Supporting Information

Targeted Protein Degradation in the Mitochondrial Matrix and Its Application to Chemical Control of Mitochondrial Morphology

Wakana Yamada,[1] Shusuke Tomoshige,[1,*] Sho Nakamura,[1] Shinichi Sato,[1,2] Minoru Ishikawa[1,*]

[1] Graduate School of Life Sciences, Tohoku University, 2-1-1 Katahira, Aoba-ku, Sendai, Miyagi 980-8577, Japan
[2] Frontier Research Institute for Interdisciplinary Sciences, Tohoku University, 2-1-1 Katahira, Aoba-ku, Sendai, Miyagi 980-8577, Japan

Corresponding Authors
Shusuke Tomoshige: stomohi@tohoku.ac.jp
Minoru Ishikawa: minoru.ishikawa.e4@tohoku.ac.jp

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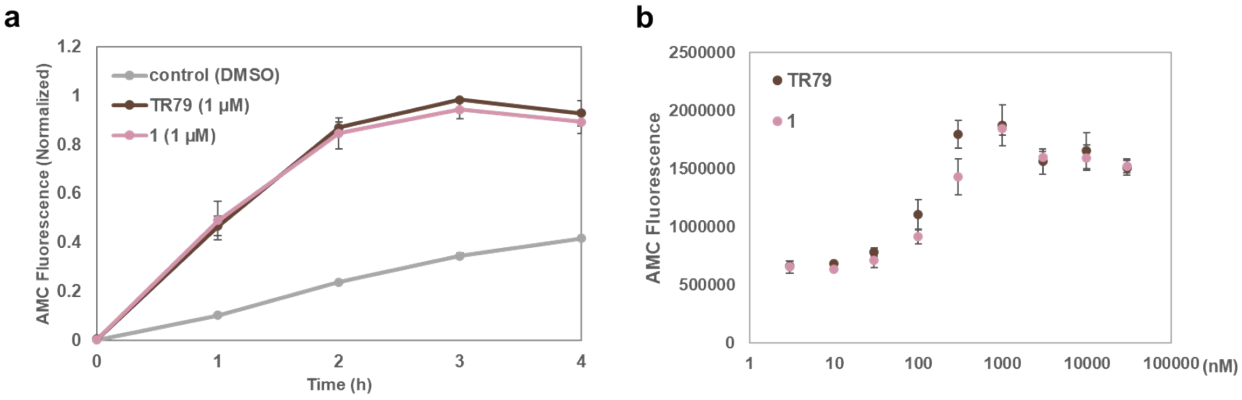


Figure S1. ClpP activation activity assay in vitro

a. Time course of Ac-WLA-AMC (7-amino-4-methylcoumarin) degradation. The fluorescence of the hydrolysis product, AMC, was determined as a measure of ClpP activation. The data were normalized by taking the maximum value as 1. Error bars indicate the SEM (n = 3 measurements).

b. Ac-WLA-AMC degradation assay of WY165 (**1**) and TR79 at various concentrations. EC_{50} values are 157 nM (WY165) and 105 nM (TR79). Error bars show the SEM (n = 3 measurements).

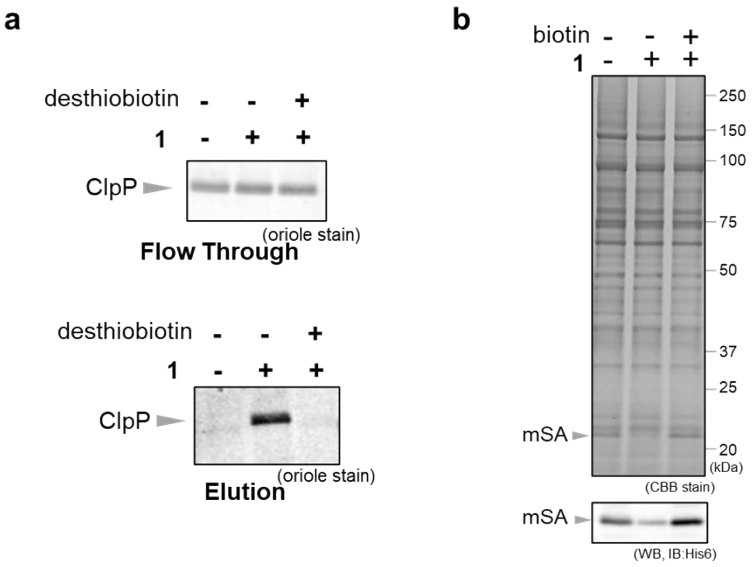


Figure S2. Analysis of WY165 (1)-induced ternary complex formation and confirmation of mSA selectivity by SDS-PAGE and western blotting.

a. Pull-down of ClpP using magnetic beads carrying streptavidin.

b. Confirmation of the selectivity of **1** for mSA degradation by **1** by CBB staining of total proteins in HEK293FT lysate.

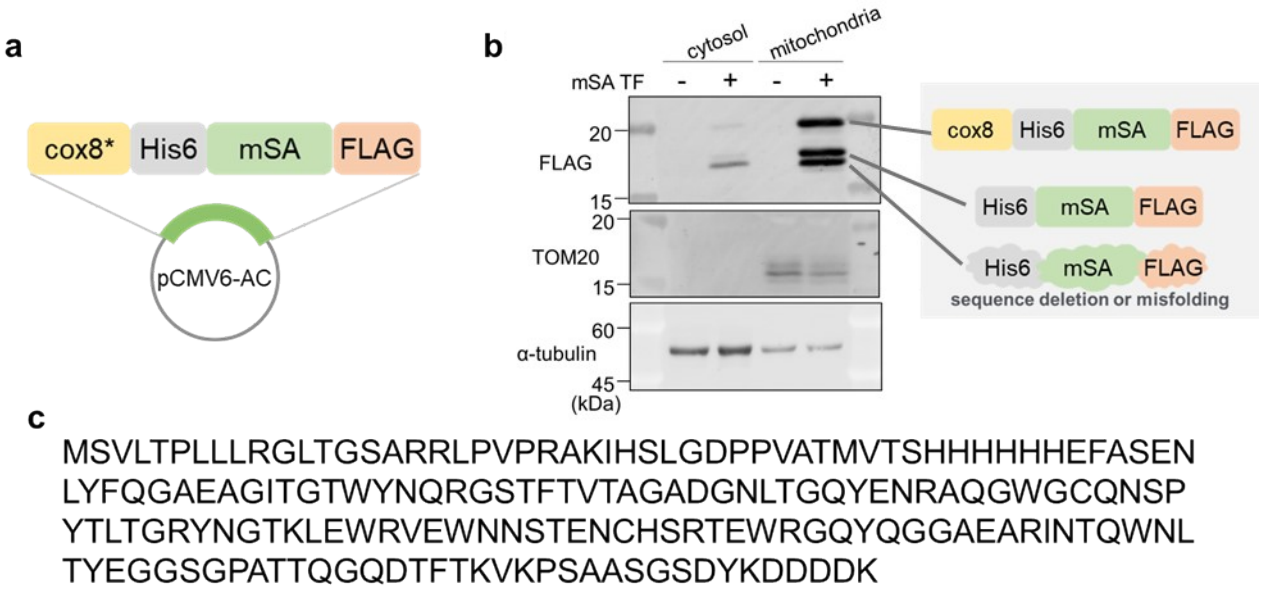


Figure S3. Construction of cox8-His6-mSA-FLAG plasmid and confirmation of mSA expression in mitochondria.

a. Schematic diagram of pCMV-cox8-His6-mSA-FLAG plasmid encoding C-terminally FLAG-tagged mSA fused with mitochondria targeting sequence (MTS) cox8 and His6 at the N-terminus.

b. Confirmation of cox8-mSA-FLAG expression in mitochondria. The diagrams on the right of the western blot image are the putative sequences of the three FLAG-positive bands. TF stands for transfection.

c. Amino acid sequence of full-length cox8-His6-mSA-FLAG.

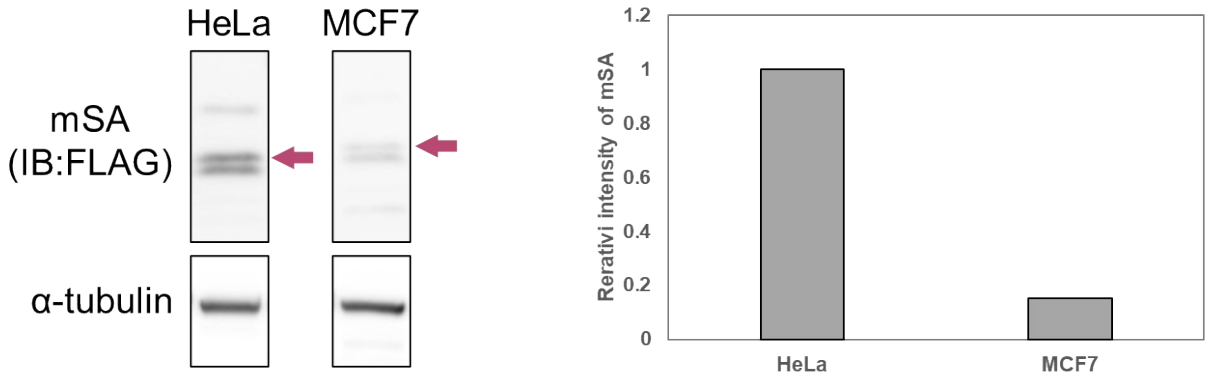


Figure S4. Comparison of mSA expression levels. Western blot images were captured from the same membrane. The quantitative results for the bands indicated by the arrows are shown as a bar graph on the right. The intensity is normalized to that of the HeLa sample, taken as 1.

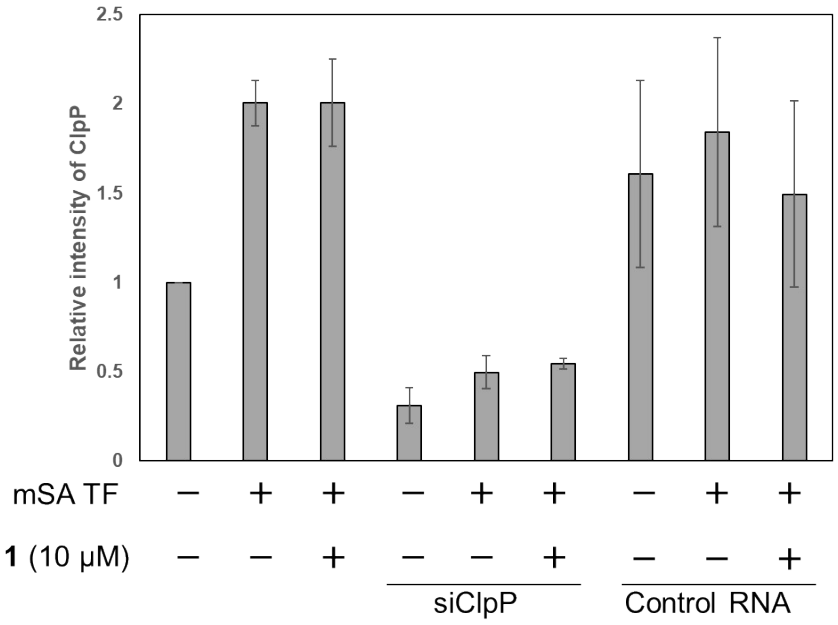


Figure S5. Quantitative results of western blot analysis of ClpP knockdown.
Intensities are normalized to that of the control (a sample not transfected and not treated with WY165 (**1**)), taken as 1. Error bars indicate SEM (n = 3 measurements).

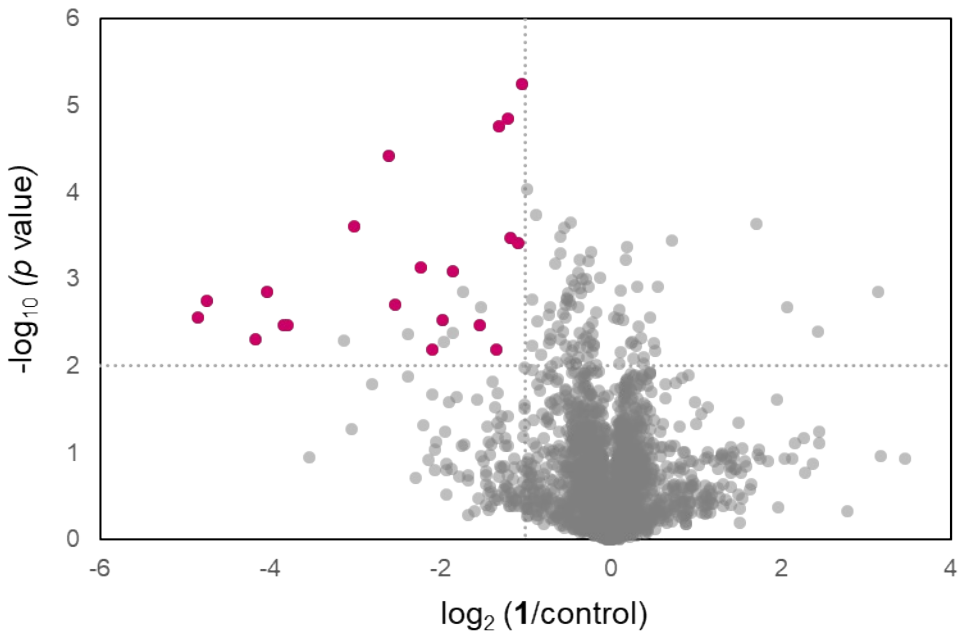
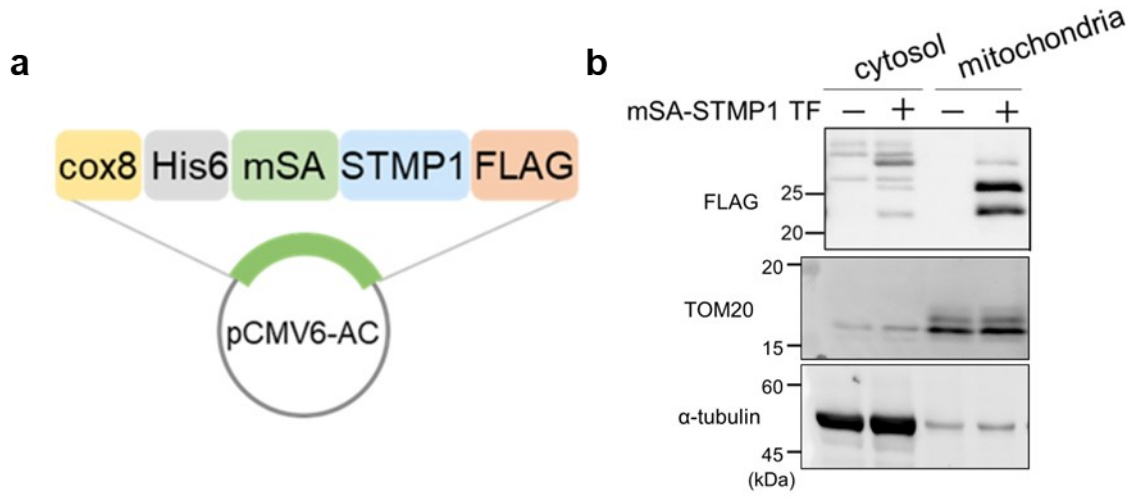


Figure S6. Proteome-wide analysis of WY165 (**1**)-induced changes in protein levels. HeLa cells expressing cox8-mSA-FLAG were treated with **1** or DMSO (control) for 12 h and analyzed by LC-MS/MS. Relative protein abundances in **1**-treated cells and control cells are shown as a volcano plot. The vertical axis represents the $-\log_{10}$ of the *p*-value (n = 3 measurements) (dotted line: $p = 0.01$), and the horizontal axis represents the $\log_2$ fold changes of protein abundance (dotted line: 0.5-fold changes). Proteins meeting the criteria of $p<0.01$ and >50% reduction are listed in Table S2. Among the proteins, mitochondrial proteins are highlighted in red. mSA was not significantly reduced in this plot, probably because LC-MS/MS analysis using the digested sample is not able to distinguish the three mSA variants detected by western blotting.



c

MSVLTPLLLRGLTGSARRLPVPRAKIHSLGDPPVATMVTSHHHHHHEFASENLYF
QGAEAGITGTWYNQRGSTFTVTAGADGNLTGQYENRAQGWGCQNSPYTLTGR
YNGTKLEWRVEWNNSTENCHSRTEWRGQYQGGAEARINTQWNLTYEGGSGPA
TTQGQDTFTKVKPSAASGSMLQFLLGFTLGNVVGMYLAQNYDIPNLAKKLEEIKK
DLDAKKKPPSASDYKDDDDK*

Figure S7. Construction of cox8-His6-mSA-STMP1-FLAG plasmid and confirmation of mSA-STMP1 expression in mitochondria.

a. Schematic diagram of pCMV-cox8-His6-mSA-STMP1-FLAG plasmid encoding C-terminally FLAG-tagged mSA-STMP1 fused with cox8 and His6 at the N-terminus of mSA-STMP1-FLAG.
b. Confirmation of mSA-STMP1 expression in mitochondria.
c. Amino acid sequence of full-length cox8-His6-mSA-STMP1-FLAG.

