Supporting Information

Highly Efficient One-Pot Ligation and Desulfurization

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Experimental Methods

General

SPPS was carried out on Rink amide resin (0.44 mmol/g loading) manually in syringes, equipped with teflon filters, purchased from Torviq or by using an automated peptide synthesizer (CS336X, CSBIO). If it is not differently described, all reactions were carried out at room temperature.

Analytical HPLC was performed on a Thermo instrument (Spectra System P4000) using an analytical column (Jupiter 5 micron, C18/C4 300 Å 150 x 4.6 mm) at a flow rate of 1.2 mL/min.

Preparative HPLC was performed on a Waters instrument using a semi-preparative column (Jupiter 10 micron, C4 300 Å, 250 x 10 mm and a flow rate of 5 mL/min or a preparative column (Jupiter 10 micron, C18/C4 300 Å, 250 x 22.4 mm) at a flow rate of 20 mL/min.

Buffer A: 0.1% TFA in water; buffer B: 0.1% TFA in acetonitrile.

Mass spectrometry analysis was carried out using LCQ Fleet Ion Trap (Thermo Scientific).

Commercial reagents were used without further purification. Resins, protected amino acids and HBTU, HCTU, HATU, were purchased from Novabiochem, Aapptec and Chem-Impex. DMF was purchased in biotech grade.

For the capture of bifunctional reagent, aminomethyl ChemMetrix resin (loading: 1.0 mmol/g) was used which was purchased from Sigma-Aldrich.

Starting materials and reagents used for the synthesis of small molecules were procured from Sigma Aldrich and Alfa-Aesar.

One pot ligation and desulfurization general protocol:

Cys-peptide and peptide-MPAA in equivalent amounts were each dissolved in 6 M Gn·HCl, 200 mM phosphate buffer containing TCEP (15 equivalents), pH ~ 7 and were mixed together (final concentration: 2 mM). The reaction was kept at 37 °C and monitored using C4 analytical HPLC with a linear gradient of 15-60% B over 30 min. Subsequently, desulfurization was carried out by adding VA-044 (40 equivalents) in 200 µL 6 M Gn·HCl, 200 mM phosphate buffer, TCEP (0.25 mM) and *tert*-BuSH (10% v/v). The reaction was kept at 42 °C and was monitored using C4 analytical HPLC with a linear gradient of 15-60 % B over 30 min.

Ligation and one-pot desulfurization for model peptides

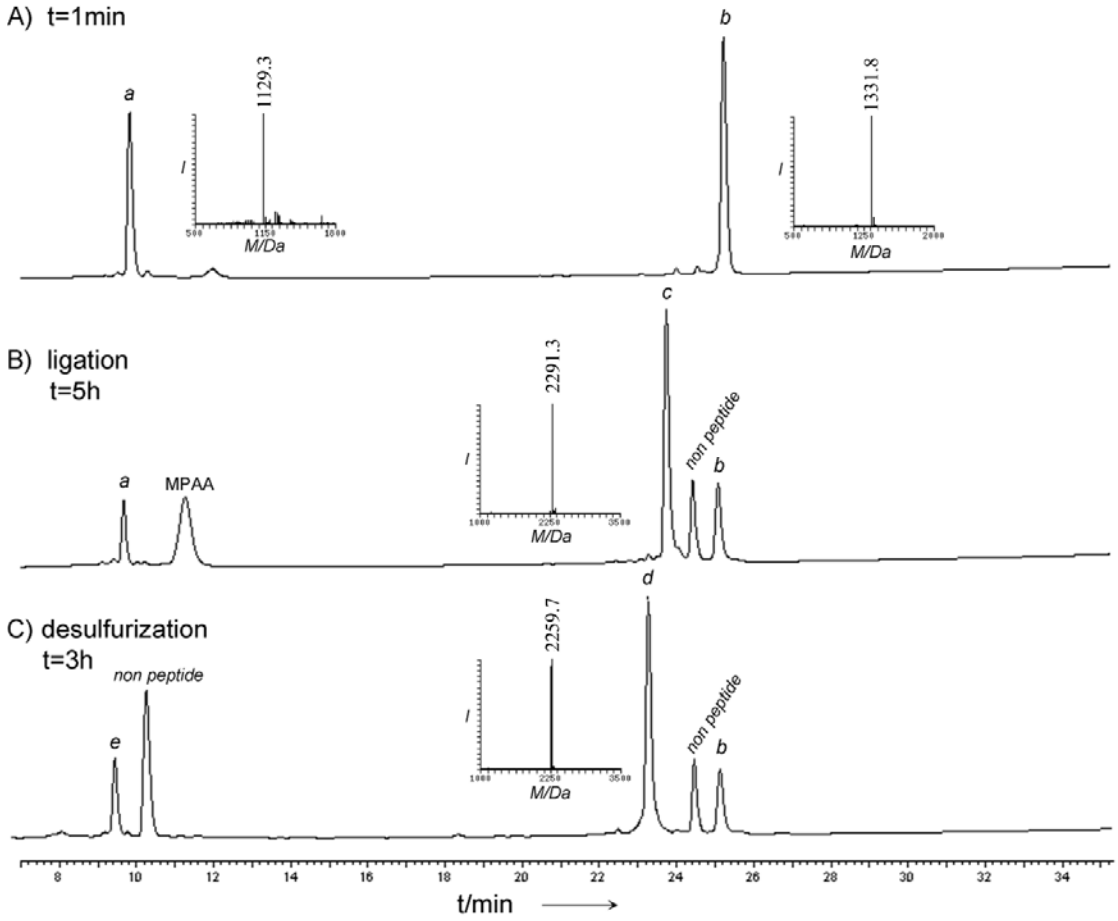


Figure S1: Analytical HPLC/ESI-MS of one-pot ligation and desulfurization of LYRAGLYRA**V**-"thiophenylester. A) Analytical HPLC trace of the ligation reaction at t = 1min. Peak a corresponds to **C**YRAGLYRAG with the observed mass 1129.3 Da (calcd 1129.3 Da); peak b corresponds to LYRAGLYRA**V**-MPAA with the observed mass 1331.8 Da (calcd 1332.4 Da). B) Analytical HPLC analysis of the ligation reaction after 5 h. Peak c corresponds to the ligation product (LYRAGLYRA**VC**YRAGLYRAG) with the observed mass 2291.3 Da (calcd 2291.7 Da). C) Analytical HPLC trace of the desulfurization reaction after 3 h. Peak d corresponds to the desulfurization product (LYRAGLYRA**VA**YRAGLYRAG) with the observed mass 2259.7 Da (calcd 2259.6 Da); peak e corresponds to AYRAGLYRAG.

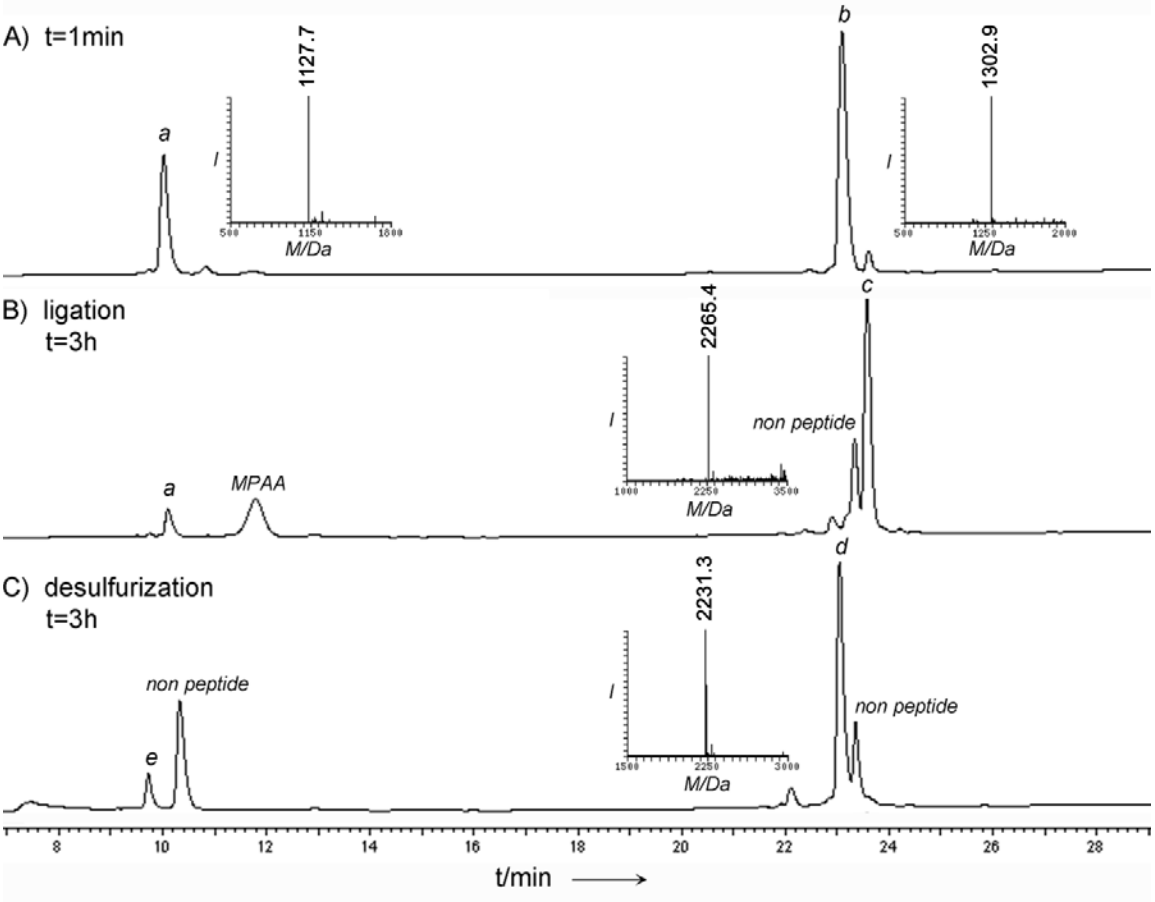


Figure S2: Analytical HPLC/ESI-MS of one-pot ligation and desulfurization of LYRAGLYRA**A**-ᵅthiophenylester. A) Analytical HPLC trace of the ligation reaction at t = 1min. Peak a corresponds to CYRAGLYRAG with the observed mass 1127.7 Da (calcd 1129.3 Da); peak b corresponds to LYRAGLYRA**A**-MPAA with the observed mass 1302.9 Da (calcd 1304.3 Da). B) Analytical HPLC trace of the ligation reaction after 1 h. Peak c corresponds to the ligation product (LYRAGLYRA<u>AC</u>YRAGLYRAG) with the observed mass 2265.4 Da (calcd 2263.6 Da). C) Analytical HPLC trace of the desulfurization reaction after 3 h. Peak d corresponds to the desulfurization product (LYRAGLYRA<u>AA</u>YRAGLYRAG) with the observed mass 2231.3 Da (calcd 2231.6 Da); peak e corresponds to AYRAGLYRAG.

