Discovery and development of inhibitors selective for human constitutive proteasome and immunoproteasome active sites

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Selective two-step activity-based protein profiling (ABPP) of different proteasome subunits

8.1 Introduction

The processing and degradation of damaged and obsolete proteins is crucial for cell viability.[1] This process is carried out in part by the ubiquitin-proteasome system (UPS), which degrades up to 90% of all proteins in the nucleus and cytoplasm of eukaryotic cells. Central to the UPS is the proteolysis of proteins by the proteasome, a large multi catalytic complex that contains three catalytically active subunits, β1c, β2c and β5c, which differ in their substrate specificities.[2] In immunoproteasomes, three distinct catalytic activities, termed β1i, β2i and β5i, replace their constitutive counterparts, β1c, β2c and β5c respectively.[3,4] In total, and including the thymus-specific thymoproteasome[5] (harboring besides β1i, β2i the thymus-specific β5t particle as catalytically active protein subunits), mammalian tissue may express up to seven unique proteasome active sites, distributed over constitutive proteasomes, immunoproteasomes, thymoproteasomes as well as a variety of mixed proteasomes.[6]

Compounds that selectively inhibit a single catalytic proteasome subunit are important tools to investigate the role of proteasomal protein degradation in antigen presentation[7] (the generation of MHC class I antigenic peptides) and may help in establishing which of the activities are to be inhibited for an ideal proteasome directed cancer treatment regime. In case the inhibitors are covalent and irreversible, they can be modified to contain reporter entities for activity-based proteasome profiling purposes. Recently, a set of inhibitors selective for one of each of the six catalytic subunits of human constitutive proteasomes and immunoproteasomes was reported (Figure 1).[8] Chapter 5 describes a detailed report on the rational design of β5c selective inhibitors (**5**). Chapter 6 and 7 present the discovery of specific inhibitors for β2c (**3**) and β2i (**4**) subunits, respectively. Taking together, with the set of subunit-selective inhibitors **1**-**7** (Figure 1), which includes the results of the research described in chapters 5-7, each human proteasome activity can be inhibited at will. The activity and selectivity of these compounds and structural analogues can be assessed using an activity-based protein profiling assay using three activity-based probes, which are selective for β1c/β1i, β2c/β2i and β5c/β5i, respectively, in other words, three compounds that are able to detect two out of the six catalytic activities of human constitutive proteasomes and immunoproteasomes combined. Activity-based probes selective for one out of the six activities and that complement selective inhibitors however are scare and subject of this chapter.

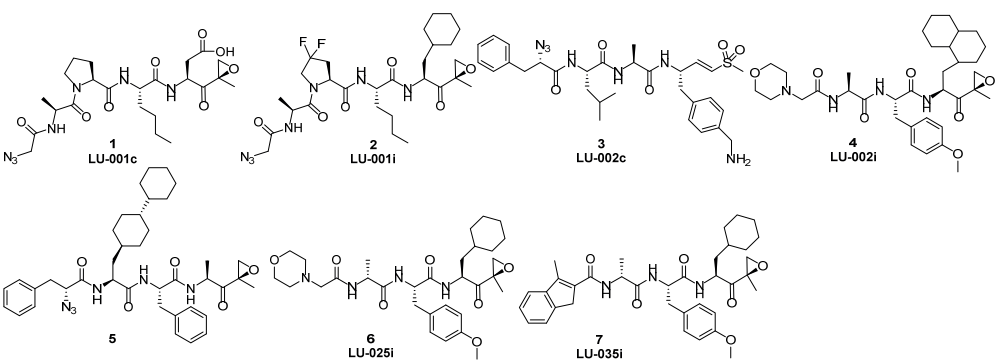


Figure 1. Structures of subunit-specific inhibitors of proteasomes: compound **1**, targeting β1c; compound **2**, targeting β1i; compound **3**, targeting β2c; compound **4**, targeting β2i; compound **5**, targeting β5c and compounds **6** and **7**, targeting β5i.

Bioorthogonal chemistry, or the reaction of two functional groups that are ideally inert in physiological samples, allows the selective modification of biomolecules in complex biological samples.[9] One application of bioorthogonal chemistry is in two-step activity-based protein profiling (two-step ABPP).[10] In two-step ABPP protocols a covalent and irreversible inhibitor containing a bioorthogonal functional group (normally an azide, an alkyne, or electron-rich alkene) reacts with an active site residue of a target enzyme. Next, the reporter moiety (a fluorophore, a biotin or a combination of the two) is installed by means of a bioorthogonal

reaction, commonly either an azide-alkyne [2+3] cycloaddition 'click' reaction[11] (which can be copper(I)-catalyzed or copper-free, strain-promoted), a modified Staudinger reaction between an azide and a phosphine,[12] or an inverse-electron-demand Diels-Alder (IEDDA) reaction between an electron-rich dienophile and an electron-poor diene.[13] Two-step ABPP is preferred over one-step ABPP when the presence of a reporter group in one-step ABP interferes with cell-permeability or, as is the case here, when the reporter moiety is detrimental to enzyme selectivity. Arguably, two-step ABPP using the set of subunit-selective inhibitors **1**-**7** as a basis and implementing bioorthogonal chemistry should allow for selective labeling of each catalytic activity of human constitutive proteasomes and human immunoproteasomes individually. In this chapter the results of research aimed at validating this hypothesis is described.

Compounds **1**, **2**, **3** and **5** all feature a bioorthogonal azide in their structure. These compounds are therefore on paper suitable for two-step ABPP protocols, in which the reporter moiety is installed following proteasome labeling by means of azide-alkyne cycloaddition (click) reactions (either copper(I)-catalyzed or strain-promoted varieties) or Staudinger-Bertozzi ligation. At the onset of the studies reported here, however, it had become apparent that IEDDA ligation is perhaps the most reliable bioorthogonal ligation strategy, at least in terms of efficiency and selectivity. This holds true especially in cases where the size of the bioorthogonal tag (which in most applications of bioorthogonal chemistry is ideally kept as small as possible) is not the most decisive factor. The latter holds true to a certain extend for proteasome inhibitors and therefore it was decided to focus also on two-step ABPP protocols using proteasome inhibitors, based on compounds **1**-**7**, but featuring an electron-rich, strained norbornene as the dienophile for IEDDA ligations. This led to the design of potential two-step ABPs **8**-**14** (Figure 2) as strained, electron-rich alkenes for bioorthogonal labeling using inverse-electron-demand Diels-Alder (IEDDA) as the ligation strategy.

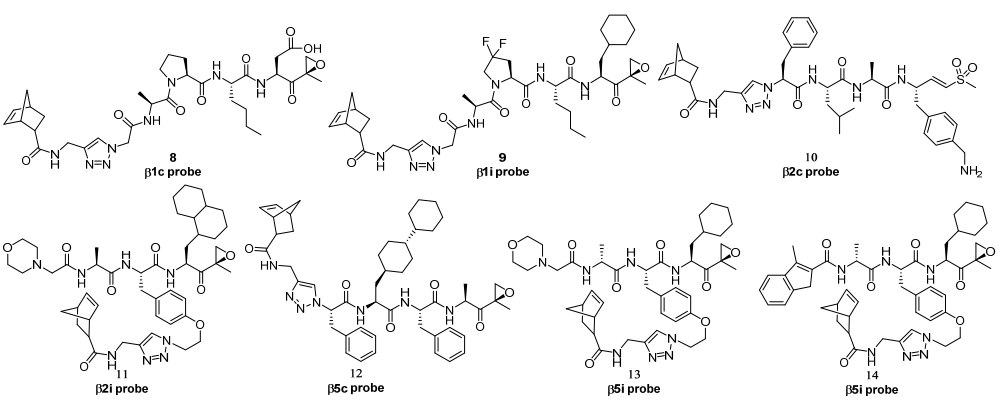


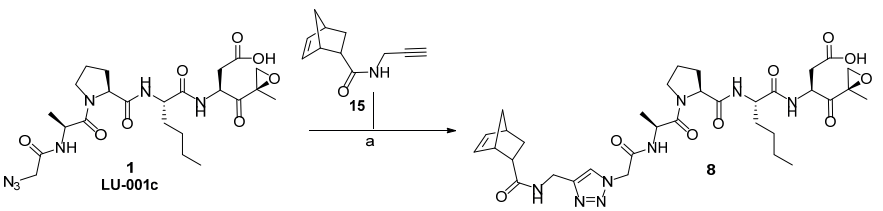
Figure 2. Structures of two-step ABPs **8**-**14** subject of the studies described in this Chapter.

8.2 Results and Discussion

Synthesis

Compounds **8**, **9**, **10**, and **12** were prepared by means of a copper(I)-catalyzed [2+3] azide-alkyne cycloaddition 'click' reaction between the corresponding azide-modified subunit-selective inhibitors **1**, **2**, **3**, **5** and norbornene-alkyne **15**.[13c] As a representative example of the synthetic strategy followed, the synthesis of compound **8** is depicted in Scheme 1 (see for details on the synthesis of compounds **9**, **10** and **12** following the same strategy the experimental section). The synthesis of norbornene-ABPs **11**, **13** and **14** are also based on click ligation of norbornene-alkyne **15** to their corresponding azide precursors (Figure 3). The synthesis of **16** is described in Chapter 7 and the preparation of compounds **17** and **18** are described in the experimental section.

