Supplementary Material (ESI) for Chemical Communications

Electronic Supplementary Information

Synthesis of K48-linked Diubiquitin Using Dual Native Chemical Ligation at Lysine

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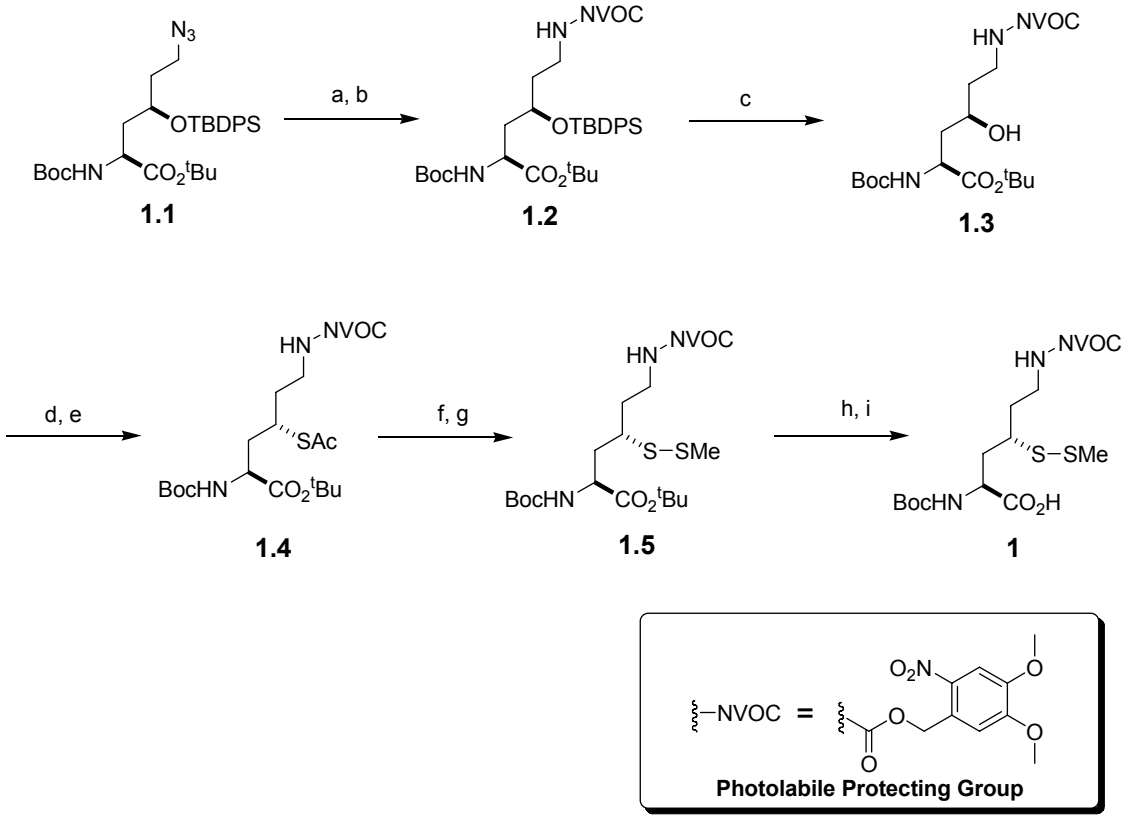
General methods:

Unless otherwise noted, all reactions were carried out in oven dried glassware under an atmosphere of nitrogen and distilled solvents were transferred by syringe. Solvents and reagents were purified according to the standard procedure prior to use. Evaporation of organic solutions was achieved by rotary evaporation with a water bath temperature below 40 °C. Product purification by flash column chromatography was accomplished using silica gel 60 (0.010-0.063 nm). Technical grade solvents were used for chromatography and distilled prior to use. NMR spectra were recorded at room temperature on a 300 MHz Bruker ACF 300, 400 MHz Bruker DPX 400 and 500 MHz Bruker AMX 500 NMR spectrometers, respectively. The residual solvent signals were taken as the reference (7.26 ppm for ^{1}H NMR spectroscopy and 77.0 ppm for ^{13}C NMR spectroscopy). Chemical shift (δ) is referred in terms of ppm, coupling constants (J) are given in Hz. Following abbreviations classify the multiplicity: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet or unresolved, br = broad signal. Infrared spectra were recorded on a Bio-RAD FTS 165 FT-IR Spectrometer and reported in cm^{-1}. Samples were prepared in thin film technique. HRMS (ESI) spectra were recorded on a Finnigan/MAT LCQ quadrupole ion trap mass spectrometer, coupled with the TSP4000 HPLC system and the Crystal 310 CE system.

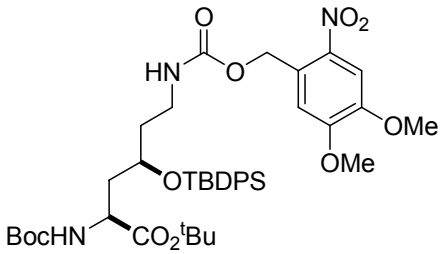
Amino acid derivatives, coupling reagents and resins were purchased from GL Biochem (Shanghai, China), Novabiochem and Chemimpex. All the other chemical reagents were purchased from Alfa Aesar, Sigma-Aldrich Chemical Company, Fisher

Scientific, Acros Organics. PCR kit, restriction enzymes, and chitin beads for construction of ubiquitin-intein expression vector and the purification of intein-CBD fusion proteins were obtained from New England Biolabs. The antibody FK2H was purchased from Enzo life sciences. C18 and C8 analytic HPLC analyses were performed by using an Agilent 1100 series instrument equipped with a Jupiter C18 (5 microm, 4.6 x 250 mm) and a Vydac MS C8 (5 microm, 4.6 x 250 mm) reverse-phase column with a flow rate of 1.0 mL/min. C4 analytic HPLC analyses were performed by using an Shimadzu UFLC system equipped with a Vydac 214MS C4 (5 microm, 4.6 x 250 mm) reverse-phase column with a flow rate of 1.0 mL/min. Detection was achieved with a UV-VIS-detector at wavelength λ = 220 nm. Semi-preparative purifications were performed with a Shimadzu system equipped with a Jupitar C18 column (5 microm, 10 x 250 mm) with a flow rate of 2.5 mL/min. Preparative purifications were performed with a Waters system equipped with a Prosphere C18 column (10 microm, 22 x 250 mm) with a flow rate of 10 mL/min. The buffer system for all the analysis was buffer A – H_2O (containing 0.045% TFA) and buffer B – 90% acetonitrile in H_2O (containing 0.04% TFA). Peptide masses were measured using a Thermo FINNIGAN LCQ Deca XP MAX equipped with ESI ion source.



General scheme for the synthesis of Nε-NVOC protected 4-mercaptolysine derivative **1**. For the synthesis of **1**, Boc-Asp-OtBu was used as the starting material. All the steps before **1.1** were the same as previously reported (*J. Am. Chem. Soc.*, 2009, **131**, 13592). Reagents and conditions: (a) H_2, Pd/C, ethyl acetate, rt , 95%; (b) NVOC-Cl, Na_2CO_3, dioxane:water (2:1), 0 °C, 85%; (c) TBAF, THF, 0 °C, 75%; (d) Ms-Cl, DIPEA, 0 °C; (e) CH_3COSK, DMF, 40 °C, two steps 70% yield; (f) NaOH, MeOH, rt; (g) *S*-methyl methanethiosulfonate (MMTS), triethylamine, CH_2Cl_2, rt, two steps 60%; (h) TFA, H_2O, rt; (i) Boc_2O/TEA, MeOH, rt, two steps 73% yield.

(7R,9S)-tert-butyl-7-(tert-butyldiphenylsilyloxy)-1-(4,5-dimethoxy-2-nitrophenyl)-13,13-dimethyl-3,11-dioxo-2,12-dioxa-4,10-diazatetradecane-9-carboxylate (1.2):



1.2

To a solution of azide **1.1** (0.5 g) in ethyl acetate (4.5 mL, 0.2M) was added 10% Pd/C (0.15 g) at room temperature. The reaction mixture was stirred for 6 h under an H_2 atmosphere at room temperature then filtered through a short pad of celite and washed with chloroform\methanol (1:1). The solvent was removed under reduced pressure (quantitative yield). This compound was direct taken for the next step without further purification.

Amine (0.45 g, 0.8 mmol) was dissolved in 2:1 ratio of dioxane\water (10 mL:5 mL) and cooled to 0 °C. Sodium carbonate (0.57 g, 1.76 mmol) was introduced portions wise at the same temperature. Then NVOC-Cl (0.24 g, 0.88 mmol) was added slowly to the reaction mixture. The reaction mixture was allowed to reach room temperature and stirred for 4 h. After being cooled to 0 °C, the reaction mixture was quenched by the addition of 0.5 N HCl (2 mL) and stirred for a further 5 min. The solution was dissolved in ethyl acetate and washed with water, and brine. The organic layer was dried over sodium sulphate and

concentrated *in vacuo*, which was purified by column chromatography on silica gel to give desired product **1.2** (0.64 g, 85 %) as a pale yellow solid.

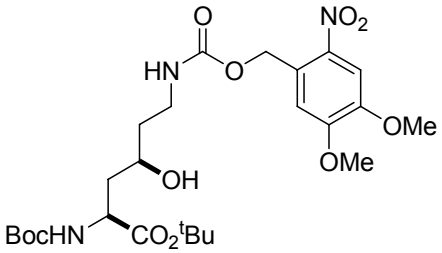
^{1}H NMR (500 MHz, THF-D$_8$): δ in ppm = 7.61-7.55 (m, 5H, H-Ph), 7.29-7.21 (m, 6H, H-Ph), 6.93 (s, 1H, H-Ph), 6.16-6.15 (m, 1H, NH), 6.07 (d, J = 8.1 Hz, 1H, NH), 5.26-5.19 (m, 2H, CH$_2$-Ph), 4.09-4.04 (m, 1H, CH-NH), 3.75 (bs, 1H, CH-OH), 3.73 (s, 6H, OCH$_3$), 2.91-2.86 (m, 2H, CH$_2$-NH), 1.86-1.65 (m, 2H, CH$_2$), 1.56-1.50 (m, 2H, CH$_2$), 1.30 (s, 9H, 3CH$_3$), 1.26 (s, 9H, 3CH$_3$), 0.93 (s, 9H, 3CH$_3$).

^{13}C NMR (100 MHz, THF-D$_8$): δ in ppm = 171.7 (C=O), 155.7 (C=O), 155.4 (C=O), 153.4 (Ph), 148.1 (Ph), 139.8 (Ph), 135.8 (Ph), 135.8 (Ph), 133.7 (Ph), 133.1 (Ph), 129.9 (Ph), 129.8 (Ph), 128.1 (Ph), 127.7 (Ph), 127.7 (Ph), 110.4 (Ph), 108.1 (Ph), 81.6 (CMe$_3$), 79.5 (CMe$_3$), 69.2 (CH-OTBDPS), 63.3 (CH$_2$-Ph), 56.3 (OCH$_3$), 51.9 (CH-NH), 38.2 (CH$_2$-NH), 37.1 (CH$_2$), 36.0 (CH$_2$), 28.3 (3CH$_3$), 27.8 (3CH$_3$), 26.9 (3CH$_3$), 19.3 (C-Si).

IR (CHCl$_3$): ν_{max} = 3018, 1710, 1521, 1215, 669 cm^{-1}.

HRMS (ESI): *m/z*: calcd for $C_{41}H_{58}N_3O_{11}Si$: 796.3841; $[M]^+$ found: 796.3835.

(7R,9S)-tert-butyl-1-(4,5-dimethoxy-2-nitrophenyl)-7-hydroxy-13,13-dimethyl-3,11-dioxo-2,12-dioxa-4,10-diazatetradecane-9-carboxylate (1.3):



1.3

Compound **1.2** (0.1 g, 0.12 mmol) was dissolved in dry THF (3 mL, 0.04M) and cooled to 0 °C. 1 M solution of TBAF (0.19 ml, 0.19 mmol) was introduced via syringe at the same temperature. The reaction mixture was stirred for 10 h at 0 °C and quenched with saturated ammonium chloride solution. Evaporation of the solvent gave a residue, which was dissolved in ethyl acetate. The solution was washed with water, and brine. The organic layer was dried over sodium sulphate and concentrated *in vacuo*, which was purified by column chromatography on silica gel to give desired product **1.3** (0.053 g, 75 %) as a colorless oil.

^{1}H NMR (500 MHz, $CDCl_3$): δ in ppm = 7.70 (s, 1H, Ph), 7.02 (s, 1H, Ph) 5.71 (bs, 1H, NH), 5.50 (s, 2H, CH_2-Ph), 5.42 (d, J = 7.6, 1H, OH), 4.60 (bs, 1H, C<u>H</u>-OH), 4.34 (d, J = 8.8, 1H, C<u>H</u>-NH), 3.98 (s, 3H, OC<u>H</u>$_3$), 3.94 (s, 3H, OC<u>H</u>$_3$), 3.71 (bs, 1H, C<u>H</u>$_2$-NH), 3.55-3.49 (m, 1H, C<u>H</u>$_2$-NH), 3.25-3.24 (m, 1H, <u>C</u>H$_2$), 1.63 (d, J = 11.2, 1H, C<u>H</u>$_2$), 1.67-1.65 (m, 2H, C<u>H</u>$_2$), 1.46 (s, 9H, 3C<u>H</u>$_3$), 1.44 (s, 9H, 3C<u>H</u>$_3$).

