A Chemical Biology Toolbox for the Study of Protein Methyltransferases and Epigenetic Signaling

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Figure S1. Western blot analysis of cognate target engagement by chemoproteomic affinity probes. Related to **Figure 3.**

(A) Western blot analysis of EED enrichment by (A-395)-biotin from G401 cell lysate, and competition by pre-treatment with (A-395)-biotin, (A-395N)-biotin or DMSO control (all 20 μM); TCL = total cell lysate.

(B) Western blot analysis of PRMT4 enrichment by MTM7172 from HEK293 cell lysate, and competition by pre-treatment with MS023, MS094 or DMSO control; TCL = total cell lysate.

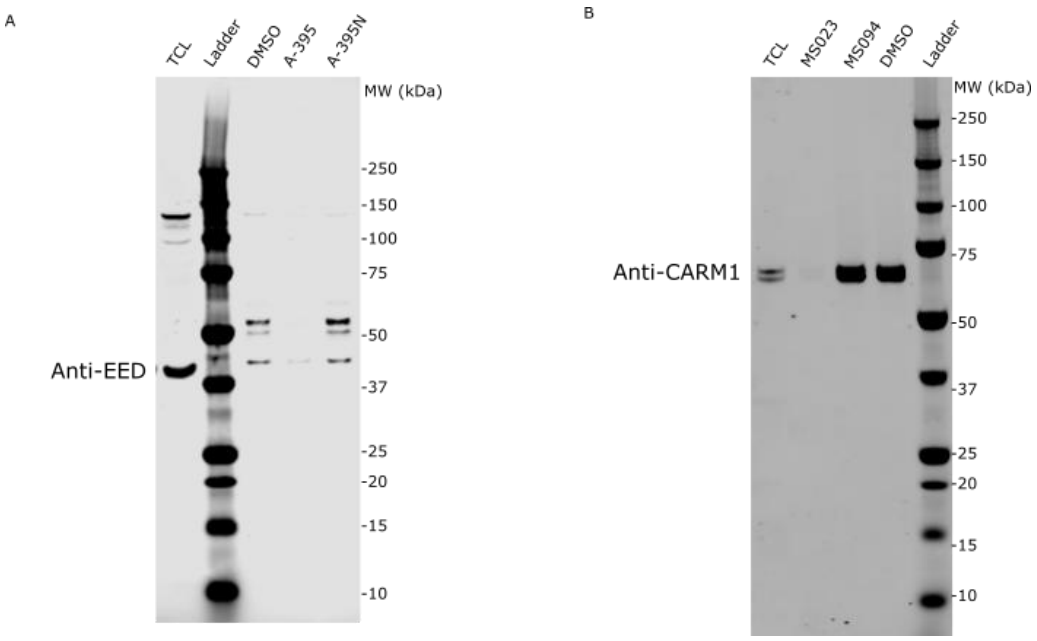


Figure S2. FACS analysis of Th1 polarized cells in the presence of indicated compounds. Naive CD4+ T cells of IFN-γ-YFP reporter mice were cultured for 4 days under Th0 or Th1 cell polarizing conditions in the presence or absence (Th0, Th1) of indicated compounds (1 µM) and analysed by FACS. Data shown is gated on viable CD4+ cells. x-axis: YFP reporter for IFN-γ, y-axis: CD4. Related to **Figures 4A and 4B.**

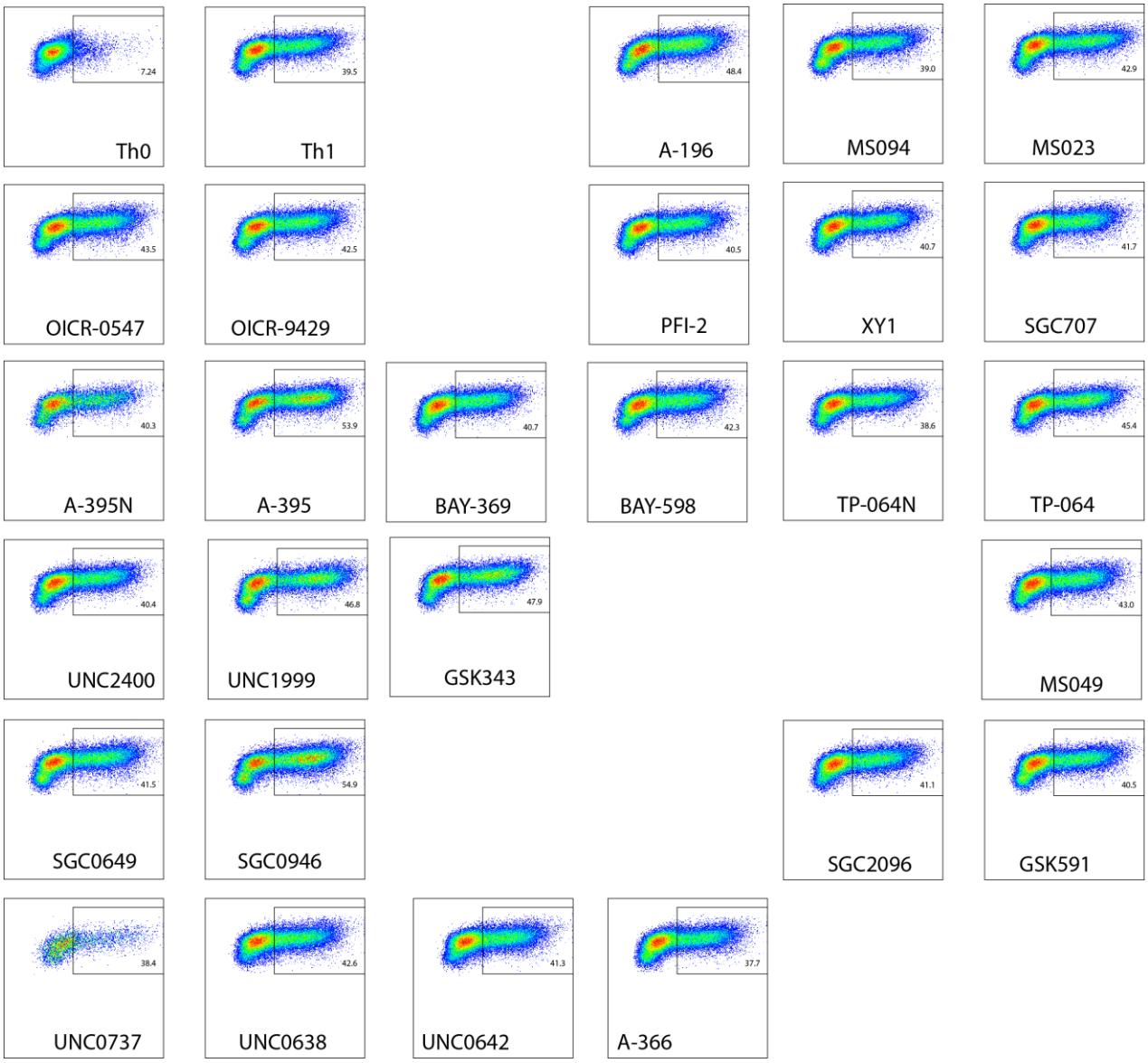


Figure S3. Figure S3. Dose- and temporal response analyses of the DOT1L chemical probe (SGC0946) and control (SGC0649) in the context of IFN-γ production, cell viability and proliferation. Related to **Figures 5A and 5B.**

(A,B) Dose-response analysis for CD4+ T cells cultured for 4 days under Th0 or Th1 cell polarizing conditions in the absence (Th0, Th1) or presence of SGC0649 (Th1) or SGC0946 (Th1) at indicated concentrations regarding (A) frequency or (B) viability of IFN-γ+ CD4+ T cells. Data shown is representative for two independent experiments.

(C) Time dependency of inhibition by the chemical probe (SGC0946) and control (SGC0649) under Th1 polarizing conditions. The probes were added to the culture at indicated time points and analyzed at day 4. Data shown is representative for two independent experiments.

(D) Proliferation of CD4+ T cells under Th1 polarizing conditions for 3 days in the absence (Th0, Th1) or presence of SGC0649 or SGC0946 at 1 µM. The proliferation was assessed by CFSE stain by flow cytometry. The proliferation index in (D) was calculated using FlowJo (v10). Data shown is representative for three independent experiments. Statistical significance between conditions was determined using one-way ANOVA (**p ≤ 0.01).

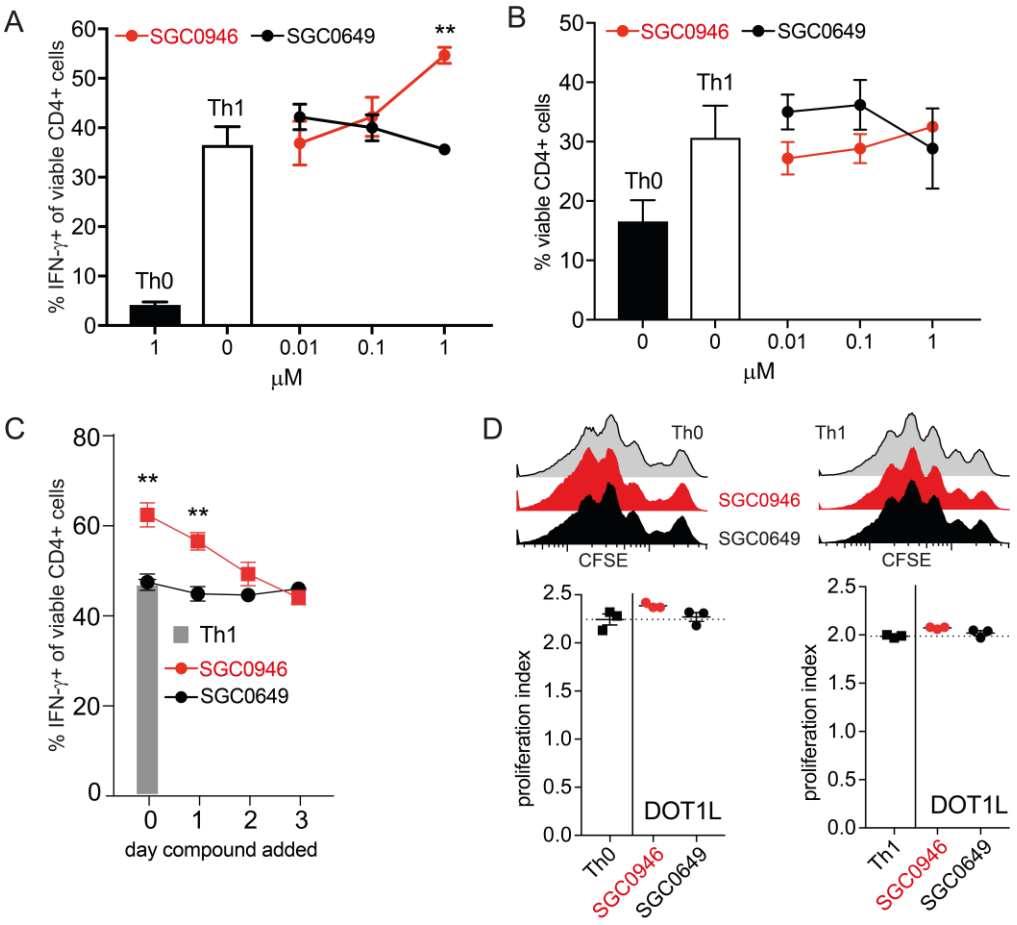


Figure S4. FACS analysis of human Th1 polarized cells in the presence of indicated compounds or their corresponding controls. Naive CD4 T cells were isolated from PBMCs of 3 healthy donors and cultured for 4 days under Th0 or Th1 cell polarizing conditions in the presence of indicated compounds and their controls (1µM) and analyzed by FACS. Data shown is gated on viable CD4+ T cells. Related to **Figures 4C and 4D.**

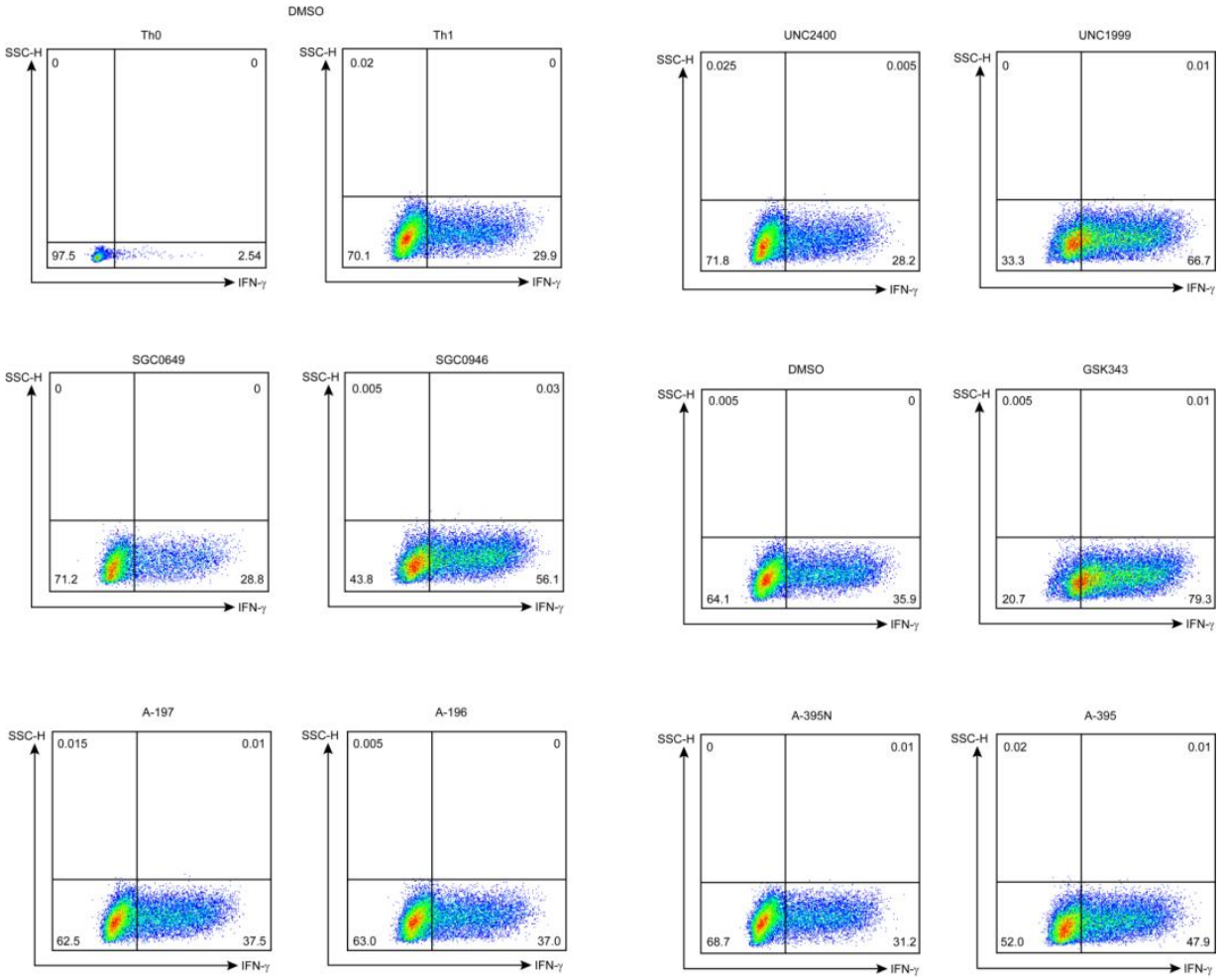


Figure S5. Cell viability after 4 days under indicated polarizing conditions in the presence of PRMT5 inhibitors LLY-283 or GSK591 and their control compounds LLY-284 and SGC2096, respectively. Statistical significance between conditions was determined using one-way ANOVA (**p ≤ 0.01, ***p ≤ 0.001). Data shown is from 3 independent experiments. Related to **Figure 6.**

