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

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Chapter 3

A cell-permeable inhibitor and activity based probe for the caspase-like activity of the proteasome[1]

Introduction

The proteasome is a multi-catalytic protease that degrades the majority of cytosolic and nuclear proteins to oligopeptides.[2] This degradation is a highly regulated process, and a small portion of the oligopeptides produced escape further degradation and are loaded on MHC class I molecules for presentation to the immune system.[3] Proteasomal protein degradation takes place in a barrel shaped structure that consists of four rings of seven subunits each. Three of the β subunits in the middle two rings, β1, β2 and β5, display protease activity. Each catalytic subunit has a different substrate specificity. The β1 subunit cleaves preferentially after acidic residues and is therefore referred to as the caspase-like subunit. The β2 subunit has a preference for peptide bond cleavage after basic residues and is responsible for the trypsin-like activity. The β5 subunit displays a chymotrypsin-like activity and cleaves after large, hydrophobic residues. In the interferon-γ-inducible immunoproteasome, the catalytic β1, β2 and β5 subunits are replaced by the β1i, β2i and

β5i immunosubunits. The immunosubunits display a substrate specificity slightly different from to that of the respective constitutive subunits. As a result, the immunoproteasome and proteasome generate a different spectrum of oligopeptides from the same protein, which effects a different spectrum of antigens presented on MHC class I molecules.[4]

Inhibitor design

To gain more insight in the role of the different proteasomal subunits in the process of protein degradation and antigen presentation, it would be highly useful to have tools to switch off one of the proteasome activities selectively. Some inhibitors with selectivity for either the trypsin-like or the chymotrypsin-like subunits have been reported.[5] In contrast, no cell-permeable selective inhibitor or activity based probe for the caspase-like proteasomal subunits has been reported to date. In order to develop such tools, precedents from the literature were used to select an appropriate recognition sequence and electrophilic trap. In this respect, it appeared that the selective reversible inhibitor **1** (Figure 1) of the caspase-like subunit of the proteasome was a suitable starting point for the development of an irreversible inhibitor.[6] Furthermore, it has been shown before that vinyl sulfones are suitable electrophilic traps to alkylate the N-terminal threonine residue, the active site nucleophile of the proteasome. The ability of vinyl sulfones to alkylate proteasomal catalytic residues depends on the functional group downstream of the vinyl sulfone moiety. For example, Bogyo and coworkers showed that in case of GL_3-vinyl sulfone, phenolic vinyl sulfone (**2**; Figure 1) was a better inhibitor of the caspase-like site than the corresponding methyl vinyl sulfone (**3**; Figure 1).[7] Thus, a phenolic vinyl sulfone seemed to be the obvious electrophilic trap to be employed.

To avoid the elaborate synthesis of a suitably protected aspartic acid vinyl sulfone moiety that would be required

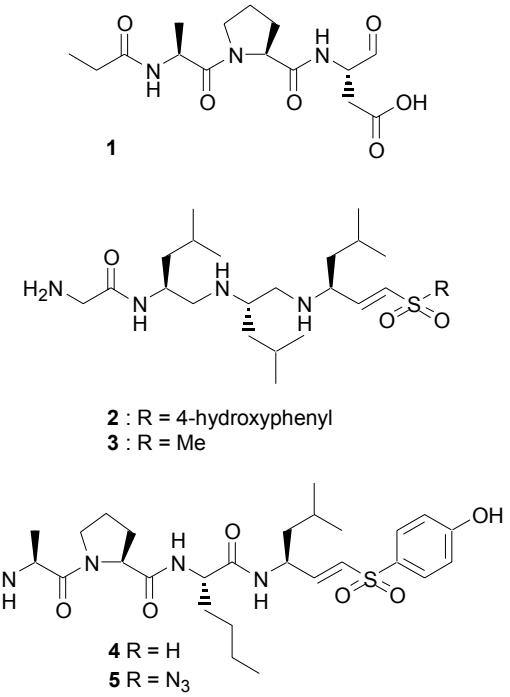


Figure 1. Relevant reversible and irreversible proteasome inhibitors.

for block coupling of the peptide vinyl sulfone building block to a peptide sequence, the P1 aspartic acid was replaced by leucine, thus generating inhibitor **4** (Figure 1). It is known that the caspase-like subunit also cleaves after hydrophobic branched chain amino acids,[6,8,9,10] and this concept has been used in irreversible inhibitor design. An epoxyketone based inhibitor with leucine in the P1 position can inhibit the caspase-like subunit with significant, albeit not absolute, specificity.[11] Furthermore, the leucine residue renders the inhibitor more apolar and thus possibly better cell-permeable.

In order to convert inhibitor **4** into an activity probe for the caspase-like subunit of the proteasome, an azide moiety was introduced on the N-terminal acetyl function to give **4** (Figure 1). In Chapter 2 of this thesis, an approach employing the Staudinger ligation to biotinylate proteasome modified with an azide-containing probe in a two-step labeling fashion is described. Based on literature evidence,[12] direct attachment of a biotin moiety to the probe was expected to influence both cell permeability and subunit specificity.

