Active-Site-Directed Probe

Chemistry in Living Cells: Detection of Active Proteasomes by a Two-Step Labeling Strategy**

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With the sequencing of the human genome and the genetic material of most relevant human pathogens nearly at an end, the focus in biomedical and biological sciences is shifting toward the global assessment of expression levels and function of the gene products. The reason for the renewed interest in protein activity is clear: It is at the protein level that biological processes are modulated in health and disease. Approaches that report on transcription levels are not informative in terms of the levels of activity of the products encoded by these transcripts. Equally importantly, the relevant activities are those in living cells and not those measured in vitro. At the same time, the global assessment of highly complex and dynamic protein mixtures as found in intact cells is a much more arduous task than that of the relatively static genome. This holds true especially when insight into the activity of proteins rather than their expression levels is desired.

Chemistry-based functional-proteomics approaches[1,2] have been developed based on the use of synthetic compounds that modify a selected subset of proteins covalently and irreversibly. These methodologies include the attractive feature that the complex proteome is simplified by selecting protein families on the basis of their function.[3] For instance, broad-spectrum, irreversible protease inhibitors have been used in the profiling of serine proteases,[4] cysteine proteases,[5] and the catalytically active subunits of the proteasome.[6,7] The inhibitors are equipped with either a radioisotope, a biotin moiety, or a fluorescent tag, to allow

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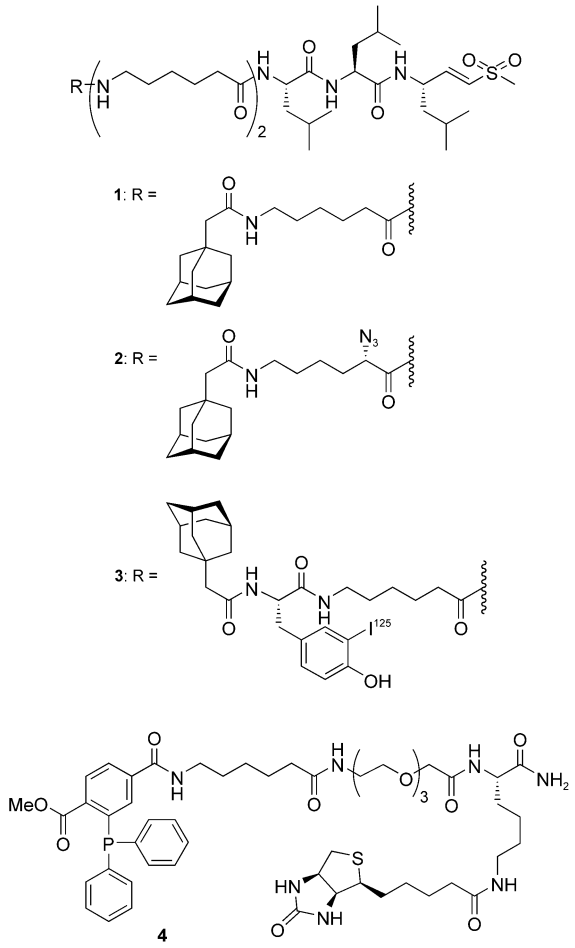
visualization, isolation, and quantification of the proteases. The cell impermeability of the compounds used in these examples[8] limits their use in living-cell systems, and this limitation is an unavoidable consequence of the use of such probes.

Herein we describe a new functional-proteomics strategy that allows two-step labeling of the catalytically active subunits of the proteasome in living cells. The proteasome is a multi-catalytic proteinase that accounts for the bulk of cytosolic and nuclear proteolysis. We have previously reported the development of a set of extended, peptide-based, irreversible proteasome inhibitors.[6] The most potent of these, $AdaAhx_3L_3VS$ **1** (Scheme 1), is unique in that it



Scheme 1. Structure of the broad-spectrum cell-permeable proteasome inhibitor **1**, the cell-impermeable radioiodinated derivative **3**, and the Staudinger ligation partners **2** and **4** described herein.

targets all catalytically active β subunits of both the constitutive- and the interferon-γ-inducible immunoproteasome with approximately equal efficiency. Although $AdaAhx_3L_3VS$ **1** proved to be cell permeable, it lacks a label that allows easy detection. We demonstrate herein that the modification of **1** with an azide group (to give **2**; Scheme 1) neither interferes with its inhibition profile nor with its cell permeability.[9] Labeling of whole cells with **2** decorates the catalytically active β subunits of the proteasome with an azide as a latent ligation handle. After cell lysis and the retrieval and denaturation of cellular protein content, the azido groups can be subjected to a modified Staudinger ligation with the biotinylated phosphane reagent **4**, in a method developed by Bertozzi and co-workers (Figure 1).[10,11]