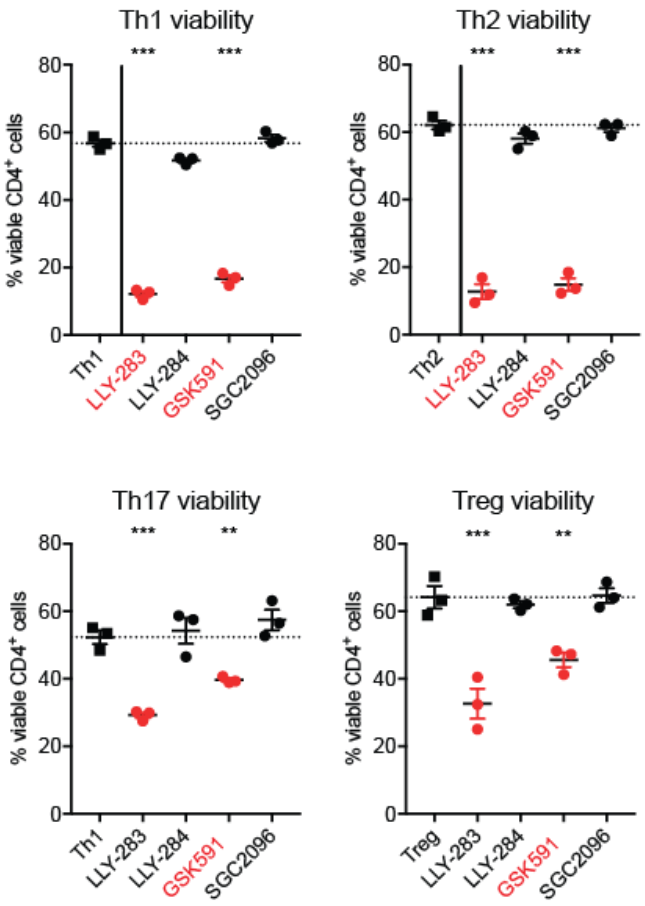
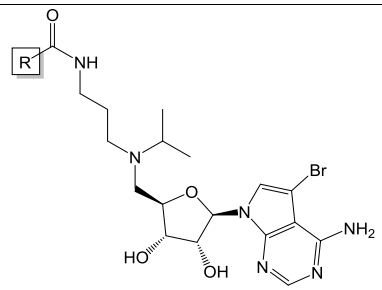
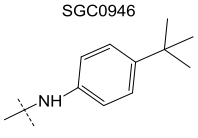
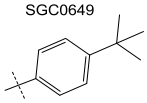
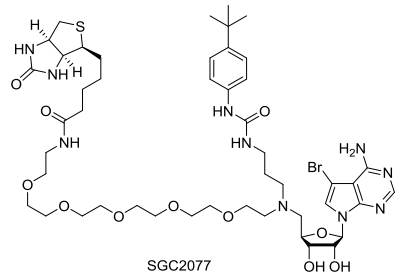
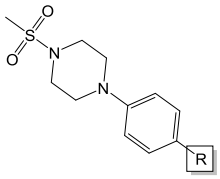
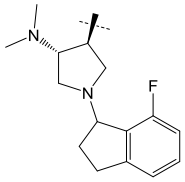
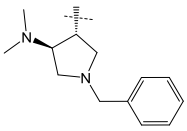
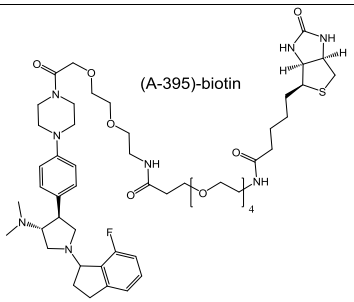
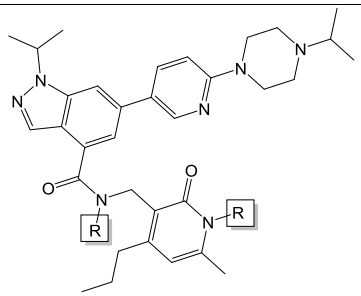
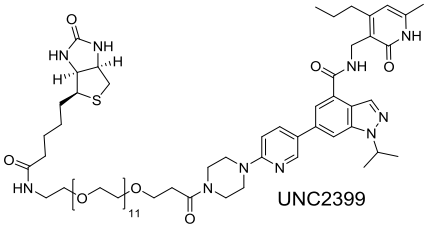
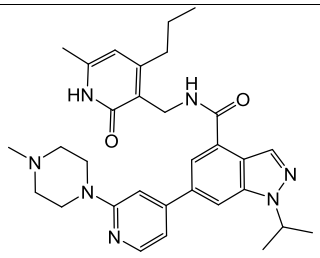
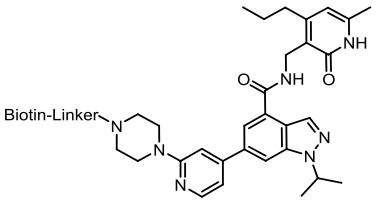


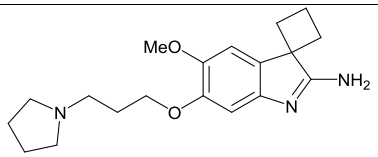
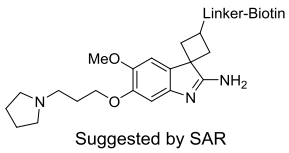
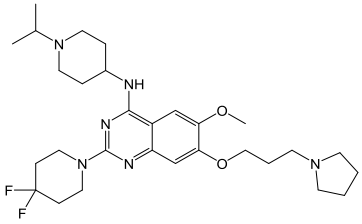
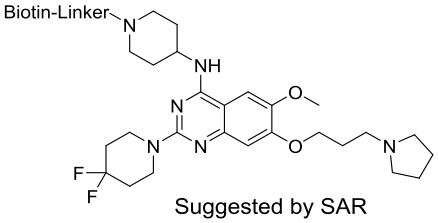
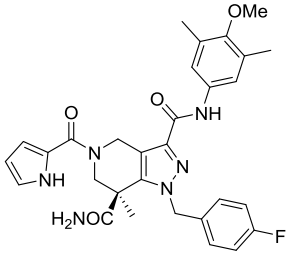
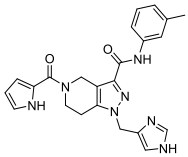
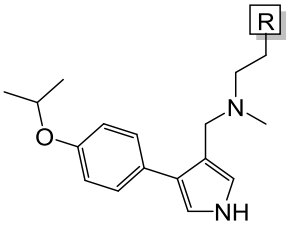
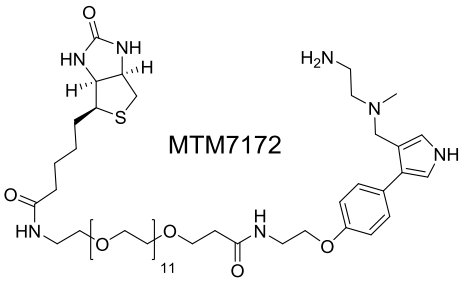
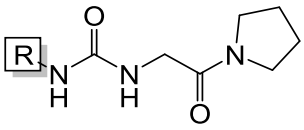
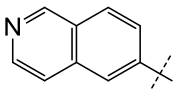
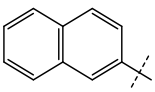
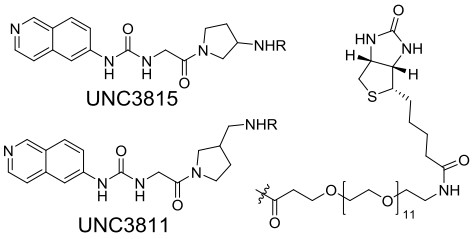
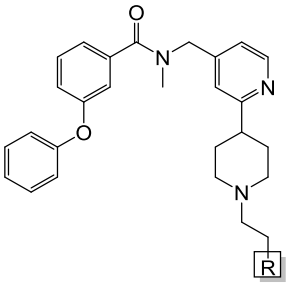
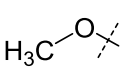
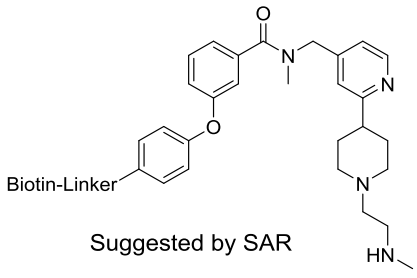
Table S1. A summary of the number of off-target proteins screened and the number of 'hits' with in vitro IC_{50} values less than 1 μM. The panel includes methyltransferases, ion channels (Eurofins (https://www.eurofinsdiscoveryservices.com) and PDSP (https://pdspdb.unc.edu/pdspWeb/)), kinases (Eurofins). Related to **Figure 1C**.

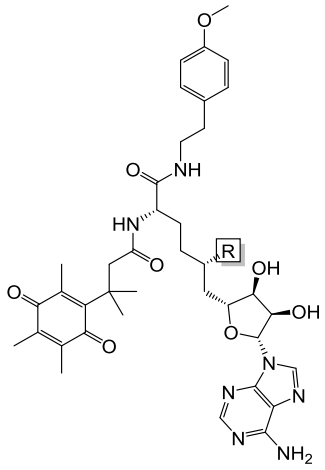
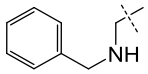
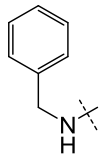
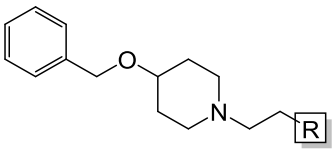
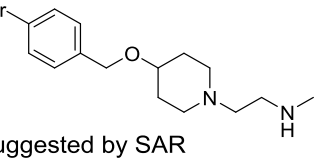
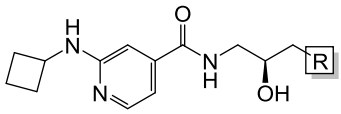
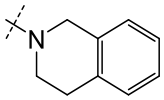
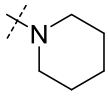
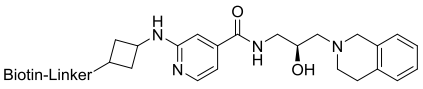
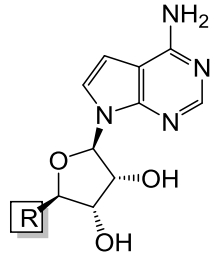
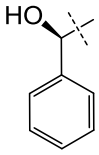
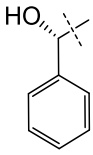
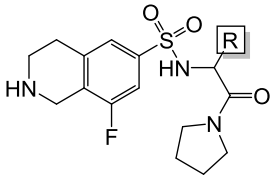
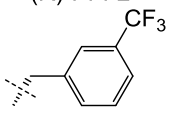
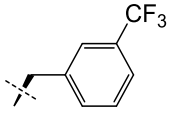
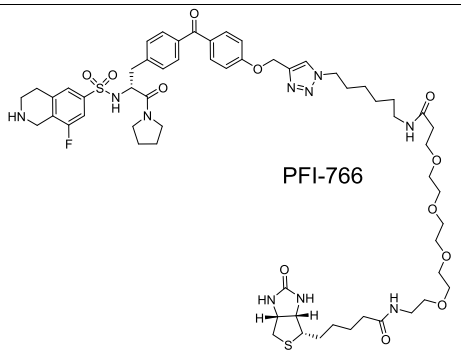
Probes	Controls	PDSP and Eurofins	Methyltransferase panel at the SGC	Total off-targets screened	Hits with Kd ≤ 1 μM	Off-targets Eurofins, PDSP[2]
UNC0642		NT	34	34	0	NT
A-366		NT	34	34	0	NT
A-395*		98	33	139	11	5-HT2B, Alpha-1A, -1B, -1D, -2A, -2B, -2C, H3, NET, SERT, Sigma 2
	A-395N*	95	34	137	1	NA
GSK343		NT	33	33	0	NA
UNC1999		NT	33	33	0	NA
	UNC2400	NT	34	34	0	NA
OICR-9429*		87	34	121	2	5-HT2B, H3
	OICR-0547*	87	34	121	0	NA
BAY-598		78	34	112	2	NK2, A3 (h)
	BAY-369	NT	34	34	0	NA
(R)-PFI-2		118	34	152	0	NA
	(S)-PFI-2	NT	34	34	1	NA
A-196		180	34	214	3	A1(h), A2A(h), PBR
	A-197	118	34	152	0	NA
	SGC2043	NT	34	34	0	NA
MS023		137	34	171	1	Sigma 1
	MS094	55	34	89	0	NA
SGC707		140	34	174	1	NA
	XY1	95	34	129	0	NA
MS049		143	34	177	3	H3, Sigma1, Sigma 2
	MS049N	NT	34	34	0	NA
TP-064		53	34	87	0	NA
	TP-064N	51	34	85	0	NA
SGC0946[1]		130	34	164	7	5-HT transporter (h), 5-HT2A (h), 5-HT2C, alpha 1B (h), alpha 2C (h), Na+ channel (site 2)
	SGC0649	NT	34	34	0	NA
GSK591		135	34	169	8	5-HT1A, 5-HT2B, 5-HT6, Alpha-2A, -2B, -2C, NET, Sigma 1
	SGC2096	NT	34	34	0	NA
LLY-283		53	34	87	0	NA
	LLY-284	51	34	85	1	NA

*These targets have also been screened against 8 methyl lysine binding domains and no activity was measured.
[1] Methyltransferase off-target activity PRMT4 (IC_{50} = 500 nM)
[2] Eurofins and PDSP assays are binding assays only and may not translate into functional activities.
NT – not tested; 'NA' – not applicable

Table S2. Chemical structures of probes, their chemotype-matched controls, and chemical biology reagents (or optimal site of derivatization). The full structure for the probe or control is the 'Core' plus the moiety in the 'Probe' or 'Control' column, respectively. The point of attachment of the moiety on the core is demarcated by the 'R' within a square. The point of attachment to the moiety in 'Probe' and 'Control' columns is the dashed line. See also **Figure 1B, C**.

Protein	Core	Probe	Control	Reagents for chemical biology
DOT1L		SGC0946 	SGC0649 	 SGC2077
EED		A-395 	A-395N 	 (A-395)-biotin The chemical structure for (A-395N)-biotin is shown in the synthetic methods.
EZH2/H1		UNC1999 H	UNC2400 CH_3	 UNC2399 Konze et. al. 2013a
EZH2		GSK343		 Biotin-Linker Suggested by SAR

G9a/GLP		A-366		 Suggested by SAR
G9a/GLP		UNC0642		 Suggested by SAR
IDH1 mutant		GSK864	 GSK990	
PRMT type I		MS023 —NH_2	MS094 —OH	 MTM7172
PRMT3		SGC707 	XY1 	 UNC3815 UNC3811
PRMT4		TP-064 	TP-064N 	 Suggested by SAR

PRMT4		SKI-73 	SKI-73N 	
PRMT4/6		MS049 NHMe 	MS049N OMe 	Biotin-Linker  Suggested by SAR
PRMT5		GSK591 	SGC2096 	 Biotin-Linker Suggested by SAR
PRMT5		LLY-283 	LLY-284 	
SETD7		(R)-PFI-2 	(S)-PFI-2 	 PFI-766

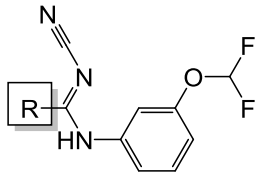
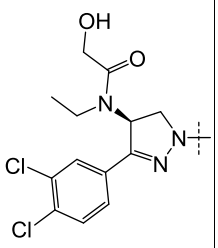
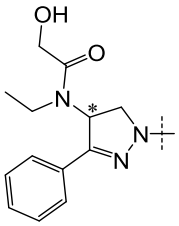
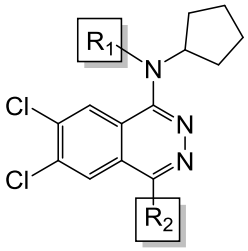
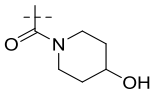
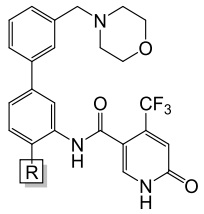
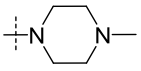
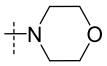
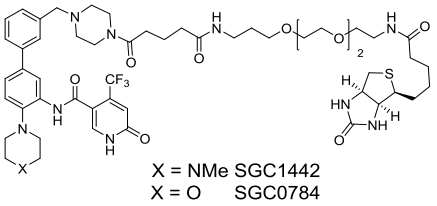
SMYD2		BAY-598 	BAY-369 	
SUV420H1/H2		A-196 R_1 = H R_2 = 4-pyridyl	SGC2043 R_1 = Me R_2 = 4-pyridyl A-197 R_1=H R_2= 	
WDR5		OICR-9429 	OICR-0547 	 X = NMe SGC1442 X = O SGC0784

Table S3. Detailed conditions used in the methyltransferase activity assays. See also **Figure 1C**.