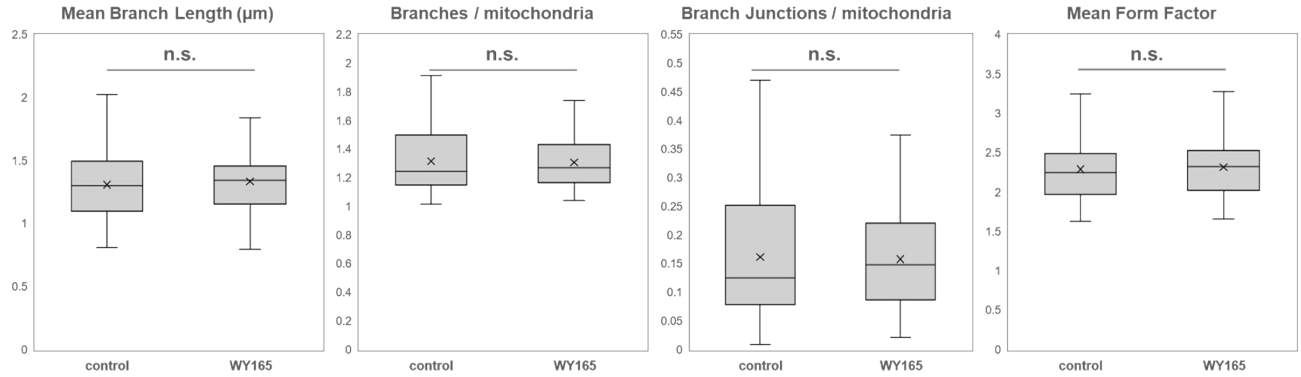


Figure S8. WY165 does not affect mitochondrial morphology in cells which do not express mSA-STMP1. Box-and-whisker plot of mitochondrial morphology in microscopic images are shown. Mean branch length: total branch length divided by number of branches. Branches/mitochondria: total number of branches in the image, normalized to mitochondria count. Branch junctions/mitochondria: number of junctions within all skeletons in the image, normalized to mitochondria count. Junctions are points where 2 or more branches meet. Mean form factor: a measure of shape, where the value 1 indicates a round object and increases with elongation. Control: 72 cells, WY165-treated: 73 cells. Steel-Dwass test (n.s., not significant)

a **His6-mSA-FLAG**:

MTSHHHHHHEFASENLYFQGAEAGITGTWYNQRGSTFTVTAGADGNLTGQY
ENRAQGWGCQNSPYTLTGRYNGTKLEWRVEWNNSTENCHSRTEWRGQYQ
GGAEARINTQWNLTYEGGSGPATTQGQDTFTKVKPSAASGSDYKDDDDK

b

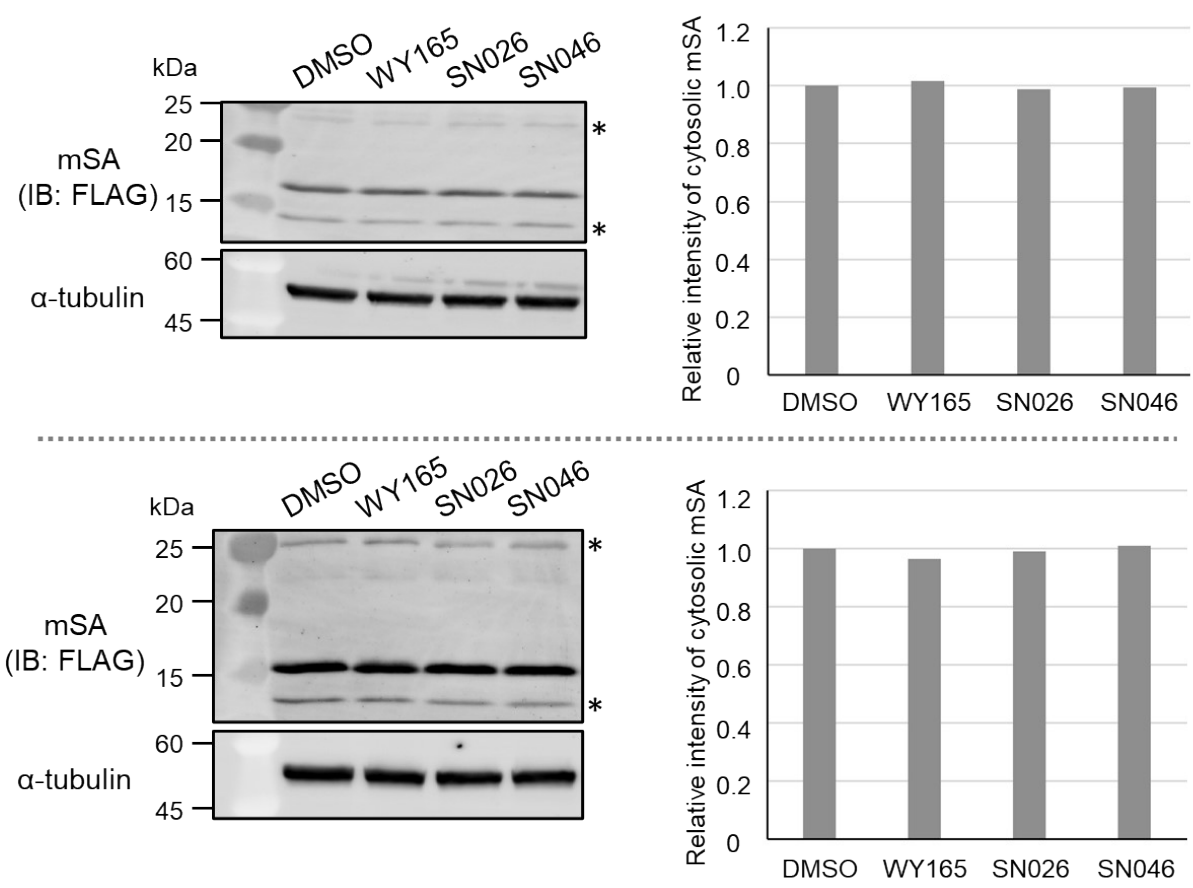


Figure S9. The degraders do not reduce the levels of His6-mSA-FLAG which lacks mitochondria targeting signal sequence.

a. The amino acid sequence of His6-mSA-FLAG. The plasmid for expression of His6-mSA-FLAG was constructed by VectorBuilder, Inc.

b. Two independent experiments showed no degradation of His6-mSA-FLAG by the degraders developed in this study. Intensities are normalized to that of the control (DMSO-treated sample), taken as 1.

Table S1. Proteomics analysis to confirm the selectivity in vitro.

Selectivity of WY165 (**1**)-induced degradation was evaluated using cell lysates supplemented with mSA and ClpP. The 34 proteins that were decreased in the presence of **1** are listed. The proteins highlighted in red are potential off-targets of **1** (see the text).

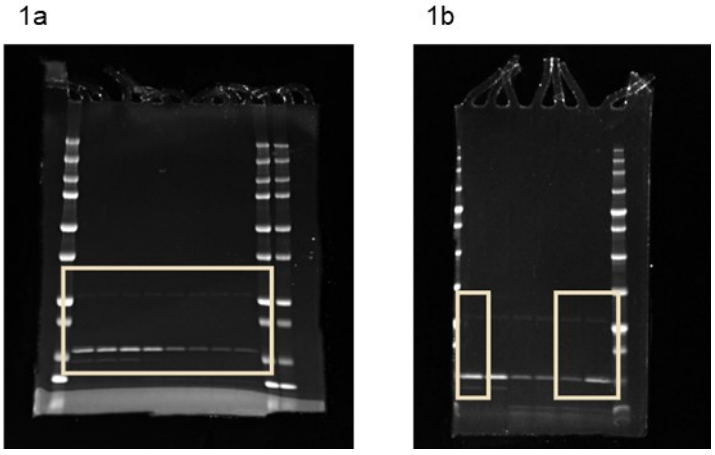
-log10 (P-value)'	difference	protein	subcellular location	MW (kDa)
3.51	-5.09	Eukaryotic translation initiation factor 4H	cytosol	27
2.55	-4.07	SAP domain-containing ribonucleoprotein	nucleus	23
3.45	-3.56	Splicing factor 3B subunit 2	nucleus	100
2.63	-3.43	Neuroblast differentiation-associated protein AHNAK	nucleus	629
2.92	-2.83	BUB3-interacting and GLEBS motif-containing protein ZNF207	nucleus	50
3.46	-2.80	Basigin	acrosomal membrane	42
4.03	-2.77	U1 small nuclear ribonucleoprotein C	nucleus	17
2.00	-2.42	Reticulocalbin-1	endoplasmic reticulum lumen	331
3.99	-2.36	Cytochrome b5 type B	mitokonndoria outer memblene	16
2.16	-2.31	Perilipin-3	Lipid droplet	47
2.11	-2.25	Ataxin-2-like protein	cytosol	113
2.07	-2.21	Calumenin	endoplasmic reticulum membrane	37
4.98	-2.09	mSA		
2.77	-1.86	Microtubule-associated protein 4	cyoplasm, cytoskeleton	121
4.57	-1.70	Elongation factor 1-beta	cytosol	24
5.69	-1.64	10 kDa heat shock protein, mitochondrial	mitochondria matrix	10
2.18	-1.47	Activated RNA polymerase II transcriptional coactivator p15	nucleus	14
6.50	-1.41	60 kDa heat shock protein, mitochondrial	mitochondria matrix	61
4.38	-1.35	Caldesmon	cytoskeleton	93
2.94	-1.33	Zyxin	cytosol	61
4.03	-1.33	Stathmin	cytoskeleton	17
2.69	-1.30	PEST proteolytic signal-containing nuclear protein	nucleus	18
3.48	-1.24	Protein NipSnap homolog 2	cytosol, mitochondria outer membrene	34
3.26	-1.22	Stathmin-2	cytoplasm	21
2.14	-1.18	CCHC-type zinc finger nucleic acid binding protein	nucleus, cytosol	19
2.20	-1.17	Myosin light polypeptide 6, Myosin light chain	cytosol	17
3.53	-1.14	Coiled-coil domain-containing protein 124	cytoskeleton	25
2.41	-1.12	Serine/arginine-rich splicing factor 2, Serine/arginine-rich splicing factor 8	nucleus	25, 32
2.93	-1.11	WD repeat and HMG-box DNA-binding protein 1	nucleus	125
3.35	-1.07	SERPINE1 mRNA-binding protein 1	nucleus, cytosol	44
3.62	-1.06	Heterogeneous nuclear ribonucleoprotein D-like	nucleus, cytosol	46
2.12	-1.05	Heterogeneous nuclear ribonucleoproteins A2/B1	nucleus, cytosol	37
2.36	-1.02	Protein CDV3 homolog	cytosol	27
3.75	-1.00	Far upstream element-binding protein 2	nucleus, cytosol	73

Table S2. List of proteins significantly reduced by WY165 (**1**). Proteins meeting the criteria of $p<0.01$ and >50% reduction (Figure S6) are listed. Mitochondrial proteins are highlighted in red.

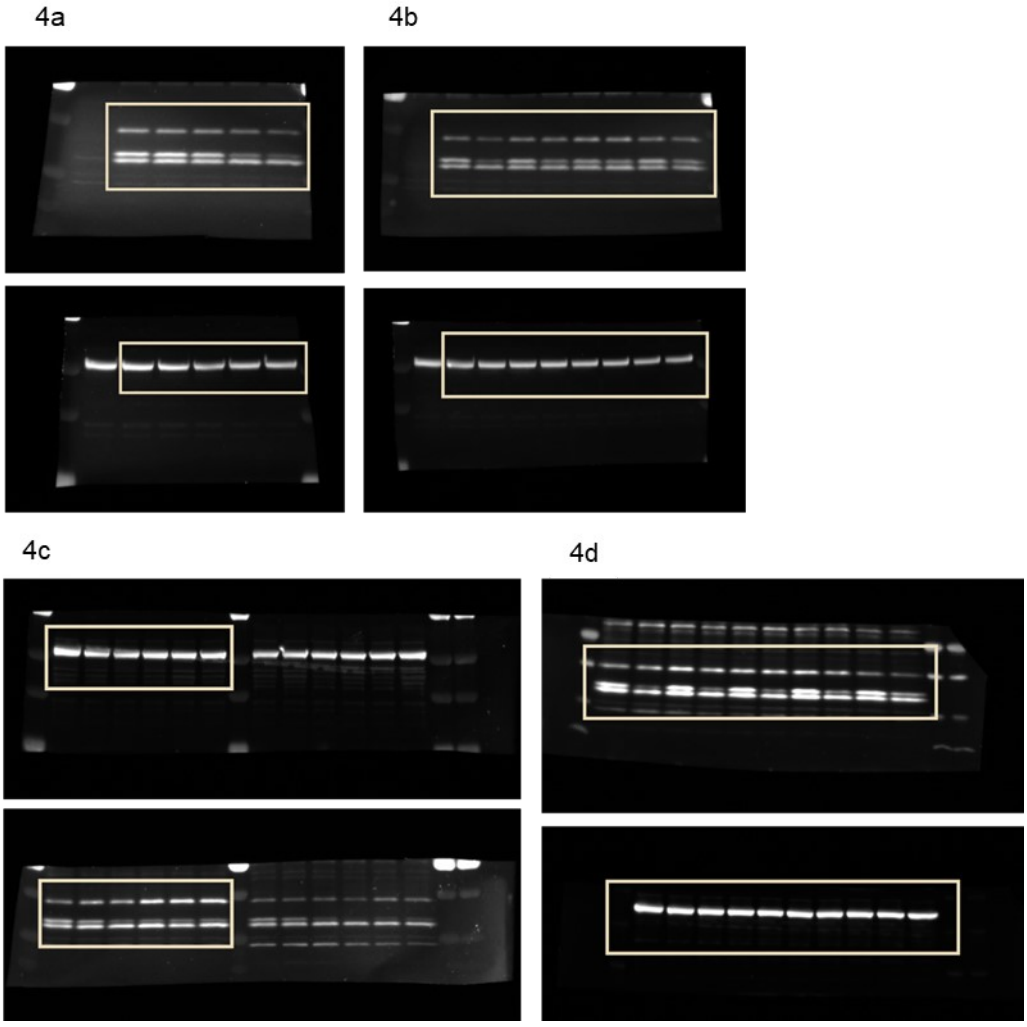
-log10 (P-value)	difference	protein	subcellular location	MW (kDa)
5.245135149	-1.032853444	Aconitate hydratase, mitochondrial	mitochondria	85
4.8495257	-1.207420349	Elongation factor Tu, mitochondrial	mitochondria	50
4.764211877	-1.310590108	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	mitochondria inner membrane	73
4.427678987	-2.598734538	GrpE protein homolog 1, mitochondrial	mitochondria matrix	24
3.605636102	-3.005639394	Mimitin, mitochondrial	mitochondria	20
3.47589052	-1.179000219	2-oxoglutarate dehydrogenase, mitochondrial	mitochondria	116
3.422703411	-1.088703791	LETM1 and EF-hand domain-containing protein 1, mitochondrial	mitochondria inner membrane	83
3.135060757	-2.228588104	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 9	mitochondria inner membrane	22
3.091708653	-1.856168747	Complex III assembly factor LYRM7	mitochondria matrix	12
2.862201931	-1.738595327	Pre-mRNA-splicing factor SLU7	nucleus	68
2.860364359	-4.041547139	28S ribosomal protein S22, mitochondrial	mitochondria	41
2.750704624	-4.750598272	Ribosome-recycling factor, mitochondrial	mitochondria	29
2.702638782	-2.532911936	28S ribosomal protein S31, mitochondrial	mitochondria	45
2.672086593	-1.526894252	Exosome complex component RRP4	cytoplasm	33
2.555283921	-4.847026189	39S ribosomal protein L12, mitochondrial	mitochondria matrix	21
2.538735178	-1.972066879	28S ribosomal protein S2, mitochondrial	mitochondria	33
2.473274147	-3.837106069	Elongation factor Ts, mitochondrial	mitochondria	35
2.471126359	-1.539598465	G-rich sequence factor 1	mitochondria matrix	53
2.465541625	-3.797537486	Isocitrate dehydrogenase [NAD] subunit gamma, mitochondrial	mitochondria	43
2.382094272	-1.850881577	SAP30-binding protein	nucleus	34
2.373859972	-2.386291504	Brain-specific angiogenesis inhibitor 1-associated protein 2-like protein 1	cytoplasm	57
2.302545227	-4.175934474	ATP-dependent Clp protease ATP-binding subunit clpX-like, mitochondrial	mitochondria	69
2.290860956	-3.131174723	Protein FAM192A	nucleus	29
2.279421659	-1.962650299	Ubiquitin-conjugating enzyme E2 D3;Ubiquitin-conjugating enzyme E2 D2	cell membrane, cytosol	17
2.198451672	-1.344062805	Polyribonucleotide nucleotidyltransferase 1, mitochondrial	cytoplasm, mitochondria	86
2.18737791	-2.092157364	Translational activator of cytochrome c oxidase 1	mitochondria	32