Ligation of Ub2-MPAA with Ub1-$CONH_2$ with or without additional MPAA

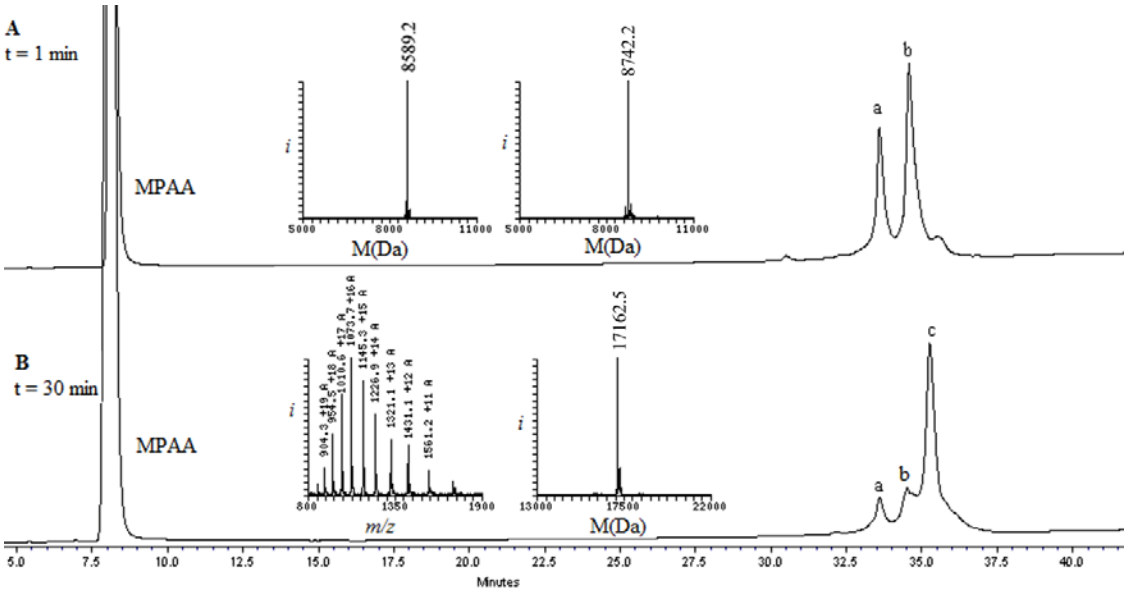


Figure S3: Analytical HPLC/ESI-MS of ligation reaction between Ub2-MPAA (1 equivalent) and Ub1-$CONH_2$ (1.1 equivalent) **in the presence of 30 equivalents of MPAA**. A) Analytical HPLC trace of the ligation reaction at t = 1 min. Peak a corresponds to Ub1-$CONH_2$(K*48) with the observed mass 8589.2 Da (calcd 8588.6 Da); peak b corresponds to Ub2-MPAA with the observed mass 8742.2 Da (calcd 8742.6 Da). B) Analytical HPLC trace of the ligation reaction after 30 min. Peak c corresponds to the ligation product with the observed mass 17,162.5 Da (calcd 17,161.2 Da).

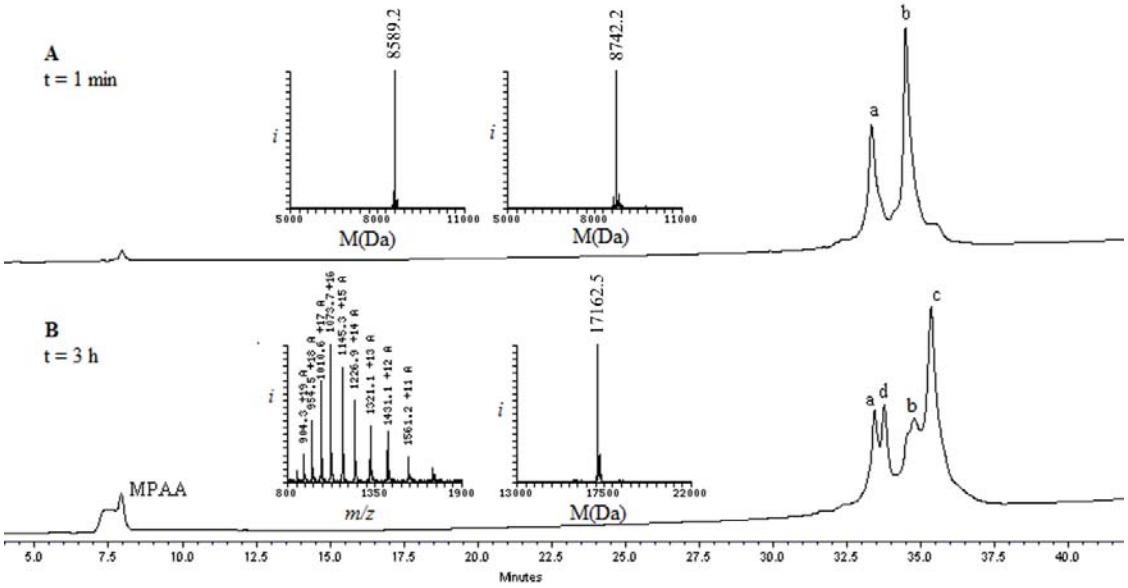
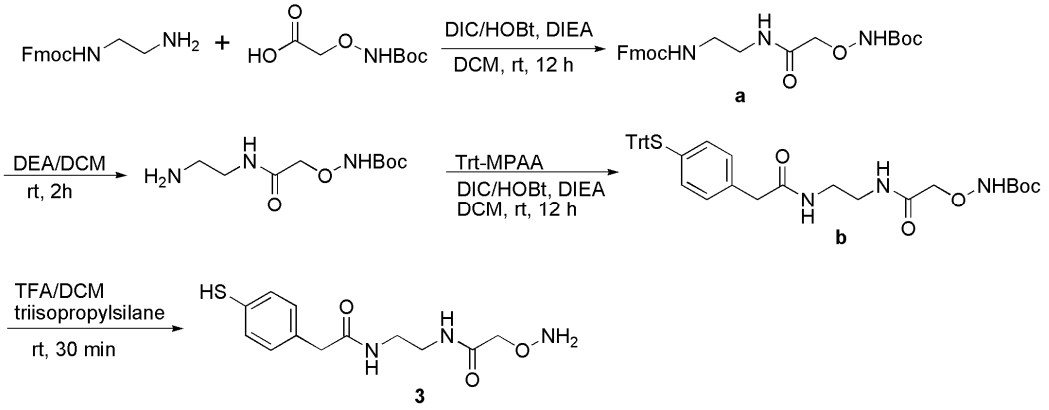


Figure S4: Analytical HPLC/ESI-MS of ligation of Ub2-MPAA with Ub1-NH_2 **without the addition of extra MPAA**. A) Analytical HPLC trace of the ligation reaction at t = 1 min. Peaks a and b are the starting peptides. B) Analytical HPLC trace of the ligation reaction after 3 h. Peak c corresponds to the ligation product with the observed mass 17,162.5 Da (calcd 17,161.2 Da); peak d corresponds to the hydrolysis of Ub2-MPAA.

Preparation of bifunctional reagents:

Bifunctional reagent 3



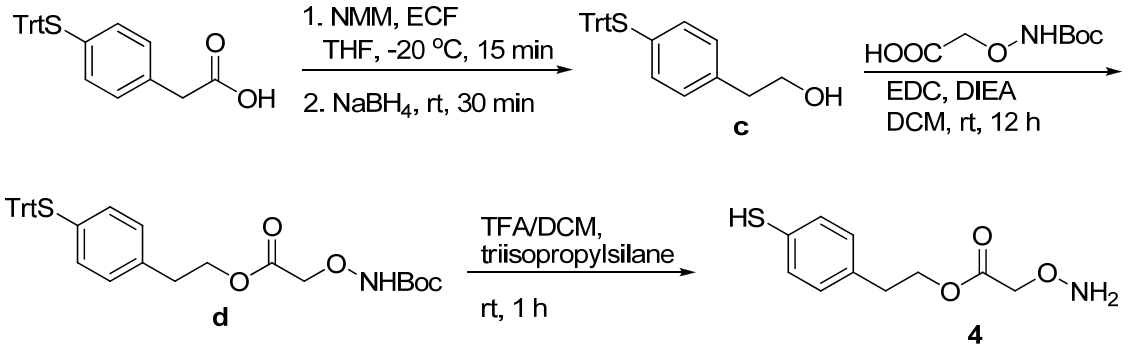
Step 1: *N*-Fmoc-1,2-diaminoethane (1 g, 3.55 mmol) was dissolved in DCM (20 mL) to which was added a solution of Boc-aminooxyacetic acid (1.35 g, 7.10 mmol) activated (for 4 min) with DIC (1.1 mL, 7.10 mmol), HOBt (957 mg, 7.10 mmol) and DIEA (2.5 mL, 14.20 mmol) in DCM (20 mL). The reaction was stirred at room temperature for 12 h. Subsequently it was concentrated under reduced pressure and the crude product was purified via column chromatography (40-60% EtOAc in hexane) to afford compound **a** as a white solid (1.09 g, 67% yield).

Step 2: The Fmoc group from compound **a** (1 g, 2.2 mmol) was removed by treatment with 1:1 solution of DEA/DCM (15 mL) for 1 h. The reaction mixture was then concentrated under reduced pressure and coupled to Trt-MPAA (1.8 g, 4.4 mmol) using DIC (687 µL, 4.4 mmol), HOBt (594 mg, 4.4 mmol) and DIEA (1.5 mL, 8.8 mmol) in DCM (15 mL). After stirring the reaction mixture for 12 h at room temperature, the solvent was evaporated and purified by column chromatography (40-60% EtOAc in hexane) to yield compound **b** as a white solid (481 mg, 35% yield over two steps).