Protein		MT (nM)	Substrate	Concentration (µM)		Buffer (20 mM, pH 8)	DTT (mM)	TCEP (mM)
				Substrate	SAM			
G9a		5	Biot-H3 (1-25)	1	10	potassium phosphate	0	0
GLP		5	Biot-H3 (1-25)	1	10	potassium phosphate	0	0
SUV39H1		10	Biot-H3 (1-25)	0.2	5	Tris-HCl	5	0
SUV39H2		10	Biot-H3 (1-25)	1	2	Tris-HCl	5	0
SETDB1		2	Biot-H3 (1-25)	0.1	5	Tris-HCl	5	0
PRMT1		15	Biot-H4 (1-24)	0.13	5	Tris-HCl	5	0
PRMT3		20	Biot-H4 (1-24)	1	28	Tris-HCl	5	0
PRMT4		75	Biot-H3 (1-25)	1	2	Tris-HCl	5	0
PRMT5-MEP50 Complex	PRMT5	15	Biot-H4 (1-24)	0.12	1	Tris-HCl	5	0
	MEP50							
PRMT6		50	Biot-H4 (1-24)	1	2	Tris-HCl	5	0
PRMT7		25	Biot-H2B (23-37)	0.3	1	Tris-HCl	5	0
PRMT8		20	Biot-H4 (1-24)	1	2	Tris-HCl	5	0
PRMT9		10	Biot-SAP145 (490-529)	0.08	20	Tris-HCl	5	0
SETD8		50	Biot-H4 (1-24)	20	30	Tris-HCl	5	0
SUV420H1		100	Biot-H4K20me1	3	10	Tris-HCl	5	0
SUV420H2		500	Biot-H4K20me1	1	10	Tris-HCl	5	0
SMYD2		30	Biot-p53 (361-380)	3	0.5	Tris-HCl	5	0
SMYD3		10	Biot-MAP3K2 [(249-273)	15	0.5	Tris-HCl	5	0
BCDIN3D		50	Biot-microRNA-145 (23bp)	0.2	2	Tris-HCl	5	0
DNMT1		100	Biot-Hemimethylated ds-DNA	0.6	2	Tris-HCl	5	0
DNMT3A/3L		10	poly(dI-dC)	0.2	0.3	Tris-HCl	5	0
DNMT3B/3L		20	poly(dI-dC)	0.3	1	Tris-HCl	5	0
NSD1		20	Chicken Nucleosume	0.2	2	Tris-HCl	0	2
NSD2		50	Chicken Nucleosume	0.4	3	Tris-HCl	0	2
NSD3		50	Chicken Nucleosume	0.3	3	Tris-HCl	0	2
ASH1L		100	Chicken Nucleosume	0.3	3	Tris-HCl	0	2
SETD2		150	Biot-H3 (21-44)	1	5	Tris-HCl	5	0
DOT1L		10	Chicken Nucleosume	1	1	Tris-HCl	5	0
PRC2 Complex-EZH1	EED	10	Biot-H3 (21-44)	1	4	Tris-HCl	5	0
	EZH1							
	SUZ12							
	RBBP4							
	AEBP2							
PRC2 Complex-EZH2	EED	20	Biot-H3 (21-44)	1	1	Tris-HCl	5	0
	EZH2							
	SUZ12							
PRDM9		1	Biot-H3 (1-25)	1	60	Tris-HCl	5	0
SETD7		10	Biot-H3 (1-25)	1	1	Tris-HCl	5	0
MLL1 Complex	MLL1	20	Biot-H3 (1-25)	2	2	Tris-HCl	5	0
	RBBP5							
	WDR5							
MLL3 Complex	MLL3	100	Biot-H3 (1-25)	12	55	Tris-HCl	5	0
	ASH2L							
	RBBP5							
	WDR5							
	DPY30							

CONTACT FOR REAGENT AND RESOURCE SHARING[#]

Domain: Protein target	Probe	Supplier[‡]	Control	Supplier	PubMed ID
MT: DOT1L	SGC0946[†]	C,M,T	SGC0649	SGC	23250418
WD: EED	A-395	M	A-395N	M	28135237
MT: EZH2	GSK343	C,M,T	-	-	24900432
MT: EZH2/H1	UNC1999	C,M,T	UNC2400	T	23614352
MT: G9a/GLP	A-366	C,M,T	-	-	24900801
	UNC0642	C,M,T	-	-	24102134
MT: PRMT type 1	MS023	C,M,T	MS094	SGC	26598975
MT: PRMT3	SGC707	C,M,T	XY-1	C,T	25728001
MT: PRMT4/6	MS049	C,M,T	MS049N	SGC	27584694
MT: PRMT4	TP-064	M,T	TP-064N	SGC	
	SKI-73*	SGC	SKI-73N	SGC	
MT: PRMT5	GSK591	C,M,T	SGC2096	SGC	26985292
	LLY-283	SGC	LLY-284	SGC	
MT: SETD7	(R)-PFI-2	C,M,T	(S)-PFI-2	C,M,T	25136132
MT: SMYD2	BAY-598	C,M	BAY-369	SGC	27075367
MT: SUV420H1/H2	A-196	C,M,T	A-197, SGC2043	SGC	28114273
WD: WDR5	OICR-9429	C,M,T	OICR-0547	SGC	26167872

*Prodrug of SKI-72
[‡]Cayman Chemical (C), Millipore-Sigma (M), Tocris (T)
[#]SGC Probe collection sold by Cayman Chemical (Cat # 17748)
†Biotinylated probe (SGC2077) is available from the SGC.
Further information about resources and reagents should be directed to Peter Brown (peterj.brown@utoronto.ca).

METHOD DETAILS

- Selectivity assays
- Mice used in the study
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    - Data analysis
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    - Procedure for the Preparation of SGC2043
    - Procedure for the Preparation of SGC2096
    - Procedure for the Preparation of SGC0649
    - Procedure for the preparation of SGC2077
    - Procedure for the Preparation of MTM7172
    - Procedure for the Preparation of UNC3815
    - Procedure for the Preparation of UNC3811
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    - Procedure for the preparation of (A-395)-biotin.
      Procedure for the preparation of (A395N)-biotin

METHOD DETAILS

Selectivity assays

The selectivity assays were performed as previously described (Shen et al., 2016). The effect of test compounds on the methyltransferase activities of G9a, GLP, SUV39H1, SUV39H2, SUV420H1, SUV420H2, SETD2, SETD8, SETDB1, SETD7, MLL1 trimeric complex, MLL3 pentameric complex, EZH1 (PRC2) pentameric complex, EZH2 (PRC2) trimeric complex, PRMT1, PRMT3, PRMT4, PRMT5/ MEP50 complex, PRMT6, PRMT7, PRMT8, PRMT9, PRDM9, SMYD2, SMYD3, DNMT1 and BCDIN3D was assessed by monitoring the incorporation of a tritium-labeled methyl group into substrates (Supplementary Table 3) using scintillation proximity assay (SPA). Briefly, a 10-µl reaction containing 3H-SAM and substrate at concentrations close to the apparent Km values for each enzyme was prepared. The reactions were quenched with 10 µl of 7.5 M guanidine hydrochloride; 180 µl of 20 mM Tris buffer (pH 8.0) were added, and the mixture was transferred to a 96-well FlashPlate followed by incubation for 1 h. The counts per minute (CPM) was measured on a TopCount plate reader; the CPM in the absence of compound or enzyme was defined as 100% activity and background (0%), respectively, for each dataset.

For DNMT1, the double-stranded DNA substrate was prepared by annealing two complementary strands (biotinylated forward strand: B-GAGCCCGTAAGCCCGTTCAGGTCG and

reverse strand: CGACCTGAACGGGCTTACGGGCTC) that were synthesized by Eurofins MWG Operon (Louisville, KY, USA).

A filter-based assay was used for DOT1L, NSD1, NSD2, NSD3, ASH1L, DNMT3A/3L, and DNMT3B/3L in which 10 µl of reaction mixture were incubated at 23°C for 1 h, followed by addition of 50 µl of 10% trichloroacetic acid (TCA). The mixture was transferred to filter plates (Millipore, Billerica, MA, USA) that were centrifuged at 2000 rpm (Allegra X-15R; Beckman Coulter, Brea, CA, USA) for 2 min, washed twice with 10% TCA and once with ethanol (180 µl), and centrifuged. After drying, 100 µl MicroScint-O (Perkin Elmer) was added to each well and the plates were centrifuged to remove the liquid. A 70-µl volume of MicroScint-O was added and the CPM was measured with a TopCount plate reader.

Mice

C57BL/6 mice and reporter mice for IFN-γ(Reinhardt et al., 2009), IL-4 (Mohrs et al., 2001) or Foxp3 (Wan and Flavell, 2005) on C57BL/6 background were used for the polarization assays, where possible. Where unavailable, intracellular staining for IFN-γ (XMG1.2), IL-13 (eBio13A), IL-17 (eBio17B7) or Foxp3 (FJK-16S) was performed. All animal experiments were approved by the Monash University Animal Care Committee.

T cell polarization, proliferation and flow cytometry for mouse and human samples

Mouse CD4+ T cells were isolated with the CD4+ T Cell-Negative Isolation Kit (Stemcell Technologies) and polarized for optimal results for 4 days under Th0, Th1, Th2, Th17 and Treg conditions as previously described (Antignano et al., 2014). In short, 175.000 naïve CD4+ T cells were cultured under Th0 (IL-2 [10 ng/ml)), Th1 (IL-2, IL-12 [10 ng/ml each], anti-IL-4 [10 µg/ml)), Th2 (IL-2, IL-4 [10 ng/ml each), anti IFN-γ [10 µg/ml]), Th17 (IL-23, IL-1β, TNF-α [10 ng/ml each], IL-6 [20 ng/ml], TGF-β [1 ng/ml], anti-IL-4 and anti-IFN-γ [10 µg/ml each] or Treg conditions (IL-2 and TGF-β [10 ng/ml each] in 96-well plates (pre-coated overnight with 1 µg/ml of each anti-CD3 and anti-CD28) in 200 µl complete RPMI media in the absence or presence of indicated amounts of the chemical probes. Viability of the cells was determined using fixable viability dye. Proliferation assay of CFSE labelled mouse CD4+ T cells was performed under Th0 or Th1 polarizing conditions and analyzed at day 3. Naïve CD4+ T cells were isolated from blood samples of three healthy donors using the human naïve CD4+ T Cell Isolation Kit II (Miltenyi). One hundred twenty thousand naïve CD4+ T cells were polarized towards Th1 cells using anti-CD3/CD28 antibodies (1 bead:5 cells ratio) in the presence of recombinant IL-12 [10 ng/mL], anti-IL4 antibody [10 mg/mL] and recombinant IL-2 [10 ng/mL] for 4 days in the presence of chemical probes or their respective probe controls at a concentration of 1µM which is in the range of the cellular IC50-IC90 of most of the active chemical probes. The media was not changed and compounds were not replenished over the duration of the experiment. Naïve CD4+ T cells were maintained in culture in the presence of IL-2 [10 ng/mL] for 4 days and used as a control.

Cells were stained with SYTOX™ Blue Dead Cell Stain (Thermo Fisher Scientific), fixed and permeabilized, followed by the intracellular staining with anti-IFN-γ antibody.

RNA-Seq and bioinformatics

Naïve $CD4^+$ T cells (CD44neg, CD62Lhi) and CD4+ T cells positive (IFN-γ^+) or negative (IFN-γ) for YFP after Th1 polarization were sorted and total RNA was extracted using the Nucleospin RNA kit (Macherey-Nagel), according to the manufacturer's instructions. RNA was isolated with an mRNA kit (TruSeq Stranded; Illumina, San Diego, CA, USA) and sequenced on a MiSeq paired-end run (75 x 75, v3; Illumina). Samples were aligned to the mm10 transcript reference using TopHat2, and differential expression was assessed using Cufflinks (Illumina). Visualization of the data was performed using DEGUST (https://github.com/drpowell/degust) and represent the average expression from 3 biological replicates (x-axis) and the Log2-fold change of SGC0946-treated cells over SGC0649-treated cells (y-axis). The RNA-Seq datasets described in this article are available at the National Center for Biotechnology Information (accession number GSE106978).

Western blot assays

Enriched $CD4^+$ T cells were cultured in the absence (Th1) or presence of indicated chemical probes for 4 days (or as indicated in the experiment) under Th1 polarizing conditions. Cells were harvested and pellets were frozen at -80˚C. Histones were extracted from frozen cell pellets by incubating in 0.2 N HCl overnight at 4˚C. Supernatants were run on 12% SDS-PAGE gels. H3K27me3 and H3K79me2 were detected using clones ab6002 and ab3594 (Abcam), respectively. A pan H3 antibody (ab1791, Abcam) was used as a loading control.

Chemical proteomics

Cell lines

G401 and HEK293T were obtained from ATCC (Virginia, USA) and cultured at 37 °C in a humidified 5% CO2 atmosphere in McCoy's Medium containing 10% FBS and DMEM Medium containing 10% FBS, respectively. For cell lysate experiments, cells were grown until approximately 80% confluency before being pelleted and washed with PBS. Cell pellets were subsequently lysed in buffer A (50 mM Tris pH 7.5, 0.8% v/v NP-40, 5% v/v glycerol, 1.5 mM $MgCl_2$, 100 mM NaCl, 25 mM NaF, 1mM Na_3VO_4, 1mM PMSF, 1mM DTT, 10 µg/mL TLCK, 1 µg/mL Leupeptin, 1 µg/mL Aprotinin, 1 µg/mL soy bean trypsin) and lysates prepared and cleared by ultracentrifugation, as previously described(Grebien et al., 2015). Protein concentration was determined (and lysates stored at -80 °C until use.

Western blotting

Eluted proteins were separated on polyacrylamide gels with SDS running buffer (50 mM MES, 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.3) and transferred to nitrocellulose blotting membranes. Membranes were blocked with blocking buffer (2.5% (m/v) BLOT-QuickBlocker (Merck) in PBST

(Phosphate-buffered saline with Tween: 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl 2.7 mM KCl, 0.05% (v/v) Tween 20) before probing with antibodies. Quantitative analysis of the western blots was performed using ImageQuant TL and represents signal strength as percentage of day zero.

Antibodies

Mouse-anti-EED, clone GT671, ThermoFisher MA5-16314, CARM1 Bethyl Laboratories inc. Catalog No. A300-421A.

Drug affinity chromatography

Amine derivatized compounds were coupled to NHS-activated Sepharose 4 fast flow beads (GE Healthcare) as described previously (Huber and Superti-Furga, 2016). Briefly, 100 µL of bead slurry (50% in isopropanol) were used for each pulldown experiment. Beads were washed with DMSO (3 × 500 µL), and resuspended in DMSO (50 µL), to which the amine (0.025 µmol) and triethylamine (0.75 µL) were added. The beads were incubated at room temperature for 16 hours, and depletion of free amine from the supernatant determined by LC-MS analysis. Ethanolamine (2.5 µL) was then added to block any unreacted NHS sites, and the beads incubated for a further 16 hours. Derivatized beads were then washed with DMSO (3 × 500 µL), buffer A (3 × 1 mL), and incubated with cell lysates (2 mg of protein per pulldown, at 6 mg/mL) that had been pre-treated with either compound (20 µM) or DMSO control for 30 minutes at 4 °C. Beads and treated lysates were incubated for 2 hours at 4 °C, before being washed with buffer A (5 mL), Buffer B (50 mM HEPES pH 7.5, 100 mM NaCl, 500 µM EDTA, 2.5 mL), and eluted with formic acid (100 mM, 250 µL). Samples were neutralised with triethylammoniumbicarbonate (TEAB, 1M, 62.5 µL) and stored at -20°C until preparation for proteomic analysis.

Biotin derivatized compounds were coupled to UltraLink Immobilized Streptavidin Plus beads (GE healthcare) as described previously (Grebien et al., 2015). Briefly, 100 µL of bead slurry (50% in isopropanol) were used for each pulldown experiment. Beads were washed with buffer A (3 x 500 µL) and resuspended (1 mL), to which the biotinylated compound was added (0.05 µmol) and incubated for 30 minutes at 4°C followed by a final wash step with Buffer A (2 × 1 mL). Lysates were precleared by the addition of 100 µL of bead slurry (50% in isopropanol) and incubated for 30 minutes at 4 °C. After preclearing, lysates were treated with either compound at the indicated concentration or DMSO control for 30 minutes at 4 °C followed by incubation with the affinity matrices for two hours at 4 °C. Affinity matrices were washed with buffer A (5 mL), Buffer B (50 mM HEPES pH 7.5, 100 mM NaCl, 500 µM EDTA, 2.5 mL), and proteins eluted with Buffer C (3M Urea, 50mM formic acid, 10 mM DTT, 250 µL). Samples were neutralised with triethylammoniumbicarbonate (TEAB, 100mM, 30 µL) and stored at -20°C until preparation for proteomic analysis. For Western blot experiments bound proteins were eluted by addition of 100 µL of 2x sample buffer (65.8 mM Tris-HCl pH6.8, 26.3% (w/v) glycerol, 2.1% SDS, 0.01% bromophenol Blue, 50 mM DTT).