Scheme 1. Synthesis of compound **8**.



Reagents and conditions: (a) $CuSO_4$, sodium ascorbate, DMF.

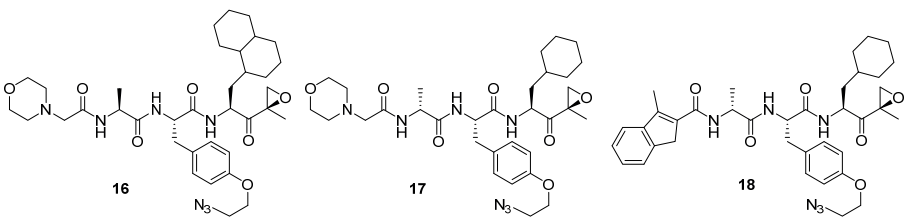
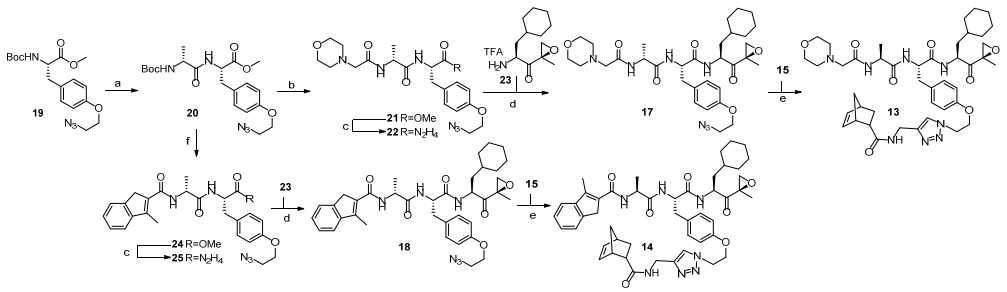


Figure 3. Structures of azide precursors **16**, **17** and **18**.

The synthesis of ABPs **13** and **14** is depicted in Scheme 2. Removal of the Boc protecting group in compound **19** using trifluoroacetic acid and subsequent condensation with Boc-D-Ala-OH gave **20** in 80% yield. Boc removal and condensation with 2-morpholinoacetic acid yielded compound **21** in 90% yield. Treatment of **21** with hydrazine monohydrate followed by treatment of the resulting hydrazoic acid **22** with tert-butylnitrite gave *in situ* the corresponding acyl azide, which was condensed with H-Cha-EK **23**[14] (as the TFA salt) to give peptide epoxyketone **17** in 11% yield. Azide-alkyne click reaction of **17** with **15** provided ABP **13** in 13% yield. Compound **24** was prepared by removal of the Boc protecting group in **20** and condensation with 3-methylindene-2-carboxylic acid (80% yield) and transformed into two-step ABP **14** following the same sequence of events as outlined for two-step ABP **13**.

Scheme 2. Synthesis of compounds **13** and **14**.



Reagents and conditions: (a) i) TFA; ii) Boc-D-Ala-OH, HCTU, DiPEA, DCM; (b) i) TFA; ii) 2-morpholinoacetic acid, HCTU, DiPEA, DCM; (c) hydrazine monohydrate, MeOH; (d) i) *t*BuONO, HCl, DMF/DCM (1:1, v/v), -30 °C; ii) **23**, DiPEA, DMF; (e) $CuSO_4$, sodium ascorbate, DMF; (f) i) TFA; ii) 3-methylindene-2-carboxylic acid, HCTU, DiPEA, DCM.

Biological evaluation

In total, taking into account the previously reported azides **1**, **2**, **3**, **5** and the newly synthesised norbornenes **8**-**14**, a set of 11 potential subunit-selective two-step proteasome ABPs are available. In the first instance, attention on establishment of their subunit-selectivity and activity was established using competitive two-step ABPP assays making use of the activity-based proteasome profiling assay described in chapter 5. The activity, selectivity and cell permeability of azides **1**, **2**, **3**, **5** in such assays had already been described, compounds **3** and **5** in chapters 5 and 6 and compounds **1** and **2** in the literature.[8] The results on the behaviour of norbornenes **8**-**14** in competitive ABPP, in particular their activity, selectivity and cell-permenability are summarized in Figure 3, Table 1 and Table 2.

In the first instance the activity of compounds **8**-**14** was assessed in extracts of Raji cells (a human B-cell lymphoma cell line that expresses both constitutive protesomes and immunoproteasomes). As can be seen (Figure 3 and Table 1), compound **8** potently and selectively inhibits β1c (apparent IC_{50}: 0.022 μM) and does not inhibit any of the remaining active subunits at concentrations up to 10 μM. At 0.3 μM final concentration of **8**, β1c labeling appears completely abolished whereas the remaining 5 activities are equally well tagged by the three-probe mixture as in the control experiment in which no inhibitor was included (Figure 3A, upper gel, left lane). Compound **9** selectively inhibits β1i at sub-micromolar concentrations (IC_{50}: 0.041 μM) and co-targets β5c and β5i at higher concentrations. The optimal concentration for *in vitro* two-step ABPP using **9** appears to be 0.3 μM - a concentration at which β1i labeling is completely abolished while labeling of the remaining active subunits is largely unimpaired. Compound **10** appeared not selective for β2c, and, in contrast to its β2c-selective parent compound **3** (LU-002c), proved to favor β5c/i. Presumably, the relatively bulky and hydrophobic norbornene moiety is causative of this loss of

β2c-selectivity. Compound **11** selectively inhibits β2i at sub-micromolar concentrations (IC_{50}: 0.24 μM) and modifies β2c at higher concentrations. At 1.0 μM final concentration, norbornene **11** completely abolished β2i labeling while leaving β2c largely untouched**.** Norbornene **12** is a potent and selective β5c inhibitor (IC_{50} 0.008 μM) with as potential optimal concentration for use in *in vitro* two-step ABPP established at 0.1 μM. Norbornene **14** finally appears to be the most effective β5i-selective inhibitor, more so than close analogue **13**, and at 0.3 μM final concentration β5i activity is blocked while the remaining five activities are as in the non-inhibitor-treated samples (as visualized in the three-probe assay).

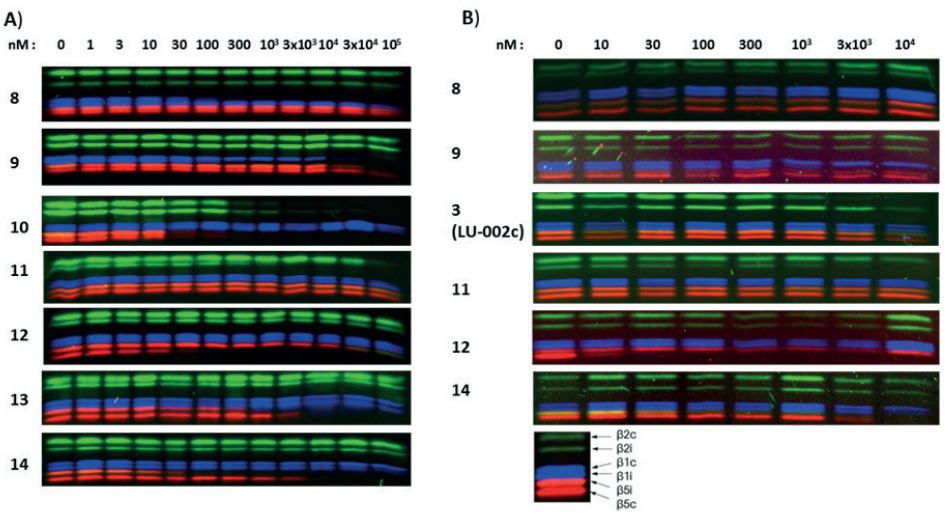


Figure 3. A) Inhibition profiles of compounds **8**-**14**, determined in Raji cell lysates; B) Inhibition profiles of ABP **8**, **9**, **3** (LU-002c), **11**, **12** and **14**, determined in intact Raji cells.

Apparent IC_{50} (μM) and pIC_{50} values												
	β1c		β1i		β2c		β2i		β5c		β5i	
Compd	IC_{50} (μM)	pIC_{50}	IC_{50} (μM)	pIC_{50}	IC_{50} (μM)	pIC_{50}	IC_{50} (μM)	pIC_{50}	IC_{50} (μM)	pIC_{50}	IC_{50} (μM)	pIC_{50}
8	0,022	7.67±0.09	>100	<4.0	>100	<4.0	>100	<4.0	>100	<4.0	>100	<4.0
9	64.9	4.18±0.11	0,041	7.39±0.08	>100	<4.0	>100	<4.0	>100	<4.0	9.67	5.02±0.13
10	>100	<4.0	>100	<4.0	0,071	7.14±0.07	0,14	6.85±0.08	0,0085	8.48±0.09	0,015	7.84±0.08
11	>100	<4.0	>100	<4.0	>100	<4.0	0,24	6.623±0.06	>100	<4.0	>100	<4.0
12	>100	<4.0	14.03	4.85±0.12	>100	<4.0	>100	<4.0	3.28	5.48±0.08	0,015	7.83±0.07
13	>100	<4.0	>100	<4.0	>100	<4.0	>100	<4.0	2.11	5.68±0.10	0,097	7.01±0.10
14	>100	<4.0	>100	<4.0	>100	<4.0	>100	<4.0	0,008	8.09±0.06	11.4	4.94±0.08

Table 1. IC_{50} values and pIC_{50} values with standard deviations for compounds **8**-**14**, determined in Raji cell lysates.

