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Elucidating the Catalytic Subunit Composition of Distinct Proteasome Subtypes: A Crosslinking Approach Employing Bifunctional Activity-Based Probes

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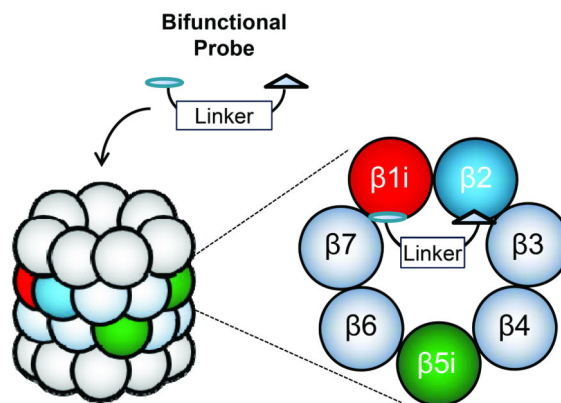
Abstract

In addition to two well-recognized proteasome subtypes—constitutive proteasomes and immunoproteasomes—mounting evidence also suggests the existence of intermediate proteasome subtypes containing unconventional mixtures of catalytic subunits. While they appear to play unique biological roles, the lack of practical methods for detecting distinct proteasome subtypes has limited functional investigations. Here, we report the development of activity-based probes that crosslink two catalytic subunits within intact proteasome complexes. Identification of the crosslinked subunit pairs provides direct evidence on the catalytic subunit composition of proteasomes. Using these probes, we found that U266 multiple myeloma cells contain intermediate proteasomes comprising both $\beta 1i$ and $\beta 2$, but not $\beta 1$ and $\beta 2i$, consistent with previous findings with other cell types. Our bifunctional probes can be utilized in functional investigations of distinct proteasome subtypes in various biological settings.

Graphical abstract

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Supporting information for this article is given via a link at the end of the document.



Keywords

Proteasome subtype; constitutive proteasome; immunoproteasome; intermediate proteasome; crosslinking agents

Introduction

Proteasomes process and degrade numerous cellular proteins, thereby serving as crucial regulators of a wide array of cellular processes including cell cycle progression,^[1] apoptosis,^[2] and immune responses.^[3] These multiprotease complexes are of particular interest due to their validation as effective therapeutic targets by the FDA approval of the proteasome inhibitor drugs bortezomib and carfilzomib for the treatment of multiple myeloma.^[4] Studies investigating the utility of proteasome inhibitors in the treatment of additional cancer types as well as autoimmune and inflammatory diseases are ongoing.^[5]

Proteasomes structurally comprise a cylindrically-shaped 20S catalytic core particle, which may be capped on one or both ends by regulatory particles.^[6] The 20S core particle is formed by 28 subunits arranged in four stacked rings, each containing seven subunits. Three different catalytically active proteasome subunits reside in each of the two inner rings, known as the β -rings.^[7] These catalytic subunits are synthesized as inactive precursors with N-terminal propeptides, which are removed during the final steps of 20S proteasome assembly to expose their catalytic threonine residues.^[8] Unique combinations of these catalytically active subunits can incorporate into otherwise identical 20S core particles, thereby forming distinct proteasome subtypes. For a number of years, the prevailing model of proteasome regulation acknowledged the predominant existence of two proteasome subtypes: constitutive proteasomes and immunoproteasomes. Constitutive proteasomes contain catalytic subunits $\beta 1$, $\beta 2$, and $\beta 5$ and are expressed in most cell types, whereas immunoproteasomes contain a homologous set of catalytic immunosubunits— $\beta 1i$, $\beta 2i$, and $\beta 5i$ —and are constitutively expressed in hematopoietic cells^[9] and inducible in other cell types following exposure to cytokines such as interferon- γ .^[10] The immunosubunits replace their constitutive counterparts during proteasome assembly, leading to alterations in proteolytic activities suggested to promote the production of MHC class I antigens.^[11] More recently, an immunoproteasome variant known as the thymoproteasome was discovered in

cortical thymic epithelial cells. The thymoproteasome contains immunosubunits $\beta 1i$ and $\beta 2i$, together with the thymoproteasome-specific subunit $\beta 5t$, and was shown to function in the positive selection of T cells.^[12]

Additionally, results from an increasing number of studies suggest the existence of intermediate proteasome subtypes comprising non-prototypical mixtures of constitutive proteasome and immunoproteasome catalytic subunits. Since their initial discovery in murine tissues,^[13] the presence of intermediate proteasome subtypes has been indicated in a variety of non-diseased tissues and cancer cells of murine and human origin.^[14] Proteasome subtypes with distinct catalytic subunit compositions appear to have unique proteolytic activity profiles as well as tissue and subcellular distribution patterns, suggesting that they may have specialized functions.^[13–15] Consistent with these observations, results from several investigations of antigenic peptide production suggested that intermediate proteasome subtypes serve to enhance the diversity of peptides presented on MHC class I molecules to CD8⁺ T cells.^[14b, 16] The exclusive production of specific tumor antigens by intermediate proteasome subtypes demonstrated the importance of identifying proteasome subtypes present in cells targeted by immunotherapy.^[14b] Furthermore, distinguishing proteasome compositions were associated with pathological conditions such as Crohn's disease and ulcerative colitis and were suggested as potential biomarkers for these diseases.^[17] Distinct proteasome subtypes are also linked to differential sensitivity to specific proteasome inhibitors;^[15b, 15c] thus, identifying proteasome subtypes present in diseased cells may ultimately facilitate the selection of therapeutic approaches which target the relevant subtypes with improved efficacy or with minimal toxicity to non-diseased cells.^[15c] However, much regarding the unique functions of each subtype or their roles as drug targets remains undiscovered, largely due to limitations in methods currently available for determining the catalytic subunit composition of individual proteasome subtypes within cells and tissues.

