Supporting Information

Detection of Cannabinoid Receptor Type 2 in Native Cells and Zebrafish with a Highly Potent, Cell-Permeable Fluorescent Probe

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ABSTRACT: Despite its essential role in the (patho)physiology of several diseases, CB_2R tissue expression profiles and signaling mechanisms are not yet fully understood. We report the development of a highly potent, fluorescent CB_2R agonist probe based on structure-based reverse design. It commences with a highly potent, preclinically validated ligand, which is conjugated to a silicon-rhodamine fluorophore, enabling cell permeability. The probe is the first to preserve interspecies affinity and selectivity for both mouse and human CB_2R.Extensive cross-validation (FACS, TR-FRET and confocal microscopy) was followed by CB_2R detection in endogenously expressing living cells along with zebrafish larvae. It is anticipated that this will benefit clinical translatability of CB_2R based drugs.

CONTENTS

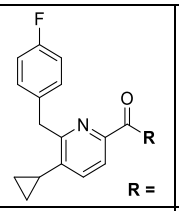
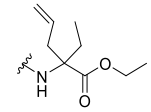
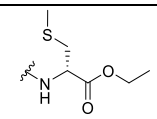
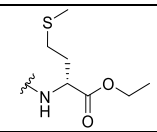
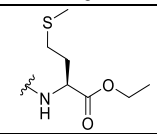
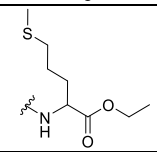
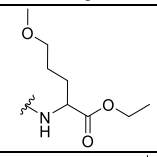
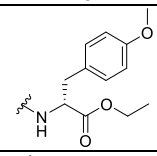
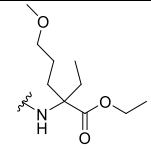
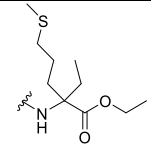
SUPPLEMENTARY TABLES

Supplementary table S-1. Key pharmacological characteristics of pyridine 1, labeling precursors and fluorescent probes. Calculated lipophilicity (AlogP), binding affinity (K_i), cAMP potency and efficacy (EC_{50} (%eff.)), and absorption and emission ranges of fluorescent probes (Abs/Ems).

Entry	Cmpd	Dye	Alog P[a]	K_i [nM][b, c]				hK_i ratio CB_1R/CB_2R	cAMP EC_{50} [nM] (%Eff.)[d, e]			hEC_{50} ratio CB_1R/CB_2R	Abs (max)/ Ems (max) [nm]
				hCB_2R	hCB_1R	mCB_2R	mCB_1R		hCB_2R	hCB_1R	mCB_2R		
1	**1**	n.a.	5.2	0.2 ± 0.2	5.4 ± 2.2	1.8 ± 0.2	8.5[e]	27	0.7 (102)	4.4 (99)	1.9 (99)	6.3	n.a.
2	**(*S*)-5**	n.a. (NH_2)	4.5	2.7 ± 0.9	64 ± 37	2.6 ± 1.0	229[e]	23	0.5 (91)	57 (84)	3.2 (91)	114	n.a.
3	**(*R*)-5**	n.a. (NH_2)	4.5	4.6 ± 5.4	180 ± 141	13 ± 5.0	163[e]	39	11 (81)	>10'000	424 (61)	>909	n.a.
4	**(*S*)-6**	NBD	6.3	9.1 ± 4.8	617 ± 129	33 ± 3.6	691[e]	68	2.2 (72)	>10'000	21 (95)	>4'545	474/550[f]
5	**(*R*)-6**	NBD	6.3	159[e]	4'925[e]	622[e]	n.d.	31	17 (84)	>10'000	1'093 (69)	>588	474/550[f]
6	**(*S*)-7**	Alexa488	5.3	44 ± 328	321 ± 2'383	28 ± 228	>10'000[e]	7	1.3 (100)	86 (109)	30.2 (80)	64	494/526[f]
7	**(*R*)-7**	Alexa488	5.3	62 ± 25	1'114 ± 219	104 ± 33	n.d.	18	12 (101)	343 (104)	14 (109)	28	494/526[f]
8	**(*S*)-8**	SiR	10.6	62 ± 102	114 ± 105	117 ± 55	1'892[e]	2	67 (96)	>10'000	66 (93)	>149	652/674[g]
9	***(S)*-SI-1**	Cy5.5	13.7	14 ± 1.4	108 ± 22	50 ± 5.9	n.d.	8	140 (110)	726 (118)	512 (119)	5	690/730[h]

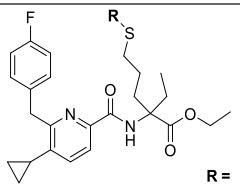
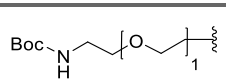
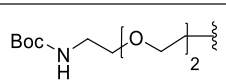
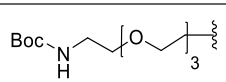
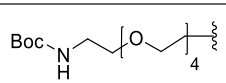
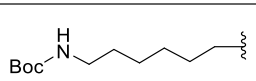
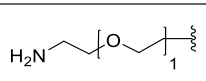
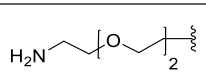
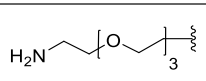
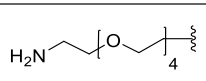
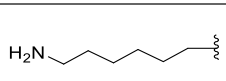
Cmpd – compound; h – human; m – mouse; n.a. – not applicable; n.d. – not determined. [a] The AlogP is a calculated value based on the contribution of each atom to the lipophilicity; [b] K_i values are the mean of at least two independent experiments performed in triplicate. Assay description and reference ligands data described in the SI, page S-10; [c] mCB_1R data generated using mouse brain membranes; [d] Functional potency (EC_{50}), percentage efficacy given in parenthesis; [e] Single experiment performed in triplicate, reference ligands data described in the SI, page S-10; [f] Fluorescence excitation and emission maxima measured in aqueous solution (DPBS, Dulbecco's phosphate buffered saline); [g] Values derived from literature;[1] [h] Fluorescence excitation and emission maxima measured in DMSO.

