Electronic Supplementary Information

Mitochondria-targeted inhibitors of the human SIRT3 histone deacetylase

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SUPPORTING FIGURES

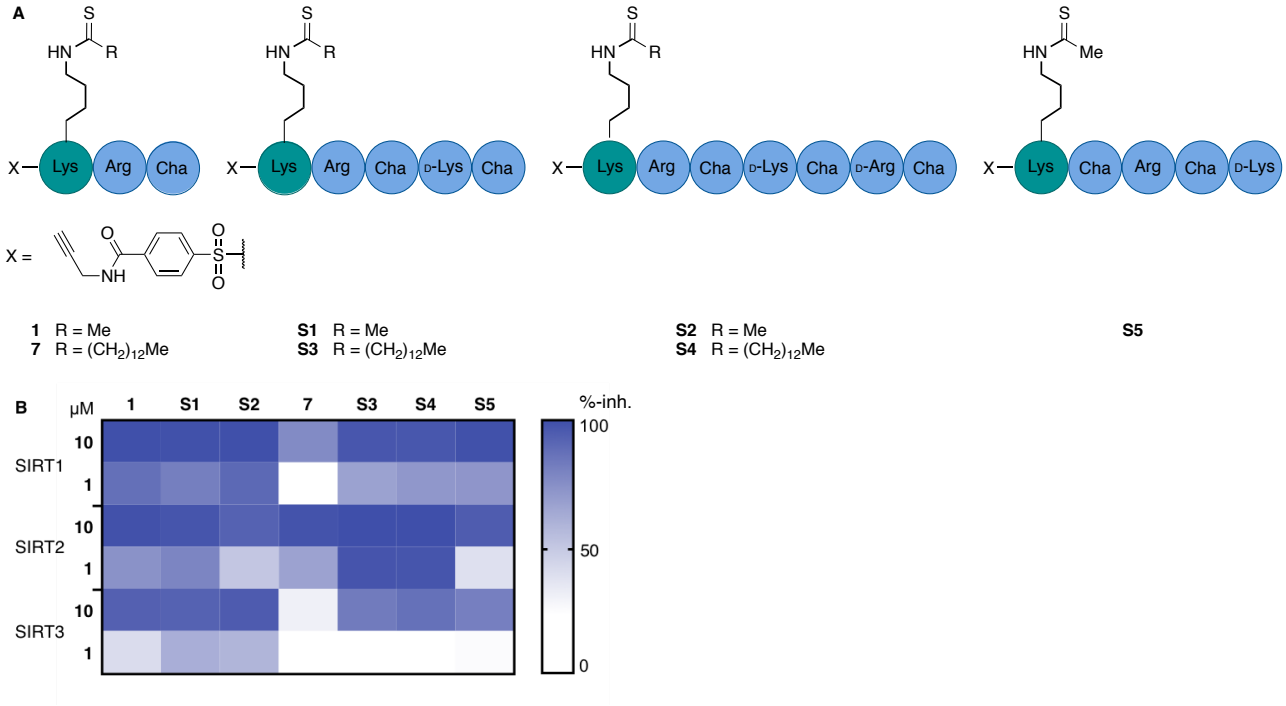


Figure S1. Mitochondrial-targeting sequence evaluation. (A) Structures of compounds **1**, **7** and **S1**–**S5**. (B) Heatmap summarizing potencies of compounds **1**, **7 and S1**–**S5** against SIRT1–3 deacetylation based on %-inhibition. All assays were performed at least twice in duplicate and the values can be found in Table S1.

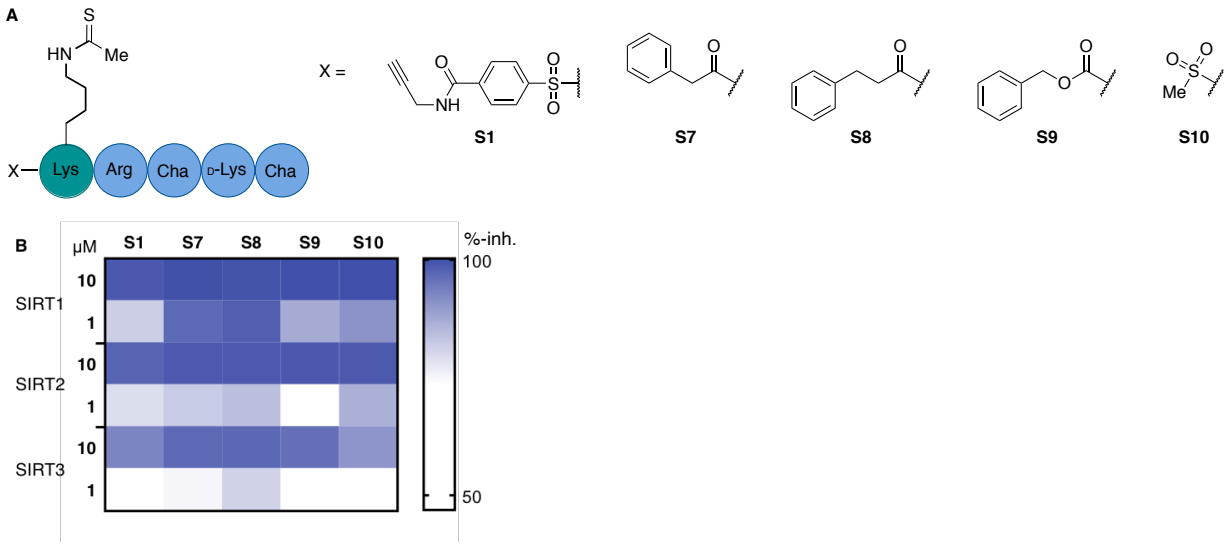


Figure S2. N-terminal evaluation. (A) Structures of compounds **S1** and **S7**–**S10**. (B) Heatmap summarizing potencies of compounds **S1** and **S7**–**S10** against SIRT1–3 based on %-inhibition. All assays were performed at least twice in duplicate and the values can be found in Table S1.

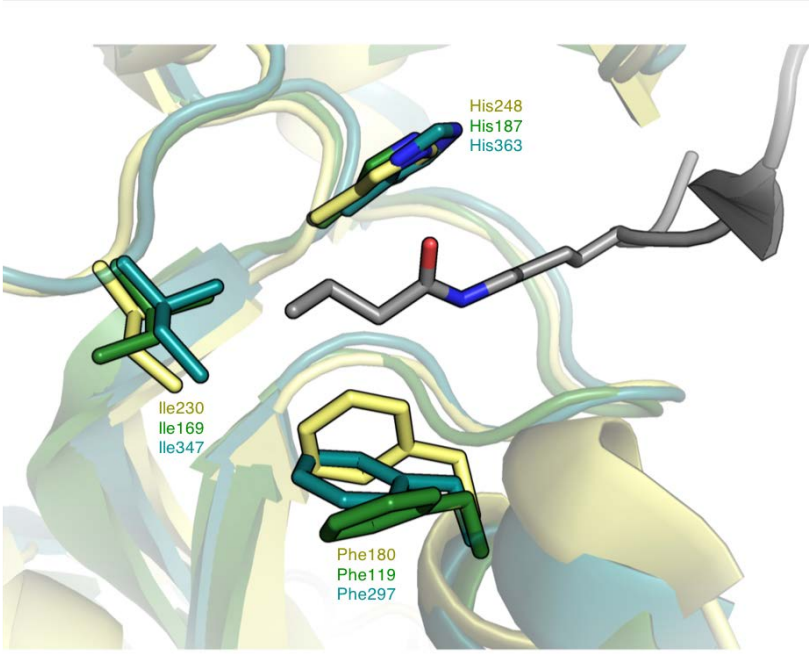


Figure S3. Overlay of SIRT1–3 active site. PyMol illustration of the structural similarity of the SIRT1 (yellow), SIRT2 (green) and SIRT3 (teal) active sites with crotonylated lysine (Kcr) in the pocket. The distance between the substrate Kcr and Phe180 is 3.3 Å. PDBs SIRT1 = 4V1C, SIRT2 = 4R8M, SIRT3 = 5BTR.

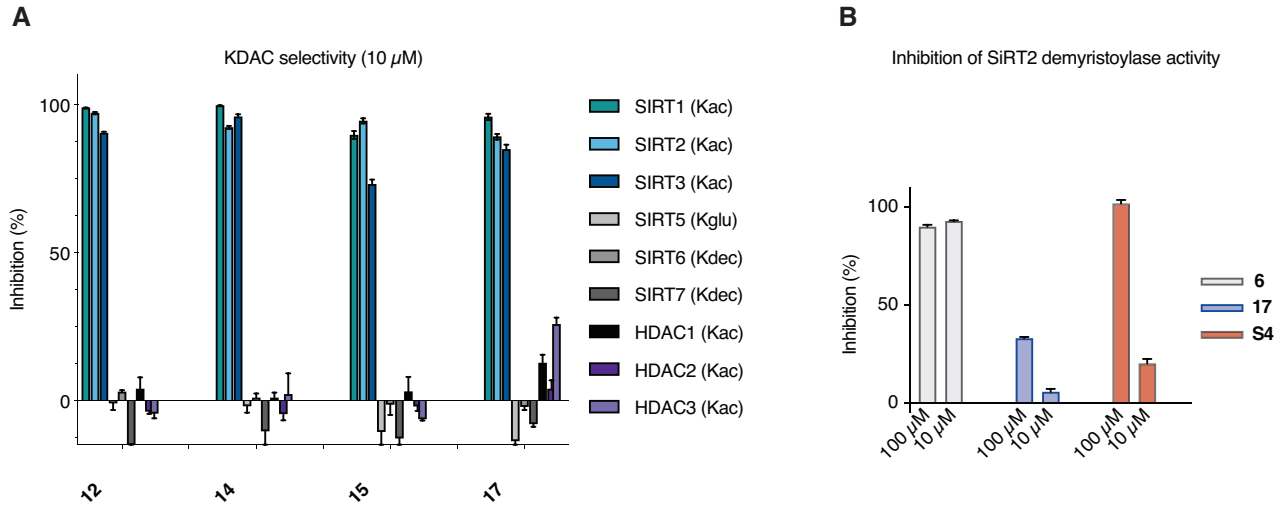


Figure S4. "KDAC" selectivity of SIRT3 inhibitors. (A) Selectivity of compounds **12**, **14**, **15** and **17**, measured at an inhibitor concentration of 10 µM. The specific acyl-lysine motifs of the used substrates are indicated for each individual KDAC. (B) Inhibition of the demyristoylase activity of SIRT2 by **6**, **17**, and **S4**. All experiments in A,B were performed at least twice in duplicate (see Methods section for details).

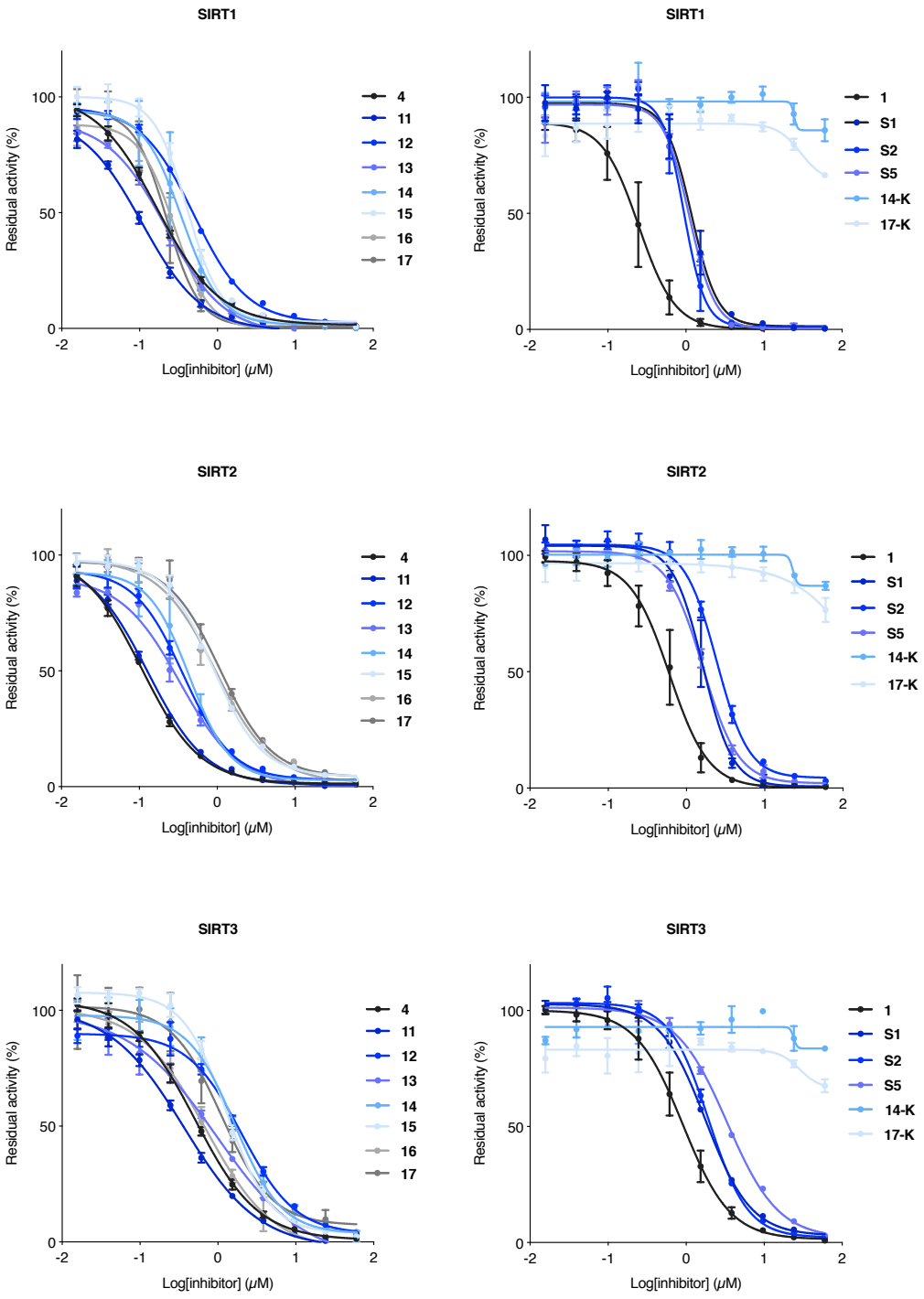


Figure S5. Dose-response curves of selected inhibitors against SIRT1–3. Concentration–response curves against inhibition of SIRT1–3 deacetylation for representative compounds using QPKKac as substrate. IC_{50} values are reported in Table S1. All assays were performed at least twice in duplicate.

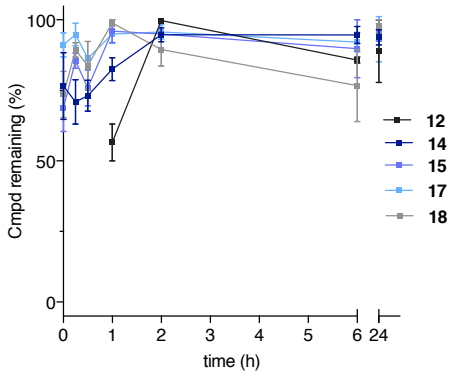


Figure S6. Stability of SIRT3 inhibitors in cell media. Stability of compound **12**, **14**, **15**, **17** and **S14** in cell media (DMEM, 10% (v/v) FBS, 1% (v/v) penicillin-streptomycin) at 37 °C over 24 h. All assays were performed at in duplicates.

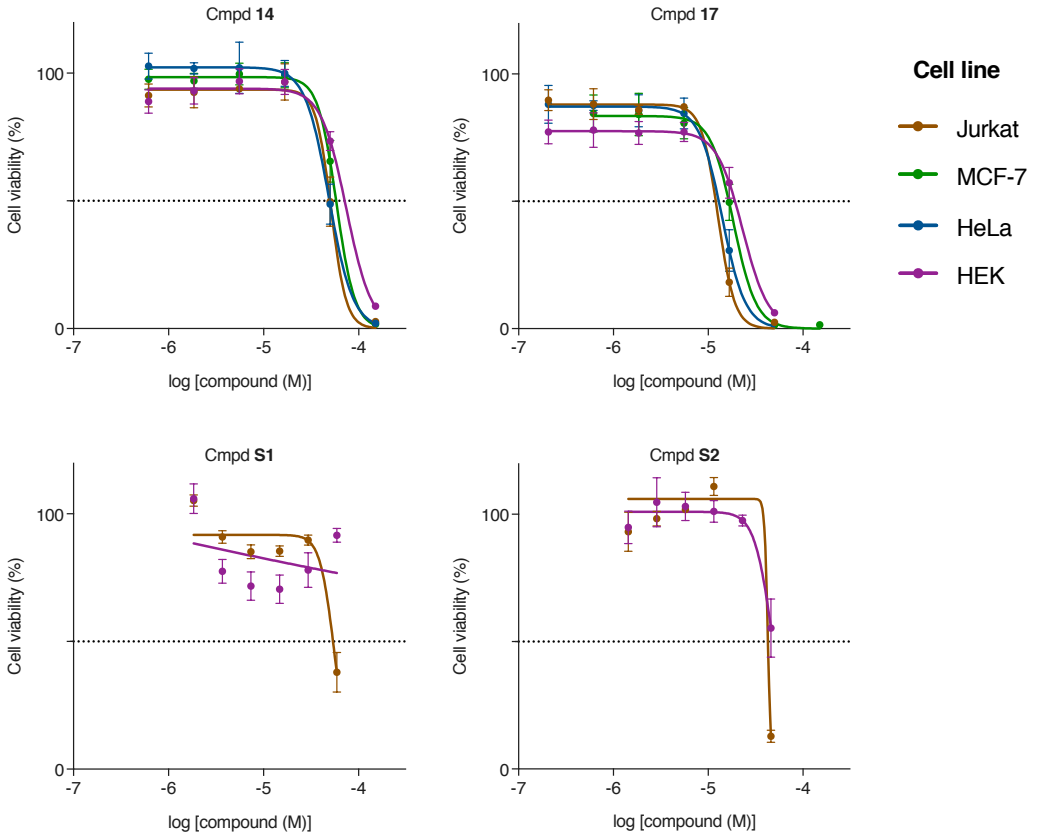


Figure S7: Cell viability assays. Concentration-response curves of cell viability (MTT) assays against different cell lines. All assays were performed three times in duplicate.

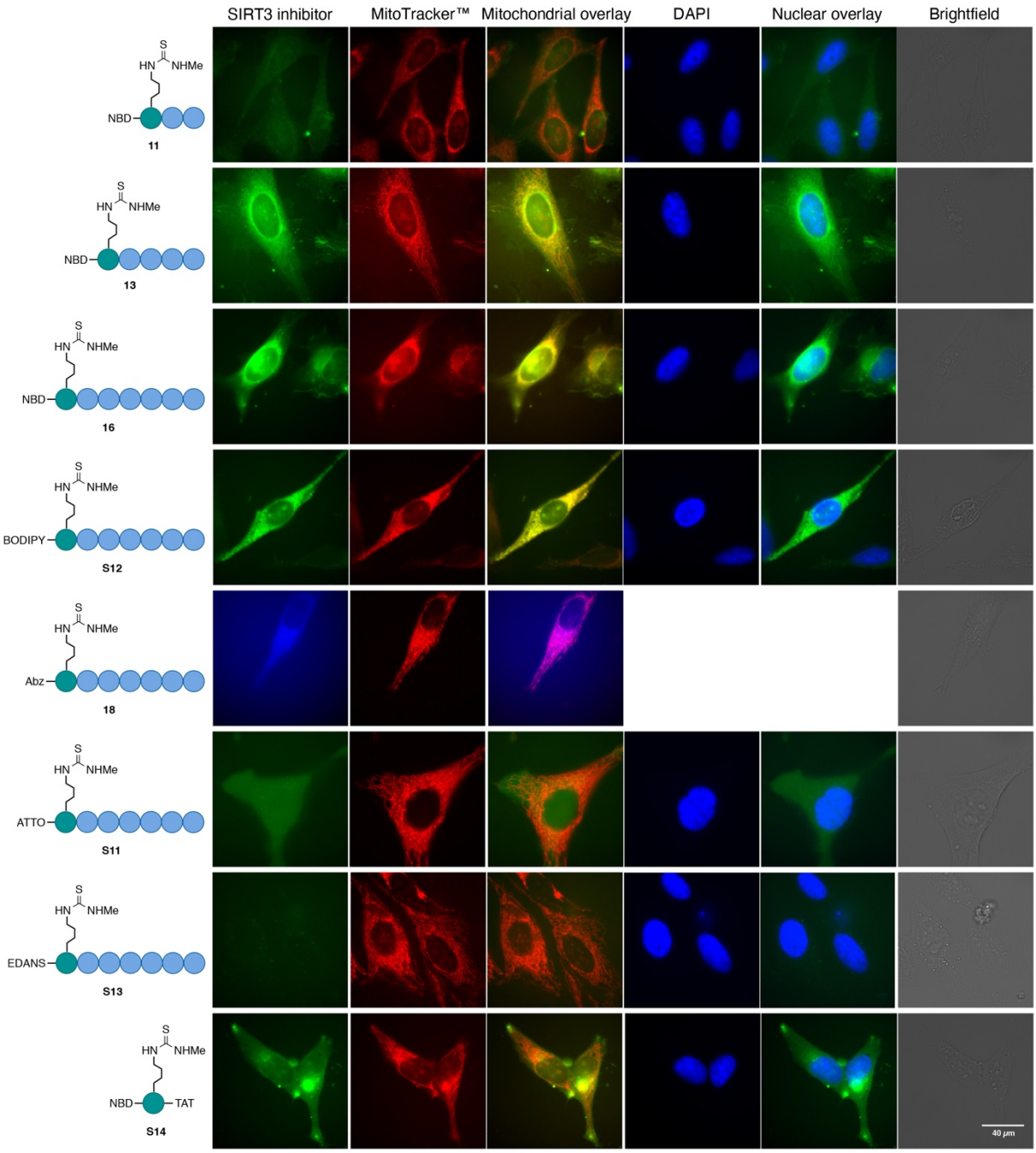


Figure S8. Cellular localization of fluorophore labelled inhibitors. Fluorescence images of HeLa cells treated with fluorophore labelled inhibitors **11**, **13**, **16**, **S11**–**S14** (15 μM) or Tat control peptide **S15** (15 μM), cotreated with NucBlue and MitoTracker (red, 10 nM) for 30 min. Pearson correlation coefficients (r): **11** = –0.66, **13** = 0.59, **16** = 0.46, **S11** = –0.05, **S12** = –0.04, **S13** = 0.96, **S14** = 0.81, **S15** = 0.13.

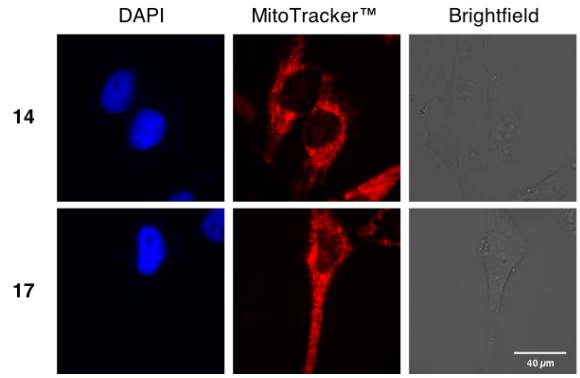


Figure S9. Images of non-fluorophore-conjugated inhibitors. Fluorescence images of HeLa cells treated with **14** or **17** (15 µM) and cotreated with NucBlue and MitoTracker (10 nM) for 30 min.

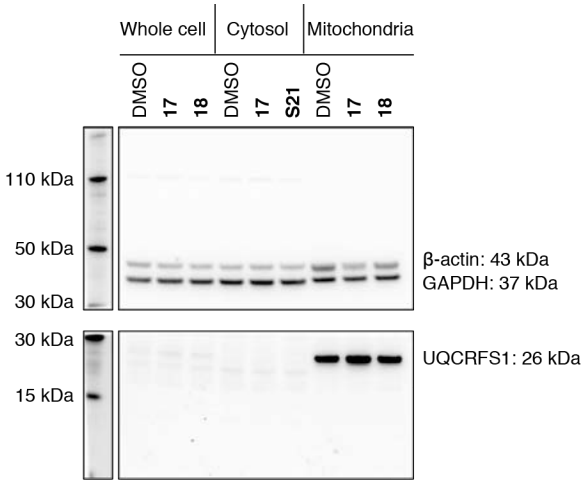


Figure S10. Western blot analysis of cell lysates after mitochondrial enrichment. Western blot analysis of enrichment lysates (HEK293T) blotted against loading control β-actin, cytosolic marker GAPDH and mitochondrial marker UQCRFS1.

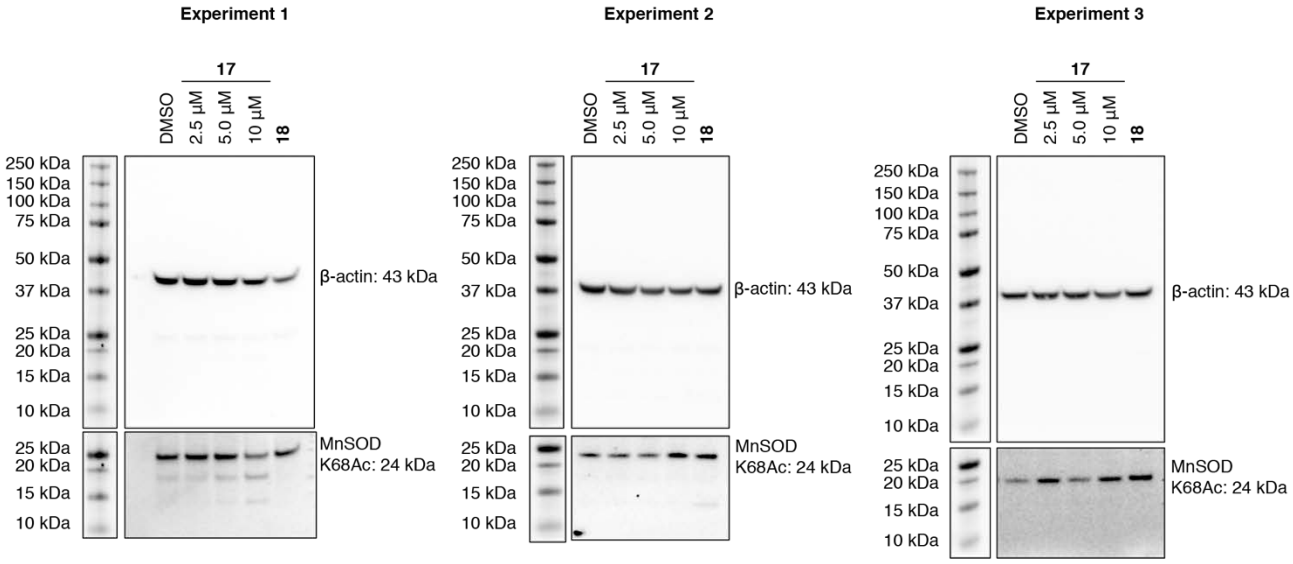


Figure S11. Western blot analysis of MnSOD K68Ac levels. Western blot analysis of mitochondrial enriched lysates (HEK293T) after 5 h treatment with DMSO, **17** (2.5 µM, 5 µM, 10 µM) or acetylating reagent **18** (0.5 mM) performed in triplicate. Full image of membranes showing levels of β-actin (loading control) and MnSOD K68Ac.

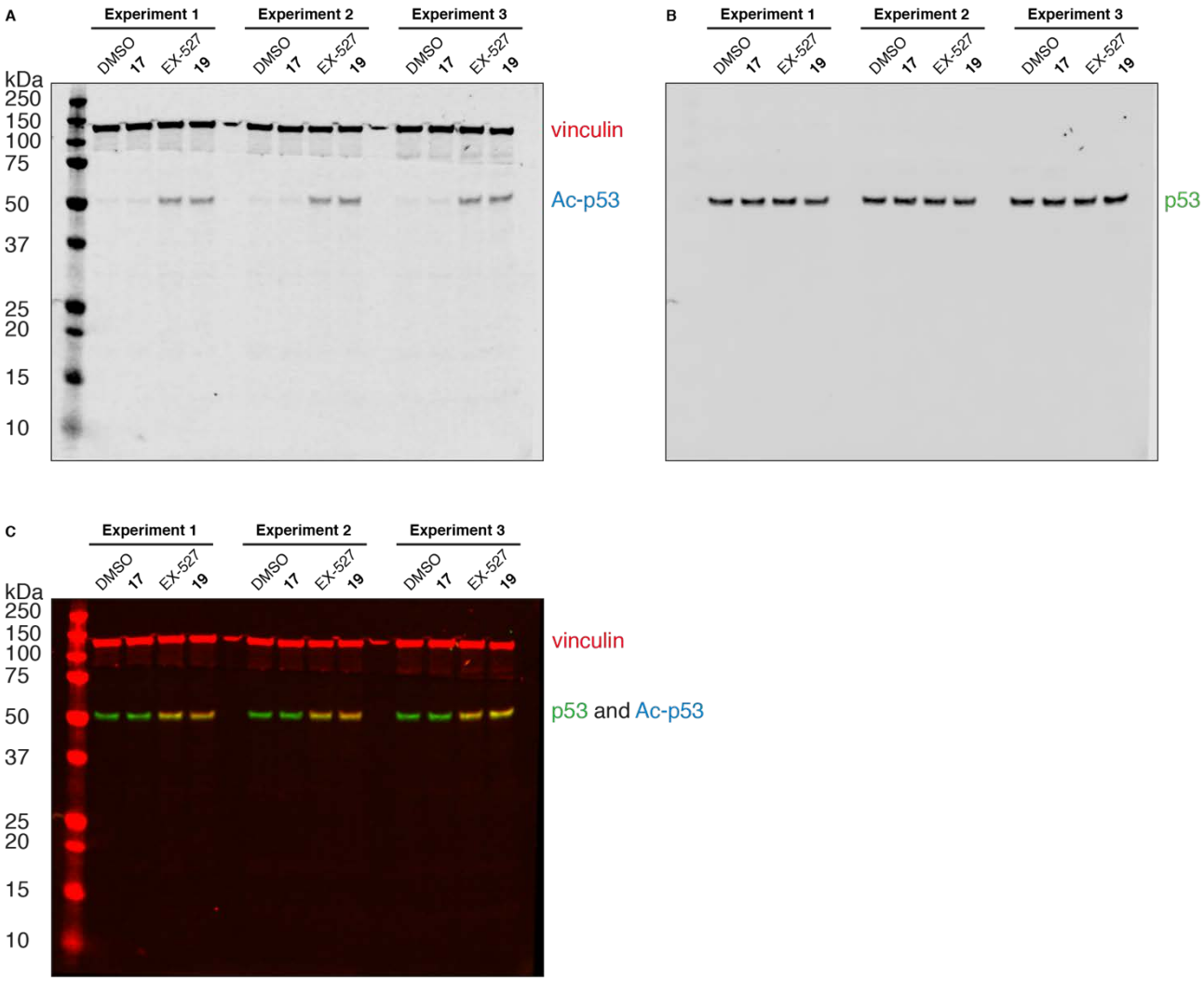


Figure S12. Western blot analysis of Ac-p53 levels. Western blot analysis of whole-cell lysates (HEK293T) after 6 h treatment with inhibitors **17,** EX-527, and **19** (10 µM), cotreated with TSA (1 µM). (A) Full image of membranes showing levels of Ac-p53 (K382) and vinculin (loading control), analyzed at 700 nm (anti-rabbit secondary antibody). (B) Full image of membranes showing levels of p53, analyzed at 800 nm (anti-mouse secondary antibody). (C). Overlay of 700 nm and 800 nm channels, showing levels of Ac-p53 (K382, red at ~50 kDa) and vinculin (loading control, red at ~125 kDa) and p53 (green). Data from three independent experiments.

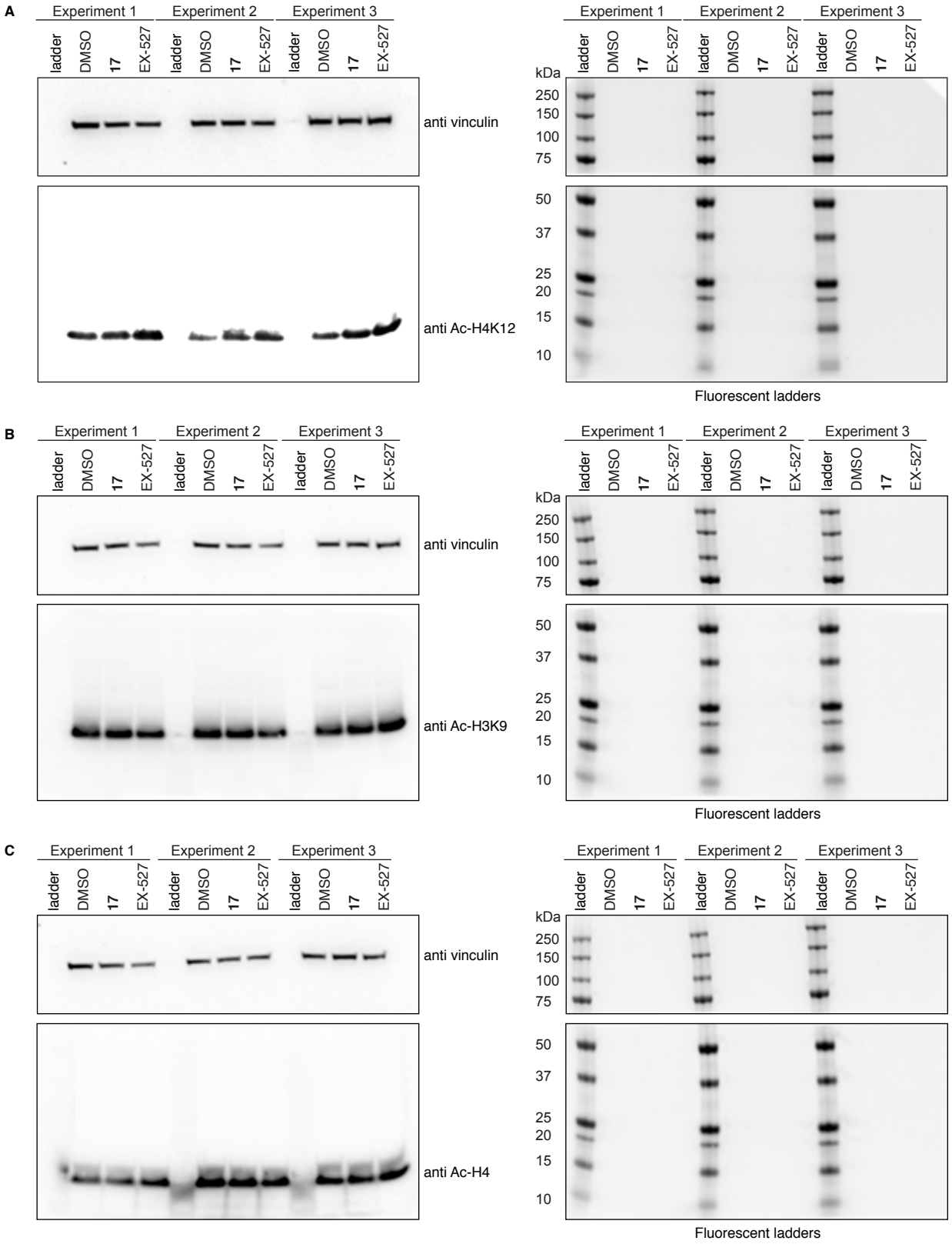


Figure S13. Western blot analysis of histone acetylation levels. Triplicate analysis of whole-cell lysates (HEK293T) after 5 h treatment with inhibitors **17** and EX-527 (10 μM), cotreated with TSA (1 μM). (A) Ac-H4K12 levels and vinculin as loading control. (B) Ac-H3K9 levels and vinculin as loading control. (C) Ac-H4 levels and vinculin as loading control.

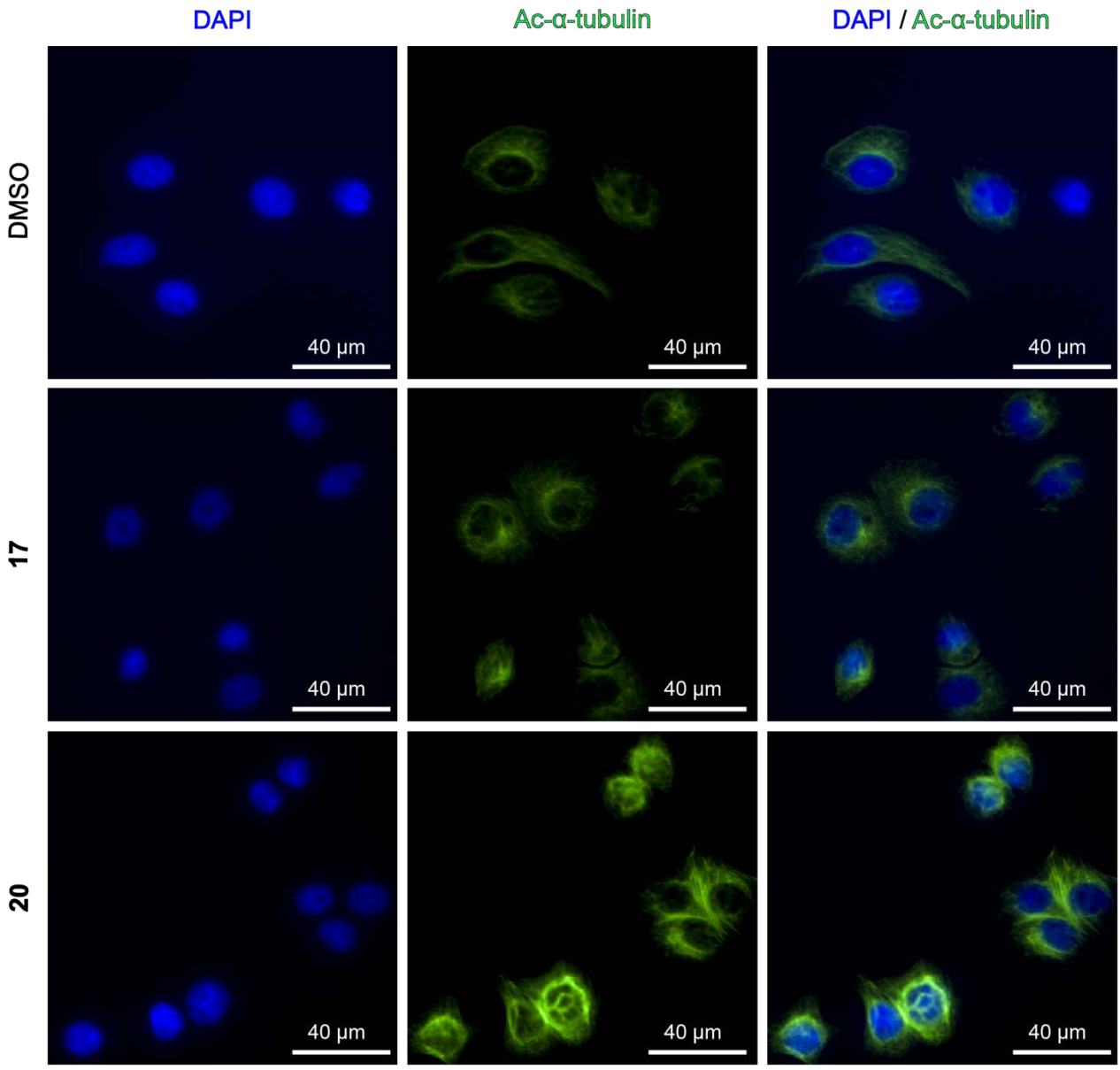


Figure S14. Qualitative analysis of perinuclear α-tubulin acetylation. Immunofluorescence images (20×) of HEK293T cells subjected to 6 h treatment with inhibitor (10 µM for **17**, 5 µM for **20**) or DMSO (vehicle). DAPI (blue, nuclear counterstain) and Ac-α-tubulin (green). The data are representative images from two individual experiments.

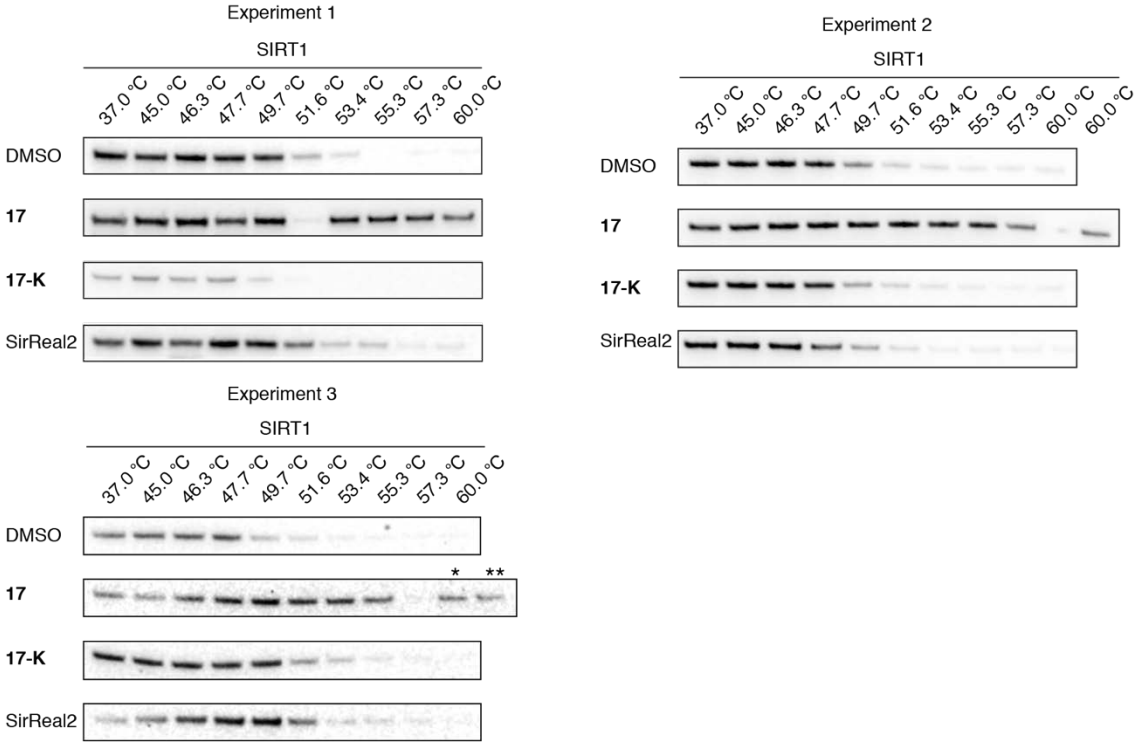


Figure S15. Triplicate analysis of SIRT1 levels in cellular thermal shift assays using DMSO, 17, 17-K, and SirReal2. Western blot analysis of whole cell lysates from HEK293T cells after 5 h treatment with DMSO, **17** (10 µM), **17-K** (10 µM), or SirReal2 (10 µM), respectively followed by heat treatment. *57.3 °C. ** 60.0 °C. For full blots and protein marker see the full western blot section.

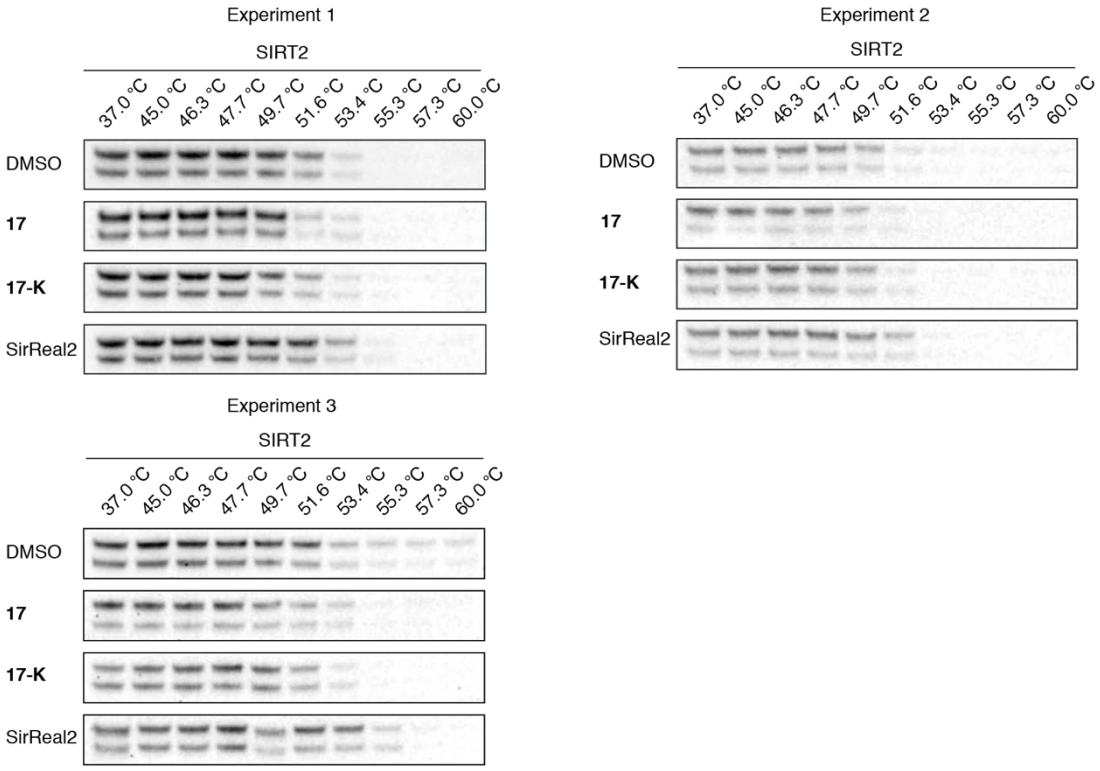


Figure S16. Triplicate analysis of SIRT2 levels in cellular thermal shift assays using DMSO, 17, 17-K, and SirReal2. Western blot analysis of whole cell lysates from HEK293T cells after 5 h treatment with DMSO, **17** (10 µM), **17-K** (10 µM), or SirReal2 (10 µM), respectively followed by heat treatment. For full blots and protein marker see the full western blot section.

