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

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Supplemental Information

Proteasome Activity Assays:

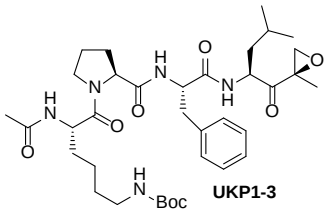
Proteasome activity assays in U266 cell lysates were conducted as described in the main text for purified proteasomes with the following exceptions: U266 cell lysates (5 μg of protein/well) were incubated with DMSO, 20 μM epoxomicin (used as a positive control for inhibition of β1i, β2, β2i, β5, and β5i activity), 10 μM YU-102 (used as a positive control for inhibition of β1 activity), or increasing concentrations of each bifunctional probe (0.5-10 μM) for 1 hr at room temperature in 20S proteasome assay buffer. 10 μL of each substrate diluted in assay buffer was then added to obtain a final volume of 100 μL per well. Boc-LRR-AMC (β2/β2i-selective) and, Suc-LLVY-AMC (β5/β5i-selective),[1] were each used at a final concentration of 100 μM. Fluorescence produced by the release of AMC was measured once per min over 90 min on a SpectraMax M5 microplate reader (Molecular Devices) using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Non-proteasomal hydrolysis of each substrate was measured following treatment with epoxomicin (for all substrates except for Ac-nLPnLD-AMC) or YU-102 (for Ac-nLPnLD-AMC) and subtracted from each measurement of proteasome activity following treatment with DMSO or bifunctional probes. Reaction velocities were determined using linear regression in GraphPad Prism and reported as a percentage of that of the DMSO-treated control.

Synthesis of Bifunctional Compounds:

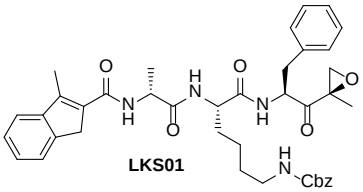
General Methods:

All commercially available reagents were purchased and used without purification unless otherwise noted. All reactions were carried out in flame-dried glassware with anhydrous solvent under nitrogen atmosphere. Flash column chromatography was performed on a Biotage SP-1 chromatography system. TLC plates were visualized by exposure to ultraviolet light (254 nm) and/or by immersion in a staining solution of phosphomolybdic acid (PMA), followed by heating on a hot plate. Mass analysis was performed using Bruker Daltonics MALDI-TOFMS.

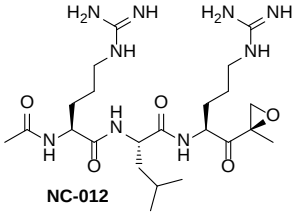
UKP1-3, LKS01, NC-012, and PR-825 were used as reference compounds in the synthesis of bifunctional probes **1-4**.[3] For the synthesis of bifunctional probes **3** and **4**, NC-012 and PR-825 were initially amine-functionalized following synthetic procedures previously reported.[3a, 3b]



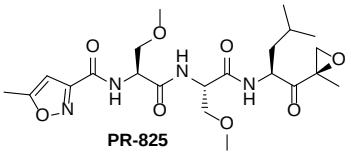
UKP1-3



LKS01

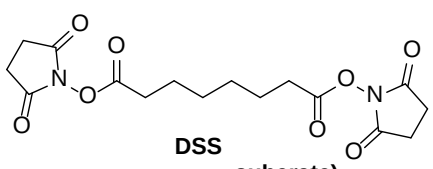


NC-012

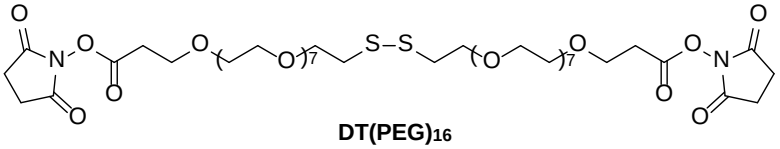


PR-825

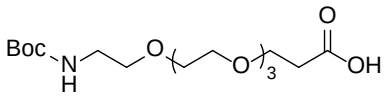
DSS and DT(PEG)$_{16}$ were used as linkers in the synthesis of bifunctional probes **1** and **2**, respectively. Boc-(PEG)$_4$-OH was used in the synthesis of bifunctional probes **3** and **4**. Additionally, BS(PEG)$_9$ was used in the synthesis of bifunctional probe **4**.