Apparent IC_{50} (μM) and pIC_{50} values												
	β1c		β1i		β2c		β2i		β5c		β5i	
Compd	IC_{50} (μM)	pIC_{50}	IC_{50} (μM)	pIC_{50}	IC_{50} (μM)	pIC_{50}	IC_{50} (μM)	pIC_{50}	IC_{50} (μM)	pIC_{50}	IC_{50} (μM)	pIC_{50}
8	> 10	< 4.0	> 10	< 4.0	> 10	< 4.0	> 10	< 4.0	> 10	< 4.0	> 10	< 4.0
9	> 10	< 4.0	0.73	6.14±0.14	> 100	< 4.0	> 100	< 4.0	> 100	< 4.0	> 100	< 4.0
3	> 10	< 4.0	> 10	< 4.0	0.54	6.27±0.16	> 10	< 4.0	> 10	< 4.0	> 10	< 4.0
11	> 10	< 4.0	> 10	< 4.0	> 10	< 4.0	0.14	6.86±0.19	> 10	< 4.0	> 10	< 4.0
12	> 10	< 4.0	> 10	< 4.0	> 10	< 4.0	> 10	< 4.0	> 10	< 4.0	0.018	7.75±0.08
14	> 10	< 4.0	> 10	< 4.0	> 10	< 4.0	> 10	< 4.0	<0.01	>8	> 10	< 4.0

Table 2. IC_{50} values and pIC_{50} values with standard deviations for compounds **3**, **8, 9, 11, 12** and **14**, as determined in living Raji cells.

Of the norbornenes, compounds **8** (β1c), **9** (β1i), **11** (β2i), **12** (β5c) and **14** (β5i) appear suitable for *in vitro* two-step ABPP. Azide **3** (previously described in the literature[8] as name LU-002c) was included in the two-step ABPP experiments described below instead of norbornene **10** (which as described above is not targeting β2c but instead modifies β5c/i)) to complete the set of potential two-step ABPs for each of the six catalytic activities of human constitutive proteasomes and immunoproteasomes. For azide **3**, 0.1 μM[8] is used as the optimal concentration for *in vitro* two-step ABPP, which allows the efficient labeling of β2c and minimizes the co-labeling of β2i. The results of the two-step ABPP experiments are depicted in Figure 4. Raji cell extracts were treated with compound **3**, **8**, **9**, **11**, **12**, or **14** at final concentrations as determined above for optimal blocking of the target activity in terms of potency and selectivity. In the next step and prior denaturation of the protein sample bioorthogonal ligation with either tetrazine-BODIPY-TMR **25**[13c] (in case of norbornene ABPs **8**, **9**, **11**, **12**, or **14**) or alkyne-BODIPY-FL **26** (in case of azide ABP **3**) using the appropriate IEDDA or click ligation protocol and in which reagents **25** or **26** were added at increasing concentrations (Figure 4E). Norbornene-epoxomicin **27** and azido-epoxomicin **28**, both of which are broad-spectrum proteasome inhibitors, were included in these experiments so that possible subunit-selective two-step ABP could be offset against broad-spectrum two-step ABPP. As negative controls reagents **25** or **26** and the accompanying bioorthogonal ligation chemistries were applied to cell extracts that had not been treated with any of the two-step ABPs.

As can be seen (Figure 4), β1c can be selectively modified by first treatment of Raji cell extracts with norbornene **8** and next bioorthogonal ligation with tetrazine **25**. Labeling of β1c became apparent at 5 μM final concentration of **25** and could be strengthened by increasing the final concentration of **25** (Figure 4A). At these high concentrations however an unspecific band appeared which runs at the same height as the proteasome β1i/5c/5i subunits on the SDS

PAGE gels. This unspecific labeling also appeared in the negative control, in which cell extracts were treated with **25** only, and features in all IEDDA two-step ABPP experiments. This caveat aside, most norbornene two-step ABPs behave as would be expected from their proteasome inhibition profiles. Norbornene **9** in combination with tetrazine **25** (from 10 μM final concentration upwards) visualizes β1i selectively. Likewise, treatment of cell extracts with norbornene **11** followed by **25** (10 μM final concentration) reveals β2i, whereas at higher concentrations of tetrazine **25** labeling of β5c/5i subunits becomes apparent besides the off-target labeling described above. In a similar vein, β5c and β5i can be visualized using norbornenes **12** and **14** respectively, in combination with tetrazine **25**. Two-step ABP **3** (LU-002c, final concentration 0.1 μM) finally appeared suitable for labeling selectively β2c in combination with CuAAC ligation with alkyne-BODYPI-FL (**26**) at increasing concentrations (Figure 4C).

As the next research objective, the inhibition profiles of compounds **3** (LU-002c), **8**, **9**, **11**, **12** and **14** in living Raji cells were determined, again by competitive ABPP using the three-probe mixture first introduced in chapter 5 of this Thesis. The experiments were carried out following essentially the same steps as were conducted for the *in vitro* assays, but now living cells were treated with the respective inhibitors at varying concentrations prior to cell lysis and exposure to the ABP set. The results are summarized in Figure 3B and Table 2. Compound **8** proved to be inactive in living Raji cells (Figure 3B, upper gel, right lane), which can be attributed to the negatively charged aspartic acid side chain at P1 that may prohibit crossing of the cell membrane. Compound **9** proved to be cell permeable and selectively inhibits β1i also *in situ* (IC_{50} 0.73 μM), with complete and selective blocking of this subunit reached at 3.0 μM. Compound **3** (LU-002c) inhibits β2c in living Raji cells at 1.0 μM while leaving the other subunits untouched. Complete inhibition of β2c is achieved at 3.0 μM but at this final concentration some co-inhibition of β2i can be observed, and it is concluded that for this compound 1.0 μM final concentration may be optimal for *in situ* two-step ABPP. Norbornene **11** blocks β2i subunit *in situ*, with 3.0 μM final concentration appearing suitable for *in situ* two-step ABPP. Compound **12** and **14** finally appear potent and selective for β5c and β5c subunits *in situ*, with concentrations of 1.0 μM and 0.1 μM, respectively, for two-step ABPP experiments in living cells. Thus, of the set of norbornene ABPs, compounds **9** (β1i), **11** (β2i), **12** (β5c) and **14** (β5i) appear suitable for *in situ* two-step ABPP and the same holds true for azide **3** (LU-002c).

As the final research objective, *in situ* two-step ABPP experiments as a means to report on individual proteasome active subunits in living cells were executed. The results are summarized in Figure 4B and D. When Raji cells were treated with compound **9** at 3.0 μM concentration, then lysed, incubated with 5 μM of **25**, followed by denaturation and resolving the protein content on SDS PAGE, selective β1i labeling was o**bserved** (Figure 4B). Similarly, β2i could be detected after *in situ* treatment of Raji cells with norbornene **11**, β5c with

norbornene **12** and β5i with norbornene **14**, respectively. In all cases, two-step ABPP in living Raji cells proved more effective than the corresponding two-step ABPP experiments conducted in cell extracts in terms of selectivity (compare the respective lanes, which all look cleaner in the *in situ* experiments). Azide **3** finally gave clean β2c labeling in a similar experiment: after treatment of Raji cells with this ABP, then cell lysis, then treatment of the protein mixture with alkyne-BODIPY-FL (**26**) under azide alkyne [2+3] cycloaddition click conditions at increasing concentrations, followed by the same denaturation and resolving the protein content on SDS PAGE, selective β2c labeling was observed (Figure 4D).

