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Two-Step Bioorthogonal Activity-Based Protein Profiling of Individual Human Proteasome Catalytic Sites

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Bioorthogonal chemistry allows the selective modification of biomolecules in complex biological samples. One application of this methodology is in two-step activity-based protein profiling (ABPP), a methodology that is particularly attractive where direct ABPP using fluorescent or biotinylated probes is ineffective. Herein we describe a set of norbornene-modified, mechanism-based proteasome inhibitors aimed to be selective for each of the six catalytic sites of human constitutive proteasomes and immunoproteasomes. The probes developed for β1i, β2i, β5c, and β5i proved to be useful two-step ABPs that effectively label their developed proteasome subunits in both Raji cell extracts and living Raji cells through inverse-electron-demand Diels–Alder (iEDDA) ligation. The compound developed for β1c proved incapable of penetrating the cell membrane, but effectively labels β1c in vitro. The compound developed for β2c proved not selective, but its azide-containing analogue LU-002c proved effective in labeling of β2c via azide–alkyne click ligation chemistry both in vitro and in situ. In total, our results contribute to the growing list of proteasome activity tools to include five subunit-selective activity-based proteasome probes, four of which report on proteasome activities in living cells.

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 the thymus-specific β5t particle, in addition to β1i and β2i, 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, we disclosed a set of inhibitors selective for one of each of the six catalytic subunits of human constitutive proteasomes and immunoproteasomes (Figure 1),[8a–c] whereas activity-based proteasome probes have also been reported by other research groups.[8d–j] With this set of subunit-selective inhibitors **1**–**7** (Figure 1 A), 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 (ABPP) assay using three activity-based probes (Figure 1 B), which are selective for β1c/β1i (Cy5-NC-001), β2c/β2i (BODIPY(FL)-LU-112), and β5c/β5i (BODIPY(TMR)-NC-005-VS): three compounds that are able to detect two out of the six catalytic activities of human constitutive proteasomes and immunoproteasomes combined.[8a] However, activity-based probes selective for one out of the six activities and that complement selective inhibitors are scare, and are the subject of this paper.

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 a covalent and irreversible inhibitor containing a bioorthogonal functional group (normally an azide, an alkyne, or electron-rich, strained alkene) reacts with an active