Earlier efforts to examine proteasome composition typically involved measuring the abundance of proteasome catalytic subunits in cell extracts or purified 20S proteasomes using various methods such as immunoblotting or 2D-PAGE^[8b, 14c, 15b, 15e, 17–18] as well as label-free LC-MS-based or ELISA-based quantification.^[14b, 14d, 15b, 15d] In these studies, the relative abundance of each catalytic subunit was then used to deduce the proteasome subtypes present within the cells or tissues being examined. However, examination of overall catalytic subunit abundance is insufficient to conclusively define the catalytic subunit composition of individual proteasome subtypes, especially if multiple subtypes are present.^[13]

More rigorous studies of distinct proteasome subtypes involved separation of purified 20S proteasome complexes by anion-exchange or hydrophobic interaction chromatography,^[13–14, 14e, 15c, 15f] isoelectric focusing-free flow electrophoresis,^[15a] or immunoprecipitation of exogenously expressed tagged forms of catalytic β -subunits,^[8b, 18] followed by evaluation of catalytic subunit composition by immunoblotting^[8b, 13, 14e, 15a, 18] or 2D-PAGE.^[14a, 15c, 15f] For example, Guillaume et al.^[14b] developed antibodies recognizing specific catalytic β -subunits in their native states, facilitating the identification of proteasome subtypes by sequential immunoprecipitation steps to deplete proteasomes

containing specific catalytic subunits and subsequent immunoblotting analysis. Using this approach, they confirmed the existence of two intermediate proteasome subtypes: one comprised of constitutive subunits $\beta 1$ and $\beta 2$ with immunosubunit $\beta 5i$ and the other comprised of constitutive subunit $\beta 2$ with immunosubunits $\beta 1i$ and $\beta 5i$.^[14b] While these techniques are considered improvements in detecting different proteasome subtypes, they require multiple technically-challenging steps and depend on the availability and affinity of antibodies that can bind to catalytic proteasome subunits in their native states.

An unmet need therefore exists to develop a more practical method for determining the catalytic subunit composition of distinct proteasome subtypes present within cell or tissue samples. We recently reported a fluorescence resonance energy transfer (FRET)-based approach for assessing the catalytic subunit composition of proteasomes.^[19] Expanding upon the designs of reversibly-binding bifunctional proteasome inhibitors previously reported by Moroder and colleagues,^[20] and subsequently by Vidal, Reboud-Ravaux and colleagues,^[21] we now report the development of an alternative approach in which irreversibly-binding bifunctional activity-based probes are utilized to crosslink pairs of catalytic subunits within individual 20S proteasomes, and thus to provide direct evidence of the catalytic subunit composition of each subtype (Figure 1).

Several proteasome inhibitors containing two reactive groups were previously developed from reversibly-binding inhibitors in effort to obtain highly potent or subunit-selective proteasome inhibitors.^[20–22] Indeed, joining of two peptide aldehyde proteasome inhibitors,^[20] or of two tripeptide linear mimics of the noncovalent proteasome inhibitor TMC-95A,^[21] by polyethylene glycol (PEG) or polyaminohexanoic acid spacers gave rise to bifunctional inhibitors displaying substantial increases in inhibitory potency of up to 2 orders of magnitude over their respective monomeric parent inhibitors. Unlike these reversible bifunctional inhibitors, the bifunctional probes reported here were synthesized by coupling two irreversible, subunit-selective peptide epoxyketone proteasome inhibitors via hydrocarbon or PEG linkers (Figure 1). The crystal structure-derived distances between catalytic threonine O^γ atoms of different subunit pairs within the mammalian 20S proteasome guided our selection of linkers of the appropriate lengths to allow crosslinking of the desired catalytic subunit pairs.^[23] The epoxyketone pharmacophore binds specifically to the catalytic threonine residues of proteasome subunits covalently and irreversibly,^[24] facilitating the detection of these crosslinked subunit pairs by immunoblotting analysis and the subsequent identification of distinct proteasome subtypes present within cells.

Results

$\beta 1$ - $\beta 2$ / $\beta 2i$ Crosslinking

Our laboratory and others have developed several subunit-selective proteasome inhibitors.^[19, 25] To determine the feasibility of our crosslinking strategy, we first utilized two of these inhibitors readily available in our laboratory—the $\beta 1$ / $\beta 1i$ -selective inhibitor UKP1-3^[19] and the $\beta 5$ / $\beta 5i$ -selective inhibitor LKS01^[25c]—to generate the first peptide epoxyketone-based bifunctional proteasome probe. UKP1-3 and LKS01 are derivatives of the $\beta 1$ / $\beta 1i$ -selective inhibitor YU-102^[25a, 26] and the $\beta 5i$ -selective inhibitor IPSI,^[25b] respectively, in which the P4 glycine side chain of YU-102 and the P2 tryptophan side chain

of IPSI were replaced with that of lysine to facilitate their coupling via an amine-reactive linker. Modifications at these positions do not alter the subunit selectivities of these inhibitors.^[19, 25c, 25e] Coupling of UKP1-3 and LKS01 via a short hydrocarbon linker with an estimated length of ~11.4 Å produced UKP13-C₆-LKS01 (probe **1**) (Figure 2, Scheme 1).

We first ensured that probe **1** displays the expected inhibitory profile by conducting proteasome activity assays with subunit-selective fluorogenic substrates^[27] in purified human 20S proteasomes and in U266 multiple myeloma cell lysates, which abundantly express all six constitutive proteasome and immunoproteasome catalytic subunits.^[28] As expected, this probe was found to preferentially inhibit the β1i and β5i subunits of the purified immunoproteasome (Table 1). Preferential inhibition of β1 and β5 of the purified constitutive proteasome was also observed. In contrast, concentrations of 10 μM or higher were required to achieve 50% inhibition of the β2 and β2i activity (Table 1). Consistent with these findings, treatment of U266 cell lysates with 1 μM of probe **1** resulted in nearly complete inhibition of all β1/β1i and β5/β5i activity, whereas a concentration of 10 μM was required to achieve ~50% inhibition of β2/β2i activity (Supplemental Table 1). Together, these results indicate that the subunit binding preferences of the parent inhibitors were in fact maintained by bifunctional probe **1**.

We next assessed the ability of probe **1** to crosslink two proteasome catalytic subunits via immunoblotting with antibodies recognizing proteasome catalytic subunits. In these assays, crosslinked subunits can be visualized by the detection of immunoreactive bands between 40 and 50 kDa (the combined molecular weight of two proteasome catalytic subunits). As shown in Figure 3A, treatment of the purified immunoproteasome with probe **1** produced an anti-β1i-immunoreactive band at ~45 kDa—consistent with the crosslinked β1i subunit. We further validated these findings by treating U266 cell lysates with probe **1** and were able to detect two bands at ~45–50 kDa on a β1i immunoblot (Figure 3B), in contrast to the single band observed in this molecular weight range following treatment of the purified immunoproteasome with this probe. The appearance of two crosslinked β1i bands suggested that β1i was likely crosslinked with two different subunits by probe **1**. In order to identify the other subunits crosslinked with β1i, we pretreated U266 lysates with the subunit-selective inhibitors NC-012 (β2/β2i-selective)^[25f] or IPSI (β5i-selective)^[25b] prior to treatment with probe **1**. Pretreatment with NC-012, but not with IPSI, was able to completely block β1i crosslinking, as indicated by the complete depletion of these two crosslinked β1i bands (Figure 3B), demonstrating that the subunits crosslinked with β1i by probe **1** are β2 and β2i. These results indicate that, in addition to proteasome subtypes containing both β1i and β2i, intermediate proteasome subtypes containing the immunosubunit β1i together with the constitutive subunit β2 are present in U266 multiple myeloma cells. It should be noted that probe **1** was found to crosslink β1i with β2/β2i, despite its preferential inhibition of β1/β1i and β5/β5i as revealed by proteasome activity assay results (Supplemental Table 1). This apparent discrepancy between the inhibitory and crosslinking preferences of probe **1** is likely due to the spatial constraint incurred by the C₆ hydrocarbon linker, driving crosslinking between adjacent subunits.