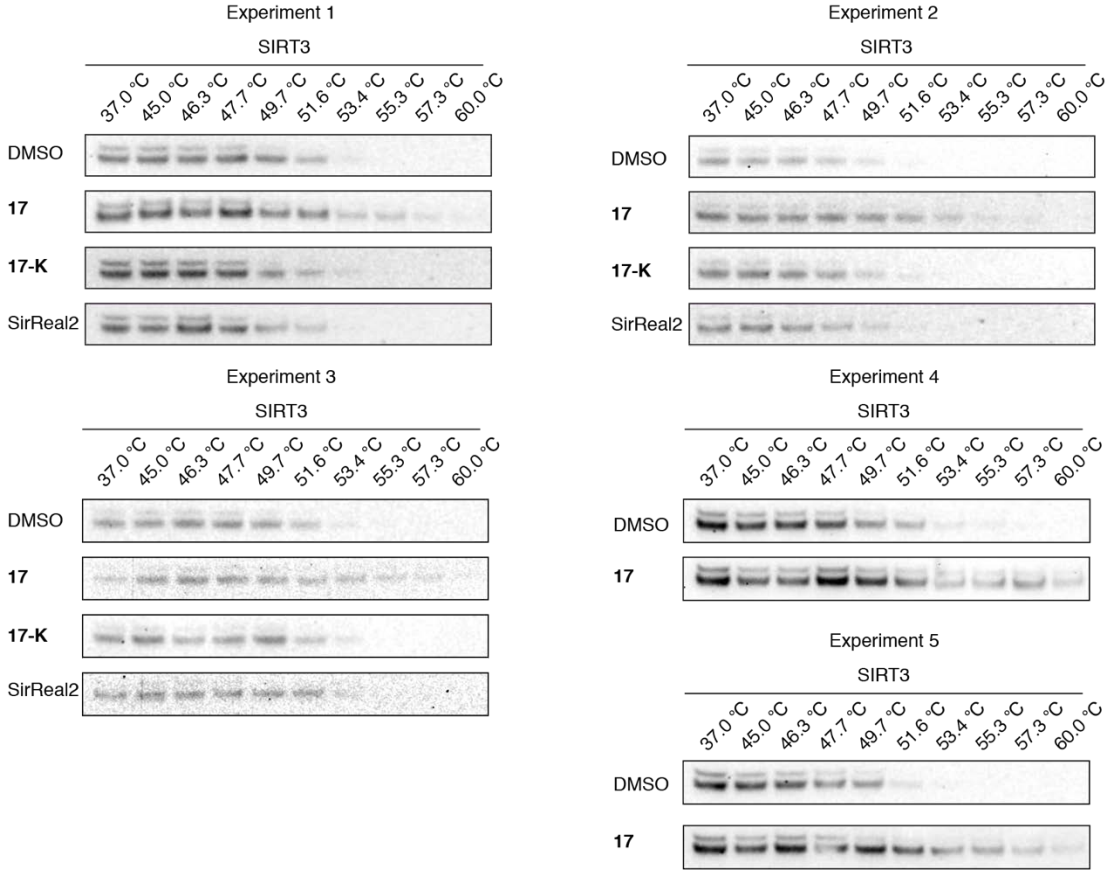


Figure S17. Triplicate or fivefold analysis of SIRT3 levels in cellular thermal shift assays using 17-K and SirReal2 or DMSO and 17, respectively. Western blot analysis of whole cell lysates from HEK293T cells after 5 h treatment with DMSO, **17** (10 µM), **17-K** (10 µM), or SirReal2 (10 µM), respectively followed by heat treatment. For full blots and protein marker see the full western blot section.

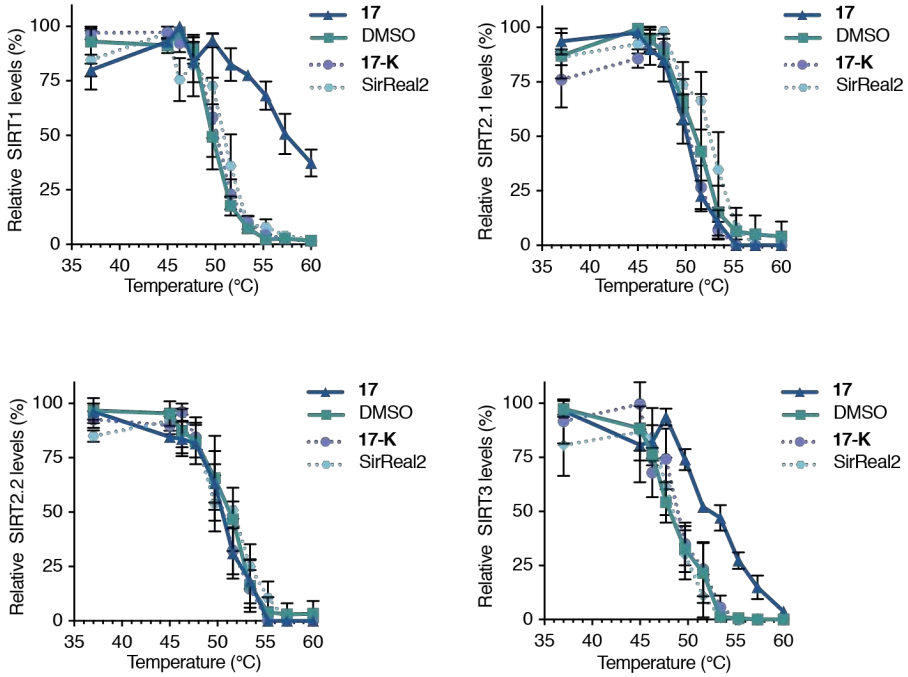


Figure S18. Quantification of cellular thermal shift assays using DMSO, 17, 17-K, and SirReal2. Western blots against SIRT1, SIRT2, and SIRT3 were quantified and plotted against the temperature gradient used. The data was fitted to a non-linear Log(inhibitor) – response curve with variable slope using the GraphPad Prism software. For full blots and protein marker see the full western blot section.

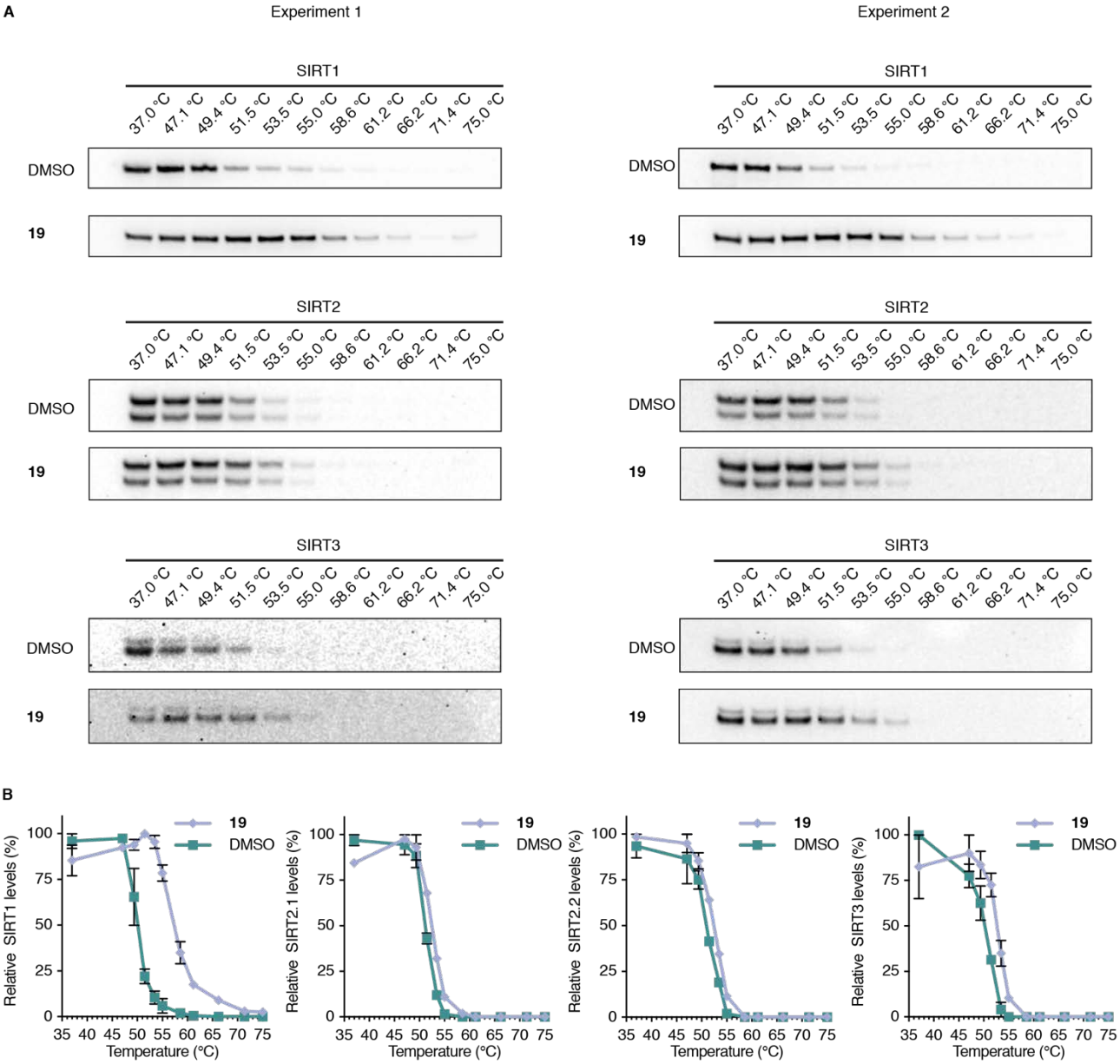


Figure S19. Duplicate analysis and quantification of SIRT1, SIRT2, and SIRT3 levels in cellular thermal shift assays using DMSO and 19. HEK293T cells were treated with DMSO or **19** (10 µM) for 90 minutes before heat treatment according to the gradient shown in panel A. The western blots were quantified and plotted against the temperature gradient. The data was fitted to a non-linear Log(inhibitor) – response curve with variable slope using the GraphPad Prism software. For full blots and protein marker see the full western blot section.

SUPPORTING TABLES

Table S1. Inhibitor potencies for all synthesized compounds against SIRT1–3[a]

Cmpd	SIRT1	SIRT2	SIRT3
1	0.25 ± 0.20 μM	0.61 ± 0.15 μM	0.91 ± 0.08 μM
2	19% [1 μM]	16% [1 μM]	19% [1 μM]
3	54% [1 μM]	32% [1 μM]	8% [1 μM]
4	017 ± 0.04 μM	0.11 ± 0.05 μM	0.51 ± 0.04 μM
5	78% [1 μM]	90% [1 μM]	11% [1 μM]
6	97% [1 μM]	98% [1 μM]	57% [1 μM]
7	25% [1 μM]	67% [1 μM]	18% [1 μM]
8	38% [1 μM]	62% [1 μM]	23% [1 μM]
9	83% [1 μM]	86% [1 μM]	21% [1 μM]
10	70% [1 μM]	97% [1 μM]	43% [1 μM]
11	0.11 ± 0.06 μM	0.13 ± 0.05 μM	0.37 ± 0.04 μM
12	0.50 ± 0.03 μM	0.37 ± 0.03 μM	2.02 ± 0.20 μM
13	0.22 ± 0.02 μM	0.33 ± 0.06 μM	0.96 ± 0.17 μM
14	0.35 ± 0.06 μM	0.44 ± 0.06 μM	1.64 ± 0.22 μM
14-K	NI [60 μM]	NI [60 μM]	NI [60 μM]
15	0.41 ± 0.03 μM	0.89 ± 0.08 μM	1.30 ± 0.10 μM
16	0.28 ± 0.03 μM	0.96 ± 0.14 μM	0.70 ± 0.11 μM
17	0.22 ± 0.04 μM	1.08 ± 0.15 μM	1.11 ± 0.34 μM
17-K	NI [60 μM]	NI [60 μM]	NI [60 μM]
19	0.59 ± 0.08 μM[1]	74% [10 μM][1]	12% [10 μM][1]
S1	1.18 ± 0.12 μM	1.62 ± 0.16 μM	1.77 ± 0.08 μM
S2	0.94 ± 0.09 μM	2.44 ± 0.17 μM	1.95 ± 0.11 μM
S3	67% [1 μM]	98% [1 μM]	31% [1 μM]
S4	72% [1 μM]	98% [1 μM]	25% [1 μM]
S5	1.11 ± 0.11 μM	1.62 ± 0.09 μM	3.37 ± 0.26 μM
S7	97% [1 μM]	82% [1 μM]	75% [1 μM]
S8	98% [1 μM]	84% [1 μM]	81% [1 μM]
S9	87% [1 μM]	47% [1 μM]	62% [1 μM]
S10	91% [1 μM]	86% [1 μM]	54% [1 μM]

[a]%-inhibition at given concentrations [1 μM or 10 μM] or IC_{50} values from SIRT1–3 deacetylation assays. Data are based on at least two individual experiments performed in duplicate. NI = no inhibition, denotes less than 50% inhibition at the highest inhibitor concentration [60 μM] applied.

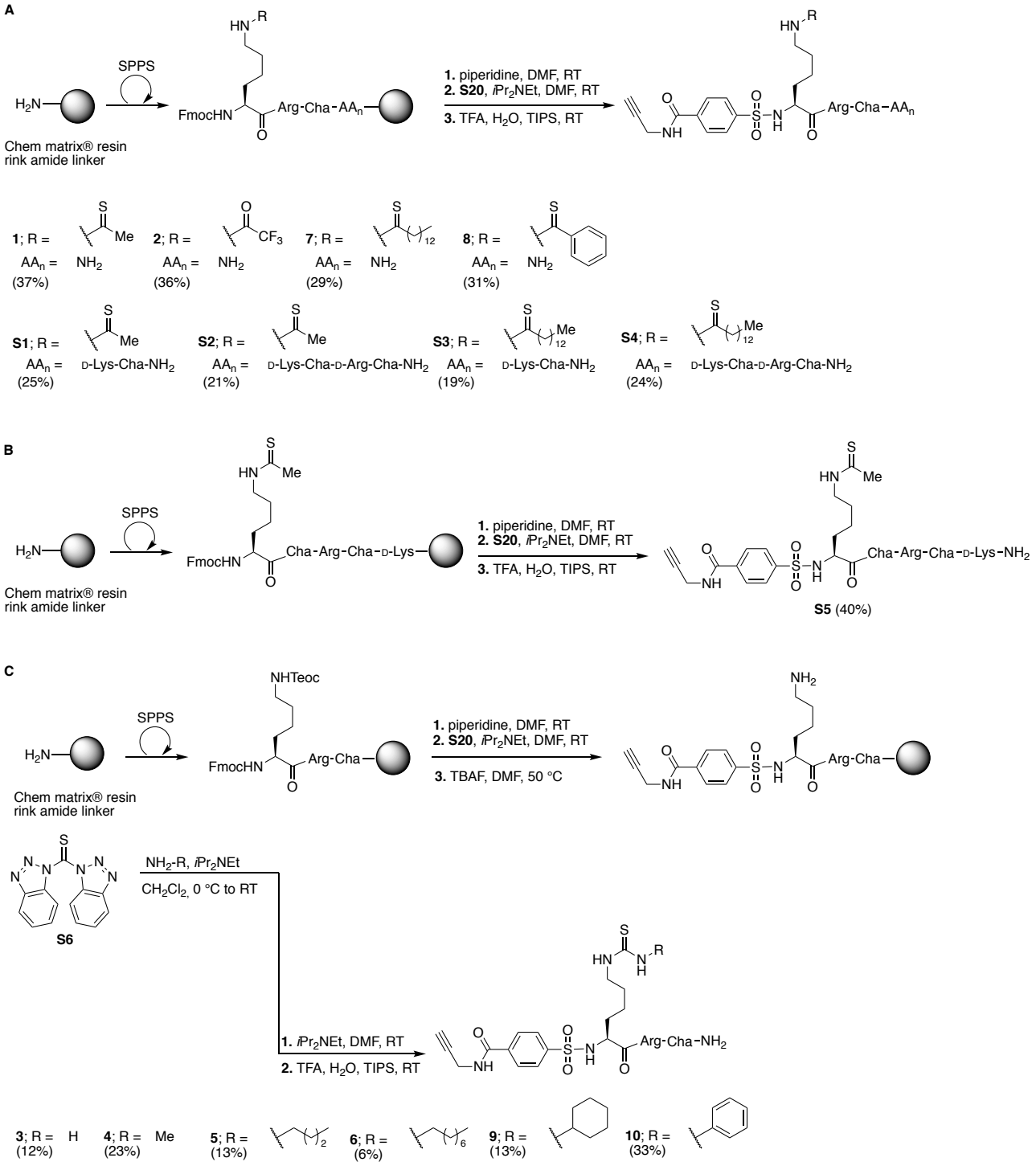
Table S2. EC_{50} values of SIRT3 inhibitors in cell viability assays[a]

Cmpd	HEK293T	Jurkat	HeLa	MCF-7
14	74.1 µM (65.9–83.4 µM)	51.2 µM (46.5–56.4 µM)	48.7 µM (44.9–52.8 µM)	58.3 µM (51.3–66.1 µM)
17	23.1 µM (20.5–26.2 µM)	12.7 µM (9.2–17.5 µM)	14.1 µM (12.2–16.1 µM)	18.5 µM (16.5–20.7 µM)
S1	55.5 µM (49.9–61.9 µM)	>60 µM	ND	ND
S2	48.0 µM (43.8–52.6 µM)	42.5 µM (17.3–67.7 µM)	ND	ND

[a]EC_{50} values and 95 % confidence intervals from cell viability (MTT) assays. Data are based on three individual experiments performed in duplicate. ND = not determined.

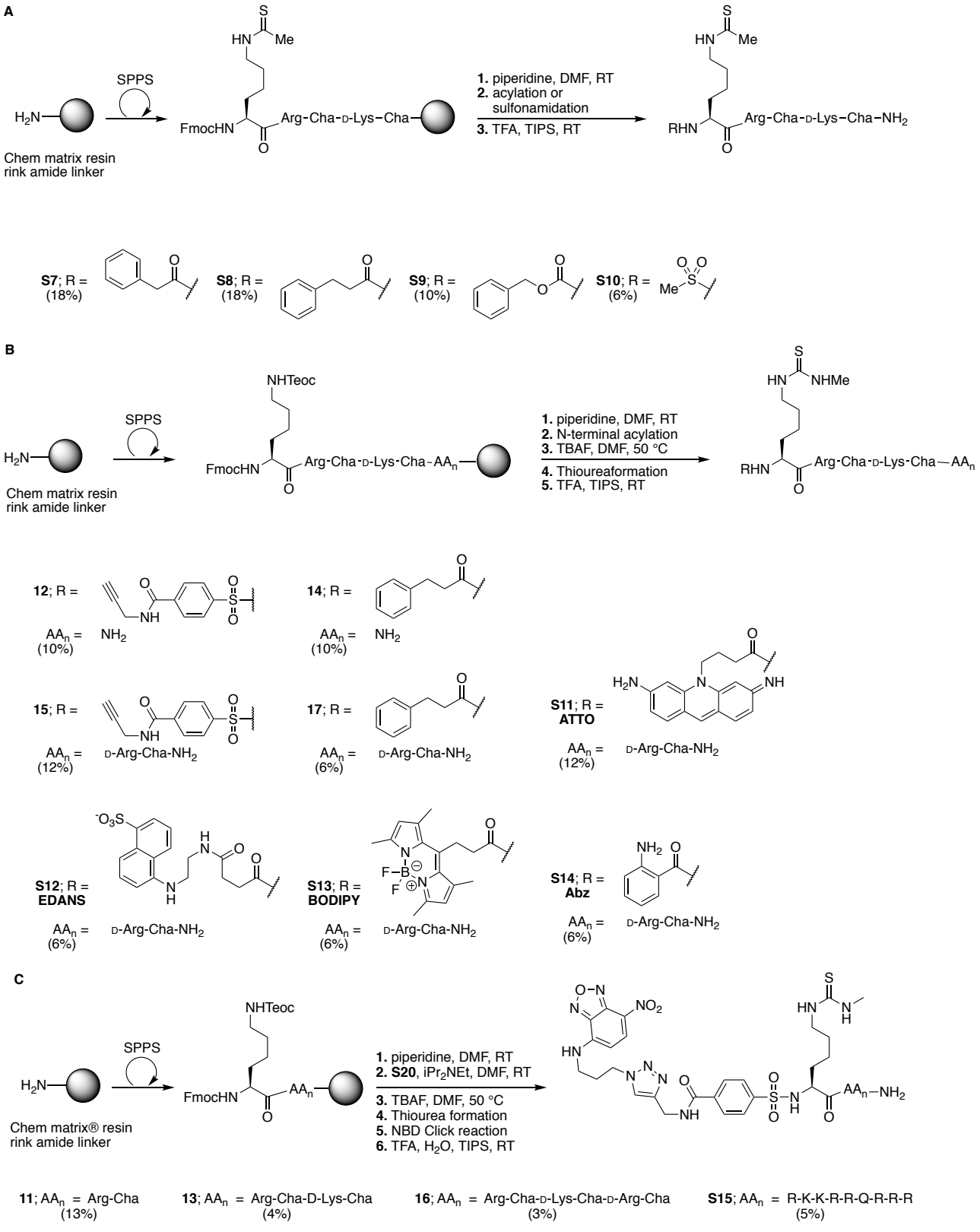
SUPPORTING SCHEMES

Scheme S1. Synthesis of compounds containing lysine side chain modifications. [a]

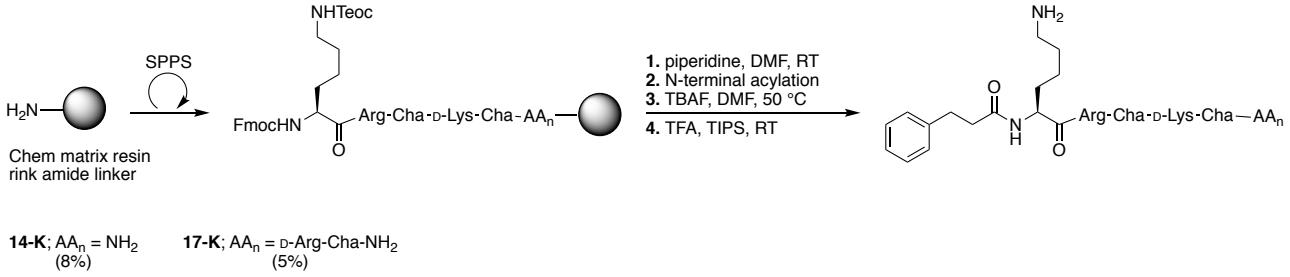


[a]Compounds **S6** was synthesized as previously reported.[2]

Scheme S2. Synthesis of compounds containing *N*-terminal modifications.

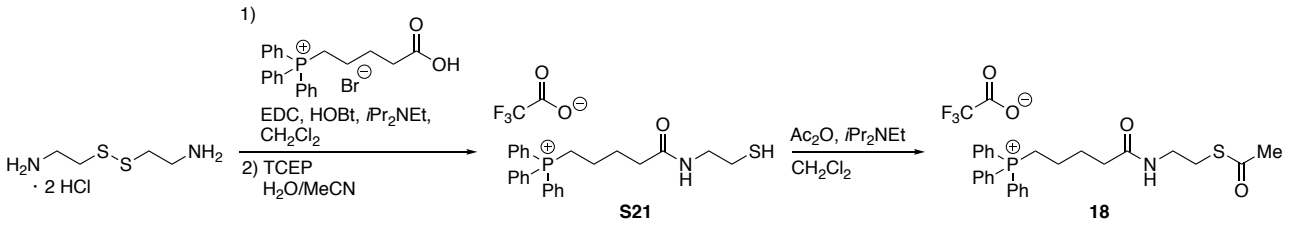


Scheme S3. Synthesis of control compounds **14-K** and **17-K**.

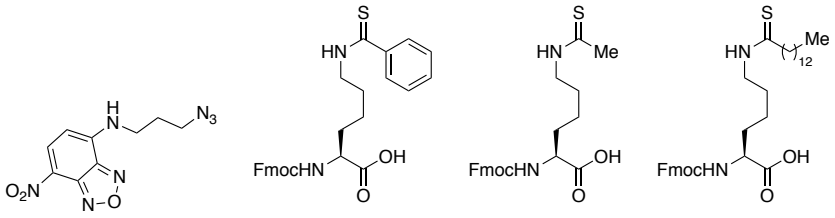


14-K; $AA_n = NH_2$ (8%) **17-K**; AA_n = D-Arg-Cha-NH_2 (5%)

Scheme S4. Synthesis of mitochondria-targeting acetylating reagent **18**.



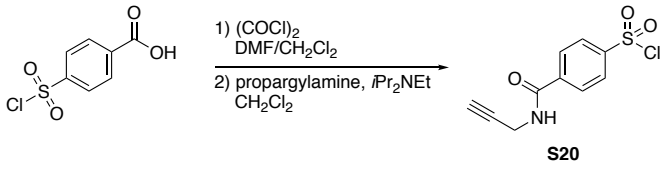
Scheme S5. Structures of previously prepared building blocks. [a]



S16 **S17** **S18** **S19**

[a]Compounds **S16**[3] and **S18**–**S19**[4] were synthesized as previously reported. Please consult the Experimental Section for details on the synthesis and characterization of **S16** and **S17**.

Scheme S6. Synthesis of capping group **S20** for incorporation of click chemistry handle.



S20

EXPERIMENTAL SECTION

Materials and methods for sirtuin deacylase assays

Fluorescence-based in vitro sirtuin deacylase assays. *Materials:* SIRT1 (aa 193–741 with *N*-terminal GST-tag, ≥60% purity; cat. #50012), SIRT2 (aa 50–356 with C-terminal His-tag, ≥90% purity; cat. #50013), SIRT3 (aa 102–399 with *N*-terminal GST-tag; ≥64% purity; cat. #50014), SIRT6 (full length with *N*-terminal GST-tag, ≥75% purity; cat. #50017), HDAC1 (full length with C-terminal His-tag, C-terminal FLAG-tag, ≥65% purity) cat. #50051), HDAC2 (full length with C-terminal His-tag, ≥88% purity; cat. #50002) and HDAC3/NCoR2 (full length with C-terminal His-tag, ≥80% purity; cat. #50003) were acquired from BPS Biosciences (San Diego, CA); SIRT5 (aa 37–310 with *N*-terminal His-tag, ≥95% purity; cat. #BML-SE555-0050) was purchased from Enzo Life Sciences (Farmingdale, NY); and SIRT7 was a gift from Julie E. bolding. Purities were based on Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis (SDS–PAGE) and Coomassie blue stain according to the supplier and all enzyme concentrations given were based on the stock concentrations determined by the supplier. Sirtuin and HDAC substrates where acquired in previous reports: Ac-Gln-Pro-Lys-Lys(Ac)-AMC (QPKKac)[5], Ac-Gln-Pro-Lys-Lys(Glu)-AMC (QPKKglu)[2], Ac-Gln-Pro-Lys-Lys(Dec)-AMC (QPKKdec)[2], Ac-Leu-Gly-Lys(Ac)-AMC (LGKac). Assay buffer was prepared as described in Biomol International product sheets (BML-KI-143; Tris HCl (50 mM), NaCl (137 mM), KCl (2.7 mM), $MgCl_2$ (1 mM), pH 8.0) with addition of BSA (1.0 mg/mL) unless stated otherwise. Trypsin (10,000 units/mg, TPCK treated from bovine pancreas; cat. #T1426) was purchased from Sigma (Steinheim, Germany). All chemicals and solvents were of analytical grade were and used without further purification as obtained from commercial suppliers.

All reactions were performed in black low binding 96-well microtiter plates (Corning half area wells), with duplicate series in each assay and each assay performed at least twice. All reactions were performed in assay buffer, with appropriate concentrations of substrates and inhibitors obtained by dilution from 2–50 mM stock solutions in either water or DMSO and appropriate concentration of enzyme obtained by dilution of the stock provided by the supplier. DMSO concentration in the final assay solution did not exceed 1% (v/v) and control wells without either enzyme (negative control) or inhibitor (positive control) were included in each plate. Plates were analyzed using a Perkin Elmer Enspire plate reader with excitation at 360 nm and detecting emission at 460 nm. Fluorescence measurements (RFU) were converted to [AMC] concentrations based on an [AMC]–fluorescence standard curve and all data analysis was performed using GraphPad Prism (vers 8.1.2).

End-point inhibition assays were performed as previously described.[2] In brief, the relevant substrate, NAD^+ and inhibitor were added to each well and the experiment was initiated by addition of a freshly prepared solution of relevant KDAC, for a final volume of 25 µL per well. The following final concentrations were used: SIRT enzyme (100 nM SIRT1–3; for selectivity screen: 200 nM for SIRT1 and SIRT2; 400 nM for SIRT3; 100 nM for SIRT5; 500 nM for SIRT6 and SIRT7; 1 nM for HDAC1 and HDAC2; 2.5 nM for HDAC3), substrate (50 µM; LGKac: 20 µM), NAD^+ (500 µM), (for SIRT7 yeast tRNA from ThermoFisher (cat. #AM-7119) (75 µg/mL)) and inhibitor (1, 10, or 100 µM or 2- or 3-fold dilution series for dose–response assays). The plate was incubated at 37 °C for 60 min (30 min for HDACs, 120 min for SIRT7), then a solution of trypsin and NAM (25 µL, 5 mg/mL and 4 mM, respectively; final concentration 2.5 mg/mL and 2 mM, respectively, trypsin 0.2 mg/mL for HDACs) was added and the assay development was allowed to proceed for 90 min at RT (10 min for HDACs), before fluorescence measurement and calculation of residual activity. For concentration-response assays, IC_{50} values were obtained by fitting the resulting data to the concentration–response equation using GraphPad Prism (version 8.1.2).

Cell culture. All cell culture media contained 10% (v/v) FBS (ThermoFisher; cat. #26140079) and 1% penicillin-streptomycin (Sigma-Aldrich; cat. #P4333) unless stated otherwise and cultured at 37 °C with 5% CO_2 in a humidified incubator. MCF-7 (Sigma-Aldrich; cat. #86012803) and HeLa (Sigma-Aldrich; cat. #93021013) cells were maintained in Minimum Essential Medium Eagle (MEM, Sigma-Aldrich; cat. #M2279) supplemented with L-glutamine (2.0 mM, Sigma-Aldrich; cat. #G7513) and MEM non-essential amino acid solution (1%, Sigma-Aldrich; cat. #P7145). Jurkat (Sigma-Aldrich; cat. #88042803) cells were maintained in Roswell Park Memorial Institute medium (RPMI-1640, Sigma-Aldrich; cat. #R0883). HEK293T (ATCC; cat. #CRL-1573) cells were maintained in Dulbecco's modified Eagle's medium (DMEM, ThermoFisher; cat. #11965118). Cell lines were sub-cultured every 2–4 days.

Cell viability assays. Cell viability was assessed using MTT cell growth kits (Merck Millipore; cat. #CT02) as previously described.[6] In short, cells were seeded into flat 96-well plates at 5,000–10,000 cells per well. After 24 h, test compounds were added to final concentrations ranging from 100–0.02 µM and incubated for 72 h. Cell viability was measured following the manufacturer's protocol. The relative cell viability in presence of test compounds was measured at 570 nm normalized to the DMSO-treated controls after background subtraction at 630 nm on a PerkinElmer EnSpire plate reader. All viability assays were performed as duplicates of triplicates. GraphPad Prism (vers. 8.1.2) was used to determine EC_{50} and 95% CI values.

Immunocytochemistry. MCF-7 or HEK293T cells (2500/well) were plated in Ninc Lab-Tek Permanox Plastic Chamber slide system (ThermoFisher, cat. #177445) and incubated overnight. After 24 h, the cells were treated with inhibitor at the noted concentration or DMSO (control) for 6 h, after which the cells were fixed in 4% formaldehyde for 15 min at RT. Cells were rinsed three times with PBS (pH 7.4) before blocking for 1 h with blocking buffer (5% goat-serum in PBS-T (PBS + 0.1% Triton-×100)) at RT and were then incubated with anti-mouse acetylated α-tubulin antibody (1:300, Santa Cruz Biotechnology; cat. #sc-23950 AC) in 5% goat-serum in PBS-T overnight at 4 ˚C. The cells were washed in PBS three times and the fluorophore conjugated antibody diluted in blocking buffer (goat-anti mouse Alexa 488) was added 1:800 and incubated 1 h in the dark at RT. After three washes with PBS, the slides were mounted using ProLong® gold antifade mountant with DAPI (ThermoFisher; cat. #P36941) and cells were visualized using an inverted fluorescence microscope EVOS™ M5000 Imaging System with an EVOS™ at 20X. Images were processed using ImageJ (version 1.8).

Chemical stability assays. 400 µL supplemented Dulbecco's modified Eagle's medium (DMEM, ThermoScientific; cat. #11965118) was incubated at 37 °C for 15 min. The medium was spiked with a DMSO-stock solution of the respective inhibitor to reach a final concentration of 150 µM. The mixture was shaken at 750 rpm in an incubator at 37 °C. Samples (45 µL) were taken out at time points (0 min, 15 min, 30 min, 1 h, 2 h, 6 h and 24 h) and quenched with 50 µL 6 M urea and incubated for 10 min at 4 °C. Ice-cold acetonitrile (100 µL) was added to the sample and incubated for another 10 min at 4 °C. The samples were centrifuged for 60 min at 20,000 g and filtered (0.50 µM) before analysis by HPLC and subsequent integration of the peak areas of recovered compound over time. Each assay was performed at least twice.

Mitochondrial localization assay. HeLa cells (P < 20) were plated in 3 cm glass bottom dishes (50.000 cells per dish) and allowed to adhere overnight. The media was removed and the cells were co-treated with inhibitor (15 µM), NucBlue™ Live ReadyProbes™ Reagent (ThermoFisher cat. #R37605) (0.125 drop/mL) and MitoTracker™ Orange CMTMRos (cat. #M7510) (10 nM) in media for 30 min. The stain solution was removed and the cells were either washed with media (2×2 mL, 37 °C) and 1 mL media was added before acquiring images or the cells were fixed in 4% formaldehyde for 15 min at RT followed by three washes with PBS and mounting ProLong® gold antifade mountant (ThermoFisher; cat. #P10144). The images were acquired on an EVOS™ M5000 Imaging System with an EVOS™ 100× Oil Objective, fluorite, coverslip-corrected - AMEP4696. The fluorescence images were analyzed with the colocalization tool in ImageJ (version 1.51) to determine Pearson's R values.

Mitochondrial enrichment. HEK293T cells were cultured in T175 flasks upon reaching approx. 80-90% confluency, the cells were treated in T175 flask with **17**, **18** or respective volume of DMSO for 5 h. After incubation, cells were washed in phosphate-buffered saline (PBS, pH 7.4, ThermoFisher; cat. #10010023) and collected by scraping in PBS. The cells were centrifuged (600*g*, 5 min, 4 °C) and after removing the PBS the cells were resuspended in 1 mL supplemented mitochondrial lysis buffer (10 mM TRIS/MOPS, 1 mM EGTA, 0.2 M sucrose, 10 mM nicotinamide (NAM, Sigma-Aldrich; cat. #N5535), 1 µM TSA (Selleckchem; cat. #S1045), pH 7.4). The cells were centrifuged at (600*g*, 10 min, 4 °C) and after removing the supernatant the cells were resuspended in 600 µL mitochondrial lysis buffer and homogenized using a dounce tissue grinder set (Sigma; cat. #D8938). The cell membranes were pelleted by centrifugation (1000*g*, 10 min, 4 °C × 3) discarding the pellet between spins. A sample (40 µL) was taken for whole cell lysate before separating the mitochondria and cytosol by centrifugation (12600*g*, 10 min, 4°C). The supernatant was collected as cytosolic fraction and the pellet was washed twice with mitochondrial lysis buffer (500 µL, 10000*g*, 10 min, 4°C). The pellet (mitochondria) was resuspended in mitochondrial lysis buffer (40 µL). To all three samples (whole cell, cytosol, mitochondria) protease inhibitor (Sigma-Aldrich; cat. #P8340) was added and the samples were sonicated for 1 min (2 s pulse, 2 s pause). The protein concentration was determined using a Bicinchoninic Acid Kit for Protein Determination (BCA assay, Sigma-Aldrich; cat. #BCA1).

Cellular thermal shift assay.[7-8] HEK293T cells were seeded in 10 cm^2 dishes and grown to 80–90% confluency. Media was aspirated and fresh medium supplemented with compound (10 µM), or the respective volume of DMSO was added to the respective plates. The cells were treated for 5 h, followed by removal of the medium by aspiration. Cells were collected in PBS (pH 7.4, ThermoFisher; cat. #10010023) by scraping, and pelleted by centrifugation (300*g*, 5 min). The cell pellets were resuspended in PBS and spun down again. The washed cell pellets were suspended in PBS supplemented with cOmplete EDTA-free protease inhibitor cocktail (COEDTAF-RO, Sigma-Aldrich, 800 µL/cell treatment). The cell suspensions were aliquoted into PCR tubes (60 µL) and heated to 37.0 °C, 45.0 °C, 46.3 °C, 47.7 °C, 49.7 °C, 51.6 °C, 53.4 °C, 55.3 °C, 57.3 °C, and 63.8 °C for 3 min and then 3 min at 25 °C in a thermal cycler (Eppendorf Mastercycler Nexus Thermal Cycler). The cellular suspensions were then lysed by three freeze/thaw cycles, snap-freezing in a dry-ice/acetone bath followed by thawing at 25 °C in the thermal cycler and subsequent vortexing. The suspensions were subjected to centrifugation (20,000*g*, 20 min) at 4 °C and the supernatants were collected as whole-cell lysate. The isolated lysates were resolved by SDS-PAGE

in NuPAGE gels (4–12% Bis-Tris, ThermoFisher; cat. #P0322BOX) with MES running buffer (ThermoFisher, cat. #NP000202) followed by analysis by Western blot.

Preparation of whole cell lysate after compound treatment of HEK293T cells. HEK293T cells were seeded in 6 or 12-well plates and grown to 80–90% confluency. Cells were treated with 10 µM of either EX-527 (Sigma-Aldrich; cat. #E7034), **17**, **19** or respective volume of DMSO together with 1 µM TSA for 5 or 6 h. After incubation, cells were washed in phosphate-buffered saline (PBS, pH 7.4, Thermo Scientific; cat. #10010023) and collected in lysis buffer (1% Triton X-100, 0.2% SDS and cOmplete EDTA-free protease inhibitor cocktail (COEDTAF-RO, Sigma-Aldrich) in PBS, 100 or 200 µL/well by scraping. The suspensions were sonicated with a Bandelin Sonopuls mini20 (2 s on, 2 s off, 80% amplitude, 1 min), centrifuged (14000*g*, 10 min, 4 °C), and protein concentrations of the supernatants were determined by a bicinchoninic acid assay (Sigma-Aldrich; cat. #BCA1).

Western blot analysis. Equal amounts of protein samples (20–40 µg) were denatured by mixing with NuPAGE LDS sample buffer (ThermoFisher, cat. #NP0007) and sample reducing agent (ThermoFisher, NP0004) followed by heating to 95 °C for 10 min. Samples were then resolved by SDS-PAGE using NuPAGE gels (4–12% Bis-Tris, ThermoFisher, cat. #NP0322BOX or 10% Bis-Tris, ThermoFisher, cat. #NP0303BOX) with MES running buffer (ThermoFisher, cat. #NP000202). Protein bands were transferred onto an PVDF membrane (ThermoFisher, cat. #IB24001) using an iBlot 2 gel transfer device. Membranes for visualization with chemiluminescence were blocked in 5% skim milk in Tris-buffered saline containing 0.1% tween-20 (TBS-T, 20 mM Tris, 150 mM NaCl, pH 7.6) for 1 h at RT. Subsequently the membranes were washed with TBS-T (3×5 min) followed by incubation with primary antibody in 5% bovine serum albumin in TBS-T (1:1000) overnight at 4 °C. After another three cycles of washing with TBS-T the membrane was incubated with HRP conjugated secondary antibody in 2% skim milk in TBS-T (1:10,000) for 1 h at RT. After washing with TBS-T (3×5 min) and TBS (1×5 min), the membranes were visualized using enhanced chemiluminescent reagents (Pierce ECL Western Blotting Substrate, ThermoFisher, cat. #32106) on a syngene PXi4 image analysis system. Membranes visualized with fluorescence were blocked in LI-COR blocking buffer for 1 h at RT and probed with primary antibody in LI-COR blocking buffer containing 0.1% tween-20 (1:1000) overnight at 4 °C. After 3×5 min washes in TBS-T the membranes were incubated with anti-rabbit and anti-mouse IgG secondary antibodies (1:15,000 diluted in LI-COR blocking buffer containing 0.1% tween-20) for 1 h at RT. Membranes were washed with TBS-T (3×5 min) and dried before visualization using the Odyssey® Fc Imaging System. Band intensities were determined using Image Studio Lite software from Li-Cor (version 5.2.5) or ImageJ (version 1.51). Antibodies: rabbit anti-vinculin (Cell Signaling Technology (CST); cat. #13901), mouse anti-p53 (CST; cat. #12524),

rabbit anti-acetyl-p53 (Lys382) (CST; cat. #2525), mouse anti-SIRT1 (Santa Cruz Biotechnology; cat. #sc-74504), rabbit anti-SIRT2 (CST; cat. #12650), rabbit anti-SIRT3 (CST; cat. #5490), rabbit anti-MnSOD (acetyl K68) (Abcam; cat. #ab137037), mouse anti-GAPDH (Abcam; cat. #ab8245), mouse anti-β-actin (Santa Cruz Biotechnology; cat. #sc-47778), rabbit anti-UQCRFS1 (ThermoFisher; cat. #PA5-48253), Goat anti-rabbit IgG (H+L) HRP-conjugated (ThermoFisher; cat. #31466), Goat anti-mouse poly-HRP (ThermoFisher; cat. #32230), rabbit anti-acetyl histone H3 (Lys9) (Merck Millipore cat. #07-352), mouse anti-acetyl histone H4 (Santa Cruz Biotechnology; cat. #sc-377520), rabbit anti-acetyl histone H4 (Lys12) (Santa Cruz Biotechnology; cat. #sc-8661-R), DyLight 680 anti-rabbit IgG (CST; cat. #5366) and DyLight 800 anti-mouse IgG (CST; cat. #5357).

General methods (chemistry)

All reagents and solvents were of analytical grade and used without further purification as obtained from commercial suppliers. Anhydrous solvents were obtained from a PureSolv-system. Reactions were conducted under an atmosphere of nitrogen whenever anhydrous solvents were used. All reactions were monitored by thin-layer chromatography (TLC) using silica gel coated plates (analytical SiO_2-60, F-254). TLC plates were visualized under UV light and by dipping in either (a) a solution of potassium permanganate (10 g/L), potassium carbonate (67 g/L) and sodium hydroxide (0.83 g/L) in water, (b) a solution of ninhydrin (3 g/L) in 3% acetic acid in water (v/v), or (c) a solution of molybdate-phosphoric acid (12.5 g/L) and cerium(IV)sulfate (5 g/L) in 3% conc. sulfuric acid in water (v/v) followed by heating with a heat gun. Vacuum liquid chromatography (VLC) was performed with silica gel 60 (particle size 15-40 µM). After column chromatography, appropriate fractions were pooled and dried at high vacuum (<2 mbar) for at least 12 h to give obtained products in high purity (>95%) unless otherwise stated. Evaporation of solvents was carried out under reduced pressure at a temperature below 40 °C. Preparative reversed-phase HPLC purification was performed on a C18 Phenomenex Luna column (5 µM, 100 Å, 250×20 mm) or a C8(2) Luna column (5 µM, 100 Å, 250×21.2 mm) using an Agilent 1260 LC system equipped with a diode array UV detector and an evaporative light scattering detector (ELSD). Gradient A with eluent I (H_2O/MeCN/TFA, 95:5:0.1, v:v) and eluent II (0.1% TFA in MeCN) rising linearly from 0-30% to 95% of IV during t = 5–45 min *or* t = 5–65 min, at a flow rate of 20 mL/min. Analytical HPLC was performed on a C18 phenomenex Luna column (3 µM, 100 Å, 150×4.60 mm) or a C8 phenomenex Luna column (5 µM, 100 Å, 250×4.60 mm) using an Agilent 1100 series system equipped with a diode array UV detector. Gradient B using eluent I and eluent II, rising linearly from 0% to 95% of IV during t = 5–25 min at a flow rate of 1.2 mL/min. UPLC–MS analyses were performed on a Phenomenex Kinetex column (1.7 µM, 50×2.10 mm) using a Waters Acquity ultra high-performance liquid chromatography

(UPLC) system. Gradient C with eluent III (0.1% HCOOH in H_2O) and eluent IV (0.1% HCOOH in MeCN) rising linearly from 0% to 95% of IV during t = 0.00-5.20 min was applied at a flow rate of 0.6 mL/min. High-resolution mass spectrometry (HRMS) measurements were recorded either on a maXis G3 quadrupole time-of-flight (TOF) mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray (ESI) source or on an Agilent 1290 UHPLC equipped with a diode array detector and coupled to Agilent 6550 QTOF mass spectrometer operated in positive electrospray or on a Bruker Solarix WR by either matrix assisted laser desorption/ionization, or electrospray ionization (ESI). Nuclear magnetic resonance (NMR) spectra were recorded either on a Bruker Avance III HD equipped with a cryogenically cooled probe (^{1}H NMR and ^{13}C NMR recorded at 600 and 151 MHz, respectively), a Bruker Avance III (^{1}H NMR, ^{13}C NMR and ^{19}F NMR recorded at 400, 101 and 377 MHz, respectively). All spectra were recorded at 298 K. Chemical shifts are reported in ppm relative to deuterated solvent as internal standard (δ_H DMSO-d_6 2.50 ppm; δ_C DMSO-d_6 39.52 ppm; δ_H $CDCl_3$ 7.26 ppm; δ_C $CDCl_3$ 77.16 ppm; δ_H MeOD 3.31 ppm; δ_C MeOD 49.0 ppm). Assignments of NMR spectra are based on 2D correlation spectroscopy (COSY, HSQC, TOCSY and HMBC spectra).

