Supporting Information for

Cell-Permeable Activity-Based Ubiquitin Probes Enable Intracellular Profiling of Human Deubiquitinases

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Supplemental Figures and Tables

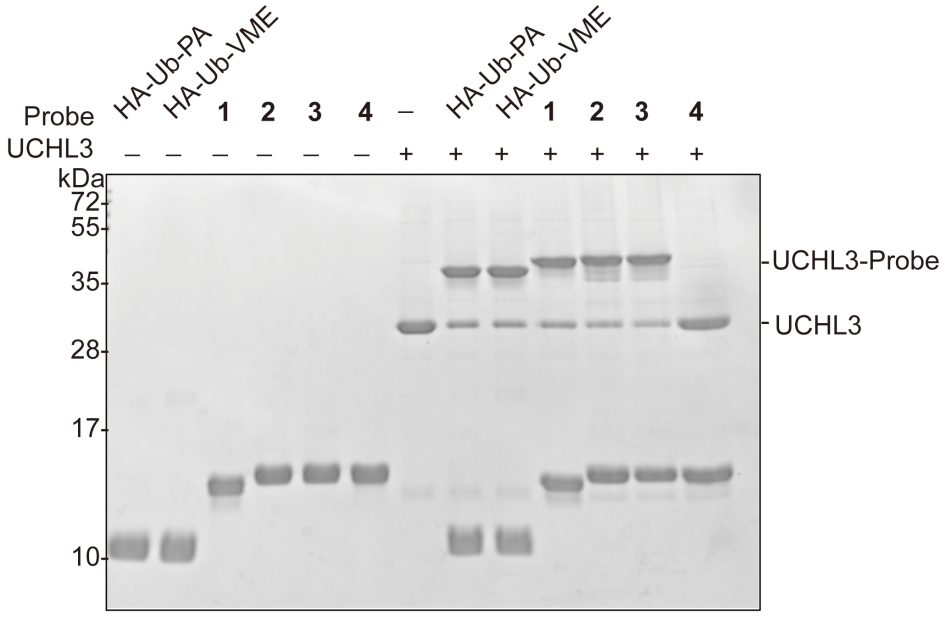


Figure S1. In vitro labeling of UCHL3 using 15 μM Ub-based DUB ABPs analyzed using 20% SDS-PAGE gel and stained with Coomassie brilliant blue.

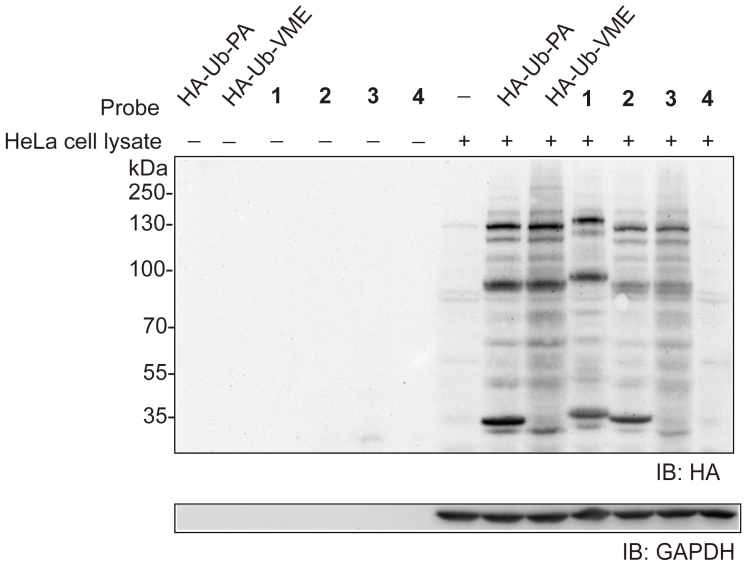


Figure S2. HeLa cell lysate DUB profiling using 15 μM Ub-based DUB ABPs. Labeled proteins were resolved using 12% SDS-PAGE gel and analyzed by immunoblotting using an anti-HA antibody. GAPDH was utilized as a loading control.

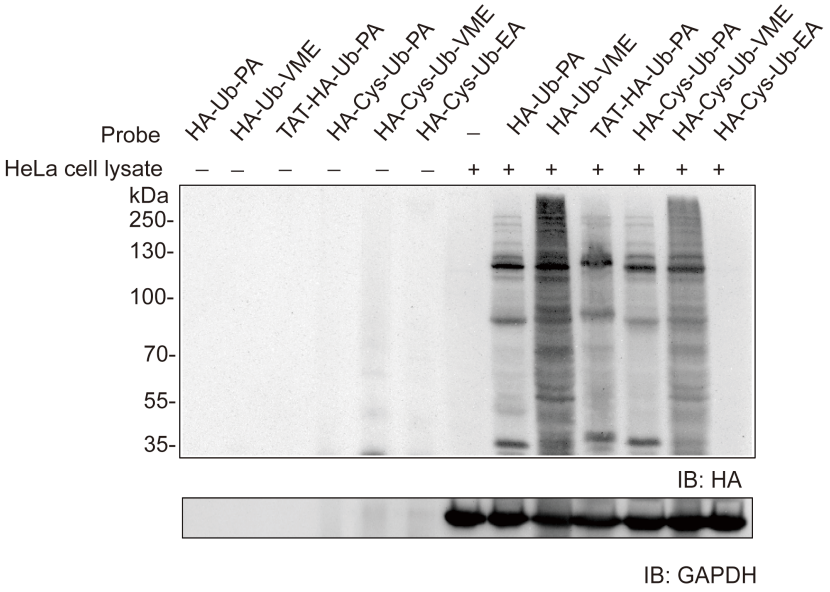


Figure S3. HeLa cell lysate DUB profiling using 15 μM HA-Cys-Ub DUB ABPs compared with HA-Ub-PA and HA-Ub-VME. Labeled proteins were resolved using 12% SDS-PAGE gel and analyzed by immunoblotting using an anti-HA antibody. GAPDH was utilized as a loading control.

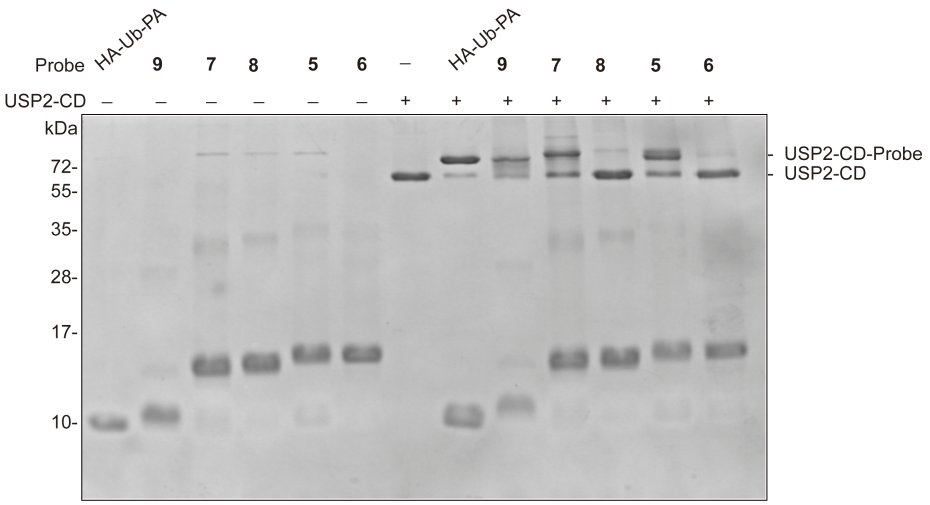


Figure S4. In vitro labeling of USP2-CD using 15 μM fluorescent Ub-based DUB ABPs analyzed using 20% SDS-PAGE gel and stained with Coomassie brilliant blue. HA-Ub-PA was utilized in a control labeling experiment and shown for comparison.

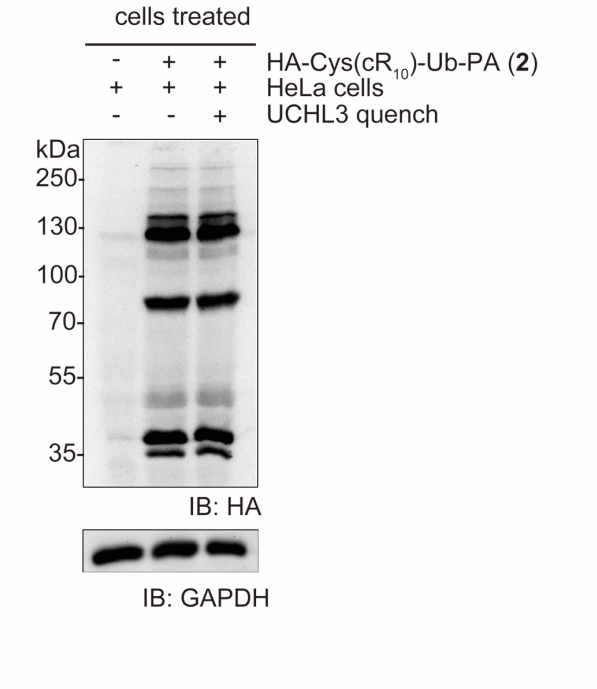


Figure S5. Comparison of intracellular DUB profiling of HeLa cells using 15 μM HA-Cys(cR_{10})-Ub-PA (**2**) with or without added UCHL3 following trypsin-EDTA solution rinse and PBS buffer wash. Labeled proteins were resolved using 10% SDS-PAGE gel and analyzed by immunoblotting using anti-HA antibody. GAPDH was used as a loading control.

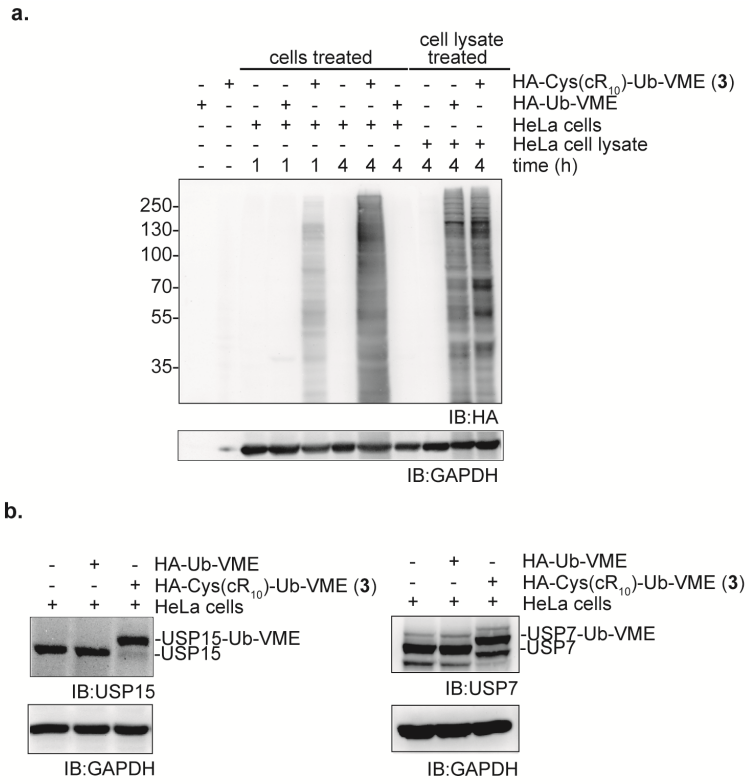


Figure S6. Intracellular HeLa cells DUB profiling using 15 μM HA-Cys(cR_{10})-Ub-VME (**3**). (a) DUB profiling in HeLa cells using HA-Cys(cR_{10})-Ub-VME (**3**) or HA-Ub-VME treated for indicated time at 15 μM. Profiling of DUBs in HeLa cell lysate using HA-Cys(cR_{10})-Ub-VME (**3**) and HA-Ub-VME (4 h, 15 μM) is also shown for comparison. (b) Intracellular labeling of USP15 and USP7 using HA-Cys(cR_{10})-Ub-VME or HA-Ub-VME for 4h at 15 μM. Immunoblotting performed using indicated antibodies, and GAPDH was used as a loading control.

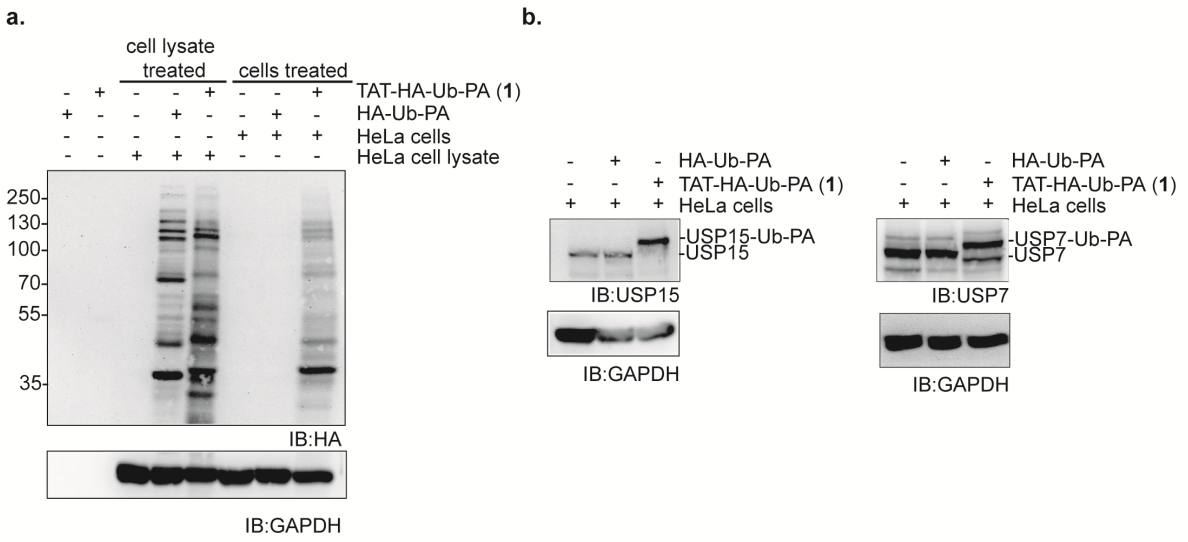


Figure S7. Intracellular HeLa cells DUB profiling using 15 μM TAT-HA-Ub-PA (**1**). (a) Intracellular DUB profiling in HeLa cells with 15 μM TAT-HA-Ub-PA (**1**) or 15 μM HA-Ub-PA for 4 h. Profiling of DUBs in HeLa cell lysate using TAT-HA-Ub-PA (**1**) and HA-Ub-PA (4 h, 15 μM) was also included for comparison. (b) Intracellular labeling of USP15 and USP7 using 15 μM TAT-HA-Ub-PA (**1**) or HA-Ub-PA for 4 h. Immunoblotting was performed using indicated antibodies, and GAPDH was used as a loading control.

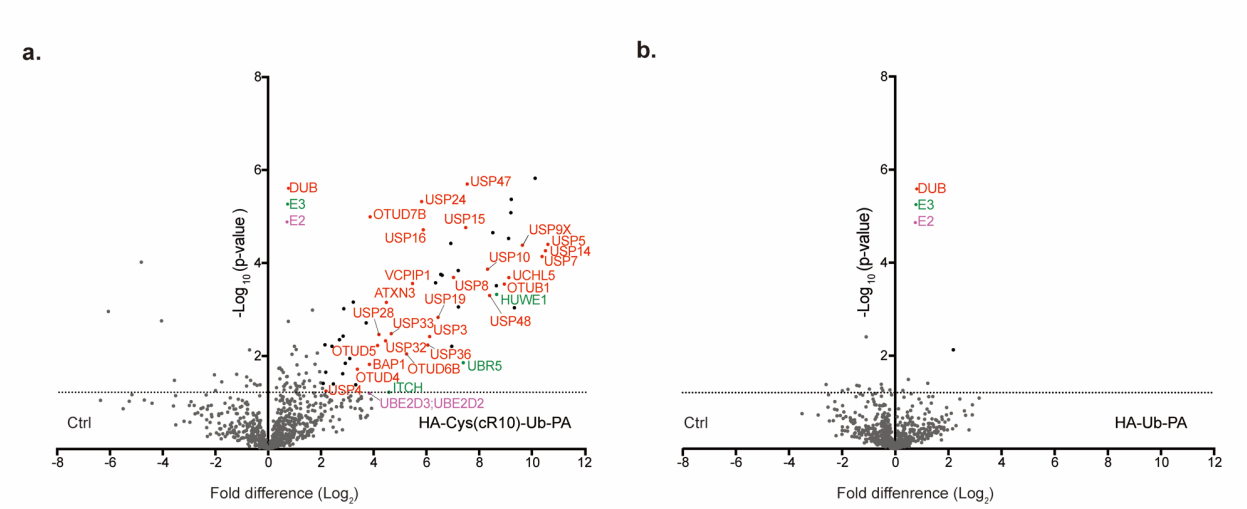


Figure S8: Enrichment of DUBs using 15 µM HA-Cys(cR_{10})-Ub-PA (**2**) for HeLa cells intracellular DUB profiling. (a) Volcano plot of pairwise comparison of protein groups pulled down with 15 µM HA-Cys(cR_{10})-Ub-PA (**2**) relative to bead control. DUBs that were captured by probe **2** with $\log_2$ (fold difference) >2, *p*-value < 0.05 are labeled and colored red. Similarly, E3s are labeled and colored green, and E2s are labeled and colored in purple. (b) Volcano plot of pairwise comparison of protein groups pulled down with HA-Ub-PA relative to bead control. LFQ intensity scores were obtained using MaxQuant and analyzed using Perseus.

Table S1. Label-free quantification (LFQ) intensity scores for DUBs captured in intracellular HeLa cells proteome-wide DUB profiling using 15 μM HA-Cys(cR$_{10}$)-Ub-PA (**2**), HA-Cys(cR10)-Ub-EA (**4**) and HA-Ub-PA respectively.

	Control			HA-Cys(cR$_{10}$)-Ub-PA			HA-Cys(cR$_{10}$)-Ub-EA			HA-Ub-PA		
Gene names	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
ATXN3	--	--	--	23.467	23.9675	22.8822	--	--	--	--	--	--
BAP1	--	--	--	24.0464	25.2925	22.2273	--	--	--	--	--	--
OTUB1	--	--	--	27.6319	27.7484	27.7277	--	--	--	--	--	--
OTUD4	--	--	--	23.2687	22.8087	22.1191	--	--	--	--	--	--
OTUD5	--	--	--	24.3707	24.2859	22.0572	--	--	--	--	--	--
OTUD6B	--	--	--	24.7508	24.9694	22.8084	--	--	--	--	--	--
OTUD7B	--	--	--	23.039	22.9976	22.9428	--	--	--	22.2257	--	--
UCHL3	--	--	--	--	--	--	--	21.6719	23.2304	20.7098	--	--
UCHL5	--	--	--	28.2165	28.5314	27.667	--	--	--	--	--	--
USP10	--	--	--	27.7519	27.8358	26.4138	--	--	--	--	--	--
USP11	--	--	--	24.2467	24.3879	--	--	--	--	--	--	--
USP14	--	--	--	29.9123	29.997	29.2104	--	--	--	--	--	--
USP15	--	--	--	26.9106	27.0341	26.5434	--	--	--	--	--	--
USP16	--	--	--	24.3416	24.9971	24.2524	--	--	--	--	--	--
USP19	--	--	--	26.4083	26.1064	24.7186	--	--	--	--	--	--
USP22;USP27X	--	--	--	--	--	19.8587	--	--	--	--	--	--
USP24	--	--	--	25.0513	25.1969	24.6339	--	--	--	--	--	--
USP28	--	--	--	23.8055	23.5471	22.1726	--	--	--	--	--	--
USP3	--	--	--	25.668	26.3169	23.1748	--	--	--	--	--	--
USP30	--	--	--	--	20.8467	--	--	--	--	--	--	--
USP32	--	--	--	24.4643	25.2071	22.4297	--	--	--	--	--	--
USP33	--	--	--	25.2344	25.1464	23.014	--	--	--	--	--	--
USP34	--	--	--	22.222	--	--	--	--	--	--	--	--
USP36	--	--	--	25.6652	26.0933	23.6225	--	--	--	--	--	--
USP37	--	--	--	--	19.4215	--	--	--	--	--	--	--
USP4	--	--	--	21.056	21.538	19.9438	--	--	--	--	--	--
USP42	--	--	--	--	--	--	--	21.9436	21.5584	--	22.4218	--
USP47	--	--	--	26.4254	26.5085	26.1043	--	--	--	--	--	--
USP48	--	--	--	27.7461	27.8699	26.4968	--	--	--	--	--	--
USP5	--	--	--	29.3508	29.3305	29.3812	--	--	--	22.6294	20.4664	18.8577
USP7	--	--	--	29.3763	29.4161	28.879	--	--	--	--	--	--
USP8	--	--	--	24.9997	25.387	23.7196	--	--	--	--	--	--
USP9X	--	--	--	28.1925	28.4993	27.5468	--	--	--	17.4533	--	--
VCPIP1	--	--	--	24.3408	24.3508	24.1352	--	--	--	--	--	--

(--), protein groups not detected. Protein groups captured by probe **2** in two or more repeats (out of the three repeats) are shown as boldface.

Table S2. Label-free quantification (LFQ) intensity scores for E2s and E3s captured in intracellular HeLa cells proteome-wide DUB profiling using 15 μM HA-Cys(cR$_{10}$)-Ub-PA (**2**), HA-Cys(cR10)-Ub-EA (**4**) and HA-Ub-PA respectively.

	Control			HA-Cys(cR$_{10}$)-Ub-PA			HA-Cys(cR$_{10}$)-Ub-EA			HA-Ub-PA		
Gene names	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
CUL4A	19.3277	20.1718	21.4455	--	--	19.5817	--	--	--	19.2424	19.9049	20.3285
CUL4B	20.7566	18.642	20.4146	19.6562	19.5171	20.5353	--	--	--	--	19.639	19.4637
HECTD3	--	--	--	--	22.8251	--	--	--	--	--	--	--
HERC2	--	--	--	24.4375	24.7989	--	--	--	--	--	--	--
HERC4	--	--	--	25.1958	25.4726	17.952	--	--	--	--	--	--
HUWE1	--	--	--	27.7221	28.0663	26.6092	--	--	--	--	--	--
ITCH	--	--	--	26.1278	26.1708	--	--	--	--	--	--	--
NEDD4	--	--	--	--	--	23.9849	--	--	--	23.8638	--	--
NEDD4L	--	--	--	21.349	--	--	--	--	--	--	--	--
RANBP2	--	--	--	--	--	21.5507	--	--	--	--	--	--
RBBP6	23.9412	24.1781	24.9529	24.5683	24.6946	23.7211	--	--	--	24.2389	24.4754	24.2674
RBX1	--	20.1546	21.3864	--	20.4213	--	--	--	--	--	20.4219	--
TOPORS	--	--	--	18.8472	--	--	--	--	--	--	18.9265	19.1969
TRIM21	--	--	19.9767	--	--	--	--	--	--	--	--	--
TRIM25	--	--	--	22.334	22.3809	--	--	--	--	--	25.7125	--
TRIP12	21.3482	--	--	19.3461	20.7355	20.6871	--	--	--	21.2344	--	--
UBE3A	--	--	--	25.2539	25.5148	18.2531	--	--	--	--	--	--
UBE3C	--	--	--	24.6713	25.0503	--	--	--	--	--	--	--
UBR5	--	--	--	28.35	28.5172	23.2259	--	--	--	--	--	--
UHRF1;UHRF2	--	--	--	21.3716	21.319	--	--	--	--	--	--	20.5627
WWP1;WWP2	--	--	--	21.442	--	--	--	--	--	--	--	--
UBE2D3;UBE2D2	--	--	--	--	23.6323	24.4665	--	--	--	--	--	--
UBE2N;UBE2NL	--	--	21.3585	--	--	21.2471	--	--	--	--	--	20.6644

(--), protein groups not detected. Protein groups captured by probe **2** in two or more repeats (out of the three repeats) are shown as boldface.