Step 3: Compound **b** (481 mg, 0.77 mmol) was dissolved in 1:1 TFA:ACN (10 mL) and triisopropylsilane (TIS, 237 µL, 1.15 mmol) was added. The reaction was stirred at room temperature for 1 h and was concentrated under reduced pressure to obtain the crude product, which was purified using column chromatography (0-10% methanol in chloroform) to afford bifunctional reagent **3** as a white solid (100 mg, 46% yield). *^{1}H NMR (400 MHz, $CDCl_3$)* δ 7.15 (d, 2H, *Ar*), 7.07 (d, 2H, *Ar*), 4.22 (s, 2H, -C*H*$_2$ONH$_2$), 3.36 (s, 2H, Ar-C*H*$_2$-CO-), 3.25 (br t, 4H, -NH-*CH*$_2$-*CH*$_2$-NH-); *^{13}C NMR (400 MHz, $CDCl_3$)* δ

174.6, 166.2, 138.7, 138.7, 130.9, 130.4, 130.2, 73.6, 43.2, 40.0; m/z: Calculated for $[C_{12}H_{18}N_3O_3S]^+$: 284.1 Da and observed: 284.1 Da.

Bifunctional reagent 4



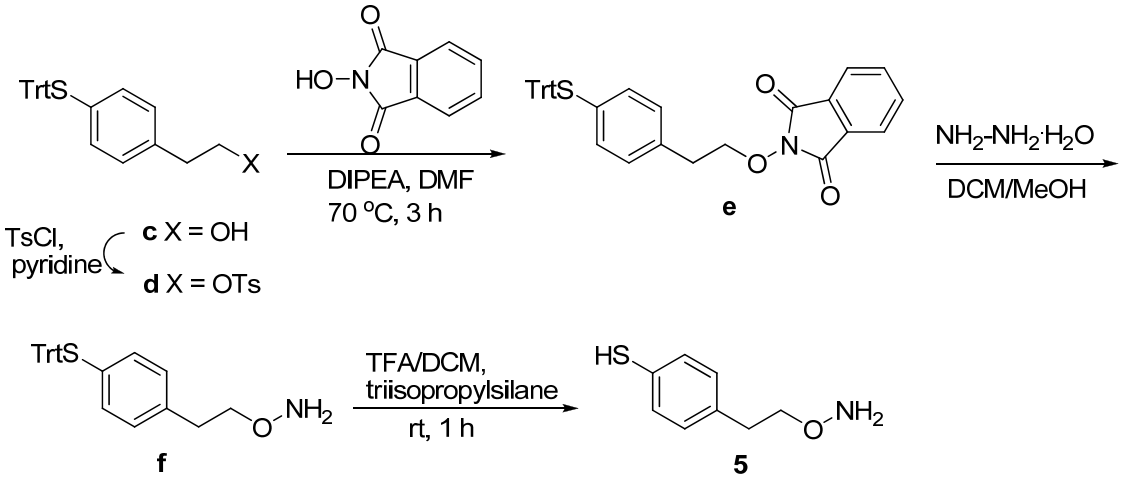
Step 1: A solution of Trt-MPAA (250 mg, 0.63 mmol) in THF was cooled to -20 °C and N-methylmorpholine (NMM, 0.95 mmol) and ethylchloroformate (ECF, 0.95 mmol) were added. After stirring for 15 min, the reaction mixture was treated with $NaBH_4$ (1.25 mmol) taken in minimum quantity of water. After an extraction work-up with EtOAc and drying over Na_2SO_4 and evaporation of solvent, the corresponding hydroxy compound **c** was obtained, which was used for the next step without further purification.

Step 2: The crude hydroxyl compound **c** (200 mg, 0.5 mmol) was coupled to Boc-aminooxyacetic acid (210 mg, 1.1 mmol) employing EDC (1.5 mmol) and DIEA (3 mmol) in dry DCM (5 mL) for 12 h. After completion of reaction (TLC analysis), the reaction mixture was diluted with DCM and was washed with 10% citric acid solution, water, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude product was column chromatographed (using 40% EtOAc in hexane) to afford compound **d** (250 mg, 83% yield).

Step 3: Compound **d** (200 mg) in a mixture of DCM:TFA (1:1, 5 mL) was stirred along with TIS (143 µL, 2.0 mmol) for an hour at room temperature after which it was concentrated under reduced pressure and purified using column chromatography (0-10% methanol in chloroform) to afford bifunctional reagent **4** as a white solid (64 mg, 80% yield).

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.06-7.41 (Ar, 4H), 4.33 (t, *J* = 4.0 Hz, 2H, -CH_2-C*H*$_2$-O-), 4.19 (s, 2H, -CO-C*H*$_2$-O-), 3.39 (s, 1H, Ar-S*H*), 2.89 (t, *J* = 8.0 Hz, 2H, Ar-C*H*$_2$-CH_2-); *^{13}C NMR (400 MHz, $CDCl_3$)*: δ 170.5, 135.0, 129.7, 129.5, 127.8, 72.1, 64.8, 34.3; m/z: Calculated for $[C_{10}H_{13}NO_3S]^+$: 227.0 Da and observed: 227.0 Da; for $[C_{10}H_{13}KNO_3S]^+$: 266.0 Da and observed 267.1 Da.

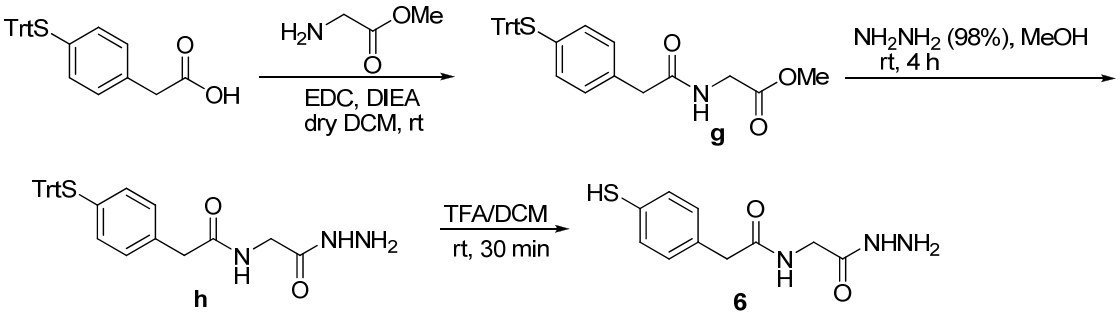
Bifunctional reagent 5



Compound **c** (300 mg, 0.75 mmol) was treated with TsCl (1.1 mmol) in pyridine (6 mL) to convert it to the corresponding tosylate. After completion of reaction (TLC analysis), it was evaporated and the tosyl compound was isolated via a simple aqueous workup. It was then reacted with *N*-hydroxyphthalimide (1.1 mmol, 1.5 eq.) in the presence of DIPEA (1.5 mmol, 2 eq.) in DMF (4 mL) at 90 ºC for 4 h. It was cooled, extracted into EtOAc to afford compound **e** (220 mg, 53% yield) after chromatographic purification (Silica gel, 20% EtOAc in hexane; Zlotorzynska *et al*., *Org. Lett.* **2008**, *10*, 5083). In the next step, hydrazine monohydrate (36 µL, 72 mmol) was added to a solution of compound **e** (200 mg, 0.37 mmol) in a mixture of CH_2Cl_2 (5 mL) and MeOH (0.5 mL) and stirred at room temperature for 4 h. The precipitate was filtered-off and the filtrate was evaporated (Ishikawa et al., *J. Antibiotics* **2000**, *53*, 1053) under vacuum and the crude compound **f** was stirred in a mixture of TFA-DCM in presence of TIS to remove the trityl group. The solvent was evaporated and, finally, it was purified using column chromatography (8% methanol in chloroform) to afford bifunctional reagent **5** as white fluffy solid (41 mg, 66%).

^{1}H NMR (400 MHz, CD_3OD) δ 2.91 (t, *J* = 4 Hz, 2H, Ar-C*H*$_2$-CH_2-), 4.16 (t, *J* = 4 Hz, 2H, -CH_2-C*H*$_2$-O-), 7.12-7.21 (Ar, 4H) ppm; 13C NMR (400 MHz, CD_3OD) δ 32.8, 75.2, 128.6, 129.1, 129.7, 129.8; m/z: Calculated for $[C_8H_{11}NKOS]^+$: 208.3 Da and observed: 208 Da.

Bifunctional reagent 6



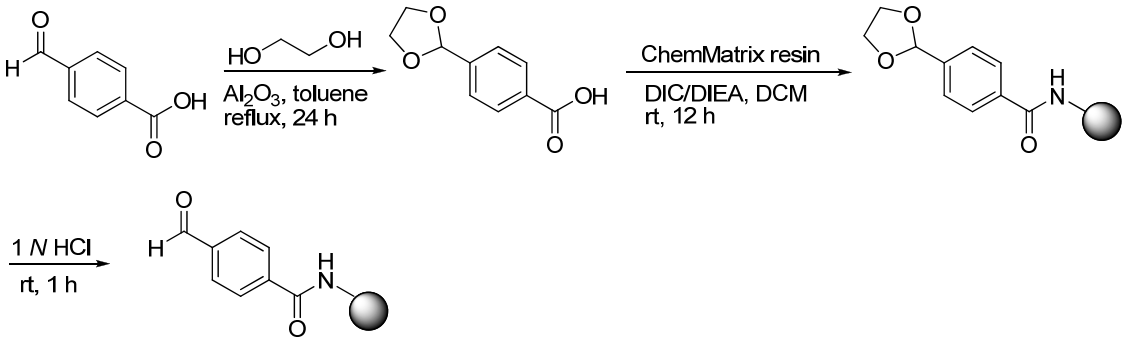
To a solution of Trt-MPAA (300 mg, 0.73 mmol) in dry DCM (5 mL), Gly-OMe (0.94 mmol, 1.3 eq) was added followed by EDC (1.0 mmol) and DIEA (1.5 mmol). The reaction mixture was stirred at room temperature for 4 h and compound **g** was isolated after a simple aqueous work up. The material was taken on to the next step without further purification.

In the second step, compound **g** (340 mg, 0.7 mmol) was suspended in MeOH (6 mL) and hydrazine (98%, 50 eq) was added and the reaction mixture was stirred at ambient temperature until it became clear. TLC analysis (5% MeOH in $CHCl_3$) showed complete consumption of the starting material. The reaction mixture was diluted with DCM (20 mL), washed with water, dried over anhydrous Na_2SO_4 and evaporated. Finally, the crude material **h** was re-dissolved in DCM and treated with TFA to remove the trityl group. After stirring for 30 minutes at room temperature, the reaction mixture was evaporated and purified via column chromatography (5-10% methanol in chloroform) to obtain bifunctional reagent **6** as off-white solid (85 mg, 50% yield).

