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Development of a traceable linker containing a thiol-responsive amino acid for the enrichment and selective labelling of target proteins†

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A traceable linker that is potentially applicable to identification of a target protein of bioactive compounds was developed. It enabled not only thiol-induced cleavage of the linker for enrichment of the target protein but also selective labelling to pick out the target from contaminated non-target proteins for facile identification.

A wide variety of biologically active organic molecules, including natural products, peptides, and synthetic small molecules, exert their biological activity through specific interactions with a target biological macromolecule. Among the macromolecules present in living systems, proteins represent one of the most important target classes, with enzymes, receptors, and ion channels being of particular interest. In the fields of chemical biology and drug discovery, the identification of novel protein targets with which organic molecules such as biologically active ligands can interact is critical to understanding complex biological signalling pathways and developing novel therapeutic agents, although studies of this type can be time-consuming and laborious. The identification of a protein target involves a sequence of specific processes, including (1) effectively '*fishing*' for a target protein using a biologically active ligand as a bait; (2) enrichment of the hooked target; and (3) sequence analysis of the target by the Edman degradation or mass spectrometry.[1] For the first step, photo-affinity labelling[1a,b,2] and activity-based probe technology,[1c–e] which allow the covalent attachment of a ligand to a target of interest by photo-irradiation or chemical reaction respectively, have shown good potential in terms of their application to low affinity binding pairs. Using these approaches, the hooked target can be connected to a biotinylated linker molecule that allows it to be purified over streptavidin beads using the highly specific biotin–streptavidin interaction.[1,3] The immobilized target can then be released from the streptavidin beads for subsequent sequence analysis by attenuating the biotin–streptavidin interaction. The high affinity of this interaction ($K_d = 10^{-15}$ M),[4] however, sometimes hampers the liberation of the target from the beads, and several alternatives have been developed for the liberation of the target, including the use of a cleavable linker between the bait and biotin.[5] The use of such a linker allows for the efficient elution of the target protein from the beads *via* linker cleavage, but this approach can be limited by the contamination of the target protein with non-target proteins, which can prevent the identification of the target.[6] The development of a method that allows for the cleavage of the linker moiety to be conducted under mild conditions with generation of an orthogonal functional group that is not seen in proteins is therefore strongly desired. The use of an orthogonal functional group in this context should allow for the introduction of an isotopic or fluorescent tag that would facilitate identification of the tagged target by mass spectrometry (isotopic tag) or sodium dodecyl sulfate (SDS)-PAGE (fluorescent tag).

We recently developed a trimethyl lock[7]-based stimulus-responsive amino acid that we applied to a nuclear-cytoplasmic shuttle peptide and a hypoxia-responsive fluorophore.[8] The molecular basis of this amino acid-induced amide cleavage is shown in Fig. 1. Peptide **1**, bearing an *O*-protected stimulus-responsive amino acid, was converted to phenol **2** by exposure to an appropriate stimulus (*e.g.*, **PG** = *o*-nitrobenzyl; stimulus = UV irradiation). Subsequent lactonization of the phenol led to the cleavage of peptide **2** to give fragments **3** and **4**.

It was envisioned that the incorporation of this amide scission system into a cleavable linker would allow for the enrichment and identification of target proteins, although two additional requirements would need to be considered, including (1) the cleavage of the linker should be mediated by a reagent that does not react irreversibly with the target mole-

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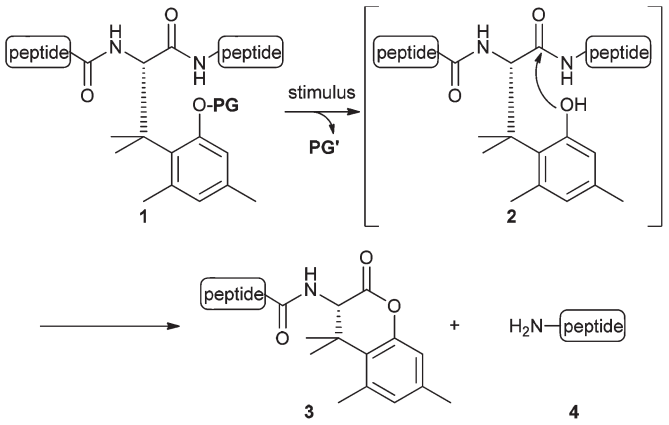


Fig. 1 Stimulus-responsive amide bond cleavage induced by a stimulus-responsive amino acid (**PG**: a protective group removable by an appropriate stimulus).

