating graphs for different values of β (0, 0.5, 1.0, and 2.0).

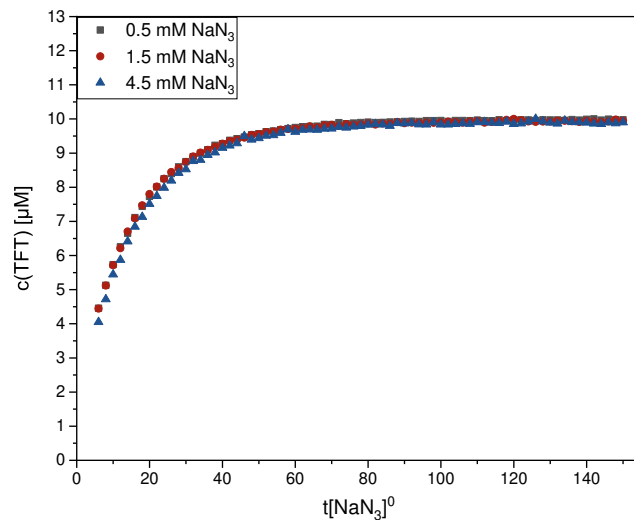


Figure E.54: VTNA analysis for $\beta = 0$.

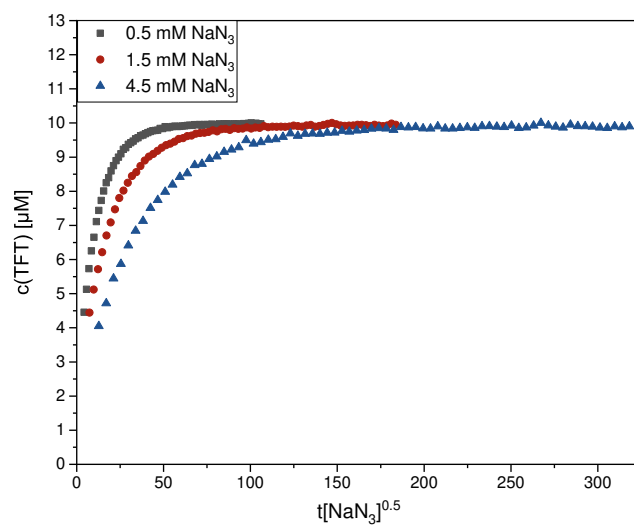


Figure E.55: VTNA analysis for $\beta = 0.5$.

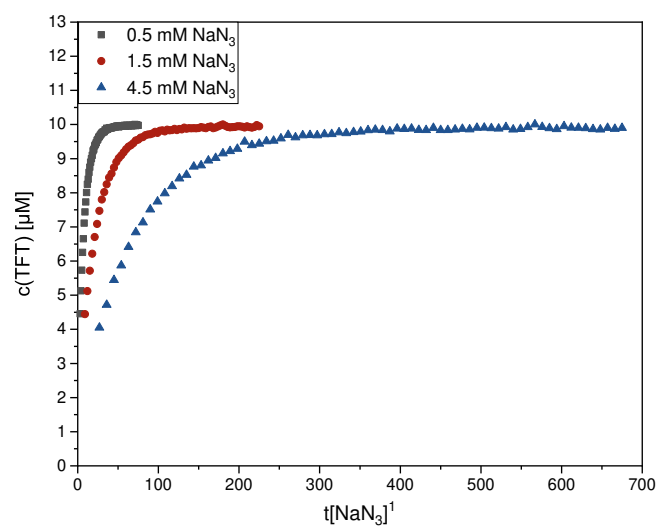


Figure E.56: VTNA analysis for $\beta = 1.0$.

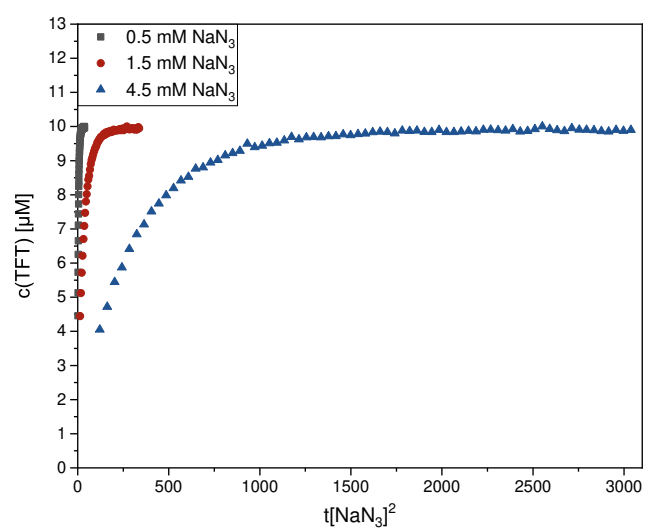
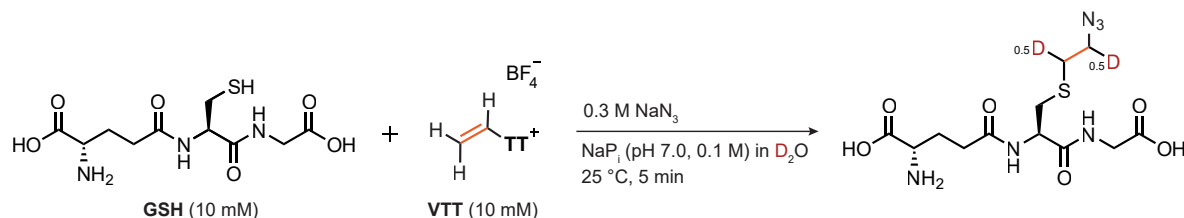


Figure E.57: VTNA analysis for $\beta = 2.0$.

Deuterium incorporation experiments

Bioconjugation with VTT and VTFT

Reaction between **GSH**, NaN_3 , and **VTT** or **VTFT** was performed in deuterated sodium phosphate buffer (D-NaP_i , pH 7.0, $c = 100 \text{ mM}$). 3-(Trimethylsilyl)propionic-2,2,3,3- d_4 acid sodium salt (**TSP**) was used as an internal standard. The peaks at 3.58 ppm and 2.85 ppm corresponding to the introduced ethylene bridge were integrated to determine the deuterium incorporation in the product.



Stock solutions of **GSH** (50 mM), **TSP** (50 mM), and NaN_3 (1.5 M) were prepared in D_2O . D-NaP_i (100 μL , pH 7.0, $c = 0.5 \text{ M}$), D_2O (95 μL), stock solutions of NaN_3 (100 μL), **TSP** (100 μL), and **GSH** (100 μL) were pipetted into an Eppendorf tube and mixed by pipetting the mixture up and down. Subsequently, **VTT** stock solution in DMF-d_7 (5.0 μL , 1.0 M) was added to the tube to obtain a mixture containing **GSH** (10 mM, 1.0 equiv.), **TSP** (10 mM, 1.0 equiv.), NaN_3 (300 mM, 30.0 equiv.), and **VTT** (10 mM, 1.0 equiv.). The ^1H NMR spectrum of the mixture was acquired 5 minutes after addition of **VTT**.

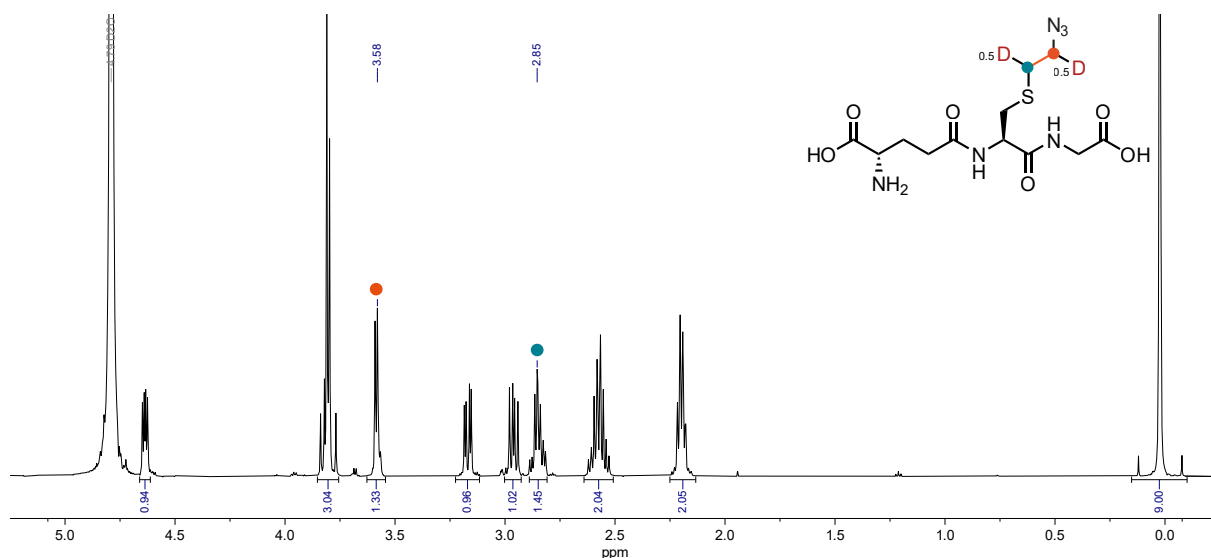
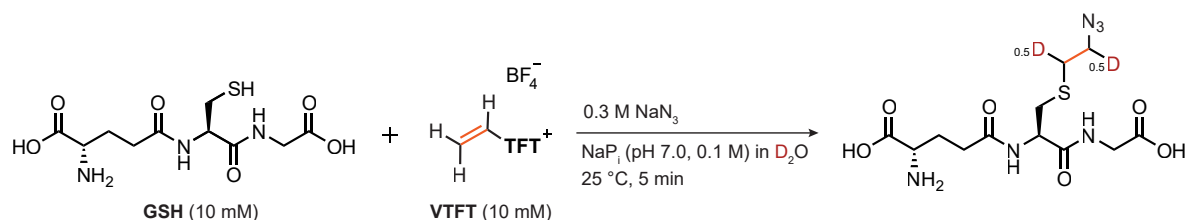


Figure E.58: ^1H NMR spectrum of **GSH** (10 mM, 1.0 equiv.) in D-NaP_i (pH 7.0, 100 mM) in presence of sodium azide (300 mM, 30.0 equiv.) and **TSP** (10 mM, 1.0 equiv.) as internal standard after addition of **VTT** (10 mM, 1.0 equiv.).



Stock solutions of **GSH** (50 mM), **TSP** (50 mM), and NaN_3 (1.5 M) were prepared in D_2O . D- NaPi (100 μL , pH 7.0, $c = 0.5\text{ M}$), D_2O (95 μL), stock solutions of NaN_3 (100 μL), **TSP** (100 μL), and **GSH** (100 μL) were pipetted into an Eppendorf tube and mixed by pipetting the mixture up and down. Subsequently, a stock solution of **VTFT** in DMF-d_7 (5.0 μL , $c = 1.0\text{ M}$) was added to the tube to obtain a mixture containing **GSH** (10 mM, 1.0 equiv.), **TSP** (10 mM, 1.0 equiv.), NaN_3 (300 mM, 30.0 equiv.), and **VTFT** (10 mM, 1.0 equiv.). The ^1H NMR spectrum of the mixture was acquired 5 minutes after addition of **VTFT**.

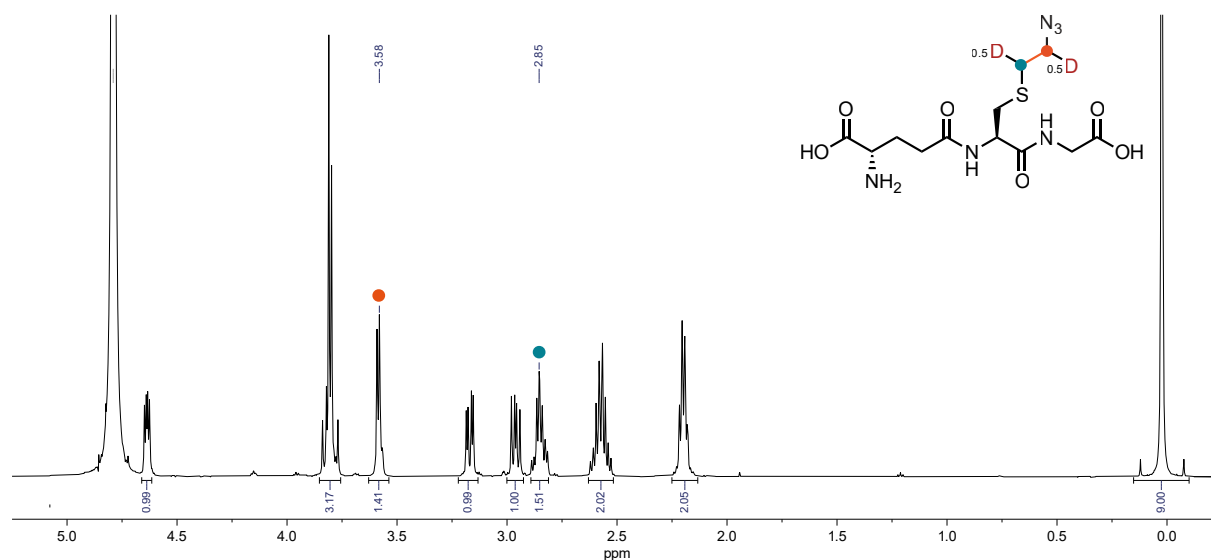
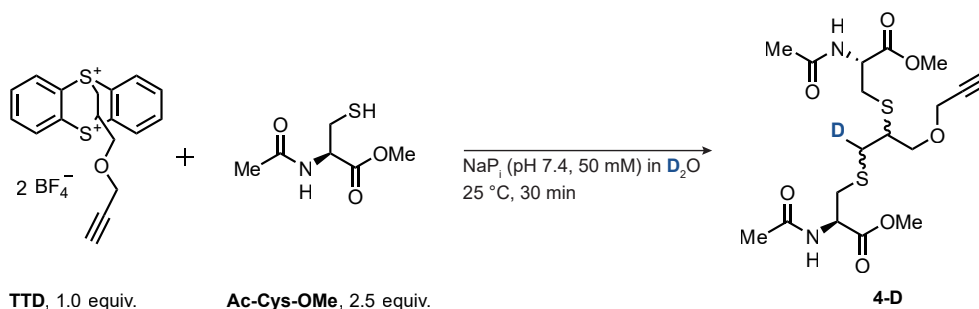


Figure E.59: ^1H NMR spectrum of **GSH** (10 mM, 1.0 equiv.) in D- NaPi (pH 7.0, 100 mM) in presence of sodium azide (300 mM, 30.0 equiv.) and **TSP** (10 mM, 1.0 equiv.) as internal standard after addition of **VTFT** (10 mM, 1.0 equiv.).

Bioconjugation with TTD

Product characterization using NMR



The reaction between **TTD** and Ac-Cys-OMe was performed in deuterated buffer (D-NaP_i, pH 7.4, c = 50 mM) and the reaction mixture was analyzed with NMR using 3-(trimethylsilyl)propionic-2,2,3,3-d₄ acid sodium salt (**TSP**) as an internal standard: NaP_i buffer in D₂O (50 μL, pH 7.4, c = 0.5 M), D₂O (295 μL), a freshly prepared stock solution of Ac-Cys-OMe in D₂O (100 μL, c = 50 mM), a stock solution of **TSP** in D₂O (50 μL, c = 50 mM) were pipetted into an Eppendorf tube and mixed by pipetting the mixture up and down. Subsequently, a freshly prepared stock solution of **TTD** in DMF-d₇ (5.0 μL, c = 0.50 M) was added to the tube to obtain a mixture containing **TTD** (5.0 mM, 1.0 equiv.), Ac-Cys-OMe (10 mM, 2.0 equiv.), **TSP** (5.0 mM, 1.0 equiv.) in D-NaP_i (50 mM). The reaction mixture was vortexed for 15 seconds, incubated at 25 °C for 30 minutes at 600 rpm, and centrifuged for 5 minutes at 17,000 g. The obtained supernatant was used for the NMR measurements.

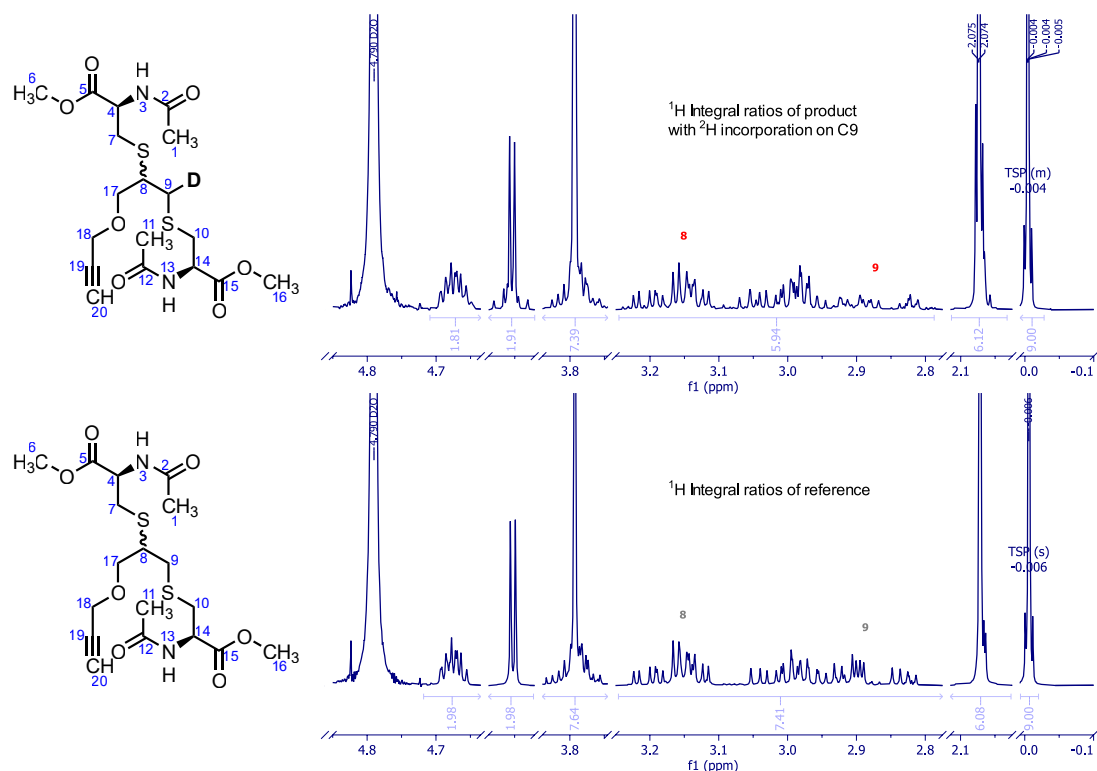


Figure E.60: Top: integrated ¹H NMR spectrum of the mixture containing **TTD** (5.0 mM), Ac-Cys-OMe (10 mM), and **TSP** (5.0 mM) in D-NaP_i (pH 7.4, 50 mM). Bottom: integrated ¹H NMR spectrum of the mixture containing reference compound **4** (5.0 mM) and **TSP** (5.0 mM) in D-NaP_i (pH 7.4, 50 mM).

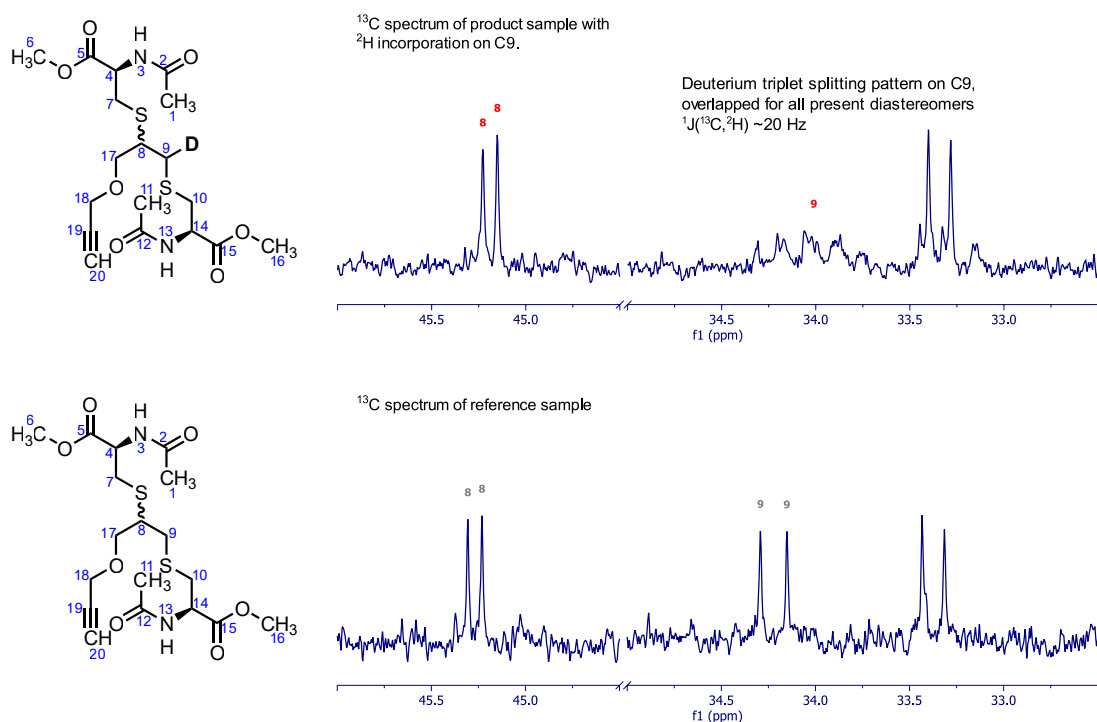


Figure E.61: Top: ¹³C NMR spectrum of the mixture containing **TTD** (5.0 mM), Ac-Cys-OMe (10 mM), and **TSP** (5.0 mM) in D-NaP_i (pH 7.4, 50 mM). Bottom: ¹³C NMR spectrum of the mixture containing reference compound **4** (5.0 mM) and **TSP** (5.0 mM) in D-NaP_i (pH 7.4, 50 mM).

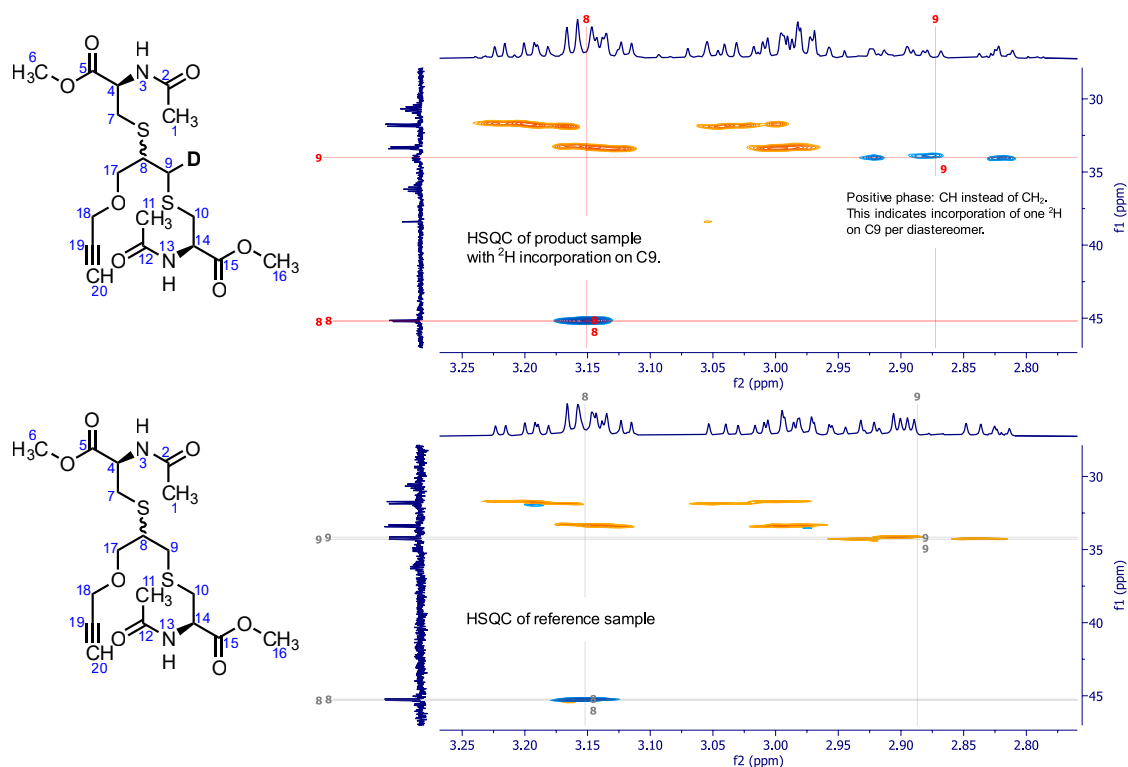


Figure E.62: Top: HSQC NMR spectrum of the mixture containing **TTD** (5.0 mM), Ac-Cys-OMe (10 mM), and **TSP** (5.0 mM) in D-NaP_i (pH 7.4, 50 mM). Bottom: HSQC NMR spectrum of the mixture containing reference compound **4** (5.0 mM) and **TSP** (5.0 mM) in D-NaP_i (pH 7.4, 50 mM).

Product characterization using MS

NaP_i buffer (50 μ L, pH 7.4, c = 0.5 M), H₂O (320 μ L), and a freshly prepared stock solution of Ac-Cys-OMe in H₂O (125 μ L, c = 50 mM) were pipetted into an Eppendorf tube and mixed by pipetting the mixture up and down. Subsequently, a freshly prepared stock solution of **TTD** in DMF (5.0 μ L, c = 0.50 M) was added to the tube to obtain a mixture containing **TTD** (5.0 mM, 1.0 equiv.) and Ac-Cys-OMe (13 mM, 2.5 equiv.) in NaP_i (50 mM). The reaction mixture was vortexed for 15 seconds, incubated at 25 °C for 30 minutes at 600 rpm, and centrifuged for 5 minutes at 17,000 xg. The obtained supernatant was used for the HRMS-measurement.

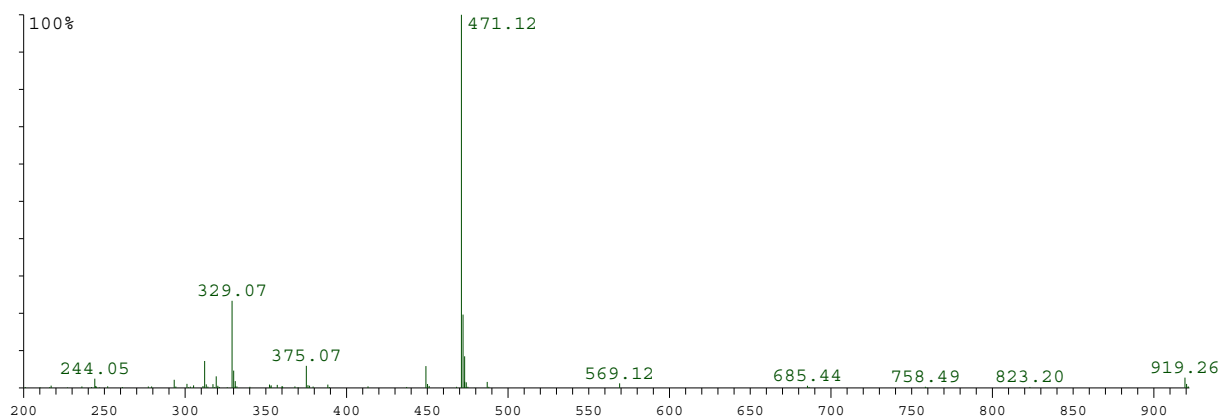


Figure E.63: HRMS analysis of the mixture containing 5.0 mM **TTD** and 13 mM Ac-Cys-OMe in NaP_i (pH 7.4, 50 mM). Calculated mass for C₁₈H₂₈N₂O₇S₂, [M+Na]⁺: 471.1230; detected: 471.1229. Deviation: -0.2 ppm.

NaP_i buffer in D₂O (50 μ L, pH 7.4, c = 0.5 M), D₂O (320 μ L), and a freshly prepared stock solution of Ac-Cys-OMe in D₂O (125 μ L, c = 50 mM) were pipetted into an Eppendorf tube and mixed by pipetting the mixture up and down. Subsequently, a freshly prepared stock solution of **TTD** in DMF-d₇ (5.0 μ L, c = 0.50 M) was added to the tube to obtain a mixture containing **TTD** (5.0 mM, 1.0 equiv.) and Ac-Cys-OMe (13 mM, 2.5 equiv.) in D-NaP_i (50 mM). The reaction mixture was vortexed for 15 seconds, incubated at 25 °C for 30 minutes at 600 rpm, and centrifuged for 5 minutes at 17,000 xg. The obtained supernatant was used for the HRMS-measurement.

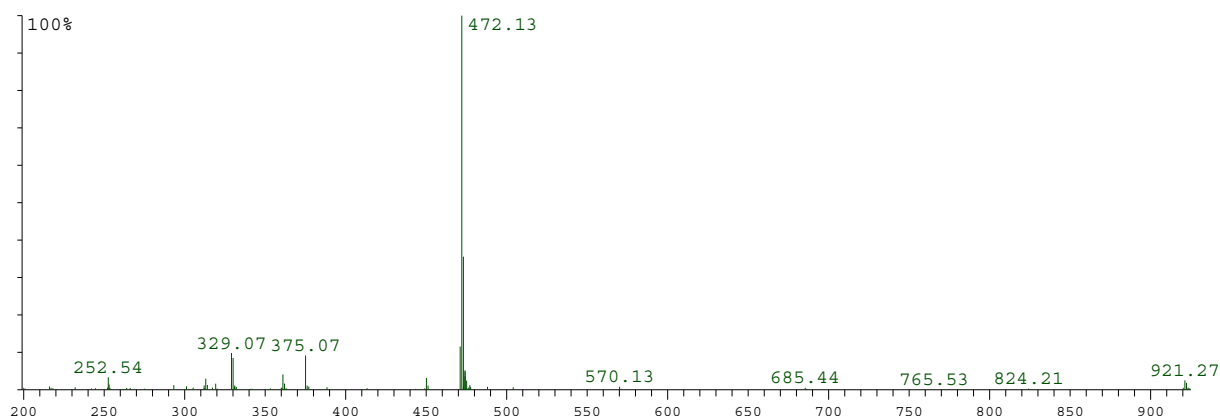


Figure E.64: HRMS analysis of the mixture containing 5.0 mM **TTD** and 13 mM Ac-Cys-OMe in D-NaP_i (pH 7.4, 50 mM). Calculated mass for C₁₈H₂₇DN₂O₇S₂, [M+Na]⁺: 472.1293; detected: 472.1292. Deviation: -0.2 ppm.

Kinetic isotope effect studies

Determination of KIE for VTT-mediated bioconjugation

The KIE was determined by introducing **GSH** in reaction with 5-fold excess of both **VTT** and $^2\text{H}_3$ -**VTT** in presence of sodium azide and determining the product ratio of the products. A reference reaction with 10-fold excess of **VTT** was performed to determine the integral value of product formed at the same reaction conditions that were used for the competition experiment.

