

Supporting Information

for

Synthesis of *N*-Hydroxy Isopeptide Containing Proteins

Suman Kumar Maity, Shimrit Ohayon and Ashraf Brik*

Schulich Faculty of Chemistry Technion—Israel Institute of Technology, 3200008
Haifa, Israel

abrik@technion.ac.il

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Experimental Section

Materials and methods

General methods

^1H and ^{13}C NMR spectra were recorded using CDCl_3 as a solvent. Chemical shifts were reported in δ units (ppm) with reference to TMS as an internal standard, and J values are given in Hz. ^1H and ^{13}C -NMR spectra were recorded on a Bruker AMX-400 MHz and Bruker AMX-500 MHz spectrometers. Mass determination of the materials was carried out using an LCQ Fleet Ion Trap (Thermo Scientific). Flash column chromatography was carried out with silica gel (220–440 mesh). The reactions were carried out in oven-dried glassware under nitrogen. Chemicals were purchased from Aldrich, Fluka and Alfa Aesar. Commercial reagents were used without further purification. Analytical thin-layer chromatography (TLC) was performed on pre-coated plates (0.25 mm, silica gel 60 F254). Compound spots were visualized by UV light (254 nm) and were stained with 12-molybdophosphoric acid hydrate.

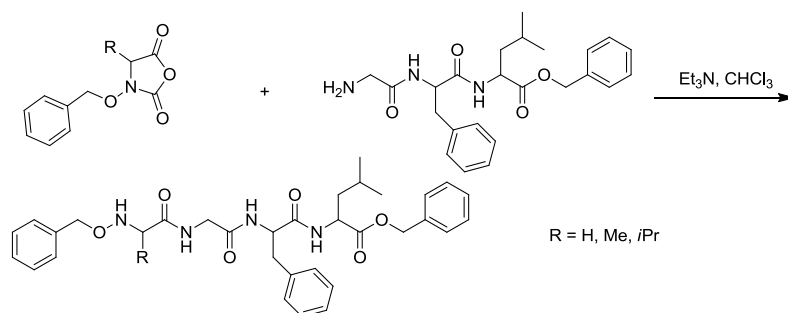
Solid Phase Peptide Synthesis (SPPS): Resins were purchased from Creosalus, protected amino acids were purchased from GL Biochem and activating reagents [2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), hydroxybenzotriazole (HOBt), [(6-chlorobenzotriazol-1-yl)oxy-(dimethylamino)methylidene]-dimethylazanium;hexafluorophosphate (HCTU), 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU)] were purchased from Luxembourg Bio Technologies. DMF and piperidine were purchased from Bio-Lab ltd. Peptide synthesis was carried out manually in syringes equipped with teflon filters, purchased from Torviq or by using automated peptide synthesizer (CS Bio Co. CS336x). If not described differently, all reactions were carried out at room temperature.

Peptide analysis: Analytical HPLC for monitoring the progress of reactions and peptide purity was performed on a Thermo instrument (Spectra System p4000) using analytical columns (Xbridge BEH300 C4 3.5 μm , 4.6 \times 150 mm and XSELECT CSH C18 3.5 μm , 4.6 \times 150 mm) at a flow rate of 1.2 mL/min. Preparative HPLC was performed on a

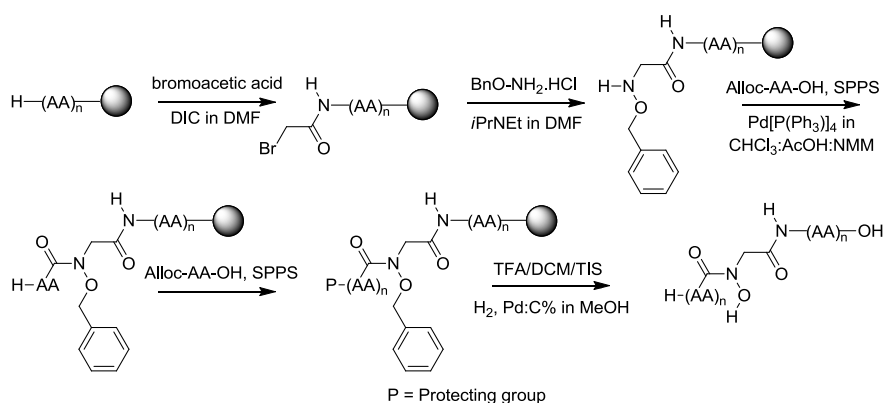
Waters instrument using XSELECT CSH 10 μm , 19 \times 250 mm and Jupiter 10 μm , 300 \AA , 250 \times 10 mm C18 columns, at flow rate of 15 mL/min. Semi preparative HPLC was performed on Thermo scientific instrument (spectra system SCM1000) using Xbridge BEH300 C4 10 μm , 10 \times 150 mm column with a flow rate 4 mL/min. Mass spectrometry analysis was carried out using LCQ Fleet Ion Trap (Thermo Scientific). Buffer A: 0.1% TFA in water; Buffer B: 0.1% TFA in acetonitrile.

Previously reported synthetic procedures for *N*-hydroxy amide containing peptides and *N*-hydroxy amino acids:

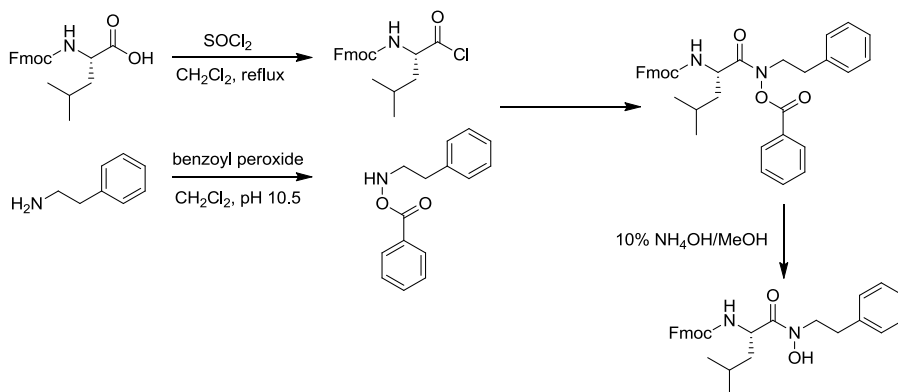
1. Reference 10.



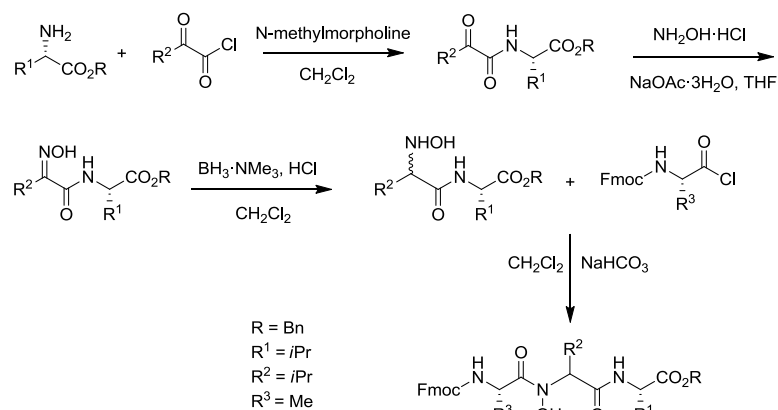
2. Reference 7a.



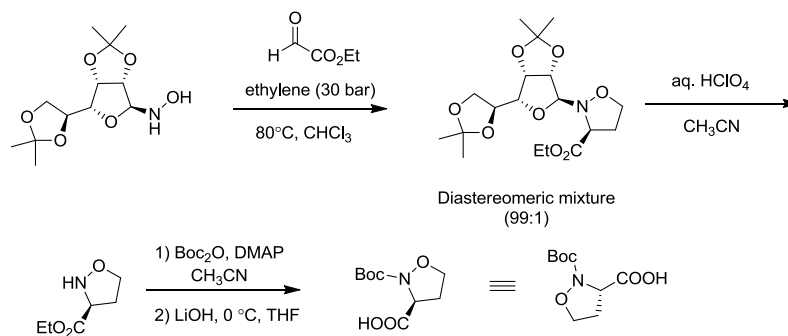
3. Reference 11.



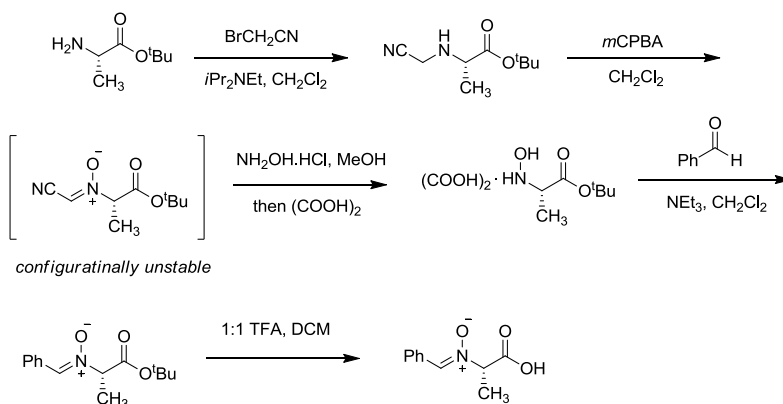
4. Reference 12.



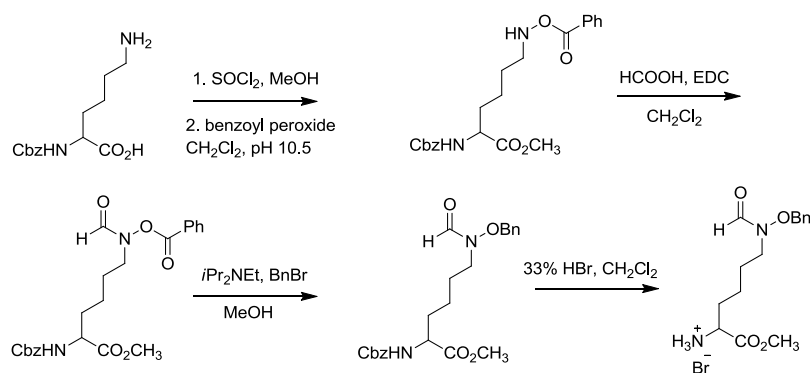
5. Reference 13a.



6. Reference 13b.

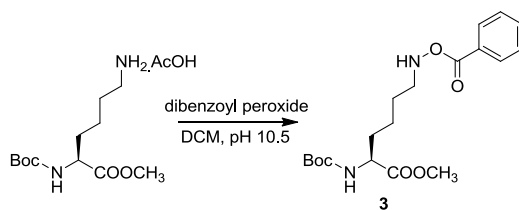


7. Reference 14.



Scheme S1: Schematic presentation of the synthesis of *N*-hydroxy amide containing peptides and *N*-hydroxy amino acids.

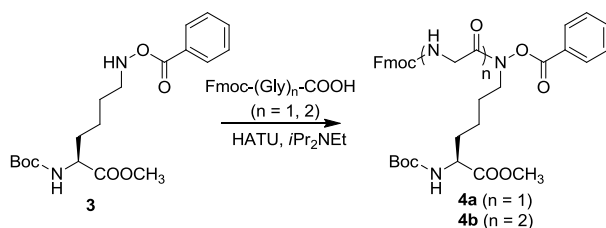
Synthesis of compound 3:



(*S*)-Methyl 6-amino-2-((*tert*-butoxycarbonyl)amino)hexanoate acetate (2 g, 6.24 mmol) was dissolved in 30 mL of CH₂Cl₂. The resulting solution was diluted with 30 mL of an aqueous NaHCO₃/NaOH buffer (pH 10.5). Dibenzoyl peroxide (3 g, 9.36 mmol) was added to the biphasic solution and the resulting mixture was stirred vigorously for 4 h. The phases were separated and the aqueous layer was extracted with CH₂Cl₂ (3 × 30 mL).

The combined organic layers were washed with an aqueous solution of 10% $\text{Na}_2\text{S}_2\text{O}_3$ (2×50 mL), dried over anhydrous Na_2SO_4 , filtered and the solvent was removed under reduced pressure. The resulting crude material was purified by flash column chromatography (25% EtOAc : hexanes) to afford **3** as a gummy liquid (1.6 g, 70% yield). ^1H NMR (400 MHz, CDCl_3 , δ ppm): 1.43 (s, 9H), 1.46-1.89 (m, 6H), 3.12 (t, $J = 8\text{ Hz}$, 2H), 3.73 (s, 3H), 4.29-4.34 (m, 1H), 5.04 (d, $J = 8\text{ Hz}$, 1H), 7.43-7.47 (m, 2H), 7.56-7.60 (m, 1H), 7.89 (br s, 1H), 8.00-8.02 (m, 2H); ^{13}C NMR (100 MHz, CDCl_3 , δ ppm): 23.02, 27.06, 28.64, 32.78, 52.39, 52.55, 53.42, 80.08, 128.52, 128.70, 129.41, 129.60, 133.58, 155.53, 167.07, 173.43. ESI-MS: Calculated for $\text{C}_{19}\text{H}_{28}\text{N}_2\text{NaO}_6$: 403.2 Da, Observed: 403.1 Da $[\text{M}+\text{Na}]$.

Synthesis of compounds **4a** and **4b**:

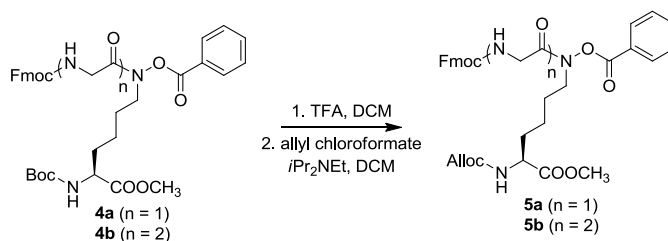


To a stirred solution of compound **3** (1.5 g, 3.9 mmol) in dry CH_2Cl_2 , Fmoc-Gly-OH (1.7 g, 5.8 mmol), HATU (2.2 g, 5.8 mmol) in 5 mL DMF and *N,N*-Diisopropylethylamine (DIEA) (2 mL, 11.7 mmol) were added at room temperature. Stirring was continued for 15 h. DCM was evaporated under vacuum and the residue was dissolved in ethylacetate followed by washing with 2 N HCl (3×50 mL), 1 M NaHCO_3 (3×50 mL) and brine solution, respectively. The combined organic layers were dried over Na_2SO_4 , filtered and solvent was removed under reduced pressure. The resulting crude material was purified by flash column chromatography (30% EtOAc : hexanes) to afford compound **4a** as a white solid (1.8 g, 73% yield). ^1H NMR (400 MHz, CDCl_3 , δ ppm): 1.43 (s, 9H), 1.47-1.88 (m, 6H), 3.72 (s, 3H), 3.84-3.86 (m, 2H), 4.04 (d, $J = 4\text{ Hz}$, 2H), 4.22 (t, $J = 7.2\text{ Hz}$, 1H), 4.27-4.32 (m, 1H), 4.36 (d, $J = 7.2\text{ Hz}$, 2H), 5.09 (d, $J = 8\text{ Hz}$, 1H), 5.60 (br s, 1H), 7.31 (t, $J = 7.6\text{ Hz}$, 2H), 7.39 (t, $J = 7.2\text{ Hz}$, 2H), 7.53 (t, $J = 7.6\text{ Hz}$, 2H), 7.60 (d, $J = 7.2\text{ Hz}$, 2H), 7.70 (t, $J = 7.6\text{ Hz}$, 1H), 7.76 (d, $J = 7.2\text{ Hz}$, 2H), 8.08 (d, $J = 7.6\text{ Hz}$, 2H);

^{13}C NMR (100 MHz, CDCl_3 δ ppm): 22.51, 28.40, 28.54, 32.40, 42.21, 47.25, 52.40, 53.37, 67.42, 80.12, 120.03, 120.23, 125.24, 125.40, 126.19, 127.25, 127.86, 129.17, 130.23, 130.47, 141.43, 144.01, 155.58, 156.39, 164.37, 173.33. Calculated for $\text{C}_{36}\text{H}_{41}\text{N}_3\text{NaO}_9$: 682.3 Da, Observed: 682.2 Da $[\text{M}+\text{Na}]$.

Compound **4b** was synthesized similarly by using Fmoc-Gly-Gly-OH instead of Fmoc-Gly-OH. The resulting crude was purified by flash column chromatography (65% EtOAc : hexanes) to afford compound **4b** as a white solid (1.56 g, 57% yield). ^1H NMR (400 MHz, CDCl_3 , δ ppm): 1.42 (s, 9H), 1.47-1.93 (m, 6H), 3.70 (s, 3H), 3.80-3.82 (m, 2H), 3.93 (d, $J = 4$ Hz, 2H), 4.08-4.10 (m, 2H), 4.22 (t, $J = 6.8$ Hz, 1H), 4.26-4.31 (m, 1H), 4.40 (d, $J = 8$ Hz, 2H), 5.13 (d, $J = 8$ Hz, 1H), 5.63 (br s, 1H), 6.84 (br s, 1H), 7.29 (t, $J = 7.2$ Hz, 2H), 7.38 (t, $J = 7.6$ Hz, 2H), 7.52 (t, $J = 7.6$ Hz, 2H), 7.59 (d, $J = 6.8$ Hz, 2H), 7.69 (t, $J = 7.2$ Hz, 1H), 7.74 (d, $J = 7.6$ Hz, 2H), 8.07 (d, $J = 7.6$ Hz, 2H); ^{13}C NMR (100 MHz, CDCl_3 δ ppm): 22.35, 26.27, 28.14, 28.33, 32.18, 40.44, 44.27, 47.05, 52.15, 53.11, 67.16, 79.88, 119.74, 120.05, 124.93, 125.88, 127.03, 127.69, 129.19, 129.95, 130.30, 141.21, 143.73, 155.36, 156.50, 164.12, 169.05, 173.13. Calculated for $\text{C}_{38}\text{H}_{44}\text{N}_4\text{NaO}_{10}$: 739.3 Da $[\text{M}^+]$, Observed: 739.3 Da $[\text{M}+\text{Na}]$.

