Supporting Information

1. Materials and general methods

Chemical materials: All chemicals and solvents were purchased from *Sigma-Aldrich, ChemImpex or J. T. Baker* and used without further purification. Reactions were monitored on *Merck* Silica 60 F254 TLC plates. Flash column chromatography was performed with flash silica gel (230-400 mesh) from *Fisher Scientific Inc*. The synthesized compounds were re-purified on the 971-FP Flash Purification System (*Agilent Technologies*). Detection was done by irradiation with UV light (254 nm or 366 nm) and/or staining with ninhydrin solution (0.3% w/v in glacial acetic acid/n-butanol 3: 97). NMR spectra were recorded on the following spectrometers: *Agilent* DD2 400 (G8303A). The chemical shifts (δ) are given in ppm, the coupling constants (*J*) in Hz. The peak assignments were done using 2D spectra. Mass spectra were recorded on the following machines: *Agilent* LCMS Q ESMS (6120B).

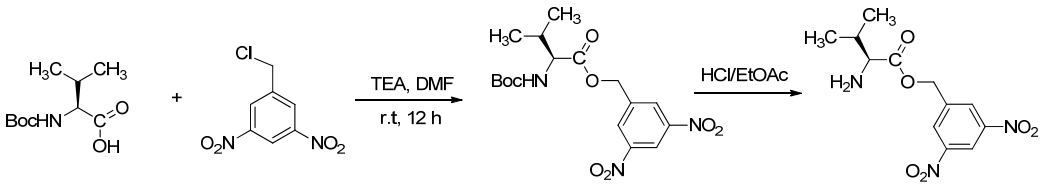
Biological materials: The commercial human histone H3 was purchased from BioLabs (Catalog #: M2507S). The MagicMark XP Western Protein Standard was taken from Life Technologies. The west-blotting of antibody against histone H3 (3H1) Rabbit mAb was from Cell Signaling (Catalog #:5192, HRP Conjugate), was tested against every histone modifications (H3wt, H3ThioAcK9, H3ThioAcK14, H3ThioAcK36 and H3ThioAcK56, H3AcK14/K56ThioAcK, H3ThioAcK14/K56ThioAcK, H3ThioAcK36/K56ThioAcK). Anti-acetyl histone H3K9 (Catalog #: 9671) and H3K56 (Catalog #: 4243) were purchased from Cell Signaling (Polyclonal Antibody). Proteins were visualized using a Donkey Anti-Rabbit IgG secondary antibody (Catalog #: AP182P, MILLIPORE Inc.) conjugated to HRP. The Western blotting was performed following the procedures recommended by Cell Signaling Inc.

LC-MS/MS analyses

The coomassie blue stained SDS-gel protein was de-stained, washed twice for 10 min with water, and cut in small fragments. The gel slices of proteins containing histone H3 were trypsin digested by a standard in-gel digestion protocol, and analyzed by LC-MS/MS on a LTQ Orbitrap XL (Thermo Scientific) equipped with a nanoACQUITY UPLC system (Waters). A Symmetry C18 trap column (180 µm x 20 mm; Waters) and a nanoACQUITY UPLC column (1.7 µm, 100 µm x 250 mm, 35°C) were used for peptide separation. Trapping was done at 15 µL min^{-1}, 99% buffer A (water with formic acid (0.1 %)) for 1 min. Peptide separation was performed at 300 nL min^{-1} with buffer A and buffer B (CH_3CN containing 0.1% formic acid). The linear gradient (51 min) was from 5% buffer B to 50% B in 50 min, to 85% B at 51 min. MS data were acquired in the Orbitrap with one microscan, and a maximum inject time of 900 ms followed by data-dependent MS/MS acquisitions in the ion trap (through collision induced dissociation, CID). The Mascot search algorithm was used to search for the appropriate non-canonical substitution (Matrix Science, Boston, MA).

2. Synthesis of the compounds

2.1 Valine 3,5-dinitrobenzyl ester (1):



3,5-dinitrobenzyl chloride

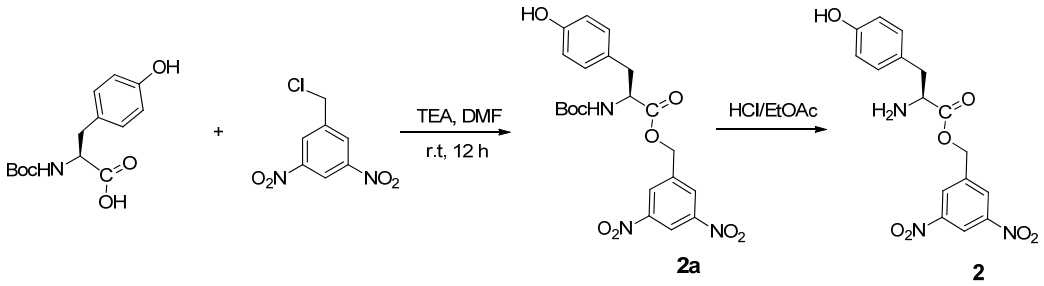
1a **1**

General procedure for synthesis of valine-DBE was modified slightly as previously described: [1]

A mixture of N^{α}-Boc-valine (1.3 g, 6 mmol), triethylamine (1.0 g, 10 mmol) and 3,5-dinitrobenzyl chloride (1.1 mg, 5 mmol) in 1 mL of dimethylformamide was stirred vigorously in the dark at r.t for 12 h. And then, 90 mL of diethylether was added and the solution was washed with 0.5 M HCl (20 mL x 3), saturated $NaHCO_3$ (20 mL x 3) and saturated NaCl (20 mL), and the organic layer was dried over $MgSO_4$. The solvent was evaporated to dryness, and the residue was applied to FC (silica gel, column 20 × 3 cm, eluted with CH_2Cl_2/MeOH 98 : 2 → 95 : 5). The solvent was evaporated to dryness and to give **1a** (1.23 g, 62%).

All of the above purified compound **1a** was dissolved in 20 mL of 4 M HCl/EtOAc and incubated for 20 min at room temperature The solution was concentrated under vacuum and the remained HCl was removed by repeating the addition of diethylether (20 mL x 2) and was evaporated to dryness. The product was precipitated by the addition of diethylether (30 mL) and the precipitants were filtered in 89% yield of **1** (820 mg, 2.76 mmol). ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 8.80 (s, 1H), 8.74 (br, 3H), 8.71 (s, 2H), 5.50 (s, 2H), 3.99 (s, J = 4.6 Hz, 1H), 2.24 (m, 1H), 1.00 (d, J = 12.0 Hz, 3H), 0.96 (d, J = 8.0 Hz, 3H).

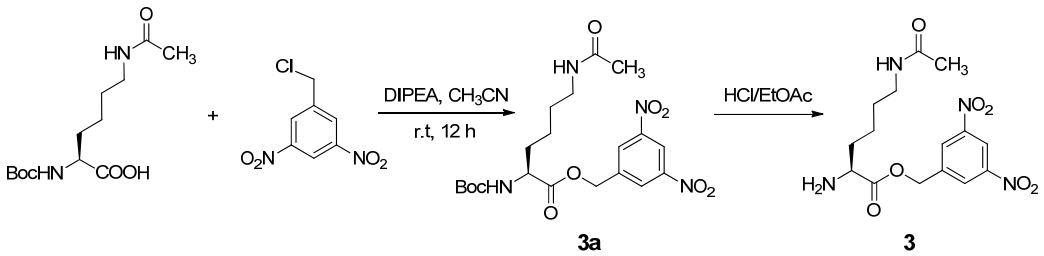
2.2 Valine 3,5-dinitrobenzyl ester (2):



A mixture of N^{α}-Boc-tyrosine (8.5 g, 30 mmol), triethylamine (5.1 g, 50 mmol) and 3,5-dinitrobenzyl chloride (5.4 mg, 25 mmol) in 15 mL of dimethylformamide was stirred vigorously in the dark at room temperature for 12 h. To stop reaction, 150 mL of diethylether was added and the solution was washed with 0.5 M HCl (50 mL x 2), saturated $NaHCO_3$ (50 mL x 2) and saturated NaCl (50 mL), and the organic layer was dried over $MgSO_4$. The solvent was evaporated to dryness, and the residue was applied to FC (silica gel, column 8 × 3 cm, eluted with CH_2Cl_2/MeOH 98 : 2 → 95 : 5). The solvent was evaporated to dryness and to give **2a** as pale-yellow powder (8.2 g, 71%). ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 8.96 (s, 1H), 8.36 (s, 2H), 6.92-6.94 (br, 2H), 6.63-6.65 (br, 2H), 5.20 (s, 2H), 4.54-4.99 (m, 1H), 2.93 (m, 1H), 3.01 (m, 1H), 1.40 (s, 9H); ^{13}C-NMR (400 MHz, DMSO-d_6) δ: 171.9, 155.2, 148.5, 139.5, 130.2, 128.0, 127.2, 118.6, 115.5, 80.6, 64.4, 55.0, 37.6, 28.2.

The purified compound **2a** (350 mg, 0.76 mmol) was dissolved in 8 mL of 4 M HCl/EtOAc and the mixture was stirred for 4 h at room temperature The solution was concentrated under vacuum and the remained HCl was removed by repeating the addition of diethylether (10 mL x 3). The product was precipitated by the addition of diethylether (10 mL) and the precipitants were filtered in 92% yield of **2** (280 mg, 0.70 mmol). ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 9.29 (s, 1H), 8.56 (s, 2H), 6.92-6.94 (br, 2H), 6.53-6.55 (br, 2H), 5.37 (s, 2H), 4.27 (m, 1H), 2.93 (m, 1H), 3.08 (m, 1H).

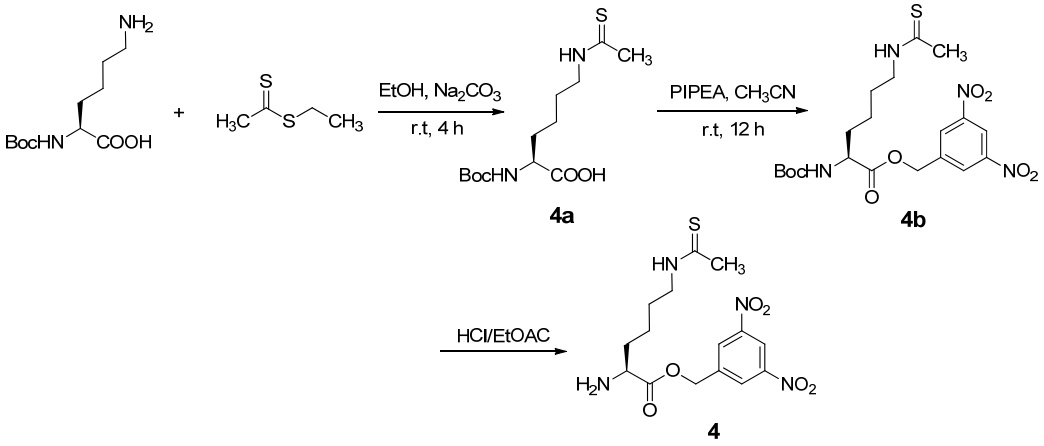
2.3 N^{ε}-acetyl-lysine 3,5-dinitrobenzyl ester (3):



N^{α}-Boc-N^{ε}-acetyl-L-lysine (8.65 g, 30 mmol) was dissolved in 80 mL of CH_3CN and treated with 14 mL (80 mmol) of *N*,*N*-diisopropylethylamine (DIPEA) followed by 3,5-dinitrobenzyl chloride (7.8 g, 36 mmol). The reaction mixture was stirred at r.t for 12 h and then treated with 100 mL of ethyl acetate. The organic phase was washed successively with a saturated $NaHCO_3$ (25 mL x 2) and saturated NaCl, and then dried (Na_2SO_4) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (20 x 3 cm) and eluted with EtOAc/hexane 40 : 60 → 80 : 20 → 100 : 0 to afford dinitrobenzyl ester **3a** as a white powder: yield 9.2 g (65%). ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 8.77 (s, 1H), 8.61 (s, 2H), 7.75 (s, 1H), 7.34-7.36 (d, J = 8 Hz, 1 H), 5.38 (s, 2H), 3.98 (m, 1H), 2.97 (m, 2H), 1.74 (s, 3H), 1.61 (m, 2H), 1.33 (s, 9H), 1.30 (m, 2 x 2H); ^{13}C-NMR (400 MHz, DMSO-d_6) δ: 172.9, 169.3, 156.1, 148.5, 141.1, 128.3, 118.5, 78.8, 64.3, 54.2, 38.6, 30.6, 29.2, 28.5, 23.5, 23.0; HPLC/MS (API-ES) calcd for $C_{20}H_{28}N_4O_9Na_2$ $(M + 2Na^+ - H)^-$ 513.2, m/z found 513.0.

The purified compound **3a** (1.9 g, 4 mmol) was dissolved in 20 mL of 4 M HCl/EtOAc and the mixture was stirred for 1.5 h at room temperature The solution was concentrated under vacuum and the remained HCl was removed by repeating the addition of diethylether (20 mL x 3). The product was precipitated by the addition of diethylether (10 mL) and the precipitants were filtered in 91% yield of **2** (1.5 g, 3.7 mmol). ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 8.79 (s, 1H), 8.73-8.76 (m, 2H), 7.94 (s, 1H), 5.48 (s, 2H), 4.08-4.09 (m, 1H), 2.96 (m, 2H), 1.82-1.88 (m, 2H), 1.75 (s, 3H), 1.34-1.39 (m, 2 x 2H); ^{13}C-NMR (400 MHz, DMSO-d_6) δ: 169.6, 169.5, 148.5, 140.1, 128.9, 118.8, 64.3, 52.3, 38.4, 30.0, 28.9, 28.5, 22.9, 22.1; HPLC/MS (API-ES) calcd for $C_{15}H_{20}N_4O_7Na_2$ $(M + 2Na^+ - H)^-$ 413.3, m/z found 413.0.

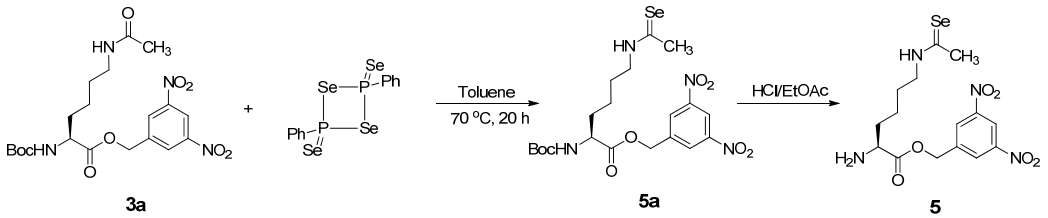
2.4 N^{ε}-thioacetyl-lysine 3,5-dinitrobenzyl ester (4):



To a stirred suspension of N^{α}-Boc-L-lysine (3.0 g, 12 mmol) in EtOH (25 mL) was added dropwise at 0°C a 5% (w/v) aqueous solution of Na_2CO_3 (25 mL). Ethyl dithioacetate (1.5 mL, 13.2 mmol) was then added dropwise at 0°C. After the addition was complete, the reaction mixture was stirred at r.t for 4 h before the addition of a 50% (v/v) solution of EtOH in DDT water (3 mL). The ethanol was removed under reduced pressure and the residue was acidified with 6 N HCl to pH 1~2 and extracted with CH_2Cl_2. The combined organics were washed with saturated NaCl, dried over anhydrous Na_2SO4, filtered, and evaporated to dryness, affording an oily residue. The product was isolated on silica gel column chromatography as white solid **4a** (3.0 g, 68%): ^{1}H-NMR (400 MHz, d-$CDCl_3$) δ: 9.34 (br, 1H), 7.95 (s, 1H), 4.29 (m, 1H), 3.64 (m, 2H), 2.55 (s, 3H), 1.48–1.87 (m, 6H); 1.44 (s, 9H); ^{13}C-NMR (400 MHz, $CDCl_3$) δ: 200.8, 176.4, 156.0, 80.6, 52.9, 46.1, 34.0, 32.3, 28.3, 27.0, 22.7; HPLC/MS (API-ES) calcd for $C_{13}H_{24}N_2O_4S$ (M - H)$^-$ 303.2, m/z found 303.3.

N^{α}-Boc-N^{ε}-thioacetyl-L-lysine (2.2 g, 7.2 mmol) was dissolved in 19 mL of CH_3CN and treated with 3.4 mL of DIPEA followed by 3,5-dinitrobenzyl chloride (1.88 g, 8.6 mmol). The reaction mixture was stirred at r.t for 12 h and then treated with 40 mL of ethyl acetate. The organic phase was washed successively with a saturated $NaHCO_3$ (10 mL x 2) and saturated NaCl and then dried (Na_2SO_4) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (8 x 3 cm) and eluted with CH_3OH/CH_2Cl_2 5 : 95 → 10 : 90 to afford dinitrobenzyl ester **4b** as a white powder: yield 2.1 g (60%). The purified compound **4b** (2.1 g, 4.3 mmol) was dissolved in 1 mL of 4M HCl/EtOAc and the mixture was stirred for 1.5 h at room temperature The solution was concentrated under vacuum and the remained HCl was removed by repeating the addition of diethylether (10 mL x 3). The product was precipitated by the addition of diethylether (5 mL) and the precipitants were filtered in 91% yield of **4** (1.5 g, 3.9 mmol). ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 9.9 (s, 1H), 8.81 (m, 1H), 8.71 (m, 2H), 8.49 (br, 2H), 5.49 (s, 2H), 4.16 (m, 1H), 3.40 (m, 2H), 2.33 (s, 3H), 1.82-1.85 (m, 2H), 1.33-1.54 (m, 2 x 2H); ^{13}C-NMR (400 MHz, DMSO-d_6) δ: 199.4, 169.6, 148.5, 139.9, 128.9, 119.0, 65.5, 52.3, 45.3, 33.2, 30.1, 27.1, 22.3; HPLC/MS (API-ES) calcd for $C_{15}H_{20}N_4O_6S$ (M + H)$^+$ 385.1, m/z found 385.0.

2.5 N^{ε}-selenoacetyl-lysine 3,5-dinitrobenzyl ester (5):



To a stirred suspension of N^{α}-Boc-N^{ε}-acetyl-L-lysine dinitrobenzyl ester **3a** (2.0 g, 4.2 mmol) in 30 mL of toluene was

added partly Woollin's reagent (679 mg, 1.3 mmol) at room temperature After the addition was complete, the reaction mixture was stirred at 70°C for 20 h. The solvent was removed under reduced pressure and the product was isolated on silica gel column chromatography as orange solid **5a** (1.2 g, 53%): ^{1}H-NMR (400 MHz, d-$CDCl_3$) δ: 9.01 (m, 1H), 8.55 (m, 2H), 8.23 (br, 1H), 5.37 (s, 2H), 4.37 (m, 1H), 3.70 (m, 2H), 2.61 (s, 3H), 1.50–1.90 (m, 6H); 1.43 (s, 9H); ^{13}C-NMR (400 MHz, $CDCl_3$) δ: 205.3, 172.3, 155.8, 148.7, 139.7, 127.8, 118.6, 80.4, 64.6, 52.9, 49.4, 38.4, 32.5, 28.3, 26.9, 22.8; HPLC/MS (API-ES) calcd for $C_{20}H_{28}N_4O_8Se$ $(M - H)^-$ 531.1, m/z found 531.0.

The purified compound **5a** (1.1 g, 2.0 mmol) was dissolved in 20 mL of 4M HCl/EtOAc and the mixture was stirred for 1.5 h at room temperature The solution was concentrated under vacuum and the remained HCl was removed by repeating the addition of diethylether (10 mL x 3). The product was precipitated by the addition of diethylether (10 mL) and the precipitants were filtered in 92% yield of **5** (900 mg, 2.1 mmol). ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.8 (s, 1H), 8.80 (m, 1H), 8.73 (m, 2H), 8.64 (br, 2H), 5.49 (s, 2H), 4.14 (m, 1H), 3.47 (m, 2H), 2.43 (s, 3H), 1.83-1.86 (m, 2H), 1.35-1.58 (m, 2 x 2H); ^{13}C-NMR (400 MHz, DMSO-d_6) δ: 202.8, 169.5, 148.4, 139.9, 128.9, 118.8, 65.5, 52.3, 49.0, 37.0, 30.1, 26.8, 22.2; HPLC/MS (API-ES) calcd for $C_{15}H_{20}N_4O_6Se$ $(M + H)^+$ 433.1, m/z found 433.0.

3. Construction of plasmids and tRNA variants

Oligonucleotide synthesis, DNA sequencing, and LC-MS/MS were performed by the Keck Foundation Biotechnology Resource Laboratory at Yale University. *E. coli* TOP10 and BL21 cells were used for general cloning and selection experiments. All cloning was performed using the Gibson Assembly kit (New England Biolabs).

3.1 Construction of pBAD-sfGFPwt and variants

The plasmid pBAD-sfGFP was derived from the pET-sfGFP plasmid[2]. The codon-optimized sfGFP gene was cloned into the pBAD plasmid and placed under the control of the inducible arabinose promoter. Two stop codon mutations, UAG2 and UAG151 were introduced to the *sfGFP* gene with the QuikChange II mutagenesis kit (Agilent Life Sciences).

sfGFPwt sequence:

MSKGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGKLTLKFICTTGKLPVPWPTLVTTLTYGVQCFSRYPDHMKR
HDFFKSAMPEGYVQERTISFKDDGTYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNFNSHNVYITADKQKNGIK
ANFKIRHNVEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSVLSKDPNEKRDHMVLLEFVTAAGITHGMDELYKGS

3.2 Construction of H3wt and variants

This human H3wt gene fragment was purchased from Life Technologies by optimizing human gene to express in *E. coli*. The plasmid pET-H3wt was derived from the plasmid pET-Duet1 (Invitrogen). Two stop codon mutations, H3K9 and H3K56 were introduced to the pET-H3wt gene with the QuikChange II mutagenesis kit (Agilent Life Sciences).

Human H3wt sequence:

MARTKQTARKSTGGKAPRKQLATKAARKSAPSTGGVKKPHRYRPGTVALREIRRYQKSTELLIRKLPFQRLVREIAQDFK
TDLRFQSAAIGALQEASEAYLVGLFEDTNLCAIHAKRVTIMPKDIQLARRIRGERA

3.3 Protein production and purification (sfGFPwt)

E. coli BL21(DE3) cells harboring pET-sfGFP-pylT were recovered in 1 mL of lysogeny broth (LB) medium for 1 h at 37 ^{o}C before being placed on LB agar plate containing 50 μg/mL Kan and 100 μg/mL Amp. A single colony was selected and grown overnight in a 10mL culture. The overnight culture was used to inoculate in 200 mL M9 medium supplemented with 1% glycerol, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 50 μg/mL Kan and 100 μg/mL Amp at 37 ^{o}C. Cells were grown at 37 ºC in an incubator (250 r.p.m.) and protein production was induced when absorbance at 600 nm reached 0.7, and IPTG was added to a final concentration of 1 mM. After 6 h induction, cells were harvested, suspended in a lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) and sonicated. The cell lysate was clarified by centrifugation (60 min, 11,000 g, 4 ºC). The supernatant was loaded onto a 5 mL Ni-NTA column (Qiagen) and washed with 25 mL lysis buffer. Proteins were eluted with 5 mL of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). Pure fractions of sfGFPwt proteins were collected and concentrated. The buffer was exchanged with PBS buffer using an Amicon Ultra-15 Centrifugal Filter Devices (10,000 MWCO) (Millopore). The purified proteins were analyzed by 12% SDS-PAGE.

3.4 Preparation and purification of tRNA gene transcripts

All *in vitro* T7 RNA polymerase run-off transcription was prepared as previously described[3]. tRNA genes together with the T7 promoter were constructed from overlapping oligonucleotides and was cloned in the vector pUC18. To generate tRNA transcripts with a 3'-CCA end, a BstNI restriction site was placed at the 3' end of tRNA gene sequence. The *in vitro* transcription reaction was performed at 37°C for 12 h in a buffer containing 40 mM Tris HCl (pH 8.1), 22 mM $MgCl_2$, 5 mM DTT, 2 mM spermidine, 16 mM guanosine monophosphate, 4 mM of each nucleoside triphosphate, pyrophosphatase (Roche 1 mg/ml), RNase inhibitor (Roche 40 U/μl), BstNI digested vector containing the template DNA (0.1 μg/μl), and 1 mM T7 RNA polymerase. U73A-$tRNA^{Sep}$ mutation was cloned from $tRNA^{Sep}$ gene with the QuikChange II mutagenesis kit (Agilent Life Sciences). $tRNA^{fMetE}$ and dinitro-flexizyme (dFx) were prepared as previously described[1]. The tRNA transcripts were purified by electrophoresis on denaturing polyacrylamide gels and full-length tRNAs extracted by 250 mM NaOAc in 75% EtOH.

