Supplementary Information

Mapping sites of aspirin-induced acetylations in live cells by quantitative acid-cleavable activity-based protein profiling (QA-ABPP)

Jigang Wang[1†], Chong-Jing Zhang[2†], Jianbin Zhang[3†], Yingke He[4†], Yew Mun Lee[1], Songbi Chen[2], Teck Kwang Lim[1], Shukie Ng[3], Han-Ming Shen[3], Qingsong Lin[1*]*

[1] Department of Biological Sciences, 14 Science Drive 4, National University of Singapore, Singapore, 117543

[2] Tropical Crops Genetic Resources Institute, Chinese Academy of Tropical Agricultural Science, Baodao Xincun, Danzhou, Hainan, P.R. China, 571737,

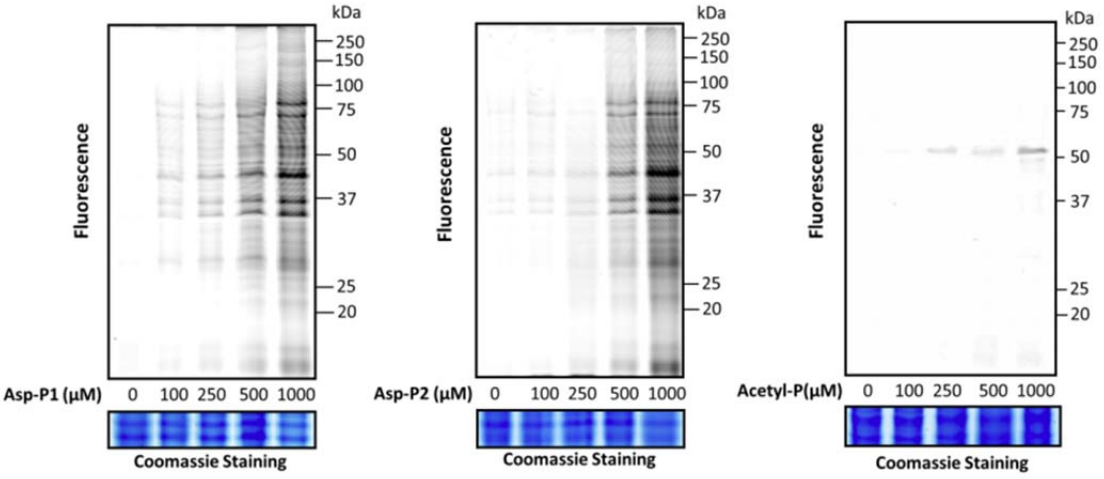
[3] Department of Physiology, Yong Loo Lin School of Medicine, Block MD9, 2 Medical Drive, National University of Singapore, Singapore 117597

[4] Department of Anaesthesia, Singapore General Hospital ，Outram Road, Singapore, 169608

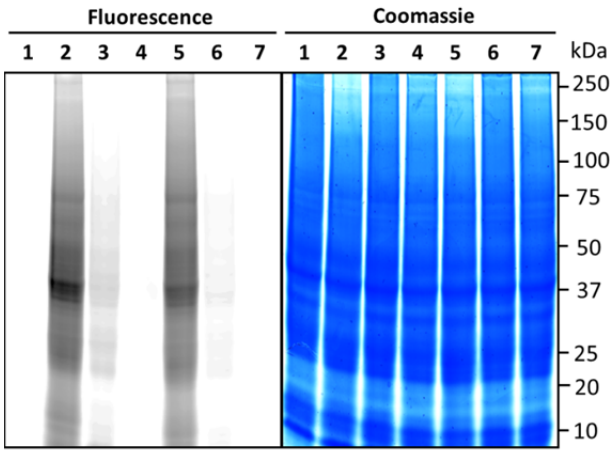
E-mail: *Qingsong Lin:* dbslinqs@nus.edu.sg
Han-Ming Shen: han-ming_shen@nuhs.edu.sg

Keywords: proteomics • activity-based probes • target identification • aspirin • binding site

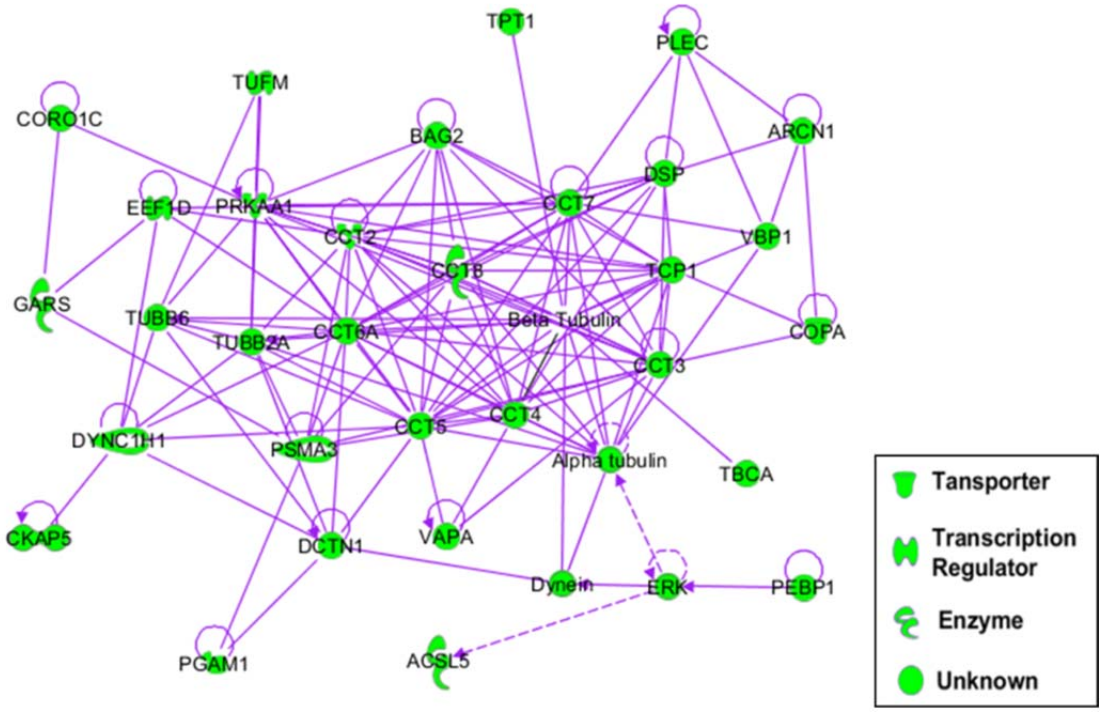
[†] These authors contributed equally to this work



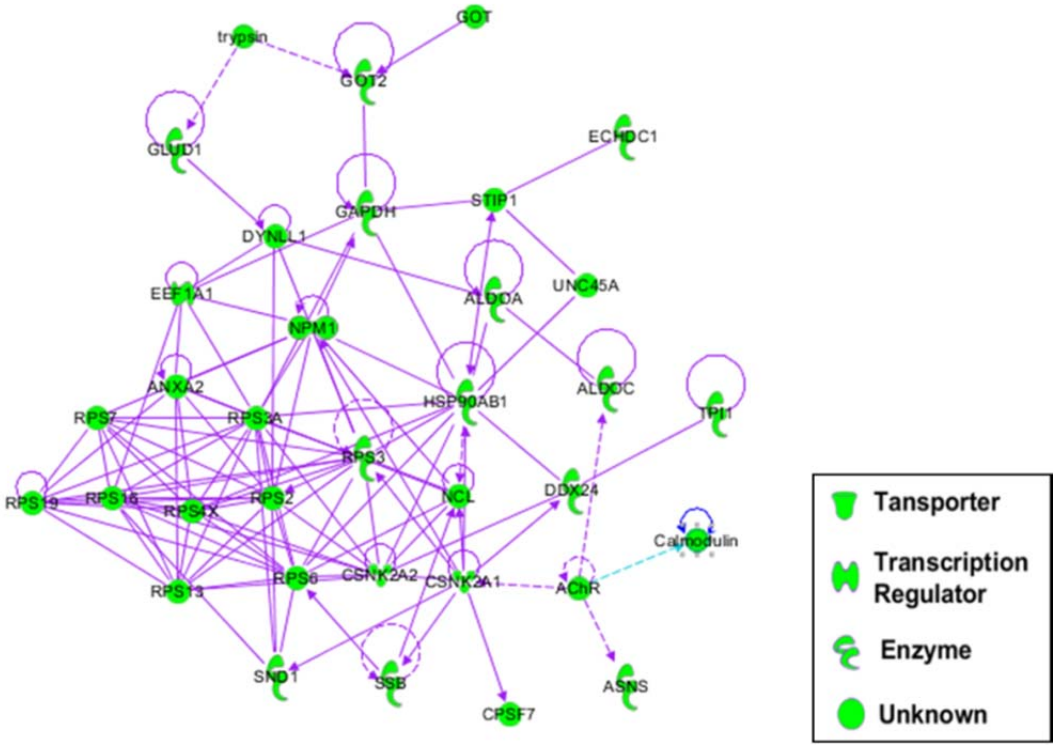
Supplementary Figure 1. The *in situ* fluorescent labeling of HCT116 cells using increasing concentrations of probes (100-1000 μM)**.** Different concentrations of **Asp-P1/2** and **Acetyl-P** were tested for *in situ* fluorescent profiling to determine the optimal working concentration. Probe labeled proteomes were visualized by click conjugation to the Cy3-azide tag followed by SDS-gel separation and fluorescence scanning. The results showed that 1000 μM of **Asp-P1/2** is sufficient to achieve an optimal target labeling, whereas the labeling efficiency of **Acetyl-P** is much lower than **Asp-P1/2**.