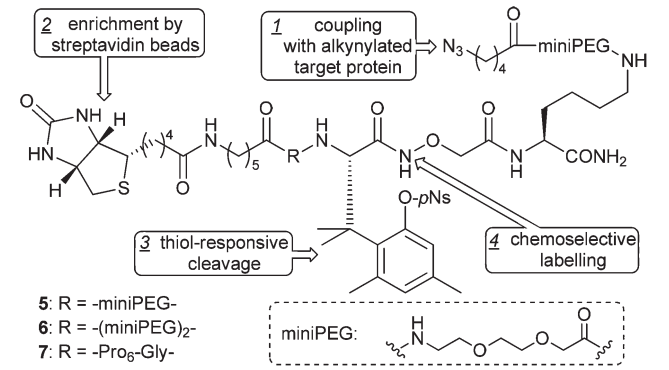
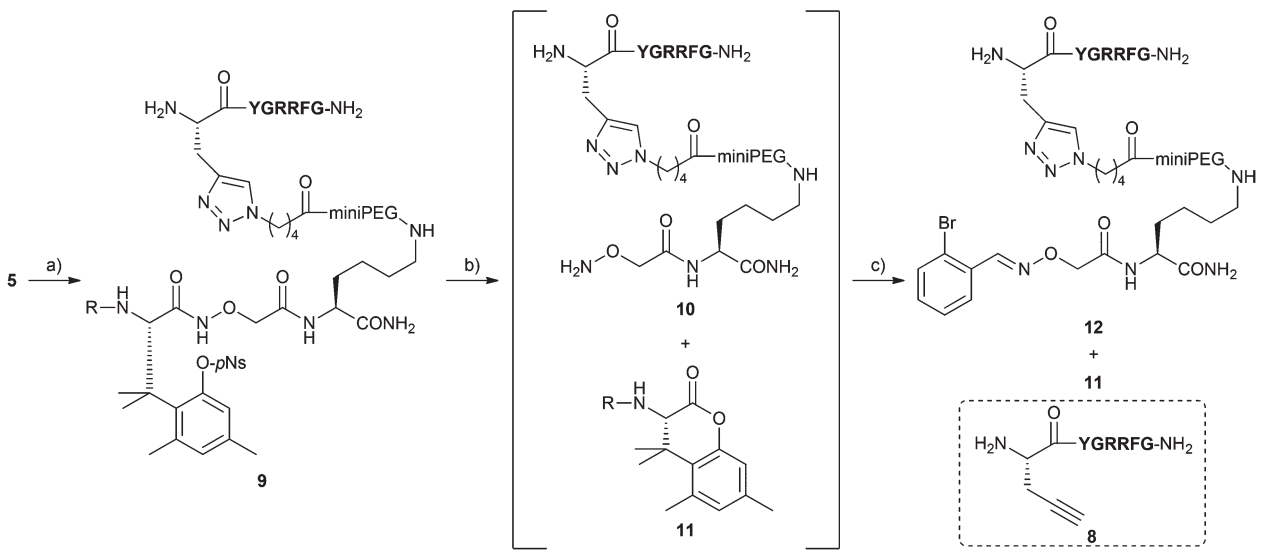


Fig. 2 Structure of traceable linkers (*p*Ns: *p*-nitrobenzenesulfonyl).