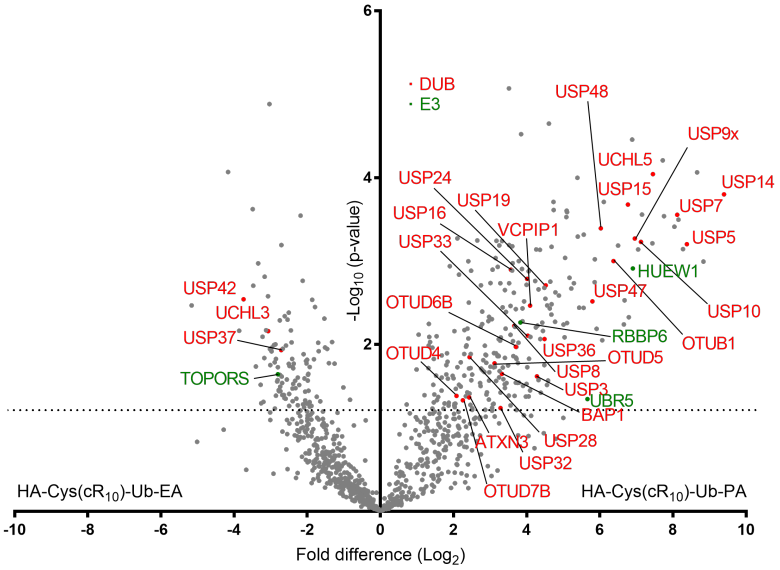


Figure S9: Enrichment of DUBs upon cellular treatment of HeLa cells using15 µM HA-Cys(cR_{10})-Ub-PA (**2**) relative to HA-Cys(cR_{10})-Ub-EA (**4**) using MS-based LFQ. Volcano plot of pairwise comparison of protein groups pulled down with 15 µM HA-Cys(cR_{10})-Ub-PA (**2**) relative to HA-Cys(cR_{10})-Ub-EA (**4**) in HeLa cells. DUBs that were captured by probe **2** with $\log_2$ (fold difference) >2, *p*-value < 0.05 are labeled and colored red. Similarly, E3s are labeled and colored green. DUBs were also identified to the left of the y-axis, indicating enrichment by probe **4**. Two of them, UCHL3 and USP42, were reliably identified, while USP37 was deemed unreliable based on the LFQ values in Table S1. LFQ intensity scores were obtained using MaxQuant and analyzed using Perseus software.

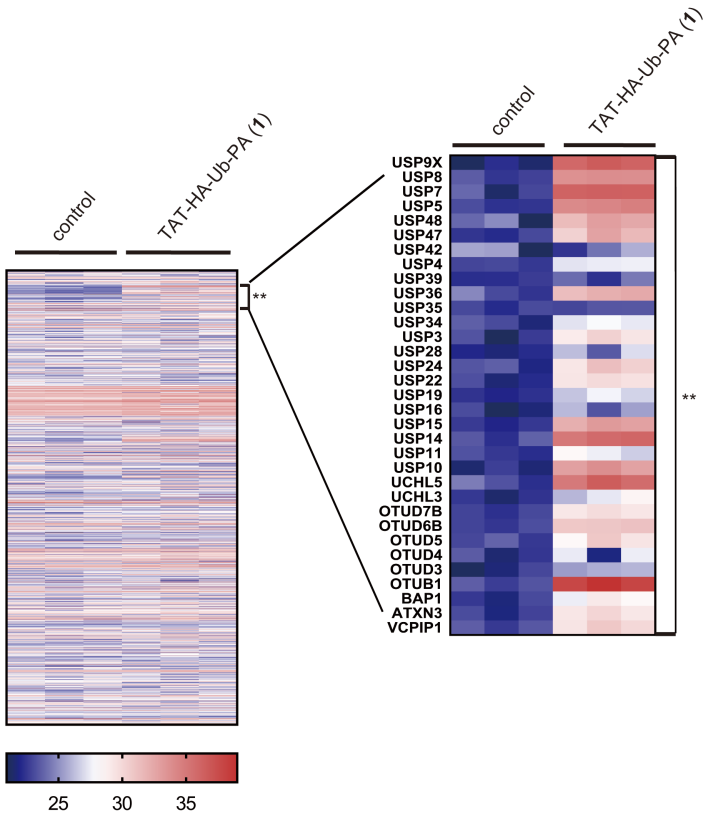


Figure S10: Enrichment of DUBs upon cellular treatment of HeLa cell using15 μM TAT-HA-Ub-PA (**1**) obtained using MS-based LFQ. Heat map representing the LFQ intensity scores of 1,335 identified protein groups from samples corresponding to the control, cellular treatment of 15 μM TAT-HA-Ub-PA (**1**). Each subcolumn represents an independent experiment (3 per group). Asterisks (**) denote the region corresponding to the DUBs. Inset shows a zoomed in view of the DUBs within the heat map. Red represents enrichment (higher LFQ intensity (log_2)), where lack of enrichment (lower LFQ intensity (log_2)) is shown in blue.

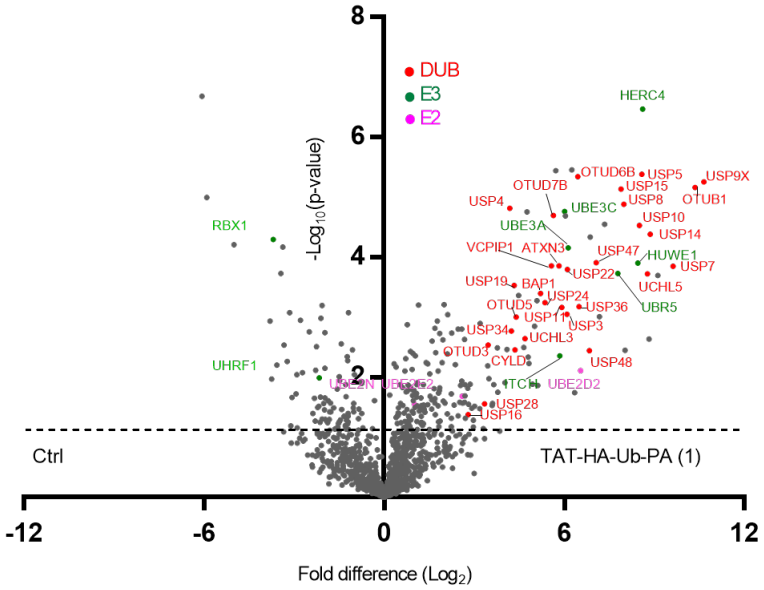


Figure S11: Enrichment of DUBs upon cellular treatment of HeLa cells using15 μM TAT-HA-Ub-PA (**1**) using MS-based LFQ. Volcano plot of pairwise comparison of protein groups pulled down with 15 μM TAT-HA-Ub-PA (**1**) relative to beads control. DUBs that were captured by probe **2** with $\log_2$ (fold difference) >2, *p*-value < 0.05 are labeled and colored red. Similarly, E3s are labeled and colored green, and E2s are labeled and colored purple. LFQ intensity scores were obtained using MaxQuant and analyzed using Perseus software.

Table S3. Label-free quantification (LFQ) intensity scores for DUBs captured in intracellular HeLa cells proteome-wide DUB profiling using 15 μM TAT-HA-Ub-PA (**1**) vs control.

	Control			TAT-HA-Ub-PA		
Gene names	**1**	**2**	**3**	**1**	**2**	3
ATXN3	--	--	--	30.1927	30.7046	30.1679
BAP1	--	--	--	29.022	30.12	29.6705
CYLD	--	--	--	28.3769	29.4959	28.7157
OTUB1	--	24.6162	25.2427	35.2216	35.9021	35.2836
OTUD3	--	--	--	27.0562	27.4629	27.6528
OTUD4	--	--	--	28.9507	23.8424	29.016
OTUD5	--	--	--	29.6759	31.1223	30.2745
OTUD6B	--	--	--	31.1297	31.1782	31.3072
OTUD7B	--	--	--	30.1676	30.5355	30.1975
UCHL3	--	--	--	27.6954	28.8832	29.7948
UCHL5	--	--	--	33.5211	34.468	33.9855
USP10	--	--	--	32.3097	32.884	32.237
USP11	--	--	--	29.6741	29.0477	28.2503
USP14	25.3534	--	--	33.5898	34.0333	34.1581
USP15	--	--	--	31.8624	32.4935	32.2837
USP16	--	--	--	27.7753	--	27.1601
USP19	--	--	--	28.0067	29.1822	28.3695
USP22	--	--	--	30.2443	30.5702	30.3691
USP24	--	--	--	30.193	31.3961	30.8561
USP28	--	--	--	27.8455	--	28.6168
USP3	--	--	--	30.0363	30.8227	30.2425
USP34	--	--	--	28.7486	29.3033	28.9137
USP35	--	--	--	24.8581	25.3063	--
USP36	--	--	--	31.4405	31.8644	32.0137
USP39	--	--	--	25.8428	--	26.1932
USP4	--	--	--	28.771	28.9749	29.0688
USP42	27.2523	27.1297	--	--	26.084	27.5191
USP47	--	--	--	30.8195	32.2501	31.555
USP48	--	--	--	31.5071	32.382	32.0568
USP5	25.0371	--	--	33.0096	33.1565	33.3387
USP7	--	--	--	34.2567	34.3636	34.3478
USP8	--	--	24.7494	32.7894	32.8958	32.8814
USP9X	23.1719	--	--	34.0877	34.5072	34.2608
VCPIP1	--	--	--	30.2827	31.1077	30.6224

(--), protein groups not detected. Protein groups captured by probe **2** in two or more repeats (out of the three repeats) are shown as boldface.

Table S4. Label-free quantification (LFQ) intensity scores for E2s and E3s captured in intracellular HeLa cells proteome-wide DUB profiling using 15 μM TAT-HA-Ub-PA (**1**) vs control.

	Control			TAT-HA-Ub-PA		
Gene names	**1**	**2**	**3**	**1**	**2**	3
CUL1	26.7988	28.1609	28.4202	--	--	--
CUL4A	29.0195	28.7317	29.0487	29.468	29.7651	28.6224
CUL4B	27.1451	26.6513	26.8429	26.1709	21.6459	24.7377
HERC4	--	--	--	32.3104	32.208	32.1009
HUWE1	23.9534	--	23.9404	33.0896	32.0919	33.292
NEDD4L	--	--	--	25.8414	--	25.8992
ITCH	--	--	--	29.1361	30.9637	30.1488
RBBP6	28.6571	30.1021	30.335	29.7937	--	27.5264
RBX1	28.5242	28.6616	29.0895	--	--	--
TRIM21	29.6783	29.6263	29.559	28.9087	28.8881	28.0053
TRIM25	26.6604	26.5686	--	--	--	27.7373
TRIP12	--	25.2183	24.898	24.2661	--	--
UBE3A	--	--	--	30.7924	31.6587	31.0561
UBE3C	--	--	--	29.5288	30.3467	30.0081
UBR5	--	--	--	31.9925	32.4166	32.2273
UHRF1	27.279	27.1655	27.0121	--	--	--
UBE2D2	27.8493	--	25.95	32.2613	32.5314	31.9353
UBE2E1;UBE2E2	--	--	--	29.5395	29.7372	29.4268
UBE2I	28.6581	28.559	28.8054	28.1986	29.3245	28.1014
UBE2N;UBE2NL	28.335	27.9397	28.063	28.9286	29.6532	28.7631
UBE2S	26.8278	--	26.9294	26.7945	--	27.7367
UBE2T	--	25.7871	26.5655	--	--	--

(--), protein groups not detected. Protein groups captured by probe **2** in two or more repeats (out of the three repeats) are shown as boldface.

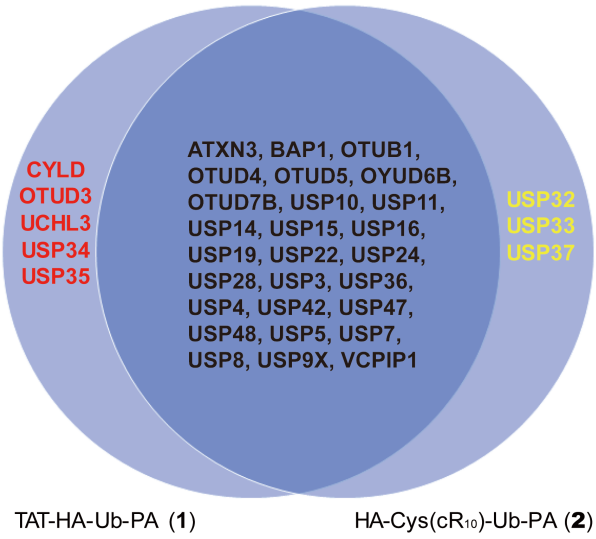


Figure S12: Intracellular HeLa cells proteome-wide DUBs profiling comparison of DUBs captured by 15 μM TAT-HA-Ub-PA (**1**) and 15 μM HA-Cys(cR$_{10}$)-Ub-PA (**2**). Intracellular DUBs exclusively pulldown by probe **1** and **2** in HeLa cells are colored as red and yellow respectively.

Table S5. Label-free quantification (LFQ) intensity scores for DUBs and ligases captured in intracellular HeLa cells proteome-wide DUB profiling using different concentrations of TAT-HA-Ub-PA (**1**) and HA-Cys(cR$_{10}$)-Ub-PA (**2**).

	Control	TAT-HA-Ub-PA			HA-Cys(cR10)-Ub-PA		
Gene Name	1	1 μM	5 μM	10 μM	1 μM	5 μM	10 μM
CYLD	--	--	--	--	--	--	27.2758
UCHL3	--	28.9547	--	--	26.6326	--	26.8905
UCHL5	--	--	--	26.3513	--	--	32.3213
USP10	--	--	--	--	--	--	28.5808
USP11	--	--	--	--	--	--	26.7261
USP14	--	--	--	28.9333	--	--	33.5107
USP15	--	--	--	32.0606	--	--	32.5838
USP16	--	--	--	27.2243	--	--	29.7399
USP19	--	--	--	29.2053	--	--	31.5493
USP24	--	--	--	24.3189	--	--	29.6076
USP3	--	--	--	--	--	--	26.7962
USP32;USP6	--	--	--	--	--	--	25.5156
USP33	--	--	--	--	--	--	27.2889
USP34	--	--	--	25.1337	--	--	28.6271
USP36	--	--	--	--	--	25.1957	--
USP38	--	--	--	--	--	--	25.1635
USP39	--	26.652	26.3987	--	27.2342	28.6049	--
USP4	--	--	--	28.1253	--	--	29.5221
USP42	--	--	--	--	--	--	--
USP47	--	--	--	--	--	--	30.38
USP5	--	29.3138	--	32.0935	25.7778	--	35.6704
USP7	--	--	--	28.8173	--	--	32.6755
USP8	--	--	--	28.6837	--	28.5965	31.3888
USP9X	--	--	--	27.3807	--	--	32.1247
HERC4	--	--	--	--	--	--	26.3606
HUWE1	--	--	--	--	--	--	27.0924
UBE2D2;UBE2D3	--	28.33	28.1059	29.3255	28.1428	29.0803	30.3245

(--), protein groups not detected.

Table S6. Label-free quantification (LFQ) intensity scores for DUBs, E1s, E2s and E3s captured in HeLa cell lysate proteome-wide DUB profiling using 15 μM HA-Cys(cR$_{10}$)-Ub-PA (**2**).

Gene Name	Control	HA-Cys(cR$_{10}$)-Ub-PA
ATXN3	--	27.3032
USP10	--	29.2377
USP14	--	30.458
USP15	--	23.9772
USP24	--	23.9198
USP42	26.3955	--
USP5	22.969	30.5312
USP7	--	29.2767
USP8	--	26.1127
USP19	--	25.0841
UCHL3	26.2385	--
UCHL5	--	30.3661
OTUB1	--	29.7214
USP9X	--	--
HUWE1	--	29.0615
NEDD4	--	24.2471
RNF213	22.1303	24.1444
TRIM21	--	23.9359
UBA2	--	26.4127
UBE2O	--	26.5489
UBE2N;UBE2NL	--	23.4486
UBA1	--	24.8436

(--), protein group was not detected

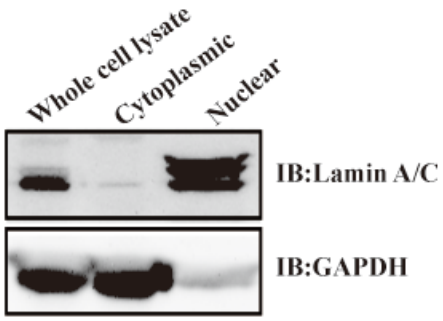


Figure S13: Nuclear and cytoplasmic extraction of HeLa cells. Immunoblotting using anti-GAPDH and anti-Lamin A/C to detect the separation efficiency.

Table S7. Label-free quantification (LFQ) intensity scores for DUBs captured in cytoplasmic fraction of HeLa cells proteome-wide DUB profiling using 15 μM HA-Cys(cR10)-Ub-PA (**2**).

	HA-Cys(cR_{10})-Ub-PA		Control
Gene Name	**1**	**2**	**1**
OTUB1	31.7888	31.864	25.9612
OTUD4	23.5577	24.6452	--
OTUD6B	28.0328	28.2806	27.9235
OTUD7B	28.177	27.8589	--
UCHL5	28.5195	28.5941	--
USP14	28.67	29.2791	--
USP15	26.475	25.988	--
USP16	--	25.7302	--
USP24	25.5855	--	--
USP5	33.024	31.7401	27.6797
USP8	29.9754	30.2256	28.5009
USP9X	--	25.2102	--

(--), protein groups not detected. Captured DUBs predominantly localized in cytosol are colored as orange.

Table S8. Label-free quantification (LFQ) intensity scores for DUBs captured nuclear fraction of HeLa cells proteome-wide DUB profiling using 15 μM HA-Cys(cR10)-Ub-PA (**2**).

	HA-Cys(cR_{10})-Ub-PA		Control
Gene Name	1	2	1
BAP1	27.22	26.2924	--
UCHL5	25.043	25.0404	--
USP36	22.7195	--	--
USP14	27.9753	27.7024	--
USP7	26.2316	--	--
USP5	28.629	28.4988	--
USP8	26.993	26.898	--
USP9X;USP9Y	27.1919	26.4903	--

(--), protein groups not detected. Captured DUBs predominantly localized in nucleus are colored as red.

Supplemental Methods

Plasmid Construction and Gene Cloning. First, the yeast $Ub_{(1-75)}$ gene was mutated using QuikChange polymerase chain reaction (PCR) protocol to generate the humanized $Ub_{(1-75)}$ (S19P, D24E, and S28A) and used throughout this work. To generate N-terminal HA-tagged $Ub_{(1-75)}$, the DNA sequence encoding the HA-tag (TAC CCA TAC GAT GTT CCA GAT GTT CCA TAC GCT) was added after the NdeI restriction site in the forward DNA primer utilized to amplify the Ub gene. Humanized $Ub_{(1-75)}$ was utilized as a template and amplified by PCR. The amplified gene product was cloned into *Escherichia coli* expression vector pTYB1 (New England Biolabs, Ipswich, MA) using the restriction sites SapI and NdeI. The N-terminal TAT-tag was added to HA-$Ub_{(1-75)}$ using a similar procedure. The DNA sequence encoding the TAT-tag (AGG AAG AAG CGG AGA CAG CGA CGA AGA) was added after the Ndel restriction site in the forward DNA primer utilized to amplify the HA-$Ub_{(1-75)}$ gene. HA-$Ub_{(1-75)}$ gene was utilized as a template and amplified by PCR. The amplified gene product was cloned into the *Escherichia coli* expression vector pTYB1 (New England Biolabs, Ipswich, MA) using the restriction sites SapI and NdeI to generate HA-Cys-$Ub_{(1-75)}$.

Protein Expression and Purification. A standard protein expression protocol described below was used for all proteins unless otherwise stated. Plasmids were transformed into BL21(DE3) cells (Novagen Limited, Hornsby Westfield, Australia) and cultured at 37 °C until OD_{600} reached 0.3-0.4. Then the incubation temperature was decreased to 16 °C for the cell culture until OD_{600} reached 0.4-0.6. Protein expression was then induced with 0.5-1 mM IPTG. Cells were cultured for an additional 15 h at 16 °C following the induction and harvested at 6,000 rpm for 10 min at 4 °C. Cells expressing HA-Ub, HA-Cys-Ub, or TAT-HA-Ub were sonicated in lysis buffer (20 mM Tris, 200 mM NaCl, 5% glycerol and 1 mM EDTA, 1 mM PMSF, pH 7.5) and cell free extract was batch bound to chitin resin (New England Biolabs, Ipswich, MA) for overnight at 4 °C. After removal of unbound proteins via centrifugation (2,000 rpm at 4 °C), the resin was washed with a high salt buffer containing 20 mM Tris (pH 7.5), 0.5 M NaCl, 5% glycerol, 1 mM EDTA. Then the resin was washed with a low salt buffer containing 20 mM MES (pH 6.5), 100 mM NaCl, and then incubated with cleavage buffer 20 mM MES (pH 6.5), 100 mM NaCl, 80 mM MESNA for 15 h at room temperature. Protein was eluted, and concentrated to desired concentration using Amicon Ultra-15 Centrifugal Filter Units (Thermo Fisher Scientific, Waltham, MA). Purity of the proteins were estimated to be 90% or greater by SDS-PAGE and Coomassie Blue staining. Protein concentrations were determined by Bradford assay. Protein samples were flash frozen and stored at -80 °C. USP2-CD and UCHL3 were expressed and purified as previously described.[1]

Mammalian Cell Culture. Human cervical carcinoma cell line, HeLa, (ATCC CCL-2) was cultured in Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific Inc., Waltham, MA) supplemented with 10% FBS, 100 units/mL penicillin and 0.1 mg/mL streptomycin (Thermo Fisher Scientific Inc., Waltham, MA). Cells were maintained at 37 °C and 5% CO_2 in a humidified incubator.

Live-cell Imaging Confocal Microscopy. HeLa cells were plated in Nunc™ Cell-Culture 6-well rectangular plates (Thermo Fisher Inc., Waltham, MA) at 50% confluence and allowed to adhere for 12 h at 37 °C and 5% CO_2 in DMEM (Thermo Fisher Scientific Inc., Waltham, MA) supplemented with 10% FBS, 100 units/mL penicillin and 0.1 mg/mL streptomycin (Thermo Fisher Scientific Inc., Waltham, MA). Cells were then treated with 15 μM of indicated Ub-based probe for 4 h at 37 °C and 5% CO_2 in medium (DMEM) (Thermo Fisher Scientific Inc., Waltham, MA) supplemented with 10% FBS in the absence of antibiotics. Post treatment, cells were washed with cold Dulbecco's Phosphate-Buffered Salt solution with calcium and magnesium (DPBS) (Mediatech, Inc., Manassas, VA). Then 0.25% trypsin, 0.1% EDTA in HBSS without calcium, magnesium, and sodium bicarbonate (Mediatech, Inc., Manassas, VA) was added to cells and allowed to incubate for approximately 1 to 5 mins at room temperature. Trypsin solution was then aspirated, and cells were washed three times with cold DPBS buffer. Cells were then washed with Live Cell Imaging Solution (Thermo Fisher Inc., Waltham, MA) and treated with NucBlue™ Live ReadyProbes™ Reagent (Thermo Fisher Inc., Waltham, MA) according to manufacturer's instructions. Images were captured on a Zeiss 880 Confocal microscope with a full environmental enclosure (temperature, humidity, and CO_2 control) optimized for live cell imaging and analyzed using ImageJ software (NIH, Bethesda, MD).

Immunoblotting Analysis of DUB labeling by Ub-based DUB ABPs. Intracellular DUB labeling experiments were performed using the following general protocol unless otherwise indicated. HeLa cells were plated in Corning™ Costar™ Flat Bottom Cell Culture 6-well plates (Mediatech, Inc., Manassas, VA) at 50% confluence and allowed to adhere for 12 h at 37 °C and 5% CO_2 in DMEM (Thermo Fisher Scientific Inc., Waltham, MA) supplemented with 10% FBS, 100 units/mL penicillin and 0.1 mg/mL streptomycin (Thermo Fisher Scientific Inc., Waltham, MA). Cells were then treated in the absence of antibiotics with 15 μM of indicated Ub-based probe for 4 h at 37 °C at 5% CO_2 in medium (DMEM) (Thermo Fisher Scientific Inc., Waltham, MA) supplemented with 10% FBS. Post treatment, cells were washed with cold Dulbecco's Phosphate-Buffered Salt solution with calcium and magnesium (DPBS). Then 0.25% trypsin, 0.1% EDTA in HBSS without calcium, magnesium, and sodium bicarbonate (Mediatech, Inc., Manassas, VA) was added to cells and allowed to incubate for approximately 1 to 5 mins at room temperature. Trypsin

solution was then aspirated, and cells were washed three times with cold DPBS buffer. Cells were then harvested and washed with DPBS buffer and used immediately for experiments or stored at -80°C until use.