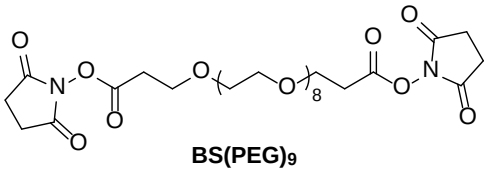
DSS
(Disuccinimidyl suberate)



DT(PEG)$_{16}$
(4,7,10,13,16,19,22,25,32,35,38,41,44,47,50,53-Hexadecaoxa-28,29-dithiahexapentacontanedioic acid di-N-succinimidyl ester)

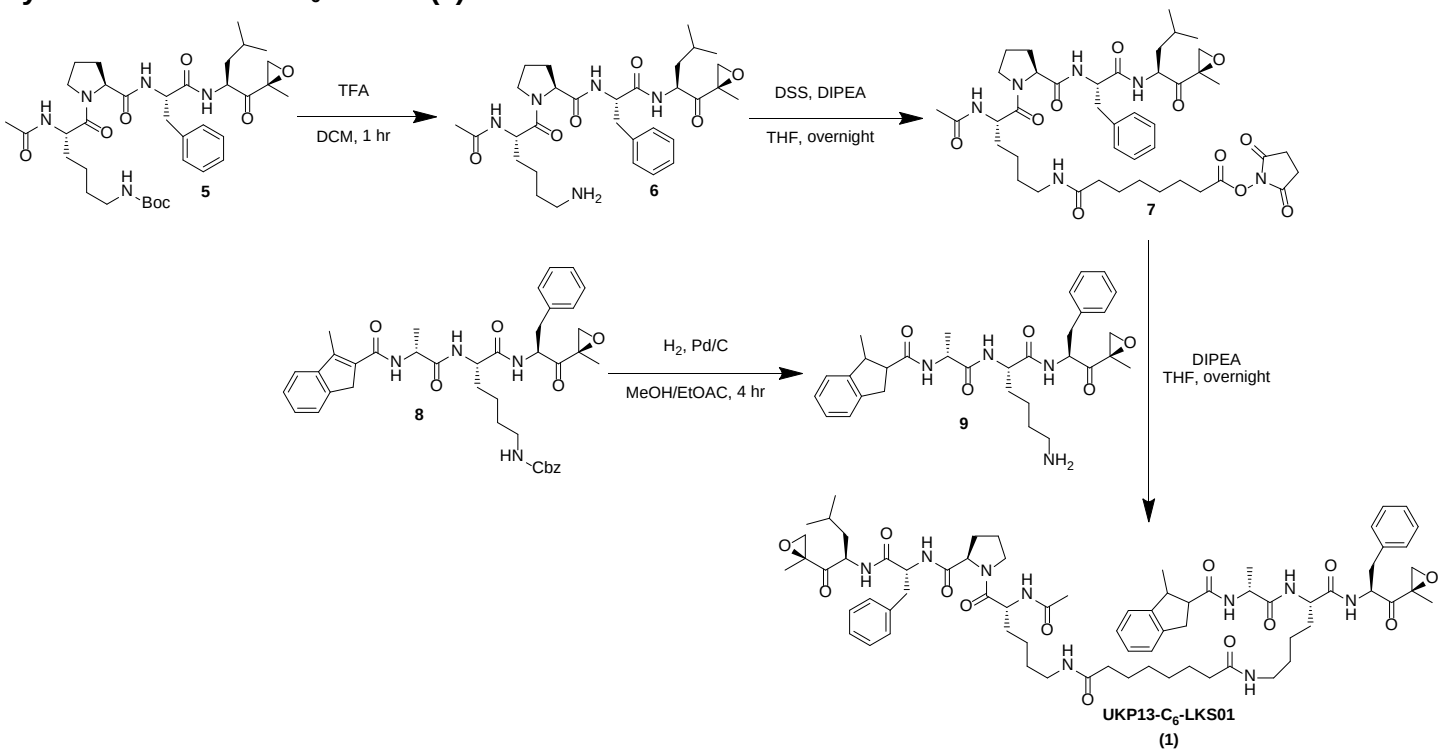


Boc-(PEG)$_4$-OH
(15-(Boc-amino)-4,7,10,13-tetraoxapentadecanoic acid)



BS(PEG)$_9$
(Bis(succinimidyl) nona(ethylene glycol))

Synthesis of UKP13-C$_6$-LKS01 (1):