To further verify that the higher-molecular-weight bands observed on our immunoblots following treatment with probe **1** in fact result from crosslinking of proteasome catalytic

subunits, we synthesized the bifunctional proteasome probe UKP13-SS-LKS01 (probe **2**) in which the C₆ linker of probe **1** was replaced with a PEG linker containing a scissile disulfide bond (Figure 2, Supplemental Information). Cleavage of this disulfide bond by adding a reducing agent, such as β -mercaptoethanol, to probe **2**-treated samples should reverse the crosslinks formed by this probe. Assessment of the subunit selectivity of this probe in the purified immunoproteasome revealed selective binding to β 5i and β 1i (Table 1). Likewise, β 1 and β 5 were preferred binding targets of this probe in the purified constitutive proteasome. Similar to probe **1** the IC₅₀ values of probe **2** for β 2 and β 2i were significantly higher than those for β 1/ β 1i and β 5/ β 5i (Table 1).

We treated U266 cell lysates with probe **2** and denatured the proteins in the presence or absence of β -mercaptoethanol. In the absence of β -mercaptoethanol, two major bands between 37 and 50 kDa were observed on a β 1i immunoblot (Figure 3C), indicating that this probe crosslinks β 1i. These bands were no longer detected following disulfide bond cleavage by β -mercaptoethanol (Figure 3C), confirming that they resulted from β 1i crosslinking by probe **2**. (Of note, although it is possible that this disulfide bond may be cleaved by intracellular glutathione present in cell lysates, the observance of bands consistent with crosslinked β 1i in the absence of β -mercaptoethanol indicates that the linker remains intact under these experimental conditions.) These results further confirm that the immunoreactive bands in the 40–50 kDa range observed following treatment with our bifunctional probes result from crosslinking of proteasome catalytic subunits.

Encouraged by the successful crosslinking of β 1i with β 2/ β 2i by probe **1** we synthesized UKP13-(PEG)₄-NC012 (probe **3**), in which, in effort to improve the β 1/ β 1i- β 2/ β 2i crosslinking efficiency, we replaced the LKS01 inhibitor and short C₆ hydrocarbon linker of probe **1** with the β 2/ β 2i-selective inhibitor NC-012 [25f] and a more flexible and water-soluble PEG linker, respectively. The N-terminus of NC-012 was derivatized to facilitate linker attachment without compromising the inhibitor's subunit selectivity [25f]. Given the relatively short (~28 Å) distance between the catalytic Thr10^y atoms of β 1/ β 1i and β 2/ β 2i within a β -ring (Figure 1), [23] we selected a short (PEG)₄ linker with an estimated length of ~23.2 Å (Figure 2). Using proteasome activity assays, we verified that probe **3** maintained comparable β 1/ β 1i inhibition to that exhibited by probe **1** (Table 1). Conversely, in comparison with probe **1**, probe **3** displayed a markedly enhanced preference for β 2/ β 2i inhibition and a reduced preference for inhibition of β 5/ β 5i (Table 1). Data obtained in U266 cell lysates are in agreement with these altered binding preferences (Supplemental Table 1).

Treatment of either the purified constitutive proteasome (Supplemental Figure 1A) or U266 cell lysates (Figure 3D) with probe **3** followed by immunoblotting analysis led to the appearance of an anti- β 1-immunoreactive band slightly below 50 kDa, consistent with β 1- β 2 crosslinking. A competition assay with the subunit-selective inhibitors IPSI (β 5i-selective), [25b] Ac-PAL-ek (β 1i-selective), and NC-012 (β 2/ β 2i-selective) [25f] in the purified immunoproteasome verified that probe **3** also crosslinks β 1i with β 2i, but not with β 5i (Supplemental Figure 1B). β 1i crosslinking was also observed in U266 cell lysates following treatment with probe **3**. Immunoblotting analysis revealed two anti- β 1i-immunoreactive bands at ~45–50 kDa (Figure 3E), similar to the results obtained using probe **1** strongly indicating β 1i- β 2/ β 2i crosslinking by probe **3**.

Taken together, the results obtained with probes **1** and **3** indicate that U266 cells harbor proteasome subtypes containing both $\beta 1i$ and $\beta 2i$, as well as those containing both $\beta 1i$ and $\beta 2$. Interestingly, we did not detect proteasome subtypes containing constitutive subunit $\beta 1$ with immunosubunit $\beta 2i$, despite the results indicating that proteasomes containing both $\beta 1$ and $\beta 2$ are also present in this cell line (Figure 3D).

$\beta 1/\beta 1i$ - $\beta 5/\beta 5i$ Crosslinking

Next, we sought to develop a bifunctional proteasome probe capable of crosslinking $\beta 1/\beta 1i$ with $\beta 5/\beta 5i$. To this end, we coupled the $\beta 1/\beta 1i$ -selective inhibitor UKP1-3 with a derivative of the $\beta 5$ -selective inhibitor PR-825^[25g, 25h] in which the N-cap of PR-825 was replaced by a 6-aminohexanoyl group to provide UKP13-(PEG)₄₉₄-6Ahx-PR825 (probe **4**) (Figure 2). The N-terminal amine of this PR-825 derivative served as the attachment point for coupling to UKP1-3 via a PEG linker with an estimated length of ~ 83.9 Å, which we expected to be more than sufficient for crosslinking $\beta 1/\beta 1i$ with $\beta 5/\beta 5i$, whose catalytic residues are separated by ~ 62 Å (Figure 1).^[23] Activity assays demonstrated that probe **4** binds preferentially to the $\beta 5$ and $\beta 1i$ subunits of the purified constitutive proteasome, and to the $\beta 1i$ and $\beta 5i$ subunits of the purified immunoproteasome (Table 1). Treatment with 5 μ M of probe **4** completely inhibits $\beta 1/\beta 1i$ and $\beta 5/\beta 5i$ activity, as expected (Supplemental Table 1).

