Supplementary Information

Chemical proteomic profiling of lysine crotonylation by minimalist bioorthogonal probes in mammalian cells

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1. Supplementary Tables

Table S1. Results of the crotonylation sites identified by Cr-alkyne and quantitative proteomics unveiling crotonylation sites regulated by HDACs using Cr-alkyne.

2. Supporting information figures

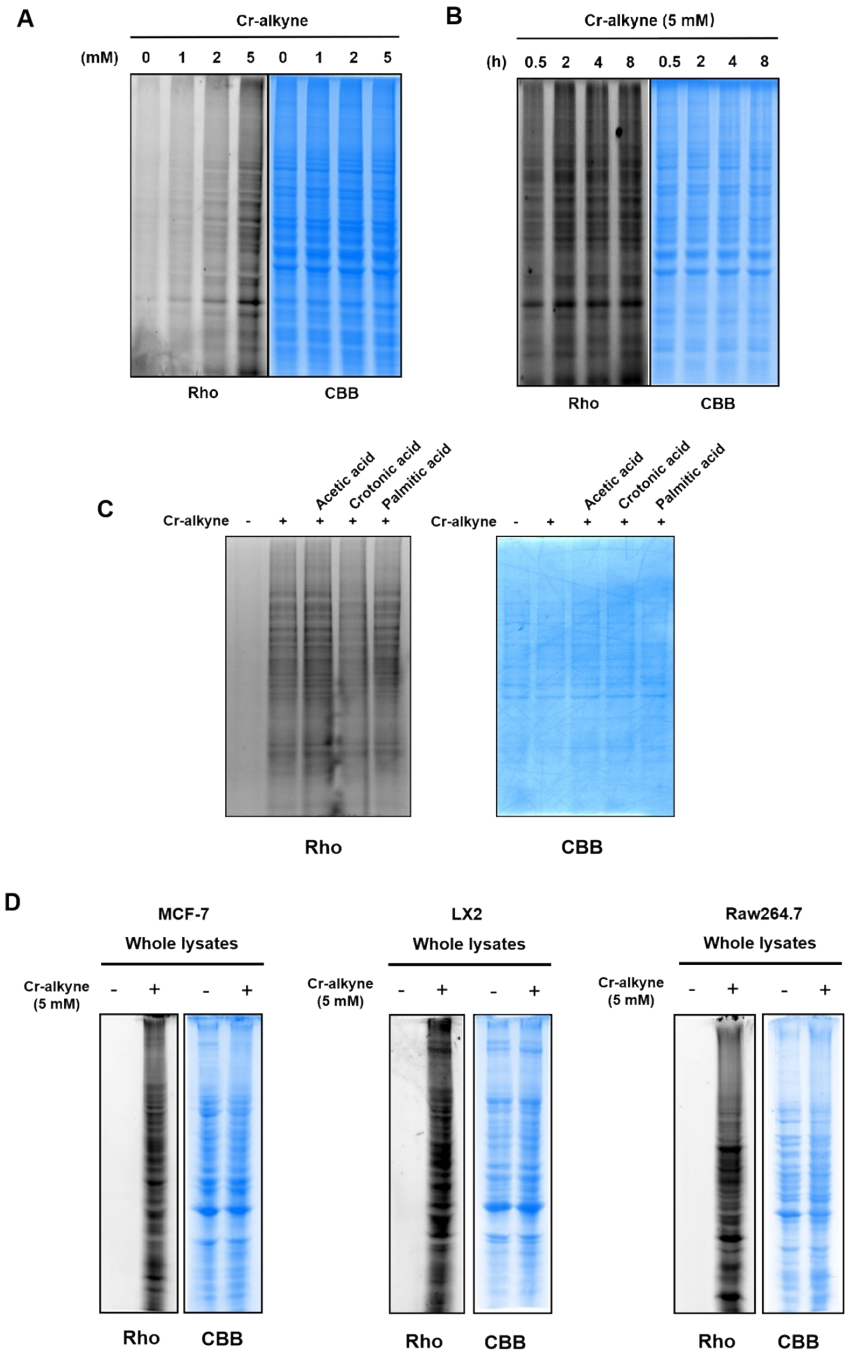
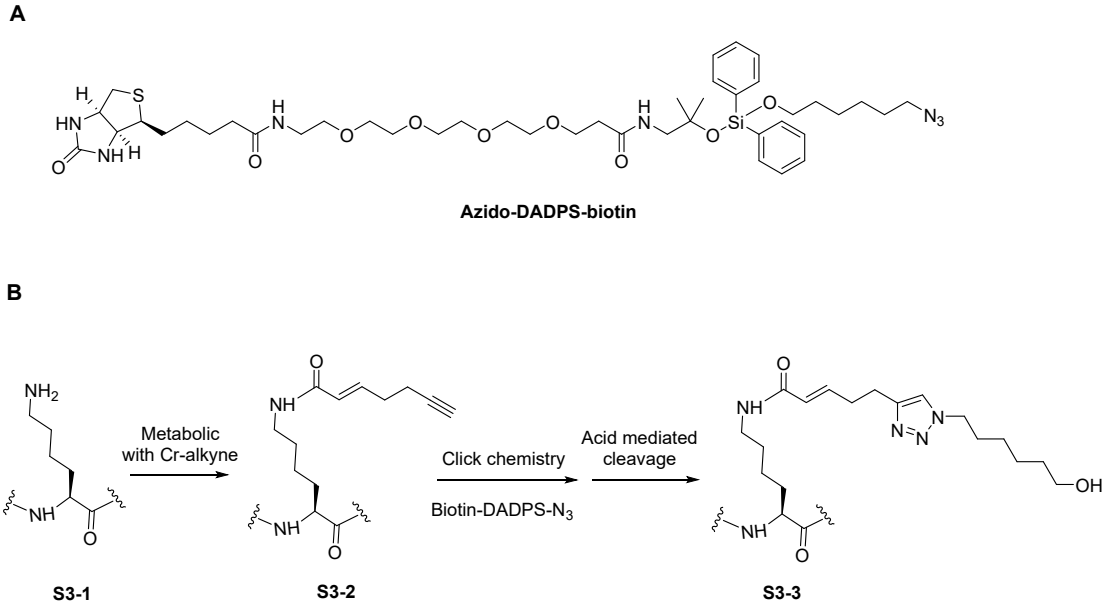


Figure S1. Detection of histone lysine crotonylation by Cr-alkyne. (A) Concentration and (B) time dependent protein labeling by Cr-alkyne (5 mM) in live cells. (C) Acetic acid (10X), crotonic acid (10X) and palmitic acid (10X) as competitor to Cr-alkyne (5 mM). (D) Proteome labelling by Cr-alkyne (5 mM) in MCF-7, LX2 and Raw264.7 cells.



$K_{variable\ modification}$ = MW (S3-3) - MW (S1-1) = 249.1477

Figure S2. Reagents and masses used for chemoproteomic analysis of crotonylation sites by Cr-alkyne. (A) Structure of the acid cleavable azido-DADPS-biotin. (B) Structure of Cr-alkyne-labeled lysine residue (**S3-2**) following click reaction and acid cleavage to form (**S3-3**).

A

Identified Kcr Sites	Acetylation	Lactylation	Identified Kcr Sites	Acetylation	Lactylation
H1-K20	X	X	H2B-K20	√	√
H1-K158	√	X	H2B-K23	√	√
H1-K167	√	√	H2B-K24	√	X
H1-K177	X	X	H2B-K85	√	√
H1-K190	√	√	H3-K4	√	X
H2A-K5	√	√	H3-K9	√	√
H2A-K9	√	√	H3-K14	√	√
H2A-K118	√	√	H3-K18	√	√
H2A-K119	X	√	H3-K23	√	√
H2A-K125	X	X	H3-K27	√	√
H2B-K5	√	√	H4-K5	√	√
H2B-K11	√	√	H4-K8	√	√
H2B-K12	√	√	H4-K12	√	√
H2B-K16	√	√	H4-K16	√	√

B

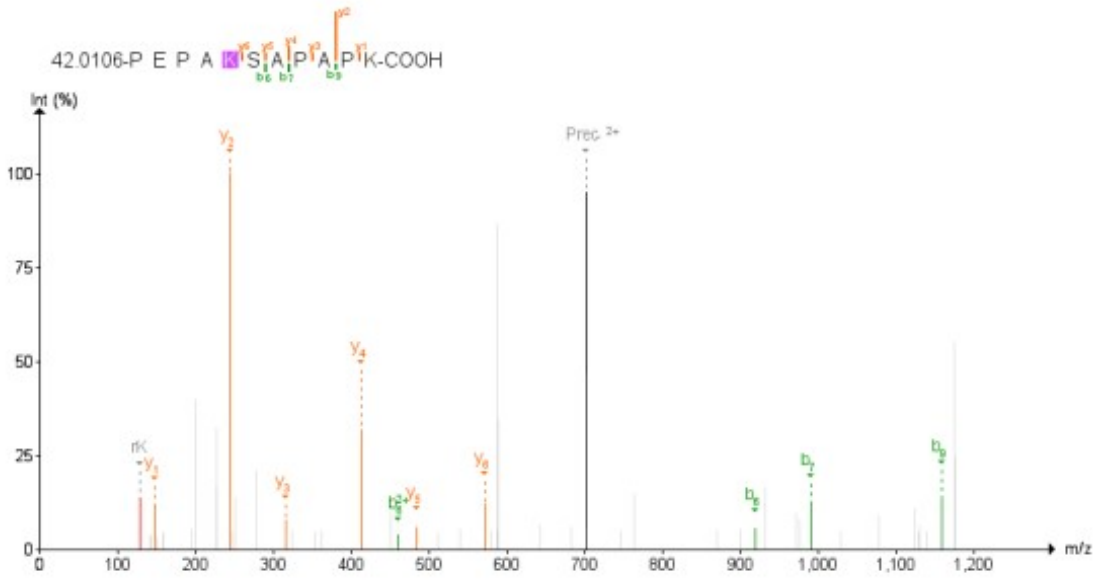


Figure S3. Detailed comparison with reported known acetylation and lactylation sites (A) and potential Kaca-alkyne modification at H2BK5 (B).

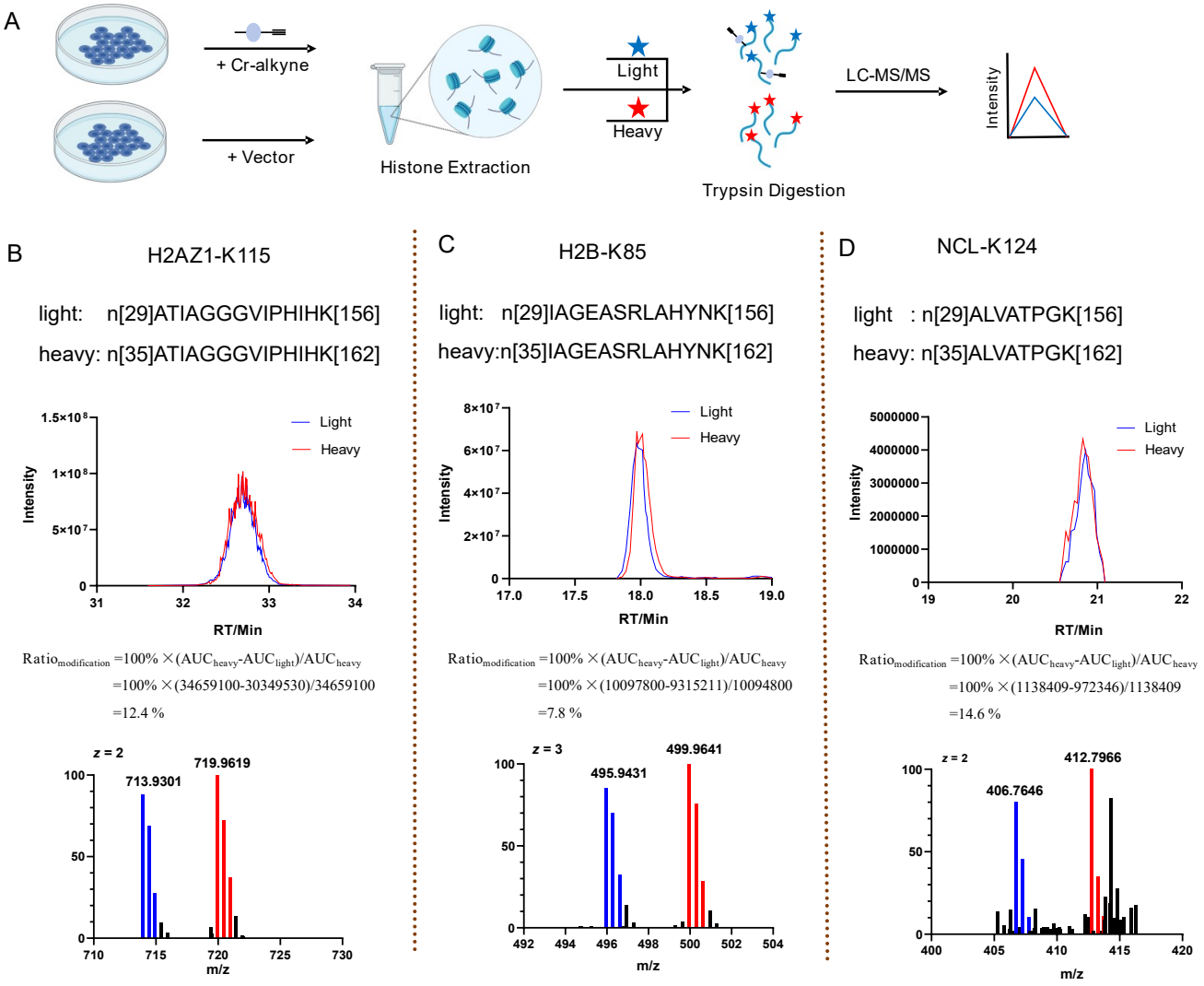
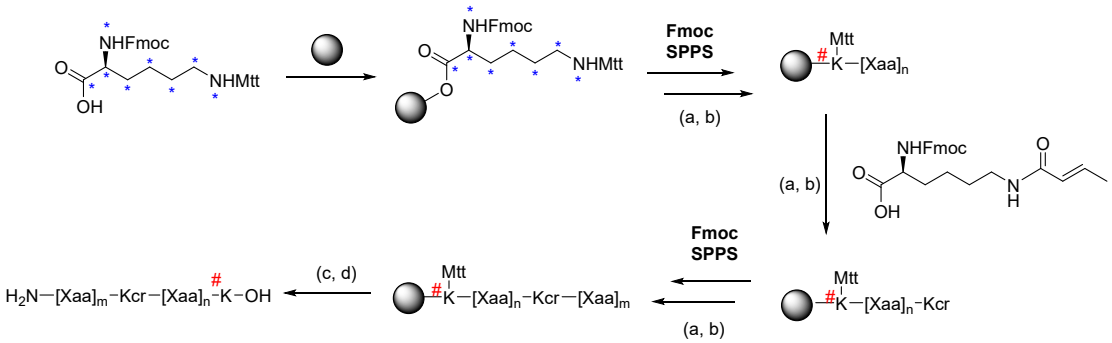


Figure S4. Quantitative analysis using dimethyl labeling to determine the percentage of metabolic crotonylation. (A) Experimental scheme; (B, C, D) Diagram illustrating the pattern of metabolic modification rates of Cr-alkyne of H2AZ1-K115, H2B-K85 and NCL-K124.

A



*** = ^{13}C or ^{15}N; n and m stand for different numbers of amino acids; $^{\#}K$ (K+8 Da)**

Reaction conditions: (a) HATU, HOAt, DIEA; (b) Piperidine/DMF (20:80, v/v); (c) TFA/TIPS/DCM (3:2.5:94.5); (d) TFA

B

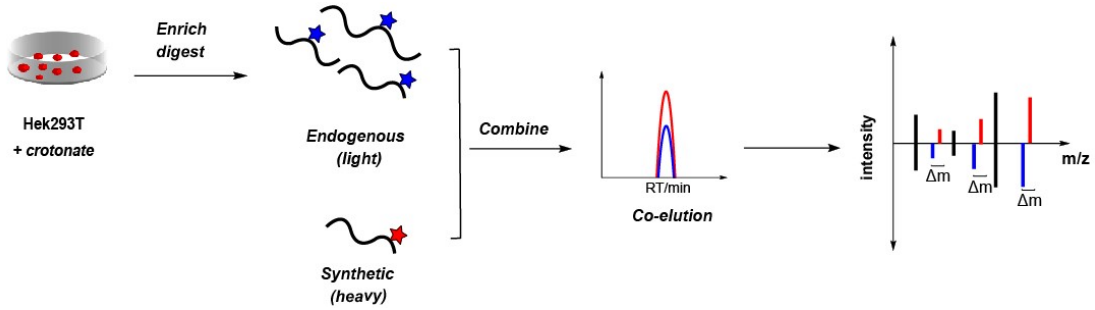


Figure S5. Validation of crotonylation peptides by coelution with synthetic standards. (A) Synthesis of standard peptides. Detailed procedure and confirmations of the final product by HPLC and MS were shown in “Procedures for solid-phase peptide synthesis”. (B) Workflow of the coelution experiment with endogenous modified peptides (blue) extracted from crotonate-treated HEK293T cells and isotopically labeled synthetic crotonylated peptide standards (red).