General protocol for automated and manual solid phase peptide synthesis (SPPS). The peptides were synthesized on a ChemMatrix® or TentaGel®-resin using a Rink amide (RAM) linker by standard solid-phase peptide synthesis. The resin loading was determined spectrophotometrically, quantifying the amount of released fluorene upon cleavage of the Fmoc group from a small sample.[2] The mitochondrial targeting sequences were synthesized by automated peptide synthesis using standard Fmoc SPPS chemistry on a Biotage SyroWave™ synthesizer. The following commercially available Fmoc-protected amino acids with side chain protecting groups were used: Fmoc-Cha-OH, Fmoc-Arg(Pbf)-OH, Fmoc-D-Arg(Pbf)-OH, Fmoc-Lys(Boc)-OH, Fmoc-D-Lys(Boc)-OH, Fmoc-Lys(Teoc)-OH, Fmoc-Gln(Trt)-OH.

SPPS was performed on 0.04-0.08 mmol scale. Automated Fmoc deprotection was performed in two stages: 1) piperidine in DMF (2:3, v/v) for 3 min and 2) piperidine in DMF (1:4, v/v) for 2 × 8 min. The deprotection was followed by washing with DMF (2 × 45 s), CH_2Cl_2 (45 s) and DMF (2 × 45 s). The automated coupling reactions were performed as single couplings using Fmoc-Xaa-OH (5.0 equiv. to the resin loading, 2.5 equiv. for Fmoc-Cha-OH and Fmoc-D-Arg(Pbf)-OH), HBTU (5 equiv.) and *i*-Pr_2NEt (10 equiv., 2.0 M in NMP) in DMF (final concentration = 0.2 M) for 2 h followed by washing with DMF (2 × 45 s), CH_2Cl_2 (45 s) and DMF (2 × 45 s).

Manual couplings on resin were performed as single couplings using Fmoc-Xaa-OH (3.0 equiv. to the resin loading), HATU (3 equiv.) and *i*Pr_2NEt (6 equiv.) in DMF for 2 h followed by washing with

DMF (3 × 4 mL), MeOH (3 × 4 mL), CH_2Cl_2 (3 × 4 mL), DMF (3 × 4 mL). Manual Fmoc deprotection was performed with piperidine in DMF (1:4, v/v) for 30 min followed by washing with DMF (3 × 4 mL), MeOH (3 × 4 mL), CH_2Cl_2 (3 × 4 mL), DMF (3 × 4 mL).

General procedure for global deprotection and cleavage from the resin. Peptides were cleaved from the resin with TFA/H_2O/TIPS (95:2.5:2.5 (v/v), 2.0 mL; 2 h), TFA was removed under a stream of nitrogen and the resulting crude triturated with ice-cold diethyl ether and purified by preparative reversed-phase HPLC. Yields were determined based on resin loading.

General procedure for on-resin Teoc deprotection. A solution of TBAF trihydrate (10 equiv.) in DMF (4.0 mL/0.1 mmol resin) was added to the fritted syringe containing the resin bound peptide and the reaction mixture was agitated for 2 h at 50 ˚C. The resin was then washed with DMF (3×4.0 mL) and CH_2Cl_2 (3×4.0 mL).

General on-resin capping procedure. Compound **S20** (3 equiv.) and *i*Pr_2NEt (6 equiv.) were dissolved in DMF (1.0 mL/0.01 mmol resin) and added to the fritted syringe containing the resin-bound peptide and the reaction mixture was agitated for 15–18 h at ambient temperature. After washing with DMF (3×4.0 mL) and CH_2Cl_2 (3×4.0 mL) the reaction progress was evaluated with a test cleavage.

General on-resin thiourea formation procedure. A solution of the desired amine (2 equiv.) and *i*Pr_2NEt (3 equiv.) in CH_2Cl_2 (3.0 mL) was added dropwise over 5 minutes to a solution of bis(1-benzotriazolyl)methanethione (2 equiv.) in CH_2Cl_2 (5 mL) at 0 °C leading to a color change from yellow to pale yellow. The reaction mixture was concentrated under reduced pressure and the resulting crude residue and *i*Pr_2NEt (2 equiv.) were dissolved in DMF (4.0 mL/0.1 mmol resin) and then added to the fritted syringe containing the resin bound peptide. The reaction mixture was agitated for 15-18 h at ambient temperature. After washing with DMF (3 × 4.0 mL) and CH_2Cl_2 (3 × 4.0 mL) the reaction progress was evaluated with a test cleavage.

General on-resin click reaction procedure. CuI (0.5 equiv.) was dissolved in CH_3CN (1 mL/0.02 mmol resin) and degassed with nitrogen. Aqueous sodium ascorbate (100 mM, 0.5 equiv.), 2,6-lutidine (2 equiv.), NBD-N_3 (**S16**, 100 mM in DMSO, 1 equiv.) and DMF (2 mL/0.1 mmol resin) were added and the solution was degassed with nitrogen and added to the fritted syringe containing the resin-bound peptide. The reaction mixture was agitated for 16-19 h at ambient temperature. After washing with DMF (3×4.0 mL) and CH_2Cl_2 (3×4.0 mL) the reaction progress was evaluated with a test cleavage.

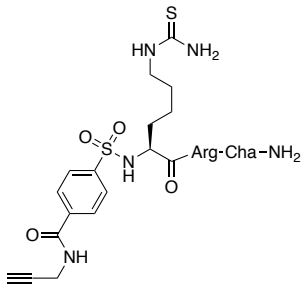
Syntheses and characterization data

((4-(prop-2-yn-1-ylcarbamoyl)phenyl)sulfonyl)-Lys(ThioAc)-Arg-Cha-NH_2 (1). Starting from H-Arg(Pbf)-Cha-resin (20 µmol, estimated loading: 0.47 mmol/g) synthesized from Fmoc-Cha-OH and Fmoc-Arg(Pbf)-OH by automated SPPS, the title compound was synthesized by on-resin coupling of Fmoc-Lys(ThioAc)-OH followed by Fmoc deprotection and capping with **S20** according to the general on-resin capping procedure. Global deprotection and cleavage from the resin, followed by preparative reversed-phase HPLC purification afforded the desired trimer **1** (5 mg, 37% based on resin loading) as a colorless fluffy material after lyophilization. ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.89 (t, J = 5.3 Hz, 1H, $NH_{\varepsilon,Lys}$), 9.16 (t, J = 5.5 Hz, 1H, NH_{alkyne}), 8.14 (d, J = 7.8 Hz, 1H, $NH_{\alpha,Arg}$), 8.10 (d, J = 8.6 Hz, 1H, $NH_{\alpha,Lys}$), 7.99 (d, J = 8.5 Hz, 2H, $H3_{Phenyl}$, $H5_{Phenyl}$), 7.84 (d, J = 8.5 Hz, 2H, $H2_{Phenyl}$, $H6_{Phenyl}$), 7.78 (d, J = 8.3 Hz, 1H, $NH_{\alpha,Cha}$), 7.53 (t, J = 5.8 Hz, 1H, $NH_{\varepsilon,Arg}$), 7.28 (d, J = 2.0 Hz, 1H, $CONH_{2,A}$), 6.93 (s, 1H, $CONH_{2,B}$), 4.25–4.18 (m, 1H, $H_{\alpha,Cha}$), 4.08 (dd, J = 5.5, 2.5 Hz, 2H, $CH_{2,alkyne}$), 4.05–3.98 (m, 1H, $H_{\alpha,Arg}$), 3.83–3.77 (m, 1H, $H_{\alpha,Lys}$), 3.37–3.30 (m, 2H, $H_{\varepsilon,Lys}$), 3.14 (t, J = 2.5 Hz, 1H, CH_{alkyne}), 3.05 (q, J = 6.6 Hz, 2H, $H_{\delta,Arg}$), 2.35 (s, 3H, CH_3), 1.70–0.98 (m, 21H), 0.92–0.69 (m, 2H) ($H_{\beta,\gamma,\delta,Lys}$, $H_{\beta,\gamma,Arg}$, $H_{\beta,\delta,\varepsilon,\xi,Cha}$, $CH_{\gamma,Cha}$). ^{13}C NMR (151 MHz, DMSO) δ 199.2 (CS), 174.5 (CO_{Cha}), 171.1(CO_{Lys}), 171.1 (CO_{Arg}), 165.4 (CO_{alkyne}), 158.8 (q, J = 32.2 Hz, residual CO_{TFA}), 157.2 (NHC(=NH)NH_2), 144.1 ($C1_{Phenyl}$), 137.3 ($C4_{Phenyl}$), 128.3 (C3, $C5_{Phenyl}$), 127.1 (C2, $C6_{Phenyl}$), 117.3 (q, J = 298.0 Hz, $CF_{3,TFA}$), 81.4 ($\underline{C}CH_{alkyne}$), 73.6 ($\underline{C}HC_{alkyne}$), 56.2 ($C_{\alpha,Lys}$) 52.5 ($C_{\alpha,Arg}$), 50.5 ($C_{\alpha,Cha}$), 45.7 ($C_{\varepsilon,Lys}$), 40.9 ($C_{\delta,Arg}$), 40.0 (overlap with solvent peak, $C_{\beta,Cha}$), 33.9 ($C_{\gamma,Cha}$), 33.7, 33.2 (CH_3), 33.0 ($C_{\beta,Lys}$), 32.2, 29.4 ($C_{\beta,Arg}$), 29.1 ($CH_{2,alkyne}$), 27.2 ($C_{\delta,Lys}$), 26.5, 26.3, 26.0, 25.3 ($C_{\gamma,Arg}$), 23.1 ($C_{\gamma,Lys}$), ($C_{\delta,\varepsilon,\xi,Cha}$). Analytical HPLC gradient 5-95% eluent II in eluent I (20 min total runtime), t_R 11.9 min (>95%, UV_{254}). HRMS m/z 734.3475 ($[M+H]^+$, $C_{33}H_{52}N_9O_6S_2^+$ Calcd 734.3476).

((4-(prop-2-yn-1-ylcarbamoyl)phenyl)sulfonyl)-Lys(2,2,2-trifluoroacetyl)-Arg-Cha-NH_2 (2). Starting from H-Arg(Pbf)-Cha-resin (20 µmol, estimated loading: 0.47 mmol/g) synthesized from Fmoc-Cha-OH and Fmoc-Arg(Pbf)-OH by automated SPPS, the title compound was synthesized by on-resin coupling of Fmoc-Lys(TFA)-OH followed by Fmoc deprotection and capping with **S20** according to the general on-resin capping procedure. Global deprotection and cleavage from the resin, followed by preparative reversed-phase HPLC purification afforded the desired trimer **2** (6 mg, 36% based on resin loading) as a colorless fluffy material after lyophilization. ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.37 (t, J = 5.7 Hz,

1H, $NH_{\varepsilon,Lys}$), 9.16 (t, *J* = 5.5 Hz, 1H, NH_{alkyne}), 8.14 (d, *J* = 7.8 Hz, 1H, $NH_{\alpha,Arg}$), 8.10 (d, *J* = 8.6 Hz, 1H, $NH_{\alpha,Lys}$), 8.02–7.94 (m, 2H, $H2_{Phenyl}$, $H6_{Phenyl}$), 7.88–7.80 (m, 2H, $H3_{Phenyl}$, $H5_{Phenyl}$), 7.76 (d, *J* = 8.3 Hz, 1H, $NH_{\alpha,Cha}$), 7.53 (t, *J* = 5.8 Hz, 1H, $NH_{\varepsilon,Arg}$), 7.28 (d, *J* = 2.1 Hz, 1H, $CONH_{2,A}$), 6.96–6.90 (m, 1H, $CONH_{2,B}$), 4.26–4.18 (m, 1H, $H_{\alpha,Cha}$), 4.08 (dd, *J* = 5.5, 2.5 Hz, 2H, $CH_{2,alkyne}$), 4.05–3.98 (m, 1H, $H_{\alpha,Arg}$), 3.82–3.77 (m, 1H, $H_{\alpha,Lys}$), 3.13 (t, *J* = 2.5 Hz, 1H, CH_{alkyne}), 3.10–2.99 (m, 4H, $CH_{2,\delta,Arg}$, $CH_{2,\varepsilon,Lys}$), 1.73–1.00 (m, 21H), 0.94–0.71 (m, 2H) ($H_{\beta,\gamma,\delta,Lys}$, $H_{\beta,\gamma,Arg}$, $H_{\beta,\delta,\varepsilon,\xi,Cha}$, $CH_{\gamma,Cha}$). ^{13}C NMR (151 MHz, DMSO-d_6) δ 174.0 (CO_{Cha}), 170.6 (CO_{Arg}), 170.6 (CO_{Lys}), 164.9 (CO_{alkyne}), 158.8–157.6 (m, residual CO_{TFA}), 156.7 ($NHC(=NH)NH_2$), 156.1 (d, *J* = 35.9 Hz, $\underline{C}OCF_3$), 143.6 ($C1_{Phenyl}$), 136.9 ($C4_{Phenyl}$), 127.9 (C2, $C6_{Phenyl}$), 126.6 (C2, $C6_{Phenyl}$), 117.0 (d, *J* = 299.0 Hz, residual $CF_{3,TFA}$), 115.9 (d, *J* = 288.2 Hz, CF_3), 80.9 ($\underline{C}CH_{alkyne}$), 73.1 ($\underline{C}HC_{alkyne}$), 55.7 ($C_{\alpha,Lys}$), 52.0 ($C_{\alpha,Arg}$), 50.0 ($C_{\alpha,Cha}$), 39.6 (overlap with solvent peak, $C_{\varepsilon,Lys}$), 39.5 (overlap with solvent peak, $C_{\beta,Cha}$), 33.4 ($C_{\gamma,Cha}$), 33.2, 32.4 ($C_{\beta,Lys}$), 31.7, 28.9 ($C_{\beta,Arg}$), 28.7 ($CH_{2,alkyne}$), 27.8, 26.0, 25.8, 25.5, 24.8 ($C_{\gamma,Arg}$), 22.3 ($C_{\gamma,Lys}$) ($C_{\delta,Lys}$, $C_{\delta,\varepsilon,\xi,Cha}$). Analytical HPLC gradient 5-95% eluent II in eluent I (20 min total runtime), t_R 14.3 min (>98%, UV_{230}). HRMS *m/z* 772.3423 ($[M+H]^+$, $C_{33}H_{49}F_3N_9O_7S^+$ Calcd 772.3422).

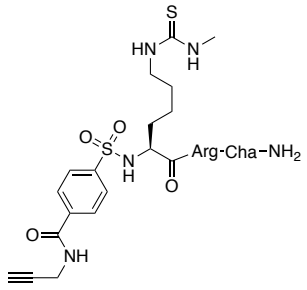
((4-(prop-2-yn-1-ylcarbamoyl)phenyl)sulfonyl)-Lys(carbamothioyl)-Arg-Cha-NH_2 (3). Starting



from H-Arg(Pbf)-Cha-resin (20 µmol, estimated loading: 0.47 mmol/g) synthesized from Fmoc-Cha-OH and Fmoc-Arg(Pbf)-OH by automated SPPS, the capped trimer was synthesized by on-resin coupling of Fmoc-Lys(Teoc)-OH followed by Fmoc deprotection and capping with **S20** according to the general on-resin capping procedure. The Teoc-group was then removed according to the general procedure for on-resin Teoc deprotection, and the title compound was synthesized by on-resin lysine side chain modification with Bis(1*H*-benzo[*d*][1,2,3]triazol-1-yl)methanethione (0.9 equiv.) and *i*Pr_2NEt (3 equiv.) in 2 mL anhydrous DMF for 1 h at -12 °C. The resin was washed with DMF (3×4.0 mL) and CH_2Cl_2 (3×4.0 mL) and a solution of ammonia (25% v/v, 30 µL, 20 equiv.) in anhydrous 1 mL DMF was added to the resin and agitated for 2.5 h at ambient temperature. The resin was washed with DMF (3×4.0 mL) and CH_2Cl_2 (3×4.0 mL) and global deprotection and cleavage from the resin, followed by preparative reversed-phase HPLC purification afforded the desired trimer **3** (2 mg, 12% based on resin loading) as a colorless fluffy material after lyophilization. ^1H NMR (600 MHz, DMSO-d_6) δ 9.17 (t, *J* = 5.5 Hz, 1H, NH_{alkyne}), 8.14 (d, *J* = 7.8 Hz, 1H, $NH_{\alpha,Arg}$), 8.10 (d, *J* = 8.6 Hz, 1H, $NH_{\alpha,Lys}$), 8.03–7.95 (m, 2H, $H3_{Phenyl}$, $H5_{Phenyl}$), 7.87–7.82 (m, 2H, $H2_{Phenyl}$, $H6_{Phenyl}$), 7.80 (d, *J* = 7.6 Hz, 1H, $NH_{\alpha,Cha}$), 7.63–7.49 (m, 2H, $NH_{\varepsilon,Arg}$, $NH_{\varepsilon,Lys}$), 7.29 (s, 1H, $CONH_{2,A}$), 6.99–6.80 (m, 3H, $CONH_{2,B}$, $CSNH_2$), 4.25–4.18 (m, 1H, $H_{\alpha,Cha}$), 4.08 (dd, *J* = 5.5, 2.5 Hz, 2H, $CH_{2,alkyne}$), 4.04–3.97 (m, 1H, $H_{\alpha,Arg}$), 3.80

(q, J = 7.8 Hz, 1H, $H_{\alpha,Lys}$), 3.22 (s, 2H, $H_{\varepsilon,Lys}$), 3.14 (t, J = 2.5 Hz, 1H, $CH_{,alkyne}$), 3.05 (q, J = 6.6 Hz, 2H, $H_{\delta,Arg}$), 1.70–1.03 (m, 21H), 0.92–0.73 (m, 2H) ($H_{\beta,\gamma,\delta,Lys}$, $H_{\beta,\gamma,Arg}$, $H_{\beta,\delta,\varepsilon,\xi,Cha}$, $CH_{\gamma,Cha}$). ^{13}C NMR (151 MHz, DMSO-d_6) δ 174.0 (CO_{Cha}), 170.7 (CO_{Lys}), 170.7 (CO_{Arg}), 165.0 (CO_{alkyne}), 158.1 (q, J = 31.3 Hz, residual CO_{TFA}), 156.7 ($NHC(=NH)NH_2$), 143.6 ($C1_{Phenyl}$), 136.9 ($C4_{Phenyl}$), 127.9 ($C3_{Phenyl}$, $C5_{Phenyl}$), 126.6 ($C2_{Phenyl}$, $C6_{Phenyl}$), 117.2 (q, J = 299.9 Hz, residual $CF_{3,TFA}$), 80.9 ($\underline{C}CH_{alkyne}$), 73.1 ($\underline{C}HC_{alkyne}$), 69.8, 55.8 ($C_{\alpha,Lys}$), 52.0 ($C_{\alpha,Arg}$), 50.0 ($C_{\alpha,Cha}$), 43.7 ($C_{\varepsilon,Lys}$), 40.4 ($C_{\delta,Arg}$), 39.4 (overlap with solvent peak, $C_{\beta,Cha}$), 33.4 ($C_{\gamma,Cha}$), 33.2, 32.5, 31.7, 28.9 ($C_{\beta,Arg}$), 28.7, 26.0, 25.8, 25.5, 24.8 ($C_{,\gamma,Arg}$), 22.5 ($C_{\gamma,Lys}$), ($C_{\beta,\delta,Lys}$, $C_{\delta,\varepsilon,\xi,Cha}$). The peak for C=S was not visible in ^{13}C NMR and the peaks for $C_{\varepsilon,Lys}$ was broad and of low intensity in ^{13}C NMR, probably due to fast quadrupolar relaxation via nearby ^{14}N-nuclei. Analytical HPLC gradient 5-95% eluent II in eluent I (20 min total runtime), t_R 12.9 min (>96%, UV_{230}). HRMS m/z 735.3426 ($[M+H]^+$, $C_{32}H_{51}N_{10}O_6S_2^+$ Calcd 735.3429).

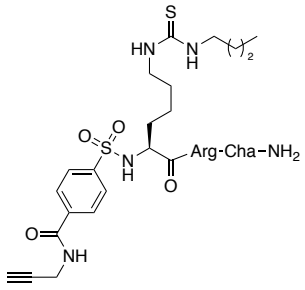
((4-(prop-2-yn-1-ylcarbamoyl)phenyl)sulfonyl)-Lys(methylcarbamothioyl)-Arg-Cha-NH_2 (4).



Starting from H-Arg(Pbf)-Cha-resin (20 µmol, estimated loading: 0.47 mmol/g) synthesized from Fmoc-Cha-OH and Fmoc-Arg(Pbf)-OH by automated SPPS, the capped trimer was synthesized by on-resin coupling of Fmoc-Lys(Teoc)-OH followed by Fmoc deprotection and capping with **S20** according to the general on-resin capping procedure. The Teoc-group was then removed according to the general procedure for on-resin Teoc deprotection, and the title compound was synthesized by on-resin thiourea formation as described in the general procedures using methylamine (33% in EtOH) as the desired amine. Global deprotection and cleavage from the resin, followed by preparative reversed-phase HPLC purification afforded the desired trimer **4** (4 mg, 23% based on resin loading) as a colorless fluffy material after lyophilization. ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.17 (t, J = 5.5 Hz, 1H, NH_{alkyne}), 8.14 (d, J = 7.8 Hz, 1H, $NH_{\alpha,Arg}$), 8.09 (d, J = 8.6 Hz, 1H, $NH_{\alpha,Lys}$), 8.03–7.97 (m, 2H, $H3_{Phenyl}$, $H5_{Phenyl}$), 7.88–7.81 (m, 2H, $H2_{Phenyl}$, $H6_{Phenyl}$), 7.79 (d, J = 8.2 Hz, 1H, $NH_{\alpha,Cha}$), 7.53 (t, J = 5.8 Hz, 1H, $NH_{\varepsilon,Arg}$), 7.43–7.32 (m, 2H, $NH_{\varepsilon,Lys}$, $CSN\underline{H}CH_3$), 7.30–7.28 (m, 1H, $CONH_{2,A}$), 6.96–6.92 (m, 1H, $CONH_{2,B}$), 4.25–4.18 (m, 1H, $H_{\alpha,Cha}$), 4.08 (dd, J = 5.5, 2.5 Hz, 2H, $CH_{2,alkyne}$), 4.02 (q, J = 7.1 Hz, 1H, $H_{\alpha,Arg}$), 3.83–3.76 (m, 1H, $H_{\alpha,Lys}$), 3.21 (s, 2H, $H_{\varepsilon,Lys}$), 3.14 (t, J = 2.5 Hz, 1H, $CH_{,alkyne}$), 3.05 (q, J = 6.5 Hz, 2H, $H_{\delta,Arg}$), 2.79 (s, 3H, CH_3), 1.71–1.03 (m, 21H), 0.92–0.73 (m, 2H) ($H_{\beta,\gamma,\delta,Lys}$, $H_{\beta,\gamma,Arg}$, $H_{\beta,\delta,\varepsilon,\xi,Cha}$, $CH_{\gamma,Cha}$). ^{13}C NMR (151 MHz, DMSO-d_6) δ 174.0 (CO_{Cha}), 170.7 (CO_{Lys}), 170.7 (CO_{Arg}), 165.0 (CO_{alkyne}), 158.2 (q, J = 31.3 Hz, residual CO_{TFA}), 156.7 ($NHC(=NH)NH_2$), 143.6 ($C1_{Phenyl}$), 136.9 ($C4_{Phenyl}$), 127.9 ($C3_{Phenyl}$, $C5_{Phenyl}$), 126.6 ($C2_{Phenyl}$, $C6_{Phenyl}$), 117.2 (q, J = 299.7 Hz, residual $CF_{3,TFA}$), 80.9 ($\underline{C}CH_{alkyne}$), 73.1 ($\underline{C}HC_{alkyne}$), 69.8, 55.8 ($C_{\alpha,Lys}$), 52.0 ($C_{\alpha,Arg}$), 50.1 ($C_{\alpha,Cha}$), 40.4 ($C_{\delta,Arg}$), 39.2 (overlap with solvent

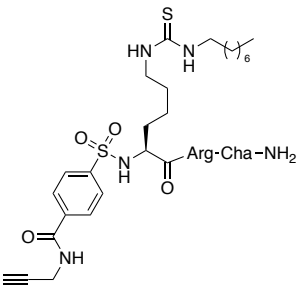
peak, $C_{\beta,Cha}$), 33.4 ($C_{\gamma,Cha}$), 33.2, 32.5 ($C_{\beta,Lys}$), 31.7, 28.9 ($C_{\beta,Arg}$), 28.7, 28.3 ($CH_{2,alkyne}$), 26.0, 25.8, 25.5, 24.8 ($C_{,\gamma,Arg}$), 22.5 ($C_{\gamma,Lys}$), ($C_{\delta,Lys}$, $C_{\delta,\varepsilon,\xi,Cha}$). The peak for C=S was not visible in ^{13}C NMR and the peaks for $C_{\varepsilon,Lys}$ and CSNH<u>C</u>H_3 were broad and of low intensity in ^{13}C NMR, probably due to fast quadrupolar relaxation via nearby ^{14}N-nuclei. Analytical HPLC gradient 5-95% eluent II in eluent I (20 min total runtime), t_R 13.3 min (>97%, UV_{230}). HRMS *m/z* 749.3583 ($[M+H]^+$, $C_{33}H_{52}N_{10}O_6S_2^+$ Calcd 749.3585).

((4-(prop-2-yn-1-ylcarbamoyl)phenyl)sulfonyl)-Lys(butylcarbamothioyl)-Arg-Cha-NH_2 (5).



Starting from H-Arg(Pbf)-Cha-resin (20 µmol, estimated loading: 0.47 mmol/g) synthesized from Fmoc-Cha-OH and Fmoc-Arg(Pbf)-OH by automated SPPS, the capped trimer was synthesized by on-resin coupling of Fmoc-Lys(Teoc)-OH followed by Fmoc deprotection and capping with **S20** according to the general on-resin capping procedure. The Teoc-group was then removed according to the general procedure for on-resin Teoc deprotection, and the title compound was synthesized by on-resin thiourea formation as described in the general procedures using butylamine to form the benzotriazole coupling reagent. Global deprotection and cleavage from the resin, followed by preparative reversed-phase HPLC purification afforded the desired trimer **5** (2 mg, 13% based on resin loading) as a colorless fluffy material after lyophilization.^1H NMR (600 MHz, DMSO-d_6) δ 9.15 (t, *J* = 5.5 Hz, 1H, NH_{alkyne}), 8.13 (d, *J* = 7.8 Hz, 1H, $NH_{\alpha,Arg}$), 8.09 (d, *J* = 8.6 Hz, 1H, $NH_{\alpha,Lys}$), 8.01–7.96 (m, 2H, $H3_{Phenyl}$, $H5_{Phenyl}$), 7.87–7.82 (m, 2H, $H2_{Phenyl}$, $H6_{Phenyl}$), 7.78 (d, *J* = 8.2 Hz, 1H, $NH_{\alpha,Cha}$), 7.45 (t, *J* = 5.8 Hz, 1H, $NH_{\varepsilon,Arg}$), 7.32–7.25 (m, 2H, $CONH_{2,A}$, $NH_{\varepsilon,Lys}$), 6.96–6.93 (m, 1H, $CONH_{2,B}$), 4.26–4.19 (m, 1H, $H_{\alpha,Cha}$), 4.08 (dd, *J* = 5.5, 2.5 Hz, 2H, $CH_{2,alkyne}$), 4.03 (q, *J* = 7.1 Hz, 1H, $H_{\alpha,Arg}$), 3.83–3.77 (m, 1H, $H_{\alpha,Lys}$), 3.15 (t, *J* = 2.5 Hz, 1H, $CH_{,alkyne}$), 3.05 (q, *J* = 6.6 Hz, 2H, $CH_{2,\delta,Arg}$), 1.69–1.02 (m, 27H), 0.91–0.73 (m, 5H, CH_3), (($\underline{C}H_2)_3CH_3$, $H_{\beta,\gamma,\delta,Lys}$, $H_{\beta,\gamma,Arg}$, $H_{\beta,\delta,\varepsilon,\xi,Cha}$, $CH_{\gamma,Cha}$). ^{13}C NMR (151 MHz, DMSO-d_6) δ 174.0 (CO_{Cha}), 170.7 (CO_{Lys}, CO_{Arg}), 165.0 (CO_{alkyne}), 156.6 ($NHC(=NH)NH_2$), 143.6 ($C1_{Phenyl}$), 136.9 ($C4_{Phenyl}$), 127.9 ($C3_{Phenyl}$, $C5_{Phenyl}$), 126.6 ($C2_{Phenyl}$, $C6_{Phenyl}$), 80.9 ($\underline{C}CH_{alkyne}$), 73.1 ($\underline{C}HC_{alkyne}$), 55.8 ($C_{\alpha,Lys}$), 52.0 ($C_{\alpha,Arg}$), 50.0 ($C_{\alpha,Cha}$), 40.4 ($C_{\delta,Arg}$), 39.3 (overlap with solvent peak, $C_{\beta,Cha}$), 33.4 ($C_{\gamma,Cha}$), 33.2, 32.6 ($C_{\beta,Lys}$), 31.7, 30.9 ($\underline{C}H_2CH_2CH_3$), 28.9 ($C_{\beta,Arg}$), 28.7 ($CH_{2,alkyne}$), 26.0, 25.8, 25.5, 24.8 ($C_{,\gamma,Arg}$), 22.5 ($C_{\gamma,Lys}$), 19.6 ($\underline{C}H_2CH_3$), 13.7 (CH_3), ($C_{\delta,Lys}$, $C_{\delta,\varepsilon,\xi,Cha}$). The peak for C=S was not visible in ^{13}C NMR and the peaks for $C_{\varepsilon,Lys}$ and CSNH<u>C</u>H_2 were broad and of low intensity in ^{13}C NMR, probably due to fast quadrupolar relaxation via nearby ^{14}N-nuclei. Analytical HPLC gradient 5-95% eluent II in eluent I (20 min total runtime), t_R 15.0 min (>97%, UV_{230}). HRMS *m/z* 791.4053 ($[M+H]^+$, $C_{36}H_{59}N_{10}O_6S_2^+$ Calcd 791.4055).

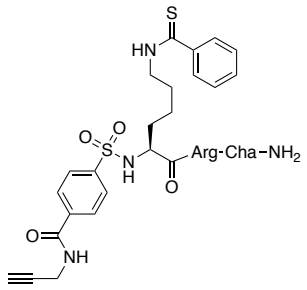
((4-(prop-2-yn-1-ylcarbamoyl)phenyl)sulfonyl)-Lys(hexylcarbamothioyl)-Arg-Cha-NH_2 (6).



Starting from H-Arg(Pbf)-Cha-resin (20 µmol, estimated loading: 0.47 mmol/g) synthesized from Fmoc-Cha-OH and Fmoc-Arg(Pbf)-OH by automated SPPS, the capped trimer was synthesized by on-resin coupling of Fmoc-Lys(Teoc)-OH followed by Fmoc deprotection and capping with **S20** according to the general on-resin capping procedure. The Teoc-group was then removed according to the general procedure for on-resin Teoc deprotection, and the title compound was synthesized by on-resin thiourea formation as described in the general procedures using octylamine as the desired amine to form the benzotriazole coupling reagent. Global deprotection and cleavage from the resin, followed by preparative reversed-phase HPLC purification afforded the desired trimer **6** (1 mg, 6% based on resin loading) as a colorless fluffy material after lyophilization. ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.15 (t, J = 5.5 Hz, 1H, NH_{alkyne}), 8.13 (d, J = 7.9 Hz, 1H, $NH_{\alpha,Arg}$), 8.09 (d, J = 8.6 Hz, 1H, $NH_{\alpha,Lys}$), 8.01–7.96 (m, 2H, $H3_{Phenyl}$, $H5_{Phenyl}$), 7.87–7.82 (m, 2H, $H2_{Phenyl}$, $H6_{Phenyl}$), 7.77 (d, J = 8.2 Hz, 1H, $NH_{\alpha,Cha}$), 7.45 (t, J = 5.8 Hz, 1H, $NH_{\varepsilon,Arg}$), 7.31–7.25 (m, 2H, $CONH_{2,A}$, $NH_{\varepsilon,Lys}$), 6.96–6.93 (m, 1H, $CONH_{2,B}$), 4.26–4.19 (m, 1H, $H_{\alpha,Cha}$), 4.08 (dd, J = 5.5, 2.6 Hz, 2H, $CH_{2,alkyne}$), 4.03 (q, J = 7.1 Hz, 1H, $H_{\alpha,Arg}$), 3.84–3.77 (m, 1H, $H_{\alpha,Lys}$), 3.14 (t, J = 2.5 Hz, 1H, $CH_{,alkyne}$), 3.05 (q, J = 6.6 Hz, 2H, $CH_{2,\delta,Arg}$), 1.70–1.01 (m, 35H, $(C\underline{H}_2)_7CH_3$, $H_{\beta,\gamma,\delta,Lys}$, $H_{\beta,\gamma,Arg}$, $H_{\beta,\delta,\varepsilon,\xi,Cha}$, $CH_{\gamma,Cha}$), 0.90–0.73 (m, 5H, CH_3). ^{13}C NMR (151 MHz, DMSO-d_6) δ 174.0 (CO_{Cha}), 170.7 (CO_{Lys}, CO_{Arg}), 165.0 (CO_{alkyne}), 156.6 ($NHC(=NH)NH_2$), 143.6 ($C1_{Phenyl}$), 136.9 ($C4_{Phenyl}$), 127.9 ($C3_{Phenyl}$, $C5_{Phenyl}$), 126.6 ($C2_{Phenyl}$, $C6_{Phenyl}$), 80.9 ($\underline{C}CH_{alkyne}$), 73.1 ($\underline{C}HC_{alkyne}$), 55.8 ($C_{\alpha,Lys}$), 52.0 ($C_{\alpha,Arg}$), 50.0 ($C_{\alpha,Cha}$), 40.4 ($C_{\delta,Arg}$), 39.3 (overlap with solvent peak, $C_{\beta,Cha}$), 33.4 ($C_{\gamma,Cha}$), 33.2, 32.6, 31.7, 31.2, 28.7, 28.7, 28.7, 26.4, 26.0, 25.8, 25.5, 24.8 ($C_{,\gamma,Arg}$), 22.5 ($C_{\gamma,Lys}$), 22.1 ($\underline{C}H_2CH_3$), 14.0 (CH_3), ($\underline{C}H_2)_7CH_3$, $C_{\beta,Arg}$, $C_{\beta,\delta,Lys}$, $C_{\delta,\varepsilon,\xi,Cha}$). The peak for C=S was not visible in ^{13}C NMR and the peaks for $C_{\varepsilon,Lys}$ and $CSNH\underline{C}H_2$ were broad and of low intensity in ^{13}C NMR, probably due to fast quadrupolar relaxation via nearby ^{14}N-nuclei. Analytical HPLC gradient 5-95% eluent II in eluent I (20 min total runtime), t_R 17.5 min (>95%, UV_{230}). HRMS m/z 847.4694 ($[M+H]^+$, $C_{40}H_{67}N_{10}O_6S_2^+$ Calcd 847.4681).

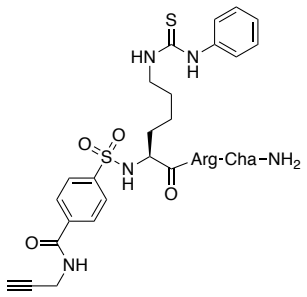
((4-(prop-2-yn-1-ylcarbamoyl)phenyl)sulfonyl)-Lys(ThioMyr)-Arg-Cha-NH_2 (7). Starting from H-Arg(Pbf)-Cha-resin (20 µmol, estimated loading: 0.47 mmol/g) synthesized from Fmoc-Cha-OH and Fmoc-Arg(Pbf)-OH by automated SPPS, the title compound was synthesized by on-resin coupling of Fmoc-Lys(ThioMyr)-OH followed by Fmoc deprotection and capping with **S20**. Global deprotection and cleavage from the resin, followed by preparative reversed-phase HPLC purification afforded the desired trimer **7** (5 mg, 29% based on resin loading), as a colorless fluffy material after lyophilization. ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.82 (t, J = 5.4 Hz, 1H, $NH_{\varepsilon,Lys}$), 9.16 (t, J = 5.5 Hz, 1H, NH_{alkyne}), 8.15 (d, J = 7.9 Hz, 1H, $NH_{\alpha,Arg}$), 8.09 (d, J = 8.7 Hz, 1H, $NH_{\alpha,Lys}$), 8.01–7.97 (m, 2H, $H3_{Phenyl}$, $H5_{Phenyl}$), 7.86–7.82 (m, 2H, $H2_{Phenyl}$, $H6_{Phenyl}$), 7.78 (d, J = 8.3 Hz, 1H, $NH_{\alpha,Cha}$), 7.53 (t, J = 5.8 Hz, 1H, $NH_{\varepsilon,Arg}$), 7.28 (d, J = 2.1 Hz, 1H, $CONH_{2,A}$), 6.95–6.92 (m, 1H, $CONH_{2,B}$), 4.25–4.19 (m, 1H, $H_{\alpha,Cha}$), 4.08 (dd, J = 5.5, 2.5 Hz, 2H, $CH_{2,alkyne}$), 4.02 (q, J = 7.1 Hz, 1H, $H_{\alpha,Arg}$), 3.84–3.78 (m, 1H, $H_{\alpha,Lys}$), 3.35 (q, J = 6.8 Hz, 2H, $CH_{2,\varepsilon,Lys}$), 3.13 (t, J = 2.5 Hz, 1H, CH_{alkyne}), 3.05 (q, J = 6.6 Hz, 2H, $CH_{2,\delta,Arg}$), 2.49–2.45 (m, 2H, $CSCH_2$), 1.71–1.01 (m, 43H), 0.91–0.70 (m, 5H, CH_3) ($CH_{2,Myr}$, $H_{\beta,\gamma,\delta,\ Lys}$, $H_{\beta,\gamma,Arg}$, $H_{\beta,\delta,\varepsilon,\xi,Cha}$, $CH_{\gamma,Cha}$). ^{13}C NMR (151 MHz, DMSO-d_6) δ 203.6 (CS), 174.0 (CO_{Cha}), 170.6 (CO_{Lys}, CO_{Arg}), 164.9 (CO_{alkyne}), 158.3 (q, J = 31.0 Hz, residual CO_{TFA}), 156.7 ($NHC(=NH)NH_2$), 143.6 ($C1_{Phenyl}$), 136.8 ($C4_{Phenyl}$), 127.8 (C3, $C5_{Phenyl}$), 126.6 (C2, $C6_{Phenyl}$), 117.2 (d, J = 300.0 Hz, residual $CF_{3,TFA}$), 80.9 ($\underline{C}CH_{alkyne}$), 73.1 ($\underline{C}HC_{alkyne}$), 55.7 ($C_{\alpha,Lys}$), 52.0 ($C_{\alpha,Arg}$), 50.0 ($C_{\alpha,Cha}$), 45.0 ($CS\underline{C}H_2$), 44.9 ($C_{\varepsilon,Lys}$), 40.4 ($C_{\delta,Arg}$), 39.3 (overlap with solvent peak, $C_{\beta,Cha}$), 33.4 ($C_{\gamma,Cha}$), 33.2, 32.5 ($C_{\beta,Lys}$), 31.7, 31.3, 29.04, 29.02, 29.00, 28.9 ($C_{\beta,Arg}$), 28.8, 28.7, 28.7, 28.2 ($CH_{2,alkyne}$), 26.7 ($C_{\delta,Lys}$), 26.0, 25.8, 25.5, 24.8 ($C_{\gamma,Arg}$) 22.6 ($C_{\gamma,Lys}$), 22.1, 13.9 (CH_3), ($C_{\beta,\delta,\varepsilon,\xi,Cha}$, $C_{\gamma,Cha}$, $CH_{2,Myr}$). Analytical HPLC gradient 5-95% eluent II in eluent I (20 min total runtime), t_R 20.5 min (>97%, UV_{230}). HRMS m/z 902.5352 ($[M+H]^+$, $C_{45}H_{76}N_9O_6S_2^+$ Calcd 902.5354).

((4-(prop-2-yn-1-ylcarbamoyl)phenyl)sulfonyl)-Lys(Thiobenzoyl)-Arg-Cha-NH_2 (8). Starting



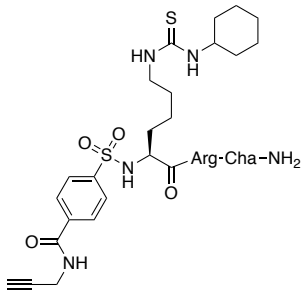
from H-Arg(Pbf)-Cha-resin (20 µmol, estimated loading: 0.47 mmol/g) synthesized from Fmoc-Cha-OH and Fmoc-Arg(Pbf)-OH by automated SPPS, the title compound was synthesized by on-resin coupling of Fmoc-Lys(Thiobenzoyl)-OH (1.5 equiv.) followed by Fmoc deprotection and capping with **S20**. Global deprotection and cleavage from the resin, followed by preparative reversed-phase HPLC purification afforded the desired trimer **8** (6 mg, 31% based on resin loading) as a colorless fluffy material after lyophilization. ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.19 (t, J = 5.4 Hz, 1H, $NH_{\varepsilon,Lys}$), 9.17 (t, J = 5.5 Hz, 1H, NH_{alkyne}), 8.15 (d, J = 7.9 Hz, 1H, $NH_{\alpha,Arg}$), 8.12 (d, J = 8.6 Hz, 1H, $NH_{\alpha,Lys}$), 8.03–7.95 (m, 2H, $H3_{Phenyl}$, $H5_{Phenyl}$), 7.88–7.83 (m, 2H, $H2_{Phenyl}$, $H6_{Phenyl}$), 7.78 (d, J = 8.3 Hz, 1H, $NH_{\alpha,Cha}$), 7.73–7.67 (m, 2H, $H2_{Thioenzoyl}$, $H6_{Thiobenzoyl}$), 7.51 (t, J = 5.8 Hz, 1H, $NH_{\varepsilon,Arg}$), 7.49–7.44 (m, 1H, $H4_{Thiobenzoyl}$), 7.43–7.37 (m, 2H, $H3_{Thiobenzoyl}$, $H5_{Thiobenzoyl}$), 7.29 (d, J = 2.2 Hz, 1H, $CONH_{2,A}$), 6.94 (d, J = 2.1 Hz, 1H, $CONH_{2,B}$), 4.25–4.19 (m, 1H, $H_{\alpha,Cha}$), 4.08 (dd, J = 5.5, 2.5 Hz, 2H, $CH_{2,alkyne}$), 4.06–4.01 (m, 1H, $H_{\alpha,Arg}$), 3.86–3.81 (m, 1H, $H_{\alpha,Lys}$), 3.60–3.55 (m, 2H, $H_{\varepsilon,Lys}$), 3.14 (t, J = 2.5 Hz, 1H, $CH_{,alkyne}$), 3.05 (q, J = 6.6 Hz, 2H, $H_{\delta,Arg}$), 1.71–1.02 (m, 21H), 0.91–0.73 (m, 2H) ($H_{\beta,\gamma,\delta,Lys}$, $H_{\beta,\gamma,Arg}$, $H_{\beta,\delta,\varepsilon,\xi,Cha}$, $CH_{\gamma,Cha}$). ^{13}C NMR (151 MHz, DMSO-d_6) δ 197.0 (CS), 174.0 (CO_{Cha}), 170.7 (CO_{Lys}), 170.6 (CO_{Arg}), 164.9 (CO_{alkyne}), 158.2 (q, J = 32.5 Hz, residual CO_{TFA}), 156.7 ($NHC(=NH)NH_2$), 143.6 ($C1_{Phenyl}$), 141.4 ($C1_{Thioenzoyl}$), 136.9 ($C4_{Phenyl}$), 130.5 ($C4_{Thioenzoyl}$), 127.9 ($C3_{Thiobenzoyl}$, $C5_{Thiobenzoyl}$), 127.9 ($C3_{Phenyl}$, $C5_{Phenyl}$), 127.1 ($C2_{Thiobenzoyl}$, $C6_{Thiobenzoyl}$), 126.6 ($C2_{Phenyl}$, $C6_{Phenyl}$), 116.9 (q, J = 298.1 Hz, residual $CF_{3,TFA}$), 80.9 ($\underline{C}CH_{alkyne}$), 73.1 ($\underline{C}HC_{alkyne}$), 55.8 ($C_{\alpha,Lys}$), 52.0 ($C_{\alpha,Arg}$), 50.0 ($C_{\alpha,Cha}$), 45.9 ($C_{\varepsilon,Lys}$), 40.4 ($C_{\delta,Arg}$), 39.3 (overlap with solvent peak, $C_{\beta,Cha}$), 33.4 ($C_{\gamma,Cha}$), 33.2, 32.6 ($C_{\beta,Lys}$), 31.7, 28.9 ($C_{\beta,Arg}$), 28.7 ($CH_{2,alkyne}$), 26.7 ($C_{\delta,Lys}$), 26.0, 25.8, 25.6, 24.8 ($C_{,\gamma,Arg}$), 22.7 ($C_{\gamma,Lys}$), ($C_{\delta,\varepsilon,\xi,Cha}$). Analytical HPLC gradient 5-95% eluent II in eluent I (20 min total runtime), t_R 15.4 min (>99%, UV_{230}). HRMS m/z 796.3630 ($[M+H]^+$, $C_{38}H_{54}N_9O_6S_2^+$ Calcd 796.3633).