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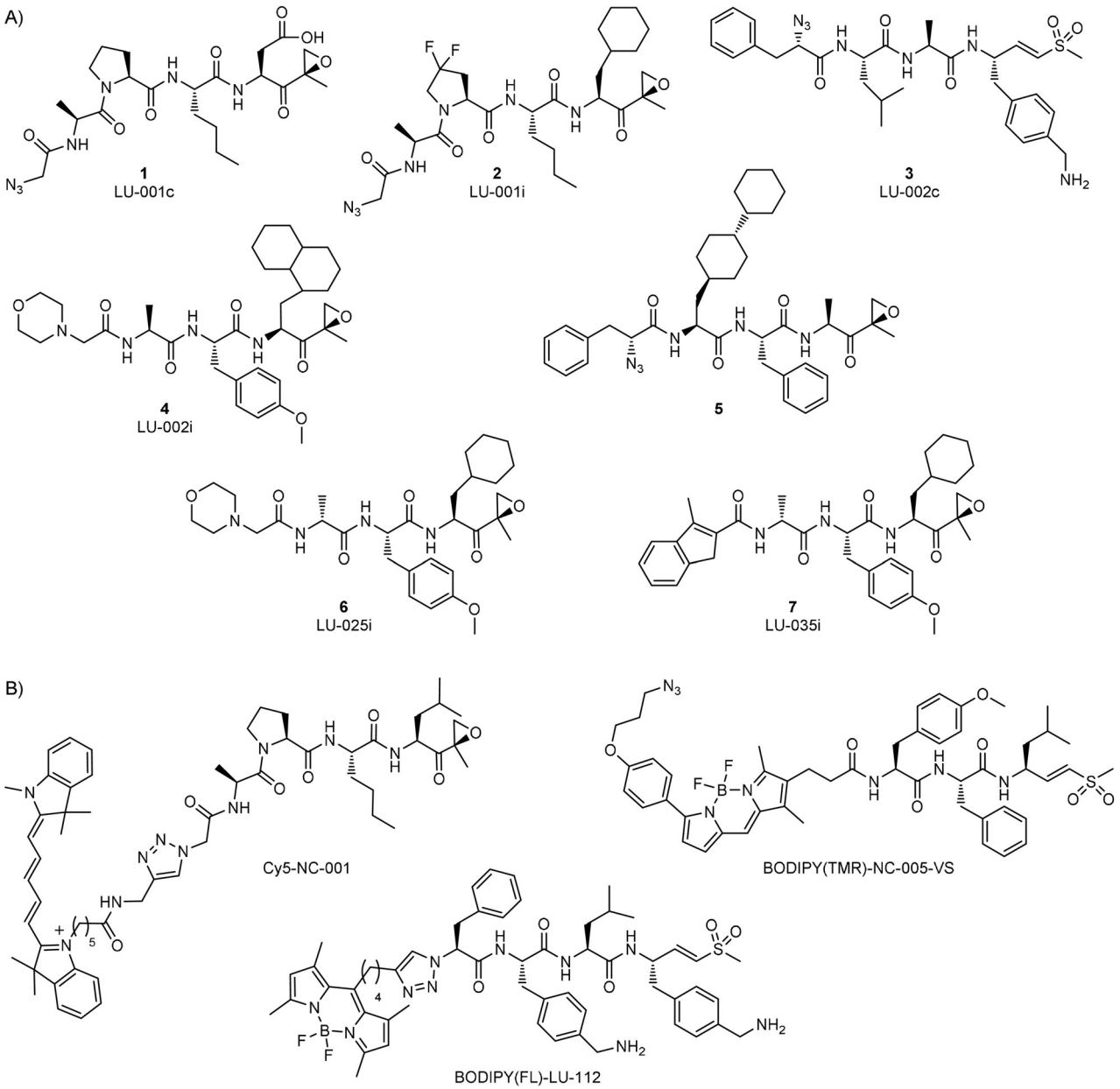

Figure 1. A) Structures of subunit-specific inhibitors of proteasomes: compound **1**, targeting β1c; compound **2**, targeting β1i; compound **3**, targeting β2c; compound **4**, targeting β2i; compound **5**, targeting β5c; and compounds **6** and **7**, targeting β5i. B) structures of cocktail ABPs: Cy5-NC-001 targeting β1c/β1i, BODIPY(FL)-LU-112 targeting β2c/β2i, and BODIPY(TMR)-NC-005-VS targeting β5c/β5i.

site residue of a target enzyme. Next, the reporter moiety (a fluorophore, 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 either 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 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 selective labeling of each catalytic activity of human constitutive proteasomes and human immunoproteasomes individually, a feat we cannot accomplish with our current set of activity-based proteasome probes.[8a] In this report, the results of research aimed at validating this hypothesis is described.

Results and Discussion

Compounds **1**, **2**, **3**, and **5** all feature a bioorthogonal azide in their structure. These compounds are therefore on paper suit-

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able 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 herein, 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 extent for proteasome inhibitors, and therefore it was decided to focus 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 development of potential two-step ABPs **8**–**14** (Figure 2) as strained, electron-rich alkenes for bioorthogonal labeling using iEDDA as the ligation strategy. Compound **12** was included in the research of bioorthogonal iEDDA reactions between non-strained vinylboronic acids and pyridyl-substituted tetrazines.[8j]

Compounds **8**, **9**, **10**, and **12** were prepared by means of a copper(I)-catalyzed [2+3] azide–alkyne cycloaddition (CuAAC) 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 the Supporting Information for details on the synthesis of compounds **9**, **10**, and **12** following the same strategy). 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** has been reported[8c] and the preparation of compounds **17** and **18** are described in the Supporting Information.