Treatment of the purified immunoproteasome with probe **4** led to the appearance of two immunoreactive bands between ~ 40 – 50 kDa on a $\beta 1i$ immunoblot (Figure 4A, left panel). Upon similar analysis using a $\beta 5i$ antibody, a crosslinked $\beta 5i$ band was detected at an apparent molecular weight comparable to that of the major crosslinked $\beta 1i$ band (Figure 4A, middle panel), suggesting that it represents $\beta 1i$ - $\beta 5i$ crosslinking. The minor immunoreactive crosslinked $\beta 1i$ band likely represents $\beta 1i$ - $\beta 2i$ crosslinking, based on the immunoblotting results obtained using a $\beta 2i$ antibody (Figure 4A, right panel). These results indicate that probe **4** preferentially crosslinks $\beta 1i$ with $\beta 5i$, and crosslinks $\beta 1i$ with $\beta 2i$ to a much lesser extent. Competition assays were conducted to confirm these results. Specifically, pretreatment of the purified immunoproteasome with the $\beta 5i$ -selective inhibitor IPSI^[25b] prior to treatment with probe **4** competed away the higher-molecular-weight anti- $\beta 5i$ -immunoreactive band (Figure 4B), confirming that this band indeed represents crosslinked $\beta 5i$. Pretreatment with the $\beta 1i$ -selective inhibitor Ac-PAL-ek also effectively depleted this band (Figure 4B), confirming that the subunit crosslinked with $\beta 5i$ is $\beta 1i$.

To further examine the subunits crosslinked by probe **4** we next treated the purified constitutive proteasome with this probe and, upon immunoblotting analysis, observed the appearance of two anti- $\beta 1$ -immunoreactive bands resulting from $\beta 1$ crosslinking (Figure 4C, left panel). When a similar analysis was performed using a $\beta 5$ antibody, a crosslinked $\beta 5$ band was detected at an apparent molecular weight comparable to the lower crosslinked $\beta 1$ band (Figure 4C, right panel), suggesting $\beta 1$ - $\beta 5$ crosslinking by probe **4**. Alternatively, alignment of the upper crosslinked $\beta 1$ band observed for the probe **4**-treated constitutive proteasome with the band resulting from $\beta 1$ crosslinking by probe **3** (Supplemental Figure 2A) suggests that this band represents $\beta 1$ - $\beta 2$ crosslinking. Competition assays were again conducted to verify the identities of the subunits crosslinked by probe **4**. Pretreatment of the purified constitutive proteasome with the $\beta 1$ -selective inhibitor Ac-nLPnLD-CHO prior to

treatment with probe **4** substantially attenuated both of the higher-molecular-weight anti- β 1-immunoreactive bands (Figure 4D and Supplemental Figure 2B), confirming that they both represent crosslinked β 1. Conversely, pretreatment with the β 5/ β 5i-selective inhibitor carfilzomib^[29] selectively competes away only the lower crosslinked β 1 band (Figure 4D), while pretreatment with the β 2/ β 2i-selective inhibitor NC-012^[25f] selectively competes away only the upper crosslinked β 1 band (Supplemental Figure 2B), confirming that these bands result from β 1- β 5 and β 1- β 2 crosslinking, respectively.

Discussion

Here, we report for the first time the development of irreversibly-binding bifunctional activity-based probes which can be used in a crosslinking strategy to determine the catalytic subunit composition of intact proteasome complexes present within cells. These probes, generated by coupling two epoxyketone pharmacophore-based proteasome inhibitors via linkers of varying lengths and chemical compositions, crosslink different pairs of proteasome catalytic subunits. Several bifunctional proteasome-targeting compounds were previously developed from reversible inhibitors in effort to improve inhibitory potency and subunit selectivity over the monomeric inhibitors of which they are comprised.^[20–21] In contrast to these reversible bifunctional inhibitors, our probes are designed to irreversibly crosslink specific pairs of catalytic subunits within individual proteasome complexes, permitting identification of the two probe-bound subunits by SDS-PAGE and immunoblotting. Results obtained using our bifunctional probes provide direct evidence of catalytic subunit colocalization within individual 20S proteasome core complexes, thereby allowing proteasomes of distinct compositions to be identified.

While the inhibitory preferences of each bifunctional probe are largely determined by those of their respective monomeric inhibitors, the length and composition of the linker by which these inhibitors are coupled appears to be an important determinant of the crosslinking efficiency of a particular catalytic subunit pair. For example, although probe **1** preferentially inhibits the activities of β 1/ β 1i and β 5/ β 5i, it preferentially crosslinks β 1i with β 2 and with β 2i (Figure 3A and B). Alternatively, crosslinking of β 1/ β 1i with β 5/ β 5i could be achieved by coupling inhibitors targeting these subunits by a significantly longer PEG linker, as observed with probe **4** (Figures 2 and 4). In addition, probe **4** also crosslinks β 1/ β 1i with β 2/ β 2i (Figure 4 and Supplemental Figure 2), demonstrating that the long flexible PEG linker allows crosslinking of multiple catalytic subunit pairs within the 20S proteasome complex.

Compared with previous approaches in which the identities of proteasome subtypes present within a particular cell or tissue type are indirectly deduced from the overall expression levels of individual proteasome catalytic subunits,^[8b, 14b–d, 15b, 15d, 15e, 17–18] our crosslinking approach allows proteasomes with distinct compositions to be clearly identified, even when multiple subtypes are present.^[13] Additionally, in contrast to approaches requiring separation of subtypes via chromatographic methods, isoelectric focusing-free flow electrophoresis, or immunoprecipitation,^[8b, 13–14, 14e, 15a, 15c, 15f, 18] our crosslinking strategy does not require the purification of proteasomes or separation of individual proteasome subtypes; incubation of our bifunctional probes with cell extracts can

be immediately followed by protein denaturation and immunoblotting analysis without additional processing steps. Furthermore, in contrast to our previously-reported FRET-based approach,^[19] the crosslinking approach reported here does not require the careful selection of subunit-specific concentrations of each probe for accurate interpretation of the experimental results.