((4-(Prop-2-yn-1-ylcarbamoyl)phenyl)sulfonyl)-Lys(phenylcarbamothioyl)-Arg-Cha-NH_2 (9).



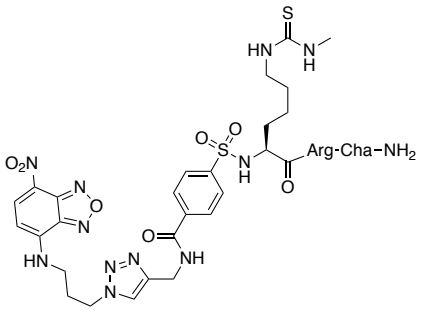
Starting from H-Arg(Pbf)-Cha-resin (20 µmol, estimated loading: 0.47 mmol/g) synthesized from Fmoc-Cha-OH and Fmoc-Arg(Pbf)-OH by automated SPPS, the capped trimer was synthesized by on-resin coupling of Fmoc-Lys(Teoc)-OH followed by Fmoc deprotection and capping with **S20** according to the general on-resin capping procedure. The Teoc-group was then removed according to the general procedure for on-resin Teoc deprotection, and the title compound was synthesized by on-resin thiourea formation as described in the general procedures using aniline to form the benzotriazole coupling reagent. Global deprotection and cleavage from the resin, followed by preparative reversed-phase HPLC purification afforded the desired trimer **9** (6 mg, 33% based on resin loading), as a colorless fluffy material after lyophilization. ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.54 (s, 1H, CSNHC), 9.17 (t, J = 5.5 Hz, 1H, NH_{alkyne}), 8.15 (d, J = 7.8 Hz, 1H, $NH_{\alpha,Arg}$), 8.11 (d, J = 8.6 Hz, 1H, $NH_{\alpha,Lys}$), 8.02–7.97 (m, 2H, $H3_{Phenyl}$, $H5_{Phenyl}$), 7.87–7.83 (m, 2H, $H2_{Phenyl}$, $H6_{Phenyl}$), 7.79 (d, J = 8.3 Hz, 2H, $NH_{\alpha,Cha}$, $NH_{\varepsilon,Lys}$), 7.52 (t, J = 5.8 Hz, 1H, $NH_{\varepsilon,Arg}$), 7.43–7.38 (m, 2H, $H2_{Thioureaphenyl}$, $H6_{Thioureaphenyl}$), 7.33–7.27 (m, 3H, $H3_{Thioureaphenyl}$, $H5_{Thioureaphenyl}$, $CONH_{2,A}$), 7.11–7.06 (m, 1H, $H4_{Thioureaphenyl}$), 6.95–6.92 (m, 1H, $CONH_{2,B}$), 4.26–4.19 (m, 1H, $H_{\alpha,Cha}$), 4.08 (dd, J = 5.6, 2.5 Hz, 2H, $CH_{2,alkyne}$), 4.03 (q, J = 7.2 Hz, 1H, $H_{\alpha,Arg}$), 3.86–3.77 (m, 1H, $H_{\alpha,Lys}$), 3.34 (d, J = 7.7 Hz, 2H, $H_{\varepsilon,Lys}$), 3.14 (t, J = 2.5 Hz, 1H, $CH_{,alkyne}$), 3.05 (q, J = 6.6 Hz, 2H, $H_{\delta,Arg}$), 1.71–1.00 (m, 21H), 0.93–0.72 (m, 2H), ($H_{\beta,\gamma,\delta,Lys}$, $H_{\beta,\gamma,Arg}$, $H_{\beta,\delta,\varepsilon,\xi,Cha}$, $CH_{\gamma,Cha}$). ^{13}C NMR (151 MHz, DMSO-d_6) δ 180.2 (CS), 174.0 (CO_{Cha}), 170.7 (CO_{Lys}), 170.7 (CO_{Arg}), 164.9 (CO_{alkyne}), 158.2 (q, J = 31.3 Hz, residual CO_{TFA}), 156.7 (NHC(=NH)NH_2), 143.6 ($C1_{Phenyl}$), 139.3 ($C1_{Thioureaphenyl}$), 136.9 ($C4_{Phenyl}$), 128.5 ($C3_{Thioureaphenyl}$, $C5_{Thioureaphenyl}$), 127.9 ($C3_{Phenyl}$, $C5_{Phenyl}$), 126.6 ($C2_{Phenyl}$, $C6_{Phenyl}$), 123.9 ($C4_{Thioureaphenyl}$), 122.8 ($C2_{Thioureaphenyl}$, $C6_{Thioureaphenyl}$), 117.0 (q, J = 299.0 Hz, residual $CF_{3,TFA}$), 80.9 (CCH_{alkyne}), 73.1 (CHC_{alkyne}), 55.9 ($C_{\alpha,Lys}$), 52.0 ($C_{\alpha,Arg}$), 50.0 ($C_{\alpha,Cha}$), 43.6 ($C_{\varepsilon,Lys}$), 40.4 ($C_{\delta,Arg}$), 39.3 (overlap with solvent peak, $C_{\beta,Cha}$), 33.4 ($C_{\gamma,Cha}$), 33.2, 32.6 ($C_{\beta,Lys}$), 31.7, 28.9 ($C_{\beta,Arg}$), 28.7, 28.0 ($CH_{2,alkyne}$), 26.0, 25.8, 25.5, 24.8 ($C_{,\gamma,Arg}$), 22.6 ($C_{\gamma,Lys}$), ($C_{\delta,Lys}$, $C_{\delta,\varepsilon,\xi,Cha}$). Analytical HPLC gradient 5-95% eluent II in eluent I (20 min total runtime), t_R 14.8 min (>95%, UV_{230}). HRMS m/z 811.3730 ($[M+H]^+$, $C_{38}H_{55}N_{10}O_6S_2^+$ Calcd 811.3742).

((4-(prop-2-yn-1-ylcarbamoyl)phenyl)sulfonyl)-Lys(cyclohexylcarbamothioyl)-Arg-Cha-NH_2



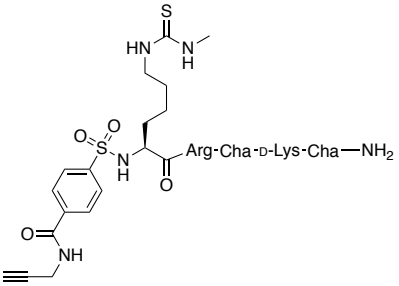
(10). Starting from H-Arg(Pbf)-Cha-resin (20 µmol, estimated loading: 0.47 mmol/g) synthesized from Fmoc-Cha-OH and Fmoc-Arg(Pbf)-OH by automated SPPS, the capped trimer was synthesized by on-resin coupling of Fmoc-Lys(Teoc)-OH followed by Fmoc deprotection and capping with **S20** according to the general on-resin capping procedure. The Teoc-group was then removed according to the general procedure for on-resin Teoc deprotection, and the title compound was synthesized by on-resin thiourea formation as described in the general procedures using cyclohexylamine to form the benzotriazole coupling reagent. Global deprotection and cleavage from the resin, followed by preparative reversed-phase HPLC purification afforded the desired trimer **10** (2 mg, 13% based on resin loading), as a colorless fluffy material after lyophilization. ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.18 (t, J = 5.5 Hz, 1H, NH_{alkyne}), 8.15 (d, J = 7.8 Hz, 1H, $NH_{\alpha,Arg}$), 8.09 (d, J = 8.6 Hz, 1H, $NH_{\alpha,Lys}$), 8.01–7.97 (m, 2H, $H3_{Phenyl}$, $H5_{Phenyl}$), 7.87–7.82 (m, 2H, $H2_{Phenyl}$, $H6_{Phenyl}$), 7.79 (d, J = 8.2 Hz, 1H, $NH_{\alpha,Cha}$), 7.53 (t, J = 5.8 Hz, 1H, $NH_{\varepsilon,Arg}$), 7.30–7.27 (m, 1H, $CONH_{2,A}$), 7.26–7.16 (m, 2H, $NH_{\varepsilon,Lys}$, CSN<u>H</u>CH), 6.96–6.93 (m, 1H, $CONH_{2,B}$), 4.25–4.18 (m, 1H, $H_{\alpha,Cha}$), 4.08 (dd, J = 5.5, 2.5 Hz, 2H, $CH_{2,alkyne}$), 4.01 (q, J = 7.2 Hz, 1H, $H_{\alpha,Arg}$), 3.84–3.77 (m, 1H, $H_{\alpha,Lys}$), 3.23 (s, 2H, $H_{\varepsilon,Lys}$), 3.14 (t, J = 2.5 Hz, 1H, $CH_{,alkyne}$), 3.05 (q, J = 6.6 Hz, 2H, $H_{\delta,Arg}$), 1.84–1.79 (m, 2H), 1.70–1.00 (m, 30H), 0.90–0.74 (m, 2H), ($H_{\beta,\gamma,\delta,Lys}$, $H_{\beta,\gamma,Arg}$, $H_{\beta,\delta,\varepsilon,\xi,Cha}$, $CH_{\gamma,Cha}$), (CH_2)$_{5,cyclohexyl}$, $CH_{cyclohexyl}$). ^{13}C NMR (151 MHz, DMSO-d_6) δ 174.0 (CO_{Cha}), 170.7 (CO_{Lys}), 170.7 (CO_{Arg}), 165.0 (CO_{alkyne}), 158.1 (q, J = 32.4 Hz, residual CO_{TFA}), 156.7 (NHC(=NH)NH_2), 143.6 ($C1_{Phenyl}$), 136.9 ($C4_{Phenyl}$), 127.9 ($C3_{Phenyl}$, $C5_{Phenyl}$), 126.6 ($C2_{Phenyl}$, $C6_{Phenyl}$), 116.7 (q, J = 297.5 Hz, residual $CF_{3,TFA}$), 80.9 (<u>C</u>CH_{alkyne}), 73.1 (<u>C</u>HC_{alkyne}), 55.8 ($C_{\alpha,Lys}$), 52.0 ($C_{\alpha,Arg}$), 50.0 ($C_{\alpha,Cha}$), 40.4 ($C_{\delta,Arg}$), 39.2 (overlap with solvent peak, $C_{\beta,Cha}$), 33.4 ($C_{\gamma,Cha}$), 33.2, 32.6 ($C_{\beta,Lys}$), 32.3, 31.7, 28.9 ($C_{\beta,Arg}$), 28.7, 28.4 ($CH_{2,alkyne}$), 26.0, 25.8, 25.5, 24.8 ($C_{,\gamma,Arg}$), 24.5, 22.5 ($C_{\gamma,Lys}$), ($C_{\delta,Lys}$, $C_{\delta,\varepsilon,\xi,Cha}$, C1-$6_{cyclohexyl}$). The peak for C=S was not visible in ^{13}C NMR and the peaks for $C_{\varepsilon,Lys}$ and CSNH<u>C</u>H were broad and of low intensity in ^{13}C NMR, probably due to fast quadrupolar relaxation via nearby ^{14}N-nuclei. Analytical HPLC gradient 5-95% eluent II in eluent I (20 min total runtime), t_R 15.6 min (>95%, UV_{230}). HRMS m/z 817.4218 ($[M+H]^+$, $C_{38}H_{61}N_{10}O_6S_2^+$ Calcd 817.4211).

((4-(((1-(3-((7-Nitrobenzo[*c*][1,2,5]oxadiazol-4-yl)amino)propyl)-1*H*-1,2,3-triazol-4-yl)methyl)carbamoyl) phenyl)sulfonyl)-Lys(methylcarbamothioyl)-Arg-Cha-NH_2 (11). Starting



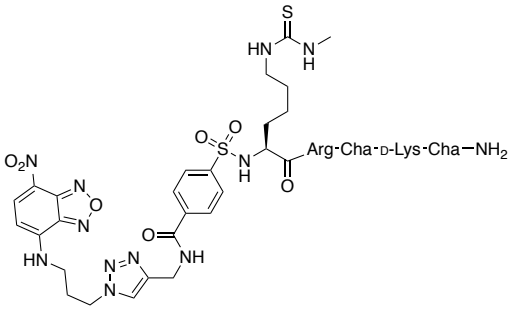
from compound **4** linked to the resin (25 µmol, estimated loading: 0.47 mmol/g) the title compound was synthesized by on-resin click reaction of NBD-N_3 as described in the general procedures. Global deprotection and cleavage from the resin, followed by preparative reversed-phase HPLC purification afforded the desired trimer **11** (4 mg, 13% based on resin loading) as an orange fluffy material after lyophilization. ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.51 (s, 1H, NH_{NBD}), 9.24 (t, J = 5.8 Hz, 1H, $NH_{benzamide}$), 8.52 (d, J = 8.9 Hz, 1H, $H6_{NBD}$), 8.13 (d, J = 7.8 Hz, 1H, $NH_{\alpha,Arg}$), 8.09 (d, J = 8.5 Hz, 1H, $NH_{\alpha,Lys}$), 8.04 (s, 1H, $H5_{triazole}$), 8.03–7.98 (m, 2H, $H3_{Phenyl}$, $H5_{Phenyl}$), 7.84 (d, J = 8.4 Hz, 2H, $H2_{Phenyl}$, $H6_{Phenyl}$), 7.78 (d, J = 8.2 Hz, 1H, $NH_{\alpha,Cha}$), 7.49 (t, J = 5.9 Hz, 1H, $NH_{\varepsilon,Arg}$), 7.39–7.24 (m, 3H, $NH_{\varepsilon,Lys}$, $CSNHCH_3$, $CONH_{2,A}$), 6.95 (s, 1H, $CONH_{2,B}$), 6.36 (d, J = 8.8 Hz, 1H, $H5_{NBD}$), 4.52 (d, J = 5.7 Hz, 2H, $CH_2NH_{Benzamide}$), 4.48 (t, J = 7.0 Hz, 2H, $CH_2N_{Triazole}$), 4.26–4.18 (m, 1H, $H_{\alpha,Cha}$), 4.04 (q, J = 7.1 Hz, 1H, $H_{\alpha,Arg}$), 3.81–3.75 (m, 1H, $H_{\alpha,Lys}$), 3.51 (s, 2H, CH_2NH_{NBD}), 3.18 (br s, 2H, $H_{\varepsilon,Lys}$), 3.05 (q, J = 6.6 Hz, 2H, $H_{\delta,Arg}$), 2.85–2.69 (m, 3H, CH_3), 2.24 (p, J = 7.0 Hz, 2H, $CH_2CH_2CH_2$), 1.69–1.02 (m, 21H), 0.92–0.73 (m, 2H) ($H_{\beta,\gamma,\delta,Lys}$, $H_{\beta,\gamma,Arg}$, $H_{\beta,\delta,\varepsilon,\xi,Cha}$, $CH_{\gamma,Cha}$). ^{13}C NMR (151 MHz, DMSO-d_6) δ 174.0 (CO_{Cha}), 170.7 (CO_{Lys}), 170.7 (CO_{Arg}), 165.1 ($CO_{Benzamide}$), 158.1 (q, J = 33.7 Hz, residual CO_{TFA}), 156.7 ($NHC(=NH)NH_2$), 144.7 ($C4_{Triazole}$), 143.5 ($C1_{Phenyl}$), 137.9 ($C6_{NBD}$), 137.2 ($C4_{Phenyl}$), 127.9 ($C3_{Phenyl}$, $C5_{Phenyl}$), 126.5 ($C2_{Phenyl}$, $C6_{Phenyl}$), 123.2 ($C5_{Triazole}$), 116.4 (d, J = 296.5 Hz, residual $CF_{3,TFA}$), 99.2 ($C5_{NBD}$), 55.9 ($C_{\alpha,Lys}$), 52.0 ($C_{\alpha,Arg}$), 50.0 ($C_{\alpha,Cha}$), 47.0 ($CH_2N_{Triazole}$), 40.4 (CH_2NH_{NBD}), 40.1 ($C_{\delta,Arg}$), 39.3 (overlap with solvent peak, $C_{\beta,Cha}$), 35.0 ($CH_2NH_{Benzamide}$), 33.4 ($C_{\gamma,Cha}$), 33.2, 32.5 ($C_{\beta,Lys}$), 31.7, 28.9 ($C_{\beta,Arg}$), 28.3 ($CH_2CH_2CH_2$), 26.0, 25.8, 25.5, 24.8 ($C_{,\gamma,Arg}$), 22.4 ($C_{\gamma,Lys}$), ($C_{\delta,Lys}$, $C_{\delta,\varepsilon,\xi,Cha}$). The peak for C=S was not visible in ^{13}C NMR and the peaks for $C_{\varepsilon,Lys}$ and $CSNHCH_3$ were broad and of low intensity in ^{13}C NMR, probably due to fast quadrupolar relaxation via nearby ^{14}N-nuclei. Analytical HPLC gradient 5-95% eluent II in eluent I (20 min total runtime), t_R 14.3 min (>64%, UV_{254}). HRMS m/z 1012.4343 ($[M+H]^+$, $C_{42}H_{62}N_{17}O_9S_2^+$ Calcd 1012.4352).

(4-(prop-2-yn-1-ylcarbamoyl)phenyl)sulfonyl-Lys(methylcarbamothioyl)-Arg-Cha-D-Lys-Cha-NH_2 (12). The title compound was synthesized on resin (50 µmol, estimated loading: 0.47 mmol/g) from Fmoc-Cha-OH, Fmoc-D-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH and Fmoc-Lys(Teoc)-OH by automated SPPS, followed by Fmoc deprotection and capping with **S20**. The lysine side chain was Teoc deprotected and modified by



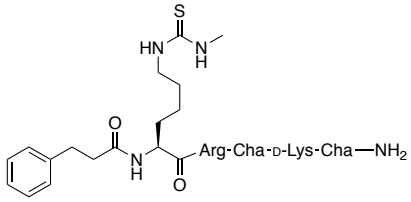
on-resin thiourea formation using methylamine (33% in EtOH) as described in the general procedures. Global deprotection and cleavage from the resin, followed by preparative reversed-phase HPLC purification afforded the desired pentamer **12** (6 mg, 10% based on resin loading) as a colorless fluffy material after lyophilization. Analytical HPLC gradient 5-95% eluent II in eluent I (20 min total runtime), t_R 13.6 min (>96%, UV_{230}). HRMS *m*/*z* 1030.5696 ($[M+H]^+$, $C_{48}H_{80}N_{13}O_8S_2^+$ Calcd 1030.5689).

((4-(((1-(3-((7-nitrobenzo[*c*][1,2,5]oxadiazol-4-yl)amino)propyl)-1*H*-1,2,3-triazol-4-yl)methyl)carbamoyl) phenyl)sulfonyl-Lys(methylcarbamothioyl)-Arg-Cha-D-Lys-Cha-NH_2



(13). Starting from compound **12** linked to the resin (50 µmol, estimated loading: 0.47 mmol/g) the title compound was synthesized by on-resin click reaction of NBD-N_3 as described in the general procedures. Global deprotection and cleavage from the resin, followed by preparative reversed-phase HPLC purification afforded the desired pentamer **13** (3 mg, 4% based on resin loading), as an orange fluffy material after lyophilization. Analytical HPLC gradient 5-95% eluent II in eluent I (20 min total runtime), t_R 14.5 min (>82%, UV_{230}). HRMS *m*/*z* 647.3261 ($[M+2H]^{2+}$, $C_{57}H_{90}N_{20}O_{11}S_2^{2+}$ Calcd 647.3264).

Phenylpropanoyl-Lys(methylcarbamothioyl)-Arg-Cha-D-Lys-Cha-NH_2 (14). The title compound



was synthesized on resin (50 µmol, estimated loading: 0.47 mmol/g) from Fmoc-Cha-OH, Fmoc-D-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH and Fmoc-Lys(Teoc)-OH by automated SPPS, followed by Fmoc deprotection and capping with phenyl propanoyl chloride (3 equiv.) in 2 mL anhydrous DMF for 19 h at ambient temperature. The lysine side chain was Teoc deprotected and modified by on-resin thiourea formation using methylamine (33% in EtOH) as described in the general procedures. Global deprotection and cleavage from the resin, followed by preparative reversed-phase HPLC purification afforded the desired pentamer **14** (6 mg, 10% based on resin loading) as a colorless fluffy material after lyophilization. Analytical HPLC gradient 5-95% eluent II in eluent I (20 min total runtime), t_R 15.0 min (>96%, UV_{230}). HRMS *m*/*z* 941.6115 ($[M+H]^+$, $C_{47}H_{81}N_{12}O_6S^+$ Calcd 941.6117).

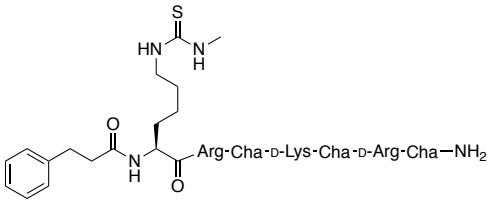
Phenylpropanoyl-Lys-Arg-Cha-D-Lys-Cha-NH_2 (14-K). The title compound was synthesized on resin (80 µmol, estimated loading: 0.47 mmol/g) from Fmoc-Cha-OH, Fmoc-D-Arg(Pbf)-OH, Fmoc-D-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH and Fmoc-Lys(Teoc)-OH by automated SPPS, followed by Fmoc deprotection and capping with phenyl propanoyl chloride (4 equiv.) and *i*Pr_2NEt (6 equiv.) in 3 mL anhydrous CH_2Cl_2 for 16 h at ambient temperature. The lysine side chain was Teoc deprotected. Global deprotection and cleavage from the resin, followed by preparative reversed-phase HPLC purification afforded the desired pentamer **14-K** (8 mg, 8% based on resin loading), as a colorless fluffy material after lyophilization. Analytical HPLC gradient 5-95% eluent II in eluent I (20 min total runtime), t_R 13.9 min (>98%, UV_{230}). HRMS *m/z* 1177.8296 ($[M+H]^+$, $C_{60}H_{105}N_{16}O_8^+$ Calcd 1177.8295). Analytical HPLC gradient 5-95% eluent II in eluent I (20 min total runtime), t_R 14.0 min (>99%, UV_{230}). HRMS *m/z* 868.6133 ($[M+H]^+$, $C_{45}H_{78}N_{11}O_6^+$ Calcd 868.6130).

(4-(Prop-2-yn-1-ylcarbamoyl)phenyl)sulfonyl-Lys(methylcarbamothioyl)-Arg-Cha-D-Lys-Cha-D-Arg-Cha-NH_2 (15). The title compound was synthesized on resin (40 µmol, estimated loading: 0.39 mmol/g) from Fmoc-Cha-OH, Fmoc-D-Arg(Pbf)-OH, Fmoc-D-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH and Fmoc-Lys(Teoc)-OH by automated SPPS, followed by Fmoc deprotection and capping with **S20**. The lysine side chain was Teoc deprotected and modified by on-resin thiourea formation as described in the general procedures. Global deprotection and cleavage from the resin, followed by preparative reversed-phase HPLC purification afforded the desired pentamer **15** (8 mg, 12% based on resin loading) as a colorless fluffy material after lyophilization. Analytical HPLC gradient 5-95% eluent II in eluent I (20 min total runtime), t_R 13.6 min (>95%, UV_{230}). HRMS *m/z* 670.3961 ($[M+2H]^{2+}$, $C_{63}H_{108}N_{18}O_{10}S_2^{2+}$ Calcd 670.3963).

((4-(((1-(3-((7-Nitrobenzo[*c*][1,2,5]oxadiazol-4-yl)amino)propyl)-1*H*-1,2,3-triazol-4-yl)methyl)carbamoyl) phenyl)sulfonyl)-Lys(methylcarbamothioyl)-Arg-Cha-D-Lys-Cha-D-Arg-Cha-NH_2 (16). Starting from compound **15** linked to the resin (50 µmol, estimated loading: 0.47 mmol/g) the title compound was synthesized by on-resin click reaction of NBD-N_3 as described in the general procedures. Global deprotection and cleavage from the resin, followed by preparative reversed-phase

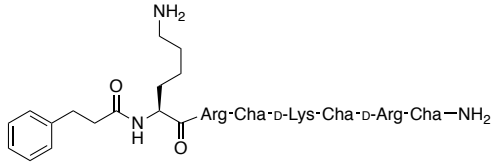
HPLC purification afforded the desired heptamer **16** (3 mg, 3% based on resin loading) as an orange fluffy material after lyophilization. Analytical HPLC gradient 5-95% eluent II in eluent I (20 min total runtime), t_R 14.3 min (>84%, UV_{230}). HRMS *m/z* 801.9344 ($[M+2H]^{2+}$, $C_{72}H_{117}N_{25}O_{13}S_2^{2+}$ Calcd 801.9347).

Phenylpropanoyl-Lys(methylcarbamothioyl)-Arg-Cha-D-Lys-Cha-D-Arg-Cha-NH_2 (17). The



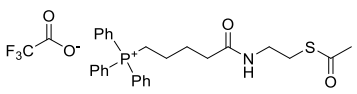
title compound was synthesized on resin (40 µmol, estimated loading: 0.47 mmol/g) from Fmoc-Cha-OH, Fmoc-D-Arg(Pbf)-OH, Fmoc-D-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH and Fmoc-Lys(Teoc)-OH by automated SPPS, followed by Fmoc deprotection and capping with phenyl propanoyl chloride (3 equiv.) in 2 mL anhydrous CH_2Cl_2 for 22 h at ambient temperature. The lysine side chain was Teoc deprotected and modified by on-resin thiourea formation using methylamine (33% in EtOH) as described under general procedures. Global deprotection and cleavage from the resin, followed by preparative reversed-phase HPLC purification afforded the desired pentamer **17** (4 mg, 6% based on resin loading) as a colorless fluffy material after lyophilization. Analytical HPLC gradient 5-95% eluent II in eluent I (20 min total runtime), t_R 14.7 min (>95%, UV_{230}). HRMS *m/z* 625.9166 ($[M+2H]^{2+}$, $C_{62}H_{109}N_{17}O_8S^{2+}$ Calcd 625.9177).

Phenylpropanoyl-Lys-Arg-Cha-D-Lys-Cha-D-Arg-Cha-NH_2 (17-K). The title compound was



synthesized on resin (80 µmol, estimated loading: 0.47 mmol/g) from Fmoc-Cha-OH, Fmoc-D-Arg(Pbf)-OH, Fmoc-D-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH and Fmoc-Lys(Teoc)-OH by automated SPPS, followed by Fmoc deprotection and capping with phenyl propanoyl chloride (4 equiv.) and *i*Pr_2NEt (6 equiv.) in 3 mL anhydrous CH_2Cl_2 for 16 h at ambient temperature. The lysine side chain was Teoc deprotected. Global deprotection and cleavage from the resin, followed by preparative reversed-phase HPLC purification afforded the desired heptamer **17-K** (6 mg, 5% based on resin loading), as a colorless fluffy material after lyophilization. Analytical HPLC gradient 5-95% eluent II in eluent I (20 min total runtime), t_R 13.9 min (>98%, UV_{230}). HRMS *m/z* 1177.8296 ($[M+H]^+$, $C_{60}H_{105}N_{16}O_8^+$ Calcd 1177.8295).

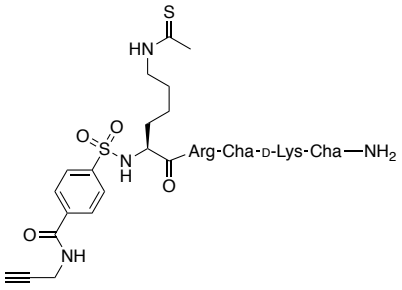
(5-((2-(acetylthio)ethyl)amino)-5-oxopentyl)triphenylphosphonium·TFA (18). Ac_2O (71 µL,



0.75 mmol) was added dropwise to a solution of thiol **S21** (104 mg, 0.19 mmol) and *i*Pr_2NEt (130 µL, 0.75 mmol) in CH_2Cl_2 (5.0 mL). The reaction mixture was stirred at ambient temperature for 45 min and was then concentrated under reduced pressure. Purification by preparative reverse-phase HPLC, afforded the desired thioester

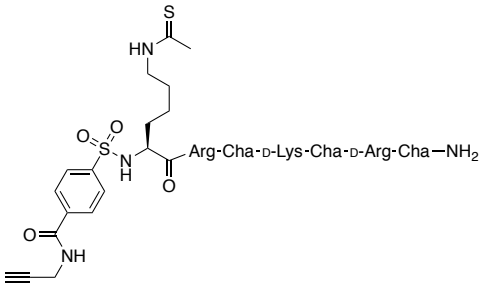
18 (52 mg, 48%) as a clear oil. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.00 (t, J = 5.8 Hz, 1H, NHCH$_2$), 7.93–7.86 (m, 3H, H4$_{Ph}$), 7.84–7.68 (m, 12H, H2$_{Ph}$, H3$_{Ph}$, H5$_{Ph}$, H6$_{Ph}$), 3.62–3.50 (m, 2H, CH$_2$PPh$_3$), 3.20–3.05 (m, 2H, NHCH$_2$), 2.81 (t, J = 6.9 Hz, 2H, NHCH$_2$CH$_2$), 2.30 (s, 3H, CH$_3$), 2.10 (t, J = 7.2 Hz, 2H, CH$_2$(CH$_2$)$_3$PPh$_3$), 1.69 (p, J = 7.3 Hz, 2H, CH$_2$(CH$_2$)$_2$PPh$_3$), 1.57–1.48 (m, 2H, CH$_2$CH$_2$PPh$_3$). ^{13}C NMR (151 MHz, DMSO-d_6) δ 195.0 (SC=O), 171.6 (NHC=O), 157.9 (q, J = 33.8 Hz, CO$_{TFA}$), 134.9 (d, J = 2.9 Hz, C4$_{Ph}$), 133.5 (d, J = 10.0 Hz, C2$_{Ph}$, C6$_{Ph}$), 130.2 (d, J = 12.3 Hz, C3$_{Ph}$, C5$_{Ph}$), 118.5 (d, J = 85.8 Hz, C1$_{Ph}$), 116.4 (q, J = 295.7 Hz, CF$_{3,TFA}$), 38.0 (NHCH$_2$), 34.2 (CH$_2$(CH$_2$)$_3$PPh$_3$), 30.5 (CH$_3$), 28.3 (NHCH$_2$CH$_2$), 26.0 (d, J = 17.7 Hz, CH$_2$(CH$_2$)$_2$PPh$_3$), 21.3 (d, J = 4.0 Hz, CH$_2$CH$_2$PPh$_3$), 20.0 (d, J = 50.2 Hz, CH$_2$PPh$_3$). UPLC-MS t_R 1.96 min, m/z 464.2 ([M+H]$^+$, $C_{27}H_{31}NO_2PS^+$ Calcd 464.2); HRMS m/z 464.1810 ([M+H]$^+$, $C_{27}H_{31}NO_2PS^+$ Calcd 464.1808).

((4-(prop-2-yn-1-ylcarbamoyl)phenyl)sulfonyl)-Lys(ThioAc)-Arg-Cha-D-Lys-Cha-NH$_2$ (S1). The



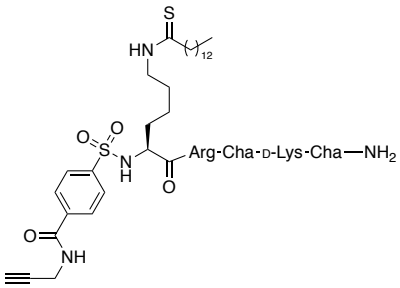
title compound was synthesized on resin (20 µmol, estimated loading: 0.47 mmol/g) from Fmoc-Cha-OH, Fmoc-D-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH and Fmoc-Lys(ThioAc)-OH by automated SPPS, followed by Fmoc deprotection and capping with **S20**. Global deprotection and cleavage from the resin, followed by preparative reversed-phase HPLC purification afforded the desired pentamer **S1** (6 mg, 25% based on resin loading), as a colorless fluffy material after lyophilization. Analytical HPLC gradient 5-95% eluent II in eluent I (20 min total runtime), t_R 13.4 min (>95%, UV$_{230}$). HRMS m/z 1015.5566 ([M+H]$^+$, $C_{48}H_{79}N_{12}O_8S_2^+$ Calcd 1015.5580).

((4-(prop-2-yn-1-ylcarbamoyl)phenyl)sulfonyl)-Lys(ThioAc)-Arg-Cha-D-Lys-Cha-D-Arg-Cha-



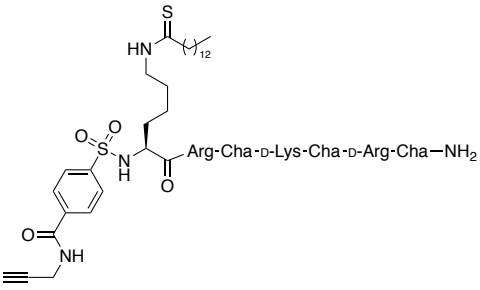
NH$_2$ (S2). The title compound was synthesized on resin (20 µmol, estimated loading: 0.47 mmol/g) from Fmoc-Cha-OH, Fmoc-D-Arg(Pbf)-OH, Fmoc-D-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH and Fmoc-Lys(ThioAc)-OH by automated SPPS, followed by Fmoc deprotection and capping with **S20**. Global deprotection and cleavage from the resin, followed by preparative reversed-phase HPLC purification afforded the desired heptamer **S2** (7 mg, 21% based on resin loading) as a colorless fluffy material after lyophilization. Analytical HPLC gradient 5-95% eluent II in eluent I (20 min total runtime), t_R 14.0 min (>96%, UV$_{254}$). HRMS m/z 1324.7710 ([M+H]$^+$, $C_{63}H_{106}N_{17}O_{10}S_2^+$ Calcd 1324.7745).

((4-(prop-2-yn-1-ylcarbamoyl)phenyl)sulfonyl)-Lys(ThioMyr)-Arg-Cha-D-Lys-Cha-NH_2 (S3)

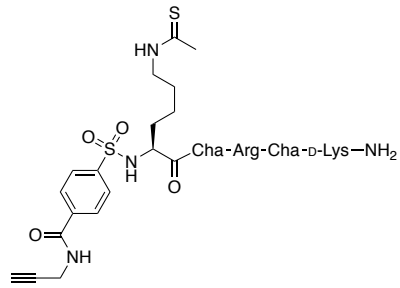


The title compound was synthesized on resin (20 µmol, estimated loading: 0.47 mmol/g) from Fmoc-Cha-OH, Fmoc-D-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH and Fmoc-Lys(ThioMyr)-OH by automated SPPS, followed by Fmoc deprotection and capping with **S20**. Global deprotection and cleavage from the resin, followed by preparative reversed-phase HPLC purification afforded the desired pentamer **S3** (5 mg, 19% based on resin loading), as a colorless fluffy material after lyophilization. Analytical HPLC gradient 5-95% eluent II in eluent I (20 min total runtime), t_R 19.3 min (>99%, UV_{230}). HRMS *m/z* 1183.7493 ($[M+H]^+$, $C_{60}H_{103}N_{12}O_8S_2^+$ Calcd 1183.7458).

((4-(prop-2-yn-1-ylcarbamoyl)phenyl)sulfonyl)-Lys(ThioMyr)-Arg-Cha-D-Lys-Cha-D-Arg-Cha-NH_2 (S4). The title compound was synthesized on resin (20 µmol, estimated loading: 0.47 mmol/g) from Fmoc-Cha-OH, Fmoc-D-Arg(Pbf)-OH, Fmoc-D-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH and Fmoc-Lys(ThioMyr)-OH by automated SPPS, followed by Fmoc deprotection and capping with **S20**. Global deprotection and cleavage from the resin, followed by preparative reversed-phase HPLC purification afforded the desired heptamer **S4** (9 mg, 24% based on resin loading), as a colorless fluffy material after lyophilization. Analytical HPLC gradient 5-95% eluent II in eluent I (20 min total runtime), t_R 18.8 min (>95%, UV_{230}). HRMS *m/z* 1492.9637 ($[M+H]^+$, $C_{75}H_{130}N_{17}O_{10}S_2^+$ Calcd 1492.9623).



((4-(prop-2-yn-1-ylcarbamoyl)phenyl)sulfonyl)-Lys(ThioAc)-Cha-Arg-Cha-D-Lys-NH_2 (S5). The title compound was synthesized on resin (20 µmol, estimated loading: 0.47 mmol/g) from Fmoc-Cha-OH, Fmoc-D-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH and Fmoc-Lys(ThioAc)-OH by automated SPPS, followed by Fmoc deprotection and capping with **S20**. Global deprotection and cleavage from the resin, followed by preparative reversed-phase HPLC purification afforded the desired pentamer **S5** (10 mg, 40% based on resin loading), as a colorless fluffy material after lyophilization. Analytical HPLC gradient 5-95% eluent II in eluent I (20 min total runtime), t_R 14.0 min (>97%, UV_{230}). HRMS *m/z* 1015.5580 ($[M+H]^+$, $C_{48}H_{79}N_{12}O_8S_2^+$ Calcd 1015.5580).



Phenylacetyl-Lys(ethanethioyl)-Arg-Cha-D-Lys-Cha-NH_2 (S7). The title compound was synthesized on resin (15 µmol, estimated loading: 0.24 mmol/g) from Fmoc-Cha-OH, Fmoc-D-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH and Fmoc-Lys(ThioAc)-OH by automated SPPS, followed by Fmoc deprotection and capping with phenyl acetyl chloride (3 equiv.) in 1 mL anhydrous CH_2Cl_2 for 21 h at ambient temperature. Global deprotection and cleavage from the resin, followed by preparative reversed-phase HPLC purification afforded the desired pentamer **S7** (3 mg, 18% based on resin loading) as a colorless fluffy material after lyophilization. Analytical HPLC gradient 5-95% eluent II in eluent I (20 min total runtime), t_R 14.6 min (>95%, UV_{230}). HRMS *m/z* 912.5840 ($[M+H]^+$, $C_{46}H_{78}N_{11}O_6S_2^+$ Calcd 912.5852).

Phenylpropanoyl-Lys(ThioAc)-Arg-Cha-D-Lys-Cha-NH_2 (S8). The title compound was synthesized on resin (15 µmol, estimated loading: 0.24 mmol/g) from Fmoc-Cha-OH, Fmoc-D-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH and Fmoc-Lys(ThioAc)-OH by automated SPPS, followed by Fmoc deprotection and capping with phenyl propanoyl chloride (3 equiv.) in 1 mL anhydrous $CH_2Cl_2$for 21 h at ambient temperature. Global deprotection and cleavage from the resin, followed by preparative reversed-phase HPLC purification afforded the desired pentamer **S8** (3 mg, 18% based on resin loading), as a colorless fluffy material after lyophilization. Analytical HPLC gradient 5-95% eluent II in eluent I (20 min total runtime), t_R 15.1 min (>95%, UV_{230}). HRMS *m/z* 926.5996 ($[M+H]^+$, $C_{47}H_{80}N_{11}O_6S^+$ Calcd 926.6008).

(Benzyloxy)carbonyl-Lys(ThioAc)-Arg-Cha-D-Lys-Cha-NH_2 (S9). The title compound was synthesized on resin (15 µmol, estimated loading: 0.24 mmol/g) from Fmoc-Cha-OH, Fmoc-D-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH and Fmoc-Lys(ThioAc)-OH by automated SPPS, followed by Fmoc deprotection and capping with Cbz-Cl (3 equiv.) in 1 mL anhydrous CH_2Cl_2 for 21 h at ambient temperature. Global deprotection and cleavage from the resin, followed by preparative reversed-phase HPLC purification afforded the desired pentamer **S9** (2 mg, 10% based on resin loading) as a colorless fluffy material after lyophilization. Analytical HPLC gradient 5-95% eluent II in eluent I (20 min total runtime), t_R 14.9 min (>96%, UV_{230}). HRMS *m/z* 928.5793 ($[M+H]^+$, $C_{46}H_{78}N_{11}O_7S^+$ Calcd 928.5801).

Methylsulfonyl-Lys(ThioAc)-Arg-Cha-D-Lys-Cha-NH_2 (S10). The title compound was synthesized on resin (15 µmol, estimated loading: 0.24 mmol/g) from Fmoc-Cha-OH, Fmoc-D-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH and Fmoc-Lys(ThioAc)-OH by automated SPPS, followed by Fmoc deprotection and capping with CH_3SO_2Cl (3 equiv.) and *i*Pr_2NEt (6 equiv.) in 1 mL anhydrous CH_2Cl_2 for 21 h at ambient temperature. Global deprotection and cleavage from the resin, followed by preparative reversed-phase HPLC purification afforded the desired pentamer **S10** (1 mg, 6% based on resin loading) as a colorless fluffy material after lyophilization. Analytical HPLC gradient 5-95% eluent II in eluent I (20 min total runtime), t_R 13.2 min (>90%, UV_{254}). HRMS *m*/*z* 872.5202 ($[M+H]^+$, $C_{39}H_{74}N_{11}O_7S_2^+$ Calcd 872.5209).

(4-(6-amino-3-iminoacridin-10(3*H*)-yl)butanoyl)-Lys(methylcarbamothioyl)-Arg-Cha-D-Lys-Cha-D-Arg-Cha-NH_2 (S11). The title compound was synthesized on resin (20 µmol, estimated loading: 0.47 mmol/g) from Fmoc-Cha-OH, Fmoc-D-Arg(Pbf)-OH, Fmoc-D-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH and Fmoc-Lys(Teoc)-OH by automated SPPS, followed by Fmoc deprotection and capping with ATTO-NHS (0.65 equiv.) and *i*Pr_2NEt (4 equiv.) in 1 mL anhydrous DMF for 16 h at ambient temperature . The lysine side chain was Teoc deprotected and modified by on-resin thiourea formation as described in the general procedures. Global deprotection and cleavage from the resin, followed by preparative reversed-phase HPLC purification afforded the desired heptamer **S11** (1 mg, 3% based on resin loading), as an orange fluffy material after lyophilization. Analytical HPLC gradient 5-95% eluent II in eluent I (20 min total runtime), t_R 13.5 min (>76%, UV_{230}). HRMS *m*/*z* 698.4499 ($[M+2H]^{2+}$, $C_{70}H_{116}N_{20}O_8S^{2+}$ Calcd 698.4497).

(3-(5,5-difluoro-1,3,7,9-tetramethyl-5*H*-4λ⁴,5λ⁴-dipyrrolo[1,2-*c*:2',1'-*f*][1,3,2]diazaborinin-10-yl)propanoyl)-Lys(methylcarbamothioyl)-Arg-Cha-D-Lys-Cha-D-Arg-Cha-NH_2 (S12). The title compound was synthesized on resin (30 µmol, estimated loading: 0.47 mmol/g) from Fmoc-Cha-OH, Fmoc-D-Arg(Pbf)-OH, Fmoc-D-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH and Fmoc-Lys(Teoc)-OH by automated SPPS, followed by Fmoc deprotection and capping with BODIPY-NHS (1.5 equiv.) and *i*Pr_2NEt (4 equiv.) in 1.5 mL anhydrous DMF for 16 h at ambient temperature. The lysine side chain was Teoc

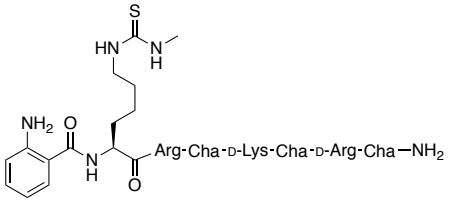
deprotected and modified by on-resin thiourea formation as described in the general procedures. Global deprotection and cleavage from the resin, followed by preparative reversed-phase HPLC purification afforded the desired heptamer **S12** (2 mg, 5% based on resin loading), as an orange fluffy material after lyophilization. Analytical HPLC gradient 5-95% eluent II in eluent I (20 min total runtime), t_R 14.0 min (>68%, UV_{230}). HRMS *m/z* 686.9605 ($[M+2H-BF_2]^{2+}$, $C_{69}H_{119}N_{19}O_8S^{2+}$ Calcd 686.9573).