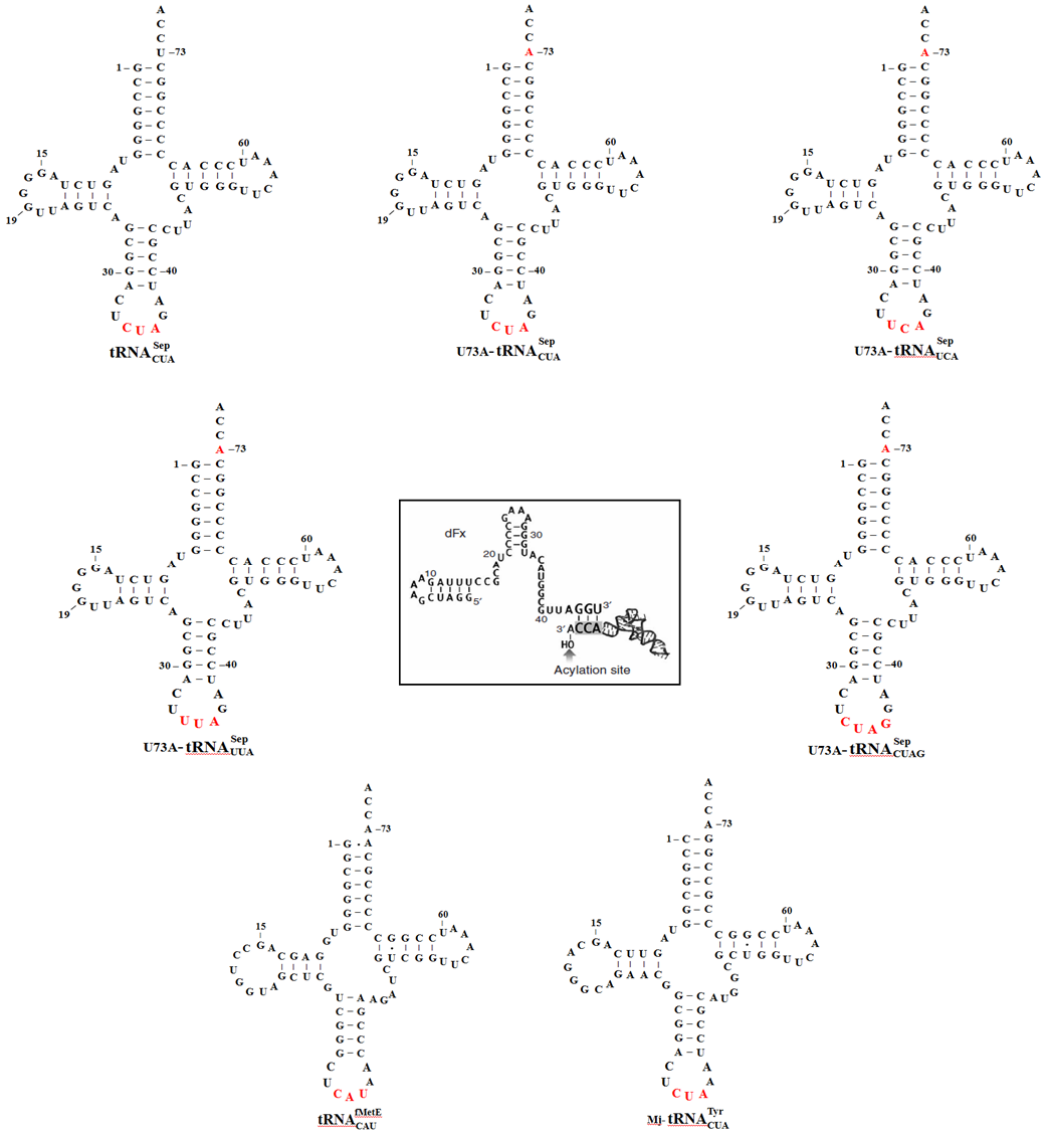


Figure S1. dFx system induced aminoacylation of tRNA anticodon stems containing $tRNA^{Sep}$ mutations, $tRNA^{fMetE}_{CAU}$ and Mj-$tRNA^{Tyr}_{CUA}$. Cloverleaf structure of $tRNA^{Sep}$ mutants selected for efficient incorporation in response to the stop codons (UAG, UAA and UGA) and quadruplet codon as well as $tRNA^{fMetE}$ and Mj-$tRNA^{Tyr}$. The anticodon sequences are shown in red. Secondary structure of dFx showing tRNA aminoacylation at the tRNA 3´-hydroxyl group by dFx[1].

All RNAs of this study were listed as follows:

$tRNA^{Sep}_{CUA}$:

5'-GCCGGGGTAGTCTAGGGGTTAGGCAGCGGACTCTAGATCCGCCTTACGTGGGTTCAAATCCCACCCCCGGCTCCA-3';

U73A-$tRNA^{Sep}_{CUA}$:

5'-GCCGGGGTAGTCTAGGGGTTAGGCAGCGGACTCTAGATCCGCCTTACGTGGGTTCAAATCCCACCCCCGGCAC

CA-3';

J17-CUA=Mj-tRNA^{Tyr}:

5'-CCGGCGGTAGTTCAGCAGGGCAGAACGGCGGACTCTAAATCCGCATGGCGCTGGTTCAAATCCGGCCCGCCGG ACCA-3';

tRNA$^{fMetE}_{CAU}$:

5'-GGCGGGGTGGAGCAGCCTGGTAGCTCGTCGGGCTCATAACCCGAAGATCGTCGGTTCAAATCCGGCCCCCGCA ACCA-3';

dFx: 5'-GGATCGAAAGATTTCCGCATCCCCGAAAGGGTACATGGCGTTAGGT-3'

4. Aminoacylation assay of tRNA by dinitro-Flexizyme (dFx)

Aminoacylation reaction of tRNA using the dFx were done as previous reported with the following modificaitons[4]: The mixture of 1 μL of 500 mM HEPES-KOH buffer (pH 7.2), 1 μL of 250 μM dFx, 1 μL of 250 μM tRNA (U73A-tRNA$^{Sep}_{CUA}$, tRNA$^{Sep}_{CUA}$, J17-CUA or tRNA$^{fMetE}_{CAU}$) and 3 μL of nuclease free H_2O was heated at 95°C for 3 min and slowly cool at room temperature for 5 min. A 2 μL of 3 M $MgCl_2$ was added into the above mixture, and incubated at room temperature for 5 min followed by incubation on ice for 3 min. The acylation mixture was initiated by addition of 2 μL of 25 mM of acid substrate in DMSO and was incubated on ice for 6 h. The reaction was stopped by addition of 20 μL of 0.6 M sodium acetate and the RNA recovered by ethanol precipitation. Centrifuge the samples at 14,000 rpm for 15 min at 18°C. The pellet was rinsed twice with 70 % ethanol containing 0.1 M NaCl and dissolved in 10 μL of 10 mM sodium acetate. 1 μL of this solution was mixed with 1 μL of acid PAGE loading buffer, and analyzed by 12 % denaturing PAGE by using sodium acetate (0.1 M) as running buffer. The gel was washed with 50 ml of 1× TBE by gently shaking for 10 min. Then the gel was stained with 20 ml of ethidium bromide gel-staining solution by gently shaking for 10 min, washed briefly with 50 ml of RNase-free water and with 50 ml of 1× TBE by gently shaking for 5 min. Finally, the gel image was scanned by a fluorescent gel scanner. To determine the yield of acylation, the bands were quantified by corresponding to free and acylated microhelix RNA.

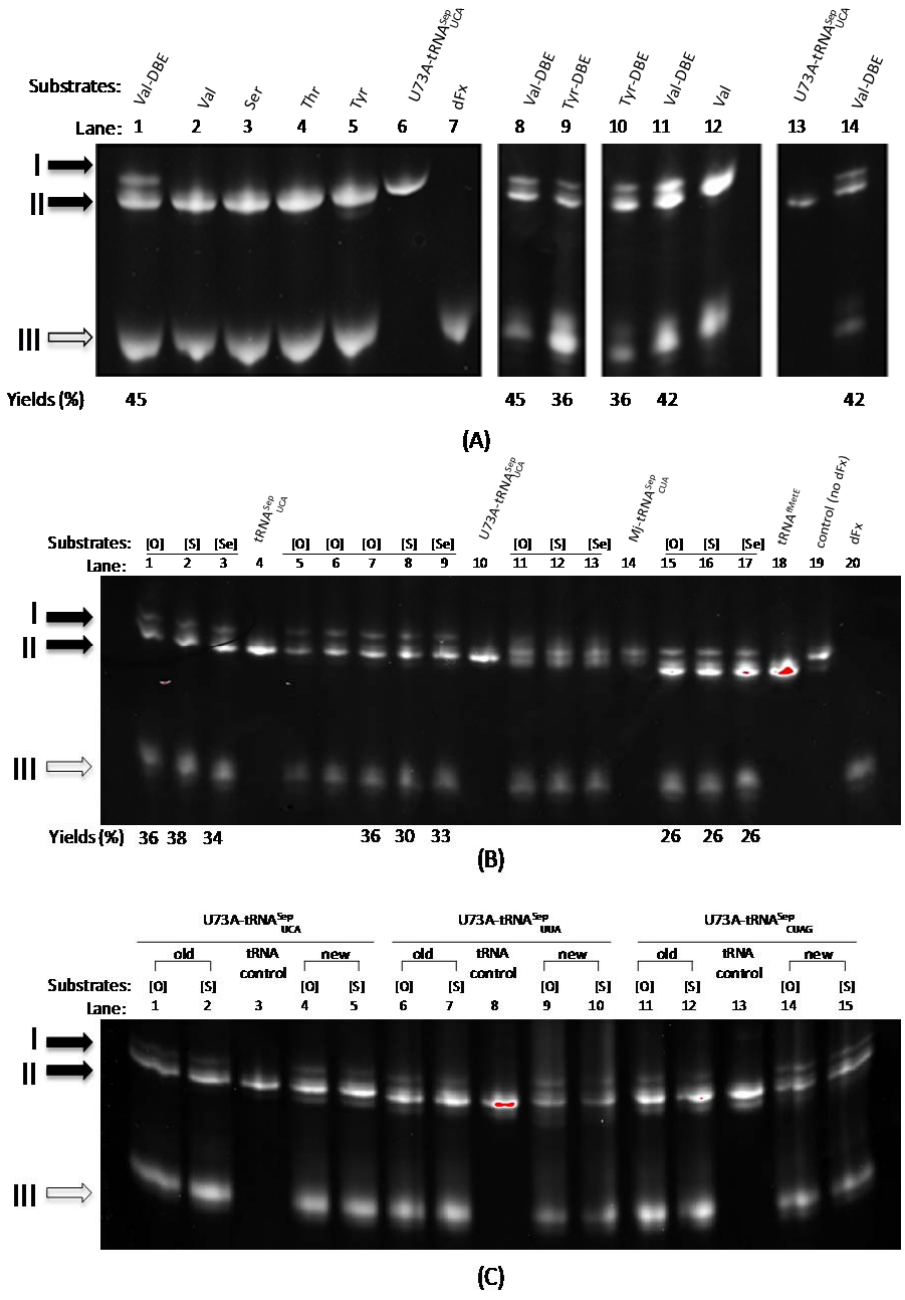


Figure S2. dFx system induced aminoacylation of tRNA anticodon stems containing $tRNA^{Sep}_{CUA}$ mutations, $tRNA^{fMetE}_{CAU}$ and Mj-$tRNA^{Tyr}_{CUA}$. (A) Acylation of U73A-$tRNA^{Sep}_{CUA}$ and different amino acids by dFx. (B) dFx induced acylation of different tRNA and AcK-DBE variants (3-5). (C) Acylation of suppressor U73A-$tRNA^{Sep}_{CUA}$ and AcK-DBE or ThioAcK-DBE by dFx. Abbreviations: [O], AcK-DBE; [S], ThioAcK-DBE; [Se], SeAcK-DBE; The word named "old" meant that aminoacyl-tRNA were stabled when they were stored at -80^{o}C for 6 months; while "new" was freshly prepared. Bands I–III are as follows: I. acyl-RNA; II. tRNA; III, dFx. The yields were calculated based on the intensity of I (acyl-tRNA) and II (tRNA), present as (I)/[(I)+(II)].

5 Incorporation of AcK or ThioAcK into sfGFP at the site of 2 or 151

5.1 RF1 deleted cell-free translation

Protein synthesis reactions of noncanonical amino acids were carried out using the PURExpress ΔRF123 Kit (E6850, BioLabs Inc.).

a) Protocol

The reactions were assembled on ice (10 μL of Solution A, 7.5 μL of Solution B, 0.5 μl of RF1 or RF2 and 0.5 μl of RF3), and 75 pmol of crude acyl-tRNAs synthesized by flexizyme was added into the above mixture. All reactions were had a final volume of 25 μL. Tubes were mixed, pulse centrifuged to collect the mixture and incubated at 37°C for 4 hr. Reactions were stopped by placing tube on ice. Samples were used directly for analysis or kept at -20°C for use at a later time. The products were analyzed by 12% SDS-PAGE. For the sfGFP proteins, all of the 25 μl samples were diluted to 50 μl and measured with excitation wavelength in 485 nm and emission wavelength 528 nm.

b) Generation of DNA template by the designed primers

Gene specific primers are used to add adaptor sequences (homologous to part of the regulatory region DNA) to the 5´ and 3´ ends of the gene of interest.

5'-UTR sequence of Forward Primer

5'-GCGAATTAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATATGAGTAAAGGAGAAGAACTTTTCACTGGA

3'-UTR sequence of Reverse Primer

5'-AAACCCCTCCGTTTAGAGAGGGGTTATGCTAGTTAGGATCCTTTGTAGAGCTCATCCATGCC

5.2 Fluorescence read-through screen

To verify whether the acyl-tRNAs prepared by dFx can function in protein translation, a cell-free PURExpress (BioLabs Inc.) reaction, based on the PURE system technology originally developed by the Ueda group, was performed *in vitro*[5]. For fluorescence read-through screen, lysine analogues were incorporated to sfGFP bearing a TAG codon at position 151 (sfGFP-151TAG) and at position 2 (sfGFP-2TAG). First, we tested the blank control in reaction series 1, it demonstrated Flexizyme substrates had no background on the sfGFP expression (Figure 2). In present of RF1 we found that the fluorescence intensity had no change with adding U73A-tRNA$^{Sep}_{CUA}$ or not by comparing to the reaction series 3 and 4, as well as 11 and 12. It excluded the possibility that designed U73A-tRNA$^{Sep}_{CUA}$ could charge other amino acids. The result was in agreement with earlier data that total *E. coli* aaRSs could not be discriminated with tRNA$^{Sep}_{CUA}$[6]**.** In the PURExpress system eliminated the UAG-recognizing RF1, translation of AcK or ThioAcK was probably 36-40% and 26% for SeAcK by comparing to wt-sfGFP, and their fluorescence intensity was similar or higher than valine or tyrosine incorporating in sfGFP-151 (Figure 2). These results demonstrated that incorporation efficiency at UAG at sfGFP-151 or sfGFP-2 was notably increased in the absence of RF1 competition. This was consistent with previous reports that background misacylated of amber suppressor tRNAs may be effectively outcompeted in the presence of an efficient near-cognate[7]. Furthermore, Flexizyme-mediated aminoacylation of tRNAs contains high salt which may affect the PURE system reactions, so that the expression level of series 5 and 13 involving the suppression of UAG is higher in the absence of aa-tRNA than in the presence of aa-tRNA (Figure S3). Without RF1, the products were from near cognate suppression of UAG codon, and they are usually Tyr, Trp, Gln, and Lys residues[7,8].

5.3 Western blotting antibodies and reagents.

The antibody against GFP was from Life Technologies (A10260, GFP Rabbit IgG Antibody Fraction, HRP Conjugate). The MagicMark™ XP Western Protein Standard was from Life Technologies. The Western blotting was performed following the procedures recommended by Life Technologies. As shown in Figure S3, the samples of sfGFP modifications in lanes 4-11 can be completely labeled using the universal antibodies (A10260) when compared to purified sfGFP.

Western blotting analysis showed similar incorporation efficiency of AcK and its analogues (about 250 mg/L) and quantitative analysis of western blotting showed the incorporation efficiency of AcK and analogues were much higher than that of valine and tyrosine, in agreement with fluorescence intensity read-through results.

Reaction series	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Template DNA	-	GFP-wt	GFP-Y151TAG								GFP-S2TAG			
RF1	-	-	+	+	-						+	+	-	
$tRNA^{Sep}$-U73A	-	-	-	+	-						-	+	-	
Acyl-tRNA	-	-	-	-	-	Val	Tyr	AcK	ThioAcK	SelAcK	-	-	-	SelAcK
Fluorescence (average)	9	8317	16	17	3531	1280	2634	3370	3043	2196	153	167	2554	2260

Note: The reaction system was eluted 2 folds to 50 μL.
Ex: 485 nm; Em: 528 nm

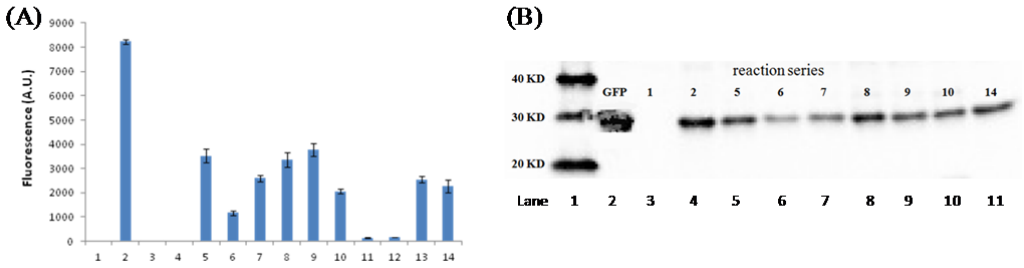


Figure S3. The single site-specific incorporation of different NcAAs at the sfGFP-Y151 and sfGFP-S2 using amber suppression. (A) Series 1, no template DNA and acyl-tRNA as blank; series 2, sfGFP-wt expression; series 3-4, in present of RF1 and no acyl-tRNA as comparison; series 5 and 13, no acyl-tRNA as control; series 6-10, dFx induced compounds 1-5 to charge with U73A-$tRNA^{Sep}_{CUA}$ and expressed in sfGFP-Y151; series 11-12, in present of RF1 and no acyl-tRNA as comparison; series 13, no acyl-tRNA as control; series 14, dFx induced U73A-$tRNA^{Sep}_{CUA}$ to acylate ThioAcK and expression of sfGFP-S2. The reaction systems were eluted 2 folds to 50 μL. The fluorescence intensity was measured with excitation wavelength in 485 nm and emission wavelength 528 nm. (B) Western blotting of the synthetic sfGFP variants Y151Valine, Y151Tyrosine, Y151AcK, Y151ThioAcK, Y151SeAcK and S2ThioAcK using antibody against sfGFP.

5.4 HPLC-MS/MS of demonstrated the incorporation of AcK or ThioAcK at the sfGFP-Y151TAG

In order to confirm the incorporation of AcK and its analogues at the site of Y151-TAG, we analyzed the in-gel trypsin digests of the sfGFP proteins by LC-MS/MS. The MS/MS analyses of the corresponding modified sfGFP peptides LEYNFNSHNV(K)ITADK gave the correct mass spectra for Y151AcK (ppm: -0.6) and Y151ThioAcK (ppm: 1.6), respectively (Figure S4-S5 and Table S1-S2). As fragmentation of the peptide can occur starting either with the N-terminus (b ion series) or the C-terminus (y ion series) both ion series can always be found. While the SeAcK was not detected due to its lability, we found the corresponding modification SeAcK was chemically changed into AcK (data not shown).

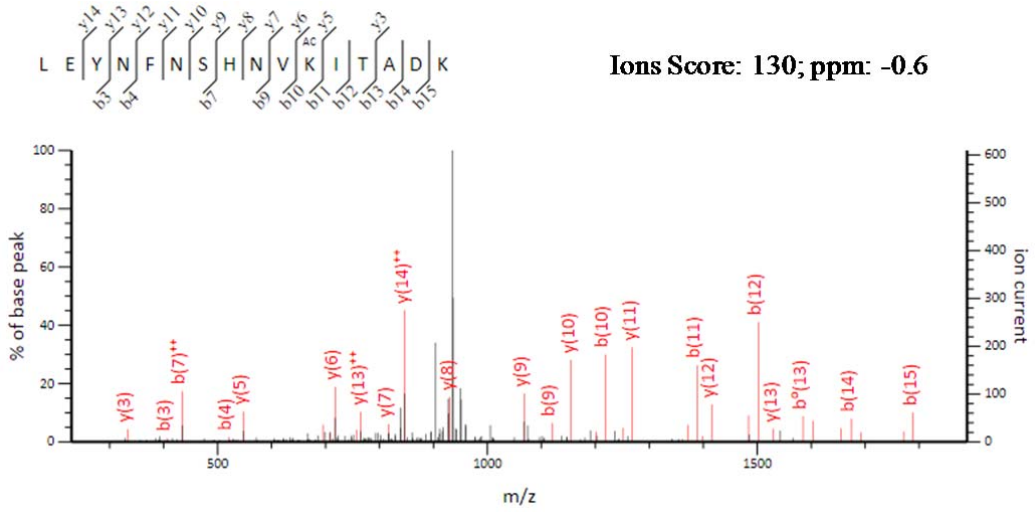


Figure S4. MS/MS spectrum of the tryptic peptide LEYNFNSHNV(AcK)ITADK of the sfGFP-Y151 digestion.

Table S1. Assigned MS/MS fragments resulting from fragmentation of the tryptic peptide LEYNFNSHNV(AcK)ITADK of the sfGFP-Y151 digestion.

#	b	b^{++}	b*	b^{*++}	b^0	b^{0++}	Seq.	y	y^{++}	y*	y^{*++}	y^0	y^{0++}	#
1	114.0913	57.5493					L							16
2	243.1339	122.0706			225.1234	113.0653	E	1821.8766	911.4419	1804.8501	902.9287	1803.8660	902.4367	15
3	**406.1973**	203.6023			388.1867	194.5970	Y	***1692.8340***	**846.9206**	1675.8075	838.4074	1674.8234	837.9154	14
4	**520.2402**	260.6237	503.2136	252.1105	502.2296	251.6185	N	***1529.7707***	**765.3890**	1512.7441	756.8757	1511.7601	756.3837	13
5	667.3086	334.1579	650.2821	325.6447	649.2980	325.1527	F	***1415.7278***	**708.3675**	1398.7012	699.8542	1397.7172	699.3622	12
6	781.3515	391.1794	764.3250	382.6661	763.3410	382.1741	N	***1268.6593***	634.8333	1251.6328	626.3200	1250.6488	625.8280	11
7	868.3836	434.6954	851.3570	426.1821	850.3730	425.6901	S	***1154.6164***	577.8118	1137.5899	569.2986	1136.6058	568.8066	10
8	1005.4425	503.2249	988.4159	494.7116	987.4319	494.2196	H	***1067.5844***	534.2958	1050.5578	525.7826	1049.5738	525.2905	9
9	**1119.4854**	560.2463	1102.4588	551.7331	1101.4748	551.2411	N	***930.5255***	465.7664	913.4989	457.2531	912.5149	456.7611	8
10	**1218.5538**	609.7805	1201.5273	601.2673	1200.5432	600.7753	V	***816.4825***	408.7449	799.4560	400.2316	798.4720	399.7396	7
11	**1388.6593**	694.8333	1371.6328	686.3200	1370.6488	685.8280	K	***717.4141***	359.2107	700.3876	350.6974	699.4036	350.2054	6
12	**1501.7434**	751.3753	1484.7169	742.8621	1483.7328	742.3701	I	***547.3086***	274.1579	530.2821	265.6447	529.2980	265.1527	5
13	**1602.7911**	801.8992	1585.7645	793.3859	1584.7805	792.8939	T	***434.2245***	217.6159	417.1980	209.1026	416.2140	208.6106	4
14	**1673.8282**	837.4177	1656.8016	828.9045	1655.8176	828.4125	A	***333.1769***	167.0921	316.1503	158.5788	315.1663	158.0868	3
15	**1788.8551**	894.9312	1771.8286	886.4179	1770.8446	885.9259	D	262.1397	131.5735	245.1132	123.0602	244.1292	122.5682	2
16							K	147.1128	74.0600	130.0863	65.5468			1

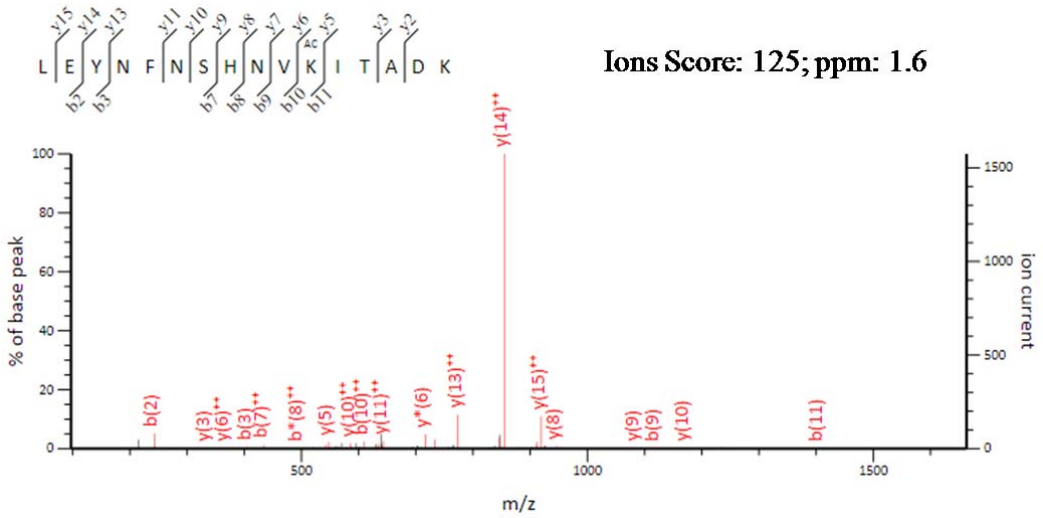


Figure S5. MS/MS spectrum of the tryptic peptide LEYNFNSHNV(ThioAcK)ITADK of the sfGFP-Y151 digestion.

