Electronic supplementary information

A chemical probe for proteome analysis and visualization of intracellular localization of lysine-succinylated proteins

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1. Synthesis

General considerations

All chemical reagents and solvents for synthesis were purchased from commercial suppliers (Fujifilm Wako Pure Chemical, Tokyo Kasei Industry and Sigma-Aldrich Chemical) and were used without further purification. All solvents for HPLC were purchased from Fujifilm Wako Pure Chemical and Kanto Chemical. All moisture-sensitive reactions were carried out under an atmosphere of argon.

^{1}H NMR and ^{13}C NMR spectra were recorded on an AVANCE III 400 Nanobay (Bruker, 400 MHz for ^{1}H, 101 MHz for ^{13}C) at room temperature. All chemical shifts (*d*) reported in ppm are relative to internal standard tetramethylsilane (*d* = 0.0 ppm), or relative to the signals of residual solvent $CDCl_3$ (7.26 ppm for ^{1}H, 77.16 ppm for ^{13}C), and coupling constants are given in Hz. High-resolution mass spectra (HRMS) were measured on a MicrOTOF (Bruker) with ESI-TOF (electron spray ionization–time-of-flight) or a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) equipped with a nano ESI source. Flash chromatography separation was undertaken using a YFLC-Al-560 chromatograph (Yamazen Co. Ltd.). HPLC purification was performed on a Jasco PU-1587 system (JASCO International Co.) equipped with a reverse-phase column (GL Sciences, Inertsil ODS-3, 10 mm x 250 mm). Eluent A (H_2O containing 1% acetonitrile and 0.1% trifluoroacetic acid) and eluent B (acetonitrile containing 1% H_2O) were used for HPLC purification. The synthesis of MalAM-yne was performed according to the previous literature.[1]

Methyl 2-acetylpent-4-ynoate (2) [2]

Methyl 2-acetylpent-4-ynoate was synthesized according to the previous literature.[2] In brief, methyl acetoacetate (**1**, 2.0 mL, 18.6 mmol, 1 eq.) was dissolved in anhydrous MeOH (20 mL) and the solution was stirred on ice. Sodium methoxide (2.52 g, 46.5 mmol, 2.5 eq.) in anhydrous MeOH (5 ml) and propargyl bromide (1.7 mL, 22.3 mmol, 1.2 eq.) were subsequently added to the solution, and the

reaction mixture was refluxed overnight, then filtered through a Celite pad, and water was added to the filtrate. The aqueous solution was extracted with ethyl acetate (x2), and the combined organic phase was washed with water and brine, dried over sodium sulfate, filtered, and evaporated. The resulted residue was purified by silica gel column chromatography (eluent: *n*-hexane/ethyl acetate = 98/2 to 60/40, v/v) to afford methyl 2-acetylpent-4-ynoate (**2**, 1.54 g, 54%) as a colorless liquid. ^{1}H NMR ($CDCl_3$): δ 3.77 (s, 3H), 3.72 (t, J = 7.5 Hz, 1H), 2.73 (dt, J = 7.5 Hz, 2.6 Hz, 2H), 2.31 (s, 3H), 2.01 (t, J = 2.6 Hz, 1H). ^{13}C NMR ($CDCl_3$): δ 201.00, 168.57, 80.33, 70.38, 58.03, 52.77, 29.65, 17.46. HRMS-ESI (*m/z*): $[M+H]^+$ calcd for $C_8H_{11}O_3$: 155.0703; found: 155.0706 (0.3 mmu, 1.9 ppm).

Dimethyl 2-prop-2-ynylbutanedioate (3)

Methyl 2-acetylpent-4-ynoate (**2**, 1.00 g, 6.49 mmol, 1 eq.) was dissolved in anhydrous THF (10 ml) and the solution was stirred on ice. Sodium hydride (60% assay, 876 mg, 9.73 mmol, 1.5 eq.) was added portionwise to the solution and the stirring was continued for further 30 minutes on ice. Methyl bromoacetate (720 µL, 7.81 mmol, 1.2 eq.) was added to the reaction mixture, then the solution was refluxed for 2 hours. After cooling to room temperature, methanol was added dropwise on ice, followed by the addition of water. The aqueous solution was extracted with ethyl acetate (x2), and the combined organic phase was washed with water and brine, dried over sodium sulfate, filtered, and evaporated. The resulted residue was purified by silica gel column chromatography (eluent: *n*-hexane/ethyl acetate = 98/2 to 60/40, v/v) to afford dimethyl 2-prop-2-ynylbutanedioate (**3**, 406 mg, 34%) as a colorless liquid. ^{1}H NMR ($CDCl_3$): δ 3.73 (s, 3H), 3.70 (s, 3H), 3.05 (tt, J = 7.8 Hz, 5.6 Hz, 1H), 2.86 (dd, J = 16.9 Hz, 8.0 Hz, 1H), 2.71 (dd, J =16.9 Hz, 5.7 Hz, 1H), 2.62 (ddd, J = 16.9 Hz, 5.0 Hz, 2.7 Hz, 1H), 2.55 (ddd, J = 16.9 Hz, 7.5 Hz, 2.7 Hz, 1H), 2.03 (t, J = 2.7 Hz, 1H).; ^{13}C NMR ($CDCl_3$): δ 173.38, 172.19, 80.37, 71.04, 52.39, 52.02, 40.06, 34.46, 20.88. HRMS-ESI (*m/z*): $[M+H]^+$ calcd for $C_9H_{13}O_4$: 185.0808; found: 185.0809 (0.1 mmu, 0.5 ppm).

Bis(acetoxymethyl) 2-(prop-2-yn-1-yl)succinate (SucAM-yne)

Dimethyl 2-prop-2-ynylbutanedioate (**3**, 446 mg, 2.42 mmol, 1 eq.) was dissolved in acetonitrile (2 mL) and 5 N NaOH (1.6 mL, 8.0 mmol, 3.3 eq.) on ice, and the reaction mixture was stirred at room temperature for 2 hours. Concentrated HCl aq. (*ca.* 1 mL) was carefully added dropwise to the reaction mixture on ice to acidify the solution (pH < 1). Then the aqueous solution was extracted with chloroform/isopropanol (4/1, v/v) 5 times, and the combined organic phase was dried over magnesium sulfate, filtered, and evaporated. The resulting residue (408 mg) was used for the next reaction without further purification. The residue was dissolved in anhydrous acetonitrile (15 mL), followed by the addition of *N,N*-diisopropylethylamine (1.0 mL, 5.75 mmol, 2.4 eq.). Bromomethyl acetate (500 μL, 5.30 mmol, 2.2 eq.) was added dropwise to the solution on ice, and the stirring was continued overnight at room temperature. The reaction mixture was diluted with ethyl acetate, and the organic phase was washed with saturated NH_4Cl aqueous solution and brine, dried over sodium sulfate, filtered, and evaporated. The resulted residue was purified by silica gel column chromatography (eluent: *n*-hexane/ethyl acetate = 70/30 to 50/50, v/v) to afford bis(acetoxymethyl) 2-(prop-2-yn-1-yl)succinate (**SucAM-yne**, 270 mg, 37%) as a colorless liquid. ^{1}H NMR ($CDCl_3$): δ 5.79 – 5.72 (m, 4H), 3.12 – 3.06 (m, 1H), 2.93 (dd, J = 17.4 Hz, 8.1 Hz, 1H), 2.77 (dd, J = 17.4 Hz, 5.4 Hz, 1H), 2.68 – 2.55 (m, 2H), 2.13 (s, 3H), 2.12 (s, 3H), 2.06 (d, J = 2.6 Hz, 1H).; ^{13}C NMR ($CDCl_3$): δ 171.35, 170.28, 169.68, 169.64, 79.57, 79.41, 79.31, 71.53, 39.60, 33.98, 20.78, 20.74, 20.50.; HRMS-ESI (*m/z*): $[M+H]^+$ calcd for $C_{13}H_{17}O_8$: 301.09179; found: 301.0919 (0.1 mmu, 0.3 ppm).

2. Biological experiments

Immunoblot analysis

Immunoblot analysis was performed according to the previous report.[3] Briefly, proteins on a gel prepared as mentioned above were transferred to an Immobilon-P PVDF membrane (0.45 μm pore size, Millipore) at 150 V for 1 hour, and after washing with distilled water several times, proteins on a membrane were stained with Ponceau S (Thermo Fisher Scientific). The membrane was then soaked in 5% BSA or 5% non-fat dry milk in TBS-T (Tris-buffered saline and 0.05% Tween-20, pH 7.6) for 30 minutes at room temperature for the purpose of destaining and blocking, followed by the addition of primary antibodies and incubated at 4°C overnight. After washing with TBS-T (5 minutes x3), the membrane was incubated with HRP-conjugated secondary antibodies in 5% BSA or 5% non-fat dry milk in TBS-T for 2 hours at room temperature. Immunoreactive bands were visualized using Lumitera (Cosmo Bio) according to the manufacturer's instructions, and detected using a luminoimage analyzer (Fusion Solo, Vilber Lourmat). The information of antibodies used was summarized in Table S5.

Cell viability test

HEK293T cells (ca. 1 x 10^5 cells/well) were seeded on a 96-well plate and cultured overnight at 37 °C in a 5%/95% CO_2/air incubator. Then the cells were incubated for 1 hours in DMEM containing 0 – 1000 μM SucAM-yne (0.5% DMSO) at 37 °C in a 5%/95% CO_2/air incubator. After washing with DMEM, and a cell viability test was performed by means of CCK assay (Cell Counting Kit-8, Dojin Chemicals) according to the manufacture's procedure.

RNA interference (RNAi) experiments

HEK293T cells seeded on a 24-well plate (*ca.* 90% confluency) were used for RNAi experiments. Small interfering RNA (siRNA) of SIRT5 was performed using Lipofectamine™ RNAiMAX

(Invitrogen) according to the manufacturer's instructions. In brief, 5 pmol RNAi duplex diluted with 25 μL Opti-MEM I medium (Invitrogen) was mixed with 1 μL Lipofectamine™ RNAiMAX in 25 μL Opti-MEM I medium and incubated for 2–3 minutes at room temperature. The mixture (50 μL total) was added to the each well of the HEK293T-seeded 24-well plate for transfection, and cells were incubated for 3 days at 37 °C under a 5% CO_2 atmosphere and subjected to assays. Silencer® select pre-designed siRNA for SIRT5 (siRNA ID: s23761 and s23762, Thermo Fisher Scientific) were used for RNAi experiments, and an equal mixture of s23761 and s23762 was used. The details of these siRNA were follows. ID s23761; Sense: 5'-GCA GAU UUU CGA AAG UUU UTT-3', Antisense: 5'-AAA ACU UUC GAA AAU CUG CCA-3'. ID s23762; Sense: 5'-GAG UCC AAU UUG UCC AGC UTT-3', Antisense: 5'-AGC UGG ACA AAU UGG ACU CTT-3'. BLOCK-iT™ Alexa Fluor® red fluorescent oligo (Thermo Fisher Scientific) was used as a control oligo in mock experiments.