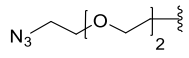
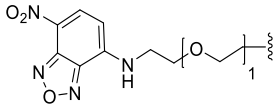
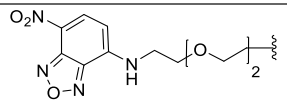
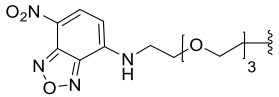
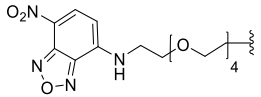
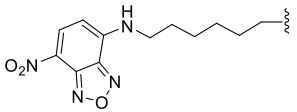
Supplementary table S-2. SAR studies around pyridine1: Exploration of elongation options at the α-position of the amino acid residue.

Entry	Compound	 R =	K_i hCB_2R [nM]	K_i hCB_1R [nM]	hK_i ratio CB_1R/CB_2R	MW [g/mol]	PSA [a] [Å²]	AlogP [b]	logD [c]	Kinetic solubility [d] [µg/mL]	PAMPA P_{eff} [e] [10^{-6} cm/s] %Acceptor/ %Membrane/ %Donor [f]
1	SI-2		0.8	2.9	3	424.5	53	3.5	n.d.	3.1	n.d.
2	SI-3		3.2	19	6	416.5	54	4.1	n.d.	<0.1	n.d.
3	SI-4		12	70	6	430.5	55	4.2	n.d.	<0.1	1.12 2 / 67 / 31
4	SI-5		65	153	2	430.5	55	4.2	n.d.	n.d.	00 0 / 57 / 43
5	SI-6		1‘263	4‘688	4	444.6	56	4.8	n.d.	1.4	0 0 / 61 / 40
6	SI-7		17	99	6	428.5	65	4.0	3.3	0.6	0.66 1 / 75 / 24
7	SI-8		34	600	18	476.5	64	5.4	n.d.	1.6	n.d.
8	SI-9		1.5	5.6	4	456.6	63	4.9	n.d.	<0.1	n.d.
9	SI-10		1.3	8.5	7	472.6	54	5.6	n.d.	2.9	n.d.

h – human; m – mouse; MW – molecular weight; PSA – polar surface area; n.d. – not determined. [a] Surface sum of all polar atoms in the molecule; [b] Calculated partition coefficient values (ALogP) based on the contribution of each atom to the logP; [c] Distribution coefficient values; [d] Solubility of the compound when diluted into aqueous environment from DMSO superstock; [e] Parallel artificial membrane permeability assay (PAMPA) was used to determine membrane permeation coefficient values (Peff);[2] [f] Percentage of compound found in acceptor, membrane and donor. Reference ligands data described in the *In-vitro pharmacology and ADME profile* section.

Supplementary table S-3. Linker studies on pyridine 1 seed structure: Elongation vector at the α-position of the amino acid residue.

En-try	Cmpd		K_i [nM]			hK_i ratio CB_1R / CB_2R	cAMP EC_{50}[a][nM] (%eff.)			MW [g/ mol]	PSA[b] [Å^2]	Alog P[c]	log D [d]	Kinetic solubility [e] [µg/mL]	PAMPA P_{eff}[f] [10^{-6} cm/s] %Acceptor/ %Membrane/ %Donor[g]
			h CB_2R	h CB_1R	m CB_2R		hCB_2 R	hCB_1 R	mCB_2 R						
1	**SI-11**		112	1’297	389	12	3.2 (87)	>10’000	15 (90)	645.8	94	6.2	n.d.	<0.3	n.d.
2	**SI-12**		164	1’141	213	7	36 (93)	>10’000	131 (81)	689.9	102	6.1	n.d.	<0.1	0 0 / 79 / 21
3	**SI-13**		138	589	117	4	10 (91)	>10’000	21 (87)	733.9	111	6.0	n.d.	<0.2	n.d.
4	**SI-14**		140	731	93	5	13 (91)	>10’000	31 (84)	778.0	120	5.9	n.d.	<0.2	n.d.
5	**SI-15**		140	1’385	367	10	7.7 (85)	>10’000	78 (78)	657.9	86	7.9	n.d.	<0.1	n.d.
6	**SI-16**		60	200	10	3	8.2 (93)	0.6 (52)	13 (92)	545.7	84	4.6	2.7	98	0.29 1 / 48 / 51
7	**rac-5**		38	433	9.8	11	28 (92)	822 (96)	45 (93)	589.8	93	4.5	2.6	210	0 0 / 64 / 36
8	**SI-17**		18	724	2.8	41	9.9 (93)	146 (72)	14 (93)	633.8	101	4.4	2.7	168	0.28 1 / 41 / 58
9	**SI-18**		2.6	164	n.d.	63	n.d.	n.d.	n.d.	677.9	110	4.3	n.d.	>723	n.d.
10	**SI-19**		2.7	204	n.d.	75	n.d.	n.d.	n.d.	557.8	76	6.3	n.d.	0.3	n.d.

