Supporting Information

Chemical Proteomic Profiling of Protein *N*-homocysteinylation with Thioester Probe

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Experimental Procedures

BIOLOGICAL METHODS

Reagents and antibodies

Biotin-PEG4-azide (Product no. 271606) was purchased from ChemPep Inc. and PC-Biotin-Azide (Product no. 1119) was obtained from Click chemistry tools. Pierce Streptavidin Agarose Resins (catalog no. 20361, Thermo scientific), RNaseA, Pancreatic (Product no. 0675-250mg, Amresco), Myoglobin from equine skeletal muscle (Product no. M0630-250mg, Sigma), Lysozyme (Product no. 0665-5g, Amresco), Bovine Serum Albumin (BSA) (Fraction V) (Product no. BP1600-100, *L*-homocysteine thiolactone hydrochloride, HTL (H6503-100mg, Sigma) and other chemical or biological reagents were obtained from commercial suppliers without any manipulation. The antibodies used for immunoblotting were Pierce Streptavidin, Horseradish Peroxidase Conjugated (Product no. 21126, Thermo scientific), Anti-mouse IgG, HRP-linked Antibody (catalog no. 7076S, Cellular signalling technology), Anti-rabbit IgG, HRP-linked Antibody (catalog no. CW0103, CoWin Biosciences), anti-GAPDH antibody (EPR16891) (catalog no. ab181602, Abcam), anti-PKM1/2 antibody (catalog no. 3186, Cellular signalling technology) and anti-ATP5A antibody (catalog no. ab14748, Abcam).

Cell culture

HeLa cells were obtained from China Center for Type Culture Collection (Wuhan, China) and cultured at 37 °C under a humidified 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific), and 1% penicillin-streptomycin (Thermo Fisher Scientific).

MTT assays for determining cytotoxicity of HTL

HeLa cells were plated in a 96-well plate at 10^4 cells/well for 48h. After being washed twice with PBS, cells were incubated with HTL at indicated concentrations in serum-free DMEM at 37 ˚C for 24 hours. After removal of HTL, cells were incubated with MTS in 120 μl DMEM media at 37 ˚C for 2 hours. The solution was incubated at 37 ˚C overnight and absorption at 490 nm was measured with a microplate reader (Bio-Rad).

Small molecule experiments

To evaluate the reactivity of different thioester probes, small molecule reactions with B-hcy were performed. 1mM of AT-1, AT-2 or AT-3 were added into 3ml of PBS (0.2mM EDTA, 8M urea, 20mM TCEP, pH 7.00) containing 0.1mM B-hcy. The reaction mixtures were incubated at 25 °C for 0, 10, 20, 30, 40, 50 and 60min with agitation. At each time point, 0.1mL of reaction mixtures were aspirated into fresh tubes. Hydroxylamine (2.5%) and IAA (50mM) were added to quench the reactions and block thiols for 1h at 25 °C. After 1h, 0.6ml of precooled acetonitrile were added and the mixtures were left at -20 °C for 1h. Then the mixtures were centrifuged (20,000*g*, 10min) at 4°C to separate the liquid and organic phase. 0.4ml of the organic solution were aspirated and dried in vacuum. For LCQ Fleet analysis, the samples were dissolved into 0.1ml of H_2O containing 10μM of the internal standard compound (ethyl benzoyltyrosinate). Under the positive and negative ion mode, full-scan mass spectra were acquired over the m/z range from 100 to 1000 using the Ion trap mass analyzer. Extracted ion chromatogram of product a (exact mass 361.15), b (exact mass 384.15) and internal standard (exact mass 313.13) from all of data were integrated and the overall intensity were compared.

Labeling of purified proteins and sites identification

250μM of Lysozyme, Mb, RNaseA and BSA were prepared in PBS (0.2mM EDTA). Either H_2O or HTL (0.2mM) was added into 0.5ml of protein solutions and the mixtures were incubated at 25 °C for 18h. After induction, proteins were filtered with NAP-5 columns (GE Healthcare) to remove small molecule species and diluted to 25μM for further analysis. 50μL of Lysozyme, Mb and RNaseA were desalted with Zeba Spin Desalting column (Thermo Fisher Scientific) for intact mass analysis by LCQ Fleet.

For gel-based labeling experiments, control or HTL-induced Lysozymes were denatured in 8M of urea/PBS with 20mM of TCEP. The pHs were adjusted to 5.00, 6.00 and 7.00 respectively. 1mM of AT-3 probe were used

and the reaction mixtures were incubated at 25 °C for 0.5, 1, 1.5 and 2h. At each time point, 200μL of proteins were aspirated into fresh tubes with addition of hydroxylamine (2.5%) and IAA (50mM). The mixtures were incubated with agitation at 25 °C for 1h, followed by acetone precipitation. The protein pellets were washed twice with 70% acetone and resuspended in 100μL of SDS/PBS (0.6%) with sonication. Samples were centrifuged (20,000*g*, 3min) and 50μL of samples were aspirated for click chemistry (200μM Rhodamine-azide, 100μM Tris (benzyl triazolylmethyl)amine (TBTA), 1mM $CuSO_4$ and 1mM TCEP). Samples were allowed to react for 1h at room temperature and quenched with 14μL of 5*SDS-PAGE loading buffer. The prepared samples were analyzed by SDS-PAGE (15% polyacrylamide; 10μL of sample/lane) and visualized by in-gel fluorescence using Rhodamine channel (BioRad ChemiDoc MP).

Protein solutions of control and HTL-induced Mb, RNaseA and BSA were also prepared as described above. The pHs were adjusted to ~ 6.00 for labeling with 1mM of AT-3 probe (25 °C, 30min). The rest of labeling procedures were identical to that of Lysozyme.

For site identification, 40μL of samples resuspended in SDS/PBS (0.6%) were aspirated into fresh tubes and 10μL of 5*SDS-PAGE loading buffer were added directly. The samples were separated by SDS-PAGE (15% polyacrylamide; 10μL of sample/lane) and subjected to in-gel trypsin digestion. The tryptic peptides of four proteins (control and HTL-induced) were collected and dried in vacuum. Finally, the peptides samples were reconstituted in 10 μL of 0.1% (v/v) formic acid in water for MS analysis. The MS methods and data analysis were described as below. In brief, cysteine residues were searched with a static modification for carboxyamidomethylation (+57.02146Da). Lysine residues were searched with up to one differential modification for alkyne labeling (+254.07251Da) as shown in Fig.1B. Peptides were required to have at least one tryptic terminus and to contain the labeling modification. The searching results were filtered by DTASelect with restriction to fully tryptic peptides and a defined spectrum false positive rate of 1%.

Proteome preparation for MS-based analyses

For *in vitro* MS profiling experiments, HeLa cells ($8*10^6$ cell per ml) were seeded in 15-cm dishes and cultured at 37 °C for 60h. Cells were harvested by scraping in PBS (2*10ml); cell pellets were transferred to 1.5ml Eppendorf tubes in PBS and pelleted again by centrifugation (2500rpm, 3min). Cell pellets were flash frozen in liquid nitrogen and stored in -80℃ for use later. PBS (0.2mM EDTA) were bubbled with argon gas for 30min and precooled on the ice. The cell pellets were resuspended in cold PBS with 0.2mM EDTA and protease inhibitor cocktail (catalog no. 4693132001, Roche) (~700μL) and lysed by probe sonication using a Vibra cell probe sonicator (5 pulses, 40% duty cycle). Cell lysates were then centrifuged (20,000*g*, 30min, 4℃) to collect soluble fractions. Protein concentrations were determined using Pierce BCA protein assay kit (Thermo Fisher Scientific) and a microplate reader (Bio-Rad) against a bovine serum albumin standard. Cell lysates were normalized to 1.5mg/ml and incubated either water or 200μM HTL for 18h at 25℃. After induction, cell lysates were filtered with NAP-10 columns (GE Healthcare) to remove small molecule species and the final concentration of obtained cell lysates was 1mg/ml.

For *in situ* MS profiling experiments, HeLa cells ($8*10^6$ cell per ml) were seeded in 15-cm dishes and cultured at 37 °C for 60h. Cells were washed twice with PBS and then incubated in 15ml of DMEM with addition of water or HTL (500μM). After 24h, cells were harvested by scraping in PBS (2*10ml); cell pellets were transferred to 1.5ml Eppendorf tubes in PBS and pelleted again by centrifugation (2500rpm, 3min). Cell pellets were flash frozen in liquid nitrogen and stored in -80℃for use. Cells were lysed, centrifuged and filtered with NAP-10 columns to remove small molecule species. The final concentration of obtained cell lysates was 1mg/ml. Proteomes were denatured in 8M of urea/PBS with 20mM of TCEP. The pH of proteome (control or HTL-induced) was adjusted to 6.00 for labeling.