Synthesis of compounds **5a** and **5b**:

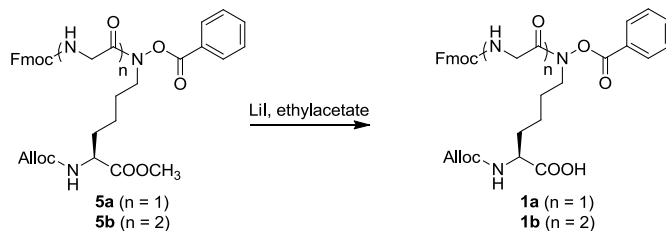


0.84 g (1.3 mmol) of compound **4a** was treated with 8 mL of TFA : DCM (1:1) and stirred for 1 h at room temperature. Subsequently all volatiles were evaporated and dried under high vacuum for overnight. To this material, 0.415 mL (3.9 mmol) allylchloroformate and 0.68 mL (3.9 mmol) DIEA were added in dry DCM under nitrogen atmosphere and stirred at room temperature for 3 h. Solvent and other volatiles were evaporated under reduced pressure and the material was subjected to flash column

chromatography (30% EtOAc : hexanes) to afford compound **5a** as a white solid (0.74 g, 90% yield). ¹H NMR (400 MHz, CDCl₃, δ ppm): 1.40-1.91 (m, 6H), 3.72 (s, 3H), 3.80-3.90 (m, 2H), 4.04 (d, *J* = 4 Hz, 2H), 4.22 (t, *J* = 7.2 Hz, 1H), 4.27-4.32 (m, 1H), 4.36 (d, *J* = 7.2 Hz, 2H), 4.54 (d, *J* = 5.6 Hz, 2H), 5.17-5.19 (m, 1H), 5.25-5.30 (m, 1H), 5.48 (d, *J* = 8 Hz, 1H), 5.66 (br s, 1H), 5.83-5.93 (m, 1H), 7.30 (t, *J* = 7.6 Hz, 2H), 7.39 (t, *J* = 7.2 Hz, 2H), 7.52 (t, *J* = 7.6 Hz, 2H), 7.60 (d, *J* = 7.2 Hz, 2H), 7.68 (t, *J* = 7.6 Hz, 1H), 7.75 (d, *J* = 7.2 Hz, 2H), 8.08 (d, *J* = 7.6 Hz, 2H); ¹³C NMR (100 MHz, CDCl₃ δ ppm): 22.32, 26.38, 32.03, 42.12, 47.17, 48.23, 52.42, 53.69, 65.92, 67.33, 117.97, 119.96, 125.18, 126.12, 127.17, 127.78, 129.10, 130.15, 132.75, 134.93, 141.34, 143.94, 155.95, 156.40, 164.29, 169.31, 173.33. Calculated for C₃₅H₃₇N₃NaO₉: 666.2 Da, Observed: 667.4 Da [M+Na].

Compound **5b** was synthesized similarly from **4b** (0.660 g, 0.94 mmol) and purified by flash column chromatography (70% EtOAc : hexanes) with 86% yield (0.58 g). ¹H NMR (500 MHz, CDCl₃, δ ppm): 1.37-1.86 (m, 6H), 3.70 (s, 3H), 3.80-3.84 (m, 2H), 3.93 (d, *J* = 5 Hz, 2H), 4.08-4.10 (m, 2H), 4.20 (t, *J* = 7 Hz, 1H), 4.31-4.35 (m, 1H), 4.38 (d, *J* = 7 Hz, 2H), 4.53 (d, *J* = 5.5 Hz, 2H), 5.16-5.19 (m, 1H), 5.25-5.29 (m, 1H), 5.60 (d, *J* = 8 Hz, 2H), 5.81-5.9 (m, 2H), 6.99 (br s, 1H), 7.28 (t, *J* = 7.5 Hz, 2H), 7.37 (t, *J* = 7 Hz, 2H), 7.51 (t, *J* = 7.5 Hz, 2H), 7.58 (d, *J* = 7 Hz, 2H), 7.67 (t, *J* = 7.5 Hz, 1H), 7.73 (d, *J* = 7.5 Hz, 2H), 8.06 (d, *J* = 7.5 Hz, 2H); ¹³C NMR (125 MHz, CDCl₃ δ ppm): 22.15, 26.18, 31.77, 40.35, 44.16, 46.98, 48.08, 52.32, 53.52, 65.70, 67.07, 117.74, 119.84, 125.02, 125.83, 126.96, 127.57, 128.95, 130.05, 132.53, 134.85, 141.14, 143.70, 155.80, 156.51, 164.09, 168.89, 169.28, 172.80. Calculated for C₃₇H₄₀N₄NaO₁₀: 723.3 Da, Observed: 723.4 Da [M+Na].

Synthesis of compounds **1a** and **1b**:

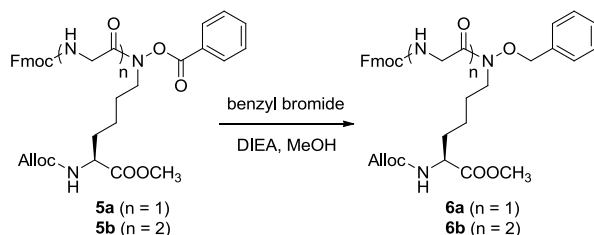


Lithium iodide (0.722 g, 5.4 mmol) was added to a stirred solution of the methyl ester **5a** (0.350 g, 0.54 mmol) in dry ethyl acetate (20 mL) under nitrogen atmosphere. The mixture was heated under reflux for additional 25 h. The reaction was then quenched by the addition of 20 mL 1 M HCl. The resulting mixture was extracted with EtOAc (15 mL \times 2). The combined organic extracts were sequentially washed with 5% aqueous Na₂S₂O₃ solution (8 mL \times 3), 1 M HCl (15 mL) and saturated NaCl solution (15 mL \times 2), dried over anhydrous Na₂SO₄, filtered and evaporated under vacuum. The residue was purified by flash column chromatography (80% EtOAc : hexanes) to afford the target compound **1a** (white solid, 0.254 g, yield 75%). ¹H NMR (500 MHz, CDCl₃, δ ppm): 1.43-1.93 (m, 6H), 3.84-3.93 (m, 2H), 4.04 (br s, 2H), 4.22 (t, $J = 7$ Hz, 1H), 4.35-4.40 (m, 3H), 4.53 (d, $J = 5.5$ Hz, 2H), 5.17 (d, $J = 10.5$ Hz, 1H), 5.25-5.29 (m, 1H), 5.56 (d, $J = 7.5$ Hz, 1H), 5.71 (br s, 1H), 5.83-5.91 (m, 1H), 7.30 (t, $J = 7.5$ Hz, 2H), 7.38 (t, $J = 7.5$ Hz, 2H), 7.52 (t, $J = 7.5$ Hz, 2H), 7.59 (d, $J = 7.5$ Hz, 2H), 7.68 (t, $J = 7.5$ Hz, 1H), 7.74 (d, $J = 7.5$ Hz, 2H), 8.07 (d, $J = 7.5$ Hz, 2H); ¹³C NMR (125 MHz, CDCl₃, δ ppm): 22.06, 26.27, 31.70, 42.16, 47.21, 48.11, 53.63, 66.09, 67.61, 118.10, 120.12, 125.34, 126.14, 127.27, 127.88, 129.24, 130.35, 132.72, 135.11, 141.54, 143.97, 156.19, 156.87, 164.34, 169.56, 175.29. Calculated for C₃₄H₃₅N₃NaO₉: 652.2 Da, Observed: 652.2 Da [M+Na].

Compound **1b** was similarly synthesized from **5b** (0.5 g, 0.71 mmol) and was purified by column chromatography (85% EtOAc : hexanes) with 65% yield (0.316 g). ¹H NMR (400 MHz, CDCl₃, δ ppm): 1.41-1.88 (m, 6H), 3.86 (m, 2H), 3.94 (d, $J = 4.8$ Hz, 2H), 4.08 (m, 2H), 4.19 (t, $J = 6.8$ Hz, 1H), 4.35-4.40 (m, 3H), 4.54 (d, $J = 5.2$ Hz, 2H), 5.16-5.19 (m, 1H), 5.25-5.29 (m, 1H), 5.71 (d, $J = 7.6$, 1H), 5.83-5.93 (m, 2H), 7.07 (br s, 1H), 7.28 (t, $J = 7.2$ Hz, 2H), 7.37 (m, 2H), 7.52 (t, $J = 7.6$ Hz, 2H), 7.58 (d, $J = 7.6$ Hz, 2H),

7.69 (t, $J = 7.6$ Hz, 1H), 7.73 (d, $J = 7.6$ Hz, 2H), 8.07 (d, $J = 7.6$ Hz, 2H); ^{13}C NMR (125 MHz, CDCl_3 δ ppm): 21.43, 25.91, 31.37, 40.35, 44.32, 47.03, 47.49, 53.55, 65.81, 67.32, 117.84, 119.95, 125.10, 125.88, 127.08, 127.71, 129.08, 130.18, 132.63, 134.98, 141.25, 143.70, 155.93, 156.94, 164.12, 170.22, 174.42. Calculated for $\text{C}_{36}\text{H}_{38}\text{N}_4\text{NaO}_{10}$: 709.2 Da, Observed: 709.4 Da $[\text{M}+\text{Na}]$.

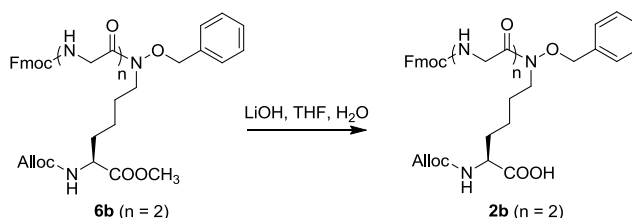
Synthesis of compounds **6b** and **2a**:



Compound **5b** (0.3 g, 0.43 mmol) was dissolved in 15 mL of anhydrous MeOH. 150 μL (0.86 mmol) of DIEA and 152 μL (1.284 mmol) of benzyl bromide were added to this solution and reaction was stirred for 16 h. MeOH was removed under reduced pressure and the residue was dissolved in 50 mL ethyl acetate. After 30 min a white precipitate was formed and removed by filtration. The filtrate was diluted with 50 mL EtOAc and washed with water and brine. The organic layer was dried over anhydrous Na_2SO_4 , filtered and evaporated under vacuum. The residue was purified by flash column chromatography (75% EtOAc : hexanes) to afford the target compound **6b** (white solid, 0.25 g, yield 85%). ^1H NMR (500 MHz, CDCl_3 , δ ppm): 1.28-1.84 (m, 6H), 3.70 (s, 3H), 3.80-3.84 (m, 2H), 3.95 (d, $J = 4.5$ Hz, 2H), 4.15 (m, 2H), 4.22 (t, $J = 7$ Hz, 1H), 4.29-4.34 (m, 1H), 4.39 (d, $J = 7$ Hz, 2H), 4.54 (d, $J = 5.5$ Hz, 2H), 4.83 (s, 2H), 5.18-5.20 (m, 1H), 5.27-5.30 (m, 1H), 5.52 (d, $J = 7.5$ Hz, 1H), 5.75 (t, $J = 5$ Hz, 1H), 5.86-5.91 (m, 1H), 6.86 (br s, 1H), 7.29 (t, $J = 7.5$ Hz, 2H), 7.36-7.40 (m, 7H), 7.59 (d, $J = 7$ Hz, 2H), 7.75 (d, $J = 7.5$ Hz, 2H); ^{13}C NMR (125 MHz, CDCl_3 , δ ppm): 22.15, 25.99, 31.76, 40.78, 44.22, 45.16, 47.03, 52.33, 53.58, 65.75, 67.14, 117.84, 119.89, 125.07, 126.74, 127.63, 128.80, 129.25, 132.57, 133.77, 141.20, 143.76, 155.80, 156.51, 169.13, 170.04, 172.85. Calculated for $\text{C}_{37}\text{H}_{42}\text{N}_4\text{NaO}_9$: 709.3 Da, Observed: 709.3 Da $[\text{M}+\text{Na}]$.

Compound **6a** was synthesized similarly from **5a** (0.23 g, 0.35 mmol) and subsequently subjected to ester hydrolysis. Compound **6a** was dissolved in 6 mL THF-water (5:1) at 0 °C and treated with 0.3 M solution of LiOH (0.032 g, 0.77 mmol) in water in small portions over 10 min. After stirring at 0 °C for 1 h, the reaction mixture pH was adjusted to 4 by adding 10% (w/v) citric acid solution and extracted with ethyl acetate. The combined organic layers were dried over anhydrous Na₂SO₄, filtered, evaporated under vacuum and purified using flash column chromatography (80% EtOAc : hexanes) to afford compound **2a** as a white solid (0.13 g, 60% yield over two steps). ¹H NMR (400 MHz, CDCl₃, δ ppm): 1.31-1.89 (m, 6H), 3.59-3.74 (m, 2H), 4.10-4.12 (m, 2H), 4.24 (t, *J* = 7.2 Hz, 1H), 4.33-4.39 (m, 3H), 4.52 (m, 2H), 4.83 (s, 2H), 5.16 (d, *J* = 10.4 Hz, 1H), 5.24-5.28 (m, 1H), 5.68 (br s, 1H), 5.81-5.91 (m, 2H), 7.31 (t, *J* = 7.6 Hz, 2H), 7.37-7.41 (m, 7H), 7.62 (d, *J* = 7.6 Hz, 2H), 7.75 (d, *J* = 7.6 Hz, 2H); ¹³C NMR (125 MHz, CDCl₃, δ ppm): 22.02, 26.06, 31.62, 42.60, 45.02, 47.23, 53.70, 66.04, 67.45, 76.73, 118.06, 120.09, 125.39, 127.24, 127.84, 129.02, 129.48, 132.72, 133.95, 141.41, 144.03, 156.25, 156.99, 171.05, 175.49. Calculated for C₃₄H₃₇N₃NaO₈: 639.2 Da, Observed: 639.3 Da [M+Na].

Synthesis of compound **2b**:



Compound **6b** (0.24 g, 0.35 mmol) was dissolved in 6 mL THF-water (5:1) at 0 °C and treated with 0.3 M solution of LiOH (0.033 g, 0.78 mmol) in water in small portions over 10 min. After stirring at 0 °C for 1 h, the reaction mixture pH was adjusted to 4 by adding 10% (w/v) citric acid solution and extracted with ethyl acetate. The combined organic layers were dried over anhydrous Na₂SO₄, filtered, evaporated under vacuum and purified using flash column chromatography (80% EtOAc : hexanes) to afford compound **2b** as a white solid (0.18 g, 75%). ¹H NMR (400 MHz, CDCl₃, δ ppm): 1.34-1.84 (m,

6H), 3.59-3.70 (m, 2H), 3.94 (d, $J = 4.8$ Hz, 2H), 4.09-4.13 (m, 2H), 4.19 (t, $J = 7.2$ Hz, 1H), 4.32-4.34 (m, 3H), 4.51 (d, $J = 5.2$ Hz, 2H), 4.80 (s, 2H), 5.15 (d, $J = 10.4$ Hz, 1H), 5.25 (d, $J = 17.2$ Hz, 1H), 5.79-5.90 (m, 2H), 6.10 (br s, 1H), 7.27 (t, $J = 7.2$ Hz, 2H), 7.35-7.38 (m, 7H), 7.58 (d, $J = 7.2$ Hz, 2H), 7.73 (d, $J = 7.6$ Hz, 2H); ^{13}C NMR (100 MHz, CDCl_3 , δ ppm): 21.60, 25.80, 31.35, 40.60, 44.16, 44.67, 46.98, 53.51, 65.75, 67.26, 76.50, 117.81, 119.80, 120.00, 125.06, 127.05, 127.66, 128.81, 129.30, 132.64, 133.72, 141.19, 143.73, 156.03, 156.89, 170.27, 170.51, 174.63. Calculated for $\text{C}_{36}\text{H}_{40}\text{N}_4\text{NaO}_9$: 695.3 Da, Observed: 695.3 Da [M+Na].

Synthesis of *N*-hydroxy isopeptide containing peptide fragments using benzoyl protecting group:

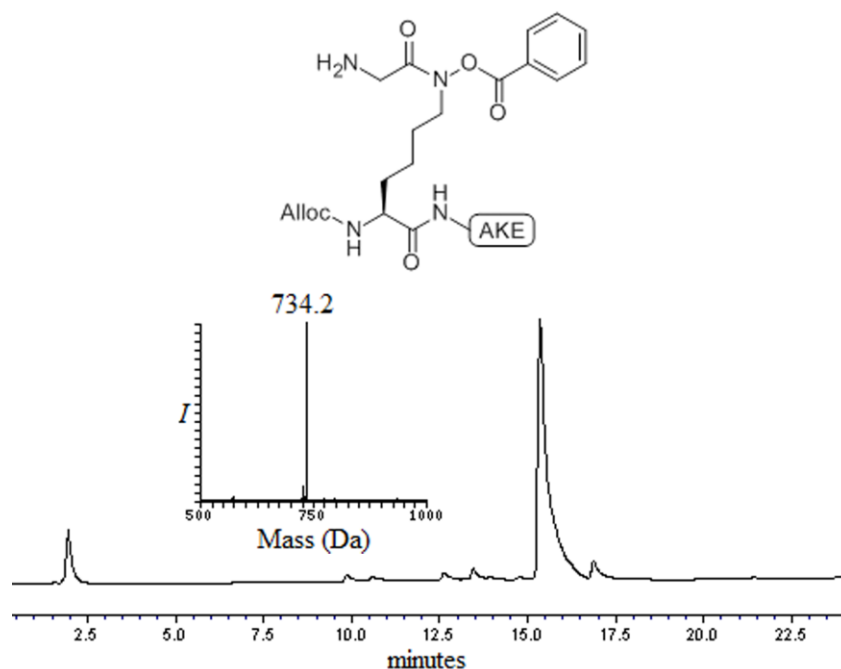


Figure S1: Analytical HPLC and mass analyses of the synthesis of *N*-hydroxy isopeptide containing peptide fragment using compound **1a**. Major peak shows the same observed mass 734.2 Da (calcd 734.6 Da) as the starting material, indicates no coupling.