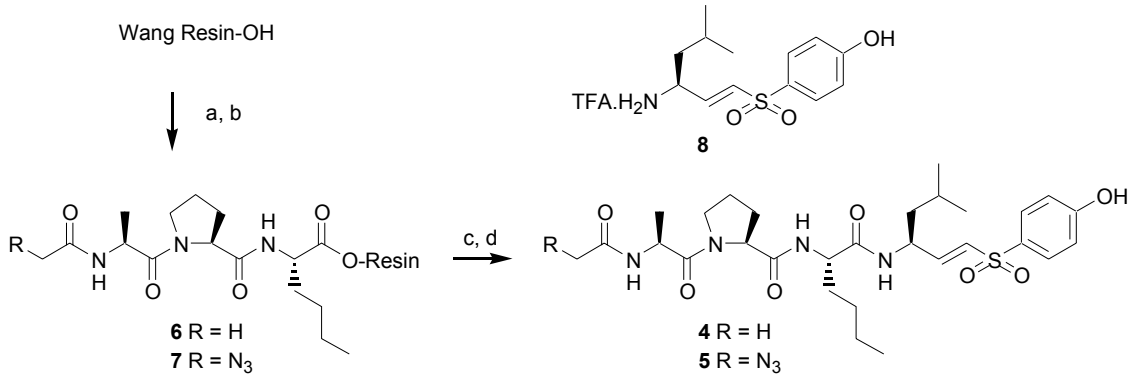
Here, the first highly specific, cell-permeable inhibitors of the caspase-like activities of the proteasome (**4** and **5**; Figure 1) are presented. At low concentrations, these compounds show selectivity towards the caspase-like subunit of the immunoproteasome in favour of the corresponding caspase-like activity of the constitutive proteasome. Activity probe **5** allows two step labeling of active caspase-like subunits of the immunoproteasome and, at higher concentrations, of the constitutive proteasome. Labeled subunits can then be visualized *via* post-lysis ligation and Western blotting.

Synthesis

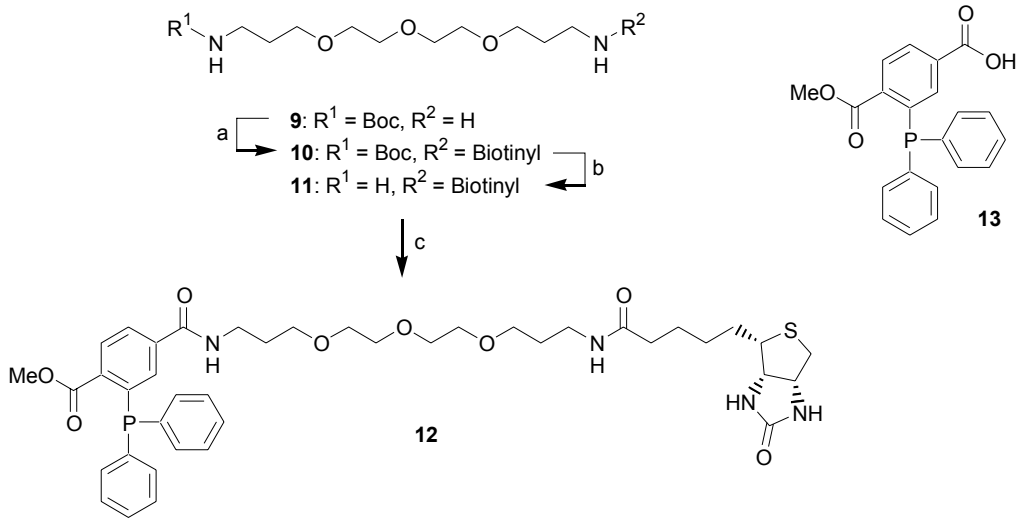
The synthesis of inhibitor **4** started with the synthesis of tripeptide **6** on acid-labile Wang resin (Scheme 1). Acidolysis of the peptide from the resin and subsequent solution phase condensation with vinyl sulfone **8**[7] afforded the target compound in 45% overall yield after RP-HPLC purification. The synthesis of activity probe **5** was accomplished in a similar fashion. Azide-functionalized peptide **7** was synthesized on Wang resin. Cleavage of the peptide from the solid support and coupling to **8** gave target compound **5** in 12% yield after RP-HPLC purification.

For the Staudinger ligation, new biotinylation agent **12** (Scheme 2) was designed. The relatively facile synthesis of **12**, as well as the possibility of purification using silica gel chromatography, allow for production of larger amounts than the comparable reagent

presented in Chapter 3. The synthesis starts with biotinylation of monoprotected bisamine **9** using a standard peptide coupling method. After removal of the Boc protecting group with 5% water in TFA, the liberated amine functionality was acylated with phosphane **13**.[13] Both the use of the EDC/HOBt system to activate the acid moiety and a protective argon atmosphere proved necessary to suppress premature oxidation of phosphane **12** to the corresponding phosphane oxide.



Scheme 1. Synthesis of inhibitor 4 and activity probe **5**. Reagents and conditions: a) Fmoc-Nle-OH, DIC, DMAP, CH_2Cl_2; b) Repeated cycles of SPPS: Fmoc cleavage: 20% piperidine in DMF; amino acid condensation: Fmoc protected amino acid, HCTU, DiPEA, NMP. Fmoc protected amino acid building blocks employed in consecutive order: Fmoc-Pro-OH, Fmoc-Ala-OH, acetic anhydride or azidoacetic acid; c) TFA/H_2O (95/5 v/v); d) **8**, HATU, DiPEA, DMF, overall yield 18% and 26% for **4** and **5** respectively.