Full blot images.

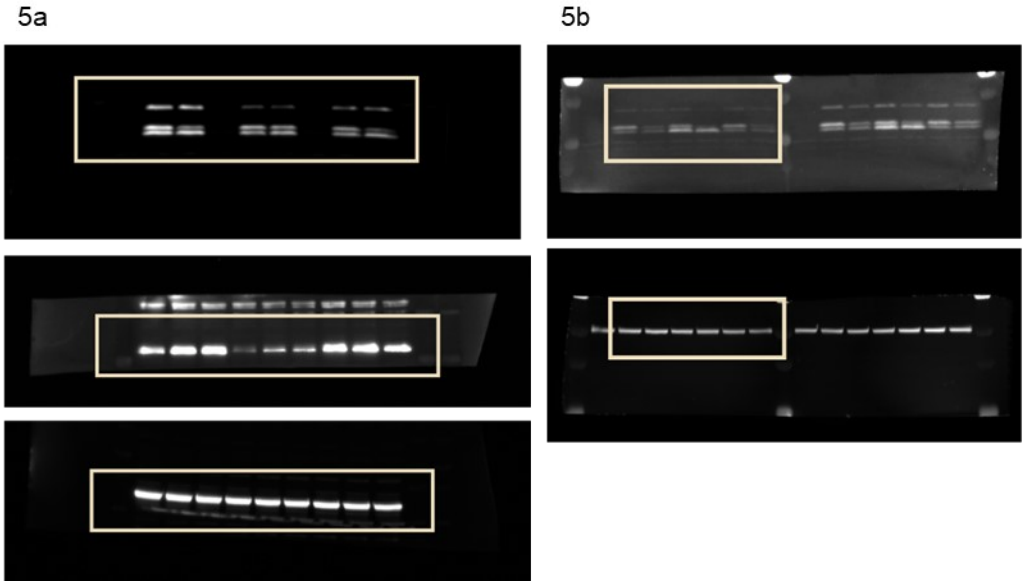
Uncropped western blot images for Fig. 1a,b.



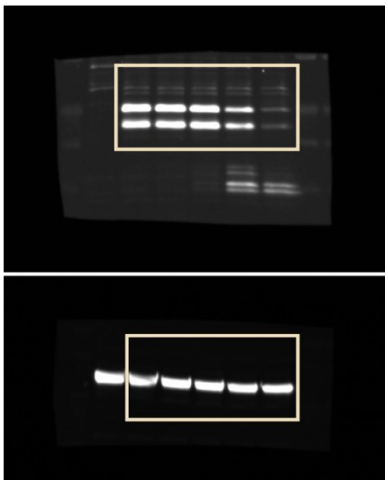
Uncropped western blot images for Fig. 4a-d.



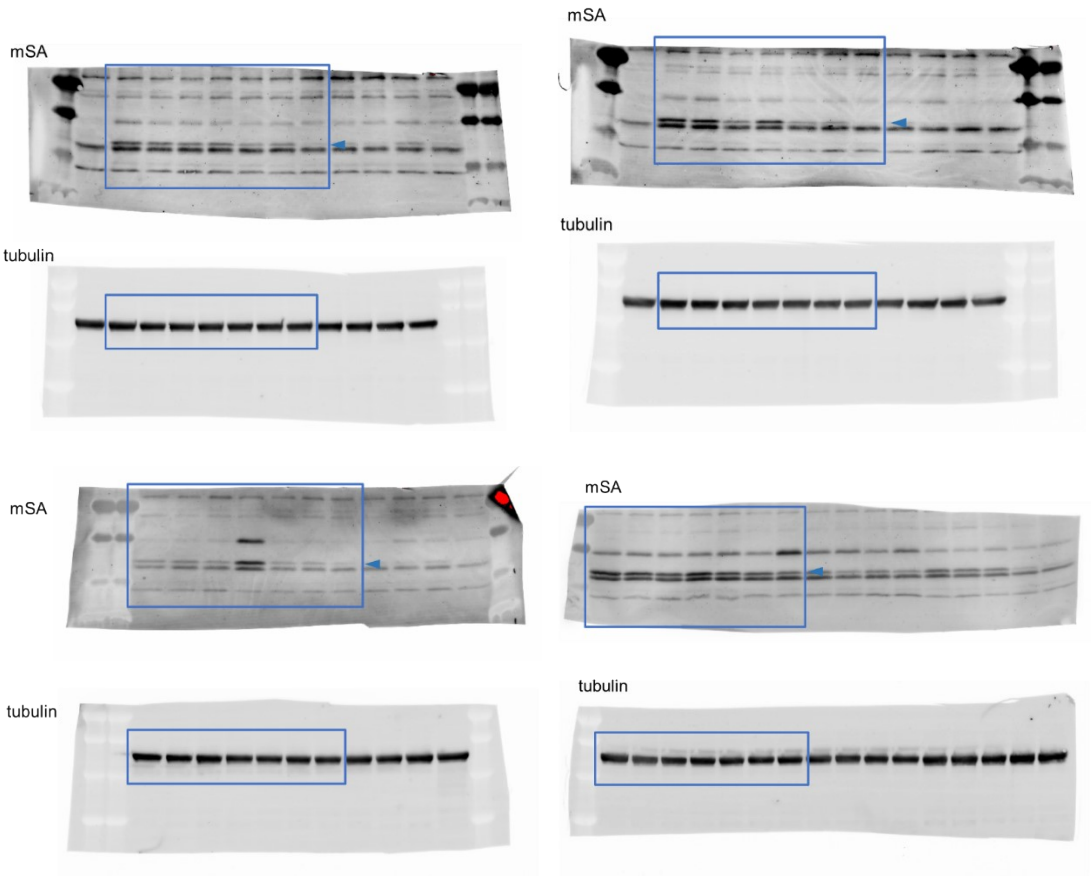
Uncropped western blot images for Fig. 5a,b.



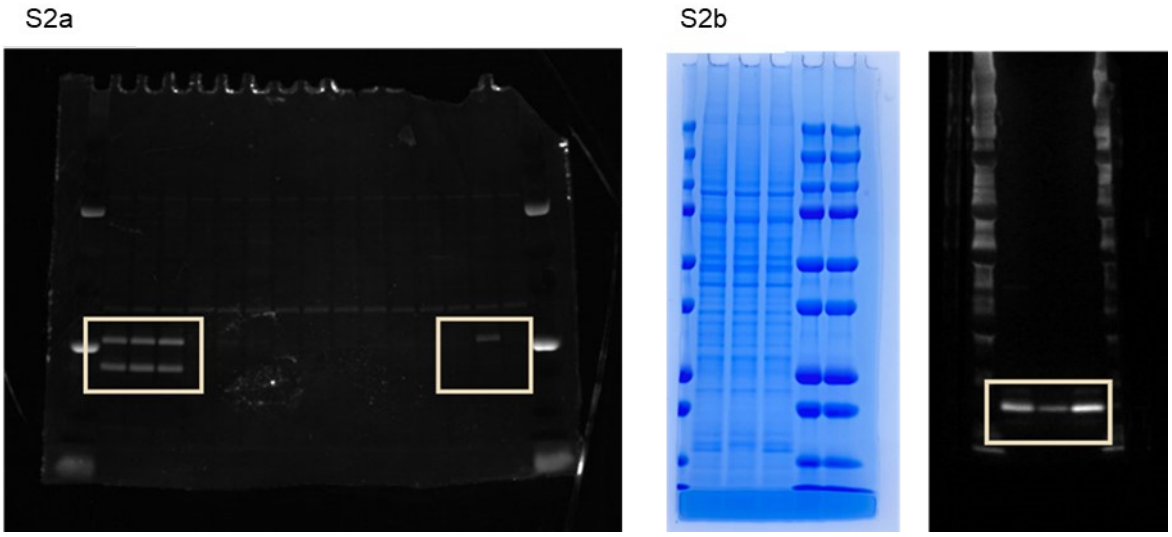
Uncropped western blot images for Fig. 6a.



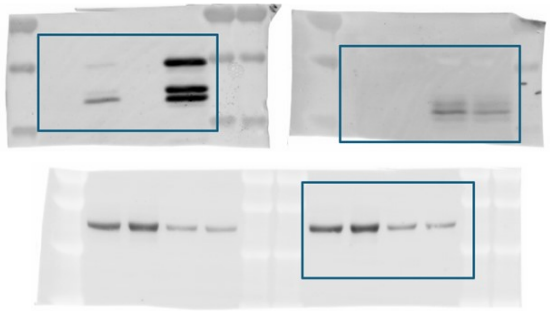
Uncropped western blot images for Fig. 7. (The bar graph in Fig. 7 shows the results of quantitative analysis of mSA band intensity (indicated by blue arrowhead). Tubulin band intensity was used for normalization (n = 4 measurements).



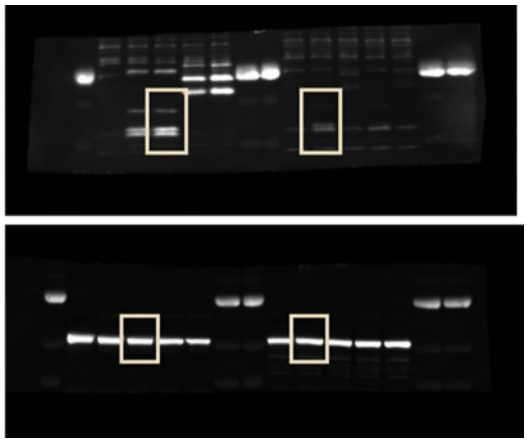
Uncropped western blot images for Fig. S2a,b.



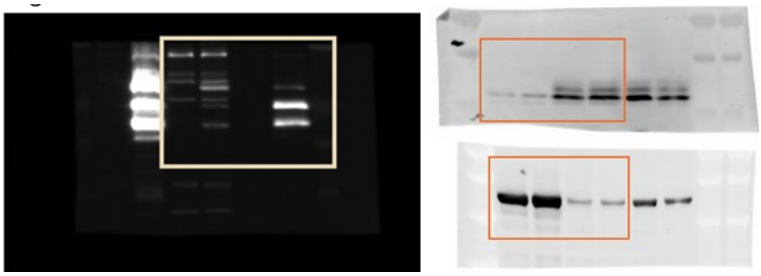
Uncropped western blot images for Fig. S3b.



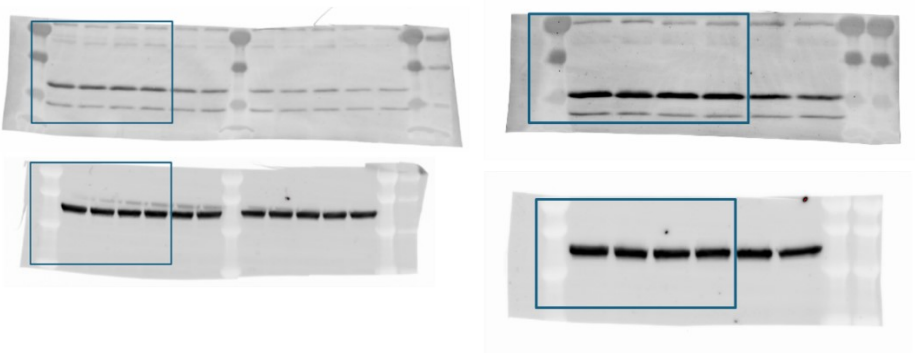
Uncropped western blot images for Fig. S4.



Uncropped western blot images for Fig. S7b.



Uncropped western blot images for Fig. S8b.



Experimental section

Chemistry

General

Reactions were performed in oven-dried glassware under positive nitrogen pressure, except where otherwise noted. All chemicals were obtained from commercial sources (Nacalai Tesque Inc., FUJIFILM Wako Pure Chemical Co., Tokyo Chemical Industry Co. Ltd., Sigma-Aldrich Co. LLC, Biosynth. Intl., Oakwood Products, Inc., Thermo Scientific) and used without further purification except in the cases of *N,N*-dimethylformamide (DMF), toluene, and ethanol. DMF and toluene were dried by passage through molecular sieves 4 Å (Nacalai Tesque Inc.). Ethanol was dried by passage through molecular sieves 3 Å (Nacalai Tesque Inc.).

NMR spectra were recorded on a JEOL JNM ECA-600 spectrometer. Chemical shifts (δ) are reported in parts per million (ppm) relative to the residual solvent signals ($CDCl_3$: 7.26 for ^1H NMR, 77.16 for ^{13}C NMR, CD_3OD: 3.31 for ^1H NMR, 49.00 for ^{13}C NMR) or tetramethylsilane (TMS 0.00). Multiplicities are reported using the following abbreviations: s, singlet; d, doublet; t; triplet; q, quartet; quin, quintet; dd, double doublet; dt, double triplet; td, triple doublet; tt, triple triplet; m, multiplet; br, broad; *J*, coupling constants in hertz.