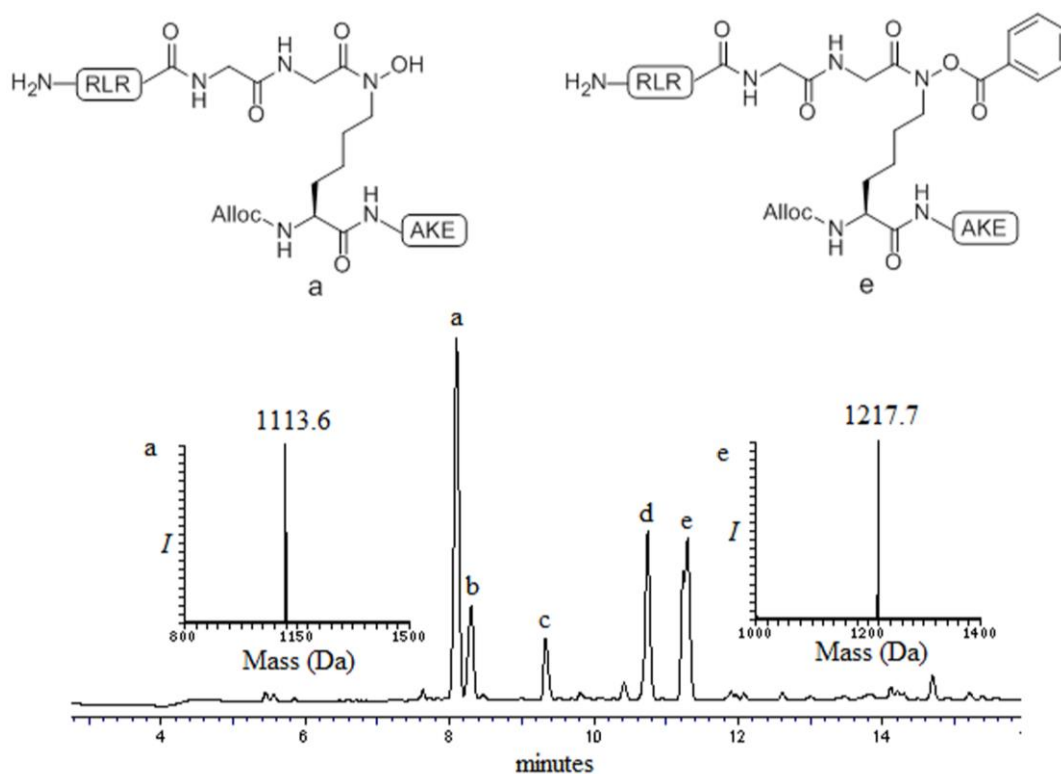


Figure S2: Analytical HPLC and mass analyses of the synthesis of *N*-hydroxy Ub(72-76) fragment. Peak a corresponds to product with benzoyl removal, having observed mass 1113.6 Da (calcd 1113.2 Da); peak b shows 18 Da mass less than peak a; peak c shows 67 Da mass extra than peak a and peak d shows 163 Da mass extra than that of the peak a. Peak e corresponds to the benzoyl-protected product having observed mass 1217.7 (calcd 1217.2 Da).

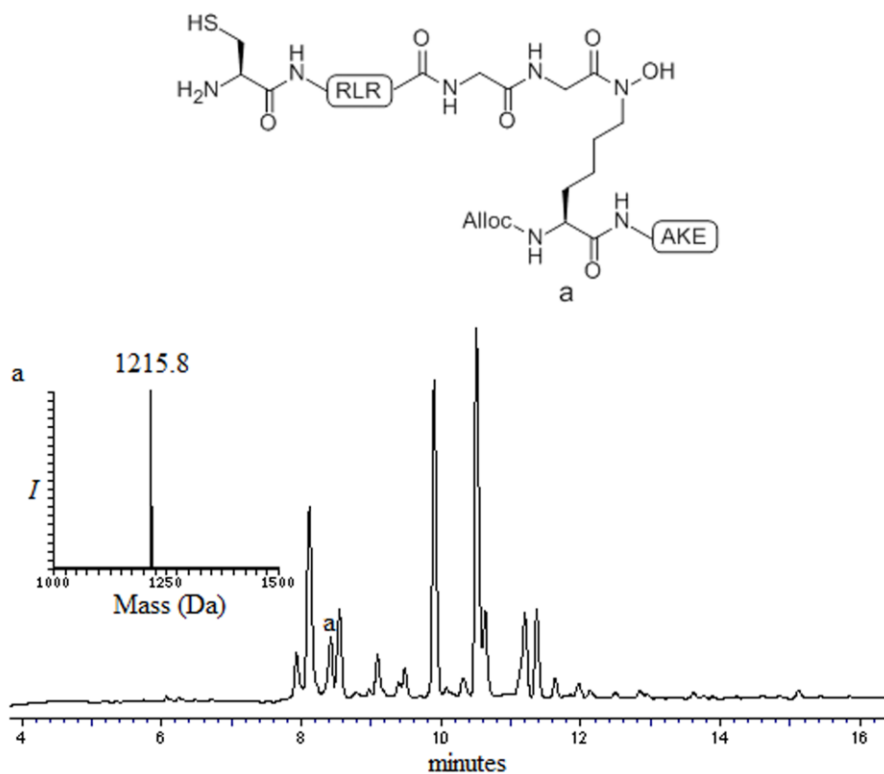


Figure S3: Analytical HPLC and mass spectra of the synthesis of *N*-hydroxy Cys-Ub(72-76) fragment. Peak a corresponds to the product with benzoyl removal, having observed mass 1215.8 Da (calcd 1216.3 Da). Other peaks correspond to unidentified side products.

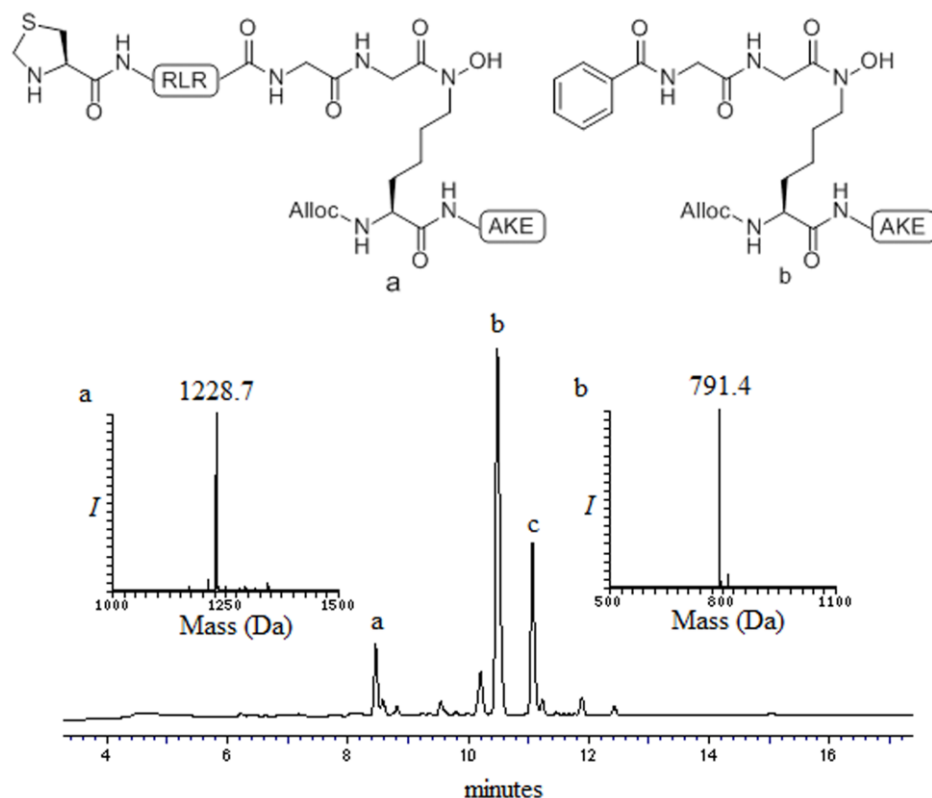
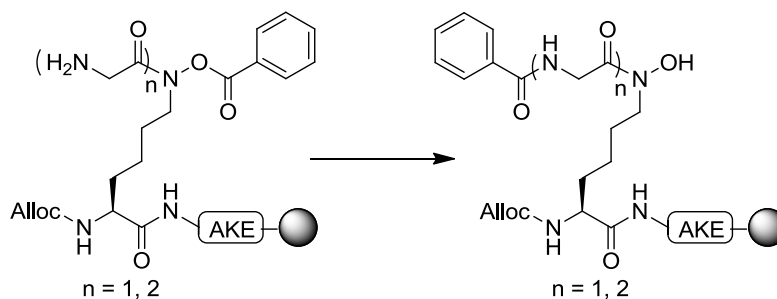


Figure S4: Analytical HPLC and spectra of the synthesis of *N*-hydroxy Thz-Ub(72-76) fragment. Peak a corresponds to benzoyl removed product having observed mass 1228.7 Da (calcd 1228.3) while peak b corresponds to unreacted starting material (capped by benzoyl transfer from oxygen to nitrogen) having observed mass 791.4 Da (calcd 791.6 Da). Peak c corresponds to unidentified side product.



Scheme S2: Proposed side reaction of the transfer of benzoyl group from oxygen to free amine. This rearrangement makes the amine unavailable for next coupling.

Synthesis of *N*-hydroxy Cys-Ub(47-76) fragment: The synthesis of *N*-hydroxy Cys-Ub(47-76) fragment was attempted using Rink amide resin (0.27 mmol/g, 0.1 mmol scale). The pre-swelled resin was treated with 20% piperidine in DMF containing 0.1 mmol HOBt (5-7-5 min) for Fmoc removal. Unless mentioned otherwise, all the amino acids were coupled manually using 4 equiv of amino acid, 8 equiv of *N,N*-diisopropylethylamine (DIEA) and 4 equiv of HCTU to the initial loading of the resin. Compound **2b** (2.5 equiv) was coupled using 2.5 equiv of HATU and 5 equiv of DIEA for 1.5 h. Pseudoproline dipeptides Fmoc-Leu-Ser($\psi^{\text{Me}},^{\text{Me}}\text{pro}$)-OH and Fmoc-Asp(O^tBu)-(Dmb)Gly-OH were coupled at positions Leu⁵⁶-Ser⁵⁷ and Asp⁵²-Gly⁵³, respectively, using 2.5 equiv of dipeptides, 2.5 equiv of HATU and 5 equiv of DIEA for 1.5 h. The *N* ^{α} -alloc protecting group of compound **2b** was removed using 0.2 equiv tetrakis(triphenylphosphine)palladium(0) (Pd[P(C₆H₅)₃]₄) and 20 equiv phenylsilane in dry DCM followed by coupling of Ser and Leu.

Analytical cleavage of the peptide: Analytical amount of dried peptide-resin was treated with 1 mL mixture of TFA : triisopropylsilane(TIS) : water (95:2.5:2.5, v/v) and the reaction mixture was shaken for 1 h at RT. The resin was removed by filtration and the peptide was precipitated from cold ether by centrifugation. The supernatant was drained out and the residue was dried by purging with argon.

To remove the benzyl protecting group, the crude was further treated with 1 mL mixture of TFA : TFMSA : *m*-cresol (10:2:2; v/v) and was shaken for 1 hr at RT. The peptide was precipitated from cold ether by centrifugation and was dissolved in acetonitrile-water for analytical HPLC.

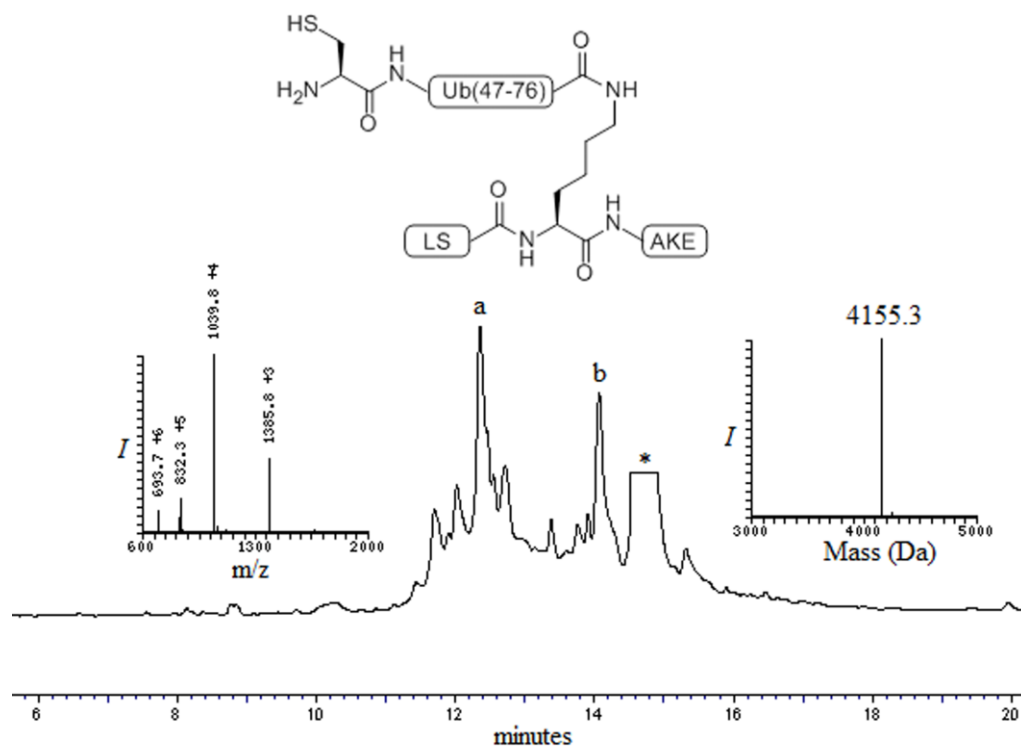
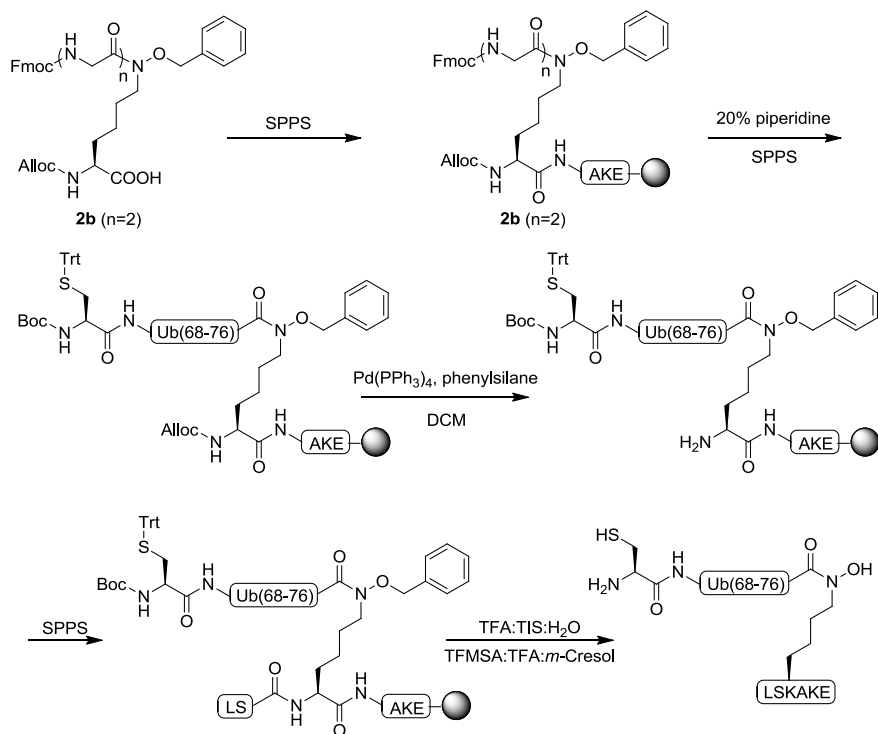


Figure S5: Analytical HPLC and mass spectra of the synthesis of *N*-hydroxy Cys-Ub(47-76) fragment. Peak a and b exhibited the same mass 4155.3 Da (calcd 4172.7 Da).

Synthesis of *N*-hydroxy Cys-Ub(68-76) fragment using benzyl protecting group:



Scheme S3: Preparation of *N*-hydroxy Cys-Ub(68-76) fragment.

Synthesis of *N*-hydroxy Cys-Ub(68-76) fragment: The synthesis of *N*-hydroxy Cys-Ub(68-76) fragment was carried out using Rink amide resin (0.27 mmol/g, 0.1 mmol scale) similarly, as described for *N*-hydroxy Cys-Ub(47-76) fragment.

Cleavage of the peptide from resin: The resin was washed with DMF, methanol, DCM and dried. The peptide was cleaved using TFA : triisopropylsilane : water (95:2.5:2.5, v/v) cocktail for 2 h. The cleavage mixture was filtered and the combined filtrate was added drop-wise to a 10-fold volume of cold ether and centrifuged. The precipitated crude peptide was dissolved in acetonitrile-water (1:3) and lyophilized. To remove the benzyl protecting group, the dried crude peptide was treated with mixture of TFA : TFMSA : *m*-cresol (10:2:2; v/v) for 2 h at room temperature and the peptide was precipitated from cold ether followed by dissolution in 25% acetonitrile-water. HPLC analysis was carried out on an analytical C18 column using a gradient 5-70% B over 30

min. Purification was done on a semi preparative C18 column using a gradient 5-60% B over 40 min to afford the pure peptide in ~ 20% yield.

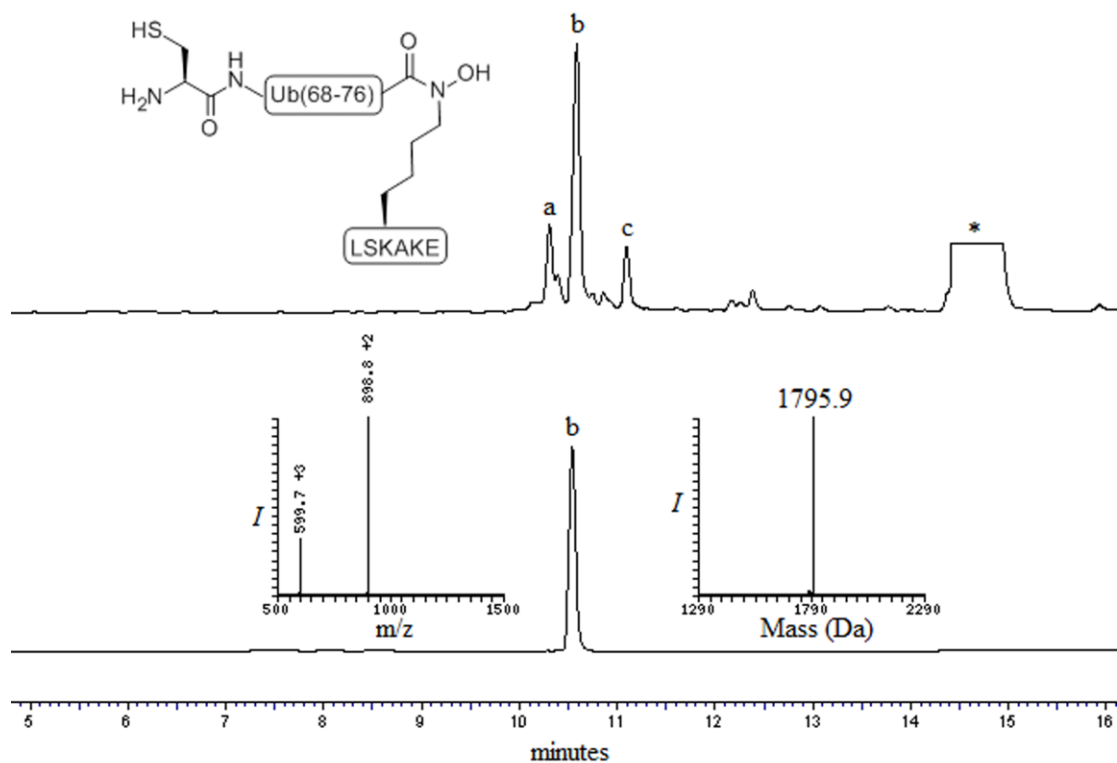
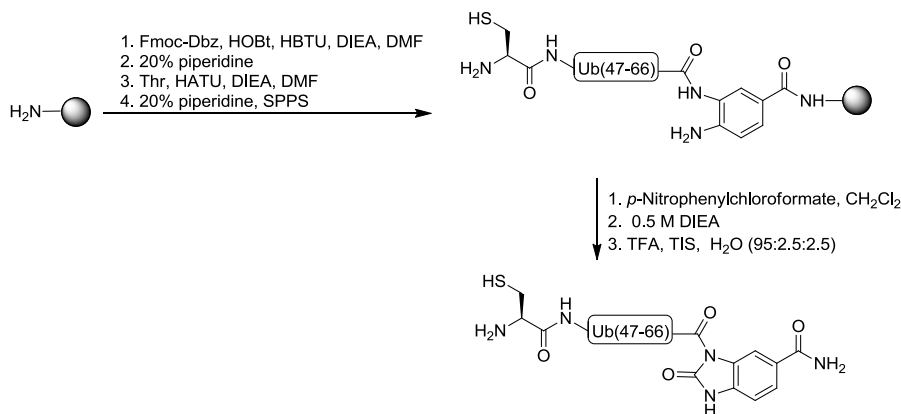


Figure S6: Analytical HPLC (upper trace: crude; lower trace: pure) and mass spectra of the synthesis of *N*-hydroxy Cys-Ub(68-76) fragment. Peak b corresponds to the desired product with the observed mass 1795.9 Da (calcd 1795.2 Da), peak a shows the same mass as peak b and peak c shows extra 163 Da mass. * Corresponds to small molecule impurity from cleavage mixture.