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 the corresponding acyl azide in situ, 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**

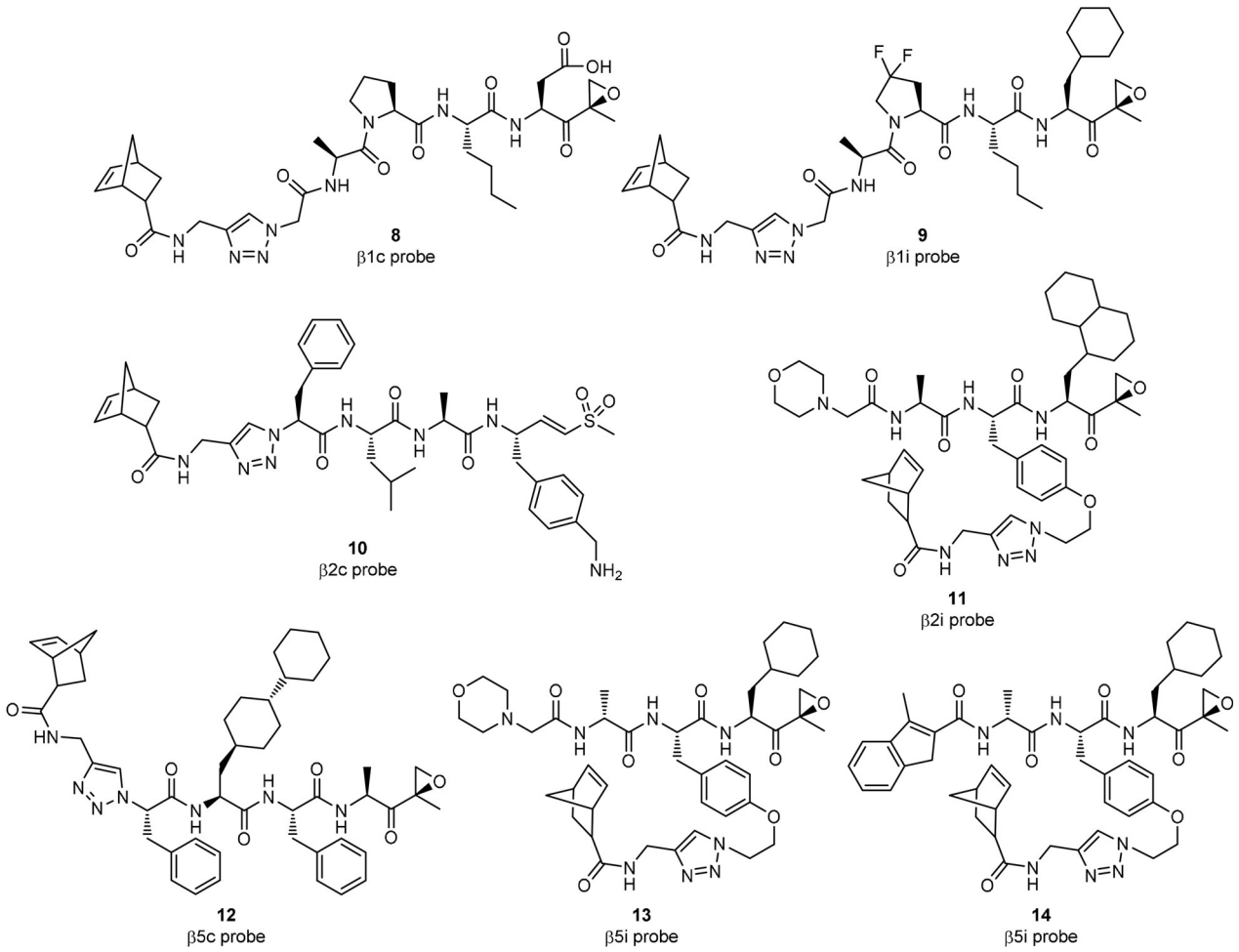

Figure 2. Structures of two-step ABPs **8**–**14**, subjects of the studies described herein.