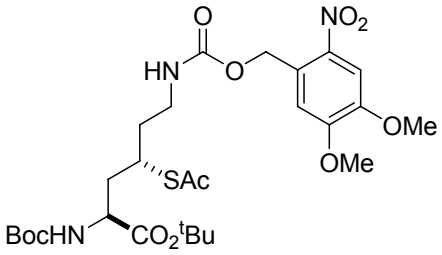
^{13}C NMR (100 MHz, $CDCl_3$): δ in ppm = 171.6 (C=O), 157.0 (C=O), 156.1 (C=O), 153.5 (Ph), 147.9 (Ph), 139.6 (Ph), 128.6 (Ph), 109.9 (Ph), 108.0 (Ph), 82.5 (C), 80.6 (C), 66.8

(CH_2-Ph), 63.3 (<u>C</u>H-OH), 56.4 (O<u>C</u>H_3), 56.3 (O<u>C</u>H_3), 51.0 (<u>C</u>H-NH), 41.9 (<u>C</u>H_2), 39.3 (CH_2), 35.7 (3<u>C</u>H_3), 28.2 (3CH_3), 27.9 (3CH_3).

IR ($CHCl_3$): ν_{max} = 3421, 3018, 1710, 1508, 1215, 669 cm^{-1}.

HRMS (ESI): *m/z*: calcd for $C_{25}H_{40}N_3O_{11}$: 558.2654; $[M]^+$ found: 558.2663.

(7S,9S)-*tert*-butyl-7-(acetylthio)-1-(4,5-dimethoxy-2-nitrophenyl)-13,13-dimethyl-3,11-dioxo-2,12-dioxa-4,10-diazatetradecane-9-carboxylate (1.4) :



1.4

0.05 mL (0.27 mmol) of diisopropylethylamine (DIPEA) was added to a solution of 0.1 g (0.18 mmol) of alcohol in 3 mL (0.06M) of dichloromethane at room temperature. The solution was cooled to 0 °C, and 0.017 mL (0.21 mmol) of methanesulfonyl chloride was added. The reaction mixture was allowed to reach room temperature and stirred for 2 h. The reaction mixture was quenched with saturated ammonium chloride solution at 0 °C and diluted with dichloromethane. The layers were separated, the aqueous layer was extracted with dichloromethane two times, and the combined organic extract was washed with water and brine. Drying over anhydrous sodium sulphate and removal of solvent under vacuum resulted in a colorless oily residue which was dissolved in DMF (3 mL, 0.05 M). A total of 0.06 g (0.5 mmol) of potassium thioacetate was added to the solution, which was heated to 40 °C for 12 h. After allowed to reach room temperature, water was added to the solution, which was extracted twice with ethyl acetate. The combined organic layer was washed with water and brine, dried over sodium sulphate and evoporated. The crude product was purified by column chromatography on silica gel to give desired product **1.4** (0.077 g, 70 %) as a

pale brown oil.

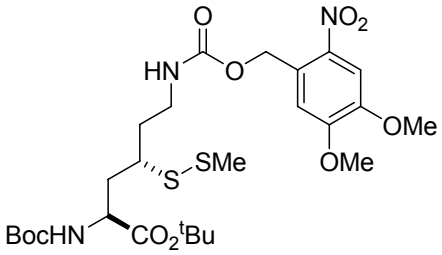
^{1}H NMR (400 MHz, $CDCl_3$): δ in ppm = 7.66 (s, 1H, Ph), 7.01 (s, 1H, Ph), 5.64 (bs, 1H, NH), 5.51-5.40 (m, 2H, CH_2-Ph), 5.24-5.22 (m, 1H, NH), 4.23-4.21 (m, 1H, C<u>H</u>-NH), 3.94 (s, 3H, OC<u>H</u>$_3$), 3.90 (s, 3H, OC<u>H</u>$_3$), 3.63-3.61 (m, 1H, C<u>H</u>-S), 3.37-3.34 (m, 1H, C<u>H</u>$_2$-NH), 3.14 (bs, 1H, C<u>H</u>$_2$-NH), 2.28 (s, 3H, CH_3), 2.04-2.01 (m, 2H, CH_2), 1.84-1.80 (m, 1H, C<u>H</u>$_2$), 1.67-1.57 (m, 1H, CH_2) 1.41 (s, 9H, 3C<u>H</u>$_3$), 1.40 (s, 9H, 3C<u>H</u>$_3$).

^{13}C NMR (100 MHz, $CDCl_3$): δ in ppm = 195.8 ($COCH_3$), 171.0 (C=O), 155.9 (C=O), 155.7 (C=O), 153.5 (Ph), 147.9 (Ph), 128.7 (Ph), 109.8 (Ph), 108.0 (Ph), 82.5 (C), 80.1 (C), 63.2 (CH_2-Ph), 56.4 (O<u>C</u>H$_3$), 56.3 (O<u>C</u>H$_3$), 52.0 (<u>C</u>H-NH), 39.8 (<u>C</u>H$_2$-NH), 38.3 (<u>C</u>H$_2$), 38.0 (CH_2), 33.5 (CH-S), 30.7 (CH_3) 28.2 (3<u>C</u>H$_3$), 27.9 ($3CH_3$).

IR ($CHCl_3$): ν_{max} = 3427, 3018, 1689, 1521, 1215, 669 cm^{-1}.

HRMS (ESI): *m/z*: calcd for $C_{27}H_{41}N_3O_{11}SNa$: 638.2360; $[M+Na]^+$ found: 638.2350.

(7S,9S)-tert-butyl-1-(4,5-dimethoxy-2-nitrophenyl)-13,13-dimethyl-7-(methyldisulfanyl)-3,11-dioxo-2,12-dioxa-4,10-diazatetradecane-9-carboxylate (1.5) :



1.5

0.020 g of compound **1.4** was dissolved in 0.6 mL of methanol. 0.175 mL of 1 N NaOH was added. After 30 min at room temperature, the mixture was neutralized carefully with 1 N HCl at 0 °C. The mixture was concentrated and extracted with ethyl acetate three times. The combined organic layer was washed with water and brine, dried over sodium sulphate and evoporated.

The residue was dissolved in 0.4 mL of DCM and added dropwise to a mixture containing 0.4 mL of DCM, 0.006 mL of Et_3N and 0.015 mL of S-methyl methanethiosulfonate (MMTS) while stirring at room temperature. The mixture was continuously stirred for 30 min. The solvent was removed and the residue was dissolved in methanol/H_2O (3:1) and purified by C18 semi-preparative HPLC followed by lyophilization to give desired product **1.5** (12 mg) as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$): δ in ppm = 7.59 (s, 1H, Ph), 7.03 (s, 1H, Ph), 6.43-6.42 (m, 1H, NH), 6.27 (d, J = 7.6 Hz, 1H, NH), 5.30 (s, 2H, CH_2-Ph), 4.09-4.04 (m, 1H, C<u>H</u>-NH), 3.80 (s, 3H, OC<u>H</u>$_3$), 3.76 (s, 3H, OC<u>H</u>$_3$), 3.47 (bs, 1H, C<u>H</u>-S), 3.21-3.05 (m, 1H, C<u>H</u>$_2$-NH),

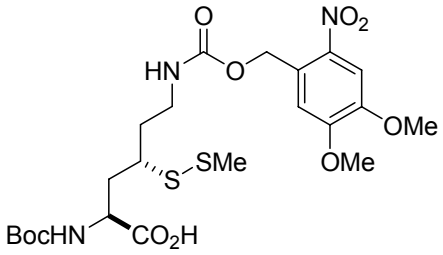
3.02-2.97 (m, 1H, C<u>H</u>), 2.17 (s, 3H, CH_3), 2.00-1.85 (m, 2H, C<u>H_2</u>), 1.74-1.62 (m, 2H, C<u>H_2</u>) 1.32 (s, 9H, 3C<u>H_3</u>), 1.31 (s, 9H, 3C<u>H_3</u>).

^{13}C NMR (100 MHz, $CDCl_3$): δ in ppm = 193.9, 171.0 (C=O), 155.5 (C=O), 153.8 (C=O), 148.3 (Ph), 139.7 (Ph), 128.5 (Ph), 110.1 (Ph), 107.9 (Ph), 80.6 (C), 78.2 (C), 62.4 (CH_2-Ph), 55.5 (O<u>C</u>H_3), 55.4 (O<u>C</u>H_3), 52.1 (<u>C</u>H-NH), 33.8 (<u>CH_3</u>-S), 29.5 (<u>CH_2</u>), 27.6 (3<u>C</u>H_3), 27.1 (3<u>C</u>H_3), 24.8 (CH-S).

IR ($CHCl_3$): ν_{max} = 3423, 3018, 1215, 758, 669 cm^{-1}.

HRMS (ESI): *m/z*: calcd for $C_{26}H_{41}N_3O_{10}S_2Na$: 642.2131; $[M+Na]^+$ found: 642.2121.

(7S,9S)-1-(4,5-dimethoxy-2-nitrophenyl)-13,13-dimethyl-7-(methyldisulfanyl)-3,11-dioxo-2,12-dioxa-4,10-diazatetradecane-9-carboxylic acid (1) :



1

The white powder of **1.5** was dissolved in 0.5 mL 95% TFA. After 1.5 h at room temperature, the TFA was removed by evaporation. The residue was dissolved in 1 mL methanol/H_2O mixture (3:1). Adjust pH to about 8 with Et_3N. 0.02 mL of Boc_2O was added. After 3 h at room temperature, the sample was subjected to C18 semi-preparative HPLC. After lyophilization 8 mg of **1** were isolated.

^{1}H NMR (400 MHz, $CDCl_3$): δ in ppm = 10.72 (bs, 1H, COOH), 7.58 (s, 1H, Ph), 7.02 (s, 1H, Ph), 6.54 (bs, 1H, NH), 5.34 (d, J = 7.2 Hz, 1H, NH), 5.34-5.25 (m, 2H, CH_2-Ph), 4.25-4.21 (m, 1H, C<u>H</u>-NH), 3.79 (s, 3H, OC<u>H</u>$_3$), 3.75 (s, 3H, OC<u>H</u>$_3$), 3.28-3.22 (m, 1H, C<u>H</u>$_2$), 3.15-3.06 (m, 1H, C<u>H</u>), 2.78-2.76 (m, 1H), 2.29 (s, 3H, CH_3), 2.29-1.87 (m, 1H, C<u>H</u>$_2$), 1.85-1.83 (m, 2H, C<u>H</u>$_2$), 1.73-1.61 (m, 1H, C<u>H</u>$_2$), 1.32 (s, 9H, 3C<u>H</u>$_3$).

^{13}C NMR (100 MHz, $CDCl_3$): δ in ppm = 172.8 (C=O), 155.6 (C=O), 153.8 (C=O), 148.3 (Ph), 139.7 (Ph), 128.2 (Ph), 128.5 (Ph), 110.1 (Ph), 107.9 (Ph), 78.2 (C), 62.4 (CH_2-Ph), 55.5, 55.4, 51.1 (<u>C</u>H-NH), 45.3 (<u>C</u>H$_2$-NH), 38.5 (<u>CH$_3$</u>-S), 32.8 (CH_2), 27.6 (3<u>C</u>H$_3$), 23.2 (CH-S).

IR ($CHCl_3$): ν_{max} = 3444, 3427, 3018, 1708, 1635, 1215 cm^{-1}.

HRMS (ESI): *m/z*: calcd for $C_{22}H_{34}N_3O_{10}S_2$: 564.1686; $[M]^+$; found 564.1678.

Solid phase peptide synthesis:

Synthesis of Ub(L1-K27)-$COSCH_2CH_2CONH_2$ (**2**) and Ub(Thz28-G47)-$COSCH_2CH_2CONH_2$ (**3**). **2** and **3** were synthesized manually employing standard tert-Butyloxycarbonyl (Boc) chemistry. The synthesis was started with 0.5 g of MBHA resin (0.9 mmol/g). S-trityl mercaptopropanoic acid (4 eq. PyBOP, 4 eq. acid, 12 eq. DIEA, preactivated in DCM for 2 min) was coupled onto the resin and served as the thiol linker. Trityl group with removed by repeated treatment with TFA/β-mercaptoethanol/TIS/DCM (5/2.5/2.5/90). The amino acids were then coupled one by one in a way similar to the loading of S-trityl mercaptopropanoic acid. The coupling was monitored with ninhydrin test. The amino acid derivatives used were Boc-Lys(2Cl-Z)-OH, Boc-Asn(Xan)-OH, Boc-Glu(OcHx)-OH, Boc-Thr(Bzl)-OH, Boc-Asp(OcHx)-OH, Boc-Ser(Bzl)-OH, Boc-Gln(Trt)-OH, Boc-Arg(Tos)-OH, Boc-Thz-OH. After sequence assembly, the Boc group was removed by treated with 30 % TFA before cleavage. The peptide thioesters were cleaved by HF/p-cresol/anisole/ (9:0.5:0.5) for 1 h at 0 ^{o}C. The crude was harvested by ether precipitation and centrifugation. The peptides were purified by C18 preparative HPLC. The desired products were characterized with C18 analytic HPLC and ESI-MS.

Synthesis of K48(4-SSMe, NVOC)-G76-OH (**4**). The peptide was synthesized manually employing standard fluorenylmethyloxycarbonyl (Fmoc) chemistry started with 1 g of Wang resin (0.44 mmol/g). The C-terminal Gly was loaded by using 8 eq. DCC, 0.8 eq DMAP, 8 eq. Fmoc-Gly-OH in dry DCM/DMF overnight. The loading was repeated for another 5 h and the resin was then capped with Ac_2O for 1 h. Fmoc group was removed

with 20% piperidine in DMF. The following amino acids were coupled using 4 eq. PyBOP, 4. eq. amino acid, 8 eq. DIEA preactivated in DMF. On average, each coupling reaction lasted for 1.5 h. The coupling was monitored with ninhydrin test. The *Nα*-Fmoc protected amino acids used were Fmoc-Arg(Pbf)-OH, Fmoc-His(Trt)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Asp(OtBu)-OH. The Gly at the AspGly junction was coupled using Fmoc-(Hmb)Gly-OH (2 eq. PyBOP, 2 eq. amino acid, 4 eq. DIEA). The N-terminal Lys was coupled using Boc-Lys(4-SSMe, NVOC)-OH (**6**). For the coupling of **6**, 40 mg of **6**, 10.6 mg of HOBt and 16 mg of DCC were dissolved in minimum amount of dry DCM/DMF. The mixture was reacted with 300 mg of peptide resin for 3 h. After sequence assembly, the resin was treated with 20 % piperidine in DMF for 20 min to hydrolyze any possible acylation at hydroxyl group of Hmb group of (Hmb)Gly residue. The resin was then cleaved with TFA/TIS/H_2O (95/2.5/2.5) for 2.5 h. The crude peptide was harvested by ether precipitation and purified with C18 preparative HPLC. The desired product was analyzed with C18 analytic HPLC and ESI-MS.

