Supporting Information for

Synthesis of Branched Tri-Ubiquitin Active-Site Directed Probes

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General Information

^{1}H and ^{13}C NMR spectra were recorded on a Bruker AV400 NMR Spectrometer. Chemical shifts are reported in ppm on the □ scale relative to residual $CHCl_3$ (δ = 7.24 for ^{1}H NMR and δ = 77.0 for ^{13}C NMR) as an internal reference. Preparative FPLC was carried out on a GE Healthcare Life Sciences ÄKTA pure chromatography system. ESI-MS spectra were measured on a Bruker MicrOTOF ESI-TOF and Thermo Scientific Orbitrap Fusion Tribrid Mass Spectrometer (for HRMS). MALDI-TOF MS data were obtained with a Bruker MicroFlex LRF MALDI-TOF.

Chemical reagents were obtained from Fisher, Sigma-Aldrich, Alfa and Acros and were of the highest available grade, and thus used without further purification. The pOPINS-OTUB1*(UBE2D2(full-length, aa 1-147)-OTUB1 (aa 16-271)), pOPINK-OTUD1 (full-length, aa 1-481), pOPINS-AREL1 (aa 436–823), pOPINS-UBE3C (aa 693–1083), UBCH5C and UBC13:MM22 constructs were all obtained from Addgene. Recombinant Human His6-Ubiquitin E1 Enzyme was obtained from Bio-Techne. A vector encoding NIel (aa 170-782) was a kind gift from Prof. Jue Chen.

1. Protein Expression and Purification

1.1. Expression and purification of $Ub_{(1-75)}$-MESNA (**7**) and Avi-$Ub_{(1-75)}$-MESNA (**7a**)

Ubiquitin$_{(1-75)}$ was cloned upstream of intein/chitin binding domain in the pTXB1 vector (NEB). This protein was expressed and purified from *E. coli* Rosetta™ 2(DE3)pLysS cells (Novagen) as described. In brief, cells were grown at 37 °C in 12 L 2x YT media supplemented with ampicillin (100 μg/mL) with shaking at 200 rpm until OD_{600}~0.75. Protein expression was induced with isopropyl β-D-1-thiogalactopysranoside (IPTG, 0.5 mM). Cells were then grown overnight at 16 °C and then harvested by centrifugation for 30 min at 8,000 x g, 4 °C. The cell pellet was resuspended in lysis buffer (20 mM NaH_2PO_4, pH 6.0, 200 mM NaCl, 1 mM EDTA) and then lysed by sonication. The lysate was clarified by centrifugation for 30 min at 30,000 x g, 4 °C. $Ub_{(1-75)}$-Intein was then incubated with 60 mL chitin resin for 4 h at 4 °C. The resin was washed with 5 column volumes of lysis buffer and high salt buffer (20 mM NaH_2PO_4, pH 6.0, 1 M NaCl, 1 mM EDTA). The resin was then incubated with 2 column volumes of cleavage buffer (20 mM Na_2HPO_4, pH 6.0, 200 mM NaCl,1 mM EDTA, 100 mM MESNa) for 40 h at room temperature with shaking. This mixture was centrifuged for 10 min at 1,000 x G, 4 °C and the supernatant was removed. The resin was washed with 1 column volume of wash buffer and centrifuged as described for the previous step. The supernatants were combined and then concentrated using Amicon Ultra-15 3K MWCO filters and buffer exchanged into MiliQ water, followed by lyophilization to afford 160 mg $Ub_{(1-75)}$-MESNA (**7**) (**Figure S1**), observed deconvoluted m/z ratio for $[M+H]^+$ is 8631.99; calculated m/z is 8631.56, as a white solid. The Avi tag was fused to the N-terminus of Ub $_{(1-75)}$ using site-directed mutagenesis (New England Biolabs). The primers (IDT technologies) used here are listed in **Table S1**. Avi-$Ub_{(1-75)}$-MESNA (**7a**) was expressed and purified using the same protocol just described (**Figure S1**).

1.2. Expression and purification of Ub variants.

Lysine-to-cysteine mutations or lysine-to-arginine mutations were introduced at specified sites in the DNA sequence of Ubiquitin (Ub $_{1-76}$) using splice overlap extension. Primers containing the TGC mutation or CGC mutation were inserted at the desired codon position. D77 was encoded in the reverse primer to afford UbK48CD77, which were ligated into a pET22b vector (Novagen). Ub variants were expressed and purified from *E. coli* RosettaTM 2(DE3)pLysS cells (Novagen) as previously described[1]. In brief, cells were grown at 37 °C to an OD600 between 0.5 and 0.9, expression was induced with 300 μM IPTG, and expression was allowed to proceed for 4 h at 37 °C. Cells were pelleted at 5000 x g, resuspended in 120 mL of Ub lysis buffer (50 mM Tris, 5 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM DTT, 0.02 % IGPAL, pH 7.5), and lysed

by sonication. Cell lysate was clarified at 8000 x g for 20 min, and proteins were precipitated by the addition of 1.2 mL of 70 % perchloric acid. Precipitated proteins were centrifuged at 8000 x g for 20 min, and the soluble portion was dialyzed against buffer A (50 mM NH_4OAc, 2 mM EDTA, 1 mM DTT, pH 4.4). Dialyzed protein was then applied to Sepharose Fast Flow MonoS resin pre-equilibrated with buffer A. The resin was washed with 25 mL of 5 % buffer B ((50 mM NH4OAc, 2 mM EDTA, 1 mM DTT, 1 M NaCl, pH 4.4), 25 mL of 10 % buffer B, and protein was eluted using 50 mL 25 % buffer B. Fractions containing ubiquitin as determined by SDS-PAGE were collected, buffer exchanged into water, concentrated, and lyophilized.

1.3. Expression and purification of Enzymes.

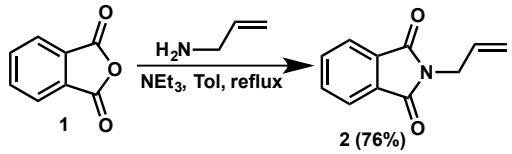
E1[2], NleL[3], UbcH5C[3], Ubc13:Mms2[4], OTUB1*[5], OTUD1, UBE3C[6] and AREL1[6] were expressed and purified as previously described. The alanine mutants of OTUB1*, UBE3C and AREL1 were prepared through Phusion site directed mutagenesis (New England Biolabs). The primers (IDT technologies) used here are listed in **Table S1**.

Table S1. Primers used in this study (**p** is phosphorylated primer)

Primers	Sequence (5'-3')
AVI-$Ub_{(1-75)}$ Intein	
AVI_f	pAAA ATT GAA TGG CAT GAA GGT GGT AGT ATG CAG ATC TTC GTC
AFI_r	pCTG CGC TTC AAA AAT ATC GTT CAG GCC CAT ATG TAT ATC TCC TTC
OTUB1*	
C91A_f	pCCT GAC GGC AAC GCG TTC TAT CGG GCT
C91A_r	pCCT GGT CTT GCG GAT GTA CGA GTA CTT TTT GTG GAG GTC CTT GAT CTT
UBE3C	
C1051A_f	pACCGCTTCCACGGCGATGAATCTGCTG
C1051A_r	pCGGCAGACGTTCCAGATCACTGCCACC
AREL1	
C790A_f	CACACAGCGTTTAACCAGCTGTGCCTCCCTACATATGACTCC
C790A_r	GTTAAACGCTGCGTGTGCAGTAGGCAGCGTGCTATGG

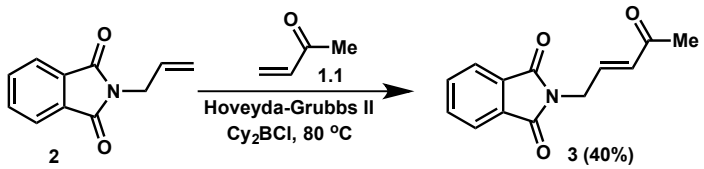
2. General procedure for the preparation of chemical probes

2-allylisoindoline-1,3-dione (**2**)



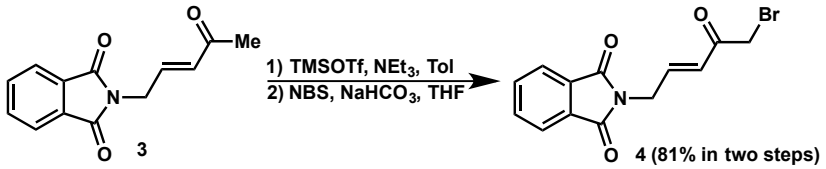
Under N_2 atmosphere, to a stirred mixture of **1** (29.60 g, 0.20 mol) and anhydrous triethylamine (2.80 mL, 0.02 mol) in anhydrous toluene with a Dean-Stark apparatus, allylamine (16.50 mL, 0.22 mol) was added and the mixture was refluxed at 110 °C for 4 h. The solution was cooled to room temperature and poured over a silica gel pad. The filtrate was collected and concentrated to afford crude **2** (28.50 g, y. 76%), as a white solid. The spectroscopic data corresponded to those reported in literature.

(E)-2-(4-oxopent-2-en-1-yl)isoindoline-1,3-dione (**3**)



Compound **3** was prepared according to a modified method of Vedrenne *et al* [7].
Under N_2 atmosphere, to a stirred mixture of **2** (4.68 g, 25 mM), methyl vinyl ketone (2.24 mL, 27.5 mmol), and Cy_2BCl (0.46 g, 3.0 mmol) in anhydrous toluene (40 mL), Hoveyda-Grubbs 2nd generation catalyst (0.24 g, 0.38 mmol) was added and the mixture was stirred at 80 °C for another 2.5 h. The mixture was cooled to room temperature and filtered through a pad of Celite. The mother liquor was concentrated to afford crude **3**, which was purified with silica gel chromatography (25% to 33% Hexane/Ethyl Acetate) to afford **3** (2.29 g, y. 40%), as a yellow solid. The spectroscopic data corresponded to those reported in literature.