3. LC-MS/MS analysis

Nano liquid chromatography-tandem mass spectrometry (nanoLC-MS/MS)

For proteomic analysis, nanoLC-MS/MS analyses were performed on an Ultimate 3000 RSLCnano system (Thermo Fisher Scientific) coupled to a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) equipped with a nano ESI source. The nanoLC system was equipped with a trap column (C18 PepMap 100, 0.3 × 5 mm, 5 μm, Thermo Fisher Scientific) and an analytical column (NTCC-360/75-3-125, Nikkyo Technos, Tokyo, Japan). Peptide separation was performed using 60 min gradient of water containing 0.1% formic acid (mobile phase A) and acetonitrile containing 0.1% formic acid (mobile phase B) at a flow rate of 300 nL/min. The elution was set as follows: 0–3 min, 2% B; 3–63 min, 2%–40% B; 63–65 min, 40%–95% B; 65–75 min, 95% B; 75–77 min, 95%–2% B; 77–90 min, 2% B. The mass spectrometer was operated in data-dependent acquisition mode. MS parameters were set as follows: spray voltage, 2.0 kV; capillary temperature,

275 °C; S-lens RF level, 50; scan type, full MS; scan range, *m/z* 350–1500; resolution, 70,000; polarity, positive; automatic gain control target, 3×10^6; maximum injection time, 100 msec. MS/MS parameters were set as follows: resolution, 17,500; automatic gain control target, 1×10^5; maximum injection time, 60 msec; normalized collision energy, 27; dynamic exclusion, 15 sec; loop count, 10; isolation window, 1.6 *m*/*z*; charge exclusion, unassigned, 1, 8, >8.

Protein identification

Protein identification was performed using PEAKS XPro (PEAKS Studio 10.6 build 20201015, Bioinformatics Solutions Inc.). The analytical parameters were set as follows: search engine, sequest HT; protein database, Swissprot (Homo Sapiens); enzyme name, trypsin; parent mass error tolerance, 10.0 ppm; fragment mass error tolerance, 0.02 Da; precursor mass search type, monoisotopic; enzyme, trypsin; max missed cleavages, 4; fixed modifications, carbamidomethylation (cysteine, +57.02 Da); variable modifications, oxidation (methionine, +15.99 Da), 2-(1-(3-aminopropyl)-1*H*-1,2,3-triazol-4-yl)methyl modification (lysine, +238.11 Da); max variable PTM per peptide, 3; false discovery rate (FDR) ≤ 1%; unique peptides ≥ 1. Proteins belonging to contaminants in keratin and fetal bovine serum albumin were excluded from the identification results. In the case that multiple isoforms were identified, they were counted as one protein and the total number of identified proteins was determined based on the Venn diagram in Fig. 3b. For identification of lysine-modified peptide sequences, the following analytical parameters were changed: unique peptides ≥ 0; PTM Ascore ≥ 50. The obtained MS/MS spectra were centroided and deconvoluted by the “preprocess” command in PEAKS XPro (Figs. 2d and S10).

Bioinformatic analyses

Bioinformatic analyses (Gene ontology term enrichment (GO term), Kyoto Encyclopedia of Genes

and Genomes (KEGG) pathway analysis) were performed by DAVID Knowledgebase (v2022q2), NIAID/NIH (https://david.ncifcrf.gov/tools.jsp). By uploading protein IDs (uniport-accession) of the neutravidin-enriched proteins identified only in HEK293T cells in the presence of the SucAM-yne, relevant GO terms and KEGG pathways composed of proteins enriched in the given list followed by statistical evaluation were extracted, and classified according to KEGG Pathway Database (https://www.genome.jp/kegg/pathway.html). Another pathway analysis was performed using KeyMolnet ver. 6.2 (KM Data, Tokyo, Japan). The list "Gene name" of the identified proteins mentioned above was uploaded into KeyMolnet, and "Interaction search" algorism was used to generate a network of molecular interactions within a single path from the starting points, including direct activation/inactivation, transcriptional activation/repression, and the complex formation. Canonical pathways associated with identified proteins were extracted from the KeyMolnet knowledge base.

4. References

1 X. Bao, Q. Zhao, T. Yang, Y. M. E. Fung, X. D. Li, *Angew. Chem. Int. Ed.* **2013**, *52*, 4883-4886.

2 K. Heinz Dötz, M. Popall, *Tetrahedron* **1985**, *41*, 5797-5802.

3 Y. Miura, A. Hayakawa, S. Kikuchi, H. Tsumoto, K. Umezawa, Y. Chiba, Y. Soejima, M. Sawabe, K. Fukui, Y. Akimoto, T. Endo, *Arch. Biochem. Biophys.* **2019**, *678*, 108167.

Supplementary Tables

Table S1 List of the proteins identified only in SucAM-yne-treated cell lysate by protein-based enrichment method (compiled in Table S1-3.xlsx)

Table S2 List of the succinylated peptides and derived proteins identified in SucAM-yne-treated cell lysate by peptide-based enrichment method (compiled in Table S1-3.xlsx)

Table S3 Entire data of KEGG pathway analysis of enriched proteins. (compiled in Table S1-3.xlsx)

Table S4 Pathway analysis of enriched and identified proteins by KeyMolnet 6.2.

rank	name	score	score(p)
1	Spliceosome assembly	80.385	6.33E-25
2	Alternative splicing regulation	45.864	1.56E-14
3	Huntingtin signaling pathway	36.375	1.12E-11
4	ARF family signaling pathway	35.353	2.28E-11
5	Rab family signaling pathway	34.922	3.07E-11
6	SNARE signaling pathway	33.566	7.87E-11
7	Arginine methylation	33.344	9.17E-11
8	14-3-3 signaling pathway	32.633	1.50E-10
9	CIITA signaling pathway	30.882	5.05E-10
10	ATM/ATR signaling pathway	29.773	1.09E-09
11	ESCRT signaling pathway	29.569	1.26E-09
12	CaMK signaling pathway	26.552	1.02E-08
13	PAK signaling pathway	26.474	1.07E-08
14	Rho family signaling pathway	24.86	3.28E-08
15	estrogen signaling pathway	24.567	4.02E-08
16	Succinate signaling pathway	23.811	6.80E-08
17	p160 SRC signaling pathway	23.799	6.85E-08
18	Transcriptional regulation by MRF	23.557	8.10E-08
19	alpha-Catenin signaling pathway	22.514	1.67E-07
20	BET family signaling pathway	22.386	1.83E-07

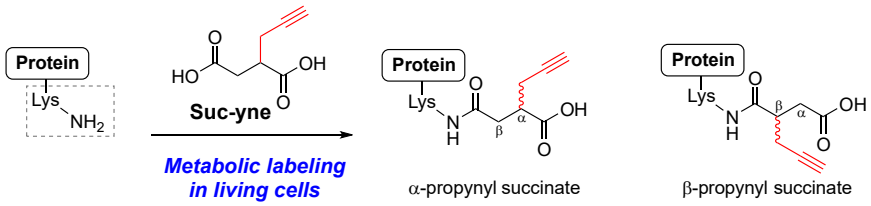
Pathological events that scored >20 were considered significantly associated with protein succinylation. Top 20 scoring events were listed. Score(p) indicates the P-value of the pathway.

Table S5 Antibodies used for immunoblotting analysis.

Antibody	Host	Clone type	Industry	Catalog ID	Lot	Dilution factor	Blocking
1st Abs							
Anti-SDHA	Rabbit	monoclonal (EPR9043(B))	Abcam	ab137040	GR269900-1	1:1,000	5% non-fat dry milk
Anti-FH	Rabbit	monoclonal (EPR11648)	Abcam	ab171948	YK020512CS	1:1,000	5% non-fat dry milk
Anti-CTSB	Mouse	monoclonal (H-5)	Santa Cruz	sc365558	G1521	1:1,000	5% non-fat dry milk
Anti-SIRT3	Rabbit	monoclonal (D22A3)	Cell Signaling	5490S	2	1:1,000	5% non-fat dry milk
Anti-biotin	Goat	polyclonal	Sigma	B3640	not provided	1:50,000	2% BSA
2nd Abs (HRP conjugate)							
Anti-rabbit IgG (H+L chain)	Goat	polyclonal	Cell signaling	7074S	25	1:5,000	5% non-fat dry milk
Anti-mouse IgG (H+L chain)	Goat	polyclonal	MBL	330	350	1:5,000	5% non-fat dry milk
Anti-goat IgG	Donkey	polyclonal	Santa Cruz	sc2354	not provided	1:20,000	2% BSA

SDHA: Succinate dehydrogenase complex, subunit A
FA: Fumarate hydratase
CTSB: Cathepsin B
SIRT3: Sirtuin 3

Supplementary Scheme



Scheme S1 Possible structures of Suc-yne-adducted lysine residue. α- and β-alknynlated adducts were conceivable in fact, but their ratio could not be determined because they could not be isolated. In the main text and the Table of Contents, one of them is described as a representative of succinylation for simplifying the scheme, but the above possibilities should be taken into account.

Supplementary Figures

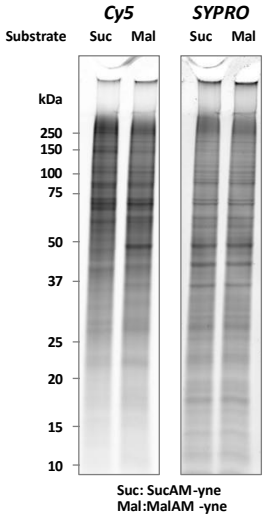


Fig. S1 Comparison of labeled proteins using different substrates (SucAM-yne or MalAM-yne). HEK293T cells were incubated with SucAM-yne or MalAM-yne independently (500 μM, 1 hour). The lysates were reacted with Cy5-azide and analyzed by fluorescence scanning (left). Total proteins were stained and visualized with SYPRO Ruby (right). The image of the Cy5 signal was highlighted in Fig. 1b.