(5-((2-(4-oxo-4λ³-butanamido)ethyl)amino)naphthalene-1-sulfonate)-Lys(methylcarbamothioyl)-Arg-Cha-D-Lys-Cha-D-Arg-Cha-NH_2 (S13). The title compound was synthesized on resin (30 µmol, estimated loading: 0.47 mmol/g) from Fmoc-Cha-OH, Fmoc-D-Arg(Pbf)-OH, Fmoc-D-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH and Fmoc-Lys(Teoc)-OH by automated SPPS, followed by Fmoc deprotection. EDANS salt (2.8 equiv.) was dissolved in 0.65 mL anhydrous DMF and *i*Pr_2NEt (6 equiv.) and succinic anhydride (2.8 equiv.) were added. The solution was stirred for 1 h at ambient temperature and full consumption of was starting material was observed by LCMS. To the solution HATU (2.8 equiv.) and 1 mL anhydrous DMF was added. The capping solution was added to the resin and agitated for 16 h at ambient temperature. The lysine side chain was Teoc deprotected and modified by on-resin thiourea formation as described in the general procedures. Global deprotection and cleavage from the resin, followed by preparative reversed-phase HPLC purification afforded the desired heptamer **S13** (2 mg, 4% based on resin loading), as a colorless fluffy material after lyophilization. Analytical HPLC gradient 5-95% eluent II in eluent I (20 min total runtime), t_R 13.4 min (>96%, UV_{230}). HRMS *m/z* 1464.8353 ($[M+H]^+$, $C_{69}H_{114}N_{19}O_{12}S_2^+$ Calcd 1464.8329).

((4-(((1-(3-((7-nitrobenzo[*c*][1,2,5]oxadiazol-4-yl)amino)propyl)-1*H*-1,2,3-triazol-4-yl)methyl)carbamoyl) phenyl)sulfonyl)-Lys(methylcarbamothioyl)-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-NH_2 (S14). The title compound was synthesized on resin (40 µmol, estimated loading: 0.47 mmol/g) from Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Gln(Trt)-OH and Fmoc-Lys(Teoc)-OH by automated SPPS, followed by Fmoc deprotection and capping with **S20**. The lysine side chain was Teoc deprotected and modified by on-resin thiourea formation as described in the general procedures. Followed by on-resin click reaction of NBD-N_3 as described in the general procedures. Global

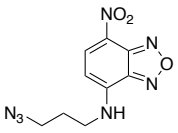
deprotection and cleavage from the resin, followed by preparative reversed-phase HPLC purification afforded the desired TAT sequence **S14** (6 mg, 5% based on resin loading) as an orange fluffy material after lyophilization. Analytical HPLC gradient 5-95% eluent II in eluent I (20 min total runtime), t_R 10.9 min (>95%, UV_{230}). HRMS *m*/*z* 506.7733 ($[M+4H]^{4+}$, $C_{80}H_{142}N_{42}O_{17}S_2^{4+}$ Calcd 506.7740).

2-Aminobenzoyl-Lys(methylcarbamothioyl)-Arg-Cha-D-Lys-Cha-D-Arg-Cha-NH_2 (S15). The



title compound was synthesized on resin (30 µmol, estimated loading: 0.47 mmol/g) from Fmoc-Cha-OH, Fmoc-D-Arg(Pbf)-OH, Fmoc-D-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH and Fmoc-Lys(Teoc)-OH by automated SPPS, followed by Fmoc deprotection and capping with 2-amonobenzoic acid (4 equiv.), HATU (4 equiv.) and *i*Pr_2NEt (4 equiv.) in 1.5 mL anhydrous DMF for 16 h at ambient temperature . The lysine side chain was Teoc deprotected and modified by on-resin thiourea formation as described in the general procedures. Global deprotection and cleavage from the resin, followed by preparative reversed-phase HPLC purification afforded the desired heptamer **18** (3 mg, 6% based on resin loading) as a colorless fluffy material after lyophilization. Analytical HPLC gradient 5-95% eluent II in eluent I (20 min total runtime), t_R 13.8 min (>95%, UV_{230}). HRMS *m*/*z* 1237.8089 ($[M+H]^+$, $C_{60}H_{105}N_{18}O_8S^+$ Calcd 1237.8077).

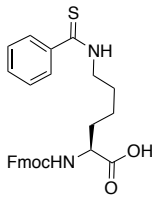
***N*-(3-azidopropyl)-7-nitrobenzo[*c*][1,2,5]oxadiazol-4-amine (S16).**[3] 4-chloro-7-nitrobenzo[c]-



[1,2,5]oxadiazol (200 mg, 1.0 mmol) was dissolved in anhydrous DMF (12.5 mL). 3-bromopropan-1-amine hydrobromide (6.24 g, 1.1 mmol) was added to the reaction, followed by triethylamine (0.18 mL, 1.3 mmol). The reaction was stirred for 2 h at room temperature. 25 mL of water was added to the reaction and the solution was extracted with EtOAc (4×15 mL). The combined organic layers were dried with Na_2SO_4 and concentrated under reduced pressure. The crude residue was purified by column chromatography (0 → 40% EtOAc in heptane) and yielded the desired bromide as an orange solid (150 mg, 50%, purity 63%) which was used without further purification [^{1}H NMR (600 MHz, MeOD) δ 8.51 (d, *J* = 8.8 Hz, 1H, $H6_{oxadiazol}$), 6.38 (d, *J* = 8.8 Hz, 1H, $H5_{oxadiazol}$), 3.73 (t, *J* = 6.4 Hz, 2H, $C\underline{H_2}NH$), 3.59 (t, *J* = 6.4 Hz, 2H, CH_2Br), 2.31 (p, *J* = 13.1, 6.4 Hz, 2H, $C\underline{H_2}CH_2$). ^{13}C NMR (151 MHz, MeOD) δ 146.6 ($C_{oxadiazol}$), 145.9 ($C_{oxadiazol}$), 145.5 ($C_{oxadiazol}$), 138.3 ($C6_{oxadiazol}$), 123.5 (impurity), 99.7 ($C5_{oxadiazol}$), 43.0 (CH_2NH), 32.4 ($CH_2\underline{C}H_2CH_2$), 31.0 (CH_2Br)].The semi pure intermediate (140 mg, 0.46 mmol) was dissolved in DMSO (7 mL) and sodium azide (39 mg, 0.60 mmol) was added. The reaction was stirred overnight at room temperature. The reaction mixture was diluted with 15 mL of water and extracted with EtOAc

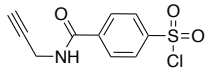
(4×15 mL). The combined organic layers were dried with Na_2SO_4 and concentrated under reduced pressure. The crude residue was purified by column chromatography (0 → 30% EtOAc in heptane) to provide the product **S16** as an orange solid (86 mg, 33%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.49 (s, 1H, NH), 8.49 (d, J = 8.9 Hz, 1H, $H6_{oxadiazol}$), 6.41 (d, J = 8.9 Hz, 1H, $H5_{oxadiazol}$), 3.63–3.43 (m, 4H, CH_2NH, CH_2N_3), 1.93 (p, J = 6.8 Hz, 2H, $C\underline{H}_2CH_2$). ^{13}C NMR (151 MHz, DMSO-d_6) δ 145.1 ($C_{oxadiazol}$), 144.5 ($C_{oxadiazol}$), 144.1($C_{oxadiazol}$), 137.8 ($C6_{oxadiazol}$), 120.8 ($C7_{oxadiazol}$), 99.2 ($C5_{oxadiazol}$), 48.3 (CH_2N_3), 40.7 (CH_2NH), 27.0 ($\underline{C}H_2CH_2$).

***N*^{2}-(((9*H*-fluoren-9-yl)methoxy)carbonyl)-*N*^{6}-(phenylcarbonothioyl)-*L*-lysine (S17).** Fmoc-



Lys(Bz)-OH (120 mg, 0.2 mmol) was co-evaporated with toluene (2×3 mL) and dioxane (4 mL). The residue was dissolved in dioxane (4 mL) and heated to reflux. Lawesson's reagent was added (49 mg, 0.12 mmol) and the reaction was monitored with LCMS. After 4 h additional Lawesson's reagent (25 mg, 0.06 mmol) was added and the solution was stirred for 1 h at ambient temperature. The solution was poured in aq. HCl (25 mL, 1M) followed by extraction with CH_2Cl_2 (2×20 mL) the combined organic layers were concentrated under reduced pressure. The crude residue was purified by preparative reversed-phase HPLC (C18 column, 5 → 95% eluent II in eluent I) and afforded the desired thioamide **S17** (22.1 mg, 22%) as a colorless fluffy material after lyophilization. ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.24 (t, J = 5.4 Hz, 1H, NH_ε), 7.89 (d, J = 7.5 Hz, 2H, $H2_{Phenyl}$, $H6_{Phenyl}$), 7.74–7.69 (m, 4H, $H_{aryl,Fmoc}$), 7.64 (d, J = 8.1 Hz, 1H, NH_α), 7.46 (tt, 1H, $H4_{Phenyl}$), 7.44–7.37 (m, 4H, $H_{aryl,Fmoc}$), 7.32 (td, J = 7.4, 1.1 Hz, 2H, $H3_{Phenyl}$, $H5_{Phenyl}$), 4.32–4.25 (m, 2H ($CH_{2,Fmoc}$), 4.22 (t, J = 7.2 Hz, 1H, CH_{Fmoc}), 3.98–3.91 (m, 1H, H_α), 3.73–3.64 (m, 2H, H_ε), 1.83–1.60 (m, 4H, $H_{\beta,\delta}$), 1.50–1.33 (m, 2H, H_γ). ^{13}C NMR (151 MHz, DMSO-d_6) δ 197.1 (CS), 173.9 (COOH), 156.1 (CONH), 143.8, 143.8, 141.4, 140.7, 130.4, 127.6, 127.2, 127.0, 125.3, 125.2, 120.1, 120.1 (C_{Ph} and C_{Fmoc}), 65.6 ($CH_{2,Fmoc}$), 53.7 (C_α), 46.7($C9_{Fmoc}$), 45.9 (C_ε), 30.5 (C_β), 26.7 (C_δ), 23.3 (C_γ).

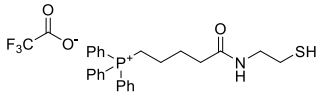
4-(prop-2-yn-1-ylcarbamoyl)benzenesulfonyl chloride (S20). 4-(chlorosulfonyl) benzoic acid



(775 mg, 3.5 mmol) was suspended in anhydrous CH_2Cl_2 (30 mL) and cooled to 0 °C. Oxalyl chloride (0.65 mL) was added followed by 4 drops of DMF. The solution was stirred at ambient temperature for 1 h. TLC confirmed full conversion of starting material and the solution was concentrated. The crude residue was redissolved in anhydrous CH_2Cl_2 (25 mL) and cooled to -20 °C. Propargylamine (0.25 mL, 3.85 mmol) was dissolved in anhydrous CH_2Cl_2 and added to the solution followed by dropwise addition of iPr$_2$NEt (0.83 mL, 4.66 mmol) and the clear orange solution was stirred at ambient temperature overnight. The solution was washed with 1 M HCl (2×50 mL) and the aqueous phase was extracted with CH_2Cl_2 (2×40 mL). The combined organic

phases were dried over Na_2SO_4 and was then concentrated under reduced pressure. The crude residue was purified by column chromatography (0 → 50% EtOAc in heptane) affording the desired amide (450 mg, 50%) as an off-white solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.94 (t, J = 5.6 Hz, 1H, NH), 7.84–7.77 (m, 2H, $H3_{benzene}$, $H5_{benzene}$), 7.70–7.63 (m, 2H, $H2_{benzene}$, $H6_{benzene}$), 4.08–4.01 (m, 2H, CH_2), 3.10 (t, J = 2.5 Hz, 1H, CH). ^{13}C NMR (151 MHz, DMSO-d_6) δ 165.6 (CO), 150.9 ($C1_{benzene}$), 133.7 ($C4_{benzene}$), 126.9 ($C3_{benzene}$, $C5_{benzene}$), 125.5 ($C2_{benzene}$, $C6_{benzene}$), 81.3 (d, J = 3.3 Hz, C_{alkyne}), 72.8 (CH_{alkyne}), 28.5 (CH_2).

(5-((2-mercaptoethyl)amino)-5-oxopentyl)triphenylphosphonium·TFA (S21). Cysteamine



dihydrochloride (113 mg, 0.50 mmol), (4-carboxybutyl) triphenylphosphonium bromide (665 mg, 1.50 mmol), HOBt (201 mg, 1.50 mmol) and iPr$_2$NEt (262 µL, 1.50 mmol) were dissolved in anhydrous CH_2Cl_2 (10 mL) and cooled to 0 °C. EDC (289 mg, 1.51 mmol) was added and the reaction mixture was stirred at 0 °C for 5 min and was then stirred overnight at ambient temperature. The reaction mixture was concentrated under reduced pressure and the crude residue was dissolved in H_2O/MeCN (2:1, 15 mL). An aqueous solution of TCEP (5 mL, 0.5 M, pH 7) was added and the reaction mixture was stirred at RT for 10 min. Purification by preparative reverse-phase HPLC afforded the desired thiol **S21** (122 mg, 46%) as a clear oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ 11.26 (s, 2H, CO_2H_{TFA}), 8.30 (t, J = 5.8 Hz, 1H, N$\underline{H}$CH$_2$), 7.86–7.79 (m, 3H, $H4_{Ph}$), 7.73–7.62 (m, 12H, $H2_{Ph}$, $H3_{Ph}$, $H5_{Ph}$, $H6_{Ph}$), 3.39–3.23 (m, 4H, C$\underline{H}_2$PPh$_3$, NHC$\underline{H}_2$), 2.60–2.52 (m, 2H, C$\underline{H}_2$SH), 2.48 (t, J = 7.2 Hz, 2H, C$\underline{H}_2$(CH$_2$)$_3$PPh$_3$), 1.92 (p, J = 7.0 Hz, 2H, C$\underline{H}_2$(CH$_2$)$_2$PPh$_3$), 1.77–1.66 (m, 2H, C$\underline{H}_2$CH$_2$PPh$_3$), 1.45 (t, J = 8.4 Hz, 1H, CH$_2$S$\underline{H}$). ^{13}C NMR (151 MHz, $CDCl_3$) δ 174.7 (CO), 160.2 (q, J = 38.4 Hz, CO_{TFA}), 135.49 (d, J = 3.2 Hz, $C4_{Ph}$), 133.50 (d, J = 9.9 Hz, $C2_{Ph}$, $C6_{Ph}$), 130.73 (d, J = 12.6 Hz, $C3_{Ph}$, $C5_{Ph}$), 117.95 (d, J = 86.3 Hz, $C1_{Ph}$), 115.7 (q, J = 288.8 Hz, $CF_{3,TFA}$), 43.1 (NH$\underline{C}$H$_2$), 34.0 ($\underline{C}$H$_2$(CH$_2$)$_3$PPh$_3$), 26.40 (d, J = 17.1 Hz, $\underline{C}$H$_2$CH$_2$PPh$_3$), 23.8 (CH$_2$SH), 22.2 (d, J = 51.7 Hz, $\underline{C}$H$_2$PPh$_3$), 21.4 (d, J = 4.3 Hz, $\underline{C}$H$_2$(CH$_2$)$_2$PPh$_3$). UPLC-MS t_R 1.23 min, m/z 422.2 ([M+H]$^+$, $C_{25}H_{29}NOPS^+$ Calcd 422.2).

Supporting references

[1] N. Rajabi, A. L. Nielsen, C. A. Olsen, *ACS Med. Chem. Lett.* **2020**, *11*, 1886-1892.

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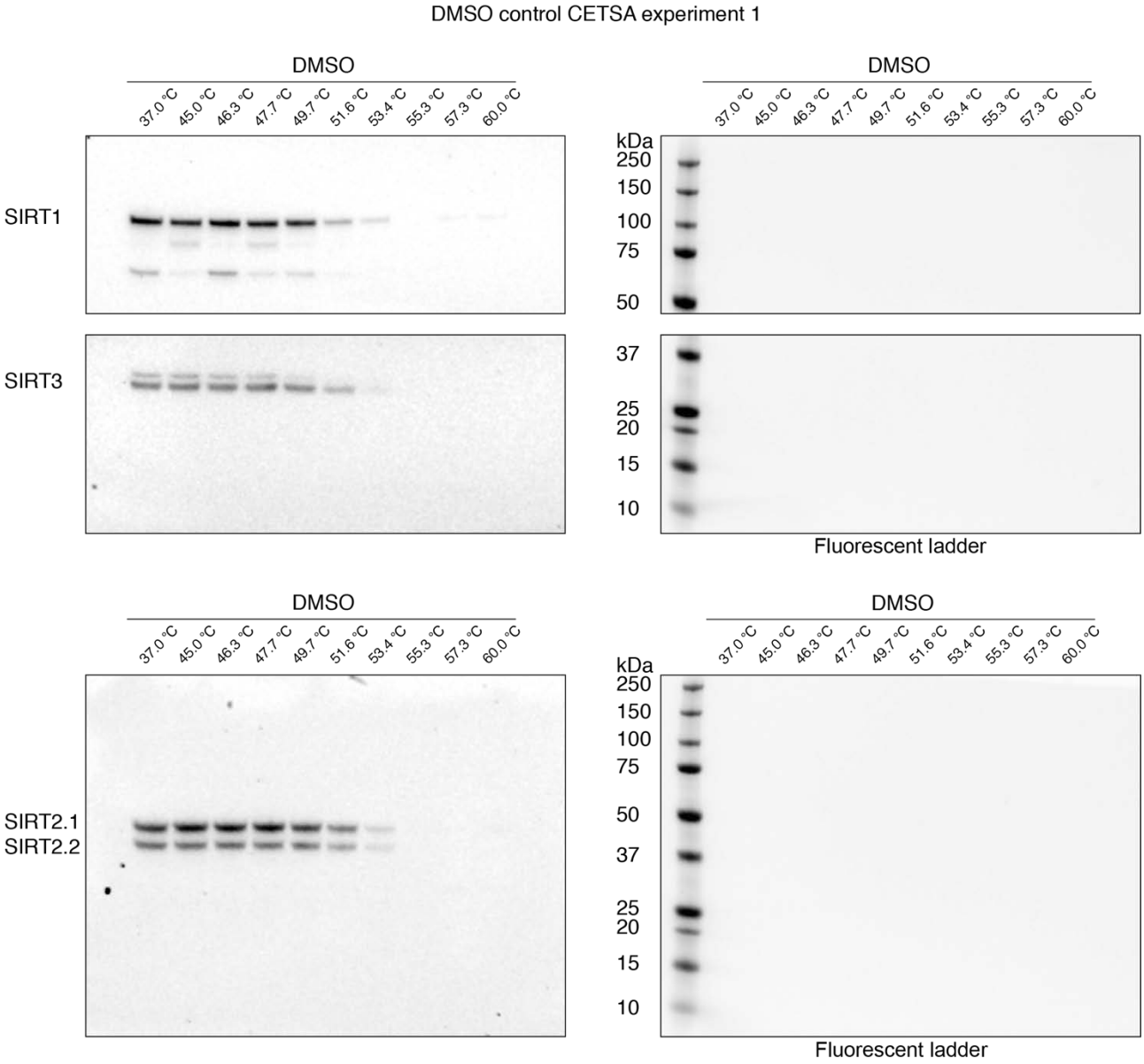
SUPPORTING DATA

Full western blots



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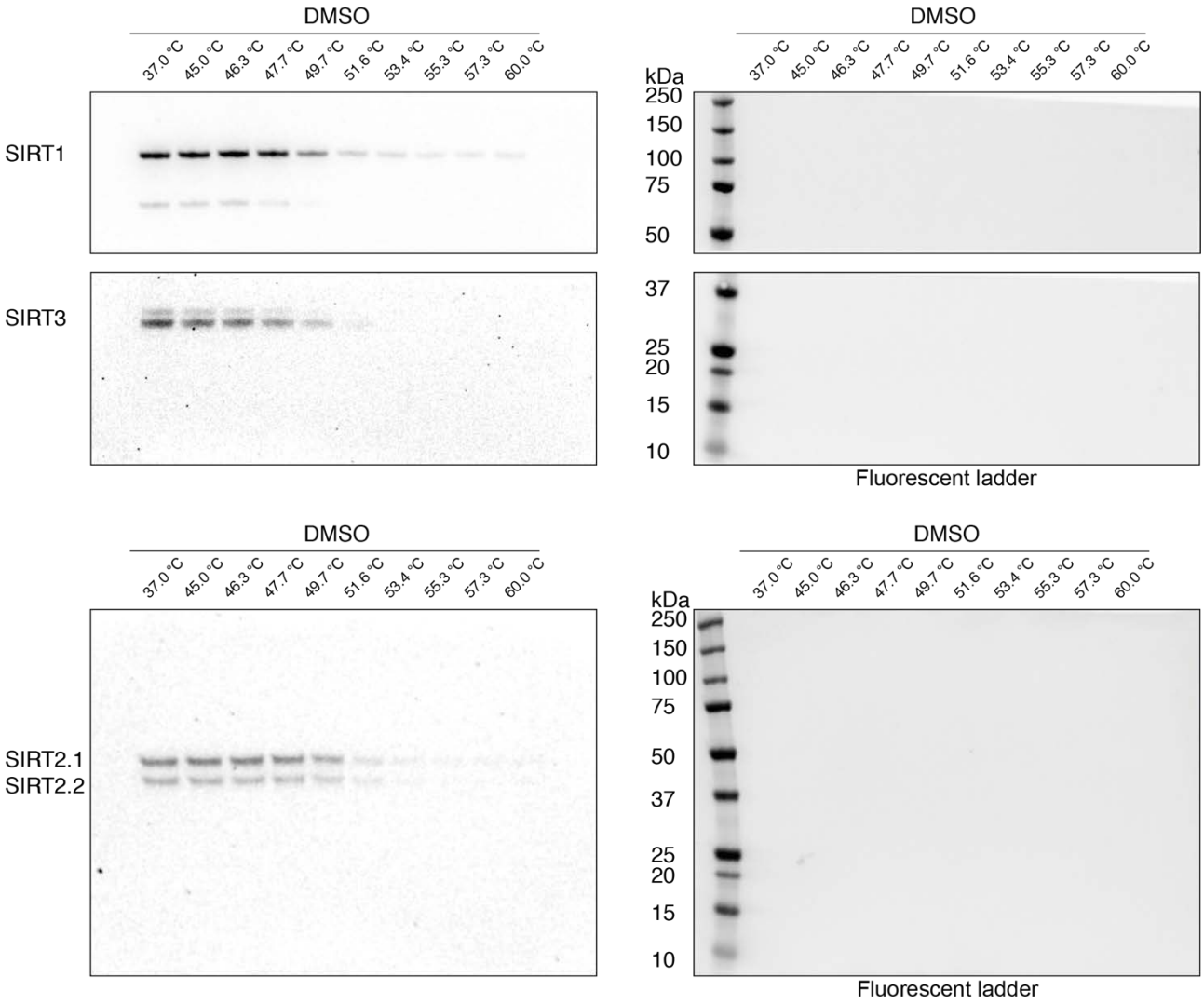

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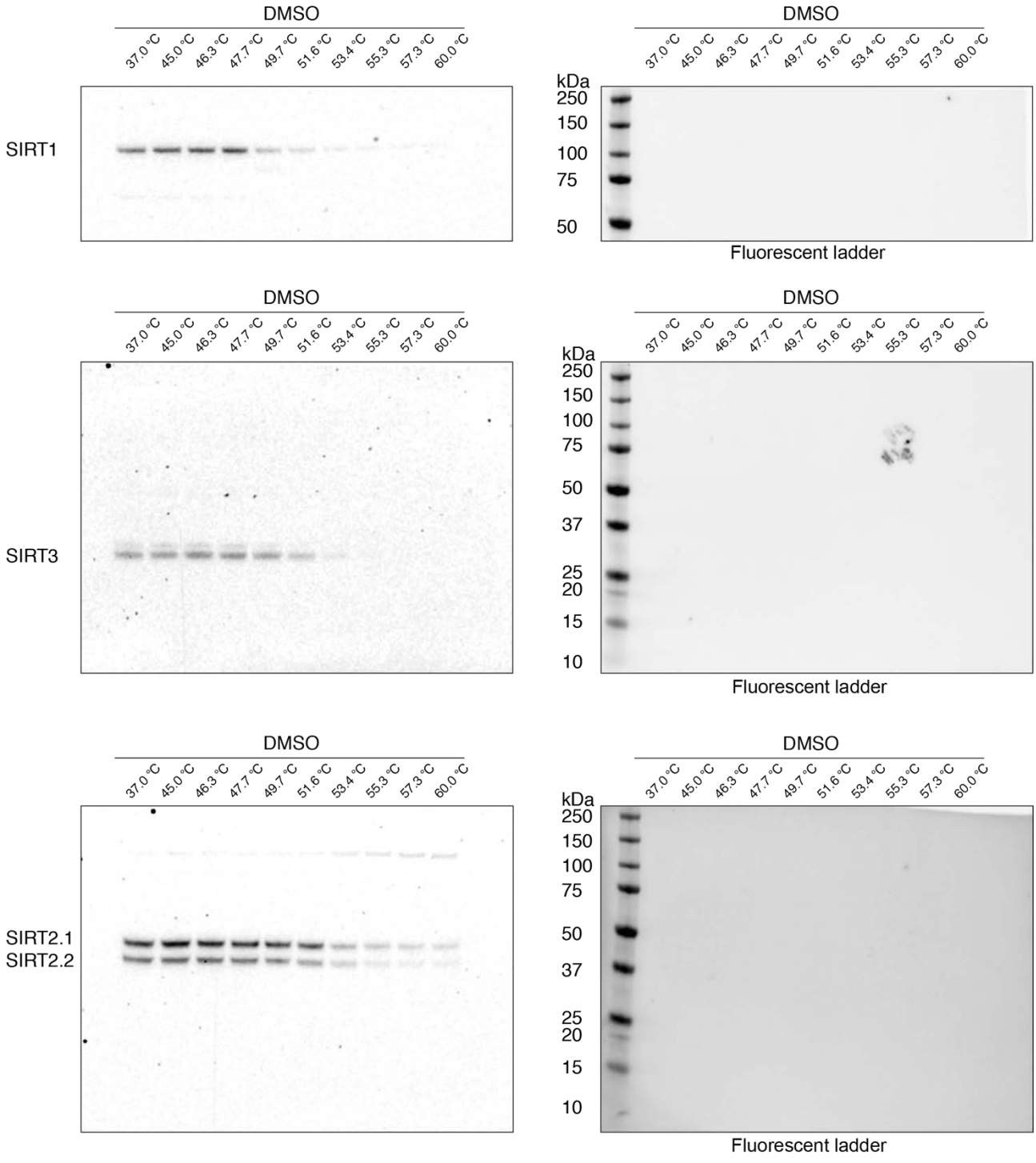

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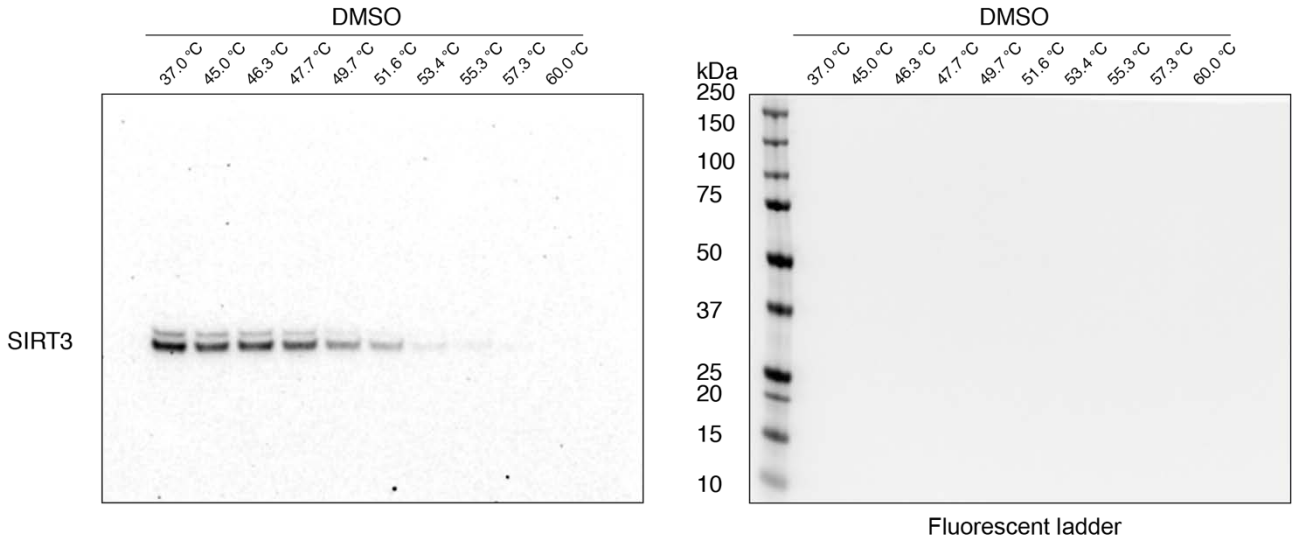

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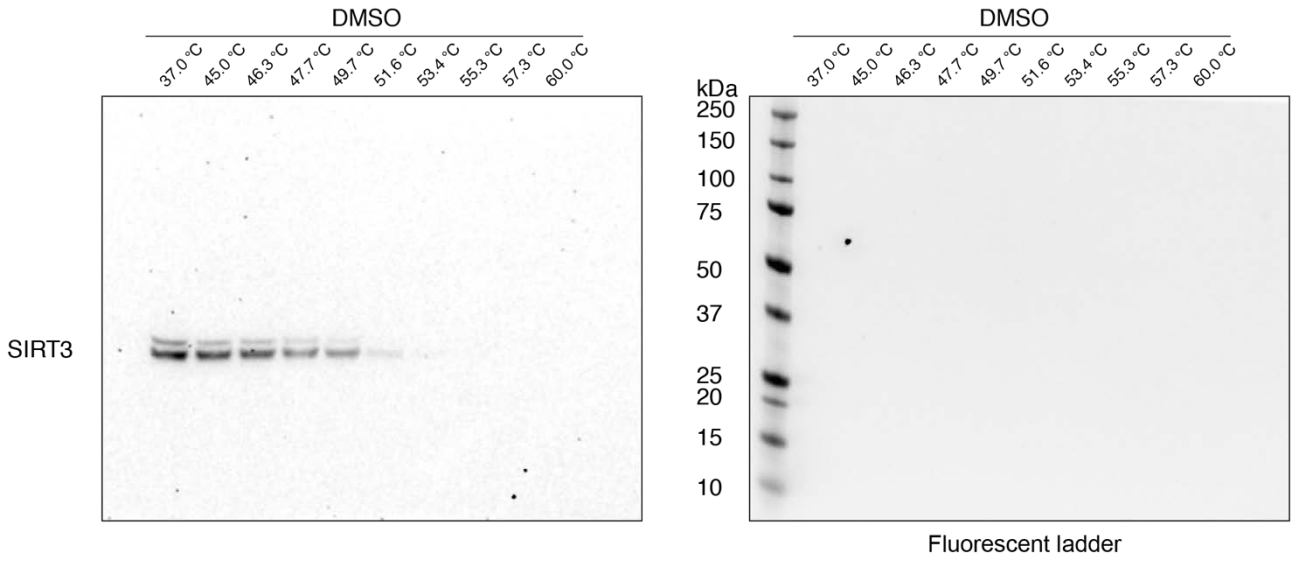

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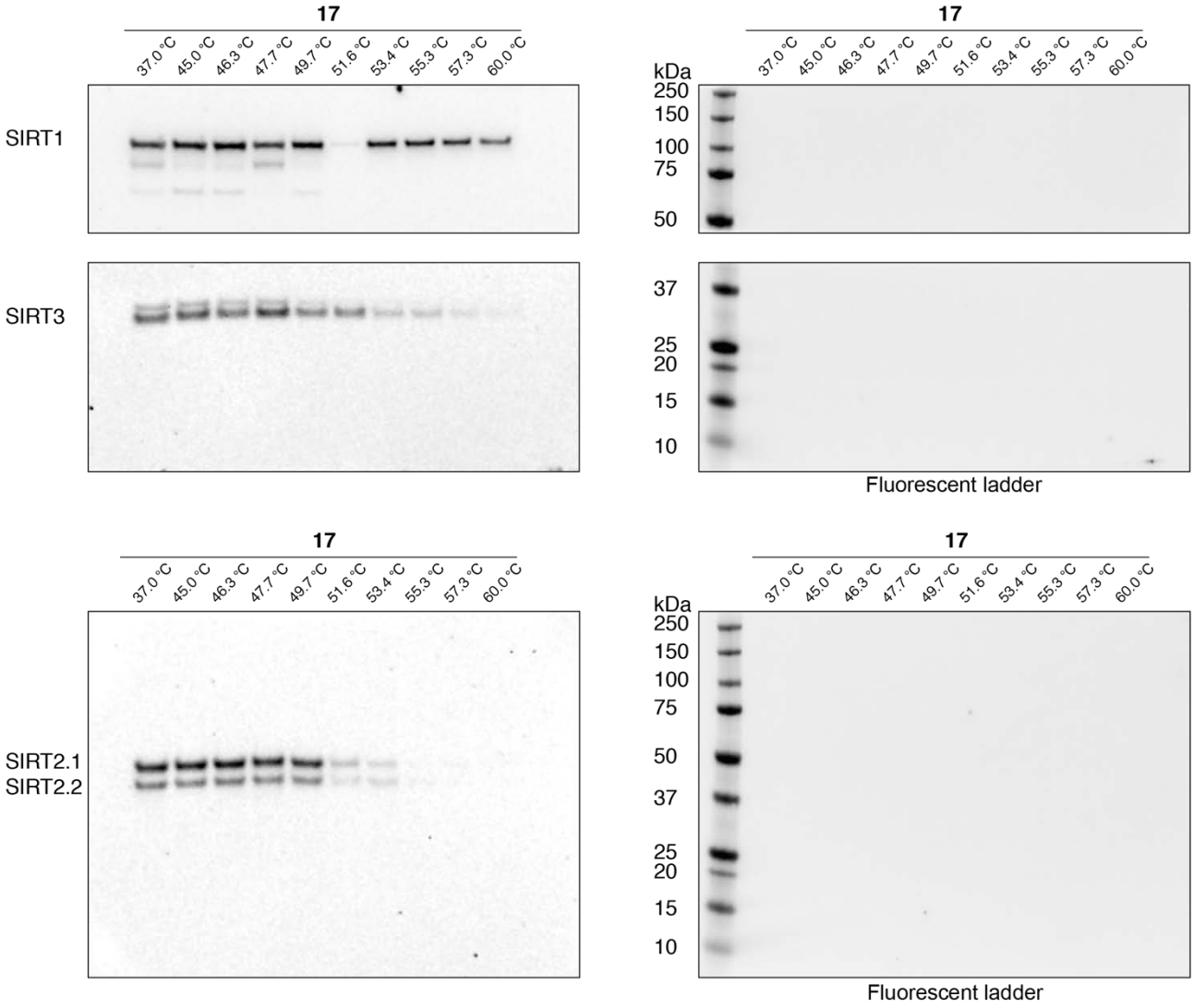

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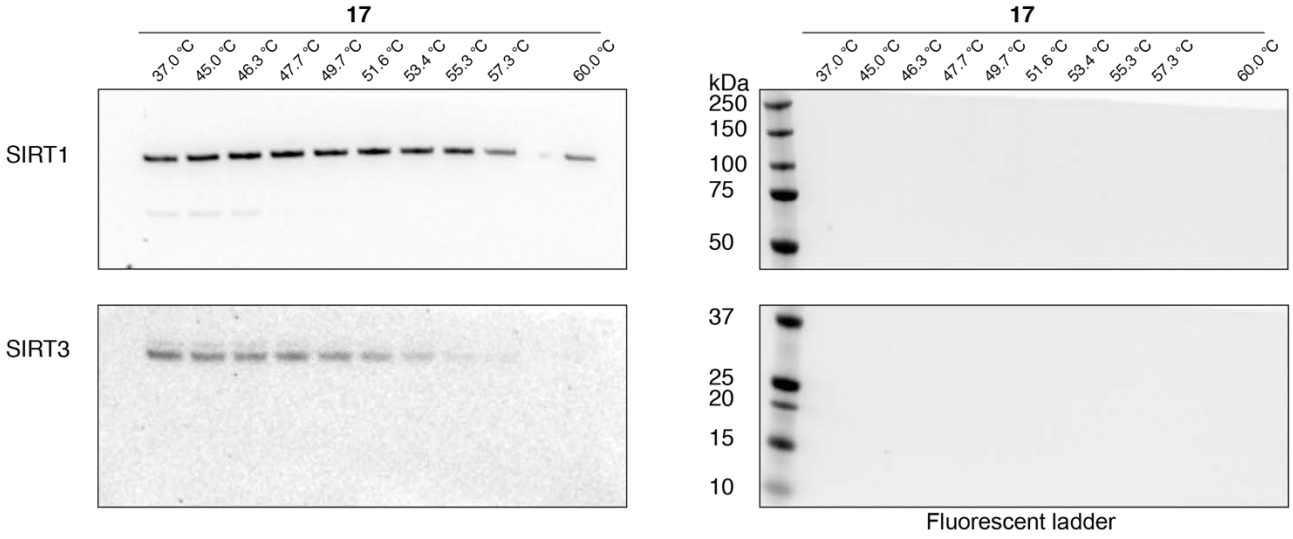

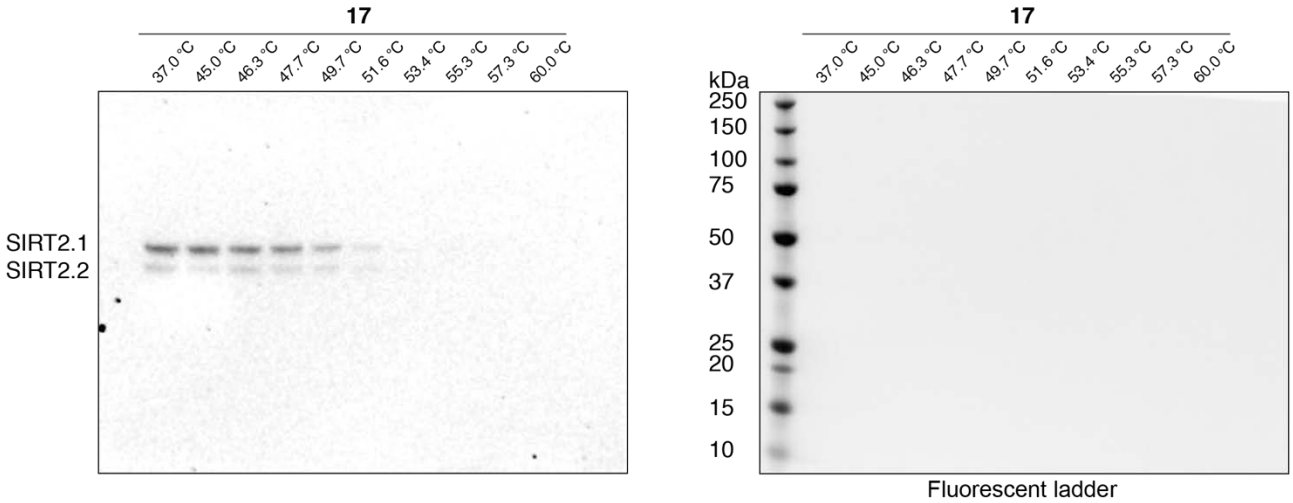

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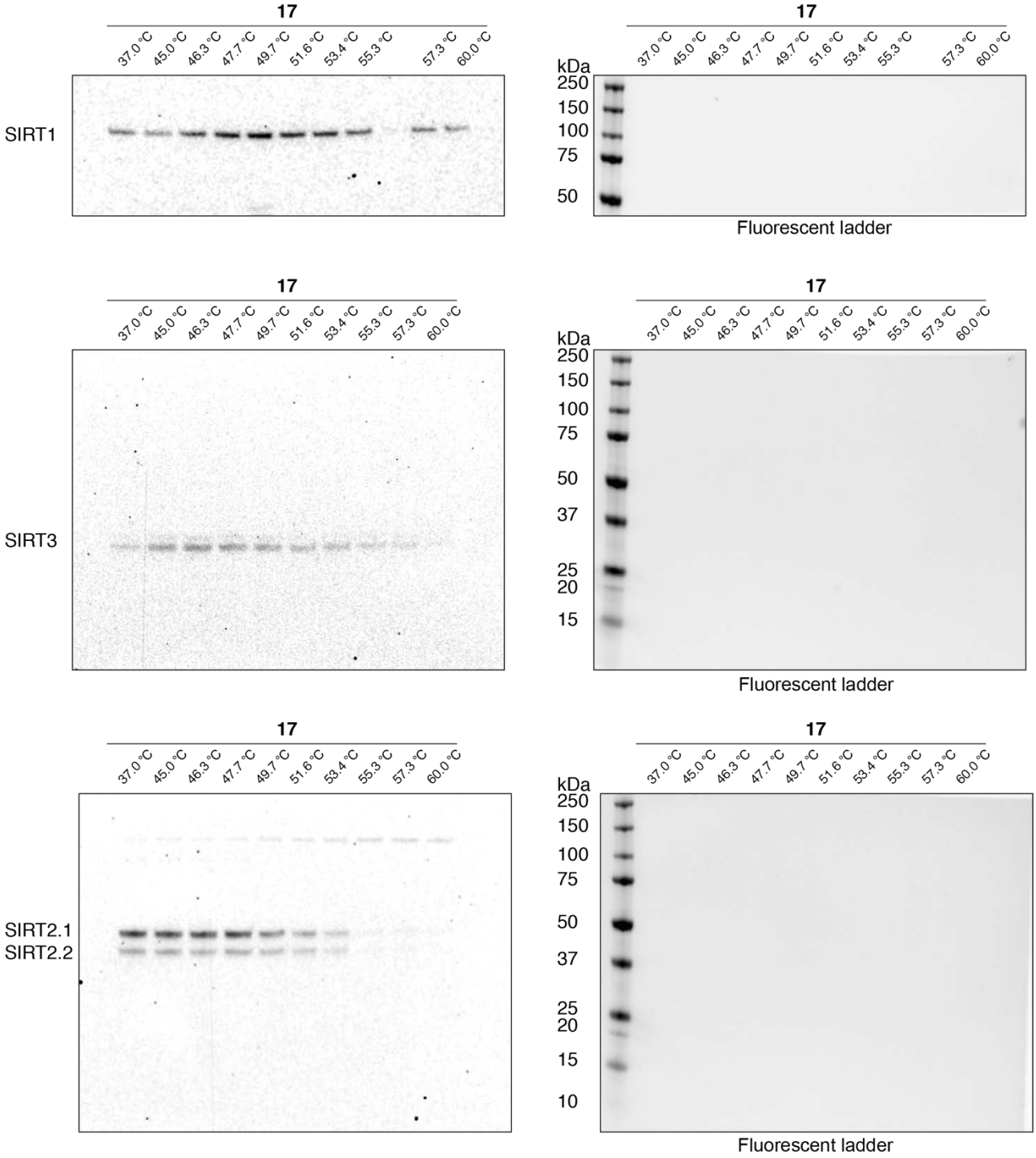

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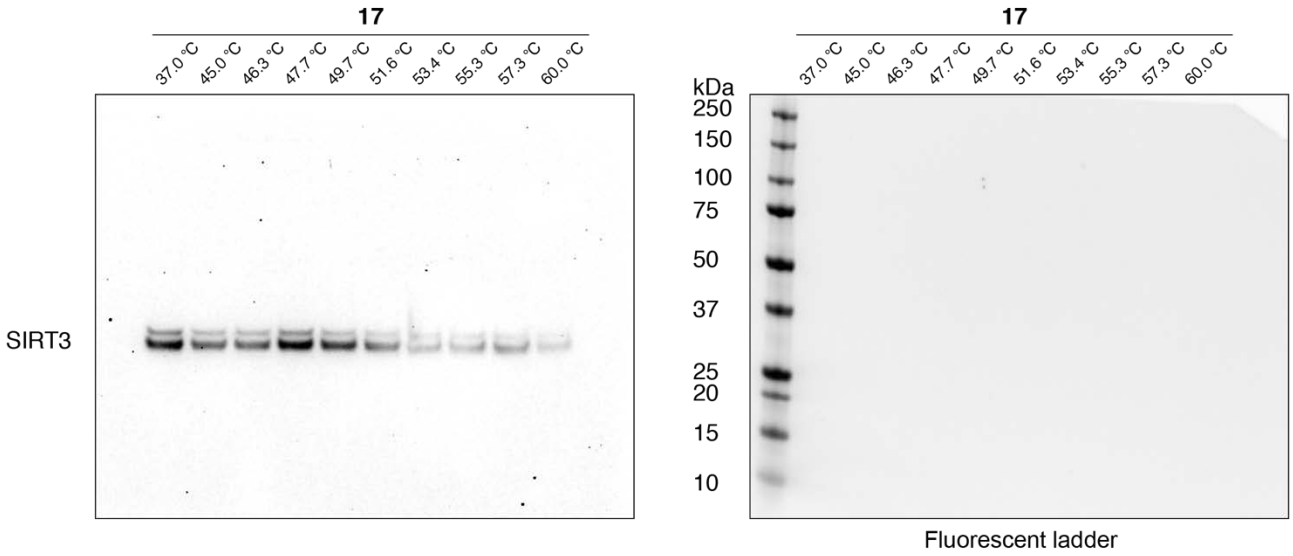

