Scalable synthesis of γ-thiolysine starting from lysine and a side by side comparison with δ-thiolysine in non-enzymatic ubiquitination

Remco Merkx,[a] Gerjan de Bruin,[a] Art Kruithof,[a] Toine van den Bergh,[b] Erwin Snip,[b] Martin Lutz,[c] Farid El Oualid,[a] Huib Ovaa*[a]

[a] *Division of Cell Biology, Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands. E-mail: h.ovaa@nki.nl.*

[b] *Mercachem B.V. Kerkenbos 1013, 6546 BB Nijmegen, The Netherlands*

[c] *Bijvoet Centre for Biomolecular Research, Crystal and Structural Chemistry, Utrecht University, Padualaan 8, 3584 CH Utrecht, the Netherlands*

SUPPORTING INFORMATION

General info

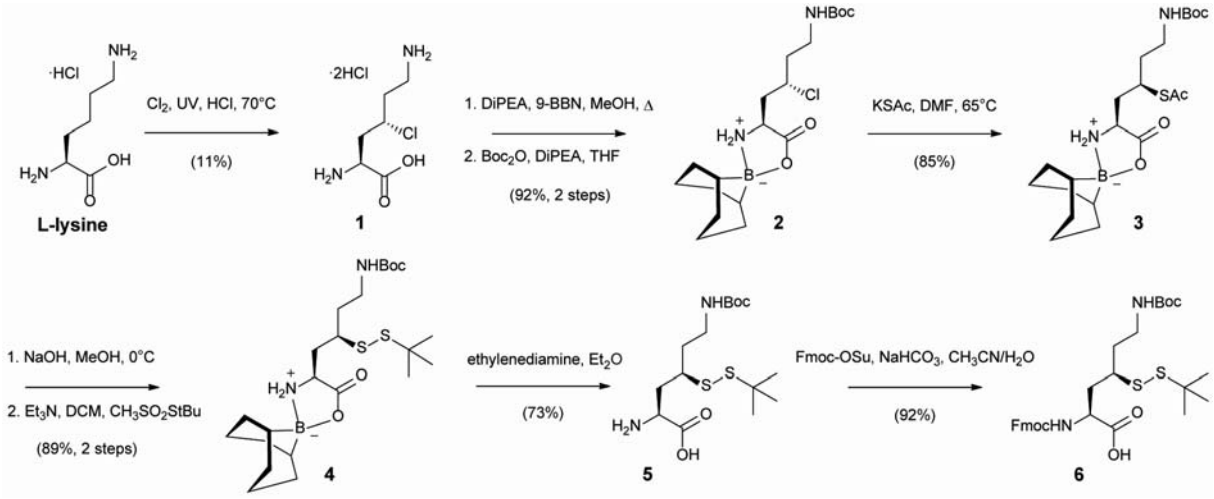
General reagents were obtained from Sigma Aldrich, Fluka and Acros and used as received. Solvents were purchased from BIOSOLVE. Peptide synthesis reagents were purchased from Novabiochem. Analytical thin layer chromatography was performed on aluminium sheets precoated with silica gel 60 F_{254}, spots were visualized using 20% ninhydrin in ethanol and heating by a heatgun. Column chromatography was carried out on silica gel (0.035-0.070 mm, 90Å, Acros). Nuclear magnetic resonance spectra (^{1}H-NMR, ^{13}C-NMR and COSY) were determined in DMSO-d_6 (^{1}H δ 2.50 ppm, ^{13}C δ 39.5 ppm) or $CDCl_3$ (^{1}H δ 7.26 ppm, ^{13}C δ 77.2 ppm) using a Bruker Avance 300 spectrometer (^{1}H: 300 MHz, ^{13}C: 100 MHz) at 298 K. Peak shapes in NMR spectra are indicated with the symbols 'd' (doublet), 'dd' (double doublet), 's' (singlet), 't' (triplet) and 'm' (multiplet). Chemical shifts (δ) are given in ppm and coupling constants *J* in Hz. LC-MS measurements were performed on a system equipped with a Waters 2795 Seperation Module (Alliance HT), Waters 2996 Photodiode Array Detector (190-750 nm) Phenomenex Kinetex C18 (2.1x50, 2.6 µm) and LCT^{TM} Orthogonal Acceleration Time of Flight Mass Spectrometer. Samples were run using 2 mobile phases: A = 1% CH_3CN, 0.1% formic acid in water and B = 1% water and 0.1% formic acid in CH_3CN. Data processing was performed using Waters MassLynx Mass Spectrometry Software 4.1 (deconvulation with Maxent1 function).

LC-MS programs

Program 1: Phenomenex Kinetex C18, (2.1×50 mm), 2.6 µM); flow rate= 0.8 mL/min, runtime= 6 min, column T= 40°C. Gradient: 0 – 0.5 min: 5% B; 0.5 – 4 min: 5 → 95% B; 4 – 5.5 min: 95% B.

Program 2: Waters Symmetry300^{TM} C4, 2.1×100 mm, 3.5 µM; flow rate = 0.5 mL/min, runtime = 30 min, column T = 20°C. Gradient: 0 - 2 min: → 5%B; 2 – 3 min: → 10% B; 3 – 17 min: → 90% B; 17 – 30 min: → 95% B.

Synthesis of suitably protected γ-thiolysine for Fmoc SPPS



Scheme S1 Synthesis of γ-thiolysine starting from lysine

Synthesis of γ-chloro-L-lysine dihydrochloride (1)

The synthesis of **1** was performed following the procedure as essentially was described by Kollonitsch *et al.*[1] In a photochemical reactor, a suspension of (S)-2,6-diaminohexanoic acid hydrochloride (137 g, 750 mmol) in concentrated hydrochloric acid (450 ml) was heated to 70°C to give a clear solution. Chlorine (74 g, 1044 mmol) was bubbled through the reaction mixture at a rate of about 40 g/h, while the mixture was irradiated using a medium pressure mercury lamp. After addition of the chlorine was complete, the reaction mixture was cooled to 20°C using a cryostat. The clear reaction mixture was transferred to a round bottom flask and stirred at room temperature. After 16h a precipitate formed which was isolated by filtration. The residue was washed with concentrated hydrochloric acid (10 mL), coevaporated three times from MeOH and triturated with MeOH (90 mL). The product was obtained after crystallization from concentrated HCl (200 mL) as a white solid (20.52 g, 11%); ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.81 (br s, 3H, αNH_3), 8.43 (br s, 3H, εNH_3), 4.48 (m, 1H, γCH), 3.96 (br s, αCH, 1H), 2.94 (m, εCH_2, 2H), 2.31 (m, total 3H, βCH_2 + δCH), 2.04 (m, 1H, δCH); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 169.9 (C=O), 56.6 (γCH), 49.6 (αCH), 38.4 (εCH_2), 36.1 (βCH_2), 35.1 (δCH_2); LC-MS (program 1): R_t = 1.05 min, MS ES+ (amu): 180.94 $[M+H]^+$.

Analysis of crude γ-chloro-L-lysine dihydrochloride (**1**)

The crude reaction product was analyzed by 1H-NMR and LC-MS. The major peaks in the 1H NMR spectrum of the crude product (Figure S1) correspond to the 1H spectrum of the final product (Figure S2). Although the crude product did contain some impurities, we were not able to find evidence for the formation of the other diastereomer in substantial amounts. The HPLC-MS spectrum of the crude product (Figure S3) predominantly showed the mass of the final product (m/z 180), in addition a mass which corresponded to a doubly chlorinated lysine side product was found (m/z 215). This presumed dichlorolysine side product was present in about 2% (calculations based on the extracted-ion chromatograms) and could be removed by crystallization from HCl.

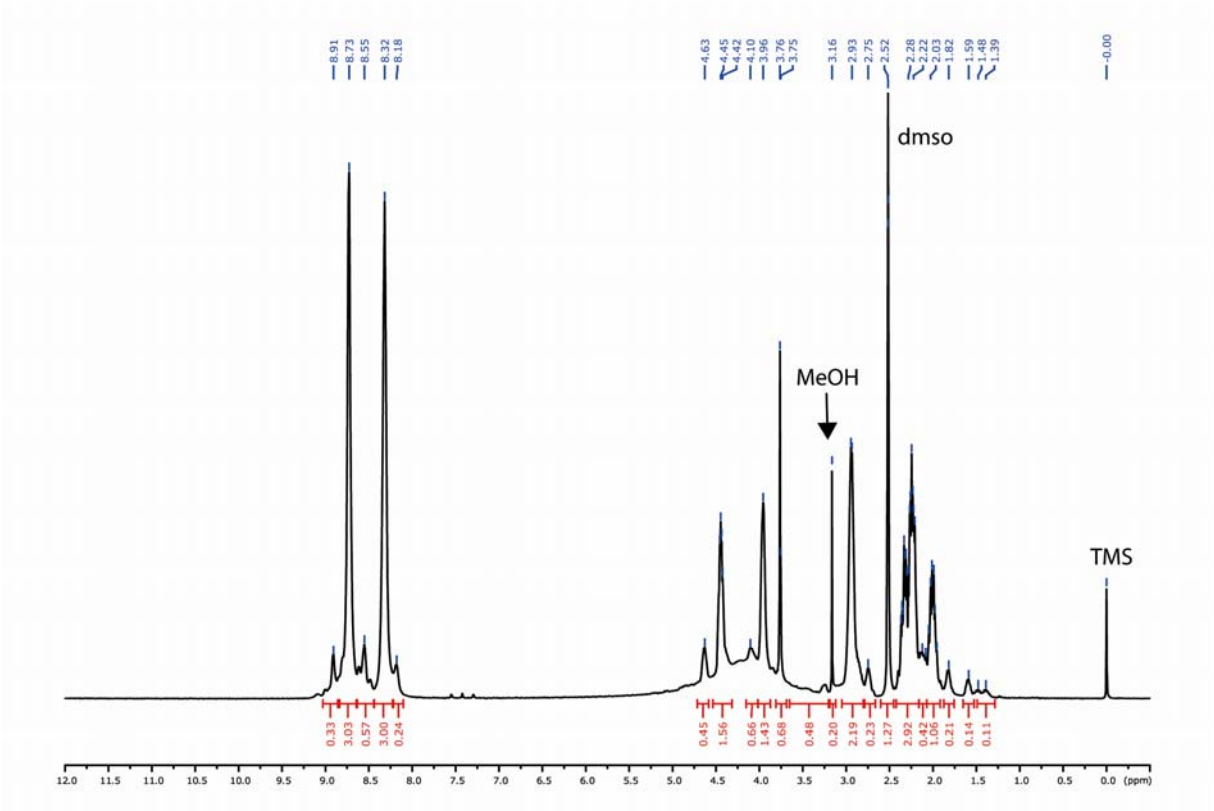


Figure S1 1H-NMR spectrum of the crude reaction product (DMSO-d_6).

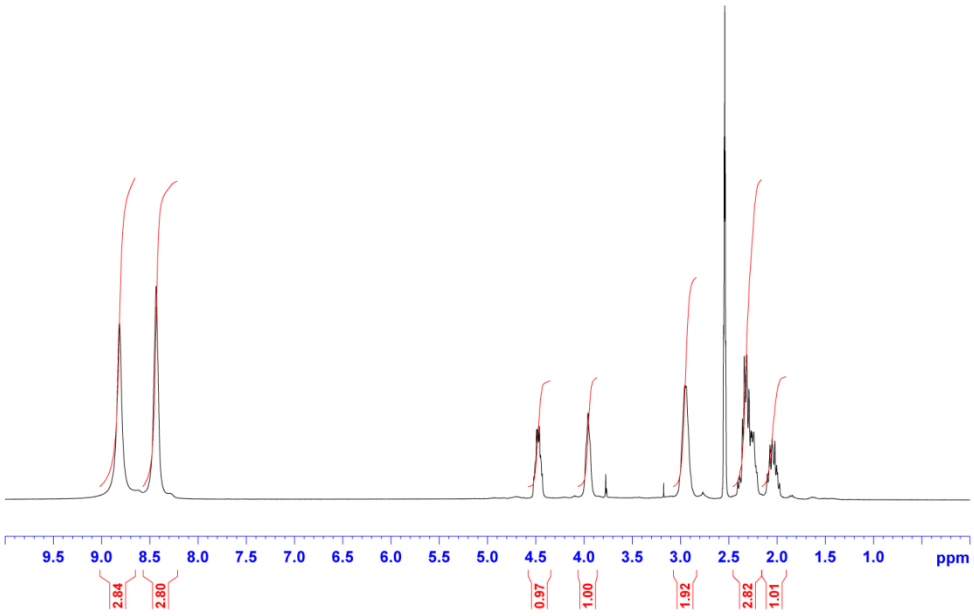
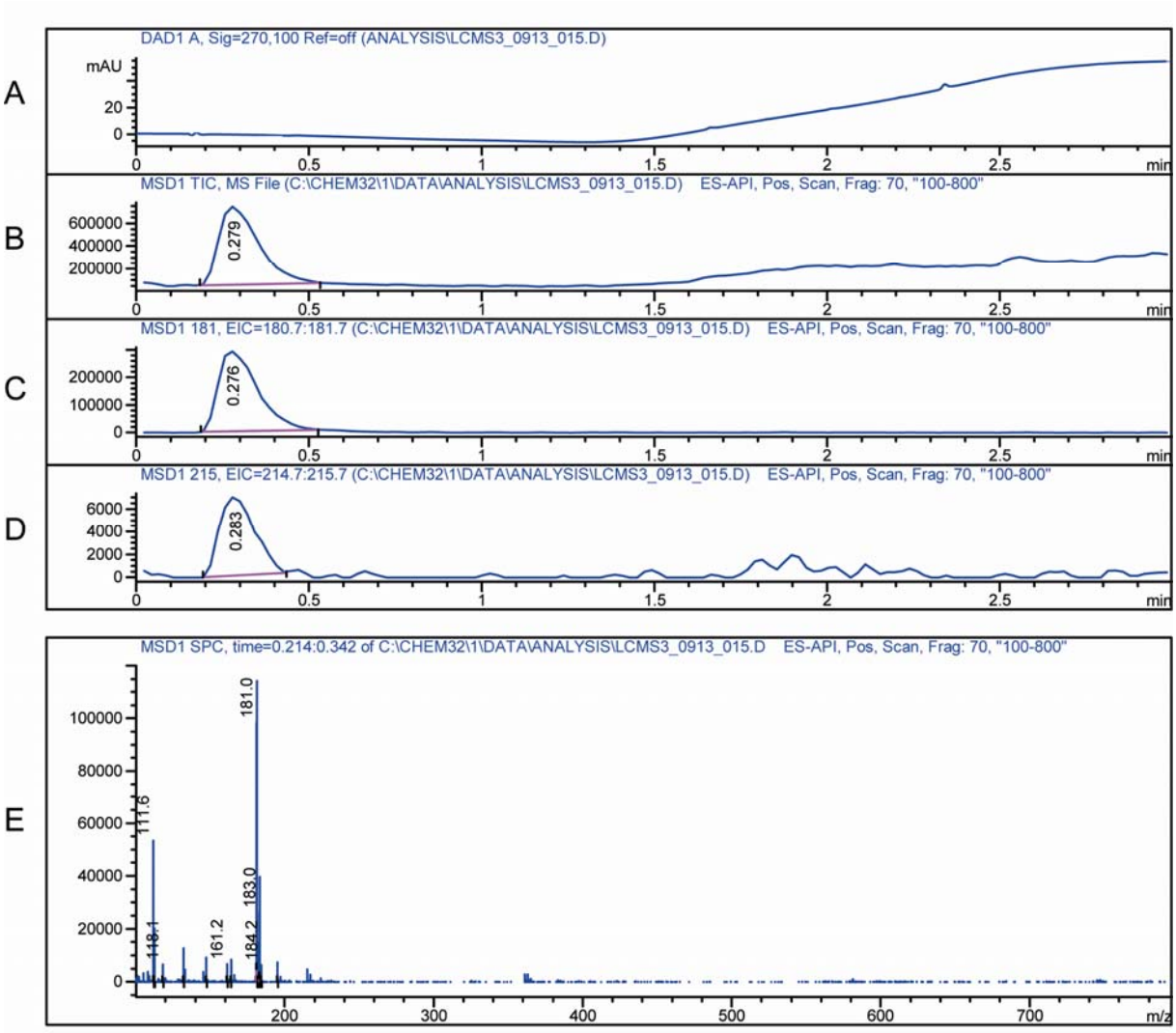


Figure S2 1H-NMR spectrum of the purified final product (DMSO-d_6).



