Electronic Supplementary Material (ESI) for ChemComm.

Electronic Supplementary Information

Native Chemical Ubiquitination Using a Genetically Incorporated Azidonorleucine

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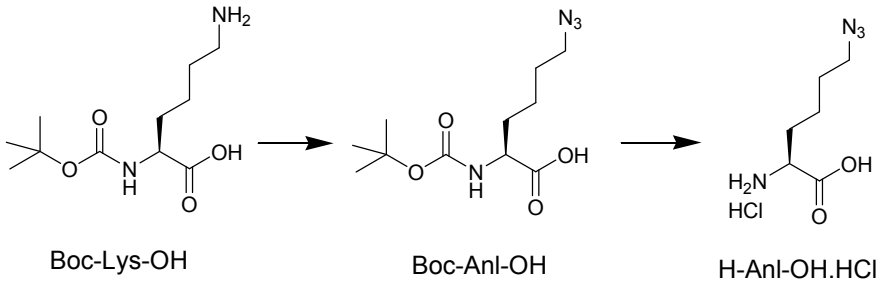
General methods.

Chemical reagents were purchased from Alfa Aesar, Sigma-Aldrich, Merck, Fisher Scientific, and Acros Organics. Analytical HPLC analyses were performed using a Shimadzu HPLC system equipped with a Jupiter C18 (5 µm, 4.6 x 250 mm) or a Vydac C8 (5 µm, 4.6 x 250 mm) reverse-phase column with a flow rate of 1.0 mL/min. Semi-preparative HPLC were performed using a semi-preparative HPLC column (Jupiter C18, 5 µm, 10 x 250 mm) on a Shimadzu system with a flow rate of 2.5 mL/min. Preparative HPLC analyses were performed on Waters 600 systems equipped with a Vydac C4 column (10 µm, 22 x 250 mm) with a flow rate of 10 mL/min. Detection was done with a UV-VIS-detector at 220 nm. The buffer system for all the analyses was buffer A – H_2O (containing 0.045% TFA) and buffer B – 90% acetonitrile (ACN) in H_2O (containing 0.045% TFA). Peptide and protein masses were measured using a Thermo FINNIGAN LCQ Deca XP MAX equipped with ESI ion source. The raw spectra were deconvoluted with MagTran software. NMR studies were performed on a 400 mHz Bruce NMR equipment with $CDCl_3$ as the solvent.

Chemical synthesis:

Synthesis of Azidonorleucine (Anl):



Nα-Boc-(L)-Lys-OH was transferred into the corresponding ε-azide following the diazotransfer method. The synthesis procedures were following Holland-Nell and Meldal's approach[1] with minor revisions. Briefly summarized as the following:

Triflic azide preparation: sodium azide (55 mmol) was dissolved in H_2O (15 mL). Dichloromethane (DCM, 10 mL) was added to the solution and cooled on ice bath. Trifluoromethanesulfonic anhydride (1.86 mL) was added slowly while stirring. After 2 h, after phase separation, the aqueous phase was extracted with DCM (7.5 mL x 2). The combined organic phase was washed with saturated Na_2CO_3. The triflic azide prepared was used directly for next step reaction without further purification.

Diazotransfer to the amino acid: Boc-(L)-Lys-OH (6 mmol, 1.5 g) was suspended in MeOH (60 mL) and H_2O (20 mL). $CuSO_4 \bullet 5H_2O$ (30 mg) was then added. K_2CO_3 (900 mg) was added to the mixture to adjust the pH to about 9.5. The triflic azide solution was added to the mixture while stirring. After overnight reaction at room temperature, DCM (150 mL) was added to enhance phase separation. The organic phase was extracted with H_2O (50 mL x 4). The combined aqueous phase was acidified to pH 2 with hydrochloric acid. The solution was then extracted with DCM (50 mL x 6). The extract was dried over Na_2SO_4 and concentrated under reduced pressure. The resulting azide (1.60 g, 98% yield) was analytically pure and directly used for next step without further purification.

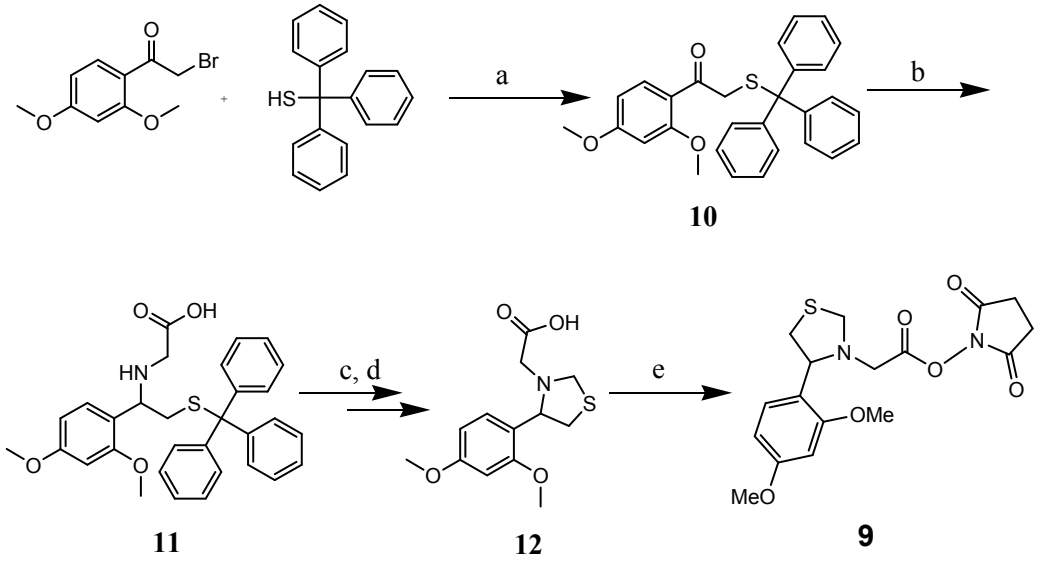
^{1}H NMR (400 MHz, $CDCl_3$): δ = 9.23 (1H, COOH), 6.70-5.14 (m, 1H of CH_2), 4.34-4.15 (m, 1H of CH_2), 3.30 (2H, t, J = 6.6 Hz, CH_2), 1.89 (m, 1H of CH_2), 1.74-1.70 (m, 1H of CH_2), 1.65-1.62 (m, 2H, CH_2), 1.46 (9H, s, CH_3 x 3).

^{13}C NMR (100 MHz, $CDCl_3$): 177.04 (COOH), 155.65 (CO), 80.34 (qC), 53.12 (CH), 51.12 (CH_2), 32.00 (CH_2), 28.40 (CH_2), 28.28 (CH_3 x 3), 22.52 (CH_2).

ESI-MS: Monoisotopic mass calculated 272.15, $[M+H]^+$ observed 273.08.

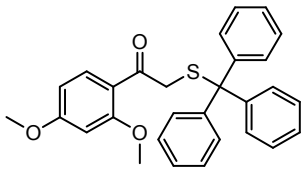
To synthesize Anl, Boc-Anl-OH (1.6 g, 5.88 mmol) was dissolved in 4 N HCl in MeOH (50 mL). After stirring for 4 h at room temperature, TLC analysis showed that the reaction was completed. The reaction mixture was concentrated under reduced pressure. The residues were dissolved in 50% aqueous ACN and lyophilized to get a yellow solid. The product was directly used for genetic incorporation experiment without further purification.

Chemical synthesis of N-(N'-(4-(2,4-dimethoxyphenyl)thiazolidinyl))glycinyl succinimide (9):



Reagents and conditions: Steps a and b were following reference 2 with minor revisions. a: DIEA, DCM, 91.2%; b: $NaCNBH_3$, glycine, MeOH, 78%; c: TFA, TIS, DCM; d: EtOH, H_2O, NaOH, HCHO, 61% (two steps); e: DCC, HOSu, DCM, 73.4%.

2-(S-trityl)mercapto-2',4'-dimethoxyacetophenone (10):



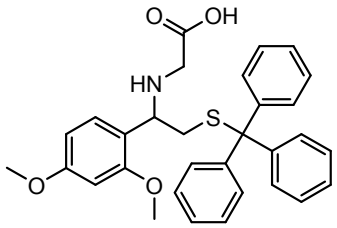
2-bromo-2',4'-dimethoxyacetophenone (1 g, 3.86 mmol) and triphenylmethanethiol (1.28 g, 4.63 mmol) were dissolved in DCM (25 mL). N-ethyldiisopropylamine (DIEA, 808 µL, 4.64 mmol) was added to the solution while stirring. The reaction mixture was stirred at room temperature for 2 h. The reaction mixture was then concentrated under reduced pressure and purified by silica gel column chromatography (hexane:ethyl acetate, 10:1 to 5:1). 1.6 g (3.52 mmol) of compound **10** was isolated with a yield of 91.2%.

^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.78 (1H, d, J = 8.8 Hz, DMB), 7.50-7.48 (6H, m, Trt), 7.33-7.29 (6H, m, Trt), 7.25-7.22 (3H, m, Trt), 6.52 (1H, dd, J1 = 8.3 Hz, J2 = 2 Hz, DMB), 6.36 (1H, d, J1 = 1.6 Hz, DMB), 3.84 (3H, s, OMe), 3.68 (2H, s, CH_2), 3.67 (3H, s, OMe).

^{13}C NMR (100 MHz, $CDCl_3$): 194.53, 164.80, 160.75, 144.82, (qC x 4), 133.17, 129.68, 127.93, 126.62, (CH x 16), 119.60 (qC), 105.36, 98.12, (CH x 2), 66.08 (qC), 55.57, 55.33, (CH_3 x 2), 45.40 (CH_2).

ESI-MS: Monoisotopic mass calculated 454.16, $[M+Na]^+$ calculated 477.15, observed 477.20.

N-(1-(2,4-dimethoxyphenyl)-2-S-tritylmercapto)ethylglycine (11):



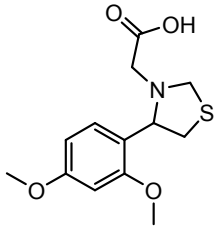
Compound **10** (0.9 g, 2.0 mmol) was suspended in MeOH (15 mL). $NaCNBH_3$ (90 mg, 1.43 mmol) and glycine (1.5g, 20 mmol) were added to the reaction mixture. After reflux for 24 h, another 90 mg of $NaCNBH_3$ and 1.5 g of glycine were added. The reaction was continued under reflux condition for another 48 h. The reaction mixture was then concentrated and purified by silica gel column chromatography (ethyl acetate to ethyl acetate:MeOH 5:2). 0.8 g (1.56 mmol) of compound **11** was isolated (yield 78%).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.41 (1H, d, J = 8.0 Hz, DMB), 7.34-7.27 (6H, m, Trt), 7.24-7.10 (9H, m, Trt), 6.47-6.37 (2H, m, DMB), 3.89-3.82 (1H, m, benzylic CH), 3.78 (3H, s, OMe), 3.64 (3H, s, OMe), 2.94-2.70 (4H, m, CH_2 x 2).

^{13}C NMR (100 MHz, $CDCl_3$): 171.18, 161.07, 158.58, 144.68, (qC x 6), 129.65, 127.90, 126.59, (CH_{Ar} x 16), 116.32 (qC), 104.64, 98.70, (CH_{Ar} x 2), 66.89 (qC), 60.42 (CH), 55.40, 55.34, (CH_3 x 2), 48.90, 34.38 (CH_2 x 2).

ESI-MS: Monoisotopic mass calculated 513.20, $[M+H]^+$ observed 514.14.

N-(4-(2,4-dimethoxyphenyl)thiazolidinyl)glycine (12):



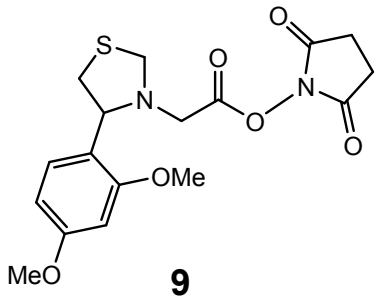
0.54 g (1.05 mmol) of compound **11** was dissolved in DCM (15 mL). 1.5 mL of trifluoroacetic acid (TFA) was added. A bright yellow color was developed. After 5 min, 1.5 mL of triisopropylsilane (TIS) was added to the mixture. After 20 min, the reaction mixture was concentrated under reduced pressure. The residue was dissolved in EtOH/H_2O (3:1, 15 mL). After adjusting pH to 8 with 1N NaOH, 83 µL of 37% aqueous formaldehyde was added. After 1.5 h, the mixture was concentrated under reduced pressure and purified by silica gel column chromatography (ethyl acetate/hexane/acetic acid, 100:20:1). 0.18 g of compound **12** (0.64 mmol) was obtained (yield 61%).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 9.70 (1H, COOH), 7.31 (1H, d, J = 8.8 Hz, DMB), 6.46-6.44 (2H, m, DMB), 4.56 (1H, t, J = 6.2, benzylic CH), 4.14 (1H, d, J = 8.8, 1H of CH_2), 3.96 (1H, d, J = 9.2, 1H of CH_2), 3.80 (3H, s, OMe), 3.78 (3H, s, OMe), 3.26 (2H, d, J = 8.4, CH_2), 3.21-3.11 (2H, m, CH_2).