cule; and (2) it should be possible for the cleavage products to react chemoselectively during the post-cleavage labelling process to allow for the target protein to be discriminated from the contaminated non-target proteins. With these requirements in mind, we designed a new linker molecule, as shown in Fig. 2. A thiol compatible with biomolecules was used to trigger the cleavage of the linker.[9] An aminooxy moiety was used for the chemoselective labelling of the eluted target, because this functionality would react selectively with the aldehyde group on the labelling reagent.[10] An azide group was placed at one end of the linker and biotin was placed at the other to allow for the introduction of the linker into the target protein–alkyne conjugate and the enrichment of the hooked target with streptavidin beads, respectively. A general scheme depicting different functional aspects of the linker is shown in Fig. 2. The linker is initially introduced into the target protein bearing an alkynylated bait using click chemistry.[11] Following enrichment of the hooked target on streptavidin beads, the target would be eluted through a thiol-induced linker cleavage. This cleavage process would generate an aminooxy group on the target that could be chemoselectively labelled with an aldehyde derivative. Given that these linker molecules would allow for the target proteins to be traced, we named them traceable linkers. The traceable linkers would be more stable and easier to be cleaved on demand than equilibrium-based hydrazone type linkers[12] which were also designed for purification and labelling of the targets. In this study, we prepared the flexible miniPEG derivatives **5** and **6**, as well as a rigid proline-rod derivative **7**.[13]

We initially investigated the use of an enantioselective synthesis for the construction of the traceable linkers composed of a thiol-responsive amino acid. We previously reported the preparation of a racemic thiol-responsive amino acid bearing a thiol-removable *p*-nitrobenzenesulfonyl (*p*Ns) group as the



Scheme 1 Click chemistry of traceable linker **5** with model peptide **8** followed by thiol-responsive cleavage and selective labelling. Reagents and conditions: (a) **8**, $CuSO_4$, Na ascorbate, TBTA, PBS, DMSO, *tert*-BuOH; (b) 2-mercaptoethanol, NP40, Na phosphate buffer (pH 7.8), DMSO, 37 °C; (c) *o*-bromobenzaldehyde, aniline. (R: biotin–$NH(CH_2)_5CO$-miniPEG-; **F**: phenylalanine; **G**: glycine; **R**: arginine; **Y**: tyrosine).

phenolic protective group.[14] The enantioselective synthesis was therefore achieved starting from the chiral intermediate **S1** (Scheme S1 in ESI†).[15] The thiol-responsive amino acid was then incorporated into the traceable linkers using Fmoc-based solid phase peptide synthesis (Fmoc SPPS) using a 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl (ivDde) group as an ε-amino protective group.

A model reaction was conducted prior to the enrichment and selective labelling of the alkynylated protein using alkynylated peptide **8** instead of the protein (Scheme 1). Click chemistry was performed between the traceable linker **5** and the model peptide **8** in the presence of tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA)[16] in phosphate buffered saline (PBS) with organic co-solvents. After 1 h of the reaction, the traceable linker–peptide conjugate **9** was generated in high purity (Fig. S1 in ESI†). We then proceeded to investigate the thiol-induced cleavage and labelling of the model peptide conjugate in a one-pot manner. The conjugate **9** was treated with 2-mercaptoethanol at 37 °C in a mixture of sodium phosphate buffer–DMSO with NP40.[17] The cleavage of the linker reached completion within 24 h to yield the model peptide **10** bearing an aminooxy group and the biotin derivative **11**. *o*-Bromobenzaldehyde was then added to the reaction mixture as a bromine-based labelling reagent[6b,18] followed by aniline, which was used to accelerate the formation of the oxime.[19] Following a reaction time of 1 h, the selective labelling of the model peptide was complete to yield the labelled peptide **12** and intact **11**.

Because the traceable linker **5** has been shown to be responsive to the thiol-induced cleavage and selective labelling processes, we proceeded to evaluate the applicability of this traceable linker strategy to the enrichment and selective labelling of an alkynylated protein. A general scheme is shown in Fig. 3A. In this study, an alkynylated enolase was used as the target protein covalently modified with the alkyne bearing substrate. The thiol-intact linker **15** was synthesized as a negative control for the thiol-induced release of the protein from the streptavidin beads (Fig. 3B). The click chemistry of linkers **5–7** and **15** was initially examined. A mixture of the alkynylated enolase and an intact one prepared according to the literature[12b] was treated with the linker in the presence of $CuSO_4$, sodium ascorbate and TBTA. SDS was also added for solubilisation of the enolase. Following a reaction time of 1 h and subsequent purification by SDS-PAGE, the biotinylated proteins and all proteins were visualized by western blot analysis using a streptavidin-horseradish peroxidase conjugate (SAv-HRP) and Lumitein staining, respectively. As shown in Fig. 4A, the linkers were successfully introduced into the enolase in all cases. It was also observed that the efficiency of the click chemistry depended on the structure of the R moiety and the amino acid on the aminooxy group. Although reason for the difference of the reactivity is not clear at present, the result suggests that optimization of not only the reaction conditions but also the structure of the linker is essential for efficient introduction of the linker onto a target protein. After the click chemistry, the proteins were treated with streptavidin beads