Signal 1: DAD1 A, Sig=270,100 Ref=off

#	Meas. Ret.	Height	Area	Area %

Signal 2: MSD1 TIC, MS File

#	Meas. Ret.	Height	Area	Area %
1	0.279	691887.06	5555886.00	100.00

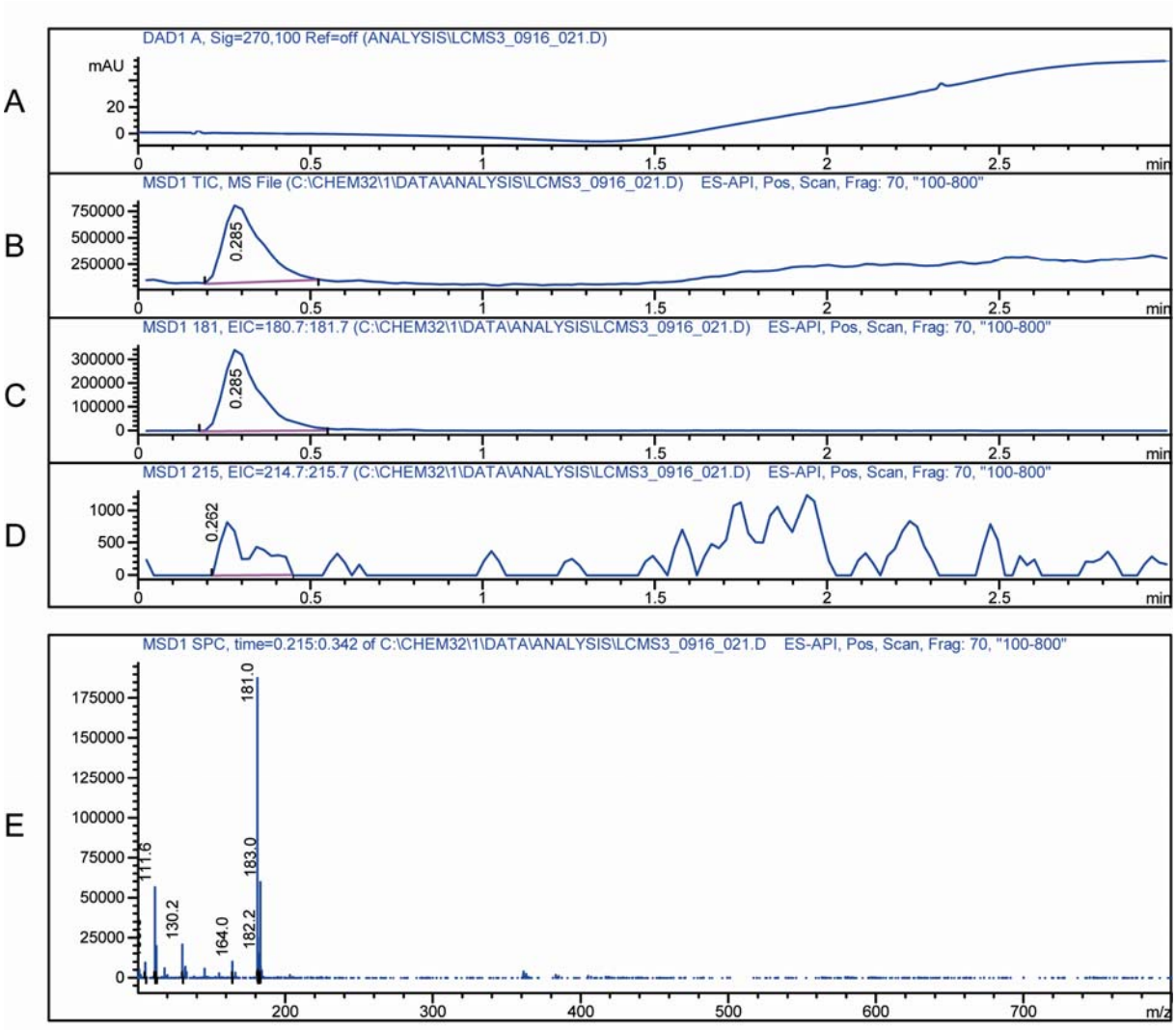
Signal 3: MSD1 181, EIC=180.7:181.7

#	Meas. Ret.	Height	Area	Area %
1	0.276	292535.69	2315632.75	100.00

Signal 4: MSD1 215, EIC=214.7:215.7

#	Meas. Ret.	Height	Area	Area %
1	0.283	6970.75	49620.11	100.00

Figure S3 HPLC-MS analysis of the crude reaction product. A) UV trace, B) Total ion chromatogram, C) Extracted-ion chromatogram (m/z 181), D) Extracted-ion chromatogram (m/z 215), E) Mass spectrum. Conditions; Column: Waters XSelect (C18, 30x2.1mm, 3.5µ), Flow: 1 ml/min Column temp: 35°C, Eluent A: 0.1% Formic acid in acetonitrile, Eluent B: 0.1% Formic acid in water, Lin. Gradient: t=0 min 2% A, t=1.6 min 98% A, t=3 min 98%A, Detection: DAD (220 - 320 nm), Detection: MSD (ESI pos/neg) mass range: 100 – 800.



Signal 1: DAD1 A, Sig=270,100 Ref=off

#	Meas. Ret.	Height	Area	Area %

Signal 2: MSD1 TIC, MS File

#	Meas. Ret.	Height	Area	Area %
1	0.285	745675.81	5640286.50	100.00

Signal 3: MSD1 181, EIC=180.7:181.7

#	Meas. Ret.	Height	Area	Area %
1	0.285	350897.44	2555194.00	100.00

Signal 4: MSD1 215, EIC=214.7:215.7

#	Meas. Ret.	Height	Area	Area %
1	0.262	847.69	5338.60	100.00

Figure S4 HPLC-MS analysis of the purified final product. A) UV trace, B) Total ion chromatogram, C) Extracted-ion chromatogram (m/z 181), D) Extracted-ion chromatogram (m/z 215), E) Mass spectrum. Conditions; Column: Waters XSelect (C18, 30x2.1mm, 3.5µ), Flow: 1 ml/min Column temp: 35°C, Eluent A: 0.1% Formic acid in acetonitrile, Eluent B: 0.1% Formic acid in water, Lin. Gradient: t=0 min 2% A, t=1.6 min 98% A, t=3 min 98%A, Detection: DAD (220 - 320 nm), Detection: MSD (ESI pos/neg) mass range: 100 – 800.

Synthesis of N^{ε}-*tert*-butoxycarbonyl-L-lysinato-bicyclononylboron (2).

DiPEA (1.75 mL, 10.0 mmol) was added to a stirred solution of **1** (1.27 g, 5.0 mmol) in dry MeOH (25 mL). After 5 minutes the reaction mixture turned turbid and 9-BBN (1.40 g, 5.75 mmol) was added. The suspension was heated at 70°C under nitrogen for 2 h until the reaction mixture turned clear and LC-MS analysis confirmed complete conversion to the borane protected product (program 2: R_t= 6.73 min, MS ES+ (amu): 301.0 $[M+H]^+$). The solvent was removed *in vacuo* and the residue was coevapporated with DCM (twice). The residue was dissolved in dry THF (25 mL) and DiPEA (1.75 mL, 10.0 mmol) and Boc_2O (1.09 g, 5.0 mmol) were added. The reaction mixture was stirred for 3 h before 1N $KHSO_4$ (25 mL) was added. The THF was removed *in vacuo*, and the remaining aqueous phase was extracted with EtOAc. The organic phase was washed with 1N $KHSO_4$ and brine, dried (Na_2SO_4) and concentrated. The product was isolated as a white foam (1.84 g, 92%) by flash column chromatography (eluent: 1:4 → 1:2 EtOAc:hexane (v/v)).

On a 187.7 mmol scale, 59.3 g (79%) of product was obtained.

R_f = 0.25 (eluent: 1:1 EtOAc:hexane (v/v)); ^{1}H-NMR (300 MHz, $CDCl_3$) δ 5.64 (m, 1H, αNH), 5.07 – 4.87 (m, total 2H, αNH + εNH), 4.31 (m, 1H, γCH), 3.88 (m, 1H, αCH), 3.21 (m, 2H, εCH_2), 2.55 – 2.43 (m, 1H, βCH), 2.19 – 1.29 (m, total 15H, βCH + δCH_2 + 6×CH_2 borane), 1.37 (s, 9H, 3×CH_3 tBu), 0.54 (br s, 2H, 2×CH borane); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 173.9 (αC=O), 156.3 (C=O Boc), 79.8 (Cq tBu), 58.9 (γCH), 53.8 (αCH), 39.5 (βCH_2), 38.5 (δCH_2), 37.7 (εCH_2), 31.8 (CH_2 borane), 31.5 (CH_2 borane), 31.2 (CH_2 borane), 31.1 (CH_2 borane), 28.4 (3×CH_3 tBu), 24.4 (CH_2 borane), 24.1 (CH borane), 23.8 (CH_2 borane), 22.6 (CH borane). LC-MS (program 2): R_t = 10.74 min, MS ES+ (amu): 401.0 $[M+H]^+$.

Synthesis of N^{ε}-*tert*-butoxycarbonyl-4-(S-acetyl)-L-lysinato-bicyclononylboron (3).

KSAc (122 mg, 1.07 mmol) was added to a solution of **2** (244 mg, 0.61 mmol) in DMF (10 mL). The reaction mixture was stirred for 3 h at 65°C before the solvent was removed *in vacuo*. The residue was redissolved in EtOAc, washed with brine, dried (Na_2SO_4) and concentrated. The product (229 mg, 85%) was isolated as a white foam by flash column chromatography (eluent: 1:4→1:2 EtOAc:hexane (v/v)).

On a 147.9 mmol scale, 47.1 g (72%) of product was obtained.

R_f = 0.17 (1:1 EtOAc/hexane v/v); 1H-NMR (300 MHz, $CDCl_3$) δ 5.42 – 5.17 (m, 2H, αNH_2), 5.04 (br s, 1H, εNH), 3.68 – 3.47 (m, total 2H, αCH + γCH), 3.35 (m, 1H, εCH), 3.15 – 3.01 (m, 1H, εCH), 2.47 – 2.30 (m, 1H, βCH), 2.38 (s, 3H, CH_3 SAc), 2.21 – 1.36 (m, total 15H, βCH + δCH_2 + 6×CH_2 borane), 1.44 (s, 9H, 3×CH_3 tBu), 0.64 (br s, 1H, CH borane), 0.51 (br s, 1H, CH, borane); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 199.2 (C=O, SAc), 173.5 (αC=O), 157.3 (C=O, Boc), 80.04 (Cq, Boc), 53.6 (αCH), 38.4 (γCH), 37.7 (εCH_2), 37.1 (δCH_2), 36.2 (βCH_2), 32.0 (CH_2 borane), 31.4 (CH_2 borane), 31.3 (CH_2 borane), 31.0 (CH_2 borane), 30.7 (CH_3 SAc), 28.3 (3×CH3, tBu), 24.5 (CH_2, borane), 24.0 (CH, borane), 23.9 (CH_2, borane), 22.5 (CH, borane). LC-MS (program 2): R_t = 11.14 min, MS ES+ (amu): 441.0 $[M+H]^+$.

Synthesis of N^{ε}-*tert*-butoxycarbonyl-4-(*tert*-butyldisulfanyl)-L-lysinato-bicyclononylboron (4).

1N NaOH (1.36 mL) was added to a cooled (0°C) solution of thioacetate **3** (487 mg, 1.11 mmol) in methanol (12 mL) the resulting mixture was stirred under nitrogen at 0°C. After 30 min the reaction mixture was neutralized by the addition of equimolar amounts of AcOH and concentrated. The residue was redissolved in ethyl acetate and washed with 1N $KHSO_4$ and brine, dried (Na_2SO_4), and concentrated to afford the crude thiol as a white foam. In a separate flask, a mixture of S-*tert*-butyl methanesulfonothioate (558 mg, 3.3 mmol) and Et_3N (1.39 µL, 9.9 mmol) in degassed DCM (5 mL) was stirred under nitrogen at room temperature before a solution of the crude thiol in degassed DCM (5 mL) was added dropwise over a period of 30 min. The reaction mixture was stirred for an additional hour and concentrated. The residue was redissolved in ethyl acetate and washed with 1N $KHSO_4$ and brine, dried (Na_2SO_4), and concentrated. The product was isolated by flash column chromatography (eluent: DCM→EtOAc/DCM 1:4 v/v) as a white foam (481 mg, 89%).

On a 106.9 mmol scale, 25.3 g (48%) of product was obtained. In the large scale procedure crude S-*tert*-butyl methanesulfonothioate was used without isolation from the reaction mixture after its synthesis from mesylchloride and 2-methyl-2-propanethiol in the presence of Et_3N in DCM.

^{1}H-NMR (300 MHz, $CDCl_3$) δ 5.40 (m, 1H, αNH), 5.16 (m, 1H, αNH), 5.03 (m, 1H, εNH), 4.17 – 3.99 (m, 1H, αCH), 6.54 (m, 1H, εCH), 3.18 – 2.99 (m, 1H, εCH), 2.90 (m, 1H, γCH), 2.24 (m, 2H, βCH_2), 2.07 – 1.27 (m, total 14H, δCH_2 + 6×CH_2 borane), 1.39 (s, 9H, 3×CH_3 tBu Boc), 1.32 (s, 9H, 3×CH_3 SStBu), 0.62 (s, total 2H, 2×CH_2 borane); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 173.7 (αC=O), 157.0 (C=O Boc), 79.9 (Cq tBu Boc), 53.5 (αCH), 48.5 (γCH), 48.4 (Cq SStBu), 37.9 (εCH_2), 36.9 (δCH_2), 35.8 (βCH_2), 32.2 (CH_2 borane), 31.3 (CH_2 borane), 31.2 (CH_2 borane), 30.9 (CH_2 borane), 29.9 (3×CH_3 SStBu), 28.3 (3×CH_3 tBu Boc), 24.3 (CH_2 borane), 24.0 (CH borane), 23.8 (CH_2 borane), 22.5 (CH borane). LC-MS (program 2): R_t = 12.58 min, MS ES+ (amu): 487.0 $[M+H]^+$.

Synthesis of N^{ε}-*tert*-butoxycarbonyl-4-(*tert*-butyldisulfanyl)-L-lysine (5).

Ethylenediamine (1050 µL, 15.4 mmol) was added to a solution of **4** (500 mg, 1.02 mmol) in ether (15 mL). The resulting mixture was stirred for 4 hours and concentrated *in vacuo*. The product (280 mg, 73%) was isolated by flash column chromatography (SiO_2, eluent: 9:1 → 6:4 DCM/MeOH v/v) as a white solid.

On a 52.0 mmol scale, 16.5 g (87%) of product was obtained. In addition, 4.4 g of the borane starting material could be retrieved which was converted to 2.4 g (72%) of **5** in a separate reaction.

^{1}H-NMR (300 MHz, $CDCl_3$) δ 3.73 (t, *J* = 6.9, 1H, αCH), 3.28-3.22 (m, 2H, εCH_2), 3.09 – 3.01 (m, 1H, γCH), 2.39 – 2.29 (m, 1H, βCH), 2.00 – 1.85 (m, total 3H, βCH+ δCH_2), 1.45 (s, 9H, 3×CH_3 SStBu), 1.35 (s, 9H, 3×CH_3 tBu Boc); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 174.0 (αC=O), 158.7 (C=O Boc), 80.3 (Cq Boc), 54.2 (γCH), 47.9 (αCH), 38.9 (εCH_2), 37.9 (δ CH_2), 35.3 (βCH_2), 30.8 (3xCH_3 SStBu), 28.9 (3xCH_3 tBu Boc). LC-MS (program 2): R_t = 7.41 min, MS ES+ (amu): 366.9 $[M+H]^+$.

Synthesis of N^{α}-(fluoren-9-ylmethoxycarbonyl)-N^{ε}-*tert*-butoxycarbonyl-4-(*tert*-butyldisulfanyl)-L-lysine (6).

A solution of Fmoc-OSu (320 mg, 0.95 mmol) in MeCN (5 mL) was added to a solution of 5 (280 mg, 0.76 mmol) and $NaHCO_3$ (128 mg, 1.52 mmol) in 1:1 H2O/MeCN (50 mL). After stirring for 3 hours, the organic layer was evaporated and 0.5 N KHSO4 (50 mL) was added. The resulting suspension was extracted with EtOAc (3x50 mL) and the organic layer was dried (Na_2SO_4) and concentrated. The product (414 mg 92%) was isolated as a white foam by column chromatography (eluent: 2→5% MeOH in DCM).

On a 45.0 mmol scale, 14.3 g (54%) of product was obtained. In addition, on a 45.0 mmol scale, 3.0 g (78%) of product was obtained. Thus in total 17.3 gram of pure **6** could be obtained starting from 47.6 g of **2**.

R_f=0.25 (5% MeOH in DCM). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 8.78 (br s, 1H, COOH), 7.74 – 7.29 (m, total 8H Hz, Fmoc), 6.45 – 6.31 (br s, 1H, αNH), 4.92 (m, 1H, εNH), 4.71 (m, 1H, αCH), 4.43 – 4.17 (m, 3H, CH + CH_2 Fmoc), 3.30 – 3.27 (m, 2H, εCH_2), 2.98 (m, 1H, γCH), 2.27 –1.72 (m, 3H, βCH_2 + δCH), 1.75 (1H, δCH), 1.43 (br s, 9H, SStBu), 1.30 (s, 9H, tBu Boc); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 174.0 (αC=O), 158.7 (C=O Boc), 80.3 (Cq Boc), 54.2 (γCH), 47.9 (αCH), 38.9 (εCH_2), 37.9 (δCH_2), 35.3 (βCH_2), 30.8 (3xCH_3 SStBu), 28.9 (3xCH_3 tBu Boc).

Synthesis of N^{α},N^{ε}-*bis*-*tert*-butoxycarbonyl-4-(*tert*-butyldisulfanyl)-L-lysine (7).