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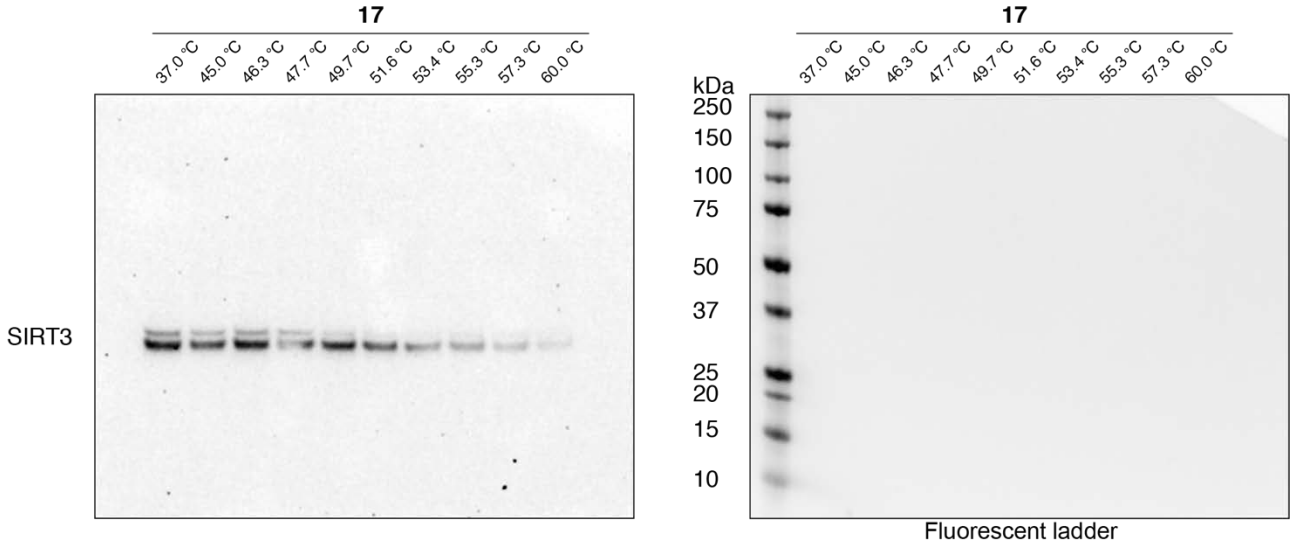

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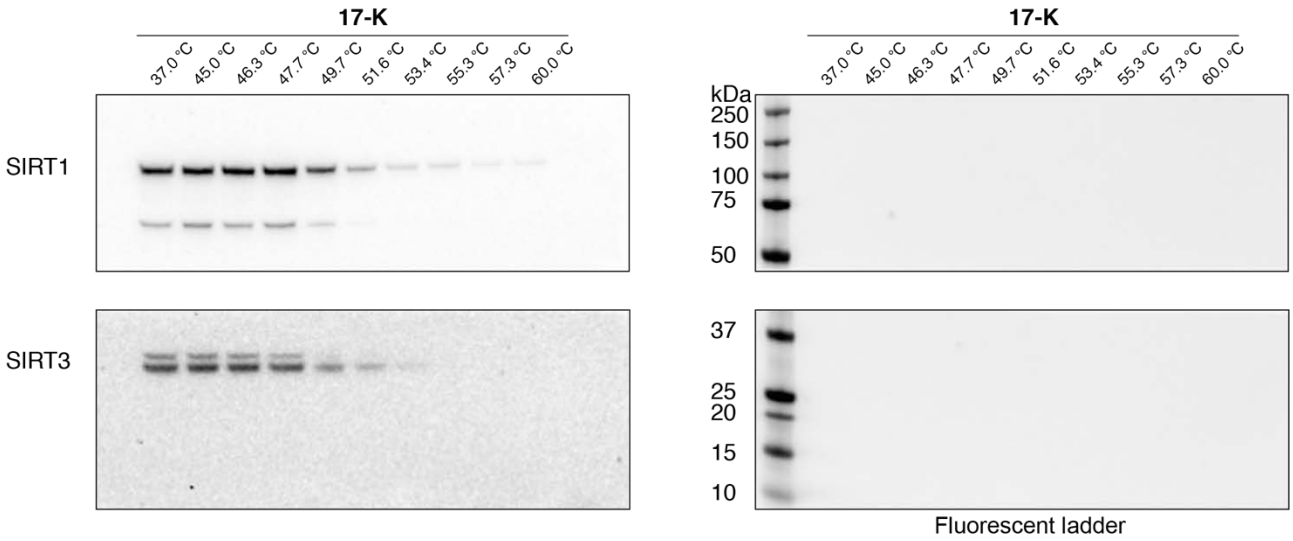

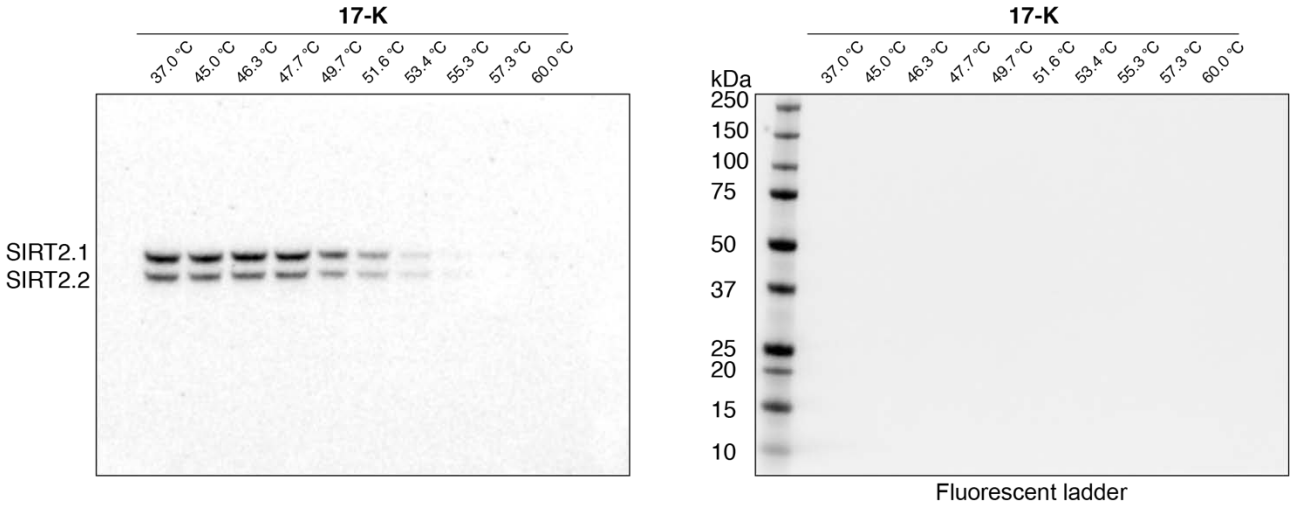

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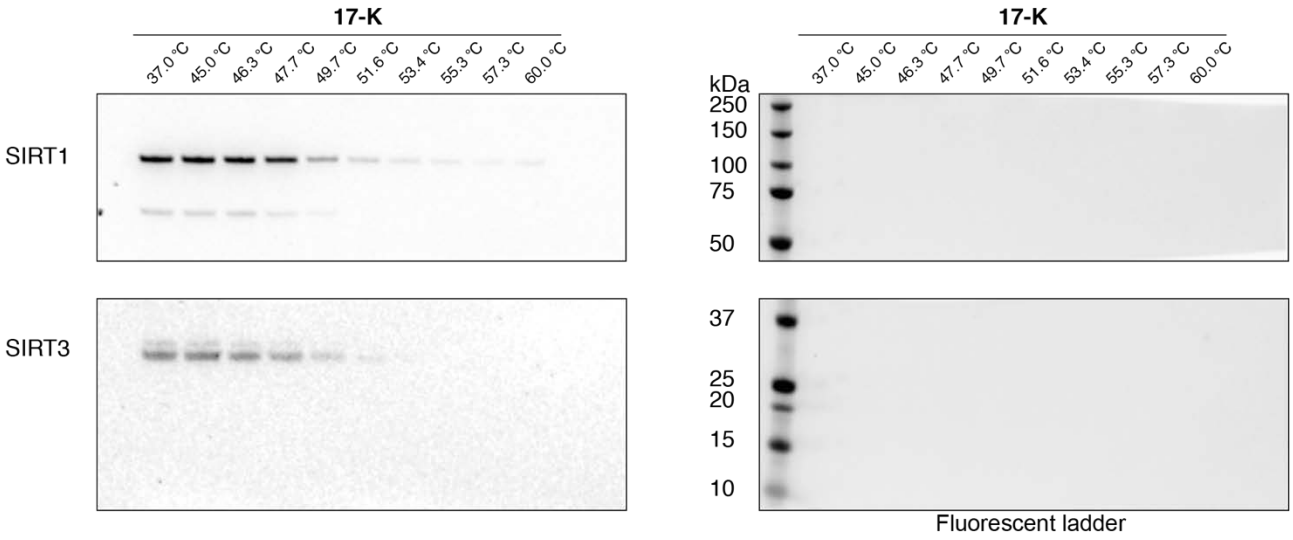

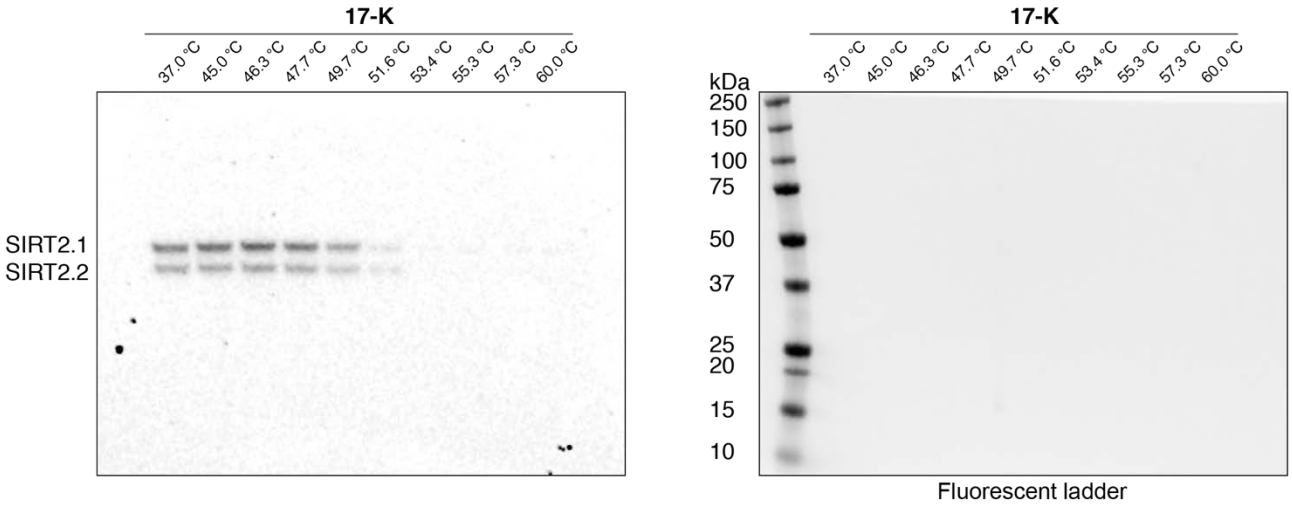

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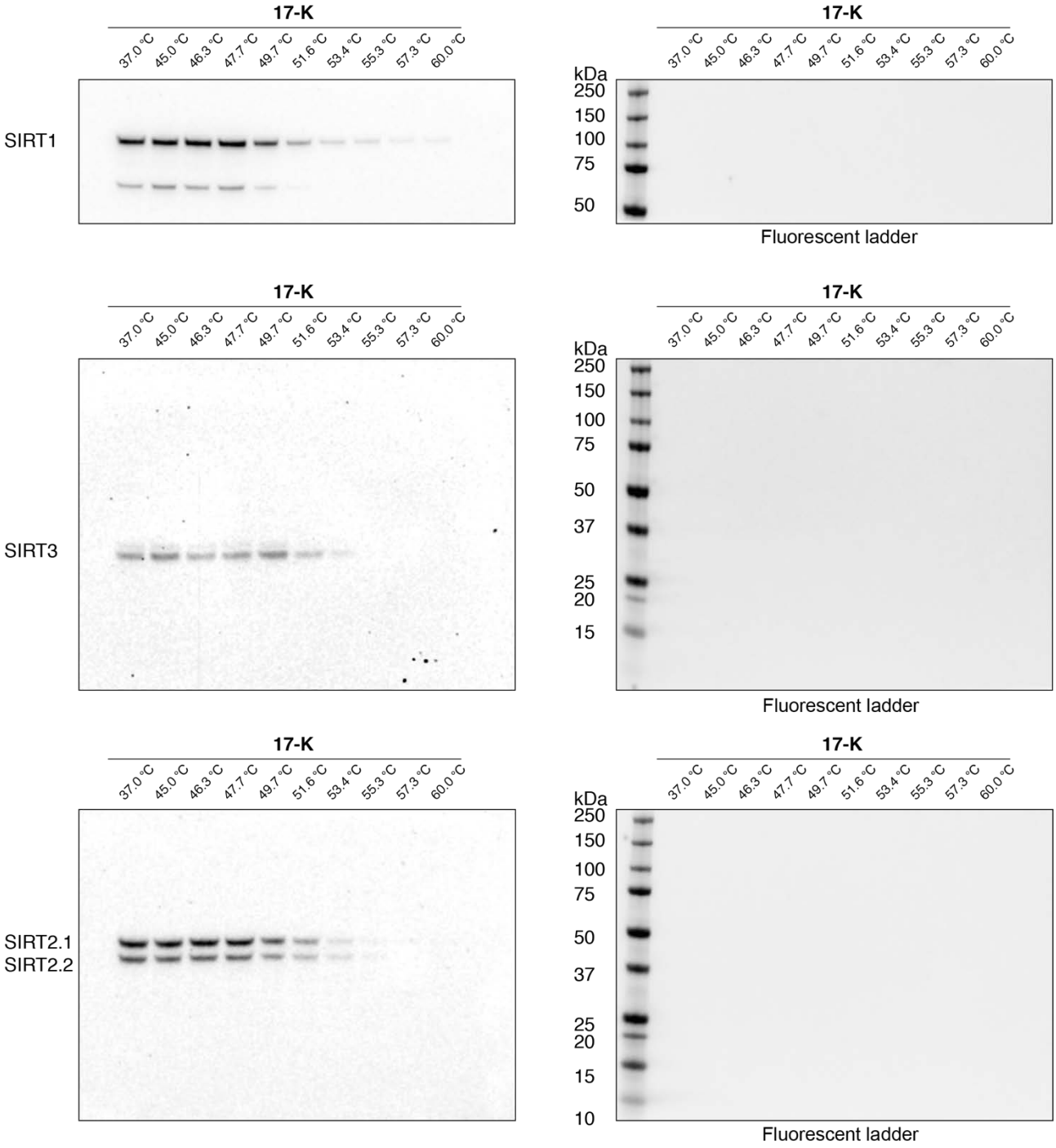

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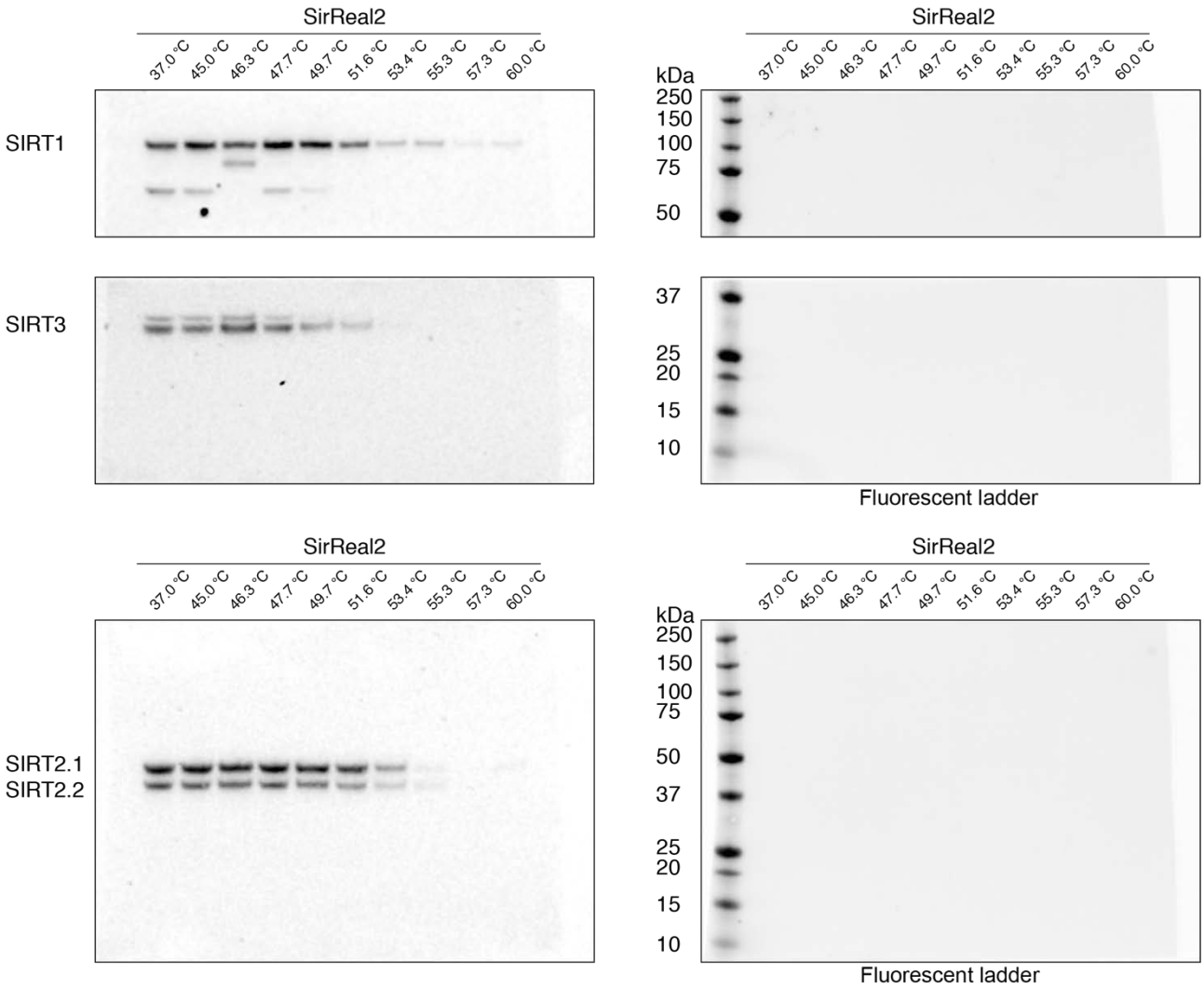

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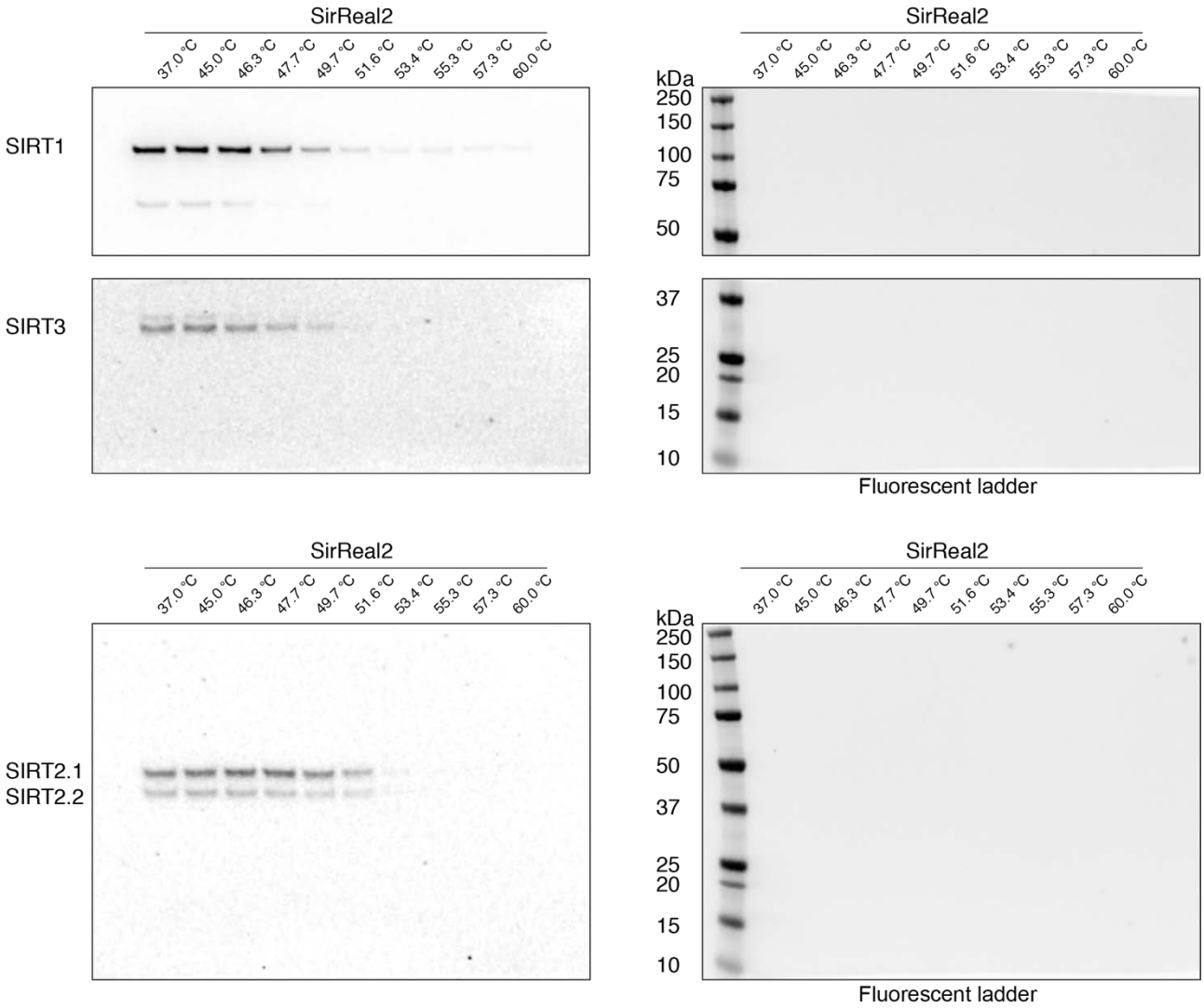

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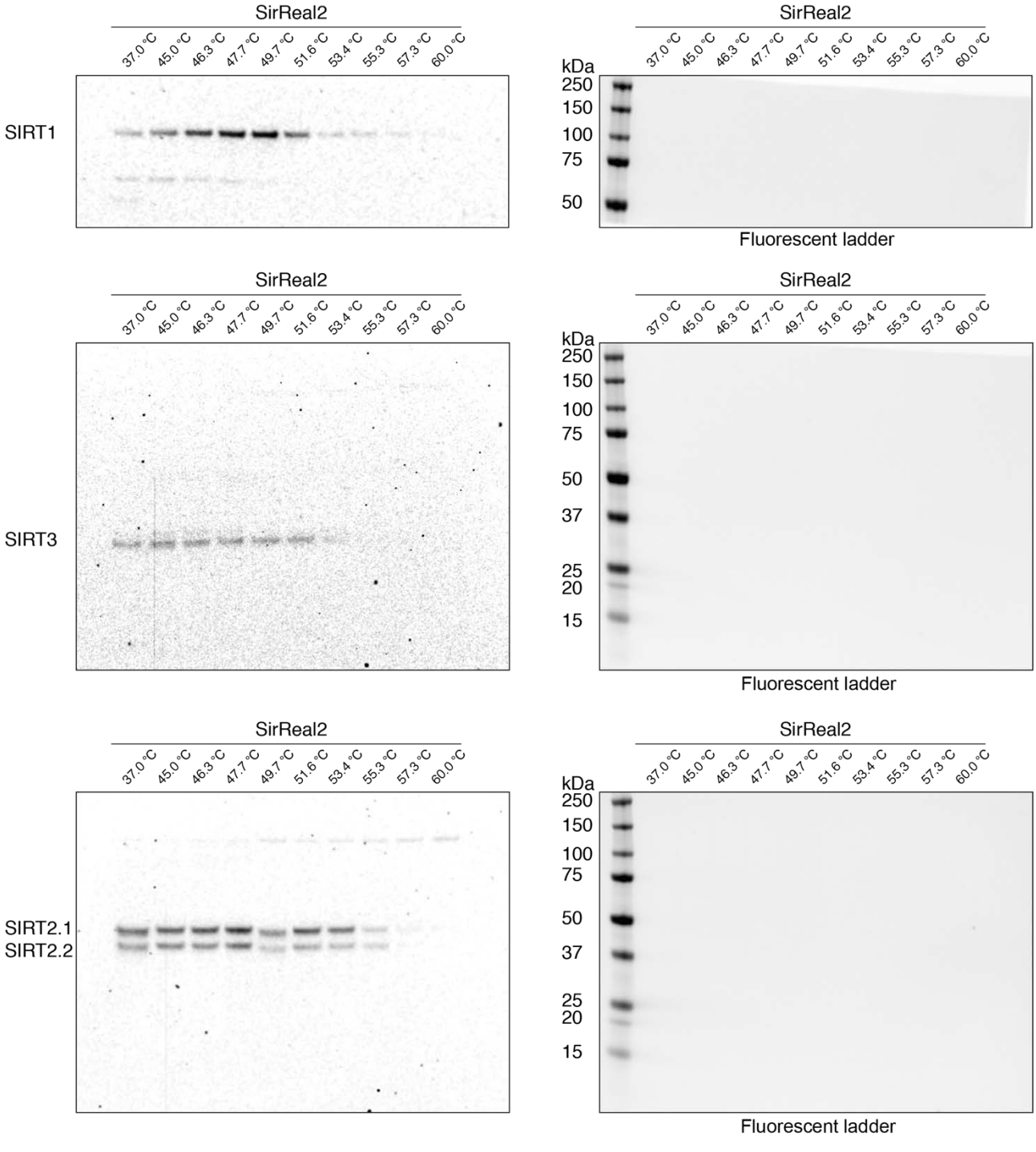

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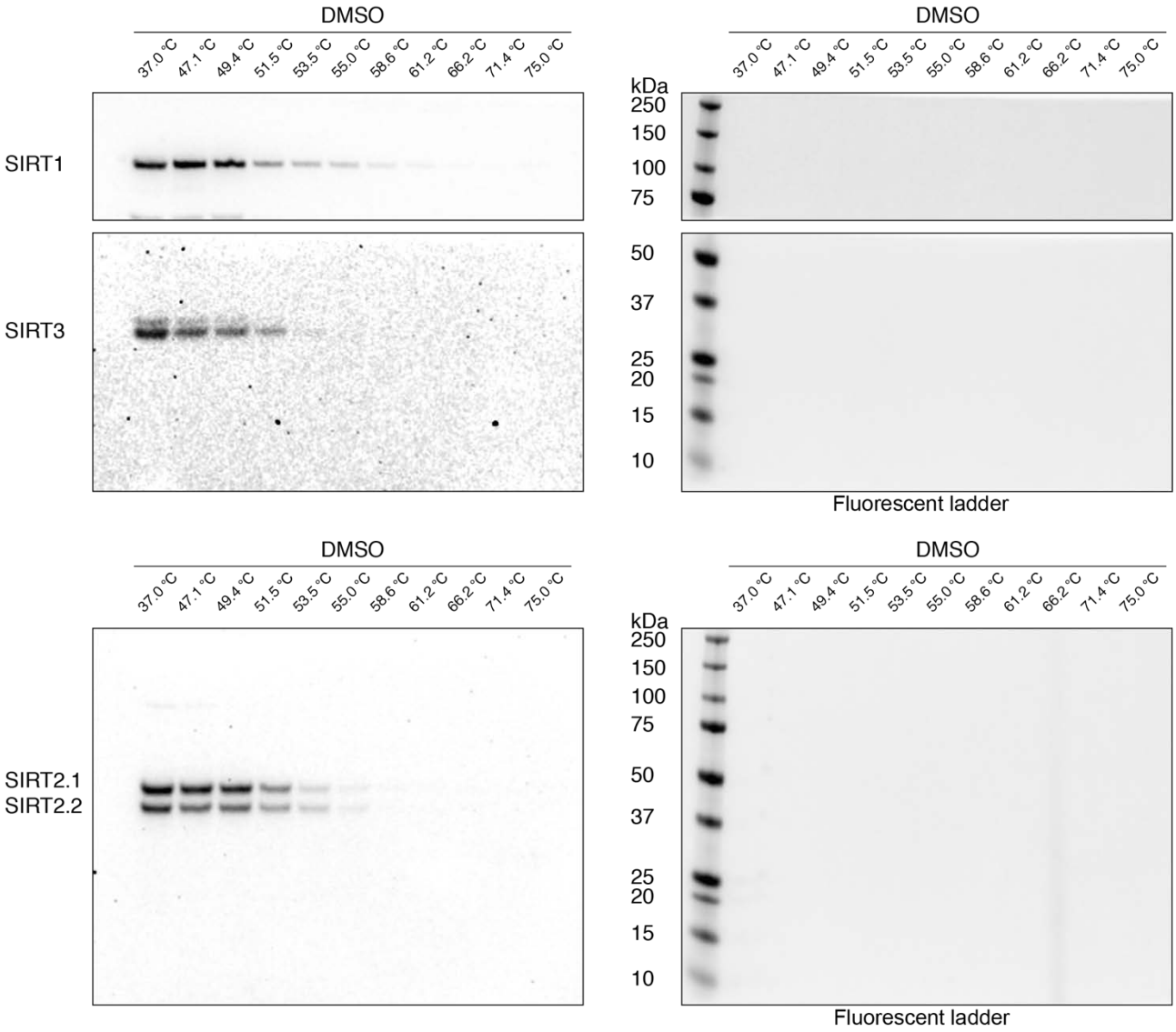

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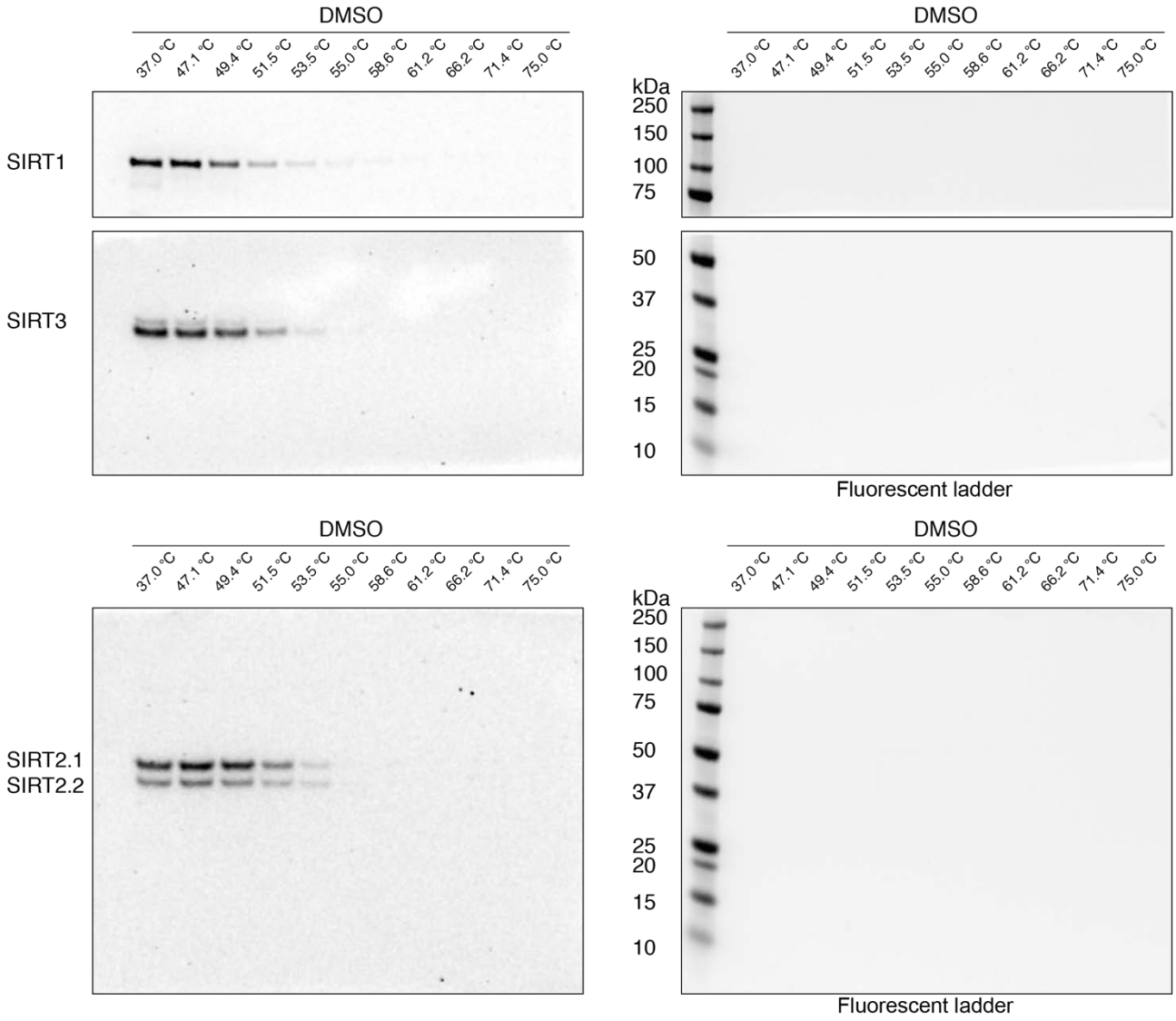

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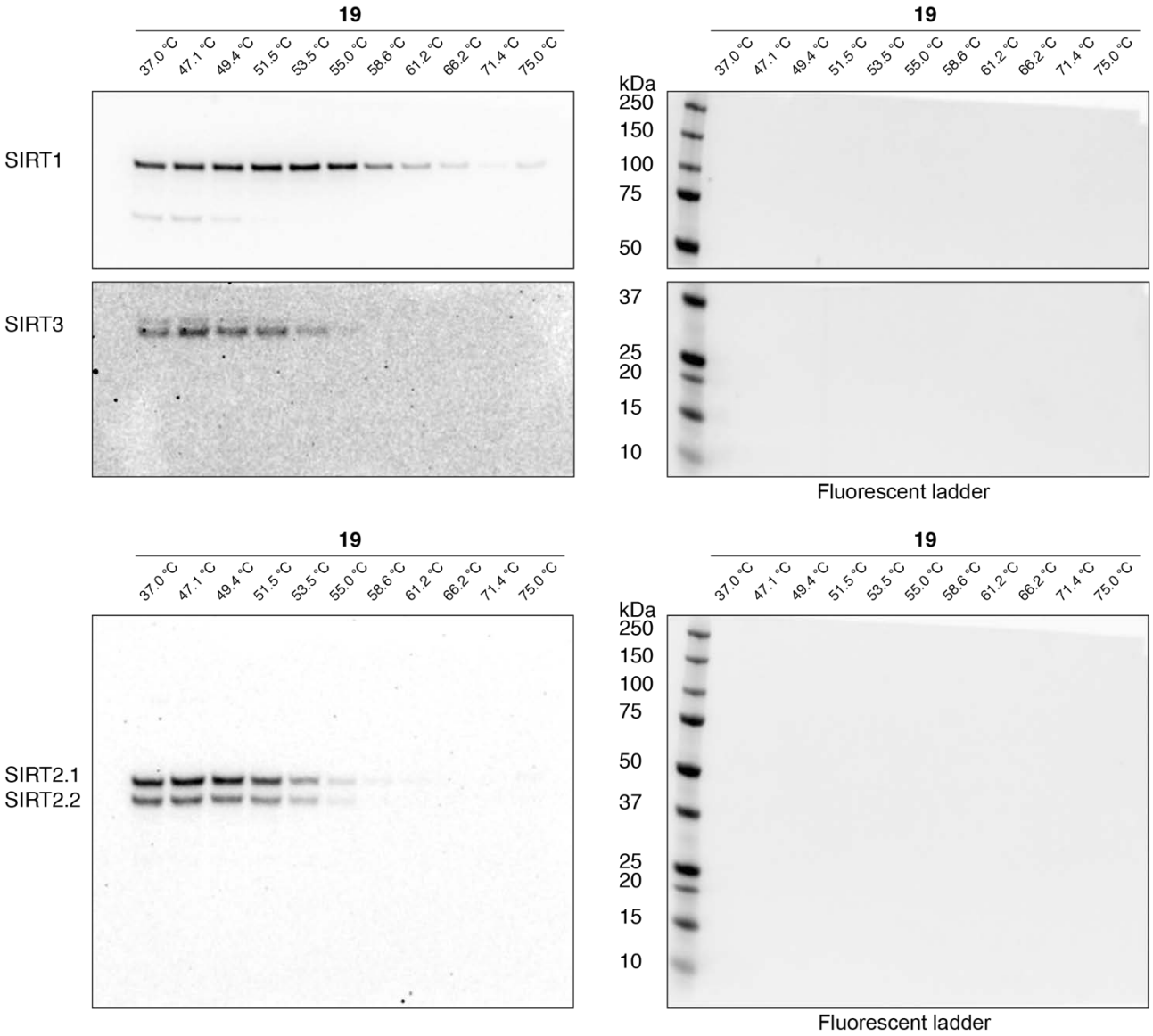

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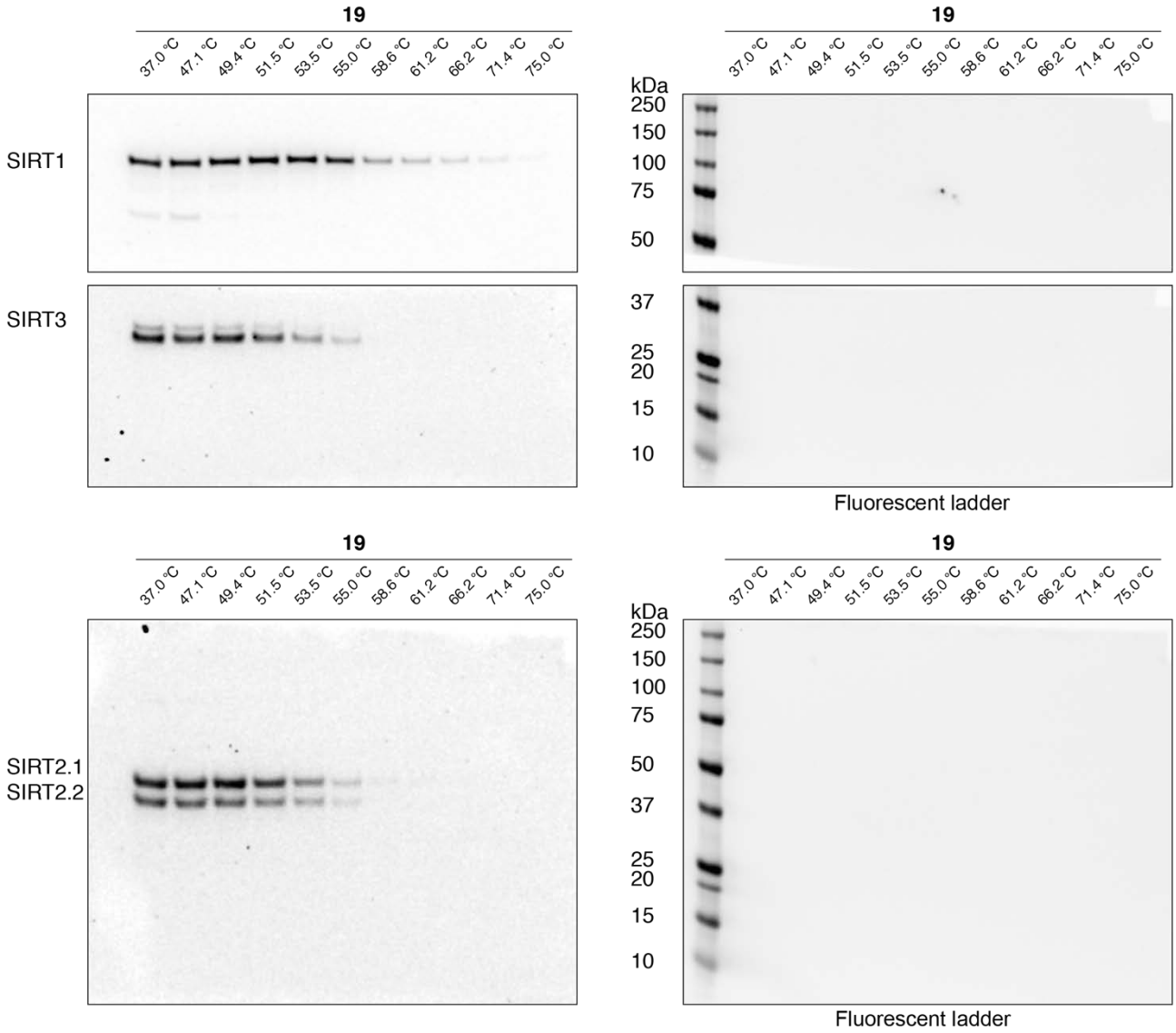

HPLC traces of final compounds

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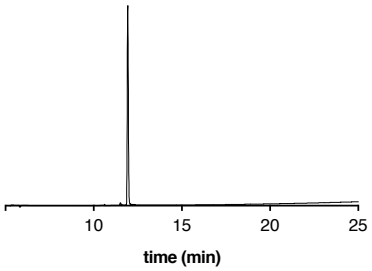

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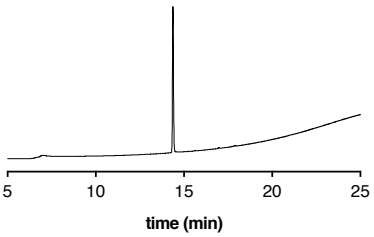

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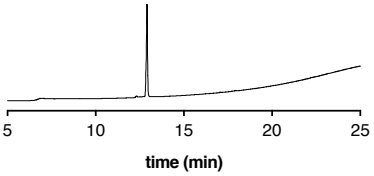

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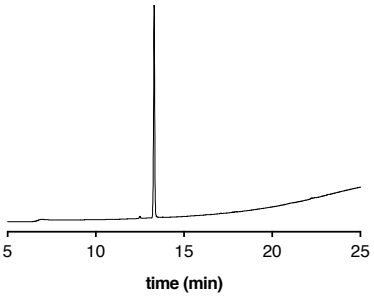

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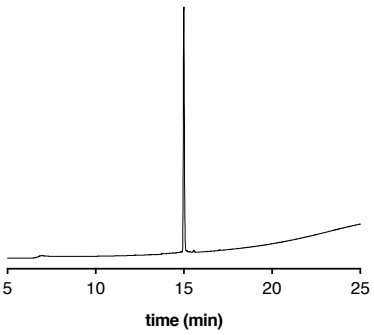

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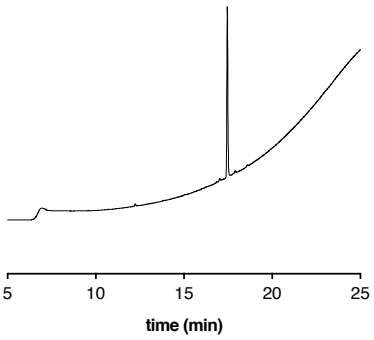

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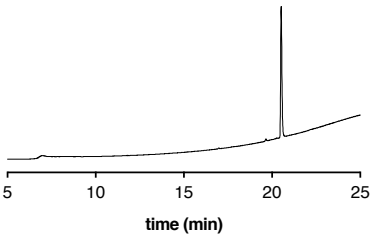

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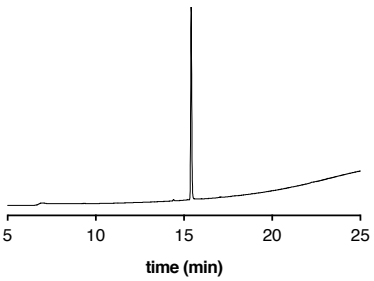

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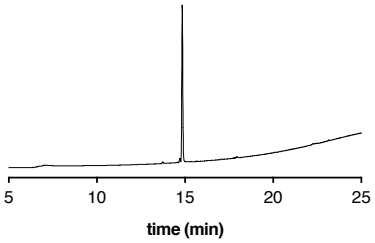

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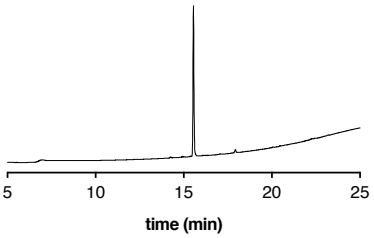

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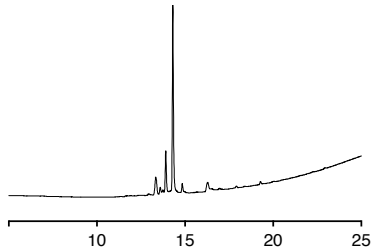

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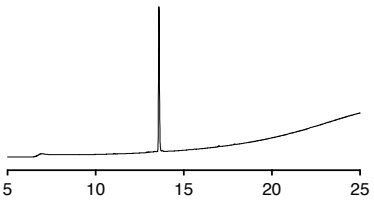

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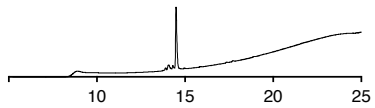

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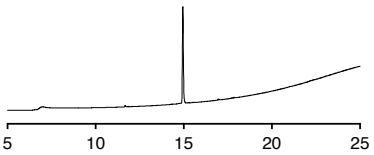

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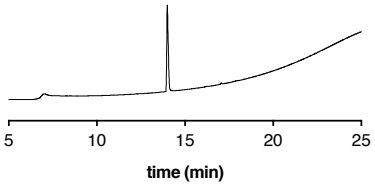