The reaction between **GSH**, NaN_3 and an excess of **VTT** was performed in deuterated buffer (D-NaP_i , pH 7.0, $c = 100 \text{ mM}$) and the reaction mixture was analyzed with ^1H NMR using **TSP** as an internal standard. Stock solutions of **GSH** (50 mM), **TSP** (50 mM), and NaN_3 (1.0 M) were prepared in D_2O . D-NaP_i (100 μL , pH 7.0, $c = 0.5 \text{ M}$), D_2O (200 μL), stock solutions of NaN_3 (75 μL), **TSP** (50 μL), and **GSH** (50 μL) were pipetted into an Eppendorf tube and mixed by pipetting the mixture up and down. Subsequently, a stock solution of **VTT** in DMF-d_7 (25 μL , $c = 1.0 \text{ M}$) was added to the tube to obtain a mixture containing **GSH** (5.0 mM, 1.0 equiv.), **TSP** (5.0 mM, 1.0 equiv.), NaN_3 (150 mM, 30.0 equiv.), and **VTT** (50 mM, 10 equiv.). The ^1H NMR spectrum of the mixture was acquired 5 minutes after addition of **VTT**:

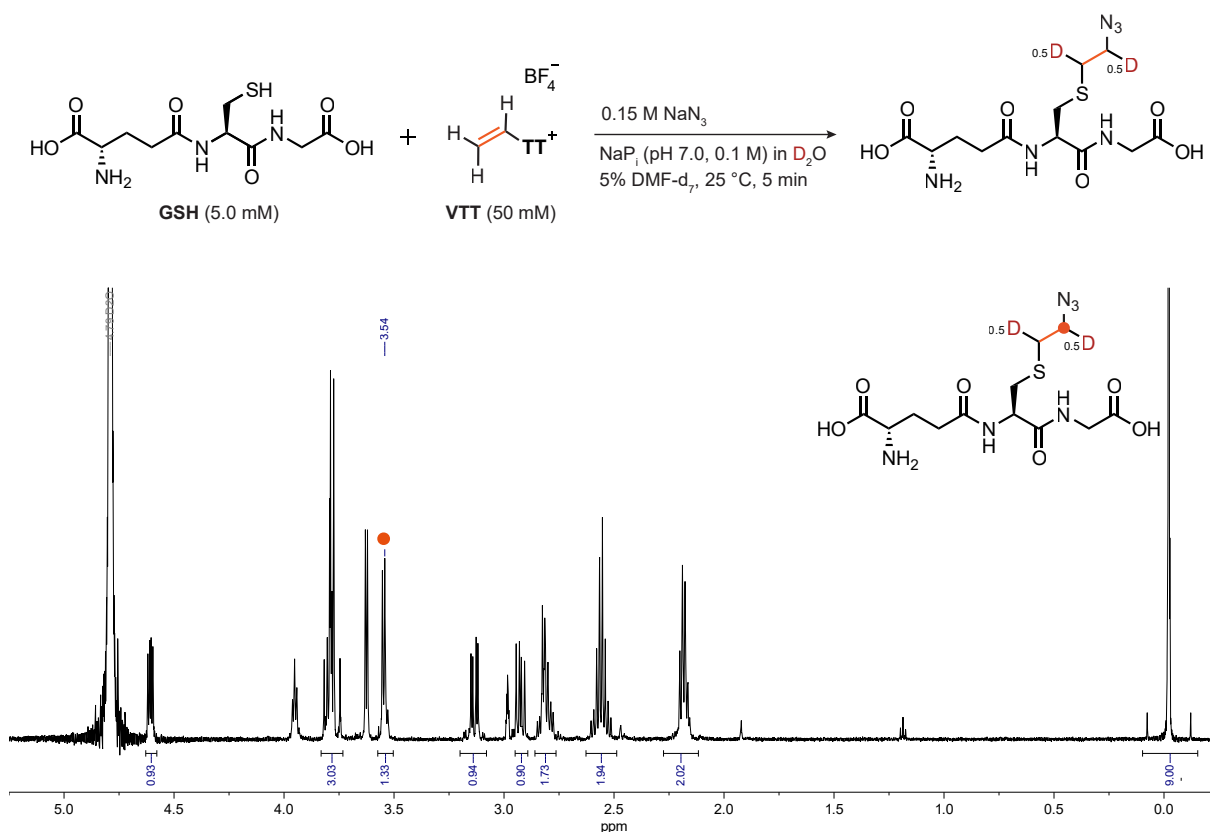


Figure E.65: ^1H NMR spectrum of **GSH** (5.0 mM, 1.0 equiv.) in D-NaP_i (pH 7.0, 100 mM) after addition of 10 equiv. **VTT** in presence of 30 equiv. NaN_3 with **TSP** as internal standard.

The reaction between **GSH**, NaN_3 and an excess of **VTT** and $^2\text{H}_3$ -**VTT** was performed in deuterated buffer (D-NaP_i , pH 7.0, $c = 100 \text{ mM}$) and the reaction mixture was analyzed with ^1H NMR using **TSP** as an internal standard: Stock solutions of **GSH** (50 mM), **TSP** (50 mM), and NaN_3 (1.0 M) were prepared in D_2O . D-NaP_i (100 μL , 500 mM), D_2O (200 μL), stock solutions of NaN_3 (75 μL), **TSP** (50 μL), and **GSH** (50 μL) were pipetted into an Eppendorf tube and mixed by pipetting the mixture up and down. Subsequently, 25 μL of a solution containing **VTT** (0.5 M) and $^2\text{H}_3$ -**VTT** (0.5 M) in DMF-d_7 was added to the tube to obtain a mixture containing **GSH**

(5.0 mM, 1.0 equiv.), **TSP** (5.0 mM, 1.0 equiv.), NaN_3 (150 mM, 30.0 equiv.), **VTT** (25 mM, 5.0 equiv.), and $^2\text{H}_3\text{-VTT}$ (25 mM, 5.0 equiv.). The ^1H NMR spectrum of the mixture was acquired 5 minutes after addition of the mixture containing **VTT** and $^2\text{H}_3\text{-VTT}$:

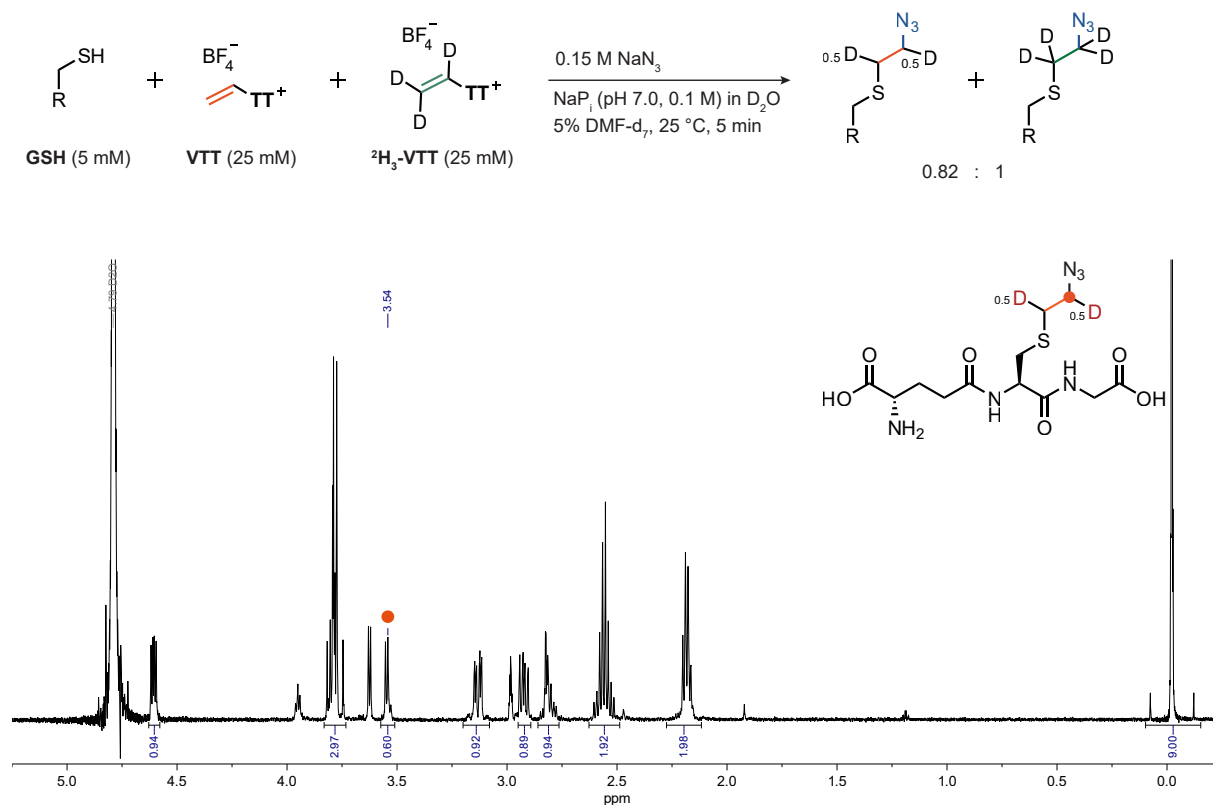


Figure E.66: ^1H NMR spectrum of **GSH** (5.0 mM, 1.0 equiv.) in D- NaPi (pH 7.0, 50 mM) after addition of 5.0 equiv. **VTT** and 5.0 equiv. $^2\text{H}_3\text{-VTT}$ in presence of 30 equiv. NaN_3 with **TSP** as internal standard.

The ratio of the obtained products formed in reaction with either **VTT** or $^2\text{H}_3\text{-VTT}$ was determined by calculating the deuterium incorporation in the ethylene bridge for both reactions. The peak at 3.54 ppm corresponds to the methylene group alpha to the azide in the product obtained with **VTT** and was integrated for both spectra to calculate the ratio of the products formed with either **VTT** or $^2\text{H}_3\text{-VTT}$. Based on the integral values:

$$\frac{k_2(^2\text{H}_3\text{-VTT})}{k_2(\text{VTT})} = \frac{0.60}{1.33 - 0.60} = \frac{0.60}{0.73} = 0.82$$

Determination of KIE for VTFT

The KIE was determined by introducing **GSH** in reaction with 5-fold excess of both **VTFT** and $^2\text{H}_3\text{-VTFT}$ in presence of sodium azide and determining the product ratio of the products. A reference reaction with 10-fold excess of **VTFT** was performed to determine the integral value of product formed at the same reaction conditions that were used for the competition experiment. The reaction between **GSH**, NaN_3 and an excess of **VTFT** was performed in deuterated buffer (D-NaP_i , pH 7.0, $c = 100 \text{ mM}$) and the reaction mixture was analyzed with ^1H NMR using **TSP** as an internal standard. Stock solutions of **GSH** (50 mM), **TSP** (50 mM), and NaN_3 (1.0 M) were prepared in D_2O . D-NaP_i (100 μL , pH 7.0, $c = 0.5 \text{ M}$), D_2O (175 μL), stock solutions of NaN_3 (75 μL), **TSP** (50 μL), and **GSH** (50 μL) were pipetted into an Eppendorf tube and mixed by pipetting the mixture up and down. Subsequently, a stock solution of **VTFT** in DMF-d_7 (50 μL , $c = 0.5 \text{ M}$) was added to the tube to obtain a mixture containing **GSH** (5.0 mM, 1.0 equiv.), **TSP** (5.0 mM, 1.0 equiv.), NaN_3 (150 mM, 30.0 equiv.), and **VTFT** (50 mM, 10 equiv.). The ^1H NMR spectrum of the mixture was acquired 5 minutes after addition of **VTFT**:

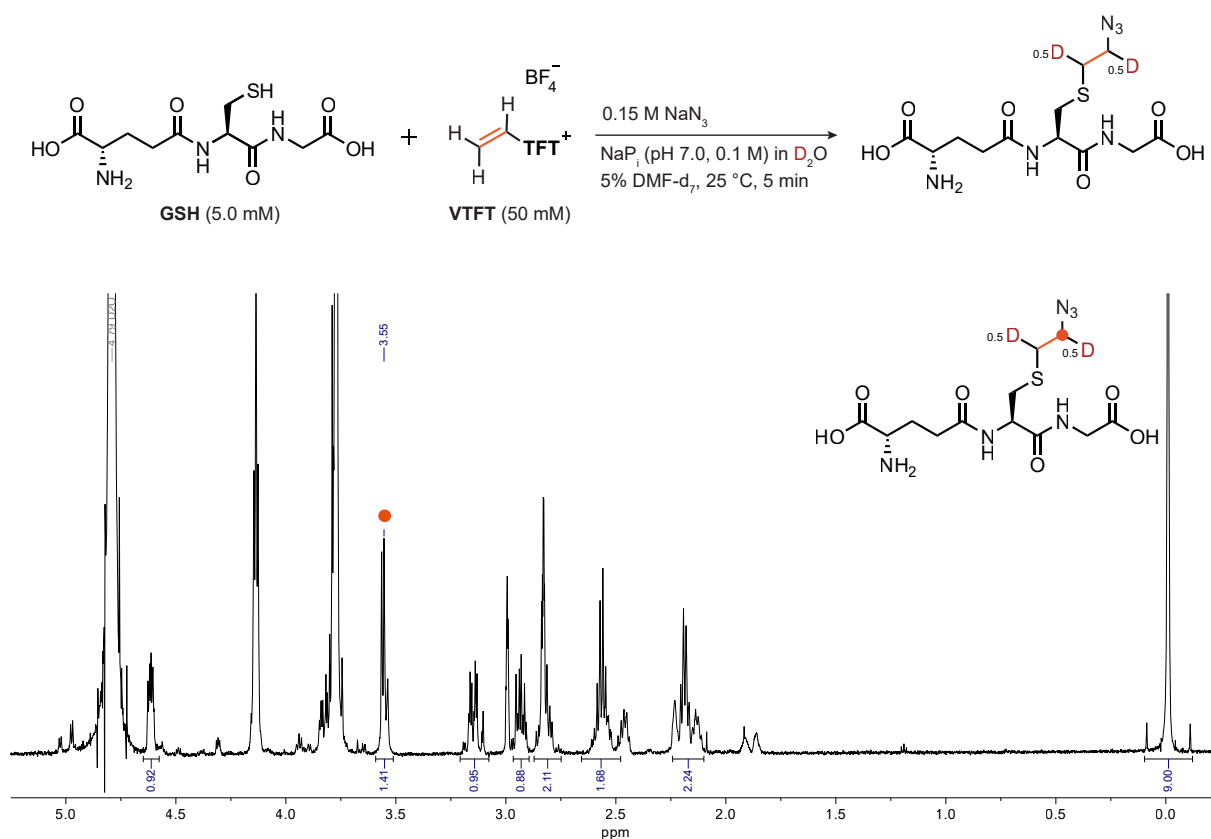


Figure E.67: ^1H NMR spectrum of **GSH** (5.0 mM, 1.00 equiv.) in D-NaP_i (pH 7.0, 50 mM) after addition of 10 equiv. **VTFT** in presence of 30.0 equiv. NaN_3 with **TSP** as internal standard.

The reaction between **GSH**, NaN_3 and an excess of **VTFT** and $^2\text{H}_3\text{-VTFT}$ was performed in deuterated buffer (D-NaP_i , pH 7.0, $c = 100 \text{ mM}$) and the reaction mixture was analyzed with ^1H NMR using **TSP** as an internal standard. Stock solutions of **GSH** (50 mM), **TSP** (50 mM), and NaN_3 (1.0 M) were prepared in D_2O . D-NaP_i (100 μL , pH 7.0, $c = 0.5 \text{ M}$), D_2O (175 μL), stock solutions of NaN_3 (75 μL), **TSP** (50 μL), and **GSH** (50 μL) were pipetted into an Eppendorf tube and mixed by pipetting the mixture up and down. Subsequently, 50 μL of a solution containing **VTFT** (0.25 M) and $^2\text{H}_3\text{-VTFT}$ (0.25 M) in DMF-d_7 was added to the tube to obtain a mixture containing **GSH** (5.0 mM, 1.0 equiv.), **TSP** (5.0 mM, 1.0 equiv.), NaN_3 (150 mM, 30.0 equiv.),

VTFT (25 mM, 5.0 equiv.), and **²H₃-VTFT** (25 mM, 5.0 equiv.). The ¹H NMR spectrum of the mixture was acquired 5 minutes after addition of the mixture containing **VTFT** and **²H₃-VTFT**:

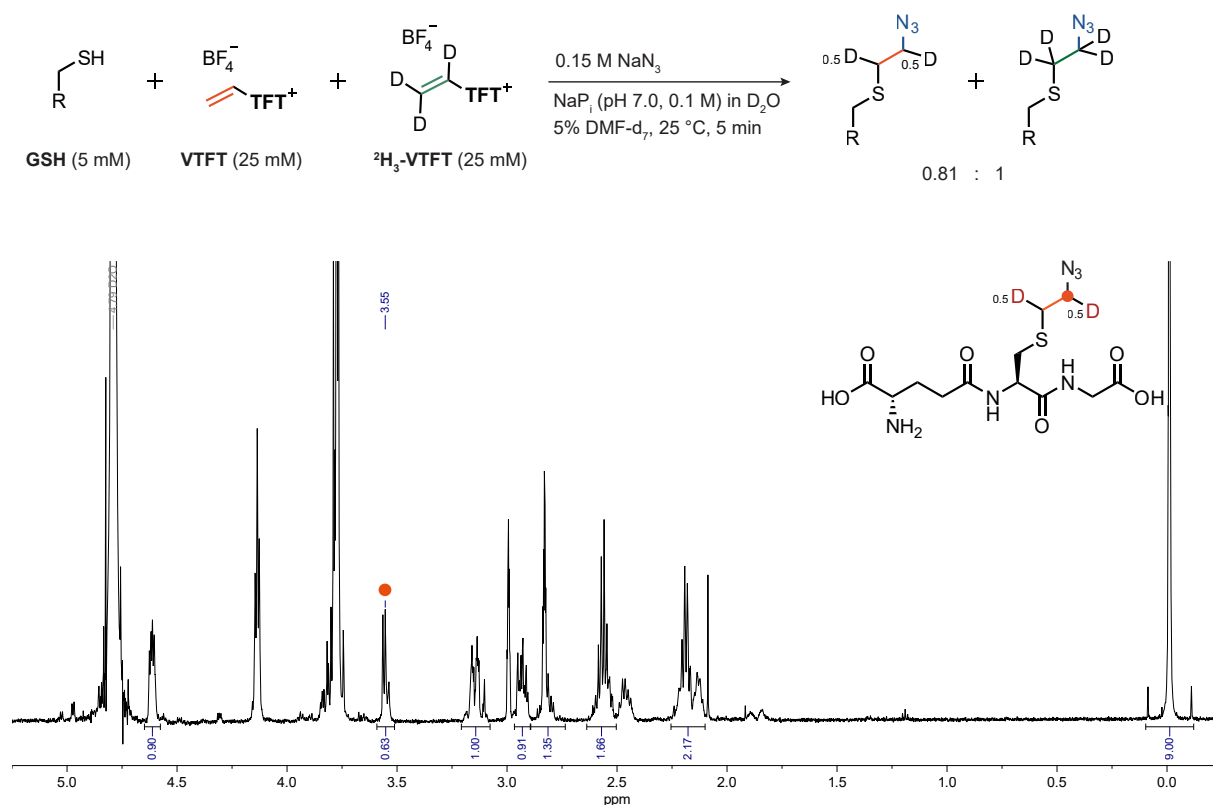


Figure E.68: ¹H NMR spectrum of **GSH** (5.0 mM, 1.00 equiv.) in D-NaPi (pH 7.0, 50 mM) after addition of 5.0 equiv. **VTFT** and 5.0 equiv. **²H₃-VTFT** in presence of 30.0 equiv. NaN_3 with **TSP** as internal standard

The ratio of the obtained products formed in reaction with either **VTFT** or **²H₃-VTFT** was determined by calculating the deuterium incorporation in the ethylene bridge for both reactions. The peak at 3.55 ppm corresponds to the methylene group alpha to the azide of the product obtained with **VTFT** and was integrated for both spectra to calculate the ratio of the products formed with either **VTFT** or **²H₃-VTFT**. Based on the integral values:

$$\frac{k_2(^2\text{H}_3\text{-VTFT})}{k_2(\text{VTFT})} = \frac{0.63}{1.41 - 0.63} = \frac{0.63}{0.78} = 0.81$$

Determination of rate constants

Bioconjugation with VTT

The consumption of **VTT** over time was determined by measuring the absorbance of **VTT** at 307 nm. The reaction mixtures contained equal concentrations of **VTT** and **GSH** (30 μ M) and an excess of NaN_3 (0.9 mM). Sodium phosphate buffer (pH 7.4, $c = 50$ mM) was used as solvent. Stock solutions of **GSH** (1.68 mM) and NaN_3 (13.8 mM) were prepared in NaP_i (pH 7.4, $c = 50$ mM). NaP_i (1.82 mL, pH 7.4, $c = 50$ mM), stock solutions of NaN_3 (0.13 mL), and **GSH** (36 μ L) were pipetted into a quartz cuvette charged with a Teflon-coated magnetic stirring bar. The cuvette was thermostated for 5 minutes at (25 ± 0.1) $^{\circ}\text{C}$ in the cell holder. After performing the background measurement, a stock solution of **VTT** in DMF (10 μ L, $c = 6.0$ mM) was added to the cuvette. The reaction mixture was homogenized by pipetting the mixture up and down. Then, the cuvette was capped and the absorbance of **VTT** at 307 nm was measured while stirring. Because formation of **TT** precipitate was observed after 5 minutes at these reaction conditions, each measurement used for the determination of the rate constant was stopped after 300 seconds. The reaction mixture was then transferred from the cuvette into a vial charged with a stirring bar and stirred at 25 $^{\circ}\text{C}$ for 24 hours. The suspension was filtered through a syringe filter and the absorbance of the clear solution at 307 nm was determined. This value was used to define the total range in which the absorbance of the reaction mixture at 307 nm changed during the reaction. The second-order initial rate constant was determined by plotting $1/c(\text{VTT})$ against time and determining the slope of the linear fit for each graph.

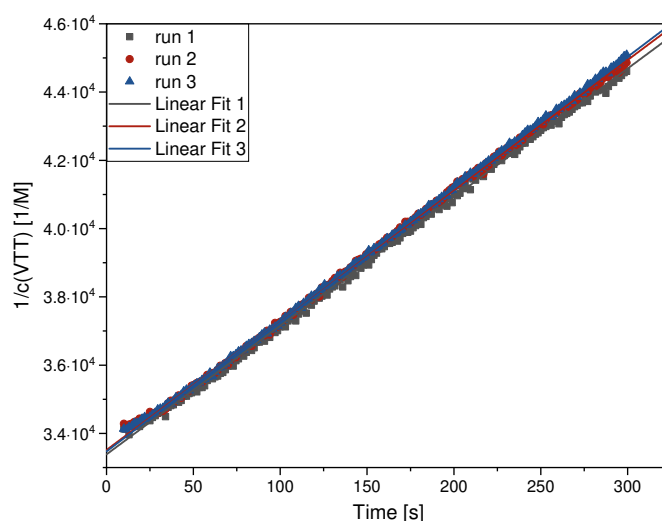


Figure E.69: Plots obtained from the **VTT** concentration decay in reaction with equimolar **GSH** in the presence of NaN_3 . The linear plots were obtained for 25% conversion.

Run	$k_2, \text{M}^{-1}\text{s}^{-1}$	R^2
1	37.7 ± 0.1	0.9991
2	38.1 ± 0.1	0.9993
3	38.5 ± 0.1	0.9996

Based on the data from three runs: $k_2(\text{VTT}) = 38.1 \pm 0.1 \text{ M}^{-1}\text{s}^{-1}$.

Bioconjugation with $^2\text{H}_3\text{-VTT}$

The consumption of $^2\text{H}_3\text{-VTT}$ over time was determined by measuring the absorbance of $^2\text{H}_3\text{-VTT}$ at 307 nm. The reaction mixtures contained equal concentrations of $^2\text{H}_3\text{-VTT}$ and **GSH** (30 μM) and an excess of NaN_3 (0.9 mM). Sodium phosphate buffer (pH 7.4, $c = 50$ mM) was used as solvent. Stock solutions of **GSH** (1.68 mM) and NaN_3 (13.8 mM) were prepared in NaP_i (pH 7.4, $c = 50$ mM). NaP_i (1.82 mL, pH 7.4, $c = 50$ mM), stock solutions of NaN_3 (0.13 mL), and **GSH** (36 μL) were pipetted into a quartz cuvette charged with a Teflon-coated magnetic stirring bar. The cuvette was thermostated for 5 minutes at (25 ± 0.1) $^\circ\text{C}$ in the cell holder. After performing the background measurement, a stock solution of $^2\text{H}_3\text{-VTT}$ in DMF (10 μL , $c = 6.0$ mM) was added to the cuvette. The reaction mixture was homogenized by pipetting the mixture up and down. Then, the cuvette was capped and the absorbance of $^2\text{H}_3\text{-VTT}$ at 307 nm was measured while stirring for 5 minutes. The reaction mixture was then transferred from the cuvette into a vial charged with a stirring bar and stirred at 25 $^\circ\text{C}$ for 24 hours. The suspension was filtered through a syringe filter and the absorbance of the clear solution at 307 nm was determined. This value was used to define the total range in which the absorbance of the reaction mixture at 307 nm changed during the reaction. The second-order initial rate constant was determined by plotting $1/c(^2\text{H}_3\text{-VTT})$ against time and determining the slope of the linear fit for each graph.

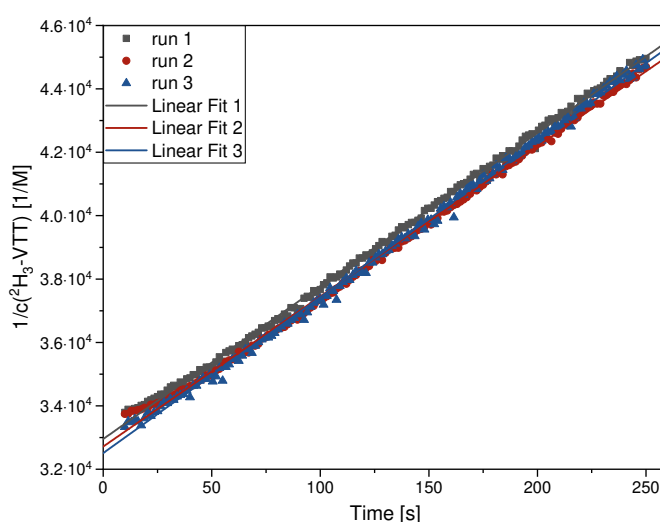


Figure E.70: Plots obtained from the $^2\text{H}_3\text{-VTT}$ concentration decay in reaction with equimolar **GSH** in the presence of NaN_3 . The linear plots were obtained for 25% conversion.

Run	$k_2, \text{M}^{-1}\text{s}^{-1}$	R^2
1	48.3 ± 0.1	0.9988
2	47.4 ± 0.2	0.9982
3	49.2 ± 0.2	0.9982

Based on the data from three runs: $k_2(^2\text{H}_3\text{-VTT}) = 48.3 \pm 0.1 \text{ M}^{-1}\text{s}^{-1}$.

Bioconjugation with VTFT

The consumption of **VTFT** over time was determined by measuring the absorbance of **VTFT** at 306 nm. The reaction mixtures contained equal concentrations of **VTFT** and **GSH** (30 μM) and an excess of NaN_3 (0.9 mM). Sodium phosphate buffer (pH 7.4, 50 mM) was used as solvent. Stock solutions of **GSH** (1.74 mM) and NaN_3 (14.2 mM) were prepared in NaP_i (pH 7.4, 50 mM). NaP_i (1.83 mL), stock solutions of NaN_3 (127 μL), and **GSH** (35 μL) were pipetted into a quartz cuvette and charged with a Teflon-coated magnetic stirring bar. The cuvette was thermostated for 5 minutes at 25 $^\circ\text{C}$ in the cell holder. After performing the background measurement, a **VTFT** stock solution in DMF (10 μL , $c = 6.0 \text{ mM}$) was added to the cuvette and the absorbance of **VTFT** at 306 nm was measured while stirring.

Because of the fast formation of **TFT** precipitate in the mixture, the absorbance change was measured for the first 11 seconds after addition of **VTFT** and before formation of **TFT** was observed. After that, the measurement was stopped, the reaction mixture was transferred from the cuvette into a vial charged with a stirring bar and stirred at 25 $^\circ\text{C}$ for 24 hours. The suspension was then filtered through a syringe filter, and the absorbance of the clear solution at 306 nm was determined. This value was used to define the total range in which the absorbance of the reaction mixture at 306 nm changed during the reaction. The second-order initial rate constant was determined by plotting $1/c(\text{VTFT})$ against time and determining the slope of the linear fit of each graph.

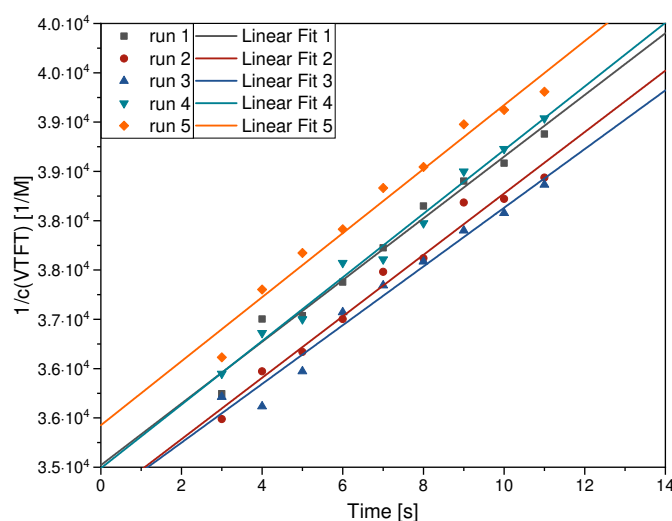


Figure E.71: Plots obtained from the **VTFT** concentration decay in reaction with equimolar **GSH** in the presence of NaN_3 . The linear plots were obtained for 15% conversion.

Run	$k_2, \text{M}^{-1}\text{s}^{-1}$	R^2
1	313 ± 18	0.9778
2	311 ± 16	0.9809
3	297 ± 19	0.9725
4	322 ± 14	0.9876
5	325 ± 21	0.9727

Based on the data from five runs: $k_2(\text{VTFT}) = 310 \pm 8 \text{ M}^{-1}\text{s}^{-1}$.

Reaction between VTT and TCEP

The consumption of **VTT** over time was determined by measuring the absorbance of **VTT** at 307 nm. The reaction mixtures contained equal concentrations of **VTT** and **TCEP** (30 μM). Sodium phosphate buffer (pH 7.4, $c = 50 \text{ mM}$) was used as solvent. Stock solution of TCEP (3.80 mM) was prepared in NaP_i (50 mM, pH 7.4). NaP_i (1.97 mL) and TCEP (16 μL) stock solutions were pipetted into a quartz cuvette charged with a Teflon-coated magnetic stirring bar. The cuvette was thermostated for 5 minutes at $(25 \pm 0.1)^\circ\text{C}$ in the cell holder. After performing the background measurement, a stock solution of **VTT** in DMF (10 μL , $c = 6.0 \text{ mM}$) was added to the cuvette. The reaction mixture was homogenized by pipetting the mixture up and down. Then, the cuvette was capped and the absorbance of **VTT** at 307 nm was measured while stirring. Because formation of **TT** precipitate was observed after 7 minutes at these reaction conditions, each measurement used for the determination of the rate constant was stopped after 400 seconds. The reaction mixture was then transferred from the cuvette into a vial charged with a stirring bar and stirred at 25°C for 2 hours. The suspension was filtered through a syringe filter and the absorbance of the clear solution at 307 nm was determined. This value was used to define the total range in which the absorbance of the reaction mixture at 307 nm changed during the reaction. The second-order initial rate constant was determined by plotting $1/c(\text{VTT})$ against time and determining the slope of the linear fit for each graph.

