SUPPORTING INFORMATION

Chemical proteomic profiling of human methyltransferases

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I. SUPPLEMENTARY TABLES AND FIGURES

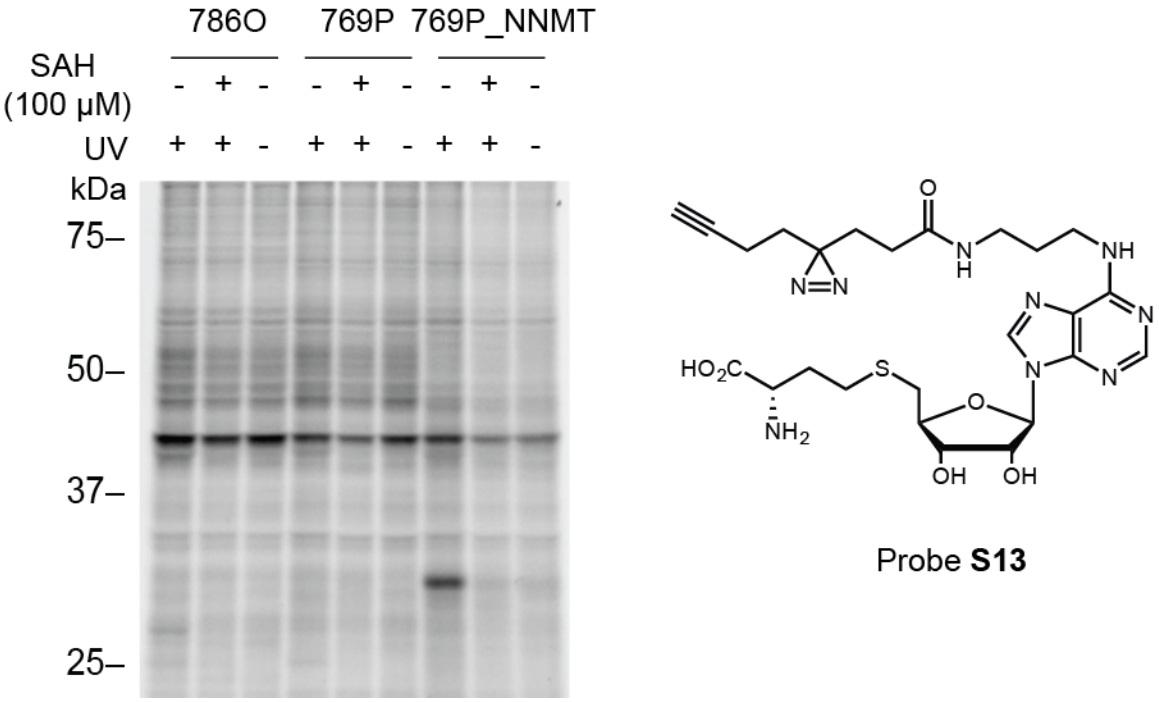


Figure S1. An alkyne SAH photoaffinity probe **S13** (25 μM) shows significant UV-independent reactivity with proteins in experiments performed with several cancer cell line lysates (1 mg protein/mL) under standard CuAAC conditions.

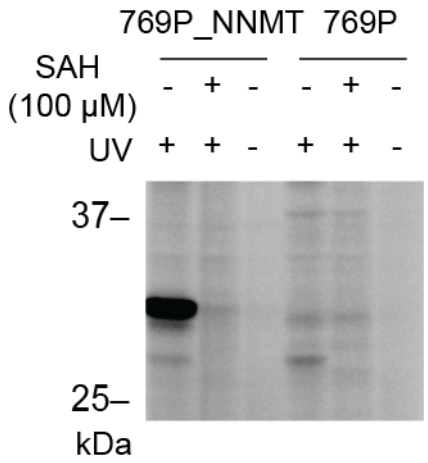


Figure S2. Probe **1** (25 μM) labels a ~30 kDa protein in lysates (1 μg protein/μL) from 769P cells engineered to express NNMT (29.6 kDa predicted MW).[1] Probe **1** labeling of this protein is UV-dependent and competed by excess SAH (100 μM).

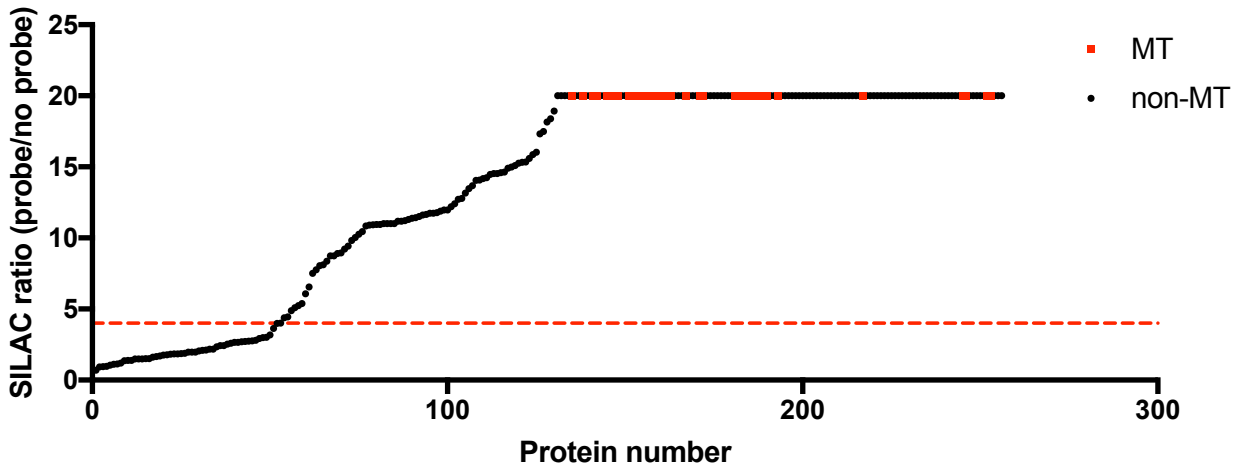


Figure S3. SILAC ratio plot for proteins identified in human cancer cell lysates treated with probe **2** (light: 25 μM) or without probe **2** (heavy) and then exposed to UV light. Results are average enrichment values (probe/no probe) for 4 aggregated experiments performed with 769P_NNMT cell lysates. MTs are marked with red dots. Dashed red lines designate four-fold changes in ratio values used to categorized proteins as enriched.

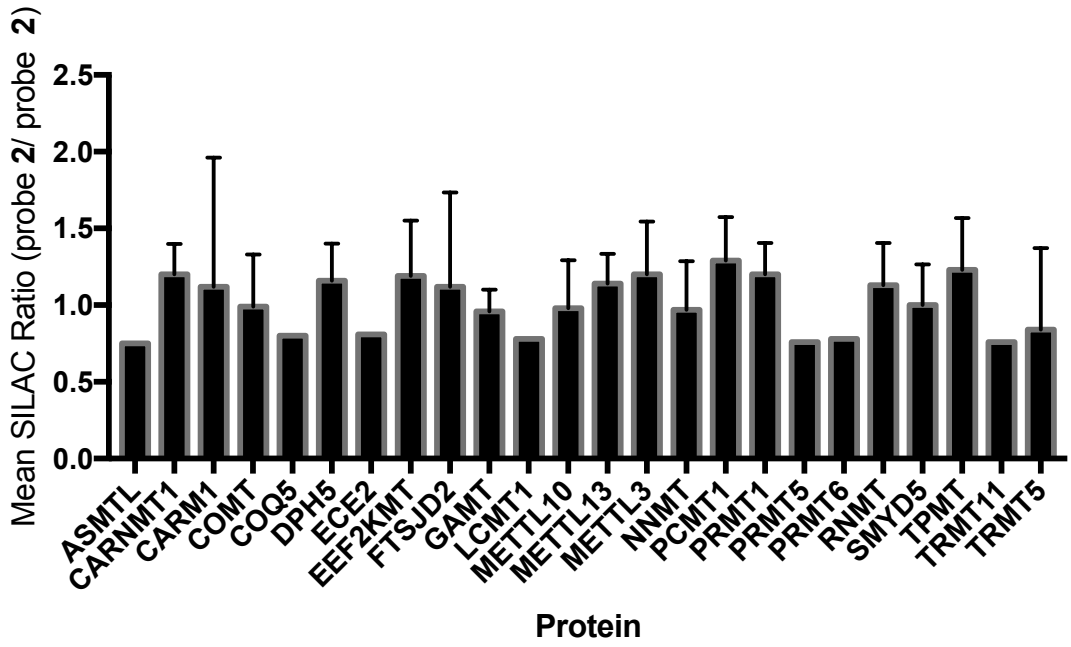
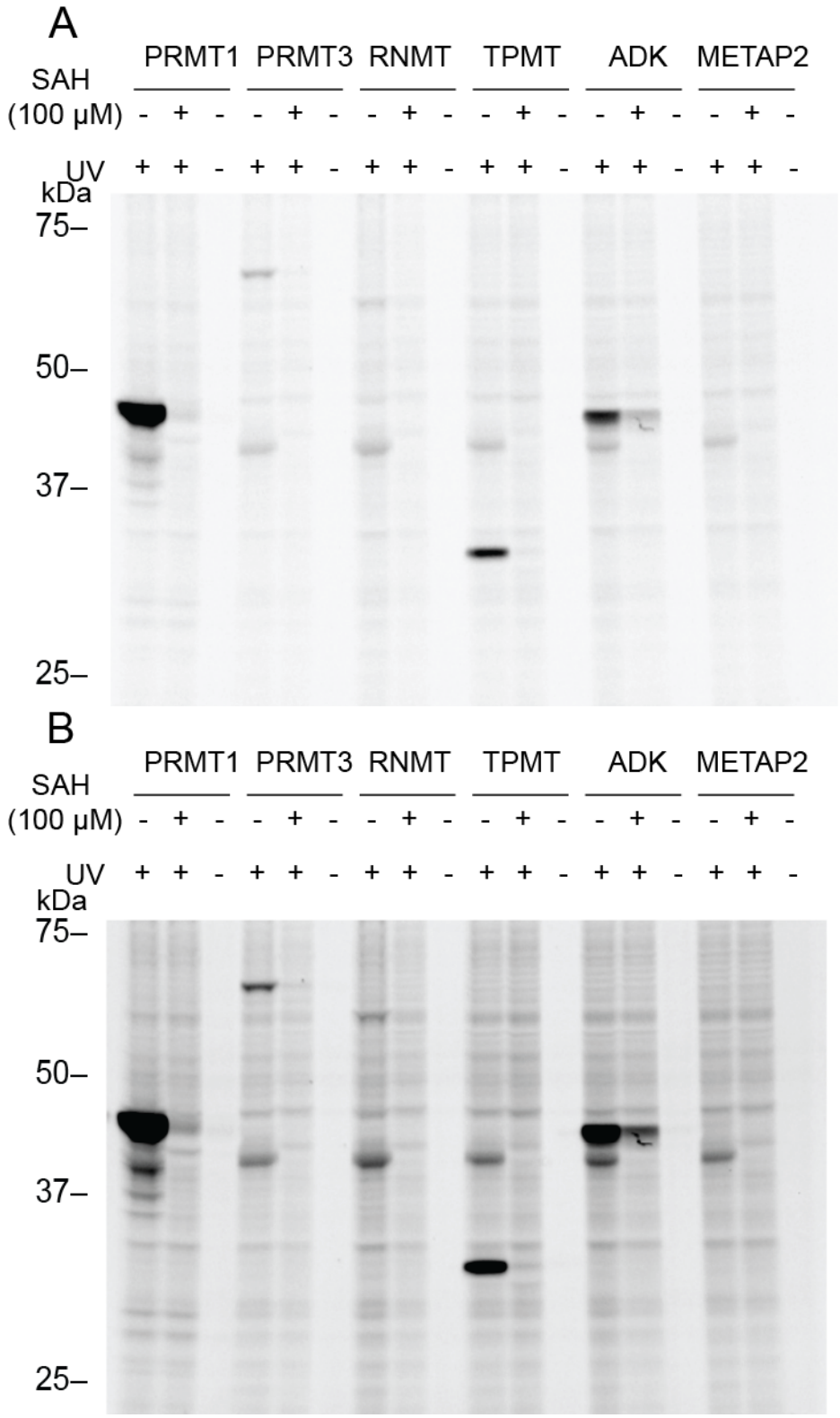
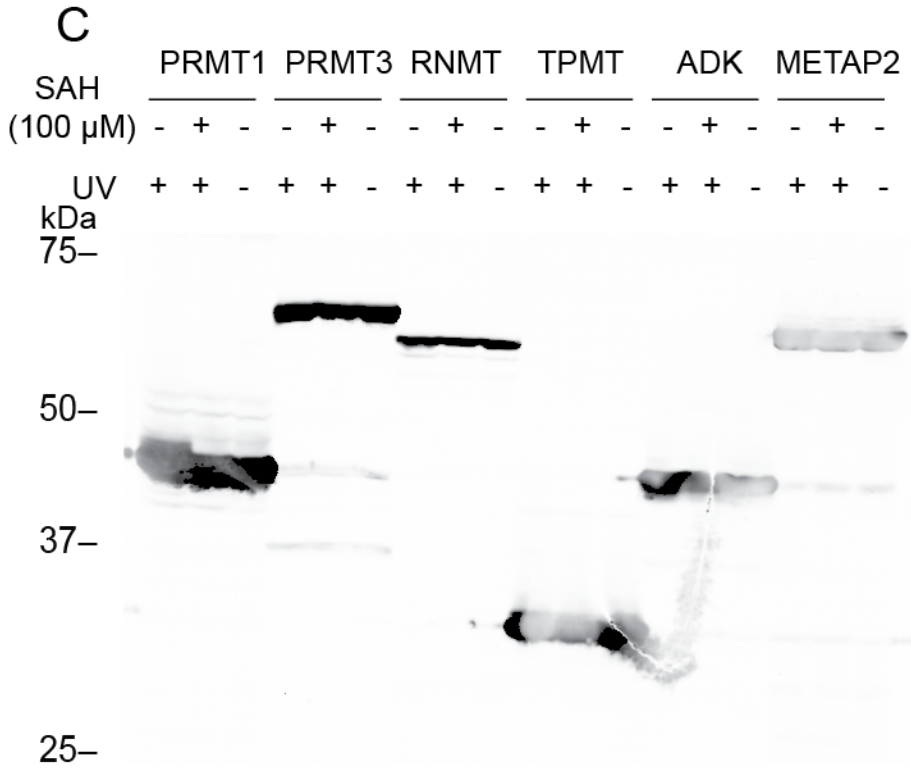


Figure S4. Representative results for control experiments where heavy and light-amino acid-labeled cell lysates were both treated with equal concentrations of probe **2** (25 μM), exposed to UV light, processed, and analyzed by quantitative MS. Results are average enrichment values (light/heavy) +/- SD for four experiments performed with 769P_NNMT cell lysates. Note that the quantified MT targets of probe **2** all show mean SILAC ratio values of ~1, as expected for equivalent enrichment in both heavy and light cell lysates.

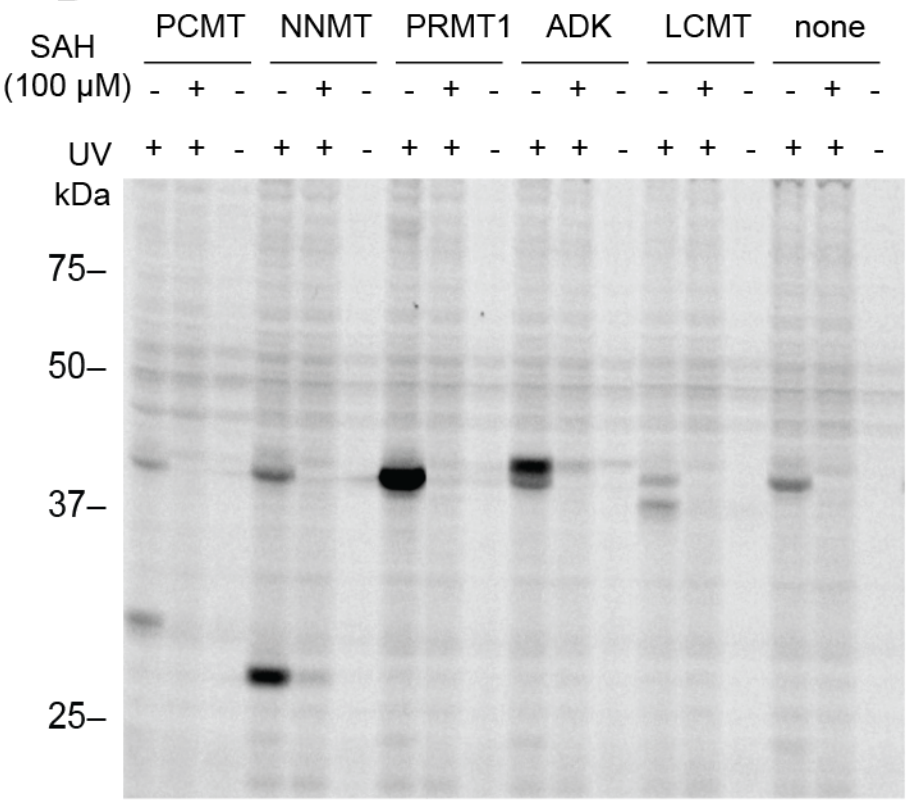
1
MASPGAGR**APPELPER**NCGYR**EVEYWDQRYQGAADSAPYDWFGDFSSFRALLEPELRPEDR**ILV
65
LGCGNSALSYELFLGGFPNVTSVDYSSVVVAAMQARHAHVPQLRWETMDVR**KLDFPSASFDVVL**
129
EKGTLDALLAGERDPWTVSSEGVHTVDQVLSEVGFQKGTRQLLGSRTQLELVLAGASLLLAALL
193
LGCLVALGVQYHRDPSHSTCLTEACIRVAGKILESLDRGVSPCEDFYQFSCGGWIRRNPLPDGR
257
SRWNTFNSLWDQNQAILKHLLENTTFNSSSEAEQKTQRFYLSCLQVERIEELGAQPLRDLIEKI
321
GGWNITGPWDQDNFMEVLKAVAGTYRATPFFTVYISADSKSSNSNVIQVDQSGLFLPSRDYYLN
385
RTANEKVLTAYLDYMEELGMLLGGRPTSTREQMQQVLELEIQLANITVPQDQRRDEEKIYHKMS
449
ISELQALAPSMDWLEFLSFLLSPLELSDSEPVVVYGMDYLQQVSELINRTEPSILNNYLIWNLV
513
QKTTSSLDRRFESAQEKLLETLYGTKKSCVPRWQTCISNTDDALGFALGSLFVKATFDRQSKEI
577
AEGMISEIRTAFEEALGQLVWMDEKTRQAAKEKADAIYDMIGFPDFILEPKELDDVYDGYEISE
641
DSFFQNMLNLYNFSAKVMADQLRKPPSRDQWSMTPQTVNAYYLPTKNEIVFPAGILQAPFYARN
705
HPKALNFGGIGVVMGHELTHAFDDQGREYDKEGNLRPWWQNESLAAFRNHTACMEEQYNQYQVN
769
GERLNGRQTLGENIADNGGLKAAYNAYKAWLRKHGEEQQLPAVGLTNHQLFFVGFAQVWCSVRT
833
PESSHEGLVTDPHSPARFRVLGTLSNSRDFLRHFGCPVGSPMNPGQLCEVW

Figure S5. Tryptic peptides from ECE2 identified by MS in probe **2**-treated lysates (highlighted in bold, red font, and underlined). Amino acids 1-160 are defined by Uniprot as the MT domain of ECE2 (http://www.uniprot.org/uniprot/O60344).