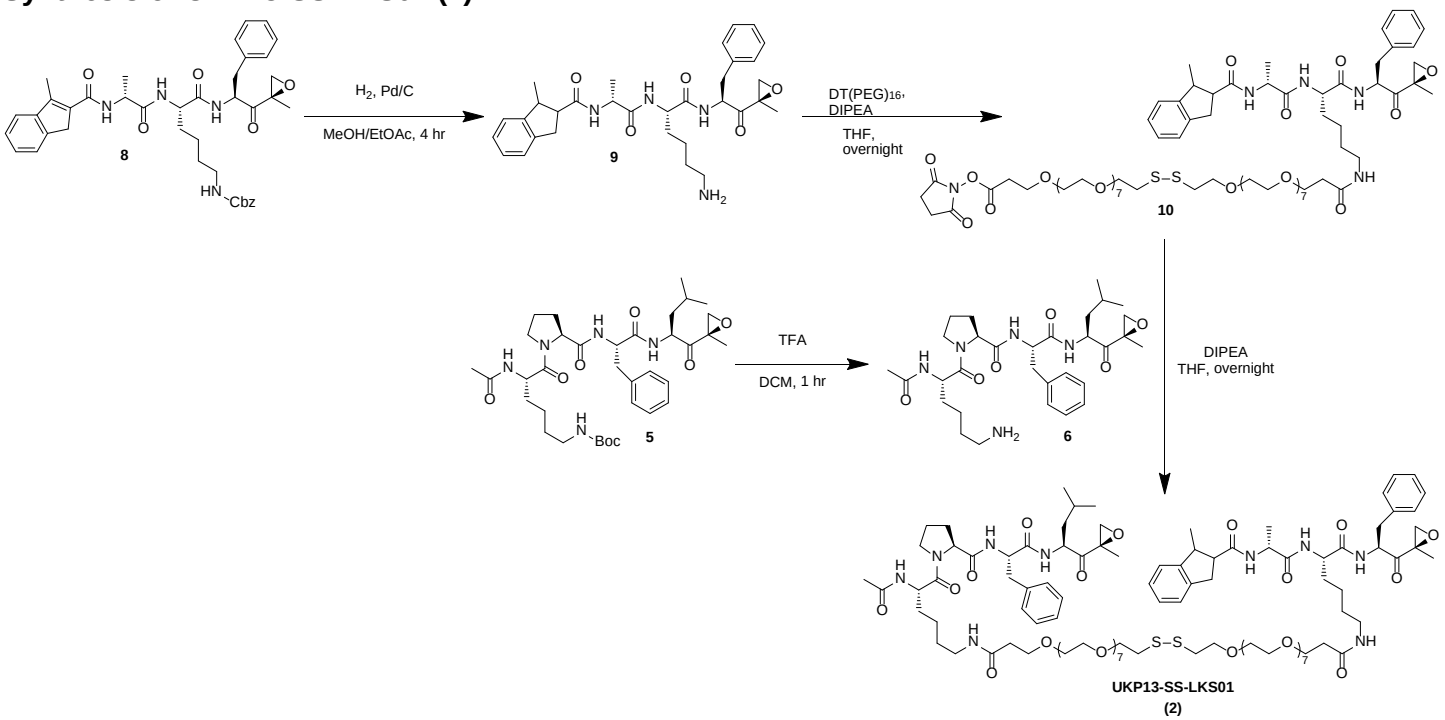


Scheme 1

UKP1-3 (**5**) (10.0 mg, 0.01 mmol) was treated with a trifluoroacetic acid (TFA)/dichloromethane (DCM) (1:1) solution (2.0 mL). The reaction mixture was stirred for 1 hr and concentrated to dryness. The crude was taken forward for the next reaction without purification. DSS (6.0 mg, 0.02 mmol) dissolved in THF (1.0 ml) was added to it, which was followed by dropwise addition of *N,N*-diisopropylethylamine (DIPEA) (0.1 mL). The reaction mixture was stirred overnight at room temperature, then concentrated to dryness and directly loaded onto a column for purification. The methanol (MeOH)/DCM solvent system was used for purification to obtain compound **7** (8.0 mg, 66% yield for 2 steps).

LKS01 (**8**) (7.0 mg, 0.01 mmol) was dissolved in a MeOH/ethyl acetate (EtOAc) solution (1:1, 4.0 mL), and Pd/C (2.0 mg) was added to it. The reaction mixture was stirred for 4 hr at rt under a bubbling hydrogen atmosphere. The reaction mixture was filtered on celite and then concentrated to dryness. The crude was then dissolved in tetrahydrofuran (THF) (2.0 mL) and added to a round bottom flask containing compound **7** (8.0 mg, 0.01 mmol). To this, DIPEA (0.05 mL) was added, and the reaction mixture was stirred overnight at room temperature. Then, it was concentrated to dryness and directly loaded onto a column for purification. The MeOH/DCM solvent system (0-5% gradient) was used for purification to obtain UKP13-C$_6$-LKS01 (probe **1**) (10.0 mg, 77% yield for 2 steps). Mass analysis (MALDI-TOFMS): M+Na = 1309 (observed), 1309 (calculated).