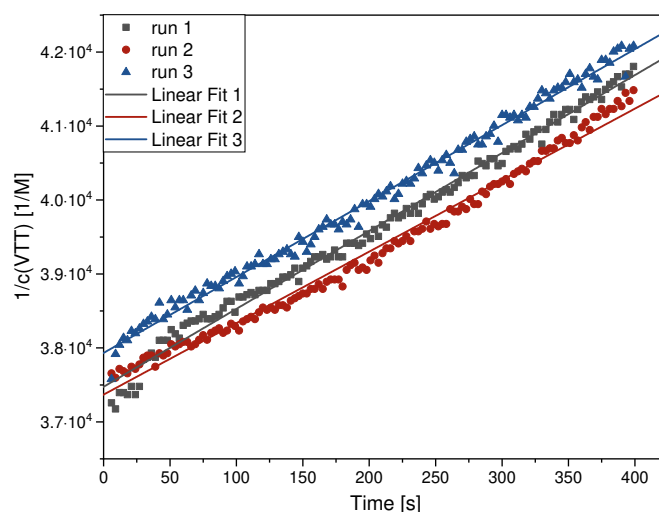
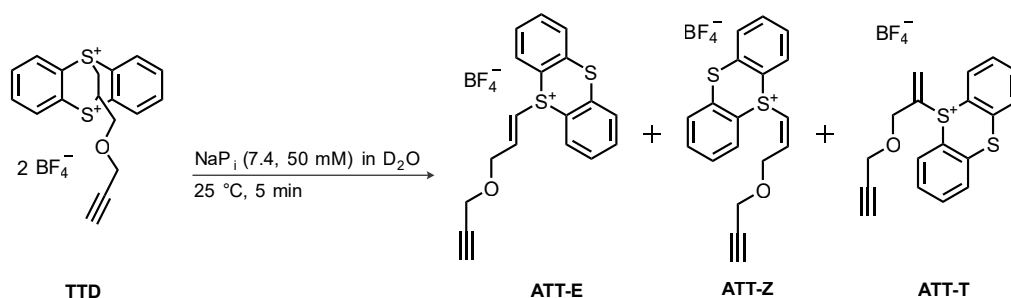


Figure E.72: Plots obtained from the **VTT** concentration decay in reaction with equimolar TCEP. The linear plots were obtained for 20% conversion.

Run	$k_2, \text{M}^{-1}\text{s}^{-1}$	R^2
1	10.5 ± 0.1	0.9913
2	9.7 ± 0.1	0.9885
3	10.3 ± 0.1	0.9927

Based on the data from three runs: $k_2(\text{VTT}) = 10.2 \pm 0.1 \text{ M}^{-1}\text{s}^{-1}$.

Determination of the deprotonation product ratio



At ambient temperature, a NaP_i solution (50 μ L, pH 7.4, 0.5 M) in D₂O was mixed with 400 μ L of D₂O in a 1.5 mL tube. Then, a freshly prepared solution of **T****T****D** in D₂O (50 μ L, 50 mM) was added to the tube, the mixture was vortexed for 10 seconds, and NMR spectra were acquired. The product ratios were determined by integration of characteristic ¹H signals that were sufficiently resolved and the respective integral values were averaged. The resulting integral ratios are 4.4 (**AAT-E**) : 1.4 (**AAT-Z**) : 1.0 (**AAT-T**).

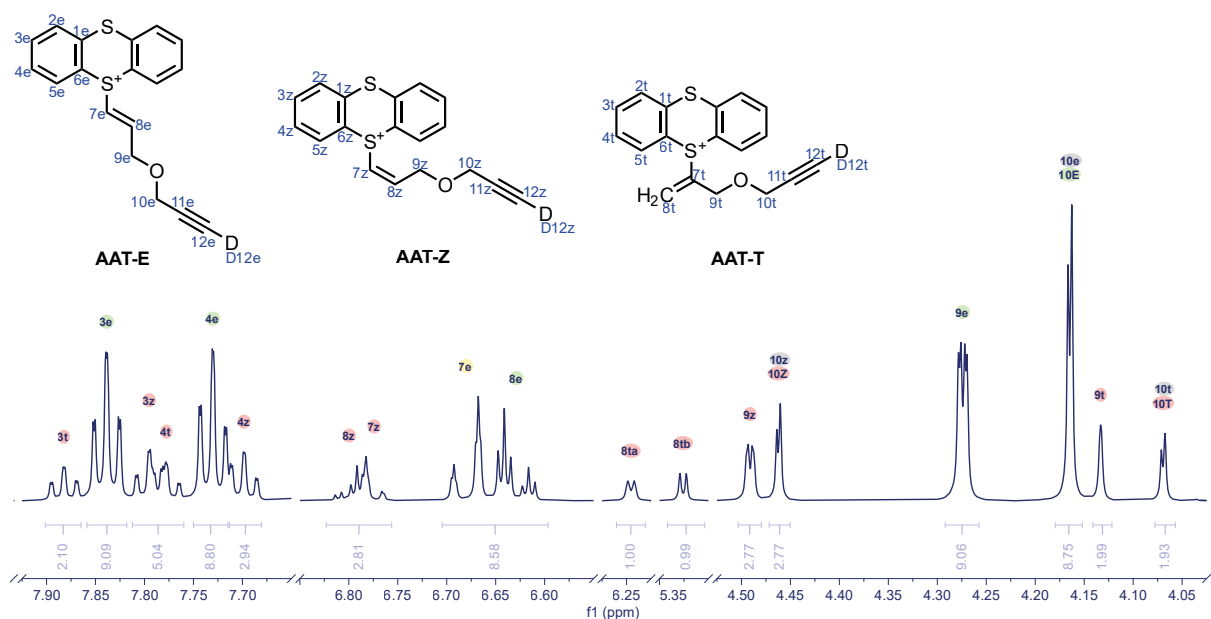


Figure E.73: ^1H NMR spectrum of the peak areas used for the quantification of the product ratio. See ref.²⁰⁶ for full NMR characterization.

Determination of the deprotonation rate

The consumption of **TTD** over time was determined by measuring the absorbance change of **TTD** at 307 nm. Sodium phosphate buffer (pH 7.4, c = 50 mM) containing 0.140 M NaCl was used as solvent. The NaP_i (1990 µL) stock solution was pipetted into a quartz cuvette charged with a Teflon-coated magnetic stirring bar. The cuvette was thermostated for 5 minutes at (25 ± 0.1) °C in the cell holder. After performing the background measurement, a stock solution of **TTD** in H₂O (10.0 µL, c = 10.0 mM) was added to the cuvette. The reaction mixture was homogenized by pipetting the mixture up and down. Then, the absorbance of **TTD** at 307 nm was measured while stirring. The first-order rate constant for the deprotonation of **TTD** in buffer

was determined by plotting $\ln[\Delta\text{Abs}_{307\text{nm}}]$ against time and determining the slope of the linear fit for each graph.

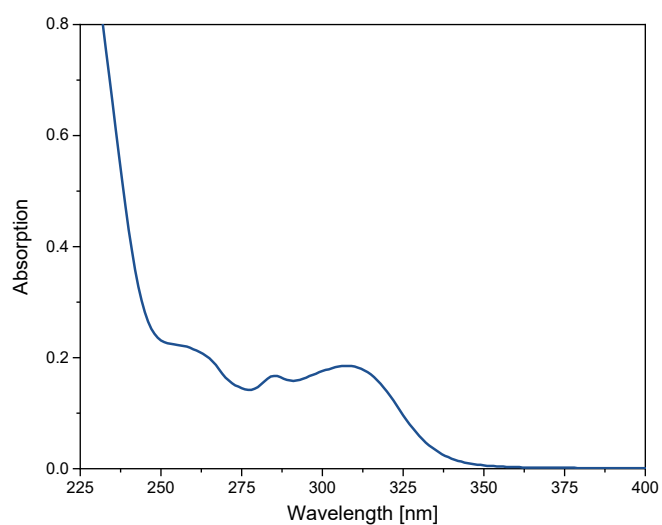


Figure E.74: Absorbance spectrum of **TTD** (50 μM) in water.

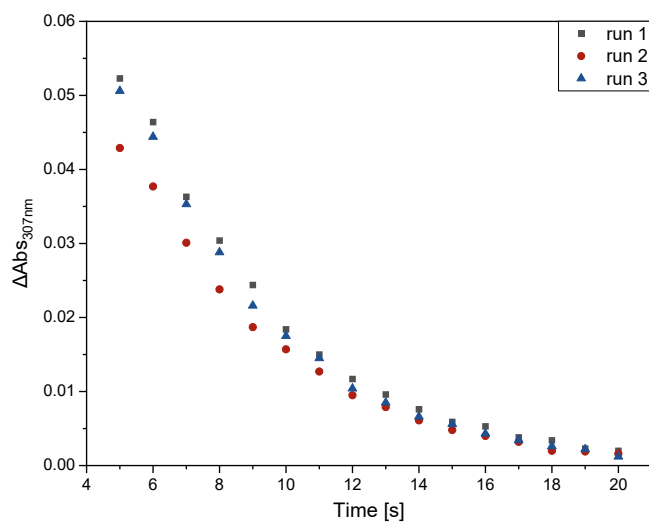


Figure E.75: Absorbance decay of **TTD** (50 μM) in NaPi .

Run	k_1, s^{-1}	$t_{1/2}, \text{s}$	R^2
1	0.223 ± 0.003	3.1	0.998
2	0.228 ± 0.004	3.0	0.996
3	0.239 ± 0.005	2.9	0.994

Based on the data from three runs: $k_1(\text{TTD}) = 0.23 \pm 0.005 \text{ s}^{-1}$ and $t_{1/2} = \ln 2 / k_1 = 3.0 \pm 0.1 \text{ s}$.

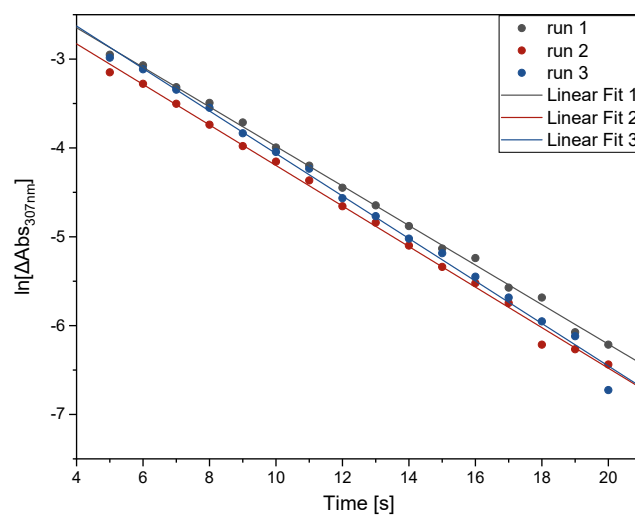


Figure E.76: Absorbance decay of **TTD** (50 μM) in NaPi_i .

Experiments with cells

***In cellulo* alkylation and crosslinking with VTT**

The author, together with Dr. Philipp Hartmann, Dr. Dario Marchionni, and Dr. Christian Preisinger performed experiments with mammalian cells. Dr. Lara Vogelsang performed experiments with bacterial, plant, and yeast cells, as well as protein overexpression in E. coli cells. The author performed in-gel protein digestion for the analysis of the in cellulo crosslinked GrxC5 samples. The author and Dr. Philipp Hartmann performed competition experiments between VTT and NEM in mammalian cells. The author performed competition experiments between VTT and TTD in E. coli cells.

K562 cells

9 mL of K562 cells (1.4×10^6 cells / mL) in 1x Gibco RPMI 1640 Medium supplemented with 10% of heat-inactivated FCS was centrifuged for 5 min at 1,500 xg at 4 °C in a 15 mL Falcon tube, the supernatant was removed, the cells were resuspended in 20 mL of DPBS, centrifuged for 5 min at 1,500 xg at 4 °C, the supernatant was discarded, the cells were washed again by resuspending with 20 mL of DPBS and centrifuging for 5 min at 1,500 xg at 4 °C, and the supernatant was discarded. The cell pellet was resuspended in 3 mL of DPBS and transferred to a 6-well culture plate (1 mL suspension / experiment). To each well, 10 µL of a 25 mM stock solution of **VTT** in DMSO was added within 1 s and the cells were incubated for 30 min at 37 °C in 5% CO₂ in a humidified atmosphere. Next, 10 µL of freshly prepared 25 mM DTT stock solution in H₂O was added and the cells were incubated for another 15 min at 37 °C. Afterwards, 272 µL of RIPA lysis buffer was added to obtain final concentrations of 1% NP-40, 0.5% SDS, 0.5% sodium deoxycholate, and Tris (pH 8.0, 50 mM), followed by addition of 12.3 µL of protease inhibitor stock solution (Protease Inhibitor Mix M from Serva) in DMSO. The mixtures were mixed by pipetting and incubated at 4 °C for 30 min. Then, the mixtures were transferred to 1.5 mL tubes, centrifuged for 15 min at 4 °C at 21,000 xg. The mixtures were frozen and kept at –78 °C until further processing. For LC–MS/MS sample preparation, 0.1 µL of 5 kU Pierce™ nuclease stock solution was added to the thawed samples, the mixtures were incubated at 25 °C for 15 min at 500 rpm, centrifuged for 15 min at 21,000 xg at 4 °C, the supernatants were transferred to separate tubes and protein concentration was determined via BCA assay. The protein digest was performed according to the general SP3 protocol and analyzed using LC–MS/MS method A for the modification search or LC–MS/MS method D for the crosslink search.

HEK cells

Confluent (≈95%) HEK cells in 1x Gibco DMEM + GlutaMAX Medium supplemented with 10% of heat-inactivated FCS in a 6-well (9.6 cm²) culture plate were washed 2 times with 2 mL of DPBS and 1 mL of DPBS was added. Next, 10 µL of 25 mM stock solution of **VTT** in DMSO was added within 1 s and the cells were incubated for 30 min at 37 °C in 5% CO₂ in a humidified atmosphere. Next, 10 µL of freshly prepared 25 mM DTT stock solution in H₂O was added and the cells were incubated for another 15 min at 37 °C. Afterwards, 272 µL of RIPA lysis buffer was added to obtain final concentrations of 1% NP-40, 0.5% SDS, 0.5% sodium deoxycholate, and Tris (pH 8.0, 50 mM), followed by addition of 12.3 µL of protease inhibitor stock solution (Protease Inhibitor Mix M from Serva) in DMSO. The mixtures were mixed by pipetting and incubated at 4 °C for 30 min. Then, the mixtures were transferred to 1.5 mL tubes, centrifuged for 15 min at 4 °C at 21,000 xg. The mixtures were frozen and kept at –78 °C until further processing. For

LC–MS/MS sample preparation, 0.1 µL of 5 kU Pierce™ nuclease stock solution was added to the thawed samples, the mixtures were incubated at 25 °C for 15 min at 500 rpm, centrifuged for 15 min at 21,000 xg at 4 °C, the supernatants were transferred to separate tubes and protein concentration was determined via BCA assay. The protein digest was performed according to the general SP3 protocol and analyzed using LC–MS/MS method A for the modification search or LC–MS/MS method D for the crosslink search.

Arabidopsis thaliana

3 mL of an *Arabidopsis thaliana* cell culture in MS-Medium (4.3 g/L MS salts (Sigma M5524); 1.0 mg/L 2,4-dichlorophenoxyacetic acid; 1:1,000 (v/v) 1000× Gamborg's vitamin stock (Sigma G1019); 30 g/L sucrose; pH 5.7, adjusted with KOH) that was incubated for 7 days after cell passage were transferred into an autoclaved Erlenmeyer flask. Next, 30 µL of a 0.10 M **VTT** solution ($c_{\text{final}} = 1.0$ mM) or 30 µL of a 0.50 M **VTT** solution ($c_{\text{final}} = 5.0$ mM) in DMSO was added within 1 s and the cells were incubated under gentle agitation at 26 °C for 60 min. Afterwards, the cells were transferred into a centrifugation tube and centrifuged for 30 min with 4,600 xg at 4 °C. Next, the supernatant was discarded and the cells were resuspended in 8 mL HEPES (pH 7.0, 50 mM) buffer. The suspension was again centrifuged for 10 min with 4,600 xg at 4 °C. The supernatant was discarded, and the cells were frozen in liquid nitrogen and stored until further use. For complete lysis, the frozen pellet was resuspended in 750 µL HEPES (pH 7.0, 50 mM) buffer and homogenized in a Precellys system with 7,500 rpm, 3 × 50 s with 50 s breaks. Next, the lysates were centrifuged for 20 min with 20,000 xg at 4 °C and the clear supernatant was transferred into a new 1.5 mL reaction tube. The lysates were frozen in liquid nitrogen and stored at –78 °C for later LC–MS/MS sample preparation. For LC–MS/MS sample preparation, the thawed samples were centrifuged for 15 min at 21,000 xg at 4 °C, the supernatants were transferred to separate tubes and protein concentration was determined via BCA assay. The protein digest was performed according to the general SP3 protocol and analyzed using LC–MS/MS method A for the modification search (samples treated with 1.0 mM and 5.0 mM **VTT**) or LC–MS/MS method D for the crosslink search (samples treated with 5.0 mM **VTT**).

Bacillus subtilis

3 mL of a *Bacillus subtilis* cell culture in Miller-LB medium with an OD₆₀₀-value of 1.9 were transferred into an autoclaved Erlenmeyer flask. Next, 30 µL of 0.10 M **VTT** solution in DMSO ($c_{\text{final}} = 1.0$ mM) was added within 1 s and the cells were incubated under gentle agitation at 37 °C for 30 min. Afterwards, the cells were transferred into a centrifugation tube and centrifuged for 30 min with 4,600 xg at 4 °C. Then, the supernatant was discarded and the cells were resuspended in 5 mL HEPES (pH 7.0, 50 mM) buffer. The suspension was again centrifuged for 10 min with 4,600 xg at 4 °C. The supernatant was discarded and the cells were frozen in liquid nitrogen and stored until lysis. For complete lysis, the frozen pellet was resuspended in 750 µL HEPES (pH 7.0, 50 mM) buffer. Next, the mixture was sonicated 2 × 20 s with 70% power at 20–25 °C and subsequently 5 min with 100% power in an ice bath. Afterwards, the tube was centrifuged for 15 min with 21,000 xg at 4 °C and the clear supernatant was transferred into a new 1.5 mL reaction tube. The lysate was frozen in liquid nitrogen and stored at –78 °C for later LC–MS/MS sample preparation. For LC–MS/MS sample preparation, the thawed samples were centrifuged for 15 min at 21,000 xg at 4 °C, the supernatants were transferred to separate tubes, and protein concentration was determined via BCA assay. The protein digest

was performed according to the general SP3 protocol and analyzed using LC–MS/MS method A for the modification search or LC–MS/MS method D for the crosslink search.

Beta vulgaris

3 mL of a *Beta vulgaris* cell culture in MS-Medium (4.3 g/L MS salts (Sigma M5524); 120 g/L sucrose; 1.0 µg/L 1-naphthalene acetic acid; 0.5 µg/L 6-benzyladenine; 2.0 µg/L vitamin C; pH 5.8, adjusted with KOH) that was incubated for 7 days after cell passage were transferred into an autoclaved Erlenmeyer flask. Next, 30 µL of 0.10 M **VTT** solution in DMSO ($c_{\text{final}} = 1.0$ mM) was added within 1 s and the cells were incubated under gentle agitation at 26 °C for 60 min. Afterwards, the cells were transferred into a centrifugation tube and centrifuged for 30 min with 4,600 xg at 4 °C. The cells did not pellet but remained in the supernatant after the centrifugation. The supernatant with the cells was carefully removed (approximately 2–3 mL), and the cells were resuspended in 6 mL of HEPES buffer (pH 7.0, 50 mM). The suspension was centrifuged for 30 min with 4,600 xg at 4 °C, after which a pellet formed. The cells were resuspended in 8 mL HEPES (pH 7.0, 50 mM) buffer. The suspension was again centrifuged for 10 min with 4,600 xg at 4 °C. The supernatant was discarded and the cells were frozen in liquid nitrogen and stored until further use. For complete lysis, the frozen pellet was resuspended in 750 µL HEPES (pH 7.0, 50 mM) buffer and homogenized in a Precellys system with 7,500 rpm, 3 × 50 s with 50 s breaks. Next, the lysates were centrifuged for 20 min with 20,000 xg at 4 °C and the clear supernatant was transferred into a new 1.5 mL reaction tube. The lysates were frozen in liquid nitrogen and stored at –78 °C for later LC–MS/MS sample preparation. For LC–MS/MS sample preparation, the thawed samples were centrifuged for 15 min at 21,000 xg at 4 °C, the supernatants were transferred to separate tubes and protein concentration was determined via BCA assay. The protein digest was performed according to the general SP3 protocol and analyzed using LC–MS/MS method A.

Saccharomyces cerevisiae

3 mL of a *Saccharomyces cerevisiae* cell culture (BY4742, MAT α ; his3 Δ 1; leu2 Δ 0; lys2 Δ 0; ura3 Δ 0) with an OD₆₀₀-value of 2.9 in YPD-medium were transferred into an autoclaved Erlenmeyer flask. Next, 30 µL of a 0.10 M **VTT** solution ($c_{\text{final}} = 1.0$ mM) or 30 µL of a 0.50 M **VTT** solution ($c_{\text{final}} = 5.0$ mM) in DMSO was added within 1 s and the cells were incubated under gentle agitation at 28 °C for 60 min. Afterwards, the cells were transferred into a centrifugation tube and centrifuged for 30 min with 4,600 xg at 4 °C. Afterwards, the supernatant was discarded and the cells were resuspended in 5 mL HEPES (pH 7.0, 50 mM) buffer. The suspension was again centrifuged for 10 min with 4,600 xg at 4 °C. The supernatant was discarded and the cells were frozen in liquid nitrogen and stored until further use. For complete lysis, the frozen pellet was resuspended in 750 µL HEPES (pH 7.0, 50 mM) buffer and homogenized in a Precellys system with 7,500 rpm, 3 × 50 s with 50 s breaks. Next, the lysates were centrifuged for 20 min with 20,000 xg at 4 °C and the clear supernatant was transferred into a new 1.5 mL reaction tube. The lysates were frozen in liquid nitrogen and stored at –78 °C for later LC–MS/MS sample preparation. For LC–MS/MS sample preparation, the thawed samples were centrifuged for 15 min at 21,000 xg at 4 °C, the supernatants were transferred to separate tubes and protein concentration was determined via BCA assay. The protein digest was performed according to the general SP3 protocol and analyzed using LC–MS/MS method A for the modification search (samples treated with 1.0 mM and 5.0 mM **VTT**) or LC–MS/MS method D for the crosslink search

(samples treated with 5.0 mM **VTT**).

Nicotiana tabacum

3 mL of a *Nicotiana tabacum* BY2 cell culture in MS-Medium (4.3 g/L MS salts (Sigma M5524); 30 g/L sucrose; 0.2 µg/L 2,4-dichlorophenoxyacetic acid; 0.56 mM myo-inositol; 3.0 µM thiamine; 1.9 mM KH₂PO₄; pH 5.0, adjusted with KOH) that was incubated for 7 days after cell passage were transferred into an autoclaved Erlenmeyer flask. Next, 30 µL of a 0.10 M **VTT** solution ($c_{\text{final}} = 1.0$ mM) or 30 µL of a 0.50 M **VTT** solution ($c_{\text{final}} = 5.0$ mM) in DMSO was added within 1 s and the cells were incubated under gentle agitation at 26 °C for 60 min. Afterwards, the cells were transferred into a centrifugation tube and centrifuged for 30 min with 4,600 xg at 4 °C. Afterwards, the supernatant was discarded and the cells were resuspended in 8 mL HEPES (pH 7.0, 50 mM) buffer. The suspension was again centrifuged for 10 min with 4,600 xg at 4 °C. The supernatant was discarded and the cells were frozen in liquid nitrogen and stored until lysis. For complete lysis, the frozen pellet was resuspended in 750 µL HEPES (pH 7.0, 50 mM). The cells were lysed in a Precellys system with 7,500 rpm, 3 × 50 s with 50 s breaks. Next, the lysates were centrifuged for 20 min with 20,000 xg at 4 °C and the clear supernatant was transferred into a new 1.5 mL reaction tube. The lysates were frozen in liquid nitrogen and stored at –78 °C for later LC–MS/MS sample preparation. For LC–MS/MS sample preparation, the thawed samples were centrifuged for 15 min at 21,000 xg at 4 °C, the supernatants were transferred to separate tubes and protein concentration was determined via BCA assay. The protein digest was performed according to the general SP3 protocol and analyzed using LC–MS/MS method A.

Escherichia coli

3 mL of an *E. coli* cell culture in Miller-LB medium with an OD₆₀₀-value of 2.5 were transferred into an autoclaved Erlenmeyer flask. Next, 30 µL of 0.10 M **VTT** solution in DMSO ($c_{\text{final}} = 1.0$ mM) was added within 1 s and the cells were incubated under gentle agitation at 37 °C for 30 min. Afterwards, the cells were transferred into a centrifugation tube and centrifuged for 30 min with 4,600 xg at 4 °C. Then, the supernatant was discarded and the cells were resuspended in 5 mL HEPES (pH 7.0, 50 mM) buffer. The cells were again centrifuged for 10 min with 4,600 xg at 4 °C. The supernatant was discarded and the cells were frozen in liquid nitrogen and stored until lysis. For complete lysis, the frozen pellet was resuspended in 320 µL of NEB Express lysis reagent mix. The mixture was incubated for 1 h at 20–25 °C. Next, the lysate was centrifuged for 15 min with 21,000 xg at 20–25 °C and the clear supernatant was transferred into a new 1.5 mL reaction tube. The lysates were frozen in liquid nitrogen and stored at –78 °C for later LC–MS/MS sample preparation. For LC–MS/MS sample preparation, the thawed samples were centrifuged for 15 min at 21,000 xg at 4 °C, the supernatants were transferred to separate tubes and protein concentration was determined via BCA assay. The protein digest was performed according to the general SP3 protocol and analyzed using LC–MS/MS method A.

Competition experiment between VTT and NEM

32 mL of K562 cells (1.1×10^6 cells / mL) in 1x Gibco RPMI 1640 Medium supplemented with 10% of heat-inactivated FCS were centrifuged for 5 min at 1,500 xg at 4 °C in a 50 mL Falcon tube, the supernatant was removed, the cells were resuspended in 25 mL of DPBS, centrifuged for 5 min at 1,500 xg at 4 °C, the supernatant was discarded, the cells were washed again with 25 mL of DPBS and the supernatant was discarded. The cell pellet was resuspended in 30 mL of DPBS and transferred to Petri dishes (5 mL suspension / experiment).

Control experiment with $^2\text{H}_3$ -VTT

50 μL of a 1.5 M NaN_3 solution in DPBS was added to three dishes with K562 cells in DPBS, the dishes were gently swirled and incubated for 15 min at 37 °C in 5% CO_2 in a humidified atmosphere. Then, 50 μL of a 0.1 M **VTT** stock solution in DMSO was added to each mixture, the dishes were gently swirled and incubated for 5 min at 37 °C in 5% CO_2 in a humidified atmosphere. Next, 50 μL of freshly prepared 0.1 M DTT stock solution in H_2O was added and the mixtures and the cells were incubated for another 15 min at 37 °C. Afterwards, 1.3 mL of RIPA lysis buffer was added to obtain final concentrations of 1% NP-40, 0.5% SDS, 0.5% sodium deoxycholate, and Tris (pH 8.0, 50 mM), followed by addition of 63 μL of protease inhibitor stock solution (Protease Inhibitor Mix M from Serva) in DMSO. The mixtures were mixed by pipetting and incubated at 4 °C for 30 min. Then, the mixtures were transferred to 15 mL Falcon tubes, frozen and kept at -78 °C. For the follow-up sample preparation, 0.5 μL of 5 kU Pierce™ nuclease stock solution was added to each of the thawed samples, the mixtures were incubated at 25 °C for 15 min and filtered 5 times with HEPES (pH 7.0, 50 mM) containing 1x cOmplete protease inhibitor with 10 kDa Amicon filters. The obtained mixtures were centrifuged for 15 min at 21,000 xg at 4 °C, the supernatant was transferred to new tubes and the protein concentration was determined via BCA assay.

Competition experiment between VTT and NEM

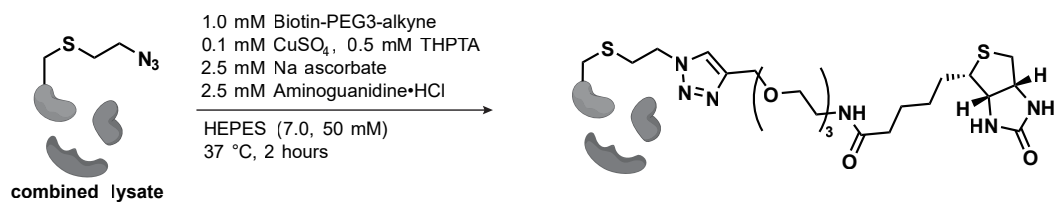
50 μL of a 1.5 M NaN_3 solution in DPBS was added to three dishes with K562 cells in DPBS. The dishes were gently swirled and incubated for 15 min at 37 °C in 5% CO_2 in a humidified atmosphere. Then, 50 μL of a freshly prepared DMSO solution containing 0.1 M **VTT** and 0.1 M **NEM** was added to each mixture, the dishes were gently swirled and incubated for 5 min at 37 °C in 5% CO_2 in a humidified atmosphere. Next, 50 μL of freshly prepared 0.1 M DTT stock solution in H_2O was added and the mixtures and the cells were incubated for another 15 min at 37 °C.

Afterwards, 1.3 mL of RIPA lysis buffer was added to obtain final concentrations of 1% NP-40, 0.5% SDS, 0.5% sodium deoxycholate, and Tris (pH 8.0, 50 mM), followed by addition of 63 μL of protease inhibitor stock solution (Protease Inhibitor Mix M from Serva) in DMSO. The mixtures were mixed by pipetting and incubated at 4 °C for 30 min. Then, the mixtures were transferred to 15 mL Falcon tubes, frozen and kept at -78 °C.

For the follow-up sample preparation, 0.5 μL of 5 kU Pierce™ nuclease stock solution was added to each of the thawed samples, the mixtures were incubated at 25 °C for 15 min and filtered 5 times with HEPES (pH 7.0, 50 mM) containing 1x cOmplete protease inhibitor with 10 kDa Amicon filters. The obtained mixtures were centrifuged for 15 min at 21,000 xg at 4 °C, the supernatant was transferred to new tubes and the protein concentration was determined via

BCA assay.