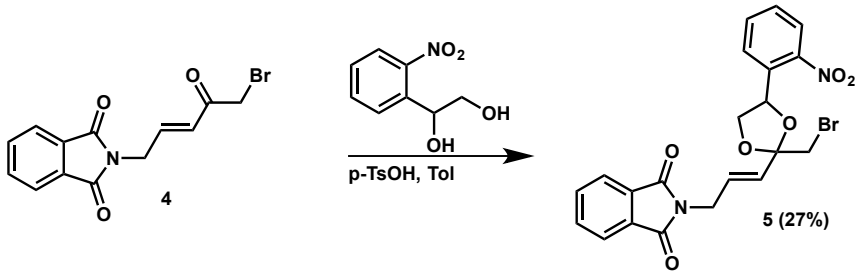
(E)-2-(5-bromo-4-oxopent-2-en-1-yl)isoindoline-1,3-dione (**4**)



Compound **4** was prepared according to a modified method of Li, G *et al* [8].
Under N_2 atmosphere, to a stirred mixture of **3** (2.0 g, 8.72 mmol), anhydrous triethylamine (1.81 mL, 13.08 mmol) in anhydrous toluene (20 mL), trimethylsilyl triflate (2.05 mL, 11.35 mmol) in anhydrous toluene (10 mL) was added dropwise over a period of 10 min under ice-water bath and then the mixture was allowed to warm to room temperature with stirring for another 3 h. Next, the reaction mixture was

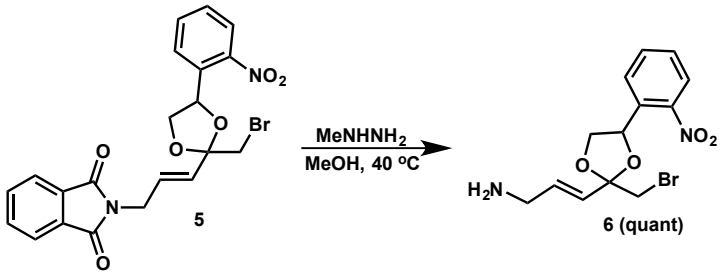
quenched with saturated aqueous $NaHCO_3$ (40 mL) under ice-water bath. The aqueous layer was extracted with ethyl acetate (30 mL x 2), and the combined organic layers were dried over $MgSO_4$, filtered, and concentrated under reduced pressure to afford a yellow solid. The crude product without further purification was dissolved in THF (20 mL) and 1.1 g $NaHCO_3$ (13.08 mmol) was added under ice-water bath. Then, NBS (1.71 g, 9.59 mmol) was added in portions and stirred for another 3 h. The reaction mixture was quenched with saturated aqueous $NaHCO_3$ (40 mL). The aqueous layer was extracted with ethyl acetate (30 mL x 2), and the combined organic layers were dried over $MgSO_4$, filtered, and concentrated under reduced pressure to afford crude **4**, which was purified with silica gel chromatography (33% to 50% Hexane/Ethyl Acetate) to afford **4** (2.18 g, y. 81%), as a yellow solid. The spectroscopic data corresponded to those reported in literature.

(E)-2-(3-(2-(bromomethyl)-4-(2-nitrophenyl)-1,3-dioxolan-2-yl)allyl)isoindoline-1,3-dione(**5**)



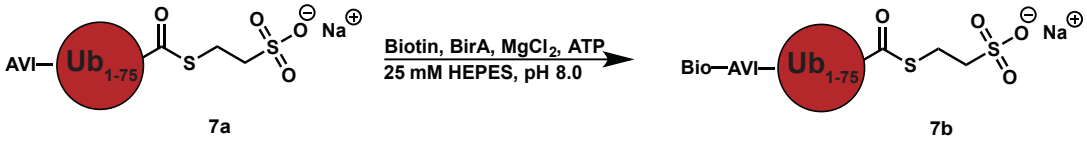
Under N_2 atmosphere, to a stirred solution of **4** (1.60 g, 5.23 mmol) in toluene (25 mL), 1-(2-nitrophenyl)ethane-1,2-diol (1.91 g, 10.46 mmol) and p-TsOH (90.09 mg, 0.52 mmol) were added, and the mixture was stirred at 80 ^oC for 4 h. The solution was cooled to r.t and filtered to recover 1-(2-nitrophenyl)ethane-1,2-diol. The mother liquor was concentrated to afford crude **5**, which was purified by silica gel chromatography (25% to 33% Hexane/Ethyl Acetate) to afford **5** (0.60 mg, y. 22%) as white solid and **4** (1.00 g). ^1H NMR ($CDCl_3$, 400 MHz) δ 8.21 (d, J = 7.9 Hz, 1H), 8.05 (d, J = 8.1 Hz, 1H), 7.85-7.87 (m, 2H), 7.72-7.74 (m, 2H), 7.70 (t, J = 7.9, 7.5 Hz, 1H), 7.47 (t, J = 8.1, 7.5 Hz, 1H), 6.09-6.16 (m, 1H), 5.75 (d, J = 15.4 Hz, 1H), 5.55 (t, J = 7.0, 7.0 Hz, 1H), 4.62 (t, J = 7.6, 7.5 Hz, 1H), 4.37 (d, J = 5.7 Hz, 2H), 3.84 (t, J = 7.6, 7.5 Hz, 1H), 3.63 (q, J = 11.4, 2H). ^{13}C NMR ($CDCl_3$, 100 MHz) 167.1, 147.1, 135.4, 134.2, 134.2, 134.1, 132.0, 129.9, 128.7, 128.4, 128.0, 124.7, 123.4, 123.4, 106.4, 74.6, 72.5, 38.5, 36.0. ESI-HRMS: m/z calcd for $C_{21}H_{17}BrN_2O_6$ $[M+Na]^+$: 495.1068. Found: 495.1065.

(E)-3-(2-(bromomethyl)-4-(2-nitrophenyl)-1,3-dioxolan-2-yl)prop-2-en-1-amine (**6**)



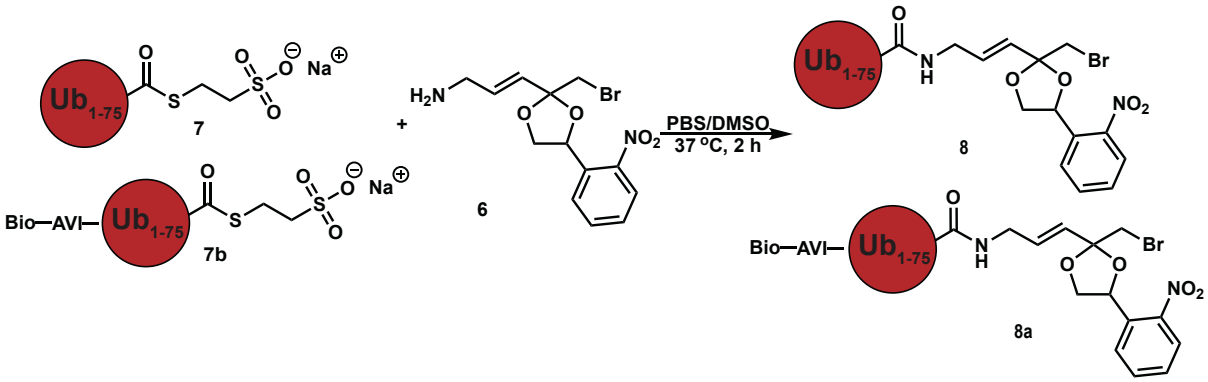
Under N_2 atmosphere, to a stirred mixture of **5** (0.43 g, 0.91 mmol) in MeOH (3 mL), $MeNH_2NH_2$ (1 mL) was added dropwise. The mixture was stirred 40 °C overnight, then concentrated. CH_2Cl_2 (30 mL) and 0.01 M NaOH (20 mL) were added to the residue. The aqueous layer was extracted with CH_2Cl_2 (30 mL x 2), and the combined organic layers were dried over $MgSO_4$, filtered, and the concentrated under reduced pressure to afford crude **6** (0.37 g, quant), as a yellow oil, which was used in the next reaction without further purification. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.25 (d, J = 7.9 Hz, 1H), 8.06 (d, J = 8.1 Hz, 1H), 7.72 (t, J = 7.9, 7.5 Hz, 1H), 7.48 (t, J = 8.1, 7.5 Hz, 1H), 6.17-6.24 (m, 1H), 5.72 (d, J = 15.6 Hz, 1H), 5.59 (t, J = 7.0, 7.0 Hz, 1H), 4.65 (t, J = 7.7, 7.4 Hz, 1H), 3.86 (t, J = 7.8, 7.2 Hz, 1H), 3.67 (q, J = 11.4, 2H), 3.47 (d, J = 4.7 Hz, 2H). ^{13}C NMR ($CDCl_3$, 100 MHz) 147.2, 135.5 134.8, 134.2, 128.7, 128.1, 126.7, 124.6, 106.7, 74.4, 72.5, 42.5, 36.3. ESI-HRMS: m/z calcd for $C_{13}H_{15}BrN_2O_4$ $[M+H]^+$: 343.0293. Found: 343.0289.

N- terminally biotinylated AVI-$Ub_{(1-75)}$-MESNA (**7b**)



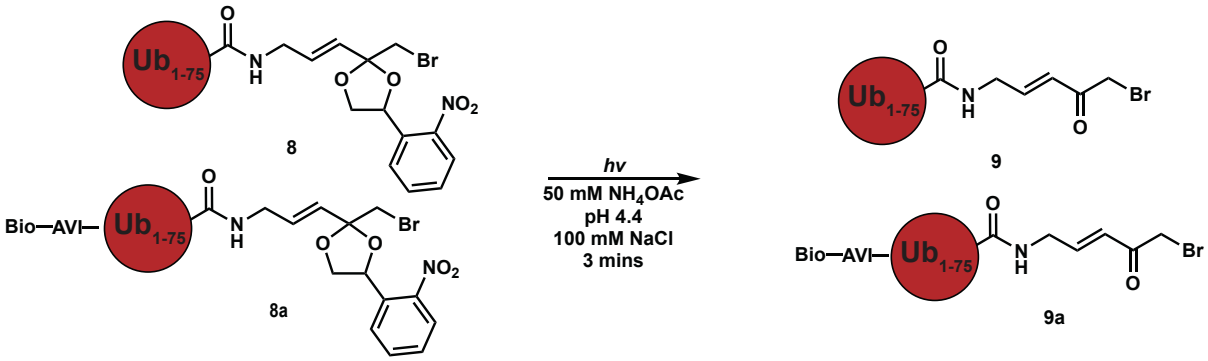
50 μM Avi-Ub1-75-MESNa was incubated with 0.35 μM BirA in 25 mM HEPES (pH 8.0), 5 mM MgCl2, 150 μM biotin, 2 mM ATP, while shaking at 37 °C for 1 h. Then the reaction product was purified by using size exclusion chromatography on an ÄKTA purifier. The running buffer was 50 mM HEPES, pH 6.0, 50 mM NaCl. The molecular weight of the **7b** was determined by ESI-MS to 10869.25 Da (theoretical MW is 10869.43 Da) (**Figure S2**).

Ubiquitin species **8** and **8a**



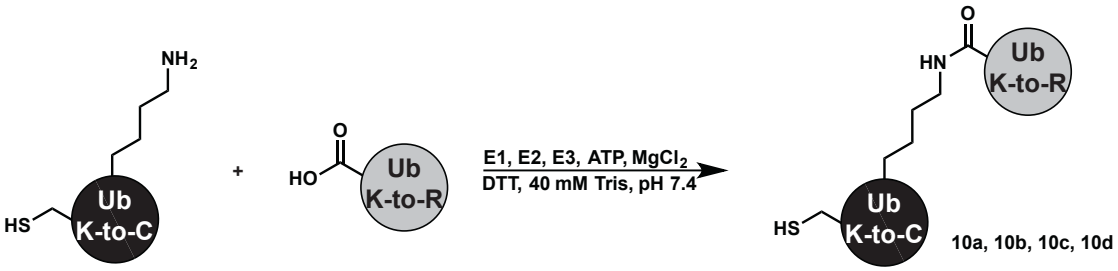
To an ice water chilled solution of Ub_{1-75}-MESNa (**7**) (2 mM) in PBS, was added compound **6** (62.5 mM) in DMSO. The mixture was shaken at room temperature for 2 h and the reaction was monitored by MALDI-TOF (**Figure S3A, B**). Then the resulting product was centrifuged and the supernatant was diluted with 9x the volume of MilliQ water, followed by concentrating using Amicon Ultra-15 3K MWCO to remove the excess of compound **6** and to obtain compound **8**. Compound **8a** was prepared and purified by the same method as described for **8**.