Studies of proteasome assembly pathways indicate that catalytic subunits are not incorporated at random; there appear to be regulatory mechanisms in place to restrict the combinations of catalytic subunits that exist together within mature 20S core particles. These studies suggested that incorporation of $\beta 2i$ into proteasome complexes requires the presence of $\beta 1i$,^[8a, 8b, 30] thus precluding the formation of intermediate proteasomes containing $\beta 2i$ without $\beta 1i$. Furthermore, $\beta 5i$ is required for the proper maturation of proteasomes containing either $\beta 1i$ or $\beta 2i$. Consequently, intermediate proteasomes comprised of $\beta 1i$ and/or $\beta 2i$ with $\beta 5$ cannot form, while the incorporation of $\beta 5i$ into proteasomes containing $\beta 1i$ and $\beta 2i$ leads to the formation of mature immunoproteasomes.^[8a, 8b, 31]

However, in contrast to the requirement of $\beta 1i$ for $\beta 2i$'s incorporation, $\beta 1i$ can incorporate into proteasomes without $\beta 2i$.^[8b] Additionally, $\beta 5i$ can efficiently incorporate into proteasomes comprised of either constitutive subunits or immunosubunits.^[8a, 8b, 30–31] Therefore, in addition to pure constitutive proteasomes and pure immunoproteasomes, intermediate proteasomes comprised of $\beta 1i$ - $\beta 2$ - $\beta 5i$ and $\beta 1$ - $\beta 2$ - $\beta 5i$ can also form. Although these subtypes were originally identified in transfected cell lines and cells from knockout mice,^[8a, 8b, 30–31] their endogenous formation in murine and human cells has been confirmed.^[13, 14b] $\beta 1i$ -containing subtypes ($\beta 1i$ - $\beta 2$ - $\beta 5i$ and $\beta 1i$ - $\beta 2i$ - $\beta 5i$) can be distinguished from one another by their discrete combinations of $\beta 1i$ with $\beta 2$ or $\beta 2i$. Likewise, $\beta 1$ -containing subtypes ($\beta 1$ - $\beta 2$ - $\beta 5$ and $\beta 1$ - $\beta 2$ - $\beta 5i$) can be distinguished from one another by their discrete combinations of $\beta 1$ with $\beta 5$ or $\beta 5i$. In this regard, our bifunctional probes that crosslink these subunit pairs can be utilized to detect the presence of these distinct proteasome subtypes. Results obtained using our probes in the current study indicate that, in addition to the two conventional proteasome subtypes (pure constitutive proteasomes and immunoproteasomes), U266 cells contain intermediate proteasome subtypes of composition $\beta 1i$ - $\beta 2$ - $\beta 5i$ (Figure 3 B and E). In contrast, we were unable to detect the presence of subtypes containing both $\beta 1$ and $\beta 2i$ (Figure 3D), consistent with previous reports suggesting that coinorporation of these subunits is not preferred.^[8a, 14b, 30]

Conclusion

In summary, it is clear that recent advances in understanding of proteasome biology have led to an increased interest in dissecting the unique functions of each proteasome subtype. This in turn necessitates the development of improved methods to readily identify the subtypes present within complex biological samples. Our crosslinking strategy, which allows the direct analysis of proteasome catalytic subunit composition in cell or tissue lysates without requiring their prior purification or separation, is well suited for this application. Studies employing this new strategy will facilitate evaluations of the functional roles of distinct proteasome subtypes and their relevance as drug targets or disease biomarkers.

Experimental Section

Cell Culture and Whole Cell Lysis

U266 human multiple myeloma cells were purchased from the American Type Culture Collection and cultured under the recommended conditions at 37°C and 5% CO₂. The cells were lysed in Passive Lysis Buffer (Promega) according to the manufacturer's instructions, and the protein concentration of the lysate was determined using the Bio-Rad Protein Assay (Bio-Rad).

Proteasome Activity Assays

Purified human 20S constitutive proteasome or immunoproteasome (Boston Biochem) (50 ng/well) were incubated with DMSO or increasing concentrations of each bifunctional probe for 1 hr at room temperature in 20S proteasome assay buffer (20 mM Tris/Cl, pH 8.0, 0.5 mM EDTA, 0.035% SDS) in 96-well plates (total volume/well = 90 μ L). (For the trypsin-like substrate, SDS was omitted from the assay buffer.) 10 μ L of each substrate diluted in assay buffer was then added to obtain a final volume of 100 μ L/well. The following subunit-selective fluorogenic substrates were used at a final concentration of 100 μ M: Ac-nLPnLD-AMC (β 1-selective),^[27a] Ac-PAL-ek (β 1i-selective), Ac-ANW-AMC (β 5i-selective),^[27b] Ac-RLR-AMC (β 2/ β 2i-selective)^[27a] and Ac-WLA-AMC (β 5-selective)^[27b] were used at a final concentration of 20 μ M. Fluorescence produced by the release of AMC was measured once per min over 1hr on a SpectraMax M5 microplate reader (Molecular Devices) using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Reaction velocities were determined using linear regression in GraphPad Prism, and those of the DMSO-treated controls were set as 100%. IC₅₀ values were determined by nonlinear regression using GraphPad Prism and represent the averages of 3 replicates.

Western Blotting

U266 cell lysates were diluted to a concentration of 1 μ g/ μ L in Passive Lysis Buffer (Promega). Alternatively, purified human 20S constitutive proteasome or immunoproteasome (Boston Biochem) was diluted to a concentration of 5–15 ng/ μ L in 20S proteasome assay buffer (20 mM Tris/Cl, pH 8.0, 0.5 mM EDTA, 0.035% SDS). Samples were then treated with DMSO, 5 μ M epoxomicin (a broadly-acting proteasome inhibitor used as a positive control for covalent modification of β 1i, β 2, β 2i, β 5, and β 5i), 10 μ M YU-102 (a β 1/ β 1i-selective inhibitor^[25a, 26] used as a positive control for covalent modification of β 1), or 1–10 μ M of each bifunctional probe for 4 hrs at room temperature. Where indicated, samples were treated with DMSO, 3 μ M Ac-nLPnLD-CHO (β 1-selective, unpublished results), 3 μ M NC-012 (β 2/ β 2i-selective),^[25f] 0.02 μ M carfilzomib (β 5/ β 5i-selective),^[29] 1 μ M Ac-PAL-ek (β 1i-selective, unpublished results), or 0.1 μ M IPSI (β 5i-selective)^[25b] for 1 hr at room temperature prior to the addition of bifunctional probes. 2X Laemmli sample buffer (Sigma-Aldrich) was added to each sample, and proteins were denatured at 100°C for 10 min. An equivalent protein amount of each sample was resolved by 14% SDS-PAGE, and proteins were subsequently transferred onto PVDF membranes (Bio-Rad). Membranes were incubated in 5% non-fat dry milk (Bio-Rad) in Tris-buffered saline with 0.05% Tween-20 (TBST) for 1 hr at room temperature. The following primary antibodies were diluted in TBST solutions containing 3% BSA: β 1i and β 5i (Abcam), β 1

(Enzo Life Sciences), $\beta 5$ (Thermo Scientific). Alternatively, the $\beta 2i$ antibody (Santa Cruz) was diluted in 3% milk in TBST. Membranes were incubated in these primary antibody solutions overnight at 4°C. Anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibodies (GE Healthcare) were diluted 1:20,000 in 3% milk in TBST, and membranes were incubated with these antibodies for 1 hr at room temperature. SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific) and X-ray film (Thermo Scientific) were utilized for protein visualization.