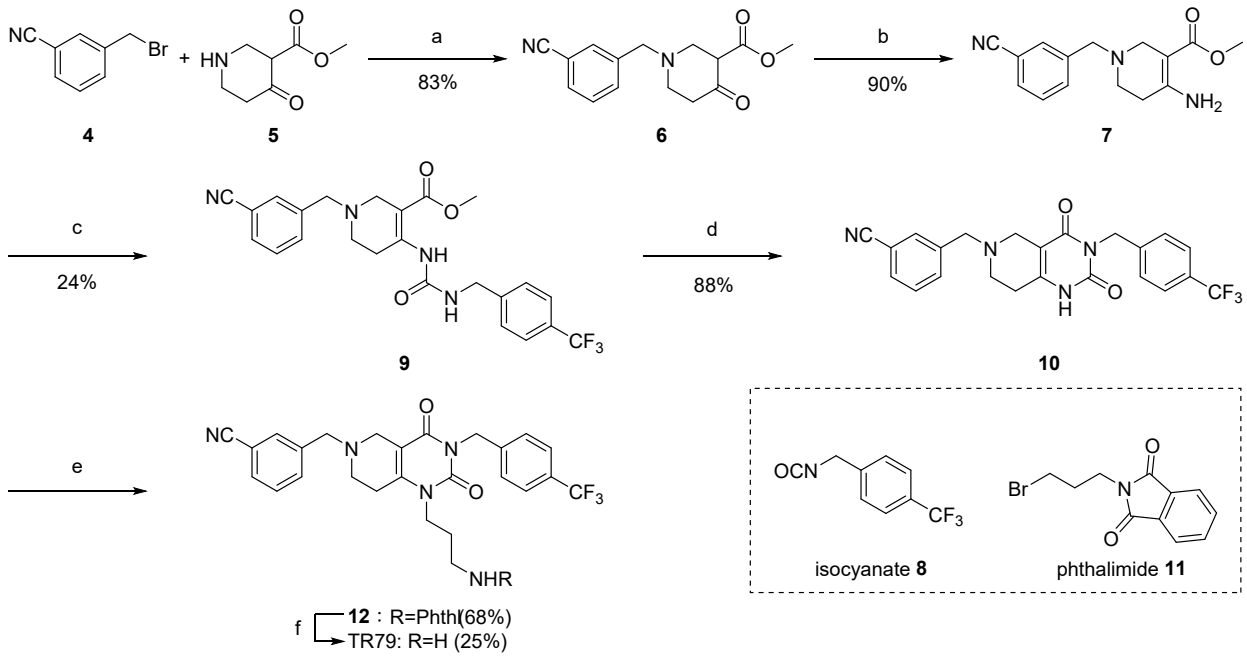
Analytical thin layer chromatography (TLC) was performed on glass plates of silica gel 60 F_{254} (Merck). Preparative thin layer chromatography (PTLC) was performed on glass plates of PLC silica gel 60 F_{254} (Merck). Flash chromatography was performed using forced flow of the indicated solvent system (Isolera, Biotage) on Silica gel 60N (Kanto Chemical. Co. Inc., Silica gel 60N, 40-50 μm). Solvent systems were hexane/AcOEt (H/A) or $CHCl_3$/MeOH (C/M).

Routine mass spectra (MS) and high-resolution mass spectra (HRMS) were recorded on Bruker ESI-TOF-MS (micrOTOF II).

Analytical HPLC was carried out using a JASCO PU-980 HPLC pump, and SSC-3315 degassing unit with a JASCO MD-2018 Plus photodiode array detector, SSC-2120 column oven, and JASCO LC-NetII/ADC interface box, with a C18 reverse phase column (Inertsil ODS-4, 150 × 4.6 mm, 5 μm (GL Science Inc.)). HPLC conditions: mobile phase A: 0.1% formic acid in H_2O, mobile phase B: MeOH (0-5 min: 5% B, 5-27 min: 5-100% B, 27-32 min: 100% B). The injection volume was 10 μL, the flow rate was 1.0 mL/min, and the column temperature was 40 °C. Compounds were detected and their purity was analyzed by measuring the absorbance at 280 nm.

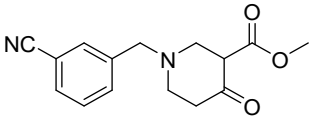
Preparative HPLC was carried out on a JASCO PU-4086 HPLC pump, JASCO LC-NetII/ADC interface box with a C18 reverse phase column (InertSustain, 20 × 250 mm, 5 μm (GL Science Inc.)), JASCO FV-4000-06 fraction valve unit, JASCO FCC fraction collector controller, JASCO UV-4075 UV/Vis detector, and JASCO CHF122sc fraction collector. HPLC conditions: mobile phase A: 0.1% FA in H_2O, mobile phase B: MeOH (0-5 min: 30% B, 5-50 min: 30-100% B, 50-70 min: 100% B). The injection volume was 10 μL, the flow rate was 1.0 mL/min, and the column temperature was room temperature. Compounds were detected by measuring the absorbance at the wavelength of 254 nm.

Synthesis



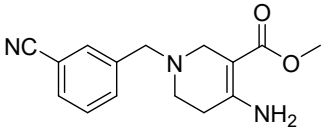
Scheme S1. Synthesis of TR79. (a) triethylamine, DMF, rt; (b) NH_3 aq., H_2O, 70 °C; (c) isocyanate **8**, triethylamine, toluene, 80 °C; (d) NaOMe, MeOH, reflux; (e) phthalimide **11**, K_2CO_3, KI, DMF, 100 °C; (f) $H_2NNH_2 \cdot H_2O$, EtOH, reflux.

Methyl 1-(3-cyanobenzyl)-4-oxopiperidine-3-carboxylate (6)



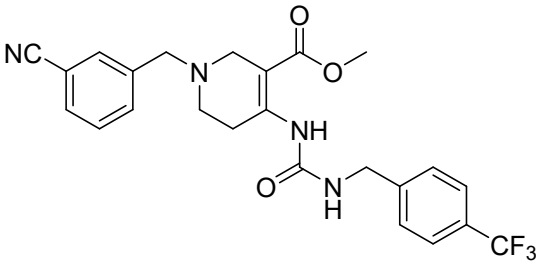
To a solution of methyl 4-oxopiperidine-3-carboxylate (**4**, 512 mg, 2.6 mmol) and 3-(bromomethyl)benzonitrile (**5**, 509 mg, 2.6 mmol) in DMF (15 mL) was added TEA (910 μL, 5.3 mmol), and the resultant solution was stirred at room temperature for 14 h. The reaction was quenched with brine, and the mixture was extracted with DCM (×2). The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by silica gel chromatography (H/A = 90/10 to 30/70) to afford **6** (594 mg, 83 %) as a colorless oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.67 (s, 1H), 7.60-7.56 (m, 2H), 7.44 (q, J = 7.8 Hz, 1H), 3.77 (s, 1H), 3.74 (s, 3H), 3.65 (s, 2H), 3.14 (t, J = 1.8 Hz, 2H), 2.63 (t, J = 6.6 Hz, 2H), 2.43 (t, J = 5.7 Hz, 2H). MS (ESI-TOF): 273 $[M + H]^+$.

Methyl 4-amino-1-(3-cyanobenzyl)-1,2,5,6-tetrahydropyridine-3-carboxylate (7)



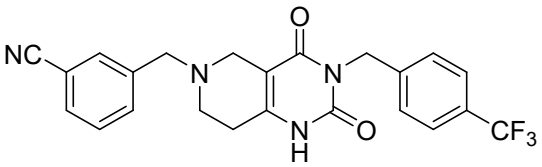
To a solution of **6** (303 mg, 1.11 mmol) in EtOH (5 mL) was added NH_3 aq. (28%, 3 mL), and the resultant solution was stirred at 70 °C for 14 h. The mixture was concentrated, diluted with water, and extracted with DCM (×4). The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to afford **7** (273 mg, 90%) as a yellow oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.68 (s, 1H), 7.60 (d, *J* = 8.3 Hz, 1H), 7.56 (d, *J* = 7.8 Hz, 1H), 7.31 (t, *J* = 7.8Hz, 1H), 3.66 (s, 3H), 3.64 (s, 2H), 3.18 (s, 2H), 2.56 (t, *J* = 5.7 Hz, 2H), 2.36 (t, *J* = 5.7 Hz, 2H). MS (ESI-TOF): 272 $[M + H]^+$

Methyl 1-(3-cyanobenzyl)-4-(3-(4-(trifluoromethyl)benzyl)ureido)-1,2,5,6-tetrahydropyridine-3-carboxylate (9)



To a solution of **7** (245 mg, 509 μmol) and TEA (189 μL, 1.358 mmol) in toluene (8 mL) was added 1-(isocyanatomethyl)-4-(trifluoromethyl)benzene (**8**, 182 mg, 509 μmol), and the resultant solution was stirred at 80 °C for 12 h. To the reaction mixture was added **8** (60.7 mg, 170 μmol), and the mixture was stirred at 80 °C for a further 8 h, then cooled to room temperature. The white solid was filtered off, and the filtrate was concentrated. The crude product was purified by silica gel chromatography (H/A = 84/16 to 0/100) to afford **7** (101 mg, 24%) as a yellow oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.66 (s, 1H), 7.61-7.56 (m, 4H), 7.45-7.38 (m, 3H), 4.46 (d, *J* = 6.0 Hz, 2H), 3.67 (s, 3H), 3.62 (s, 2H), 3.18 (s, 2H), 3.14 (t, *J* = 5.7 Hz, 2H), 2.57 (t, *J* = 6.0 Hz, 2H).

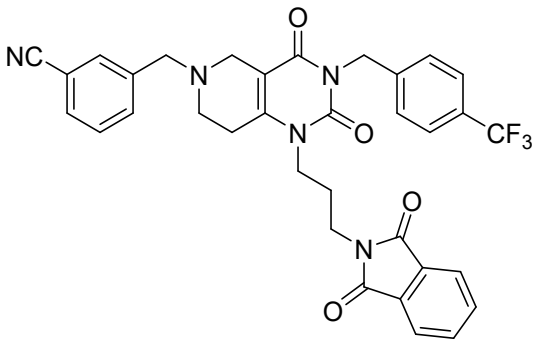
3-((2,4-Dioxo-3-(4-(trifluoromethyl)benzyl)-1,3,4,5,7,8-hexahydropyrido[4,3-d]pyrimidin-6(2*H*)-yl)methyl)benzonitrile (10)



To a solution of **9** (52.1 mg, 110 μmol) in MeOH (3 mL) was added NaOMe (28% MeOH solution, 800 μL, 551 μmol), and the resultant solution was stirred at reflux for 16 h. The mixture was concentrated, diluted with water, adjusted to pH 4.0 by the addition of 1 M aqueous HCl solution, and extracted with AcOEt (×4). The combined organic layers were dried over Na_2SO_4. Concentration of the filtrate under reduced pressure afforded **10** (42.9 mg, 88%) as a yellow oil. ^{1}H NMR (600 MHz, CD_3OD) δ 7.75 (s, 1H), 7.70 (d, *J* = 7.8 Hz, 1H), 7.65 (dt, *J* = 9.0, 1.2 1H), 7.59 (d, *J* = 8.3 Hz, 2H), 7.55-7.50 (m,3H), 5.12 (s, 2H), 3.77 (s, 2H), 3.25 (s, 2H), 2.76 (t, *J* = 6.0 Hz, 2H), 2.57 (t, *J* = 5.7 Hz,

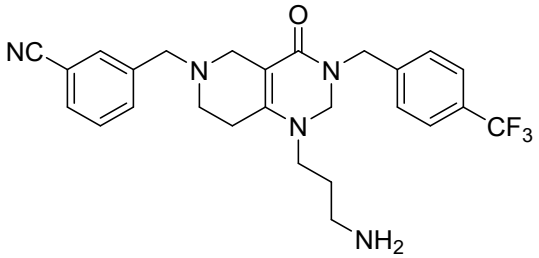
2H).

3-((1-(3-(1,3-Dioxoisoindolin-2-yl)propyl)-2,4-dioxo-3-(4-(trifluoromethyl)benzyl)-1,3,4,5,7,8-hexahydropyrido[4,3-d]pyrimidin-6(2*H*)-yl)methyl)benzonitrile (12)

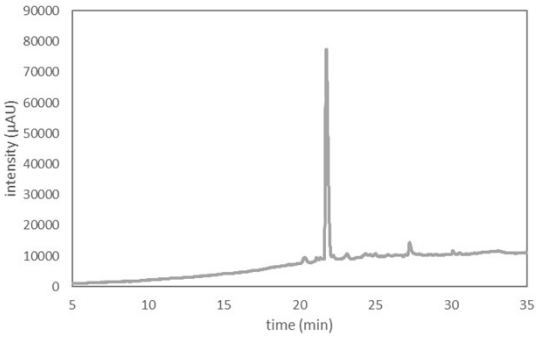


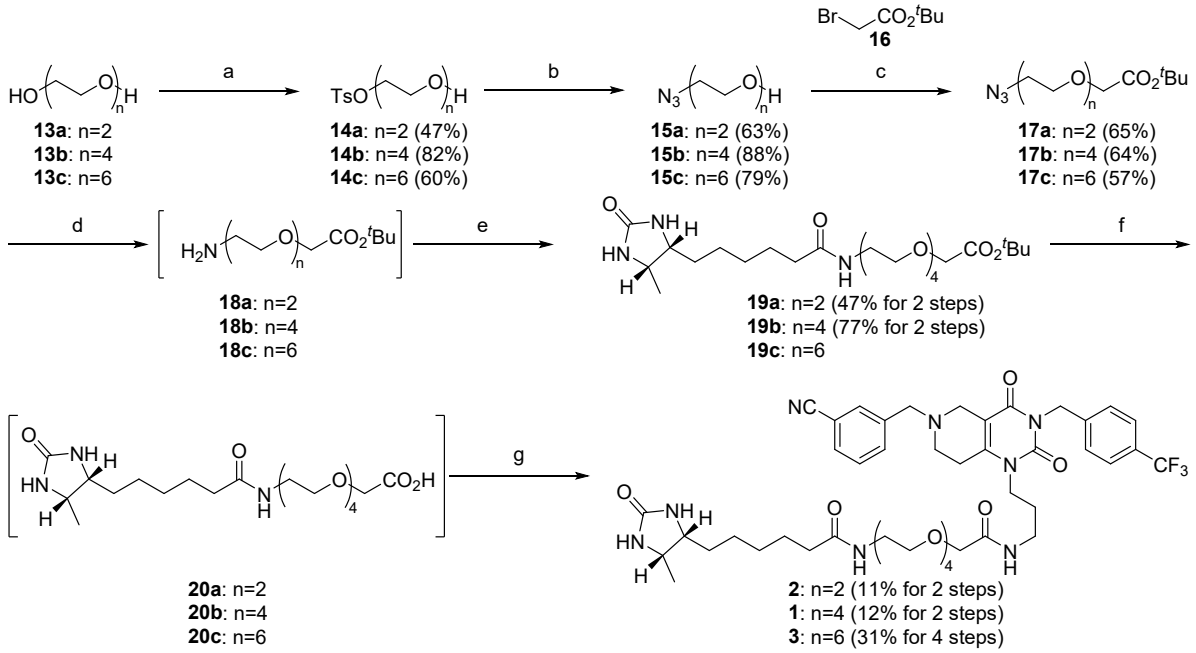
To a solution of **10** (23.7 mg, 53.8 μmol) and 2-(3-bromopropyl)isoindoline-1,3-dione (**11**, 15.9 mg, 59.3 μmol) in DMF (2 mL) were added potassium carbonate (8.0 mg, 58.0 μmol) and potassium iodide (18.2 mg, 110 μmol), and the mixture was stirred at 100 °C for 4 h. To the reaction mixture was added **9** (5.4 μg, 20.1 μmol), and the resultant mixture was stirred at 100 °C for a further 15 h. The reaction was quenched with water, and the mixture was extracted with EtOAc (×7). The combined organic layers were washed with brine (×2), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The crude product was purified by silica gel chromatography (H/A = 84/16 to 0/100) to afford **10** (23.0 mg, 68%) as a yellow oil. ^{1}H NMR (600 MHz, CD_3OD) δ 7.85-7.83 (m, 2H), 7.79-7.78 (m, 2H), 7.74 (s, 1H), 7.68 (d, J = 7.8 Hz, 1H), 7.65 (dt, *J* = 7.3 Hz, 1H), 7.56-7.51 (m, 3H), 7.46 (d, *J* = 8.3 Hz, 2H), 5.10 (s, 2H), 3.94 (t, *J* = 7.5 Hz, 2H), 3.74-3.73 (m, 4H), 3.25 (s, 2H), 2.75 (s, 4H), 2.07-2.03 (m, 1H). MS (ESI-TOF): 650 $[M + Na]^+$.