Chlorolysine **2** (4 g, 15.78 mmol, 1 eq.) was slowly added to a cooled solution (0 °C) of thionyl chloride (11.46 mL, 157.8 mmol, 10 eq.) in MeOH (200 mL). The resulting reaction mixture was refluxed at 75 °C for 4h until LC-MS analysis indicated complete conversion to the methyl ester. The reaction mixture was allowed to cool down to r.t. and the reaction mixture was concentrated *in vacuo*. The crude methyl ester (**2**) was used without further purification. ESMS Calcd for $C_7H_{15}N_2O_2Cl$ $[M+H]^+$, 195.09, found 195.00. R_t = 0.45 min. Crude **2** (15.76 mmol, 1 eq.) was suspended in 140 mL MeCN and Et_3N (8.8 mL, 63.04 mmol, 4 eq.) was added. Next, a solution of Boc_2O (12 g, 55.16 mmol, 3.5 eq.) in MeCN (20 mL) was added. The resulting reaction mixture was stirred at r.t. for 18h. The reaction mixture was concentrated to dryness and the residue was taken up in EtOAc. The organic layer was washed with 1M $KHSO_4$ (3x) and $NaHCO_3$ (sat.) (3x). The organic layer was dried over Na_2SO_4, filtered and concentrated. The product was isolated by flash column chromatography (SiO_2, eluent: 1:3 → 1:2 EtOAc/hexane) to give **3** (6.09 g, 15.42 mmol, 98%) as a colorless oil. R_f = 0.64 (EtOAc/hexane 1:2). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 5.29 (br s, 1H, NH), 4.82 (br s, 1H, NH), 4.43 (m, 1H, αCH), 4.03 (m, 1H, γCH), 3.30 (m, 2H, εCH_2), 2.34 – 2.03 (m, total 3H, βCH_2 + δCH), 1.44 (s, tBu Boc, 9H), 1.42 (s, tBu Boc, 9H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 172.2 (C=O), 156.0 (C=O), 155.4 (C=O), 80.3 (Cq Boc), 79.2 (Cq Boc), 56.3 (γCH), 52.6 (OCH_3), 51.3 (αCH), 41.6 (βCH_2), 37.8 (double line δCH_2 + εCH_2), 28.4 (3xCH_3 tBu Boc) 28.3 (3xCH_3 tBu Boc); LC-MS (program 2): R_t = 3.57 min, MS ES+ (amu):417.0 $[M+H]^+$.

A small portion of **7** was crystallized from DCM/hexanes for X-ray crystal structure determination.

X-ray crystal structure determination of N^{α},N^{ε}-*bis*-*tert*-butoxycarbonyl-4-(*tert*-butyldisulfanyl)-L-lysine (7)

$C_{17}H_{31}ClN_2O_6$, Fw = 394.89, colourless needle, 0.50 × 0.03 × 0.03 mm^3, trigonal, $P3_1$ (no. 144), a = b = 32.591(3), c = 5.1877(5) Å, V = 4772.1(9) Å^3, Z = 9, D_x = 1.237 g/cm^3, μ = 1.88 mm^{-1}. 2995 Reflections were measured on a Bruker Proteum diffractometer with rotating anode (λ = 1.54184 Å) at a temperature of 100(2) K up to a resolution of $(\sin\theta/\lambda)_{max}$ = 0.57 Å^{-1}. Intensity data were integrated with the Saint software.[2] Absorption correction and scaling was performed with SADABS[3] (correction range 0.59-0.75). 9698 Reflections were unique (R_{int} = 0.062), of which 8880 were observed [I>2σ(I)]. The structure was solved with the program SHELXT.[4] Least-squares refinement was performed with SHELXL-2013[5] against F^2 of all reflections. Non-hydrogen atoms were refined freely with anisotropic displacement parameters. All hydrogen atoms were located in difference Fourier maps and refined with a riding model. 725 Parameters were refined with one restraint (floating origin). R1/wR2 [I > 2σ(I)]: 0.0438 / 0.1168. R1/wR2 [all refl.]: 0.0506 / 0.1211. S = 1.117. Flack parameter[6] x = -0.006(15). Residual electron density between -0.28 and 0.35 e/Å^3. Geometry calculations and checking for higher symmetry was performed with the PLATON program.[7]

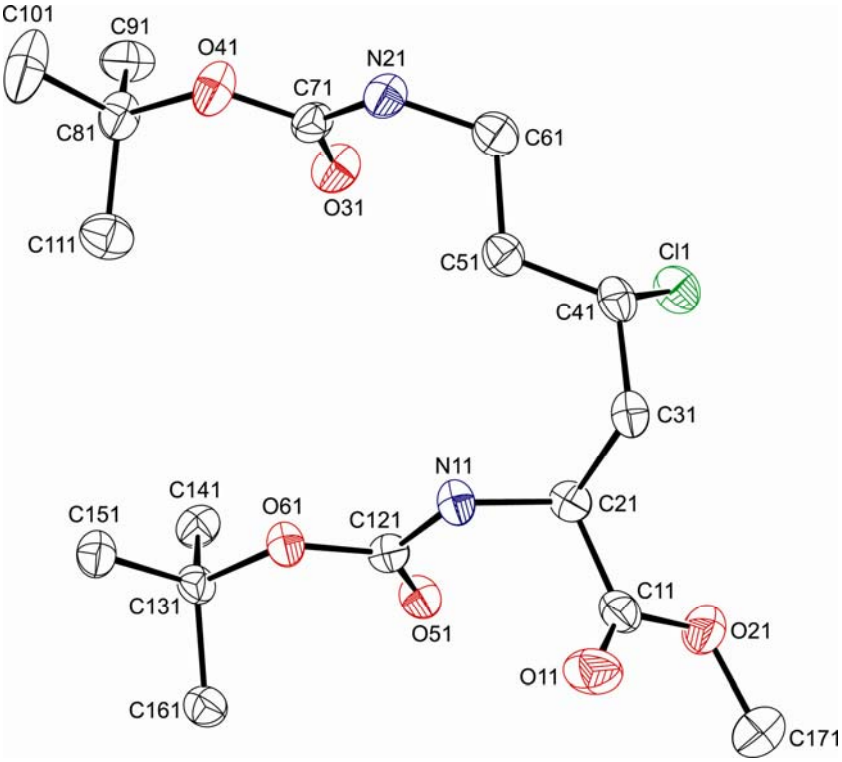


Figure S5 ORTEP representation of the structure of *bis*-Boc protected γ-chlorolysine methylester (**7**), drawn at the 50% probability level. Only one of three independent molecules is shown. Hydrogen atoms are omitted for clarity.

Synthesis ubiquitin δ/γ-thiolysine mutants

General Fmoc SPPS Strategy

SPPS was performed on a Syro II MultiSyntech Automated Peptide synthesizer using standard 9-fluorenylmethoxycarbonyl (Fmoc) based solid phase peptide chemistry at 25 µmol scale, using fourfold excess of amino acids relative to pre-loaded Fmoc amino acid Wang type resin (0.2 mmol/g, Applied Biosystems®) or pre-loaded Fmoc amino acid trityl resin (0.2 mmol/g, Rapp Polymere GmbH). The Ub (mutant) peptide sequences were synthesized on resin following the procedures as described before.[8]

Purification of ubiquitin thiolysine mutants.

The crude Ub thiolysine mutant was taken up in a minimal amount of warm DMSO and dilluted with 50 mM NaOAc pH 4.5 while the final DMSO concentration was kept as low as possible (2 – 10%). Next, the peptide was purified by cation chromatography using a MonoS column and a 0 → 1 M NaCl gradient in 50 mM NaOAc pH 4.5. Fractions that contained the were pooled and further purified by prep-HPLC using 2 mobile phases: A=0.05% TFA in MQ and B: 0.05% TFA in CH_3CN. Prep-HPLC program: Waters C18-Symmetry 80 Å (10x250 mm); flowrate: 7.5 mL/min. Gradient: 0 – 5 min: 5% B; 5 – 8 min: 5 → 25% B; 8 – 30 min: 25 → 60% B; 30 – 33 min: 60 → 95% B. Pure fractions were pooled and lyophilized.

Ub(K48δ-thiolysine): LC-MS (program 1): R_t = 2.35, ES MS+ (amu) calcd: 8643, found 8642

Ub(K48γ-thiolysine): LC-MS (program 1): R_t = 2.32, ES MS+ (amu) calcd: 8685, found 8690

Ub(K33δ-thiolysine): LC-MS (program 1): R_t = 2.50, ES MS+ (amu) calcd: 8643, found 8646

Ub(K33γ-thiolysine): LC-MS (program 1): R_t = 2.57, ES MS+ (amu)calcd 8685, found 8687

Synthesis of K48δ/γ-thiolysine linked diUbs

In a sealed reaction vessel under a nitrogen atmosphere, a solution of the K48Ub δ/γ-thiolysine mutant (0.9 mg, 0.58 mM) in 0.2 M sodium phosphate buffer (pH 7) containing 6M Gdn·HCl was incubated with TCEP (150 mM) at 37°C for 40 minutes. Next, a solution of UbMESNa thioester (1.25 equiv) in 0.2 M sodium phosphate buffer (pH 7) containing 6M Gdn·HCl and MPAA (100 mM) was added and the mixture was incubated at 37°C. After 6 hours, an additional portion of UbMESNa (1 equiv) was added and the reaction mixture was further incubated at 37°C over night. After a total reaction time of 24 hours, the reaction mixture was spun down to remove the precipitate that had formed and most of the small molecules (MPAA, TCEP) were removed from the supernatant by a buffer exchange for 0.2 M sodium phosphate buffer (pH 7) containing 6M Gdn·HCl over a 3 kDa cutoff spin-column (Amicon® Ultra). The obtained mixture was purified by prep-HPLC using 2 mobile phases: A=0.05% TFA in MQ and B: 0.05% TFA in CH_3CN. Prep-HPLC program: Phenomenex Jupiter 5u C18 300 Å (50x4.6 mm); flowrate: 1 mL/min. Gradient: 0 – 5 min: 5% B; 5 – 8 min: 5 → 25% B; 8 – 30 min: 25 → 45% B; 30 – 33 min: 45 → 95% B.

K48δ-thiolysine linked Ub_2: 0.92 mg (53%), LC-MS (program 1): R_t = 2.45, ES MS+ (amu) calcd 17143, found 17142

K48γ-thiolysine linked Ub_2: 0.90 mg (48%), LC-MS (program 1): R_t = 2.45, ES MS+ (amu) calcd 17143, found 17143

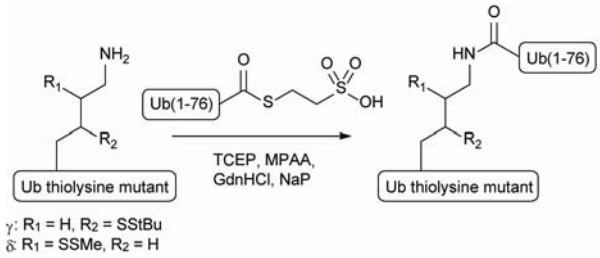
Synthesis of K33δ/γ-thiolysine linked diUbs

In a sealed reaction vessel under a nitrogen atmosphere, a solution of the K33Ub δ/γ-thiolysine mutant (2 mg, 0.58 mM) in 0.2 M sodium phosphate buffer (pH 7) containing 6M Gdn·HCl was incubated with TCEP (150 mM) at 37°C for 40 minutes. Next, a solution of UbMESNa thioester (1.25 equiv) in 0.2 M sodium phosphate buffer (pH 7) containing 6M Gdn·HCl and MPAA (100 mM) was added and the mixture was incubated at 37°C. After 3 hours, an additional portion of UbMESNa (1 equiv) was added and the reaction mixture was further incubated at 37°C over night followed by the addition of another portion of UbMESNa (0.5 equiv). After a total reaction time of 24 hours, the reaction mixture was spun down to remove the precipitate that had formed and most of the small molecules (MPAA, TCEP) were removed from the supernatant by a buffer exchange for 0.2 M sodium phosphate buffer (pH 7) containing 6M Gdn·HCl over a 3 kDa cutoff spin-column (Amicon® Ultra). The obtained mixture was purified by prep-HPLC using 2 mobile phases: A=0.05% TFA in MQ and B: 0.05% TFA in CH_3CN. Prep-HPLC program: Phenomenex Jupiter 5u C18 300 Å (50x4.6 mm); flowrate: 1 mL/min. Gradient: 0 – 5 min: 5% B; 5 – 8 min: 5 → 25% B; 8 – 30 min: 25 → 45% B; 30 – 33 min: 45 → 95% B.

K33δ-thiolysine linked Ub_2: LC-MS (program 1): R_t = 2.60, ES MS+ (amu) calcd 17143, found 17141

K33γ-thiolysine linked Ub_2: LC-MS (program 1): R_t = 2.60, ES MS+ (amu) calcd 17143, found 17148

SDS-PAGE analysis of Ub ligation reactions



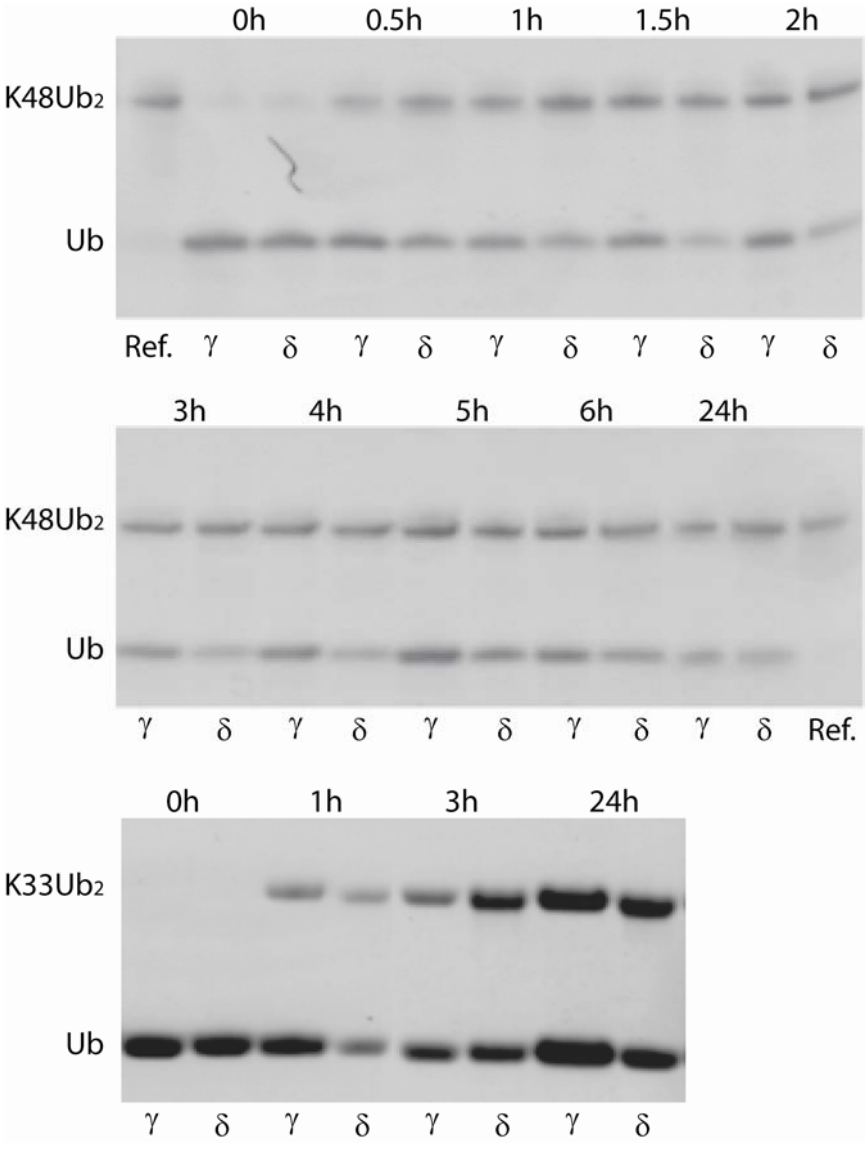


Figure S6 Gel analysis of the Ub ligation reactions (SDS PAGE gel 12%, MES buffer).

Quantification of gel bands

The gel band ratios between Ub2 and Ub were quantified at different time-points after background correction using the ImageJ (NHI, ver. 1.47) software package.

T (h)	0	0	0.5	0.5	1	1	1.5	1.5	2	2
	gamma	delta	gamma	delta	gamma	delta	gamma	delta	gamma	delta
k48Ub2	1599	2081	6970	15932	14110	18542	16792	16294	15470	18697
Ub	18375	17231	16332	15730	18009	12377	16001	9217	16214	9371
T (h)	3	3	4	4	5	5	6	6	24	24
	gamma	delta	gamma	delta	gamma	delta	gamma	delta	gamma	delta
k48Ub2	16729	17958	18324	17405	18766	17345	17654	19106	21329	21843
Ub	15532	9740	17374	9348	18817	15491	16051	15268	15337	12529
T (h)	0	0	1	1	3	3	24	24		
	gamma	delta	gamma	delta	gamma	delta	gamma	delta		
k33Ub2	478	165	6700	8424	10622	18767	24194	21816		
Ub	24954	23217	21552	17987	18989	19698	29396	22758		

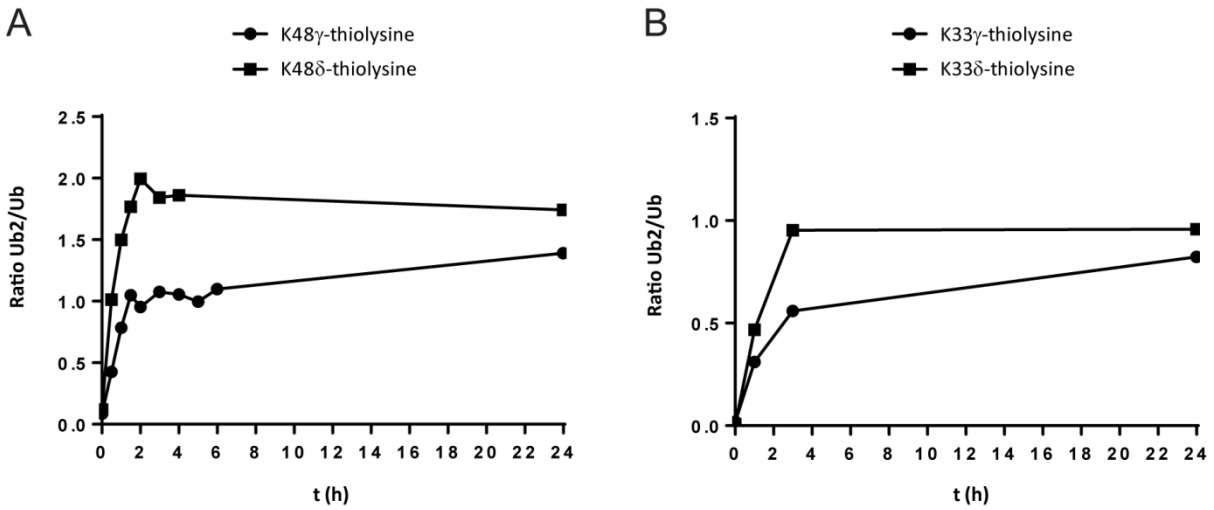


Figure S7 Plot of gel bands ratios between Ub2 and Ub for K48δ/γ-thiolysine (A)* and K33δ/γ-thiolysine (B) mediated ligations at different time-points.

