

A Mild Chemically Cleavable Linker System for Functional Proteomic Applications**

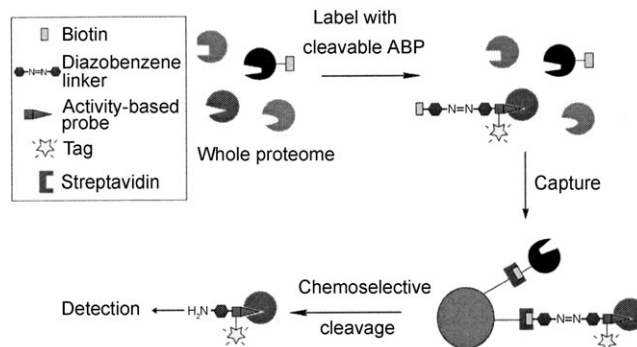
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One of the primary goals in the field of proteomics is finding ways to enrich specific protein targets from complex mixtures. Generally, this is accomplished with activity-based probes (ABPs) that allow specific modification by the formation of a stable covalent bond with reactive groups on a target protein.^[1] Such proteomic probes often carry biotin tags and can either be generally reactive towards free nucleophiles, such as thiols (i.e., isotope-coded affinity tagging (ICAT) reagents),^[2] or react through a specific enzymatic process with a key catalytic residue.^[3] Although enrichment by immobilized streptavidin allows efficient isolation of even highly dilute targets, one of the primary limitations is the need for harsh, denaturing conditions to disrupt the biotin–streptavidin interaction. This elution generally results in contamination of the desired probe-labeled proteins with avidin monomers, proteins that were nonselectively bound to the streptavidin, and endogenously biotinylated proteins. Hence, additional purification techniques such as gel electrophoresis are required prior to identification with MS.

The incorporation of a cleavable linker between the biotin tag and the site of attachment to the target protease provides a significant advance as it allows specific elution of probe-labeled proteins or peptides. Recently, a number of cleavable linkers have been reported with a focus on applications in MS and ICAT.^[4–6] However, these reagents require a strong acid (trifluoroacetic acid), making cleavage of labeled proteins directly from a streptavidin resin problematic as nonselective release can also occur.^[6] Disulfide cleavable linker systems, though useful for a variety of applications, are subject to disulfide exchange and show premature cleavage in cellular systems and reducing buffer solutions.^[7]

Herein, we report a novel diazobenzene derivative and its application as a chemoselective cleavable linker system

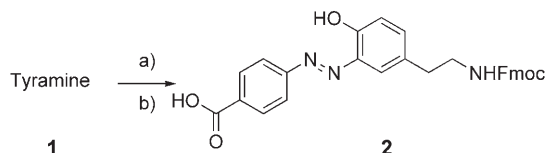
(Scheme 1). This linker can be easily incorporated into small-molecule probes that allow the isolation of specific protein targets by streptavidin affinity purification



Scheme 1. General strategy of a cleavable activity-based probe. Enrichment of specifically labeled proteases is followed by a mild chemoselective cleavage, which only releases the target proteases.

(Scheme 1). Preliminary MS data show highly selective identification of target proteases with virtually no signals observed from background proteins. We chose to use diazobenzenes for the linker strategy as they can be cleaved under mild reducing conditions by using sodium dithionite. Importantly, the cleavage reaction conditions are compatible with biochemical systems, as exemplified by the use of diazobenzenes as cleavable cross-linking reagents for proteins^[8] and, more recently, in the functionalization of a tyrosine residue on the surface of viral capsids.^[9]

Diazobenzene derivatives are readily accessible from diazonium salts and phenols. This led us to the design of the cleavable-linker building block Fmoc-CL-OH (**2**, Fmoc = 9-fluorenylmethoxycarbonyl, CL = cleavable linker; Scheme 2), which was made by a diazonium coupling of



Scheme 2. a) 4-Carboxybenzenediazonium chloride (2 equiv), overnight treatment with aqueous NaHCO₃, then b) Fmoc-chloride (1.1 equiv), 59% (two steps).

