Protein ubiquitination via Dehydroalanine: Development and Insights into the Diastereoselective 1,4-Addition Step

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Supporting Information

Table of Content

1. General Methods

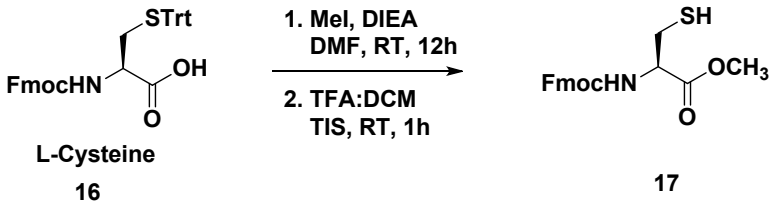
SPPS was carried out manually in syringes, equipped with teflon filters, purchased from Torviq or by using an automated peptide synthesizer (CS336X, CSBIO). Analytical grade DMF was purchased from Biotech. Resins were purchased from Creosalus, protected amino acids were purchased from GL Biochem and 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. Analytical HPLC was performed on a Thermo instrument (Dionex Ultimate 3000) using analytical columns Xbridge (waters, BEH300 C4, 3.5µm, 4.6 × 150 mm) and XSelect (waters, CSH C18, 3.5 µm, 4.6 × 150 mm) at flow rate of 1.2 mL/min. Semi preparative HPLC was performed on a Thermo Scientific instrument (Spectra System SCM1000) using Jupiter C4 10 µm, 300 Å, 250 × 10 mm column at flow rate 4 mL/min. Preparative HPLC was performed on a Waters instrument using XSelect C18 10µm 19 × 250 mm at flow rate of 25 mL/min. Synthetic products were purified by HPLC and characterized by mass spectrometry using LCQ Fleet Ion Trap (Thermo Scientific). For all HPLC based purifications water with 0.1% trifluoroacetic acid was used as a solvent A and acetonitrile with 0.1% trifluoroacetic acid as solvent B. All calculated masses have been reported as an average isotope composition.

^{1}H and ^{13}C-NMR spectra were recorded on a Bruker AMX-400 MHz using CD_3OD as a solvent. Chemical shifts (δ) were reported in ppm and *J* values are given in Hz. 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.

2. Synthesis of compound 19

2.1 Synthesis of compound 17

(*R*)-2-(((9H-fluoren-9-yl) Methoxy) carbonylamino)-3-(tritylthio) propanoic acid (**Fmoc-L-Cys(Trt)-OH**) (2.5 g, 4.3 mmol) was dissolved in 10 mL of DMF. To this solution diisopropylethylamine (DIEA) (1.1 g, 8.6 mmol) and methyl iodide (1.2 g, 8.6 mmol) were added. The reaction mixture was stirred for 12 h and the progress of the reaction was monitored by TLC. After completion of reaction, the reaction mixture was poured in water (75 mL) and extracted with ethyl acetate (30 mL × 3). The combined organic layer was washed with brine, dried over Na_2SO_4 and concentrated under reduced pressure to afford Fmoc-L-Cys(Trt)-OMe as gummy liquid (2.3 g, 90% yield). The obtained product was treated with 30 mL of TFA : DCM mixture, triisopropylsilane (1 mL) and stirred for 1 h at room temperature . After completion of the reaction (monitored by TLC) all volatiles were evaporated and dried under high vacuum for overnight. The obtained trityl deprotected Fmoc-Cys-OMe **17** was used for the next step without purification.



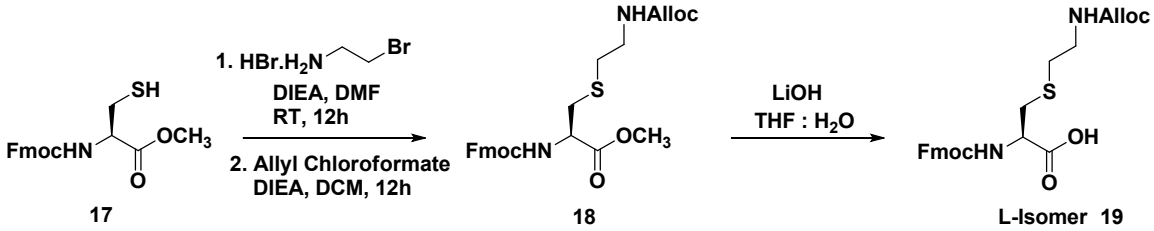
2.2 Synthesis of compound 19

The Fmoc-Cys-OMe **17** was dissolved in DMF (12 mL). To this solution the 2-bromoethylamine hydrobromide (0.93 g, 4.5mmol) and DIEA (0.98 g, 7.6 mmol) were added. The reaction mixture was stirred for 12 h. Subsequently it was poured in water (75 mL) and extracted with ethyl acetate (30 mL × 3). The combined organic layer was washed with brine, dried over Na_2SO_4 and evaporated under reduced pressure. The obtained S-substituted ethylamine Cys was dried under high vacuum for overnight.

The product was dissolved in dry DCM and treated with allyl chloroformate (0.46 g, 3.8 mmol) and DIEA (0.98 g, 7.6 mmol). The reaction mixture was stirred for 12 h

followed by all volatiles were evaporated under reduced pressure. The residue was dissolved in ethyl acetate (75 mL), washed with water, brine, and dried over anhydrous Na_2SO_4. The organic layer was evaporated under reduced pressure and product was purified by flash column chromatography (50% EtOAc : n-Hexane) to yield **18** (1.2 g, 60% yield over 3 steps).

The obtained product **18** was dissolved in 48 mL of THF : Water (5 : 1) and cooled at 0 °C. To this reaction mixture, 0.3*M* solution of LiOH (0.23 g in 18 mL H_2O) was added dropwise over the period of 15 min. The progress of reaction was monitored by TLC. After completion of the reaction, the reaction mixture was acidified to pH~3-4 using 10 % citric acid and extracted with ethyl acetate (25 mL × 3). The combined organic layer was washed with brine, dried over Na_2SO_4 and concentrated under reduced pressure to afford final product **19** (0.81 g, 70% yield).



^{1}H NMR (400 MHz, CD_3OD) δ 7.71 (dd, J = 15.1, 7.4 Hz, 2H), 7.60 (dd, J = 15.9, 6.8 Hz, 2H), 7.32 (dd, J = 14.0, 6.7 Hz, 2H), 7.28 – 7.18 (m, 2H), 5.95 – 5.76 (m, 1H), 5.22 (d, J = 17.1 Hz, 1H), 5.09 (d, J = 10.3 Hz, 1H), 4.48 (dd, J = 23.2, 4.9 Hz, 2H), 4.37 – 4.06 (m, 4H), 3.29 – 3.19 (m, 2H), 3.08 – 2.75 (m, 2H), 2.62 (t, J = 6.7 Hz, 1H) ^{13}C NMR (101 MHz, CD_3OD) δ 173.9, 158.5, 145.2, 142.5 134.4, 128.7, 128.1, 126.3, 120.9, 117.4, 68.1, 66.3, 55.4, 54.6, 41.4, 41.0, 34.6, 32.9, 23.6, 14.4.Calculated mass for $C_{24}H_{27}N_2O_6S$, 471.16, Observed mass: 471.0 [M + H]

3. Synthesis of compound 21

Starting with Fmoc-D-Cys(Trt)-OH, similar procedure (as mentioned for the synthesis of compound **19**) was followed for the synthesis of D-Isomer **21** and the desired product was obtained in overall 38% yield (0.76 g)

^{1}H NMR (400 MHz, CD_3OD) δ 7.70 (dd, J = 15.1, 7.5 Hz, 2H), 7.59 (dd, J = 15.9, 7.1 Hz, 2H), 7.31 (dd, J = 14.1, 6.8 Hz, 2H), 7.27 – 7.21 (m, 2H), 5.94 – 5.76 (m, 1H), 5.21 (d, J = 17.3 Hz, 1H), 5.09 (d, J = 10.3 Hz, 1H), 4.48 (dd, J = 13.9, 9.5 Hz, 2H),

4.36 – 4.10 (m, 4H), 3.24 (dd, *J* = 11.5, 4.0 Hz, 2H), 3.06 – 2.76 (m, 3H), 2.62 (t, *J* = 6.7 Hz, 1H); ^{13}C NMR (101 MHz, CD_3OD) δ 174.0, 158.5, 145.1, 142.5, 134.4, 128.7, 128.1, 126.3, 120.9, 117.5, 68.1, 66.3, 55.4, 54.6, 41.4, 41.0, 34.6, 33.1, 32.7, 23.6, 14.4. Calculated mass for $C_{24}H_{27}N_2O_6S$, 471.16, Observed mass: 471.83 [M + H]

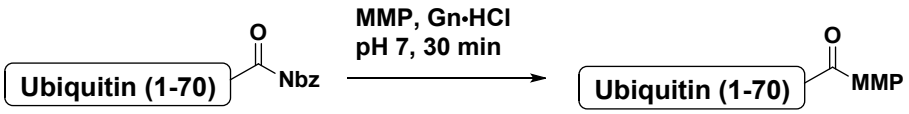
4. Synthesis of peptides

All peptides were synthesized using standard SPPS protocol. The coupling agents were used in 4-fold and DIEA in 8-fold excess to the initial loading of the resin. For deprotection steps treatment with 20% piperidine in DMF was applied.