CuAAC reaction for biotin enrichment



Each of the three filtered lysates from the control experiments with ²H₃-VTT (0.25 mg protein each) was combined with a filtered lysate from the competition experiment (0.25 mg protein each), respectively. HEPES (pH 7.0, 50 mM) was added to obtain the mixture volume of 470 µL in each case. Then, 13 µL of a 40 mM biotin-PEG3-alkyne stock solution in DMSO was added ($c_{\text{final}} = 1.0$ mM) and each mixture was vortexed for 1 s. Next, 10 µL of Cu-THPTA mixture (5 mM CuSO₄ and 25 mM THPTA in H₂O) premixed at 37 °C for 10 min was added ($c_{\text{final}}(\text{CuSO}_4) = 0.1$ mM; $c_{\text{final}}(\text{THPTA}) = 0.5$ mM). Then, 5.0 µL of an aminoguanidinium hydrochloride stock solution (0.25 M in H₂O) was added ($c_{\text{final}} = 2.5$ mM), followed by addition of 5.0 µL of a sodium ascorbate stock solution (0.25 M in H₂O; $c_{\text{final}} = 2.5$ mM). The mixtures were vortexed for 2 s, transferred into a Thermocycler preheated at 37 °C, and incubated at 37 °C for 2 hours at 1000 rpm. The samples were used for protein digest and biotin enrichment.

Competition experiment between VTT and TTD

Control experiments with $^2\text{H}_3$ -VTT

Experiment with incubation for 5 minutes

For each of three biological replicates, 2 mL of an *E. coli* cell culture in Miller-LB medium with an OD_{600} of 2.4 was transferred into a 2 mL reaction tube. Next, 20 μL of a freshly prepared DMSO solution containing 0.1 M $^2\text{H}_3$ -VTT was added to each mixture. The mixtures were incubated at 37 °C at 500 rpm for 5 minutes. Then, a freshly prepared stock solution of DTT in H_2O was added to each mixture, and the mixtures were incubated for another 15 minutes at 37 °C at 500 rpm. Next, the mixtures were centrifuged for 30 min with 4,600 xg at 4 °C. Then, the supernatants were discarded and the resulting pellets were resuspended in 1 mL HEPES buffer (pH 7.0, 50 mM). The cells were again centrifuged for 15 min with 4,600 xg at 4 °C. The supernatant was discarded and the cells were washed two more times with 1 mL HEPES buffer. After discarding the supernatant, the pellets were resuspended in 250 μL of NEB Express lysis reagent mix and incubated for 1 hour at 25 °C at 500 rpm. The lysates were centrifuged at 25 °C for 20 min at 19,000 xg, and the clear supernatants were transferred into fresh 1.5 mL reaction tubes. The lysates were frozen in liquid nitrogen and stored at –78 °C for later LC–MS/MS sample preparation.

Experiment with incubation for 1 hour

For each of three biological replicates, 2 mL of an *E. coli* cell culture in Miller-LB medium with an OD_{600} of 2.4 was transferred into a 2 mL reaction tube. Next, 20 μL of a freshly prepared DMSO solution containing 0.1 M $^2\text{H}_3$ -VTT was added to each mixture. The mixtures were incubated at 37 °C at 500 rpm for 1 hour. Next, the mixtures were centrifuged for 30 min with 4,600 xg at 4 °C. Then, the supernatants were discarded and the resulting pellets were resuspended in 1 mL HEPES buffer (pH 7.0, 50 mM). The cells were again centrifuged for 15 min with 4,600 xg at 4 °C. The supernatant was discarded and the cells were washed two more times with 1 mL HEPES buffer. After discarding the supernatant, the pellets were resuspended in 250 μL of NEB Express lysis reagent mix and incubated for 1 hour at 25 °C at 500 rpm. The lysates were centrifuged at 25 °C for 20 min at 19,000 xg, and the clear supernatants were transferred into fresh 1.5 mL reaction tubes. The lysates were frozen in liquid nitrogen and stored at –78 °C for later LC–MS/MS sample preparation.

Competition experiments between VTT and TTD

Experiment with incubation for 5 minutes

For each of three biological replicates, 2 mL of an *E. coli* cell culture in Miller-LB medium with an OD_{600} of 2.4 was transferred into a 2 mL reaction tube. Next, 20 μL of a freshly prepared DMSO solution containing 0.1 M VTT and 0.1 M TTD was added to each mixture. The mixtures were incubated at 37 °C at 500 rpm for 5 minutes. Then, a freshly prepared stock solution of DTT in H_2O was added to each mixture, and the mixtures were incubated for another 15 minutes at 37 °C at 500 rpm. Next, the mixtures were centrifuged for 30 min with 4,600 xg at 4 °C. Then, the supernatants were discarded and the resulting pellets were resuspended in 1 mL HEPES buffer (pH 7.0, 50 mM). The cells were again centrifuged for 15 min with 4,600 xg at 4 °C. The supernatant was discarded and the cells were washed two more times with 1 mL HEPES buffer. After discarding the supernatant, the pellets were resuspended in 250 μL of NEB Express lysis reagent mix and incubated for 1 hour at 25 °C at 500 rpm. The lysates were centrifuged at 25 °C

for 20 min at 19,000 xg, and the clear supernatants were transferred into fresh 1.5 mL reaction tubes. The lysates were frozen in liquid nitrogen and stored at -78 °C for later LC-MS/MS sample preparation.

Experiment with incubation for 1 hour

For each of three biological replicates, 2 mL of an *E. coli* cell culture in Miller-LB medium with an OD₆₀₀ of 2.4 was transferred into a 2 mL reaction tube. Next, 20 µL of a freshly prepared DMSO solution containing 0.1 M **VTT** and 0.1 M **TTD** was added to each mixture. The mixtures were incubated at 37 °C at 500 rpm for 1 hour. Next, the mixtures were centrifuged for 30 min with 4,600 xg at 4 °C. Then, the supernatants were discarded and the resulting pellets were resuspended in 1 mL HEPES buffer (pH 7.0, 50 mM). The cells were again centrifuged for 15 min with 4,600 xg at 4 °C. The supernatant was discarded and the cells were washed two more times with 1 mL HEPES buffer. After discarding the supernatant, the pellets were resuspended in 250 µL of NEB Express lysis reagent mix and incubated for 1 hour at 25 °C at 500 rpm. The lysates were centrifuged at 25 °C for 20 min at 19,000 xg, and the clear supernatants were transferred into fresh 1.5 mL reaction tubes. The lysates were frozen in liquid nitrogen and stored at -78 °C for later LC-MS/MS sample preparation.

Sample mixing for LC-MS/MS analysis

The protein concentration of thawed samples was determined via BCA assay, each of the three lysates from the control experiments was combined with a lysate from the competition experiment as follows:

- ²H₃-**VTT** control experiment samples and samples from **VTT/TTD** competition experiment performed for 5 minutes;
- ²H₃-**VTT** control experiment samples and samples from **VTT/TTD** competition experiment performed for 1 hour;

The samples were digested according to the general SP3 protocol and analyzed using LC-MS/MS method E.

***In cellulo* crosslinking of GrxC5**

Plasmid generation and amplification

The coding sequence of *A. thaliana* GRXC5 (AT4G28730), excluding the chloroplast transit peptide, was amplified from leaf cDNA using gene-specific primers. The resulting PCR product was cloned into the pEXP-NT-TOPO vector (Invitrogen) via thymidine-adenosine (TA) cloning and verified by sequencing²⁰⁷.

Forward: 5'-ACTTCTTCTTCTTCGGCTGCATC-3'

Reverse: 5'-AACTCTGACCGTTTTTACCGTTG-3'

The plasmid was transformed into One Shot™ TOP10 Chemically Competent *E. coli* (Invitrogen) by a heat shock protocol and grown overnight on agar plates supplemented with kanamycin (50 µg/ml). A single colony was transferred to 20 mL of Miller-LB medium (50 µg/ml kanamycin) and incubated at 37 °C overnight. Plasmid purification was performed by NucleoSpin Plasmid kit (Macherey-Nagel) following the manufacturer's instructions.

***In cellulo* crosslinking with VTT and protein purification**

The experiment was performed in three biological replicates. *E. coli* NiCO₂1 (DE3) cells were transformed with the plasmid DNA (GrxC5 in pEXP5-NT/TOPO). A 50 mL overnight culture in Miller-LB medium supplemented with 100 µg/mL ampicillin was incubated at 37 °C with shaking (120 rpm). On the next day, 100 mL of fresh LB medium containing 100 µg/mL ampicillin was inoculated with 5 mL of the overnight culture and incubated at 37 °C until the culture reached an OD₆₀₀ of 0.6. Protein expression was induced by the addition of 40 µL of 1.0 M IPTG stock solution in H₂O, followed by an incubation at 19 °C overnight under continuous shaking. On the following day, the OD₆₀₀ was determined (2.54), and 1 mL of a 0.10 M **VTT** stock solution in DMSO was added to the culture, followed by an incubation for 15 min at 37 °C with gentle shaking. Cells were harvested by centrifugation at 4,600 xg for 30 min at 4 °C. The supernatant was discarded, and the pellet was washed once with HEPES buffer (pH 7.0, 50 mM), followed by a second centrifugation step (10 min; 4,600 xg, 4 °C). The supernatant was discarded and the pellet was frozen in liquid nitrogen. For lysis, the pellet was resuspended in 9.3 mL of NEB Express Cell Lysis Reagent (NEB) and incubated for 30 min at room temperature. The suspension was then supplemented with 150 µL lysozyme solution (10 mg/mL) and further incubated for 30 min at 20–25 °C under gentle shaking. Subsequently, the mixture was subjected to sonication (1 × 3 min, 70% amplitude) and centrifuged at 20,830 xg for 90 min at 4 °C. The clear supernatant was incubated with 1.5 mL Ni-NTA agarose beads (ROTI®Garose-His/Ni-NTA, Carl Roth), which were pre-equilibrated with lysis buffer (Tris-HCl (pH 8.0, 50 mM), 0.30 M NaCl, 5 mM imidazole). The mixture was incubated for 1 hour at 4 °C under gentle rotation. Afterwards, the lysate-bead mixture was loaded onto a gravity-flow column. The beads were washed with four column volumes of wash buffer I (Tris-HCl (pH 8.0, 50 mM), 0.30 M NaCl and 10 mM imidazole), followed by one column volume of wash buffer II (Tris-HCl (pH 8.0, 50 mM), 0.30 M NaCl and 20 mM imidazole) and two additional column volumes of wash buffer I. Bound proteins were eluted by incubating the beads twice with 1.5 mL of elution buffer (Tris-HCl (pH 8.0, 50 mM), 0.30 M NaCl, 0.25 M imidazole) for 10 min each prior to elution. The eluates were combined (3 mL total) and dialyzed against 2 L of HEPES buffer (pH 8.0, 50 mM) at 4 °C. After 3 hours, the buffer was replaced with 2 L of HEPES buffer (pH 8.0, 50 mM), and dialysis was continued overnight. On the following day, the dialysis was stopped, and the purified protein was aliquoted into reaction tubes and stored at –78 °C for subsequent LC–MS/MS sample preparation.

In-gel protein digestion

The protein concentration of each sample was determined via BCA assay, 15 µg protein was applied for SDS-PAGE separation. The gel was stained with Coomassie for 13 hours and washed with water for 3 hours. The bands corresponding to the overexpressed protein were cut into small ($\approx 1\text{ mm}^3$) cubes and transferred to 1.5 mL tubes. The gel pieces were washed twice with 0.5 mL of H₂O, destained 2 times by addition of 100 µL 50% MeCN in 50 mM NH₄HCO₃, incubation for 15 min at 25 °C. The washing solutions were discarded. Then, 0.5 mL of MeCN was added and the samples were incubated for 10 min at 25 °C. The solvent was discarded, 100 µL of a freshly prepared 10 mM TCEP solution in 100 mM NH₄HCO₃ was added to each sample, the samples were transferred to a thermocycler preheated to 56 °C, and the samples were incubated for 30 min at 1000 rpm. Then, the solution was discarded and the gel pieces were dehydrated with 0.5 mL MeCN for 10 min at 25 °C. After removal of the liquid, 100 µL of a freshly prepared 55 mM chloroacetamide solution in 100 mM NH₄HCO₃ was added to each

sample, and the samples were incubated at 25 °C for 60 min at 1000 rpm. Then, the solution was discarded and the gel pieces were dehydrated with 0.5 mL MeCN for 10 min at 25 °C. After removal of the liquid, 100 µL of a freshly prepared trypsin (Promega; #V5111) solution (2.5 ng/µL) in 100 mM NH_4HCO_3 was added to each sample and the samples were incubated for 90 min at 4 °C. Then, the samples were transferred to an incubator preheated at 37 °C and incubated for 19 hours. 0.2 mL of a 1:2 (v/v) 5% formic acid in H_2O : MeCN solution was added to each sample and the samples were incubated for 15 min at 37 °C at 500 rpm. The solutions were transferred to new tubes, the samples were evaporated using SpeedVac and analyzed using LC–MS/MS method C.

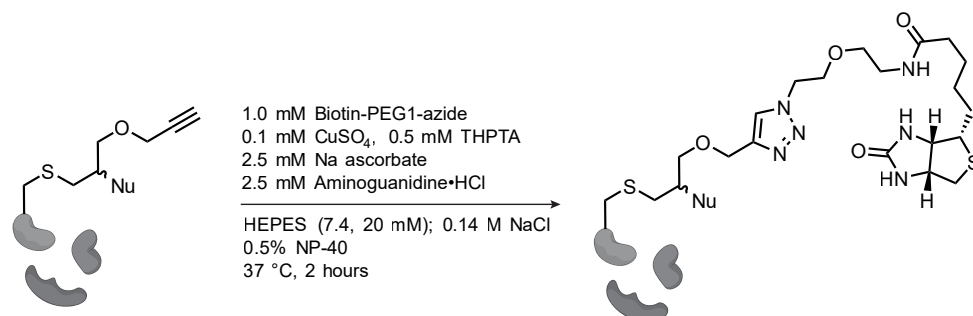
***In cellulo* alkylation and crosslinking with TTD**

Experiments with HEK cells

The experiment was performed with three biological replicates. In each case, confluent ($\approx 95\%$) HEK cells in 1x Gibco DMEM + GlutaMAX Medium supplemented with 10% of heat-inactivated FCS in a 10 cm culture plate were washed 2 times with 5 mL of DPBS and 10 mL of DPBS was added. Next, 100 μL of a freshly prepared 0.1 M stock solution of **TTD** in DMSO was added within 5 s ($c_{\text{final}} = 1.0 \text{ mM}$) and the cells were incubated for 1 hour at 37 °C in 5% CO_2 in a humidified atmosphere. Next, each cell-containing mixture was transferred to a 15 mL Falcon tube and centrifuged for 5 minutes at 500 xg at 4 °C. The supernatants were discarded, 2 mL of DPBS was added to each tube, and the cell pellets were resuspended by gentle shaking of the tube. Then, each mixture was centrifuged for 5 minutes at 500 xg at 4 °C. The cells were washed two more times with 2 mL DPBS, the last centrifugation step was performed for 5 minutes at 1,500 xg at 4 °C, the supernatants were discarded, and the cell pellets were frozen and kept at $-78 \text{ }^\circ\text{C}$ until further processing.

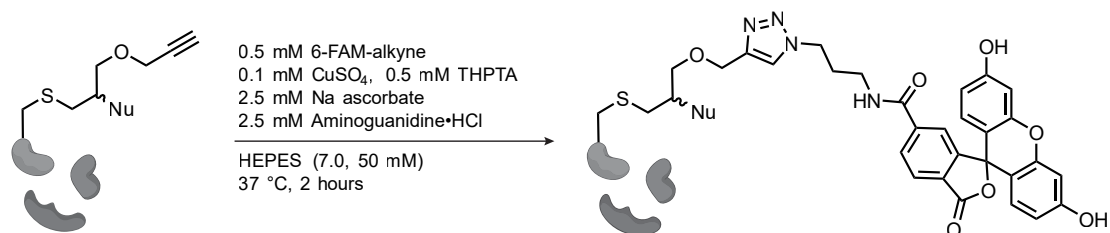
To lyse the cells, the cell pellets were thawed, and each cell pellet was resuspended in 1.0 mL of the lysis buffer (HEPES (pH 7.4, 20 mM); 0.14 M NaCl; 0.5% (v/v) NP-40; 1 $\times$ cComplete™, Mini, EDTA-free Protease Inhibitor Cocktail) by pipetting. The mixtures were incubated for 30 minutes at 4 °C after addition of the lysis buffer. The mixtures were vortexed for 10 seconds immediately, 10 minutes, and 20 minutes after addition of the lysis buffer. The tubes were centrifuged for 15 minutes at 21,000 xg at 4 °C, the supernatants were collected, and the protein concentration was determined via BCA assay.

CuAAC reaction for biotin enrichment



Lysis buffer (HEPES (pH 7.4, 20 mM); 0.14 M NaCl; 0.5% (v/v) NP-40; 1 $\times$ cComplete™, Mini, EDTA-free Protease Inhibitor Cocktail) was added to 1.5 mL tubes containing lysates to obtain mixtures with 0.5 mg protein in 234 μL lysis buffer. Then, 13 μL of a 40 mM biotin-PEG1-azide stock solution in DMSO was added ($c_{\text{final}} = 1.0 \text{ mM}$) and each mixture was vortexed for 1 s. Next, 10 μL of Cu-THPTA mixture (5 mM CuSO_4 and 25 mM THPTA in H_2O) premixed at 37 °C for 10 minutes was added ($c_{\text{final}}(\text{CuSO}_4) = 0.1 \text{ mM}$; $c_{\text{final}}(\text{THPTA}) = 0.5 \text{ mM}$). Then, 5.0 μL of an aminoguanidinium hydrochloride stock solution (0.25 M in H_2O) was added ($c_{\text{final}} = 2.5 \text{ mM}$), followed by addition of 5.0 μL of a sodium ascorbate stock solution (0.25 M in H_2O ; $c_{\text{final}} = 2.5 \text{ mM}$). The mixtures were vortexed for 2 s, transferred into a Thermocycler preheated at 37 °C, and incubated at 37 °C for 2 hours at 1000 rpm. The obtained samples were used for biotin enrichment.

CuAAC reaction for SDS-PAGE analysis



In each case, the reactions were performed as follows: At 20–25 °C, HEPES (pH 7.0; 50 mM) buffer was added to the lysate to obtain final volume of 94 μ L. Then, 2.1 μ L of a 24 mM 6-FAM azide stock solution in DMSO was added ($c_{\text{final}} = 0.5$ mM). Next, 2.0 μ L of a Cu-THPTA mixture (5 mM CuSO₄ and 25 mM THPTA in H₂O) premixed at 37 °C for 10 minutes was added ($c_{\text{final}}(\text{CuSO}_4) = 0.1$ mM; $c_{\text{final}}(\text{THPTA}) = 0.5$ mM). Next, 1.0 μ L of a sodium ascorbate stock solution (0.25 M in H₂O) was added ($c_{\text{final}} = 2.5$ mM), followed by addition of 1.0 μ L of an aminoguanidinium hydrochloride stock solution (0.25 M in H₂O, $c_{\text{final}} = 2.5$ mM). The mixture was vortexed for 2 s, transferred into a Thermocycler preheated at 37 °C, and incubated at 37 °C for 2 hours at 600 rpm. Next, the samples were rebuffed five times in HEPES (pH 7.0, 50 mM) utilizing 3 kDa Amicon filter units. After rebuffing, the sample volumes were adjusted to 45 μ L by addition of the HEPES buffer and the reaction mixtures were analyzed via SDS-PAGE analysis loading 10 μ g protein per lane.

Labeling of lysates

The experiments with lysates were performed by the author.

Generation of K562 lysates

K562 cells (1×10^6 cells / mL) were washed 2 times with DPBS buffer, the supernatant was discarded, the cells were split in tubes (50 μ L each) and the pellets were frozen and stored at -78 °C. 150 μ L of HEPES (pH 7.0, 50 mM) buffer containing 1 $\times$ cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail was added to thawed K562 cell pellets and resuspended via pipetting. The tubes were frozen in liquid nitrogen, and after 1 min transferred to a Thermocycler pre-cooled to 4 °C. The mixtures were incubated for 13 min at 300 rpm, vortexed for 3 s, and frozen in liquid nitrogen. The freeze-thaw cycle was repeated 2 more times, the tubes were centrifuged for 15 min at 21,000 xg at 4 °C. Then, the supernatant was collected, transferred to new tubes, and protein concentration was determined via BCA assay.

Labeling of K562 lysates with VTT

The experiment was performed with three biological replicates. In each case, 4.1 μ L of the K562 lysate (12.3 mg/mL) was added to a tube containing 45 μ L of PBS buffer to obtain final protein concentration of 1.0 mg/mL and the mixture was vortexed for 1 s. Then, 0.5 μ L of a 0.25 mM **VTT** stock solution in DMSO was added to the mixture, vortexed for 1 s, and the tube was transferred to a Thermocycler preheated at 37 °C. The mixture was incubated for 30 min at 600 rpm and 0.5 μ L of a 0.25 mM DTT stock solution in H₂O was added to the mixture. The mixture was incubated for 15 min at 37 °C at 600 rpm, centrifuged for 10 min at 21,000 xg at 4 °C, the supernatant was collected and transferred to a new tube for the LC–MS/MS sample preparation. The protein digest was performed according to the general SP3 protocol and analyzed using LC–MS/MS method A.

Sample preparation for LC–MS/MS analysis

The LC–MS/MS sample preparation using the general SP3 protocol was performed by the author and Dr. Philipp Hartmann. Preparation of the non-enriched HEK samples after treatment with TTD was performed by the author. The author performed the digestion and enrichment of the samples obtained from the competition experiment between VTT and NEM. The author and Sven Müller performed the digestion and enrichment of the samples obtained after treatment of HEK cells with TTD.

SP3 protocol

General SP3 protocol

The LC–MS/MS sample preparation was performed via slightly modified SP3 workflow²⁰⁸. HEPES (pH 8.0, 0.5 M) was added to the lysates to obtain a HEPES concentration of 100 mM. Then, a 10% (w/w) stock solution of SDS in H₂O and a 1.0 M stock solution of TCEP in H₂O were added to the sample to obtain final concentrations of 1% (v/v) SDS and 10 mM TCEP. The mixtures were incubated for 30 min at 60 °C at 1000 rpm, allowed to cool down to 20–25 °C, and alkylated with chloroacetamide ($c_{\text{final}} = 40$ mM) for 1 hour at 37 °C at 1000 rpm. For processing 20 µg of protein starting material, 4.0 µL of the magnetic beads stock (combined 1:1 Sera-Mag SpeedBeads (GE Healthcare, cat. no. 45152105050250) and Sera-Mag SpeedBeads (GE Healthcare, cat. no. 65152105050250)) were added to the reduced and alkylated lysates, the beads were resuspended by vortexing the mixtures for 1 s, followed by addition of abs. EtOH to obtain 50% (v/v) EtOH mixture. The mixtures were incubated for 5 min at 24 °C at 1000 rpm, transferred to a magnetic rack, and the beads were washed three times with 400 µL of 80% (v/v) EtOH in H₂O according to the protocol²⁰⁸. The on-bead digestion was performed in 100 µL of a freshly prepared 100 mM NH₄HCO₃ pH 8.0 buffer containing 0.4 µg of Trypsin/LysC mix (Promega, #V5072) at 37 °C at 1000 rpm for 18 hours. The samples were centrifuged for 3 min at 17,000 xg, the supernatants were transferred to new tubes, evaporated using SpeedVac, and submitted for LC–MS/MS measurements.

SP3 protocol for non-enriched HEK samples after treatment with TTD

The LC–MS/MS sample preparation was performed via slightly modified SP3 workflow²⁰⁸. HEPES (pH 8.0, 1.0 M) was added to the lysates to obtain a HEPES concentration of 100 mM. Then, a 20% (w/w) stock solution of SDS in H₂O and a 0.5 M stock solution of TCEP in H₂O were added to the samples to obtain final concentrations of 1% (v/v) SDS and 10 mM TCEP. The mixtures were incubated for 30 min at 37 °C at 1000 rpm, allowed to cool down to 20–25 °C, and alkylated with chloroacetamide ($c_{\text{final}} = 40$ mM) for 1 hour at 37 °C at 1000 rpm. For processing 200 µg of protein starting material, 40 µL of the magnetic beads stock (combined 1:1 Sera-Mag SpeedBeads (GE Healthcare, cat. no. 45152105050250) and Sera-Mag SpeedBeads (GE Healthcare, cat. no. 65152105050250)) were added to the reduced and alkylated lysates, the beads were resuspended by vortexing the mixtures for 1 s, followed by addition of abs. EtOH to obtain 50% (v/v) EtOH mixture. The mixtures were incubated for 10 min at 24 °C at 1000 rpm, transferred to a magnetic rack, and the beads were washed three times with 1 mL of 80% (v/v) EtOH in H₂O according to the protocol²⁰⁸. The on-bead digestion was performed in 200 µL of a freshly prepared 100 mM NH₄HCO₃ pH 8.0 buffer containing 4 µg of Trypsin/LysC mix (Promega, #V5072) at 37 °C at 1000 rpm for 18 hours. The samples were centrifuged for 3 min at 17,000 xg, the supernatants were transferred to new tubes, evaporated using SpeedVac,

and submitted for LC–MS/MS measurements that were performed with LC–MS/MS method D.

SP3-digest for biotin enrichment

Competition experiment between VTT and NEM

1. **Reduction and alkylation:** 65 μ L of HEPES (pH 8.0, 1.0 M) was added to the lysates after CuAAC reaction (each mixture contained 0.5 mg protein in 500 μ L). Then, 33 μ L of a 20% (w/w) stock solution of SDS in H₂O and a 13 μ L of a 0.5 M stock solution of TCEP in H₂O were added to the samples. The mixtures were incubated for 30 min at 37 °C at 1000 rpm, 40 μ L of a 0.66 M chloroacetamide stock solution in H₂O were added, and the mixtures were incubated for 1 hour at 37 °C at 1000 rpm.
2. **SP3-digestion:** 100 μ L of the magnetic beads stock (combined 1:1 Sera-Mag Speed-Beads (GE Healthcare, cat. no. 45152105050250) and Sera-Mag SpeedBeads (GE Healthcare, cat. no. 65152105050250)) were added to the reduced and alkylated lysates, the beads were resuspended by vortexing the mixtures for 1 s, followed by addition of 750 μ L of abs. EtOH. The mixtures were incubated for 10 min at 24 °C at 1000 rpm, transferred to a magnetic rack, and the beads were washed three times with 1000 μ L of 80% (v/v) EtOH in H₂O according to the SP3-protocol²⁰⁸. The on-bead digestion was performed in 400 μ L of HEPES buffer (pH 8.0, 50 mM) containing 10 μ g of Trypsin/LysC mix (Promega, #V5072) at 37 °C at 1000 rpm for 18 hours.
3. **Heat treatment:** The samples were centrifuged for 10 min at 21,000 xg, the supernatants were transferred to new tubes and incubated for 10 min at 95 °C at 1000 rpm to ensure the inactivation of trypsin prior to addition to NeutrAvidin beads.
4. **Biotin-NeutrAvidin binding:** 125 μ L of NeutrAvidin beads slurry (ThermoFischer, #29200) were washed 3 times with 1 mL of PBS. Each washing step included addition of the buffer to the beads, vortexing the mixtures for 1 s, centrifugation at 1,700 xg for 2 min at 20–25 °C, and discarding the supernatant. Then, the heat-treated peptide mixtures were added to the tubes with washed NeutrAvidin beads at 20–25 °C, the mixtures were vortexed for 2 s and transferred to a Thermocycler pre-cooled to 25 °C. The mixtures were incubated for 2 hours at 25 °C at 1000 rpm.
5. **Washing step:** The mixtures were centrifuged at 1,700 xg for 2 min at 20–25 °C, the supernatant was discarded. The beads were washed 5 times with 1 mL of PBS and 4 times with 1 mL of H₂O. Each washing step included addition of the buffer/water to the beads, vortexing the mixtures for 3 s, centrifugation at 1,700 xg for 2 min at 20–25 °C, and discarding the supernatant.
6. **Peptide release:** The washed beads were resuspended in 150 μ L of the elution buffer (80% MeCN, 0.1% formic acid (v/v) in H₂O), vortexed for 3 s, and incubated for 10 min at 25 °C at 1000 rpm¹⁵¹. The mixtures were centrifuged at 1,700 xg for 2 min at 20–25 °C, the supernatant was transferred to new tubes. Then, the beads were resuspended in 150 μ L of the elution buffer and incubated for 10 min at 72 °C at 1000 rpm. The mixtures were centrifuged at 1,700 xg for 2 min at 20–25 °C, the supernatant was combined with the mixtures from the first elution step.
7. **Washing step:** The beads were washed 2 times with 100 μ L of the release buffer. Each

washing step included addition of the release buffer (80% MeCN, 0.1% formic acid (v/v) in H₂O) to the beads, vortexing the mixtures for 3 s, centrifugation at 1,700 xg for 2 min at 20–25 °C, and combining the supernatant with the mixtures from step 6. The combined mixtures in the elution buffer were evaporated using SpeedVac.

8. **Separation of the remaining beads:** The pellets obtained in step 7 were washed 3 times with H₂O. First, the pellets were resuspended in 47 µL of H₂O, vortexed for 15 s, and centrifuged for 5 min at 21,000 xg at 20–25 °C. Then, 40 µL of the supernatant was carefully transferred to a new tube. The washing step was repeated two more times with addition and transferring 30 µL of H₂O each time. The combined mixtures without remaining beads were evaporated using SpeedVac and analyzed using LC–MS/MS method B.

Treatment of HEK cells with TTD

1. **Reduction and alkylation:** 65 µL of HEPES (pH 8.0, 1.0 M) was added to a lysate after CuAAC reaction (each mixture contained 0.5 mg protein in 500 µL). Then, 33 µL of a 20% (w/w) stock solution of SDS in H₂O and a 9.3 µL of a 0.70 M stock solution of TCEP in H₂O were added to the sample. The mixture was incubated for 30 minutes at 37 °C at 1000 rpm, 43 µL of a 0.60 M chloroacetamide stock solution in H₂O was added, and the mixture was incubated for 1 hour at 37 °C at 1000 rpm.
2. **SP3-digestion:** 100 µL of the magnetic beads stock (combined 1:1 Sera-Mag SpeedBeads (GE Healthcare, cat. no. 45152105050250) and Sera-Mag SpeedBeads (GE Healthcare, cat. no. 65152105050250)) was added to the reduced and alkylated lysate, the beads were resuspended by vortexing the mixture for 1 s, followed by addition of 750 µL of abs. EtOH. The mixture was incubated for 10 minutes at 24 °C at 1000 rpm, transferred to a magnetic rack, and the beads were washed three times with 1000 µL of 80% (v/v) EtOH in H₂O according to the SP3-protocol²⁰⁸. The on-bead digestion was performed in 400 µL of HEPES buffer (pH 8.0, 50 mM) containing 10 µg of Trypsin (Promega, #V5111) at 37 °C at 1000 rpm for 18 hours.
3. **Heat treatment:** The sample was centrifuged for 10 minutes at 21,000 xg, the supernatant was transferred to a new tube and incubated for 10 minutes at 95 °C at 1000 rpm to ensure the inactivation of trypsin prior to addition to NeutrAvidin beads.
4. **Biotin-NeutrAvidin binding:** 125 µL of NeutrAvidin beads slurry (ThermoFischer, #29200) was washed 3 times with 1 mL of PBS. Each washing step included adding the buffer to the beads, vortexing the mixtures for 1 s, centrifuging at 1,700 xg for 2 minutes at 20–25 °C, and discarding the supernatant. Then, the heat-treated peptide mixture was added to the tube with washed NeutrAvidin beads at 20–25 °C, the mixture was vortexed for 2 s, and transferred to a Thermocycler pre-cooled to 25 °C. The mixture was incubated for 2 hours at 25 °C at 1000 rpm.
5. **Washing step:** The mixture was centrifuged at 1,700 xg for 2 minutes at 20–25 °C, the supernatant was discarded. The beads were washed five times with 1 mL of PBS and four times with 1 mL of H₂O. Each washing step included adding PBS or water to the beads, vortexing the mixture for 3 s, centrifuging at 1,700 xg for 2 minutes at 20–25 °C, and discarding the supernatant.