Proteome labeling, samples preparation and MS analysis

For quantitative profiling by reductive dimethylation, 3ml of proteome (control or HTL-induced, prepared as described above) were treated with DMSO or 1mM of AT-3 probe and incubated for 30min at 25°C with rotation (1000rpm), followed by treatment with 2.5% of hydroxylamine for 1h. The labeled proteomes were then subjected to methanol-chloroform precipitation (3ml of PBS, 7.5ml of methanol-chloroform (4/1, v/v). The resulting mixtures were vortexed and centrifuged (3000g, 10min). The organic and aqueous layers were aspirated leaving a protein disc that had formed between the layers. The disc was dissolved into cold methanol (2*1ml) and then sonicated. Methanol was removed by centrifugation (10000g, 5min). The remaining pellet was dissolved into 1ml of SDS/PBS (0.6%) with sonication for click chemistry (200μM Biotin-PEG4-azide, 100μM Tris (benzyl triazolylmethyl)amine (TBTA), 1mM $CuSO_4$ and 1mM TCEP). The mixtures were left at room temperature with rotation for 1h. Then a second methanol-chloroform precipitation (1ml of PBS, 2.5ml of methanol-chloroform (4/1, v/v)) was performed to remove the click reagents. The protein pellets were washed with cold methanol and resuspended into 1ml of SDS/PBS (1.2%) with sonication, followed by incubation at 90°C for 5min. The solution was centrifuged (20,000g) for 3min to remove insoluble species. The solubilized proteins was added into 5ml of PBS to give 0.2% SDS for enrichment. Streptavidin beads (100μL of slurry) were washed with PBS (3*5ml) and added into each sample. The mixtures were incubated at room temperature for 3h with rotation. After being washed with 3*5ml of PBS and water sequentially, the beads were denatured in 6M urea/PBS, reduced with 10mM dithiothreitol (DTT) at 37°C for 30min and alkylated with 20mM iodoacetamide (IAA) at 35°C for 30min in dark. Then the beads were washed with 4*1ml of 100mM Triethylammonium bicarbonate (TEAB) buffer (Sigma). Finally, the beads were incubated with 200μL of 100mM TEAB buffer and 4μL of trypsin (100μg reconstituted in 200μL of 100mM TEAB buffer, Promega) at 37 °C with agitation overnight. After trypsin digestion, 8μL of 4% heavy ($D^{13}CDO$, Sigma) or light (HCHO, Sigma) formaldehyde were added, respectively. Then 8μL of 0.6M sodium cyanoborohydride was added and the reaction mixtures were left at room temperature with agitation for 1h. 32μL of 1% ammonia was added to quench the dimethyl labeling reactions. Finally, 16μL of 5% formic acid was added and the samples labeled with Heavy or Light formaldehyde were combined into a fresh LoBind tube through a Micro Bio-Spin column (Bio-Rad).

The dimethyl labeled peptide samples were dried under vacuum and resuspended into 100μL of water. Then the samples were centrifuged (100,000*g*, 10min, 4°C) and fractionated with a fast sequencing workflow by dual reverse phase high performance liquid chromatography (RP-HPLC). The first dimension of high pH RP chromatography was performed on an Agilent 1260 infinity quaternary LC by using a durashell RP column (5μM, 150Å, 250mm * 4.6mm i.d., Agela). Mobile phase A (2% acetonitrile, adjusted pH to 10.0 using $NH_3•H_2O$) and B (98% acetonitrile, adjusted pH to 10.0 using $NH_3•H_2O$) were used to develop a gradient. The solvent gradient was set as follows:

Time/min	A%	B%
0.10	95.0	5.0
2.00	92.0	8.0
13.00	82.0	18.0
22.00	68.0	32.0
23.00	5.0	95.0
24.00	5.0	95.0
26.00	85.0	15.0
27.00	85.0	15.0

The peptides were separated at an eluent flow rate of 1.5 ml/min and monitored by UV at 214 nm. The

temperature of column oven was set as 45°C. Eluents were collected every minute. The samples were dried under vacuum, combined into 10 fractions and reconstituted in 10 μL of 0.1% (v/v) formic acid in water. The second dimension of low pH RP chromatography was performed on an Ultimate 3000 LC system coupled with a Q-Exactive plus Orbitrap mass spectrometer (Thermo Fisher Scientific) for MS/MS analysis. Each fraction from the first dimension was separated on a C18 column (75 μm inner-diameter, 360 μm outer-diameter × 15 cm, 3 μm C18). Mobile phase A consisted of 0.1% formic acid in water solution, and mobile phase B consisted of 0.1% formic acid in acetonitrile solution; according to the hydrophobicity of each fraction, an adjusted linear gradient was applied with a flow rate of 350 nL/min. The MS conditions are as the followings: Under the positive-ion mode, full-scan mass spectra were acquired over the m/z range from 350 to 1800 using the Orbitrap mass analyzer with a resolution of 70,000. MS/MS fragmentation was performed in the data-dependent mode, in which the 20 most intense ions were selected from each full-scan mass spectrum for fragmentation by high-energy collision induced dissociation (HCD). MS/MS spectra were acquired with a resolution of 17,500 using the Orbitrap analyzer. Some other parameters in the centroid format: isolation window, 2.0 m/z units; default charge, 2+; normalized collision energy, 28%; maximum IT, 50 ms; dynamic exclusion, 20.0 s.

For site identification, 9ml of HTL-induced proteomes (prepared as described above) were treated with 1mM of AT-3 probe and incubated for 30min at 25°C with rotation (1000rpm), followed by 2.5% of hydroxylamine for 1h. The labeled proteomes were then subjected to methanol-chloroform precipitation, conjugation with photo-cleavable biotin azide via click chemistry, methanol-chloroform precipitation and enrichment sequentially. The beads were denatured in 6M urea/PBS, reduced with 10mM dithiothreitol (DTT) at 37°C for 30min and alkylated with 20mM iodoacetamide (IAA) at 35°C for 30min in dark. Then the beads were incubated with 200μL of 2M urea/PBS buffer, 2μL of 100mM $CaCl_2$ and 4μL of trypsin (100μg reconstituted in 200μL of resuspend buffer) at 37 °C with agitation overnight. After trypsin digestion, the beads were washed with 3*PBS and 3*water. Then 1ml of methanol-H_2O (7/3, v/v) were added to the beads and the mixtures were irradiated with UV 365nm (5000*100μJ/cm^2) for 1h on ice to release the adducted peptides. The mixtures were transferred to LoBind tube and centrifuged to collect the solvent containing cleaved peptides. The beads were washed with 2*0.5ml of methanol-H_2O (7/3, v/v) and the peptides solutions were combined. After being dried in vacuum, the peptides samples were reconstituted in 10 μL of 0.1% (v/v) formic acid in water for MS analysis.

Peptide identification and quantification

The MS/MS spectra were extracted from the raw file using RAW Xtractor into a ms2 format, which were searched using the ProLuCID algorithm using a reverse concatenated, nonredundant variant of the Human UniProt database (release-2012_11).[1]

For quantitative profiling of *N*-homocysteinylated proteins by dimethyl labeling, searches included a static modification for carboxyamidomethylation (+57.02146Da) of cysteine and differential oxidation of methionine (+15.9949Da). Peptides were searched with a static modification for dimethylation of lysine or the protein N-terminus (+28.0313Da). Each raw data was additionally searched with a heavy parameter file, in which static modification of lysine (+34.06312Da) and the N-terminus (+34.06312Da) were set. Peptides were required to have at least one cognate tryptic. ProLuCID data was filtered through DTASelect (version 2.0)[2] to achieve a peptide false-positive rate below 1%. Ratio of heavy/light (HTL-induced protein/control) peaks were calculated by the in-house CIMAGE software as described before[3]. The MS1 ion chromatograms (+/−10 p.p.m.) from light and heavy peptide masses were extracted when one peptide ion was selected for MS/MS fragmentation and identified. The heavy/light peptide ratios were calculated according to the peak areas of the light and heavy chromatographs with some computational filters, including co-elution correlation score filter and envelope correlation score filter. Peak pairs that have poor co-elution scores (R^2 correlation value less than 0.7), or poor envelope correlation score (R^2 correlation value less than 0.7) are eliminated from consideration. For example,