Ubiquitin species **9** and **9a**



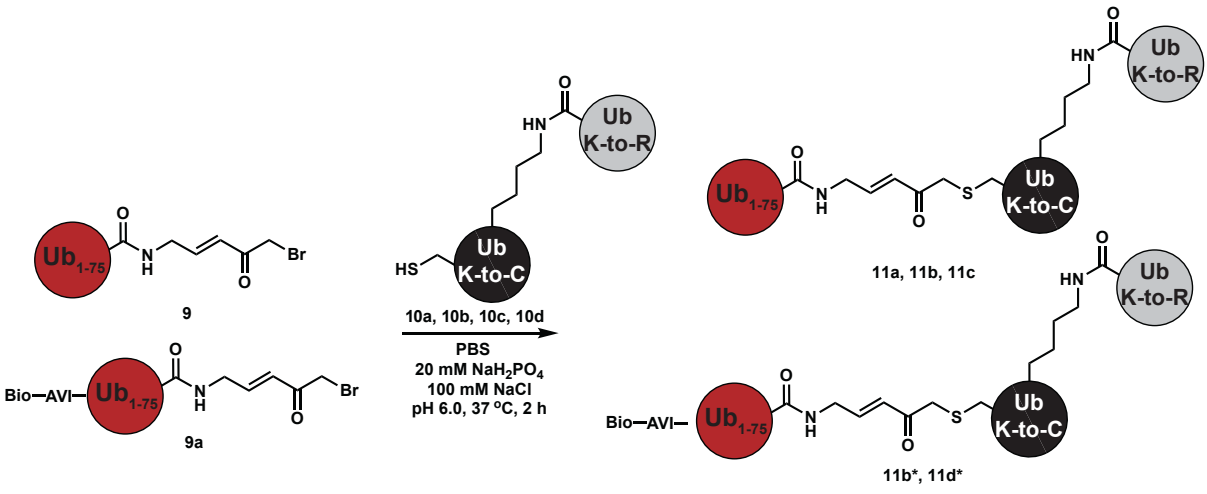
A solution of **8** (4 mg/mL) in 50 mM NH_4OAc, pH 4.4, 100 mM NaCl was irradiated for 3 min at 350 nm and the reaction was monitored by MALDI-TOF (**Figure S3B**) The resulting product **9** was buffer exchanged with 20 mM NaH_2PO_4, pH 6.0, 100 mM NaCl, which was used in the next reaction without further purification. Compound **9a** was prepared and purified by the same method as described for **9**.

Ubiquitin species **10a, 10b, 10c** and **10d**



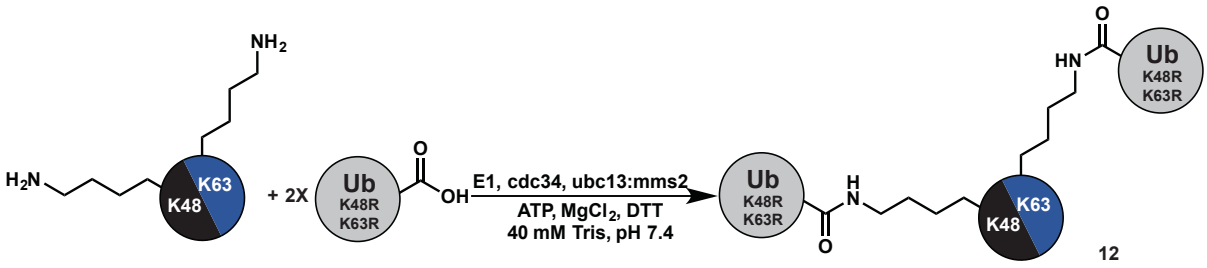
These native dimers were prepared as described previously[1,3]. 1) To prepare K63 Ub_2 with Ub D77 at the proximal site. 2 mM of Ub K48C-D77 (or Ub K6C-D77) and 2 mM Ub K63R(or Ub K6R/K48R) were reacted with 2 μM E1 and 20 μM Ubc13:Mms2 in a buffered solution containing 40 mM Tris, 10 mM $MgCl_2$, 10 mM ATP, and 0.6 mM DTT at pH 7.4. The reaction was incubated at 37 °C overnight. 2) For K6 Ub_2 with UbD77 at the proximal site. 2 mM of Ub K48C-D77 and 2 mM Ub K6R/K48Rwere reacted with 0.2 μM E1 and 1.5 μM NleL, 7.5 μM UbcH5C in a buffered solution containing 40 mM Tris, 10 mM $MgCl_2$, 10 mM ATP, and 0.6 mM DTT at pH 7.4. Again, the reaction was incubated at 37°C overnight. 3) To prepare K48 Ub_2 with Ub D77 at the proximal site. 1 mM of Ub K63C-D77 and 1 mM Ub K48R were reacted with 1 μM E1 and 10 μM Cdc34 in a buffered solution containing 40 mM Tris, 10 mM $MgCl_2$, 10 mM ATP, and 0.6 mM DTT at pH 7.4. The reaction was incubated at 37°C overnight as well. The reactions were quenched using acetic acid and centrifugated to remove precipitate. The residues were first buffer exchanged into buffer A (100 mM ammonium acetate, 100 mM NaCl, pH 4.4, 1 mM DTT) using a Centricon (3 kDa MWCO). The samples were loaded to a preequilibrated SP column and then eluted at a flow rate of 2 mL/min using a gradient of 0 to 65% buffer B (100 mM ammonium acetate, 1 M NaCl, pH 4.4, 1 mM DTT). Fractions were collected in 2.2 mL volume, and those containing pure dimer **10a, 10b, 10c and 10d** were pooled and concentrated. The molecular weight of the dimer **10a, 10b, 10c and 10d** were determined by ESI-MS **(Figure S4A, B, C, D**).

Ubiquitin species **11a, 11b, 11b*, 11c, 11d***



To an ice water chilled solution of **9** (4 mg/mL) in 20 mM NaH_2PO_4, pH 6.0 , was added 100 mM NaCl, K6 Ub_2 **10a** (4 mg/mL) in PBS. The solution was incubated at 37 ^oC for 2 h. SDS-PAGE gel was used to detect the formation of triUb (**Figure 2A**). Then the reaction product was purified by using size exclusion chromatography on an ÄKTA purifier. The running buffer was 50 mM HEPES, pH 7.4, 50 mM NaCl. The molecular weight of the trimer **11a** was determined by ESI-MS to 25845.8 Da (theoretical MW is 25842.6 Da) (**Figure 2A**). **11b**,**11b*** **11c** and **11d*** were prepared and purified by the same method as described for **11a** (**Figure 2B**, **Figure S6**).

Native branched tri-ubiquitin K48/K63 (**12**)



This native branched trimer was prepared as described previously. 2 mM of Ub D77 and 4 mM Ub K48R/K63R were reacted with 2 μM E1, 10 μM Cdc34 and 20 μM Ubc13:Mms2 in a buffered solution containing 40 mM Tris, 10 mM $MgCl_2$, 10 mM ATP, and 0.6 mM DTT at pH 7.4. The reaction was incubated at 37 ^oC overnight. The reaction was quenched using acetic acid and centrifugated to remove precipitate. The residue was first buffer exchanged into buffer A (100 mM ammonium acetate, 100 mM NaCl, pH 4.4, 1 mM DTT) using a Centricon (3 kDa MWCO). The sample was loaded to a preequilibrated SP column and then eluted at a flow rate of 2 mL/min using a gradient of 0 to 65% buffer B (100 mM ammonium acetate, 1 M NaCl, pH 4.4, 1 mM DTT). Fractions were collected in 2.2 mL volume, and those containing pure native branched trimer were pooled and concentrated.

3. DUBs and E3 Ligases labeling assays

3.1. Reactivity of tri-Ub probes towards different DUBs and E3 ligases

3.1.1
DUBs (OTUB1 variants; 5 μM, or OTUD1 variants; 0.5 μM) were incubated with different probes (25 μM or 2 μM) at r.t or 37 ºC for 5 min or 30 min in 50 mM HEPES, pH 7.4 containing 50 NaCl. Then the reaction was quenched by the addition of the SDS-PAGE 3X loading solution. The resulting samples were separated by SDS-PAGE and stained with Coomassie brilliant blue or SYPRO Ruby for analysis.

3.1.2
HECT E3 ligases (UBE3C variants and AREL1 variants; 5 μM) were incubated with different probes (25 μM) at 37 ºC for 15 min or 30 min in 50 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$. The addition of 5 % (v/v) glycerol in the reaction buffer was important to prevent AREL1 precipitation during the reaction. The reactions were quenched by the addition of the SDS-PAGE 3X loading solution. The resulting samples were separated by SDS-PAGE and stained with Coomassie brilliant blue for analysis.

3.2. Time-course assays

3.2.1 OTUB1* with tri-Ub probe (**11b**) and native isopeptide-linked tri-Ub
OTUB1* (0.5 μM) was incubated with **11b** (5 μM) at 37 ºC for 20 min in 50 mM HEPES pH 7.4 containing 50 mM NaCl. Samples were taken at different time points (1, 2, 5, 10, 20 min). The reactions were quenched by the addition of the SDS-PAGE 3X loading solution. The resulting samples were separated by SDS-PAGE and stained with SYPRO Ruby for analysis. The same condition was used in OTUB1* time-course assay with native isopeptide-linked K48/K63 tri-Ub (**12**).

3.2.2 OTUD1 with tri-Ub probes (**11b**, **11b*** and **11d***)
OTUD1 (0.5 μM) was incubated with **11b**, **11b*** and **11d*** (5 μM) at 37 ºC for 30 min in 50 mM HEPES pH 7.4 containing 50 mM NaCl. Samples were taken at different time points (5, 15, 30 min). The reactions were quenched by the addition of the SDS-PAGE 3X loading solution. The resulting samples were separated by SDS-PAGE and stained with SYPRO Ruby for analysis.

3.2.3 HECT E3 ligases with tri-Ub probes (**11a**, **11b**, **11c** and **11d***)
UBE3C was incubated with **11a**, **11b**, **11c** and **11d***) (5 μM) at 37 ºC for 15 min in 50 mM HEPES pH 7.4 containing 50 mM NaCl. Samples were taken at different time points (0, 1, 5 , 10, 15 min). The reactions were quenched by the addition of the SDS-

PAGE 3X loading solution The resulting samples were separated by SDS-PAGE and stained with Coomassie brilliant blue for analysis.

AREL1 was incubated with **11a**, **11b**, **11c** and **11d***) (5 μM) at 37 ºC for 60 min in 50 mM HEPES pH 7.4 containing 50 mM NaCl and 5% glycerol. Samples were taken at different time points (0, 5 ,15, 30, 60 min). The reactions were quenched by the addition of the SDS-PAGE 3X loading solution. The resulting samples were separated by SDS-PAGE and stained with Coomassie brilliant blue for analysis.