MS sample preparation

Samples were reduced with DTT (10 mM final concentration) for 30 minutes at room temperature, alkylated with iodoacetamide (55 mM final concentration) for 30 minutes at room temperature, diluted to 300 µL with TEAB, and incubated with trypsin (6 µL, 0.2 mg/mL) overnight at 37 °C. The digests were then desalted using SEPAC lite columns (Waters), eluted with 69% v/v MeCN , 0.1% v/v FA in H2O (1 mL) and dried in vacuo. Dried peptides were stored at -20°C before resuspension in 2% V/V MeCN,).1% v/v FA in H2O (20 µL) for LC-MS/MS analysis

LC-MS/MS data acquisition

Mass spectrometry data was acquired at the Discovery Proteomics Facility (University of Oxford). Digested samples were analysed by nano-UPLC–MS/MS using a Dionex Ultimate 3000 nano UPLC with EASY spray column (75 µm × 500 mm, 2 µm particle size, Thermo Scientific) with a 60 min gradient of 0.1% (v/v) formic acid in 5% (v/v) DMSO to 0.1% (v/v) formic acid with 35% (v/v) acetonitrile in 5% (v/v) DMSO at a flow rate of approximately 250 nL min−1 (600 bar per 40 °C column temperature). Mass spectrometry data were acquired either with an Orbitrap Q Exactive (Survey scans acquired at a resolution of 70,000 @ 200m/z and the 15 most abundant precursors were selected for HCD fragmentation), or an Orbitrap Q Exactive High Field (HF) instrument (survey scans were acquired at a resolution of 60,000 at 400 m/z and the 20 most abundant precursors were selected for CID fragmentation.)

Data analysis

Raw data was processed using MaxQuant version 1.5.0.253 and the reference complete human proteome FASTA file (Uniprot). Label Free Quantification (LFQ) and Match Between Runs were selected; replicates were collated into parameter groups to ensure matching between replicates only. Cysteine carbamidomethylation was selected as a fixed modification, and methionine oxidation as a variable modification. Default settings for identification and quantification were used. Specifically, a minimum peptide length of 7, a maximum of 2 missed cleavage sites, and a maximum of 3 labelled amino acids per peptide were employed. Peptides and proteins were identified utilising a 0.01 false discovery rate, with “Unique and razor peptides” mode selected for both identification and quantification of proteins (razor peptides are uniquely assigned to protein groups and not to individual proteins). At least 2 razor + unique peptides were required for valid quantification. Processed data was further analysed using Perseus version 1.5.0.9 and Microsoft Excel 2010. Peptides categorised by MaxQuant as ‘potential contaminants’, ‘only identified by site’ or ‘reverse’ were filtered, and the LFQ intensities transformed by Log2. Experimental replicates were grouped, and 2 valid LFQ values were required in at least one experimental group. Missing values were imputed using default settings, and the data distribution visually inspected to ensure that a normal distribution was maintained. Statistically significant competition was determined through the application of P2 tests, using a permutation-based FDR of 0.05 and an S0 of 2, and visualised in volcano plots. Significantly competed targets were further analysed in STRING (http://string-db.org) and protein interaction networks generated.

Data deposition

The mass spectrometry proteomics data have deposited to the ProteomeXchange Consortium via the PRIDE partner repository (Vizcaino et al., 2016) with the data set identifier XXXXXX.

Synthesis of reagents

Procedure for the Preparation of PFI-766

Procedure for the Preparation of SGC2043

Procedure for the Preparation of SGC2096

Procedure for the Preparation of SGC0649

Procedure for the preparation of SGC2077

Procedure for the Preparation of MTM7172

Procedure for the Preparation of UNC3815

Procedure for the Preparation of UNC3811

Procedure for the preparation of SGC1442 and SGC0784

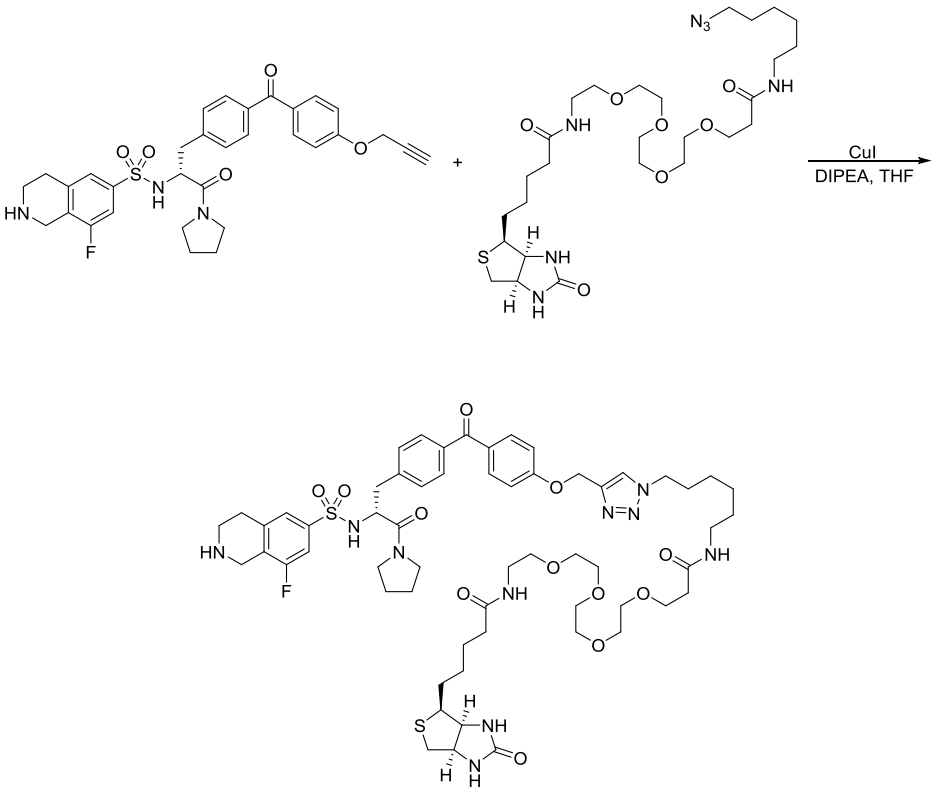
Procedure for the preparation of (A-395)-biotin.

Procedure for the preparation of (A395N)-biotin

Chemistry General Procedures: Analytical thin-layer chromatography (TLC) was performed employing EMD Milipore 210-270 μm 60-F254 silica gel plates. The plates were visualized by exposure to UV light. Flash column chromatography was performed on a Teledyne ISCO Combi*Flash* Rf^+ system equipped with a variable wavelength UV detector and a fraction collector using Redi*Sep* Rf normal or reverse phase silica columns. Nuclear Magnetic Resonance (NMR) spectra were acquired on a Bruker DRX-600 spectrometer or on a Varian Mercury spectrometer at 400 MHz. Chemical shifts are reported in parts per million (ppm, δ) scale relative to solvent residual peak (chloroform-d, ^{1}H: 7.26 ppm; methanol-d_4, ^{1}H: 3.31 ppm). ^{1}H NMR data are reported as follows: chemical shift, multiplicity (s = singlet, br s = broad singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet, app = apparent), coupling constant, and integration. HPLC spectra for all compounds were acquired using an Agilent 6110 series system with a UV detector set to 254 nm. Samples were injected (5 μL) onto an Agilent Eclipse Plus, 4.6 Å~ 50 mm, 1.8 μM, C18 column at room temperature. Either with a linear gradient from 50% to 100% B (MeOH + 0.1%acetic acid) in 5.0 min was followed by pumping 100% B for another 2 min with A being H_2O + 0.1% acetic acid or by a linear gradient from 10% to 100% B (MeOH + 0.1% acetic acid) in 5.0 min was followed by pumping 100% B for another 2 min with A being H_2O + 0.1% acetic acid. Preparative HPLC was performed on an Agilent Prep 1200 series with a UV detector set to 254 nm. Samples were injected onto a Phenomenex Luna, 75×30 mm, 5μM, C18column at room temperature. The flow rate was 40 mL/min. A linear gradient was used with 10% (or 50%) MeOH (A) in 0.1% TFA in H_2O (B) to 100% MeOH (A). Mass spectrometry (MS) data were acquired in positive ion mode using an Agilent 6110 single-quadrupole mass spectrometer with an electrospray ionization (ESI) source. HRMS analysis was

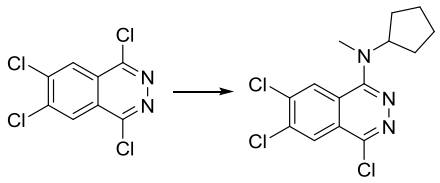
conducted on an Agilent Technologies G1969A high-resolution API-TOF mass spectrometer attached to an Agilent Technologies 1200 HPLC system. Samples were ionized by electrospray ionization (ESI) in positive mode. All biologically evaluated compounds had > 95% purity using the HPLC methods described above.

Procedure for the Preparation of PFI-766

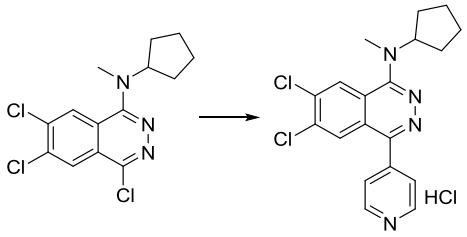


Procedure involves dissolving (*R*)-8-fluoro-*N*-(1-oxo-3-(4-(4-(prop-2-yn-1-yloxy)benzoyl)phenyl)-1-(pyrrolidin-1-yl)propan-2-yl)-1,2,3,4-tetrahydroisoquinoline-6-sulfonamide[1] (152 mg, 0.258 mmol) in THF (1.7 mL) at room temperature, followed by the addition of *N*-(6-azidohexyl)-1-(5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamido)-3,6,9,12-tetraoxapentadecan-15-amide (170 mg, 0.271 mmol), DIPEA (220 μL, 1.29 mmol), and CuI (10 mg, 0.052 mmol). The resulting solution was stirred for 2 h prior to filtration of the entire mixture through a small plug of Celite® and subsequent removal of all solvents under reduced pressure. The residue was purified by column chromatography on silica gel using 0-100% MeOH/EtOAc as the eluent to yield **PFI-766** (90 mg, 29%). LRMS (ESI): 1206.2 $[M+H]^+$.

Procedure for the Preparation of SGC2043

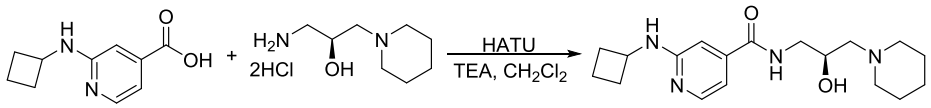


1,4,6,7-Tetrachlorophthalazine (1.35 g, 5.04 mmol) was dissolved in DMSO (13 mL) at room temperature, followed by the addition of *N*,*N*-diisopropylethylamine (1.14 mL, 6.55 mmol). The resulting mixture was then heated to 80 °C, followed by the dropwise addition of *N*-methylcyclopentanamine (0.500 g, 5.04 mmol) in DMSO (1 mL). After addition, the resulting solution was stirred for an additional 2 h prior to cooling to room temperature. The solution was stirred for 16 h at room temperature before dilution with brine (50 mL), and extraction with EtOAc (3 x 20 mL). The combined organic extracts were dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The residue of 4,6,7-trichloro-*N*-cyclopentyl-*N*-methylphthalazin-1-amine (1.62 g, 97%) was then dried under vacuum for 16 h, and used in the subsequent reaction without further purification. LRMS (ESI): 331.6 $[M+H]^+$.



4,6,7-Trichloro-*N*-cyclopentyl-*N*-methylphthalazin-1-amine (400 mg, 1.21 mmol) was dissolved in dioxane/H_2O (4:1, 25 mL) at room temperature, followed by the addition of 4-pyridinylboronic acid (149 mg, 1.21 mmol), potassium carbonate (501 mg, 3.63 mmol), and solid-supported tetrakis(triphenylphosphine)palladium (0) (Aldrich # 511579, 0.5-0.9 mmol/g loading, 0.242 mg, 0.121 mmol). The resulting solution was then heated to 80 °C for 16 h prior to cooling to room temperature, dilution with brine (40 mL), and extraction with EtOAc (3 x 30 mL). The combined organic extracts were dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (10-100% EtOAc/hexanes), and the appropriate product fraction collected prior to removal of all solvents under reduced pressure. The residue was then dissolved in aqueous HCl solution (1 N, 10 mL) before being washed with EtOAc (2 x 20 mL) to remove some triphenylphosphine contaminant. The aqueous layer was subsequently frozen and lyophilized over 16 h to yield 6,7-dichloro-*N*-cyclopentyl-*N*-methyl-4-(pyridin-4-yl)phthalazin-1-amine hydrochloride (**SGC2043**) (116 mg, 23%). LRMS (ESI): 374.2 $[M+H]^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.01 (d, *J* = 5.7 Hz, 2H), 8.43 (s, 1H), 8.17 (d, *J* = 5.6 Hz, 2H), 8.15 (s, 1H), 4.66-4.59 (m, 1H), 3.22 (s, 3H), 2.09-2.00 (m, 2H), 1.88-1.73 (m, 4H), 1.67-1.56 (m, 2H).

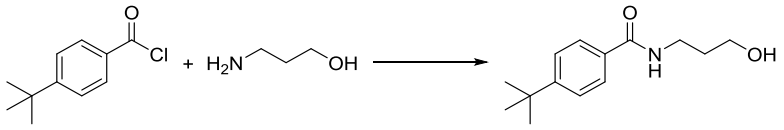
Procedure for the Preparation of SGC2096



Procedure involves suspending 2-(cyclobutylamino)isonicotinic acid (270 mg, 1.40 mmol) in CH_2Cl_2 (6 mL) at room temperature, followed by the addition of (*S*)-1-amino-3-(piperidin-1-yl)propan-2-ol dihydrochloride (390 mg, 1.69 mmol), HATU (532 mg, 1.40 mmol), and DMF (2 mL). Triethylamine (0.59 mL, 4.20 mmol) was then added, and the resulting solution stirred for 16 h prior to dilution with brine (15 mL), adjustment to pH = 13 with aqueous sodium hydroxide solution, and extraction of the aqueous layer with EtOAc (3 x 20 mL). The combined organic extracts were dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel using 0-40% MeOH/EtOAc as the eluent to yield (*S*)-2-(cyclobutylamino)-*N*-(2-hydroxy-3-(piperidin-1-yl)propyl)isonicotinamide (235 mg, 51%). LRMS (ESI): 333.2 $[M+H]^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.43 (br t, 1H), 8.03 (d, *J* = 5.0 Hz, 1H), 6.96 (d, *J* = 7.5 Hz, 1H), 6.81 (d, *J* = 5.0 Hz, 1H), 6.77 (s, 1H), 4.34-4.21 (m, 1H), 3.85-3.75 (m, 1H), 3.38-3.30 (m, 2H), 3.21-3.12 (m, 1H), 2.48-2.35 (br m, 4H), 2.34-2.22 (br m, 4H), 1.93-1.82 (m, 2H), 1.75-1.64 (m, 2H), 1.55-1.44 (br m, 4H), 1.41-1.32 (br s, 2H).

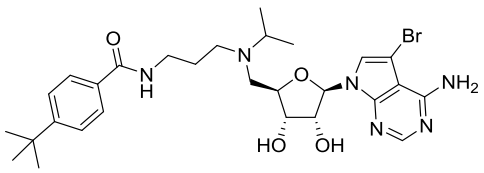
Procedure for the Preparation of SGC0649

The procedure described for the synthesis of **SGC0946** (Supplementary Information, *Nature Communications*, DOI: 10.1038/ncomms2304) was also used for the synthesis of **SGC0649**. The only difference between the two procedures involved replacement of 1-(4-(*tert*-butyl)phenyl)-3-(3-hydroxypropyl)urea with 4-(*tert*-butyl)-*N*-(3-hydroxypropyl)benzamide, the synthesis of which is described below.



To a solution of 3-aminopropan-1-ol (7.78 mL, 101.7 mmol) and triethylamine (3.54 mL, 25.4 mmol) in CH_2Cl_2 (50 mL) at 0 °C was added 4-(*tert*-butyl)benzoyl chloride (4.97 mL, 25.4 mmol) dropwise over 10 minutes. The resulting solution was warmed to room temperature, and stirred for an additional 16 hours before being diluted with H_2O (50 mL). After the layers were separated, the organic layer was washed with HCl solution (1 M, 2 x 25 mL), dried with anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure to afford the title compound as a colorless oil (4.61 g, 77%). The purity of this material was assessed as >95% by ^{1}H NMR, and used in the subsequent reaction without further purification. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.74 (d, *J* = 8.4 Hz, 2H), 7.43 (d, *J* = 8.5 Hz, 2H), 7.06 (s, 1H), 3.74 – 3.68 (m, 2H), 3.64 – 3.59 (m, 2H), 1.78 (dt, *J* = 11.6, 5.8 Hz, 2H), 1.33 (s, 9H). LRMS (ESI): 236.2 $[M+H]^+$.