To generate the cell lysates for immunoblotting, the cell pellets were first washed in Gibco™ Hank's Balanced Salt Solution (HBSS) with calcium and magnesium, but no phenol red (Life Technologies Corporation, Carlsbad, CA). Cells were then resuspended in Pierce RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific Inc., Waltham, MA) supplemented with 1X Complete Protease Cocktail Inhibitor (Thermo Fisher Scientific Inc., Waltham, MA), and allowed to incubate on ice for 1 h. Cells were then centrifuged 14,000 rpm at 4 °C for 10 min to remove cell debris. Total protein concentration was determined using Bradford assay (Thermo Fisher Scientific Inc., Waltham, MA). Prepared cell lysates were used immediately for experiments or stored at -80°C until use. 100 μg samples were prepared using a 6X gel loading dye (35 mM Tris, 10% SDS, 30% glycerol and 0.81 M, pH 6.8) resolved on a 10% SDS-PAGE gel followed by transfer onto a PDVF membrane (Thermo Fisher Scientific Inc., Waltham, MA) and immunoblotted with an anti-HA antibody HRP-conjugated anti-mouse antibody was used as the secondary antibody. To detect specific DUB labeling, 50 μg samples were prepared using a 6X gel loading dye and resolved by a 12% SDS-PAGE gel followed by transfer onto a PDVF membrane (Thermo Fisher Scientific Inc., Waltham, MA). Following the transfer, membranes were immunoblotted using DUB-specific antibodies (USP7 and USP15). HRP-conjugated anti-mouse antibody was used as the secondary antibody for anti-USP15. HRP-conjugated anti-rabbit antibody was used as the secondary antibody for anti-USP7. GAPDH was utilized as a loading control using an anti-GAPDH antibody HRP-conjugated anti-rabbit antibody was used as the secondary antibody for GAPDH. Signals were detected using ECL Western blotting substrate (Thermo Fisher Scientific Inc., Waltham, MA).

To generate cell lysates for the cell lysate-based DUB profiling experiments, cells were harvested and washed extensively with DPBS and used immediately for experiments or stored at -80°C until use. To generate the cell lysates, the cell pellets were resuspended in lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 5 mM DTT, 2 mM ATP, 0.5% NP-40, 10% glycerol and incubated on ice for 30 min. Cells were lysed on ice using sonication, and centrifuged at 14,000 rpm at 4°C for 10 min to remove cell debris. Total protein concentration was determined using a Bradford assay (Thermo Fisher Scientific Inc., Waltham, MA) and aliquoted. Prepared cell lysates were used immediately for experiments or stored at -80 °C until use. In cell lysate-based DUB profiling experiments, 50 μg cell lysate was incubated with 15 μM of indicated Ub-based DUB ABP at 37 °C for 3 h in a labeling solution containing 50 mM Tris (pH 7.0), 5 mM $MgCl_2$, 0.5 mM EDTA, 5 mM DTT, 2 mM ATP and 250 mM sucrose. Samples were separated by a 10% SDS-PAGE gel, transferred onto a PDVF membrane and blotted with anti-HA antibody. HRP-conjugated anti-mouse antibody was used as the secondary antibody. Signals were detected using ECL Western blotting substrate.

Pulldown and Mass Spectrometry Analysis. To generate untreated, HA-Ub-PA, or HA-Cys(cR_{10})-Ub-PA treated cells for intracellular proteome-wide DUB profiling using MS-based LFQ, 400 μg sample of indicated treatment was incubated with 100 μL anti-HA magnetic beads in RIPA Lysis and Extraction Buffer supplemented with 1X Complete Protease Cocktail Inhibitor at room temperature for 2 h. Unbound proteins were aspirated and anti-HA beads were washed 3X with Tris-buffered saline, 0.1% Tween-20 (TBST). Enriched samples were eluted with 50 mM NaOH (twice). Eluate was adjusted to pH 8.0 using a 1M Tris (pH 8.0) solution and reduced with 10 mM DTT incubating at 60 °C water bath for 1 h. The sample was then subsequently alkylated with 40 mM iodoacetamide at room temperature for 30 min at room temperature (protected from light). The alkylation reaction was quenched by addition of 10 mM DTT prior to digestion with 4 μg of trypsin protease at 37 °C for 18 h. Tryptic digestion was quenched by 0.1% formic acid and centrifuged at 13,000 g at room temperature for 10 min to remove residual precipitation. Digested peptide sample was desalted with C18 resin stage-tip made in house following previous publication[2] and lyophilized prior to LC-MS/MS analysis.

Lyophilized tryptic digests were dissolved in 0.1% formic acid in ddH_2O prior to LC-MS/MS (15 cm x 75 m reverse phase nano-LC (Thermo Fisher Scientific, Waltham, MA)) analysis using Orbitrap Q-Exactive (positive polarity mode, collision induced dissociation) (Thermo Fisher Scentific, Waltham, MA) with a nano-electrospray ion source. A linear gradient from 5% to 60% acetonitrile in 0.1% formic acid water solution was used to separate peptides for 150 minutes at a constant flow of 200 nL/min. The system was set to operate in a data-dependent mode with MS/MS scan of the six most abundant peaks from a full MS scan. Full scans were acquired between 300 to 1800 m/z with a resolution of 60,000.

Nine raw files (three experiments, with three independent replicates each) were analyzed together using MaxQuant software version 1.4.0.6. Peak list was searched with built-in Andromeda search engine against the UniProt human database downloaded from Uniprot website (www.uniprot.org/proteomes/UP000005640). MaxQuant parameters were set as follow unless otherwise indicated. Multiplicity was set to one (no isotope labeling), and trypsin was set to cleave after lysine and arginine, unless followed by a proline. Maximum two missed cleavages were allowed, and maximum charge of peptide was set to 7. Seven amino acids was set to be the minimum peptide length. Carbamidomethylation of cysteine was a fixed modification. N-terminal acetylation, methionine oxidation, phosphorylation (serine, threonine, and tyrosine), and ubiquitin diglycine remnant were set as variable modifications.

Label-free Quantification (LFQ) module was enabled with a minimum ratio count of 1, and second peptide search and re-quantify functions were enabled. The maximum number of modifications was set to 5 per peptide. The option of requiring MS/MS for LFQ comparisons was enabled. Target decoy approach was used to filter peptide spectrum matches (PSM), and a false discovery rate (FDR) of 1% was used for protein identification. The output text file "protein groups" was imported and analyzed using Perseus (software version 1.5.4.1). Proteins categorized as "only identified by modified peptides" were eliminated. Proteins identified with reversed decoy mode and the common contaminant proteins were excluded. Experimental replicates (3 total) were grouped. Proteins with at least one valid LFQ values (LFQ intensity >0) in at least one group were kept. Missing values were imputed using the normal distribution of the total matrix using the default parameters (0.3 width and 1.8 down shift). Two-sample unpaired *t*-test was performed using the default parameters. To generate volcano plot, the $-\log_{10}(p\text{-}value)$ (y-axis) and $\log_2$(fold difference of LFQ values between paired samples) (x-axis) were plotted in GraphPad Prism (version 7).

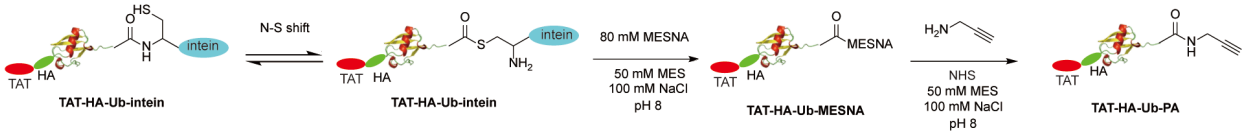
Nuclear and cytoplasmic extraction and Pulldown-MS analysis. For pulldown-MS analysis, HeLa cells were treated with 15 μM probe **2** for 4 h at 37 °C as described above. The nuclear and cytoplasmic protein extraction was performed using Thermo Scientific NE-PER™ Kit (Catalog number 78833). 480 μl CER I buffer was added to 40 μl harvested HeLa cells (packed cell volume) and vortexed for 15 seconds, followed by incubation on ice for 10 min. 48 μl CER II buffer was added to the resuspended HeLa cells and vortexed for 5 seconds, followed by centrifugation for 5 min at 13,000 rpm. The supernatant (cytoplasmic extraction) was transferred into a new tube. Then 200 μl NER buffer was added to the remaining pellet and vortexed for 15 seconds five times with a 10 min interval. Following centrifugation for 5 min at 13,000 rpm, the supernatant (nuclear extraction) was transferred to a new tube. Successful cytoplasmic and nuclear protein extraction was verified by Western blotting against GAPDH and Lamin A/C, respectively. For pulldown, 300 μg cytoplasmic fraction and 100 μg nuclear fraction were incubated with 75 and 25 μL anti-HA magnetic beads, respectively, in RIPA Lysis and Extraction Buffer supplemented with 1X Complete Protease Cocktail Inhibitor at room temperature for 2 h. Then the same procedure was followed for subsequent sample preparation and data analysis as described above.

General information.

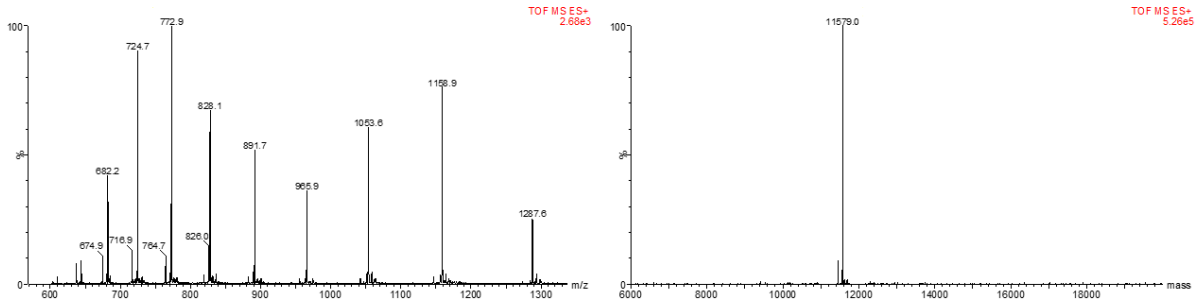
Chemical reagents were obtained from Fisher, Sigma-Aldrich, Alfa and Acros of the highest available grade and used without further purification. ^{1}H and ^{13}C NMR spectra were recorded on Bruker AV400 NMR Spectrometer with a CryoProbe. Chemical shifts are reported in δ (ppm) units using ^{13}C and residual ^{1}H signals from deuterated solvents as references. Mass spectra for small molecules were recorded on ACQUITY UPLC H-Class/SQD2 instrument equipped with an electrospray ionization (ESI) source. Mass spectra for proteins were recorded on Xevo G2-S QTof instrument equipped with an electrospray ionization (ESI) source. Mass spectra for proteomic study using LC-MS/MS (15 cm x 75 m reverse phase nano-HUPLC) analysis using Orbitrap Q-Exactive (positive polarity mode, collision induced dissociation) (Thermo Fisher Scentific, Waltham, MA) with a nano-electrospray ion source. Analytical thin layer chromatography (TLC) was performed on silica gel 60 GF254 (Merck). Column chromatography was conducted on silica gel (230-400 mesh). Reversed phase HPLC was performed on VARIAN ProStar HPLC systems (with Applied Biosystems as the solvent delivery units). For probes purification, Jupiter C18 (10 ×250 mm 10 micron) column was used at flow rates of 4 mL/min. The UV absorption at 214 nm and were monitored for the injections. Water (with 0.1% formic acid) and acetonitrile (with 0.1% formic acid) were chosen as the solvents. The gradient of the solvents was optimized for each probe.

GAPDH, UCHL3, and USP15 antibodies were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX). USP7 antibody was purchased from Bethyl Labs (Montgomery, TX). HA-tag and HRP-conjugated anti-mouse antibodies were purchased from (MilliporeSigma, Bilerica, MA). HRP-conjugated anti-rabbit antibody was purchased from Abcam (Cambridge, MA). Pierce Anti-HA magnetic beads and trypsin protease (MS grade) were purchased from ThermoFisher Scientific (Waltham, MA). Restriction enzymes and Quick DNA ligase was purchased from New England BioLabs (NEB) (Ipswitch, MA)

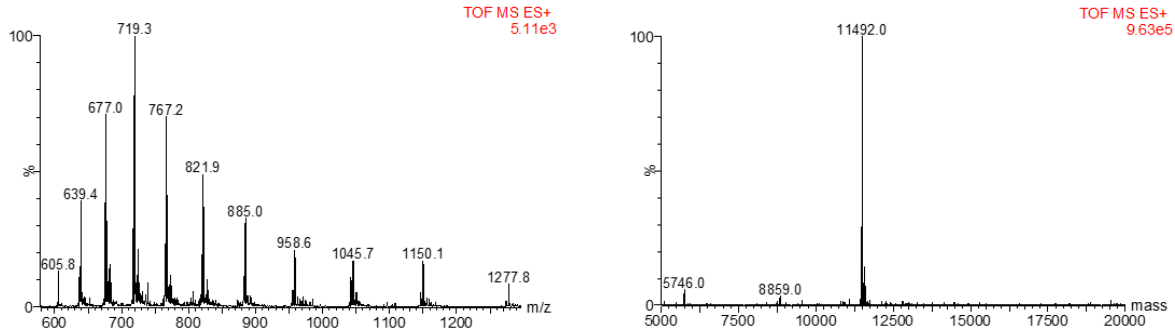
Scheme S1. Generation of TAT-HA-Ub-PA (**1**)



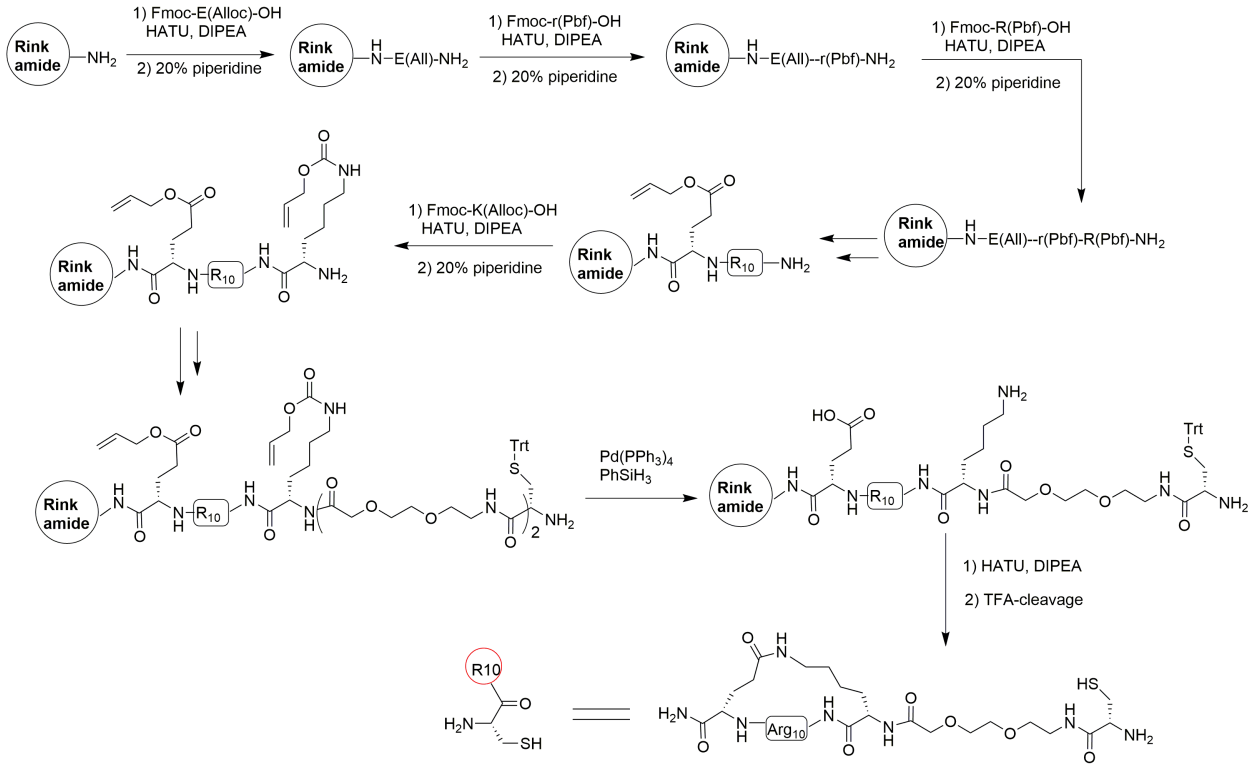
Generation of TAT-HA-Ub–MESNA. The procedure of TAT-HA-Ub–MESNA generation is described in protein expression and purification section. The molecular weight of the TAT-HA-Ub-MESNA species was determined by ESI -MS. (Calculated mass: 11,578 Da, Observed mass: 11,579 Da).



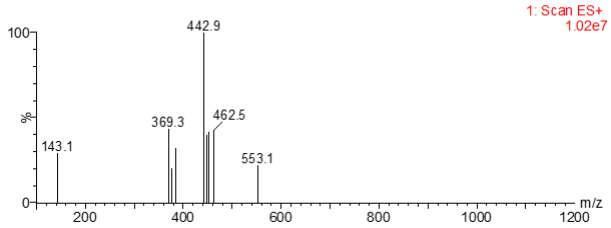
Generation of TAT-HA-Ub–PA. To a solution of NHS (500 µL, 2 M solution in ddH_2O, 1 mmol, 1000 eq.), prop-2-yn-1-amine (64 µL, 15.6 M, 1 mmol, 1000 eq.) was added TAT-HA-Ub-MESNA (10 mg/mL, 1 mL, 1 µmol) in MES buffer (20 mM MES, 100 mM NaCl, pH 6.5). The final pH of the reaction solution was approximately 8.0. The mixture was immediately vortexed and reacted at room temperature for 4 h. The reaction mixture was then buffer exchanged into MES buffer (20 mM MES, 100 mM NaCl, pH 6.5). The final concentration of the product was determined by Bradford assay. The molecular weight of TAT-HA-Ub-PA was determined by ESI-MS. (Calculated mass: 11,492 Da, Observed mass: 11,492 Da).



Scheme S2. Synthesis of cyclic polyarginine 10 (cR_{10}) peptide.

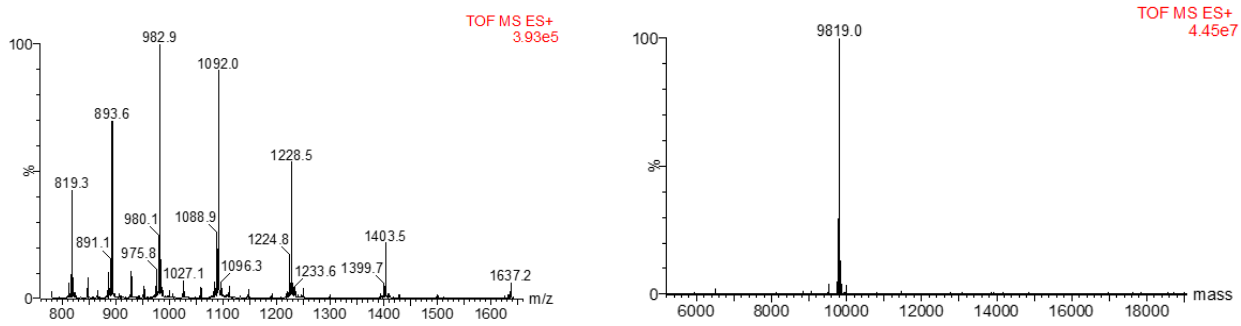


Synthesis of cyclic polyarginine peptide (cR_{10}) was carried out according to a previous publication.[3] A linear sequence of the peptide (Boc)(Trt)C(PEG)$_2$(Alloc)KRrRrRrRrRr(OAll)E was synthesized on a Rink amide resin (0.066 mmol, 0.33 mmol/g). Upper case letters correspond to L-, lower case letters to D-amino acids. The coupling reactions were performed using 2.5 eq. of amino acid, 2.5 eq. of HATU and 5.0 eq. of DIEA in NMP solution. The Alloc- and OAll protecting groups were removed using $Pd(PPh_3)_4$ (7.7 mg, 0.1 eq.) and $PhSiH_3$ (181 mg, 203 µL, 25 eq.) in dry DCM for 30 min at ambient temperature under argon atmosphere. To remove the Pd catalyst afterwards, the resin was washed with 0.2 M DIPEA/NMP. The cyclization of the peptide was carried out using 1 eq. HATU and 2 eq. DIPEA in 8 mL NMP for 2 h at room temperature. After washing and drying, the peptide was cleaved from the solid support (5 h in 5.4 mL 95% TFA, 2.5% TIS, 2.5% DTT), TFA evaporated via N_2-stream and the peptide precipitated in 40 mL diethyl ether. Then the precipitate was dissolved in ddH_2O and used in next step without further purification. The molecular weight of the cR_{10} peptide was determined by ACQUITY UPLC H-Class/SQD2. (Calculated mass: 2210 Da, $[M+5H]^{5+}$ 443, Observed mass: $[M+5H]^{5+}$ 442.9).

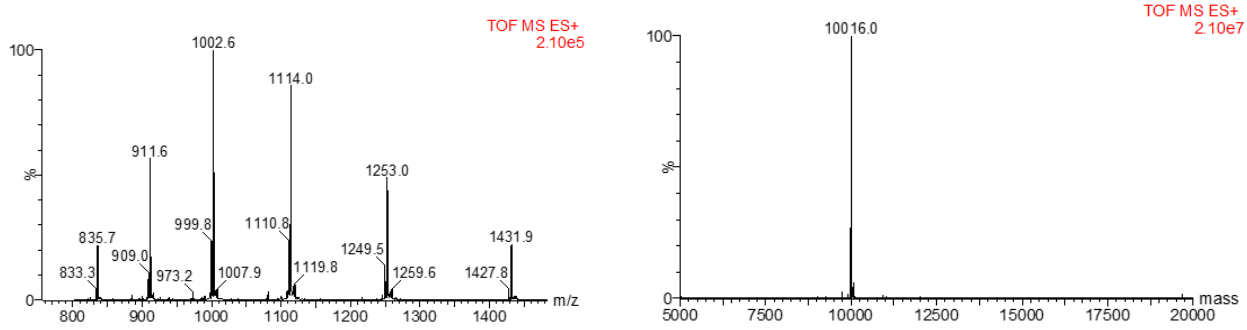


Generation of HA-Cys(cR$_{10}$)-Ub-PA (2).

Generation of HA-Cys-Ub-MESNA. The procedure of HA-Cys-Ub–MESNA generation is described in protein expression and purification section. The molecular weight of the HA-Cys-Ub–MESNA species was determined by ESI-MS (Calculated mass: 9,818 Da, Observed mass: 9,819 Da).

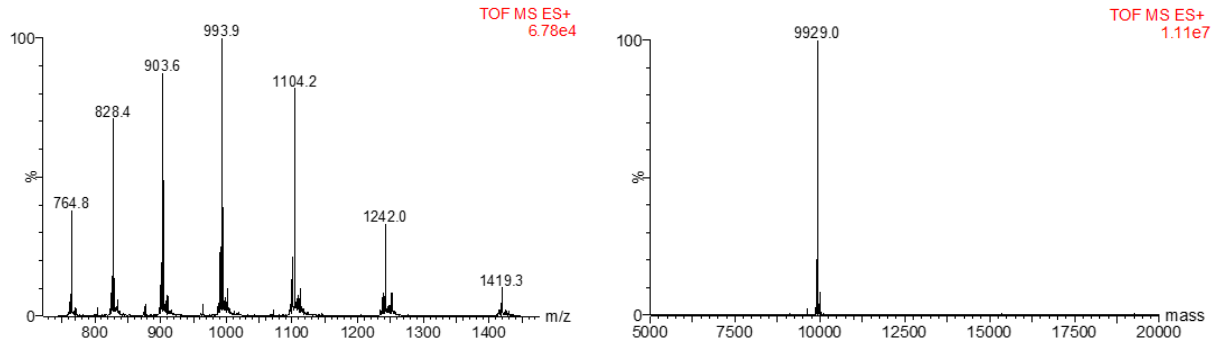


Generation of HA-Cys(TNB)-Ub-MESNA. To a solution of HA-Cys-Ub-MESNA (10 mg/mL, 2 mL, 2 μmol) in MES buffer (20mM MES, 100mM NaCl, pH 6.5) was added 5,5′-Dithiobis(2-nitrobenzoic acid) (4 mL, 10 mM solution in ddH_2O, 40 μmol, 20 eq.) to the solution. The mixture was immediately vortexed and reacted at room temperature for 0.5 h. Then the reaction mixture was used in next step without further purification. The molecular weight of HA-Cys(TNB)-Ub-PA was determined by ESI-MS (Calculated mass: 10,015 Da, Observed mass: 10,016 Da).