Scheme 2. Synthesis of Staudinger-reagent **12**. Reagents and conditions: a) biotin, HCTU, DiPEA, DMF; b) TFA/H_2O (95/5 v/v), 56% yield over two steps; c) **13**, HOBt, EDC, DiPEA, CH_2Cl_2, argon atmosphere, 22% yield.

Evaluation of inhibitory activity

The subunit specificity of newly synthesized inhibitor **4** was determined as follows. Purified rabbit muscle 26S proteasome was incubated in the presence or absence of 50 μM inhibitor, and aliquots of the mixture were assayed with fluorogenic substrates for the respective active site. The effect of the inhibitor on the proteasomal activity was determined by relative fluorescence in the presence or absence of inhibitor.[14] Figure 2 shows that **4** selectively blocks the caspase-like site of the proteasome. Furthermore, an increased activity of the trypsin-like site is observed.

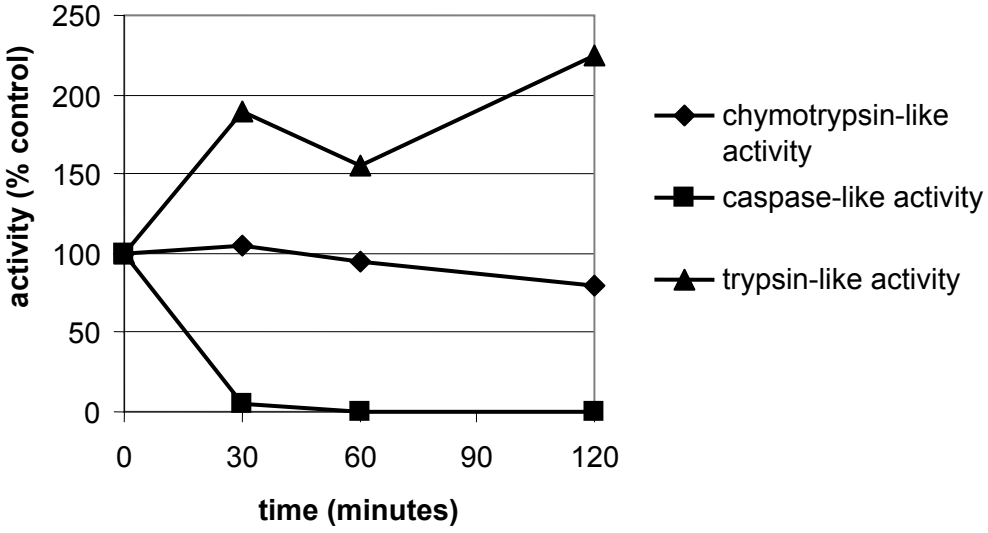


Figure 2. Protease assay with purified proteasome shows specificity of **4** for the caspase-like site of the proteasome. Proteasome was incubated with 50 μM inhibitor and protease activity was measured by the amount of fluorescence generated by degradation of 7-amido-4-methylcoumarin (amc) substrates for the three proteasomal subunits. Suc-LLVY-amc was used for the chymotrypsin-like site, Ac-nLPnLD-amc for the caspase-like site and Ac-RLR-amc for the trypsin-like site.

Next, the effects of compound **4** and **5** on proteasomes in cultured cells were measured. Mouse T-cell lymphoma EL-4 cells, human multiple myelome RMPI8226 cells and human immortalized B-lymphocytes (LG2 clone) were incubated for 16 h with different concentrations of inhibitors **4** and **5**. Cytosol was squeezed out of digitonin-permeabilized cells by centrifugation, and the activity of all three types of active sites was determined by a fluorogenic substrate assay (Figure 3).[15] Results of experiments with **4** in