^{1}H NMR (400 MHz, DMSO-d_6): δ 2.5 (d, 2H, -NH-N*H*$_2$), 3.44 (s, 2H, Ar-C*H*$_2$-CO-), 3.80 (d, *J* = 6 Hz, 2H, -NH-C*H*$_2$-CO-), 5.32 (br, 1H, Ar-S*H*), 7.15-7.23 (Ar, 4H), 8.53 (d, *J* = 4Hz, 1H, -CO-N*H*-); *^{13}C NMR (400 MHz, DMSO-d_6)*: δ 170.9, 168.9, 130.6, 130.2, 128.6,

127.8, 41.6, 40.9; m/z: Calculated for $[C_{10}H_{13}N_3O_2S]^+$: 240.0 Da and observed: 240.1 Da; for $[C_{10}H_{13}N_3NaO_2S]^+$: 262.0 and observed 262.1.

Preparation of aldehyde-ChemMatrix resin



Terephthalaldehydic acid was protected as corresponding diacetal by reacting with ethylene glycol according to Kamitori et al., (*Tetrahedron Lett*, **1985**, *26*, 4767). Briefly, to a solution of terephthalaldehydic acid (1 mmol) in toluene (5 mL), ethylene glycol (5 mmol) and Al_2O_3 (200 mg) were added and the mixture was refluxed for 24 h. It was filtered, washed with CH_2Cl_2 and combined filtrate was washed with water, dried over anhydrous Na_2SO_4 and evaporated under vacuum. It was used as such for next step. Subsequently, the acid (12 mg, 0.062 mmol) was activated in DCM (500 µL) using DIC (10 µL, 0.062 mmol) and DIEA (21 µL, 0.125 mmol) and added to pre-swollen aminomethyl ChemMetrix resin 1.0 mmol/g (25 mg, 0.025 mmol). The reaction was kept at room temperature for 12 h after which it was washed with DCM. To regenerate the aldehyde functionality, the resin was treated with 1 *N* HCl for an hour; washed with 6 M guanidine·HCl (Gn·HCl, pH 4.0) and used as such for capturing the bifunctional reagent.

One-pot ligation, capture and desulfurization using bifunctional reagent

Cys-Ub(47-76) (1 mg, 0.28 µmol) and Ub(1-45)-MMP (1.6 mg, 0.30 µmol) were dissolved in 143 µL 6 M Gn·HCl, 200 mM phosphate buffer (pH 7.2), along with bifunctional reagent **6** (2.05 mg, 8.57 µmol) and TCEP (1.2 mg, 4.20 µmol). The reaction was incubated at 37 ˚C for 1.5 h at which time HPLC analysis showed completion of the ligation. The pH of the reaction mixture was adjusted to 4.0 and the mixture was added to the aldehyde

functionalized resin (17.5 mg, 17.14 µmol based on original loading) for 2 h. The completion of this capture step was confirmed by analytical HPLC. The reaction mixture was eluted from the resin, followed by washing with 100 µL 6 M Gn·HCl and the combined mixture was subjected to radical desulfurization employing VA-044 (1.8 mg, 5.57 µmol), TCEP (0.25 M) and *tert*-BuSH (10% v/v) for 2 h at 40 ˚C at which time the reaction was complete as monitored by analytical HPLC.

Analytical data for ligation of Cys-Ub(47-76) and Ub(1-45)-MMP, capture and desulfurization

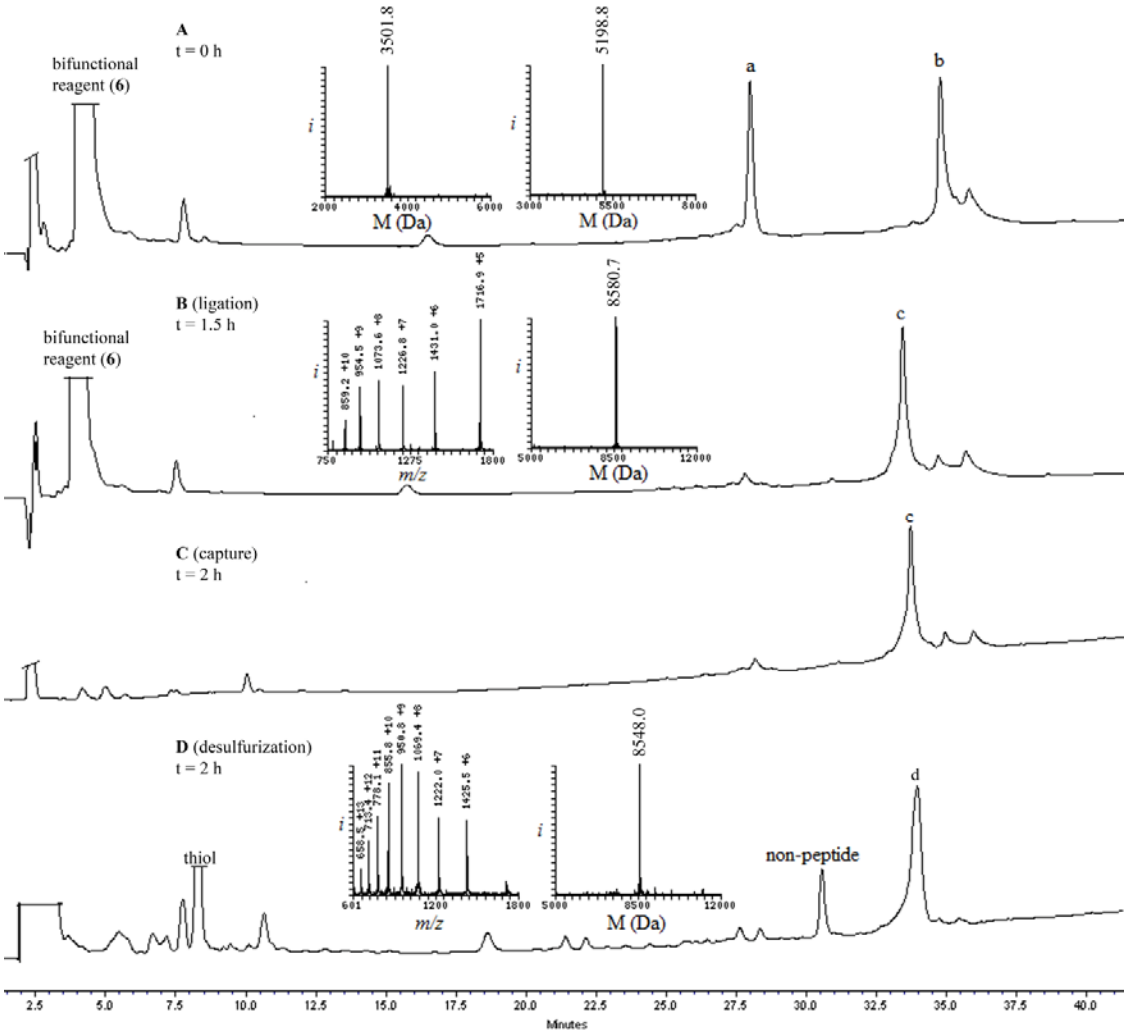


Figure S5: Analytical HPLC/ESI-MS of one-pot ligation and desulfurization of UbN-MMP and UbC. A) Analytical HPLC traces of the ligation reaction at t = 0 h. Peak a corresponds to UbC with the observed mass 3501.8 Da (calcd 3501.0 Da); peak b corresponds to UbN-MMP with the observed mass 5198.8 Da (calcd 5199.9 Da). B) Analytical HPLC traces of the ligation reaction after 1.5 h. Peak c corresponds to the ligation

product with the observed mass 8580.7 Da (calcd 8579.9 Da). C) Analytical HPLC trace of ligation mixture after the capturing step. D) Analytical HPLC trace of the desulfurization reaction after 2 h. Peak d corresponds to the desulfurization product with the observed mass 8548.0 Da (calcd 8547.8 Da).