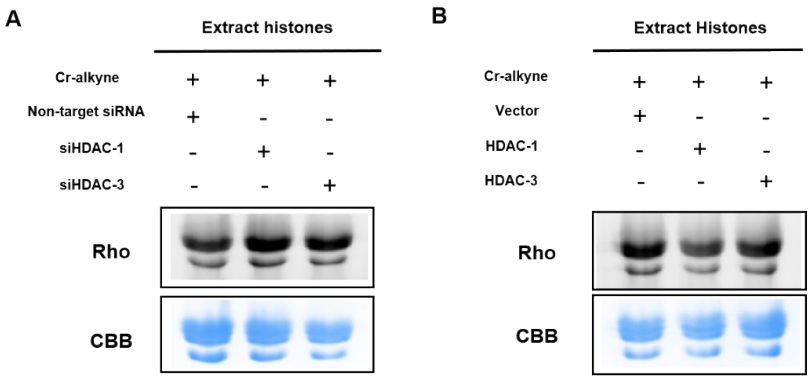


Figure S6. In-gel fluorescence results of Cr-alkyne-labeled histones of HEK293T cells with HDAC1/3 perturbation. (A) HDAC1/3 knockdown；(B) HDAC1/3 over expression. HEK293T cells were labeled with 5 mM Cr-alkyne for additional 8 h. Cells were then lysed to separate the core histones for click reactions with azide-rho and in-gel fluorescence analysis.

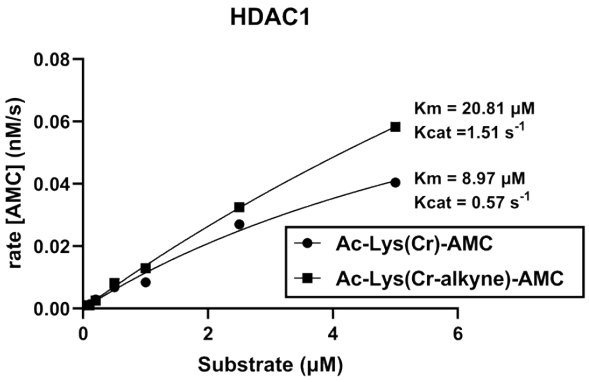


Figure S7. Kinetic study for HDAC1 to decronylated two substrates: Ac-Lys(Cr)-AMC and Ac-Lys(Cr-alkyne)-AMC. Michaels-Menten plots are shown with Kcat and Km calculated.

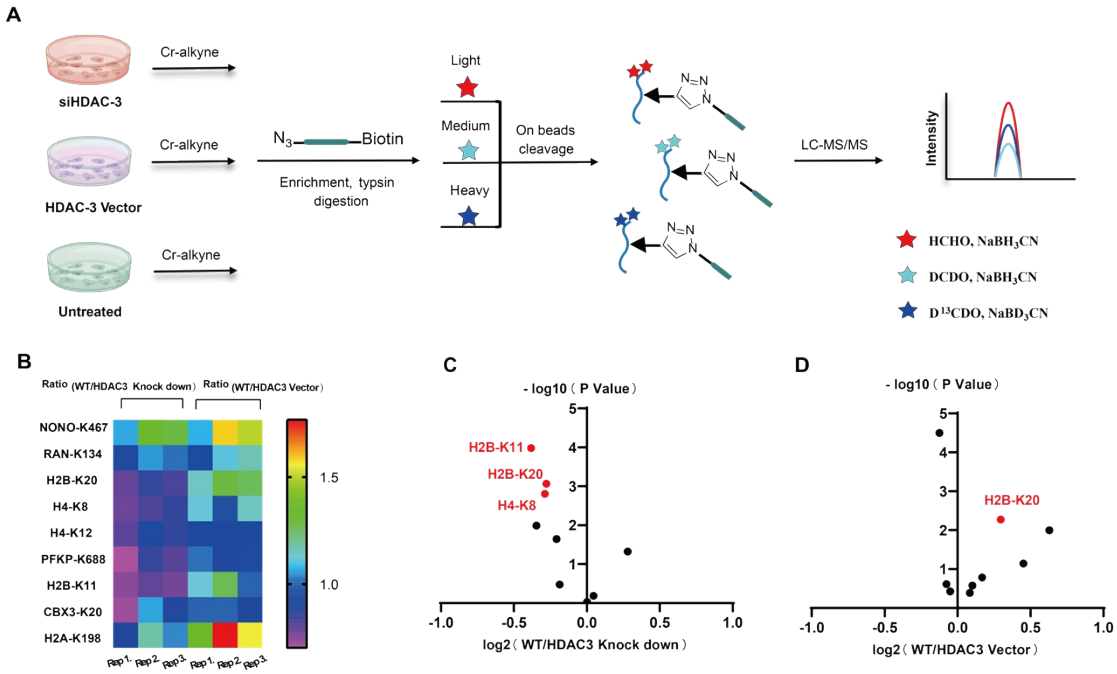


Figure S8. Quantitative chemoproteomics unveils crotonylation sites regulated by HDAC3. (A) Proteomes from HDAC3 knock down, over expression and untreated cells were treated by the Cr-alkyne probe and then subjected to the rdTOP-ABPP procedures. (B) Perturbed of Cr-alkyne-labeled lysine based on quantified rdTOP-ABPP ratios repeated in three groups. (C) Volcano plot of the rdTOP-ABPP ratios for Cr-alkyne-labeled lysine quantified in the WT HEK293T cells as compared to that in HDAC3 knock down cells. Highlighted in red are Cr-alkyne-labeled lysines with significantly suppressed reactivity during HDAC3 knock down. (D) Volcano plot of the rdTOP-ABPP ratios for Cr-alkyne-labeled lysine quantified in the WT HEK293T cells as compared to that in HDAC3 overexpression cells. Highlighted in red are Cr-alkyne-labeled lysines with significantly up-regulated during HDAC3 overexpression.

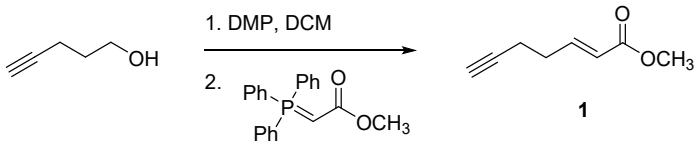
3. General methods and materials

3.1 General Information

All reactions were carried out under an argon atmosphere with dry solvents under anhydrous conditions, unless otherwise noted. Reagents were purchased at the highest commercial quality and used without further purification, unless otherwise stated. Solvents for chromatography were used as supplied by Merck chemicals. Reactions were monitored by thin layer chromatography (TLC) carried out on 0.2 mm Merck gel plates (60F-254) using UV light as visualizing agent and aqueous ammonium cerium nitrate/ammonium molybdate as developing agent. Merck silica gel (60, particle size 0.040–0.063mm) was used for flash column chromatography. NMR spectra were recorded on Bruker AM 400 MHz. The spectra were calibrated by using residual undeuterated solvents (for ^{1}H NMR) and deuterated solvents (for ^{13}C NMR) as internal references: chloroform (δ_H = 7.26 ppm) and $CDCl_3$ (δ_C = 77.16 ppm). The following abbreviations are used to designate multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, quint = quintet, br = broad. High-resolution mass spectra (HR-FT-MS) were recorded on Bruker FT-ICR-MS. Azido-rhodamine, azido-biotin and Azido-DADPS-biotin was purchased from Click Chemistry Tools. Oligonucleotide primers and gene fragments were synthesized by Tsingke. Plasmid DNA isolation was carried out with the Plasmid Mini Kit (Omega). Polyethylenimine (PEI) was purchased from Polysciences. Protease inhibitor cocktail was purchased from Roche (cOmplete ULTRA mini Tablets, EDTA-free). In-gel fluorescence and western blotting analyses were recorded on a Chemidoc MP imaging system (Biorad). Confocal fluorescence imaging was performed with a Nikon A1R confocal fluorescence microscope.

3.2 Synthesis of probes

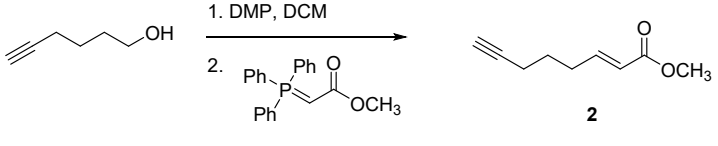
Synthesis of compound 1



1

The 4-pentyn-1-ol (2.0 g, 24 mmol) was dissolved in DCM (2 mL), and Dess-Martin periodinane (20 g, 48 mmol) was added at room temperature for 30 min, and then Methyl (triphenylphosphoranylidene) acetate (7.9 g, 24 mmol) was added and reacted for 2 hours. The mixture was quenched with saturated aq. solution of $NaHCO_3$ and extracted with EtOAc (3 × 300 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered and concentrated under vacuum. The residue was purified by a flash column chromatography on silica gel (CH_2Cl_2: EtOAc=10:1) to produce compound 1 as colorless oil (2.6 g, yield 80 %).^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.98 (dt, *J* = 15.7, 6.6 Hz, 1H), 5.96 – 5.84 (m, 1H), 2.48 – 2.40 (m, 2H), 2.38 – 2.33 (m, 2H), 2.03 (t, *J* = 2.6 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.61, 146.56, 122.04, 82.55, 69.47, 51.40, 30.93, 17.32. HR-FT-MS (m/z): [M + H]+ calcd for $C_9H_{13}O_2$ 153.0910, found 153.0910.

Synthesis of compound 2

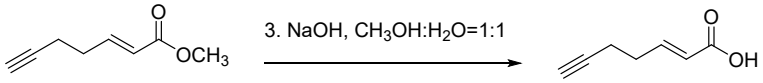


2

The 5-hexyn-1-ol (2.0 g, 18 mmol) was dissolved in DCM (2 mL), and Dess-Martin periodinane (18 g, 36

mmol) was added at room temperature for 30 min, and then Methyl (triphenylphosphoranylidene) acetate (6.8 g, 18 mmol) was added and reacted for 2 hours. The mixture was quenched with saturated aq. solution of $NaHCO_3$ and extracted with EtOAc (3 × 300 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered and concentrated under vacuum. The residue was purified by a flash column chromatography on silica gel (CH_2Cl_2: EtOAc=10:1) to produce compound **2** as colorless oil (2.6 g, yield 84 %). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.95 (dt, *J* = 15.6, 7.0 Hz, 1H), 5.87 (dt, *J* = 15.6, 1.6 Hz, 1H), 2.34 (qd, *J* = 7.2, 1.5 Hz, 2H), 2.23 (td, *J* = 7.0, 2.7 Hz, 2H), 1.99 (t, *J* = 2.6 Hz, 1H), 1.70 (p, *J* = 7.2 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.90, 148.14, 121.64, 83.43, 69.04, 51.41, 30.91, 26.67, 17.81. HR-FT-MS (m/z): [M + H]+ calcd for $C_8H_{11}O_2$ 139.0753, found 139.0753.

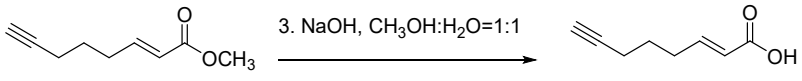
Synthesis of compound 3



1 **3**

The compound **1** (1.4 g, 10 mmol) was dissolved in methanol/H_2O (3 mL, 1:1) and added NaOH (1.0 g, 50 mmol) at room temperature. The mixture was quenched with 1M HCl and extracted with EtOAc (3 × 300 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered and concentrated under vacuum. The residue was purified by a flash column chromatography on silica gel (CH_2Cl_2: EtOAc=3:1) to produce compound **3** as white solid (1.0 g, yield 81 %). ^{1}H NMR (400 Hz, Chloroform-*d*) δ 12.19 (s, 1H), 7.11 (dt, *J* = 15.7, 6.7 Hz, 1H), 5.91 (d, *J* = 15.7 Hz, 1H), 2.47 (qd, *J* = 6.7, 3.2 Hz, 2H), 2.41 – 2.35 (m, 2H), 2.02 (t, *J* = 2.6 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.95, 149.34, 121.91, 82.45, 69.62, 31.06, 17.28. HR-FT-MS (m/z): [M - H]$^-$ calcd for $C_7H_7O_2$ 123.0452, found 123.0452.

Synthesis of compound 4



2 **4**

The compound **2** (1.52 g, 10 mmol) was dissolved in methanol/H_2O (3 mL, 1:1) and added NaOH (1.0 g, 50 mmol) at room temperature. The mixture was quenched with 1M HCl and extracted with EtOAc (3 × 300 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered and concentrated under vacuum. The residue was purified by a flash column chromatography on silica gel (CH_2Cl_2: EtOAc=3:1) to produce compound **4** as white solid (1.2 g, yield 87 %). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 11.87 (s, 1H), 7.08 (dt, *J* = 15.6, 7.0 Hz, 1H), 5.87 (dt, *J* = 15.6, 1.6 Hz, 1H), 2.38 (qd, *J* = 7.1, 1.5 Hz, 2H), 2.24 (td, *J* = 7.0, 2.7 Hz, 2H), 1.99 (t, *J* = 2.7 Hz, 1H), 1.71 (p, *J* = 7.2 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.09, 150.97, 121.42, 83.37, 69.16, 31.04, 26.54, 17.88. HR-FT-MS (m/z): [M - H]$^-$ calcd for $C_8H_9O_2$ 137.0608, found 137.0609.

Preparation of Cr-alkyne and Cr-alkyne-1

3 or **4** were transferred to EP tube and add aqueous solution of $NaHCO_3$ (1 eq.). The mixture was filtered

by 0.45 μm membrane, frozen by liquid nitrogen, and lyophilized to obtain white powdered Cr-alkyne or Cr-alkyne-1.

3.3 Procedures for solid-phase peptide synthesis

Deprotection

Peptide resin was treated with piperidine/DMF (20:80, *v/v*) for 2 times, and washed with DMF (× 3), DCM (× 3) and DMF (× 3).

Isotope lysine coupling

Isotope labeled Fmoc-Lys(Mtt)-OH (1.2 equiv), HATU (1.2 equiv) were dissolved in 4 mL of DMF, to which DIEA (2.4 equiv) was added. The amino acid was preactivated for 1 min, then the solution was added to the resin. After agitated for 2 h, the resin was washed with DMF (× 3), DCM (× 3) and DMF (× 3).

Automated solid-phase peptide synthesis

Automated peptide synthesis was performed on a CEM peptide synthesizer (Liberty Blue). Peptides were synthesized following the general protocol using DMF as solvent, deblock for 2 min in piperidine/DMF (20/80, v/v), couple for 5 min using excess amino acids (4 equiv) and DIC/Oxyma (1:1, 4 equiv) as coupling reagents. The needed $^{\alpha}$*N*-Fmoc or $^{\alpha}$*N*-Boc-protected amino acids from Novabiochem, GL Biochem or CS Bio were employed in SPPS.

Preparation and characterization of isotopic (K*) crotonyl-peptides

NH_2-ATIAGGGVIPHIHKcrSLIGK*-COOH (peptide **1**)

Peptide **1** was prepared according to Procedure **3.3** using the Fmoc-Wang resin (0.42 mmol/g, 0.05 mmol) and other standard $^{\alpha}$*N*-Fmoc or $^{\alpha}$*N*-Boc amino acids. After global deprotection using TFA, the crude peptide was dissolved in 15 mL of CH_3CN/H_2O/AcOH (20/75/5, *v/v/v*) and further purified using RP-HPLC. The fractions were collected and lyophilized to provide peptide **1** (5.3 mg, 25.5 %) as a fluffy white solid.

