Technische Universität München
Lehrstuhl für Chemie der Biopolymere

Design of cleavable linkers and applications in chemical proteomics

Yinliang Yang

Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

genehmigten Dissertation.

Vorsitzende:	Univ.-Prof. Dr. D. Langosch
Prüfer der Dissertation:	1. TUM Junior Fellow Dr. S. H. L. Verhelst
	2. Univ.-Prof. Dr. B. Küster

Die Dissertation wurde am 19. 12. 2013 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftzentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 17. 02. 2014 angenommen.

Table of contents

Abstract

Small molecule probes are useful tools to characterize protein targets and assign protein function. The development of bioorthogonal chemistry allows the application of tandem labeling for protein target identification *in situ*. The application of chemical probes is often combined with mass spectrometry analysis in order to identify the protein targets. During the workflow, immobilization and elution is a necessary step in the enrichment of target proteins. The biotin-streptavidin system is a widely applied strategy used for this purpose. Due to the strong interaction of biotin and streptavidin, harsh conditions are necessary in the elution process. The endogenously biotinylated proteins and non-specifically bound proteins will release simultaneously with the target proteins. Some strategies have been developed to solve these problems such as optimization of the washing steps, synthesis of different biotin analogs or mutation of streptavidin to reduce the binding affinity between biotin and streptavidin. Another promising tool to solve this problem is cleavable linker.

To develop a simple and efficient cleavable linker for activity based probes, a cleavable building block derived from tartaric acid was constructed and subsequently incorporated into an activity based probe as well as clickable biotin reagent for tandem labeling. Papain-like cysteine proteases were labeled and captured with streptavidin beads followed by chemoselective elution under a mild condition. The amount of background proteins in chemical elution was much lower compared with on-bead digestion. In addition, the linker is compatible with tandem labeling and target identification in living cells. More cathepsin targets were identified in living cells compare to cell extracts indicating that the activity of proteases may get lost while making cell extracts and emphasizes the necessity to use whole cells for target identification.

In addition, we designed and synthesized five different cleavable trifunctional biotin reagents containing diol, diazobenzene, hydrazone, disulfide and dde based cleavable linkers for labeling and purification of alkyne-probe modified proteins. All the reagents except the disulfide linker can selectively react with alkyne-E64 modified cathepsins. The labeled proteins were enriched with streptavidin beads and eluted with their respective elution conditions. The efficiency of immobilization and elution was profiled with fluorescent based SDS-PAGE assays.

Zusammenfassung

Kleine molekulare Sonden sind nützliche Werkzeuge um Zielproteine zu charakterisieren und ihnen eine Funktion zuzuordnen. Die Entwicklung der bioorthogonalen Chemie erlaubte die Anwendung von Tandem-Markierungsreaktionen für die Identifizierung von Zielproteinen *in situ*. Die Anwendung von chemischen Sonden wird oft mit einer massenspektrometrischen Analyse kombiniert, um Zielproteine zu identifizieren. Während des Arbeitsablaufes sind Immobilisation und Elution wichtige Schritte in der Anreicherung der Zielproteine. Das Biotin-streptavidin-system ist eine weit verbreitete Strategie, die für diesen Zweck verwendet wird. Aufgrund der starken Interaktion zwischen Biotin und Streptavidin sind stringente Bedingungen während der Elution nötig. Endogen-biotinylierte Proteine und unspezifisch gebundene Proteine werden dabei mit den Zielproteinen zusammen eluiert. Einige Verfahren wurden entwickelt um diese Probleme zu lösen, indem die Bindungsaffinität zwischen Biotin und Streptavidin reduziert wird, etwa durch Optimierung des Waschschrittes, der Synthese eines Biotin-Analogs oder einer Streptavidin-Mutante. Ein weiteres vielversprechendes Verfahren dieses Problem zu lösen ist die Verwendung eines spaltbaren Verbindungsstücks.

Um ein einfach und effizient spaltbares Verbindungsstück für aktivitätsbasierte Sonden zu entwickeln, wurde ein spaltbarer Baustein synthetisiert, der sich von 2,3-Dihydroxybutandisäure ableitet. Dieser Baustein wurde sowohl in eine Aktivitätsbasierte Sonde als auch in ein klickbares Biotin-Reagenz für Tandem-Markierungsversuche eingebaut. Papain-artige Cysteinproteasen wurden markiert und an Streptavidin-Agarose-Kügelchen immobilisiert, was dann chemoselektive Elution unter milden Bedingungen ermöglichte. Die Menge an Hintergrundproteinen waren bei der chemischen Elution viel geringer verglichen mit dem Verdau direkt am Agarose-Kügelchen. Zusätzlich ist das Verbindungsstück mit Tandem-Markierungsreaktionen und der Zielproteinbestimmung in lebenden Zellen kompatibel. Es wurden mehr Cathepsin-Zielproteine in lebenden Zellen identifiziert als in Zellextrakten. Dieses Ergebnis zeigt, dass die Aktivität von Proteasen bei der Herstellung von Zellextrakten verloren gehen kann verdeutlicht dass es wichtig ist, ganze Zellen für die Zielproteinidentifikation zu verwenden.

Zusätzlich haben wir fünf verschiedene trifunktionale Biotin-Reagenzien entwickelt und synthetisiert, die diol, diazobenzen, hydrazon, disulfid und dde-basierte spaltbare Verbindungsstücke für die Markierung und Reinigung von durch Alkyinsonden modifizierte Proteine haben. Alle Reagenzien mit Ausnahme der Disulfid-Verbindungsstücke können selektiv mit Alkin-E64-modifizierten Cathepsinen reagieren. Die markierten Proteine wurden mit Streptavidin-Agarose-Kügelchen angereichert und unter den spezifischen Bedingungen eluiert. Die Effizienz der Immobilisation und der Elution wurde mit Hilfe eines fluoreszenzbasierten SDS-PAGE Assays analysiert.

1 Introduction

1.1 Chemical probes for protein profiling

Genome sequencing projects have provided the chance to decode the genome of several kinds of eukaryotes and prokaryotes. The big challenge in the post-genomic era is the characterization of functions of proteins encoded by the genome. The function of proteins is related to the sequence and structure of these proteins. It is well appreciated that post-translational modifications enhance the structural and functional complexity of proteins. Furthermore, the protein function may be regulated by the interaction with DNA, lipids or other proteins. However these regulatory effects are not considered during the characterization of protein function with its purified form *in vitro*. To overcome this drawback, chemical proteomics is one of the systematic ways to study protein function in complex biological systems (1). Chemical probes based on bioactive molecules are useful tools for detection, location and isolation of proteins in chemical proteomics (2). Mass spectrometry instrumentation and bioinformatic tools facilitate the downstream identification and quantification of isolated proteins.

Chemical probes are designed based on the interactions between small molecules and proteins. The three necessary components of covalent chemical probes are a covalent binding motif, a detection and/or purification tag and a linker (3, 4) (Figure 1). A binding motif is responsible for the interaction between probes and target proteins. The tag is utilized for visualization and/or purification. The tags can be attached to the ABPs by a linker which can have different functions such as cleavability or increase of the solubility of probes. In tandem labeling, a binding motif is functionalized with bioorthogonal handle so the probe-protein complex can be subsequently labeled with tags under the influence of bioorthogonal chemistry.

Using chemical probes, protein targets can be visualized and profiled by gel based assays or imaged in living cells or organisms (5). Chemical probes can also be used in high throughput screening for discovery of new inhibitors of enzymes (6, 7). The utilization of chemical probes

has also facilitate the discovery of biochemical pathways (8) and function of the enzyme (9). In addition, chemical probes are useful tools for decoding the mechanism of phenotypic changes in cells or organisms induced by small molecules (10). The unknown protein targets of small molecule can be labeled and isolated from proteomes and sequenced by MS analysis The technology afforded the chance to develop drugs and identify off-targets (11).

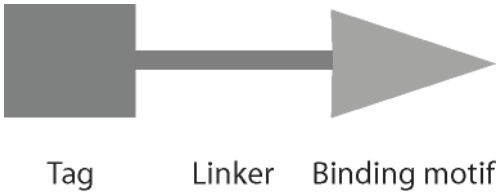


Figure 1: General elements of chemical probes. 1) Binding motif is responsible for the interaction with target protein; 2) Tag is used for visualization and affinity purification of probe-protein complex; 3) Binding motif and tag are connected by a linker.

1.1.1 Activity based probes

Activity based probes (ABPs) are chemical probes which rely on the catalytic mechanism of proteins and covalently bind to the active site of their targets. They specifically bind to the active forms but not to the inactive forms of enzymes. The binding moiety of ABPs consists of two structural elements: (1) a reactive motif named warhead to react with a specific residue of the target proteins. (2) a group that tunes the selectivity (3, 12). ABPs are utilized in activity based protein profiling (ABPP) to study enzyme function (3, 4).

1.1.1.1 Warhead

Electrophiles or latent electrophiles are key motifs in warheads responsible for covalent binding to target proteins. The development of warheads has benefited from discovery of protein reactive natural products and the research of irreversible inhibitors (13). For example, Cravatt et al designed a broad-spectrum ABP for serine hydrolases based on fluorophosphonate reagents which are irreversible inhibitor for serine hydrolases (14). DCG-

04 is an ABP for papain-like cathepsins derived from E64, a natural product isolated from mold Aspergillus japonicas (15). Since then, more electrophilic warheads have been developed for different classes of enzymes (4, 13). Quinone methide, a latent electrophile, is also a useful tool for the development of warheads for tyrosine phosphatases (16) and glycosidases (17). The latent electrophile transforms into a reactive intermediate upon reaction of the warhead in the active center of the enzyme (Figure 2).

1.1.1.2 Regulation of specificity

The specificity of ABPs is regulated by the recognition element which is usually derived from the substrate sequences of target enzymes. The recognition element has the ability to drive the probe to the active centre of the enzyme. Then the catalytic machinery of the enzyme enables the formation of a covalent bond between the warhead and the active site (3). For example, the selectivity ABP derived from diphenyl phosphonates is tuned by attachment of different natural peptide sequences (18, 19). To broaden the possibility to find selective recognition element, non-natural amino acids have also been incorporated (20).

1.1.2 Metabolic labeling based probes

Chemical probes derived from alkyne- or azido-functionalized metabolites such as amino acids (21), lipids (22-26) and glycan (27, 28) can be incorporated into proteins by the metabolic machinery of the cell. Identifications and quantifications of newly synthesized proteins (21, 29) and posttranslational modifications such as lipidation and glycosylation benefited from the development of these probes (30, 31) (Figure 2).

1.1.3 Affinity based probes

The recognition of affinity based probes by protein partners is based on the non-covalent interaction. Since the interaction is reversible, incorporation of photoreactive functionalities facilitates labeling and identification of low abundance and low affinity of protein partners (32, 33). Upon UV irradiation, highly reactive intermediates are generated which covalently, non-specifically react with neighboring molecules. In addition, stringent washing can be carried out in affinity purification of covalent probe-protein mixture due to the high stability of the covalent interaction (Figure 2).

1.1.4 Linkers

The incorporation of linkers to chemical probes is not only utilized to reduce the steric effects of the tag on activity of probes. The steric effect of the target protein to affinity beads during immobilization can also be reduced. Polymethylene and polyethyleneglycol (PEG) are mostly used as flexible linkers in chemical probes. PEG linker is more hydrophilic and hence increases the solubility of chemical probes and reduces the non-specific binding proteins (11). In addition, rigid linkers such as polyproline (34) and triazoyl-phenyl linker (35) were exploited to enable the binding of chemical probes to bulky proteins.

1.1.5 Tags

The tags are necessary elements for the subsequent detection after labeling of target proteins. Fluorophores are incorporated into ABP for easy and sensitive visualization. The labeling targets can be detected in cell or whole animal by fluorescence microscopy (36). The proteins can also be separated by 1D or 2D SDS-PAGE for gel based visualization and purification. New tools for chemical proteomics have been developed based on fluorophore imagining. Quenched activity based probes (37) and activity-based near-infrared glucuronide trapping probes (38) are designed as useful tools for *in vivo* and *in situ* imaging. Due to the different wavelength of Cy3 and Cy5, the combination of these fluorophores in chemical probes enables to distinguish target proteins and background proteins (39). Since the availability of antibodies for fluorophores, they have be used as an affinity handles for enrichment of fluorophores and subsequently analyzed by MS (40).

Biotin can also be used for visualization of tagged proteins by biotin-streptavidin blot. However, biotin is mostly used as an affinity handle for enrichment of labeled protein with streptavidin beads. The strong interaction between biotin and streptavidin allows enrichment of low abundant proteins by this system. Generally, harsh elution conditions are required for elution. Alternatively, peptide sequences have been exploited as tag in chemical probes. Hexahistidine-tag has been used as a tag in a cysteine reactive probes and the enrichment of labeled proteins was achieved by incubation with nickel-NTA resin (41). In addition, incorporation of a hemagglutinin tag in ubiquitin protein derived probes allowed the labeled proteins to be detected by western blot with an anti-hemagglutinin antibody and purification by immunoprecipitation (42).

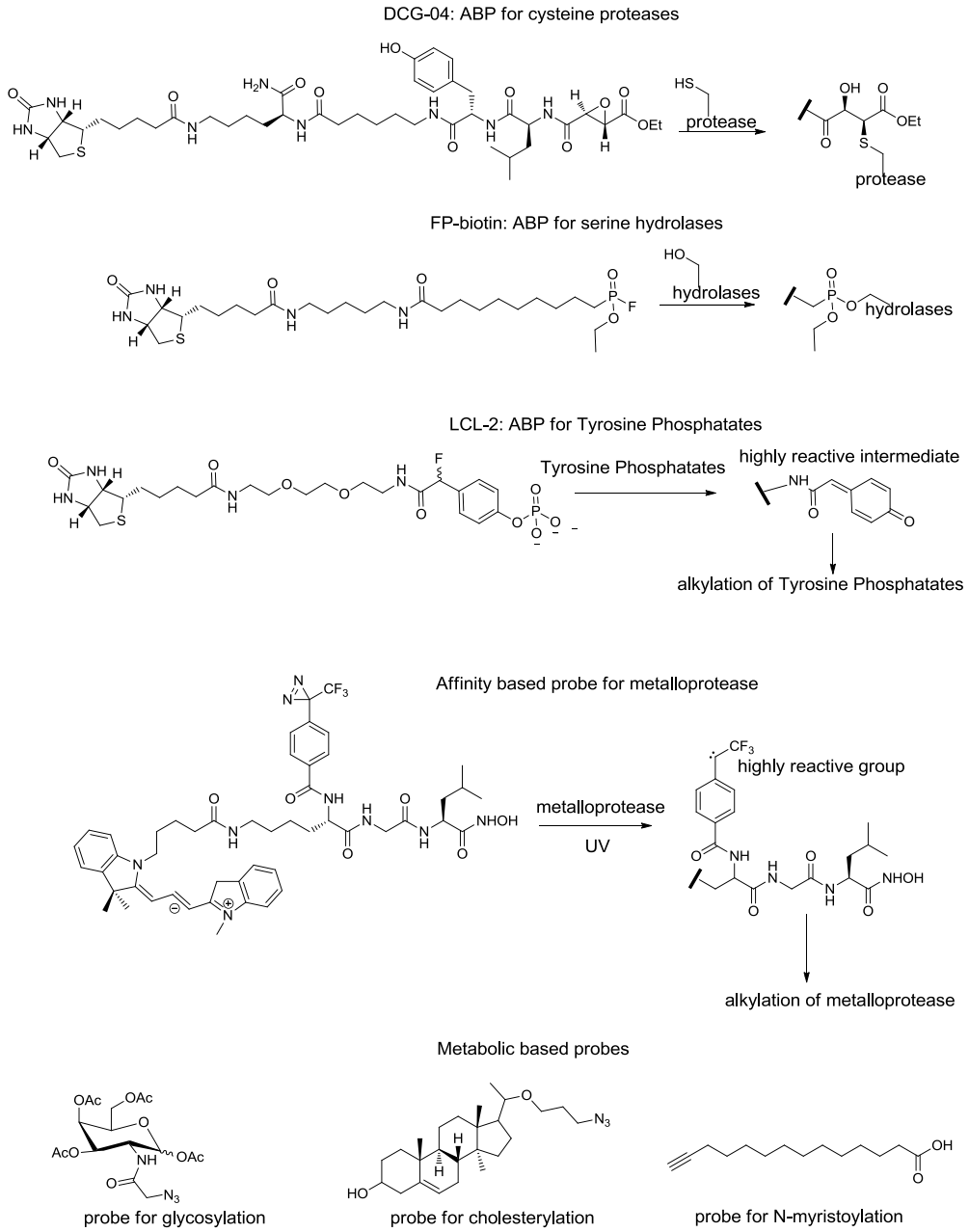


Figure 2: Representative chemical probes for chemical proteomics.

Radioisotope tags such as ^{125}I were incorporated into ABPs for gel based profiling (15). The minimal structural modification of probes has no negative effect on the activity of probes. Cu-64 labeled activity based probe has been used to image cathepsins in solid tumor in mice using positron emission tomography (PET) imaging (43). Stable isotope-coded affinity tags (ICAT)

are utilized to label cysteine-containing proteins and quantify their abundance (44). Recently, the reactivity of cysteine in cellular condition is quantified using isotopically labeled clickable biotin reagents (45, 46).

1.2 Detection of protein targets in living cells

Usually, target discovery of chemical probes is carried out in cell extracts or tissue homogenates. But functions of proteins, especially enzymes, often rely on protein-protein interactions. The possible alteration of conformation of proteins occurring during the generation of cell lysate may lead to loss of binding affinity between probes and target proteins. Comparisons of labeling efficiency of phenyl sulfonate ester activity-based probes in

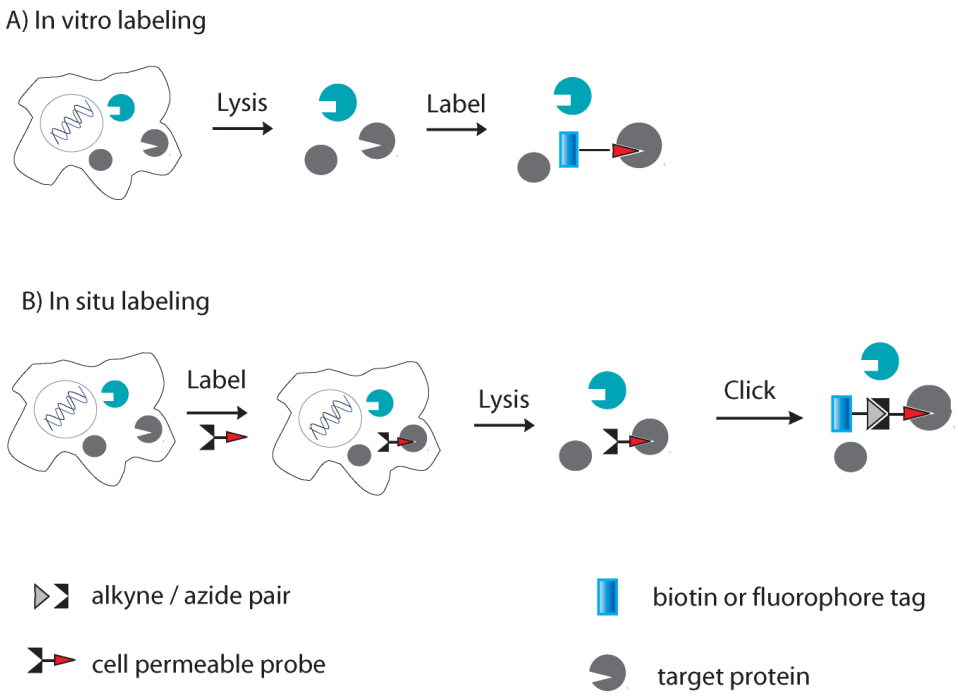


Figure 3: The labeling of protein target *in vitro* and *situ.* A) in *vitro* labeling: cell lysate is generated and subsequently incubated with probes. B) in *situ* labeling: cells are treated with probes and subsequently lysed. Biotin or fluorescent tag can be attached to probe-protein complex under the influence of Copper-catalyzed azide alkyne cycloaddition (CuAAC).

lysate and living cancer cells revealed that the activities of some enzymes were weaker in lysate and some enzymes were only identified in living cells (47). Hence, target identification

in living cells or organisms overcomes the drawbacks of *in vitro* experiments.

There were several successful examples showing that fluorophore tagged probes can be used to labeling targets in living cells (48, 49). However, treatment with high concentration of probes is usually required and it may lead to non-specific binding. In addition, the low solubility of biotin and fluorophore tagged probes is a problem to obtain high concentration of probes. Hence, target labeling with biotin and fluorophore tagged probes is mainly restricted to *in vitro* experiments.

Several different strategies have been developed to circumvent this problem. Latex beads have been used as tools to increase the uptake and allow detection the activity of cathepsins with DCG-04 (50). The incorporation of cell-penetrating peptides into chemical probes is also a promising strategy for cellular targets detection (36). Tandem labeling strategy is the most widely applied method for labeling of intact cells (47, 51). The strategy benefits from the development of bio-orthogonal chemistries such as Staudinger ligation and CuAAC. Appendage of a small tandem labeling tag to chemical probes has a minimal steric effect on the activity and cell permeability of probes (52). Biotin and fluorophore tags can be subsequently conjugated to probe-modified proteins in a lysate (Figure 3).

1.3 Chemoselective ligation reaction in chemical proteomics

1.3.1 Hydrazone and oxime ligation

The ligation reaction of aldehydes or ketones with hydrazides and aminooxy compounds forms hydrazone or oxime moieties. Since aldehydes can be generated on N-glycoprotein by periodate oxidation, hydrazide beads and biotin reagents are widely used to capture glycoproteins in complex proteomes (53). Hahne and coworkers developed carbonyl-reactive tandem mass tags to label and quantify N-linked glycans based on the ligation (54). The proteins modified by 4-hydroxy-2-nonenal, a lipid peroxidation end-product, were tagged with biotin hydrazides and enriched with streptavidin beads for MS analysis (55). Since the moiety of hydrazone is not very stable, labeling was followed by reduction with $NaCNBH_3$ to avoid the loss of label. Since the oxime is more stable than a hydrazone, the oxime ligation is employed to label and visualization of sialylated glycoproteins in living cells (56). The drawback of slow kinetics is overcome by using catalysts such as aniline (57), m-phecylenediamine (58) and 4-amino-phenylalanine (59). Due to the existence of metabolite

bearing aldehydes and ketones in natural biological systems, hydrazone and oxime ligations are not ideal choices for target identification of small molecules.

1.3.2 Copper(I)-catalyzed azide-alkyne cycloaddition

Azide-alkyne cycloaddition can give triazoles, stable five-membered heterocycles, but the reaction rate is low. To circumvent this problem, Sharpless and coworkers found that catalysis of Cu(I) enhances the reaction kinetics (Copper-catalyzed azide alkyne cycloaddition) (60). The reaction was further optimized by adding tris(benzyl-triazolylmethyl)amine (TBTA). TBTA can accelerate the reaction rate, stabilize the Cu(I) oxidation state and reduce the harmful effect of copper species in aqueous solution to protein and living cells (61). THPTA (62) and BTTAA (63) have been developed later and show different reaction kinetics. THPTA and BTTAA had better aqueous solubility and can be used at a higher concentration. Validation of application of CuAAC for tandem labeling was carried out by Speers and Cravatt (47, 51). The installation of azide or alkyne to probes is easily performed. Small reactive group leads to minimal structural steric effect on the activity of chemical probes. The rare occurrence of these two moieties in nature makes them suitable for two step labeling in biological systems. Since then, it has been widely used in target identification *in situ* and *in vivo*. The disadvantages of copper catalysis are the cellular toxicity and possible denaturation of protein targets. Some copper-free ligation strategies were also used in chemical proteomics.

1.3.3 Staudinger ligation

The ligation of an azide with a phosphine resulting in a stable amide bond is called the Staudinger ligation. The compatibility of the Staudinger ligation with bioconjugation has been validated by Saxon and Bertozzi who reported the labeling and visualization of metabolically incorporated azide-containing glycans in living cells (28). The applications of the Staudinger ligation to identify targets of ABPs are also reported by different groups (64, 65). Due to the different size and stability of the reactive partners, the azide moiety is incorporated into probes for cellular labeling and the phosphine part is attached to affinity or fluorophore tags.

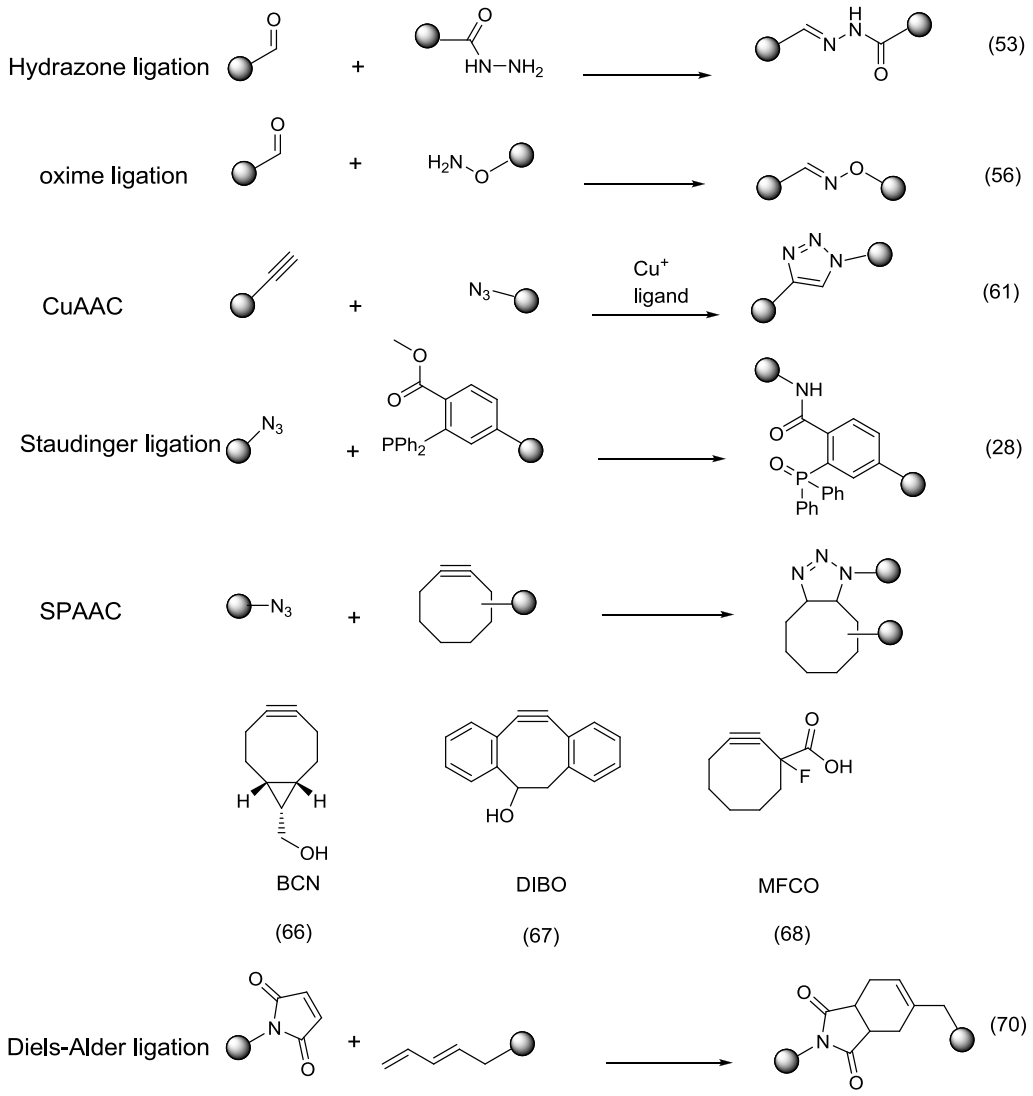


Figure 4: Chemoselective ligation reaction in chemical proteomics.

1.3.4 Strain-promoted alkyne-azide cycloaddition (SPAAC)

Copper catalysis is not required in the reaction between cyclooctynes and azides. But the reaction rate is quite slow. Structural modifications generated a series of more reactive compounds such as DIBO (66), BCN (67) and MFCO (68). The efficiencies of biotin reagents containing DIBO, BCN or MFCO for activity based proteasome labeling in cell extracts were compared by Overkleeft and coworkers (69). Background labeling observed for all of these reagents indicates that the selectivity of cyclooctyne reagents needs improvement.

1.3.5 Diels-Alder reaction

Overkleeft and coworkers validated the Diels-Alder strategy in two steps labeling for ABPP (70). Diene-derivatized proteasome probes or diene-derivatized cathepsins probe modified proteins could react with maleimide-functionalized fluorophore tag. Alkylation of cysteine is a necessary step prior to Diels-Alder labeling due to the side reaction of Michael addition between cysteine and maleimide.

1.4 Capture and release strategy in chemical proteomics

Nowadays mass spectrometry instrumentations are quite sensitive and enable identification of several thousand proteins from complex proteomes. However, it is still a challenge to directly profile targets of small molecules by mass spectrometry analysis. Isolation of the protein targets and reducing the complexity of samples is key step before MS analysis.

1.4.1 Affinity matrix with ligands

The ligands can be directly immobilized onto affinity beads which can be used to capture protein partners of ligands in lysate from cell or organ. The simple affinity strategy has been widely used to profile reversible binding targets of natural products and drugs (2, 71). During this strategy, the interaction between proteins and ligands is mostly non-covalent binding. Incorporation of diazirine-based photo reactive to affinity beads could allow the immobilization of compounds to beads under covalent interaction (72). The proteins are eluted with sample buffer or by free ligands. For elution of strong non-covalent binding partners, sample buffer in combination with boiling should be used. In addition, label-free quantification and quantitative proteomics based on isotope labeling have been utilized for identification and profiling the activity of putative protein targets (73).

1.4.2 Affinity matrix with chemical ligation handle

The immobilization of chemical ligation handle for affinity purification has benefited from the development of the bioorthogonal ligation methods. Hydrazide beads are simple and efficient tools for the capture of proteins and peptides modified by aldehyde and ketone (53). Finn and co-workers developed clickable agarose beads for immobilization of molecules under the influence of CuAAC (74). In a recent combination with metabolic labeling, alkyne-resin has been employed to capture azide-N-acetylglucosamine modified proteins in complex cell

extract (27, 75). Cleavable linkers have been incorporated between matrix and ligation handle for elution of proteins under mild conditions (76, 77).

1.4.3 Biotin-streptavidin interaction

The interaction between biotin and streptavidin is one of the strongest non-covalent interactions known. Biotin-streptavidin based affinity system is widely used to immobilize low abundant biotinylated proteins or peptides from complex mixtures. Target proteins can be biotinylated by direct modification with biotinylated probes or clickable probes followed by reaction with clickable biotin reagents. Harsh, denaturing conditions (SDS boiling) are required to disrupt the interaction between biotin and streptavidin to release biotinylated target proteins. Non-specific binding proteins, endogenously biotinylated proteins and streptavidin will release simultaneously and lead to the contamination of the samples for subsequent MS analysis. Gel based purification is necessary to reduce the background proteins and target bands are excised for tryptic digestion (78). To circumvent this problem, streptavidin mutants and several different biotin analogs such as 2-iminobiotin (79), 2'-thiobiotin (80), desthiobiotin (81) and N3'ethyl biotin (82) were developed to reduce the binding affinity of streptavidin and biotin. The lower binding affinity allowed the immobilized proteins released under more mild conditions. Fox example, N3'ethyl biotin modified proteins could be released by incubation with buffers containing 2 mM of biotin. However, the lower binding affinity may lead to the inefficient immobilization of low abundant target proteins. In addition, the targets may get lost during the stringent washing steps for cleaning of background.

1.5 Cleavable linker in chemical proteomics

1.5.1 Application of cleavable linker in chemical proteomics

Application of cleavable linkers is a promising strategy for reducing background proteins (83, 84). Incorporation of cleavable linkers to affinity matrix or biotin reagents allows the elution of protein under mild conditions. Mild elution conditions can reduce the contamination of non-specifically bound proteins and endogenously biotinylated proteins. The proteins released by mild conditions can be submitted to solution digestion for subsequent MS analysis without gel based purification. Furthermore release of biotin is helpful for identification of from probe-modified peptides (83). An ideal linker should be stable enough in cell extract and different

buffer systems. The cleavage condition should be mild and not denature the streptavidin and background proteins.

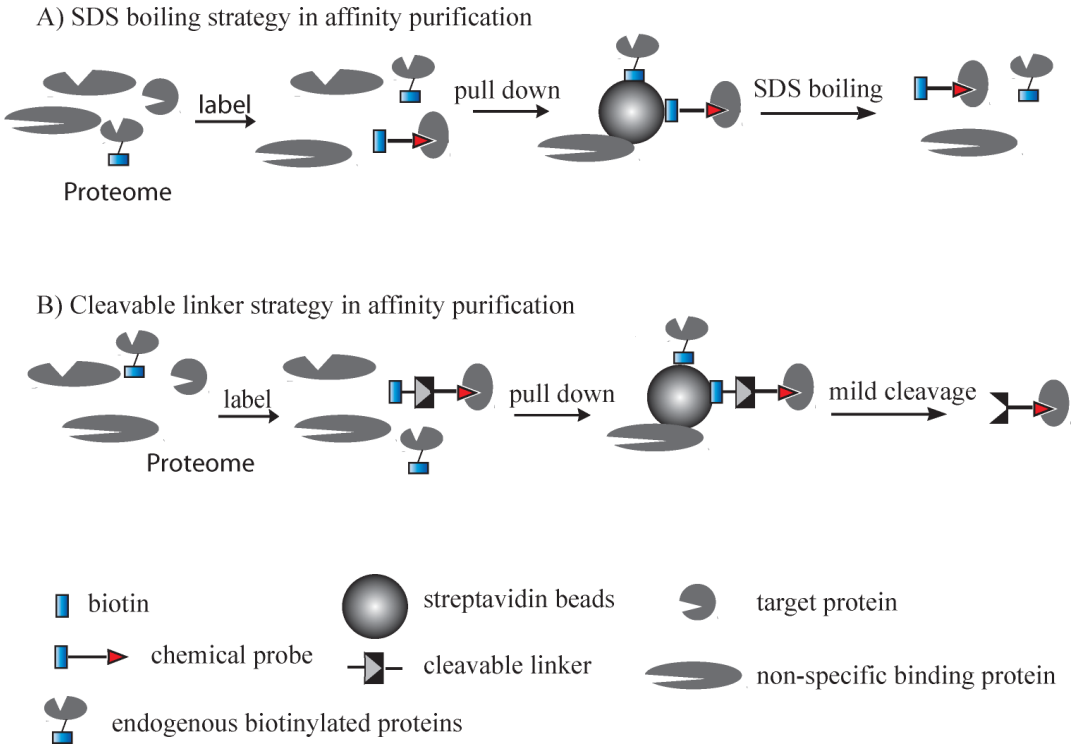


Figure 5: Comparison of SDS boiling elution with chemical selective elution. A) SDS boiling strategy: Target protein is labeled with biotinylated probe. During the incubation with streptavidin beads, probe-protein complex, endogenously biotinylated proteins and non-specific binding proteins bound to streptavidin beads. SDS boiling leads to the elution of all the proteins together. B) Cleavable linker strategy: Target protein is labeled with cleavable probe. During the incubation with streptavidin beads, probe-protein complex, endogenously biotinylated proteins and non-specific binding proteins bound to streptavidin beads. Mild cleavage condition leads to the elution of only probe-protein complex.

1.5.2 Chemical cleavable linker

Disulfide based linkers are sensitive to mild reducing reagents such as DTT, TCEP and BME which are biocompatible (21, 85). Clickable biotin reagents containing disulfide have also been utilized to identify the protein targets in living cell (21). The liability of the linker to free thiols such as free cysteine residues may lead to loss of target proteins and an increase in non-

specific labeling. Alkylation of the free cysteines with iodoacetamide prior to labeling is a way to circumvent this drawback (85). In addition, sterically hindered modifications have been introduced to the disulfide linker to increase its stability, resulting in sensitivity to TCEP reduction but not to DTT reduction (86).

The application of diazobenzene-derived linker in chemical proteomics has been reported by different groups (84, 87). In addition, clickable diazobenzene derivatives were attached to an agarose matrix which was functionalized with affinity ligands for protein capture and release (76). Structural optimization has led to new diazobenzene linkers such as the 2-(2-alkoxy-4-hydroxy-phenylazo) benzoic acid scaffold which is more sensitive to sodium dithionite (88).

The ester bond-based cleavable linker in the biotinylated probe allowed the immobilized proteins to be released with an alkaline buffer (pH=11) (89). More alkaline liable cleavable linkers based on benzoyl esters were developed and introduced to thiol reactive biotinylated probes to enrich cysteinyl peptides. The linker is stable in pH=4.5 buffer but rapidly hydrolyzed at higher pH (90). The high sensitivity of this linker to pH may limit its applications. In addition, the ester-bond can be cleaved by nucleophiles such as hydroxyl amine. The drawback of above ester bond-based cleavable linker is premature cleavage due to the alkaline and nucleophiles liability. The levulinoyl ester was successfully developed as a cleavable linker to overcome these drawbacks. The intrinsic base-lability was avoided by application of ortho-isopropyl groups to the ester. The novel linker is stable in cell extracts and different buffer systems and compatible with tandem labeling (91). However, the multistep organic synthesis is a drawback for the generation of this linker.

TFA cleavable linkers have also been applied in chemical proteomics (92-94). The high concentration of TFA will denature proteins and may lead to the release of non-specific binding proteins. The efficiency of TFA cleavable linkers in reduction of non-specific binding proteins has not been reported yet. A more acids liable linker based on diphenyldialkoxysilane was developed (85). The immobilized proteins were nearly quantitatively recovered in 30min with 10% formic acid. Although this condition is milder than the TFA solution, SDS solution is preferable used to wash beads to recover precipitated proteins due to low pH.

Acylhydrazone is a motif used in drug delivery systems since it is stable under neutral conditions but hydrolyses under moderately acidic conditions. The acylhydrazone bond is acquired by bio-conjugation of aldehyde with acylhydrazine. The bio-conjugation is widely used in immobilization and elution of aldehyde containing proteins with hydrazine beads (53,

95). The incorporation of this motif into an azido biotin reagent was carried out by Kohn et al. Azido-acylhydrazone-biotin labeled proteins were selectively released through hydrazone exchange with acylhydrazides (96). The drawback of this linker is the premature cleavage induced by hydrolysis which makes it not suitable for bioconjugation. Another exchangeable biocompatible linker based on bisaryl hydrazones overcomes this drawback. It is stable in mild acidic solution but rapidly cleaves by hydroxylamine under the catalysis of aniline (97). Both of above hydrazone-based cleavable linkers allowed the released proteins to be modified by conjugation of nucleophiles. For example, isotope and fluorescent tags could be incorporated into the released protein for subsequent detection.

A novel cleavable linkers based on the malondialdehyde-indole condensation reaction was utilized to affinity purify the targets of bosutinib, a kinase inhibitor (98). The immobilized proteins were eluted with 100 mM of pyrrolidine. However, acidic conditions should be avoided with the cleavable beads due to the presence of an acylhydrazone which may lead to hydrolysis.