Preparation of Ub(1-76)-MES：

The construction of ubiquitin-intein fusion protein expression plasmid pTYB1-Ubi, the expression and MESNa thiolysis of ubiquitin-intein were the same as we reported in the previous work (*J. Am. Chem. Soc.*, 2009, **131**, 13592). The purified product was analyzed with C4 analytic HPLC and ESI-MS.

Free radical mediated desulfurization:

The desulfurization was performed under N_2. All the solutions were prepared under N_2 immediately before use. 0.8 mg of sulfur containing diubiquitin was dissolved in 300 μL buffer containing 6 M Gdn•HCl, 0.1 M phosphate, pH 6.5. 100 μL of 0.5 M TCEP solution (neutralized with NaOH) was added. 25 μL of 10 mM glutathione was added. 10 μL of 0.2M VA-044 was added. The solution was stirred at 37 ^{o}C for 6 h. 10 μL of 0.2M VA-044 was added to the mixture and the solution was continuously stirred for another 3 h. The final K48-linked diubiquitin (**8**) was purified by C18 semi-preparative HPLC.

Western blot analysis of K48-linked diubiquitin (8):

8 was dissolved in 8 M urea and analyzed with 18% SDS-PAGE. The samples on the gel were then electrotransferred to PVDF (polyvinylidene difluoride) membrane. The membrane was blocked by 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. A 1: 3000 diluted FK2H was used to detect **8** as visualized by chemilluminescence (SuperSignal West Dura Trial Kit, Pierce, USA).

Circular Dichroism (CD) measurement of K48-linked diubiquitin (8):

For the folding of K48-linked diubiquitin (**8**), 0.7 mg of **8** was dissolved in 0.1 mL buffer (6 M Gdn·HCl, 10 mM phosphate, 100 mM NaCl, pH 7.4) and serially dialyzed against 10 mM phosphate, 100 mM NaCl, pH 7.4 buffer containing decreasing

concentration of Gdn·HCl. After folding, the CD of **8** was measured with Chirascan spectrometer with the final dialysis solution as baseline. The scan was performed between 180-260 nm and the passlength was 0.1 mm.

Ubiquitin C-terminal hydrolase assay of K48-linked diubiquitin (8):

12 μL of 1μg/μL of Ubiquitin C-terminal hydrolase UCH-L3 (ENZO life sciences) was prereduced for 20 min at room temperature in a buffer containing 50 mM Tris, 150 mM NaCl, 12 mM DTT, pH 8.0. 80 μL (5 μg/μL) of folded diubiquitin **8** was then added. The mixture was incubated at 37 °C. The reaction was checked with C4 analytic HPLC at 5.5 and 13.5 h, respectively.

Ub(L1-K27)-COSCH$_2$CH$_2$CONH$_2$ (2)

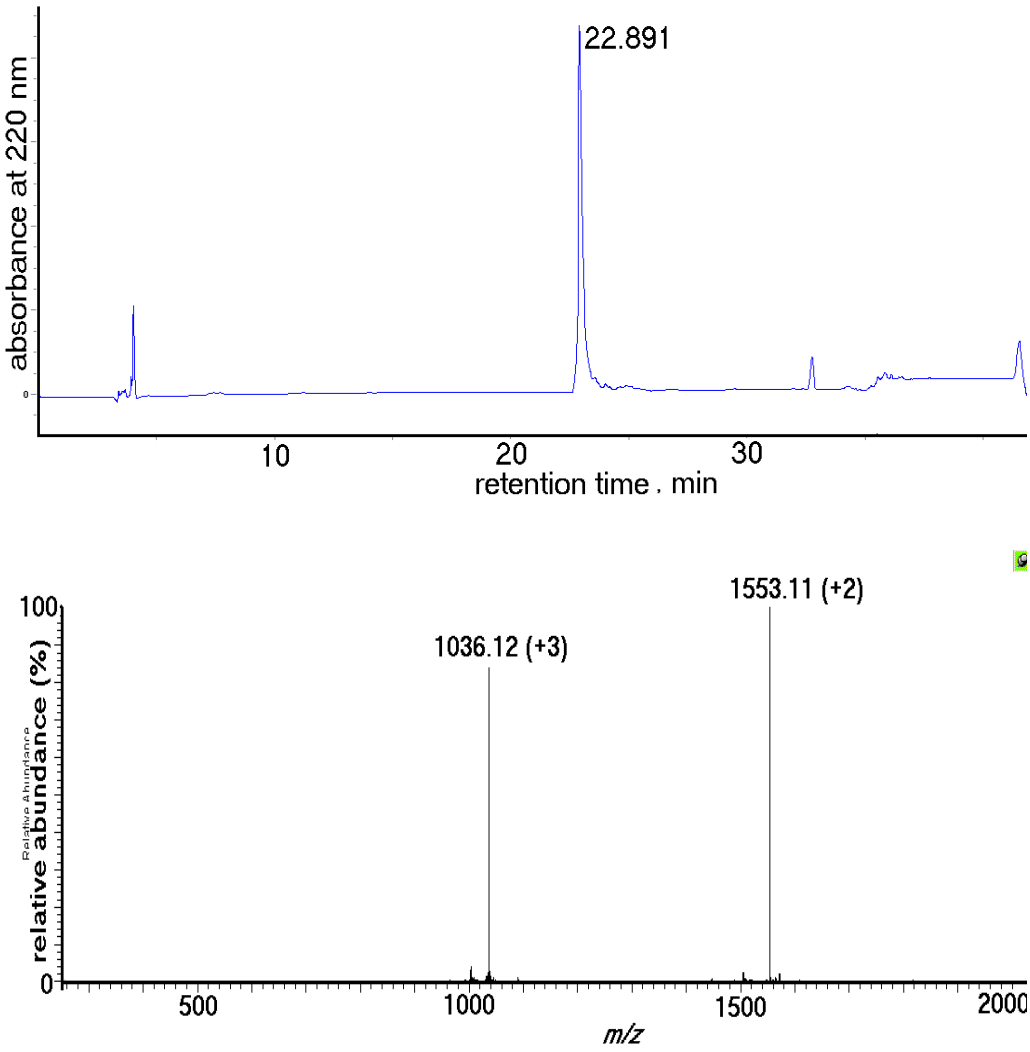


Figure S1: C18 analytic HPLC and ESI-MS of purified Ub(L1-K27)-COSCH$_2$CH$_2$CONH$_2$ (**2**). HPLC gradient: 0-60% B in 30 min. Calculated $[M+H]^+$ = 3105.6, found $[M+2H]^{2+}$ = 1553.11, $[M+3H]^{3+}$ = 1036.12.

Ub(Thz28-G47)-COSCH$_2$CH$_2$CONH$_2$ (3):

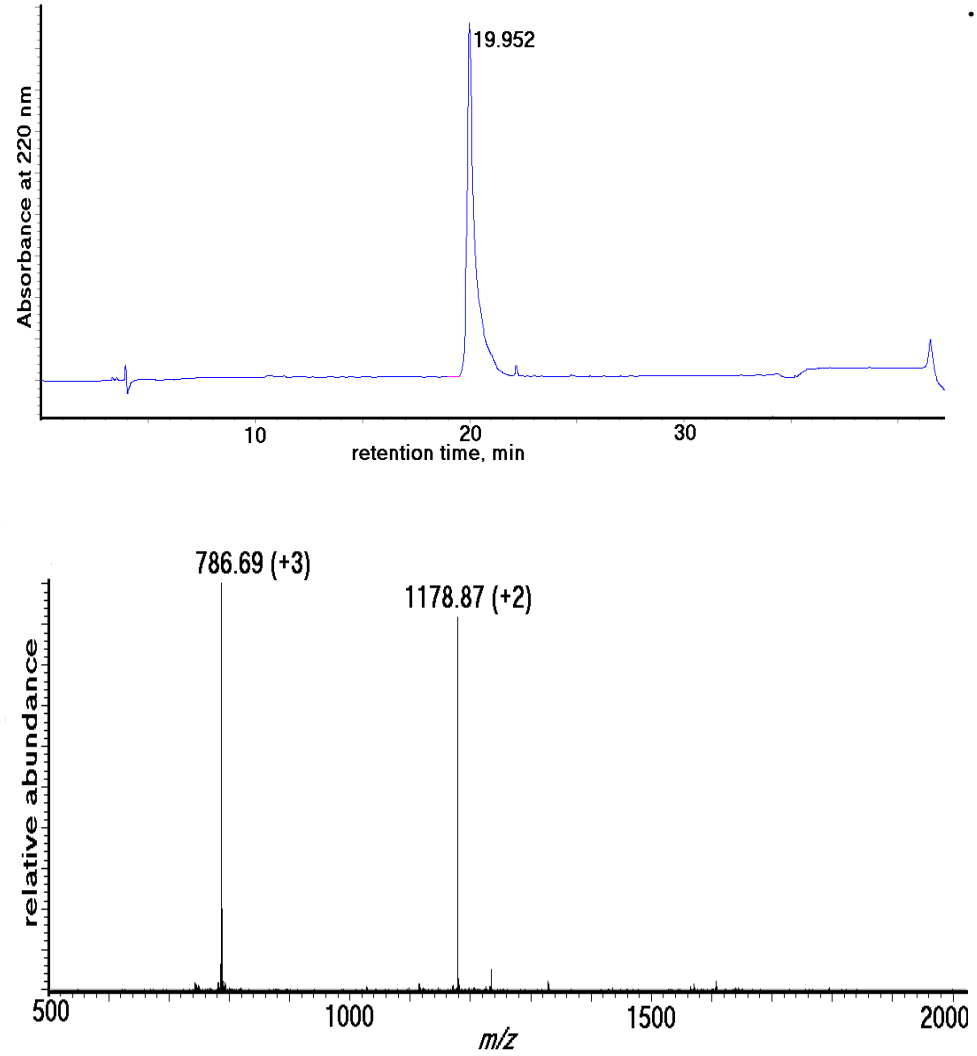


Figure S2: C18 analytic HPLC and ESI-MS of purified Ub(Thz28-G47)-COSCH$_2$CH$_2$CONH$_2$ (**3**). HPLC gradient: 0-60% B in 30 min. Calculated $[M+H]^+$ = 2356.7, found $[M+2H]^{2+}$ =1178.87, $[M+3H]^{3+}$ =786.69.

K48(4-SSMe, NVOC)-G76-OH (4):

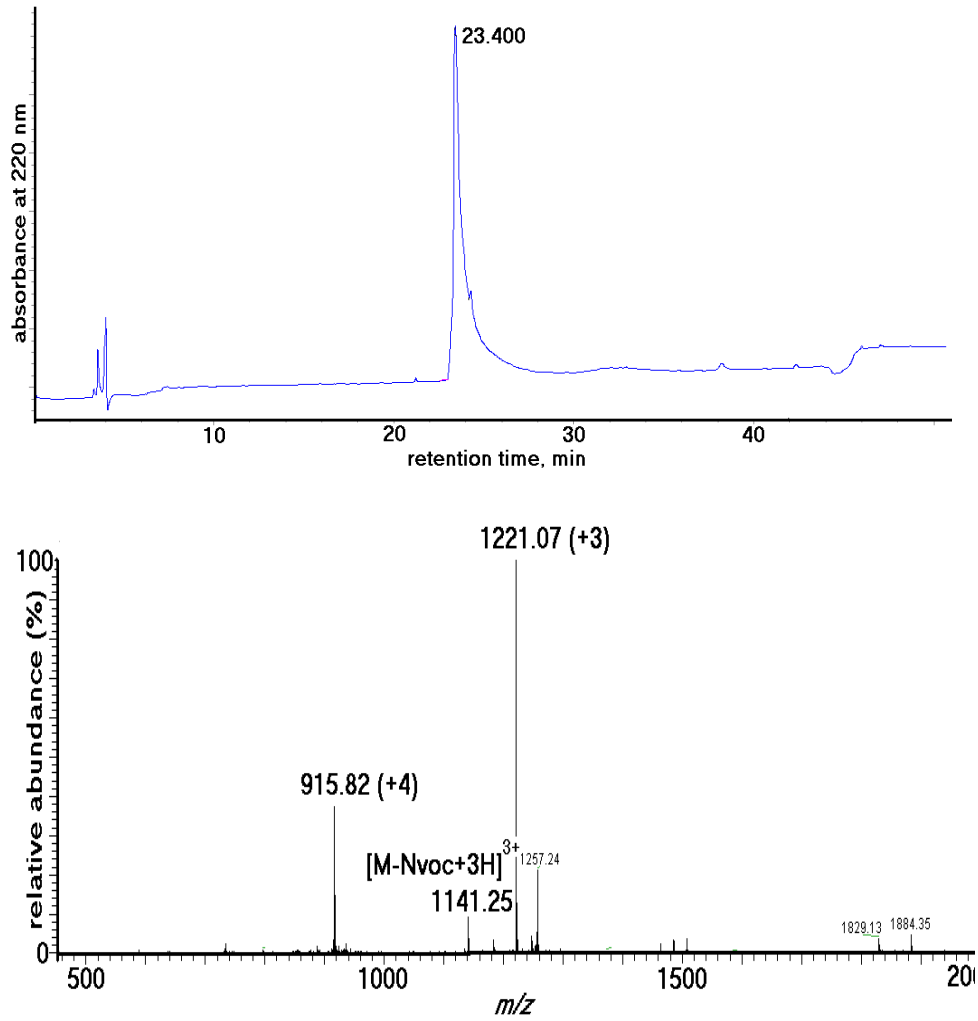


Figure S3: C18 analytic HPLC and ESI-MS of purified K48(4-SSMe, NVOC)-G76-OH (**4**). HPLC gradient: 0-80% B in 40 min. Calculated $[M+H]^{+}$ = 3659.0, found $[M+3H]^{3+}$ =1221.07, $[M+4H]^{4+}$ =915.82, $[M\text{-}NVOC+3H]^{3+}$ = 1141.25.