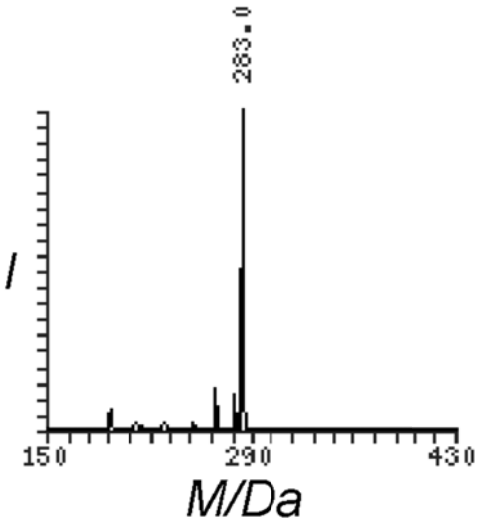


Figure S6: ESIMS of bifunctional reagent **3**

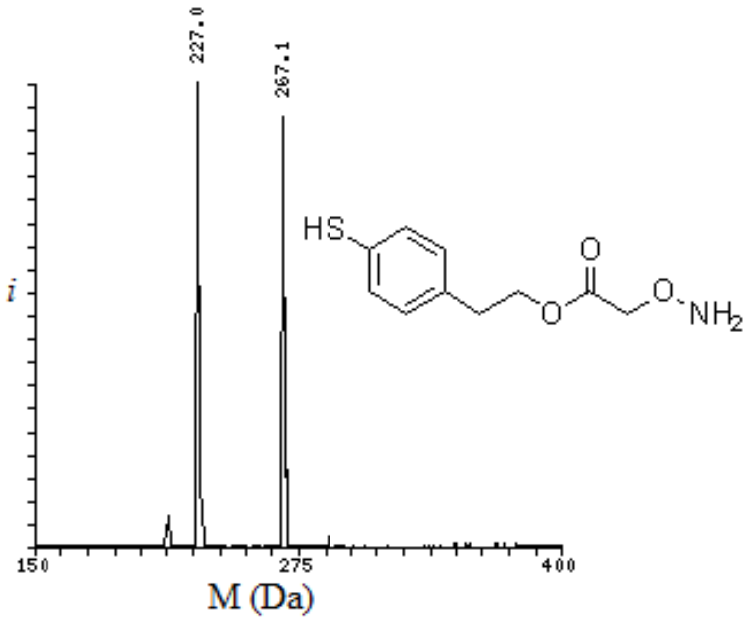


Figure S7: ESIMS of bifunctional reagent **4**

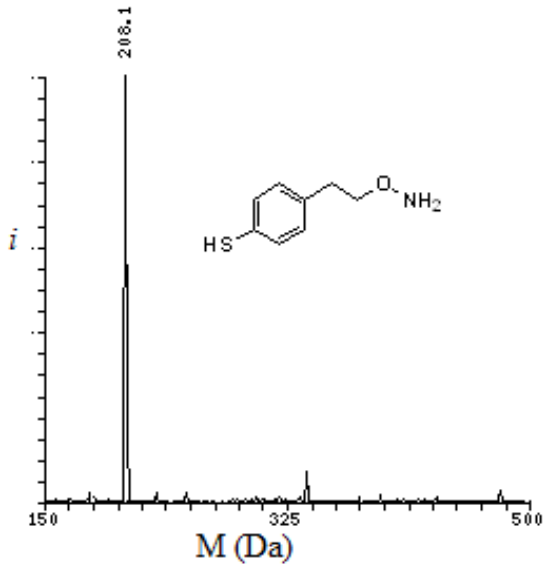


Figure S8: ESIMS of bifunctional reagent **5**

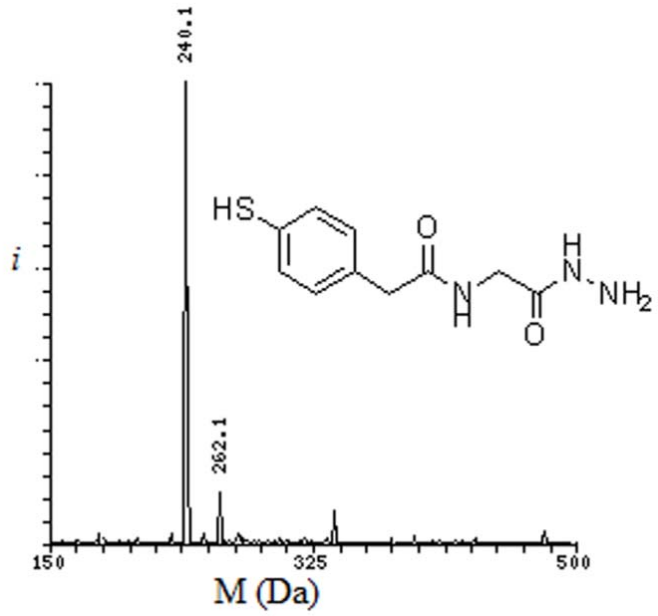


Figure S9: ESIMS of bifunctional reagent **6**

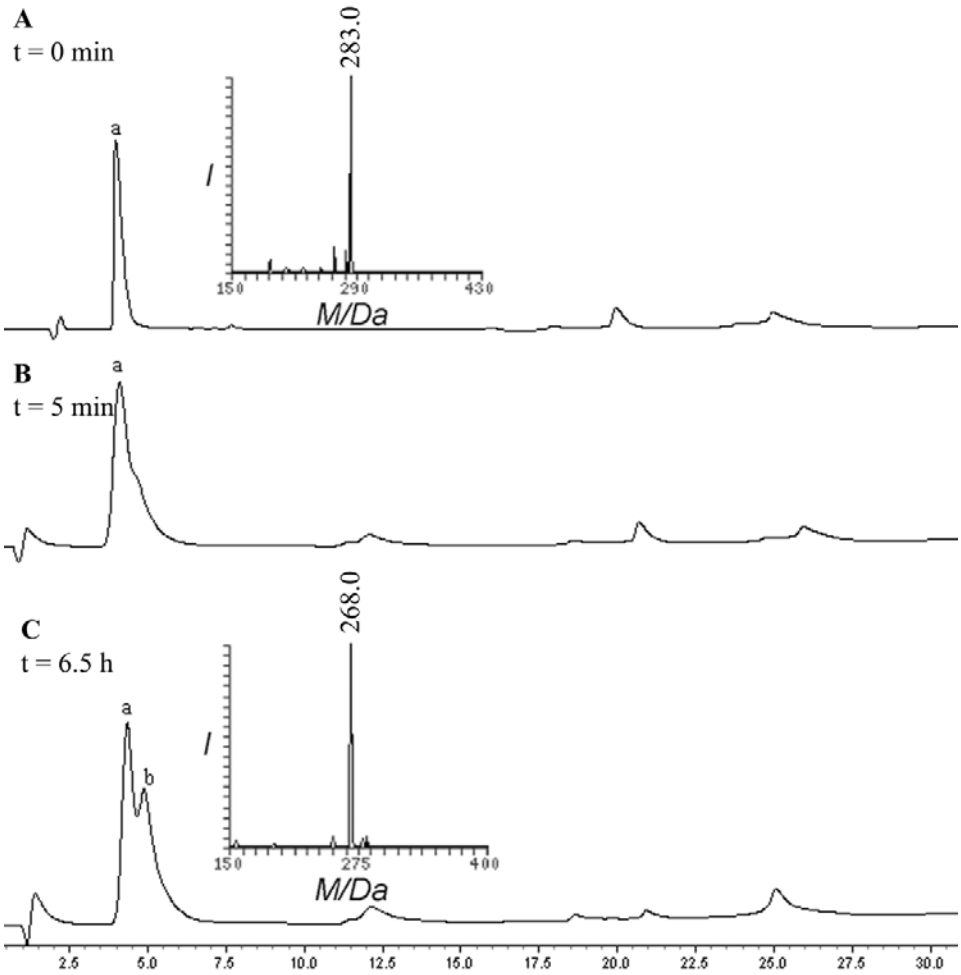
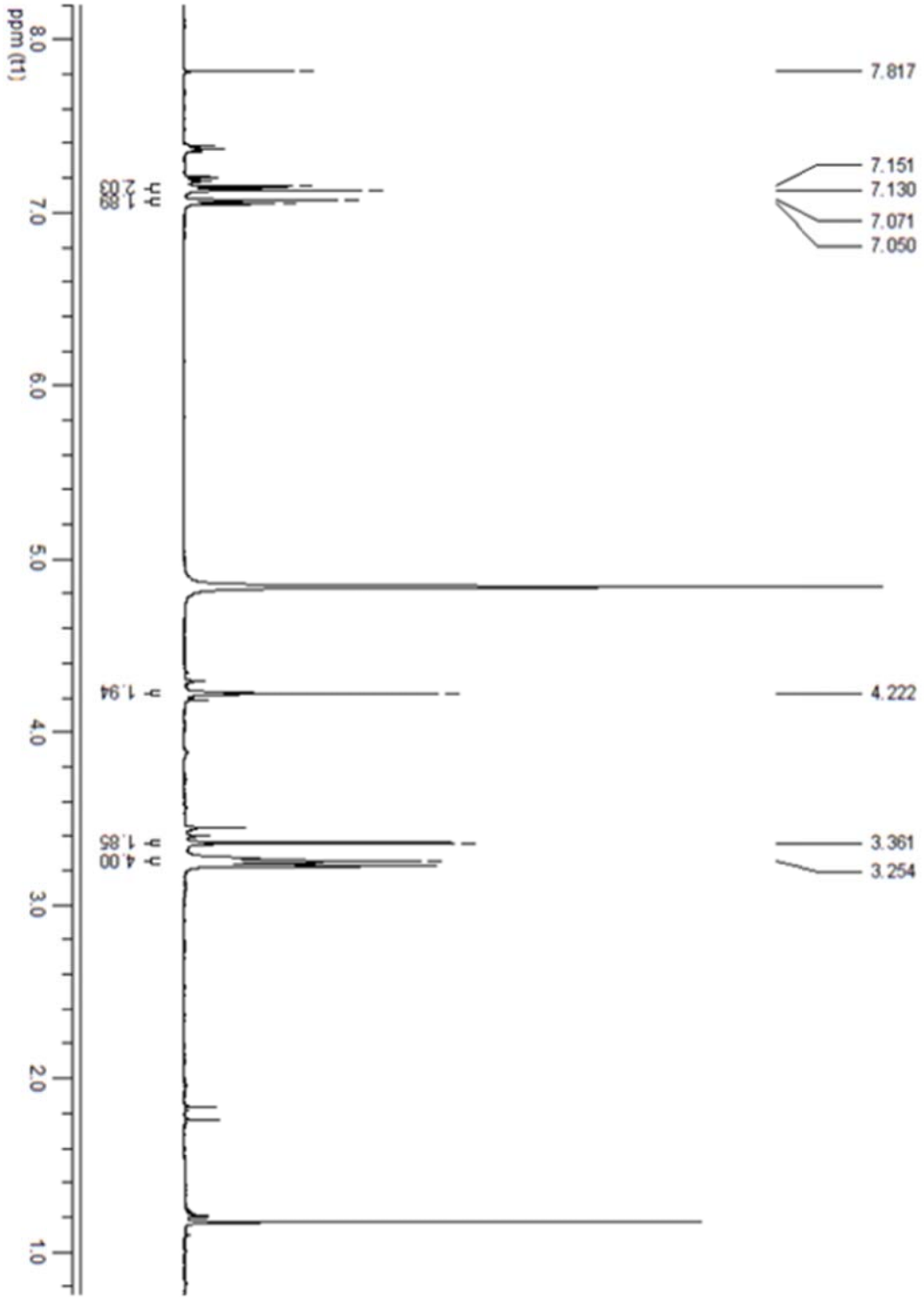
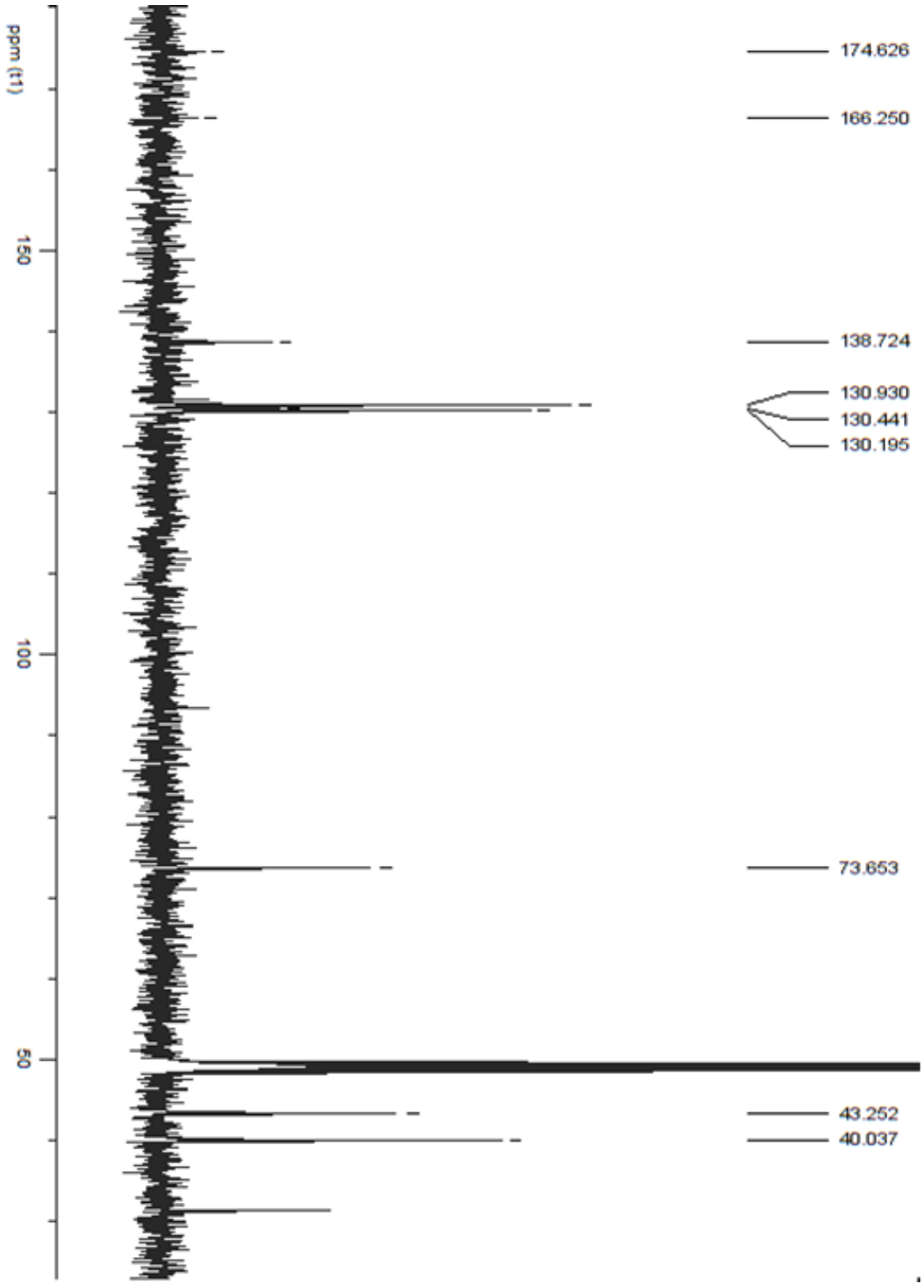