Fukuyama et al developed a cleavable linker based on the motif of nitrobenzenesulfonamide. The linker was more easily cleaved by BME under mild alkaline buffer. To validate the application of this linker in chemical proteomics, it was introduced to a photoaffinity probe to label γ-secretase in HeLa cell lysate (99). The labeled protein was fully enriched on streptavidin beads but only partly eluted by BME. More efficient sulfonamide-based cleavable linker was developed for diazirinyl photoprobes. The linker is stable but becomes liable to hydroxylamine after the activation by iodoacetonitrile (100).

1.5.3 Photo cleavable linker

In 1995, a photo cleavable biotin derivative based on ortho-nitrobenzyl compound was synthesized to capture biomolecules. UV ($\lambda > 300$ nm) exposure can fully photolyse this structure in 5 min (101). The advantages of the photo cleavable linker such as high selectivity, time economical and no need for chemical led to these derivatives to be widely applied in chemical proteomics. The drawback of the linker is the undesired modification of target proteins by highly reactive aromatic nitroso compounds during cleavage. An UV cleavable biotin reagent containing benzoin ester based cleavable linker was applied to study the modification site of 4-hydroxynonenal on plasma proteins (102). Alkaline hydrolysis based cleavage may lead to premature cleavage of the linker (90). A phenacyl ester based cleavable

linker was introduced to clickable biotin reagents. Evaluation of cleavage efficiency indicated that the linker was readily cleaved when exposed to wavelengths at 250 nm (103). However, these wavelengths can lead to photo damage. Hence the linker has not been used in protein purification yet.

1.5.4 Enzyme cleavable linkers

Besides photo and chemically cleavable linkers, enzymatically cleavable linkers also represent useful tools in chemical proteomics. One of the drawbacks of on-bead digestion is the missing of peptides modified with biotinylated probes due to the strong interaction between biotin and streptavidin. The incorporation of a trypsin cleavable linker into biotin reagents could circumvent this problem (104). However, target proteins, non-specific binding proteins and endogenously biotinylated proteins would be digested together during on-bead digestion. The peptide sequences from background proteins complicate the subsequent analysis. Clickable biotin tags with a tobacco etch virus protease (TEV) cleavable site were tested in activity based profiling by Carvatt et al (83, 105). The high selectivity of this protease enabled the target proteins to be selectively released from the beads. The length between biotin and the TEV cleavage site can affect the release efficiency. However, the TEV cleavable linker was not very efficient in a new capture-and-released system based on the ligation between the Halo Tag fusion protein and hexylchloride (106). The liability of protease cleavable linker to endogenous proteases should be considered when applied in chemical proteomics. Hatanaka et al introduced a V8 protease cleavable linker to a photoaffinity probe of chymotrypsin (107). To prevent the premature cleavage of this linker, the carboxylic acid side chain of glutamic acid was protected with methyl ester. The enzymatic cleavage could only be carried out after the alkaline hydrolysis of methyl ester with aqueous of NaOH. Besides peptide sequences as cleavable linkers, DNA-based linkers have been applied in the biotin-streptavidin system (108). Recently, the incorporation of automatically synthesized single strand DNA-based cleavable linker in biotin reagents was used to label and identify alkyne-tagged glycoproteins by exploiting CuAAC (109).

Table 1: Cleavable linkers for chemical proteomics

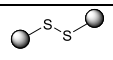
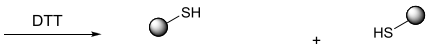
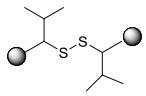
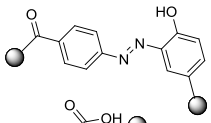
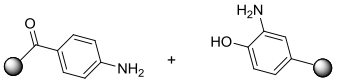
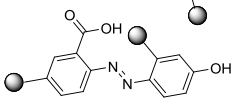
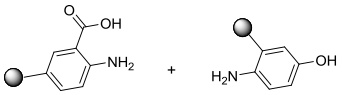
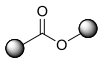
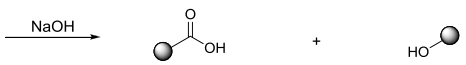
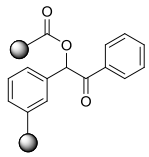
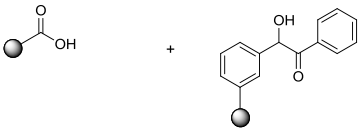
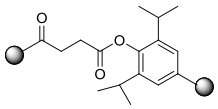
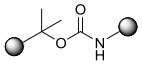
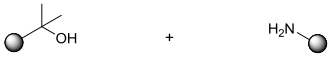
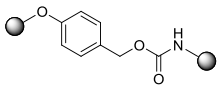
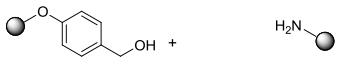
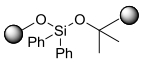
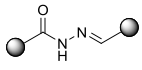
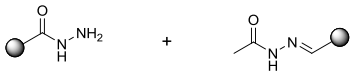
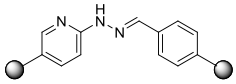
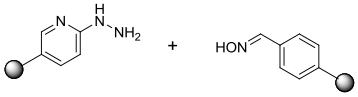
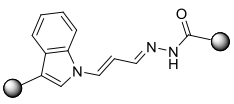
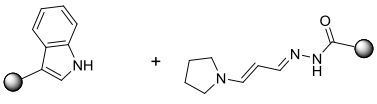
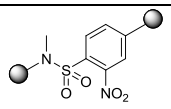
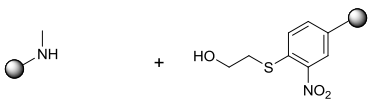
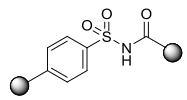
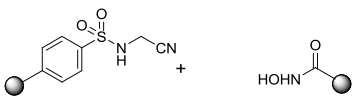
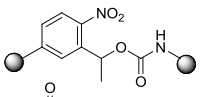
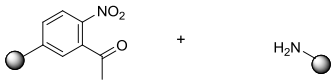
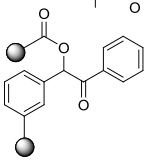
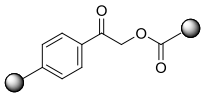
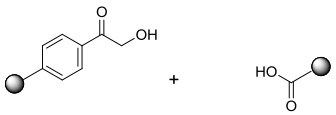
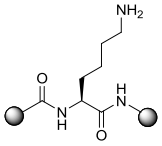
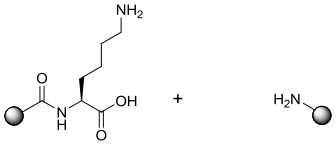
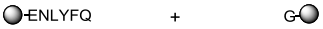
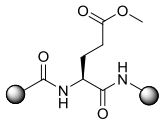
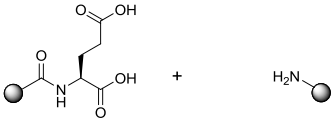
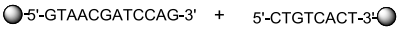
Linkers	Cleavable products	Refs
a) 		(21, 85)
b) 		(86)
c) 		(84)
d) 		(88)
e) 		(89)
f) 		(90)
g) 		(91)
h) 		(92-93)
i) 		(94)
j) 		(85)
k) 		(95, 96)
l) 		(97)
m) 		(98)

Table 1: Cleavable linkers for chemical proteomics (continued)

Linkers	Cleavable products	Refs
n) 		(99)
o) 		(100)
p) 		(101)
q) 		(102)
r) 		(103)
s) 		(104)
t) 		(83, 105)
u) 		(107)
v) 		(109)

2 Aims of this work

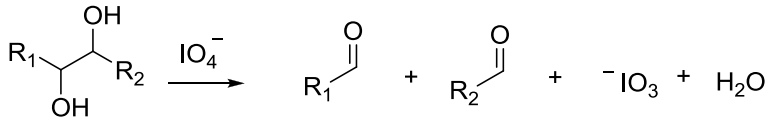
Application of cleavable linkers afforded the chance to reduce background proteins in biotin-streptavidin system and several cleavable linkers have been developed. But simple and efficient cleavable linkers are still called for. The overall goal of this thesis was the development and application of cleavable linkers in chemical proteomics. Firstly, we report the application of vicinal diol cleavable linker, based on tartrate, in chemical proteomics. The diol cleavable building block was synthesized in two steps and introduced into the activity based probe DCG-04 by solid phase synthesis. To show the compatibility of the linker with tandem labeling strategy, a clickable biotin reagent containing the diol linker was synthesized. The efficiency and chemoselectivity of chemical elution of target proteins was compared with SDS boiling elution. We evaluated the efficiency of this linker in reducing background proteins by comparison of chemical release and on-bead digestion. The cathepsin targets of azido-E64 in RAW cell extract and in living RAW cells were compared.

Secondly, since tandem labeling strategies are helpful to study protein targets *in situ* and *in vivo*, different cleavable trifunctional biotin reagents were designed and synthesized. Each reagent contains an azido-handle to react with alkyne-probe modified proteins, a biotin for purification by streptavidin beads, a fluorescent tag for sensitive visualization of target proteins and a PEG linker to increase the solubility of tags. We studied the efficiency and selectivity for labeling of alkye-E64 modified protein in cell extract with different trifunctional reagents. We then investigated the efficiency of immobilization of labeled proteins. The enriched proteins were eluted under mild chemical conditions and SDS boiling to see the elution efficiency and chemical selectivity.

3 Results and discussion

3.1 Application of diol cleavable linker in chemical proteomics

The cleavage of a vicinal diol with $NaIO_4$ is shown in Scheme 1 (110). The cleavage results in the formation of two aldehyde parts. Due to the mild cleavage conditions and high cleavage efficiency, this reaction has many applications in chemical biology research. For example, it was used as a cleavable-cross linker for the detection of protein-protein interactions (111). This linker has also been used to synthesize peptoids (112). Furthermore, this linker was used as a hydrophilic linker to reduce non-specific binding proteins in affinity chromatography (113).



Scheme 1: Oxidation of $NaIO_4$ leads to the cleavage of vicinal diol.

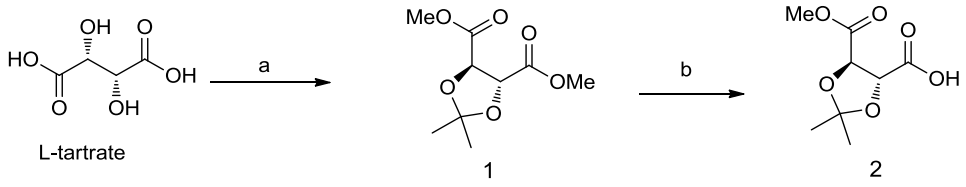
3.1.1 Synthesis of a diol cleavable linker and incorporation into chemical probes

3.1.1.1 Synthesis of cleavable building block

L-tartaric acid is a commercially available, inexpensive compound. In order to incorporate the L-tartrate-based cleavable linker into chemical probes and clickable biotin reagents, we transformed L-tartrate to methyl-2,3-*O*-isopropylidene-L-tartrate as a cleavable building block in two straightforward protecting group manipulation steps (Scheme 2). Since the reaction is

easily handled and the starting material is inexpensive, the building block can be synthesized on a large scale.

In order to convert L-tartrate into a synthetic building block, two hydroxyl groups and one of the carboxyl groups should be protected. Acetonide is the most common protection group for 1,2-diols. It is easily removed by hydrolysis with aqueous acids. The stability of the acetonide is compatible with Fmoc-based solid phase peptide synthesis. Furthermore the deprotection of acetonide can be achieved simultaneously with the removal of the peptide from a resin with TFA solution. Methyl ester was selected as carboxyl protective group since it can be introduced in the same reaction as the acetonide. These two different kinds of protective group were introduced via reaction with methanol, 2, 2-dimethoxypropane, and a catalytic amount of *p*-toluenesulfonic acid at elevated temperature. After work-up, the fully protected tartrate was purified by means of vacuum distillation and obtained as light yellow oil in 74% yield. Next, one methyl ester was saponified through reaction with one equivalent of potassium hydroxide in MeOH. Building block **2** was isolated via extraction as colorless oil in 63% yield. Building block **2** can be used in subsequent reaction without further purification.



Scheme 2: Synthesis of the diol cleavable building block, a) dimethoxy-propane, pTsOH, 74%; b) 1 eq of KOH in MeOH. 63%.

3.1.1.2 Design of diol cleavable linker containing probes and clickable biotin reagents

Synthetic building block **2** can be used both in solution and solid phase synthesis. To illustrate this, we introduced this diol cleavable building block to DCG-04 (15), an activity based probe for cysteine proteases, by solid phase synthesis. The structure of DCG-04 is shown in Figure 2.

Diol-DCG-04 is a cleavable version of DCG-04 which allows the enriched proteins to be chemically released from streptavidin beads (**5**, Scheme 4). A diol-DCG-04 with a TMR fluorophore (**7**, Scheme 5) was also synthesized to make the released proteins sensitively detectable by in gel fluorescent scanning (84). We also synthesized biotin-diol-azide (**8**, Scheme 6) by solid phase and biotin-diol-alkyne reagent (**11**, Scheme 7) in solution phase for ligation with azide or alkyne modified proteins, respectively.

3.1.1.3 Synthesis of diol-DCG-04

These compounds are easily assembled via Fmoc-based solid phase synthesis which has some advantages over solution phase synthesis: the byproducts and coupling reagents can be easily filtered away and only one purification step is performed to get the final products. Rink amide resin was selected as solid support for solid phase synthesis. The Fmoc group was removed by 20% piperidine in DMF (Figure 6) and the resin was washed with DMF. The resulting amine was subsequently coupled to the next building block. The deprotection and coupling steps were repeated until the final product was assembled. The products were cleaved from resin using TFA solution.

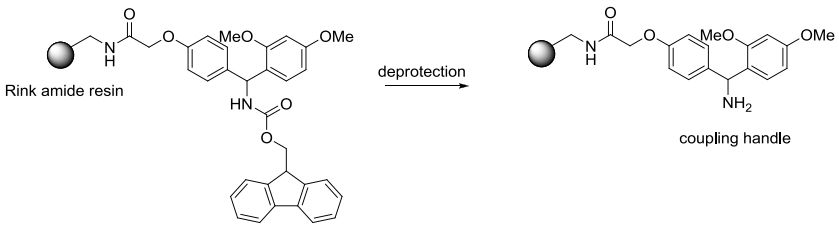
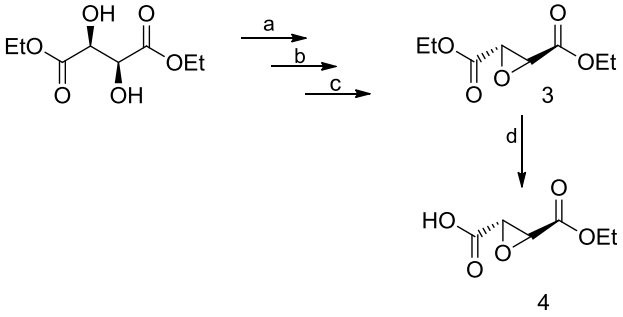


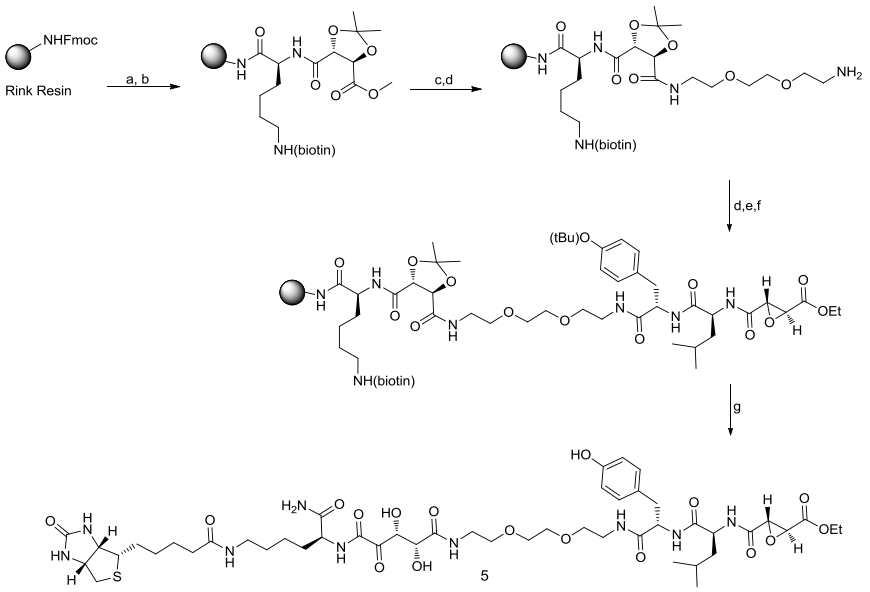
Figure 6: Fmoc deprotection of Rink amide resin with 20% piperidine in DMF.

The warhead of diol-DCG-04 and TMR-diol-DCG-04 is ethyl (2S, 3S)-epoxysuccinate (**4**) which was synthesized starting from diethyl-D-tartrate as described (15). For synthetic details, the reader is referred to the material and methods part.

Elongation of Rink resin with Fmoc-biotinyl-lysine and cleavable building block **2** under the influence of DIC/HOBt was followed by treatment with neat 1,8-diamino-3,6-dioxaoctane, which replaced the methyl ester of the tartrate under formation of an amide bond. Further elongation was achieved with Fmoc-tyrosine, Fmoc-leucine, and ethyl (2S, 3S)-epoxysuccinate (**4**). Diol-DCG-04 (**5**) was cleaved from the resin and purified via HPLC.



Scheme 3: Synthesis of ethyl (2S, 3S)-epoxysuccinate. a) 33% HBr/AcOH, overnight; b) acetyl chloride, reflux, 6 h; c) DBU, 2 h; d) 1 eq of KOH.



Scheme 4: Synthesis of diol-DCG-04 (**5**). a) 20% piperdine in DMF, then Fmoc-Lys(biotin)-OH, DIC, HOBt and 20% piperdine in DMF; b) diol cleavable building block **2**, DIC, HOBt; c) 1,8-diamino-3,6-dioxaoctane, overnight; d) Fmoc-Tyr(tBu)-OH, DIC, HOBt then 20% piperdine in DMF; e) Fmoc-Leu-OH, DIC, HOBt then 20% piperdine in DMF ; f) ethyl (2S, 3S)-epoxysuccinate, DIC, HOBt; g) TFA : TIS: H_2O (90% : 2.5% : 7.5%) 3h, then precipitation with cold diethyl ether and purification by HPLC; 17% after purification.

3.1.2 Detection of cleavage of diol-DCG-04 by LC-MS

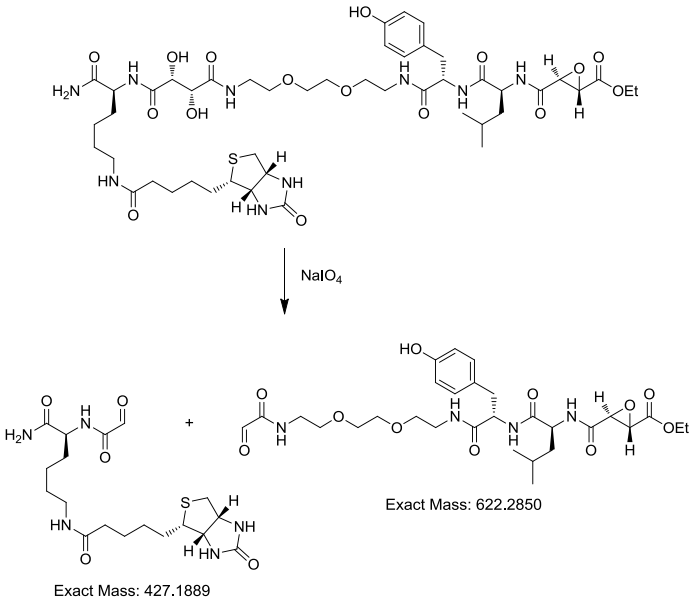


Figure 7: Structures of cleavage products of diol-DCG-04.

We tested whether the diol cleavable linker within the context of a probe could be cleaved by periodate. To this end, we incubated diol-DCG-04 with 10 mM sodium periodate in SP buffer (pH 7.4) for 2 h. The cleavage products were purified with a C18 spin column and dissolved in 0.1% FA in 50% ACN/H_2O. The nature of the cleavage products was analyzed with LC-MS. We observed formation of the aldehydes, which are the expected cleavage product, as well as the hydrate of the aldehydes (Figure 8A).

Aldehydes can react with primary amines resulting in imines. If aldehyde groups of the chemically released proteins react with amines of streptavidin, the protein will fail to be released from streptavidin beads. In order to check if this reaction occurs between the aldehyde parts generated from the probes and proteins or peptides, the peptide bradykinin was taken as a model peptide to test this. As a control, bradykinin was treated with 10 mM sodium periodate for 2 h. The LC-MS spectra showed there were no side effects to bradykinin (Figure 8B). In order to see if the cleavage product of diol-DCG-04 reacted with bradykinin, a mixture of diol-DCG-04 (2 µM or 10 µM) and bradykinin (2 µM or 10µM) was treated with $NaIO_4$ (10 mM) for 2 h. There was no reaction observed between the cleaved diol-DCG-04 and the amino

functions of bradykinin (Figure 9A and Figure 10). The sample was tested again after 24 h incubation at room temperature (Figure 9B), with no indication of side products. This result means that chemical release and tryptic digest samples can be safely stored before analysis takes place.

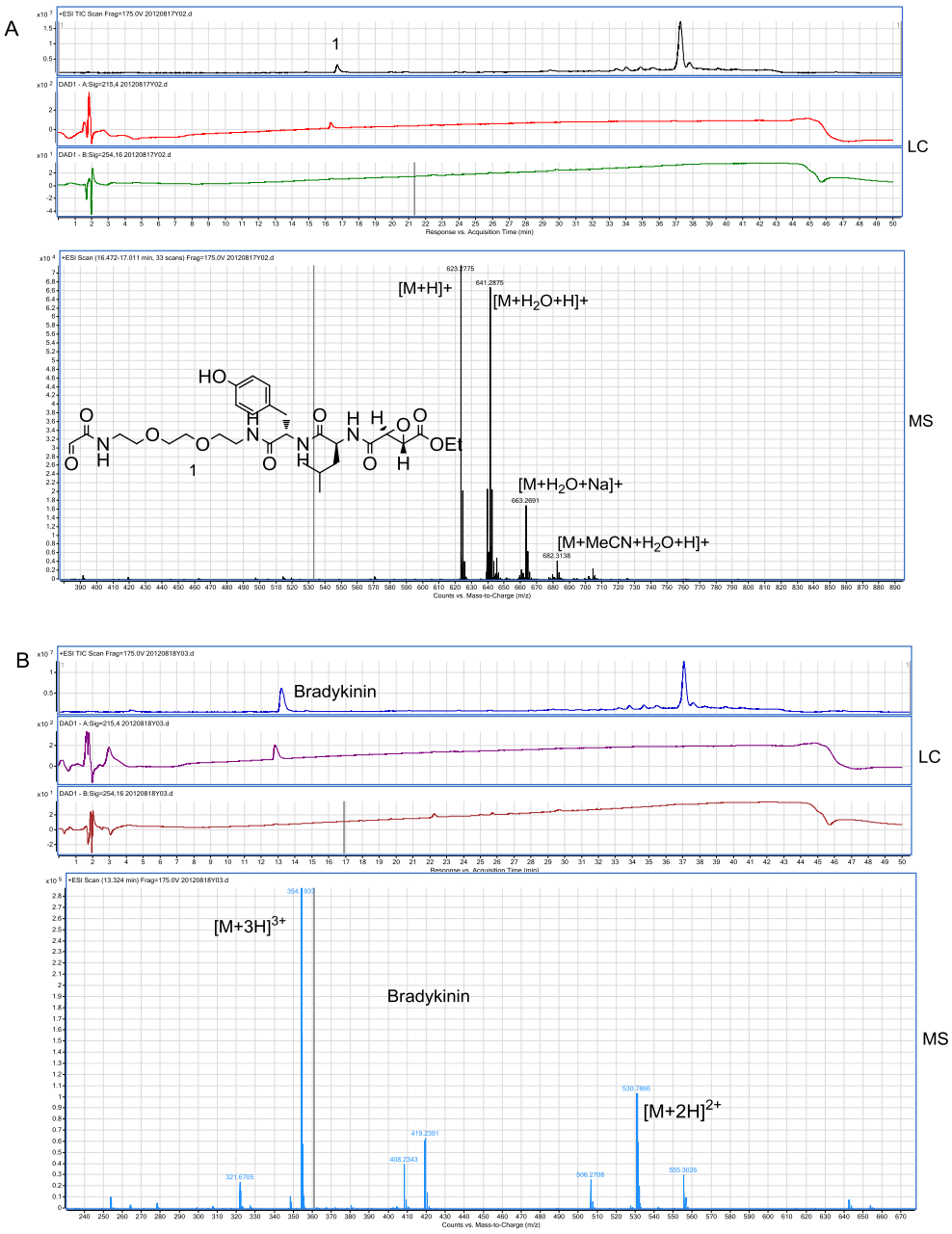


Figure 8. LC-MS analysis of effect of $NaIO_4$ on diol-DCG-04 and bradykinin. A) Cleavage of diol-DCG-04 by 10 mM of $NaIO_4$ in SP buffer. B) Bradykinin was treated with 10 mM of $NaIO_4$ in SP buffer.

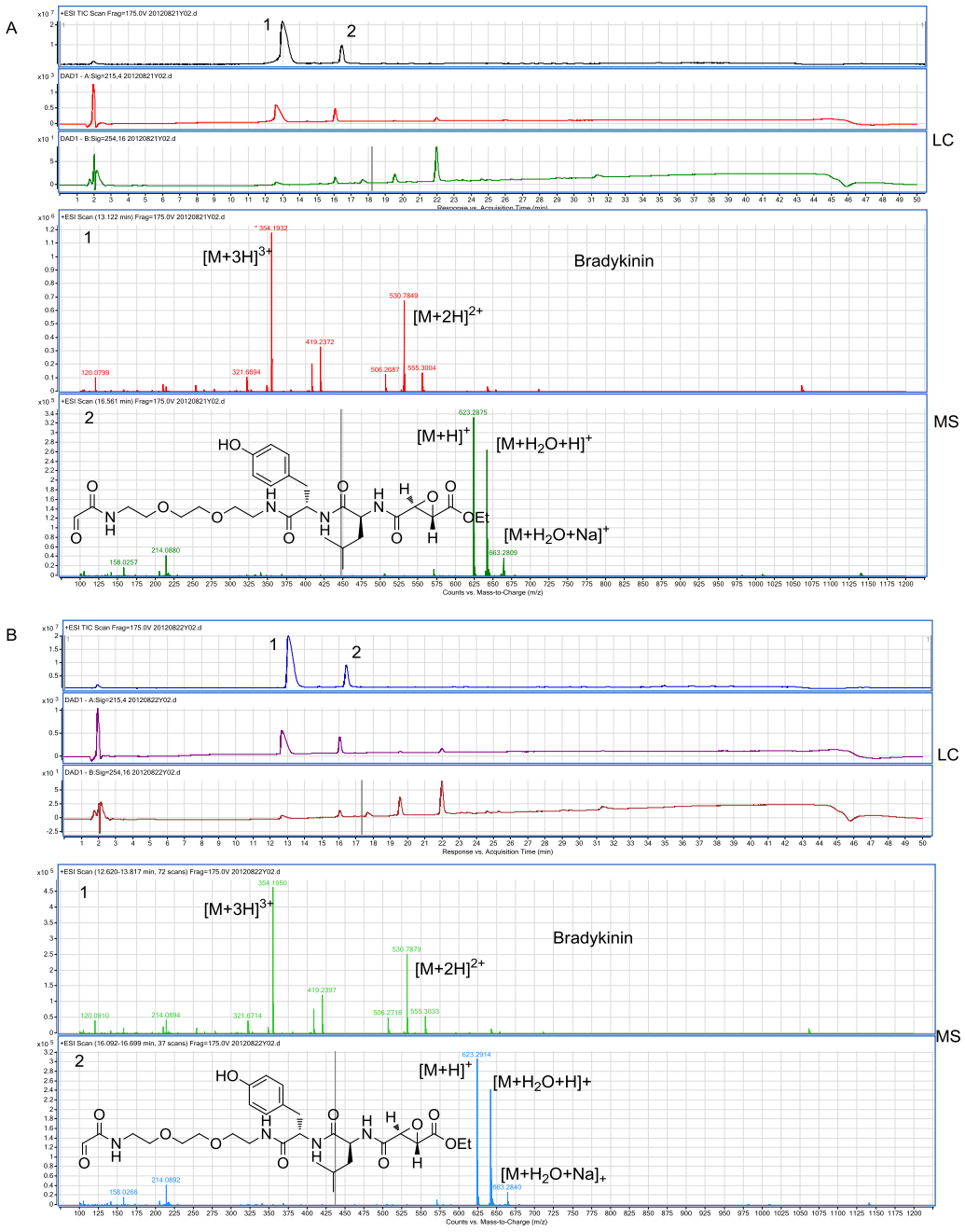


Figure 9: LC-MS analysis of $NaIO_4$ on the effect of mixture of diol-DCG-04 (2 µM) and bradykinin (2 µM) in SP buffer. A) The mixture of bradykinin and diol-DCG-04 was treated with $NaIO_4$. B) The sample was kept overnight at room temperature and analyzed again.

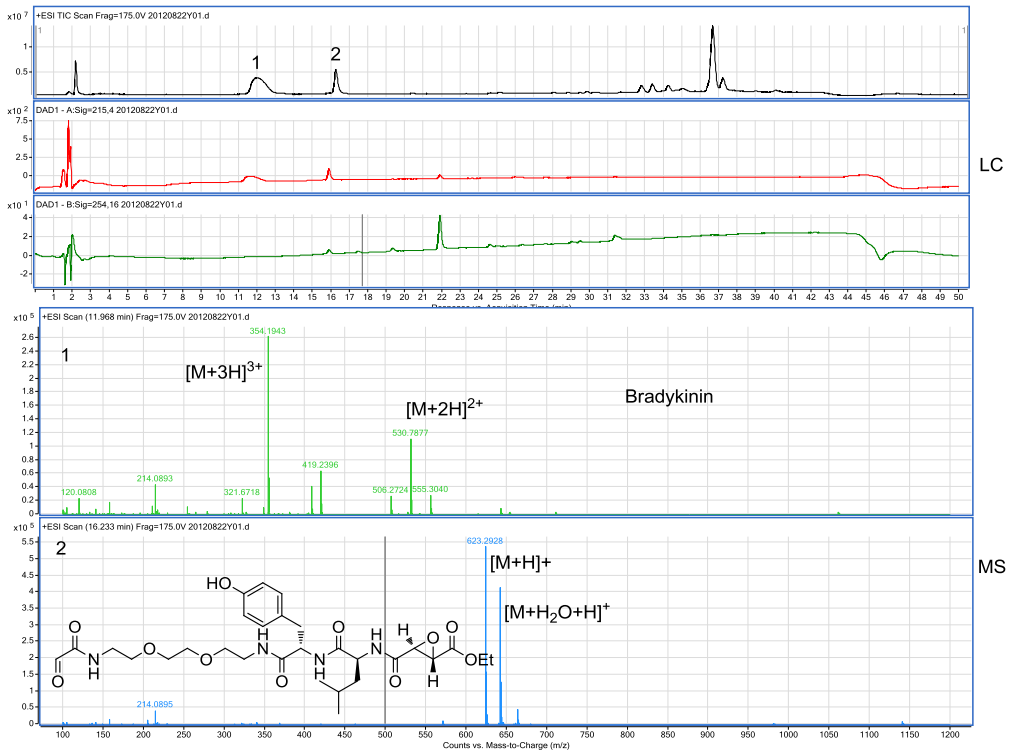


Figure 10: LC-MS analysis of $NaIO_4$ on the effect of mixture of diol-DCG-04 (10 µM) and bradykinin (10 µM) in SP buffer.

3.1.3 Labeling of cathepsins in rat liver proteome

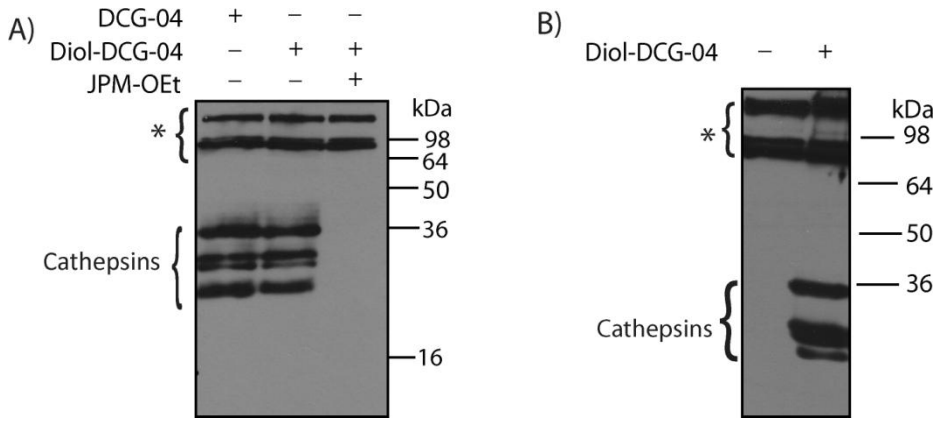


Figure 11: Diol-DCG-04 and DCG-04 labeled cathepsins in rat liver lysate. A) Rat liver lysate was treated with diol-DCG-04 and DCG-04 (1μM, 0.5h). Labeled cathepsins in rat liver lysate and the proteins were visualized by streptavidin western blot. The diol-DCG-04 labeled cathepsin bands are competed by pretreatment with the cysteine protease inhibitor JPM-OEt (50 μM, 0.5h). B) Rat liver lysate with or without labeling with diol-DCG-04 (1μM, 0.5h) were separated by SDS-PAGE and proteins were visualized with streptavidin western blot.

To demonstrate that the diol-containing cathepsin ABP diol-DCG-04 retains the activity of the parent compound DCG-04, we first made a direct comparison of diol-DCG-04 with DCG-04. In a rat liver proteome, which contains a variety of previously identified cathepsins, both probes yielded a similar, activity-based labeling pattern. Pretreatment of JPM-OEt, an inhibitor of papain like cathepsins (114), can fully inhibit the activity of cathepsins and diol-DCG-04 failed to label them, indicating that the cleavable linker does not influence the specificity and potency of the ABP (Figure 11A). In order to make sure the top two bands are endogenously biotinylated proteins, rat liver lysate without any labeling was loaded on SDS-PAGE. Biotin-streptavidin western blot clearly shows that the top two bands are endogenous biotinylated proteins (Figure 11B). The high abundance of endogenous biotinylated proteins highlights the importance of the application of cleavable linkers.

3.1.4 Test of capture and release efficiency of diol-DCG-04 with small amount of proteome

From the results in above experiments, we know diol-DCG-04 can label the same cathepsins as DCG-04 in rat liver lysate. Next, we would like to see if diol-DCG-04 can be used for efficient affinity capture of cathepsins from a complex proteome. In addition, the elution efficiency of the chemical cleavage was compared to a standard release by boiling in SDS sample buffer. The endogenously biotinylated proteins and probe-labeled proteins were visualized by biotin-streptavidin blot. Both probe-labeled cathepsins were fully enriched onto streptavidin beads since there were no cathepsins bands visible in the supernatant lane. The immobilized streptavidin beads were washed with PBS to make sure no cathepsins non-specifically bind to the beads. Afterwards the immobilized streptavidin beads were divided into equal amounts. One part was treated with 1 mM of $NaIO_4$ and subsequent boiled to check the efficiency of the chemical release. One part was directly boiled as a control. SDS boiling can efficiently elute all probe-labeled cathepsin from the beads. For both probes, there were no cathepsin bands in the $NaIO_4$ treatment lanes but the involved mechanisms are different. For DCG-04 samples, subsequent boiling show the same intensity of cathepsin bands as directly boiling. These results confirm that $NaIO_4$ does not disturb the interaction between biotin and streptavidin. For diol-DCG-04, subsequent boiling yields no cathepsins bands. It means diol-DCG-04-labeled cathepsins had already been released from the beads by $NaIO_4$. Due to cleavage of the diol linker, biotin still retained on the streptavidin beads. The released cathepsins cannot be visualized by biotin streptavidin blot due to the loss of biotin. Collectively, these results confirm that diol-DCG-04 enables the capture of cathepsins from a proteome. $NaIO_4$ selectively elutes diol-DCG-04 labeled cathepsins without disrupting the interaction of biotin and streptavidin (Figure 12).

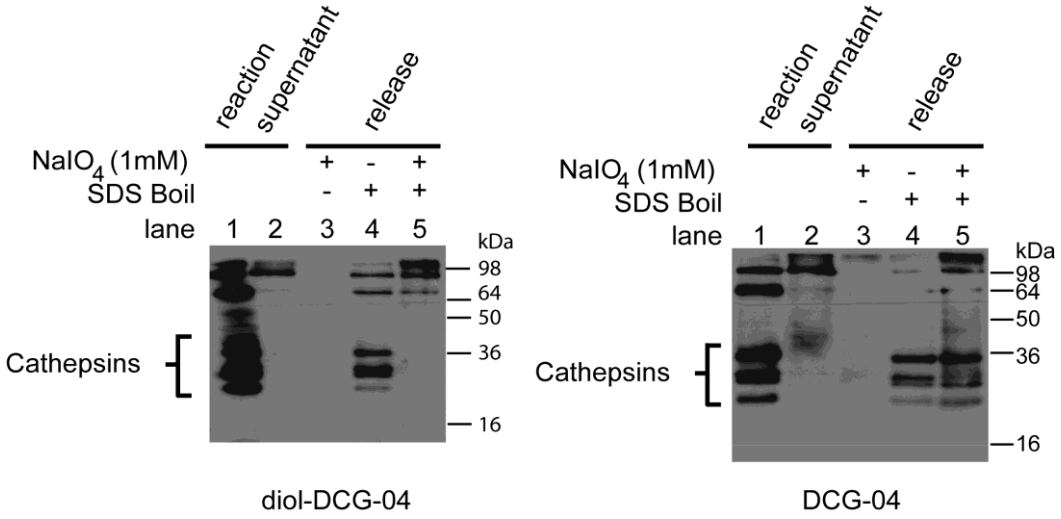
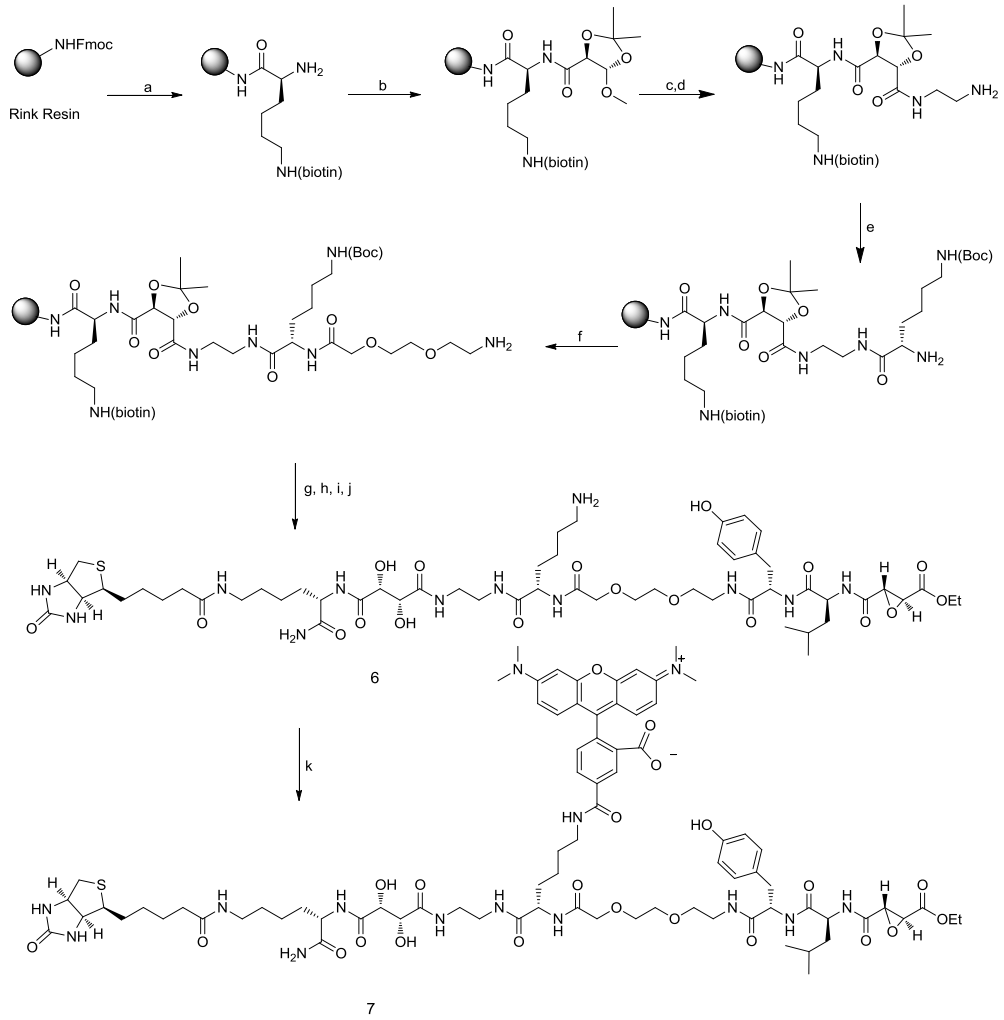


Figure 12: Enrichment of cathepsins from rat liver lysate and elution from streptavidin beads. Cathepsins labeled by diol-DCG-04 (left gel) or DCG-04 (right gel) can be efficiently pulled down by streptavidin beads (compare lanes 1 and 2). They are released by boiling (Lanes 4). Treatment with $NaIO_4$ (lanes 3) does not show any cathepsin bands, since they have lost their biotin (diol-DCG-04; left gel) or are not eluted (DCG-04; right gel). Subsequent boiling of these samples releases the remaining biotinylated proteins. For DCG-04, both endogenously biotinylated proteins and probe labeled cathepsins are eluted in the subsequent boiling. For diol-DCG-04, only endogenously biotinylated are eluted, indicating efficient release of diol-DCG-04 labeled proteins by $NaIO_4$.