A
PRMT1
PRMT3
RNMT
TPMT
ADK
METAP2
SAH
(100 μM)
UV
kDa
75–
50–
37–
25–
B
PRMT1
PRMT3
RNMT
TPMT
ADK
METAP2
SAH
(100 μM)
UV
kDa
75–
50–
37–
25–


C
PRMT1
PRMT3
RNMT
TPMT
ADK
METAP2
SAH
(100 μM)
UV
kDa
75–
50–
37–
25–

D


PCMT
NNMT
PRMT1
ADK
LCMT
none
SAH
(100 μM)
UV
kDa
75–
50–
37–
25–

E

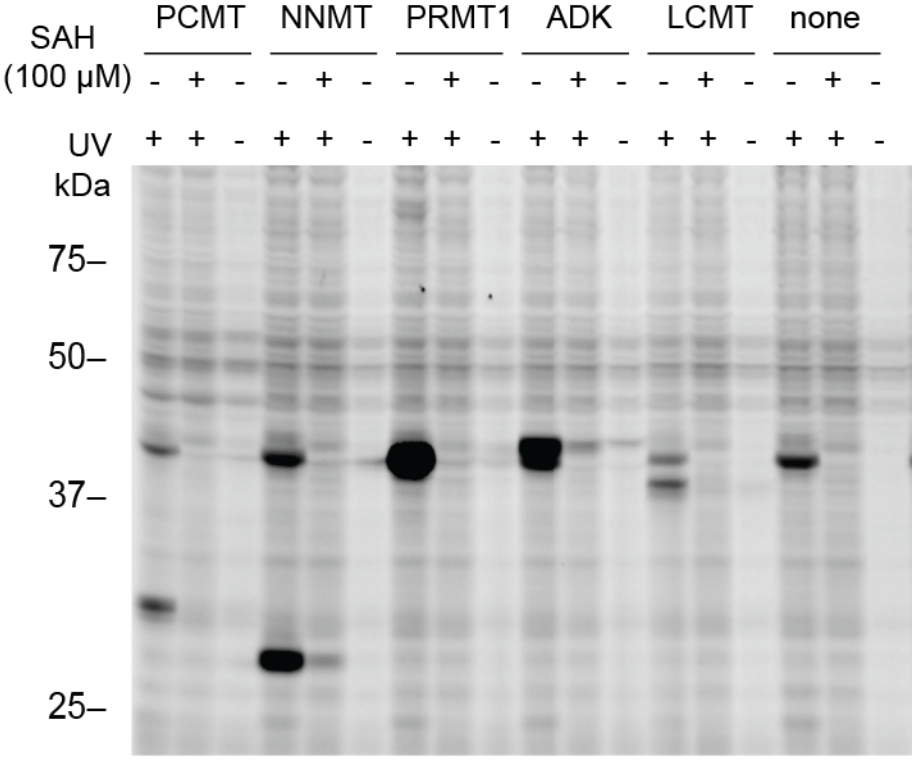

PCMT
NNMT
PRMT1
ADK
LCMT
none
SAH
(100 μM)
UV
kDa
75–
50–
37–
25–

Figure S6. Full length gels for data shown in Figure 4. (A, D) Low and (B, E) high intensity scans of full gels for probe **2**-labeling of proteins expressed by transient transfection in HEK293T cells (A, B, see panel C for anti-FLAG western blot) or overexpressed in bacterial systems and doped into HEK293T lysates (D, E). METAP2, a non-MT, was used a control protein for comparison to MT and other SAH-binding proteins.

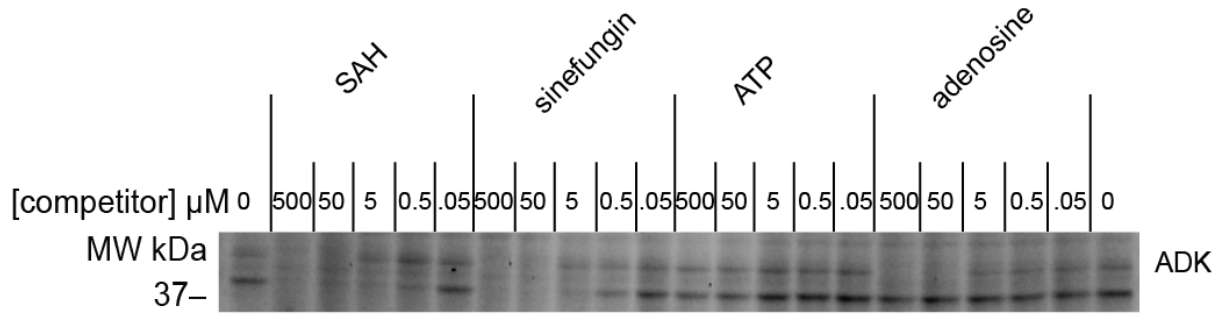


Figure S7. Effect of various small-molecule competitors on probe **1** labeling of adenosine kinase (ADK) in transfected HEK293T cell lysates. Cell lysates (2 mg/mL) were cotreated with various adenosyl-containing competitors (at the concentrations shown, in PBS) and probe **1** (10 μM). Probe **1**-labeled ADK migrates just above a distinct endogenous MT protein (that is also competed by SAH and sinefungin, but not ATP or adenosine).

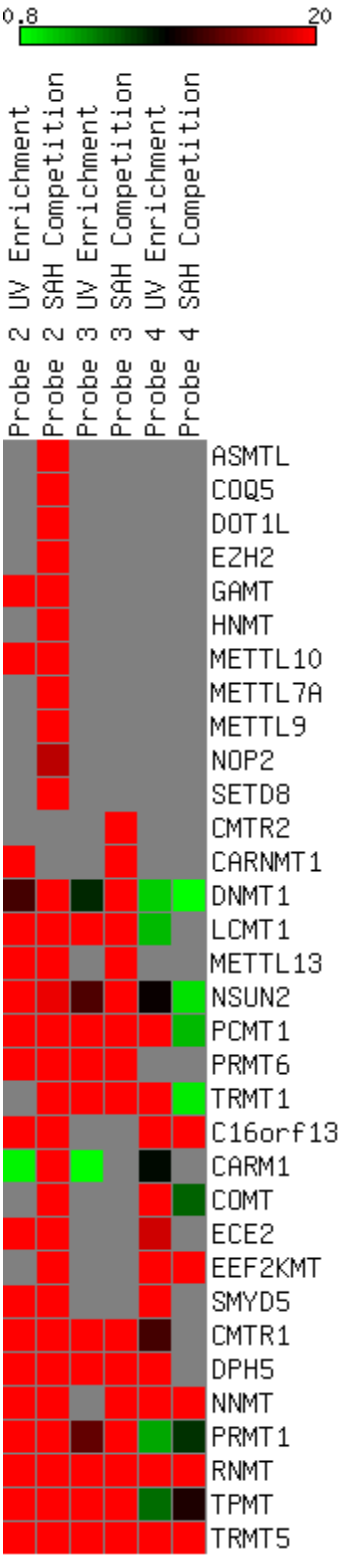


Figure S8. Heatmap representation of average SILAC ratios (+ UV/- UV or – SAH/+ SAH) across different experiment types and probes (n = 3-4 per group) for all MTs competed by at least one probe in K562 soluble lysates. Grey panels indicate MT was not detected in that set of experiments.

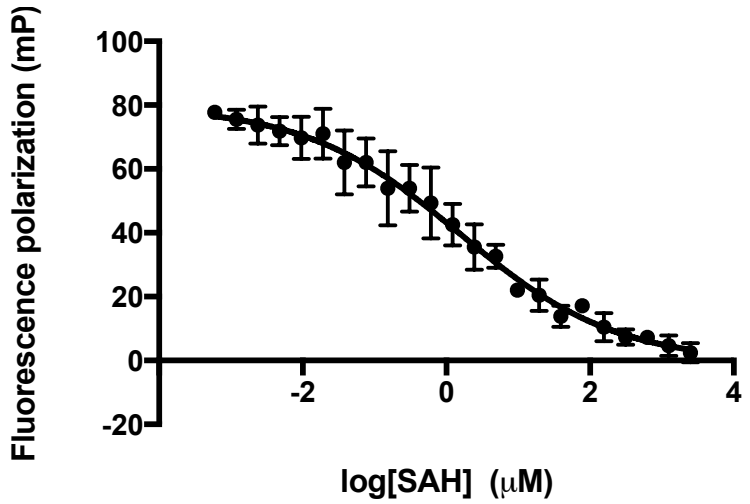


Figure S9. SAH competition of fluorescent polarization signal derived from probe **1** (1 μM) incubated with purified, recombinant human NNMT (6 μM). Data represent mean values +/- standard deviation; n = 4 per group.

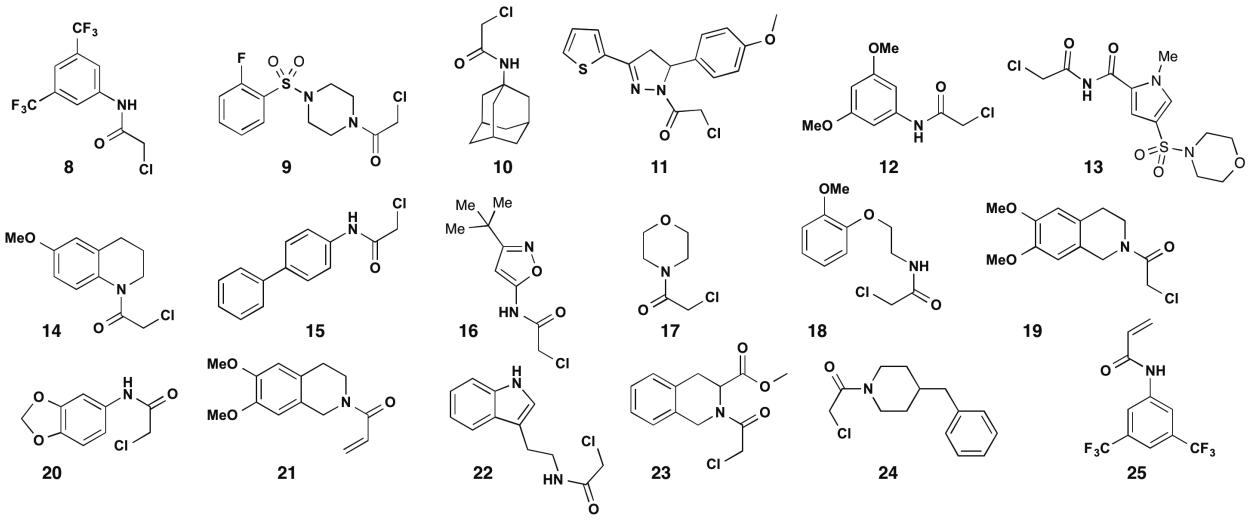


Figure S10. Electrophilic fragments screened in the competitive fluorescence polarization assay shown in **Figure 6C**.

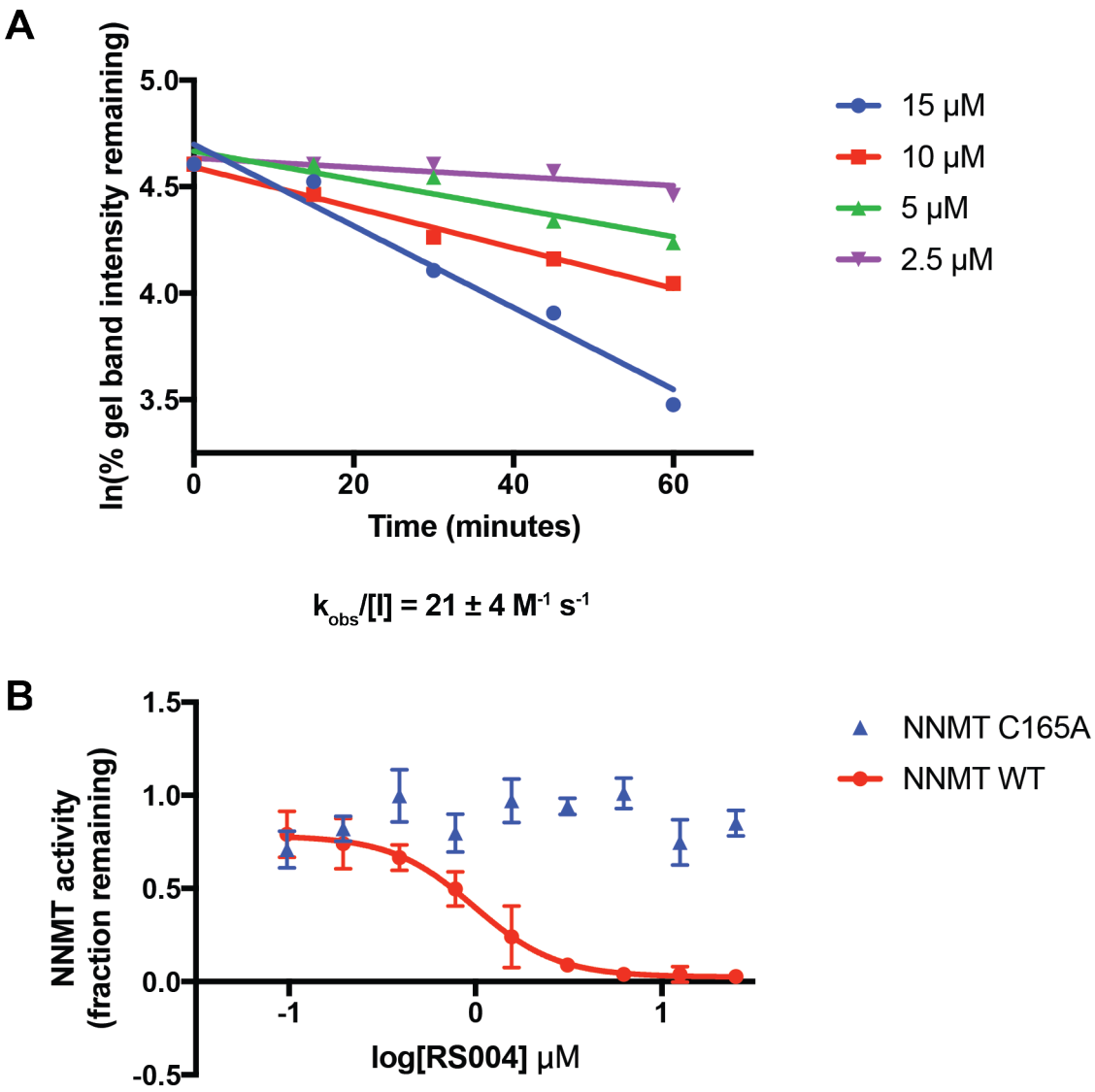


Figure S11. **(A** Time-dependent inhibition of NNMT by RS004 (**31**) with calculated $k_{obs}/[I]$ value as measured by competitive labeling with probe **2** following the indicated pre-treatment times with inhibitors. Calculated $k_{obs}/[I]$ value is expressed ± s. d.**)** (B) RS004 (**31**) inhibits NNMT activity in a concentration-dependent manner for WT but not C165A mutant NNMT as measured with a nicotinamide substrate assay. Data represent average values +/- standard deviation, n = 4 per group.

Supplementary Table S1. Complete proteomics data sets. See attached Excel file.

Tab 1 ('Probe Targets'). Mean SILAC ratios for high-confidence probe targets identified in Probe **2** treatments of 769P (+/- NNMT), 786O and K562 cell lysates, comparing heavy and light amino acid labeled cells.

Tab 2 ('UV Enriched'). Mean SILAC ratios for all proteins with ratios > 4 identified in UV enrichment experiments with Probe **2.**

Tab 3 ('No-Probe Enriched'). Mean SILAC ratios for all proteins with ratios > 4 identified in probe-vs-no-probe enrichment experiments with Probe **2**.

Tab 4 ('Competed'). Mean SILAC ratios for all proteins with ratios > 4 identified in SAH competition experiments with Probe **2**.

Tab 5 ('Enriched Not Competed'). Mean SILAC ratios for all proteins that showed UV enrichment ratios > 4 and SAH competition ratios < 4.

Tab 6 ('Medium Confidence Targets'). SILAC ratios for medium-confidence targets that were enriched or competed by Probe **2**.

Tab 7 ('Figure 5 Data'). Tabulated data used to generate Figure 5. Mean SILAC ratios for methyltransferases identified in UV enrichment and SAH competition studies from K562 soluble lysates from Probes **2**, **3** and **4**.

Tab 8 ('Probe 2 SAH Competition'). Median SILAC ratios and number of quantified peptides for individual datasets for all proteins identified in SAH competition experiments using Probe **2**.

Tab 9 ('Probe 2 UV Enrichment'). Median SILAC ratios and number of quantified peptides for individual datasets for all proteins identified in UV Enrichment experiments using Probe **2**.

Tab 10 ('Probe 2 No-Probe Enrichment'). Median SILAC ratios and number of quantified peptides for individual datasets for all proteins identified in No-probe enrichment experiments using Probe **2**.

Tab 11 ('Probe 3 SAH Competition'). Median SILAC ratios and number of quantified peptides for individual datasets for all proteins identified in SAH competition experiments using Probe **3**.

Tab 12 ('Probe 3 UV Enrichment'). Median SILAC ratios and number of quantified peptides for individual datasets for all proteins identified in UV Enrichment experiments using Probe **3**.

Tab 13 ('Probe 4 SAH Competition'). Median SILAC ratios and number of quantified peptides for individual datasets for all proteins identified in SAH competition experiments using Probe **4**.

Tab 14 ('Probe 4 UV Enrichment'). Median SILAC ratios and number of quantified peptides for individual datasets for all proteins identified in UV Enrichment experiments using Probe **14**.

Tab 15 ('Probe 2 Probe-vs-Probe'). Median SILAC ratios and number of quantified peptides for individual datasets for all proteins identified in probe-vs-probe experiments using Probe **2**.

Tab 16 ('Probe 2 RS004 competition'). Median SILAC rations and number of quantified peptides for individual datasets for all proteins identified in experiments comparing Probe **2** enrichment with and without RS004 pretreatment (25 μM for 30 minutes).

II. BIOLOGICAL METHODS

Materials

All materials were obtained from commercial suppliers and used without further purification. Probes **1**-**4** and inhibitors **26-31** were synthesized according to procedures outlined below. Fragment electrophiles **8-25** were produced according to Backus *et*. *al*.[2] UV-mediated crosslinking was performed in a Stratagene UV Stratalinker 1800 Crosslinker equipped with 365 nm light bulbs.

Cell culture

All cell lines were obtained from the ATCC. Low-passage human embryonic kidney (HEK293T) cells were maintained in DMEM (Mediatech), while human renal cancer (769P, 786O) and human chronic myelogenous leukemia (K562) cells were maintained in RPMI-1640 media (Mediatech), each supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 U/mL),

streptomycin (100 μg/mL), and L-glutamine (2mM), at 37 ^{O}C under a humidified 5% CO_2 atmosphere. For SILAC (Stable Isotope Labeling of Amino acids in Cell culture) experiments,[3,4] each cell line was passaged a minimum of six times in lysine- and arginine-free RPMI (Thermo) containing dialyzed FBS supplemented with penicillin, streptomycin and L-glutamine (concentrations as above) and either isotopically enriched L-[$^{13}C_6{}^{15}N_2$]-lysine hydrochloride and L-[$^{13}C_6{}^{15}N_4$]-arginine hydrochloride or natural abundance isotopologues (100 μg/mL each, Sigma). Heavy and light cells were maintained in parallel and cell aliquots were frozen after six passages in SILAC media and stored in liquid N_2 until required. Whenever thawed, cells were passaged at least three times before use in experiments.

Proteome preparation for gel- and MS-based analyses

Cells were harvested by scraping in cold DPBS (2 x 8 mL); cell pellets were isolated by centrifugation (1,400*g*, 3 min, 4 ºC), transferred to Eppendorf tubes in cold DPBS (2 x 0.8 mL), and pelleted again by centrifugation (1,400*g*, 3 min, 4 ºC). Cold DPBS (100-1000 μL) was added to the cell pellets and the cells were lysed by probe sonication using a Branson Sonifier probe sonicator (6-7 pulses, 50% duty cycle, output setting = 3). When analyzing soluble and membrane components separately, cell lysates were then centrifuged (100,000*g*, 45 min, 4 ºC) to provide soluble (supernatant) and particulate (pellet) fractions. Protein concentrations were determined using the DC protein assay (Bio-Rad) and a microplate reader (Tecan, Infinite F500) against a bovine serum albumin standard.