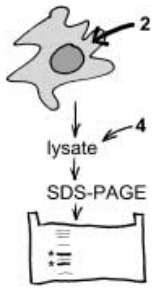
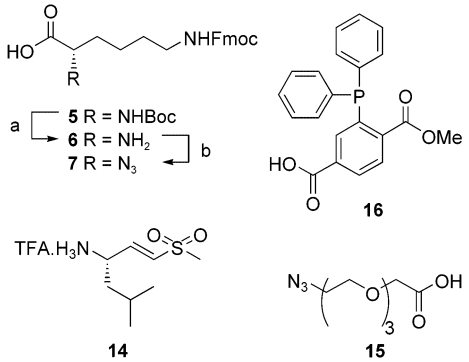


Figure 1. General strategy: irreversible proteasome inhibition and subsequent Staudinger-ligation-mediated biotinylation to enable the visualization of proteasomes in an activity-based manner. SDS-PAGE = sodium dodecylsulfate–polyacrylamide gel electrophoresis.

The synthesis of the azide-containing proteasome inhibitor **2** was carried out as follows. The treatment of *N*-α-Boc,*N*-ε-Fmoc-L-lysine (**5**) with trifluoroacetic acid and subjection of the product to diazotransfer conditions (TfN_3, $CuSO_4$)[12] readily afforded (2*S*)-2-azido-6-(fluorenylmethyloxycarbonylamino)hexanoic acid (**7**; Scheme 2). Standard Fmoc-based solid-phase peptide synthesis (SPPS) was carried out on acid-labile Wang resin

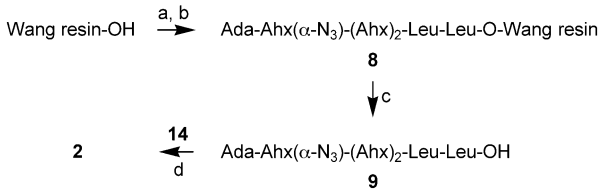


Scheme 2. Nonstandard building blocks used in the synthesis of **2** and **4**. Conditions: a) 50% TFA/CH_2Cl_2, 92%; b) TfN_3, $CuSO_4$, H_2O/MeOH, 89%. Boc = *tert*-butyloxycarbonyl, Fmoc = 9-fluorenylmethoxycarbonyl, TFA = trifluoroacetic acid, TfN_3 = trifluoromethanesulfonyl azide.

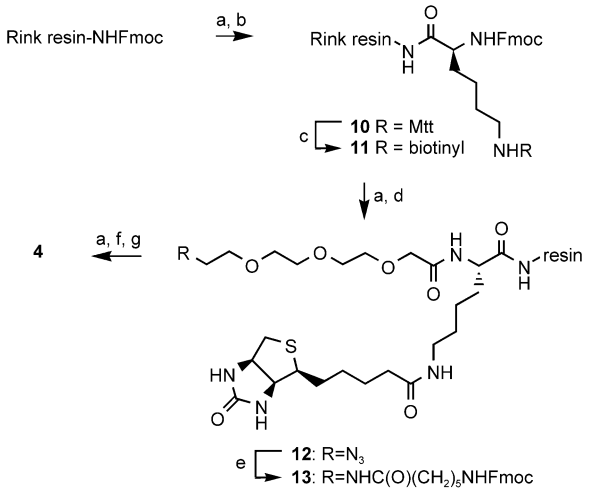
and led to immobilized peptide **8** from **7**. Cleavage from the resin and solution-phase condensation of resulting carboxylate **9** with leucine vinyl sulfone **14**[13] afforded target compound **2**, which was purified by silica-gel chromatography (58% overall yield; Scheme 3).

The synthesis of phosphane reagent **4** commenced with the condensation of the Rink amide linker with *N*-α-Boc,*N*-ε-Mtt-L-lysine.[14] Deprotection of the side-chain protective group in **10** was followed by condensation with biotin to afford immobilized biocytin **11**. Standard solid-phase peptide synthesis with sequential addition of azido acid **15**,[15] 6-(Fmoc-amino)hexanoic acid, and phosphane **16**, followed by acidic cleavage from the resin and HPLC purification gave the target compound **4** in 16% yield (Scheme 4).

