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Cleavable trifunctional biotin reagents for protein labelling, capture and release†

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Trifunctional biotin reagents incorporating cleavable linkers are evaluated for their usage in protein enrichment. A linker based on the Dde protecting group leads to efficient release of protein targets under mild conditions. It additionally contains a masked trypsin cleavage site, which eliminates the majority of the tag during tryptic digestion.

Site-specific modification of proteins by small molecule chemical probes is one of the main techniques in chemical proteomics.[1] Examples include lipid and carbohydrate derivatives for metabolic labelling of post-translationally modified proteins[2] and activity-based probes (ABPs) for the tagging of active enzymes.[3] Ideally, the labelling of protein targets is carried out in models that resemble physiological conditions as well as possible, such as living cells or animals. Bioorthogonal ligation methods have provided opportunities to study proteins *in situ* and *in vivo*. To this end, probes containing alkyne or azide moieties are functionalized after protein modification by Cu(I)-catalyzed azide–alkyne cycloaddition, Staudinger ligation or Cu-free cycloaddition.[4] Trifunctional tags, incorporating a ligation handle, a biotin and a fluorophore, have become popular, since they can be applied for both visualization and affinity purification.[5–7] However, due to the strong interaction between biotin and streptavidin harsh conditions are necessary for the elution of enriched proteins. This usually leads to contamination of the sample with non-specifically bound proteins and endogenously biotinlytated proteins, which complicates target identification. Cleavable linkers[8] have been employed to solve this problem.

In this study, we design trifunctional reagents with cleavable linkers in order to facilitate mild release of target proteins during enrichment from proteomes (Fig. 1). We compare five linkers that cleave under different conditions, evaluate their compatibility with bioorthogonal ligation, and determine the efficiency of the release. We also report on a new linker based on the 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde) protecting group,[9] which has – to our knowledge – not yet been used in chemical proteomics applications. We show here that this linker leads to efficient protein elution under very mild conditions. Additionally, in the trifunctional reagent with the Dde linker, a masked trypsin cleavable bond eliminates the fluorescent tag upon tryptic digestion, reducing the molecular weight of the final modification when compared to other trifunctional tags.

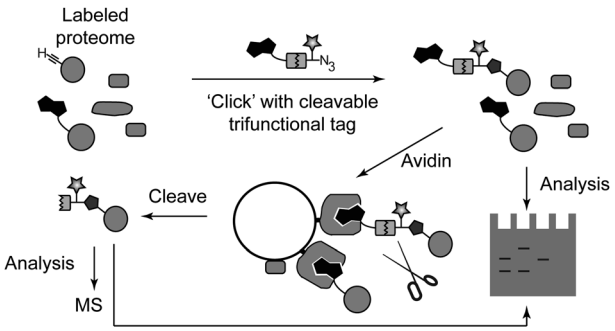

Fig. 1 Cleavable trifunctional tags in chemical proteomics serve the purpose of sensitive detection, enrichment of targets and selective elution.

The cleavable trifunctional tags were designed as follows (Fig. 2). All tags incorporate an azide group to enable click chemistry-mediated derivatization of alkyne-labelled proteins. A PEG spacer is introduced to increase the solubility. A carboxytetramethylrhodamine (Rh) fluorophore, which remains attached to the protein after cleavage of the linker, serves the purpose of sensitive detection. Finally, the biotin enables efficient protein enrichment. As cleavable linkers (Fig. 2) we use:

1 A vicinal diol synthesized from tartrate,[10,11] which can be cleaved under oxidative conditions.

2 A diazobenzene derivative,[12–14] which is cleavable under reductive conditions (sodium dithionite).

3 A bisaryl hydrazone. This biocompatible linker can be cleaved by hydroxylamine.[15–17] It has not yet been used in combination with bioorthogonal ligations.