*Data points at 5h and 6h were outliers and are therefore omitted from the graph.

Desulfurization of K48δ/γ-thiolysine modified diUbs

To a solution of the diUb conjugate (0.9 mg) in 900 µL degassed 0.2 M sodium phosphate buffer (pH 6.5) containing 6M Gdn·HCl, GSH (40 mM) and TCEP (200 mM), was added VA-044 (final conc 40 mM). The mixture was incubated under Ar at 37°C. In most cases desulfurizations were complete within 4 h, incomplete reactions were treated with fresh portions of VA-044 and GSH to ensure full conversion to the desulfurized product. The mixtures were purified by prep-HPLC using 2 mobile phases: mobile phases: A=0.05% TFA in MQ water and B: 0.05% TFA in CH_3CN. Phenomenex Jupiter 5u C18 300 Å (50x4.6 mm); flowrate: 1 mL/min. Gradient: 0 – 5 min: 5% B; 5 – 8 min: 5 → 25% B; 8 – 30 min: 25 → 45% B; 30 – 33 min: 45 → 95% B.

K48 linked Ub_2 synthesized *via* δ-thiolysine: 0.30 mg (30%), LC-MS (program 1): R_t = 2.50, ES MS+ (amu) calcd 17112, found 17110

K48 linked Ub_2 synthesized *via* γ-thiolysine: 0.30 mg (30%), LC-MS (program 1): R_t = 2.48, ES MS+ (amu) calcd 17112, found 17110

Desulfurization of K33δ/γ-thiolysine modified diUbs

To a solution of the diUb conjugate in 2 mL degassed 0.2 M sodium phosphate buffer (pH 6.5) containing 6M Gdn·HCl, GSH (40 mM) and TCEP (200 mM), was added VA-044 (final conc 40 mM). The mixture was incubated under Ar at 37°C. In most cases desulfurizations were complete within 4 h, incomplete reactions were treated with fresh portions of VA-044 and GSH to ensure full conversion to the desulfurized product. The mixtures were purified by prep-HPLC using 2 mobile phases: mobile phases: A=0.05% TFA in MQ water and B: 0.05% TFA in CH_3CN. Phenomenex Jupiter 5u C18 300 Å (50x4.6 mm); flowrate: 1 mL/min. Gradient: 0 – 5 min: 5% B; 5 – 8 min: 5 → 25% B; 8 – 30 min: 25 → 45% B; 30 – 33 min: 45 → 95% B.

K33 linked Ub_2 synthesized *via* δ-thiolysine: 1.4 mg (35%, 2 steps), LC-MS (program 1): R_t = 2.70, ES MS+ (amu) calcd 17112, found 17107

K33 linked Ub_2 synthesized *via* γ-thiolysine: 1.1 mg (28%, 2 steps), LC-MS (program 1): R_t = 2.65, MS+ (amu) calcd 17112, found 17107

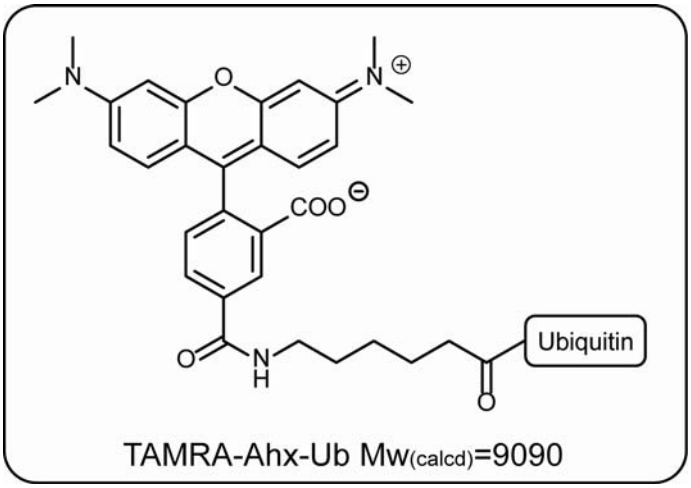
Stability of TMR-Ahx-Ub under free-radical desulfurization conditions

To investigate the stability of TAMRA-Ahx-Ub under free-radical desulfurization conditions, the fluorescent Ub mutant was incubated under different conditions using two radical initiators.

conditions a: V-50 (200 mM), T=60°C, TCEP (200 mM), GSH (40 mM), Gdn·HCl (6M), pH 6.5

conditions b: VA-044 (40 mM), T=37°C, TCEP (200 mM), GSH (40 mM), Gdn·HCl (6M), pH 6.5

It was found that TAMRA-Ahx-Ub was not stable when incubated at elevated temperature (T=60°C) using high concentration of radical initiator V-50 (conditions A). The reaction mixture changed color over time and mass analysis of the reaction mixture after 4.5h showed a mass that was indicative for loss of a methyl group (-15 Da) from the TAMRA label. In contrast, TAMRA-Ahx-Ub remained stable when it was incubated under mild temperature and low radical initiator concentration (conditions b) as was confirmed by ESMS analysis of the reaction mixture after 4.5h and 22h.



TAMRA-Ahx-Ub $Mw_{(calcd)}$=9090

M/z (observed)	M/z (calcd)	Compound
9090	9090	TAMRA-Ahx-Ub
9106	9106	TAMRA-Ahx-Ub + O (oxidation)
9075	9075	TAMRA-Ahx-Ub – CH_3 (demethylation)

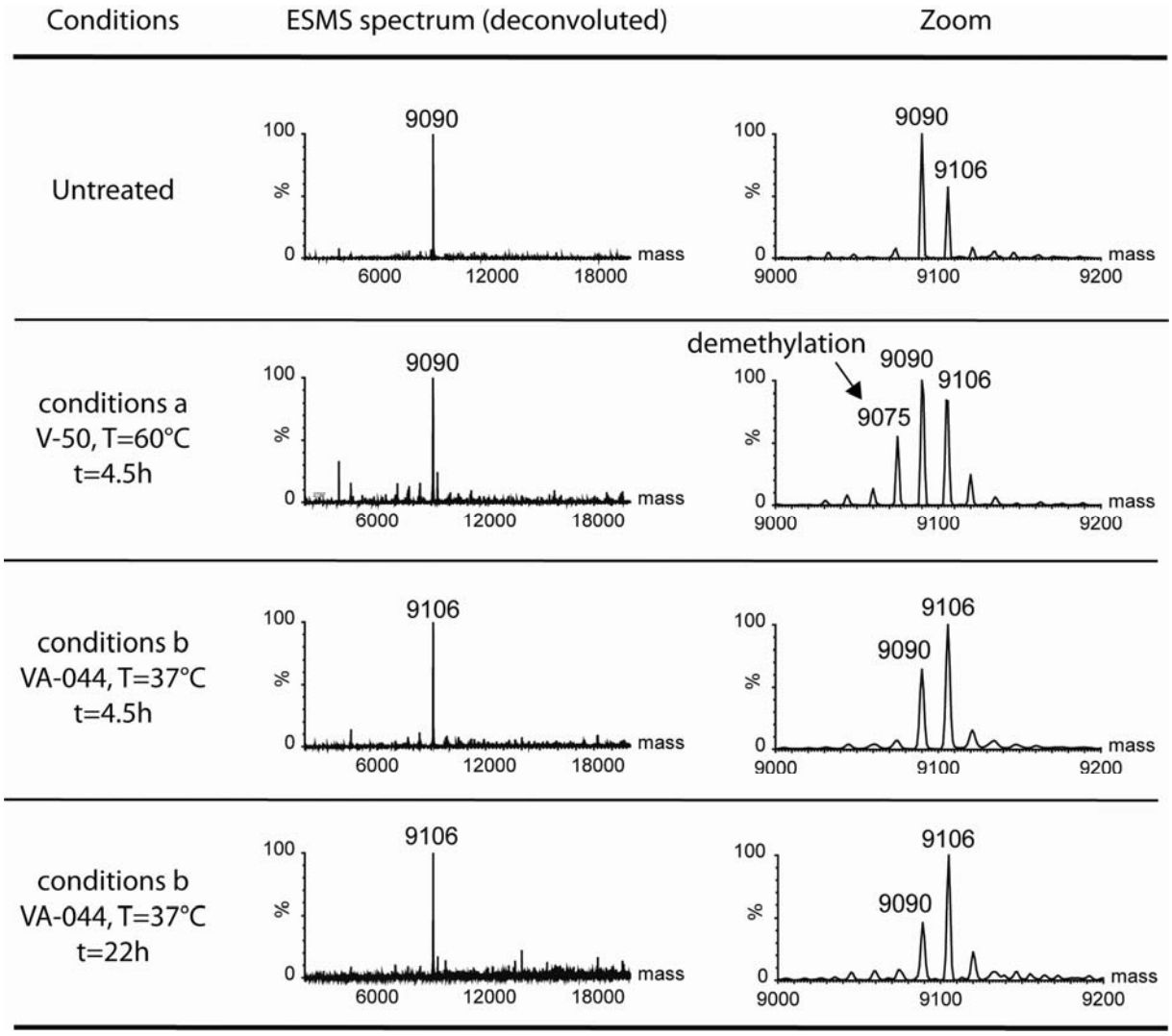


Figure S8 ESMS analysis of TAMRA-Ahx-Ub stability under free-radical desulfurization conditions.

Desulfurization of K6δ/γ-thiolysine linked Ub_2

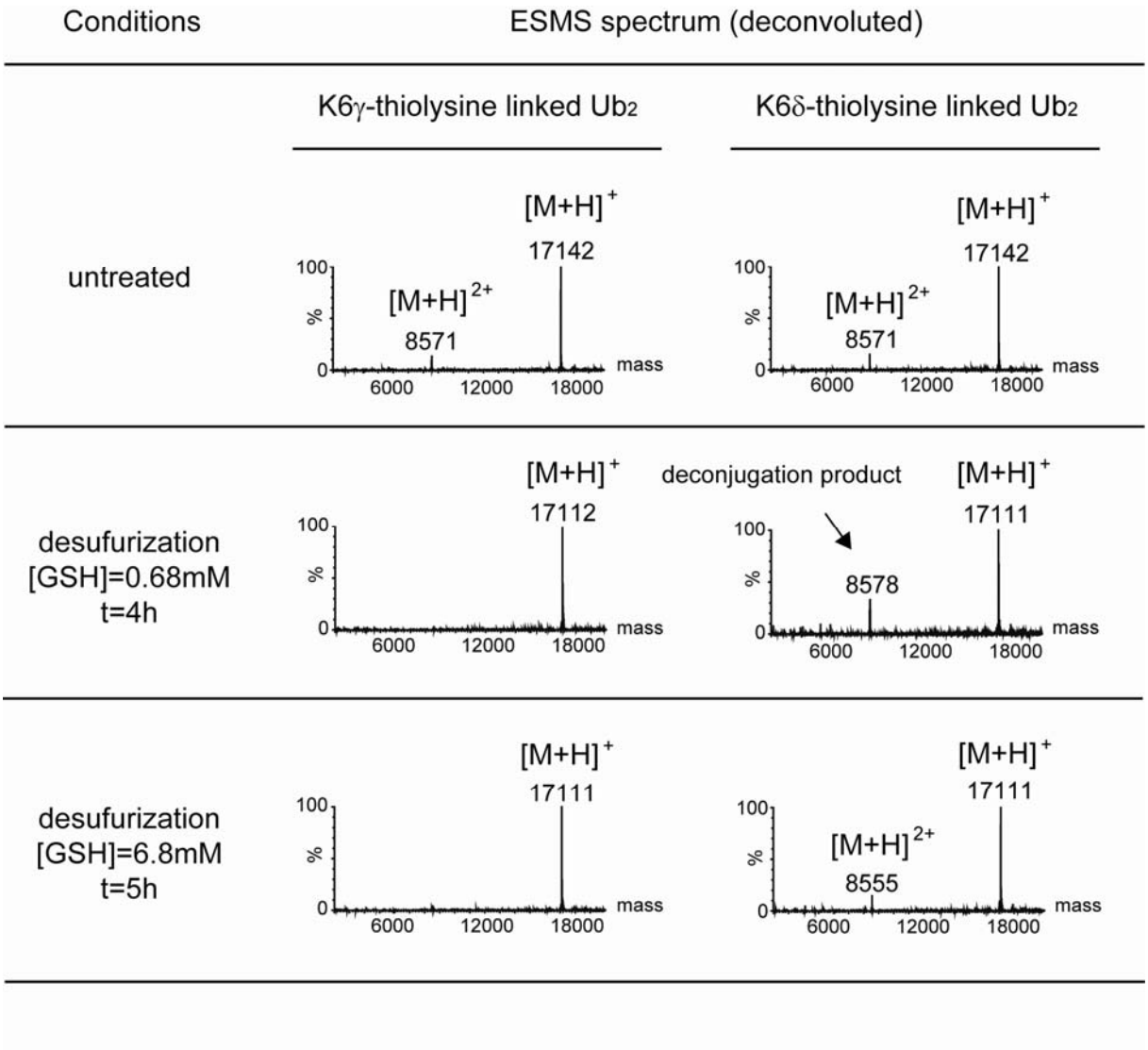


Figure S9 ESMS analysis of the reaction mixture during free-radical desulfurization of K6γ-thiolysine and K6δ-thiolysine linked diUb in the presence of different concentrations of GSH. Conditions: diUb (2 mg/mL), 0.2 M sodium phosphate (pH 6.5), 6M Gdn·HCl, GSH (0.68 or 6.8 mM), TCEP (200 mM), VA-044 (40 mM).

Circular Dichroism (CD) Measurements

CD spectra were recorded at 298 K on a JASCO J-810 spectrometer equipped with a JASCO CDF-426S temperature control unit using 0.1 cm path length quartz cells. The CD spectra are an average of three scans, collected at 0.2 nm intervals between 185 and 260 nm. Ellipticity is reported as the mean residue ellipticity [θ] in degrees cm^2 $dmol^{-1}$. Synthetic diUbs were compared to native Ub which was commercially obtained from Boston Biochem.

Sample preparation:

The purified diUb samples were dissolved in dmso and then diluted with 15 mL 20 mM $NaPO_4$ (pH 6.8). Next, the dmso was removed by replacing the buffer for 20 mM $NaPO_4$ (pH 6.8) over a 3.5 KDa Millipore spin filter. The samples were resuspended in 500 µL 20 mM $NaPO_4$ (pH 6.8) buffer and the diUb concentrations were calculated using the BCA assay (K33 linked Ub_2: 50 ug/mL, K48 linked Ub_2: 40 ug/mL, mono Ub: 150 ug/mL).

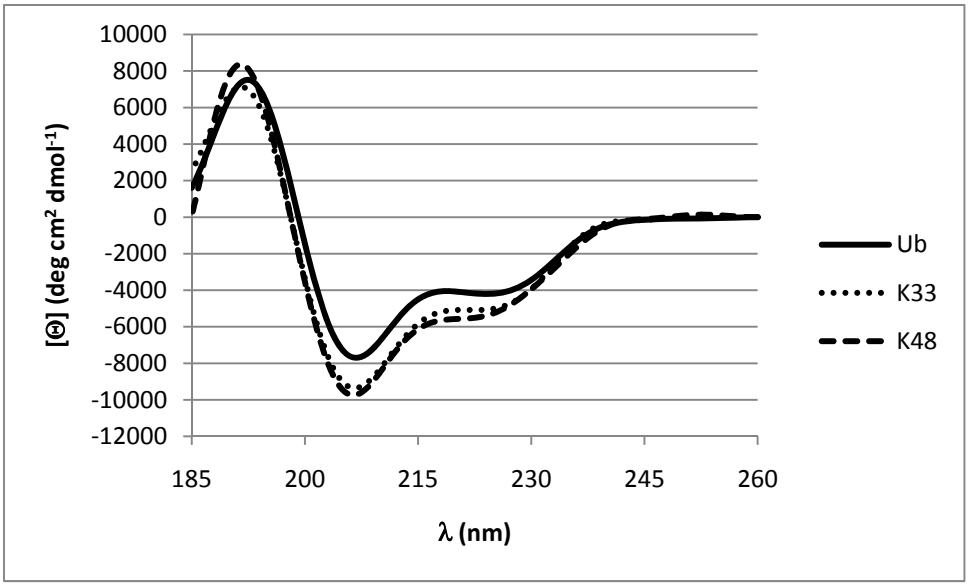


Figure S10 CD spectra of synthetic K33 linked Ub_2, synthetic K48 linked Ub_2 and native mono Ub.

Enzymatic hydrolysis of synthetic diUbs by DUBs

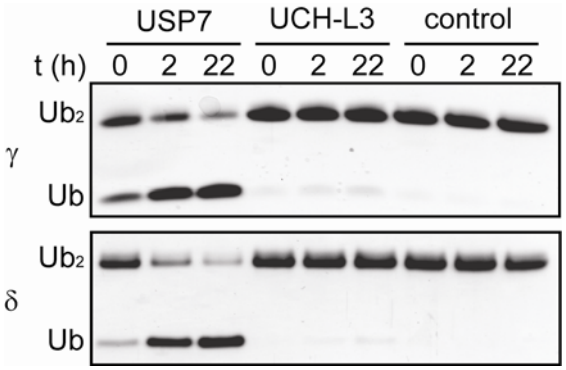


Figure S11 Treatment of synthetic K33 linked di-Ub and K48 linked di-Ub with USP7 and UCH-L3. Conditions: 40 nM DUB, 15 µM diUb, 20 mM Tris (pH=7.5), 50 mM NaCl, 5 mM DTT.