Cleavage from the resin: The resin was washed with DMF, MeOH, DCM and dried. The peptide was cleaved with mixture of TFA:triisopropylsilane (TIS):water (95:2.5:2.5) for 2h at RT. The resin was removed by filtration and then washed again with the cleavage mixture. The combined filtrate was precipitated by drop-wise addition to 10-fold volume of cold ether. Then ether was decanted followed by dissolution of the peptide in acetonitrile-water. The HPLC analysis was carried out on a C18 analytical column using a gradient of 0-60% B over 40 min. For preparative HPLC, the same gradient was used to purify the desired product.

5. Synthesis of ubiquitin (1-70)-MMP 7

The synthesis was carried out on Knorr resin (0.27 mmol/g, 0.1 mmol scale) using automatic peptide synthesizer. First, 3-(Fmoc)-4-diaminobenzoic acid (Fmoc-Dbz) was coupled to the resin employing HCTU/DIEA/DMF (1 h × 2). Then standard Fmoc-SPPS protocol was applied to complete the sequence. Finally, Dbz cyclization was applied on the peptide using *p*-nitrochloroformate and N,N-diisopropylethylamine (DIEA) in DMF as previously reported[1] followed by cleavage and lyophilization. The crude peptide was dissolved in 6M Gn·HCl buffer pH~7, containing 200 equivalents of methyl 3-mercaptopropionate (MMP) and reaction was kept at room temperature for 30 minutes to afford ubiquitin (1-70)-MMP.



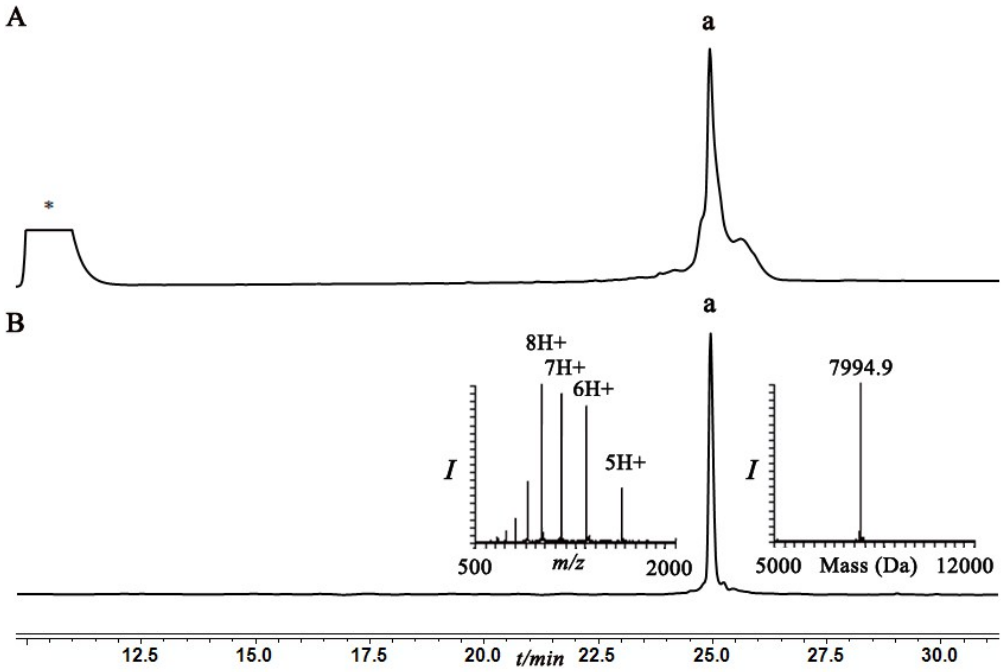


Figure S1: HPLC and mass profile for: A) Crude ubiquitin(1-70)-MMP and B) Purified ubiquitin (1-70)-MMP with observed mass 7994.9±0.5 Da, calcd. 7997.0 Da. * corresponds to MMP (methyl 3-mercaptopropionate).

6. Isolation of α-globin from hemoglobin sample

Commercially available (Sigma) lyophilized sample of human hemoglobin 30 mg was dissolved in 6M Gn·HCl buffer (1 mL, pH ~ 6) containing TCEP (7 mg). Purification was carried out using preparative HPLC using C4 column with the gradient 30-70% B over 50 min to obtain the desired α-globin (~ 9 mg).

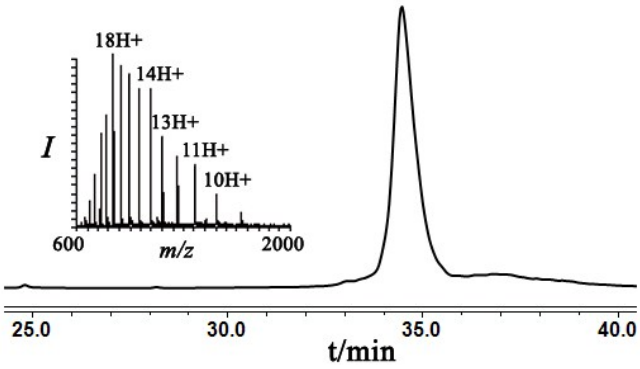


Figure S2: HPLC and mass analysis for pure α-globin with the observed mass 15123.5±1.4 Da, calcd. 15125.3 Da.

7. Synthesis of ubiquitin-Dha 9 and α-globin-Dha

Ubiquitin (10 mg), carrying Cys at defined position was dissolved in 450 µl 6M Gn·HCl buffer pH 8. A solution of α, α"-di-bromo-adipyl(bis)amide reagent[2] was prepared by dissolving 37.9 mg (100 eq) in 150 µl DMF. This was added to ubiquitin solution and the reaction was continued for 30 min at room temperature, followed by 2.5 h at 37 ºC. The reaction was followed by analytical HPLC using C18 analytical column and gradient of 5-50% B over 40 min. For preparative HPLC, the same gradient was used to purify the desired product in 45% yield (4.5 mg)

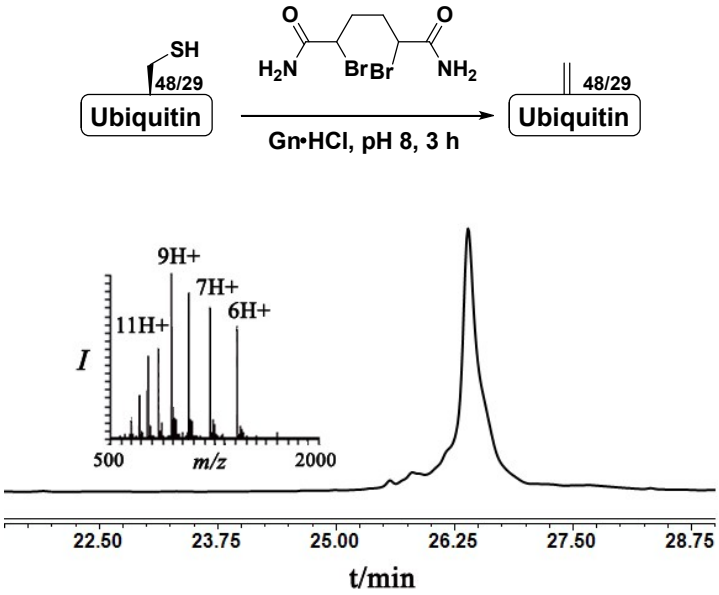


Figure S3: HPLC and mass analyses for ubiquitin-Dha48, **9**, with the observed mass 8485.9±0.5 Da, calcd. 8487.8.