4 A disulfide linker, which is commercially available and can be cleaved under reductive conditions.[18]

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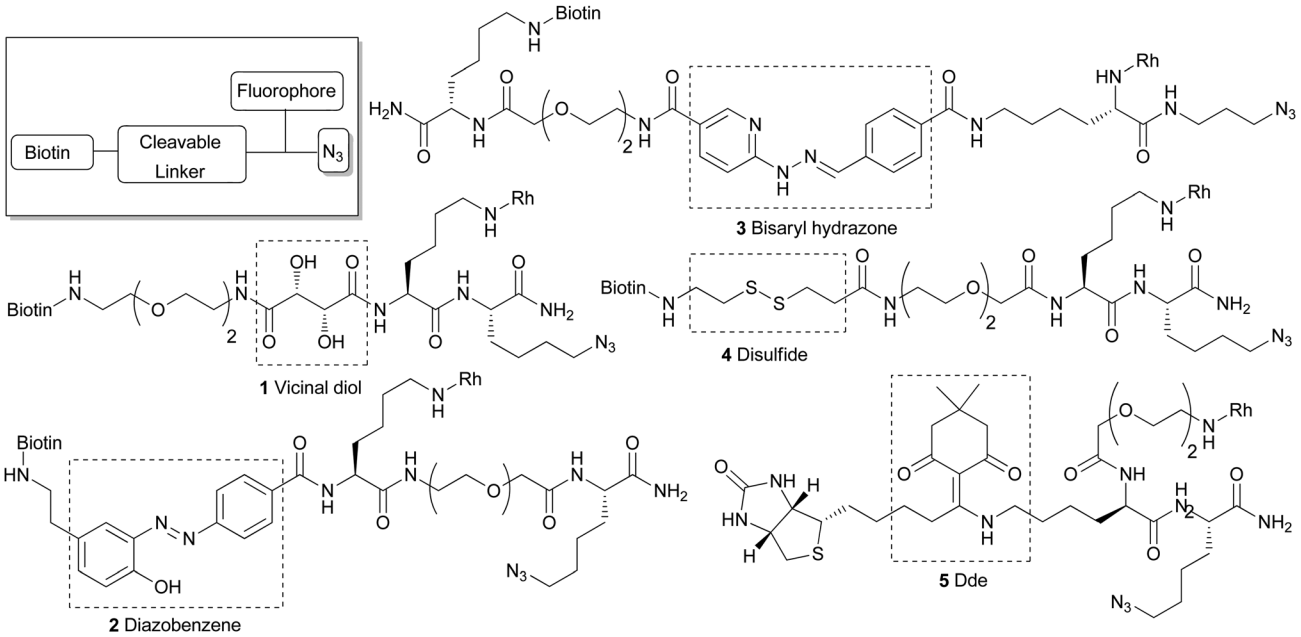

Fig. 2 The structures of cleavable trifunctional tags **1–5**. All contain an azide, a Rh fluorophore, a cleavable linker (boxed) and a biotin.

5 Besides the four above-mentioned cleavable linkers, we designed a linker that is novel in chemical proteomics. It is based on the Dde group, which is used as a protecting group for the ε-amino group of lysine in solid phase peptide synthesis (SPPS). The conventional way of deprotecting the Dde group consists of using 2% hydrazine.[9]

All reagents were synthesized by a combination of SPPS and solution phase synthesis (Schemes S1–S5, ESI†). Having the cleavable tags **1–5** in hand (Fig. 2), we first tested whether they can be used for the detection of alkyne modified proteins in a complex proteome. To this end, we treated a lysate of the macrophage cell line RAW264.1 with alkyne-E-64 (Fig. 4), an activity-based probe for cathepsin proteases.[19] We then attached the cleavable tags to the alkyne-E-64 labelled proteins by Cu(I)-catalyzed click chemistry, and the lysate was resolved on a gel. As determined before by mass spectrometry, cathepsin Z and B are the two main targets of E-64 in the lysate.[10,19] All tags display similar labelling compared with a fluorophore-azide derivative, illustrating the compatibility of the cleavable tags with the bioorthogonal labelling strategy (Fig. 3a). They also showed efficient depletion of the labelled cathepsins from a proteome by using immobilized streptavidin (Fig 3b, middle lanes and Table S1, ESI†), while boiling released the targets again (right lanes). The pull down efficiency was slightly lower for the disulfide and the Dde linker, possibly due to premature cleavage or steric hindrance around the biotin.

At this point, we investigated the release of the tagged proteins. For each individual linker, the specific elution was optimized. In all experiments, we determined the efficiency of the elution using fluorescent gel band densitometry by comparison of the proteins selectively eluted and residually bound to the immobilized streptavidin (Table S1, ESI†). We also tested whether elution conditions were specific (*i.e.* if they resulted in cleavage of the biotin from the tagged proteins; ESI,† Fig. S1–S5).

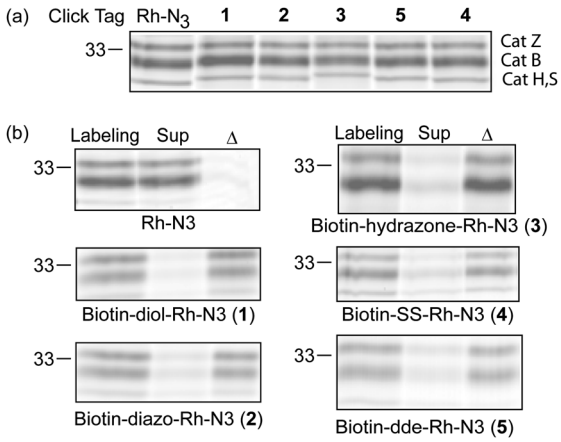

Fig. 3 (a) Labelling of alkyne-E-64 labelled cathepsins in RAW 264.7 cell lysate with a tetramethylrhodamine fluorophore and with trifunctional tags. (b) Pull down of labelled cathepsins by streptavidin beads. Left lanes: labelling after click chemistry with carboxy-tetramethylrhodamine fluorophore or trifunctional tags. Middle lanes: supernatant (sup) after capture with streptavidin beads. Right lane: release of immobilized proteins by boiling with SDS sample buffer (Δ). A carboxytetramethylrhodamine coupled 3-aminopropylazide (Rh–N_3) was used as a control to show that no non-specific binding of labelled cathepsins to streptavidin beads occurs.