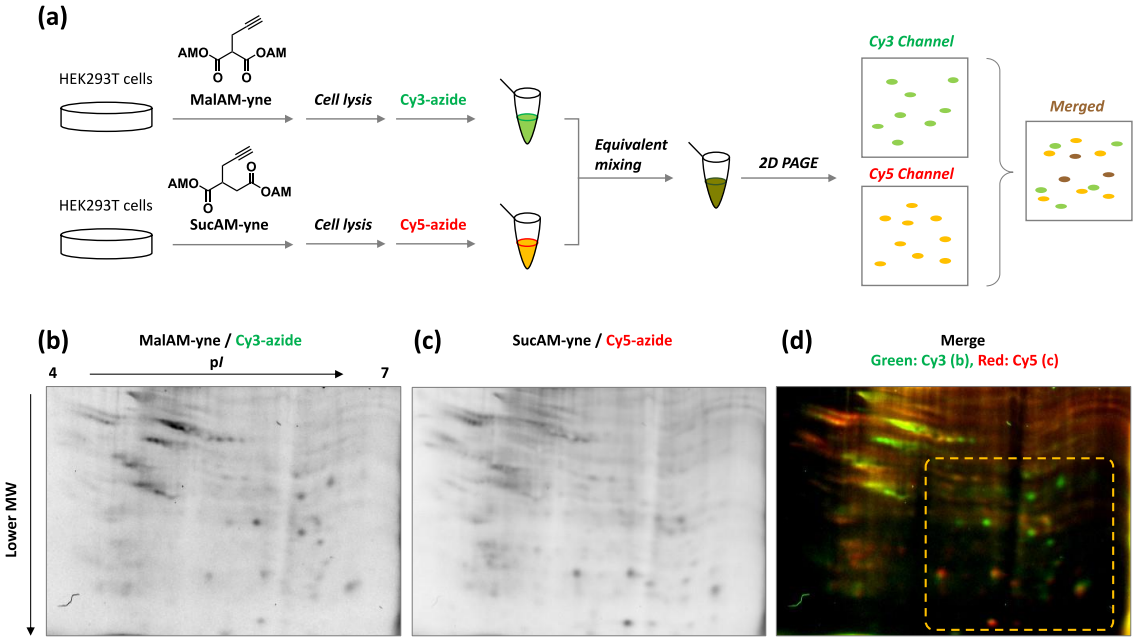


Fig. S2 Comparison of labeled proteins using different substrates (SucAM-yne and MalAM-yne) analyzed by 2D-PAGE. (a) Schematic diagram for 2D-PAGE analysis. (b-d) In-gel fluorescence images of Cy3-labeled (b) and Cy5-labeled (c) proteins, and their merged image (d, The rectangular area in the orange box was highlighted in Fig. 1c).

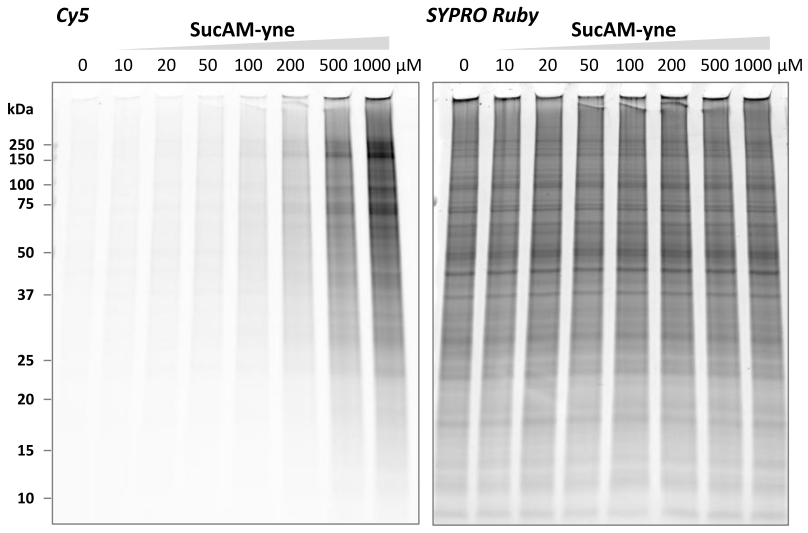


Fig. S3 Dose-dependent efficiency of metabolic labeling of HEK293T cells with SucAM-yne (0 – 1000 μM, 1 hour). The lysates were reacted with Cy5-azide and analyzed by fluorescence scanning (left). Total proteins were stained and visualized with SYPRO Ruby (right).

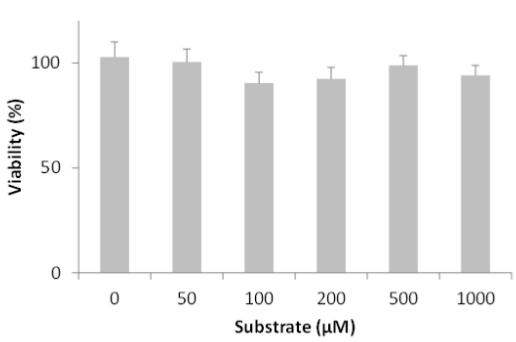


Fig S4 Viability of HEK293T cells treated with SucAM-yne (0 – 1000 μM, 1 hour) measured by CCK-8 assay kit (n = 10).

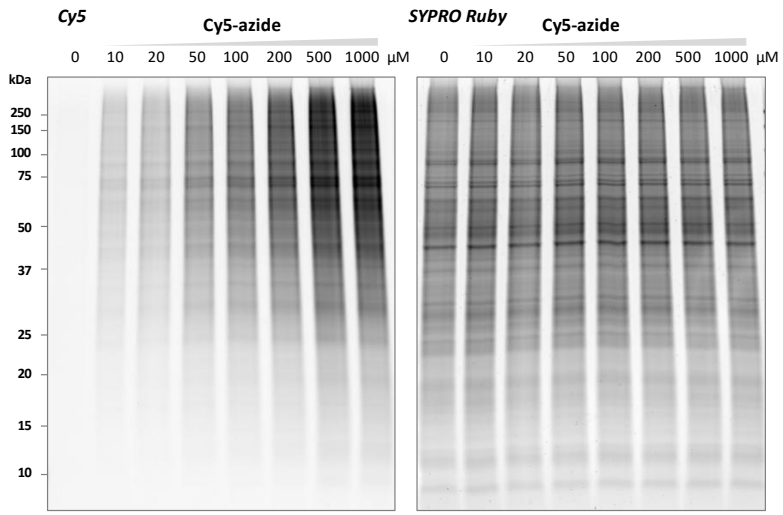


Fig. S5 Optimization of Cy5-azide concentration for Click ligation. The lysates of HEK293T cells incubated with SucAM-yne (500 μM, 1 hour) were reacted with various concentrations of Cy5-azide and analyzed by fluorescence scanning (left). Total proteins were stained and visualized with SYPRO Ruby (right).

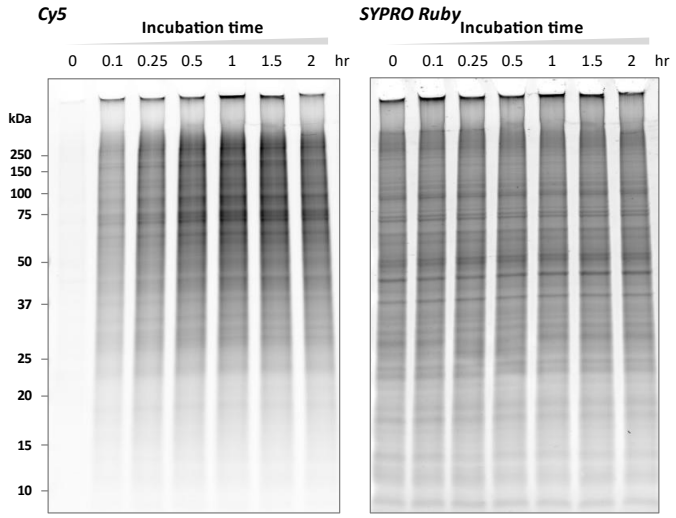


Fig. S6 Time-dependent efficiency of metabolic labeling of HEK293T cells with SucAM-yne. HEK293T cells were with SucAM-yne (500 μM) for the indicated time. The lysates were reacted with Cy5-azide and analyzed by fluorescence scanning. Total proteins were stained and visualized with SYPRO Ruby.

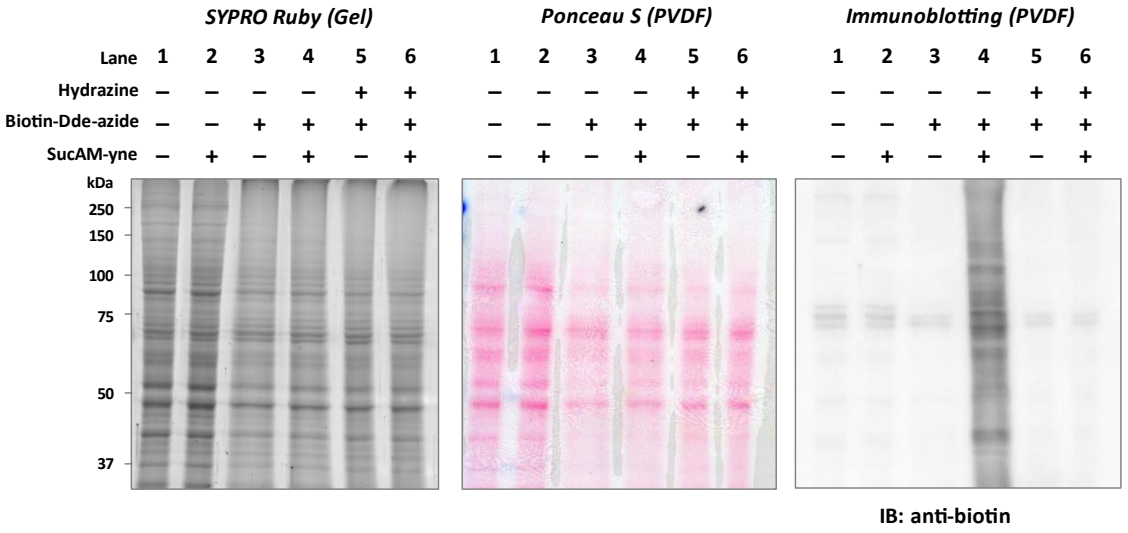


Fig. S7 Confirmation of biotinylation of succinylated proteins. The lysates of HEK293T cells incubated with SucAM-yne (500 μM, 1 hour) were reacted with biotin-Dde-azide, and the proteins were purified by acetone precipitation. In addition, hydrazine-based linker cleavage was undertaken (2% hydrazine, rt, 30 min), and each sample was applied for SDS-PAGE separation (10% acrylamide gel) and subsequent immunoblotting analysis. Total proteins on a gel or on a PVDF membrane were stained and visualized with SYPRO Ruby or Ponceau S (Thermo Fisher Scientific), respectively, and biotinylated proteins on a PVDF membrane were detected by enhanced chemiluminescence scanning using the anti-biotin primary antibody in accordance with the procedure in the supporting information.

