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Chemical tools for the study of proteolytic activities associated with antigen presentation

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Citation
Swieten, P. F. van. (2007, January 18). *Chemical tools for the study of proteolytic activities associated with antigen presentation*. Retrieved from https://hdl.handle.net/1887/9143

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Chapter 2

Detection of active proteasomes by a two-step labeling strategy[1]

Introduction

Major efforts in unraveling of the human genome and the genetic material of most relevant human pathogens, enabled the focus in biomedical and biological sciences to shift towards the global assessment of expression levels and function of the gene products. The reason for the renewed interest in protein activity is obvious: biological processes are modulated at the protein level in health and disease. Approaches that report on transcription levels are not informative for the levels of activities of the products encoded by these transcripts. Equally important, the relevant activities are those in living cells and not what is 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 aiming for insight in the activity of proteins rather than for their expression levels.

Chemistry-based functional proteomics approaches[2,3] have been developed based on the use of synthetic compounds that modify a selected subset of proteins covalently and

irreversibly. These methodologies combine the attractive features of simplifying the complex proteome by selecting protein families on the basis of their function.[4] For instance, broad-spectrum, irreversible protease inhibitors have been used in the profiling of serine proteases,[5] cysteine proteases,[6] and the catalytically active subunits of the proteasome.[7,8] These inhibitors are equipped with either a radioisotope, a biotin moiety or a fluorescent tag, to allow respectively visualization, isolation and quantification of the proteases. The cell-impermeability of the compounds used in these examples limits their use to in vitro applications and this limitation is an unavoidable consequence of the use of such probes.

Two-step labeling strategy

Here, a new functional proteomics strategy is described, that allows two-step labeling of the catalytically active subunits of the proteasome in living cells.[9] The proteasome is a multi-catalytic protease that accounts for the bulk of cytosolic and nuclear proteolysis.[10] Previously, a set of extended, peptide-based, irreversible proteasome inhibitors have been reported.[7] The most potent of these, AdaAhx$_3$L$_3$VS **1** (Figure 1), is unique in that it targets all catalytically active β-subunits of both the constitutive- and the interferon-γ-inducible immunoproteasome with approximately equal efficiency. Although AdaAhx$_3$L$_3$VS **1** proved to be cell permeable, it lacks a label that allows easy detection.