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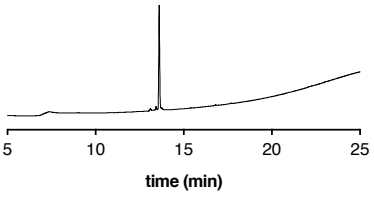

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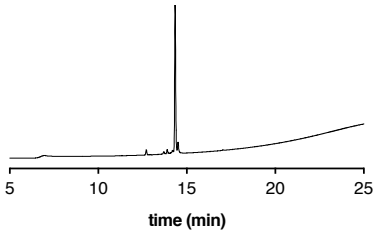

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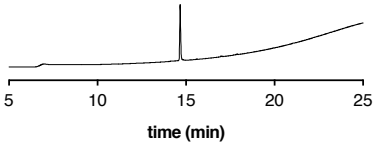

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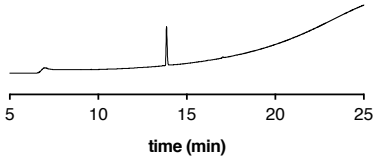

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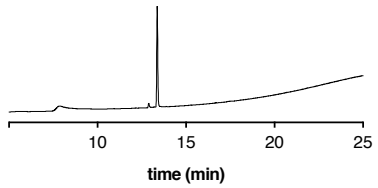

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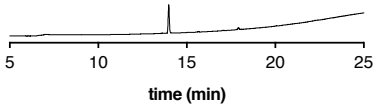
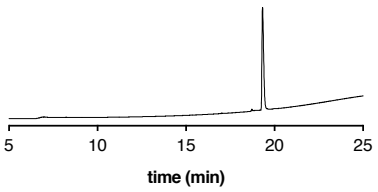
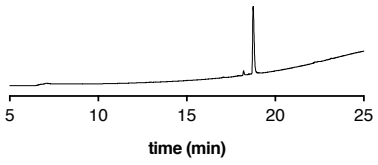


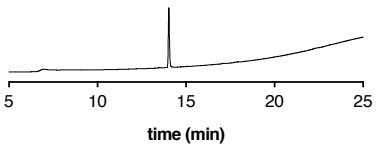
cmpd S3



cmpd S4



cmpd S5



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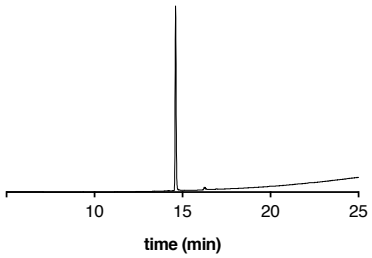

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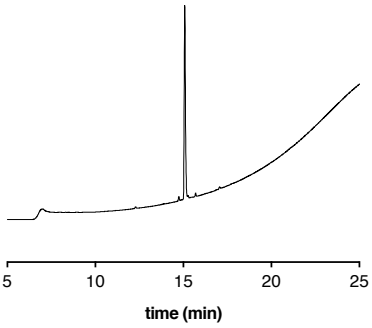

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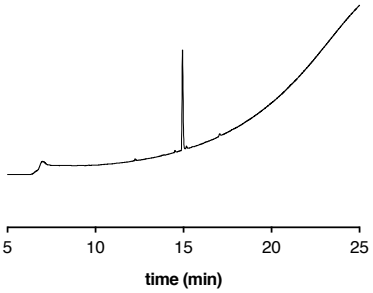
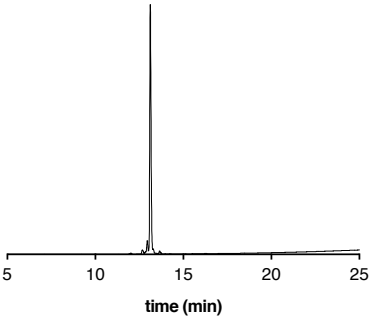
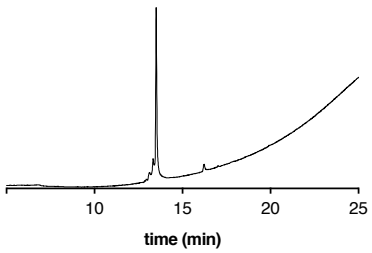