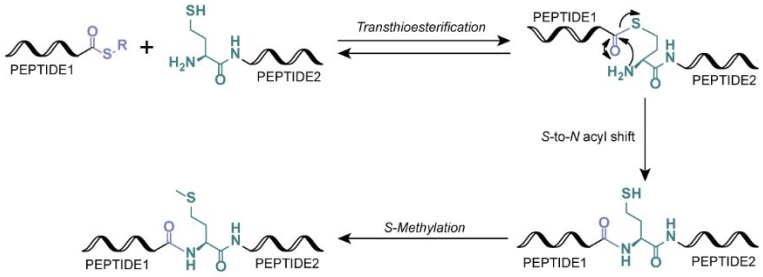
isotopic peptides that have the incorrect monoisotopic mass or charge, or whose isotopic envelopes are not well correlated with the predicted envelope are excluded. After ratios for unique peptide entries are calculated from each LC fractions, the median ratio of peptides from a specific protein was reported as the final protein ratio. In the “singleton” cases where only heavy peaks exist due to no enrichment of control proteins, the singleton MS1 chromatographic peaks were aligned with the corresponding peptide sequence to validate the charge state and monoisotopic mass using the envelope correlation score filter (R^2 correlation value ≥ 0.7). Then the candidate peak was cross-checked to ensure there was no other peaks co-eluting around the same retention time window. Only after all these criteria are met, the “singleton” peptide was assigned with an artificial threshold ratio of 15.

For *N*-homocysteinylated sites identification, cysteine residues were searched with a static modification for carboxyamidomethylation (+57.02146Da). Lysine residues were searched with up to one differential modification for photo-cleavable tags (+354.14741Da) as shown in Fig. 4A. The ProLuCID search results were filtered by DTASelect with restriction to fully tryptic peptides and a defined spectrum false positive rate of 1%.

Supplementary tables, schemes and figures

Table S1 List of reported human proteins with *N*-homocysteinylated sites up to data.

Protein	Site identification	Reference
Human albumin	Yes	4-6
Human fibrinogen	Yes	4
Human antitrypsin, γ-Globulin, Transferrin, LDL, HDL	No	7
Hemoglobin	Yes	8
Histone H3	Yes	9



Scheme S1 Methionine ligation. The thioester at the *C*-terminus of a synthetic peptide 1 is ligated to the *N*-terminal homocysteine of a peptide 2 via intermolecular transthioesterification. The intermediate is converted to a stable conjugation through a rapid S-to-N acyl shift. Finally, selective methylation is done to produce methionine.

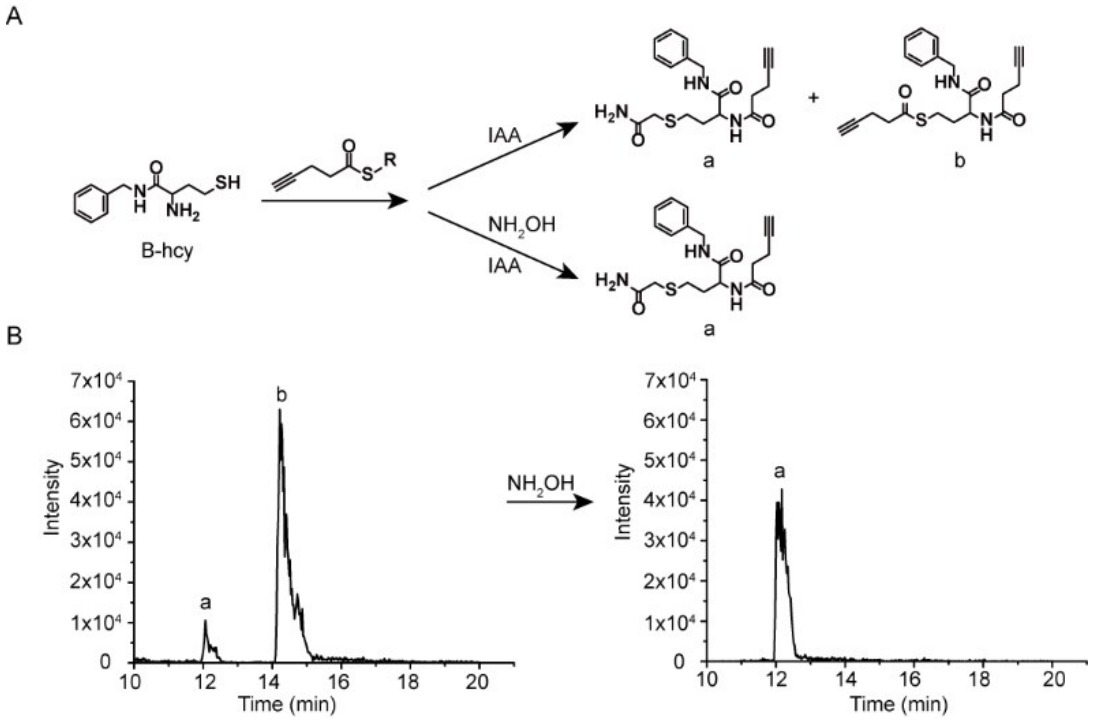


Fig. S1 Small molecule reaction model between B-hcy and thioester probes. (A) The reactions were monitored by LC-MS and two major products (a and b) were detected. (a) was the desired product and (b) was produced by B-hcy reacting with two equivalents of thioester probes. To remove (b) and excessive thioester probes, NH_2OH was added and only product (a) was detected. (B) Extracted MS1 chromatograms of product (a) and (b). (b) was removed completely with the addition of NH_2OH.

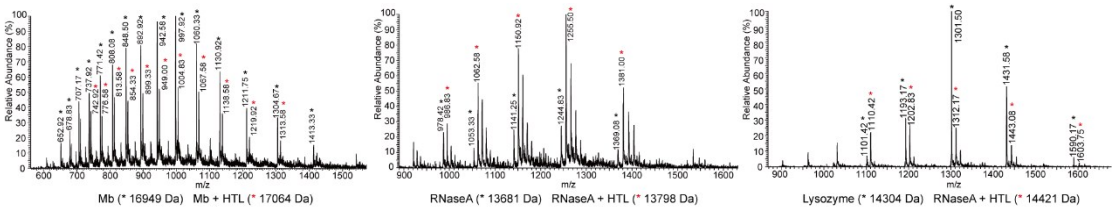


Fig. S2 Full mass-spectra of intact of Mb, RNaseA and Lysozyme treated with HTL. The observed mass of unmodified form (labeled with black asterisks) are 16949 Da, 13681 Da and 14304 Da, respectively. The observed mass of one HTL-modified form (labeled with red asterisks) are 17064 Da, 13798 Da and 14421 Da, respectively.

Table S2 Non-specific labeling of lysine residues of lysozyme in four conditions.

Condition	Labeled residues
pH 5, 2h	K31, K51, K114, K134,
pH 6, 0.5h	K31, K51, K134,
pH 6, 1h	K31, K51, K114, K115, K134,
pH 7, 0.5h	K51, K114,

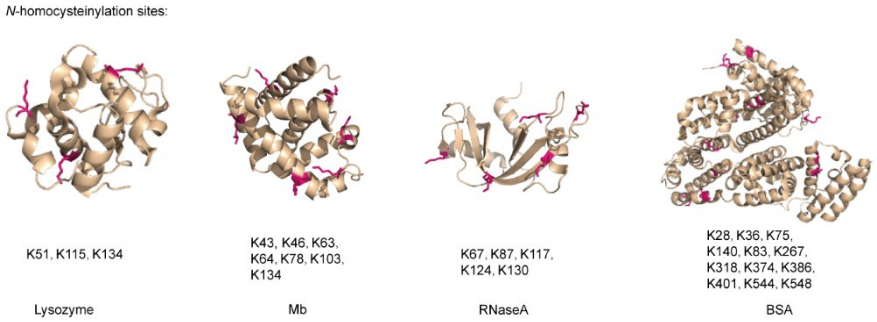


Fig. S3 Structures of Lysozyme (PDB: 5JEN), Mb (PDB: 2FRF), RNaseA (PDB: 2E3W) and BSA (PDB: 4F5S). Lysine residues identified with *N*-homocysteinylation modification are shown in pink with their residue numbers listed below each structure.