^{13}C NMR (100 MHz, $CDCl_3$): 172.71 (COOH), 161.01, 158.31, 128.34, 117.87, 104.52, 98.84 (C_{Ar} x 6), 65.63 (CH), 57.53 (CH_2), 55.53, 55.39, (CH_3 x 2), 55.12 (CH_2), 33.51 (CH_2).

ESI-MS: Monoisotopic mass calculated 283.09, $[M+H]^+$ observed 284.16.

N-(N'-(4-(2,4-dimethoxyphenyl)thiazolidinyl))glycinyl succinimide (9):



9

0.18 g (0.64 mmol) of compound **12** (0.64 mmol) was dissolved in DCM (5 mL). N,N'-dicyclohexylcarbodiimide (DCC, 144 mg, 0.7 mmol) and N-hydroxysuccinimide (HOSu, 80 mg, 0.7 mmol) were added to the reaction mixture. The reaction was completed after stirring at room temperature for 1.5 h. The reaction mixture was filtered and the flow through was concentrated and purified by silica gel column chromatography (ethyl acetate/hexane, 1:1 to 2:1). 0.18 g (0.47 mmol) of compound **9** was obtained (yield 73.4%).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.39 (1H, d, J = 8.4 Hz, DMB), 6.51-6.47 (2H, m, DMB), 4.54 (1H, t, J = 7, benzylic CH), 4.31 (1H, d, J = 8, 1H of CH_2), 4.00 (1H, d, J = 8.4, 1H of

CH_2), 3.81 (6H, s, OMe x 2), 3.74 (1H, d, J = 17.2, 1H of CH_2), 3.57 (1H, d, J = 17.6, 1H of CH_2), 3.26 (1H, t, J = 8.6, 1H of CH_2), 2.96 (1H, t, J = 9, 1H of CH_2), 2.34 (4H, br, CH_2 x 2).

^{13}C NMR (100 MHz, $CDCl_3$): 168.93 (COON), 165.88 (CON x 2), 160.58, 158.59, 127.94, 119.42, 104.64, 98.72 (C_{Ar} x 6), 64.03 (benzylic CH), 56.51 (CH_2), 55.51, 55.38, (CH_3 x 2), 51.45 (CH_2), 36.01 (CH_2), 25.58 (CH_2 x 2).

ESI-MS: Monoisotopic mass calculated 380.10, $[M+H]^+$ observed 381.04.

Construction of UbK48Anl (ub 1) expression vector:

All PCR reactions were carried out using KAPA HiFi *pfu* Polymerase (KAPA). All restriction enzymes were purchased from New England Biolabs (Beverly, MA). All oligonucleotide PCR primers were from Integrated DNA Technologies. The 2.5 kb methionyl-tRNA synthetase (MetRS) expression cassette (GenBank accession number X55791) was amplified from *E. coli* DH5α by primer pRep4-EZ-MetRS-AvaI-F and pRep4-EZ-MetRS-Bsu36I-R. This fragment was then ligated into linearized pRep4 (Qiagen) digested by AvaI and Bsu36I to generate pRep4-MetRS. The plasmid pRep4-MetRS-L13A encoding the L13A mutant of MetRS was generated using QuickChange site-directed mutagenesis with primers MetRS-L13A-F and MetRS-L13A-R. Plasmid pQLN was constructed in two steps. In the first step, the fragment coding lacI repressor was obtained from plasmid pQE80L by digestion using NheI and NdeI and then this fragment was ligated with linearized pQE60 digested by the same enzymes. In the second step, the original NcoI site in plasmid pQE80L was destroyed by site-directed mutagenesis method according to the manufacture's recommendation using primers pQE80L-D NcoI F and pQE80L-D NcoI R. The gene coding for MASUb76 was amplified from plasmid pET-Ubi in PCR reaction using primers MASUb76-pQE60-NcoI F and MASUb76-pQE60-HindIII R. This fragment was then ligated into linearized pQLN digested by NcoI and HindIII to generate pQLN-MASUbi. The plasmid pQLN-MASUbiK48M encoding the K48M mutant of MASUbi was generated using QuickChange site-directed mutagenesis with the primers UbiK48M F and UbiK48M R. The integrity of all the constructs was confirmed by DNA sequencing.

Primer sequences used:

pRep4-EZ-MetRS-AvaI-F:

5'-GAGTTCTTCGCCCACCCCGGGCCTGAAGAATATAGAGAAGTAC-3'

pRep4-EZ-MetRS-Bsu36I-R:

5'-CGCGAGGTCGTCCAGCCTCAGGTTGAGAGAGCCCGTTACC-3'.

MetRS-L13A-F:

5'-CTG GTG ACG TGC GCA GCG CCG TAC GCT AAC GGC-3'

MetRS-L13A-R:

5’-GCC GTT AGC GTA CGG CGC TGC GCA CGT CAC CAG-3’

pQE80L-D NcoI F:

5’-CGCCCCCGTTTTCAgCATGGGCAAATATT-3’

pQE80L-D NcoI R:

5’-AATATTTGCCCATGcTGAAAACGGGGGCG-3’

MASUb76-pQE60-NcoI F:

5’-CATGCCATGGCAAGTCAGATCTTCGTCAAGACGT-3’

MASUb76-pQE60-HindIII R:

5’-CCCAAGCTTTCAACCACCTCTTAGTCTTA-3’

UbiK48M F:

5’-GATCTTTGCCGGTATGCAGCTCGAGGACG-3’

UbiK48M R:

5’-CGTCCTCGAGCTGCATACCGGCAAAGATC-3’

Expression and Purification of Ub 1:

The plasmids pRep4-MetRS-L13A and pQLN-MASUbiK48M were transformed into methionine auxotrophic *E. coli* strain CAG18491 (Yale CGSC). The overnight cultures of *E. coli* was grown in 6 L of M9 media supplied with 20 natural amino acids with a concentration of about 40 mg/L each and two antibiotics (ampicillin 100mg/L and kanamycin 25 mg/L) at 37^{o}C. When OD_{600} reached 0.9, the cells were harvested by centrifugation and washed twice with M9 media without amino acids. The cells were then resuspended in 6 L of fresh M9 media supplied with 19 amino acids (40 mg/L) and Anl (1 mM) and the two antibiotics mentioned. The cells were grown at 37^{o}C. After 15 min, isopropyl β-D-1-thiogalactopyranoside (IPTG, 1 mM) was added to the media to induce protein expression. After 4 h, the cells were harvested by centrifugation. The cell pellet was resuspended in 300 mL lysis buffer (50 mM Tris-HCl, pH 7.6). The cells were lyzed through sonication. The supernatant was collected through centrifugation at 23,000 g for 25 min. To precipitate non-ubiquitin proteins, 2.1 mL of 70% perchloric acid was added to the supernatant while stirring.

After 10 min, the precipitate was removed after centrifugation at 23,000 g for 25 min. The supernatant was dialyzed against FPLC buffer A (50 mM ammonium acetate, pH 4.5) for 6 h. After buffer exchange, the supernatant was subjected to FPLC purification with HiTrap SP FF 5 mL column (GE healthcare) with a gradient of 0-100% buffer B (50 mM ammonium acetate, 0.5 M NaCl, pH 4.5) in 100 min. Ubiquitin was usually eluted at 0.2-0.3 M NaCl. The FPLC fractions were checked with MALDI-TOF after desalting with C4 ziptip (Merck Millipore). The fractions containing desired protein were combined and purified with C4-preparative HPLC with a gradient of 10-70% of HPLC buffer B in buffer A for 60 min. HPLC fractions were analyzed by ESI-MS. Fractions containing pure ub **1** were combined and lyophilized. About 60 mg of ub **1** was obtained from 6 L cell culture.

Construction of Ub(1-75)-MES expression vector:

The human ubiquitin gene was amplified by PCR using the following primers 5'-GGTCATATGCAGATCTTTGTGAAGAC-3' (forward) and 5'-GTTGCTCTTCCGCAACCTCGCAGGCGCAACACC-3' (reverse). The PCR product was purified and ligated into the T-easy vector (Promega). After digested with NdeI and SapI, the product was purified and ligated into the identically digested pTYB1 vector (New England Biolabs). Then the correct insert was confirmed by sequencing.

Expression and Purification of Ub(1-75)-MES:

The expression vector for Ub(1-75)-MES was transformed into *E. coli* BL21 (DE3) cells. The overnight culture of cells was grown in 3 L of LB media containing ampicillin (100 mg/L) at 37°C. When OD_{600} reached 0.8, the cells were induced with IPTG (0.05 mM) for 16 h at 16°C. The cells were then harvested through centrifugation at 6,500 g. The cells were then resuspended in 150 mL lysis buffer (20 mM HEPES, 50 mM sodium acetate, 75 mM NaCl, pH 6.5). The cells were lyzed by sonication. The cell lysate was centrifuged at 23,000 g for 25 min. For the binding of the ubiquitin-intein fusion protein to the chitin beads, the supernatant were mixed with 10 ml of chitin beads (New England Biolabs) which had been pre-equilibrated with lysis buffer. The mixture was stirred at 4°C for 2 h. The beads was then collected by filtration through a slica pad and washed extensively with lysis buffer. The thiolysis of the ubiquitin-intein fusion protein was performed by mixing the beads with 20 mL of lysis buffer containing 0.2 M MESNa (pH 7.3). After overnight cleavage at 37°C, the cleavage solution was subjected to C4-preparative HPLC purification. The fractions were

analyzed by ESI-MS. The desired product was lyophilized. The yield of ub(1-75)-MES was about 5 mg/L.

Synthesis of Ub 5 from Ub 1

8.8 mg of ub **1** was dissolved in 300 μL of dimethyl sulfoxide (DMSO). 3.6 μL of Boc anhydride was added. 1.6 μL of N-ethyldiisopropylamine (DIEA) was added. The reaction mixture was kept at room temperature for 1 h and diethyl ether was added to precipitate out the Boc-protected form ub **2**. The ether-precipitate was next dissolved in 300 μL DMSO followed by addition of 30 μL of 1 M aqueous TCEP. After 3 h reduction reaction at room temperature, ether was added to the reaction mixture to precipitate the reduced product ub **3**. The precipitated ub **3** was then dissolved in 330 μL DMSO, and 2.6 mg of compound **9** and 6 μL of DIEA were added. After 45 min, the crude product ub **4** was obtained by ether precipitation. Finally, the crude protein **4** was treated with 200 μL of TFA/TIS/H_2O (95/2.5/2.5) for 20 min for global Boc deprotection. After ether precipitation, the crude deprotection product was purified by reverse-phase HPLC to give 4.9 mg of **5** (56% overall yield starting from ub **1**).

Deubiquitinase assay:

To refold the chemically synthesized diubiquitin **8**, 2.5 mg of diubiquitin **8** was dissolved in 3 mL of unfolding buffer (6 M Gdn•HCl, 100 mM Na_2HPO_4, 500 mM NaCl, pH 7.4). The solution was dialyzed over night against refolding buffer (20 mM Na_2HPO_4, 100 mM NaCl, pH 7.4). The final concentration of **8** was determined to be 500 μg/mL.

For the deubiquitinase assay catalyzed by isopeptidase T (IsoT) (BostonBiochem, MA), 250 μg of refolded **8** (500 μL) was added to 100 μL 10X DUB buffer (500 mM Tris-HCl, 500 mM NaCl, 50 mM DTT, pH 7.5) and topped up to 670 μL with H_2O. 4 μg of IsoT was added to 330 μL of DUB activation buffer (25 mM Tris-HCl, 150 mM NaCl, 10 mM DTT, pH 7.5). After pre-activation for 10 min at room temperature, the enzyme solution was added to the diubiquitin solution. The hydrolysis assay was performed at 37°C. A small aliquot was taken and mixed with 5 μL 6X SDS page loading dye at 0, 2.5, 10, 30, 60 and 90 min, respectively. The samples were analyzed by 5-15% SDS-PAGE. At 120 min, the reaction mixture was analyzed by C8 analytical HPLC.