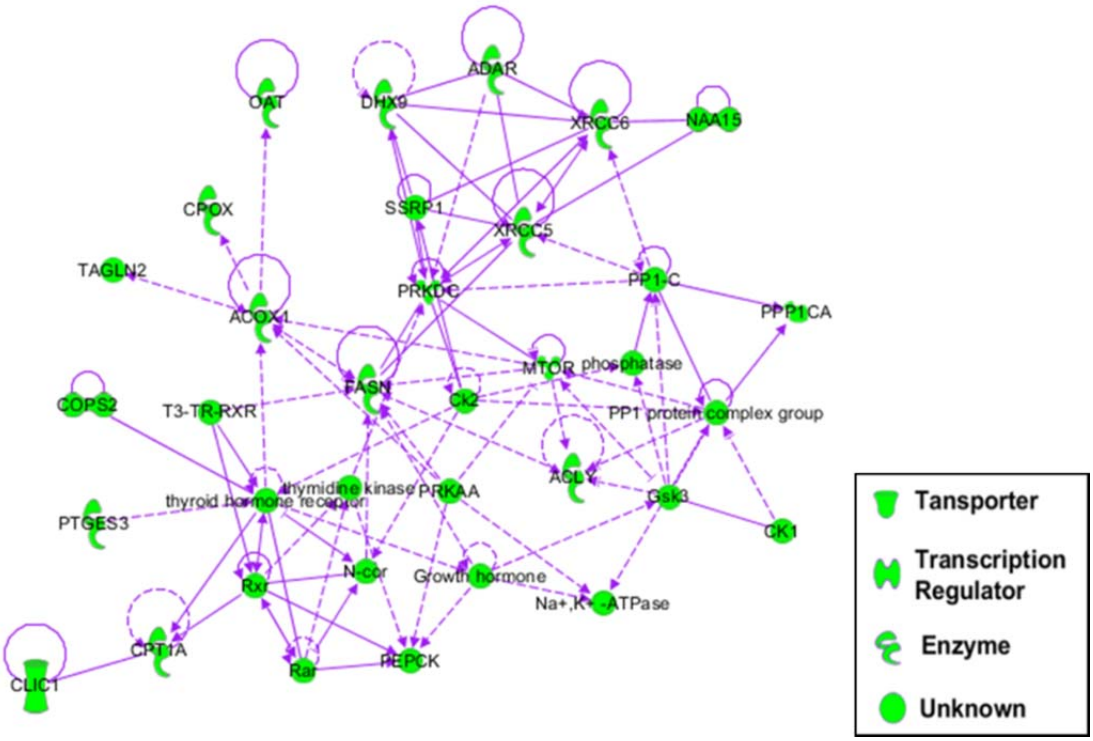
Supplementary Figure S2. The *in vitro* competition assay by in-gel fluorescence scanning. The HCT116 cell lysates were pretreated with aspirin (1 mM or 2mM) for two hrs. Then the lysates were incubated for another 12 hrs together with aspirin probes (500μM). Probe labeled proteomes were visualized by click conjugation to the Cy3-azide tag followed by SDS-gel separation and fluorescence scanning. **1**: DMSO; **2**: **Asp-P1**; **3**: **Asp-P1**+ 1 mM aspirin; **4**: **Asp-P1**+ 2 mM aspirin; **5**: **Asp-P2**; **6**: **Asp-P2**+ 1 mM asprin; **7**: **Asp-P2**+ 2mM aspirin.



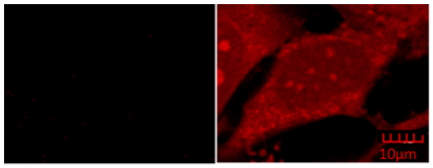
Supplementary Figure 3. The network revealing that aspirin affects cell death and survival processes; derived from Ingenuity Pathway Analysis (IPA) study of the aspirin target proteins. All proteins shown in green nodes were identified as specific targets of aspirin.



Supplementary Figure 4. The network revealing that aspirin affects protein synthesis, cancer cell death; derived from Ingenuity Pathway Analysis (IPA) study of the aspirin target proteins. All proteins shown in green nodes were identified as specific targets of aspirin.



Supplementary Figure 5. The network revealing that aspirin affects cellular assembly and organization, cellular function and maintenance, DNA replication, recombination, and repair; derived from Ingenuity Pathway Analysis (IPA) study of the aspirin target proteins. All proteins shown in green nodes were identified as specific targets of aspirin.

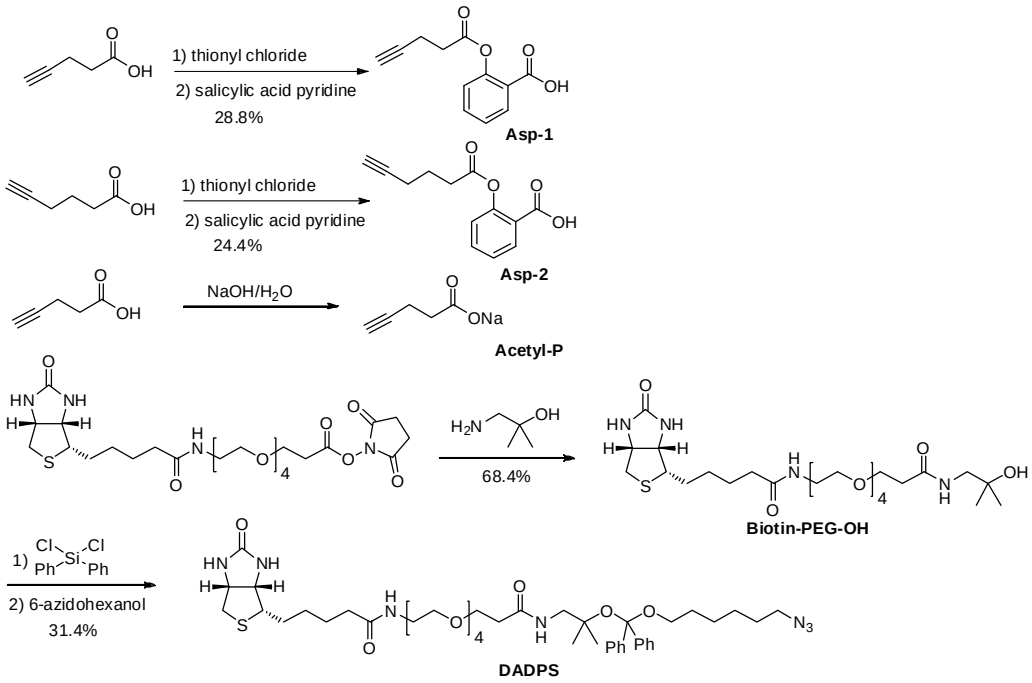


Supplementary Figure 6. **Cellular imaging of MEFs incubated with 1mM Asp-P2.** MEFs were firstly treated with **Asp-P2** (1 mM) for 16 hrs, and then fixed and permeabilized for the click chemistry reaction. Finally, the cellular fluorescence image was acquired using confocal microscopy. Scale bars, 10 μm.

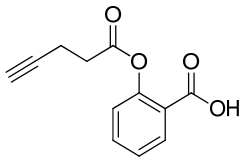
Materials and Methods:

1. Synthesis

All chemicals were purchased from commercial vendors and used without further purification, unless otherwise noted. Dichloromethane (DCM) was distilled over CaH_2. All non-aqueous reactions were carried out under nitrogen atmosphere in oven-dried glassware. All ^{1}H-NMR and ^{13}C-NMR spectra were taken on a Bruker ACF-300/500 MHz NMR spectrometer using $CDCl_3$ or $(CD_3)_2SO$ as the solvent. Chemical shifts are reported in parts per million referenced with respect to residual solvent ($CDCl_3$ = 7.26 ppm and $(CD_3)_2SO$ = 2.50 ppm) for ^{1}H-NMR, ($CDCl_3$ = 77.0 ppm and $(CD_3)_2SO$ = 40.0 ppm) for ^{13}C-NMR. LC-MS spectra were recorded using Shimadzu LC-MS (ESI). Extent of reaction was monitored by thin layer chromatography (TLC) using Merck 60 F254 pre-coated silica gel plates with fluorescent indicator UV254. After the plates were subjected to elution in the TLC chamber, the spots were visualized under UV light or with the appropriate stain (I_2, $KMnO_4$, ninhydrin, ceric ammonium molybdate (CAM)). Flash column chromatography was carried out using Merck silica gel (0.040-0.063).

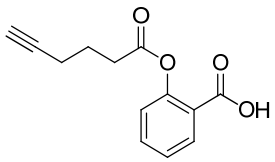


Supplementary Figure 7. Synthesis of different probes and linker (DADPS)



(Asp-1)

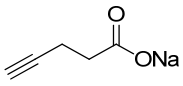
Pent-4-ynoic acid (0.9 g, 9.18 mmol) was dissolved in thionyl chloride (3.0 ml). The solution was heated at reflux for 2 hrs before all the solvent was removed under reduced pressure. The desired residue was added slowly into the solution of salicylic acid (633 mg, 4.6 mmol) in pyridine (15 ml) at 0 ^{o}C. The mixture was stirred for 30 min at the same temperature and then quenched with ice. EA (200 ml) and HCl aqueous solution (1 N, 100 ml) were subsequently added to reaction mixture. The organics was taken, washed further with HCl aqueous solution (1 N, 100 ml×2) and brine (100 ml), dried over sodium sulfite, filtrated and concentrated, purified with chromatography (Hex/EA = 20/1-5/1) to give Asp-1 (290 mg, 28.8%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.13 (d, J = 7.5 Hz, 1H), 7.63 (t, J = 7.5 Hz, 1H), 7.36 (t, J = 7.5 Hz, 1H), 7.16 (d, J = 7.5 Hz, 1H), 2.89 (t, J = 7.5 Hz, 2H), 2.67 (td, J_1 = 2.5 Hz, J_2 = 7.5 Hz, 2H), 2.04 (t, J = 2.5 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.4, 169.3, 151.0, 134.9, 132.5, 126.2, 124.0, 122.0, 82.2, 69.3, 33.4, 14.2; ESI calcd for $[M+Na]^{+}$: 241.0477, found: 241.0596.



(Asp-2)

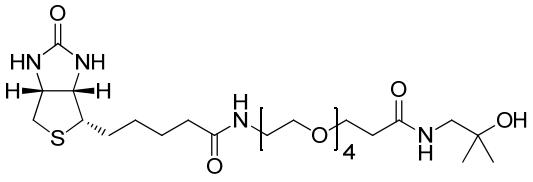
Hex-5-ynoic acid (1.68 g, 15 mmol) was dissolved in thionyl chloride (3.0 ml). The solution was heated at reflux for 2 hrs before all the solvent was removed under reduced pressure. The desired residue was added slowly into the solution of salicylic acid (1.0 g, 7.24 mmol) in pyridine (20 ml) at 0 ^{o}C. The mixture was stirred for 30 min at the same temperature and then quenched with ice. EA (200 ml) and HCl aqueous solution (1 N, 100 ml) were added to reaction mixture. The organics was taken, washed further with HCl aqueous solution (1 N, 100 ml×2) and brine (100 ml),

dried over sodium sulfite, filtrated and concentrated, purified with chromatography (Hex/EA = 20/1-5/1) to give Asp-2 (410 mg, 24.4%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.66 (brs, 1H), 8.13 (dd, J_1 = 1.5 Hz, J_2 = 7.8 Hz, 1H), 7.66 (td, J_1 = 1.5 Hz, J_2 = 7.5 Hz, 1H), 7.37 (m, 1H), 7.41 (d, J = 8.1 Hz, 1H), 2.78 (d, J = 7.2 Hz, 2H), 2.40 (td, J_1 = 2.4 Hz, J_2 = 6.9 Hz, 2H), 1.97-2.05 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.6, 170.0, 151.1, 134.8, 132.5, 126.1, 123.9, 122.2, 83.1, 69.3, 32.7, 23.2, 17.8; ESI calcd for $[M+Na]^+$: 255.0633, found: 255.0818.