Table S2. Assigned MS/MS fragments resulting from fragmentation of the tryptic peptide LEYNFNSHNV(ThioAcK)ITADK of the sfGFP-Y151 digestion.

#	b	b^{++}	b*	b^{*++}	b^{0}	b^{0++}	Seq.	y	y^{++}	y*	y^{*++}	y^{0}	y^{0++}	#
1	114.0913	57.5493					L							16
2	**243.1339**	122.0706			225.1234	113.0653	E	1837.8538	***919.4305***	1820.8272	910.9172	1819.8432	910.4252	15
3	**406.1973**	203.6023			388.1867	194.5970	Y	1708.8112	***854.9092***	1691.7846	846.3959	1690.8006	845.9039	14
4	520.2402	260.6237	503.2136	252.1105	502.2296	251.6185	N	1545.7478	***773.3776***	1528.7213	764.8643	1527.7373	764.3723	13
5	667.3086	334.1579	650.2821	325.6447	649.2980	325.1527	F	1431.7049	***716.3561***	1414.6784	707.8428	1413.6943	707.3508	12
6	781.3515	391.1794	764.3250	382.6661	763.3410	382.1741	N	1284.6365	***642.8219***	1267.6099	634.3086	1266.6259	633.8166	11
7	868.3836	434.6954	851.3570	426.1821	850.3730	425.6901	S	***1170.5936***	***585.8004***	1153.5670	577.2871	1152.5830	576.7951	10
8	1005.4425	503.2249	988.4159	494.7116	987.4319	494.2196	H	***1083.5615***	***542.2844***	1066.5350	533.7711	1065.5510	533.2791	9
9	**1119.4854**	560.2463	1102.4588	551.7331	1101.4748	551.2411	N	***946.5026***	473.7550	929.4761	465.2417	928.4921	464.7497	8
10	1218.5538	609.7805	1201.5273	601.2673	1200.5432	600.7753	V	832.4597	***416.7335***	815.4332	408.2202	814.4491	407.7282	7
11	**1404.6365**	702.8219	1387.6099	694.3086	1386.6259	693.8166	K	***733.3913***	***367.1993***	716.3647	358.6860	715.3807	358.1940	6
12	1517.7206	759.3639	1500.6940	750.8506	1499.7100	750.3586	I	***547.3086***	274.1579	530.2821	265.6447	529.2980	265.1527	5
13	1618.7682	809.8878	1601.7417	801.3745	1600.7577	800.8825	T	***434.2245***	217.6159	417.1980	209.1026	416.2140	208.6106	4
14	1689.8054	845.4063	1672.7788	836.8930	1671.7948	836.4010	A	***333.1769***	167.0921	316.1503	158.5788	315.1663	158.0868	3
15	1804.8323	902.9198	1787.8057	894.4065	1786.8217	893.9145	D	***262.1397***	131.5735	245.1132	123.0602	244.1292	122.5682	2
16							K	147.1128	74.0600	130.0863	65.5468			1

6. Specific-site incorporations of AcK and/or ThioAcK into human histone H3

6.1 Cell-based expression of optimized histone H3wt

The pET-Duet-1 plasmid containing the histone H3wt gene fragment was transformed into BL21(DE3) cells, and protein expression performed in LB containing ampicillin (100 mg/L). The transformed cells were grown at 37°C for 3 h to OD_{600} = 0.6～0.8. Protein expression was then induced with IPTG (1mM) at 37°C for 5 h (no adding IPTG as control). The 15 μL of cell culture were harvested by centrifuging (10,000 rpm, 25 min, 4°C), and directly suspended in loading buffer including β-mercaptoethanol without adding any protease inhibitors or NAM. The sample was heated at 95°C for 3 minutes and cooled to room temperature., and then was analyzed by 12% SDS-gel. In this case 150～200 mg of human H3wt per liter medium can be obtained with comparing and calculating the loading 2.5 μg of commercial human H3 (Figure S6).

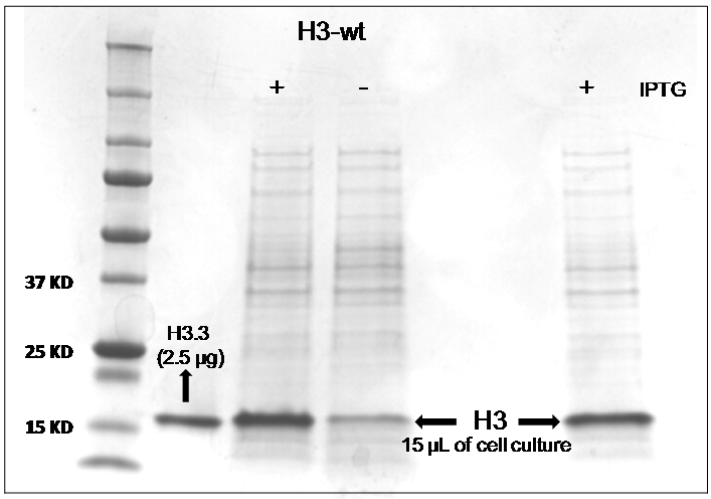


Figure S6. Cell-based expression of optimized human histone H3wt.

6.2 Cell-free translation: incorporation of AcK and ThioAcK into human H3

Protein synthesis reactions of noncanonical amino acids were carried out using the PURExpress® ΔRF123 Kit (E6850, BioLabs Inc.).

Generation of DNA template by the designed primers

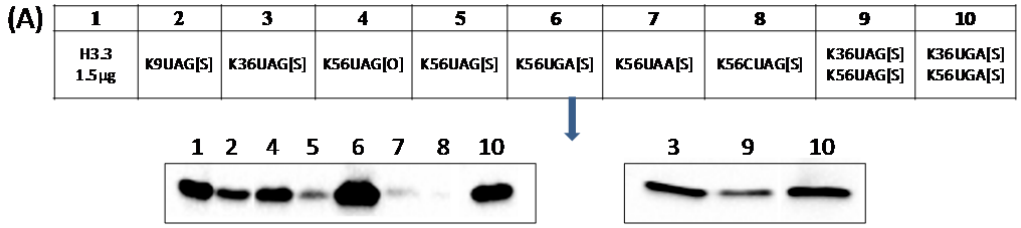
5'-UTR sequence of Forward Primer

5'-GCGAATTAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATATGGCACGTACCAAACAGACCGCACGTAAA

3'-UTR sequence of Reverse Primer

5'-AAACCCCTCCGTTTAGAGAGGGGTTATGCTAGTTATGCACGTTCACCACGAATACGACGTGC

6.2.1 Western blotting (against H3 histone antibody)



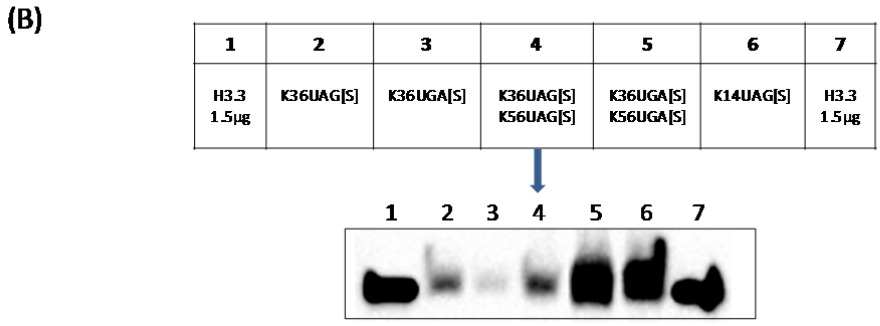


Figure S7. The histone H3 variants incorporated AcK and/or ThioAcK at the positions (K9, K14, K36, K56 or K36/K56) were synthesized by amber or opal suppression, and a same amount of loading samples was monitored in western blotting. Commercial H3 was used as a standard control.

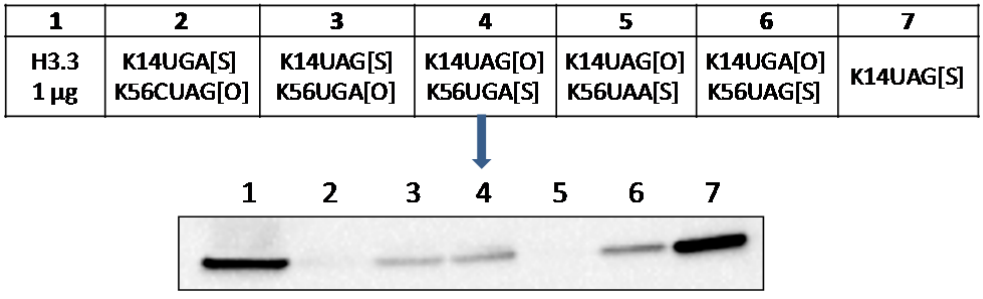


Figure S8. By the combination of amber and opal suppressions, histone H3 variants incorporating AcK and ThioAcK at multiple-sites of K14/K56 were prepared. A same amount of loading samples was monitored in western blotting, and commercial H3 was used as a standard control.

6.2.2 Western blotting (anti-acetyl histone H3K14 and H3K56 antibodies)

Anti-acetyl histone H3K14 and H3K56 antibodies are special site-selectivity for the acetylated histone modifications, and they can be performed to distinguish some acetylated histone variants (lanes 2-13, Figure S9A; lanes 2-7, Figure S9B) and non-acetylated histone proteins such as commercial histone H3.3 (lane 1) in Figure S9.

1	2	3	4	5	6	7	8	9	10	11	12	13
H3.3 1 μg	K14UAG [O]	K14UAG [S]	K14UGA [S]	K14UAA [S]	K14CUGA [S]	K14UAG [S] K56UAG [S]	K14UGA [S] K56UGA [S]	K14UAG [O] K56CUAG [S]	K14UGA [O] K56UGA [O]	K14UGA [S] K56UAG [O]	K14UGA [S] K56UAA [O]	K14UGA [O] K56UAA [S]

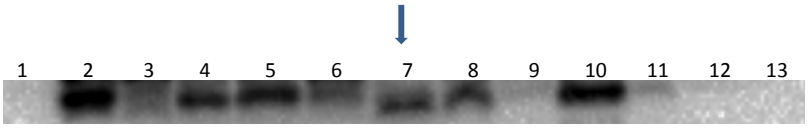


Figure S9. Western blotting of the synthetic histone H3 variants. Anti-acetyl histone H3K14 antibody.

6.2.3 HPLC-MS/MS of demonstrated the incorporation of AcK or ThioAcK in histone H3

H3K56ThioAcK(UGA)

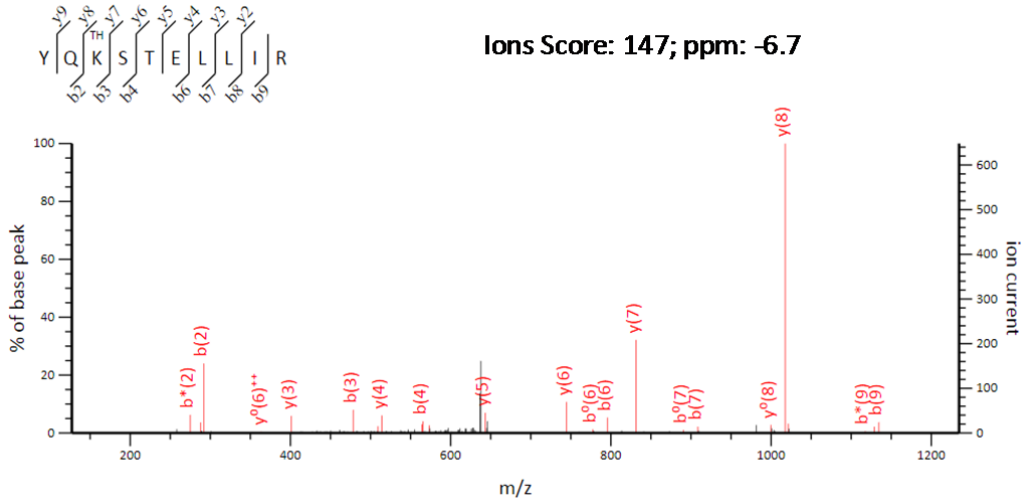


Figure S10. Opal suppression of histone H3 modification incorporating ThioAcK at position of K56. MS/MS spectrum of the tryptic peptide YQ(ThioAcK)STELLIR of the H3K56ThioAcK digestion.

Table S3. Assigned MS/MS fragments resulting from fragmentation of the tryptic peptide YQ(ThioAcK)STELLIR of the H3K56ThioAcK digestion.

#	b	b^{++}	b*	b^{*++}	b^{0}	b^{0++}	Seq.	y	y^{++}	y*	y^{*++}	y^{0}	y^{0++}	#
1	164.0706	82.5389					Y							10
2	***292.1292***	146.5682	275.1026	138.0550			Q	1145.6347	**573.3210**	1128.6082	564.8077	1127.6241	564.3157	9
3	***478.2119***	239.6096	461.1853	231.0963			K	***1017.5761***	**509.2917**	1000.5496	500.7784	999.5656	500.2864	8
4	***565.2439***	283.1256	548.2173	274.6123	547.2333	274.1203	S	***831.4934***	416.2504	814.4669	407.7371	813.4829	407.2451	7
5	666.2916	333.6494	649.2650	325.1362	648.2810	324.6441	T	***744.4614***	372.7343	727.4349	364.2211	726.4509	363.7291	6
6	***795.3342***	398.1707	778.3076	389.6574	777.3236	389.1654	E	***643.4137***	322.2105	626.3872	313.6972	625.4032	313.2052	5
7	***908.4182***	454.7128	891.3917	446.1995	890.4077	445.7075	L	***514.3711***	257.6892	497.3446	249.1759			4
8	***1021.5023***	511.2548	1004.4757	502.7415	1003.4917	502.2495	L	***401.2871***	201.1472	384.2605	192.6339			3
9	***1134.5864***	567.7968	1117.5598	559.2835	1116.5758	558.7915	I	***288.2030***	144.6051	271.1765	136.0919			2
10							R	175.1190	88.0631	158.0924	79.5498			1

H3K14ThioAcK(UGA)

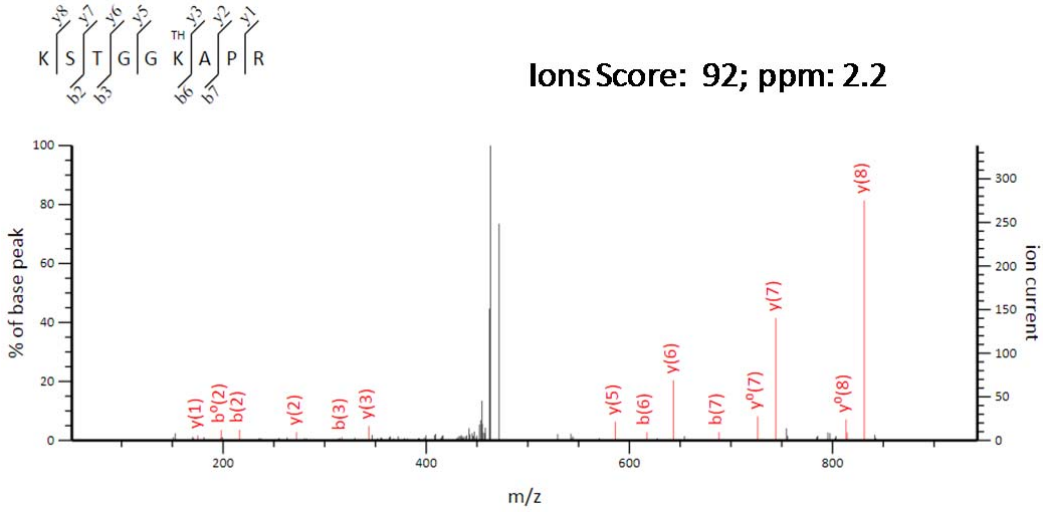
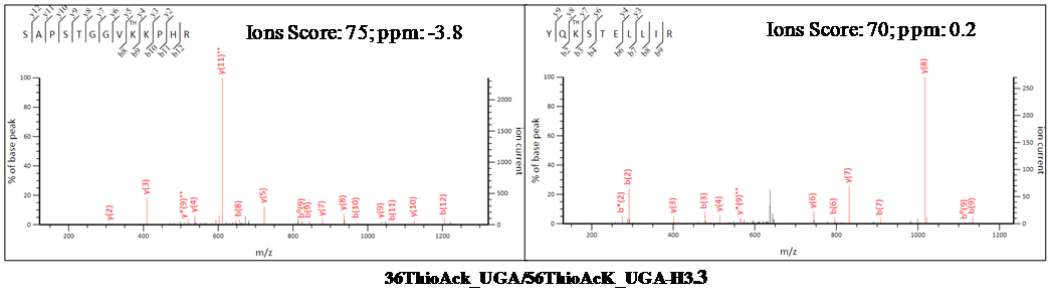


Figure S11. Opal suppression of histone H3 modification incorporating ThioAcK at position of K14. MS/MS spectrum of the tryptic peptide KSTGG(ThioAcK)APR of the histone H3K14ThioAcK digestion.

Table S4. Assigned MS/MS fragments resulting from fragmentation of the tryptic peptide KSTGG(ThioAcK)APR of the histone H3K14ThioAcK digestion.

#	b	b^{++}	b*	b^{*++}	b^{0}	b^{0++}	Seq.	y	y^{++}	y*	y^{*++}	y^{0}	y^{0++}	#
1	129.1022	65.0548	112.0757	56.5415			K							9
2	**216.1343**	108.5708	199.1077	100.0575	198.1237	99.5655	S	***831.4141***	416.2107	814.3876	407.6974	813.4036	407.2054	8
3	**317.1819**	159.0946	300.1554	150.5813	299.1714	150.0893	T	***744.3821***	372.6947	727.3556	364.1814	726.3716	363.6894	7
4	374.2034	187.6053	357.1769	179.0921	356.1928	178.6001	G	***643.3344***	322.1709	626.3079	313.6576			6
5	431.2249	216.1161	414.1983	207.6028	413.2143	207.1108	G	***586.3130***	293.6601	569.2864	285.1469			5
6	**617.3076**	309.1574	600.2810	300.6441	599.2970	300.1521	K	529.2915	265.1494	512.2650	256.6361			4
7	**688.3447**	344.6760	671.3181	336.1627	670.3341	335.6707	A	***343.2088***	172.1081	326.1823	163.5948			3
8	785.3974	393.2024	768.3709	384.6891	767.3869	384.1971	P	***272.1717***	136.5895	255.1452	128.0762			2
9							R	***175.1190***	88.0631	158.0924	79.5498			1

H3K36ThioAcK(UGA)/K56ThioAcK(UGA)



36ThioAck_UGA/56ThioAcK_UGA-H3.3

Figure S12. The multiple site-specific lysine (thio)acetylation on the H3K36/K56. MS/MS spectra of two tryptic peptides SAPSTGGV(ThioAcK)KPHR at position of K36 and YQ(ThioAcK)STELLIR at position of K56.

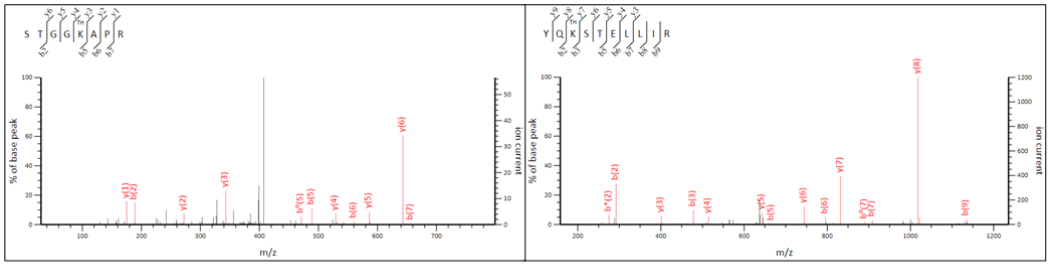
Table S5. Assigned MS/MS fragments resulting from fragmentation of two tryptic peptides SAPSTGGV(ThioAcK)KPHR at position of K36 and YQ(ThioAcK)STELLIR at position of K56 of the histone H3K36/K56 digestion.

#	b	b^{++}	b*	b^{*++}	b^{0}	b^{0++}	Seq.	y	y^{++}	y*	y^{*++}	y^{0}	y^{0++}	#
1	88.0393	44.5233			70.0287	35.5180	S							13
2	159.0764	80.0418			141.0659	71.0366	A	1292.6892	**646.8482**	1275.6627	638.3350	1274.6786	637.8430	12
3	256.1292	128.5682			238.1186	119.5629	P	***1221.6521***	**611.3297**	1204.6255	602.8164	1203.6415	602.3244	11
4	343.1612	172.0842			325.1506	163.0790	S	***1124.5993***	562.8033	1107.5728	554.2900	1106.5888	553.7980	10
5	444.2089	222.6081			426.1983	213.6028	T	***1037.5673***	**519.2873**	1020.5407	510.7740	1019.5567	510.2820	9
6	501.2304	251.1188			483.2198	242.1135	G	***936.5196***	468.7634	919.4931	460.2502			8
7	558.2518	279.6295			540.2413	270.6243	G	***879.4982***	440.2527	862.4716	431.7394			7
8	**657.3202**	329.1638			639.3097	320.1585	V	***822.4767***	411.7420	805.4501	403.2287			6
9	**843.4029**	422.2051	826.3764	413.6918	825.3923	413.1998	K	***723.4083***	362.2078	706.3817	353.6945			5
10	**971.4979**	486.2526	954.4713	477.7393	953.4873	477.2473	K	***537.3256***	269.1664	520.2990	260.6532			4
11	**1068.5506**	534.7790	1051.5241	526.2657	1050.5401	525.7737	P	***409.2306***	205.1190	392.2041	196.6057			3
12	**1205.6096**	603.3084	1188.5830	594.7951	1187.5990	594.3031	H	***312.1779***	156.5926	295.1513	148.0793			2
13							R	175.1190	88.0631	158.0924	79.5498			1

36ThioAck_UGA/56ThioAcK_UGA-H3.3

#	b	b^{++}	b*	b^{*++}	b^{0}	b^{0++}	Seq.	y	y^{++}	y*	y^{*++}	y^{0}	y^{0++}	#
1	164.0706	82.5389					Y							10
2	***292.1292***	146.5682	275.1026	138.0550			Q	1145.6347	573.3210	1128.6082	564.8077	1127.6241	564.3157	9
3	***478.2119***	239.6096	461.1853	231.0963			K	**1017.5761**	509.2917	1000.5496	500.7784	999.5656	500.2864	8
4	***565.2439***	283.1256	548.2173	274.6123	547.2333	274.1203	S	**831.4934**	416.2504	814.4669	407.7371	813.4829	407.2451	7
5	666.2916	333.6494	649.2650	325.1362	648.2810	324.6441	T	**744.4614**	372.7343	727.4349	364.2211	726.4509	363.7291	6
6	***795.3342***	398.1707	778.3076	389.6574	777.3236	389.1654	E	643.4137	322.2105	626.3872	313.6972	625.4032	313.2052	5
7	***908.4182***	454.7128	891.3917	446.1995	890.4077	445.7075	L	**514.3711**	257.6892	497.3446	249.1759			4
8	***1021.5023***	511.2548	1004.4757	502.7415	1003.4917	502.2495	L	**401.2871**	201.1472	384.2605	192.6339			3
9	***1134.5864***	567.7968	1117.5598	559.2835	1116.5758	558.7915	I	288.2030	144.6051	271.1765	136.0919			2
10							R	175.1190	88.0631	158.0924	79.5498			1

H3K14ThioAcK(UGA)/K56ThioAcK(UGA)



14ThioAck_UGA/56ThioAcK_UGA-H3.3

Figure S13. The multiple site-specific lysine (thio)acetylation on the H3K14/K56. MS/MS spectra of two tryptic peptides STGG(ThioAcK)APR at position of K14 and YQ(ThioAcK)STELLIR at position of K56.