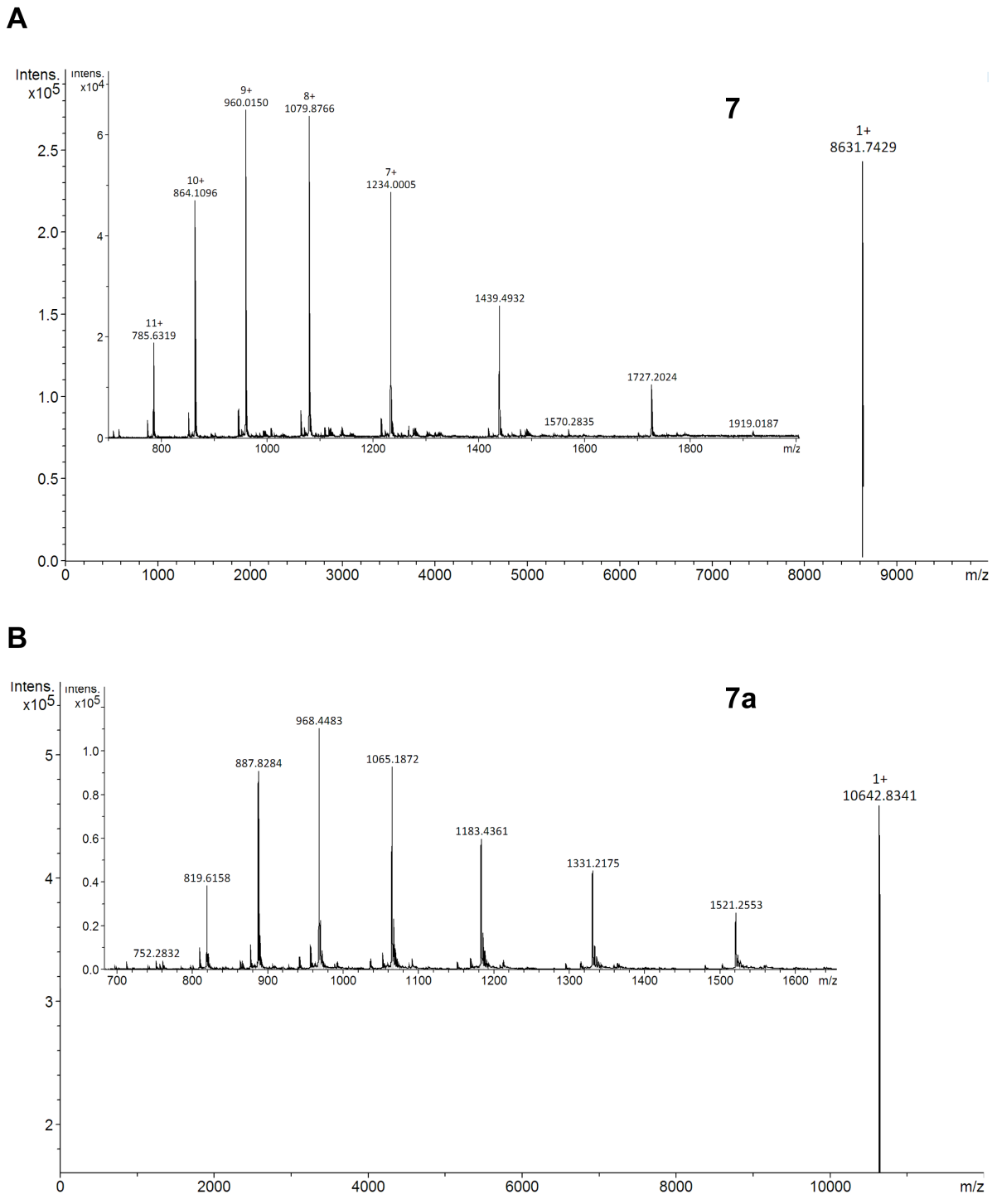


Figure S1. ESI mass spectrum of Ub(1-75)-MES (**7**) and AVI-Ub(1-75)-MES (**7a**): **7**: observed deconvoluted m/z ratio for $[M+H]^+$ is 8631.99; calculated m/z is 8631.56; **7a**: observed deconvoluted m/z ratio for $[M+H]^+$ is 10642.83; calculated m/z is 10643.14.

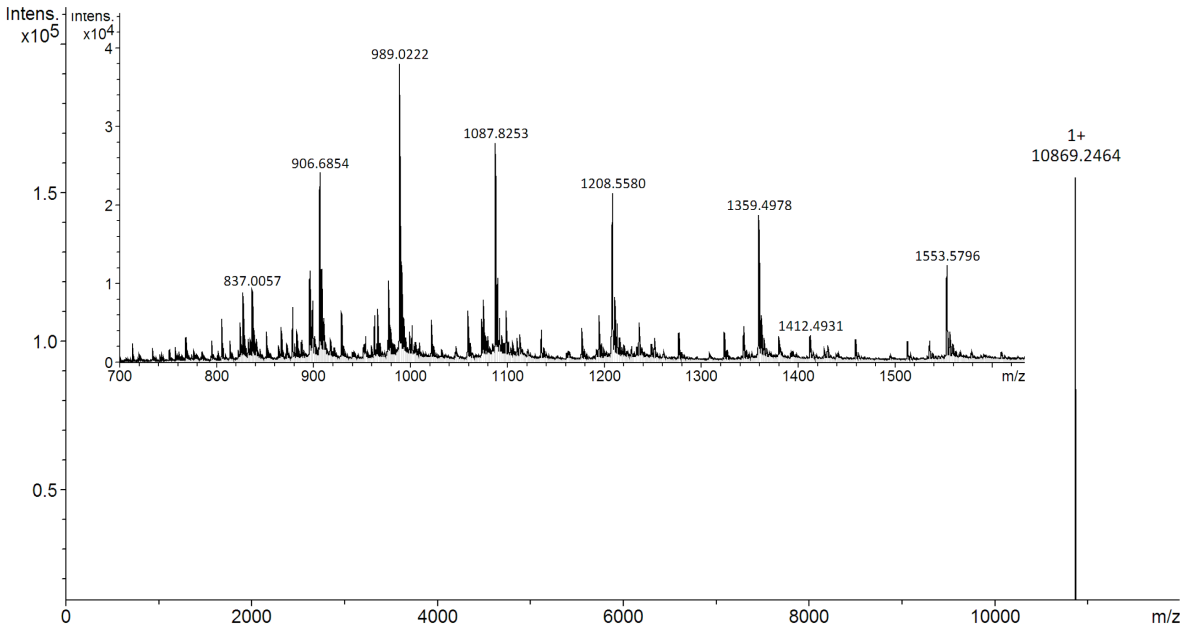


Figure S2. ESI mass spectrum of Bio-Avi-Ub(1-75)-MES (**7b**): observed deconvoluted m/z ratio for $[M+H]^+$ is 10869.25; calculated m/z is 10869.43.

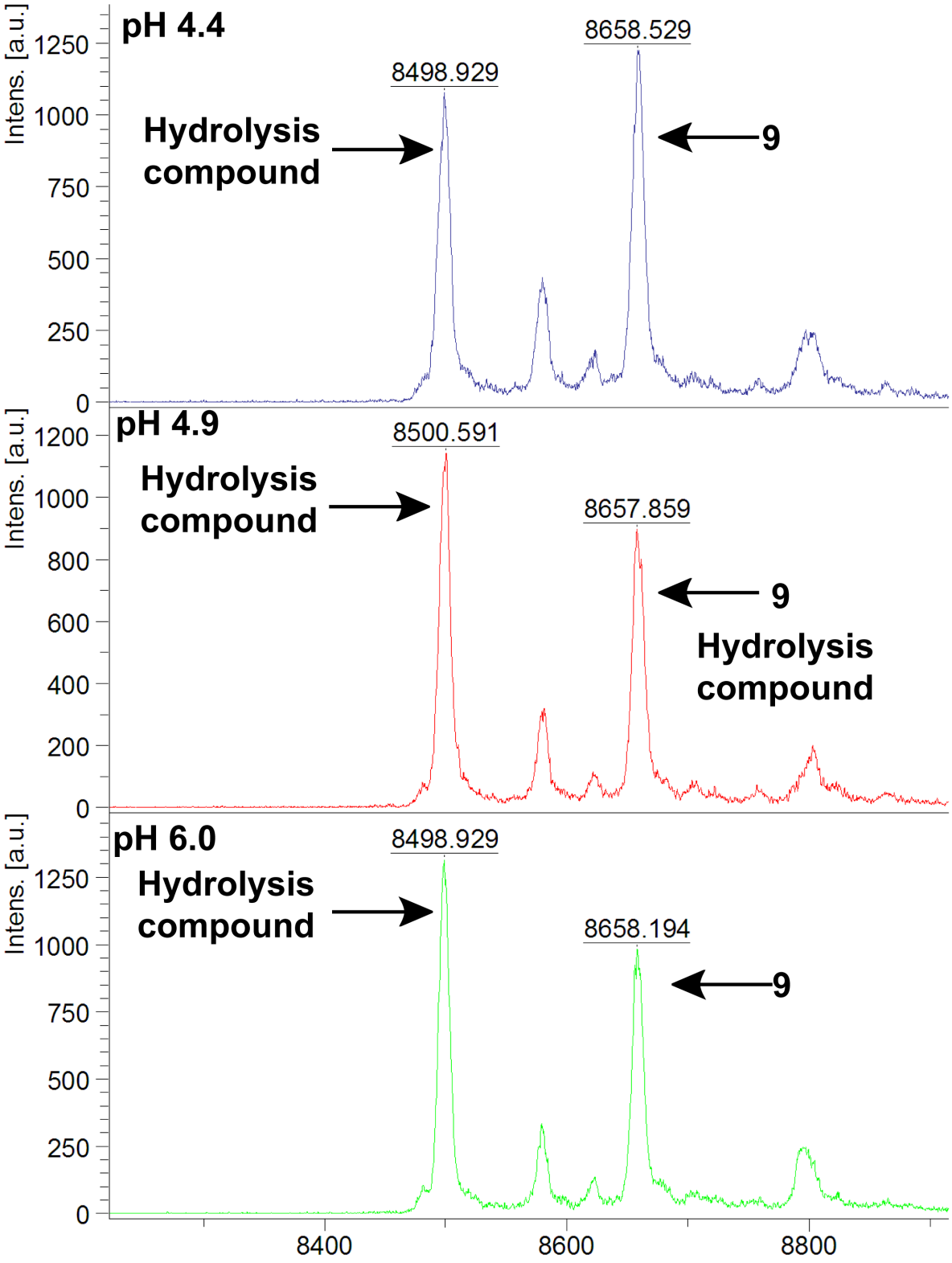


Figure S3. Monitoring the deprotection of compound **8** at different pHs (pH 4.4, pH 4.9, and pH 6.0) using MALDI MS.