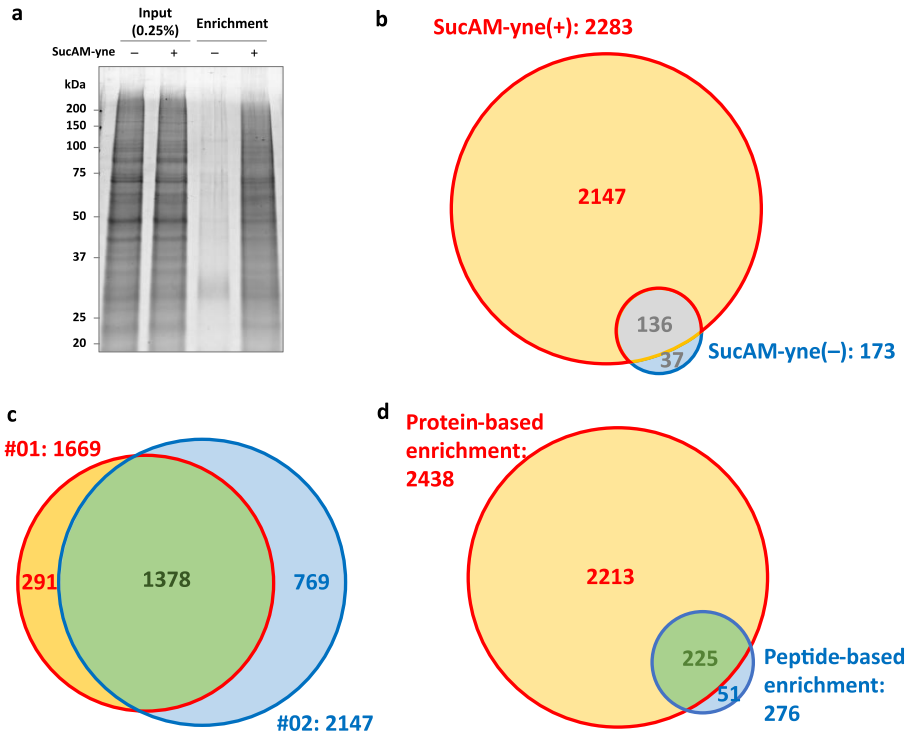


Fig. S8 (a, b) Evaluation of the reproducibility of the experiments in Figs. 2a and b. The same experiments (cell culture, metabolic labeling of SucAM-yne, Click ligation, protein enrichment and proteomic analysis) were independently performed on different days. (a) Confirmation of the enrichment of succinylated proteins by in-gel fluorescence scanning stained with SYPRO Ruby. (b) Venn diagram of identified enriched proteins. (c) Confirmation of the biological replicates. Venn diagram of identified proteins in Fig. 2b (#01) and Fig. S8b (#02) showed that 57% of the identified proteins were merged. (d) Comparison of proteins identified by using different enrichment protocols (protein-based and peptide-based methods, as listed in Table S1 and S2, respectively.)

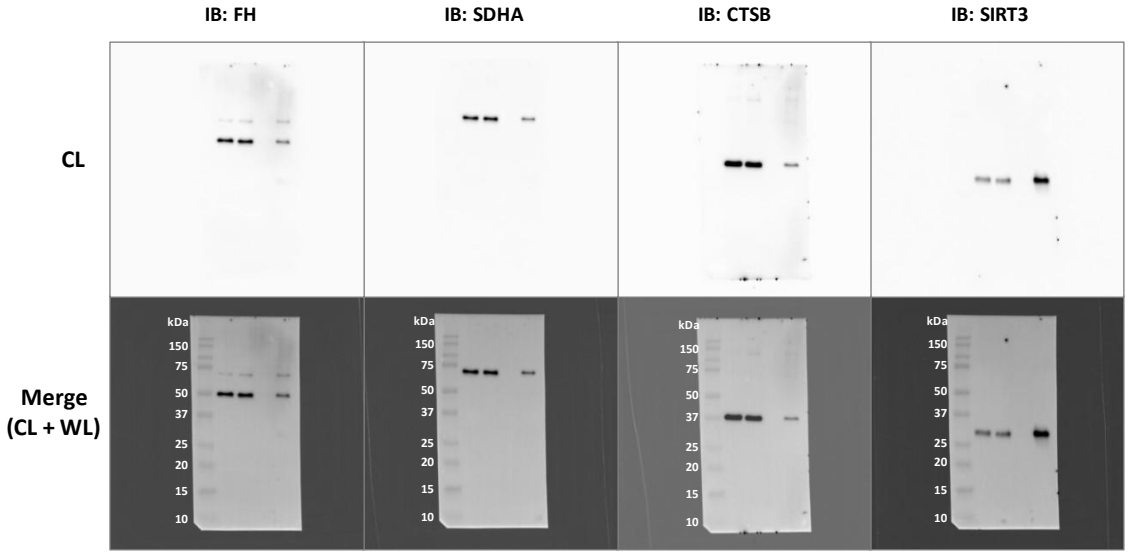
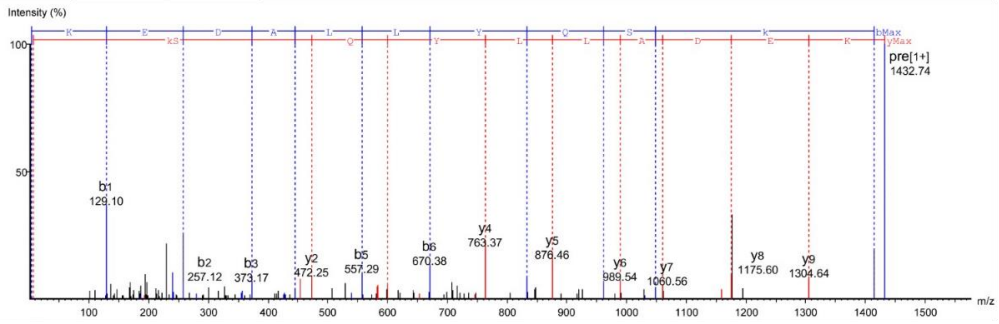


Fig. S9 Original data of the immunoblotting images corresponding to the experiment in Fig. 2c. CL: chemiluminescence images, WL: white-light images. IB represents the primary antibodies used.

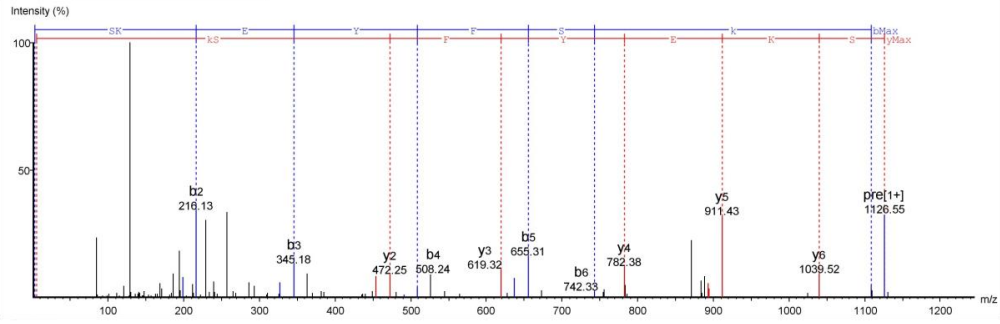
(a) P50402|EMD_HUMAN Emerin



79 K E D A L L Y Q S k 88

#	b	b−H2O	b−NH3	b (2+)	Seq	y	y−H2O	y−NH3	y (2+)	#
1	129.10	111.09	112.08	65.05	K					10
2	258.15	240.13	241.12	129.57	E	1304.64	1286.64	1287.62	652.83	9
3	373.17	355.16	356.15	187.07	D	1175.60	1157.59	1158.59	588.30	8
4	444.21	426.20	427.18	222.60	A	1060.56	1042.57	1043.55	530.79	7
5	557.29	539.28	540.27	279.13	L	989.54	971.53	972.51	495.27	6
6	670.38	652.35	653.35	335.69	L	876.46	858.45	859.43	438.73	5
7	833.44	815.43	816.41	417.22	Y	763.37	745.36	746.36	382.19	4
8	961.51	943.49	944.47	481.25	Q	600.31	582.30	583.28	300.65	3
9	1048.52	1030.50	1031.50	524.77	S	472.25	454.24	455.22	236.63	2
10					K(+238.11)	385.22	367.21	368.19	193.10	1

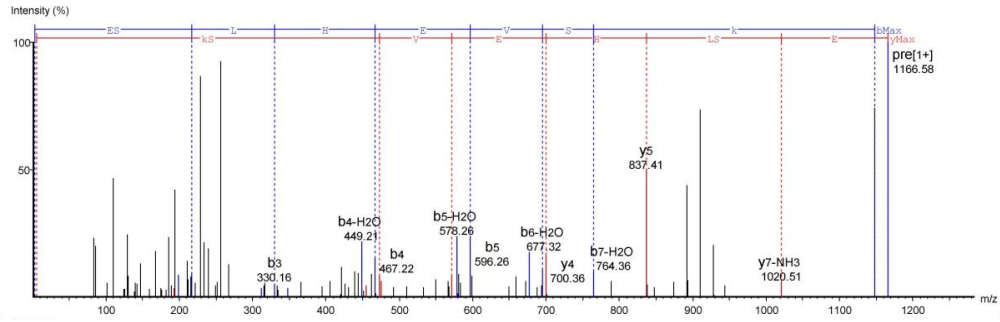
(b) Q06830|PRDX1_HUMAN Peroxiredoxin-1



191 S K E Y F S k 197

#	b	b−H2O	b−NH3	b (2+)	Seq	y	y−H2O	y−NH3	y (2+)	#
1	88.04	70.03	71.01	44.52	S					7
2	216.13	198.12	199.11	108.57	K	1039.52	1021.51	1022.49	520.26	6
3	345.18	327.17	328.15	173.09	E	911.43	893.41	894.41	456.21	5
4	508.24	490.23	491.23	254.62	Y	782.38	764.37	765.36	391.69	4
5	655.31	637.30	638.28	328.15	F	619.32	601.31	602.29	310.16	3
6	742.33	724.33	725.31	371.67	S	472.25	454.24	455.22	236.63	2
7					K(+238.11)	385.22	367.21	368.19	193.11	1