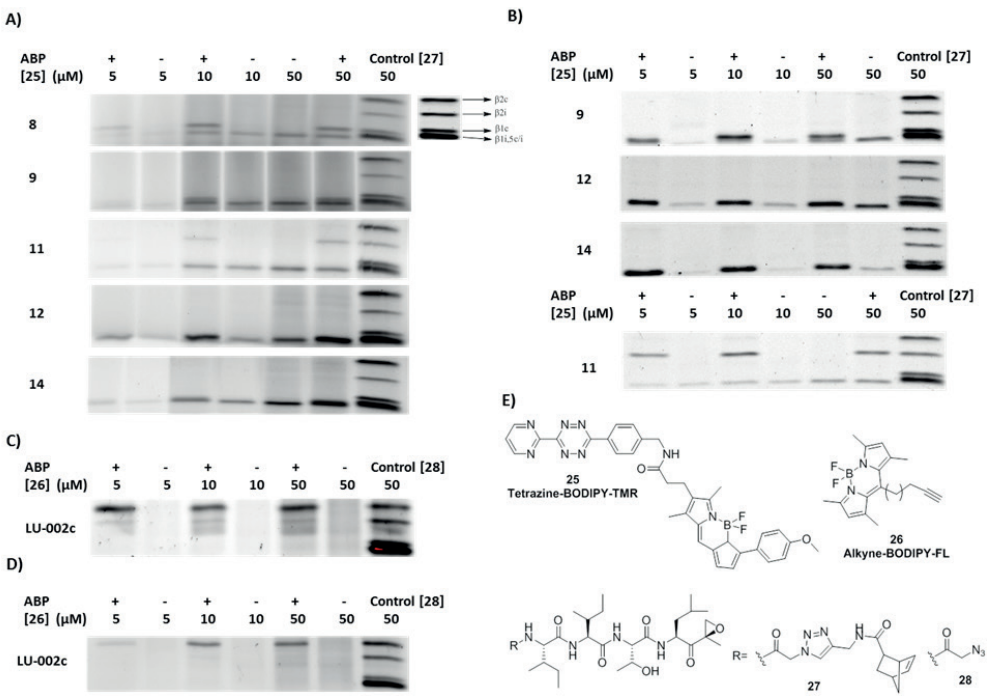


Figure 4. A) Two-step ABPP in Raji cell lysates using tetrazine ligation strategy; B) two-step ABPP in living Raji cells using tetrazine ligation strategy; C) two-step ABPP in Raji cell lysates using CuAAC strategy; D)) two-step ABPP in living Raji cells using CuAAC strategy; E) structures of ligation tags (**25**, **26**) and position controls (**27**, **28**).

8.3 Conclusions

In this chapter, a set of seven norbornene modified peptide vinyl sulfone and peptide epoxyketones have been designed and synthesized. From these, compounds **9** (β1i), **11** (β2i), **12** (β5c) and **14** (β5i) proved useful two-step ABPs to label selectively their projected proteasome targets in both Raji cell extracts and living Raji cells. In contrast, norbornene derivative **10** proved not selective for β2c, as was projected based on its parent compound (LU-002c, **3**), but instead gave preferential inhibition of the chymotryptic sites of both constitutive proteasomes and immunoproteasomes. Two-step ABPP of β2c could however be

achieved using azide-containing inhibitor **3,** and employing azide-alkyne click ligation chemistry. Compound **8** (β1c) proved effective to inhibit and tag (by means of IEDDA chemistry) β1c *in vitro* but not *in situ*, the latter likely caused by cell impermeability inherent to the acidic nature of the compound. In total, the research described here has expanded the proteasome ABP toolset to include five new *in vitro* ABPs, of which four can also be applied to monitor proteasome activity profiles in living cells. Optimizations, especially regarding the background labeling, but also to procure ABPs selective for the remaining proteasome activities, need however to be conducted to make the two-step ABPP set ready for use by the proteasome biochemistry community, in the same vein as the three-probe cocktail (chapter 5) is currently used.

8.4 Experimental section

Synthesis

General procedures

All reagents were of commercial grade and used as received unless indicate otherwise. The purity of all tested compounds is >95% on the basis of LC-MS and NMR. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AV-400 (400 MHz), AV-600 (600 MHz) or AV-850 (850 MHz) spectrometer. Chemical shifts are given in ppm (δ) relative to CD_3OD or $CDCl_3$ as internal standard. Coupling constants are given in Hz and peak assignments are based on 2D ^{1}H COSY and ^{13}C HSQC NMR experiments. All presented ^{13}C APT spectra are proton decoupled. LC-MS analysis was performed on a Finnigan Surveyor HPLC system with a Gemini C18 50 × 4.60 mm column (detection at 200−600 nm) coupled to a Finnigan LCQ Advantage Max mass spectrometer with ESI. Methods used are: 15 min (0-0.5 min: 10% MeCN; 0.5-10.5 min: 10% to 90% MeCN; 10.5-12.5 min: 90% MeCN; 12.5-15 min: 90% to 10% MeCN) or 12.5 min (0-0.5 min: 10% MeCN; 0.5-8.5 min: 10% to 90% MeCN; 8.5-10.5 min: 90% MeCN; 10.5-12.5 min: 90% to 10% MeCN). HRMS was recorded on a LTQ Orbitrap (ThermoFinnigan). For reverse phase HPLC purification, an automated Gilson HPLC system equipped with a C18 semiprep column (Phenomenex Gemini C18, 5µm 250×10 mm) and a GX281 fraction collector.

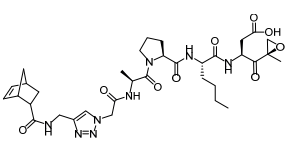
General procedure A

Azide containing compound and Norborbene-alkyne **15** (1.2 eq.) were dissolved in DMF. An aqueous solution of sodium ascorbate (100 µL, 0.75 eq.) and an aqueous solution of $CuSO_4$ (100 µL, 0.5 eq.) were added. The reaction mixture was stirred at r.t. overnight. The mixture was concentrated *in vacuo* and purification by HPLC yielded the title compound.

General procedure B

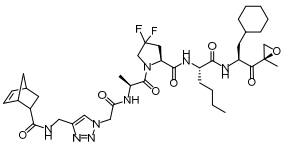
The appropriate Boc-protected compound was dissolved in TFA and stirred for 20 min. Co-evaporation with toluene (3x) afforded the TFA-salt, which was used directly in the next step. The deprotected amine TFA salt (1.0 eq.) and free acid (1.2 eq.) were dissolved in DCM, followed by addition of HCTU (1.2 eq.) and DiPEA (3.5 eq.). After stirring overnight, the reaction mixture was concentrated *in vacuo* and re-dissolved in EtOAc, washed with 1M HCl (2x), sat. aq. $NaHCO_3$ (3x) and brine (in case of 2-morpholinoacetic acid coupling, no 1M HCl washing). The organic layer was dried over $MgSO_4$ and concentrated *in vacuo*. Purification by silica gel flash column chromatography yielded the title compound.

Synthesis of final compounds via "click" reaction

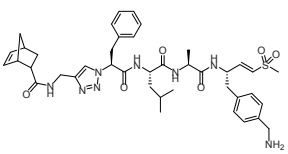


Nor-Ala-Pro-Nle-Asp-EK (8). This compound was prepared according to the general procedure A on a 10 µmol scale using compound LU-001c as starting material. Purification by HPLC (32%-42% MeCN-H_2O) yielded the title compound (2.1 mg, 2.9 µmol, 29%). ^{1}H NMR (600 MHz, MeOD) δ 7.87 (s, 1H), 6.18 (dd, *J* = 5.6, 3.1 Hz, 1H), 5.92-5.86 (m, 1H), 5.22-5.15 (m, 2H), 4.82-4.74 (m, 1H), 4.64-4.62 (m, 1H), 4.47-4.44 (m, 1H), 4.42-4.40 (m, 2H), 4.29-4.27 (m, 1H), 3.80-3.76 (m, 1H), 3.67-3.64 (m, 1H), 3.25 (d, *J* = 5.0 Hz, 1H), 3.20-3.17 (m, 1H), 2.98-2.87 (m, 4H), 2.83-2.78 (m, 1H), 2.74-2.70 (m, 1H), 2.27-2.15 (m, 1H), 2.10-1.96 (m, 4H), 1.93-1.77 (m, 3H), 1.66-1.61 (m, 1H), 1.51 (s, 3H), 1.46-1.25 (m, 15H), 0.95 (t, *J* = 7.0 Hz, 4H). ^{13}C NMR (150 MHz, MeOD) δ 207.12, 176.93, 174.23, 174.09, 173.21, 167.50, 138.55, 133.05, 125.79, 61.47, 60.32, 54.64, 53.25, 52.70, 50.79, 50.49, 49.57, 47.61, 45.30, 43.99, 36.10, 35.68, 32.79, 30.46, 29.87, 28.91,

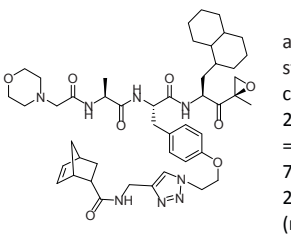
26.01, 23.45, 17.16, 16.76, 14.28. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 15.0 min): R_t (min): 5.39 (ESI-MS (m/z): 713.20, $(M+H^+)$). HRMS calculated for $C_{34}H_{48}N_8O_9$ 713.36170 $[M+H]^+$; found 713.36167.