For the deubiquitinase assay catalyzed by $A20_{CD}$ (BostonBiochem, MA), 62.5 μg of refolded **8** (125 μL) was added to 25 μL 10X DUB and topped up to 168 μL with H_2O. 7 μg of $A20_{CD}$

was added to 82 μL of DUB activation buffer. After pre-activation for 10 min at room temperature, the enzyme was added to the diubiquitin solution. The hydrolysis assay was performed at 37°C. A small aliquot was taken and mixed with 5 μL 6X SDS page loading dye at 0, 2.5, 10, 30, 60 and 90 min, respectively. The samples were analyzed by 5-15% SDS-PAGE. At 120 min and 240 min, the reaction mixture was analyzed by C8 analytical HPLC.

Western blot:

About 4 μg of ub **1** and 4 μg of diubiquitin **8** were analyzed by 5-15% SDS-PAGE. The proteins on the gel were then electrotransferred to PVDF (polyvinylidene difluoride) membrane. The membrane was blocked by 20 mL of 5% w/v non-fat milk powder in TBS buffer (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) containing 0.1% Tween-20 for 1 h at room temperature. A 1:6700 dilution of ubiquitin mAb P4D1 (HRP conjugated) (Enzo Life Sciences) was added to the blocking mixture and incubated for 2 h at room temperature. The mono- and di- ubiquitin were visualized by chemilluminescence (SuperSignal West Dura Trial Kit, Pierce, USA).

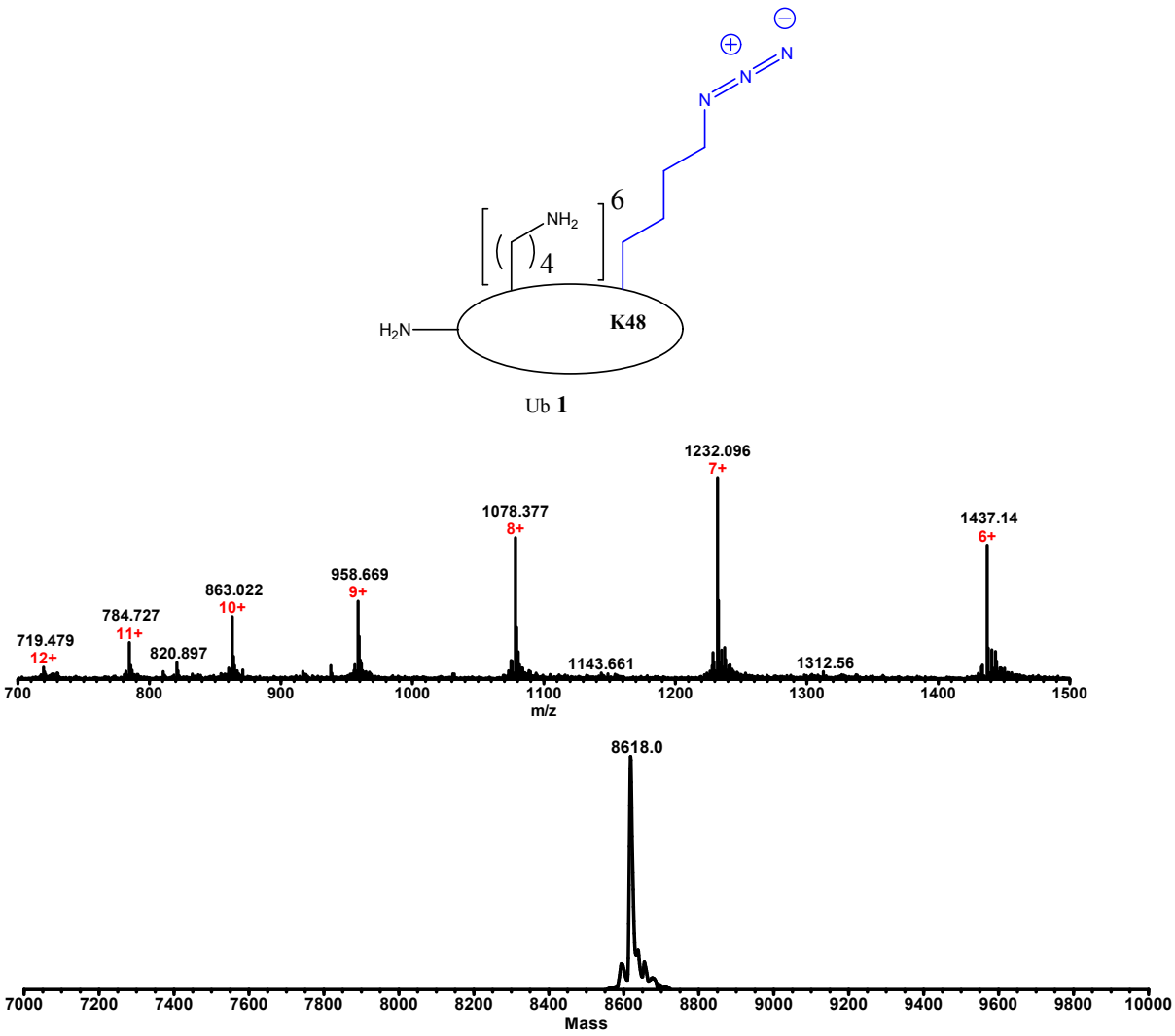


Figure S1. Raw and deconvoluted ESI-MS data of purified ub **1** (Average molecular mass calculated 8617.70; found 8618.0.

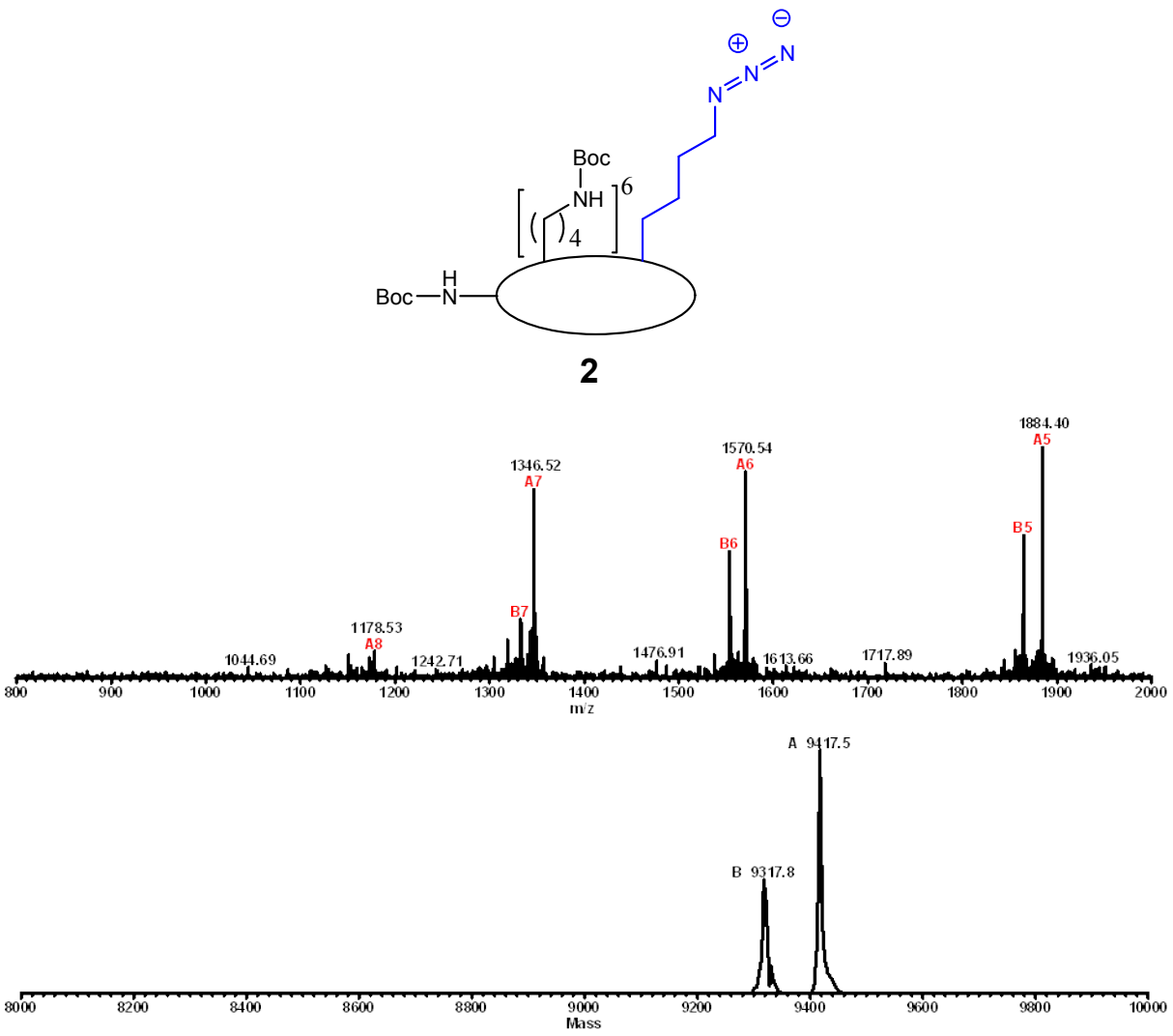


Figure S2. Raw and deconvoluted ESI-MS data of crude ub **2**. (ub**1**Boc$_8$: average mass calculated 9418.62, found 9417.5; ub**1**Boc$_7$: average mass calculated 9318.50, found 9317.8).

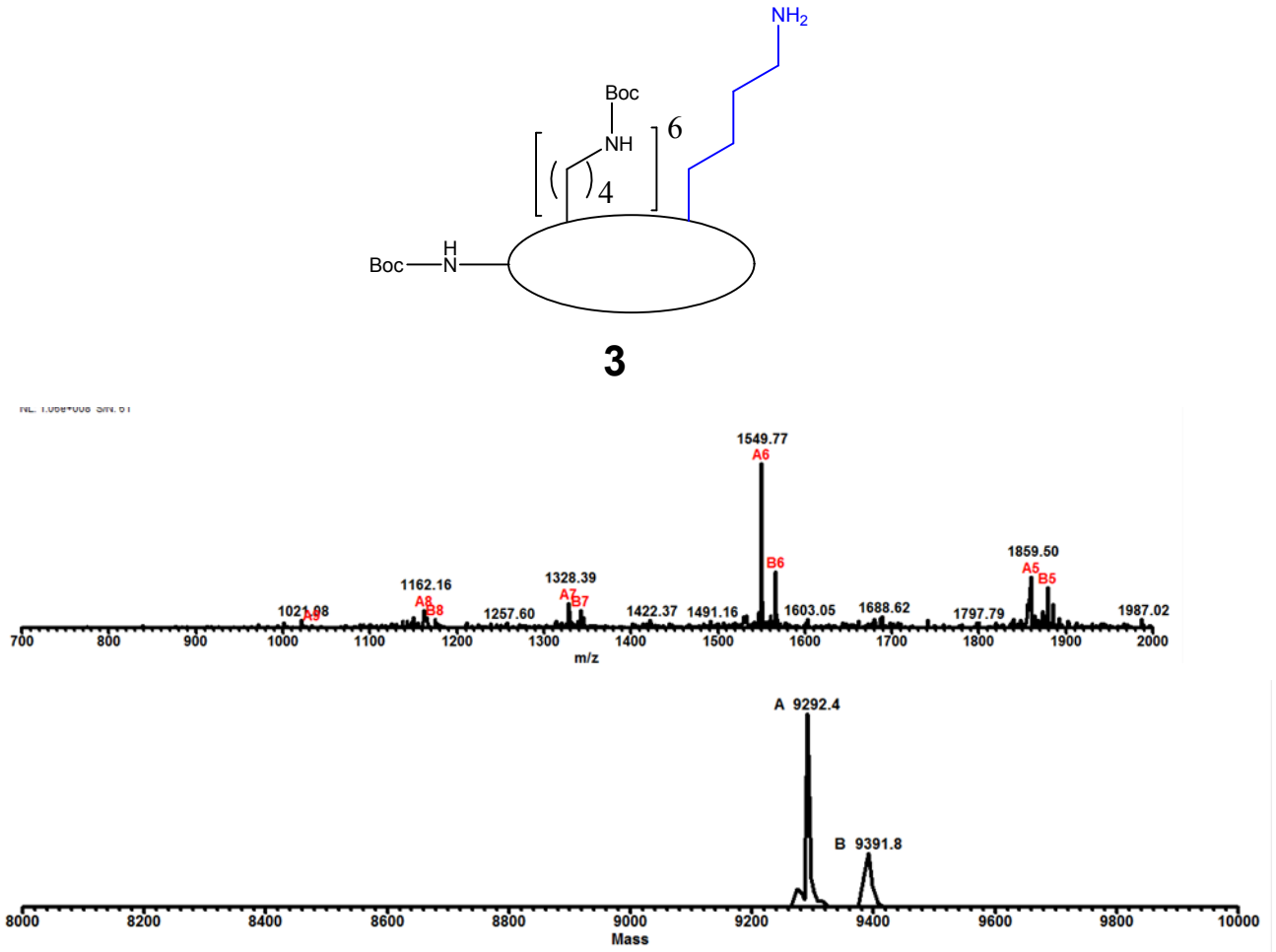


Figure S3. Raw and deconvoluted ESI-MS data of crude ub **3**. (ubK48Boc$_8$: average mass calculated 9392.63, found 9391.8; ubK48Boc$_7$: average mass calculated 9292.51, found 9292.4).

