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Supporting Information

A Pro-Fluorescent Ubiquitin-Based Probe to Monitor Cysteine-Based E3 Ligase Activity

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Supporting Information for

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In memory of Professor Huib Ovaa, his passion for science will always be an inspiration to us.

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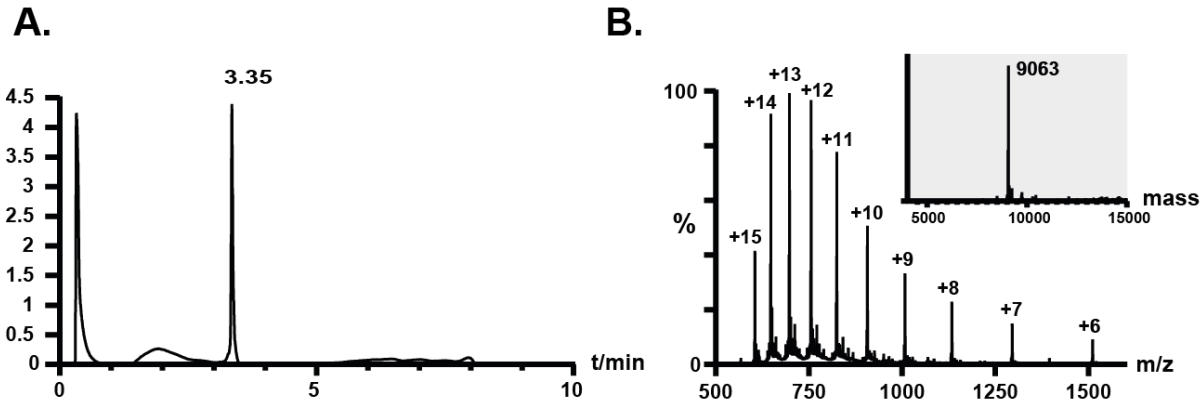
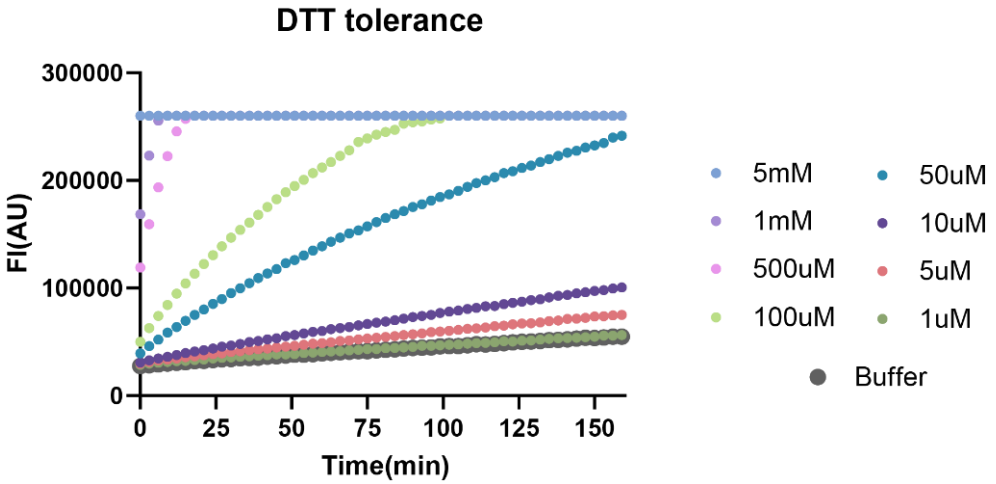
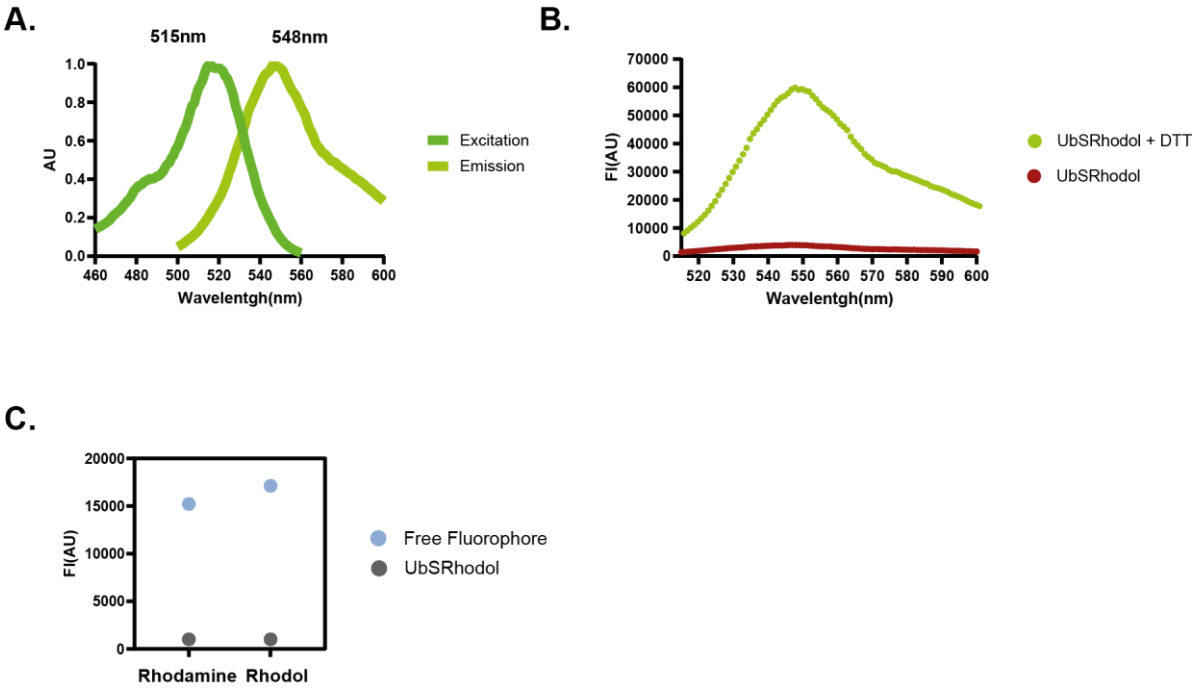


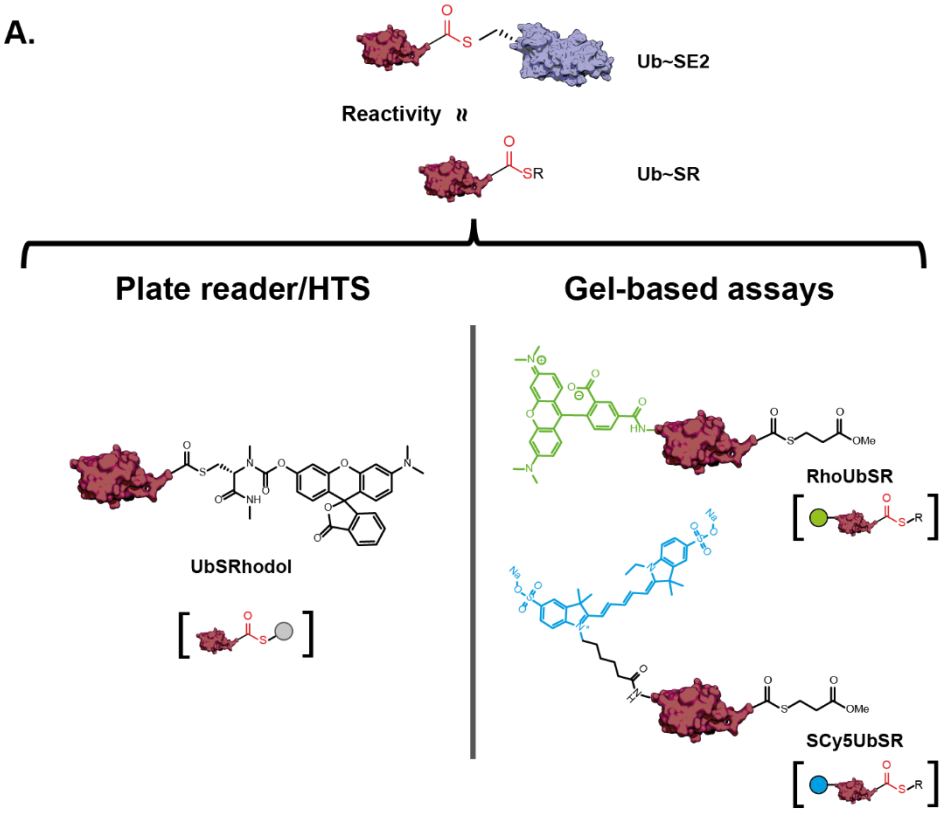
Supporting Information Figure 1. LC-MS characterization of UbSRhodol. A) Liquid chromatography trace (3.35min, 10^7 scale) and **B)** Mass charge (m/z) envelope and deconvoluted mass spectra (ESI MS+ (amu) calcd: 9063, found 9063 (deconv.)).



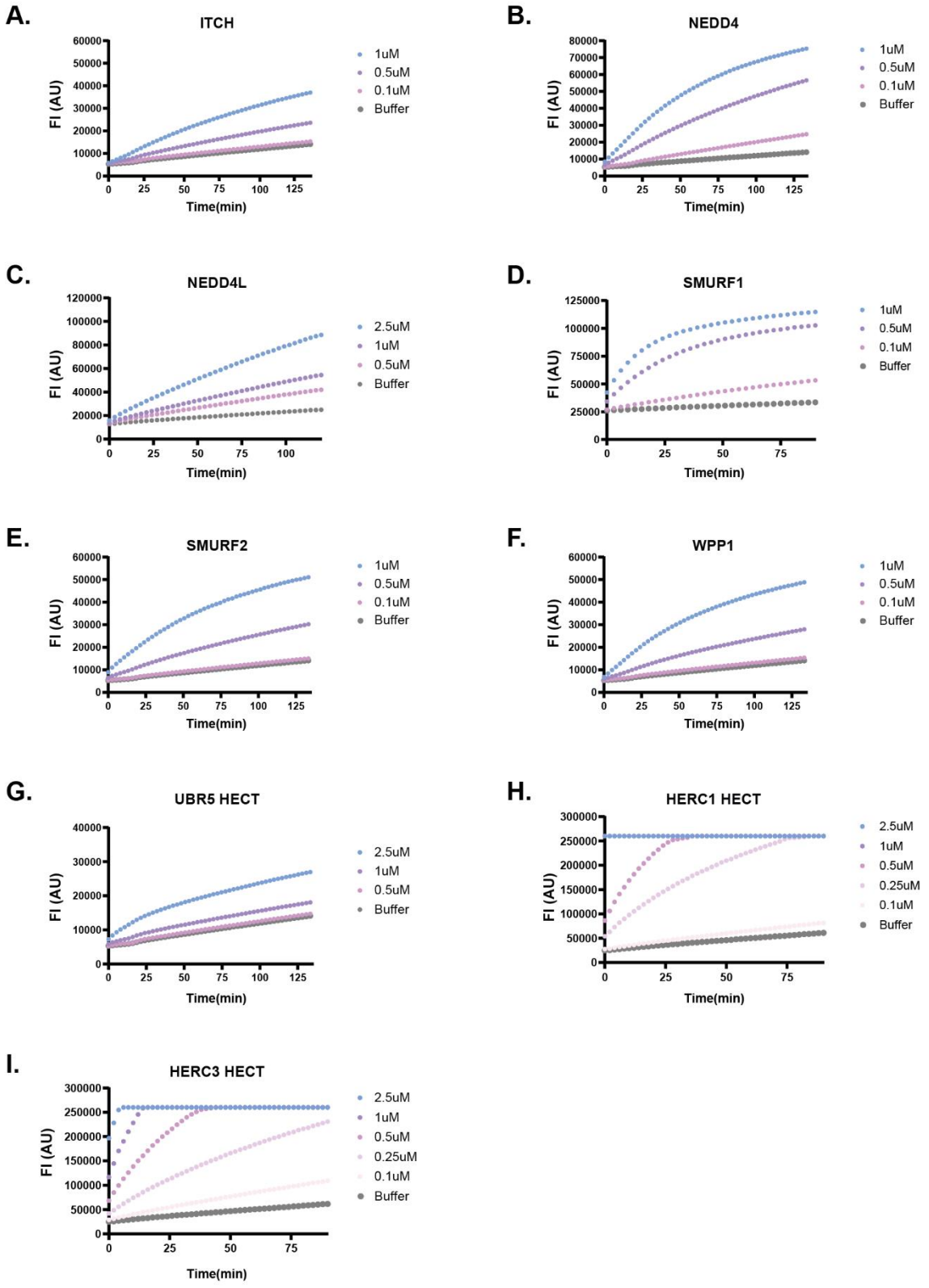
Supporting Information Figure 2. DTT titration UbSRhodol. Incubation of UbSRhodol with the reducing agent DTT (5-0.001 mM) shows hydrolysis of the thioester of the probe over time.



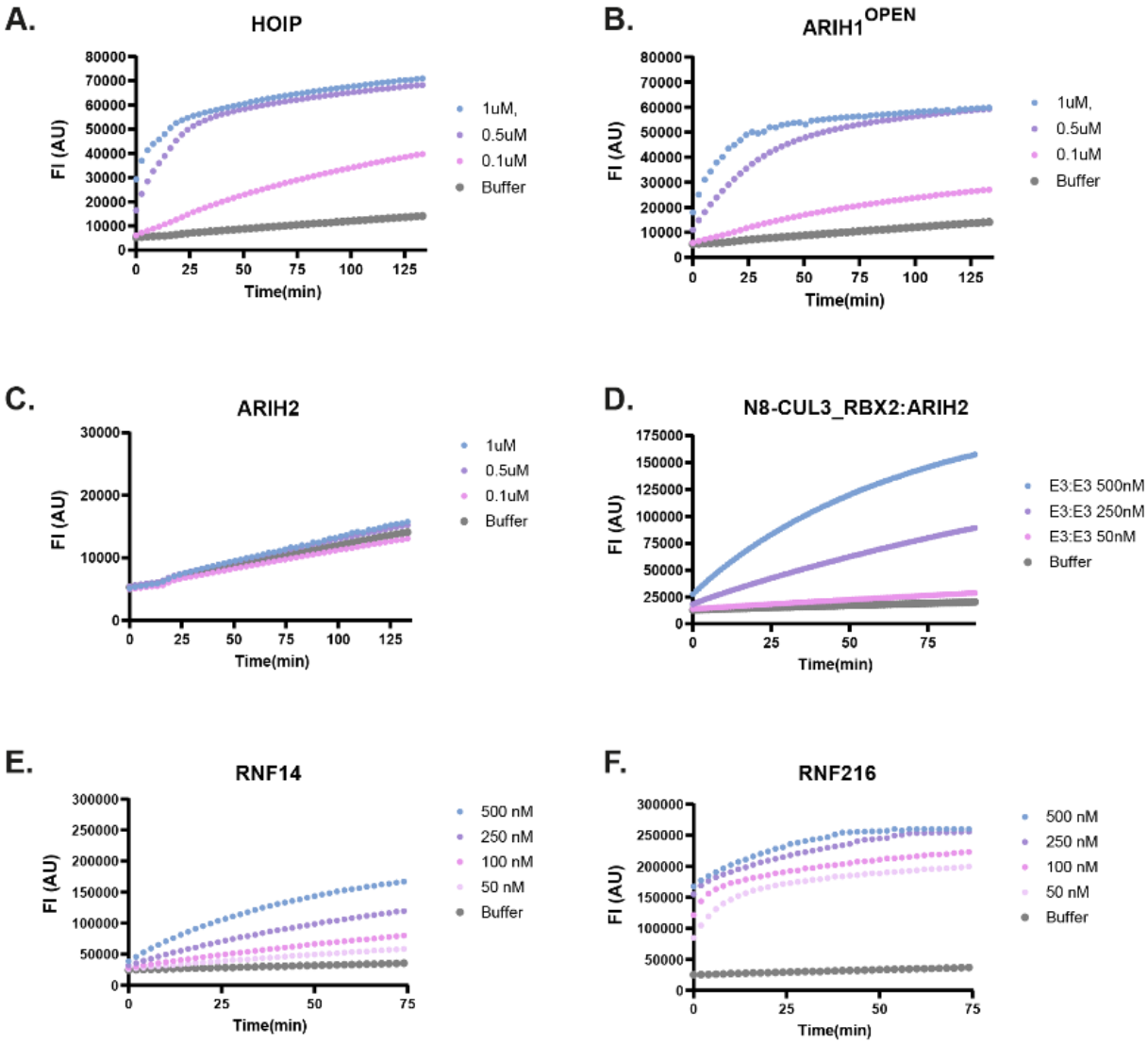
Supporting Information Figure 3. Spectroscopic characterization of Rhodol and UbSRhodol upon DTT transthiolation. A) Spectral scan of excitation and emission of the Rhodol dye **B**) Fluorescence intensity (FI) increase of 16 fold after DTT (5Mm) processing of UbSRhodol **C**) FI increase of UbSRhodol after DTT processing at Rhodamine and Rhodol channel (Rhodamine ($\lambda_{ex/em}$): 490/520nm; Rhodol($\lambda_{ex/em}$): 515/548nm). FI fold increase is comparable for both wavelengths (17/15.2 FI fold, Rhodol/Rhodamine).



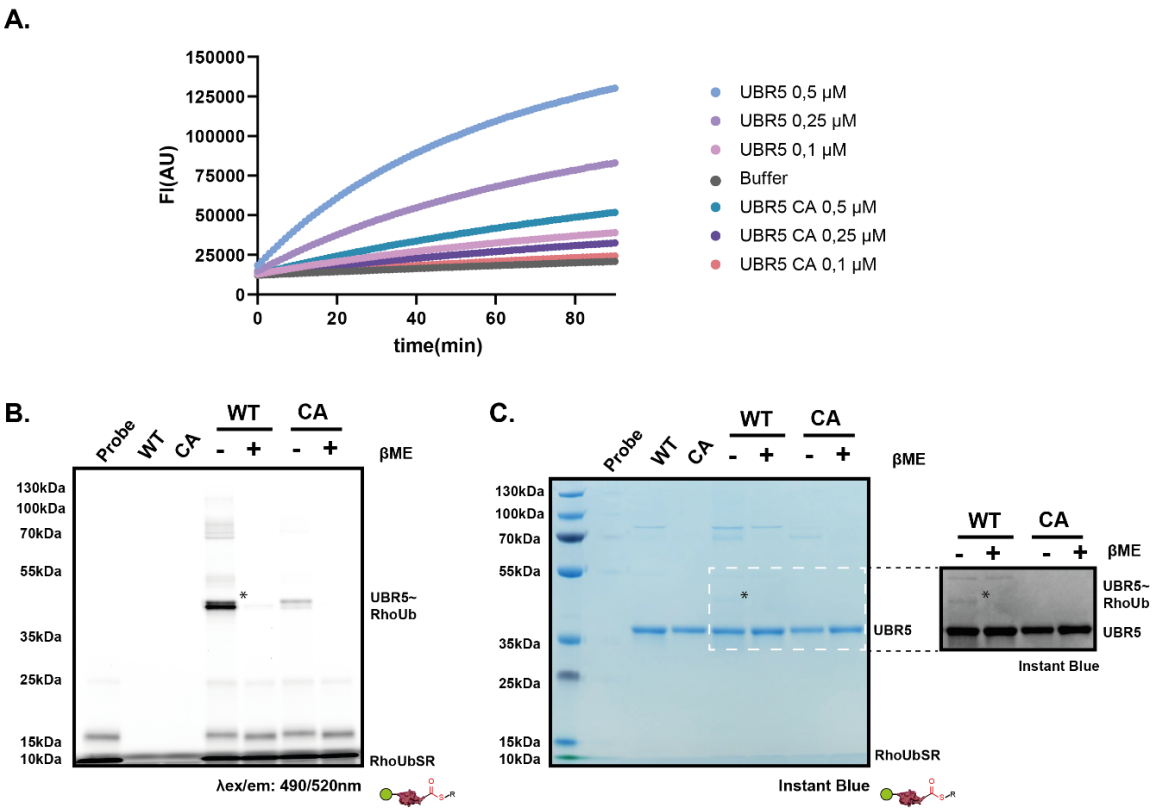
Supporting Information Figure 4. Bypass probes for plate reader versus gel-based assays UbSRhodol facilitates plate reader based assays, whereas N-terminal labeled fluorophore based probes (RhoUbSR and SCy5UbSR) will ease gel-based analysis.



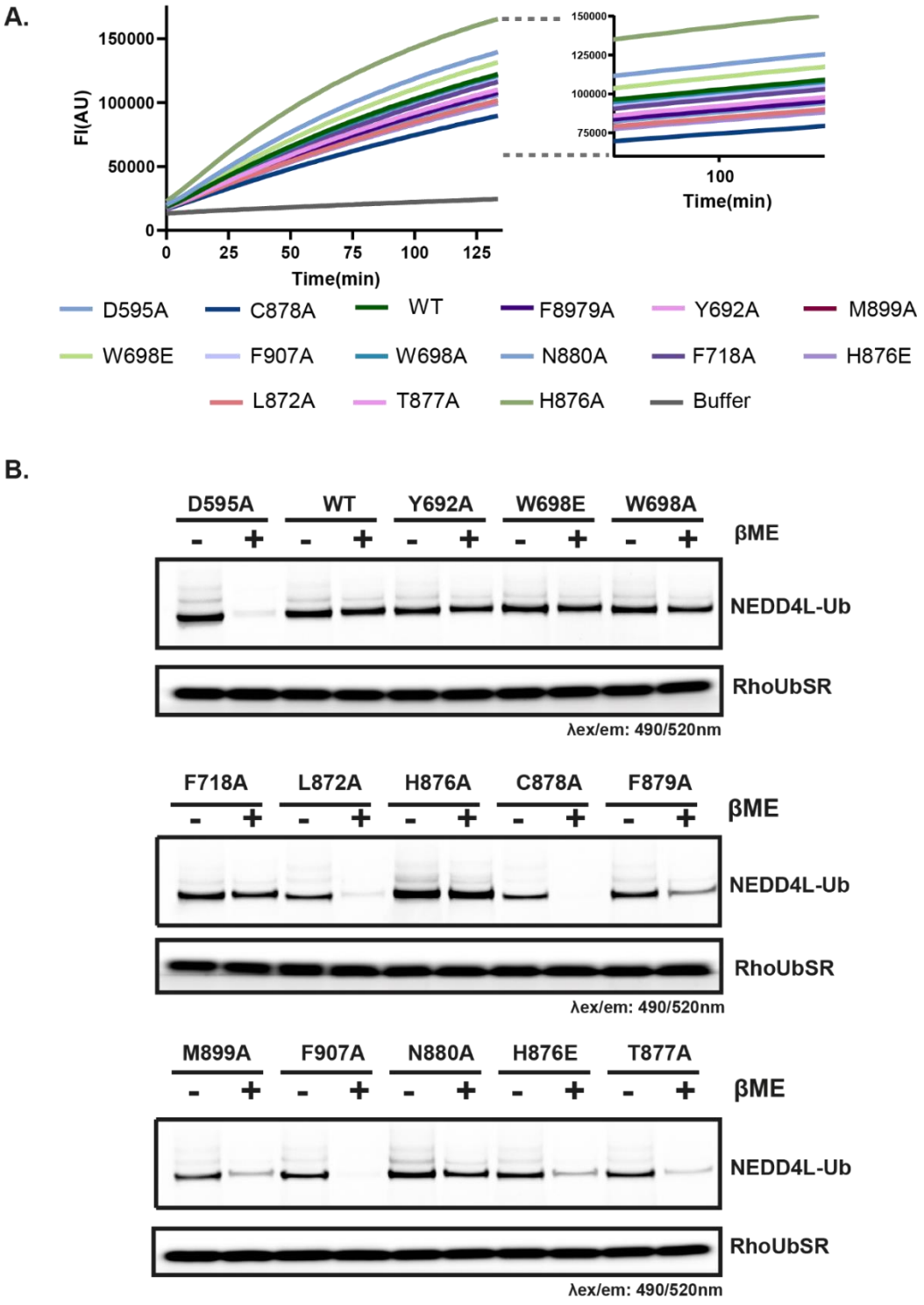
Supporting Information Figure 5. Transthiolation profile of HECT E3 ligases panel profiled by UbSRhodol. HECT NEDD4 family ligases: **A.** ITCH; **B.** NEDD4; **C.** NEDD4L; **D.** SMURF1; **E.** SMURF2 and **F.** WPP1. Other HECTs: **G**.: UBR5 (HECT domain only) and HERC family ligases: **H.** HERC1 and **I.** HERC3 (HECT domain only)



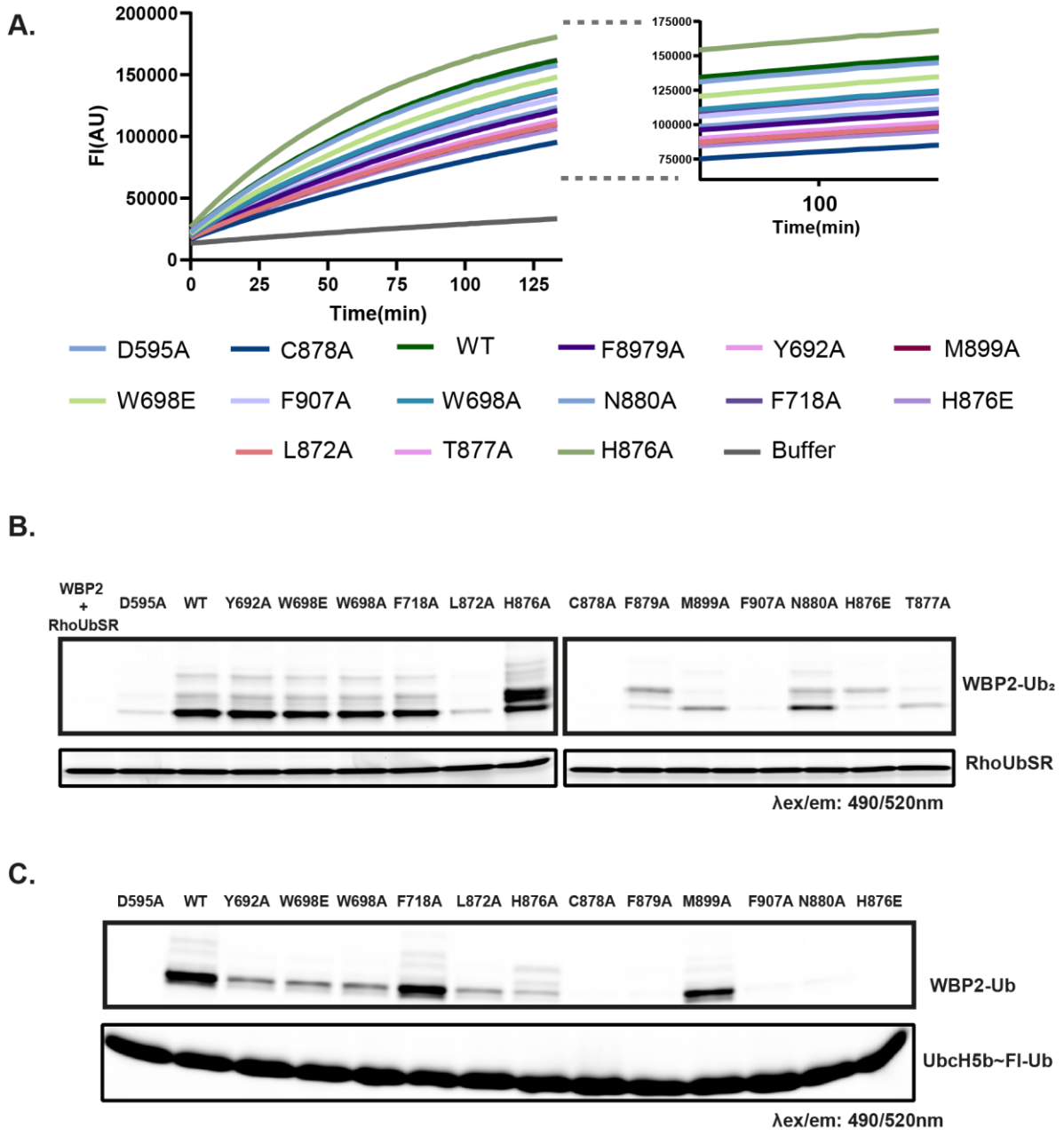
Supporting Information Figure 6. Transthiolation profile of RBR E3 ligases panel profiled by. UbSRhodol. A. HOIP; **B.** ARIH1^{OPEN}; **C.** ARIH2 (not active) versus **D.** ARIH2 in a E3:E3 super assembly with Neddylated CUL3-RBX2 (active); **E.** RNF14; **F.** RNF216; were profiled.



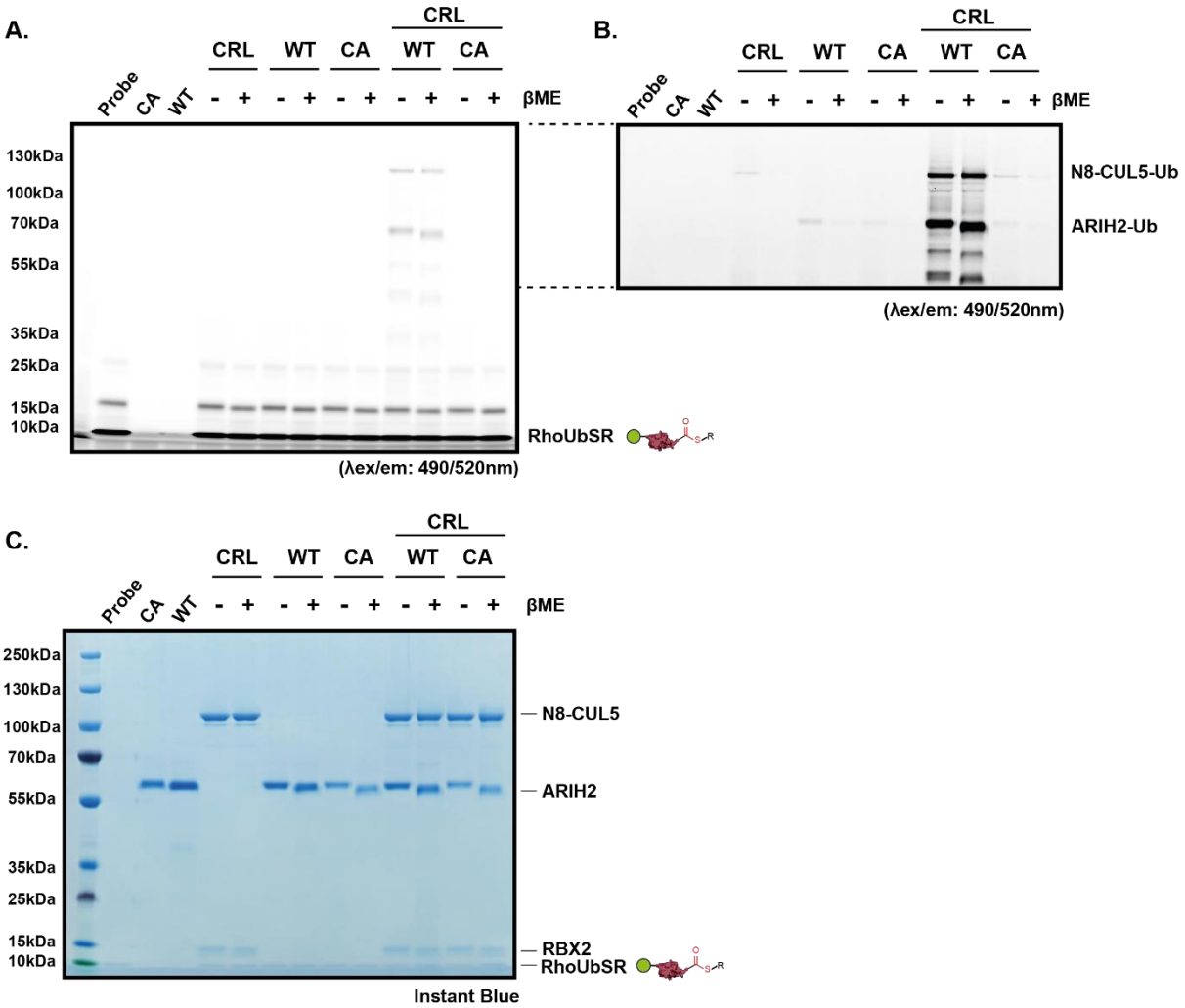
Supporting Information Figure 7. UBR5 HECT dependent transthiolation and product formation with bypass probes. A) Transthiolation graph of UBR5 HECT domain wild type (WT) and catalytic cysteine 2768 mutated to alanine (CA) mutant using UbSRhodol (500nM). **B**) Alternatively, RhoUbSR harboring a fluorophore on its N-terminus, was used to evaluate product formation by gel-based assays. Samples were untreated or boiled under the presence of βME to assess thioester formation (marked with asterisk, *). C) Instant blue stain of gel and higher exposure of the square area to observe minimal thioester formation.