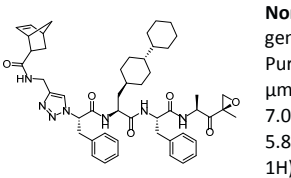
Nor-Ala-4,4-F_2Pro()-Nle-Cha-EK (9). This compound was prepared according to the general procedure **A** on a 8.5 µmol scale using compound LU-001i as starting material. Purification by HPLC (50%-65% MeCN-H_2O) yielded the title compound (2.5 mg, 3.2 µmol, 38%). ^{1}H NMR (600 MHz, MeOD) δ 7.83 (s, 1H), 6.19-6.17 (m, 1H), 5.99-5.84 (m, 1H), 5.24-5.11 (m, 2H), 4.69-4.66 (m, 1H), 4.60-4.52 (m, 2H), 4.41 (d, *J* = 1.8 Hz, 2H), 4.35-4.25 (m, 2H), 4.06-4.00 (m, 1H), 3.26 (d, *J* = 5.2 Hz, 1H), 3.18-3.16 (m, 1H), 2.99-2.86 (m, 3H), 2.79-2.71 (m, 1H), 2.58-2.37 (m, 1H), 1.91-1.53 (m, 9H), 1.48 (s, 3H), 1.46-1.18 (m, 15H), 1.04-0.88 (m, 5H). ^{13}C NMR (150 MHz, MeOD) δ 209.69, 176.97, 174.26, 173.53, 171.95, 167.63, 138.54, 133.06, 129.47, 127.82, 126.18, 60.13, 59.09, 54.75, 53.06, 52.63, 50.79, 47.61, 45.33, 43.99, 38.64, 38.24, 38.08, 37.92, 35.69, 35.57, 35.14, 33.07, 32.89, 29.89, 28.85, 27.56, 27.36, 27.07, 23.51, 17.06, 16.63, 14.30. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 15.0 min): R_t (min): 7.63 (ESI-MS (m/z): 787.20, $(M+H^+)$). HRMS calculated for $C_{39}H_{56}F_2N_8O_7$ 787.43128 $[M+H]^+$; found 787.43163.



Nor-Phe-Leu-Ala-Phe(4-CH_2NH_2)-VS (10). This compound was prepared according to the general procedure **A** on a 16.2 µmol scale using compound LU-002c as starting material. Purification by HPLC (50%-55% MeCN-H_2O) yielded the title compound (2.3 mg, 2.5 µmol, 16%). ^{1}H NMR (600 MHz, MeOD) δ 7.92 (d, *J* = 1.5 Hz, 1H), 7.45-7.33 (m, 4H), 7.28-7.17 (m, 3H), 7.17-7.11 (m, 2H), 6.84-6.81 (m, 1H), 6.62-6.60 (m, 1H), 6.17 (dd, *J* = 5.7, 3.1 Hz, 1H), 5.84-5.81 (m, 1H), 5.64-5.61 (m, 1H), 4.87-4.84 (m, 1H), 4.40-4.29 (m, 3H), 4.25-4.21 (m, 1H), 4.11 (s, 2H), 3.53 (dd, *J* = 14.3, 5.5 Hz, 1H), 3.41 (dd, *J* = 14.3, 10.3 Hz, 1H), 3.16-3.13 (m, 1H), 3.06-2.99 (m, 2H), 2.96 (s, 3H), 2.94-2.88 (m, 2H), 1.90-1.86 (m, 1H), 1.65-1.56 (m, 3H), 1.43-1.33 (m, 6H), 0.95 (d, *J* = 5.8 Hz, 3H), 0.87 (d, *J* = 5.8 Hz, 3H). ^{13}C NMR (150 MHz, MeOD) δ 176.94, 174.45, 174.12, 169.98, 146.68, 146.61, 139.63, 138.59, 136.95, 133.04, 133.03, 131.72, 131.30, 130.25, 130.00, 129.99, 129.66, 128.23, 123.99, 123.92, 65.99, 53.71, 52.41, 50.86, 50.84, 50.81, 50.78, 49.85, 49.57, 47.65, 47.59, 45.27, 45.22, 44.03, 43.99, 43.98, 42.71, 41.39, 40.27, 39.19, 35.60, 29.86, 25.84, 23.47, 21.79, 18.10, 18.08. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 15.0 min): R_t (min): 6.85 (ESI-MS (m/z): 786.07, $(M+H^+)$). HRMS calculated for $C_{41}H_{54}N_8O_6S$ 787.39598 $[M+H]^+$; found 787.39523.

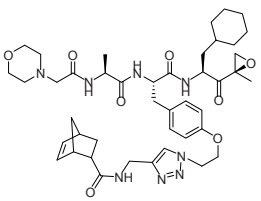


Morp-Ala-Try(C_2H_4-triazole-Nor)-1-DecAla-EK (11). This compound was prepared according to the general procedure **A** on a 33 µmol scale using compound **16** as starting material. Purification by HPLC (45%-55% MeCN-H_2O) yielded the title compound (3.22 mg, 3.6µmol, 11%). ^{1}H NMR (600 MHz, MeOD) δ 7.20 (d, *J* = 8.1 Hz, 2H), 6.87 (d, *J* = 8.3 Hz, 2H), 6.22 (s, 1H), 5.91 (s, 1H), 4.84 (t, *J* = 4.5 Hz, 2H), 4.66 (t, *J* = 9.7 Hz, 1H), 4.57 (t, *J* = 10.9 Hz, 1H), 4.44 (dd, *J* = 16.5, 7.6 Hz, 6H), 4.24-3.68 (m, 7H), 3.24 (d, *J* = 19.8 Hz, 3H), 3.10 (dd, *J* = 13.8, 5.4 Hz, 1H), 3.05-2.94 (m, 3H), 2.94-2.83 (m, 1H), 1.96-1.92 (m, 2H), 1.89-1.70 (m, 5H), 1.70-1.55 (m, 6H), 1.55-1.44 (m, 9H), 1.40 (d, *J* = 7.6 Hz, 7H), 1.35-0.96 (m, 8H). ^{13}C NMR (151 MHz, MeOD) δ 209.81, 209.47, 176.99, 174.09, 173.32, 173.26, 164.80, 161.87, 158.50, 158.42, 146.77, 138.61, 133.00, 131.58, 130.97, 130.95, 130.79, 125.01, 115.62, 115.58, 67.68, 67.58, 64.76, 60.08, 60.05, 59.91, 58.26, 55.57, 55.55, 55.53, 53.89, 53.86, 52.91, 52.80, 51.00, 50.97, 50.80, 50.79, 50.29, 49.57, 49.43, 49.28, 49.19, 49.14, 49.00, 48.86, 48.72, 48.57, 47.59, 45.31, 43.99, 43.08, 39.12, 38.25, 38.19, 36.45, 35.68, 34.83, 33.85, 33.73, 29.92, 29.91, 29.05, 27.99, 27.92, 27.69, 27.62, 26.74, 26.60, 26.54, 22.38, 22.32, 21.22, 20.22, 18.11, 16.89. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 15.0 min): R_t (min): 6.77 (ESI-MS (m/z): 871.47, $(M+H^+)$). LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 15.0 min): R_t (min): 6.77 (ESI-MS (m/z): 871.47, $(M+H^+)$). HRMS calculated for $C_{47}H_{66}N_8O_8$ 871.50764 $[M+H]^+$; found 871.50791.

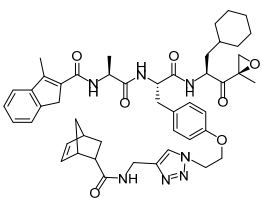


Nor-Phe-trans-BiCha-Phe-Ala-EK (12). This compound was prepared according to the general procedure **A** on a 5.5 µmol scale using compound LU-005c as starting material. Purification by HPLC (75%-85% MeCN-H_2O) yielded the title compound (1.2 mg, 1.4 µmol, 22%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.54 (d, *J* = 4.0 Hz, 1H), 7.24-7.13 (m, 5H), 7.01-6.87 (m, 2H), 6.57 (d, *J* = 5.2 Hz, 1H), 6.43 (d, *J* = 5.2 Hz, 1H), 6.23-6.12 (m, 2H), 5.86-5.83 (m, 1H), 5.27-5.23 (m, 1H), 4.61-4.58 (m, 1H), 4.48-4.33 (m, 3H), 3.55-3.43 (m, 1H), 3.42-3.31 (m, 1H), 3.14-3.09 (m, 2H), 2.99 (d, *J* = 7.0 Hz, 2H), 2.95-2.80 (m, 3H), 1.67-1.54 (m, 8H), 1.51 (s, 3H), 1.46-1.26 (m, 5H), 1.22 (d, *J* = 7.1 Hz, 3H), 1.19-0.74 (m, 13H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 207.80, 174.66, 171.21, 170.08, 167.36, 145.04, 138.01, 137.99, 136.38, 135.22, 132.33, 132.23, 129.42, 128.95, 128.88, 128.76, 127.61, 127.23, 123.26, 123.14, 65.92, 58.99, 54.34, 52.50, 51.91, 50.17, 48.12, 46.29, 44.77, 43.32, 42.84, 39.39, 39.04, 38.18, 34.89, 34.47, 33.69, 32.96, 30.35, 30.02, 29.90, 29.71, 29.65, 26.93, 17.32, 16.90,

0.15. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 15.0 min): R_t (min): 9.68 (ESI-MS (m/z): 860.20, $(M+H^+)$). HRMS calculated for $C_{50}H_{65}N_7O_6$ 860.50691 $[M+H]^+$; found 860.50719.