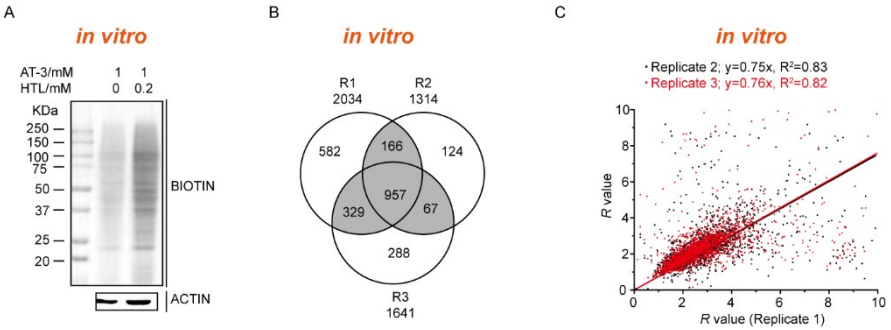


Fig. S4 Profiling of *N*-homocysteinylated proteins from HeLa proteomes *in vitro* treated with HTL. (A) Proteomes prepared from HeLa cell lysates treated with HTL (0.2mM) directly were selectively labeled with AT-3. Samples were conjugated with biotin azide via CuAAC and analyzed by Western blots. (B) The number of quantified proteins with *N*-homocysteinylation from three replicate experiments. (C) Comparison of protein ratios (*R* values) quantified between replicate 1, 2 and 3.

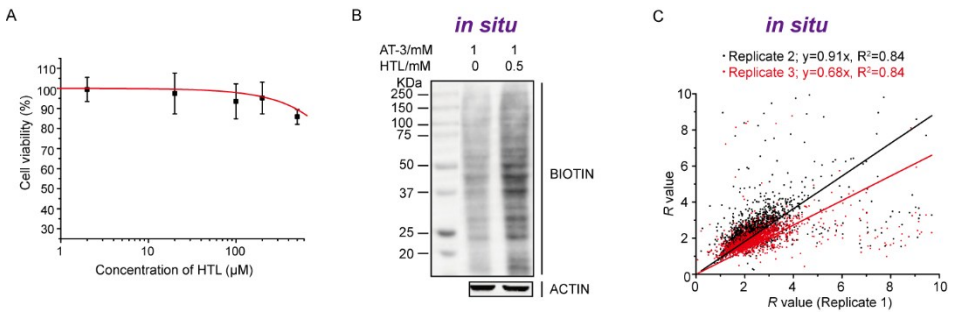


Fig. S5 Profiling of *N*-homocysteinylated proteins from HeLa cells *in situ* treated with HTL. (A) Cell viability assays with HTL. HeLa cells were treated with HTL for 24h. (B) Proteomes prepared from HeLa cells treated with HTL (0.5mM) were selectively labeled with AT-3. Samples were conjugated with biotin azide via CuAAC and analyzed by Western blotting. (C) Comparison of protein ratios (*R* values) quantified between replicate 1, 2 and 3.

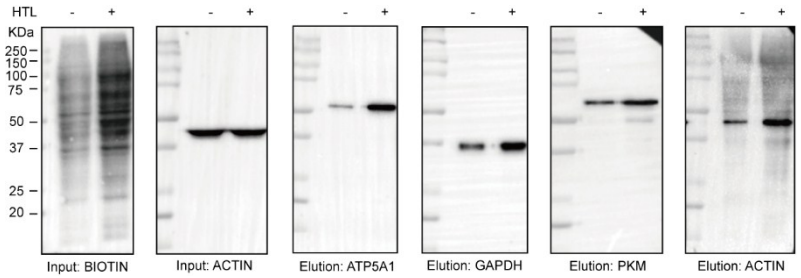


Fig. S6 Western blots confirming selected proteins are enriched by AT-3 in HTL-treated samples.

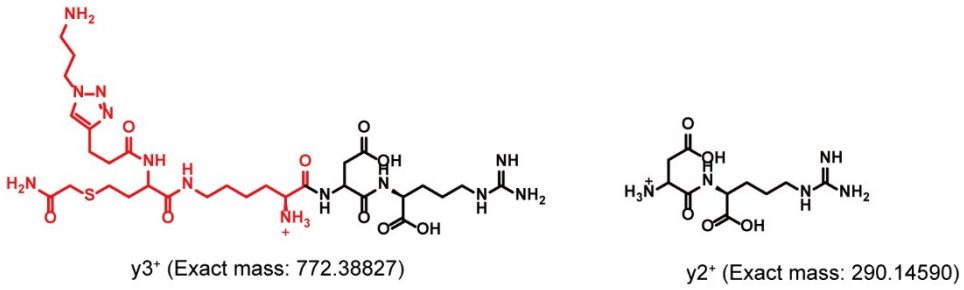


Fig. S7 Structures of $y3^+$ and $y2^+$. Exact mass of $y3^+$ and $y2^+$ are 772.38827 and 290.14590, respectively. The calculated m/z difference is 482.24237 and the observed m/z difference is 482.2426. Red structure denotes the modified lysine residue.

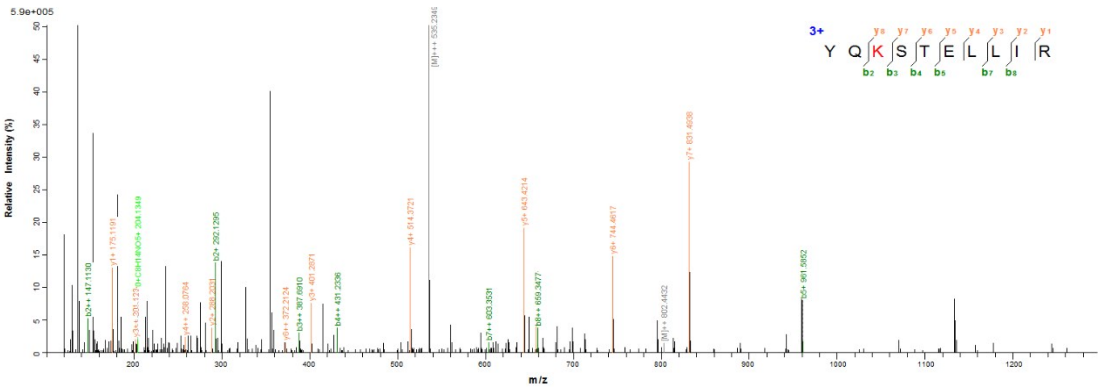


Fig. S8 MS/MS fragmentation spectrum of YQKSTELLIR from Histone H3. All b and y ions are labeled. Red K denotes *N*-homocysteinylation modification.

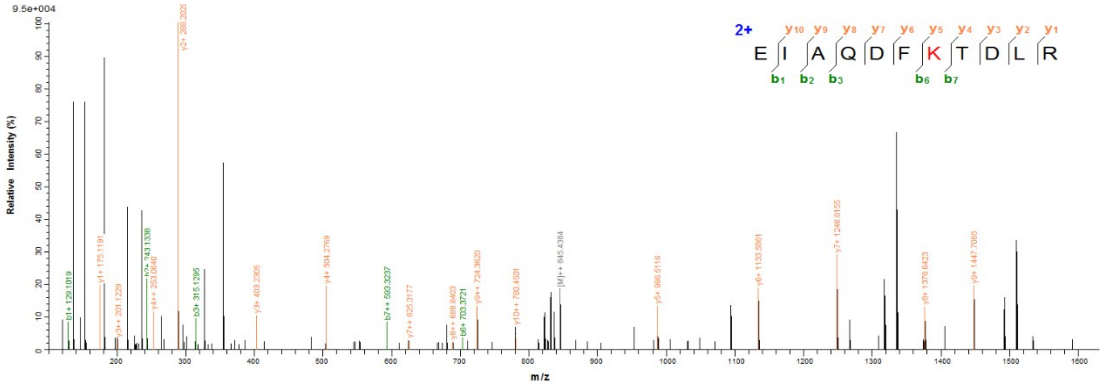


Fig. S9 MS/MS fragmentation spectrum of EIAQDFKTDLR from Histone H3. All b and y ions are labeled. Red K denotes *N*-homocysteinylation modification.