6. **Peptide release:** The washed beads were resuspended in 150 μL of the elution buffer (80% MeCN, 0.1% formic acid (v/v) in H_2O), vortexed for 3 s, and incubated for 10 minutes at 25 $^{\circ}\text{C}$ at 1000 rpm¹⁵¹. The mixture was centrifuged at 1,700 xg for 2 minutes at 20–25 $^{\circ}\text{C}$, the supernatant was transferred to a new tube. Then, the beads were resuspended in 150 μL of the elution buffer and incubated for 10 minutes at 72 $^{\circ}\text{C}$ at 1000 rpm. The mixture was centrifuged at 1,700 xg for 2 minutes at 20–25 $^{\circ}\text{C}$, the supernatant was combined with the mixture from the first elution step.
7. **Washing step:** The beads were washed 2 times with 100 μL of the release buffer. Each washing step included adding the release buffer (80% MeCN, 0.1% formic acid (v/v) in H_2O) to the beads, vortexing the mixtures for 3 s, centrifuging at 1,700 xg for 2 minutes at 20–25 $^{\circ}\text{C}$, and combining the supernatant with the mixture from step 6. The combined mixture in the elution buffer was evaporated using SpeedVac.
8. **Separation of the remaining beads:** The pellet obtained in step 7 was washed 3 times with H_2O . First, the pellet was resuspended in 35 μL of H_2O , vortexed for 15 s, and centrifuged for 5 min at 21,000 xg at 20–25 $^{\circ}\text{C}$. Then, 30 μL of the supernatant was carefully transferred to a new tube. The washing step was repeated two more times with the addition and transferring 30 μL of H_2O each time. The combined mixtures without remaining beads were evaporated using SpeedVac and analyzed using LC–MS/MS method D.

Cytotoxicity studies

The author and Dr. Philipp Hartmann performed the membrane integrity assays with **VTT**. The author performed the membrane integrity assays with **TTD**. The author performed the viability assays. The author and Dr. Philipp Hartmann analyzed the data.

Membrane integrity assays

CellTox™ assay with VTT and K562 cells

5 mL of K562 cells (1.2×10^6 cells / mL) in 1x Gibco RPMI 1640 Medium supplemented with 10% of heat-inactivated FCS was centrifuged for 5 min at 1,500 xg at 4 °C in a 15 mL Falcon tube, the supernatant was removed, the cells were resuspended in 5 mL of DPBS, centrifuged for 5 min at 1,500 xg at 4 °C, the supernatant was discarded, the cells were washed again with 5 mL of DPBS, and the supernatant was discarded. The cell pellet was resuspended in 30 mL of DPBS and transferred to a black / clear bottom 96-well plate (50 μ L suspension / well). To each well, 50 μ L of the CellTox™ reagent mixture (freshly prepared by diluting the CellTox™ (Promega, #G8741) stock solution 1:1000 (v/v) with DPBS) was added. Then, 1.0 μ L of a DMSO stock solution containing various concentrations of **VTT** was added to the wells. In case of the control experiment with the lysis buffer, 4.0 μ L of the Lysis Solution from the CellTox™ kit was added to the wells. The last control experiment involved no addition of DMSO. Each experiment was performed in four replicates. The plate was incubated for 60 min at 37 °C in 5% CO₂ in a humidified atmosphere and the fluorescence measurement was performed at $\lambda_{\text{ex}} = 487$ nm and $\lambda_{\text{em}} = 535$ nm. Then, the plate was incubated for another 60 min at 37 °C in 5% CO₂ in a humidified atmosphere and another measurement was taken.

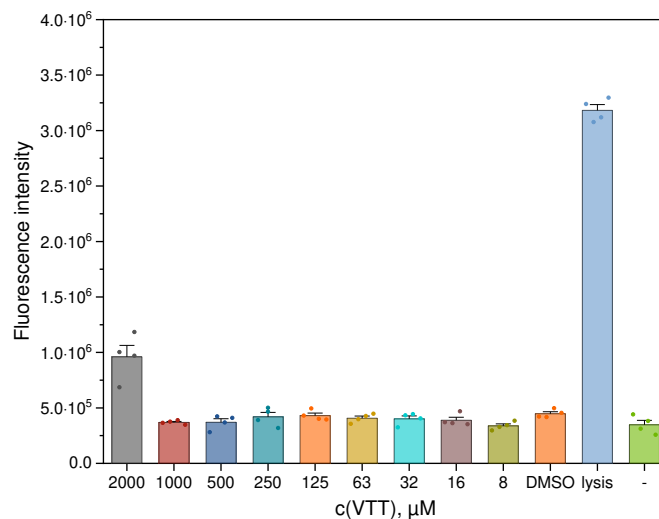


Figure E.77: Fluorescence intensities obtained after incubation of K562 cells supplemented with CellTox™ reagent with solutions containing various concentrations of **VTT** in DMSO / 1% (v/v) DMSO / lysis buffer (lysis) / no DMSO (-) for 1 hour. Individual data points are shown as dots, bars represent mean values, and error bars indicate $\pm$ standard error.

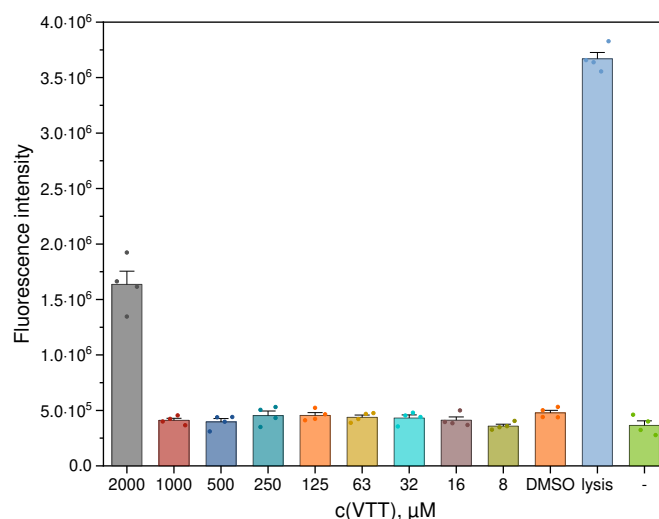


Figure E.78: Fluorescence intensities obtained after incubation of K562 cells supplemented with CellTox™ reagent with solutions containing various concentrations of **VTT** in DMSO / 1% (v/v) DMSO / lysis buffer (lysis) / no DMSO (-) for 2 hours. Individual data points are shown as dots, bars represent mean values, and error bars indicate $\pm$ standard error.

CellTox assay with VTT and HEK cells

1.7 mL of confluent HEK cells (8.9×10^5 cells / mL) in 1x Gibco DMEM + GlutaMAX Medium supplemented with 10% of FCS was diluted and resuspended in 28.3 mL of the medium and transferred to a black / clear bottom 96-well plate (100 μL suspension / well). The plate was incubated for 20 hours at 37 °C in 5% CO₂ in a humidified atmosphere. The growth medium was removed and the cells were washed two times with 100 μL of DPBS. Then, 100 μL of the CellTox™ reagent mixture (freshly prepared by diluting the CellTox™ (Promega, #G8741) stock solution 1:500 (v/v) with DPBS) was added. Then, 1.0 μL of a DMSO stock solution containing various concentrations of **VTT** was added to the wells. In case of the control experiment with the lysis buffer, 4.0 μL of the Lysis Solution from the CellTox™ kit was added to the wells. The last control experiment involved no addition of DMSO. Each experiment was performed in four replicates. The plate was incubated for 60 min at 37 °C in 5% CO₂ in a humidified atmosphere and the fluorescence measurement was performed at $\lambda_{\text{ex}} = 487$ nm and $\lambda_{\text{em}} = 535$ nm.

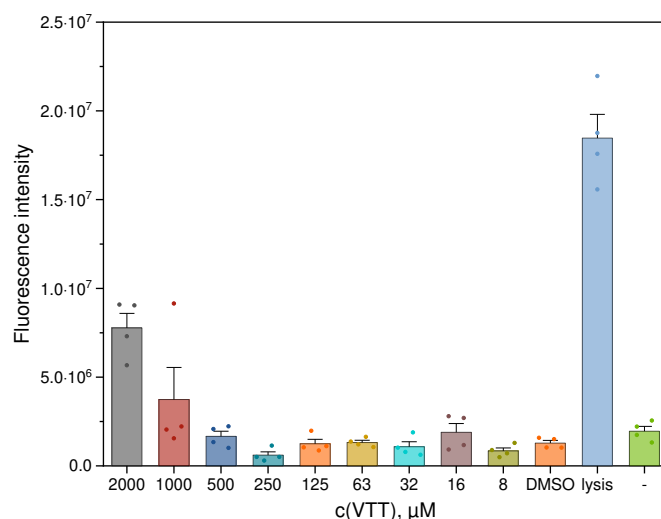


Figure E.79: Fluorescence intensities obtained after incubation of HEK cells supplemented with Cell-Tox™ reagent with solutions containing various concentrations of **VTT** in DMSO / 1% (v/v) DMSO / lysis buffer (lysis) / no DMSO (-) for 1 hour. Individual data points are shown as dots, bars represent mean values, and error bars indicate $\pm$ standard error.

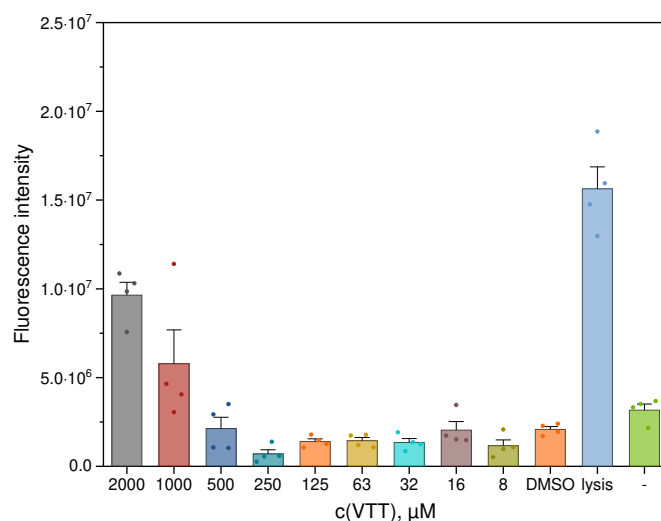


Figure E.80: Fluorescence intensities obtained after incubation of HEK cells supplemented with Cell-Tox™ reagent with solutions containing various concentrations of **VTT** in DMSO / 1% (v/v) DMSO / lysis buffer (lysis) / no DMSO (-) for 2 hours. Individual data points are shown as dots, bars represent mean values, and error bars indicate $\pm$ standard error.

CellTox assay with TTD and HEK cells

Confluent ($\approx 95\%$) HEK cells in 1x Gibco DMEM + GlutaMAX Medium supplemented with 10% of heat-inactivated FCS in a 10 cm culture plate were washed 2 times with 5 mL of DPBS and resuspended in DPBS to obtain a 5 mL suspension with 3.4×10^6 cells / mL. The suspension was centrifuged for 5 min at 1,500 xg at 4 °C in a 15 mL Falcon tube, the supernatant was removed, the cells were resuspended in DPBS to obtain 10 mL of suspension containing 0.1×10^6 cells / mL. 10 μL of CellTox™ (Promega, #G8741) stock solution was added to the cell suspension and the obtained mixture was added to a black / clear bottom 96-well plate (100 μL suspension / well). The wells contained 1 μL of a freshly prepared stock solution of **TTD** in DMSO, 1 μL DMSO, or no DMSO as a control. Each experiment was performed in four replicates. The plate was

incubated at 37 °C in 5% CO₂ in a humidified atmosphere and the fluorescence measurements ($\lambda_{\text{ex}} = 487 \text{ nm}$ and $\lambda_{\text{em}} = 535 \text{ nm}$) were performed 60 minutes, 90 minutes, and 120 minutes after addition of the cells to the wells. To obtain the value for the lysed cells, 1 μL of NP-40 stock solution was added to the wells containing cells and DMSO (1%, v/v) 5 hours after the addition of the cells to the plate, the plate was incubated for 1 hour at 37 °C in 5% CO₂ in a humidified atmosphere, and the fluorescence measurements were performed.

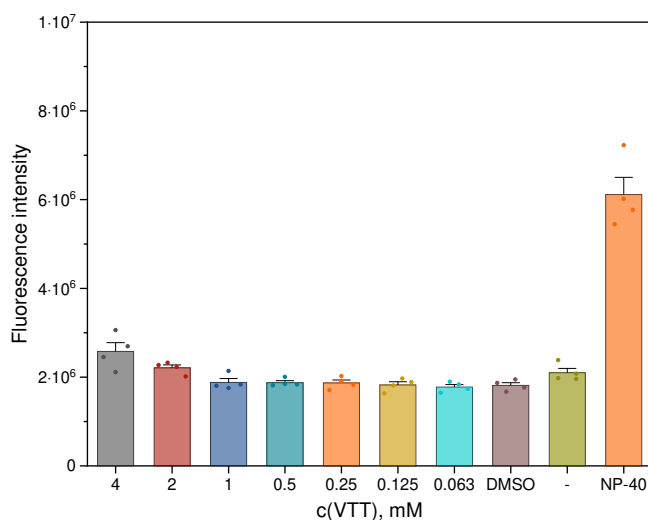


Figure E.81: Fluorescence intensities obtained after incubation of HEK cells supplemented with Cell-Tox™ reagent with solutions containing various concentrations of **TTD** in DMSO / 1% (v/v) DMSO / no DMSO (-) for 1 hour. The reference value with NP-40 as a lysis buffer was obtained after incubating cell suspensions containing DMSO with 1% (v/v) NP-40 for 1 hour. Individual data points are shown as dots, bars represent mean values, and error bars indicate $\pm$ standard error.

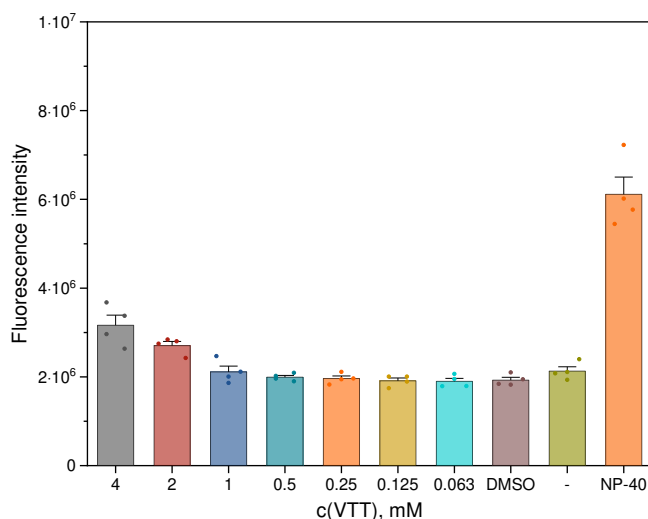


Figure E.82: Fluorescence intensities obtained after incubation of HEK cells supplemented with Cell-Tox™ reagent with solutions containing various concentrations of **TTD** in DMSO / 1% (v/v) DMSO / no DMSO (-) for 1.5 hours. The reference value with NP-40 as a lysis buffer was obtained after incubating cell suspensions containing DMSO with 1% (v/v) NP-40 for 1 hour. Individual data points are shown as dots, bars represent mean values, and error bars indicate $\pm$ standard error.

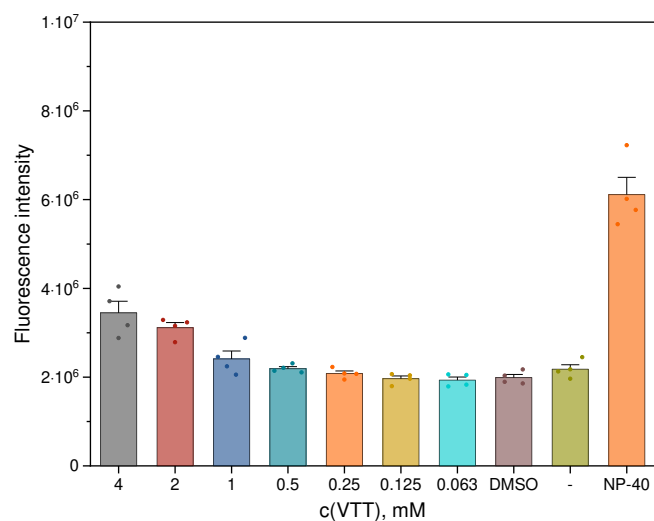


Figure E.83: Fluorescence intensities obtained after incubation of HEK cells supplemented with Cell-Tox™ reagent with solutions containing various concentrations of **TTD** in DMSO / 1% (v/v) DMSO / no DMSO (-) for 2 hours. The reference value with NP-40 as a lysis buffer was obtained after incubating cell suspensions containing DMSO with 1% (v/v) NP-40 for 1 hour. Individual data points are shown as dots, bars represent mean values, and error bars indicate $\pm$ standard error.

Resazurin viability assays

Viability assay with VTT and K562 cells

Resazurin (PrestoBlue™ HS) was added either immediately or 25 minutes after addition of the alkylating reagents to assess the acute effects of the alkylating reagents on cellular redox homeostasis within the depicted timeframe used for cell labeling experiments with **VTT**. The observed variations in fluorescence intensities between measurements in the two experimental setups – despite equal incubation times – suggest that the obtained values represent cellular stress response rather than actual cell viability.

Assay with a delayed addition of resazurin reagent

45 mL of K562 cells (1.19×10^6 cells / mL) in 1x Gibco RPMI 1640 Medium supplemented with 10% of heat-inactivated FCS was split in two parts and centrifuged for 5 min at 1,500 xg at 4 °C in two 50 mL Falcon tubes. In each tube, the supernatants were removed, the cells were resuspended in 25 mL of DPBS, centrifuged for 5 min at 1,500 xg at 4 °C, the supernatant was discarded, the cells were washed again with 25 mL of DPBS, and the supernatant was discarded. The cell pellets were combined and resuspended in 10.8 mL of DPBS and transferred to a black / clear bottom 96-well plate (80 μ L suspension / well) containing 0.8 μ L of freshly prepared DMSO solutions of **VTT**, **NEM**, **IAA** alkyne, 0.8 μ L of DMSO, or no DMSO, respectively, as control in the wells. Each experiment was performed in four replicates. The plate was incubated for 25 minutes at 37 °C in 5% CO₂ in a humidified atmosphere, 20 μ L of Presto-Blue™ HS reagent (Invitrogen, #P50200) diluted 1:1 (v/v) with DPBS was added to each well, and the plate was incubated at 37 °C in 5% CO₂ in a humidified atmosphere. The fluorescence measurements were performed at various time points at $\lambda_{\text{ex}} = 560$ nm and $\lambda_{\text{em}} = 610$ nm.

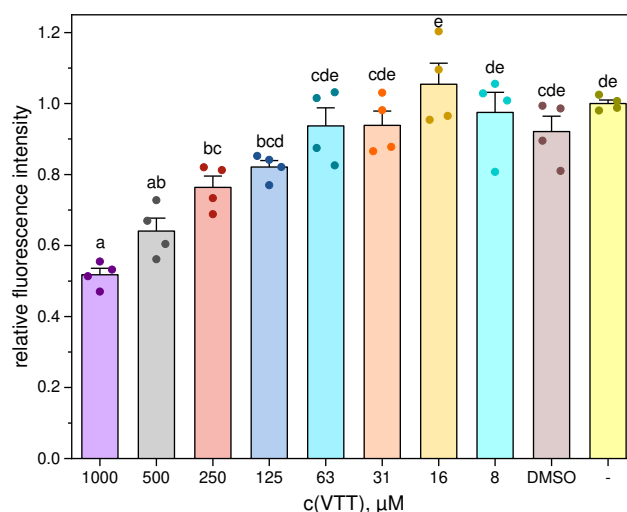


Figure E.84: Fluorescence intensities obtained after incubation of K562 cells with solutions containing various concentrations of **VTT** / 1% DMSO / no DMSO (-) and PrestoBlue™ reagent. PrestoBlue™ reagent was added 25 minutes after addition of the cells to the wells with alkylating reagents. The measurement was performed 15 minutes after addition of the PrestoBlue™ reagent to the wells. Individual data points are shown as dots, bars represent mean values, and error bars indicate $\pm$ standard error. The significance of the difference was determined using a one-way ANOVA, followed by Tukey's post hoc test with $p < 0.05$.

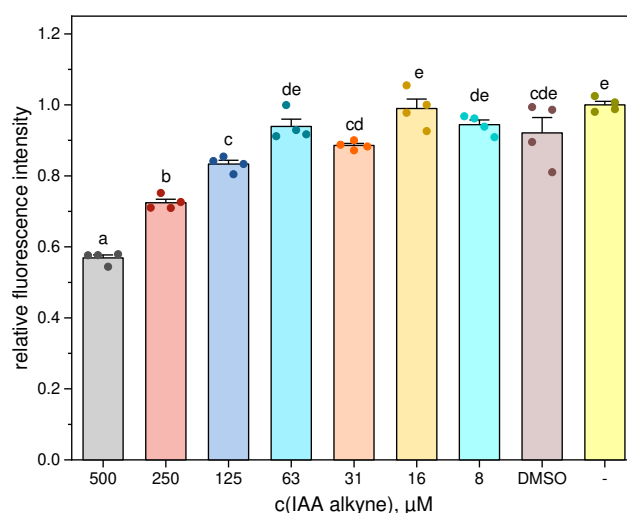


Figure E.85: Fluorescence intensities obtained after incubation of K562 cells with solutions containing various concentrations of **IAA** alkyne / 1% DMSO / no DMSO (-) and PrestoBlue™ reagent. PrestoBlue™ reagent was added 25 minutes after addition of the cells to the wells with alkylating reagents. The measurement was performed 15 minutes after addition of the PrestoBlue™ reagent to the wells. Individual data points are shown as dots, bars represent mean values, and error bars indicate $\pm$ standard error. The significance of the difference was determined using a one-way ANOVA, followed by Tukey's post hoc test with $p < 0.05$.

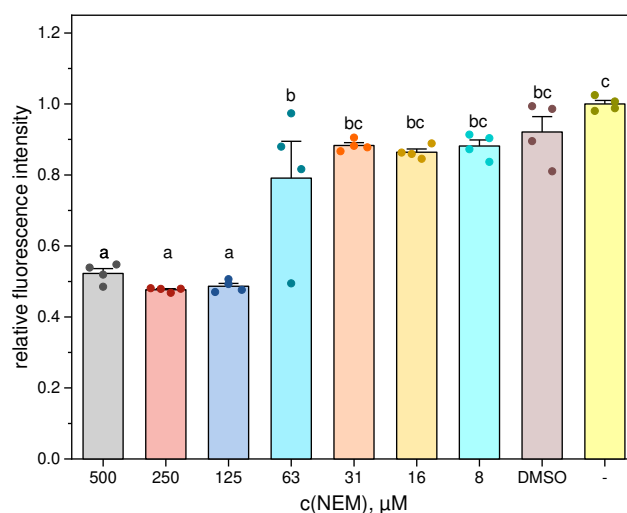


Figure E.86: Fluorescence intensities obtained after incubation of K562 cells with solutions containing various concentrations of **NEM** / 1% DMSO / no DMSO (-) and PrestoBlue™ reagent. PrestoBlue™ reagent was added 25 minutes after addition of the cells to the wells with alkylating reagents. The measurement was performed 15 minutes after addition of the PrestoBlue™ reagent to the wells. Individual data points are shown as dots, bars represent mean values, and error bars indicate $\pm$ standard error. The significance of the difference was determined using a one-way ANOVA, followed by Tukey's post hoc test with $p < 0.05$.

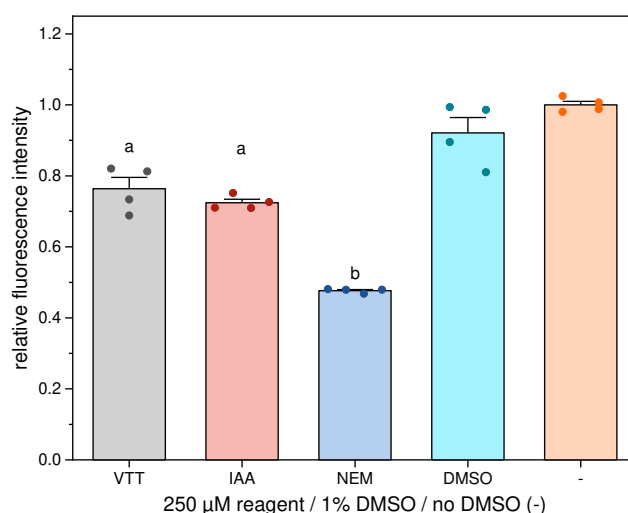


Figure E.87: Fluorescence intensities obtained after incubation of K562 cells with solutions containing 0.25 mM of **VTT** / **IAA** alkyne (IAA) / **NEM** / 1% DMSO / no DMSO (-) and PrestoBlue™ reagent. PrestoBlue™ reagent was added 25 minutes after addition of the cells to the wells with alkylating reagents. The measurement was performed 15 minutes after addition of the PrestoBlue™ reagent to the wells. Individual data points are shown as dots, bars represent mean values, and error bars indicate $\pm$ standard error. The significance of the difference was determined using a one-way ANOVA, followed by Tukey's post hoc test with $p < 0.05$.

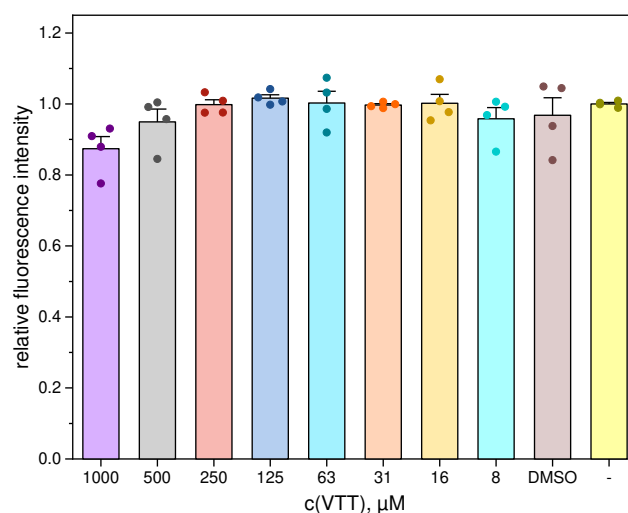


Figure E.88: Fluorescence intensities obtained after incubation of K562 cells with solutions containing various concentrations of **VTT** / 1% DMSO / no DMSO (-) and PrestoBlue™ reagent. PrestoBlue™ reagent was added 25 minutes after addition of the cells to the wells with alkylating reagents. The measurement was performed 4 hours after addition of the PrestoBlue™ reagent to the wells. Individual data points are shown as dots, bars represent mean values, and error bars indicate $\pm$ standard error.

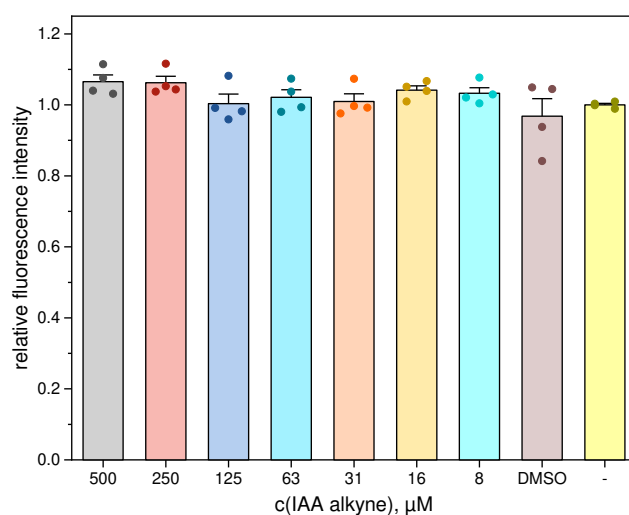


Figure E.89: Fluorescence intensities obtained after incubation of K562 cells with solutions containing various concentrations of **IAA** alkyne / 1% DMSO / no DMSO (-) and PrestoBlue™ reagent. PrestoBlue™ reagent was added 25 minutes after addition of the cells to the wells with alkylating reagents. The measurement was performed 4 hours after addition of the PrestoBlue™ reagent to the wells. Individual data points are shown as dots, bars represent mean values, and error bars indicate $\pm$ standard error.

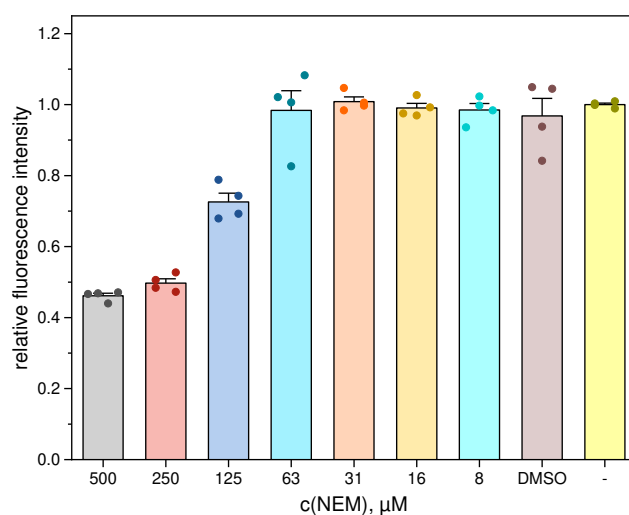


Figure E.90: Fluorescence intensities obtained after incubation of K562 cells with solutions containing various concentrations of **NEM** / 1% DMSO / no DMSO (-) and PrestoBlue™ reagent. PrestoBlue™ reagent was added 25 minutes after addition of the cells to the wells with alkylating reagents. The measurement was performed 4 hours after addition of the PrestoBlue™ reagent to the wells. Individual data points are shown as dots, bars represent mean values, and error bars indicate $\pm$ standard error.