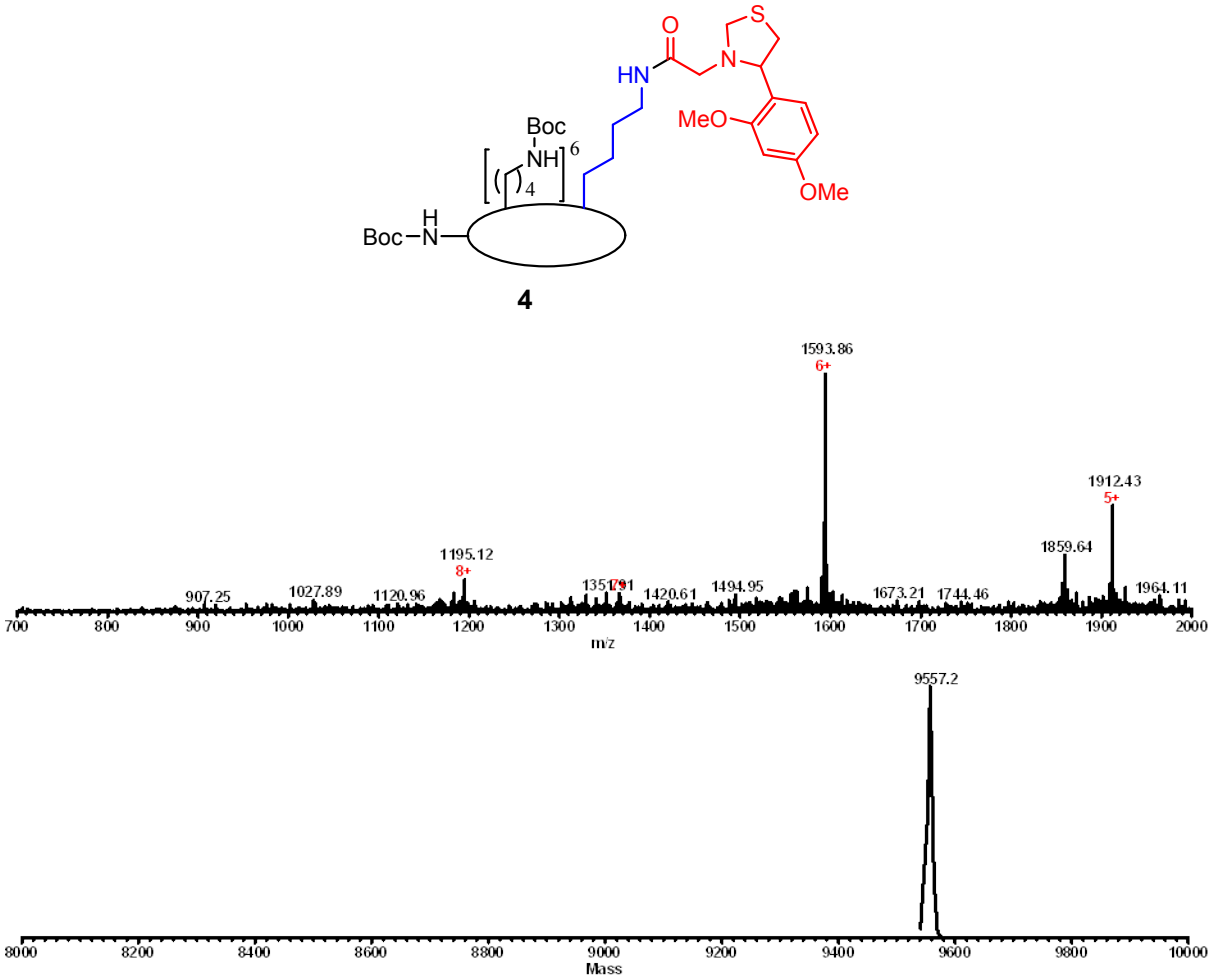


Figure S4. Raw and deconvoluted ESI-MS data of crude ub **4**. (Average mass calculated 9557.84; found 9557.2).

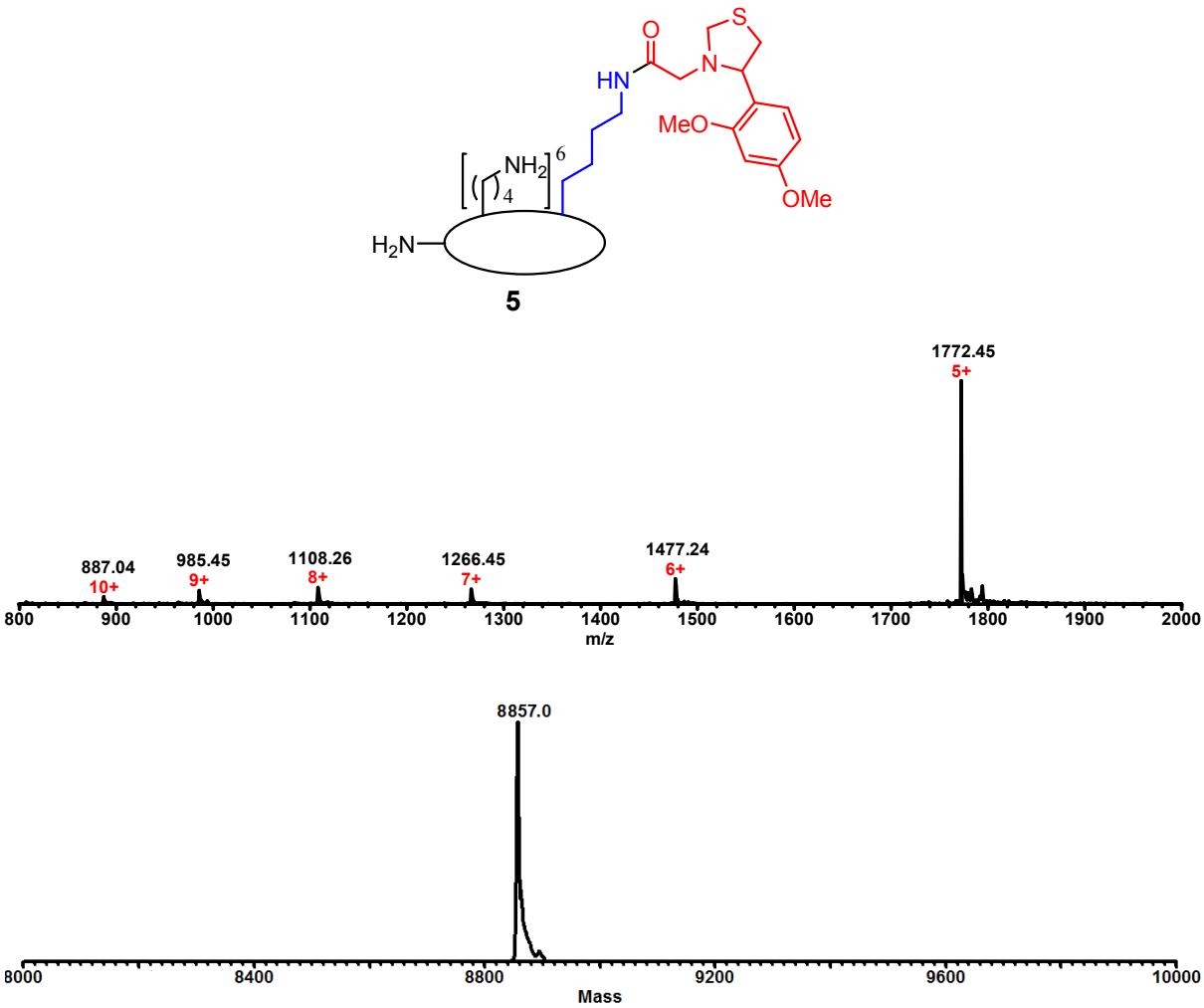


Figure S5. Raw and deconvoluted ESI-MS data of purified ub **5**. (Mass calculated 8857.03; found 8857.0).

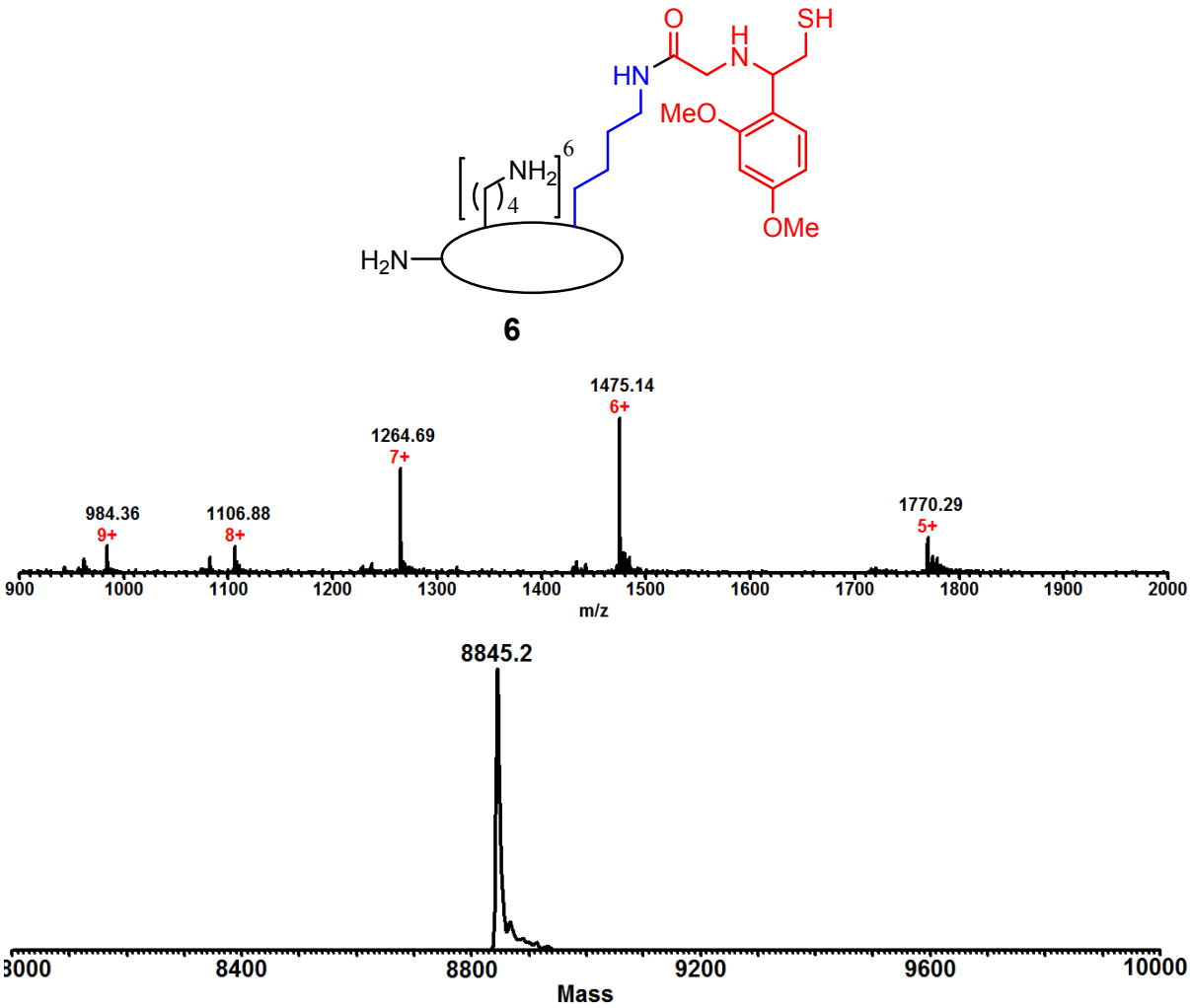


Figure S6. Raw and deconvoluted ESI-MS data of purified ub **6**. (Average mass calculated 8845.02; found 8845.2).