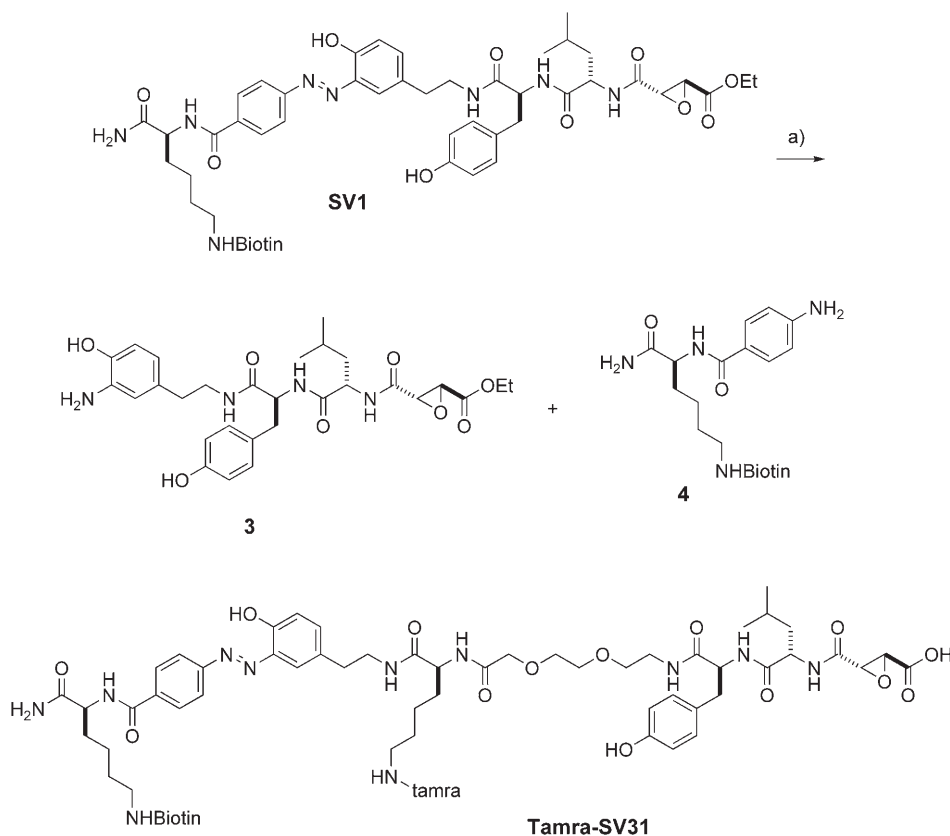
tyramine (**1**) and 4-carboxy-benzenediazonium chloride followed by the installation of a Fmoc group.

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[**] This work was financially supported by a National Technology Center for Networks and Pathways grant (U54 RR020843) and a NIH grant R01-EB005011 (to M.B.) and a postdoctoral fellowship from the Netherlands Organization for Scientific Research (to S.V.).

Supporting information for this article is available on the WWW under <http://www.angewandte.org> or from the author.

By using protocols for solid-phase peptide synthesis, the diazobenzene cleavable linker was incorporated into the activity-based probe **SV1** (Scheme 3), which was analogous to the previously reported general papain-family-probe DCG-04.^[10] We initially tested the cleavage of **SV1** by incubation with 100 mM Na₂S₂O₄. After 30 min, full cleavage of **SV1** into the two aniline fragments **3** and **4** was observed by using LC–MS analysis (Scheme 3, and Figure S1 in the Supporting Information).



Scheme 3. Probes with the benzenediazonium linker and their chemoselective cleavage. a) Na₂S₂O₄ (100 mM).

We next investigated the capture and release of proteases selectively labeled in a complex proteome. For this analysis, the related probe **SV31** was constructed to contain a poly(ethylene glycol) (PEG) linker and a free epoxysuccinate for increased hydrophilicity. In addition, a fluorophore was introduced on the side of the probe that remains attached to the target enzymes. This feature allows rapid and sensitive in-gel fluorescent detection after on-bead cleavage of the diazobenzene linker and subsequent resolution by SDS-PAGE. Moreover, it overcomes the need for additional staining techniques.

Accordingly, rat liver homogenate, which contains a number of previously characterized cathepsin activities,^[11] was incubated with tetramethylrhodamine (tamra)-tagged **SV31** for 1 h and excess probe was removed by filtration by using a size-exclusion column. The resulting eluate showed clean labeling of the four major cathepsins present in the

proteome that could be detected by fluorescent scanning (Figure 1, lane 1). Incubation with immobilized streptavidin efficiently depleted labeled enzymes from the supernatant (Figure 1, lane 2). Nonselective cleavage by boiling in SDS buffer solution resulted in near-quantitative release of the labeled proteases (Figure 1, lane 3).