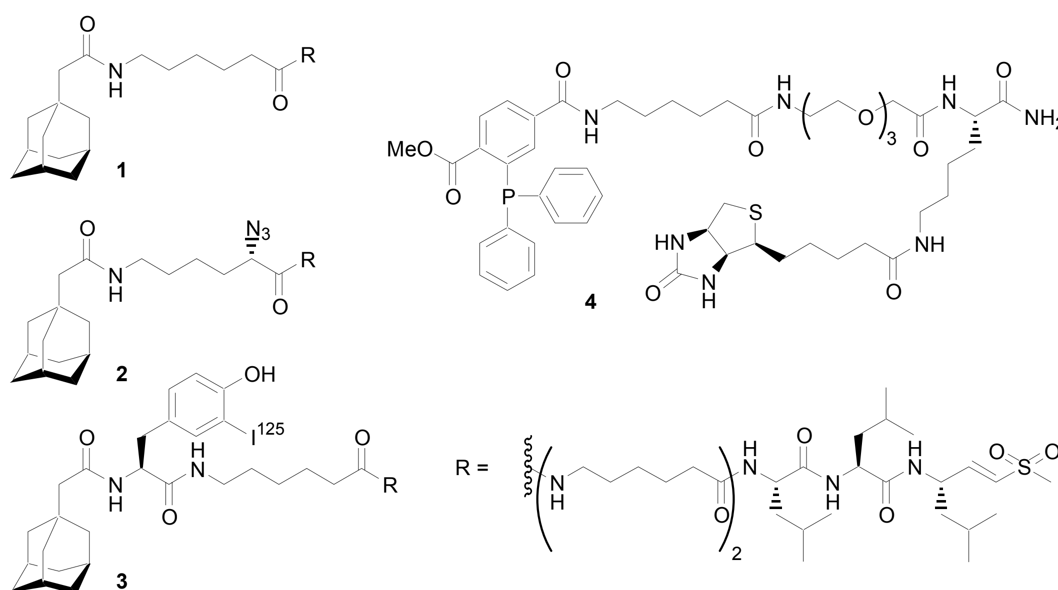


Figure 1. Structure of the broad-spectrum cell-permeable proteasome inhibitor **1**, the cell-impermeable radioiodinated derivative **3**, and the Staudinger ligation partners **2** and **4** described here.

Here, it is demonstrated that modification of **1** with an azide group (to give **2**; Figure 1) neither interferes with its inhibitory profile nor with its cell permeability.[11] 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, retrieval and denaturation of cellular protein content, the azido groups can be addressed by the biotinylated phosphane reagent **4** in a modified Staudinger ligation, as developed by Bertozzi and coworkers (Figure 2).[12,13]

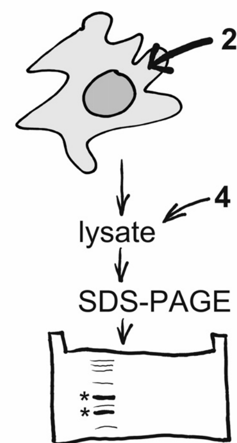
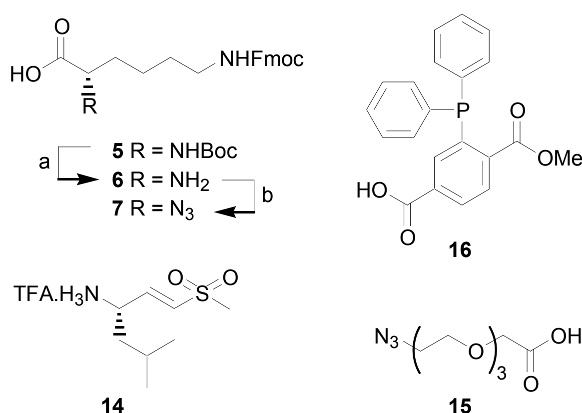


Figure 2. General strategy: irreversible proteasome inhibition and subsequent Staudinger ligation-mediated biotinylation to enable visualization of proteasomes in an activity-based manner.

Synthesis

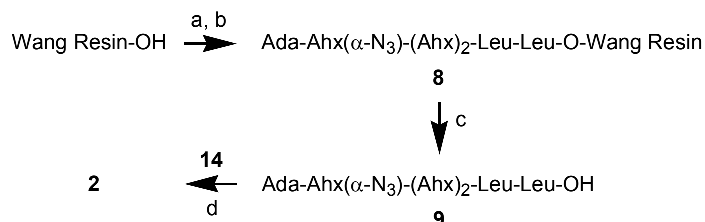
The synthesis of the azide-containing proteasome inhibitor **2** was accomplished as follows (Scheme 1 and 2). Treatment of Boc-Lys(Fmoc)-OH (**5**) with trifluoroacetic acid, followed by subjection to diazotransfer conditions (TfN_3, $CuSO_4$)[14] readily afforded (2*S*)-2-azido-6-(fluorenylmethyloxycarbonylamino)-hexanoic acid (**7**; Scheme 1). Standard Fmoc-based solid phase peptide synthesis (SPPS) afforded immobilized peptide **8** on acid-labile Wang resin (Scheme 2). Cleavage from the resin and solution-phase condensation of carboxylate **9** with leucine vinyl sulfone **14**[15] afforded target compound **2** that was purified by silica gel chromatography (58% overall yield).