Ub(1-76)-MES:

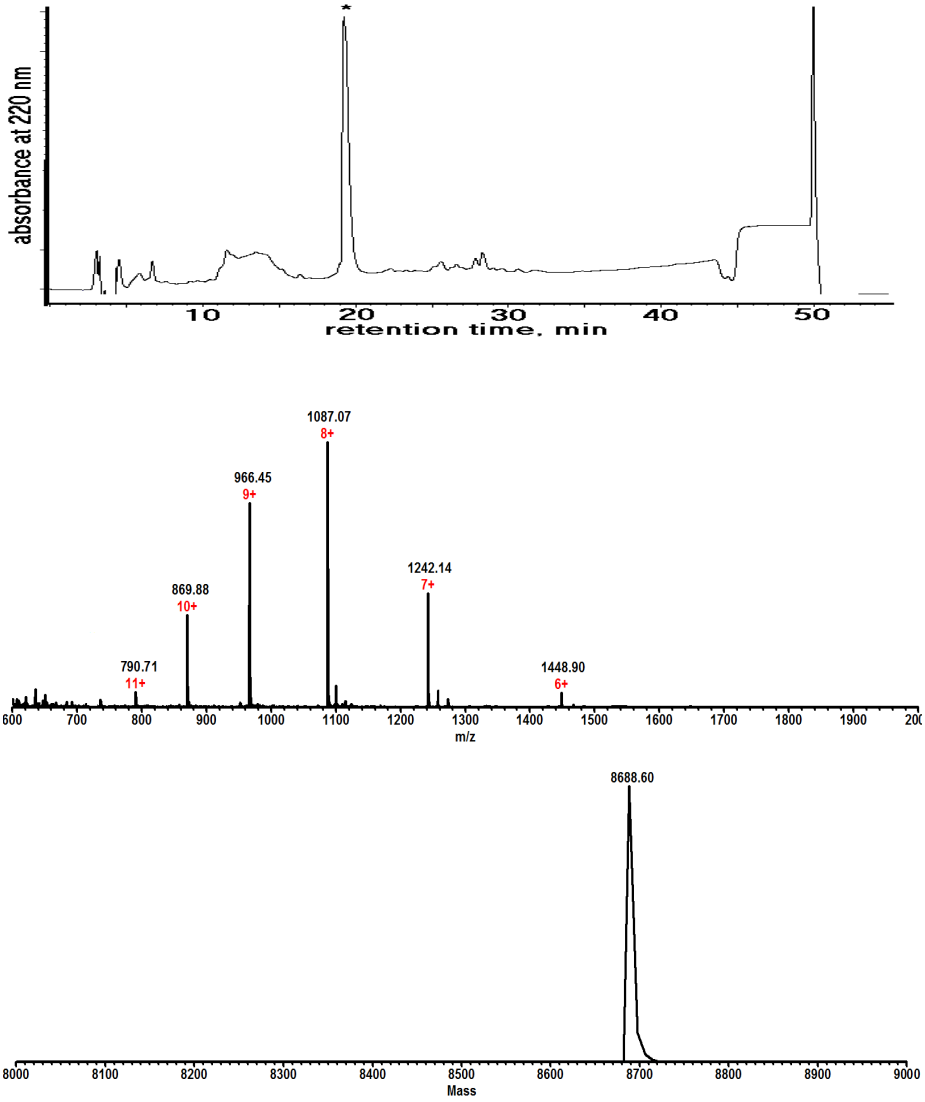


Figure S4: C4 analytic HPLC and ESI-MS of purified Ub(1-76)-MES. HPLC gradient: 0-80% B in 40 min. "*" indicates the desired product. Calculated M = 8688.9 Da, deconvoluted M = 8688.6 Da.

ESI-MS of some key intermediates during the synthesis of **6**:

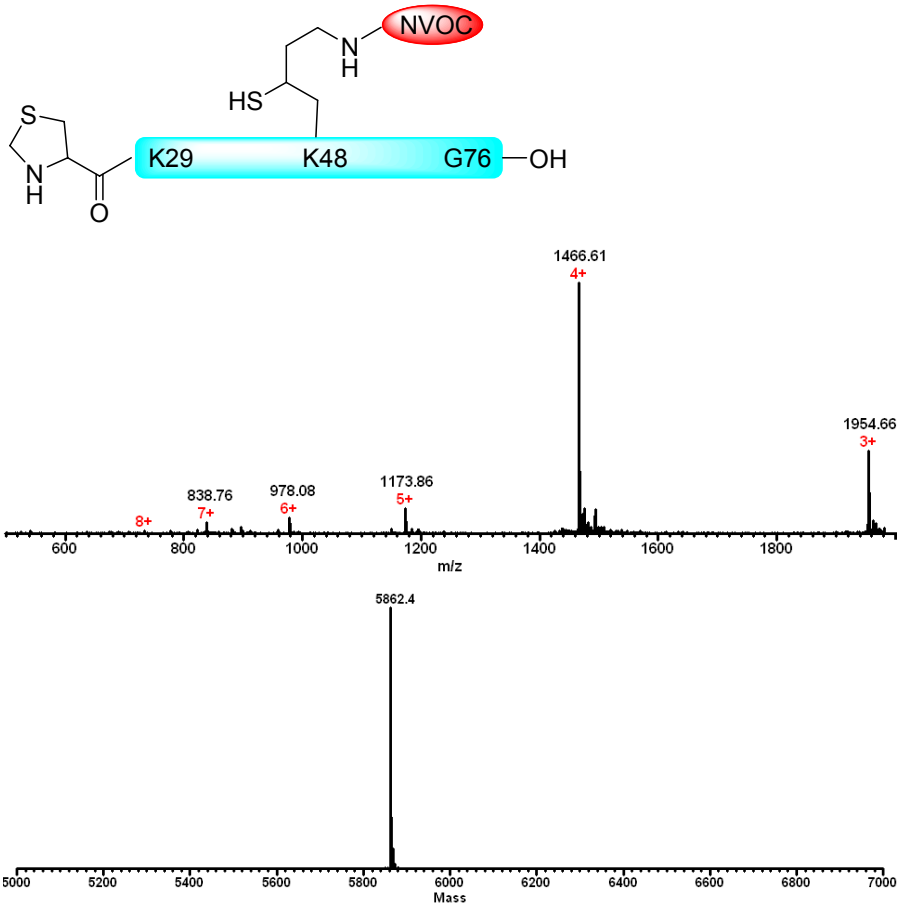


Figure S5: ESI-MS of Thz28-K48(4-SH, NVOC)-G76-OH, the ligation product of **3** and **4**. calculated M = 5861.6 Da, deconvoluted M = 5862.4 Da.

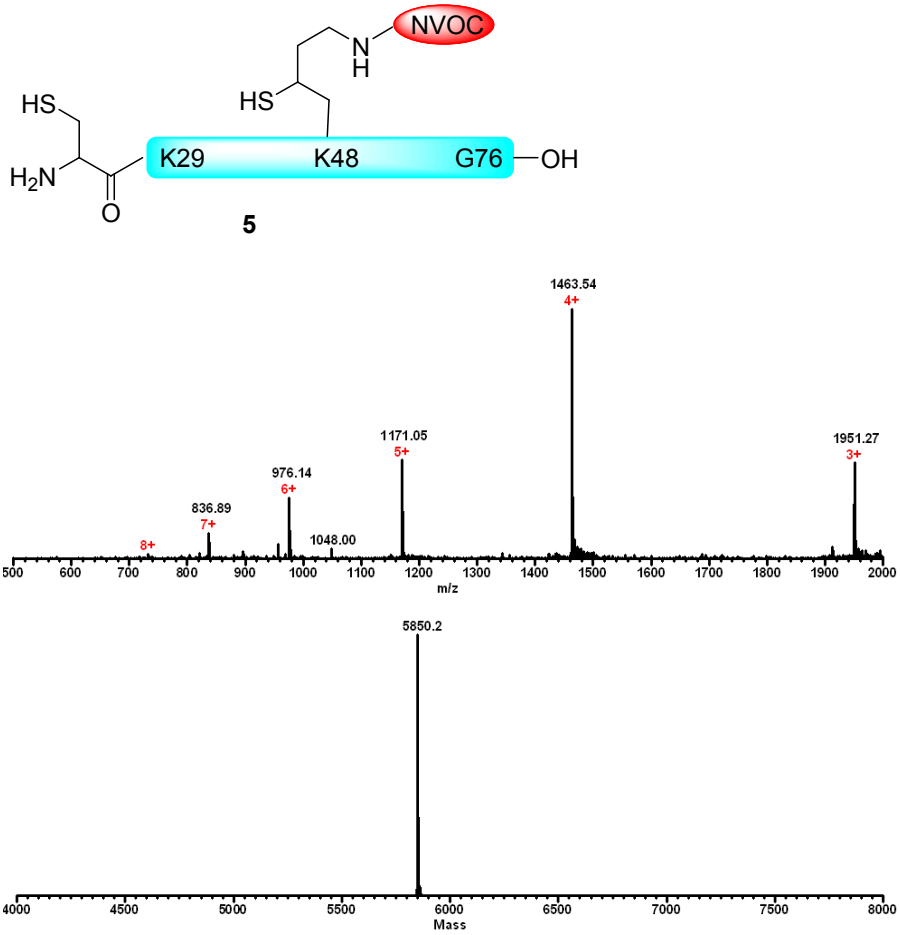
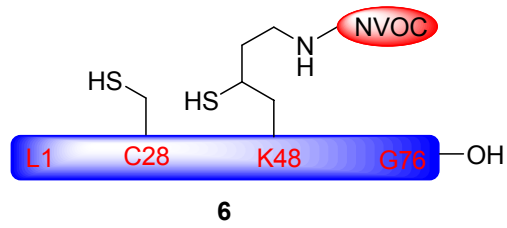


Figure S6: ESI-MS of C28-K48(4-SH, NVOC)-G76-OH (**5**), the Thz deprotection product.

Calculated M = 5849.6 Da, deconvoluted M = 5850.2 Da.



6

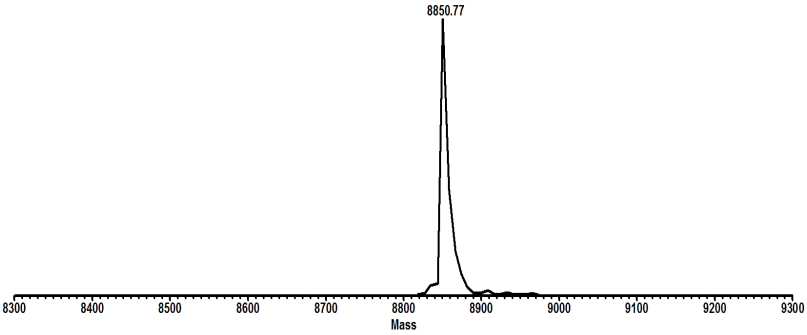


Figure S7: The deconvoluted ESI-MS profile of **6**. Calculated M = 8850.0 Da, deconvoluted M = 8850.8 Da. The ESI-MS before deconvolution was shown in main text (**Fig.3**, left panel).

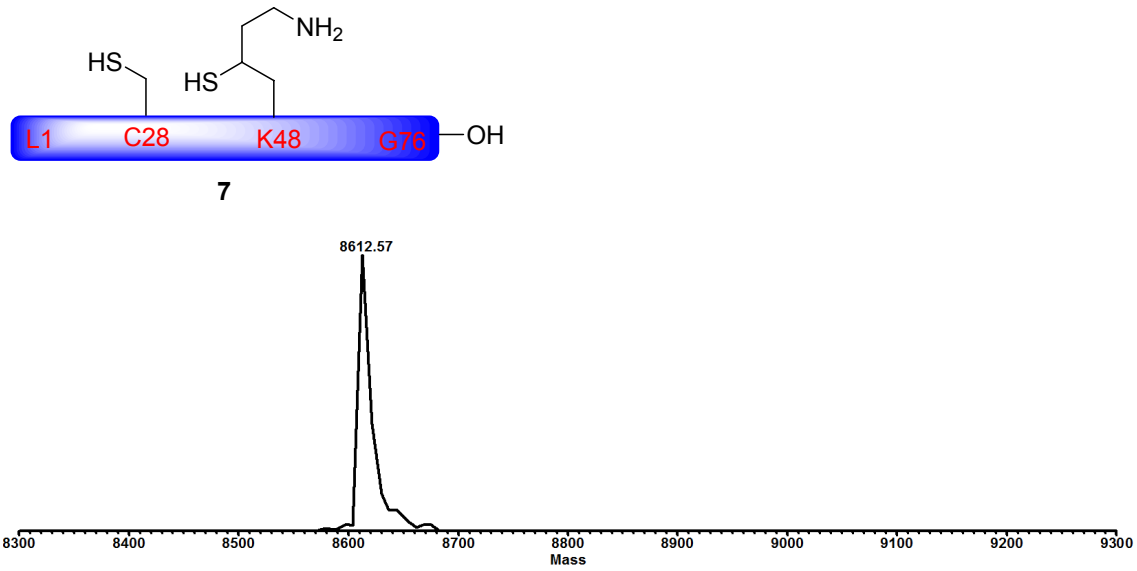


Figure S8: The deconvoluted ESI-MS profile of **7**. Calculated M = 8610.8 Da, deconvoluted M= 8612.6 Da. The ESI-MS before deconvolution was shown in main text (**Fig. 3**, right panel).