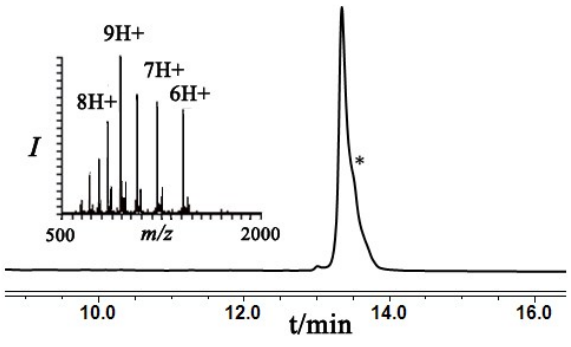


Figure S4: HPLC and mass analysis for ubiquitin-Dha29, **9** with the observed mass 8485.9±0.6 Da, calcd. 8487.8 Da. * corresponds to unidentified compound.

A similar procedure was followed for the preparation of α-globin-Dha.

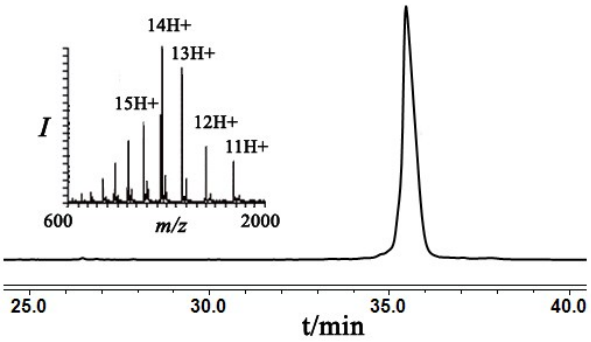
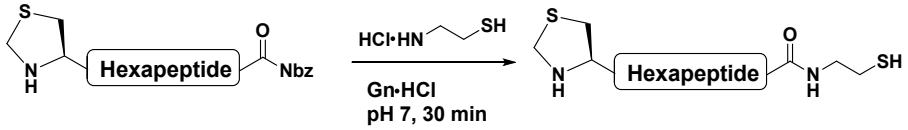


Figure S5: HPLC and mass analysis for α-globin-Dha with the observed mass of 15090.5±1.6 Da, calcd. 15093 Da.

8. Synthesis of hexapeptide-thiol 4

The synthesis was carried out manually in syringes, equipped with teflon filters, purchased from Torviq using Fmoc-SPPS on Rink amide resin (0.43 mmol/g, 0.4 mmol scale). Fmoc-Dbz was coupled to the resin, as described above, followed by SPPS to complete the sequence. The N-terminal Cys carries thiazolidine (Thz) protecting group. The Dbz cyclization was performed on the peptide, followed by cleavage and lyophilization. The peptide was purified using C18 column on preparative HPLC with a gradient of 0-40% B over 60 min. Next, the purified hexapeptide-Nbz (10 mg, 50 mM) was dissolved in 6M Gn·HCl buffer pH 7 in the presence of 69 mg (50 eq) cysteamine and incubated for 30 minutes at RT. The desired hexapeptide-thiol **4** was purified using preparative HPLC with a gradient of 0-40% B over 60 min to give the desired product in 62% isolated yield (5.5 mg).



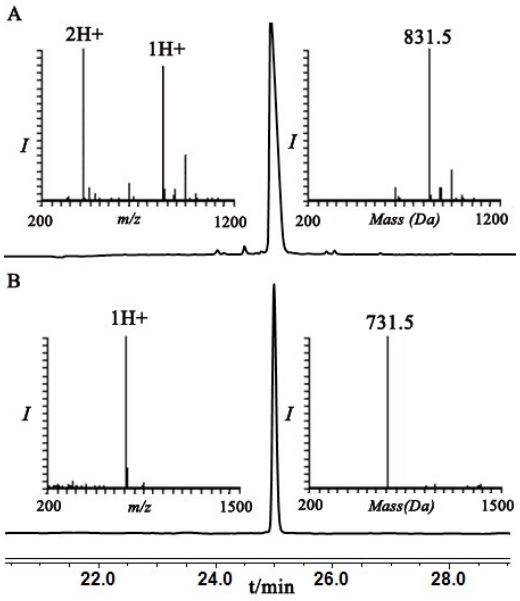
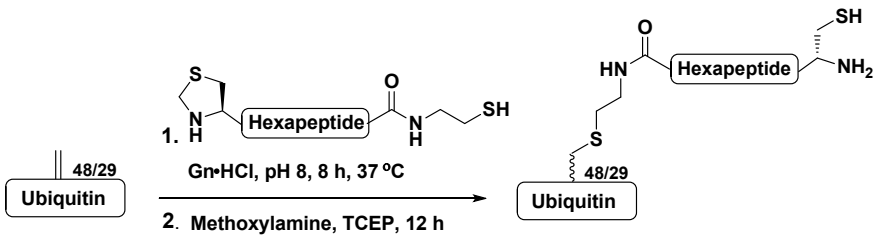


Figure S6: HPLC and mass profile for pure: A) Hexapeptide-Nbz with observed mass of 831.5±0.3, calcd. 831.8 B) Hexapeptide thiol **4** with the observed mass of 731.5±0.4 Da, calcd. 732.8 Da.

9. Conjugation of hexapeptide-thiol 4 to ubiquitin-Dha 9

Under denaturating conditions: Ubiquitin-Dha **9** (4 mg, 2mM) was dissolved in 240 µl Gn·HCl buffer pH 8. To this solution, 14 mg hexapeptide-thiol **4** (40 eq.) was added and the reaction was incubated for 8 h at 37 ºC. The reaction was followed by analytical HPLC with C18 column and a gradient of 5-50B over 40 minutes. Subsequently, 1.9 mg (50 eq.) of methoxylamine and 3.3 mg (25 eq.) of TCEP were added to this solution and incubated for additional 12 h at 37 ºC to unmask the Thz protection. Finally, the conjugated product was purified by HPLC using gradient of 5-50B over 40 minutes to give the desired product in ~57% isolated yield. Similarly, conjugation of hexapeptide-thiol **4** to α-globin-Dha (10 mg) was carried out to afford hexapeptide conjugated α-globin in ~50% yield (5.2 mg).



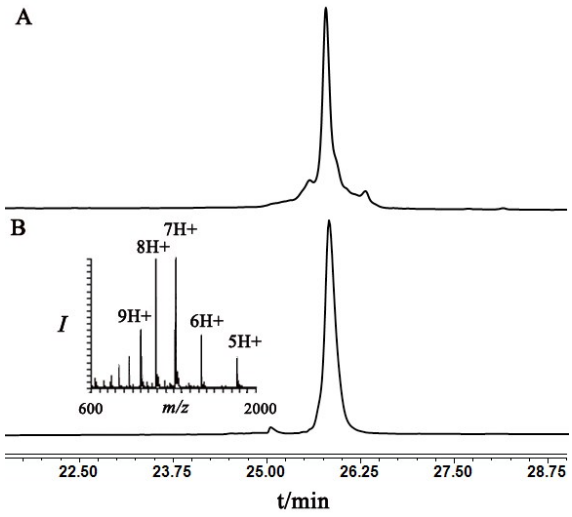


Figure S7: HPLC and mass analyses for **10** crude (A) and pure (B), with the observed mass 9206.3±0.6 Da, calcd. 9206.6 Da.

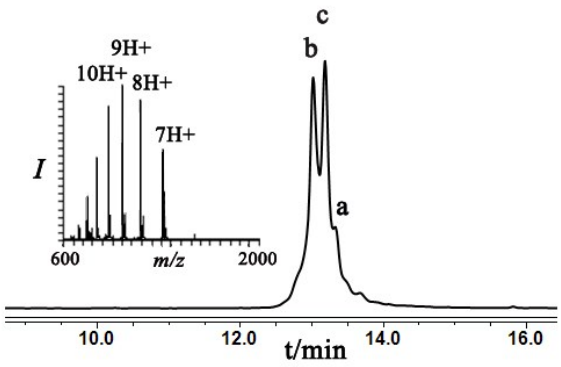


Figure S8: HPLC and mass analysis for conjugation of hexapeptide-thiol **4** to ubiquitin-Dha29 **9** under denaturating conditions. Peak **b** and **c** correspond to the conjugation products with the observed mass 9206.1±0.5 Da, calcd. 9206.6. Da Peak **a** corresponds to unreacted ubiquitin-Dha29 **9**.