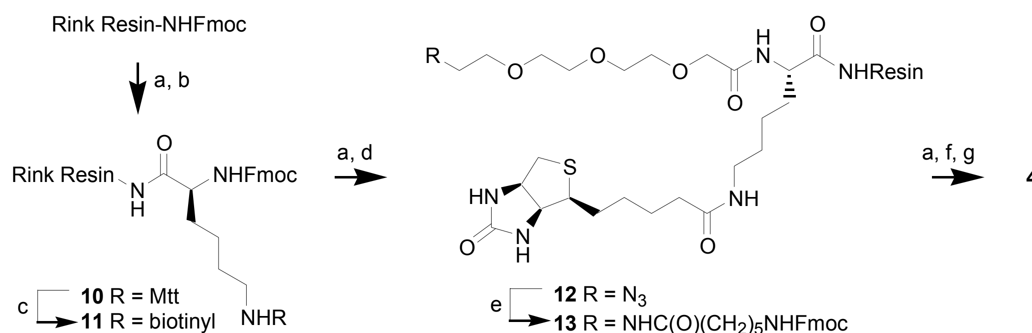
Scheme 1. Non-standard building blocks used in the synthesis of **2** and **4**. Reagents and conditions: a) 50% TFA/CH_2Cl_2, *92%*; b) TfN_3, $CuSO_4$, H_2O/MeOH, *89%*.

The synthesis of phosphane reagent **4** commenced with the condensation of Rink amide linker with Fmoc-Lys(Mtt)-OH (Scheme 3).[16] The side chain protective group in **10** was removed, followed by condensation with biotin to afford immobilized biocytin **11**. Standard solid phase peptide synthesis employing azido acid

15,[17] Fmoc-aminohexanoic acid and phosphane **16**, respectively, followed by acidic cleavage from the resin and HPLC purification gave the homogeneous target compound **4** in 16% yield.



Scheme 2. Synthesis of azide-containing proteasome inhibitor **2**. Reagents and conditions: a) Fmoc-Leu-OH, DIC, DMAP, CH_2Cl_2; b) Repeated cycles of SPPS: Fmoc cleavage: 20% piperidine in DMF; amino acid condensation: Fmoc protected amino acid, PyBOP, DiPEA, DMF. Fmoc protected amino acids building blocks employed in consecutive order: Fmoc-Leu-OH, Fmoc-Ahx-OH, Fmoc-Ahx-OH, **7**, adamantane acetic acid; c) 10% H_2O/TFA; d) HBTU, DiPEA, DMF, 58% overall yield.



Scheme 3. Synthesis of biotinylated phosphane reagent **4**. Reagents and conditions: a) 20% piperidine in DMF; b) Fmoc-Lys(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 Fmoc-Ahx-OH, PyBOP, DiPEA, DMF; f) **16**, EDC, HOBt, CH_2Cl_2; g) 50% TFA/CH_2Cl_2, 16% overall yield.

Inhibitory results

In order to establish the inhibition profile of **2**, a set of competition experiments was performed. The cell line used was 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, containing, in total, 6 distinct catalytically active β-subunits. The cell line HeLa, derived from a human cervical carcinoma, was also used. HeLa expresses only the constitutive proteasome (3 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 radio-iodinated peptide vinyl sulfone **3** (Figure 1), it was observed (Figure 3A) that labeling of the six individual subunits was abolished at final inhibitor concentrations of 10-30 μM, demonstrating **2** to be a proteasome inhibitor of equal potency as **1**.[7] The ability of peptide vinyl sulfone **2** to disable the proteasome in living cells