Peptide **1**

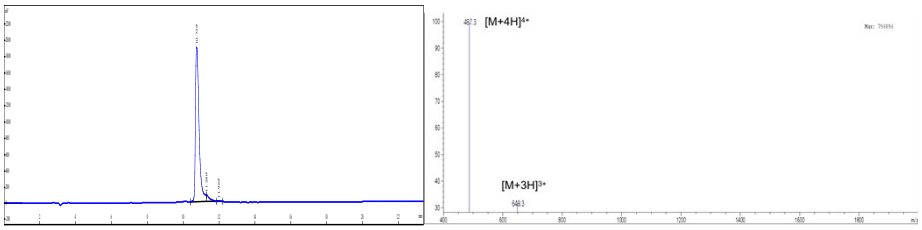


Figure S9. HPLC (left) and MS (right) analysis of peptide **1**

NH_2-GKcrVGRPTASK*-COOH (peptide **2**)

Peptide **2** was prepared according to Procedure **3.3** using the Fmoc-Wang resin (0.42 mmol/g, 0.05 mmol) and other standard $^{\alpha}$*N*-Fmoc or $^{\alpha}$*N*-Boc amino acids. After global deprotection using TFA, the crude peptide was dissolved in 15 mL of CH_3CN/H_2O/AcOH (20/75/5, *v/v/v*) and further purified using RP-HPLC. The fractions were collected and lyophilized to provide peptide **2** (5.1 mg, 30.1 %) as a fluffy white solid.

Peptide **2**

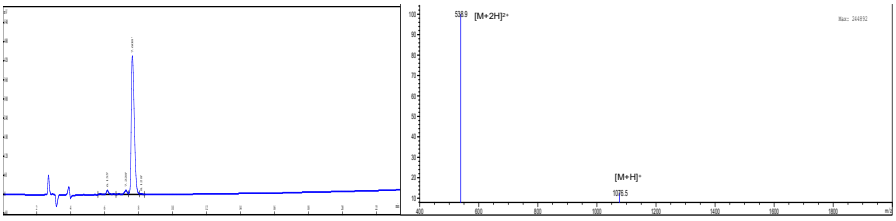
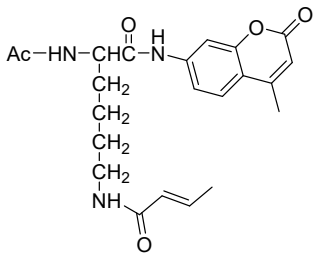
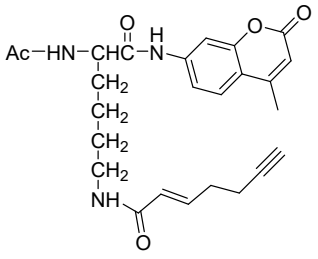


Figure S10. HPLC (left) and MS (right) analysis of peptide **2.**

3.4 Structure of Ac-Lys(cr)-AMC and Ac-Lys(cr-alkyne)-AMC.



Ac-Lys(cr)-AMC



Ac-Lys(cr-alkyne)-AMC

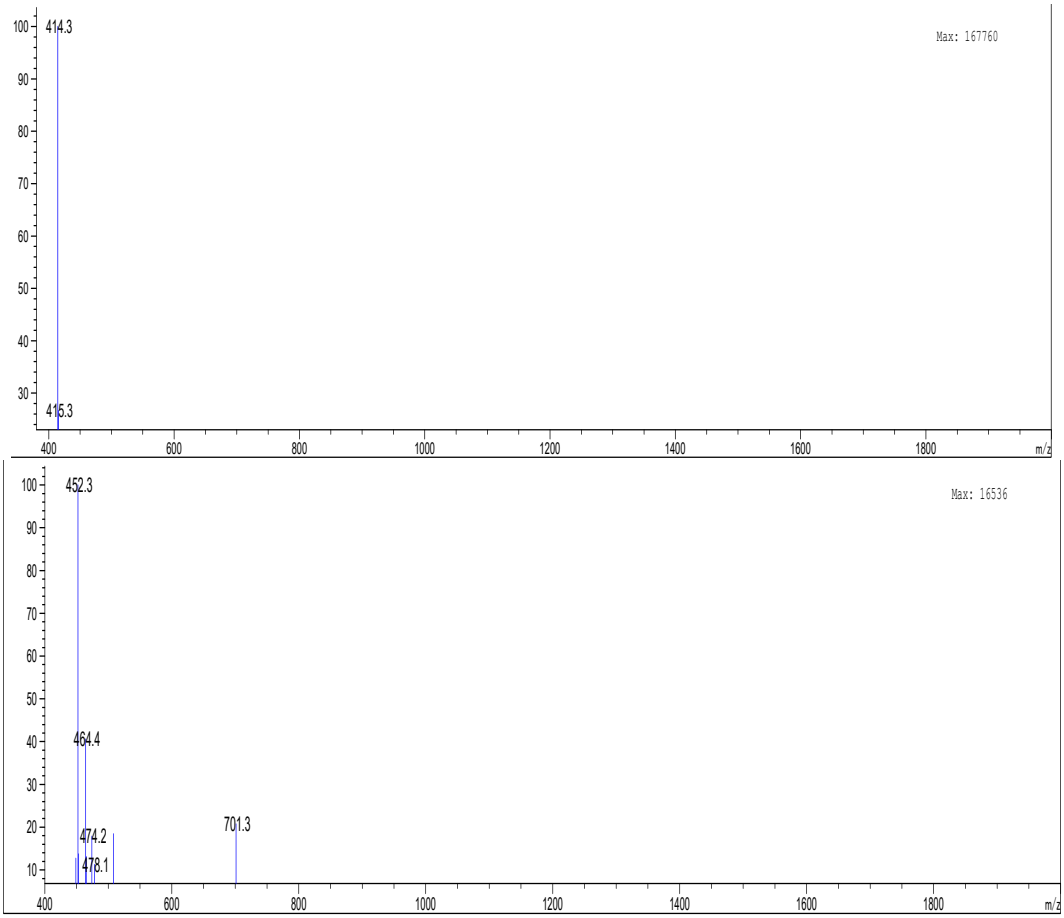


Figure S11. Structure and mass of Ac-Lys(cr)-AMC (top) and Ac-Lys(cr-alkyne)-AMC (bottom)**.**

4. Materials and Methods

4.1 Cell culture

HEK293T, MCF-7, LX2 and Raw264.7 were obtained from ATCC and maintained in DMEM (Thermo Fisher Scientific) supplemented with 10% (vol/vol) dialyzed FBS (Thermo Fisher Scientific), 100 U/mL penicillin, and 100 mg/mL streptomycin in a humidified atmosphere at 37°C with 5% CO_2. For transfection, cells were grown on cell culture dishes or plates to 70% confluence and transfected with indicated plasmids

using PEI (Polysciences) at a ~2.5:1 ratio of transfection reagent/DNA in Opti-MEM media (ThermoFisher) for about 18-24 h.

4.2 Plasmids and cloning

Total RNAs were extracted following the instructions of Eastep Super Total RNA Extraction (LS1040, Promega). The reverse transcription were adapted from protocols of RevertAid First Strand cDNA Synthesis Kit (#cat: K1622, Thermo Fisher Scientific). Full-length encoding proteins of interest (HMGB1, YWHAE, TMPO, HDAC1, HDAC3) were from cDNAs and cloned into the pCMV-FLAG vector.

4.3 Metabolic labeling in mammalian cells with Cr-alkyne and Cr-alkyne-1

Bioorthogonal probes (Cr-alkyne and Cr-alkyne-1) were dissolved in PBS to make the 1 M stock solutions. For metabolic labeling of cellular proteins, cells were incubated with the probes at desired concentrations in DMEM supplemented with 10% FBS for indicated time periods at 37 °C. Generally, Cr-alkyne was incubated with cells at 5 mM for 8 h, unless otherwise indicated. For cellular competition experiments, sodium crotonate was dissolved in PBS to make the 10 M stock solution. Cells were pre-treated with sodium crotonate at indicated concentrations for 8 h, and then co-incubated with the probes and sodium crotonate at indicated concentrations in DMEM supplemented with 10% FBS for 8 h. Probe-labeled cells were harvested, washed with cold PBS, and flash-frozen in liquid nitrogen before stored at -80 °C.

4.4 CuAAC click reaction and in-gel fluorescence analysis

To take 100 μL of cell lysates (2 mg/mL) and then reacted with 11 μL freshly prepared reagents containing azido-rhodamine (1 μL, 20 mM stock in DMSO), tris-(2-carboxyethyl) phosphine hydrochloride (TCEP, 2 μL, 50 mM freshly prepared stock in ddH_2O), tris-[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA, 6 μL, 10 mM stock solution in DMSO/*t*-butanol), and $CuSO_4$ (2 μL, 50 mM freshly prepared stock solution in ddH_2O) for 1 h at room temperature in the dark. The click reactions were terminated by Methanol chloroform precipitation, and then centrifuged at 20,000g for 15 min at 4 °C to obtain the proteins. The protein pellets were washed with ice-cold methanol twice and air-dried. The resulting protein pellets were resuspended with 35 μL of SDS lysis buffer (4% SDS, 150 mM NaCl, 50 mM triethanolamine, pH 7.4), and diluted with 12.5 μL 5X SDS-loading buffer (40% glycerol, 200 mM Tris-HCl pH 6.8, 8% SDS, 0.4% bromophenol blue) and 2.5 μL 2-mercaptoethanol. The resulting samples were heated for 5 min at 95 °C before loaded onto 10 % PAGE gels for SDS-PAGE separation. Generally, 30 μg of protein per gel lane is loaded for in-gel fluorescence visualization. For in-gel fluorescence, gels were scanned on a ChemiDoc MP Imager (BioRad) with the rhodamine filter. After in-gel fluorescence scanning, gels were stained with Coomassie Brilliant Blue staining reagent.

4.5 Fluorescence imaging

HEK293T cells were cultured on sterilized coverslips and incubated with Cr-alkyne (5 mM, 1 M stock solution in PBS) in culture medium. The same volume of PBS was used as the vehicle control. After 12 h of labeling at 37 °C, the cells were washed once with warmed PBS, fixed with 4% formaldehyde in PBS for 10 min at room temperature, and then washed twice with ice-cold PBS. Cells were permeabilized with 0.3% Triton X-100 in PBS for 10 min at room temperature, blocked with 3% BSA in PBS for 30min at room temperature, and washed with PBS (3 x 3 min with gentle agitation). The cells were then treated with a freshly premixed click reaction cocktail (50 μM azido-rhodamine, 1 mM TCEP, 100 μM TBTA, and 1 mM $CuSO_4$) in

PBST (0.1% Tween-20 in PBS) for 1 h at room temperature. After gentle washes three times with 1% Tween-20 in PBS, cells were then stained with Hoechst 33342 (Beyotime, cat#C1029) and imaged on a Nikon A1R confocal fluorescence microscope. For Hoechst channel, the 405 nm laser was used as the excitation, and emission was collected between 425 nm to 475 nm. For rhodamine channel, the 561 nm laser was used as the excitation, and emission was collected between 570 nm to 620 nm.

4.6 Histone extraction

For extraction of core histones, a standard acid-extraction protocol was adapted. The nuclear pellets were resuspended in 0.4 N H_2SO_4 and shaked overnight on a rotator at 4 °C. The nuclear debris was pelleted by centrifugation at 16000g for 10 min at 4 °C. The supernatant containing core histones was collected and then precipitated with MeOH at −80 °C overnight. Precipitated histone proteins were centrifuged at 16000g for 10 min at 4 °C and washed twice with ice-cold MeOH. Protein pellets were air-dried at room temperature and resuspended in ddH_2O. Protein concentrations were generally determined by the BCA assay (Pierce).

4.7 RNAi Experiments

30 nM of HDAC1 siRNA , HDAC3 siRNA was transfected into HEK293T cell lines with Lipofectamine 2000 Transfection Reagent (Thermo Scientific), according to the manufacturer's instructions. Corresponding concentrations of control siRNA were used as negative controls. Following transfection, cells were then maintained in a humidified 37°C incubator with 5% CO_2 for another 48 h.

4.8 Pull-down and immunoprecipitation

For pull-down experiments, HEK293T cells were transfected with plasmids expressing FLAG-tagged proteins of interest for 18-24 h, metabolically labeled with Cr-alkyne (5 mM) for another 8 h, and harvested. Cell lysates were prepared in SDS lysis buffer (1% SDS, 150 mM NaCl, 50 mM HEPES, pH 7.4, supplemented with benzonase) with brief vortexing. Cell lysates (2 mg protein) were incubated with freshly prepared click reaction cocktail containing 100 μM azido-biotin, 1 mM TCEP, 100 μM TBTA, and 1 mM $CuSO_4$ at 37 °C for 1 h in the dark. After protein precipitation and resuspension as described above, the biotinylated proteins were incubated with streptavidin agarose beads (ThermoFisher) at room temperature on a rotator for 2 h. The beads were washed six times with 1 mL of 1% SDS lysis buffer and the proteins were eluted with SDS-PAGE sample loading buffer (~30 μL) containing 70% SDS buffer (4% SDS, 150 mM NaCl, 50 mM triethanolamine, pH 7.4), 20% 5X SDS loadingbuffer, and 5% 2-mercaptoethanol after heating at 95 °C for 5 min. The supernatant (20 μL per gel lane) was separated by SDS-PAGE for Western blotting analysis. For immunoprecipitation experiments, HEK293T cells were transfected with plasmids expressing FLAG-tagged proteins of interest for 18-24 h and harvested. The cells were lysed with RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris, pH 7.4, supplemented with EDTA-free protease inhibitor cocktail) with vigorous vortexing. The resulting cell lysates were centrifuged at 16,000g for 10 min at 4 °C to remove cellular debris. Protein concentrations were determined by the BCA assay. Equal amounts of cell lysates (~1 mg) were incubated with anti-FLAG agarose beads (ThermoFisher) on a rotator at 4 °C overnight. The beads were washed six times with 1 mL of chilled 1% Triton X-100 buffer and then resuspended in SDS-PAGE sample loading buffer (~30 μL) with heating at 95 °C for 5 min. The supernatant (20 μL per gel lane) was separated by SDS-PAGE for Western blotting analysis.

4.9 Immunoaffinity enrichment using anti-crotonyllysine antibody

Immunoaffinity enrichment was performed by using agarose-conjugated anti-crotonyllysine antibody (PTM BioLab Co. Ltd., China). Briefly, the tryptic histone peptides were desalted and resolubilized in NETN buffer (50 mM Tris·HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% NP40). The peptides were incubated with 20 μL antibody-immobilized beads at 4 °C for 6 h with gentle rotation. The beads were washed three times with NETN buffer, twice with ETN buffer (50 mM Tris·HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA) and once with water. The bound peptides were eluted from the beads by washing three times with 50 μL of 0.5% trifluoroacetic acid. Eluates were combined and dried in a SpeedVac. The resulting peptides were cleaned in C18 tips prior to LC-MS/MS analysis.

4.10 Western blotting

Gels were transferred to PVDF membranes using Bio-Rad Trans-Blot Turbo Transfer System. The membranes were blocked with PBST (0.05% Tween-20 in PBS) containing 5% nonfat milk for 30 min at room temperature and then incubated with primary antibodies at 4 °C overnight. Membranes were washed with PBST three times, incubated with appropriate secondary antibodies, and developed using Bio-Rad Clarity Western ECL substrate. Membranes were imaged with a ChemiDoc MP Imager (Bio-Rad). Anti-FLAG-HRP conjugate was purchased from proteintech for anti-FLAG blots. Pan anti-crotonyllysine antibody (PTM-501) and site-specific anti-crotonyllysine antibody H4K8cr (PTM-0522RM), H2BK20cr (PTM-0534) were purchased from PTM Biolabs for anti-crotonyl blots. Goat anti-rabbit and anti-mouse HRP secondary antibodies were purchased from proteintech.

4.11 Chemoproteomic profiling of crotonated proteins

HEK293T cells were treated with 5 mM Cr-alkyne for 8 h. Cells were harvested, lysed and centrifuged at 20,000g for 10 min to remove cellular debris. Protein concentrations were determined by the BCA assay (Pierce). The cell lysates (2 mg protein) were then clicked with acid cleavable azido-DADPS-biotin (Click Chemistry Tools, cat#1330) in the presence of TCEP, TBTA, and $CuSO_4$ as described above. Methanol precipitated and washed protein pellets were again resuspended in 4% SDS buffer. Protein concentrations were determined and equal amounts of each protein sample were diluted by volume with 50 mM triethanolamine buffer to 1% SDS (~1.6 mg/mL protein). Then pre-washed streptavidin agarose beads (~50 μL slurry; ThermoFisher) were added to each sample. The protein and beads mixtures were incubated at room temperature on a rotator for 4 h. The beads were then washed 3 times with PBS and then 3 times with ddH_2O and transferred into spin-columns (ThermoFisher). The beads were then added with 6 M urea and then incubated with 10 mM DTT for 0.5 h, followed by treatment with 20 mM iodoacetamide for another 0.5 h in the dark. After that, the beads were washed with PBS and digested with 0.5 μg of trypsin in ABC buffer at 37 °C overnight. The beads were again washed 6 times with PBS and ddH_2O. Finally, the beads were resuspended with 2 % formic acid in ddH_2O (200 μL) to cleave the DADPS linker. The elution was repeated twice and the supernatants were pooled and dried by SpeedVac for LC-MS/MS analysis.