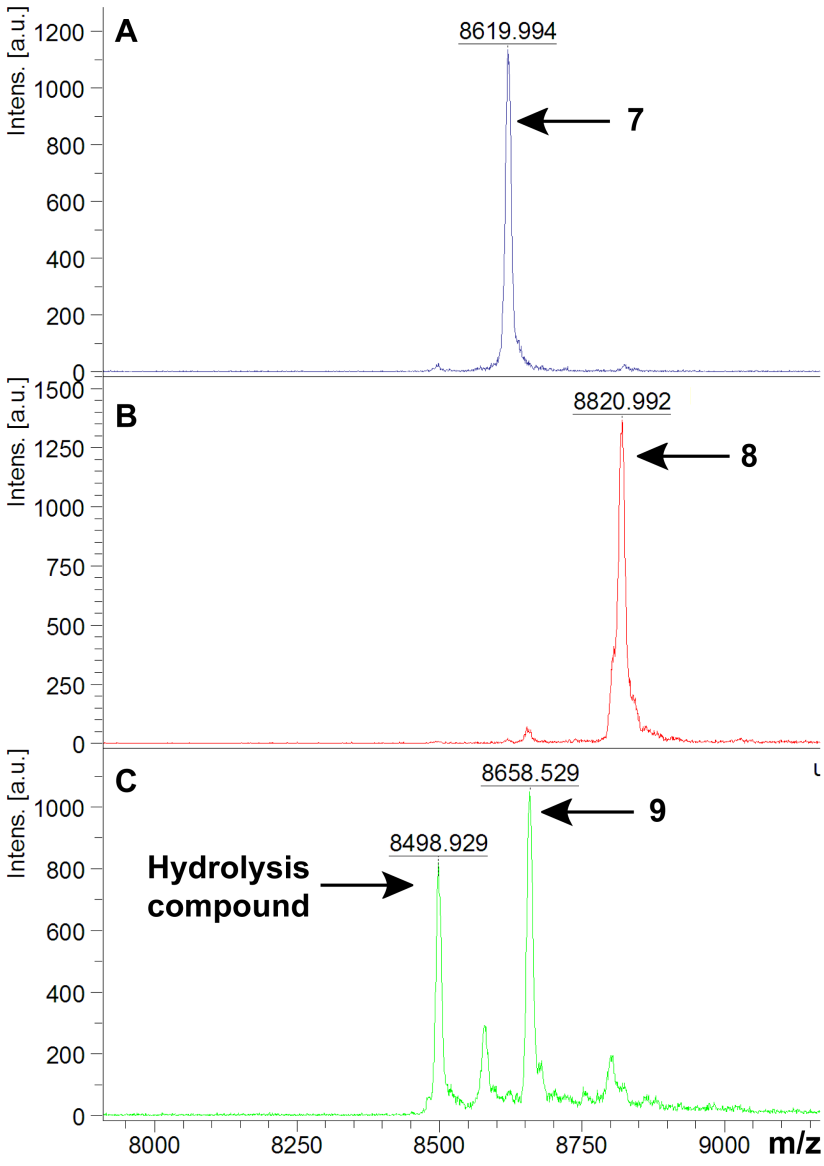
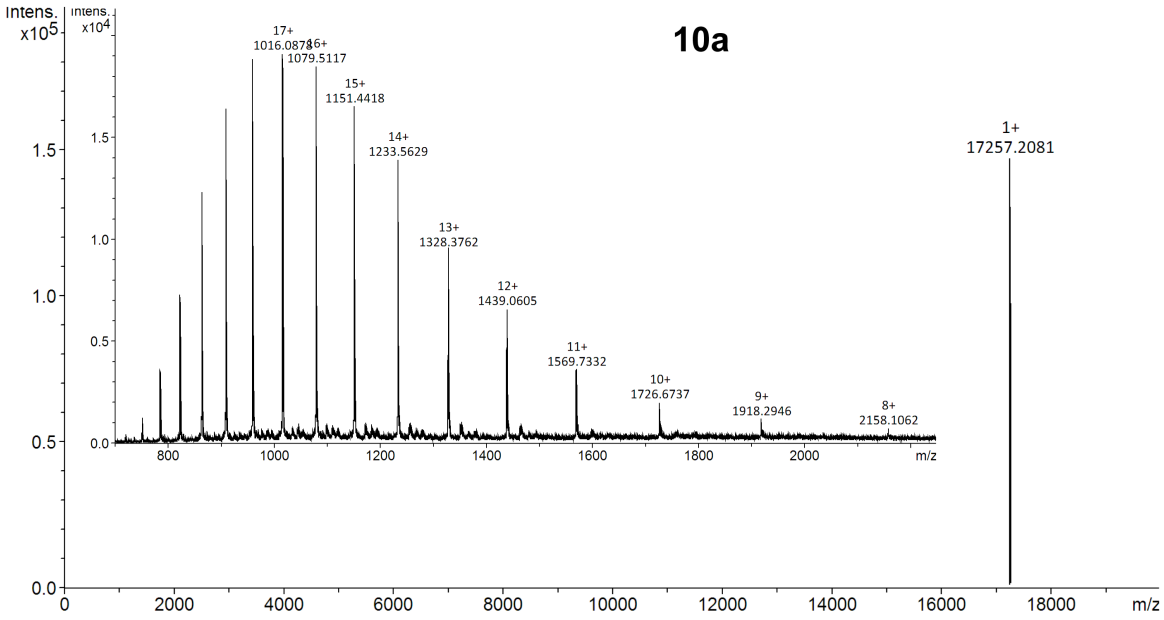
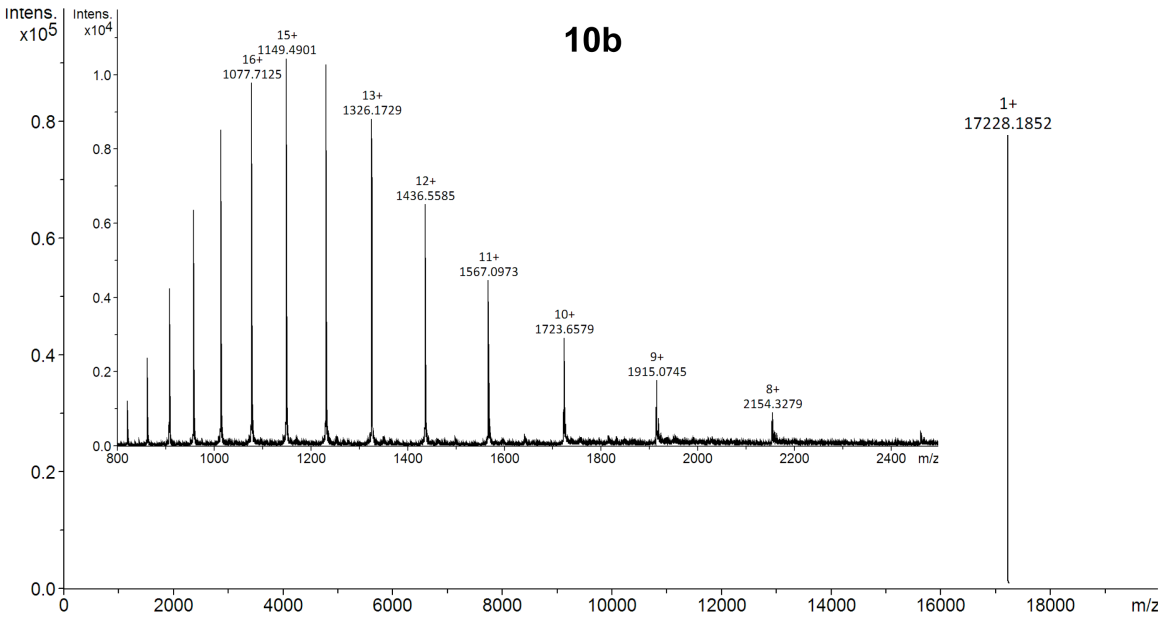


Figure S4. Monitoring the formation of **8** and Ub-BVK (**9**) using MALDI MS.


10a
Intens.
x10^5
Intens.
x10^4
17+
1016.0878
16+
1079.5117
15+
1151.4418
14+
1233.5629
13+
1328.3762
12+
1439.0605
11+
1569.7332
10+
1726.6737
9+
1918.2946
8+
2158.1062
1+
17257.2081
m/z


10b
Intens.
x10^5
Intens.
x10^4
15+
1149.4901
16+
1077.7125
13+
1326.1729
12+
1436.5585
11+
1567.0973
10+
1723.6579
9+
1915.0745
8+
2154.3279
1+
17228.1852
m/z