3.1.5 Synthesis and evaluation of TMR-diol-DCG-04

In the last section, the capture and release efficiency of diol-DCG-04 was analyzed by streptavidin blot. Though streptavidin blot is sensitive for the detection of biotinylated proteins, diol-DCG-04-labeled cathepsins released by $NaIO_4$ could not be visualized due to the absence of biotin. Hence, TMR-diol-DCG-04 was designed and synthesized. Compared with diol-DCG-04, a fluorescent tag was incorporated between the cleavable linker and the reactive group. The biotin of released protein still gets lost but remains attached to the fluorescent tag which allows the protein to be visualized by in-gel fluorescent scanning (84).

3.1.5.1 Synthesis of TMR-diol-DCG-04



Scheme 5: Synthesis of TMR-diol-DCG-04. a) 20% piperdine in DMF, then Fmoc-Lys(biotin)-OH, DIC, HOBt and 20% piperdine in DMF; b) diol cleavable building block 2, PyBOP, DIEA; c) 0.25M KOH in EtOH, 2 x 20 min; Then wash with 1% AcOH in EtOH and DMF; d) mono Fmoc-ethylene diamine, PyBOP, DIEA; then 20% piperdine in DMF; e) Fmoc-Lys(Boc)-OH, DIC, HOBt; then 20% piperdine in DMF; f) Fmoc-8-amino-3, 6-dioxaoctanoic acid, DIC, HOBt; then 20% piperdine in DMF; g) Fmoc-Tyr(tBu)-OH, DIC, HOBt; then 20% piperdine in DMF; h) Fmoc-Leu-OH, DIC, HOBt; then 20% piperdine in DMF; i) ethyl(2S, 3S)-epoxysuccinate, DIC, HOBt; j) TFA: TIS: H_2O (90%: 2.5%: 7.5%) 3h. then precipitation with cold diethyl ether and purification with by HPLC. k) cleavable building block **6**, 5(6)-carboxytetramethylrhodamine succinimidyl ester and DIEA; 56% HPLC purification.

TMR-diol-DCG-04 was synthesized by a combination of solution and solid phase synthesis. After the coupling of Fmoc-Lys(biotin)-OH and cleavable building block, the resin bound intermediate was treated with 0.25 M KOH to deprotect the methyl ester protecting group, which was followed by washing with 1% AcOH in EtOH and DMF. Fmoc-Lys(Boc)-OH was introduced to provide an amine function for later coupling of 5(6)-carboxytetramethylrhodamine succinimidyl ester in solution. The other elements were introduced by standard solid phase synthesis. The build block was removed from resin and 5(6)-carboxytetramethylrhodamine succinimidyl ester was coupled to amine of lysine in solution which was purified with HPLC.

3.1.5.2 Capture and release cathepsins in RAW cell lysate

The active cathepsins were labeled by TMR-diol-DCG-04 and immobilized by streptavidin beads. SDS boiling enables release of immobilized proteins which can be visualized by biotin-streptavidin blot and fluorescent gel. $NaIO_4$ released most of the immobilized cathepsins, which is shown in the fluorescent gel but not in the streptavidin blot (Figure 13). These results

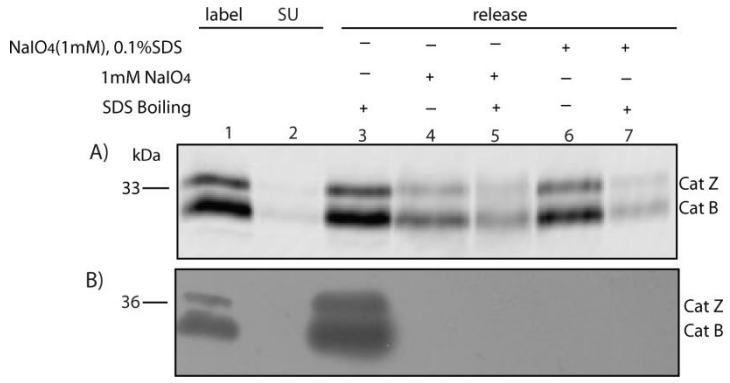


Figure 13: Enrichment of cathepsins from RAW cell lysate with TMR-diol-DCG-04 and elution from streptavidin beads. Cathepsins are labeled by TMR-diol-DCG-04 (1µM for 1h) (Lane 1) can be efficiently pulled down by streptavidin beads (compare Lanes 1and 2). They are released by boiling (Lanes 3). Treatment with $NaIO_4$ (1 mM) in SP (Lanes 4) or $NaIO_4$ in SP with 0.1% SDS (Lane 6) show the cathepsin bands in fluorescent gel but not in western blotting, since they have lost their biotin but carried a fluorescent tag. Subsequent boiling of these samples release the remaining labeled proteins (lane 5 and lane 7). Upper gel: fluorescent gel scanning. Lower gel: biotin-streptavidin western blotting.

indicated that the elution by $NaIO_4$ is induced by cleavage of the diol linker. However, the release was not very efficient since there was a little protein in the subsequent boiling lane in fluorescent gel but not in western blot gel. It means the cleavable linker indeed cleaves by $NaIO_4$ but the released proteins bind to beads or tubes. A previous publication showed usage of SDS to help keeping the released proteins in solutions (87). In our study, adding of 0.1% SDS in cleavage solution released more protein than $NaIO_4$ alone. Subsequent boiling lanes did not yield any biotinylated cathepsins for both samples. 0.1% SDS has no effect on the cleavage efficiency of $NaIO_4$ treatment. The addition of 0.1% SDS recovered a little more proteins should be due to the fact that it aids the solubilization of proteins or prevents them from sticking to plastics (e.g. eppendorf tubes).

3.1.6 Capture and release cathepsins from rat liver proteome

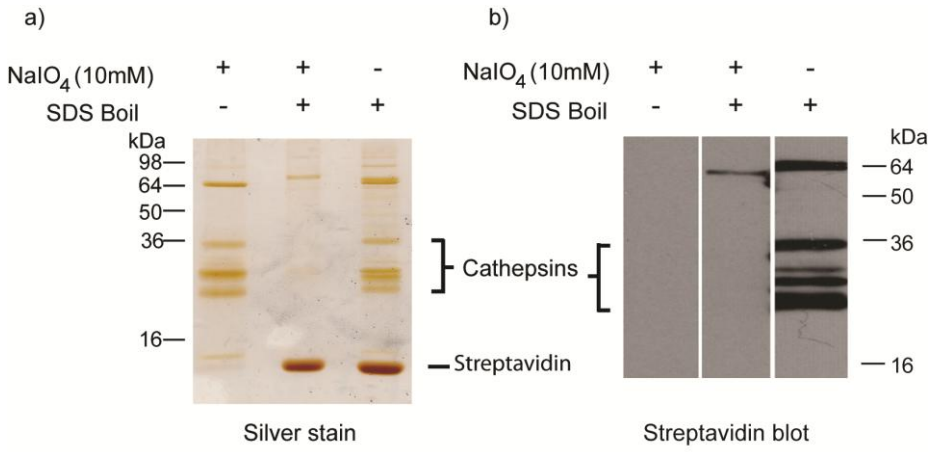


Figure 14: Comparison of chemical elution and SDS boiling elution of enriched rat liver lysate cathepsins. (A) Selective (10 mM $NaIO_4$) or non-selective **(**SDS sample buffer boil) release of cathepsins from streptavidin beads detected by silver staining. Boiling of the beads after selective release does not elute additional cathepsin proteins. (B) Streptavidin western blot of the same samples as in the silver staining. Note that the $NaIO_4$ cleaved proteins are invisible due to the chemoselective removal of the biotin part.

To evaluate the application of diol-DCG-04 for large scale protein capture and release in a complex proteome, cathepsins in rat liver lysate were labeled with diol-DCG-04. The labeled

cathepsins were captured by incubation with streptavidin beads. The enriched proteins were eluted under two conditions: either selective elution with 10 mM $NaIO_4$ or non-selective elution by boiling in SDS sample buffer. The eluted proteins were separated by SDS-PAGE and visualized by silver staining. Both elution samples show almost the same intensity of cathepsins bands. Subsequent boiling of the periodate-treated sample didn't release any additional cathepsins, indicating quantitative elution induced by $NaIO_4$ treatment. The background protein of approximately 65 kDa was visible in both samples. However, a large amount of free streptavidin along with a faint smear of non-selectively bound high molecular weight proteins was detected in the SDS-treated sample, indicating that SDS boiling elution results in more background proteins than chemical release (84, 96). The samples of each lane were also subjected to SDS-PAGE and visualized with biotin streptavidin blot. Biotinylated proteins were detected in SDS treated sample but not in $NaIO_4$ treated sample indicating that the cleavage of the diol linker led to proteins release in the $NaIO_4$ treated sample (Figure 14).
To show if a lower concentration of $NaIO_4$ could release proteins efficiently, the immobilized probe-cathepsin complexes were treated with 1mM for 2 h at room temperature (Figure 15) or 1mM overnight at 4°C (Figure 16). Biotin-streptavidin blot shows that diol-DCG-04 labeled cathepsins were fully immobilized by streptavidin beads. Proteins were released by $NaIO_4$ treatment and subsequent SDS boiling to see the efficiency of chemical elution. Unfortunately, proteins only partially eluted under both conditions.

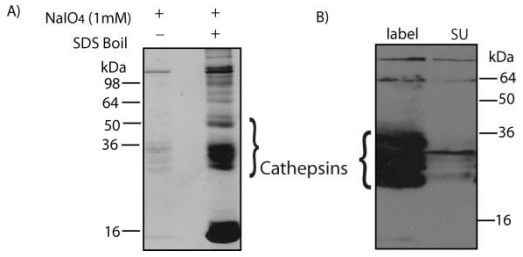


Figure 15: Elution of enriched rat liver lysate cathepsins with 1 mM of $NaIO_4$ at room temperature (2h). A) Selective ($NaIO_4$, 1mM) release of cathepsins from streptavidin beads detected by silver staining (left lane). Subsequent boiling of the beads after selective release elutes additional cathepsin proteins (right lane). B) Biotin-streptavidin western blot to detect the pull down efficiency. Cathepsins labeled by diol-DCG-04 (left lane) can be efficiently pulled down by streptavidin beads (compare left lane and right lane).

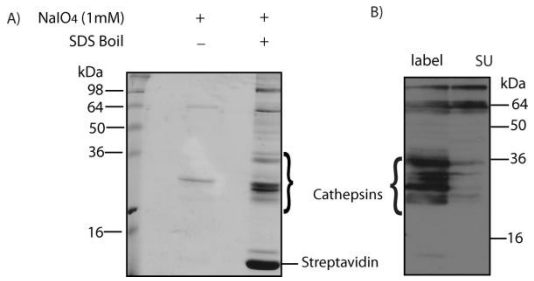


Figure 16: Elution of enriched rat liver lysate cathepsins with 1 mM of $NaIO_4$ at 4°C (overnight). A) Selective ($NaIO_4$, 1mM) release of cathepsins from streptavidin beads detected by silver staining (left lane). Subsequent boiling of the beads after selective release elutes additional cathepsin proteins (right lane). B) Biotin-streptavidin western blot to detect the pull down efficiency. Cathepsins labeled by diol-DCG-04 (left lane) can be efficiently pulled down by streptavidin beads (compare left lane and right lane).

3.1.7 Identification of protein targets by mass spectrometry in rat liver proteome

3.1.7.1 On-bead digestion and chemical elution based solution digestion

Traditionally, identification of protein targets enriched by streptavidin beads can be performed by elution with SDS boiling followed by SDS-PAGE and in-gel digestion (Figure 17). In addition, on-bead tryptic digestion has also been carried out (64, 115). However, endogenously biotinylated proteins, non-specifically bound proteins and streptavidin will digest together with the target proteins during on-bead digestion. The digested peptides originating from the background proteins can be a problem for the detection of low abundance target protein identification. Cleavable linker based chemical release and solution digestion is an attractive method as an alternative to on-bead digestion (83, 84). The elution from chemical release can be digested with trypsin in solution (78, 83) or submitted to SDS-PAGE for in-gel digestion (87, 116) (Figure 17). Gel based assay showed that periodate elution has less contaminations than SDS boiling elution. The protein compatible elution buffer and the low elution volume make it suitable for in-solution digestion. To further quantify the efficiency of

background protein reduction, the samples of on-bead digestion and periodate release followed by in-solution digestion were compared by LC-MS/MS.

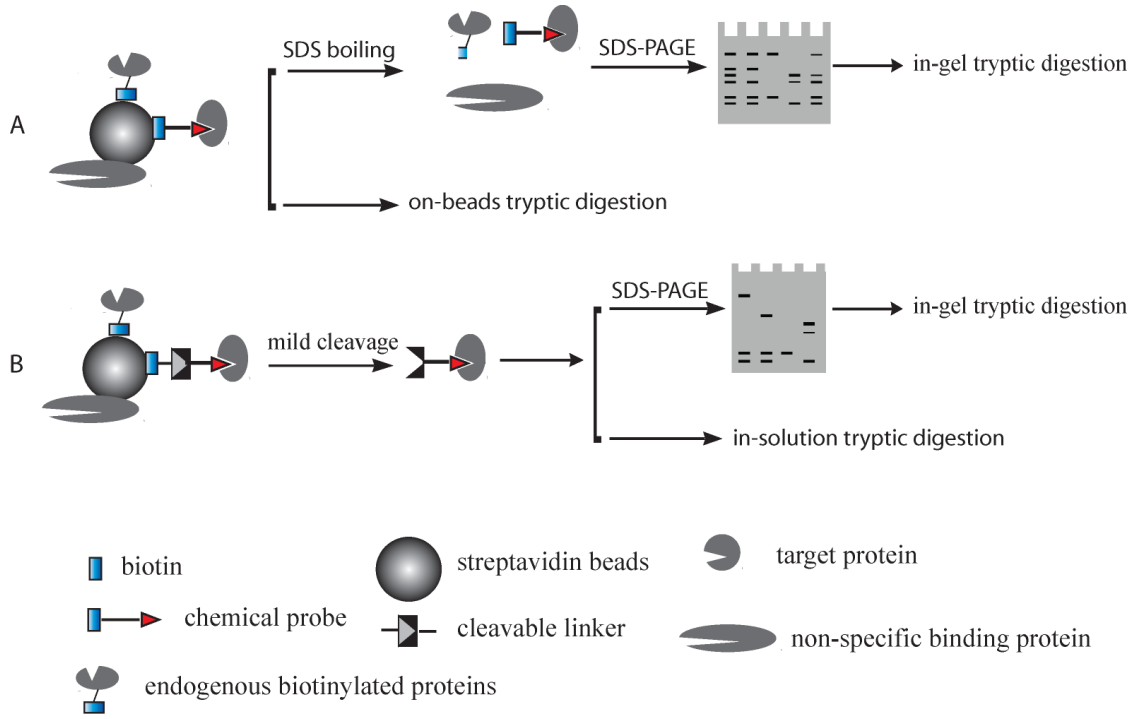


Figure 17: The flow charts for elution and tryptic digestion of immobilized proteins for subsequent MS analysis. A) For non-cleavable chemical probe: the immobilized proteins can be eluted with SDS boiling, separated by SDS-PAGE and subjected to in-gel digestion. The immobilized proteins can also be directly digested on beads. B) For cleavable chemical probe: the immobilized proteins can be eluted under mild conditions and directly digested in solution. If necessary, the elution can be further purified with SDS-PAGE and subjected to in-gel digestion.

3.1.7.2 Identification of targets of diol-DCG-04 in rat liver lysate

To show the application of the diol cleavable linker in gel free methods, protein targets of the cleavable version of DCG-04 in rat liver lysate were enriched for comparison of on-bead digestion and chemical release-based gel-free digestion. Hence, diol-DCG-04 was incubated with rat liver lysate and probe-modified cathepsins were enriched with streptavidin beads. The pull down efficiency was detected by biotin-streptavidin blot (Figure 18).

To reduce the amount of non-specifically bound proteins, stringent washing steps with detergent containing buffers are performed prior to chemical release or on-bead digestion (78, 85, 91). Therefore the beads were extensively washed by 1% SDS, 4M urea, 1M NaCl, 10% EtOH and PBS to remove most non-specifically bound proteins. The streptavidin beads were then divided into two equal aliquots. For on-bead digestion, proteins were digested with trypsin followed by reduction of disulfide bonds and alkylation with iodoacetamide. For chemical release and solution digestion, the beads were treated with $NaIO_4$ cleavage buffer to elute diol-DCG-04 labeled proteins. The elution was subjected to zeba spin column in order to get rid of $NaIO_4$ which may disturb the activity of trypsin. The elution from zeba spin column was subjected to reduction and alkylation followed by trypsin digestion. The peptides were purified by C18 column and subjected to LC-MS/MS analysis.

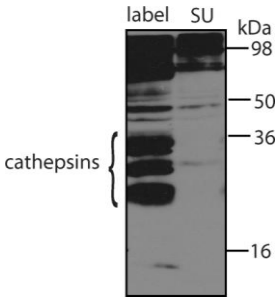


Figure 18: Enrichment of cathepsins from a rat liver lysate for MS analysis. Cathepsins labeled by diol-DCG-04 (left lane) were efficiently pulled down by streptavidin beads (compare left lane and right lane).

The identified proteins contain targets of diol-DCG-04 and background protein containing endogenously biotinylated proteins and non-specific binding proteins. LC-MS/MS analysis revealed the presence of 7 cathepsins in both chemical release and on-bead digestion samples. Cat S and Cat L1 were identified in our research but not detected in the previous reports (117). The sequence coverage of Cat L1 in the chemical release sample was less than on-bead digestion, but the coverages of other cathepsins are nearly the same (Table 2). The mild elution condition successfully avoids the contamination of endogenously biotinylated proteins. The endogenously biotinylated proteins propionyl-CoA carboxylase alpha chain, carbamoyl-phosphate synthase and pyruvate carboxylase were only detected in the on-bead digestion with many unique peptides and high protein sequence coverage, but not in the chemoselectively released sample (Table 3). This is direct evidence that the interaction of biotin and streptavidin

was not disturbed by $NaIO_4$ treatment. In the on-bead digestion sample, also a lot of non-specifically bound proteins were found. Highly abundant cytoskeletal proteins, such as actin, keratins and tubulins were identified in both samples. Overall, samples obtained by chemical cleavage showed a nearly 80% reduction in background proteins compared to on-bead digestion (Figure 22).

Table 2: Cathepsins identified in rat liver lysate. Listed are the number of unique peptides and the sequence coverage (in brackets) of all cathepsin targets of diol-DCG-04 identified from a rat liver lysate.

Protein name	Accession number	Chemical cleavage	On-bead digestion
Cathepsin Z	Q9R1T3	10 (32%)	11 (30%)
Cathepsin B	P00787	9 (24%)	11 (28%)
Cathepsin C	P80067	7 (22%)	8 (23%)
Cathepsin H	P00786	9 (26%)	8 (26%)
Cathepsin S	Q02765	3 (15%)	5 (17%)
Cathepsin J	Q63088	2 (9.3%)	4 (14%)
Cathepsin L1	P07154	2 (5.4%)	10 (32%)

Table 3**:** Endogenous biotinylated proteins identified in rat liver lysate. Listed are the number of unique peptides and the sequence coverage (in brackets).

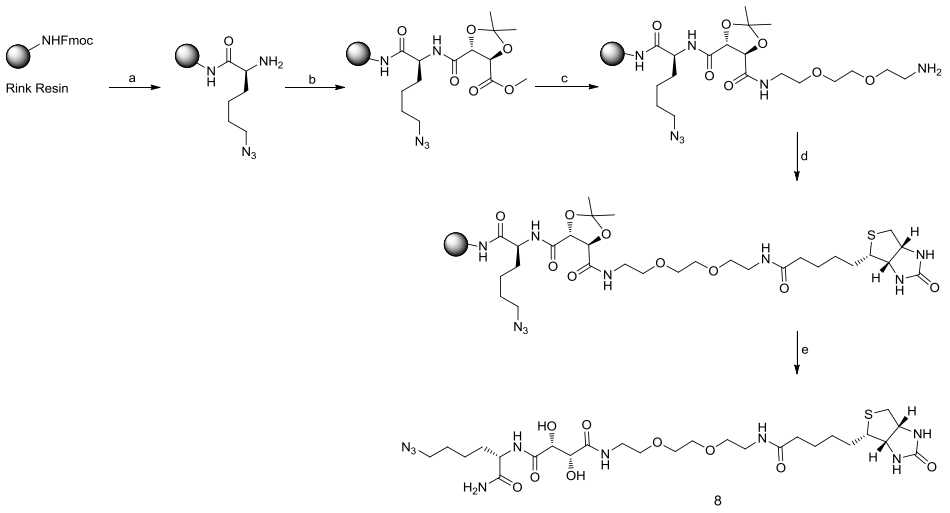
Protein name	On-bead digestion	Chemical cleavage
Propionyl-CoA carboxylase alpha chain	52 (60%)	0 (0%)
Carbamoyl-phosphate synthase	24 (19%)	0 (0%)
Pyruvate carboxylase	13 (11%)	0 (0%)

3.1.8 Application of diol cleavable linker in tandem labeling

Biotinylated chemical probes afford the chance for detection and purification of protein targets in complex proteome. But most of these probes can only be used in cell or organ extracts and not in living cells due to the low cell permeability. To overcome this problem, the tandem labeling strategy developed by Cravatt allows wide application of chemical probes in cells or *in vivo* (47, 51). To combine the tandem labeling strategy and cleavable linker strategy, several cleavable linkers were introduced to clickable biotin reagents for identification the cellular targets (83, 85, 87, 96, 102, 109).

In order to show the application of the diol linker in tandem labeling, we synthesized biotin-diol-azide (**8**) and biotin-diol-alkyne (**11**) for ligation with the clickable probe-protein complex. E64 derived probes, azido-E64 (**14**) and alkyne-E64 (**16**) were synthesized and label cathepsins in complex proteome to validate the applications of biotin diol reagents.

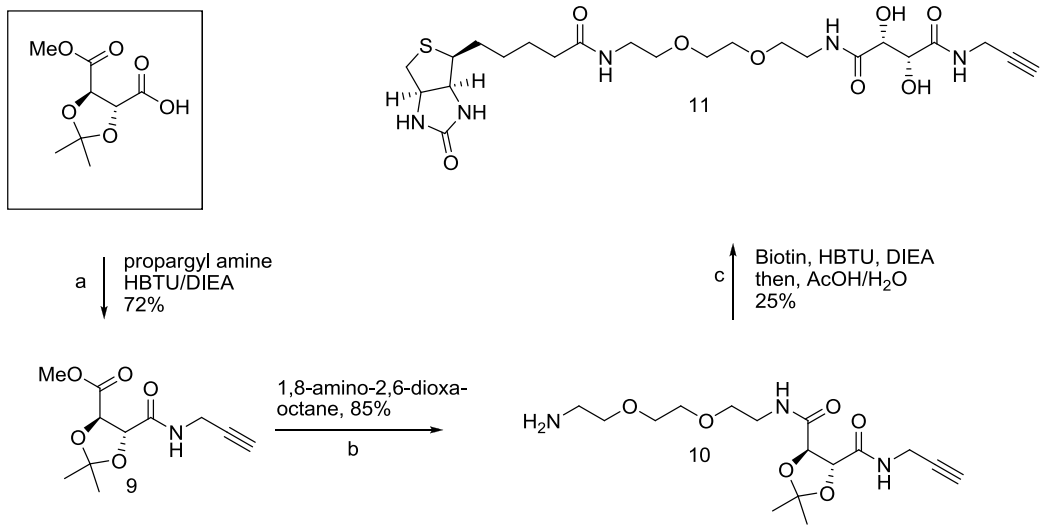
3.1.8.1 Synthesis of biotin-diol-azide



Scheme 6: Synthesis of biotin-diol-azide. a) 20% piperdine in DMF, then Fmoc-Lys(azide)-OH, DIC, HOBt; 20% piperdine in DMF; b) diol cleavable building block **2**, DIC, HOBt; c) 1,8-diamino-3,6-dioxaoctane, overnight; d) biotin, DIEA, PyBOP; e) TFA : TIS: H_2O (90% : 2.5% : 7.5%) 3 h, then precipitation with cold diethyl ether and purification by HPLC.

Solid phase synthesis enables synthesis of only micromole scale of product but it is easily handled. Since Fmoc-Lys(azide)-OH is commercially available, we show that biotin click tag can be assembled on solid phase similar to the synthesis of diol-DCG-04 (Scheme 6). The biotin tag was cleaved from resin and purified with a 20% yield. The sample synthesis may allow the reagent widely used in biology laboratories.

3.1.8.2 Synthesis of biotin-diol-alkyne

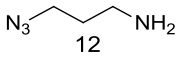


Scheme 7: Synthesis of biotin-diol-alkyne. a) diol cleavable building block **2**, DIEA, HBTU; 72% after silica column chromatograph purification; b) 1,8-diamino-3,6-dioxaoctane, 80 ^{o}C overnight; 85% after silica column chromatography purification; c) biotin, HBTU, DIEA overnight; 25% after HPLC purification.

We have shown that the diol cleavable building block can be used to synthesize clickable biotin reagent on solid phase. To show the cleavable building block can be used to synthesize clickable biotin reagent in large scale, we synthesized biotin-diol-alkyne in solution (Scheme 7). Diol cleavable building block was coupled to propargylamine with HBTU/DIEA. After extraction and silica column purification, the product was reacted with an excess of 1,8-diamino-3,6-dioxaoctane in toluene at 80 °C. The resulting compound was purified (silica column chromatography) and coupled to biotin under the influence of HBTU and DIEA in

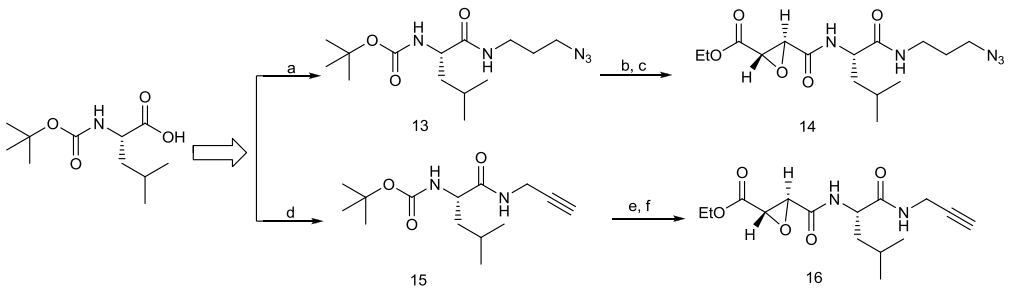
dimethylformamide. The solvent was evaporated and the residue heated in 90% acetic acid for deprotection of the isopropylidene group. The solvent was removed, and the final product was purified via HPLC.

3.1.8.3 Synthesis of alkyne-E64 and azido-E64



Azido-proplyamine **12** was acquired by reaction between chloropropylamine and sodium azide in water. The resulting compound **12** was extracted and can be used in subsequent experiments without purification.

Alkyne-E64 and azido-E64 were synthesized following a literature procedure (118). In brief, anhydrides derived from Boc-Leu-OH and isobutyl chloroformate were coupled with propargylamine or azido-proplyamine. The Boc protection group of the resulting building blocks **13** and **15** was removed with TFA/DCM. After evaporation, the residue was reacted with ethyl (2S, 3S)-epoxysuccinate under the influence of DIEA/HBTU. The resulting compound was purified (silica column chromatography) (Scheme 8).



Scheme 8: Synthesis of azido-E64 and alkyne-E64. a) BCF and NMM, 15 min at -20°C; then 4-methylmorpholine, azido-proplyamine for another 3 h at room temperature; b) 25% TFA/DCM, 1h; c) ethyl (2S, 3S)-epoxysuccinate, HBTU, DIEA, overnight; d) BCF and NMM, 15 min at -20°C; then NMM, propargylamine for another 3 h at room temperature; e) 25% TFA/DCM, 1h; f) ethyl (2S, 3S)-epoxysuccinate, HBTU, DIEA, overnight.

3.1.8.4 Labeling of cathepsins in RAW cell lysate and RAW cells

Cell permeability of azido-E64 was evaluated by a competition experiment. Raw cells were treated with azido-E64 at indicated concentrations and time. Lysate from these treated cells were then incubated with DCG-04 to detect the residually active cathepsins. The labeled proteome was separated with SDS-PAGE and visualized by biotin streptavidin blot. The intensity of DCG-04 labeled cathepsins from control cells was higher than cells pre-treated with azido-E64. It means the azido-64 blocked all the active cathepsins in the cell (Figure 19). The cell permeability of alkye-E64 was detected with a similar method (Figure 21).

To visualize the target proteins of azido-E64 in RAW cell lysate, the lysate was incubated with azido-E64 for 1h and then applied to a Zeba gel filtration spin column to get rid of free probe. The elution was subjected to click chemistry with biotin-diol-alkyne under the influence of $CuSO_4$, TCEP and TBTA. Biotin streptavidin blot showed azido-E64 labels Cat Z and Cat B in the cell lysate which are the same protein targets as DCG-04 (Figure 20A). To show the cellular targets of azido-E64, a cell lysate from azido-E64 treated live cells was subjected to click chemistry and the proteins were visualized. There are four biotin blot bands for the two different labeling conditions (20 μM, 0.5 h and 5 μM, 1h). Treatment of RAW cells with JPM-OEt, a cell permeable cathepsin inhibitor, prior to the addition of azido-E64 inhibits the labeling of these protein targets (Fig 20B). Hence, more protein targets were labeled by azido-E64 in living cells than in cell lysate.

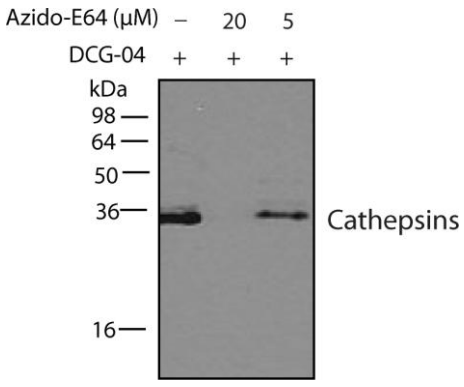


Figure 19: Streptavidin western blots of testing the cell permeability of azido-E64 in RAW 264.7 cells. A. Cells were treated with azido-E64 (20 μM, 0.5h or 5 μM, 1h). Lysates were prepared and labeled with DCG-04 to detect residual active cathepsins.

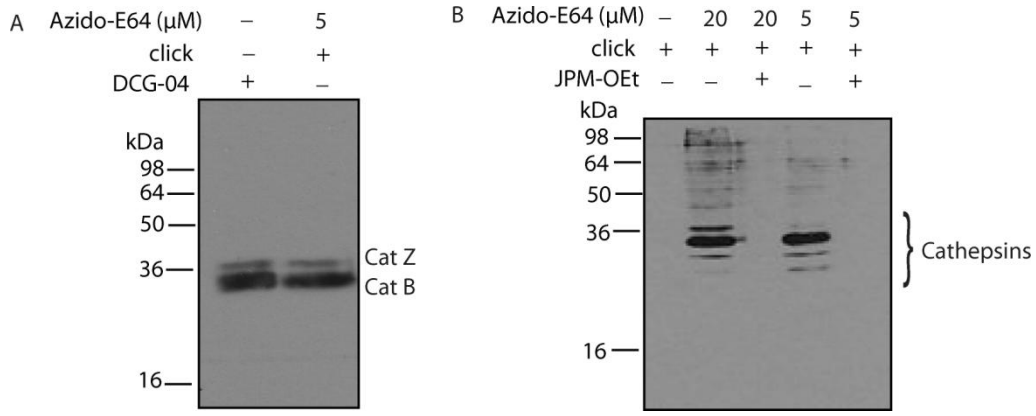


Figure 20: Labeling of cathepsins in RAW cell lysate and RAW cells. A) RAW cell lysate was incubated with azido-E64 at indicated concentration for 1h. After running zeba spin column to get rid of free azido-E64, the cell lysate reacted with biotin-diol-alkyne under the influence of $CuSO_4$ (1 mM), TBTA (50 μM) and TCEP (1 mM). B) RAW cells were treated azido-E64 (20 μM, 0.5h or 5 μM, 1h). To show the labeled proteins are cathepsins, RAW cells pretreated with JPM-OEt (50 μM) for 1h were treated azido-E64 (20 μM, 0.5h or 5 μM, 1h). The cell lysate reacted with biotin-diol-alkyne under the influence of $CuSO_4$ (1 mM), TBTA (50 μM) and TCEP (1 mM). The samples were loaded on SDS-PAGE and proteins were visualized with biotin-streptavidin blot.

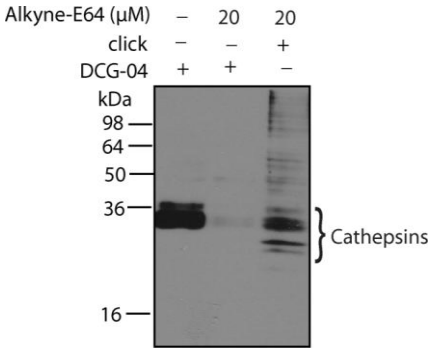


Figure 21: Streptavidin western blots of testing the cell permeability test of alkyne-E64 and labeling of protein targets in RAW cells. Left lane: RAW cell lysate was treated with DCG-04. Middle lane: cells were treated with alkyne-E64 (20 μM, 0.5h). Lysates were prepared and labeled with DCG-04 to detect residual active cathepsins. Right lane: cells were treated with alkyne-E64 (20 μM, 0.5h). Lysate was subjected to click chemistry with biotin-diol-azide. Proteins were separated by SDS-PAGE and visualized by biotin-streptavidin blot.

Using the same methods, we showed that alkyne-E64 is also cell permeable. Cell lysate from RAW cells treated with alkyne-E64 and reacted with biotin-diol-azide resulted in labeling of the same protein targets as azido-E64 (Figure 21).

3.1.8.5 Identification of targets of azido-E64 in RAW cell lysate and living RAW cells

We next performed identification of the protein targets of azido-E64 in RAW cell lysate and RAW cells with tandem labeling and LC-MS/MS. For *in vitro* labeling, RAW cell lysate was incubated with azido-E64 (10 μM, 1h) and free probe was removed by PD-10 column. The elution was subjected to click chemistry with biotin-diol-alkyne. For cellular targets, RAW 264.7 macrophages were treated with azido-E64 (5 μM, 1h), washed, and lysed. The cell proteome extract can be labeled with biotin-diol-alkyne under the same click chemistry conditions as described before. The reaction mixture was run over a PD-10 column to get rid of free biotin-diol-alkyne. The elution was incubated with streptavidin beads and an aliquot of the supernatant on western blot showed the probe-labeled-cathepsins were fully immobilized onto beads in both samples. Labeling in whole cells resulted in the immobilization of more protein target than in cell lysate.

Following the same workflow as during the identification of targets of diol-DCG-04 in rat liver lysate, chemical elution was followed by in solution digestion and compared with on-bead digestion by LC-MS/MS. Efficiency for reduction of background proteins was comparable to the above experiment. More than 85% of background proteins are reduced by chemical elution compared with on-bead digestion (Figure 22).

Tandem mass spectrometry identified cathepsin Z and B both *in vitro* and *in situ* (Table 3 and 4, respectively). Cathepsins F, H, S and L1 were only identified in intact cells. These proteases correspond to the lower running gel bands, which match the molecular weights of the mature cathepsins H, S and L1 (24, 24 and 19 kDa, respectively). Cathepsin F was only identified in on-bead digestion but the coverage was just 4.5%. Taken together, the coverages of cathepsins identified *in vitro* and *vivo* are comparable in chemical release and on-bead digestion, indicating the chemical elution based solution digestion and on-bead digestion have similar

efficiency. The endogenous biotinylated proteins in RAW cell were only found in on-bead digestion with a lot of unique peptides but not in the chemical release sample (Table 5).

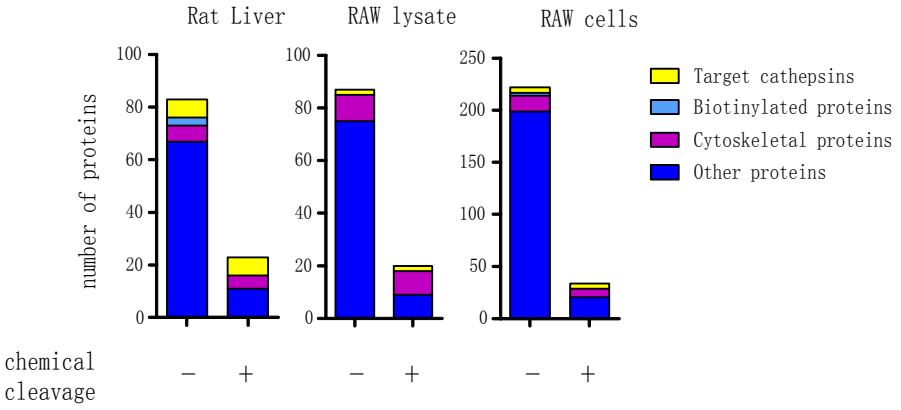


Figure 22: Overview of the background reduction in the proteomic analysis of ABP targets in rat liver, RAW cell lysate, and RAW cells. After labeling and removal of free probe, proteomes were incubated with streptavidin beads. Beads were collected, washed, and subjected to either on-bead digestion or chemical cleavage followed by digestion. Tryptic peptides were analyzed via LC-MS/MS. Contaminating non-rat or non-mouse proteins are not depicted. Proteins are classified as target cathepsins and background originating from endogenously biotinylated proteins, cytoskeletal proteins, and other proteins.