Synthesis of Thz-Ub(47-66)-Nbz fragment:

The synthesis was carried out according to the following scheme:



The synthesis of Thz-Ub(47-66)-Nbz was carried out on Rink amide resin (0.27 mmol/g, 0.1 mmol scale). Unless specified otherwise, for each coupling cycle, amino acids and HCTU were used in 4-fold excess, while DIEA was used in 8-fold excess to the initial loading of the resin. For the synthesis of Thz-Ub(47-66)-Nbz, the pre-swelled resin was treated with 20% piperidine in DMF containing 0.1 mmol HOBt (5-7-5 min) to remove the Fmoc protecting group. Fmoc-3,4-diaminobenzoic acid (Fmoc-Dbz) was coupled to the resin using HBTU/HOBt for 1 h, (2 cycles). Subsequently, the resin was washed with DMF (3 × 5 mL). Following Fmoc removal, the Thr⁶⁶ was manually coupled using HATU for 1 h (2 cycles). The remaining amino acids were coupled manually. Pseudoproline dipeptides Fmoc-Leu-Ser(ψ^{Me} , Me^{pro})-OH and Fmoc-Asp(O^tBu)-(Dmb)Gly-OH were coupled at positions Leu⁵⁶-Ser⁵⁷ and Asp⁵²-Gly⁵³, respectively, by using 2.5 equiv of dipeptides, 2.5 equiv of HATU and 5 equiv of DIEA for 1.5 h. Analytical cleavage and HPLC analysis were performed after these couplings to ensure complete reactions.

Dbz cyclization: The resin was washed with DCM and a solution of *p*-nitrophenyl chloroformate (100 mg, 5 equiv) in 4 mL of dry DCM was added and shaken for 30 min at room temperature and washed with DCM (3 × 5 mL). Following this, the resin was treated with a solution of 0.5 M DIEA in DMF (2 mL) and was shaken for additional 10 min for complete cyclization and washed with DMF (X2).

Cleavage of the peptide from resin: The resin was washed with DMF, methanol, DCM and dried. The peptide was cleaved using TFA : triisopropylsilane : water (95:2.5:2.5, v/v) cocktail for 2 h. The cleavage mixture was filtered and the combined filtrate was added drop-wise to a 10-fold volume of cold ether and centrifuged. The precipitated crude peptide was dissolved in acetonitrile-water (1:3) and lyophilized. The HPLC analysis was carried out on a C18 analytical column using a gradient of 5-70% B over 30 min. Purification was done on a preparative C18 column using a gradient 20-60% B over 60 min to afford the pure peptide in ~ 45% yield.

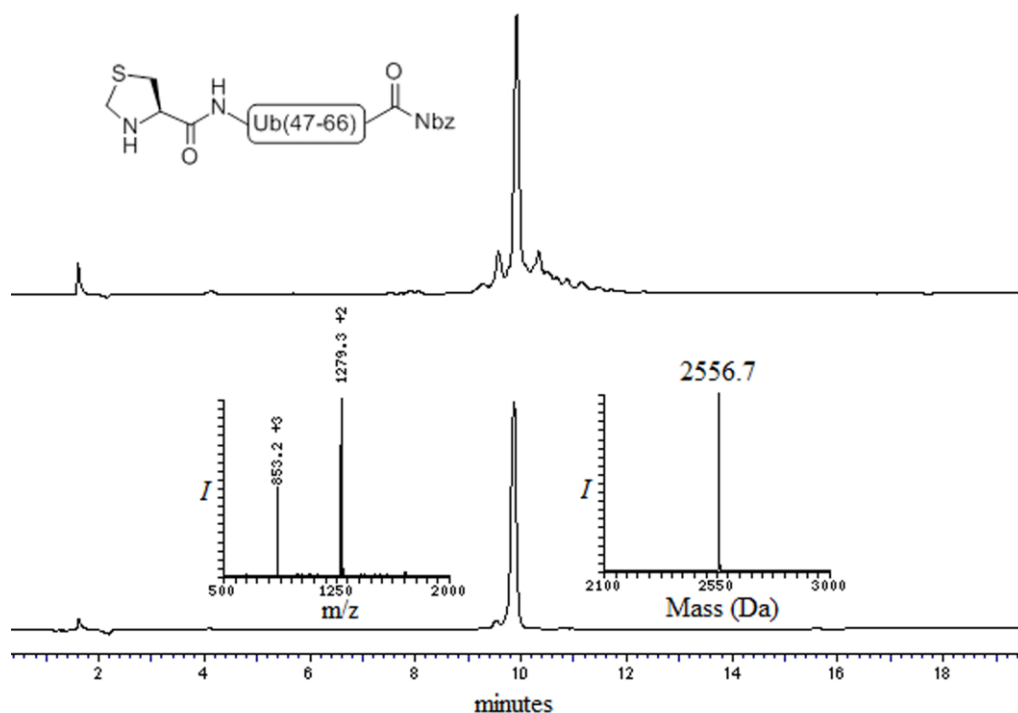
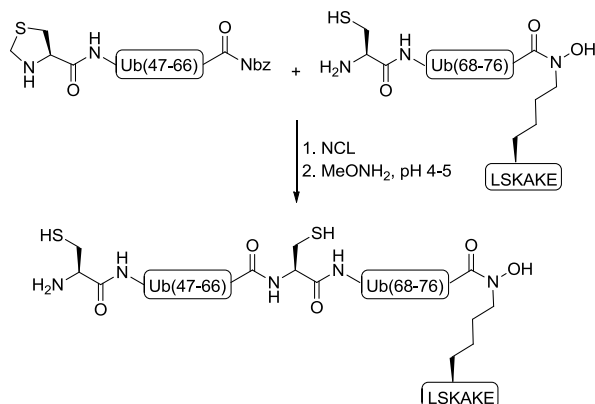


Figure S7: Analytical HPLC (upper trace: crude; lower trace: pure) and mass spectra of the synthesis of Thz-Ub(47-66)-Nbz fragment. Major peak shows the mass of the desired product 2556.7 Da (calcd 2556.6 Da).

Synthesis of *N*-hydroxy Cys-Ub(47-76) from two fragments:



In a typical ligation, Thz-Ub(47-66)-Nbz (1.4 mg, 0.56 μ mol) and *N*-hydroxy Cys-Ub(68-76) fragments (1 mg, 0.56 μ mol) were dissolved in argon purged 6 M Gn·HCl, 200 mM phosphate buffer (278 μ L, 2 mM) containing 30 equiv of MPAA and 15 equiv of TCEP, pH 7.3. The reaction mixture was incubated at 37 °C for 5 h. Progress of the reaction was monitored by using analytical HPLC (C4 column) with a gradient of 5-70% B over 30 min. After completion of the reaction, Thz deprotection was carried out by adding 30 equiv of MeONH₂ and 15 equiv TCEP at pH 4 and incubated at 37 °C for 8 h. Progress of the reaction was monitored by analytical HPLC (C4 column) with a gradient of 5-70% B over 30 min. For semi-preparative HPLC, the same gradient was used to isolate the product in 45% yield (1 mg).

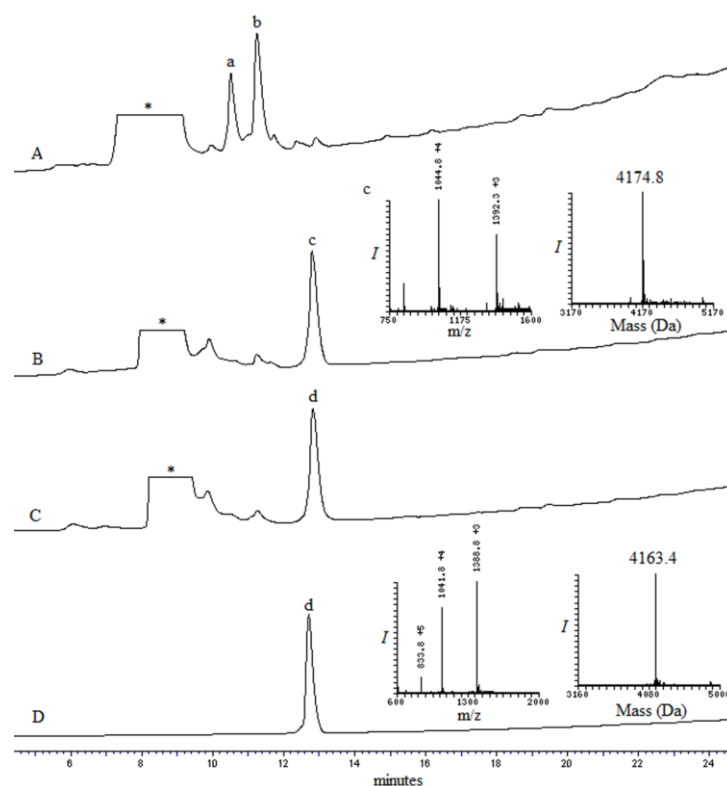
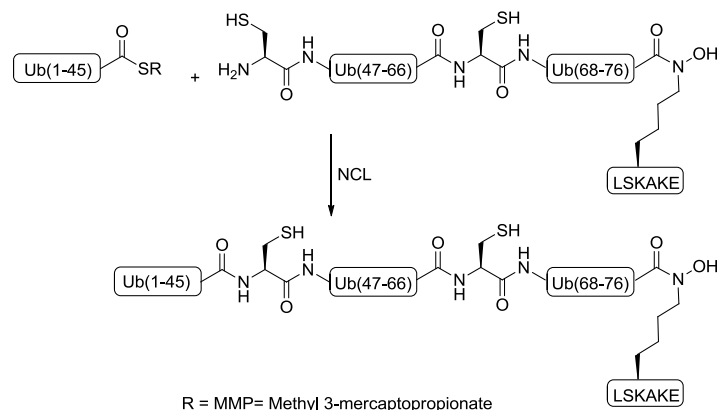


Figure S8: Synthesis of *N*-hydroxy Cys-Ub(47-76). (A) Analytical HPLC and mass spectra of the ligation at time zero; peak a corresponds to Thz-Ub(47-66)-Nbz fragment and peak b corresponds to *N*-hydroxy Cys-Ub(68-76) fragment. (B) Ligation after 5 h; peak c corresponds to ligation product with the observed mass 4174.8 Da (calcd 4174.7 Da). (C) Analytical HPLC of the reaction after methoxyl amine treatment for 8 h. Peak d corresponds to Thz opened product with the observed mass 4163.4 Da (calcd 4162.7 Da). (D) Analytical HPLC and mass spectra after purification. * Corresponds to MPAA.

Synthesis of *N*-hydroxy ubiquitinated peptide:



In a typical ligation, *N*-hydroxy Cys-Ub(47-76)-LSKAKE fragment (1 mg, 0.24 μ mol) and Ub(1-45)-MMP (1.2 mg, 0.24 μ mol) were dissolved in argon purged 6 M Gn·HCl, 200 mM phosphate buffer (120 μ L, 2 mM) containing 30 equiv of MPAA and 15 equiv of TCEP, pH 7.3. The reaction mixture was incubated at 37 $^{\circ}$ C for 5 h. Progress of the reaction was monitored by using analytical HPLC (C4 column) with a gradient of 5-70% B over 30 min. For semi-preparative HPLC, the same gradient was used to isolate the ligation product in 55% yield (~1.3 mg).

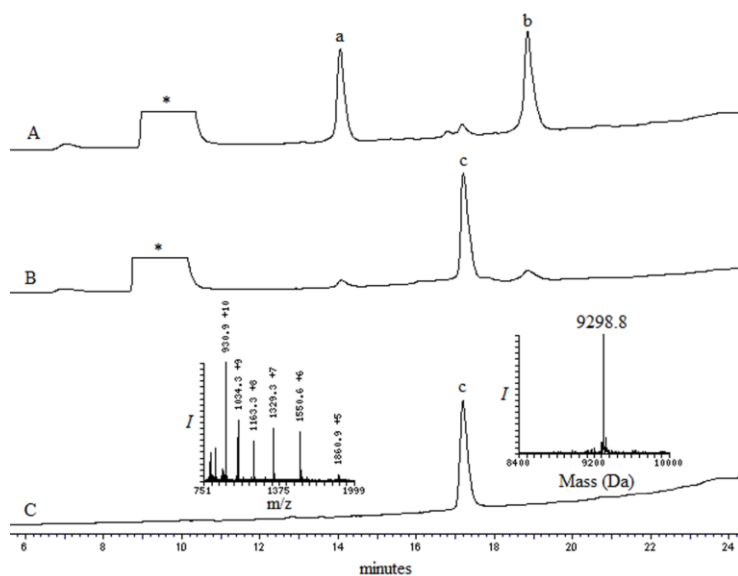


Figure S9: Synthesis of *N*-hydroxy ubiquitinated peptide. (A) Analytical HPLC and mass spectra of the ligation at time zero; peak a corresponds to *N*-hydroxy Ub(47-76) fragment and peak b corresponds Ub(1-45)-MMP fragment. (B) Ligation after 5 h. (C) Analytical HPLC and mass spectra after purification; peak c corresponds to ligation product with the observed mass 9298.8 Da (calcd 9297.7 Da). * Corresponds to MPAA.

Synthesis of WT Cys-Ub(47-76): The synthesis of WT Cys-Ub(47-76) was carried out using Rink amide resin (0.27 mmol/g, 0.1 mmol scale). The pre-swelled resin was treated with 20% piperidine in DMF containing 0.1 mmol HOBt (5-7-5 min) to remove the Fmoc protecting group. Unless mentioned otherwise, all amino acids were coupled manually using 4 equiv of amino acid, 8 equiv of DIEA and 4 equiv of HCTU to the initial loading

of the resin. N^α -Fmoc- N^ϵ -Alloc-Lys-OH (2.5 equiv) was coupled using 2.5 equiv of HATU and 5 equiv of DIEA for 1.5 h. Following Fmoc removal, Ser and Leu were coupled. The Alloc protecting group was removed using 0.2 equiv tetrakis(triphenylphosphine)palladium(0) ($\text{Pd}[\text{P}(\text{C}_6\text{H}_5)_3]_4$) and 20 equiv phenylsilane in dry DCM. Then other amino acids of ubiquitin were coupled as described above where Leu⁶⁷ was mutated to Cys. Pseudoproline dipeptides Fmoc-Leu-Ser($\psi^{\text{Me}}, \text{Me}_{\text{pro}}$)-OH and Fmoc-Asp(O^tBu)-(Dmb)Gly-OH were coupled at positions Leu⁵⁶-Ser⁵⁷ and Asp⁵²-Gly⁵³ respectively by using 2.5 equiv of dipeptides, 2.5 equiv of HATU and 5 equiv of DIEA for 1.5 h. Analytical cleavage and HPLC analysis were performed to ensure complete reactions.

Cleavage of the peptide from resin: Peptide was cleaved from resin as described above for Thz-Ub(47-66)-Nbz fragment and purified on a preparative C18 column using a gradient 20-60% B over 60 min to afford WT Cys-Ub(47-76) in ~ 40% yield .