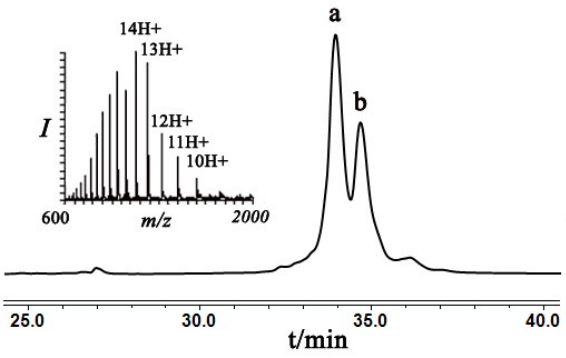


Figure S9: HPLC and mass analysis for conjugation of hexapeptide-thiol **4** to α-globin-Dha under denaturating conditions. Peaks **a** and **b** correspond to conjugation products with the observed mass 15811.9±2.0 Da and 15812.2±2.1 Da, calcd. 15813.1 Da.

Under folded conditions: Ubiquitin-Dha (~0.5 mg) was first dissolved in 10 µl 6M Urea pH 7 and diluted with 190 µl 20 mM Tris buffer, containing 150 mM NaCl, pH 7.3. The folding of protein was confirmed by circular dichroism spectroscopy. Finally 2.1 mg (40 eq.) of hexapeptide-thiol **4** was added to the reaction solution and the reaction was incubated for 8 h at 37 ºC. The progress of the reaction was monitored by HPLC using gradient of 5-50B over 20 minutes and the same gradient was used to purify the desired protein.

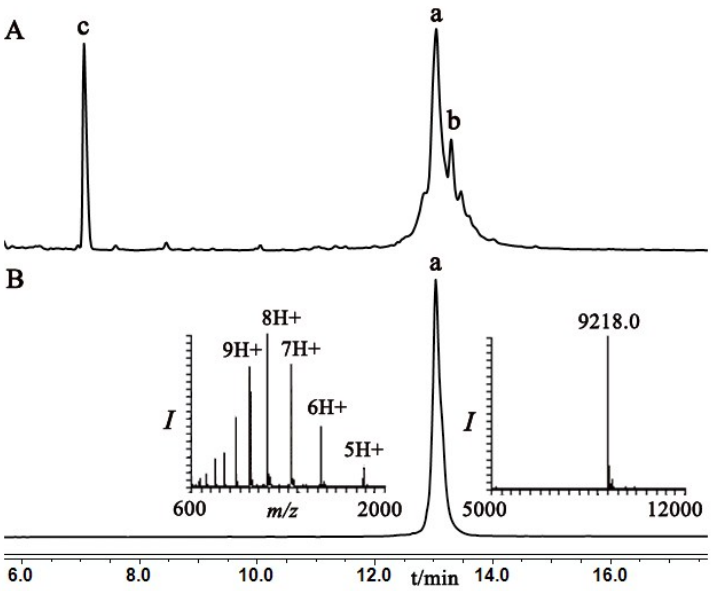


Figure S10: HPLC and mass profile for: A) Conjugation of hexapeptide-thiol **4** with ubiquitin48-Dha **9** in folded conditions. Peak **a** corresponds to conjugated product ubiquitin48-hexapeptide **10**. Peak **b** corresponds to unreacted ubiquitin48-Dha **9** and peak **c** corresponds to hexapeptide-thiol **4**. B) Purified ubiquitin48-hexapeptide **10** with observed mass 9218.0±0.6 Da, calcd. 9218.6 Da.

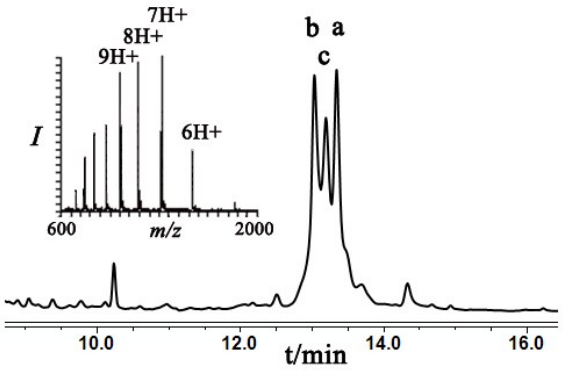


Figure S11: HPLC and mass analysis for conjugation of hexapeptide-thiol **4** to ubiquitin29-Dha **9** under folding conditions. Peak **b** and **c** correspond to the conjugation products with the observed mass 9206.1±0.5 Da, calcd. 9206.6 Da. Peak **a** corresponds to unreacted ubiquitin29-Dha **9**.

10. Circular dichroism spectroscopy

The Ubiquitin-Dha was first dissolved in 6M urea solution (5% of the total volume) and then diluted with 20 mM Tris buffer, containing 150 mM NaCl, pH 7.3. With this solution circular dichroism spectrum was recorded in a Chirascan (Applied Photophysics) instrument.

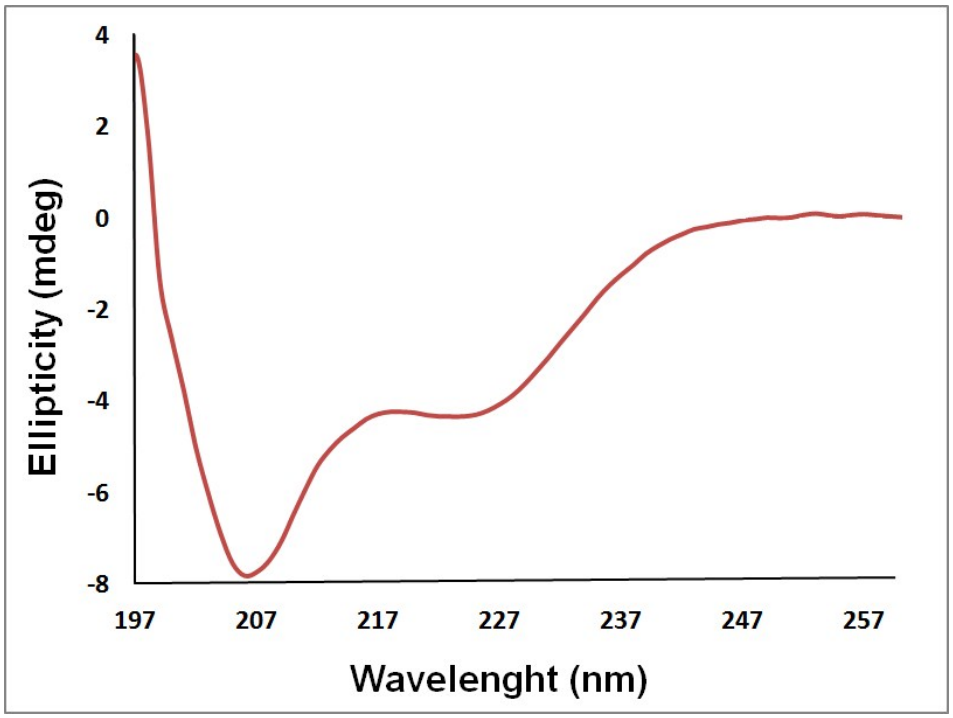
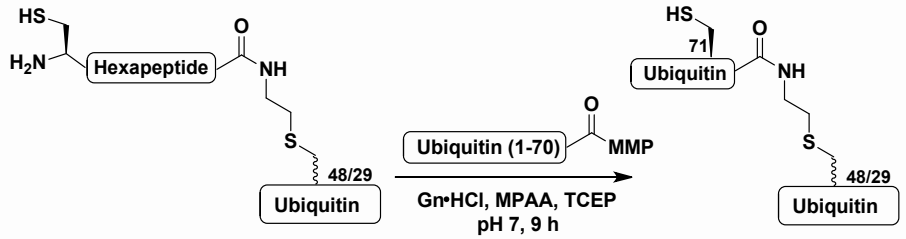


Figure S12: CD spectra of folded ubiquitin48-Dha.