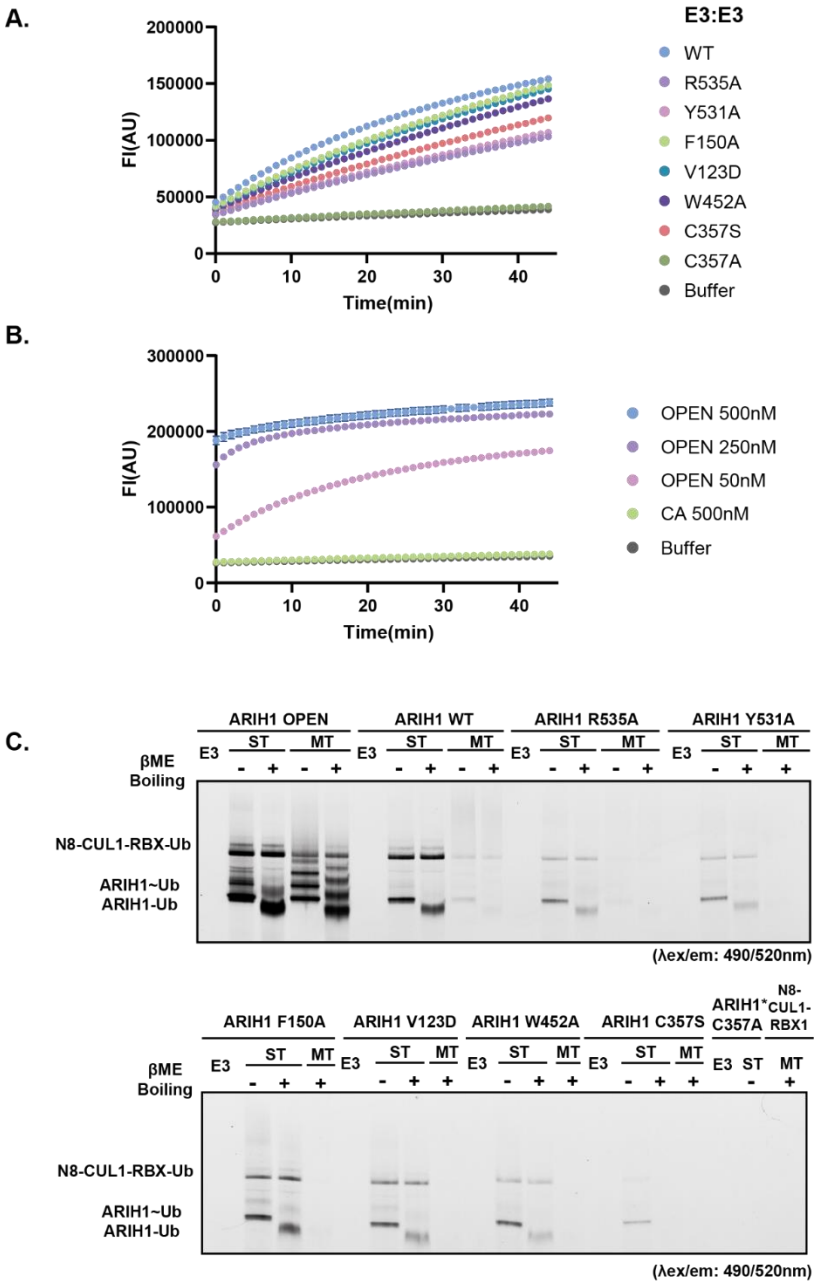
Supporting Information Figure 8. Transthiolation profile of (ΔC2)NEDD4L isoform 2 mutants with UbSRhodol under ST conditions (2.5 µm E3 vs 500 nM UbSRhodol). A) (ΔC2)NEDD4L mutant-dependent fluorescence intensity build-up over time with UbSRhodol under ST conditions (2.5 µM E3 vs 500 nM UbSRhodol). Zoom in around 100 minutes time point used for the standardization of the mutants WT(100%) and C878A(0%). **B)** RhoUbSR was used under the same conditions to evaluate thioester and product formation by gel-based assays measuring at the rhodamine channel. Samples were untreated or boiled in the presence of βME to assess thioester formation. (See Supporting Information Fig. 30 for full gel).



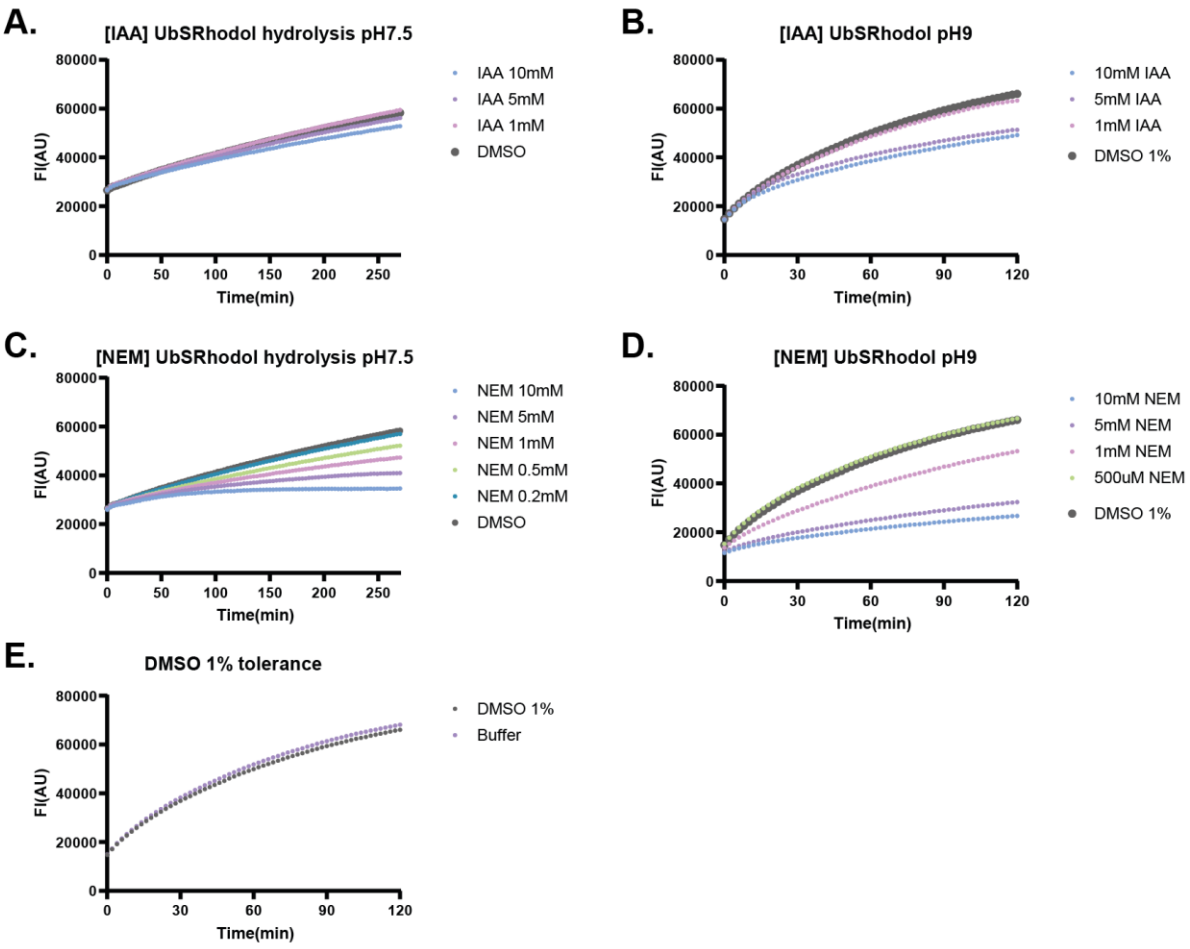
Supporting Information Figure 9. WBP2 ubiquitination profile of all (ΔC2)NEDD4L isoform 2 mutants with UbSRhodol under ST conditions (2.5μM E3 vs 500nM UbSRhodol) and native ubiquitination cascade. A.) (ΔC2)NEDD4L mutant-dependent fluorescence intensity build-up over time with UbSRhodol under ST conditions (2.5 μM E3, 500 nM WBP2 vs 500 nM UbSRhodol). Zoom in around 100 minutes time point used for the standardization of the mutants WT(100%) and C878A(0%). B.) RhoUbSR was used under the same conditions to evaluate WBP2 ubiquitination by gel-based assays measuring at the rhodamine channel. Samples were βME treated and boiled. C.)Pulse-chase assay of Ubch7 loaded fluorescein ubiquitin as thioester (Ubch5b~Fl-Ub, ~ for thioester) (ΔC2)NEDD4L and ubiquitination of WBP2 with native cascade 0.5 μM UbcH5b~ubiquitin, 0.2 μM NEDD4L and 10 μM WBP2. Reactions were quenched after 5 minutes. (See Supporting Information Fig. 31 for full gel).



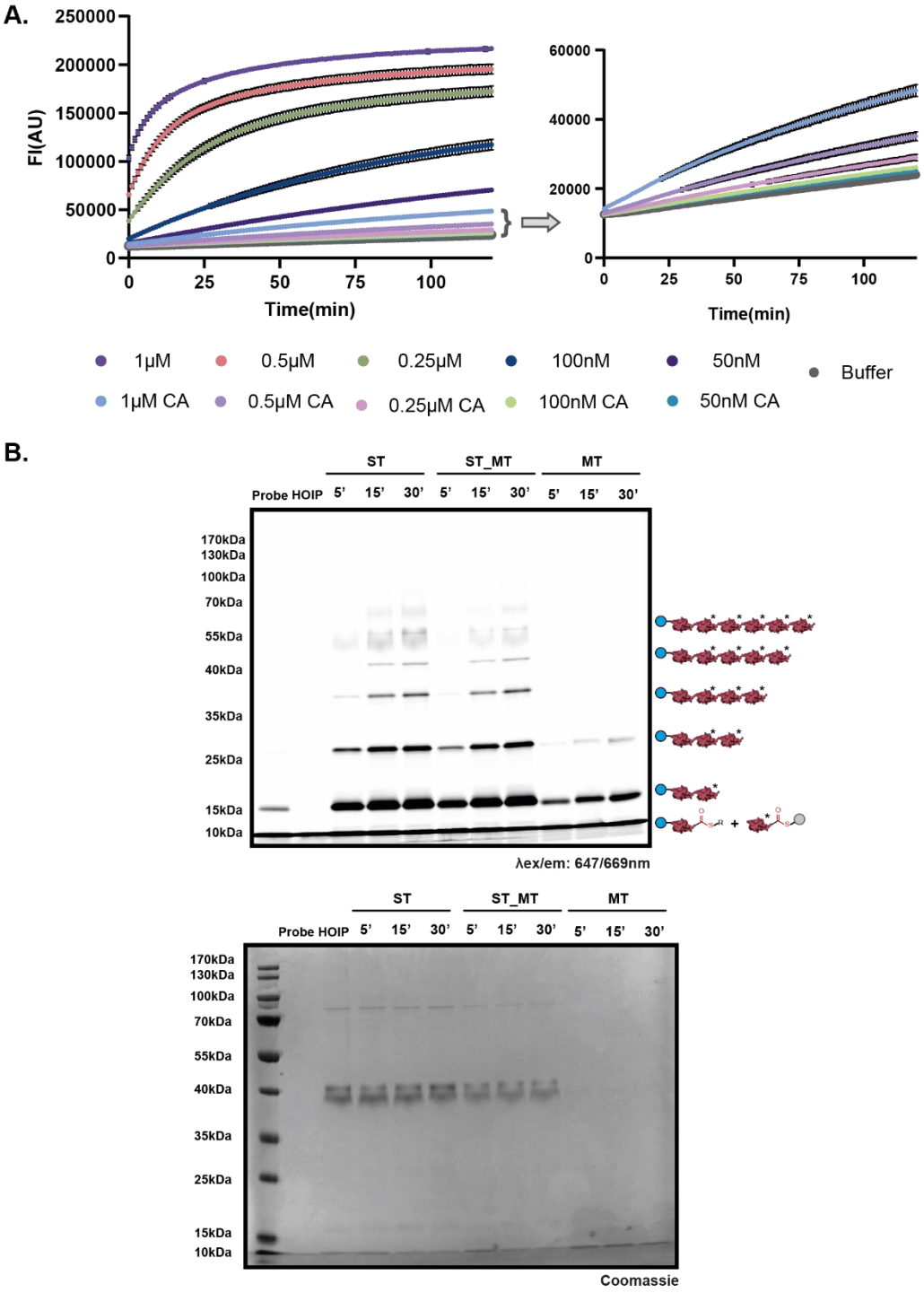
Supporting Information Figure 10. Gel-based analysis of ARIH2 E3:E3 super assembly formation. RhoUbSR was incubated at the same UbSRhodol conditions (500 nM of E3 or E3:E3 and 500 nM of probe) with ARIH2 and CA mutant both alone or in complex with another E3 CRL (N8-CUL5-RBX2). Samples were untreated or boiled in the presence of βME. A) Fluorescence scan, B) Higher exposed fluorescence scan and C) Instant Blue stain.



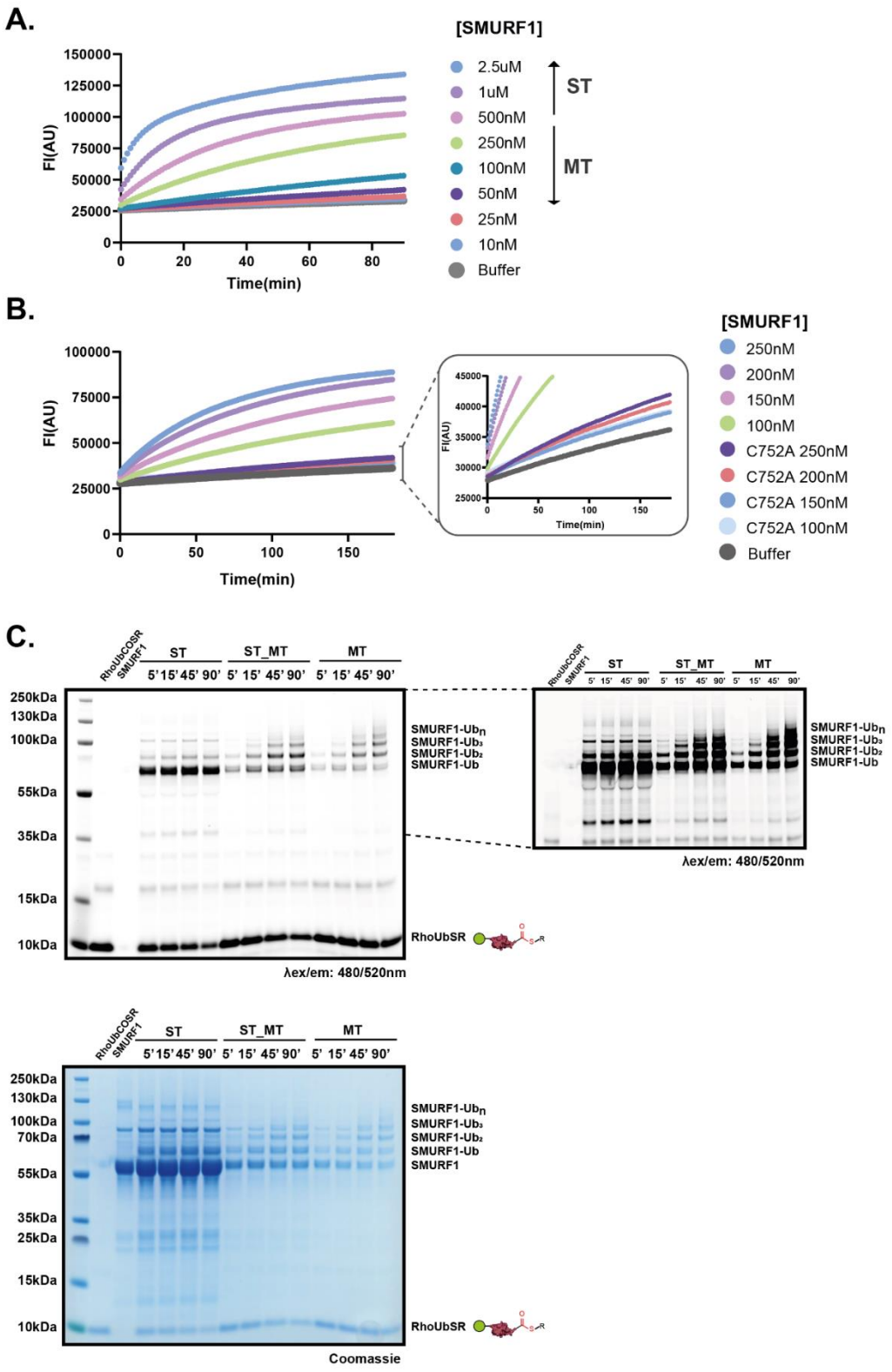
Supporting Information Figure 11. Profiling ARIH1 and mutants in E3:E3 superassembly formation. A) E3:E3 super-assembly transthiolation profile of defective ligation mutants of ARIH1 (500 nM) with UbSRhodol (500 nM) under Single Turnover conditions (ST). **B)** ARIH1^{OPEN} concentration dependent transthiolation profile with UbSRhodol (500 nM) **C)** RhoUbSR was incubated at the same UbSRhodol conditions (E3:E3 and probe 500nM) with N8-CUL1-RBX1:ARIH1 mutants to evaluate product formation by gel-based assays under ST (500nM E3, 1:1 E3 vs probe) or MT (50nM E3, 1:10 E3 vs probe) conditions. Hyperactivated ARIH1^{OPEN} showed product formation (autoubiquitination) under MT conditions while the other mutants showed diminished ligation activity as compared with ARIH1 WT.



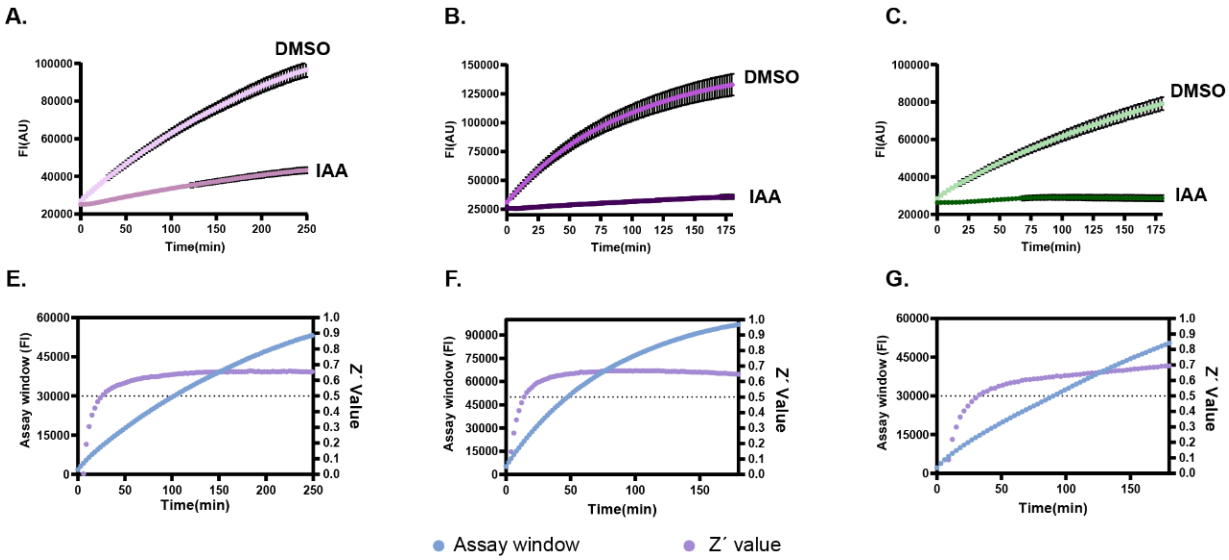
Supporting Information Figure 12. Examining effect of positive controls (IAA and NEM) and DMSO on intramolecular cyclization of UbSRhodol. **A**) IAA titration against UbSRhodol hydrolysis at pH7.5 or **B**) pH 9 **C**.) NEM titration against UbSRhodol hydrolysis at pH7.5 or **D**) pH 9 to select concentrations of positive control **E**) Effect of 1% DMSO on cyclisation.



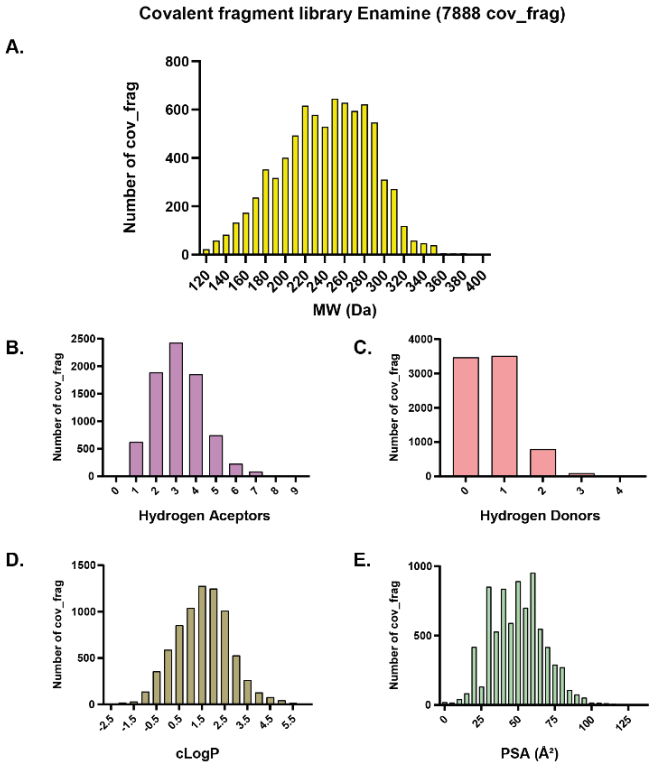
Supporting Information Figure 13. HOIP bypass system and product formation. A) Activity profile of HOIP using UbSRhodol (1µM). UbSRhodol transthiolation of HOIP is exclusive of catalytic cysteine 885 at low concentrations (0.5µM to 50nM) as observed with the C885A mutant. **B**) UbSRhodol (500nM) and SCy5UbSR (500nM) were incubated at different ST and MT conditions (ST: 1:1, ST_MT: 0.25 fold or MT 0.05 fold E3 over total probe (1 µM) to evaluate product formation by gel-based assays. Samples were collected at different timepoints and boiled under the presence of βME.A clear anchored linear ubiquitin chain build-up was observed over time. *Note:* Combination of bypass probes was required to observe chain building as SCy5UbSR has a blocked N-terminus.



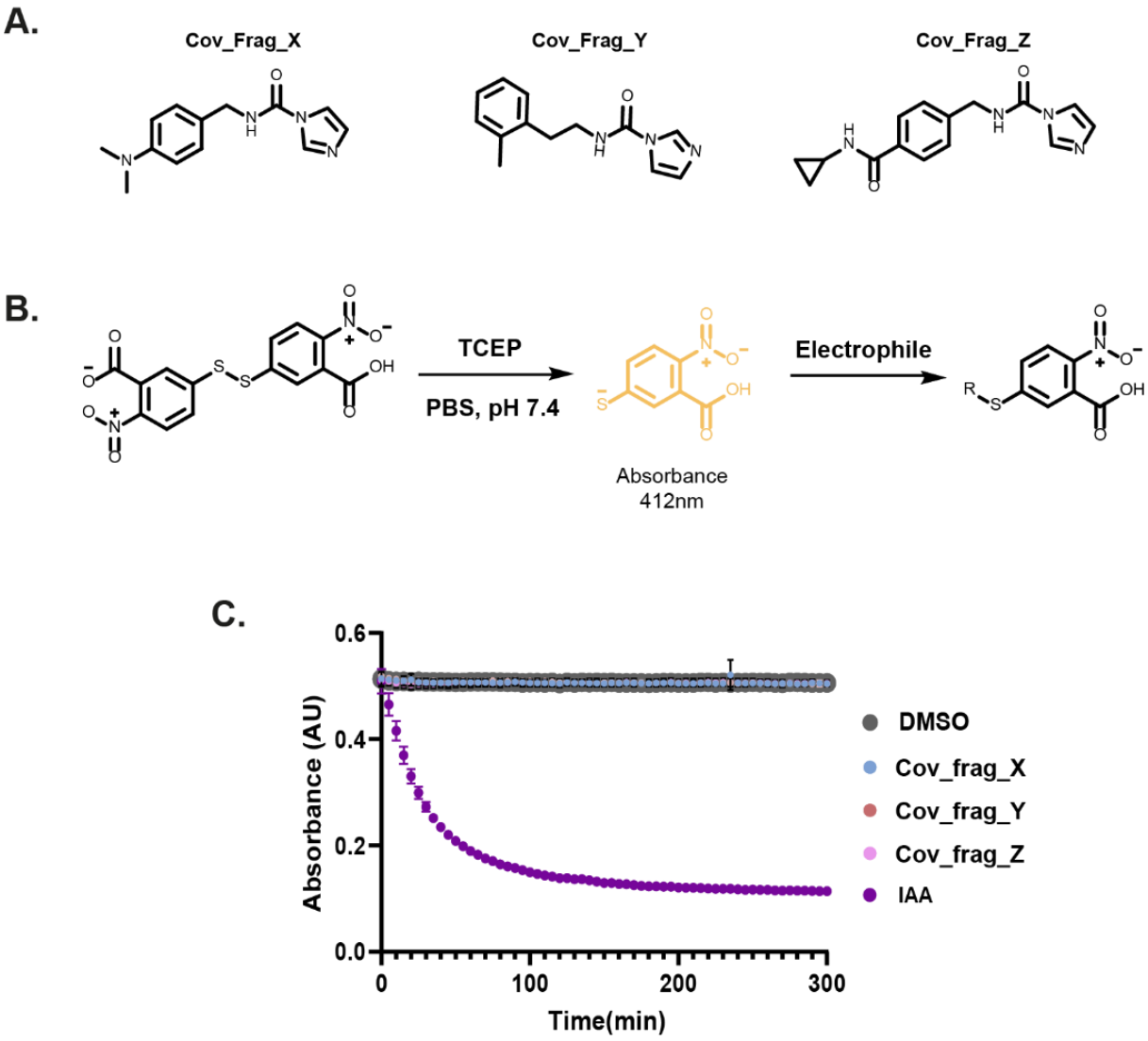
Supporting Information Figure 14. SMURF1 bypass system and product formation. A) Activity profile of (ΔC2)SMURF1 using UbSRhodol (500nM). **B**) UbSRhodol transthiolation (ΔC2)SMURF1 is exclusive of catalytic cysteine 752 as observed with the C752A mutant. **C**) RhoUbSR was incubated different ST and MT conditions (ST: 5 fold, ST_MT: 0.5 fold or MT 0.2 fold E3 over probe (500nM) to evaluate product formation by gel-based assays. Samples were collected at different timepoints and boiled under the presence of βME.



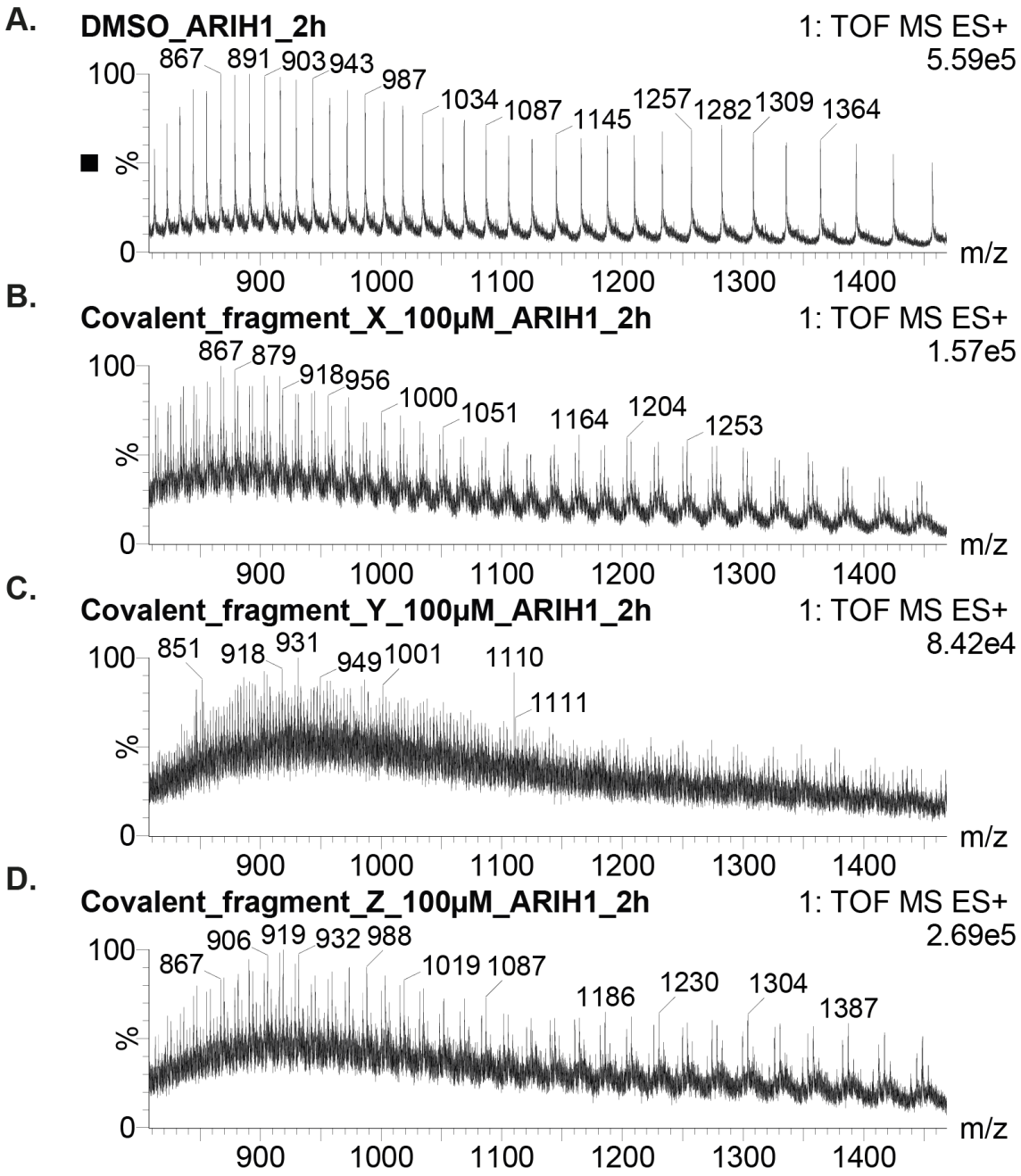
Supporting Information Figure 15. HTS descriptors for E3 ligase screening (1536-well plate). UbSRhodol E3 transthiolation profile using positive and negative controls (1mM IAA and 1% DMSO, n_{pos}=n_{neg}=128) of **A)** HOIP 25nM; **B)** ARIH1^{OPEN} 15nM; **C)** SMURF1 150nM. Z' values (purple) and assay window in (AU)(blue) relationship with time for **E)** HOIP; **F)** ARIH1^{OPEN} ; **G)** SMURF1.