***N*-(3-((((2*R*,3*S*,4*R*,5*R*)-5-(4-Amino-5-bromo-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl)(isopropyl)amino)propyl)-4-(*tert*-butyl)benzamide (SGC0649)**



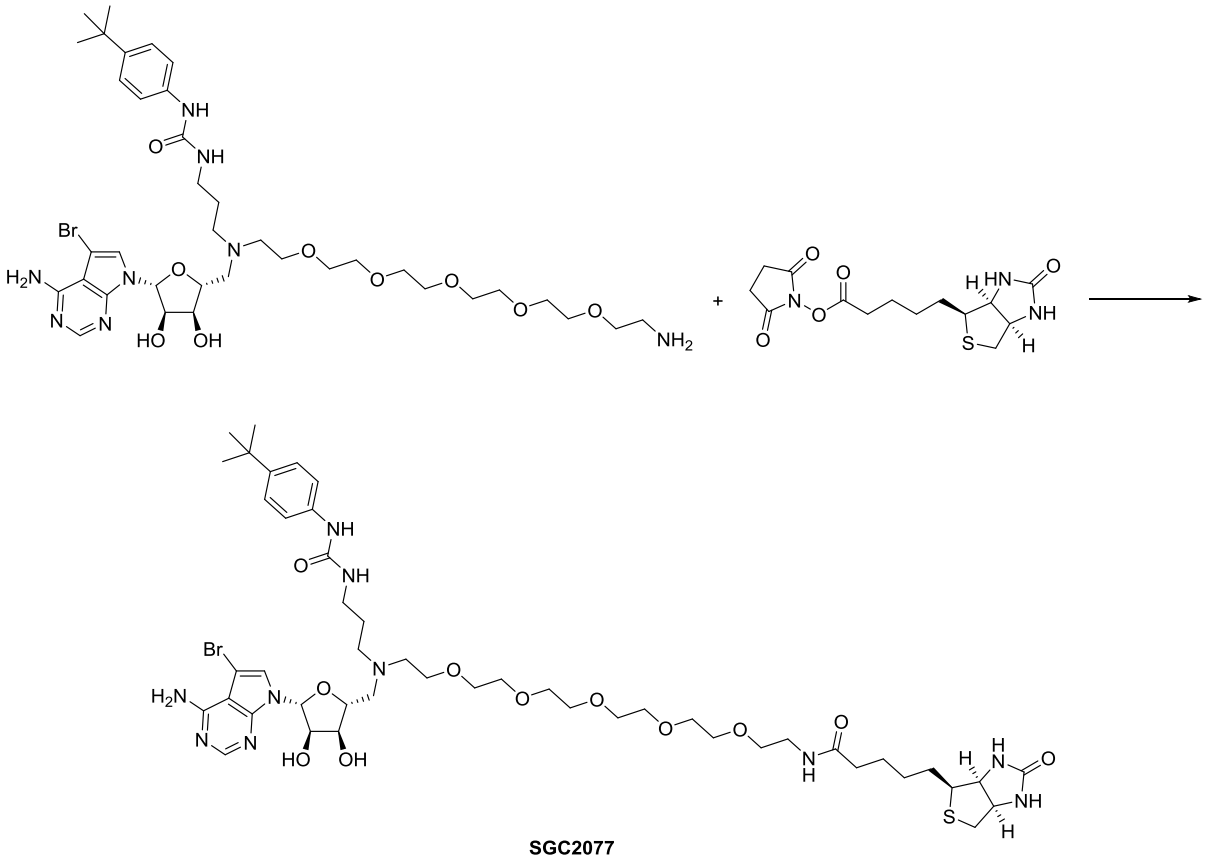
SGC0649

The title compound was isolated as a white powder. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.43 (br s, 1H), 8.11 (s, 1H), 7.74 (d, *J* = 8.3 Hz, 2H), 7.69 (s, 1H), 7.41 (d, *J* = 8.3 Hz, 2H), 6.81 (br s, 1H), 6.09 (d, *J* = 5.5 Hz, 1H), 5.48 (br s, 1H), 4.42 (br s, 1H), 4.20 – 3.91 (m, 2H), 3.34 – 3.21 (m, 2H), 3.17 (s, 1H), 2.64 (br s, 2H), 1.92 (s, 2H), 1.72 (br s, 2H), 1.28 (s, 9H), 1.04 (s, 3H), 0.98 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 172.46, 158.60 (q, *J* = 30.8 Hz), 157.42, 154.22, 153.03, 150.27, 132.32, 127.41 (2), 125.38 (2), 122.29, 117.78 (q, *J* = 300.2 Hz). 101.53, 87.61, 73.42, 72.13, 52.75, 49.06, 48.81, 37.95, 35.00, 31.41 (3), 21.54, 17.24 (2). LRMS (ESI): 603.8 $[M+H]^+$.

Procedure for the preparation of SGC2077

The procedure described for the synthesis of 1-(1-amino-18-(((2*R*,3*S*,4*R*,5*R*)-5-(4-amino-5-bromo-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl)-3,6,9,12,15-pentaoxa-18-azahenicosan-21-yl)-3-(4-(*tert*-butyl)phenyl)urea (**12**) (Supplementary Information, *Nature Structural & Molecular Biology*, DOI: 10.1038/nsmb.3249) was also used for the synthesis of **SGC2077**. The amine (**12**) was prepared, and then biotinylated according to the procedure described below.

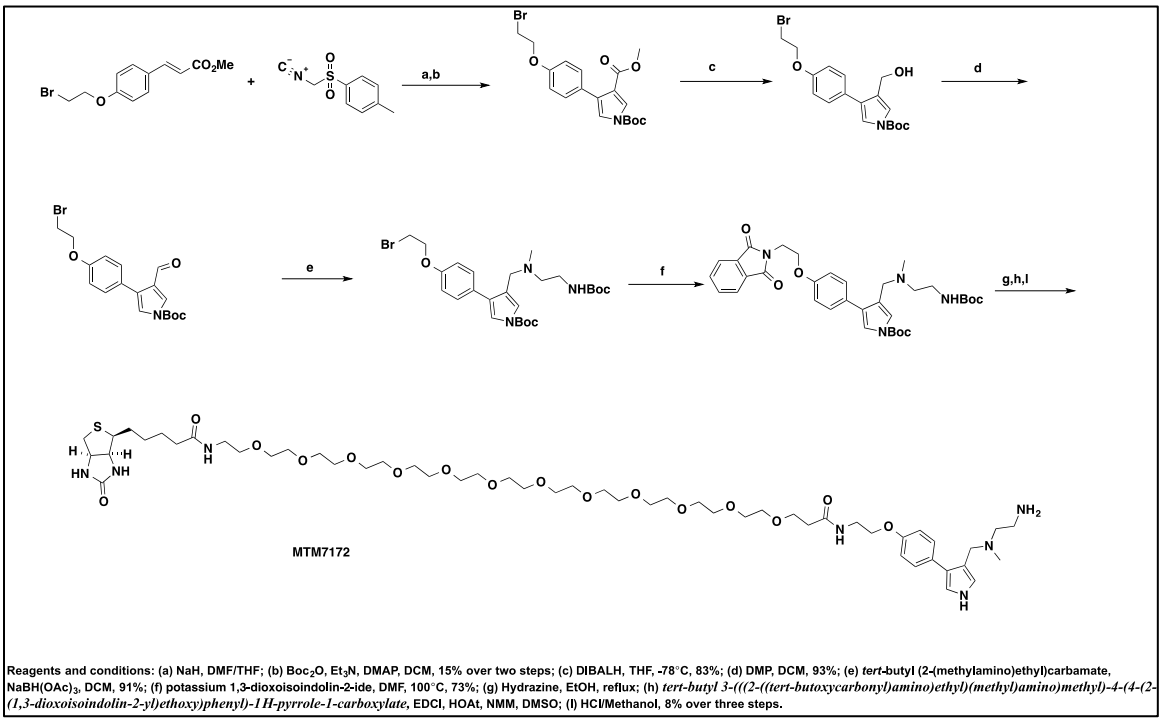
***N*-(6-(((2*R*,3*S*,4*R*,5*R*)-5-(4-Amino-5-bromo-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl)-1-((4-(*tert*-butyl)phenyl)amino)-1-oxo-9,12,15,18,21-pentaoxa-2,6-diazatricosan-23-yl)-5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1H-thieno[3,4-*d*]imidazol-4-yl)pentanamide (SGC2077)**



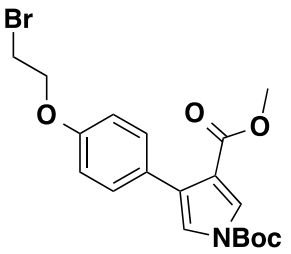
SGC2077

To a solution of 1-(1-amino-18-(((2*R*,3*S*,4*R*,5*R*)-5-(4-amino-5-bromo-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl)-3,6,9,12,15-pentaoxa-18-azahenicosan-21-yl)-3-(4-(*tert*-butyl)phenyl)urea (0.404 g, 0.481 mmol) in DMF (5.7 mL) at room temperature was added triethylamine (1 mL) until pH = 9. Biotin-OSu (0.197 g, 0.577 mmol) was then added, and the resulting solution stirred for 2 hours. The reaction mixture was diluted with saturated aqueous $NaHCO_3$ solution (25 mL), and extracted with CH_2Cl_2 (3 x 15 mL). The combined organic extracts were dried with anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel (Biotage SNAP 25 g column, 5-40% MeOH/EtOAc as the eluent, 26 CV) to afford the title compound as a white powder (0.220 g, 43%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.45 (s, 1H), 8.11 (s, 1H), 7.83 (s, 1H), 7.66 (s, 1H), 7.29 (d, J = 8.5 Hz, 2H), 7.21 (d, J = 8.4 Hz, 2H), 6.79 (br s, 2H), 6.42 (s, 1H), 6.36 (s, 1H), 6.25 (s, 1H), 6.06 (d, J = 5.5 Hz, 1H), 5.42 (br s, 1H), 5.29 (br s, 1H), 4.39 – 4.33 (m, 1H), 4.33 – 4.27 (m, 1H), 4.15 – 4.10 (m, 1H), 4.04 – 4.00 (m, 1H), 3.94 – 3.88 (m, 1H), 3.51 – 3.45 (m, 19H), 3.39 (t, J = 5.8 Hz, 3H), 3.23 – 3.15 (m, 2H), 3.13 – 3.04 (m, 3H), 2.80 (td, J = 12.4, 5.1 Hz, 2H), 2.67 – 2.60 (m, 3H), 2.58 (d, J = 12.5 Hz, 1H), 2.06 (t, J = 7.3 Hz, 2H), 1.66 – 1.41 (m, 6H), 1.34 – 1.26 (m, 2H), 1.25 (s, 9H). LRMS (ESI): 1067.8 $[M+H]^+$.

Procedure for the Preparation of MTM7172

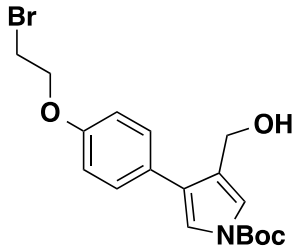


Reagents and conditions: (a) NaH, DMF/THF; (b) Boc_2O, Et_3N, DMAP, DCM, 15% over two steps; (c) DIBALH, THF, -78°C, 83%; (d) DMP, DCM, 93%; (e) *tert*-butyl (2-(methylamino)ethyl)carbamate, $NaBH(OAc)_3$, DCM, 91%; (f) potassium 1,3-dioxoisoindolin-2-ide, DMF, 100°C, 73%; (g) Hydrazine, EtOH, reflux; (h) *tert-butyl 3-(((2-((tert-butoxycarbonyl)amino)ethyl)(methyl)amino)methyl)-4-(4-(2-(1,3-dioxoisoindolin-2-yl)ethoxy)phenyl)-1H-pyrrole-1-carboxylate*, EDCI, HOAt, NMM, DMSO; (I) HCl/Methanol, 8% over three steps.

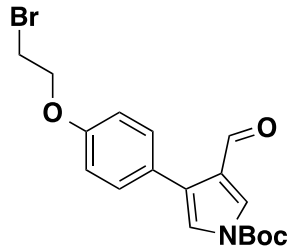


Synthesis of 1-(*tert*-butyl) 3-methyl 4-(4-(2-bromoethoxy)phenyl)-1*H*-pyrrole-1,3-dicarboxylate: To a mixture of sodium hydride (380 mg, 9.47 mmol, 60% in mineral oil) in 8 mL of tetrahydrofuran (THF) and 8 mL of N, N-dimethylformaldehyde (DMF) cooled by ice bath, was added dropwise during 15 min the solution of methyl (*E*)-3-(4-(2-bromoethoxy)phenyl)acrylate (900 mg, 3.15 mmol), 1-((isocyanomethyl)sulfonyl)-4-methylbenzene (616 mg, 3.15 mmol) in 8 mL of THF and 8 mL of DMF. The mixture was stirred for additional 1 h before carefully adding water to quench the reaction. The solution was extracted with ethyl acetate (EtOAc) (3 x 20 mL), and combined organic phase was dried over anhydrous sodium sulfate and concentrated under reduced pressure. The residue was used for next step without purification. The residue was dissolved in 50 mL of dichloromethane, and triethylamine (0.4 mL, 3.15 mmol), and 4-(dimethylamino)pyridine (38 mg, 0.31 mmol) were added. To the cooled solution in ice bath, was added di-*tert*-butyl dicarbonate (686 mg, 3.15 mmol) in portions. The solution was stirred overnight and quenched by water. The mixture was extracted with dichloromethane (3 x 20 mL), combined organic phase was dried over anhydrous sodium sulfate and concentrated under reduced pressure. The residue was purified by flash chromatography on silica gel column with eluent (EtOAc/hexane, 0-30%) to give oil (200 mg, 15%). ^{1}H NMR (600 MHz, chloroform-d) δ 7.88 (s,

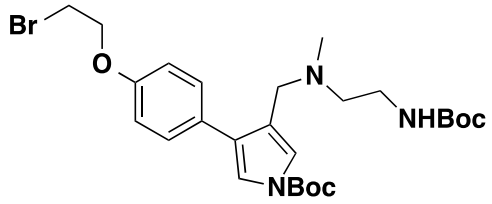
1H), 7.40 (d, *J* = 8.7 Hz, 2H), 7.19 (s, 1H), 6.91 (d, *J* = 8.7 Hz, 2H), 4.31 (t, *J* = 6.3 Hz, 2H), 3.76 (s, 3H), 3.65 (t, *J* = 6.3, 3.2 Hz, 2H), 1.62 (s, 9H).



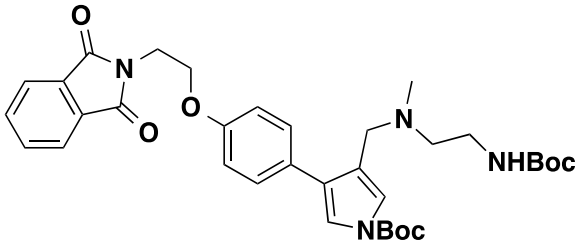
Synthesis of *tert*-butyl 3-(4-(2-bromoethoxy)phenyl)-4-(hydroxymethyl)-1*H*-pyrrole-1-carboxylate: To a solution of 1-(*tert*-butyl) 3-methyl 4-(4-(2-bromoethoxy)phenyl)-1*H*-pyrrole-1,3-dicarboxylate (200 mg, 0.47 mmol) in 10 mL of tetrahydrofuran cooled at -78°C, was added dropwise diisobutylaluminium hydride (DIBAL-H)(3 mL, 1 M in hexane). After completion of the reaction monitored by TLC, 1 mL of methanol was added cautiously to quench the reaction. Then 10 mL of saturated aqueous sodium potassium tartrate was added and the resulting mixture was stirred until transparent phase observed. The solution extracted with ethyl acetate and dried over anhydrous sodium sulfate, concentrated under reduced pressure. The residue was purified by flash chromatography on silica gel column with eluent (EtOAc/hexane, 0-50%) to give oil (150 mg, 83%). ^{1}H NMR (600 MHz, chloroform-d) δ 7.47 (d, *J* = 8.7 Hz, 2H), 7.34 – 7.21 (m, 2H), 6.94 (d, *J* = 8.8 Hz, 2H), 4.60 (s, 2H), 4.31 (t, *J* = 6.3 Hz, 2H), 3.65 (t, *J* = 6.3 Hz, 2H), 1.60 (s, 9H).