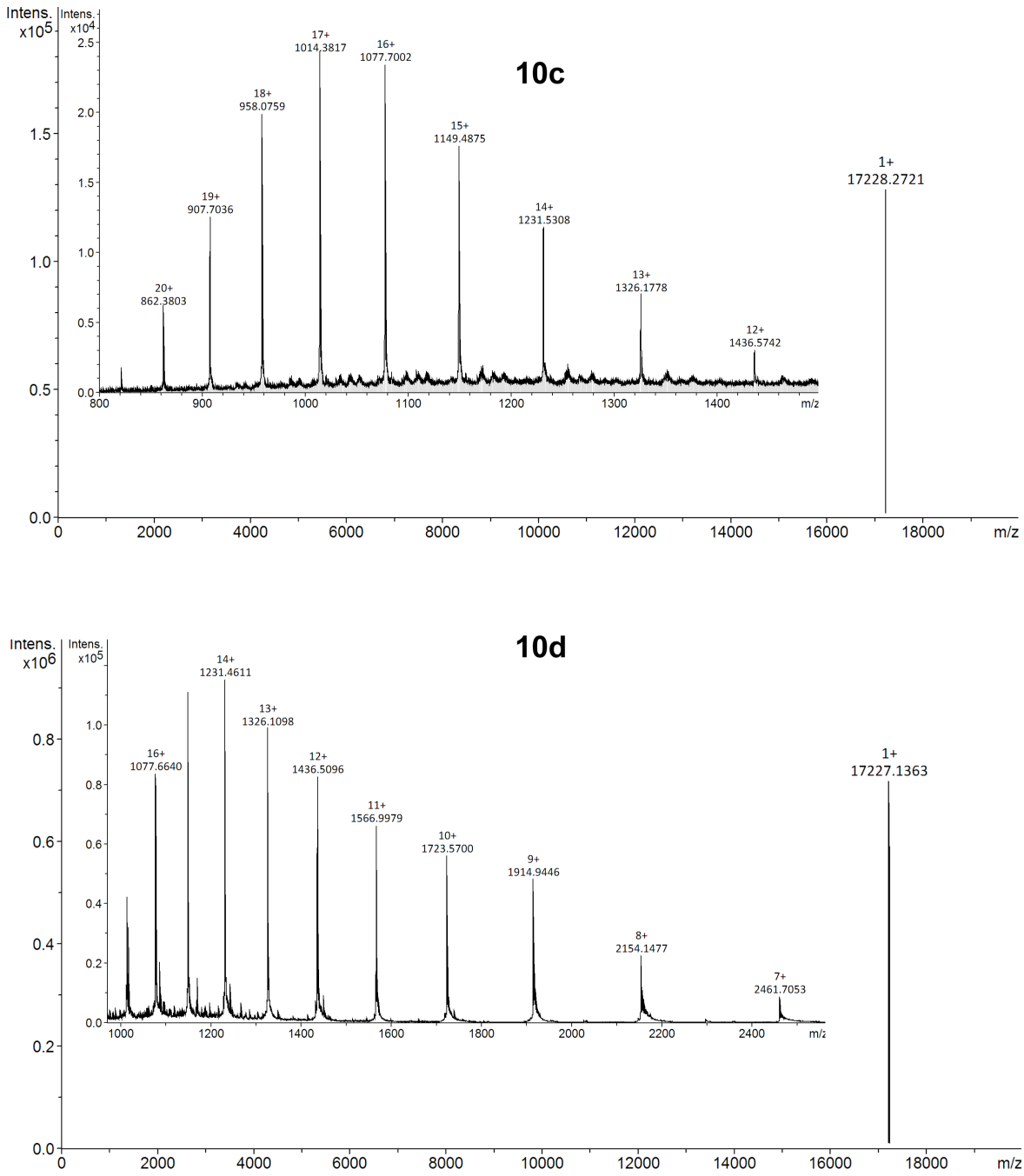


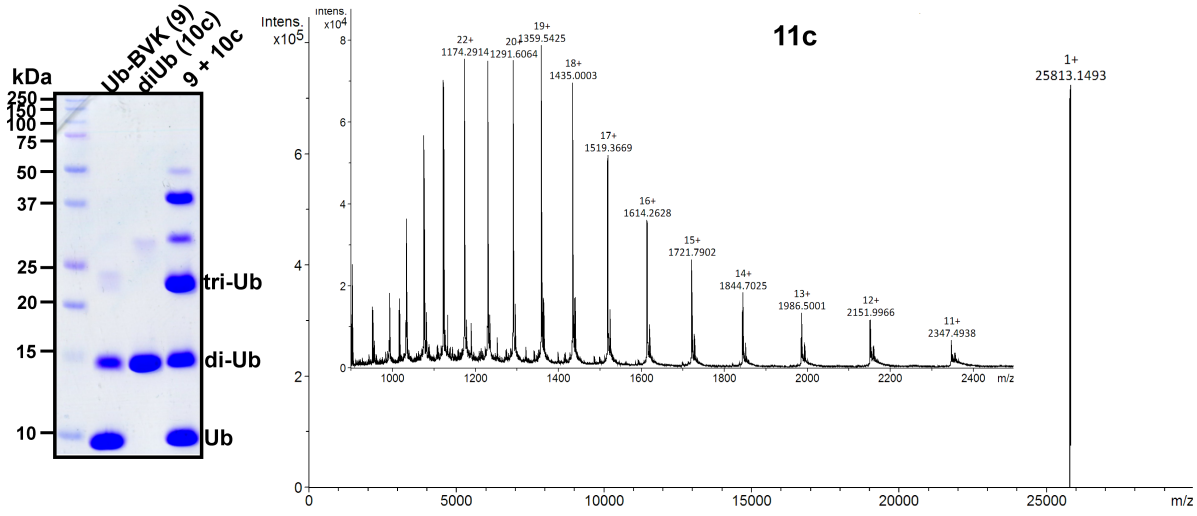
Figure S5. ESI mass spectrum of diUb:

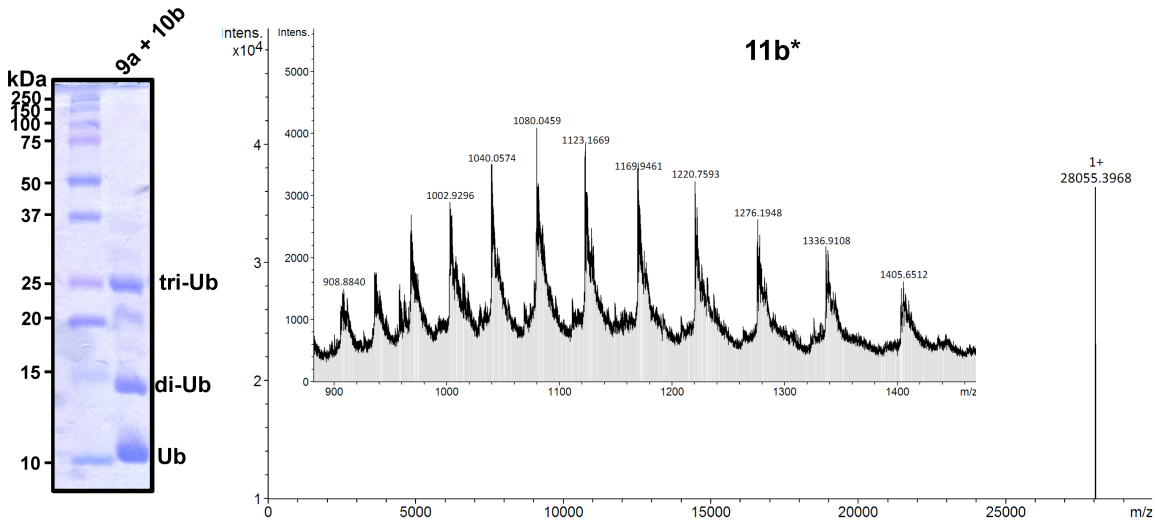
10a: observed deconvoluted m/z ratio for $[M+H]^+$ is 17257.2; calculated m/z is 17258.8;

10b: observed deconvoluted m/z ratio for $[M+H]^+$ is 17228.2; calculated m/z is 17230.7;

10c: observed deconvoluted m/z ratio for $[M+H]^+$ is 17228.3; calculated m/z is 17230.7;

10d: observed deconvoluted m/z ratio for $[M+H]^+$ is 17227.1; calculated m/z is 17230.7.


kDa
Ub-BVK (9)
diUb (10c)
9 + 10c
250
150
100
75
50
37
25
20
15
10
tri-Ub
di-Ub
Ub
11c
Intens.
x10^4
x10^5
22+
1174.2914
20+
1291.6064
19+
1359.5425
18+
1435.0003
17+
1519.3669
16+
1614.2628
15+
1721.7902
14+
1844.7025
13+
1986.5001
12+
2151.9966
11+
2347.4938
1+
25813.1493
m/z


9a + 10b
kDa
250
150
100
75
50
37
25
20
15
10
tri-Ub
di-Ub
Ub
11b*
Intens.
x10^4
908.8840
1002.9296
1040.0574
1080.0459
1123.1669
1169.9461
1220.7593
1276.1948
1336.9108
1405.6512
1+
28055.3968
m/z

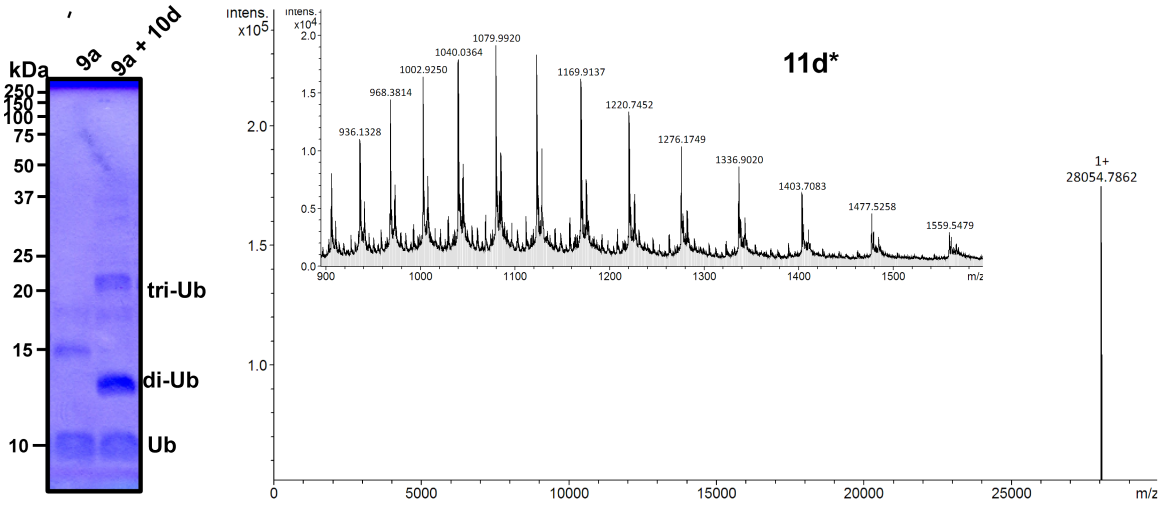


Figure S6. ESI mass spectrum of branched tri-Ub probes:

11c: observed deconvoluted m/z ratio for $[M+H]^+$ is 25813.2; calculated m/z is 25817.1;

11b*: observed deconvoluted m/z ratio for $[M+H]^+$ is 28055.4; calculated m/z is 28055.8;

11d*: observed deconvoluted m/z ratio for $[M+H]^+$ is 28055.8; calculated m/z is 28055.8.

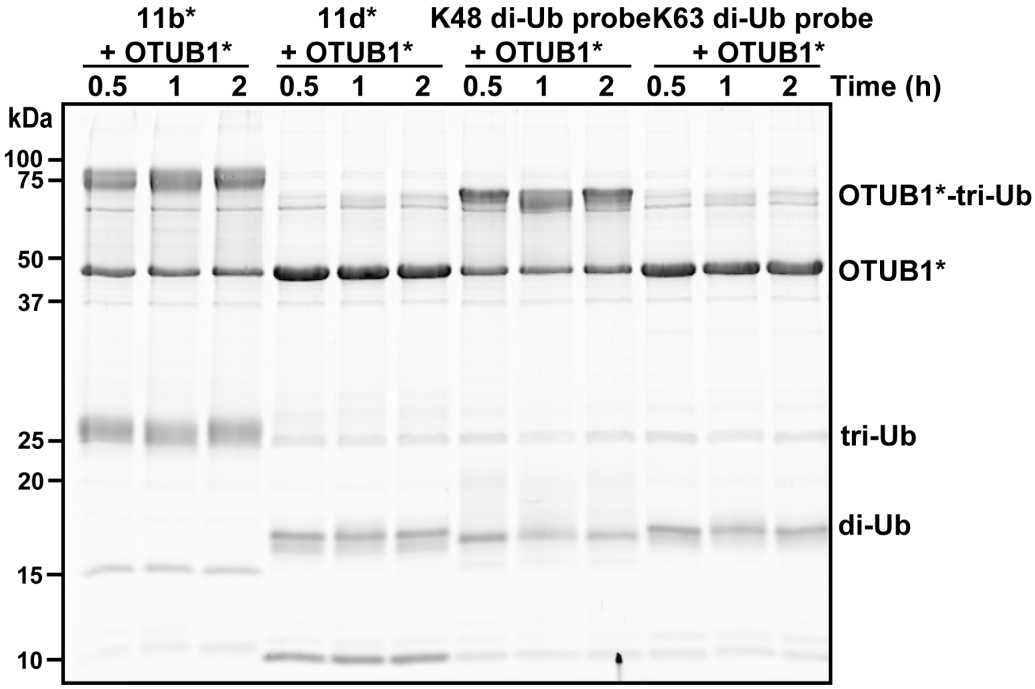


Figure S7. OTUB1* does not react with probe **11d** or the Lys63 di-Ub probe. Label does occur with probes containing the warhead at position-48, e.g., **11b** and Lys48 di-Ub probe.