MS-based analysis of crosslinked proteins

Cell lysates (prepared as described above), in 0.4 mL in DPBS diluted to 1.5-2.0 mg/mL (equivalent concentrations and volumes for both isotopically heavy and light proteomes), were treated with 50 μL of 5 mM SAH (giving 500 μM final, for competition experiments) or 50 μL DPBS (for competition control or enrichment) followed by 50 μL 250 μM probe (or DPBS for probe/no probe experiments), mixed, and incubated for 1-10 minutes. Samples (with caps open to allow light to penetrate) were photocrosslinked for 10 min. at 4 ^{O}C. Heavy and light samples were then combined into methanol (2 mL) and chloroform (0.5 mL), vortexed, then DPBS (1 mL) was added and the resulting cloudy mixture was vortexed and centrifuged (5,000*g*, 15 min, 4 ºC). The organic and aqueous layers were aspirated leaving a protein disc that had formed between the layers. The disc was washed with cold 1:1 methanol:chloroform (3 x 1 mL) and then suspended in 2 mL methanol by sonication. Chloroform (0.5 mL) was then added, the

proteins were pelleted via centrifugation (5,000*g*, 10 min, 4 ºC), and the organic layer was removed via aspiration. The remaining pellet was dissolved in a freshly prepared solution of urea (500 µL, 6M in DPBS). A mixture of TCEP (100 mM) and K_2CO_3 (300 mM) was added (50 µL), and the solution was incubated at 37 ^{O}C with shaking for 30 min. Samples were then treated with iodoacetamide (70 µL, 400 mM) and incubated in the dark for 30 min at room temperature. Proteomes were diluted with 140 µL SDS (10% in DPBS) followed by 5.5 mL DPBS, then treated with DPBS-washed avidin A9207-5mL or Pierce 20349 streptavidin beads (100 µL, 1:1, Aldrich [avidin] or Pierce [streptavidin]) and rotated at ambient temperature for 90 min. The beads were then pelleted by centrifugation (1,400*g*, <1 min) and sequentially washed with 0.25% SDS in DPBS (3 x 10 mL), DPBS (3 x 10 mL) and ddH_2O (3 x 10 mL). The beads were then transferred to a Protein LoBind tube (Eppendorf) and the immobilized proteins were digested by addition of urea (200 µL, 2.0M in DPBS), $CaCl_2$ (2.0 µL, 100 mM in H_2O) and sequencing grade modified porcine trypsin (2 µg; Promega). After digesting overnight at 37 ^{O}C with shaking, the beads and supernatant were filtered through a Micro Bio-Spin column (Bio-Rad) into a fresh LoBind tube, acidified with formic acid (16 µL) and stored at -20 ^{O}C until analysis by LC/LC-MS/MS.

Proteomic samples were analyzed using a Thermo Orbitrap Velos mass spectrometer according to previously described methods.[5] Peptides from on-bead tryptic digests were pressure loaded onto a 250 µm (inner diameter) fused silica capillary column packed with 4 cm C18 resin (5 µm, Phenomenex). Peptides were then eluted onto a 100 µm (inner diameter) fused silica capillary column packed with 3 cm strong cation exchange (SCX) resin followed by 10 cm C18 resin. Chromatographic separation of the peptide mixture was achieved using a 5-step multidimensional LC-MS (MuDPIT)[6] protocol consisting of 0%, 25%, 50%, 80% and 100% salt bumps of NH_4OAc (500 mM) followed by an increasing gradient of CH_3CN (containing 0.1% formic acid) in H_2O. Peptides were analyzed in data-dependent acquisition mode where one MS1 microscan [400-1800 mass to charge ratio (m/z)] was followed by 30 data-dependent fragmentation (MS2) scans. Dynamic exclusion (repeat count of 1, exclusion duration of 20 s) and a monoisotopic precursor selection were enabled, whereas all other parameters were left at default values.

Peptide identification

MS2 spectra were extracted from raw data files using RAW Xtractor (version 1.9.9.2; 2004 release; publically available at http://fields.scripps.edu/yates/wp/?page_id=17). Each .ms2 file

was searched using the ProLuCID algorithm against a reverse-concatenated, nonredundant database of the human proteome (UniProt release –11/05/2012) and filtered using DTASelect 2.0 within the Integrated Proteomics Pipeline (IP2) software. The precursor-ion mass tolerance was set to 50 ppm and the fragment-ion mass tolerance was the default assignment of 0. Cysteine residues were specified with a static modification (an invariable mass shift) for carbamidomethylation (+57.0215 Da) and one methionine residue per peptide (if found) was allowed variable oxidation (+15.9949 Da). Heavy peptides were searched separately from light peptides with static modifications on lysine (+8.0142) and arginine (+10.0082). Matched MS2 spectra from ProLuCID searches were assembled by protein and filtered using DTASelect (version 2.0.47) which allowed only half-tryptic or fully-tryptic peptides for identification and downstream quantification. Peptides were restricted to a specified false positive rate of 1%.[7] Redundant peptide identifications, if common between multiple proteins, were allowed, as database entries were limited to a single consensus splice variant. SILAC ratios were determined using in-house software (CIMAGE).[5] Briefly, MS1 ion chromatograms (±10 ppm) from 'light' and 'heavy' target peptide masses (m/z) were generated using a retention time window (±10 min) centered at the time the peptide ion was selected for MS/MS fragmentation, and subsequently identified. The ratio of 'light' and 'heavy' peptide peak areas were then calculated. To ensure the correct peak-pair is used for quantification, CIMAGE applies a co-elution correlation score filter ($R^2 \geq 0.8$) for heavy and light peptide peaks to exclude target peptides with bad co-elution profiles. Furthermore, an 'envelope correlation score' filter is applied to ensure the experimentally observed high-resolution MS1 spectrum matches ($R^2 > 0.8$) the predicted isotopic distribution. Peptides detected as singletons, where only the heavy or light isotopically labeled peptide was detected and sequenced, but which passed all other filters described above, were given a standard ratio of 20, which is the maximum SILAC ratio reported here.

Categorization of SAM-dependent methyltransferases

A UniProt search for E.C.: 2.1.1.* and organism: Homo sapiens (Human) [9606] was performed, and the resulting list was manually parsed to remove non-SAM-dependent MTs (e.g. TRMT112, MTR) that were found in proteomics datasets. The list was then cross-references against Petrossian *et. al.*[8] to generate a final list of UniProt IDs used to categorize proteins as methyltransferases for the purposes of this study.

Transient protein expression in HEK293T cells

Full-length cDNAs encoding for each protein of interest were subcloned into FLAG-tagged (N-terminal) pRK5 vectors (Addgene). HEK293T cells were grown to ~70% confluence under standard growth conditions before adding the appropriate cDNA and polyethyleneimine (PEI) 'MAX' (MW 40,000, Polysciences Inc.) as a transfection reagent under standard transfection conditions [3:1 vector/PEI (w/w) ratio]. Cells were incubated for ~48h before harvesting and labeling (as described above).

Recombinant ADK, PCMT and PRMT1 expression and purification

Full-length human ADK was subcloned into pET45b(+) vector (Novagen) (N-terminal His-tagged). PCMT in pET30b(+) was provided by the Clarke laboratory. Following transformation of *Escherichia coli* strain BL21(DE3)star with appropriate vectors, a single colony was isolated and grown in Terrific Broth containing carbenicillin (100 mg/L) at 37 ºC to an OD_{600} of 0.5. Protein expression was induced with IPTG (1.0 mM) and the cells were incubated at 18 ^{O}C with shaking overnight. After centrifugation, cell pellets were resuspended in cold buffer containing HEPES (0.05M), NaCl (0.03 M), imidazole (5.0 mM), DNase (0.1 mg/mL), $MgCl_2$ (1.0 mM), $CaCl_2$ (1.0 mM) and EDTA-free cOmplete Protease Inhibitor Cocktail (Sigma) and lysed using a microfluidizer. Cell lysates were centrifuged (8,000*g*, 30 min, 4 ºC) and the supernatant was stirred with Talon cobalt affinity resin (0.4 mL/g cells) (Clontech) for 1 hour at 4 ºC. The suspension was applied to a column and the retained resin was washed with HEPES buffer containing 500 mM NaCl until the eluent was clean of DNA and protein ($A_{280/260,}$ NanoDrop) and then with HEPES buffer containing 10 mM imidazole (5 mL/1 mL resin). Protein was then slowly eluted with HEPES buffer containing 50 mM imidazole in 1.0 mL fractions and the combined fractions containing desired protein were passed through a Sephadex **G**-25M column to remove imidazole and concentrated in an Amicon centrifugal filter device (Millipore). These conditions produced PCMT at approximately 69 mg/L of culture and ADK at approximately 47 mg/L of culture. PRMT1 was expressed and purified from bacteria generally as described in Backus *et. al.*[2]

Recombinant LCMT and NNMT Expression and Purification

The full-length gene for human LCMT was codon-optimized and synthesized by GenScript. The gene product was cloned into pET45b(+) at AgeI and SacI restriction sites and the LCMT expression plasmid was transformed into One Shot BL21 Star (DE3) Chemically Competent *E.*

coli (ThermoFisher). The recombinant protein (~40 kDa) contained an additional 12 amino acids appended to the *N*-terminus (MAH_6VGTG) to allow for detection and purification. Conversely, the full-length gene for NNMT was obtained from Open BioSystems (BC000234) in pOTB7 was subcloned into pET-45b (+) vector and transformed into the same expression strain of *E. coli*. Preparation of the plasmids, growth of the expression strains, and purification of the protein were conducted as described previously[9] and adapted as follows. Cultures were grown at 37 °C in rich LB broth [35 g/L tryptone, 20 g/L yeast extract, 5 g/L NaCl, and 0.1 g/L carbenicillin (pH 7.0)] to an optical density at 600 nm of 0.6–0.8. They were cooled rapidly by incubation on ice for 30 min prior to addition of IPTG (1 mM). Following induction, cultures were grown at 15–18 ºC for an additional 16–20 h and cells were harvested by centrifugation (8,000 *g*, 30 min, 4 ºC). The cell paste was flash-frozen in liquid N_2 and stored at –80 ºC. A typical yield was ~5 g of wet cell paste per liter of culture. The frozen paste was resuspended (5 mL/g) in 50 mM Na-HEPES buffer (pH 7.5) containing 300 mM NaCl, 5 mM imidazole, 0.1 mg/mL Dnase I, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.1 mM phenylmethanesulfonyl fluoride (NNMT had a protease cocktail I believe). The cells were lysed at 4 ºC by a single passage through a microfluidizer at 16,000 psi and the cell debris was pelleted by centrifugation (10,000 *g*, 20 min, 4 ºC). The supernatant was slowly stirred with Ni-NTA resin [0.5 mL column volume (CV)/g cells] for 30 min at 4 ºC. The slurry was loaded into a column and washed with 50 mM Na-HEPES buffer (pH 7.5) containing 300 mM NaCl and 5 mM imidazole until the A_{280} and A_{260} were both ~0 (~10 CVs). Protein was eluted from the resin with 50 mM Na-HEPES buffer (pH 7.5) containing 100 mM NaCl and 250 mM imidazole. Protein fractions were pooled and dialyzed against 50 mM Na-HEPES buffer (pH 7.5) containing 150 mM NaCl and 1 mM DTT (NNMT only). Following dialysis, the protein was concentrated to ~30 mg/mL and ~17 mg/mL for LCMT and NNMT, respectively, prior to being flash-frozen with 20% glycerol and stored at –80 ºC. The yield was ~2 mg or ~ 7 mg protein for LCMT and NNMT, respectively per g of cell paste.

FluoPol assay optimization

FluoPol conditions were optimized by screening a matrix of conditions varying concentrations of NNMT and probe **1** in DPBS containing 0.05% Pluronic F-127. Competition of FluoPol signal was then achieved by adding varying amounts of SAH (see figure **S9**). Optimal conditions were selected for approximately 80% probe occupancy, and were found to be: 6 μM NNMT, 1 μM probe **1**, 0.05 % Pluronic F-127 in DPBS.

FluoPol assay

A solution of NNMT (40 µL of 6.75 µM in PBS with 0.05% Pluronic F-127 and 1 mM beta-mercaptoethanol) was added to Eppendorf tubes and 4.1 µL of PBS was added, followed by 0.9 µL of the inhibitor in DMSO at 50x concentration. NNMT was incubated with inhibitors for 1 hour, then 5 µL of 10 µM Probe **1** in PBS (with 0.05% Pluronic) was added, the mixture was pipette-mixed and then aliquoted into Greiner 384-well plates and fluorescence polarization was measured by detecting fluorescence intensity through polarized 595 nm filters.

NNMT activity assay

NNMT activity assays were performed according to published protocols.[1] Briefly, NNMT (ca. 32 mg/mL in 50% glycerol/DPBS) was diluted to 0.013 mg/mL (400 nM) in 50 mM Tris·HCl (pH 8.0) and 39.2 µL of NNMT stock was added to 0.8 µL of 50x compound stock and incubated for 1 hour. Reaction mix (7.04 µL) containing 200 µM d_4-nicotinamide (d_4-NA) and 50 µM *S*-adenosyl-L-methionine (SAM) was added and aliquots (20 µL) were quenched into 20 µL of methanol (containing 5 µM 1-methyl nicotinamide as internal standard) at 5 and 10 minutes. Formation of d_4-1-methyl nicotinamide (d_4-MNA) was followed by targeted LC/MS analysis. Separation of analytes was achieved with a Luna-NH_2 column (5 µm, 100 Å, 50 x 4.6 mm, Phenomenex) together with a precolumn (NH_2, 4 x 3.0 mm). Mobile phase A consisted of 100% CH_3CN containing 0.1% formic acid, and mobile phase B contained 95:5 (v/v) H_2O:CH_3CN supplemented with 50 mM NH_4OAc and 0.2% NH_4OH. The flow rate started at 0.1 mL/min, and the gradient consisted of 5 min 0% B, a linear increase to 100% B over 15 min at a flow rate of 0.4 mL/min, followed by isocratic elution of 100% B for 15 min at 0.5 mL/min before equilibrating for 5 min at 0% B at 0.4 mL/min (40 min total). For each run, the ejection volume was 20 µL. MS spectra were obtained on an Agilent G64B10 tandem mass spectrometer with ESI source. The dwell time for d_4-1MNA was set to 100 ms. The capillary was set to 4 kV, and the fragmentor was set to 100 V. The drying gas temperature was 350 ^{o}C, the drying gas flow rate was 11 L/min and the nebulizer pressure was 35 psi. The mass spectrometer was run in MRM mode, monitoring the transition of *m/z* from 141 to 98 for d_4-1MNA (positive ionization mode).

Determination of k_{obs}/[I]

Following established procedures,[10,11] 768O cell lysates (soluble fraction, which expresses high levels of NNMT) were treated with varying concentrations of RS004 (**31**) for 0-60 minutes at 15 minute intervals and quenched with 25 µM Probe **1**, exposed to UV light, and analyzed by SDS-

PAGE according to procedures outlined above. The gel bands corresponding to NNMT were quantified using Image Lab (Bio-Rad) and fit using the following equation:

$$(1)\ E_f(t) = E_f(0)e^{-k_{obs}t}$$

where E_f is the fraction of free enzyme remaining at time (t) or (0), and k_{obs} is the observed rate of inhibition of the enzyme by an irreversible inhibitor. Rearranging equation (1) we get:

$$(2)\ ln\left(\frac{E_f(t)}{E_f(0)}\right) = -k_{obs}t$$

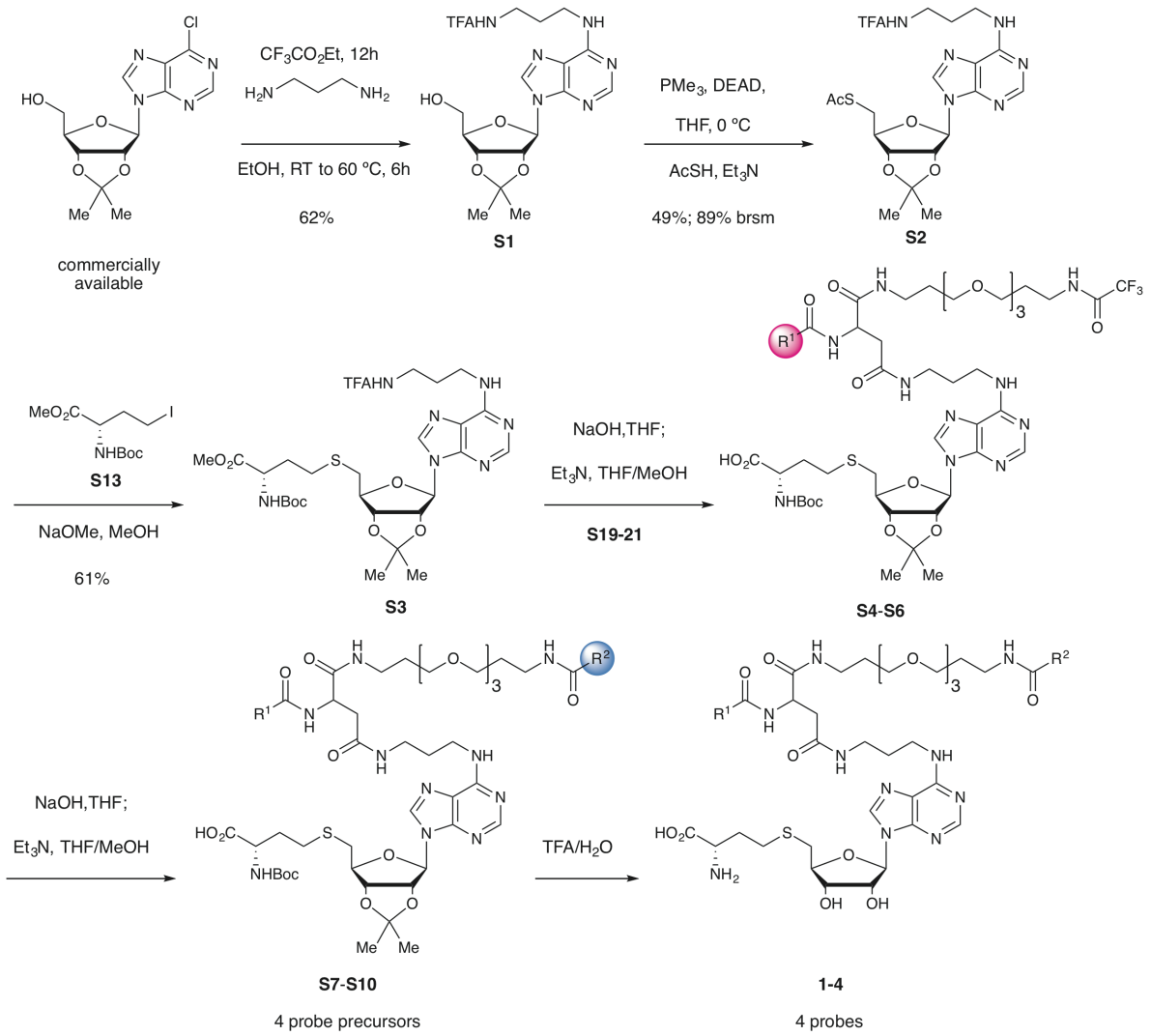
We determined k_{obs} at varying inhibitor concentrations, then plotted k_{obs} as a function of inhibitor concentration [I], the slope of which functions as an approximation of k_{inact}/K_I, according to equation 3:

$$(3)\ \frac{k_{obs}}{[I]} = \frac{k_{inact}}{K_I}$$

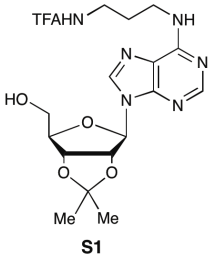
III. SYNTHETIC METHODS AND COMPOUNDS CHARACTERIZATION

General Methods

All chemicals, including anhydrous solvents, were obtained from commercial suppliers and used without further purification. Merck silica gel TLC plates (0.25 mm, 60 F_{254}) were used to monitor reaction progress and were visualized under UV light (254 nm) or by staining with potassium permanganate ($KMnO_4$). Flash chromatography was performed using SiliaFlash® F60 silica gel (SiO_2, 40-60 µM, 60 Å), loaded with dichloromethane unless otherwise noted. NMR spectra were recorded at ambient temperature on Bruker DRX-500, Varian Inova-400, Bruker DPX-400, Brukler AV-400 or Bruker AV-600 (5 mm DCH cryoprobe) instruments. Chemical shifts are recorded in ppm relative to tetramethylsilane (TMS, ^1H, 0 ppm) or solvent signals: $CDCl_3$ (^1H, 7.26; ^{13}C, 77.16 ppm), MeOD-d_4 (^1H, 3.31; ^{13}C 49.00), D_2O (^1H 4.80). Peaks are reported as follows: chemical shift, multiplicity (s = singlet, bs = broad singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constant(s) (Hz). High-resolution mass spectra (HRMS) were obtained on an Agilent LC/MSD TOF mass spectrometer by electrospray ionization-time-of-flight (ESI-TOF). NHS esters for preparing **S13 precursor**, **S19, S20 and S21**, were commercially available (2,5-dioxopyrrolidin-1-yl 3-(3-methyl-3*H*-diazirin-3-yl)propanoate, 2,5-dioxopyrrolidin-1-yl 4-benzoylbenzoate, 2,5-dioxopyrrolidin-1-yl 4-azidobenzoate) or prepared according to literature procedures (2,5-dioxopyrrolidin-1-yl 3-(3-(but-3-yn-1-yl)-3*H*-diazirin-3-yl)propanoate[12]).