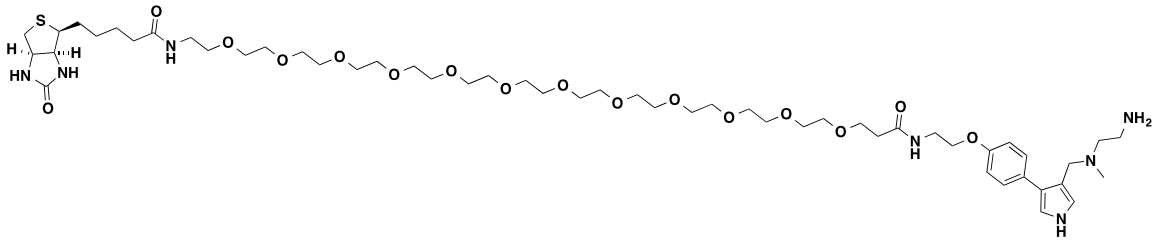
Synthesis of *tert*-butyl 3-(4-(2-bromoethoxy)phenyl)-4-formyl-1*H*-pyrrole-1-carboxylate: To a solution of *tert*-butyl 3-(4-(2-bromoethoxy)phenyl)-4-(hydroxymethyl)-1*H*-pyrrole-1-carboxylate (200 mg, 0.5 mmol) in 10 mL of dichloromethane, was added Dess-Martin periodinane (318 mg, 0.75 mmol) in portion. After completion of the reaction as TLC showed, the mixture was purified by flash chromatography on silica gel column with eluent (EtOAc/hexane, 0-40%) to give oil (140 mg, 93%). ^{1}H NMR (600 MHz, chloroform-d) δ 9.91 (s, 1H), 7.91 (s, 1H), 7.44 (d, 2H), 7.27 (s, 1H), 6.95 (d, *J* = 7.9 Hz, 2H), 4.33 (t, *J* = 6.3 Hz, 2H), 3.66 (t, *J* = 6.3 Hz, 2H), 1.64 (s, 9H).



Synthesis of *tert*-butyl 3-(4-(2-bromoethoxy)phenyl)-4-(((2-((*tert*-butoxycarbonyl)amino)ethyl)(methyl)amino)methyl)-1*H*-pyrrole-1-carboxylate: To a solution of *tert*-butyl 3-(4-(2-bromoethoxy)phenyl)-4-formyl-1*H*-pyrrole-1-carboxylate (140 mg, 0.36 mmol) and *tert*-butyl (2-(methylamino)ethyl)carbamate (72.8 mg, 0.42 mmol) in 10 mL of dichloromethane, was added sodium triacetoxyborohydride (127 mg, 0.6 mmol). The mixture was stirred overnight. Saturated aqueous sodium bicarbonate was added and extracted with dichloromethane. The combined organic phase was dried over sodium sulfate and concentrated under reduced pressure. The residue was purified by flash chromatography on silica gel column with eluent (EtOAc/hexane, 0-50%) to give oil (180 mg, 91%). ^{1}H NMR (600 MHz, chloroform-d) δ 7.48 (d, *J* = 8.6 Hz, 2H), 7.24 (s, 1H), 7.15 (s, 1H), 6.94 (d, *J* = 8.3 Hz, 2H), 4.78 (s, 1H), 4.31 (t, *J* = 6.4 Hz, 2H), 3.64 (t, *J* = 6.3 Hz, 2H), 3.36 (s, 2H), 3.18 – 3.12 (m, 2H), 2.45 (t, *J* = 6.0 Hz, 2H), 2.16 (s, 3H), 1.60 (s, 9H), 1.43 (s, 9H).

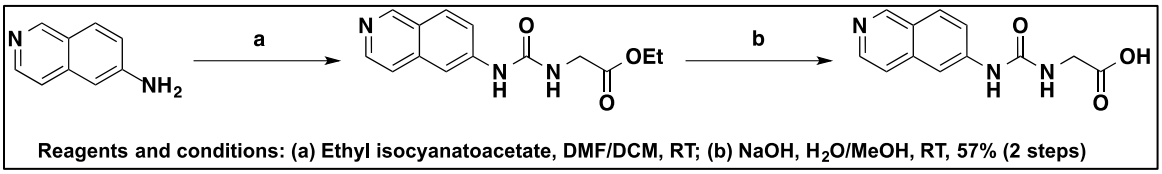


Synthesis of *tert*-butyl 3-(((2-((*tert*-butoxycarbonyl)amino)ethyl)(methyl)amino)methyl)-4-(4-(2-(1,3-dioxoisoindolin-2-yl)ethoxy)phenyl)-1*H*-pyrrole-1-carboxylate: *tert*-butyl 3-(4-(2-bromoethoxy)phenyl)-4-(((2-((*tert*-butoxycarbonyl)amino)ethyl)(methyl)amino)methyl)-1*H*-pyrrole-1-carboxylate (180 mg, 0.33 mmol) and potassium 1,3-dioxoisoindolin-2-ide (57.4 mg, 0.31 mmol) were dissolved into N,N-dimethylformaldehyde (DMF). The solution was heated to 100 °C and stirred overnight before the addition of water. The solution was extracted with ethyl acetate and combined organic phase was dried over anhydrous sodium sulfate, concentrated under reduced pressure. The residue was purified by flash chromatography on silica gel with eluent (EtOAc/hexane, 0-50%) to give solid (140 mg, 73%). ^{1}H NMR (600 MHz, chloroform-d) δ 7.85 (dd, *J* = 5.4, 3.0 Hz, 2H), 7.71 (dd, *J* = 5.5, 3.0 Hz, 2H), 7.42 (d, *J* = 8.3 Hz, 2H), 7.19 (s, 1H), 7.13 (s, 1H), 6.89 (d, *J* = 8.8 Hz, 2H), 4.77 (s, 1H), 4.24 (t, *J* = 5.8 Hz, 2H), 4.11 (t, *J* = 6.6, 4.8 Hz, 2H), 3.33 (s, 2H), 3.17 – 3.09 (m, 2H), 2.42 (t, *J* = 5.9 Hz, 2H), 2.14 (s, 3H), 1.59 (s, 9H), 1.39 (s, 9H).



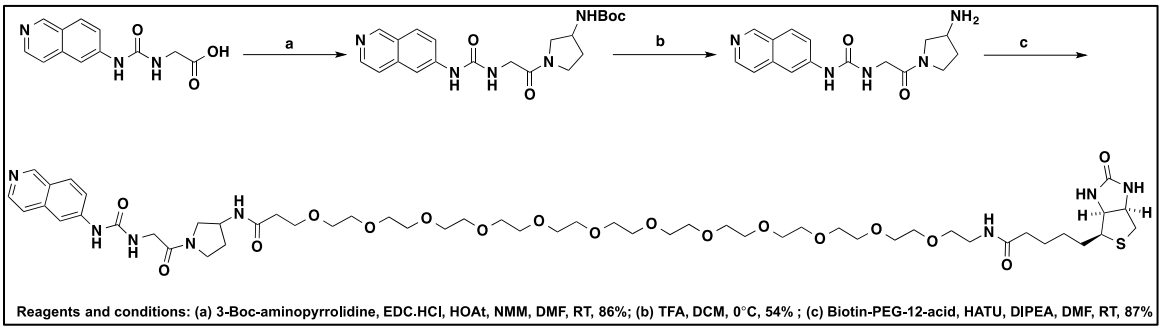
Synthesis of MTM7172: To a solution of *tert*-butyl 3-(((2-((*tert*-butoxycarbonyl)amino)ethyl)(methyl)amino)methyl)-4-(4-(2-(1,3-dioxoisoindolin-2-yl)ethoxy)phenyl)-1*H*-pyrrole-1-carboxylate (140 mg, 0.23 mmol) in 5 mL of ethanol, was added aqueous hydrazine (45.7 mg, 0.5 mmol, 35 wt. %), and the mixture was heated to reflux for 2 h. The volatile was removed under reduced pressure. The residue was used for next step without purification. To a solution of the crude residue, Biotin-PEG12-acid (84 mg, 0.1 mmol), (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) (29 mg, 0.15 mmol), and 1-hydroxy-7-azabenzo-triazole (20 mg, 0.15 mmol) in 5 mL of dimethylsulfoxide, was added 4-methylmorpholine (30 mg, 0.3 mmol). The solution was stirred overnight and purified by preparative HPLC (10%-100% methanol / 0.1% TFA in H_2O) to give oil. The oil was treated with 5 mL of 3 M methanolic hydrochloric acid for 3 hours before the volatile was removed. The residue was lyophilized into solid (20 mg, 8%). ^{1}H NMR (600 MHz, Methanol-d_4) δ 7.31 (d, J = 8.0 Hz, 2H), 7.23 (s, 1H), 7.02 (d, J = 8.0 Hz, 2H), 6.89 (s, 1H), 4.61 – 3.94 (m, 6H), 3.82 – 3.42 (m, 52H), 3.39 – 3.33 (m, 2H), 3.23 (d, 3H), 3.09 – 2.91 (m, 2H), 2.76 – 2.42 (m, 5H), 2.23 (s, 2H), 1.78 – 1.38 (m, 6H). HRMS (m/z) for $C_{53}H_{92}N_7O_{16}S^+$ $[M + H]^+$: calculated 1114.6316, found 1114.6276.

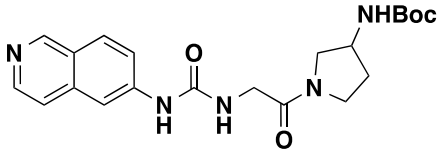
Procedure for the Preparation of UNC3815



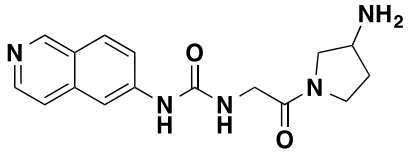
Synthesis of (isoquinolin-6-ylcarbamoyl)glycine: To a stirring solution of 6-aminoisoquinoline (1.2 g, 8.32 mmol) in mixture of dichloromethane (DCM) and DMF (30 mL & 10 mL) was added ethyl isocyanatoacetate (2.80 mL, 25 mmol) and the resulting mixture was stirred 18 hours at room temperature. After removal of volatiles, crude mixture was purified by flash column chromatography (gradient from 100% dichloromethane to 10% methanol in dichloromethane) to yield desired ethyl ester as pale yellow solid which was re-suspended in Methanol (48 mL) and water (16 mL) followed by addition of 1N solution of NaOH (24 mL). The resulting clear mixture was then stirred at room temperature 16 hours. After concentration of the mixture under reduced pressure crude mixture was purified by reverse phase flash column chromatography (gradient from 100% water with 0.1 %TFA to 10% methanol) to yield desired (isoquinolin-6-ylcarbamoyl)glycine as a TFA salt (2.04 g, 57% over 2 steps). ^{1}H NMR (400 MHz, Methanol-d_4) δ 9.40 (s, 1H), 8.46 (d, J = 2.1 Hz, 1H), 8.36-8.30 (m, 2H),

8.15 (d, J = 6.7 Hz, 1H), 7.86 (dd, J = 9.0, 2.1 Hz, 1H), 4.01 (s, 2H). MS (ESI) *m/z* $[M+H]^+$ for $C_{12}H_{12}N_3O_3^+$: calculated 246.1, found 246.1.



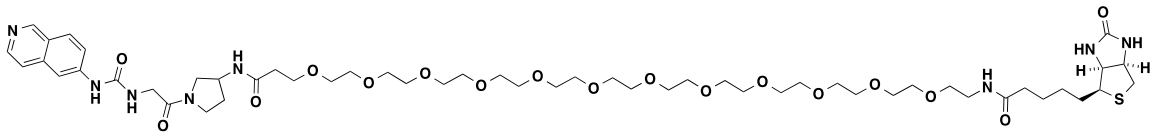


Synthesis of *tert*-butyl (1-((isoquinolin-6-ylcarbamoyl)glycyl)pyrrolidin-3-yl)carbamate: To a stirring mixture of (isoquinolin-6-ylcarbamoyl)glycine (60 mg, 0.17 mmol) in DMF (1.5 mL) was added N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC.HCl) (64.0 mg, 0.33 mmol); 1-Hydroxy-7-azabenzotriazole (HOAt) (35 mg, 0.25 mmol) and 3-Boc-aminopyrrolidine (62.2 mg, 0.33 mmol) followed by N-Methylmorpholine (NMM) (38 µL, 0.34 mmol) and resulting mixture was stirred 18 hours at room temperature. The volatiles were removed under reduced pressure and the crude reaction mixture was purified by flash column chromatography to give white solid (52.7 mg, 76%). ^{1}H NMR (400 MHz, Methanol-d_4) δ 9.01 (d, J = 0.9 Hz, 1H), 8.27 (d, J = 5.9 Hz, 1H), 8.06 (d, J = 2.0 Hz, 1H), 7.94 (d, J = 8.9 Hz, 1H), 7.60 (d, J = 5.9 Hz, 1H), 7.56 (dd, J = 8.9, 2.1 Hz, 1H), 4.24-4.10 (m, 1H), 4.10-3.96 (m, 2H), 3.78-3.49 (m, 3H), 3.41-3.34 (m, 1H), 2.27-2.07 (m, 1H), 2.02-1.84 (m, 1H), 1.45 (br s, 9H). MS (ESI) *m/z* $[M+H]^+$ for $C_{21}H_{28}N_5O_4^+$: calculated 414.2, found 414.2.



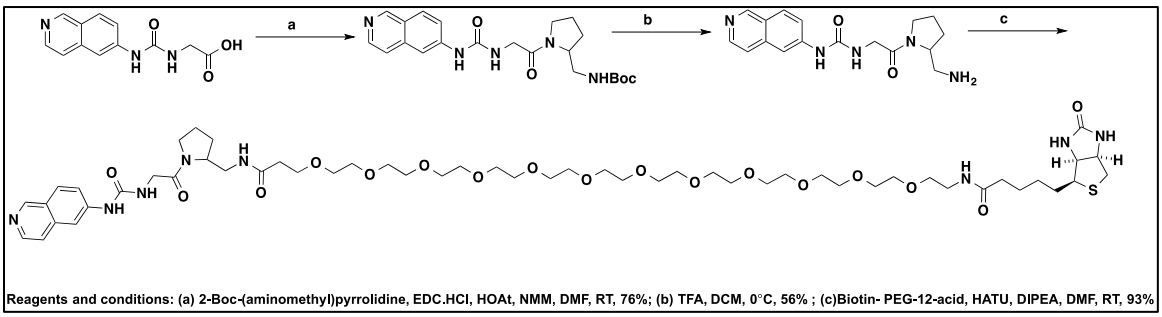
Synthesis of 1-(2-(3-aminopyrrolidin-1-yl)-2-oxoethyl)-3-(isoquinolin-6-yl)urea: To the stirring mixture of *tert*-butyl (1-((isoquinolin-6-ylcarbamoyl)glycyl)pyrrolidin-3-yl)carbamate (52.7 mg, 0.127 mmol) in dicholoromethane (1 mL) was added trifluoroacetic acid (TFA) (0.25 mL) at 0 °C and resulting mixture was stirred at 0 °C for 3.5 hrs. The volatiles were removed under reduced pressure and the crude reaction mixture

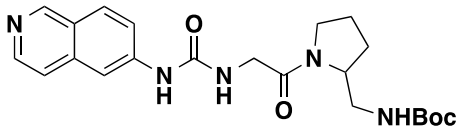
was purified by flash column chromatography to give yellow solid (37 mg, 54%). ^{1}H NMR (400 MHz, Methanol-d_4) δ 9.18 (s, 1H), 8.29 (d, J = 5.6 Hz, 1H), 8.20 (s, 1H), 8.10 (d, J = 8.9 Hz, 1H), 7.82 (d, J = 6.3 Hz, 1H), 7.69 (dd, J = 9.0, 2.2 Hz, 1H), 4.15-3.89 (m, 3H), 3.82-3.63 (m, 4H), 2.54- 2.31 (m, 1H), 2.28-2.03 (m, 1H). MS (ESI) *m/z* $[M+H]^+$ for $C_{16}H_{20}N_5O_2^+$: calculated 314.2, found 314.2.



Synthesis of UNC3815: To the stirring mixture of above product (20.0 mg, 0.037 mmol) in DMF (0.3 mL) was added Biotin-PEG12-acid (28.3 mg, 0.034 mmol) followed by 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) (15.3 mg, 0.040 mmol) at room tempreture to give a clear yellow solution. To this solution was added diisopropylethylamine (DIPEA) (23 μL, 0.134 mmol) and resulting mixture was stirred at room temperature for 18 hours. The volatiles were removed under reduced pressure and the crude reaction mixture was purified by HPLC to give desired product as colorless oil (37.0 mg, 87%). ^{1}H NMR (400 MHz, Methanol-d_4) δ 9.44 (s, 1H), 8.48 (s, 1H), 8.38-8.33 (m, 2H), 8.19 (d, J = 6.7 Hz, 1H), 7.89 (dt, J = 9.0, 2.0 Hz, 1H), 4.52- 4.35 (m, 2H), 4.30 (dd, J = 7.9, 4.5 Hz, 1H), 4.17-4.00 (m, 2H), 3.83-3.43 (m, 52H), 3.36-3.33 (m, 2H), 3.23-3.18 (m, 1H), 2.93 (dd, J = 12.7, 5.0 Hz, 1H), 2.71 (d, J = 12.7 Hz, 1H), 2.49-2.43 (m, 2H), 2.32-2.13 (m, 3H), 2.09-1.93 (m, 1H), 1.80-1.53 (m, 4H), 1.47-1.39 (m, 2H). HRMS (m/z) for $C_{53}H_{87}N_8O_{17}S^+$ $[M + H]^+$: calculated 1139.5904, found 1139.5897.