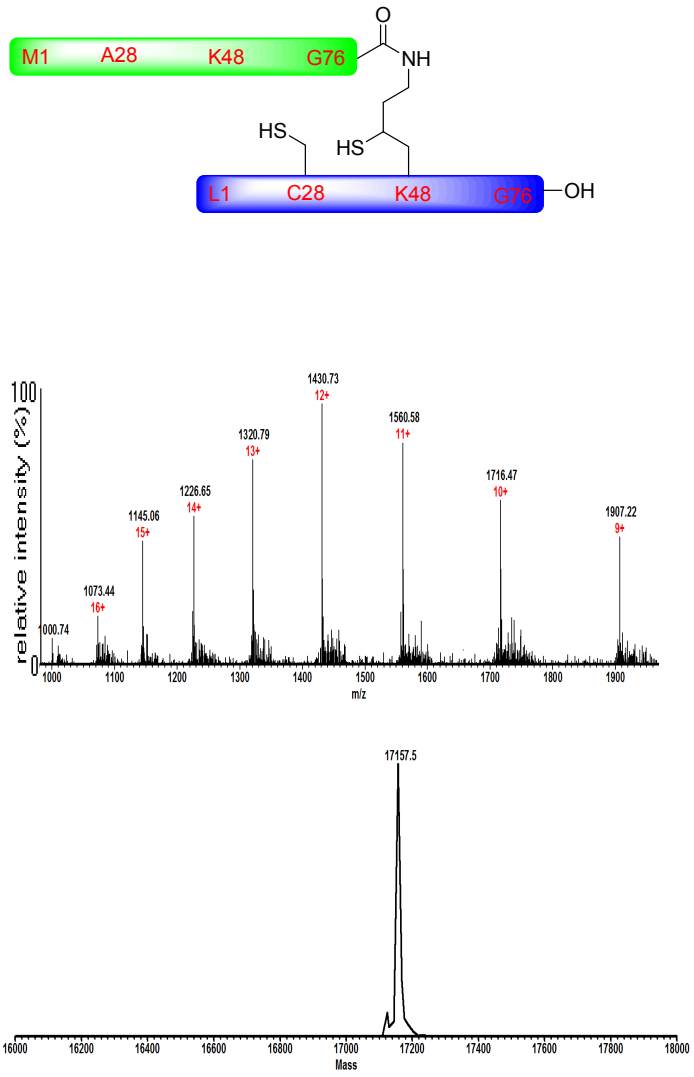


Figure S9: ESI-MS of sulfur containing diubiquitin (before desulfurization). Calculated M = 17157.6 Da, deconvoluted M = 17157.5 Da.

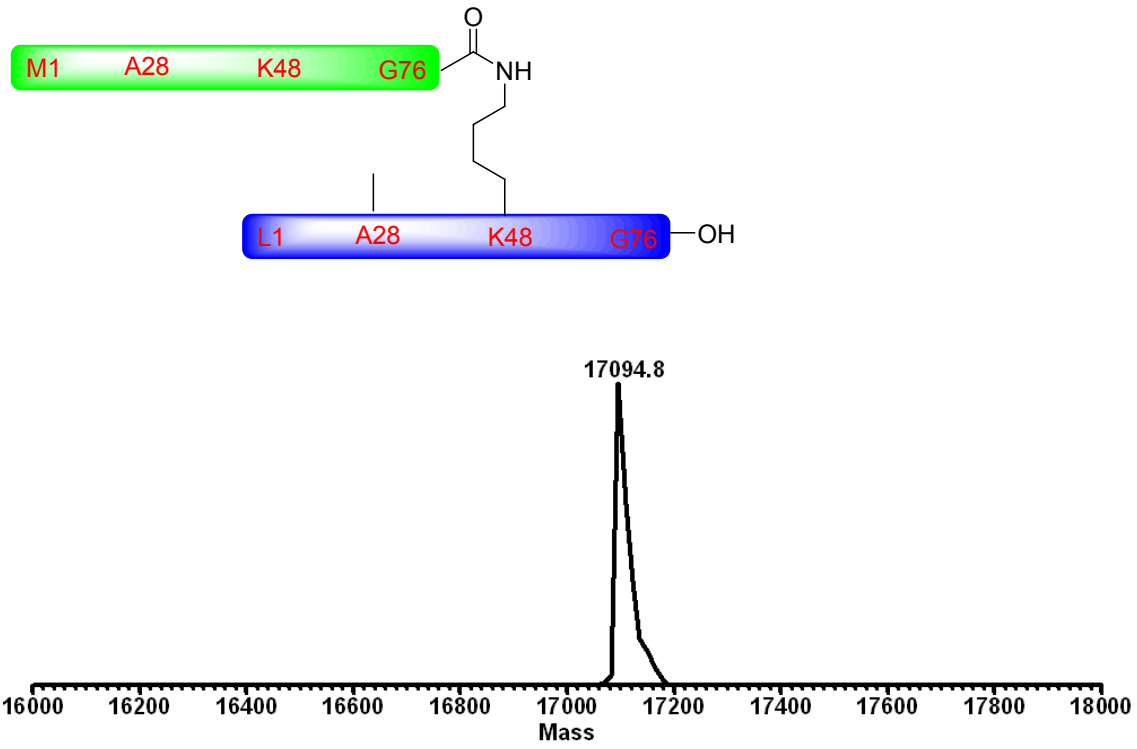


Figure S10: The deconvoluted ESI-MS of K48-linked diubiquitin **8**. Calculated molecular weight 17093.4 Da, deconvoluted molecular weight 17094.8 Da. The ESI-MS data before deconvolution is shown in Fig. 5C in the main text.

Ubiquitin C-terminal hydrolase assay:

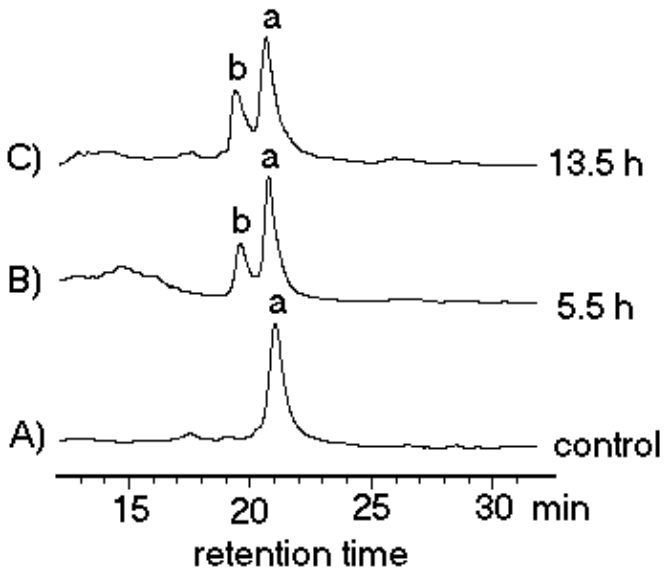
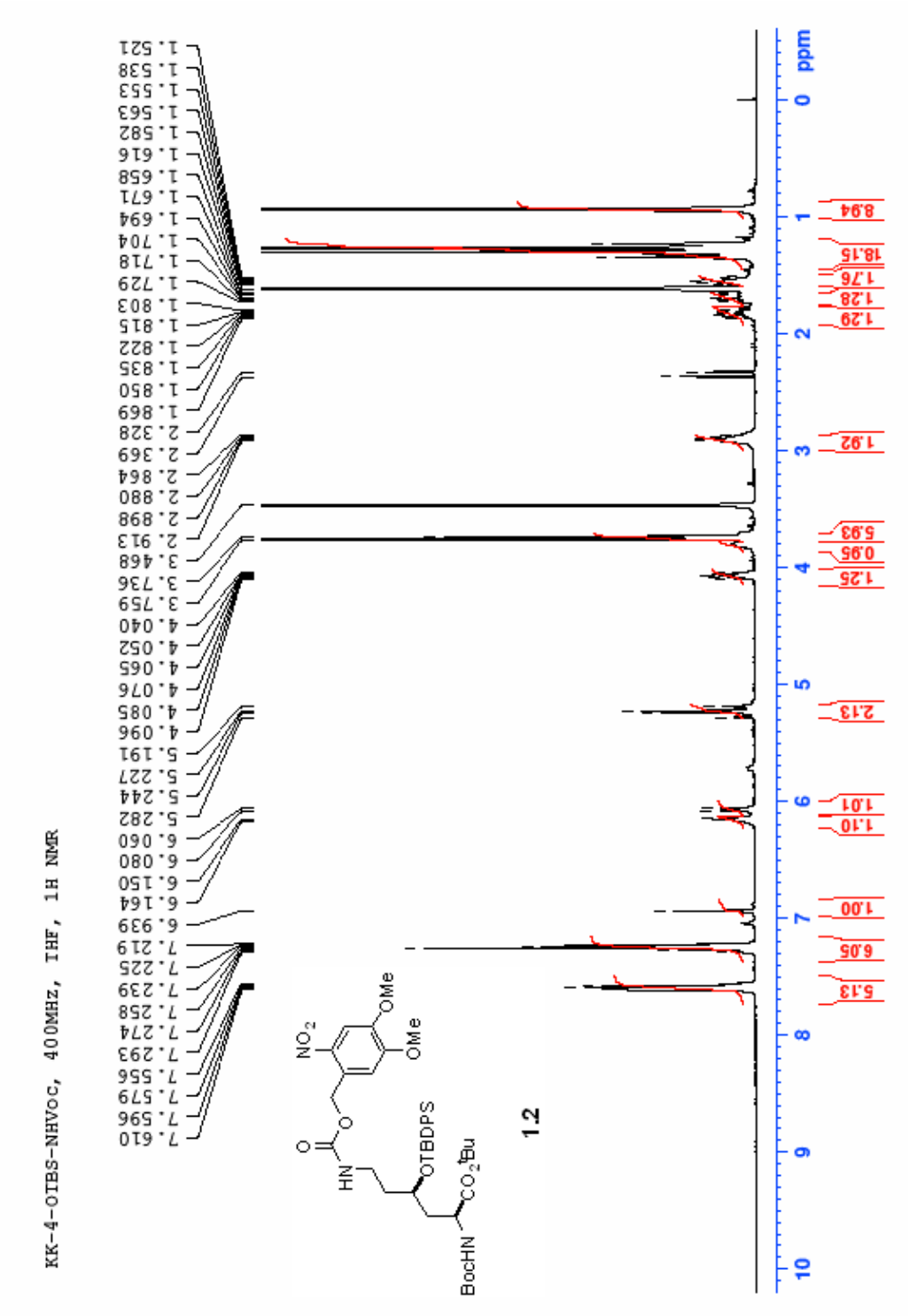


Figure S11: C4 analytic HPLC monitored the hydrolysis of K48-linked diubiquitin catalyzed by ubiquitin C-terminal hydrolase, UCH-L3, at 0 h, 5.5 h and 13.5 h, respectively. Peak a, K48-linked diubiquitin; peak b, the mixture of ubiquitin (M1-G76)-OH and ubiquitin (L1-G76)-OH. The left proportion of peak b is ubiquitin (M1-G76)-OH (calculated MW = 8564.7 Da, MOLAI-TOF detected $[M+H]^+$ = 8566.1) and the right proportion of the peak b is ubiquitin (L1-G76)-OH (calculated MW = 8546.7 Da, MOLAI-TOF detected $[M+H]^+$ = 8547.3).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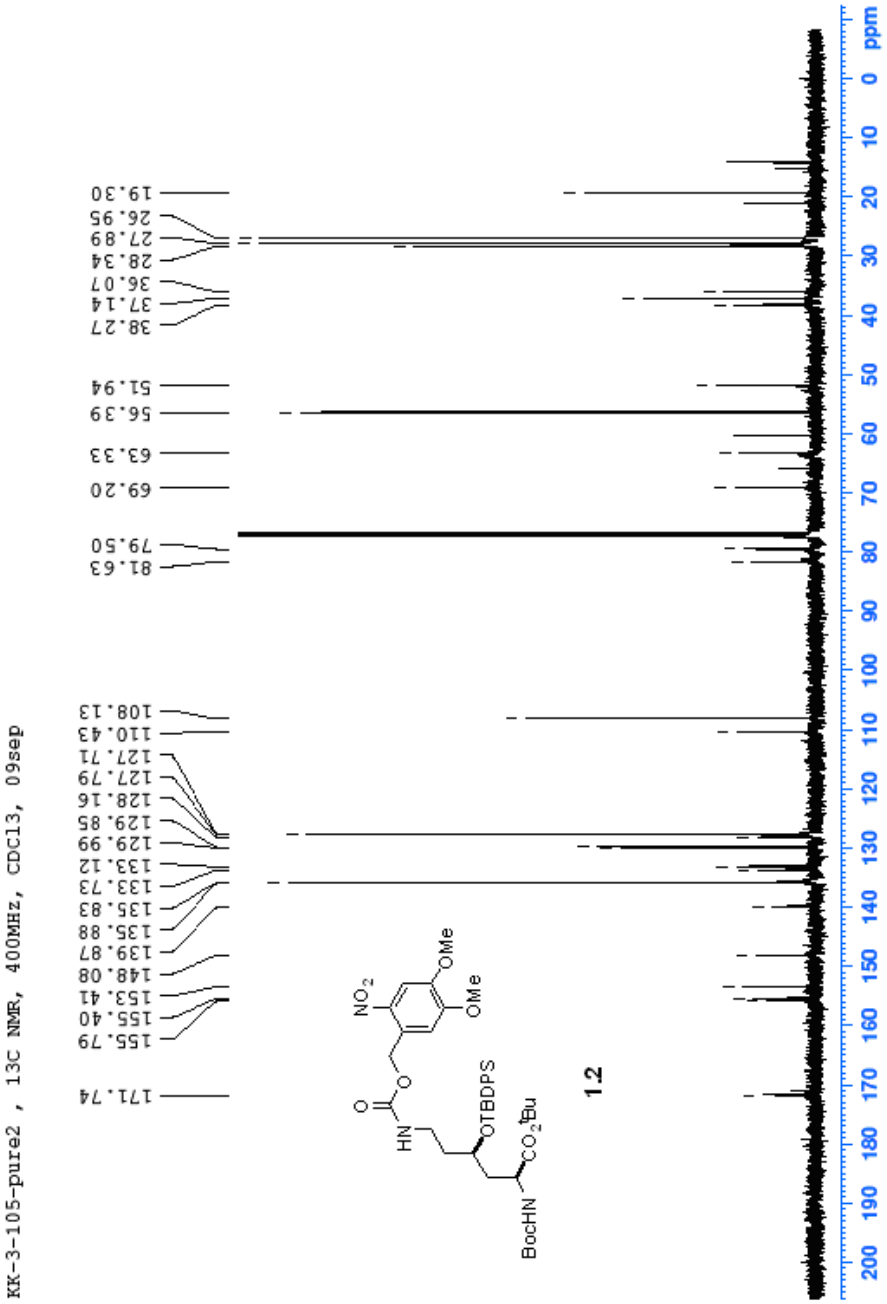
1.2
ppm