4.12 Detection of the Cr-alkyne metabolic modification rate in histones.

Two groups were set up: one group of HEK293T cells were treated with probe and another group of HEK293T cells were without probe treatment. Histones were extracted from both groups, and SP3 beads were used to exchange the buffer to TBTA. The samples were then digested overnight with trypsin (37℃, 16-17 h). The resulting peptides were labeled with dimethyl tags (light formaldehyde for the probe-treated group and heavy formaldehyde for the control group). After desalting, the samples were analyzed on the Orbitrap

Eclipse™ Tribrid™ Mass Spectrometer (Thermo Fisher Scientific) for identification. Search of peptides was performed by using FragPipe GUI v16.0 with MSFragger1 (version 3.3), Philosopher2 (version 4.0.0), and IonQuant3 (version 1.7.5). Precursor mass tolerance was set as 0-500 ppm. Missed cleavages were allowed up to 2. Peptide length was set 6 to 50, and peptide mass range was set 500 to 5000. Cysteine carboxyamidomethylation (+57.02146 Da) was set as fixed modification. 15.9949 Da is set as variable modification on methionine. For the search of quantitative data, the dimethylation fixation of lysine residues and N-terminal amino groups were set as light (+ 28.0313 Da), heavy (+ 34.06312) respectively. Peptides were required to achieve a peptide false-positive rate below 1%.

4.13 Quantitative proteomics unveiling crotonylation sites regulated by HDACs

HDAC1 knock down cells, HDAC1 overexpressing cells, and HEK293T cells were treated with 5 mM Cr-alkyne for 8 h. Cells were harvested, lysed and centrifuged at 20,000g for 10 min to remove cellular debris. Protein concentrations were determined by the BCA assay (Pierce). The whole lysates were undergoing clicked with azido-DADPS-biotin, enrichment with streptavidin and on-bead trypsin digestion as mentioned above. The probe-adducted peptides from the knock down, over expression and untreated cells were isotopically labeled by light (HCHO, $NaBH_3CN$), medium (DCDO, $NaBH_3CN$) and heavy ($D^{13}CDO$, $NaBD_3CN$) dimethylation regents according to rdTOP-ABPP procedures. Finally, to combine the beads of light, medium and heavy groups and then to resuspended it with 2 % formic acid in ddH_2O (200 μL) to cleave the DADPS linker. The elution was repeated twice and the supernatants were pooled and dried by SpeedVac for LC-MS/MS analysis. For HDAC3 group, the sample preparation process is similar to that of HDAC1 mentioned above.

4.14 LC-MS/MS analysis

LC-MS/MS was performed on a Q-Exactive plus Orbitrap mass spectrometer (Thermo Fisher Scientific) coupled with Ultimate 3000 LC system. Mobile phase A was 0.1% formic acid in H_2O, and mobile phase B was 0.1% formic acid, 80% acetonitrile in H_2O. Flow rate was 3 μL/min for loading and 0.3 μL/min for eluting. 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 mass resolution of 70000. MS/MS fragmentation is performed in a data-dependent mode, of which 20 most intense ions are selected for MS/MS analysis a resolution of 17500 using collision mode of HCD. Other important mass parameters: isolation window, 1.6 m/z units; default charge, 2+; normalized collision energy, 28%; maximum IT, 100 ms; dynamic exclusion, 20.0 s.

4.15 Fluorogenic Michaelis-Menten assays

Kinetic parameters were determined through rate experiments using Ac-Lys(Cr)-AMC and Ac-Lys(Cr-alkyne)-AMC substrates in Tris buffer (10 mM Tris, 150 mM NaCl, 10% glycine, pH=8.0). Various concentrations of substrates were incubated with 200 nM HDAC1 and 50 μg/mL trypsin. The *in situ* release of fluorophore was monitored using a FLUOstar Omega microplate reader (excitation: 355 nm, emission: 450 nm) by measuring fluorescence at 1 minute intervals for 30 minutes at 25°C. Initial conversion rates were determined for each substrate concentration, and the data were fitted to the Michaelis-Menten equation to determine Km and Kcat values.

4.16 Data analysis

Closed search of probe modified sites was performed by using FragPipe GUI v16.0 with MSFragger[1] (version 3.3), Philosopher[2] (version 4.0.0), and IonQuant[3] (version 1.7.5). Precursor mass tolerance was set

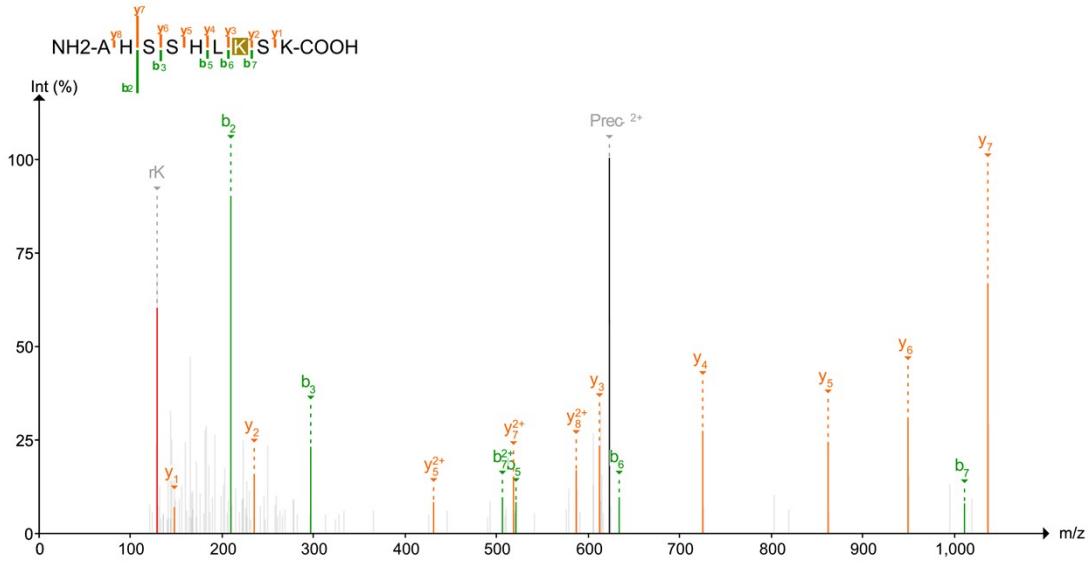
as 0-500 ppm. Missed cleavages were allowed up to 2. Peptide length was set 6 to 50, and peptide mass range was set 500 to 5000. Cysteine carboxyamidomethylation (+57.02146 Da) was set as fixed modification. **249.1477** Da was set as variable modification on lysine. 15.9949 Da is set as variable modification on methionine. For the search of quantitative data, the dimethylation fixation of lysine residues and N-terminal amino groups were set as light (+ 28.0313 Da), Medium (+ 32.0564) and heavy (+ 36.0757) respectively. Peptides were required to achieve a peptide false-positive rate below 1%.

4.17 Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the iProX[4,5] partner repository with the dataset identifier PXD053632. The data that support the findings of this study are available from the corresponding authors on reasonable request.

5. MS/MS analysis of representative Cr-alkyne-modified peptides in this study.

A

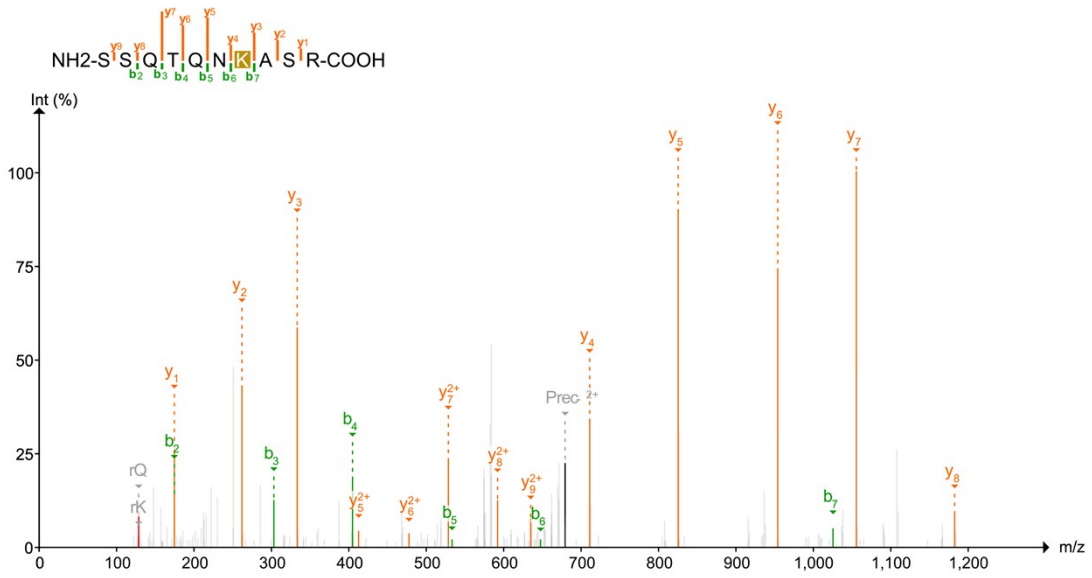


B

	b	b++		y	y++	
1	72.04439025	36.52583336	A			9
2	209.1033021	105.0552893	H	1172.65344	586.8303583	8
3	296.1353305	148.5713035	S	1035.594528	518.3009023	7
4	383.1673589	192.0873177	S	948.5624998	474.7848881	6
5	520.2262708	260.6167736	H	861.5304714	431.2688739	5
6	633.3103348	317.1588056	L	724.4715596	362.739418	4
7	1010.552998	505.7801371	K	611.3874956	306.197386	3
8	1097.585026	549.2961513	S	234.1448326	117.5760545	2
9			K	147.1128042	74.06004032	1

Figure S12. MS/MS analysis of a representative Cr-alkyne-modified peptide from P53. (A) The MS/MS peptide spectrum showing the identification of Cr-alkyne modification on the K372 residue of P53. (B) Summary of the assigned MS/MS fragment ion peaks of the Cr-alkyne modified P53 peptide shown in (A).

A

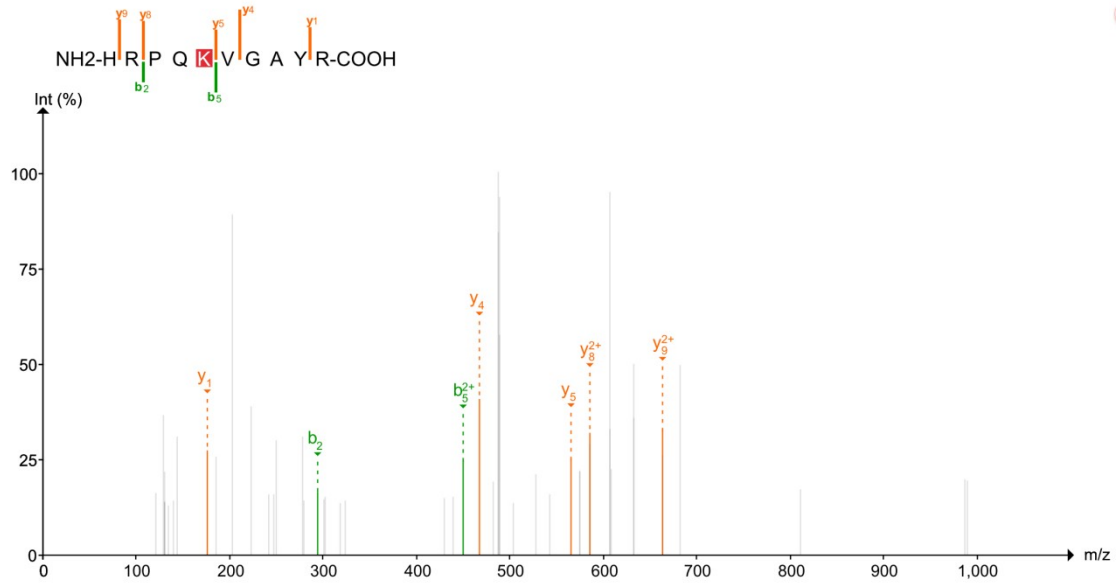


B

	b	b++		y	y++	
1	88.03930487	44.52329067	S			10
2	175.0713333	88.03930487	S	1268.670547	634.8389116	9
3	303.1299108	152.0685936	Q	1181.638518	591.3228974	8
4	404.1775892	202.5924329	T	1053.579941	527.2936086	7
5	532.2361668	266.6217216	Q	952.5322623	476.7697694	6
6	646.2790942	323.6431853	N	824.4736848	412.7404806	5
7	1023.521757	512.2645168	K	710.4307574	355.7190169	4
8	1094.558871	547.7830737	A	333.1880944	167.0976854	3
9	1181.590899	591.2990879	S	262.1509806	131.5791285	2
10			R	175.1189522	88.06311432	1

Figure S13. MS/MS analysis of a representative Cr-alkyne-modified peptide from SETLP. (A) The MS/MS peptide spectrum showing the identification of Cr-alkyne modification on the K199 residue of SETLP. (B) Summary of the assigned MS/MS fragment ion peaks of the Cr-alkyne modified SETLP peptide shown in (A).

A

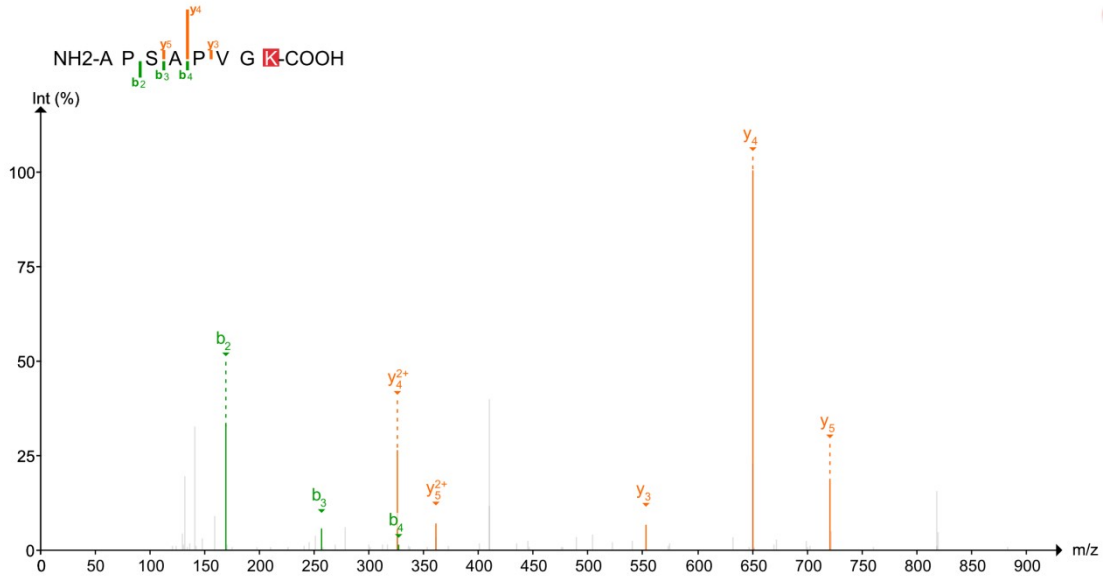


B

	b	b++		y	y++	
1	138.0661883	69.5367324	H			10
2	294.1672993	147.5872879	R	1323.764388	662.385832	9
3	391.2200632	196.1136698	P	1167.663276	584.3352765	8
4	519.2786407	260.1429586	Q	1070.610513	535.8088946	7
5	896.5213037	448.7642901	K	942.5519351	471.7796058	6
6	995.5897176	498.298497	V	565.3092721	283.1582743	5
7	1052.611181	526.8092289	G	466.2408582	233.6240673	4
8	1123.648295	562.3277858	A	409.2193945	205.1133355	3
9	1286.711624	643.8594501	Y	338.1822807	169.5947786	2
10			R	175.1189522	88.06311432	1

Figure S14. MS/MS analysis of a representative Cr-alkyne-modified peptide from WIZ. (A) The MS/MS peptide spectrum showing the identification of Cr-alkyne modification on the K1448 residue of WIZ. (B) Summary of the assigned MS/MS fragment ion peaks of the Cr-alkyne modified WIZ peptide shown in (A).