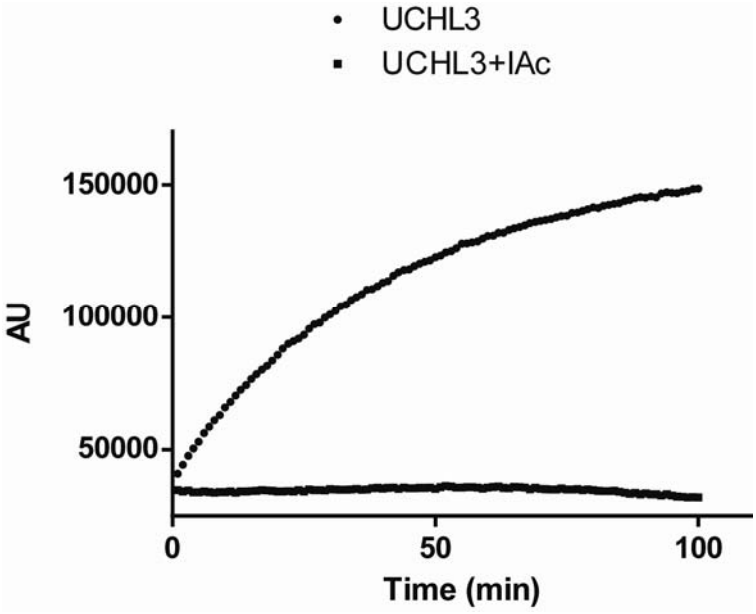


Figure S12 UbAMC (400 nM) hydrolysis by UCH-L3 (5 pM), iodoacetamide (IAc) was added as negative controll.

HPLC-MS chromatograms and ESMS spectra

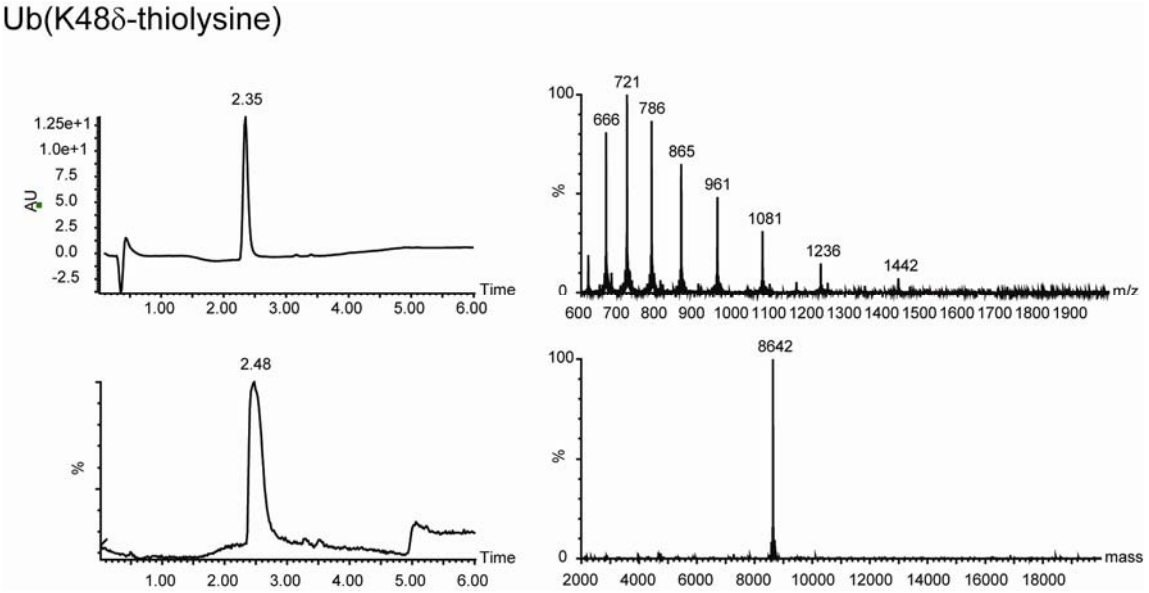


Figure S13 HPLC-MS analysis Ub with Lys48 mutated to SSMe protected δ-thiolysine. (top left) HPLC chromatogram, (bottom left) MS chromatogram, (top right) ESMS spectrum, (bottom right) deconvoluted mass of peak at 2.48 min from MS chromatogram.

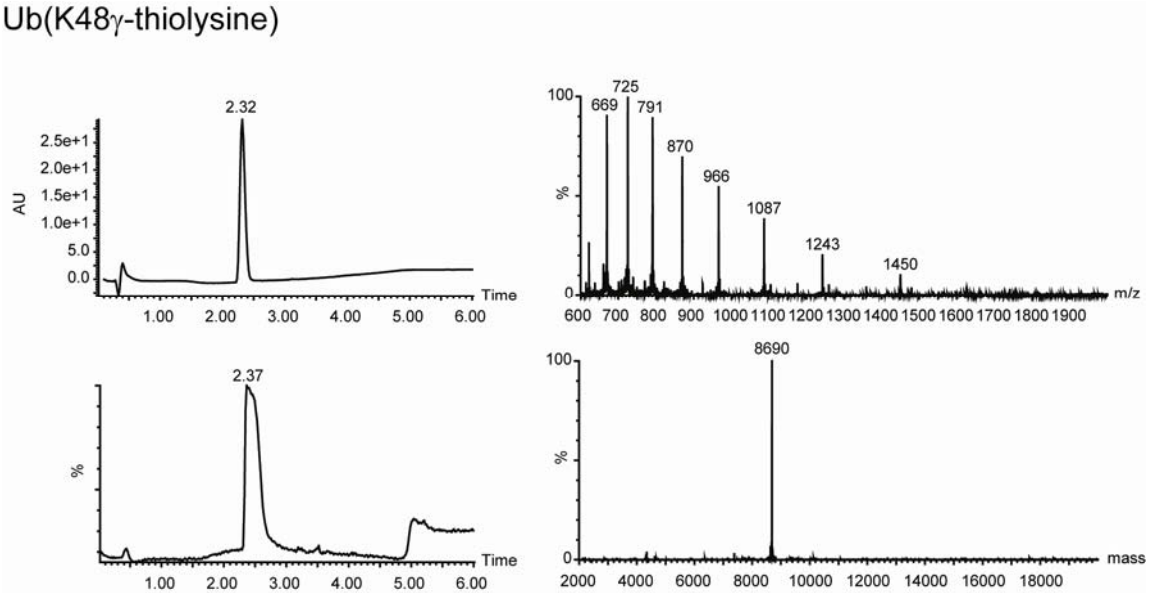


Figure S14 HPLC-MS analysis Ub with Lys48 mutated to SStBu protected γ-thiolysine. (top left) HPLC chromatogram, (bottom left) MS chromatogram, (top right) ESMS spectrum, (bottom right) deconvoluted mass of peak at 2.37 min from MS chromatogram.

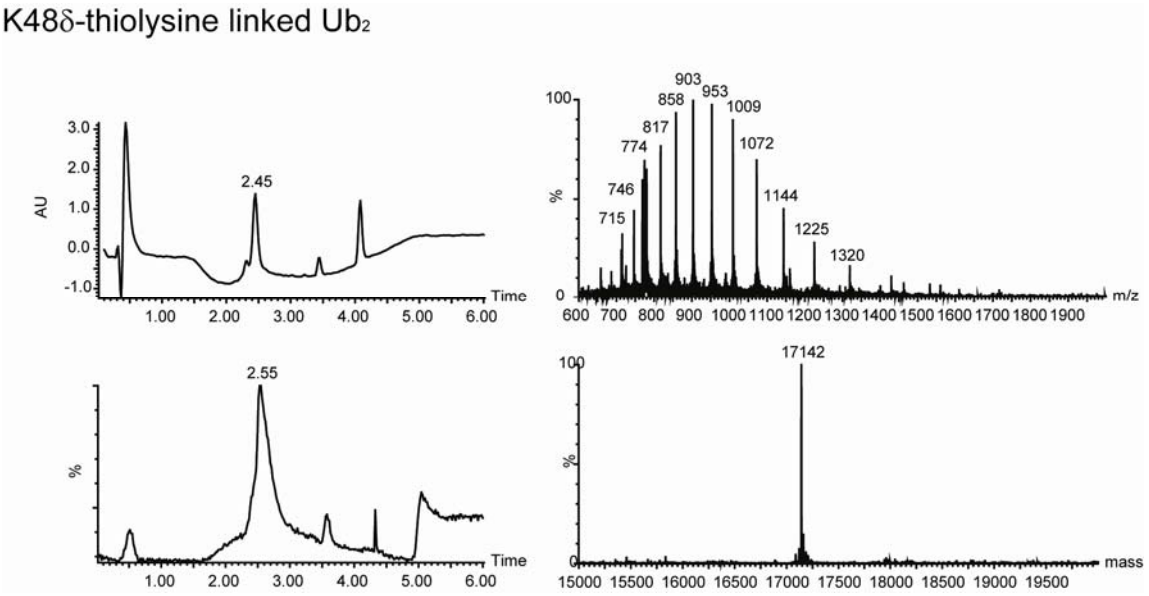


Figure S15 HPLC-MS analysis Lys48δ-thiolysine linked Ub_2. (top left) HPLC chromatogram, (bottom left) MS chromatogram, (top right) ESMS spectrum, (bottom right) deconvoluted mass of peak at 2.55 min from MS chromatogram.

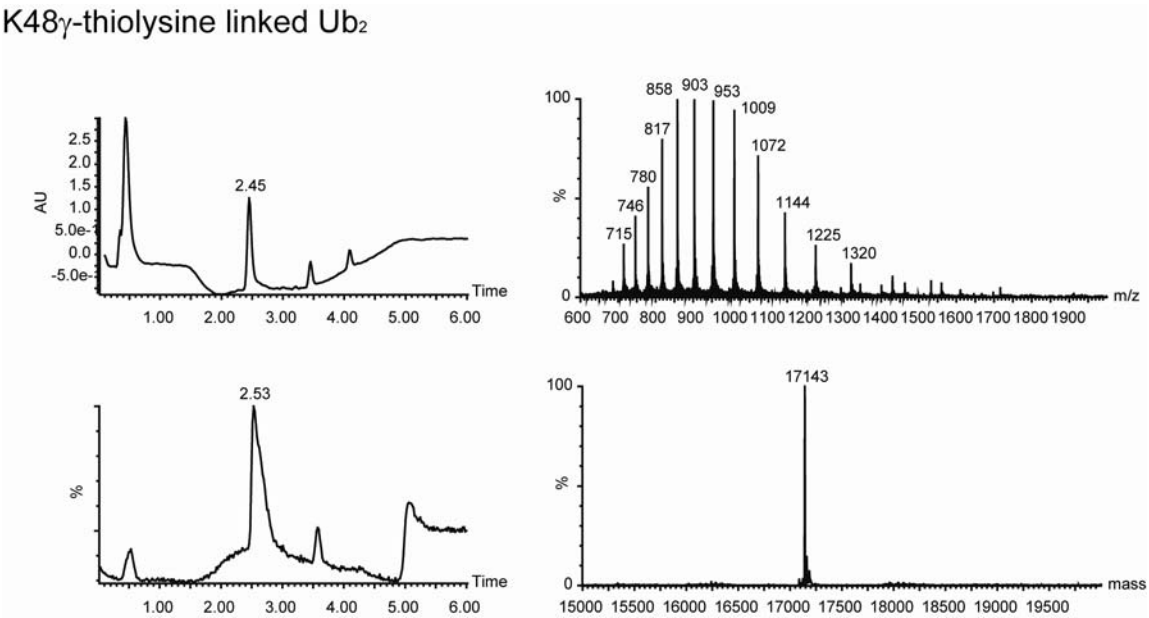


Figure S16 HPLC-MS analysis Lys48γ-thiolysine linked Ub_2. (top left) HPLC chromatogram, (bottom left) MS chromatogram, (top right) ESMS spectrum, (bottom right) deconvoluted mass of peak at 2.53 min from MS chromatogram.

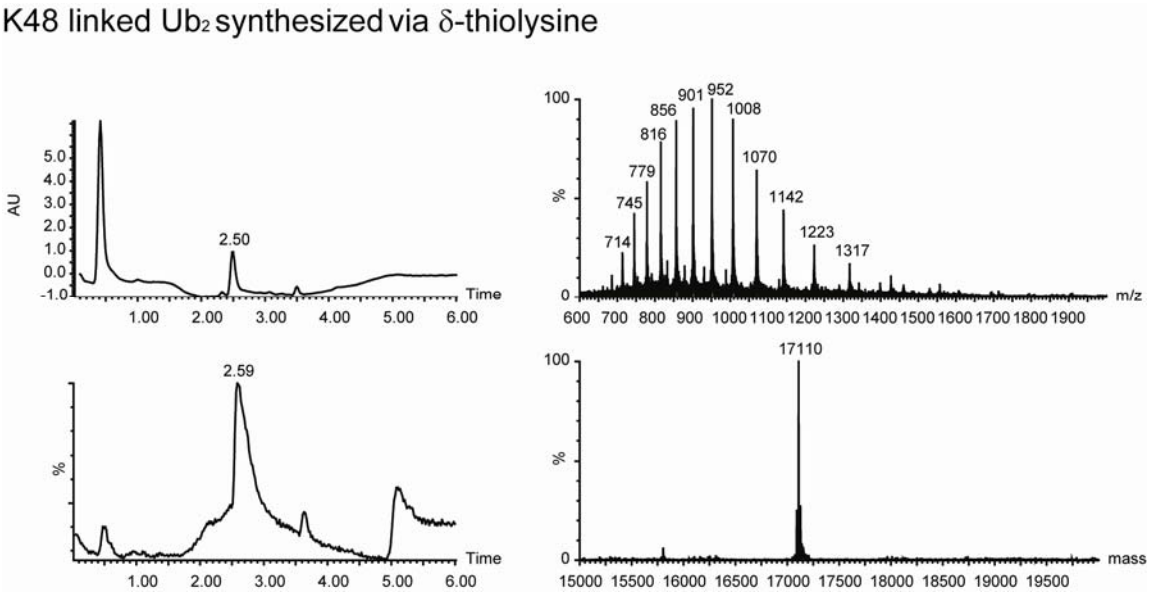


Figure S17 HPLC-MS analysis Lys48 linked Ub_2 synthesized via δ-thiolysine mediated ligation. (top left) HPLC chromatogram, (bottom left) MS chromatogram, (top right) ESMS spectrum, (bottom right) deconvoluted mass of peak at 2.59 min from MS chromatogram.

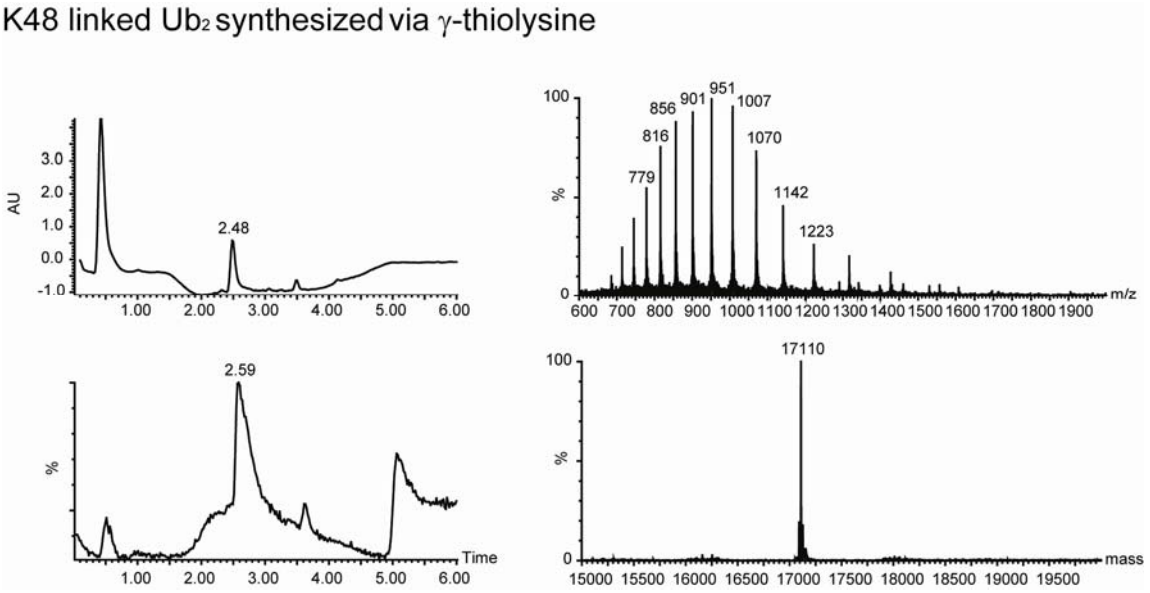


Figure S18 HPLC-MS analysis Lys48 linked Ub_2 synthesized via γ-thiolysine mediated ligation. (top left) HPLC chromatogram, (bottom left) MS chromatogram, (top right) ESMS spectrum, (bottom right) deconvoluted mass of peak at 2.59 min from MS chromatogram.