Table S6. Assigned MS/MS fragments resulting from fragmentation of two tryptic peptides STGG(ThioAcK)APR at position of K14 and YQ(ThioAcK)STELLIR at position of K56 of the histone H3K14/K56 digestion.

#	b	b^{++}	b*	b^{*++}	b^{0}	b^{0++}	Seq.	y	y^{++}	y*	y^{*++}	y^{0}	y^{0++}	#
1	88.0393	44.5233			70.0287	35.5180	S							8
2	189.0870	95.0471			171.0764	86.0418	T	744.3821	372.6947	727.3556	364.1814	726.3716	363.6894	7
3	246.1084	123.5579			228.0979	114.5526	G	643.3344	322.1709	626.3079	313.6576			6
4	303.1299	152.0686			285.1193	143.0633	G	586.3130	293.6601	569.2864	285.1469			5
5	489.2126	245.1099	472.1860	236.5967	471.2020	236.1047	K	529.2915	265.1494	512.2650	256.6361			4
6	560.2497	280.6285	543.2232	272.1152	542.2391	271.6232	A	343.2088	172.1081	326.1823	163.5948			3
7	657.3025	329.1549	640.2759	320.6416	639.2919	320.1496	P	272.1717	136.5895	255.1452	128.0762			2
8							R	175.1190	88.0631	158.0924	79.5498			1

14ThioAck_UGA/56ThioAcK_UGA-H3.3

#	b	b^{++}	b*	b^{*++}	b^{0}	b^{0++}	Seq.	y	y^{++}	y*	y^{*++}	y^{0}	y^{0++}	#
1	164.0706	82.5389					Y							10
2	292.1292	146.5682	275.1026	138.0550			Q	1145.6347	573.3210	1128.6082	564.8077	1127.6241	564.3157	9
3	478.2119	239.6096	461.1853	231.0963			K	1017.5761	509.2917	1000.5496	500.7784	999.5656	500.2864	8
4	565.2439	283.1256	548.2173	274.6123	547.2333	274.1203	S	831.4934	416.2504	814.4669	407.7371	813.4829	407.2451	7
5	666.2916	333.6494	649.2650	325.1362	648.2810	324.6441	T	744.4614	372.7343	727.4349	364.2211	726.4509	363.7291	6
6	795.3342	398.1707	778.3076	389.6574	777.3236	389.1654	E	643.4137	322.2105	626.3872	313.6972	625.4032	313.2052	5
7	908.4182	454.7128	891.3917	446.1995	890.4077	445.7075	L	514.3711	257.6892	497.3446	249.1759			4
8	1021.5023	511.2548	1004.4757	502.7415	1003.4917	502.2495	L	401.2871	201.1472	384.2605	192.6339			3
9	1134.5864	567.7968	1117.5598	559.2835	1116.5758	558.7915	I	288.2030	144.6051	271.1765	136.0919			2
10							R	175.1190	88.0631	158.0924	79.5498			1

6.3 Histone deacetylase (HDAC) assay

A time course assay solution had the following components: 20 mM Tris–HCl (pH 7.5), 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mM β-NAD^+, 5 μL of PURExpress solution, and 2 μL of human GST-SIRT1 (Cat.#BML-SE239-0100, Enzo Life Sciences Inc.). An enzymatic reaction on histone modification containing ThioAcK56 was initiated by the addition of enzyme at 37°C and was allowed to be incubated at 37°C until quenched at different time points (0-180 min). The assay solutions were analyzed by western blotting (anti-acetyl histone H3K56). Under the same assay conditions, histone modification containing AcK at the site of K56 was performed as a comparison. Non-enzymatic reactions were used as control.

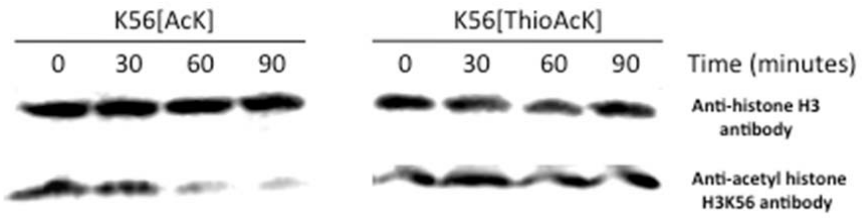


Figure S14. HDAC assays performed on histone H3K56AcK and H3K56ThioAcK. Deacetylation and de(thio-)acetylation reactions were quantified by Western blot analysis of histone H3 using anti-acetyl histone H3K56 antibody. Non-enzymatic reactions were used as time zero controls.

7. References

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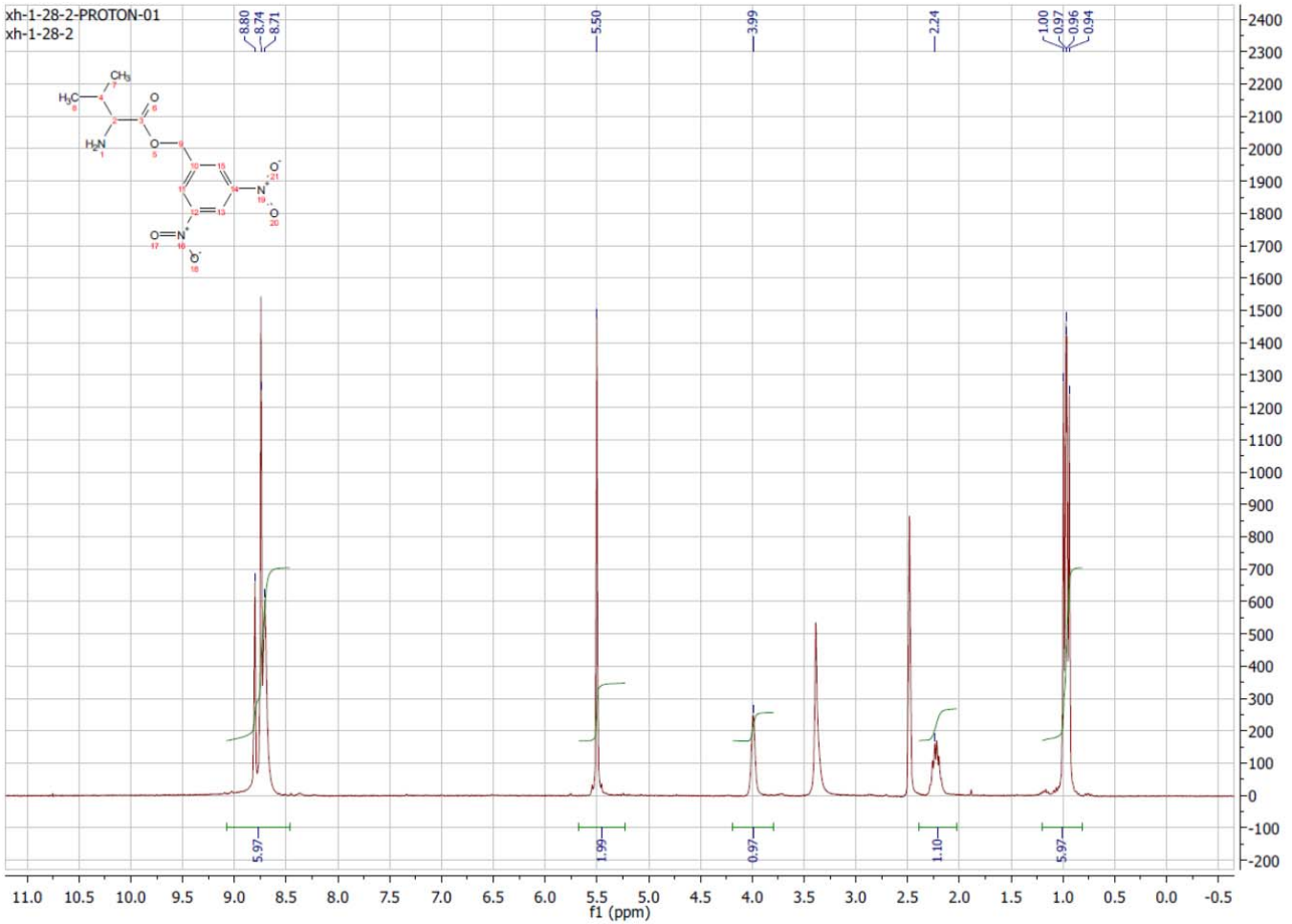