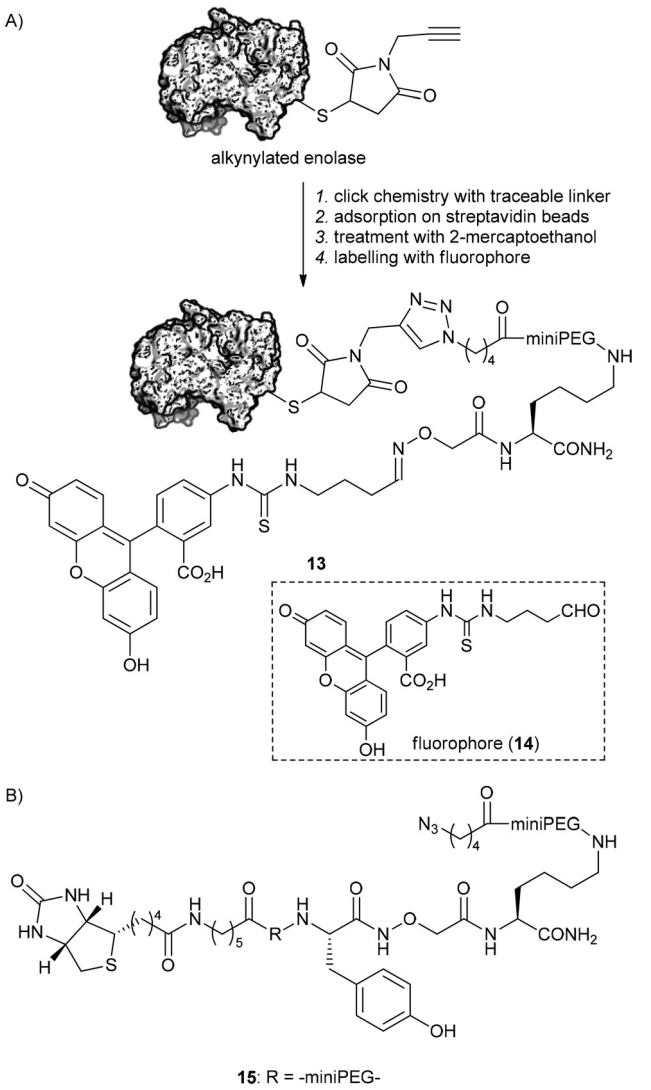


Fig. 3 (A) Schematic representation of enrichment and labelling of alkynylated enolase by traceable linker. (B) Structure of thiol-intact negative control linker **15**.

for 24 h. Following a period of washing, the beads were reacted with 2-mercaptoethanol in sodium phosphate buffer (pH 7.8) containing NP40 at 37 °C for 24 h (Fig. 3A and 4B). The resulting product was centrifuged and the supernatant was treated with the fluorophore **14**[20] in the presence of aniline for 24 h. When the traceable linker **5**, **6**, or **7** was employed, the thiol-induced elution and labelling of the enolase proceeded successfully, whereas small amounts of proteins remained on the beads after the thiol treatment (elution efficiency calculated based on Lumitein staining in Fig. 4B: 68% for **5**, 66% for **6**, and 63% for **7**).[21] In the case of the thiol-inert negative control **15**, although the enolase was successfully adsorbed onto the bead, it was not released by the thiol treatment. These observations suggested that the traceable linkers enabled the thiol-responsive release of the target protein in a similar manner to conventional cleavable linkers to allow for subsequent labelling of the aminooxy group of the