3-((1-(3-Aminopropyl)-2,4-dioxo-3-(4-(trifluoromethyl)benzyl)-1,3,4,5,7,8-hexahydropyrido[4,3-d]pyrimidin-6(2*H*)-yl)methyl)benzonitrile (TR79)



To a solution of **12** (12.2 mg, 19.4 μmol) in EtOH (300 μL) was added hydrazine hydrate (11.8 μL, 194 μmol), and the resultant solution was stirred at reflux for 2 h. The mixture was concentrated, diluted with water, and extracted with DCM (×4). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The crude product was purified by preparative HPLC to afford TR79 (2.4 mg, 25%) as a yellow oil. ^{1}H NMR (600 MHz, CD_3OD) was consistent with that reported[1]. Purity, 93%.





Scheme S2. Synthesis of WY165 (1), SN026 (2), and SN046 (3). (a) TsCl, NaOH, THF, 0 ºC to rt; (b) NaN_3, DMF, 60 ºC; (c) **16**, NaH, DMF, 0 ºC; (d) PPh_3, H_2O, THF, rt, or Pd/C, H_2, THF, rt; (e) desthiobiotin, EDC·HCl, DMAP, DMF, rt; (f) TFA, DCM, rt; (g) TR79, EDC·HCl, DMAP, $HOBt·H_2O$, DMF, rt.

General Procedure A: Mono-tosylation.

A solution of ethylene glycol **13** (1 eq.) and NaOH (2 eq.) in THF (0.2 M) was stirred on ice. To this mixture was added dropwise a solution of 4-methylbenzenesulfonyl chloride (1 eq.) in THF, and the reaction mixture was stirred at room temperature. The reaction was quenched with ice water, and the mixture was extracted with AcOEt. The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The crude product was purified by silica gel chromatography (hexane/ethyl acetate) to afford **14**.

General Procedure B: S_N2 reaction with sodium azide.

To a solution of **14** (1 eq.) in DMF was added sodium azide (1.5 eq.), and the resultant solution was

stirred at 60 °C. The precipitate was filtered off, and the filtrate was concentrated under reduced pressure. The crude product was purified by silica gel chromatography (hexane/ethyl acetate) to afford **15**.

General Procedure C: S_N2 reaction with *tert*-butyl bromoacetate (16).

To a solution of **15** (1 eq.) in THF was added NaH (4.5 eq.), and the resultant mixture was stirred on ice for 30 min. To this mixture, *tert*-butyl bromoacetate (**16**) (1.2 eq.) was added, and the slurry was stirred at room temperature for 19 h. The reaction was quenched with water, and the mixture was extracted with AcOEt. The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by silica gel chromatography (hexane/ethyl acetate) to afford **17**.

General Procedure D: Amide bond formation with desthiobiotin

A solution of desthiobiotin (2 eq.), EDC·HCl (2.5 eq.), and DMAP (4 eq.) in DMF was stirred on ice for 30 min. To this solution was added a solution of crude amine **18** (1 eq.) in DMF and the mixture was stirred at room temperature for a further 21 h. The mixture was concentrated under reduced pressure. The crude product was purified by silica gel chromatography (chloroform/methanol) to afford **19**.

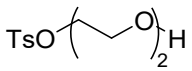
General Procedure E: Deprotection of tert-butyl ester.

To a solution of **19** in DCM was added excess TFA on ice. The resultant solution was allowed to warm to room temperature, stirred for 3 h, and then concentrated under reduced pressure. The crude carboxylic acid **20** was subjected to the next reaction without further purification.

General Procedure F: Amide bond formation with TR79

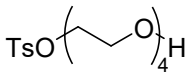
A solution of crude **20** (1 eq.), EDC·HCl (2 eq.), DIEA (5 eq.), HOBt·H_2O (4 eq.) and DMAP (1 eq.) in DMF was stirred on ice for 30 min. To the mixture was added a solution of TR79 (1 eq.) in DMF. The resulting mixture was stirred at room temperature, concentrated, diluted with water, and extracted with DCM. The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by HPLC (MeOH/0.1% FA aq.) to afford **1-3**.

2-(2-Hydroxyethoxy)ethyl 4-methylbenzenesulfonate (14a)



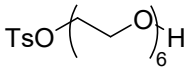
Compound **14a** (2.58 g, 47%), a colorless oil, was synthesized from **13a** (2.2 g, 21 mmol) by general procedure A: ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.83 (dd, *J* = 6.4, 1.8 Hz, 2H), 7.37 (d, *J* = 8.3 Hz, 2H), 4.23-4.21 (m, 2H), 3.72-3.68 (m, 4H), 3.56 (dd, *J* = 5.3, 3.9 Hz, 2H), 2.47 (s, 3H).

2-(2-(2-(2-Hydroxyethoxy)ethoxy)ethoxy)ethyl 4-methylbenzenesulfonate (14b)



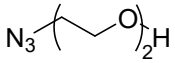
A solution of tetraethylene glycol **13b** (5.1 mg, 26 mmol) and NaOH (373 mg, 9.3 mmol) in THF (1 mL) was stirred on ice. To this mixture was added dropwise a solution of 4-methylbenzenesulfonyl chloride (943 mg, 4.9 mmol) in THF (10 mL), and the reaction mixture was stirred at room temperature for 2.5 h. The reaction was quenched with ice water, and the mixture was extracted with AcOEt. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The crude product was purified by silica gel chromatography (hexane/ethyl acetate) to afford **14b** (1.42 g, 82%) as a colorless oil: ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.80 (dd, *J* = 6.6, 1.8 Hz, 2H), 7.35 (d, *J* = 8.3 Hz, 2H) 4.17 (t, *J* = 4.8 Hz, 2H), 3.72-3.69 (m, 4H), 3.67-3.66 (m, 2H), 3.64-3.63 (m, 2H), 3.61-3.60 (m, 6H), 2.45 (s, 3H). MS (ESI-TOF): 371 $[M + Na]^+$.

17-Hydroxy-3,6,9,12,15-pentaoxaheptadecyl 4-methylbenzenesulfonate (14c)



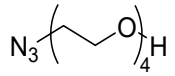
Compound **14c** (2.08 g, 60%), a colorless oil, was synthesized from **13c** (2.3 g, 8 mmol) by general procedure A: ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.80 (dd, *J* = 6.4, 1.8 Hz, 2H), 7.34 (d, *J* = 7.8 Hz, 2H), 4.16 (dd, *J* = 5.3, 4.4, 2H), 3.72-3.59 (m, 22H), 2.45 (s, 3H).

2-(2-Hydroxyethoxy)ethyl 4-methylbenzenesulfonate (15a)



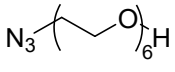
Compound **15a** (383.4 mg, 63%), a colorless oil, was synthesized from **14a** (1.2 g, 4.6 mmol) by general procedure B: ^{1}H NMR (600 MHz, $CDCl_3$) δ 3.80-3.77 (m, 2H), 3.74-3.72 (m, 2H), 3.65-3.64 (m, 2H), 3.44 (t, *J* = 4.8 Hz, 2H), 2.06 (t, *J* = 6.2 Hz, 1H).

2-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy)ethan-1-ol (15b)



Compound **15b** (321 mg, 88%), a colorless oil, was synthesized from **14b** (583 mg, 1.67 mmol) by general procedure B: ^{1}H NMR (600 MHz, $CDCl_3$) δ 3.75-3.72 (m, 2H), 3.70-3.67 (m, 10H), 3.63-3.61 (m, 2H), 3.41 (t, *J* = 5.0 Hz, 2H). MS (ESI-TOF): 242 $[M + Na]^+$.

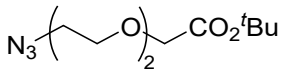
17-Azido-3,6,9,12,15-pentaoxaheptadecan-1-ol (15c)



Compound **15c** (295.9 mg, 79%), a colorless oil, was synthesized from **14c** (530 mg, 1.21 mmol) by

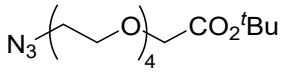
general procedure B: ^{1}H NMR (600 MHz, $CDCl_3$) δ 3.72 (s, 2H), 3.70-3.65 (m, 18H), 3.61 (td, *J* = 3.7, 1.7 Hz, 2H), 3.39 (t, *J* = 5.0 Hz, 2H).

***tert*-Butyl 2-(2-(2-azidoethoxy)ethoxy)acetate (17a)**



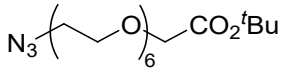
Compound **17a** (136.8 mg, 65%), a colorless oil, was synthesized from **15a** (113.3 mg, 864 μmol) by general procedure C: ^{1}H NMR (600 MHz, $CDCl_3$) δ 4.04 (s, 2H), 3.74-3.69 (m, 6H), 3.41 (t, *J* = 4.8 Hz, 6H), 1.48 (s, 9H).

***tert*-Butyl 14-azido-3,6,9,12-tetraoxatetradecanoate (17b)**



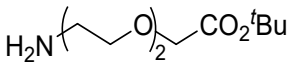
Compound **17b** (75.8 mg, 64%), a colorless oil, was synthesized from **15b** (78.5 mg, 358 μmol) by general procedure C: ^{1}H NMR (600 MHz, $CDCl_3$) δ 4.02 (s, 2H), 3.72-3.66 (m, 14H), 3.39 (t, *J* = 5.6 Hz, 2H), 1.48 (s, 9H). MS (ESI-TOF): 356 $[M + Na]^+$.

***tert*-Butyl 20-azido-3,6,9,12,15,18-hexaoxaicosanoate (17c)**



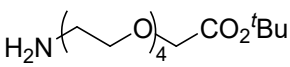
Compound **17c** (235 mg, 57%), a colorless oil, was synthesized from **15c** (300 mg, 976 μmol) by general procedure C: ^{1}H NMR (600 MHz, $CDCl_3$) δ 4.02 (s, 2H), 3.71-3.66 (m, 22H), 3.39 (t, *J* = 5.1 Hz, 2H), 1.48 (s, 9H).

***tert*-Butyl 2-(2-(2-aminoethoxy)ethoxy)acetate (18a)**



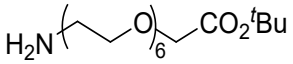
A solution of **17a** (100 mg, 408 μmol) and 10% palladium on carbon (10 mg, 94.0 μmol) in THF (5 mL) was stirred at rt for 7 h. The mixture was filtered through a Celite pad and the filtrate was concentrated under reduced pressure. The crude product was subjected to the next reaction without further purification.

***tert*-Butyl 14-amino-3,6,9,12-tetraoxatetradecanoate (18b)**



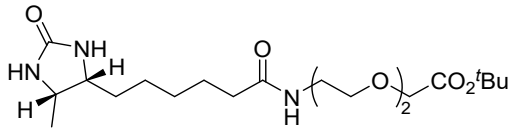
A solution of **17b** (22.7 mg, 68.1 μmol) and triphenylphosphine (19.6 mg, 74.9 μmol) in THF (1 mL) and water (600 μL) was stirred at room temperature for 2 days. The mixture was concentrated under reduced pressure. The crude product was subjected to the next reaction without further purification.

***tert*-Butyl 20-amino-3,6,9,12,15,18-hexaoxaicosanoate (18c)**



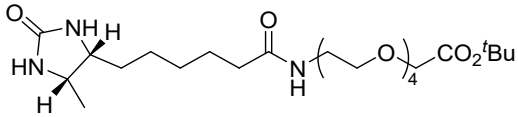
A solution of **17c** (229.4 mg, 544 μmol) and palladium on carbon (23.6 mg, 199 μmol) in THF (10 mL) was stirred at rt for 5.5 h. The mixture was filtered through a Celite pad and the filtrate was concentrated under reduced pressure. The crude product was subjected to the next reaction without further purification.

***tert*-Butyl 2-(2-(2-(6-(5-methyl-2-oxoimidazolidin-4-yl)hexanamido)ethoxy)ethoxy)acetate (19a)**



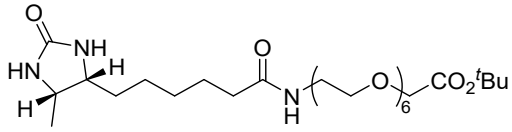
Compound **19a** (70.1 mg, 47% for 2 steps), a yellow oil, was synthesized from **18a** (79.0 mg, 360 μmol) by general procedure D: ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.56 (s, 1H), 5.07 (s, 1H), 4.55 (s, 1H), 4.01 (s, 2H), 3.83 (quin, *J* = 6.9 Hz, 1H), 3.72-3.66 (m, 4H), 3.59 (t, *J* = 5.1 Hz, 2H), 3.49 (s, 1H), -3.46 (q, *J* = 5.2 Hz, 2H), 2.21 (t, *J* = 7.3 Hz, 2H), 1.66 (t, *J* = 7.2 Hz,2H), 1.51-1.24 (m, 15H), 1.13 (d, *J* = 6.4 Hz, 3H).

***tert*-Butyl 21-((4*R*,5*S*)-5-methyl-2-oxoimidazolidin-4-yl)-16-oxo-3,6,9,12-tetraoxa-15-azahenicosanoate (19b)**



Compound **19b** (26.5 mg, 77% for 2 steps), a white solid, was synthesized from **18b** (20.9 mg, 68.0 μmol) by general procedure D: ^{1}H NMR (600 MHz, $CDCl_3$) 4.00 (s, 2H), 3.82 (t, *J* = 6.9 Hz, 1H), 3.71-3.60 (m, 13H), 3.55 (t, *J* = 5.0 Hz, 2H), 3.43 (q, *J* = 5.2 Hz, 2H), 2.18 (t, *J* = 7.6 Hz, 2H), 1.64 (quin, *J* = 7.3 Hz, 2H), 1.46 (s, 9H), 1.39-1.32 (m, 3H), 1.28-1.23 (m, 1H), 1.11 (d, *J* = 6.4 Hz, 3H). MS (ESI-TOF): 526 $[M + Na]^+$.

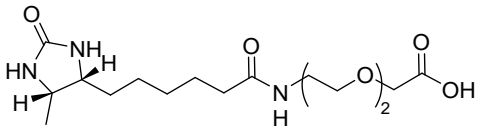
***tert*-Butyl 27-(5-methyl-2-oxoimidazolidin-4-yl)-22-oxo-3,6,9,12,15,18-hexaoxa-21-azaheptacosanoate (19c)**



Crude **19c** was obtained from **18c** (210 mg, 531 μmol) by general procedure D. The crude material was used in the next step without further purification: ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.42 (br, 1H), 4.98 (br, 1H), 4.47 (br, 1H), 4.02 (s, 2H), 3.84 (quin, *J* = 6.9 Hz, 1H), 3.72-3.61 (m, 20H), 3.56 (t, *J* =

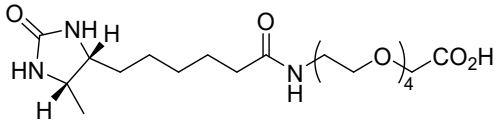
5.0 Hz, 2H), 3.44 (q, *J* = 5.2 Hz, 2H), 2.19 (t, *J* = 7.5 Hz, 2H), 1.66 (quin, *J* = 7.2 Hz, 2H), 1.53-1.23 (m, 15H), 1.12 (d, J = 6.6 Hz, 3H).

