Proteomics

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A Cleavable Linker Based on the Levulinoyl Ester for Activity-Based Protein Profiling**

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Activity-based protein profiling has recently come to the fore as an attractive strategy to disclose enzyme activity in the context of complex biological samples.[1] An activity-based profiling probe (ABP) normally consists of a recognition element targeted at a specific enzyme (family), and is equipped with a reactive group through which the targeted enzymes are covalently and irreversibly modified.[2,3] A third essential element of ABPs is the affinity/identification tag, which can be either incorporated into the ABP directly or alternatively installed through a bioorthogonal process in so-called two-step activity-based protein profiling strategies.[4] Visualization/identification tags come in several types, including fluorophores and immune epitope tags. The most popular tag in this context is the biotin group. It is relatively small, easy to manipulate, and has strong binding affinity to streptavidin. As a consequence a range of biochemical tools have become available over the decades for either the visualization of biotinylated biomolecules or their pull-down.

The strong affinity of streptavidin for biotin makes the release of biotinylated proteins captured by, for example, streptavidin-coated beads difficult. Most effective procedures rely on relatively harsh conditions, such as boiling of the sample in denaturing conditions and often in the presence of unmodified biotin. As a consequence, next to (denatured) streptavidin, the eluted protein pool is often contaminated with endogenously biotinylated biomolecules.

The introduction of a specific linker between biotin and the ABP reactive group, which can be cleaved chemoselectively so that only the ABP bound material is released, is an attractive strategy to circumvent this problem (Figure 1 a). Examples of such cleavable linkers (CL) are the disulfide linkage,[5] enzymatically (Tev) cleavable,[6] acid-cleavable,[7] diazobenzene-derived (cleavable with $Na_2S_2O_4$),[8] and hydrazone-based linkers.[9] An ideal cleavable linker is stable towards the various conditions (acidic, basic, reductive, including generally applied buffer systems) to which the biological sample may be exposed, depending on the nature of the experiment, and can also withstand the reactive (nucleophilic) species that are present in a cell extract. At the same time, it is susceptible to mild cleavage conditions. With this reasoning in mind our attention was focused on the levulinoyl ester, a versatile protecting group often applied in synthetic organic chemistry. It is acid stable and can be removed selectively with respect to other esters by treatment with hydrazine. A shortcoming is its intrinsic base-lability and we reasoned that this drawback can be rectified by choosing the alcohol with which the levulinolate group is condensed such that it is both electron-rich (to reduce its leaving-group properties) and sterically congested (to minimize intermolecular nucleophilic attack). Altogether, we came to the design of the epoxomicin-based,[10] levulinolate-modified

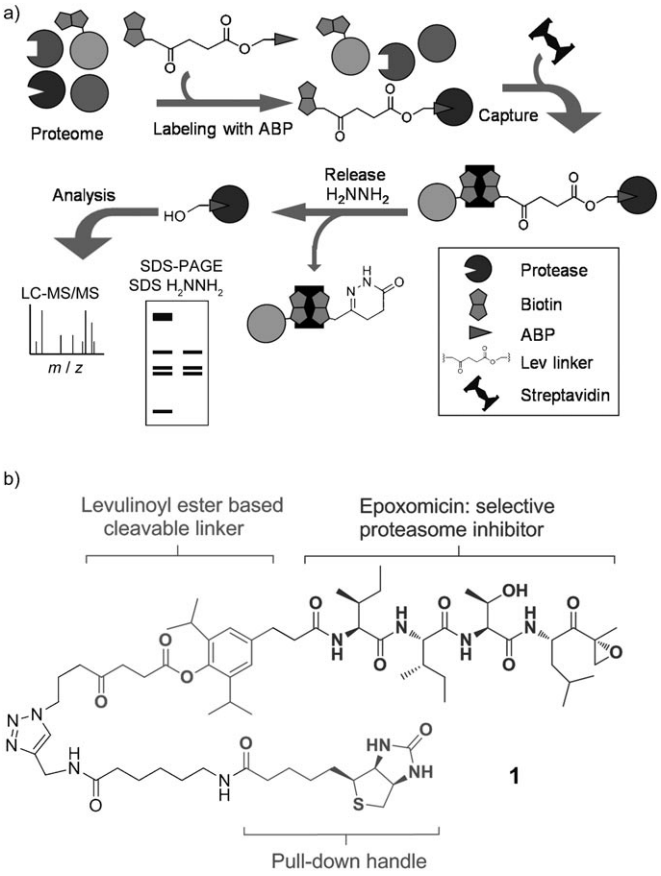

Figure 1. a) Schematic representation of an activity-based enrichment experiment using the cleavable linker based on a levulinoyl ester. After labeling of the target enzyme and pull-down the linker is (chemo)-selectively cleaved with hydrazine. b) Structure of target proteasome probe **1** containing the cleavable linker based on a levulinoyl ester.