(c) O43615|TIM44_HUMAN Mitochondrial import inner membrane translocase subunit TIM44

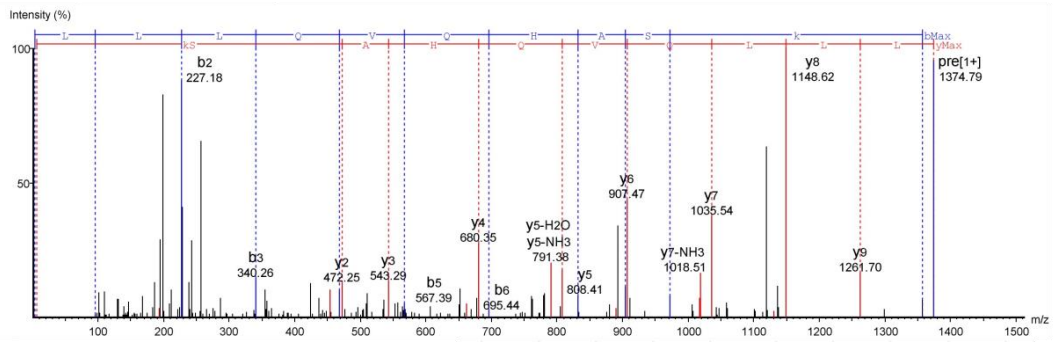


131 E S L H E V S k 138

#	b	b−H2O	b−NH3	b (2+)	Seq	y	y−H2O	y−NH3	y (2+)	#
1	130.05	112.04	113.02	65.53	E					8
2	217.08	199.07	200.06	109.04	S	1037.54	1019.53	1020.51	519.27	7
3	330.16	312.16	313.14	165.58	L	950.51	932.49	933.48	475.75	6
4	467.22	449.21	450.20	234.11	H	837.41	819.41	820.39	419.21	5
5	596.26	578.26	579.26	298.63	E	700.36	682.35	683.34	350.68	4
6	695.33	677.32	678.31	348.16	V	571.32	553.31	554.29	286.16	3
7	782.37	764.36	765.34	391.68	S	472.25	454.24	455.22	236.63	2
8					K(+238.11)	385.22	367.21	368.19	193.10	1

Fig. S10 (continued)

(d) P12236|ADT3_HUMAN ADP/ATP translocase 3

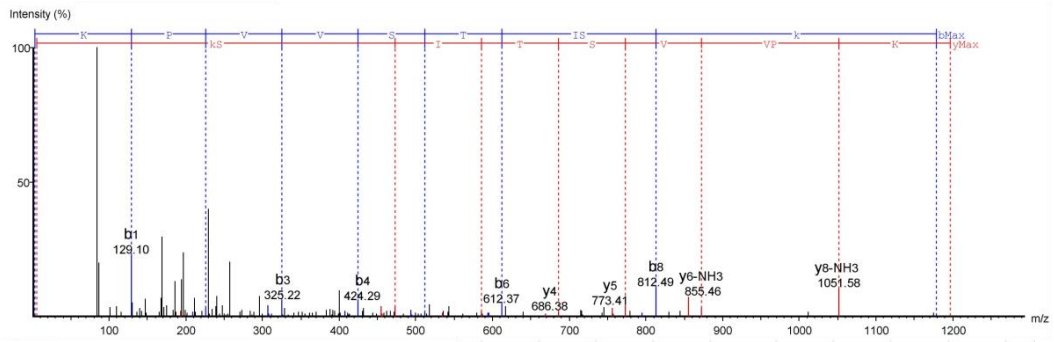


34 43

L L L Q V Q H A S k

#	b	b−H2O	b−NH3	b (2+)	Seq	y	y−H2O	y−NH3	y (2+)	#
1	114.09	96.08	97.06	57.55	L					10
2	227.18	209.17	210.15	114.09	L	1261.70	1243.69	1244.67	631.35	9
3	340.26	322.25	323.23	170.63	L	1148.62	1130.60	1131.59	574.81	8
4	468.32	450.31	451.29	234.66	Q	1035.54	1017.52	1018.51	518.27	7
5	567.39	549.38	550.36	284.19	V	907.47	889.45	890.45	454.24	6
6	695.44	677.43	678.42	348.22	Q	808.41	790.38	791.38	404.70	5
7	832.50	814.49	815.48	416.75	H	680.35	662.33	663.32	340.67	4
8	903.54	885.53	886.51	452.27	A	543.29	525.28	526.26	272.14	3
9	990.57	972.56	973.56	495.79	S	472.25	454.24	455.22	236.63	2
10					K(+238.11)	385.22	367.21	368.19	193.10	1

(e) Q9UPY5|XCT_HUMAN Cystine/glutamate transporter



4 12

K P V V S T I S k

#	b	b−H2O	b−NH3	b (2+)	Seq	y	y−H2O	y−NH3	y (2+)	#
1	129.10	111.09	112.08	65.05	K					9
2	226.15	208.14	209.13	113.58	P	1068.60	1050.59	1051.58	534.80	8
3	325.22	307.21	308.20	163.11	V	971.55	953.54	954.53	486.28	7
4	424.29	406.28	407.26	212.65	V	872.49	854.47	855.46	436.74	6
5	511.32	493.31	494.31	256.16	S	773.41	755.40	756.39	387.21	5
6	612.37	594.36	595.35	306.69	T	686.38	668.37	669.34	343.69	4
7	725.46	707.45	708.43	363.23	I	585.33	567.32	568.31	293.17	3
8	812.49	794.49	795.46	406.74	S	472.25	454.24	455.23	236.63	2
9					K(+238.11)	385.22	367.21	368.19	193.10	1

Fig. S10 MS/MS spectra and identification data of lysine-modified peptides of some identified proteins (a-e). The lowercase letter "k" in the peptide sequence denotes the lysine modified with 2-(1-(3-aminopropyl)-1*H*-1,2,3-triazol-4-yl)methyl modification (lysine; +238.11 Da), and the numbers in blue and red in the table are the b- and y-ions identified with errors within 0.02 Da, respectively. b-/y-H_2O, b-/y-NH_3, and b/y (2+) denote dehydrated b-/y- ions, deaminated b-/y- ions, and divalent b-/y- ions, respectively.

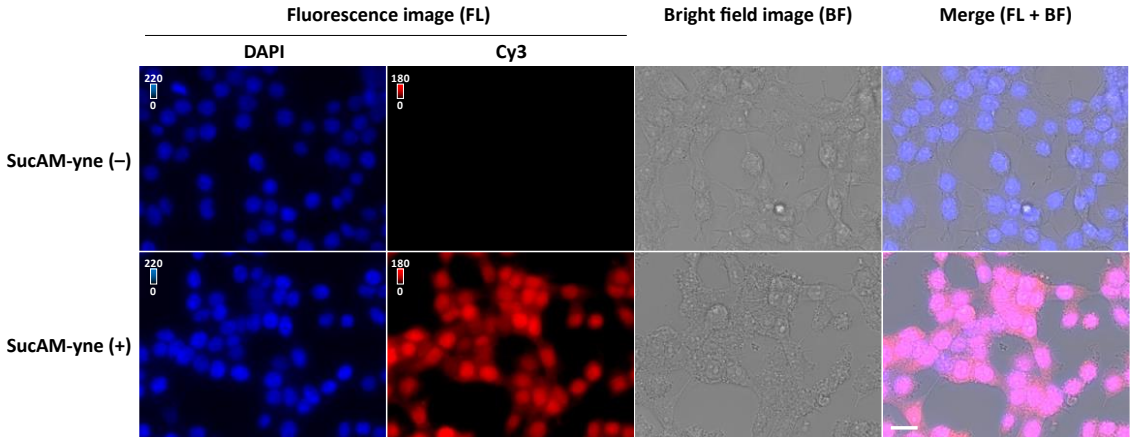


Fig. S11 Epi-fluorescence images of Cy3-azide-clicked HEK293T cells treated with DMSO (upper) or SucAM-yne (bottom). Scale bar: 20 μm.

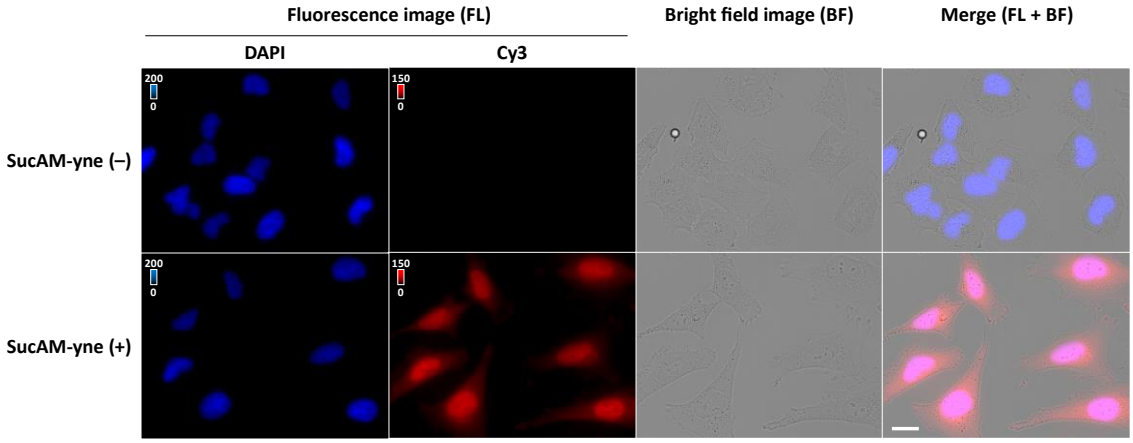


Fig. S12 Epi-fluorescence images of Cy3-azide-clicked HeLa cells treated with DMSO (upper) or SucAM-yne (bottom). Scale bar: 20 μm.