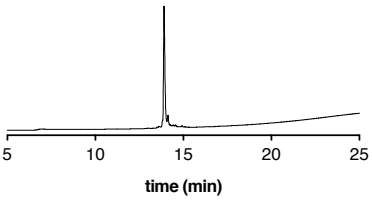
cmpd S10



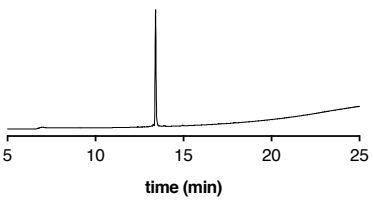
cmpd S11



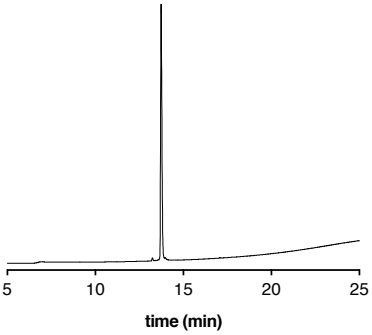
cmpd S12



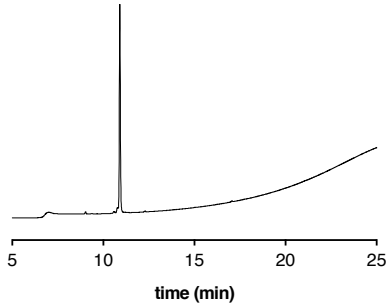
cmpd S13



cmpd S14

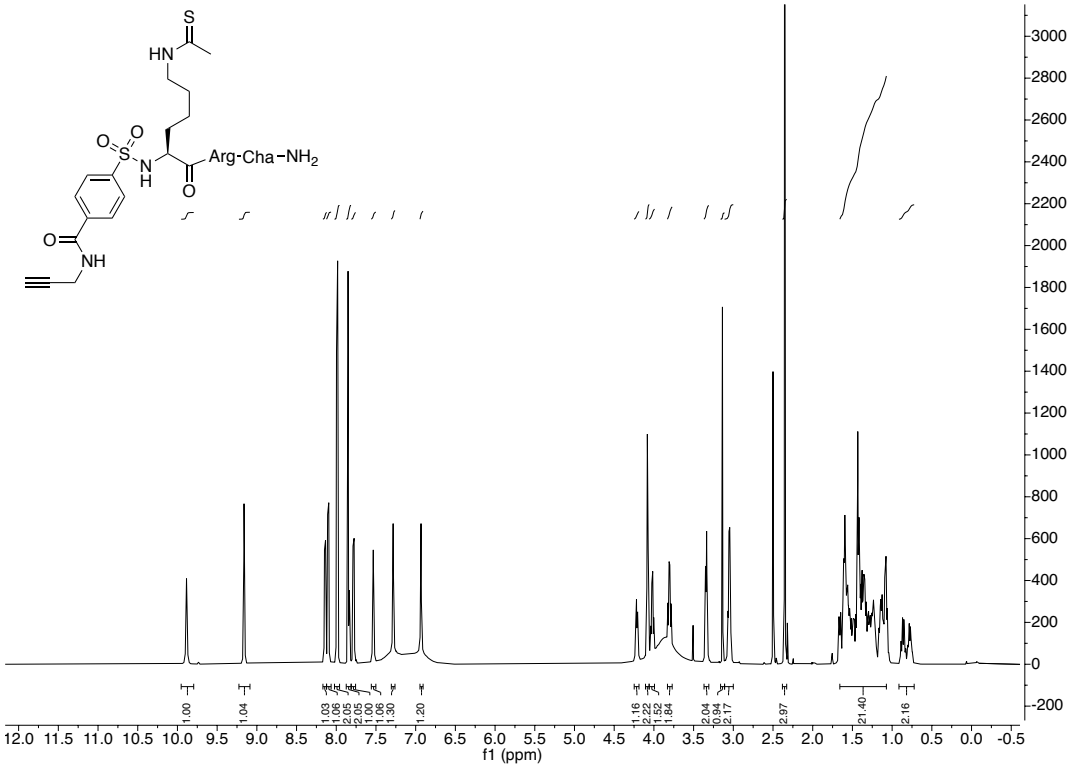


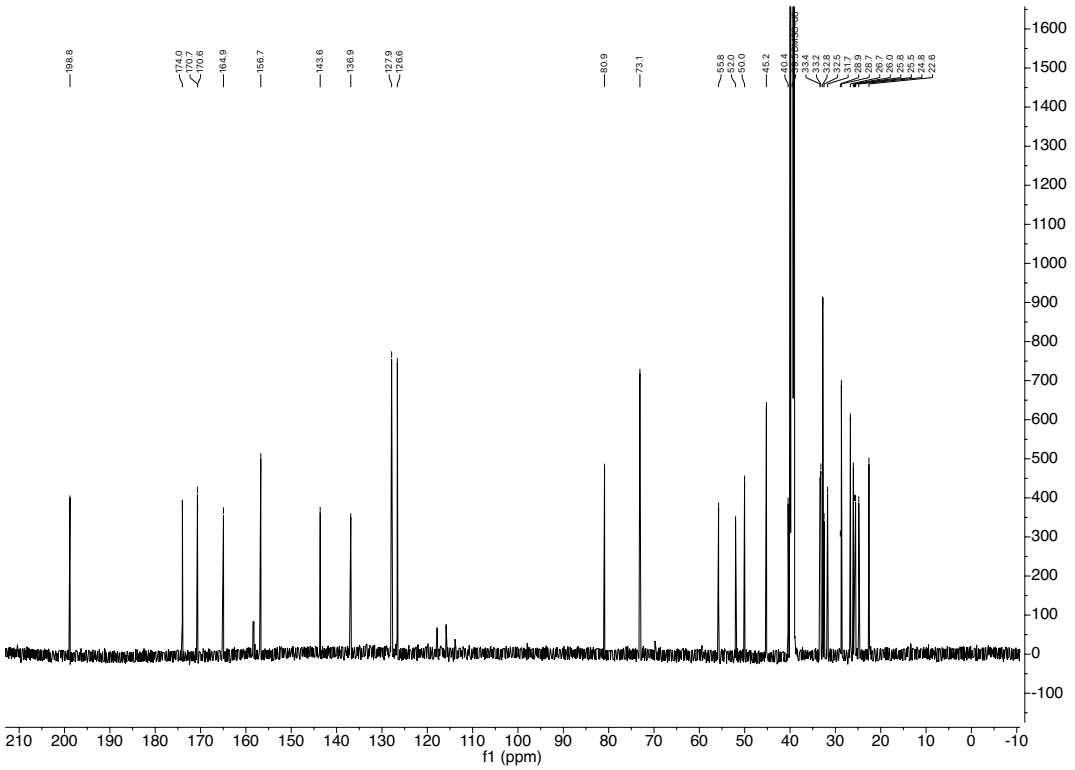
cmpd S15



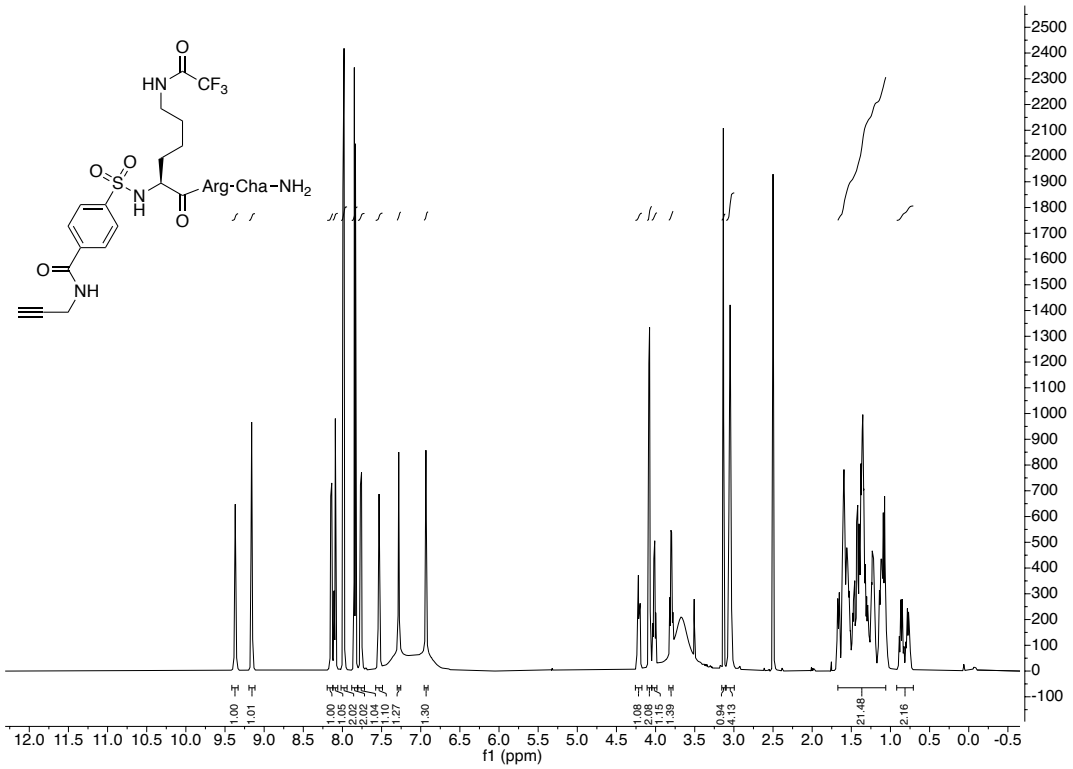
NMR spectra

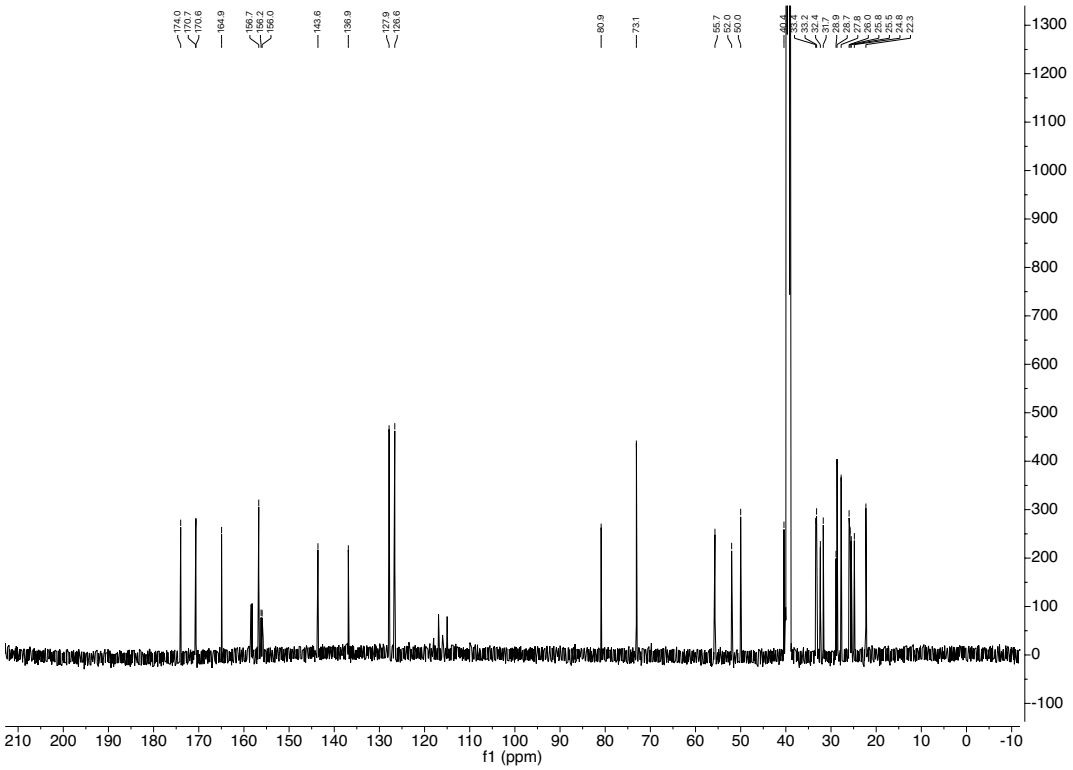
^{1}H and ^{13}C spectra of compound 1



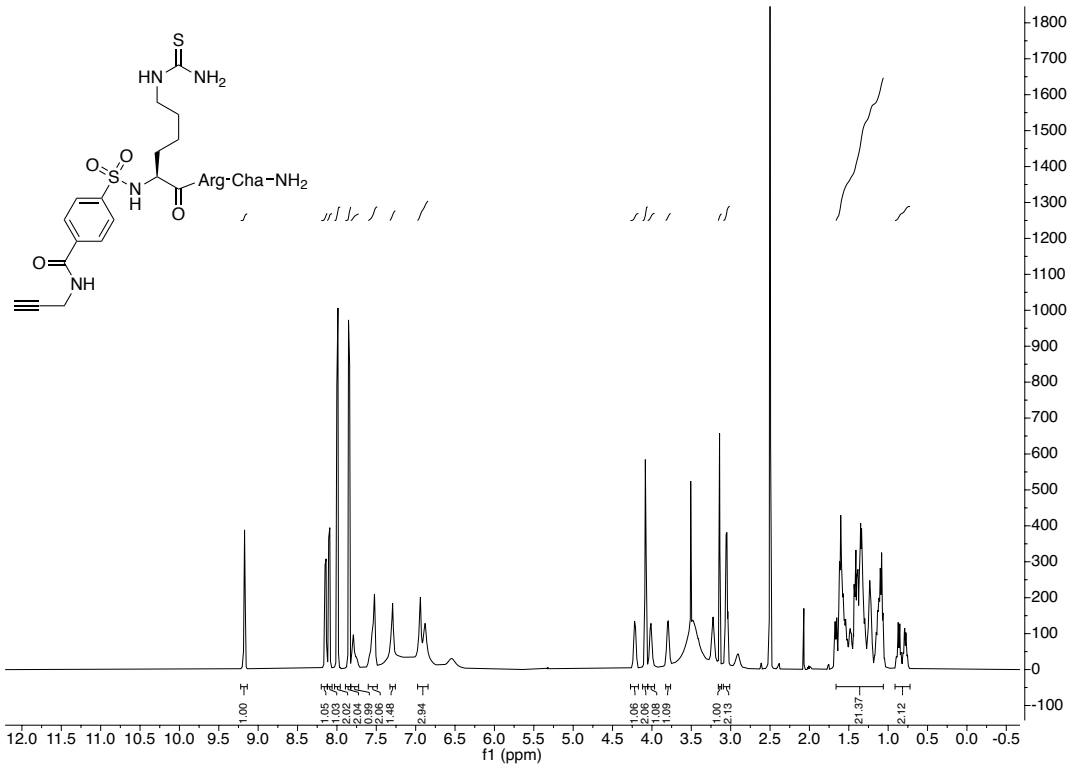


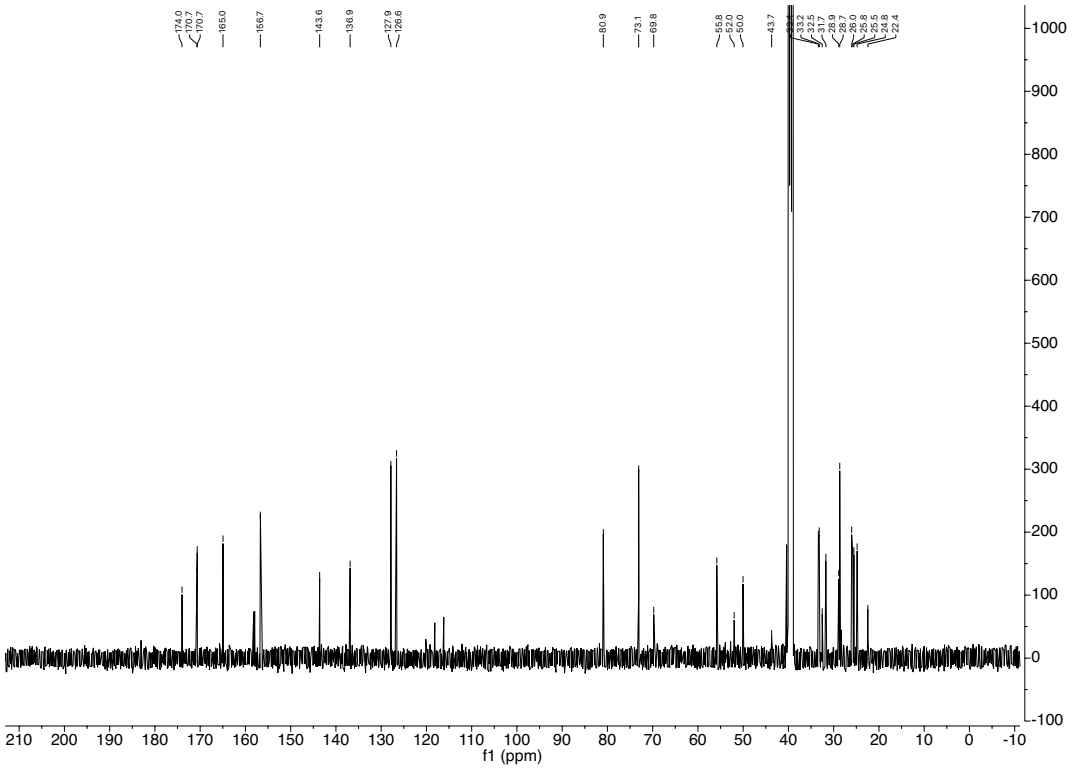
^{1}H and ^{13}C spectra of compound 2



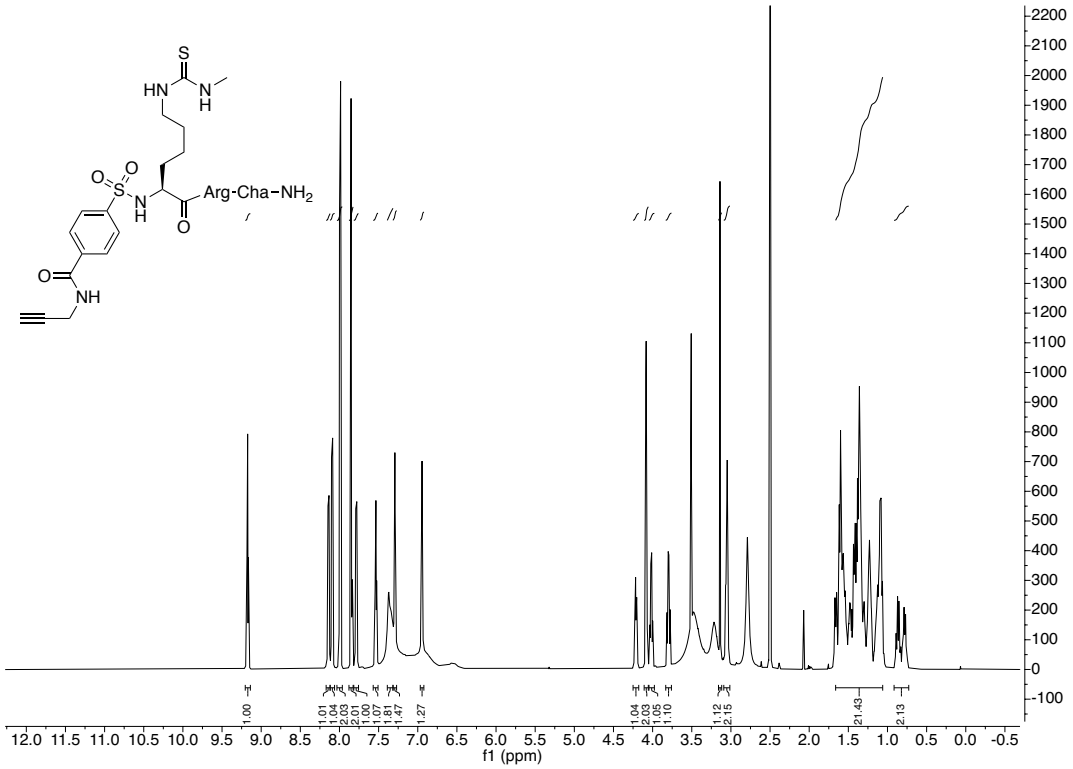


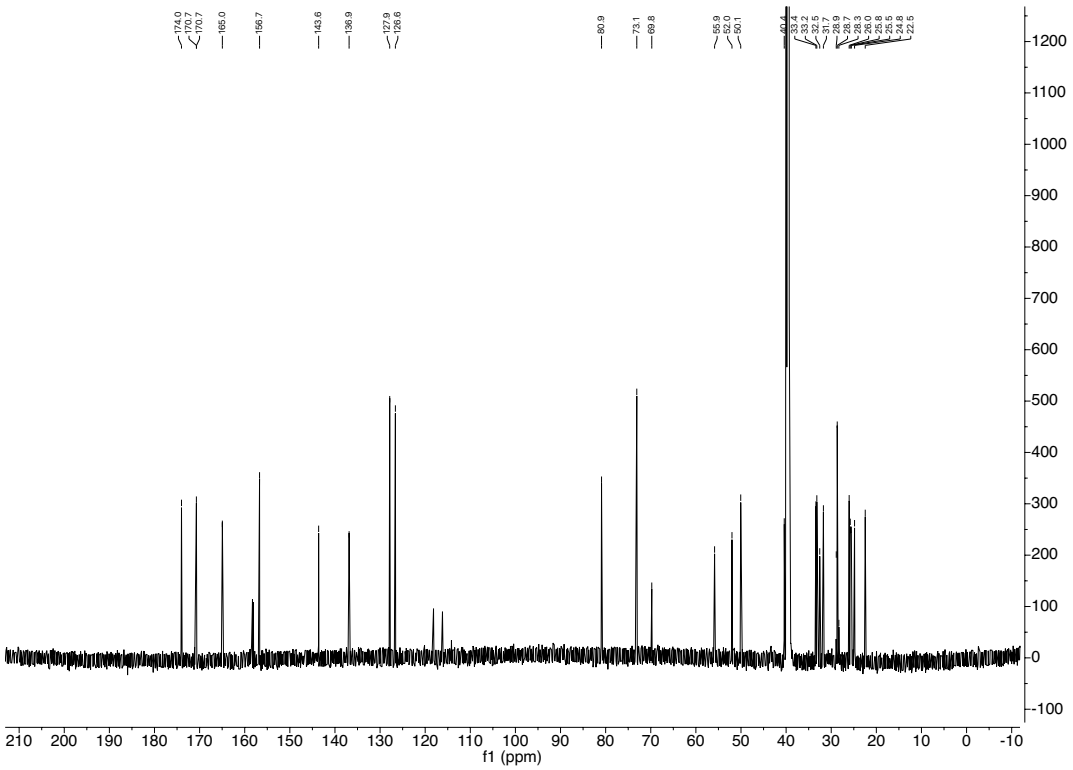
^{1}H and ^{13}C spectra of compound 3



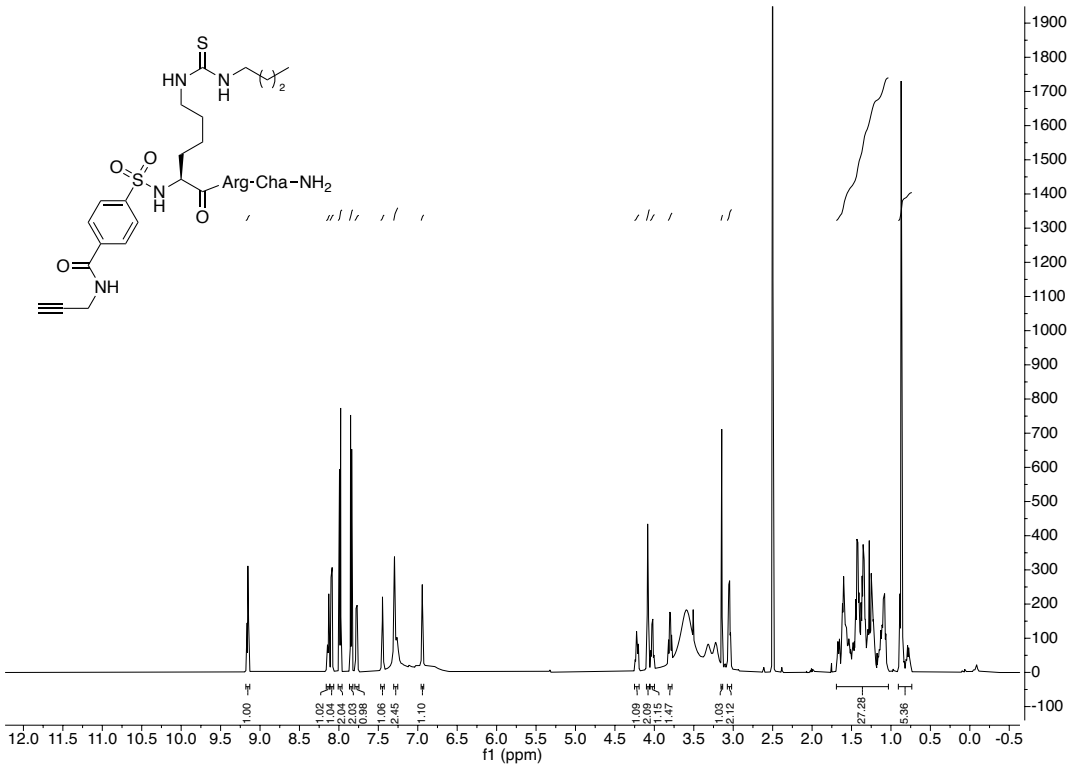


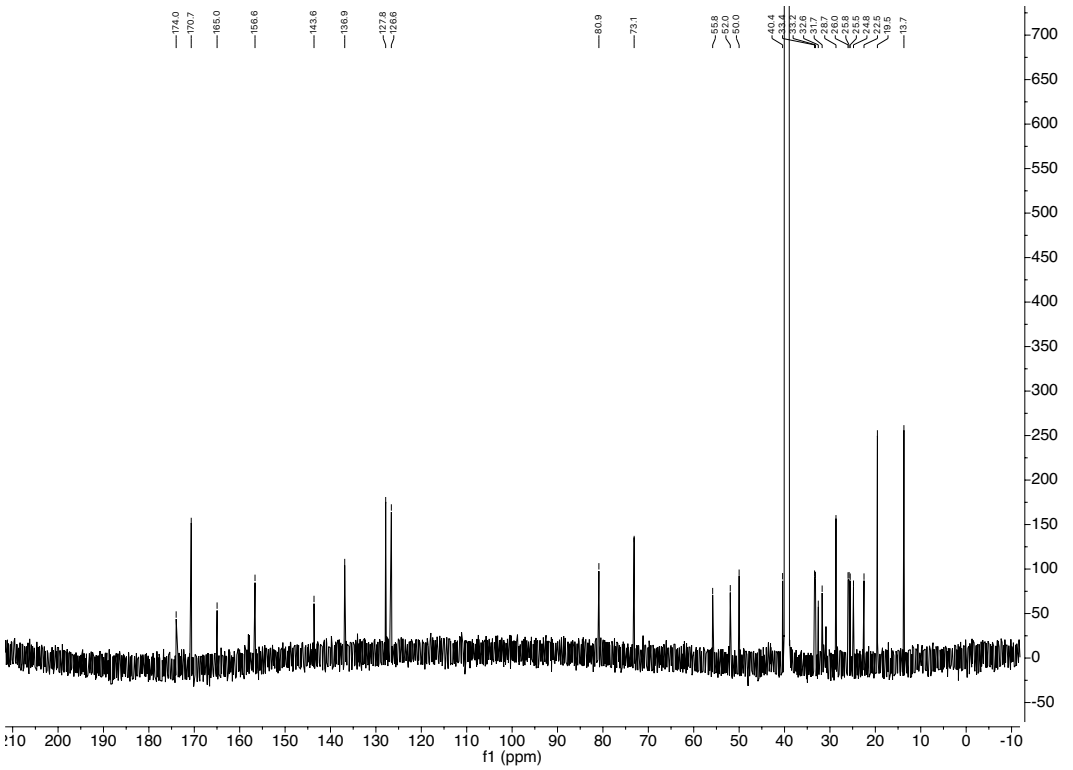
^{1}H and ^{13}C spectra of compound 4



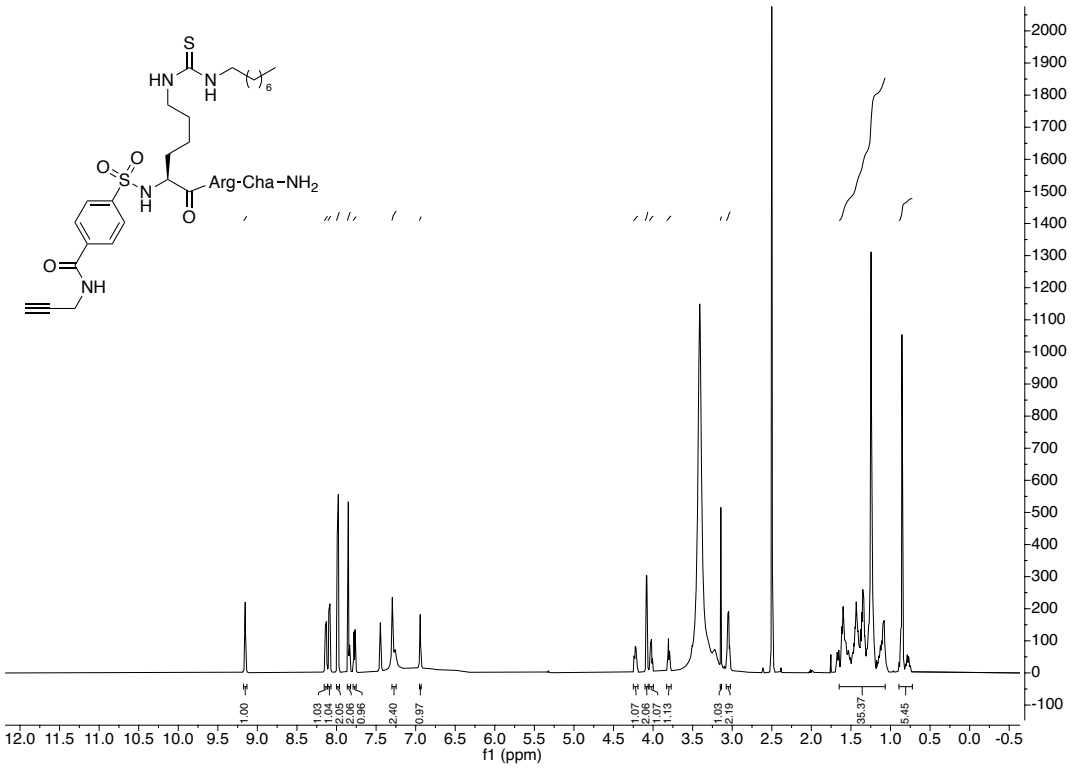


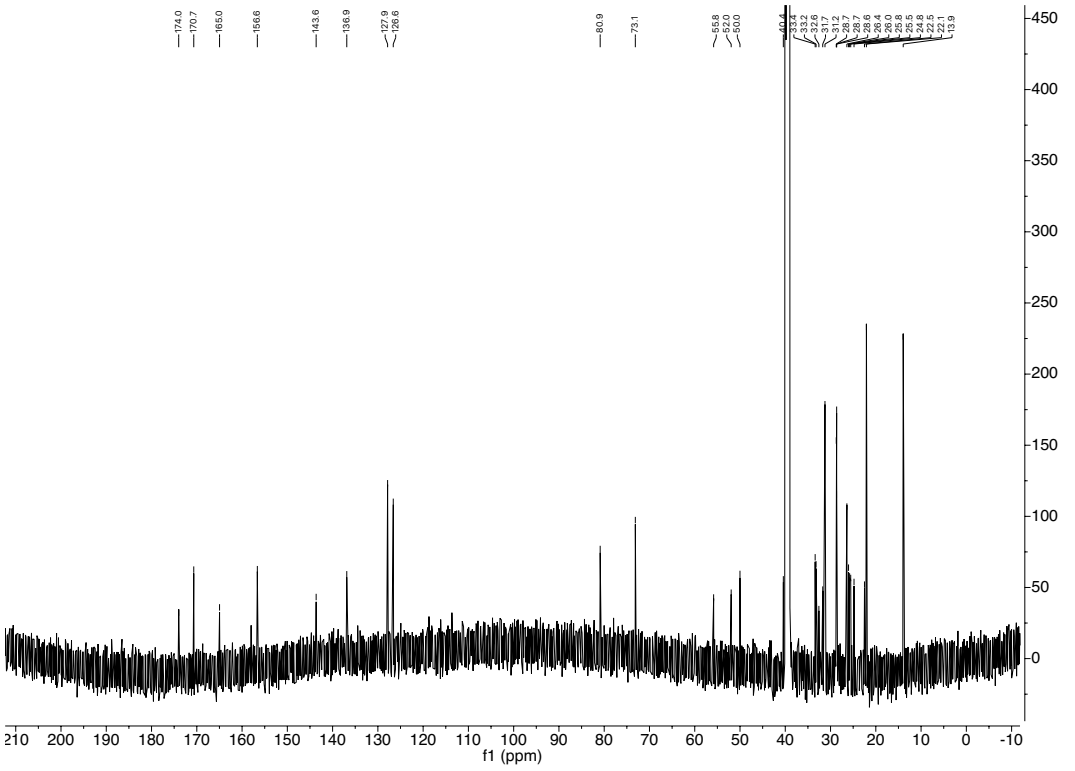
^{1}H and ^{13}C spectra of compound 5



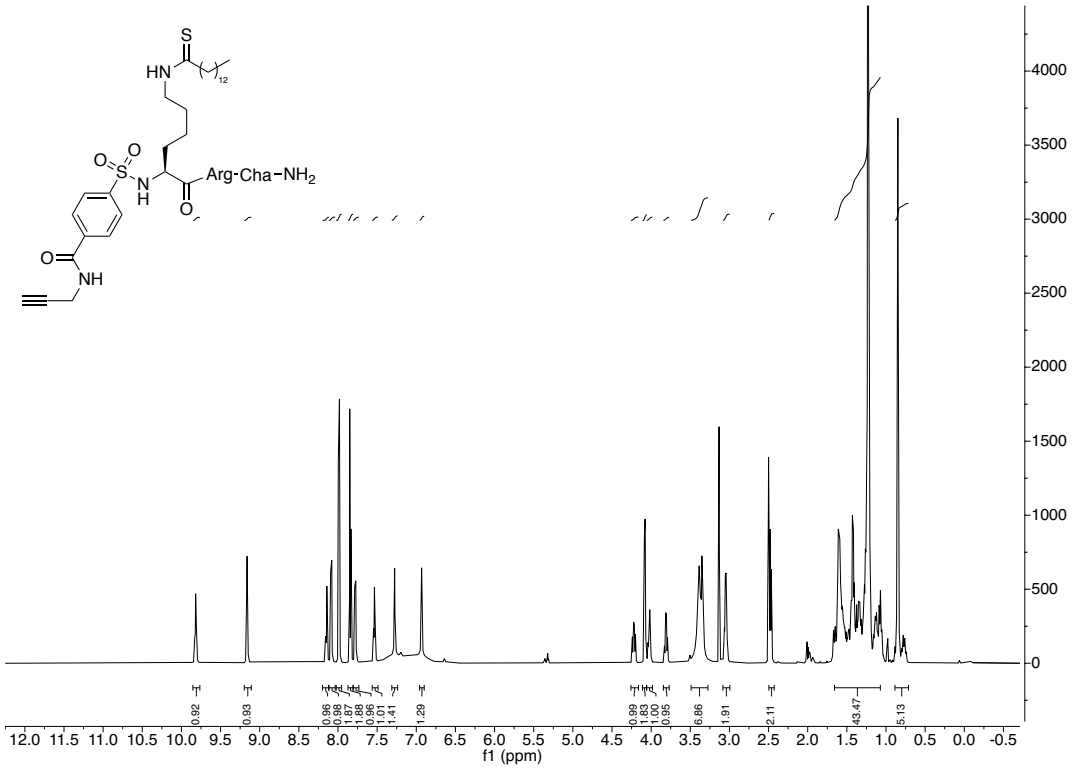


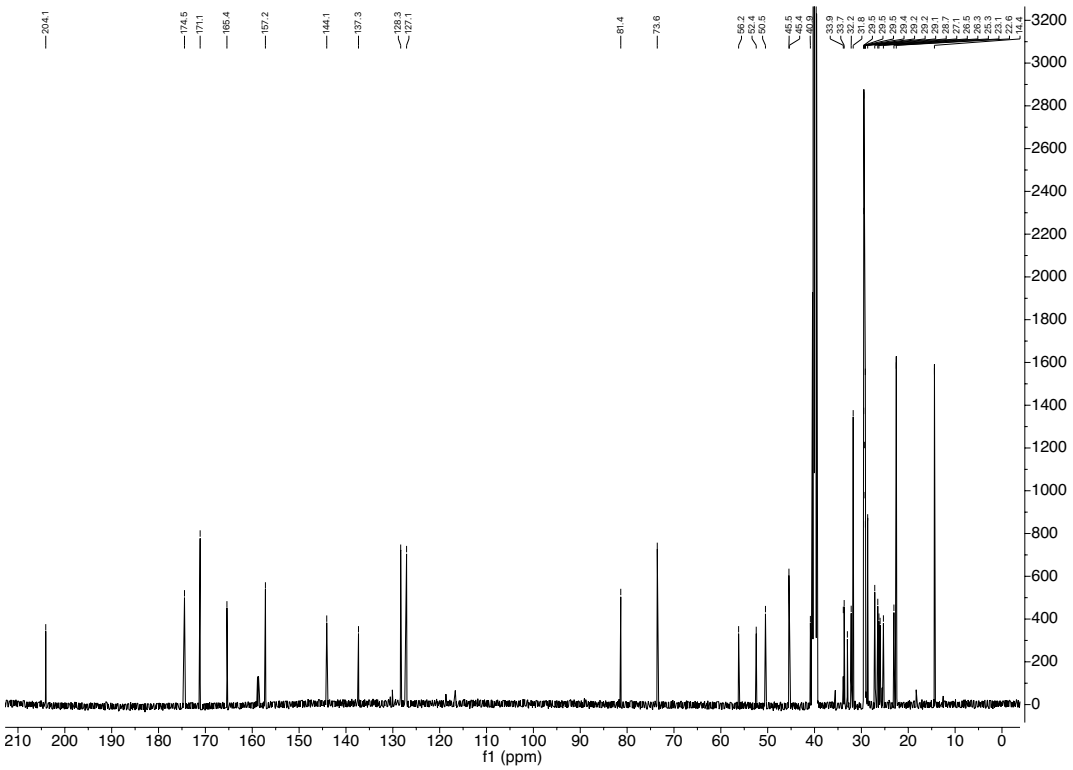
^{1}H and ^{13}C spectra of compound 6



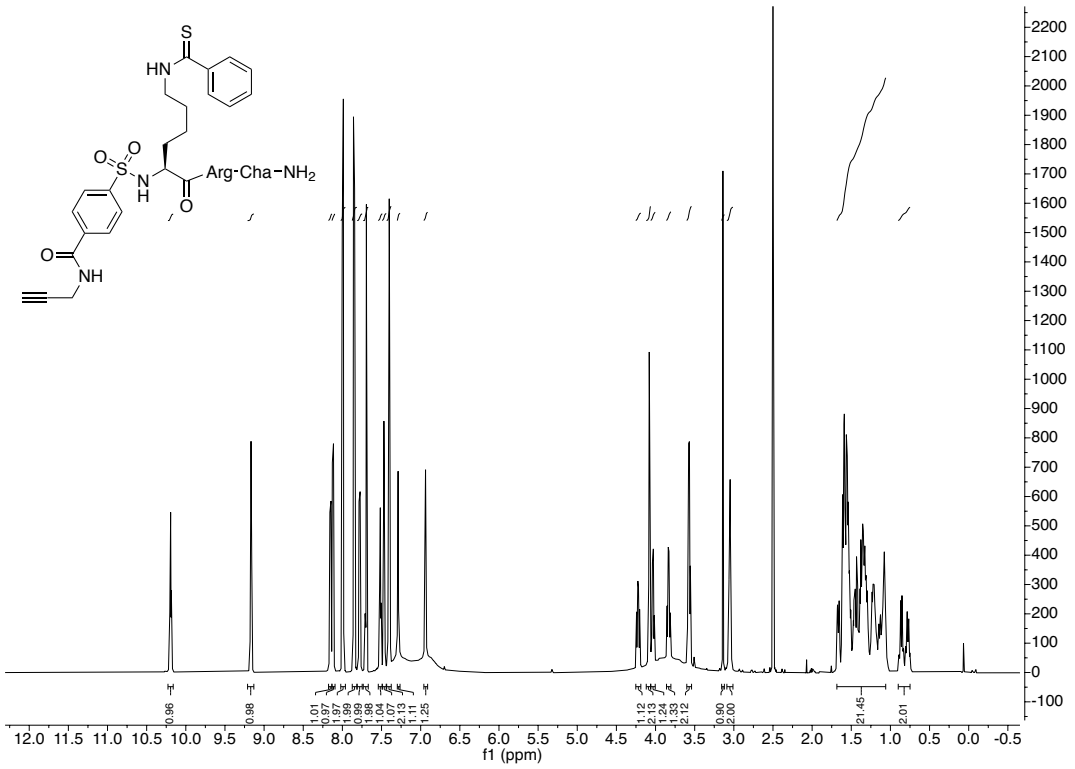


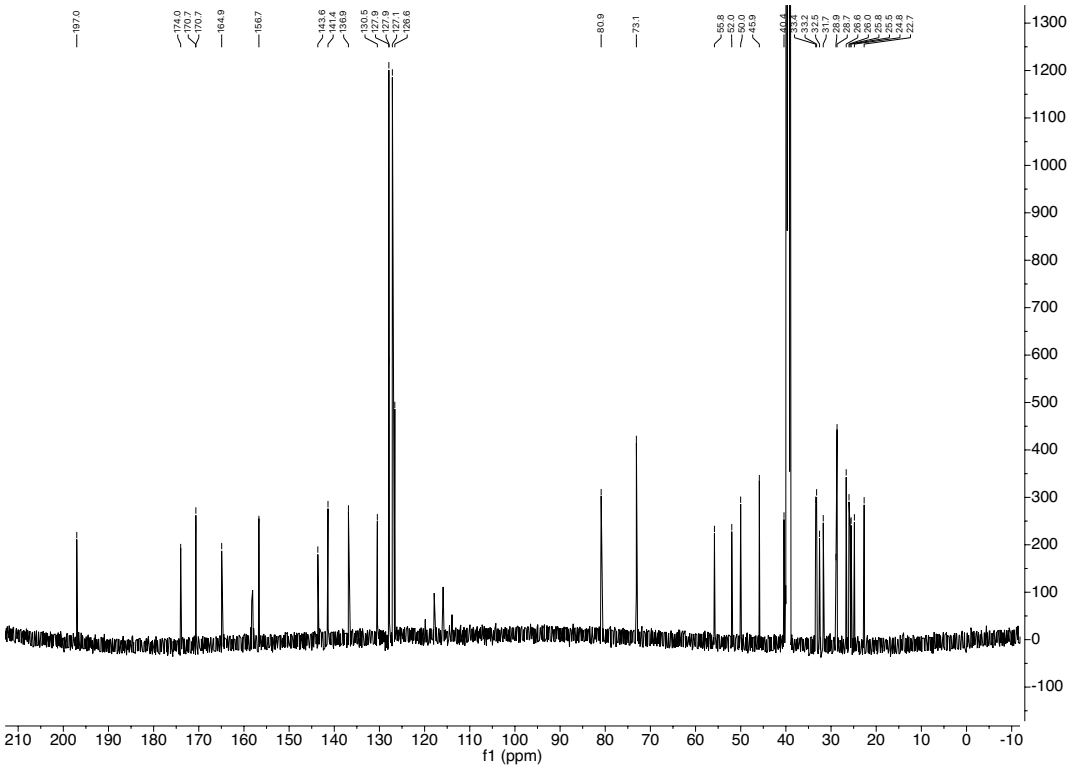
^{1}H and ^{13}C spectra of compound 7



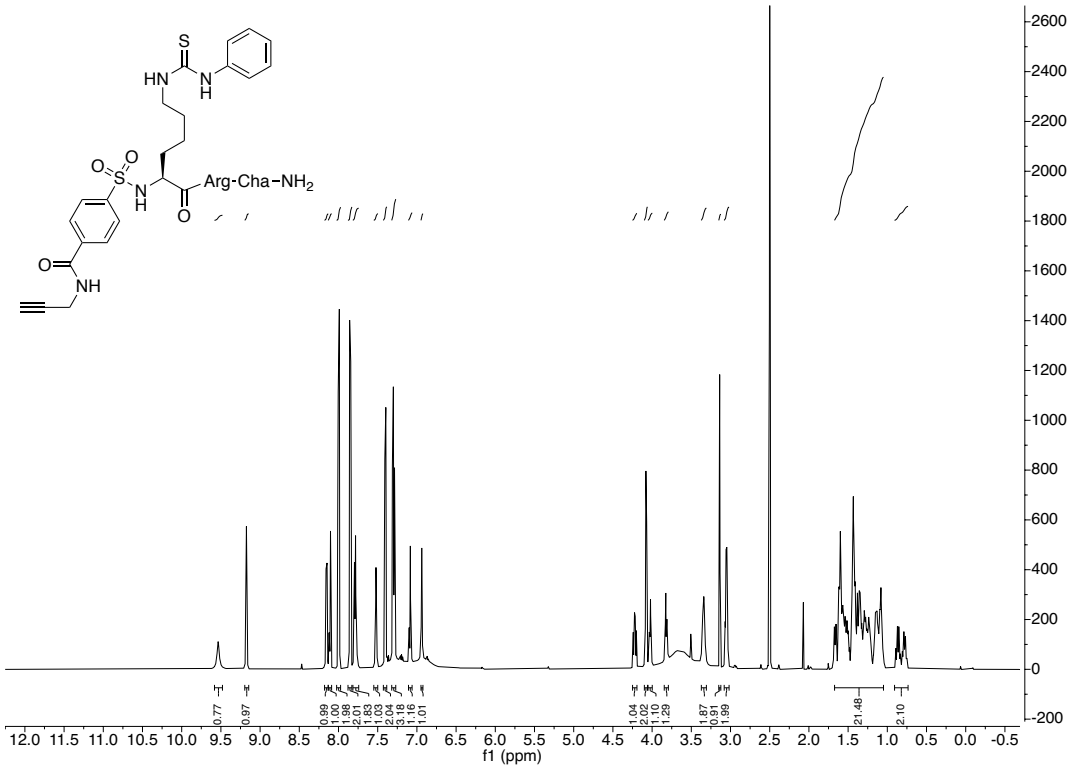


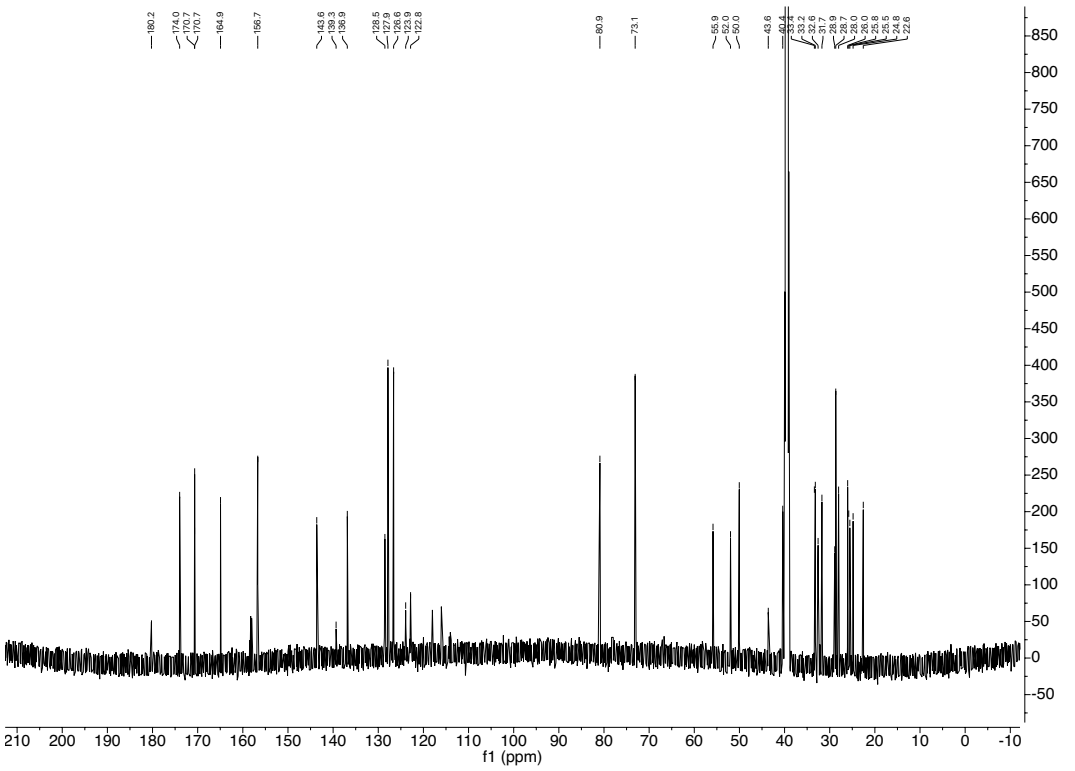
^{1}H and ^{13}C spectra of compound 8



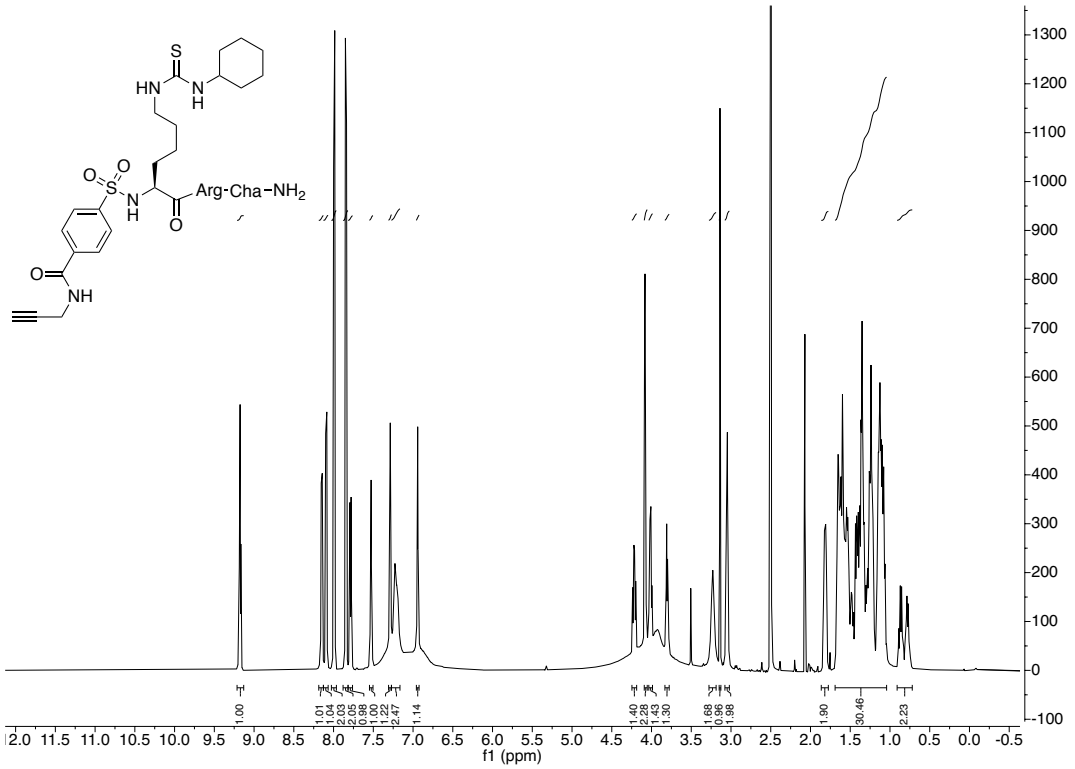


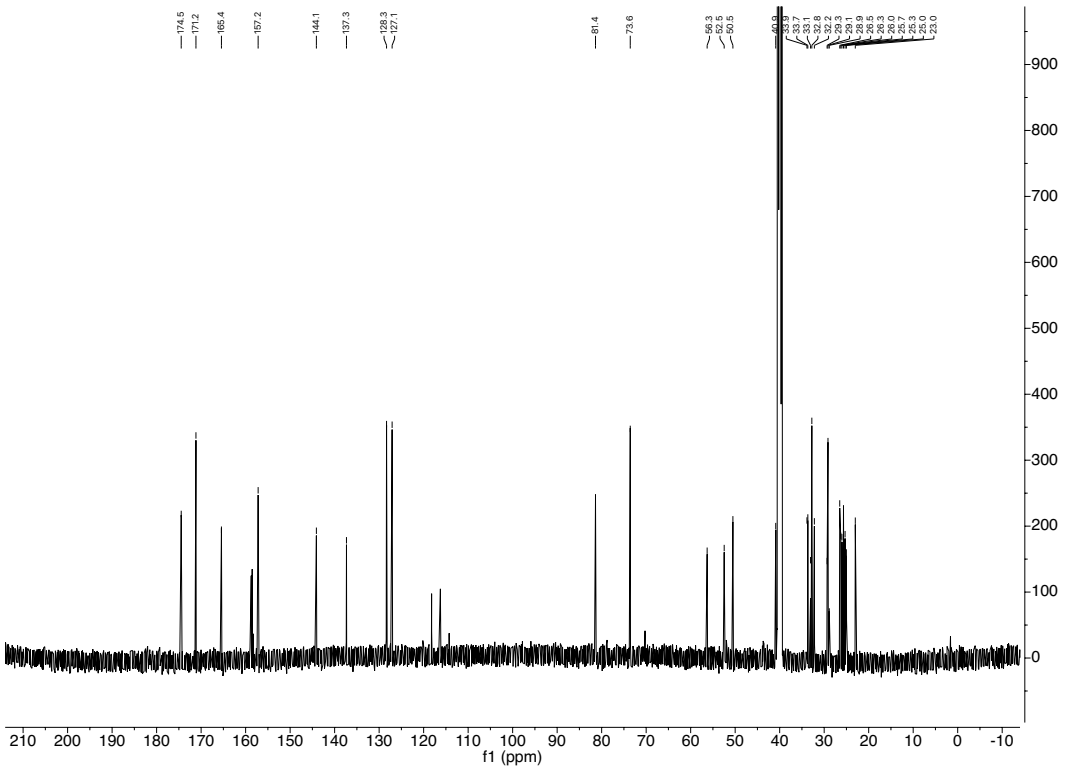
^{1}H and ^{13}C spectra of compound 9



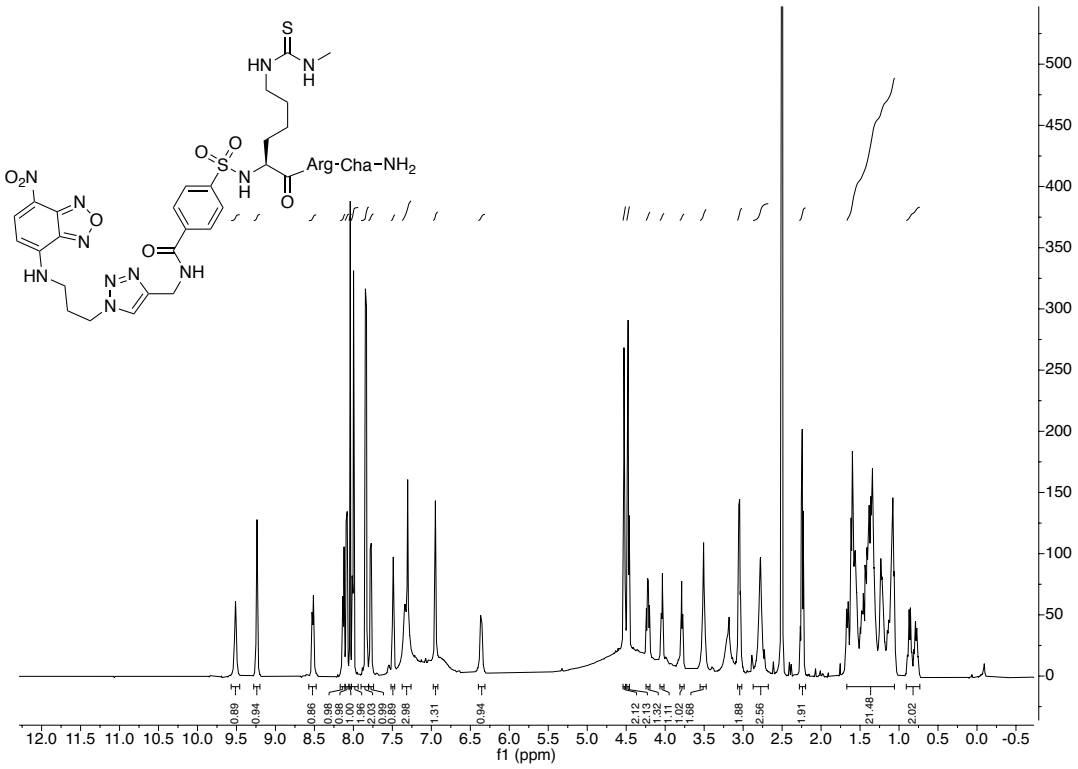


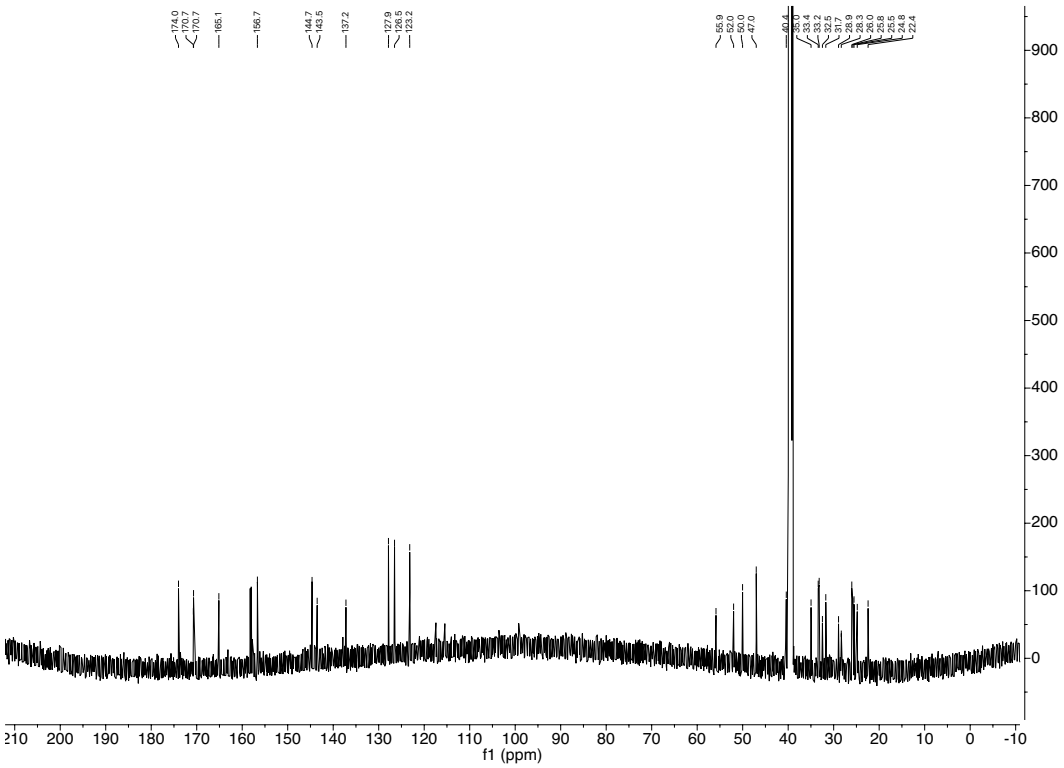
^{1}H and ^{13}C spectra of compound 10



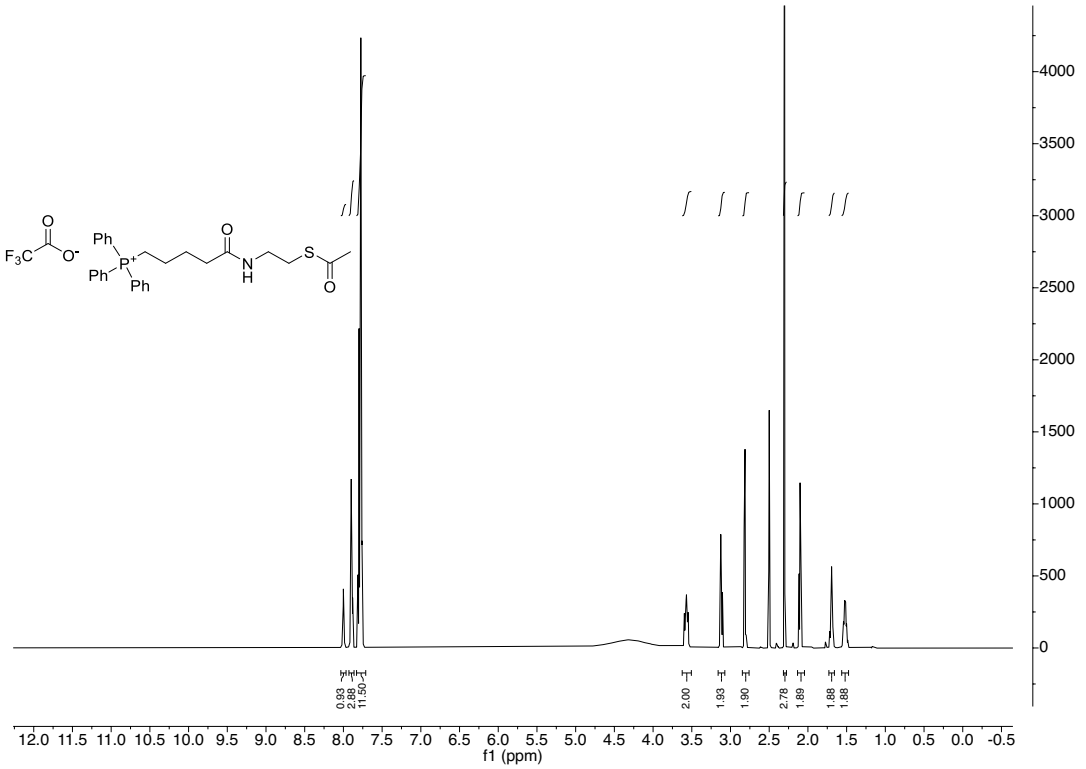


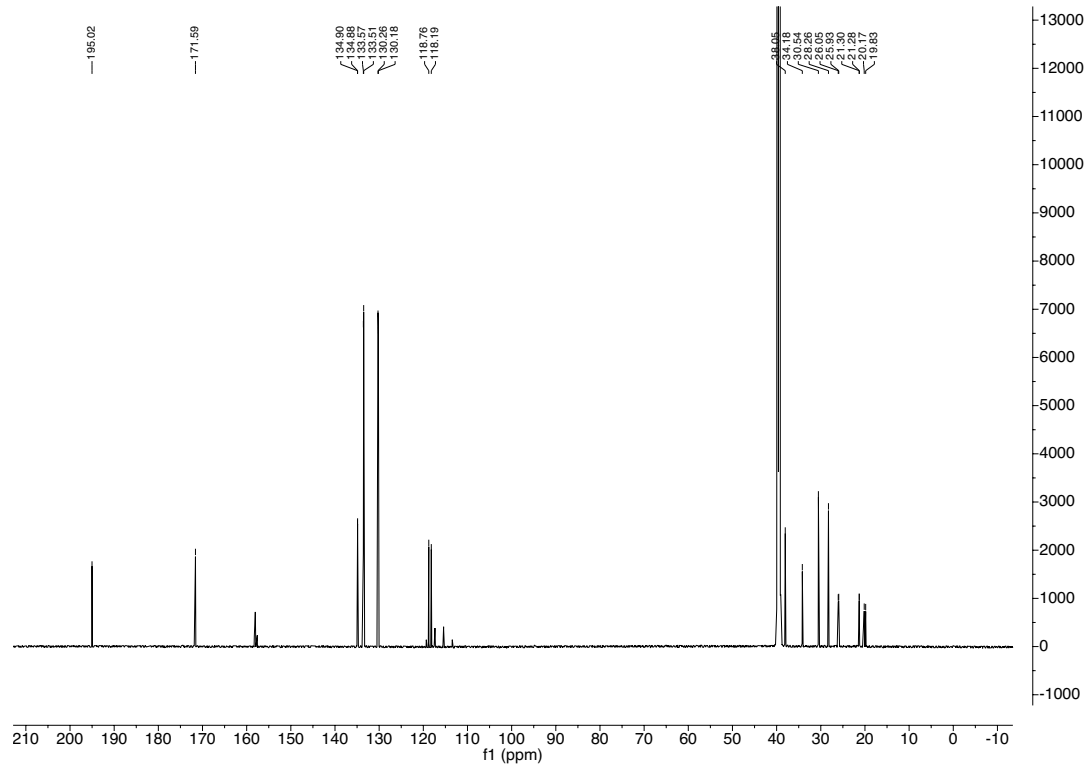
^{1}H and ^{13}C spectra of compound 11



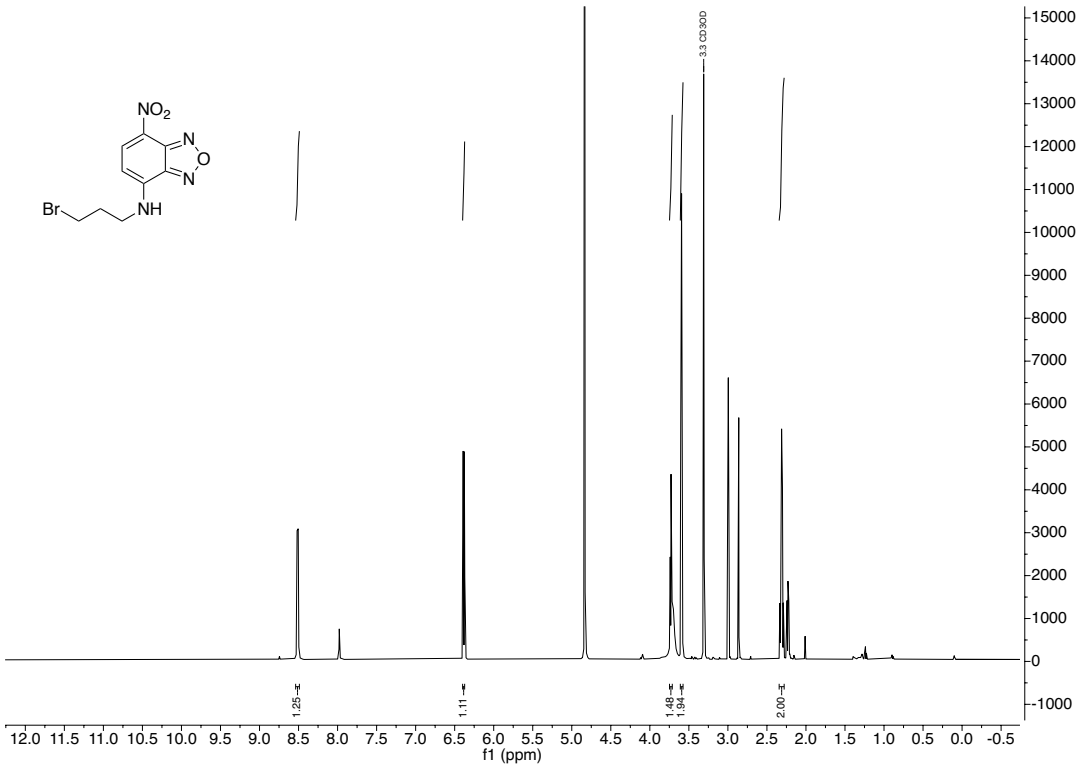


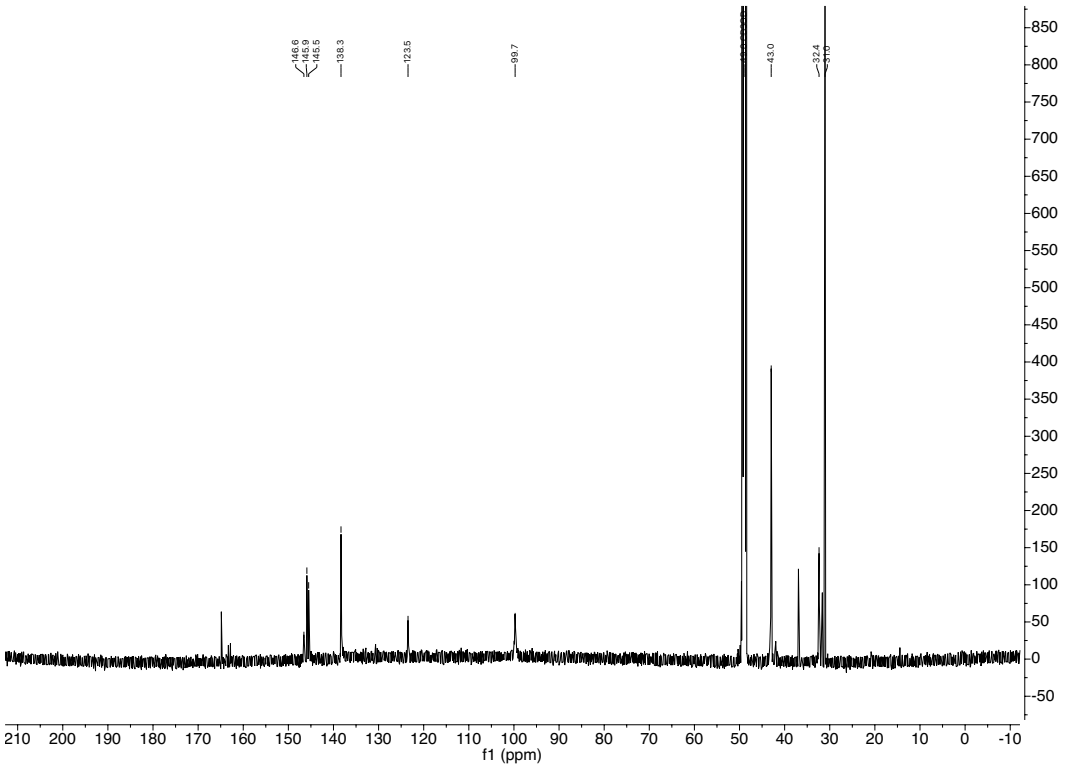
^{1}H and ^{13}C spectra of compound 18



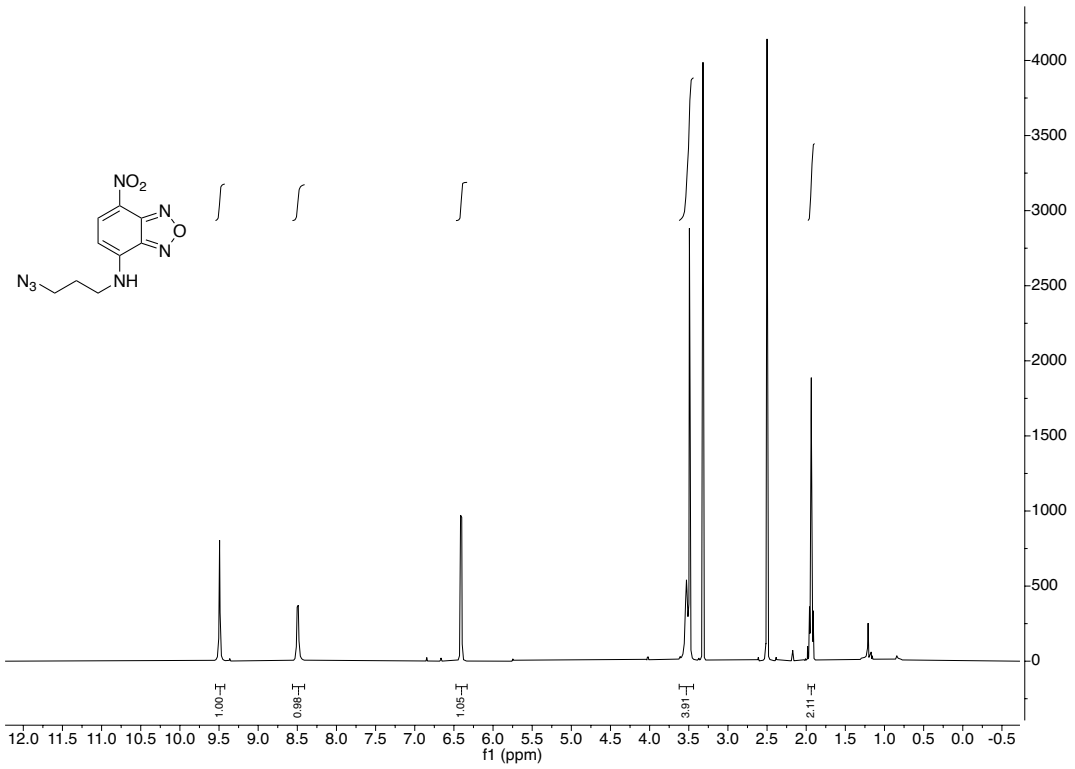


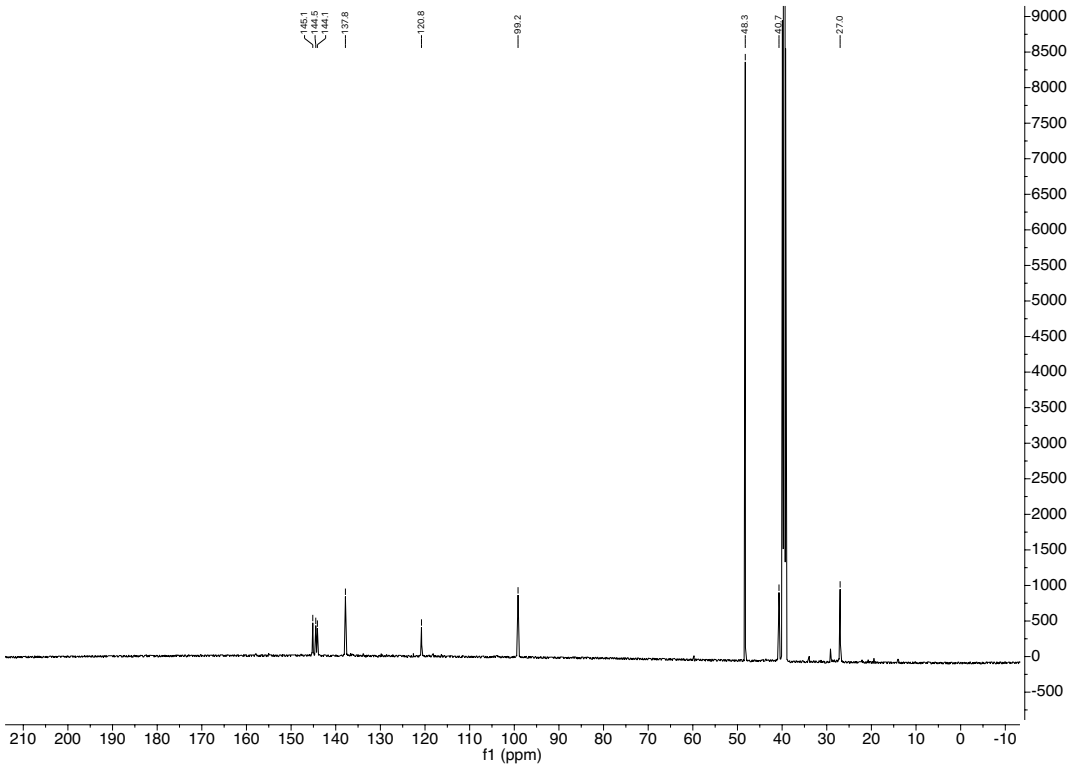
^{1}H and ^{13}C spectra of compound S16-intermediate



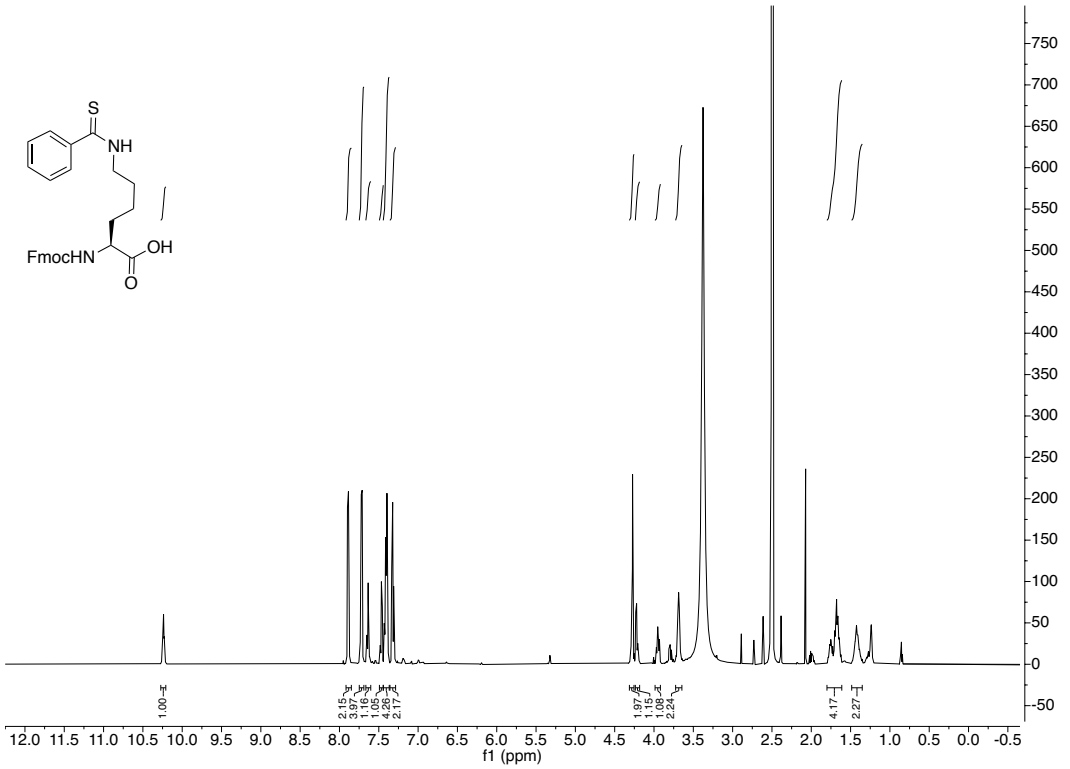


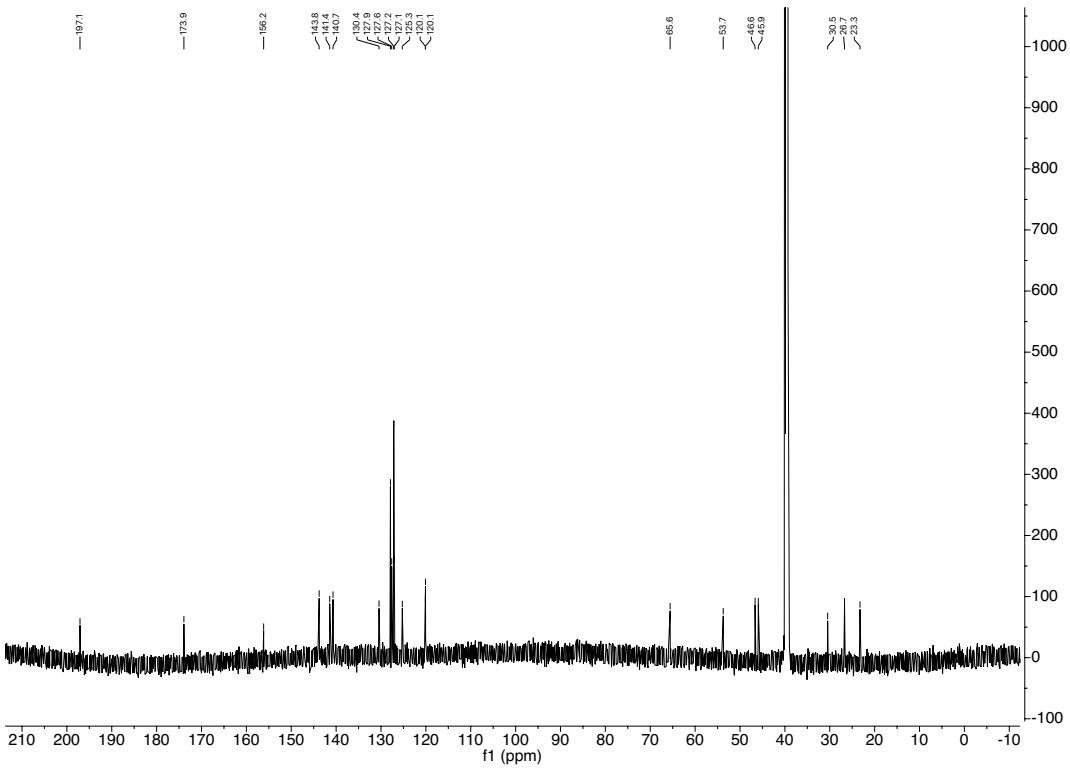
^{1}H and ^{13}C spectra of compound S16



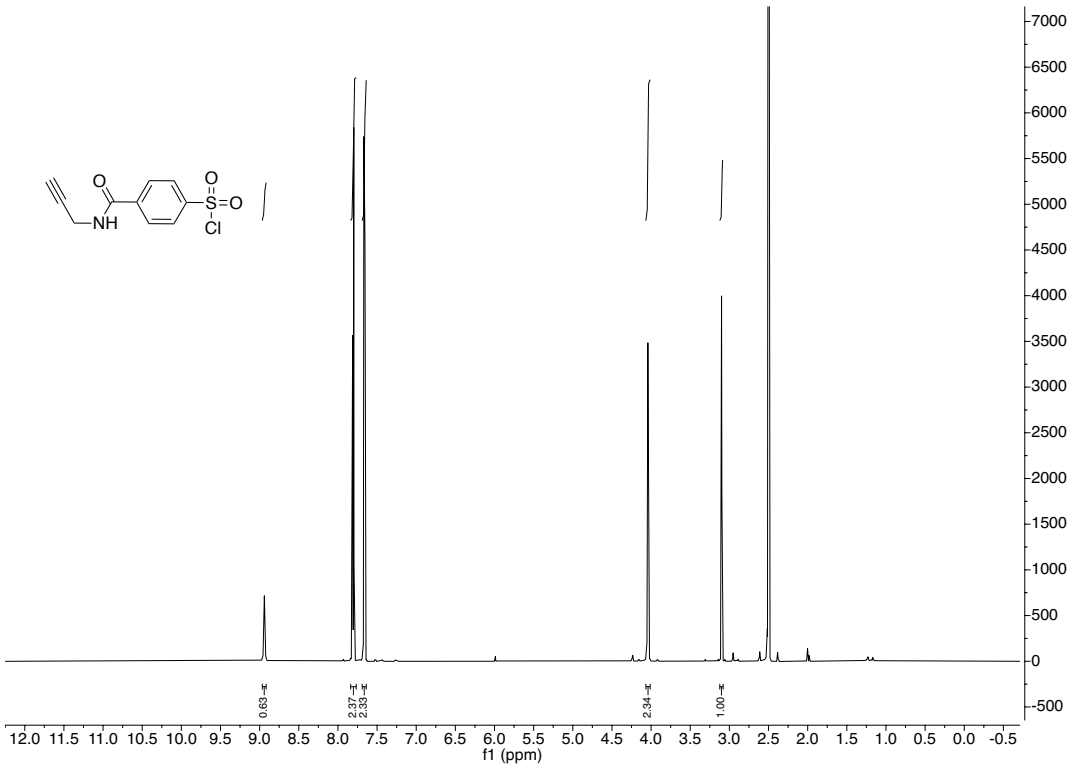


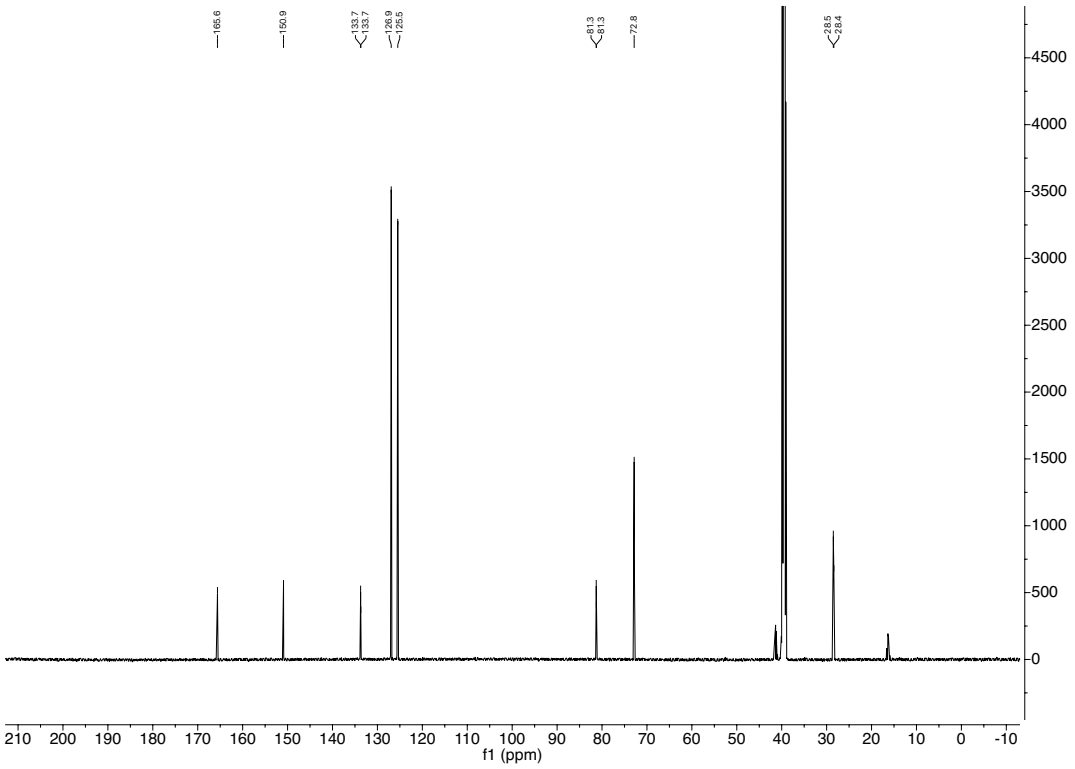
^{1}H and ^{13}C spectra of compound S17





^{1}H and ^{13}C spectra of compound S20





^{1}H and ^{13}C spectra of compound S21