Figure S15. ^{1}H-NMR spectrum of compound **1**

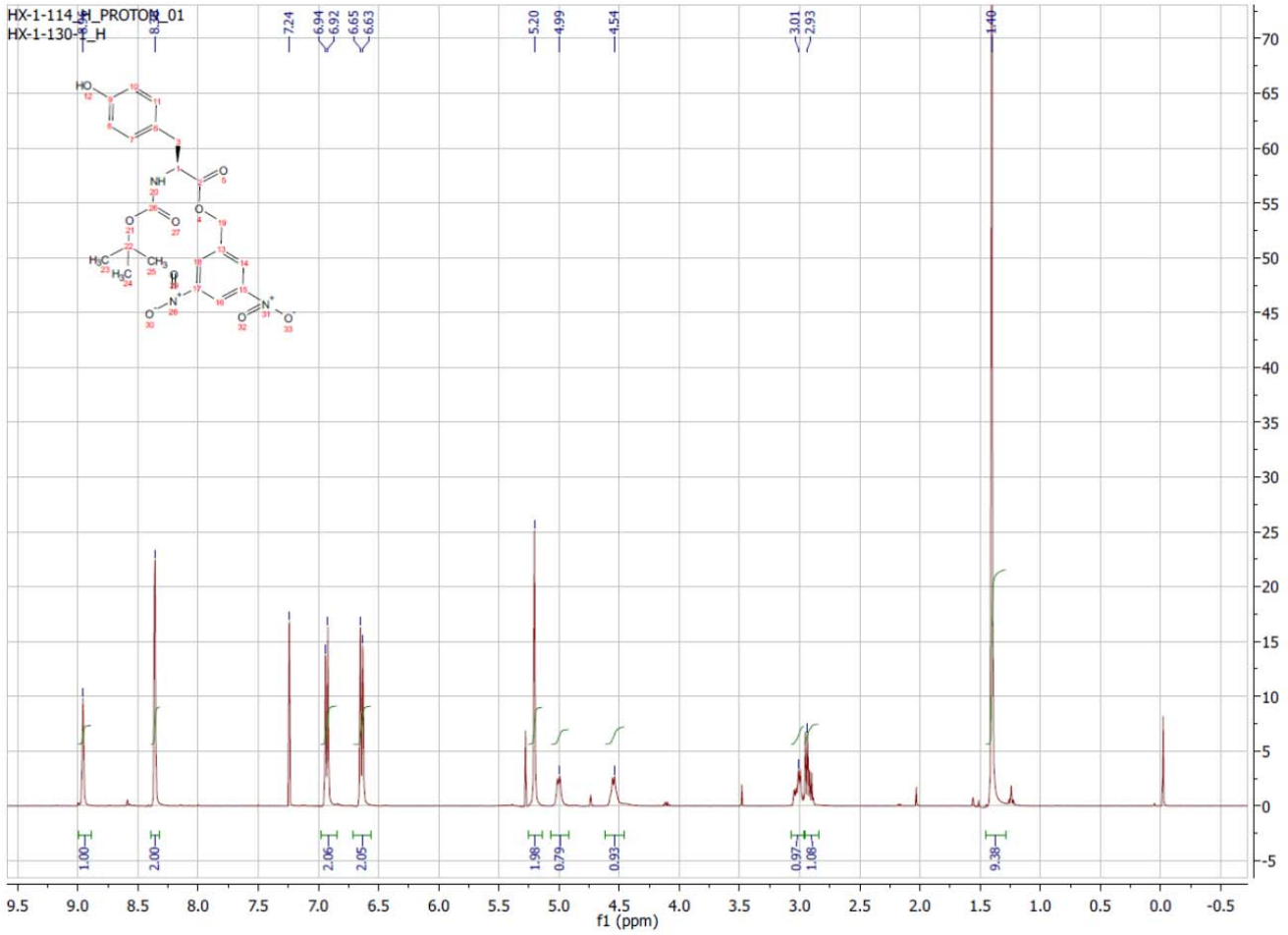


Figure S16. ^{1}H-NMR spectrum of compound **2a**

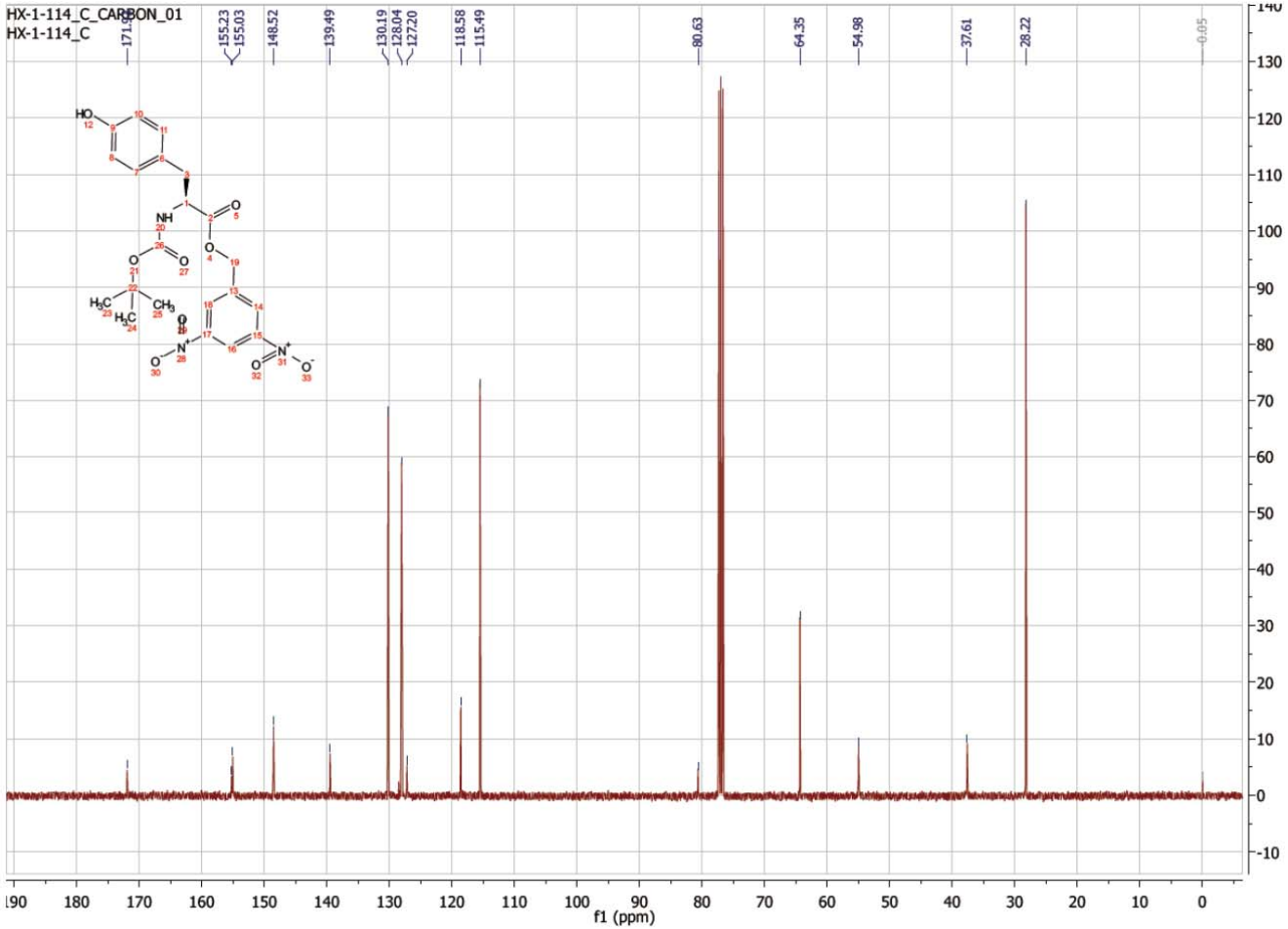


Figure S17. ^{13}C-NMR spectrum of compound **2a**

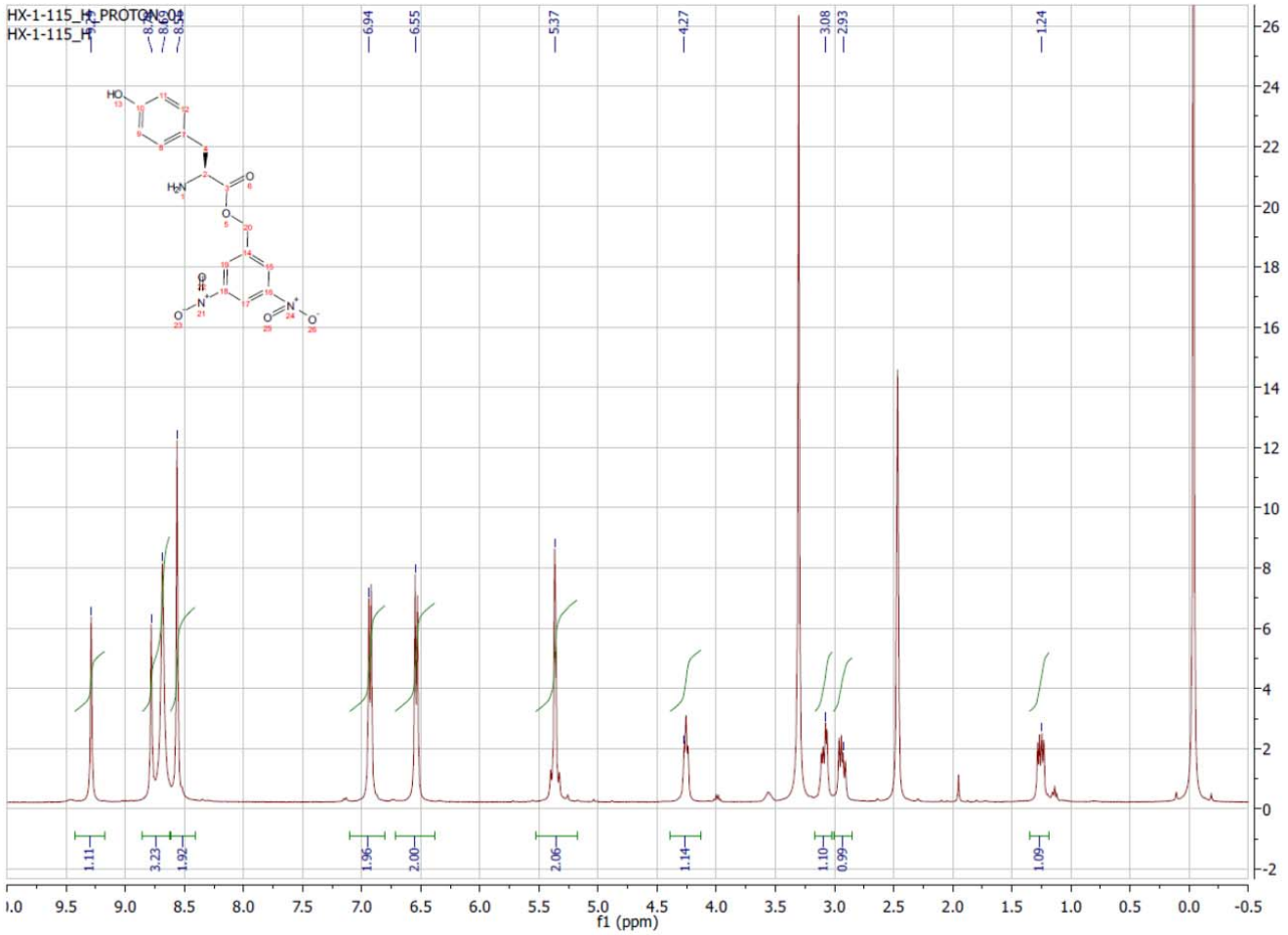


Figure S18. ^{1}H-NMR spectrum of compound **2**

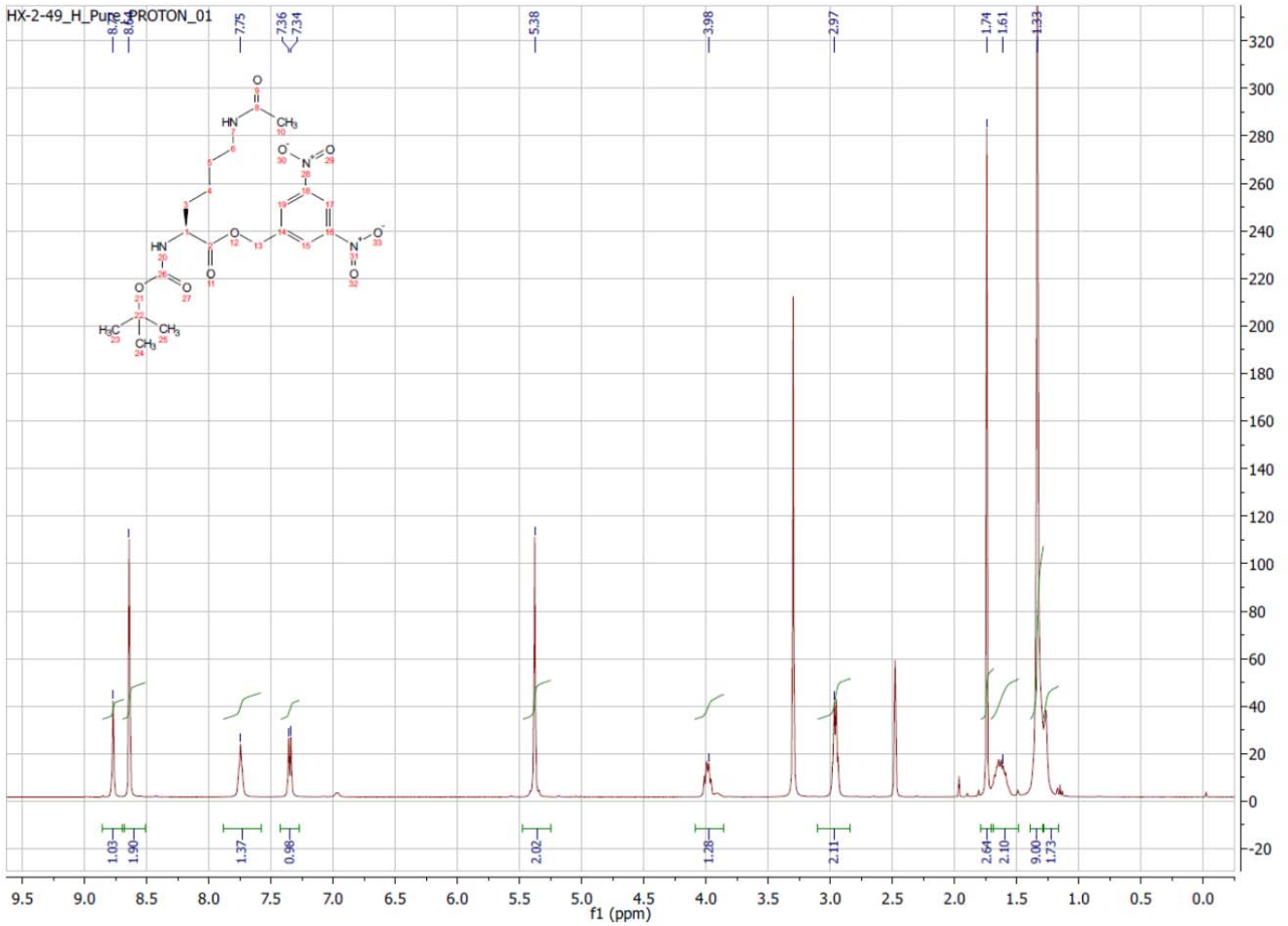


Figure S19. ^{1}H-NMR spectrum of compound **3a**

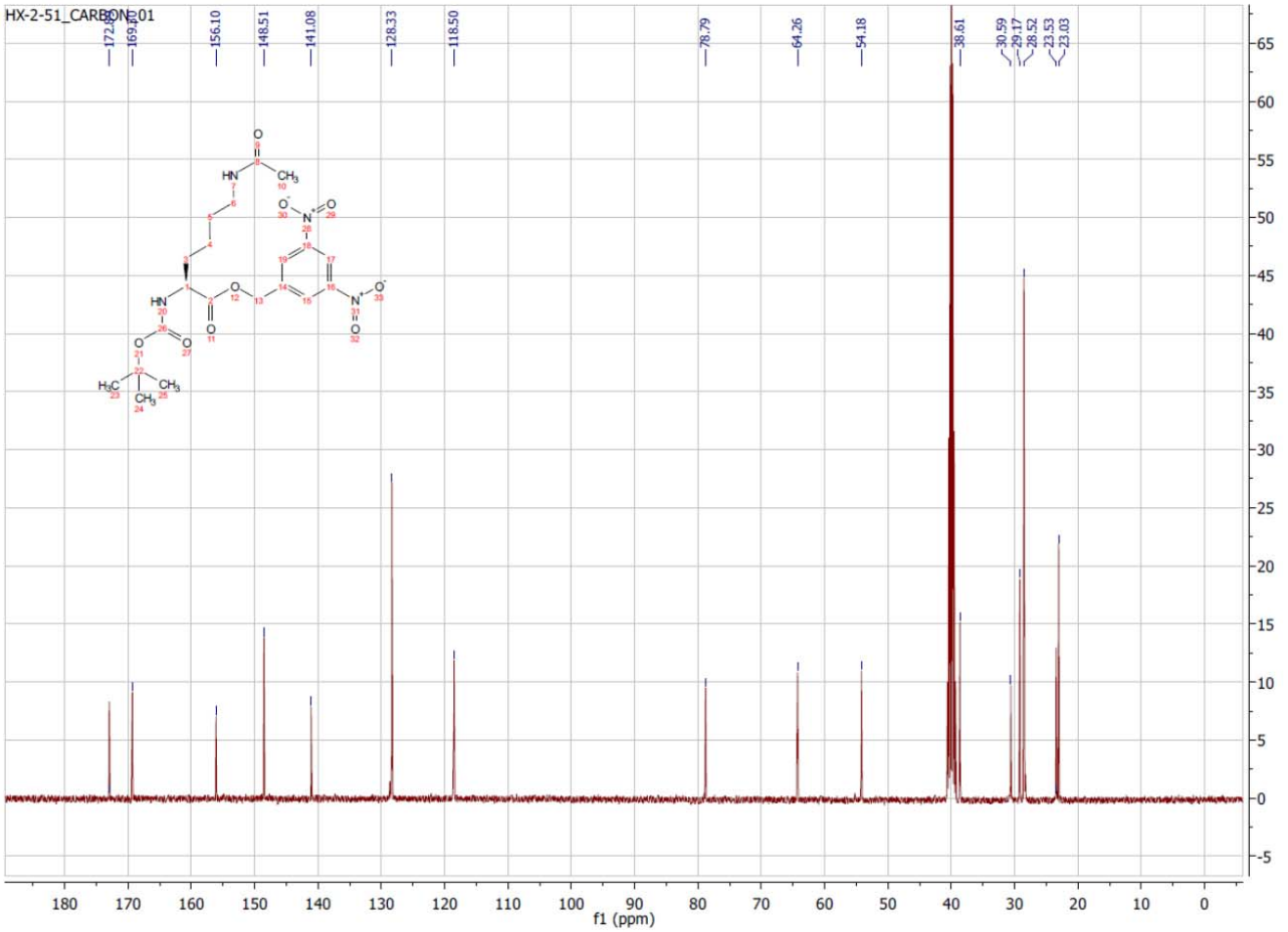


Figure S20. ^{13}C-NMR spectrum of compound **3a**

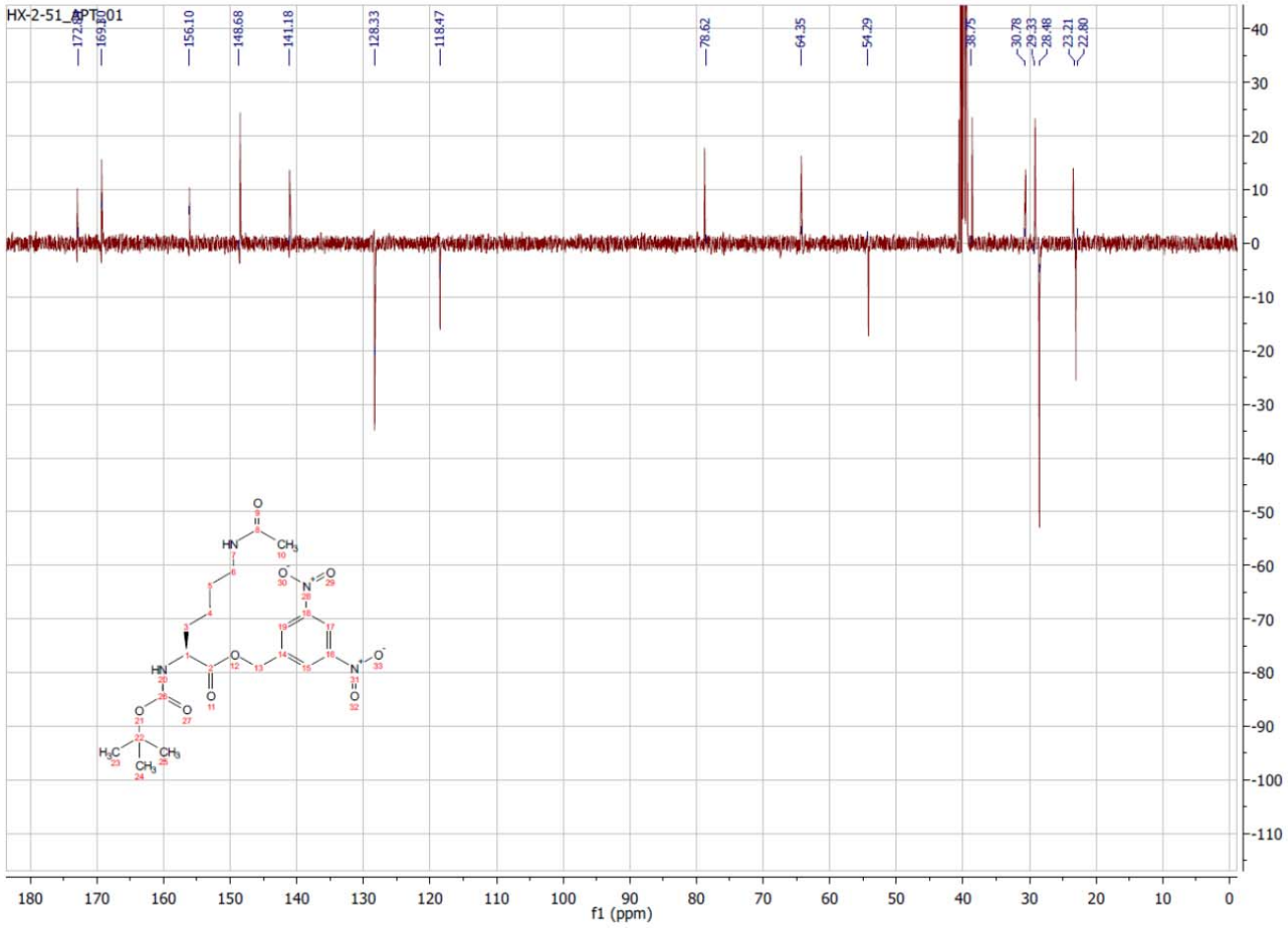


Figure S21. APT-NMR spectrum of compound **3a**

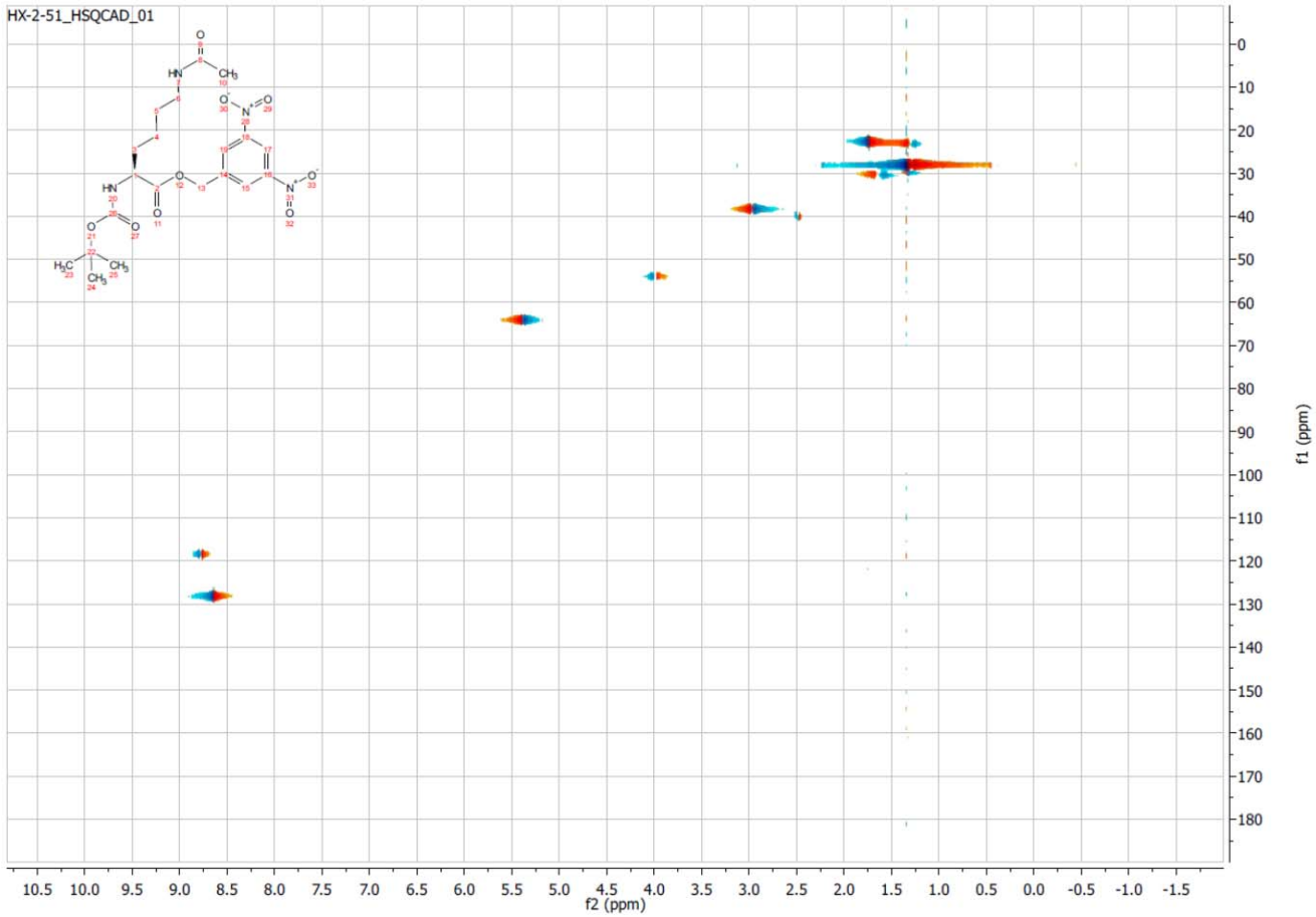
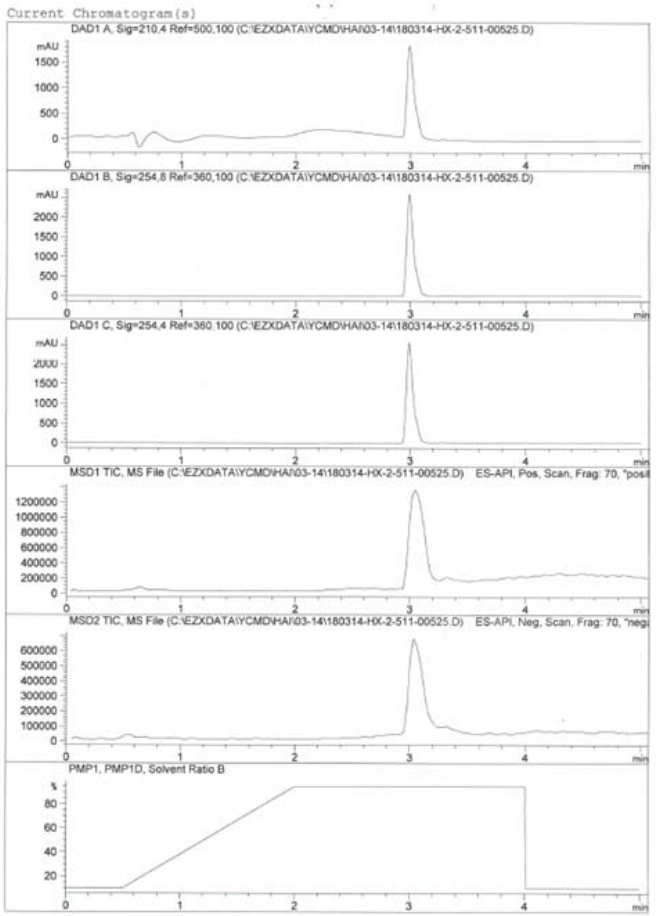
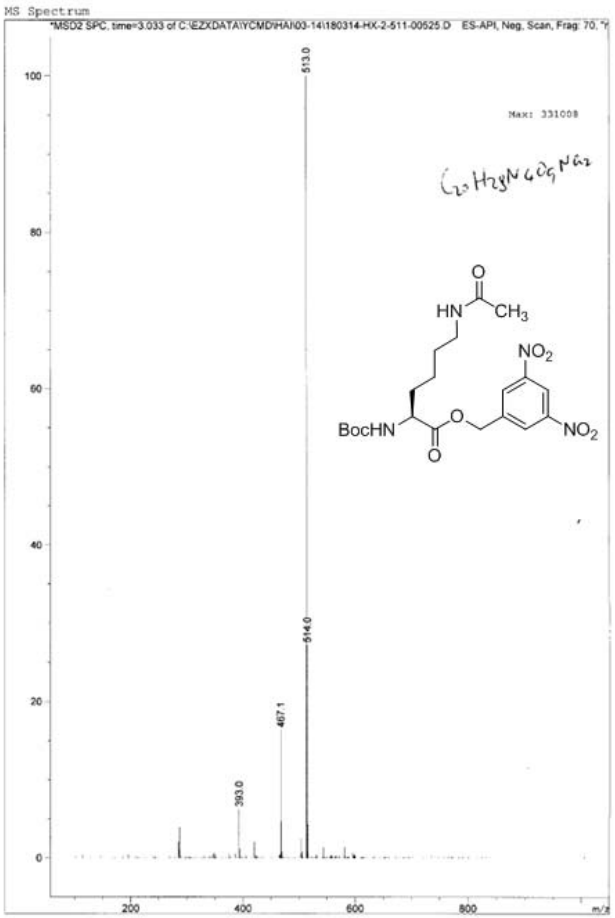


Figure S22. HSQCAD-NMR spectrum of compound **3a**





Nᵅ-Boc-Nᵋ-acetyl-lysine 3,5-dinitrobenzyl ester (3a)