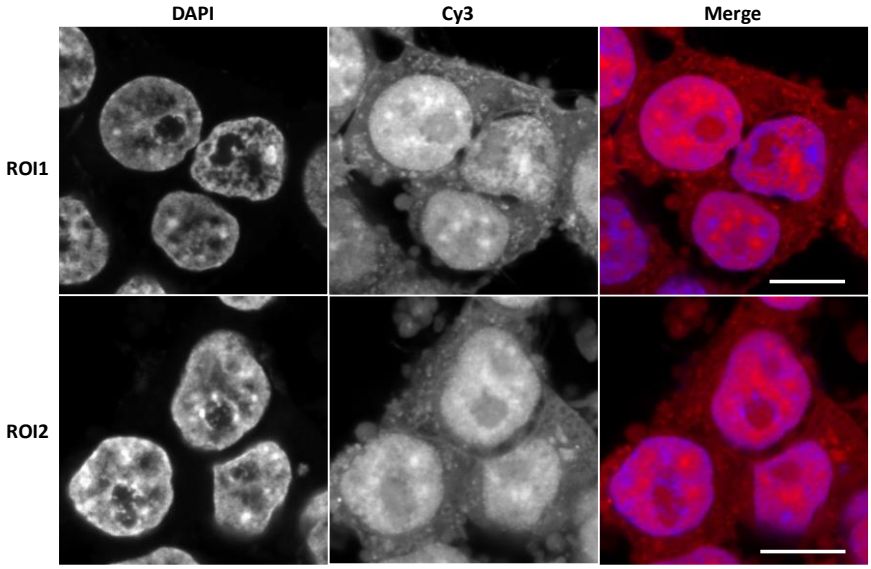


Fig. S13 Confocal images of succinylated proteins in HEK293T cells. After incubation with SucAM-yne (500 μM, 1 hour), cells were fixed and permeabilized, followed by Click ligation with Cy3-azide and subsequent staining with DAPI. Scale bar: 10 μm. Fluorescence images in some of the regions of interest (ROIs) were taken.

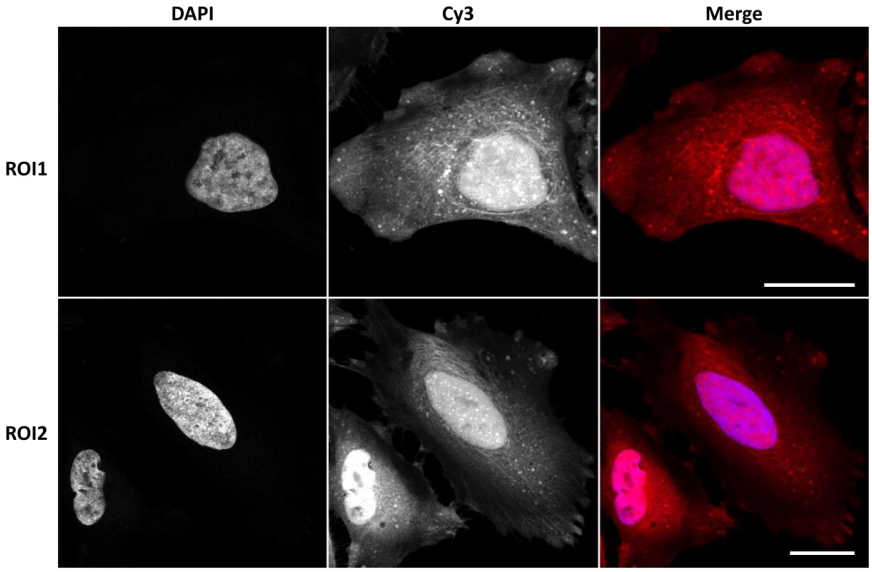


Fig. S14 Confocal images of succinylated proteins in HeLa cells corresponding to the images in Fig. 4. After incubation with SucAM-yne (500 μM, 1 hour), cells were fixed and permeabilized, followed by Click ligation with Cy3-azide and subsequent staining with DAPI. Scale bar: 25 μm. Fluorescence images in some of the regions of interest (ROIs) were taken. ROI1 and ROI2 images of Cy3 were also indicated in Fig. 4.

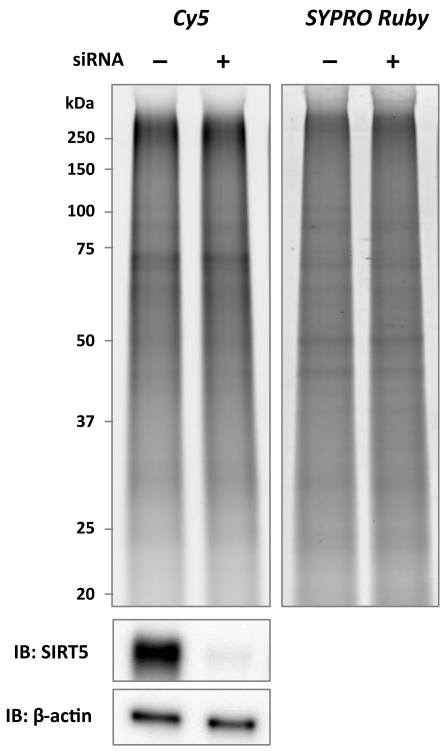


Fig. S15 Evaluation of metabolic labeling of the proteins in SIRT5-downregulated HEK293T cells by RNA interference. HEK293T cells with/without siRNA were incubated with SucAM-yne (500 μM, 1 hour) and lysed, then the lysates were reacted with Cy5-azide and analyzed by fluorescent scanning.

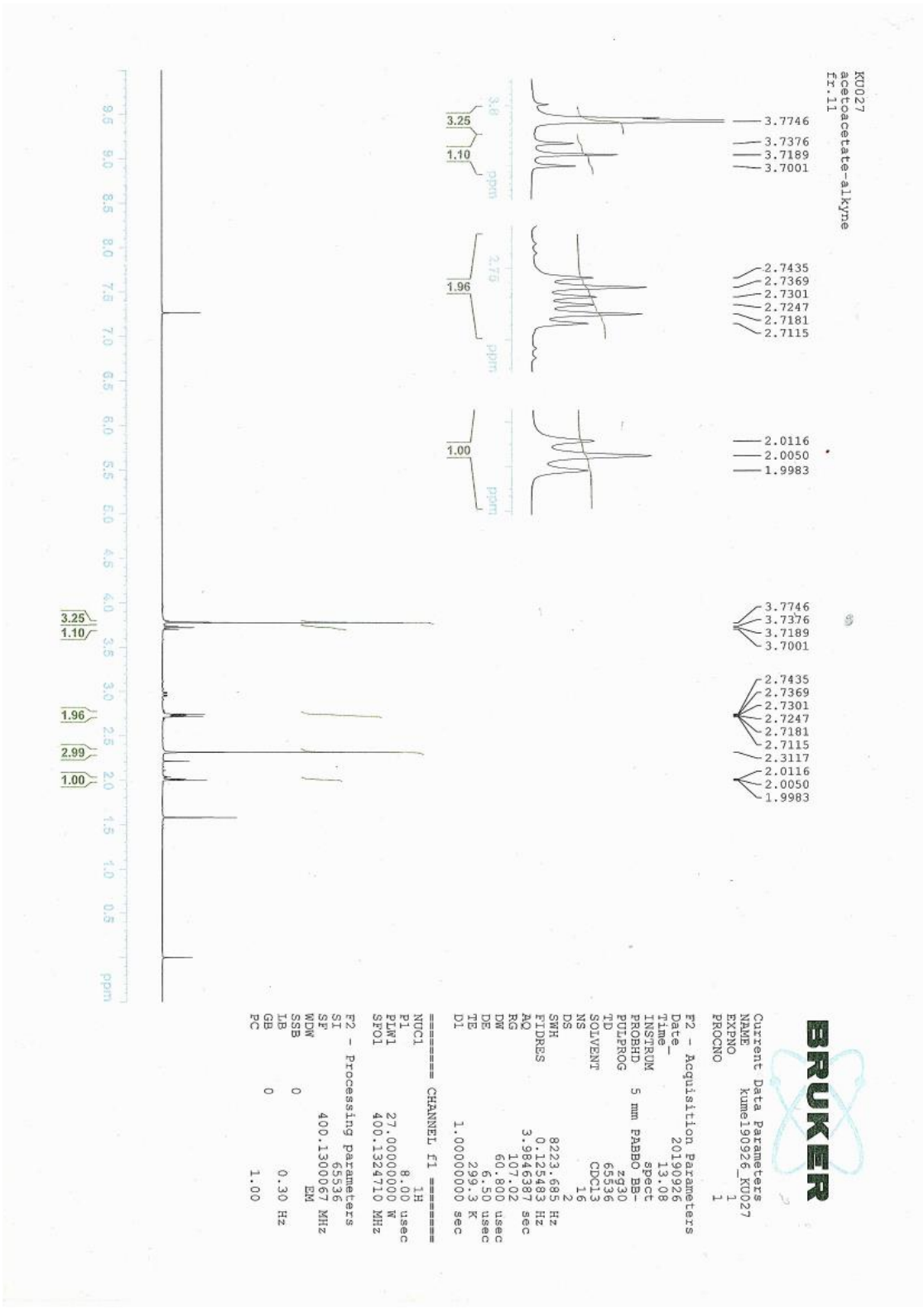


Fig. S16 ^{1}H-NMR spectrum of compound 2.