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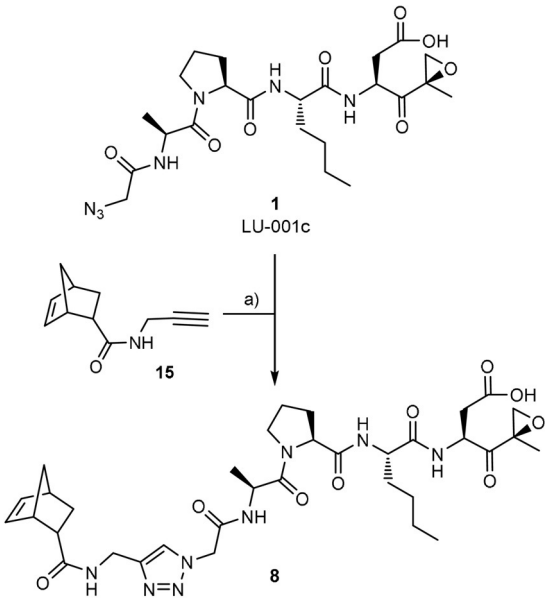


Scheme 1. Synthesis of compound **8**. a) $CuSO_4$, sodium ascorbate, DMF.

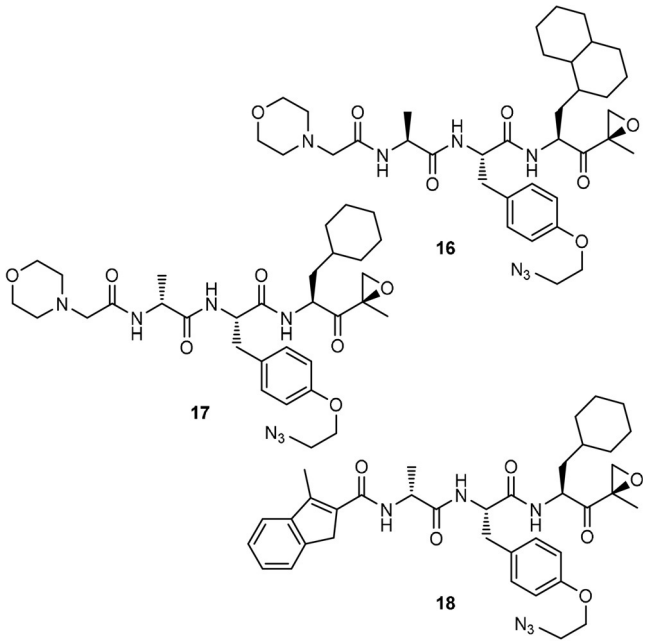
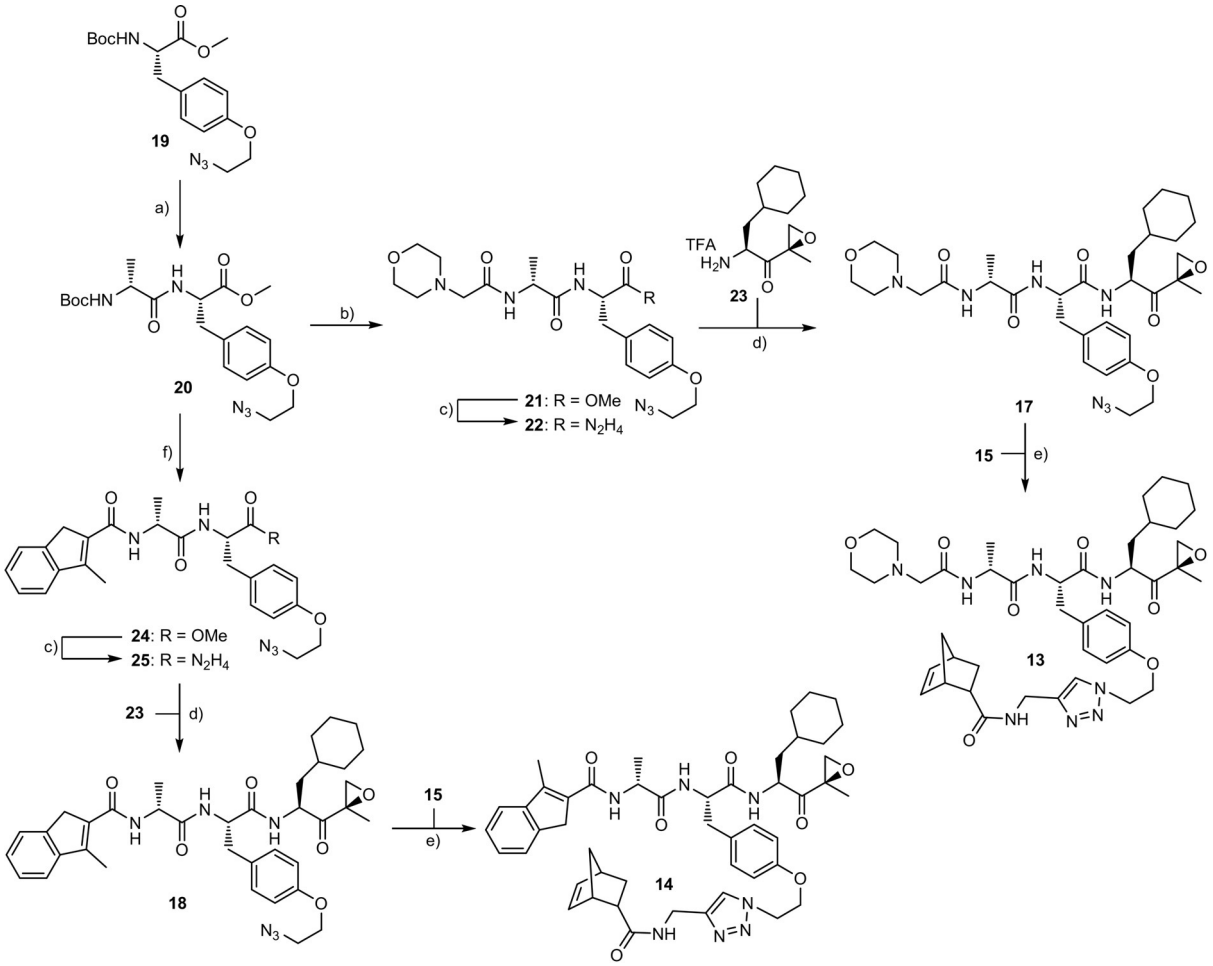