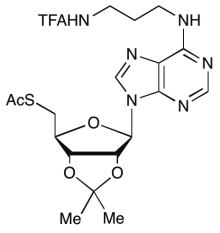
Scheme S1. Synthetic route to generate SAH-derived photoaffinity probes. See **Figure 1** for definitions of R groups.



S1

2,2,2-trifluoro-*N*-(3-((9-((3a*R*,4*R*,6*R*,6a*R*)-6-(hydroxymethyl)-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxol-4-yl)-9*H*-purin-6-yl)amino)propyl)acetamide (S1). To 426 µL of 1,3-diaminobutane (5.1 mmol, 1.85 equiv) in ethanol (56 mL, 0.05 M with respect to purine) was added 695 µL of ethyl trifluoroacetate (5.82 mmol, 2.1 equiv) and the resulting solution was stirred for 1 hour, at which point 916 mg of ((3a*R*,4*R*,6*R*,6a*R*)-6-(6-chloro-9*H*-

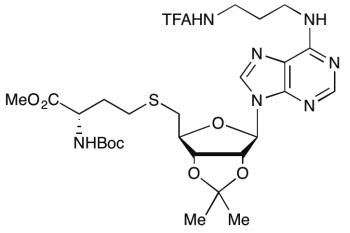
purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxol-4-yl)methanol (2.77 mmol, 1 equiv) was added, followed by 1.17 mL of trimethylamine (8.4 mmol, 3 equiv) and the reaction was stirred overnight. The reaction was subsequently concentrated and purified directly by flash column chromatography (gradient elution: 50 to 100 % ethyl acetate in hexanes, followed by 1% methanol in ethyl acetate) to provide 1.01g of **S1** as a white foam (78% yield): ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.90 (s, 1H), 8.31 (s, 1H), 7.84 (s, 1H), 6.44 (d, *J* = 10.8 Hz, 1H), 6.30 (s, 1H), 5.87 (d, *J* = 4.8 Hz, 1H), 5.21 (s, 1H), 5.13 (d, *J* = 5.8 Hz, 1H), 4.56 (s, 1H), 3.98 (d, *J* = 12.8 Hz, 1H), 3.86 – 3.76 (m, 3H), 3.46 – 3.39 (m, 2H), 1.89 (s, 2H), 1.66 (s, 3H), 1.39 (s, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 157.41 (q, *J* = 36.6 Hz), 155.68, 152.46, 147.40, 139.91, 121.03, 116.18 (q, *J* = 287.8 Hz), 114.13, 94.05, 86.18, 83.14, 81.64, 65.84, 63.28, 35.84, 29.22, 27.59, 25.23. HRMS *m/z* calc'd for $C_{18}H_{23}F_3N_6O_5S$ $[M + H]^+$ 461.1755, found: 461.1755.



S2

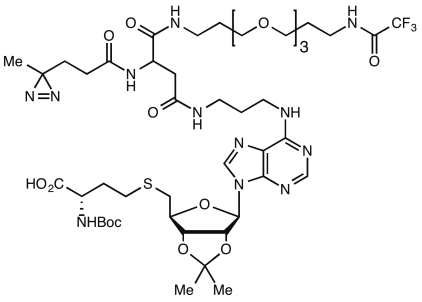
***S*-(((3a*S*,4*S*,6*R*,6a*R*)-2,2-dimethyl-6-(6-((3-(2,2,2-trifluoroacetamido)propyl)amino)-9*H*-purin-9-yl)tetrahydrofuro[3,4-*d*][1,3]dioxol-4-yl)methyl) ethanethioate (S2).** To an oven-dried flask under nitrogen was added trimethylphosphine (7.75 mL of a 1.0 M solution in THF, 7.75 mmol, 3.0 equiv) followed by THF (13 mL) and the solution was cooled to -10 ºC. Diethylazodicarboxylate (3.53 mL of a 40% [wt/wt] solution in toluene, 7.75 mmol, 3.0 equiv) and the resulting yellow solution was stirred for 1 h while maintaining temperature. At this point, a solution of **S1** (1.19g, 2.6 mmol, 1.0 equiv) in THF (13 mL) was added and the resulting solution was stirred for 20 min, at which point thioacetic acid was added (551 µL, 7.75 mmol, 3.0 equiv) and the solution was allowed to warm to room temperature overnight. The reaction mixture was subsequently concentrated and purified directly via flash column chromatography (gradient elution, 50 to 100% ethyl acetate in hexanes) to provide the title compound (**S2**), 658 mg as a colorless oil (49% yield), along with 532 mg of starting material (45%): ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.19 (s, 1H), 8.36 (s, 1H), 7.91 (s, 1H), 6.30 (s, 1H), 6.09 (d, *J* = 2.2 Hz, 1H), 5.51 (dd, *J* = 6.4, 2.2 Hz, 1H), 4.99 (dd, *J* = 6.4, 3.1 Hz, 1H), 4.37 (td, *J* = 6.9, 3.2 Hz, 1H), 3.80 (s, 2H), 3.44 (d, *J* = 6.1 Hz, 2H), 3.36 – 3.15 (m, 2H), 2.38 (s, 3H), 1.91 (s, 2H), 1.68 – 1.58 (m, 3H), 1.41 (d, *J* = 0.7 Hz, 3H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 194.0, 157.1, 156.9 (q, *J* = 36.3, 35.6 Hz), 156.7, 156.4, 152.4, 139.3, 116.7 (q, *J* = 389.9 Hz), 114.1, 90.5, 85.7, 83.8,

83.2, 30.8, 30.1, 29.1, 26.6, 24.9, 22.4, 22.0. HRMS *m/z* calc'd for $C_{20}H_{25}F_3N_6O_5S$ $[M + H]^+$ 519.1632, found: 519.1635.



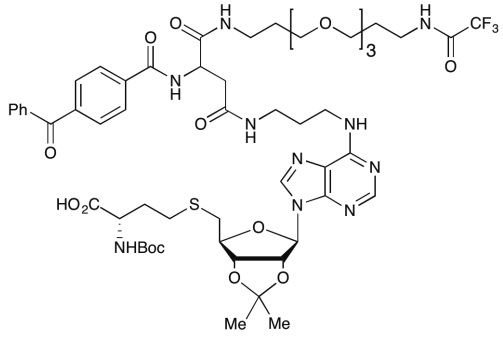
S3

methyl *N*-(*tert*-butoxycarbonyl)-*S*-(((3a*S*,4*S*,6*R*,6a*R*)-2,2-dimethyl-6-(6-((3-(2,2,2-trifluoroacetamido)propyl)amino)-9*H*-purin-9-yl)tetrahydrofuro[3,4-*d*][1,3]dioxol-4-yl)methyl)-*L*-homocysteinate (S3). To a solution of **S2** (658 mg, 1.27 mmol, 1.0 equiv) and methyl (*S*)-2-((*tert*-butoxycarbonyl)amino)-4-iodobutanoate (**S12**) (480 mg, 1.4 mmol, 1.1 equiv) in 6.35 mL degassed methanol was added 6.35 mL of 0.2 M sodium methoxide in methanol (generated by adding 6.35 mL degassed methanol to 56 mg of 40% [wt/wt] sodium hydride, 1.4 mmol, 1.1 equiv) and the resulting solution was stirred for 30 min. Acetic acid (1.0 mL, excess) was then added and the solution was concentrated and purified by flash column chromatography (gradient elution, 20 to 35 to 50% acetone in hexanes) to provide 556 mg of the title compound as a white solid (63% yield): ^{1}H NMR (600 MHz, Chloroform-*d*) δ 9.03 (s, 1H), 8.31 (s, 1H), 7.92 (s, 1H), 6.83 (s, 2H), 6.59 (s, 1H), 6.06 (d, *J* = 2.2 Hz, 1H), 5.46 (d, *J* = 6.3 Hz, 1H), 5.23 – 5.10 (m, 1H), 5.03 (dd, *J* = 6.4, 3.2 Hz, 1H), 4.37 (td, *J* = 6.8, 3.2 Hz, 2H), 3.71 (s, 3H), 3.41 (d, *J* = 7.0 Hz, 2H), 2.81 (ddd, *J* = 49.2, 13.7, 6.6 Hz, 2H), 2.55 (t, *J* = 7.5 Hz, 2H), 2.16 (d, *J* = 4.3 Hz, 1H), 1.94 – 1.77 (m, 3H), 1.60 (s, 3H), 1.41 (s, 9H), 1.38 (s, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 172.80, 157.32 (q, *J* = 36.3 Hz), 155.59, 153.02, 139.82, 120.38, 119.16, 116.30 (q, *J* = 288.0 Hz), 115.35, 114.63, 90.95, 86.74, 84.13, 83.83, 80.14, 69.61, 62.25, 53.96, 52.68, 52.50, 34.36, 32.68, 31.83, 29.37, 28.35, 27.16, 25.37, 14.49. HRMS *m/z* calc'd for $C_{28}H_{40}F_3N_7O_8S$ $[M + H]^+$ 692.2684, found: 692.2685.



S4

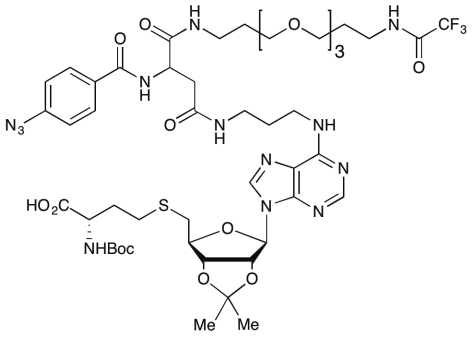
Probe 1/2 precursor S4. To a solution of **S3** (142.1 mg, 205 μmol, 1 equiv) in 4 mL THF (0.05 M) was added 1 mL of 1 M $NaOH_{(aq)}$ (5 equiv) and the resulting solution was stirred at ambient temperature overnight. After LCMS analysis indicated complete deprotection of the methyl ester and trifluoroacetamide, acetic acid (118 μL, 10 equiv) was added and the reaction was concentrated under a stream of nitrogen, taken up in ~5 mL ethyl acetate and concentrated twice. Triethylamine (284 μL, 10 equiv) was added, followed by a solution of **S19** (131.2 mg, 1 equiv) in 5 mL of 1:1 THF:MeOH. The reaction was stirred overnight at ambient temperature until LCMS analysis indicated complete conversion of the amine, at which point the reaction was concentrated twice from ethyl acetate. Acetic acid (80 μL) was added, and the crude mixture was purified by flash column chromatography (gradient elution, 50% acetone in hexanes to 100% acetone to 1% acetic acid in acetone) to provide 94.2 mg of the title compound (45% yield) as a pale yellow gum: ^{1}H NMR (400 MHz, Methanol-d_4) δ 8.29 (s, 1H), 8.25 (s, 1H), 6.18 (d, *J* = 2.5 Hz, 1H), 5.53 (d, *J* = 6.4 Hz, 1H), 5.07 (dd, *J* = 6.2, 2.8 Hz, 1H), 4.70 (t, *J* = 6.7 Hz, 1H), 4.35 (td, *J* = 6.9, 2.9 Hz, 1H), 4.12 (s, 1H), 3.68 – 3.45 (m, 10H), 3.37 (dd, *J* = 12.5, 5.6 Hz, 2H), 2.81 (t, *J* = 6.6 Hz, 1H), 2.68 (s, 4H), 2.63 (s, 3H), 2.58 (t, *J* = 9.6 Hz, 2H), 2.18 (s, 4H), 2.14 (dd, *J* = 8.6, 6.9 Hz, 2H), 1.89 – 1.78 (m, 3H), 1.74 (t, *J* = 6.4 Hz, 1H), 1.66 (dd, *J* = 8.6, 6.9 Hz, 2H), 1.59 (s, 3H), 1.42 (s, 9H), 1.25 (s, 9H), 0.98 (s, 2H). HRMS *m/z* calc'd for $C_{46}H_{71}F_3N_{12}O_{14}S$ $[M + H]^+$ 1105.4958, found: 1105.4959.



S5

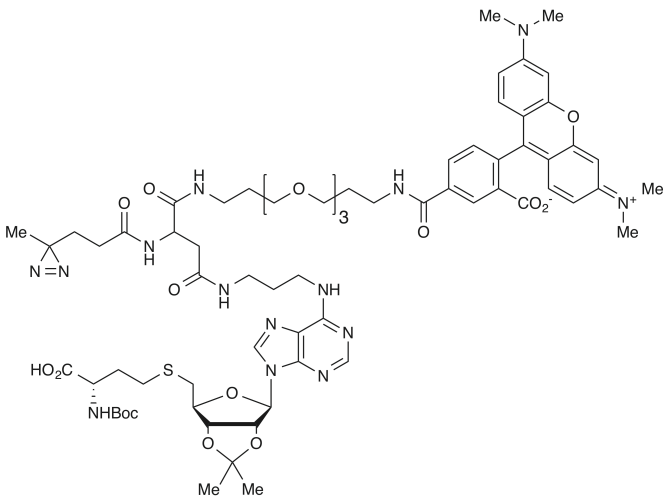
Probe 3 precursor S5. To a solution of deprotected **S3** (generated as for **S4**) (25.4 μmol, 1 equiv) in 500 μL 1:1 THF:MeOH was added 35 μL triethylamine (10 equiv) and the resulting solution was added to 18.7 mg of **S21** (25.4 μmol, 1 equiv) and stirred overnight at ambient temperature. The reaction was subsequently concentrated and purified by flash column chromatography (gradient elution, 50% acetone in hexanes to 1% acetic acid in acetone) to provide 11.8 mg of the title compounds as a colorless amorphous solid (39% yield): ^{1}H NMR (600 MHz, Methanol-d_4) δ 8.19 (s, 3H), 7.94 (dd, *J* = 8.1, 6.4 Hz, 2H), 7.78 – 7.66 (m, 4H), 7.62 (t, *J* = 7.4 Hz, 1H), 7.49 (t, *J* = 7.6 Hz, 2H), 6.13 (s, 1H), 6.09 (s, 1H), 5.44 (s, 1H), 5.01 (s, 1H),

4.92 (t, *J* = 7.2 Hz, 1H), 4.35 – 4.22 (m, 2H), 4.04 (s, 2H), 3.60 – 3.48 (m, 9H), 3.48 – 3.43 (m, 4H), 3.27 (p, *J* = 1.7 Hz, 13H), 3.17 (q, *J* = 7.3 Hz, 5H), 2.89 – 2.68 (m, 4H), 2.52 (s, 3H), 1.86 – 1.68 (m, 8H), 1.38 (d, *J* = 4.0 Hz, 10H). HRMS *m/z* calc'd for $C_{55}H_{73}F_3N_{10}O_{15}S$ $[M + H]^+$ 1203.5002, found: 1203.5001.



S6

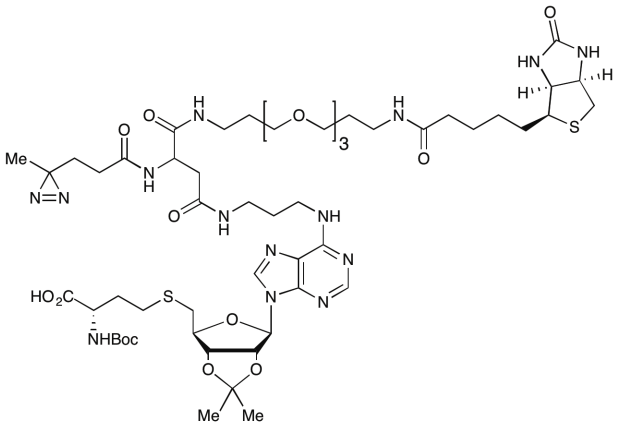
Probe 4 precursor S6. To a solution of deprotected **S3** (generated as for **S4**) (18.7 µmol, 1 equiv) in 374 µL 1:1 THF:MeOH was added 26 µL triethylamine (187 µmol, 10 equiv) and the resulting solution was added to 12.6 mg of **S20** (18.7 µmol, 1 equiv). After stirring overnight, the reaction was concentrated and purified by flash column chromatography (gradient elution, 50% acetone in hexanes to 1% acetic acid in acetone) to provide 9.2 mg of the title compound as a white solid (64% yield): ^{1}H NMR (500 MHz, Methanol-d_4) δ 8.32 (s, 2H), 7.94 (d, *J* = 8.3 Hz, 2H), 7.13 (d, *J* = 8.4 Hz, 2H), 6.24 (s, 1H), 5.58 (s, 1H), 5.16 – 5.11 (m, 1H), 3.75 (dd, *J* = 5.5, 4.0 Hz, 10H), 3.64 (td, *J* = 5.4, 4.4, 2.5 Hz, 16H), 3.59 (ddd, *J* = 12.4, 5.8, 2.4 Hz, 4H), 3.47 – 3.42 (m, 2H), 2.89 (ddd, *J* = 23.5, 16.0, 7.7 Hz, 5H), 2.76 (s, 3H), 2.71 (s, 4H), 2.26 (s, 6H), 1.96 – 1.76 (m, 6H), 1.67 (d, *J* = 2.2 Hz, 4H), 1.54 – 1.48 (m, 12H), 1.37 (d, *J* = 3.0 Hz, 3H). HRMS *m/z* calc'd for $C_{48}H_{68}F_3N_{13}O_{14}S$ $[M + H]^+$ 1140.4754, found: 1140.4741.