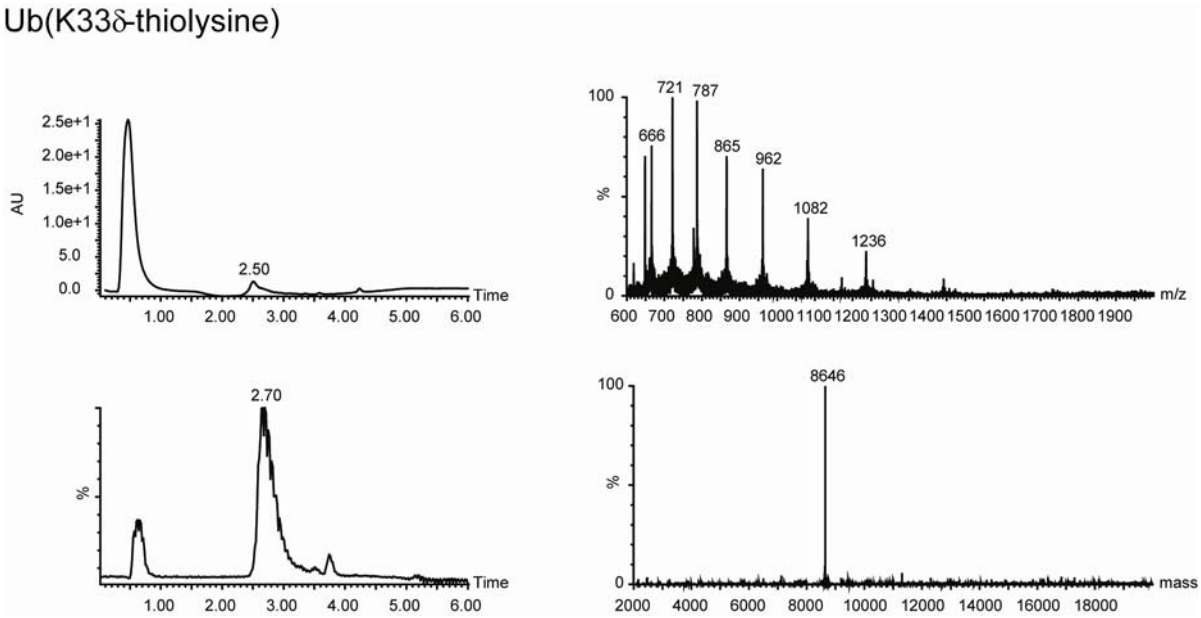


Figure S19 HPLC-MS analysis Ub with Lys33 mutated to SSMe protected δ-thiolysine. (top left) HPLC chromatogram, (bottom left) MS chromatogram, (top right) ESMS spectrum, (bottom right) deconvoluted mass of peak at 2.70 min from the MS chromatogram.

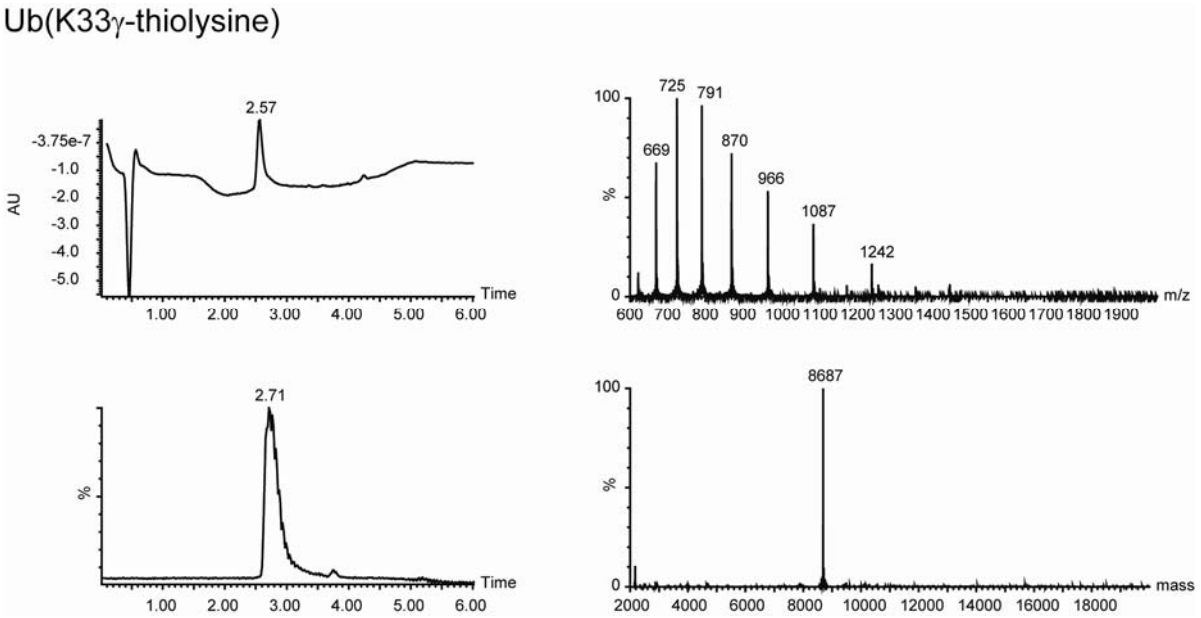


Figure S20 HPLC-MS analysis Ub with Lys33 mutated to SStBu protected γ-thiolysine. (top left) HPLC chromatogram, (bottom left) MS chromatogram, (top right) ESMS spectrum, (bottom right) deconvoluted mass of peak at 2.71 min from the MS chromatogram.

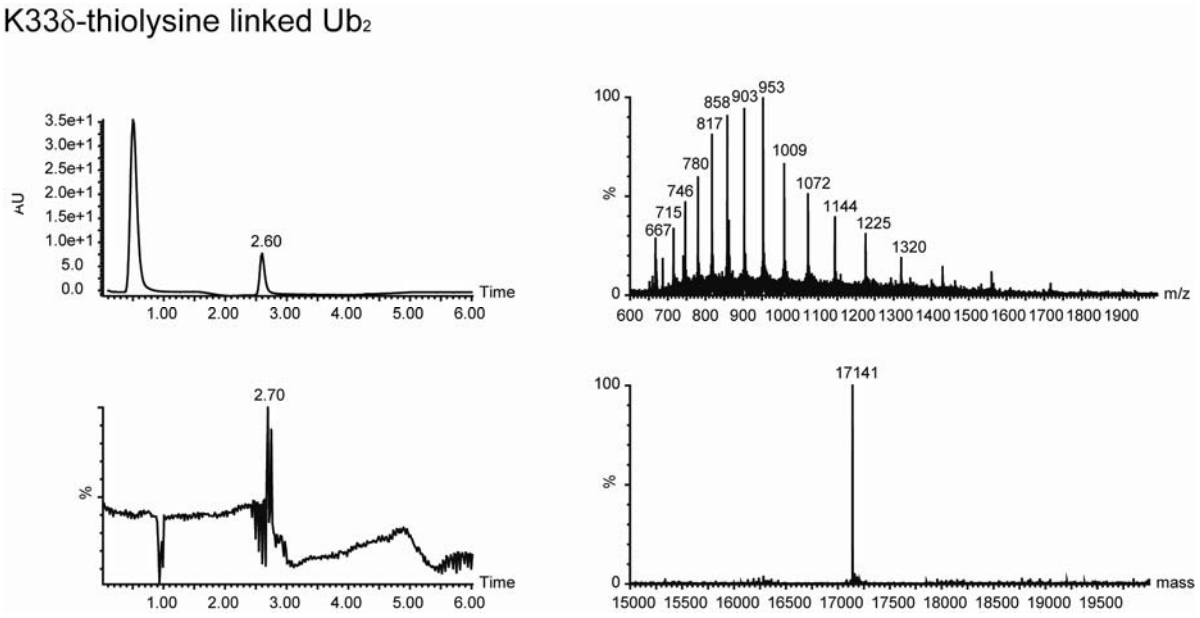


Figure S21 HPLC-MS analysis Lys33δ-thiolysine linked Ub_2. (top left) HPLC chromatogram, (bottom left) MS chromatogram, (top right) ESMS spectrum, (bottom right) deconvoluted mass of peak at 2.70 min from the MS chromatogram.

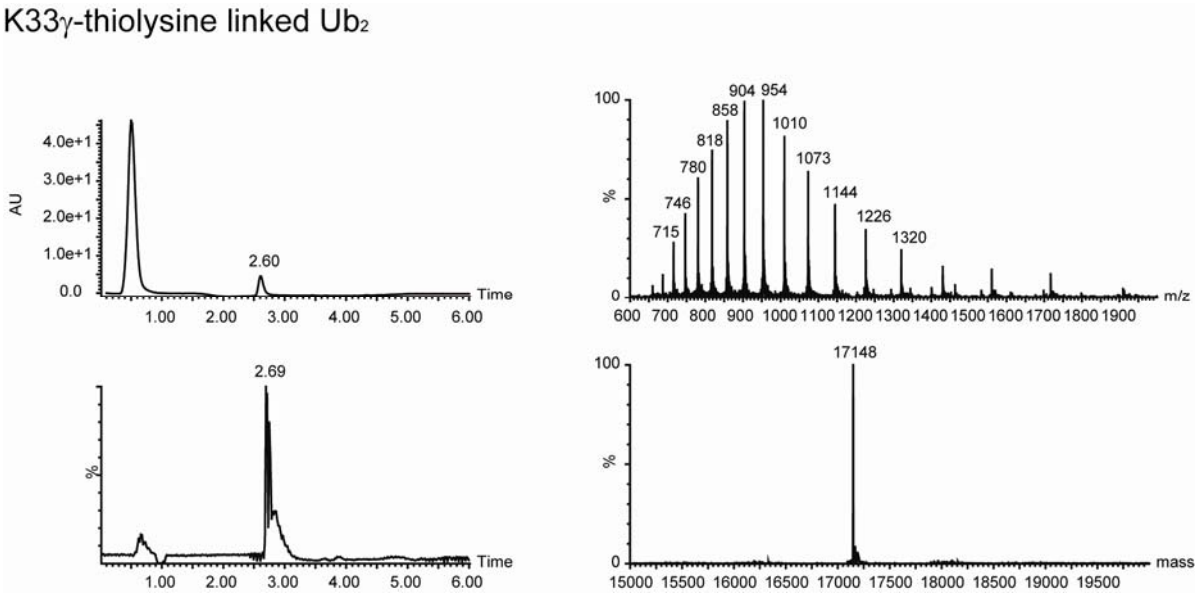


Figure S22 HPLC-MS analysis Lys33γ-thiolysine linked Ub_2. (top left) HPLC chromatogram, (bottom left) MS chromatogram, (top right) ESMS spectrum, (bottom right) deconvoluted mass of peak at 2.69 min from the MS chromatogram.

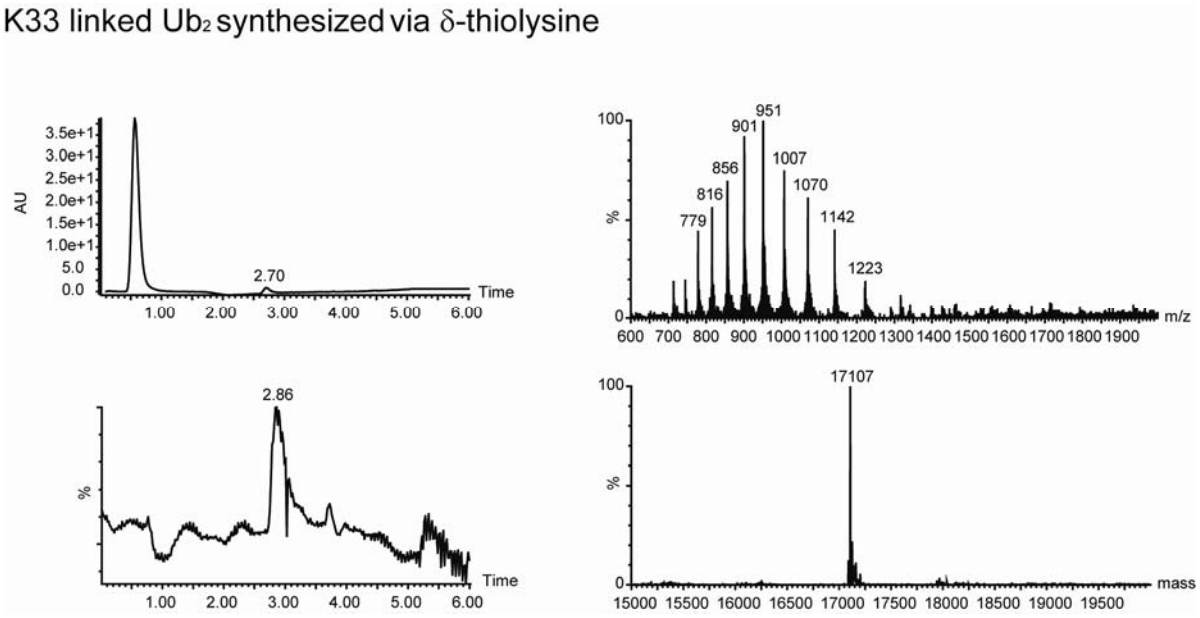


Figure S23 HPLC-MS analysis Lys48 linked Ub_2 synthesized via δ-thiolysine mediated ligation. (top left) HPLC chromatogram, (bottom left) MS chromatogram, (top right) ESMS spectrum, (bottom right) deconvoluted mass of peak at 2.86 min from MS chromatogram.

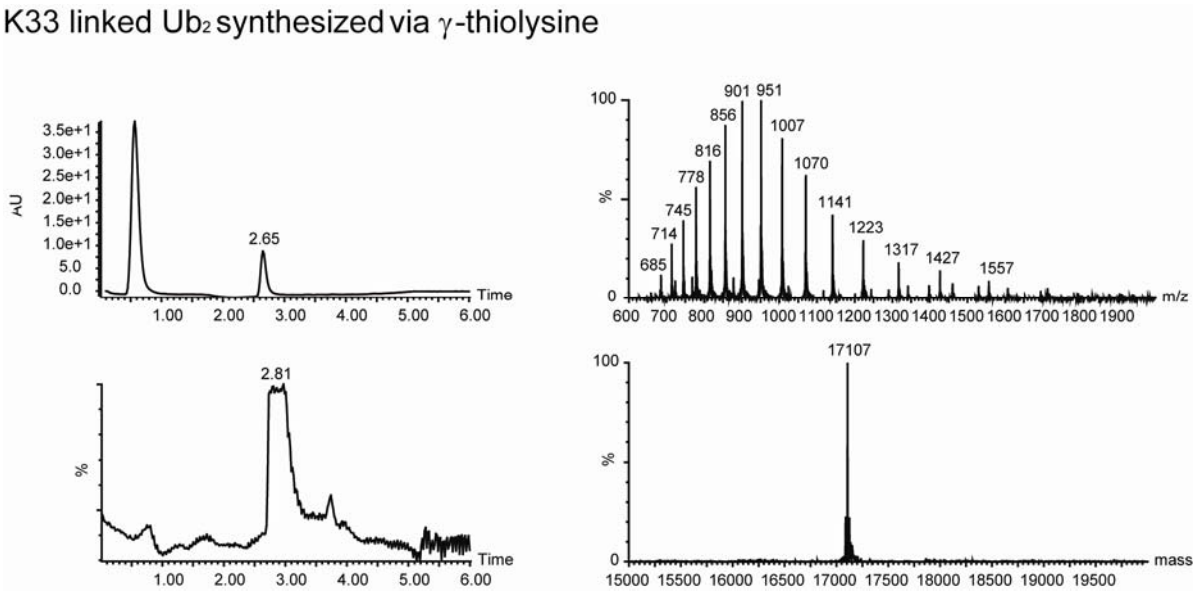


Figure S24 HPLC-MS analysis Lys48 linked Ub_2 synthesized via γ-thiolysine mediated ligation. (top left) HPLC chromatogram, (bottom left) MS chromatogram, (top right) ESMS spectrum, (bottom right) deconvoluted mass of peak at 2.81 min from MS chromatogram.

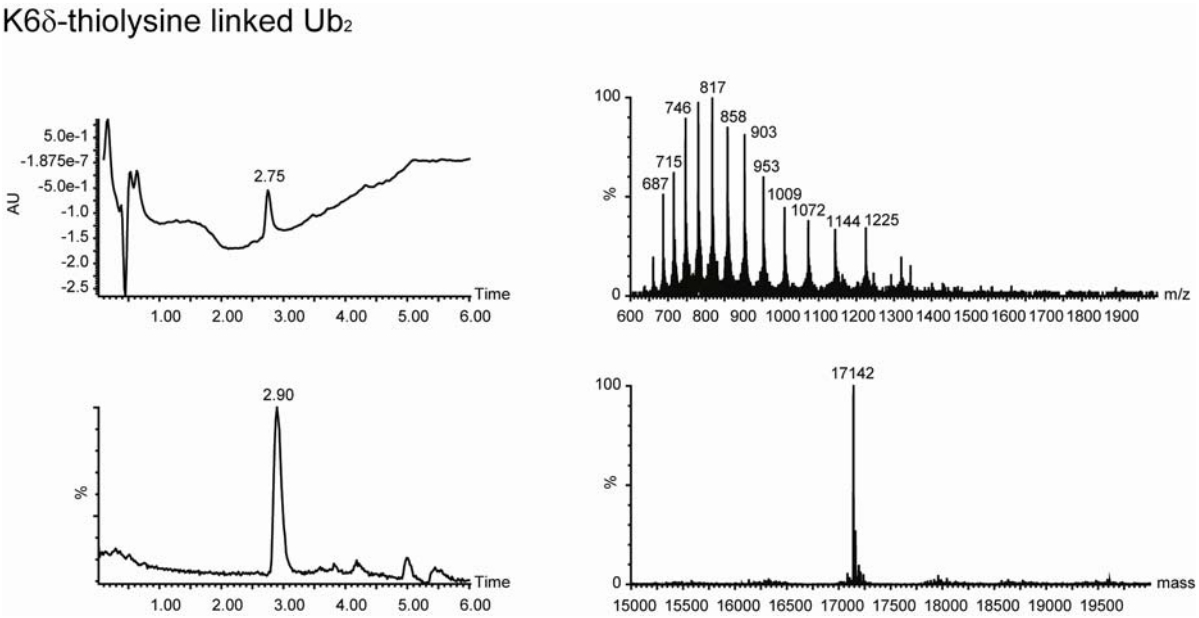


Figure S25 HPLC-MS analysis Lys6δ-thiolysine linked Ub_2. (top left) HPLC chromatogram, (bottom left) MS chromatogram, (top right) ESMS spectrum, (bottom right) deconvoluted mass of peak at 2.90 min from the MS chromatogram.