11. Preparation of di-ubiquitin analogues.

11.1 Preparation of cysteine containing analogue 11

Ubiquitin-hexapeptide **10** (1 mg) and of ubiquitin(1-70)-MMP **7** (1.13 mg) were dissolved in 54 µl 6M Gn·HCl buffer pH ~7. To this solution 0.91 mg (50 eq.) of 4-mercaptophenylacetic acid (MPAA) and 0.78 mg (25 eq.) of tris(2-carboxyethyl)phosphine hydrochloride **(**TCEP) were added. The reaction was incubated for ~ 9 h at 37 ºC. The ligation was monitored by analytical HPLC followed by isolation in semipreparative HPLC using C4 column and a gradient of 5-50B over 40 minutes, affording **11** in 39% of isolated yield (0.72 mg).



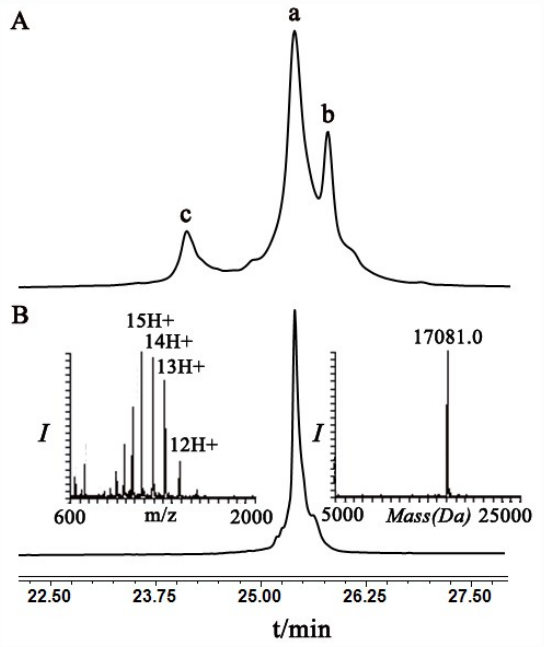


Figure S13: HPLC and mass analyses for NCL of ubiquitin(1-70)-thioester **7** with **10**, where peak **a** corresponds to the ligated product **11** with the observed mass 17082.1±2 Da, calcd. 17082.6 Da, peak **b** corresponds to unreacted **10**, peak **c** corresponds to unreacted ubiquitin(1-70)-thioester **7** with the observed mass 8043.4±0.7 Da, calcd. 8045.0 Da.

11.2 Preparation of desulfurized analogue 12

The previous ligation reaction mixture was dialyzed in a Slide-A-Lyzer 3.5K dialysis cassette (Thermo scientific, 0.1-0.5 mL) in 6M Gn·HCl, 200 mM phosphate buffer (500 mL) for overnight and then radical induced desulfurization was applied.[3] To the reaction mixture TCEP (~17 mg, 250 mmol), VA-044 (0.95 mg, 50 eqiv) and *tert*-butyl thiol (25 µl) were added and incubated at 37 °C for 6 h. The desired product was isolated using semipreparative HPLC with C4 column and a gradient of 5-50B over 40 minutes, affording analogue **12** in 35% isolated yield (0.35 mg).

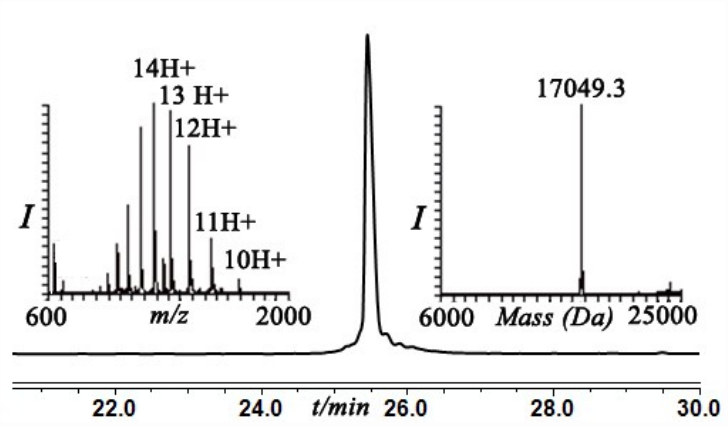


Figure S14: HPLC and mass profile for **12** with observed mass 17049.3±2.3 Da, calcd. 17050.6 Da.

11.3 Preparation of alkylated analogue 13

Di-ubiquitin **11** (~0.7 mg, 4×10^{-5} mmol) was dissolved in 10 µl 6M Gn·HCl 200 mM phosphate buffer (pH 7.5) and diluted with 90 µl of 50 mM Tris buffer (pH 7.5) containing 0.1 mM EDTA. Solution of the iodoacetamide was prepared by dissolving 0.4 mg (~50 eq) in 20 µl of 50% acetonitrile/water and added to the di-ubiquitin reaction mixture, followed by incubation for 1 h at 37 °C. The desired product was isolated using semipreparative HPLC and C4 column, 5-50% B over 40 min.

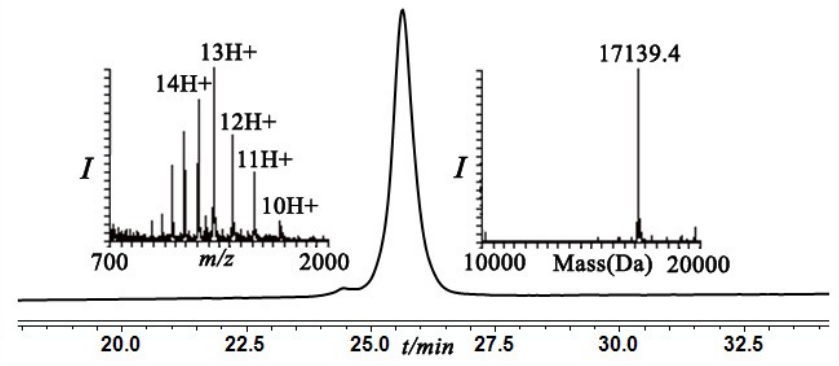


Figure S15: HPLC and mass profile for **13** with observed mass 17139.4±2.9 Da, calcd. 17139.6 Da.

Similarly the preparation of α-globin-ubiquitin was carried out, starting with hexapeptide conjugated α-globin (1 mg) affording the desired product in 15% isolated yield.

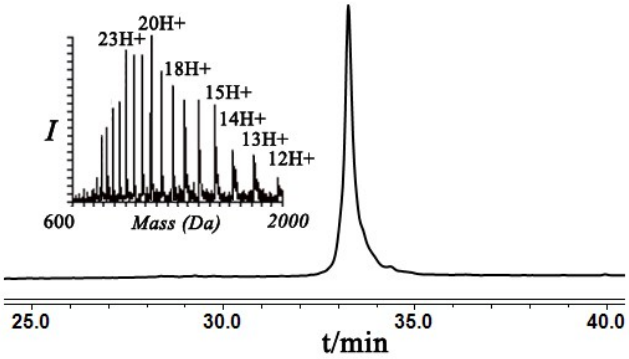


Figure S16: HPLC and mass analysis for the ligation product of hexapeptide conjugated α-globin and ubiquitin(1-70)-thioester with the observed mass of 23686.3±2.2 Da, calcd. 23689.1 Da.

12. Synthesis of wild type di-ubiquitin

Lys48 linked di-ubiquitin was synthesized as previously reported.[4]

13. Enzymatic studies with USP2

The di-ubiquitin analogues were dissolved in 6M Gn·HCl 200 mM phosphate buffer (pH 7.5) and diluted 95% (v/v) with 50 mM Tris buffer (pH 7.5) containing 0.1mM EDTA. The exact final concentration of each protein solution was determined using Pierce® BCA Protein Assay Kit (Thermo scientific) and diluted to a final concentration of 6.66 μM. A stock solution of the USP2 (human catalytic domain, obtained form BostonBiochem, 2 μM) was prepared by diluting 50 μg of the enzyme in 50 mM Tris buffer (pH 7.54) containing 0.1 mM EDTA and 5 mM DTT. The enzymatic assay was initiated by incubating 5 μM solution of each substrate with USP2 (100 nM) for 30 min at 37 ºC. The cleavage pattern was monitored by SDS-PAGE.