Figure 3. Structures of azide precursors **16**, **17**, and **18**.



Scheme 2. Synthesis of compounds **13** and **14**. a) i: TFA; ii: Boc-D-Ala-OH, HCTU, DiPEA, CH_2Cl_2; b) i: TFA; ii: 2-morpholinoacetic acid, HCTU, DiPEA, CH_2Cl_2; c) hydrazine monohydrate, MeOH; d) i: *t*BuONO, HCl, DMF/CH_2Cl_2 (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, CH_2Cl_2.

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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**.

In total, taking into account the previously reported azides **1**, **2**, **3**, **5** as well as the newly synthesized norbornenes **8**–**14**, a set of 11 potential subunit-selective two-step proteasome ABPs are available. In the first instance, we assessed their subunit-selectivity and activity by competitive ABPP making use of the activity-based proteasome profiling assay and the structures of these probes are shown in Figure 1B.[8a] The activity, selectivity and cell permeability of azides **1**, **2**,[8a] **3**,[8c] and **5**[8b] in such assays had already been described. The results on the behavior of norbornenes **8**–**14** in competitive ABPP, in particular their activity, selectivity, and cell permeability are summarized in Figure 4 and Tables 1 and 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 proteasomes and immunoproteasomes). As can be seen (Figure 4 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 five activities are equally well tagged by the three-probe mixture as in the control experiment in which no inhibitor was included (Figure 4A, 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 the cause for 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 the 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

Table 1. IC_{50} values for compounds **8**–**14**, determined in Raji cell lysates.