We performed a set of competition experiments to establish the inhibition profile of **4**. We used the cell line



Scheme 3. Synthesis of azide-containing proteasome inhibitor **2**: a) FmocLeuOH, DIC, DMAP, DMF; b) Repeated cycles of SPPS: Fmoc cleavage: 20% piperidine in DMF; amino acid condensation: Fmoc-protected amino acid, PyBOP, DiPEA, DMF; Fmoc-protected amino acid building blocks were used in the following order: FmocLeuOH, FmocAhxOH, FmocAhxOH, **7**, adamantane acetic acid; c) 10% H_2O/TFA; d) HBTU, DiPEA, DMF, 58% overall yield. Ada = adamantane acetyl, Ahx = 1-amino-6-hexanoyl, DIC = *N*,*N*′-diisopropylcarbodiimide, DiPEA = *N*,*N*-diisopropylethylamine, DMAP = 4-(dimethylamino)pyridine, DMF = *N*,*N*-dimethylformamide, HBTU = 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, Leu = leucine, PyBOP = benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate.



Scheme 4. Synthesis of biotinylated phosphane reagent **4**: a) 20% piperidine in DMF; b) FmocLys(Mtt)OH, PyBOP, DiPEA, DMF; c) 1% TFA/CH_2Cl_2, then biotin, PyBOP, DiPEA, DMF; d) **15**, PyBOP, DiPEA, DMF; e) Me_3P, 20% H_2O/dioxane, then FmocAhxOH, PyBOP, DiPEA, DMF; f) **16**, EDC, HOBt, CH_2Cl_2; g) 50% TFA/CH_2Cl_2, 16% overall yield. EDC = 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, HOBt = *N*-hydroxybenzotriazole, Mtt = 4-methyltrityl.

EL-4, derived from a murine thymoma. This cell line is advantageous because it expresses both the constitutive proteasome and the interferon-γ-inducible immunoproteasome, which contain a total of six distinct catalytically active β subunits. We also used the cell line HeLa, derived from a human cervical carcinoma. HeLa expresses only the constitutive proteasome (three catalytically active β subunits). When lysates of EL-4 cells were incubated with azide-containing proteasome inhibitor **2** at different concentrations, prior to treatment with radioiodinated peptide vinyl sulfone **3**, we observed that labeling of the six individual subunits was abolished at final inhibitor concentrations of 10–30 μM (Figure 2a), thus demonstrating **2** to be a proteasome inhibitor of equal potency to **1**.[6] The ability of peptide

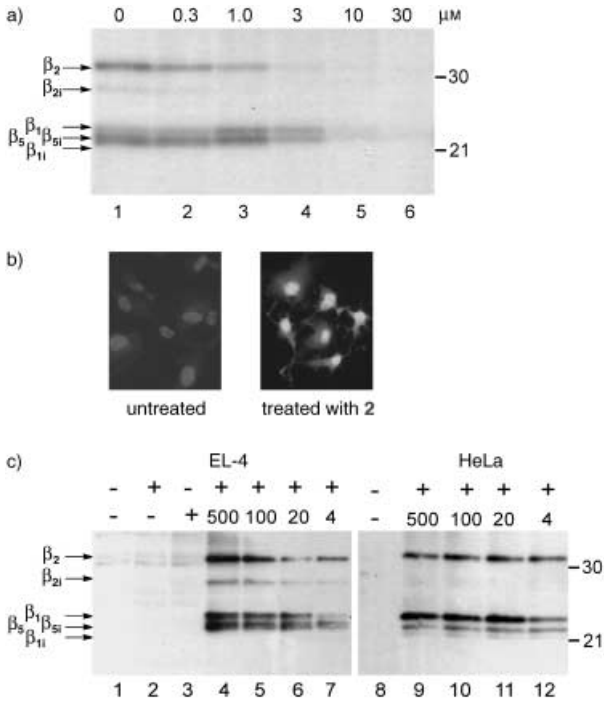


Figure 2. a) Cell lysate prepared from EL-4 cells was incubated with **2** at concentrations ranging from 0 to 30 μM. Residual unmodified subunits were labeled by subsequent incubation with radioiodinated inhibitor **3**. Labeled subunits were resolved by SDS-PAGE and visualized by autoradiography. b) Ub-R-GFP accumulates when proteasomal degradation is blocked. Cells were incubated with either a solvent control or **2** (50 μM final concentration) for 8 h and fixed, followed by blue nuclear staining of the DNA with 4,6-diamidino-2-phenylindole (DAPI blue). Confocal laser-scanning microscopy revealed **2** to be a cell-permeable proteasome inhibitor. c) Lysates from EL-4 and HeLa were treated with **2** at 37 °C for 1 h and then boiled in the presence of sodium dodecylsulfate to cause protein denaturation and exposure of the azido moieties of conjugated **2**. The azido moieties were biotinylated through a Staudinger ligation by adding an aqueous solution of reagent **4** to the reaction mixture, followed by incubation at 37 °C for 2 h. Samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane. Incubation with streptavidin–horseradish peroxidase (strept–HRP) conjugates allowed the visualization of active proteasomal β subunits by chemiluminescence.