11	**SI-20**		60	464	52	8	1.9 (93)	>10'000	5.4 (93)	615.8	116	6.1	n.d.	<0.1	n.d.
12	**SI-21**		22	418	59	19	171 (121)	2'059 (152)	181 (118)	708.8	151	6.4	n.d.	2	n.d.
13	**rac-6**		22	383	73	17	4.7 (100)	600 (126)	102 (123)	752.9	160	6.3	2.8	9	n.d.
14	**SI-22**		27	460	85	17	9.2 (100)	789 (96)	74 (113)	796.9	169	6.1	n.d.	14	0.65 2 / 38 / 61
15	**SI-23**		38	617	118	16	20 (102)	1'121 (110)	192 (117)	841.0	177	6.0	n.d.	6.1	0.70 2 / 45 / 53
16	**SI-24**		55	1'072	144	19	9.9 (100)	928 (122)	142 (122)	720.9	143	8.1	n.d.	14	0.50 2 / 12 / 87

h – human; m – mouse; MW – molecular weight; PSA – polar surface area; n.d. – not determined. [a] Functional potency (cAMP assay), percentage efficacy given in parenthesis; [b] Surface sum of all polar atoms in the molecule; [c] Calculated partition coefficient values (ALogP) based on the contribution of each atom to the logP; [d] Distribution coefficient values; [e] Solubility of the compound when diluted into aqueous environment from DMSO superstock; [f] Parallel artificial membrane permeability assay (PAMPA) was used to determine membrane permeation coefficient values (Peff);[2] [g] Percentage of compound found in acceptor, membrane and donor. Reference ligands data described in the *In-vitro pharmacology and ADME profile* section.

Supplementary table S-4. *In vitro* pharmacology data of 7-Alexa 488 for a representative set of common off-targets.

Assay name	Readout	Value (% inhibition)
MAO-A (h)	Enzymatic activity	1
5-HT transporter (h) (antagonist radioligand)	Specific binding	-8
5-HT1A (h) (agonist radioligand)	Specific binding	1
5-HT2A (h) (agonist radioligand)	Specific binding	5
5-HT2B (h) (agonist radioligand)	Specific binding	2
5-HT3 (h) (antagonist radioligand)	Specific binding	6
A1 (h) (agonist radioligand)	Specific binding	2
A3 (h) (agonist radioligand)	Specific binding	77
Abl kinase (h)	Enzymatic activity	13
ACE (h)	Enzymatic activity	-22
acetylcholinesterase (h)	Enzymatic activity	-5
alpha 1A (h) (antagonist radioligand)	Specific binding	0
alpha 2A (h) (antagonist radioligand)	Specific binding	5
AR(h) (agonist radioligand)	Specific binding	1
AT1 (h) (antagonist radioligand)	Specific binding	0
beta 1 (h) (agonist radioligand)	Specific binding	9
beta 2 (h) (antagonist radioligand)	Specific binding	7
BZD (central) (agonist radioligand)	Specific binding	-1
Ca2+ channel (L, diltiazem site) (benzothiazepines) (antagonist radioligand)	Specific binding	6
CB1 (h) (agonist radioligand)	Specific binding	41
CCK1 (CCKA) (h) (agonist radioligand)	Specific binding	23
CDK2 (h) (cycA)	Enzymatic activity	5
Cl- channel (GABA-gated) (antagonist radioligand)	Specific binding	30
COX2(h)	Enzymatic activity	-5
D1 (h) (antagonist radioligand)	Specific binding	10
D2S (h) (agonist radioligand)	Specific binding	-11
Estrogen ER alpha (h) (agonist radioligand)	Specific binding	0
FP (h) (agonist radioligand)	Specific binding	14
glycine (strychnine-insensitive) (antagonist radioligand)	Specific binding	8
GR (h) (agonist radioligand)	Specific binding	33
GSK3alpha (h)	Enzymatic activity	-13
GSK3beta (h)	Enzymatic activity	-10
H1 (h) (antagonist radioligand)	Specific binding	2
H2 (h) (antagonist radioligand)	Specific binding	15
H3 (h) (agonist radioligand)	Specific binding	-1
HIV-1 protease	Enzymatic activity	47
ZAP70 kinase (h)	Specific binding	35
M1 (h) (antagonist radioligand)	Specific binding	-5
M2 (h) (antagonist radioligand)	Specific binding	8
MMP-9 (h)	Enzymatic activity	21
mu (MOP) (h) (agonist radioligand)	Specific binding	0

N muscle-type (h) (antagonist radioligand)	Specific binding	14
N neuronal alpha 4beta 2 (h) (agonist radioligand)	Specific binding	-7
norepinephrine transporter (h) (antagonist radioligand)	Specific binding	9
PCP (antagonist radioligand)	Specific binding	4
PDE3B (h)	Enzymatic activity	15
PDE4D2 (h)	Enzymatic activity	32
PPAR gamma (h) (agonist radioligand)	Specific binding	29
xanthine oxidase/ superoxide O2- scavenging	Enzymatic activity	-6
ZAP70 kinase (h)	Enzymatic activity	39

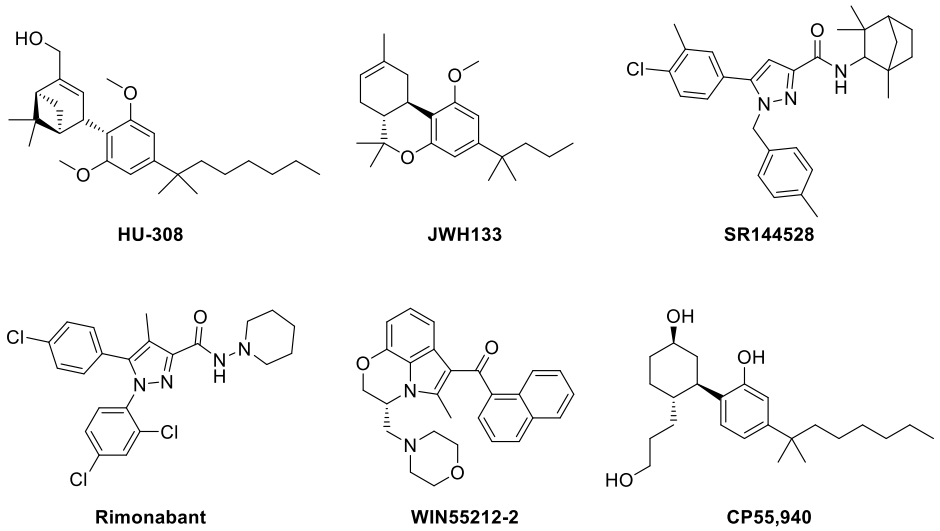
Representative off target selection.[3] Data shown is the mean percentage of inhibition for binding assays and the mean percentage of inhibition for enzyme and cell-based assays at a test concentration of 10 μM (n=2). Data were generated at Eurofins Cerep (France).