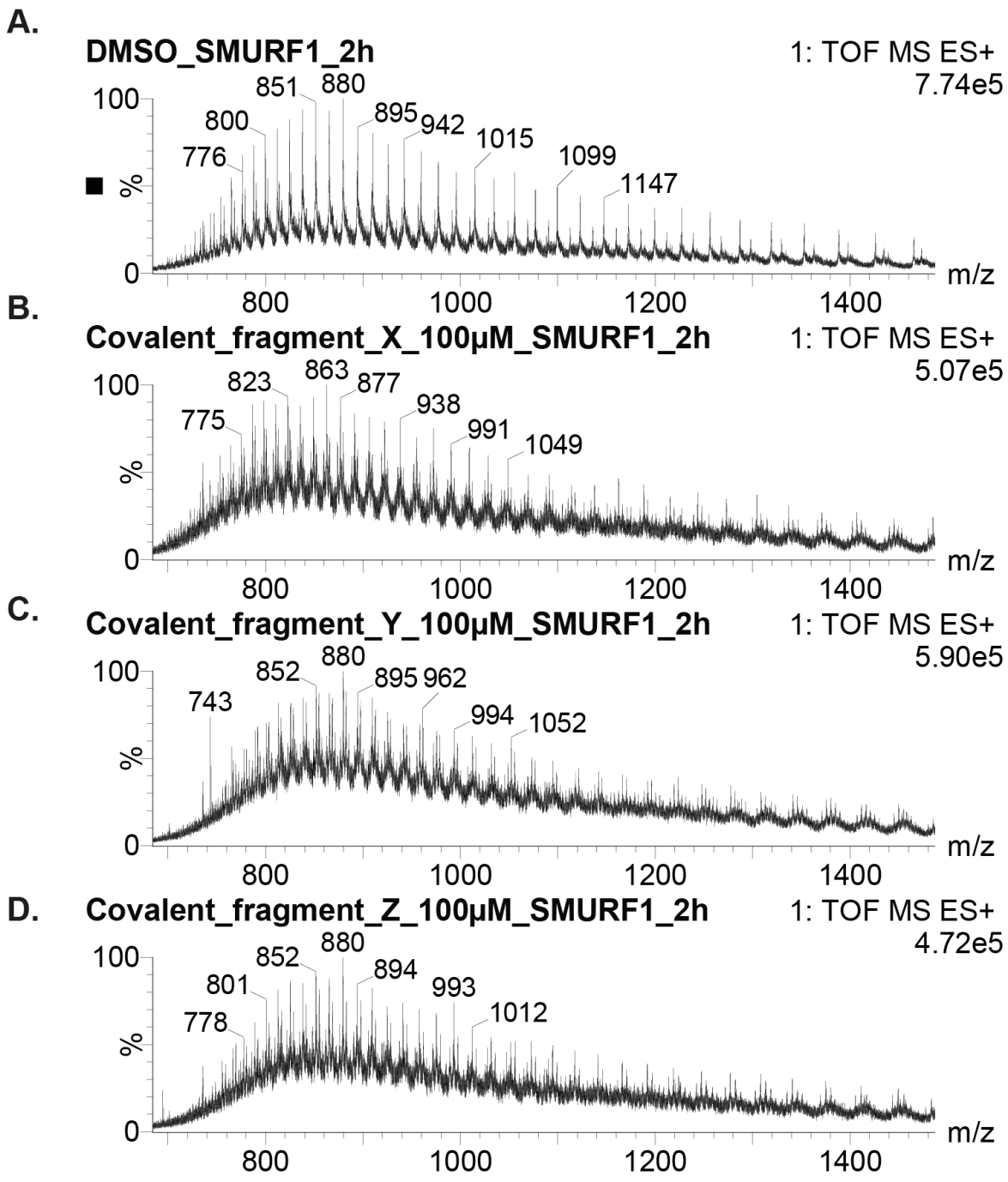
Supporting Information Figure 16. Enamine covalent library descriptors. A.) Distribution of molecular weight (Da) of covalent fragment library. **B.**) Distribution of hydrogen acceptors of covalent fragment library. **C.**) Distribution of hydrogen donors of covalent fragment library. **D.**) Distribution of partition coefficient (clogP) of covalent fragment library **E.**) Distribution of Polar surface area (PSA) of covalent fragment library. Descriptors were calculated using CHEMDRAW software 20.0.0.41 with the SMILE codes.



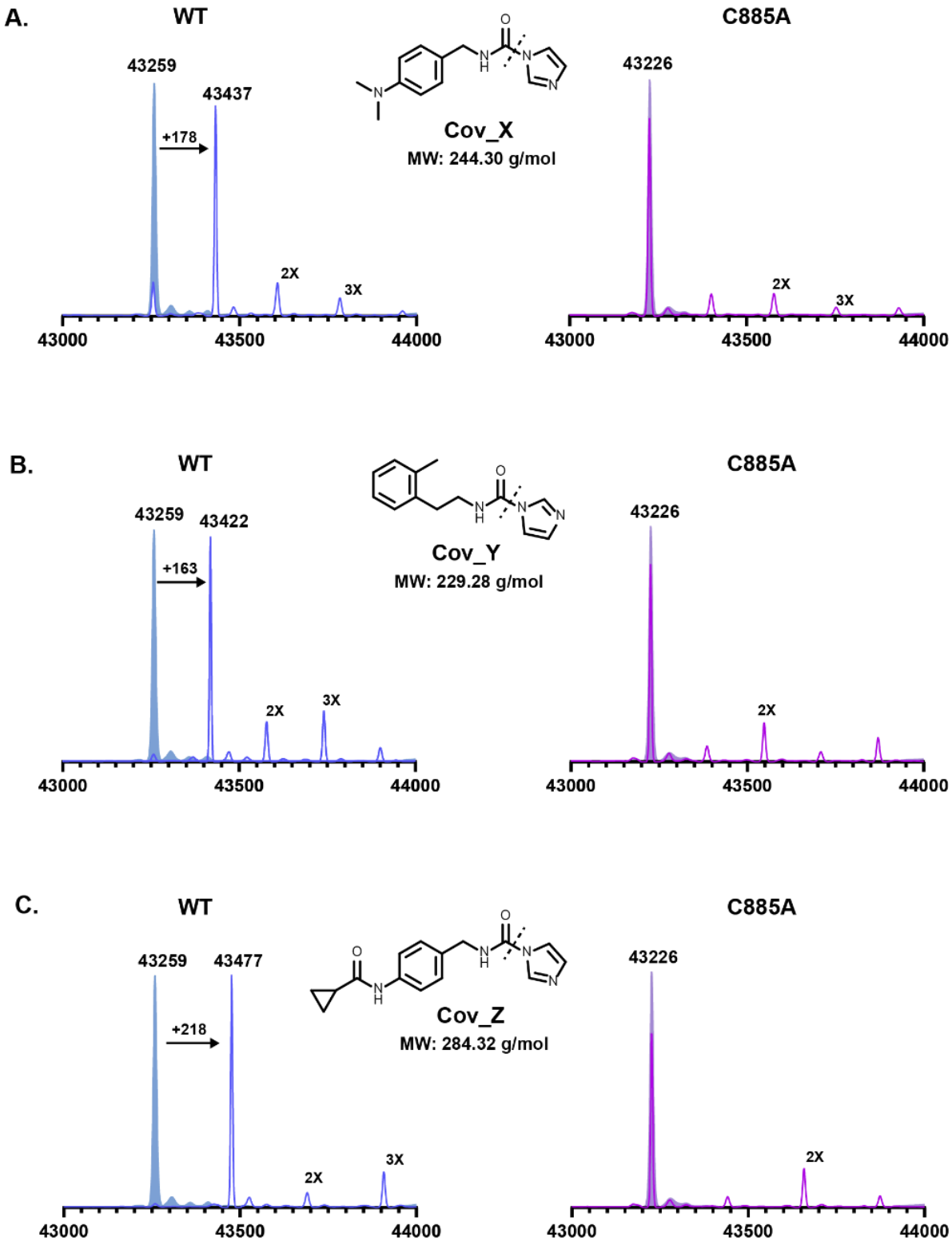
Supporting Information Figure 17. Elmann's reagent experiment to access intrinsic reactivity of carbonylimidazole serie fragment X,Y,Z. A) Chemical structures of fragments X,Y and Z. **B**) Scheme of Elmann's reactionMechanism of reactivity assay using Elmann's reagent. Absorbance at 412nM is measured overtime after adding an electrophile. If the electrophile is reactive it will attack the nucleophilic sulfur of the reagent, thereby reducing its absorbance **C**) Fragments X,Y and Z are incubated with the elmann's reagent and the reaction is measured overtime at 412nM.



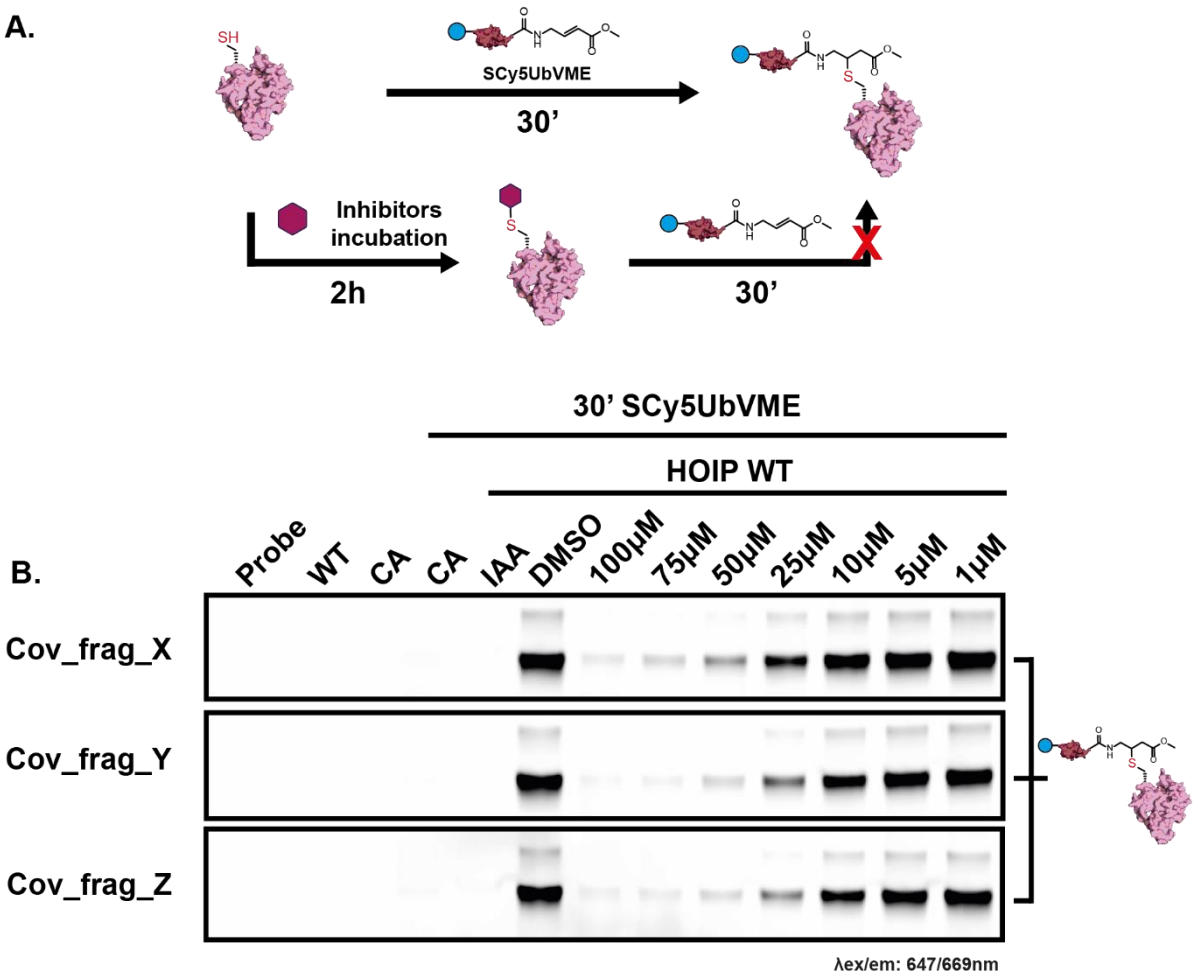
Supporting Information Figure 18. MS adduct formation experiments of fragments X,Y and Z with ARIH1. Mass charge envelopes of ARIH1 (1 µM) incubated for 2 hours at room temperature with 100µM of covalent fragment or DMSO. Multiple labelling of at least 4 molecules was observed. **A**) DMSO **B**) Covalent fragment X **C**) Covalent fragment Y and **D**) Covalent fragment Z



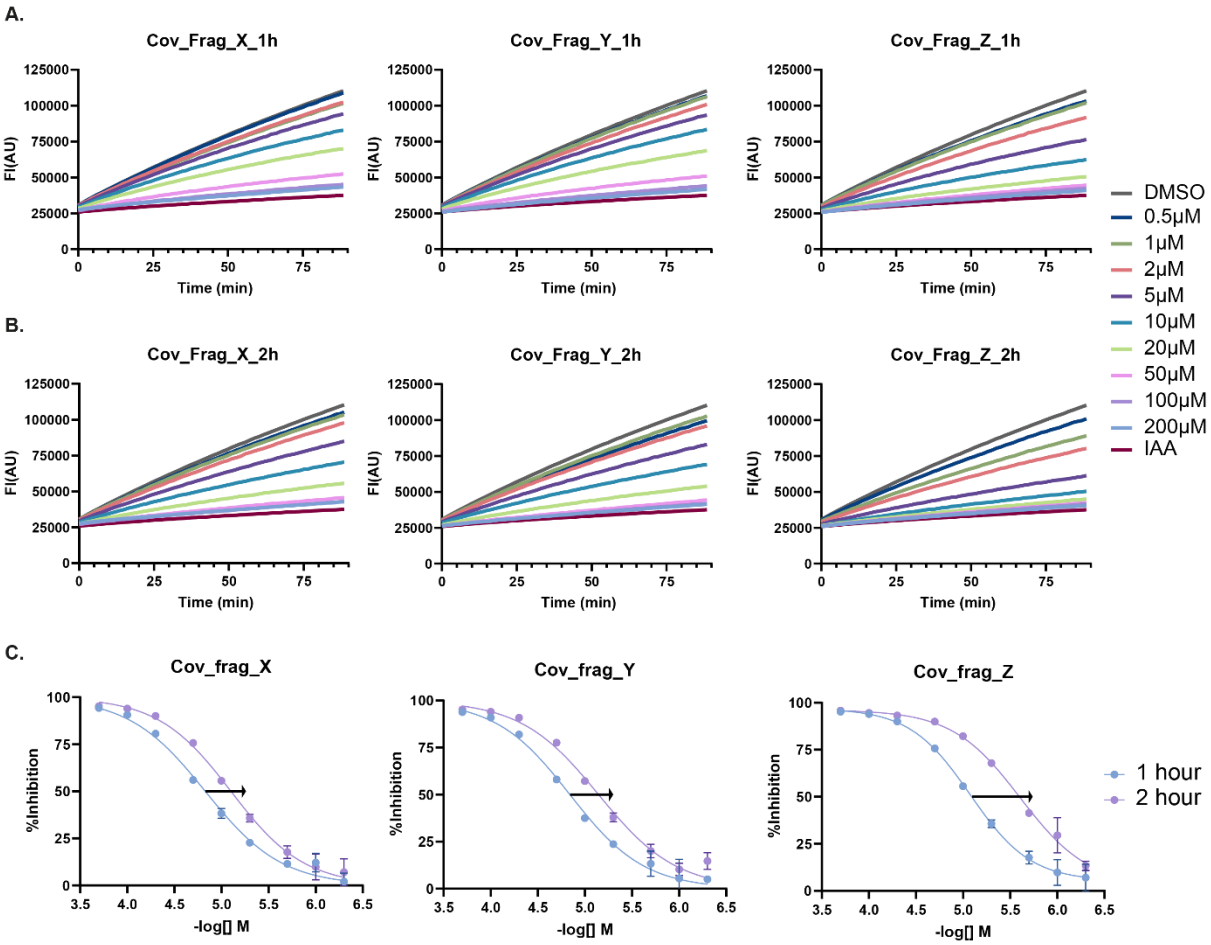
Supporting Information Figure 19. MS adduct formation experiments of fragments X,Y and Z with SMURF1. Mass charge envelopes of SMURF1 (1 µM) incubated for 2 hours at room temperature with 100µM of covalent fragment or DMSO. Multiple labelling of at least 4 molecules was observed. **A**) DMSO **B**) Covalent fragment X **C**) Covalent fragment Y and **D**) Covalent fragment Z



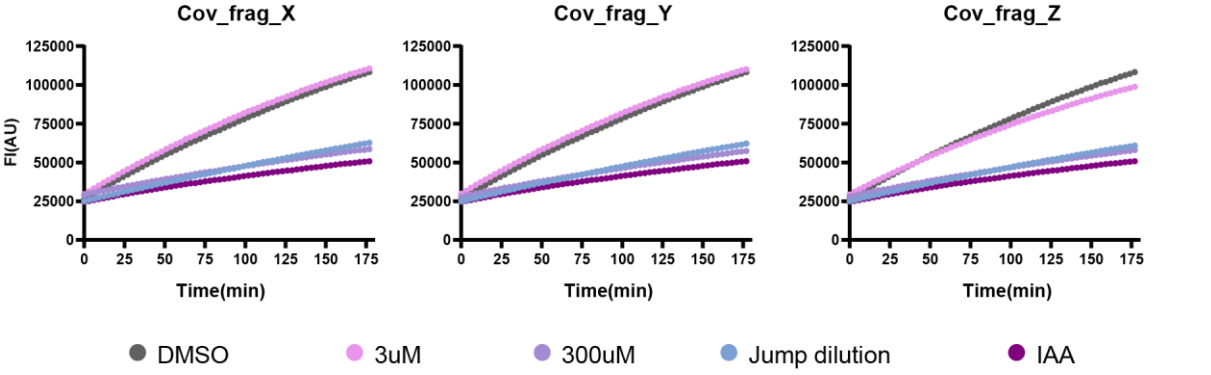
Supporting Information Figure 20. MS adduct formation experiments of series of fragments X,Y and Z against HOIP and catalytically dead HOIP C885A. Deconvoluted mass of HOIP and HOIP C885A (1 µM) incubated for 24 hours at 4°C with 100 µM of covalent fragment.



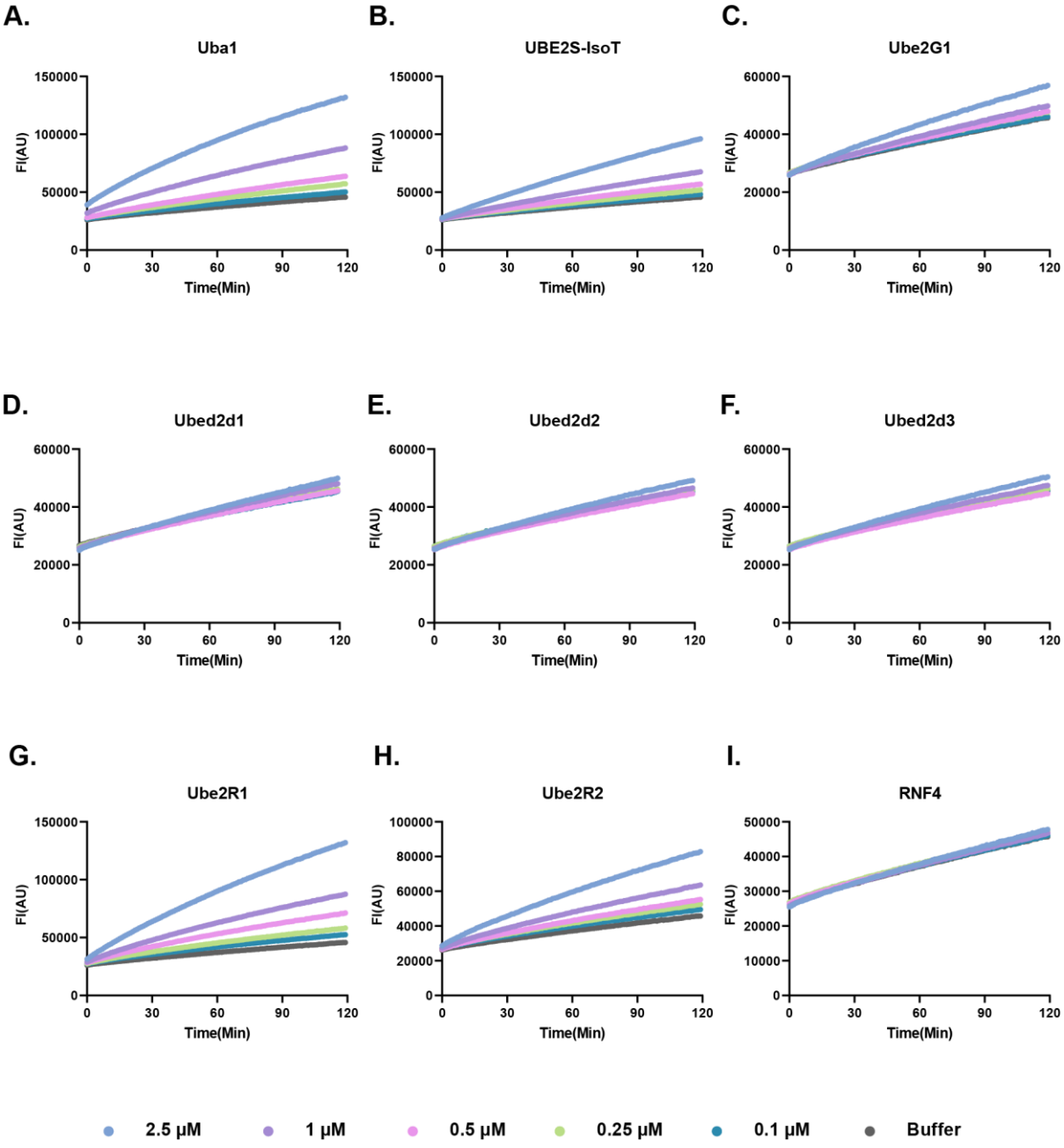
Supporting Information Figure 21. HOIP competition experiment with SCy5UbVME of fragments X,Y and Z. A) Schematic of competition assay. The catalytic cysteine of HOIP will be labelled by the suicide probe SCy5UbVME unless any small molecule impedes its labelling (purple hexagon) by blocking the active site of HOIP. **B**.) Concentration range of covalent fragments X,Y and Z in competition with SCy5UbVME (10µM) to label HOIP (1µM). HOIP C885A mutant and IAA (1 mM) are taken along as negative controls.



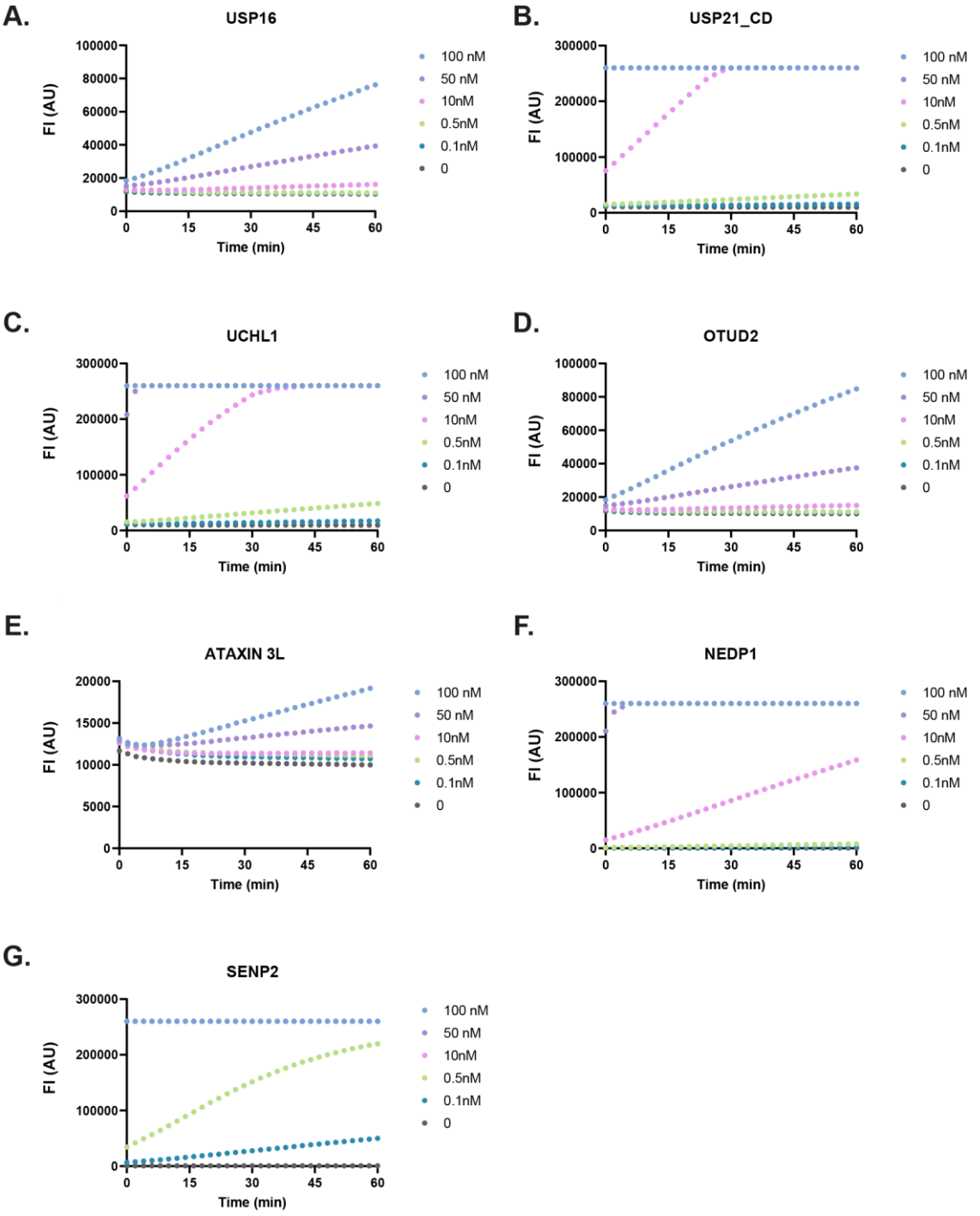
Supporting Information Figure 22. HOIP IC_{50} determination of fragments X,Y and Z. A) HOIP-dependent fluorescence intensity increase over time measured with UbSRhodol of depicted cov_frag concentrations after 1h incubation and **B**) after 2h incubation of depicted cov_frag concentrations. **C**) IC_{50} determination of cov_frag. An incubation time-dependent IC_{50} shift (arrow 1h to 2h) is observed indicating a covalent mode of action. (IC_{50} : **Cov_frag_X** 1h: 14.84 µM, 2h: 7.92 µM; **Cov_frag_Y** 1h: 14.42 µM, 2h: 7.16 µM; **Cov_frag_Z** 1h: 7.92 µM, 2h: 2.57 µM).



Supporting Information Figure 23. Jump dilution assays of fragments X, Y and Z with HOIP. (100x dilution) Jump dilution curves overlaps with highest concentration inhibitor (300µM).

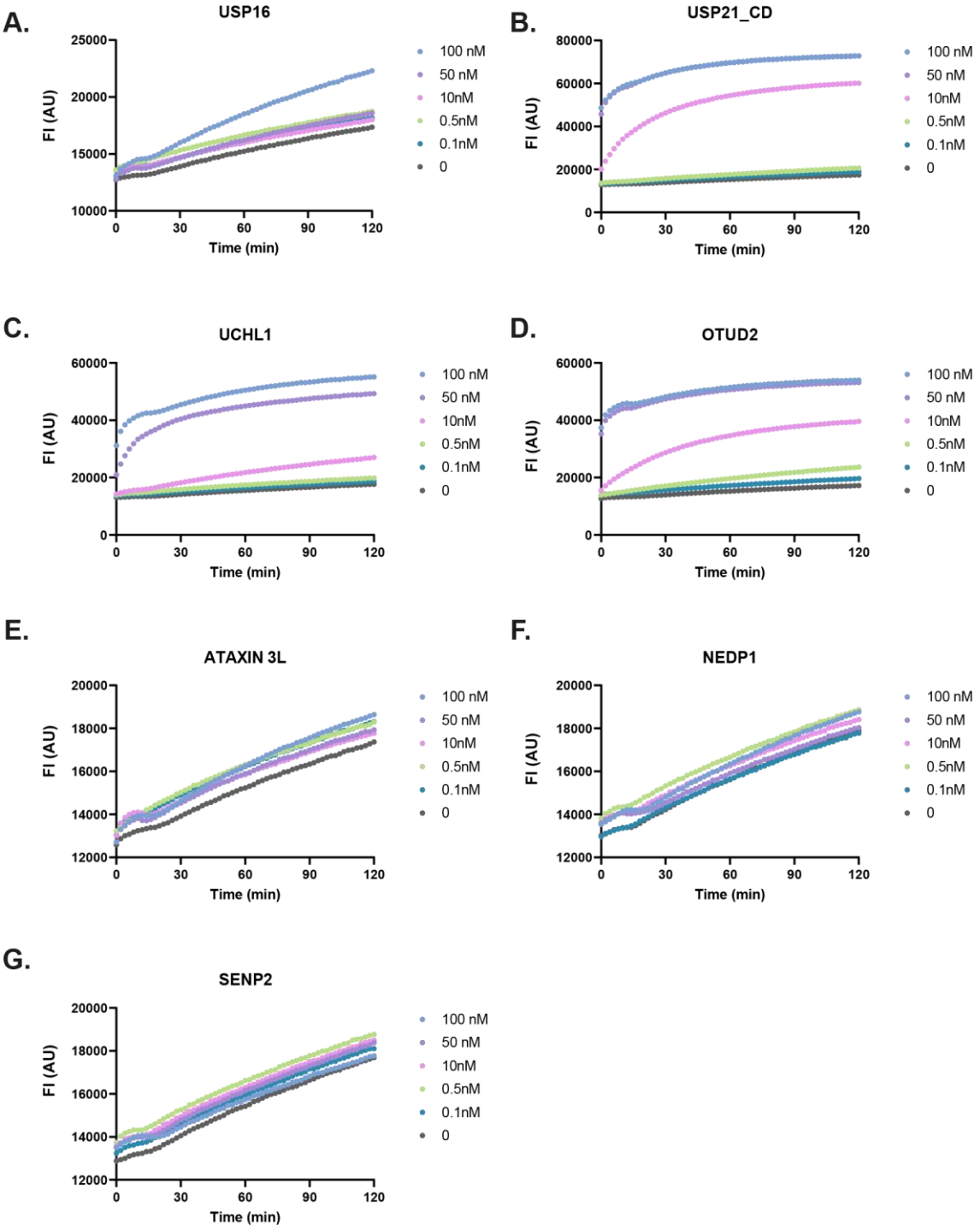


Supporting Information Figure 24. UbSRhodol transthiolation profile of Ub cascade enzymes. A) Uba1 (E1) **B)** UBE2S-IsoT **C**) Ube2G1 **D)** Ubed2d1 **E)** Ubed2d2 **F)** Ubed2d3 **G)** Ube2R1 **H)** Ube2R2 **I)** RNF4 (RING E3).