Figure S10: A case study to test the stability of bifunctional reagent **3** under ligation conditions. A is the reaction at t = 0 min; peak a corresponds to bifunctional reagent **3**. C. Analytical HPLC trace of ligation after 6.5 h; peak b corresponds to byproduct of bifunctional reagent **3** due to ammonia elimination.

^{1}H and ^{13}C NMR for bifunctional reagent 3

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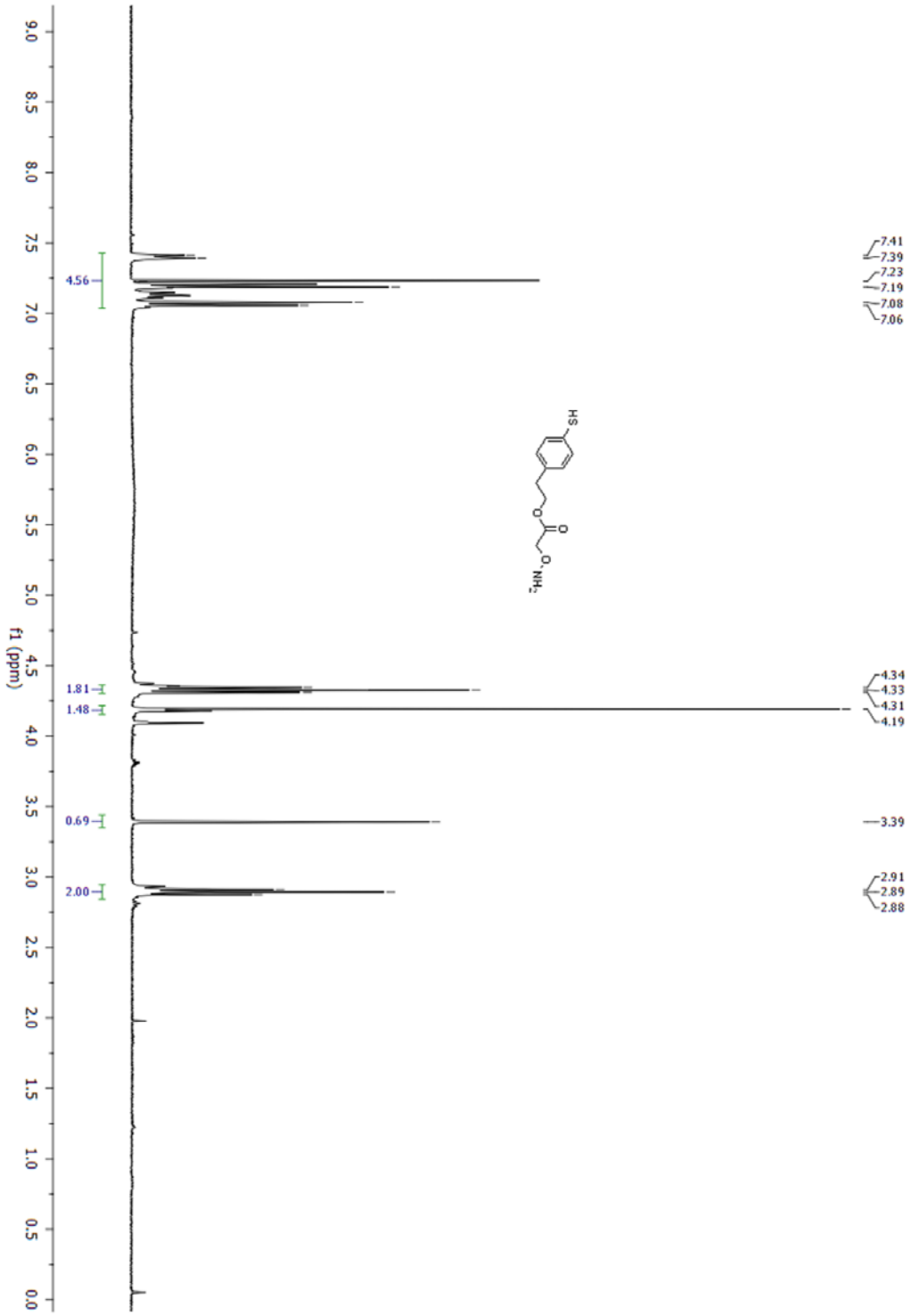
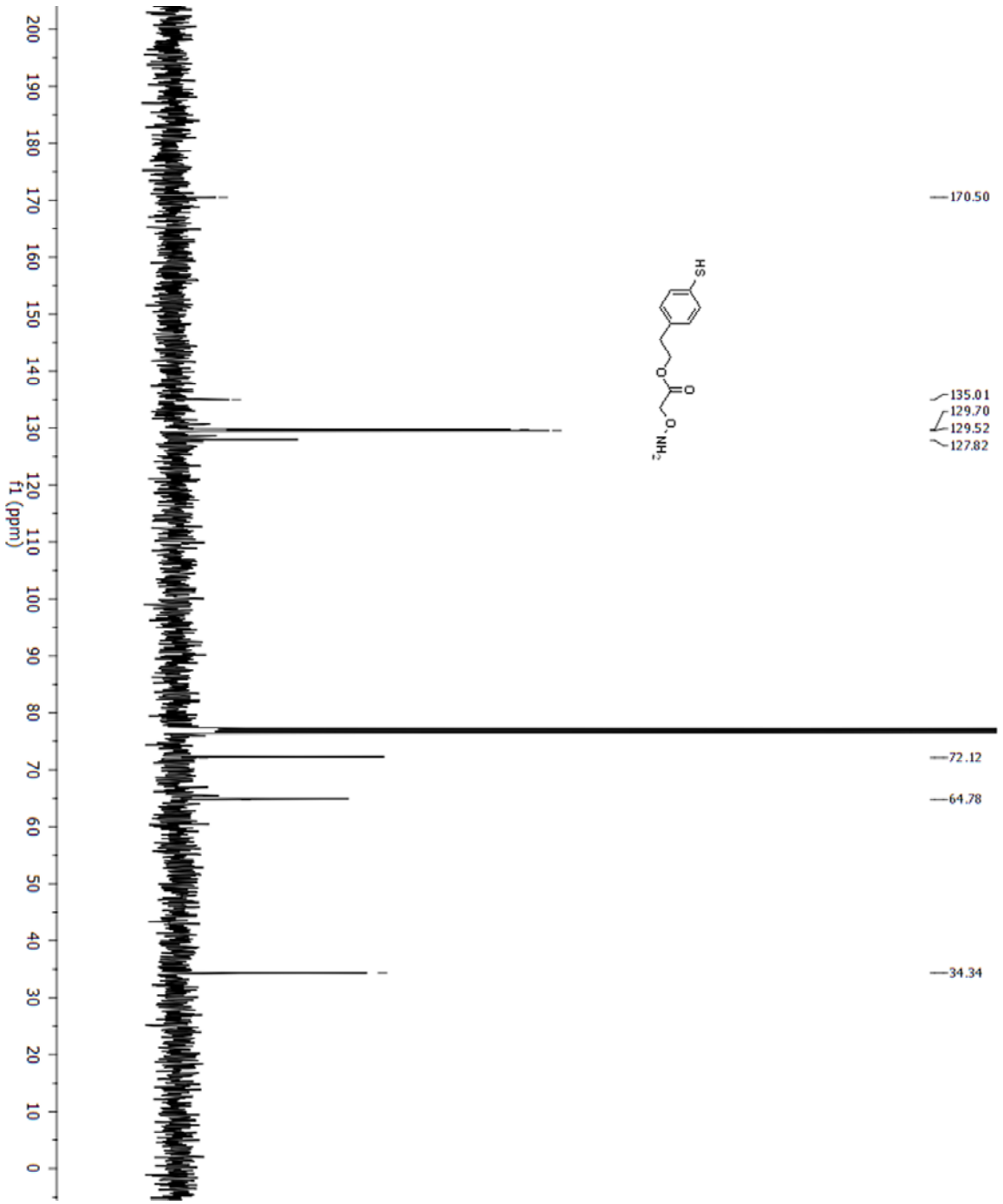
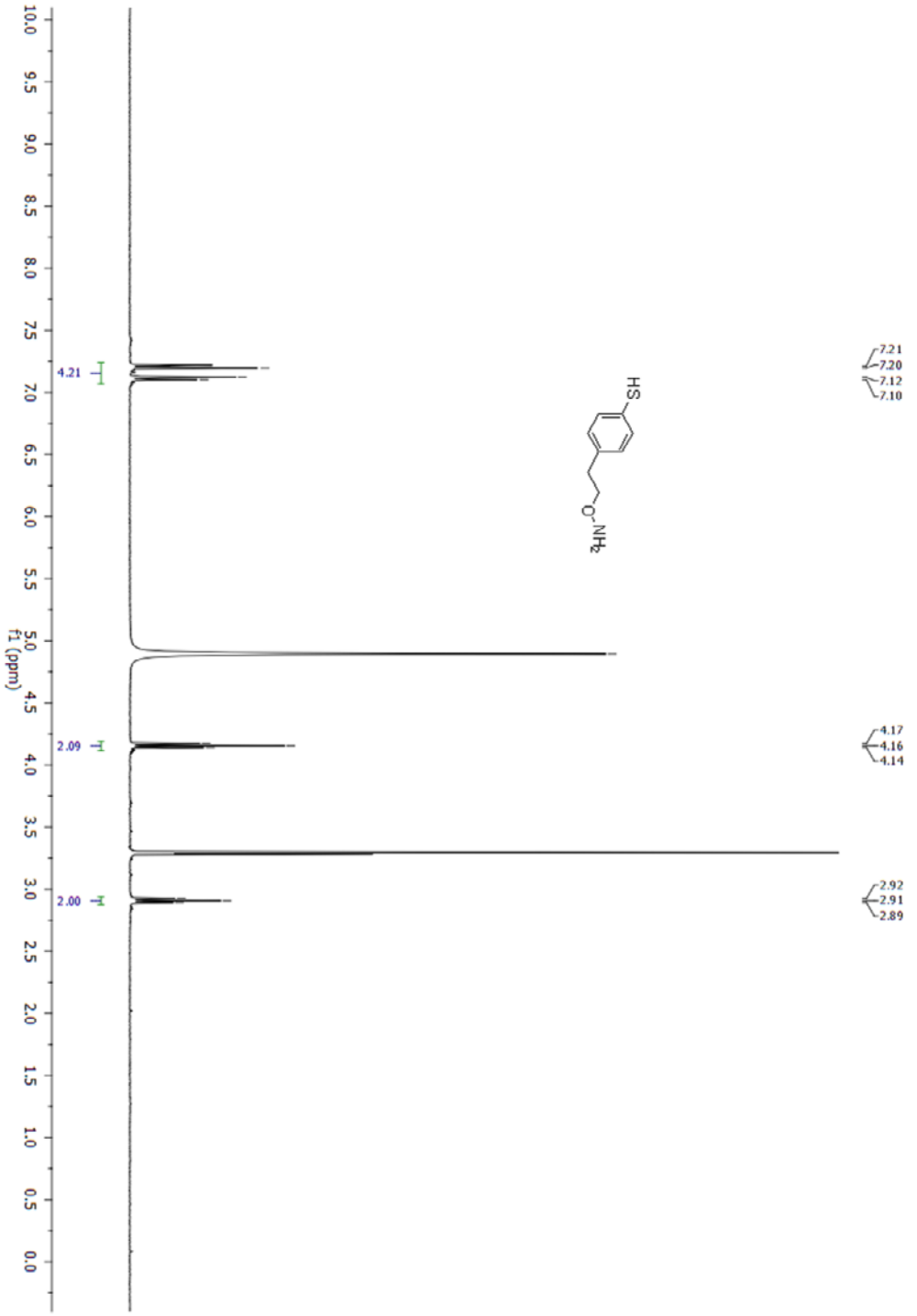
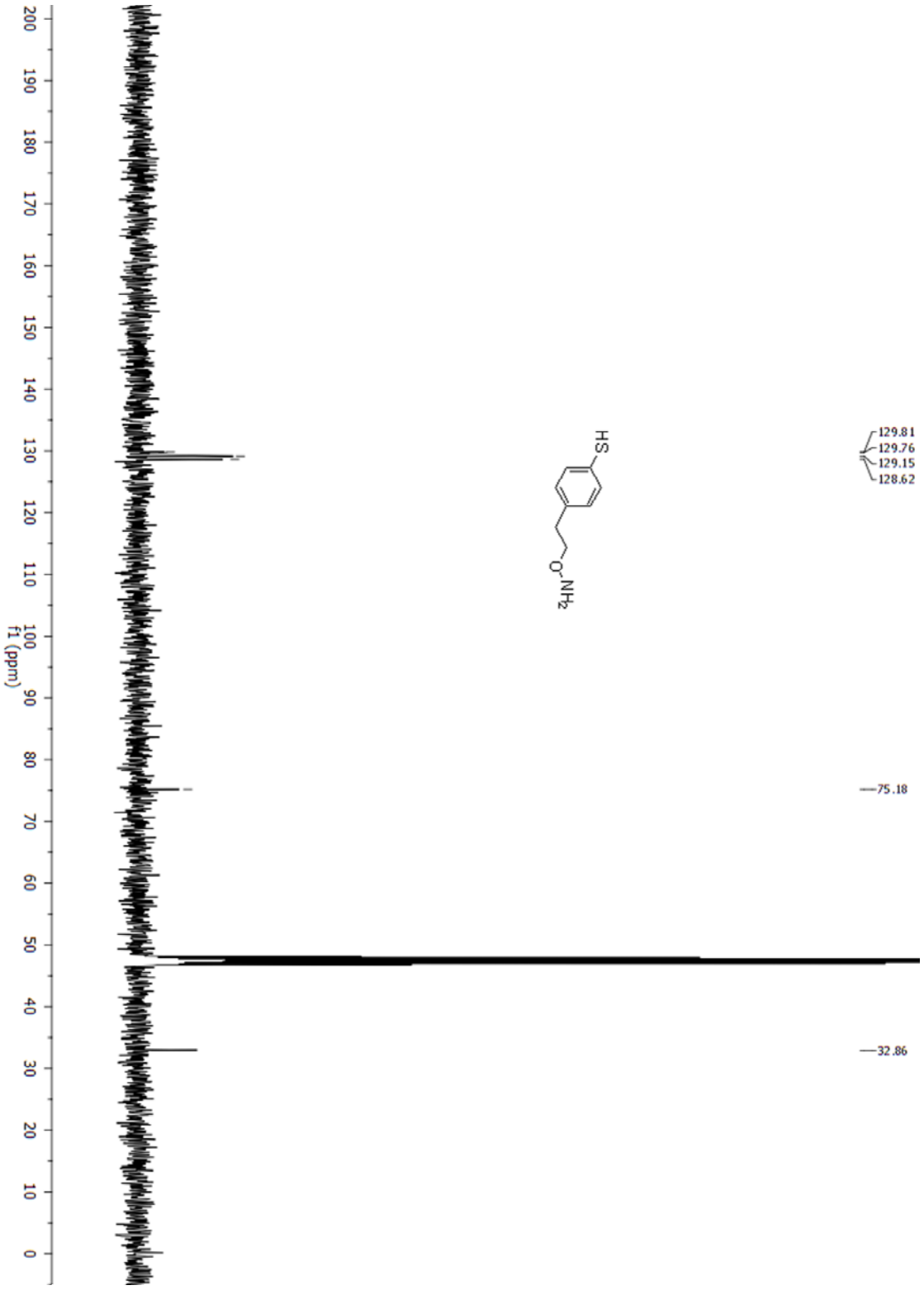
^{1}H and ^{13}C NMR for bifunctional reagent 4