A

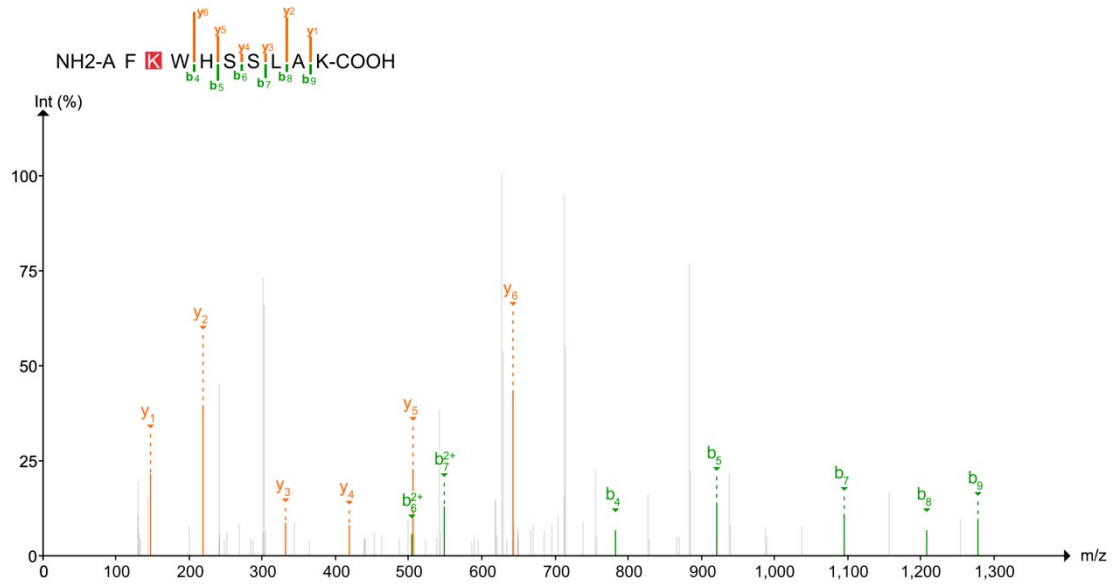


B

	b	b++		y	y++	
1	72.04439025	36.52583336	A			8
2	169.0971541	85.05221528	P	904.5250517	452.7661641	7
3	256.1291825	128.5682295	S	807.4722878	404.2397822	6
4	327.1662963	164.0867864	A	720.4402594	360.7237679	5
5	424.2190601	212.6131683	P	649.4031456	325.2052111	4
6	523.2874741	262.1473753	V	552.3503818	276.6788291	3
7	580.3089378	290.6581071	G	453.2819679	227.1446222	2
8			K	396.2605042	198.6338903	1

Figure S15. MS/MS analysis of a representative Cr-alkyne-modified peptide from PI2R. (A) The MS/MS peptide spectrum showing the identification of Cr-alkyne modification on the K342 residue of PI2R. (B) Summary of the assigned MS/MS fragment ion peaks of the Cr-alkyne modified PI2R peptide shown in (A).

A

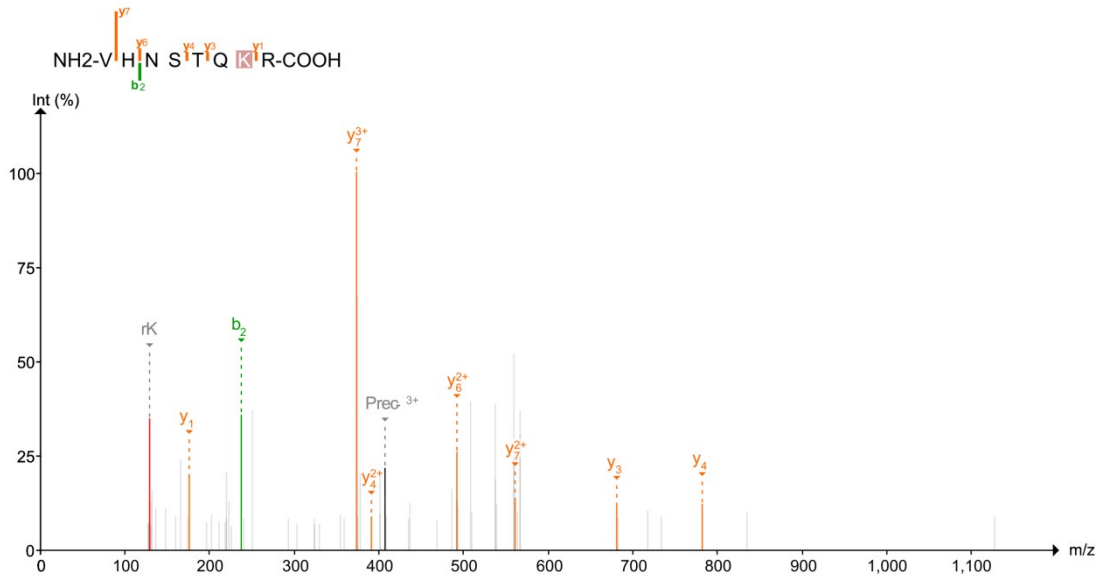


B

	b	b++		y	y++	
1	72.04439025	36.52583336	A			10
2	219.1128042	110.0600403	F	1352.74734	676.8773085	9
3	596.3554672	298.6813718	K	1205.678927	603.3431015	8
4	782.4347801	391.7210283	W	828.4362635	414.72177	7
5	919.493692	460.2504842	H	642.3569506	321.6821135	6
6	1006.52572	503.7664984	S	505.2980387	253.1526576	5
7	1093.557749	547.2825126	S	418.2660103	209.6366434	4
8	1206.641813	603.8245446	L	331.2339819	166.1206292	3
9	1277.678927	639.3431015	A	218.1499179	109.5785972	2
10			K	147.1128042	74.06004032	1

Figure S16. MS/MS analysis of a representative Cr-alkyne-modified peptide from ZN735. (A) The MS/MS peptide spectrum showing the identification of Cr-alkyne modification on the K391 residue of ZN735. (B) Summary of the assigned MS/MS fragment ion peaks of the Cr-alkyne modified ZN735 peptide shown in (A).

A

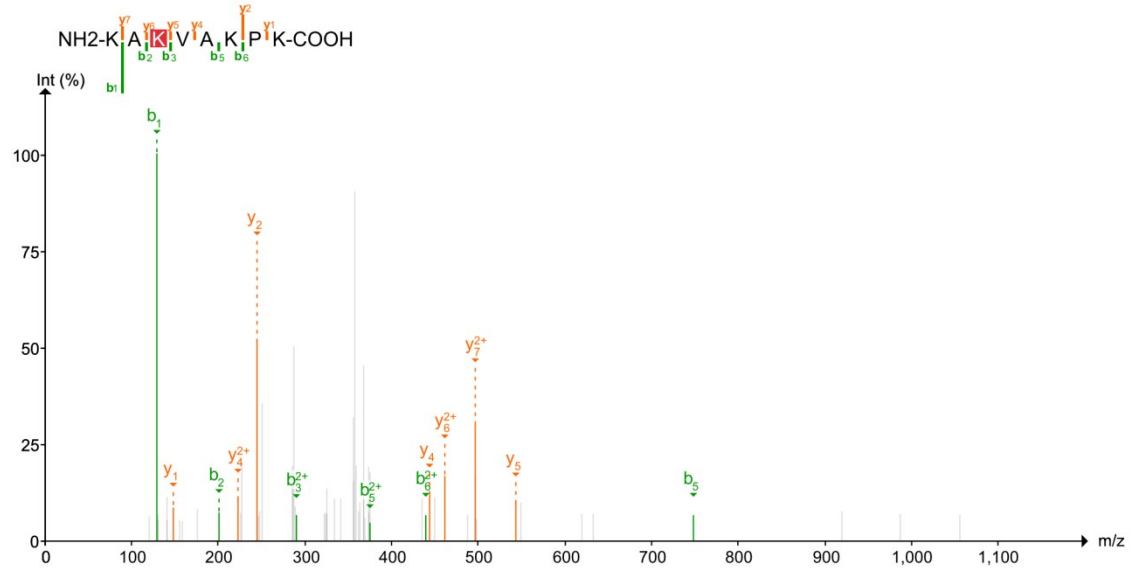


B

	b	b++		y	y++	
1	100.0756904	50.54148342	V			8
2	237.1346022	119.0709394	H	1119.601739	560.3045077	7
3	351.1775297	176.0924031	N	982.542827	491.7750517	6
4	438.2095581	219.6084173	S	868.4998996	434.753588	5
5	539.2572366	270.1322565	T	781.4678712	391.2375738	4
6	667.3158141	334.1615453	Q	680.4201927	340.7137346	3
7	1044.558477	522.7828768	K	552.3616152	276.6844458	2
8			R	175.1189522	88.06311432	1

Figure S17. MS/MS analysis of a representative Cr-alkyne-modified peptide from UBE2T. (A) The MS/MS peptide spectrum showing the identification of Cr-alkyne modification on the K180 residue of UBE2T. (B) Summary of the assigned MS/MS fragment ion peaks of the Cr-alkyne modified UBE2T peptide shown in (A).

A

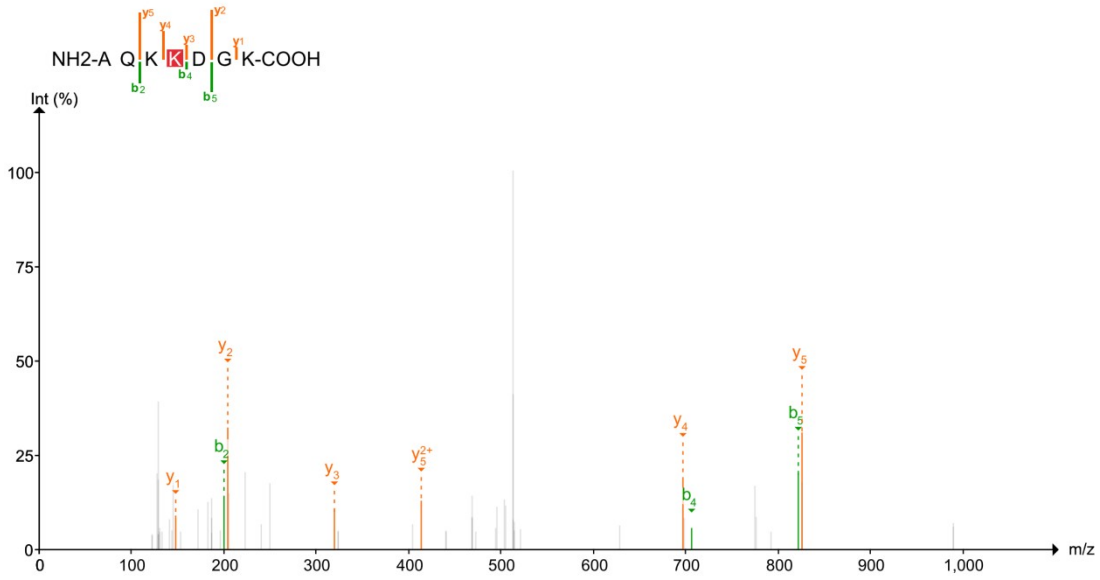


B

	b	b++		y	y++	
1	129.1022395	65.05475797	K			8
2	200.1393533	100.5733149	A	990.6458355	495.826556	7
3	577.3820163	289.1946464	K	919.6087217	460.3079991	6
4	676.4504302	338.7288533	V	542.3660587	271.6866676	5
5	747.487544	374.2474102	A	443.2976448	222.1524606	4
6	875.582507	438.2948917	K	372.260531	186.6339037	3
7	972.6352708	486.8212737	P	244.165568	122.5864222	2
8			K	147.1128042	74.06004032	1

Figure S18. MS/MS analysis of a new Cr-alkyne-modified peptide at H1-K177. (A) The MS/MS peptide spectrum showing the identification of Cr-alkyne modification on the K177 residue of H1. (B) Summary of the assigned MS/MS fragment ion peaks of the Cr-alkyne modified H1 peptide shown in (A).

A

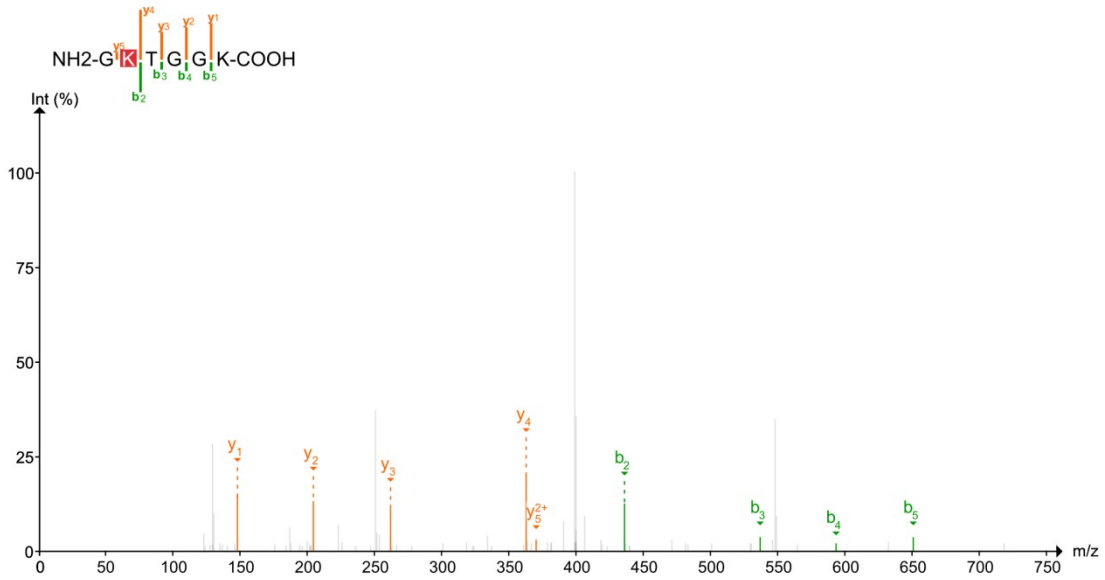


B

	b	b++		y	y++	
1	72.04439025	36.52583336	A			7
2	200.1029678	100.5551221	Q	952.5574144	476.7823455	6
3	328.1979308	164.6026036	K	824.4988369	412.7530567	5
4	705.4405938	353.2239351	K	696.4038739	348.7055752	4
5	820.4675368	410.7374066	D	319.1612109	160.0842437	3
6	877.4890005	439.2481385	G	204.1342679	102.5707722	2
7			K	147.1128042	74.06004032	1

Figure S19. MS/MS analysis of a new Cr-alkyne-modified peptide at H2B-K24. (A) The MS/MS peptide spectrum showing the identification of Cr-alkyne modification on the K24 residue of H2B. (B) Summary of the assigned MS/MS fragment ion peaks of the Cr-alkyne modified H2B peptide shown in (A).

A

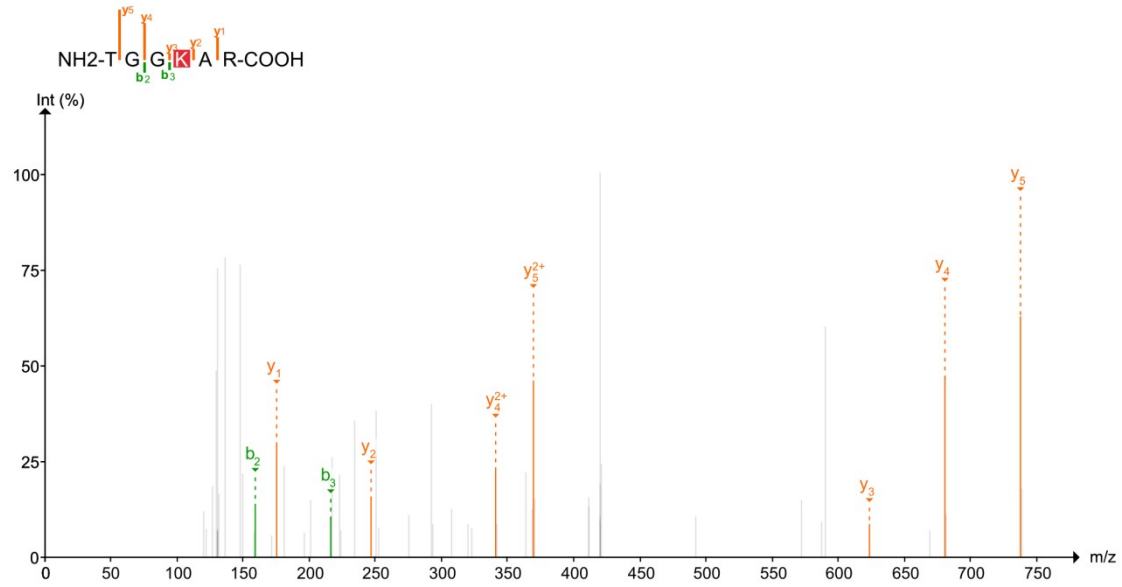


B

	b	b++		y	y++	
1	58.02874019	29.51800833	G			6
2	435.2714032	218.1393398	K	739.4460731	370.2266748	5
3	536.3190817	268.6631791	T	362.2034101	181.6053433	4
4	593.3405454	297.1739109	G	261.1557316	131.081504	3
5	650.3620091	325.6846428	G	204.1342679	102.5707722	2
6			K	147.1128042	74.06004032	1

Figure S20. MS/MS analysis of a new Cr-alkyne-modified peptide at H2AX-K5. (A) The MS/MS peptide spectrum showing the identification of Cr-alkyne modification on the K5 residue of H2AX. (B) Summary of the assigned MS/MS fragment ion peaks of the Cr-alkyne modified H2AX peptide shown in (A).