Assay with direct addition of resazurin reagent

48 mL of K562 cells (1.12×10^6 cells / mL) in 1x Gibco RPMI 1640 Medium supplemented with 10% of heat-inactivated FCS was split in two parts and centrifuged for 5 min at 1,500 xg at 4 °C in two 50 mL Falcon tubes. In each tube, the supernatants were removed, the cells were resuspended in 25 mL of DPBS, centrifuged for 5 min at 1,500 xg at 4 °C, the supernatant was discarded, the cells were washed again with 25 mL of DPBS, and the supernatant was discarded. The cell pellets were combined and resuspended in 10.8 mL of DPBS and transferred to a black / clear bottom 96-well plate (80 μ L suspension / well) containing 1.0 μ L of freshly prepared DMSO solutions of **VTT**, **NEM**, **IAA** alkyne, 1.0 μ L of DMSO, or no DMSO, respectively, as control in the wells. 20 μ L of PrestoBlue™ HS reagent (Invitrogen, #P50200) diluted 1:1 (v/v) with DPBS was added immediately after addition of the cell suspension to the wells. Each experiment was performed in four replicates. The plate was incubated at 37 °C in 5% CO₂ in a humidified atmosphere and the fluorescence measurements were performed at various time points λ_{ex} = 560 nm and λ_{em} = 610 nm.

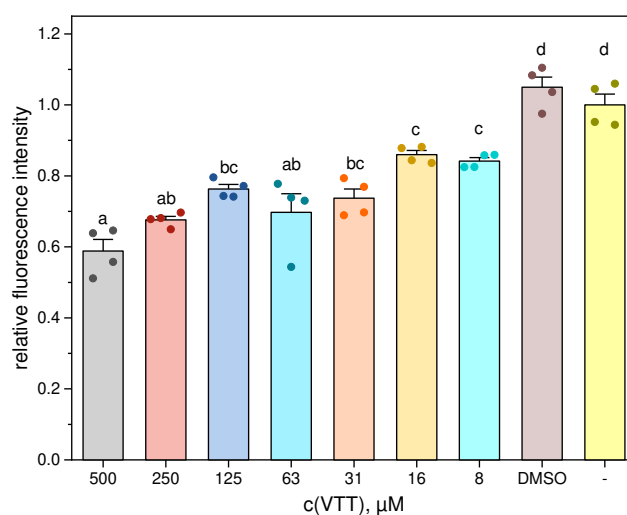


Figure E.91: Normalized fluorescence intensities obtained after incubation of K562 cells with solutions containing various concentrations of **VTT** / 1% DMSO / no DMSO (-) and PrestoBlue™ reagent. The measurement was performed 15 minutes after addition of the PrestoBlue™ reagent to the wells. Individual data points are shown as dots, bars represent mean values, and error bars indicate $\pm$ standard error. The significance of the difference was determined using a one-way ANOVA, followed by Tukey's post hoc test with $p < 0.05$.

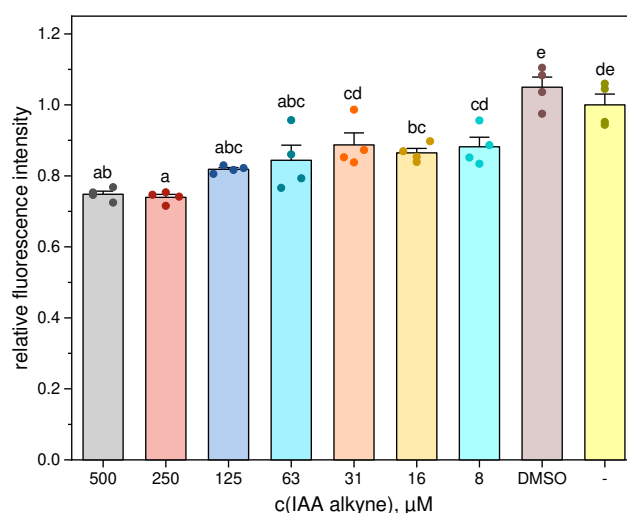


Figure E.92: Normalized fluorescence intensities obtained after incubation of K562 cells with solutions containing various concentrations of **IAA** alkyne / 1% DMSO / no DMSO (-) and PrestoBlue™ reagent. The measurement was performed 15 minutes after addition of the PrestoBlue™ reagent to the wells. Individual data points are shown as dots, bars represent mean values, and error bars indicate $\pm$ standard error. The significance of the difference was determined using a one-way ANOVA, followed by Tukey's post hoc test with $p < 0.05$.

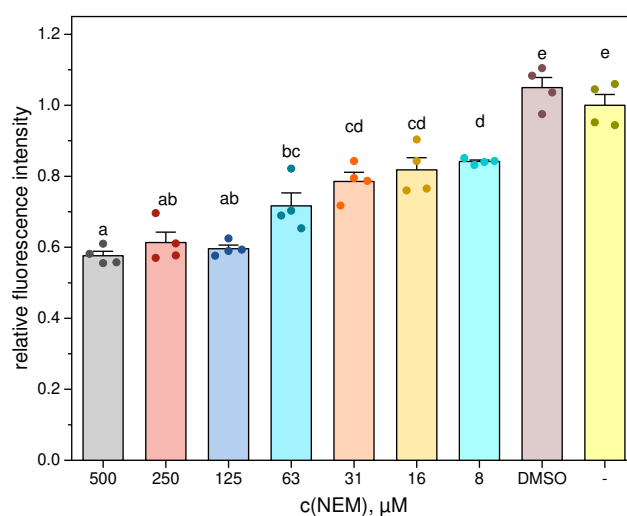


Figure E.93: Normalized fluorescence intensities obtained after incubation of K562 cells with solutions containing various concentrations of **NEM** / 1% DMSO / no DMSO (-) and PrestoBlue™ reagent. The measurement was performed 15 minutes after addition of the PrestoBlue™ reagent to the wells. Individual data points are shown as dots, bars represent mean values, and error bars indicate $\pm$ standard error. The significance of the difference was determined using a one-way ANOVA, followed by Tukey's post hoc test with $p < 0.05$.

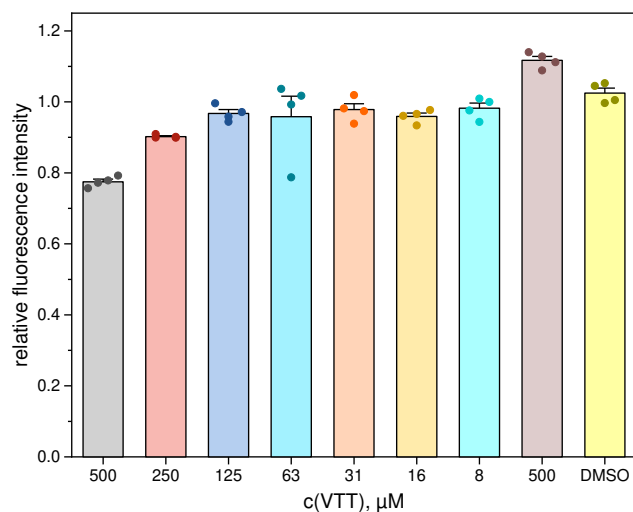


Figure E.94: Normalized fluorescence intensities obtained after incubation of K562 cells with solutions containing various concentrations of **VTT** / 1% DMSO / no DMSO (-) and PrestoBlue™ reagent. The measurement was performed 4 hours after addition of the PrestoBlue™ reagent to the wells. Individual data points are shown as dots, bars represent mean values, and error bars indicate $\pm$ standard error.

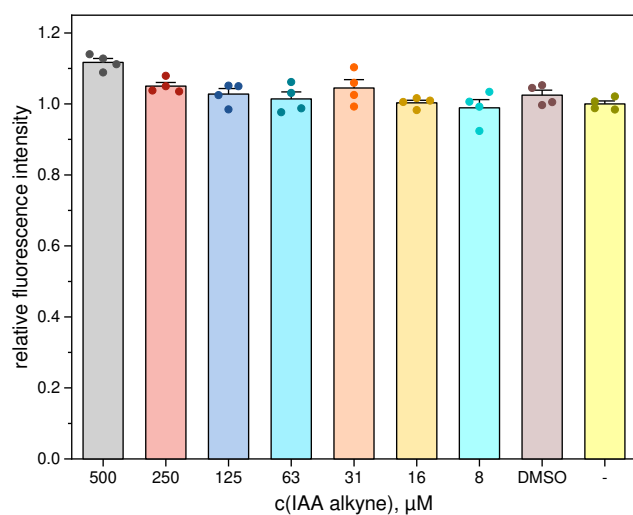


Figure E.95: Normalized fluorescence intensities obtained after incubation of K562 cells with solutions containing various concentrations of **IAA** alkyne / 1% DMSO / no DMSO (-) and PrestoBlue™ reagent. The measurement was performed 4 hours after addition of the PrestoBlue™ reagent to the wells. Individual data points are shown as dots, bars represent mean values, and error bars indicate $\pm$ standard error.

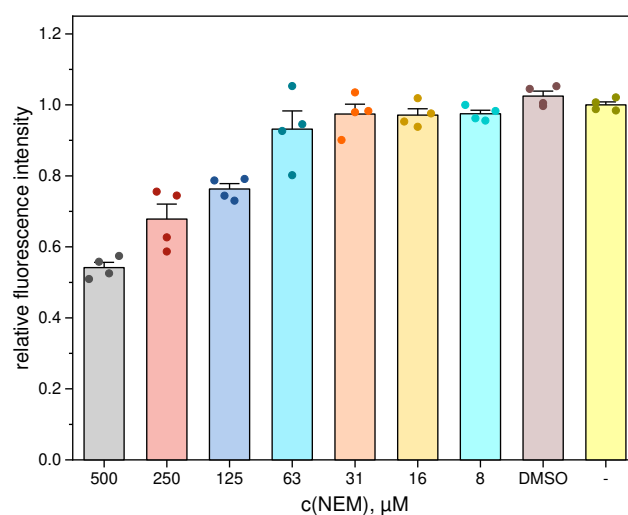


Figure E.96: Normalized fluorescence intensities obtained after incubation of K562 cells with solutions containing various concentrations of **NEM** / 1% DMSO / no DMSO (-) and PrestoBlue™ reagent. The measurement was performed 4 hours after addition of the PrestoBlue™ reagent to the wells. Individual data points are shown as dots, bars represent mean values, and error bars indicate $\pm$ standard error.

Viability assay with VTT/TTD and HEK cells

Confluent ($\approx 95\%$) HEK cells in 1x Gibco DMEM + GlutaMAX Medium supplemented with 10% of heat-inactivated FCS in six 10 cm culture plates were washed each 2 times with 5 mL of DPBS and resuspended in DPBS to obtain a 30 mL suspension with 2.8×10^6 cells / mL. The suspension was centrifuged for 5 min at 1,500 xg at 4 °C in a 50 mL Falcon tube, the supernatant was removed, the cells were resuspended in DPBS to obtain 67 mL of suspension containing 1.25×10^6 cells / mL. The obtained suspension was added to a black / clear bottom 96-well plate (80 μ L suspension / well). The wells contained 0.8 μ L of a freshly prepared stock solutions of **TTD** or **VTT** in DMSO, 0.8 μ L DMSO, or no DMSO as a control. Each experiment was performed in four replicates. 5 minutes after addition of the cell suspension to the wells, 20 μ L of PrestoBlue™ HS reagent (Invitrogen, #P50200) diluted 1:1 (v/v) with DPBS was added to each well, and the plate was incubated at 37 °C in 5% CO₂ in a humidified atmosphere. The fluorescence measurements were performed at various time points at $\lambda_{\text{ex}} = 540$ nm and $\lambda_{\text{em}} = 580$ nm.

The significant variation in fluorescence intensities observed after 1 hour of incubation, particularly in cells treated with **TTD**, indicated that this incubation time was insufficient for obtaining reliable absolute viability values with the used setup, since even a small deviation in the measured concentration of resorufin can lead to substantial discrepancies in the obtained results. Extending the incubation time to a total of 20 hours resulted in increased fluorescence intensity and more reliable measurements. Although the values obtained after 20 hours also cannot be used to estimate absolute viability due to the prolonged incubation of adherent cells in suspension, the results provide additional evidence that cells treated with 1 mM **TTD** exhibit approximately twice the viability of those treated with **VTT** under the same conditions, consistent with the data obtained after 1 hour of incubation. The results additionally suggest that using suspension cells may be a more suitable approach for performing crosslinking experiments in PBS if the goal is to ensure high cell viability.

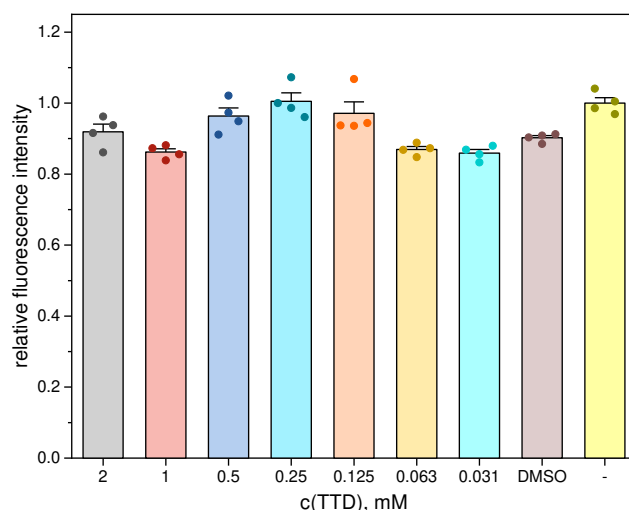


Figure E.97: Fluorescence intensities obtained after incubation of HEK cells with solutions containing various concentrations of **TTD** / 1% DMSO / no DMSO (-) and PrestoBlue™ reagent. The measurement was performed 1 hour after addition of the PrestoBlue™ reagent to the wells. Individual data points are shown as dots, bars represent mean values, and error bars indicate $\pm$ standard error.

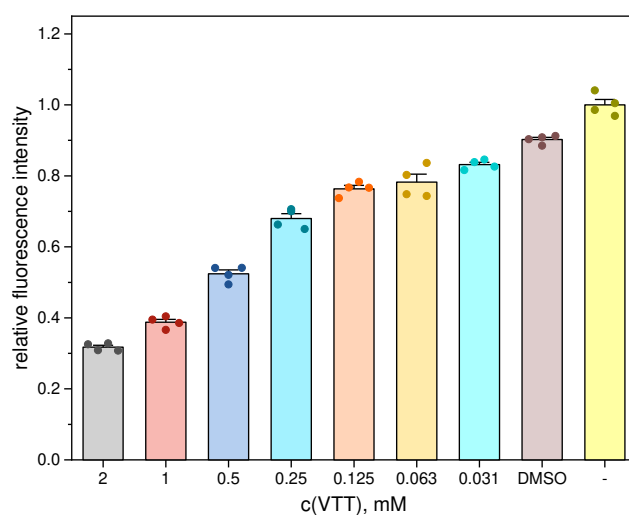


Figure E.98: Fluorescence intensities obtained after incubation of HEK cells with solutions containing various concentrations of **VTT** / 1% DMSO / no DMSO (-) and PrestoBlue™ reagent. The measurement was performed 1 hour after addition of the PrestoBlue™ reagent to the wells. Individual data points are shown as dots, bars represent mean values, and error bars indicate $\pm$ standard error.

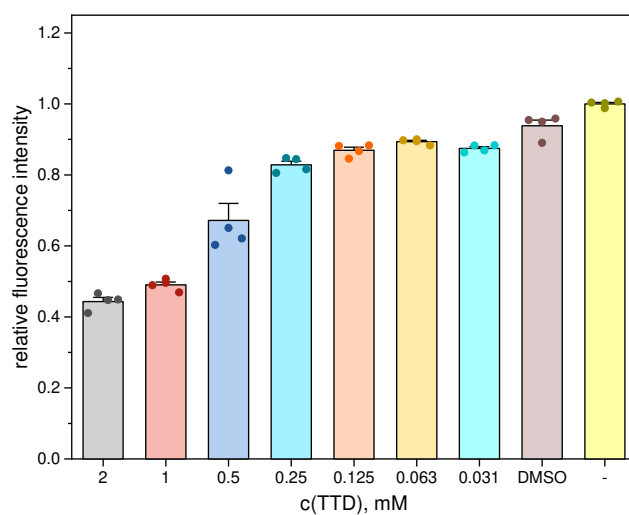


Figure E.99: Fluorescence intensities obtained after incubation of HEK cells with solutions containing various concentrations of **TTD** / 1% DMSO / no DMSO (-) and PrestoBlue™ reagent. The measurement was performed 20 hours after addition of the PrestoBlue™ reagent to the wells. Individual data points are shown as dots, bars represent mean values, and error bars indicate $\pm$ standard error.

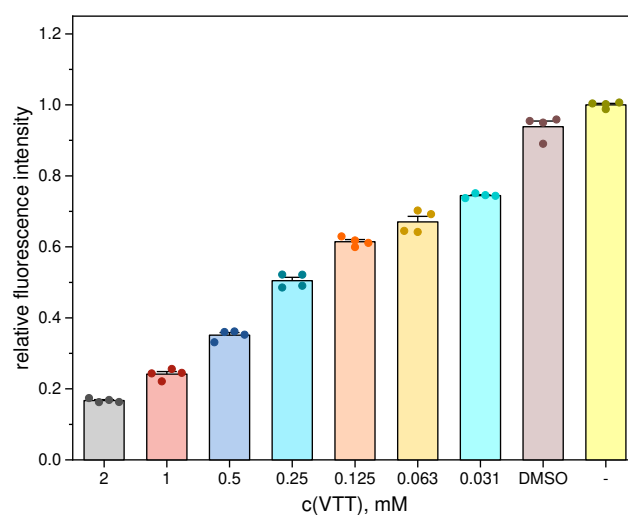


Figure E.100: Fluorescence intensities obtained after incubation of HEK cells with solutions containing various concentrations of **VTT** / 1% DMSO / no DMSO (-) and PrestoBlue™ reagent. The measurement was performed 20 hours after addition of the PrestoBlue™ reagent to the wells. Individual data points are shown as dots, bars represent mean values, and error bars indicate $\pm$ standard error.

Modification searches and labeling efficiencies

The LC–MS/MS data were acquired by Dr. Christian Preisinger and Dr. Alessandro Vetere. The author performed the database searches and the data analysis.

VTT-mediated alkylation: modification search

Processing of raw data was performed using MaxQuant 2.6.3.0²⁰⁹ on the HPC system Raven at the Max Planck Computing and Data Facility. MS² spectra were assigned to the reference proteomes from UniProt (*H. sapiens*: UP000005640; *A. thaliana*: UP000006548; *B. subtilis*: UP000001570; *E. coli*: UP000000625; *B. vulgaris*: UP000035740; *S. cerevisiae*: UP000002311; *N. tabacum*: UP000084051). Trypsin specificity was required and a maximum of two missed cleavages was allowed. Carbamidomethylation of cysteine, oxidation of methionine, and protein N-terminal acetylation were set as variable modifications. The **VTT**-based modification was defined as a variable modification on Cys with the formula C₂H₄O for the episulfonium hydrolysis product formed with **VTT**. A false discovery rate of 1% for peptide spectrum matches and proteins was applied. The total number of peptides of interest was extracted after filtering off the decoy peptides and potential contaminants from the output table.

Labeling efficiencies in different cell types

The labeling efficiencies were calculated by normalizing the number of the identified Cys-modified peptides by the number of Cys-containing peptides detected in each run.

Cell type	c(VTT), mM	# Replicate	# Cys peptides	# Cys–C ₂ H ₄ OH peptides	Labeling efficiency
<i>H. sapiens</i> , K562	0.25	1	1198	314	26.00%
		2	1201	298	25.00%
		3	1154	355	31.00%
<i>H. sapiens</i> , HEK	0.25	1	1244	319	26.00%
		2	1015	245	24.00%
		3	1231	276	22.00%
<i>A. thaliana</i>	1.0	1	661	44	7.00%
		2	426	20	5.00%
		3	751	44	6.00%
<i>A. thaliana</i>	5.0	1	612	205	33.00%
		2	1150	188	16.00%
		3	1223	222	18.00%
<i>B. subtilis</i>	1.0	1	579	150	25.90%
		2	668	174	26.00%
		3	642	165	25.70%
<i>B. vulgaris</i>	1.0	1	260	79	30.00%
		2	195	58	30.00%
		3	282	69	24.00%
<i>S. cerevisiae</i>	1.0	1	546	50	9.00%
		2	427	31	7.00%
		3	278	16	6.00%

Cell type	c(VTT), mM	# Replicate	# Cys peptides	# Cys–C ₂ H ₄ OH peptides	Labeling efficiency
<i>S. cerevisiae</i>	5.0	1	325	64	19.70%
		2	315	57	18.10%
		3	217	40	18.40%
<i>N. tabacum</i>	1.0	1	584	69	12.00%
		2	842	50	6.00%
		3	804	49	6.00%
<i>N. tabacum</i>	5.0	1	730	154	21.00%
		2	631	137	22.00%
		3	742	121	16.00%
<i>E. coli</i>	1.0	1	722	177	25.00%
		2	673	190	28.00%
		3	756	175	23.00%

Labeling efficiency *in vitro* vs *in cellulo*

To compare peptide identities between several sample groups, the lists of detected peptides with zero missed cleavages that were not decoys or potential contaminants were extracted from the output table after database search with MaxQuant and matches between unique peptides from both groups were identified. The overlaps between peptides detected in all biological replicates were visualized using a Venn diagram. A detailed analysis for overlaps between all identified peptides in each biological replicate were obtained via UpSet plot²¹⁰.

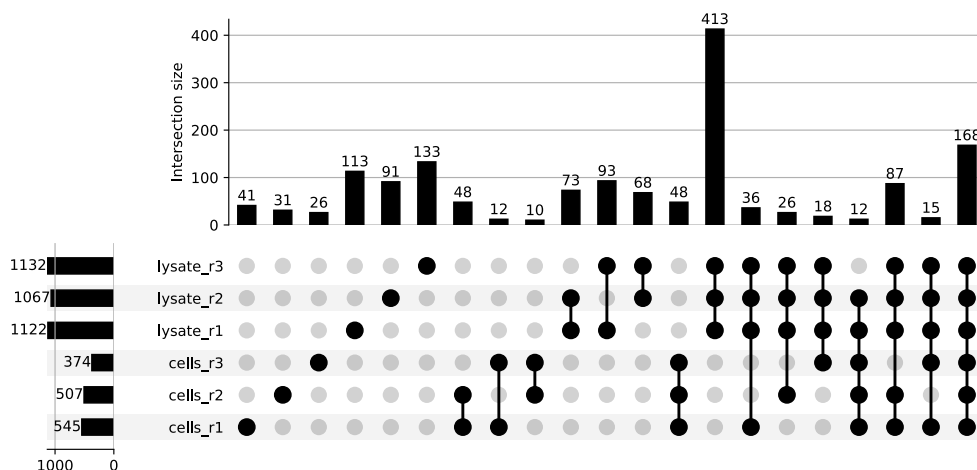


Figure E.101: Intersection analysis of modified peptides identified in the biological replicates (r1, r2, r3) after protein modification *in cellulo* in K562 cells (cells) or *in vitro* in K562 lysates (lysate). Each vertical bar represents a unique combination of input sets (indicated by filled dots in the matrix below the x-axis); the bar height equals the number of elements shared exclusively by that combination. Horizontal bars to the left show the total size of each individual set. Intersections containing fewer than ten elements were omitted for clarity.

Competition experiments with VTT

Competition experiment between VTT and NEM

For quantification of the intensity ratios, a database search using the quantification feature of MaxQuant²⁰⁹ was performed. MS² spectra were assigned to the reference proteome from UniProt (*H. sapiens*: UP000005640). Trypsin specificity was required and a maximum of two missed cleavages was allowed. The light label was specified as C₂₁H₃₄N₆O₅S (biotin-enriched product formed with **VTT** after CuAAC reaction); The heavy label was specified as C₂₁H₃₁D₃N₆O₅S (biotin-enriched product formed with **²H₃-VTT** after CuAAC reaction). Both labels were specified with Cys-selectivity. N-acetylation and methionine oxidation were specified as variable modifications. The list of the median H/L ratios for each replicate was obtained after excluding hits that corresponded to decoy (reverse) peptides and potential contaminants.

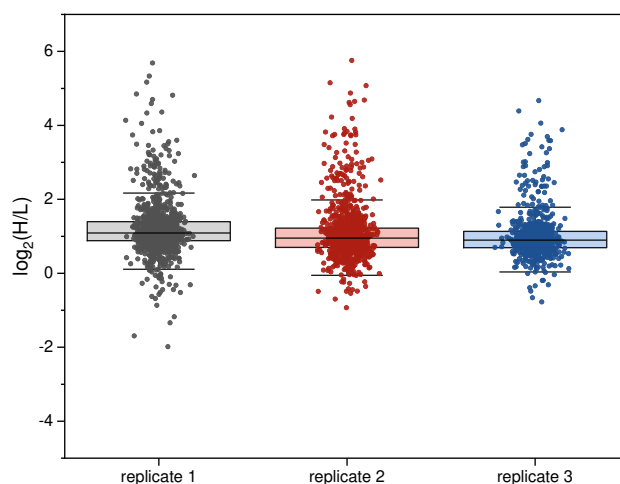


Figure E.102: Box plots showing the log₂-ratios of MS¹ intensities between heavy-labeled **²H₃-VTT** (H) and unlabeled **VTT** (L) across biological replicates. The box spans from the first quartile (the 25th percentile) to the third quartile (the 75th percentile), to capture the interquartile range, which contains the middle 50% of the data. Horizontal line indicates median, whiskers extend to 1.5 × interquartile range from box boundaries.

Replicate	# quantified peptide pairs	Median of H/L ratios
1	1078	2.13
2	1007	1.93
3	1057	1.88

The mean of the median H/L ratios obtained for three biological replicates is 2.0 ± 0.1 .

Competition experiment between VTT and TTD

For quantification of the intensity ratios, a database search using the quantification feature of MaxQuant²⁰⁹ was performed. MS² spectra were assigned to the reference proteome from UniProt (*E. coli*: UP000000625). Trypsin specificity was required and a maximum of two missed cleavages was allowed. The light label was specified as C₂H₄O (product formed with **VTT**); The heavy label was specified as C₂D₃HO (product formed with **²H₃-VTT**). Both labels were specified with Cys-selectivity. N-acetylation and methionine oxidation were specified as variable modifications. The list of the median H/L ratios for each replicate was obtained after excluding hits that corresponded to decoy (reverse) peptides and potential contaminants.

Competition experiment performed for 5 minutes:

Replicate	# quantified peptide pairs	Median of H/L ratios
1	59	1.09
2	53	1.19
3	48	1.25

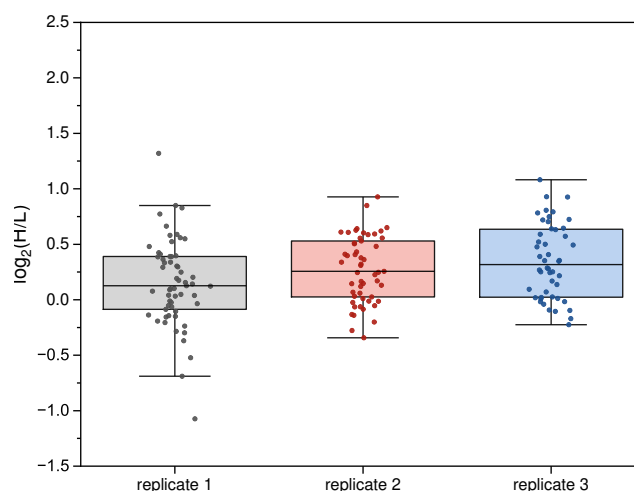


Figure E.103: Box plots showing the $\log_2$ -ratios of MS¹ intensities between heavy-labeled **²H₃-VTT** (H) and unlabeled **VTT** (L) across biological replicates. The box spans from the first quartile (the 25th percentile) to the third quartile (the 75th percentile), to capture the interquartile range, which contains the middle 50% of the data. Horizontal line indicates median, whiskers extend to 1.5 × interquartile range from box boundaries.

The mean of the median H/L ratios obtained for three biological replicates is 1.18 ± 0.05 .

Competition experiment performed for 1 hour:

Replicate	# quantified peptide pairs	Median of H/L ratios
1	66	1.319
2	79	1.310
3	79	1.310

The mean of the median H/L ratios obtained for three biological replicates is 1.313 ± 0.003 .

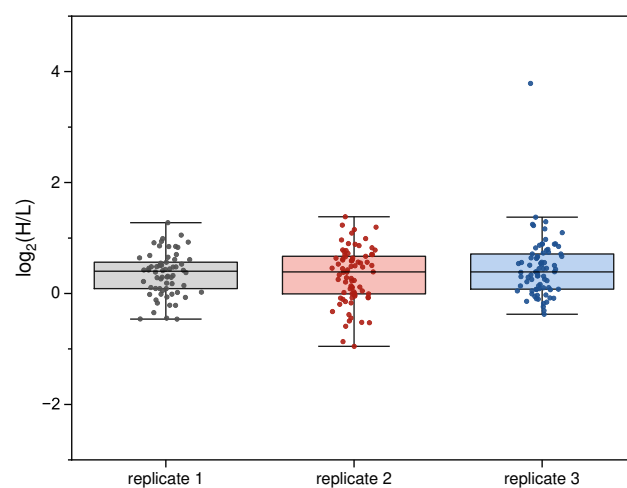


Figure E.104: Box plots showing the $\log_2$ -ratios of MS^1 intensities between heavy-labeled 2H_3 -VTT (H) and unlabeled VTT (L) across biological replicates. The box spans from the first quartile (the 25th percentile) to the third quartile (the 75th percentile), to capture the interquartile range, which contains the middle 50% of the data. Horizontal line indicates median, whiskers extend to $1.5 \times$ interquartile range from box boundaries.

TTD-mediated alkylation: modification search

Processing of raw data was performed using MaxQuant 2.6.3.0²⁰⁹ on the HPC system Raven at the Max Planck Computing and Data Facility. MS² spectra were assigned to the reference proteome from UniProt (*H. sapiens*: UP000005640). Trypsin specificity was required and a maximum of two missed cleavages was allowed. Carbamidomethylation of cysteine, oxidation of methionine, and protein N-terminal acetylation were set as variable modifications. The **TTD**-based modification formed via episulfonium hydrolysis followed by biotin-enrichment of the obtained products was defined as a variable modification on one of the nucleophilic amino acids (Cys, Lys, His, Ser, Thr, Glu, Asp, Arg, Tyr) with formula C₂₀H₃₂N₆O₅S and H₂O as a neutral loss.

A false discovery rate of 1% for peptide spectrum matches and proteins was applied. The total number of peptides of interest was obtained after filtering off the decoy peptides and potential contaminants from the output table:

replicate	# All peptides	# modified peptides	% unmodified peptides
1	5903	5446	7.7%
2	5755	5353	7.0%
3	5967	5512	7.6%

For the analysis of the modification selectivity, only modified peptides with localization probability of at least 95% were considered:

AA	# mod. peptides, r. 1	# mod. peptides, r. 2	# mod. peptides, r. 3
Cys	1324	1290	1297
Lys	79	84	62
Arg	43	35	34
Glu	34	20	20
His	24	25	23
Ser	14	21	24
Asp	14	19	16
Thr	11	8	11
Tyr	6	4	7

Modification search in enriched and non-enriched samples

Trypsin specificity was required and a maximum of two missed cleavages was allowed. Carbamidomethylation of cysteine, oxidation of methionine, and protein N-terminal acetylation were set as variable modifications. The enriched **TTD**-based modification was defined as a variable modification on Cys with formula C₂₀H₃₂N₆O₅S and H₂O as a neutral loss. The non-enriched **TTD**-based modification was defined as a variable modification on Cys with formula C₆H₈O₂ and H₂O as a neutral loss.