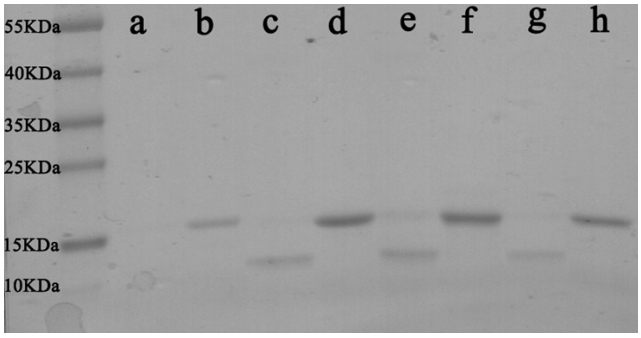


Figure S17: SDS-PAGE of the hydrolysis reaction of the prepared Lys48-linked di-ubiquitin analogues **11-13** by USP2 compared to the wild type reaction. Lines **a** and **b** correspond to WT di-ubiquitin with and without USP2, respectively. Lines **c** and **d** correspond to di-ubiquitin **12** with and without USP2, respectively. Lines **e** and **f** correspond to di-ubiquitin **11** with and without USP2, respectively. Lines **g** and **h** correspond to di-ubiquitin **13** with and without USP2, respectively.

14. Trypsin digestion of conjugated products 10

The conjugate **10** (~0.1 mg, 1.1×10^{-5} mmol) was dissolved in 15 µl of 6M Gn·HCl 200 mM phosphate buffer (pH 7.7) and then diluted with 83 µl of 50 mM Tris buffer (pH 7.7), containing 150 mM NaCl. To this solution 2 µl (2 mg) of trypsin stock from porcine pancreas was added and the reaction was incubated for overnight at 37 °C. Finally, trypsinized solution was analyzed with analytical HPLC using C18 column with gradient 0-40B over 40 minutes. α-globin-hexapeptide was also trypsinized using the same protocol.

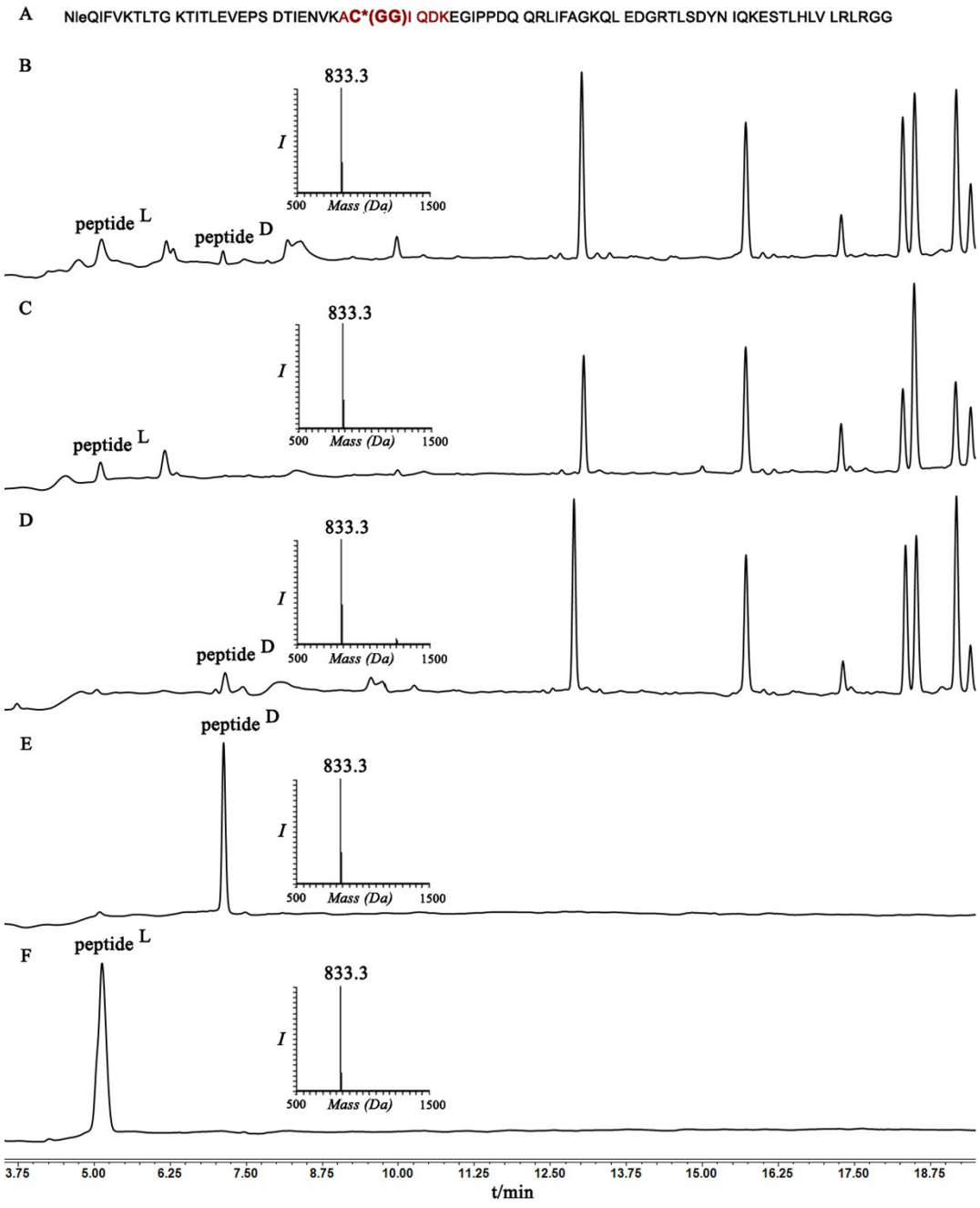


Figure S18: A) Sequence of full ubiquitin, highlighting products of trypsinization in D- and L-forms. * indicates the isopeptide bond mimic (shown in bold). HPLC and mass analysis for: B) Trypsinization of ubiquitin29-hexapeptide **10**. Observed mass of L- and D- peptides is 833.3±0 Da, calcd. 834.4 Da. C) Trypsinization of the first eluted product of conjugation contains the L-peptide with observed mass of 833.3±0 Da, (calcd. 834.4 Da). D) Trypsinization of the second eluted product of conjugation contains the D-peptide with observed mass of 833.3±0 Da, (calcd. 834.4 Da). E) and F) Synthetically prepared peptides correspond to peptide-D and L diastereomers

obtained from trypsinization respectively with the observed mass 833.3±0 Da, (calcd. 834.4 Da).