A

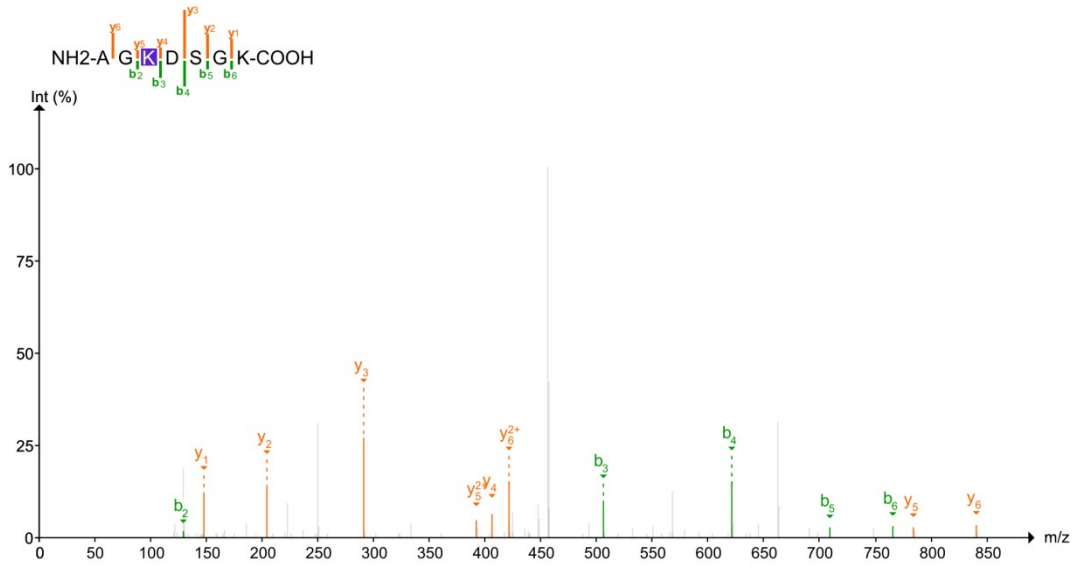


B

	b	b++		y	y++	
1	102.0549549	51.5311157	T			6
2	159.0764187	80.04184756	G	737.4416564	369.2244664	5
3	216.0978824	108.5525794	G	680.4201927	340.7137346	4
4	593.3405454	297.1739109	K	623.398729	312.2030027	3
5	664.3776592	332.6924678	A	246.156066	123.5816712	2
6			R	175.1189522	88.06311432	1

Figure S21. MS/MS analysis of a new Cr-alkyne-modified peptide at H2AX-K9. (A) The MS/MS peptide spectrum showing the identification of Cr-alkyne modification on the K9 residue of H2AX. (B) Summary of the assigned MS/MS fragment ion peaks of the Cr-alkyne modified H2AX peptide shown in (A).

A

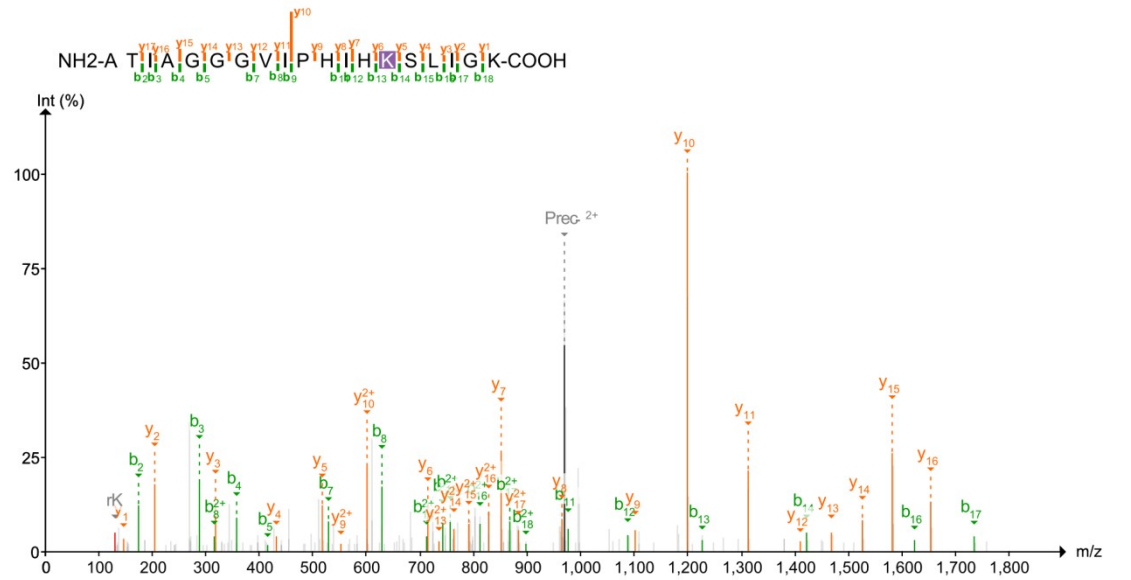


B

	b	b++		y	y++	
1	72.04439025	36.52583336	A			7
2	129.065854	65.03656522	G	840.457366	420.7323213	6
3	506.308517	253.6578967	K	783.4359023	392.2215894	5
4	621.33546	311.1713682	D	406.1932393	203.6002579	4
5	708.3674884	354.6873824	S	291.1662963	146.0867864	3
6	765.3889521	383.1981143	G	204.1342679	102.5707722	2
7			K	147.1128042	74.06004032	1

Figure S22. MS/MS analysis of a new Cr-alkyne-modified peptide at H2AZ1-K7. (A) The MS/MS peptide spectrum showing the identification of Cr-alkyne modification on the K7 residue of H2AZ1. (B) Summary of the assigned MS/MS fragment ion peaks of the Cr-alkyne modified H2AZ1 peptide shown in (A).

A

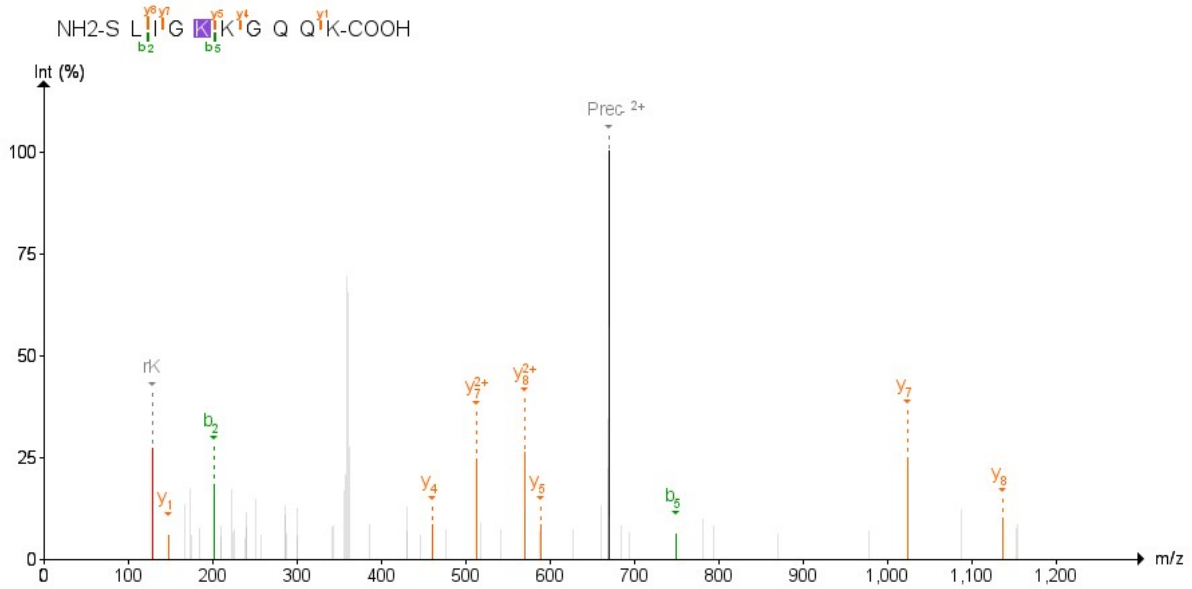


B

	b	b++		y	y++	
1	72.04439025	36.52583336	A			19
2	173.0920687	87.04967259	T	1866.095964	933.5516203	18
3	286.1761327	143.5917046	I	1765.048286	883.027781	17
4	357.2132465	179.1102615	A	1651.964222	826.4857491	16
5	414.2347102	207.6209933	G	1580.927108	790.9671922	15
6	471.2561739	236.1317252	G	1523.905644	762.4564603	14
7	528.2776376	264.6424571	G	1466.88418	733.9457284	13
8	627.3460516	314.176664	V	1409.862717	705.4349966	12
9	740.4301155	370.718696	I	1310.794303	655.9007896	11
10	837.4828794	419.2450779	P	1197.710239	599.3587576	10
11	974.5417912	487.7745339	H	1100.657475	550.8323757	9
12	1087.625855	544.3165658	I	963.5985631	482.3029198	8
13	1224.684767	612.8460218	H	850.5144991	425.7608878	7
14	1420.80593	710.9066033	K	713.4555873	357.2314319	6
15	1507.837958	754.4226175	S	517.3344242	259.1708504	5
16	1620.922022	810.9646495	L	430.3023958	215.6548362	4
17	1734.006086	867.5066815	I	317.2183319	159.1128042	3
18	1791.02755	896.0174133	G	204.1342679	102.5707722	2
19			K	147.1128042	74.06004032	1

Figure S23. MS/MS analysis of a new Cr-alkyne-modified peptide at H2AZ1-K115. (A) The MS/MS peptide spectrum showing the identification of Cr-alkyne modification on the K115 residue of H2AZ1. (B) Summary of the assigned MS/MS fragment ion peaks of the Cr-alkyne modified H2AZ1 peptide shown in (A).

A

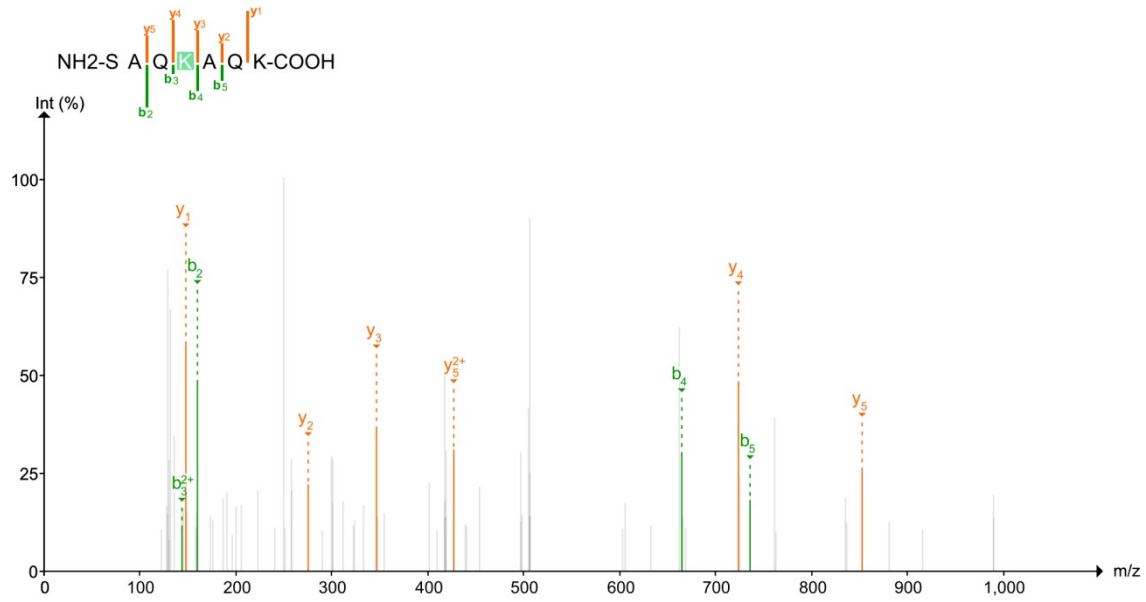


B

	b	b++		y	y++	
1	88.03930487	44.52329067	S			10
2	201.1233688	101.0653227	L	1276.773541	638.8904085	9
3	314.2074328	157.6073546	I	1163.689477	582.3483765	8
4	371.2288965	186.1180865	G	1050.605413	525.8063446	7
5	776.4664596	388.736868	K	993.5839489	497.2956127	6
6	904.5614226	452.7843495	K	588.3463859	294.6768312	5
7	961.5828863	481.2950814	G	460.2514229	230.6293497	4
8	1089.641464	545.3243701	Q	403.2299592	202.1186178	3
9	1217.700041	609.3536589	Q	275.1713817	138.0893291	2
10			K	147.1128042	74.06004032	1

Figure S24. MS/MS analysis of a new Cr-alkyne-modified peptide at H2AZ1-K120. (A) The MS/MS peptide spectrum showing the identification of Cr-alkyne modification on the K120 residue of H2AZ1. (B) Summary of the assigned MS/MS fragment ion peaks of the Cr-alkyne modified H2AZ1 peptide shown in (A).

A

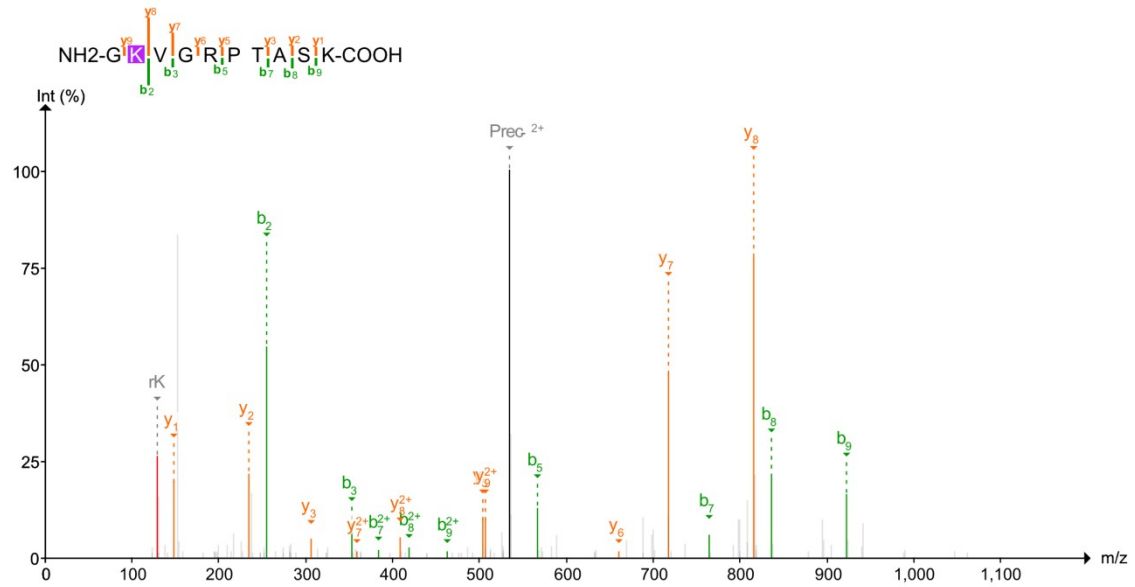


B

	b	b++		y	y++	
1	88.03930487	44.52329067	S			7
2	159.0764187	80.04184756	A	922.5468498	461.7770631	6
3	287.1349962	144.0711363	Q	851.509736	426.2585062	5
4	664.3776592	332.6924678	K	723.4511585	362.2292175	4
5	735.414773	368.2110247	A	346.2084955	173.607886	3
6	863.4733505	432.2403135	Q	275.1713817	138.0893291	2
7			K	147.1128042	74.06004032	1

Figure S25. MS/MS analysis of a new Cr-alkyne-modified peptide at EEF1A1-K457. (A) The MS/MS peptide spectrum showing the identification of Cr-alkyne modification on the K457 residue of EEF1A1. (B) Summary of the assigned MS/MS fragment ion peaks of the Cr-alkyne modified EEF1A1 peptide shown in (A).