Table 3**:** Cathepsins identified in RAW cell lysate. Listed are the number of unique peptides and the sequence coverage (in brackets) of all cathepsin targets of azido-E64 identified from a RAW cell lysate.

Protein name	Accession number	On-bead digestion	Chemical cleavage
Cathepsin Z	Q9WUU7	9 (26%)	9 (25%)
Cathepsin B	P10605	11 (26%)	11 (26%)

Table 4: Cathepsins identified in RAW cells. Listed are the number of unique peptides and the sequence coverage (in brackets) of all cathepsin targets of azido-E64 identified from experiments with living RAW 264.7 cells.

Protein name	Accession number	On-bead digestion	Chemical cleavage
Cathepsin Z	Q9WUU7	8 (25%)	8 (21%)
Cathepsin B	P10605	11 (26%)	11 (26%)
Cathepsin H	P49935	6 (24%)	8 (29%)
Cathepsin S	O70370	5 (19 %)	10 (36%)
Cathepsin L1	P06797	6 (24%)	8 (29%)
Cathepsin F	Q9R013	3 (4.5%)	0 (0%)

Table 5: Endogenously biotinylated proteins identified in RAW cells. Listed are the number of unique peptides and the sequence coverage (in brackets).

Protein name	On-bead digestion	Chemical cleavage
Pyruvate carboxylase	23 (22%)	0 (0%)
Propionyl-CoA carboxylase alpha chain	23 (40%)	0 (0%)

It is interesting that more cathepsin targets identified in living cell than in lysate. A previous study has shown that the activity of Cat L1 is compromised when making a lysate (119). Indeed, we only identified the Cat L1 with the activity based probe azido-E64 in living RAW cell. Cathepsins H and S also failed to be identified in RAW cell lysate, probably because their activity became weak upon cell lysis. One possibility may be that the lysis buffer is different from the cellular condition. In addition, cytoplasmic inhibitors may react with these enzymes. Speers and co-workers also identified more enzyme targets of phenyl sulfonate ester based

ABP *in situ* than *in vitro* since these enzymes may be sensitive to the regulation of posttranslational modifications (47).

3.1.9 Identification of probe-modified peptides

Another application of the cleavable linker strategy is the identification of the modification site on the protein. One drawback of identification of modified peptides with conventional elution is that the biotin tag increases the mass of the modified peptide, reduces peptide ionization, and gives rise to fragmentation in the MS/MS (102). SDS-PAGE based visualization confirmed that $NaIO_4$ can remove the biotin in labeled cathepsins. The probe-peptide adducts were shown in Figure 23.

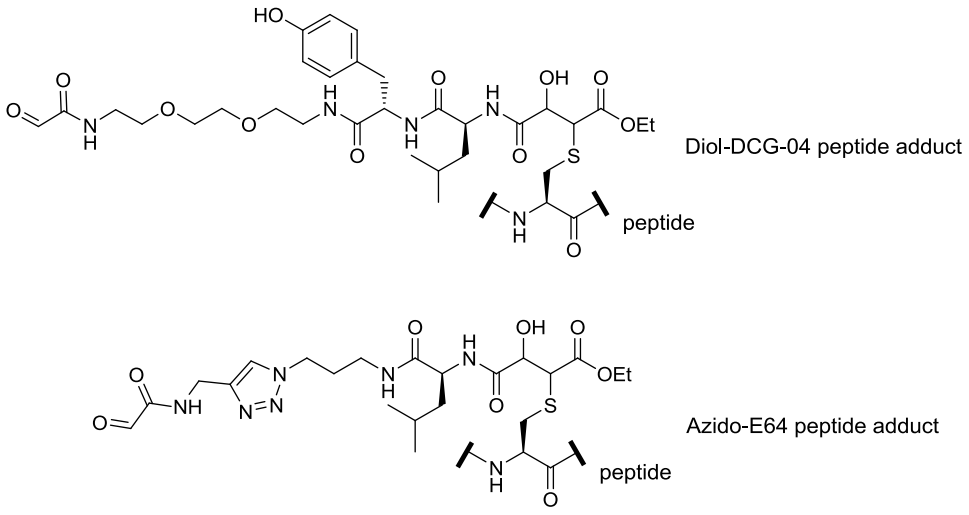


Figure 23: Probe peptide adducts.

However, the modification site of the probe in cathepsins has not been found. This is more inherent to the cathepsin protease targets than to the method. After tryptic digest, cathepsin active site peptides are very large and are very difficult to identify. To our knowledge, only one paper has successfully identified modified cathepsin active site peptides making use of (a relatively high) amount of purified cathepsin B (78).

3.1.10 The influence of oxidation on the identification of target proteins

Some concerns should be considered when using the diol cleavable linker. The methionines of proteins will be easily oxidized by periodate. To look into this, we analyzed the tryptic peptides of the cathepsin targets in rat liver lysate. Indeed, there were more oxidized methionines in the sample from chemical elution than in the sample from on-bead digestion (Table 6 and 7). However, the oxidation didn't have an influence on the identification of the target proteins.

$NaIO_4$ can potentially lead to oxidative cleavage of N-terminal serine and threonine residues. However, proteins with an N-terminal serine or threonine rarely occur (53). Tryptic digestion can generate peptide sequences with a serine or threonine at their N-terminus. Therefore, the $NaIO_4$ oxidizing agent should be removed prior to the digestion. In order to show that these side reactions do not happen during the tryptic digest, we looked at the identification of sequences of peptides with N-terminal of serine and threonine residues in the cathepsins identified by chemical elution digestion and on-bead digestion. One peptide with an N-terminal threonine from Cat S in rat liver lysate was only identified in the on-bead digestion. However, two peptides with N-terminal serine or threonine from Cat L1 in RAW cells were only found in the chemical cleavage sample. In addition, 7 other peptides were found in both samples (Table 8). Overall, the removal of $NaIO_4$ seems to be an effective way to prevent that N-terminal serine and threonine containing peptides are damaged.

The oxidative cleavage of the diol linker generates a reactive aldehyde. In addition, many cathepsins are N-glycosylated (120) and will also generate aldehydes after treatment with $NaIO_4$. The N-terminal of serine and threonine will also be oxidized to aldehydes. One concern is the aldehydes will generate a Schiff base with streptavidin which will prevent the elution of proteins. However, the intensity of cathepsins released by $NaIO_4$ was nearly the same as released by SDS boiling, indicating this process has no effect on the efficiency of chemical elution (Figure 14).

We also concern if the aldehyde containing peptides will react with amines of the N-terminus and the lysine side chain in eluted proteins and trypsin. It may lead to complex peptide mixtures and will affect the MS identification. To test this, a mixture of diol-DCG-04 and bradykinin was treated with $NaIO_4$. Previous experiments have shown (Figure 9 and 10) that

Table 6: The number of total and oxidized methionine residues in all identified peptides of the cathepsin targets in rat liver proteome.

	On-bead digestion		Chemical digestion	
Cathepsins	Total Met	Oxidized Met	Total Met	Oxidized Met
Cathepsin Z	26	3	30	9
Cathepsin B	24	6	18	12
Cathepsin H	6	0	6	1
Cathepsin L	4	0	0	0
Cathepsin C	3	0	3	2
Cathepsin J	0	0	0	0

Table 7: Spectral counts of selected cathepsin peptides in oxidized and non-oxidized form from rat liver proteome.

		On-bead digestion		Chemical cleavage	
	Selected peptides[1]	Non-oxidized	oxidized	Non-oxidized	oxidized
CatB	HEAGDV**M**GGHAIR	3	2	1	3
	SGVYKHEAGDV**M**GGHAIR	3	1	2	2
Cat Z	M**M**AEIYANGPISCGIMATER	2	1	1	4
Cat C	NQESCGSCYSFASLG**M**LEAR	2	0	1	1
Cat H	GI**M**GEDSYPYIGK	2	0	2	1

[1]Oxidized methionines are indicated in bold

Table 8: Identified peptides with N-terminal of Serine or Threonine of cathepsin targets.

Samples	Protein name	Peptide sequence	On-bead digestion	Chemical cleavage
Rat liver lysate	Cat S	TGKLVSLSAQNLVDCSTEEK	+	-
	Cat H	SGVYSSNSCHKTPDK	+	+
	Cat B	SGVYKHEAGDVMGGHAIR	+	+
RAW cell lysate	Cat B	SCEAGYSPSYK	+	+
		SGVYKHEAGDMMGGHAIR	+	+
Living RAW cell	Cat B	SCEAGYSPSYK	+	+
		SGVYKHEAGDMMGGHAIR	+	+
	Cat L1	TLPDTVDWR	+	+
		SGVYDDPSCTGNVNHGVLVVGYGTLDGKDYWLVK	-	+
		TGKLISLSAQANLVDCSNEEK	-	+

the cleavage products have no reaction with bradykinin at a concentration of 10 µM. Furthermore the comparable sequence coverage for cathepsins identified by chemical elution digestion and on-bead digestion confirms that these potential side reactions, if they occur at all, have no affect on the sequence coverage.

Overall, we compared the chemical elution based solution digestion and on-bead digestion. Cathepsins identified by chemical release and on-bead digestion showed nearly the same sequence coverage. Mild elution conditions facilitate more reduction in background protein identification of more than 80% compared to on-bead digestion. The probe-modified peptides have not been identified, which is likely due to the large size of the digested peptide. Though oxidation of $NaIO_4$ can lead to some side reactions, it has no effect on the efficiency of target identification.

3.2 Cleavable trifunctional biotin reagents for protein labeling, capture and release

The importance of the target characterization of chemical probes in cellular condition was illustrated by the finding from the previous section, where we identified more targets of azido-E64 in living RAW cells than in lysate. Trifunctional biotin reagents have been developed to detect probe-modified proteins in living system (25, 51). However, efficient cleavable forms of them are still missing. In this section, we designed and synthesized trifunctional reagents with five different cleavable linkers in order to facilitate purification of target proteins with the biotin streptavidin system under mild release conditions (Figure 24). These reagents were successfully assembled combining solid phase and solution phase synthesis. Their compatibility with bioorthogonal ligation and the efficiency were evaluated by labeling, immobilization and subsequent release of alkyne-E64 modified cathepsins in RAW cell lysate.

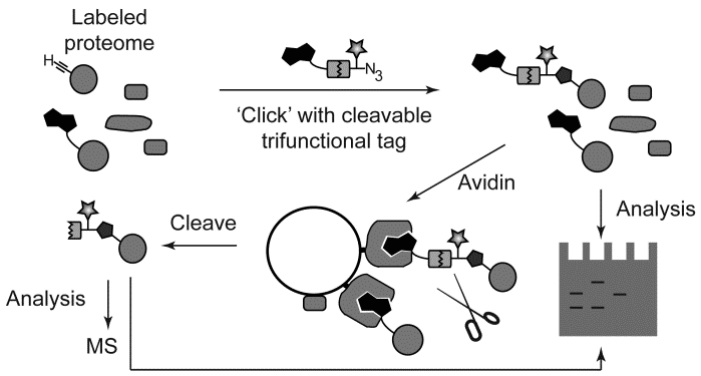


Figure 24: Cleavable trifunctional tags in chemical proteomics serve sensitive detection, enrichment of targets and selective elution. Alkyne-probe-modified proteins react with cleavable trifunctional tags. The labeled proteins can be analyzed in gel fluorescent scanning and enriched with streptavidin beads. The elution induced by chemical cleavage can be analyzed in gel fluorescent scanning or mass spectrometry.

3.2.1 Design of trifunctional biotin reagents

Each of the cleavable trifunctional tags has several different functional elements (Figure 25). An azide group is introduced for conjugation with alkyne-probe-labeled proteins. A PEG linker is introduced to increase the solubility of the trifunctional reagents in aqueous system. Biotin enables efficient protein enrichment with streptavidin beads. A tetramethylrhodamine

(TMR) fluorophore is used for the visualization of released proteins. Incorporation of cleavable linkers enables the selective release of immobilized proteins by the respective release conditions. Five different cleavable linkers were studied in this project.

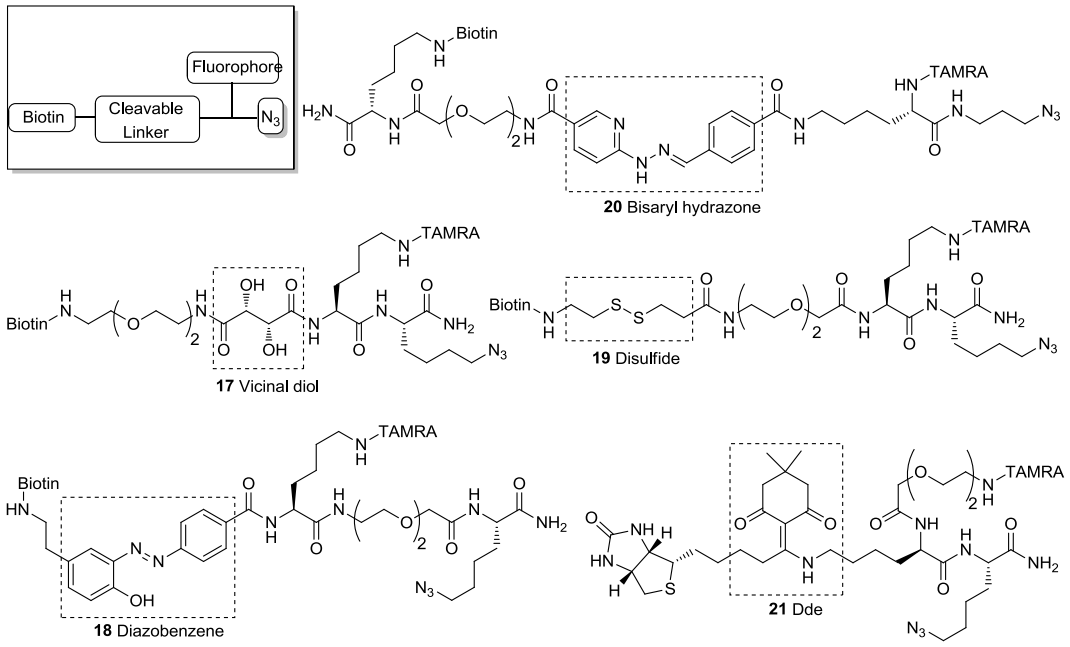


Figure 25: The structures of cleavable trifunctional tags **17-21**. All contain an azide, a TMR fluorophore, a cleavable linker (boxed) and a biotin.

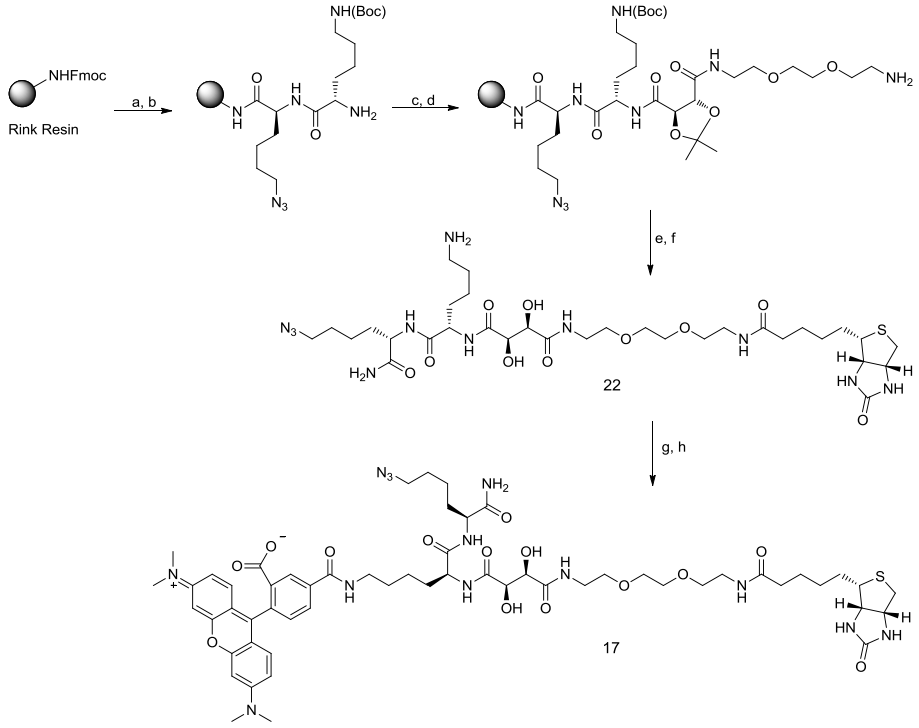
In the last section, we have shown that the diol cleavable linker is easily acquired and incorporated into probes and biotin reagents. The application of this linker enabled the reduction of background proteins. Furthermore, we have shown that the oxidation condition is compatible with in-gel fluorescent scanning. These advantages make the diol linker a good choice for trifunctional biotin reagents. Diazobenzene based linkers are biocompatible and cleaved by mild reducing conditions such as dithionite (84). They have been introduced into intact probes and clickable biotin reagents to aid the identification of protein targets (84, 87). The compatibility of dithionite release conditions was confirmed by previous reports (84). The disulfide linker is sensitive to reductive conditions such as DTT or TCEP. The mild release conditions and commercial availability allow the disulfide linker to be widely applied in

chemical biology (121). Bisaryl hydrazone based linkers have been reported as biocompatible linkers which can be cleaved by nucleophiles such as hydroxylamine (97). The usage of this linker in biotin reagents in combination with bioorthogonal ligations has not been studied. We also developed a new linker based on the 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (dde) group which is used as a protecting group for the ε-amino group of lysine in solid phase peptide synthesis (SPPS) (122). The conventional way of deprotecting the dde group is with a low percentage of hydrazine.

3.2.2 Synthesis of cleavable trifunctional biotin reagents

3.2.2.1 Synthesis of biotin-diol-TMR–azide

The synthesis of biotin-diol-TMR-azide was started with the coupling of Fmoc-Lys(azide)-OH to rink amide resin. Fmoc-Lys(Boc)-OH was introduced as an amino containing building block for coupling with TMR in solution (Scheme 9). After the coupling of the diol cleavable building block, the resin was treated with 1,8-diamino-3,6-dioxaoctane. This PEG linker increases the solubility of the tag in aqueous solution. Biotin was incorporated under the influence of DIC/HOBt. The cleavable building block **22** was cleaved from the resin with 90% TFA, purified by HPLC and reacted with 5(6)-carboxytetramethylrhodamine succinimidyl ester. The final product was detected by LC-MS and purified by HPLC (Scheme 9).

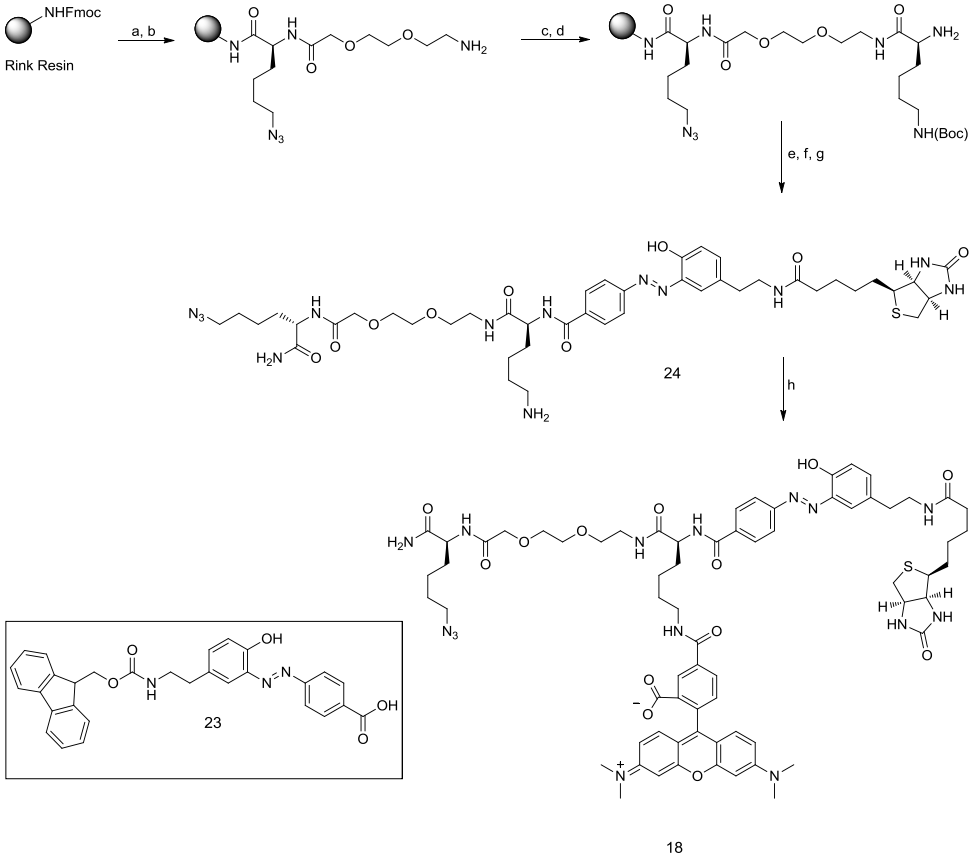


Scheme 9: Synthesis of biotin-diol-TMR-azide. a) 20% piperdine in DMF, then Fmoc-Lys(azide)-OH, DIC, HOBt; 20% piperdine in DMF; b) Fmoc-Lys(Boc)-OH, DIC, HOBt; then 20% piperdine in DMF; c) diol cleavable building block, DIC, HOBt; d) 1,8-diamino-3,6-dioxaoctane, overnight; e) biotin, DIC, HOBt; f) TFA : TIS: H_2O (90% : 2.5% : 7.5%) 3h, then precipitation with cold diethyl ether and purification by HPLC; g) 5(6)-carboxytetramethylrhodamine succinimidyl ester, DIEA; h) HPLC purification.

3.2.2.2 Synthesis of biotin-diazo-TMR-azide

The diazobenzene derived cleavable building block **23** was synthesized by Dr. Steven Verhelst. Biotin-diazo-TMR-azide was synthesized similar to biotin-diol-TMR-azide. Fmoc-Lys(azide)-OH was first bounded to the resin. Fmoc-8-amino-3,6-dioxaoctanoic acid was then introduced as a PEG linker to increase the water solubility. Since the reactivity of carboxylic acids in diazobenzene derived cleavable building blocks **23** is lower than natural amino acids, HBTU/DIEA was used in this coupling step. The Fmoc of the cleavable building block was removed followed by coupling of biotin under the influence of DIC/HOBt. It should be noted

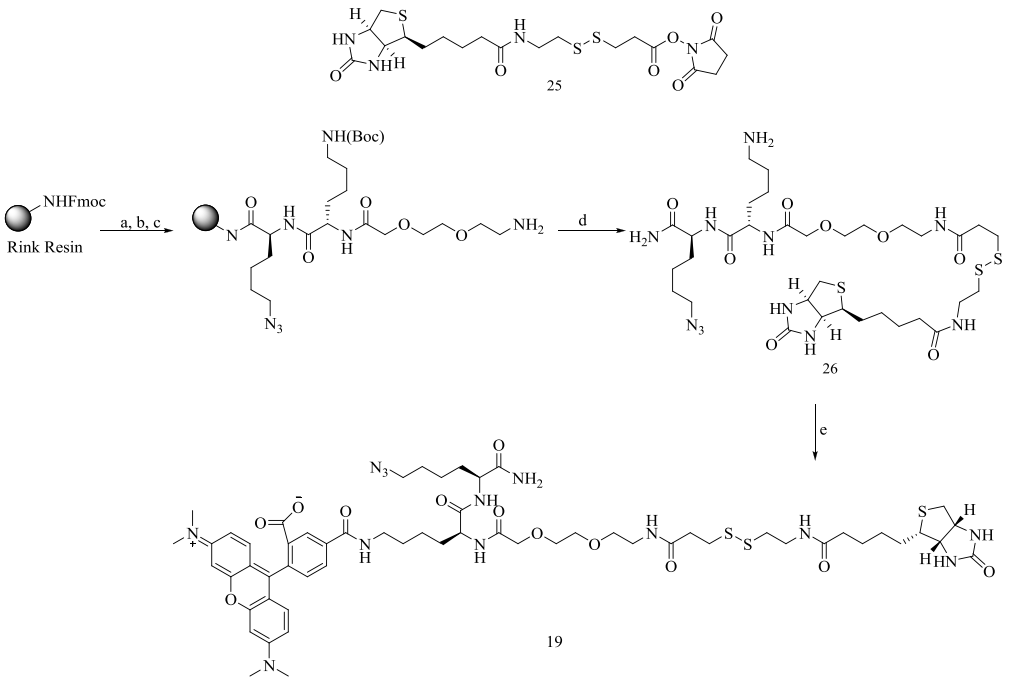
that the strong coupling reagent PyBOP/DIEA should be avoided in this step, since we have observed byproducts derived from the side reaction between biotin and PyBOP with the hydroxyl group of the diazobenzene building block. Finally, building block **24** was reacted with 5(6)-carboxytetramethylrhodamine succinimidyl ester in DMSO and purified by HPLC (Scheme 10).



Scheme 10: Synthesis of biotin-diazo-TMR-azide. a) 20% piperdine in DMF, then Fmoc-Lys(azide)-OH, DIC, HOBt; b) 20% piperdine in DMF, Fmoc-8-amino-3,6-dioxaoctanoic acid, DIC, HOBt; c) 20% piperdine in DMF; then Fmoc-Lys(Boc)-OH, DIC, HOBt; d) 20% piperdine in DMF; e) building block **23**, DIEA, HBTU; f) 20% piperdine in DMF, then biotin, DIC, HOBt; g) TFA : TIS: H_2O (95% : 2.5% : 2.5%) 2h, Then precipitation with cold diethyl ether and purification by HPLC; h) 5(6)-carboxytetramethylrhodamine succinimidyl ester, DIEA. Purification with HPLC.

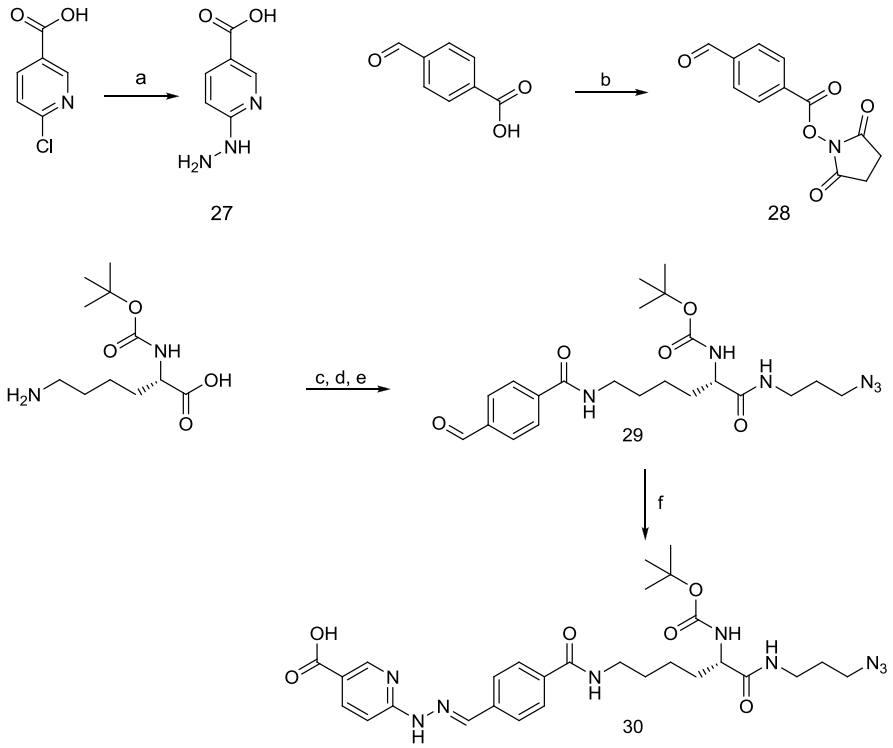
3.2.2.3 Synthesis of biotin-S-S-TMR-azide

The synthesis of biotin-S-S-TMR-N_3 is straightforward since Sulfo-NHS-SS-biotin (**25,** Scheme 11), cleavable biotin building block, is commercially available. After coupling of Fmoc-Lys(azide)-OH, Fmoc-Lys(Boc)-OH and Fmoc-8-amino-3,6-dioxaoctanoic acid with DIC/HOBt respectively, the Sulfo-NHS-SS-biotin cleavable building block **25** was introduced by incubation with the resin-bound intermediate in DMF without coupling reagents. The resulting building block **26** was reacted with 5(6)-carboxytetramethylrhodamine succinimidyl ester in DMSO solution.



Scheme 11: Synthesis of biotin-S-S-TMR-N_3 (19). a) 20% piperdine in DMF, then Fmoc-Lys(azide)-OH, DIC, HOBt; b) 20% piperdine in DMF then Fmoc-Lys(Boc)-OH, DIC, HOBt; c) 20% piperdine in DMF, Fmoc-8-amino-3,6-dioxaoctanoic acid, DIC, HOBt; 20% piperdine in DMF; d) Sulfo-NHS-S-S-biotin, DIEA; Then TFA : TIS: H_2O (95% : 2.5% : 2.5%) 2h, then precipitation with cold diethyl ether and lyophilization; e) 5(6)-carboxytetramethylrhodamine succinimidyl ester, DIEA, then HPLC purification.

3.2.2.4 Synthesis of biotin-hydrazone-TMR-azide

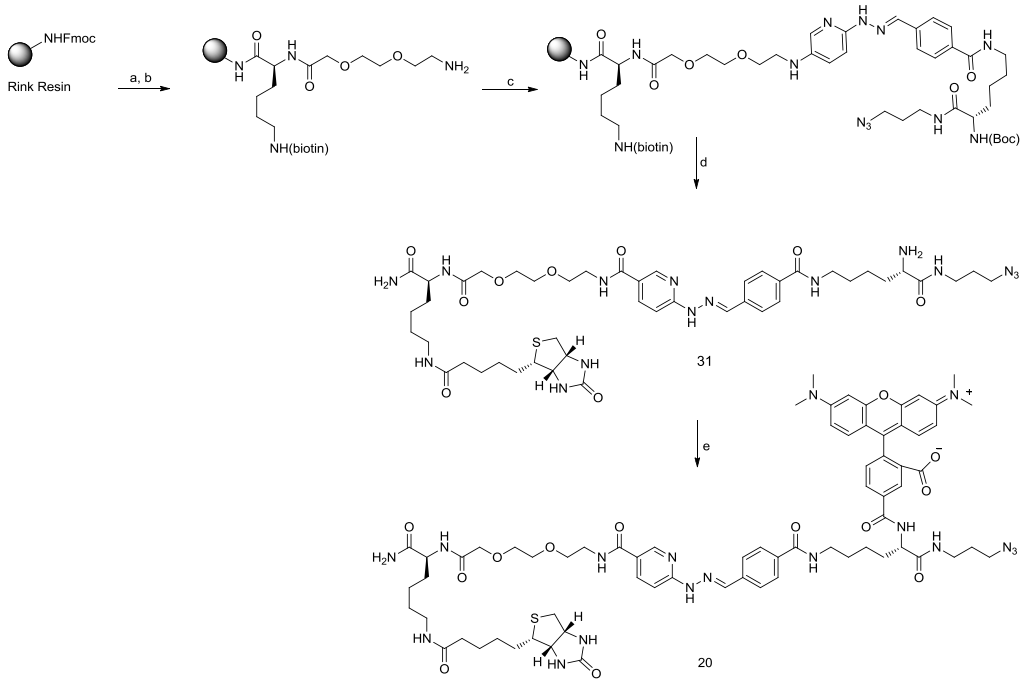


Scheme 12: Synthesis of hydrazone cleavable building block. a) 80% hydrazine in H_2O, 100 °C, 4h; b) EDCI, *N*-hydroxysuccinimide; c) 4-Formyl-benzoic acid NHS ester, DIEA; d) EDCI, *N*-hydroxysuccinimide; e) DIEA, 3-azido-propylamine; f) 6-hydrazinonicotinic acid, overnight in the dark.

6-hydrazinonicotinic acid (**27**) was acquired by treating 6-chloronicotinic acid with aqueous hydrazine. The resulting compound was easily isolated by precipitation in acidic solution (pH 5.5) and washing with 95% ethanol and ether (123). After lyophilisation, it was used in the subsequent experiment without further purification (Scheme 12).

4-Formyl-benzoic acid NHS ester (**28**) was synthesized by activating carboxylbenzaldehyde with *N*-hydroxysuccinimide under the influence of EDCI. After workup, compound **28** was reacted with Boc-Lys-OH. The resulting compound was transformed to *N*-hydroxysuccinimide-activated ester under the influence of EDCI. After work-up, the residue reacted with azido-propylamine. Purification with silica column chromatography afforded compound **29**. The building block **30** was assembled using hydrazone chemical ligation in

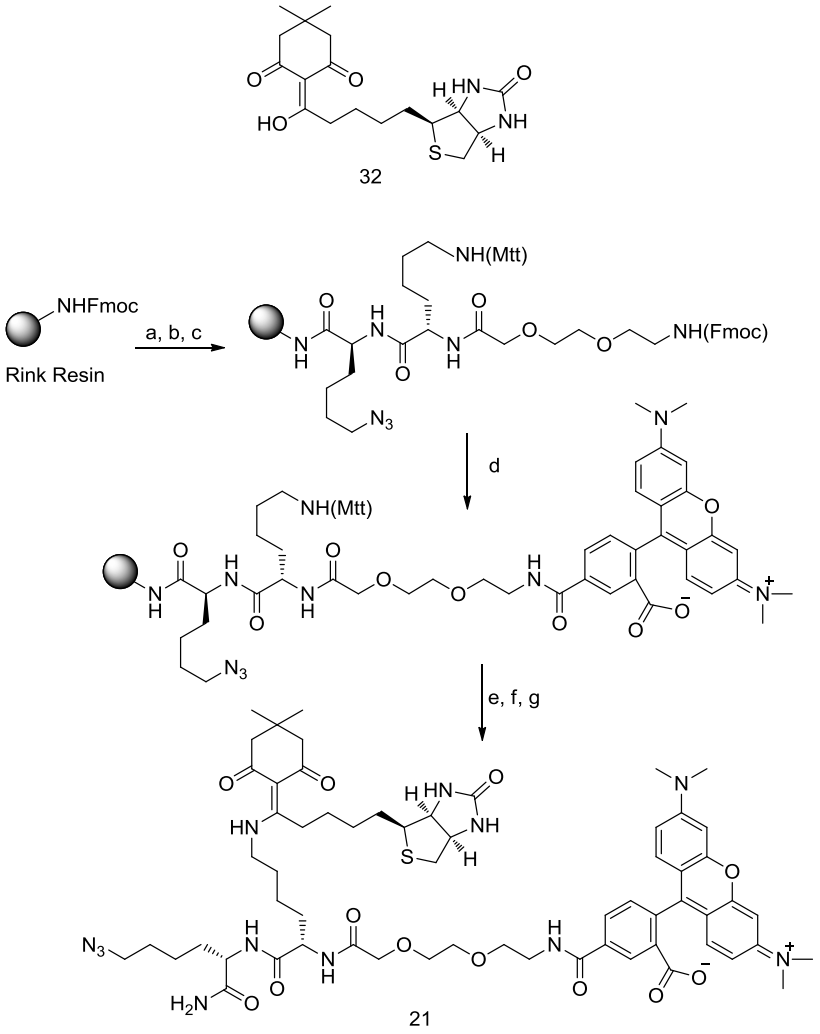
DMF. Evaporation of DMF resulted in cleavable building block **30** which was incorporated in cleavable building block **31** in subsequent solid phase synthesis without further purification (Scheme 12).



Scheme 13: Synthesis of biotin-hydrazone-TMR-N_3. a) 20% piperdine in DMF, then Fmoc-Lys(biotin)-OH, DIC, HOBt and 20% piperdine in DMF; b) Fmoc-8-amino-3,6-dioxaoctanoic acid, DIC, HOBt, then 20% piperdine in DMF. c) building block **30**, DIEA, HBTU; d) TFA : TIS: H_2O (95% : 2.5% : 2.5%) 2h, then precipitation with cold diethyl ether and purification by HPLC; e) 5(6)-carboxytetramethylrhodamine succinimidyl ester, DIEA, then HPLC purification.

Since the cleavable building block **30** already contained an azide group, which is different from the above described cleavable building blocks, Fmoc-Lys(biotin)-OH was coupled to rink resin in the first step. After coupling of Fmoc-8-amino-3,6-dioxaoctanoic acid with DIC/HOBt, hydrazone cleavable building block **30** was introduced under the influence of DIEA/HBTU. The resulting building block **31** was cleaved from the resin and purified by HPLC. Biotin-hydrazone-TMR-N_3 was acquired by coupling of cleavable building block **31** and 5(6)-carboxytetramethylrhodamine succinimidyl ester (Scheme 13).

3.2.2.5 Synthesis of biotin-dde-TMR-azide



Scheme 14: Synthesis of biotin-dde-TMR-azide; a) 20% piperdine in DMF, then Fmoc-Lys(azide)-OH, DIC, HOBt; b) 20% piperdine in DMF, Fmoc-Lys(Mtt)-OH, DIC, HOBt; c) 20% piperdine in DMF, Fmoc-8-amino-3,6-dioxaoctanoic acid, DIC, HOBt; d) 20% piperdine in DMF, 5(6)-carboxytetramethylrhodamine succinimidyl ester, DIEA e) 1% TFA in DCM (10 min x 8), then biotin(dde), DIEA; f) TFA : TIS: H_2O (95% : 2.5% : 2.5%) 2h, g) evaporation and purification by HPLC.

Biotin-dde (**32**) was synthesized by coupling of biotin and dimedone under the influence of DIC/DMAP (124).

The above described four linkers were synthesized by solid phase synthesis of the main cleavable building blocks which were reacted with 5(6)-carboxytetramethylrhodamine

succinimidyl ester in solution. Biotin-dde-TMR-N_3 was completely assembled on solid support. Fmoc-Lys(azide)-OH, Fmoc-Lys(Mtt)-OH and Fmoc-8-amino-3,6-dioxaoctanoic acid were coupled respectively under the influence of DIC/HOBt. It has been shown that dde protecting group could migrate to unprotected amine during the removal of Fmoc (125). To avoid the possibility of migration of the biotin-dde, the Fmoc of the resin bound mixture was first removed and the resulting amine was reacted with 5(6)-carboxytetramethylrhodamine succinimidyl ester. Subsequently, the Mtt was removed by treatment with 1% TFA followed by coupling of biotin-dde. Cleavage and purification resulted in the target compound with a yield 51% which is a satisfying result for 5 coupling steps on solid phase synthesis.