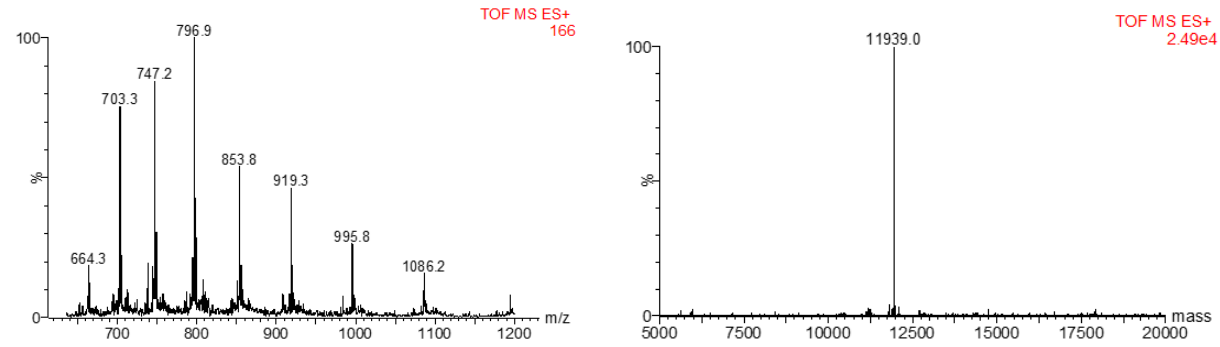


Generation of HA-Cys(TNB)-Ub-PA. To a solution of NHS (375 μL, 2 M solution in ddH_2O, 0.75 mmol, 1000 eq.) and prop-2-yn-1-amine (15.6 M, 48 μL, 0.75 mmol, 1000 eq.) was added HA-Cys(TNB)-Ub-

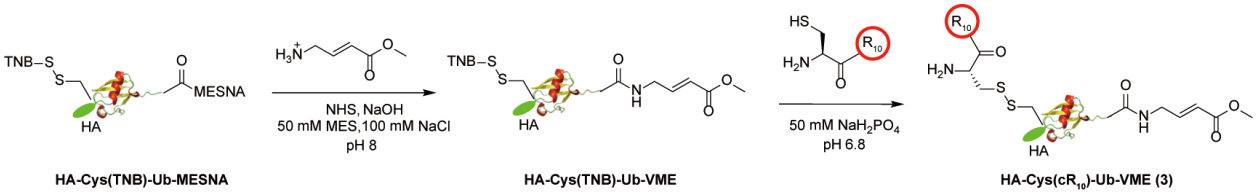
MESNA (3 mg/mL, 2.5 mL, 0.75 μmol) in MES buffer (20 mM MES, 100 mM NaCl, pH 6.5). The final pH of the reaction was approximately 8.0. The mixture was immediately vortexed and reacted at room temperature for 4 h. Then the reaction mixture was buffer exchanged into MES buffer (20 mM MES, 100 mM NaCl, pH 6.5). The final concentration of the product was determined by Bradford assay. The molecular weight of HA-Cys(TNB)-Ub-PA was determined by ESI-MS (Calculated mass: 9,929 Da, Observed mass: 9,929 Da).



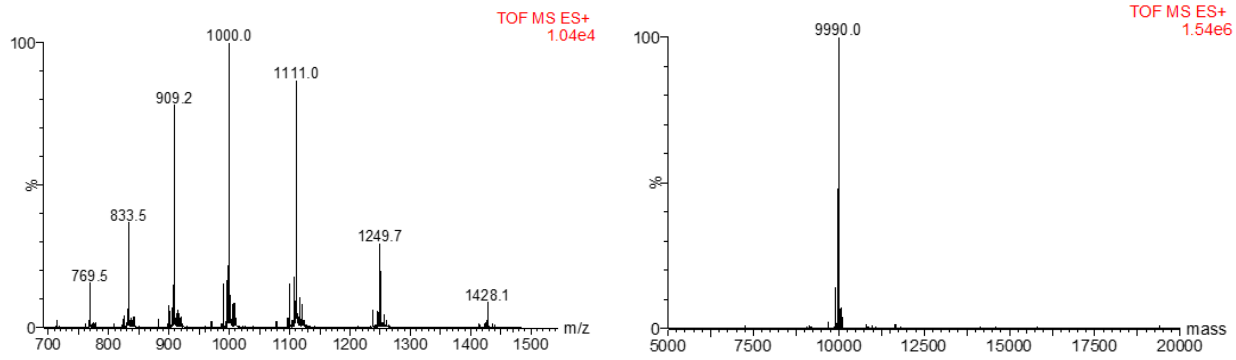
Generation of HA-Cys(cR$_{10}$)-Ub-PA. cR$_{10}$ peptide (100 mg/mL, 0.14 mL, 6.4 μmoL, 20 eq.) was added to a 50 mM NaH_2PO_4 buffer (pH 6.8) solution containing HA-Cys(TNB)-Ub-PA (2 mg/mL, 1.6 mL, 0.32 μmol). The mixture immediately became yellow, then vortexed and reacted at room temperature for 2 h. The reaction mixture was then diluted with MES buffer (20 mM MES, 100 mM NaCl, pH 6.5) and concentrated to a total volume of 0.5 mL. C18 HPLC column was utilized to purify the product. Jupiter 10 μm C18 300A 10 x 250 mm running at 4 mL/min. Solvent A is consisted of water with 0.1% formic acid, Solvent B is consisted of acetonitrile with 0.1% formic acid. Gradient in time → % B: 0 min → 5%, 10 min → 30%, 70 min → 40%, 79 min→ 95%, 80 min. The product was eluted at approximately 25% buffer B. Fractions containing product were pooled and lyophilized overnight. The pure product was dissolved in 6 M guanidine hydrochloride (pH 6.0) then buffer exchanged into MES buffer (20 mM MES, 100 mM NaCl, pH 6.5) for four times. The molecular weight of HA-Cys(cR$_{10}$)-Ub-PA was determined by ESI-MS (Calculated mass: 11,939 Da, Observed mass: 11,939 Da).



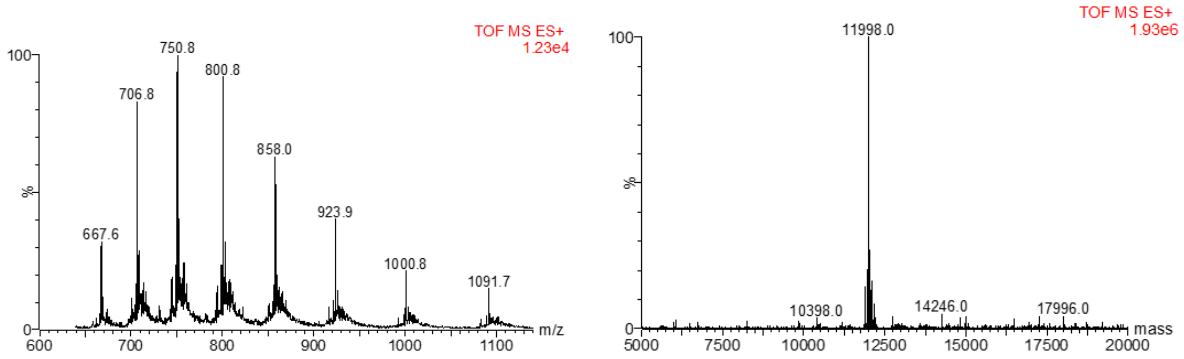
Scheme S3. Generation of HA-Cys(cR$_{10}$)-Ub-VME (**3**).



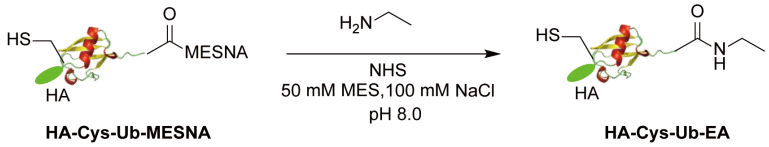
Generation of HA-Cys(TNB)-Ub-VME. To a solution of NHS (375 µL, 2 M solution in ddH$_2$O, 0.75 mmol, 1000 eq.), (E)-4-methoxy-4-oxobut-2-en-1-aminium (375 µL, 2 M solution in ddH$_2$O, 0.75 mmol, 1000 eq.) and NaOH (375 µL, 2 M solution in ddH$_2$O, 0.75 mmol, 1000 eq.) was added to HA-Cys(TNB)-Ub-MESNA (3 mg/mL, 2.5 mL, 0.75 µmol) in MES buffer (20 mM MES, 100 mM NaCl, pH 6.5). The mixture was immediately vortexed and reacted at room temperature for 4 h. The reaction mixture was buffer exchanged into MES buffer (20 mM MES, 100 mM NaCl, pH 6.5). The final concentration of the product was determined by Bradford assay. The molecular weight of HA-Cys(TNB)-Ub-VME was determined by ESI-MS (Calculated mass: 9,990 Da, Observed mass: 9,990 Da).



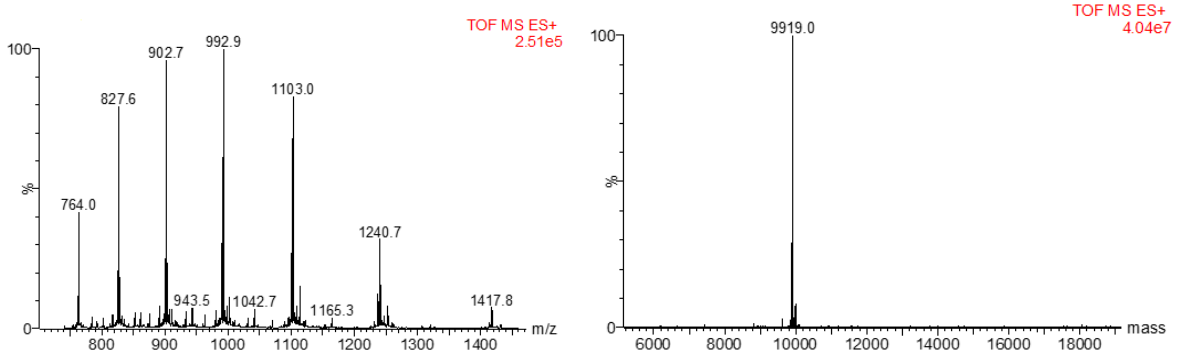
Generation of HA-Cys(cR$_{10}$)-Ub-VME. cR$_{10}$ peptide (100 mg/mL, 0.53 mL, 24 µmol, 20 eq.) was added to HA-Cys(TNB)-Ub-VME (6 mg/mL, 2 mL, 1.2 µmol) in 50 mM NaH$_2$PO$_4$ (pH 6.8). The mixture immediately became yellow, then was vortexed and reacted at room temperature for 2 h. The reaction mixture was diluted with MES buffer (20 mM MES, 100 mM NaCl, pH 6.5) and concentrated to a total volume of 0.5 mL. C18 HPLC column was utilized to purify the product. Jupiter 10 µm C18 300A 10 x 250 mm running at 4 mL/min. Solvent A is consisted of water with 0.1% formic acid. Solvent B is consisted of acetonitrile with 0.1% formic acid. Gradient in time → % B: 0 min → 5%, 10 min → 30%, 70 min → 40%, 79 min→ 95%, 80 min. Product was eluted at 25% Buffer B. Fractions containing product were pooled and lyophilized. The pure product was dissolved in 6 M guanidine hydrochloride (pH 6.0), then buffer exchanged into MES buffer (20 mM MES, 100 mM NaCl, pH 6.5) for four times. The molecular weight of HA-Cys(cR$_{10}$)-Ub-VME was determined by ESI-MS (Calculated mass: 12,000 Da, Observed mass: 11,998 Da).



Scheme S4. Generation of HA-Cys(cR$_{10}$)-Ub-EA (**4**).

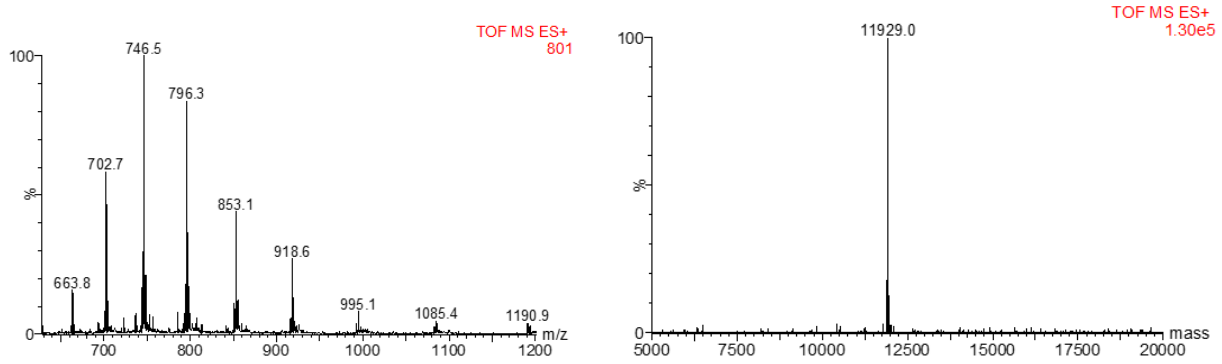


Generation of HA-Cys(TNB)-Ub-EA. To a solution of NHS (1 mL, 2 M solution in ddH_2O, 2 mmol, 1000 eq.), ethanamine (190 µL, 10.5 M solution in ddH_2O, 2 mmol, 1000 eq.) was added HA-Cys(TNB)-Ub-MESNA (8 mg/mL, 2.5 mL, 2 µmol) in MES buffer (20 mM MES, 100 mM NaCl, pH 6.5). The final pH of the reaction was approximately 8.0. The mixture was immediately vortexed, and reacted at room temperature for 4 h. The reaction mixture was buffer exchanged into MES buffer (20 mM MES, 100 mM NaCl, pH 6.5). The final concentration of the product was determined by Bradford assay. The molecular weight of HA-Cys(TNB)-Ub-EA was determined by ESI-MS (Calculated mass: 9,919 Da, Observed mass: 9,919 Da).

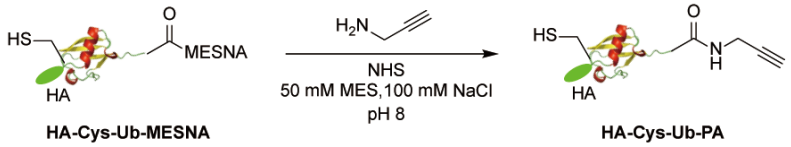


Generation of HA-Cys(cR$_{10}$)-Ub-EA. cR$_{10}$ peptide (100 mg/mL, 0.53 mL, 24 µmol, 20 eq.) was added to a solution of HA-Cys(TNB)-Ub-EA (9 mg/mL, 1.3 mL, 1.2 µmol) in 50 mM NaH_2PO_4 (pH 6.8). The mixture immediately became yellow then vortexed and reacted at room temperature for 2 h. The reaction

mixture was diluted with MES buffer (20 mM MES, 100 mM NaCl, pH 6.5) and concentrated to a total volume of 0.5 mL. C18 HPLC column was used to purify the product. Jupiter 10 µm C18 300A 10 x 250 mm running at 4 mL/min. Solvent A is consisted of water with 0.1% formic acid, Solvent B is consisted of acetonitrile with 0.1% formic acid. Gradient in time → %B: 0 min → 5%, 10 min → 30%, 70 min → 40%, 79 min→ 95%, 80 min. Product was eluted at approximately 25% Buffer B. Fractions containing the product were pooled and lyophilized. The pure product was dissolved in 6 M guanidine hydrochloride (pH 6.0), then buffer exchanged into MES buffer (20 mM MES, 100 mM NaCl, pH 6.5). The molecular weight of HA-Cys(cR_{10})-Ub-EA was determined by ESI-MS (Calculated mass: 11,929 Da, Observed mass: 11,929 Da).

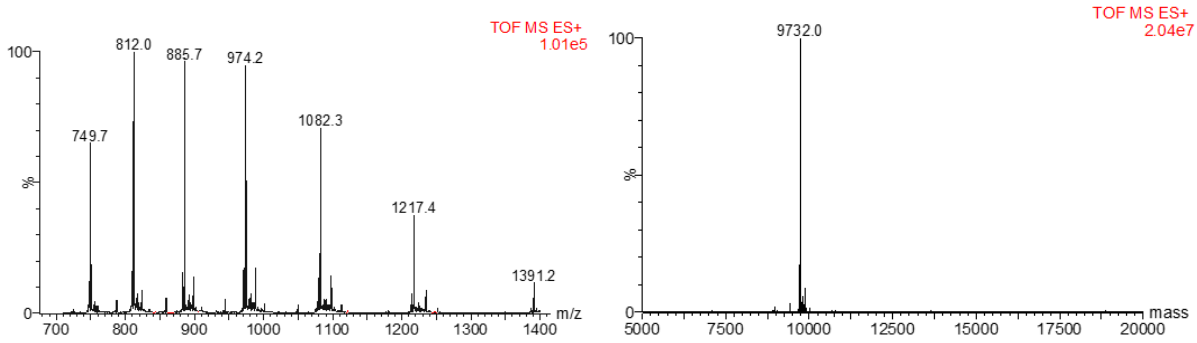


Scheme S5. Generation of HA-Cys-Ub-PA.

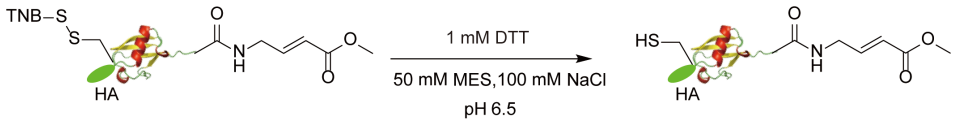


Generation of HA-Cys-Ub-PA. To a solution of NHS (1 mL, 2 M solution in ddH_2O, 2 mmol, 1000 eq.), prop-2-yn-1-amine (190 µL, 10.5 M solution in ddH_2O, 2 mmol, 1000 eq.) was added HA-Cys-Ub-MESNA (8 mg/mL, 2.5 mL, 2 µmol) in MES buffer (20 mM MES, 100 mM NaCl, pH 6.5). The final pH of the reaction was approximately 8.0. The mixture was immediately vortexed, and reacted at room temperature for 4 h. The reaction mixture was diluted with MES buffer (20 mM MES, 100 mM NaCl, pH 6.5) and concentrated to a total volume of 0.5 mL. C18 HPLC column was used to purify the product. Jupiter 10 µm C18 300A 10 x 250 mm running at 4 mL/min. Solvent A is consisted of water with 0.1% formic acid, Solvent B is consisted of acetonitrile with 0.1% formic acid. Gradient in time → %B: 0 min → 5%, 10 min → 30%, 70 min → 40%, 79 min→ 95%, 80 min. Product eluted at approximately 25% Buffer B. Fractions containing the product were pooled and lyophilized. The pure product was dissolved in 6 M guanidine hydrochloride (pH 6.0), then buffer exchanged into MES buffer (20 mM MES, 100 mM NaCl, pH 6.5).

The final concentration of the product was determined by Bradford assay. The molecular weight of HA-Cys-Ub-PA was determined by ESI-MS (Calculated mass: 9,732 Da, Observed mass: 9,732 Da).

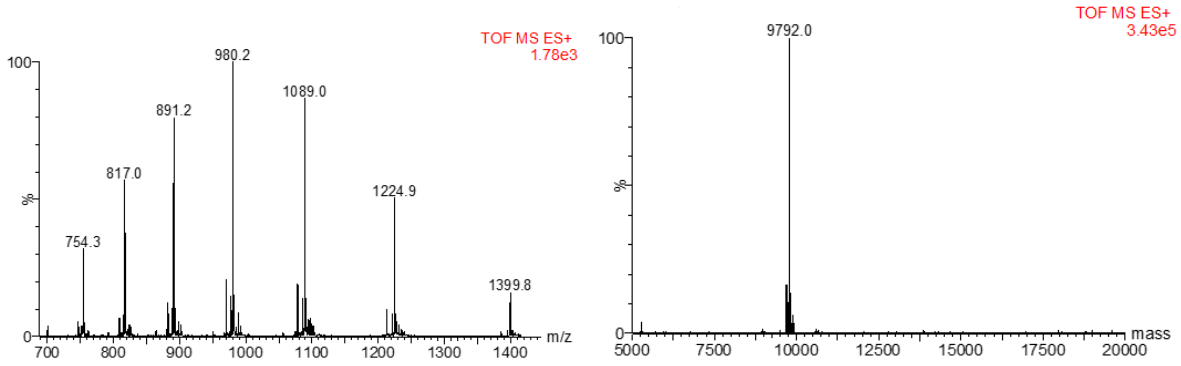


Scheme S6. Generation of HA-Cys-Ub-VME

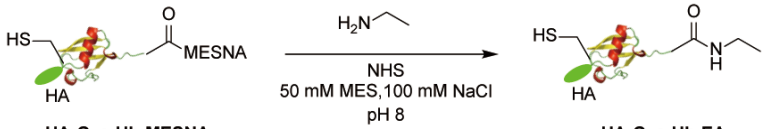


HA-Cys(TNB)-Ub-VME **HA-Cys-Ub-VME**

Generation of HA-Cys-Ub-VME. To a solution of HA-Cys(TNB)-Ub-MESNA (3 mg/mL, 2.5 mL, 0.75 μmol) in MES buffer (20 mM MES, 100 mM NaCl, pH 6.5), was added DTT (10 mM, 0.28 μL). The mixture was immediately vortexed and reacted at room temperature for 1 h. The reaction mixture was diluted with MES buffer (20 mM MES, 100 mM NaCl, pH 6.5) and buffer exchanged. The final concentration of the product was determined by Bradford assay. The molecular weight of HA-Cys-Ub-VME was determined by ESI-MS (Calculated mass: 9,792 Da, Observed mass: 9,792 Da).

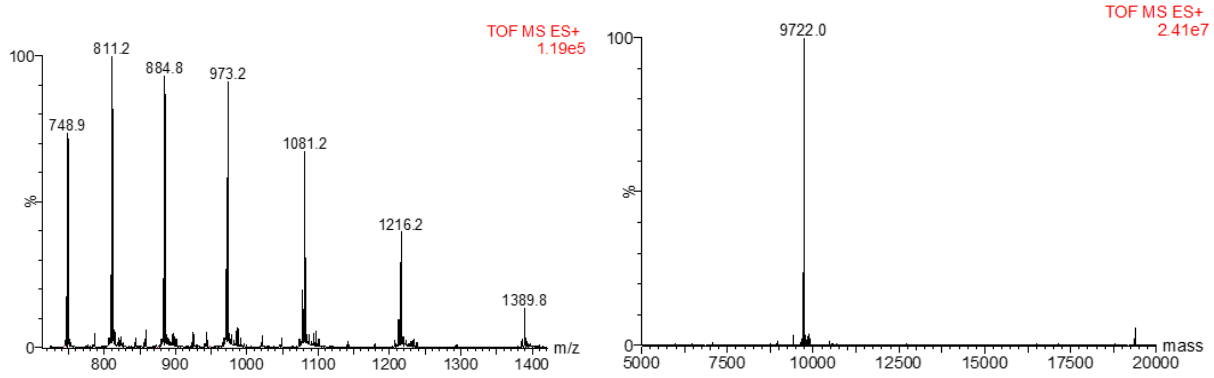


Scheme S7. Generation of HA-Cys-Ub-EA

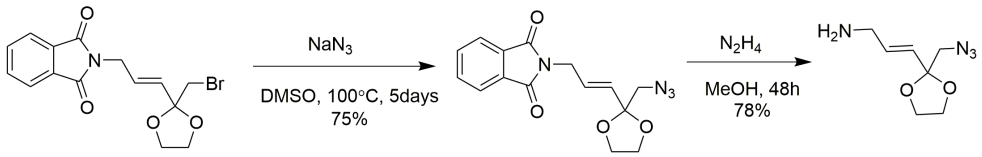


HA-Cys-Ub-MESNA **HA-Cys-Ub-EA**

Generation of HA-Cys(TNB)-Ub-EA. To a solution of NHS (1 mL, 2 M solution in ddH_2O, 2 mmol, 1000 eq.), ethanamine (190 µL, 10.5 M solution in ddH_2O, 2 mmol, 1000 eq.) was added HA-Cys-Ub-MESNA (8 mg/mL, 2.5 mL, 2 µmol) in MES buffer (20 mM MES, 100 mM NaCl, pH 6.5). The final pH of the reaction was approximately 8.0. The mixture was immediately vortexed, and reacted at room temperature for 4 h. The reaction mixture was diluted with MES buffer (20 mM MES, 100 mM NaCl, pH 6.5) and concentrated to a total volume of 0.5 mL. C18 HPLC column was used to purify the product. Jupiter 10 µm C18 300A 10 x 250 mm running at 4 mL/min. Solvent A is consisted of water with 0.1% formic acid, Solvent B is consisted of acetonitrile with 0.1% formic acid. Gradient in time → %B: 0 min → 5%, 10 min → 30%, 70 min → 40%, 79 min→ 95%, 80 min. Product was eluted at approximately 25% Buffer B. Fractions containing the product were pooled and lyophilized. The pure product was dissolved in 6 M guanidine hydrochloride (pH 6.0), then buffer exchanged into MES buffer (20 mM MES, 100 mM NaCl, pH 6.5). The final concentration of the product was determined by Bradford assay. The molecular weight of HA-Cys-Ub-EA was determined by ESI-MS (Calculated mass: 9,722 Da, Observed mass: 9,722 Da).