(Acetyl-P)

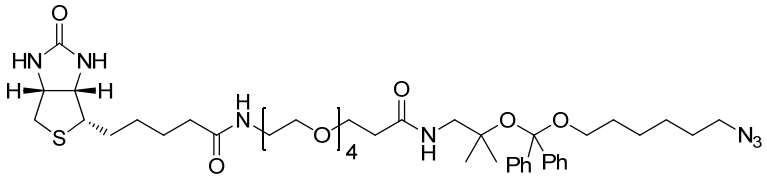
4-pentynoic acid (291 mg, 2.97 mmol) was dissolved in 10 ml ddH_2O. Aqueous NaOH solution (3 mmol, ~0.18 N) was added dropwise to reaction mixture while stirring. The reaction mixture was then filtered through 0.45 μm membrane, frozen in liquid nitrogen and lyophilized to dryness to give Acetyl-P. ESI calcd for $[M+Na]^+$: 143.0085, found: 143.0099.



(Biotin-PEG-OH)

To the solution of biotin-peg-NHS (950 mg, 1.61 mmol) in DCM (30 ml) was added 1-amino-2-methylpropan-2-ol (171 mg, 1.93 mmol) and TEA (200 mg, 1.98 mmol). The resulting mixture was stirred at room temperature for 4 hrs before all the solvent was removed under reduced pressure. The desired residue was purified with chromatography (DCM/MeOH = 30/1-15/1) to give the product (620 mg, 68.4%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.27 (t, J = 5.5 Hz, 1H), 7.22 (t, J = 6.0 Hz, 1H), 6.51 (s, 1H), 6.03 (s, 1H), 5.24 (s, 1H), 4.56 (brs, 1H), 4.44 (dd, J_1 = 5.0 Hz, J_2 = 7.0 Hz, 1H), 4.25 (m, 1H), 3.94 (brs, 6H), 3.70 (t, J = 6.0 Hz, 2H), 3.56 (m, 12H), 3.48 (t, J =

5.0 Hz, 2H), 3.35 (dd, J_1 = 5.0 Hz, J_2 = 10.0 Hz, 2H), 3.17 (d, J = 6.0 Hz, 2H), 3.09 (m, 1H), 2.84 (dd, J_1 = 5.0 Hz, J_2 = 12.5 Hz, 1H), 2.68 (d, J = 12.5 Hz, 1H), 2.60 (s, 1H), 2.45 (t, J = 6.0 Hz, 2H), 2.45 (t, J = 7.5 Hz, 2H), 1.53-1.67 (m, 4H), 1.38 (m, 2H), 1.24 (t, J = 7.5 Hz, 2H), 1.11 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.7, 173.4, 172.1, 164.1, 70.4, 70.16, 70.14, 70.11, 70.06, 69.9, 69.74, 69.73, 67.2, 61.7, 60.0, 55.5, 53.3, 50.0, 45.5, 40.2, 38.9, 36.7, 36.5, 28.1, 27.8, 26.8, 25.4, 25.2, 8.51.



(DADPS)

To the solution of dichlorodiphenylsilane (0.60 ml, 2.70 mmol) and TEA (740 mg, 7.32 mmol) in anhydrous DCM (50 ml) was added Biotin-PEG-OH (300 mg, 0.54 mmol). After 10 h, 6-azidohexanol (900 mg, 6.6 mmol) was added. The reaction mixture was stirred at room temperature overnight before being loaded to silica gel column and then eluted with (DCM/MeOH = 10/1). The crude product was further purified again with chromatography (DCM/MeOH = 30/1-10/1, containing 1% of TEA) to give the DADPS (150 mg, 31.4%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.23-7.65 (m, 10H), 6.85 (m, 1H), 6.48 (m, 1H), 6.41 (m, 1H), 5.48 (m, 1H), 4.48 (m, 1H), 4.30 (m, 1H), 3.72-3.77 (m, 2H), 3.50-3.66 (m, 14), 3.45 (m, 2H), 3.33 (d, J = 6.0 Hz, 2H), 3.24 (d, J = 7.0 Hz, 2H), 3.13 (m, 2H), 3.01 (dd, J_1 = 7.5 Hz, J_2 = 14.5 Hz, 1H), 2.90 (dd, J_1 = 5.0 Hz, J_2 = 15.5 Hz, 1H), 2.73 (d, J = 13.5 Hz, 1H), 2.42 (t, J = 6.0 Hz, 2H), 2.23 (m, 2H), 1.66-1.70 (m, 3H), 1.60 (dd, J_1 = 7.0 Hz, J_2 = 14.0 Hz, 2H), 1.34-1.45 (m, 5H), 1.27 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.2, 171.1, 163.7, 134.7, 134.6 (m, 6C), 130.1, 127.8, 127.6 (m, 2C), 75.6, 70.4, 70.36, 70.33, 70.2, 70.0, 69.9, 67.2, 62.9, 61.7, 60.1, 55.4, 51.3, 50.3, 45.8, 40.4, 39.1, 37.0, 35.8, 32.1, 28.7, 28.1, 28.0, 27.5, 26.3, 25.5, 25.3; ESI calcd for $[M+H]^+$: 886.4569, found: 886.4612.

2. Biochemical experiments

2.1 Reagents and antibodies

Besides the chemicals synthesized in the synthesis section, other reagents used in our experiments are listed below. Aspirin (99%), streptavidin beads, acetonitrile (ACN), methanol, Trifluoroacetic acid (TFA), urea, dithiothreitol (DTT), phosphoric acid, and Dimethyl sulfoxide (DMSO), tris [(1-benzyl-1H-1,2,3-triazol-4-yl) methyl]amine (TBTA), Tris(2-carboxyethyl) phosphine (TCEP), $CuSO_4$, were purchased from Sigma-Aldrich (St. Louis, MO). Cy3-azide and Rhodamine B alkyne were obtained from a commercial source (Jinglan Co, Guangzhou, China). Methyl methanethiosulfonate (MMTS) were purchased from Pierce (Rockford, IL). Dulbecco's Modified Eagle Medium (DMEM containing 4500 mg/l D-glucose, without L-glutamine, sodium pyruvate, L-methionine, and L-cystine, Click-iT® AHA (L-azidohomoalanine) reagent, dialyzed fetal bovine serum, 4% formaldehyde in PBS, 0.5% TritonTMX-100 in PBS, 3% Bovine serum albumin (BSA) in PBS (3% BSA in PBS, pH 7.4), 1% SDS in 50 mM Tris-HCl (pH 8.0), and amino acid-free medium were purchased from Invitrogen (Carlsbad, CA). Sequencing grade trypsin was obtained from Promega (Madison, WI). Protease and phosphatase inhibitor cocktails were purchased from Roche. Ultrapure water used for all experiments was purified with an ELGA water system. Unless otherwise indicated, all the other reagents used for the biochemical methods were purchased from Sigma-Aldrich. The antibodies used in our experiments included: microtubule-associated protein 1 light chain 3 (LC3), p62 and β-actin from Sigma Aldrich, phospho-S6 (Ser235/236) from Cell Signaling Technology (Danvers, MA).

2.2 Cell culture

HCT116 and MEFs cell lines were purchased from ATCC (Manassas, VA). Both cell lines were cultured in Dulbecco's Modified Eagle Medium (Sigma Aldrich) supplemented with 10% fetal bovine serum (Invitrogen) and 1 × antibiotic/antimycotic (Invitrogen). Cells were maintained at 37 ℃ in a humidified incubator supplemented with 5% CO_2.

2.3 *In situ* fluorescence labeling using Asp-1/2 and Acetyl-P and competition assay

HCT116 cells were grown to 80-90% confluence in 6-well plates. After the media was removed, cells were washed twice with PBS. Asp-P1/P2 or Acetyl-P (1000 μM) in 2 ml medium (1% DMSO) were added, and cells were incubated for 12 hrs at 37 °C and 5% CO_2. An equal volume of DMSO was also used as a negative control. For the concentration optimization experiment (Supplementary Figure 1 in Supporting Information), increasing concentrations (100-1000 μM) of probes were used to culture the cells for 12 hrs. Subsequently, the medium was removed, and cells were washed with PBS and detached with trypsin. The cell pellet was resuspended in PBS and washed before sonication in 150 μl of PBS to lyse cells. The resultant cell lysate was cleared by centrifuging at 13,000 rpm for 30 min. Protein concentrations of the cell lysates were determined using Bradford assay. Equal amounts (100 μg) of different treatment samples were used for subsequent fluorescent labeling. For each reaction, Cy3-azide (20 μM), TCEP (1 mM, 100 × fresh stock in water), TBTA ligand (100 μM, 100 × stock in DMSO), and $CuSO_4$ (1 mM, 100 × stock in water) were added to the lysate. The samples were incubated at room temperature for 2 hrs. Next, clicked proteins were precipitated by acetone and air dried. 1× SDS loading buffer (100 μl) was added to dissolve the sample, and 50 μl of sample was separated by SDS-PAGE on 12.5% polyacrylamide gel. After SDS-PAGE, gels were visualized using a Typhoon 9410 laser scanner (GE Healthcare; Buckinghamshire, UK) and images were analyzed by TotalLab software. For competition assay, the HCT116 cell lysate were pretreated with aspirin (1 mM or 2mM) for two hrs. Then the lysate were incubated for another 12 hrs together with aspirin probes (500μM). Probe labeled proteomes were visualized by click conjugation to the Cy3-azide tag followed by SDS-gel separation and fluorescence scanning.

2.4 Using QA-ABPP to identify targets of aspirin-induced acetylation

2.4.1 Cell labeling

In the subsequent QA-ABPP study, two biological duplicate of Asp-1/2 treated and two DMSO treated samples were pulled down and digested in parallel. The two biological duplicate of Asp-1/2 treated samples were labeled with iTRAQ reagent and quantified by iTRAQ ratios against the DMSO control samples. Briefly, HCT116 cells were grown to 80-90% confluence in T75 flasks. Used medium was then aspirated and the cells were washed twice with PBS. Asp-1/2 (1000 μM) in 10 ml medium (1% DMSO) was added to the cells in the flasks and incubated for 12 hrs in the CO_2 incubator. Culture medium containing 1% DMSO was used as negative control. Subsequently, Asp-1/2 and DMSO-containing media were removed before the cells were washed with PBS and detached with trypsin. The cell pellet was resuspended in PBS, washed and lysed by sonication in PBS. The cell lysates were clarified by centrifugation at 13,000 rpm for 30 min followed by Bradford assay. Equal amount (5 mg) of cell lysates (4 probe treated and 2 DMSO treated samples) were used for subsequent click chemistry to conjugate proteins with the DADPS tag separately. For each reaction, DADPS (20 μM), TCEP (1 mM, 100 × fresh stock in water), TBTA ligand (100 μM, 100 × stock in DMSO), and $CuSO_4$ (1 mM, 100 × stock in water) were added to the cell lysates and incubated at room temperature for 4 hrs. Next, clicked proteins were subjected to precipitation with acetone and air dried. Subsequently, the pellet was dissolved in 1 ml of 0.1% SDS in PBS and incubated with 50 μl of Streptavidin beads (Sigma-Aldrich) under gentle mixing for 2 hrs at room temperature.