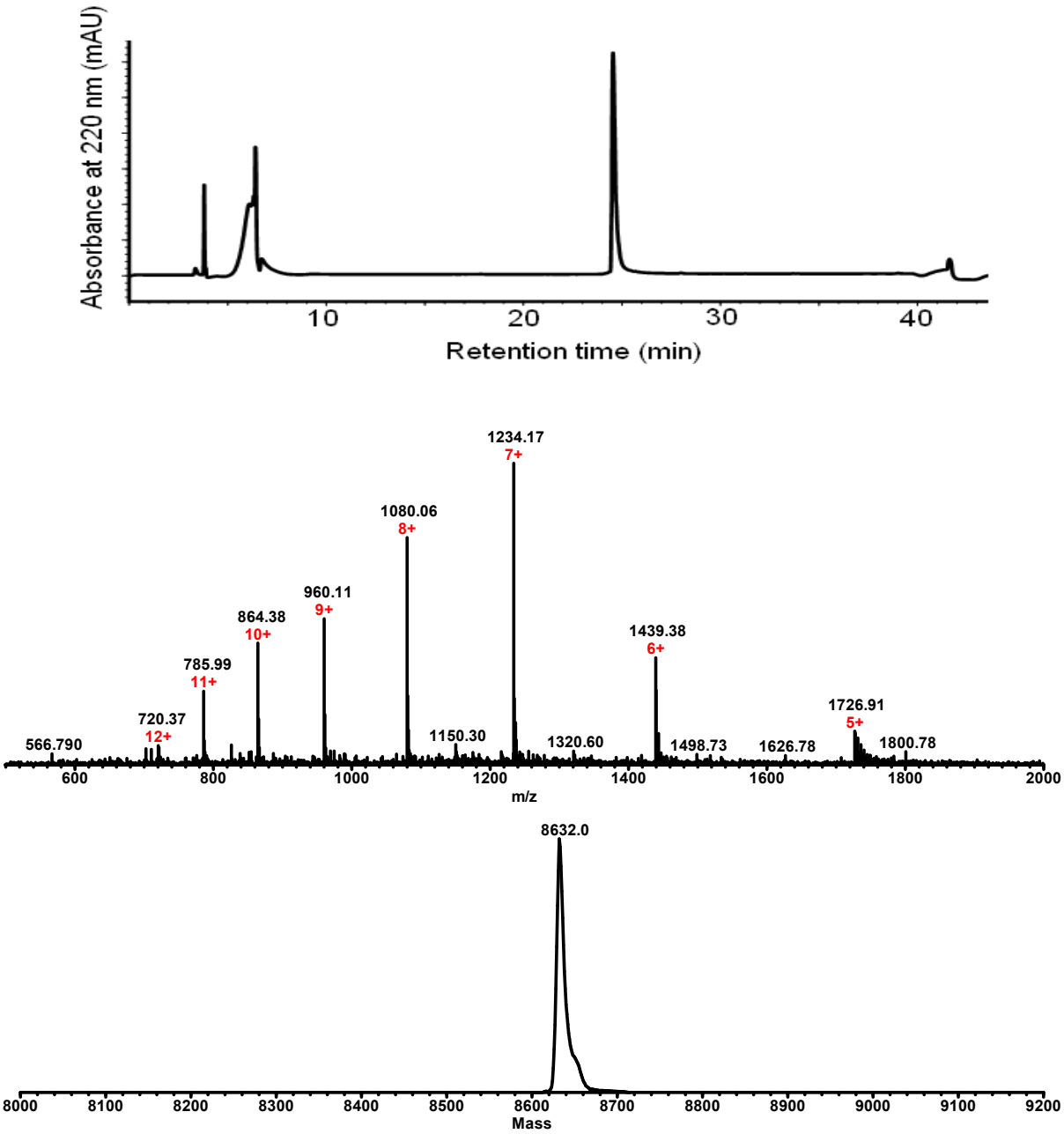


Figure S7. C18 analytical HPLC, raw and deconvoluted ESI-MS data of ub(1-75)-MES. Average mass calculated 8630.87, found 8632.0.

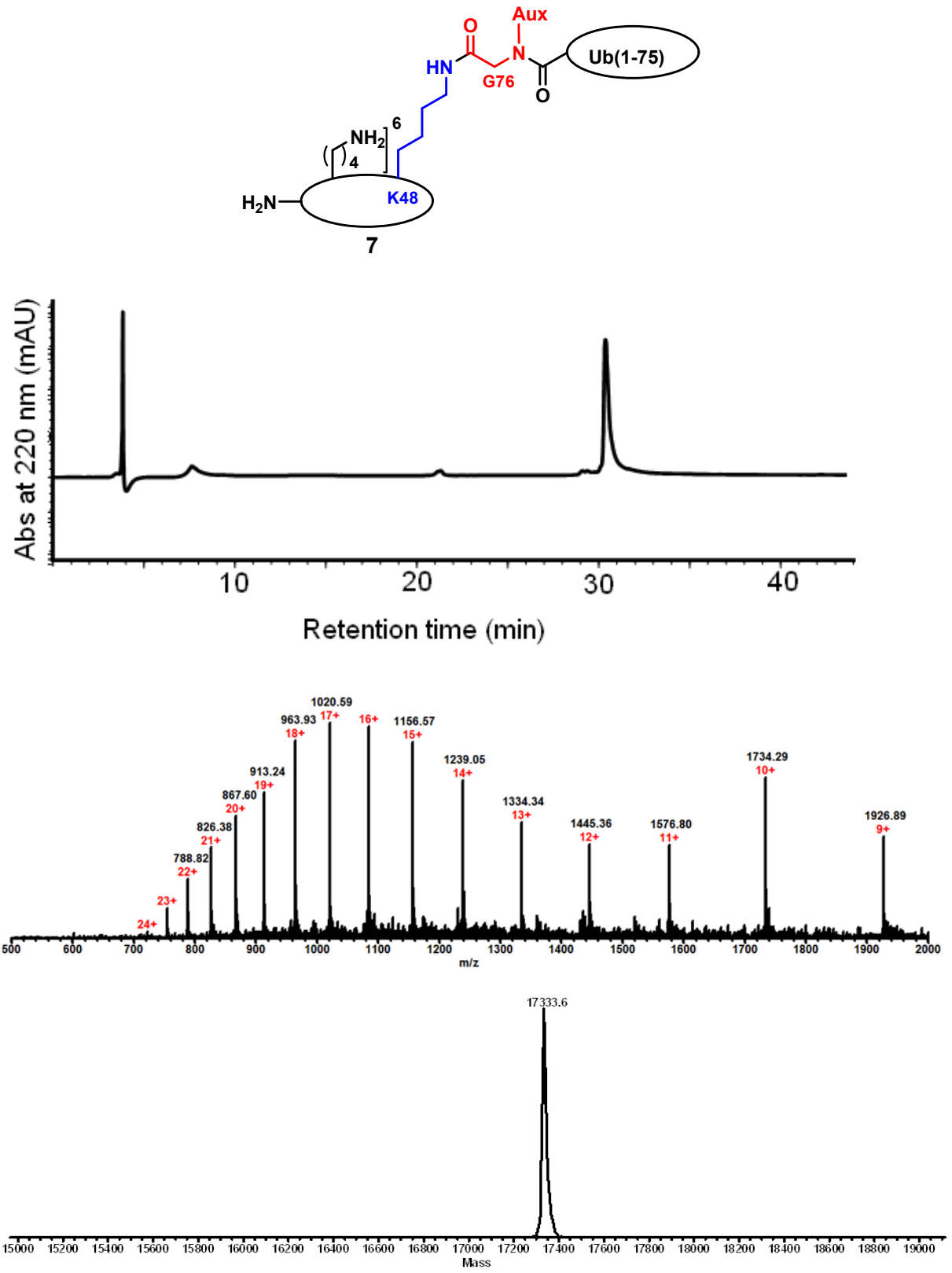


Figure S8. C8 analytical HPLC, raw and deconvoluted ESI-MS data of the purified ligation product ub **7**. (Average mass calculated 17334.69; found 17333.6).

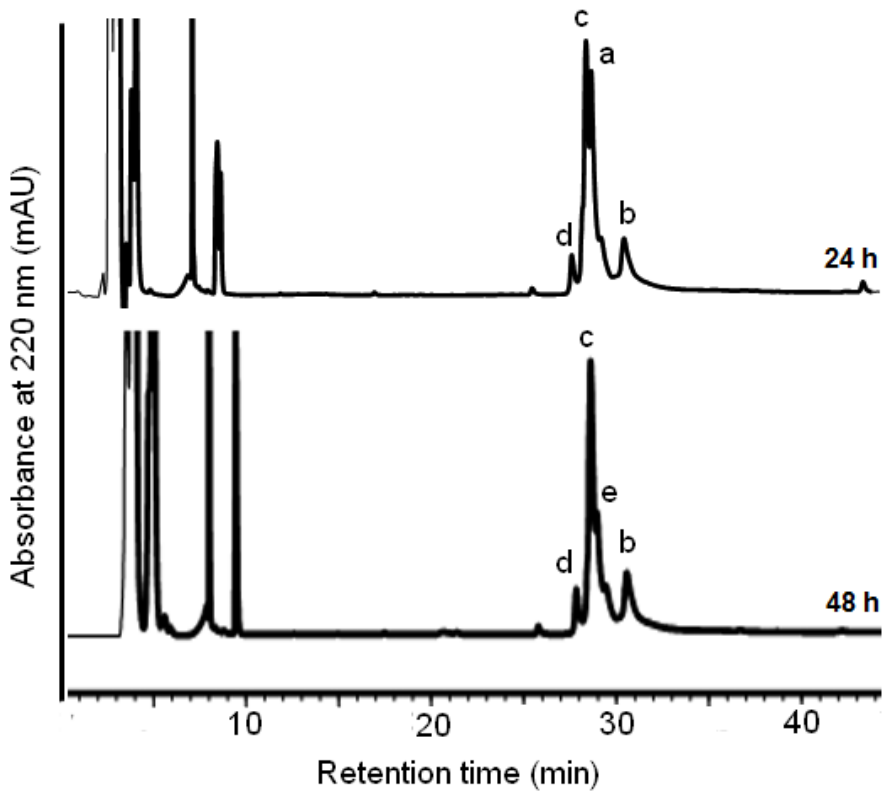


Figure S9. C8 analytical HPLC monitored ligation reaction between ub **6** and Ub(1-75)-MES with MESNa as the thiol additive at 24 h and 48 h, respectively. Peak a: the mixture of the two starting materials, ub **6** and Ub(1-75)-MES; peak b: ligation product ub **7**; peak c: Ub(1-75)-OH; peak d: Ub(1-75)-SH; peak e: ub **6**.

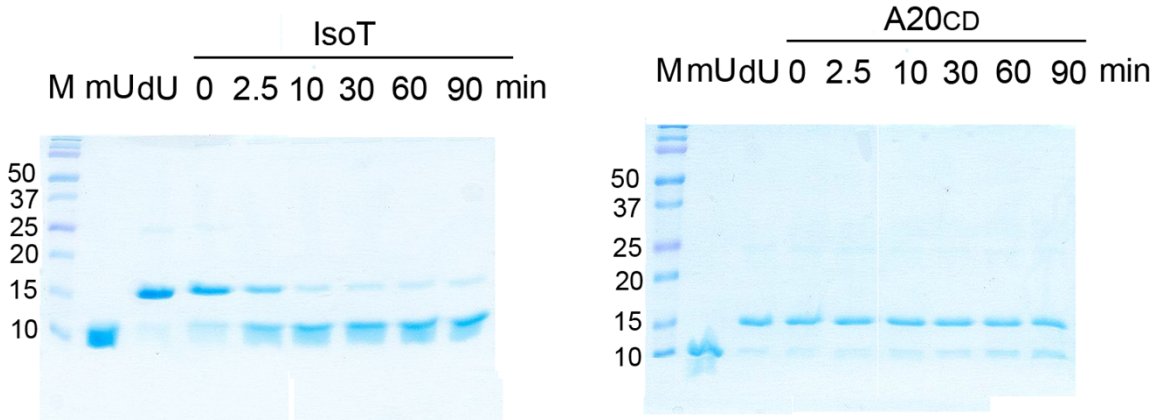
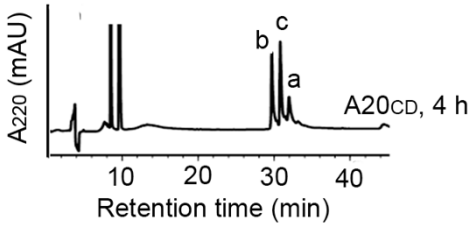
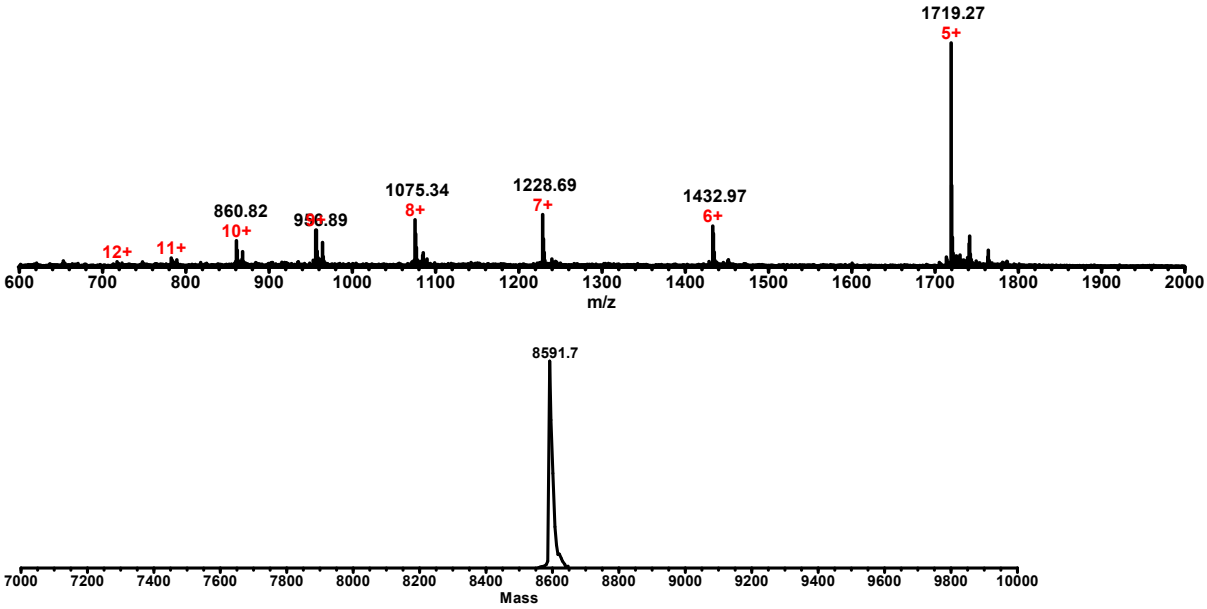