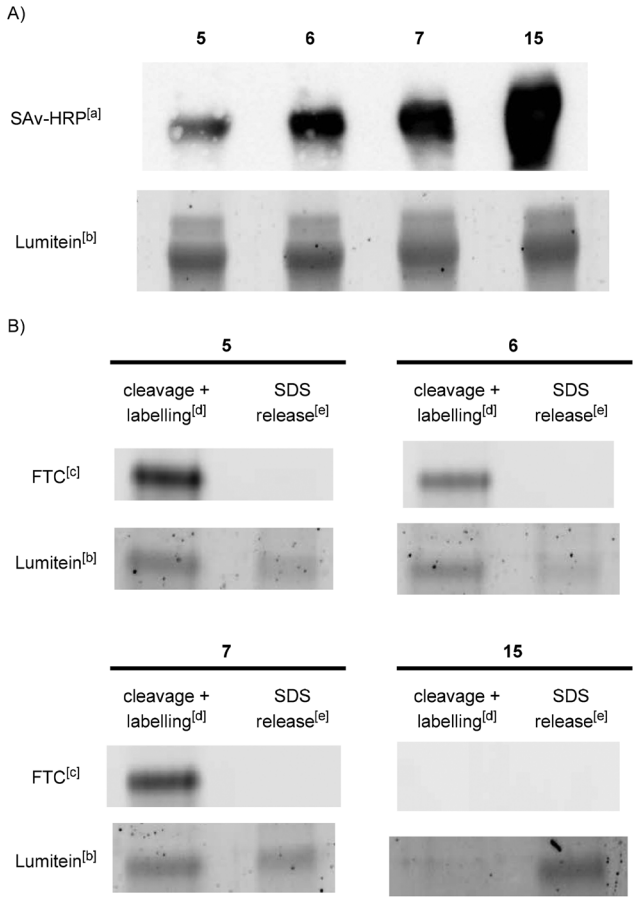


Fig. 4 Monitoring of the reactions shown in Fig. 3A using SDS-PAGE. (A) After click chemistry. Linker **5–7** or **15** (0.10 mM) was introduced into the alkynylated enolase (0.50 g L^{-1}) using a mixture of $CuSO_4$ (1.0 mM), Na ascorbate (0.50 mM), TBTA (0.10 mM), SDS (1% (w/v)), PBS and co-solvents over 1 h. (B) Adsorption on streptavidin beads followed by thiol-induced elution and labelling. Proteins were treated with streptavidin beads for 24 h following the click chemistry. After washing, the beads were reacted with 2-mercaptoethanol (100 mM) and NP40 (1% (v/v)) in Na phosphate buffer (10 mM, pH 7.8) at 37 °C for 24 h. The product was centrifuged and the supernatant was treated with fluorophore **14** (0.10 mM) and aniline (100 mM) for labelling. The reaction mixture was stirred for 24 h. [a] Biotinylated proteins were detected by western blotting analysis using a SAv-HRP. [b] All proteins were visualized by Lumitein staining. [c] Fluorescein-labelled proteins were detected at λ_{ex} = 488 nm and λ_{em} = 530 nm without staining. [d] Proteins after thiol treatment followed by the labelling reaction. [e] Proteins remaining on streptavidin beads after the thiol treatment. The beads after centrifugation followed by removal of the supernatant was suspended in SDS-PAGE sample loading buffer, and the resulting mixture was heated at 100 °C for 5 min. After centrifugation, the supernatant was analysed.

cleavage products. Following the enrichment and labelling processes, the traceable linker **5** gave the brightest band of all of the traceable linkers for fluorescein-labelled enolase, and this linker was therefore used in all of the subsequent experiments.

We then proceeded to investigate the orthogonal nature of the aminooxy group generated by the cleavage of the linker. The enolase–linker conjugate derived from the click chemistry with the traceable linker **5** or control **15** was treated with 2-mercaptoethanol followed by the fluorophore **14** in a similar manner to that shown in the footnote of Fig. 4. Following purification by SDS-PAGE, the labelled enolase was detected using its fluorescence tag. As shown in Fig. S3 in ESI,† labelling was only observed when the traceable linker was used. This result demonstrated that the cleaved traceable linker was suitable for the selective labelling of the target protein in the presence of other proteins.