2-(2-(2-(6-(5-Methyl-2-oxoimidazolidin-4-yl)hexanamido)ethoxy)ethoxy)acetic acid (20a)



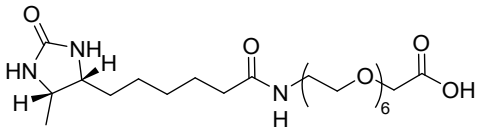
Crude **20a** was obtained from **19a** (46.7 mg, 112 μmol) by general procedure E. The crude material was used in the next step without further purification.

21-((4*R*,5*S*)-5-Methyl-2-oxoimidazolidin-4-yl)-16-oxo-3,6,9,12-tetraoxa-15-azahenicosanoic acid (20b)



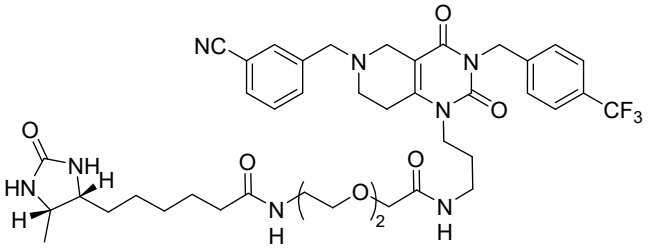
Crude **20b** was obtained from **19a** (26.5 mg, 52.6 μmol) by general procedure E. The crude material was used in the next step without further purification.

27-(5-Methyl-2-oxoimidazolidin-4-yl)-22-oxo-3,6,9,12,15,18-hexaoxa-21-azaheptacosanoic acid (20c)



Crude **20c** was obtained from crude **19c** (58.4 mg, 98.7 μmol) by general procedure E. The crude material was used in the next step without further purification.

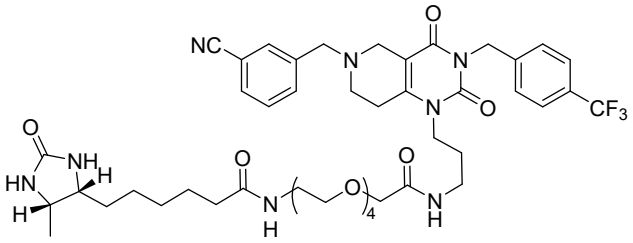
SN026 (2)



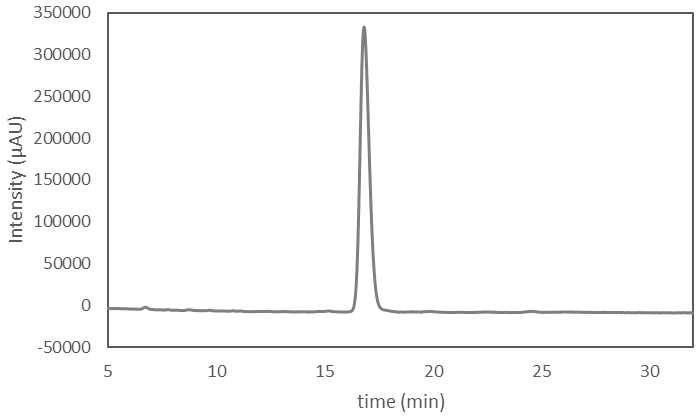
SN026 (6.8 mg, 11% for 2 steps), a yellow oil, was synthesized from **20a** (36.0 mg, 72.4 μmol) by general procedure F: ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.67 (s, 1H), 7.58-7.51 (m, 5H), 7.44 (t, *J* = 7.8 Hz, 1H), 6.2 (t, *J* = 5.3 Hz, 1H), 5.15 (d, *J* = 8.7 Hz, 2H), 4.01 (s, 2H), 3.89 (t, *J* = 7.3 Hz, 1H), 3.82 (quin, *J* = 7.3 Hz, 1H), 3.70-3.63 (m, 6H), 3.54 (t, *J* = 5.3 Hz, 2H), 3.45-3.42 (m, 2H), 3.35 (t, *J* = 6.0 Hz, 2H), 3.33 (br, 2H), 2.73 (t, *J* = 5.3 Hz, 1H), 2.69 (d, *J* = 4.6 Hz, 1H), 2.17 (t, *J* = 7.3 Hz, 2H), 1.88

(quin, J = 7.1 Hz, 2H), 1.63 (quin, J = 7.2 Hz, 2H), 1.49-1.24 (m, 8H), 1.11 (d, J = 6.6 Hz, 3H); ^{13}C NMR (600 MHz, $CDCl_3$) δ 173.1, 170.2, 163.2, 161.1, 151.8, 146.6, 140.7, 139.3, 133.2, 132.3, 131.2, 129.3, 129.0, 129.0, 125.4, 125.4, 118.8, 112.7, 107.8, 71.1, 70.7, 70.2, 70.1, 61.3, 55.9, 51.4, 49.5, 49.0, 44.1, 41.8, 39.1, 36.1, 35.9, 29.7, 29.5, 29.2, 28.8, 26.9, 26.0, 25.3, 15.8; HRMS (ESI-TOF): calcd for $C_{42}H_{53}F_3N_8O_7Na^+$ $[M+Na]^+$: 861.3882; found: 861.3894; Purity, 96%.

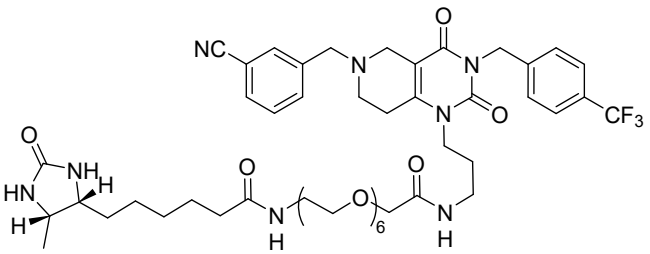
WY165 (1)



WY165 (7.9 mg, 16% for 2 steps), a yellow oil, was synthesized from **20b** (23.5 mg, 52.5 μmol) by general procedure F: ^{1}H NMR (600 MHz, CD_3OD) δ 7.77 (s, 1H), 7.70 (d, J = 7.8 Hz, 1H), 7.66 (d, J = 7.8 Hz, 1H), 7.59 (d, J = 8.3 Hz, 2H), 7.55-7.50 (m, 3H), 5.17 (s, 2H), 3.99 (s, 2H), 3.92 (t, J = 7.6 Hz, 2H), 3.80 (dd, J = 7.8, 6.4 Hz, 1H), 3.76 (s, 2H), 3.69-3.56 (m, 14H), 3.50 (t, J = 5.5 Hz, 2H), 3.34 (t, J = 5.5 Hz, 1H), 3.30-3.28 (m, 1H), 2.78 (d, J = 6.9 Hz, 4H), 2.18 (t, J = 7.6 Hz, 2H), 1.87 (quin, J = 7.3 Hz, 2H), 1.63-1.59 (quin, 2H), 1.49-1.27 (m, 6H), 1.09 (d, J = 6.4 Hz, 3H); ^{13}C NMR (600 MHz, CD_3OD) δ 175.0, 174.9, 171.6, 164.8, 161.8, 151.6, 148.4, 141.6, 139.7, 133.7, 132.4, 131.0, 129.3, 128.3, 125.0, 125.0, 118.4, 112.2, 106.5, 70.7, 70.2, 70.1, 70.1, 69.9, 69.9, 69.3, 60.5, 56.0, 51.4, 49.0, 48.7, 43.7, 41.8, 39.1, 39.0, 35.9, 35.8, 35.6, 35.6, 29.4, 28.9, 28.4, 26.2, 25.8, 25.5, 14.3; HRMS (ESI-TOF): calcd for $C_{46}H_{61}F_3N_8O_9$ $[M + Na]^+$: 949.4406; found: 949.4404; Purity, 95%.



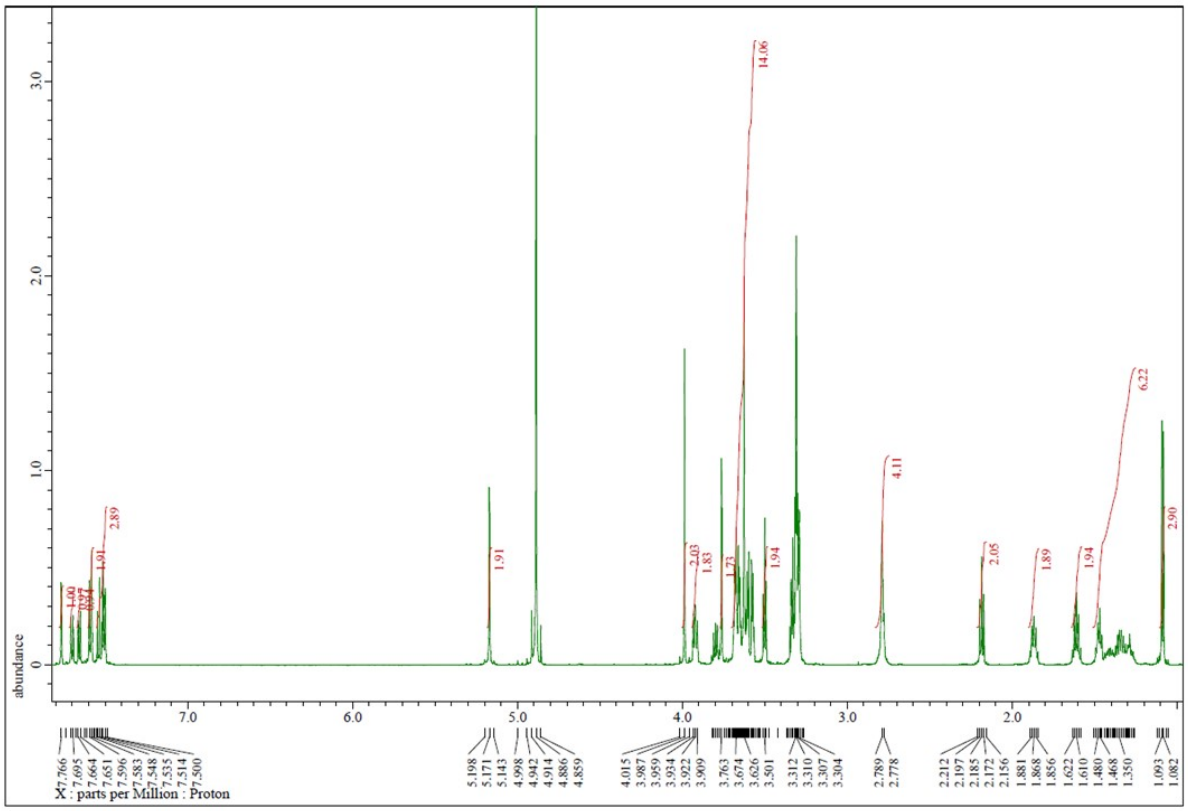
SN046 (3)



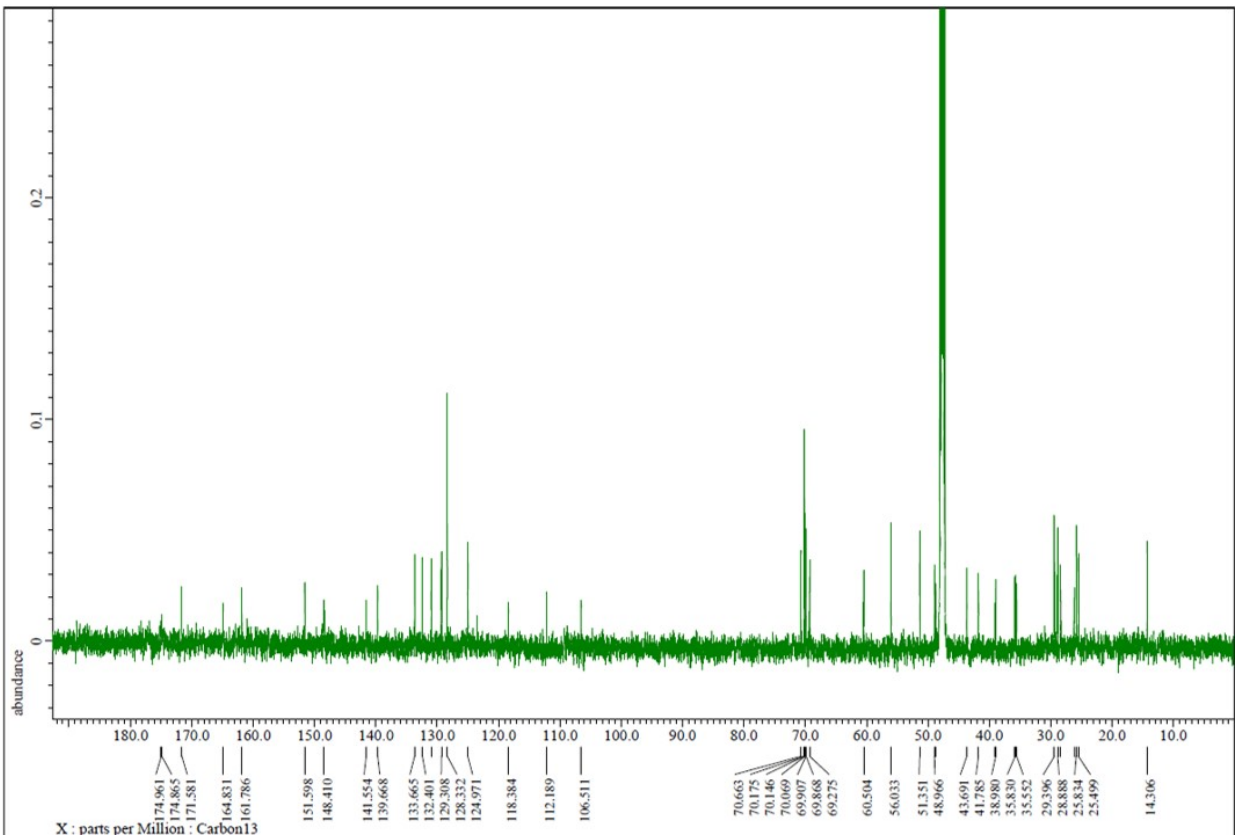
SN046 (22.7 mg, 31 % for 4 steps), a yellow oil, was synthesized from **20c** (52.9 mg, 71.2 μmol) by general procedure F: ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.67 (s, 1H), 7.58-7.54 (m, 5H), 7.44 (t, J = 7.6 Hz, 2H), 6.56-6.43 (br, 1H), 5.14 (s, 1H), 4.17 (s, 1H), 3.99 (s, 1H), 3.88-3.82 (m, 2H), 3.75-3.60 (m, 34H), 3.56-3.54 (m, 2H), 3.46-3.42 (m, 2H), 3.34-3.32 (m, 2H), 2.70 (dd, *J* = 20.4, 4.8 Hz, 2H), 2.19 (td, *J* = 7.3, 4.6 Hz, 2H), 1.87 (quin, *J* = 7.2 Hz, 2H), 1.50-1.45 (m, 2H), 1.38-1.34 (m, 2H), 1.29-1.22 (m, 2H), 1.12 (t, *J* = 6.6 Hz, 3H); ^{13}C NMR (600 MHz, $CDCl_3$) δ 173.2, 170.9, 170.4, 163.5, 161.1, 151.5, 146.6, 139.3, 133.2, 132.3, 131.2, 129.3, 129.1, 125.3, 125.3, 112.6, 107.4, 71.0, 70.9, 70.6, 70.5, 70.5, 70.3, 70.1, 70.0, 68.6, 61.3, 56.1, 56.0, 51.8, 51.4, 49.5, 49.1, 44.1, 42.0, 39.2, 36.0, 29.5, 29.2, 28.8, 26.8, 26.0, 25.9 25.3, 15.8; HRMS (ESI-TOF): calcd for $C_{50}H_{69}F_3N_8O_{11}$ $[M+Na]^+$: 1037.4930; found: 1037.4924; Purity, 95%.