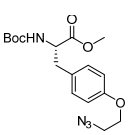


Morph-D-Ala-Tyr(C_2H_4-triazole-Nor)-Cha-EK (**13**). This compound was prepared according to the general procedure **A** on a 50 µmol scale using compound **17** as starting material. Purification by HPLC (41% MeCN-H_2O) yielded the title compound (5.13 mg, 6.2 µmol, 13%). ^{1}H NMR (400 MHz, MeOD) δ 7.20 (d, *J* = 8.6 Hz, 1H), 6.89 (d, *J* = 8.7 Hz, 1H), 6.33-6.16 (m, 1H), 5.91 (dd, *J* = 5.6, 2.8 Hz, 1H), 4.91-4.74 (m, 1H), 4.66 (dd, *J* = 9.5, 3.9 Hz, 1H), 4.57-4.32 (m, 2H), 4.26-3.67 (m, 3H), 3.30 (dd, *J* = 16.1, 4.8 Hz, 1H), 3.26-3.17 (m, 1H), 3.00 (dd, *J* = 13.1, 4.4 Hz, 1H), 2.95 (s, 1H), 2.81 (dd, *J* = 14.1, 10.1 Hz, 1H), 2.00-1.84 (m, 1H), 1.84-1.69 (m, 2H), 1.64 (d, *J* = 7.2 Hz, 1H), 1.54 (s, 1H), 1.51-1.43 (m, 2H), 1.40 (d, *J* = 8.1 Hz, 1H), 1.31 (t, *J* = 10.5 Hz, 2H), 1.20 (d, *J* = 7.1 Hz, 1H), 1.14-0.90 (m, 1H). ^{13}C NMR (100 MHz, MeOD) δ 209.89, 176.97, 174.27, 173.34, 165.05, 158.47, 138.59, 132.99, 131.48, 131.24, 124.97, 115.59, 67.66, 64.90, 60.10, 58.16, 55.46, 53.87, 53.01, 50.99, 50.91, 50.79, 49.64, 49.43, 49.21, 49.00, 48.79, 48.57, 48.36, 47.58, 45.29, 43.98, 38.77, 37.92, 35.66, 35.64, 35.09, 33.01, 29.90, 27.52, 27.34, 27.08, 17.67, 16.97. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 15.0 min): R_t (min): 6.07 (ESI-MS (m/z): 817.33, $(M+H^+)$). HRMS calculated for $C_{43}H_{60}N_8O_8$ 817.46069 $[M+H]^+$; found 817.46065.

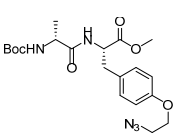


3MeIndAc-Ala-Tyr(O-C_2H_4-triazole-Nor)-Cha-EK (14). This compound was prepared according to the general procedure A on a 50 µmol scale using compound **18** as starting material. Purification by HPLC (60%-70% MeCN-H_2O) yielded the title compound (11.6 mg, 13.7 µmol, 27%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (s, 1H), 7.55-7.42 (m, 2H), 7.35 (p, *J* = 7.2 Hz, 2H), 7.27 (s, 1H), 7.09 (d, *J* = 8.5 Hz, 1H), 7.01 (dd, *J* = 15.5, 8.2 Hz, 1H), 6.71 (d, *J* = 8.5 Hz, 1H), 6.67-6.51 (m, 2H), 6.47 (d, *J* = 6.9 Hz, 1H), 6.19 (s, 1H), 5.85 (d, *J* = 5.6 Hz, 1H), 4.75-4.58 (m, 2H), 4.58-4.50 (m, 1H), 4.44 (t, *J* = 7.1 Hz, 2H), 4.18 (td, *J* = 12.6, 10.6, 5.5 Hz, 2H), 3.58 (s, 1H), 3.24 (d, *J* = 5.0 Hz, 1H), 3.12 (s, 1H), 3.02 (d, *J* = 6.6 Hz, 1H), 2.96-2.77 (m, 3H), 2.50 (d, J = 2.0 Hz, 3H), 1.97-1.82 (m, 1H), 1.82-1.69 (m, 1H), 1.69-1.47 (m, 5H), 1.43 (d, *J* = 8.5 Hz, 3H), 1.36 (d, *J* = 6.9 Hz, 2H), 1.28 (d, *J* = 8.1 Hz, 3H), 1.22-0.98 (m, 4H), 0.98-0.66 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 208.44, 175.31, 172.80, 171.05, 166.37, 156.87, 148.99, 145.49, 144.50, 142.24, 138.13, 132.14, 131.10, 130.73, 129.50, 127.68, 127.00, 123.97, 121.05, 114.70, 77.48, 77.16, 76.84, 65.99, 59.16, 54.41, 52.51, 50.28, 50.15, 46.33, 44.77, 42.83, 38.45, 38.25, 36.92, 34.41, 33.97, 31.95, 29.89, 26.40, 26.25, 25.98, 18.12, 16.82, 12.54. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 15.0 min): R_t (min): 8.66 (ESI-MS (m/z): 846.27, $(M+H^+)$). HRMS calculated for $C_{48}H_{59}N_7O_7$ 846.45487$[M+H]^+$; found 846.45490.

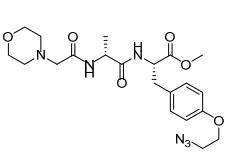
Synthesis of tripeptide hydrazide



Boc-Tyr($C_2H_4N_3$)-OMe (19). Boc-Tyr-OMe (442 mg, 1.5 mmol) was dissolved in anhydrous DMF，followed by the addition of 2-azidoethyl 4-methylbenzenesulfonate[15] (433 mg, 1.8 mmol, 1.2 eq.) and K_2CO_3 (830 mg, 6.0 mmol, 4.0 eq.). The reaction mixture was stirred over 48h at 80 ºC. The reaction mixture was concentrated *in vacuo,* re-dissovled with EtOAc, washed with H_2O (3x) and brine, dried over $MgSO_4$ and concentrated *in vacuo*. Purification by flash column chromatography (4% EtOAc-Pentane →20% EtOAc-Pentane) yielded the titled compound (283 mg, 0.77 mmol, 51%). 1H NMR (400 MHz, $CDCl_3$) δ 7.05 (d, *J* = 8.1 Hz, 2H), 6.84 (d, *J* = 7.8 Hz, 2H), 5.19 (d, *J* = 7.3 Hz, 1H), 4.52 (d, *J* = 6.7 Hz, 1H), 4.16-4.00 (m, 2H), 3.69 (s, 3H), 3.53 (d, *J* = 4.3 Hz, 2H), 3.16-2.86 (m, 2H), 1.41 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.18, 171.52, 157.10, 154.96, 130.18, 130.09, 128.64, 128.48, 114.42, 79.56, 77.48, 77.16, 76.84, 66.77, 63.93, 63.50, 61.10, 60.15, 54.43, 53.22, 51.95, 49.91, 49.35, 37.69, 37.16, 29.45, 28.08. HRMS calculated for $C_{17}H_{24}N_4O_5$ 365.18195 $[M+H]^+$; found 365.18204.

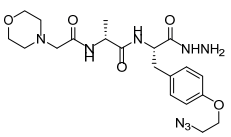


Boc-D-Ala-tyr($C_2H_4N_3$)-OMe (20). This compound was prepared according to general procedure **B** on 0.77 mmol scale using compound **19** and Boc-D-Ala-OH as starting materials. Purification by flash column chromatography (5% EtOAc-Pentane → 50% EtOAc-Pentane) yielded the titled compound (263 mg, 0.61 mmol, 80%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.04 (dd, *J* = 9.1, 2.8 Hz, 2H), 6.96-6.70 (m, 2H), 5.40 (d, *J* = 7.9 Hz, 1H), 4.81 (dt, *J* = 8.0, 6.1 Hz, 1H), 4.22 (qt, *J* = 11.2, 6.3 Hz, 1H), 4.10 (t, *J* = 4.9 Hz, 2H), 3.69 (s, 3H), 3.56 (t, *J* = 4.9 Hz, 2H), 3.03 (dtd, *J* = 29.3, 15.3, 14.7, 7.2 Hz, 2H), 1.42 (s, 9H), 1.35-1.15 (m, 4H). ^{13}C NMR (101 MHz, CDCl3) δ 172.68, 171.96, 171.79, 157.32, 155.48, 130.39, 128.61, 114.63, 79.89, 77.60, 77.48, 77.28, 76.96, 66.95, 63.86, 53.30, 52.32, 50.14, 49.93, 49.53, 37.02, 29.67, 28.32, 18.53. HRMS calculated for $C_{20}H_{29}N_5O_6$ 436.21906 $[M+H]^+$; found 436.21921.