For cleavage of the diol linker, we used 1 and 10 mM $NaIO_4$ (3 × 20 min; directly followed by quenching of the periodate with sample buffer). Under both conditions, the target proteins were efficiently released (Fig. 4 and Fig. S1, ESI†). The 1 mM $NaIO_4$ treatment gave rise to 74% cleavage efficiency. For the diazobenzene linker, which was treated with 200 mM $Na_2S_2O_4$, the cleavage efficiency was 58%. The bisaryl hydrazone linker could not be cleaved by 100 mM hydroxylamine (Fig. S3, ESI†), but the addition of aniline, a reported catalyst in the cleavage of these linkers, led to a release of 47% of the proteins (Fig. 4).

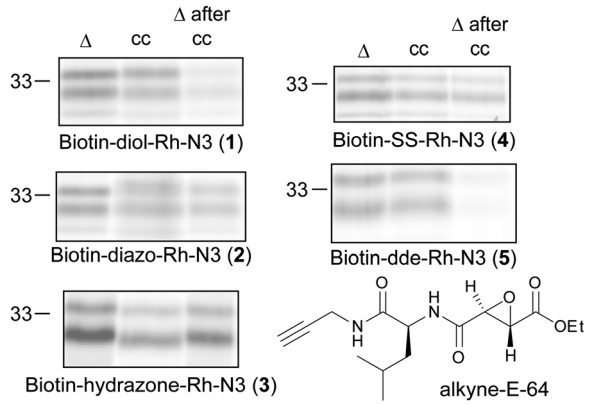

Fig. 4 Evaluation of the elution of alkyne-E-64 targets from streptavidin beads with the cleavable trifunctional tags. Left lane: direct release by boiling with SDS sample buffer (Δ). Middle lane: chemical cleavage (cc). (1) 1 mM $NaIO_4$, 100 mM phosphate, pH 7.4, 3 × 20 min. (2) 200 mM $Na_2S_2O_4$, 100 mM phosphate, pH 7.4, 3 × 20 min. (3) 100 mM NH_2OH and 100 mM aniline 100 mM phosphate, pH 4.6, 4 h. (4) 0.5 M DTT in H_2O, 1 h. (5) 0.05% SDS, 200 mM Tris, pH 8.5, 2 h. Right lane: (Δ after cc), boiling the streptavidin beads with SDS sample buffer after chemical release to elute unreleased proteins.

Unfortunately, a further increase in the concentration of hydroxylamine did not lead to full release (Fig. S3, ESI†). This is in accordance with recent experiments by Claessen *et al.*, who reported poor elution of targets enriched from a whole proteome using a similar bisaryl hydrazone linker.[17] The disulfide linker is a widely used and commercially available linker, which can be cleaved by reducing agents. However, one problem of a disulfide linker is the potential disulfide exchange. This has been reported before in the labelling of biomolecules by clickable disulfide cleavable linkers.[20] We indeed observed a high degree of disulfide exchange when analysing samples in non-reducing gels (Fig. S4, ESI†). The cleavage efficiency was only moderately effective with 60% after treatment with 0.5 M DTT (Fig. 4).

Besides the four above-mentioned cleavable linkers, which have previously been applied in the capture and release of proteins, we designed a linker based on the Dde protecting group (**5**, Fig. 2). The conventional way of deprotecting the Dde group in peptide synthesis consists of using 2% hydrazine. This turned out to be not compatible with fluorescent scanning (Fig. S5a, ESI†). However, the protein targets were released and visualized almost quantitatively (92%) with a solution of 0.05% SDS and 200 mM Tris at pH 8.5 (Fig. 4). Both reagents are necessary for efficient cleavage. Other mild conditions did not lead to the release of target proteins (Fig. S5b, ESI†), whereas other linkers are unaffected by the Tris-SDS treatment (Fig S5c, ESI†), indicating that the release is dependent on the presence of the Dde linker. LC-MS analysis showed that linker **5** treated with a Tris-SDS solution releases the biotin–Dde moiety by hydrolysis, which is likely the mechanism by which the proteins are released (Fig. S6, ESI†). Interestingly, the cleavage of the biotin–Dde group unmasks a 'hidden' trypsin cleavable bond. As a result, the majority of the trifunctional tag is eliminated during a tryptic digestion (Table S1 and Fig. S7, ESI†): both the biotin and the fluorophore are released and only the azidolysine residue of the trifunctional tag will be left behind on a target protein. Modifications that have a large size and undergo fragmentation in tandem MS experiments are undesired for the identification of probe-labelled tryptic peptides. Hence, this secret trypsin site may aid in future identification of the modification sites of probes and post-translational modifications.

In conclusion, we have synthesized and analyzed five different cleavable trifunctional biotin tags for tandem labelling of proteins and subsequent detection or enrichment. Overall, the trifunctional tags containing a diol or Dde linker were the most efficient in the chemical release experiments. The Dde group represents to our knowledge a novel cleavable linker in protein enrichment strategies. This linker can be easily synthesized and cleaved under mild conditions. Moreover, after tryptic digestion, only a small residual modification results. We expect this linker to find application in proteomics experiments aimed at the identification of modification sites. Further studies along these lines will be reported in due course.

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