The synthesis of the bifunctional probes and proteasome activity assays are described in the Supplemental Information section.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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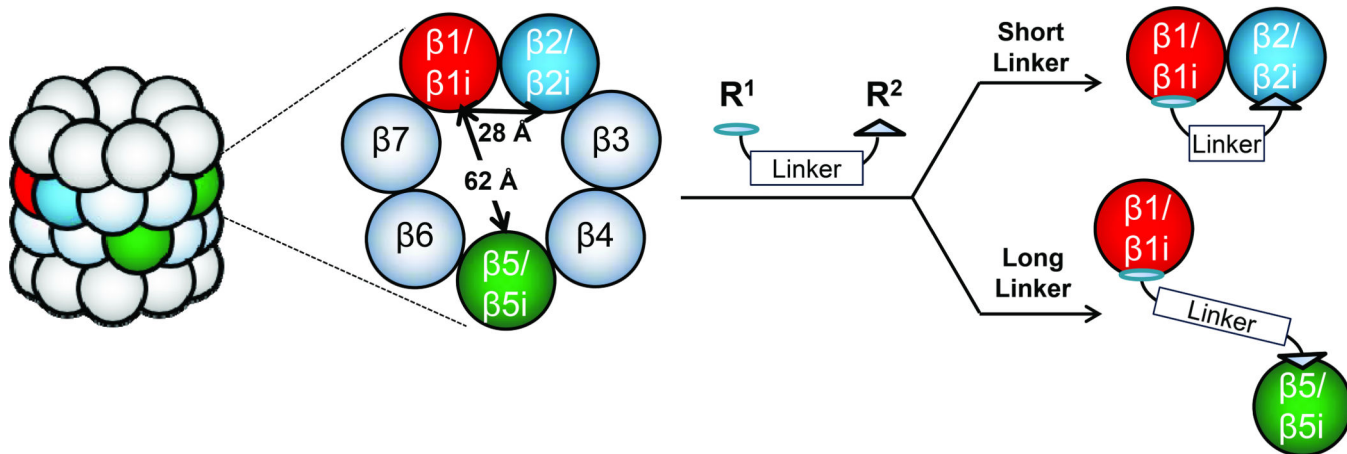
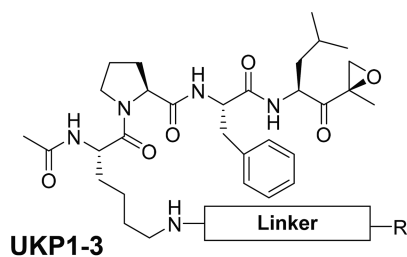
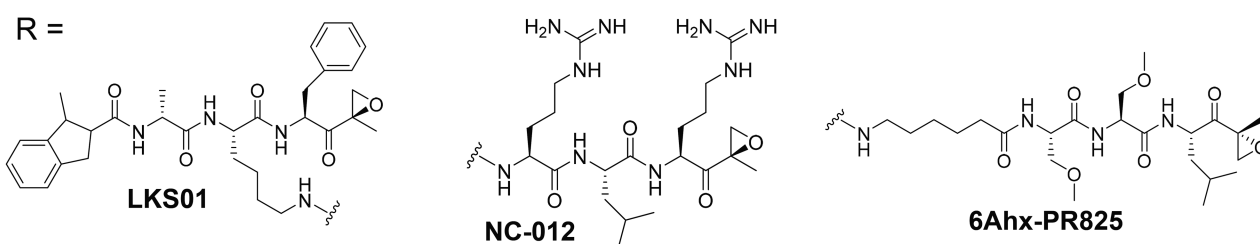
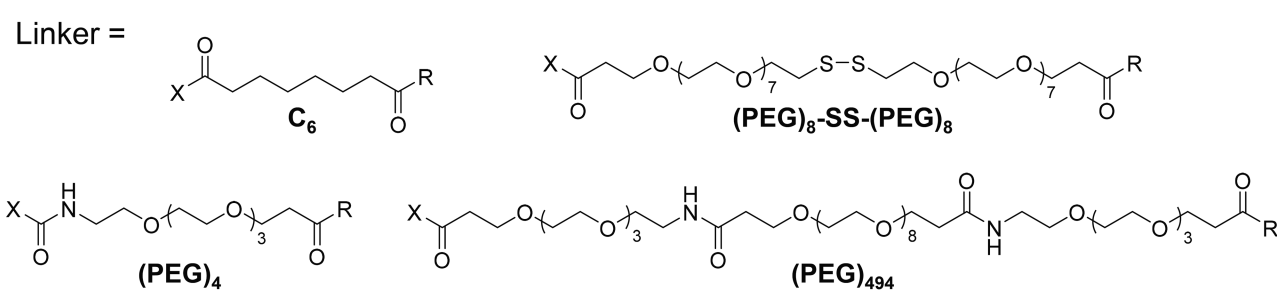


Figure 1.

Crosslinking strategy for proteasome subtype identification. Bifunctional proteasome probes, comprised of two peptide epoxyketone proteasome inhibitors coupled by a linker, crosslink different pairs of proteasome catalytic subunits when they are present within the same 20S core complex. Crosslinking efficiency of a particular subunit pair is determined by the subunit selectivity of the two inhibitors as well as the length of the linker by which they are coupled. Following treatment of cell lysates or purified 20S proteasomes with bifunctional proteasome probes, the crosslinked subunit pairs are identified by immunoblotting analysis.

A)

Probe	R	Linker
1	LKS01	C ₆
2	LKS01	(PEG) ₈ -SS-(PEG) ₈
3	NC-012	(PEG) ₄
4	6Ahx-PR825	(PEG) ₄₉₄

B)**C)****X = UKP1-3****Figure 2.**

Structures of bifunctional proteasome probes, each comprising the β 1/ β 1i-selective proteasome inhibitor UKP1-3 (**A**) coupled with another peptide epoxyketone proteasome inhibitor (**B**) via a hydrocarbon or polyethylene glycol linker (**C**).