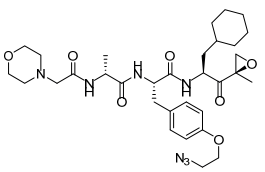


Morph-D-Ala-Tyr($C_2H_4N_3$)-OMe (21). This compound was prepared according to general procedure **B** on 0.61 mmol scale using compound **20** and 2-morpholinoacetic acid as starting materials. Purification by flash column chromatography (1% MeOH-DCM → 3% MEOH-DCM) yielded the titled compound (256 mg, 0.55 mmol, 90%). ^{1}H NMR (400 MHz,

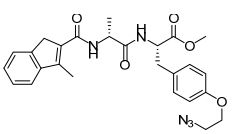
CDCl3) δ 7.80-7.49 (m, 2H), 7.33 (s, 1H), 7.09 (dd, *J* = 18.9, 8.3 Hz, 3H), 6.84 (d, *J* = 8.6 Hz, 2H), 4.78 (q, *J* = 6.5, 5.7 Hz, 1H), 4.70-4.46 (m, 1H), 4.46-4.17 (m, 1H), 4.17-4.00 (m, 2H), 3.71 (s, 3H), 3.57 (t, *J* = 4.9 Hz, 2H), 3.10 (d, *J* = 14.0 Hz, 1H), 3.06-2.89 (m, 3H), 2.50 (d, *J* = 4.8 Hz, 4H), 1.29 (d, *J* = 7.0 Hz, 6H), 0.87 (d, *J* = 5.1 Hz, 2H). ^{13}C NMR (101 MHz, CDCl3) δ 171.88, 171.79, 171.74, 171.72, 169.77, 169.74, 167.60, 157.24, 157.18, 132.20, 130.33, 130.30, 128.60, 128.57, 114.53, 114.50, 77.48, 77.16, 76.84, 66.89, 66.86, 66.81, 66.11, 65.80, 64.23, 63.77, 61.70, 61.65, 53.66, 53.22, 52.25, 50.08, 50.06, 49.47, 47.99, 38.53, 36.90, 36.72, 35.31, 29.59, 18.53. HRMS calculated for $C_{21}H_{30}N_6O_6$ 463.22966 $[M+H]^+$; found 463.22961.



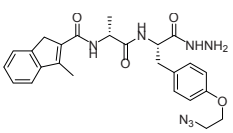
Morph-D-Ala-Tyr($C_2H_4N_3$)-NHNH$_2$ (22). Compound **21** (256 mg, 0.55 mmol) was dissolved in MeOH, followed by the addition of hydrazine monohydrate (0.85 mL, 16.5 mmol, 30.0 eq.). The reaction mixture was stirred overnight at room temperature and then refluxed at 70 ºC until TLC analysis showed complete consumption of the starting material. The reaction mixture was concentrated *in vacuo* and the crude was co-evaporated with toluene (3x) which gave the titled compound in quantitative yield.



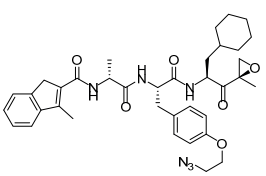
Morph-D-Ala-Tyr($C_2H_4N_3$)-Cha-EK (17). The hydrazide **22** (23 mg, 50.0 µmol) was dissolved in 1:1 DMF:DCM (v/v) and cooled to -30 ºC. *t*BuONO (7.2 µL, 60.0 µmol, 1.2 eq.) and HCl (35.0 µL, 140 µmol, 4M solution in 1,4-dioxane, 2.8 eq.) were added, and the mixture was stirred for 3 h at -30 ºC after which TLC analysis showed complete consumption of the starting material. The warhead H-Cha-EK TFA salt **23** (17.9 mg, 55.0 µmol, 1.1 eq.) was added to the reaction mixture as a solution in DMF with DiPEA (252 µmol, 44 µL, 4.5 eq.) and this mixture was allowed to warm up to room temperature slowly overnight. The mixture was diluted with EtOAc and washed with H_2O (2x) and brine. The organic layer was dried over $MgSO_4$ and concentrated *in vacuo*. Purification by HPLC (38%-45% MeCN-H_2O) yielded the title compound (3.65 mg, 5.7 µmol, 11%). ^{1}H NMR (400 MHz, MeOD) δ 7.23 (d, *J* = 8.6 Hz, 1H), 6.94 (d, *J* = 8.6 Hz, 1H), 4.68 (m, *J* = 9.8, 5.1, 4.3 Hz, 1H), 4.27-4.15 (m, 1H), 4.15-3.80 (m, 3H), 3.72-3.61 (m, 1H), 3.36-3.23 (m, 1H), 3.10-2.93 (m, 1H), 1.94-1.69 (m, 2H), 1.69-1.57 (m, 1H), 1.55 (s, 1H), 1.54-1.37 (m, 1H), 1.37-1.24 (m, 1H), 1.21 (d, *J* = 7.1 Hz, 1H), 1.15-0.84 (m, 1H). ^{13}C NMR (100 MHz, MeOD) δ 209.90, 174.37, 173.40, 158.77, 131.46, 131.42, 130.96, 115.52, 115.48, 68.40, 65.03, 60.11, 58.29, 55.50, 53.91, 53.03, 51.40, 51.33, 50.92, 49.64, 49.42, 49.21, 49.00, 48.79, 48.57, 48.36, 38.76, 37.92, 35.64, 35.10, 33.00, 27.52, 27.34, 27.08, 17.66, 16.96. HRMS calculated for $C_{32}H_{47}N_7O_7$ 642.36097 $[M+H]^+$; found 642.36095.



3MeIndAc-D-Ala-Tyr($C_2H_4N_3$)-OMe (24). This compound was prepared according to general procedure **B** on 0.67 mmol scale using compound **20** and 3-Methylindene-2-carboxylic acid as starting materials. Purification by flash column chromatography (10% EtOAc-Pentane → 20% EtOAc-Pentane) yielded the titled compound (260 mg, 0.52 mmol, 80%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.53-7.38 (m, 2H), 7.38-7.23 (m, 3H), 7.20 (d, *J* = 8.1 Hz, 1H), 7.05 (t, *J* = 7.6 Hz, 2H), 6.75 (d, *J* = 8.6 Hz, 2H), 6.65 (d, *J* = 7.4 Hz, 1H), 4.90-4.77 (m, 1H), 4.71 (q, *J* = 7.1, 6.4 Hz, 1H), 3.98-3.88 (m, *J* = 23.7, 10.0, 5.0 Hz, 2H), 3.67 (s, 3H), 3.53 (s, 2H), 3.48 (d, *J* = 4.9 Hz, 2H), 3.11 (dd, *J* = 14.0, 5.4 Hz, 1H), 3.01 (dd, *J* = 13.9, 7.0 Hz, 1H), 2.47 (s, 3H), 1.43 (t, *J* = 3.4 Hz, 1H), 1.38 (d, *J* = 7.0 Hz, 3H), 1.26 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.51, 172.45, 172.01, 171.85, 165.80, 157.31, 157.17, 147.93, 147.74, 145.48, 145.44, 142.17, 142.15, 131.66, 131.55, 130.39, 130.31, 128.57, 128.55, 128.42, 127.27, 126.77, 126.73, 123.82, 120.76, 114.55, 114.45, 114.09, 77.48, 77.16, 76.84, 66.78, 66.59, 64.64, 63.96, 53.30, 52.39, 50.09, 50.00, 49.52, 48.76, 38.22, 38.16, 36.88, 33.82, 31.92, 29.69, 29.66, 29.61, 29.50, 29.36, 29.15, 28.94, 22.70, 18.65, 12.25. HRMS calculated for $C_{20}H_{29}N_5O_5$ 492.22415$[M+H]^+$; found 492.22402.



3MeIndAc-D-Ala-Tyr($C_2H_4N_3$)-NHNH$_2$ (25). The titled compound was prepared on 0.52 mmol scale according to the procedure as described above for the preparation of compound **22** using compound **24** as starting material with quantitative yield.



3MeIndAc-D-Ala-Tyr($C_2H_4N_3$)-Cha-EK (18). The titled compound was prepared on 50 µmol scale according to the procedure as described above for the preparation of compound **17** using compound **25** as starting material. Purification by HPLC (70% MeCN-H_2O) yielded the title compound (3.41 mg, 5.0 µmol, 10%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.54-7.42 (m, 2H), 7.35 (pd, *J* = 7.4, 1.0 Hz, 2H), 7.26 (s, 3H), 7.14 (d, *J* = 8.6 Hz, 2H), 6.81 (d, *J* = 8.6 Hz, 3H), 6.39-6.20 (m, 2H), 4.61 (q, *J* = 6.5 Hz, 1H), 4.58-4.48 (m, 2H), 3.978-4.06 (m, 2H), 3.63-3.56 (m, 2H), 3.54 (t, *J* = 5.0 Hz, 2H), 3.23 (d, *J* = 5.0 Hz, 1H), 3.14-2.95 (m, 2H), 2.86 (d, *J* = 4.9 Hz, 1H), 2.52 (t, *J* = 2.1 Hz, 3H), 1.80-1.52 (m, 5H), 1.52-1.43 (m, 4H), 1.43-1.34 (m, 3H), 1.34-1.22 (m, 1H), 1.22-0.96 (m, 5H), 0.96-0.73 (m, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 208.35, 172.51, 170.83, 166.28, 157.50, 148.85, 145.55, 142.22, 131.18, 130.68, 128.98,

127.62, 126.98, 123.96, 121.03, 114.84, 77.48, 77.16, 76.84, 67.02, 59.14, 54.42, 52.51, 50.27, 49.86, 49.21, 38.64, 38.29, 36.96, 34.37, 34.00, 32.00, 26.42, 26.27, 26.00, 18.15, 16.80, 12.53. HRMS calculated for $C_{37}H_{46}N_6O_6$ 671.35516 $[M+H]^+$; found 671.35529.