KK-3-105-pure2 , 13C NMR, 400MHz, CDCl3, 09sep
19.30
26.95
27.89
28.34
36.07
37.14
38.27
51.94
56.39
63.33
69.20
79.50
81.63
108.13
110.43
127.71
127.79
128.16
129.85
129.99
133.12
133.73
135.83
135.88
139.87
148.08
153.41
155.40
155.79
171.74
ppm
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
NO2
OMe
OMe
O
O
HN
OTBDPS
CO2tBu
BocHN
12

Elemental Composition Report

Single Mass Analysis

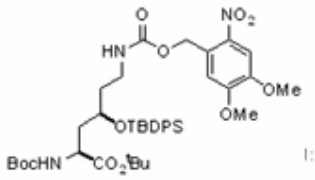
Tolerance = 50.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
127 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)
Elements Used:
C: 41-41 H: 58-58 N: 3-3 O: 11-11 152Gd: 0-1 154Gd: 0-1 155Gd: 0-1 156Gd: 0-1 157Gd: 0-1 158Gd: 0-1
160Gd: 0-1 Si: 0-1

C41H57N3O11Si, MW795

HAB333

Lys-OTBS 5 (0.119) Cm (5:9)



1.2

11:18:18
07-Oct-2009
I: TOF MS ES+
5.75e+003

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Maximum:		5.0	50.0	50.0			
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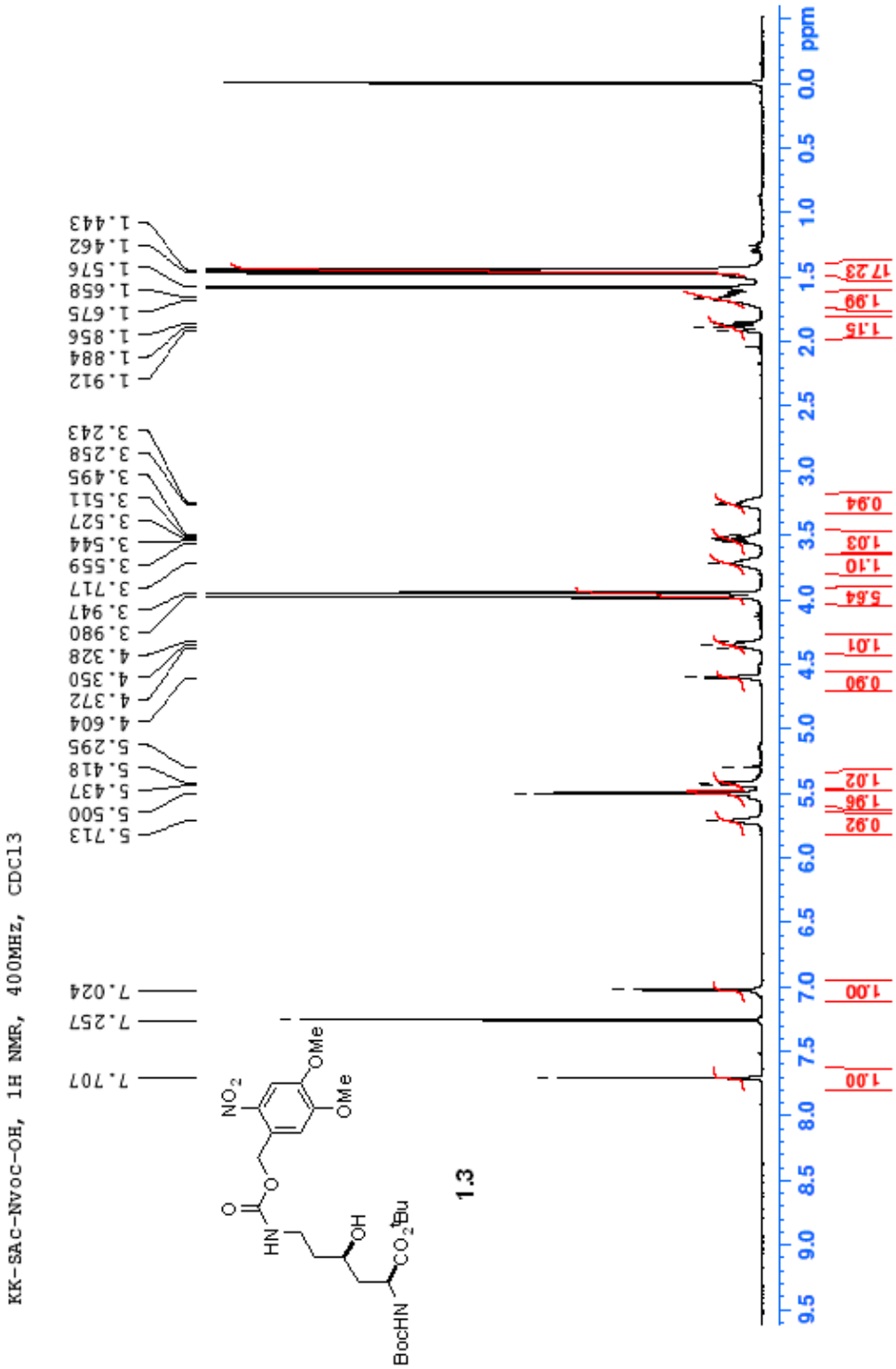
KK-SAc-Nvoc-OH, 1H NMR, 400MHz, CDCl3
1.443
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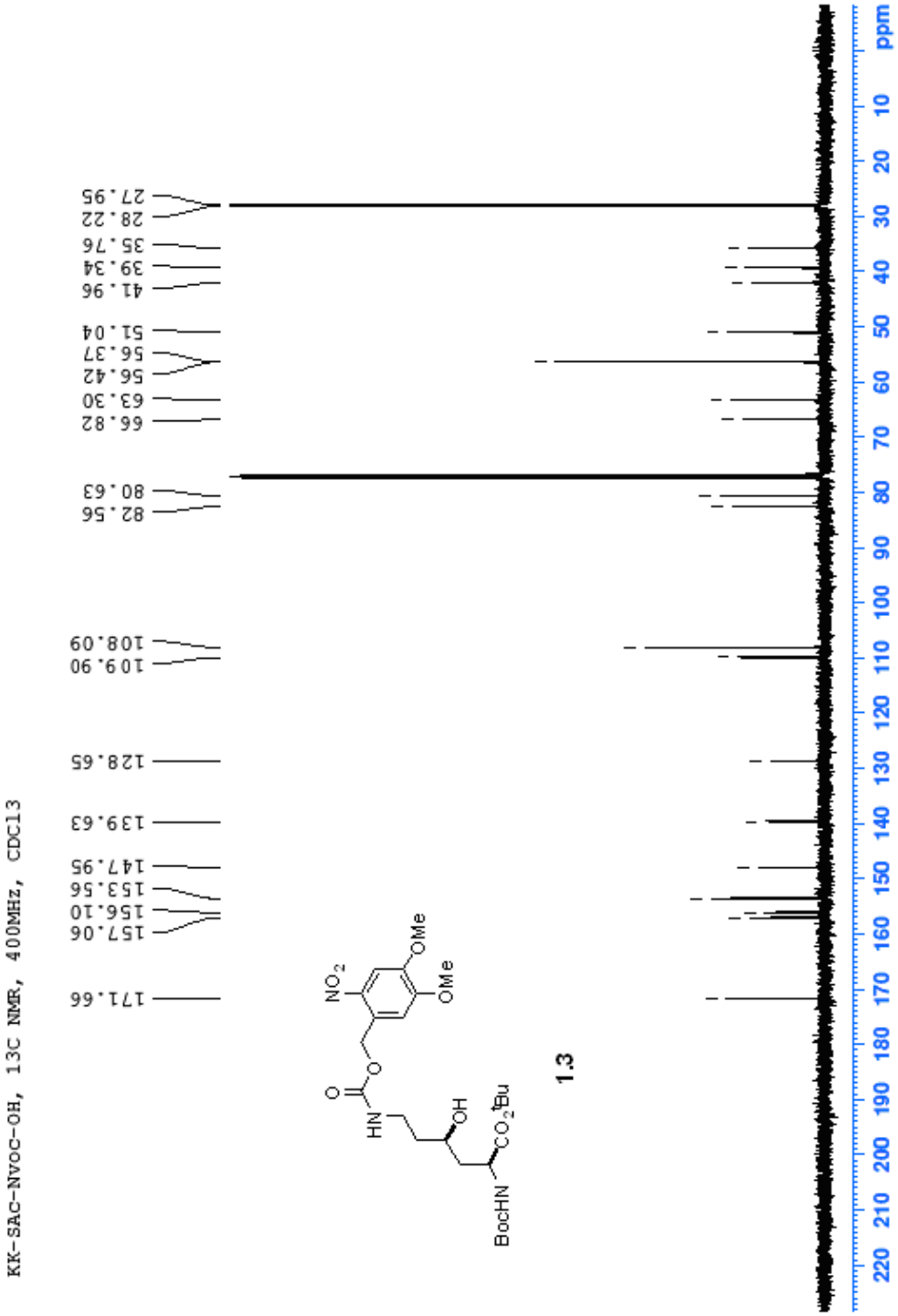
1.576
1.658
1.675
1.856
1.884
1.912
3.243
3.258
3.495
3.511
3.527
3.544
3.559
3.717
3.947
3.980
4.328
4.350
4.372
4.604
5.295
5.418
5.437
5.500
5.713
7.024
7.257
7.707
1.3
NO2
OMe
OMe
O
HN
O
OH
CO2tBu
BocHN
ppm
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
17.23
1.99
1.15
0.94
1.03
1.10
5.84
1.01
0.90
0.92
1.96
1.02
1.00
1.00


KK-SAC-NVOC-OH, 13C NMR, 400MHz, CDCl3
27.95
28.22
35.76
39.34
41.96
51.04
56.37
56.42
63.30
66.82
80.63
82.56
108.09
109.90
128.65
139.63
147.95
153.56
156.10
157.06
171.66
ppm
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
210
220
NO2
OMe
OMe
O
HN
O
OH
CO2tBu
BocHN
1.3

Elemental Composition Report

Single Mass Analysis

Tolerance = 50.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
29 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)
Elements Used:
C: 25-25 H: 40-40 N: 3-3 O: 11-11 152Gd: 0-1 154Gd: 0-1 155Gd: 0-1 156Gd: 0-1 157Gd: 0-1 158Gd: 0-1
160Gd: 0-1

C25H39N3O11, MW557

HAB333

11:29:22
07-Oct-2009
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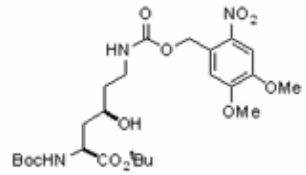
9.34e+003

Lys-OH 3 (0.082) Cm (2:8)