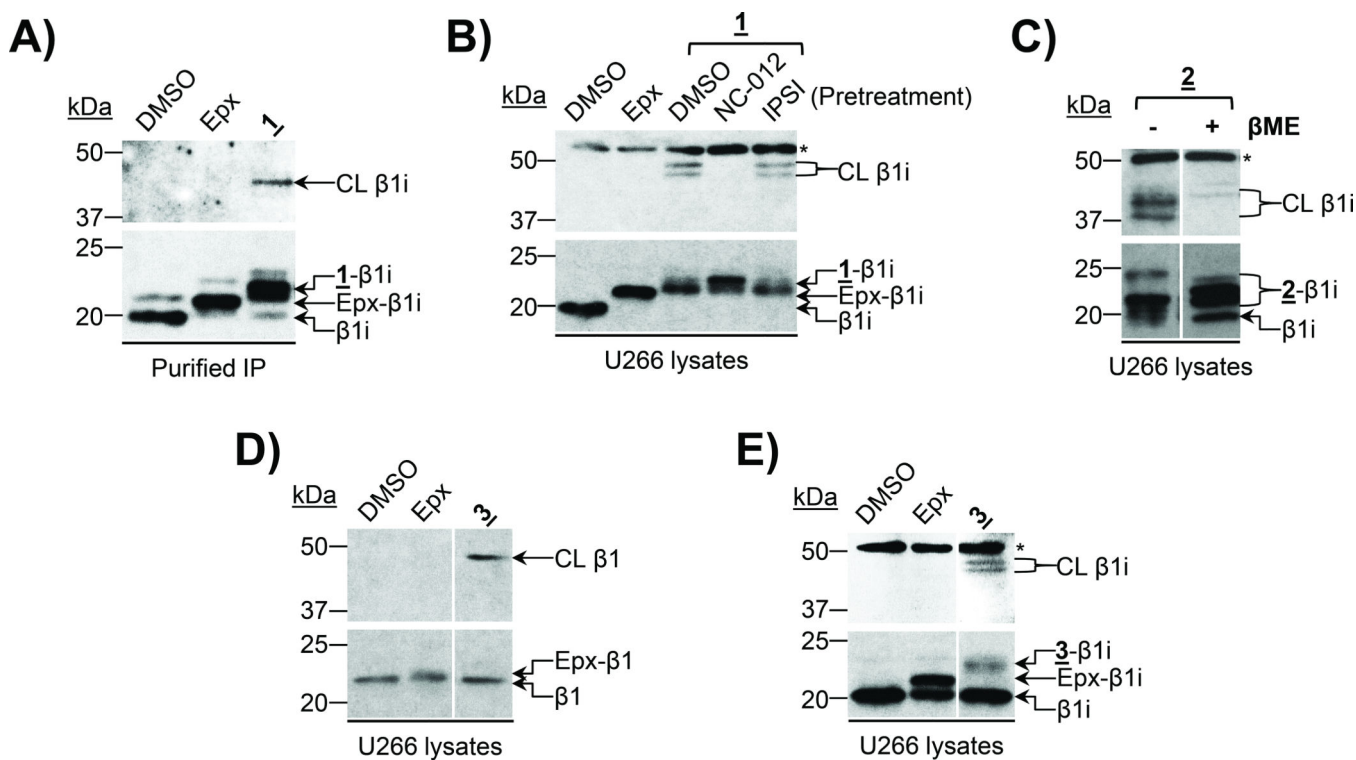


Figure 3. UKP13- C_6 -LKS01 (probe **1**) and UKP13-(PEG) $_4$ -NC012 (probe **3**) crosslink $\beta 1/\beta 1i$ with $\beta 2/\beta 2i$. **A**) The purified 20S immunoproteasome (IP) was treated with DMSO, epoxomicin (Epx), or probe **1** prior to immunoblotting for $\beta 1i$. **B**) U266 cell lysates were treated with DMSO or epoxomicin, or pretreated with DMSO or the indicated subunit-selective inhibitors prior to treatment with probe **1** as indicated. $\beta 1i$ was detected by immunoblotting. **C**) High-molecular-weight immunoblot signals detected following treatment with bifunctional proteasome probes result from crosslinking of proteasome catalytic subunits. U266 cell lysates were treated with probe **2** and proteins were subsequently denatured in sample buffer with or without β -mercaptoethanol (β ME), which cleaves the internal disulfide bond of the linker. $\beta 1i$ was detected by immunoblotting. **D**) U266 cell lysates were incubated with DMSO, epoxomicin, or probe **3** prior to immunoblotting for $\beta 1$. **E**) U266 cell lysates were incubated with DMSO, epoxomicin, or probe **3** prior to immunoblotting for $\beta 1i$. Irrelevant lanes were cropped out of blots shown in **C**–**E**. CL = crosslinked β -subunit, * = non-specific band.