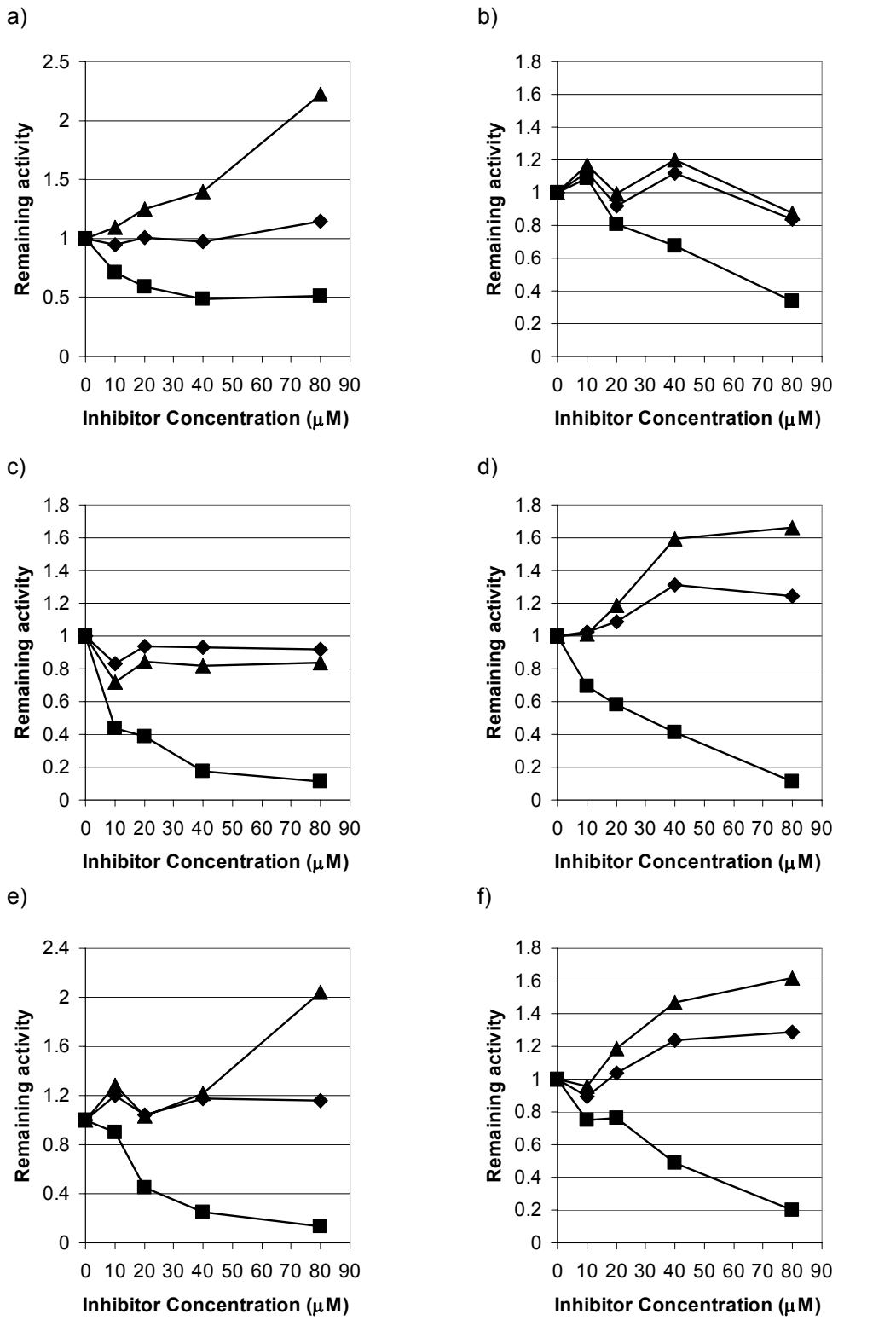


Figure 3. Protease activity measured in cell extracts after treatment with inhibitor **4** (a,c,e) or **5** (b,d,f). EL-4 cells (a,b), RPMI 8226 cells (c,d) and LG2 cells (e,f) were incubated 16-18 h with a concentration range of **4** or **5**. Cells were lysed, and remaining proteasomal activity was addressed by performing a protease assay.

EL-4 cells (Figure 3a) show a plateau at 50% inhibition of the caspase-like activity. As EL-4 cells express both proteasome and immunoproteasome in equal amounts, this could indicate that **4** targets only one of the caspase-like sites. Also, the trypsin-like activity is increased over two-fold, indicating allosteric activation or an increase in proteasome expression due to inhibition of the caspase-like site. In RMPI cells, a plateau in caspase-like activity occurs between 10 and 20 μM **4** (Figure 3c), and at higher concentrations all caspase-like subunits can be blocked. In this cell type, no activation of the trypsin-like site is observed. Inhibitor **5** is less potent in these cells and does not show a plateau (Figure 3d), but some activation of the other subunits is observed. In LG2 cells (Figure 3e and 3f), inhibitor **4** shows a plateau around 10 μM, and at higher concentrations activation of the trypsin-like activity occurs. Azide-containing inhibitor **5** shows a plateau at higher concentrations, and less activation of the other subunits.

Labeling of caspase-like subunits

In order to visualize the subunits that are modified by inhibitor **5**, the azide moieties of labeled proteasomal subunits were modified with a biotin moiety by Staudinger ligation with biotinylated phosphane **12**. In short, cells were incubated for 16 h with different concentrations of **5**, permeabilized with digitonin, and cytosol was squeezed out by centrifugation, followed by extraction of organellar proteins by sonication. Both extracts were treated with phosphane **12** to introduce a biotin moiety, separated on SDS-PAGE and transferred onto PVDF membranes. Biotinylated proteins were visualized using a streptavidin-IRdye 800CW conjugate (Figure 4). In all cell lines, at lower concentration only β1i is modified, whereas at higher concentration of **5**, both caspase-like subunits (β1 and β1i) are targeted. The lack of labeling of the other proteasomal subunits confirms the specificity for **5** for the caspase-like site as found in the fluorogenic peptidase assays described above.

Because the proteasomal subunits are not always resolved on SDS-PAGE, a 2D separation was performed, which is known to separate all catalytic active proteasomal subunits. Cells were incubated with 40 μM and 80 μM of inhibitor **5**. Cytosolic extracts were prepared as described above, incubated with phosphane **12** and separated on a 2D gel (Figure 5). At both concentrations, the caspase-like subunit of the immunoproteasome is labeled exclusively.