3.2.3 Cleavable trifunctional tags label alkyne-E64 modified cathepsins in RAW cell lysate and RAW cell

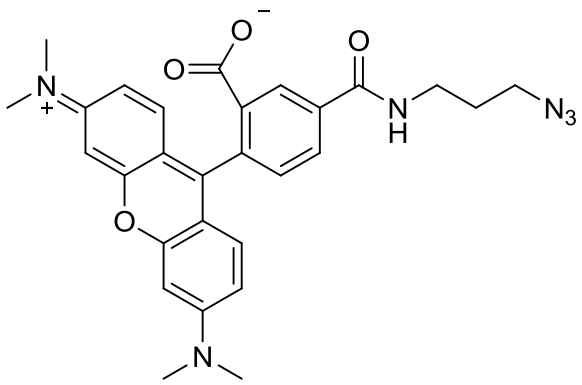


Figure 26: Structure of TMR-N_3.

TMR-N_3 (Figure 26) is widely used in tandem labeling to visualize protein modified by alkyne containing probes. To show that the different trifunctional tags can detect those proteins in a complex proteome as efficiently as TMR-N_3, a lysate of the macrophage cell line RAW 264.7 labeled with alkyne-E64 was subjected to click chemistry with these tags under the influence of $CuSO_4$, TBTA and TCEP. Figure 27 shows that trifunctional tags label alkyne-E64 as efficiently as TMR-N_3 indicating that structural differences have no effect on the bioconjugation efficiency. We also observed that intact probes are more efficient than tandem labeling since the intensity of cathepsin Z and B labeled by TMR-DCG-04 was stronger than labeling by alkyne-E64 followed by click chemistry with azide tags (Figure 27). To expand

the application of cleavable trifunctional tags to label protein targets in living cells, alkyne-E64 was incubated with RAW cells for the indicated time, and the lysate was then subjected to click chemistry with different tags. All the trifunctional tags labeled the alkyne-E64 modified cathepsins in living RAW cells (Figure 28). Consistent with the result in the previous section, the activity of some cathepsins is weaker in lysate since the intensity of Cat Z and Cat B are comparable. But the other two bands are quite stronger *in situ* than *in vitro*.

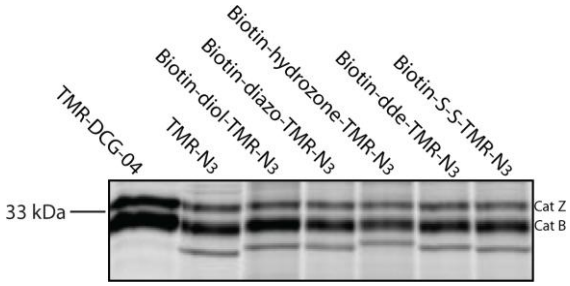


Figure 27: Labeling of alkyne-E64 labeled cathepsins in RAW 264.7 cell lysate with TMR-N_3 and with trifunctional tags. RAW cell lysate was incubated with alkyne-E64 (10 µM for 2h). The reaction was stopped by running the samples over PD-10 column. The protein concentration was adjusted to 1 mg/ml. Click chemistry was kept for 1h at room temperature. The labeled cathepsins were visualized by in-gel fluorescent scanning. As a control, cathepsins in RAW cell lysate were also labeled by TMR-DCG-04 (1 µM, 1h).

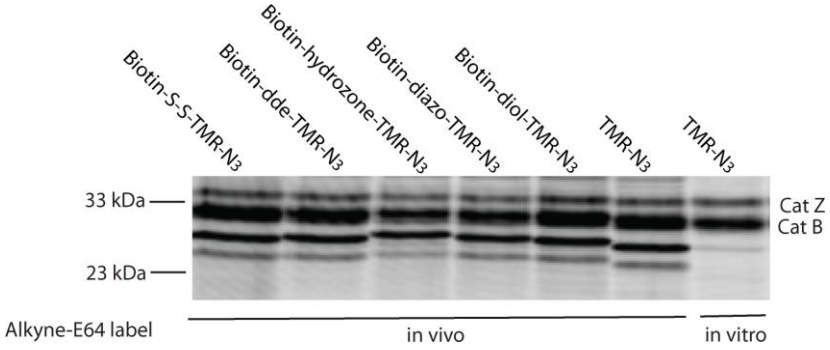


Figure 28: Labeling of alkyne-E64 labeled cathepsins in living RAW 264.7 cell with TMR-N_3 and with trifunctional tags. RAW cells were incubated with alkyne-E64 (10 µM for 1h). The cells were collected and made into lysate. The lysate was subjected to click chemistry. Cathepsins in RAW cell lysate were labeled with Alkyne-E64 (10 µM, 2h) and clicked with TMR-N_3. The labeled cathepsins were visualized by in-gel fluorescent scanning.

To test if the background labeling occurred in the labeling of disulfide based trifunctional tags,

alkyne-E64 labeled RAW cell lysate was reacted with disulfide based trifunctional tags. As a control, the labeled proteome was clicked with TMR-N_3 and biotin-N_3. The labeled proteome was separated with a non-reducing gel to prevent cleavage of the disulfide bond. Cat Z and Cat B were not separated well in the non-reducing gel. There was little background labeling for TMR-N_3 and biotin-N_3. However, disulfide trifunctional tags labeled sample show background labeling in both fluorescent scanning and western blot (Figure 29). It has been shown that the biotin-disulfide tags caused much background labeling during Cu catalysis chemistry in a complex proteome (85).

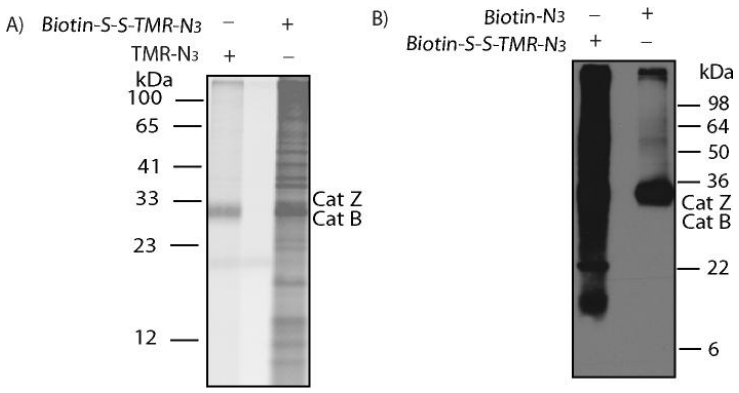


Figure 29: Labeling of alkyne-E64 labeled cathepsins in RAW 264.7 cell lysate (10 µM for 2h) with TMR-N_3, biotin-N_3 and disulfide trifunctional tags. The reaction was stopped by acetone precipitation. The protein pellet was dissolved in non-reducing sample buffer. Proteins were separated and visualized by biotin-streptavidin or in- gel fluorescent scanning. A) The labeled cathepsins were visualized by in-gel fluorescent scanning. B) The labeled cathepsins were visualized by biotin-streptavidin western blot.

Taken together, all tags efficiently labeled alkyne-modified proteins *in vitro* and in living cells, illustrating the compatibility of the cleavable tags with the bioorthogonal labeling strategy. Disulfide trifunctional tags get much background labeling which could be observed in non-reducing gels.

3.2.4 Test the click efficiency of different concentration of trifunctional tags

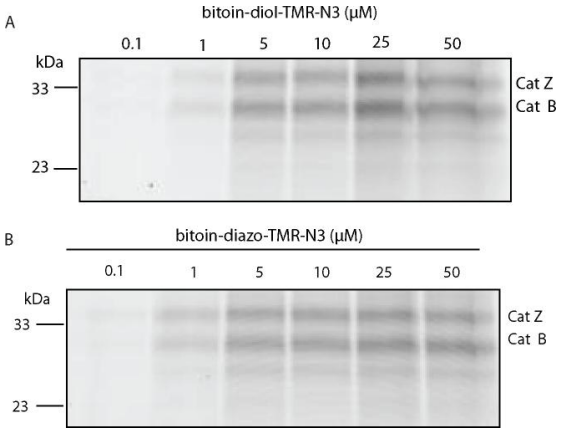


Figure 30: Labeling of alkyne-E64 labeled cathepsins in RAW 264.7 cell lysate with different concentrations of the trifunctional reagents. A) Biotin-diol-TMR-N_3 and B) biotin-diazo-TMR-N_3. The labeled cathepsins were visualized by in gel fluorescent scanning.

Alkyne-E64 labeled RAW cell lysate was clicked with different concentration of biotin-diol-TMR-N_3 and biotin-diazo-TMR-N_3. There were no fluorescently labeled cathepsins detected under the concentration of 0.1μM. The intensity of cathepsins bands increased as the concentration of tags increased up to 25 μM. The efficiency of the click reaction was not much different at the concentrations of 25 μM and 50 μM for both tags (Figure 30).

3.2.5 The efficiency of immobilization with different trifunctional reagents

To show that the trifunctional reagents can be used to capture proteins from a complex proteome, cleavable reagents were reacted with alkyne-E64 labeled RAW cell lysate. The labeled cathepsins were efficiently immobilized with streptavidin beads and directly boiling released the targets again (Figure 31). TMR-N_3 labeled cathepsins failed to be immobilized indicating that immobilization is caused by interaction between biotin-streptavidin but not by non-specific interaction between protein and streptavidin beads. The immobilization efficiency

was calculated by comparison of fluorescent intensities of cathepsins in label lane and supernatant lane (Table 9). Biotin-diol-TMR-N_3 was most efficient. The pull down efficiency

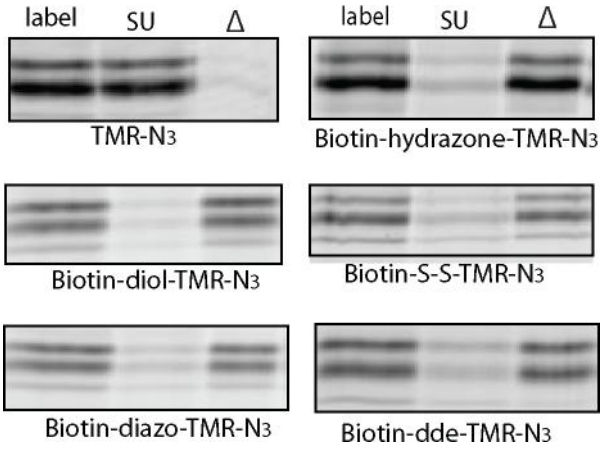


Figure 31: Immobilization of labeled cathepsins by streptavidin beads. The labeled cathepsins were visualized by in gel fluorescent scanning. Left lanes: labeling after click chemistry with TMR-N_3 or trifunctional tags. Middle lanes: supernatant (SU) after capture with streptavidin beads. Right lane: release of immobilized proteins by boiling with SDS sample buffer (Δ). TMR-N_3 was used as a control to show that no non-specific binding of labeled cathepsins to streptavidin beads occurs.

was slightly lower for the disulfide, possibly due to premature cleavage since the disulfide bridge is not stable towards free thiols in the proteome. The pull down efficiency for the dde linker was similar to the disulfide cleavable linker. The steric hindrance around the biotin maybe lead to the lower immobilization efficiency of the dde based reagent.

Table 9: Pull down efficiency of different trifunctional tags.

	diol	diazo	hydrazone	SS	dde
Pull down efficiency	82%	78%	84%	73%	72%

3.2.6 Elution efficiency of different trifunctional reagents

To investigate the release efficiency of immobilized proteins, equal amounts of immobilized beads were treated by boiling (non-selective release) or the chemical release conditions. The beads treated with the chemical release conditions were subsequently boiled to release all

residual proteins bound to the beads. The samples were loaded onto a SDS-PAGE and visualized by in-gel fluorescent scanning. We determined how efficient the elution was by means of fluorescent gel band densitometry by comparison with the proteins selectively eluted and residually bound to the immobilized streptavidin. When the linker structure allowed, the SDS gels were subjected to biotin-streptavidin western blot after fluorescent scanning to detect the selectivity of different chemical conditions. The proteins released by selective cleavage of the linker could not be visualized in biotin-streptavidin western blot.

3.2.6.1 Release efficiency of biotin-diol-TMR-N_3

For cleavage of the diol linker, we used 1 mM and 10 mM of $NaIO_4$ (3x 20 min; directly followed by quenching of the periodate with sample buffer). Under both conditions, the target proteins were efficiently released (Figure 32). The 1mM $NaIO_4$ treatment gave rise to 82% cleavage efficiency. These conditions selectively cleave the diol linker since the released protein failed visualization in the biotin-streptavidin blot.

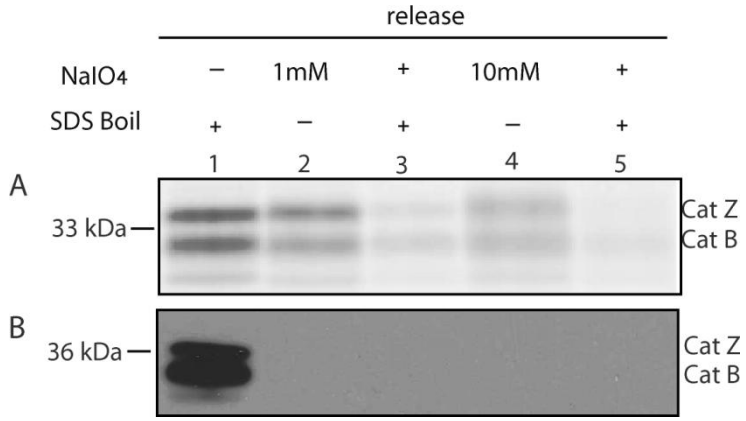


Figure 32: Evaluation of the elution of alkyne-E64 targets from streptavidin beads with the biotin-diol-TMR-N_3 trifunctional tags. A) The labeled cathepsins were visualized by in gel fluorescent scanning. B) The labeled cathepsins were visualized by biotin-streptavidin western blot. Alkyne-E64 labeled RAW cell lysate (1mg/ml) reacted with biotin-diol-TMR-N_3 and the labeled proteome was immobilized with streptavidin beads. The beads were divided into equal aliquots. One part was treated with SDS boiling (lane 1). One part was treated with $NaIO_4$ (1 mM in SP buffer, pH=7.4) for 3x 20min (lane 2) and subsequently SDS boiling (lane 3). One part was treated with $NaIO_4$ (10 mM in SP buffer, pH=7.4) for 3x 20min (lane 4) and subsequently SDS boiling (lane 5).

3.2.6.2 Release efficiency of biotin-diazo-TMR-N_3

For the diazobenzene linker, which was treated with 200 mM $Na_2S_2O_4$, the cleavage efficiency was 73%. The western blot confirmed the release of the proteins due to the cleavage of diazobenzene linker since the proteins can be detected in the fluorescent gel but not in the western blot (Figure 33). Diazobenzene linker was firstly developed and applied in chemical proteomics by Verhelst *el al* in 2007 (78, 84). Subsequently, Hang et al found that the ortho-hydroxyl substituent is essential for the efficient cleavage of the diazobenzene based linker (87). Wagner et al develop a new linker based on 2-(2-alkoxy-4-hydroxyphenylazo)benzoic acid which is more sensitive to dithionite (88). However, the synthesis of this cleavable building block is more complicated than the building block utilized in our research.

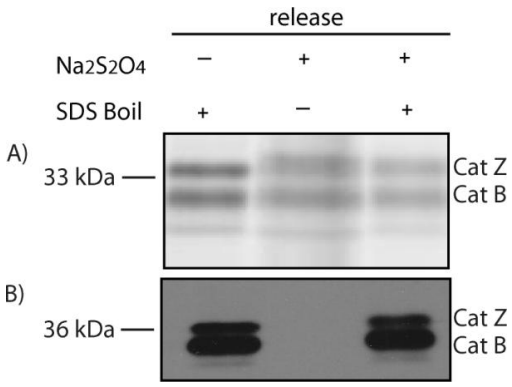


Figure 33: Evaluation of the elution of alkyne-E64 targets from streptavidin beads with the biotin-diazo-TMR-N_3 trifunctional tags. A) The labeled cathepsins were visualized by in gel fluorescent scanning. B) The labeled cathepsins were visualized by biotin-streptavidin western blot. Alkyne-E64 labeled RAW cell lysate (1mg/ml) reacted with biotin-diazo-TMR-N_3 and the labeled proteome was immobilized with streptavidin beads. The beads were divided into equal aliquots which was treated with SDS boiling (left lane) or $Na_2S_2O_4$ (200 mM in SP buffer, pH=7.4) for 3x 20min (middle lane) and subsequently SDS boiling (right lane).

3.2.6.3 Release efficiency of biotin-hydrazone-TMR-N_3

The immobilized proteins could not be eluted by 100 mM hydroxylamine (Figure 34a), whereas the release of 46% of the proteins was achieved after the addition of aniline which is a

reported catalyst in the cleavage of these linkers (Figure 34a). The release is caused by cleavage of the linker, since the released proteins were visualized by in-gel fluorescent scanning but not by biotin-streptavidin western blot. Unfortunately, a further increase of the hydroxylamine concentration didn't lead to a full release (Figure 34b). In accordance with our results, Claessen *et al* reported that a chemical probe containing a similar bisaryl hydrazone linker was not sensitive to this elution condition (126). Acylhydrazone-based cleavable linkers have been developed for isolation of proteins from complex proteomes (96). It was reported that the stability of this linker was weaker than the bisaryl hydrazone linker (97). Indeed, the elution of biotin-hydrazone-TMR-N_3 labeled proteins by SDS boiling can be detected by biotin-streptavidin blot indicating the bisaryl hydrazone linker is tolerant towards SDS boiling. However, the acylhydrazone-based cleavable linker was rapidly cleaved with a SDS solution at room temperature (96). Possible structural modifications of the bisaryl hydrazone linker may be carried out to make it stable in biological system and at the same time have a higher sensitivity to hydroxylamine/aniline cleavage.

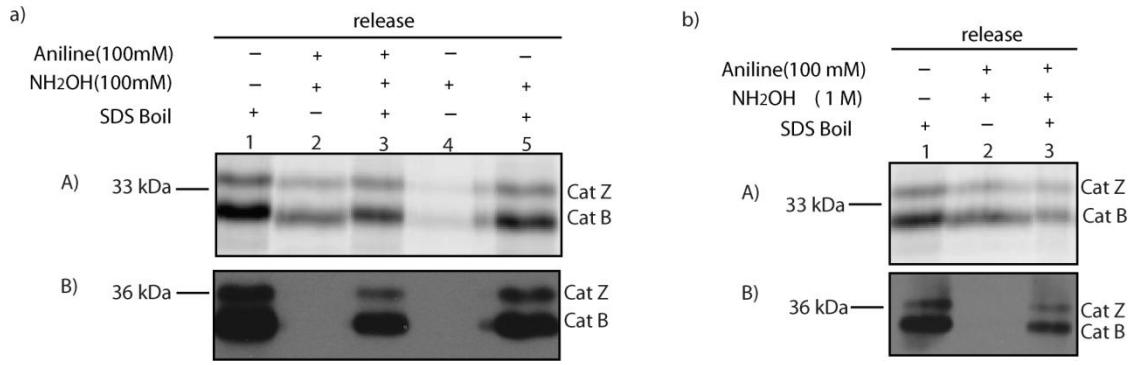


Figure 34: Evaluation of the elution of alkyne-E64 targets from streptavidin beads with the biotin-hydrazone-TMR-N_3 trifunctional tags. A) The labeled cathepsins were visualized by in gel fluorescent scanning. B) The labeled cathepsins were visualized by biotin-streptavidin western blot. Alkyne-E64 labeled RAW cell lysate (1mg/ml) reacted with biotin-hydrazone-TMR-N_3 and the labeled proteome was immobilized with streptavidin beads. The beads were divided into equal aliquots. One part was treated with SDS boiling (lane 1 in Figure 34a and Figure 34b). One part was treated with NH_2OH and aniline (100 mM NH_2OH and 100 mM of aniline in SP buffer, pH=4.6) for 4h (lane 2, Figure 34a) and subsequently SDS boiling (lane 3, Figure 34a). One part was treated with NH_2OH (100 mM in SP buffer, pH=4.6) for 4h (lane 4, Figure 34a) and subsequently SDS boiling (lane 5, Figure 34a). One part was treated with 1M NH_2OH and 100 mM of aniline in SP buffer, pH=4.6 for 4h (lane 2, Figure 34b) and subsequently SDS boiling (lane 3, Figure 34b).

3.2.6.4 Release efficiency of biotin-S-S-TMR-N_3

Disulfide linkers can be cleaved by reducing agents such as DTT, TCEP and BME. Additionally the disulfide linker building blocks are commercially available. Hence this linker is easy to apply in chemical biology research. In our study, 0.5 M DTT was used to treat the immobilized beads and the elution was loaded onto a reducing SDS-PAGE. 60% of enriched protein was released in 1h (Figure 35). The same gel was not subjected to biotin streptavidin blot due to the cleavage of disulfide bond in the reducing gel which would therefore not give any additional information.

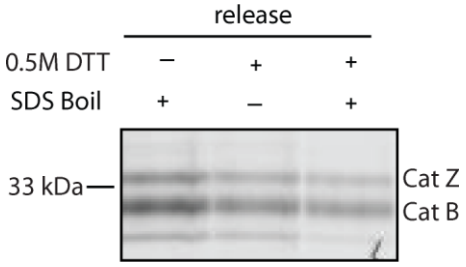


Figure 35: Evaluation of the elution of alkyne-E64 targets from streptavidin beads with the biotin-S-S-TMR-N_3 trifunctional tags in reducing gel. Alkyne-E64 labeled RAW cell lysate (1mg/ml) reacted with biotin-S-S-TMR-N_3 and the labeled proteome was immobilized with streptavidin beads. The beads were divided into equal aliquots. One part was treated with SDS boiling (lane 1). One part was treated with 0.5M of DTT in H_2O for 1h (lane 2) and subsequently SDS boiling (lane 3). The labeled cathepsins were visualized by in gel fluorescent scanning.

However, one problem of the disulfide linker is the potential disulfide exchange (85). This has been observed during the labeling of alkyne-E64 modified cathepsins in section **3.2.3** (Figure 25). In order to get further evidence that disulfide exchange leads to background labeling, the capture and release of proteins was loaded onto a non-reducing gel. Proteins were visualized by fluorescent gel and streptavidin western blot. We indeed observed a high degree of disulfide exchange when analyzing samples in non-reducing gels (Figure 36). Background proteins were visualized in labeling lanes both by in-gel fluorescent scanning and biotin streptavidin blot. Followed by immobilization with streptavidin beads, the background labeling in the supernatant was only observed in the in-gel fluorescent scanning since the

biotinylated proteins were enriched onto streptavidin beads. Subsequent SDS boiling (sample buffer without BME) released these background proteins.

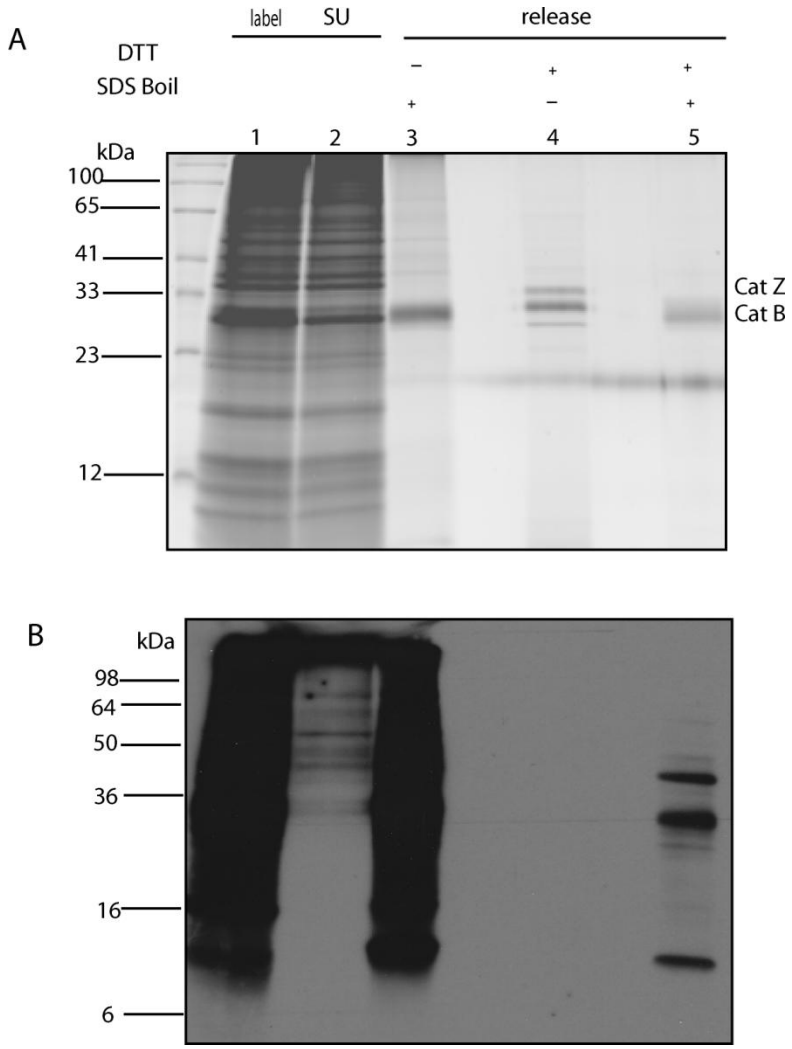


Figure 36: Evaluation of the immobilization and elution of alkyne-E64 targets from streptavidin beads with the biotin-S-S-TMR-N_3 trifunctional tags in non-reducing gel. Alkyne-E64 labeled RAW cell lysate (1mg/ml) reacted with biotin-S-S-TMR-N_3 (lane1) and the labeled proteome was immobilized with streptavidin beads (lane 2). The beads were divided into equal aliquots. One part was treated with SDS boiling (lane 3). One part was treated with 0.5M of DTT in H_2O for 1h (lane 4) and subsequently SDS boiling (lane 5). A) The labeled proteins were visualized by in gel fluorescent scanning. B) The labeled proteins were visualized by biotin-streptavidin western blot.

3.2.6.5 Release efficiency of biotin-dde-TMR-N_3

The conventional way of deprotection of the dde group in peptide synthesis consists of 2% of hydrazine (122). Here the immobilized proteins with biotin-dde-TMR-N_3 were treated with 2% hydrazine in water. However there were no fluorescently labeled proteins in the hydrazine treated sample and the proteins recovered in subsequent boiling sample. A small amount of SDS (0.05%) is necessary for the release of immobilized proteins with chemical probes containing a levulinoyl ester based cleavable linker which is sensitive to hydrazine (91). Hence a solution of 2% hydrazine containing 0.05% SDS was used to elute biotin-dde-TMR-N_3 labeled proteins. The fluorescently labeled cathepsins were fully recovered (Figure 37). These results indicated the low concentration of SDS is necessary for the elution of labeled proteins with hydrazine.

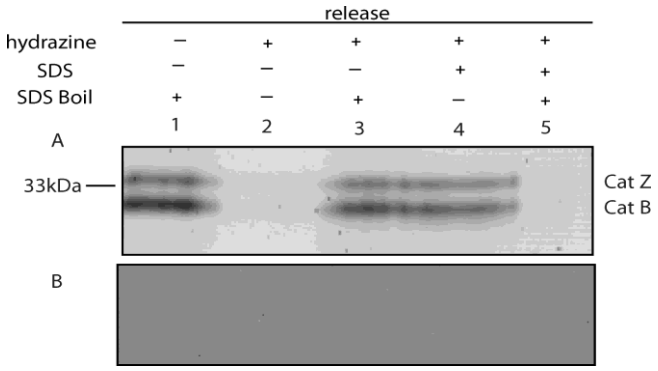


Figure 37: Elution of biotin-dde-TMR-N_3 trifunctional tags labeled proteins with a solution of hydrazine. After the immobilization, the beads were divided into equal aliquots which were treated with SDS boiling (lane 1), 2% hydrazine in H_2O for 2h (lane 2) and subsequently SDS boiling (lane 3), 2% hydrazine and 0.05% SDS for 2h (lane 4) and subsequently SDS boiling (lane 5), A) The labeled proteins were visualized by in -gel fluorescent scanning. B) The labeled proteins were visualized by biotin-streptavidin western blot.

Interestingly, a solution of 200 mM Tris, 0.05% SDS, pH = 8.5 almost quantitatively released (92%) the immobilized proteins which can be visualized by fluorescent scanning (Figure 38). Tris buffer (200 mM Tris, pH=8.5) failed to release proteins and 0.05% SDS released a little protein, confirming that both reagents are necessary for efficient elution. The elution condition

is specific for dde based linkers since it has no effect on biotin-diol-TMR-N_3 (Figure 39). LC-MS analysis showed that biotin-dde-TMR-N_3 treated with a Tris-SDS solution releases the biotin dde moiety by hydrolysis, which is likely the mechanism by which the proteins are released (Figure 42). The exact mechanism of elution with Tris-SDS should be further studied.

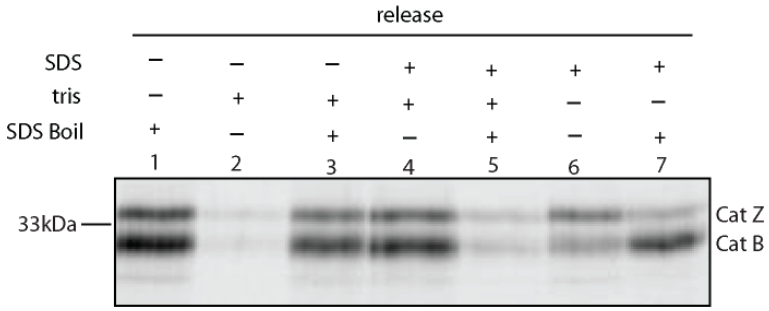


Figure 38: Evaluation of the elution of alkyne-E64 targets from streptavidin beads with the biotin-dde-TMR-N_3 trifunctional tags with Tris/SDS condition. Alkyne-E64 labeled RAW cell lysate (1mg/ml) reacted with biotin-dde-TMR-N_3 and the labeled proteome was immobilized with streptavidin beads. The beads were divided into equal aliquots. The beads were treated with SDS boiling (lane 1), 200 mM Tris, pH 8.5 for 2h (lane 2) and subsequently SDS boiling (lane 3), 200 mM Tris, 0.05% SDS, pH 8.5 for 2h (lane 4) and subsequently SDS boiling (lane 5), 0.05% SDS for 2h (lane 6) and subsequently SDS boiling (lane 7). The labeled proteins were visualized by in gel fluorescent scanning.

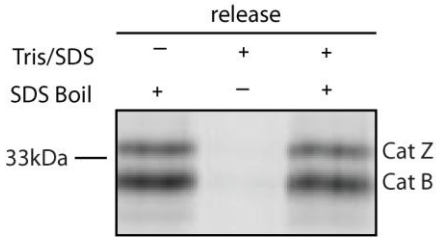


Figure 39: Test the stability of biotin-diol-TMR-N_3 trifunctional tags upon Tris/SDS condition. Alkyne-E64 labeled RAW cell lysate (1mg/ml) reacted with biotin-diol-TMR-N_3 and the labeled proteome was immobilized with streptavidin beads. The beads were divided into equal aliquots which were treated with SDS boiling (left lane), 200 mM of Tris, 0.05% SDS, pH 8.5 for 2h (middle lane) and subsequently SDS boiling (right lane). The labeled proteins were visualized by in gel fluorescent scanning.

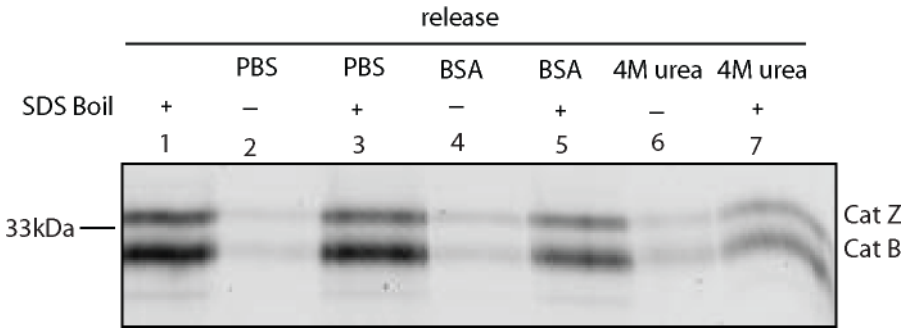


Figure 40: Test the stability of biotin-dde-TMR-N_3 trifunctional tags. After the immobilization, the beads were divided into equal aliquots which were treated with SDS boiling (lane 1), PBS, pH 7.4 for 2h (lane 2) and subsequently SDS boiling (lane3), BSA (1mg/ml) for 2h (lane 4) and subsequently SDS boiling (lane 5), 4M urea for 2h (lane 6) and subsequently SDS boiling (lane 7). The labeled proteins were visualized by in gel fluorescent scanning.

PBS and BSA (1mg/ml) didn't lead to the release of target proteins indicating the linker is stable in PBS buffer and protein solution. In addition, this linker is stable in 4M urea solution hence 4M urea solution can be used to wash the non-specifically bound proteins before chemical release (Figure 40).

Taken together, the proteins immobilized with biotin-dde-TMR-N_3 can be released under a mild condition (Table 10). The elution efficiency was better than that of the other trifunctional reagents for affinity purification of small amounts of protein in our research. Biotin-diazo-TMR-N_3 and Biotin-S-S-TMR-N_3 had comparable release efficiencies. However, there is some background labeling for Biotin-S-S-TMR-N_3 which may limit its application in targets identification in chemical proteomics.

Table 10: The elution efficiency of different trifunctional reagents.

linkers	diol	diazo	hydrazone	S-S	dde
Elution conditions	1 mM $NaIO_4$ pH = 7.4 3 X 20 min	200 mM $Na_2S_2O_4$ pH = 7.4 3 X 20 min	100 mM NH_2OH 100 mM aniline pH = 4.6 4h	0.5 M DTT 1h	200 mM Tris 0.05% SDS pH = 8.5, 2h
Elution efficiency	74%	58%	47%	60%	92%

3.2.7 Comparison of the molecular weight of the residual tag left after cleavage and tryptic digestion

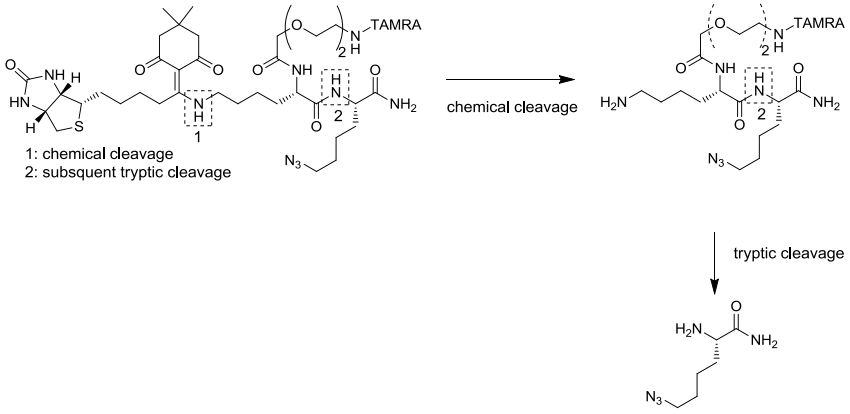


Figure 41: The 'hidden' trypsin cleavable bond in biotin-dde-TMR-N_3.

Overall, cleavable trifunctional biotin reagents are versatile tools for capture, release and visualization of target proteins. During the process of release, biotin is left on the streptavidin beads but the fluorescent group is still attached to the released proteins for subsequent visualization. In general, the purified proteins are then digested with trypsin and analysed by LC-MS/MS to identify the unknown proteins. However, the attachment of a fluorescent group makes the modification have a large size (Table 11). The large size of a modification may complicate the identification of probe-labeled tryptic peptides (85).

Table 11: Molecular weights of residual tag left after cleavage and trypsin digestion

linkers	diol	diazo	hydrazone	SS	dde
MW [a]	767.83	976.09	771.86	945.09	171.20

MW [a] : molecular weight of residual tag left after cleavage and trypsin digestion

Interestingly we noticed there was a 'hidden' trypsin cleavable bond in biotin-dde-TMR-N_3. After the cleavage of biotin-dde group, the fluorophore is eliminated during a tryptic digest (Figure 41). To validate this, the cleavage of the 'hidden' trypsin cleavable bond was checked by LC-MS. Figure 43 shows that biotin-dde is fully cleaved by hydrazine and the fluorophore

was removed by trypsin digestion (Figure 44). Compared with other cleavable trifunctional reagents, biotin-dde-TMR-N_3 has the smallest molecular weight of residual tag left after cleavage and trypsin digestion (table 11).

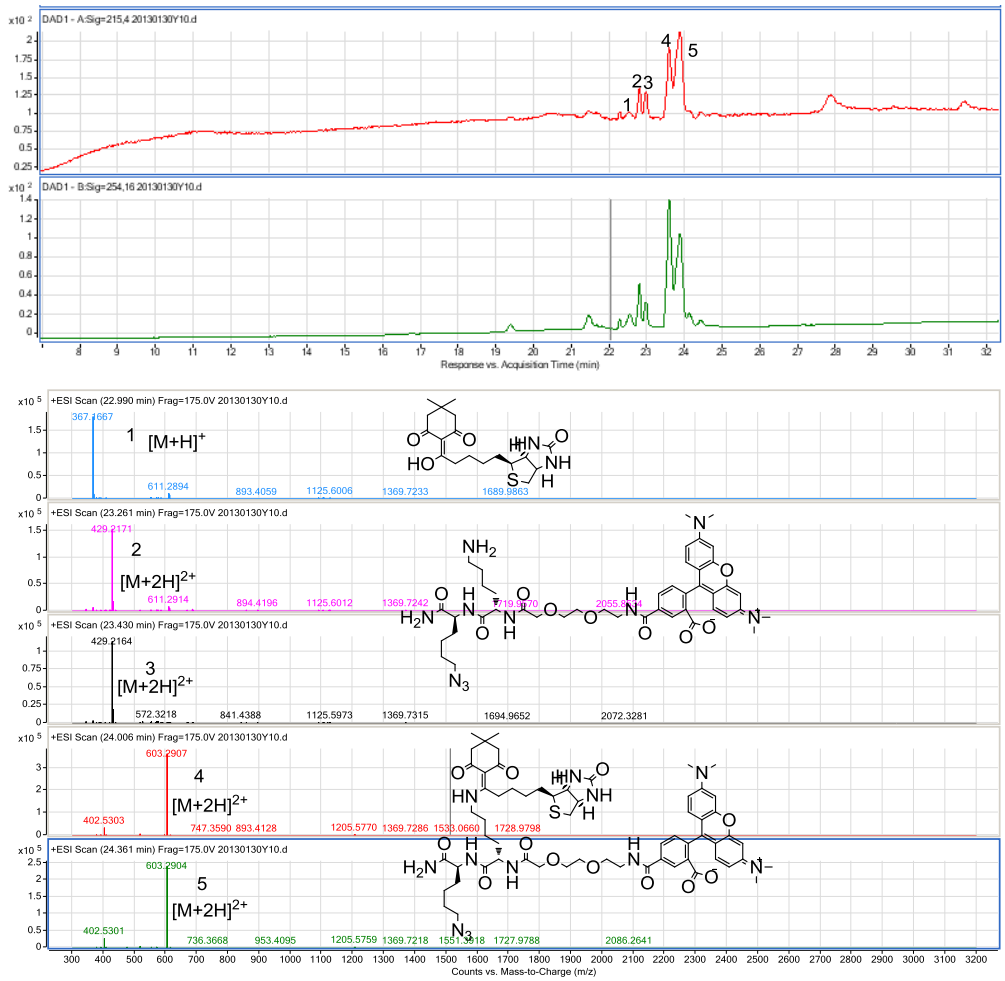


Figure 42: Treatment of the dde-trifunctional tag with Tris/SDS. The products were detected by LC-MS. Peak 1 contains the cleaved-off biotin moiety. MS shows a mass of $[M+H]^+$ 367.1667. Calculated for $C_{18}H_{27}N_2O_4S^+$: 367.1686. Peak 2 and 3 contain the cleaved-off two different regioisomers of the TMR fluorophore. Peak 4 and 5 are two different regioisomers dde-trifunctional tag.