Compd	IC_{50} [μM]					
	β1c	β1i	β2c	β2i	β5c	β5i
8	0.022	>100	>100	>100	>100	>100
9	64.9	0.041	>100	>100	>100	9.67
10	>100	>100	0.071	0.14	0.0085	0.015
11	>100	>100	>100	0.24	>100	>100
12	>100	14.03	>100	>100	3.28	0.015
13	>100	>100	>100	>100	2.11	0.097
14	>100	>100	>100	>100	0.008	11.4

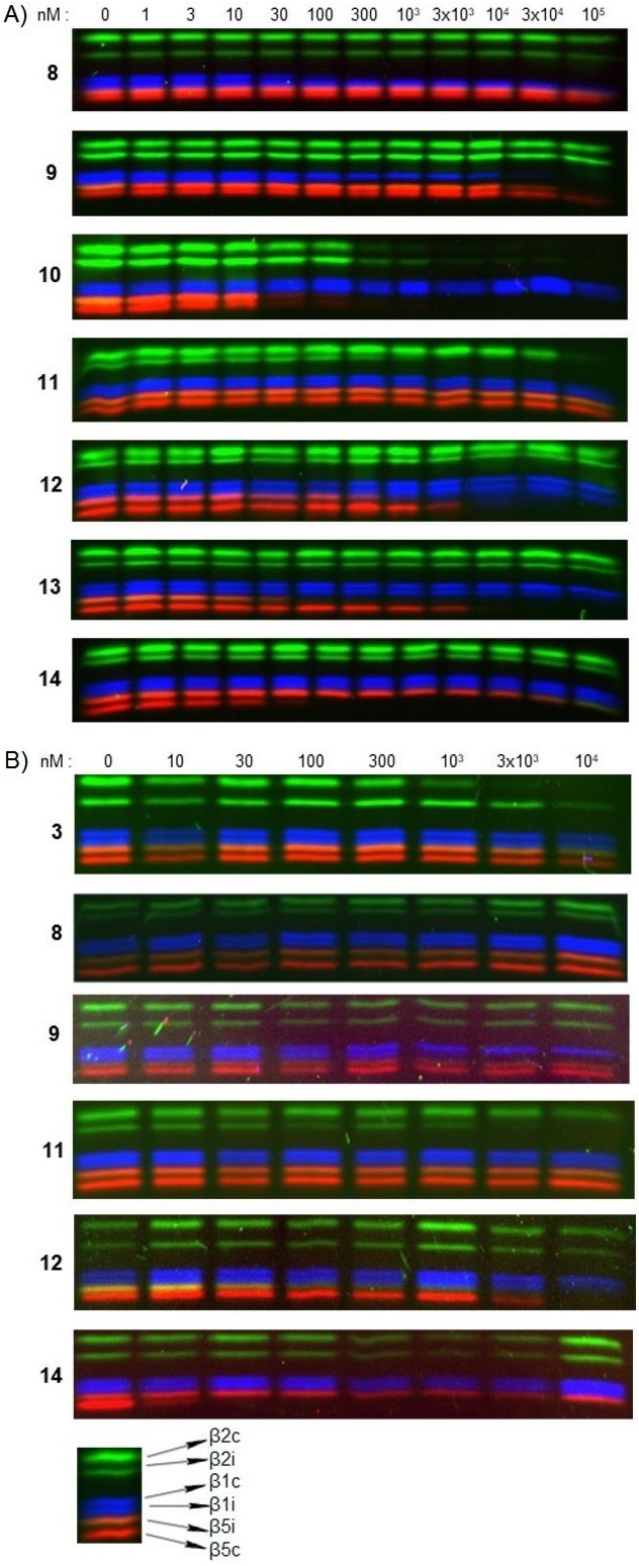

Figure 4. Inhibition profiles of competitive ABPP experiments. Probe Cys5-NC-001 labels β1c/i (blue); probe BODIPY(FL)-LU-112 labels β2c/i (green); probe BODIPY(TMR)-NC-005-VS labels β5c/i (red). A) Inhibition profiles of compounds **8**–**14**, determined in Raji cell lysates. B) Inhibition profiles of ABP **8**, **9**, **3** (LU-002c), **11**, **12**, and **14**, determined in intact Raji cells.