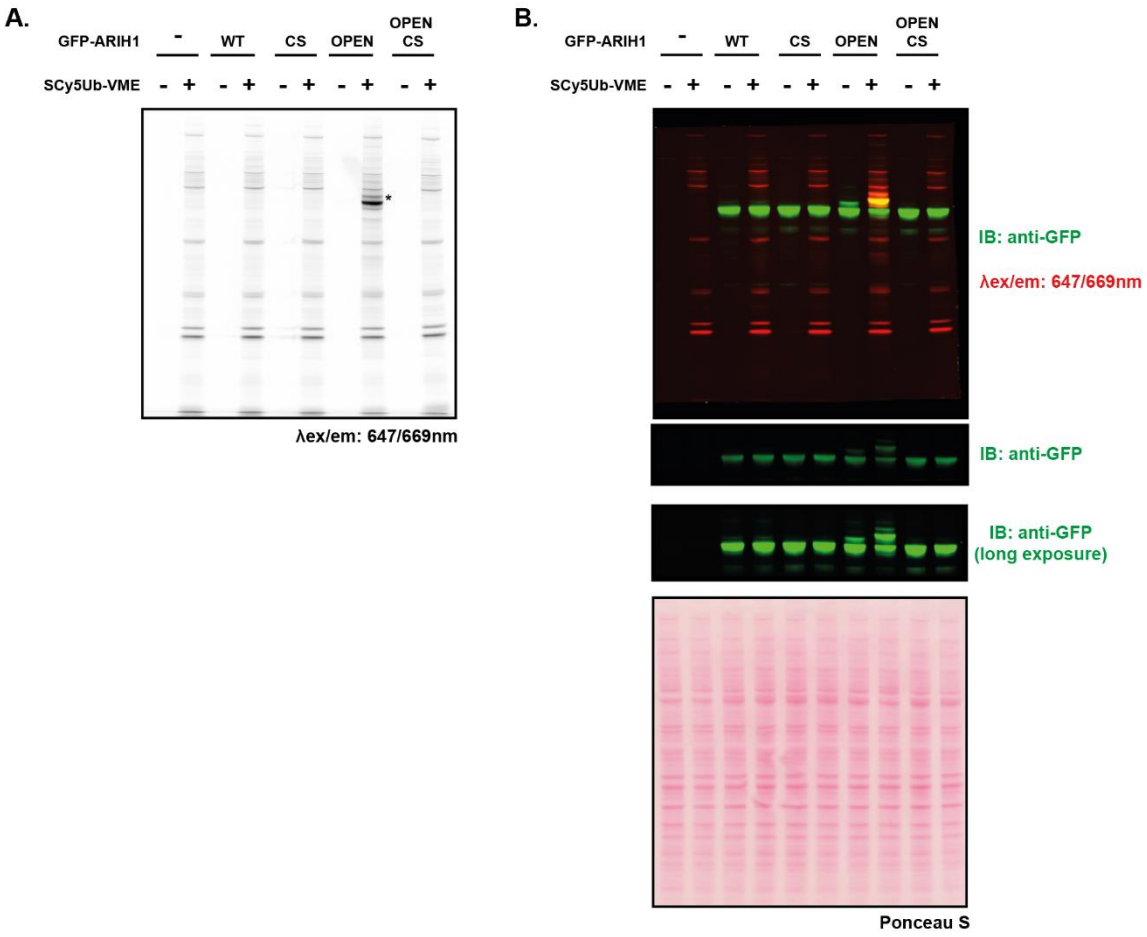


Supporting Information Figure 25. Activity assay of Ub(l) proteases using Ub(l)-Rho-morpholine to assess their capability of cleaving their cognate substrate; an isopeptide linkage.

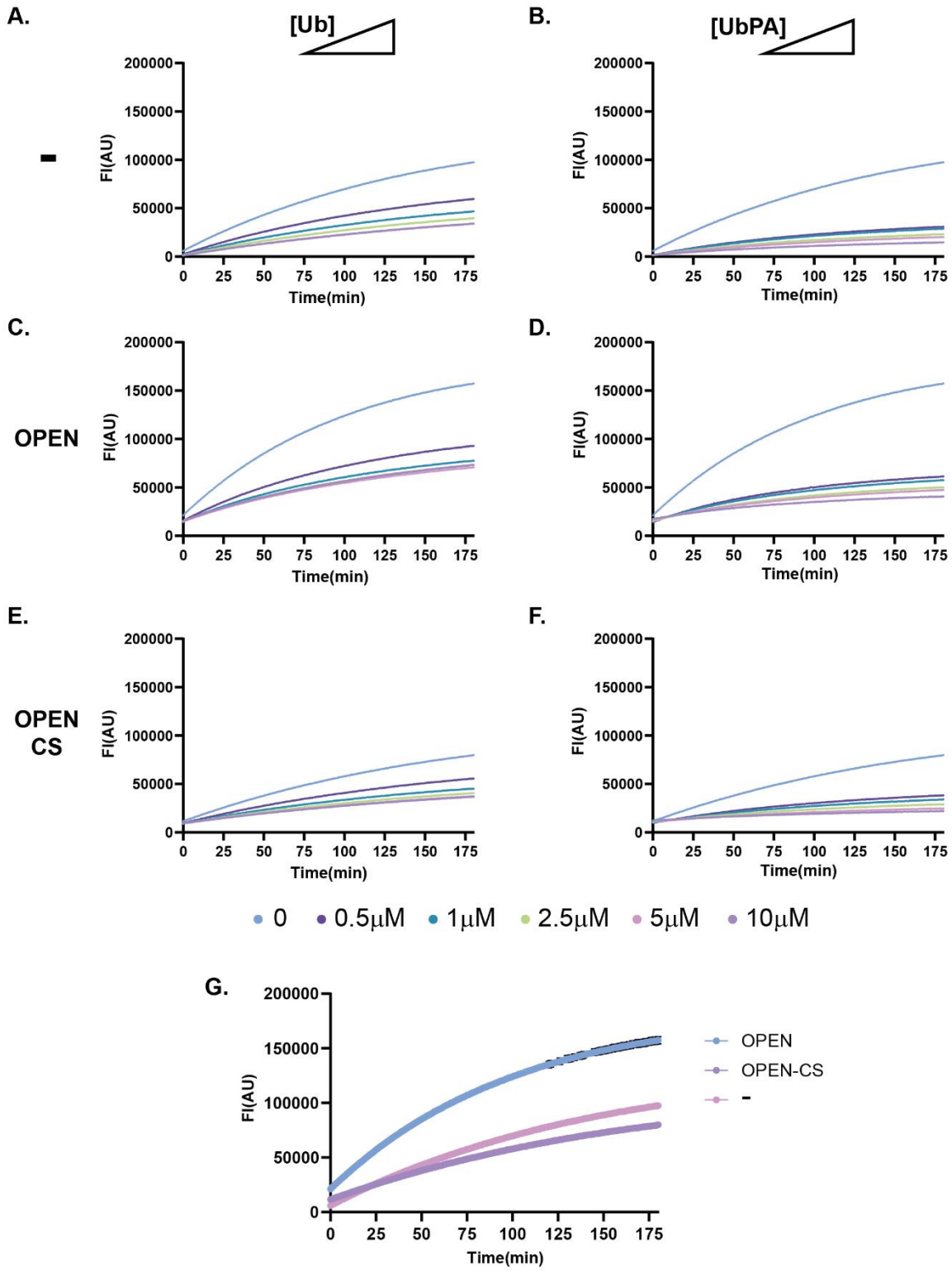
Enzyme activity was confirmed by cleavage of a C' terminal amide releasing rhodamine morpholine of each cognate substrate (Ub, Nedd8 or SUMO2) **A)** USP16 **B)** USP21_CD **C**) UCHL1 **D)** OTUD2 **E)** ATAXIN 3L **F)** NEDP1 **G)** SENP2



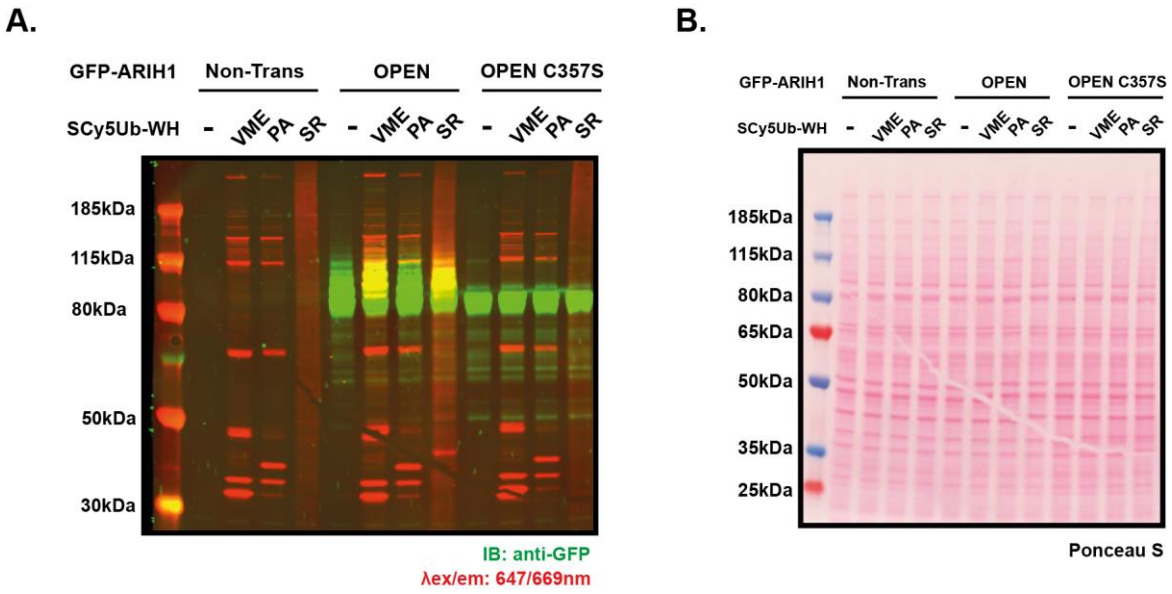
Supporting Information Figure 26. UbSRhodol transthiolation profile of Ub(l) protease panel. A) USP16 **B)** USP21_CD **C**) UCHL1 **D)** OTUD2 **E)** ATAXIN 3L **F)** NEDP1 **G)** SENP2



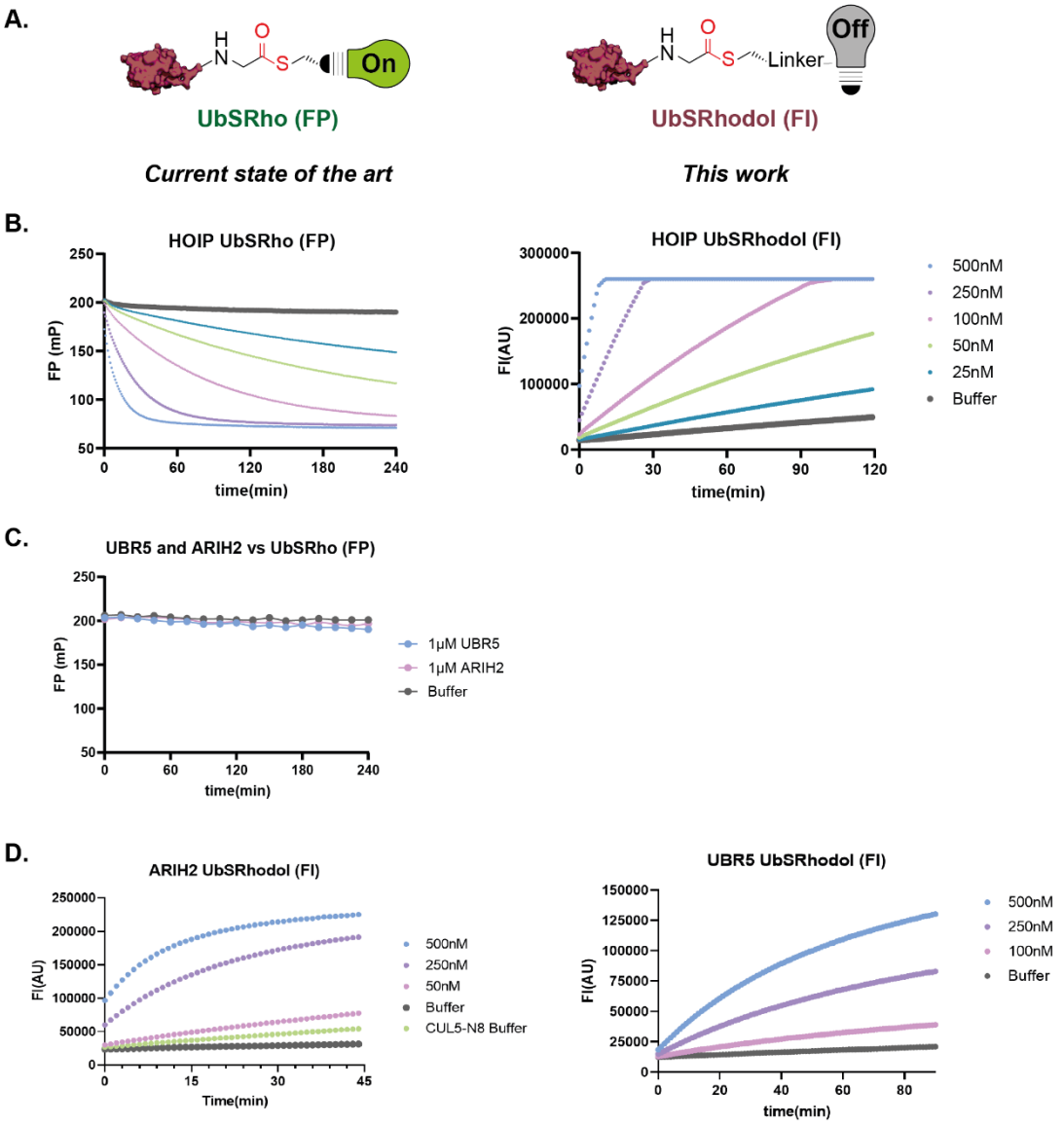
Supporting Information Figure 27. SCyUbVME probe labelling in total cell lysates of HEK293T cells overexpressing GFP-ARIH1 WT, CS, OPEN and OPEN-CS. A) Fluorescent scan after SDS-PAGE at 647/669 nM. Activity of control HEK293T cells (-) or cells overexpressing the indicated versions of GFP-ARIH was tested with the suicide probe SCy5UbVME. Only GFP-ARIH1 OPEN showed activity versus the VME probe (*) **B)** Top: Anti-GFP immunoblot (IB) blot and fluorescent scan at the Cy5 wavelength shows labelling of only ARIH1 OPEN. Middle: Anti-GFP signal only. Bottom: Ponceau S staining of the blot validating equal loading.



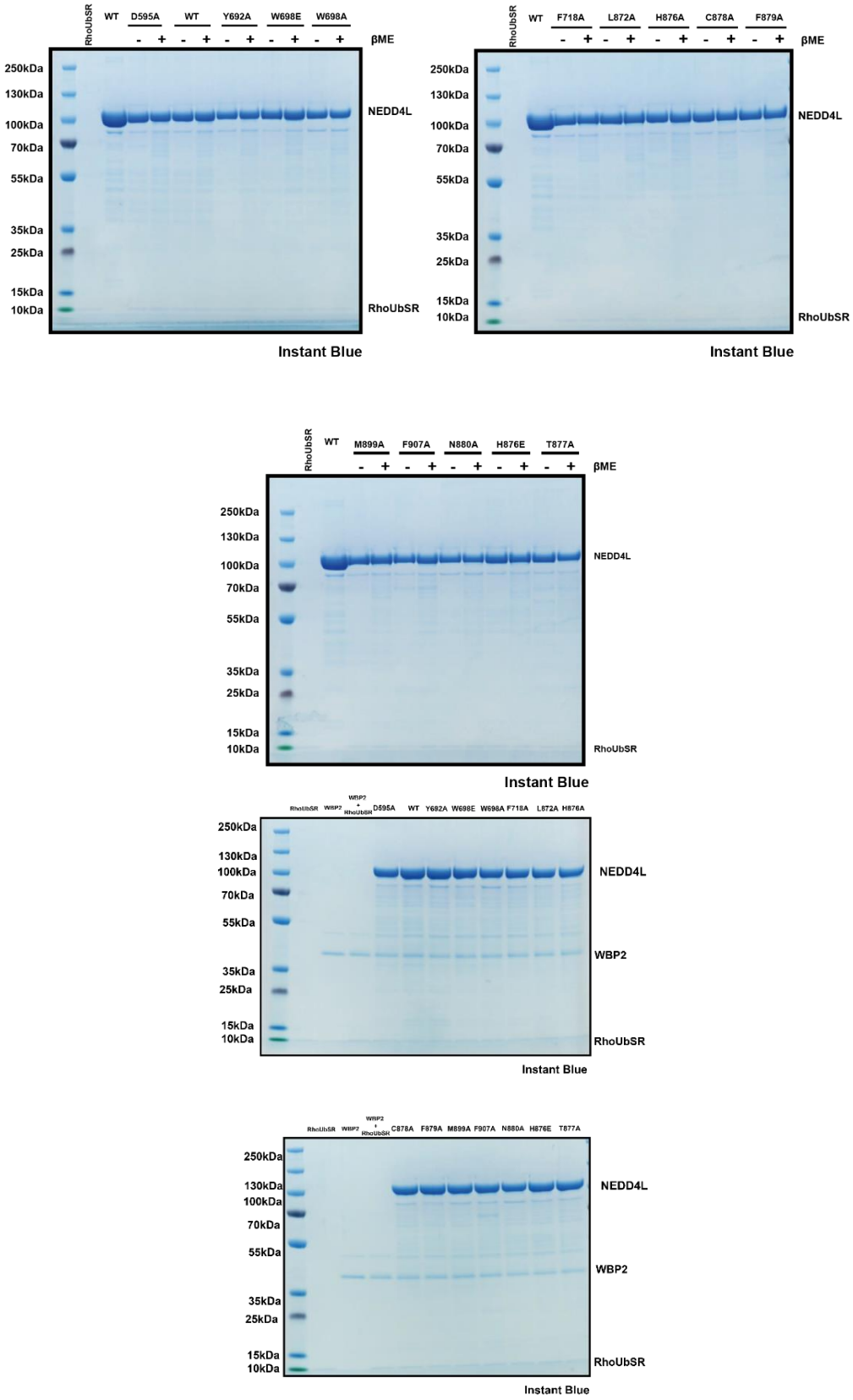
Supporting Information Figure 28. UbSRhodol mediated transthiolation in cell lysates of control HEK293T cells (-) , HEK293T cells overexpressing GFP-ARIH1 OPEN, or GFP-ARIH1 OPEN-CS. Experiment performed in the absence or presence of different concentrations of bypass probe system competitors Ub and UbPA. **A**) Untransfected Ub titration **B**) Untransfected UbPA titration **C**) ARIH1^{OPEN} Ub titration **D**) ARIH1^{OPEN} UbPA titration **E**) ARIH1$^{OPEN-CS}$ Ub titration **F**) ARIH1$^{OPEN-CS}$ UbPA titration **G**) DMSO control (concentration 0 competitors) all cell lysates.



Supporting Information Figure 29. Comparison of labelling profile for suicide ubiquitin-based probes (PA and VME) and bypass probe SCy5UbSR of overexpressed E3 ligase (GFP-ARIH1). A) Western blot high exposure **B**) Ponceau S staining.



Supporting Information Figure 30. Head-to-head comparison of chemically synthesized Fluorescence Polarization (UbSRho) and Fluorescence Intensity (UbSRhodol) probes. A) Schematic representation; **B)** HOIP E3 transthiolation measurements by FP probe UbSRho (left) and FI probe UbSRhodol (right); **C)** UBR5 and N8-CUL3:RBX2:ARIH2 E3 transthiolation measurements with UbSRho (FP) versus **D)** UbSRhodol (FI).



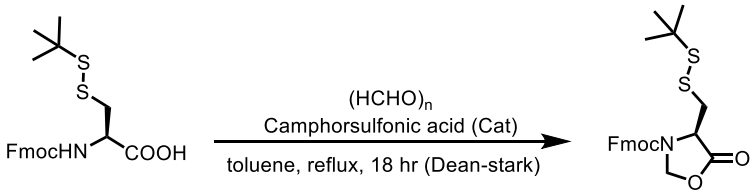
Supporting Information Figure 31. A-C) Instant Blue staining of (ΔC2)NEDD4L mutants with bypass probe RhoUbSR. (Corresponding to Supporting Information Fig. 8). **D-E)** Instant Blue staining of (ΔC2)NEDD4L mutant WBP2-ubiquitination with bypass probe RhoUbSR (Corresponding to Supporting Information Figure 9).

Chemical Synthesis

General. All commercially available reagents and solvents were used as purchased. Reported yields are given after the purification. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 300 (300 MHz for ^{1}H, 75.00 MHz for ^{13}C) using the residual solvent as internal standard (^{1}H: 7.26 ppm for $CDCl_3$, 2.50 ppm for DMSO-d_6 and 3.31 ppm for MeOD. ^{13}C: 77.16 ppm for $CDCl_3$, 39.52 ppm for DMSO-d_6 and 49.00 ppm for MeOD). Chemical shifts (δ) are given in ppm and coupling constants (*J*) are quoted in hertz (Hz). Resonances are described as s (singlet), d (doublet), t (triplet), q (quartet), p (quintet), b (broad) and m (multiplet) or combinations thereof. Thin Layer Chromatography (TLC) was performed using TLC plates from Merck (SiO_2, Kieselgel 60 F254 neutral, on aluminum with fluorescence indicator) and compounds were visualized by UV, $KMnO_4$ or ninhydrin staining. Flash Column Chromatography (FCC) purifications were performed using Grace Davisil Silica Gel (particle size 40–63 µm, pore diameter 60 Å) and the indicated eluent.

Synthesis of building block 1

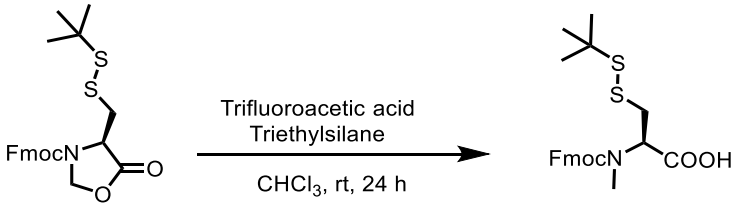
Synthesis of (9H-fluoren-9-yl)methyl (R)-4-((tert-butyldisulfaneyl)methyl)-5-oxooxazolidine-3-carboxylate **3**



2 **3**

To a room temperature solution of Fmoc-Cys(S^{t}Bu)-OH (2 g, 4.63 mmol, 1.0 eq.) in 46 mL toluene was added para-formaldehyde (0.715 mg, 23.8 mmol, 5.1 eq.) and camphorsulfonic acid (107 mg, 0.463 mmol, 0.1 eq.). The reaction was heated to reflux (oil-bath 100 C°) in a Dean-Stark apparatus and stirred for 18 hr. The reaction was concentrated to provide clear oil. After impregnation onto flash silica, the oxazolidinone **3** was purified *via* flash-chromatography (Silica gel; 0 – 50% Heptane:EtOAc) to provide **3** as a white amorphous solid. Yield: 94% (1.9 g). TLC (R_f): 0.42 (Heptane/EtOAc, 70:30).
HRMS (ESI-TOF) m/z: $[M + H]^+$ calculated for $C_{23}H_{25}NO_4S_2$ 444.1303; found 444.1309
^{1}H NMR (300 MHz, $CDCl_3$) δ 7.84 – 7.76 (m, 2H), 7.63 – 7.52 (m, 2H), 7.50 – 7.30 (m, 4H), 5.55 – 5.21 (br, m's, 2H), 4.68 – 4.55 (br, m's, 2H), 4.28 (t, *J* = 5.9 Hz, 1H), 4.07 (br, s, 1H), 3.56 (br, s, 0.5H), 3.23 (br, s, 0.5H), 3.01 (br, s, 0.5H), 2.72 (br, s, 0.5H), 1.30 (s, 9H).
^{13}C NMR (75 MHz, $CDCl_3$) δ 170.8, 152.1, 143.4, 141.3, 128.0, 127.2, 124.8, 120.1, 78.4, 67.9, 67.4, 55.4, 48.4, 47.0, 29.6.

Synthesis of N-(((9H-fluoren-9-yl)methoxy)carbonyl)-S-(tert-butylthio)-N-methyl-L-cysteine ***4***

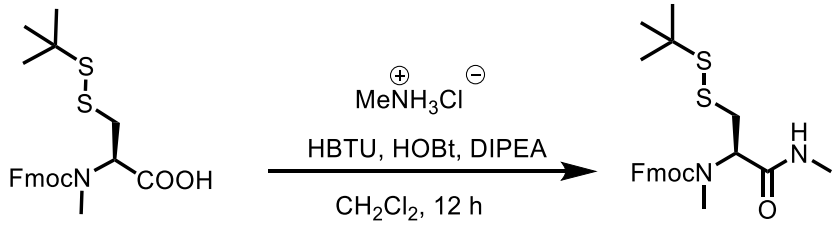


3 **4**

To a stirring solution of **3** (1.5 g, 3.38 mmol, 1.0 eq.) in 20 mL of $CHCl_3$ under room temperature was added triethylsilane (TES, 5.4 mL, 45.1 mmol, 10.0 eq.) followed by the rapid addition of trifluoroacetic acid (TFA, 10.3 mL, 135.2 mmol, 40.0 eq.). The reaction was stirred at room temperature for 16 h and then concentrated. The oil was co-evaporated several times with dichloromethane to complete removal of TFA. The crude product was further purified through flash-chromatography (Silica gel; 0 – 5% CH_2Cl_2:MeOH) was provided **4** as white solid[1]. Yield: 92% (1.39 g). TLC (R_f): 0.30 (CH_2Cl_2/MeOH, 95:5).
HRMS (ESI-TOF) m/z: $[M + H]^+$ calculated for $C_{23}H_{27}NO_4S_2$ 446.1460; found 446.1462
^{1}H NMR (300 MHz, $CDCl_3$, mixture of rotamers) δ 10.91 (s, 1H), 7.84 – 7.75 (m, 3H), 7.69 – 7.61 (m, 3H), 7.44 (td, *J* = 7.4, 1.3 Hz, 3H), 7.40 – 7.30 (m, 4H), 4.80 (ddd, *J* = 18.3, 10.3, 4.5 Hz, 2H), 4.65 – 4.52 (m, 1H), 4.52 – 4.42 (m, 2H), 4.31 (dt, *J* = 15.2, 6.5 Hz, 2H), 3.41 (dd, *J* = 14.0, 4.5 Hz, 1H), 3.33 – 3.12 (m, 2H), 3.09 (s, 3H), 3.00 (s, 2H), 1.39 (s, 9H), 1.37 (s, 5H).

^{13}C NMR (75 MHz, $CDCl_3$, mixture of rotamers) δ 175.4, 175.3, 156.6, 156.1, 143.8, 143.8, 143.7, 143.6, 141.3, 141.2, 127.8, 127.6, 127.7, 127.63, 127.1, 127.0, 125.1, 125.0, 124.8, 124.7, 119.9, 68.0, 67.8, 59.8, 58.7, 48.2, 47.0, 39.1, 38.9, 33.8, 33.0, 29.8.

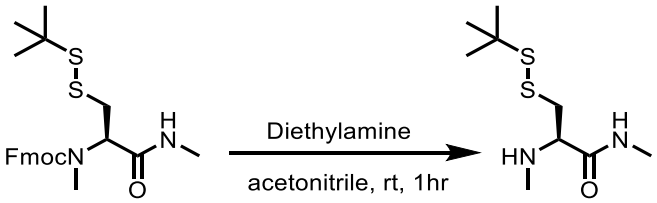
Synthesis of (9H-fluoren-9-yl)methyl (R)-(3-(tert-butyldisulfaneyl)-1-(methylamino)-1-oxopropan-2-yl)(methyl)carbamate **5**



4 **5**

To an ice cooled solution of **4** (1.2 g, 2.7 mmol, 1.0 eq) in CH_2Cl_2 (25 mL), HBTU (1.1 g, 3.0 mmol, 1.1 eq.), HOBt (400 mg, 3.0 mg, 1.1 eq.), methylamine hydrochloride (272 mg, 4.05 mmol, 1.5 eq.) and DIPEA (1.2 mL, 6.75 mmol, 2.5 eq.) were added sequentially. The reaction mixture was allowed to warm to room temperature and stirred for 12 hr. The reaction mixture was then diluted with 30 mL of CH_2Cl_2 and washed with H_2O. The aqueous layer was washed again with 20 mL CH_2Cl_2 and the combined organic layers were washed with 1N HCl (2 X 10 mL), saturated $NaHCO_3$ (2 X 10 mL) and finally with water and brine. The organic layer collected dried with Na_2SO_4, filtered, evaporated to give a crude product, and purified by flash column chromatography (Silica gel; 0 – 100% Heptane:EtOAc) to give **5** as white solid. Yield: 97% (1.2 g). TLC (R_f): 0.42 (Heptane/EtOAc, 50:50)
HRMS (ESI-TOF) m/z: $[M + H]^+$ calculated for $C_{24}H_{30}N_2O_3S_2$ 459.1776; found 459.1790
^{1}H NMR (300 MHz, $CDCl_3$, mixture of rotamers) δ 7.79 (d, *J* = 7.3 Hz, 3H), 7.62 (d, *J* = 7.4 Hz, 3H), 7.49 – 7.28 (m, 7H), 6.22 (d, *J* = 5.7 Hz, 1H), 4.90 (dd, *J* = 9.7, 5.8 Hz, 1H), 4.68 (d, *J* = 5.1 Hz, 1H), 4.62 – 4.40 (m, 2H), 4.40 – 4.22 (m, 2H), 3.31 (dd, *J* = 13.9, 5.8 Hz, 1H), 3.15 (dd, *J* = 13.9, 6.0 Hz, 1H), 3.01 (dd, *J* = 13.9, 9.8 Hz, 1H), 2.84 (s, 3H), 2.82 (s, 3H), 2.81 – 2.78 (m, 2H), 2.62 (d, *J* = 4.8 Hz, 1H), 1.35 (s, 9H), 1.31 (s, 5H).
^{13}C NMR (75 MHz, $CDCl_3$, mixture of rotamers) δ 170.0, 169.4, 157.1, 155.8, 143.9, 143.9, 143.6, 141.5, 141.4, 141.3, 141.1, 127.9, 127.8, 127.7, 127.4, 127.2, 127.1, 127.0, 125.0, 124.6, 120.1, 120.0, 120.0, 67.8, 67.1, 58.5, 58.3, 48.0, 48.0, 47.3, 47.2, 38.9, 38.6, 30.7, 29.9, 26.4, 26.3.