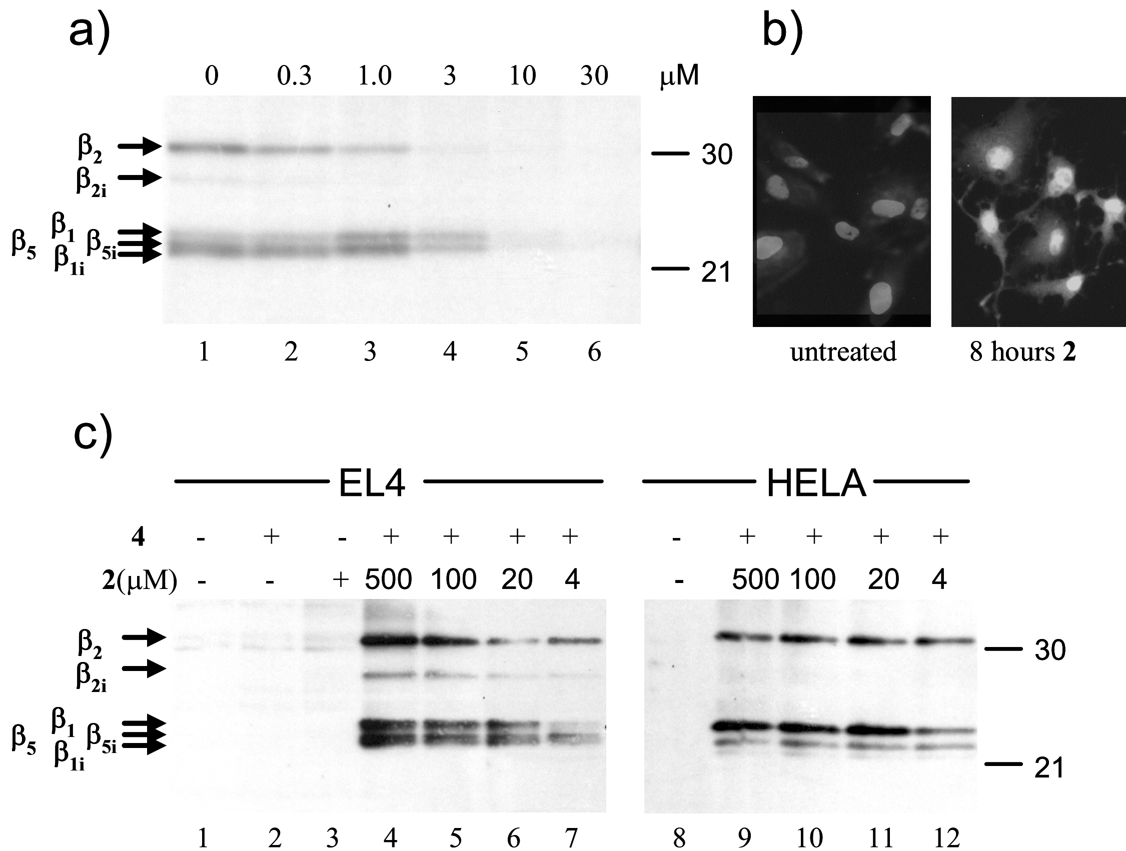


Figure 3. a) Cell lysate prepared from EL-4 cells was incubated with **2** at concentrations ranging from 0 to 30 μM. Residual unmodified subunits were labeled by subsequent incubation with radioiodinated inhibitor **3**. Labeled subunits were resolved by SDS-PAGE and visualized by autoradiography. b) Ub-R-GFP accumulates when proteasomal degradation is blocked. Cells were incubated either with a solvent control or **2** (50 μM final concentration) for 8 hours and fixed, followed by blue nuclear staining of the DNA with DAPI (blue). Confocal laser scanning microscopy revealed **2** to be a cell-permeable proteasome inhibitor. c) Lysates from EL-4 and HeLa were reacted with **2** at 37˚C for one hour and then boiled in the presence of SDS, to achieve protein denaturation and exposure of the azido moieties of conjugated **2**. The azido moieties were biotinylated *via* a Staudinger ligation by adding an aqueous solution of reagent **4** to the reaction mixture, followed by incubation for 1 h at 37˚C. 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.

was determined with the following procedure: U373 cells expressing a green fluorescent protein (GFP)-ubiquitin fusion protein[18] (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 with little green fluorescence at steady state as a result (Figure 3B). However, in cells treated with **2**, a time-dependent accumulation of fluorescence was observed, demonstrating the capacity of **2** to inactivate the proteasome in living cells.