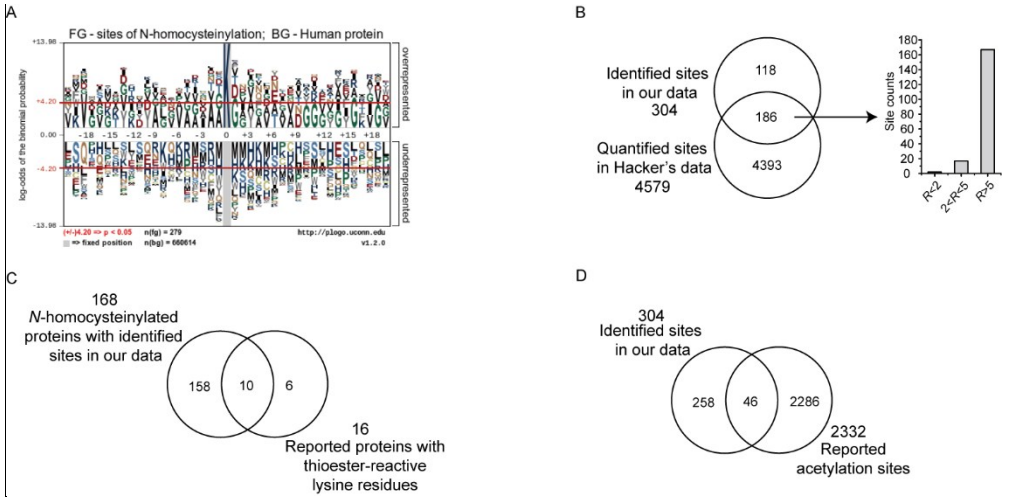


Fig. S10 Bioinformatic analysis of residue sites with *N*-homocysteinylation. (A) Frequency plot showing there is no obvious conserved motifs for *N*-homocysteinylated lysine residues. (B) Classification of *N*-homocysteinylated lysine residues according to their intrinsic reactivity as reported by Benjamin F Cravatt and colleagues[10]. (C) Venn diagram showing 10 proteins reported with thioester-reactive lysine residues[11] are found in our list. (D) Venn diagram showing 46 *N*-homocysteinylated lysines are also identified with acetylation as annotated in the Uniprot database.

Table S3 List of identified *N*-homocysteinylated proteins from HeLa proteomes *in vitro* treated with HTL.

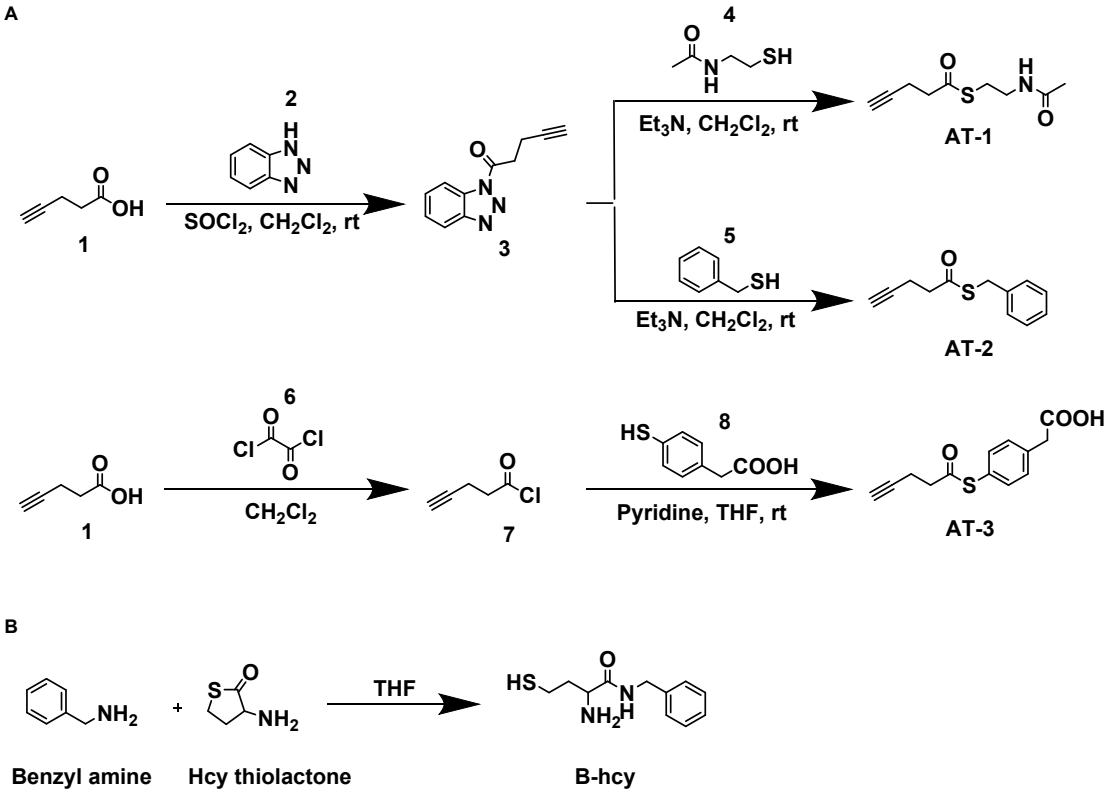
Table S4 List of identified *N*-homocysteinylated proteins from HeLa proteomes *in situ* treated with HTL and related analysis.

Table S5 List of identified sites with *N*-homocysteinylation from HeLa proteomes *in situ* treated with HTL and related analysis.

Chemical Synthesis

General Methods

Unless otherwise noted, all chemicals, including anhydrous solvents, are purchased from Sigma-Aldrich or J&K. NMR spectra are recorded on Bruker UltraShield 400 MHz spectrometer. Chemical shifts are recorded in ppm relative to tetramethylsilane (TMS, ^{1}H, 0 ppm) or solvent signals: $CDCl_3$ (^{1}H,7.26; ^{13}C, 77.16 ppm), MeOD-d_4 (^{1}H, 3.31; ^{13}C 49.00). Peaks are reported as follows: chemical shift, multiplicity (s = singlet, bs = broad singlet, d = doublet, t = triplet, q =quartet, m = multiplet), coupling constant(s) (Hz). B-hcy is purified by HPLC (Agilent 1260 infinity). High-resolution mass spectra (HRMS) were obtained on Bruker Solarix XR FTMS.



Scheme S2. Synthetic route to generate thioester probes (A) and B-hcy (B).

***S*-(2-acetamidoethyl) pent-4-ynethioate (AT-1)**

The synthesis of 3 and AT-1 was reported in the reference[11]. In brief, the intermediate compound 3 (398mg, 2mmol) and triethylamine (202mg, 2mmol) were added to a round- bottom flask containing CH_2Cl_2 (25ml) and reactant 4 (238mg, 2mmol). The reaction mixture was allowed to stir at room temperature for 3h and thin layer chromatography (TLC) indicated the reaction was completely. Then the reaction mixture was diluted with CH_2Cl_2 and washed with brine three times. The organic layer was dried over anhydrous Na_2SO_4, filtered and the solvent was removed by rotary evaporation under vacuum. After purification by silica gel chromatography, the product AT-1 was obtained as white solid (372mg, yield 90%). ^{1}H NMR (400MHz, $CDCl_3$) δ 6.12 (s, 1H), 3.44 (dd, *J* = 12.4, 6.2 Hz, 2H), 3.07 (t, *J* = 6.5Hz, 2H), 2.81 (t, *J* = 7.2 Hz, 2H), 2.55 (dd, *J* = 7.2, 2.6 Hz, 2H), 2.01 (t, *J* = 2.6Hz, 1H). ^{13}C NMR (100MHz, $CDCl_3$) δ 197.85, 170.47, 81.85, 69.61, 42.43, 39.56, 28.66, 23.25, 14.71. HRMS *m/z* calc'd for $C_9H_{13}NO_2S$ $[M+H]^+$ 200.07452, found: 200.07385.

***S*-benzyl pent-4-ynethioate (AT-2)**

AT-2 was prepared via the similar synthesis procedure of AT-1. After purification by silica gel chromatography, AT-2 was obtained as white solid (383mg, yield 82%). ^{1}H NMR (400MHz, $CDCl_3$) δ 7.36-7.18 (m, 5H), 4.15 (s, 2H), 2.80 (t, *J* = 7.4Hz, 2H), 2.55 (td, *J* = 7.4, 2.6Hz, 2H), 1.99 (t, *J* = 2.7Hz, 1H). ^{13}C NMR (100MHz, $CDCl_3$) δ 196.64, 137.31, 128.83, 127.33, 81.92, 69.42, 42.14, 33.25, 14.59. HRMS *m/z* calc'd for $C_{12}H_{12}OS$ $[M+H]^+$ 205.06871, found: 205.06822.