Synthesis of UKP13-SS-LKS01 (2):

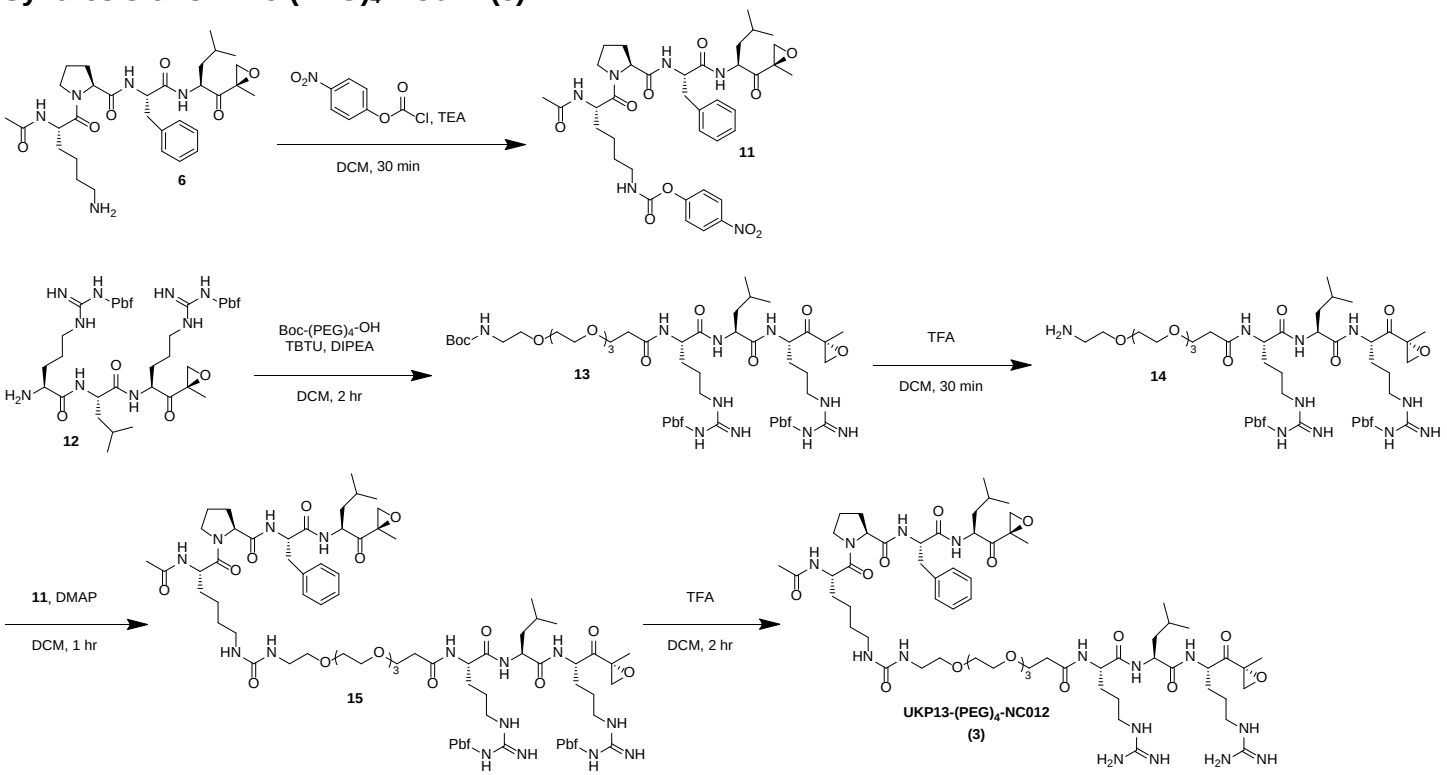


Scheme 2

To a solution of LKS01 (**8**) (10.0 mg, 0.01 mmol) in MeOH/EtOAc (1:1, 4 mL), 10% Pd/C (2.0 mg) was added. The reaction mixture was stirred for 4 hr at room temperature under a bubbling hydrogen atmosphere. The reaction mixture was filtered on celite and then concentrated to dryness. The crude was then dissolved in THF (2.0 mL) and added to a round bottom flask containing $DT(PEG)_{16}$ (10.0 mg, 0.01 mmol). To this, DIPEA (0.05 mL) was added, and the reaction mixture was stirred overnight at room temperature. It was then concentrated to dryness and directly loaded onto a column for purification. The MeOH/DCM solvent system (0-10% gradient) was used for purification to obtain compound **10**.