Scheme S8. Synthesis of pMAL-N_3 (E)-3-(2-(azidomethyl)-1,3-dioxolan-2-yl)prop-2-en-1-amine

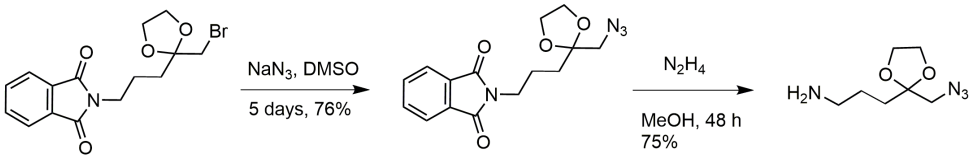


Synthesis of 2-(3-(2-(azidomethyl)-1,3-dioxolan-2-yl)propyl)isoindoline-1,3 dione. (2-(3-(2-(bromomethyl)-1,3-dioxolan-2-yl)propyl)isoindoline-1,3-dione) was synthesized according to a previous publication.[4] (2-(3-(2-(bromomethyl)-1,3-dioxolan-2-yl)propyl)isoindoline-1,3-dione) (2.00 g, 5.68 mmol) was dissolved in DMSO (30 mL), then sodium azide (1.25 g, 17.04 mmol) was added to the solution and heated at 100°C for 5 days. After the reaction completed, the dark brown reaction solution was cooled to room temperature, quenched with saturated sodium chloride solution, and extracted with ethyl acetate three

times (100 mL*3). The combined organic layer was washed with brine and dried over anhydrous Na_2SO_4. The solvent was evaporated and the residue was purified by silica gel column chromatography with Hexane/EtOAc (3:1) to provide (E)-2-(3-(2-(azidomethyl)-1,3-dioxolan-2-yl)allyl)isoindoline-1,3-dione (1.35 g, 4.26 mmol, 75%) as white solid. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.86 (dd, *J*=5.40 Hz, *J*=3.04 Hz, 2H), 7.74 (dd, *J*=5.40 Hz, *J*=3.0 Hz, 2H), 6.03 (dt, *J*=5.72 Hz, *J*=15.44 Hz,1H), 5.63 (d, *J*=15.44 Hz,1H), 4.32 (dd, *J*=5.64 Hz, *J*=1.6 Hz, 2H), 4.05-4.02 (m, 2H), 3.94-3.91 (m, 2H), 3.26 (s, 2H) ^{13}C NMR ($CDCl_3$, 100 MHz): δ 167.79, 134.12, 132.01, 130.27, 127.21, 123.42, 107.36, 65.37, 55.40, 38.49. MS (ESI, positive) *m/z* calculated for $C_{15}H_{14}N_4O_4$ $[M+H]^+$: 314.10, found: 315.10.

Synthesis of (E)-3-(2-(azidomethyl)-1,3-dioxolan-2-yl)prop-2-en-1-amine. To a mixture of (E)-2-(3-(2-(azidomethyl)-1,3-dioxolan-2-yl)allyl)isoindoline-1,3-dione (900 mg, 2.87 mmol) and 100 mL of methanol was added 1 mL hydrazine hydrate. The reaction mixture was stirred at room temperature for 48 h. The solvent was evaporated and the residue was purified by silica gel column chromatography with CH_2Cl_2/CH_3OH/Et_3N (30:1:1) to provide (E)-3-(2-(azidomethyl)-1,3-dioxolan-2-yl)prop-2-en-1-amine (412 mg, 2.24 mmol, 78 %) as a colorless oil. ^{1} H NMR (CDCl3, 400 MHz) δ 6.11 (dt, *J*=5.32 Hz, *J*=15.56 Hz,1H), 5.61 (d, J=15.72 Hz, 1H), 4.08-4.05 (m, 2H), 3.98-3.94 (m, 2H), 3.38 (dd, *J*=1.8 Hz, *J*=5.48 Hz, 2H), 3.30 (s, 2H), 2.18 (bs, 2H); ^{13}C NMR (CDCl3, 100 MHz): δ 134.08, 126.99, 107.68, 65.35, 55.58, 42.80. MS (ESI, positive) m/z calculated for $C_7H_{12}N_4O_2$ [M+H]+ : 184.10, found: 185.14.

Scheme S9. Synthesis of pNRL-N_3 3-(2-(azidomethyl)-1,3-dioxolan-2-yl)propan-1-amine

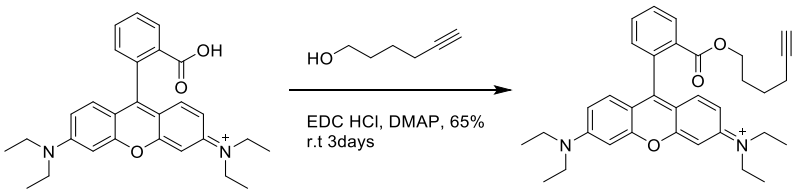


Synthesis of 2-(3-(2-(azidomethyl)-1,3-dioxolan-2-yl)propyl)isoindoline-1,3-dione. 2-(3-(2-(bromomethyl)-1,3-dioxolan-2-yl)propyl)isoindoline-1,3-dione was synthesized as described in a previous publication.[5] 2-(3-(2-(bromomethyl)-1,3-dioxolan-2-yl)propyl)isoindoline-1,3-dione (2.00 g, 5.65 mmol) was dissolved in DMSO (30 mL), then sodium azide (1.25 g, 17.04 mmol) was added to the solution and heated at 100°C for 5 days. After the reaction completed, the dark brown reaction solution was cooled to room temperature and quenched with saturated sodium chloride solution, and extracted with ethyl acetate three times (100mL*3). The combined organic layer was washed with brine and dried over anhydrous Na_2SO_4. The solvent was evaporated and the residue was purified by silica gel column chromatography with Hexane/EtOAc (3:1) to provide 2-(3-(2-(azidomethyl)-1,3-dioxolan-2-yl)propyl)isoindoline-1,3-dione (1.35 g, 4.27 mmol, 76%) as white solid. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.84 (dd, *J*=5.48 Hz, *J*=3.04 Hz, 2H), 7.72 (dd, *J*=5.48 Hz, *J*=3.04 Hz, 2H), 4.05-4.01 (m, 4H), 3.72-3.70 (m, 2H), 3.19 (s, 2H), 1.77-

1.76 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 168.42, 134.04, 133.96, 132.10, 123.24, 109.87, 65.89, 55.39, 37.86, 33.13, 22.43. MS (ESI, positive) *m/z* calculated for $C_{15}H_{14}N_4O_4$ $[M+H]^+$: 314.10, found: 314.18.

Synthesis of 3-(2-(azidomethyl)-1,3-dioxolan-2-yl)propan-1-amine. To a mixture of 2-(3-(2-(azidomethyl)-1,3-dioxolan-2-yl)propyl)isoindoline-1,3-dione (1.2 g, 3.8 mmol) in 100 mL of methanol was added 1.3 mL hydrazine hydrate. The reaction mixture was stirred at room temperature for 48 h. The solvent was evaporated and the residue was purified by silica gel column chromatography with CH_2Cl_2/CH_3OH/Et_3N (30:1:1) to provide 3-(2-(azidomethyl)-1,3-dioxolan-2-yl)propan-1-amine (544 mg, 2.93 mmol, 75%) as a colorless oil. ^{1} H NMR (CDCl3, 400 MHz) δ 4.05-3.97 (m, 4H), 3.20 (s, 2H), 2.71 (t, *J*=4.68 Hz, 2H), 1.90 (br, 2H), 1.72-1.69 (m, 2H), 1.53-1.50 (m, 2H); ^{13}C NMR (CDCl3, 100 MHz): δ 110.13, 65.76, 65.73, 55.22, 42.05, 33.22, 27.04. MS (ESI, positive) m/z calculated for $C_7H_{12}N_4O_2$ $[M+H]+$: 184.10, found: 185.23.

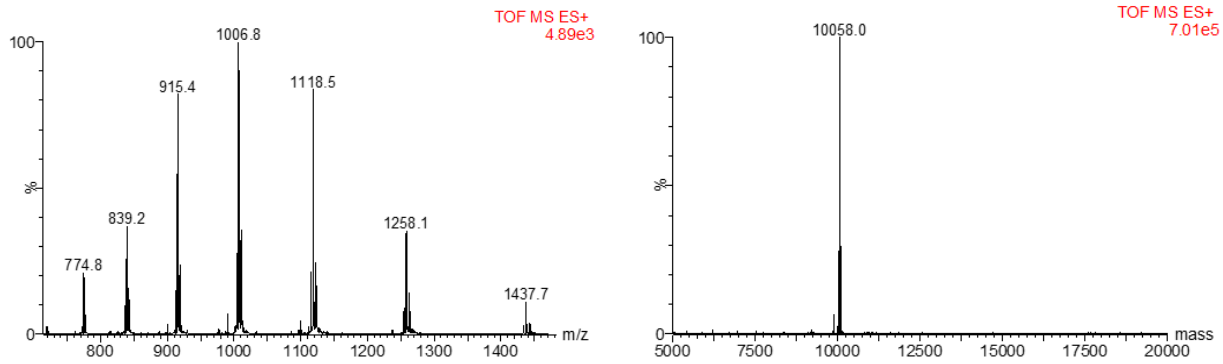
Scheme S10. Synthesis of 4-pentynyl rhodamine B ester (RhB-alkyne)



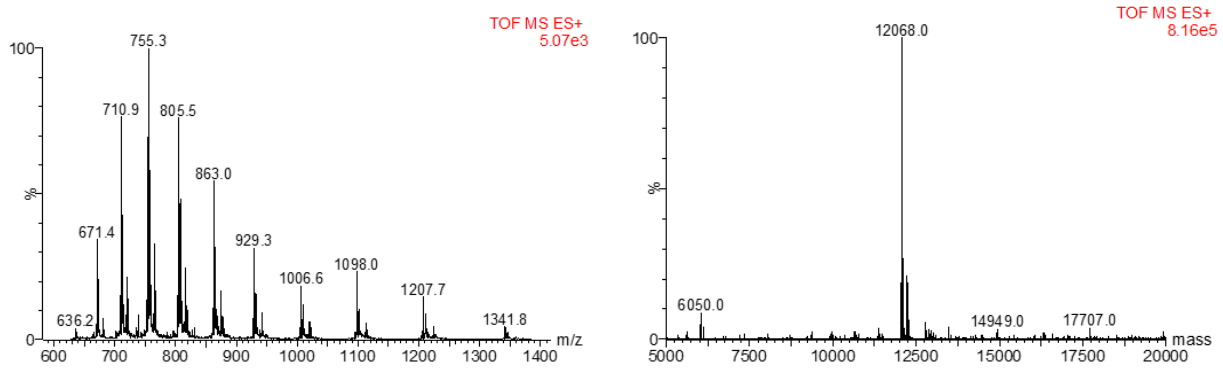
N-(6-(diethylamino)-9-(2-((hex-5-yn-1-yloxy)carbonyl)phenyl)-3H-xanthen-3-ylidene)-N-ethylethanaminium was synthesized according to a previous publication.[6] 4-Pentynyl rhodamine B ester was synthesized by the esterification of rhodamine B and 4-pentyn-1-ol. 4-Pentyn-1-ol (263 mg, 3.13 mmol) was dissolved in DCM (10 mL) and cooled to 0°C. Rhodamine B (500 mg, 1.04 mmol), EDC HCl (550 mg, 2.87 mmol), and DMAP (508 mg, 4.16 mmol) were added while stirring, and the reaction progressed at room temperature under N_2 for 48 h in the absence of light. The reaction mixture was concentrated and purified by column chromatography resulting in 0.507 g (77%) mercury-shiny solid product. ^{1} H NMR (CDCl3, 400 MHz) δ 8.18 (m, 1H), 7.71-7.68 (m, 1H), 7.65-7.60 (m, 1H), 7.26-7.17 (m, 1H), 6.98-6.94 (m, 2H), 6.84-6.80 (m, 2H), 6.69-6.67 (m, 2H), 5.20-5.19 (m, 1H), 3.94-3.90 (m, 1H), 3.57-3.32 (m, 8H), 2.01-1.96 (m, 2H), 1.49-1.44 (m, 2H), 1.30-1.12 (m, 14H); ^{13}C NMR (CDCl3, 100 MHz): δ 165.02, 158.72, 157.60, 155.44, 133.35, 133.06, 131.28, 131.24, 130.36, 130.12, 129.86, 114.25, 113.38, 96.16, 83.49, 68.85, 65.05, 53.56, 46.12, 27.32, 24.71, 17.95, 12.61. MS (ESI, positive) m/z calculated for $C_{13}H_{15}N_5O_4$ $[M+H]+$: 305.11, found: 306.15

Generation of HA-Cys(cR$_{10}$)-Ub-MAL-TER (5).

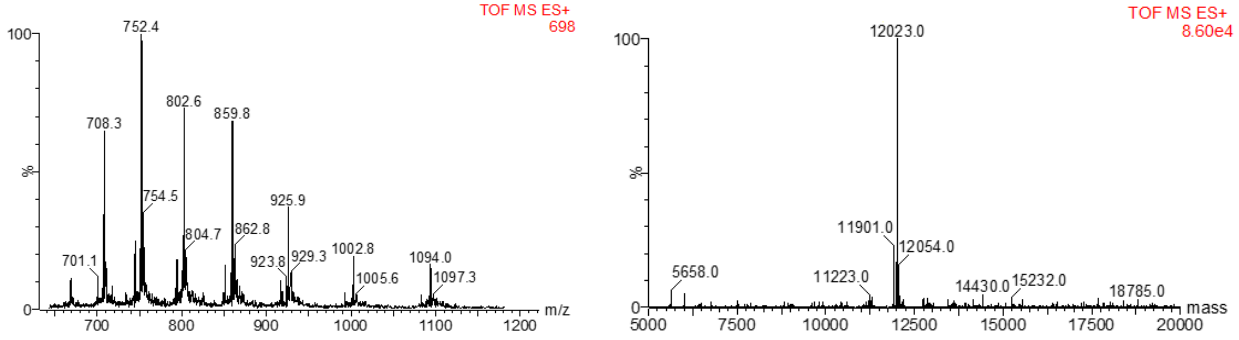
Generation of HA-Cys(TNB)-Ub-pMAL-N$_3$. To a solution of NHS (1.5 mL, 2 M solution in ddH$_2$O, 30 mmol, 1000 eq.) and (E)-3-(2-(azidomethyl)-1,3-dioxolan-2-yl)prop-2-en-1-amine (1.5 mL, 2 M in DMSO, 30 mmol, 1000 eq.) was added HA-Cys(TNB)-Ub-MESNA (10 mg/mL, 3 mL, 30 µmol) in MES buffer (20 mM MES, 100 mM NaCl, pH 6.5). The final pH of the reaction was approximately 8.0. The mixture was immediately vortexed, and reacted at room temperature for 12 h. Then the reaction mixture was buffer exchange into MES buffer (20 mM MES, 100 mM NaCl, pH 6.5). The final concentration of the product was determined by Bradford assay. The molecular weight of HA-Cys(TNB)-Ub-pMAL-N$_3$ was determined using ESI-MS (Calculated mass: 10,058 Da, Observed mass: 10,058 Da).



Generation of HA-Cys(cR$_{10}$)-Ub-pMAL-N$_3$. cR$_{10}$ peptide (100 mg/mL, 1.06 mL, 48 µmoL, 20 eq.) was added to a solution of HA-Cys(TNB)-Ub-pMAL-N$_3$ (8 mg/mL, 3 mL, 2.4 µmol) in 50 mM NaH$_2$PO$_4$ (pH 6.8), The mixture immediately became yellow and then was vortexed and reacted at room temperature for 2 h. The reaction mixture was diluted with MES buffer (20 mM MES, 100 mM NaCl, pH 6.5) then concentrated to a final volume of 0.5 mL. C18 HPLC column was used to purify the product. Jupiter 10 µm C18 300A 10 x 250 mm running at 4 mL/min. Solvent A is consisted of water with 0.1% formic acid. Solvent B is consisted of acetonitrile with 0.1% formic acid. Gradient in time → %B: 0 min → 5%, 10 min → 30%, 70 min → 40%, 79 min→ 95%, 80 min. The product eluted at approximately 20% Buffer B. Fractions containing product were pooled and lyophilized. The pure product was dissolved in 6 M guanidine hydrochloride (pH 6.0) then buffer exchanged into MES buffer (20 mM MES, 100 mM NaCl, pH 6.5). The molecular weight of HA-Cys(cR$_{10}$)-Ub-pMAL-N$_3$ was determined by ESI-MS (Calculated mass: 12,068 Da, Observed mass: 12,068 Da).

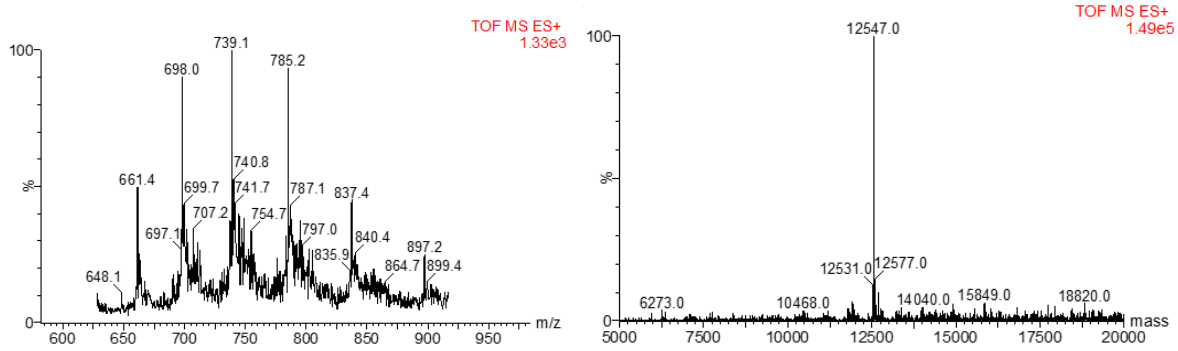


Generation of HA-Cys(cR$_{10}$)-Ub-MAL-N$_3$. 1 mL of FA, and 80 µL 1 M p-TSOH was added to HA-Cys(cR$_{10}$)-Ub-pMAL-N$_3$ (5 mg/mL, 0.6 mL, 0.3 µmol) in MES buffer (20 mM MES, 100 mM NaCl, pH 6.5). The reaction progressed at room temperature for 30 min. The crude mixture was precipitated with 20 mL of cold diethyl ether, then washed with cold diethyl ether and air dried. The crude product was dissolved in 6 M guanidine hydrochloride (pH 6.0.) then buffer exchanged into MES buffer (20 mM MES, 100 mM NaCl, pH 6.5). The final concentration of the product was determined by Bradford assay. The molecular weight of HA-Cys(cR$_{10}$)-Ub-MAL-N$_3$ was determined by ESI-MS (Calculated mass: 12,024 Da, Observed mass: 12,024 Da).

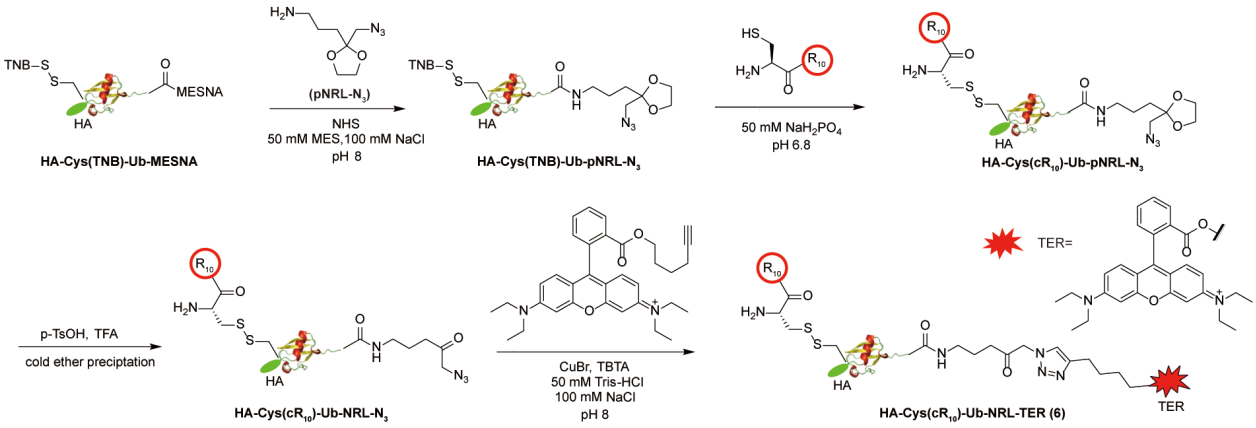


Generation of HA-Cys(cR$_{10}$)-Ub-MAL-TER. HA-Cys(cR$_{10}$)-Ub-MAL-N$_3$ (8 mg/mL, 1 mL, 0.8 µmol) in MES buffer (20 mM MES, 100 mM NaCl, pH 6.5) was added to 1.6 mL of reaction buffer (50 mM Tris-HCl, 50 mM NaCl, 5% glycerol, pH 8.0), followed by addition of 66 µL SDS (25 mM in ddH_2O solution). Then 80 µL N-(6-(diethylamino)-9-(2-((hex-5-yn-1-yloxy)carbonyl)phenyl)-3H-xanthen-3-ylidene)-N-ethylethanaminium (100 mM in MeCN solution, 8 µmol, 10 eq.) was added. To the resulting solution, 400 µL of TBTA (40 mM in MeCN, 16 µmol, 20 eq.) was added, then flushed with N_2. Then 80 µL (100 mM Cu(I)Br in MeCN, 8 umol, 10 eq.) was added, flushed with N_2 and gently vortexed, repeated in 5 minute intervals, 5 times. After reactions were finished, as judged by LC-MS (~ 0.5 h), the reaction was quenched by the addition of 16 µL of 0.5 M EDTA (pH 7.0). The reaction mixture was spun down and supernatant

was aliquoted out. The supernatant was diluted using MES buffer (20 mM MES, 100 mM NaCl, pH 6.5), and concentrated to a total volume of 0.5 mL. C18 HPLC column was used to purify the product. Jupiter 10µ C18 300A 10 x 250 mm running at 4 mL/min. Solvent A is consisted of water with 0.1% formic acid. Solvent B is contained acetonitrile with 0.1% formic acid. Gradient in time → % B: 0min → 5%, 10 min → 30%, 70 min → 40%, 79 min→ 95%, 80 min. Product eluted at 25% Buffer B. Fractions containing product were pooled and lyophilized. The pure product was dissolved in 6 M guanidine hydrochloride (pH 6.0), then buffer exchanged into MES buffer (20 mM MES, 100 mM NaCl, pH 6.5. The concentration of the final product was determined by Bradford assay. The molecular weight of HA-Cys(cR$_{10}$)-Ub-MAL-TER was determined by ESI-MS (Calculated mass: 12,547 Da, Observed mass: 12,547 Da).