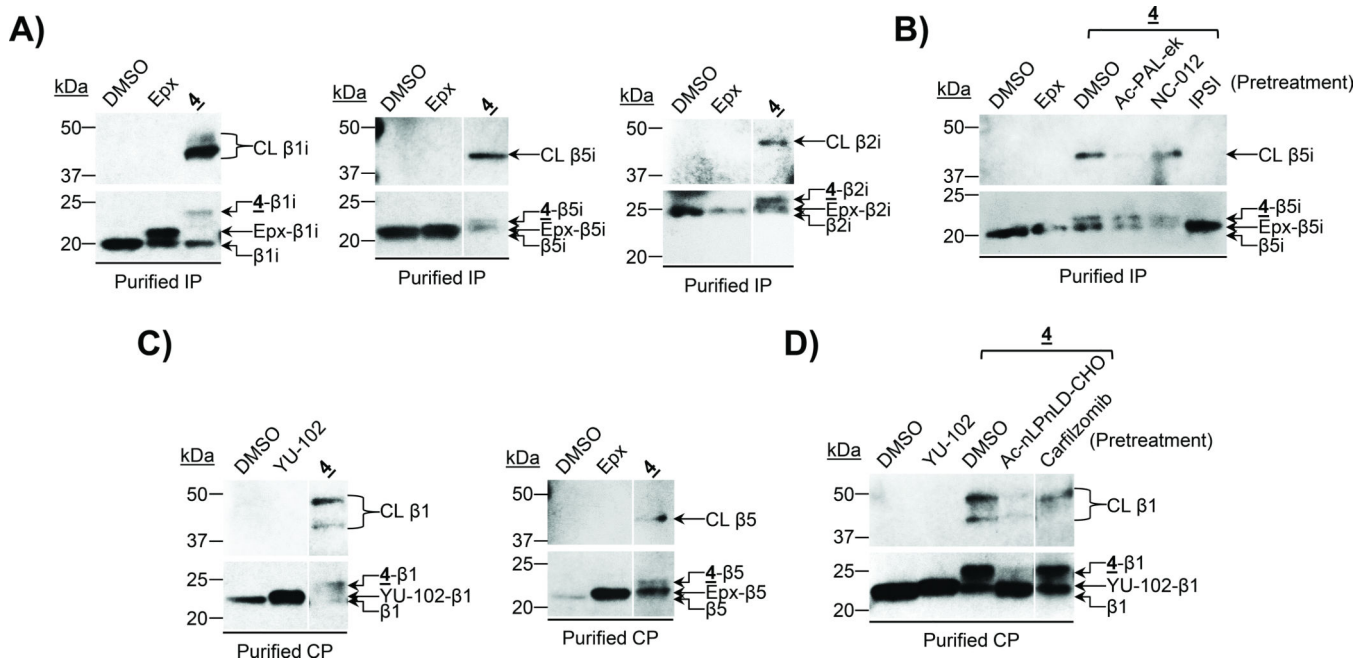
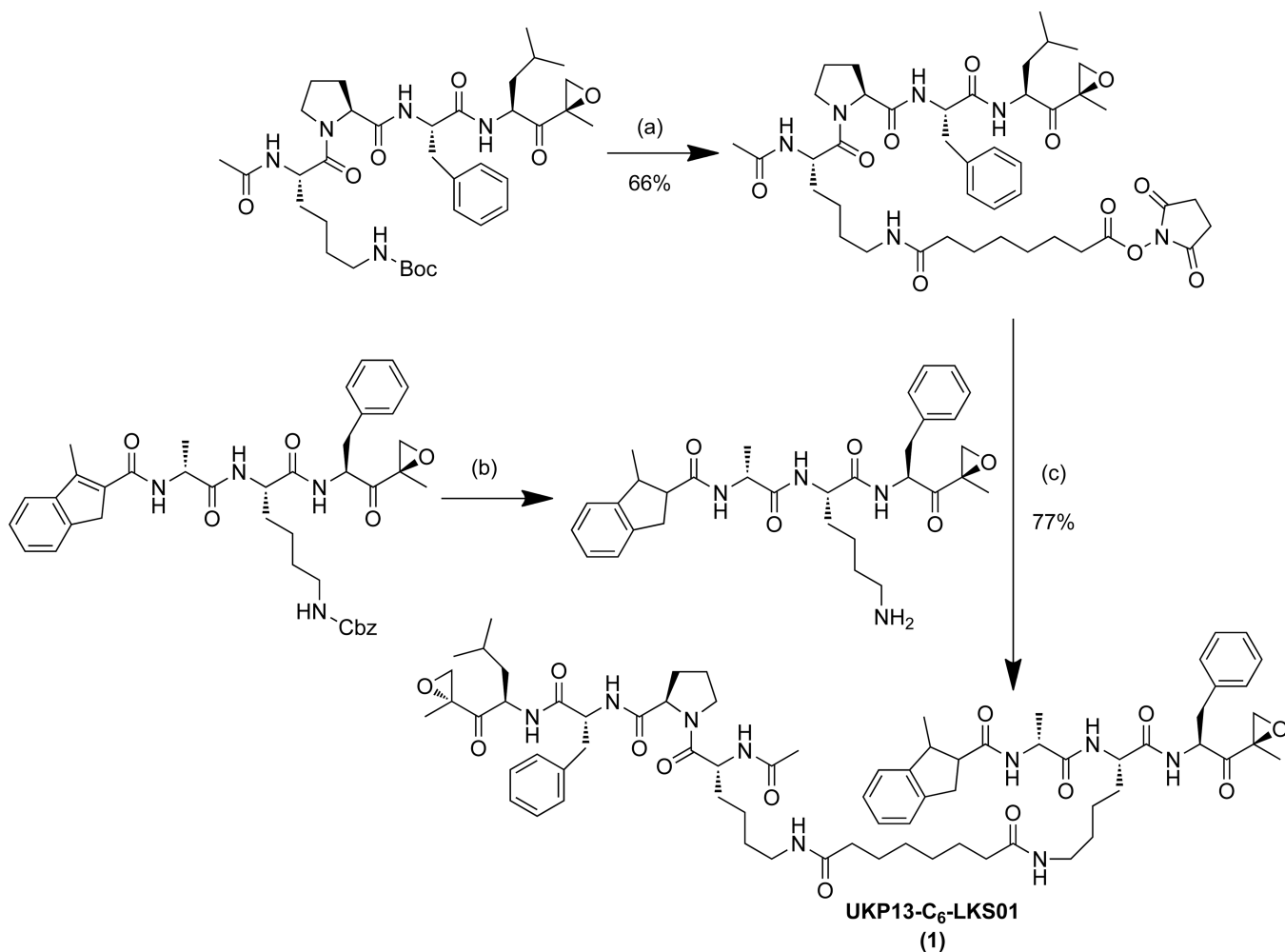


Figure 4. UKP13-(PEG)₄₉₄-6Ahx-PR825 (probe **4**) crosslinks multiple pairs of catalytic subunits. **A**) The purified 20S immunoproteasome (IP) was treated with DMSO, epoxomicin (Epx), or probe **4** prior to immunoblotting for β 1i, β 5i, or β 2i. **B**) The purified 20S immunoproteasome was treated with DMSO or epoxomicin, or pretreated with DMSO or the indicated subunit-selective inhibitors prior to treatment with probe **4** as indicated. β 5i was detected by immunoblotting. **C**) The purified 20S constitutive proteasome (CP) was treated with DMSO, YU-102, epoxomicin, or probe **4** prior to immunoblotting for β 1 or β 5. **D**) The purified 20S constitutive proteasome was treated with DMSO or YU-102, or pretreated with DMSO or the indicated subunit-selective inhibitors prior to treatment with probe **4** as indicated. β 1 was detected by immunoblotting. Irrelevant lanes were removed from blots shown in **A**, **C** and **D**. CL = crosslinked subunit.

**Scheme 1.**

Synthesis of UKP13-C₆-LKS01 (probe 1). (a) 1) TFA, dichloromethane, 1 hr; 2) DSS (Disuccinimidyl suberate), DIPEA (N,N-diisopropylethylamine), THF, overnight; (b) H₂, Pd/C, MeOH/EtOAc, 4 hr; (c) DIPEA, THF, overnight.

Inhibitory potencies (IC_{50} values, μM) of bifunctional probes **1–4** towards constitutive proteasome and immunoproteasome catalytic subunits.^[a]

Table 1

Probe	$\beta 1$	$\beta 2$	$\beta 5$	$\beta 1i$	$\beta 2i$	$\beta 5i$
1	0.655	10.64	0.895	0.402	>10	0.349
2	0.094	6.669	0.165	0.038	>10	0.017
3	1.153	0.128	3.93	0.803	0.03	4.35
4	0.188	15.28	0.075	0.130	28.07	0.38

[a] Inhibition of hydrolysis of subunit-selective fluorogenic peptide substrates was evaluated following a 1 hr incubation of the purified constitutive proteasome or immunoproteasome with each bifunctional probe.