To a solution containing UKP1-3 (**5**) (7.0 mg, 0.01 mmol) in DCM (1.0 mL), TFA (1.0 mL) was added, and the reaction mixture was stirred for 1 hr at room temperature. The mixture was then concentrated to dryness and put on high vacuum to obtain compound **6**. The crude was dissolved in THF (2.0 mL) and compound **10** (10.5 mg, 0.01 mmol) was added to it, followed by addition of DIPEA (0.05 mL). After stirring the reaction mixture overnight at room temperature, it was concentrated and loaded onto a column for purification using the MeOH/DCM solvent system (0-20% gradient) to obtain UKP13-SS-LKS01 (probe **2**). Mass analysis (MALDI-TOFMS): M+ = 2026 (observed), 2026 (calculated); M+Na = 2049 (observed), 2049 (calculated); M+K = 2065 (observed), 2065 (calculated).

Synthesis of UKP13-(PEG)$_4$-NC012 (3):



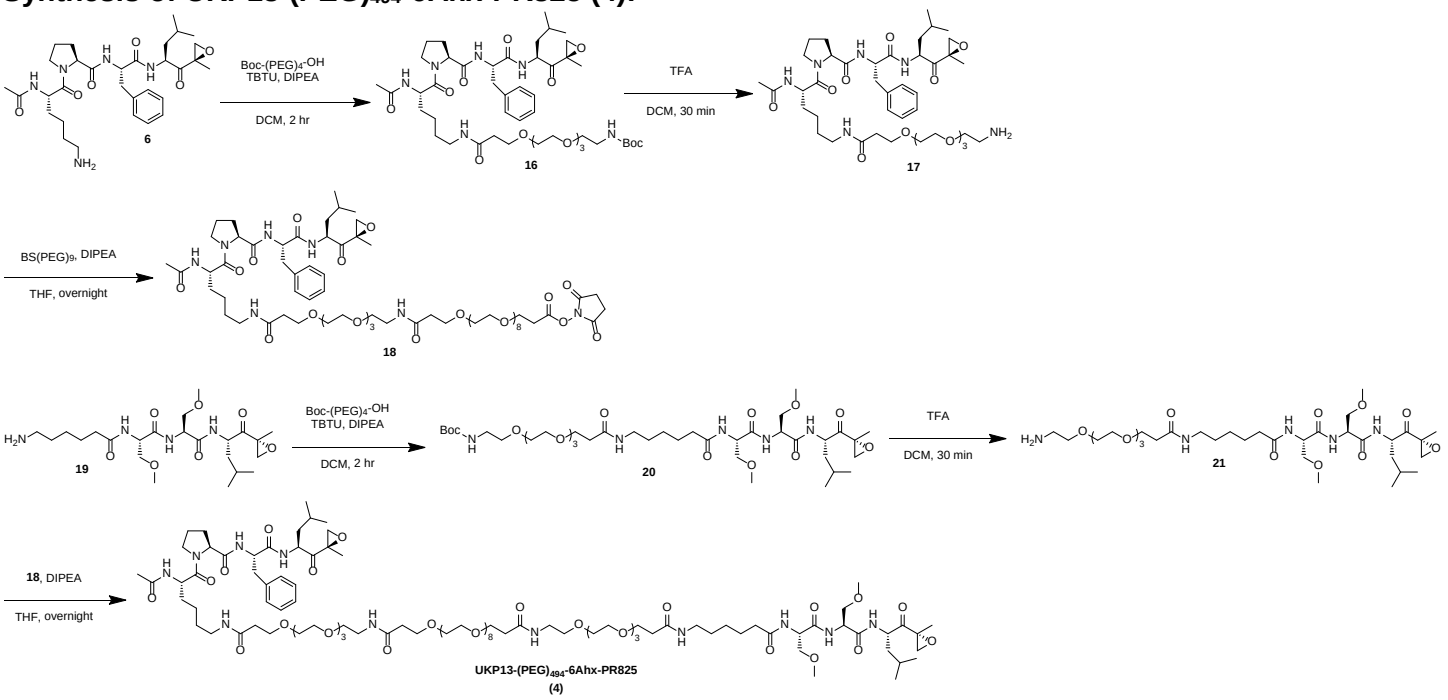
Scheme 3

Compound **6** (30.0 mg, 0.05 mmol) and 4-Nitrophenyl chloroformate (10.0 mg, 0.05 mmol) were dissolved in DCM (2.0 mL), followed by addition of triethylamine (TEA) (14.0 mL, 0.10 mmol). The resulting solution was stirred at rt for 30 min. The reaction mixture was concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel using the MeOH/DCM solvent system to afford compound **11** (31.0 mg, 81% yield).

DIPEA (0.03 mL, 0.15 mmol) was added to a solution of compound **12** (50.0 mg, 0.05 mmol), Boc-(PEG)$_4$-OH (18.0 mg, 0.05 mmol), and O-(benzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium tetrafluorobotate (TBTU) (24.0 mg, 0.08 mmol) in DCM (1.0 mL). The resulting solution was stirred at rt for 2h. The reaction mixture was concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel using the MeOH-DCM solvent system to afford compound **13** (50.0 mg, 74% yield). 15% trifluoroacetic acid (TFA) (0.5 mL) was added to a solution of compound **13** (50.0 mg, 0.04 mmol) in DCM (1.0 mL). The resulting solution was stirred at rt for 30 min. The reaction mixture was concentrated under reduced pressure to obtain compound **14**.