vinyl sulfone **2** to disable the proteasome in living cells was determined by the following procedure: U373 cells expressing the green fluorescent protein (GFP) ubiquitin-GFP fusion protein[16] (Ub-R-GFP) were treated with compound **2** at 50 μM (final concentration) and compared with untreated cells for the presence of GFP fluorescence. Ub-R-GFP is rapidly degraded by the proteasome, which results in a steady state with hardly any green fluorescence as a result (Figure 2b). However, in cells treated with **2**, a time-dependent accumulation of fluorescence was observed, thus demonstrating the capacity of **2** to inactivate the proteasome in living cells.

Encouraged by these results, we set out to establish the suitability of a Staudinger ligation for the two-step visualization of catalytically active proteasome subunits in cell lysates, as well as in living cells. In the first experiment, cell lysates from EL-4 and HeLa cells were exposed to **2** at various

concentrations, prior to denaturation of the cellular protein. The resulting mixtures were incubated with biotinylated Staudinger-ligation reagent **4** and separated by SDS-PAGE. Transfer of the separated protein mixture onto a polyvinylidene difluoride (PVDF) membrane, followed by chemiluminescence induced by horseradish peroxidase–streptavidin conjugate, resulted in a distinct labeling profile. Labeling intensity depended on the dose of **2**. The labeling pattern conforms to that established for radioiodinated probe **3**.[6] Importantly, proteasome-derived polypeptides were detected only when both inhibitor **2** and Staudinger reagent **4** were used (Figure 2c, lanes 4–7 and 9–12). These results establish the selectivity of **4** in complex physiological mixtures to target only those proteins modified with an azide functionality.

We then investigated the possibility of covalent proteasome inhibition in living cells, followed by postlysis Staudinger ligation and immunoblotting. EL-4 cells were incubated overnight with **2** (Figure 3). Subsequent glass-bead

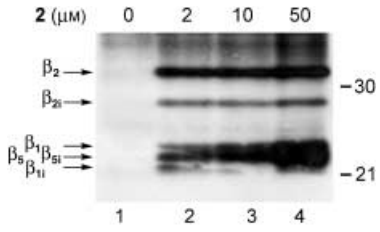


Figure 3. Proteasome labeling in living cells. Incubation of living cells (5×10^6 at 37 °C) with **2** followed by postlysis ligation and immunoblotting reveals the active proteasomal content and composition in living cells.

lysis, incubation with **4**, SDS-PAGE separation, and Western blotting afforded a labeling pattern virtually indistinguishable from that obtained for the labeling of cell lysates (Figure 2c). In vivo labeling appeared to be more effective (compare Figure 3 with the labeling patterns obtained in vitro in Figure 2c), thus indicating a more efficient targeting of all proteasomal subunits in living cells. This observation is possibly a result of partial dissociation of the proteasome particle during cell lysis and storage. We conclude that inhibitor **2** can be used in combination with biotinylation reagent **4** for the visualization of active proteasomes in living cells.

In summary, we have presented a novel strategy for the visualization of active enzymes in living cells. Compound **2** was identified as a powerful, cell-permeable inhibitor of all proteasomal activities, and **2** can subsequently undergo postlysis labeling through a chemoselective Staudinger ligation. This protocol opens the way toward the screening, in living cells, of proteasomal activity, for example, in human tissue samples. The measurement of proteasome activity in live cells remains an important goal, not only in the context of novel treatment strategies for cancer, but also in biological systems more generally. For instance, malfunction of the ubiquitin–proteasome system has been implicated in both cancer[17] and neurodegeneration.[18] Importantly, this two-step methodology (this is, covalent, irreversible enzyme modification followed by chemoselective modification) may be extended toward the development of novel chemoselective ligation partners that are compatible with desired cellular environments. We are currently pursuing the application of this strategy to the assessment of the activity of a variety of other enzyme families in living cells.[19]

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