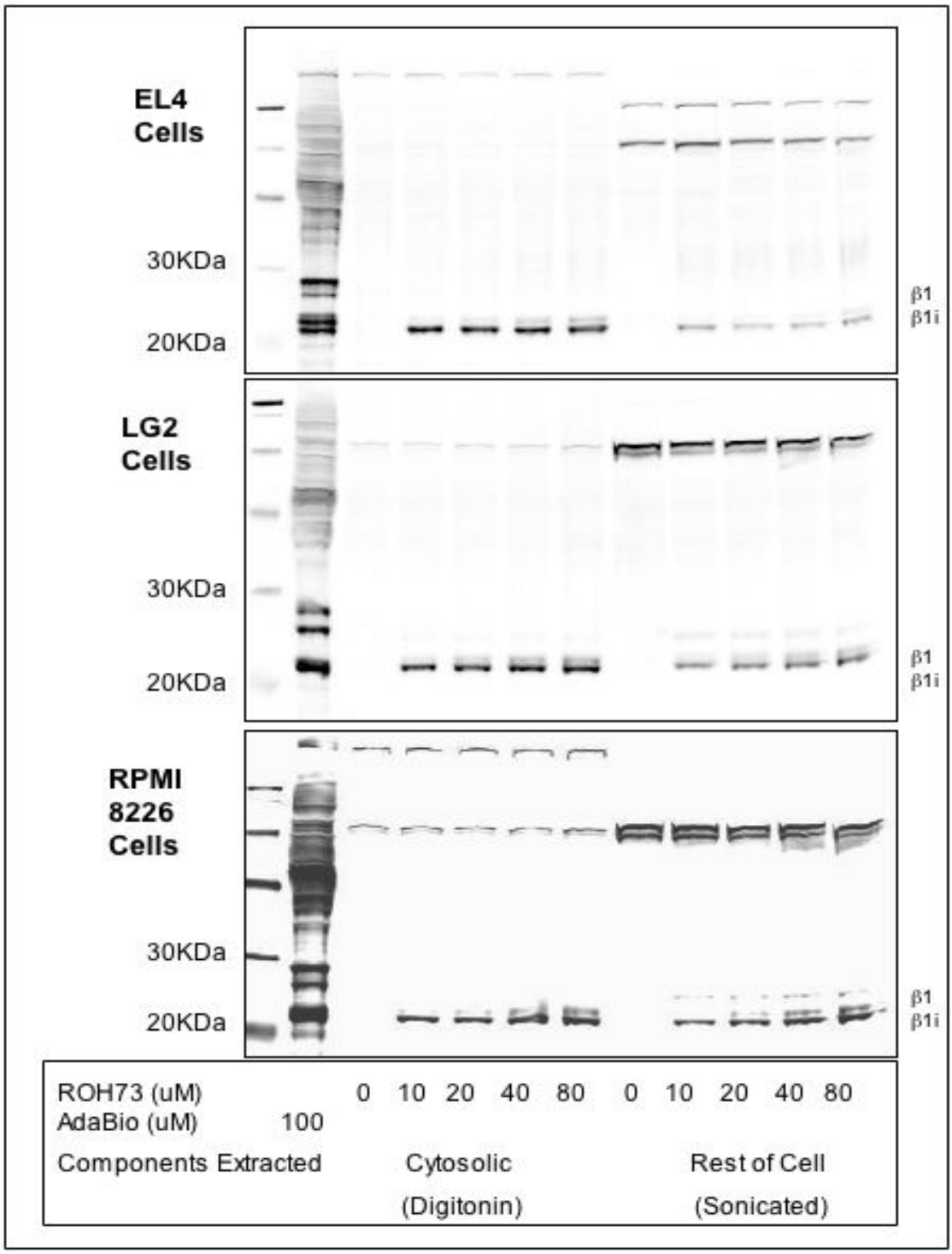


Figure 4. Two-step visualization of targets of compound **5**. EL-4 cells, RPMI 8226 cells and LG2 cells were incubated 16-18 h with different concentrations of **5**. Cells were permeabilized with digitonin, allowing separation of the cytosolic fraction from the organellar fraction. Both were treated with biotinylated phosphane **12**, followed by fractionation on SDS-PAGE and Western blotting. Labeled proteins were visualized by streptavidin-IRdye800CW.