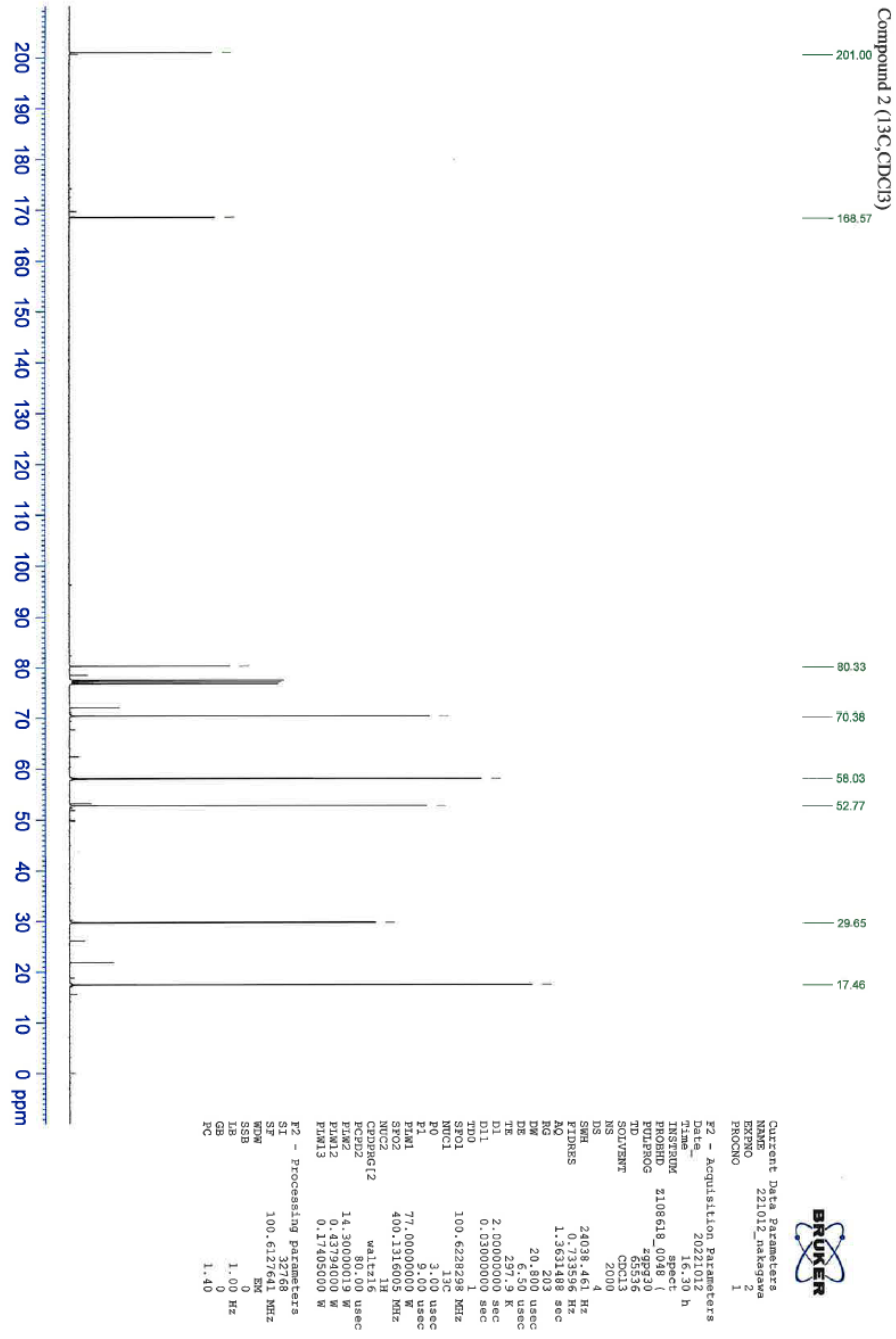


Fig. S17 ^{13}C-NMR spectrum of compound 2.

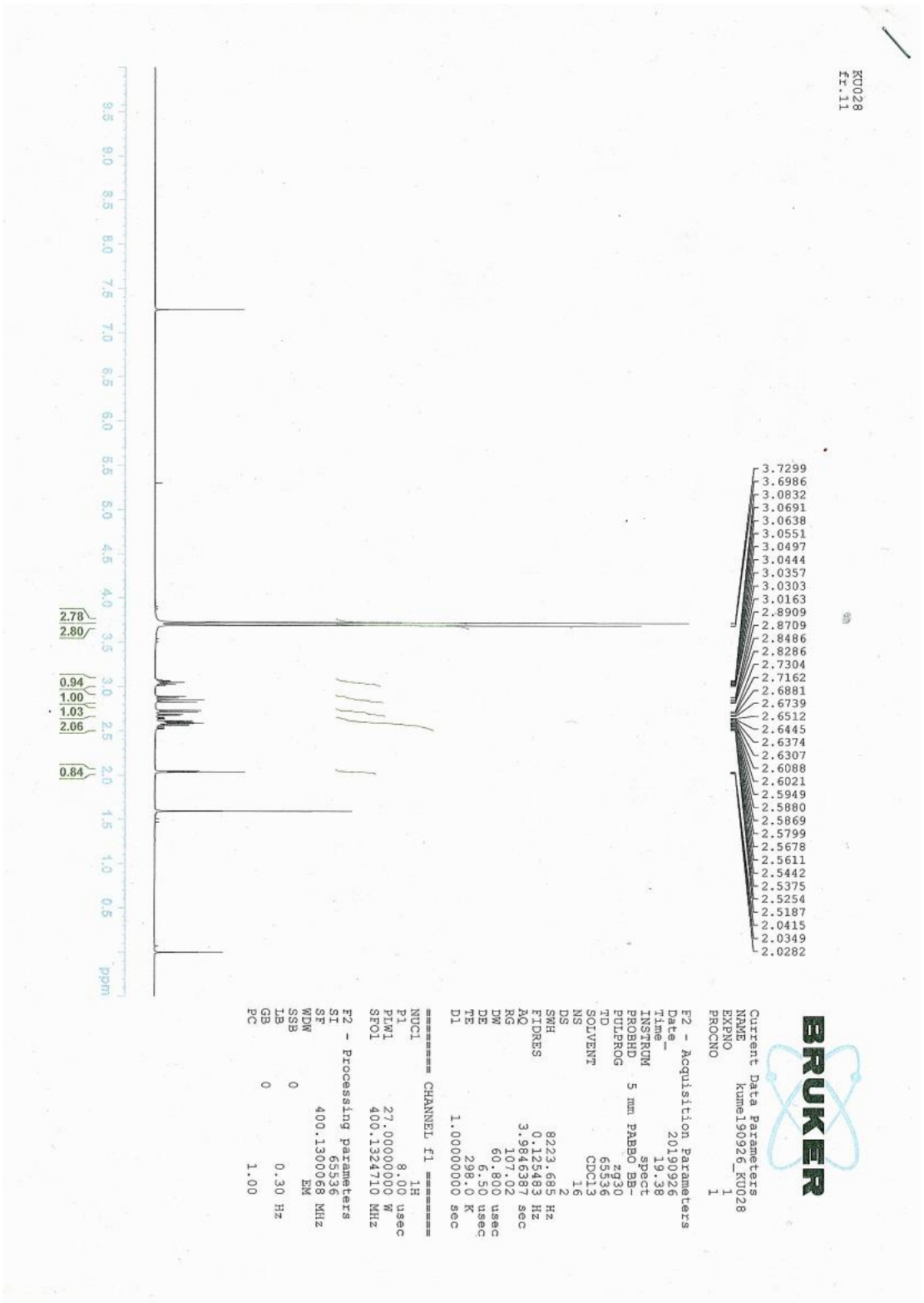


Fig. S18 ^{1}H-NMR spectrum of compound 3.

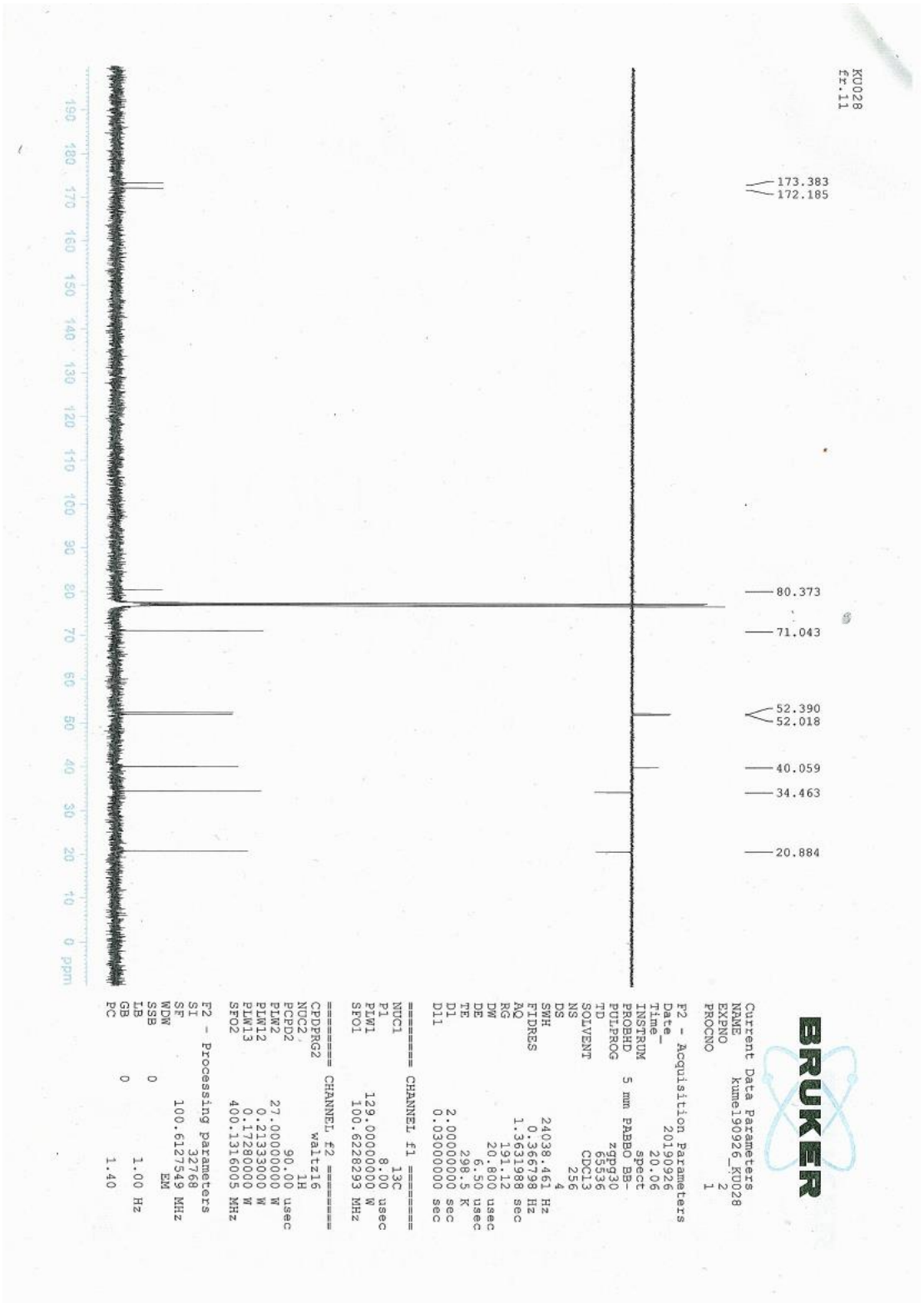


Fig. S19 ^{13}C-NMR and DEPT-135 spectra of compound 3.