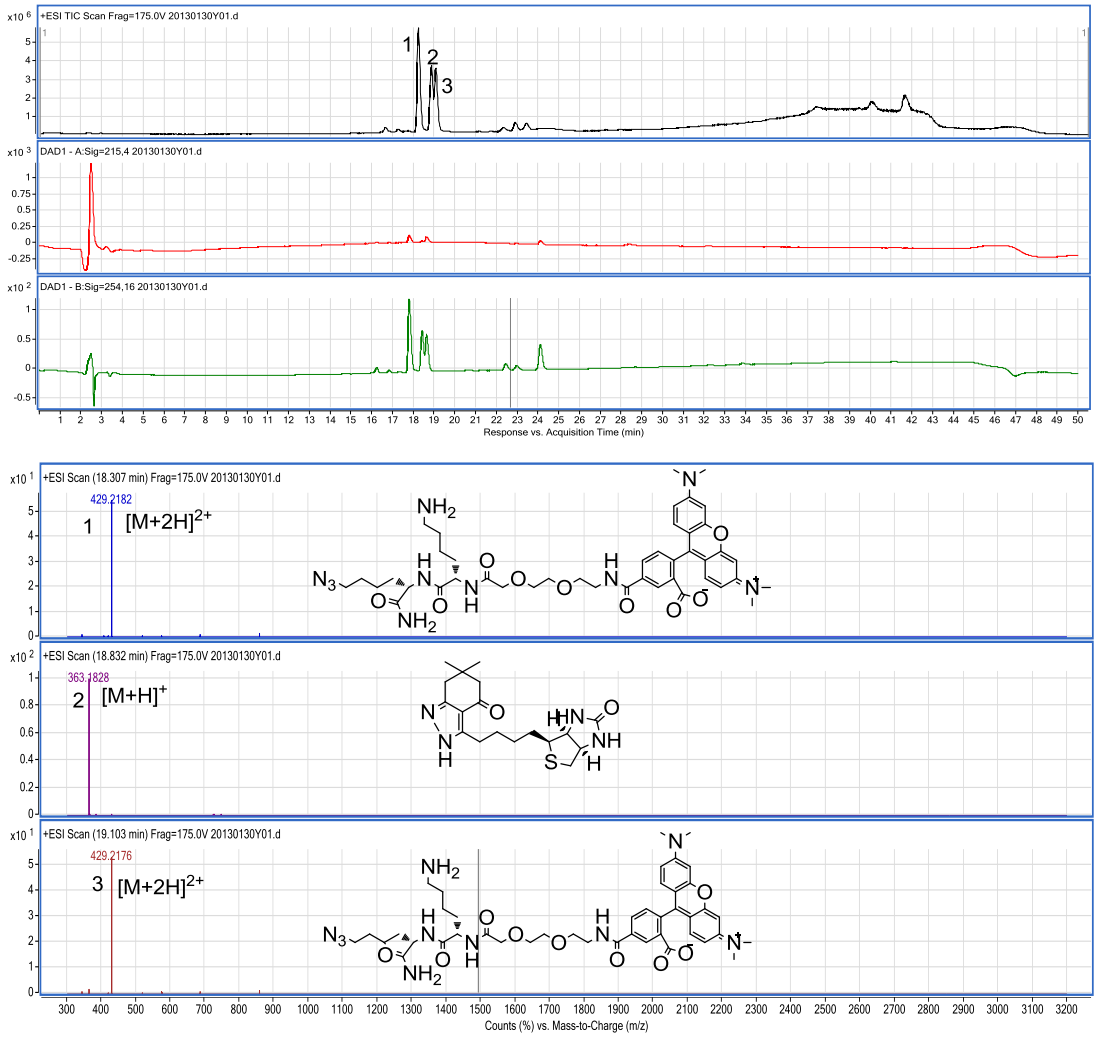


Figure 43: The dde-trifunctional tag was cleaved with hydrazine. Peak 1 and 3 contain two different regioisomers of the TMR fluorophore. Peak 2 contains the cleaved-off biotin moiety.

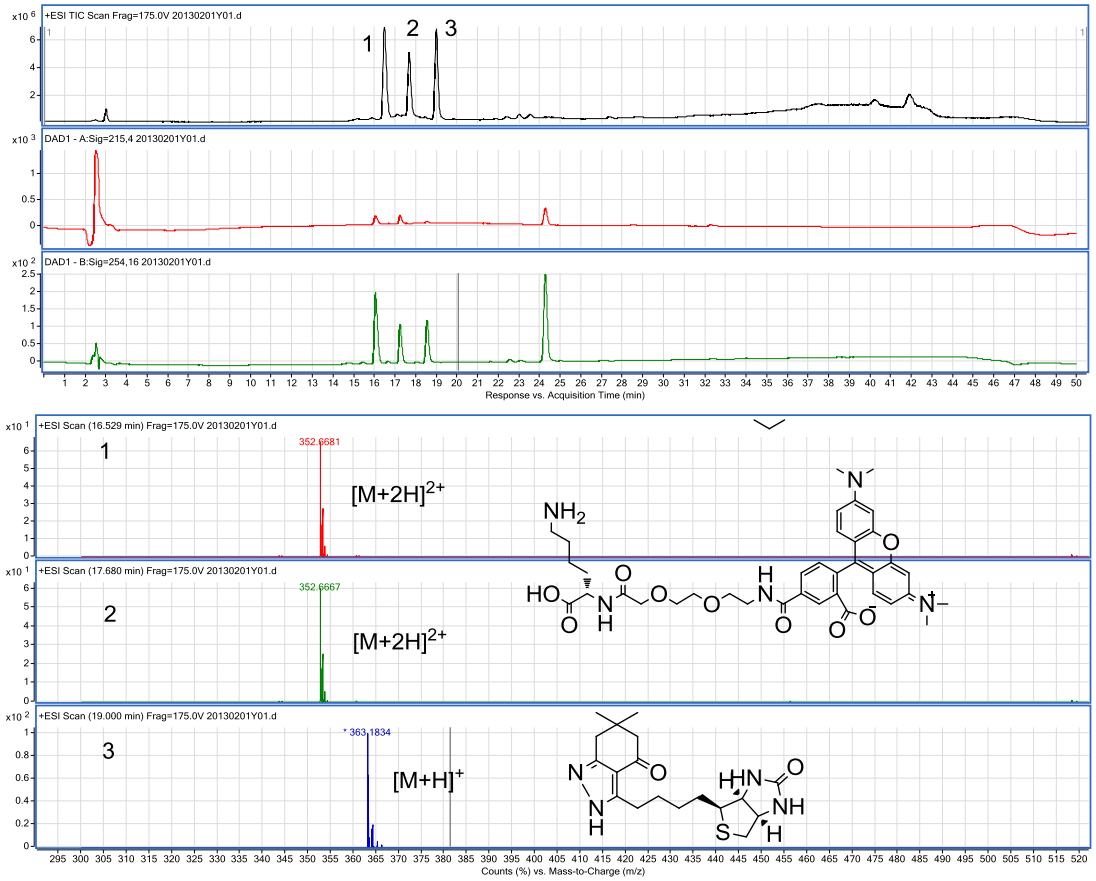


Figure 44: The dde-trifunctional tag was digested with trypsin after cleavage with hydrazine. Peak 1 and 2 contain the two regioisomers of the trypsin digested TMR tag. Peak 3 contains the cleaved off biotin.

4 Conclusion and outlook

4.1 The advantages of using a diol cleavable linker for chemical proteomics

Streptavidin beads are widely used for affinity purification of biotinylated probe-modified proteins in complex proteomes. The disadvantage of the affinity system is the contamination of non-specific binding proteins and endogenously biotinylated proteins. The cleavable linker strategy is one of the promising ways to reduce the contamination of non-specifically bound proteins in affinity purifications (78). The first part of thesis reports the usage of diol cleavable linkers for the selective release of the protein targets. In the literature, a variety of cleavable building blocks have been developed for chemical proteomics. However, some of these linkers are difficult to synthesize hence they can not be readily applied in proteomics research (86, 91). The diol cleavable building block reported here can be easily synthesized in large scale from tartrate. The building block can be incorporated into probes with Fmoc-based solid phase synthesis which may be handed in laboratories without an extensive background in organic chemistry. In addition, the stability of some linkers limits their widely application. For example, disulfide-cleavable linkers are liable to buffers containing reducing reagents such DTT and an acylhydrazone motif based linker is not compatible with acidic buffers (96, 97). The diol linker is stable under acidic, basic and reducing conditions, which makes it compatible with general buffers in proteomics research. An tartaric acid based hydrophilic spacer has been utilized to reduce the non-specific binding proteins in affinity purification (113). The hydrophilicity of diol cleavable linkers may enable increasing the solubility of ABPs and reducing the non-specific binding of background proteins during pull-down. Moreover, the diol linker is small relative to most other cleavable linkers and may have less influence on the activity of chemical probes.

4.2 Diol cleavable linker based probes specifically label the protein targets in proteomes for subsequent purification

To evaluate the efficiency of the diol cleavable linker in target identification, it was incorporated into DCG-04, a chemical probe for cathepsins. Incorporation of a diol cleavable linker had no influence on the activity of DCG-04 since diol-DCG-04 and DCG-04 were able to label the same protein targets in rat live lysate. The probe-modified proteins were fully enriched from complex proteomes in a small scale and large scale with streptavidin beads. The immobilized proteins were quantitatively eluted with SDS boiling and oxidative cleavage respectively. In comparison with SDS boiling elution, there were less contamination by non-specific binding protein, endogenously biotinylated proteins and streptavidin.

4.3 Chemical elution leads to reduction of background proteins

The combination of affinity purification and MS analysis is one of the strategies for target deconvolution of small molecules. Contamination of non-specific binding proteins is a common drawback of affinity purification (127). To overcome this challenge, some labs repeat the experiment two or more times and proteins identified in all of the experiments are considered target proteins (128). A proper negative control (pull down that is carried out in an untreated sample) can also give useful information (129). In addition, quantitative proteomics is a powerful tool to distinguish target protein and contamination (73). However, reduction of background proteins prior to tryptic digestion and MS analysis will reduce the interference in the subsequent analysis. For low abundant new and uncharacterized target proteins, it would be more important to clean these contaminations. Though stringent washing was carried out to remove the non-specifically binding proteins, there is a lot contamination in the sample from on-bead tryptic digestion which indicated the non-specific binding is tight. The mild elution conditions of the diol linker allow the reduction of background protein identification by more than 80% in comparison with on-bead digestion. Two more cathepsins were identified in rat

liver lysate than previous report (117). The sequence coverage of cathepsins is comparable in both on-bead digestion and chemical elution sample. Endogenous biotinylated proteins were only observed in on-bead digestion suggesting that cathepsins were selectively eluted without disrupting the biotin-streptavidin complex. Recently, diol cleavable linker based probes have been utilized to capture and analyze Aspartic proteases (130). Consistent with our results, contamination was also significantly reduced.

4.4 Diol cleavable linker is compatible with tandem labeling strategy

Small molecule chemical probes are utilized to characterize protein targets and unravel protein function in chemical proteomics (11, 71). Biotin is widely used for attachment to chemical probes for target protein visualization and immobilization. The limited cell permeability of biotin-containing probes restricts their application *in vitro*. However, the ideal way for protein target identification is carrying out the labeling in physiological conditions such as a cellular context or a whole organism (47, 131). The application of CuAAC based tandem labeling strategy for targets discovery *in situ* and *in vivo* was firstly validated by Cravatt (47, 51). To show the application of the diol cleavable linker, it was incorporated into clickable biotin reagents with solid phase synthesis or solution phase synthesis for ligation with alkyne-E64 and azido-E64 modified cathepsins. More cathepsins were identified in living cells than in lysate, indicating the importance to characterize the protein targets *in situ*. We assume that a combination of the diol cleavable linker with a clickable biotin tag will be widely used to uncover novel protein targets *in vivo/situ*.

4.5 The oxidation of sodium periodate has no influence on the protein elution and sequence coverage of proteins

Cleavage of the diol linker in chemical probes and glycans in cathepsins generated aldehydes during the chemical elution with sodium periodate. The possible Schiff base formation between aldehydes and streptavidin beads may lead to the inefficient elution. However, we showed the efficiency of chemical elution was as efficient as SDS boiling elution indicating

the aldehydes have no influence on the protein elution. In addition, oxidized diol probe has no reaction with bradykinin (a mode peptide) even at the concentration of 10 μM indicating the aldehydes should not react with tryptic peptides. $NaIO_4$ can also cause the oxidation of methionines. LC-MS/MS analysis confirmed that aldehyde formation and oxidation of methionines had no significant influence on the sequence coverage of cathepsins. Furthermore, treatment of $NaIO_4$ oxidizes the N-terminal serine and threonine of proteins but these proteins are quite rare. Therefore, the loss of N-terminal peptides of these proteins does not represent a problem for LC-MS/MS analysis of proteins.

4.6 The probe-modified peptides could not be identified

An additional benefit of a cleavable linker is the removal of biotin modified peptides and enhancement of the ionization. In addition, SDS boiling could disrupt the probe-protein/peptide adduct which are heat sensitive (132). Therefore cleavable linkers are ideal tools for the characterization of modification sites of proteins (83, 87). The aldehyde groups in the probe-peptide adduct have no influence on the characterization of probe-modified peptides since the reactive aldehydes 4-hydroxynonenal modified peptides were identified successfully (133). In our study, the active sites were not detected probably due to the large size of the modified peptides. In the future, we will try to identify the modification site of other families of enzymes with this cleavable biotin reagent. Instead of trypsin, other digestive methods may aid to reduce the mass of the modified peptide in the future. In addition, a higher amount of proteome can increase the abundance of modified peptides.

4.7 Cleavable trifunctional biotin reagents were designed and synthesized

Clickable biotin reagents can be used for labeling and purification of proteins modified with a clickable probe in living cells or animals (47). Visualization can then be performed using a streptavidin-western blot. In addition, clickable fluorophores afforded a straightforward way for detection of probe-modified proteins but subsequent enrichment of tagged proteins was not straightforward (13). For protein purification, biotin reagents can be used in a pull-down on

streptavidin beads and subsequent elution. Before trypsin digestion, the eluted proteins are usually separated with SDS-PAGE. Proteins are then visualized with silver staining or coomassie staining. These two protein staining methods, however, are limited in their sensitivity and fail to visualize low abundant proteins. A tag containing both a biotin and a fluorophore can overcome this problem by providing a visualization method that is less time consuming, highly sensitive and compatible with in gel digestion (47, 134). Hence, trifunctional tags, carrying a ligation handle for conjugate with the probe-protein mixture, a biotin for immobilization and a fluorophore for protein visualization have become popular, since they can be applied for both visualization and affinity purification (132, 135). Through click handles the trifunctional tags are conjugated to probe-protein complexes. The tag-probe-protein complex can be enriched with the biotin-streptavidin system. The fluorescent group makes the labeled protein sensitively detectable by in-gel fluorescent scanning, and allows it to be excised for subsequent MS analysis.

However, due to the strong interaction between biotin and streptavidin harsh conditions are necessary for the elution of enriched proteins. This usually leads to a contamination of the sample with non-specifically bound proteins and natively biotinylated proteins, which complicates target identification. To solve this problem, N3-ethyl biotin, an analog of biotin with less affinity to streptavidin, was incorporated into trifunctional reagents (82). The new reagent allowed the immobilized proteins to be released under a mild condition while N3-ethyl biotin was not cleaved from probe-protein complex. Sieber et al developed a trifunctional biotin reagent containing a cleavable linker derived from Phenacyl ester (103). However, it has not been used to enrich and release proteins in a biological system.

We designed and synthesized five different versatile biotin reagents for tandem labeling of proteins and subsequent immobilization, elution and detection. Biotin-dde-TMR-N_3 was assembled by solid phase synthesis and four other reagents were partly synthesized on solid support, except for the fluorophore coupling which was done in solution.

4.8 Cleavable trifunctional biotin reagents can be used for protein labeling, immobilization and mild elution

All the reagents except biotin-S-S-TMR-N_3 could selectively react with proteins that had been modified with alkyne-probes *in vitro* or in living cells. Background labeling occurred for biotin-S-S-TMR-N_3. This phenomenon was also observed in the labeling with other disulfide

based biotin reagents in previous reports (85). Alkylation of free cysteine in the proteome prior to conjugation of disulfide based biotin reagents enables reduction of background labeling (85).

The proteins labeled by trifunctional reagents could be immobilized by streptavidin beads efficiently. The immobilization efficiency of the disulfide linker and the dde linker was weaker compared than other linkers. For the disulfide linker, alkylation of free cysteine to prevent the premature cleavage may improve the immobilization efficiency. For elution of captured proteins, the trifunctional reagents containing a diol or a dde linker were the most efficient in chemical elution while diazo and hydrazone containing reagents are less efficient. For diazo containing reagents, more sensitive cleavable building blocks could be introduced in the future to increase its elution efficiency. For hydrazone containing reagents, appropriate structural modifications may increase the sensitivity towards hydroxylamine. In addition, increasing of cleavage time could be an alternative way to increase the elution efficiency. The dde based linker represents a novel cleavable linker in protein enrichment strategies. Biotin-dde is simply acquired by one step synthesis and easily incorporated into trifunctional reagents by solid phase synthesis. Furthermore, the biotin-dde trifunctional tag has a hidden trypsin cleavable bond and allows release of the biotin and fluorophore which leads to a small residual modification.

4.9 Outlook

Due to the inexpensive starting material, simple synthesis, mild elution condition and efficient background proteins reduction, we anticipate that the diol linker will be valuable tool in chemical proteomics. Future research could be directed towards different chemical probes and enzymes to evaluate the efficiency of the diol cleavable linker for the identification of probe-modified peptides. Beside above mentioned methods, a different workflow can also be helpful for the site identification; the probe-labeled proteome is first digested, the modified peptides are then immobilized, followed by chemical elution (102). Alternatively, the probe labeled proteome can be enriched onto streptavidin beads. On-bead digestion is then followed by washing and chemical release of the modified peptides (83, 105) (Figure 45). These workflows can reduce the effect of ion suppression by other, highly abundant tryptic peptides.

Incorporation of stable isotopes into biotin-diol-alkyne and biotin-diol-azide could also be carried out to broaden the application of the diol linker in quantitative proteomics.

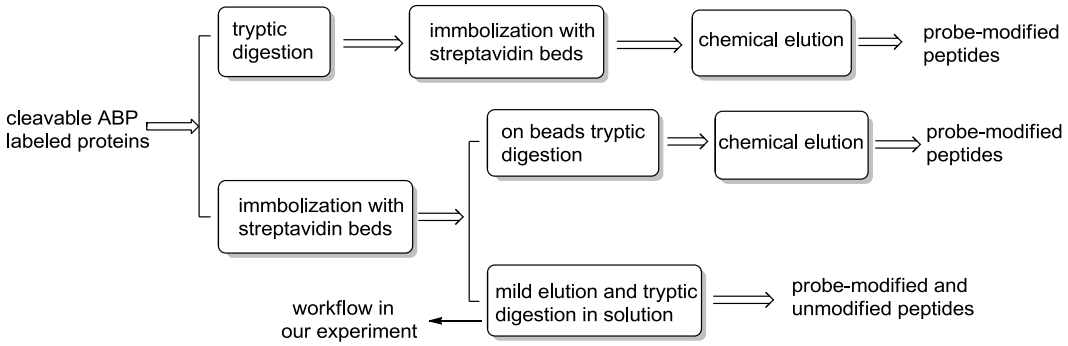


Figure 45: The flow charts for isolation of probe-modified peptides.

For trifunctional biotin reagents, further studies will be carried out to quantify the efficiency of background proteins reduction by comparison of on-bead digestion with chemical elution digestion with LC-MS/MS analysis. Extension of the length of the linker between the biotin and the dde motif can be performed to see if a reduction in the steric bulk of the dde next to the biotin improves the pull down efficiency. The advantage of the biotin-dde-TMR-N_3 for probe-modified peptides identification should also be evaluated.

5 Materials and methods

5.1 Biochemistry

5.1.1 SDS-PAGE and protein detection

5.1.1.1 Preparation of SDS-PAGE gel

All the 15% SDS PAGE gels used in this thesis were made in our laboratory. The solutions for preparation of 10 mini gels and 2 Triple-wide gels are prepared as shown in Table 1. Tetramethylethylenediamine (TEMED) is added to the solution as a last step. The solution is mixed gently and gels are poured as soon as possible. 2-propanol (200 µl for each mini gel and 1ml for each Triple-wide gels) is overlaid on top of separating gels. After polymerization and removal of 2-propanol (0.5h-1h), the stacking gels is poured. The gels are covered with wet paper and stored at 4 ^{o}C.

Table 12.Recipes for SDS gels

	For 10 mini gels		For 2 Triple-wide gels	
	Stacking gels	separating gels	Stacking gels	separating gels
Tris buffer	15 ml	20 ml	3.75 ml	12.5 ml
30% acrylmide	9.6 ml	40 ml	2.5 ml	25 ml
dd H_2O	35 ml	20 ml	8.75 ml	12.5 ml
Total	59.6 ml	80 ml	15 ml	50 ml
10% APS	450 µl	550 µl	100 µl	250 µl
TEMED	35 µl	35 µl	10 µl	25 µl

5.1.1.2 SDS-PAGE

To the samples 4X sample buffer (16 µl for 50 µl protein sample) was added. 30 µl of the mixed solution was loaded onto the SDS-PAGE. Gel electrophoresis was done at 120 V, 40

mA for 10 lane mini gels and 120V, 250 mA for triple-wide gels. The gel running was stopped when the blue dye front was at the bottom of the gel.

5.1.1.3 Proteins visualized by in-gel fluorescent scanning

Tetramethylrhodamine-labeled samples were resolved on a 15% SDS polyacrylamide gel. The glass plate of SDS-PAGE gel was washed with H_2O and dried with paper. The gel, in the glass plates, was directly scanned on a Typhoon Trio+. Raw image data were analyzed with Image J (NIH).

5.1.1.4 Biotin-streptavidin western blot

Biotinylated probes labeled samples were resolved on a 15% SDS polyacrylamide gel. Separated proteins were transferred to a nitrocellulose membrane. The membrane was blocked with 3% milk in PBST (0.1% Tween in PBS) for 1h at room temperature or overnight at 4 ^{o}C. Then the membrane was washed with PBST (3x5 min) and incubated with streptividin-peroxidase (sigma, diluted 3500 x from a 1mg/ml stock solution) for 1h. The membrane was washed with PBST (3x15min). Then the membrane was treated with ECL plus (GE healthcare; catalog # RPN 2132) 0.5-1 mL: incubation for 2 minutes) and exposed to Kodak X-omat LS film (180x240 mm).

5.1.1.5 Coomassie blue staining

SDS-PAGE gels were incubated with freshly made coomassie staining solution (ddH_2O : Methanol : Roti®-Blue = 3: 1: 1) (Roti®-Blue, colloidal Coomassie; Carl Roth) overnight. The SDS-page gels were washed with ddH_2O 5 times and incubated with ddH_2O for 30min.

5.1.1.6 Silver staining

Gels with a low amount of proteins were stained by silver staining, which is more sensitive than Coomassie blue staining. The solutions needed in this process were prepared in the lab. The gel was treated following the workflow below. 1) Incubate the gel for 1h in Fix-sln; 2)

Wash the gel with MeOH/ddH_2O (1:1) for 2x 15 min; 3) Incubate the gel in pretreat-sln for 1 min; 4) Wash the gel with ddH_2O 3x 20 s; 5) Impregnate the gel with impregnation-sln for 20 min; 6) Wash the gel with dd H_2O 2x 30 s; 7) Incubate with develop-sln until the appearance of bands. 8) Wash with stop-sln 2x 10 min.

5.1.2 Cell culture

RAW 264.7 cells were cultured in DMEM medium containing 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, and 100µg/mL streptomycin and maintained in a humidified 37 °C incubator with 5% CO_2.

5.1.3 Preparation of cell lysate and rat liver homogenate

To generate lysates, cells were washed twice with cold phosphate-buffered saline (PBS), harvested by usage of a cell scraper, and collected by centrifugation. Cell pellets were then washed with PBS and lysed with different lysate buffers on ice for 30 min. The mixture was centrifuged (15000 rpm, 4^{o}C, for 20 minutes) to remove unlysed cells and cell debris. The supernatant was snap-frozen in liquid nitrogen and stored at -80^{o}C. Protein concentration was determined by Bradford or DC protein assay (Bio-Rad).

For the rat liver homogenate, a piece of rat liver was washed with PBS to get rid of hair and fat. Then it was cut into small chunks and combined with lysis buffer (50 mM acetate pH 5.5, 5 mM $MgCl_2$, 250 mM sucrose and 2 mM DTT). The liver/buffer mixture was dounced 15x on ice. After douncing, the mixture was centrifuged (2000 rpm, 4^{o}C for 10 min) to remove unlysed tissue, then centrifuged (15000 rpm, 4^{o}C, for 20 minutes) to remove cell debris. The supernatant was collected, snap frozen in liquid nitrogen and stored at -80^{o}C. The protein concentration was determined by Bradford or DC protein assay (Bio-Rad).

5.1.4 Labeling of cathepsins *in situ* and *in vitro*

In *situ* labeling: Cells with nearly 90% confluence were incubated with fresh medium. Probes (in DMSO stock solution) were added to the medium (final concentration of DMSO is 0.1%). Cells were maintained in a humidified 37°C incubator with 5% CO_2 for the indicated time. Cells were washed with PBS (3x) and disrupted in different lysate buffers.

In vitro labeling: Cell lysate and rat liver lysate were incubated with probes for indicated time at room temperature. The reaction was stopped by adding 4xSB buffer and the proteins were separated by SDS-PAGE. Proteins were visualized by different methods dependent on the

labeling tags. For bioconjugation chemistry, the labeled proteome was run through a Zeba spin column (for small scale) or PD-10 column (for large scale) to get rid of free clickable probes. The elution was subjected to click chemistry. For pull down, the labeled proteome was run through a Zeba spin column (for small scale) or PD-10 column (for large scale) to remove free biotinylated probes. The biotinylated probes can also be removed by acetone precipitation.

5.1.5 Competition experiment

Competition experiments were used to test the activity, selectivity and cell permeability of new probes (114) (usually the probe itself has no tags for detection). In *vitro*, biotinylated probe was added to a lysate which was pretreated with new probes for the indicated time. In addition, lysate was directly incubated with biotinylated probes as a control. In living cells, new probes were added to the medium and cells were grown for the indicated time and a lysate was prepared using standard conditions. The cell lysate was labeled with biotinylated probe. Lysates from control cells were also labeled with biotinylated probe. The labeled proteomes were separated with SDS PAGE and visualized by biotin streptavidin blot.

5.1.6 Acetone precipitation

The samples were added to 4 volumes of cold acetone (pre-stored at -20 ^{o}C). The mixture was vortexed and stored at -20 ^{o}C for 30 min, then centrifuged at 4 ^{o}C, 10000-15000 g for 10 min. The supernatant was carefully removed, making sure not to dislodge the protein pellet. The acetone was evaporated at room temperature for 20-30 min. The pellet was dissolved in 1% SDS in PBS. If necessary, the SDS was diluted with PBS.

5.1.7 Click chemistry

To clickable probe labeled proteomes from living cells and elutions from Zeba spin columns or PD-10 columns the clickable tag (25-50 µM, from a 5 mM stock solution in DMSO), TBTA (50 µM, from a 5 mM solution in DMSO), $CuSO_4$ (1 mM, from a 25 mM solution in ddH_2O) and TCEP (1 mM, from a 25 mM in dd H_2O) were added. The solutions of $CuSO_4$ and TCEP were freshly made. The reaction mixture was incubated at room temperature for 0.5-1h. For pull down, free probes were removed by column (Zeba spin column for small scale and PD-10 column for large scale) or by acetone precipitation. For SDS-PAGE, 4XSB buffer was added to stop reaction and separated by SDS-PAGE. For fluorophore labeled samples, the

reaction was stopped by acetone precipitation to remove free fluorophore tags. The protein pellet was redissolved in 1X SB and loaded onto SDS-PAGE.

5.1.8 Immobilization

For probe-modified proteome, 50 µg of probe labeled proteome (no free probe present) was kept as a control to detect the efficiency of immobilization. Streptavidin beads were washed with PBS for 5-6 times. Probe labeled proteome was added to streptavidin beads and the mixture was shaken for 1-5h at room temperature. After centrifugation, the supernatant was taken out. The same amount of proteome from control and supernatant was loaded on SDS-PAGE and labeled proteins were visualized by biotin streptavidin blot. The efficiency of immobilization was determined by comparison of the labeled proteins in two samples.

5.1.9 Comparison of chemical release and SDS boiling

After immobilization, streptavidin beads were washed with indicated buffers and then divided into equal aliquots. For SDS boiling, the beads were added to 1XSB and boiled at 90 °C for 5min. The warm elution was taken out as soon as possible. For chemical release, the beads were treated with chemical release buffer and shaken for the indicted time. Next, the beads were washed with the indicated buffers. Chemical elution and wash solution were combined and 1/3 volume of 4XSB buffer was added. Subsequent boiling of the beads was carried out to see the left protein after chemical release. The final volume of chemical elution and SDS boiling should be kept equal. The same amount of the solution was loaded onto SDS PAGE. The proteins were visualized and the efficiency of chemical release and SDS boiling release was determined by comparison of the intensity of target bands.

5.1.10 On-bead digestion and chemical release based in solution digestion

The beads were sequentially washed with a series of buffers containing 1% SDS in PBS, 4 M urea in H_2O, 1 M NaCl in PBS, and 10% EtOH in PBS and finally the beads were washed with PBS buffer 3 times. After washing, beads were either used for on-bead digestion or chemical elution. For on-bead digestion, streptavidin beads with bound proteins were resuspended in 100 µl of denaturing buffer (50 mM ammonium hydrogen carbonate, 6 M urea). Bound

proteins were reduced in the presence of 10 mM DTT for 1 h. Samples were alkylated by addition of 200 mM iodoacetamide (20 µl) and incubated for 1 h in the dark. Unreacted iodoacetamide was neutralized by addition of 200 mM DTT (20 µl). The urea concentration was reduced by addition of distilled H_2O (800 µl). Samples were incubated with trypsin overnight at 37 °C and purified on a Vivapure C18 spin column (Sartorius). Chemical elution was performed by addition of elution buffer 50 µl (10 mM sodium periodate in 100 mM sodium phosphate buffer, pH 7.4) for 30 min in the dark. The cleavage buffer was removed and replaced with fresh solution. The two eluted supernatants were combined and desalted with a Zeba spin desalting column. To the eluate (100 µl; 100 mM ammonium hydrogen carbonate) was added 12 M urea (100 µl) and the sample was prepared for LC-MS/MS analysis as described for the on-bead digestion.

5.1.11 Protein identification with LC-MS/MS

(Samples were measured in Chair of proteomics and bioanalytics, TUM) Nanoflow LC-MS/MS of tryptic peptides was performed by coupling an Eksigent nanoLC-Ultra 1D+ (Eksigent, Dublin, CA) to a LTQ Orbitrap Velos (Thermo Scientific, Bremen, Germany). Tryptic peptides were eluted from the Vivapure spin columns with 50/50 water/ACN (20 µl 0.1% FA) and 10 µl were injected for each analysis. Peptides were delivered to a trap column (100 µm i.d. x 2 cm, packed with 5 µm C18 resin, Reprosil PUR AQ, Dr. Maisch, Ammerbuch, Germany) at a flow rate of 5 µL/min in 100% buffer A (0.1% FA in HPLC grade water). After 10 min of loading and washing, peptides were transferred to an analytical column (75 µmx40 cm C18 column Reprosil PUR AQ, 3µm, Dr. Maisch, Ammerbuch, Germany) and separated using a 110 minute gradient from 7% to 35% of buffer B (0.1% FA in ACN) at a flow rate of 300 nL/min. The mass spectrometer was operated in data dependent mode, automatically switching between MS and MS2. Full scan MS spectra were acquired in the Orbitrap at 30,000 resolution, and tandem MS spectra were acquired at 7500 resolution. Internal calibration was performed using the ion signal $(Si(CH_3)_2O)_6H^+$ at m/z 445.120025 present in ambient laboratory air. Tandem mass spectra were generated for up to ten peptide precursors using higher energy collision dissociation (136). Precursors were dynamically excluded from fragmentation for 20 sec, and unassigned charge states as well as singly charged ions were rejected.

5.1.12 LC-MS/MS data processing and analysis

Peak picking and processing of raw MS data was performed using the Mascot Distiller software (version 2.3, Matrix Science, London, UK) and peak list files were submitted to Mascot (version 2.3.01, Matrix Science, London, UK) for peptide and protein identification. The Mascot database search was performed against either the SwissProt database (version 57) with taxonomy restricted to *Rattus* for the rat liver lysate samples or against the UniProtKB mouse complete proteome set (download date 26/10/2010) for the RAW cell samples including protein sequences of common laboratory contaminants and with the Mascot built-in target-decoy database search option was enabled. Search parameters included a precursor tolerance of 10 ppm, a fragment tolerance of 0.02 Da, and accounted for the oxidation of methionine (+15.9949 Da), the carbamidomethylation of cysteine residues (+57.0215 Da), and the modification by cleaved diol-DCG-04 with and without hydration (622.2928 and 640.3034 Da) or clicked and cleaved azido-E64 with and without hydration (466.2254 and 484.2360 Da), all as variable modifications. The Mascot search result files were analyzed using Scaffold version 3.3.1 (Proteome Software Inc., Portland, USA). Threshold parameters (protein probability, 99%; minimum number of peptides, 2; peptide probability, 95%) were highly conservative and resulted in a peptide and protein false discovery rate of 0%.

5.1.13 Detection of the cleavage of chemical probes and tags

5.1.13.1 Detection of the cleavage of diol-DCG-04

Diol-DCG-04 (2 mM in DMSO, 20 µL) was added to 400 µl of $NaIO_4$ (10 mM in SP buffer, pH 7.4). The solution was stirred for 1 h at RT. Then solution was subsequently purified with a C18 column (Pierce). The bound material was eluted with 0.1% FA in 50% ACN/H_2O and detected by LC-MS.

5.1.13.2 Detection of the reaction between bradykinin and the cleavage products of diol-DCG-04

4µL of Diol-DCG-04 (2 mM in DMSO) and 8 µL of bradykinin (1M in DMSO) were added to

4 mL or 0.8 mL of cleavage buffer (10 mM of $NaIO_4$ in SP buffer, pH 7.4). The solution was stirred in the dark for 1 h at room temperature. Then the solution was purified with a C18 column (Pierce) following the protocol provided by the supplier. The bound material was eluted with 0.1% FA in 50% ACN/H_2O (50 µl). As a control, 10 µL of bradykinin (1M in DMSO) was added to 0.4 mL of cleavage buffer (10 mM of $NaIO_4$, SP buffer, pH 7.4). The solution was stirred for 1 h and purified with a C18 column followed by analysis with LC-MS.

5.1.13.3 Detection of the cleavage of biotin-dde-TMR-N_3

For cleavage by hydrazine, biotin-dde-TMR-N_3 (1µl of 5 mM of stock solution in DMSO) was added to hydrazine solution (100 mM of hydrazine in H_2O, 1 ml) or Tris buffer (200 mM Tris, 0.05% SDS, pH 8.5, 1ml). The solutions were stirred 14h at room temperature. The solutions were concentrated with a C18 stage tip (137) and eluted with 100 µl of 50% MeCN/H_2O, 0.1% FA. 1µl of solution was analyzed by LC-MS. To detect the hidden tryptic cleavage site, the elution from hydrazine treated sample was evaporated in a speed vac. Acetonitrile (10 µl) was added to dissolve the residue. 100 mM of NH_4HCO_3 (1 ml) and trypsin (1µg) were added. The solution was incubated overnight at 37 ^oC. The solution was concentrated with a C18 stage tip and eluted with 100 µl of 50% MeCN/H_2O, 0.1% FA. 1 µl of solution was analyzed by LC-MS.

5.2 Synthesis of chemical probes and cleavable biotin reagents

5.2.1 General methods for chemistry synthesis

Unless otherwise noted, all reagents were purchased from commercial suppliers and used without further purification. Reactions were analyzed by thin-layer chromatography or LC-MS. Target compounds were purified with HPLC and silica column chromatograph. The purity was characterized by NMR or LC-MS.

5.2.1.1 LC-MS

All LC-MS runs were performed on an Agilent 6210 LC-MS equipped with an electrospray TOF, using an Agilent Zorbax-SB C18 column (150 x 0.5 mm) and a gradient of 0-100% MeCN in water + 0.1% formic acid.

5.2.1.2 HPLC

Preparative HPLC purification was performed on a Waters 515 HPLC system using a X-bridge C18 column. Purifications were performed at room temperature and compounds were eluted with increasing concentration of acetonitrile (solvent A: 0.1% TFA in water, solvent B: 0.1% TFA in acetonitrile). The collected fractions were analyzed by direct injection of diluted faction to ESI-TOF. The solution containing products was frozen in liquid N_2 and lyophilized. The resulting powder was stored in -20 ^{o}C.

5.2.1.3 Silica column purification

Silica gel (grain distribution of 0.04-0.063 mm, pore size of 60 Å, silica gel 60 was used to prepare chromatography columns. EtOAc/PE and DCM/MeOH were solvent systems in our study. For purification of compound with free amine, 1% of NH_4OH was added to the solvent.

5.2.1.4 Thin layer chromatography

Thin layer chromatography was used to detect the reaction and silica column purification. High concentration of solution was diluted with DCM before spotted on thin layer chromatography Plate (ALUGRAM sil G/UV254 from Roth). Samples (not colored) were visualized with UV and staining solution. Four different staining solutions were prepared and used in our synthesis. Cerium-ammonium-molybdate solution is a universal staining. Ninhydrin solution is for free amines. Dinitrophenylhydrazine solution is good for aldehydes and ketones. Basic $KMnO_4$ solution is for alkynes and alkenes.