Labeling of active proteasomes

Encouraged by these results, the suitability of a Staudinger ligation was established for 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 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 pattern of labeling conforms to that established for radio-iodinated probe **3**.[7] Importantly, proteasome derived polypeptides were detected only when both inhibitor **2** and Staudinger reagent **4** were used (Figure 3C, 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.

Next, the possibility of covalent proteasome inhibition in living cells followed by post-lysis Staudinger ligation and immunoblotting was investigated. EL-4 cells were incubated overnight with **2** (Figure 4). Subsequent glass bead lysis, incubation with **4**, ensuing SDS-PAGE separation and Western blotting afforded a labeling pattern virtually indistinguishable from

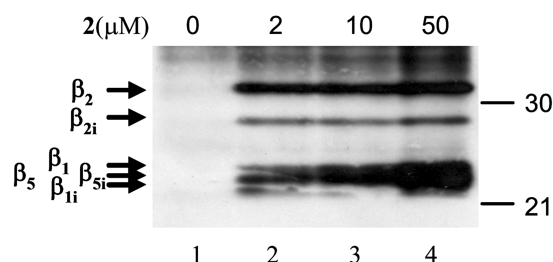


Figure 4. Proteasome labeling in living cells. Incubation of living cells (5 × 10^6 at 37°C) with **2** followed by post-lysis ligation and immunoblotting reveals the active proteasomal content and composition in living cells.

that obtained for labeling of cell lysates (Figure 3C). Importantly, *in vivo* labeling appeared to be more effective (compare Figure 4 with Figure 3C), indicating a more efficient targeting of all proteasomal subunits in living cells as compared to the labeling patterns obtained *in vitro*. The latter observation is possibly due to partial dissociation of the proteasome particle during cell lysis and storage. It was concluded that inhibitor **2** can be used in combination with biotinylation reagent **4** for the visualization of active proteasomes in living cells.

Conclusions

In summary, we have presented a novel strategy to visualize active enzymes in living cells. Compound **2** was identified as a powerful, cell-permeable inhibitor of all proteasomal activities, and **2** can subsequently be addressed by post-lysis labeling *via* a chemoselective Staudinger ligation. This protocol opens the way towards the screening, in living cells, of proteasomal activity in, for example, 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, malfunctioning of the ubiquitin-proteasome system has been implicated in both cancer[19] and neurodegeneration.[20]

Importantly, the two-step methodology (this is, covalent, irreversible enzyme modification followed by chemoselective modification) may be extended towards the development of novel chemoselective ligation partners compatible with desired cellular environments. The application of this strategy for the assessment of the activity of other protease enzyme activities in living cells is further elaborated in chapter 3.

Experimental section

General methods and materials: Solvents used in the solid phase peptide synthesis, DiPEA and TFA were all of peptide synthesis grade (Biosolve) and used as received. The protected amino acids, Wang resin (0.86 mmol/g), Rink amide MBHA resin (0.78 mmol/g) and PyBOP were obtained from NovaBiochem. Fmoc-Lys(Mtt)-OH was from Senn Chemicals, EDC and DIC from Acros and anhydrous HOBt from Neosystem. Adamantane acetic acid was purchased from Aldrich. Leucine vinyl sulfone was prepared as reported.[15] SPPS was carried out using a 180° Variable Rate Flask Shaker (St. John Associates, Inc.). LC/MS analysis was performed on a Jasco HPLC system (detection simultaneously at 214 and 254 nm) coupled to a Perkin Elmer Sciex API 165 mass

spectrometer equipped with a custom-made Electrospray Interface (ESI). An analytical Alltima C_{18} column (Alltech, 4.6 × 250 mm, 5 µm particle size) was used. Buffers: A = H_2O; B = CH_3CN; C = 0.5% aq. TFA. For RP HPLC-purification of **2** and **4** a Biocad "Vision" automated HPLC system (PerSeptive Biosystems, Inc.) was used. ^{1}H-NMR and ^{13}C-NMR spectra were recorded with a Bruker AC200 instrument at 200 and 50.1 MHz, respectively.