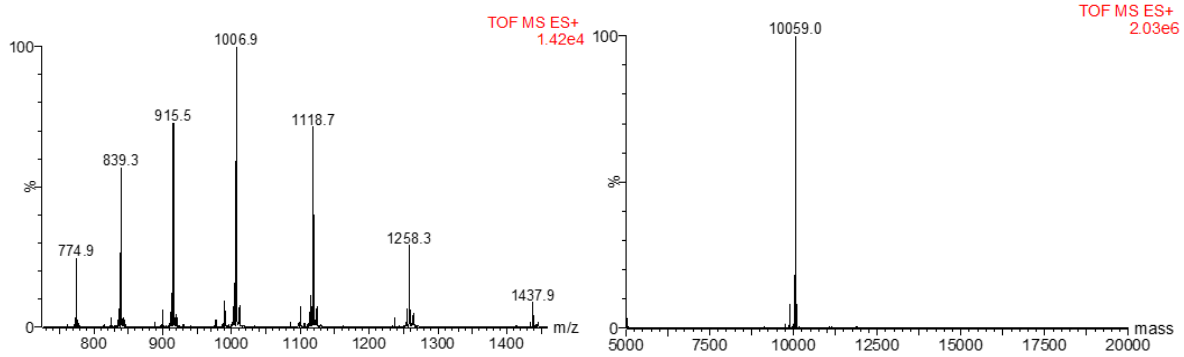


Scheme 11. Generation of HA-Cys(cR$_{10}$)-Ub-NRL-TER (**6**).

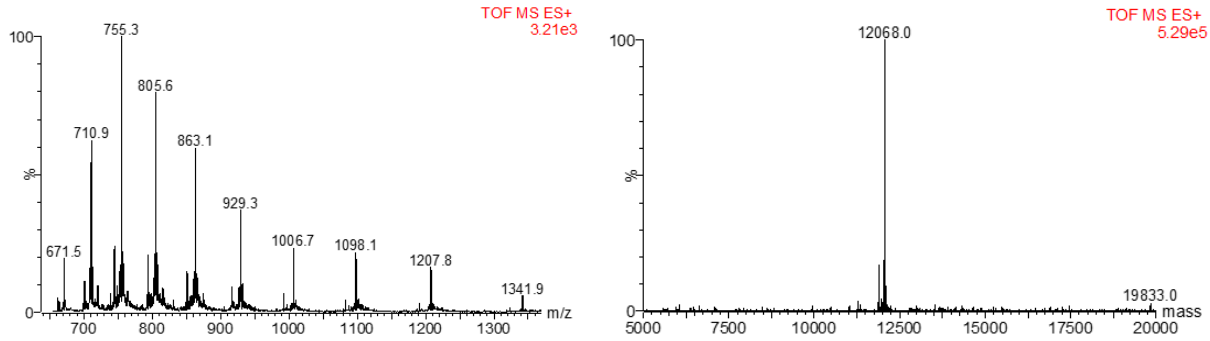


Generation of HA-Cys(TNB)-Ub-pNRL-N$_3$. To a solution of HA-Cys(TNB)-Ub-MESNA (3 mg/mL, 4 mL, 1.2 µmol) in Mes buffer (20 mM MES, 100 mM NaCl, pH 6.5) was added NHS (600 µL, 2 M solution in ddH_2O, 1.2 mmol, 1000 eq.). 3-(2-(azidomethyl)-1,3-dioxolan-2-yl)propan-1-amine (pNRL) was dissolved in DMSO to make a 1 M solution. Then the compound solution (1.2 mL, 1 M in DMSO, 1.2 mmol, 1000eq.) was added. The final pH of the reaction solution was approximately 8.0. The mixture was

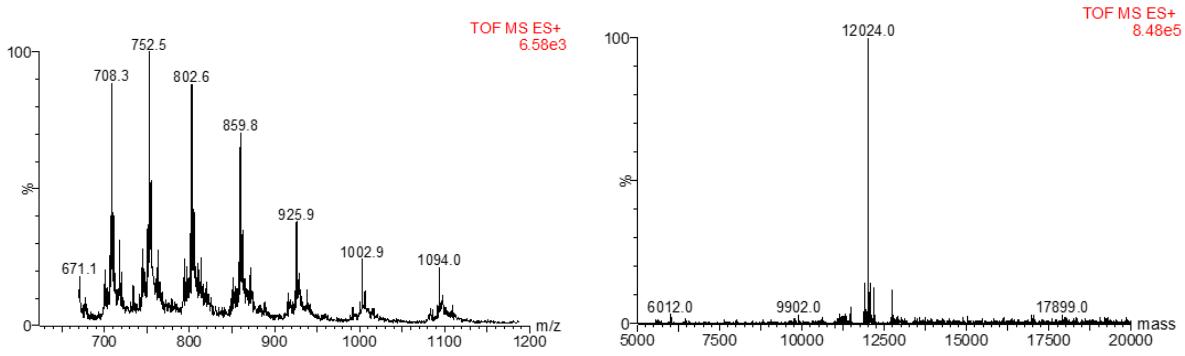
immediately vortexed and reacted at 37 C° for 24 h. Then the reaction mixture was buffer exchanged for three time with MES buffer (20 mM MES, 100 mM NaCl, pH 6.5) using 3 kDa Milipore Centrifuge Column. The final concentration of the product was determined by Bradford assay. The molecular weight of HA-Cys(TNB)-Ub-pNRL-N_3 was determined by ESI-MS (Calculated mass: 10,060 Da, Observed mass: 10,059 Da).



Generation of HA-Cys(cR_{10})-Ub-pNRL-N_3. cR_{10} peptide (100 mg/mL, 0.14 mL, 6.4 μmoL, 20 eq.) was added to a solution of HA-Cys(TNB)-Ub-pNRL-N_3 (2 mg/mL, 1.6 mL, 0.32 μmol) in 50 mM NaH_2PO_4 (pH 6.8). The mixture became yellow, vortexed, and allowed to react at room temperature for 2 h. The reaction mixture was diluted using MES buffer (20 mM MES, 100 mM NaCl, pH 6.5) and concentrated to a total volume of 0.5 mL. C18 HPLC column was used to purify the product. Jupiter 10 μm C18 300A 10 x 250 mm running at 4 mL/min. Solvent A is consisted of water with 0.1% formic acid. Solvent B is consisted of acetonitrile with 0.1% formic acid. Gradient in time → %B: 0 min → 5%, 10 min → 30%, 70 min → 40%, 79 min→ 95%, 80 min. Product eluted out at approximately 25% Buffer B. Fractions containing product were pooled and lyophilized. The pure product was dissolved in 6 M guanidine hydrochloride (pH 6.0) and then buffer exchanged into MES buffer (20 mM MES, 100 mM NaCl, pH 6.5). The concentration of the final product was determined by Bradford assay. The molecular weight of HA-Cys(cR_{10})-Ub-pNRL-N_3 was determined by ESI-MS (Calculated mass: 12,070 Da, Observed mass: 12,068 Da).

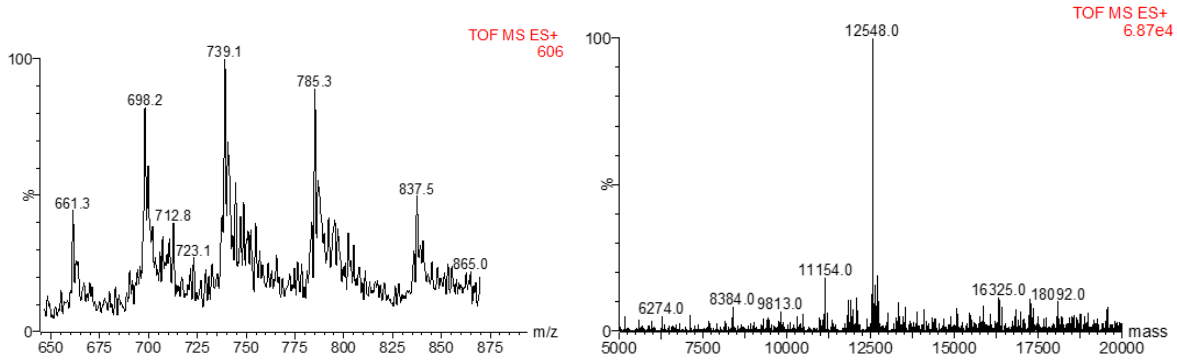


Generation of HA-Cys(cR$_{10}$)-Ub-NRL-N$_3$. 1 mL TFA, and 80 µL 1M p-TSOH was added to a solution of HA-Cys(cR$_{10}$)-Ub-pNRL-N$_3$ (5 mg/mL, 0.6 mL, 0.3 µmol). The reaction was allowed at room temperature for 12 h. The crude mixture was precipitated with 20 mL of cold diethyl ether, then washed with cold diethyl ether and air dried. The crude product was dissolved in 6 M guanidine hydrochloride (pH 6.0) and buffer exchanged into MES buffer (20 mM MES, 100 mM NaCl, pH 6.5). The final concentration of the product was determined by Bradford assay (2.5 mg/mL, 1 mL, 0.25 µmol, 83%). The molecular weight of HA-Cys(cR$_{10}$)-Ub-NRL-N$_3$ was determined by ESI-MS (Calculated mass: 12,026 Da, Observed mass: 12,024 Da).

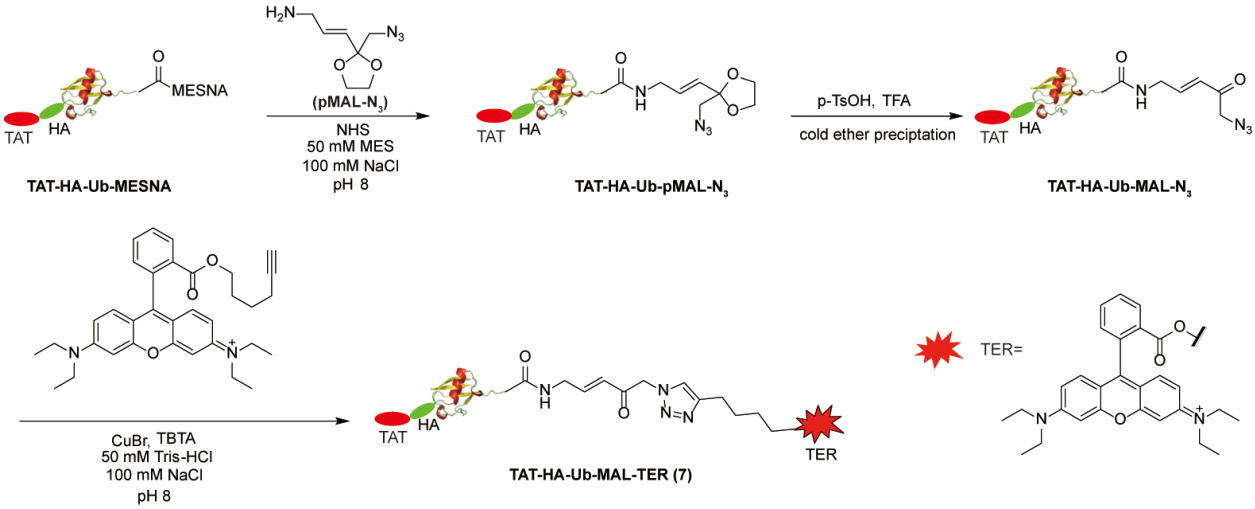


Generation of HA-Cys(cR$_{10}$)-Ub-NRL-TER. HA-Cys(cR$_{10}$)-Ub-NRL-N$_3$ (8 mg/mL, 1 mL, 0.8 µmol) in MES buffer (20 mM MES, 100 mM NaCl, pH 6.5) was added to 1.6 mL of reaction buffer (50 mM Tris-HCl, 50 mM NaCl, 5% glycerol, pH 8.0), followed by addition of 66 µL SDS (25 mM in ddH_2O solution) and 80 µL N-(6-(diethylamino)-9-(2-((hex-5-yn-1-yloxy)carbonyl)phenyl)-3H-xanthen-3-ylidene)-N-ethylethanaminium (100 mM in MeCN solution, 8 µmol, 10 eq.). To the resulting solution, 400 µL of TBTA (40 mM in MeCN, 16 µmol, 20 eq.) was added and flushed with N_2. Then 80 µL (100 mM Cu(I)Br in MeCN, 8 µmol, 10 eq.) was added, flushed with N_2, and gently vortexed. This was repeated 5 times in 5 min intervals. After reactions were finished, as judged by LC-MS (~ 0.5 h), the reaction was quenched by the addition of 16 µL of 0.5 M EDTA, pH 7.0. The reaction mixture was spun down and supernatant was aliquoted out. The supernatant was diluted using MES buffer (20 mM MES, 100 mM NaCl, pH 6.5), then concentrated to a total volume of 0.5 mL. C18 HPLC column was used to purify the product. Jupiter 10 µm C18 300 A 10 x 250 mm running at 4 mL/min. Solvent A is consisted of water with 0.1% formic acid. Solvent B is consisted of acetonitrile with 0.1% formic acid. Gradient in time → % B: 0 min → 5%, 10 min → 30%, 70 min → 40%, 79 min→ 95%, 80 min. Product eluted at 25% Buffer B. Fractions containing product were pooled and lyophilized. The pure product was dissolved in 6 M guanidine hydrochloride (pH 6.0), then buffer exchanged into MES buffer (20 mM MES, 100 mM NaCl, pH 6.5). The concentration of

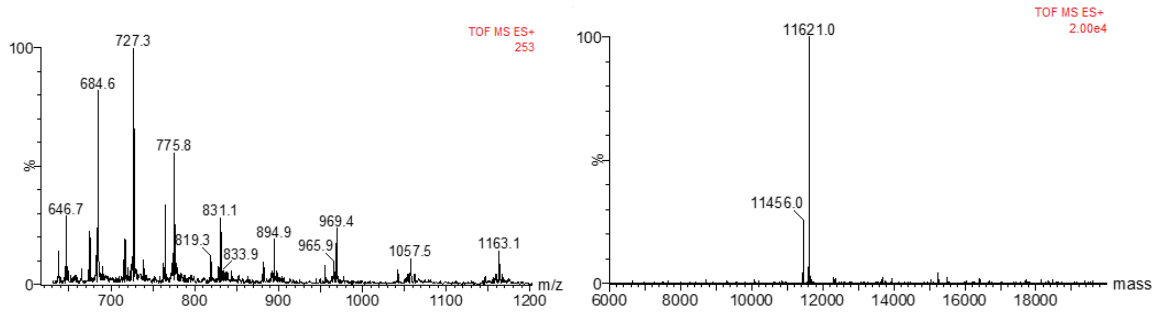
the final product was determined by Bradford assay. The molecular weight of HA-Cys(cR$_{10}$)-Ub-NRL-TER was determined by ESI-MS (Calculated mass: 12,549 Da, Observed mass: 12,548 Da).



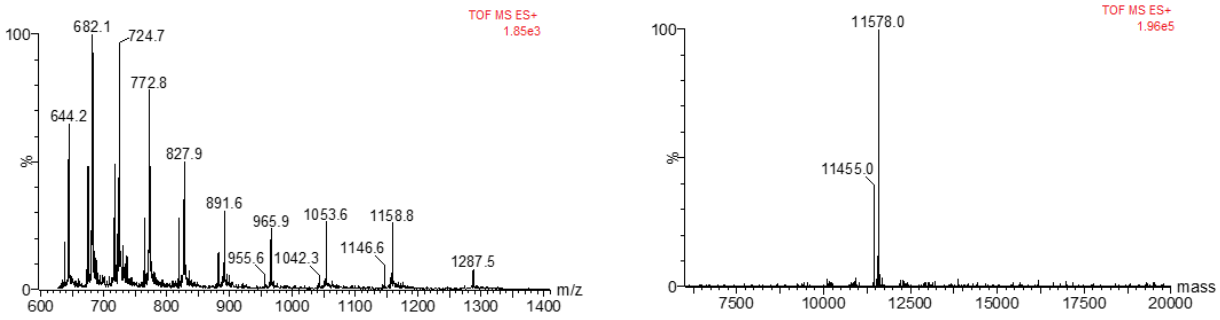
Scheme 12. Generation of TAT-HA-Ub-MAL-TER (**7**).



Generation of TAT-HA-Ub-pMAL-N$_3$. To a solution of NHS (450 µL, 2 M solution in ddH_2O, 1000 eq.) and (E)-3-(2-(azidomethyl)-1,3-dioxolan-2-yl)prop-2-en-1-amine (450 µL, 2 M in DMSO, 0.9 mmol, 1000eq.) were added TAT-HA-Ub-MESNA (3 mg/mL, 3 mL, 0.9 µmol) in MES buffer (20 mM MES, 100 mM NaCl, pH 6.5) then vortexed. The final pH of the reaction solution was approximately 8.0. The mixture was reacted at room temperature for 12 h. The reaction mixture was buffer exchanged into MES buffer (20 mM MES, 100 mM NaCl, pH 6.5). The final concentration of the product was determined by Bradford assay. The molecular weight of TAT-HA-Ub-pMAL-N$_3$ was determined by ESI-MS. (Calculated mass: 11,621 Da, Observed mass: 11,621 Da).

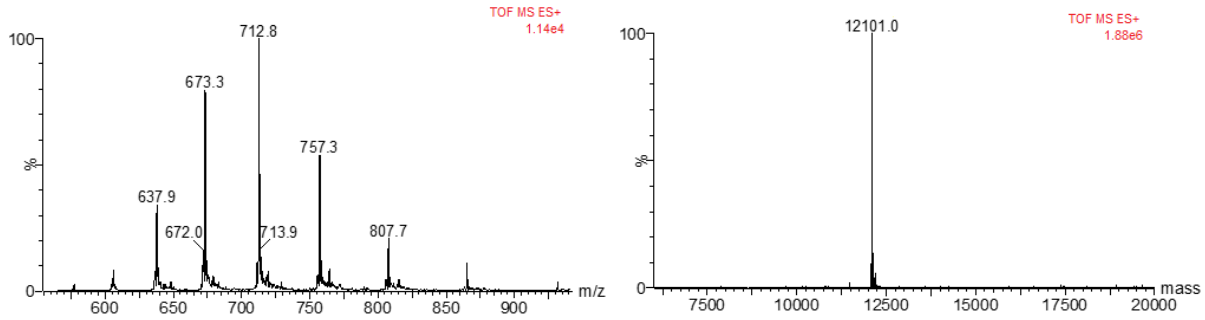


Generation of TAT-HA-Ub-MAL-N_3. To a solution of TAT-HA-Ub-pMAL-N_3 (5 mg/mL, 0.6 mL, 0.3 μmol) 0.6 mL ddH_2O, 1 mL TFA, and 80 μL 1 M p-TSOH was added. The reaction was allowed to progress at room temperature for 30 min. The crude mixture was precipitated using 20 mL of cold diethyl ether, then washed with cold diethyl ether and air dried. Then the crude product was dissolved in 6 M guanidine hydrochloride (pH 6.0) and buffer exchanged into MES buffer (20 mM MES, 100 mM NaCl, pH 6.5). The final concentration of the product was determined by Bradford assay. The molecular weight of TAT-HA-Ub-MAL-N_3 was determined by ESI-MS (Calculated mass: 11,577 Da, Observed mass: 11,578 Da).

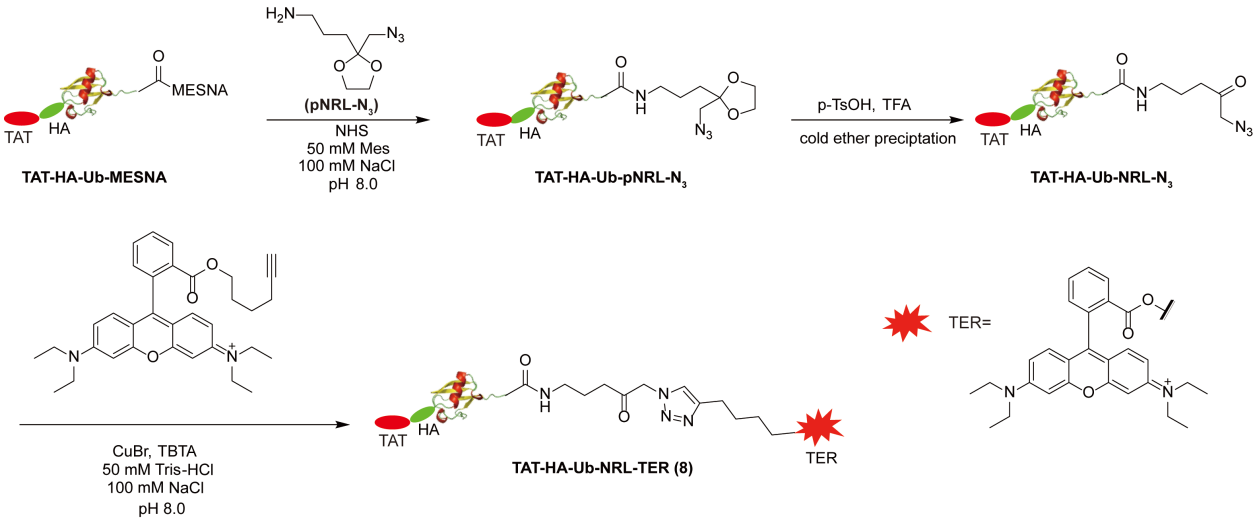


Generation of TAT-HA-Ub-MAL-TER. TAT-HA-Ub-MAL-N_3 (8 mg/mL, 1 mL, 0.8 μmol) in MES buffer (20 mM MES, 100 mM NaCl, pH 6.5) was added to 1.6 mL of reaction buffer (50 mM Tris-HCl, 50 mM NaCl, 5% Glycerol, pH 8), followed by addition of 66 μL SDS (25 mM in ddH_2O solution), and 80 μL N-(6-(diethylamino)-9-(2-((hex-5-yn-1-yloxy)carbonyl)phenyl)-3H-xanthen-3-ylidene)-N-ethylethanaminium (100 mM in MeCN solution, 8 μmol, 10 eq.). To the resulting solution, 400 μL of TBTA (40 mM in MeCN, 16 μmol, 20 eq.) was added and flushed with N_2. Then 80 μL of 100 mM Cu(I)Br in MeCN (8 μmol, 10 eq.) was added, flushed with N_2, and gently vortex. This was repeated in 5 times in 5 min intervals. After reactions were finished, as judged by LC-MS (~ 0.5 h), the reaction was quenched by the addition of 16 μL of 0.5 M EDTA (pH 7.0). The reaction mixture was spun down and supernatant was aliquoted out and diluted using MES buffer (20 mM MES, 100 mM NaCl, pH 6.5), then concentrated to a total volume of 0.5 mL. C18 HPLC column was used to purify the product. Jupiter 10 μm C18 300 A 10 x 250 mm running at 4 mL/min. Solvent A is consisted of water with 0.1% formic acid. Solvent B is consisted

of acetonitrile with 0.1% formic acid. Gradient in time → % B: 0 min → 5%, 10 min → 30%, 70 min → 40%, 79 min→ 95%, 80 min. Product eluted at 25% Buffer B. Fractions containing product were pooled and lyophilized. The pure product was dissolved in 6 M guanidine hydrochloride (pH 6.0) and buffer exchanged into MES buffer (20 mM MES, 100 mM NaCl, pH 6.5). The concentration of the final product was determined by Bradford assay. The molecular weight of TAT-HA-Ub-MAL-TER was determined by ESI-MS. (Calculated mass: 12,100 Da, Observed mass: 12,101 Da).

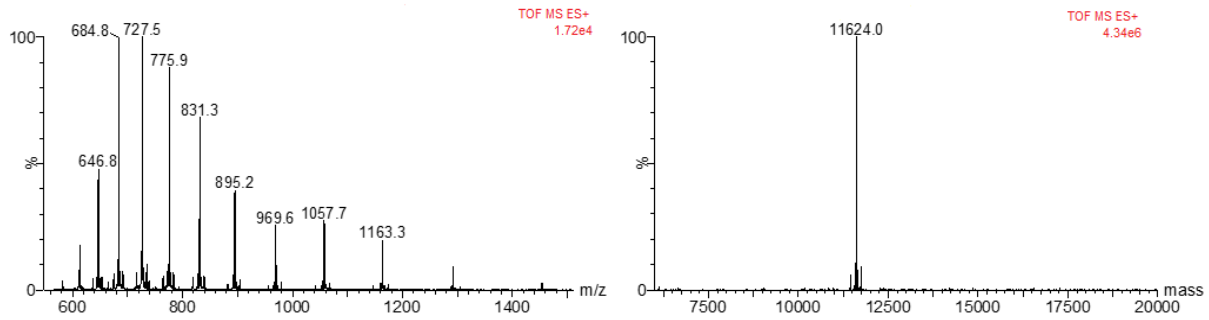


Scheme 13. Generation of TAT-HA-Ub-NRL-TER (**8**).