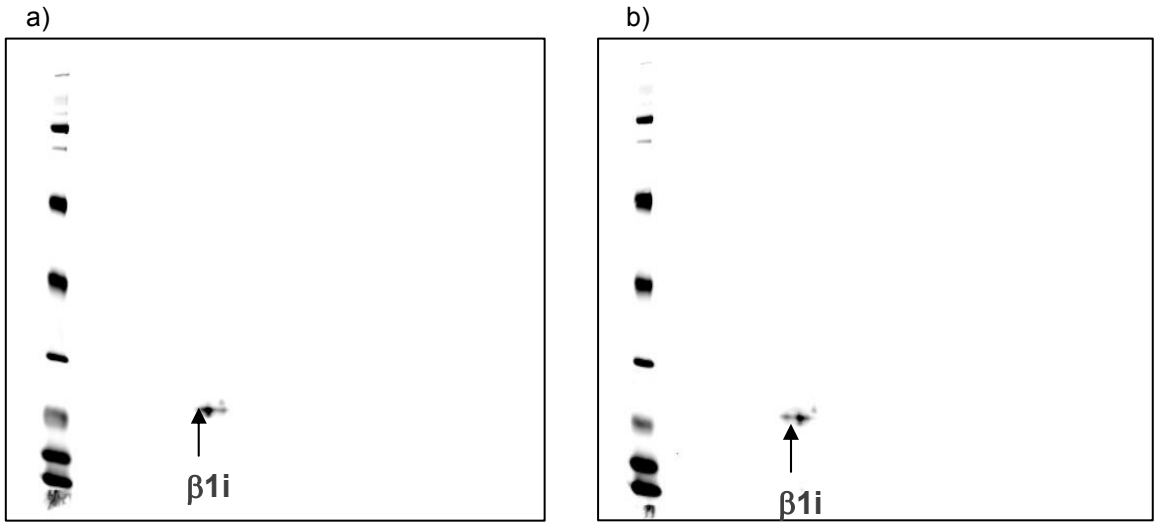


Figure 5. 2D SDS-PAGE of cytosilic extracts of labeled EL-4 cells treated with 10 μM (a) and 80 μM (b) **5**. Extracts were generated and treated as before, and separated by 2D gel electrophoresis. Proteins were stained with streptavidin-IRdye800CW indicating that only the caspase-like activities of the immunoporteasome (β1i) are labeled.

Conclusions

Two new vinyl sulfone based proteasome inhibitors are presented here that are selective inhibitors of the caspase-like subunits of the proteasome. The uniqueness of these reagents is that at lower concentrations they selectively inhibit the caspase-like sites of the immunoproteasomes. The introduction of an azide-moiety did not change the inhibition profile, and confirmed selective targeting of the caspase-like subunits by visualization *via* a Staudinger ligation followed by and SDS-PAGE and Western blotting.

Both inhibitors presented here, as well as the Staudinger ligation protocol applied, might be of value for future research aimed at the role of the caspase-like subunit in the processing of antigens. Furthermore, at lower concentration of inhibitor, the specific contribution of the immunoproteasomal caspase-like site in antigen processing can be studied.

Experimental section

General methods and materials: Solvents used in the solid phase peptide synthesis, DiPEA and TFA were all peptide synthesis grade (Biosolve) and used as received. The protected amino acids, Wang resin (0.86 mmol/g), and HCTU were obtained from NovaBiochem. 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). High resolution mass spectra were recorded on a Finnigan LTQ-FT (Thermo electron).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-purifications a Biocad "Vision" automated HPLC system (PerSeptive Biosystems, Inc.) was used. The applied buffers were A, B and C. ^{1}H-NMR and ^{13}C-NMR spectra were recorded with a Bruker AC200 instrument at 200 and 50.1 MHz, respectively. 600 MHz ^{1}H-NMR spectra were recorded with a Bruker DMX 600 instrument with chemical shifts (δ) relative to tetramethylsilane. TLC analysis was performed on Merck 25DC Plastikfolien Kieselgel 60 F_{254}, with detection by UV absorption (254 nm) and spraying with $KMnO_4$ (10 g/l in 2% aq Na_2CO_3). Fluka silica gel (230-400 mesh) was used for column chromatography.

(*S*)-4-(3-Amino-5-methyl-hex-1-ene-1-sulfonyl)-phenol (8): To a solution of KOtBu (1 g, 8.94 mmol) and (4-hydroxy-benzenesulfonylmethyl) phosphonic acid diethyl ester (1.5 g, 4.87 mmol) in THF was added known Boc-protected leucinal[16] (0.92 g, 4.1 mmol). The mixture was stirred for 16 h, the mixture was diluted with Et_2O, washed with sat. aq. $NaHCO_3$ (3×) and sat. aq. NaCl (3×), dried ($MgSO_4$), filtered and concentrated *in vacuo* yielding 1.3 g of crude Boc protected title compound. The Boc protecting group was subsequently removed by treatment with TFA/H_2O (1 ml, 95/5 v/v) for 1 h. The reaction mixture was poured into Et_2O (50 ml), and the precipitate collected yielding 0.45 g, (1.2 mmol, 29% over two steps) of the title compound, which was used without further purification.

N_3AcAlaProNle-OH: Wang Resin (2.0 g, 1.7 mmol) was condensed with Fmoc-Nle-OH (4.2 g, 12 mmol) in CH_2Cl_2 (30 ml) under the agency of DIC (1.8 ml, 12 mmol) and about a spatula tip of 4-(dimethylamino)pyridine for 16 h. The resin was filtered off and washed (DMF 3×, CH_2Cl_2 3×, Et_2O 3×) and air-dried. Loading was determined by quantification (UV) of Fmoc cleavage, and proved to be 0.77 mmol/g. The resin (130 mg, 100 μmol) was elongated using standard Fmoc-based SPPS to give resin-bound N_3AcAlaProNle-OH. Where appropriate removal of the Fmoc protecting group was accomplished by treatment of the resin-bound peptide with piperidine/DMF (1/4 v/v) for 20 min. Peptide coupling steps were performed by treatment of the resin with a premixed (5 min) solution of the appropriate acid (5 eq.), HCTU (5 eq.) and DiPEA (6 eq.) in NMP (5 ml) for 1 h unless stated otherwise. Coupling efficiencies were monitored with the Kaiser test and couplings were repeated if necessary. After coupling and deprotection steps the resin was washed with DMF (5×). After the last coupling step, the resin was washed extensively (alternating CH_2Cl_2-MeOH 3×, alternating CH_2Cl_2-Et_2O 3×). The resin was treated with TFA/H_2O (2 ml, 95/5 v/v) for 2 h, the resin was filtered into toluene, the mixture was filtered and the resin washed with TFA (2× 1 ml). The

filtrate was diluted (toluene, 10 ml) and concentrated *in vacuo*. The crude product was coevaporated (toluene, 3×).