2.4.2 On-beads digestion by trypsin

The beads were washed a total of 9 times; thrice with 1% SDS, followed by thrice with urea (6 M) and thrice with PBS. The extensively washed beads were re-suspended in 100 μl 25 mM triethylammonium bicarbonate (TEAB) and 2 μl TCEP (100 mM stock solution) was added. The beads were placed in a 65°C heat block for 60 min. Next, 1 μl MMTS (200 mM stock solution) was added, and the

samples left in the dark and allowed to react for 15 min at room temperature. After reduction and alkylation, trypsin (12.5 ng/ µl) was added and incubated at 37°C overnight. The digested peptides were separated from the beads using a filter-spin column (GE Healthcare). These digested peptides could be stored at -20 °C for several months pending iTRAQ labeling and mass spectrometry analysis.

2.4.3 On-beads treatment with formic acid to cleave the probe modified peptide

For the pull down sample using Asp-P2, the washed and filtered beads which bear the binding peptides were further washed three times using 25 mM TEAB to remove the digested unmodified peptides. The beads were then incubated with 5% formic acid for 2 hrs to cleave the acid cleavable biotin tag. The resulting cleaved probe modified peptides were separated from the beads using a filter-spin column and identified using LC-MS/MS.

2.4.4 iTRAQ Labeling of the peptides from on-beads digestion by trypsin

iTRAQ labeling was performed using iTRAQ Reagent kit (AB SCIEX; Foster City, CA) based on the vender's instruction manual with minor modifications. The two biological replicates of the negative control pull-down samples were labelled with iTRAQ reagent 113 and 114, respectively. Similarly, two biological replicate of digested pull-down samples by Asp-1/2 probes were labeled with reagent 117, 118 and 119, 121 respectively. Briefly, the on-beads digested peptides were dried and reconstituted with equal volume of dissolution buffer (0.5M TEAB). The peptides were then labeled with the respective iTRAQ reagents and incubated at room temperature for 2 hrs before all the samples were pooled together. The iTRAQ workflow is shown in Figure 1b in the main text.

2.4.5 Strong cation exchange (SCX) chromatography and C18 desalting of iTRAQ labeled samples

To remove interfering substances like SDS, isopropanol, dissolution buffer (TEAB), reducing agent (TCEP), alkylating agent (MMTS), calcium chloride and excess

iTRAQ reagents etc., the pooled iTRAQ-labeled peptide sample was subjected to strong cation exchange chromatography (SCX) using the iTRAQ Method Development Kit (AB SCIEX). The bound peptides were eluted with 5 % ammonium hydroxide (NH_4OH) in 30 % methanol. The eluate was desalted using a Sep-Pak C_{18} cartridge (Waters, Milford, MA), dried and then reconstituted with 100 µl of diluent (98% water, 2% acetonitrile (ACN), 0.05% formic acid (FA)).

2.4.6 Protein identification, quantification and Asp binding sites mapping

2.4.6.1 Nano LC−ESI-MS

The detailed methods for LC-MS/MS was described previously.[1] Briefly, separation of the iTRAQ labeled peptides or acid cleaved Asp-2 modified peptides was carried out on an Eksigent nanoLC Ultra and ChiPLC-nanoflex (Eksigent, Dublin, CA) in Trap Elute configuration. The samples were desalted with Sep-Pak tC 18 µ Elution Plate (Waters, Miltford, MA, USA) and reconstituted with 50 µl of diluent (98% Water, 2% ACN, 0.1% FA). A volume of 5 µl of the sample was loaded on a 200 µm x 0.5mm trap column and eluted on an analytical 75 µm×150mm column. Both trap and analytical columns were made of ChromXP C18-CL, 3 µm (Eksigent, Germany). Peptides were separated by a gradient formed by 2% ACN, 0.1% FA (mobile phase A) and 98% ACN, 0.1% FA (mobile phase B): 5−12% of mobile phase B (20 min), 12−30% of mobile phase B (90 min), 30−90% of mobile phase B (2 min), 90% of mobile phase B (5 min), 90−5% of mobile phase B (3 min), and 5−5% of mobile phase B (13 min), at a flow rate of 300 nl/min.

The MS analysis was performed on a TripleTOF 5600 system (AB SCIEX, Foster City, CA, USA) in Information Dependent Mode. MS spectra were acquired across the mass range of 400–1250 m/z in high resolution mode (>30000) using 250 ms accumulation time per spectrum. A maximum of 20 precursors per cycle were chosen for fragmentation from each MS spectrum with 100 ms minimum accumulation time for each precursor and dynamic exclusion for 15 s with charge state between 2 to 4. For

iTRAQ sample, tandem mass spectra were recorded in high sensitivity mode (resolution >15000) with "adjust CE when using iTRAQ Reagent" on.

2.4.6.2 ProteinPilot™ analysis

The detailed method of ProteinPilot™ analysis was described previously.[2] Briefly, the protein identification and iTRAQ quantification were performed with ProteinPilot™ 4.5 (AB SCIEX) which uses the Paragon™ algorithm to perform database searches. The database used was the SwissProt_2013_09 (total sequence 540958). The search parameters used were as follows: Cysteine alkylation with MMTS; Trypsin Digestion; TripleTOF 5600; Biological modifications (All the protein modifications available in the ProteinPilot™ search engine are taken into consideration, which include most if not all the known protein modifications. A whole list of protein modifications is provided as Supplementary information). Redundancy was eliminated by the grouping of identified proteins using the ProGroup algorithm in the software. A decoy database search strategy was used to determine the false discovery rate (FDR) for protein identification. A corresponding randomized database was generated using the Proteomics System Performance Evaluation Pipeline feature in the ProteinPilot™ Software 4.5. In this study, a stringent cut-off threshold with total unused score >1.3 was adopted as the qualification criterion, which corresponded to a protein confidence level of >95% and a false discovery rate (FDR) of 0.33%.

2.4.6.3 Quantitative iTRAQ data analysis

iTRAQ ratio for each protein was calculated using unique peptides identified only, excluding peptides with miscleavage and peptides missing an iTRAQ reagent label. To reduce the likelihood of selecting the false-positive drug targets, we chose a stringent iTRAQ ratio equivalent to 2 as the cut-off threshold to identify specific protein targets for subsequent experiments. Moreover, the targets must be identified in both Asp-P1/P2 pull down results. Using these criteria, 1110 proteins were identified. The full list of the 1110 potential targets is shown in Supplematary Table 2.

2.4.6.4 Asp-2 binding site mapping

For the identification of drug modification sites, the mass spectra data were converted into Mascot generic format (MGF). The data was searched with Mascot 2.4.0 (Matrix Science). The database used was the SwissProt_2013_09 (total sequence 540958). The search parameters used were as follows: Trypsin Digestion; TripleTOF 5600; Cysteine alkylation of MMTS; Aspirin probe modified residue mass difference 237.24 amu was specified as variable modification at lysine, serine, arginine, histidine, threonine, tyrosine, tryptophan and cysteine residues. Peptide Mass Tolerance was 10 ppm and Fragment Mass Tolerance was 0.4 Da. A decoy database search strategy was used to determine the FDR for peptide identification. We have applied MOWSE score ≥ 30 to filter the identified peptide list, with FDR of 0.15% (Decoy:Normal = 10:6770) for the whole peptide list, and FDR of 0.086% (Decoy:Normal = 4:4645) for the peptides with Aspirin modification.

2.5 Pathway analysis of aspirin targets

The specific aspirin targets—identified using the QA-ABPP approach—were analyzed using the Ingenuity Pathway Analysis software (IPA; Ingenuity® Systems, Redwood city, CA). A spreadsheet containing the list of aspirin targets was uploaded into IPA. The software mapped each of the proteins to the repository of information in the Ingenuity Pathways Knowledge base. Molecular networks and canonical pathways regulated by these drug targets were obtained using IPA core analysis.

2.6 Metabolic labeling of newly synthesized proteins with AHA

The cells with 70~80% confluency in a 6-well plate were washed with warm PBS and cultured in L-methionine-free DMEM for 30 min to deplete the intracellular methionine reserves. Following methionine depletion, the cells were labelled with AHA in 10% FBS DMEM (methionine-free) for designated time. After incubation, the cells were then harvested and fixed in 4% formaldehyde in PBS for 15 min and permeabilized with 0.25% Triton^{TM} X-100 in PBS for 20 min at room temperature. Finally, the cells were used for the click reaction. For each reaction, Rhodamine B

alkyne (10 μM), TCEP (1 mM, 100 × fresh stock in water), TBTA ligand (100 μM, 100 × stock in DMSO), and $CuSO_4$ (1 mM, 100 × stock in water) were added into the suspended cells. The samples were incubated at room temperature for 2 hrs, and then the reaction cocktail was removed and the cells were washed once with 3% BSA in PBS. After fluorescence tagging, nascent protein synthesis was assessed by flow cytometry, and AHA signal intensity was determined in the FL3 channel. We quantified the fluorescence intensity of the cells and calculated the ratio of the fluorescence intensity of aspirin treated cells to that of the control cells.

2.7 Cathepsin B and L activity assays

MEFs and HCT116 cells were both cultured in 24-well plates. After the designated treatment, cells were further loaded with Magic Red cathepsin B or cathepsin L reagents for 15 min. Fluorescence intensities of cells per sample were measured by flow cytometry using the FACS cytometer (BD Biosciences; Franklin Lakes, NJ) or observed under a confocal microscope (Olympus Fluoview FV1000).

2.8 Estimation of intralysosomal pH using LysoTracker

The intralysosomal pH was estimated using LysoTracker, following manufacturer's instructions. The fluorescence intensity was observed under a confocal microscope (Olympus Fluoview FV1000) and representative cells were selected and photographed.

2.9 Western blotting

At the end of the designated treatments, cells were lysed in Laemmli SDS buffer (62.5 mM Tris, pH 6.8, 25% glycerol, 2% SDS, phosphatase inhibitor and proteinase inhibitor cocktails). An equal amount of protein was resolved by SDS-PAGE and transferred onto PVDF membrane. After blocking with 5% non-fat milk, the membrane was probed with designated primary and secondary antibodies, developed with the enhanced chemiluminescence method and visualized with ImageQuant LAS 500 (GE Healthcare).