Supplementary table S-5. Comparison of *in vitro* pharmacology and ADME profile of *N*-Boc protected thioether SI-12 with oxidized analogues.

Entry	Compound	K_i [nM]			hK_i ratio CB_1R/CB_2R	cAMP EC_{50}[a][nM] (%eff.)			MW [g/mol]	PSA[b] [Å^2]	Alog P[c]	log D[d]	Kinetic solubility[e] [µg/mL]	PAMPA P_{eff}[f] [10*-6 cm/s] %Acceptor/ %Membrane/ %Donor[g]
		hCB_2R	hCB_1R	mCB_2R		hCB_2R	hCB_1R	mCB_2R						
1	***rac*-SI-12 (thioether)**	164	1'141	213	7	36 (93)	>10'000	131 (81)	689.9	102	6.1	n.d.	<0.1	0 0 / 79 / 21
2	**(*S*)-SI-12 (thioether)**	28	163	64	6	4.0 (93)	>10'000	19 (77)	689.9	102	6.1	n.d.	<0.4	n.d.
3	**(*R*)-SI-12 (thioether)**	151	1'533	552	10	23 (86)	>10'000	128 (44)	689.9	102	6.1	2.5	<0.4	n.d.
4	**SI-25 (sulfoxide)**	29	326	90	11	7.9 (93)	>10'000	65 (79)	705.9	118	5.0	3.2	5.2	5.4 3 / 82 / 15
5	**SI-26 (sulfone)**	45	190	100	4	17 (89)	>10'000	33 (80)	721.9	134	5.1	n.d.	<0.4	1.77 3 / 62 / 36

h – human; m – mouse; MW – molecular weight; PSA – polar surface area; n.d. – not determined. [a] Functional potency (cAMP assay), percentage efficacy given in parenthesis; [b] Surface sum of all polar atoms in the molecule; [c] Calculated partition coefficient values (ALogP) based on the contribution of each atom to the logP; [d] Distribution coefficient values; [e] Solubility of the compound when diluted into aqueous environment from DMSO superstock; [f] Parallel artificial membrane permeability assay (PAMPA) was used to determine membrane permeation coefficient values (Peff);[2] [g] Percentage of compounds found in acceptor, membrane and donor. Reference ligands data described in the *In-vitro pharmacology and ADME profile* section.

IN-VITRO PHARMACOLOGY AND ADME PROFILE



Supplementary figure S-1. Structures of reference ligands. HU-910, phytocannabinoid CB_2R agonist used in TR-FRET experiments; JWH133, CB_2R agonist, reference ligand for FACS, binding and cAMP assays; SR144528, CB_2R agonist, reference ligand for TR-FRET assay; Rimonabant, CB_1R agonist, reference ligand for binding and cAMP assays; WIN55212-2, non-selective agonist, reference ligand for binding and cAMP assays; CP55,940, non-selective agonist, reference ligand for binding and cAMP assays.

RADIOLIGAND BINDING ASSAY AND CAMP ASSAY

Radioligand binding assays and forskolin-stimulated cAMP assays were performed as described by Soethoudt *et al*.[4] The clonal cell lines CHO-DUKX_HOMSA_CNR2_Clone_90_CRE-Luc, CHO-DUKX_HOMSA_CNR1_Clone_20_CRE-Luc, CHO-K1_MUSMU_CNR2_beta arrestin (PathHunter® CHO-K1 mCNR2 β-Arrestin Cell Line DiscoverX, 93-0472C2) were cultured and membranes for radioligand binding assays were prepared in analogy to Soethoudt *et al*.[4] The corresponding B_{max} of cells lines is indicated in brackets as following: WT-CHO, hCB_2R-CHO (2.08 pmol/mg protein), hCB_1R-CHO (5.5 pmol/mg protein), and mCB_2R (11.8 pmol/mg protein). Excluding the culturing step, mouse brain_140603 membranes were prepared using the same protocol (protein concentration: 2290 µg/ml, KD: 8.291 nM, B_{max}: 9.67 pmol/ mg protein). K_i measurements were performed using 0.3 nM 3H-CP55´940 radioligand for hCB_1R, hCB_2R and mCB_2R and 1.5 nM 3H-CP55´940 radioligand for mCB_1R. Reference compounds for binding and cAMP assays were selected in accordance to literature.[4] The corresponding mean K_i and EC_{50} values, as well as standard error of the mean (SEM), are stated below. Data are means from one or two independent experiments performed in triplicate.

hCB_2R K_i: the mean K_i value of the positive control JWH133 used for each run was 34.9 nM and standard error of the mean (SEM) for this standard compound was ±3.4 nM (n=128).

hCB_1R K_i: the mean K_i value of the positive control Rimonabant used for each run was 2.8 nM and standard error of the mean (SEM) for this standard compound was ±0.2 nM (n=105).

mCB_2R K_i: the mean K_i value of the positive control WIN55212-2 used for each run was 5.8 nM and standard error of the mean (SEM) for this standard compound was ±1.3 nM (n=57).

hCB_2R, mCB_2R and hCB_1R cAMP EC_{50}: the mean EC_{50} value of the positive control CP55,940 used for each run were 0.08 nM, 0.05 nM and 0.11 nM, respectively, and standard error of the mean (SEM) for this standard compound was ±0.011 nM (n=114), ±0.007 nM (n=94) and ±0.013 nM (n=95), respectively.