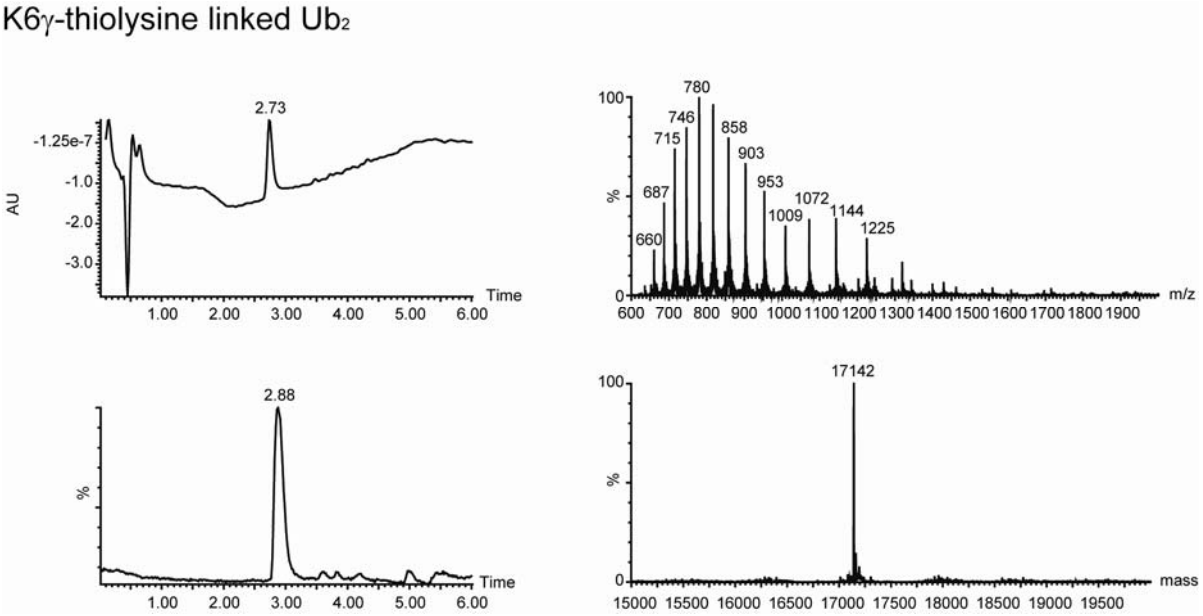
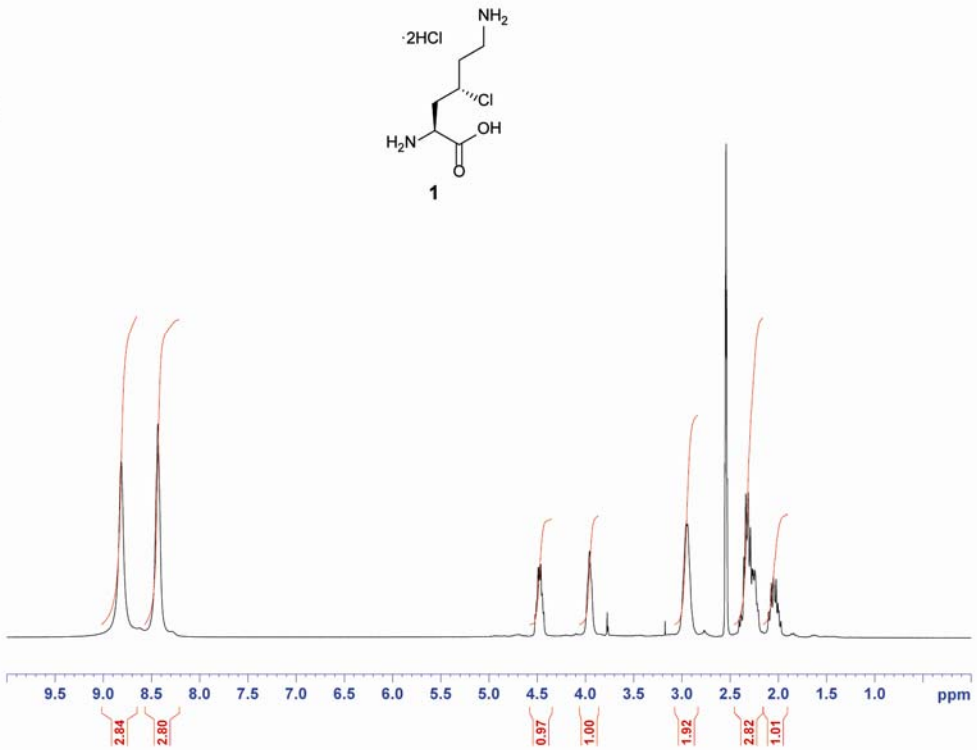


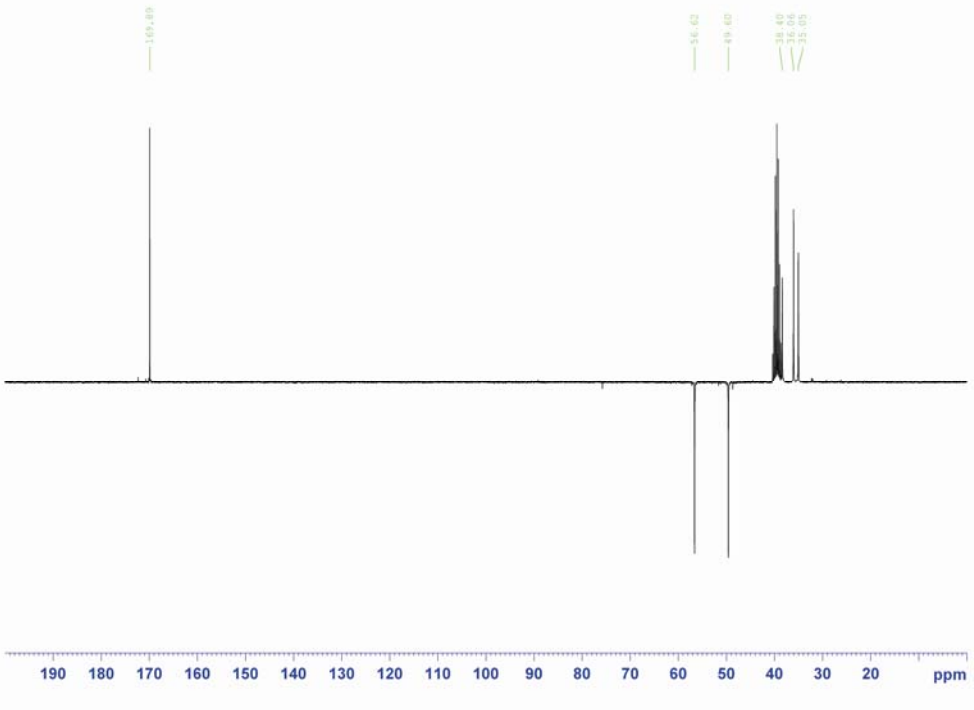
Figure S26 HPLC-MS analysis Lys6γ-thiolysine linked Ub_2. (top left) HPLC chromatogram, (bottom left) MS chromatogram, (top right) ESMS spectrum, (bottom right) deconvoluted mass of peak at 2.88 min from the MS chromatogram.

NMR spectra

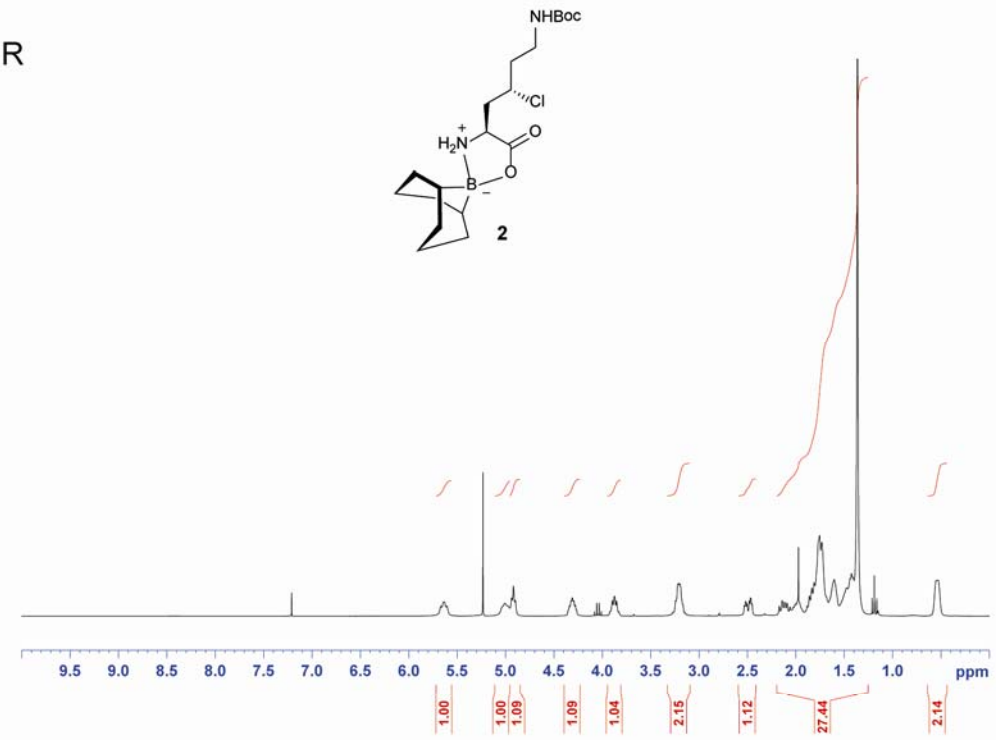
^{1}H-NMR



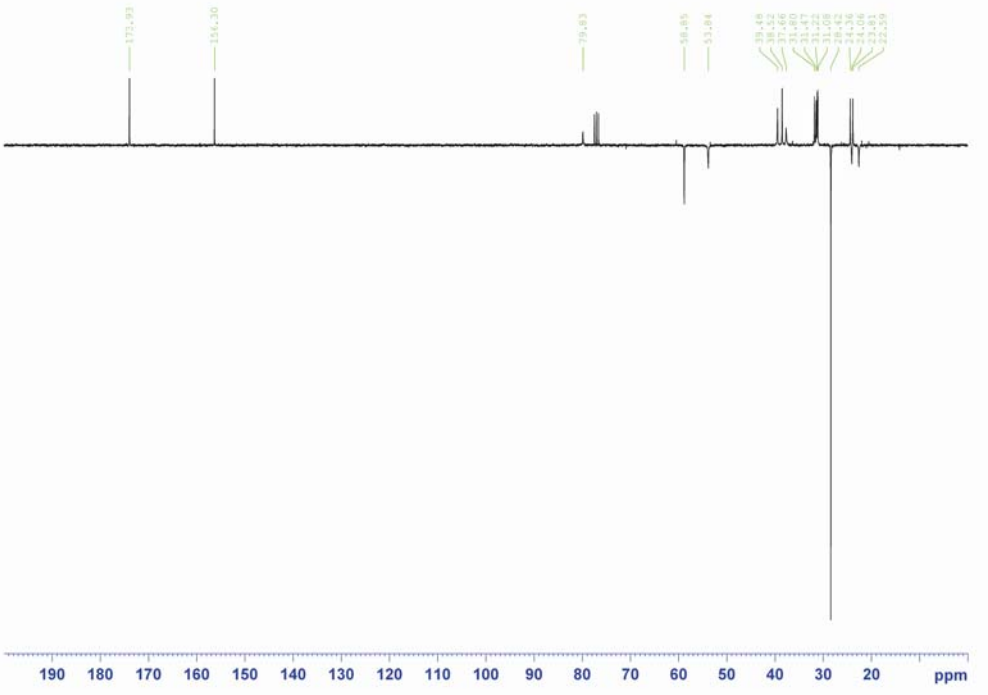
^{13}C-NMR



^{1}H-NMR



^{13}C-NMR



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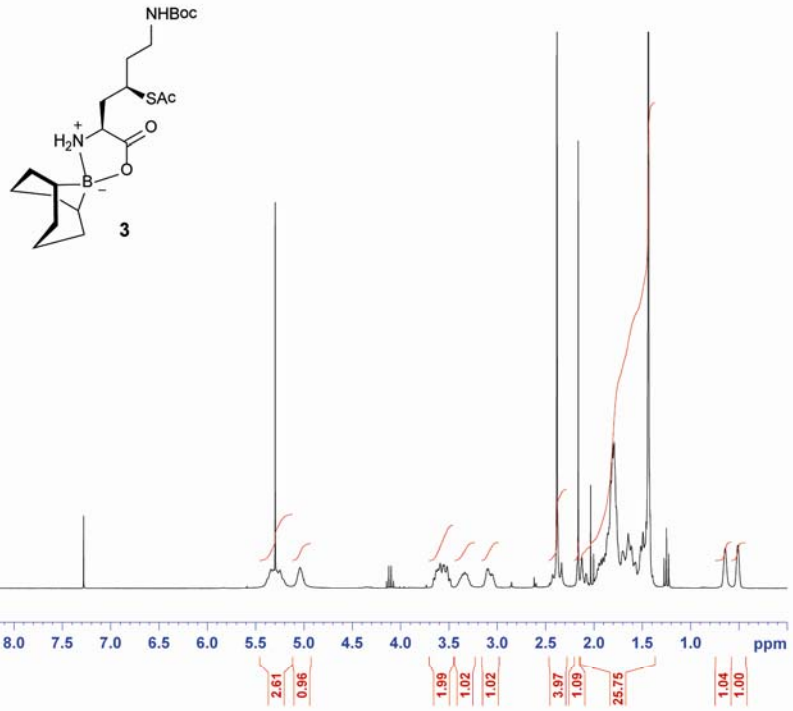

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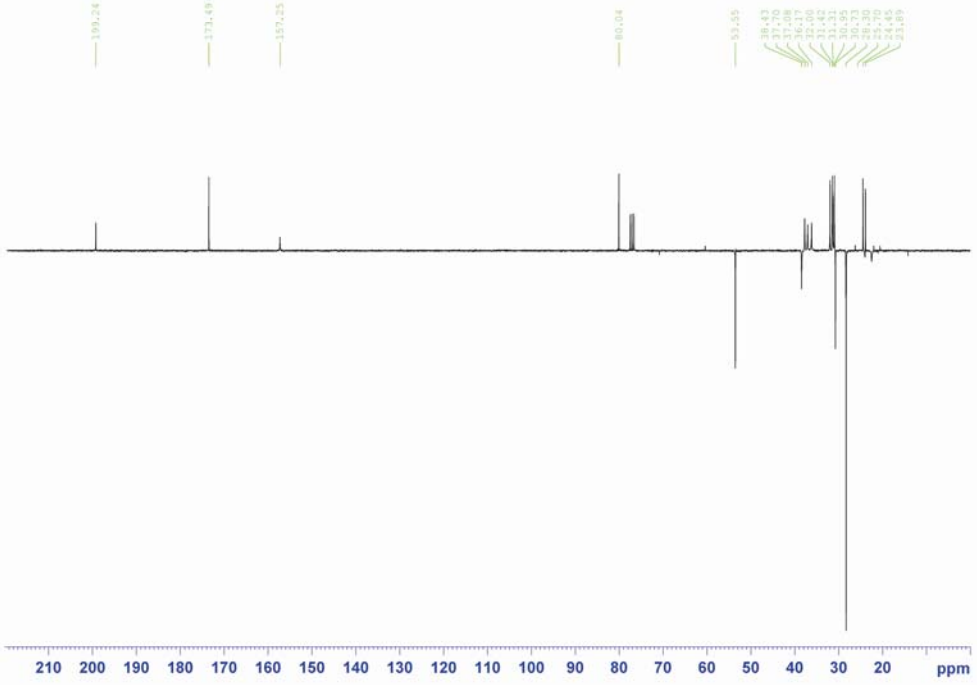

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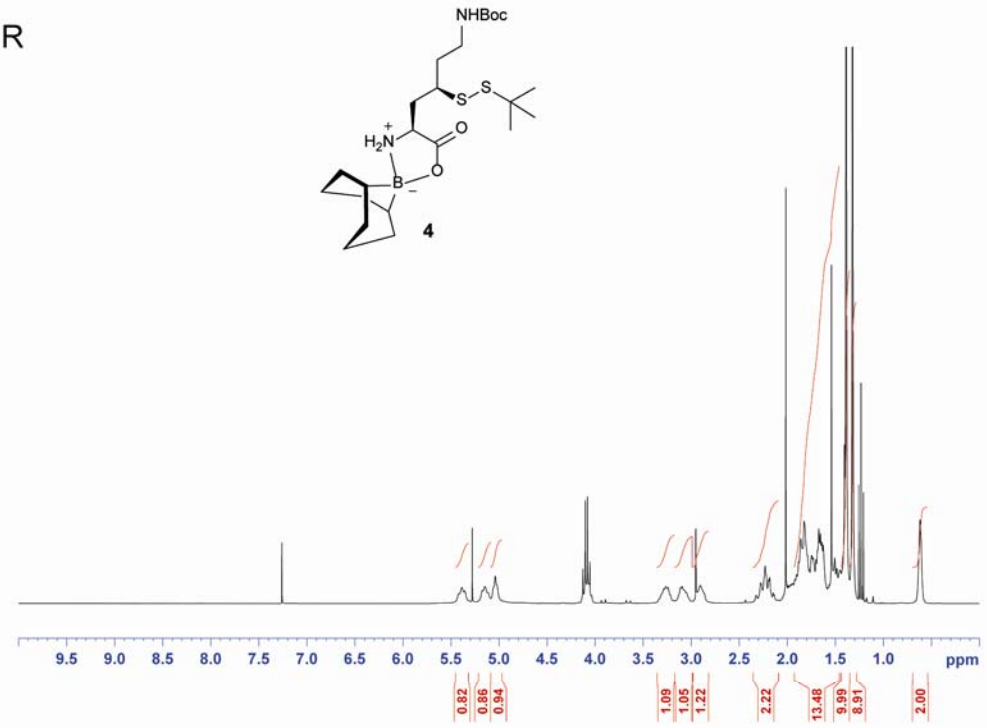