A false discovery rate of 1% for peptide spectrum matches and proteins was applied. The total number of peptides of interest was obtained after filtering off the decoy peptides and potential contaminants from the output table.

To compare peptide identities between several sample groups, the lists of detected peptides with zero missed cleavages that were not decoys or potential contaminants were extracted from the output table after database search with MaxQuant and matches between unique peptides from

both groups were identified. The overlaps between peptides detected in all biological replicates were visualized using a Venn diagram. A detailed analysis for overlaps between all identified peptides in each biological replicate were obtained via UpSet plot²¹⁰.

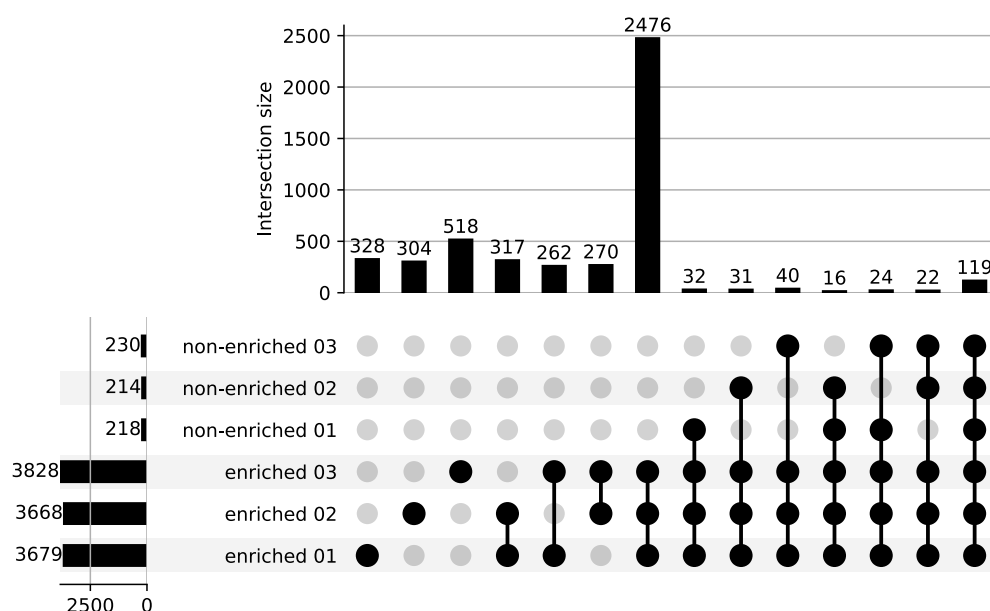


Figure E.105: Intersection analysis of modified peptides identified in the biological replicates (01, 02, 03) after biotin-enrichment (enriched) and without enrichment of modified peptides (non-enriched). Each vertical bar represents a unique combination of input sets (indicated by filled dots in the matrix below the x-axis); the bar height equals the number of elements shared exclusively by that combination. Horizontal bars to the left show the total size of each individual set. Intersections containing fewer than 30 elements are omitted for clarity.

Identification of Cys- and GSH-conjugates

The open search with MSFragger 4.1²¹¹ was performed using the standard settings²¹². The modification search was performed using MaxQuant 2.6.3.0²⁰⁹ on the HPC system Raven at the Max Planck Computing and Data Facility. MS² spectra were assigned to the reference proteome from UniProt (*H. sapiens*: UP000005640). Trypsin specificity was required and a maximum of two missed cleavages was allowed. Carbamidomethylation of cysteine, oxidation of methionine, and protein N-terminal acetylation were set as variable modifications. The **TTD**-based modifications were defined as variable modifications on one of the nucleophilic amino acids (Cys, Lys, His, Ser, Thr, Glu, Asp, Arg, Tyr) with formulas C₃₀H₄₇N₉O₁₀S₂ (**GSH**-conjugate) and C₂₃H₂₇N₇O₆S₂ (Cys-conjugate).

A false discovery rate of 1% for peptide spectrum matches and proteins was applied. The total number of peptides of interest was obtained after filtering off the decoy peptides and potential contaminants from the output table. Only modified peptides with localization probability of at least 95% were considered for the analysis.

Crosslink searches

The XL LC–MS/MS data were acquired by Dr. Christian Preisinger and Dr. Alessandro Vetere. The author performed the database searches and the data analysis.

VTT-mediated crosslinking

In cellulo crosslinking in different cell types

Raw data were processed using MSConvert 3.0.24²¹³ to convert the raw files to mzML format for the subsequent mass recalibration¹⁵⁵. An open modification search with MSFragger 4.1²¹¹ was then performed to recalibrate precursor masses, and the obtained files were converted to mgf format with MSConvert 3.0.24²¹³. The mgf spectra were split for parallel database search on the HPC system Raven at the Max Planck Computing and Data Facility. Each of the peak-files was searched separately against the corresponding proteomes from UniProt (*H. sapiens*: UP000005640; *A. thaliana*: UP000006548; *B. subtilis*: UP000001570; *E. coli*: UP000000625; *S. cerevisiae*: UP000002311) using xiSEARCH 1.8.7⁹³ with the following settings: MS¹ tolerance: 3 ppm; MS² tolerance: 5 ppm, allowing up to two missing monoisotopic peaks and three missed tryptic cleavages. Cysteine carbamidomethylation and oxidation of methionine were defined as variable modifications. $-H_2O/-NH_3$ were defined as losses. The **VTT** crosslinker was defined as follows: the linkage mass was set to 26.01565 Da and the specificity was set to C and one of the following residues: C, H, K, E, D, S, T, R, Y, C-terminus, N-terminus. The crosslink search for each of the considered crosslink combinations was performed separately. Variable modifications to account for the hydrolysis of episulfonium with water (18.0105647 Da), or formation of a crosslink within a peptide (26.01565 Da) were also defined. Non-covalent crosslink with a mass of zero was included in all searches to flag spectra potentially arising from gas-phase associated peptides²¹⁴. The output files obtained for all the crosslink combinations of the same biological replicate were combined together. The matches obtained for the non-covalent crosslink were removed prior to false-discovery-rate (FDR) estimation, which was performed using xiFDR 2.3.5⁹³. The input file was filtered to obtain peptide pairs with at least two (*H. sapiens*, *S. cerevisiae*) or three (*A. thaliana*, *B. subtilis*) matched fragment ions in each crosslinked peptide. The ambiguity on the residue pair and protein group levels was set to 1 and the results were filtered on residue pair level (1%) with the boosting feature enabled. The obtained files were uploaded to xiView.org²¹⁵ and the list of the residues in non-ambiguous crosslinks was obtained for each replicate.

For visualization of the crosslinked proteins identified in different cells, the data were filtered to obtain the list of the crosslinks that were detected in at least two biological replicates. The filtered data for proteins with the highest number of identified crosslinks were used as an input for protein structure prediction with AlphaLink2⁸⁹ using ColabFold with the following parameters: model weights: 2.2 (trained on 10 Å C α –C α); use templates: True; msa mode: MMseqs2; max recycling iters: 3; num ensembles: 1; manual seed: 42; times: 1. The obtained structures with the detected crosslinks were visualized using ChimeraX 1.9¹²¹.

Crosslinks identified in K562 cells

X-linked AA	# XLs, r. 1	# XLs, r. 2	# XLs, r. 3	relative AA frequency in the genome	mean crosslink frequency (%)	ratio between mean XL frequency and norm. AA freq.
Glu	101	102	102	15.9	20.4	1.3
Asp	84	85	84	10.6	16.9	1.6
Thr	87	82	79	12.1	16.6	1.4
Ser	79	87	78	18.7	16.3	0.9
Cys	66	67	56	5.2	12.7	2.4
Tyr	33	40	35	6	7.2	1.2
His	38	37	32	5.9	7.2	1.2
Lys	8	11	12	12.9	2.1	0.2
Arg	3	0	5	12.7	0.5	0

Representative MS²-spectra

MS²-spectra of identified crosslinked peptides that were detected in K562 samples are presented below.

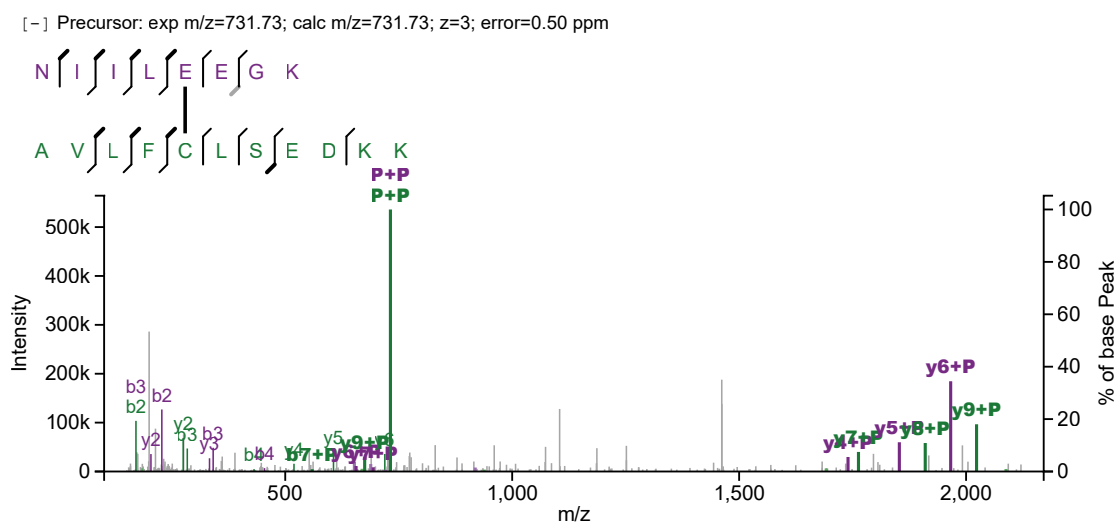


Figure E.106: Representative MS²-spectra of detected crosslinks between Cys and Glu.

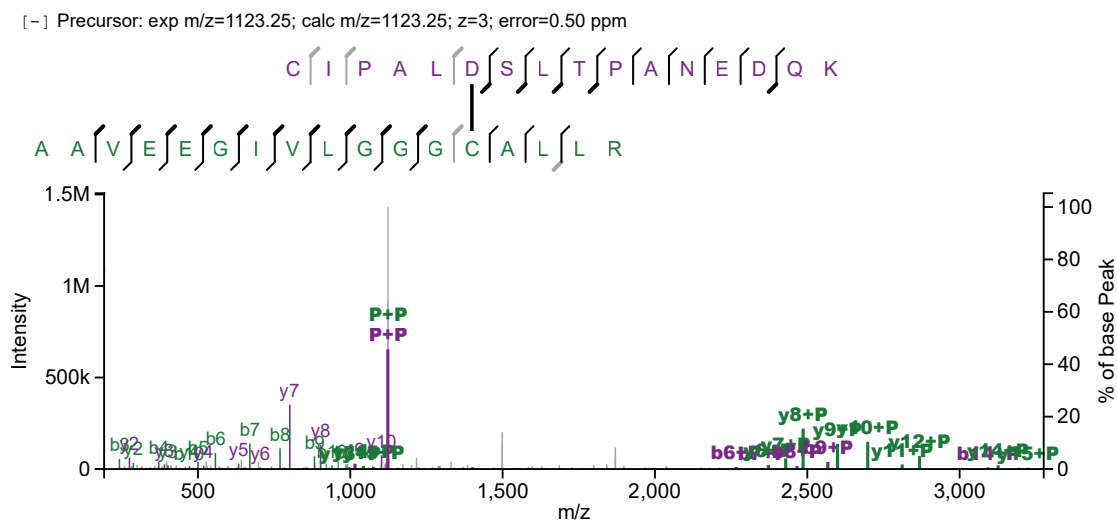


Figure E.107: Representative MS²-spectra of detected crosslinks between Cys and Asp.

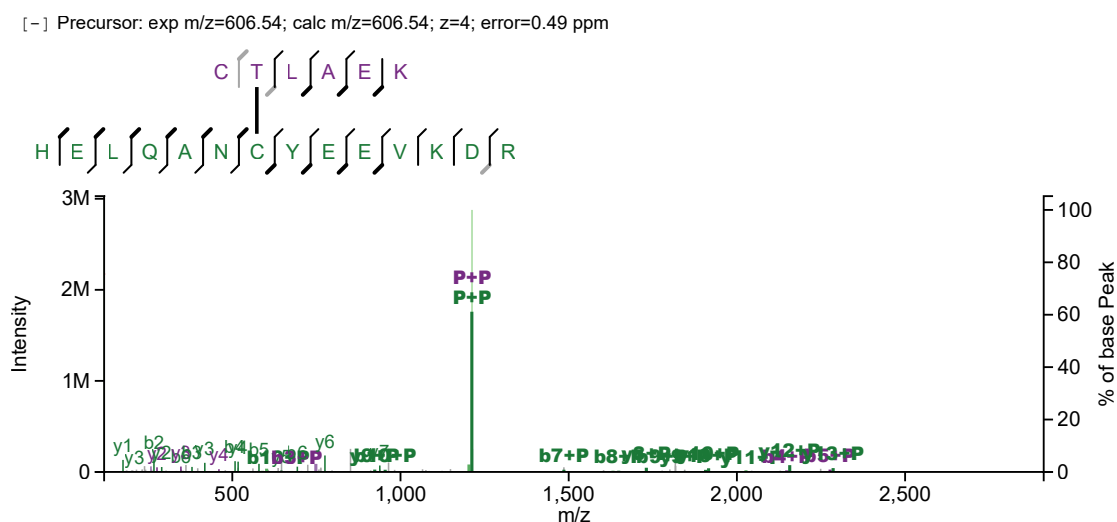


Figure E.108: Representative MS²-spectra of detected crosslinks between Cys and Thr.

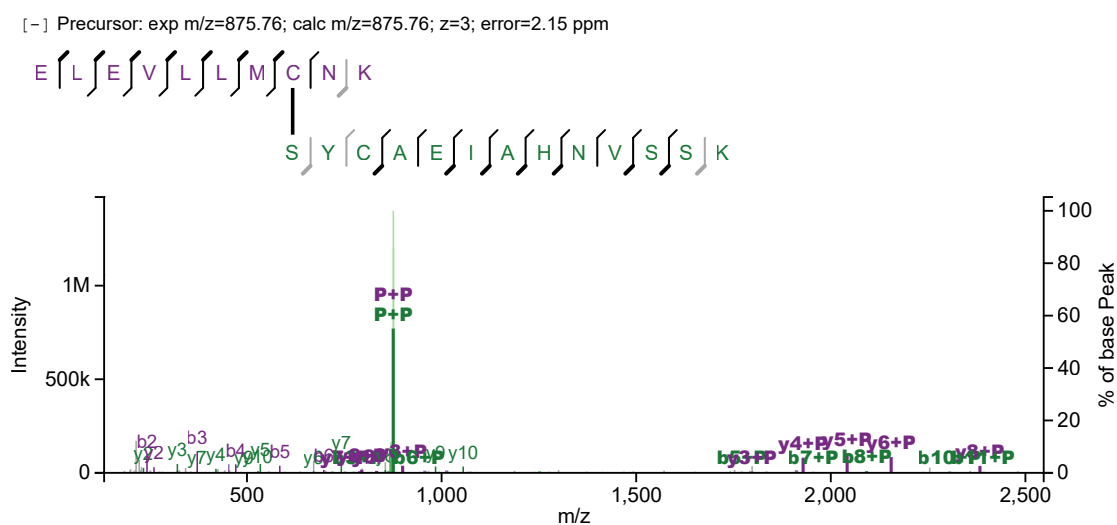
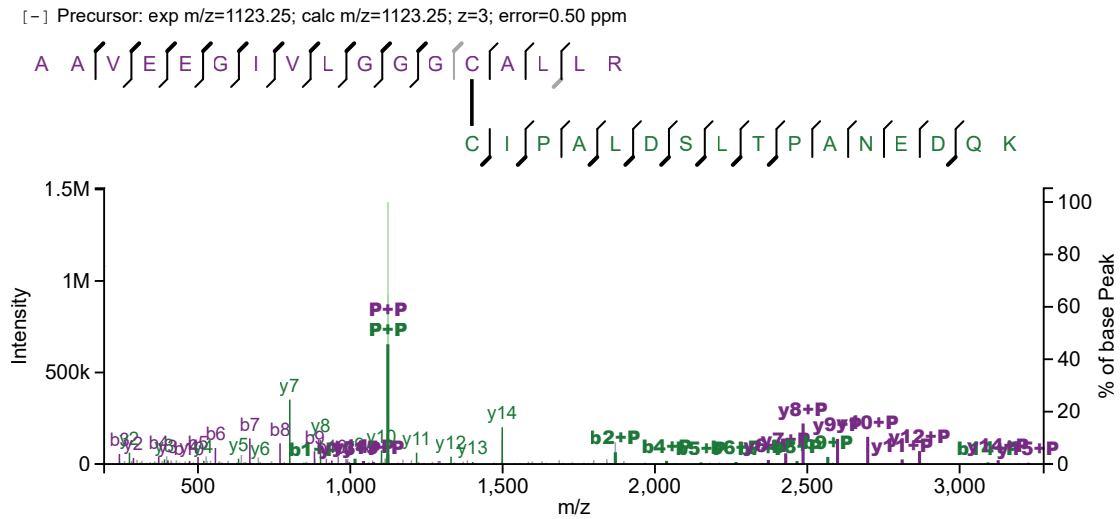


Figure E.109: Representative MS²-spectra of detected crosslinks between Cys and Ser.



Crosslink distance distribution

For the analysis of the intra-protein crosslink distances, the crosslink pairs that were identified in K562 samples were filtered to obtain the list of the crosslinks that were detected in at least two biological replicates. Proteins that are known to form homooligomers were excluded from the analysis. High resolution X-ray structures with full sequence coverage were used to obtain the C α –C α distances for the detected crosslinks. In cases where no such structure was available, AlphaFold-predicted protein structures with predicted local distance difference test (pLDDT) score ≥ 80 were used for crosslink distance mapping.

The assignment of the secondary structure for the crosslinked residues was performed using PyMOL. The obtained assignments are listed below as H (helix), S (sheet), and L (loop / coil):

Protein	Seq. Pos. 1	Seq. Pos. 2	Residue 1 name	Residue 2 name	Distance (Å)	Second. struct. 1	Second. struct. 2
O00299	178	233	CYS	TYR	21.5	H	H
O00299	24	59	CYS	CYS	18.3	L	L
O00299	27	59	SER	CYS	15.9	H	L
O00299	177	223	ASP	CYS	13.6	H	L
O00299	178	221	CYS	SER	13	H	H
O00299	178	223	CYS	CYS	11.5	H	L
O00299	178	222	CYS	THR	10.6	H	H
O00299	53	59	GLU	CYS	10.1	H	L
O00299	178	218	CYS	GLU	9.3	H	H
P00558	98	108	ASP	CYS	12	L	H
P00558	99	108	CYS	CYS	10.3	L	H
P00558	103	108	GLU	CYS	8.8	H	H
P07355	262	272	CYS	ASP	11.1	H	H
P07355	262	269	CYS	TYR	5.9	H	H
P08708	27	35	ASP	CYS	12.3	L	H
P08708	29	35	HIS	CYS	10	H	H
P08708	30	35	THR	CYS	8.7	H	H
P13639	388	462	CYS	GLU	19.6	L	L
P13639	370	388	GLU	CYS	13.5	H	L
P13639	373	388	TYR	CYS	13.5	L	L
P13639	369	389	CYS	ASP	12.6	H	L
P13639	369	388	CYS	CYS	9.8	H	L
P13639	387	466	SER	CYS	9.8	H	L
P13639	369	387	CYS	SER	9.5	H	H
P13639	388	466	CYS	CYS	6	L	L
P15880	176	188	LYS	CYS	30.7	L	S
P15880	178	229	HIS	CYS	28.9	L	L
P15880	178	188	HIS	CYS	25.6	L	S
P15880	221	229	ASP	CYS	24.5	L	L
P15880	182	229	CYS	CYS	24.5	L	L

P15880	182	230	CYS	THR	22.8	L	L
P15880	182	190	CYS	SER	22.6	L	L
P15880	179	188	THR	CYS	22.5	L	S
P15880	222	229	CYS	CYS	21.1	L	L
P15880	182	188	CYS	CYS	19.7	L	S
P15880	223	229	TYR	CYS	18.9	S	L
P15880	222	230	CYS	THR	18.2	L	L
P15880	225	229	SER	CYS	12.7	S	L
P15880	188	230	CYS	THR	10.5	S	L
P15880	188	229	CYS	CYS	10.3	S	L
P15880	190	229	SER	CYS	6.4	L	L
P17987	131	147	TYR	CYS	21.9	H	H
P17987	147	155	CYS	SER	12.5	H	H
P17987	68	76	GLU	CYS	11.1	L	H
P17987	69	76	HIS	CYS	10.8	L	H
P17987	147	154	CYS	THR	10.5	H	H
P17987	141	147	ASP	CYS	8.7	H	H
P17987	142	147	GLU	CYS	8.6	H	H
P17987	140	147	THR	CYS	6.4	H	H
P18077	37	47	ASP	CYS	19.2	H	S
P18077	40	47	GLU	CYS	14.8	H	S
P18077	39	47	THR	CYS	13.9	H	S
P18077	42	47	TYR	CYS	9.7	H	S
P21964	83	94	CYS	ASP	9.9	H	H
P21964	83	87	CYS	GLU	5	H	L
P23396	134	160	CYS	SER	15.4	S	L
P23396	134	159	CYS	HIS	11.9	S	L
P23396	129	134	SER	CYS	11	H	S
P23396	128	134	GLU	CYS	10.9	H	S
P23396	134	170	CYS	THR	9.9	S	S
P23396	134	154	CYS	ASP	5.5	S	S
P23528	39	66	CYS	ASP	23	S	L
P23528	39	68	CYS	TYR	18.7	S	H
P23528	39	70	CYS	THR	17.8	S	H
P23528	139	147	CYS	CYS	15.4	L	H
P23528	133	147	HIS	CYS	15	S	H
P23528	140	147	TYR	CYS	14	H	H
P23528	139	148	CYS	THR	13.4	L	H
P23528	141	147	GLU	CYS	11.5	H	H
P23528	144	147	LYS	CYS	8.8	H	H
P23528	39	50	CYS	GLU	5.7	S	L
P23528	145	147	ASP	CYS	5.5	H	H
P31948	26	62	CYS	CYS	19.9	H	H

P31948	458	471	ASP	CYS	18.5	L	H
P31948	460	471	SER	CYS	18	L	H
P31948	26	59	CYS	GLU	17.8	H	H
P31948	27	62	TYR	CYS	16.2	H	H
P31948	28	62	SER	CYS	16	H	H
P31948	461	471	CYS	CYS	15.6	L	H
P31948	26	60	CYS	ASP	14.8	H	H
P31948	329	339	GLU	CYS	14.4	H	H
P31948	330	339	HIS	CYS	13.6	L	H
P31948	326	339	SER	CYS	10	H	H
P36578	245	250	HIS	CYS	14.1	L	S
P39023	313	336	SER	CYS	20.5	L	L
P39023	54	336	THR	CYS	9.9	S	L
P40939	322	333	CYS	THR	16.8	H	L
P40939	322	329	CYS	GLU	10.5	H	H
P43487	132	136	CYS	GLU	11.8	L	S
P46776	68	144	SER	CYS	22.3	H	S
P46776	70	144	CYS	CYS	17.4	L	S
P46776	76	144	ASP	CYS	15.5	H	S
P46776	72	144	THR	CYS	15.1	S	S
P49368	366	373	CYS	THR	11.8	L	S
P49368	367	372	LYS	CYS	11.8	L	S
P49368	365	372	ASP	CYS	10.4	L	S
P49368	366	372	CYS	CYS	9.4	L	S
P49368	364	372	THR	CYS	9	S	S
P50990	244	261	CYS	THR	32.4	L	L
P50990	244	263	CYS	GLU	31.5	L	H
P50991	252	267	CYS	SER	28.6	L	L
P50991	252	269	CYS	TYR	27.7	L	H
P50991	252	261	CYS	ASP	18.2	L	L
P50991	252	258	CYS	THR	13.4	L	L
P55769	93	103	CYS	SER	10.1	H	S
P55769	93	102	CYS	CYS	8.2	H	S
P60520	7	15	GLU	CYS	14	H	H
P60520	8	15	ASP	CYS	13.4	H	H
P60520	9	15	HIS	CYS	9.9	L	H
P60520	10	15	SER	CYS	8.6	L	H
P60981	133	147	HIS	CYS	12.7	S	H
P60981	134	147	GLU	CYS	12.3	S	H
P60981	135	147	CYS	CYS	9.5	S	H
P61247	89	96	GLU	CYS	7.7	S	S
P61247	90	96	ASP	CYS	6.1	S	S
P62241	160	174	SER	CYS	19.2	H	S

P62241	164	174	GLU	CYS	17.9	H	S
P62280	116	130	CYS	GLU	17.9	L	S
P62280	116	131	CYS	CYS	17.3	L	L
P62280	116	124	CYS	ASP	15.5	L	S
P62280	116	127	CYS	THR	14.3	L	S
P62280	114	131	SER	CYS	13.1	L	L
P62280	112	131	HIS	CYS	9.8	S	L
P62495	115	127	GLU	CYS	20.3	L	S
P62495	113	127	ASP	CYS	15.9	S	S
P62753	12	19	CYS	ASP	22.9	S	L
P62753	12	17	CYS	GLU	17	S	S
P62857	27	36	CYS	ASP	25.5	S	L
P62857	27	38	CYS	THR	24.3	S	L
P62857	27	33	CYS	GLU	18.7	S	S
P62888	85	107	CYS	SER	21.9	H	L
P62888	85	98	CYS	ASP	14.6	H	S
P62888	52	62	CYS	TYR	14.5	L	H
P62888	75	92	SER	CYS	11.5	L	L
P62888	52	59	CYS	GLU	11.1	L	H
P62888	85	92	CYS	CYS	11	H	L
P62888	85	93	CYS	THR	10.8	H	S
P62888	73	92	HIS	CYS	10.6	S	L
P62888	80	92	GLU	CYS	9.5	H	L
P62888	74	92	TYR	CYS	9.5	S	L
P62888	52	58	CYS	SER	9.5	L	H
P62888	52	93	CYS	THR	7.6	L	S
P62888	52	92	CYS	CYS	5.7	L	L
P62910	91	101	CYS	HIS	22.7	L	L
P62910	91	98	CYS	GLU	14.4	L	S
P62910	91	96	CYS	CYS	8.8	L	S
P62910	91	95	CYS	TYR	6	L	S
P62910	91	94	CYS	SER	5.4	L	H
P62913	72	80	CYS	GLU	14.4	S	H
P62987	91	115	CYS	CYS	22.3	H	L
P62987	91	117	CYS	HIS	21.9	H	L
P62987	92	115	ASP	CYS	21.5	L	L
P62987	91	118	CYS	THR	21.1	H	L
P63220	18	56	SER	CYS	8.4	L	L
P63220	17	56	CYS	CYS	5.4	L	L
P83731	11	36	TYR	CYS	10.2	S	H
P83731	6	37	CYS	GLU	9.7	S	H
P83731	7	36	SER	CYS	8.4	L	H
P83731	6	36	CYS	CYS	8	S	H

Q02543	88	109	SER	CYS	20.9	L	H
Q02543	90	109	THR	CYS	17.1	S	H
Q02543	91	109	HIS	CYS	15.2	S	H
Q13347	76	82	CYS	GLU	18.7	S	L
Q13347	75	81	SER	CYS	18.2	S	L
Q13347	76	83	CYS	THR	18.2	S	L
Q13347	76	81	CYS	CYS	15.2	S	L
Q13347	76	80	CYS	ASP	13.8	S	S
Q15185	40	57	CYS	HIS	24.7	S	L
Q15185	40	58	CYS	CYS	23.9	S	L
Q15185	40	60	CYS	ASP	22.6	S	L
Q15185	39	58	SER	CYS	20.6	S	L
Q15185	37	58	THR	CYS	14.1	S	L
Q15185	40	52	CYS	GLU	12.4	S	S
Q16658	57	89	SER	CYS	13.4	L	H
Q16658	61	88	CYS	ASP	9.4	S	H
Q16658	61	89	CYS	CYS	5.7	S	H
Q8N1F7	391	397	TYR	CYS	7.7	H	L
Q8N1F7	392	398	CYS	ASP	6.3	H	L
Q8N1F7	392	397	CYS	CYS	4.9	H	L
Q99832	370	385	CYS	THR	16.9	S	H
Q99832	370	386	CYS	GLU	14.4	S	H
Q99832	349	364	GLU	CYS	13.1	S	L
Q99832	345	362	CYS	THR	6.2	L	S
Q99832	345	364	CYS	CYS	4.5	L	L
Q9BZZ5	234	241	CYS	CYS	10.7	H	H
Q9BZZ5	236	241	ASP	CYS	8.4	H	H
Q9NTK5	68	75	ASP	CYS	10.9	L	H
Q9NTK5	69	75	GLU	CYS	9.7	H	H
Q9UIA9	42	47	ASP	CYS	8.6	L	H
Q9UIA9	43	47	CYS	CYS	6	H	H
Q9UIA9	45	47	SER	CYS	5.5	H	H
Q9UQ80	49	58	CYS	GLU	14.3	H	H
Q9UQ80	49	53	CYS	ASP	6.2	H	H
Q9Y2R5	96	100	THR	CYS	9.8	L	L
Q9Y2R5	93	100	ASP	CYS	6.3	L	L
Q9Y696	234	246	CYS	ASP	20.8	L	H
Q9Y696	234	245	CYS	SER	18.2	L	H
Q9Y696	234	244	CYS	TYR	15.5	L	H
Q9Y696	234	239	CYS	GLU	9.9	L	H

***In cellulo* crosslinked GrxC5**

Raw data were converted to mgf format with MSConvert 3.0.24²¹³. Each of the peak files was searched separately against the protein FASTA sequence using xiSEARCH 1.8.7⁹³ with the following settings: MS¹ tolerance: 6 ppm; MS² tolerance: 20 ppm, allowing up to two missing monoisotopic peaks and three missed tryptic cleavages. Cysteine carbamidomethylation and oxidation of methionine were defined as variable modifications. $-H_2O/-NH_3$ were defined as losses.

The **VTT** crosslinker was defined as follows: the linkage mass was set to 26.01565 Da and the specificity was set to C and one of the following residues: C, H, K, E, D, S, T, R, Y, C-terminus, N-terminus. Variable modifications to account for the hydrolysis of episulfonium with water (18.0105647 Da), or formation of a crosslink within a peptide (26.01565 Da) were also defined. Non-covalent crosslink with a mass of zero was included in all searches to flag spectra potentially arising from gas-phase associated peptides²¹⁴.

The matches obtained for the non-covalent crosslink were removed prior to the FDR estimation, which was performed using xiFDR 2.3.5⁹³. The input file was filtered to obtain peptide pairs with at least two matched fragment ions in each crosslinked peptide. The ambiguity on the residue pair level was set to 1 and the results were filtered on residue pair level (1%) with the boosting feature enabled.

The obtained files were uploaded to xiView.org²¹⁵ and the crosslink pairs found in at least two of the three biological replicates were used as an input for protein structure prediction with AlphaLink2⁸⁹ using ColabFold with the following parameters: model weights: 2.2 (trained on 10 Å C α -C α); use templates: True; msa mode: MMseqs2; max recycling iters: 3; num ensembles: 1; manual seed: 42; times: 1. The obtained structure with the detected crosslinks was visualized using ChimeraX 1.9¹²¹.