2-(4-(pent-4-ynoylthio)phenyl)acetic acid (AT-3)

The synthesis of AT-3 was according to reference[12]. Oxalyl dichloride 6 was added slowly to the round-bottom flask containing CH_2Cl_2 (10ml) and pent-4-ynoic acid 1 (350mg, 3.5mmol). Then two drops of DMF were added

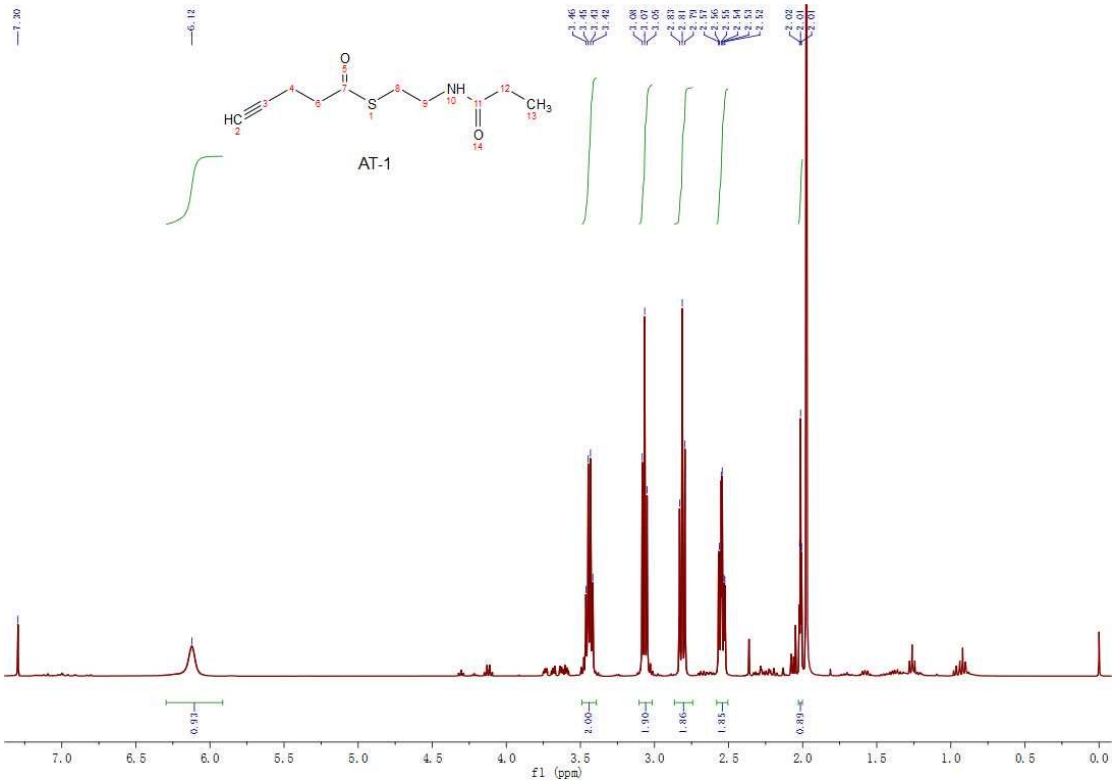
and the reaction mixture was stirred at room temperature overnight. The brown solution was evaporated under vacuum and the product pent-4-ynoyl chloride 7 was used for next step without further purification.

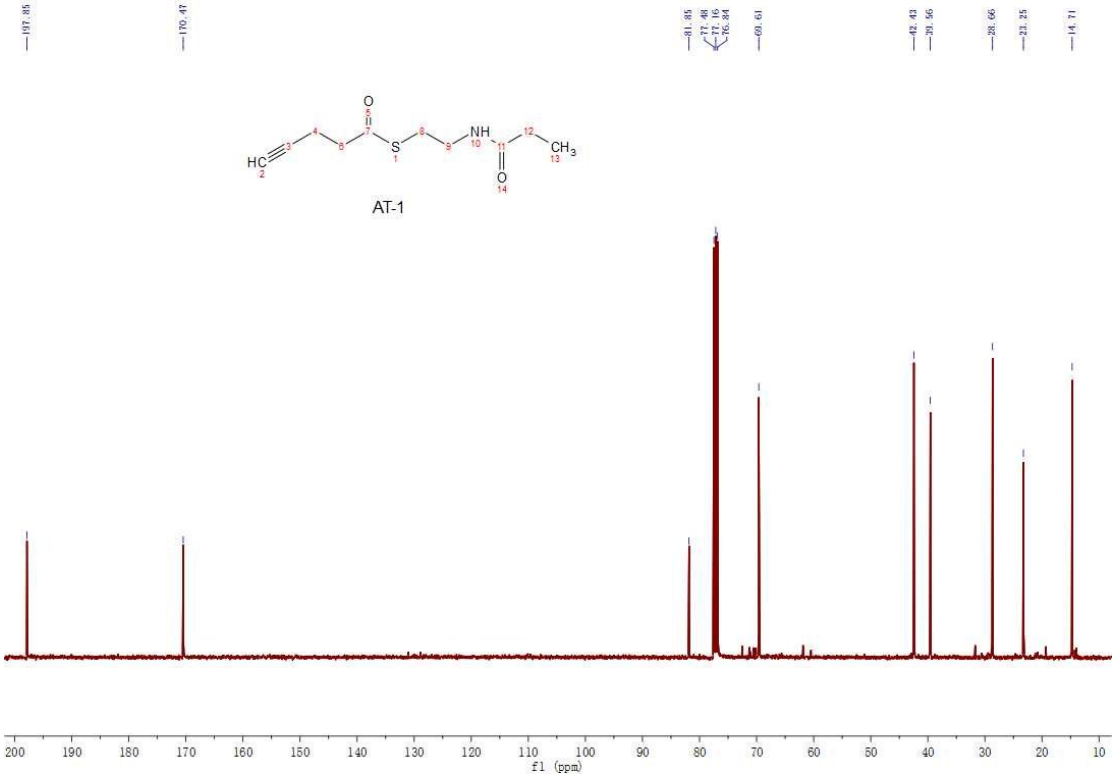
Pyridine (711mg, 9mmol) was added into the round-bottom flask containing dry THF (15ml) and 2-(4-mercaptophenyl)acetic acid 8 (500mg, 3mmol) at 0℃. The prepared pent-4-ynoyl chloride 7 was dissolved in dry THF (2ml) and added into the flask slowly. The reaction mixture was allowed to stir at room temperature for another 3h. Toluene (10ml) was added and the solvent was removed under vacuum. The crude mixture was dissolved in ethyl acetate and washed with 1N HCl, followed by brine. The organic layer was dried over anhydrous $MgSO_4$, filtered and the solvent was removed by rotary evaporation under vacuum. After purification by silica gel chromatography, the product AT-3 was obtained as white solid (460mg, yield 62%). ^{1}H NMR (400MHz, $CDCl_3$) δ 7.37 (q, *J* = 8.2 Hz,4H), 3.67 (s, 2H), 2.89 (t, *J* = 7.4Hz, 2H), 2.57 (td, *J* = 7.4, 2.6 Hz, 2H), 2.01 (t, *J* = 2.6 Hz, 1H). ^{13}C NMR (100MHz, $CDCl_3$) δ 195.59, 176.56, 135.03, 134.46, 130,46, 126.54, 81.93, 69.68, 42.21, 40.79, 14.74. HRMS *m/z* calc'd for $C_{13}H_{12}O_3S$ $[M+H]^+$ 249.05854, found: 249.05839.