AcAlaProNleLeuVSOH (4): Crude AcAlaProNle-OH (100 µmol, prepared *via* in the same method as has been described above for N_3AcAlaProNle-OH) was coupled with the TFA-salt of phenolic leucine vinyl sulfone and purified as described for N_3AcAlaProNleLeuVSOH to give 13.6 mg (23 µmol, 45%) of the title compound. ^{1}H NMR (DMSO, 300 K, DMX 600): δ, 10.58 (s, 1H), 8.39 (d, 1H, *J* = 7.4 Hz), 8.36 (d, 1H, *J* = 7.3 Hz), 7.92 (d, 1H, *J* = 5.2 Hz), 7.83-7.80 (m, 2H), 7.60 (d, 2H, *J* = 8.7 Hz), 6.92 (d, 2H, *J* = 8.7 Hz), 6.73-6.48 (m, 2H), 4.59-4.47 (m, 2H), 4.29-4.26 (m, 1H), 4.07-4.02 (m, 1H), 2.03-1.95 (m, 1H), 1.90-1.33 (m, 9H), 1.23-1.10 (m, 14H), 0.85-0.79 (m, 7H). HRMS: $C_{29}H_{44}N_4O_7S + H^+$ requires 593.3004, found 593.2995.

N_3AcAlaProNleLeuVSOH (5): Crude N_3AcAlaProNle-OH (100 µmol), HATU (38 mg, 0.1 mmol) and DiPEA (0.2 ml, 0.12 mmol) were stirred for 5 min in DMF. The TFA-salt of phenolic leucine vinyl sulfone (46 mg, 0.12 mmol) and DiPEA (0.3 ml, 0.18 mmol) were added and the mixture was stirred for 16 h. The reaction mixture was concentrated in vacuo, the residue was dissolved in EtOAc and was washed with 1N HCl, sat. aq. $NaHCO_3$ and sat. aq. NaCl. The organic layer was dried with $MgSO_4$ and concentrated *in vacuo*. The product was purified to homogeneity by RP-HPLC (linear gradient of 40-50% B in 3 column volumes) to yield 7.43 mg (12 µmol, 12%) of the title compound. ^{1}H NMR (DMSO, 300 K, DMX 600): δ, 10.58 (s, 1H), 8.13 (d, 1H, *J* = 7.5 Hz), 7.79 (d, 1H, *J* = 7.6 Hz), 7.77 (d, 1H, *J* = 8.7 Hz), 7.60 (d, 2H, *J* = 8.7 Hz), 6.92 (d, 2H, *J* = 8.7 Hz), 6.69 (dd, 1H, *J* = 15.0 Hz, *J* = 4.9 Hz), 6.55 (dd, 1H, *J* = 15.0 Hz, *J* = 1.3 Hz), 4.56-4.48 (m, 1H), 4.27-4.22 (m, 1H), 4.07-4.02 (m, 1H), 3.61-3.50 (m, 2H), 2.04-1.94 (m, 1H), 1.88-1.72 (m, 4H), 1.63-1.30 (m, 5H), 1.24-1.09 (m, 14H), 0.86-0.77 (m, 7H). HRMS: $C_{29}H_{43}N_7O_7S + H^+$ requires 633.2928, found 633.2935.

***Tert*-butyl (4,7,10-trioxa-13-(biotinylamino)tridecyl) carbamate (10)**: Biotin (488 mg, 2.2 mmol) and HCTU (826 mg, 2 mmol) were dissolved in DMF (5 ml) and DiPEA (0.40 ml, 2.4 mmol) was added, upon which the reaction mixture turned brown. (1-(tert-butyloxycarbonyl-amino)-4,7,10-trioxa-13-tridecanamine) **9** (704 mg, 2.2 mmol) was added slowly and the reaction mixture was stirred and turned red. After 2 h, TLC-analysis ($CHCl_3$/CH_3OH 5/1 v/v) indicated a completed reaction. The mixture was concentrated *in vacuo*, the residue was taken up in CH_2Cl_2 and washed (1N HCl 2×, saturated $NaHCO_3$), dried ($MgSO_4$) and filtered. The filtrate was concentrated *in vacuo* to yield 1.3 g of a brown oil, which was used without further purification.

4,7,10-Trioxa-13-(biotinylamino)tridecylammonium trifluoroacetate (11): Crude **10** (1.3 g, 2.0 mmol) was stirred in TFA (2 ml) with water (1 drop). After 1 h, TLC-analysis ($CHCl_3$/CH_3OH 5/1 v/v) indicated a completed reaction. The mixture was concentrated *in vacuo*, and coevaporated (3×

toluene and 3× methanol). The residue was taken up in 3 ml methanol and poured into Et_2O (200 ml). Slowly, a syrup formed on the surface of the flask. The mother liquor was decanted and the oil (750 mg, 56% over two steps) was collected.