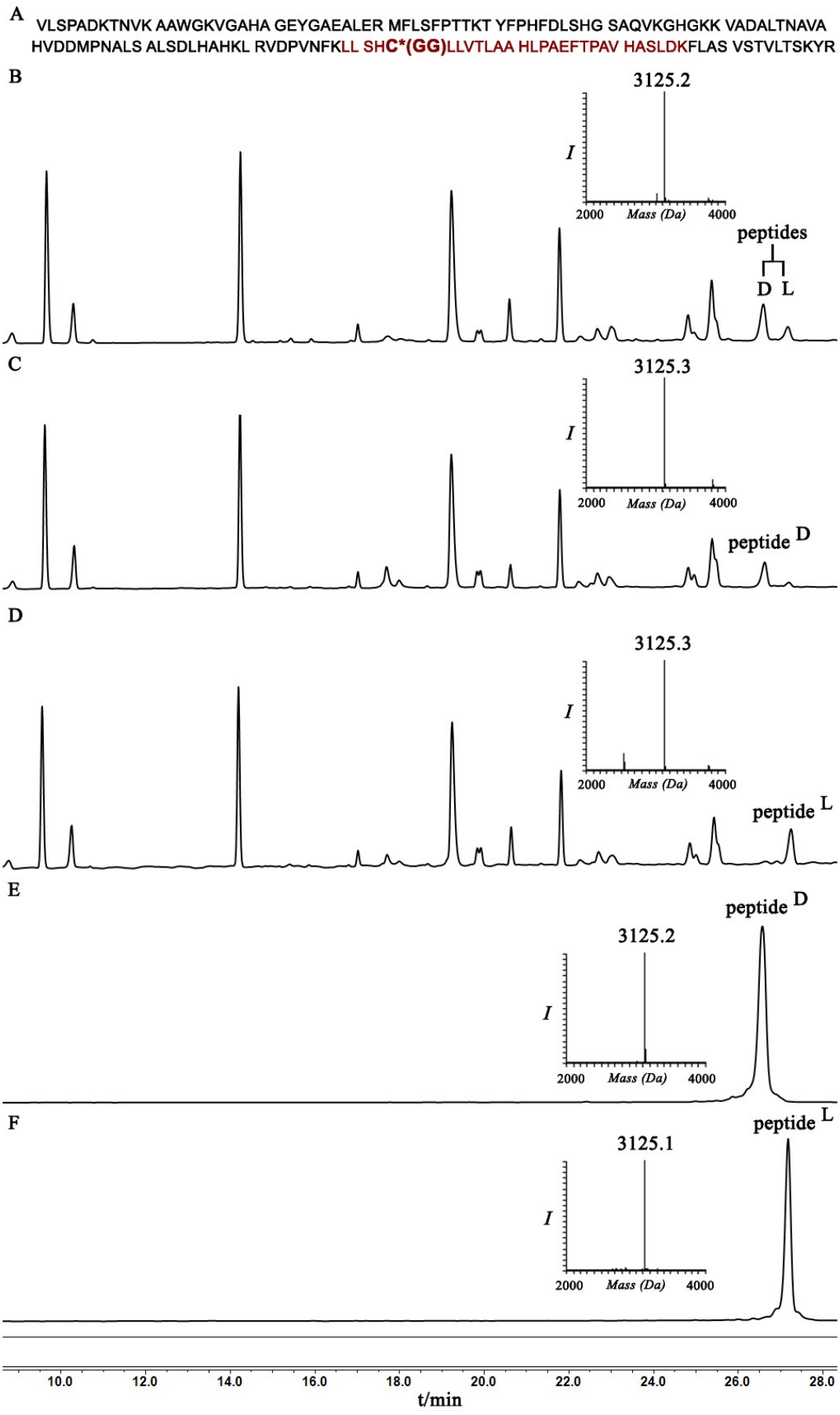

A
VLSPADKTNVK AAWGKVGAHA GEYGAEALER MFLSFPTTKT YFPHFDLSHG SAQVKGHGKK VADALTNAVA
HVDDMPNALS ALSDLHAHKL RVDPVNFKLL SHC*(GG)LLVTLAA HLPAEFTPAV HASLDKFLAS VSTVLTSKYR
B
3125.2
I
2000
Mass (Da)
4000
peptides
D L
C
3125.3
I
2000
Mass (Da)
4000
peptide D
D
3125.3
I
2000
Mass (Da)
4000
peptide L
E
peptide D
3125.2
I
2000
Mass (Da)
4000
F
peptide L
3125.1
I
2000
Mass (Da)
4000
10.0
12.0
14.0
16.0
18.0
20.0
22.0
24.0
26.0
28.0
t/min

Figure S19: A) Sequence of full α-globin, highlighting products of trypsinization in the D- and L-forms. Asterisk indicates Cys, bearing an isopeptide bond mimic with the GG from ubiquitin (shown in bold). HPLC and mass analysis for: B) Trypsinization of diastereomeric mixture of hexapeptide-conjugated α-globin. Observed mass of L- and D peptides is 3125.2 Da±0.5, calcd. 3124.6 Da. C) Trypsinization of the first eluted product of conjugation contains the D-peptide with the observed mass 3125.3±0.5 Da, (calcd. 3124.6 Da). D) Trypsinization of the second eluted product of conjugation contains the L-peptide with the observed mass 3125.3±0.5 Da, (calcd. 3124.6 Da). E) and F) The synthetically prepared peptides corresponded to the peptides D- and L-diastereomers obtained from trypsinization, respectively, with observed mass 3125.2±0.3 and 3125.1±0.3, (calcd. 3124.6 Da).

15. Copies of ^{1}H, ^{13}C NMR and mass analysis

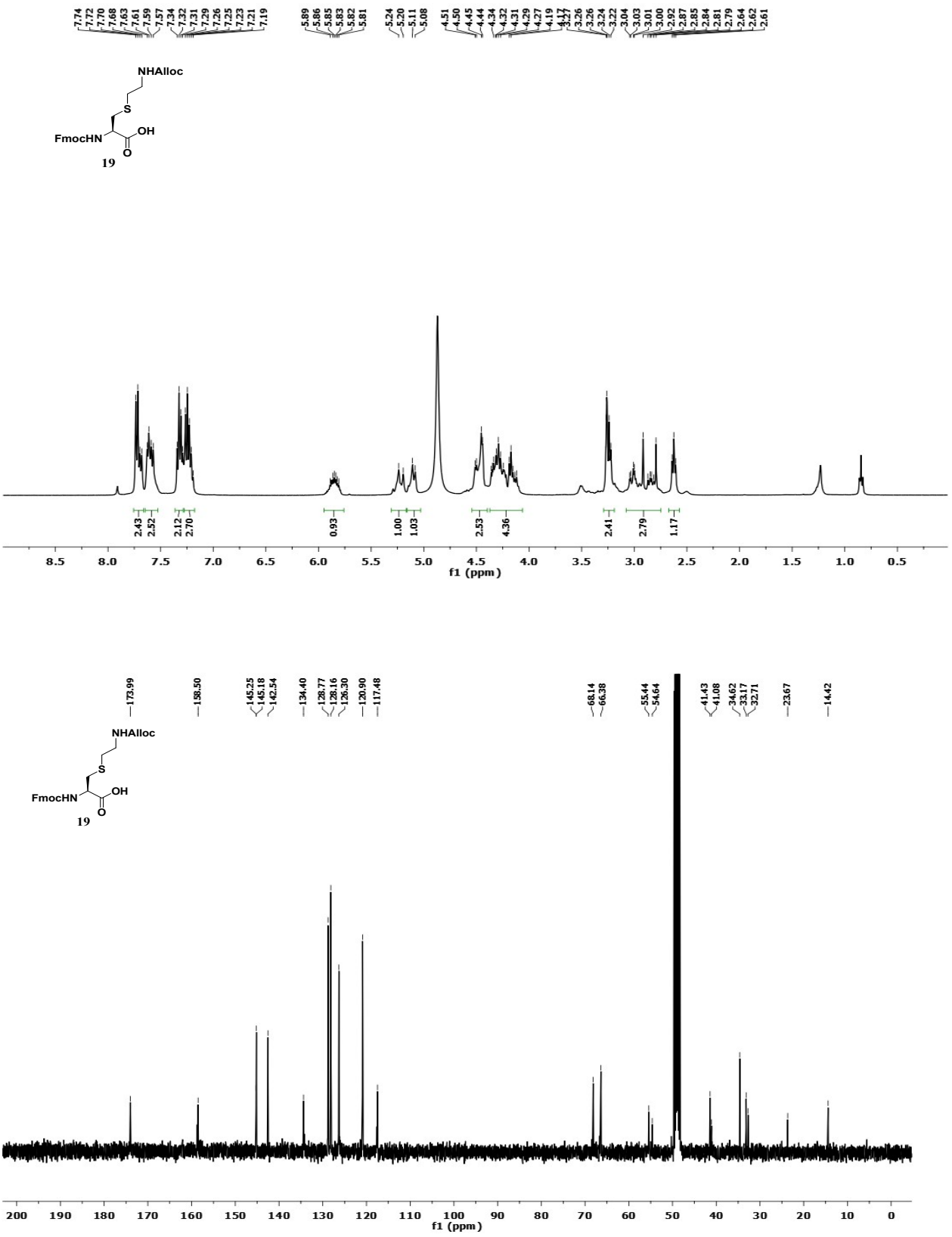


Figure S20: ^{1}H and ^{13}C NMR spectra of **19** in CD_3OD (400 MHz and 101 MHz respectively)