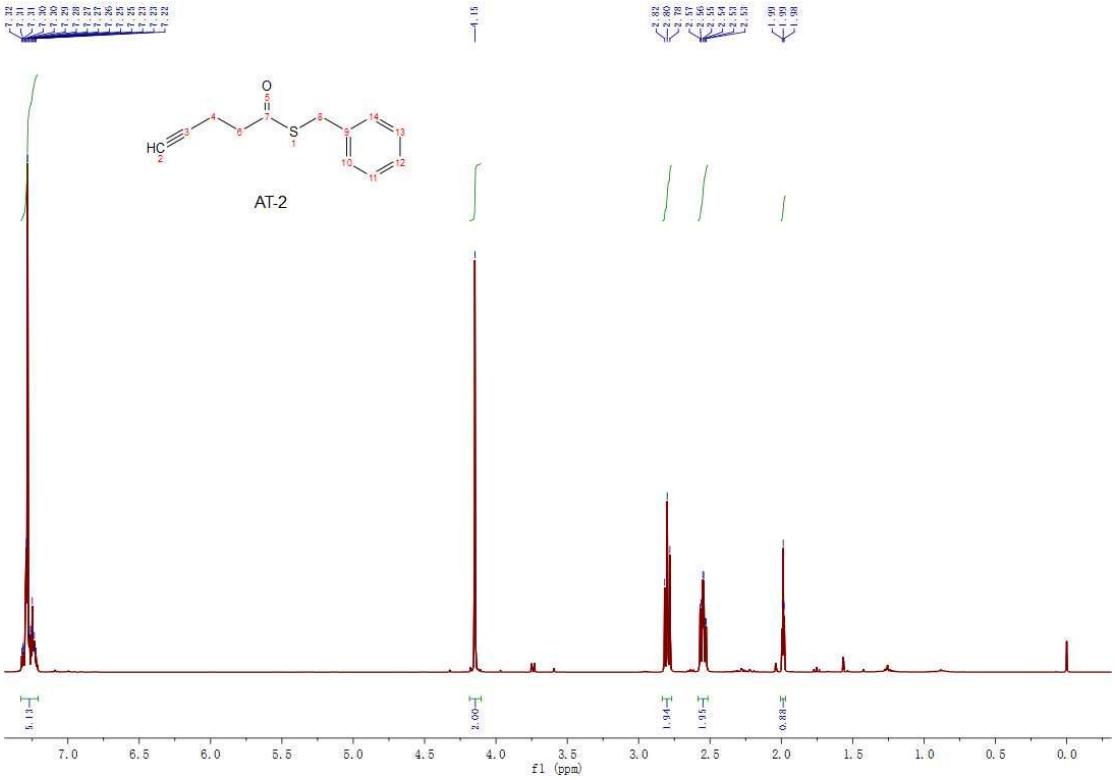
2-amino-*N*-benzyl-4-mercaptobutanamide (B-hcy)

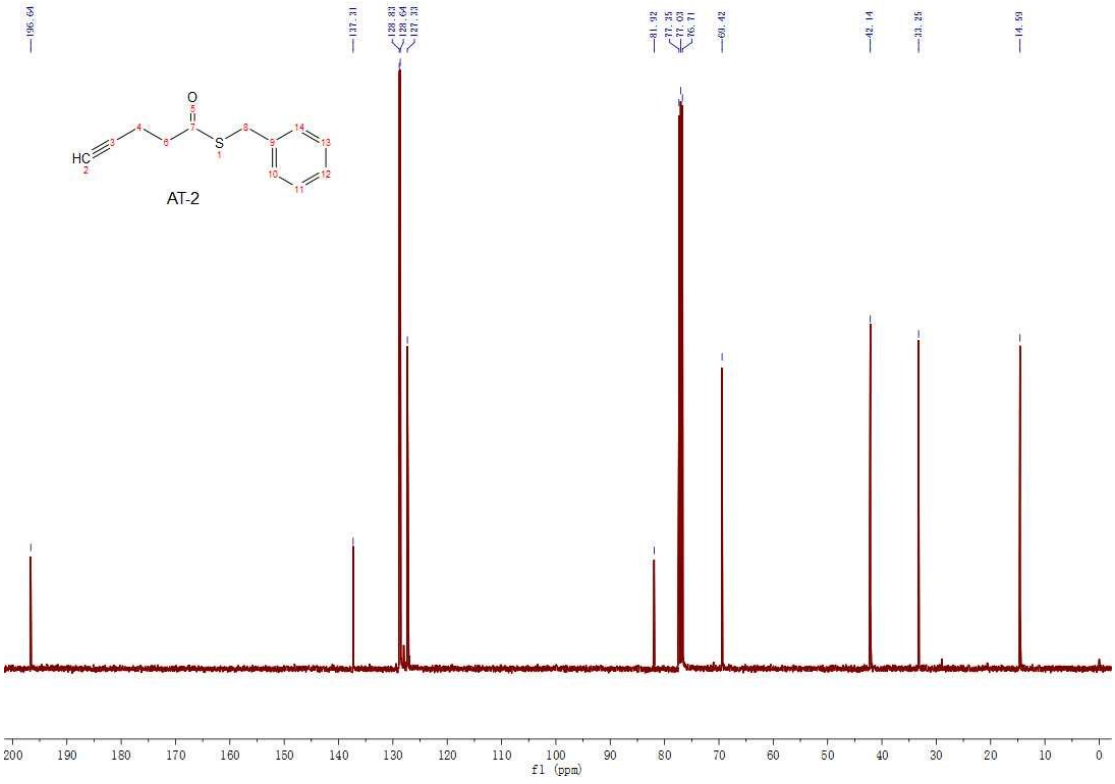
D,L-homocysteine thiolactone hydrochloride (500mg, 3.25mmol) was added into a round-bottom flask containing THF (20ml) and benzyl amine (1.85ml, 17mmol). The reaction mixture was protected under argon and stirred at room temperature for 4h. The solvent was removed by rotary evaporation under vacuum and the crude mixture was dissolved into methanol for semi-preparative HPLC. The fractions containing B-hcy were collected and dried under vacuum to give white powder (500mg, 69%). ^{1}H NMR (400MHz, Methanol-d_4) δ 7.38-7.19 (m, 5H), 4.40 (d, *J* = 3.9Hz, 2H), 4.02-3.85 (m, 1H), 2.53 (td, *J* = 7.7, 2.1 Hz, 2H), 2.23-1.98 (m, 2H). ^{13}C NMR (100MHz, Methanol-d_4) δ 169.50, 139.33, 129.69, 128.84, 128.58, 53.43, 44.41, 37.09, 20.31. HRMS *m/z* calc'd for $C_{11}H_{16}N_2OS$ $[M+H]^+$ 225.10616, found: 225.10587.