Methyl-2-(diphenylphosphino)-4-(4,7,10-trioxa-13-(biotinylamino)tridecylamino)-carbonyl)benzoic acid (12): Crude **11** (375 mg, 0.56 mmol) was treated with a preactivated solution of 2-(diphenylphosphino)-4-(methoxycarbonyl)benzoic acid (236 mg, 0.65 mmol), HOBt (88 mg, 0.65 mmol), EDC (128 mg, 0.67 mmol) in CH_2Cl_2 under argon atmosphere in the presence of DiPEA (0.1 ml, 0.67 mmol). The mixture was stirred for 16 hour under argon atmosphere and LC/MS analysis indicated a completed reaction. The reaction mixture was diluted (CH_2Cl_2) and washed (1N HCl 2×, saturated $NaHCO_3$ 2×), dried ($MgSO_4$) and filtered. The filtrate was concentrated *in vacuo* and the residue was purified (SiO_2; CH_2Cl_2/CH_3OH 16/1 v/v) to yield a yellow syrup (112 mg, 0.14 mmol, 22%). The product was taken up in CH_3CN/CH_3OH (1/1 v/v) and lyophilised into 5.4 mg aliquots that were stored at -20°C under argon atmosphere. LC/MS-analysis (10-90% B, rt=13.79 min) indicated that the purity of the product was 88%. $[M+H]^+$ = 793.5, $[M+2H]^{2+}$ = 397.3. ^{1}H NMR (200 MHz, CD_3OD): δ 7.95 (dd, *J*=3.3, 8.0, 1H), 7.67 (dd, *J*=1.7, 8.2, 1H), 7.32 (dd, 1.5, 4.0, 1H), 7.3-7.1 (m, 10H), 4.36 (dd, *J*=4.0, 8.0, 1H), 4.16 (dd, 4.4, 7.7, 1H), 3.58 (s, 3H), 3.5-3.0 (m, 18H), 2.80 (dd, *J*=4.7, 12.8, 1H), 2.58 (d, 12.8 Hz, 1H), 2.07 (t, *J*=7.1, 2H), 1.7-1.3 (m, 10H). ^{13}C NMR (50.1 MHz, CD_3OD): δ, 175.8, 169.0, 168.1, 165.9, 142.6, 142.0, 139.0, 138.6, 138.4, 137.8, 135.2, 134.8, 134.4, 131.6, 130.1, 129.8, 129.6, 127.7, 71.4, 71.2, 69.9, 69.8, 63.2, 61.5, 56.9, 52.7, 41.1, 38.7, 36.8, 30.4, 30.2, 29.7, 29.4, 26.8. ^{31}P NMR (50.1 MHz, CD_3OD): δ, -2.72. HRMS: $C_{41}H_{53}N_4O_8PS + H^+$ requires 793.3395, found 793.3384.

Continuous assay of peptidase activities on a fluorescent plate reader: An assay of proteasome activity was performed on a 96-well plate. The ssay mixture was prepared by mixing 100 µl of 2× substrate, 50 µl of buffer (or 4× inhibitor), and 50 µl of 4× enzyme (or extract) to give a final assay volume of 200 µl. Substrates used were Suc-LLVY-amc for chymotrypsin-like sites, Ac-nLPnLD-amc for caspase-like sites and Ac-RLR-amc for trypsin-like sites, all at a final concentration of 100 µM. All substrates were initially prepared as 100× stocks in DMSO. All peptidase assays of the 26S proteasome use the same assay buffer, which consists of 50 mM Tris-HCl, pH 7.5, 40 mM KCl, 5 mM $MgCl_2$, 0.5 mM ATP and 1 mM dithiothreitol (DTT). ATP is needed to prevent dissociation of the 26S proteasome into its components, to ensure maximal activity, and in a way that 26S and not 20S activity is measured. The plate was then placed in a fluorescent plate reader, and the fluorescence of 4-amino-7-methyl-coumarin reaction product was monitored continuously (excitation 380 nm and emission 460 nm were used). The reaction rates were determined from the slopes of the reaction progress curves. In order to account for the contribution of other proteases that may be present in extracts to the cleavage of proteasomal substrates, extracts were preincubated

in the presence or absence of 20 μM epoxomicin for 30 min at 37°C. For each sample, the proteasome activity was calculated by subtracting activity in the epoxomicin-treated extract from the activity in the mock-treated extracts.[14]

Determination of inhibition of proteasomes in cells: To determine the effect of the inhibitors in living cells, cells were incubated at 37°C overnight with varying concentrations of inhibitor. Cells were then washed three times in ice cold 1× PBS by centrifugation at 4°C. The cells were frozen briefly at -80°C. After thawing, the cells were re-suspended in 4 volumes of homogenization buffer (50 mM Tris-HCl, pH 7.5, 250 mM sucrose, 5 mM $MgCl_2$, 2 mM ATP, 1 mM DTT, 0.5 mM EDTA) containing 0.025% digitonin. Cells were then incubated for at least 5 minutes on ice to allow permeabilization by digitonin. The cytosol was then "squeezed out" by centrifugation at 20.000×g for 15 min (4°C). Protein concentration of the extract was determined by Bradford assay. The extract was then assayed as above or treated with 0.5 mM phosphane, and analyzed on a SDS-PAGE (12% Novex NuPAGE Bis-Tris gel with MOPS running buffer) or 2D-gels (using Invitrogen ZOOM system with pH 3-10 isoelectric focusing strips, and a 4-12% gradient NuPAGE Bis-Tris gel with MOPS buffer). In some cases, the remaining cell pellet was re-suspended in the same homogenization buffer and then sonicated to extract the remaining components. The extracts were then analyzed by SDS-PAGE exactly as described for cytosolic extracts.

References and notes

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