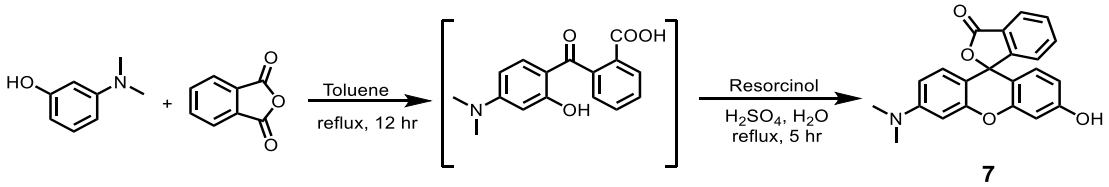
Synthesis of (R)-3-(tert-butyldisulfaneyl)-N-methyl-2-(methylamino)propenamide **6**.



5 **6**

A solution of **5** (1 g, 2.18 mmol, 1.0 eq.) in 10 mL of acetonitrile was treated with diethylamine (5.64 mL, 54.5 mmol, 25 eq.) and the reaction mixture was stirred at room temperature under nitrogen for 1 hr (TLC analysis). The solvent and volatiles were removed by rotatory evaporation. The crude product
was then purified by column chromatography (Silica gel; 0 – 5% CH_2Cl_2:MeOH) to afford **6**. Yield: 98% (505 g). TLC (R_f): 0.35 (CH_2Cl_2/MeOH, 97:3).
HRMS (ESI-TOF) m/z: $[M + H]^+$ calculated for $C_9H_{20}N_2OS_2$ 237.1095; found 237.1103
^{1}H NMR (300 MHz, $CDCl_3$) δ 7.32 (br, s, 1H), 3.23 – 3.05 (m, 2H), 2.73 (d, *J* = 5.0 Hz, 3H), 2.64 (dd, *J* = 13.1, 9.1 Hz, 1H), 2.30 (s, 3H), 1.94 (br, s, 1H), 1.24 (s, 9H).
^{13}C NMR (75 MHz, $CDCl_3$) δ 172.8, 63.4, 48.2, 43.1, 35.1, 29.8, 25.8.

Synthesis of 3'-(dimethylamino)-6'-hydroxy-3H-spiro[isobenzofuran-1,9'-xanthen]-3-one **7**



7

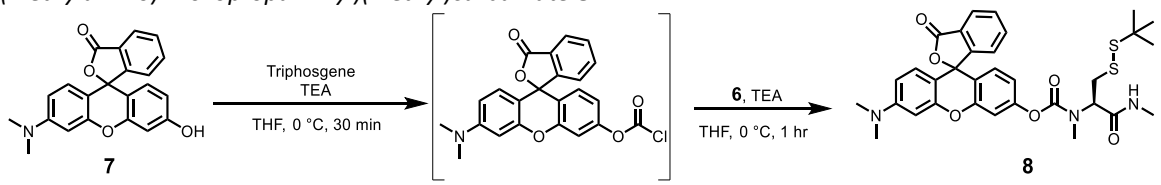
A mixture of *m*-dimethylaminophenol (6.0 g, 43.7 mmol) and phthalic anhydride (6.48 g, 43.7 mmol, 1.0 eq.) in 50 ml of toluene was refluxed for 6 h. The toluene was evaporated under vacuum and dried. To the crude intermediate 50 ml of 50% H_2SO_4, resorcinol (7.22 g, 65.6 mmol, 1.5 eq.) was added and the crude reaction mixture was heated to 120-130°C for 5 hr. Further, the reaction mixture was cooled to 0 °C and the pale orange precipitate obtained was filtered and washed with ice-cold water, dried and crystalized in chloroform. The bisulfate salt obtained was further treated with 5% Na_2CO_3 (50 mL) and filtered, the resulting solid product was neutralized with 1N HCl, washed with water and crystalized with methanol to yield desired product **7** as pale brown solid which was used directly without further purification[2]. Yield: 60% (9.43 g). TLC (R_f): 0.35 (CH_2Cl_2/MeOH, 90:10).

HRMS (ESI-TOF) m/z: $[M + H]^+$ calculated for $C_{22}H_{17}NO_4$ 360.1236; found 360.1244

^{1}H NMR (300 MHz, DMSO-d_6) δ 10.12 (s, br, 1H), 8.04 – 7.94 (m, 1H), 7.75 (dtd, *J* = 23.6, 7.4, 1.2 Hz, 2H), 7.24 (dt, *J* = 7.6, 1.0 Hz, 1H), 6.68 (t, *J* = 1.4 Hz, 1H), 6.53 (dd, *J* = 4.0, 1.5 Hz, 3H), 6.50 (d, *J* = 1.6 Hz, 2H), 2.94 (s, 6H).

^{13}C NMR (75 MHz, DMSO) δ 169.2, 159.8, 152.9, 152.5, 152.3, 152.3, 135.9, 130.4, 129.4, 128.7, 126.8, 124.9, 124.4, 112.8, 110.3, 109.6, 106.1, 102.6, 98.4, 84.2, 40.2.

Synthesis of 3'-(dimethylamino)-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-6'-yl ((R)-3-(tert-butyldisulfaneyl)-1-(methylamino)-1-oxopropan-2-yl)(methyl)carbamate **8**



7 **8**

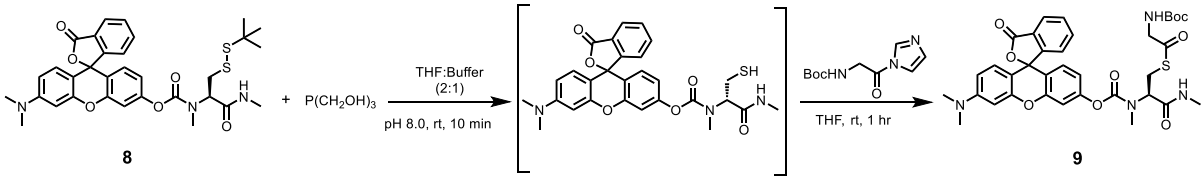
To a solution of triphosgene (165 mg, 0.55 mmol, 0.4 eq.) in dry THF (10 mL) at 0 °C, a solution of **7** (500 mg, 1.39 mmol, 1.0 eq.) with triethylamine (193 µL, 1.39 mmol, 1.0 eq.) in THF (10 mL) was added dropwise over 20 min. The reaction was stirred at room temperature for 30 min to generate the intermediate chloroformate. To this intermediate THF solution of **Rhodol 7** (330 mg, 1.39 mmol, 1.0 eq.), followed by triethylamine (193 µL, 1.39 mmol, 1.0 eq.) were added. The reaction mixture was stirred at room temperature for another 1 hr at room temperature then the solvent was evaporated and the solid reaction mixture was directly loaded onto a flash chromatography and purified (Silica gel; 0 – 100% Heptane:EtOAc) to give **8** as pale yellow solid[3]. Yield: 48% (415 mg). TLC (R_f): 0.36 (Heptane/EtOAc, 20:80).

HRMS (ESI-TOF) m/z: $[M + H]^+$ calculated for $C_{32}H_{35}N_3O_6S_2$ 622.2045; found 622.2051

^{1}H NMR (300 MHz, $CDCl_3$, mixture of rotamers) δ 7.93 (t, *J* = 1.2 Hz, 1H), 7.91 – 7.88 (m, 1H), 7.57 (t, *J* = 1.5 Hz, 0.3H), 7.54 (dt, *J* = 2.2, 1.3 Hz, 1.8H), 7.51 (t, *J* = 1.0 Hz, 1H), 7.09 (s, 0.5H), 7.08 – 7.01 (m, 4H), 6.78 – 6.70 (m, 2H), 6.70 – 6.67 (m, 1.6H), 6.65 (d, *J* = 3.7 Hz, 0.6H), 6.56 – 6.46 (m, 4H), 6.41 (d, *J* = 2.5 Hz, 2H), 6.35 (d, *J* = 2.5 Hz, 1H), 6.32 (d, *J* = 2.6 Hz, 1H), 5.01 – 4.89 (m, 0.8H), 4.82 (ddd, *J* = 9.8, 5.8, 1.4 Hz, 1H), 3.03 – 2.97 (m, 1H), 2.97 – 2.95 (m, 4H), 2.89 (s, 11H), 2.83 (s, 2H), 2.76 (d, *J* = 2.7 Hz, 1H), 2.70 (dd, *J* = 4.8, 3.4 Hz, 3H), 2.66 – 2.65 (m, 2H), 2.65 – 2.63 (m, 2H), 1.26 (s, 9H), 1.25 (s, 6H).

^{13}C NMR (75 MHz, $CDCl_3$, mixture of rotamers) δ 171.1, 169.6, 169.4, 169.4, 155.2, 154.3, 153.0, 153.0, 152.9, 152.4, 152.3, 152.3, 152.2, 152.1, 152.1, 152.0, 134.9, 129.7, 128.9, 128.9, 128.8, 128.6, 126.8, 124.9, 124.1, 124.0, 117.2, 117.1, 116.8, 116.7, 110.3, 110.3, 110.2, 109.2, 105.9, 98.5, 83.7, 59.6, 59.5, 48.3, 48.1, 40.2, 39.0, 38.7, 31.4, 31.4, 29.9, 29.9, 26.4, 26.3.

Synthesis of S-((2R)-2-((((3'-(dimethylamino)-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-6'-yl)oxy)-carbonyl)(methyl)amino)-3-(methylamino)-3-oxopropyl) 2-((tert-butoxycarbonyl)amino)ethanethioate **9**



8 9

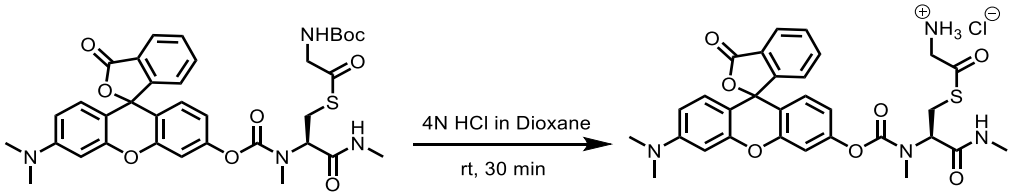
To a round bottom flask were charged Tris(3-hydroxypropyl)phosphine (THPP) (92 mg, 0.44 mmol, 1.10 eq.) and the basic buffer solution [tris-(hydroxymethyl)-aminomethane-$CaCl_2$ based buffer; pH 8.00; 0.5 mL]. The reaction mixture was stirred at room temperature for about 5 min and then to this homogenous solution was charged the solution of **5** (250 mg, 0.4 mmol, 1.00 eq.) in 1 mL of THF. This homogeneous aqueous reaction mixture was stirred for 10 mins at room temperature (until TLC showed complete disappearance of the starting material). To this solution, a preformed tert-butyl (2-(1H-imidazol-1-yl)-2-oxoethyl)carbamate[4] (4.5 g, 20 mmol, 50 eq.) was added and the reaction mixture was stirred for 1 hr at room temperature under nitrogen atmosphere. The crude reaction mixture extracted with ethyl acetate (2 X 20 mL). The organic layer collected dried with Na_2SO_4, filtered, evaporated to give a crude product, and purified by flash column chromatography (Silica gel; 0 – 100% Heptane:EtOAc) to give **9** as pale brown solid which is stored under inert atmosphere. Yield: 60% (165 mg). TLC (R_f): 0.41 (Heptane/EtOAc, 30:70).

HRMS (ESI-TOF) m/z: $[M + H]^+$ calculated for $C_{35}H_{38}N_4O_9S$ 691.2438; found 691.2471

^{1}H NMR (300 MHz, $CDCl_3$, mixture of rotamers) δ 8.02 (d, *J* = 1.7 Hz, 1H), 8.00 (d, *J* = 1.5 Hz, 1H), 7.73 – 7.56 (m, 4H), 7.14 (qd, *J* = 8.5, 7.6, 3.9 Hz, 4H), 6.87 – 6.70 (m, 4H), 6.62 (d, *J* = 8.8 Hz, 2H), 6.55 – 6.40 (m, 5H), 5.31 (s, 1H), 4.73 (dd, *J* = 9.2, 6.2 Hz, 2H), 4.07 – 3.95 (m, 3H), 3.52 (ddd, *J* = 21.1, 14.1, 6.1 Hz, 2H), 3.32 (dd, *J* = 14.1, 9.1 Hz, 2H), 3.04 (s, 4H), 2.99 (s, 11H), 2.94 (s, 2H), 2.83 (s, 3H), 2.76 (d, *J* = 4.6 Hz, 4H), 1.45 (s, 6H), 1.44 (s, 9H).

^{13}C NMR (75 MHz, $CDCl_3$, mixture of rotamers) δ 198.3, 198.0, 171.2, 169.5, 169.1, 168.9, 155.5, 155.19, 153.7, 152.8, 152.4, 152.3, 152.1, 152.0, 134.9, 129.7, 128.9, 128.9, 128.7, 126.8, 126.8, 125.0, 124.2, 124.0, 117.1, 117.1, 116.8, 110.2, 109.4, 106.3, 103.8, 98.7, 80.4, 66.9, 66.2, 60.4, 58.3, 50.3, 40.4, 31.8, 31.0, 28.3, 27.0, 26.4, 26.3.

Synthesis of 2-(((2R)-2-((((3'-(dimethylamino)-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-6'-yl)oxy)carbonyl)(methyl)amino)-3-(methylamino)-3-oxopropyl)thio)-2-oxoethan-1-aminium chloride **BB-1**.



9 **BB-1**

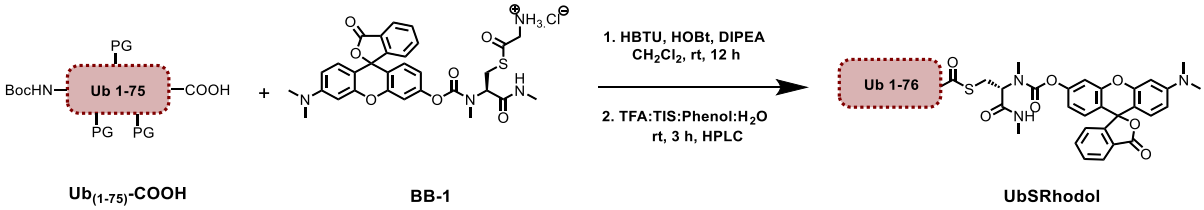
A solution of HCl/dioxane (4 mL, 4M) in a 25-mL round-bottom flask equipped with a magnetic stir-bar was cooled by an ice-water bath under argon. Compound **10** (100 mg, 0.15 mmol) was added in one portion with stirring. The ice-bath was removed and the mixture was kept stirred. After 30 min, TLC indicated that the reaction was completed. The reaction mixture was condensed by rotary evaporation under high vacuum at room temperature. The residue was then washed with dry ethyl ether and collected by filtration. The solid product **1** obtained was further lyophilized and stored under argon atmosphere. Yield: 99% (89 mg). TLC (R_f): 0.5 (CH_2Cl_2:MeOH:AcOH, 80:18:2).

HRMS (ESI-TOF) m/z: $[M + H]^+$ calculated for $C_{30}H_{30}N_4O_7S$ 591.1913; found 591.1932

^{1}H NMR (300 MHz, DMSO-d_6) δ 8.75 1(br, m, 2H), 8.11 (d, *J* = 7.4 Hz, 2H), 7.81 (dt, *J* = 21.9, 7.3 Hz, 2H), 7.48 (d, *J* = 12.4 Hz, 1H), 7.37 (d, *J* = 7.4 Hz, 1H), 7.11 (t, *J* = 8.8 Hz, 1H), 6.99 (d, *J* = 9.4 Hz, 3H), 6.83 (d, *J* = 8.9 Hz, 1H), 4.63 (td, *J* = 11.4, 10.6, 5.6 Hz, 1H), 4.09 – 4.02 (br, m, 2H), 3.68 (dd, *J* = 8.6, 4.2 Hz, 1H), 3.62 (s, 3H), 3.47 (dd, *J* = 7.6, 3.8 Hz, 1H), 2.95 (s, 6H), 2.58 (d, *J* = 4.3 Hz, 3H).

^{13}C NMR (75 MHz, DMSO) δ 193.4, 172.4, 168.3, 156.9, 156.3, 154.1, 153.2, 152.3, 151.5, 135.3, 133.6, 130.9, 130.3, 129.5, 128.5, 127.0, 124.4, 119.5, 117.4, 110.7, 110.6, 100.0, 72.5, 70.9, 53.2, 41.7, 32.3, 28.1, 26.3.

Synthesis of UbSRhodol.



$Ub_{(1-75)}$-COOH BB-1 UbSRhodol

To a solution of protected $Ub_{(1-75)}$-COOH (20 µmol)[5] in DCM (20 mL), HBTU/HOBt (39.7mg/17mg, 100µmol, 5.eq.) and DIPEA (35µL, 200 µmol, 10 eq.) were added followed by **BB-1** (15 mg, 24.5 µmol, 3.5 eq.). The reaction was allowed to stir overnight at room temperature and monitored by LC-MS. After confirmation of the desired product formation, the solvent was evaporated under vacuum and the crude peptide was further treated with TFA/TIS/H_2O/Phenol (92.5/2.5/2.5/2.5, 5mL) for 3 hours at room temperature and then the peptide was further precipitated it in cold diethylether/pentane (1:1, 30 mL) and wash it several times with diethylether with centrifugation to yield crude solid material. Pure UbSRhodol was obtained by HPLC purification* and different fractions containing the desired product were pool together and lyophilized yielding 32mg of pure UbSRhodol (yield= 17.65%).

UbSRhodol:

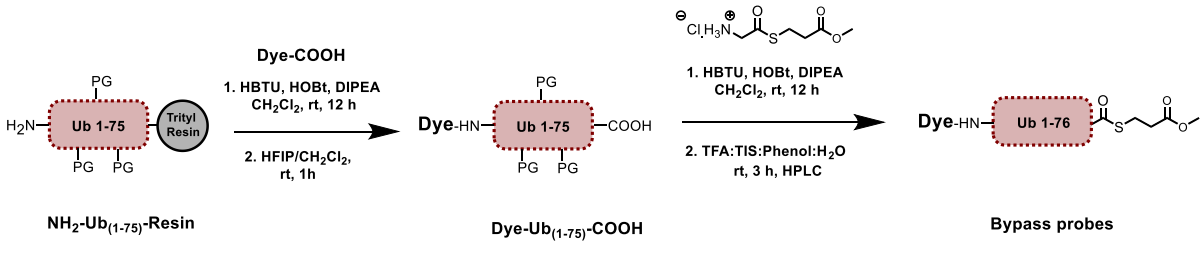
NleLIKVKTLTGKEIEIDIEPTDKVERIKERVEEKEGIPPQQQRLIYSGKQNleNDEKTAADYKILGGSVLHLVLALRGG~SRhodol (**MW_{exp}**: 9062; **MW_{obs}**: 9063 [UbSRhodol-H^+])

Please note: Nle stands for Nor-Leucine, a well-known methionine isostere.

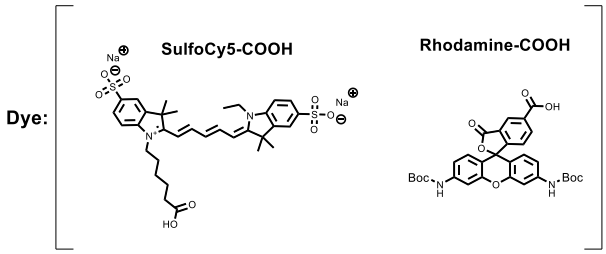
*(HPLC purification: samples were run on a Waters 2535 HPLC equipped with a Waters 2489 UV/Vis detector, Waters fraction collector III and Waters XBridge prep C18 OBD (30 mm × 150 mm, 5 µm). UbSRhodol crude mixture was dissolved in DMSO containing 2.5% TFA (5% total volume) and added to a vessel containing 0.1% formic acid in MQ, to a concentration of 5mg/ml.

Flowrate = 37.5 mL min−1. Eluents: A = H_2O, B = CH_3CN and D = 1% TFA in H_2O. Gradient: 0–5 min: 90% A, 5% B, 5% C; 5–7 min: 90% A → 75% A, 5% B → 20% B, 5% C; 7–23 min: 75% A → 50% A, 20% B → 45% B, 5% C; 23–23.5 min: 50% A → 0% A, 45% B → 95% B, 5% C; 23.5–26.5 min: 0% A, 95% B, 5% C; 26.5–26.6 min: 0% A → 90% A, 95% B → 5% B, 5% C; 26.6–30 min: 90% A, 5% B, 5% C.

Synthesis bypass probes Rho/Cy5UbSR for gel based readout.



NH_2-$Ub_{(1-75)}$-Resin Dye-$Ub_{(1-75)}$-COOH Bypass probes



The N-terminus of Ub(1-75) was modified on resin by Rhodamine or SulfoCy5. To a solution of protected $Ub_{(1-75)}$-COOH (20 µmol) in DCM (20 mL), HBTU/HOBt (39.7mg/17mg, 100µmol, 5.eq.) and DIPEA (35µL, 200 µmol,

10 eq.) were added followed by Rhodamine-COOH or SulfoCy5-COOH (3 equiv), respectively. Reactions were allowed to stir at room temperature overnight. Finally, the resin was washed with NMP, DCM and Et_2O and the C-terminus modified as following. The resin was treated with 5 mL of DCM/HFIP (4:1 v/v) for 30 min and filtered. The resin was rinsed with DCM (3x5 mL) and the combined filtrates were concentrated. The partially protected peptide residue (1 equiv) was redissolved in DCM(1umol/ml) and HBTU/HOBt (39.7mg/17mg, 100µmol, 5.eq.) and DIPEA (35µL, 200 µmol, 10 eq.) were added followed by methyl methyl 3-(glycylthio)propanoate (42.4 mg,10 equiv). The reaction mixture was stirred overnight at room temperature. The solvent was removed in vacuo and the residue treated with TFA/H_2O/phenol/iPr_3SiH (90/5/2.5/2.5 v/v/v/v) for 3 h followed by precipitation with cold Et_2O/pentane (1:1 v/v). Purification by HPLC following UbSRhodol protocol gave RhoUb(1-75)SR as a pink powder (27mg, yield= 15%) or Cy5Ub(1-75)SR as a blue powder (18mg, yield= 10%).

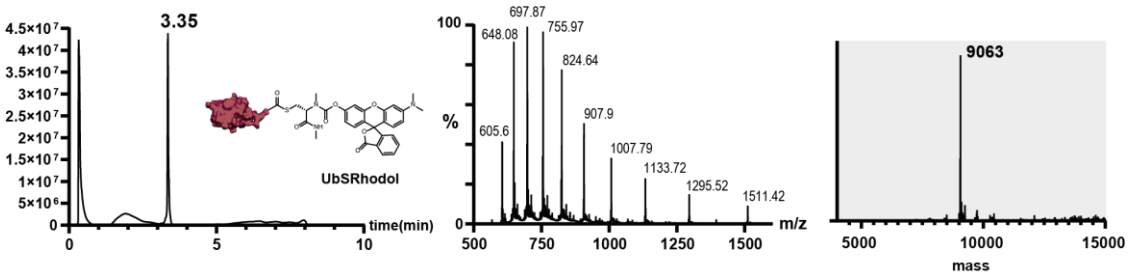
RhoUbSR:

Rhodamine-NleLIKVKTLTGKEIEIDIEPTDKVERIKERVEEKEGIPPQQQRLIYSGKQNleNDEKTAADYKILGGSVLHLVLALRG G~SR (**MW_{exp}**: 9004; **MW_{obs}**: 9005 [RhoUbSR-H^+)

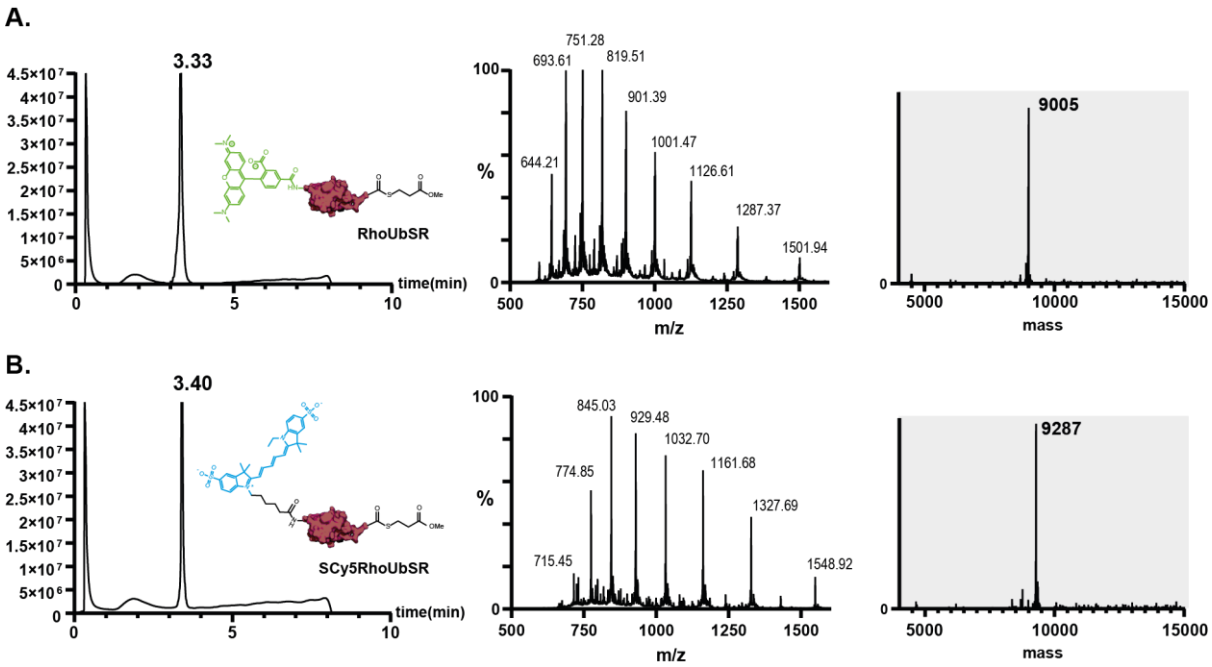
SCy5UbSR:

SulfoCy5-NleLIKVKTLTGKEIEIDIEPTDKVERIKERVEEKEGIPPQQQRLIYSGKQNleNDEKTAADYKILGGSVLHLVLALRGG~ SR (**MW_{exp}**: 9286; **MW_{obs}**: 9287 [SCy5UbSR-H^+)

Please note: Nle stands for Nor-Leucine, and R for methylmalonate.



Supporting Information Figure 32. LC-MS characterization of UbSRhodol. UV/Vis Chromatogram, charge/mass envelope and deconvoluted mass



Supporting Information Figure 33. LC-MS characterization of bypass probes: A) RhoUbSR **and B)** SCy5UbSRUbSRhodol. UV/Vis Chromatogram, charge/mass envelope and deconvoluted mass **.**

Materials and Methods

Expression of proteins

UBR5 HECT DOMAIN

Plasmids

Recombinant DNA: pRSF 6xHis-UBR5_HECT (2499-2799) and pRSF 6xHis-UBR5_HECT C2768A (2499-2799) were used.

Expression and purification

The C-terminal fraction of the wildtype HECT-domain of UBR5 (2499-2799) or the catalytic cysteine mutant C2768A respectively, were expressed with a N-terminal 6xHis-tag using BL21-CodonPlus (DE3)-RIL cells in Terrific Broth medium. Protein expression was induced with 0.6 mM IPTG with subsequent shaking overnight at 18°C. The cell pellet was resuspended in lysis buffer consisting of 25 mM Tris-HCl, pH 8.0, 200 mM NaCl, 5 mM β-Mercaptoethanole, 2.5 mM PMSF, and protease inhibitor. Lysis was performed using sonication followed by pre-clearance of the lysate using centrifugation for 40 min at 51.000 x g at 4°C. The protein was isolated by performing a gravity flow affinity purification with His-Select Nickel Affinity gel (SIGMA), subsequent anion exchange chromatography and lastly, size exclusion chromatography with the final buffer consisting of 25 mM HEPES. pH 7.5, 150 mM NaCl, 1 mM DTT. The protein was aliquoted and flash-frozen in liquid nitrogen until usage.

HERC proteins

Expression and purification

His tagged constructs of HERC1 and HERC3 HECT domains were expressed in E. coli BL21 RIL cells. Briefly, the cells were induced with 0.4 mM IPTG at 0.8 O.D. and were allowed to grow overnight at 18 degrees. After 16 hours of induction cells were harvested and resuspended in 50 mM Tris-HCl pH-7.5, 500 mM NaCl, 25 mM Imidazole and 5% glycerol (lysis buffer). The cells were lysed with sonication and insoluble fraction was pelleted down at 15000 rpm. Soluble fraction was allowed to bind to Talon beads which were preequilibrated with the lysis buffer. After binding, the beads were washed with the lysis buffer and the protein was eluted with 50 mM Tris-HCl pH-7.5, 500 mM NaCl, 250 mM Imidazole and 5% glycerol. The eluted protein was concentrated down to 1 ml and applied to Superdex 16/600 75 pg size exclusion column which was preequilibrated with 25 mM Tris-HCl pH-7.5, 200 mM NaCl and 1 mM TCEP. Following gel filtration, the protein was concentrated with the help of Centricon with 30 kDa molecular weight cut-off filter. After concentration the protein was flash frozen in liquid nitrogen and stored at -80 degrees.

NEDD4L

Plasmids and cloning

All NEDD4L constructs are listed in Supporting Information Table S1. NEDD4L WT (full-length and ΔC2) and 14 HECT domain mutants (ΔC2) were cloned into pGEX-4T1 bearing an N-terminal GST-tag followed by a Tobacco Etch Virus (TEV) cleavage site. All NEDD4L constructs were derived from NEDD4L isoform 2. Full-length WBP2 was cloned into pBTD15 containing an N-terminal His-MBP-tag followed by a TEV cleavage site. All constructs were cloned using Gibson assembly.