Solid phase peptide synthesis: Where appropriate removal of the Fmoc protecting group was accomplished by treatment of the resin-bound peptide with 20% (v/v) piperidine in DMF for 20 min. Peptide coupling steps were performed by treatment of the resin with a premixed (5 min) solution of the of the appropriate acid (5 eq.), PyBOP (5 eq.) and DiPEA (6 eq.) in DMF for 1 h. Coupling efficiencies were monitored with the Kaiser test and couplings were repeated if necessary. After coupling and deprotecting steps the resin was washed with DMF (5×).

Synthesis of Fmoc-Ahx(α-N_3)-OH (7): Boc-Lys(Fmoc)-OH (2.0 g, 4.2 mmol) was treated with TFA/CH_2Cl_2 (20 ml, 1/1 v/v) for 40 minutes. Solvents were removed *in vacuo* yielding 1.9 g (3.9 mmol, 92%) of crude TFA-salt of H-Lys(Fmoc)-OH **6**. The crude product was treated with trifluoromethanesulfonyl azide as described[14] followed by a modified work-up procedure. After removal of the organic solvents, the aqueous slurry was acidified with 1 N HCl to pH 2 and extracted with EtOAc (4×). The combined organic phases were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification of the crude product over silica gel (hexanes/EtOAc 2/1 v/v) yielded 1.4 g (3.5 mmol, 89%) of a white foam. ^{1}H NMR (200 MHz, $CDCl_3$, 25°C, TMS): δ= 7.75 (d, ^3J (H,H)=7.3 Hz, 2H; ArH), 7.57 (d, ^3J (H,H)=7.3 Hz, 2H; ArH), 7.40-7.25 (m, 4H; ArH), 4.41 (d, ^3J (H,H)=6.6 Hz, 2H; CHC*H*$_2$O), 4.19 (t, ^3J (H,H)=6.6 Hz, 1H; C*H*CH_2O), 3.92 (t, ^3J (H,H)=6.7 Hz, 1H; αCH), 3.18 (m, 2H; εCH_2), 1.7-1.3 ppm (m, 6H; βCH_2, γCH_2, $\delta$$CH_2$); ^{13}C NMR (50.1 MHz, $CDCl_3$, 25°C, TMS): δ=174.1, 156.8, 143.5, 140.9, 127.1, 126.7, 124.6, 119.6, 66.4, 62.1, 60.4, 46.7, 30.6, 28.8, 22.4 ppm; ESI-MS: *m/z* (%) = 417.2 (100) [M+Na^+], 811.5 (20) [2M+Na^+].

Synthesis of 2: Wang resin (1.0 g, 0.86 mmol) was condensed with Fmoc-Leu-OH (1.2 g, 3.4 mmol) in CH_2Cl_2 (25 ml) under the agency of DIC (0.54 ml, 3.44 mmol) and a catalytic amount of 4-(dimethylamino)pyridine for 2 h. The resin was filtered off, washed (3× CH_2Cl_2-MeOH, CH_2Cl_2 and Et_2O), and air-dried. Loading was determined by quantification (UV) of Fmoc cleavage, and proved to be 0.66 mmol/g. The resin, 260 mg (0.17 mmol) was elongated using standard Fmoc-based SPPS to give resin-bound AdaAhx(α-N_3)Ahx_2Leu_2-OH. Treatment of the immobilized peptide with TFA/H_2O (95/5 v/v) for 1 h, filtration and removal of solvent *in vacuo* was followed by a solution phase block coupling with leucine vinyl sulfone TFA salt **14** (1 eq.) under the agency of HBTU (1 eq.) and DiPEA (2.2 eq.) in DMF (1.5 ml). After evaporation of the solvent, the residue was dissolved in EtOAc. The product was precipitated by sonication. The precipitate was filtered and washed with EtOAc, Et_2O and hexanes to yield 146 mg (0.15 mmol, 88%) of the title

compound in 90% purity as judged by LMCS. Silica gel purification (0-10% MeOH in EtOAc) of 21 mg (22 μmol) of the crude product yielded 14 mg (14 μmol) of the title compound (58% overall yield) ready to use in biological experiments. LC/MS: m/z = 974.9 [$M+H^+$].