2.10 Cellular imaging of aspirin-induced acetylation

MEFs cells were seeded to a Nunc™ Lab-Tek™ coverglass slide chamber (Thermo Fisher Scientific; Waltham, MA), and after 16 hrs aspirin probe (Asp-2, 1 mM) labeling, cells were washed with PBS, then fixed with 4% paraformaldehyde in PBS for 15 min at room temperature before permeabilizing with 0.25% Triton™ X-100 in PBS for 15 min. Cells were washed with PBS and blocked with 1% BSA in PBS for 30 min, then clicked with Rhodamine B alkyne. The cells were examined and recorded using a confocal microscope (Olympus Fluoview FV1000) and representative cells were selected and photographed.

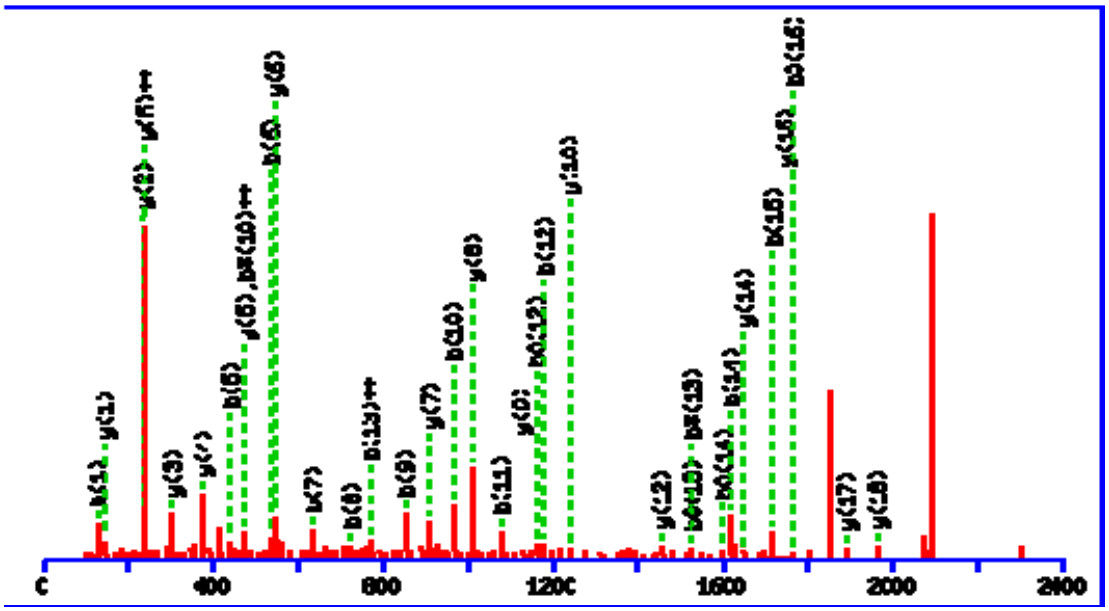
2.11 Mascot Information of KASGPPVSELITK*AVAASK

Mascot Search Results

Peptide View

MS/MS Fragmentation of **KASGPPVSELITKAVAASK**
Found in **H14_HUMAN** in **SwissProt**, Histone H1.4 OS=Homo sapiens GN=HIST1H1E PE=1 SV=2
Match to Query 15061: 2090.205072 from(697.742300,3+) rtinseconds(3287) index(14257)
Title: Locus:1.1.1.3042.15 File:"131027_JG_Spl7_Asp26_shotgun_5ul.wiff"
Data file C:\Documents and Settings\adminnus\Desktop\131027_JG_Spl7_Asp26_shotgun_5ul_MGFPeaklist.mgf
Click mouse within plot area to zoom in by factor of two about that point

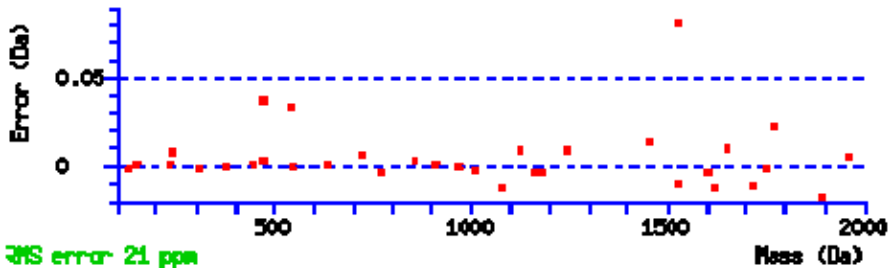
Or, Plot from 0 to 2400 Da Full range

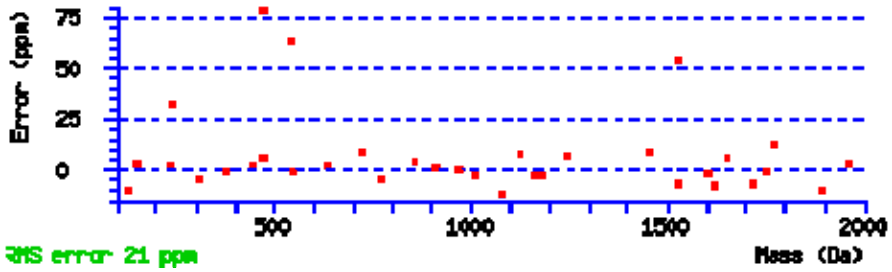


Monoisotopic mass of neutral peptide Mr(calc): 2090.2099
Variable modifications:
K13 : ASP_Lys (K)
Ions Score: 115 **Expect:** 1.5e-010
Matches : 34/210 fragment ions using 57 most intense peaks (help)

#	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							19
2	200.1394	100.5733	183.1128	92.0600			A	1963.1223	982.0648	1946.0957	973.5515	1945.1117	973.0595	18
3	287.1714	144.0893	270.1448	135.5761	269.1608	135.0840	S	1892.0851	946.5462	1875.0586	938.0329	1874.0746	937.5409	17

4	344.1928	172.6001	327.1663	164.0868	326.1823	163.5948	G	1805.0531	903.0302	1788.0266	894.5169	1787.0425	894.0249	16
5	441.2456	221.1264	424.2191	212.6132	423.2350	212.1212	P	1748.0316	874.5195	1731.0051	866.0062	1730.0211	865.5142	15
6	538.2984	269.6528	521.2718	261.1396	520.2878	260.6475	P	1650.9789	825.9931	1633.9523	817.4798	1632.9683	816.9878	14
7	637.3668	319.1870	620.3402	310.6738	619.3562	310.1817	V	1553.9261	777.4667	1536.8996	768.9534	1535.9156	768.4614	13
8	724.3988	362.7030	707.3723	354.1898	706.3882	353.6978	S	1454.8577	727.9325	1437.8312	719.4192	1436.8471	718.9272	12
9	853.4414	427.2243	836.4149	418.7111	835.4308	418.2191	E	1367.8257	684.4165	1350.7991	675.9032	1349.8151	675.4112	11
10	966.5255	483.7664	949.4989	475.2531	948.5149	474.7611	L	1238.7831	619.8952	1221.7565	611.3819	1220.7725	610.8899	10
11	1079.6095	540.3084	1062.5830	531.7951	1061.5990	531.3031	I	1125.6990	563.3531	1108.6725	554.8399	1107.6885	554.3479	9
12	1180.6572	590.8322	1163.6307	582.3190	1162.6466	581.8270	T	1012.6150	506.8111	995.5884	498.2978	994.6044	497.8058	8
13	1545.8999	773.4536	1528.8734	764.9403	1527.8893	764.4483	K	911.5673	456.2873	894.5407	447.7740	893.5567	447.2820	7
14	1616.9370	808.9721	1599.9105	800.4589	1598.9265	799.9669	A	546.3246	273.6659	529.2980	265.1527	528.3140	264.6606	6
15	1716.0054	858.5064	1698.9789	849.9931	1697.9949	849.5011	V	475.2875	238.1474	458.2609	229.6341	457.2769	229.1421	5
16	1787.0425	894.0249	1770.0160	885.5116	1769.0320	885.0196	A	376.2191	188.6132	359.1925	180.0999	358.2085	179.6079	4
17	1858.0797	929.5435	1841.0531	921.0302	1840.0691	920.5382	A	305.1819	153.0946	288.1554	144.5813	287.1714	144.0893	3
18	1945.1117	973.0595	1928.0851	964.5462	1927.1011	964.0542	S	234.1448	117.5761	217.1183	109.0628	216.1343	108.5708	2
19							K	147.1128	74.0600	130.0863	65.5468			1





NCBI **BLAST** search of KASGPPVSELITKAVAASK
(Parameters: blastp, nr protein database, expect=20000, no filter, PAM30)
Other BLAST web gateways

All matches to this query

Score	Mr(calc)	Delta	Sequence	Site Analysis
115.1	2090.2099	-0.0048	KASGPPVSELITKAVAASK	ASP_Lys K13 99.72%
89.3	2090.2099	-0.0048	KASGPPVSELITKAVAASK	ASP_Lys K19 0.26%
79.2	2090.2099	-0.0048	KASGPPVSELITKAVAASK	ASP_Lys S18 0.03%
61.0	2090.2099	-0.0048	KASGPPVSELITKAVAASK	ASP_Lys S8 0.00%
45.9	2090.2099	-0.0048	KASGPPVSELITKAVAASK	ASP_Lys S3 0.00%
25.5	2090.2099	-0.0048	KASGPPVSELITKAVAASK	ASP_Lys K1 0.00%
3.8	2090.1848	0.0203	KNVAGEIVLITGAGSGLGR	
3.7	2090.2112	-0.0062	ALSPHNILLDRKGHIK	

Mascot: http://www.matrixscience.com/

2.12 Mascot Information of KASGPPVSELITKAVAAS*K

Mascot Search Results

Peptide View

MS/MS Fragmentation of **KASGPPVSELITKAVAASK**

Found in **H14_HUMAN** in **SwissProt**, Histone H1.4 OS=Homo sapiens GN=HIST1H1E PE=1 SV=2