S7

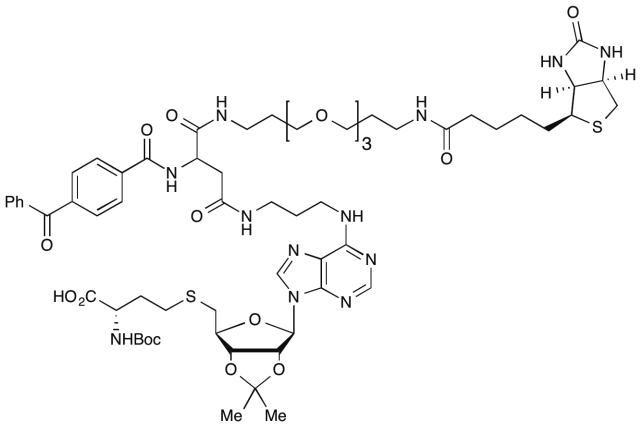
Probe 1 precursor S7. To a solution of **S4** (8.1 mg, 7.3 μmol, 1 equiv) in methanol (360 μL, 0.02M) was added 360 μL of concentrated aqueous ammonia and the resulting solution was stirred overnight. The next day, LCMS analysis indicated complete deprotection of the trifluoroacetamide. The solution was concentrated, then further concentrated twice from 10% triethylamine in ethyl acetate. The resulting amorphous solid was taken up in THF (0.73 mL) and 3.9 mg of 5-carboxy tetramethylrhodamine hydroxy-succinimidyl ester (7.3 μmol, 1 equiv), followed by 10.2 μL of triethylamine (73 μmol, 10 equiv) were added and the solution was stirred overnight. LCMS analysis indicated complete conversion of the intermediate amine. The reaction was concentrated, taken up in dichloromethane and purified by preparatory thin layer chromatography (SiO_2, 1000 micron thickness) using 2% aqueous ammonia, 18% methanol in dichloromethane as eluent to provide 3.7 mg of the title compound as a dark purple solid (36% yield): ^{1}H NMR (600 MHz, Methanol-d_4) δ 8.55 (dd, *J* = 1.9, 0.5 Hz, 1H), 8.24 (s, 2H), 8.20 (s, 1H), 8.06 (dd, *J* = 7.9, 1.9 Hz, 1H), 7.38 (dd, *J* = 7.9, 0.5 Hz, 1H), 7.24 (dd, *J* = 9.4, 1.8 Hz, 2H), 6.98 (ddd, *J* = 9.5, 3.7, 2.5 Hz, 2H), 6.88 (dd, *J* = 10.0, 2.5 Hz, 2H), 6.14 (d, *J* = 2.6 Hz, 1H), 5.48 (d, *J* = 6.4 Hz, 2H), 5.04 (dd, *J* = 6.3, 2.9 Hz, 1H), 4.67 (dd, *J* = 8.1, 5.6 Hz, 1H), 4.34 (ddd, *J* = 7.7, 6.0, 3.0 Hz, 1H), 4.02 (s, 1H), 3.77 (td, *J* = 6.8, 3.3 Hz, 1H), 3.68 – 3.60 (m, 10H), 3.59 – 3.53 (m, 5H), 3.48 (t, *J* = 6.1 Hz, 2H), 2.91 – 2.86 (m, 1H), 2.85 – 2.79 (m, 1H), 2.79 – 2.75 (m, 1H), 2.66 (dd, *J* = 14.8, 5.6 Hz, 1H), 2.60 – 2.52 (m, 3H), 2.16 (d, *J* = 0.8 Hz, 1H), 2.14 (d, *J* = 1.3 Hz, 1H), 2.13 (d, *J* = 1.2 Hz, 1H), 2.12 (q, *J* = 0.8 Hz, 1H), 1.94 (p, *J* = 6.5 Hz, 2H), 1.78 (t, *J* = 6.7 Hz, 2H), 1.74 – 1.71 (m, 2H), 1.65 – 1.62 (m, 2H), 1.60 – 1.57 (m, 5H), 1.41 (s, 7H), 1.38 (t, *J* = 1.2 Hz, 4H), 1.29 (s, 12H). ^{13}C NMR (151 MHz, MeOD) δ 181.21, 172.47, 171.22, 170.39, 167.09, 160.19, 157.11, 156.79, 152.21, 146.01, 139.27, 138.72, 135.36, 135.04, 130.73, 129.03, 127.74, 127.55, 113.57, 113.12, 112.94, 95.43, 89.71, 85.73, 83.39, 83.27, 69.69,

69.47, 69.33, 68.33, 67.93, 61.57, 61.21, 50.33, 38.98, 37.49, 36.94, 36.11, 35.91, 34.76, 34.22, 33.30, 33.13, 31.20, 29.29, 29.27, 29.02, 28.92, 28.88, 28.81, 28.61, 28.60, 28.45, 26.92, 25.98, 25.56, 23.69, 21.86, 17.89, 12.56. HRMS *m/z* calc'd for $C_{69}H_{92}N_{14}O_{17}S$ $[M + H]^+$ 1421.6558, found: 1421.6553.



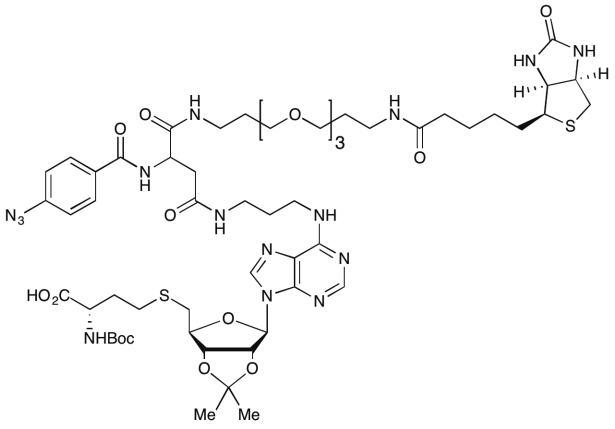
S8

Probe 2 precursor S8. To a solution of **S4** (7.5mg, 6.8 µmol) in 340 µL methanol (0.02 M) was added 340 µL of aqueous ammonia, deprotected and worked up as for **S7**. The deprotected amine was taken up in 0.7 mL THF. Biotin N-hydroxysuccinimide ester was added (2.3 mg, 1 equiv) followed by 10 µL triethylamine (~10 equiv). The solution was stirred overnight, concentrated and purified by preparatory thin layer chromatography (SiO_2, 500 micron thickness) using 2% aqueous ammonia, 18% methanol in dichloromethane as eluent to provide 2.2 mg of the title compound as a colorless solid (26% yield): ^{1}H NMR (600 MHz, Methanol-d4) δ 8.32 (s, 1H), 8.29 (s, 1H), 6.19 (d, *J* = 2.5 Hz, 1H), 5.48 (m, 1H), 5.08 (dd, *J* = 6.4, 2.9 Hz, 1H), 4.71 (dd, *J* = 7.5, 6.0 Hz, 1H), 4.48 (m, 3H), 4.38 (ddd, *J* = 7.6, 6.2, 3.0 Hz, 1H), 4.32 (dt, *J* = 7.8, 4.8 Hz, 3H), 3.56 (m, 8H), 3.52 (dt, *J* = 17.4, 6.1 Hz, 4H), 3.37 (s, 1H), 3.26 (m, 5H), 3.20 (m, 3H), 2.91 (m, 3H), 2.76 (m, 2H), 2.72 (dd, *J* = 12.7, 2.8 Hz, 4H), 2.64 (dd, *J* = 15.0, 7.6 Hz, 1H), 2.57 (td, *J* = 7.3, 6.0, 2.2 Hz, 2H), 2.28, 2.20 (m, 7H), 2.15 (m, 2H), 1.88 (t, *J* = 6.8 Hz, 2H), 1.73 (m, 6H), 1.65 (m, 5H), 1.46 (m, 4H), 1.44 (s, 10H), 1.41 (s, 3H), 1.36 ? 1.29 (m, 12H), 1.00 (s, 3H), 0.92 (t, *J* = 7.0 Hz, 1H). ^{13}C NMR (151 MHz, MeOD) δ 210.06, 179.03, 175.94, 174.35, 173.05, 166.13, 157.60, 147.99, 141.32, 128.73, 115.41, 111.40, 93.84, 87.78, 85.28, 71.53, 71.23, 69.96, 69.79, 63.36, 61.63, 56.98, 41.07, 41.04, 38.74, 37.98, 37.85, 36.87, 36.20, 35.11, 34.82, 33.08, 31.19, 31.16, 30.78, 30.67, 30.41, 30.31, 29.79, 29.49, 28.80, 27.42, 27.15, 26.90, 26.73, 26.42, 25.55, 23.74, 19.76.



S9

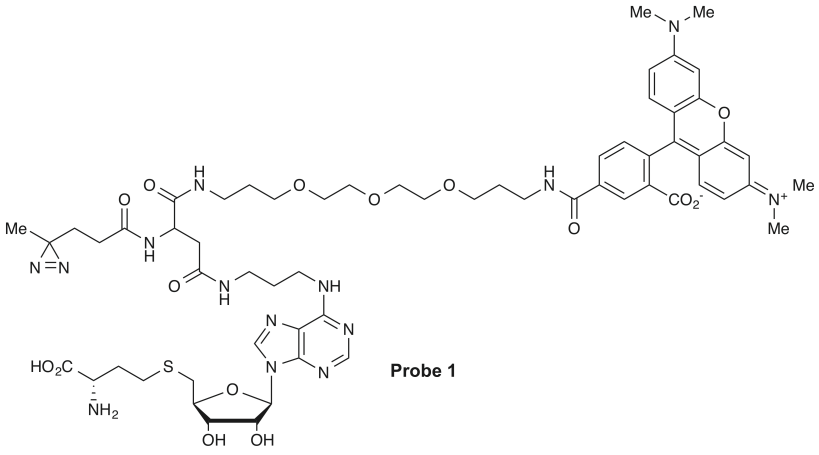
Probe 3 precursor S9. To a solution of **S5** (18.9 mg, 15.7 μmol) in methanol (0.5 mL) was added aqueous ammonia (0.5 mL) and the resulting solution was stirred overnight. The solution was concentrated and then re-concentrated from 10% triethylamine in ethyl acetate twice. The crude residue was taken up in 0.8 mL 1:1 methanol:THF and 9.0 mg biotin N-hydroxy succinimydyl ester (26 μmol, 1.67 equiv) was added, followed by 22 μL triethylamine (157 μmol, 10 equiv). The resulting solution was stirred overnight, concentrated and purified directly by preparatory thin layer chromatography using 2% aqueous ammonia, 18% methanol, 80% dichloromethane as eluent to provide 10.1 mg of the title compound as a white solid (48% yield): ^{1}H NMR (600 MHz, Methanol-d4) δ 8.21 (s, 3H), 7.98 (d, *J* = 7.9 Hz, 3H), 7.74 (t, *J* = 9.6 Hz, 4H), 7.52 (t, *J* = 7.6 Hz, 2H), 6.13 (s, 1H), 5.46 (s, 1H), 4.32 (s, 1H), 3.70 – 3.65 (m, 4H), 3.56 (tdd, *J* = 13.2, 6.3, 3.0 Hz, 13H), 3.50 (dt, *J* = 8.8, 6.1 Hz, 5H), 3.35 (t, *J* = 6.8 Hz, 3H), 2.80 (s, 5H), 2.67 (d, *J* = 5.9 Hz, 1H), 1.84 – 1.75 (m, 4H), 1.56 (s, 4H), 1.42 (s, 11H), 1.37 (d, *J* = 8.5 Hz, 5H), 1.30 (d, *J* = 13.6 Hz, 1H). HRMS *m/z* calc'd for $C_{63}H_{86}N_{12}O_{16}S_3$ $[M + H]^+$ 1333.5955, found: 1333.5962.



S10

Probe 4 precursor S10. To a solution of **S6** (22.2 mg, 19.5 μmol) in methanol (1 mL, 0.02 M) was added aqueous ammonia (1 mL) and the resulting solution was stirred overnight. The next day, the solution was concentrated, taken up in 10% triethylamine in ethyl acetate and concentrated twice, then dissolved in 0.8 mL of 1:1 THF:methanol and 6.7 mg of bionin N-hydroxy succinimydyl ester (19.6 μmol, 1 equiv) was added, followed by 13.5 μL of triethylamine (97 μmol, 5 equiv). The next day, the reaction as concentrated and purified directly by preparatory thin layer chromatography using 2% aqueous ammonia, 18% methanol in dichloromethane as eluent to provide 4.7 mg of the title compound as a white solid (19% yield): ^{1}H NMR (600 MHz, Methanol-d4) δ 8.32 – 8.24 (m, 2H), 7.91 – 7.84 (m, 2H), 7.10 – 7.03 (m, 2H), 6.17 (t, *J* = 3.1 Hz, 1H), 5.49 (d, *J* = 6.4 Hz, 1H), 5.05 (dd, *J* = 6.6, 2.8 Hz, 1H), 4.59 (s, 3H), 4.49 (td, *J* = 7.4, 4.8 Hz, 2H), 4.41 – 4.24 (m, 3H), 4.06 (s, 1H), 3.68 (dd, *J* = 5.5, 4.1 Hz, 3H), 3.61 – 3.54 (m, 9H), 3.54 – 3.47 (m, 7H), 3.27 – 3.23 (m, 2H), 3.18 (ddd, *J* = 7.0, 5.1, 2.7 Hz, 2H), 2.92 (ddd, *J* = 12.7, 10.7, 5.0 Hz, 2H), 2.85 – 2.74 (m, 4H), 2.70 (dd, *J* = 12.7, 5.8 Hz, 2H), 2.55 (d, *J* = 5.9 Hz, 2H), 2.26 – 2.15 (m, 5H), 1.87 – 1.81 (m, 2H), 1.75 (q, *J* = 6.5 Hz, 5H), 1.59 (s, 4H), 1.41 (d, *J* = 2.0 Hz, 10H), 1.39 (s, 3H), 0.90 (t, *J* = 7.0 Hz, 3H). HRMS *m/z* calc'd for $C_{56}H_{84}N_{15}O_{15}S_2$ $[M + H]^+$ 12790.5707, found: 1270.5701.

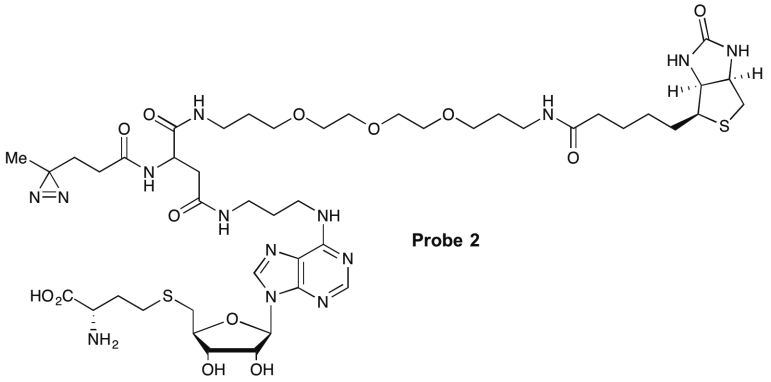
General procedure for deprotection to yield final probes: Neat probe precursor was taken up in 4:1 (v:v) trifluoroacetic acid:water at 0.01 M concentration, vortexed briefly (ca. 30 sec) and then concentrated under a stream of nitrogen. Deuterium oxide (1 mL) was added, and the solution was concentrated again under a stream of nitrogen. The resulting colorless oil was then taken up in deuterium oxide, analyzed by NMR, and directly diluted to working concentrations in DPBS.



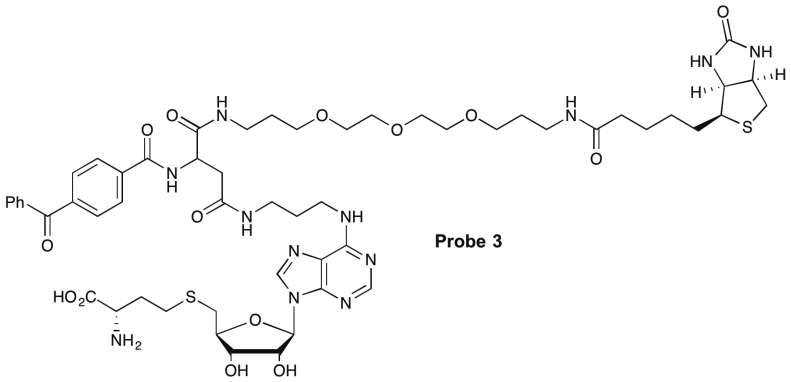
Probe 1

Probe 1. Prepared according to the general procedure from 3.7 mg of **S7** to provide 520 μL of a 5 mM stock solution: ^{1}H NMR (600 MHz, Deuterium Oxide) δ 8.52 (s, 1H), 8.23 (d, *J* = 12.5 Hz,

1H), 8.13 – 8.08 (m, 1H), 7.48 (d, *J* = 7.8 Hz, 1H), 7.00 (d, *J* = 9.5 Hz, 1H), 6.76 (d, *J* = 9.4 Hz, 2H), 6.45 (s, 2H), 5.95 (d, *J* = 4.9 Hz, 1H), 4.53 (s, 1H), 4.30 (d, *J* = 7.1 Hz, 1H), 4.20 (s, 1H), 3.98 (t, *J* = 6.4 Hz, 1H), 3.67 – 3.60 (m, 6H), 3.57 (dd, *J* = 5.8, 2.9 Hz, 2H), 3.55 – 3.48 (m, 4H), 3.41 (t, *J* = 6.3 Hz, 3H), 3.14 (t, *J* = 6.9 Hz, 3H), 3.07 (s, 11H), 2.62 (dt, *J* = 16.3, 6.5 Hz, 2H), 2.53 – 2.45 (m, 1H), 2.32 – 2.10 (m, 1H), 2.03 (t, *J* = 7.2 Hz, 2H), 1.94 – 1.87 (m, 2H), 1.78 (s, 2H), 1.68 – 1.61 (m, 2H), 1.52 (t, *J* = 7.3 Hz, 2H), 1.32 – 1.11 (m, 14H), 0.83 (d, *J* = 3.4 Hz, 3H). HRMS *m/z* calc'd for $C_{61}H_{80}N_{14}O_{15}S$ $[M + 2H]^{2+}$ 641.2897, found: 641.2893.

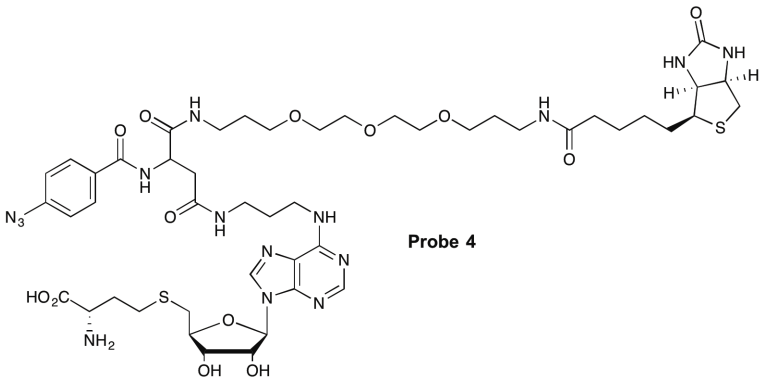


Probe 2. Prepared according to the general procedure from 2.2 mg of **S8** to provide 407 μL of a 5 mM stock solution: ^{1}H NMR (600 MHz, Deuterium Oxide) δ 8.24 (d, *J* = 30.5 Hz, 1H), 8.14 (d, *J* = 23.6 Hz, 1H), 5.85 (d, *J* = 4.8 Hz, 1H), 4.57 (s, 1H), 4.38 (d, *J* = 7.3 Hz, 1H), 4.33 (dd, *J* = 8.0, 4.9 Hz, 2H), 4.14 (ddd, *J* = 12.9, 7.6, 4.3 Hz, 4H), 4.06 (d, *J* = 6.1 Hz, 1H), 3.91 (t, *J* = 6.5 Hz, 1H), 3.35 (dd, *J* = 17.6, 6.1 Hz, 10H), 3.26 (dt, *J* = 21.3, 6.4 Hz, 4H), 3.08 – 2.94 (m, 8H), 2.81 – 2.64 (m, 4H), 2.54 – 2.44 (m, 6H), 2.36 (dd, *J* = 15.1, 8.4 Hz, 1H), 2.01 (dt, *J* = 26.0, 7.4 Hz, 6H), 1.94 – 1.82 (m, 3H), 1.68 (s, 2H), 1.47 (ddt, *J* = 21.5, 14.8, 7.0 Hz, 4H), 1.33 (ddq, *J* = 37.3, 15.2, 7.9, 7.3 Hz, 6H), 1.21 – 1.05 (m, 6H), 0.67 (s, 3H). ^{13}C NMR (151 MHz, Deuterium Oxide) δ 179.6, 176.1, 174.3, 171.4, 170.7, 164.6, 164.5, 161.48 (q, *J* = 37.1 Hz, TFA), 115.21 (q, *J* = 289.8 Hz, TFA), 73.0, 68.9, 68.7, 67.7, 67.6, 61.6, 61.5, 59.8, 59.7, 54.6, 54.5, 50.9, 50.8, 50.2, 39.2, 39.0, 38.9, 38.9, 36.7, 36.7, 35.9, 35.8, 34.6, 33.8, 32.7, 29.2, 28.9, 28.7, 27.5, 27.5, 27.2, 27.2, 26.9, 26.7, 25.5, 24.5, 24.3. HRMS *m/z* calc'd for $C_{46}H_{74}N_{14}O_{13}S_2$ $[M + 2H]^{2+}$ 548.2574, found: 548.2573.