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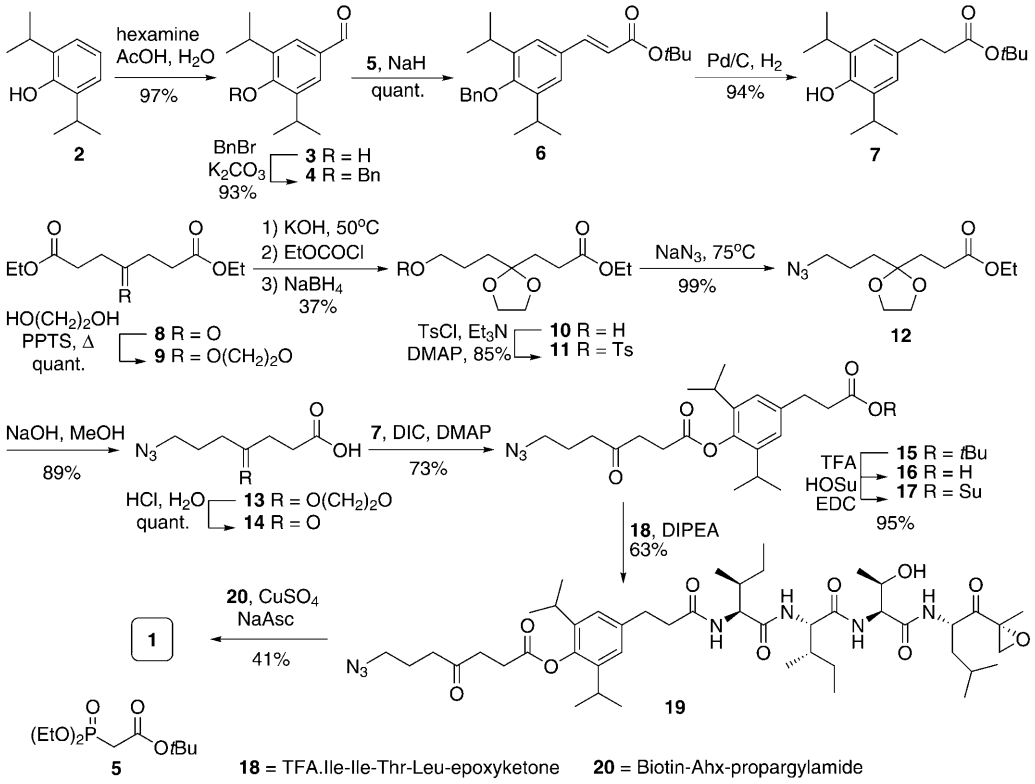
activity-based proteasome probe **1** (Figure 1b). Herein, we report its synthesis and application in the activity-based enrichment and identification of the proteasome active subunits from cell lysate.

The synthesis of ABP **1** is shown in Scheme 1. Commercially available 2,6-diisopropylphenol (**2**) was converted into benzaldehyde **3** in a Duff reaction[11] and the alcohol was subsequently protected as the benzyl ether (**4**). The ensuing Horner–Wadsworth–Emmons reaction with phosphonate **5** proceeded smoothly and gave **6**. Hydrogenation and concomitant removal of the benzyl protecting group led to alcohol **7**. Next, the ketone in diethyl 4-oxopimelate **8** was protected as ketal **9**[12] and one of the ethyl ester groups was selectively saponified. The obtained carboxylate was reduced to the alcohol **10**, which was converted into azide **12** via its tosylate. Saponification and subsequent acidic hydrolysis gave **14**, which was condensed with **7** and produced levulinoyl ester **15**. The *tert*-butyl ester was removed and the resulting carboxylic acid **16** was transformed into its activated NHS ester **17**. Condensation with **18** gave **19**, which was treated with alkyne **20** and gave the target compound **1**.

We came to the design of the linker system present in **1** after conducting a number of studies (see the Supporting Information). First, the 2,6-diisopropyl-4-alkylphenol ester was selected on the basis of a preliminary screen of a panel of esters derived from levulinic acid and different alcohols (primary, secondary, tertiary, and phenols). The isopropyl groups *ortho* to the ester appeared sterically sufficiently bulky to avoid hydrolysis under basic conditions. The 4-alkyl substituent proved to be favorable compared to a 4-carbonyl substituent in that this more electron-rich phenol is less prone to hydrolysis.[13]

The efficacy of compound **1** to select and identify proteasome catalytic activities from cell extracts in an activity-based labeling experiment followed by hydrazine-mediated linker cleavage was investigated as follows. Incubation of HEK-293 cell lysate with **1** in Tris buffer and subsequent treatment with or without hydrazine under different conditions and visualization by streptavidin Western blotting revealed that all three active subunits (β1, β2, and β5) are labeled (Figure S2 in the Supporting Information). Cleavage of the linker is dependent on the hydrazine concentration and exposure of proteasomes labeled with **1** to 50 mM hydrazine for varying periods of time revealed that full cleavage occurred after three to five hours (Figure S3 in the Supporting Information. In all ensuing cleavage studies we chose to subject the samples to hydrazine for 15 h).