Compound **11** (10.0 mg, 0.01 mmol) and compound **14** (25.0 mg, 0.02 mmol) were dissolved in DCM (0.5 mL), and 4-Dimethylaminopyridine (DMAP) (3.0 mg, 0.02 mmol) was added. The resulting solution was stirred at rt for 1h. The reaction mixture was concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel using the MeOH/DCM solvent system to afford compound **15** (18.0 mg, 73% yield). The Pbf protecting groups were removed by adding 50% TFA to a solution of compound **15** in DCM. After stirring the reaction mixture for 2 hr at room temperature, the resulting solution was extracted with water and filtered through a syringe filter. The filtrate was then lyophilized to obtain UKP13-(PEG)$_4$-NC012 (probe **3**) (7.8 mg, 60% yield); Mass analysis (MALDI-TOFMS): M+ = 1343 (observed), 1343 (calculated); M+2H_2O = 1379 (observed), 1379 (calculated).

Synthesis of UKP13-(PEG)$_{494}$-6Ahx-PR825 (4):



Scheme 4

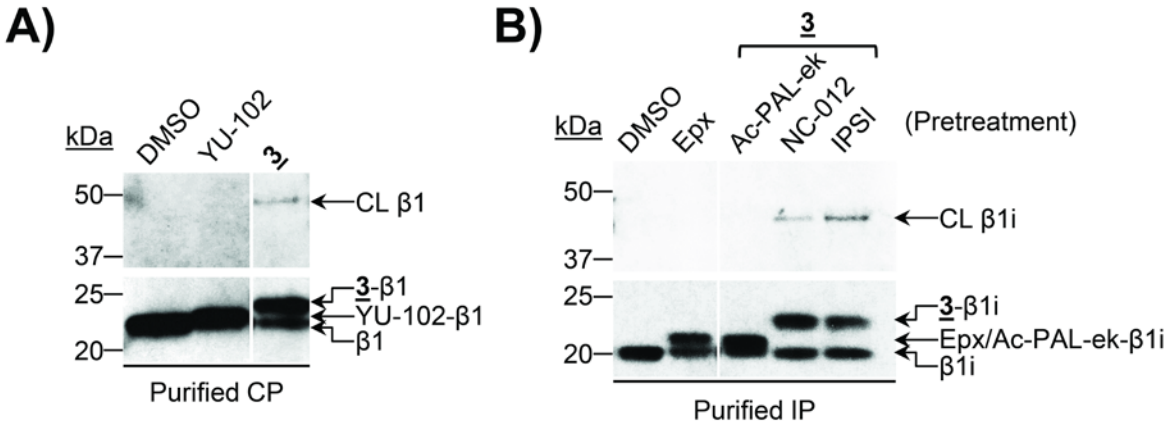
Compound **6** (27.0 mg, 0.05 mmol), Boc-(PEG)$_4$-OH (17.0 mg, 0.05 mmol), and TBTU (22.0 mg, 0.07 mmol) were dissolved in DCM (1.0 mL), and DIPEA (0.02 mL, 0.14 mmol) was added. The resulting solution was stirred at rt for 2h. The reaction mixture was concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel using the MeOH/DCM solvent system to afford compound **16** (30.0 mg, 70% yield). 15% TFA (0.5 mL) was added to a solution of compound **16** (30.0 mg, 0.03 mmol) in DCM (1.0 mL). The resulting solution was stirred at rt for 30 min. The reaction mixture was concentrated under reduced pressure to obtain compound **17**. One drop of DIPEA was added to a solution of compound **17** (8.9 mg, 0.01 mmol) and BS(PEG)$_9$ (15.0 mg, 0.02 mmol) in THF (0.5 mL). The resulting solution was stirred at rt overnight. The reaction mixture was concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel using the DCM/MeOH solvent system to afford compound **18** (10.0 mg, 67% yield).