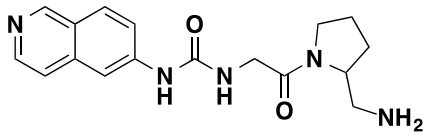
Procedure for the Preparation of UNC3811



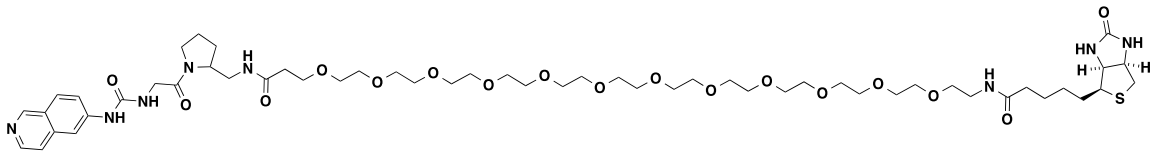


Synthesis of *tert*-butyl ((1-((isoquinolin-6-ylcarbamoyl)glycyl)pyrrolidin-2-yl)methyl)carbamate: To a stirring mixture of (isoquinolin-6-ylcarbamoyl)glycine (124 mg, 0.34 mmol, 1.0 eq) in DMF (3 mL) was added N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC.HCl) (131 mg, 0.69 mmol, 2.0 eq); 1-Hydroxy-7-azabenzotriazole (HOAt) (69 mg, 0.51 mmol, 1.5 eq) and 2-Boc-(aminomethyl)pyrrolidine (138 mg,

0.69 mmol, 2.0 eq) followed by N-Methylmorpholine (NMM) (76 μL, 0.69 mmol, 2.0 eq) and resulting mixture was stirred 18 hours at room temperature. The volatiles were removed under reduced pressure and the crude reaction mixture was purified by flash column chromatography to give white solid (124 mg, 84%). ^{1}H NMR (400 MHz, Methanol-d_4) δ 9.03 (s, 1H), 8.29 (d, J = 5.9 Hz, 1H), 8.09 (s, 1H), 7.97 (d, J = 8.9 Hz, 1H), 7.64 (d, J = 5.9 Hz, 1H), 7.59 (dd, J = 8.9, 2.0 Hz, 1H), 4.22-3.95 (m, 3H), 3.58-3.52 (m, 2H), 3.28-3.08 (m, 2H), 2.14-1.83 (m, 4H), 1.44 (br s, 9H). MS (ESI) *m/z* $[M+H]^+$ for $C_{22}H_{30}N_5O_4^+$: calculated 428.2, found 428.2.



Synthesis of 1-(2-(2-(aminomethyl)pyrrolidin-1-yl)-2-oxoethyl)-3-(isoquinolin-6-yl)urea: To the stirring mixture of the above product (65.5 mg, 0.153 mmol, 1.0 eq) in dicholoromethane (1 mL) was added trifluoroacetic acid (TFA) (0.25 mL) at 0 °C and resulting mixture was stirred at 0 °C for 3.5 hrs. The volatiles were removed under reduced pressure and the crude reaction mixture was purified by flash column chromatography to give off white solid (48 mg, 56%). ^{1}H NMR (400 MHz, Methanol-d_4) δ 9.01 (q, J = 0.9 Hz, 1H), 8.28 (d, J = 5.9 Hz, 1H), 8.07 (s, 1H), 7.96 (d, J = 8.9 Hz, 1H), 7.62 (d, J = 5.9 Hz, 1H), 7.57 (dd, J = 8.9, 2.0 Hz, 1H), 4.13-4.10 (m, 1H), 4.06 (br s, 2H), 3.59-3.53 (m, 2H), 2.86 (dd, J = 12.9, 4.9 Hz, 1H), 2.71 (dd, J = 12.9, 7.0 Hz, 1H), 2.10-1.84 (m, 4H). MS (ESI) *m/z* $[M+H]^+$ for $C_{17}H_{22}N_5O_2^+$: calculated 328.2, found 328.2.



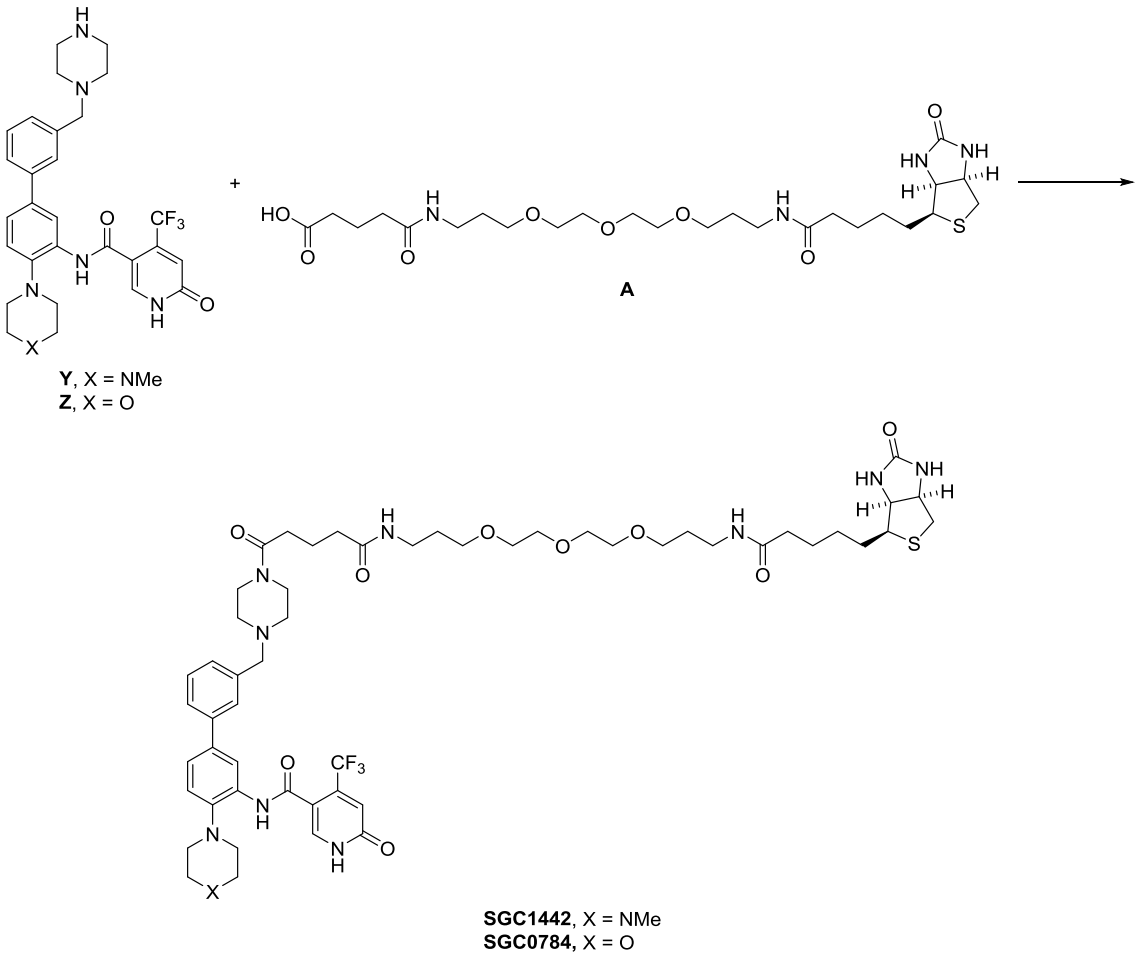
Synthesis of UNC3811: To the stirring mixture of above product (22.0 mg, 0.040 mmol) in DMF (0.3 mL) was added Biotin-PEG12-acid (30.0 mg, 0.036 mmol) followed by 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) (16.4 mg, 0.043 mmol) at room temperature to give a clear yellow solution. To this solution was added diisopropylethylamine (DIPEA) (25 μL, 0.144 mmol) and resulting mixture was stirred at room temperature for 18 hours. The volatiles were removed under reduced pressure and the crude reaction mixture was purified by HPLC to give desired product as colorless oil (45.3 mg, 93%). ^{1}H NMR (400 MHz, Methanol-d_4) δ 9.45 (d, J = 2.0 Hz, 1H), 8.48 (s, 1H), 8.40-8.32 (m, 2H), 8.19 (d, J = 7.7 Hz, 1H), 7.89 (dd, J = 9.0, 2.0 Hz, 1H), 4.53-4.47 (m, 1H), 4.32-4.16 (m, 2H), 4.08 (s, 2H), 3.76-3.52 (m, 52H), 3.36-3.33 (m, 2H), 3.23-3.16 (m, 1H), 2.92 (dd, J = 12.7, 5.0 Hz, 1H), 2.74-2.64 (m, 1H), 2.51-2.43 (m, 2H), 2.21 (t, J = 7.4 Hz, 2H), 2.16 -1.82 (m, 4H), 1.78-1.54 (m, 4H), 1.47-1.40 (m, 2H). HRMS (m/z) for $C_{54}H_{89}N_8O_{17}S^+$ $[M + H]^+$: calculated 1153.6061, found 1153.6060.

Procedure for the preparation of SGC1442 and SGC0784

The procedure described for the synthesis of **16d** (Supplementary Information, *Journal of Medicinal Chemistry*, DOI: 10.1021/acs.jmedchem.5b01630) was adapted for the synthesis of (**Y**) and (**Z**), precursors for the synthesis of **SGC1442** and **SGC0784** respectively (see below). The only changes made involved replacement of 1-methylpiperazine with morpholine for the synthesis of (**Z**), and replacement of 4-(3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)morpholine in General Procedure F with *tert*-butyl 4-(3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)piperazine-1-carboxylate for the synthesis of both (**Y**) and (**Z**).

***N*-(3'-((4-(5,21-Dioxo-25-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)-10,13,16-trioxa-6,20-diazapentacosanoyl)piperazin-1-yl)methyl)-4-(4-methylpiperazin-1-yl)-[1,1'-biphenyl]-3-yl)-6-oxo-4-(trifluoromethyl)-1,6-dihydropyridine-3-carboxamide (SGC1442)**

***N*-(3'-((4-(5,21-dioxo-25-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)-10,13,16-trioxa-6,20-diazapentacosanoyl)piperazin-1-yl)methyl)-4-morpholino-[1,1'-biphenyl]-3-yl)-6-oxo-4-(trifluoromethyl)-1,6-dihydropyridine-3-carboxamide (SGC0784)**

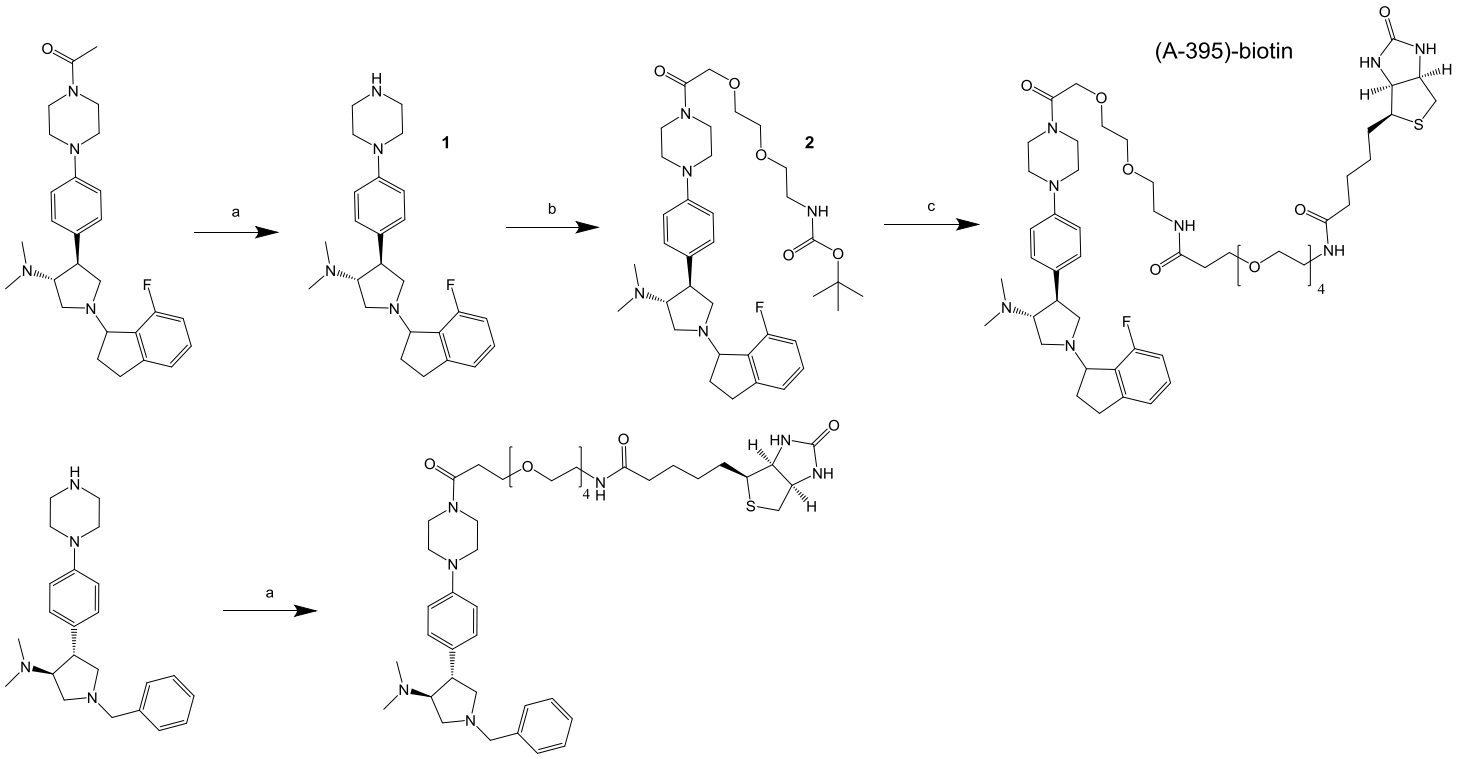


To a solution of either *N*-(4-(4-methylpiperazin-1-yl)-3'-(piperazin-1-ylmethyl)-[1,1'-biphenyl]-3-yl)-6-oxo-4-(trifluoromethyl)-1,6-dihydropyridine-3-carboxamide (**Y**) or *N*-(4-morpholino-3'-(piperazin-1-ylmethyl)-[1,1'-biphenyl]-3-yl)-6-oxo-4-(trifluoromethyl)-1,6-dihydropyridine-3-carboxamide (**Z**) (0.019 mmol) and 5,21-dioxo-25-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)-10,13,16-trioxa-6,20-diazapentacosanoic acid·DIPEA (**A**) (0.019 mmol) in DMF (0.5 mL) at room temperature was added HATU (0.019 mmol) and *N*,*N*-diisopropylethylamine (9.74 μL, 0.056 mmol). The resulting solution was stirred for 16 hours prior to removal of the solvent under reduced pressure. The residue was purified by prep HPLC (see below for conditions) followed by additional purification using a cation exchange column (PoraPak RxnCX 6 cc column, acidic PE resin, eluting with 3% NH_4OH in MeOH v/v) to afford the title compounds as white powders (>70% yield). **SGC1442** LRMS (ESI): 1098.2 $[M+H]^+$. **SGC0784** LRMS (ESI): 1085.2 $[M+H]^+$.