To evaluate the use of **1** for protein capture and release we treated HEK-293 cell lysate with this compound, followed by denaturation, cysteine bridge reduction and capping, and capturing with streptavidin-coated magnetic beads. The beads were divided into equal aliquots and the elutes from each sample, after treatment with or without hydrazine under different conditions, were resolved by SDS-PAGE and visualized by silver stain (Figure 2a). As a control experiment, the biotinylated probe Ada-Lys(biotin)Ahx$_3$Leu$_3$VS (**21**)[14] was added to the lysate, which was then treated with either standard (nonselective) elution conditions (1% SDS, 10 μM biotin, 100 °C, lanes 1 and 7) or hydrazine (lanes 2 and 8). These results show that the captured proteins from **1** can be released chemoselectively with hydrazine both at room temperature and at 37 °C (lanes 4 and 10), whereas the captured construct derived from **21** is resistant towards these conditions (lanes 2 and 8). The steptavidin blots of the same samples in Figure 2b show a



Scheme 1. Synthesis of two-step labeling probe **19** followed by a "click" reaction leading to target compound **1**. Bn = benzyl, DIC = 1,3-diisopropylcarbodiimide, DIPEA = *N*,*N*-diisopropylethylamine, DMAP = 4-dimethylaminopyridine, EDC = 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide, PPTS = pyridinium *p*-toluenesulfonate, TFA = trifluoroacetic acid, Ts = 4-toluenesulfonyl.

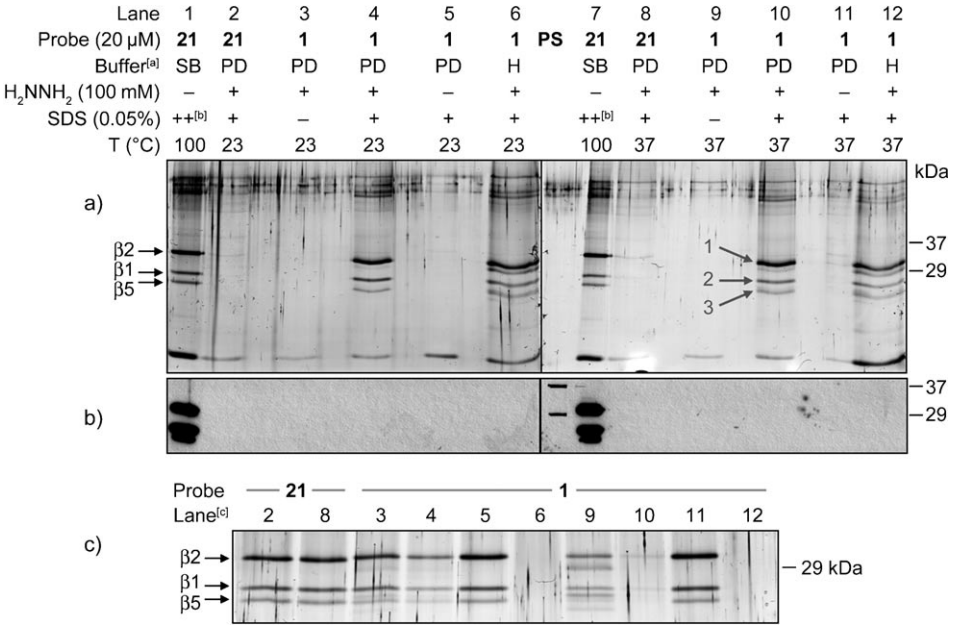


Figure 2. Enriched proteins after pull-down with ABPs **1** and **21** and cleavage from streptavidin beads under the indicated conditions for 15 hours. a) Silver stain. b) Streptavidin Western blot from the same samples. c) Determination of the cleavage efficiency: subsequent to hydrazine treatment the beads were extensively washed, after which all captured leftovers were eluted with SDS boiling and visualized by silver staining (the complete gel image is shown in Figure S4 in the Supporting Information). [a] The applied buffers are: SB (30 mM Tris·HCl pH 6.8, 1% SDS, 9% glycerol, bromophenolblue, 0.25 M β-mercaptoethanol, 10 μM biotin); PD (50 mM Tris·HCl pH 7.5, 150 mM NaCl); H (50 mM HEPES pH 5.8). [b] "++" refers to the 1% SDS present in the sample buffer (SB). [c] The lanes correspond to the same samples in the lanes above. The arrows indicate the excised bands analyzed by LC-MS/MS after in-gel tryptic digest. HEPES = 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid, SDS-PAGE = sodium dodecylsulfate–polyacrylamide gel electrophoresis, PD = pull-down buffer, PS = prestained marker low-range (Bio-Rad), SDS = sodium dodecylsulfate, Tris = tris(hydroxymethyl)aminomethane.