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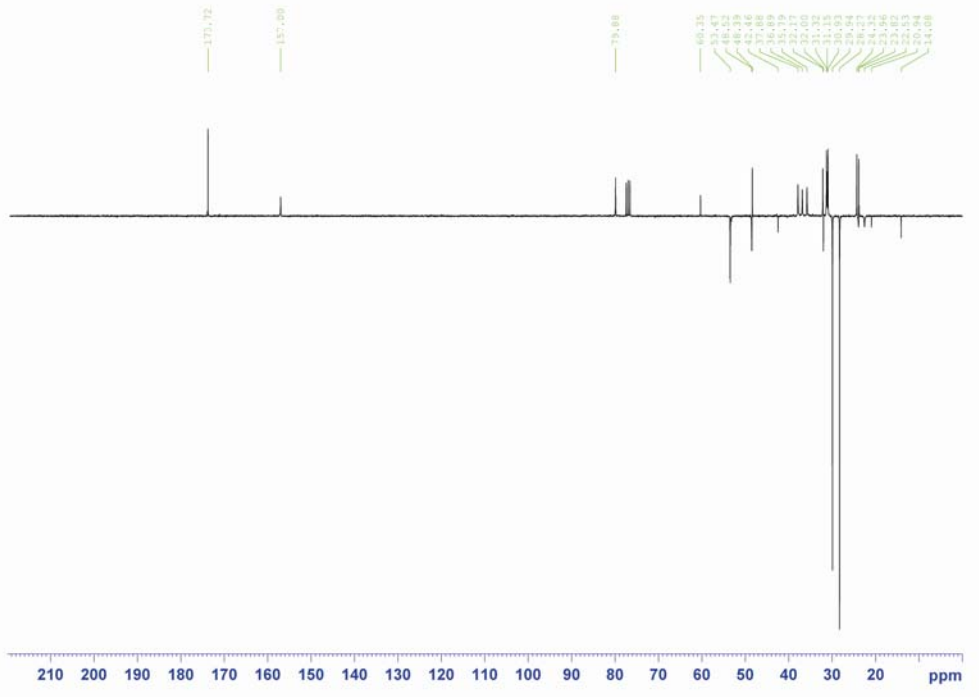

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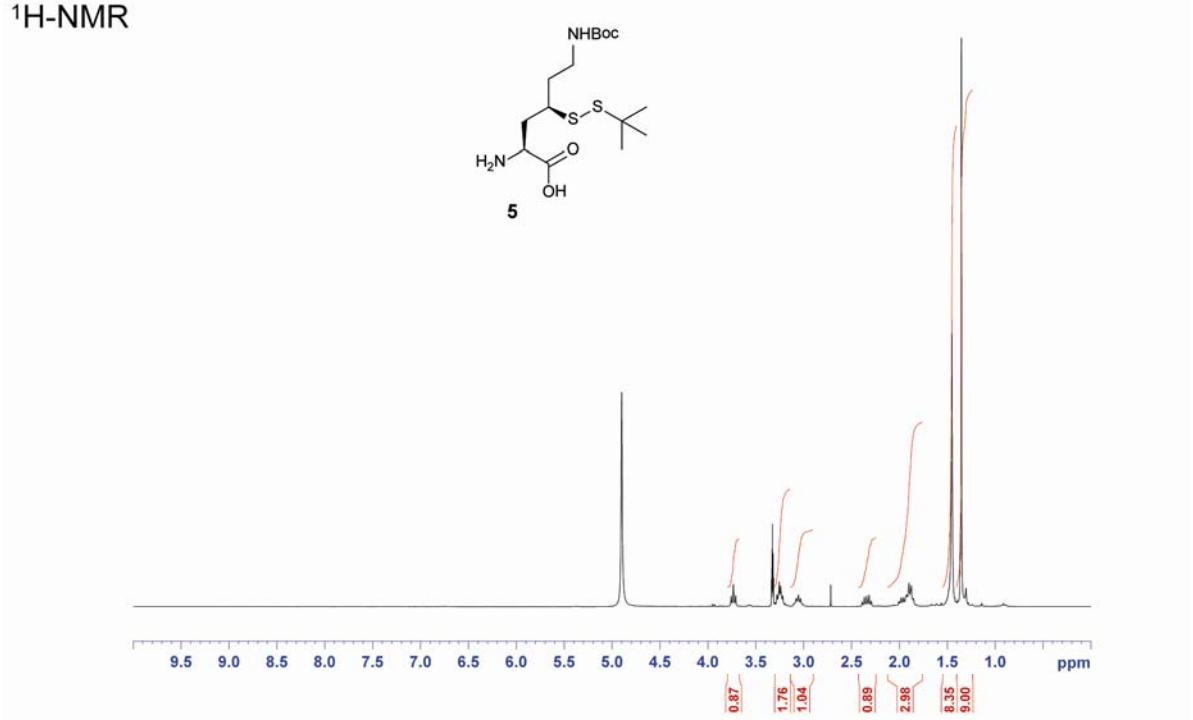

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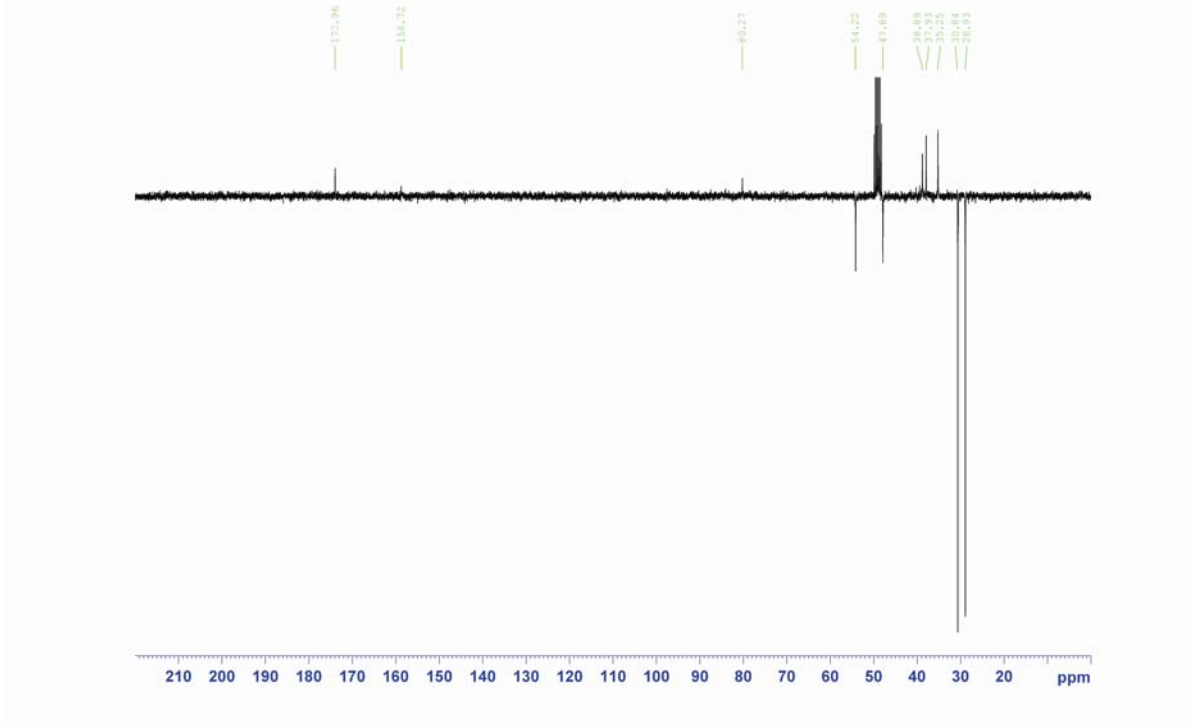

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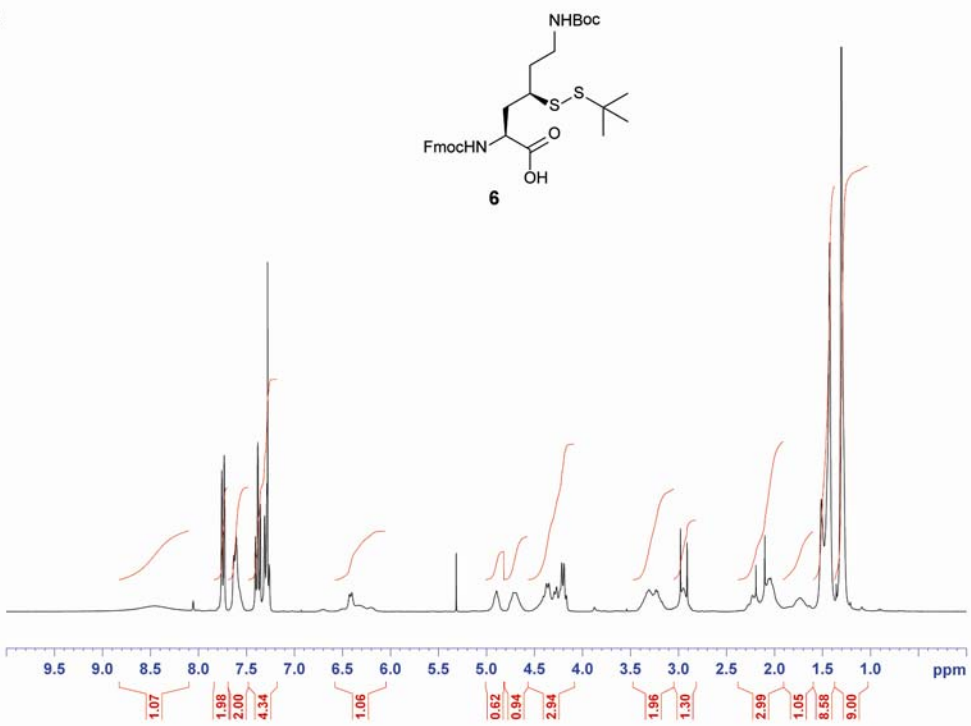

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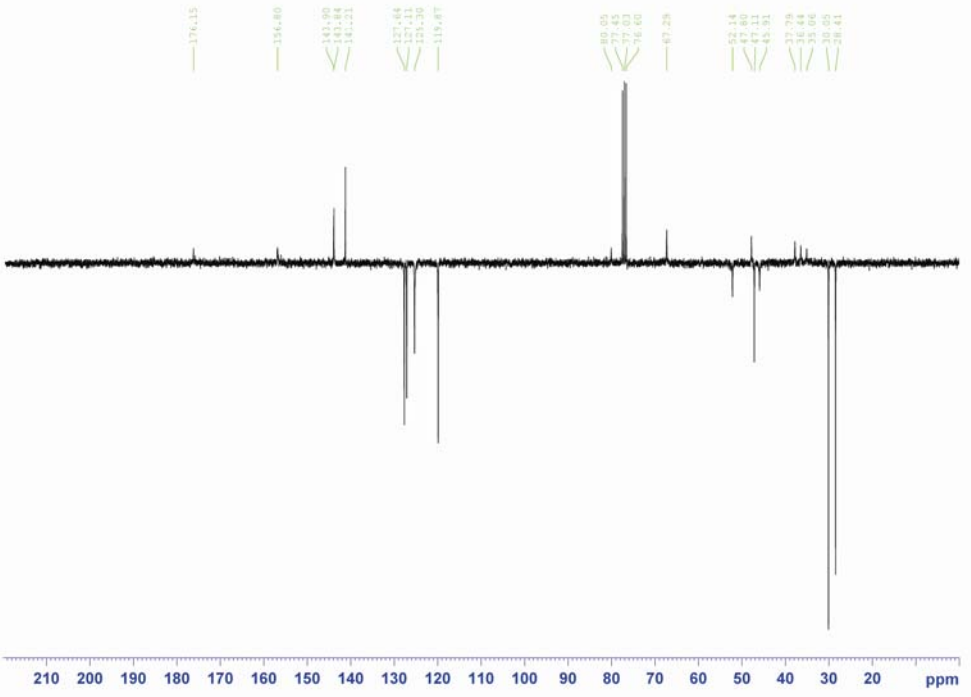

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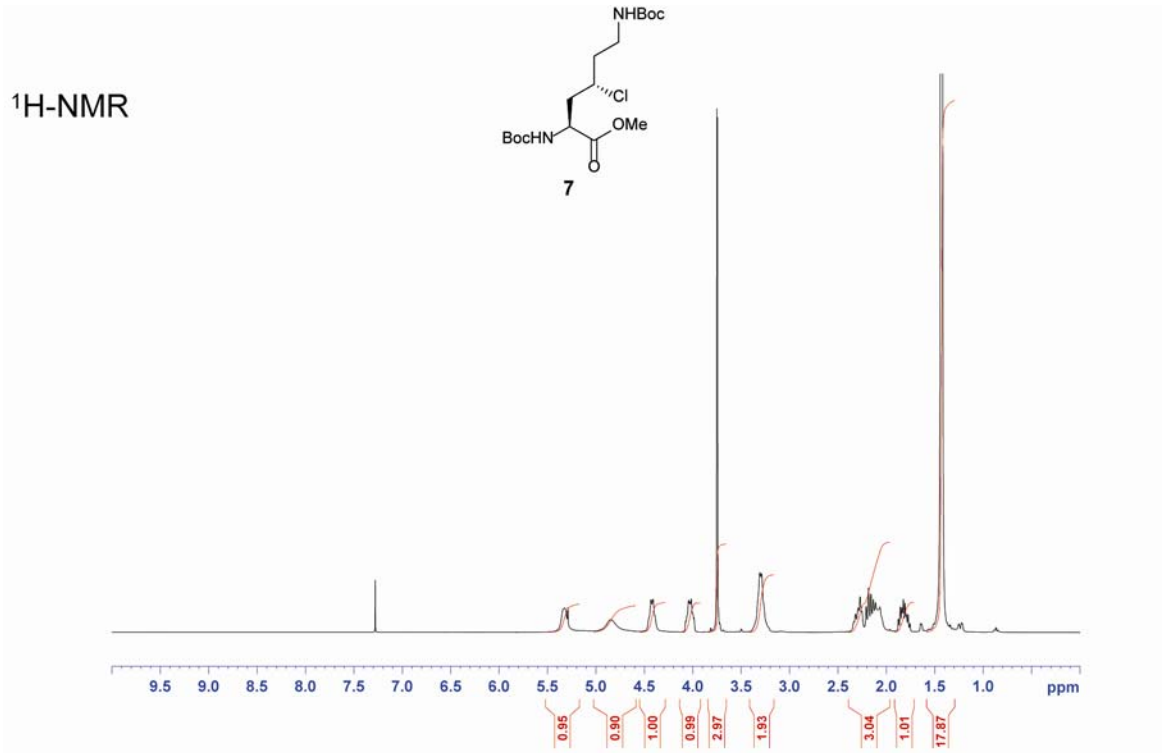

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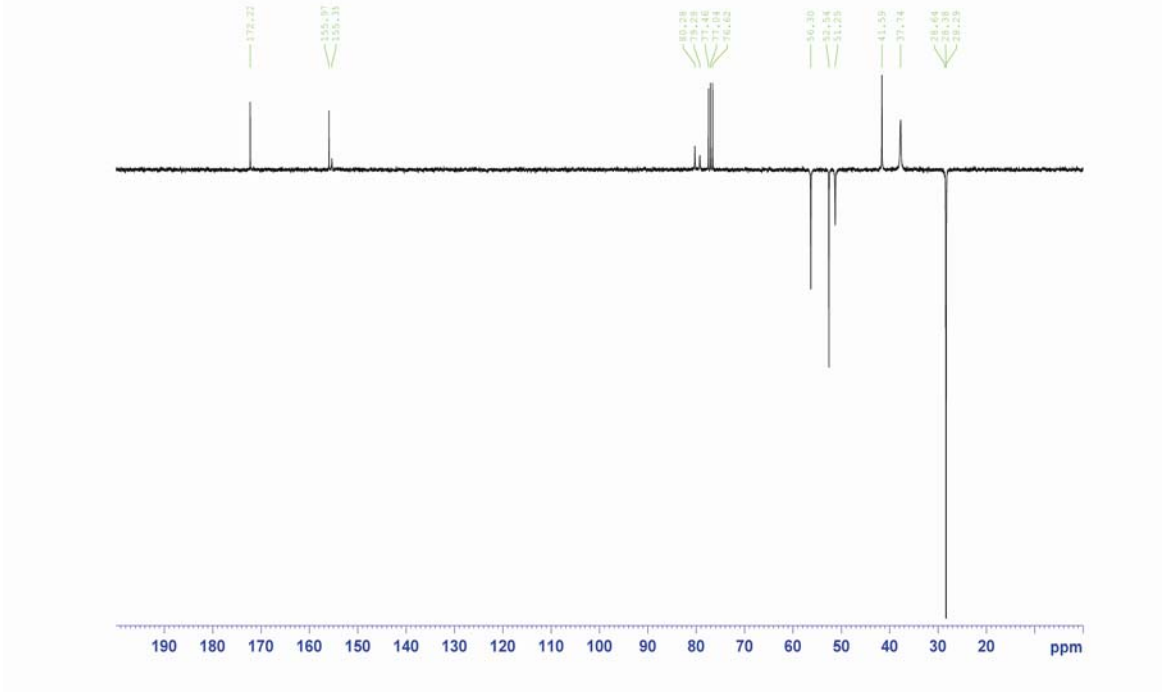

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