Figure S10. SDS-PAGE analysis of deubiquitinase assays catalyzed by IsoT and $A20_{CD}$. M: maker in KDa; mU: ub **1**; dU: K48-linked diubiquitin **8**.



Peak b:



Peak c:

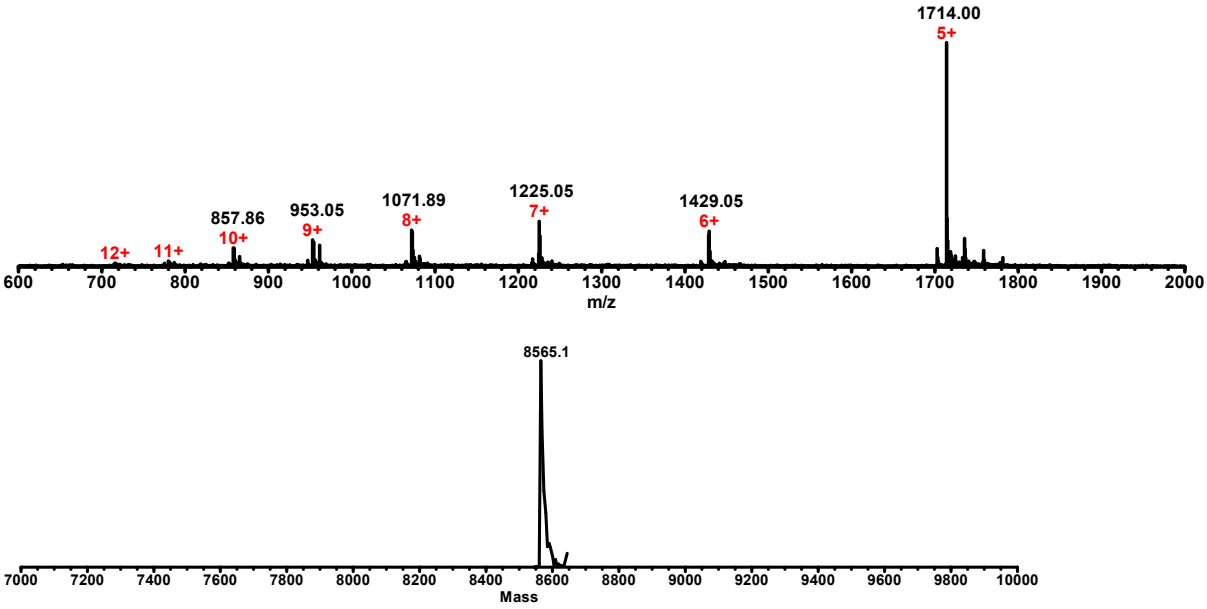
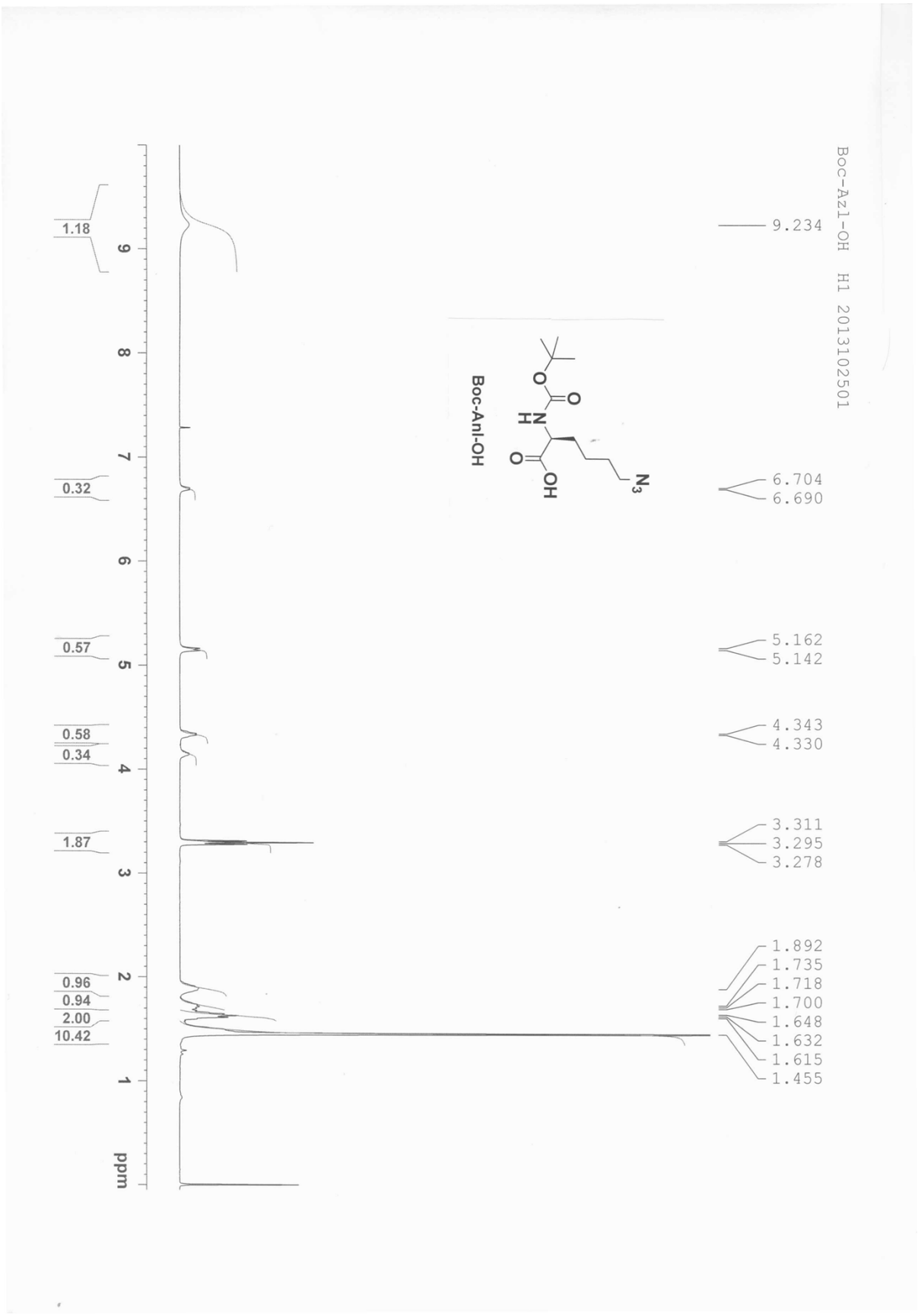


Figure S11. ESI-MS analysis of the products of deubiquitinase assays catalyzed by $A20_{CD}$. Peak b: reduced ub **1** (ASUbK48), average mass calculated 8591.70, observed 8591.7; peak c: wild type ub, average mass calculated 8564.74, observed 8565.1.