Figure S23. HPLC-MS spectrum of compound **3a**

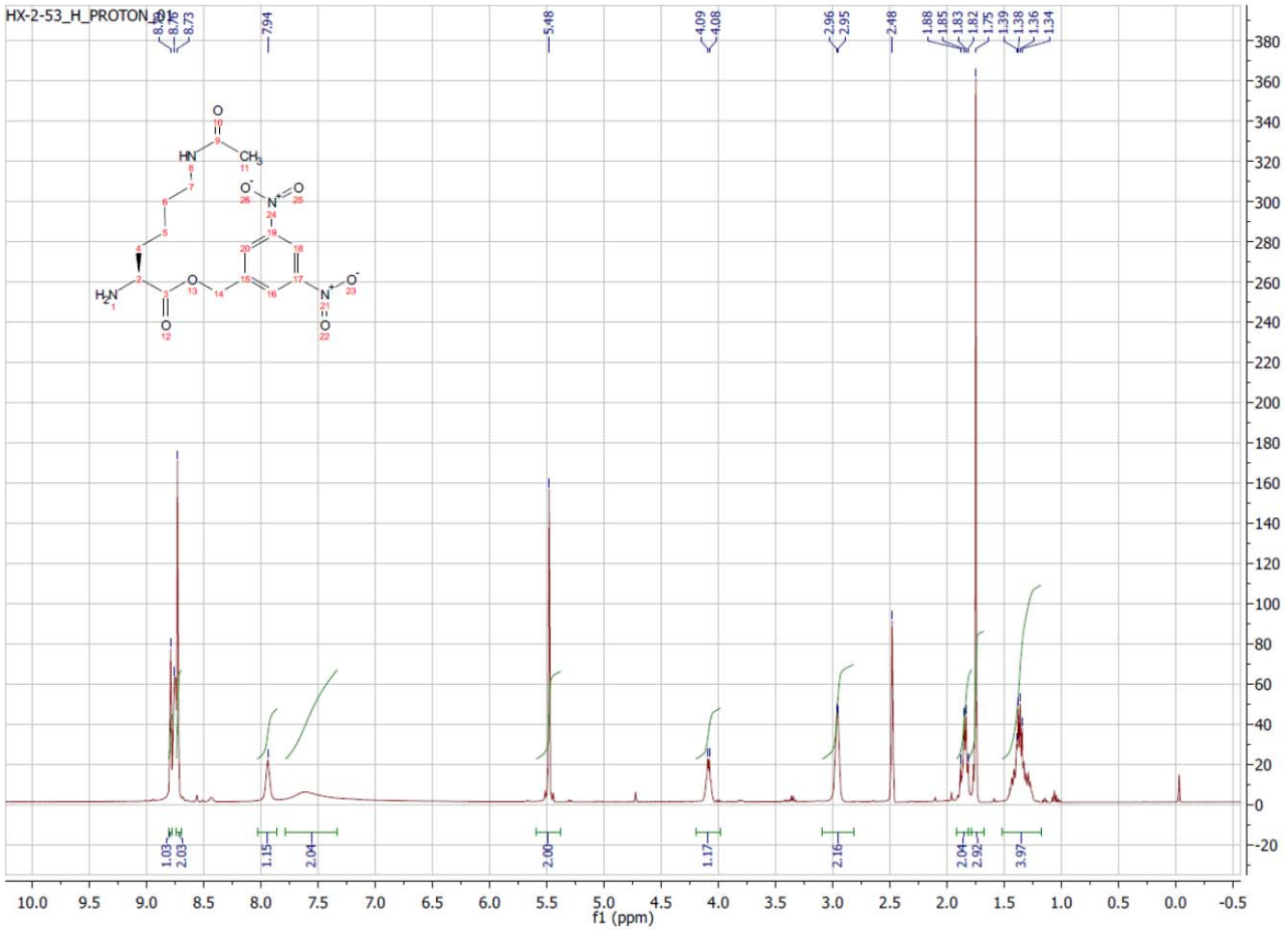


Figure S24. ^{1}H-NMR spectrum of compound **3**

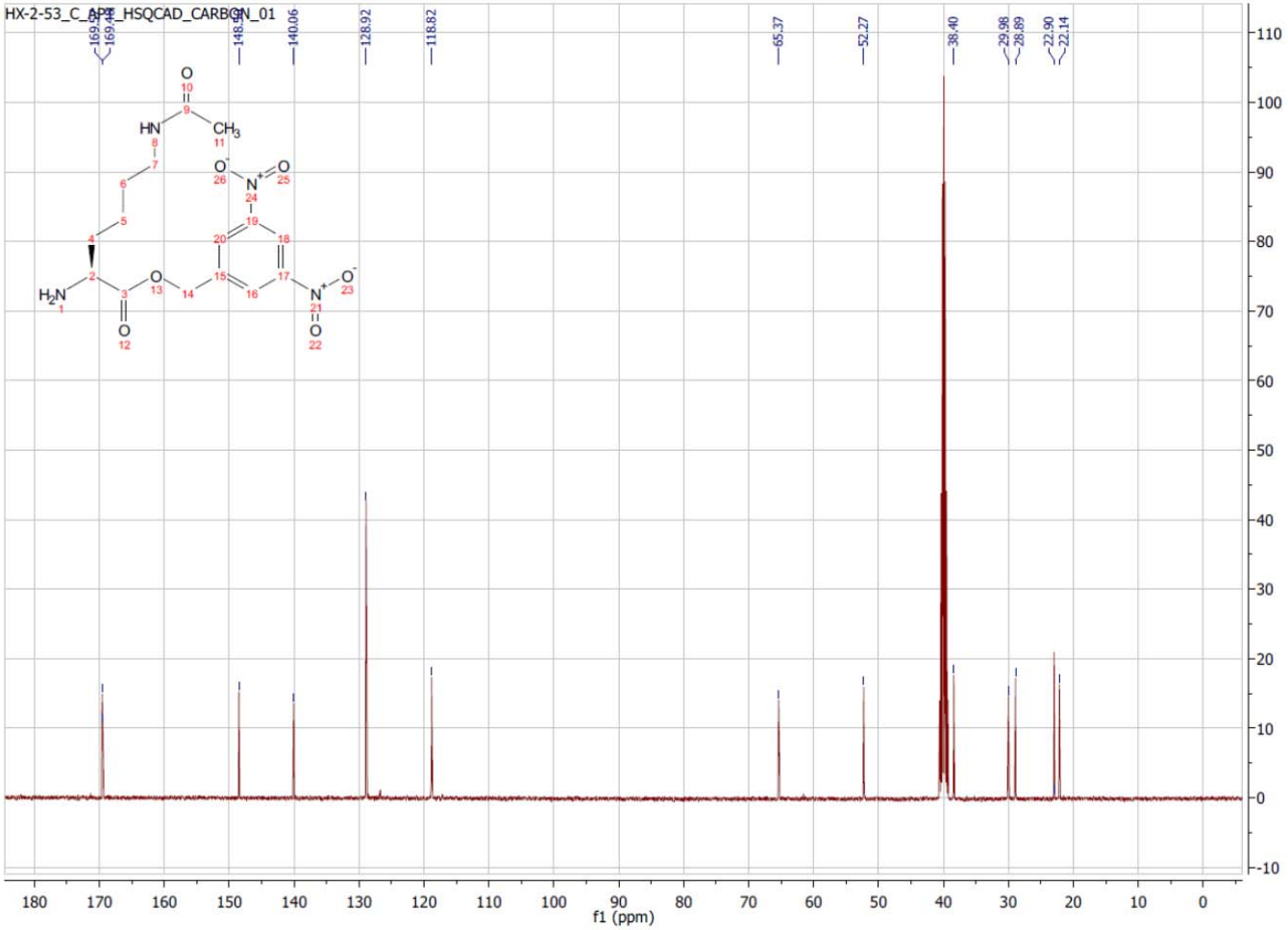


Figure S25. ^{13}C-NMR spectrum of compound **3**

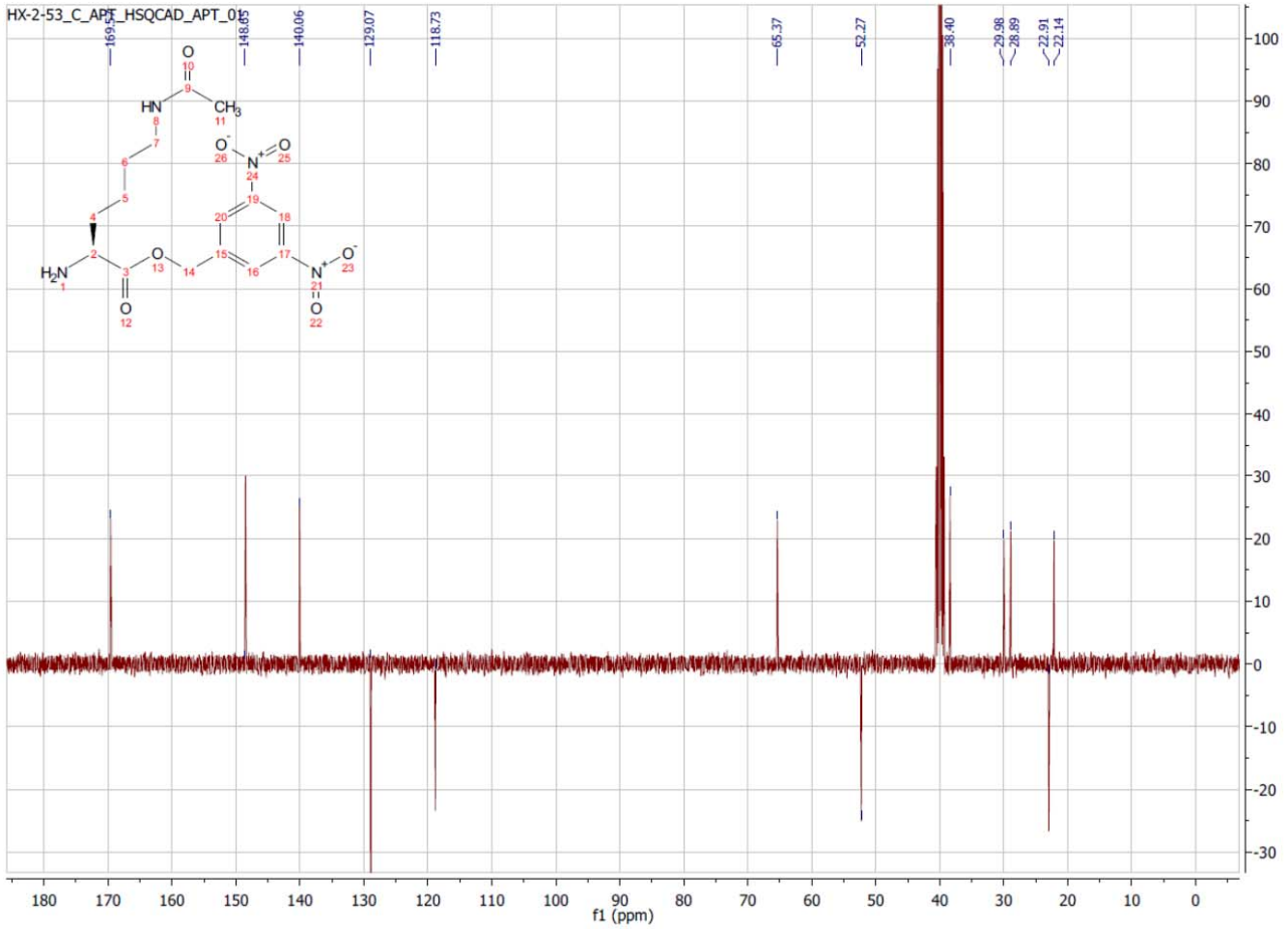


Figure S26. APT-NMR spectrum of compound **3**

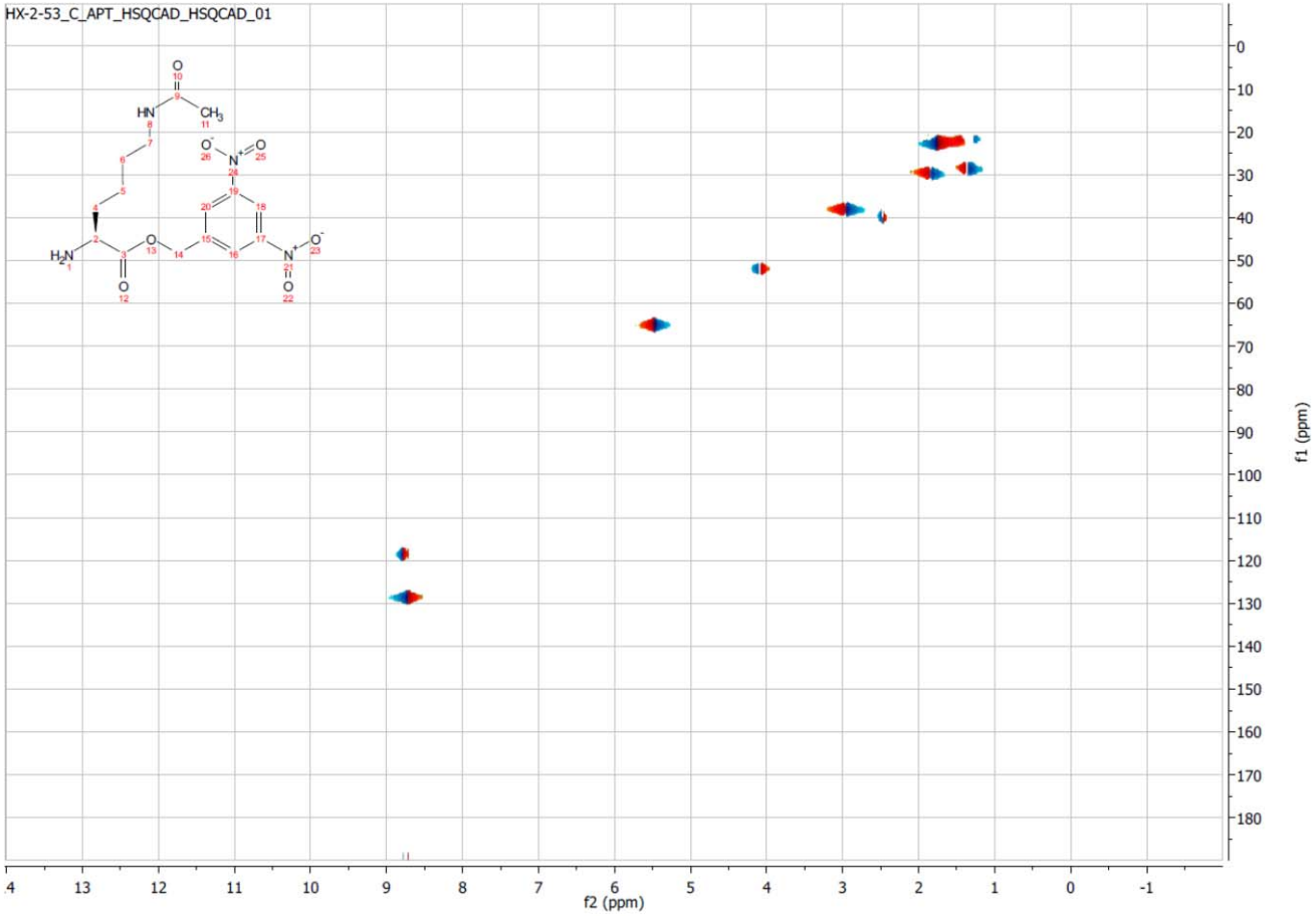
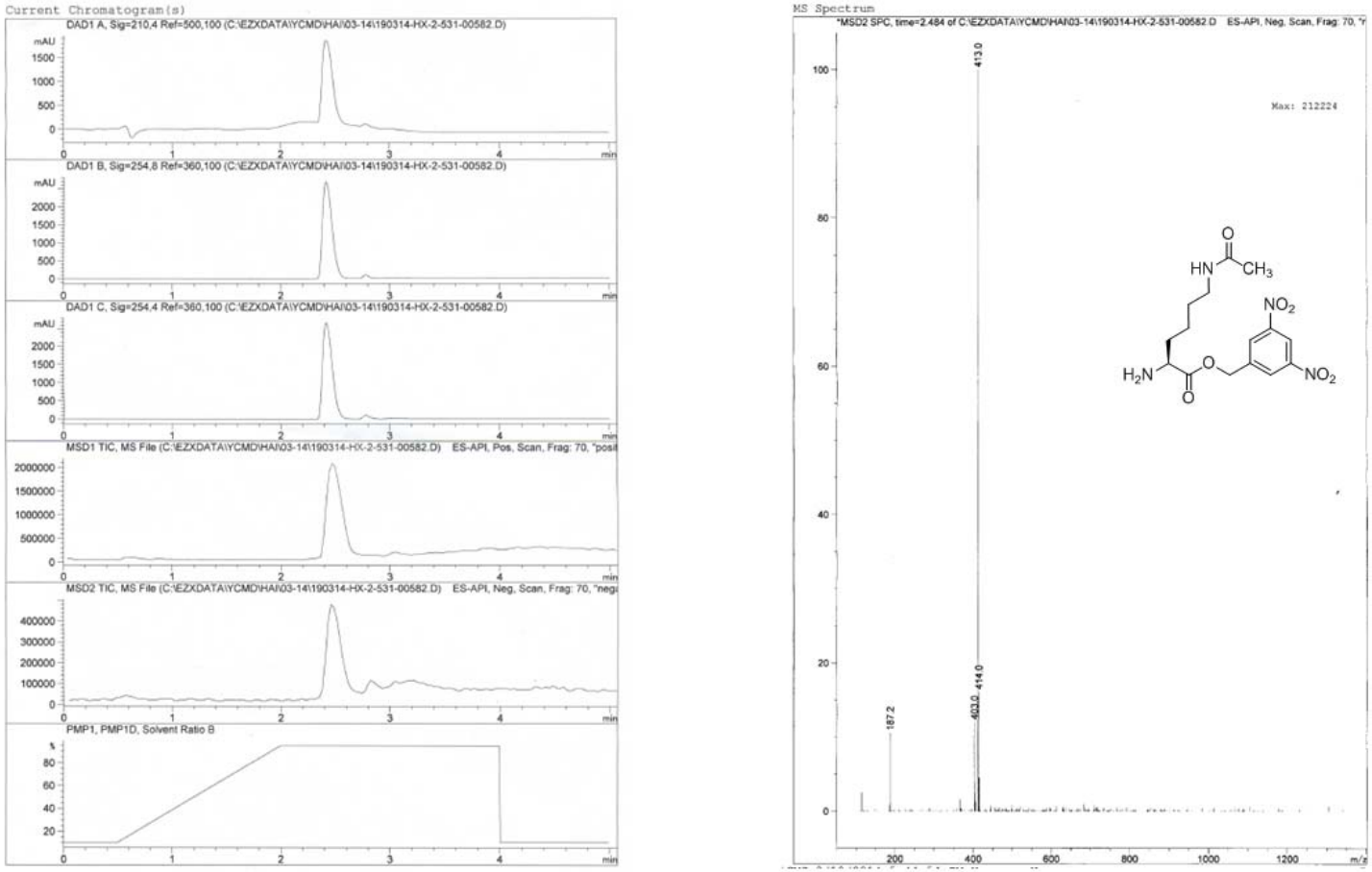


Figure S27. HSQCAD-NMR spectrum of compound **3**



Nε-acetyl-lysine 3,5-dinitrobenzyl ester (3)