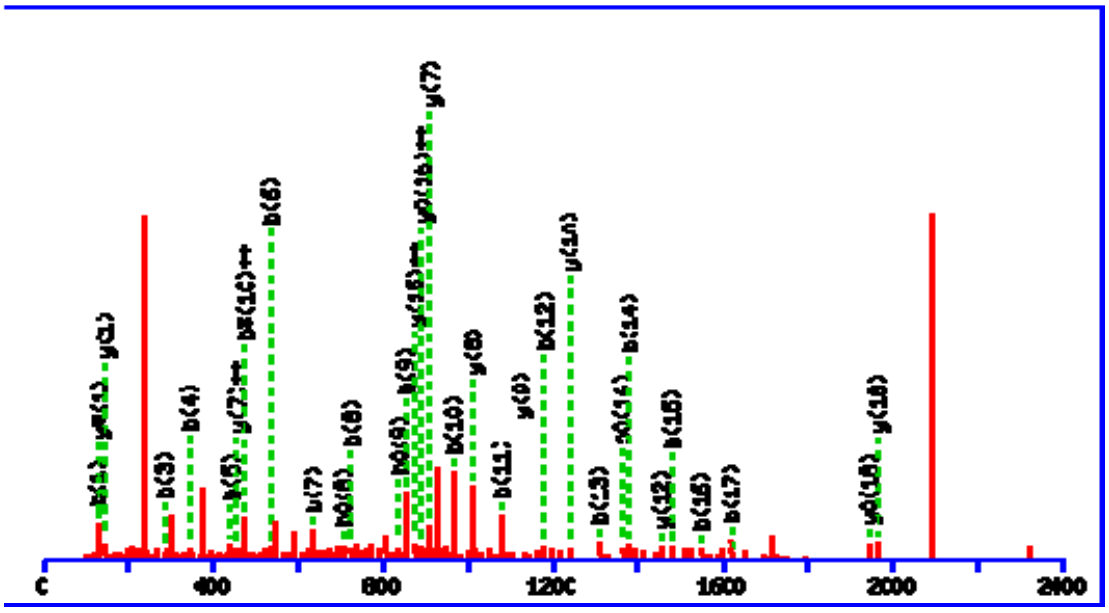
Match to Query 15062: 2090.205072 from(697.742300,3+) rtinseconds(3273) index(14157)

Title: Locus:1.1.1.3037.15 File:"131027_JG_Spl7_Asp26_shotgun_5ul.wiff"

Data file C:\Documents and Settings\adminnus\Desktop\131027_JG_Spl7_Asp26_shotgun_5ul_MGFPeaklist.mgf

Click mouse within plot area to zoom in by factor of two about that point

Or, Plot from 0 to 2400 Da Full range



Monoisotopic mass of neutral peptide Mr(calc): 2090.2099

Variable modifications:

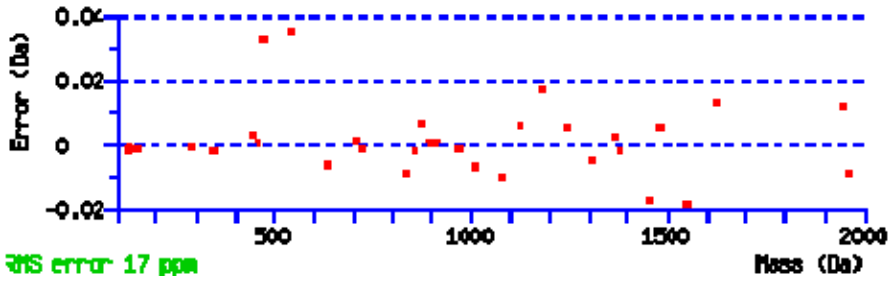
S18 : ASP_Ser (S)

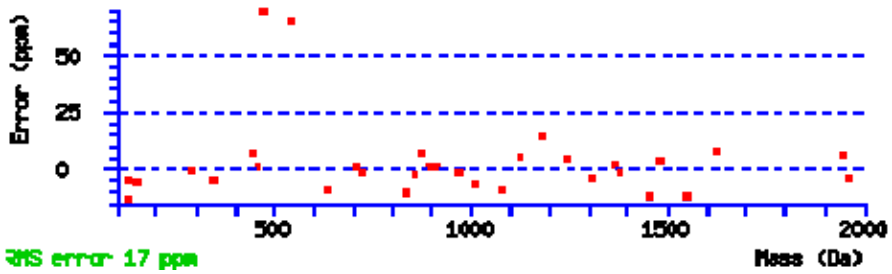
Ions Score: 101 **Expect:** 3.5e-009

Matches : 33/210 fragment ions using 87 most intense peaks (help)

#	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							19

2	200.1394	100.5733	183.1128	92.0600			A	**1963.1223**	982.0648	1946.0957	973.5515	1945.1117	973.0595	18
3	***287.1714***	144.0893	270.1448	135.5761	269.1608	135.0840	S	1892.0851	946.5462	1875.0586	938.0329	1874.0746	937.5409	17
4	***344.1928***	172.6001	327.1663	164.0868	326.1823	163.5948	G	1805.0531	903.0302	1788.0266	894.5169	1787.0425	894.0249	16
5	***441.2456***	221.1264	424.2191	212.6132	423.2350	212.1212	P	1748.0316	874.5195	1731.0051	866.0062	1730.0211	865.5142	15
6	***538.2984***	269.6528	521.2718	261.1396	520.2878	260.6475	P	1650.9789	825.9931	1633.9523	817.4798	1632.9683	816.9878	14
7	***637.3668***	319.1870	620.3402	310.6738	619.3562	310.1817	V	1553.9261	777.4667	1536.8996	768.9534	1535.9156	768.4614	13
8	***724.3988***	362.7030	707.3723	354.1898	706.3882	353.6978	S	**1454.8577**	727.9325	1437.8312	719.4192	1436.8471	718.9272	12
9	***853.4414***	427.2243	836.4149	418.7111	835.4308	418.2191	E	1367.8257	684.4165	1350.7991	675.9032	1349.8151	675.4112	11
10	***966.5255***	483.7664	949.4989	475.2531	948.5149	474.7611	L	**1238.7831**	619.8952	1221.7565	611.3819	1220.7725	610.8899	10
11	***1079.6095***	540.3084	1062.5830	531.7951	1061.5990	531.3031	I	**1125.6990**	563.3531	1108.6725	554.8399	1107.6885	554.3479	9
12	***1180.6572***	590.8322	1163.6307	582.3190	1162.6466	581.8270	T	**1012.6150**	506.8111	995.5884	498.2978	994.6044	497.8058	8
13	***1308.7522***	654.8797	1291.7256	646.3665	1290.7416	645.8744	K	**911.5673**	456.2873	894.5407	447.7740	893.5567	447.2820	7
14	***1379.7893***	690.3983	1362.7627	681.8850	1361.7787	681.3930	A	783.4723	392.2398	766.4458	383.7265	765.4618	383.2345	6
15	***1478.8577***	739.9325	1461.8312	731.4192	1460.8471	730.9272	V	712.4352	356.7212	695.4087	348.2080	694.4246	347.7160	5
16	***1549.8948***	775.4510	1532.8683	766.9378	1531.8843	766.4458	A	613.3668	307.1870	596.3402	298.6738	595.3562	298.1817	4
17	***1620.9319***	810.9696	1603.9054	802.4563	1602.9214	801.9643	A	542.3297	271.6685	525.3031	263.1552	524.3191	262.6632	3
18	***1945.1117***	973.0595	1928.0851	964.5462	1927.1011	964.0542	S	471.2926	236.1499	454.2660	227.6366	453.2820	227.1446	2
19							K	**147.1128**	74.0600	130.0863	65.5468			1





NCBI **BLAST** search of KASGPPVSELITKAVAASK

(Parameters: blastp, nr protein database, expect=20000, no filter, PAM30)

Other BLAST web gateways

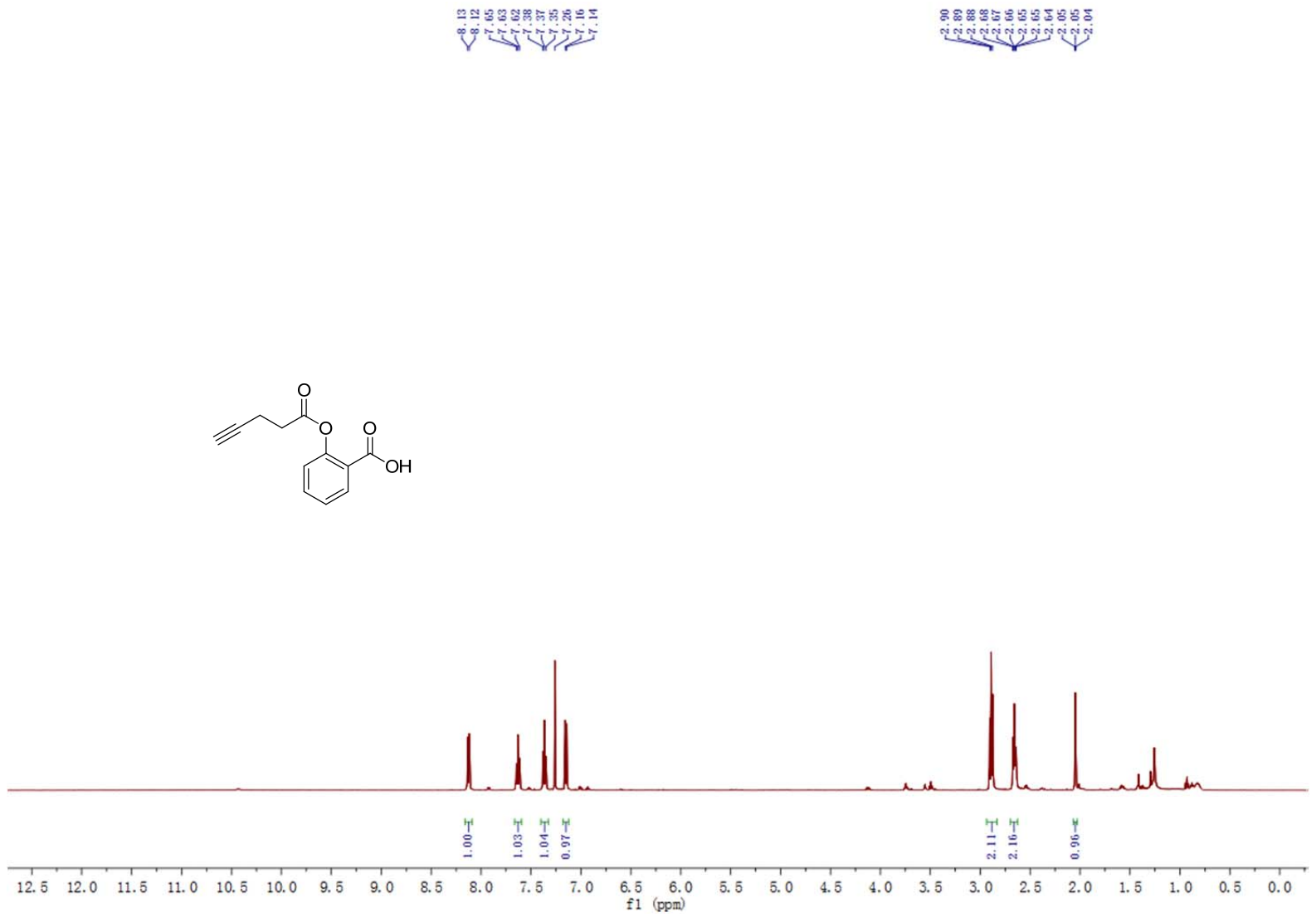
All matches to this query

Score	Mr(calc)	Delta	Sequence	Site Analysis
101.4	2090.2099	-0.0048	KASGPPVSELITKAVAASK	ASP_Ser S18 85.43%
92.8	2090.2099	-0.0048	KASGPPVSELITKAVAASK	ASP_Ser K19 11.77%
86.6	2090.2099	-0.0048	KASGPPVSELITKAVAASK	ASP_Ser K13 2.80%
50.4	2090.2099	-0.0048	KASGPPVSELITKAVAASK	ASP_Ser K1 0.00%
40.1	2090.2099	-0.0048	KASGPPVSELITKAVAASK	ASP_Ser S3 0.00%
35.8	2090.2099	-0.0048	KASGPPVSELITKAVAASK	ASP_Ser S8 0.00%
2.9	2090.1848	0.0203	KNVAGEIVLITGAGSGLGR	
1.9	2090.2112	-0.0062	ALSPHNILLDRKGHIK	
0.2	2090.2211	-0.0161	TISALKTTQRQLSK	