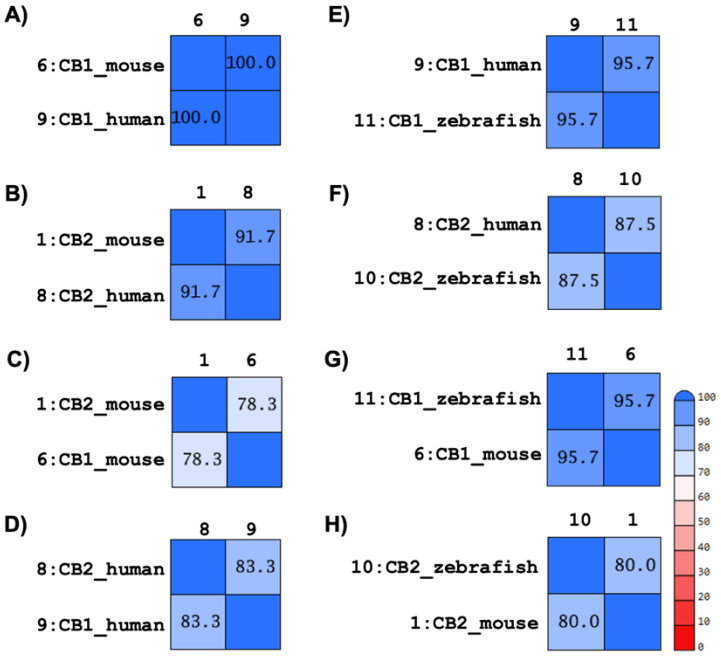
The chemical structures of reference compounds JWH133, CB_2R selective agonist, Rimonabant (CB_1R selective agonist), WIN55212-2 (non-selective agonist) and CP55,940 (non-selective agonist) are shown in Supplementary figure S-1. Lipophilicity (logD), kinetic solubility and PAMPA assays were prepared and carried out as described in literature.[4]

MOLECULAR DOCKING

The X-ray structure of active state CB_2R in complex with agonist AM12033 (PDB 6KPC) was used as template to dock CB_2R ligands.[17] The docking experiments were performed with the software MOE (*Molecular Operating Environment (MOE)*, 2020.09; Chemical Computing Group ULC, 1010 Sherbrooke St. West, Suite #910, Montreal, QC, Canada, H3A 2R7, **2020**.) with default settings. The best 10 docking poses for each compound were energy-minimized within the binding pocket using MOE and examined visually to select the most reasonable docking mode with respect to molecular interactions and internal conformational strain. Finally, the consistency of the selected poses was evaluated using the available structure-activity relationship information.

Human, mouse and zebrafish CB_1R and CB_2R sequence identity at binding site

The binding pocket amino acid sequence identity of human, mouse and zebrafish CB_1R and CB_2R were compared using MOE. All protein sequences were obtained from UniProt (www.uniprot.org). As shown in Supplementary figure S-2, no interspecies differences between human and mouse were observed at the CB_1R binding pocket (Supplementary figure S-2A). Similarly, for CB_2R, the binding sites show roughly 98% sequence identity (Supplementary figure S-2B). The binding site sequence identity between CB_1R and CB_2R remains at 78% for the mouse and 83% for the human proteins (Supplementary figures S-2C and 2D). The binding site sequence identity between CB_1R and CB_2R from human and zebrafish remains 96% for CB_1R and 88% for CB_2R (Supplementary figures S-2E and 2F). The corresponding comparison between mouse and zebrafish receptor sequence identity provides 96% for CB_1R and 80% for CB_2R (Supplementary figures S-2G and 2H). The high interspecies sequence identity at the binding site for both CB_1R and CB_2R, in human, mouse and zebrafish, suggests that ligands should display similar SARs among these three species. The significantly lower degree of sequence identities between CB_1R and CB_2R within the same species are expected to offer opportunities for subtype-selective CBR ligands.



Supplementary figure S-2. Comparison of sequence homology of human and mouse cannabinoid receptors. Binding site sequence homology between A) mCB_1R and hCB_1R, B) mCB_2R and hCB_2R, C) mCB_1R and mCB_2R, D) hCB_1R and hCB_2R, E) hCB_1R and zCB_1R, F) hCB_2R and zCB_2R, G) zCB_1R and mCB_1R, H) zCB_2R and mCB_2R. The sequence identities are colored *via* a heatmap (*see legend on the right*)**.**

ABSORPTION-EMISSION SPECTRA OF FLUORESCENT PROBES 6-NBD, 7-ALEXA 488 AND SI-1-CY5.5

UV/Vis absorbance spectra of 50 µM of probes **6**, **7**, **8** and **SI-1** in various solvents (see below) were recorded in wavelength range of 250-750 nm to determine the wavelength with the maximal absorbance signal used later for excitation of compound excitation (1 cm path length, room temperature, scan step 1 nm; Thermo Evolution 600 UV/Vis spectrophotometer, Thermo Electron Scientific Instruments LLC, Madison, WI, USA). Due to the limited solubility of the probes in DPBS, the spectra in DPBS were measured in a cuvette with 10 cm path length at 5 µM in presence of 0.1 % (v/v) DMSO. Technical excitation and emission fluorescence spectra (uncorrected for chromatic aberrations) of the fluorescent probes were measured at 10 µM compound concentration (20°C, integration time 1s scan step 2 nm, slits 2.4 mm and 2 mm in excitation and emission, respectively; ISS Inc. PC1 fluorometer, Champaign, IL, USA) in organic DMSO, DCM, acetonitrile, EtOAc and MeOH, and aqueous solvent DPBS (Dulbecco's phosphate buffered saline). Due to the limited solubility of the fluorescent probes in DPBS, the spectra in DPBS were measured at 1.0 µM in the presence of 0.1 % (v/v) DMSO and then scaled to expected fluorescence signal intensity at concentration of 10 µM by multiplication of the signal intensity by factor of 10. As expected, the observed fluorescence intensity increased with decreasing dielectric constant of the solvents: aqueous solution (PBS, ε_{25} = 79.0) < DMSO (ε_{20} = 47) < acetonitrile (ε_{20} = 36.64) ≤ MeOH (ε_{25} = 32.6) < DCM (ε_{20} = 9.08) ≤ EtOAc (ε_{25} = 6).[7, 8] Thus, the fluorescence intensity (quantum yield) of the fluorescence of probes **6**, **7**, **8** and **SI-1** is expected to increase when in close proximity of the cell membrane (ε = 3) or when bound to the hydrophobic binding site of CB_2R. The relatively lower