NMR spectra

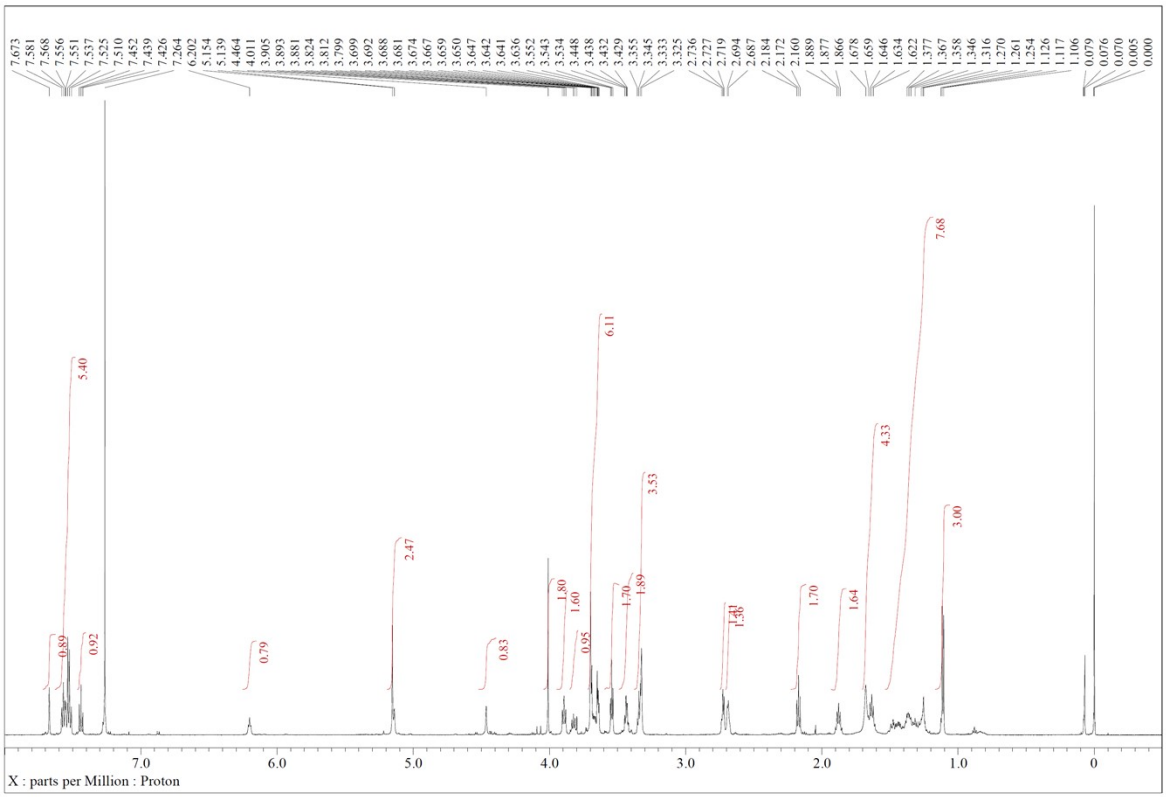
^{1}H NMR ($CDCl_3$, 600 MHz) of WY165



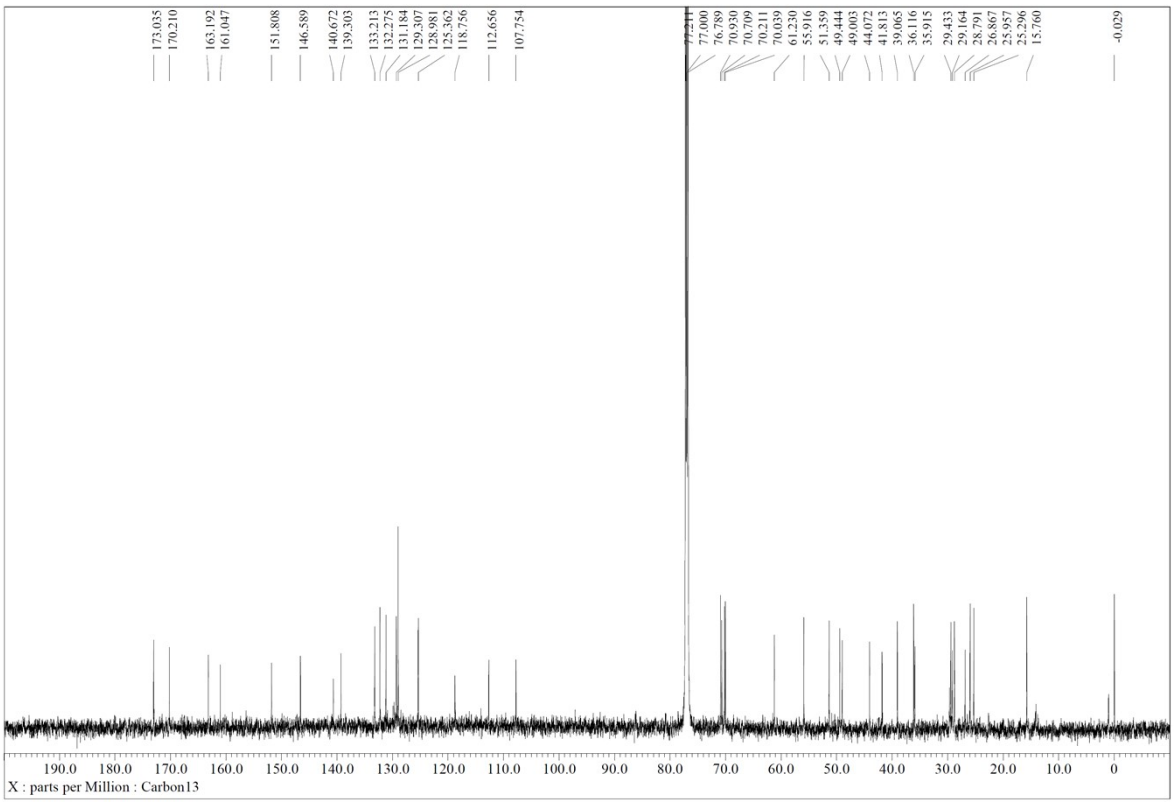
^{13}C NMR ($CDCl_3$, 150 MHz) of WY165



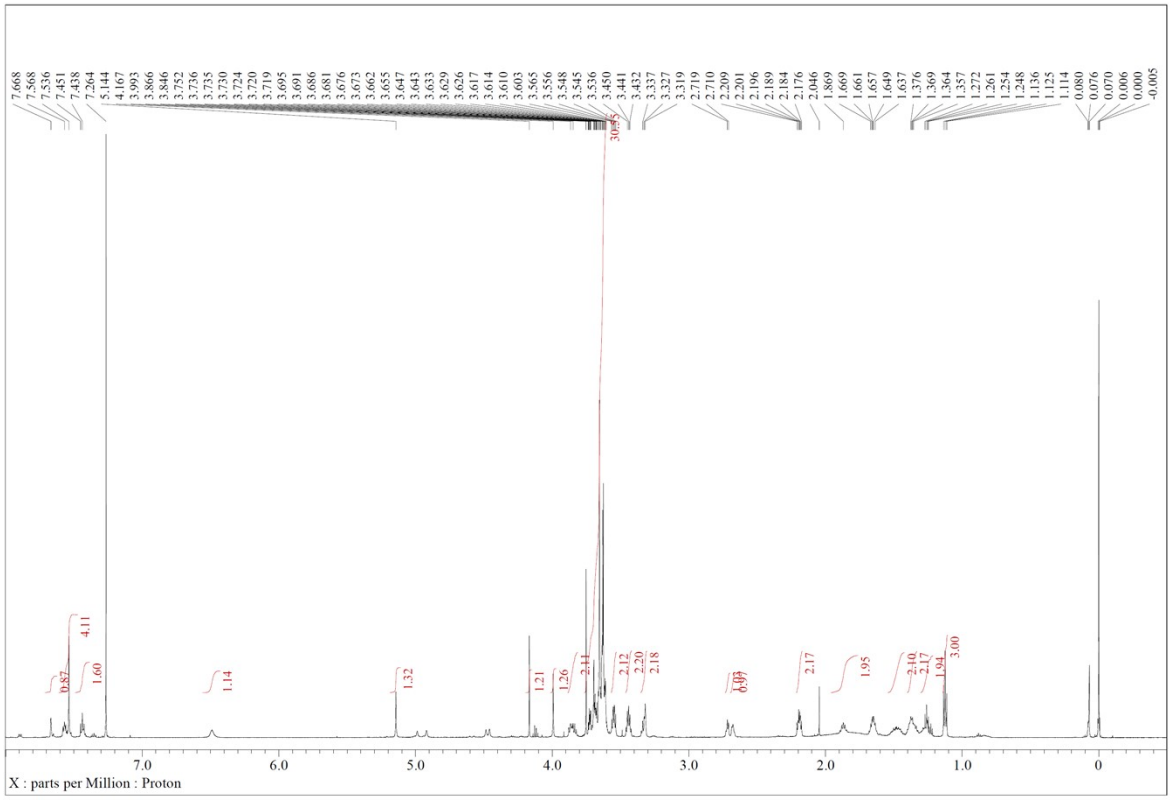
^{1}H NMR ($CDCl_3$, 600 MHz) of SN026



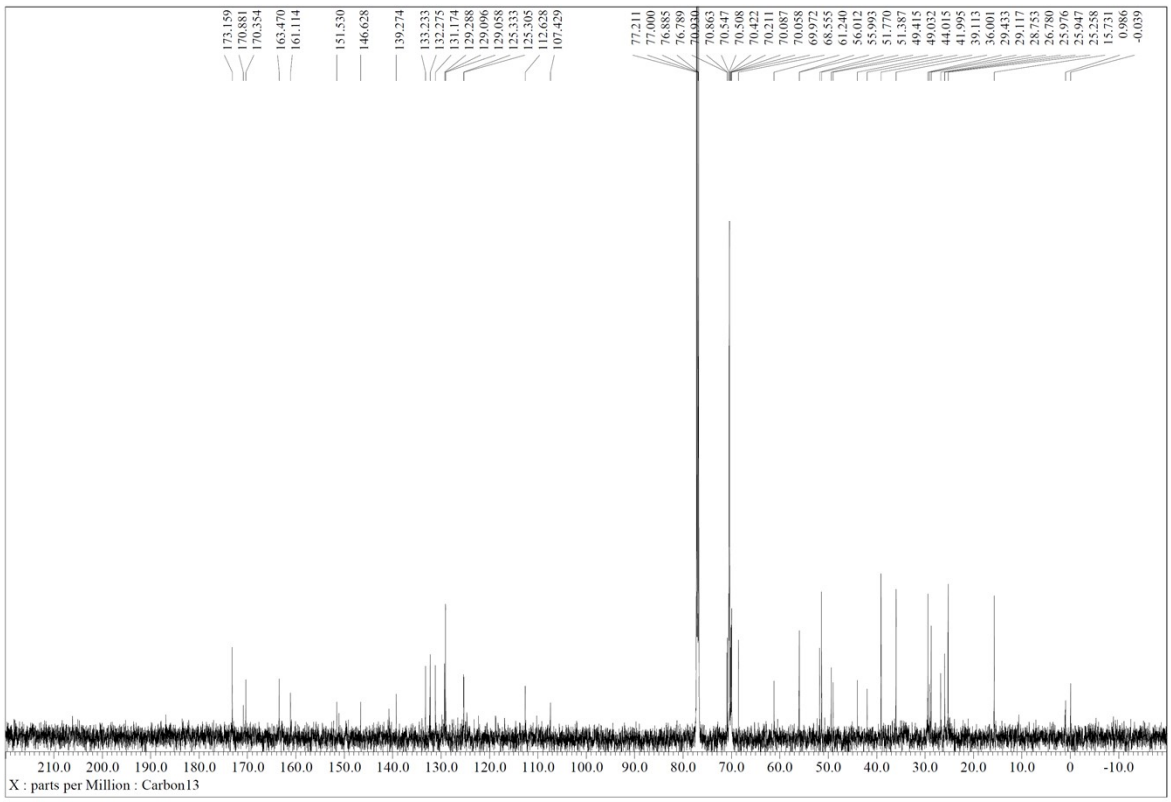
^{13}C NMR ($CDCl_3$, 150 MHz) of SN026



^{1}H NMR ($CDCl_3$, 600 MHz) of SN046



^{13}C NMR ($CDCl_3$, 150 MHz) of SN046



Biology

SDS-PAGE/Western Blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using SuperSep™ Ace 10-20 % (Wako Pure Chemical Industries). Gels were stained with a silver stain kit (Wako Pure Chemical Industries), Oriol (BioRad), or Coomassie Brilliant Blue (CBB). For western blotting, proteins in the gel were transferred onto LF PVDF membrane (BioRad). After blocking with Bullet Blocking One (Nacalai tesque), the membrane was washed with TBS-T (20 mM Tris base, 0.15 M NaCl, pH 7.6) and stained with the primary antibody (anti-FLAG M2 monoclonal antibody (Sigma, 1:1000), anti-His6 antibody (Cell Signaling Technology, 1:1000), anti-tubulin antibody (Cell Signaling Technology, 1:1000), anti-Tom20 antibody (Cell Signaling Technology, 1:1000), and anti-ClpP antibody (Cell Signaling Technology, 1:1000)) in Can Get Signal™ Immunoreaction Enhancer Solution 1 (TOYOBO) for 6 h-O/N at 4 °C. The membrane was then washed with TBS-T×3 and stained with secondary antibodies, goat anti-mouse IgG or goat anti-rabbit IgG conjugated with Star Bright 700 (BioRad, 1:5000) in Can Get Signal™ Immunoreaction Enhancer Solution Solution 2 (TOYOBO) for 6 h-overnight at 4 °C. Finally, the membrane was washed with TBS-T×3 and bands were detected with ChemiDoc Touch MP (BioRad).

Proteomics analysis for cell lysate supplemented with mSA and ClpP

NanoLC-MS/MS was carried out on a system comprising a quadrupole time-of-flight mass spectrometer (Triple TOF® 5600 system; SCIEX) equipped with a nanospray ion source and a nanoLC system (Eksigent Nano LC Ultra 1D Plus; SCIEX, Massachusetts, U.S.A.). The trap column was a NanoLC Trap ChromXP C18, 3 μm 120 Å (SCIEX) and the separation column was a 12.5 cm × 75 μm capillary column packed with 3 μm C18-silica particles (Nikkyo Technos Co., Ltd., Japan). The micropump (flow rate 300 nL/min) gradient was as follows: mobile phase A: 2% acetonitrile, 0.1% formic acid, mobile phase B: 80% acetonitrile, 0.1% formic acid aq. 0–20 min: 5–45% B, 20−21 min: 45–100% B, 21–26 min: 100% B. The nanoLC-MS/MS data were acquired in an information-dependent acquisition mode controlled by Analyst® TF 1.5.1 software (SCIEX). The settings of the data-dependent acquisition were as follows: accumulation time was 0.25 s; the full MS (MS1, TOF-MS) scan range was 400–1250 m/z; exclusion time of former target ion, 12 s; mass tolerance, 50 mDa. The top 10 signals were selected for MS2 scan per one full MS scan. The MS2 (product ion) scan accumulation time and range were 0.05 s and 100–1500 m/z. Experiments were conducted in triplicate. The MS/MS spectra were searched against the respective amino acid sequence using MaxQuant (freeware) with a default setting. A FASTA file corresponding to the protein sequence was used.