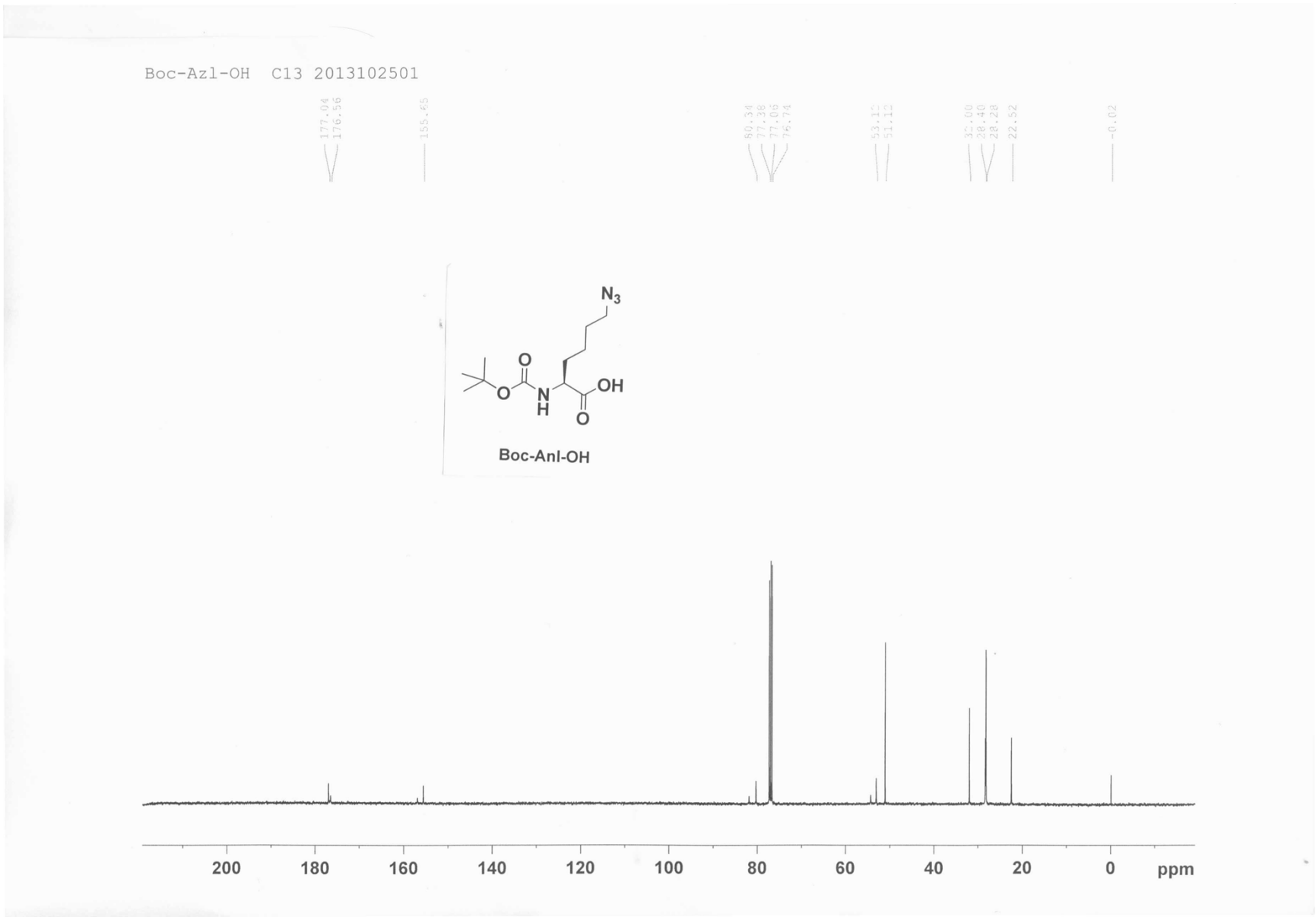
References

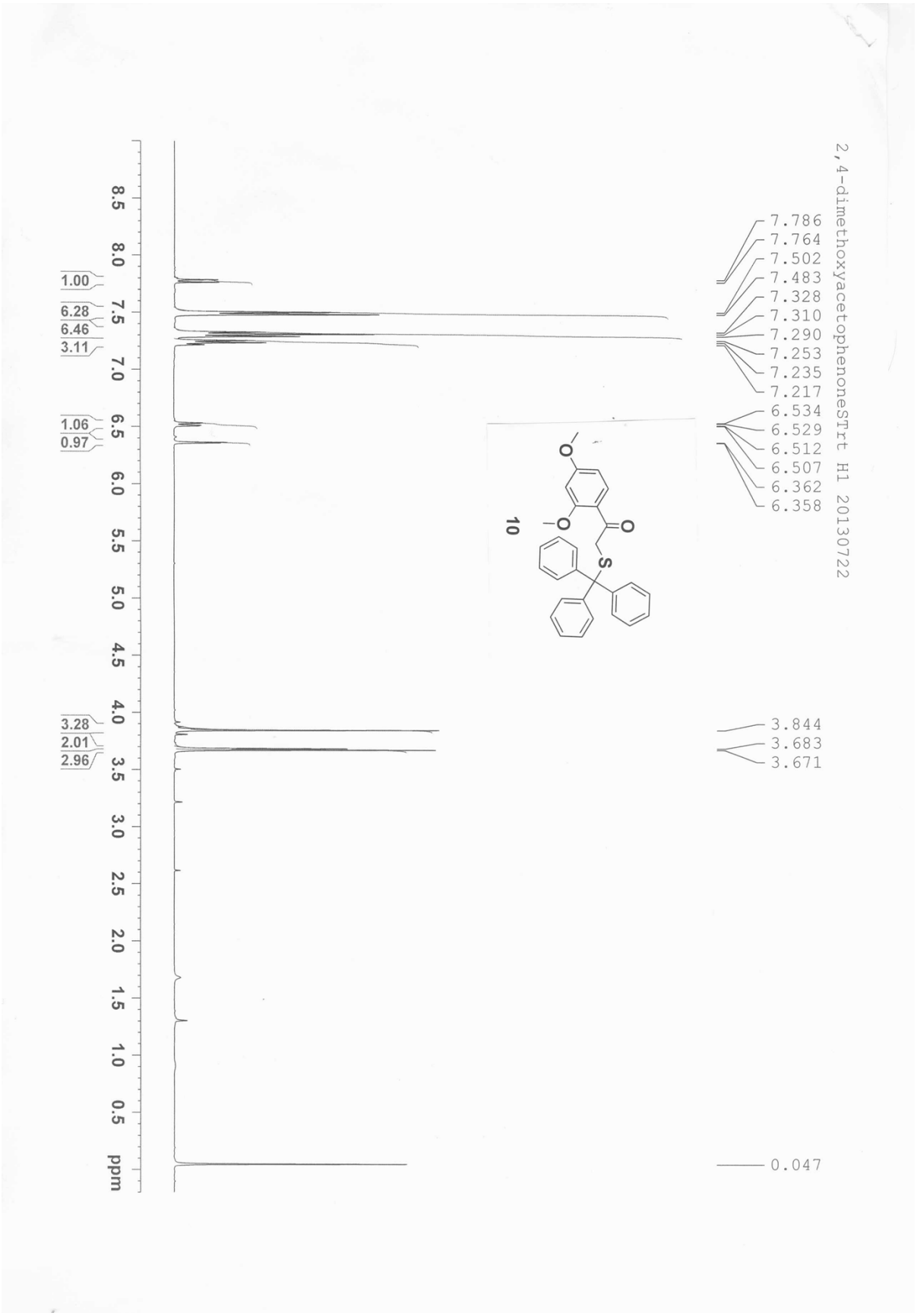
1: K. Holland-Nell and M. Meldal, *Angew. Chem., Int. Ed.* **2011**, 50, 5204 –5206.

2: D. Macmillan and D. W. Anderson*, Org. Lett.* **2004**, 6, 4659-4662.

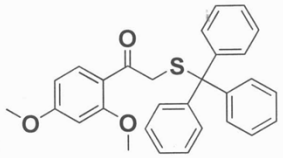

Boc-Azl-OH H1 2013102501
9.234
6.704
6.690
5.162
5.142
4.343
4.330
3.311
3.295
3.278
1.892
1.735
1.718
1.700
1.648
1.632
1.615
1.455
Boc-Anl-OH
1.18
0.32
0.57
0.58
0.34
1.87
0.96
0.94
2.00
10.42
ppm

Boc-Azl-OH C13 2013102501




2,4-dimethoxyacetophenoneSTrt H1 20130722
7.786
7.764
7.502
7.483
7.328
7.310
7.290
7.253
7.235
7.217
6.534
6.529
6.512
6.507
6.362
6.358
3.844
3.683
3.671
0.047
10
1.00
6.28
6.46
3.11
1.06
0.97
3.28
2.01
2.96
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
ppm

2,4-dimethoxyacetophenoneSTrt C13 20130722



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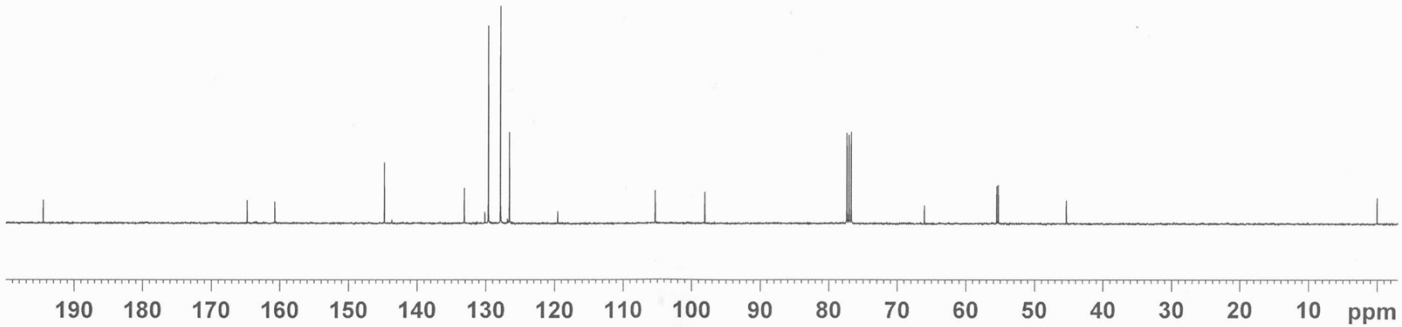
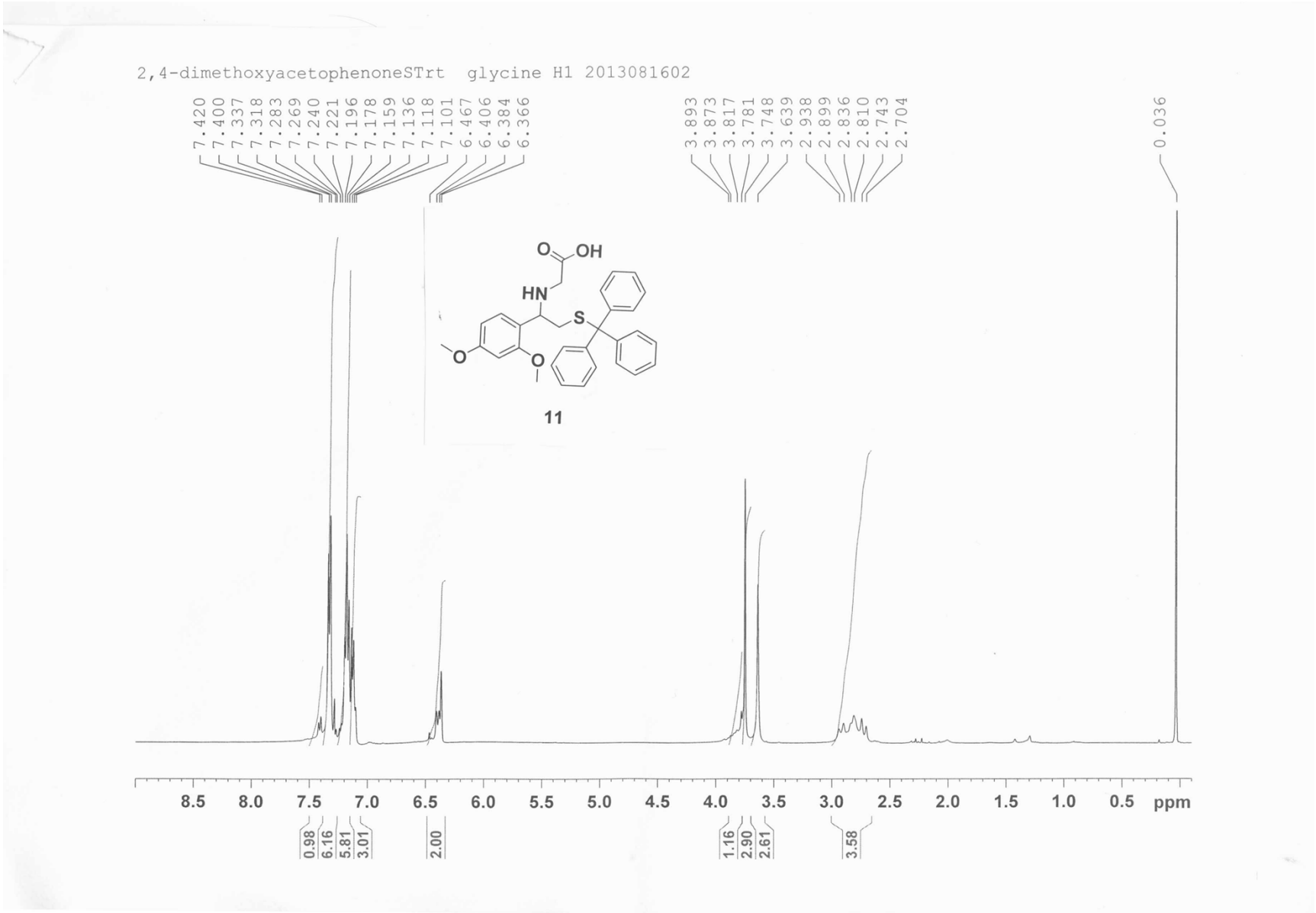
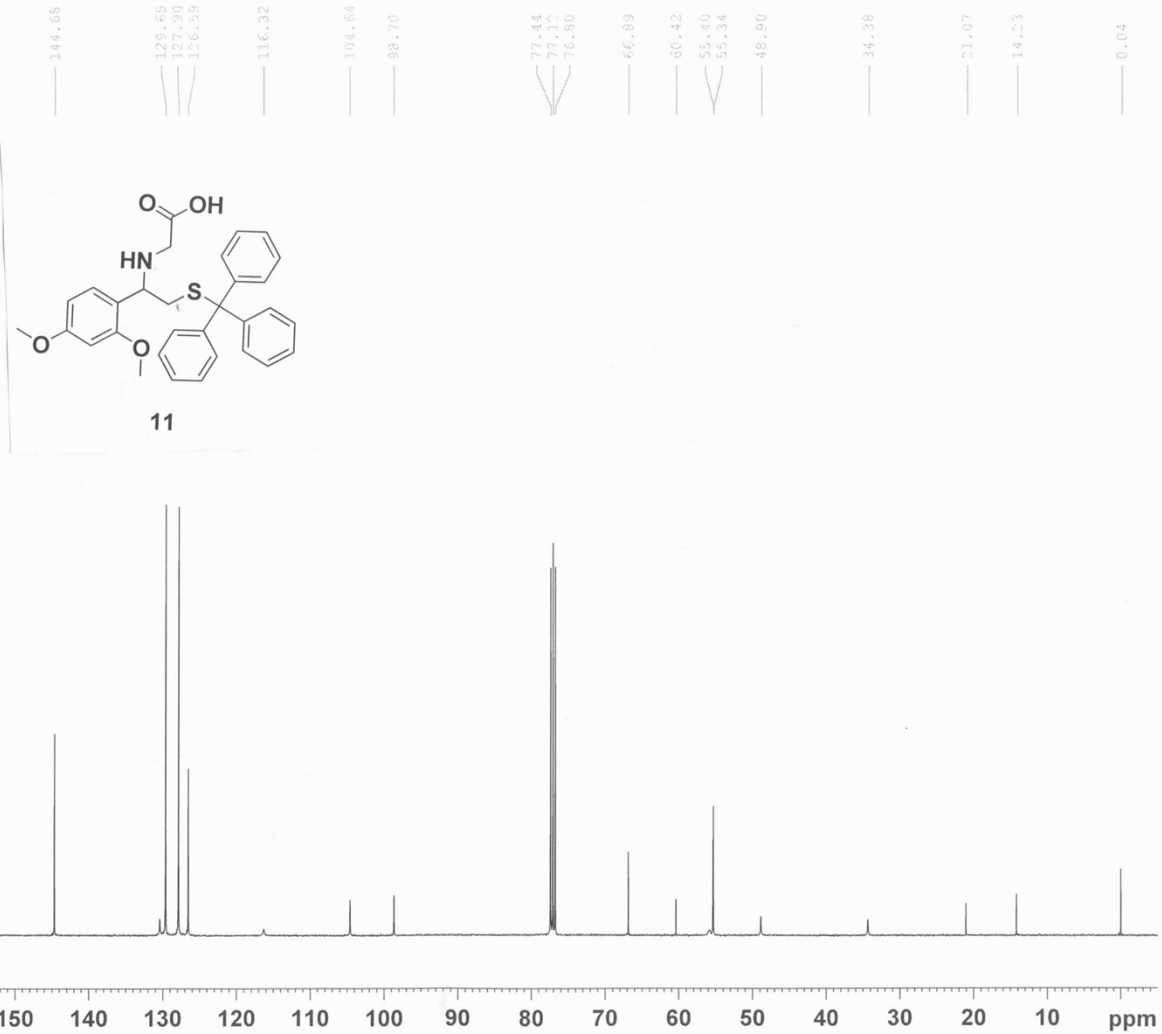
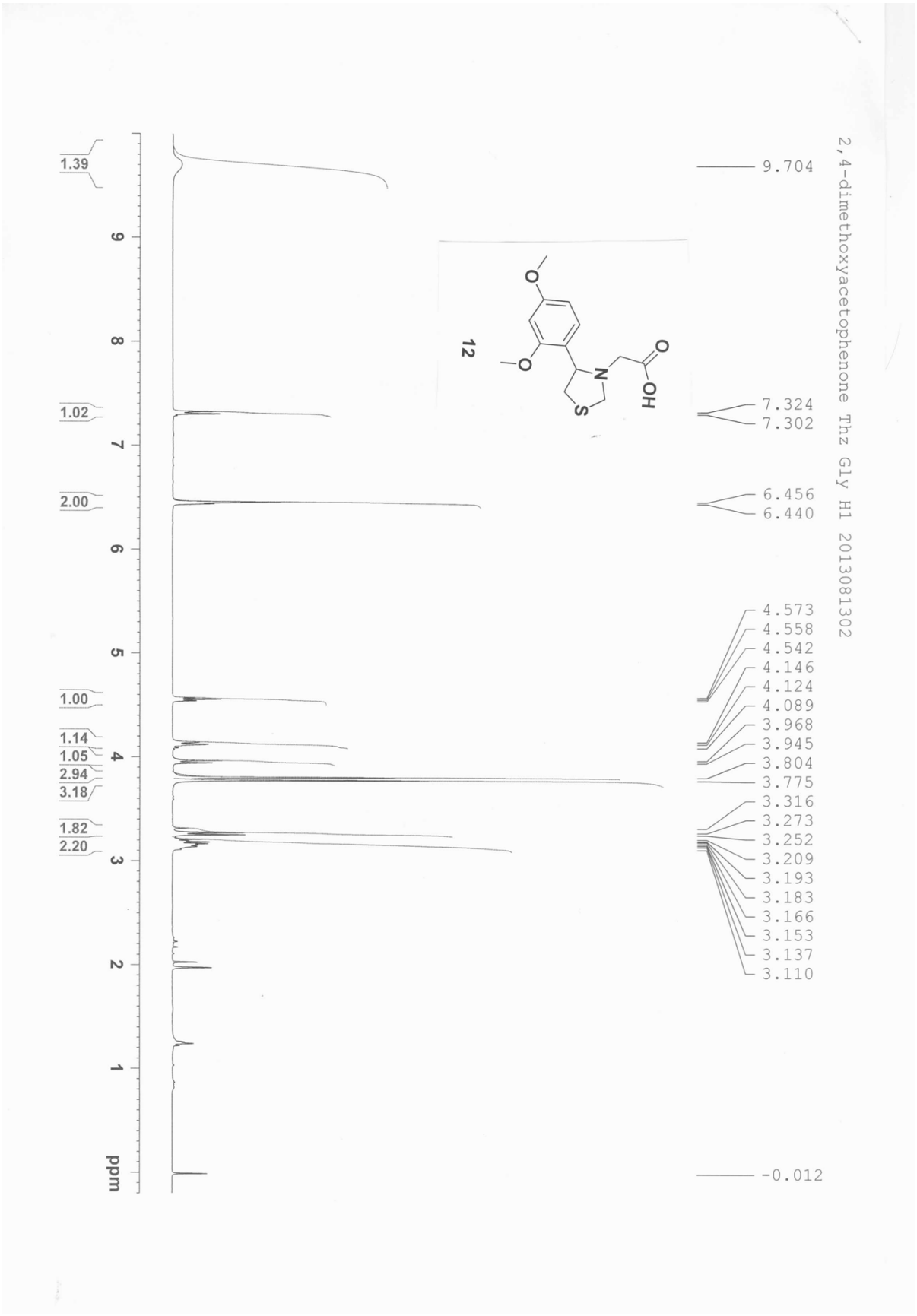