fluorescence intensity values observed for ***rac*-6** (NBD-labeled) in MeOH and EtOAc, respectively, may indicate on specific solvent effects, but overall the expected trend of increasing of fluorescence signal intensity with reduction of solvent polarity is observed. Overall, the fluorescence features of these probes enable their applications in e.g. tissue imaging for CB_2R-specific disorders.

For *rac*-NBD probe **6**, the fluorescence excitation and emission maxima of **6** were observed wavelengths ranging from 468 - 478 nm and 528 - 550 nm, respectively, depending on the applied solvent. The highest fluorescence emission signal was observed in EtOAc (Supplementary figure S-3 and Supplementary table S-6). The lowest fluorescence signal was observed in aqueous solution (DPBS, Dulbecco's phosphate buffered saline). The Stokes shift of probe **6** has values between 60 nm observed in DCM and EtOAc, and 76 nm observed in DPBS.

For Alexa 488-(*S*) probe **7**, the fluorescence excitation and emission maxima of **7** were observed at wavelengths of 494 - 512 nm and in range of 526 - 540 nm, respectively, depending on the applied solvent. The highest fluorescence emission signal was observed in MeOH and DPBS (Supplementary figure S-3 and Supplementary table S-6). The lowest fluorescence signal was observed in EtOAc. The Stokes shift of probe **7** has values between 28 nm observed in DMSO, acetonitrile, DCM and EtOAc, and 32 nm observed in methanol and DPBS.

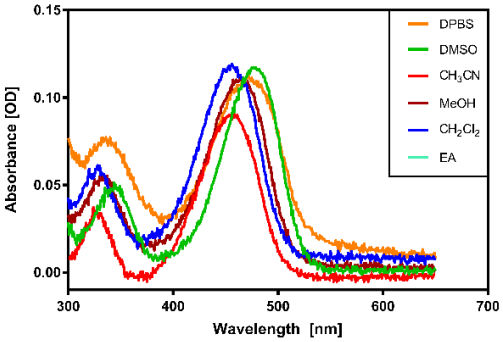
For SiR-probe **8**, the fluorescence excitation and emission maxima of **8** were observed at wavelengths of 626 - 660 nm and in range of 660 - 690 nm, respectively, depending on the applied solvent. The highest fluorescence emission signal was observed in acetonitrile (Supplementary figure S-3 and Supplementary table S-6). The lowest fluorescence signal was observed in DMSO. The Stokes shift of probe **8** has values between 20 nm observed in acetonitrile, DCM and EtOAc, and 30 nm observed in DMSO.

For Cy5.5-(*S*) probe **SI-1**, the fluorescence excitation and emission maxima of **SI-1** were observed at wavelengths of 690 nm and in range of 707 - 730 nm, respectively, depending on the applied solvent. The highest fluorescence emission signal was observed in DCM (Supplementary figure S-3 and Supplementary table S-6). The lowest fluorescence signal was observed in EtOAc. The Stokes shift of probe **SI-1** has values between 17 nm observed in acetonitrile, and 40 nm observed in DMSO.

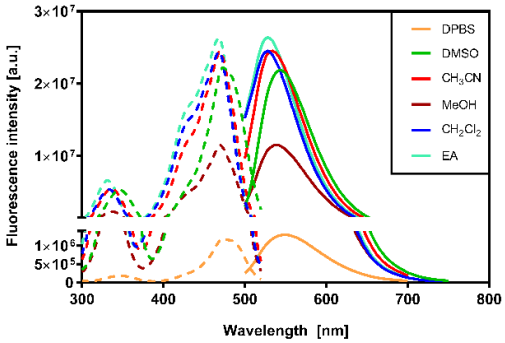
Supplementary table S-6. Fluorescence excitation and emission wavelengths for CB_2R-selective fluorescence labeled ligands with corresponding fluorescence intensities and Stokes' shifts recorded in distinct solvents.

Probe	Fluorescence data	DPBS	DMSO	Acetonitrile	MeOH	DCM	EtOAc
***rac*-NBD-6**	Excitation λ [nm]	474	478	470	470	468	468
	Emission λ [nm]	550	544	534	538	528	528
	Stokes shift [nm]	76	66	64	68	60	60
	Signal intensity $*10^6$ [a.u.]	1.2	22.2	24.5	11.5	24.5	26.4
7-Alexa 488	Excitation λ [nm]	494	512	498	494	498	498
	Emission λ [nm]	526	540	526	526	526	526
	Stokes shift [nm]	32	28	28	32	28	28
	Signal intensity $*10^6$ [a.u.]	64.2	49.9	52.9	70.7	43.2	37.0
8-SiR	Excitation λ [nm]	643	660	650	636	650	650
	Emission λ [nm]	665	690	670	660	670	670
	Stokes shift [nm]	22	30	20	24	20	20
	Signal intensity $*10^6$ [a.u.]	0.05	0.008	0.55	0.10	0.20	0.001
SI-1-Cy5.5	Excitation λ [nm]	n.t.	690	690	690	690	690
	Emission λ [nm]	n.t.	730	707	710	722	714
	Stokes shift [nm]	n.a.	40	17	20	32	24
	Signal intensity $*10^6$ [a.u.]	n.t.	2.7	3.1	2.2	2.9	3.4