Expression and purification

All proteins were expressed in *E. coli* BL21 (DE3) Gold. Cells were grown to an OD_{600} of 0.8 and expression was induced with 0.2 mM IPTG. After induction, the protein was expressed at 18°C overnight. *E. coli* cells were harvested and the cell pellet was resuspended in 1x PBS supplemented with 2.5 mM PMSF, 5 mM DTT and 0.001 mg/ml benzonase. The cells were lysed by sonication and insoluble material was separated by centrifugation at 50,000 x g for 30 min. For NEDD4L proteins, the supernatant was incubated with Glutathione Sepharose beads

(Cytiva) for 1 h. After extensive washing of the beads with buffer A (30 mM Tris pH 7.5, 200 mM NaCl, 5 mM DTT) the bound protein was eluted by incubating the beads with buffer A supplemented with 0.02 mg/ml TEV protease at 4°C overnight. The next day, the protein was concentrated using an Amicon 30 kDa MWCO centrifugal filter and loaded on a Superdex 200 10/300 Increase SEC column (GE Healthcare) with buffer B (20 mM HEPES pH 7.5, 100 mM NaCl, 1 mM TCEP) as a final polishing step. Peak fractions containing NEDD4L protein were pooled, concentrated to 70-150 μM, shock frozen in liquid nitrogen and stored at -80°C. The WBP2 containing supernatant was incubated with His-Select Nickel Affinity gel (Sigma-Aldrich) at 4°C for 30 min. After washing the beads with buffer A, the protein was eluted with buffer A supplemented with 250 mM imidazole. Protein containing fractions were concentrated and loaded on a Superdex 200 10/300 Increase SEC column (GE Healthcare) with buffer B. Peak fractions of WBP2 were pooled, concentrated to 290 μM, shock frozen in liquid nitrogen and stored at -80°C.

Supporting Information Table S1

Construct	**mutation**	**residue function**
GST-TEV-NEDD4L-ΔC2-D595A	D595A	acidic loop
GST-TEV-NEDD4L-ΔC2	WT	
GST-TEV-NEDD4L-ΔC2-Y692A	Y692A	E2 binding site
GST-TEV-NEDD4L-ΔC2-W698A	W698E	E2 binding site
GST-TEV-NEDD4L-ΔC2-W698E	W698A	E2 binding site
GST-TEV-NEDD4L-ΔC2-F718A	F718A	N-lobe/ubiquitin
GST-TEV-NEDD4L-ΔC2-L827A	L872A	C-lobe/ubiquitin
GST-TEV-NEDD4L-ΔC2-H876A	H876A	cat. loop
GST-TEV-NEDD4L-ΔC2-C878A	C878A	cat. Cys
GST-TEV-NEDD4L-ΔC2-F879A	F879A	cat. loop
GST-TEV-NEDD4L-ΔC2-M899A	M899A	C-lobe/ubiquitin
GST-TEV-NEDD4L-ΔC2-F907A	F907A	-4 F mutant
GST-TEV-NEDD4L-ΔC2-N880A	N880A	cat. loop
GST-TEV-NEDD4L-ΔC2-H876E	H876E	cat. loop
GST-TEV-NEDD4L-ΔC2-T877A	T877A	cat. loop
GST-TEV-NEDD4L	Full-length	
His-MBP-TEV-WBP2	model substrate – WBP2	

SMURF1

Plasmids and cloning

The expression plasmid (pET28a-LIC_SMURF1-W-HECT) for the W-HECT domain of SMURF1 (UNIPROT: Q9HCE7; a.a. 306-757) was a kind gift from Masoud Vedadi from the Structural Genomics Consortium Toronto. Catalytic point mutation (C725A) was performed using IVA cloning[6] with primers listed in Supporting Information table S2. All clones were sequence verified

Expression and purification

Wild-type and mutant SMURF1 plasmids were transformed into *E. coli* BL21(DE3) and grown in LB supplemented with 50 µg L^{-1} kanamycin. Cultures were grown at 37°C until OD_{600} reached 1.0, upon which the temperature was lowered to 18°C and overnight expression was induced using 0.4 mM isopropyl-d-1-thiogalactopyranoside (IPTG). Next day, cells were pelleted by centrifugation for 20 min at 4000 G and subsequently resuspended in buffer A (20 mM Tris pH8.0, 300 mM NaCl and 20 mM Imidazole).

All purification steps were carried out at 4°C. Cells of 2L expression culture were lysed using sonification and debris was spun down for 40 min at 30000 G. The supernatant was applied to 2 mL of nickel-charged NTA beads and incubated for 30 min. Beads were washed extensively using buffer A, before elution using buffer B (buffer A with 200 mM Imidazole). The eluate was concentrated and applied to an equilibrated (in 20 mM Tris pH8.0, 150 mM NaCl, 1mM DTT) S75 16/60 (GE Healthcare) size-exclusion column using a Bio-rad NGC. Fractions were analysed using SDS-PAGE and SMURF1-containing ones were pooled and concentrated before aliquoting and flash freezing in liquid nitrogen for storage at -80°C.

Supporting Information table S2

SMURF1-C725A-fw	*CCATACCgcCTTTAACC*GGATCGACATTCCACCATATGAGTCC
SMURF1-C725A-rv	*GGTTAAAGgcGGTATGG*GCCTTCGGAAGGTTGTCTGTG

RBR proteins ARIH1, ARIH2 and HOIP

Plasmids and cloning

Coding sequences for all proteins are of human origin, were obtained from Max Planck Institute of Biochemistry core facility cDNA libraries and cloned into expression vectors via Gibson assembly cloning. Mutant versions of ARIH1, ARIH2 and HOIP were generated using Quikchange system (Agilent).

Expression and purification

Neddylated versions of CUL1-RBX1 and CUL5-RBX2 were obtained as previously described[7]. ARIH1, ARIH2 and mutant versions were expressed as GST-TEV fusion proteins in *E. coli* Rosetta 2 (DE3) and induced with IPTG (0.1 mM) at an OD of 0.6-0.8 in Terrific Broth medium containing 0.1 mM $ZnCl_2$. After induction, temperature was reduced to 18˚C and expression continued overnight. Cell lysis was performed by sonication and followed by centrifugation. Fusion protein-containing supernatant was subjected to incubation with Glutathione Sepharose® beads, washed several times with wash buffer (50 mM Tris pH 7.5, 200 mM NaCl and 1 mM DTT) and incubated with TEV protease overnight on beads. Target protein was eluted with two bead volumes of wash buffer and anion exchange and size-exclusion chromatography (25 mM HEPES pH 7.5, 150 mM NaCl, 0.5 mM TCEP) were carried out. HOIP (696-1072) and its C885A mutant were expressed as His-SUMO fusion protein in *E. coli* Rosetta 2 (DE3) and cells consequently treated as for ARIH1/2. After incubation with Ni-NTA beads, beads were washed with five bead volumes of wash buffer (50mM Tris pH 8, 200 mM NaCl, 1 mM ß-Mercaptoethanol) and eluted with another five volumes of elution buffer (50 mM Tris pH 8, 200 mM NaCl, 300 mM Imidazole and 1 mM ß-Mercaptoethanol). Fusion protein was digested with SENP2 protease overnight and anion exchange and size-exclusion chromatography (25 mM HEPES pH 7.5, 150 mM NaCl, 0.5 mM TCEP) were performed the following day.
GST-TEV-RNF14 and STREP-3C-RNF216 were expressed in *Trichoplusia Ni* Hi-5™ insect cells in 0.1 mM $ZnSO_4$-containing medium. After lysis, fusion proteins were subjected to either Glutathione Sepharose® or Streptavidin Sepharose® beads, washed several times with wash buffer and eluted with either Glutathione or Desthiobiotin-containing elution buffer. Overnight cleavage was performed with either TEV or 3C protease and target proteins further purified anion exchange and size-exclusion chromatography.

E3 activity measurements with UbSRhodol (384well plate) and product formation.

Assays were performed in non-binding-surface, flat-bottom, low-flange, black 384-well plates (Corning3820) at room temperature in a buffer containing 50 mM HEPES, 150 mM NaCl at pH 7.5, 1.0 mM TCEP, 0.5 mg/mL 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid (CHAPS) in quadruplets. UbSRhodol solution was prepared by pipetting gently 1.25µl of a 2mM DMSO (2.5%TFA) stock into 1mL of buffer to yield a 1.25µM solution (2.5X). Serial dilutions of proteins (2.5 µM/1µM/0.5µM (ST conditions) and 250/100/50/25/0 nM (MT conditions)) were prepared as a 1.66X stock and 9µL were added to the assay plate. Then, 6 µL of UbSRhodol (2.5X, 500nM final concentration or 1µM in the case of HOIP) were dispensed on a Biotek MultiFlowFX dispenser, plate was centrifuged (1000 rpm, 1min) and the E3-mediated release of Rhodol was measured at the emission wavelength of 520 nm (±10 nm) after excitation at 485 nm (±10 nm) in a Pherastar plate reader (BMG LABTECH GmbH, Germany) in continuous mode for several timepoints. Gain (5 /10%) was set at the probe only well.

Product formation assays (HOIP and SMURF1)

The assays were conducted in a buffer containing 50 mM HEPES, 150 mM NaCl at pH 7.5 and 1.0 mM TCEP. RhoUbSR/SCy5UbSR solution were prepared by pipetting gently the appropriate volume of a 2mM DMSO (2.5%TFA) stock into the appropriate volume to yield a 2.5X solution of probe: 1.25µM for SMURF1 (500nM total concentration) or 2.5µM solution for HOIP (combination of 1.25µM UbSRhodol and 1.25µM SCy5UbSR) (1µM total concentration)

45µl of E3 stocks (see experimental 3. *E3 activity measurements* (1.66X, E3)) were combined with 30uL RhoUbSR/SCy5UbSR at a final concentration of 500nM or 1µM for HOIP(2.5X). Reactions were stopped at the selected timepoint by adding loading buffer with or without 5% βME to assess thioester formation. Samples containing 5% βME were boiled at 95°C for 5 minutes, cool down and centrifuge. Samples were resolved by SDS-PAGE using a 4–12% Bis-Tris gel (Invitrogen, NuPAGE) with MES or MOPS SDS running buffer (Novex, NuPAGE) for 45 min at 190 V. Gels were scanned for fluorescence on a GE Typhoon FLA 9500 using a green channel (λex/em 473/530 nm) and a red channel (λex/em 647/669 nm), followed by staining with InstantBlue Coomassie protein stain (Expedeon), after which the gel was scanned on a GE Amersham Imager 600.

NEDD4L-dependent transthiolation measurements and product formation

The assay was conducted in a buffer containing 50 mM HEPES, 150 mM NaCl at pH 7.5 and 1.0 mM TCEP , 0.5 mg/mL 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid (CHAPS) in quadruplets. UbSRhodol solution was prepared by pipetting gently the appropriate volume of a 2mM DMSO(2.5%TFA) stock into the appropriate volume to yield a 1.25µM solution (2.5X). Different dilutions of NEDD4L (2.5µM and 1µM) and NEDD4L(2.5µM and 1µM) containing 500nM of WBP2 were prepared as a 1.66X stock and 9µL were added to the assay plate. Then, 6 µL of UbSRhodol (2.5X, 500nM final concentration) were dispensed on a Biotek MultiFlowFX dispenser, plate was centrifuged (1000 rpm, 1min) and the E3-mediated release of Rhodol was measured at the emission wavelength of 520 nm (±10 nm) after excitation at 485 nm (±10 nm) in a Pherastar plate reader (BMG LABTECH GmbH, Germany) in continuous mode for several timepoints. Gain (5 /10%) was set at the probe only well. 100 minutes timepoint was selected for all the different mutants to quantify their percentage of activity by standardization between the WT (100%) and C878A(0%) and plotted in GraphPad Prism 9.3.1.

Using the E3 1.66X stocks (2.5µM) (see experimental 3. *E3 activity measurements*) the product formation assays were performed (Transthiolation and Cognate substrate ubiquitination model). 45µL of E3 were combined with 30µL of RhoUbSR (2.5X, 500nM final concentration). All the samples were incubated for 100 minutes at room temperature and stopped by adding loading buffer with or without 5% βME to asses thioester formation. Samples containing 5% βME were boiled at 95°C for 5 minutes, cool down and centrifuge. Samples were resolved by SDS-PAGE using a 4–12% Bis-Tris gel (Invitrogen, NuPAGE) with MOPS SDS running buffer (Novex, NuPAGE) for 45 min at 190 V. Gels were scanned for fluorescence on a GE Typhoon FLA 9500 using the green channel

(λex/em 473/530 nm), followed by staining with InstantBlue Coomassie protein stain (Expedeon), after which the gel was scanned on a GE Amersham Imager 600.

For native ubiquitination assays of WBP2 pulse-chase assays were performed to observe the efficiency of ubiquitin to NEDD4L and subsequently to WBP2 for different NEDD4L mutants. For ubiquitin loading, 10 μM UbcH5b were incubated with 0.2 μM E1, 0.04 mg/ml ovalbumin and 20 μM fluorescent ubiquitin for 30 min. The reaction was quenched with 2 μM apyrase. Afterwards, NEDD4L (WT and mutants) and WBP2 were added to the mixture to a final concentration of 0.5 μM UbcH5b~ubiqutin, 0.2 μM NEDD4L and 10 μM WBP2. The reaction was carried out at 4°C and 0.5 min, 2 min and **5 min** time points were quenched with SDS loading dye.

E3:E3 superassembly mutant profiling and product formation (ARIH1 and ARIH2).

UbSRhodol assay: The assay was conducted in a buffer containing 50 mM HEPES, 150 mM NaCl at pH 7.5 and 1.0 mM TCEP , 0.5 mg/mL 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid (CHAPS) in quadruplets. UbSRhodol solution was prepared by pipetting gently the appropriate volume of a 2mM DMSO(2.5%TFA) stock into the appropriate volume to yield a 1.25μM solution(2.5X). RBR alone or an equimolar solution of RBR (ARIH1 or ARIH2) and neddylated cullin (N8-CUL1:RBX1 or N8-CUL5:RBX2) were prepared as a 1.66X stock (500nM final concentration) and 9μL were added to the assay plate. Then, 6μL of UbSRhodol (2.5X, 500nM final concentration) were dispensed on a Biotek MultiFlowFX dispenser, plate was centrifuged (1000 rpm, 1min) and the E3-mediated release of Rhodol was measured at the emission wavelength of 520 nm (±10 nm) after excitation at 485 nm (±10 nm) in a Pherastar plate reader (BMG LABTECH GmbH, Germany) in continuous mode for several timepoints. Gain (5 /10%) was set at the probe only well.

RhoUbSR gel-based assay: The same stocks were used for product formation assays with N' fluorescently labelled bypass probe (RhoUbSR, 500nM final concentration). 45μL of E3 were combined with 30μL of RhoUbSR (2.5X, 500nM final concentration). All the samples were incubated for 30 minutes at room temperature and stopped by adding loading buffer with or without 5% βME to assess thioester formation. Samples containing 5% βME were boiled at 95°C for 5 minutes, cool down and centrifuge. Samples were resolved by SDS-PAGE using a 4–12% Bis-Tris gel (Invitrogen, NuPAGE) with MOPS SDS running buffer (Novex, NuPAGE) for 45 min at 190 V. Gels were scanned for fluorescence on a GE Typhoon FLA 9500 using the green channel (λex/em 473/530 nm), followed by staining with InstantBlue Coomassie protein stain (Expedeon), after which the gel was scanned on a GE Amersham Imager 600.

Determination of positive control concentration for HTS screening

Assays were performed in non-binding-surface, flat-bottom, low-flange, black 384-well plates (Corning3820) at room temperature in a buffer containing 50 mM HEPES, 150 mM NaCl at pH 7.5 or pH 9.0 , 1.0 mM TCEP, 0.5 mg/mL 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid (CHAPS) in quadruplets. Iodoacetamide and NEM were transferred from freshly prepared stocks to the empty plate using a Labcyte Echo550 acoustic dispenser and accompanying dose–response software z obtain a 5-point serial dilution (4 replicates) of 10/5/1/0.5/0.2/0 mM. A DMSO backfill was performed to obtain equal volumes of DMSO (150 nL) in each well. Then, 9μL of buffer only at different pH (7.5 or 9.0) were added to the assay plate and 6 μL of UbSRhodol (2.5X, 500nM final concentration) were dispensed on a Biotek MultiFlowFX dispenser, plate was centrifuged (1000 rpm, 1min) and the release of Rhodol was measured at the emission wavelength of 520 nm (±10 nm) after excitation at 485 nm (±10 nm) in a Pherastar plate reader (BMG LABTECH GmbH, Germany) in continuous mode for several timepoints. Gain (5 /10%) was set at the probe only well (DMSO).

Z' value calculation (1536 well plate) for HTS

Calculations of were performed in non-binding-surface, flat-bottom, low-flange, black 1536-well plates (Corning3724) at room temperature in a buffer containing 50 mM HEPES, 150 mM NaCl at pH 7.5, 1.0 mM TCEP, 0.5 mg/mL 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid (CHAPS). Iodoacetamide (IAA, 1 mM) was used a positive control (100% inhibition), and DMSO was used as a negative control (0% inhibition), both were transferred from DMSO stocks (80nL per well) to the empty plate using a Labcyte Echo550 acoustic dispenser. 128 positive and negative controls were placed out in the columns covering the great area of the

plate to minimize variability within the plate. Buffered solutions were dispensed using a Biotek MultiFlowFX dispenser. First, E3 enzyme buffer (6 µl, 1.33X enzyme stock, final concentration HOIP: 25nM, ARIH1: 15nM and SMURF1: 150nM) was dispensed, plate was shook gently for 10 seconds, centrifuged (1000rpm, 1min) and allowed to incubated for 2 hours at room temperature. Buffered UbSRhodol solution was prepared by pipetting gently the appropriate volume of a 2mM DMSO(2.5%TFA) UbSRhodol stock into the appropriate volume to yield a 2µM solution (4X UbSRhodol stock) 5 minutes prior the end of the E3-inhibitor incubation time. Then, 2 µl of this solution was dispensed, the plate was centrifuged and the E3-mediated release of Rhodol was measured at the emission wavelength of 520 nm (±10 nm) after excitation at 485 nm (±10 nm) in a Pherastar plate reader (BMG LABTECH GmbH, Germany) for 180 minutes each 3 minutes. Gain (10%) was set at the IAA control well. Z' values were calculated for each time point following Zhang's equation and plotted against time in GraphPad Prism 9.3.1 software.

$$(1)\quad Z' = 1 - \frac{3(\sigma_{pos} + \sigma_{neg})}{|\mu_{pos} - \mu_{neg}|}$$

μ_{pos} and σ_{pos} average and standard deviation of 128 positive controls, respectively

μ_{neg} and σ_{neg} average and standard deviation of 128 positive controls, respectively

High-Throughput Screening of Enamine covalent fragment library (1536 well plate)

High-throughput screenings were performed in non-binding-surface, flat-bottom, low-flange, black 1536-well plates (Corning3724) at room temperature in a buffer containing 50 mM HEPES, 150 mM NaCl at pH 7.5, 1.0 mM TCEP, 0.5 mg/mL 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid (CHAPS). Iodoacetamide (IAA, 1 mM) was used a positive control (100% inhibition), and DMSO was used as a negative control (0% inhibition), both were transferred from DMSO stocks (80nL per well) to the empty plate using a Labcyte Echo550 acoustic dispenser. Buffered solutions were dispensed using a Biotek MultiFlowFX dispenser. First, E3 enzyme buffer (6 µl, 1.33X enzyme stock, 80ml) was dispensed, plate was shook gently for 10 seconds, centrifuged (1000rpm, 1min) and allowed to incubated for 2 hours at room temperature. Buffered UbSRhodol solution was prepared by pipetting gently 30µl of a 2mM DMSO(2.5%TFA) UbSRhodol stock into 30ml of buffer to yield a 2µM solution (4X UbSRhodol stock, ca. 550µg UbSRhodol per HTS) 5 minutes prior the end of the E3-inhibitor incubation time. Then, 2 µl of this solution was dispensed, the plate was centrifuged and the E3-mediated release of Rhodol was measured at the emission wavelength of 520 nm (±10 nm) after excitation at 485 nm (±10 nm) in a Pherastar plate reader (BMG LABTECH GmbH, Germany) in a single time point. Gain (10%) was set at the IAA control well. Data was then standardized to obtain the % of inhibition and a set of hits (60% cut-off) was selected for validation by triplicates. Validated hits by triplicates to discard possible dispensing errors was conducted for each ligase to generate the venn3 diagram. For HOIP 69 hits were retrieved (0.79% hit rate), for ARIH1 72 hits were retrieved (0.92% hit rate) and 79 for SMURF1 (1.00% hit rate).

Elmann's reagent reactivity assay against fragments X, Y and Z

A 100 µM sample of DTNB was incubated with 400 µM TCEP in 20 mM sodium phosphate buffer pH 7.4 and 150 mM NaCl for 5 min at room temperature, in order to obtain TNB^{2-}. A same volume solution of carbonyl imidazoles X, Y and Z (400 µM) in the same buffer was mixed with the previous TNB^{2-} solution, to yield a final solution of 200µM of compounds and 100µM of TNB^{2-}. Compounds without DTNB were plated aswell to extract their absorbance at 412 nm at 37 °C were performed. The absorbances were acquired every 5 min for 7 h. The assay was performed in a 96-well plate (Costar) using a Clariostar plate reader(BMG LABTECH GmbH, Germany. Compounds were measured in triplicate and their absorbances were plotted in GraphPad Prism 9.3.1 software.

MS full intact protein inhibitor engagement for series X,Y and Z

The assay was conducted in a buffer containing 50 mM HEPES, 150 mM NaCl at pH 7.5 and 1.0 mM TCEP. Equal volumes of HOIP/ARIH1/SMURF1 (2µM, 50µL, 2X) and covalent fragment inhibitors (covfrag) (200µM, 50µL, 2X) were pooled together to yield a solution of 1µM HOIP/ARIH1/SMURF1 and 100 µM covfrag and incubated for 2 hours at room temperature or at 4°C overnight in the case of HOIP. Samples were then diluted 5-fold with water

containing 0.1% formic acid and analyzed by mass spectrometry by injecting 5 µL into a Waters XEVO-G2 XS Q-TOF mass spectrometer equipped with an electrospray ion source in positive mode (capillary voltage 1.2 kV, desolvation gas flow 900 L/h, T = 60 °C) with a resolution of R = 26 000. Samples were run using two mobile phases: (A) 0.1% formic acid in water and (B) 0.1% formic acid in CH3CN on a Waters Acquity UPLC protein BEH C4 column [300 Å, 1.7 µm (2.1 × 50 mm2), flow rate = 0.5 mL/min, run time = 14.00 min, column T = 60 °C, and mass detection 200–2500 Da]. Gradient: 2–100% B. Data processing was performed using Waters MassLynx mass spectrometry software 4.2, and ion peaks were deconvoluted using the built-in MaxEnt1 function. Peak annotation was plotted using GraphPad Prism 9.3.1 software

SulfoCy5UbVME competition assays for series X,Y and Z

The assay was conducted in a buffer containing 50 mM HEPES, 150 mM NaCl at pH 7.5 and 1.0 mM TCEP. Equal amounts of HOIP (2µM, 50µL, 2X) and covalent fragment inhibitors (covfrag) (200/150/100/50/20/10/2µM, 50µL, 2X) were pooled together to yield a solution of 1µM HOIP and 100/75/50/25/10/5/1 µM covfrag and incubated for 2 hours at room temperature. Then 5µL of SulfoCy5-UbVME in buffer (200µM, 20X) was added and the solution was pipet up and down gently. All the samples were incubated for 30 minutes at room temperature and stopped by addition of loading buffer containing βME. Samples were resolved by SDS-PAGE using a 4–12% Bis-Tris gel (Invitrogen, NuPAGE) with MOPS SDS running buffer (Novex, NuPAGE) for 45 min at 190 V. Gels were scanned for fluorescence on a GE Typhoon FLA 9500 using the red channel (λex/em 532/570 nm), followed by staining with InstantBlue Coomassie protein stain (Expedeon), after which the gel was scanned on a GE Amersham Imager 600.

IC_{50} determination for series X,Y and Z

IC_{50} determination assays were performed in non-binding-surface, flat-bottom, low-flange, black 384-well plates (Corning3820) at room temperature in a buffer containing 50 mM HEPES, 150 mM NaCl at pH 7.5, 1.0 mM TCEP, 0.5 mg/mL 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid (CHAPS) in triplicate. Each well had a final volume of 15 µL. All dispensing steps involving buffered solutions were performed on a Biotek MultiFlowFX dispenser. The compounds were dissolved in DMSO as 10, 1, and 0.1 mM stock solutions, and appropriate volumes were transferred from these stocks to the empty plate using a Labcyte Echo550 acoustic dispenser and accompanying dose–response software to obtain a 9-point serial dilution (3 replicates) of 0.05 to 200 µM. A DMSO backfill was performed to obtain equal volumes of DMSO (150 nL) in each well. Iodoacetamide (IAA, 1 mM) was used a positive control (100% inhibition), and DMSO was used as a negative control (0% inhibition). Enzyme (9 µL, 1.66X stock) was added, and the plate was vigorously shaken for 10 seconds and incubated either for 1h or 2h. Then, 6 µL of UbSRhodol of 2.5X stock (final concentration 500 nM) were dispensed and the plate was centrifuged and the rhodol-dependant increase in fluorescence intensity over time was recorded using a BMG Labtech CLARIOstar or PHERAstar plate reader (excitation 490 nm, emission 520 nm). The initial enzyme velocities were calculated from the slopes, normalized to the positive and negative controls, and plotted against the inhibitor concentrations (in M) using the built-in equation "[inhibitor] vs response – variable slope (four parameters), least-squares fit" with constraints "Bottom = 0" and "Top = 100" in GraphPad Prism 9.3.1 software to obtain the IC50 values.

Jump dilution assays determination for series X,Y and Z

Jump dilution assays were performed at room temperature in a buffer containing 50 mM HEPES, 150 mM NaCl at pH 7.5, 1.0 mM TCEP, 0.5 mg/mL 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid (CHAPS) in quadruplets. The final concentrations used were 30 nM HOIP, 1 µM UbSRhodol, and 300 µM or 3µM or a jump dilution of 300 µM to 3 µM of inhibitors. Samples of 20 µL containing 3 µM HOIP and 300 µM inhibitor (1% DMSO), 1% DMSO, or 1 mM Iodoacetamide (IAA) were incubated for 1 hour at room temperature. Each sample (5 µL) was then diluted into a 500 µL solution containing 1µM UbSRhodol. After a gentle mixing, 15 µL of each of these solutions was quickly transferred to a non-binding-surface, flat-bottom, low-flange, black 384-well plate (Corning3820) and the increase in fluorescence over time was recorded using a BMG Labtech PHERAstar plate reader (excitation 485 nm, emission 520 nm). As a control, samples were taken in which 40 µL of a 600 µM and 6 µM inhibitor solution in buffer (1% DMSO) was added to 35 µL of a 69 nM HOIP solution. After 1 hour of

incubation, 5 µL of a 16µM UbSRhodol solution was added, after which 15 µL of each solution was transferred to the same 384-well plate mentioned above, and the increase in fluorescence intensity was measured concomitantly. Fluorescent intensities were plotted against time using GraphPad Prism 9.3.1.

Cell lysate experiments with the activity based probes SCy5-UbVME and -UbPA, and bypass probe SCy5-UbSR

HEK293T cells were transiently transfected on 6 well or 10 cm dishes, or mock-treated, using PEI as transfection reagent according to the manufacturer's instructions. For ARIH1 overexpression pcDNA™FRT/TO-GFP-ARIH1 WT, CS, ARIH1^{OPEN} and ARIH1$^{OPEN-CS}$ were used[8]. 24h after transfection, cells were washed with ice cold PBS and harvested by scraping in lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.5% Triton X100, 2 mM TCEP and protease inhibitor tablet (Roche)), and lysate was clarified by centrifugation. Lysates were subsequently aliquoted and stored at -80. For activity based probe (ABP) assays 1 uM SCy5-labeled-ABP was added, lysates were incubated for 30 min at room temperature, reactions were stopped by boiling in 1x loading buffer containing βME, proteins were separated by SDS-PAGE on 4-12% Bis-Tris NuPage gels (Invitrogen, NuPAGE) with MOPS-SDS running buffer (Novex, NuPAGE), imaged using a GE Typhoon FLA 9500, blotted on nitrocellulose, and after blocking, incubated o/n with rabbit anti-GFP serum[9], washed, incubated 30min with LI-COR IRDye 800CW goat anti-rabbit secondary antibody, and imaged on LICOR Odyssey system v3.

Cell lysates were equalized for protein concentration by measuring A280 absorbance using a NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer.

E1,E2 and E3 activity measurements with UbSRhodol (384well plate)

Assays were performed in non-binding-surface, flat-bottom, low-flange, black 384-well plates (Corning3820) at room temperature in a buffer containing 50 mM HEPES, 150 mM NaCl at pH 7.5, 1.0 mM TCEP, 0.5 mg/mL 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid (CHAPS) in quadruplets. UbSRhodol solution was prepared by pipetting gently 1.25µl of a 2mM DMSO (2.5%TFA) stock into 1mL of buffer to yield a 1.25µM solution (2.5X). Serial dilutions of enzymes (Uba1 (E1), UBE2S-IsoT, Ube2G1, Ubed2d1, Ubed2d2, Ubed2d3, Ube2R1, Ube2R2 and RNF4 (RING E3), (2.5 µM/1µM/0.5/0.25 and 0.1µM)) were prepared as a 1.66X stock and 9µL were added to the assay plate. Then, 6 µL of UbSRhodol (2.5X, 500nM final concentration or 1µM in the case of HOIP) were dispensed on a Biotek MultiFlowFX dispenser, plate was centrifuged (1000 rpm, 1min) and the mediated release of Rhodol was measured at the emission wavelength of 520 nm (±10 nm) after excitation at 485 nm (±10 nm) in a Pherastar plate reader (BMG LABTECH GmbH, Germany) in continuous mode for several timepoints. Gain (5 /10%) was set at the probe only well.

Protease activity measurements with cognate fluorogenic substrate and UbSRhodol (384 well plate)

Assays were performed in non-binding-surface, flat-bottom, low-flange, black 384-well plates (Corning3820) at room temperature in a buffer containing 50 mM HEPES, 150 mM NaCl at pH 7.5, 1.0 mM TCEP, 0.5 mg/mL 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid (CHAPS) in quadruplets. First, all the enzymes (USP16, USP21_CD, UCHL1, OTUD2, ATAXIN 3L, NEDP1 and SENP2) were buffer exchanged with a 3kda Zeba Spin Desalting Column to remove DTT traces.

All proteases were tested against its cognate fluorogenic substrate[10], UbRhoMP, Nedd8RhoMP and SUMO2RhoMP for Ub, Nedd8 and SUMO2 proteases respectively, using the same enzyme (range) and substrate concentration (500nM total substrate) as for UbSRhodol. Here the DUBs are tested for their capability of cleaving their cognate substrate; an isopeptide.

UbSRhodol solution was prepared by pipetting gently 1.25µl of a 2mM DMSO (2.5%TFA) stock into 1mL of buffer to yield a 1.25µM solution (2.5X). Serial dilutions of enzymes (100 nM/50nM/10nM/0.5nM and 0.1nM) were prepared as a 1.66X stock and 9µL were added to the assay plate. Then, 6 µL of UbSRhodol (2.5X, 500nM final concentration) were dispensed on a Biotek MultiFlowFX dispenser.