Figure S28. HPLC-MS spectrum of compound **3**

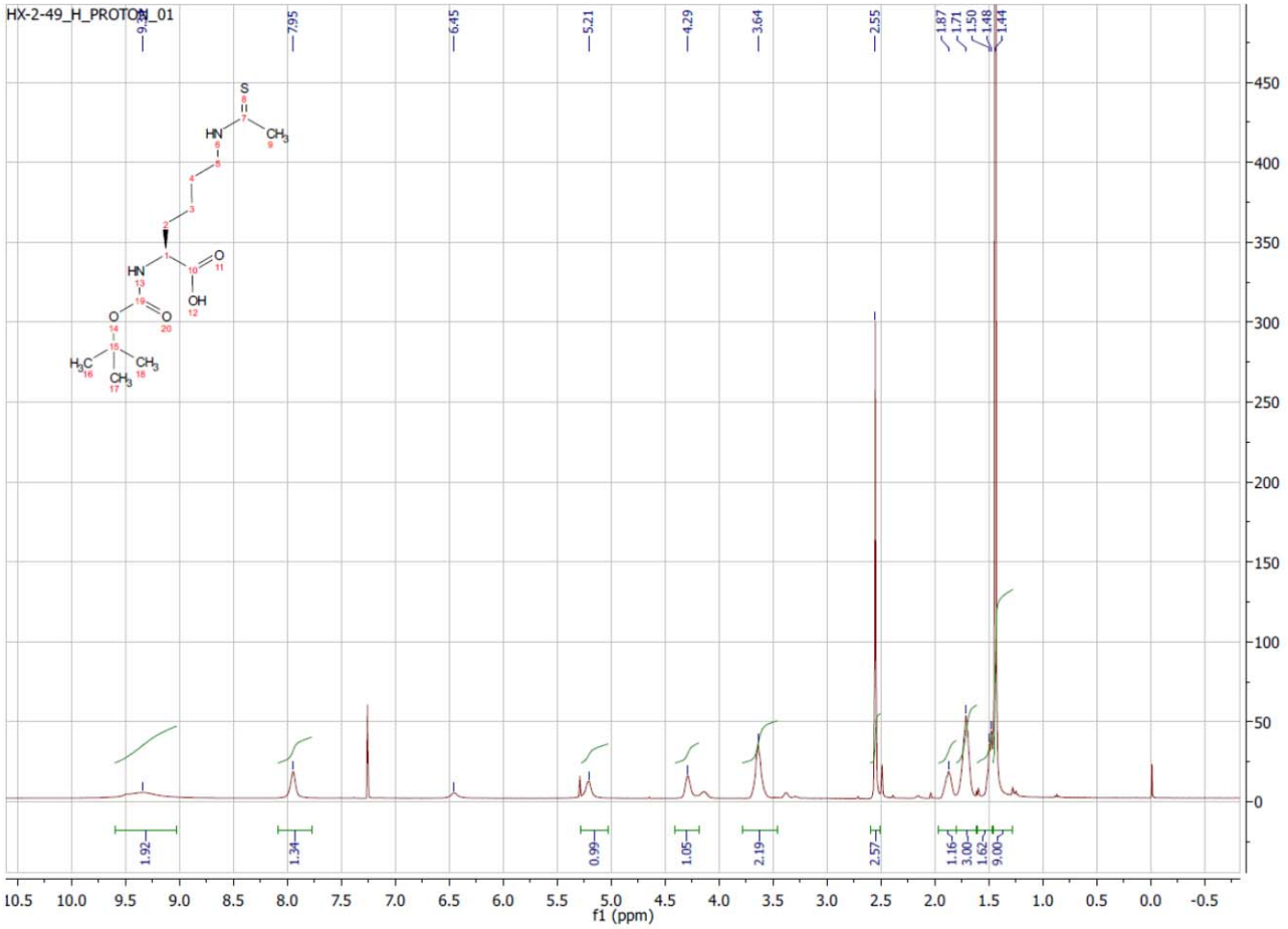


Figure S29. ^{1}H-NMR spectrum of compound **4a**

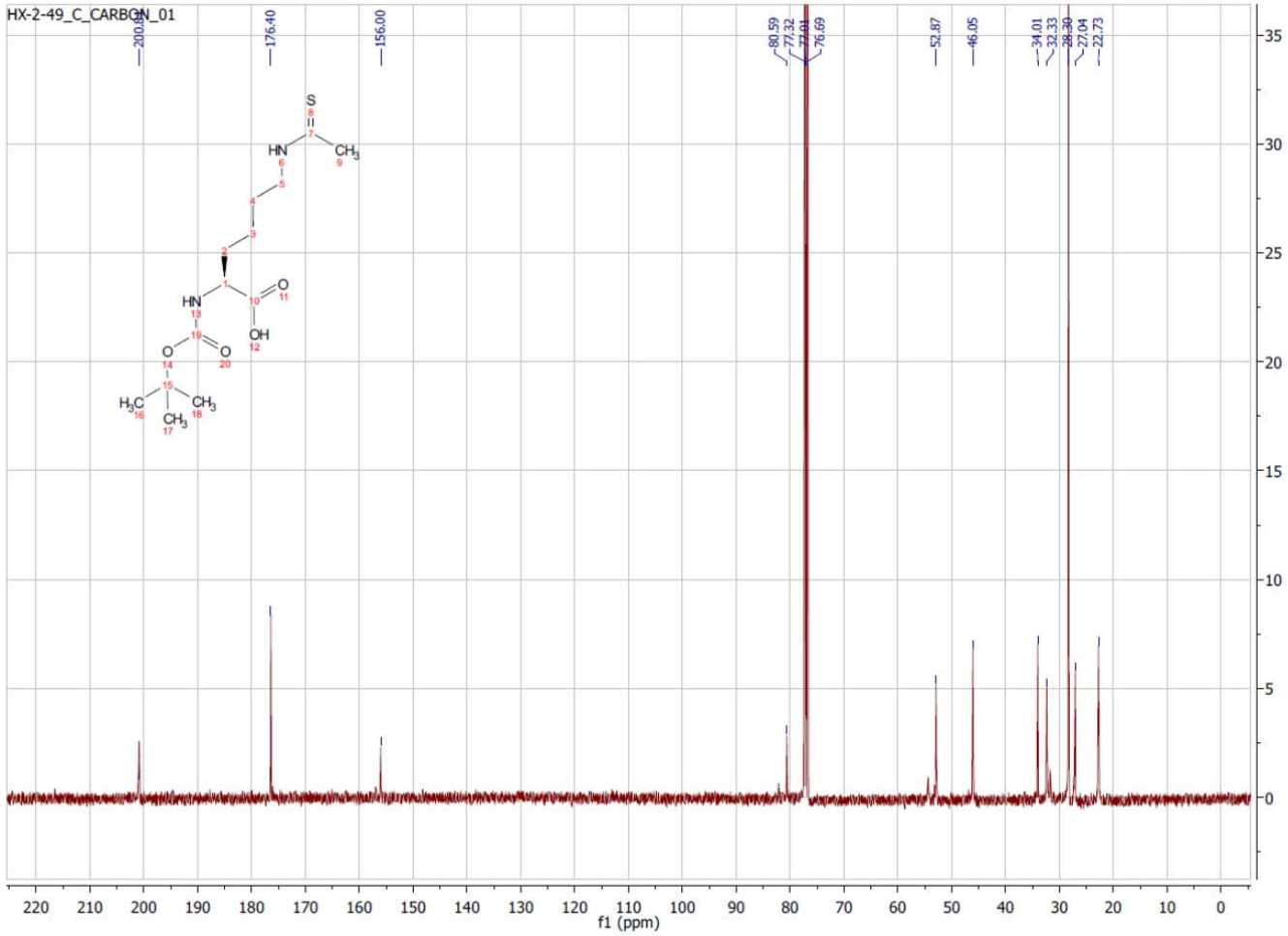


Figure S30. ^{13}C-NMR spectrum of compound **4a**

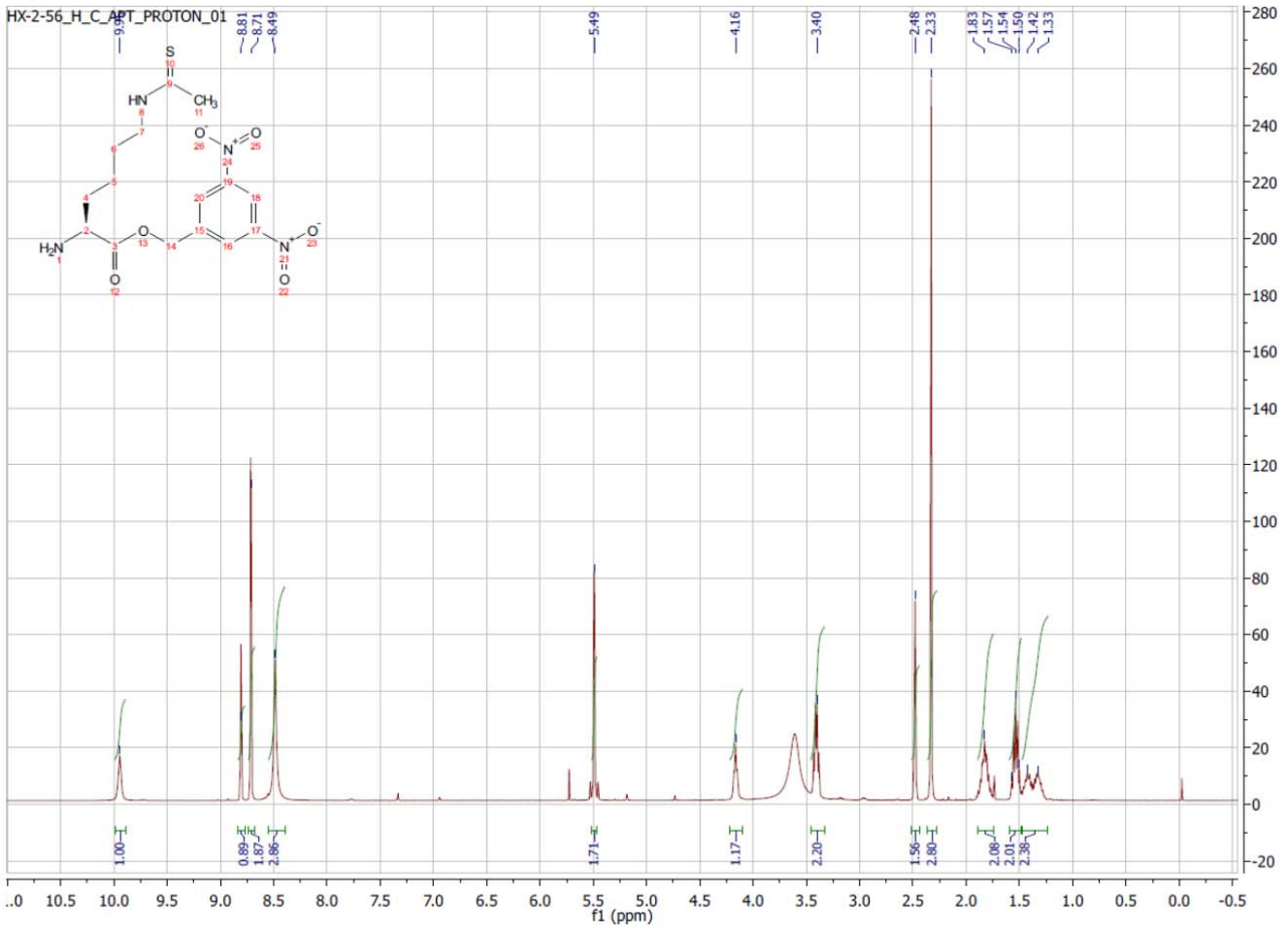


Figure S31. ^{1}H-NMR spectrum of compound **4**

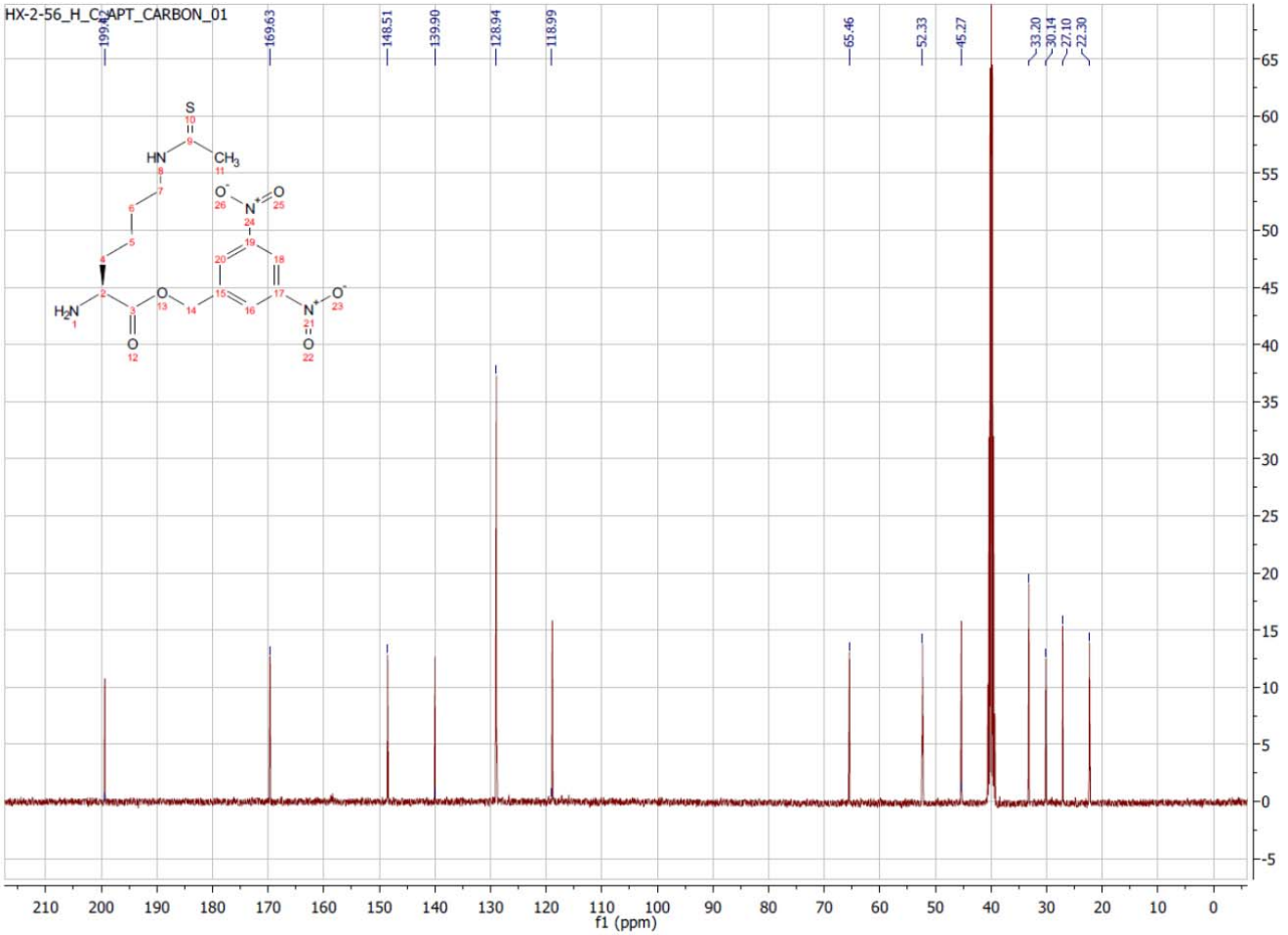


Figure S32. ^{13}C-NMR spectrum of compound **4**

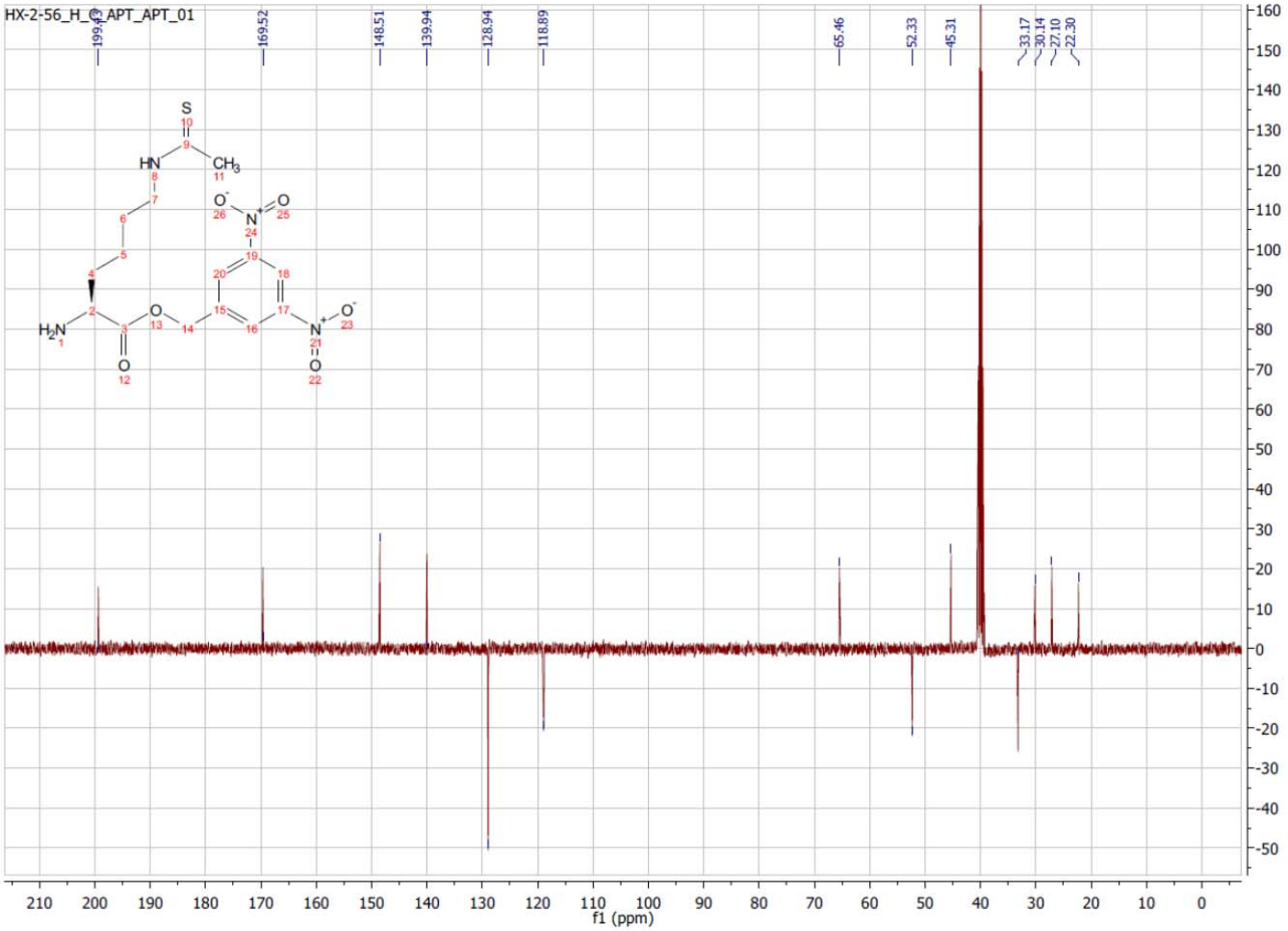


Figure S33. APT-NMR spectrum of compound **4**

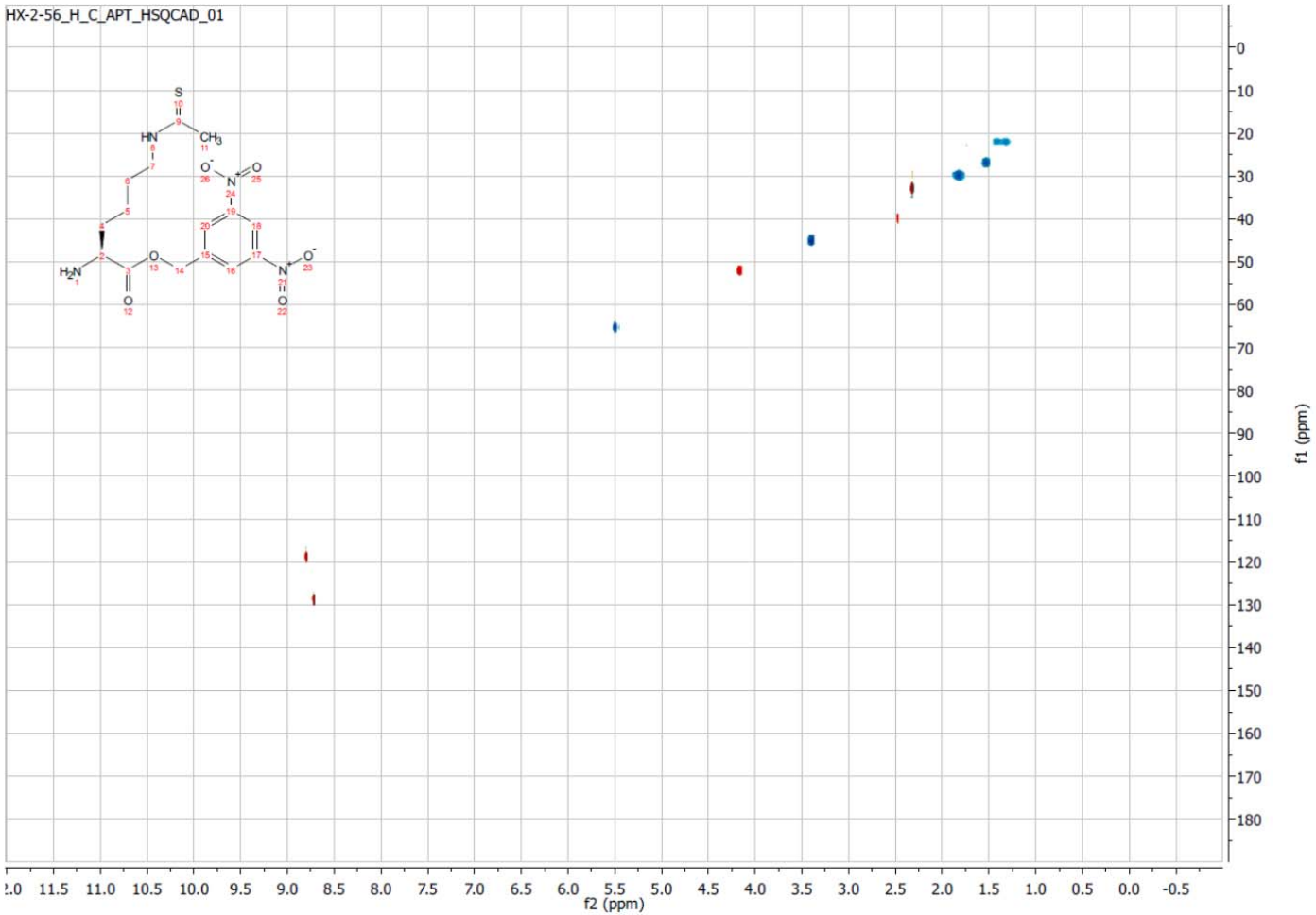
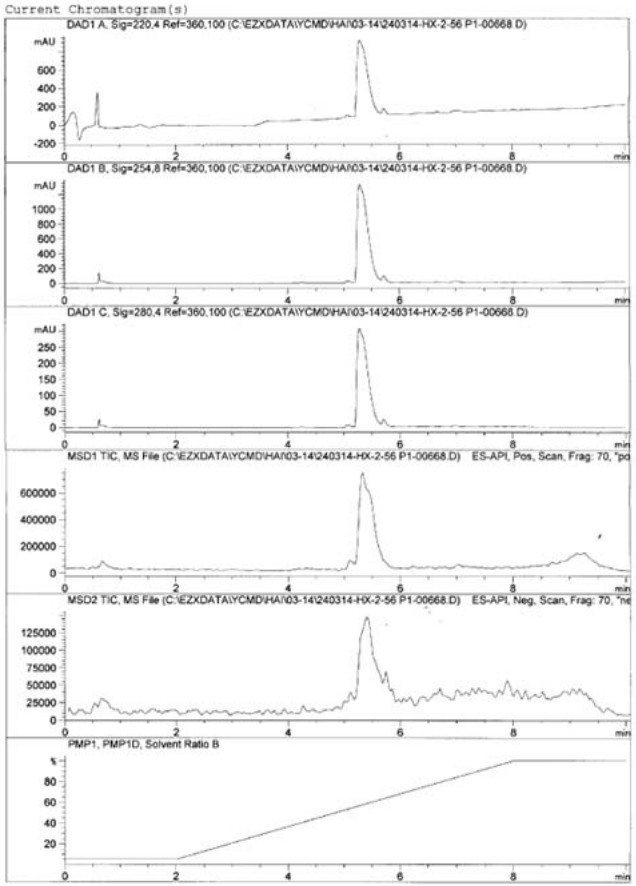
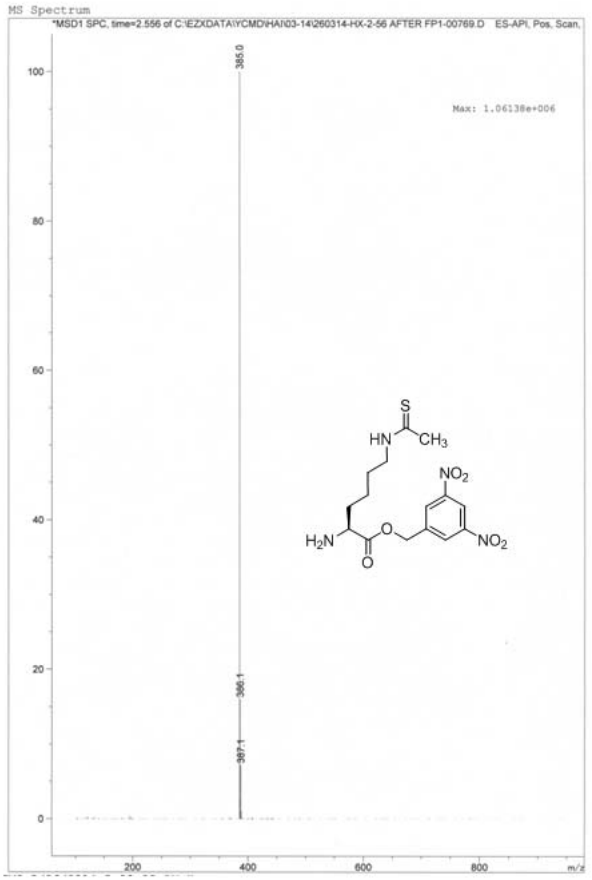


Figure S34. HSQCAD-NMR spectrum of compound **4**





N^{ε}-thioacetyl-lysine 3,5-dinitrobenzyl ester (4)