Biological analysis

General

Raji cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, GlutaMAX, penicillin, streptomycin in a 5% CO_2 humidified incubator at 37 °C. Cell lysates were prepared from cell pellets by resuspension in digitonin lysis buffer (50 mM Tris pH7.5, 5 mM $MgCl_2$, 1 mM DTT, 2 mM ATP, 0.025% digitonin; 5x pellet volume), incubation on ice for 60 min, and centrifugation for 15 min at 14000 rpm, after which the supernatants containing the cytosolic fractions were collected and the protein concentration was determined by Bradford assay. Precipitation of proteins was done using a chloroform/methanol (c/m) precipitation protocol.[8] SDS-PAGE analysis: in-gel fluorescence was measured on a Typhoon Variable Mode Imager (Amersham Biosciences) using Cy3/TAMRA settings (excitation wavelength 532 nm, emission wavelength 580 nm) or Cy2/Blue FAM settings (excitation wavelength 488 nm, emission wavelength 520 nm). As a loading control gels were stained with Coomassie Blue. Protein standards are Dual Color protein standard (DC, Bio-Rad) and biotinylated protein marker (BM, Bio-Rad).

***In vitro* competitive essay**

Raji lysates (10 µg total protein per experiment) in lysis buffer (9 µL) were exposed to the indicated concentrations of the ABP (1 µL 10x solution in DMSO) for 1 hr at 37 °C, prior to incubation with cocktail probe mixture (1.0 µL 10x solution in DMSO) for 1 hr at 37 ℃. The reaction mixtures were then boiled for 5 min at 100 ℃ with 4 µL 4x Laemmli's sample buffer containing 2- mercaptoethanol and resolved on 12.5% SDS-PAGE. In-gel visualization of the fluorescent labeling was performed in the wet gel slabs directly using Cy2/Cy3/Cy5. Intensities of bands were measured by fluorescent densitometry and divided by the intensity of bands in mock-treated extracts. Gels were stained by Coomassie Brilliant Blue, which was used to correct for gel loading differences. Average values of two or three independent experiments were plotted against inhibitor concentrations. IC_{50} (inhibitor concentrations giving 50% inhibition) values were calculated using GraphPad Prism software.

***In situ* competitive assay**

Raji cells (~$0.75x10^6$ cells per experiment) were collected per sample in a 48 well plate in 500 µL of medium after which the cells were exposed to the indicated concentration of the ABP (5 µL 100x solution in DMSO DMSO) for 2 hrs at 37 °C. Next cells were centrifuged for 5 min at 1200 rpm, and washed with PBS (1x). Afterwards cells were lysed on ice for 1 h in 30 µL of lysis buffer (50 µL, 50 mM Tris, pH 7.5, 2 mM DTT, 5 mM $MgCl_2$, 10% glycerol, 2 mM ATP, 0.05% digitonin) while vortexing every 15 min. Lysate was then centrifuged at 4 °C for 15 min at 14000 rpm while vortexing every 15 min. Lysate was then centrifuged at 4 ℃ for 15 min at maximum speed. 9 µL of cell lysate exposed to cocktail mix (1 µL, 10 x solution in DMSO) for 1 h at 37 °C. The reaction mixtures were then boiled for 5 min at 100 ℃ with 4 µL 4x Laemmli's sample buffer containing 2-mercaptoethanol and resolved on 12.5% SDS-PAGE. In-gel visualization of the fluorescent labeling was performed in the wet gel slabs directly using Cy2/Cy3/Cy5. Intensities of bands were measured by fluorescent densitometry and divided by the intensity of bands in mock-treated extracts. Gels were stained by Coomassie Brilliant Blue, which was used to correct for gel loading differences. Average values of two or three independent experiments were plotted against inhibitor concentrations. IC_{50} (inhibitor concentrations giving 50% inhibition) values were calculated using GraphPad Prism software.

***In vitro* two-step protein profiling of the proteasome**

General procedure for tetrazine ligation *in vitro*

Raji lysates (10 µg total protein per experiment) in lysis buffer (9 µL) were exposed to were exposed to the indicated concentrations the ABP (1 µL 10x solution DMSO) for 1 hr at 37 °C, followed by ligation with the indicated concentrations of tetrazine-BODIPY (1.0 µL 10x solution in DMSO) for 1 hr at 37 °C. In control experiments lysates were treated with Epoxymicin-norbornene (5 µM) (positive control) followed by ligation with the indicated concentrations of tetrazine-BODIPY (1.0 µL 10x sin olution in DMSO) for 1 h at 37 °C and subjected to tetrazine labeling in the absence of an ABP (background control). After the ligation reaction proteins were taken up in 10 µL Laemmli's sample buffer containing 2-mercaptoethanol, boiled for 5 min at 100 °C and resolved on 12.5% SDS-PAGE. Fluorescent labeling was visualized in the wet gel slabs directly using Cy5 settings.

General procedure for tetrazine ligation *in situ*

Raji cells (~$0.75x10^6$ cells per experiment) were collected per sample in a 48 well plate in 500 µL of medium after which the cells were exposed to the indicated concentration of the ABP (5 µL 100x solution in DMSO DMSO) for 2 hrs at 37 °C. After removal of the medium, the cells were washed with 3 mL medium for 30 min at 37 °C, and then

exposed to the indicated concentrations of Tetrazine-BODIPYTMR (5 µL 100x solution in DMSO) for 1 hr at 37 ℃. As a positive control, cells were exposed to Epoxymicin-norbornene (1 µM) (positive control) followed by ligation with the indicated concentrations of Tetrazine-BODIPYTMR (5.0 µL 100 x solution in DMSO) for 1 hr at 37 °C and subjected to tetrazine labeling in the absence of an ABP (background control). Next cells were centrifuged for 5 min at 1200 rpm, and washed with PBS (1x). Afterwards cells were lysed on ice for 1 h in 30 µL of lysis buffer (50 µL, 50 mM Tris, pH 7.5, 2 mM DTT, 5 mM $MgCl_2$, 10% glycerol, 2 mM ATP, 0.05% digitonin) while vortexing every 15 min. Lysate was then centrifuged at 4 °C for 15 min at 14000 rpm. The lysates (20 µg total protein per experiment) were boiled for 5 min at 100 °C with Laemmli's sample buffer containing 2-mercaptoethanol and resolved on 12.5% SDS-PAGE. Fluorescent labeling was visualized in the wet gel slabs directly using Cy5 settings.

General procedure for Copper(I)- catalysed click ligation *in vitro*

Raji lysates (10 µg total protein per experiment) in lysis buffer (9 µL) were exposed to were exposed to the indicated concentrations the ABP (1 µL 10x solution DMSO) for 1 hr at 37 °C. The mixture was then diluted with an additional 9 µL of buffer containing 10 mM $CuSO_4$, 60 mM sodium L-Ascorbate and 2 mM of THTPTA and exposed 1 h to the indicated concentration of alkyne-Cy2 (1 µL 20x solution DMSO). In control experiments lysates were treated with Epoxymicin-azide (1 µM) (positive control) followed by ligation with the indicated concentrations Alkyne-BODIPYFL **53** (1.0 µL 20x solution in DMSO) for 1 h at 37 °C and subjected to Alkyne-BODIPYFL **53** in the absence of an ABP (background control). After the ligation reaction proteins were taken up in 10 µL Laemmli's sample buffer containing 2-mercaptoethanol, boiled for 5 min at 100 °C and resolved on 12.5% SDS-PAGE. Fluorescent labeling was visualized in the wet gel slabs directly using Cy2 settings.

General procedure for Copper(I)- catalysed click ligation *in situ*

Raji cells (~$0.75x10^6$ cells per experiment) were collected per sample in a 48 well plate in 500 µL of medium after which the cells were exposed to the indicated concentration of the ABP (5 µL 100x solution in DMSO DMSO) for 2 h at 37 °C. In control experiments Raji cells were treated with Epoxymicin-azide (1 µM) (positive control) followed by ligation with the indicated concentrations Alkyne-BODIPYFL **53** (1.0 µL 20x solution in DMSO) for 1 h at 37 °C and subjected to Alkyne-BODIPYFL **53** in the absence of an ABP (background control). Next cells were centrifuged for 5 min at 1200 rpm, and washed with PBS (1x). Afterwards cells were lysed on ice for 1 h in 30 µL of lysis buffer while vortexing every 15 min. Lysate was then centrifuged at 4 °C for 15 min at maximum speed. 10 µL of cell lysate was diluted with an additional 9 µL of buffer containing 10 mM $CuSO_4$, 60 mM sodium L-Ascorbate and 2 mM of THTPTA and exposed 1 hr to the indicated concentration of alkyne-Cy2 (1 µL 20x solution DMSO). The reaction mixtures were then boiled for 5 min at 100 °C with 4 µL 4x Laemmli's sample buffer containing 2-mercaptoethanol and resolved on 12.5% SDS-PAGE. In-gel visualization of the fluorescent labeling was performed in the wet gel slabs directly using Cy2 settings.

8.5 Reference

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