NMR spectra for compounds






AT-2
f1 (ppm)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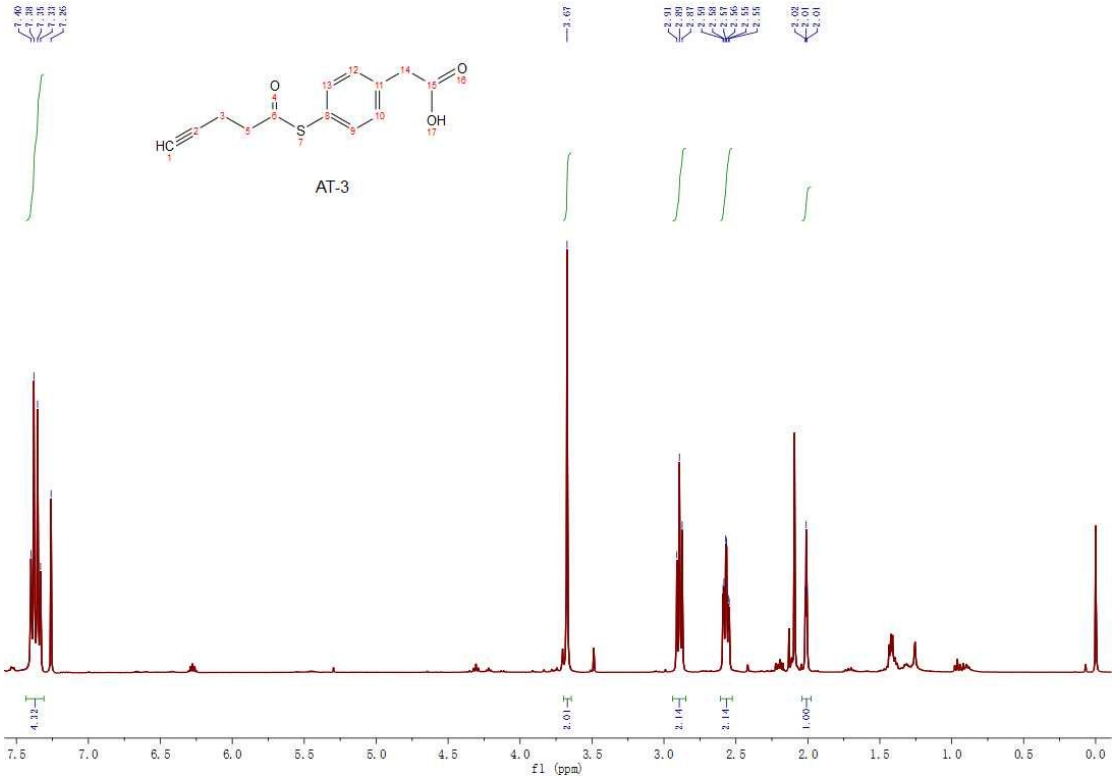
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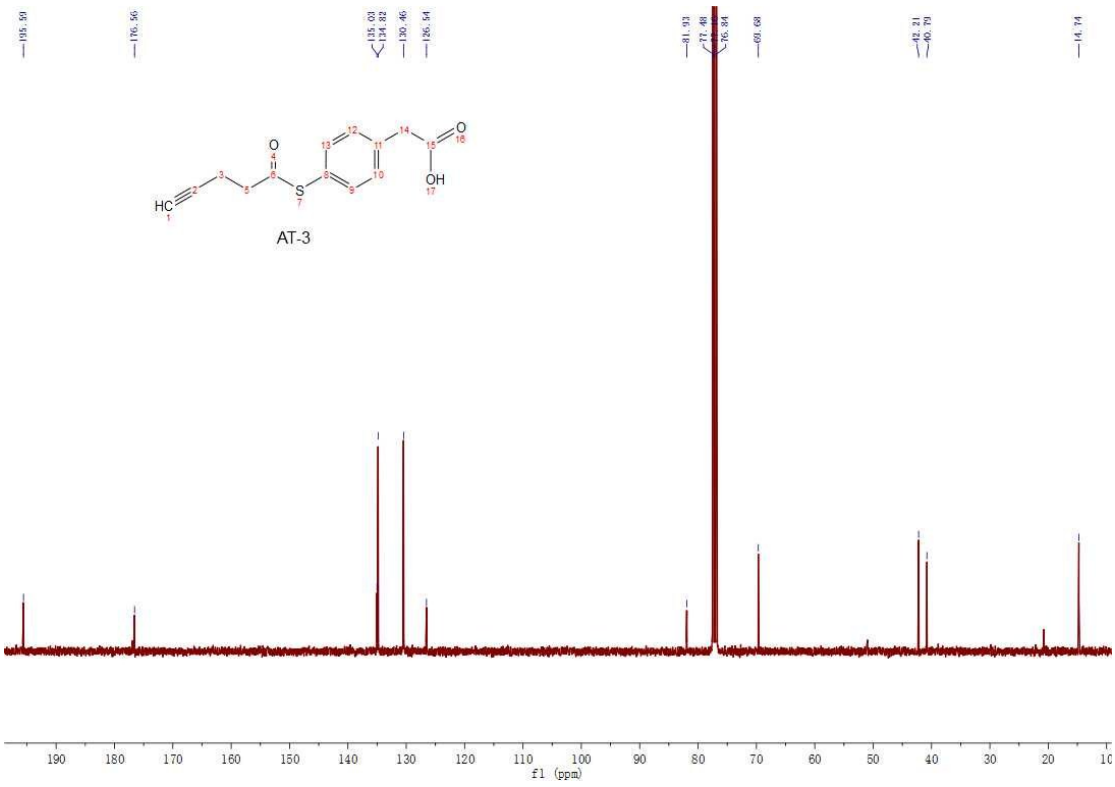
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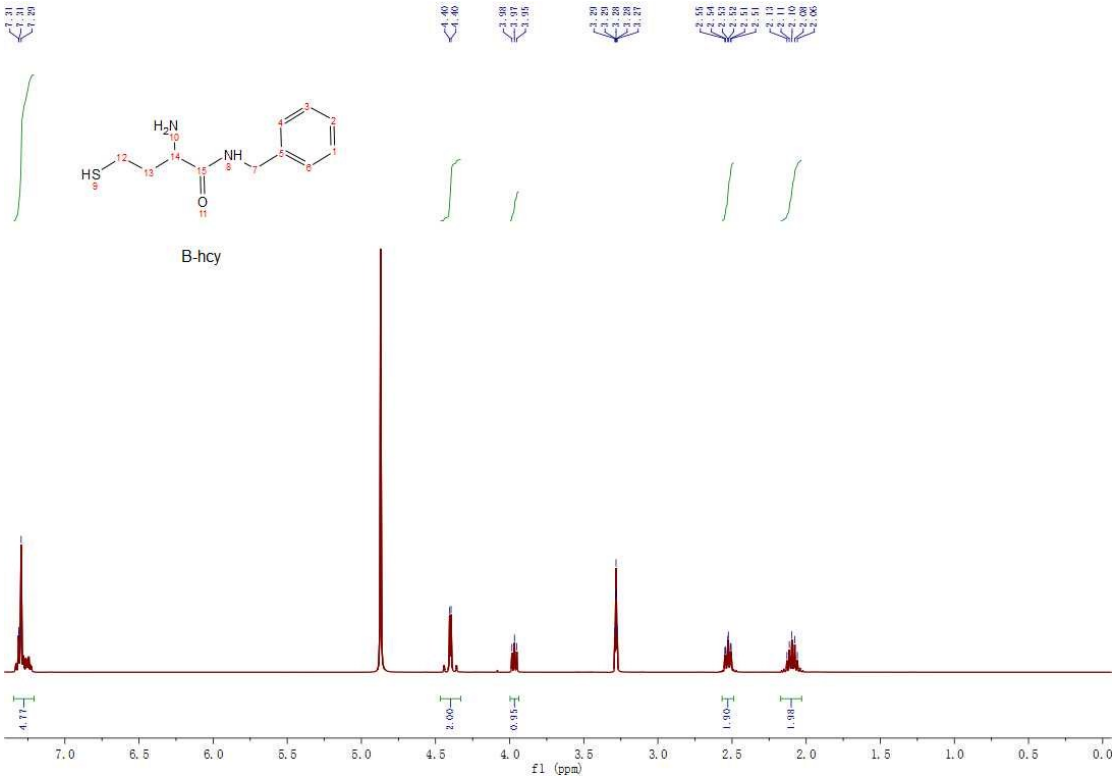
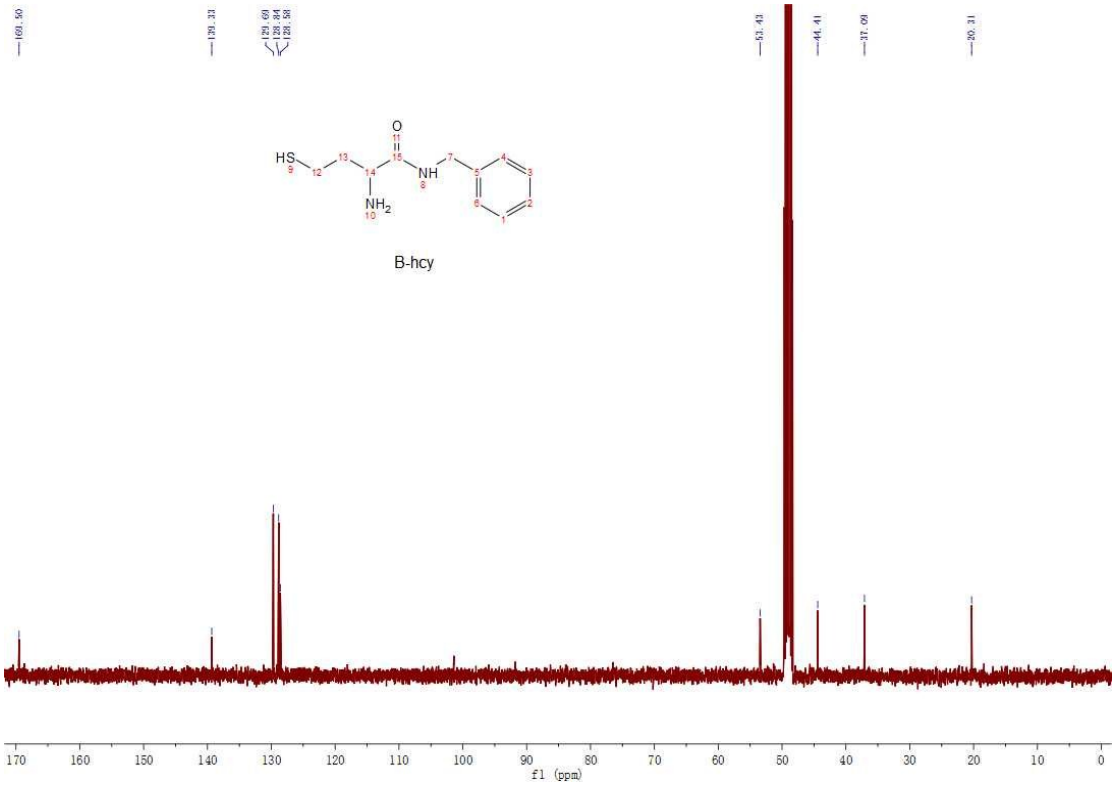

B-hcy
f1 (ppm)


B-hcy
f1 (ppm)

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