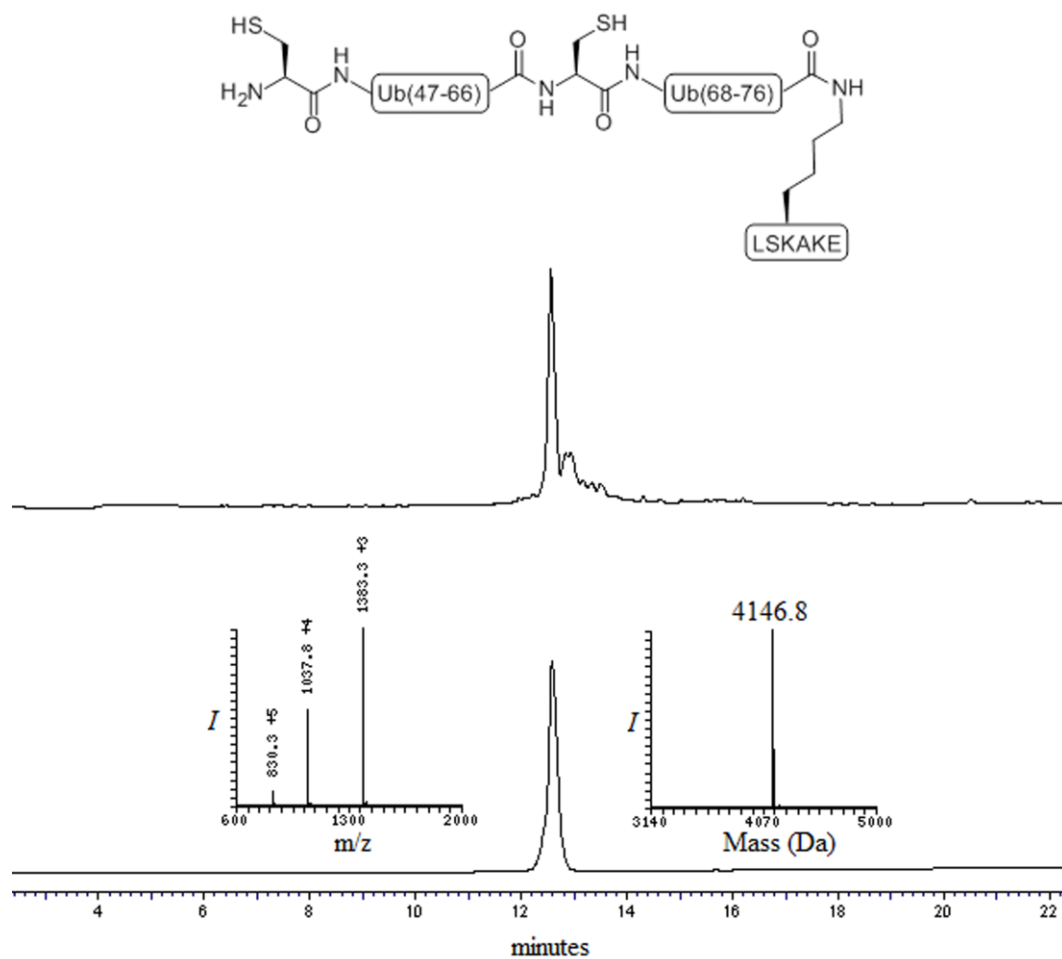
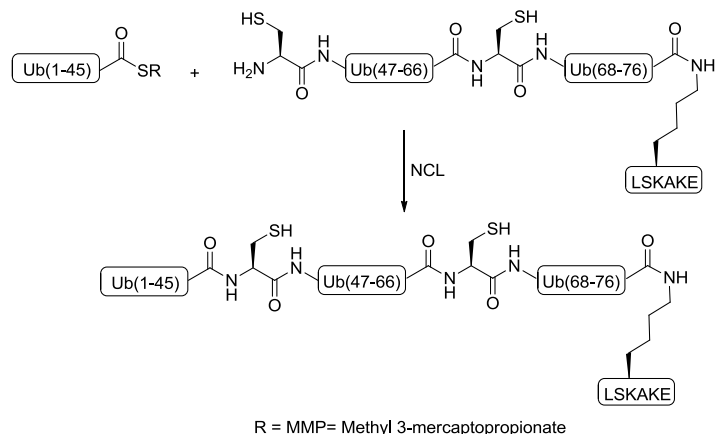


Figure S10: Analytical HPLC (upper trace: crude; lower trace: pure) and mass spectra of the synthesis of WT Cys-Ub(47-76) fragment. Major peak shows the mass of the desired product with the observed mass 4146.8 Da (calcd 4146.7 Da).

Synthesis of WT ubiquitinated peptide:



In a typical ligation, WT Cys-Ub(47-76) fragment (1.2 mg, 0.28 μ M) and Ub(1-45)-MMP (1.4 mg, 0.28 μ M) were dissolved in argon purged 6 M Gn·HCl, 200 mM phosphate buffer (120 μ L, 2 mM) containing 30 equiv of MPAA and 15 equiv of TCEP, pH 7.3. The reaction mixture was incubated at 37 $^{\circ}$ C for 5 h. Progress of the reaction was monitored by using analytical HPLC (C4 column) with a gradient of 5-70% B over 30 min. For semi-preparative HPLC, the same gradient was used to isolate the ligation product in 55% yield (1.4 mg).

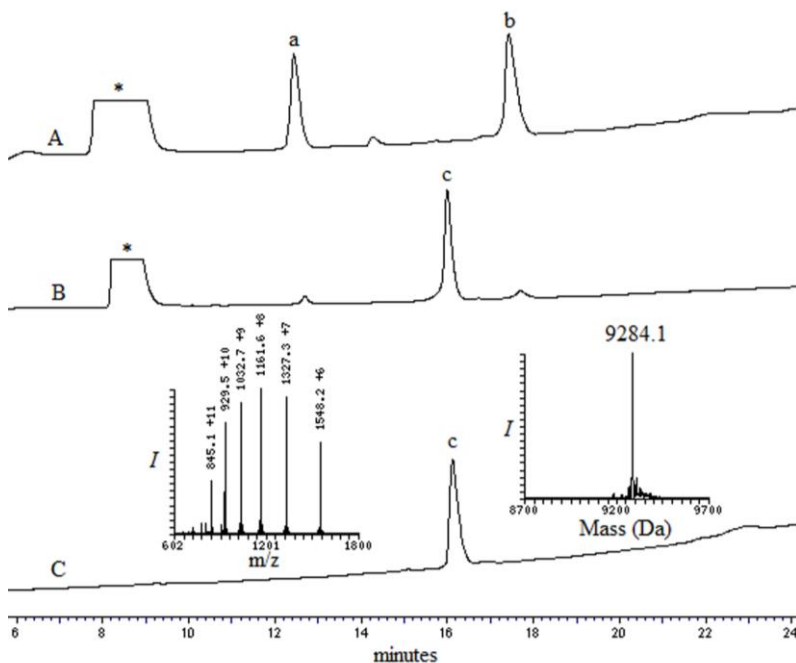
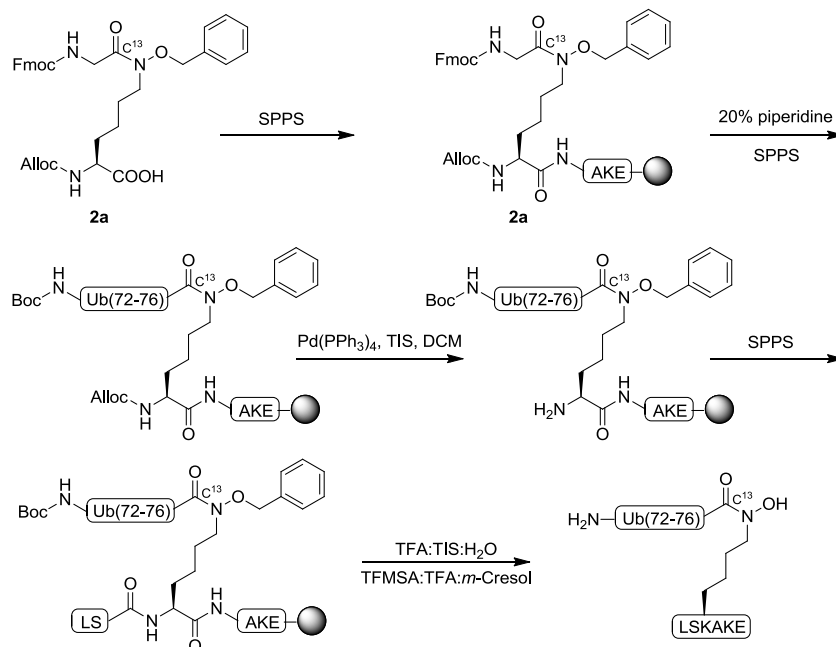


Figure S11: Synthesis of WT ubiquitinated peptide. (A) Analytical HPLC and mass analysis of the ligation at time zero; peak a corresponds to WT Cys-Ub(47-76) fragment and peak b corresponds to Ub(1-45)-MMP fragment. (B) Ligation after 5 h. (C) Analytical HPLC and mass spectra after purification. Peak c corresponds to ligation product with the observed mass 9284.1 Da (calcd 9283.7 Da). * Corresponds to MPAA.

Synthesis of *N*-hydroxy Ub(72-76) with ^{13}C labelled Gly⁷⁶ :



Synthesis of *N*-hydroxy Ub(72-76) with ^{13}C labelled Gly⁷⁶ was carried out similarly as described for the *N*-hydroxy Cys-Ub(68-76) using compound **2a** with ^{13}C labelled Gly⁷⁶.

Cleavage of the peptide: The peptide was cleaved similarly as described for *N*-hydroxy Cys-Ub(68-76).

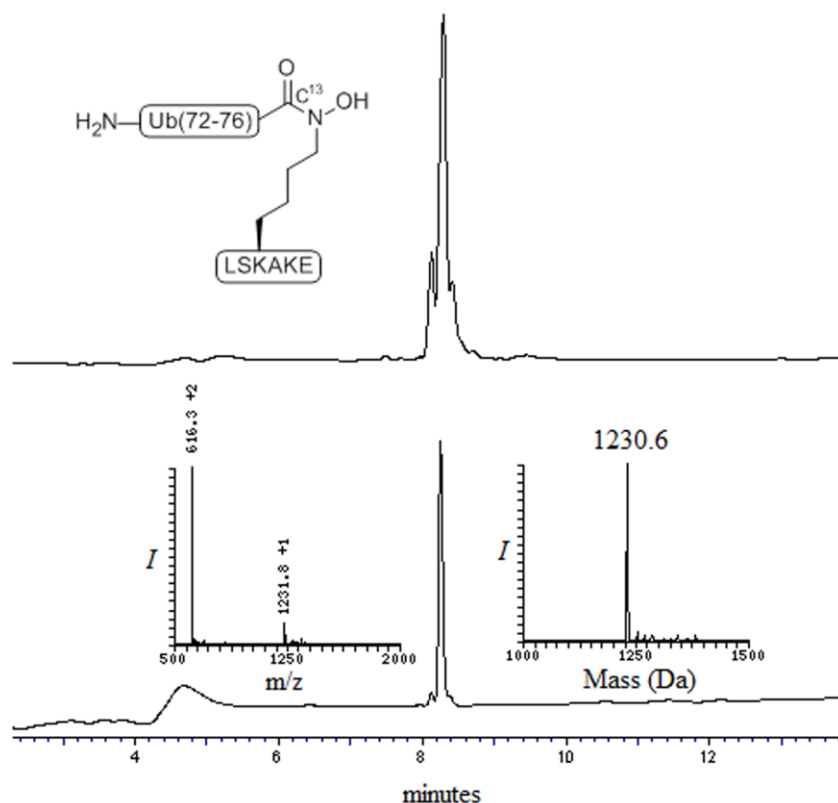


Figure S12: Analytical HPLC and mass analysis (upper trace: crude; lower trace: pure) of the synthesis of *N*-hydroxy Ub(72-76) with ^{13}C labelled Gly⁷⁶. Major peak shows the mass of desired product with the observed mass 1230.6 Da (calcd 1230.4 Da).

Synthesis of WT Ub(72-76)-LSKAKE with ^{13}C labelled Gly⁷⁶: Synthesis of WT Ub(72-76) with ^{13}C labelled Gly⁷⁶ was carried out similarly as described for WT Cys-Ub(47-76).

Cleavage of the peptide: The peptide was cleaved similarly as described for WT Cys-Ub(47-76).

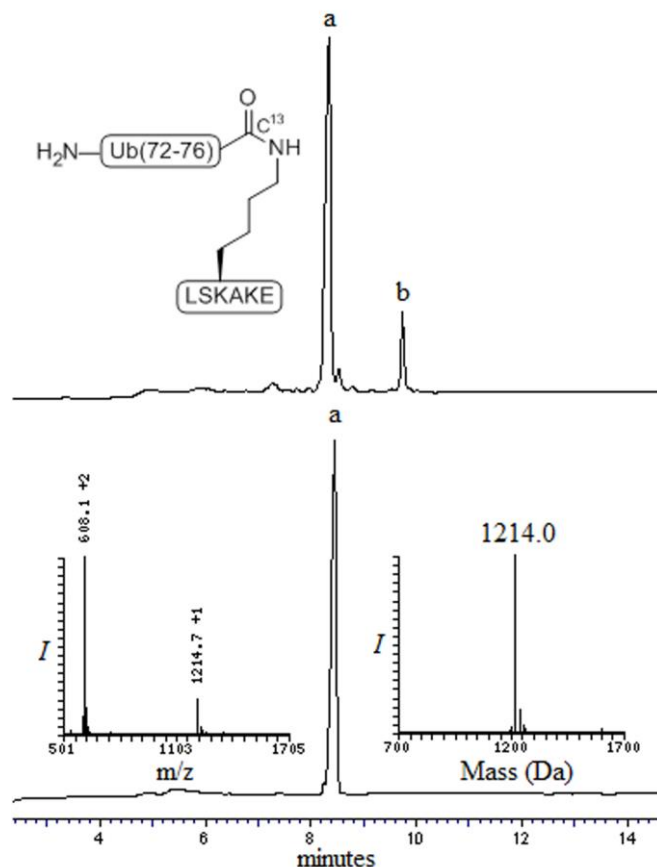


Figure S13: Analytical HPLC and mass analysis (upper trace: crude; lower trace: pure) of the synthesis of WT Ub(72-76) with ^{13}C labelled Gly⁷⁶. Major peak shows the mass of desired product with the observed mass 1214.0 Da (calcd 1214.4 Da).

Evaluation of UCH-L3 activity with *N*-hydroxy and WT ubiquitinated peptide: *N*-hydroxy and WT ubiquitinated peptides were dissolved in 6 M Gn·HCl, 200 mM phosphate buffer (pH 7.3) to 5% of the total volume and then further diluted with the assay buffer (50 mM HEPES, pH 8). The exact concentration of each protein solution was determined using Pierce® BCA Protein Assay Kit (Thermo scientific). Stock solution of 1 μM recombinant human UCH-L3 (BostonBiochem) was prepared by diluting 33.3 μM of the enzyme with enzyme buffer (50 mM HEPES buffer, 1 mM DTT, 0.5 mg/mL BSA, pH 8.0). The enzymatic reaction was carried out by incubating each substrate (20 μM , 50 μL) with UCH-L3 (48.2 nM, 50 μL) for 5 mins at 37 °C. Enzyme activity was detected by analytical HPLC using C4 column, 0-60% B over 45 min.

Evaluation of UCH-L3 activity with *N*-hydroxy and WT ubiquitinated peptide in presence of zinc (II) ion: The enzymatic assay was evaluated in presence of 5, 10 and 15 equiv of zinc (II) ion by incubating 40 μ L of 25 μ M WT and *N*-hydroxy ubiquitinated peptides with 10 μ L of 0.5, 1 and 1.5 mM zinc chloride solution (prepared from 10 mM stock solution in water) for 5 mins in room temperature, followed by incubating with UCH-L3 (48.2 nM, 50 μ L) for 5 mins at 37 $^{\circ}$ C. Enzyme activity was monitored by analytical HPLC using C4 column, 0-60% B over 45 min.

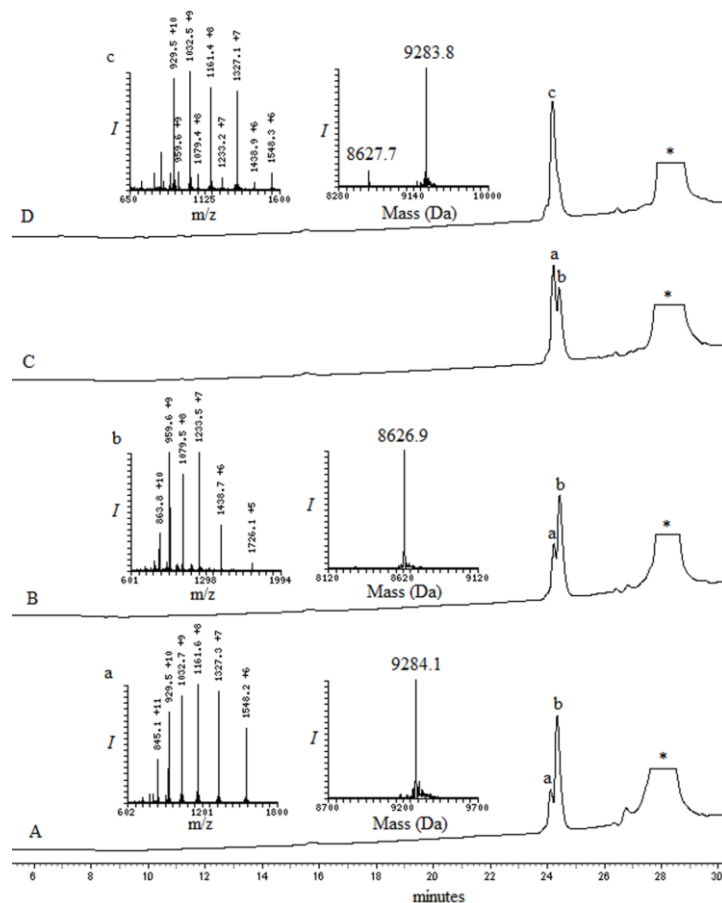


Figure S14: HPLC and mass spectra of the hydrolysis of WT ubiquitinated peptide by UCH-L3 in presence of (A) 0 equiv, (B) 5 equiv, (C) 10 equiv and (D) 15 equiv of zinc (II) ion. Peak a corresponds to WT ubiquitinated peptide with the observed mass 9284.1 Da (calcd 9283.7 Da), peak b corresponds to hydrolysed Ub with the observed mass 8626.9 Da (calcd 8626.9 Da) and peak c corresponds to the mixture of WT ubiquitinated peptide and hydrolysed Ub. * Corresponds to BSA.

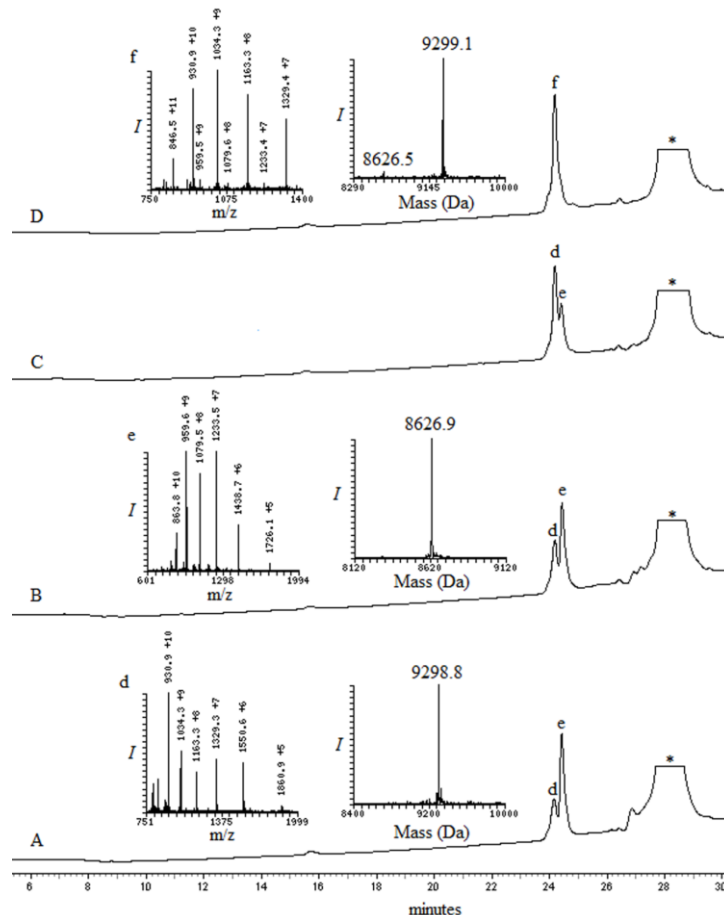


Figure S15: HPLC and mass spectra of the hydrolysis of *N*-hydroxy ubiquitinated peptide by UCH-L3 in presence of (A) 0 equiv, (B) 5 equiv, (C) 10 equiv and (D) 15 equiv of zinc (II) ion. Peak d corresponds to *N*-hydroxy ubiquitinated peptide with the observed mass 9298.8 Da (calcd 9297.7 Da), peak e corresponds to hydrolysed Ub with the observed mass 8626.9 Da (calcd 8626.9 Da) and peak f corresponds to the mixture of *N*-hydroxy ubiquitinated peptide and hydrolysed Ub. * Corresponds to BSA.