[M+H] Calc. 385.1, $[M+H]^+$ Found 385.0

Figure S35. HPLC-MS spectrum of compound **4**

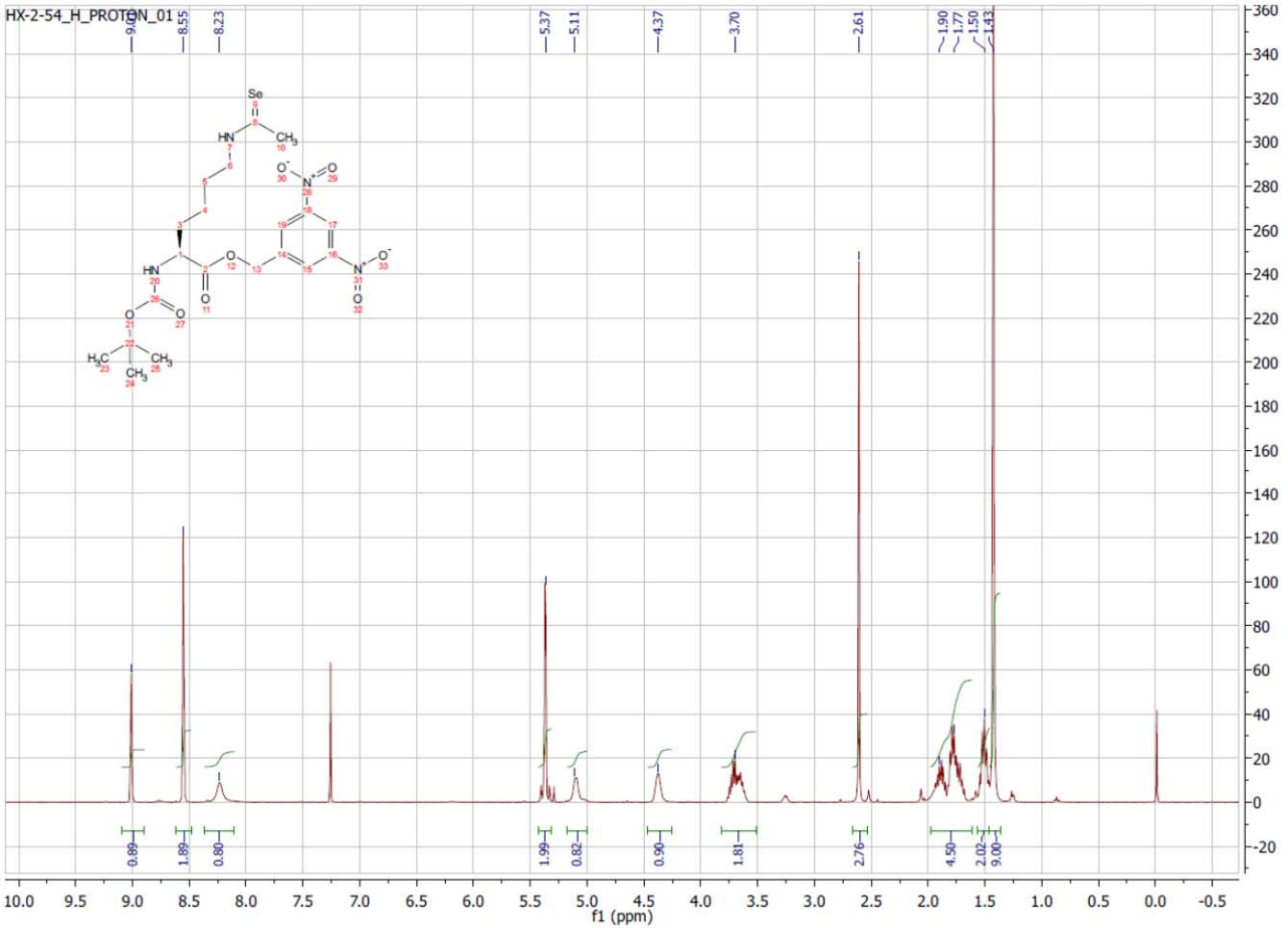


Figure S36. ^{1}H-NMR spectrum of compound **5a**

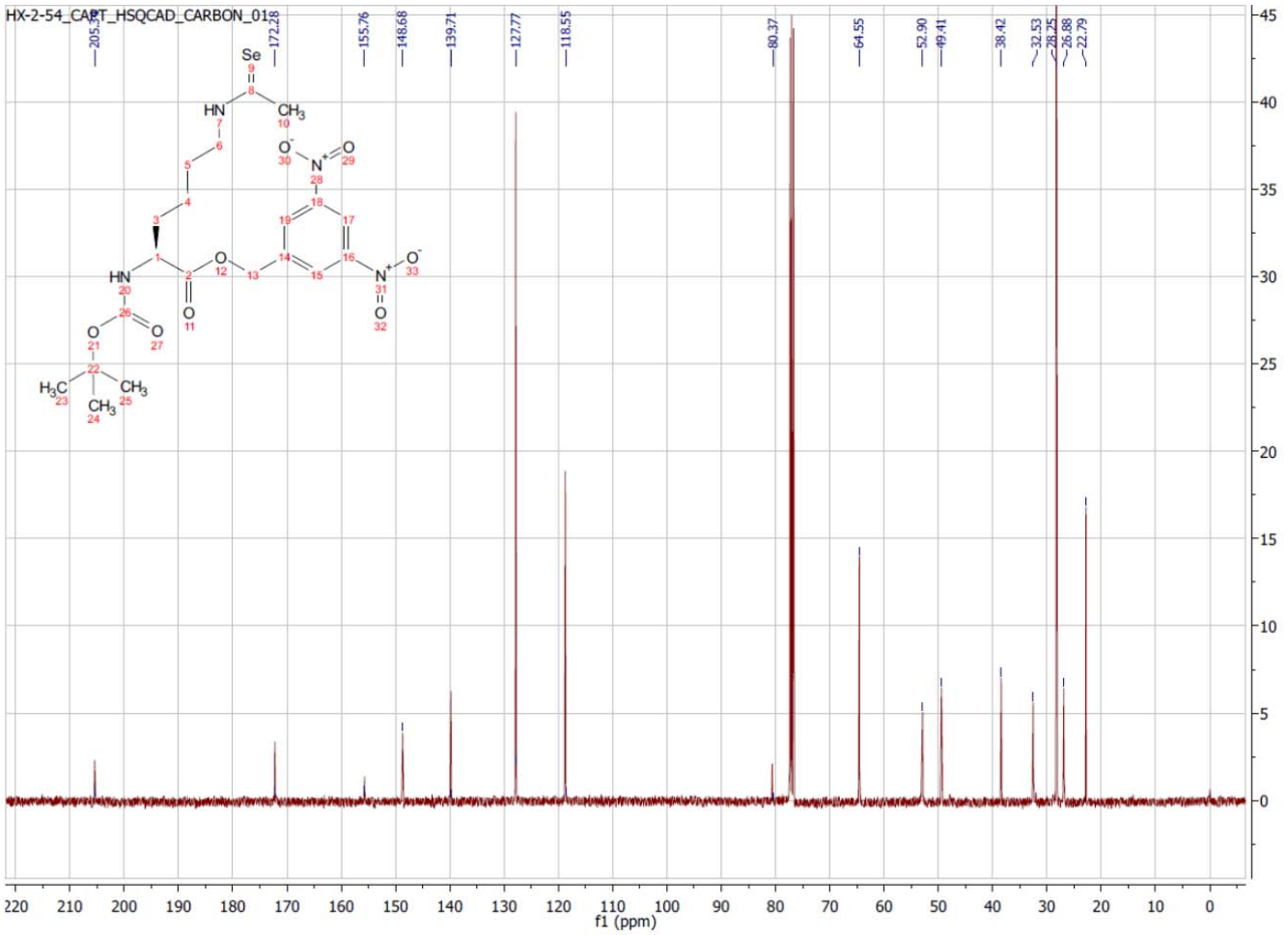


Figure S37. ^{13}C-NMR spectrum of compound **5a**

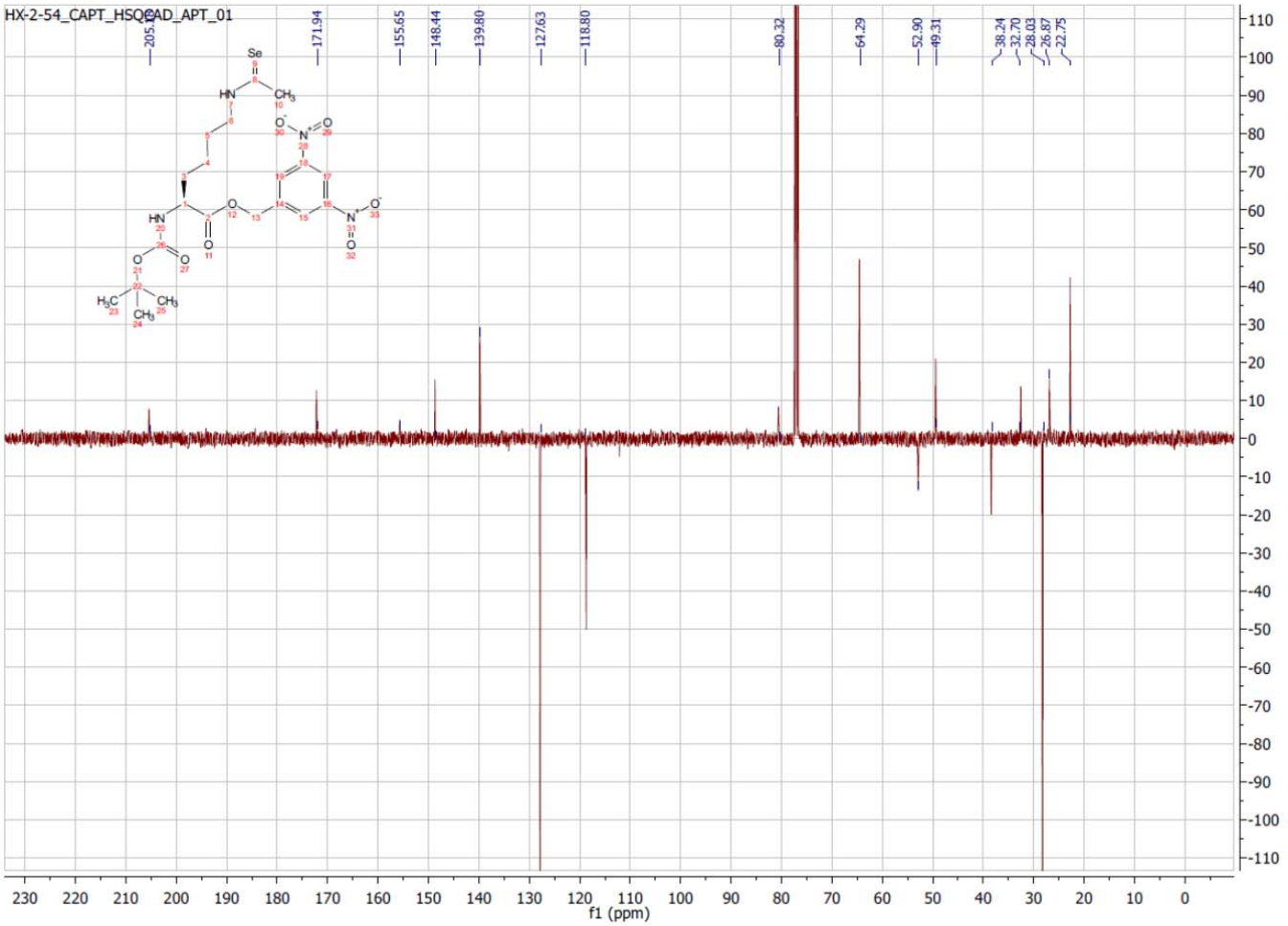


Figure S38. APT-NMR spectrum of compound **5a**

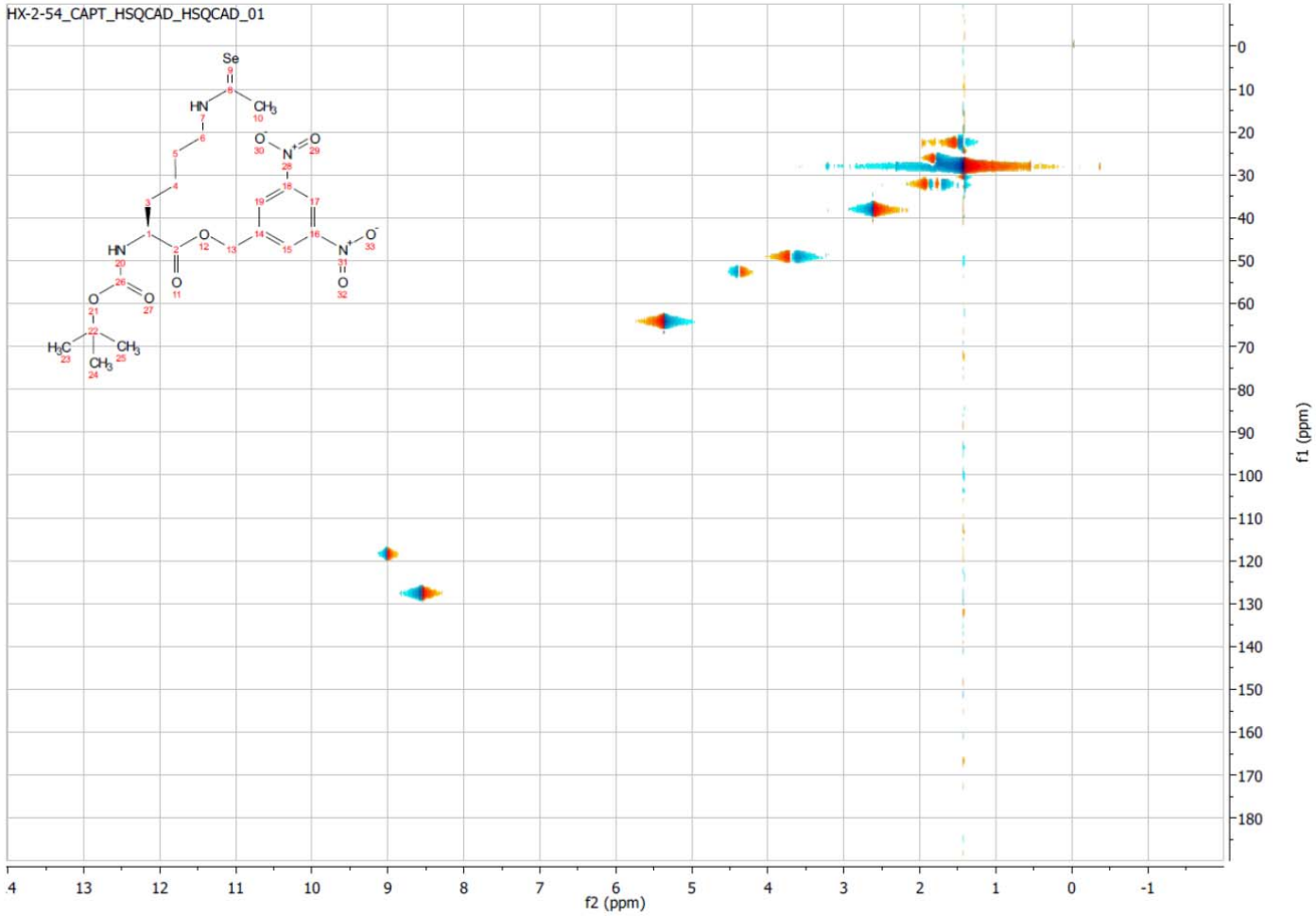


Figure S39. HSQCAD-NMR spectrum of compound **5a**

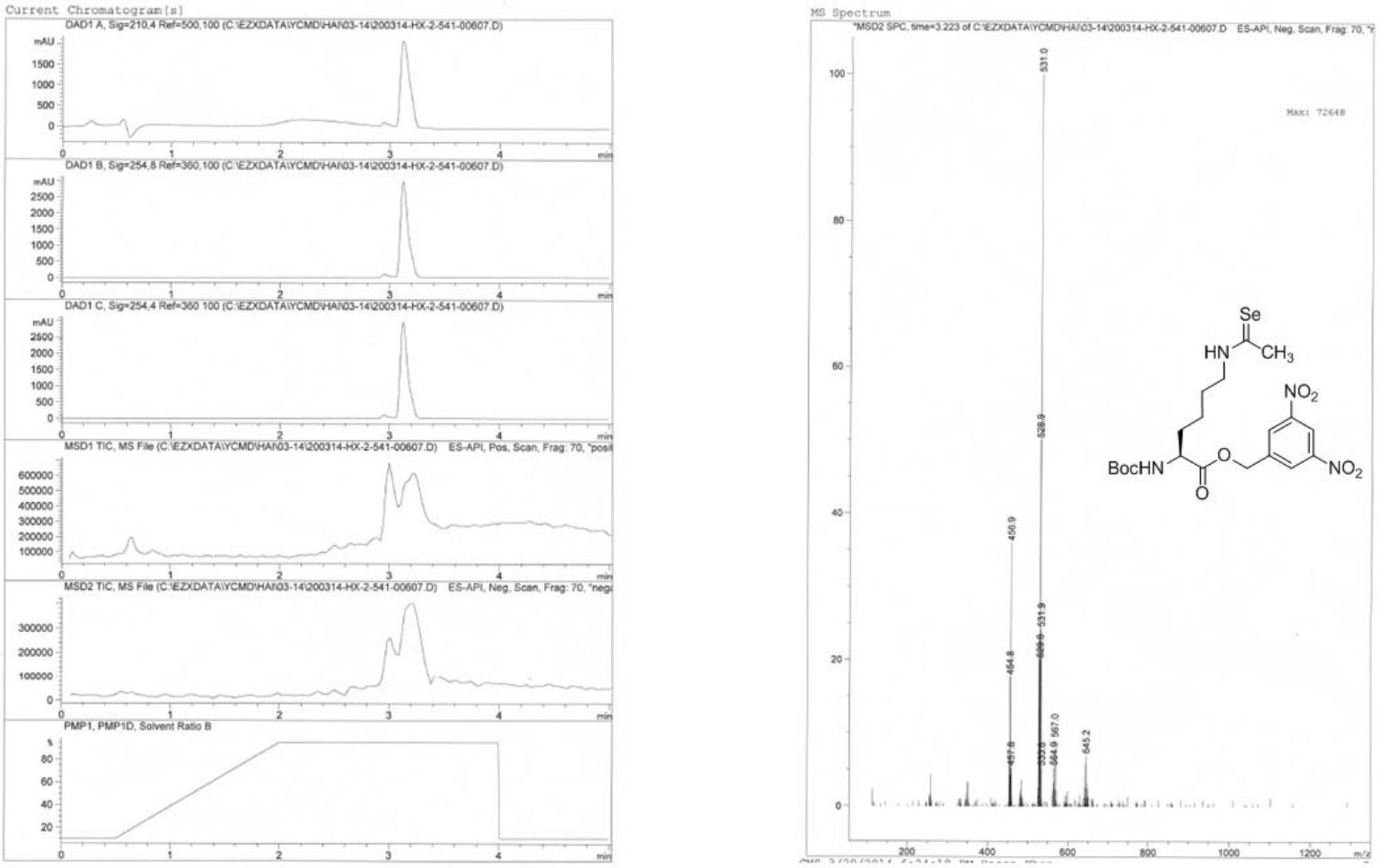


Figure S40. HPLC-MS spectrum of compound **5a**

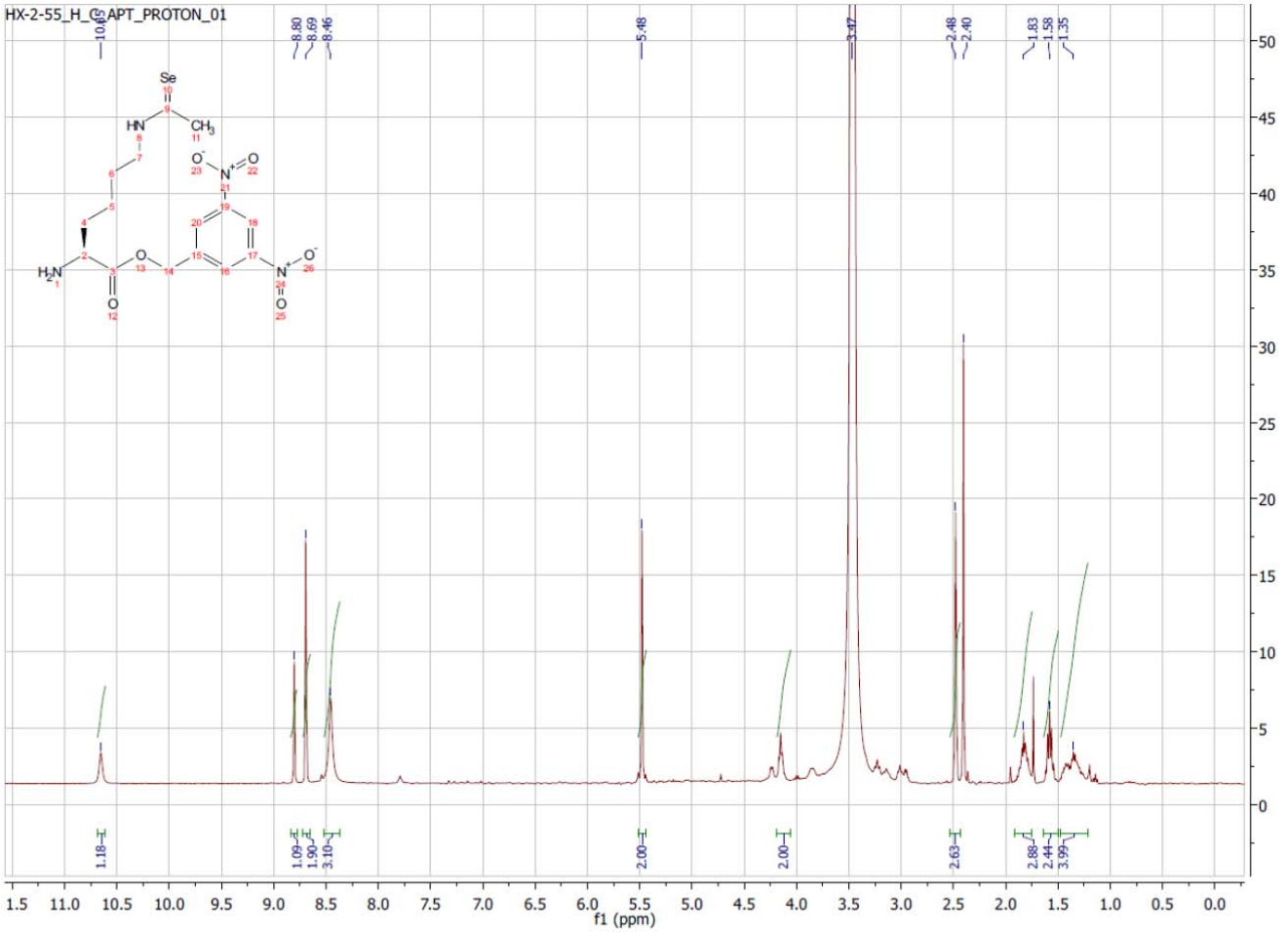


Figure S41. ^{1}H-NMR spectrum of compound **5**

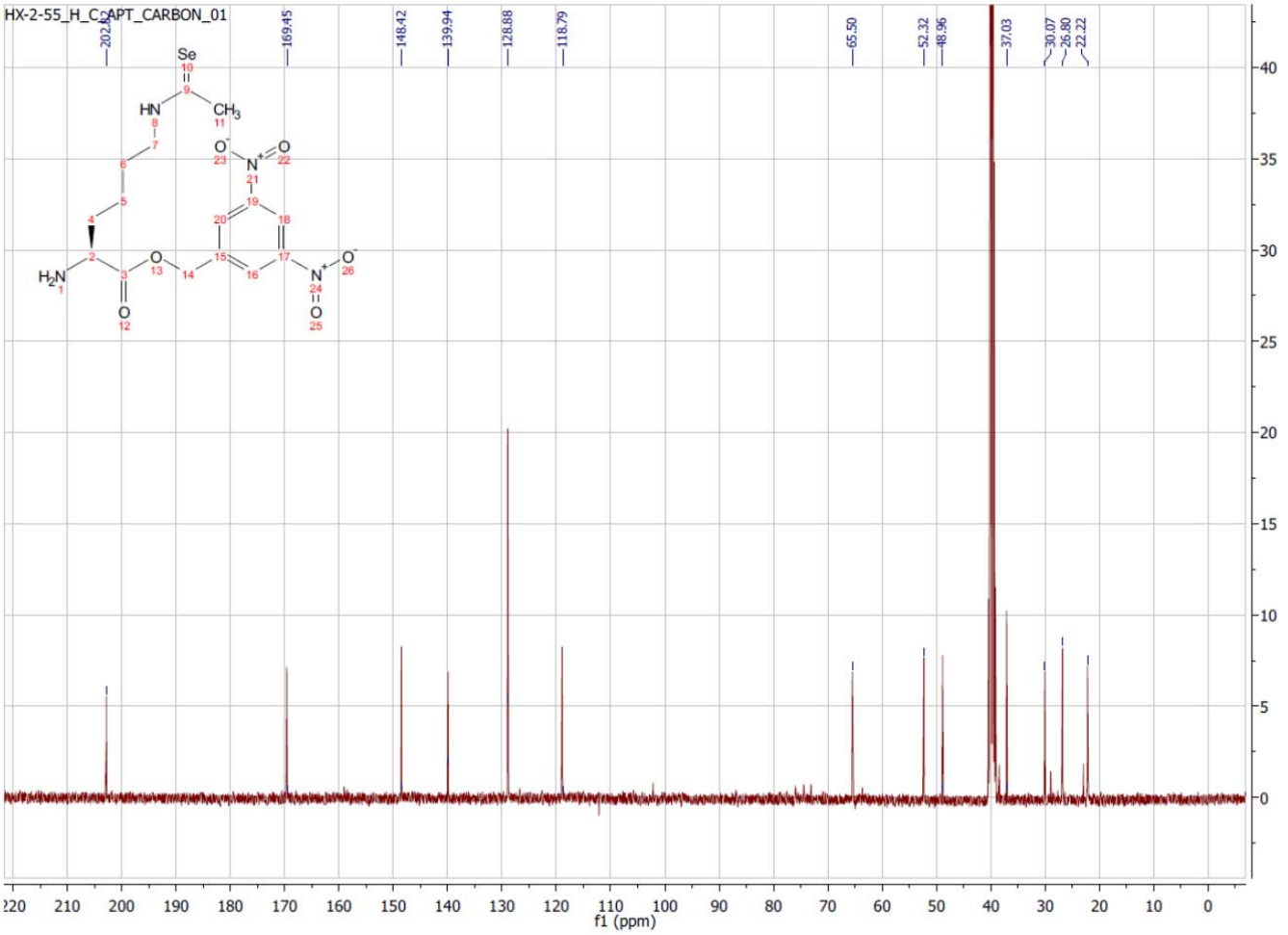


Figure S42. ^{13}C-NMR spectrum of compound **5**

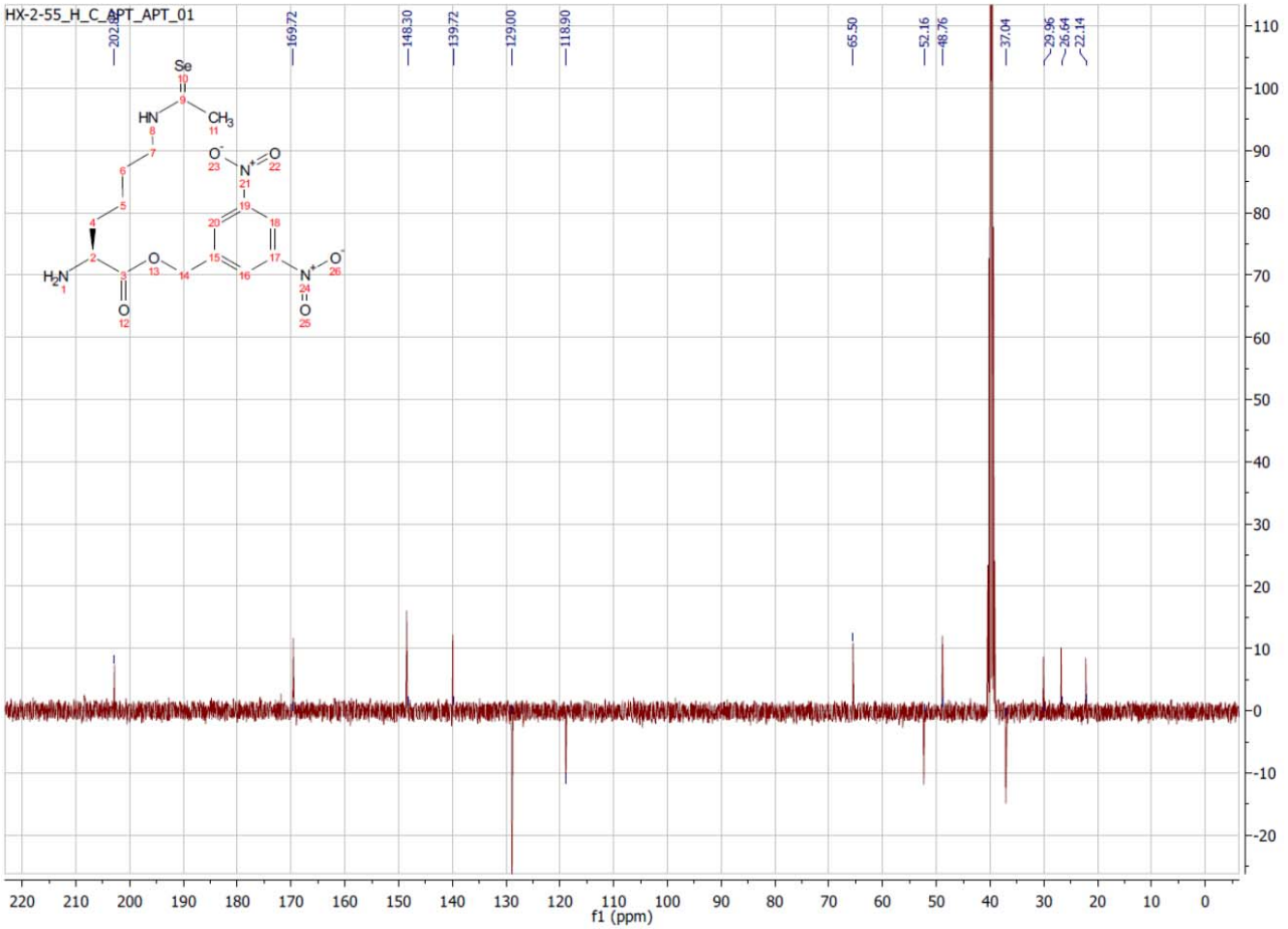


Figure S43. APT-NMR spectrum of compound **5**

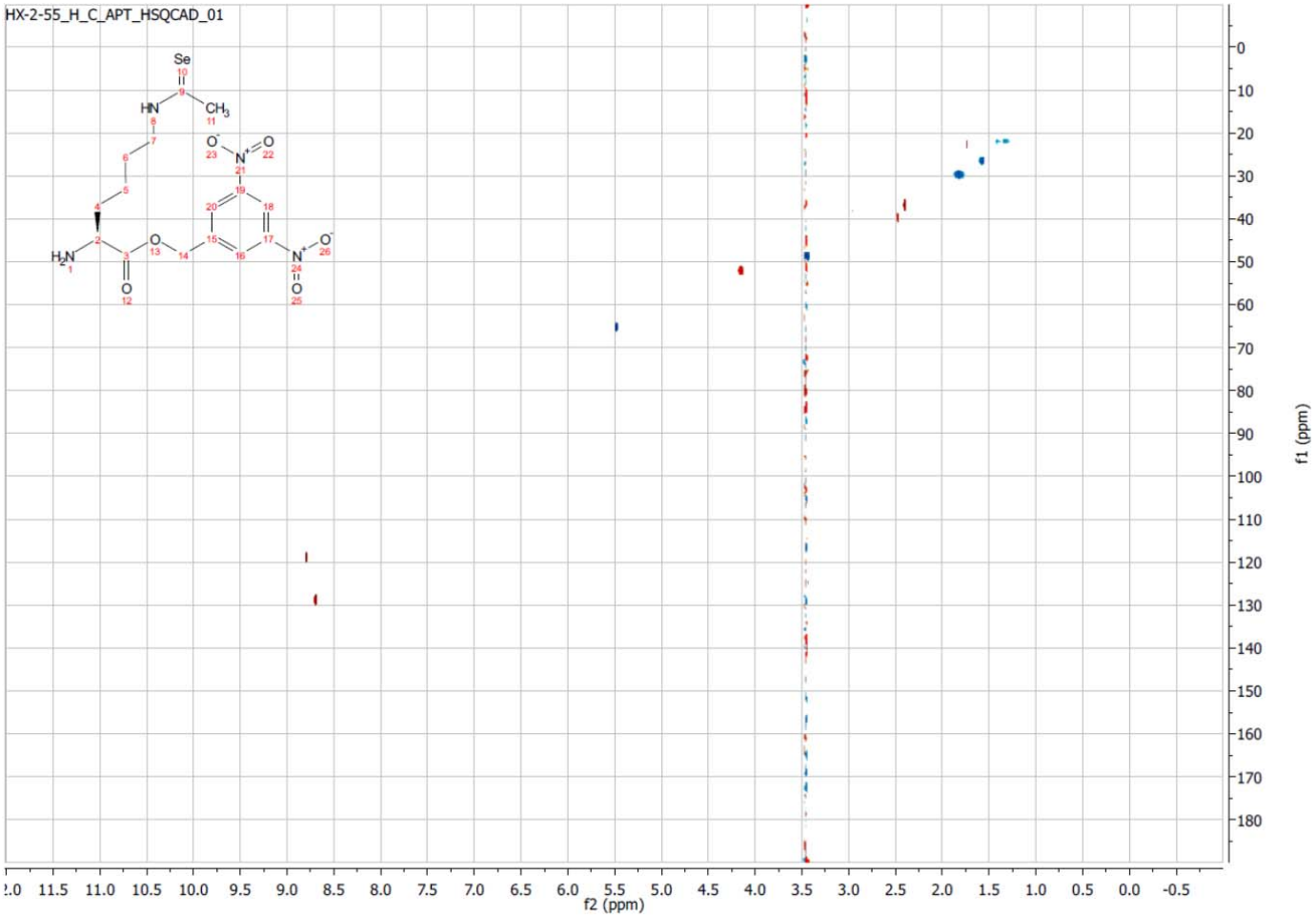


Figure S44. HSQCAD-NMR spectrum of compound **5**