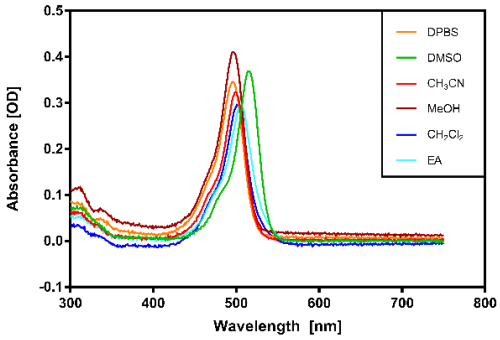
A) UV-Vis spectra of ***rac*-NBD-6**



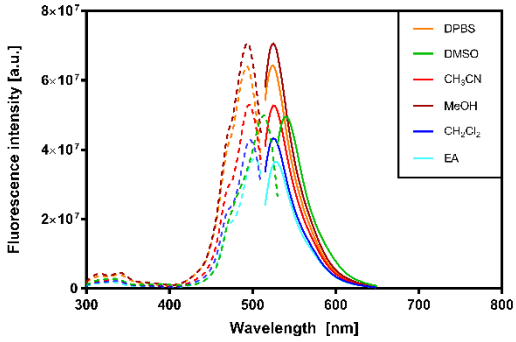
B) Fluorescence spectra of ***rac*-NBD-6**



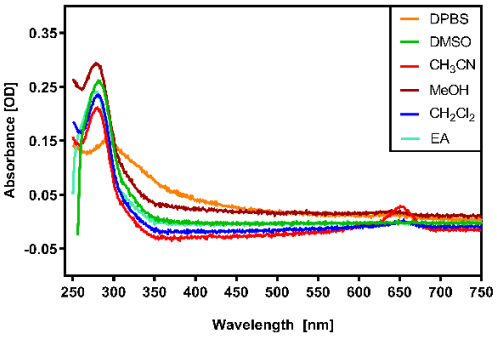
C) UV-Vis spectra of **7-Alexa 488**



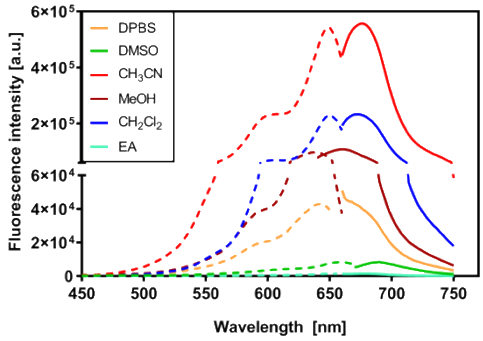
D) Fluorescence spectra of **7-Alexa 488**



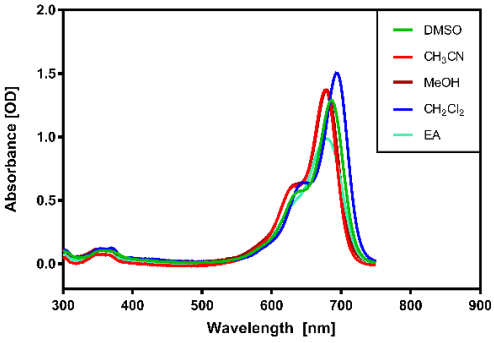
E) UV-Vis spectra of **8-SiR**



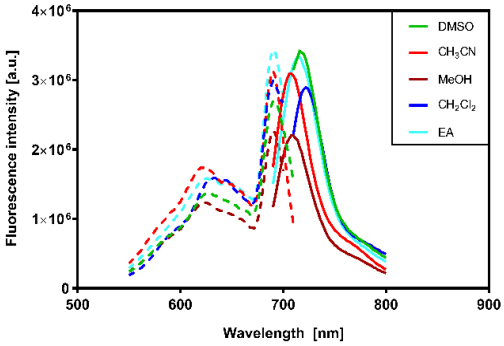
F) Fluorescence spectra of **8-Si**



G) UV-Vis spectra of **SI-1-Cy5.5**



H) Fluorescence spectra of **SI-1-Cy5.5**

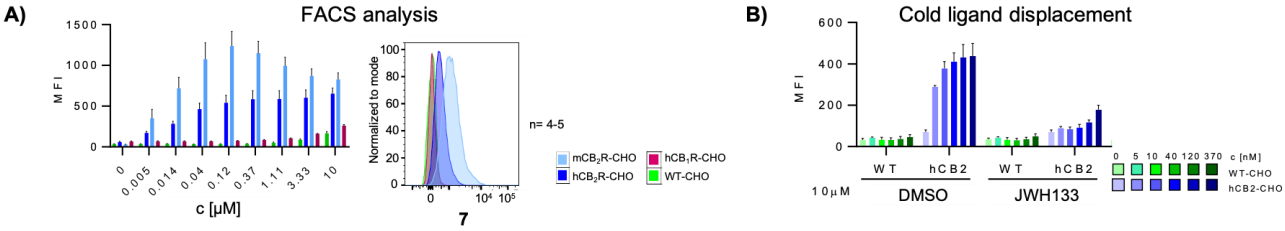


Supplementary figure S-3. Solution spectra of compounds ***rac*-6-NBD** (A and B), **7-Alexa 488** (C and D), **8-SiR** (E and F) and **SI-1-Cy5.5** (G and H). A), C), E) and G) UV-Vis spectra (50 µM probe for A, E and G; 10 µM probe for C). B), D), F) and G) technical excitation and emission fluorescence spectra not corrected for chromatic aberrations (10 µM probe) in indicated organic solvents and aqueous solution. Excitation and emission spectra are depicted with dashed or solid lines, respectively.