Table S1: Percentage of hydrolysis of the WT and *N*-hydroxy ubiquitinated peptides in presence of different equiv of zinc (II) ion.

The numbers ascribed for enzymatic hydrolysis experiments were calculated from mass spectrometric analysis where no peak separation was observed. Where there was some peak separation, extent of hydrolysis was analyzed by integration using HPLC software.

	UCH-L3	
Zn (eq.)	WT	<i>N</i> -OH
0	74.83	67.59
5	67.95	59
10	43.22	33.82
15	13	1

^{13}C NMR studies for ^{13}C labelled WT and *N*-hydroxy Ub(72-76) peptides:

^{13}C NMR spectra for WT and *N*-hydroxy Ub(72-76) were recorded at concentration 3.52 mM in 550 μL of 50 mM HEPES buffer in D_2O (pH 8.0) (containing methanol- d_4 for reference) with Bruker AMX-400 MHz spectrometer. To check the effect of zinc (II) ion, 1 equiv zinc chloride (1.9 μL of 1 M solution in D_2O) was added to the NMR tubes and spectra were recorded. To check the effect of Ga(III) ion, 10 equiv gallium sulfate (4.7 mg $\text{Ga}_2(\text{SO}_4)_3$) was added to the solution of *N*-hydroxy Ub(72-76)-LSKAKE (2.02 mM, 550 μL in D_2O with methanol- d_4) and spectrum was recorded.

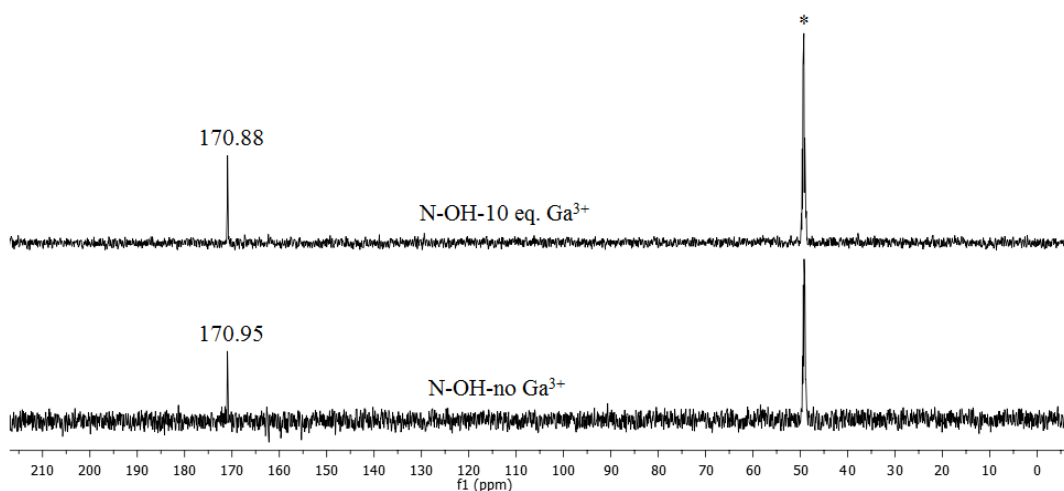


Figure S17: ^{13}C NMR (100 MHz) spectra of ^{13}C labelled *N*-hydroxy ubiquitinated peptide with and without Ga^{3+} ion.

Evaluation of UCH-L3 activity with *N*-hydroxy and WT ubiquitinated peptide in presence of Fe(III) ion:

The enzymatic assay was evaluated in presence of 15 equiv of Fe(III) ion by incubating 40 μ L of 25 μ M WT and *N*-hydroxy ubiquitinated peptides with 10 μ L of 1.5 mM of ferric nitrate solution (prepared from 10 mM stock solution in water) for 5 min at RT, followed by incubating with UCH-L3 (48.2 nM, 50 μ L) for 5 min at 37 $^{\circ}$ C. Enzyme activity was monitored by analytical HPLC using C4 column, 0-60% B over 45 min.

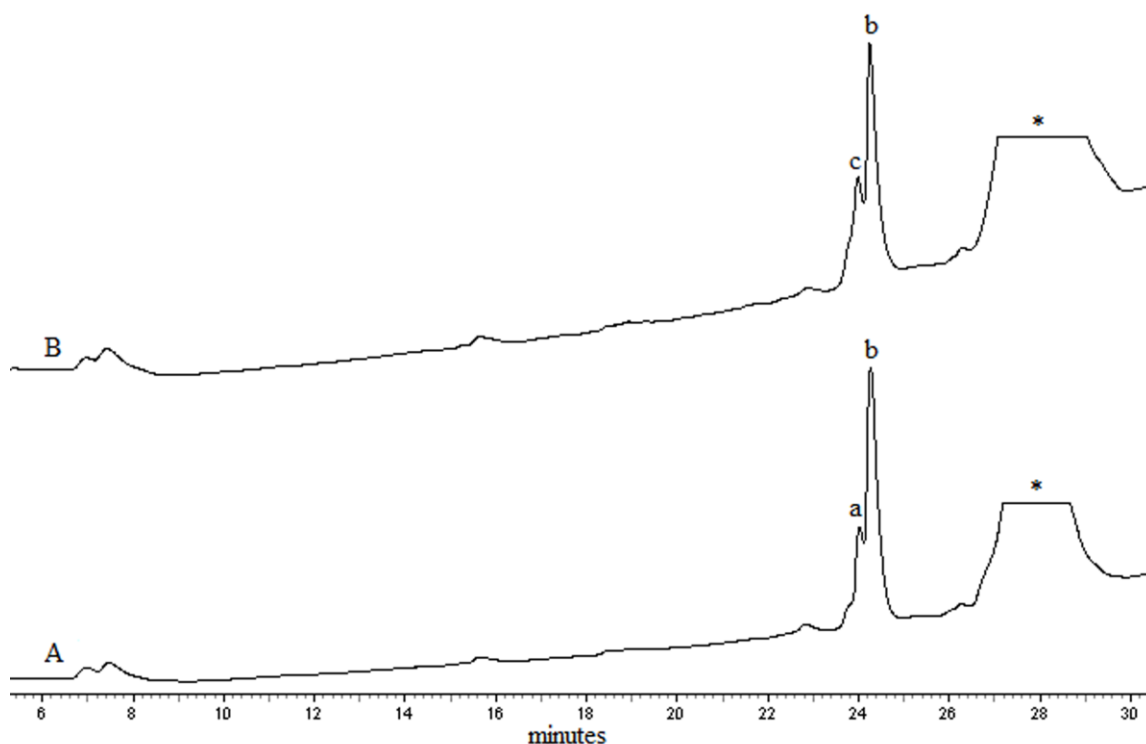


Figure S16: HPLC traces of hydrolysis of (A) WT ubiquitinated peptide and (B) *N*-hydroxy ubiquitinated peptide by UCH-L3 in presence of 15 equiv Fe(III) ion. Peak a and c correspond to WT ubiquitinated peptide and *N*-hydroxy ubiquitinated peptide, respectively; peak b corresponds to hydrolysed Ub. * Corresponds to BSA.

Evaluation of UCH-L3 activity with *N*-hydroxy and WT ubiquitinated peptide in presence of Cu(II) ion:

The enzymatic assay was evaluated in presence of 15 equiv of Cu(II) ion by incubating 40 μ L of 25 μ M WT and *N*-hydroxy ubiquitinated peptides with 10 μ L of 1.5 mM of cupric sulfate solution (prepared from 10 mM stock solution in water) for 5 min at room temperature followed by incubating with UCH-L3 (48.2 nM, 50 μ L) for 5 min at 37 $^{\circ}$ C. Enzyme activity was monitored by analytical HPLC using C4 column, 0-60% B over 45 min.

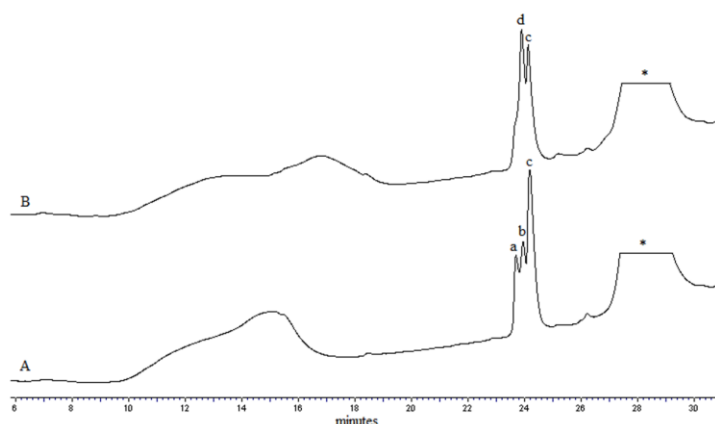
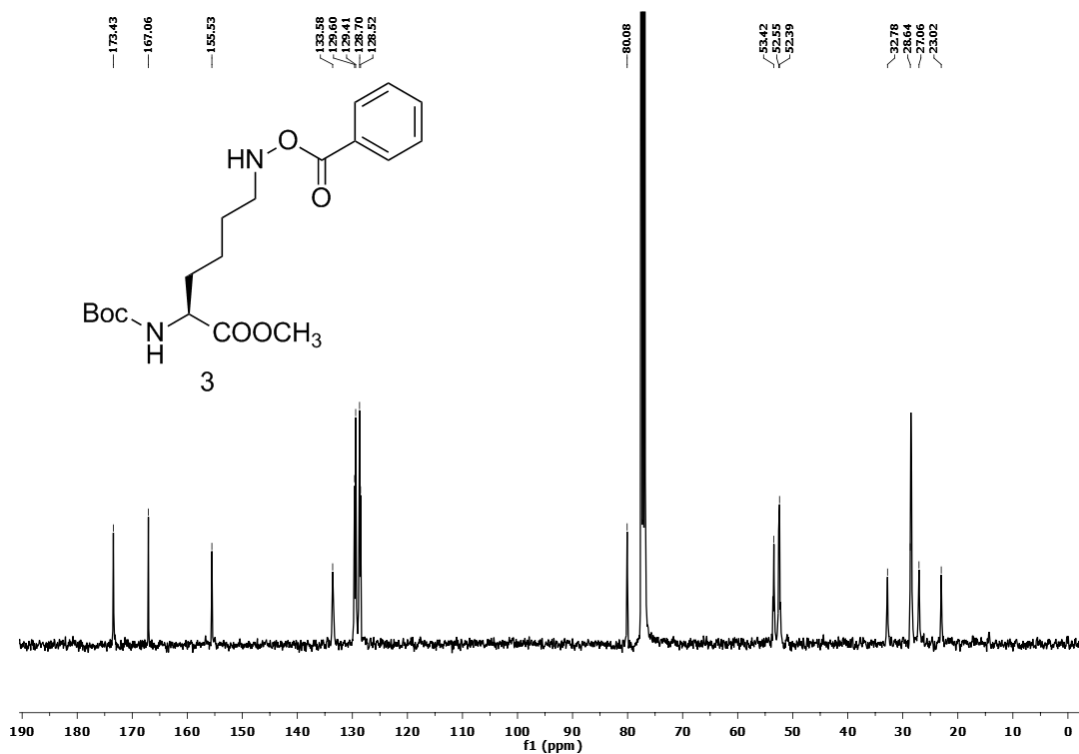
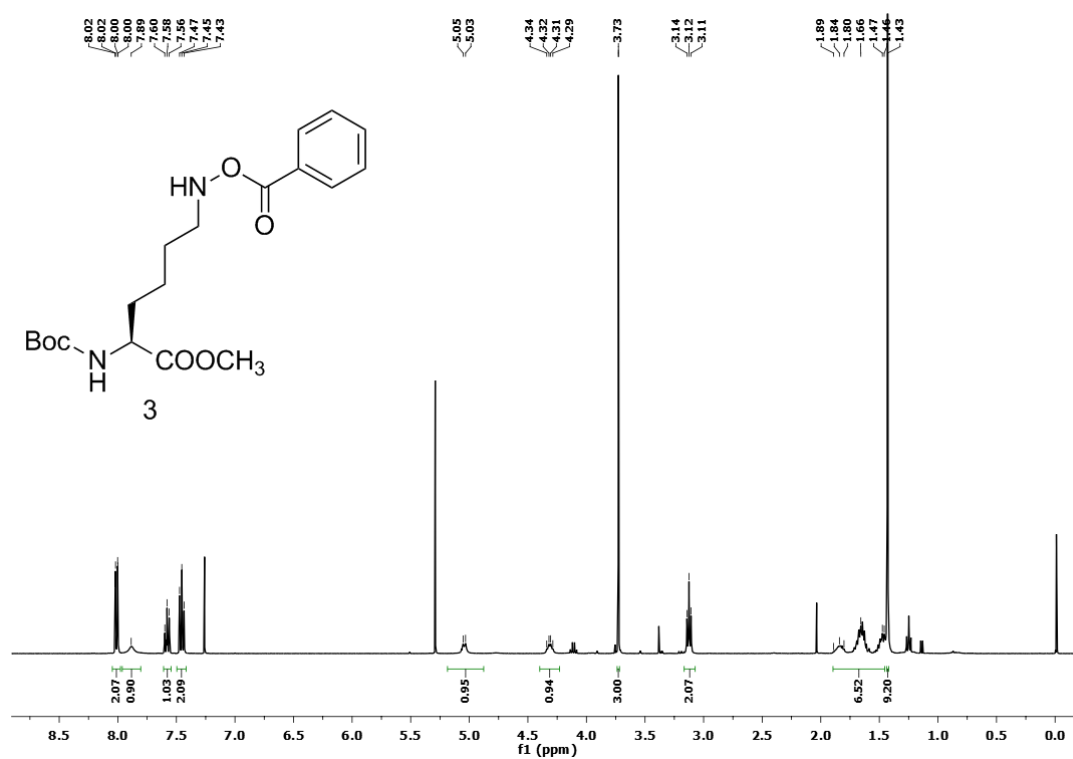
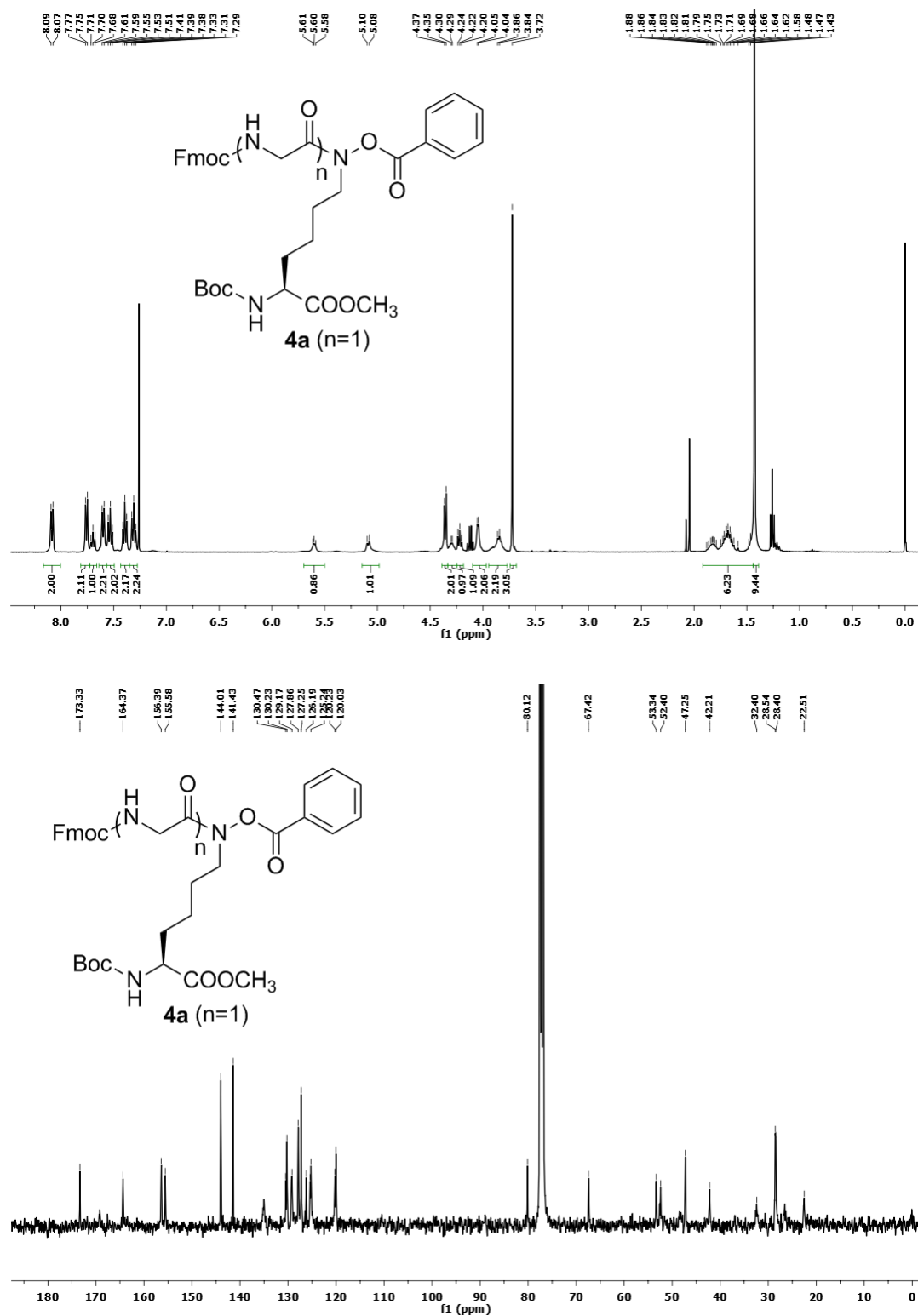