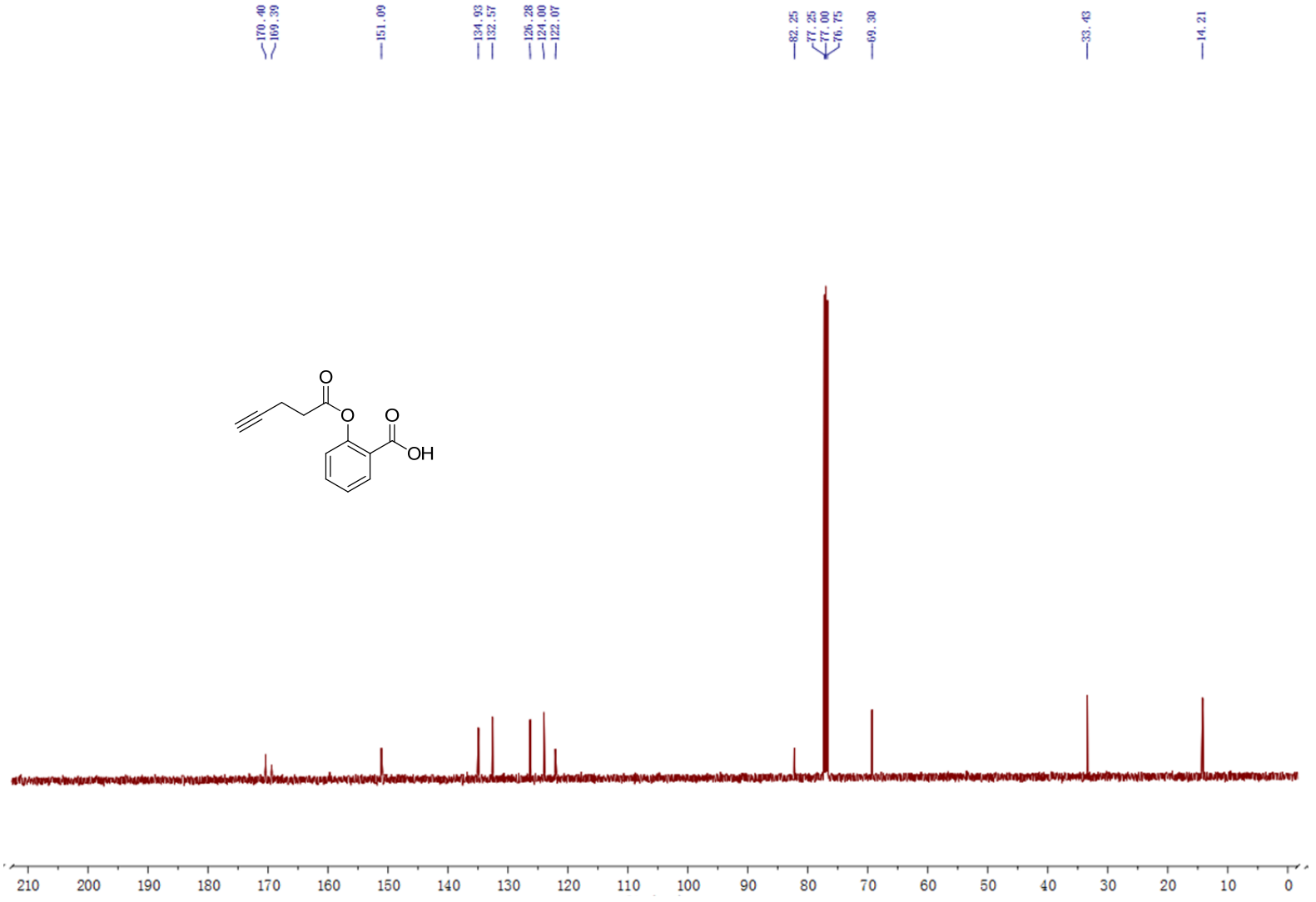
Reference:

1. Tang, L. A. L. *et al.* High-performance graphene-titania platform for detection of phosphopeptides in cancer cells. *Anal. Chem.* **84,** 6693–6700 (2012).

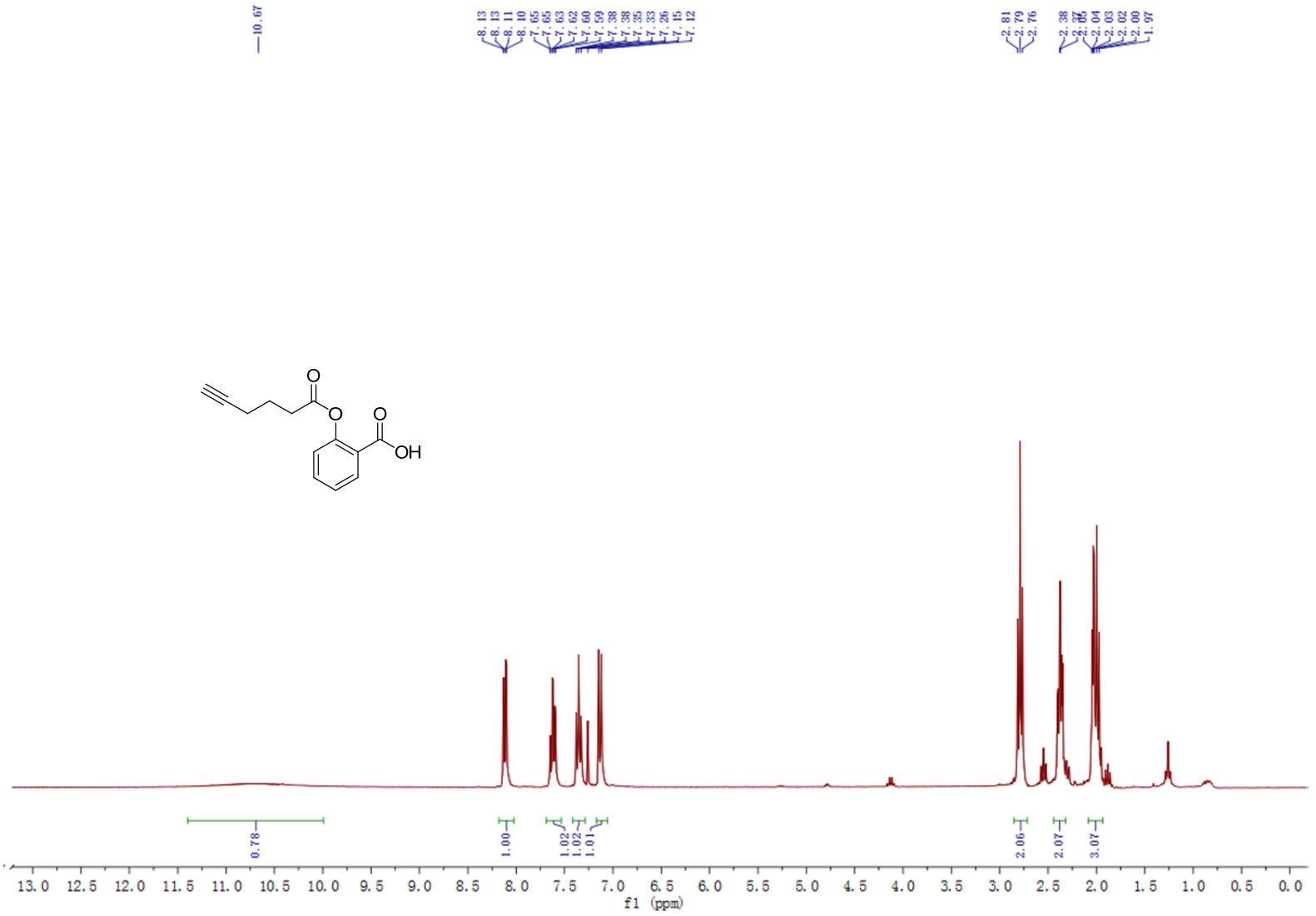
2. Ghosh, D. *et al.* Identification of Key Players for Colorectal Cancer Metastasis by iTRAQ Quantitative Proteomics Profiling of Isogenic SW480 and SW620 Cell Lines. *J. Proteome Res.* **10,** 4373–4387 (2011).