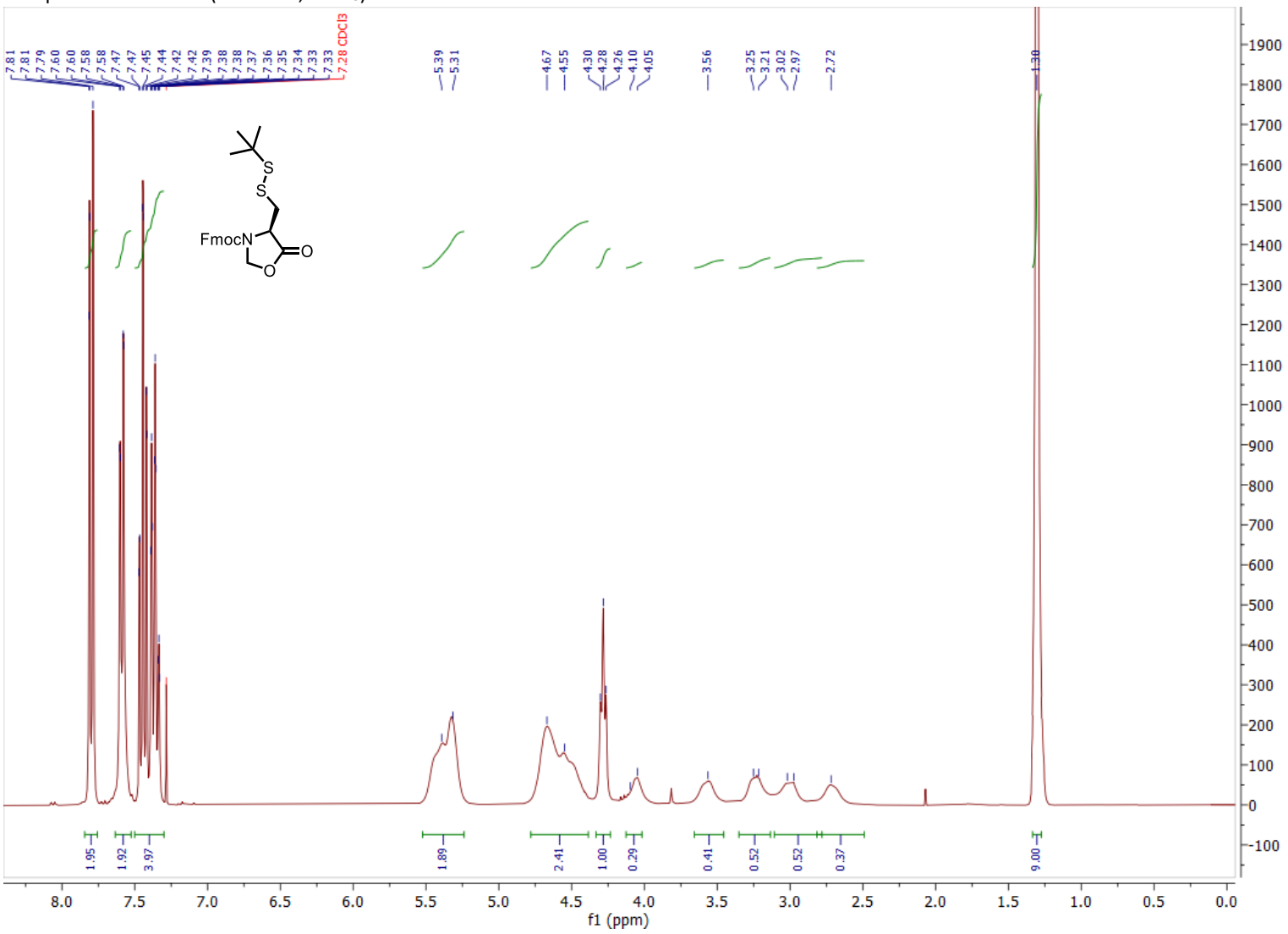
Then the plate was centrifuged (1000 rpm, 1min) and the mediated release of Rhodamine (Supporting Information Fig. 25) or Rhodol (Supporting Information Fig. 26) was measured at the emission wavelength of 520 nm (±10 nm) after excitation at 485 nm (±10 nm) in a Pherastar plate reader (BMG LABTECH GmbH, Germany) in continuous mode for several timepoints. Gain (5 /10%) was set at the probe only well.

UbSRhodol transthiolation in cell lysates

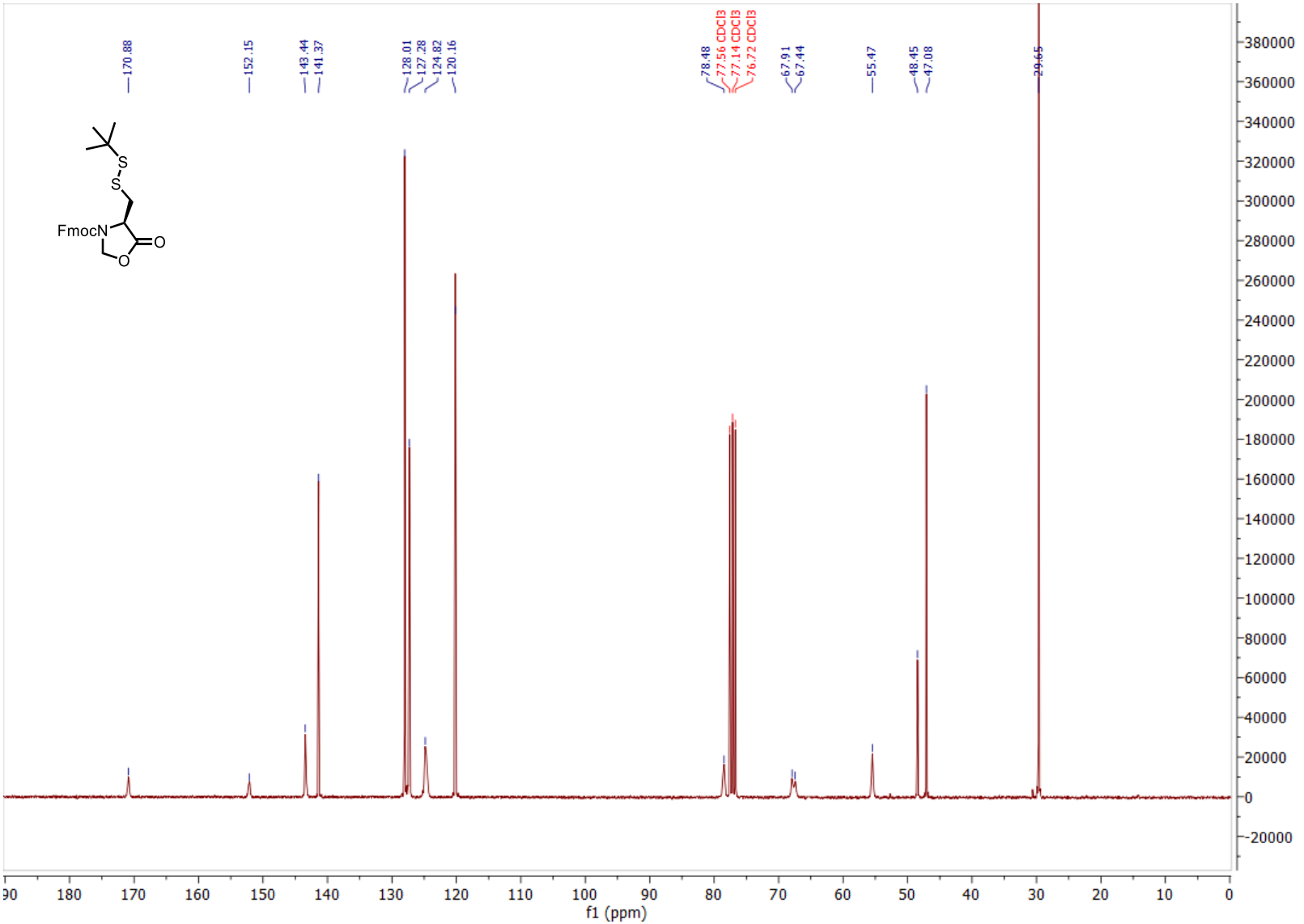
Ub76 and UbPA were transferred from DMSO stocks (2 mM) , and appropriate volumes were transferred from these stocks to the empty plate 384-well plate (Corning3820) using a Labcyte Echo550 acoustic dispenser and accompanying dose–response software to obtain a 6-point serial dilution (4 replicates) of 0/0.5/1/2.5/5/10µM. A DMSO backfill was performed to obtain equal volumes of DMSO (37.5 nL, 0.25%) in each well. Cell lysates were equalized with their intrinsic absorbance at 280nm, diluted 150 fold with the assay buffer (50 mM HEPES, 150 mM NaCl at pH 7.5, 1.0 mM TCEP, 0.5 mg/mL 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid (CHAPS)), and 9µL were dispensed to the plate, and incubated for 30 minutes at room temperature. Then, 6uL of UbSRhodol (2.5X stock, 1µM final concentration) were added with a Biotek MultiFlowFX dispenser, the plate was centrifuged (1000 rpm, 1min), and ligase-mediated release of Rhodol was measured at the emission wavelength of 520 nm (±10 nm) after excitation at 485 nm (±10 nm) in a Pherastar plate reader (BMG LABTECH GmbH, Germany) in continuous mode for several timepoints. Gain (5 /10%) was set at the probe only well.

Chemical Characterization Compounds

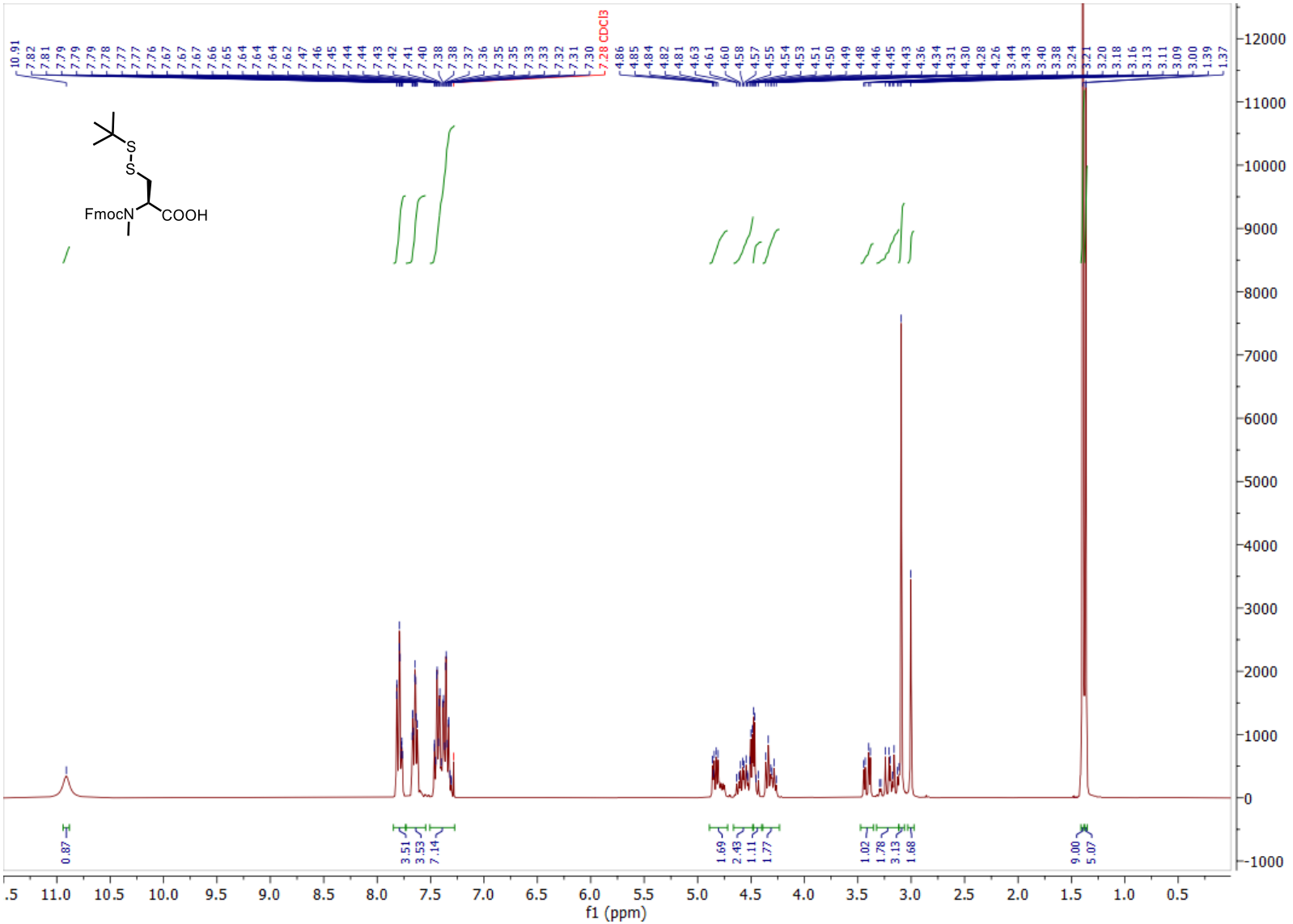
Compound **3**: ^{1}H NMR (300 MHz, $CDCl_3$)



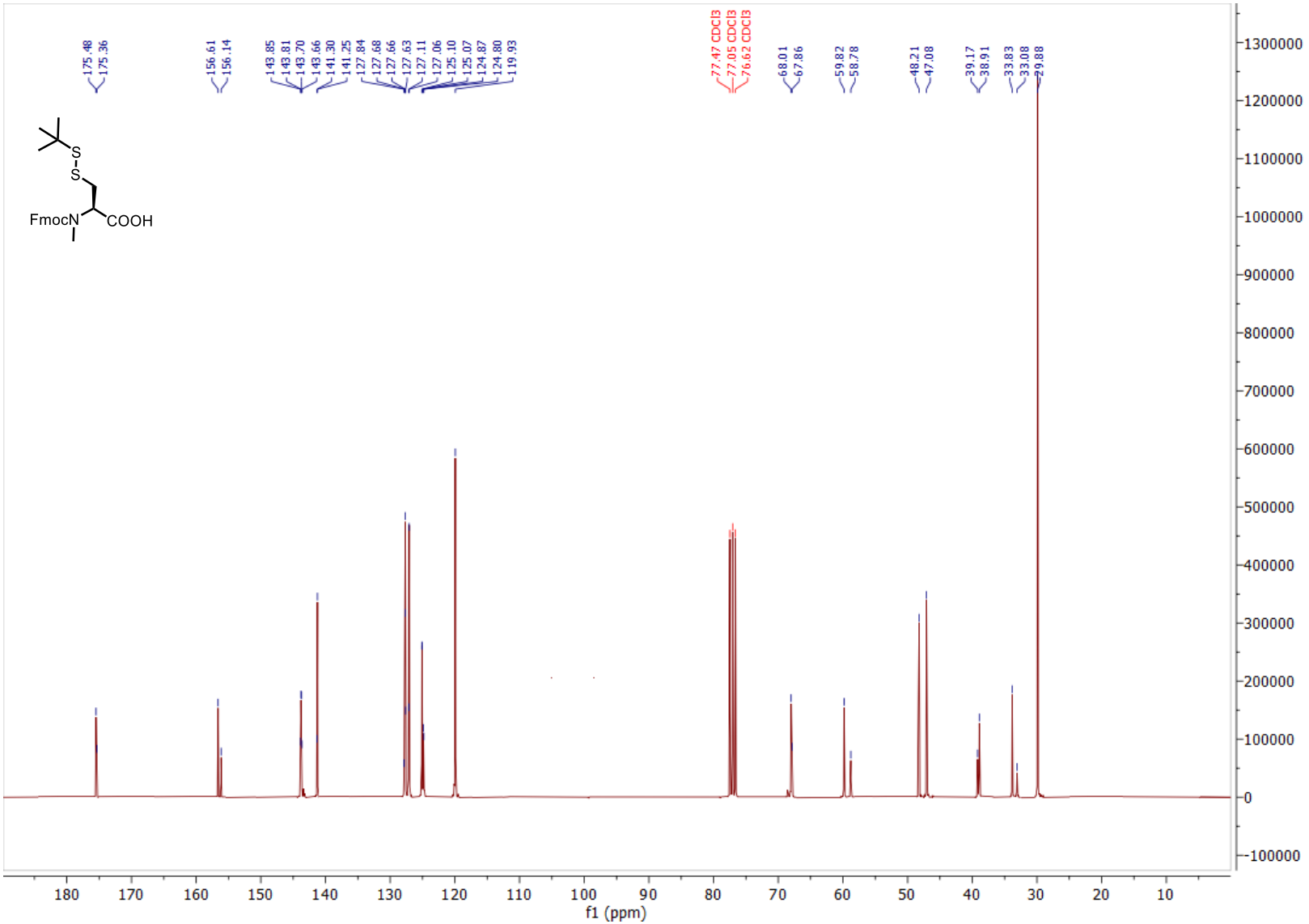
Compound **3**: ^{13}C NMR (75 MHz, $CDCl_3$)



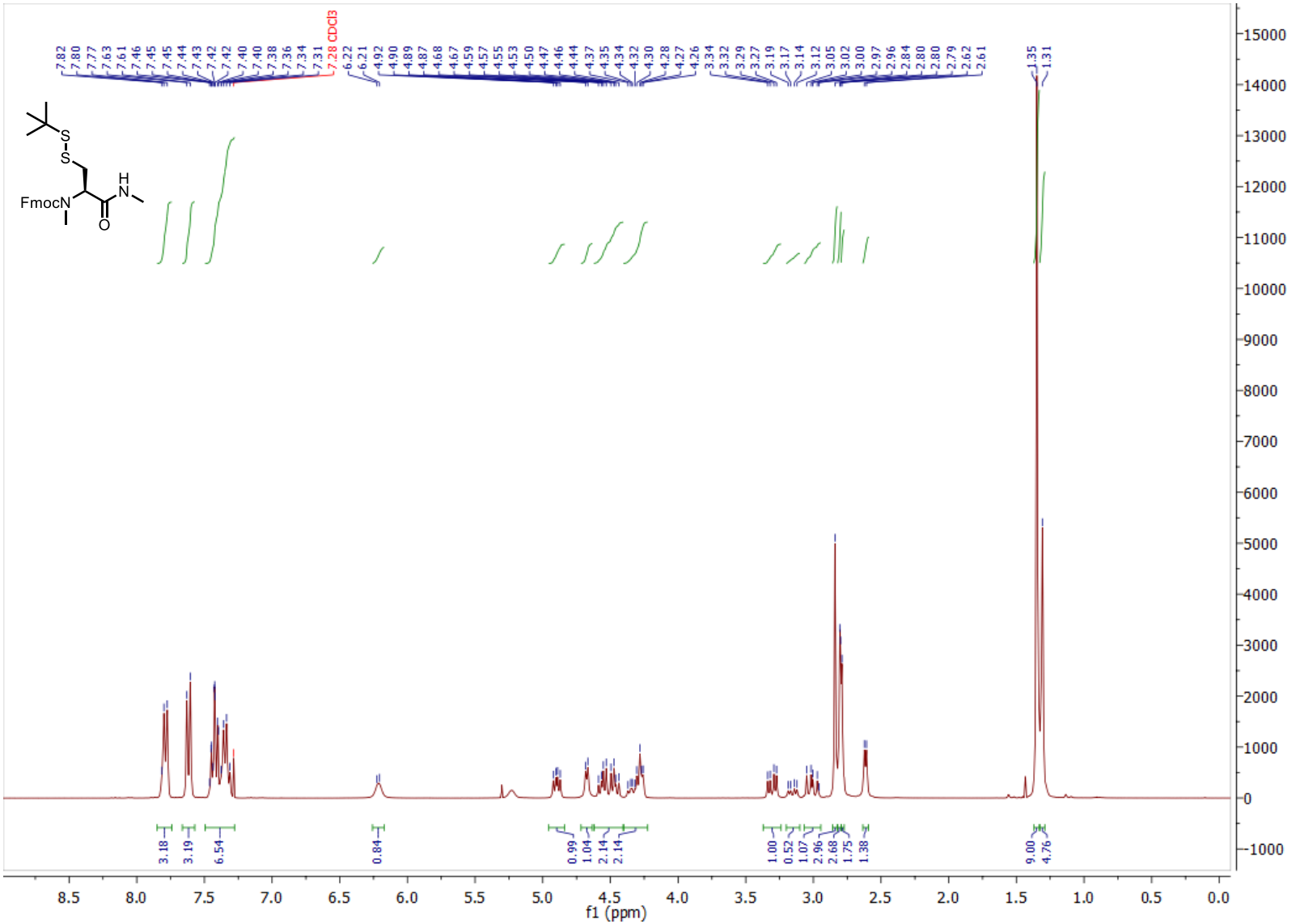
Compound **4**: ^{1}H NMR (300 MHz, $CDCl_3$)



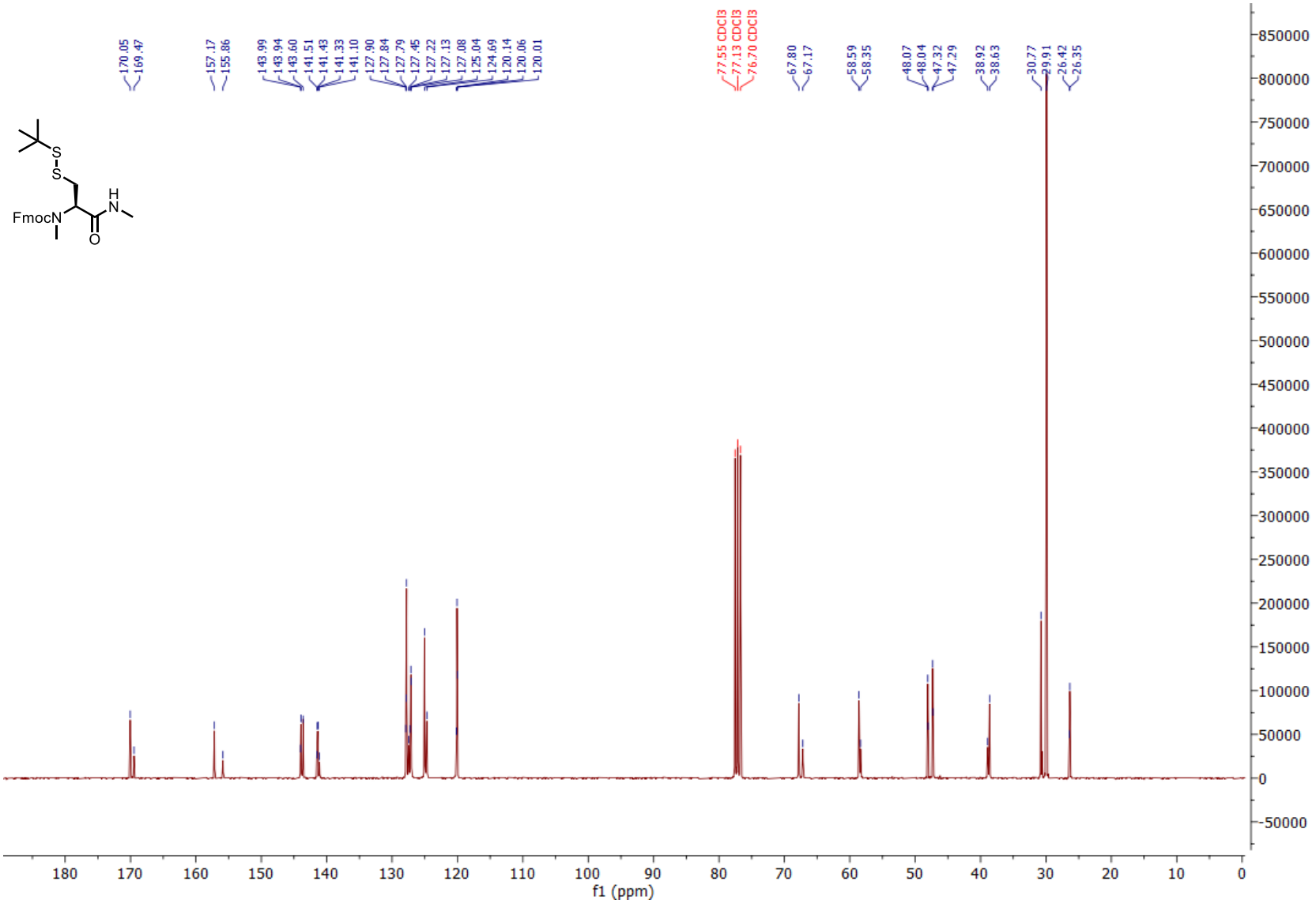
Compound **4**: ^{13}C NMR (75 MHz, $CDCl_3$)



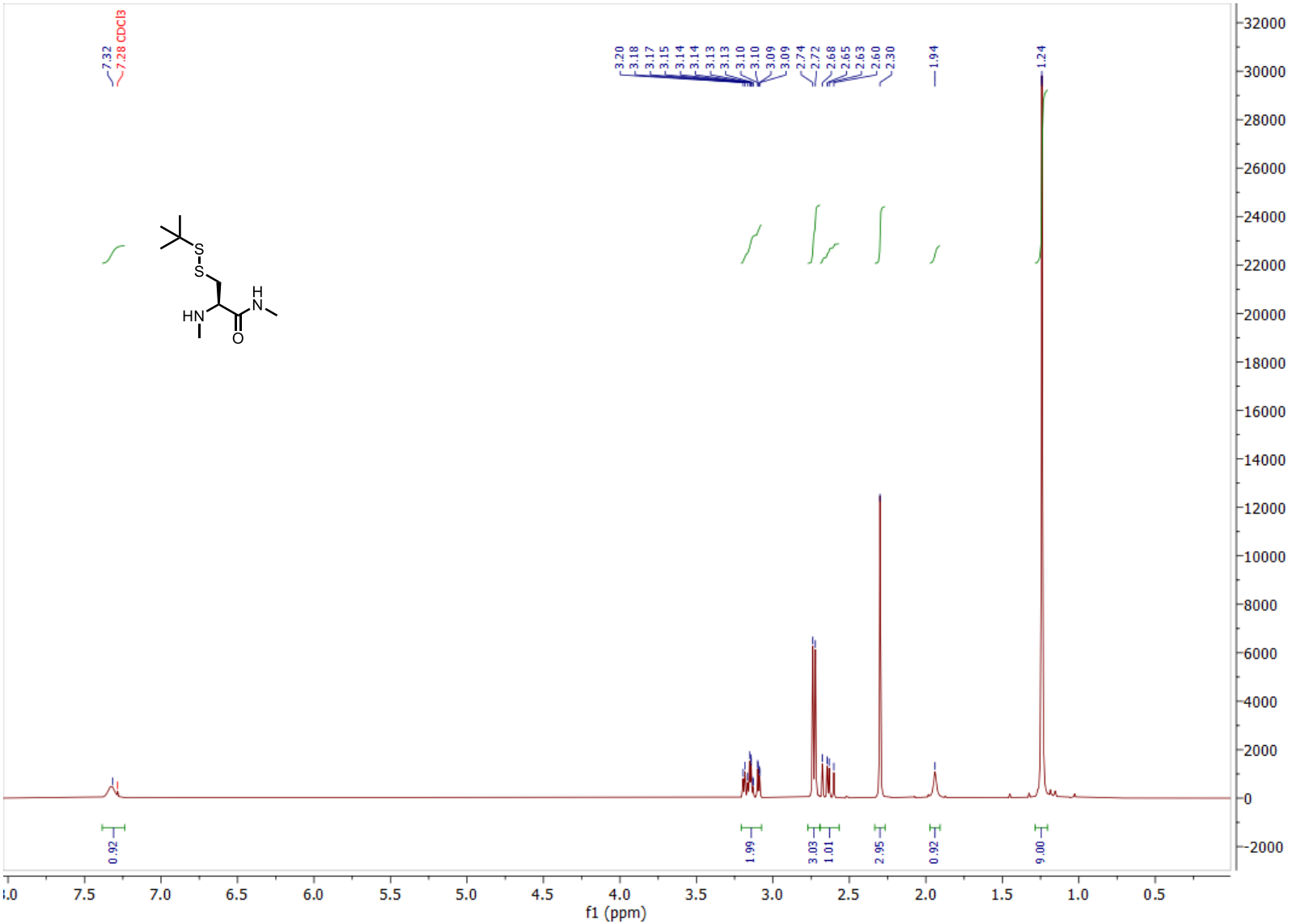
Compound **5**: ^{1}H NMR (300 MHz, $CDCl_3$)



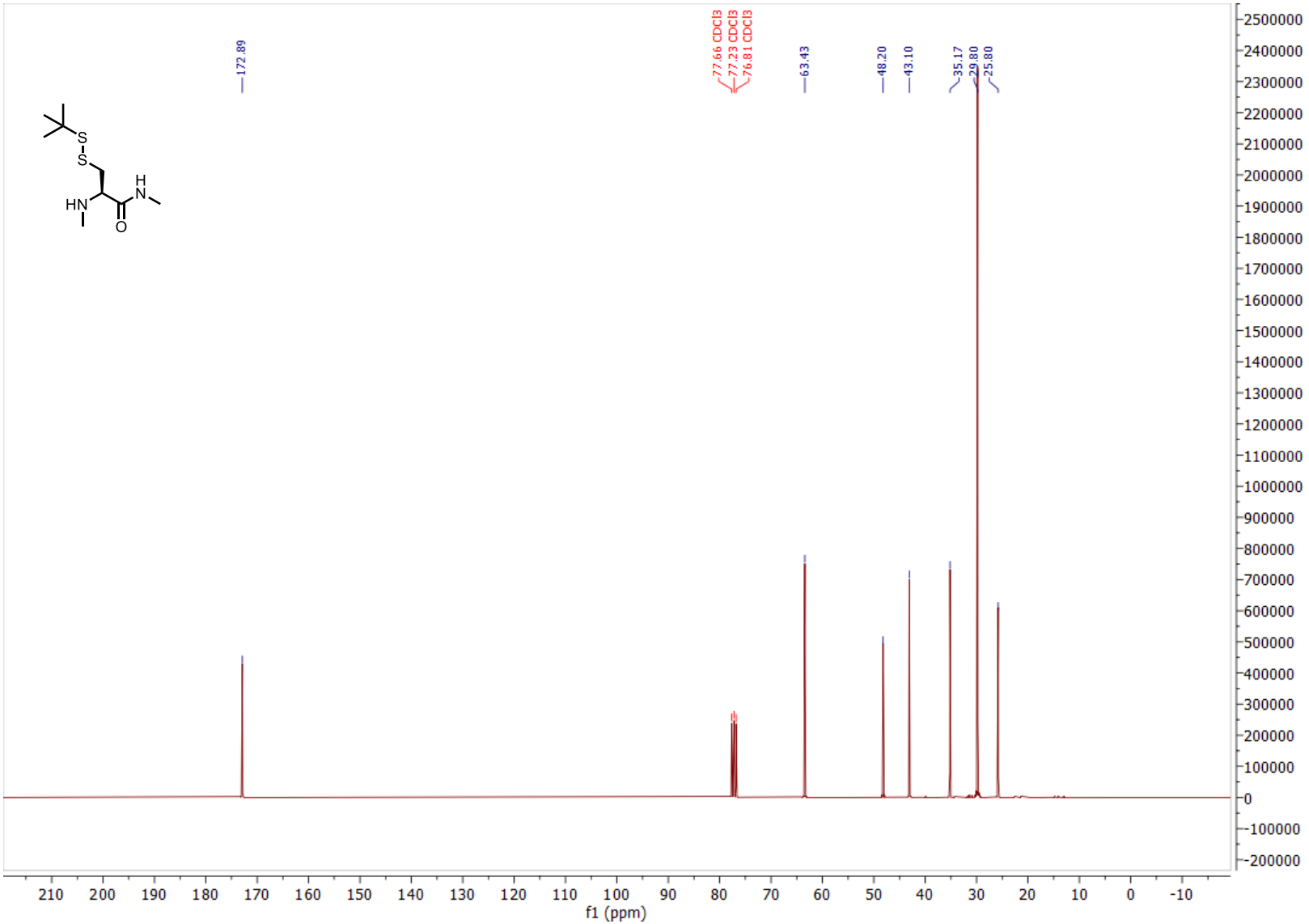
Compound **5**: ^{13}C NMR (75 MHz, $CDCl_3$)