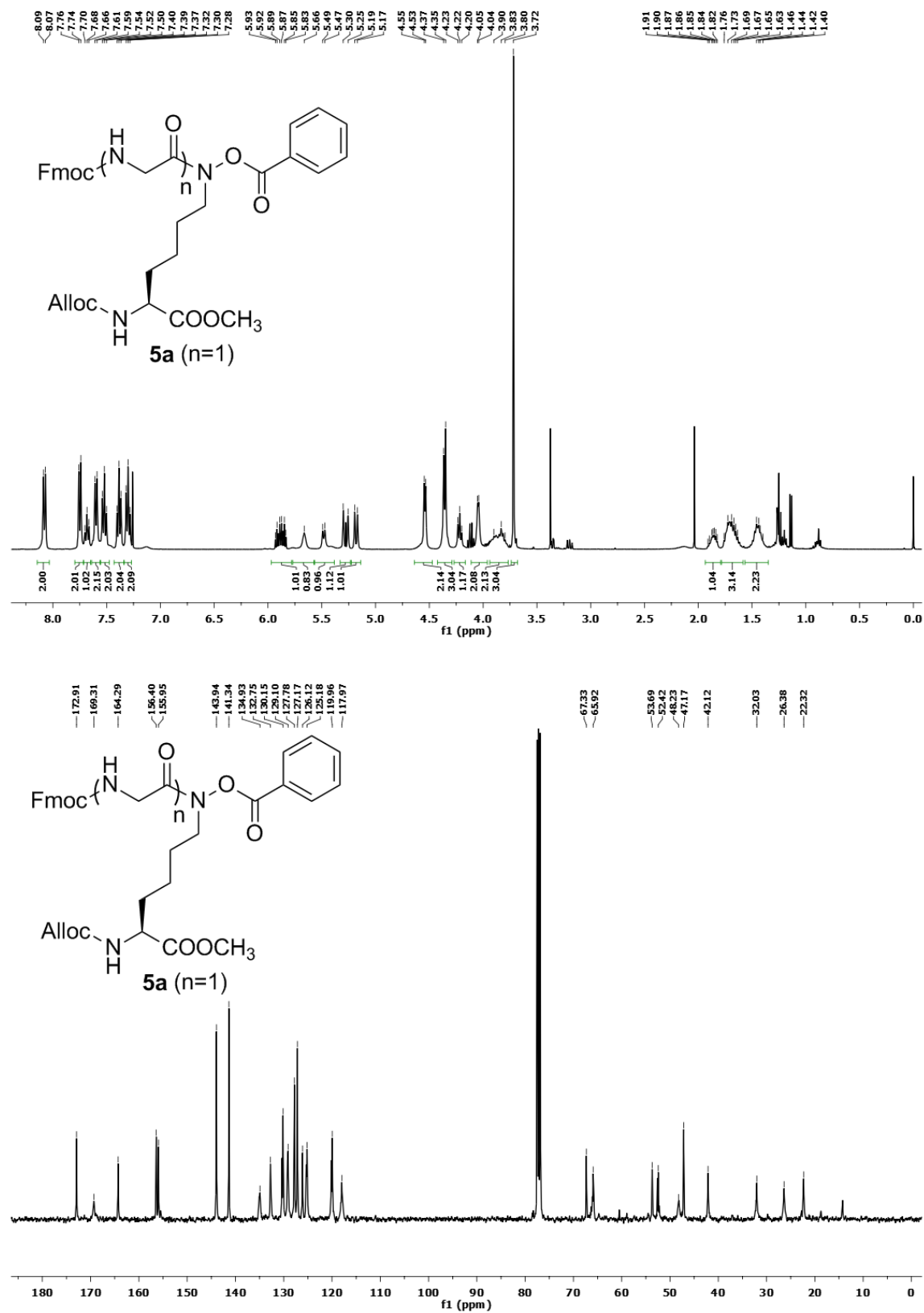
Figure S18: HPLC traces of hydrolysis of WT and *N*-hydroxy ubiquitinated peptide by UCH-L3 in presence of 15 equiv Cu(II) ion. Peak a and b correspond to WT ubiquitinated peptide, peak c corresponds to hydrolysed Ub and peak d corresponds to *N*-hydroxy ubiquitinated peptide. * Corresponds to BSA.



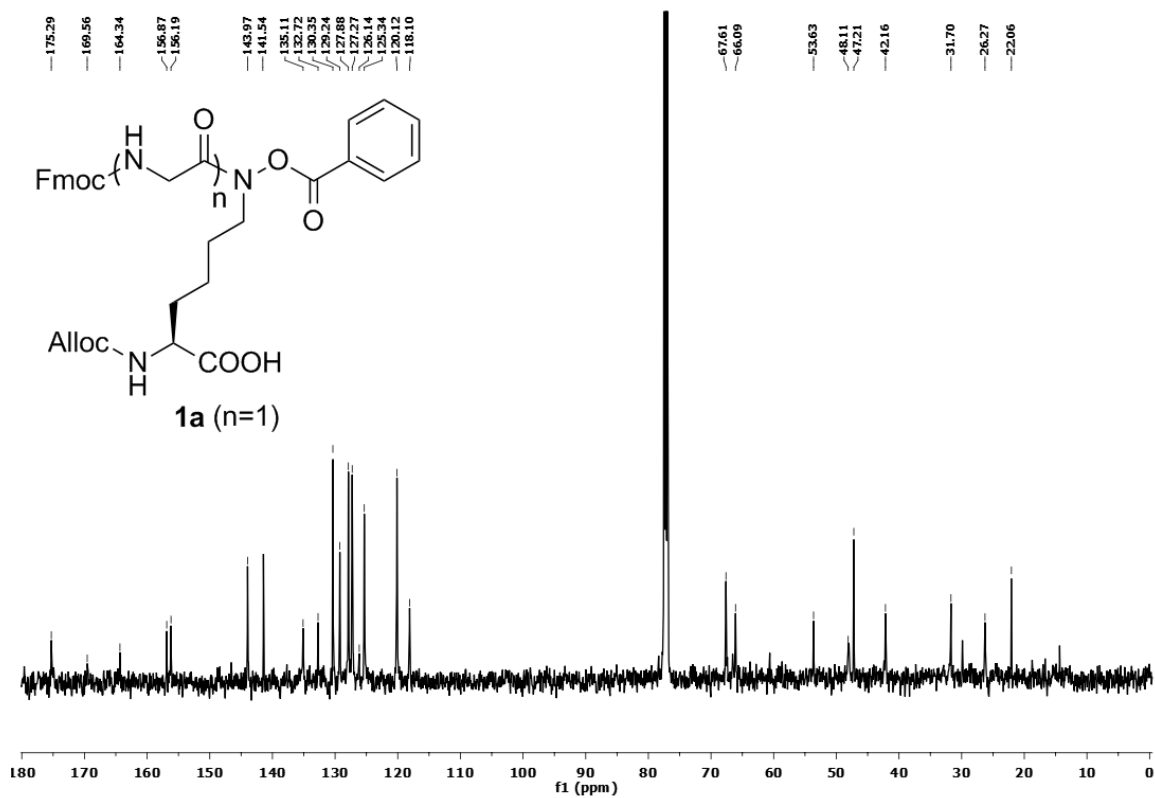
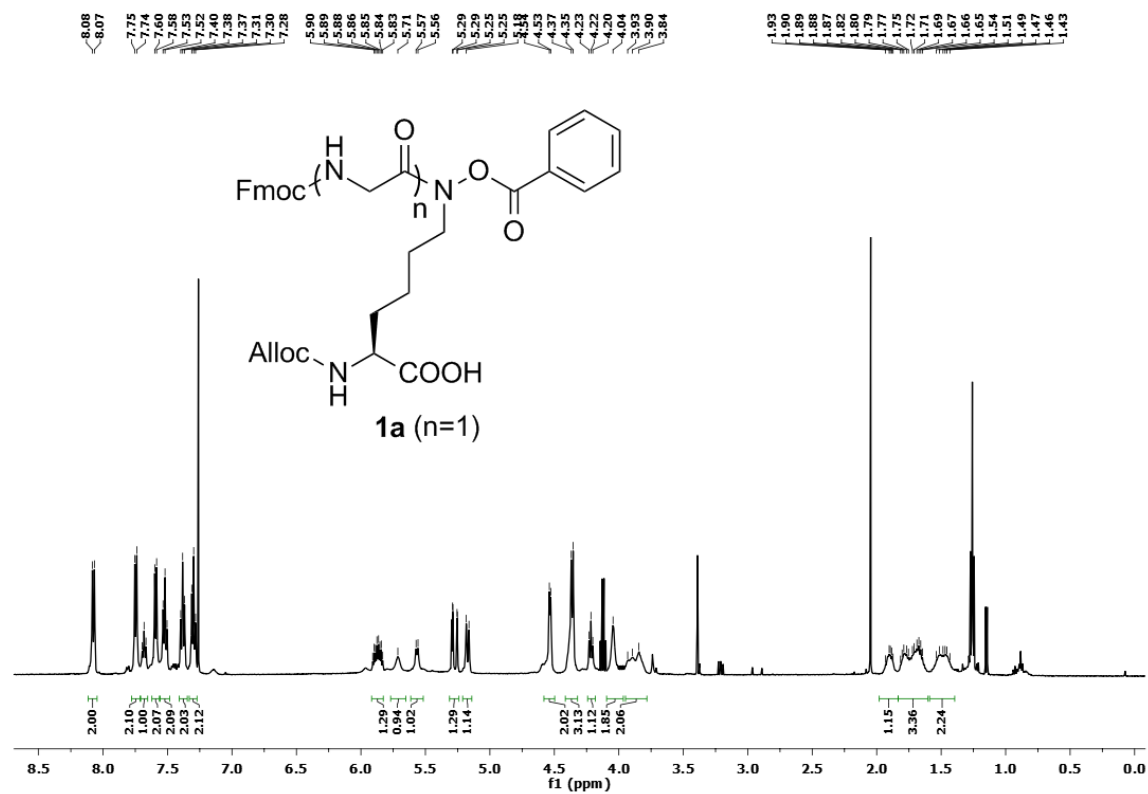
SI Figure 1: ¹H and ¹³C spectra of compound **3** in CDCl₃ (400 MHz and 100 MHz respectively).



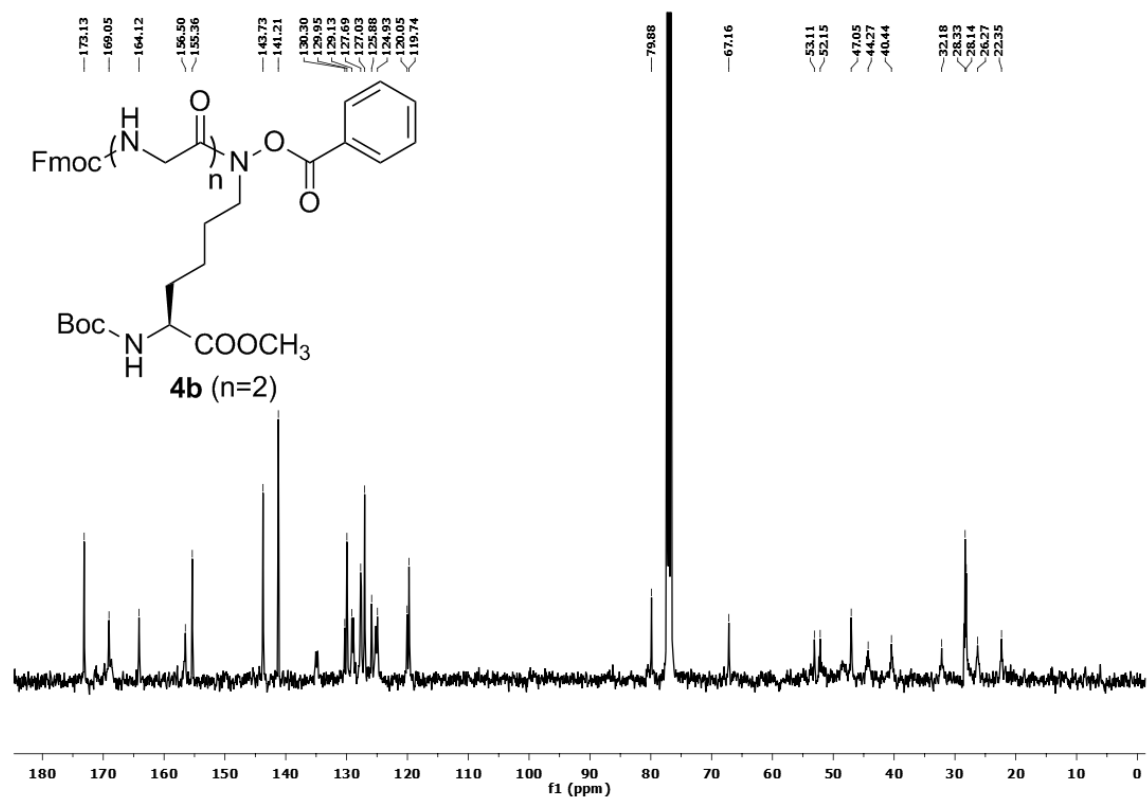
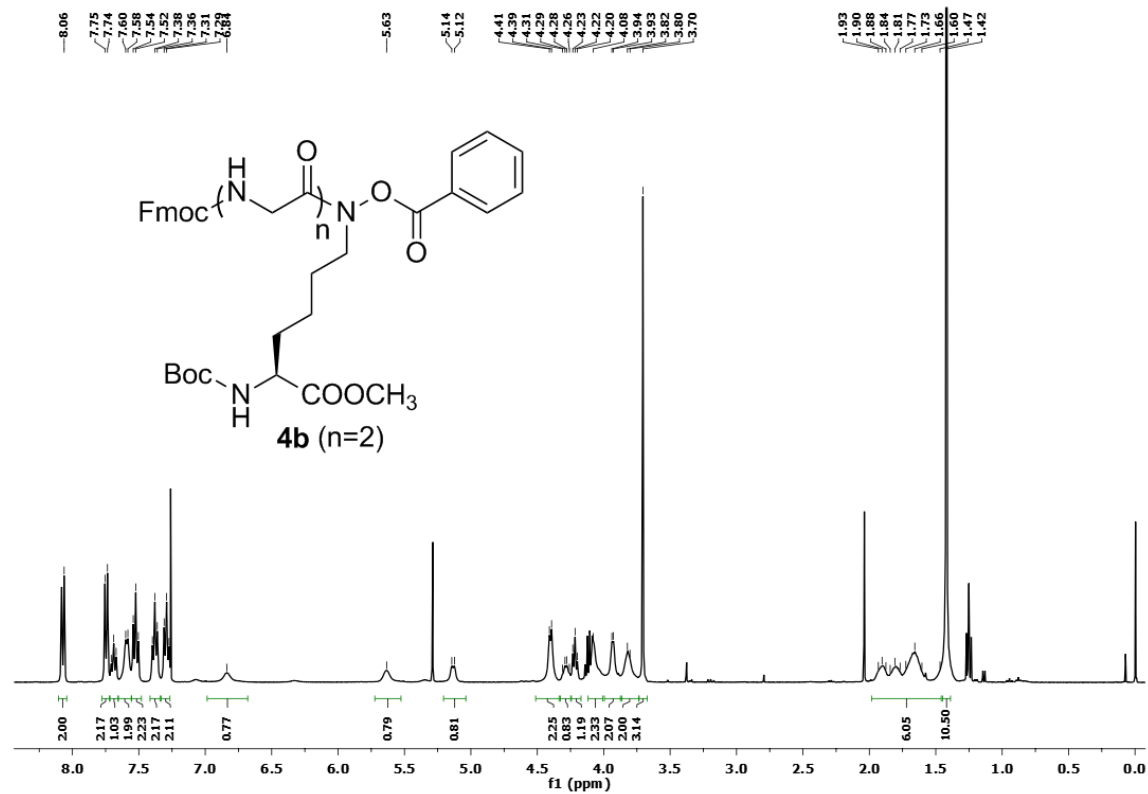
SI Figure 2: ^1H and ^{13}C spectra of compound **4a** in CDCl_3 (400 MHz and 100 MHz respectively).



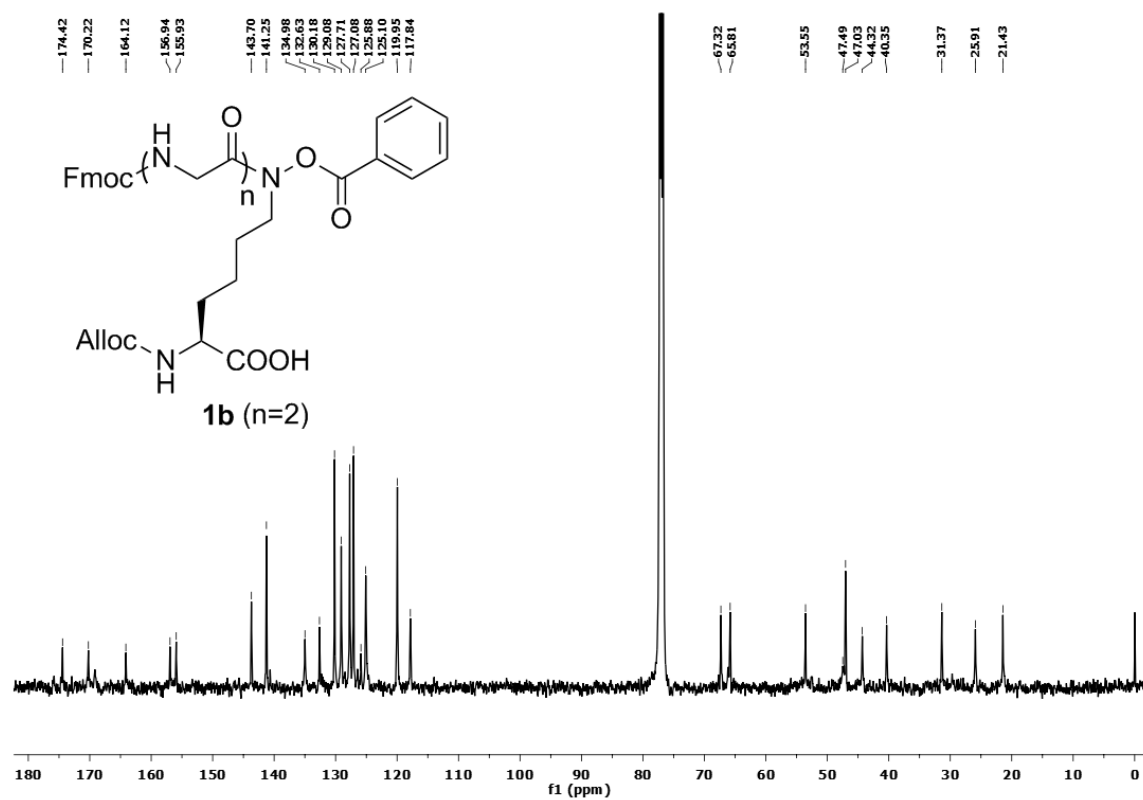
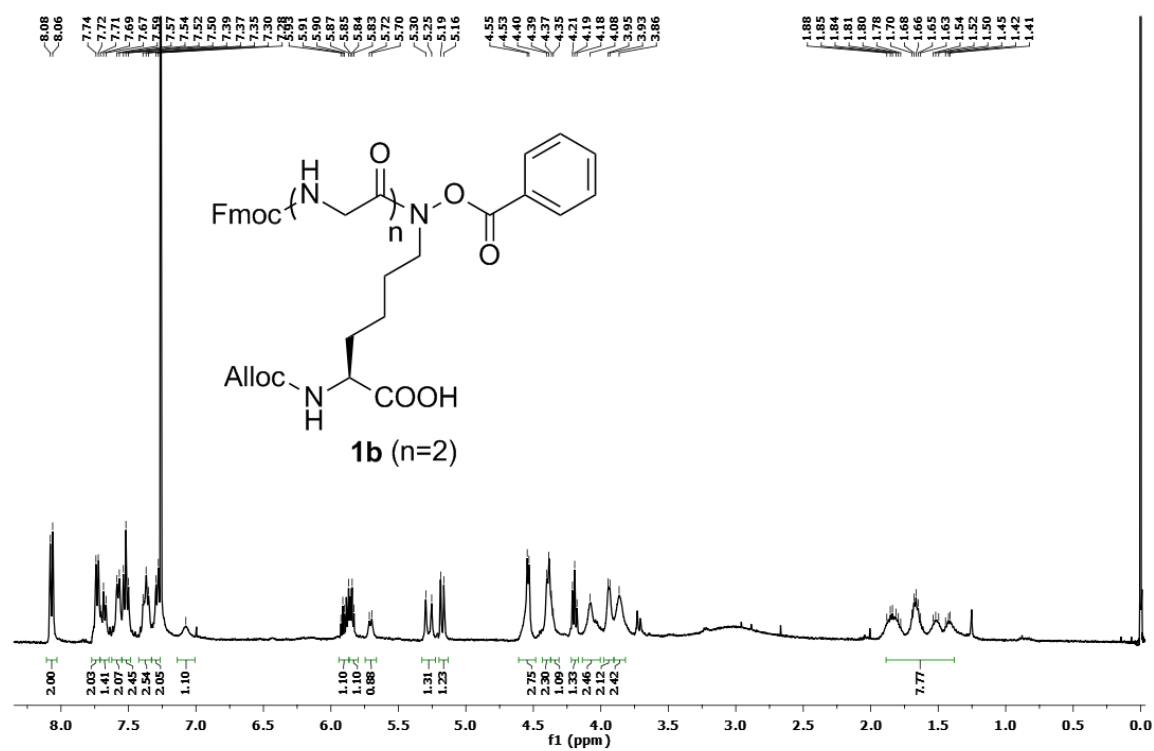
SI Figure 3: ^1H and ^{13}C spectrum of **5a** in CDCl_3 (400 MHz and 100 MHz respectively).