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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).

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[8a] as LU-002c) was included in the two-step ABPP experiments described below instead of norbornene **10** (which, as described above, does not target 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[8a] 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 5. Raji cell extracts were treated with compounds **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 to denaturation of the protein sample bioorthogonal ligation with either tetrazine-BODIPY-TMR **26**[13c] (in the case of norbornene ABPs **8**, **9**, **11**, **12**, or **14**) or alkyne-BODIPY-FL **27** (in the case of azide ABP **3**) using the appropriate iEDDA or click ligation protocol and in which reagents **26** or **27** were added at increasing concentrations (Figure 5 E). Norbornene-epoxomicin **28** and azido-epoxomicin **29**, 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 **26** or **27** 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 5), β1c can be selectively modified by first treatment of Raji cell extracts with norbornene **8** and next bioorthogonal ligation with tetrazine **26**. Labeling of β1c became apparent at 5.0 μM final concentration of **26** and could be strengthened by increasing the final concentration of **26** (Figure 5 A). At these high concentrations, however, an unspecific band appeared which runs at the same height as the proteasome β1i/5c/5i subunits on SDS-PAGE. This unspecific labeling also appeared in the negative control, in which cell extracts were treated with **26** 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 **26** (from 10.0 μM final concentration upward), visualizes β1i selectively. Likewise, treatment of cell extracts with norbornene **11** followed by **26** (10.0 μM final concentration) reveals β2i, whereas at higher concentrations of tetrazine **26** labeling of β5c/5i subunits becomes apparent besides the off-