Agilent Prep HPLC
Column: XSelect Prep C18 5 μm, 10x100 mm
Flow: 5 mL/min
Run time: 15 min
A: MeCN
B: 10 mM Ammonium Bicarbonate (3.2 g/4 L)

Time	A	B
0	10%	90%
11	65%	35%
12	90%	10%
13	90%	10%
14	10%	90%
15	10%	90%

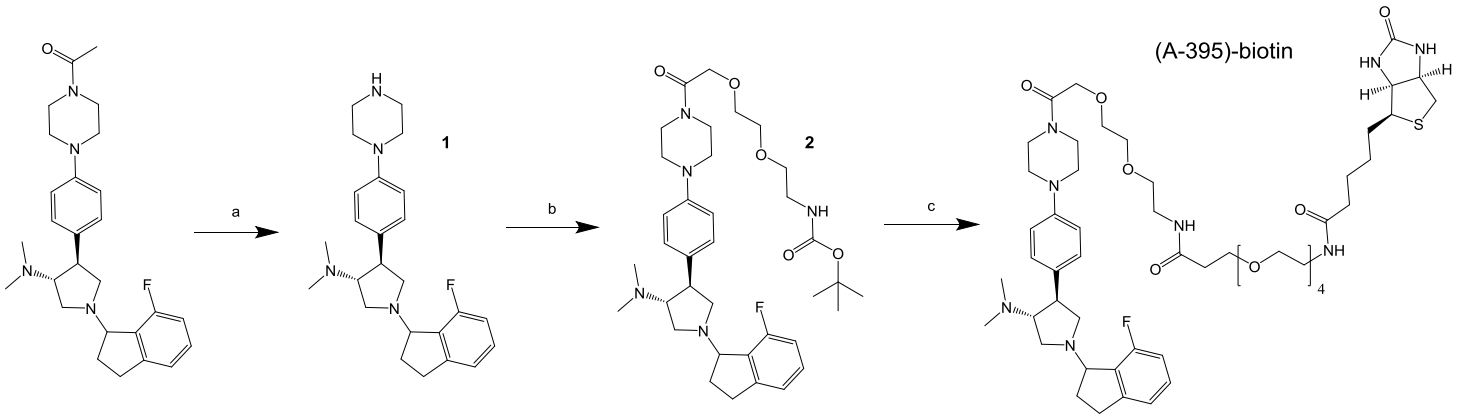
Procedure for the preparation of (A-395)-biotin.



Reagents: (a) NHS-dPEG$_4$-biotin, DIEA, DMSO, rt, 98%.

N-(15-(4-(4-((3R,4S)-1-benzyl-4-(dimethylamino)pyrrolidin-3-yl)phenyl)piperazin-1-yl)-15-oxo-3,6,9,12-tetraoxapentadecyl)-5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide bis-trifluoroacetate (A-395N)-biotin: (3S,4R)-1-benzyl-N,N-dimethyl-4-(4-(piperazin-1-yl)phenyl)pyrrolidin-3-amine tris-trifluoroacetate (100 mg, 0.142 mmol) was combined with NHS-dPEG$_4$-biotin (50 mg, 0.085 mmol) in 1 mL anhydrous DMSO. N,N-diisopropylethylamine) (74.2 µl, 0.425 mmol) was added. The reaction was shaken for 16 h, diluted to 3 mL with 90% DMSO/water and purified in one injection by RP-HPLC on a Waters Deltapak C18 200 x 25 mm column eluted with a gradient of of 5% A (0.1% TFA-water):B (MeCN) to 95% A:B to give **(A395N)-biotin** (90 mg, 98.9%) as a waxy solid: ESI-MS *m/z* 420.0 $(M+2H)^{2+}$, 838.5 $(M+H)^+$; 835.7 $(M-H)^-$; Analytical LCMS TFA Method R_t 0.72 min, APCI-MS *m/z* 838.23 $(M+H)^+$; ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 7.84 (t, *J* = 5.7 Hz, 1H), 7.65 – 7.39 (m, 7H), 7.37 (d, *J* = 8.5 Hz, 2H), 7.10 – 6.88 (m, 2H), 6.43 (s, 2H), 4.31 (dd, *J* = 7.8, 4.9 Hz, 4H), 4.28 (s, 1H), 3.72 (m, 1H), 3.62 (dt, *J* = 24.5, 5.2 Hz, 4H), 3.62 – 3.58 (m, 4H), 3.52 – 3.48 (m, 12H), 3.39 (t, *J* = 11.9 Hz, 2H), 3.22 – 2.91 (m, 8H), 2.82 (dd, *J* = 12.4, 5.1 Hz, 1H), 2.72 (s, 6H), 2.67 – 2.50 (m, 3H), 2.07 (t, *J* = 7.4 Hz, 2H), 1.62 (m, 1H), 1.56 – 1.37 (m, 3H), 1.30 (m, 3H); δ_C (125 MHz, DMSO-d_6) δ_C 172.1, 168.8, 162.68, 150.2, 130.04, 128.99, 128.81, 128.63, 127.65, 115.87, 69.66, 69.04, 67.85, 66.69, 60.97, 59.33, 59.16, 57.63, 55.37, 53.2, 48.34, 47.99, 43.02, 40.98, 40.68, 39.76, 38.38, 35.08, 32.75, 28.15, 28.0, 25.22.

<u>Procedure for the preparation of (A-395)-biotin.</u>



Reagents: (a) 6 M HCl, THF, MeOH, rt, 64%; b) HOOC-dPEG$_2$-NHBoc, PyAOP, DIEA, DMF, rt, 51%; (c) (i) TFA, rt; (ii) NHS-dPEG$_4$-biotin, DIEA, DMSO, rt, 61%.

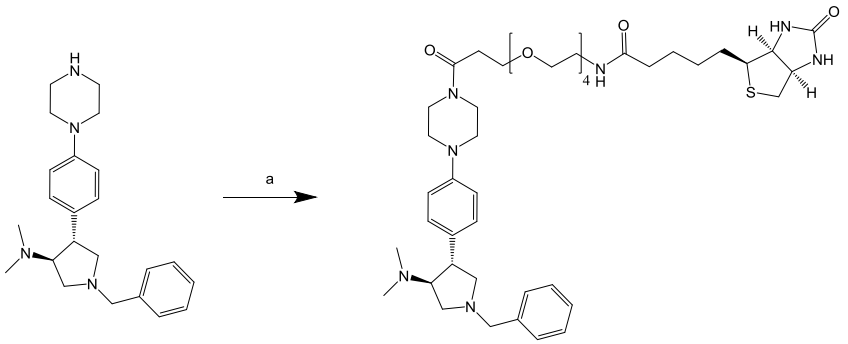
(3R,4S)-1-(7-fluoro-2,3-dihydro-1H-inden-1-yl)-N,N-dimethyl-4-(4-(piperazin-1-yl)phenyl)pyrrolidin-3-amine 1: 1-(4-(4-((3S,4R)-4-(dimethylamino)-1-(7-fluoro-2,3-dihydro-1H-inden-1-yl)pyrrolidin-3-yl)phenyl)piperazin-1-yl)ethanone (100 mg, 0.222 mmol) in THF (740 µl) and MeOH (740 µl) at room temperature was treated with 6 M HCl (444 µl, 2.66 mmol) (Curtin et al., 2017). The clear solution was stirred overnight at ambient to partial conversion, then at 60°C for 7h. The solution was added to 20 mL sat. sodium bicarbonate and extracted with 2 × 20 mL ethyl acetate. The combined organic layers were washed with brine, dried over $MgSO_4$ and concentrated. The residue was chromatographed (Biotage 10g HP SNAP cartridge, methanol/dichloromethane gradient 5-20%) to afford **1** (58 mg, 64%) as a 60:40 mixture of epimers: ESI-MS *m/z* 409.2 $(M+H)^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 7.33 – 7.17 (m, 1H), 7.17 – 7.01 (m, 3H), 6.93 (td, *J* = 8.8, 5.2 Hz, 1H), 6.78 (dd, *J* = 8.7, 3.3 Hz, 2H), 4.30 (dd, *J* = 7.4, 1.8 Hz, 1H), 4.16 (dd, *J* = 7.0, 1.7 Hz, 1H), 3.25 – 2.92 (m, 6H), 2.92 – 2.71 (m, 8H), 2.63 (dd, *J* = 9.0, 5.7 Hz, 1H), 2.45 – 2.27 (m, 1H), 2.27 – 1.76 (m, 8H).

***Tert*-butyl (2-(2-(2-(4-(4-((3S,4R)-4-(dimethylamino)-1-(7-fluoro-2,3-dihydro-1H-inden-1-yl)pyrrolidin-3-yl)phenyl)piperazin-1-yl)-2-oxoethoxy)ethoxy)ethyl)carbamate tris-trifluoroacetate 2**: (3R,4S)-1-(7-fluoro-2,3-dihydro-1H-inden-1-yl)-N,N-dimethyl-4-(4-(piperazin-1-yl)phenyl)pyrrolidin-3-amine (**1**) and 2,2-dimethyl-4-oxo-3,8,11-trioxa-5-azatridecan-13-oic acid (81 mg, 0.306 mmol) were combined in 2 mL anhydrous dimethylformamide. ((3H-[1,2,3]triazolo[4,5-b]pyridin-3-yl)oxy)tri(pyrrolidin-1-yl)phosphonium hexafluorophosphate(V) (191 mg, 0.367 mmol) and DIEA (N,N-diisopropylethylamine) (257 µl, 1.469 mmol) were added. The reaction was shaken at ambient overnight. The reaction was diluted to 6 mL with 90% DMSO/water and purified in two injections by RP-HPLC on a Waters Deltapak C18 200 x 25 mm column eluted with a gradient of of 5% A (0.1% TFA-water):B (MeCN) to 95% A:B to give **2** (51.5 mg, 39.3%) as a white solid: ESI-MS *m/z* 610.3 $(M+H)^+$; 607.7 $(M-H)^-$; **Analytical LCMS TFA Method.** Analytical LCMS was performed on a Thermo MSQ-Plus mass spectrometer and Agilent 1100/1200 HPLC system running Xcalibur

2.0.7, Open-Access 1.4, and custom login software. The mass spectrometer was operated under APCI or ESI ionization conditions as noted in experimentals. The HPLC system comprised an Agilent Binary pump, degasser, column compartment, autosampler and diode-array detector, with a Polymer Labs ELS-2100 evaporative light-scattering detector. The column used was a Phenomenex Kinetex C8, 2.6 μm 100Å (2.1mm × 30mm), at a temperature of 65°C. A gradient of 5-100% acetonitrile (B) and 0.1% trifluoroacetic acid in water (A) was used, at a flow rate of 1.5 mL/min (0-0.05 min 5% A, 0.05-1.2 min 5-100% A, 1.2-1.4 min 100% A, 1.4-1.5 min 100-5% A. 0.25 min post-run delay) to give an elution times (R_t) of 0.73 min; ESI-MS *m/z* 610.13 $(M+H)^+$.

N-(2-(2-(2-(4-(4-((3R,4S)-1-benzyl-4-(dimethylamino)pyrrolidin-3-yl)phenyl)piperazin-1-yl)-2-oxoethoxy)ethoxy)ethyl)-1-(5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)-3,6,9,12-tetraoxapentadecan-15-amide bis-trifluoroacetate (A-395)-biotin: *Tert*-butyl (2-(2-(2-(4-(4-((3S,4R)-4-(dimethylamino)-1-(7-fluoro-2,3-dihydro-1H-inden-1-yl)pyrrolidin-3-yl)phenyl)piperazin-1-yl)-2-oxoethoxy)ethoxy)ethyl)carbamate tris-trifluoroacetate (**2**) (22 mg, 0.023 mmol) was dissolved in 1 mL trifluoroacetic acid and immediately evaporated to dryness under a stream of dry nitrogen gas. The residue was combined with NHS-$dPEG_4$-biotin (25 mg, 0.042 mmol, Quantabiodesign, Product #10200) in 1 mL anhydrous DMSO to which was added 30 μL DIEA. The mixture was shaken at ambient for 16 h, diluted to 3 mL with 90% DMSO/water and purified in one injection by RP-HPLC on a Waters Deltapak C18 200 x 25 mm column eluted with a gradient of of 5% A (0.1% TFA-water):B (MeCN) to 95% A:B to give **(A395)-biotin** (15.3 mg, 60.7%) as a waxy solid: ESI-MS *m/z* 514.6 $(M+2H)^{2+}$, 1027.7 $(M+H)^+$; 1026.1 $(M-H)^-$; Analytical LCMS TFA Method R_t 0.71 min, ESI-MS *m/z* 514.07 $(M+H)^+$; 1H NMR (500 MHz, DMSO-d_6) δ ppm 7.92 (t, J = 5.6 Hz, 1H), 7.84 (t, J = 5.7 Hz, 1H), 7.40 (tdd, J = 8.2, 5.3, 3.5 Hz, 1H), 7.33 (d, J = 8.4 Hz, 2H), 7.20 (dd, J = 7.4, 2.0 Hz, 1H), 7.07 (dt, J = 17.8, 8.9 Hz, 1H), 6.97 (dd, J = 8.9, 2.4 Hz, 2H), 6.44 6.40 (m, 3H), 4.31 (dd, J = 7.8, 4.9 Hz, 1H), 4.22 4.10 (m, 4H), 3.63 3.52 (m, 9H), 3.50 (s, 6H), 3.55 3.43 (m, 7H), 3.40 (dt, J = 12.0, 5.9 Hz, 4H), 3.19 (dq, J = 8.7, 5.9 Hz, 4H), 3.15 (s, 5H), 3.15 3.06 (m, 2H), 2.94 2.78 (m, 2H), 2.72 (d, J = 11.0 Hz, 6H), 2.58 (d, J = 12.5 Hz, 1H), 2.32 (t, J = 6.5 Hz, 2H), 2.10 2.03 (m, 2H), 1.67 1.56 (m, 1H), 1.56 1.40 (m, 2H), 1.48 (s, 1H), 1.36 1.22 (m, 2H); δ_C (125 MHz, DMSO-d_6) 172.14, 170.13, 167.29, 162.68, 159.0, 149.97, 149.37, 131.72, 129.12, 128.27, 124.59, 121.2, 115.84, 113.32, 69.7, 69.58, 69.53, 69.46, 69.34, 69.07, 69.01, 66.79, 68.6, 64.15, 61.02, 59.15, 55.34, 48.2, 43.11, 40.91, 40.74, 40.59, 39.71, 38.5, 38.43, 36.01, 35.05, 30.38, 29.03, 28.34, 28.06, 25.23.

Procedure for the preparation of (A395N)-biotin



Reagents: (a) NHS-dPEG$_4$-biotin, DIEA, DMSO, rt, 98%.

N-(15-(4-(4-((3R,4S)-1-benzyl-4-(dimethylamino)pyrrolidin-3-yl)phenyl)piperazin-1-yl)-15-oxo-3,6,9,12-tetraoxapentadecyl)-5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide bis-trifluoroacetate (A-395N)-biotin: (3S,4R)-1-benzyl-N,N-dimethyl-4-(4-(piperazin-1-yl)phenyl)pyrrolidin-3-amine tris-trifluoroacetate (100 mg, 0.142 mmol) was combined with NHS-dPEG$_4$-biotin (50 mg, 0.085 mmol) in 1 mL anhydrous DMSO. N,N-diisopropylethylamine) (74.2 µl, 0.425 mmol) was added. The reaction was shaken for 16 h, diluted to 3 mL with 90% DMSO/water and purified in one injection by RP-HPLC on a Waters Deltapak C18 200 x 25 mm column eluted with a gradient of of 5% A (0.1% TFA-water):B (MeCN) to 95% A:B to give **(A395N)-biotin** (90 mg, 98.9%) as a waxy solid: ESI-MS *m/z* 420.0 $(M+2H)^{2+}$, 838.5 $(M+H)^{+}$; 835.7 $(M-H)^{-}$; Analytical LCMS TFA Method R_t 0.72 min, APCI-MS *m/z* 838.23 $(M+H)^{+}$; ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 7.84 (t, *J* = 5.7 Hz, 1H), 7.65 – 7.39 (m, 7H), 7.37 (d, *J* = 8.5 Hz, 2H), 7.10 – 6.88 (m, 2H), 6.43 (s, 2H), 4.31 (dd, *J* = 7.8, 4.9 Hz, 4H), 4.28 (s, 1H), 3.72 (m, 1H), 3.62 (dt, *J* = 24.5, 5.2 Hz, 4H), 3.62 – 3.58 (m, 4H), 3.52 – 3.48 (m, 12H), 3.39 (t, *J* = 11.9 Hz, 2H), 3.22 – 2.91 (m, 8H), 2.82 (dd, *J* = 12.4, 5.1 Hz, 1H), 2.72 (s, 6H), 2.67 – 2.50 (m, 3H), 2.07 (t, *J* = 7.4 Hz, 2H), 1.62 (m, 1H), 1.56 – 1.37 (m, 3H), 1.30 (m, 3H); δ_C (125 MHz, DMSO-d_6) δ_C 172.1, 168.8, 162.68, 150.2, 130.04, 128.99, 128.81, 128.63, 127.65, 115.87, 69.66, 69.04, 67.85, 66.69, 60.97, 59.33, 59.16, 57.63, 55.37, 53.2, 48.34, 47.99, 43.02, 40.98, 40.68, 39.76, 38.38, 35.08, 32.75, 28.15, 28.0, 25.22.

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