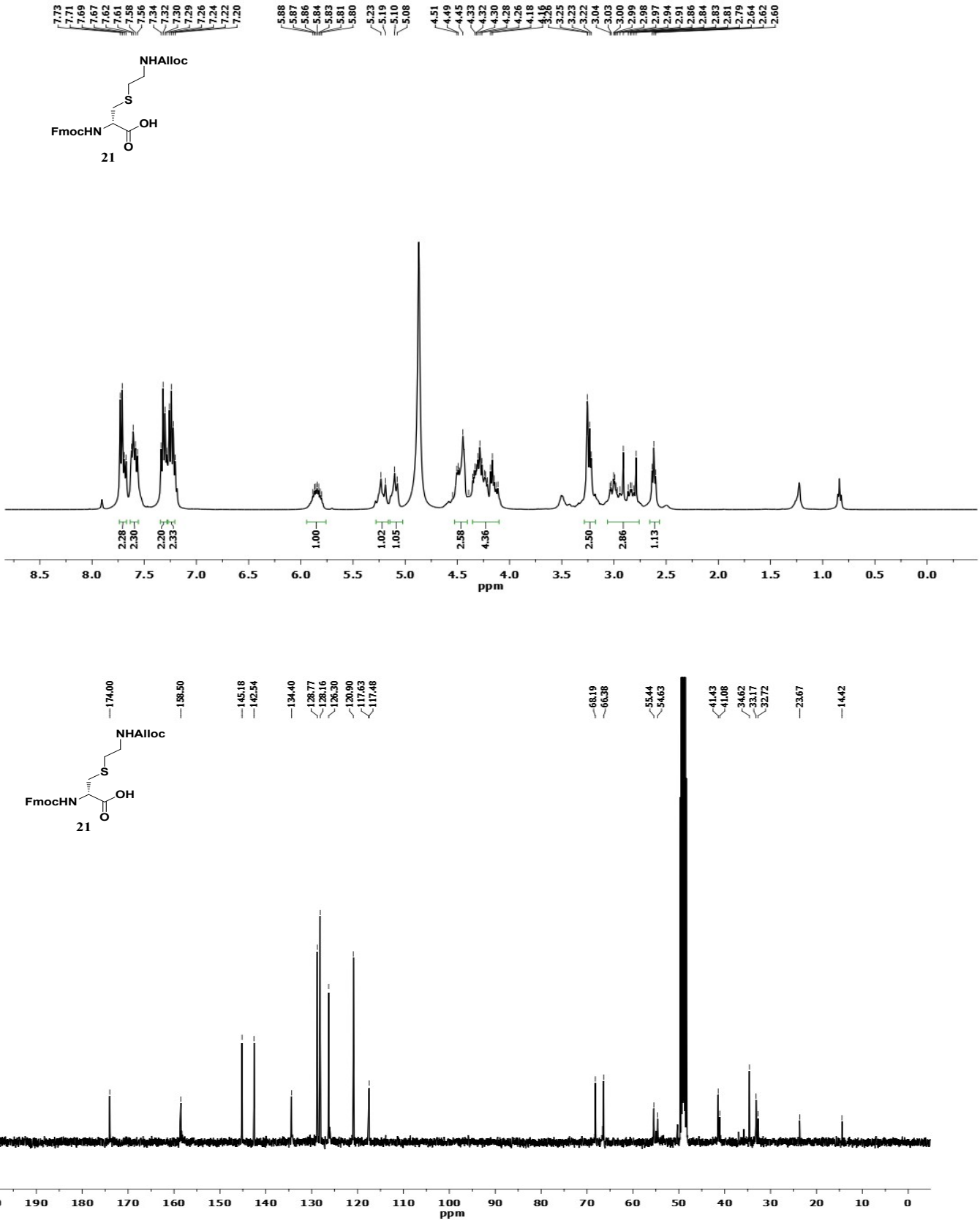
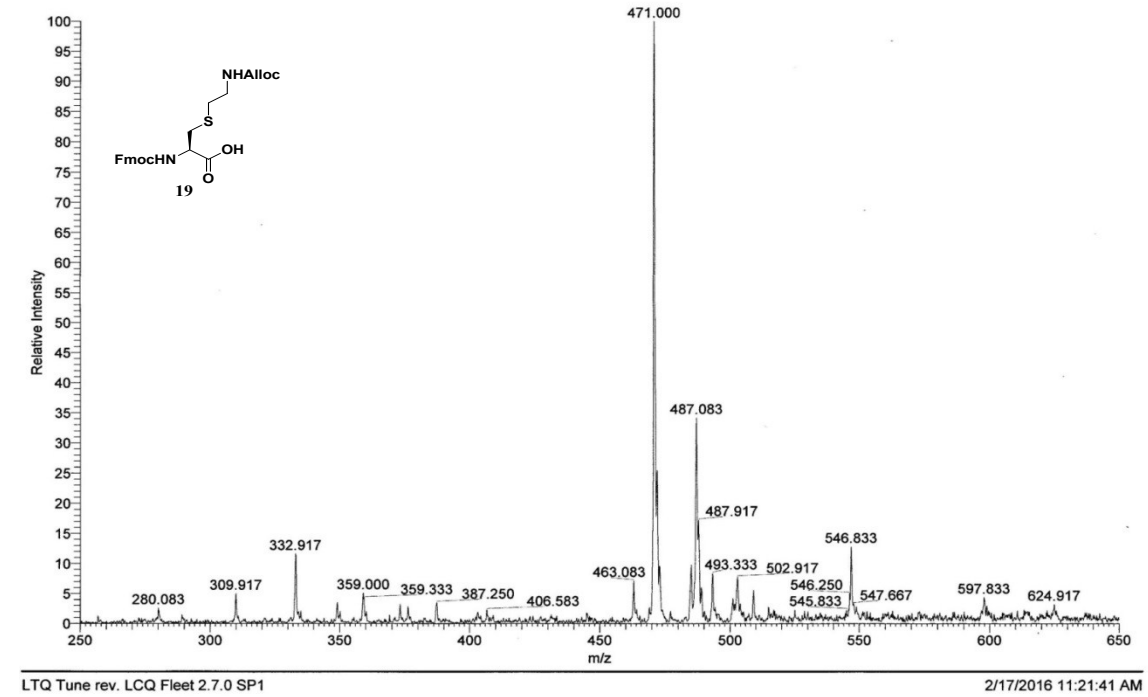


Figure S21: ^{1}H and ^{13}C NMR spectra of **21** in CD_3OD (400 MHz and 101 MHz respectively)



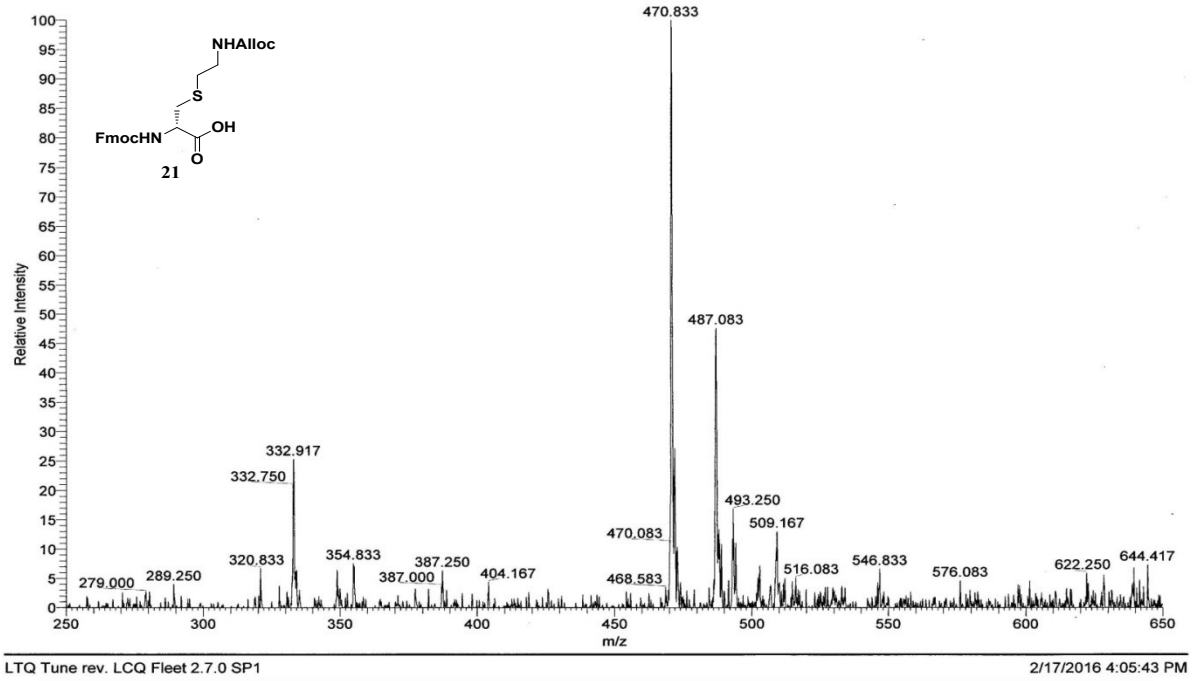


Figure S22: Mass spectras of **19** and **21** respectively

16. References

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(4) Kumar, A. K. S.; Haj-Yahya, M.; Olschewski, D.; Lashuel, H. A.; Brik, A. *Angew. Chem. Int. Ed*. **2009**, *48*, 8090.