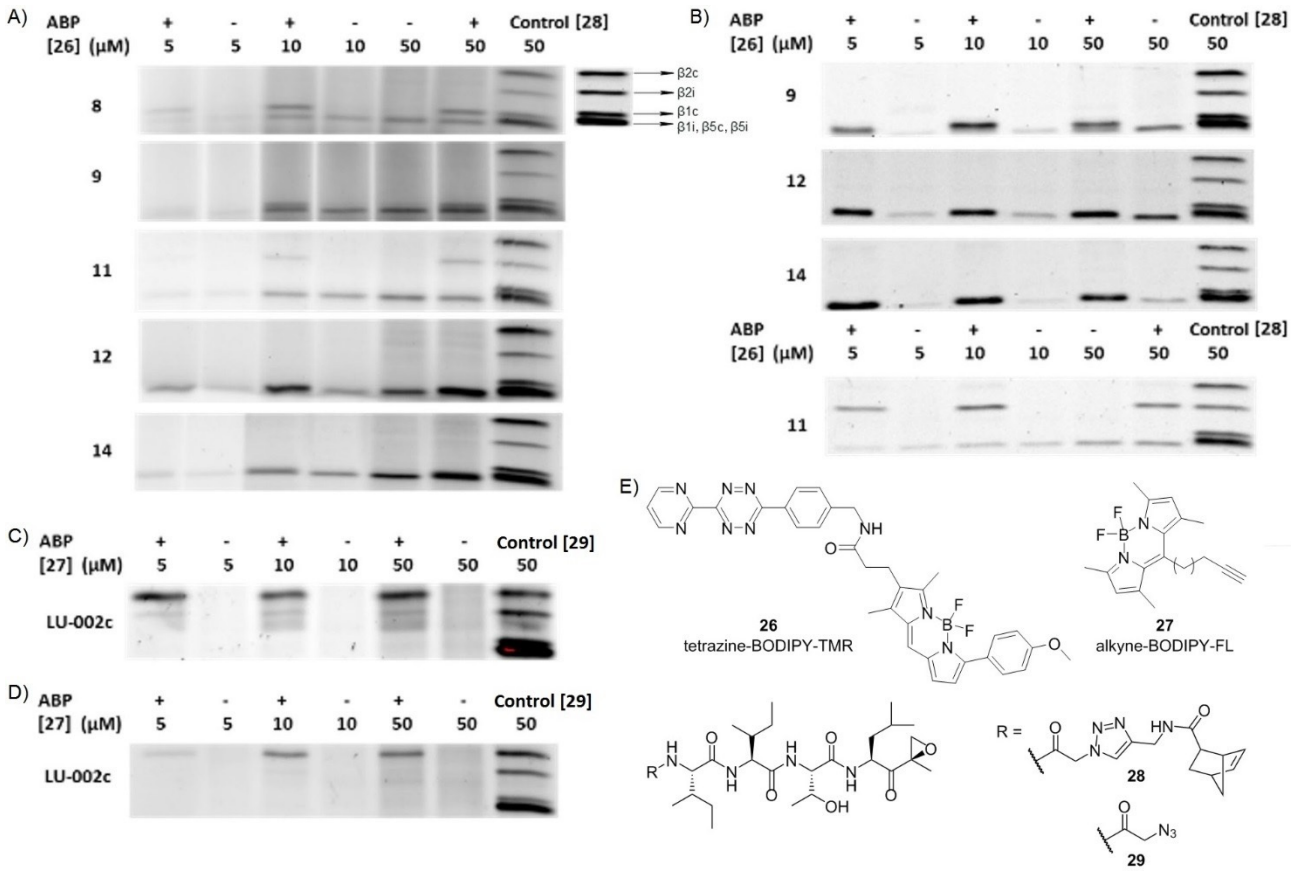

Figure 5. A) Two-step ABPP in Raji cell lysates using tetrazine ligation strategy. B) Two-step ABPP in living Raji cells using tetrazine ligation strategy. C) two-step ABPP in Raji cell lysates using CuAAC strategy. D) Two-step ABPP in living Raji cells by CuAAC-mediated ligation. E) Structures of ligation tags (**26**, **27**) and position controls (**28**, **29**).

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target labeling described above. In a similar vein, β5c and β5i can be visualized using norbornenes **12** and **14** respectively, in combination with tetrazine **26**. 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-BODIPY-FL (**27**) at increasing concentrations (Figure 5C).

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. 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 4B, 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 we conclude that for this compound 1.0 μM final concentration might 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. Compounds **12** and **14** finally appear potent and selective for β5c and β5i subunits in situ, with concentrations of 1.0 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).

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

Compd	IC_{50} [μM]					
	β1c	β1i	β2c	β2i	β5c	β5i
3	>10	>10	0.54	>10	>10	>10
8	>10	>10	>10	>10	>10	>10
9	>10	0.73	>100	>100	>100	>100
11	>10	>10	>10	0.14	>10	>10
12	>10	>10	>10	>10	>10	0.018
14	>10	>10	>10	>10	<0.01	>10

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 5B and D. When Raji cells were treated with compound **9** at 3.0 μM concentration, then lysed, incubated with 5 μM of **26**, followed by denaturation and resolving the protein content by SDS-PAGE, selective β1i labeling was observed (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 (**27**) under azide–alkyne [2+3] cycloaddition conditions at increasing concentrations, followed by the same denaturation and resolving the protein content on SDS-PAGE, selective β2c labeling was observed (Figure 5D).

Conclusions

In summary, a set of seven norbornene-modified peptide vinyl sulfones and peptide epoxyketones were developed 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 herein 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. Further studies are required to complete a toolset for labeling each immunoproteasome and constitutive proteasome activity individually, this to complement our three-probe multiplexing ABPP[8c] that is currently in use by us and others.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: activity-based probes · activity-based protein profiling · bioorthogonal chemistry · proteasomes · tetrazine ligation

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