Probe 3

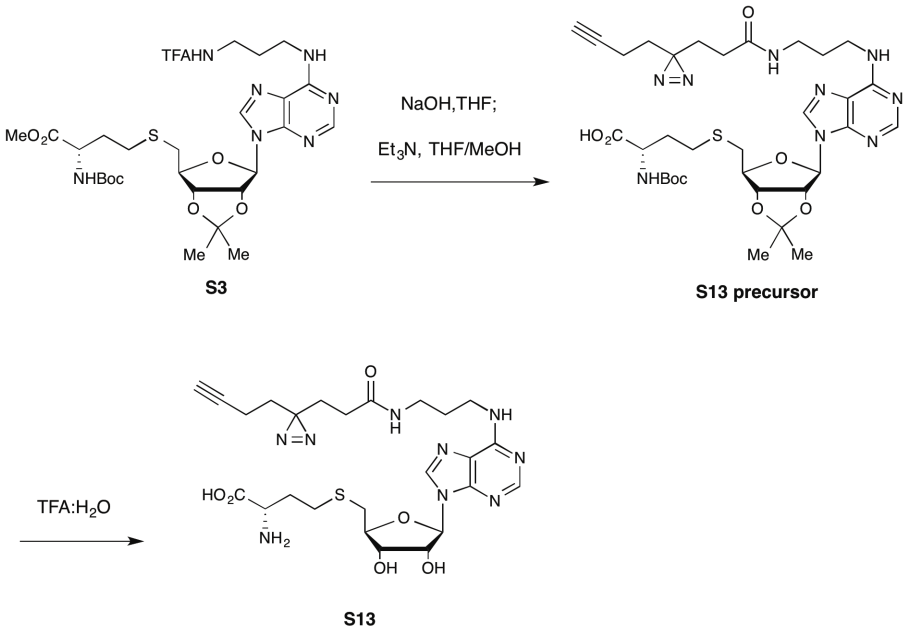
Probe 3. Prepared according to the general procedure from 10.1 mg of **S9** to provide 760 μL of a 10 mM stock solution: ^{1}H NMR (600 MHz, Deuterium Oxide) δ 8.34 (s, 1H), 8.28 (s, 1H), 7.81 (t, *J* = 12.0 Hz, 3H), 7.78 – 7.66 (m, 2H), 7.68 – 7.61 (m, 2H), 7.57 (t, *J* = 7.7 Hz, 2H), 5.98 (d, *J* = 4.6 Hz, 1H), 4.54 (dd, *J* = 8.0, 4.9 Hz, 1H), 4.37 – 4.31 (m, 2H), 4.23 (d, *J* = 6.3 Hz, 1H), 4.16 (t, *J* = 6.4 Hz, 1H), 3.73 (dd, *J* = 5.5, 3.8 Hz, 4H), 3.64 (dd, *J* = 5.5, 3.7 Hz, 4H), 3.62 – 3.52 (m, 12H), 3.49 (t, *J* = 6.4 Hz, 3H), 3.39 – 3.12 (m, 10H), 3.03 – 2.86 (m, 5H), 2.71 (dd, *J* = 11.1, 6.9 Hz, 3H), 2.28 – 2.20 (m, 1H), 2.16 (t, *J* = 7.3 Hz, 3H), 1.89 (d, *J* = 39.3 Hz, 3H), 1.79 (t, *J* = 6.4 Hz, 2H), 1.74 – 1.68 (m, 2H), 1.67 – 1.62 (m, 0H), 1.60 – 1.44 (m, 2H), 1.29 (dt, *J* = 19.1, 7.0 Hz, 4H). HRMS *m/z* calc'd for $C_{55}H_{76}N_{12}O_{14}S_2$ $[M + H]^+$ 1193.5118, found: 1193.5114.



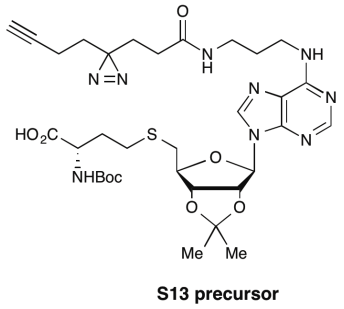
Probe 4

Probe 4. Prepared according to the general procedure from 4.7 mg of **S10** to provide 740 μL of a 5 mM solution: ^{1}H NMR (600 MHz, Deuterium Oxide) δ 8.45 (s, 1H), 8.21 (s, 1H), 7.62 (d, *J* = 8.2 Hz, 2H), 6.80 (d, *J* = 8.3 Hz, 2H), 6.06 (d, *J* = 5.1 Hz, 1H), 4.91 (d, *J* = 10.1 Hz, 1H), 4.81 (s, 1H), 4.53 (dd, *J* = 8.0, 5.0 Hz, 1H), 4.49 (dd, *J* = 7.9, 4.9 Hz, 1H), 4.39 (s, 1H), 4.35 (dd, *J* = 8.0, 4.6 Hz, 1H), 4.29 (dt, *J* = 9.3, 4.8 Hz, 2H), 4.03 (q, *J* = 5.6, 5.0 Hz, 1H), 3.68 – 3.64 (m, 2H), 3.58 – 3.55 (m, 2H), 3.51 (d, *J* = 6.1 Hz, 12H), 3.45 (q, *J* = 6.1 Hz, 6H), 3.21 (dt, *J* = 12.8, 6.4 Hz, 2H), 3.13 (td, *J* = 6.9, 2.8 Hz, 4H), 2.94 – 2.84 (m, 2H), 2.73 – 2.65 (m, 6H), 2.20 (t, *J* = 7.4 Hz, 2H), 2.12 (t, *J* = 7.3 Hz, 3H), 1.68 (dt, *J* = 18.6, 6.5 Hz, 6H), 1.61 – 1.41 (m, 4H), 1.28 (dd, *J*

= 8.6, 6.1 Hz, 2H), 1.19 (t, *J* = 7.3 Hz, 1H).HRMS *m/z* calc'd for $C_{48}H_{71}N_{15}O_{13}S_2$ $[M + H]^+$ 1130.4870, found: 1130.4864.



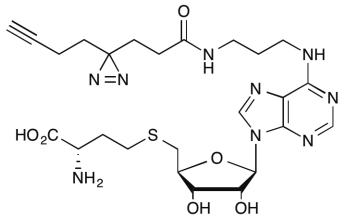
Scheme S2. Preparation of alkyne probe **S13**.



S13 precursor

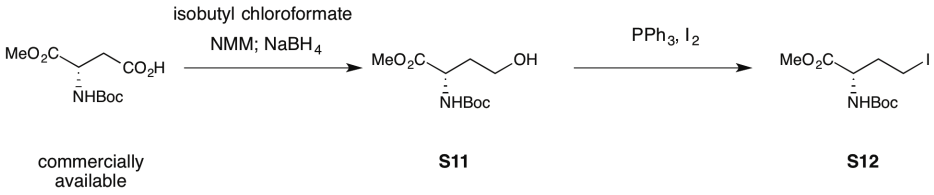
S13 precursor. To a solution of **S3** (7.3 mg, 10.6 µmol, 1 equiv) in THF (200 µL, 0.05 M) was added sodium hydroxide (34 µL of a 1 M solution, 34 µmol, 3.2 equiv) and the solution was stirred for 2 hours until LCMS analysis indicated complete deprotection of the methyl ester and trifluoroacetamide. The resulting solution was added to 2,5-dioxopyrrolidin-1-yl 3-(3-(but-3-yn-1-yl)-3*H*-diazirin-3-yl)propanoate (12.7 mg, 4.6 equiv) and stirred for 30 minutes. LCMS analysis indicated complete consumption of the deprotected amine, so the solution was concentrated and purified by flash column chromatography (1% acetic acid, 50% acetone in hexanes) to provide 5.8 mg of the title compound as a colorless solid (75% yield): ^{1}H NMR (600 MHz, Methanol-d4) δ 8.29 (s, 1H), 8.27 (s, 1H), 6.18 (d, *J* = 2.5 Hz, 1H), 5.50 (dd, *J* = 6.9, 2.7 Hz, 1H), 5.07 (dd, *J* = 6.2, 3.0 Hz, 1H), 4.35 (td, *J* = 6.9, 3.0 Hz, 1H), 4.22 (dd, *J* = 9.3, 4.2 Hz, 1H), 3.64 (d, *J* = 12.3 Hz, 2H), 3.29 (d, *J* = 6.8 Hz, 1H), 2.82 (dd, *J* = 6.9, 4.0 Hz, 2H), 2.65 – 2.60 (m, 1H), 2.54 (dt, *J* = 13.3, 7.9 Hz, 1H), 2.49 – 2.38 (m, 1H), 2.27 (q, *J* = 2.6, 2.1 Hz, 1H), 2.06 –

2.01 (m, 3H), 1.99 (s, 2H), 1.91 – 1.87 (m, 2H), 1.81 (dd, *J* = 9.3, 4.7 Hz, 1H), 1.75 (dd, *J* = 8.4, 7.0 Hz, 2H), 1.61 (d, *J* = 7.5 Hz, 1H), 1.59 (d, *J* = 2.3 Hz, 3H), 1.43 (s, 9H), 1.39 (s, 3H). HRMS *m/z* calc'd for $C_{33}H_{47}N_9O_8S$ $[M + H]^+$ 730.3341, found: 730.3343.



S13

Probe S13. Prepared according to the general procedure from 10 mg of **S13 precursor** to provide 1.37 mL of a 10 mM stock solution: ^{1}H NMR (600 MHz, Deuterium Oxide) δ 8.45 (s, 1H), 8.38 (s, 1H), 6.11 (d, *J* = 4.8 Hz, 1H), 4.43 (t, *J* = 5.1 Hz, 1H), 4.36 – 4.30 (m, 1H), 3.93 (t, *J* = 6.4 Hz, 1H), 3.74 – 3.70 (m, 1H), 3.66 – 3.60 (m, 1H), 3.34 (dd, *J* = 7.8, 4.2 Hz, 2H), 3.06 (dd, *J* = 14.2, 4.9 Hz, 1H), 2.99 (dd, *J* = 14.2, 7.0 Hz, 1H), 2.77 (s, 1H), 2.71 (t, *J* = 7.5 Hz, 2H), 2.32 (t, *J* = 2.7 Hz, 1H), 2.15 (ddt, *J* = 53.5, 14.8, 7.4 Hz, 2H), 2.05 (t, *J* = 7.3 Hz, 2H), 2.02 – 1.91 (m, 4H), 1.72 (t, *J* = 7.2 Hz, 2H), 1.61 (t, *J* = 7.2 Hz, 2H). ^{13}C NMR (151 MHz, D_2O) δ 177.28, 173.95, 163. 77 (q, *J* = 35.5 Hz, TFA), 146.36, 142.81, 117.11 (q, *J* = 518 Hz, TFA), 101.79, 97.00, 89.10, 84.98, 84.39, 81.97, 76.02, 74.38, 74.05, 73.10, 72.36, 70.59, 61.21, 53.77, 34.19, 31.65, 30.92, 30.65, 29.39, 28.31, 25.94, 13.20. HRMS *m/z* calc'd for $C_{25}H_{35}N_9O_6S$ $[M + H]^+$ 590.2504, found: 590.2502.



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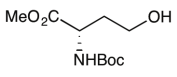
S11

S12

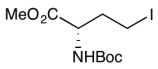
Scheme S3. Preparation of amino acid **S12**.



S11

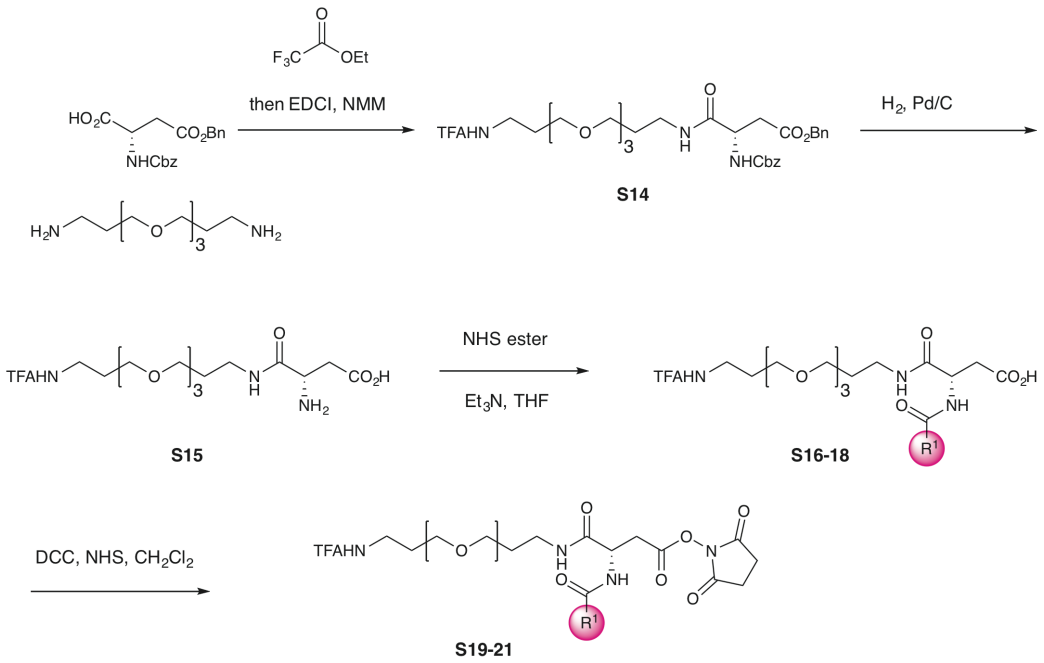
methyl (*tert*-butoxycarbonyl)-*L*-homoserinate (S11). Prepared in a similar fashion to *Dalhoff* et. al.[13] To a solution of N-Boc-L-aspartic acid 1-methyl ester (2.16g, 8.7 mmol, 1 equiv) in THF at -15 ºC was added N-methyl morpholine (0.96 mL, 8.7 mmol, 1 equiv) followed by isobutyl

chloroformate (1.13 mL, 8.7 mmol, 1 equiv) to provide a white suspension that was stirred at temperature for 10 min. Solid sodium borohydride (1 g, 26.4 mmol, 3 equiv) was added followed by careful addition of methanol (gas evolution occurs and will cause the reaction to bubble over the flask if not carefully monitored). The resulting solution was stirred for 10 min, then quenched by addition of 17 mL of 1M hydrochloric acid (6.2 mmol, 2 equiv). The organic portion was extracted with ethyl acetate, washed with with 1 M HCl, saturated $NaHCO_{3(aq),}$ water then brine, dried over magnesium sulfate and concentrated. The crude residue was purified by flash column chromatography (gradient elution, 30 to 50% ethyl acetate in hexanes) to provide 1.39g of the title compound as a colorless oil (68% yield). Spectral characteristics matched those in the literature.[14]

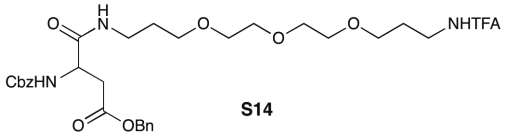


S12

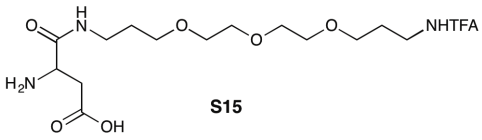
methyl (*S*)-2-((*tert*-butoxycarbonyl)amino)-4-iodobutanoate (S12). To a solution of triphenylphosphine (3.1g, 11.8 mmol, 2 equiv) in dichloromethane (30 mL) at 0 ºC was added iodine (3.0g, 11.8 mmol, 2 equiv) and the resulting solution was stirred for 30 min at temperature. A solution of **S11** (1.37g, 5.9 mmol, 1 equiv) and imidazole (820mg, 12 mmol, 2.05 equiv) in dichloromethane (30 mL) was added, the resulting solution was allowed to warm to ambient temperature and stirred for 2 h, then filtered through a short plug of silica gel eluting with 50% ethyl acetate in hexanes. The resulting solution was concentrated and purified by flash column chromatography (gradient elution, 10 to 15 to 30% ethyl acetate in hexanes) to provide 1.67 g of the title compound as a colorless oil (83% yield): ^{1}H NMR (600 MHz, Chloroform-d) δ 5.08 (s, 1H), 4.34 (d, *J* = 7.1 Hz, 1H), 3.76 (s, 3H), 3.17 (t, *J* = 7.6 Hz, 2H), 2.41 (d, *J* = 13.2 Hz, 1H), 2.22 – 2.15 (m, 1H), 1.44 (s, 9H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 171.56, 154.83, 79.82, 53.81, 52.15, 36.67, 27.83, -1.15. HRMS *m/z* calc'd for $C_{10}H_{18}INO_4$ $[M + H]^+$ 344.0353, found: 344.0354.



Scheme S4. Synthetic route for preparation of NHS esters **S19-S21.**

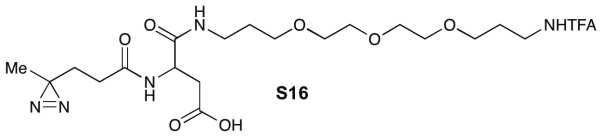


benzyl 19-(((benzyloxy)carbonyl)amino)-1,1,1-trifluoro-2,18-dioxo-7,10,13-trioxa-3,17-diazahenicosan-21-oate (S14). To a solution of 3,3'-((oxybis(ethane-2,1-diyl))bis(oxy))bis(propan-1-amine) (1.56 mL, 7.1 mmol, 1.2 equiv) in THF (30 mL, 0.2 M relative to amino acid) was added ethyl trifluoroacetate (951 μL, 8 mmol, 1.35 equiv) and the resulting solution was stirred for 3 h. LCMS analysis indicated that the majority of the diamine had been mono-protected as the trifluoroacetate. To this solution was added N-Cbz-L-Aspartic acid 4-benzyl ester (2.11 g, 5.9 mmol, 1 equiv) followed by EDCI (1.24 g, 6.5 mmol, 1.1 equiv) and HOBt (153 mg, 1.2 mmol, 0.2 equiv) and the resulting solution was stirred overnight. The solution was subsequently concentrated and purified directly by flash column chromatography (gradient elution, 20 to 30 to 40% acetone in hexanes) to provide 3.03 g of the title compound as a colorless oil (78% yield): ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.62 (s, 1H), 7.40 – 7.29 (m, 10H), 6.86 (s, 1H), 5.96 (d, *J* = 8.8 Hz, 1H), 5.23 – 5.00 (m, 4H), 4.55 (s, 1H), 3.69 – 3.43 (m, 14H), 3.33 (d, *J* = 6.1 Hz, 2H), 3.04 (dd, *J* = 17.1, 4.9 Hz, 1H), 2.77 (dd, *J* = 16.9, 6.1 Hz, 1H), 1.88 – 1.79 (m, 2H), 1.78 – 1.69 (m, 2H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 170.2, 136.1, 135.5, 128.7, 128.7, 128.5, 128.5, 128.4, 128.3, 77.4, 76.9, 70.5, 70.4, 70.3, 67.4, 67.0, 29.0, 28.1. HRMS *m/z* calc'd for $C_{31}H_{40}F_3N_3O_9$ $[M + H]^+$ 656.2789, found: 656.2790.