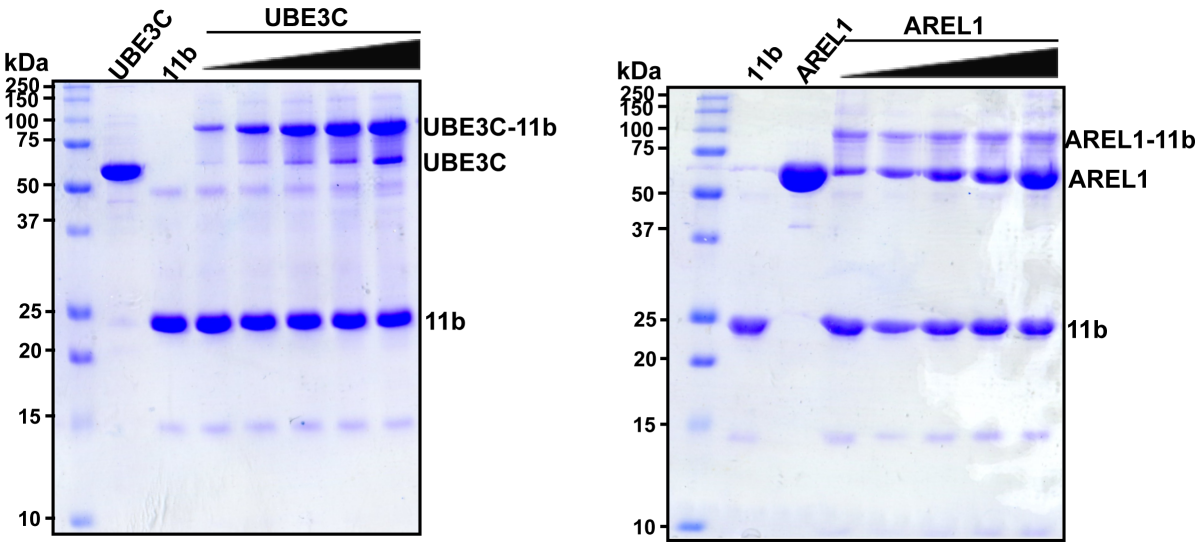


Figure S8. Extent of adduct formation depends on the concentration of E3.

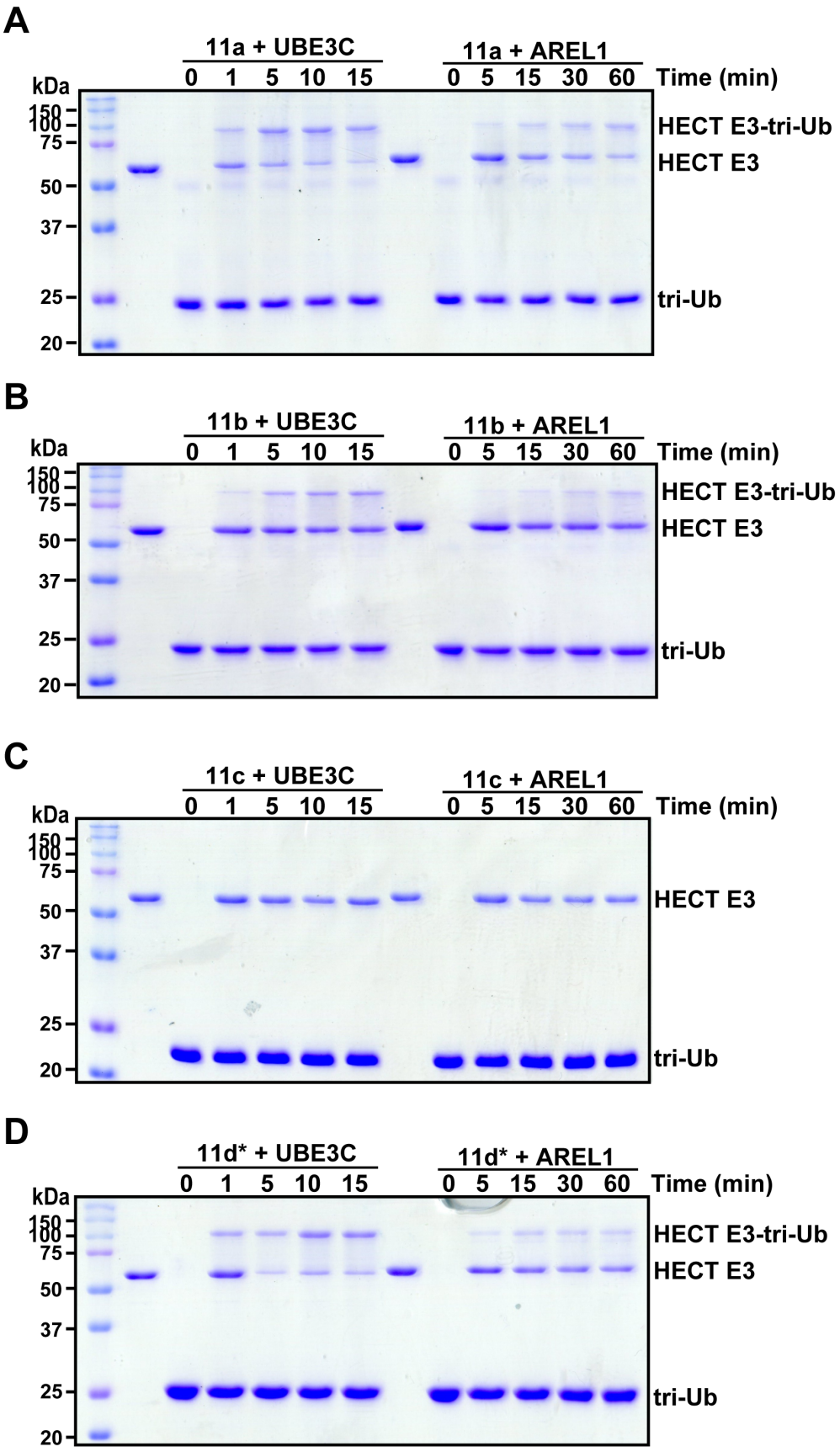

A
11a + UBE3C
11a + AREL1
0 1 5 10 15
0 5 15 30 60
Time (min)
kDa
150
100
75
50
37
25
20
HECT E3-tri-Ub
HECT E3
tri-Ub
B
11b + UBE3C
11b + AREL1
0 1 5 10 15
0 5 15 30 60
Time (min)
kDa
150
100
75
50
37
25
20
HECT E3-tri-Ub
HECT E3
tri-Ub
C
11c + UBE3C
11c + AREL1
0 1 5 10 15
0 5 15 30 60
Time (min)
kDa
150
100
75
50
37
25
20
HECT E3
tri-Ub
D
11d* + UBE3C
11d* + AREL1
0 1 5 10 15
0 5 15 30 60
Time (min)
kDa
150
100
75
50
37
25
20
HECT E3-tri-Ub
HECT E3
tri-Ub

Figure S9. Time course assay for the labeling of HECT E3s with the different probes.

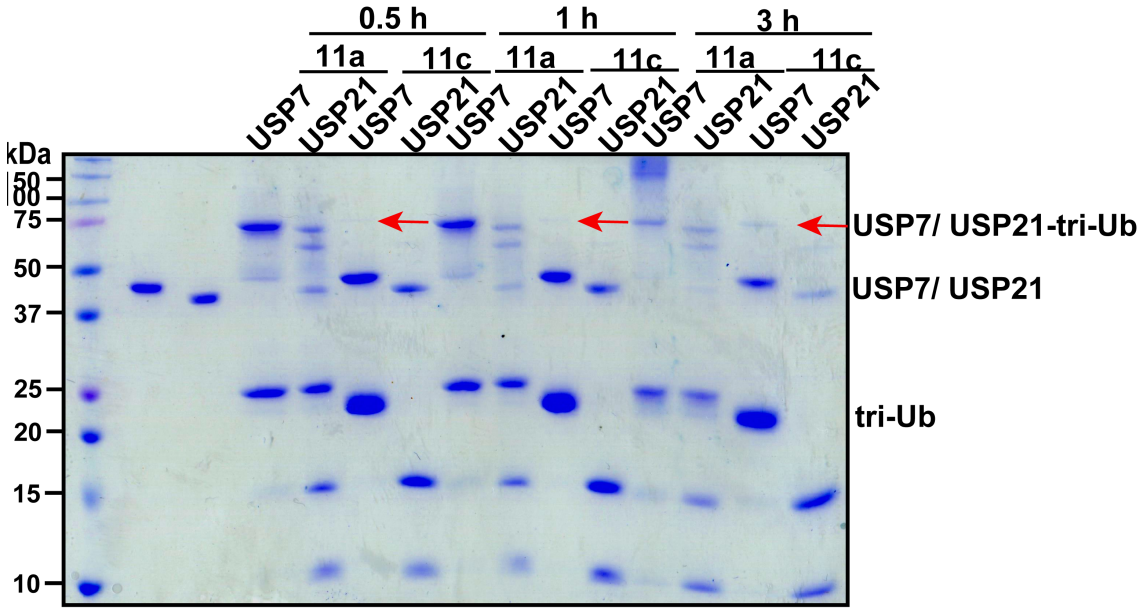


Figure S10. USP7, a DUB that cleaves many linkage types, reacts with probe **11c** (red arrow).

References

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(2) Beaudenon, S.; Huibregtse, J. M. *Methods Enzymol.* **2005**, *398* (*05*), 3–8.

(3) Hospenthal, M. K.; Freund, S. M. V.; Komander, D. *Nat. Struct. Mol. Biol.* **2013**, *20* (*5*), 555–565.

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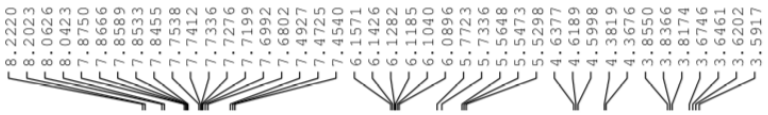
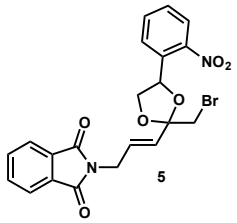
(7) Vedrenne, E.; Dupont, H.; Oualef, S.; Elkaïm, L.; Grimaud, L. *Synlett* **2005**, No. 4, 670–672.

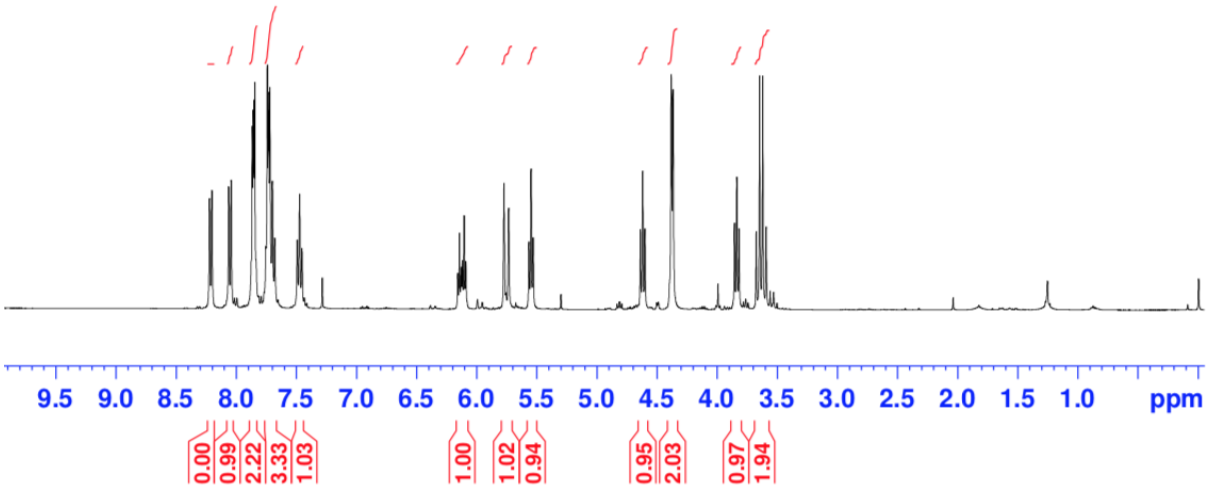
(8) Li, G.; Liang, Q.; Gong, P.; Tencer, A. H.; Zhuang, Z. *Chem. Commun.* **2014**, *50* (*2*), 216–218.