6Ahx-PR825 (**19**) (19.0 mg, 0.04 mmol), Boc-PEG$_4$-OH (14.0 mg, 0.04 mmol), and TBTU (19.0 mg, 0.06 mmol) were dissolved in DCM (1.0 mL), and DIPEA (0.02 mL, 0.12 mmol) was added. The resulting solution was stirred at rt for 2h. The reaction mixture was concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel using the MeOH/DCM solvent system to afford compound **20** (21.0 mg, 64% yield). 15% TFA (0.5 mL) was added to a solution of compound **20** (21.0 mg, 0.03 mmol) in DCM (1.0 mL). The resulting solution was stirred at rt for 30 min. The reaction mixture was concentrated under reduced pressure to obtain compound **21**. One drop of DIPEA was added to a solution of compound **21** (7.7 mg, 0.01 mmol) and compound **18** (10.0 mg, 0.01 mmol) in THF (0.5 mL). The resulting solution was stirred at rt overnight. The reaction mixture was concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel using the MeOH/DCM solvent system to afford UKP13-(PEG)$_{494}$-6Ahx-PR825 (probe **4**) (6.3 mg, 44% yield); Mass analysis (MALDI-TOFMS): M+Na = 2067 (observed), 2067 (calculated).

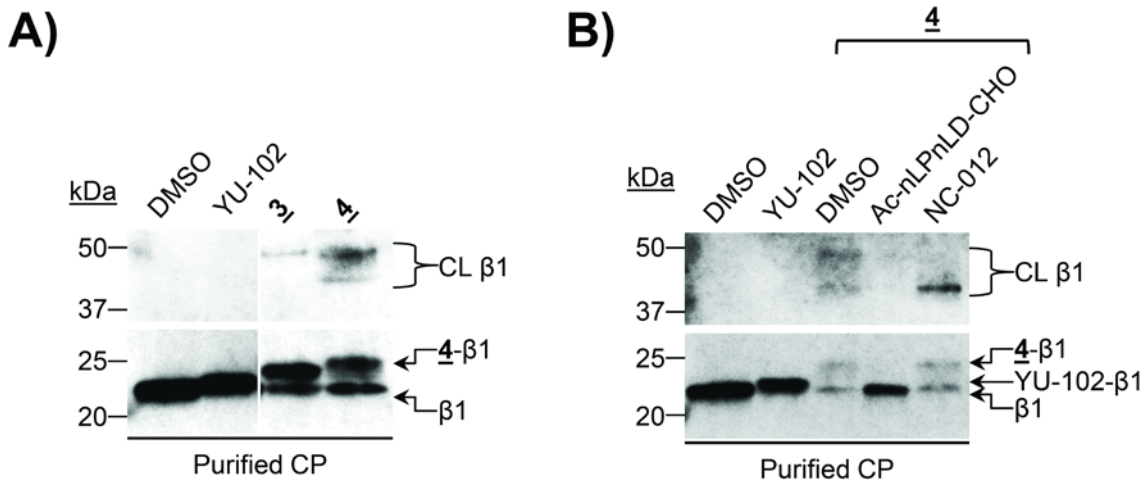
Supplemental Table and Figures:

Compound	Concentration	Relative Hydrolysis Rate (% DMSO Control)					
		Suc-LLVY-AMC (β5/β5i)	Ac-WLA-AMC (β5)	Ac-ANW-AMC (β5i)	Ac-nLPnLD-AMC (β1)	Ac-PAL-AMC (β1i)	Boc-LRR-AMC (β2/β2i)
UKP13-C_6-LKS01 (**1**)	0.5 μM	N.D.	N.D.	N.D.	N.D.	N.D.	104%
	1 μM	4%	N.D.	4%	3%	0%	91%
	5 μM	0%	N.D.	0%	0%	0%	71%
	10 μM	0%	N.D.	1%	0%	0%	47%
UKP13-$(PEG)_4$-NC012 (**3**)	1 μM	N.D.	77%	101%	15%	7%	23%
	5 μM	N.D.	33%	88%	2%	1%	6%
	10 μM	N.D.	8%	61%	0%	0%	0%
UKP13-$(PEG)_{494}$-6Ahx-PR825 (**4**)	0.5 μM	N.D.	0%	5%	5%	0%	N.D.
	1 μM	N.D.	0%	2%	3%	0%	N.D.
	5 μM	N.D.	0%	0%	0%	0%	N.D.
	10 μM	N.D.	0%	0%	0%	0%	N.D.

Supplemental Table 1. U266 cell lysates were treated with the indicated concentration of each bifunctional probe, and proteasome activity was assessed by measuring the hydrolysis of subunit-selective fluorogenic peptide substrates. DMSO, epoxomicin, and YU-102-treated lysates served as controls. Values represent the substrate hydrolysis rate as a percentage of that of the DMSO-pretreated control. N.D. = not determined.



Supplemental Figure 1. UKP13-$(PEG)_4$-NC012 (probe **3**) crosslinks β1/β1i with β2/β2i. **A)** The purified constitutive proteasome (CP) was treated with DMSO, YU-102, or probe **3** prior to immunoblotting for β1. **B)** The purified 20S immunoproteasome (IP) was pretreated with the indicated subunit-selective inhibitors prior to treatment with probe **3** as indicated. β1i was detected by immunoblotting. Irrelevant lanes were removed. CL = crosslinked β-subunit.



Supplemental Figure 2. UKP13-$(PEG)_{494}$-6Ahx-PR825 (probe **4**) crosslinks β1 with β2 in the purified constitutive proteasome. **A)** The purified 20S constitutive proteasome (CP) was treated with DMSO, YU-102, probe **3**, or probe **4** prior to immunoblotting for β1. **B)** 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. β1 was detected by immunoblotting. CL = crosslinked β-subunit.

References:

[1] A. F. Kisselev, A. L. Goldberg, in *Methods Enzymol., Vol. 398* (Ed.: J. D. Raymond), Academic Press, **2005**, pp. 364-378.

[2] C. Blackburn, K. M. Gigstad, P. Hales, K. Garcia, M. Jones, F. J. Bruzzese, C. Barrett, J. X. Liu, T. A. Soucy, D. S. Sappal, N. Bump, E. J. Olhava, P. Fleming, L. R. Dick, C. Tsu, M. D. Sintchak, J. L. Blank, *Biochem. J.* **2010**, *430*, 461-476.

[3] a) J. E. Park, Y. Wu, K. C. Carmony, Z. Miller, L. K. Sharma, D.-M. Lee, D.-Y. Kim, W. Lee, K.-B. Kim, *Mol. BioSyst.* **2014**, *10*, 196-200; b) L. K. Sharma, N.-R. Lee, E. R. Jang, B. Lei, C.-G. Zhan, W. Lee, K.-B. Kim, *ChemBioChem* **2012**, *13*, 1899-1903; c) Anne C. Mirabella, Alexandre A. Pletnev, Sondra L. Downey, Bogdan I. Florea, Tamer B. Shabaneh, M. Britton, M. Verdoes, Dmitri V. Filippov, Herman S. Overkleeft, Alexei F. Kisselev, *Chem. Biol.* **2011**, *18*, 608-618; d) H.-J. Zhou, M. A. Aujay, M. K. Bennett, M. Dajee, S. D. Demo, Y. Fang, M. N. Ho, J. Jiang, C. J. Kirk, G. J. Laidig, E. R. Lewis, Y. Lu, T. Muchamuel, F. Parlati, E. Ring, K. D. Shenk, J. Shields, P. J. Shwonek, T. Stanton, C. M. Sun, C. Sylvain, T. M. Woo, J. Yang, *J. Med. Chem.* **2009**, *52*, 3028-3038; e) T. Muchamuel, M. Basler, M. A. Aujay, E. Suzuki, K. W. Kalim, C. Lauer, C. Sylvain, E. R. Ring, J. Shields, J. Jing, P. Shwonek, F. Parlati, S. D. Demo, M. K. Bennett, C. J. Kirk, M. Groettrup, *Nat. Med.* **2009**, *15*, 781-787.