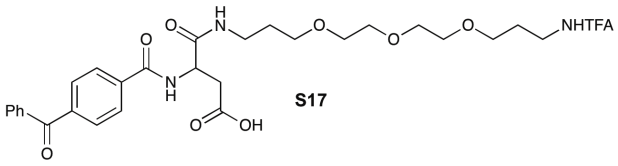
S15

19-amino-1,1,1-trifluoro-2,18-dioxo-7,10,13-trioxa-3,17-diazahenicosan-21-oic acid (S15). A solution of **S14** (727 mg, 1.1 mmol, 1 equiv) in ethanol (11 mL, 0.1 M) was degassed by bubbling through nitrogen for 30 min. Palladium on carbon (10 wt%, 50 mg) was added, and the system was charged with hydrogen by bubbling through for 30 min, then allowed to stir under a constant hydrogen atmosphere overnight. The cloudy black suspension was subsequently filtered over celite, rinsed with ethanol, methanol, and ethyl acetate, and the combined organics were concentrated to yield 445 mg of the title compound (94% yield) as a slightly brown oil: ^{1}H NMR (600 MHz, Methanol-d4) δ 4.91 (s, 2H), 4.04 (dd, *J* = 9.2, 4.5 Hz, 1H), 3.65 – 3.63 (m, 4H), 3.60 – 3.57 (m, 5H), 3.53 (td, *J* = 6.1, 2.3 Hz, 5H), 3.38 (t, *J* = 6.9 Hz, 2H), 2.69 (dd, *J* = 17.0, 4.5 Hz, 1H), 2.59 (dd, *J* = 17.0, 9.3 Hz, 1H), 1.87 – 1.73 (m, 4H). ^{13}C NMR (151 MHz, MeOD) δ 175.99, 170.11, 158.86 (q, *J* = 36.5 Hz), 118.67, 117.54 (q, *J* = 286.7 Hz), 112.29, 71.52, 71.49, 71.25, 71.20, 69.66, 69.57, 38.32, 38.21, 38.01, 30.28, 29.85. HRMS *m/z* calc'd for $C_{16}H_{28}F_3N_3O_7$ $[M + H]^+$ 432.1952, found: 432.1953.

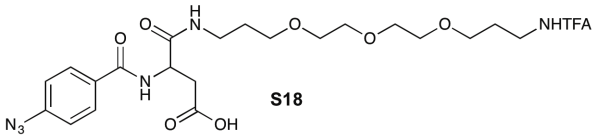


S16

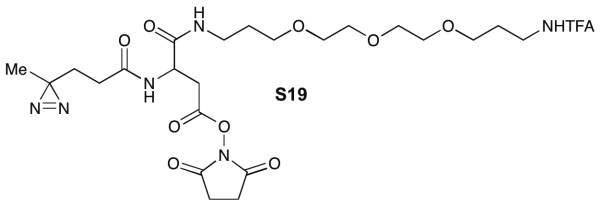
Diazirine side chain precursor S16. To a solution of **S15** (445 mg, 1.03 mmol, 1 equiv) in 1:3 (v:v) methanol:THF (10 ml, 0.1 M) was added 2,5-dioxopyrrolidin-1-yl 3-(3-methyl-3*H*-diazirin-3-yl)propanoate (232 mg, 1.03 mmol, 1 equiv) and triethylamine (286 μL, 2.06 mmol, 2 equiv) and the solution was stirred overnight. The reaction was subsequently quenched with excess acetic acid, concentrated and purified directly by flash column chromatography (gradient elution, 50 to 100% acetone in hexanes) to provide 180.4 mg of the title compound as a colorless oil (32% yield): ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.88 (s, 1H), 7.36 (d, *J* = 8.2 Hz, 1H), 7.32 (t, *J* = 5.6 Hz, 1H), 4.76 (q, *J* = 6.8 Hz, 1H), 3.75 (t, *J* = 4.4 Hz, 1H), 3.68 – 3.55 (m, 9H), 3.47 (dt, *J* = 18.2, 6.0 Hz, 3H), 3.30 (dq, *J* = 19.1, 6.8 Hz, 1H), 2.83 (dd, *J* = 16.7, 5.4 Hz, 1H), 2.71 (s, 6H), 2.09 (td, *J* = 7.6, 2.4 Hz, 1H), 1.90 – 1.81 (m, 2H), 1.81 – 1.64 (m, 3H), 0.99 (s, 3H). HRMS *m/z* calc'd for $C_{21}H_{34}F_3N_5O_8$ $[M + H]^+$ 542.2432, found: 542.2434.



Benzophenone side chain precursor S17. Prepared analogously to **S16** starting from 54.4 mg of **S15** (126 μmol) to provide 55.7 mg of the title compound as a colorless oil (69% yield): ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.06 (d, *J* = 8.0 Hz, 1H), 7.93 (d, *J* = 8.1 Hz, 2H), 7.87 (t, *J* = 5.5 Hz, 1H), 7.80 – 7.71 (m, 4H), 7.62 – 7.55 (m, 1H), 7.46 (t, *J* = 7.7 Hz, 3H), 5.06 – 4.95 (m, 1H), 3.64 – 3.48 (m, 12H), 3.42 (t, *J* = 6.2 Hz, 2H), 3.31 (ddd, *J* = 19.6, 13.3, 6.5 Hz, 1H), 3.05 – 2.76 (m, 2H), 1.78 (dt, *J* = 27.0, 6.1 Hz, 4H). HRMS *m/z* calc'd for $C_{30}H_{36}F_3N_3O_9$ $[M + H]^+$ 640.2476, found: 640.2475.

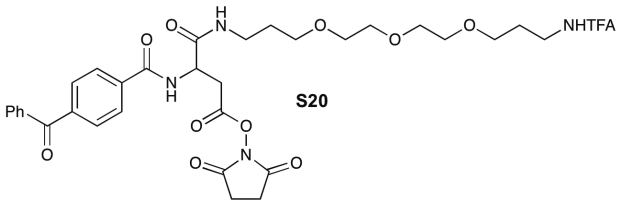


Aryl azide side chain precursor S18. Prepared analogously to **S16** starting from 84 mg of **S15** (195 μmol) to provide 38.5 mg of the title compound as a colorless oil (29% yield): ^{1}H NMR (400 MHz, Chloroform-d) δ 7.93 – 7.74 (m, 3H), 7.03 (d, *J* = 8.5 Hz, 1H), 4.96 (td, *J* = 7.5, 4.4 Hz, 1H), 3.70 – 3.36 (m, 13H), 3.30 (dt, *J* = 13.5, 6.3 Hz, 1H), 3.00 (dd, *J* = 16.7, 4.4 Hz, 1H), 2.75 (dd, *J* = 16.6, 7.5 Hz, 1H), 2.16 (d, *J* = 2.5 Hz, 2H), 1.92 – 1.80 (m, 2H), 1.81 – 1.70 (m, 1H), 1.24 (s, 2H). HRMS *m/z* calc'd for $C_{23}H_{31}F_3N_6O_8$ $[M + H]^+$ 577.2228, found: 577.2232



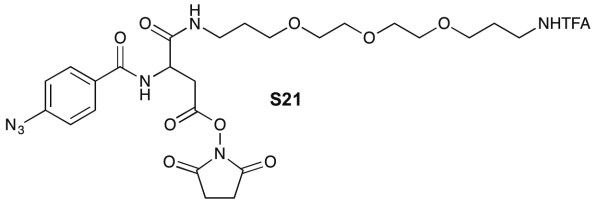
NHS ester S19. To a solution of **S16** (180.4 mg, 330 μmol, 1.0 equiv) in anhydrous dichloromethane (3.3 mL, 0.1 M) was added DCC (69 mg, 330 μmol, 1.0 equiv) followed by N-hydroxy succinimide (38.4 mg, 330 μmol, 1.0 equiv) then DMAP (8 mg, 65 μmol, 0.2 equiv) and the resulting solution was stirred for 3 h. The reaction was then cooled to 0 ºC and filtered through celite rinsing with cold dichloromethane, concentrated and purified by flash column chromatography (gradient elution, 30 to 40 to 50% acetone in hexanes) to provide 134.9 mg of the title compound as a colorless oil (64% yield): ^{1}H NMR (400 MHz, Chloroform-d) δ 7.80 (s, 1H), 7.05 (t, *J* = 5.6 Hz, 1H), 6.97 – 6.87 (m, 1H), 6.35 – 6.26 (m, 1H), 4.94 – 4.82 (m, 1H), 3.74 – 3.42 (m, 17H), 3.36 (p, *J* = 6.6 Hz, 2H), 3.22 (dd, *J* = 16.5, 5.7 Hz, 1H), 3.08 (dd, *J* = 16.1, 6.4

Hz, 1H), 2.93 (t, *J* = 6.1 Hz, 2H), 2.72 (s, 2H), 2.23 – 1.98 (m, 2H), 1.90 – 1.82 (m, 2H), 1.79 – 1.73 (m, 4H). HRMS *m/z* calc'd for $C_{25}H_{37}F_3N_6O_{10}$+ $[M + H]^+$ 639.2596, found: 639.2597.



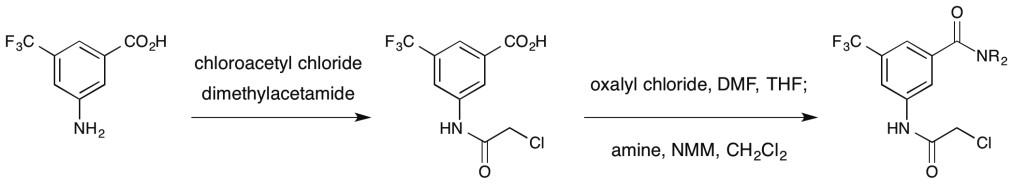
S20

NHS ester S20. Prepared analogously to **S19** starting from **S17** (55.7 mg) to provide 18.7 mg of the title compound as a colorless oil (29% yield): ^{1}H NMR (600 MHz, Chloroform-d) δ 7.96 (dd, *J* = 8.2, 2.8 Hz, 2H), 7.84 (dd, *J* = 8.3, 2.0 Hz, 2H), 7.82 – 7.76 (m, 2H), 7.72 – 7.59 (m, 2H), 7.50 (h, *J* = 5.3, 4.6 Hz, 2H), 7.19 – 7.14 (m, 1H), 5.15 – 5.06 (m, 1H), 3.70 – 3.51 (m, 12H), 3.52 – 3.34 (m, 5H), 3.21 (ddt, *J* = 13.7, 7.2, 4.0 Hz, 1H), 2.92 – 2.80 (m, 4H), 2.72 – 2.61 (m, 1H), 2.17 (dd, *J* = 4.4, 2.6 Hz, 1H), 1.92 – 1.75 (m, 3H), 1.31 – 1.22 (m, 2H). HRMS *m/z* calc'd for $C_{34}H_{39}F_3N_4O_{11}$ $[M + H]^+$ 737.2640, found: 737.2638.

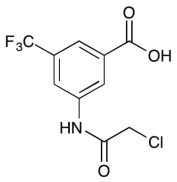


S21

NHS ester S21. Prepared analogously to **S19** starting from **S18** (46.5 mg) to provide 12.6 mg of the title compound as a colorless oil (23% yield): ^{1}H NMR (400 MHz, Chloroform-d) δ 7.85 (d, *J* = 8.6 Hz, 1H), 7.77 (s, 1H), 7.48 (d, *J* = 7.8 Hz, 1H), 7.08 (dd, *J* = 9.4, 7.3 Hz, 3H), 5.06 (q, *J* = 6.8 Hz, 1H), 3.70 – 3.31 (m, 19H), 3.16 (dd, *J* = 16.1, 6.6 Hz, 1H), 2.84 (s, 4H), 1.93 – 1.72 (m, 4H). HRMS *m/z* calc'd for $C_{27}H_{34}F_3N_7O_{10}$ $[M + H]^+$ 674.2392, found: 674.2394.



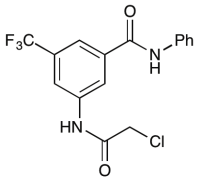
Scheme S5. Synthetic route for preparation of chloroacetamides **26-31**.



S22

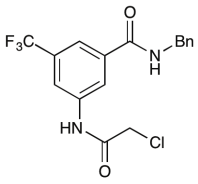
3-(2-chloroacetamido)-5-(trifluoromethyl)benzoic acid (S22). To a solution of 3-Amino-5-(trifluoromethyl)benzoic acid (500 mg, 2.44 mmol, 1 equiv) in N,N-dimethylacetamide (1.5 mL, 1.6 M) at 0 ºC was added chloroacetyl chloride (214 μL, 2.68 mmol, 1.1 equiv) dropwise over 2 minutes. The resulting solution was warmed to room temperature and stirred for 20 minutes. Ethyl acetate (40 mL) was added, followed by 30 mL of water. The mixture was basified to pH 10 using 1M NaOH, and the aqueous layer was washed with 40 mL ethyl acetate. The aqueous layer was subsequently acidified to pH 3 with concentrated $HCl_{(aq)}$ and extracted into ethyl acetate. The organic layer was washed twice with 0.1 M HCl, once with 40 mL of brine, dried over magnesium sulfate, filtered and concentrated to yield 453.5 mg of the title compound as a white crystalline solid (66% yield): ^{1}H NMR (600 MHz, Methanol-d4) δ 8.42 (dt, *J* = 1.5, 0.9 Hz, 1H), 8.28 (t, *J* = 1.4 Hz, 1H), 8.03 – 7.98 (m, 1H), 4.23 (s, 2H). ^{13}C NMR (151 MHz, MeOD) δ 165.88, 165.66, 138.80, 132.10 (q, *J* = 33 Hz), 130.43, 123.21, 120.62, 119.28, 115.08, 41.92. HRMS *m/z* calc'd for $C_{10}H_7ClF_3NO_3Na$ $[M + Na]^+$ 303.9959, found: 303.9960.

General procedure for the preparation of chloroacetamides 26-31: To a solution of **S22** in THF (0.1 M) containing 1 μL DMF was added oxalyl chloride (3 equivalents). The solution was stirred for 3 h and concentrated. The resulting acid chloride was taken up in dichloromethane (0.1 M). To this solution was added 2 equiv of the appropriate amine nucleophile followed by 2 equiv of N-methyl morpholine. The reaction was then stirred overnight. The solution was subsequently diluted with ethyl acetate, extracted twice with 1M $HCl_{(aq)}$ and twice with 1M $NaOH_{(aq)}$ then once with brine. The combined organic fractions were dried over sodium sulfate, filtered and concentrated. The resultant residue was then purified by flash column chromatography.



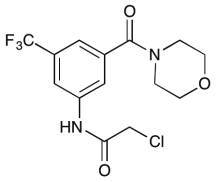
26

Chloroacetamide 26. Prepared according to the general procedure, starting from 0.1 mmol of **S22**, flash column chromatography (gradient elution, 10 to 20 to 30% ethyl acetate in hexanes) provided 15 mg of the title compound as a white solid (42% yield): ^{1}H NMR (400 MHz, Chloroform-d) δ 8.55 (s, 1H), 8.26 (s, 1H), 8.08 (s, 1H), 8.00 (s, 1H), 7.89 (s, 1H), 7.66 – 7.60 (m, 2H), 7.42 – 7.35 (m, 2H), 7.19 (td, *J* = 7.4, 1.1 Hz, 1H), 4.22 (d, *J* = 1.1 Hz, 2H). $C_{15}H_{10}Cl_2F_3N_3O_2$HRMS *m/z* calc'd for $C_{16}H_{12}ClF_3N_2O_2$ $[M + H]^+$ 357.0612, found: 357.0610.



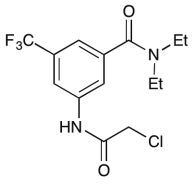
27

Chloroacetamide 27. Prepared according to the general procedure, starting from 0.1 mmol of **S22**, flash column chromatography (gradient elution, 20 to 30% ethyl acetate in hexanes) provided 9.1 of the title compound as a white solid (25% yield): ^{1}H NMR (400 MHz, Chloroform-d) δ 8.57 – 8.48 (s, 1H), 8.17 (s, 1H), 8.09 (s, 1H), 7.82 (s, 1H), 7.41 – 7.28 (m, 5H), 6.56 (s, 1H), 4.66 (d, *J* = 5.7, 2H), 4.21 (s, 2H). HRMS *m/z* calc'd for $C_{17}H_{14}ClF_3N_2O_2$ $[M + H]^+$ 371.0769, found: 371.0769.



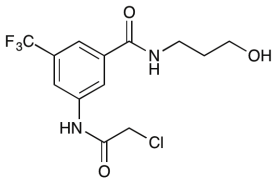
28

Chloroacetamide 28. Prepared according to the general procedure, starting from 0.1 mmol of **S22**, flash column chromatography (gradient elution, 50 to 100% ethyl acetate in hexanes) provided 3 mg of the title compound as a white solid (9% yield): ^{1}H NMR (400 MHz, Chloroform-d) δ 8.48 (s, 1H), 7.91 (t, *J* = 1.7 Hz, 1H), 7.86 (t, *J* = 1.8 Hz, 1H), 7.48 (s, 1H), 4.22 (s, 2H), 3.73 (d, *J* = 46.1 Hz, 7H), 3.46 (s, 2H). HRMS *m/z* calc'd for $C_{14}H_{14}ClF_3N_2O_3$ $[M + H]^+$ 351.0718, found: 351.0725.



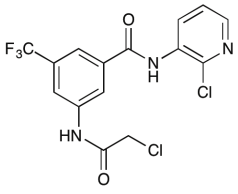
29

Chloroacetamide 29. Prepared according to the general procedure, starting from 0.1 mmol of **S22**, flash column chromatography (gradient elution, 30 to 50% ethyl acetate in hexanes) provided 3 mg of the title compound as a white solid (17% yield): ^{1}H NMR (400 MHz, Chloroform-d) δ 8.62 (s, 1H), 7.85 (t, *J* = 1.7 Hz, 1H), 7.81 (t, *J* = 1.7 Hz, 1H), 7.42 (dt, *J* = 1.6, 0.8 Hz, 1H), 4.20 (s, 2H), 3.55 (d, *J* = 8.3 Hz, 2H), 3.25 (d, *J* = 7.8 Hz, 2H), 1.35 – 1.21 (m, 3H), 1.15 (s, 3H). HRMS *m/z* calc'd for $C_{14}H_{16}ClF_3N_2O_2$ $[M + H]^+$ 337.0925, found: 337.0926.



30

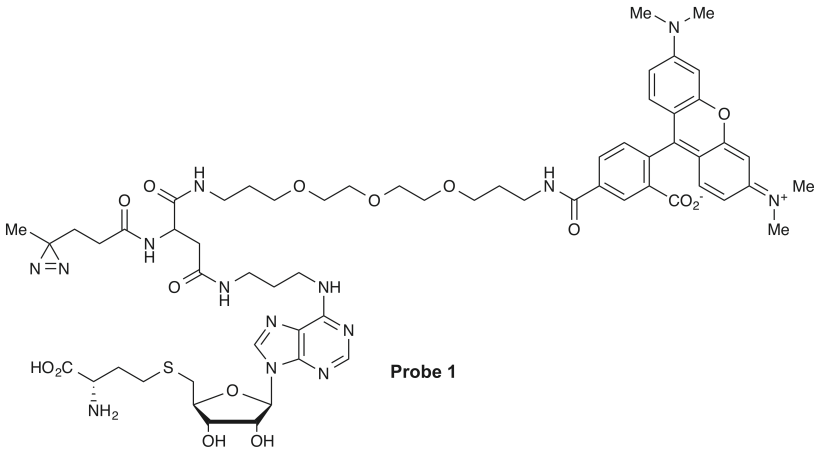
Chloroacetamide 30. Prepared according to the general procedure, starting from 0.066 mmol of **S22**, flash column chromatography (gradient elution, 50 to 100% ethyl acetate in hexanes) provided 5.6 mg of the title compound as a colorless oil (25% yield): ^{1}H NMR (400 MHz, Chloroform-d) δ 8.53 (s, 1H), 8.19 (s, 1H), 8.04 (s, 1H), 7.83 – 7.81 (m, 1H), 6.97 (s, 1H), 4.23 (d, *J* = 0.9 Hz, 2H), 3.78 (t, *J* = 5.5 Hz, 2H), 3.66 (q, *J* = 5.9 Hz, 2H), 1.91 – 1.79 (m, 2H). HRMS *m/z* calc'd for $C_{13}H_{14}ClF_3N_2O_3$ $[M + H]^+$ 339.0718, found: 339.0719.