Synthesis of 4: Fmoc Rink amide resin (128 mg, 100 μmol) was deprotected as described. Fmoc-Lys(Mtt)-OH (0.5 mmol, 312 mg) was coupled. The Mtt protecting group was removed by treatment of the resin with 1% TFA in CH_2Cl_2 for 0.5 min.[16] This treatment was repeated 9 times until no more yellow color was observed in the eluted solution. The resin was neutralized with 10% DiPEA in DMF. Biotin (0.5 mmol, 122 mg) was coupled under the agency of PyBOP (1 eq.) and DiPEA (1.2 eq.) in DMF. After removal of the Fmoc group, the resulting free amine was condensed with **15**[17] (0.5 mmol, 237 mg). After washing of the immobilized peptide with dioxane (3×), the azide moiety was reduced by treatment with Me_3P (0.6 ml of a 1 M solution in toluene, 0.6 mmol, 6 eq.) in dioxane/water (2 ml, 4/1 v/v) for 40 min[14] followed by dioxane washes (3×), and Fmoc-Ahx-OH (0.5 mmol, 180 mg) was coupled. Of the obtained resin, 50 μmol was treated with piperidine as described above to remove the Fmoc protecting group and the resin was then washed with CH_2Cl_2 (3×). Phosphane **16** (90 mg, 0.25 mmol) was activated with EDC (48 mg, 0.25 mmol) and HOBt (41 mg, 0.3 mmol) in CH_2Cl_2 (2 ml) under an argon atmosphere for 5 min, and subsequently added to the resin. The resin was agitated under argon atmosphere for 1 h, and the resin was washed (CH_2Cl_2, then DMF-MeOH alternating (3×), CH_2Cl_2-MeOH alternating (3×), and CH_2Cl_2), while keeping the resin under argon atmosphere. The immobilized peptide was liberated from the resin by treatment with TFA/CH_2Cl_2 (1/1 v/v) for 1 h. Evaporation of the solvents *in vacuo* followed by HPLC-purification of the crude product (linear gradient in B: 25-55% B in 3 column volumes) yielded 8.0 mg (8 μmol, 16%) of a white solid. LC/MS: calculated = 1020 [$M+H^+$], found = 1020 [$M+H^+$]. The product was kept as aliquots of a stock solution (1.6 mM) in degassed DMSO at –80°C.

Proteasome labeling in living cells: EL-4 cells, cultured in RPMI (Gibco, Invitrogen Corp.) supplemented with L-glutamine, fetal calf serum, penicillin and streptomycin were incubated overnight with concentrations of inhibitor **2** as indicated in Figure 3. Cells were harvested by centrifugation. After glass-bead lysis, 10 μg of protein in 33 μl of lysis buffer (50 mM Tris, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.25 mM Sucrose, pH 7.4) was denatured by the addition of 2 μl of 20% SDS followed by brief boiling. The denatured sample was further incubated with 10 μl of **4** (100 μM) in DMSO/CH_3CN/H_2O (6/1/1 v/v/v) for 1 h at 37°C. Without further heating 4× sample buffer was added and samples were run on either a 12.5% or 15% SDS-PAGE gel followed by transfer to PVDF membrane. The membrane was blocked with milk and washed briefly with PBS containing 1% TWEEN-20 (Sigma) (3×) and incubated with streptavidin-HRP (Amersham Life Sciences,

dilution 1: 10.000) for 1 h at ambient temperature. The membrane was washed as before and soaked in Western Lightning Chemiluminescence (Perkin Elmer) reagent followed by developing a film.

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