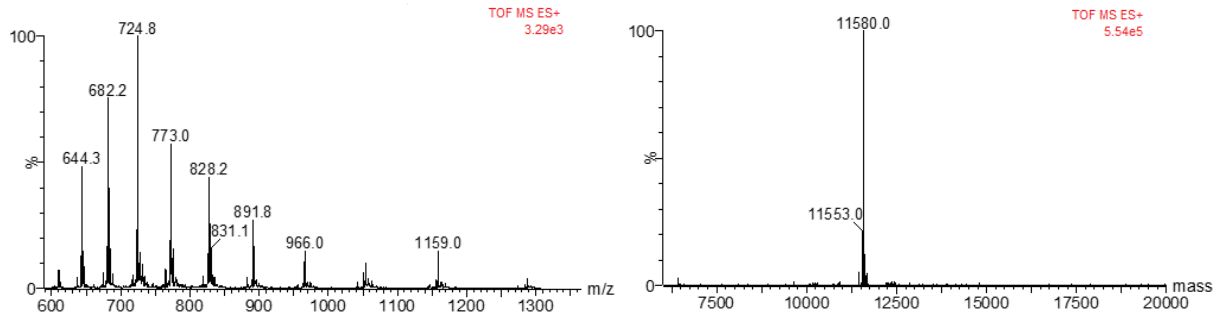


Generation of TAT-HA-Ub-pNRL-N_3. To a solution of TAT-HA-Ub-MESNA (10 mg/mL, 1.2 mL, 1.2 µmol) in MES buffer (20 mM MES, 100 mM NaCl, pH 6.5) was added NHS (600 µL, 2 M solution in ddH_2O, 1.2 mmol, 1000 eq.). 3-(2-(azidomethyl)-1,3-dioxolan-2-yl)propan-1-amine was dissolved in DMSO to make a 1 M solution. Then the compound solution (1.2 mL, 1 M in DMSO, 1.2 mmol, 1000 eq.) was added. The final pH of the reaction solution is approximately 8.0. The mixture was immediately vortexed and reacted at 37°C for 24 h. The reaction mixture was buffer exchanged into MES buffer (20 mM MES, 100 mM NaCl, pH 6.5). The final concentration of the product was determined by Bradford

assay. The molecular weight of TAT-HA-Ub-pNRL-N_3 was determined by ESI-MS (Calculated mass: 11,623 Da, Observed mass: 11,624 Da).

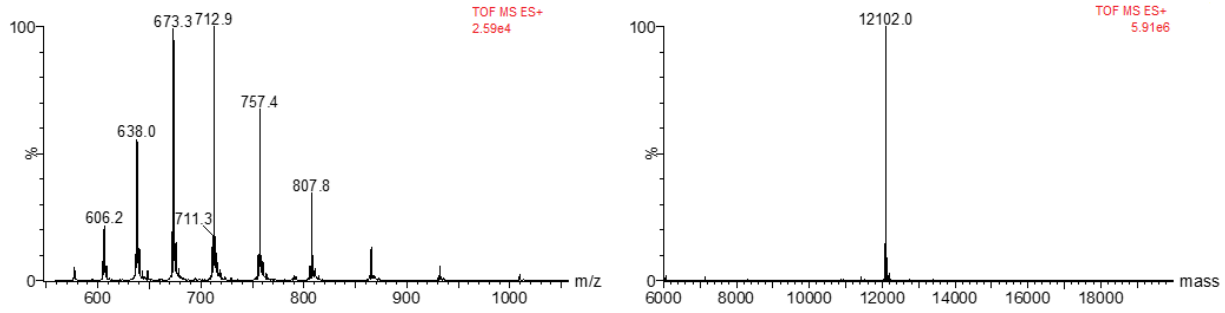


Generation of TAT-HA-Ub-NRL-N_3. 0.6 mL ddH_2O, 1 mL TFA, and 80 µL 1 M p-TS-OH was added to a solution of TAT-HA-Ub-pNRL-N_3 (5 mg/mL, 0.6 mL, 0.3 µmol). The reaction was allowed to progress at room temperature for 12 h. The crude mixture was precipitated using 20 mL of cold diethyl ether, then washed with cold diethyl ether and air-dried. The crude product was dissolved in 6 M guanidine hydrochloride (pH 6.0), then buffer exchanged into MES buffer (20 mM MES, 100 mM NaCl, pH 6.5). The final concentration of the product was determined by Bradford assay. The molecular weight of TAT-HA-Ub-NRL-N_3 was determined by ESI-MS (Calculated mass: 11,579 Da, Observed mass: 11,580 Da).

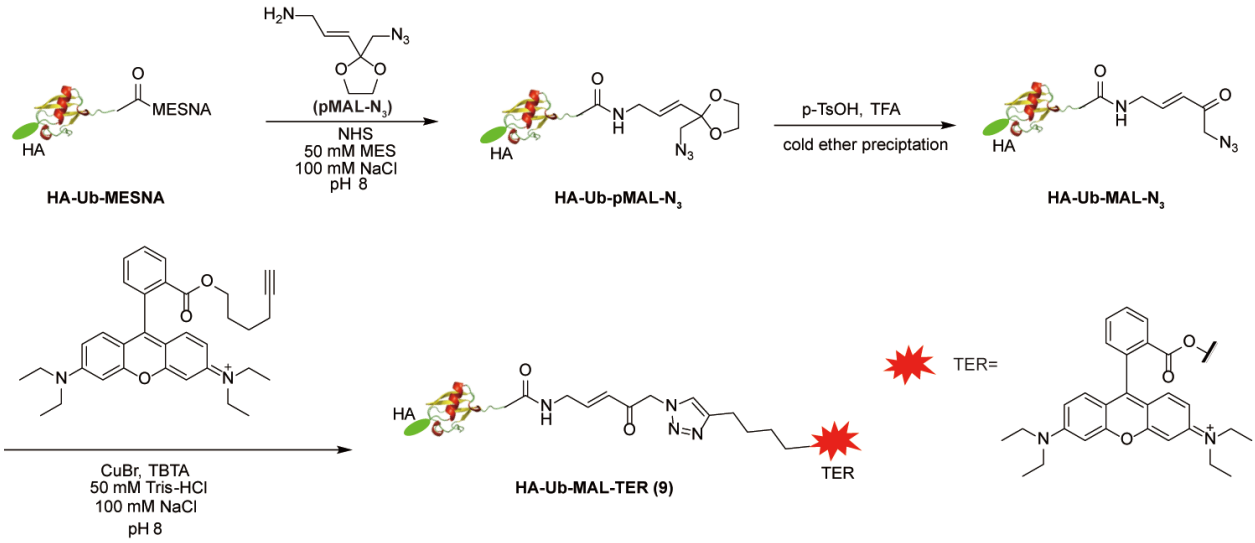


Generation of TAT-HA-Ub-NRL-TER. TAT-HA-Ub-NRL-N_3 (8 mg/mL, 1 mL, 0.8 µmol) in MES buffer (20 mM MES, 100 mM NaCl, pH 6.5) was added to 1.6 mL of reaction buffer (50 mM Tris-HCl, 50 mM NaCl, 5% glycerol, pH 8.0), followed by addition of 66 µL SDS (25 mM in ddH_2O solution) and 80 µL N-(6-(diethylamino)-9-(2-((hex-5-yn-1-yloxy)carbonyl)phenyl)-3H-xanthen-3-ylidene)-N-ethylethanaminium (100 mM in MeCN solution, 8 µmol, 10 eq.), respectively. To the resulting solution, 400 µL of TBTA (40 mM in MeCN, 16 µmol, 20 eq.) was added and flushed with N_2. Then 80 µL of 100 mM Cu(I)Br in MeCN (8 umol, 10 eq.) was added, flushed with N_2, and gently vortexed. This was repeated 5 times in 5 min. After reactions were finished, as judged by LC-MS (~ 0.5 h), the reaction was quenched by the addition of 16 µL of 0.5 M EDTA (pH 7.0). The reaction mixture was spun down and supernatant

was aliquoted out and diluted using MES buffer (20 mM MES, 100 mM NaCl, pH 6.5) and concentrated to a total volume of 0.5 mL. C18 HPLC column was used to purify the product. Jupiter 10 µm C18 300 A 10 x 250 mm running at 4 mL/min. Solvent A is consisted of ddH_2O with 0.1% formic acid. Solvent B is consisted of acetonitrile with 0.1% formic acid. Gradient in time → % B: 0 min → 5%, 10 min → 30%, 70 min → 40%, 79 min→ 95%, 80 min. Product eluted at approximately 25% Buffer B. Fractions containing product were pooled and lyophilized. The pure product was dissolved in 6 M guanidine hydrochloride buffer (pH 6.0), and buffer exchanged into MES buffer (20 mM MES, 100 mM NaCl, pH 6.5). The concentration of the final product was determined by Bradford assay. The molecular weight of TAT-HA-Ub-NRL-TER was determined by ESI-MS (Calculated mass: 12,101 Da, Observed mass: 12,102 Da).

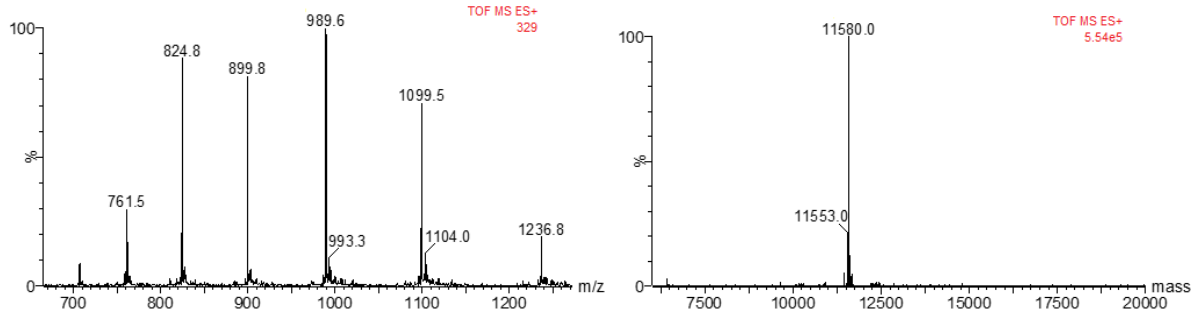


Scheme 14. Generation of HA-Ub-MAL-TER (**9**).

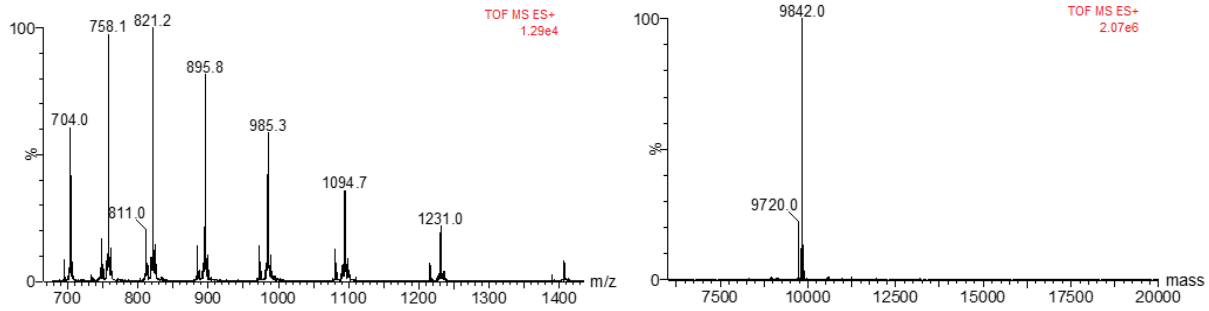


Generation of HA-Ub-pMAL-N_3. To a solution of NHS (450 µL, 2 M solution in ddH_2O, 1000 eq.) and (E)-3-(2-(azidomethyl)-1,3-dioxolan-2-yl)prop-2-en-1-amine (450 µL, 2 M in DMSO, 0.9 mmol, 1000 eq.) was added HA-Ub-MESNA (3 mg/mL, 3 mL, 0.9 µmol) in MES buffer (20 mM MES, 100 mM NaCl, pH 6.5) and vortexed. The final pH of the reaction was approximately 8.0. The mixture was reacted at room temperature for 12 h. The reaction mixture was then buffer exchanged into MES buffer (20 mM MES, 100

mM NaCl, pH 6.5). The final concentration of the product was determined by Bradford assay. The molecular weight of HA-Ub-pMAL-N_3 was determined by ESI-MS. (Calculated mass: 9,886 Da, Observed mass: 9,886 Da).

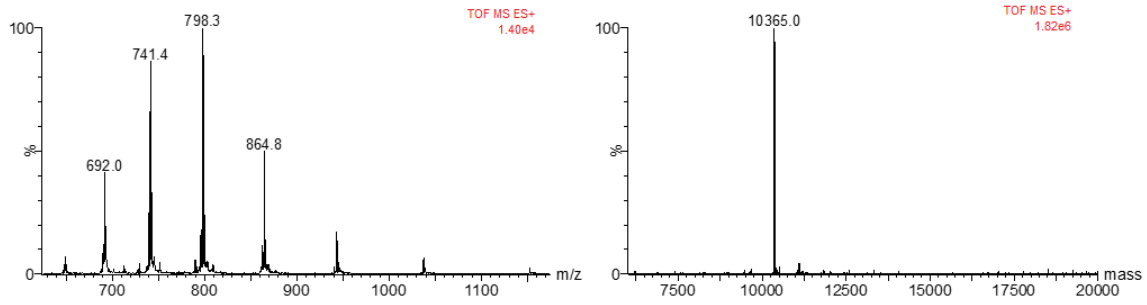


Generation of HA-Ub-MAL-N_3. To a solution of 0.6 mL ddH_2O, 1 mL TFA, and 80 µL 1 M p-TSOH was added HA-Ub-pMAL-N_3 (5 mg/mL, 0.6 mL, 0.3 µmol). The reaction was allowed to progress at room temperature for 30 min. The crude mixture was precipitated using 20 mL of cold diethyl ether, then washed with cold diethyl ether and air-dried. The crude product was dissolved in 6 M guanidine hydrochloride (pH 6.0), then buffer exchanged into MES buffer (20 mM MES, 100 mM NaCl, pH 6.5). The final concentration of the product was determined by Bradford assay. The molecular weight of HA-Ub-MAL-N_3 was determined by ESI-MS. (Calculated mass: 9,842 Da, Observed mass: 9,842 Da).



Generation of HA-Ub-MAL-TER. HA-Ub-MAL-N_3 (8 mg/mL, 1 mL, 0.8 µmol) in MES buffer (20 mM MES, 100 mM NaCl, pH 6.5) was added to 1.6 mL of reaction buffer (50 mM Tris-HCl, 50 mM NaCl, 5% glycerol, pH 8.0), followed by addition of 66 µL SDS (25 mM in ddH_2O solution) and 80 µL N-(6-(diethylamino)-9-(2-((hex-5-yn-1-yloxy)carbonyl)phenyl)-3H-xanthen-3-ylidene)-N-ethylethanaminium (100 mM in MeCN solution, 8 µmol, 10 eq.). To the resulting solution, 400 µL of TBTA (40 mM in MeCN, 16 µmol, 20 eq.) was added and flushed with N_2. Then 80 µL of 100 mM Cu(I)Br in MeCN, 8 µmol, 10 eq.) was added, flushed with N_2, and gently vortexed. This was repeated 5 times for at 5 min. After reaction was finished, as judged by LC-MS (~ 0.5 h), the reaction was quenched by the addition of 16 µL of 0.5 M EDTA (pH 7.0). The reaction mixture was spun down and supernatant was aliquoted out and diluted with

MES buffer (20 mM MES, 100 mM NaCl, pH 6.5), then concentrated to a total volume of 0.5 mL. C18 HPLC column was used to purify the product. Jupiter 10 µm C18 300 A 10 x 250 mm running at 4 mL/min. Solvent A is consisted of water with 0.1% formic acid. Solvent B is consisted of acetonitrile with 0.1% formic acid. Gradient in time → %B: 0min → 5%, 10min → 30%, 70min → 40%, 79 min→ 95%, 80 min. Product eluted at approximately 25% Buffer B. Fractions containing product were pooled and lyophilized. The pure product was dissolved in 6 M guanidine hydrochloride (pH 6.0), then buffer exchanged into MES buffer (20 mM MES, 100 mM NaCl, pH 6.5). The concentration of the final product was determined by Bradford assay. The molecular weight of HA-Ub-MAL-TER was determined by ESI-MS. (Calculated mass: 10,364 Da, Observed mass: 10,365 Da).



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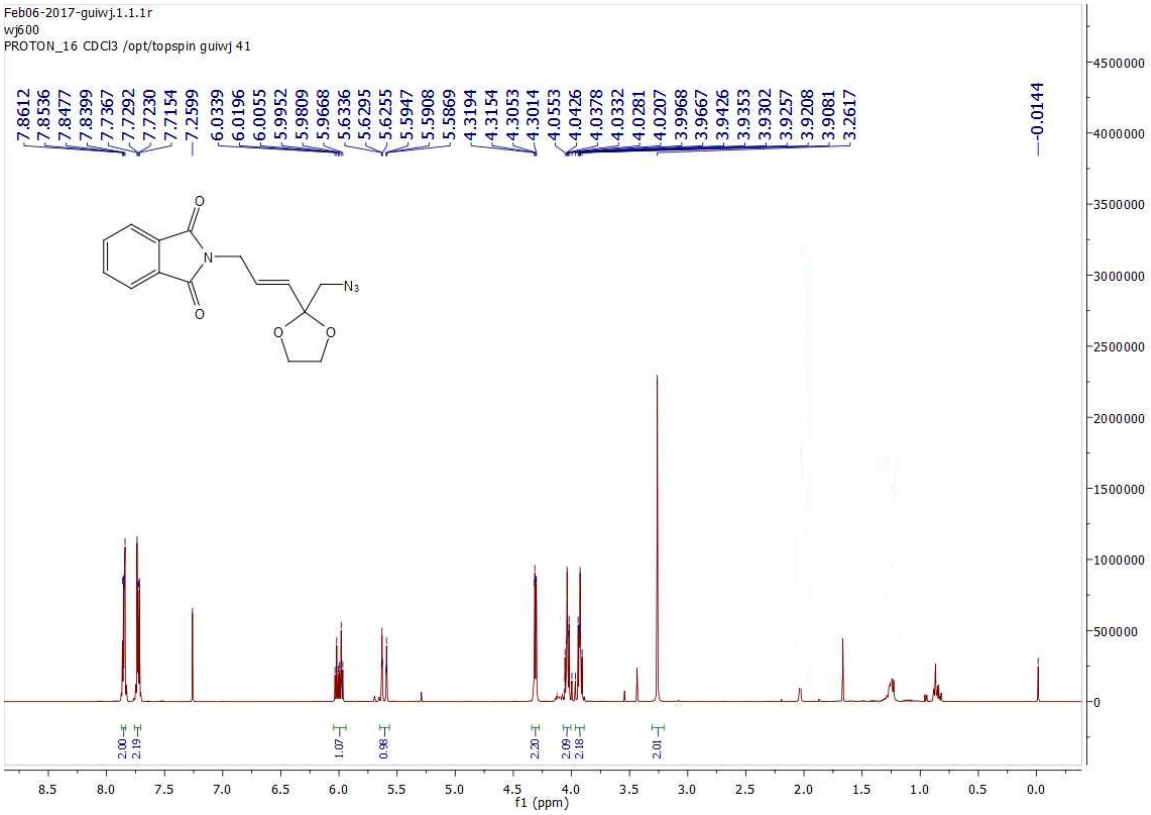
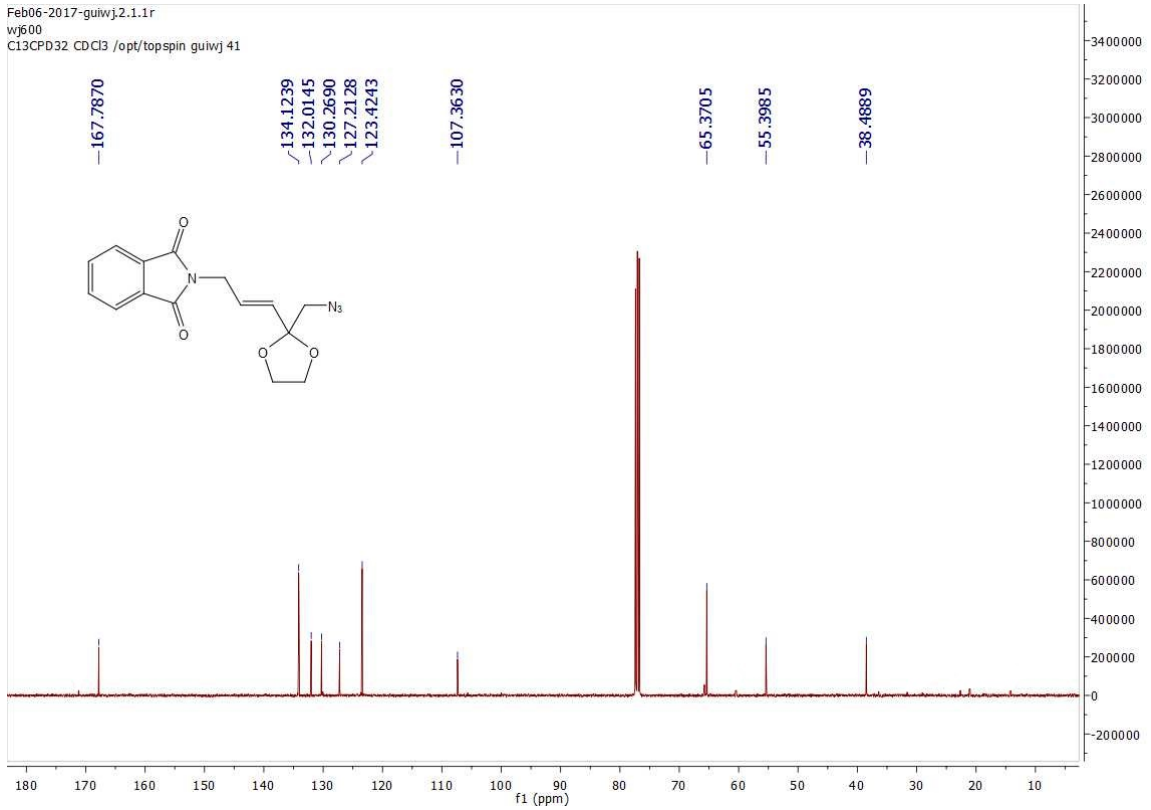
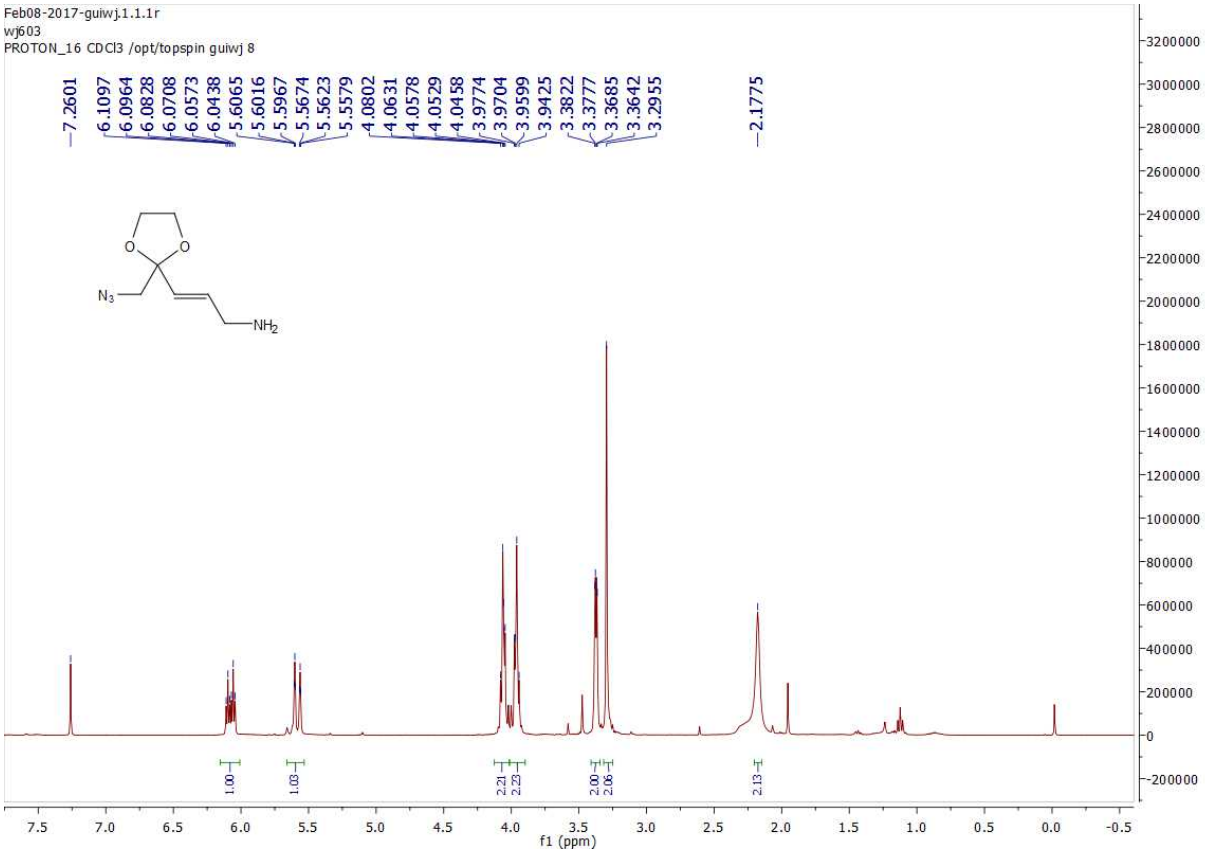
^{1}H and ^{13}C NMR Spectra