Next, we determined the cleavage efficacy at a range of pH values and buffer-solution conditions. After treatment with the chemoselective cleavage cocktail (Figure 1a, lanes a), the proteases remaining on the beads were cleaved off by boiling with SDS sample buffer solution (Figure 1a, lanes b). We tested both low-temperature (0°C) and high-temperature (90°C) conditions and found little effect on cleavage efficiency (data not shown). However, the absence of buffer solution, low pH buffer solutions (< 6.5), or high pH buffer solutions (> 8.5) resulted in incomplete cleavage (see Figure 1a, lanes 4 for pH 9). Furthermore, decreasing in dithionite concentration (Figure 1a, lanes 6) also reduced cleavage efficiency. A phosphate buffer solution at pH 7.4 (3 × 15 minutes^[8c]) and a 25 mM Na₂S₂O₄ concentration was identified as the optimal cleavage conditions (Figure 1a, lanes 5). Densitometry of the lanes by image-analysis software (NIH Image J) revealed that approximately 91% of the protein is recovered under optimized cleavage conditions (determined by the ratio of lanes a and b and averaged over three independent runs).

Although the release of probe-labeled proteins from streptavidin by using dithionite is slightly less efficient than denaturing conditions, it is likely to result in a reduction in background signals and easier identification of the target proteases. To confirm this, release of **SV1**-labeled proteases by either SDS or dithionite cleavage was performed and followed by silver staining of the resulting gel. These results confirm that the chemoselective conditions give cleaner releases of target proteins and prevented contamination by excessive amounts of streptavidin (Figure 1b).

To confirm applicability in a gel-free system, we analyzed the cleaved sample in Figure 1b by digestion with trypsin followed by LC–MS/MS analysis of all peptide products. This analysis identified multiple peptides from each of the expected cathepsin proteases without any contamination by background proteins (Table 1 and Figure S1 in the Supporting Information). These results show that the mild cleavage

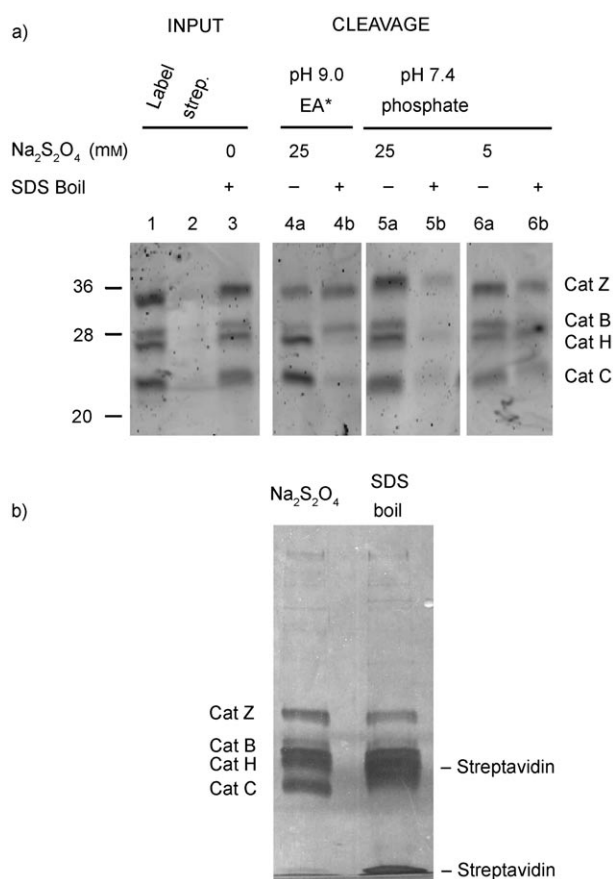


Figure 1. Optimization of the chemoselective cleavage of probe-labeled proteins. a) Fluorescent gel image showing the four major cathepsin activities in rat liver homogenate (Cats Z, B, H, and C) that were labeled by the general papain-probe **SV34** (lane 1). These proteins were removed from the mixture by incubation with streptavidin beads (strep.; lane 2) and could be released by nonselective denaturing conditions (lane 3; SDS boil). Cleavage of labeled proteins by using several pH buffer solutions and different concentrations of cleavage reagent (lanes 4–6) allow identification of the optimal cleavage conditions in lane 5 with greater than 90% cleavage efficiency. EA: ethanolamine buffer solution. b) Specific cleavage of **SV1**-labeled proteins as in (a), lane 5 a and analyzed by silver staining of eluted proteins. Note the presence of excess streptavidin in the SDS-boiled sample and the presence of several endogenously biotinylated proteins (indicated by *).

conditions are compatible with MS methods. Application of the described strategy to different probes and other proteomes is currently under investigation and will be reported in due course.

Table 1: Identified proteases targeted by **SV1** after chemoselective release from immobilized streptavidin.

Protein	$n_p^{[a]}$	Sequence coverage
cathepsin Z	4	32%
cathepsin B	2	8%
cathepsin H	5	29%
cathepsin C	3	16%

[a] n_p = number of identified peptides.

Received: September 16, 2006

Revised: November 30, 2006

Published online: January 5, 2007

Keywords: activity-based probes · affinity purification · cleavable linkers · mass spectrometry · proteomics

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