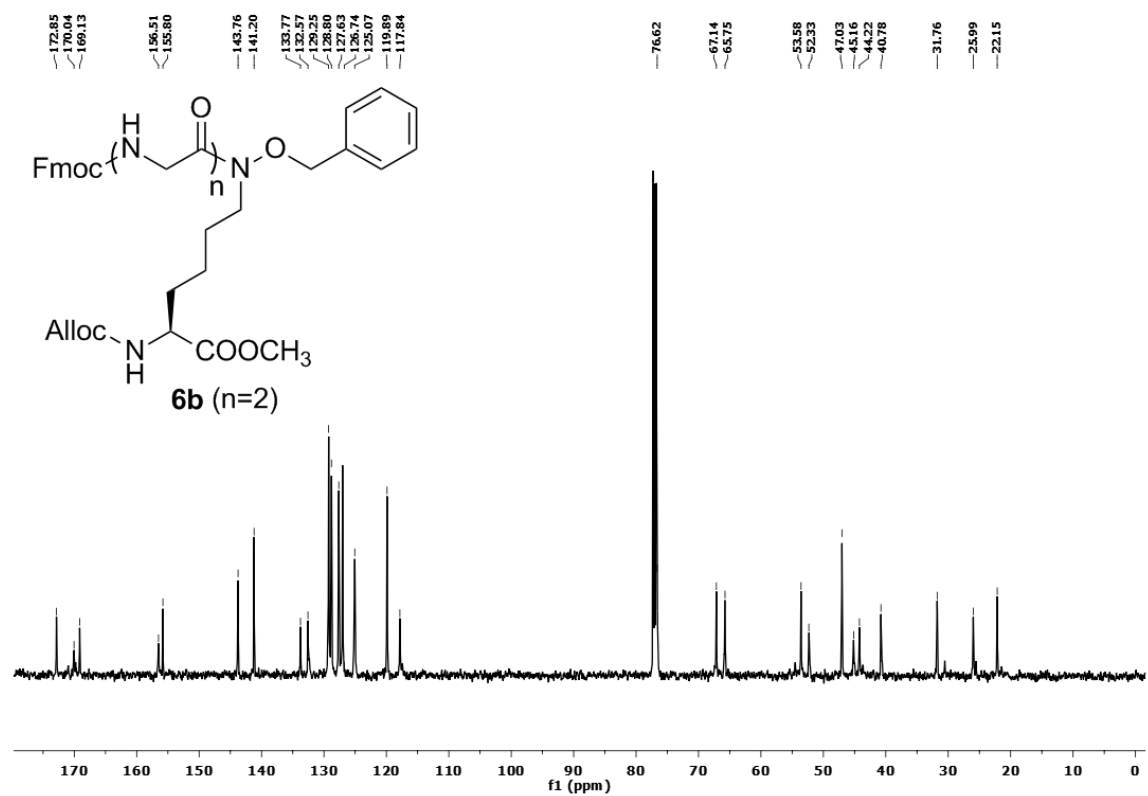
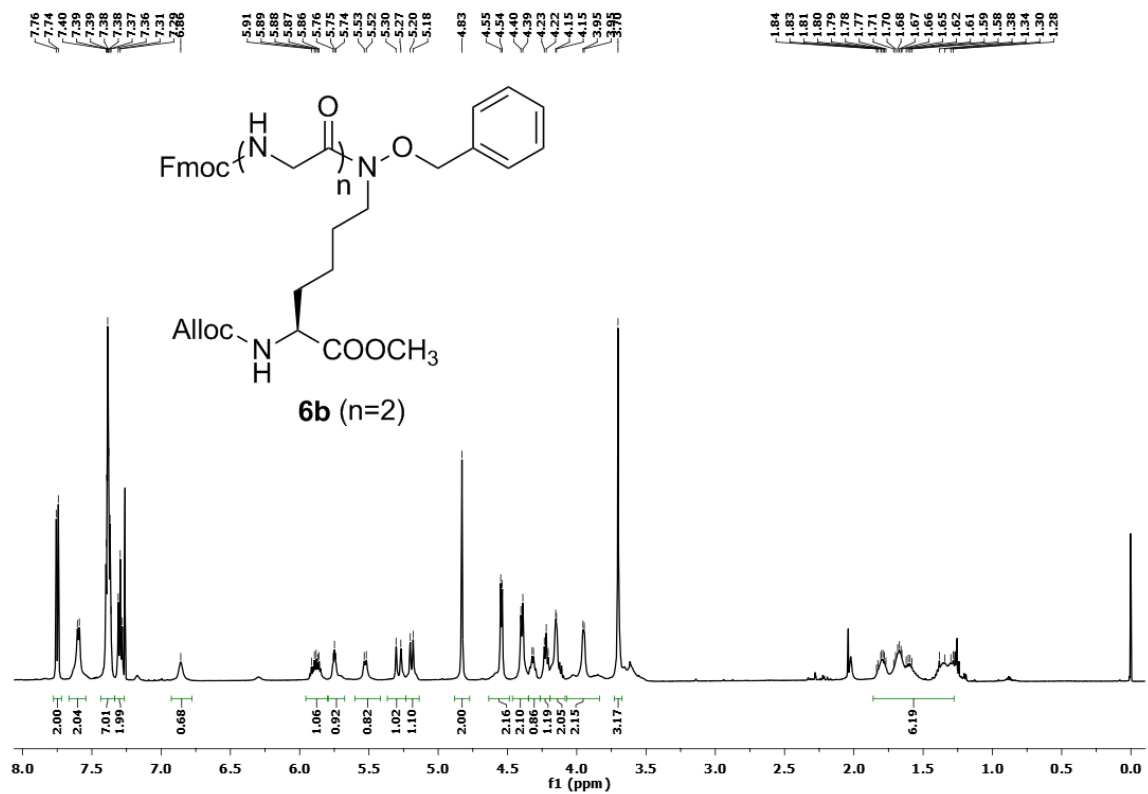
SI Figure 4: ¹H and ¹³C spectrum of **1a** in CDCl₃ (500 MHz and 125 MHz respectively).



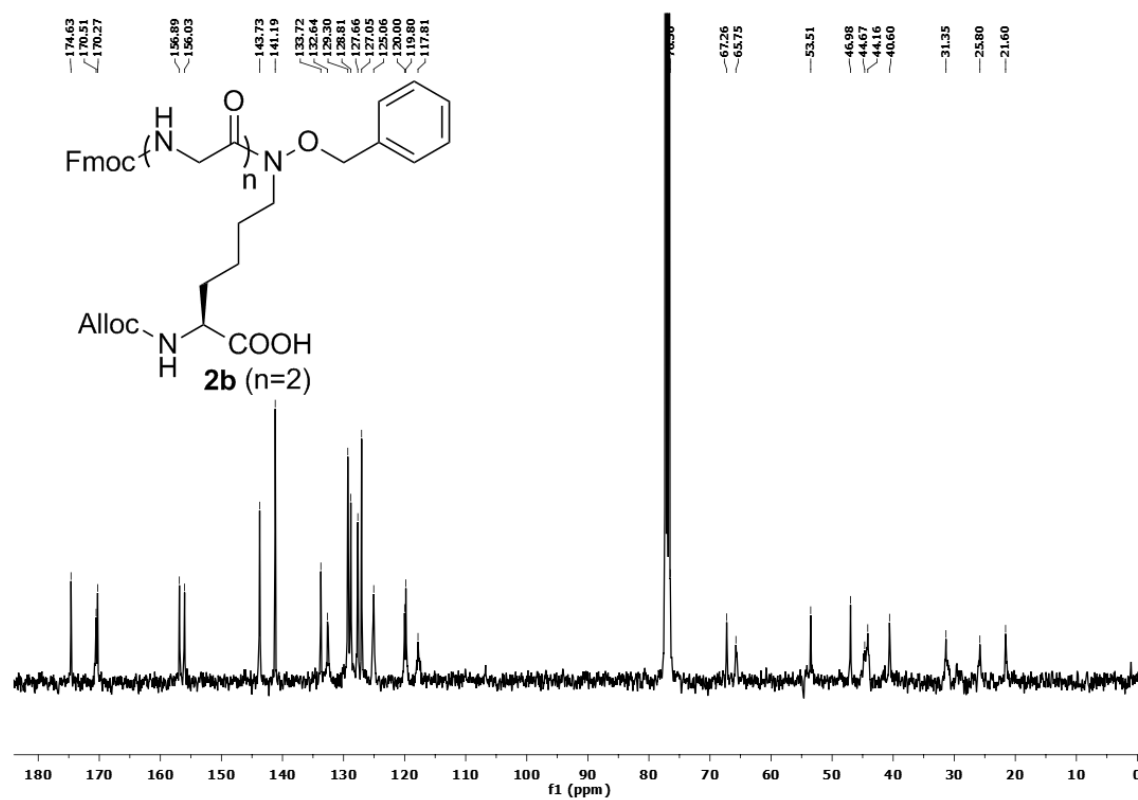
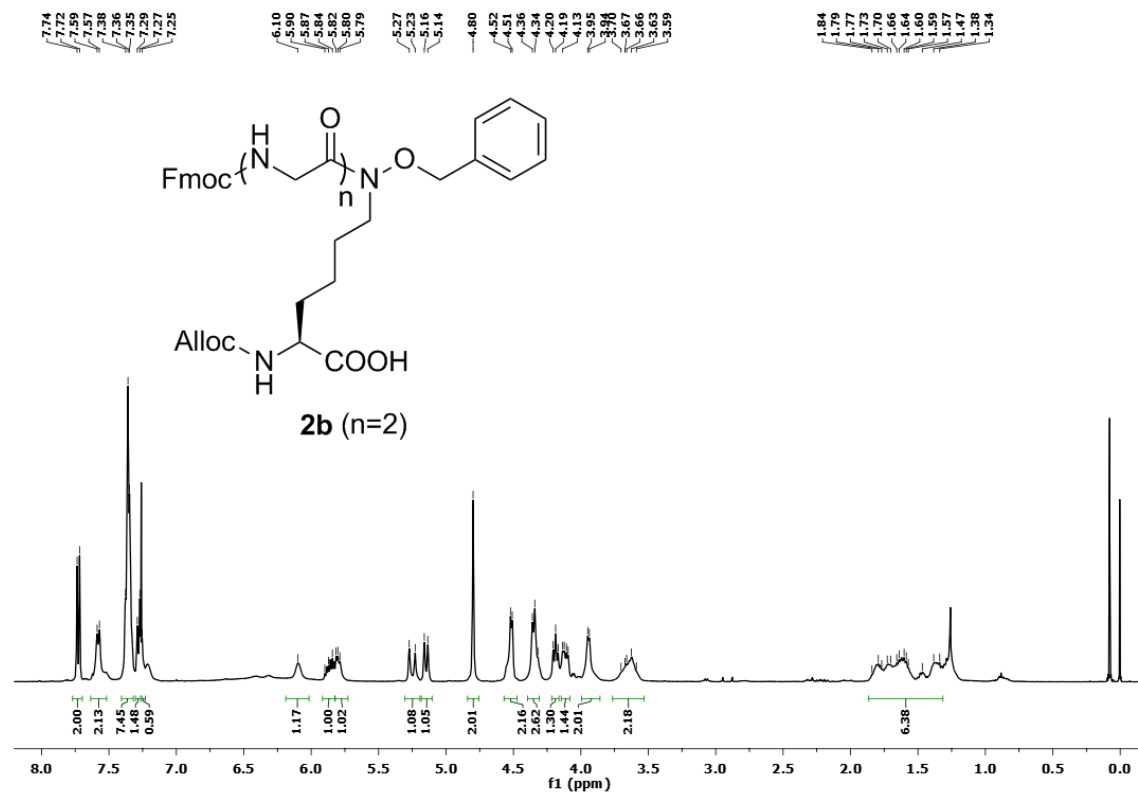
SI Figure 5: ¹H and ¹³C NMR spectrum of **4b** in CDCl₃ (400 MHz and 100 MHz respectively).



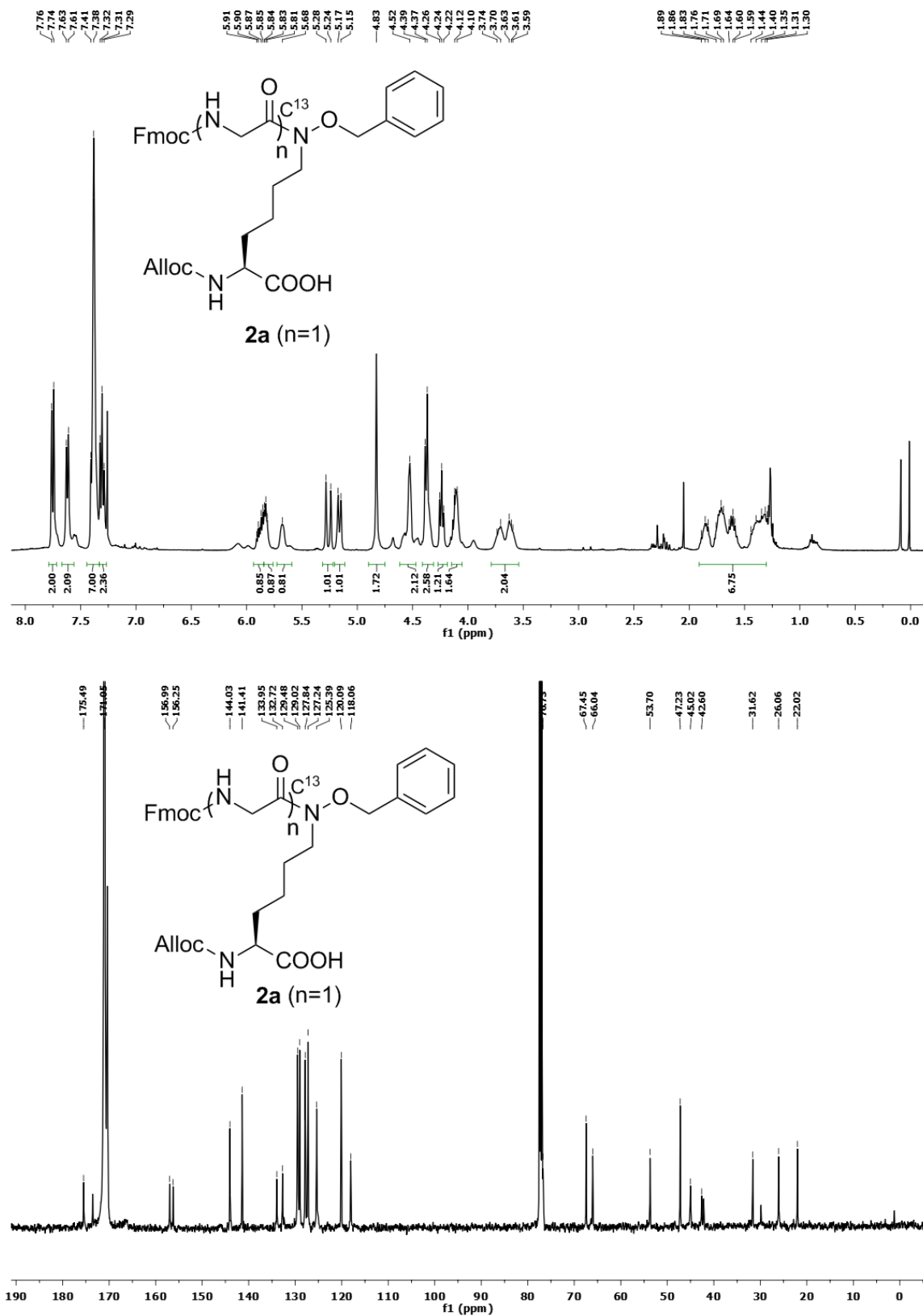
SI Figure 7: ^1H and ^{13}C NMR spectra of **1b** in CDCl_3 (400 MHz and 125 MHz respectively).



SI Figure 8: ^1H and ^{13}C NMR spectra of **6b** in CDCl_3 (500 MHz and 125 MHz respectively).



SI Figure 9: ^1H and ^{13}C NMR spectra of **2b** in CDCl_3 (400 MHz and 100 MHz respectively).



SI Figure 10: ^1H and ^{13}C NMR spectra of **2a** in CDCl_3 (400MHz and 125 MHz respectively).