5.2.1.5 NMR

^{1}H and ^{13}C NMR spectra were measured on a Bruker AM-400 spectrometer in $CDCl_3$ or DMSO-d_6 using residual non-deuterated solvent peak as an internal reference (δH = 7.26 ppm for $CDCl_3$ and δH = 2.5 ppm for DMSO-d_6). Assignment abbreviations: s (singlet), d (doublet), dd (double doublet), t (triplet), m (multiplet), bs (broad singlet). COSY and DEPT were used as a guide to assign signals in ^{1}H-spectra where required. Chemical shifts (δ) are reported in parts per million (ppm), and the coupling constants (*J*) are given in Hertz (Hz)

5.2.1.6 Solid phase synthesis

Fmoc-Rink amide resin was used as solid support to synthesized probes and biotin reagents. The reaction was carried out in polypropylene cartridge (ERC GmbH, 3 ml of polypropylene cartridge for 25 µmol scale synthesis, 6 ml of polypropylene cartridge for 50-100 µmol scale synthesis and 12 ml of polypropylene cartridge for 200 µmol scale synthesis). Polypropylene cartridge was fitted with stopcocks and connected to a 12 well port vacuum manifold that was used to drain solvents and reagents from the polypropylene cartridge. The resin was swelled in DMF. Fmoc is removed by treatment with 20% piperidine in DMF for 20 min followed by washing with DMF (3 x). The building block was coupled under the influence of coupling reagents (DIC/HOBt, DIEA/HBTU or DIEA/PyBOP). After coupling, the resin was washed with DMF (3 x) and DCM (3 x). Kaiser-test was performed to detect the coupling efficiency. If the coupling was complete, DMF was used to wash resin to get rid of DCM. New building block was loaded following the same process. After all building blocks are loaded on to resin, resin is washed with DMF (3x) and MeOH (3 x) followed by treated with cleavage cocktail. The mix was collected and resin was washed with TFA (2 x). These solutions were combined and TFA was removed by cold ether precipitation or evaporation under reduced pressure. Products were dissolved in a minimal amount of DMSO and purified by HPLC.

5.2.2 Synthesis of dimethyl 2,3-*O*-isopropylidene-L-tartrate 1

L-tartaric acid (10.1 g, 67.3 mmol) was added to a solution of methanol (4 ml) and *p*-toluenesulfonic acid (40 mg, 0.23 mmol) and 2, 2-dimethoxypropane (19 ml). The solution was stirred under reflux at 50^{o}C for 2 h. Another 10 ml 2,2-dimethoxypropane and dry cyclohexane (40 ml) was added. The solution was (heated) to 68^{o}C and refluxed for 2 days and the volatile compounds (acetone, methanol) with cyclohexane were slowly removed by simple distillation. Anhydrous potassium carbonate (100 mg) was added to neutralize the reaction. The solvent and unreacted 2,2-dimethoxypropane were removed under reduce pressure. The crude compound was purified by vacuum distillation and isolated as a light yellow oil 10.9 g (50 mmol, 74%). ^{1}H NMR (400 MHz): 4.81 (s, 2H), 3.83 (s, 6H), 1.50 (s, 6H). ^{13}C DEPT NMR (400 MHz): 76.99, 52.83, 26.30.

5.2.3 Synthesis of methyl 2,3-*O*-isopropylidene-L-tartrate 2

To a solution of dimethyl 2,3-*O*-isopropylidene-L-tartrate (2.06 g, 9.468 mmol) in CH_3OH (20 ml) was added a solution of KOH (531.24 mg, 9.468 mmol) over 1h. The reaction was stirred overnight and evaporated. The resulting residue was dissolved in Et_2O (30 ml) and washed with 10 ml saturated $NaHCO_3$ solution. The aqueous portion was acidified to pH 3.5 with 3 M HCI and extracted with Et_2O (8 x 20 ml). The combined Et_2O extracts were dried and evaporated to give a colorless oil 1.21g (63%). ^{1}H NMR (400 MHz): 4.89 (d, 1H, J = 6.0 Hz), 4.83 (d,1H, J = 5.5 Hz), 3.85 (s, 3H), 1.53 (s, 3H), 1.51 (s, 3H). ^{13}C DEPT NMR (400 MHz): 77.04, 76.46, 53.03, 26.33, 26.29.

5.2.4 Synthesis of ethyl (2S, 3S)-epoxysuccinate 4

Diethyl (2S, 3S)-epoxysuccinate (**3**): 33% HBr/AcOH (40 ml) was added to diethyl-D-tartrate dropwise (10.308 g, 50 mmol) at 0^{o}C. After the last drop of HBr/AcOH was added, the solution was stirred overnight at RT. The solution was extracted with diethyl ether and washed with water (4 x) and brine. The organic layer was dried with $MgSO_4$ and concentrated under reduced pressure. EtOH (75 ml) and acetyl chloride (2 ml) was added to the resulting oil. The solution was heated and refluxed for 6 hours. Concentration under reduced pressure yielded pale-yellow oil. The pale-yellow oil was added diethyl ether (75 ml) and put on the ice. DBU (7.46 ml, 50 mmol) was added to diethyl ether (37.5 ml). The mixed solution was added to the pare-yellow oil dropwisely at 0^{o}C. After 2 h, H_2O (12.5 ml) was added to the solution and the reaction stirred for another 30 min. Then the solution was washed with 1M $KHSO_4$ and H_2O. The organic layer was dried with $MgSO_4$ and concentrated under reduced pressure. The

resulting oil (4.3 g) was purified by vacuum distillation and isolated as light yellow oil (3.52 g, 37%).

Ethyl (2S, 3S)-epoxysuccinate (**4**): Diethyl (2S, 3S)-epoxysuccinate (1.88 g, 10 mmol) was dissolved in EtOH (25 ml) and placed in an ice bath. KOH (0.5611g, 10 mmol) was dissolved in EtOH (15 ml) and dropwise added to the colorless solution over 15 min. The reaction was stirred at RT and detected by TLC. After the reaction was complete, the solvent was removed by evaporation. Water (30 ml) was added and then the solution was washed by DCM (10 ml). The aqueous layer was acidified with concentrated HCl and NaCl (8.5 g) was added. The solution was extracted with EtOAc (4 x 30 ml). The organic layer was combined and dried by $MgSO_4$. The residue was concentrated yielding colorless oil (1.3 g, 81%).

5.2.5 Synthesis of diol-DCG-04 5

The Fmoc of Rink resin (84 mg, 50 µmol) was deprotected by 20% piperdine in DMF. Fmoc-Lys(biotin)-OH (44 mg, 75 µmol), DIC (23µl, 150 µmol), HOBt hydrate (23 mg, 150 µmol) were dissolved in 1.5 ml of NMP and the solution was added to the resin and the reaction was agitated overnight. Fmoc group was deprotected and washed. Methyl 2,3-O-isopropylidene-L-tartrate (30 mg, 147 µmol), DIC (23.6 µl, 150 µmol), HOBt hydrate (23 mg, 150 µmol) were added and the reaction was kept for 6 h. The resin was washed with DMF and 1,8-diamino-3,6-dioxaoctane (1 ml) was added for overnight. 1,8-diamino-3,6-dioxaoctane was washed with DMF followed by coupling of Fmoc-Tyr(tBu)-OH (69 mg, 150 µmol) with DIC (23.6 µl, 150 µmol), HOBt hydrate (23 mg, 150 µmol) for 3 h. The Fmoc was deprotected followed by washing and adding Fmoc-Leu-OH (52.95 mg, 150 µmol), DIC (23.6 µl, 150 µmol), HOBt hydrate (23 mg, 150 µmol). The coupling of Fmoc-Leu-OH was kept for 3 h. After remove of Fmoc, ethyl (2S, 3S)-epoxysuccinate (24 mg, 150 µmol), DIC (23.6 µl, 150 µmol) and HOBt hydrate (23 mg, 150 µmol) were added overnight. Diol-DCG-04 was cleaved from resin by incubation with a solution of TFA: TIS: H_2O (90%: 2.5%: 7.5%) for 3 hours. The peptide was precipitated in cold diethyl ether and purified by HPLC using a linear gradient of 10-70% B yield building block (9 mg, 17%). ESI-MS: $[M+H]^+$ calculated for $C_{47}H_{73}N_9O_{16}S$ 1052.4896, found 1052.4897. ^{1}H NMR (400 MHz): 8.52 (d, 1H, *J* = 4.4 Hz), 8.08 (d, 1H, *J* = 8.0 Hz), 7.9 (t, 1H, *J* = 5.6 Hz), 7.73 (t, 1H, *J* = 5.6 Hz), 7.68 (d, 1h, *J* = 8.0 Hz), 7.65 (t, 1H, *J* = 6.0 Hz), 7.35 (s, 1H), 7.16 (s, 1H), 6.97 (d, 2H, *J* = 8.4 Hz), 6.61 (d, 2H, *J* = 8.4 Hz), 6.42 (bs, 2H), 4.39 -4.27 (m, 4H), 4.24-4.11 (m, 5H), 3.71 (d, 1H, *J* = 1.6 Hz), 3.59 (d, 1H, *J* = 2.0 Hz), 3.54-3.46 (m, 4H), 3.43 (t, 2H, *J* = 6.0 Hz), 3.38- 3.07 (m, 7H), 3.01- 2.94 (m, 2H), 2.82(dd, 2H, *J*

= 5.2 Hz, *J* = 12.8 Hz), 2.71-2.65 (m, 1H), 2.57(d,1H, *J* = 12.4 Hz), 2.035 (t, 2H, *J* = 7.2 Hz), 1.75-1.28 (m, 15H), 1.23 (t, 3H, *J* = 7.2 Hz), 0.86 (d, 3H, *J* = 6.4 Hz), 0.822 (d, 3H, *J* = 6.4 Hz).

5.2.6 Synthesis of TMR-diol-DCG-04 7

The Fmoc of Rink resin (84 mg, 50 µmol) was deprotected by 20% piperdine in DMF. Fmoc-Lys(biotin)-OH (44 mg, 75 µmol), DIC (23.6 µl, 150 µmol), HOBt hydrate (23 mg, 150 µmol) were dissolved in 1.5 ml of NMP and the solution was added to the resin and the reaction was agitated overnight. Fmoc group was deprotected and washed. Methyl 2,3-O-isopropylidene-L-tartrate (32.7 mg, 160 µmol), DIEA (52 µl, 300 µmol), PyBOP (78 mg, 150 µmol) were added and the reaction was kept for 3 h. The resin was washed with DMF and incubated with 0.25M KOH in EtOH for 2 x 20 min. The resin was washed with 1% AcOH in EtOH and DMF. Mono-Fmoc-ethylenediamine (47.7 mg, 150 µmol), DIEA (52 µl, 300 µmol), PyBOP (78 mg, 150 µmol) was added and the reaction was kept for 3h. N-terminal of Fmoc was removed followed by adding Fmoc-Lys(Boc)-OH (70 mg, 150 µmol), DIC (23.6 µl, 150 µmol), HOBt hydrate (23 mg, 150 µmol). After deprotection, Fmoc-8-amino-3, 6-dioxaoctanoic acid (58 mg, 150 µmol) was introduced under the influence of DIC (23 µl, 150 µmol), HOBt hydrate (23 mg, 150 µmol). After deprotection, Fmoc-Tyr(tBu)-OH (69 mg, 150 µmol) with DIC (23.6 µl , 150 µmol), HOBt hydrate (23 mg, 150 µmol) were added for 3 h. The Fmoc was deprotected followed by washing and adding Fmoc-Leu-OH (52.95 mg), DIC (23 µl, 150 µmol), HOBt hydrate (23 mg, 150 µmol). The coupling of Fmoc-Leu-OH was kept for 3 h. After remove of Fmoc, ethyl (2S, 3S)-epoxysuccinate (24 mg, 150 µmol), DIC (23.6 µl, 150 µmol), HOBt hydrate (23 mg, 150 µmol) were added overnight. Cleavable building block **6** was cleaved from resin by incubation with a solution of TFA: TIS: H_2O (90%: 2.5%: 7.5%) for 3 hours. The peptide was precipitated in cold diethyl ether and purified by HPLC using a linear gradient of 20-60% B yield building block (3.8 mg, 6.2%). ESI-MS: $[M+H]^+$ calculated for $C_{54}H_{88}N_{12}O_{17}S$ 1237.6060, found 1237.6040. To a solution of build block **6** (1.5 mg, 1.2 µmol) in DMSO (100 µl), 5(6)-carboxytetramethylrhodamine succinimidyl ester (0.768 mg, 1.44 µmol) and DIEA (0.223 µl, 1.3 µmol) was added and kept overnight. The solution was purified by HPLC using a linear gradient of 30-70% B yield TMR-diol-DCG-04 (1.1 mg, 56%). ESI-MS: $[M+2H]^{2+}$ calculated for $C_{84}H_{108}N_{14}O_{22}S$ 825.3739, found 825.3721.

5.2.7 Synthesis of biotin-diol-azide 8

The Fmoc of Rink resin (84 mg, 50 µmol) was deprotected by 20% piperdine in DMF. Fmoc-Lys(N_3)-OH (30 mg, 75 µmol), DIC (23.6 µl, 150 µmol), HOBt hydrate(23 mg, 150 µmol) were dissolved in DMF and the solution was added to the resin and the reaction was agitated overnight. Fmoc group was deprotected and washed. Methyl 2,3-O-isopropylidene-L-tartrate (32.7 mg, 160 µmol), DIC (23.6 µl, 150, µmol), HOBt hydrate (23 mg, 150 µmol) were added and the reaction was kept for 3 h. The resin was washed with DMF and incubated with 1,8-diamino-3,6-dioxaoctane (1 ml) overnight. The resin was washed with DMF followed by adding biotin (36.5 mg, 150 µmol), DIEA (52 µl, 300 µmol), PyBOP (78 mg, 150 µmol) and the reaction was kept for 3h. Biotin-diol-azide was cleaved from resin by incubation with a solution of TFA: TIS: H_2O (90%: 2.5%: 7.5%) for 3 hours. The peptide was precipitated in cold diethyl ether and purified by HPLC using a linear gradient of 10-40% B yield building block (6.73 mg, 20%). ESI-MS: $[M+H]^+$ calculated for $C_{26}H_{45}N_9O_9S$ 660.3061, found 660.3127.

5.2.8 Synthesis of biotin-diol-alkyne 11

Compound 9: To a solution of methyl 2,3-*O*-isopropylidene-L-tartrate **2** (448 mg, 2.2 mmol) in DMF (10 ml) was added propargylamin-hydrochlorid (183 mg, 2 mmol), HBTU (758 mg, 2 mM) and DIEA (690 µl, 4 mmol), the reaction was stirred overnight and evaporated. The resulting residue was dissolved in DCM and washed with saturated $NaHCO_3$, 1 M $KHSO_4$ and brine. The organic layer was dried on $MgSO_4$ and concentrated under reduced pressure. Silica column chromatograph [10-40% EtOAc in PE] afforded the title compound (347.4 mg, 72%). ESI-MS: $[M+H]^+$ calculated for $C_{11}H_{15}NO_5$ 242.0950, found 242.1047. ^{1}H NMR (400 MHz): 6.7 (bs, 1H), 4.79 (d, 1H, J = 5.0 Hz), 4.76 (d, 1H, J = 5.0 Hz), 4.17-4.04 (m, 2H), 3.83 (s, 3H), 2.27 (t, 1H, J = 2.4 Hz), 1.51 (s, 3H), 1.49 (s, 3H). ^{13}C DEPT NMR (400 MHz): 77.68, 77.30, 52.89, 38.62, 28.88, 26.60, 26.22.

Compound 10: To a solution of compound **9** (183 mg, 0.76 mmol) in Toluene (1 ml) was added 1,8-diamino-3,6-dioxaoctane (555 µl, 3.8 mmol). The reaction was stirred at 80^{o}C overnight. The solvents were evaporated in *vacuo*, and the product was purified to homogeneity by silica gel chromatography (MeOH/DCM/NH_4OH 0/10/0.1 to 1/10/0.1) to yield compound **10** (231mg, 0.646 mmol, 85%). ESI-MS: $[M+H]^+$ calculated for $C_{16}H_{27}N_3O_6$ 358.1900, found 358.2207. ^{1}H NMR (400 MHz): 7.40 (bs, 1H), 7.22 (bs, 1H), 4.61 (d, 1H, J

= 6.8 Hz), 4.58 (d, 1H, *J* = 7.2 Hz), 4.17-4.05 (m, 2H), 3.65- 3.46 (m, 10H), 2.88 (t, 2H, *J* = 5.2 Hz), 2.25 (t, 2H, *J* = 2.4 Hz), 1.54 (bs, 2H), 1.51 (s, 3H), 1.49 (s, 3H). ^{13}C DEPT NMR (400 MHz): 77.46, 77.29, 73.48, 70.46, 70.26, 69.55, 41.75, 39.04, 28.98, 26.22, 26.07.

Synthesis of biotin-diol-alkyne 11: To a solution of compound **10** in DMF (728 µl) was added biotin (39.2 mg, 0.16 mmol), HBTU (60.9 mg, 0.16 mmol) and DIEA (27.6 µl, 0.16 mmol), and the reaction was stirred overnight. The solvents were evaporated *in vacuo*, 90% AcOH in water (3 ml) was added, and the mixture was stirred at 80°C overnight. Solvents were evaporated *in vacuo* and the product was purified by HPLC using a linear gradient of 10 -30% B yield biotin-diol-alkyne (20 mg, 25%). ESI-MS: $[M+H]^+$ calculated for $C_{26}H_{41}N_5O_8S$ 544.2363, found 544..2407; ^{1}H NMR (400 MHz): 8.06 (t, 1H, *J* = 5.6 Hz), 7.83 (t, 1H, *J* = 5.6 Hz), 7.63 (t, 1H, *J* = 6.0 Hz), 6.41 (bs, 2H), 4.30 (dd, 1H, *J* = 7.6 Hz, *J* = 5.2 Hz), 4.24 (dd, 2H, *J* = 8.8 Hz, *J* = 1.6 Hz), 4.13 (dd, 1H, *J* = 7.6 Hz, *J* = 4.4 Hz), 3.96 - 3.56 (m, 2H + DMSO), 3.51 (m, 4H), 3.44(t, 2H, *J* = 6.0 Hz), 3.40 (t, 2H, *J* = 6.0 Hz), 3.1-3.1 (m, 5H), 3.05 (d, 1H, *J* = 2.4 Hz), 2.82 (dd, 1H, *J* = 5.2 Hz, *J* = 12.4 Hz), 2.58 (d, 1H, *J* = 12.4 Hz), 2.07 (t, 2H, *J* = 7.2 Hz), 1.65-1.25 (m, 6H).

5.2.9 Synthesis of azido-E64 14

Azido-proplyamine (12): Chloropropylamine (1.3 g, 10 mmol) was dissolved in H_2O (10 ml). Sodium azide (1.95 g, 30 mmol) was added. The solution was heated at 80°C overnight. The flask was cooled on ice. EtOAc (20 ml) was added. KOH (0.8 g) was added slowly. The solution was extracted with EtOAc (3 x). The organic layer was combined and dried with $MgSO_4$. The organic solvent was evaporated and yielded colorless oil (750 mg). The compound was used in the subsequent reaction without further purification.

Compound 13: Boc-Leu-OH (462 mg, 2 mmol) was added to dry dioxane (2-3 ml) and the solvent was removed under reduced pressure. The process was repeated 2 more times to dry Boc-Leu-OH. THF (10 ml) was added and cooled to -20°C. BCF (287 µL , 2.2 mmol) and NMM (263 µl , 2.4 mmol) was added. The reaction was kept at -20°C under N_2 for 15 min. Then azido-proplyamine (220 mg, 2.2 mmol) and 4-methylmorpholine (242 mg, 2.4 mmol) were added in 2 min. The reaction was kept 3 h at RT. The solvent was evaporated and the residue was dissolved in EtOAc and washed with 1M $KHSO_4$ and saturated $NaHCO_3$ and dried by $MgSO_4$. Evaporation of EtOAc resulted in target compound (583 mg). ESI-MS: $[M+H]^+$ calculated for $C_{17}H_{27}N_5O_3$ 314.2114, found 314.2617.

Azido-E64: Boc-Leu-azide (200 mg, 0.638 mmol) was added to 25% TFA/DCM for 1h. After evaporation of TFA/DCM, toluene were added and evaporated (2 x) to get rid of TFA. The residue was dissolved in DMF (5 ml) and ethyl (2S, 3S)-epoxysuccinate (102 mg, 0.638 mmol), HBTU (266 mg, 0.7 mmol) and DIEA (122 µL, 0.7 mmol) were added. The reaction was kept overnight. The solvent was evaporated. EtOAc was added and washed with 1M $KHSO_4$, saturated $NaHCO_3$ and dried by $MgSO_4$. The solvent was removed under reduced pressure. Silica column chromatograph [40-60% EtOAc in PE] afforded the title compound (80.8 mg, 35%). ESI-MS: $[M+H]^+$ calculated for $C_{15}H_{25}N_5O_5$ 356.1856, found 356.1991.

5.2.10 Synthesis of alkyne-E64 16

Compound 15: Boc-Leu-OH (462 mg, 2 mmol) was added to dry dioxane (2-3 ml) and the solvent was removed under reduced pressure. The process was repeated 2 more times to dry Boc-Leu-OH. THF (10 ml) was added and cooled to -20°C. BCF (287 µL , 2.2 mmol) and NMM (263 µl , 2.4 mmol) were added. The reaction was kept at -20°C under N_2 for 15 min. Then propargylamine (201 mg, 2.2 mmol) and 4-methylmorpholine (242 mg, 2.4 mmol) were added in 2 min. The reaction was kept 3 h at RT. The solvent was evaporated. The residue was dissolved in EtOAc and followed by washing with 1M $KHSO_4$ and saturated $NaHCO_3$.The organic layer was dried by $MgSO_4$. Evaporation of EtOAc resulted in target compound 493 mg without further purification. ESI-MS: $[M+H]^+$ calculated for $C_{14}H_{24}N_2O_3$ 269.1787, found 269.2015.

Alkyne-E64: Compound **15** (200 mg, 0.745 mmol) was added to 25% TFA/DCM for 1 h. After evaporation of TFA/DCM, toluene was added and evaporated (2 x) to get rid of TFA. The residue was dissolved in DMF (5 ml) and ethyl (2S, 3S)-epoxysuccinate (119.28 mg, 0.745 mmol), HBTU (310.79 mg, 0.82 mmol), DIEA (142.7 µL, 0.82 mmol) were added. The reaction was kept overnight. The solvent was evaporated and EtOAc was added. The solution was washed with 1M $KHSO_4$, saturated $NaHCO_3$ and dried by $MgSO_4$. The solvent was removed under reduced pressure. Silica column chromatograph [40-60% EtOAc in PE] afforded the title compound (76.4 mg, 33%). ESI-MS: $[M+H]^+$ calculated for $C_{15}H_{22}N_2O_5$ 311.1529, found 311.1636.

5.2.11 Synthesis of biotin-diol-TMR-N_3 17

The Fmoc of Rink resin (168 mg, 100 µmol) was deprotected by 20% piperdine in DMF. Fmoc-Lys(N_3)-OH (59.1 mg, 150 µmol), DIC (23.6 µl, 150 µmol), HOBt hydrate (23 mg, 150 µmol) were added to resin in DMF. The reaction was agitated overnight. N-terminal Fmoc

group was deprotected and washed. Fmoc-Lys(Boc)-OH (140 mg, 300 µmol), DIC (47 µl, 300 µmol), HOBt hydrate (46 mg, 300 µmol) were added and the reaction was kept for 3 h. Methyl 2,3-O-isopropylidene-L-tartrate (61.2 mg, 300 µmol), DIC (47 µl, 300 µmol), HOBt hydrate (46 mg, 300 µmol) were added and the reaction was kept overnight. The resin was washed with DMF and incubated with 1,8-diamino-3,6-dioxaoctane (2 ml) overnight. The resin was washed with DMF followed by adding biotin (73 mg, 300 µmol), DIC (47 µl, 300 µmol), HOBt hydrate (46 mg, 300 µmol) and the reaction was kept overnight. The resin-bound intermediate was cleaved by incubation with a solution of TFA: TIS: H_2O (90%: 2.5%: 7.5%) for 3 hours. The peptide was precipitated in cold diethyl ether and purified by HPLC using a linear gradient of 10-40% B yielding a white solid (18 mg, 23%). ESI-MS: $[M+H]^+$ calculated for $C_{32}H_{57}N_{11}O_{10}S$ 788.4011, found 788.4051. The building block **22** (3 mg, 3.8 µmol) was reacted overnight with 5(6)-carboxytetramethylrhodamine succinimidyl ester (2.5 mg, 4.7 µmol) and DIEA (1.72 µl, 10 µmol) in DMSO (200 µl). The solution was purified by HPLC using a linear gradient of 20-60% B to give a target compound (2 mg; yield: 44%). ESI-MS: $[M+H]^+$ calculated for $C_{57}H_{77}N_{13}O_{14}S$ 1200.5434, found 1200.5389.

5.2.12 Synthesis of biotin-diazo-TMR-N_3 18

The Fmoc of Rink resin (336 mg, 200 µmol) was deprotected by 20% piperdine in DMF. Fmoc-Lys(N_3)-OH (120 mg, 300 µmol), DIC (94 µl, 600 µmol), HOBt hydrate (92 mg, 600 µmol) were added to resin in DMF. The reaction was agitated overnight. N-terminal Fmoc group was deprotected and washed. Fmoc-8-amino-3,6-dioxaoctanoic acid (154 mg, 400 µmol), DIEA (139 µl, 800 µmol) and HBTU (152 mg, 400 µmol) were added and the reaction was kept overnight. The Fmoc was removed by treating the resin with 20% piperdine in DMF. Fmoc-Lys(Boc)-OH (280 mg, 600 µmol), DIC (94 µl, 600 µmol), HOBt hydrate (92 mg, 600 µmol) were added and the reaction was kept for 3 h. The resin was washed followed by deprotection. The diazobenzene cleavable building block **23** (150 mg, 296 µmol), DIEA (139 µl, 800µmol, HBTU (152 mg, 300 µmol) were added and the reaction was kept overnight. Fmoc of the building block was removed and the resin was washed followed by adding biotin (146 mg, 600 µmol), DIC (94 µl, 600 µmol), HOBt hydrate (92 mg, 600 µmol) and the reaction was kept overnight. The resin-bound building block was cleaved by incubation with a solution of TFA: TIS: H_2O (95%: 2.5%: 2.5%) for 2 hours. The peptide was precipitated in cold diethyl ether and purified by HPLC using a linear gradient of 30-60% B yielding orange

solid (10.5 mg, yield 5.6%). ESI-MS: $[M+H]^+$ calculated for $C_{43}H_{63}N_{13}O_9S$ 938.4592, found 938.4686. The building block **24** (10 mg, 0.011 mmol) was reacted overnight in DMSO (200 µl) with 5(6)-carboxytetramethylrhodamine succinimidyl ester (4.68 mg, 0.009 mmol) and DIEA (7.6 µL, 0.044 mol). The solution was purified by HPLC using a linear gradient of 30-60% B to give a red compound (10 mg; yield 83%). ESI-MS: $[M+H]^+$ calculated for $C_{68}H_{83}N_{15}O_{13}S$ 1350.6015, found 1350.6037.

5.2.13 Synthesis of biotin-S-S-TMR-N_3 19

The Fmoc of Rink resin (50.4 mg, 30 µmol) was deprotected by 20% piperdine in DMF. Fmoc-Lys(N_3)-OH (17.7 mg, 45 µmol), DIC (14 µl, 90 µmol), HOBt hydrate (13.8 mg, 90 µmol) were added to resin in DMF. The Fmoc was removed by treating the resin with 20% piperdine in DMF. Fmoc-Lys(Boc)-OH (42 mg, 90 µmol), DIC (14 µl, 90 µmol), HOBt hydrate (13.8 mg, 90 µmol) were added and the reaction was kept for 3 h. N-terminal Fmoc group was deprotected and washed. Fmoc-8-amino-3,6-dioxaoctanoic acid (17 mg, 44 µmol), DIEA (15.7 µl, 90 µmol) and HBTU (17 mg, 45 µmol) were added and the reaction was kept overnight. The Fmoc was removed by treating the resin with 20% piperdine in DMF and washed with DMF. Sulfo-NHS-SS-Biotin **25** (27.27 mg, 45 µmol) and DIEA (15.7 µl, 90 µmol) were added and the mixture was shaken overnight. The compound was cleaved by incubation with a solution of TFA: TIS: H_2O (95%: 2.5%: 2.5%) for 2 hours and precipitated in cold diethyl ether yielding a white solid (21 mg). The crude building block **26** (3.4 mg) was dissolved in DMSO (200 µl). 5(6)-carboxytetramethylrhodamine succinimidyl ester (1.41 mg, 0.0027 mmol) and DIEA (5 µL, 0.029 mmol) were added and the reaction was shaken overnight. The final product was purified by HPLC using a linear gradient of 30-60% B to give a red compound (0.12 mg; yield 3.7%). ESI-MS: $[M+2H/]^+/2$ calculated for $C_{58}H_{80}N_{13}O_{12}S$ 623.7567, found 623.7559.

5.2.14 Synthesis of biotin-hydrazone-TMR-N_3 20

The synthesis of 6-hydrazinonicotinic acid (**27)** was performed using a reported procedure (123). 6-chloronicotinic acid (1.5 g) was added 10 ml of hydrazine hydrate (80% hydrazine in water). The reaction was kept at 100 °C for 4h. The homogeneous reaction mixture was cooled to room temperature and concentrated to give a white solid. The solid was dissolved in water and acidified to pH 5.5 with HCl. The formed precipitate was isolated by filtration. The solid

was washed with 95% ethanol and ether, and dried under vacuum resulting in 1.24 g of a light yellow compound.

4-Formyl-benzoic acid NHS ester (**28**): 4-Formyl-benzoic acid (450 mg, 3 mmol) was dissolved in a mixture of THF (15 ml) and DCM (20 ml). *N*-hydroxysuccinimide (345 mg, 3 mmol) and EDCI (1.14g, 6 mmol) were added. The reaction was maintained at room temperature for 6 h. The solvent was evaporated and DCM (20 ml) was added. The excess EDCI was removed by washing with 1M $KHSO_4$ and the organic layer was washed with brine, dried with $MgSO_4$ and concentrated *in vacuo*. The residue was directly used in the next step without purification.

Compound 29: Boc-Lys-OH (246 mg, 1 mmol) was dissolved in 10 ml DMF and 5 ml water. 4-Formyl-benzoic acid NHS ester (271 mg, 1.1 mmol) and DIEA 345 µL (2 mmol) were added. The reaction was maintained at room temperature for 5 h. The solvent was evaporated and EtOAc (20 ml) was added. The organic layer was washed with a pH 2.5 citrate solution. The organic layer was dried by $MgSO_4$ and the solvent was evaporated, resulting in 380 mg of crude material. This compound was dissolved in THF (10 ml). *N*-hydroxysuccinimide (127 mg, 1.1 mmol) and EDCI (380 mg, 2 mmol) were added. The reaction stirred for 6 h at room temperature. The solvent was evaporated and DCM was added. The organic layer was washed with 1M $KHSO_4$ and dried by $MgSO_4$. Evaporation of solvent resulted in 500 mg of crude material, which was used in the next step without further purification. It was reacted for 3h with DIEA (369 µL, 2.1 mmol) and 3-azido-propylamine (120 mg, 1.2 mmol) in DCM (20 ml). The reaction mixture was washed with 1M $KHSO_4$, saturated $NaHCO_3$ and brine. The organic layer was dried on $MgSO_4$ and concentrated under reduced pressure. Silica column chromatograph (66-100% EtOAc in PE) afforded the title compound (301 mg, 65%) overall 4 steps. ESI-MS: $[M+H]^+$ calculated for $C_{22}H_{32}N6O_5$ 461.2434, found 461.2497.

Compound 30: compound **29** (138 mg, 300 µmol) was dissolved in DMF (5 ml) and 6-hydrazinonicotinic acid (46 mg, 300 µmol) was added. The reaction was stirred overnight and the solvent was evaporated. The residue was used in solid phase synthesis without purification. ESI-MS: $[M+H]^+$ calculated for $C_{28}H_{37}N_9O_6$ 596.2867, found 596.2884.

Biotin-hydrazone-TMR-N_3 (20): The Fmoc of Rink resin (84 mg, 50 µmol) was deprotected by 20% piperdine in DMF. Fmoc-Lys(biotin)-OH (44 mg, 75 µmol), DIC (23.6 µl, 150 µmol), HOBt hydrate (23 mg, 150 µmol) were dissolved in 1.5 ml of NMP, added to the resin and the reaction was agitated overnight. Fmoc group was deprotected and washed. Fmoc-8-amino-3,6-dioxaoctanoic acid (58 mg, 150 µmol), DIC (23.6 µl, 150 µmol), HOBt (23 mg, 150 µmol) were added and the reaction was kept overnight. The resin was treated with 20% piperdine in

DMF and washed with DMF. Building block **30** (44.6 mg, 75 µmol), HBTU (28.4 mg, 75 µmol) and DIEA (26 µl, 150 µmol) were added in DMF solution which was shaken overnight. The compound was cleaved from the resin by incubation with a solution of TFA: TIS: H_2O (95%: 2.5%: 2.5%) for 2 hours, precipitated in cold diethyl ether and purified by HPLC using a linear gradient of 10-70% B yielding a yellow solid (10 mg, yield 20%). ESI-MS: $[M+H]^+$ calculated for $C_{45}H_{67}N_{15}O_9S$ 994.4967, found 994.4740. The building block **31** (5 mg, 0.005 mmol) was dissolved in DMSO (200 µl). 5(6)-carboxytetramethylrhodamine succinimidyl ester (2.65 mg, 0.005 mmol) and DIEA (1.74 µL, 0.01 mmol) were added and the reaction mixture was shaken overnight. The solution was purified by HPLC using a linear gradient of 10-70% B to give a red compound (6 mg; yield 85%). ESI-MS: $[M+3H]^+/3$ calculated for $C_{70}H_{87}N_{17}O_{13}S$ 469.5463, found 469.5505.

5.2.15 Synthesis of biotin-dde-TMR-N_3 21

Synthesis of biotin-dde (32): Biotin-dde was made according to a literature procedure (124). Dimedone (154 mg, 1.1 mmol) was dissolved in DMF (5 ml). Biotin (244 mg, 1.0 mmol), DIC (156 µl, 1.0 mmol) and DMAP (122 mg, 1.0 mmol) were added. The solution was stirred for 48h. DMF was removed under reduced pressure and EtOAc was added. The organic layer was washed with 1M $KHSO_4$ and extracted with saturated $NaHCO_3$. The solution was acidified with HCl and extracted with DCM. The organic layer was dried by $MgSO_4$ and concentrated under reduced pressure. The residue was recrystallized in MeOH/H_2O (1:1) yielding 200 mg of the target compound (55%). ESI-MS: $[M+H]^+$ calculated for $C_{18}H_{26}N_2O_4S$ 367.1613, found 367.1746.

Biotin-dde-TMR-N_3 (21): The Fmoc of Rink resin (42 mg, 25 µmol) was deprotected by 20% piperdine in DMF. Fmoc-Lys(N_3)-OH (14.7 mg, 37.5 µmol), DIC (12 µl, 75 µmol), HOBt hydrate (11.5 mg, 75 µmol) were dissolved in DMF, added to the resin and the reaction was agitated overnight. Fmoc group was deprotected and washed with DMF. Fmoc-Lys(Mtt)-OH (47 mg, 75 µmol), DIC (12 µl, 75 µmol) and HOBt (11.5 mg, 75 µmol) were added to the resin and the reaction was kept overnight. Fmoc was removed and Fmoc-8-amino-3,6-dioxaoctanoic acid (29 mg, 75 µmol), DIC (23µl, 150 µmol), HOBt (23 mg, 150 µmol) were added and the reaction was kept overnight. The resin was treated with 20% piperdine in DMF and washed with DMF. 5(6)-carboxytetramethylrhodamine succinimidyl ester (15.8 mg, 30 µmol) and DIEA (8.5 µl, 50 µmol) were added and the mixture was shaken overnight. The resin was washed with DMF and the Mtt was removed by 1% TFA in DCM for 10 min. The

process was repeated 7 more times followed by washing with DMF and addition of bioin-dde (27 mg, 75 μmol) and DIEA (25.5 μl, 150 μmol). The reaction was agitated overnight. The compound was cleaved from the resin by incubation with a solution of TFA: TIS: H_2O (95%: 2.5%: 2.5%) for 2 hours. The solvent was evaporated under reduced pressure and the residue was purified by HPLC using a linear gradient of 20-60% B to give a red compound (15.28 mg; yield 51%). ESI-MS: $[M+2H]^{2+}$ calculated for $C_{61}H_{80}N_{12}O_{12}S$ 603.2870, found 603.3004.

5.3 Buffers and solutions

TLC solutions:

Basic KMnO4

$KMnO_4$ (1.5 g)

K_2CO_3 (10 g)

NaOH (0.125 g)

ddH_2O (200 ml)

Cerium-ammonium-molybdate (CAM)

Cerium-ammonium-molybdate $(NH_4)_6Mo_7O_24.4H_2O$ (25 g)

Cerium(IV)sulfate $Ce(SO_4)_2$ (5 g)

Conc H_2SO_4 (50 ml)

ddH_2O (450 ml)

Ninhydrin

Ninhydrin (0.3 g)

Glacial acetic acid (3 ml)

n-butanol (100 ml)

Dinitrophenylhydrazine (particularly good for aldehydes and ketones)

2,2- Dinitrophenylhydrazine (12 g)

Conc H_2SO_4 (60 ml)

ddH_2O (80 ml)

EtOH (200 ml)

Silver staining solutions:

Fix solution: 50% MeOH, 12% AcOH
500 mL MeOH

120 mL AcOH

500 µL 37% formaldehyde

Bring to 1000 mL with ddH_2O

Pretreat solution: 0.02% $Na_2S_2O_3.5\ H_2O$

80 mg $Na_2S_2O_3.5\ H_2O$

400 mL water

Impregnation: 0.2% $AgNO_3$

4 mL 20% silvernitrate stock

400 mL water

300 µL 37% formaldehyde

Develop: 6% Na_2CO_3

18 g Na_2CO_3

300 mL water

150 µL 37% formaldehyde

6 mL of Fix-solution

4 mL of Pretreat-solution

4X sample buffer:

Glycerol (20 ml)

1M Tris pH6.8 (10 ml)

beta-mercaptoethanol (10 ml)

SDS (6 g)

bromophenol blue (20 mg)

Add dd H_2O up to 50ml volume

Aliquot and stored -20^oC

10 X PBS:

NaCl (80 g)

KCl (2 g)

Na_2HPO_4 (14,4g)

$KHPO_4$ (2.4 g)

ddH_2O 800 ml

adjust to pH 7.4

adjust volume to 1L.

Phosphate buffer:

NaH_2PO_4 (100 mM), pH 7.4

5 X Gel running buffer:

75 g Tris base

360 g Glycine

25 g SDS

Bring to 5 L with ddH_2O

Acetate buffer

NaOAc (50 mM)
$MgCl_2$ (5 mM)
DTT (2 mM)
pH 5.5

Rat liver lysate buffer

NaOAc (50 mM)
$MgCl_2$ (5 mM)
Sucrose (250 mM)
DTT (2 mM)
pH 5.5

PBST

0.1% Tween in 1XPBS buffer

Kaiser test solutions

Solution A: 2% KCN/H_2O (16.5 mg KCN in 25 ml H_2O) 98% Pyridine (v/v)
Solution B: 5% Ninhydrin (w/v) in ethanol
Solution C: 4:1 Phenol (w/v) in Ethanol

Buffers for preparation of SDS-PAGE gels:

Tris buffer for Stacking gels: 0.5M Tris pH 6.76 (2% bromophenol blue sodium salt 600 µl for 500 ml).

2% bromophenol blue sodium salt : 20 mg of bromophenol blue sodium salt was added to 1ml of ddH_2O.