Table 1: Proteasome active subunits identified from the indicated bands in Figure 2 after in-gel tryptic digest and LC-MS/MS analysis.[a]

Band	Subunit	n_p[b]	Sequence coverage
1	β2	6	42.7%
2	β1	5	39.0%
3	β5	7	54.4%

[a] The complete data including all peptides are shown in Table S2 in the Supporting Information. [b] n_p = number of identified peptides.

complete absence of biotin for the proteins captured with **1**, which proves that the cleavable linker is indeed cleaved. In addition, it appears that a small amount of SDS (0.05%) is necessary during cleavage (compare lanes 3 and 9 with lanes 4 and 10), whereas SDS at this concentration solely does not sustain cleavage (lanes 5 and 11). Cleavage in HEPES buffer at pH 5.8 occurs as well, but is accompanied by a higher release of undesired material (compare lanes 10 and 12). The cleavage efficiency was determined by extensive washing of the same beads, eluting all remaining captured material by boiling in SDS and silver staining after SDS-PAGE (Figure 2c). For the samples of lanes 6, 10, and 12 the cleavage efficiency was (nearly) quantitative (no proteasome characteristic bands are present) and for that of lane 4 only a small amount of active proteasome subunits was visible.

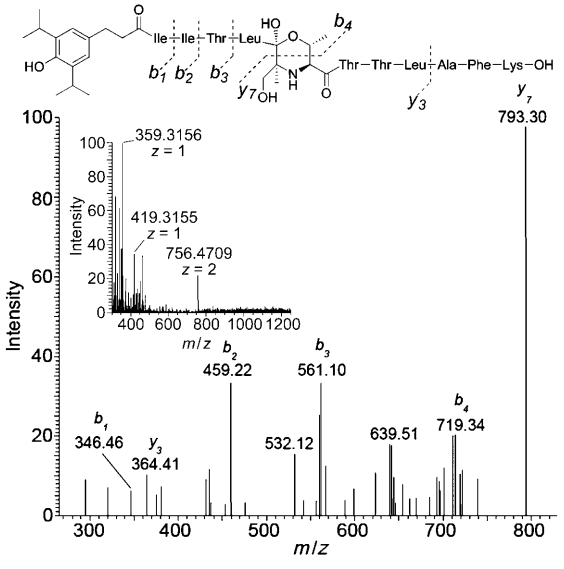


Figure 3. Tandem mass spectrometry (MS/MS) analysis of the β5 active site fragment attached to the expected cleaved probe. Insert: part of the LC-MS run showing the parent ion m/z (calcd $m/z = 756.4704$ for $z = 2$) for the peptide-inhibitor construct as shown above. The complete LC-MS/MS data for both β2 and β5 subunits is shown in the Supporting Information.

To establish that the visualized bands indeed correspond to the targeted proteasome subunits the indicated bands (arrows in Figure 2a) were cut from the gel and analyzed by LC-MS/MS after in-gel tryptic digest. In this analysis multiple characteristic peptides for each of the indicated subunits were identified (Table 1), including the modified active site fragment peptides derived from β2 and β5 (Figure 3 and the Supporting Information).

In summary, we put forward a levulinoyl-based linker system for use in activity-based protein profiling. Tuning the nature of the levulinoyl ester has produced a linker system that is robust enough to survive conditions commonly applied to cell extracts in biochemical experiments. Our linker withstands aqueous, acidic, and basic media (including the widely used Tris buffer), and is resistant towards disulfide-reducing conditions. We thus believe that our linker has some important advantages over the previously reported[5–9] linker systems. Our

results form the basis for the development of new activity-based profiling strategies, for instance two-step labeling strategies that include bioorthogonal chemistry. From our synthetic scheme it is apparent that the linker is compatible with click chemistry. In a preliminary experiment (Figure S5 in the Supporting Information) we exposed HEK-293 cell lysates to azide-containing probe **19**. Ensuing treatment with biotinylated Staudinger phosphane,[15] SDS-PAGE resolution of the protein contained, and streptavidin blotting revealed a pattern characteristic for the proteasome catalytic activities. Finally we investigated the stability of compound **1** in serum and plasma. Initial studies (see the Supporting Information) demonstrate that the levulinoyl linker is indeed stable in these environments for 15 hours at 37 °C but can be cleaved by addition of hydrazine. These last results demonstrate the viability of our levulinoyl linker for two-step ABP profiling of enzymes in general, and both for in situ and in vivo applications.

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