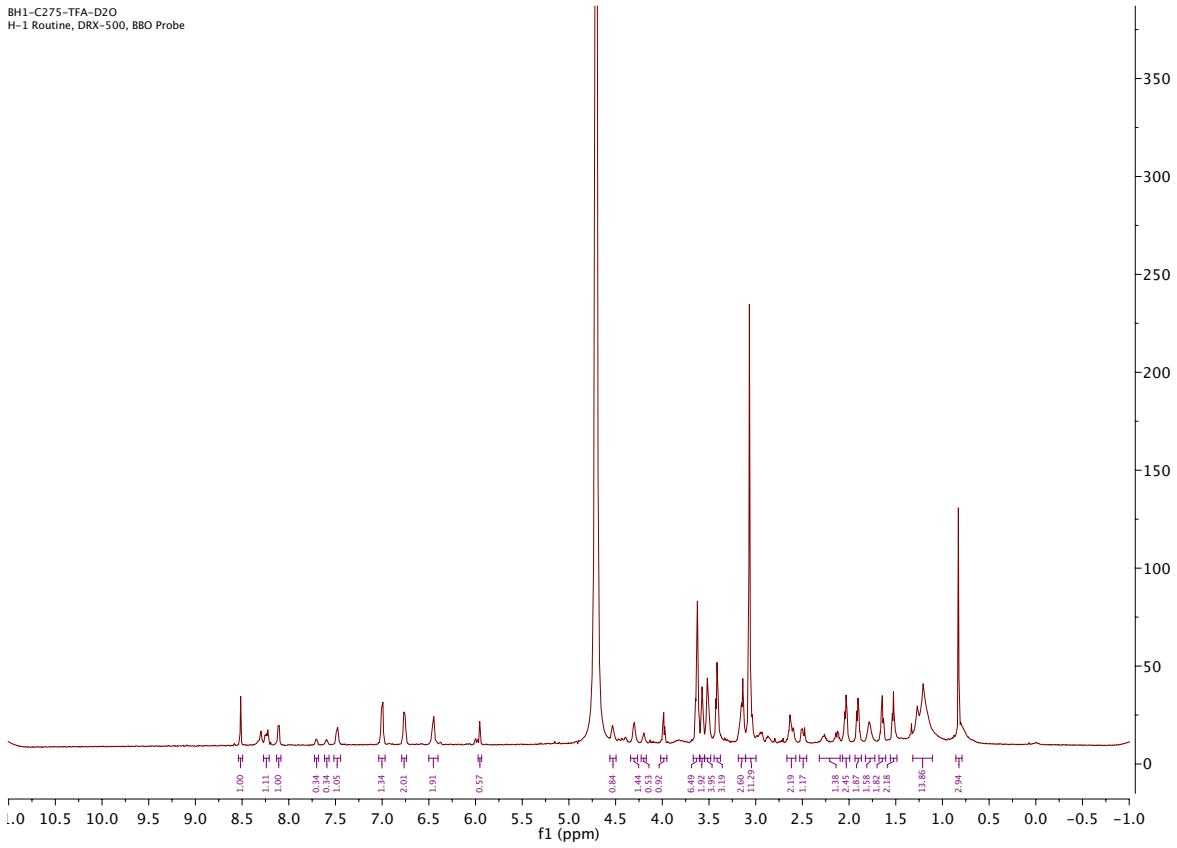
31

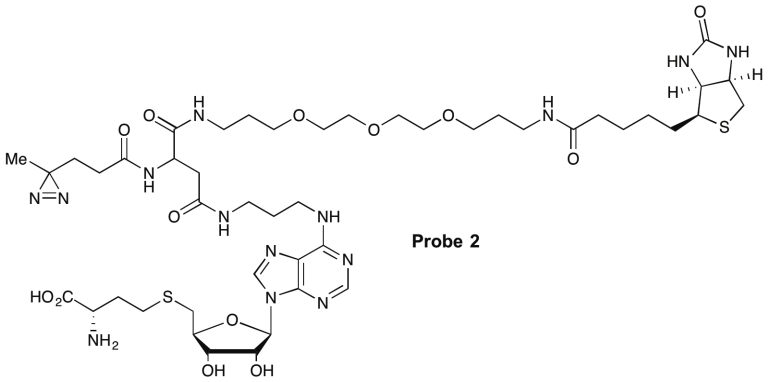
Chloroacetamide 31. Prepared according to the general procedure, starting from 0.43 mmol of **S22**, flash column chromatography (gradient elution, 20 to 28 to 35% ethyl acetate in hexanes) provided 39.2 mg of the title compound as a white solid (23% yield): ^{1}H NMR (600 MHz, Methanol-d4) δ 8.41 (t, *J* = 1.8 Hz, 1H), 8.29 – 8.24 (m, 3H), 8.02 (dd, *J* = 1.6, 0.8 Hz, 1H), 7.46 (dd, *J* = 7.8, 4.9 Hz, 1H), 4.25 (s, 2H). ^{13}C NMR (151 MHz, MeOD) δ 174.62, 166.07, 165.03, 145.66, 144.90, 139.07, 135.17, 134.95, 131.39, 130.77, 124.04, 122.67, 121.76, 119.05, 42.09. HRMS *m/z* calc'd for $C_{15}H_{10}Cl_2F_3N_3O_2$ $[M + H]^+$ 392.0175, found: 392.0172.

Copies of NMR spectra for key compounds.

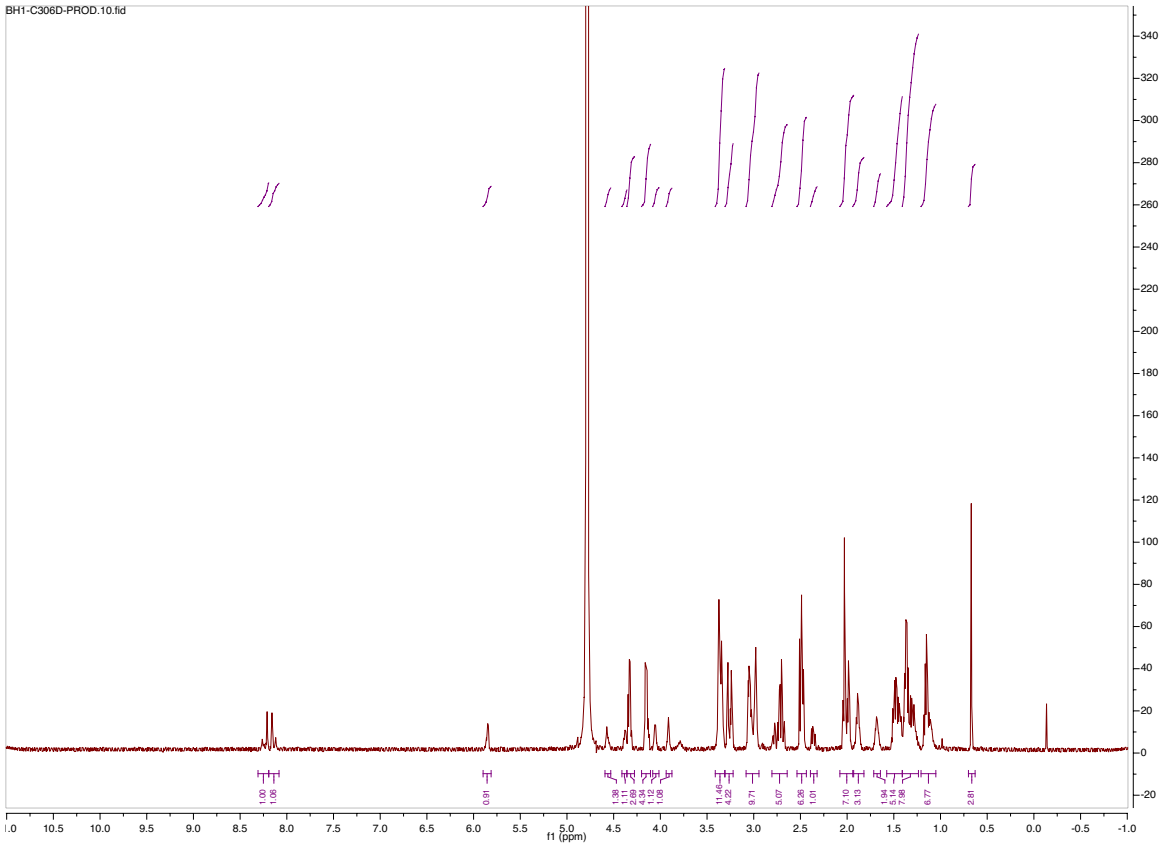


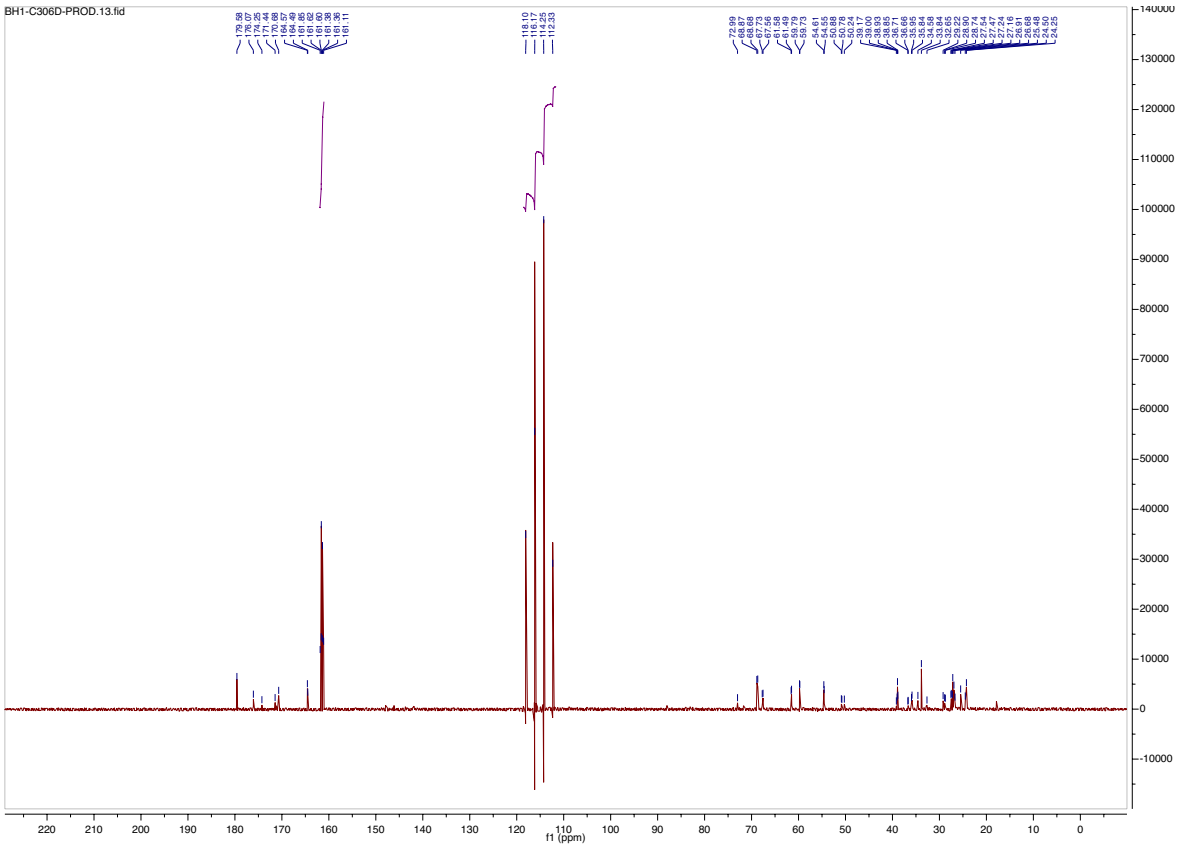
Probe 1

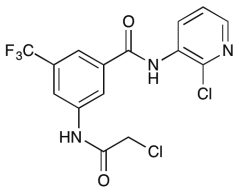




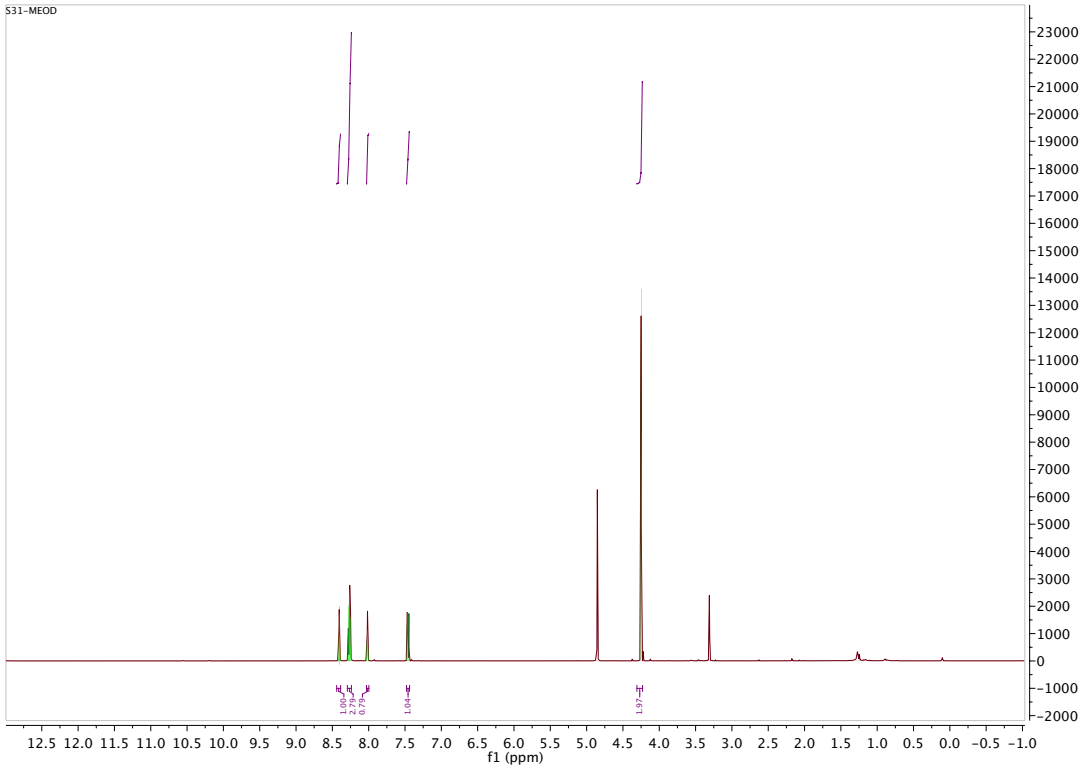
Probe 2

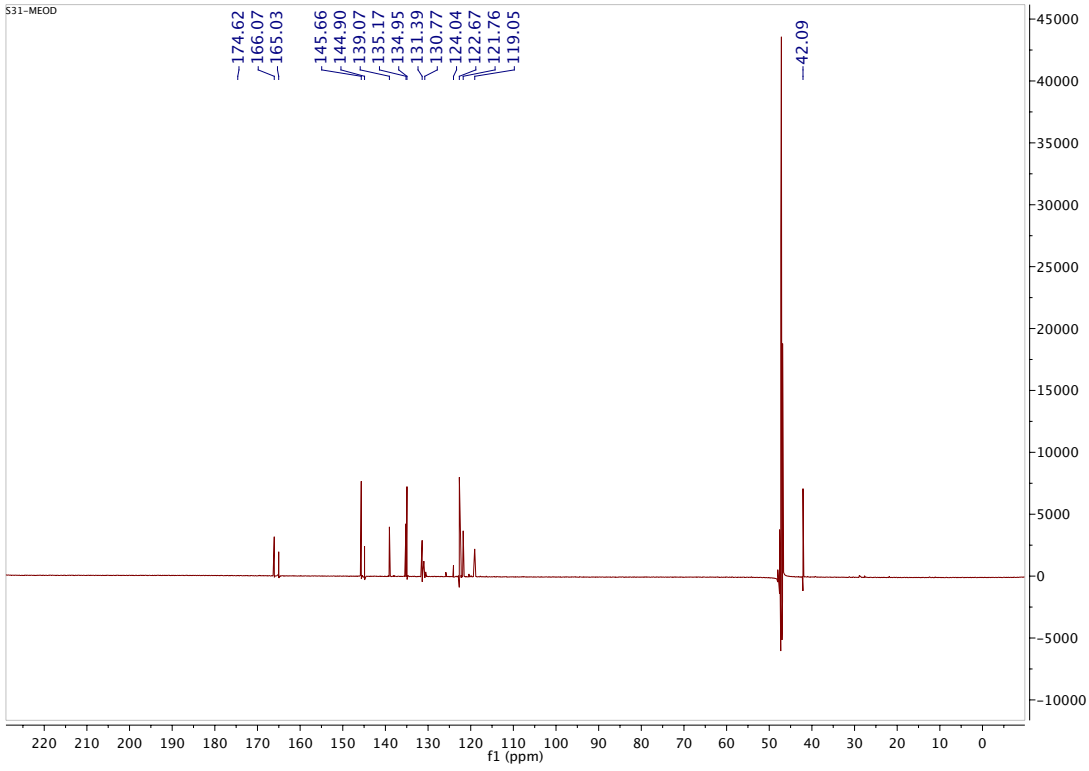






31


S31-MEOD
f1 (ppm)


S31-MEOD
174.62
166.07
165.03
145.66
144.90
139.07
135.17
134.95
131.39
130.77
124.04
122.67
121.76
119.05
42.09
f1 (ppm)

(1) Ulanovskaya, O. A.; Zuhl, A. M.; Cravatt, B. F. *Nat Chem Biol* **2013**, *9*, 300.

(2) Backus, K. M.; Correia, B. E.; Lum, K. M.; Forli, S.; Horning, B. D.; Gonzalez-Paez, G. E.; Chatterjee, S.; Lanning, B. R.; Teijaro, J. R.; Olson, A. J.; Wolan, D. W.; Cravatt, B. F. *Nature* **2016**, *534*, 570.

(3) Ong, S. E.; Blagoev, B.; Kratchmarova, I.; Kristensen, D. B.; Steen, H.; Pandey, A.; Mann, M. *Mol Cell Proteomics* **2002**, *1*, 376.

(4) Mann, M. *Nat Rev Mol Cell Biol* **2006**, *7*, 952.

(5) Weerapana, E.; Wang, C.; Simon, G. M.; Richter, F.; Khare, S.; Dillon, M. B. D.; Bachovchin, D. A.; Mowen, K.; Baker, D.; Cravatt, B. F. *Nature* **2010**, *468*, 790.

(6) Washburn, M. P.; Wolters, D.; Yates, J. R., 3rd *Nat Biotechnol* **2001**, *19*, 242.

(7) Tabb, D. L.; McDonald, W. H.; Yates, J. R., 3rd *J Proteome Res* **2002**, *1*, 21.

(8) Petrossian, T. C.; Clarke, S. G. *Mol Cell Proteomics* **2011**, *10*, M110 000976.

(9) Matthews, M. L.; Krest, C. M.; Barr, E. W.; Vaillancourt, F. H.; Walsh, C. T.; Green, M. T.; Krebs, C.; Bollinger, J. M. *Biochemistry* **2009**, *48*, 4331.

(10) Copeland, R. A. *Methods Biochem Anal* **2005**, *46*, 1.

(11) Parsons, W. H.; Kolar, M. J.; Kamat, S. S.; Cognetta, A. B., 3rd; Hulce, J. J.; Saez, E.; Kahn, B. B.; Saghatelian, A.; Cravatt, B. F. *Nat Chem Biol* **2016**, *12*, 367.

(12) Li, Z.; Hao, P.; Li, L.; Tan, C. Y.; Cheng, X.; Chen, G. Y.; Sze, S. K.; Shen, H. M.; Yao, S. Q. *Angew Chem Int Ed Engl* **2013**, *52*, 8551.

(13) Dalhoff, C.; Huben, M.; Lenz, T.; Poot, P.; Nordhoff, E.; Koster, H.; Weinhold, E. *Chembiochem* **2010**, *11*, 256.

(14) Link, A. J.; Vink, M. K.; Tirrell, D. A. *Nat Protoc* **2007**, *2*, 1884.