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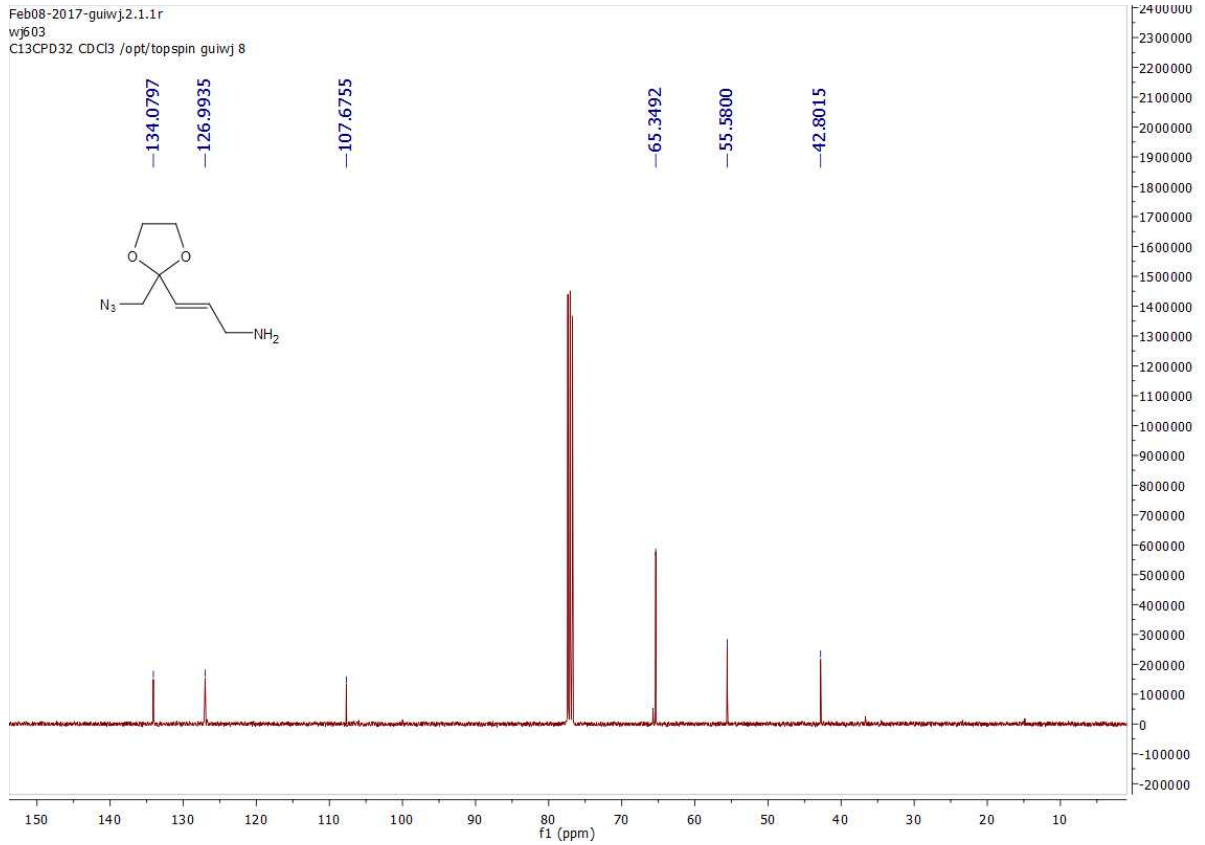
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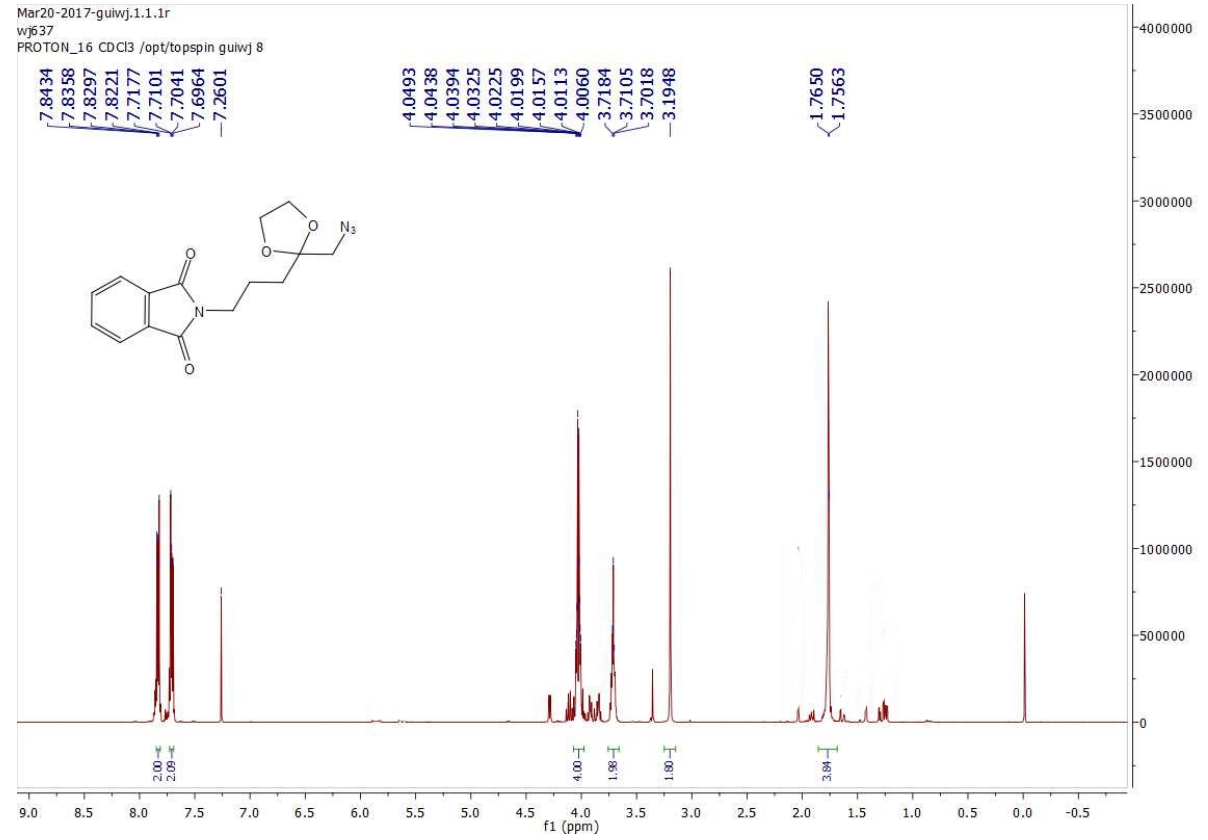
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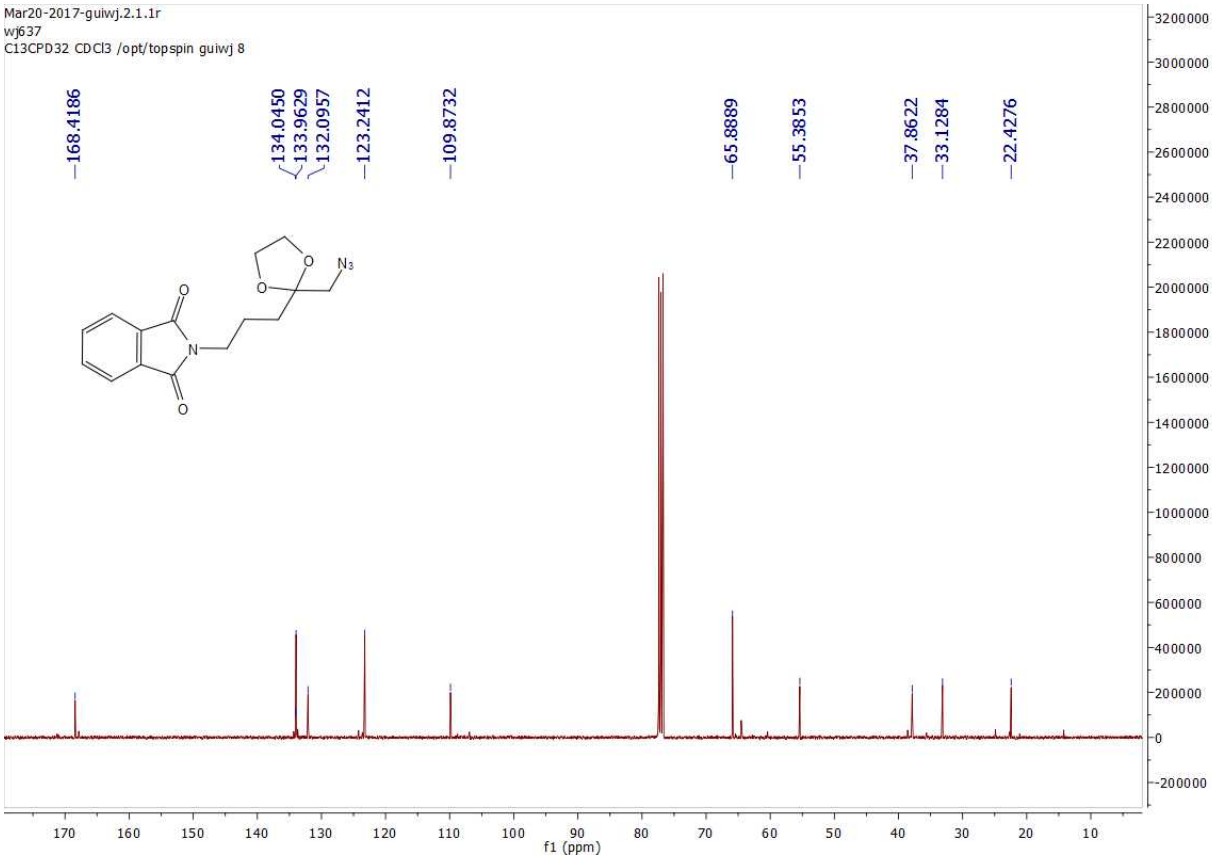
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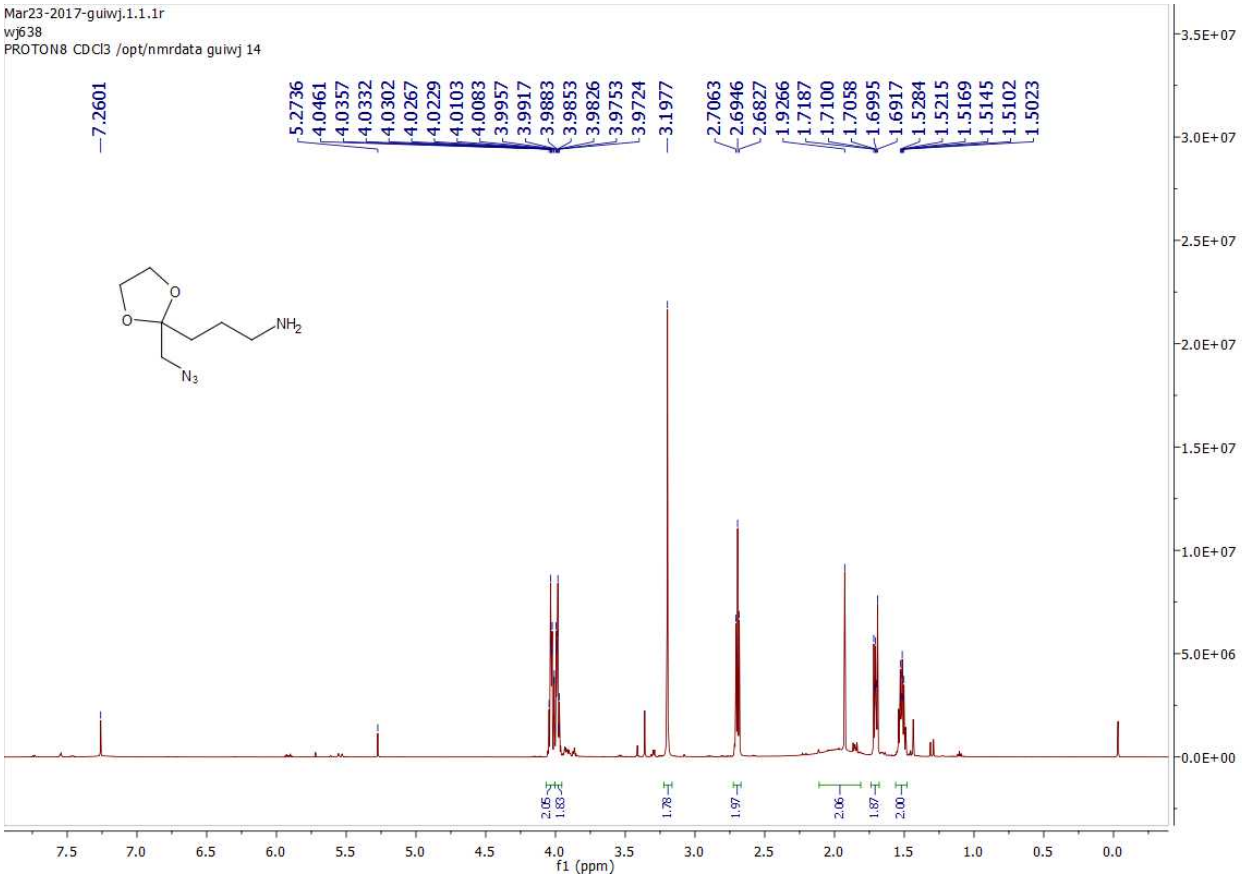
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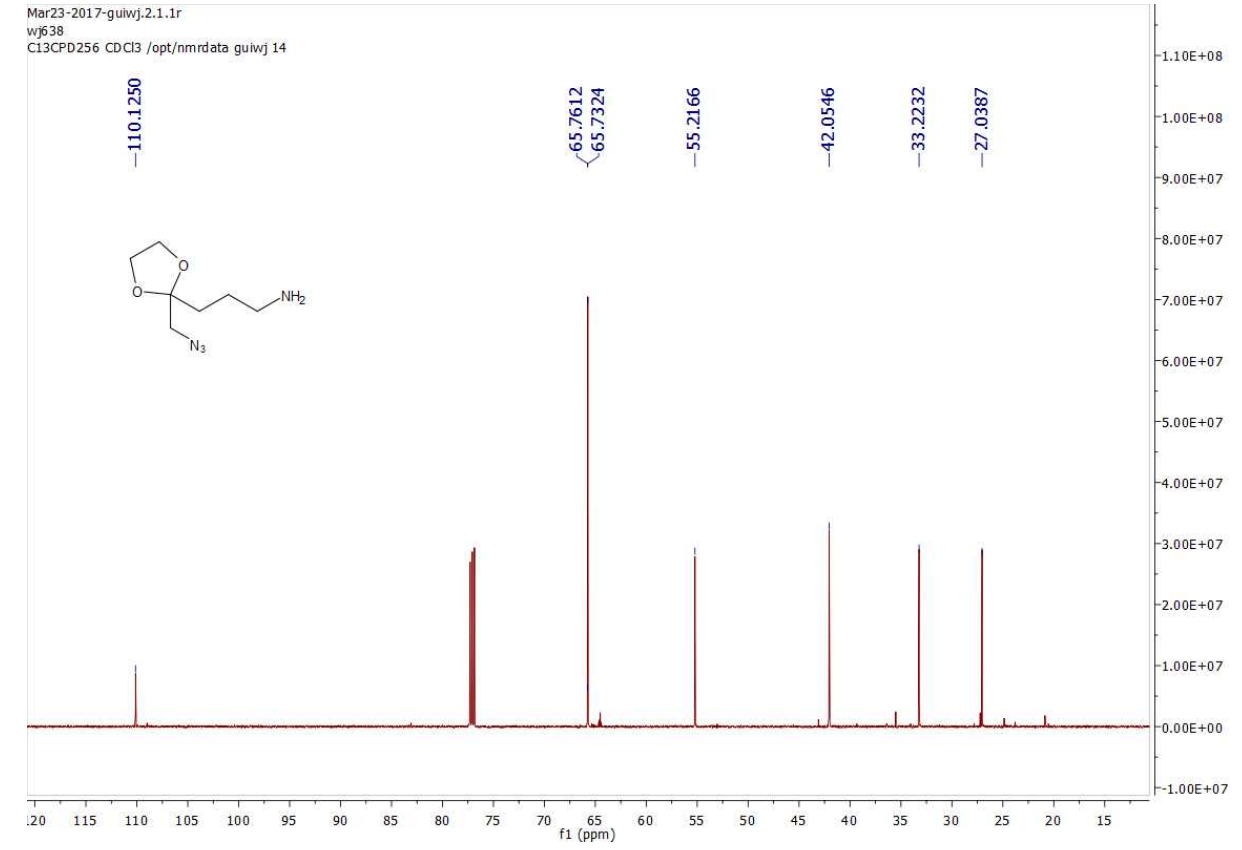
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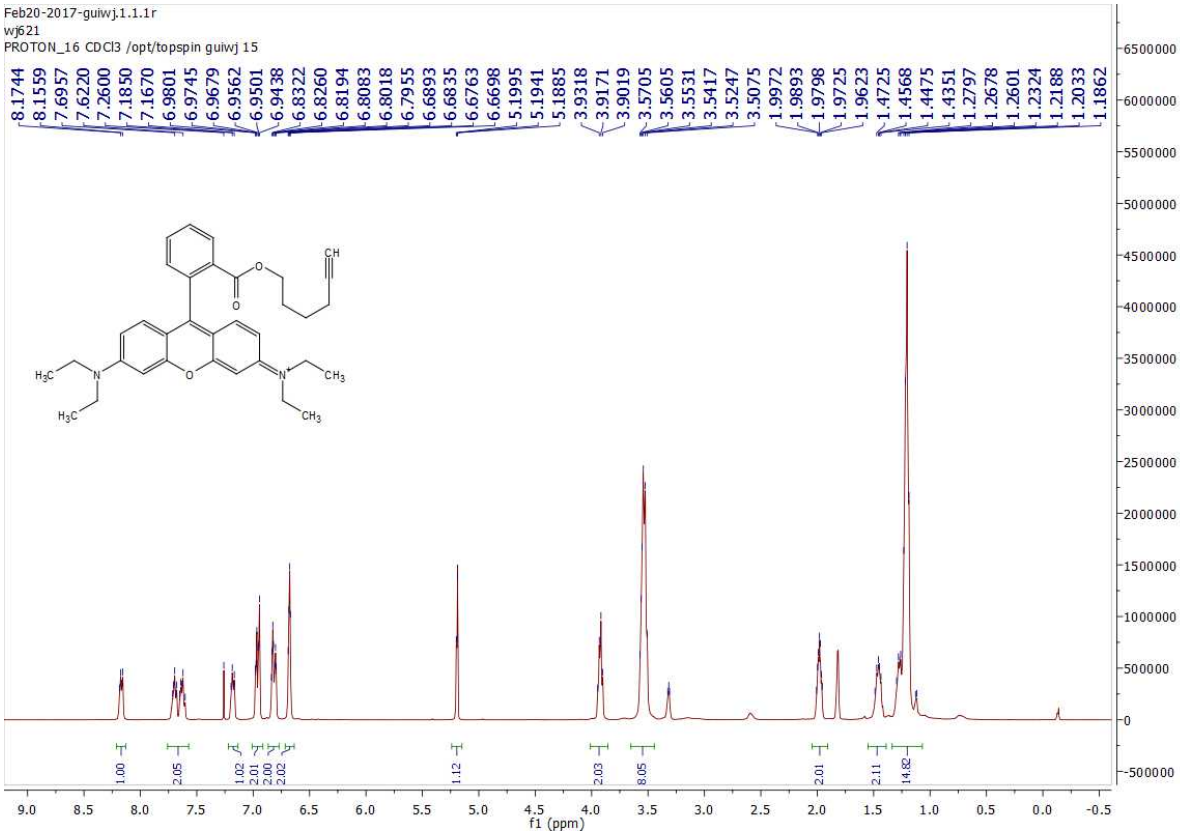
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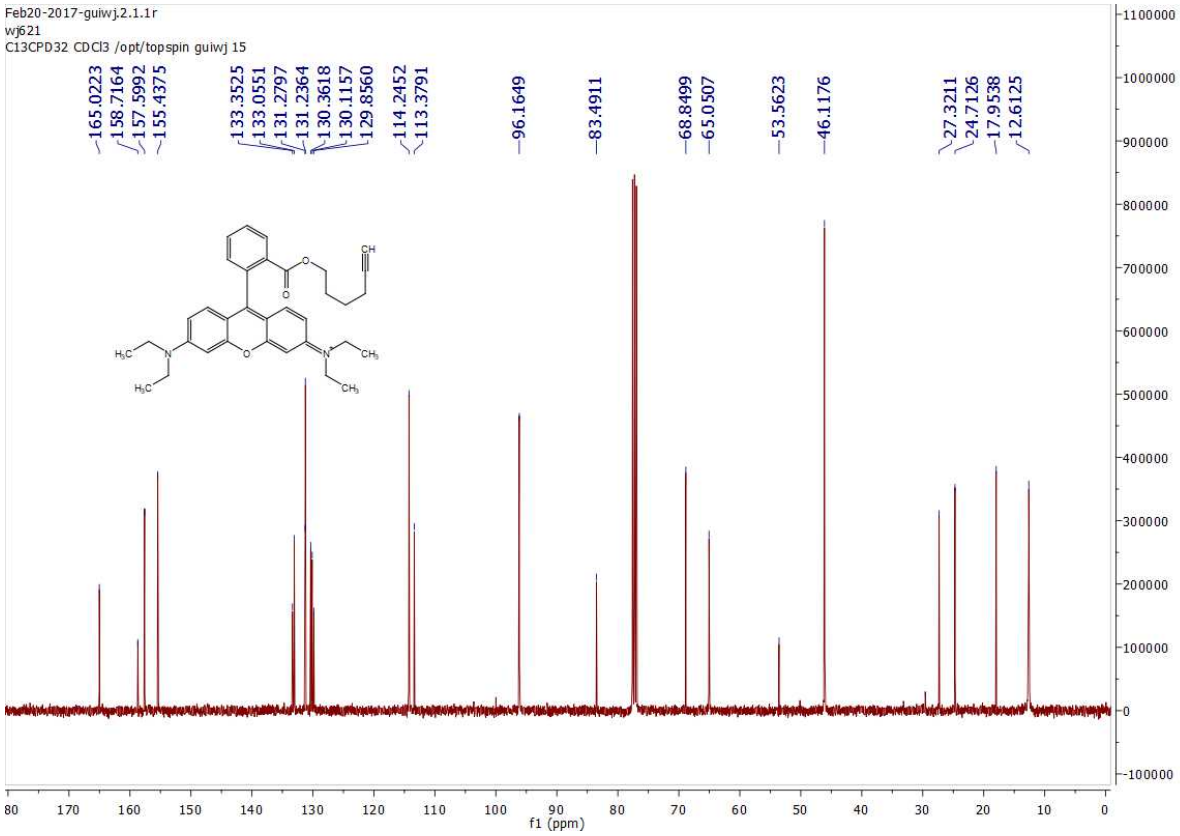
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