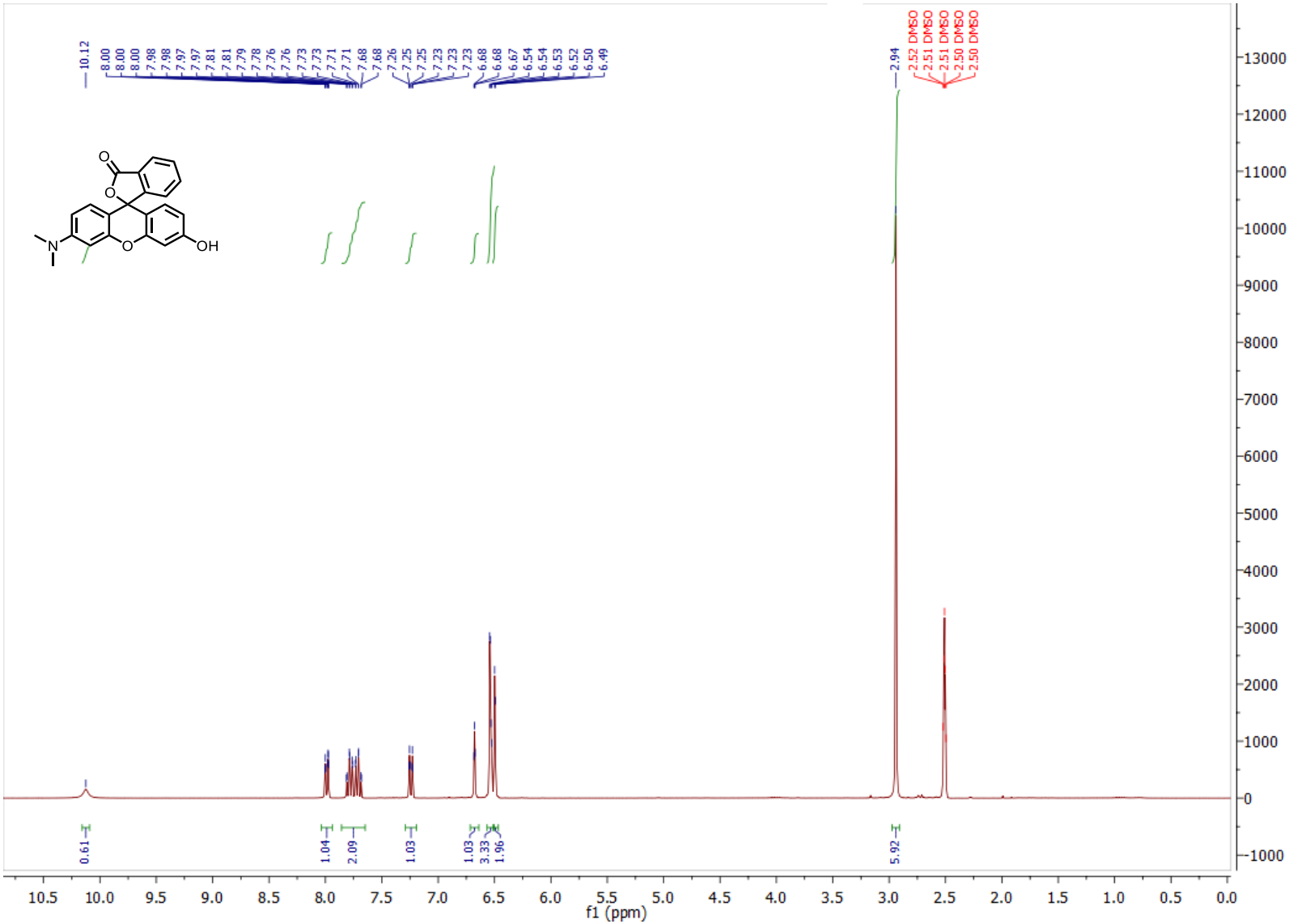
Compound **6**: ^{1}H NMR (300 MHz, $CDCl_3$)



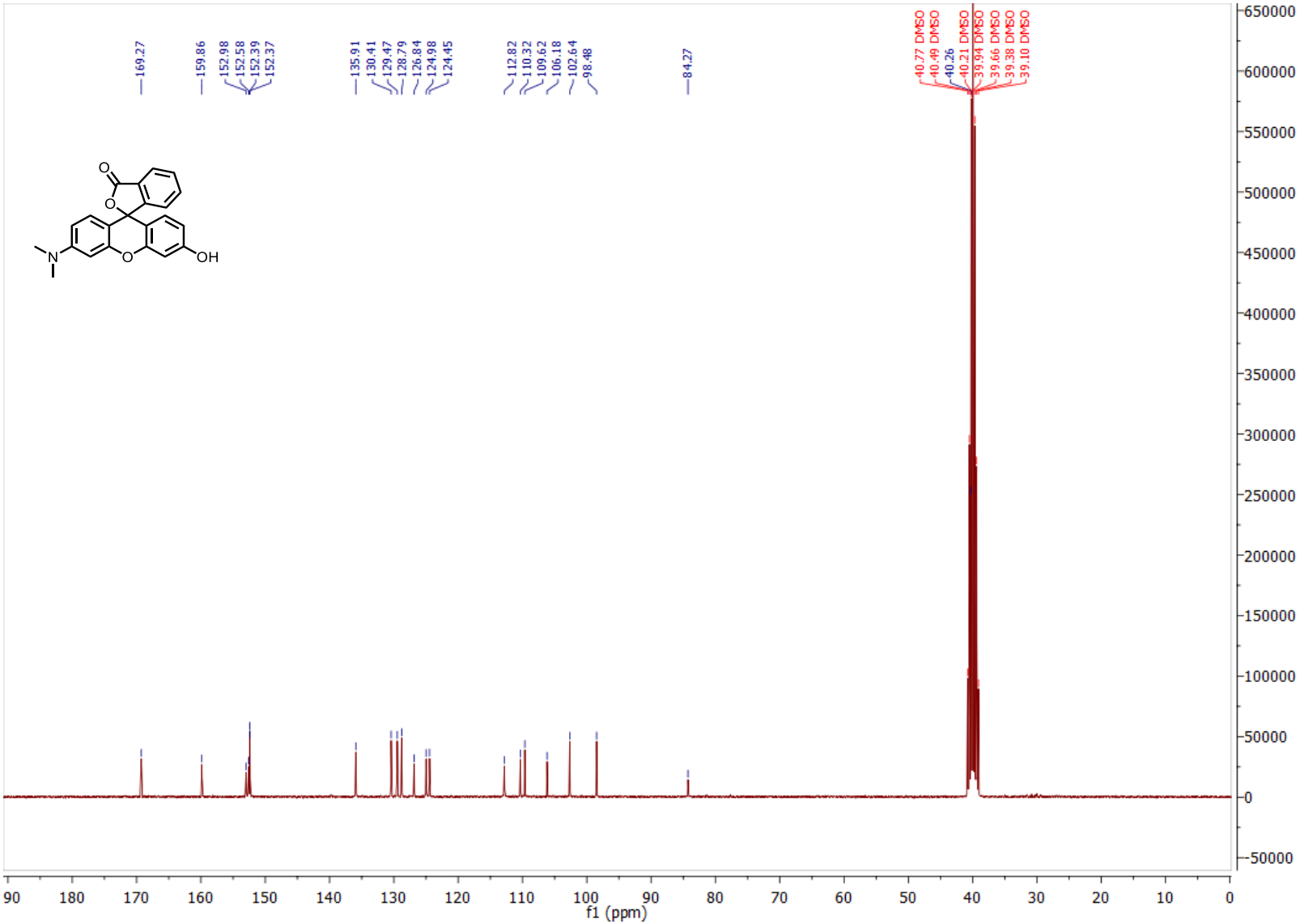
Compound **6**: ^{13}C NMR (75 MHz, $CDCl_3$)



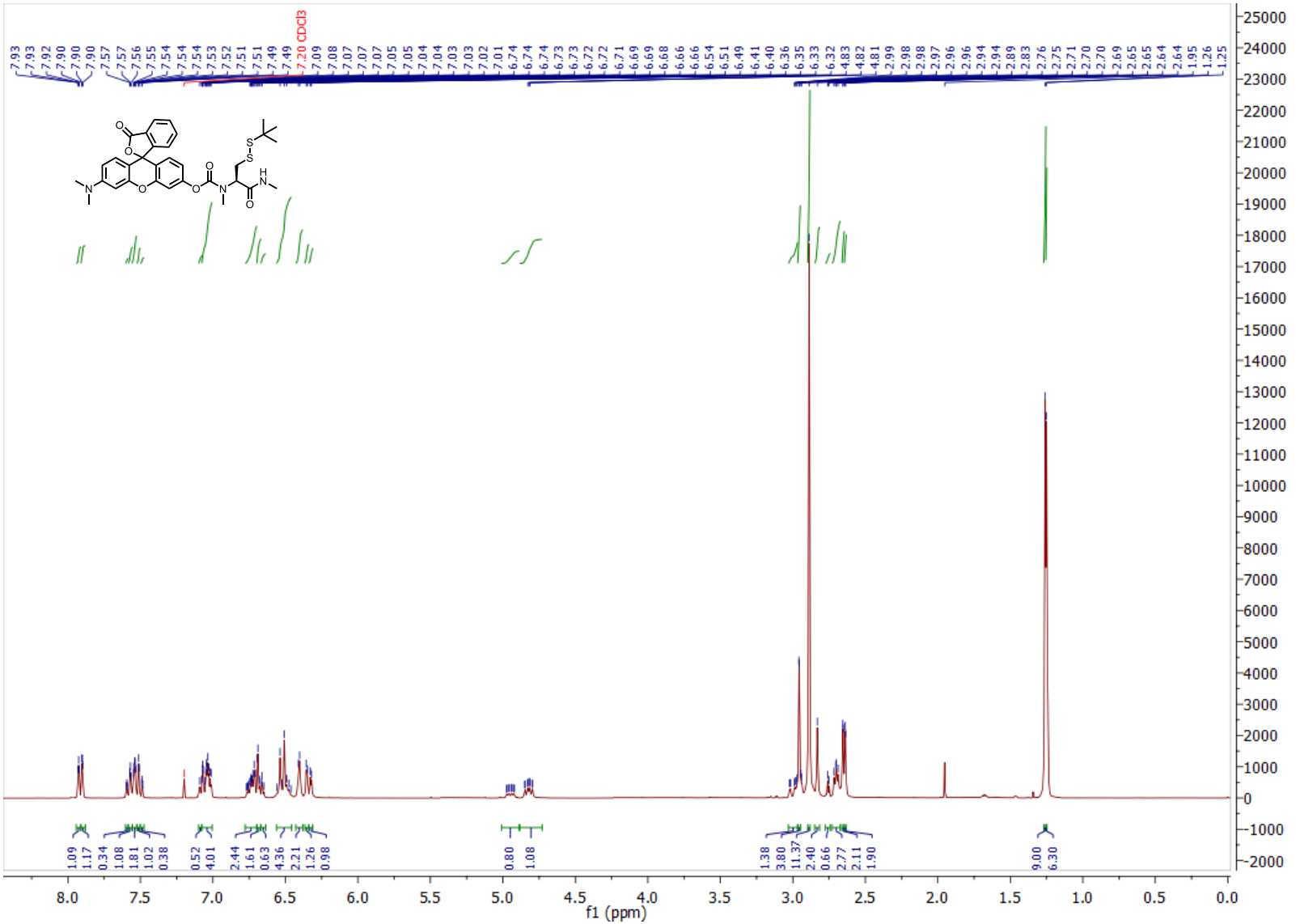
Compound **7**: ^{1}H NMR (300 MHz, DMSO-d_6)



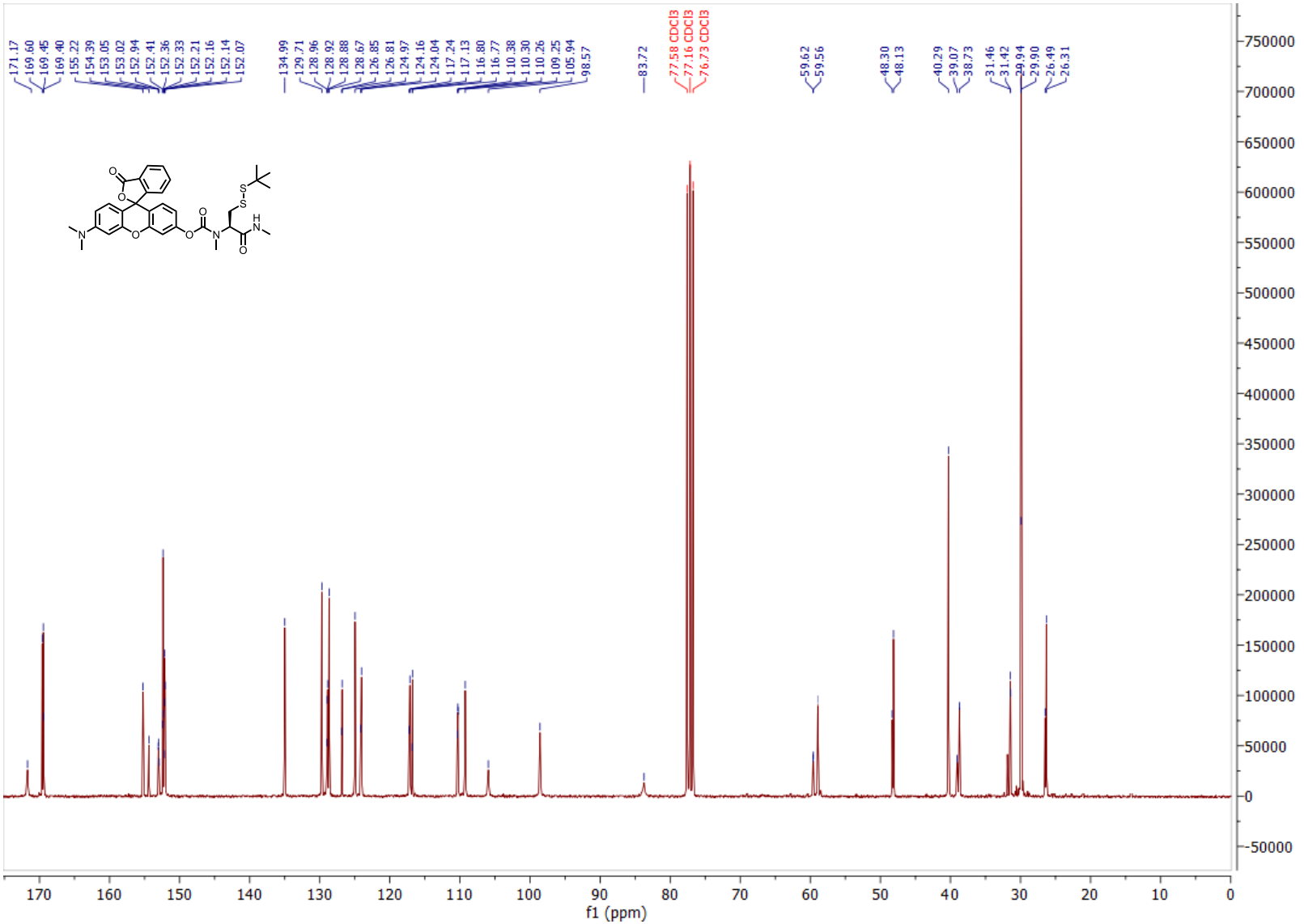
Compound **7**: ^{13}C NMR (75 MHz, DMSO-d_6)



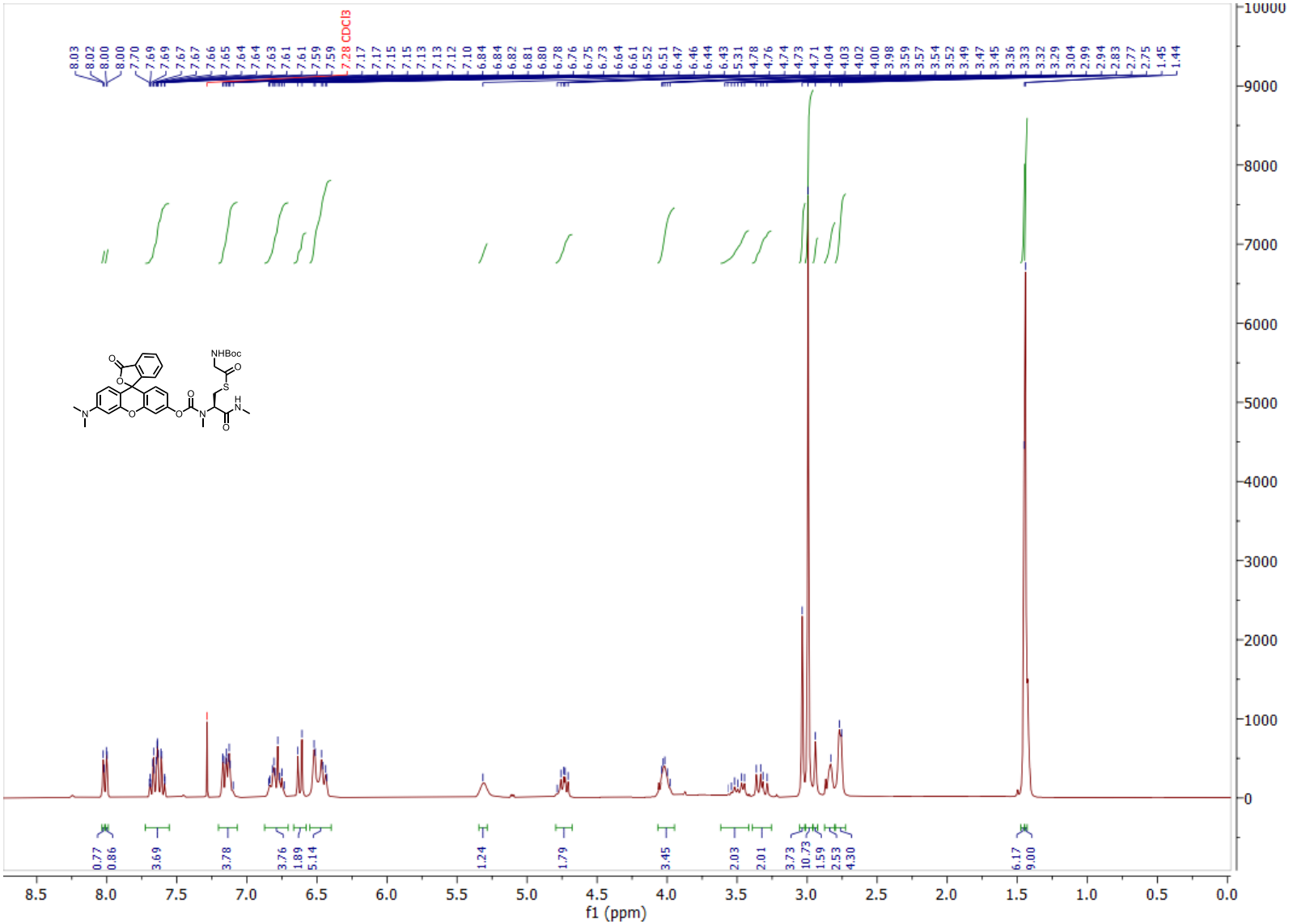
Compound **8**: ^{1}H NMR (300 MHz, $CDCl_3$)



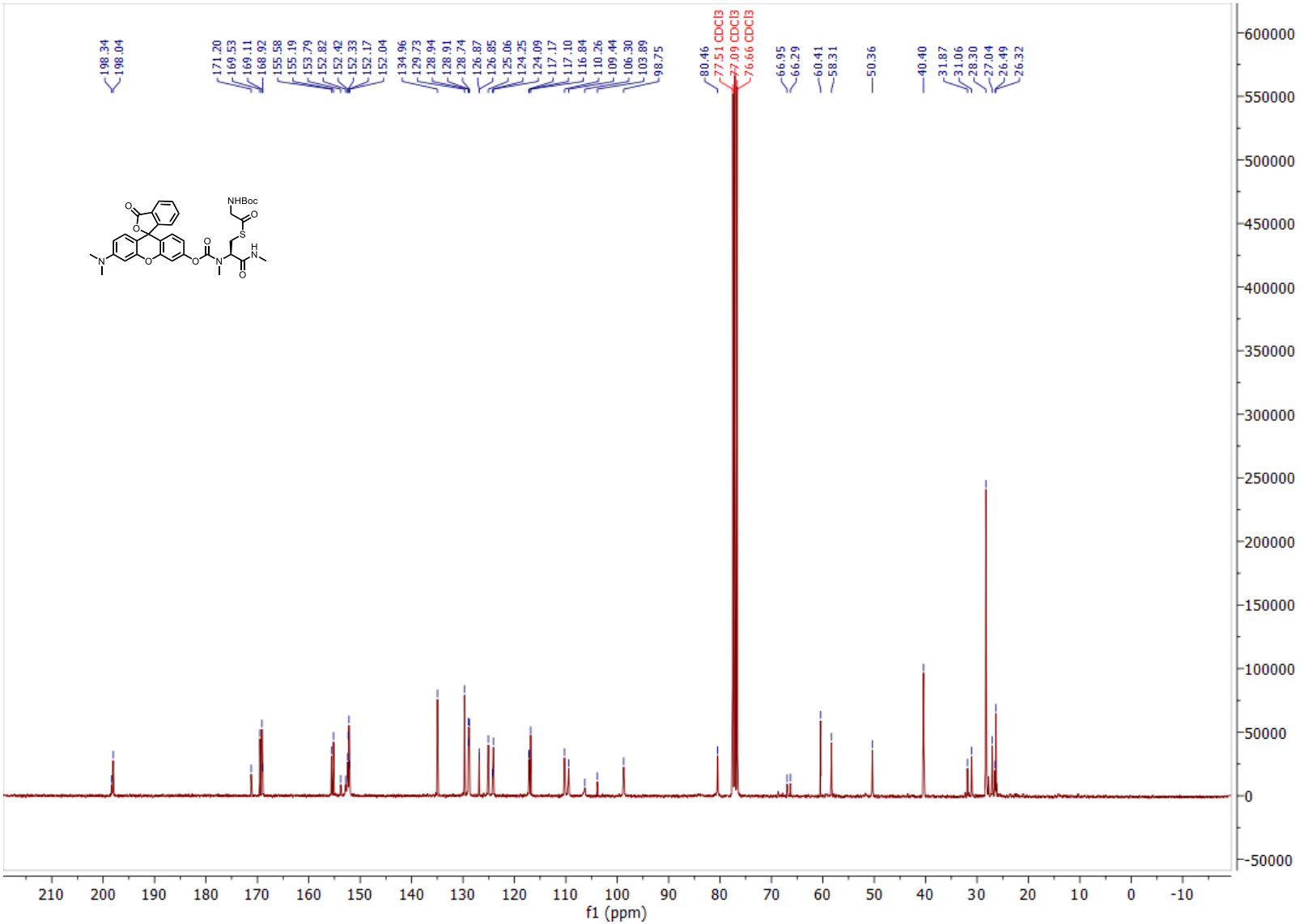
Compound **8**: ^{13}C NMR (75 MHz, $CDCl_3$)



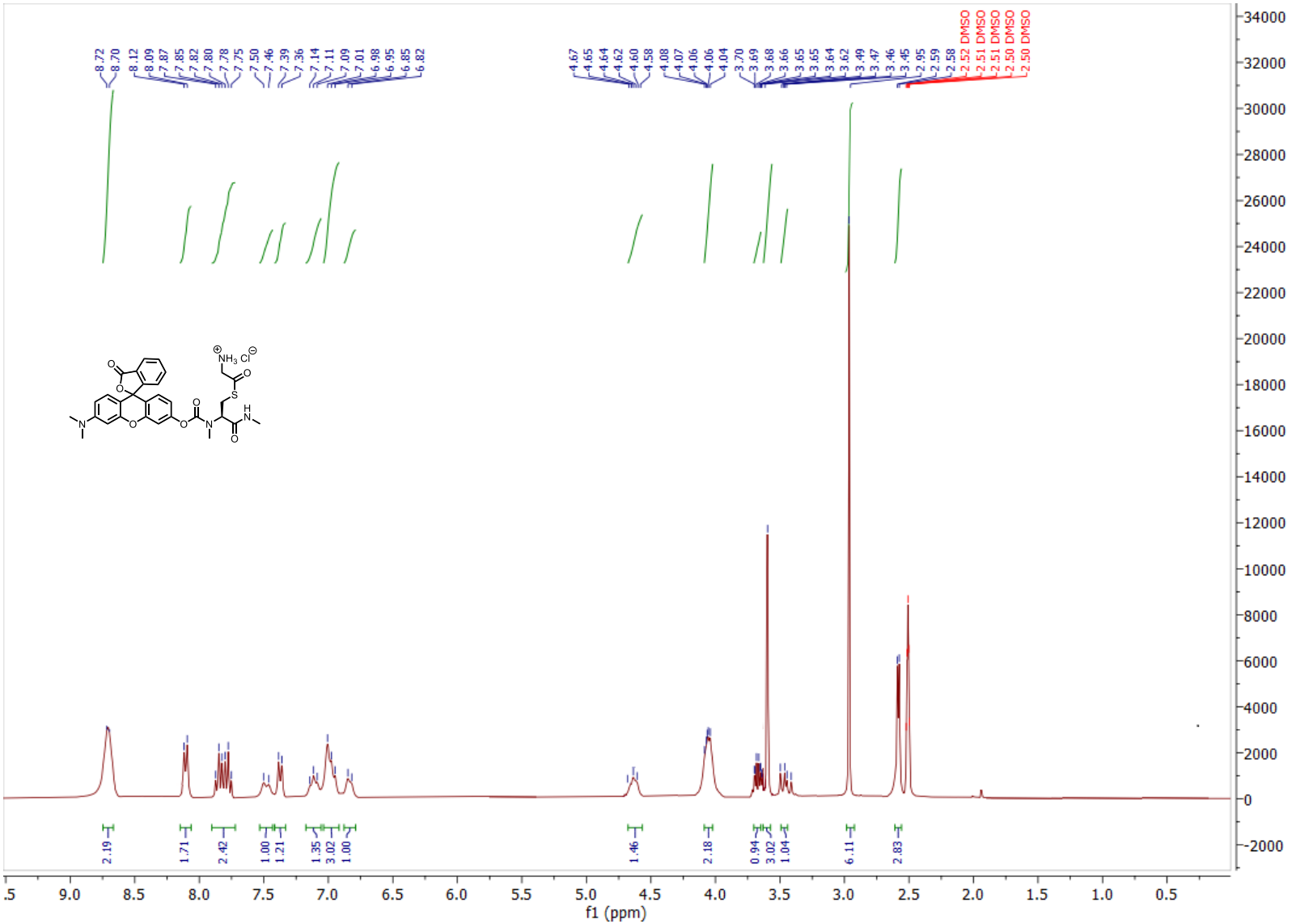
Compound **9**: ^{1}H NMR (300 MHz, $CDCl_3$)



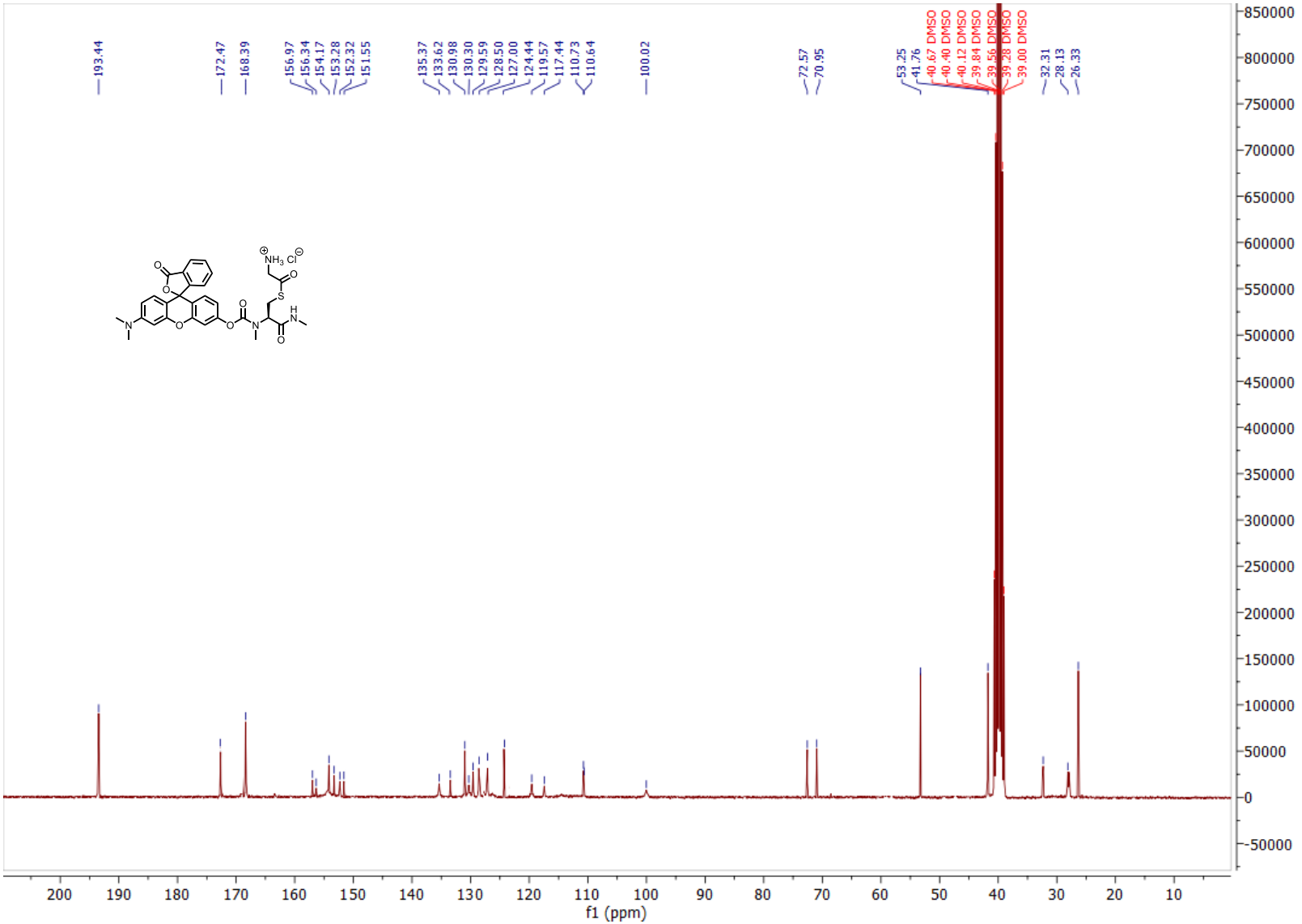
Compound **9**: ^{13}C NMR (75 MHz, $CDCl_3$)



Compound **1**: ^{1}H NMR (300 MHz, DMSO-*d*6)



Compound **1**: ^{13}C NMR (75 MHz, DMSO-*d*6)



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