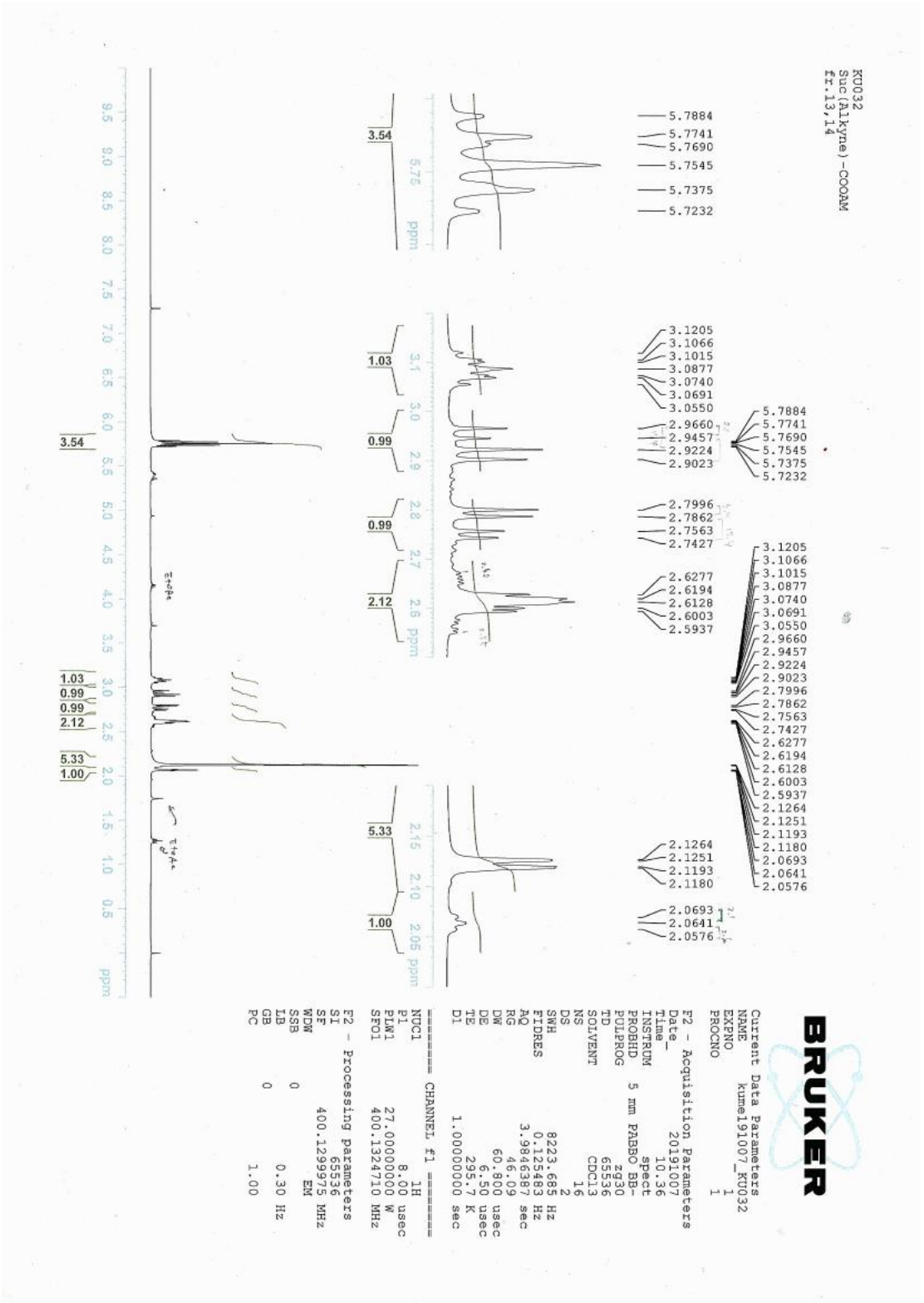


Fig. S20 ^{1}H-NMR spectrum of SucAM-yne.

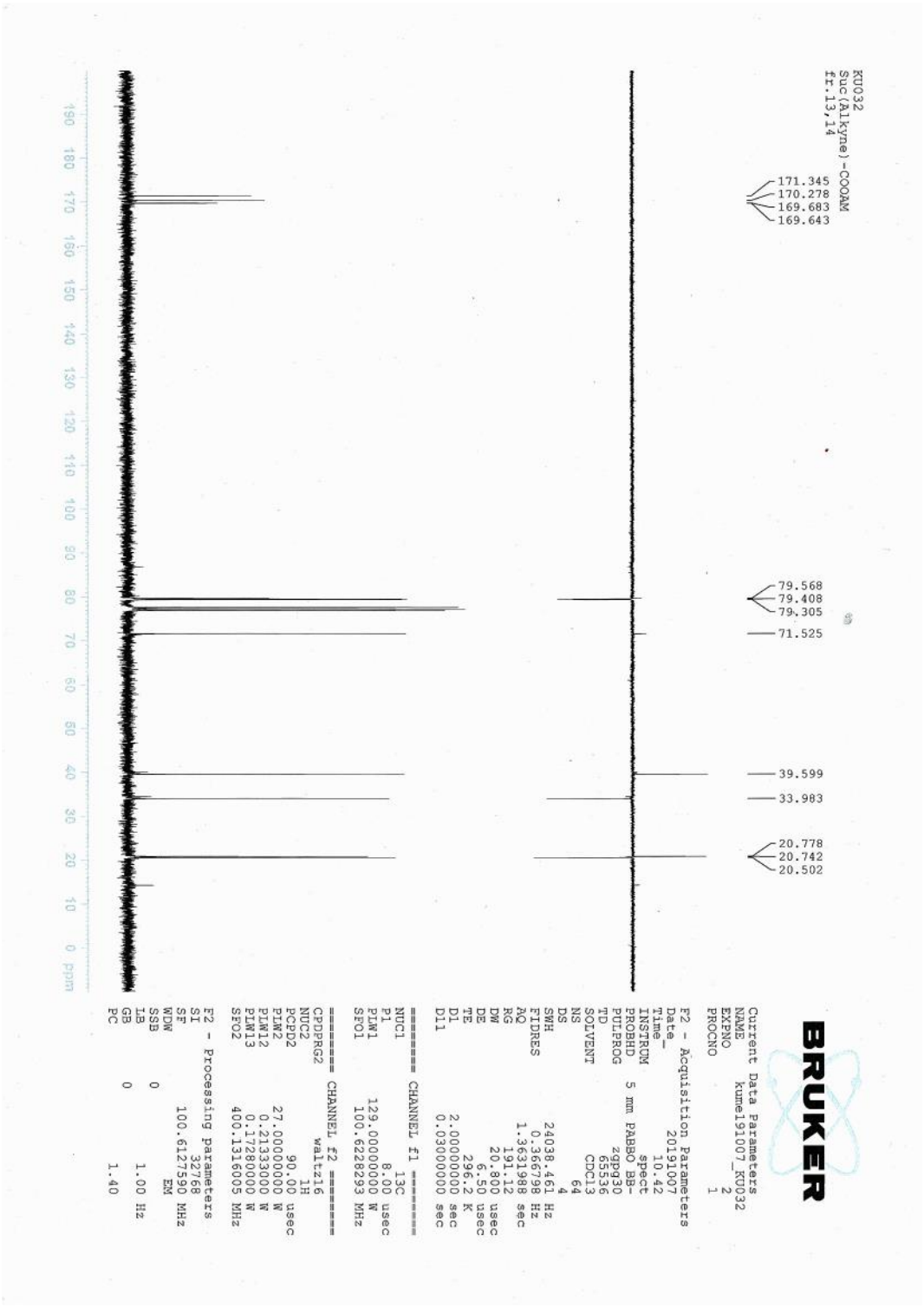


Fig. S21 ^{13}C-NMR and DEPT-135 spectra of SucAM-yne.

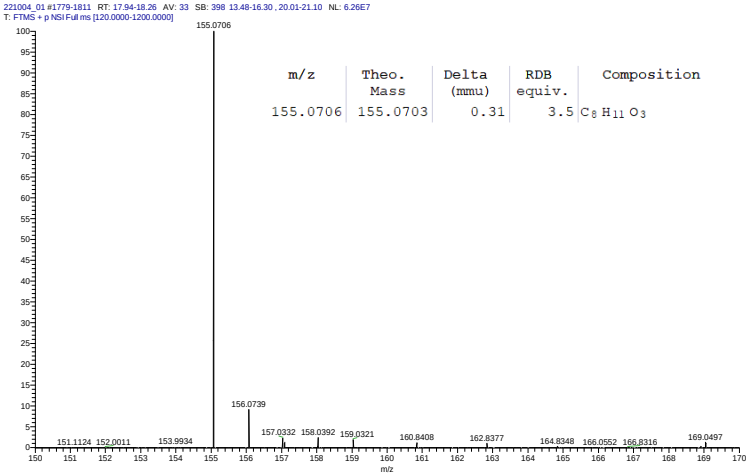


Fig. S22 HRMS spectrum of compound 2.

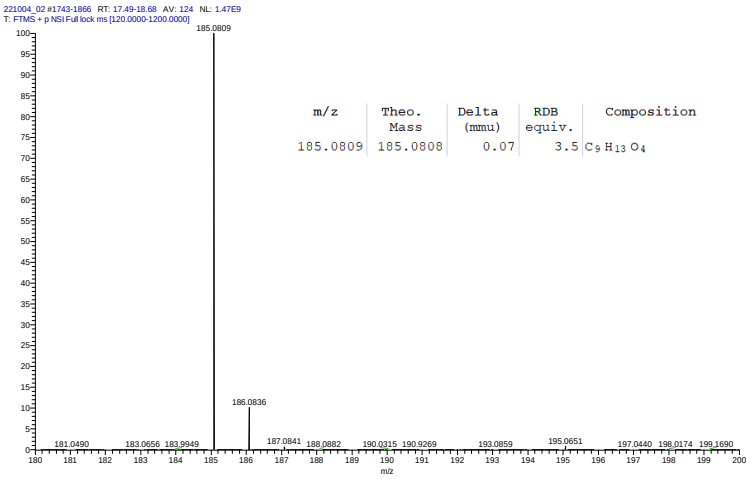


Fig. S23 HRMS spectrum of compound 3.

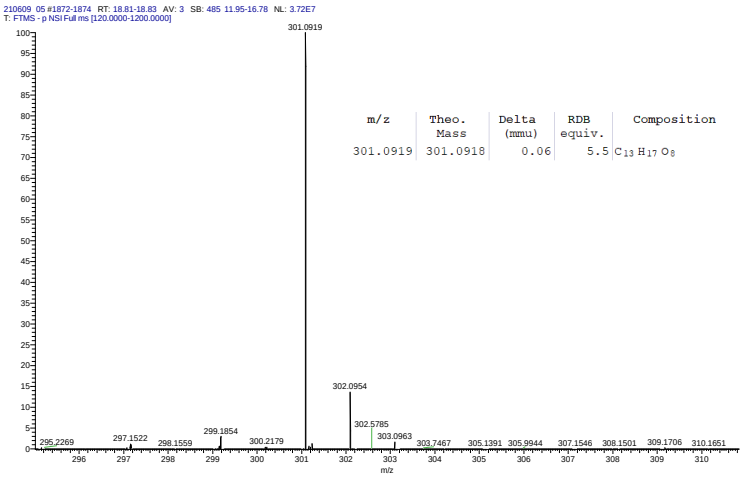


Fig. S24 HRMS spectrum of SucAM-yne.