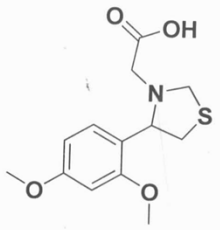

2,4-dimethoxyacetophenoneSTrt glycine H1 2013081602
7.420
7.400
7.337
7.318
7.283
7.269
7.240
7.221
7.196
7.178
7.159
7.136
7.118
7.101
6.467
6.406
6.384
6.366
3.893
3.873
3.817
3.781
3.748
3.639
2.938
2.899
2.836
2.810
2.743
2.704
0.036
O
OH
HN
S
O
O
11
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
ppm
0.98
6.16
5.81
3.01
2.00
1.16
2.90
2.61
3.58

2,4-dimethoxyacetophenoneSTrt Gly C13 2013101701




2,4-dimethoxyacetophenone Thz Gly H1 20130813O2
9.704
7.324
7.302
6.456
6.440
4.573
4.558
4.542
4.146
4.124
4.089
3.968
3.945
3.804
3.775
3.316
3.273
3.252
3.209
3.193
3.183
3.166
3.153
3.137
3.110
-0.012
12
1.39
1.02
2.00
1.00
1.14
1.05
2.94
3.18
1.82
2.20
ppm

2,4-dimethoxyacetophenone Thz Gly C13 2013081301



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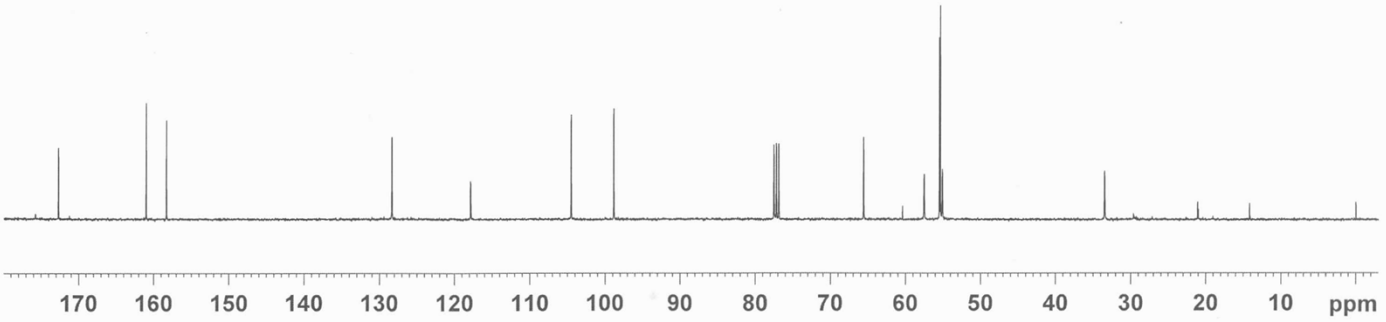
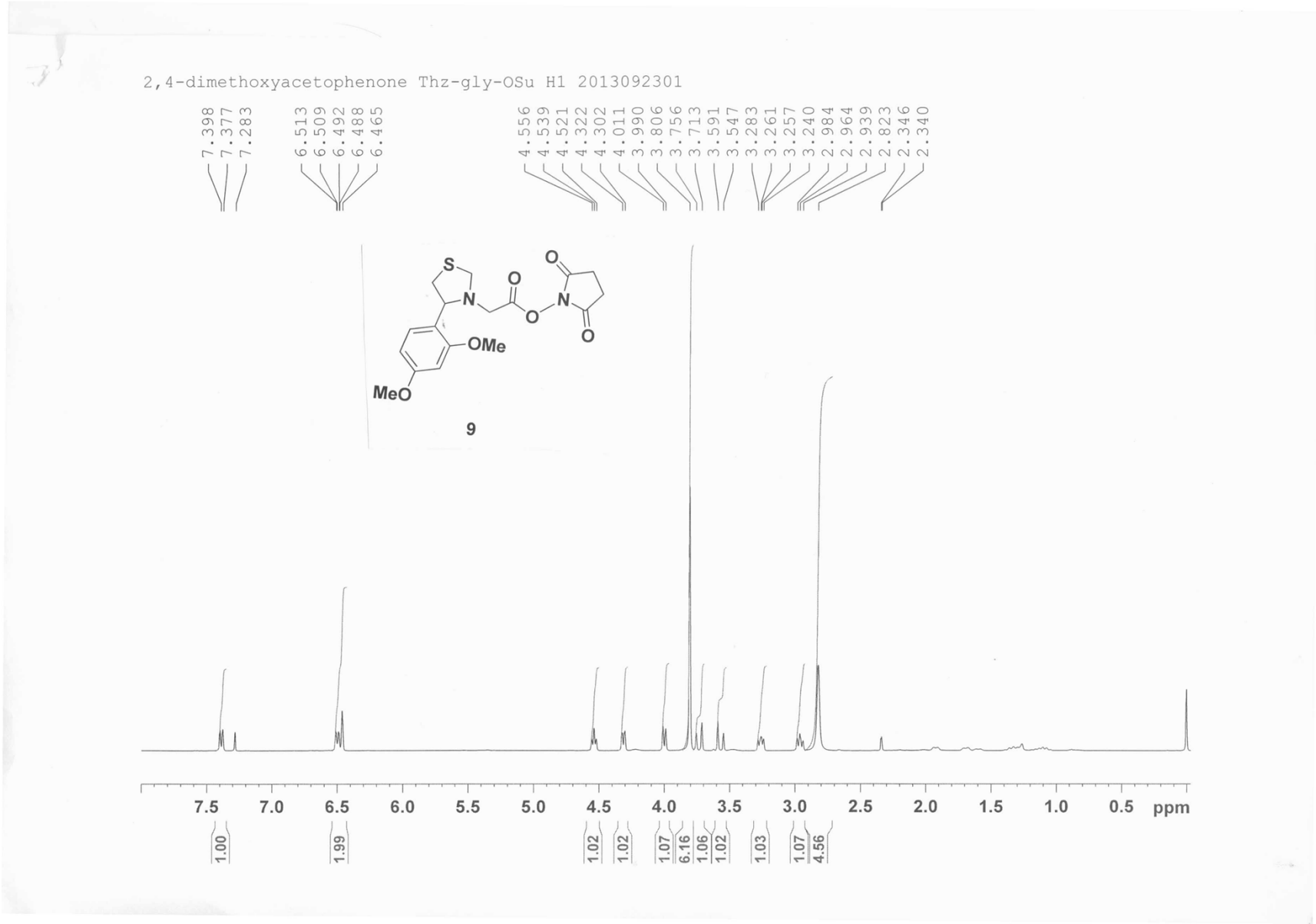
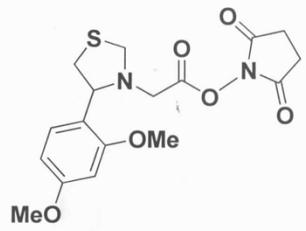



2,4-dimethoxyacetophenone Thz-gly-OSu H1 2013092301
7.398
7.377
7.283
6.513
6.509
6.492
6.488
6.465
4.556
4.539
4.521
4.322
4.302
4.011
3.990
3.806
3.756
3.713
3.591
3.547
3.283
3.261
3.257
3.240
2.984
2.964
2.939
2.823
2.346
2.340
S
N
O
O
N
O
O
OMe
MeO
9
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
ppm
1.00
1.99
1.02
1.02
1.07
6.16
1.06
1.02
1.03
1.07
4.56

2,4-dimethoxyacetophenone Thz-gly-OSu c13 2013092301

169.18
162.93
165.88
165.62
160.58
158.59
127.94
119.42
104.64
98.72
77.44
77.32
77.12
76.81
64.03
56.51
55.51
55.38
51.45
49.10
36.01
33.91
25.58
24.95
17.56
0.01



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