A



B

	b	b++		y	y++	
1	58.02874019	29.51800833	G			10
2	254.1499032	127.5785898	K	1011.59454	506.3009084	9
3	353.2183171	177.1127968	V	815.4733773	408.2403269	8
4	410.2397808	205.6235287	G	716.4049634	358.7061199	7
5	566.3408919	283.6740842	R	659.3834997	330.1953881	6
6	663.3936557	332.2004661	P	503.2823887	252.1448326	5
7	764.4413342	382.7243053	T	406.2296248	203.6184506	4
8	835.478448	418.2428622	A	305.1819464	153.0946114	3
9	922.5104764	461.7588764	S	234.1448326	117.5760545	2
10			K	147.1128042	74.06004032	1

Figure S26. MS/MS analysis of a new Cr-alkyne-modified peptide at NUCKS1-K188. (A) The MS/MS peptide spectrum showing the identification of Cr-alkyne modification on the K188 residue of NUCKS1. (B) Summary of the assigned MS/MS fragment ion peaks of the Cr-alkyne modified NUCKS1 peptide shown in (A).

6. Characterization of the compounds

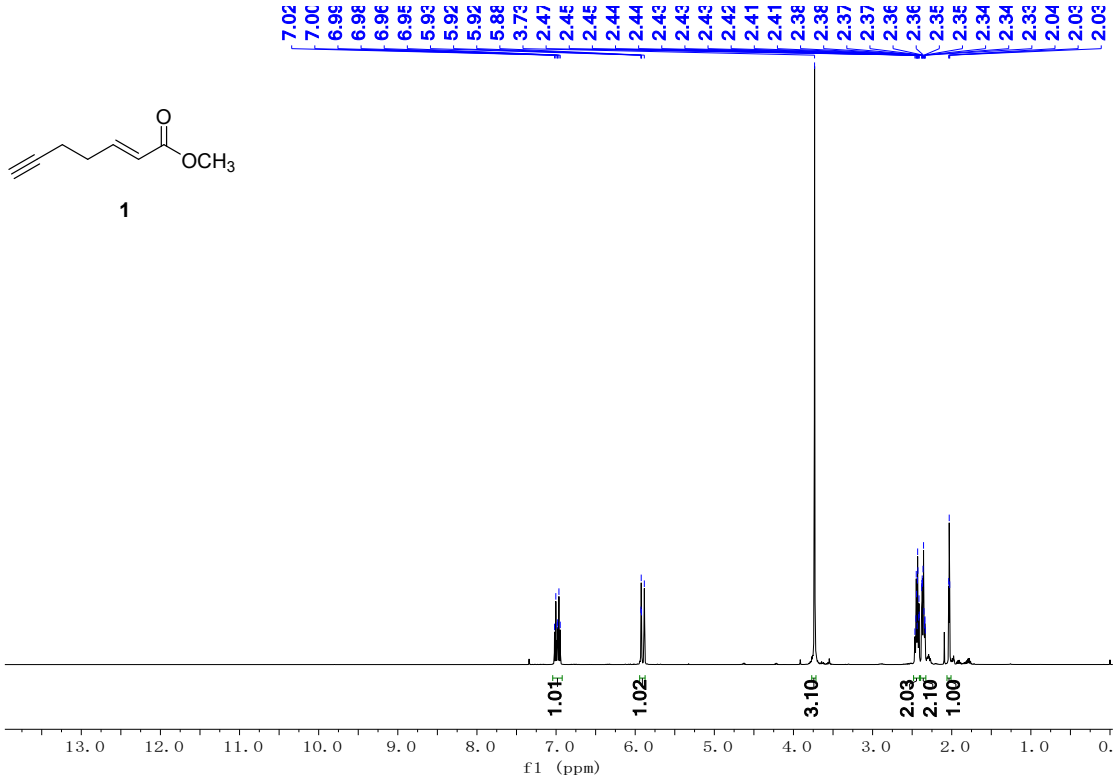


Figure S27. ^{1}H NMR (400 MHz) spectrum of compound 1 in $CDCl_3$.

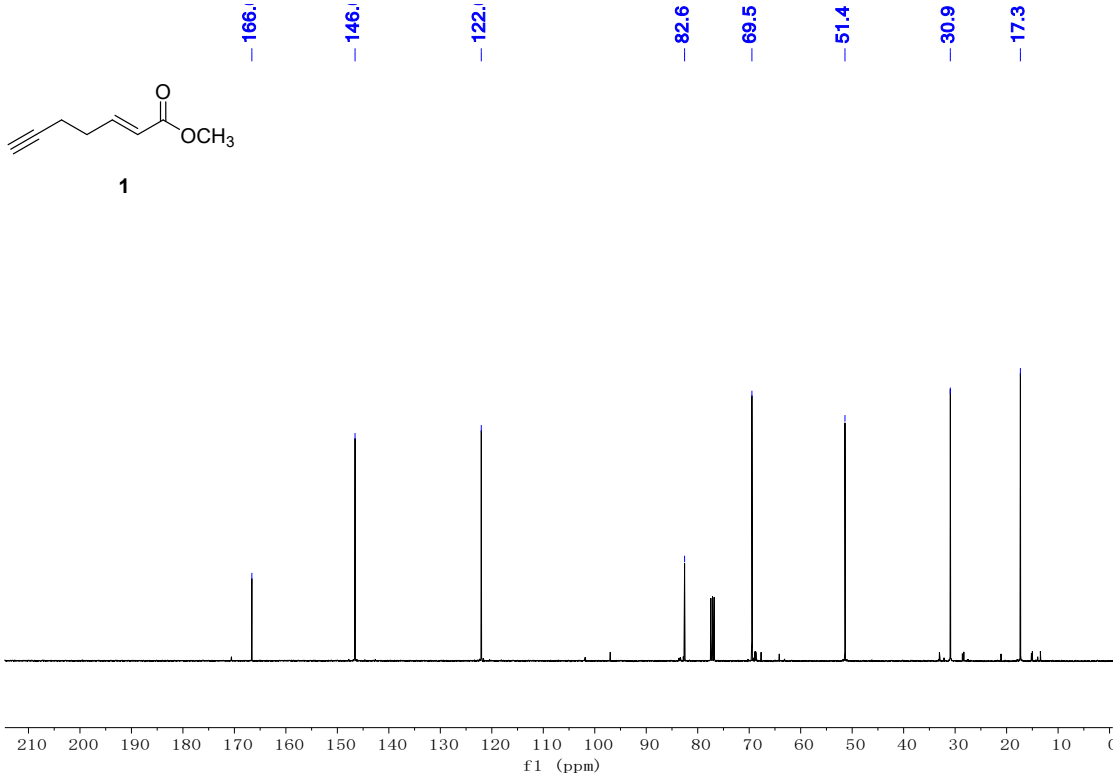


Figure S28.^{13}C NMR (101 MHz) spectrum of compound 1 in $CDCl_3$.

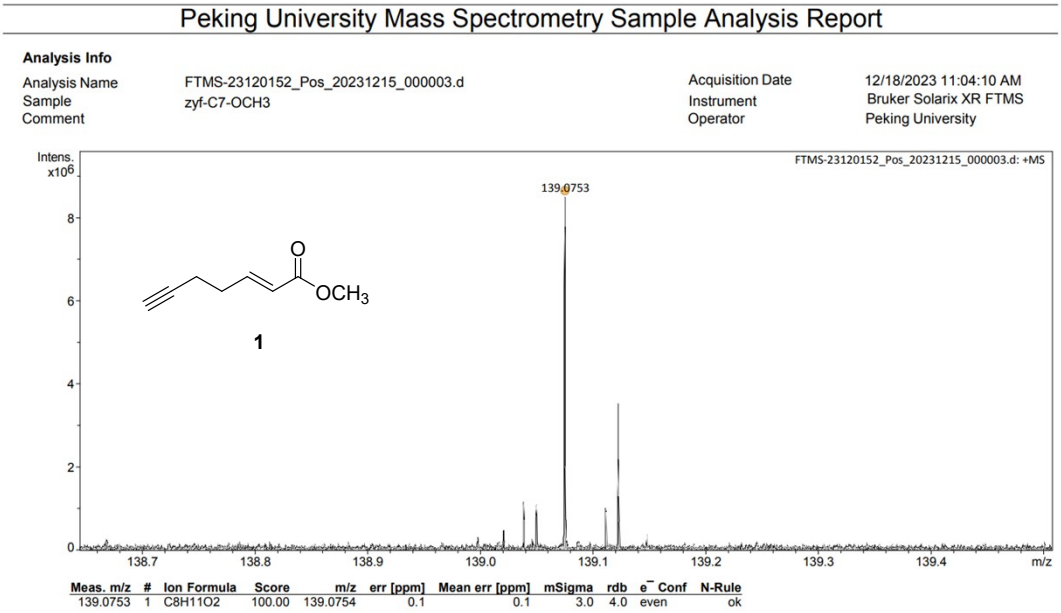


Figure S29. HR-FT-MS spectrum of compound 1.

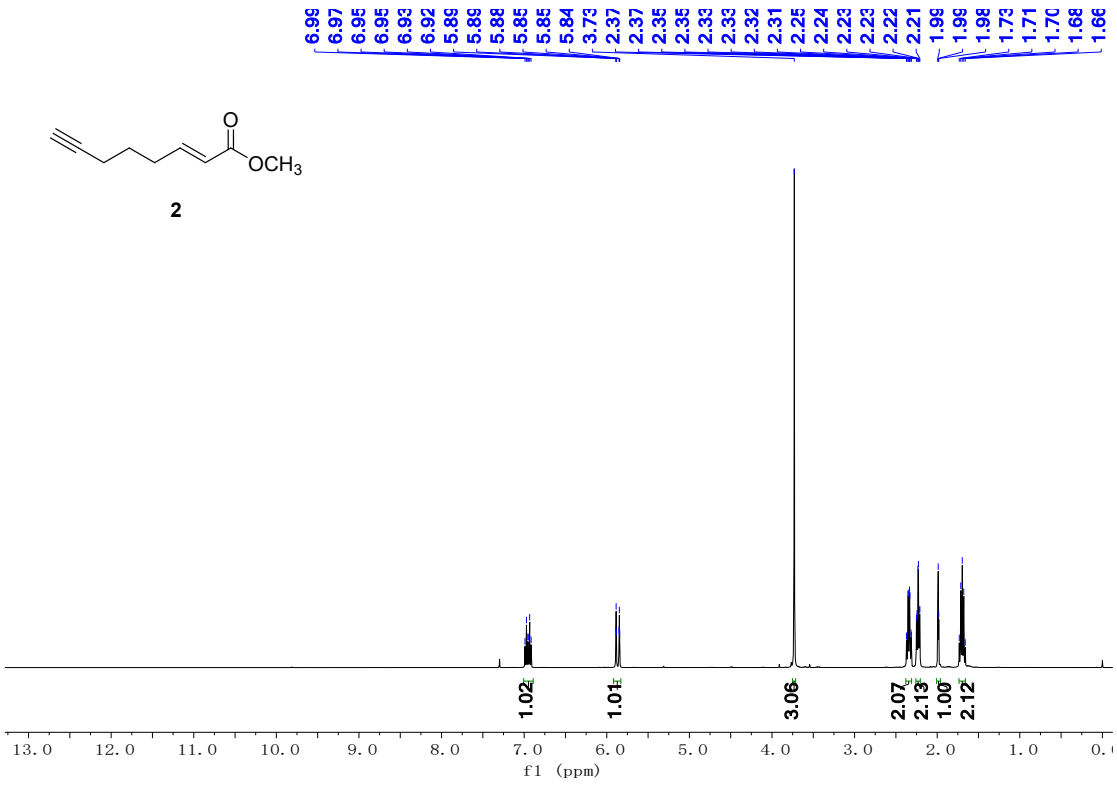


Figure S30. ^{1}H NMR (400 MHz) spectrum of compound 2 in $CDCl_3$.

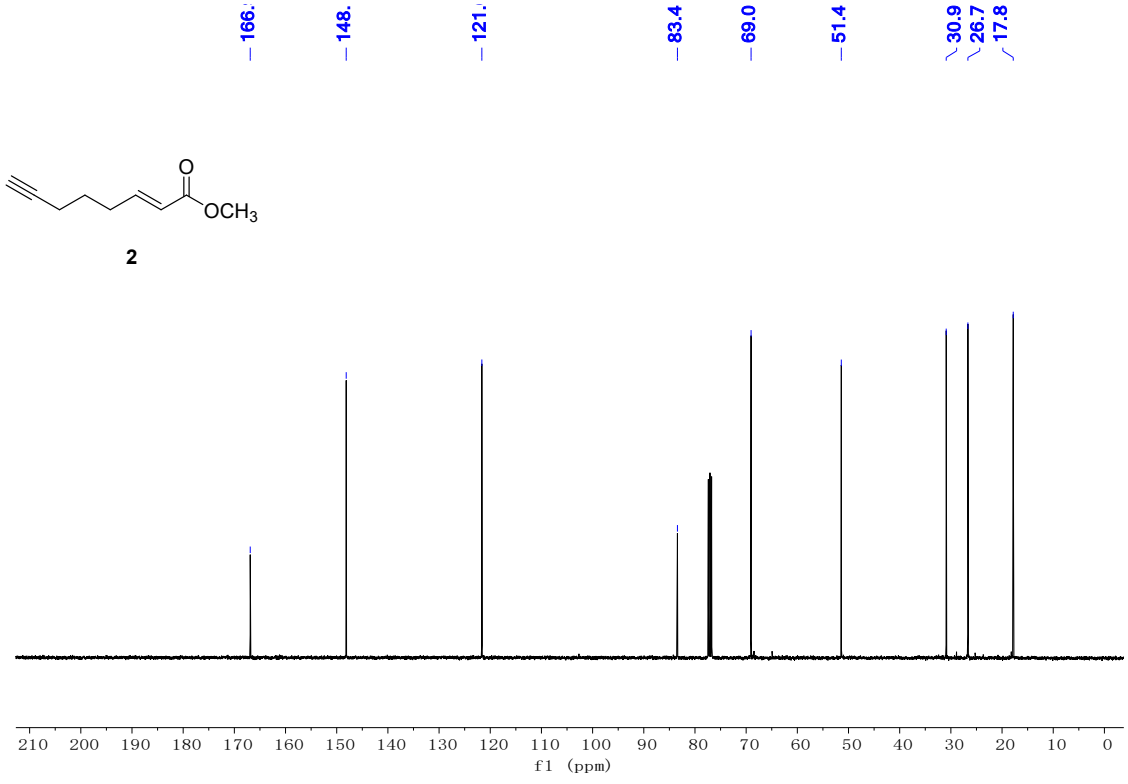


Figure S31.^{13}C NMR (101 MHz) spectrum of compound 2 in $CDCl_3$.

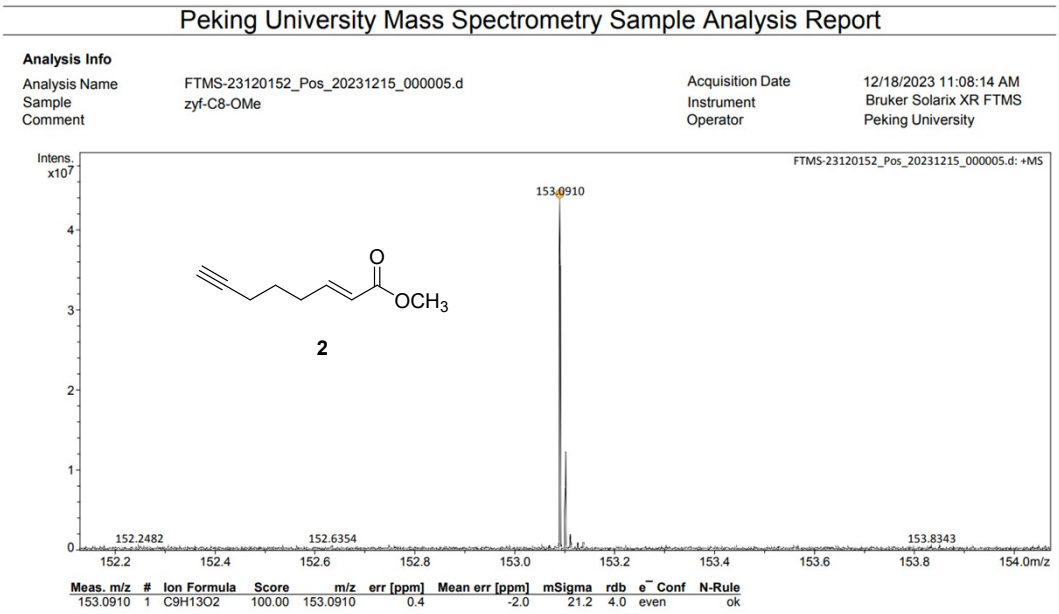


Figure S32. HR-FT-MS spectrum of compound 2.