558.2654



1.3

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Maximum:		5.0	50.0	50.0			
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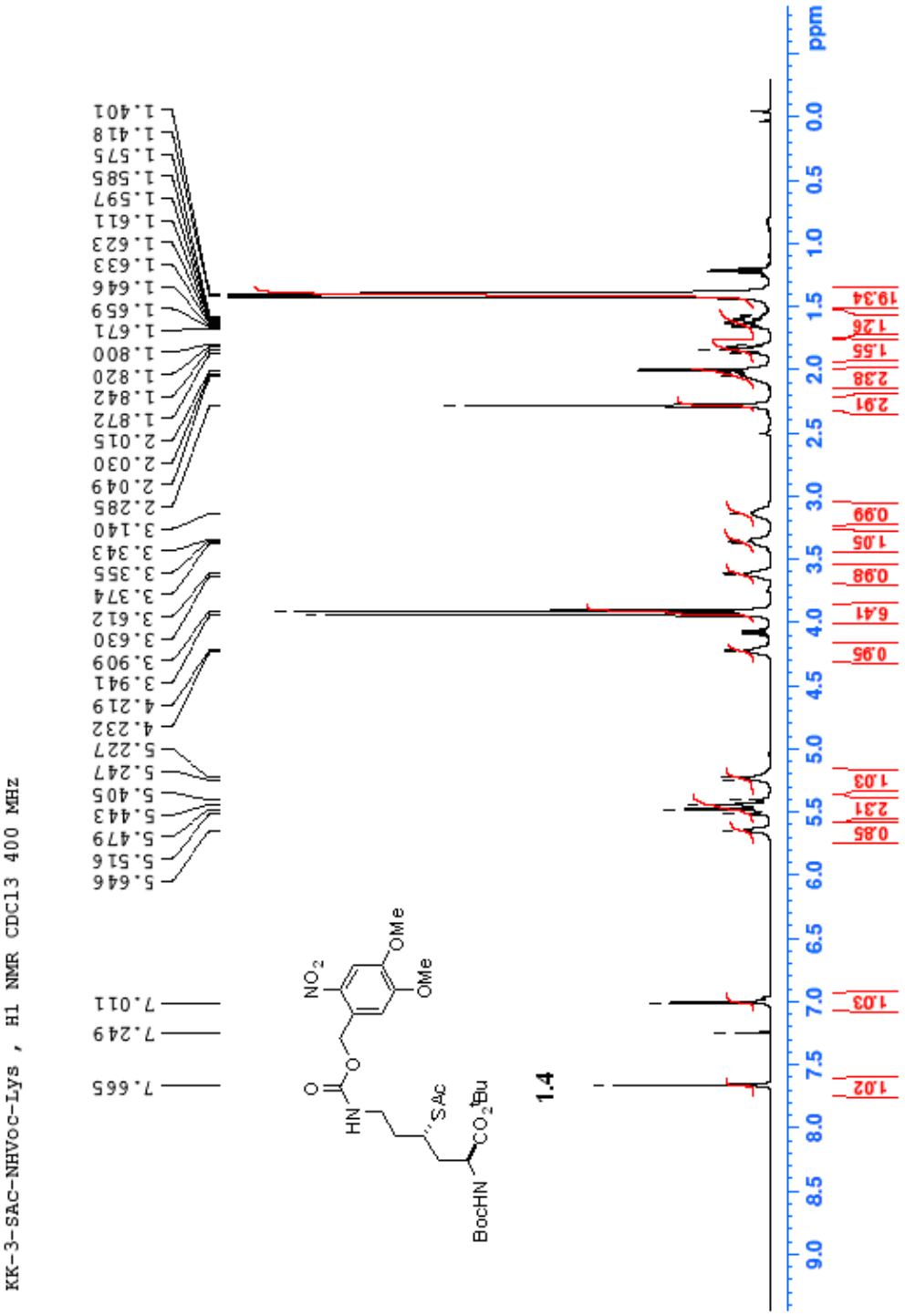
KK-3-SAc-NHVoc-Lys , H1 NMR CDCl3 400 MHz
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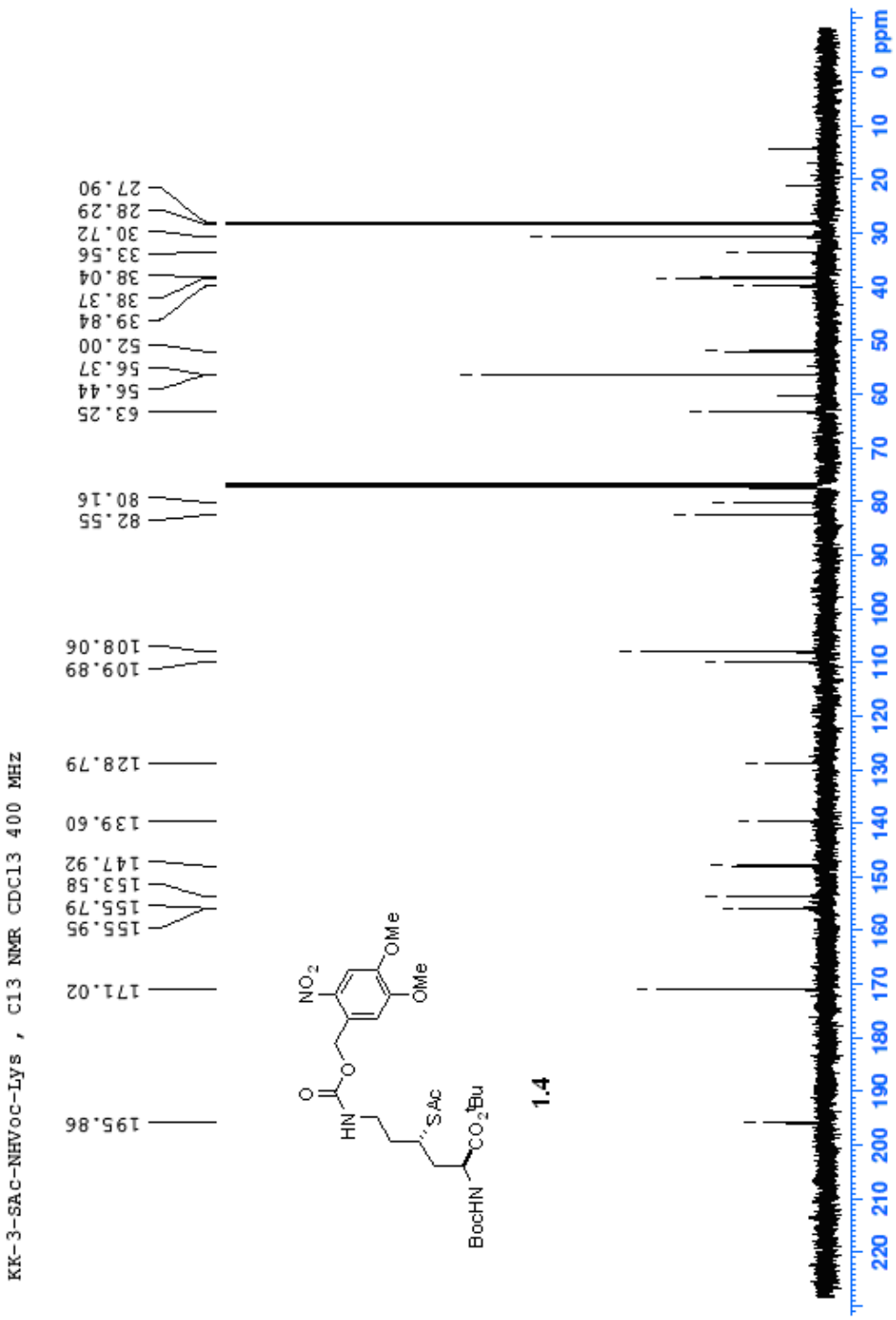
OMe
OMe
HN
O
SAc
CO2tBu
BocHN
ppm


KK-3-SAc-NHVoc-Lys , C13 NMR CDCl3 400 MHz
27.90
28.29
30.72
33.56
38.04
38.37
39.84
52.00
56.37
56.44
63.25
80.16
82.55
108.06
109.89
128.79
139.60
147.92
153.58
155.79
155.95
171.02
195.86
NO2
OMe
OMe
O
HN
SAc
CO2tBu
BocHN
1.4
0 ppm
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
210
220

Elemental Composition Report Page 1

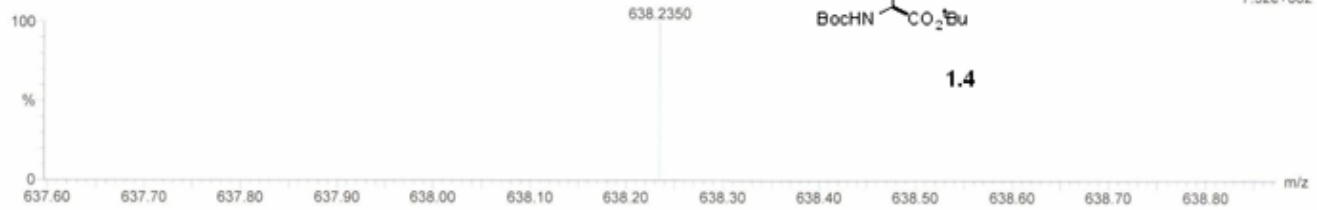
Single Mass Analysis
Tolerance = 50.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

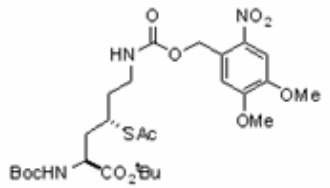
Monoisotopic Mass, Even Electron Ions
29 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)
Elements Used:
C: 21-27 H: 24-41 N: 0-3 O: 3-11 S: 1-1 Na: 1-1

C27H41N3O11S, MW615 HAB333

Lys-SAc 6 (0.138) Cm (6:10)

11:40:18
07-Oct-2009
1: TOF MS ES+
7.52e+002





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Maximum:		5.0	50.0	50.0			
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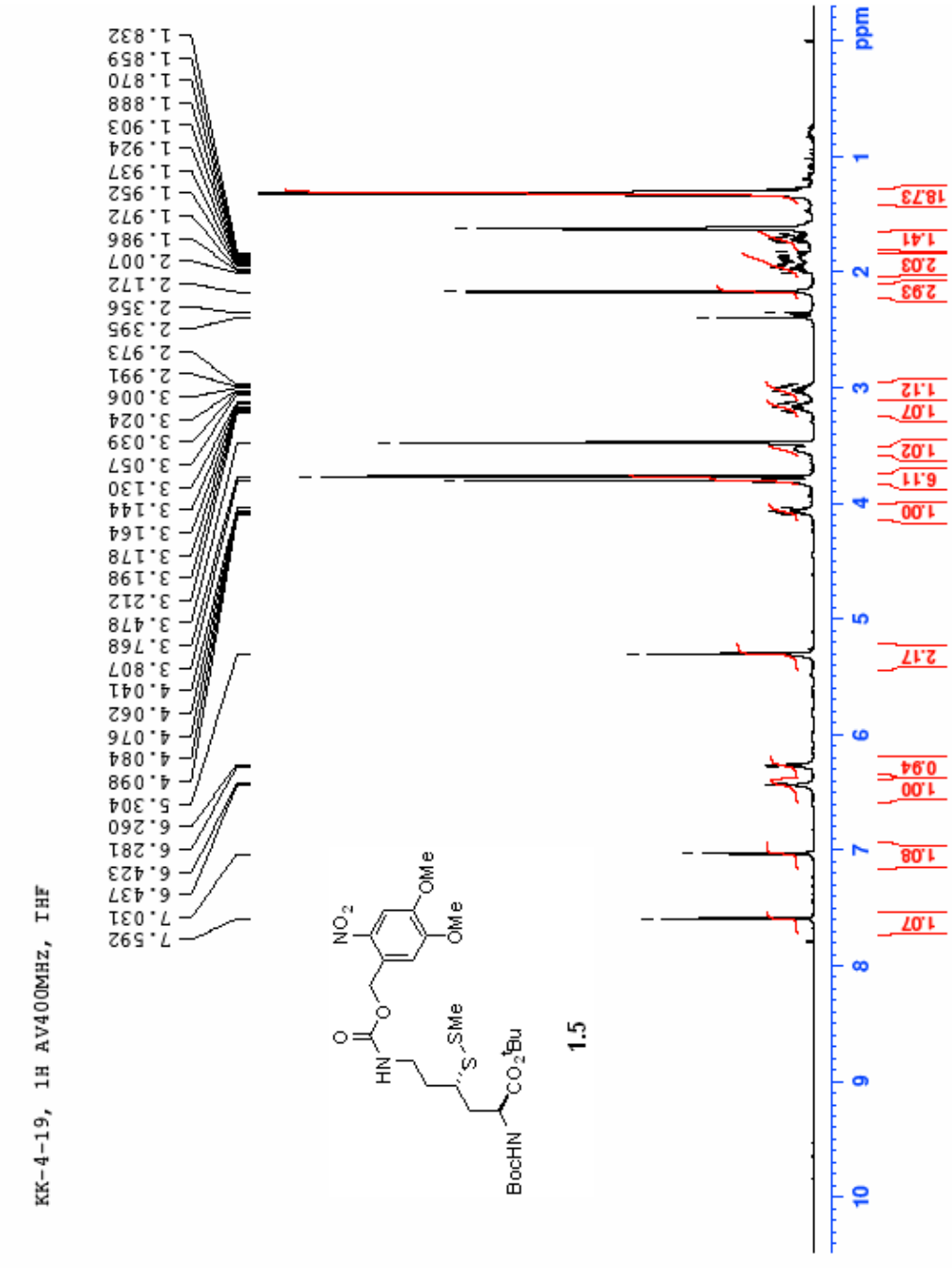
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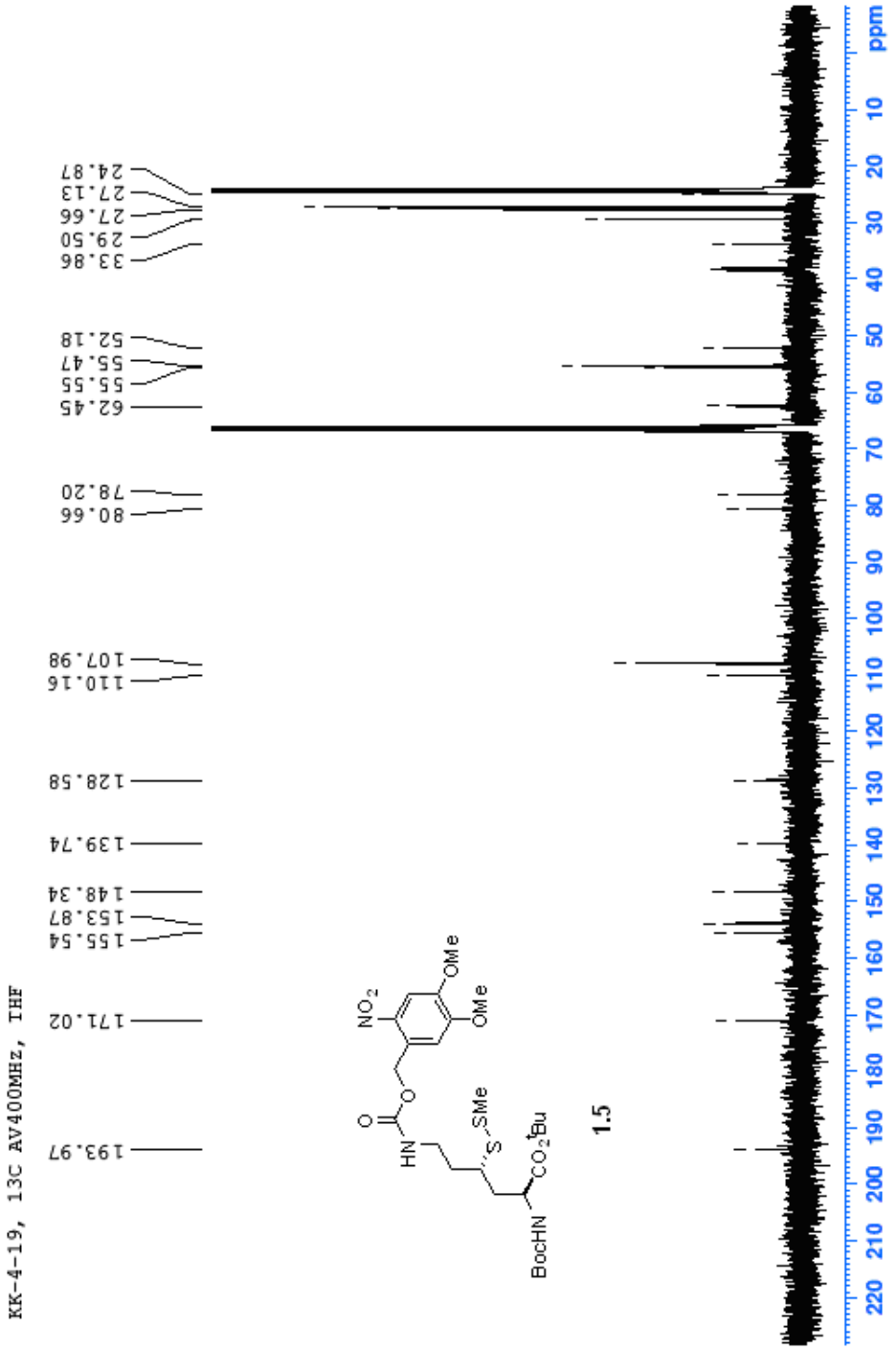
HN
SMe
OMe
OMe
NO2
ppm