^{1}H and ^{13}C NMR for bifunctional reagent 5




200
190
180
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160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)
HS
O
NH2
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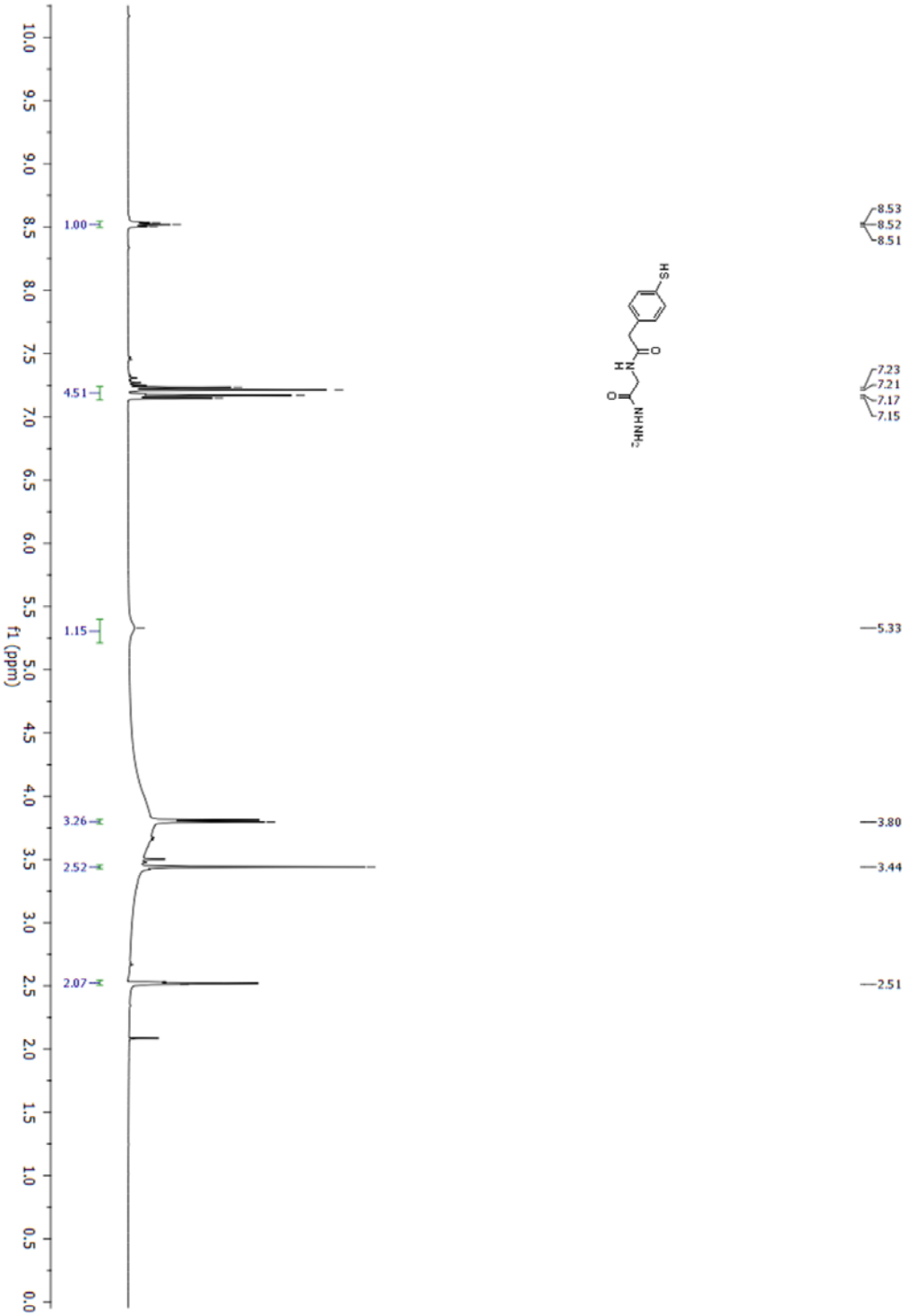
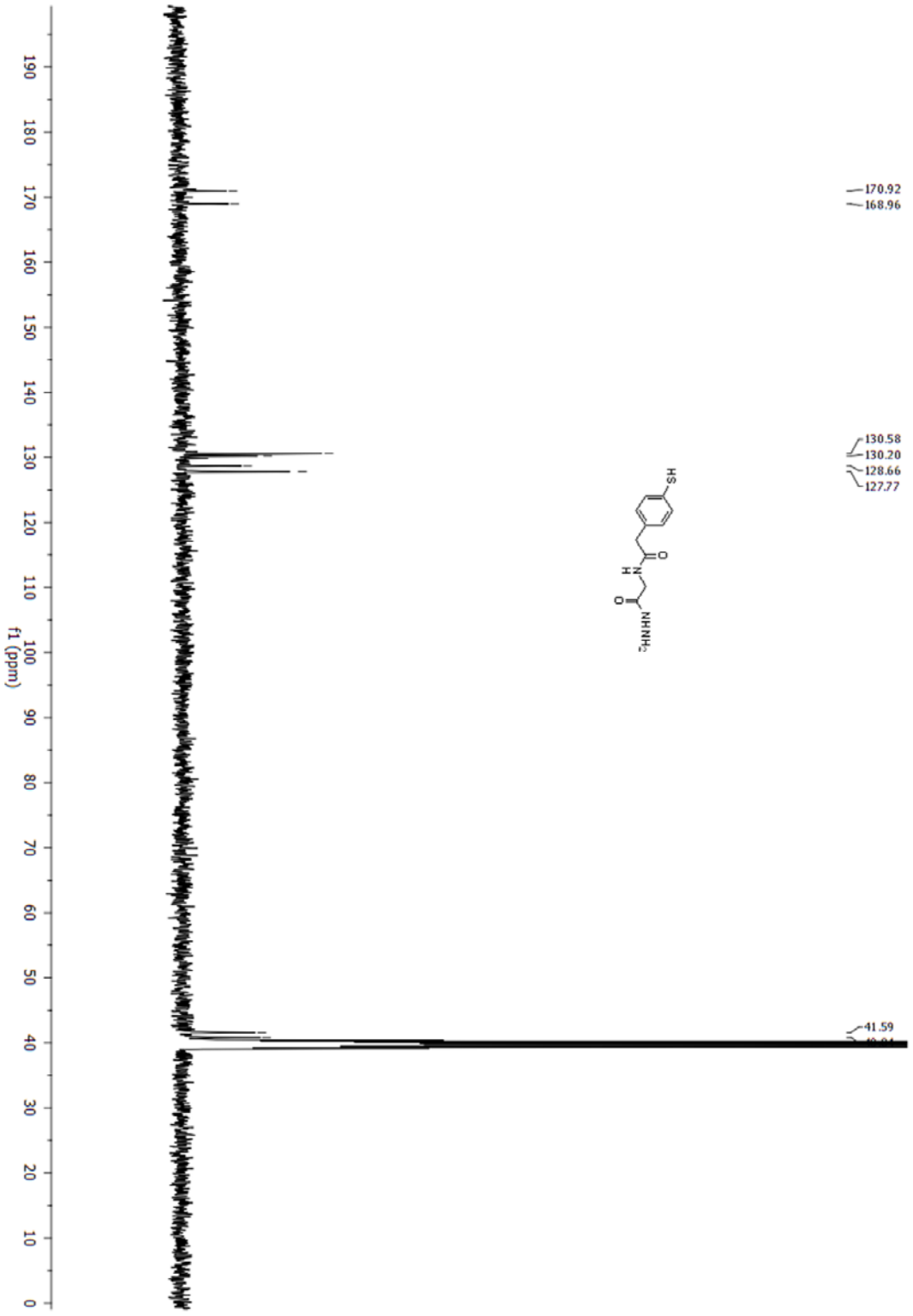
129.76
129.15
128.62
75.18
32.86

^{1}H and ^{13}C NMR for bifunctional reagent 6

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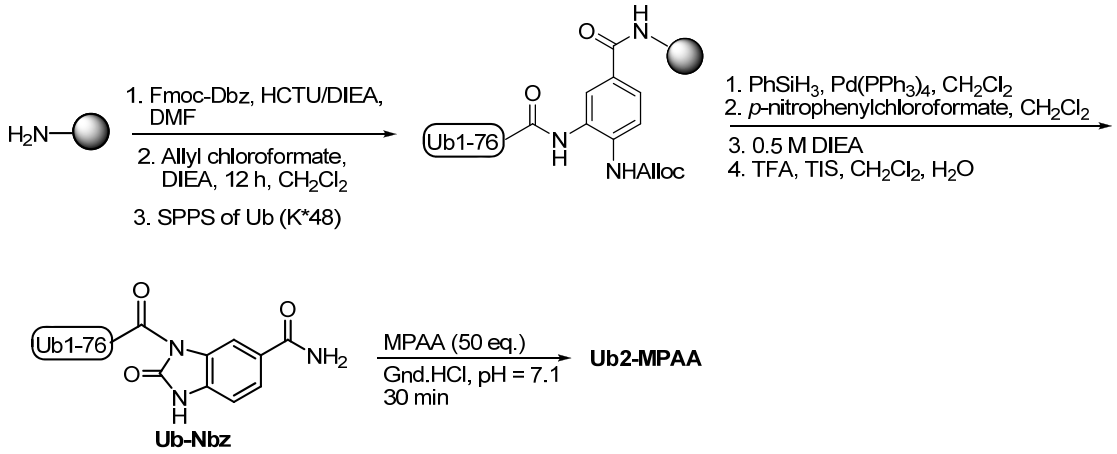
127.77
41.59
SH
O
H
N
O
NHNH2
f1 (ppm)
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0

Synthesis of Ub2-MPAA via Nbz chemistry



Pre-swollen Rink amide resin (loading: 0.44 mmol/g, 0.1 mmol scale) was treated with 20% piperidine in DMF (5-10-5 min) to remove the Fmoc-group. Mono-Fmoc-3,4-diaminobenzoic acid (Fmoc-Dbz, Blanco-Canosa and Dawson, *Angew. Chem. Int. Ed.* **2008**, *47*, 6851) was coupled to the resin employing HCTU/DIEA/DMF (1 h x 2). The free amine group on Dbz was protected with Alloc group by treating the resin with allyloxychloroformate (Alloc-Cl, 100 equivalent) and DIEA (1 equivalent corresponding to resin loading) in DCM overnight (Mahto et al., *ChemBioChem* **2011**, *12*, 2488). This was followed with Fmoc-SPPS to complete the Ub sequence. After completion, the alloc group was removed by reacting the peptide-resin with $Pd(PPh_3)_4$ (0.35 equivalent), and phenylsilane (20 equivalent) in DCM for 1 h. The resin after washing with DCM and DMF (2 x 5 min) was the treated with *p*-nitrochloroformate (5 equivalent), DIEA (0.5 M in DMF) to achieve cyclization of Nbz group followed by peptide cleavage and lyophilization. The crude peptide (50 mg) was dissolved in 500 µL of 6 M guanidine buffer (pH 7.1) containing MPAA (47 mg, ~50 eq. corresponding to crude weight) for 30 min to afford Ub-MPAA which was purified by preparative HPLC and characterized (yield: 4.1 mg, 8.2%).

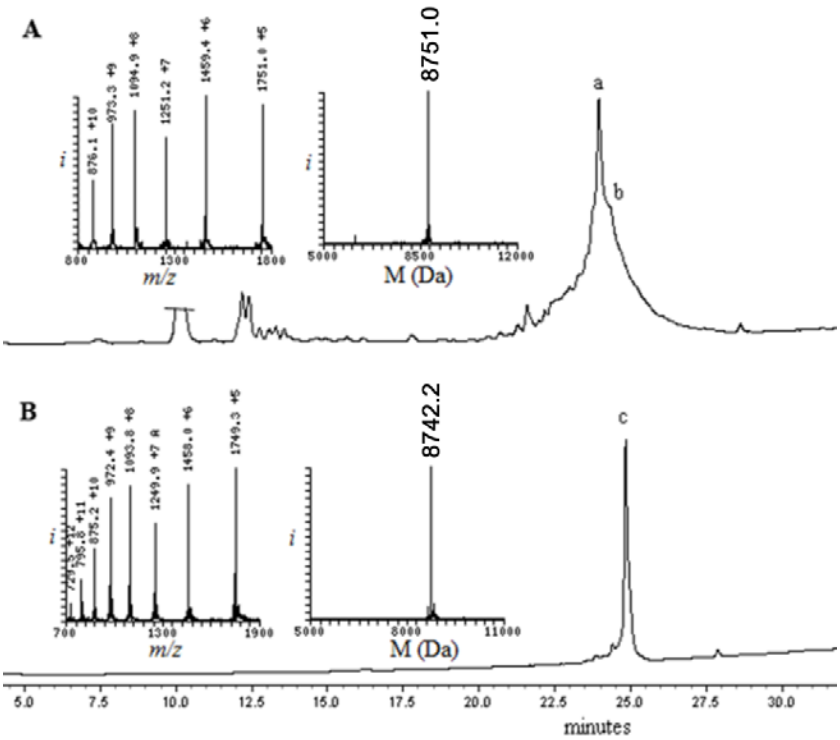


Figure S11: A. Analytical HPLC trace for crude Ub2-Nbz; peak a corresponds to Ub2-Nbz with a mass 8751.0 Da (calc 8750.6 Da); peak b corresponds to corresponding piperidine adduct (+67). B. Analytical HPLC trace for Ub2-MPAA after purification; peak c corresponds to the desired peptide-thioester with a mass 8742.2 Da (calc 8742.6 Da).