Pull-down experiments were finally conducted for the enrichment and selective labelling of the alkynylated enolase in a protein mixture, and the results are depicted in Fig. 5. A mixture consisting of the alkynylated enolase, bovine serum albumin (BSA) and ovalbumin (1/1/1 (w/w)) was subjected to enrichment (the click chemistry with the traceable linker **5**, adsorption on streptavidin beads, and thiol treatment) and selective labelling with the fluorophore **14**. A procedure similar to that described for the alkynylated enolase shown in Fig. 4 was used in this particular case and the experimental details are provided in the ESI.† After enrichment with streptavidin beads, ovalbumin was excluded from the protein mixture as shown in Fig. 5 [Lumitein staining: proteins adsorbed on the beads before the thiol-treatment (SDS release) and proteins eluted from the beads after the thiol-treatment (cleavage + labelling)]. In this experiment, the eluent was found to be contaminated with BSA, in the same way as conventional cleavable linker systems can be contaminated with non-target proteins.[6] The eluent containing the enolase and the BSA contaminant was then treated with the fluorophore **14** in the presence of

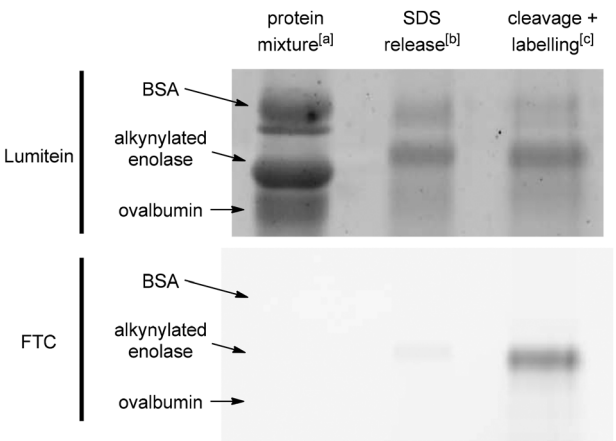


Fig. 5 Pull-down experiment for the alkynylated enolase in a protein mixture consisting of the alkynylated enolase, BSA, and ovalbumin. Enrichment (click chemistry with traceable linker **5**, adsorption on streptavidin beads, and thiol treatment) and selective labelling with fluorophore **14** were performed in a similar manner to that described in the footnote of Fig. 4. Following purification by SDS-PAGE, the labelled products or all proteins were visualized by fluorimetry without staining (FTC: λ_{ex} = 488 nm, λ_{em} = 530 nm) or Lumitein staining (Lumitein), respectively. [a] Mixture of the alkynylated enolase, BSA, and ovalbumin (1/1/1 (w/w)). [b] The beads were suspended in SDS-PAGE sample loading buffer prior to the thiol-treatment, and the mixture was heated at 100 °C for 5 min. The mixture was then centrifugated, and the supernatant was analysed. [c] Samples after enrichment and selective labelling of the alkynylated enolase.

aniline for 24 h, which resulted in the selective labelling of the enolase and allowed for the target enolase to be distinguished from the non-target contaminants (Fig. 5; FTC detection: cleavage + labelling). These results demonstrate that the traceable linker is applicable to the enrichment and selective labelling of the target protein for facile identification, even when the target protein has been contaminated with non-target proteins in the eluent obtained from the streptavidin beads.

Conclusions

In summary, we developed the traceable linker as an advanced cleavable linker. This new linker not only enabled the thiol-induced cleavage of the linker for the enrichment of the target protein in a manner similar to that of conventional cleavable linkers, but also allowed for the selective labelling of the target so that it could be distinguished from contaminated non-target proteins. Depending on an experimental design, in principle, a stimulus for cleavage of the traceable linker can be altered simply by changing a phenolic protection of the stimulus-responsive amino acid.[22] Combination of this traceable linker-based technique with the target selective introduction of an alkyne unit by the use of photo-affinity labelling or activity-based probe technology[1,2] therefore represents a new methodology for the facile clarification of the targets of the biologically active compounds, including drug candidates. The application of this traceable linker technique to the identification of the target proteins of naturally occurring bioactive compounds is currently underway in our laboratory.

Acknowledgements

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22 A fluoride-responsive traceable linker has already been developed and it will be published in due course.