KK-4-19, 13C AV400MHz, THF
24.87
27.13
27.66
29.50
33.86
52.18
55.47
55.55
62.45
78.20
80.66
107.98
110.16
128.58
139.74
148.34
153.87
155.54
171.02
193.97
ppm
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
210
220
NO2
OMe
OMe
O
O
HN
SMe
S
CO2tBu
BocHN
1.5

Elemental Composition Report Page 1

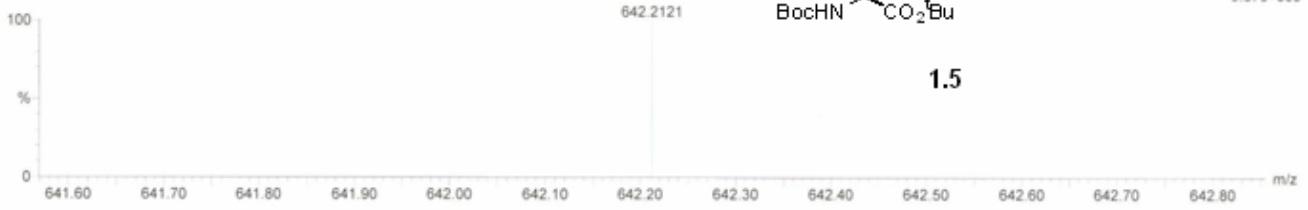
Single Mass Analysis
Tolerance = 50.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

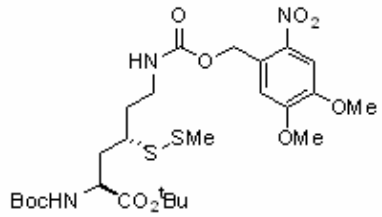
Monoisotopic Mass, Even Electron Ions
57 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)
Elements Used:
C: 21-26 H: 24-41 N: 0-3 O: 3-10 Na: 1-1 S: 1-2

C26H41N3O10S2, MW619 HAB333

Lys-SSMe 4 (0.101) Cm (4:9)

11:51:13
07-Oct-2009
1: TOF MS ES+
9.87e+003





1.5

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Maximum:		5.0	50.0	50.0			
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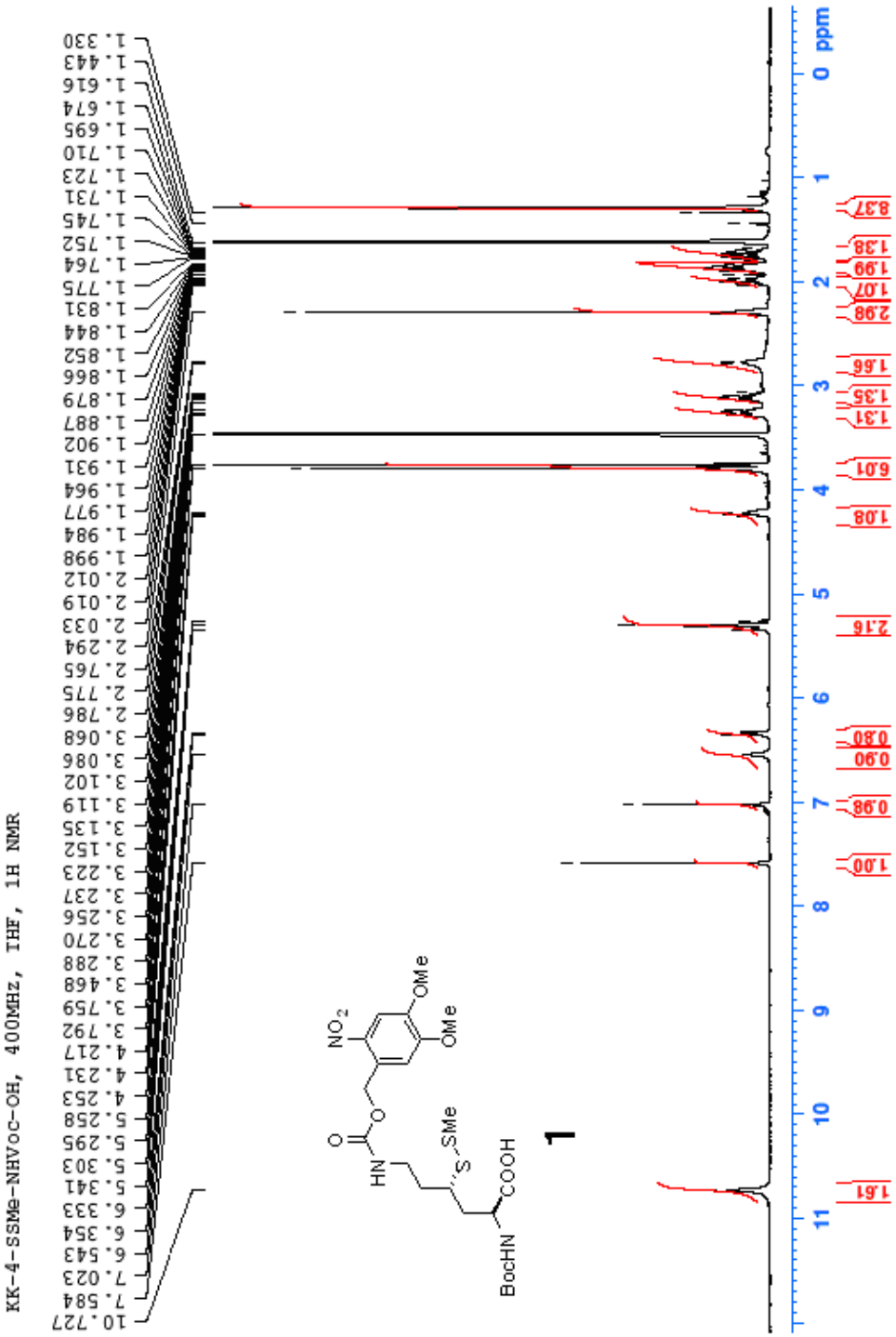
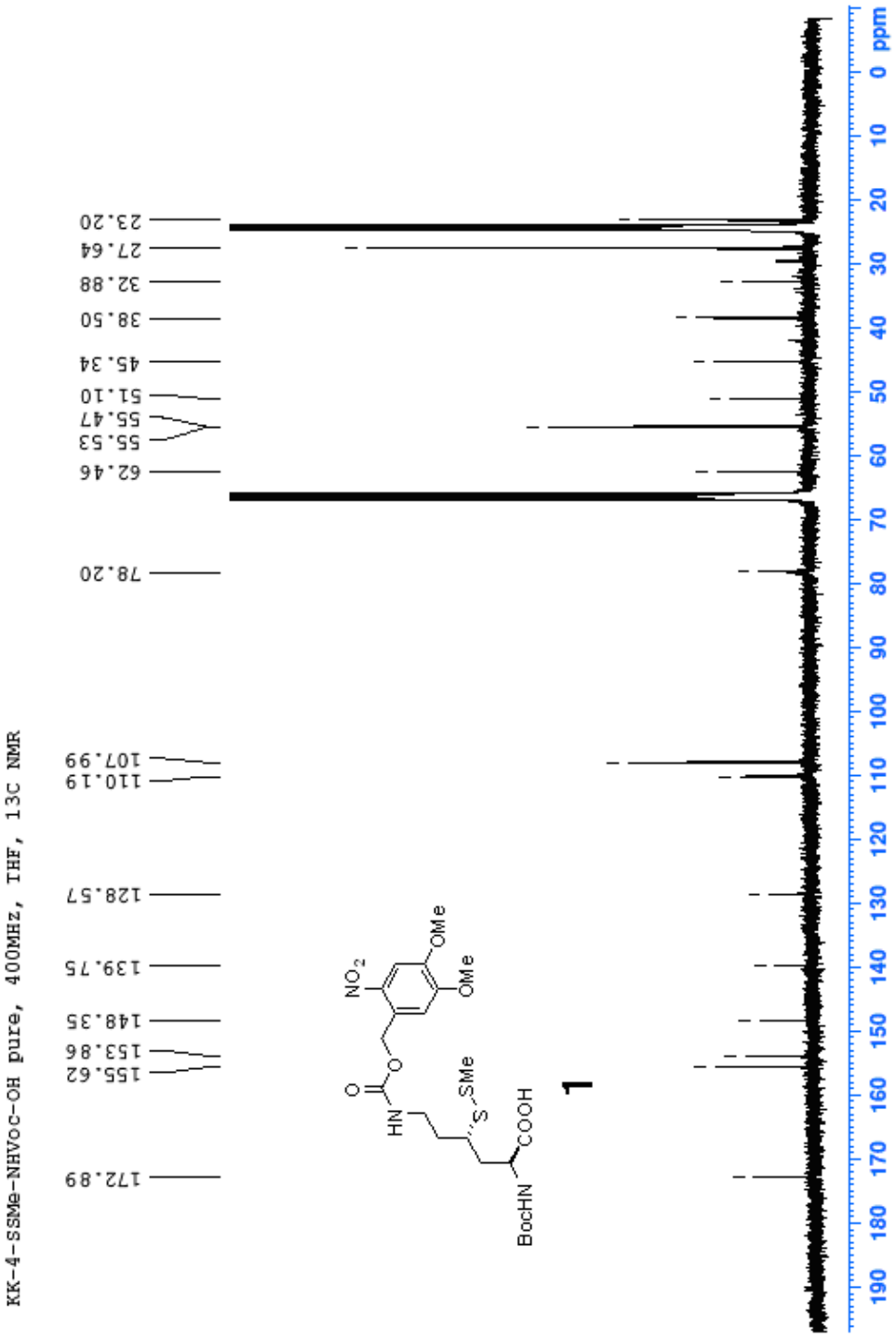

KK-4-SSMe-NHVoc-OH, 400MHz, THF, 1H NMR
ppm
8.37
1.38
1.99
1.07
2.98
1.66
1.35
1.31
6.01
1.08
2.16
0.80
0.90
0.98
1.00
1.61
NO2
OMe
OMe
O
HN
S
SMe
BocHN
COOH
1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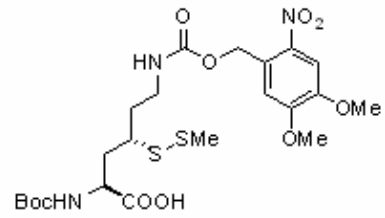
27.64
32.88
38.50
45.34
51.10
55.47
55.53
62.46
78.20
107.99
110.19
128.57
139.75
148.35
153.86
155.62
172.89
ppm
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
NO2
OMe
OMe
O
O
HN
SMe
S
COOH
BocHN
1

Elemental Composition Report Page 1

Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
257 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)
Elements Used:
C: 7-22 H: 0-35 N: 0-3 O: 0-10 Si: 0-1 S: 0-2
C18H22N2O2 MW 298
LML126 2 (0.064)



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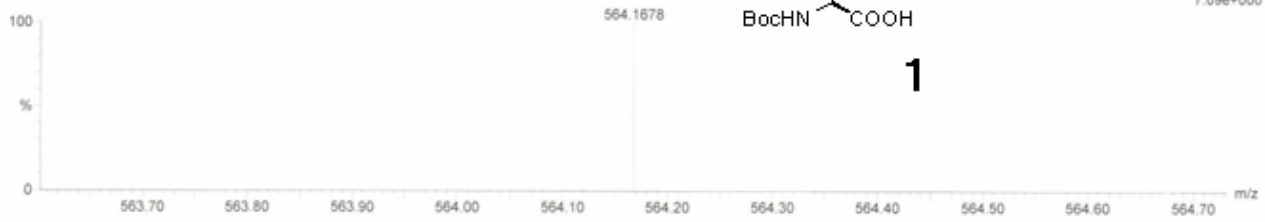

Minimum:				-1.5			
Maximum:		5.0	5.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
564.1678	564.1686	-0.8	-1.4	7.5	13.4	0.0	C22 H34 N3 O10 S2