Proteome-wide analysis of 1-incuded change in HeLa cell-derived proteins

NanoLC-MS/MS analysis was performed on a system comprising a quadrupole, orbitrap, and ion trap Tribrid mass spectrometer (Orbitrap Fusion; Thermo Fisher Scientific) equipped with a nanospray ion source and a nano HPLC system (Easy-nLC 1000; Thermo Fisher Scientific). The trap column was a

2 cm × 75 μm capillary column packed with 3 μm C18-silica particles (Thermo Fisher Scientific) and the separation column was a 12.5 cm × 75 μm capillary column packed with 3 μm C18-silica particles (Nikkyo Technos Co., Ltd.). The micropump (flow rate: 300 nL/min) gradient was as follows: mobile phase A: 0.1% formic acid; mobile phase B: 80% acetonitrile, 0.1% formic acid aq. 0–1 min: 0–5% B, 1−61 min: 5–40% B, 61−63 min: 40–95% B, 63–80 min: 95% B. The LC-MS/MS data were acquired in a data-dependent acquisition mode controlled by Xcalibur 4.3 (Thermo Fisher Scientific). The settings for the data-dependent acquisition were as follows: maximum injection time, 0.05 s; full MS (MS1, orbitrap) scan range, 375–1500 m/z, exclusion time for former target ion, 20 s; and mass tolerance, 10 ppm. The top speed cycle time was set to 3 s and the maximum MS2 scan could be acquired within that time. The MS2 (isolation: quadrupole, detection: ion trap) scan maximum injection time and range were 0.035 s and the m/z range defined based on the MS1 scan, respectively. The measurement was performed three times for each sample.

The identification and label-free quantification of the proteins were conducted by using the Proteome Discoverer 3.0 software embedded with the Sequest algorithm (Thermo Fisher Scientific). The list of the human proteins was obtained from the UniProt database (taxonomy 9606; downloaded on Sep. 13, 2023). The labled-free quantification was performed using the workflow "PWF_Tribrid_Precursor_Quan_and_LFQ_IT HCD_SequestHT_Percolator" for processing step, and "CWF_Comprehensive_Enhanced Annotation_LFQ_and_Precursor_Quan" for consensus step.

ClpP binding and activation assay *in vitro*[1]

ClpP (Cat#NBP1-72319, Novus Biologicals) enzymatic activity was measured by monitoring the release of fluorescent coumarin from the fluorogenic substrate Ac-WLA-AMC (7-amino-4-methylcoumarin) (Cat#S330, Boston Biochem, Inc.) as described previously[1]. Briefly, 12.5 μg/mL ClpP was dissolved in 24 μL of ClpP assay buffer (50 mM Tris, 10 mM $MgCl_2$, 100 mM KCl, 1 mM DTT, 4 mM ATP, 0.02% Triton X-100, and 5% glycerol, pH 8.0 (HCl)) mixed with 6 μM test compound in 5 μL of ClpP assay buffer (0.5% DMSO) and incubated at rt for 60 min. Then 250 μM Ac-WLA-AMC in 1 μL of ClpP assay buffer (2.5% DMSO) was added to 24 μL of the mixture in 96-well half-black plates and incubated at 37°C. The fluorescence of released AMC was recorded at 350 nm excitation and 460 nm emission using Envision (PerkinElmer) every 1 min for 1 h then every 1 h for 4 h. Measurements were carried out in triplicate and the activity of samples treated with the vehicle (DMSO) was measured as the background and subtracted from the experimental data.

ClpP pull-down assay

Streptavidin-conjugated beads (Dynabeads™ MyOne™ Streptavidin C1, Invitrogen) were washed with ClpP assay buffer and mixed with 1 μM ClpP and 1.85 μM dihydrofolate reductase pre-incubated with 1 μM test compound in the assay buffer (20 μL, final 0.5% DMSO) for 1 h at rt in a tube shaker. The reaction mixture was harvested as a flow-through fraction and the beads were washed with the assay buffer three times. Then 300 μM TR79 (20 μL, final 3% DMSO) was added and the mixture

was stirred at rt for 1 h. Flow-through and eluted fractions were analyzed by SDS-PAGE and bands were stained with CBB.

mSA degradation *in vitro* using compound WY165

ClpP (50 nM degradation assay, 100 nM for mechanistic analysis using WY165) and various concentrations of test compounds were dissolved in the ClpP assay buffer (25 µL) and incubated at rt for 60 min (final 0.5% DMSO). Then 2.5 µM mSA (Sigma-Aldrich, SAE0094) in 1 µL ClpP assay buffer was added and the mixture was incubated at 37 °C for 24 h. The samples were mixed with Laemmli buffer, boiled at 95 °C for 5 min and subjected to SDS-PAGE. Bands were stained with CBB.

Selectivity analysis of WY165-induced mSA degradation

HEK293FT cells were suspended in ClpP assay buffer and lysed with sonication to afford lysate, which was diluted to 5.2 mg/ml proteins. The assay was carried out in a similar way to the mSA degradation assay. Briefly, 24 µL of lysate was mixed with of 15 µM mSA (in 16 µL ClpP assay buffer) and added to a pre-incubated solution of 30 nM ClpP and test compound (in 80 µL ClpP assay buffer, final 0.01% DMSO). After 24 h incubation at 37 °C, a part of the reaction mixture was subjected to SDS-PAGE. Bands were stained with CBB. The rest of the reaction mixture was used for nanoLC-MS/MS.

LC-MS/MS sample preparation

To 100 µL of sample was added 500 mM dithiothreitol aq. (1 µL) and the mixture was incubated at 37 °C for 30 min in a tube for Cys reduction. Next, 1 M iodoacetamide aq. (2 µL) was added, and the mixture was incubated at room temperature for 30 min in the dark for Cys alkylation. Evaporation under reduced pressure gave a white pellet, which was suspended in 200 mM HEPES buffer (pH 8.0) containing 4 M urea (100 µL). To this solution, 1 µg/µL LysC (in 50 mM AcOH aq. 0.5 µL) was added. The mixture was incubated at rt for 3 h, then diluted with 100 µL of 200 mM HEPES buffer (pH 8.0). To this, 1 µg/µL trypsin solution (in 50 mM AcOH aq. 0.5 µL) was added and the mixture was incubated at 37 °C overnight. The reaction was quenched by adding TFA aq. (final conc. 0.1%) and the solution was desalted using C18 pipette tips (Agilent).

DNA work

Custom-synthesized cox8-His6-mSA-FLAG and cox8-His6-mSA-STMP1-FLAG DNA sequences (Eurofin) were amplified with primers containing restriction enzyme sites (XhoI-FW 5'-nnnnCTCGAGATGTCTGTCTTGACGCCCCTTC -3' [32mer, Tm = 60 °C, 56% GC] and FseI-RV 5'-nnnnGGCCGGCCTCATTTGTCGTCATCATCCTTGTAGTCAC -3' [41mer, Tm = 60 °C, 54% GC]) using KOD Fx neo polymerase (TOYOBO, KFX-201). The insert fragment and pCMV6-AC-mRFP (ORIGENE, CAT# PS100034) were treated with XhoI (New England Bio Lab, R0146) and FseI (New England Bio Lab, R0588). Each insert sequence was cloned downstream of the CMV promoter of the pCMV6-AC plasmid by T4 DNA ligase (TaKaRa, 2011A) and purified with a

QIAprep Spin Miniprep Kit (Qiagen) to afford pCMV6-cox8-His6-mSA-FLAG or pCMV-cox8-His6-mSA-STMP1-FLAG plasmids. The sequences were confirmed by Sanger sequencing (Eurofin).

mSA:
ATGTCTGTCTTGACGCCCCTTCTGCTCAGAGGCCTGACAGGCAGTGCCAGACGGCTTCC
CGTACCACGAGCCAAGATCCACAGCTTGGGAGATCCACCTGTTGCCACAATGGTGACTA
GCCACCATCACCATCACCACGAGTTTGCCTCCGAAAACCTGTACTTCCAAGGAGCTGAA
GCCGGGATAACCGGTACCTGGTACAACCAACGTGGGTCCACATTCACCGTCACTGCTGG
TGCGGATGGGAATCTCACAGGACAGTACGAGAATCGCGCTCAAGGATGGGGCTGTCAG
AATAGCCCGTATACCCTGACTGGGAGGTATAATGGCACTAAACTGGAGTGGAGAGTGG
AGTGGAACAACAGCACTGAGAACTGCCATTCCCGCACAGAATGGAGGGGACAGTATCA
GGGAGGCGCTGAAGCCCGGATTAACACCCAGTGGAATCTGACCTATGAGGGCGGTTCT
GGCCCTGCAACCACACAGGGTCAGGACACCTTTACGAAGGTGAAACCCTCAGCAGCAT
CAGGGAGTGACTACAAGGATGATGACGACAAATGA

mSA-STMP1:
ATGTCTGTCTTGACGCCCCTTCTGCTCAGAGGCCTGACAGGCAGTGCCAGACGGCTTCC
CGTACCACGAGCCAAGATCCACAGCTTGGGAGATCCACCTGTTGCCACAATGGTGACTA
GCCACCATCACCATCACCACGAGTTTGCCTCCGAAAACCTGTACTTCCAAGGAGCTGAA
GCCGGGATAACCGGTACCTGGTACAACCAACGTGGGTCCACATTCACCGTCACTGCTGG
TGCGGATGGGAATCTCACAGGACAGTACGAGAATCGCGCTCAAGGATGGGGCTGTCAG
AATAGCCCGTATACCCTGACTGGGAGGTATAATGGCACTAAACTGGAGTGGAGAGTGG
AGTGGAACAACAGCACTGAGAACTGCCATTCCCGCACAGAATGGAGGGGACAGTATCA
GGGAGGCGCTGAAGCCCGGATTAACACCCAGTGGAATCTGACCTATGAGGGCGGTTCT
GGCCCTGCAACCACACAGGGTCAGGACACCTTTACGAAGGTGAAACCCTCAGCAGCAT
CAGGGAGTATGCTCCAGTTCCTGCTTGGATTTACACTGGGCAACGTGGTTGGAATGTAT
CTGGCTCAGAACTATGATATACCAAACCTGGCTAAAAAACTTGAAGAAATTAAAAAGG
ACTTGGATGCCAAGAAGAAACCCCCTAGTGCAAGTGACTACAAGGATGATGACGACAA
ATGA

Cell culture

HEK293FT, HeLa, and MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), and penicillin/streptomycin mixture at 37 °C in a humidified atmosphere of 5% CO_2 in air. These cells were used in biological assays at 6 to 20 passages (HeLa) or 7 to 18 passages (MCF7).

Cell lysate and SDS-PAGE sample preparation

Cells in 12-well plates were washed with PBS and lysed in 100 μL NP-40 lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 1% NP-40) containing Protease Inhibitor Cocktail (Nacalai tesque), followed by centrifugation at 13,500 rpm, 4 °C for 5 minutes. Protein concentrations were determined using BCA protein assay and normalized by the total protein concentration in each lysate. The fluorescence intensity of EGFP was measured at 485 nm excitation and 535 nm emission using Envision to correct the protein expression level by transfection. After mixing with Laemmli buffer (0.25 % BPB, 0.5M DTT, 50 % glycerol, 10 % SDS, 0.25 M Tris-HCl, pH 6.8), each lysate was heated at 95 °C for 5 min.

mSA degradation assay

HeLa cells were seeded at 1.5 $\times 10^5$ cells/well and MCF7 cells were seeded at 2.0×10^5 cells/well in 12-well clear plates, and cultured overnight. The cells in each well were transiently transfected with 187.5 ng (HeLa), 750 ng (MCF7) pCMV6-cox8-His6-mSA-FLAG or 93.8 ng pCMV-cox8-His6-mSA-STMP1-FLAG plasmids and 62.5 ng (HeLa), 250 ng (MCF7) EGFP plasmids using 0.4 μL (HeLa), 0.8 μL (MCF7) avalanche omni (EZ Biosystems) in Opti-MEM (12.5 μL for HeLa, 25 μL for MCF7) and incubated for 12 h (HeLa) or 16 h (MCF7). Then the cells were washed with phosphate-buffered saline (PBS) and treated with test compounds in DMSO at the indicated concentrations (final 0.1% DMSO) for the indicated times (2 h-12 h).

For linker SAR, mSA degradation assay was conducted in the same way, except for the transfection protocol. HeLa cells in each well were transiently transfected with 246 ng pCMV6-cox8-His6-mSA-FLAG plasmid using 0.4 μL avalanche omni in 12.5 μL of Opti-MEM.

Proteasome or lysosome inhibition

Proteasome or lysosome inhibition assay was carried out in a similar way to the mSA degradation assay. The transfected cells were treated with proteasome inhibitor (300 nM bortezomib) or lysosome inhibitor (10 μM leupeptin and 30 mM NH_4Cl), added simultaneously with compound **1** (final 0.1% DMSO), and then incubated for 6 h.

ClpP knockdown

HeLa cells were transfected with custom-prepared ClpP-directed siRNA with the sequence[2] GUUUGGCAUCUUAGACAAGGUUCUGUU (Biologica). siRNAs were transfected at 16 nM in the presence of lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) at a 1:1.5 ratio in 100 μL Opti-MEM. After 12 h incubation, the medium was removed, the cells were washed with PBS and fresh medium was added. After a further 12 h incubation, the cells were transiently transfected with pCMV6-cox-8-His6-mSA-FLAG plasmids and EGFP using avalanche omni as described above and incubated for 12 h. The cells were then washed with PBS, incubated for 6 h, and treated with test compounds (final 0.1% DMSO) at the indicated concentrations for 6 h.

Isolation of mitochondria

1.5×10^7 HeLa cells expressing mSA or mSA-STMP1 were collected and their mitochondria were isolated using Mitochondria Isolation Kit for Cultured Cells (Thermo Scientific). The mitochondria were lysed with NP-40 lysis buffer and the protein concentration was determined by BCA protein assay and normalized by the total protein concentration in each lysate.

Sample preparation for LC-MS/MS analysis of 1-degradable proteome

HeLa cells were seeded at 1.5×10^5 cells/well in 12-well clear plates and cultured overnight. The cells in each well were transiently transfected with 750 ng pCMV6-cox8-His6-mSA-FLAG plasmids using 0.8 μL avalanche omni in 25 μL Opti-MEM and incubated for 12 h, then washed with PBS and treated with 10 mM compound **1** in DMSO at the indicated concentrations (final 10 μM compound **1** and 0.1% DMSO) for 12 h. Lysis and LC-MS/MS sample preparation were performed using an iST kit (PreOmics GmbH).

Analysis of mitochondrial morphology

To examine mitochondrial morphology, cells were seeded on a 35 mm dish and cultured overnight. The cells were transfected with 160 ng pCMV-FLAG-His6-mSA-STMP1-FLAG using 0.5 μL avalanche omni in 16 μL Opti-MEM and further cultured for 12 h, then re-seeded on a glass-bottomed dish (35 mm, poly-Lys coated). After culture for 24 h, the cells were treated with test compound (final 0.1% DMSO) at the indicated concentrations for 6 h, then stained with MitoTracker (Invitrogen), fixed with 4% paraformaldehyde, permeabilized with 0.4% Triton-100, and photographed using a microscope (Keyence Corporation, BZ-X800) (the fluorescence of MitoTracker was recorded at 545 nm excitation and 605 nm emission). Binary and morphologic skeleton images were then obtained by transformation of these original images. Cell length and total number of mitochondrial branches were measured using the Mitochondrial Network Analysis macro (Mitochondria analyzer[3]) in Fiji/ImageJ.

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