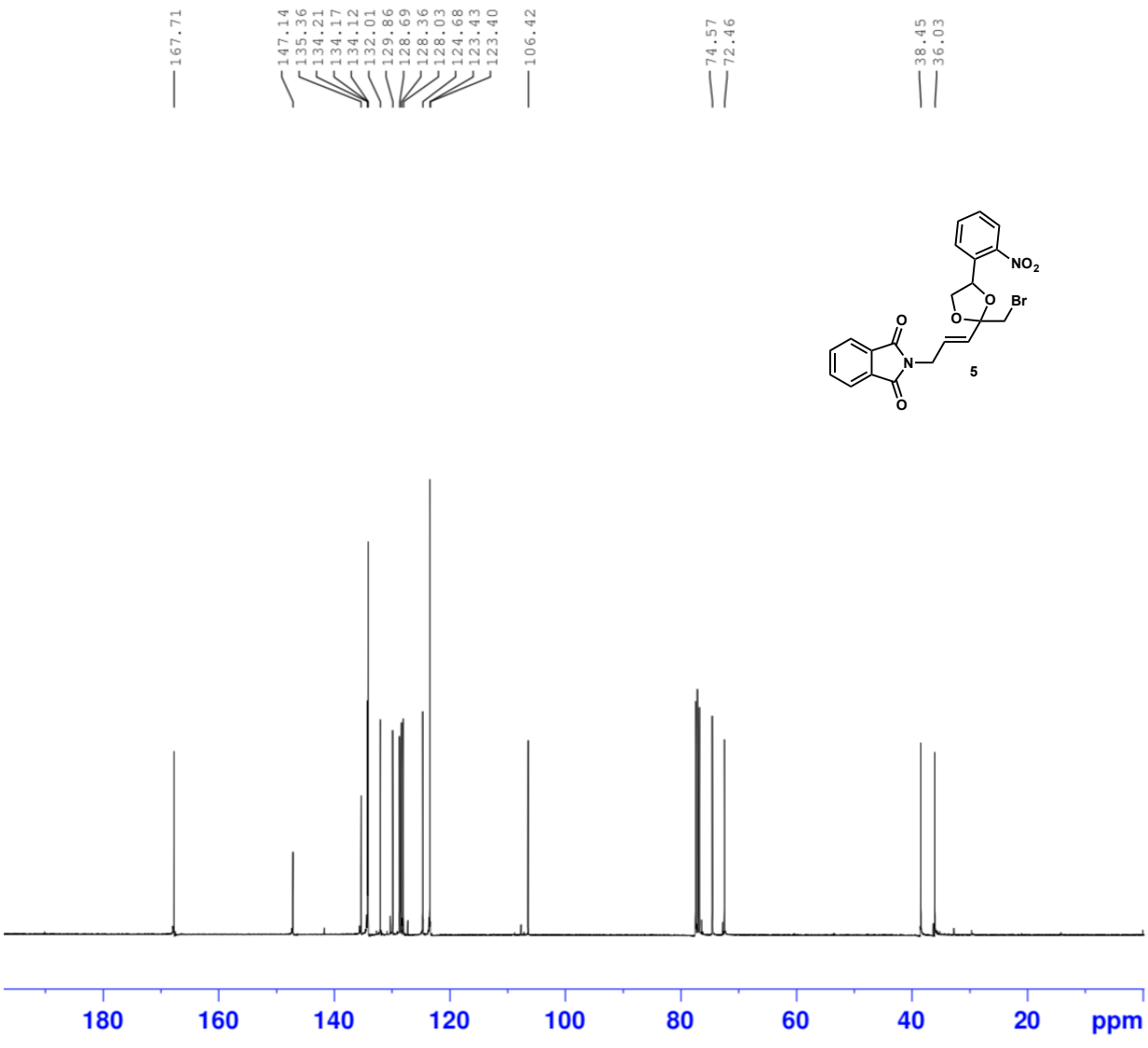
Copies of 1H and 13C-NMR spectra of

all the new compounds reported in this study





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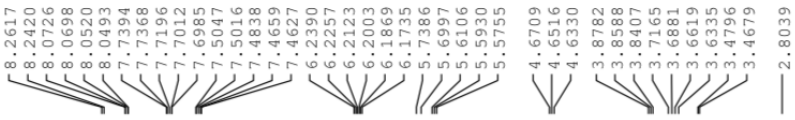
147.14
135.36
134.21
134.17
134.12
132.01
129.86
128.69
128.36
128.03
124.68
123.43
123.40
106.42
74.57
72.46
38.45
36.03
NO2
Br
O
O
O
N
O
5
180
160
140
120
100
80
60
40
20
ppm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8.2420
8.0726
8.0698
8.0520
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7.7394
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7.7196
7.7012
7.6985
7.5047
7.5016
7.4838
7.4659
7.4627
6.2390
6.2257
6.2123
6.2003
6.1869
6.1735
5.7386
5.6997
5.6106
5.5930
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4.6709
4.6516
4.6330
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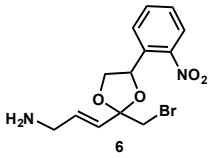
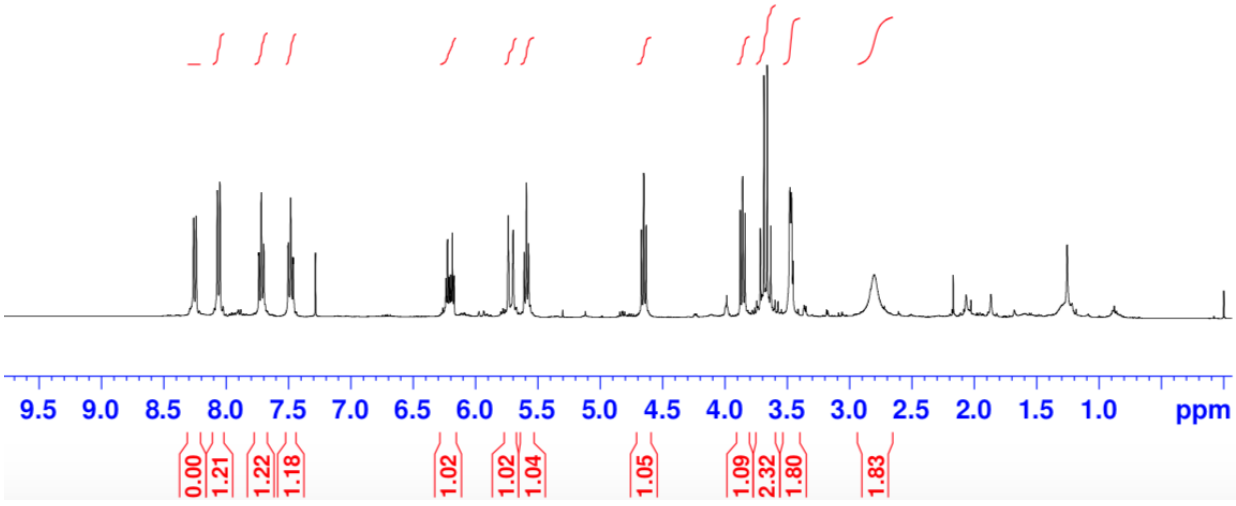
3.4679
2.8039


NO2
O
O
Br
H2N

6


9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 ppm
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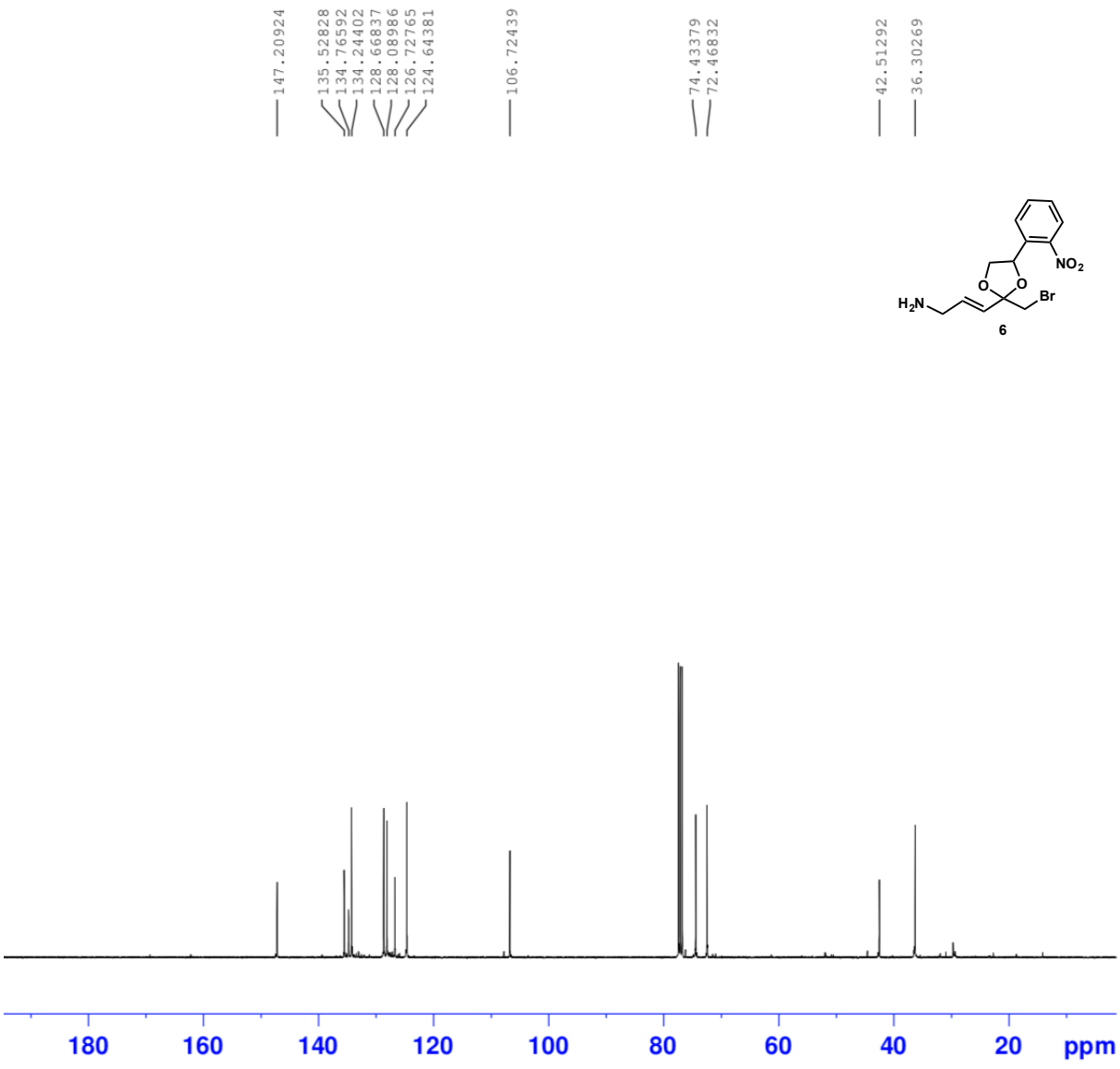
1.04
1.05
1.09
2.32
1.80
1.83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126.72765
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106.72439
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72.46832
42.51292
36.30269
NO2
O
O
Br
H2N
6
180
160
140
120
100
80
60
40
20
ppm