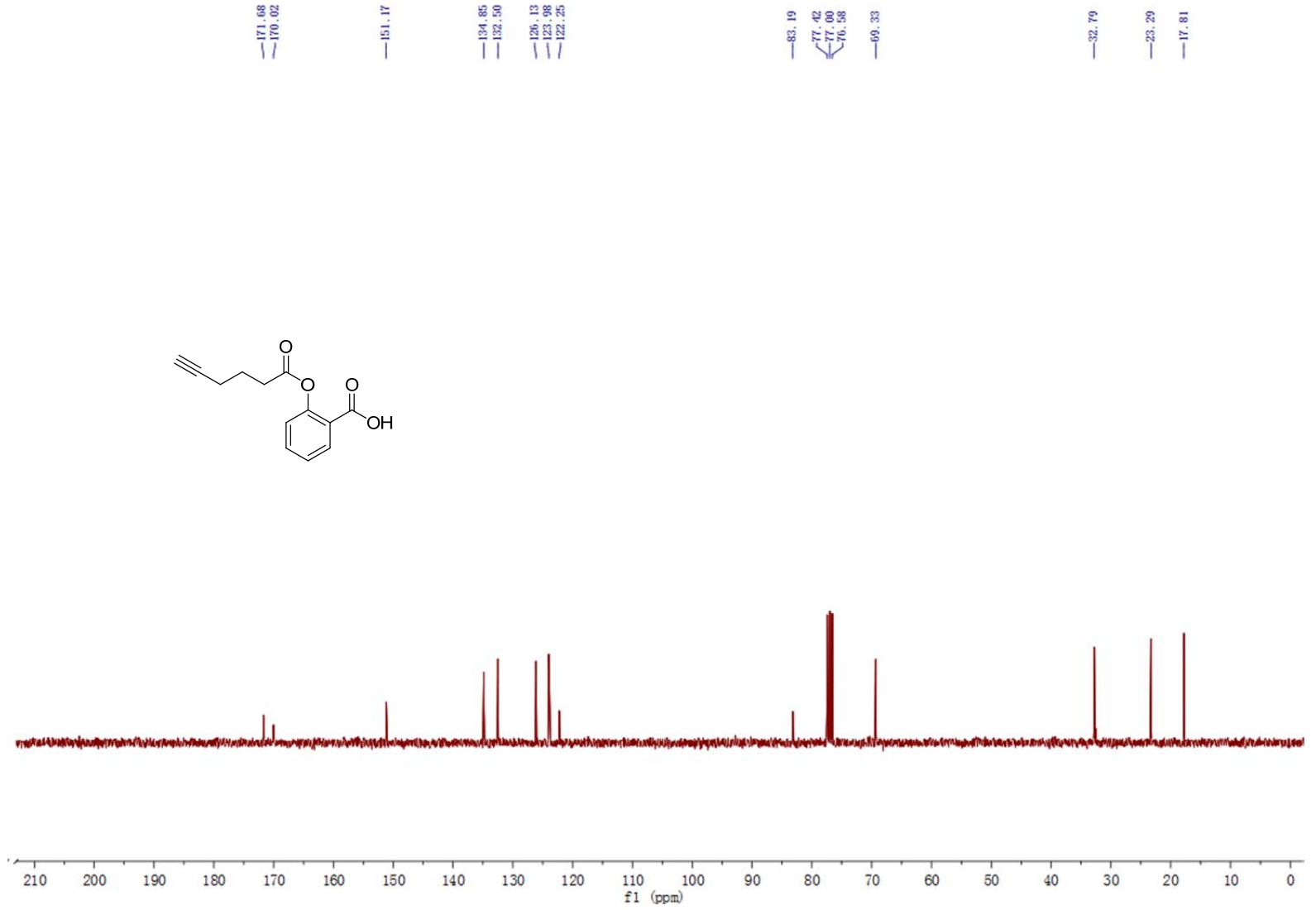
Supplementary Figure 7. ^{1}H-NMR of Asp-1



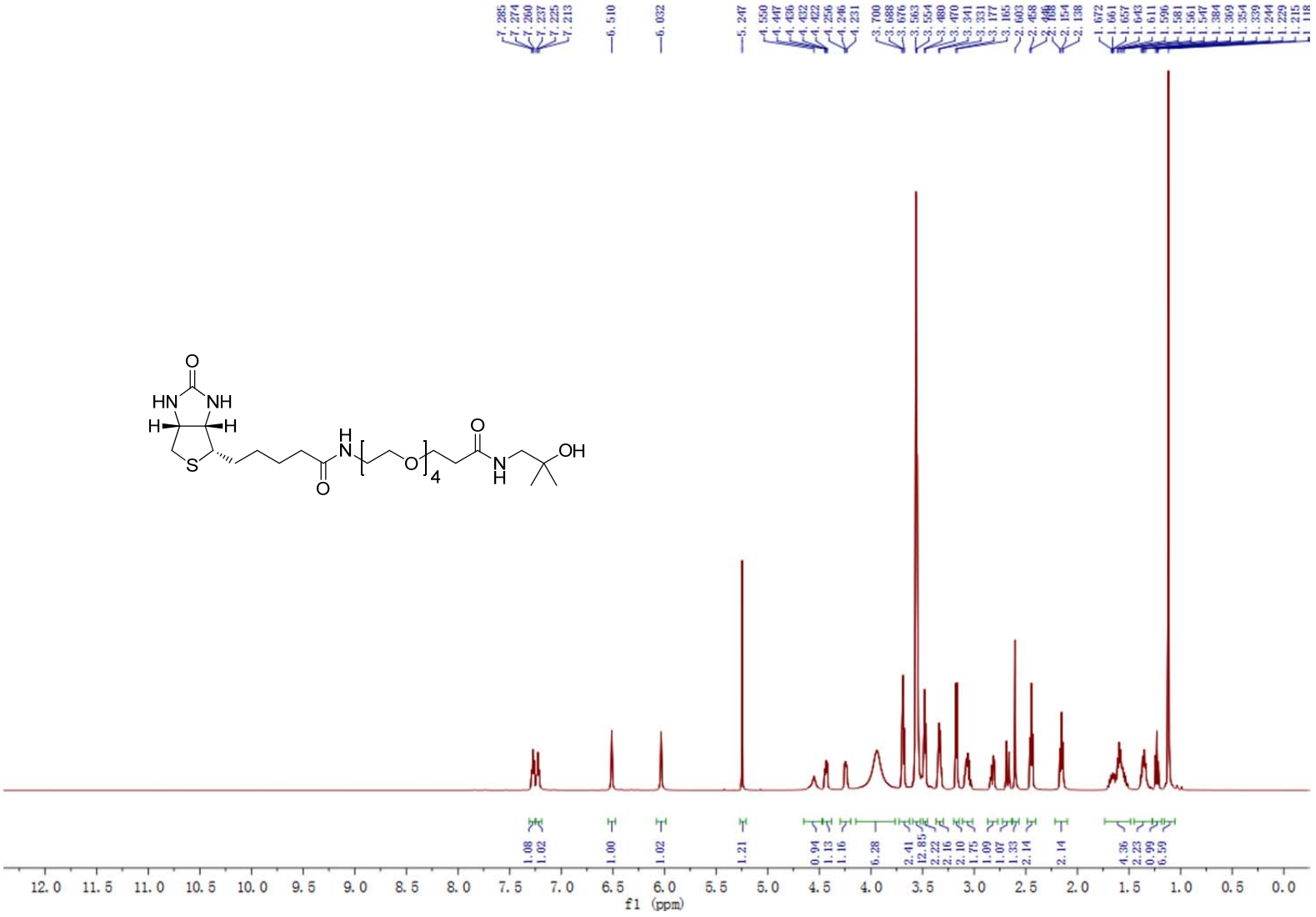
Supplementary Figure 8. ^{13}C-NMR of Asp-1



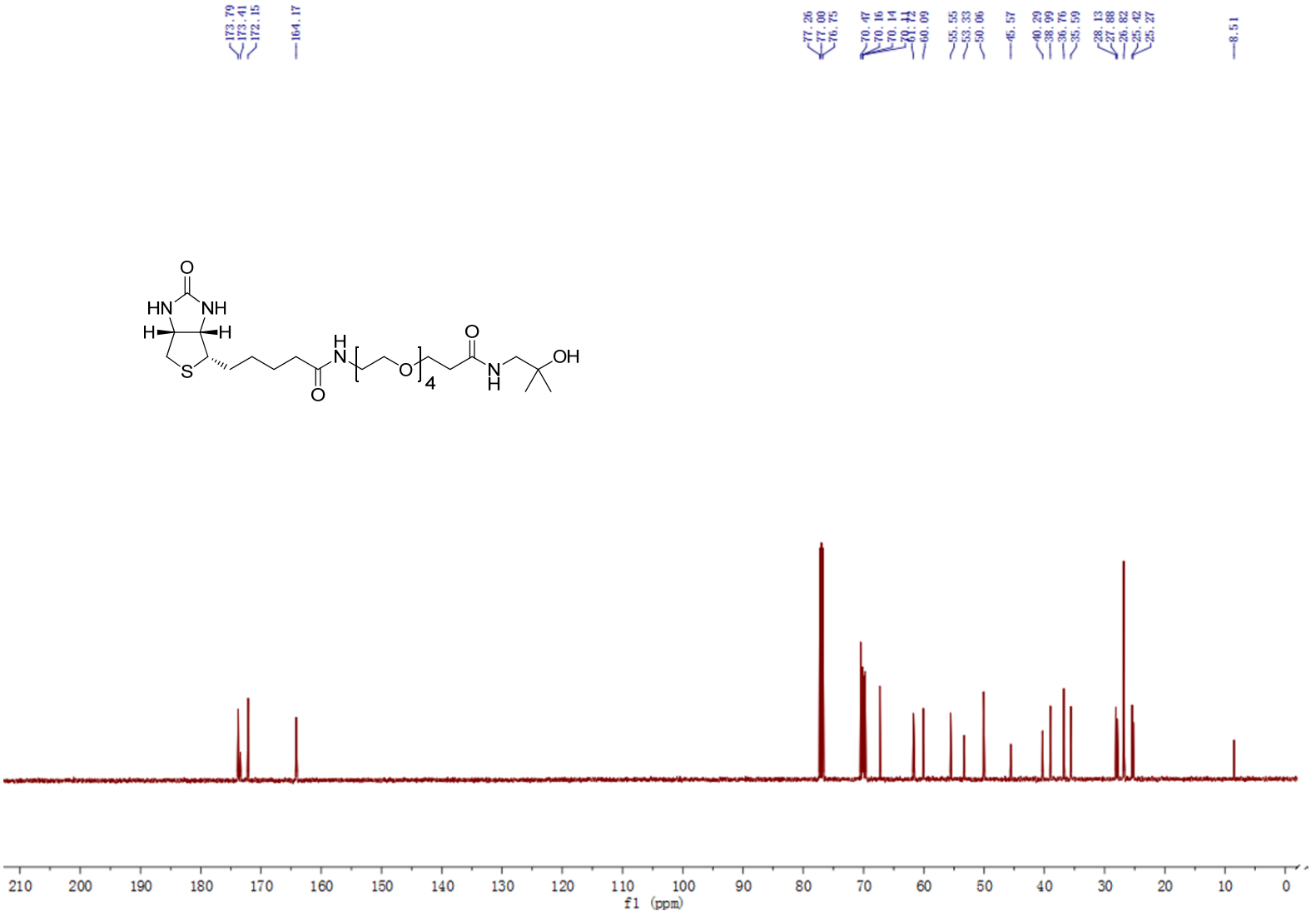
Supplementary Figure 9. ^{1}H-NMR of Asp-2



Supplementary Figure 10. ^{13}C-NMR of Asp-2



Supplementary Figure 11. ^{1}H-NMR of Biotin-PEG-OH



Supplementary Figure 12. ^{13}C-NMR of Biotin-PEG-OH