TTD-mediated crosslinking

Raw data were processed using MSConvert 3.0.24²¹³ to convert the raw files to mzML format for the subsequent mass recalibration¹⁵⁵. An open modification search with MSFragger 4.1²¹¹ was then performed to recalibrate precursor masses, and the obtained files were converted to mgf format with MSConvert 3.0.24²¹³. The mgf spectra were split for parallel database search on the HPC system Raven at the Max Planck Computing and Data Facility.

Each of the peakfiles was searched separately against the *H. sapiens* proteome (UP000005640) using xiSEARCH 1.8.7⁹³ with the following settings: MS¹ tolerance: 3 ppm; MS² tolerance: 5 ppm, allowing up to two missing monoisotopic peaks and three missed tryptic cleavages. Cysteine carbamidomethylation and oxidation of methionine were defined as variable modifications. $-H_2O/-NH_3$ were defined as losses.

The **TTD** crosslinker was defined as follows: the linkage mass was set to 450.204926 Da and the specificity was set to C and one of the following residues: C, H, K, E, D, S, T, R, Y, C-terminus, N-terminus. The crosslink search for each of the considered crosslink combinations was performed separately. Variable modifications to account for the hydrolysis of episulfonium with water (18.0105647 Da), or formation of a crosslink within a peptide (450.204926 Da) were also defined. Non-covalent crosslink with a mass of zero was included in all searches to flag spectra potentially arising from gas-phase associated peptides²¹⁴.

The output files obtained for all the crosslink combinations and biological replicates were combined together. The matches obtained for the non-covalent crosslink were removed prior to false-discovery-rate estimation, which was performed using xiFDR 2.3.5⁹³. FDR estimation was performed separately for the intramolecular and intermolecular crosslinks. In each case, the input file was filtered to obtain peptide pairs with at least three matched fragment ions in each crosslinked peptide. For the intramolecular crosslinks, the ambiguity on the residue pair and protein group levels was set to 1, and the results were filtered on residue pair level (1%) with the boosting feature enabled. For the intermolecular crosslinks, the ambiguity on the residue pair level was set to 1, and the results were filtered on residue pair level (1%) and protein pair level (1%) with the boosting feature enabled on the residue pair level. The obtained files were uploaded to xiView.org²¹⁵ and the list of the non-ambiguous crosslinks was used for the further analysis.

PPIs identified via TTD-mediated crosslinking and enrichment

Protein 1	Protein 2	# Unique Distance Restraints	STRING conf. score (functional interaction)	STRING conf. score (physical interaction)
P12956	P78527	6	0.999	0.999
Q15631	Q99598	3	0.999	0.999
P63244	Q8NC51	6	0.998	0.957
P17987	P49368	4	0.999	0.999
P48643	P78371	4	0.999	0.999
P49321	P84243	10	0.949	0.919
Q13619	Q86VP6	4	0.988	0.735
P62888	Q8NC51	6	0.987	0.915
Q8N5N7	Q9BYD3	6	0.989	0.96
Q15003	Q15021	3	0.999	0.999
P48643	Q99832	4	0.999	0.999
O43815	Q9Y3A3	6	0.999	0.98
Q13033	Q9Y3A3	6	0.999	0.98
P45880	Q9Y277	4	0.997	0.995
Q16555	Q9BPU6	2	0.99	0.965
P46821	Q15388	1	0.369	-
P63244	Q00341	7	0.774	0.508
Q6ZN66	Q99497	3	n.a.	n.a.
P63244	Q9UKY7	5	n.a.	n.a.
P07099	P10746	3	n.a.	n.a.
P40926	Q7RTU4	3	n.a.	n.a.
O43175	Q8N8E3	4	n.a.	n.a.

Calculated properties of crosslinking reagents

The author performed the data analysis.

Commonly used crosslinking reagents

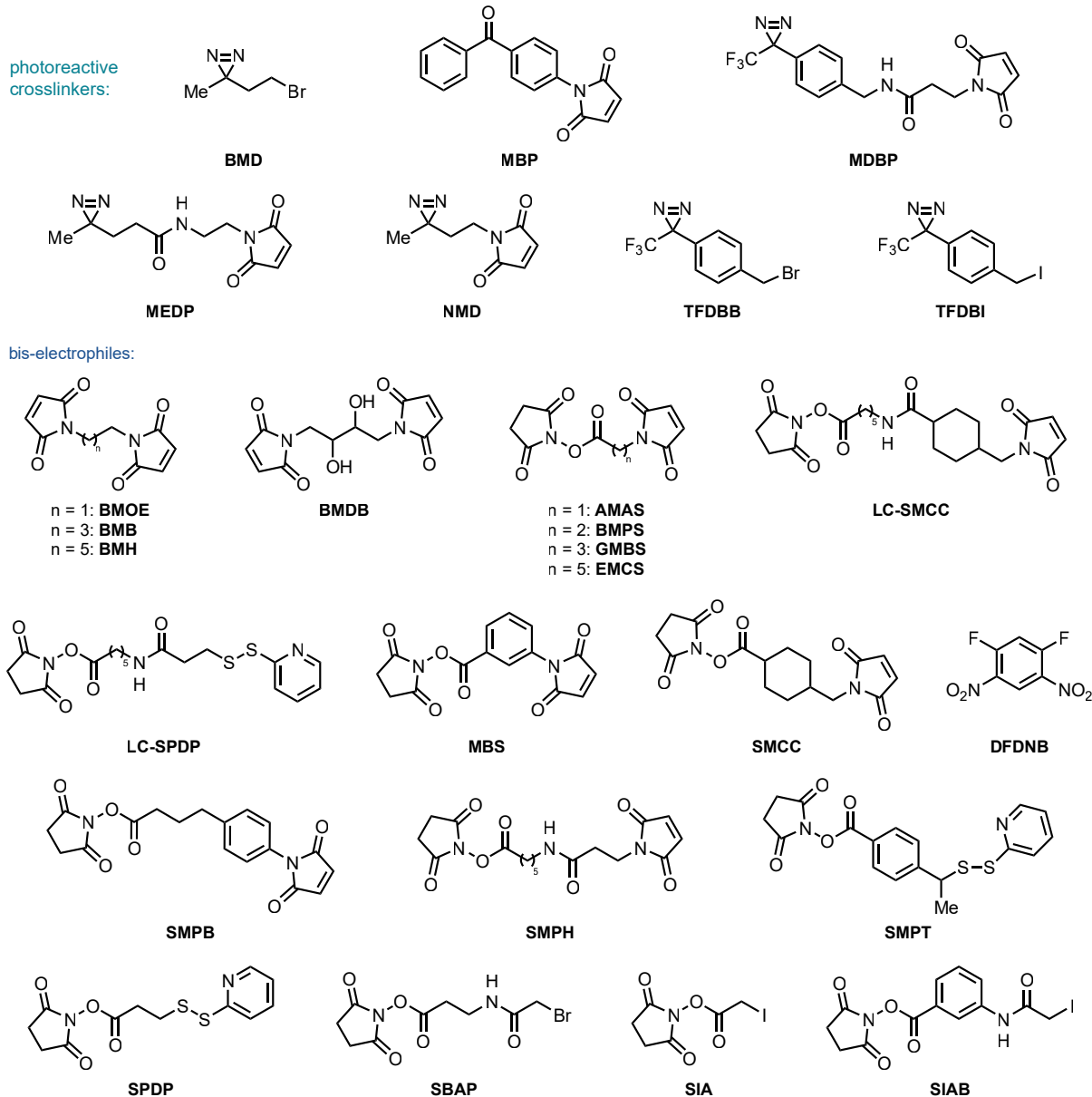


Figure E.115: Structures of the Cys-reactive crosslinking reagents used for the comparison with **VTT**. Only commercially available reagents were considered for the analysis. Reagents with PEG linkers or sulfo-derivatives were not included due to the low cell permeability of these compounds.

Calculated logP and TPSA values

The TPSA and XlogP3 values for **VTT** and other Cys-selective crosslinking reagents were calculated using SwissADME²¹⁶. The listed linker distances were previously reported²¹⁷ or calculated using Avogadro²¹⁸.

Reagent	Full name	Spacer arm (Å)	TPSA, (Å ²)	Log P
BMD	3-(2-Bromoethyl)-3-methyl-3H-diazirine	2.6	24.72	1.63
MBP	4-(N-Maleimido)-benzophenone	8.1	51.21	3
MDBP	3-(Maleimido)-N-(4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzyl)-propanamide	12.3	91.2	1.22
MEDP	N-(2-(Maleimido)ethyl)-3-(3-methyl-3H-diazirin-3-yl)propanamide	10.8	91.2	-0.63
NMD	N-maleimido-diazirine	6	62.1	0.12
TFDBB	4-[3-(Trifluoromethyl)-3H-diazirin-3-yl]benzyl Bromide	5.9	24.72	3.48
TFDBI	4-[3-(Trifluoromethyl)-3H-diazirin-3-yl]benzyl Iodide	5.9	24.72	3.93
AMAS	N- α -(Maleimidoacetoxy)-succinimide ester	4.4	101.06	-1.48
BMB	1,4-Bis-Maleimidobutane	10.9	74.76	-0.55
BMDB	1,4-Bis-Maleimidyl-2,3-dihydroxy-butane	10.2	115.22	-2.5
BMH	Bis-Maleimidohexane	16.1	74.76	0.16
BMOE	Bis-Maleimidoethane	8	74.76	-1.26
BMPS	N-(β -Maleimidopropoxy)succinimide ester	5.9	101.06	-1.58
DFDNB	1,5-Difluoro-2,4-dinitrobenzene	3	91.64	1.78
EMCS	N-(ϵ -Maleimidocaproyloxy)succinimide ester	11.8	101.06	-0.51
GMBS	N-(γ -Maleimidobutyryloxy)succinimide ester	7.3	101.06	-1.22
LC-SMCC	Succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxy-(6-amidocaproate)	16.2	130.16	0.23
LC-SPDP	Succinimidyl 6-(3'-[2-pyridyl-dithio]-propionamido)hexanoate	15.7	156.27	0.99
MBS	m-Maleimidobenzoyl-N-hydroxysuccinimide ester	7.3	101.06	0.01
SBAP	Succinimidyl 3-(bromoacetamido)-propionate	6.2	92.78	-0.73
SIA	N-succinimidyl iodoacetate	1.5	63.68	-0.09
SIAB	N-Succinimidyl-(4-iodoacetyl)-aminobenzoate	10.6	92.78	0.75
SMCC	Succinimidyl 4-(N-maleimido-methyl)-cyclohexane-1-carboxylate	8.3	101.06	-0.09

SMPB	Succinimidyl 4-(p-maleimido-phenyl)- butyrate	11.6	101.06	0.59
SMPH	Succinimidyl 6-(β -maleimidopropionamido)- hexanoate	14.2	130.16	-1.26
SMPT	4-Succinimidylloxycarbonyl- methyl- α -(2-pyridyldithio)toluene	20	127.17	2.59
SPDP	N-Succinimidyl 3-(2-pyridyldithio)- propionate	6.8	127.17	0.67
VTT	Vinylthianthrenium tetrafluoroborate	1.5	50.6	4.64

TTD crosslinker

Calculated logP and TPSA values

The TPSA and logP values for **VTT** and **TTD**-derived alkenyl thianthrenium salts were calculated using SwissADME²¹⁶.

Molecule	TPSA	XLOGP3	WLOGP	MLOGP	Silicos-IT Log P	Consensus Log P
VTT	50.6	4.64	4.33	5.01	3.33	3.46
ATT-E	59.8	4.28	4.43	4.85	3.97	3.51
ATT-Z	59.8	4.28	4.43	4.85	3.97	3.51
ATT-T	59.8	4.42	4.43	4.85	3.98	3.54

Avogadro outputs

Geometry optimization was performed using the following settings: Force field: MMFF94; Number of steps: 25,000.

TFDBB and **TFDBI**-derived crosslinks. Distance between C7 and C8: 5.9 Å

C -1.00159 2.68253 -0.42266
C -0.62351 4.00200 -0.13536
C -0.04230 1.81543 -0.96607
C 0.67709 4.44429 -0.39183
C 1.25800 2.25832 -1.22252
C 1.62917 3.57994 -0.94614
H -1.33310 4.70516 0.29699
H -0.29348 0.78113 -1.19259
H 1.98426 1.56061 -1.63444
H 0.94464 5.47069 -0.14890
C 3.03380 4.05556 -1.21513
C -2.40843 2.17423 -0.14559
C -2.71602 2.20309 1.35485
H -1.96068 1.64610 1.92057
H -3.69001 1.74539 1.55882
H -2.74007 3.22531 1.74794
C -3.48668 2.91571 -0.93693
F -3.60628 4.20935 -0.56482
F -4.69636 2.33317 -0.74654
F -3.24317 2.88571 -2.26660
H -2.45842 1.12294 -0.45940
S 4.12203 3.77290 0.21422
H 3.02201 5.12461 -1.45591
H 3.43883 3.53235 -2.08875
C 5.64842 4.45175 -0.47504
H 6.44769 4.36634 0.26591
H 5.51749 5.50800 -0.72413
H 5.94314 3.89610 -1.36906

MBP-derived crosslink. Distance between C7 and C17: 8.1 Å

C -0.87668 2.88839 -0.11585
C -0.50924 4.16599 0.33422
C 0.13652 2.12152 -0.71003
C 0.79980 4.65425 0.19588
C 1.44645 2.60638 -0.85223
C 1.80477 3.88156 -0.39554
H -1.21516 4.82845 0.82681
H -0.04721 1.12556 -1.10146
H 2.18682 1.96261 -1.32488

H 1.02145 5.65566 0.56228
C 3.23480 4.40948 -0.57066
C 3.41065 4.83331 -2.03961
H 3.30705 3.99266 -2.73477
H 4.38983 5.29975 -2.20263
H 2.66603 5.58930 -2.31982
O 3.39807 5.62425 0.18540
H 4.33122 5.66802 0.45476
C 4.29200 3.42412 -0.05188
C 5.27964 2.85291 -0.87126
C 4.28703 3.07142 1.31374
C 5.22930 2.18422 1.83667
C 6.19821 1.63129 1.00569
C 6.22333 1.96474 -0.34586
H 5.33637 3.08059 -1.93242
H 3.53271 3.49053 1.97937
H 5.20211 1.92362 2.89157
H 6.93130 0.93831 1.41002
H 6.97902 1.53030 -0.99590
C -6.45098 -0.31594 0.58729
S -4.66628 -0.45741 0.84919
H -6.96865 -0.73027 1.42708
H -6.72479 -0.84920 -0.29903
H -6.71493 0.71532 0.47894
C -4.48326 2.29653 0.63157
C -4.11610 1.01496 -0.07288
O -1.92119 0.12529 -0.51084
C -2.60977 1.08820 -0.19637
H -4.59632 0.91852 -1.02419
O -3.38199 4.39084 0.50075
C -3.29780 3.17531 0.38433
H -4.62626 2.13108 1.67898
H -5.40032 2.72067 0.27940
N -2.21589 2.39565 0.03351

BMD-derived crosslink. Distance between C2 and C5: 2.6 Å

C -2.04756 -0.21479 1.63221
C -0.64957 -0.83007 1.73467
H -2.19179 0.57149 2.38108
H -2.82020 -0.97558 1.78614
H -2.20777 0.22563 0.64240
C 0.41760 0.24819 1.46063
C -0.47596 -1.48975 3.10358
H -0.56852 -1.60865 0.96553
H -1.28676 -2.20039 3.29754

H -0.48062 -0.74506 3.90665
H 0.46302 -2.04836 3.16093
C 1.83777 -0.31597 1.40186
H 0.35702 1.02575 2.23364
H 0.18067 0.73154 0.50325
S 3.01043 0.99777 0.93356
H 1.89438 -1.12075 0.66195
H 2.13852 -0.71005 2.37668
C 4.53148 0.01969 0.94978
H 5.37530 0.66145 0.68291
H 4.46922 -0.79318 0.22163
H 4.70927 -0.39134 1.94690

MEDP-derived crosslink. Distance between C2 and C11: 10.8 Å

C -2.05883 -0.17403 1.65572
C -0.67609 -0.82689 1.72434
H -2.18144 0.58133 2.43944
H -2.84929 -0.92203 1.77888
H -2.21142 0.31384 0.68716
C 0.41511 0.23519 1.49798
C -0.51410 -1.55241 3.06091
H -0.61617 -1.57089 0.91994
H -1.33894 -2.25464 3.22378
H -0.50273 -0.84556 3.89747
H 0.41366 -2.13154 3.09048
C 1.82288 -0.35274 1.39108
H 0.38839 0.97664 2.30715
H 0.19061 0.78306 0.57315
C 2.85990 0.70115 1.04480
H 1.84399 -1.12469 0.61304
H 2.12115 -0.80901 2.34107
O 2.60967 1.89502 0.93338
N 4.12608 0.17056 0.88633
C 5.22393 1.00887 0.43503
H 4.18886 -0.82683 0.72376
C 6.58986 0.40307 0.76599
H 5.13126 2.00341 0.88691
H 5.10770 1.13463 -0.64772
N 7.67946 1.19773 0.23097
H 6.65792 -0.60666 0.34375
H 6.70418 0.31687 1.85268
C 8.12959 1.08902 -1.04983
C 8.31934 2.17968 0.93200
C 9.29030 2.88242 0.00709
C 9.29196 2.01600 -1.24526

H 10.20362 1.41042 -1.29349
H 9.18167 2.57064 -2.18104
O 8.13807 2.45377 2.10973
O 7.70397 0.32562 -1.90369
S 8.69647 4.58055 -0.32132
H 10.27828 2.91122 0.47731
C 10.18514 5.26241 -1.09132
H 10.01163 6.31330 -1.33776
H 10.42388 4.72646 -2.01315
H 11.03100 5.20392 -0.40144

MDBP-derived crosslink. Distance between C8 and C17: 12.3 Å

C 0.17593 2.90383 -0.53111
C 0.60757 4.09483 0.07077
C 0.81750 2.47555 -1.70287
C 1.64862 4.84060 -0.48811
C 1.85801 3.22217 -2.26174
C 2.27617 4.41745 -1.66541
H 0.13939 4.46179 0.98267
H 0.51330 1.55484 -2.19740
H 2.33879 2.86538 -3.17070
H 1.96665 5.75948 0.00142
C 3.41540 5.20736 -2.24928
C -0.95637 2.07423 0.05673
C -0.58839 1.55100 1.44882
H 0.35611 0.99604 1.42200
H -1.36276 0.87335 1.82423
H -0.47716 2.36335 2.17523
C -2.29422 2.81493 0.08954
F -2.28131 3.86877 0.93523
F -3.28313 1.98428 0.50234
F -2.64722 3.26660 -1.13466
H -1.10139 1.19411 -0.58396
N 4.67306 4.79497 -1.67303
H 3.27634 6.28095 -2.07464
H 3.46978 5.06736 -3.33550
C 5.84268 5.44283 -1.99393
H 4.70188 3.92752 -1.15259
O 5.88884 6.42447 -2.72637
C 7.05967 4.82517 -1.34595
C 8.33076 5.55432 -1.76555
H 7.11369 3.76943 -1.63677
H 6.93832 4.86919 -0.25728
N 9.51086 4.95202 -1.16697
H 8.28212 6.60683 -1.46239

H 8.44205 5.52314 -2.85593
C 10.15223 3.86693 -1.69555
C 10.02893 5.33041 0.03540
C 11.27840 4.54373 0.29826
C 11.23057 3.42312 -0.73051
O 9.87896 3.31656 -2.75320
O 9.59183 6.19419 0.78025
H 11.30501 4.20454 1.33735
H 12.13563 5.20081 0.11459
S 10.75283 1.81865 0.01168
H 12.17934 3.32551 -1.26715
C 12.33466 1.37194 0.76886
H 12.23691 0.39690 1.25352
H 12.62253 2.10617 1.52513
H 13.11488 1.30272 0.00649

NMD-derived crosslink. Distance between C2 and C14: 6.0 Å

C -4.76959 -0.26244 0.41492
C -3.88612 0.31704 -0.69253
H -5.80680 -0.35717 0.07625
H -4.76077 0.37571 1.30523
H -4.42333 -1.25910 0.70828
C -2.42538 0.38502 -0.21247
C -1.44790 0.84812 -1.29664
H -2.11963 -0.60974 0.13807
H -2.34864 1.05065 0.65718
N -0.07274 0.76210 -0.83722
H -1.64708 1.88937 -1.57041
H -1.56073 0.22794 -2.19278
C 0.54260 1.73868 -0.11357
C 2.04957 -0.09778 -0.34350
C 1.95947 1.33456 0.16365
H 2.62209 1.99009 -0.41155
H 2.17771 1.45217 1.22899
O 0.05671 2.80503 0.23438
O 0.43154 -1.32469 -1.68518
C 0.72654 -0.32710 -1.04134
S 2.25495 -1.30833 1.01105
H 2.85754 -0.21201 -1.07349
C 4.00637 -1.03484 1.37507
H 4.31826 -1.71775 2.16977
H 4.17501 -0.00989 1.71441
H 4.61480 -1.23432 0.48908
C -4.42506 1.68251 -1.12122
H -3.94116 -0.35950 -1.55481

H -4.32862 2.41830 -0.31574
H -5.48447 1.61430 -1.39145
H -3.89267 2.06362 -1.99787

VTT-derived crosslink. Distance between C1 and C2: 1.5 Å

C 1.40116 0.38796 0.90559
C 2.82325 0.53934 1.43876
S 3.47802 2.18683 1.00250
H 3.48180 -0.21992 1.00326
H 2.83942 0.43952 2.52944
C 5.11431 2.03179 1.75803
H 5.67525 2.95581 1.59488
H 5.66661 1.20512 1.30374
H 5.02242 1.86649 2.83461
S 0.74640 -1.25954 1.34182
H 1.38500 0.48779 -0.18510
H 0.74260 1.14721 1.34109
C -0.88977 -1.10462 0.58601
H -1.45071 -2.02865 0.74915
H -0.79770 -0.93941 -0.49057
H -1.44218 -0.27793 1.04014

Gene ontology annotation

The author performed the GO annotation analysis.

VTT-mediated alkylation

GO annotations were obtained for the proteins that had modified peptides detected in at least two biological replicates. The annotations were exported from QuickGO²¹⁹ after restricting the search to Swiss-Prot entries in the canonical reference proteome. Only manually curated annotations backed by primary literature were considered for the analysis: direct or high-throughput experimental evidence (ECO:0000269 EXP, 0314 IDA, 0315 IMP, 0316 IGI, 0270 IEP, 0353 IPI, as well as the high-throughput counterparts 0007005 / 7001 / 7003 / 7007 / 6056), traceable author statements (ECO:0000304 TAS) and curator inferences (ECO:0000305 IC). Annotations based on sequence transfer or fully automatic pipelines (ECO:0000250, 0247, 0266, 0255, 0501) and entries lacking supporting data (ECO:0000303 NAS, 0000307 ND) were excluded, as well as all negation (NOT|...) qualifiers. The filtered GO terms were then mapped to the goslim_generic subset and QuickGO's "Use these terms as an exact match" option was used.

TTD-mediated crosslinking

GO annotations for the proteins in the obtained datasets were exported from QuickGO²¹⁹ after restricting the search to Swiss-Prot entries in the canonical reference proteome. Only manually curated annotations backed by primary literature were considered for the analysis: direct or high-throughput experimental evidence (ECO:0000269 EXP, 0314 IDA, 0315 IMP, 0316 IGI, 0270 IEP, 0353 IPI, as well as the high-throughput counterparts 0007005 / 7001 / 7003 / 7007 / 6056), traceable author statements (ECO:0000304 TAS) and curator inferences (ECO:0000305 IC). Annotations based on sequence transfer or fully automatic pipelines (ECO:0000250, 0247, 0266, 0255, 0501) and entries lacking supporting data (ECO:0000303 NAS, 0000307 ND) were excluded, as well as all negation (NOT|...) qualifiers.

The filtered GO terms were then mapped to the goslim_generic subset in two distinct steps, depending on the size of each protein set. For proteins detected in the previously unreported PPIs, QuickGO's "Use these terms as a GO slim" option with relationships restricted to "is_a," "part_of" and "occurs_in" was applied to preserve broader hierarchical context. For all proteins with at least two identified crosslinks (intra- or intermolecular crosslinks), QuickGO's "Use these terms as an exact match" option was used.

Docking studies

The author performed the docking studies. The obtained models were analyzed by the author and Dr. Philipp Hartmann.

General

Docking was carried out using the HADDOCK 2.4 web server¹⁶⁰ through the expert mode interface. PDB structures were used as input for the docking studies unless no structure was available or the regions containing the crosslinked residues in the reported structures were unresolved. The distance restraints were used as unambiguous restraints between the crosslinked residues as follows: 23 Å for **TTD** and 35 Å for **DSSO**. “Center of mass” restraints were implemented in all calculations. The first model of the top-scoring cluster was used for the analysis, unless structural variability within the cluster indicated a lack of convergence. If the mean HADDOCK score ($\pm$ SD) of the top-scoring cluster overlapped with the mean HADDOCK score ($\pm$ SD) of another cluster, the first models of both clusters were analyzed.

CCT5 and CCT7

Input coordinates for both CCT5 (P48643) and CCT7 (Q99832) were extracted from the PDB structure of the Human TRiC complex (7NVL). The docking was performed in water using standard sampling parameters (1000 structures for rigid body docking; 200 structures for semi-flexible refinement; 200 structures for the final refinement and the analysis).

VDAC2 and VDAC3

Input coordinates for VDAC2 (P45880) were extracted from the PDB structure 9EII. AlphaFold-predicted structure (#AF-Q9Y277-F1-v4, pLDDT score = 95.4) was used for VDAC3 (Q9Y277). The docking was performed in DMSO using modified sampling parameters (10,000 structures for rigid body docking; 500 structures for semi-flexible refinement; 500 structures for the final refinement and the analysis) and a C2 symmetry restraint was defined in all cases.

RACK1 and HDLBP

Input coordinates for RACK1 (P63244) were extracted from the PDB structure 4AOW. AlphaFold-predicted structure (#AF-Q00341-F1-v4, pLDDT score = 75.0) was used for HDLBP (Q00341). The docking was performed in water using modified sampling parameters (10,000 structures for rigid body docking; 500 structures for semi-flexible refinement; 500 structures for the final refinement and the analysis). Two top-scoring clusters (cluster 2 and cluster 3) with overlapping HADDOCK score values were obtained:

Calculated value	cluster 2	cluster 3
HADDOCK score	-117.3 ± 5.7	-108.5 ± 4.0
Cluster size	158	25
RMSD from the overall lowest-energy structure	14.3 ± 0.2	2.9 ± 0.6
Van der Waals energy	5.8 ± 4.5	-34.5 ± 5.0
Electrostatic energy	-736.0 ± 30.5	-419.6 ± 22.2
Desolvation energy	24.1 ± 3.8	9.9 ± 5.8
Restraints violation energy	0.0 ± 0.0	0.0 ± 0.0
Buried Surface Area	1459.2 ± 81.4	1898.4 ± 160.7
Z-Score	-1.7	-1.3

The top models in both clusters place RACK1 in a proximity to the KH-14 domain of Vigilin. The structure from cluster 3 was used for the analysis because of the negative E_{vdW} , lower RMSD and E_{desolv} values, and higher BSA value consistent with the formation of a stable complex.

CEP112 and PHGDH

Input coordinates for PHGDH (O43175) were extracted from the PDB structure 7DKM. AlphaFold-predicted structure (#AF-Q8N8E3-F1-v4, pLDDT score = 76.1) was used for CEP112 (Q8N8E3). The docking was performed in water using modified sampling parameters (10,000 structures for rigid body docking; 500 structures for semi-flexible refinement; 500 structures for the final refinement and the analysis). Estimation of the binding affinity in the complex was performed using the PRODIGY Web Server^{182,183}. The obtained output includes the predicted value of the binding affinity (ΔG), the calculated value of the dissociation constant (K_d), numbers of intermolecular contacts (ICs) at the interface within the threshold distance of 5.5 Å, separately listed according to the contact properties, and percentage of the charged and apolar non-interacting surface (NIS%) of the complex:

Property	Value
ΔG (kcal mol ⁻¹)	-8.8
K_d (M) at 25 °C	3.4×10^{-7}
ICs charged–charged	13
ICs charged–polar	8
ICs charged–apolar	20
ICs polar–polar	0
ICs polar–apolar	4
ICs apolar–apolar	8
NIS charged	35.1
NIS apolar	34.33

Data availability

VTT-mediated alkylation and crosslinking

The python scripts used for the data processing, raw data from biological assays, raw NMR data, and SDS-PAGE data can be accessed via Zenodo (zenodo.org/records/15382541).

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE²²⁰ partner repository with the dataset identifier PXD063857: ebi.ac.uk/pride/archive/projects/PXD063857.

The crosslinking mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE²²⁰ partner repository with the dataset identifier PXD063839 (10.6019/PXD063839): ebi.ac.uk/pride/archive/projects/PXD063839.

TTD-mediated alkylation and crosslinking

The following data has been deposited to Zenodo (zenodo.org/records/15991853):

- Raw NMR data;
- Raw protein LC–MS data and MS peak files;
- Raw SDS-PAGE data;
- HADDOCK input and output files;
- Python scripts used for data processing;
- QuickGO annotation tables;
- xiSEARCH output tables.

The crosslinking mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE²²⁰ partner repository with the dataset identifier PXD066251 (10.6019/PXD066251): ebi.ac.uk/pride/archive/projects/PXD066251.

Miscellaneous data

Additional data have been deposited to Zenodo and can be accessed using the [link](#)).

The following data are included:

- Raw LC–MS/MS data obtained from the following experiments:
 - Treatment of K562 cells and K562 lysates with 0.25 mM **VTT**;
 - Treatment of HEK cells with **TTD**, followed by LC–MS/MS sample preparation without enrichment;
- MaxQuant output files.
- MSFragger output from the open search with data from enriched samples (experiments with **TTD**).
- Cytotoxicity assay data obtained for **TTD**.
- Identified **TTD**-modified protein sites carrying **GSH** modification and the comparison table with the data from iCysMod.

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Appendix

Declaration of Authorship

I, *Konstantin Bohdan*

declare that this thesis and the work presented herein are my own and has been generated by me as the result of my own original research.

Hiermit erkläre ich an Eides statt / I do solemnly swear that:

1. This work was done wholly or mainly while in candidature for the doctoral degree program at this faculty and university;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this university or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others or myself, this is always clearly attributed;
4. Where I have quoted from the work of others or myself, the source is always given. This thesis is entirely my own work, with the exception of such quotations;
5. I have acknowledged all major sources of assistance;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. Parts of this work have been published before (see NOTE).

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List of publications

1. **Bohdan, K.***; Hartmann, P.*; Müller, S.; Marchionni, D.; Preisinger, C.; Jacobs, J. B.; Vogelsang, L.; Sterling, M. S.; Dietz, K.-J.; Ritter, T. Thianthrenium Chemistry for Identification of Protein–Protein Interactions in Cells. *Journal of the American Chemical Society* **2025**, *147* (49), 45576–45584.
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