Tris buffer for separating gels: 1.5M Tris pH 8.8

30% acrylamide (37.5:1 acryl:bisacryl)

10% APS (5 g of APS was added to 50 ml of dd H_2O, and store in -20^oC)

Abbreviations

A	alanine or adenine
ABP	activity based probe
ABPP	activity-based protein profiling
AOMK	acyloxymethyl ketone
APS	Ammonium persulfate
BCN	bicyclononyne
BME	2-mercaptoethanol
BSA	bovine serum albumin
BTTAA	2-[4-{(bis[(1-*tert*-butyl-1H-1,2,3-triazol-4-yl)methyl]amino)methyl}-1H-1,2,3-triazol-1-yl]acetic acid
C	cytosine
Cat	cathepsin
CuAAC	Copper-catalyzed azide-alkyne cycloaddition
DCM	dichloromethane
DDE	1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl
DIBO	4-Dibenzocyclooctyol
DIC	diisopropylcarbodiimide
DIEA	N,N-Diisopropylethylamine
DMAP	4-Dimethylaminopyridine
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E	glutamic acid
ESI	electrospray ionization
EtOAc	ethyl acetate
F	phenylalanine
FA	formic acids

FBS	fetal bovine serum
Fmoc	9-fluorenylmethoxycarbonyl
G	glycine or guanine
HBTU	O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate
HOBt	hydroxybenzotriazole
HPLC	high performance liquid chromatography
ICAT	isotope-coded affinity tag
L	leucine
LC	liquid chromatography
MFCO	1-fluorocyclooct-2-ynecarboxylic acid
MS	mass spectrometry
N	asparagine
NMM	N-Methylmorpholine
NMP	N-Methyl-2-pyrrolidone
NMR	nuclear magnetic resonance spectroscopy
NTA	nitroloacetic acid
PAGE	polyacrylamide gel electrophoresis
PET	positron emission tomography
PyBOP	benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
Q	glutamine
RAW cell	mouse leukaemic monocyte macrophage cell line
SB	sample buffer
SDS	sodium dodecyl sulfate
SPAAC	strain-promoted alkyne-azide cycloadditions
SPPS	Solid phase peptide synthesis
SU	supernatant
T	thymine
TBTA	tris-(Benzyltriazolylmethyl)amine
TCEP	tris-(2-Carboxyethyl)phosphine
TEMED	tetramethylethylenediamine
TEV	nuclear inclusion protease
TFA	trifluoroacetic acid

THPTA	tris(3-hydroxypropyltriazolylmethyl)amine
TIS	triisopropylsilane
TMR	tetramethylrhodamine
TLC	thin layer chromatography
Tris	tris(hydroxymethyl)aminomethane
UV	Ultraviolet
Y	tyrosine

Bibliography

1. Saghatelian, A., and Cravatt, B. F. (2005) Assignment of protein function in the postgenomic era. *Nat Chem Biol* 1, 130-142
2. Lenz, T., Fischer, J. J., and Dreger, M. (2011) Probing small molecule-protein interactions: A new perspective for functional proteomics. *J Proteomics* 75, 100-115
3. Heal, W. P., Dang, T. H., and Tate, E. W. (2011) Activity-based probes: discovering new biology and new drug targets. *Chem Soc Rev* 40, 246-257
4. Serim, S., Haedke, U., and Verhelst, S. H. (2012) Activity-based probes for the study of proteases: recent advances and developments. *ChemMedChem* 7, 1146-1159
5. Sadaghiani, A. M., Verhelst, S. H., and Bogyo, M. (2007) Tagging and detection strategies for activity-based proteomics. *Curr Opin Chem Biol* 11, 20-28
6. Wolf, E. V., Zeissler, A., Vosyka, O., Zeiler, E., Sieber, S., and Verhelst, S. H. (2013) A new class of rhomboid protease inhibitors discovered by activity-based fluorescence polarization. *PLoS One* 8, e72307
7. Lone, A. M., Bachovchin, D. A., Westwood, D. B., Speers, A. E., Spicer, T. P., Fernandez-Vega, V., Chase, P., Hodder, P. S., Rosen, H., Cravatt, B. F., and Saghatelian, A. (2011) A substrate-free activity-based protein profiling screen for the discovery of selective PREPL inhibitors. *J Am Chem Soc* 133, 11665-11674
8. Nomura, D. K., Dix, M. M., and Cravatt, B. F. (2010) Activity-based protein profiling for biochemical pathway discovery in cancer. *Nat Rev Cancer* 10, 630-638
9. Deu, E., Leyva, M. J., Albrow, V. E., Rice, M. J., Ellman, J. A., and Bogyo, M. (2010) Functional studies of Plasmodium falciparum dipeptidyl aminopeptidase I using small molecule inhibitors and active site probes. *Chem Biol* 17, 808-819
10. Child, M. A., Hall, C. I., Beck, J. R., Ofori, L. O., Albrow, V. E., Garland, M., Bowyer, P. W., Bradley, P. J., Powers, J. C., Boothroyd, J. C., Weerapana, E., and Bogyo, M. (2013) Small-molecule inhibition of a depalmitoylase enhances Toxoplasma host-cell invasion. *Nat Chem Biol* 9, 651-656
11. Ziegler, S., Pries, V., Hedberg, C., and Waldmann, H. (2013) Target identification for small bioactive molecules: finding the needle in the haystack. *Angew Chem Int Ed Engl* 52, 2744-2792
12. Haedke, U., Kuttler, E. V., Vosyka, O., Yang, Y., and Verhelst, S. H. (2013) Tuning probe selectivity for chemical proteomics applications. *Current opinion in chemical biology* 17, 102-109
13. Evans, M. J., and Cravatt, B. F. (2006) Mechanism-based profiling of enzyme families. *Chem Rev* 106, 3279-3301
14. Liu, Y., Patricelli, M. P., and Cravatt, B. F. (1999) Activity-based protein profiling: the serine hydrolases. *Proc Natl Acad Sci U S A* 96, 14694-14699
15. Greenbaum, D., Medzihradszky, K. F., Burlingame, A., and Bogyo, M. (2000) Epoxide electrophiles as activity-dependent cysteine protease profiling and discovery tools. *Chem Biol* 7, 569-581

16. Lo, L. C., Pang, T. L., Kuo, C. H., Chiang, Y. L., Wang, H. Y., and Lin, J. J. (2002) Design and synthesis of class-selective activity probes for protein tyrosine phosphatases. *J Proteome Res* 1, 35-40
17. Tsai, C. S., Li, Y. K., and Lo, L. C. (2002) Design and synthesis of activity probes for glycosidases. *Org Lett* 4, 3607-3610
18. Serim, S., Mayer, S. V., and Verhelst, S. H. (2013) Tuning activity-based probe selectivity for serine proteases by on-resin 'click' construction of peptide diphenyl phosphonates. *Org Biomol Chem* 11, 5714-5721
19. Sabido, E., Tarrago, T., Niessen, S., Cravatt, B. F., and Giralt, E. (2009) Activity-based probes for monitoring postproline protease activity. *Chembiochem* 10, 2361-2366
20. Mahrus, S., and Craik, C. S. (2005) Selective chemical functional probes of granzymes A and B reveal granzyme B is a major effector of natural killer cell-mediated lysis of target cells. *Chem Biol* 12, 567-577
21. Howden, A. J., Geoghegan, V., Katsch, K., Efstathiou, G., Bhushan, B., Boutureira, O., Thomas, B., Trudgian, D. C., Kessler, B. M., Dieterich, D. C., Davis, B. G., and Acuto, O. (2013) QuaNCAT: quantitating proteome dynamics in primary cells. *Nat Methods* 10, 343-346
22. Hang, H. C., Geutjes, E. J., Grotenbreg, G., Pollington, A. M., Bijlmakers, M. J., and Ploegh, H. L. (2007) Chemical probes for the rapid detection of Fatty-acylated proteins in Mammalian cells. *J Am Chem Soc* 129, 2744-2745
23. Jao, C. Y., Roth, M., Welti, R., and Salic, A. (2009) Metabolic labeling and direct imaging of choline phospholipids in vivo. *Proc Natl Acad Sci U S A* 106, 15332-15337
24. Haberkant, P., Raijmakers, R., Wildwater, M., Sachsenheimer, T., Brugger, B., Maeda, K., Houweling, M., Gavin, A. C., Schultz, C., van Meer, G., Heck, A. J., and Holthuis, J. C. (2013) In vivo profiling and visualization of cellular protein-lipid interactions using bifunctional fatty acids. *Angew Chem Int Ed Engl* 52, 4033-4038
25. Heal, W. P., Jovanovic, B., Bessin, S., Wright, M. H., Magee, A. I., and Tate, E. W. (2011) Bioorthogonal chemical tagging of protein cholesterylation in living cells. *Chem Commun (Camb)* 47, 4081-4083
26. Resh, M. D. (2006) Trafficking and signaling by fatty-acylated and prenylated proteins. *Nat Chem Biol* 2, 584-590
27. Hahne, H., Sobotzki, N., Nyberg, T., Helm, D., Borodkin, V. S., van Aalten, D. M., Agnew, B., and Kuster, B. (2013) Proteome wide purification and identification of O-GlcNAc-modified proteins using click chemistry and mass spectrometry. *J Proteome Res* 12, 927-936
28. Saxon, E., and Bertozzi, C. R. (2000) Cell surface engineering by a modified Staudinger reaction. *Science* 287, 2007-2010
29. Kuhn, P. H., Koroniak, K., Hogl, S., Colombo, A., Zeitschel, U., Willem, M., Volbracht, C., Schepers, U., Imhof, A., Hoffmeister, A., Haass, C., Rossner, S., Brase, S., and Lichtenthaler, S. F. (2012) Secretome protein enrichment identifies physiological BACE1 protease substrates in neurons. *EMBO J* 31, 3157-3168
30. Grammel, M., and Hang, H. C. (2013) Chemical reporters for biological discovery. *Nat Chem Biol* 9, 475-484
31. Heal, W. P., and Tate, E. W. (2010) Getting a chemical handle on protein post-translational modification. *Org Biomol Chem* 8, 731-738

32. Chan, E. W., Chattopadhaya, S., Panicker, R. C., Huang, X., and Yao, S. Q. (2004) Developing photoactive affinity probes for proteomic profiling: hydroxamate-based probes for metalloproteases. *J Am Chem Soc* 126, 14435-14446
33. Crump, C. J., am Ende, C. W., Ballard, T. E., Pozdnyakov, N., Pettersson, M., Chau, D. M., Bales, K. R., Li, Y. M., and Johnson, D. S. (2012) Development of clickable active site-directed photoaffinity probes for gamma-secretase. *Bioorg Med Chem Lett* 22, 2997-3000
34. Sato, S., Kwon, Y., Kamisuki, S., Srivastava, N., Mao, Q., Kawazoe, Y., and Uesugi, M. (2007) Polyproline-rod approach to isolating protein targets of bioactive small molecules: isolation of a new target of indomethacin. *J Am Chem Soc* 129, 873-880
35. Tamura, S., Inomata, S., Ebine, M., Genji, T., Iwakura, I., Mukai, M., Shoji, M., Sugai, T., and Ueda, M. (2013) Triazoyl-phenyl linker system enhancing the aqueous solubility of a molecular probe and its efficiency in affinity labeling of a target protein for jasmonate glucoside. *Bioorg Med Chem Lett* 23, 188-193
36. Edgington, L. E., Berger, A. B., Blum, G., Albrow, V. E., Paulick, M. G., Lineberry, N., and Bogyo, M. (2009) Noninvasive optical imaging of apoptosis by caspase-targeted activity-based probes. *Nat Med* 15, 967-973
37. Edgington, L. E., Verdoes, M., Ortega, A., Withana, N. P., Lee, J., Syed, S., Bachmann, M. H., Blum, G., and Bogyo, M. (2013) Functional imaging of legumain in cancer using a new quenched activity-based probe. *J Am Chem Soc* 135, 174-182
38. Cheng, T. C., Roffler, S. R., Tzou, S. C., Chuang, K. H., Su, Y. C., Chuang, C. H., Kao, C. H., Chen, C. S., Harn, I. H., Liu, K. Y., Cheng, T. L., and Leu, Y. L. (2012) An activity-based near-infrared glucuronide trapping probe for imaging beta-glucuronidase expression in deep tissues. *J Am Chem Soc* 134, 3103-3110
39. Park, J., Oh, S., and Park, S. B. (2012) Discovery and target identification of an antiproliferative agent in live cells using fluorescence difference in two-dimensional gel electrophoresis. *Angew Chem Int Ed Engl* 51, 5447-5451
40. Okerberg, E. S., Wu, J., Zhang, B., Samii, B., Blackford, K., Winn, D. T., Shreder, K. R., Burbaum, J. J., and Patricelli, M. P. (2005) High-resolution functional proteomics by active-site peptide profiling. *Proc Natl Acad Sci U S A* 102, 4996-5001
41. Camerini, S., Polci, M. L., Restuccia, U., Usuelli, V., Malgaroli, A., and Bachi, A. (2007) A novel approach to identify proteins modified by nitric oxide: the HIS-TAG switch method. *J Proteome Res* 6, 3224-3231
42. Hemelaar, J., Borodovsky, A., Kessler, B. M., Reverter, D., Cook, J., Kolli, N., Gan-Erdene, T., Wilkinson, K. D., Gill, G., Lima, C. D., Ploegh, H. L., and Ovaa, H. (2004) Specific and covalent targeting of conjugating and deconjugating enzymes of ubiquitin-like proteins. *Mol Cell Biol* 24, 84-95
43. Ren, G., Blum, G., Verdoes, M., Liu, H., Syed, S., Edgington, L. E., Gheysens, O., Miao, Z., Jiang, H., Gambhir, S. S., Bogyo, M., and Cheng, Z. (2011) Non-invasive imaging of cysteine cathepsin activity in solid tumors using a 64Cu-labeled activity-based probe. *PLoS One* 6, e28029
44. Gygi, S. P., Rist, B., Gerber, S. A., Turecek, F., Gelb, M. H., and Aebersold, R. (1999) Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat Biotechnol* 17, 994-999

45. Weerapana, E., Wang, C., Simon, G. M., Richter, F., Khare, S., Dillon, M. B., Bachovchin, D. A., Mowen, K., Baker, D., and Cravatt, B. F. (2010) Quantitative reactivity profiling predicts functional cysteines in proteomes. *Nature* 468, 790-795
46. Qian, Y., Martell, J., Pace, N. J., Ballard, T. E., Johnson, D. S., and Weerapana, E. (2013) An isotopically tagged azobenzene-based cleavable linker for quantitative proteomics. *Chembiochem* 14, 1410-1414
47. Speers, A. E., and Cravatt, B. F. (2004) Profiling enzyme activities in vivo using click chemistry methods. *Chem Biol* 11, 535-546
48. Yee, M. C., Fas, S. C., Stohlmeyer, M. M., Wandless, T. J., and Cimprich, K. A. (2005) A cell-permeable, activity-based probe for protein and lipid kinases. *J Biol Chem* 280, 29053-29059
49. Florea, B. I., Verdoes, M., Li, N., van der Linden, W. A., Geurink, P. P., van den Elst, H., Hofmann, T., de Ru, A., van Veelen, P. A., Tanaka, K., Sasaki, K., Murata, S., den Dulk, H., Brouwer, J., Ossendorp, F. A., Kisselev, A. F., and Overkleeft, H. S. (2010) Activity-based profiling reveals reactivity of the murine thymoproteasome-specific subunit beta5t. *Chem Biol* 17, 795-801
50. Lennon-Dumenil, A. M., Bakker, A. H., Maehr, R., Fiebiger, E., Overkleeft, H. S., Rosemblatt, M., Ploegh, H. L., and Lagaudriere-Gesbert, C. (2002) Analysis of protease activity in live antigen-presenting cells shows regulation of the phagosomal proteolytic contents during dendritic cell activation. *J Exp Med* 196, 529-540
51. Speers, A. E., Adam, G. C., and Cravatt, B. F. (2003) Activity-based protein profiling in vivo using a copper(i)-catalyzed azide-alkyne [3 + 2] cycloaddition. *J Am Chem Soc* 125, 4686-4687
52. Willems, L. I., van der Linden, W. A., Li, N., Li, K. Y., Liu, N., Hoogendoorn, S., van der Marel, G. A., Florea, B. I., and Overkleeft, H. S. (2011) Bioorthogonal chemistry: applications in activity-based protein profiling. *Acc Chem Res* 44, 718-729
53. Zhang, H., Li, X. J., Martin, D. B., and Aebersold, R. (2003) Identification and quantification of N-linked glycoproteins using hydrazide chemistry, stable isotope labeling and mass spectrometry. *Nat Biotechnol* 21, 660-666
54. Hahne, H., Neubert, P., Kuhn, K., Etienne, C., Bomgarden, R., Rogers, J. C., and Kuster, B. (2012) Carbonyl-reactive tandem mass tags for the proteome-wide quantification of N-linked glycans. *Anal Chem* 84, 3716-3724
55. Codreanu, S. G., Zhang, B., Sobecki, S. M., Billheimer, D. D., and Liebler, D. C. (2009) Global analysis of protein damage by the lipid electrophile 4-hydroxy-2-nonenal. *Mol Cell Proteomics* 8, 670-680
56. Zeng, Y., Ramya, T. N., Dirksen, A., Dawson, P. E., and Paulson, J. C. (2009) High-efficiency labeling of sialylated glycoproteins on living cells. *Nat Methods* 6, 207-209
57. Dirksen, A., Dirksen, S., Hackeng, T. M., and Dawson, P. E. (2006) Nucleophilic catalysis of hydrazone formation and transimination: implications for dynamic covalent chemistry. *J Am Chem Soc* 128, 15602-15603
58. Rashidian, M., Mahmoodi, M. M., Shah, R., Dozier, J. K., Wagner, C. R., and Distefano, M. D. (2013) A highly efficient catalyst for oxime ligation and hydrazone-oxime exchange suitable for bioconjugation. *Bioconjug Chem* 24, 333-342

59. Blanden, A. R., Mukherjee, K., Dilek, O., Loew, M., and Bane, S. L. (2011) 4-aminophenylalanine as a biocompatible nucleophilic catalyst for hydrazone ligations at low temperature and neutral pH. *Bioconjug Chem* 22, 1954-1961
60. Rostovtsev, V. V., Green, L. G., Fokin, V. V., and Sharpless, K. B. (2002) A stepwise huisgen cycloaddition process: copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes. *Angew Chem Int Ed Engl* 41, 2596-2599
61. Wang, Q., Chan, T. R., Hilgraf, R., Fokin, V. V., Sharpless, K. B., and Finn, M. G. (2003) Bioconjugation by copper(I)-catalyzed azide-alkyne [3 + 2] cycloaddition. *J Am Chem Soc* 125, 3192-3193
62. Chan, T. R., Hilgraf, R., Sharpless, K. B., and Fokin, V. V. (2004) Polytriazoles as copper(I)-stabilizing ligands in catalysis. *Org Lett* 6, 2853-2855
63. Besanceney-Webler, C., Jiang, H., Zheng, T., Feng, L., Soriano del Amo, D., Wang, W., Klivansky, L. M., Marlow, F. L., Liu, Y., and Wu, P. (2011) Increasing the efficacy of bioorthogonal click reactions for bioconjugation: a comparative study. *Angew Chem Int Ed Engl* 50, 8051-8056
64. Hang, H. C., Loureiro, J., Spooner, E., van der Velden, A. W., Kim, Y. M., Pollington, A. M., Maehr, R., Starnbach, M. N., and Ploegh, H. L. (2006) Mechanism-based probe for the analysis of cathepsin cysteine proteases in living cells. *ACS Chem Biol* 1, 713-723
65. Ovaa, H., van Swieten, P. F., Kessler, B. M., Leeuwenburgh, M. A., Fiebiger, E., van den Nieuwendijk, A. M., Galardy, P. J., van der Marel, G. A., Ploegh, H. L., and Overkleeft, H. S. (2003) Chemistry in living cells: detection of active proteasomes by a two-step labeling strategy. *Angew Chem Int Ed Engl* 42, 3626-3629
66. Ning, X., Guo, J., Wolfert, M. A., and Boons, G. J. (2008) Visualizing metabolically labeled glycoconjugates of living cells by copper-free and fast huisgen cycloadditions. *Angew Chem Int Ed Engl* 47, 2253-2255
67. Dommerholt, J., Schmidt, S., Temming, R., Hendriks, L. J., Rutjes, F. P., van Hest, J. C., Lefeber, D. J., Friedl, P., and van Delft, F. L. (2010) Readily accessible bicyclononynes for bioorthogonal labeling and three-dimensional imaging of living cells. *Angew Chem Int Ed Engl* 49, 9422-9425
68. Schultz, M. K., Parameswarappa, S. G., and Pigge, F. C. (2010) Synthesis of a DOTA--biotin conjugate for radionuclide chelation via Cu-free click chemistry. *Org Lett* 12, 2398-2401
69. van der Linden, W. A., Li, N., Hoogendoorn, S., Ruben, M., Verdoes, M., Guo, J., Boons, G. J., van der Marel, G. A., Florea, B. I., and Overkleeft, H. S. (2012) Two-step bioorthogonal activity-based proteasome profiling using copper-free click reagents: a comparative study. *Bioorg Med Chem* 20, 662-666
70. Willems, L. I., Verdoes, M., Florea, B. I., van der Marel, G. A., and Overkleeft, H. S. (2010) Two-step labeling of endogenous enzymatic activities by Diels-Alder ligation. *Chembiochem* 11, 1769-1781
71. Leslie, B. J., and Hergenrother, P. J. (2008) Identification of the cellular targets of bioactive small organic molecules using affinity reagents. *Chem Soc Rev* 37, 1347-1360
72. Kanoh, N., Honda, K., Simizu, S., Muroi, M., and Osada, H. (2005) Photo-cross-linked small-molecule affinity matrix for facilitating forward and reverse chemical genetics. *Angew Chem Int Ed Engl* 44, 3559-3562

73. Bantscheff, M., Lemeer, S., Savitski, M. M., and Kuster, B. (2012) Quantitative mass spectrometry in proteomics: critical review update from 2007 to the present. *Anal Bioanal Chem* 404, 939-965
74. Punna, S., Kaltgrad, E., and Finn, M. G. (2005) "Clickable" agarose for affinity chromatography. *Bioconjug Chem* 16, 1536-1541
75. Slade, P. G., Hajivandi, M., Bartel, C. M., and Gorfien, S. F. (2012) Identifying the CHO secretome using mucin-type O-linked glycosylation and click-chemistry. *J Proteome Res* 11, 6175-6186
76. Landi, F., Johansson, C. M., Campopiano, D. J., and Hulme, A. N. (2010) Synthesis and application of a new cleavable linker for "click"-based affinity chromatography. *Org Biomol Chem* 8, 56-59
77. Temming, R. P., van Scherpenzeel, M., te Brinke, E., Schoffelen, S., Gloerich, J., Lefeber, D. J., and van Delft, F. L. (2012) Protein enrichment by capture-release based on strain-promoted cycloaddition of azide with bicyclononyne (BCN). *Bioorg Med Chem* 20, 655-661
78. Fonovic, M., Verhelst, S. H., Sorum, M. T., and Bogyo, M. (2007) Proteomics evaluation of chemically cleavable activity-based probes. *Mol Cell Proteomics* 6, 1761-1770
79. Garret-Flaudy, F., and Freitag, R. (2000) Use of the avidin (imino)biotin system as a general approach to affinity precipitation. *Biotechnol Bioeng* 71, 223-234
80. Flaster, H., and Kohn, H. (1981) Syntheses and spectral properties of 2-Thiobiotin and biotin derivatives. *Journal of Heterocyclic Chemistry* 18, 1425-1436
81. Hirsch, J. D., Eslamizar, L., Filanoski, B. J., Malekzadeh, N., Haugland, R. P., and Beechem, J. M. (2002) Easily reversible desthiobiotin binding to streptavidin, avidin, and other biotin-binding proteins: uses for protein labeling, detection, and isolation. *Anal Biochem* 308, 343-357
82. Ying, L. Q., and Branchaud, B. P. (2011) Design of a reversible biotin analog and applications in protein labeling, detection, and isolation. *Chem Commun (Camb)* 47, 8593-8595
83. Speers, A. E., and Cravatt, B. F. (2005) A tandem orthogonal proteolysis strategy for high-content chemical proteomics. *J Am Chem Soc* 127, 10018-10019
84. Verhelst, S. H., Fonovic, M., and Bogyo, M. (2007) A mild chemically cleavable linker system for functional proteomic applications. *Angew Chem Int Ed Engl* 46, 1284-1286
85. Szychowski, J., Mahdavi, A., Hodas, J. J., Bagert, J. D., Ngo, J. T., Landgraf, P., Dieterich, D. C., Schuman, E. M., and Tirrell, D. A. (2010) Cleavable biotin probes for labeling of biomolecules via azide-alkyne cycloaddition. *J Am Chem Soc* 132, 18351-18360
86. Gartner, C. A., Elias, J. E., Bakalarski, C. E., and Gygi, S. P. (2007) Catch-and-release reagents for broadscale quantitative proteomics analyses. *J Proteome Res* 6, 1482-1491
87. Yang, Y. Y., Grammel, M., Raghavan, A. S., Charron, G., and Hang, H. C. (2010) Comparative analysis of cleavable azobenzene-based affinity tags for bioorthogonal chemical proteomics. *Chem Biol* 17, 1212-1222
88. Budin, G., Moune-Dimala, M., Leriche, G., Saliou, J. M., Papillon, J., Sanglier-Cianferani, S., Van Dorsselaer, A., Lamour, V., Brino, L., and Wagner, A. (2010) Nondenaturing chemical proteomics for protein complex isolation and identification. *Chembiochem* 11, 2359-2361

89. Jahng, W. J., David, C., Nesnas, N., Nakanishi, K., and Rando, R. R. (2003) A cleavable affinity biotinylating agent reveals a retinoid binding role for RPE65. *Biochemistry* 42, 6159-6168
90. Lin, D., Li, J., Slebos, R. J., and Liebler, D. C. (2010) Cysteinyl peptide capture for shotgun proteomics: global assessment of chemoselective fractionation. *J Proteome Res* 9, 5461-5472
91. Geurink, P. P., Florea, B. I., Li, N., Witte, M. D., Verasdonck, J., Kuo, C. L., van der Marel, G. A., and Overkleeft, H. S. (2010) A cleavable linker based on the levulinoyl ester for activity-based protein profiling. *Angew Chem Int Ed Engl* 49, 6802-6805
92. Fauq, A. H., Kache, R., Khan, M. A., and Vega, I. E. (2006) Synthesis of acid-cleavable light isotope-coded affinity tags (ICAT-L) for potential use in proteomic expression profiling analysis. *Bioconjug Chem* 17, 248-254
93. Truong, T. H., Garcia, F. J., Seo, Y. H., and Carroll, K. S. (2011) Isotope-coded chemical reporter and acid-cleavable affinity reagents for monitoring protein sulfenic acids. *Bioorg Med Chem Lett* 21, 5015-5020
94. van der Veken, P., Dirksen, E. H., Ruijter, E., Elgersma, R. C., Heck, A. J., Rijkers, D. T., Slijper, M., and Liskamp, R. M. (2005) Development of a novel chemical probe for the selective enrichment of phosphorylated serine- and threonine-containing peptides. *Chembiochem* 6, 2271-2280
95. Nilsson, J., Ruetschi, U., Halim, A., Hesse, C., Carlsohn, E., Brinkmalm, G., and Larson, G. (2009) Enrichment of glycopeptides for glycan structure and attachment site identification. *Nat Methods* 6, 809-811
96. Park, K. D., Liu, R., and Kohn, H. (2009) Useful tools for biomolecule isolation, detection, and identification: acylhydrazone-based cleavable linkers. *Chem Biol* 16, 763-772
97. Dirksen, A., Yegneswaran, S., and Dawson, P. E. (2010) Bisaryl hydrazones as exchangeable biocompatible linkers. *Angew Chem Int Ed Engl* 49, 2023-2027
98. Sturm, M., Leitner, A., and Lindner, W. (2011) Development of an indole-based chemically cleavable linker concept for immobilizing bait compounds for protein pull-down experiments. *Bioconjug Chem* 22, 211-217
99. Yokoshima, S., Abe, Y., Watanabe, N., Kita, Y., Kan, T., Iwatsubo, T., Tomita, T., and Fukuyama, T. (2009) Development of photoaffinity probes for gamma-secretase equipped with a nitrobenzenesulfonamide-type cleavable linker. *Bioorg Med Chem Lett* 19, 6869-6871
100. Park, J. J., Sadakane, Y., Masuda, K., Tomohiro, T., Nakano, T., and Hatanaka, Y. (2005) Synthesis of diazirinyl photoprobe carrying a novel cleavable biotin. *Chembiochem* 6, 814-818
101. Olejnik, J., Sonar, S., Krzymanska-Olejnik, E., and Rothschild, K. J. (1995) Photocleavable biotin derivatives: a versatile approach for the isolation of biomolecules. *Proc Natl Acad Sci U S A* 92, 7590-7594
102. Kim, H. Y., Tallman, K. A., Liebler, D. C., and Porter, N. A. (2009) An azido-biotin reagent for use in the isolation of protein adducts of lipid-derived electrophiles by streptavidin catch and photorelease. *Mol Cell Proteomics* 8, 2080-2089
103. Orth, R., and Sieber, S. A. (2009) A photolabile linker for the mild and selective cleavage of enriched biomolecules from solid support. *J Org Chem* 74, 8476-8479
104. Dieterich, D. C., Lee, J. J., Link, A. J., Graumann, J., Tirrell, D. A., and Schuman, E. M. (2007) Labeling, detection and identification of newly synthesized proteomes with bioorthogonal non-canonical amino-acid tagging. *Nat Protoc* 2, 532-540

105. Weerapana, E., Speers, A. E., and Cravatt, B. F. (2007) Tandem orthogonal proteolysis-activity-based protein profiling (TOP-ABPP)--a general method for mapping sites of probe modification in proteomes. *Nat Protoc* 2, 1414-1425
106. Brigham, J. L., Perera, B. G., and Maly, D. J. (2013) A hexylchloride-based catch-and-release system for chemical proteomic applications. *ACS Chem Biol* 8, 691-699
107. Hashimoto, M., Okamoto, S., Nabeta, K., and Hatanaka, Y. (2004) Enzyme cleavable and biotinylated photoaffinity ligand with diazirine. *Bioorg Med Chem Lett* 14, 2447-2450
108. Santala, V., and Saviranta, P. (2004) Affinity-independent elution of antibody-displaying phages using cleavable DNA linker containing streptavidin beads. *J Immunol Methods* 284, 159-163
109. Zheng, T., Jiang, H., and Wu, P. (2013) Single-stranded DNA as a cleavable linker for bioorthogonal click chemistry-based proteomics. *Bioconjug Chem* 24, 859-864
110. Liu, B., Burdine, L., and Kodadek, T. (2006) Chemistry of periodate-mediated cross-linking of 3,4-dihydroxylphenylalanine-containing molecules to proteins. *J Am Chem Soc* 128, 15228-15235
111. Smith, R. J., Capaldi, R. A., Muchmore, D., and Dahlquist, F. (1978) Cross-linking of ubiquinone cytochrome c reductase (complex III) with periodate-cleavable bifunctional reagents. *Biochemistry* 17, 3719-3723
112. Paulick, M. G., Hart, K. M., Brinner, K. M., Tjandra, M., Charych, D. H., and Zuckermann, R. N. (2006) Cleavable hydrophilic linker for one-bead-one-compound sequencing of oligomer libraries by tandem mass spectrometry. *J Comb Chem* 8, 417-426
113. Shiyama, T., Furuya, M., Yamazaki, A., Terada, T., and Tanaka, A. (2004) Design and synthesis of novel hydrophilic spacers for the reduction of nonspecific binding proteins on affinity resins. *Bioorg Med Chem* 12, 2831-2841
114. Chehade, K. A. H., Baruch, A., Verhelst, S. H. L., and Bogyo, M. (2005) An Improved Preparation of the Activity-Based Probe JPM-OEt and In Situ Applications. *Synthesis* 2005, 240-244
115. Fukuyama, H., Ndiaye, S., Hoffmann, J., Rossier, J., Liuu, S., Vinh, J., and Verdier, Y. (2012) On-bead tryptic proteolysis: an attractive procedure for LC-MS/MS analysis of the Drosophila caspase 8 protein complex during immune response against bacteria. *J Proteomics* 75, 4610-4619
116. Wilson, J. P., Raghavan, A. S., Yang, Y. Y., Charron, G., and Hang, H. C. (2011) Proteomic analysis of fatty-acylated proteins in mammalian cells with chemical reporters reveals S-acylation of histone H3 variants. *Mol Cell Proteomics* 10, M110 001198
117. Greenbaum, D., Baruch, A., Hayrapetian, L., Darula, Z., Burlingame, A., Medzihradszky, K. F., and Bogyo, M. (2002) Chemical approaches for functionally probing the proteome. *Mol Cell Proteomics* 1, 60-68
118. Kaschani, F., Verhelst, S. H., van Swieten, P. F., Verdoes, M., Wong, C. S., Wang, Z., Kaiser, M., Overkleeft, H. S., Bogyo, M., and van der Hoorn, R. A. (2009) Minitags for small molecules: detecting targets of reactive small molecules in living plant tissues using 'click chemistry'. *Plant J* 57, 373-385
119. Wilcox, D., and Mason, R. W. (1992) Inhibition of cysteine proteinases in lysosomes and whole cells. *Biochem J* 285 (Pt 2), 495-502
120. Katunuma, N. (2010) Posttranslational processing and modification of cathepsins and cystatins. *J Signal Transduct* 2010, 375345

121. Leriche, G., Chisholm, L., and Wagner, A. (2012) Cleavable linkers in chemical biology. *Bioorg Med Chem* 20, 571-582
122. Nash, I. A., Bycroft, B. W., and Chan, W. C. (1996) Dde — A selective primary amine protecting group: A facile solid phase synthetic approach to polyamine conjugates. *Tetrahedron Letters* 37, 2625-2628
123. Abrams, M. J., Juweid, M., tenKate, C. I., Schwartz, D. A., Hauser, M. M., Gaul, F. E., Fuccello, A. J., Rubin, R. H., Strauss, H. W., and Fischman, A. J. (1990) Technetium-99m-human polyclonal IgG radiolabeled via the hydrazino nicotinamide derivative for imaging focal sites of infection in rats. *J Nucl Med* 31, 2022-2028
124. Kellam, B., Chan, W. C., Chhabra, S. R., and Bycroft, B. W. (1997) Transient affinity tags based on the Dde protection/deprotection strategy: Synthesis and application of 2-biotinyl-and 2-hexanoyldimedone. *Tetrahedron Letters* 38, 5391-5394
125. Augustyns, K., Kraas, W., and Jung, G. (1998) Investigation on the stability of the Dde protecting group used in peptide synthesis: migration to an unprotected lysine. *J Pept Res* 51, 127-133
126. Claessen, J. H., Witte, M. D., Yoder, N. C., Zhu, A. Y., Spooner, E., and Ploegh, H. L. (2013) Catch-and-release probes applied to semi-intact cells reveal ubiquitin-specific protease expression in Chlamydia trachomatis infection. *Chembiochem* 14, 343-352
127. Gingras, A. C., Gstaiger, M., Raught, B., and Aebersold, R. (2007) Analysis of protein complexes using mass spectrometry. *Nat Rev Mol Cell Biol* 8, 645-654
128. Lam, Y. W., Yuan, Y., Isaac, J., Babu, C. V., Meller, J., and Ho, S. M. (2010) Comprehensive identification and modified-site mapping of S-nitrosylated targets in prostate epithelial cells. *PLoS One* 5, e9075
129. Yang, P. Y., Wang, M., He, C. Y., and Yao, S. Q. (2012) Proteomic profiling and potential cellular target identification of K11777, a clinical cysteine protease inhibitor, in Trypanosoma brucei. *Chem Commun (Camb)* 48, 835-837
130. Maurer, A., Zeyher, C., Amin, B., and Kalbacher, H. (2013) A periodate-cleavable linker for functional proteomics under slightly acidic conditions: application for the analysis of intracellular aspartic proteases. *J Proteome Res* 12, 199-207
131. Simon, G. M., Niphakis, M. J., and Cravatt, B. F. (2013) Determining target engagement in living systems. *Nat Chem Biol* 9, 200-205
132. Battenberg, O. A., Yang, Y., Verhelst, S. H., and Sieber, S. A. (2013) Target profiling of 4-hydroxyderricin in S. aureus reveals seryl-tRNA synthetase binding and inhibition by covalent modification. *Molecular bioSystems* 9, 343-351
133. Roe, M. R., Xie, H., Bandhakavi, S., and Griffin, T. J. (2007) Proteomic mapping of 4-hydroxynonenal protein modification sites by solid-phase hydrazide chemistry and mass spectrometry. *Anal Chem* 79, 3747-3756
134. Kunzmann, M. H., Staub, I., Bottcher, T., and Sieber, S. A. (2011) Protein reactivity of natural product-derived gamma-butyrolactones. *Biochemistry* 50, 910-916
135. Staub, I., and Sieber, S. A. (2009) Beta-lactam probes as selective chemical-proteomic tools for the identification and functional characterization of resistance associated enzymes in MRSA. *J Am Chem Soc* 131, 6271-6276
136. Olsen, J. V., Macek, B., Lange, O., Makarov, A., Horning, S., and Mann, M. (2007) Higher-energy C-trap dissociation for peptide modification analysis. *Nat Methods* 4, 709-712

137. Rappsilber, J., Mann, M., and Ishihama, Y. (2007) Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. *Nat Protoc* 2, 1896-1906

Acknowledgement

I would like to thank my supervisor Dr. Steven Verhelst for accepting me as a PhD student in his lab. Thanks for invaluable supervision, constant generation of scientific ideas, patience and encouragement.

Thank Prof. Dr. Dieter Langosch for providing outstanding scientific environment and well-equipped laboratory.

Thank Prof. Dr. Bernhard Küster and Dr. Hannes Hahne in Chair of proteomics and bioanalytics for running the MS samples, analyzing the data and suggestions.

Thank Oliver, Ute, Sevner, Eliane in Verhelst lab for their help in my project and life. Thank Markus, Walter, Martina, Elke, Rashmi, Christian, Martin and other members for their advices and help.

Thank my family for their tremendous support and endless encouragement.

Publications

1 **Yang, Y**., Hahne, H., Kuster, B., and Verhelst, S. H. (2013) A simple and effective cleavable linker for chemical proteomics applications. *Mol Cell Proteomics* 12, 237-244

2 **Yang, Y**., and Verhelst, S. H. (2013) Cleavable trifunctional biotin reagents for protein labelling, capture and release. *Chem Commun (Camb)* 49, 5366-5368

3 **Yang, Y**., Yang, X., and Verhelst, S. H. (2013) Comparative analysis of click chemistry mediated activity-based protein profiling in cell lysates. *Molecules* 18, 12599-12608

4 Battenberg, O. A., **Yang, Y**., Verhelst, S. H., and Sieber, S. A. (2013) Target profiling of 4-hydroxyderricin in S. aureus reveals seryl-tRNA synthetase binding and inhibition by covalent modification. *Mol Biosyst* 9, 343-351

5 Haedke, U., Kuttler, E. V., Vosyka, O., **Yang, Y**., and Verhelst, S. H. (2013) Tuning probe selectivity for chemical proteomics applications. *Curr Opin Chem Biol* 17, 102-109

Curriculum Vitae

Personal data

Name Yinliang Yang

Contact information Verhelst lab, Lehrstuhl für Chemie der Biopolymere
Technische Universität München
Weihenstephanerberg 3
85354 Freising
Germany
Email: y.yang@tum.de

Education

01/2010-Now PhD student in Technische Universität München, Germany

09/2006- 06/2009 MSc in School of Life Science, Lanzhou University, China

09/2002-06/2006 BSc in School of Life Science, Lanzhou University, China