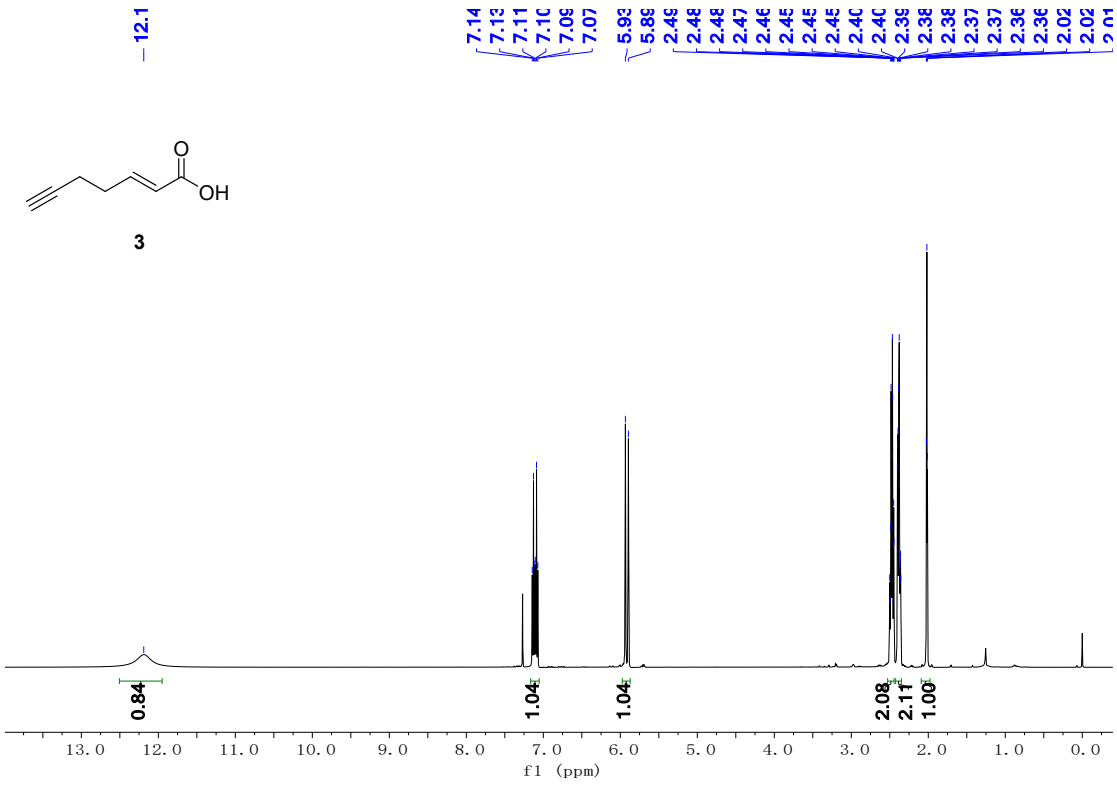


Figure S33. ^{1}H NMR (400 MHz) spectrum of compound 3 in $CDCl_3$.

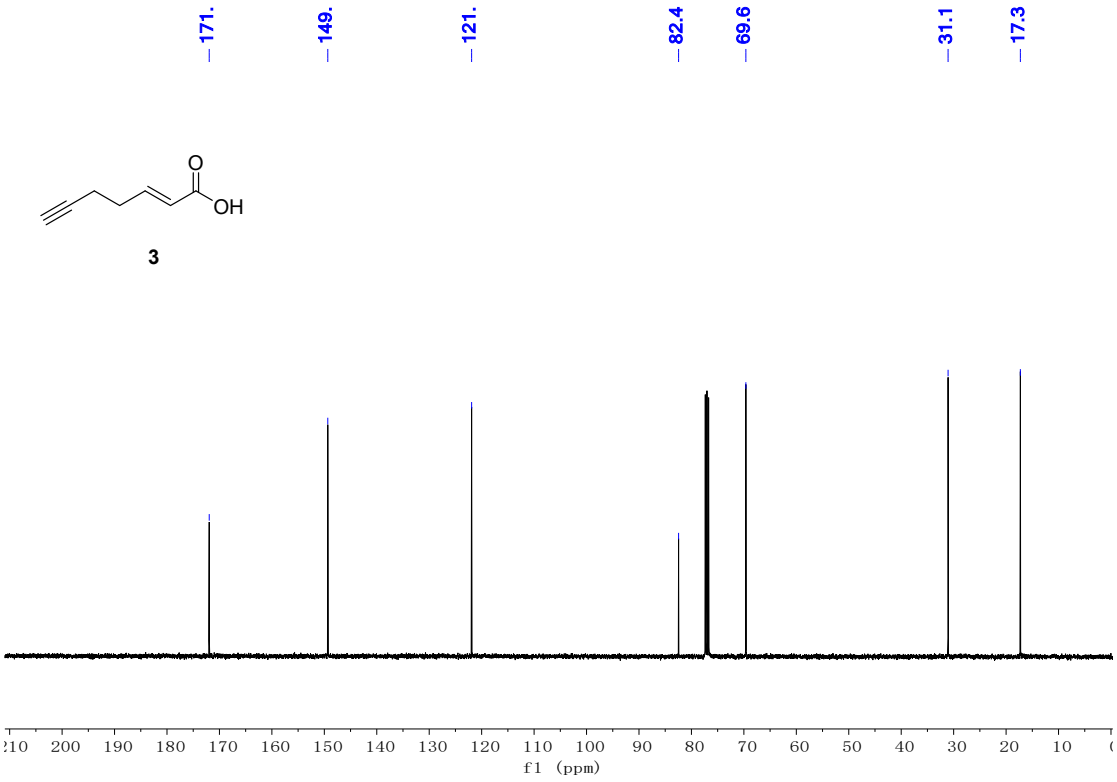


Figure S34.^{13}C NMR (101 MHz) spectrum of compound 3 in $CDCl_3$.

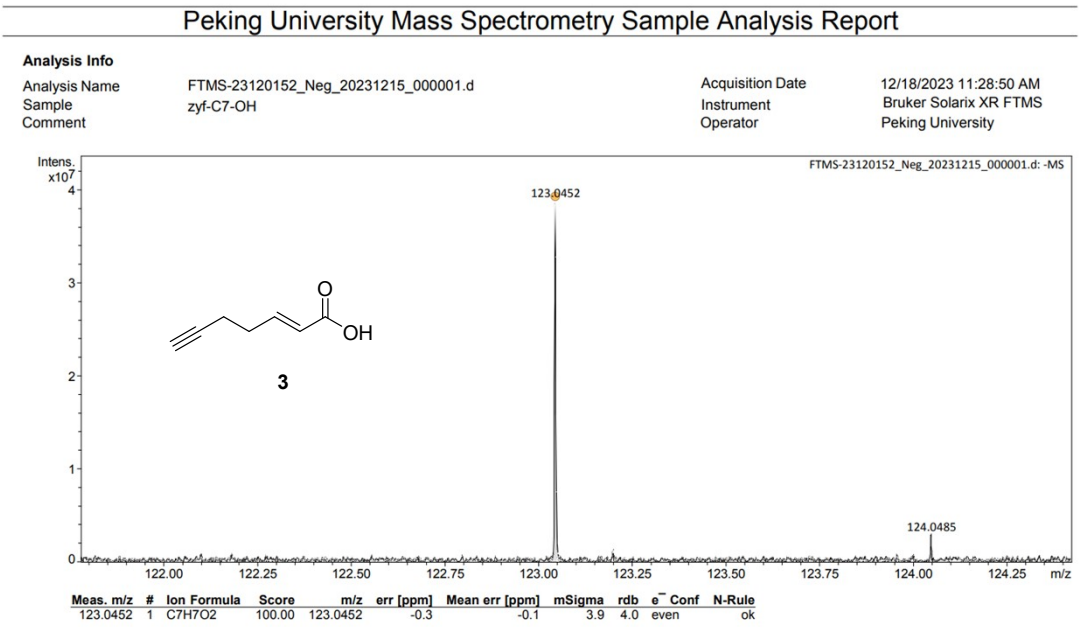


Figure S35. HR-FT-MS spectrum of compound 3.

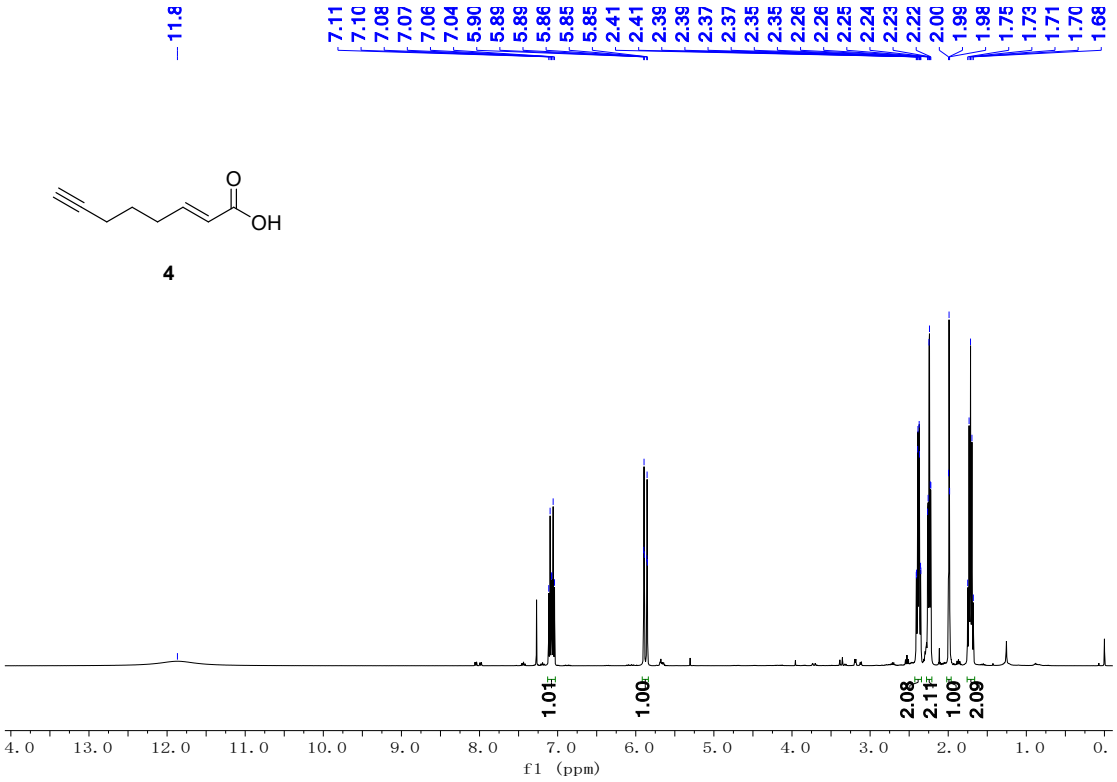


Figure S36. ^{1}H NMR (400 MHz) spectrum of compound 4 in $CDCl_3$.

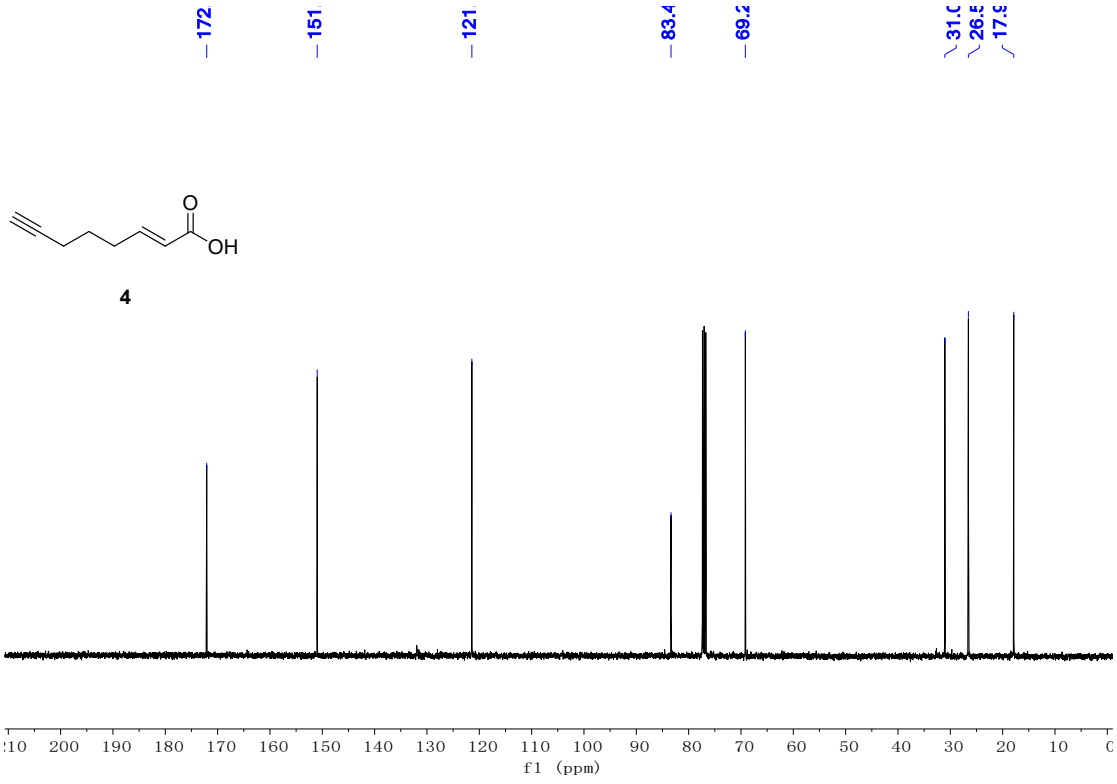


Figure S37.^{13}C NMR (101 MHz) spectrum of compound 4 in $CDCl_3$.

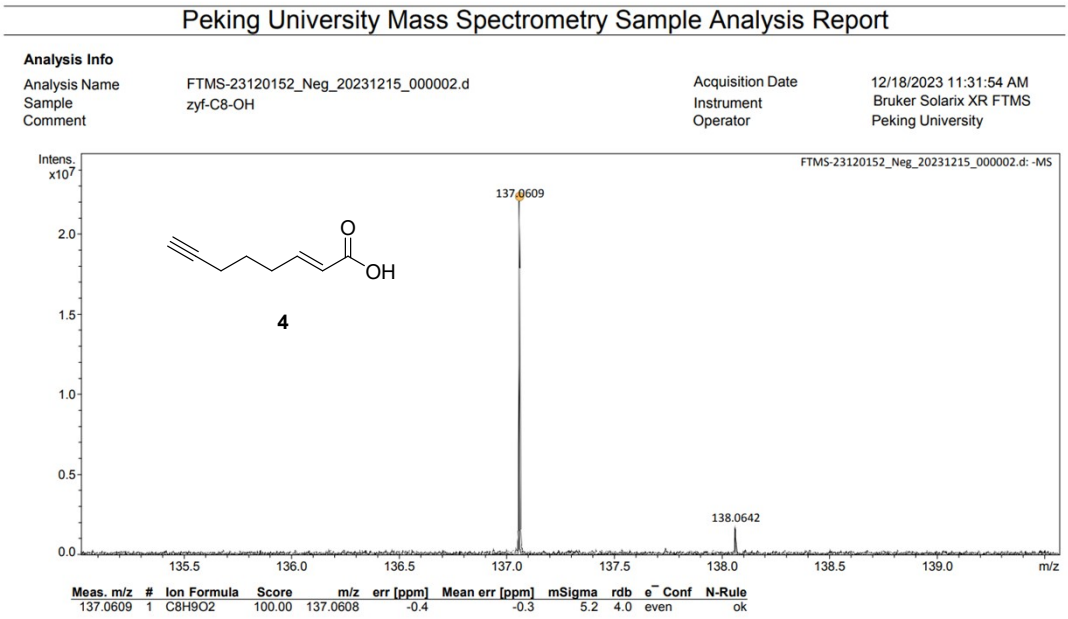


Figure S38. HR-FT-MS spectrum of compound 4.

7. References

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