FLUORESCENCE ASSAYS

FACS Assay

For the validation of CB_2R-fluoroprobes via FACS analysis, 50'000 WT-CHO (wildtype), or CHO cells overexpressing hCB_2R, mCB_2R or hCB_1R were incubated with different concentrations of **7** or **8** (0.005 µM – 10 µM) in PBS/ 0.5% BSA/ 2 mM EDTA for 30 min at 4 °C. For cold ligand replacement experiments, 50'000 WT-CHO cells or CHO cells overexpressing hCB_2R were pre-incubated with 10 µM JWH133 (*Supplementary figure S-1 for chemical structure*) in PBS/ 0.5% BSA/ 2 mM EDTA at room temperature. After 30 min, different concentrations of probes **7-AF488** or **8-SiR** were added to the cell suspensions (5 nM – 370 nM) and cells were incubated for another 30 min at 4 °C. In both experiments, after probe incubation, cells were washed 3 times with PBS/ 0.5% BSA/ 2 mM EDTA and re-suspended in PBS/ 0.5% BSA/ 2 mM EDTA containing 1:1000 AquaZombie (Biolegend 423102). After exclusion of dead cells, mean fluorescent intensity of viable cells was determined. Data are presented in mean ± SEM from a representative of 3-5 experiments.



Supplementary figure S-4. A) FACS analysis of the mean fluorescent intensity (MFI) of WT, hCB2R, mCB2R and hCB1R overexpressing CHO cells at different concentrations of 7-AF488. B) FACS analysis of the MFI of WT and hCB2R-CHO cells pre-treated with JWH133 (10 µM) and stained with different concentrations of 7-AF488.

Supplementary table S-7. Statistical analysis of data shown in Figure 2A (main text). Adjusted p-value for statistical testing using 2-way-ANOVA analysis.

Fluoroprobe CB_2R specificity testing			
Concentration (uM)	**Comparison**	**p adj**	
		8-SiR	**7-AF488**
0	hCB_2R vs. hCB_1R	>0.9999	>0.9999
	hCB_2R vs. mCB_2R	>0.9999	>0.9999
	hCB_2R vs. WT	>0.9999	>0.9999
	hCB_1R vs. mCB_2R	>0.9999	>0.9999
	hCB_1R vs. WT	>0.9999	>0.9999
	mCB_2R vs. WT	>0.9999	>0.9999
0.005	hCB_2R vs. hCB_1R	0.9995	>0.9999
	hCB_2R vs. mCB_2R	>0.9999	>0.9999
	hCB_2R vs. WT	0.9994	>0.9999
	hCB_1R vs. mCB_2R	0.9989	0.9146
	hCB_1R vs. WT	>0.9999	>0.9999
	mCB_2R vs. WT	0.9987	0.6999
0.014	hCB_2R vs. hCB_1R	0.995	>0.9999
	hCB_2R vs. mCB_2R	0.9998	0.2184
	hCB_2R vs. WT	0.9938	>0.9999
	hCB_1R vs. mCB_2R	0.9901	0.0176
	hCB_1R vs. WT	>0.9999	>0.9999
	mCB_2R vs. WT	0.9884	0.0115
0.04	hCB_2R vs. hCB_1R	0.9596	0.03
	hCB_2R vs. mCB_2R	0.992	0.1351
	hCB_2R vs. WT	0.9548	0.0166
	hCB_1R vs. mCB_2R	0.8758	<0.0001
	hCB_1R vs. WT	>0.9999	>0.9999
	mCB_2R vs. WT	0.8675	<0.0001
0.12	hCB_2R vs. hCB_1R	0.6735	0.0065
	hCB_2R vs. mCB_2R	0.9108	0.1154
	hCB_2R vs. WT	0.649	0.003
	hCB_1R vs. mCB_2R	0.3147	<0.0001
	hCB_1R vs. WT	>0.9999	>0.9999
	mCB_2R vs. WT	0.2961	<0.0001
0.37	hCB_2R vs. hCB1R	0.306	0.0026
	hCB_2R vs. mCB2R	0.4218	0.4281
	hCB_2R vs. WT	0.2645	0.001

	hCB_1R vs. mCB_2R	0.01	<0.0001
	hCB_1R vs. WT	0.9998	>0.9999
	mCB_2R vs. WT	0.0078	<0.0001
1.11	hCB_2R vs. hCB_1R	0.2047	0.0033
	hCB_2R vs. mCB_2R	0.2304	0.9528
	hCB_2R vs. WT	0.1732	0.001
	hCB_1R vs. mCB_2R	0.0014	<0.0001
	hCB_1R vs. WT	0.9998	>0.9999
	mCB_2R vs. WT	0.0011	<0.0001
3.33	hCB_2R vs. hCB_1R	0.2594	0.0092
	hCB_2R vs. mCB_2R	0.1137	>0.9999
	hCB_2R vs. WT	0.2627	0.0015
	hCB_1R vs. mCB_2R	0.0006	0.0011
	hCB_1R vs. WT	>0.9999	>0.9999
	mCB_2R vs. WT	0.0006	0.0002
10	hCB_2R vs. hCB_1R	0.37	0.0593
	hCB_2R vs. mCB_2R	0.8439	>0.9999
	hCB_2R vs. WT	0.1093	0.006
	hCB_1R vs. mCB_2R	0.0921	0.0188
	hCB_1R vs. WT	0.9142	>0.9999
	mCB_2R vs. WT	0.0186	0.0016

Supplementary table S-8. Statistical analysis of data shown in Figure 2B (main text). Adjusted p-value for statistical testing using 2-way-ANOVA analysis.

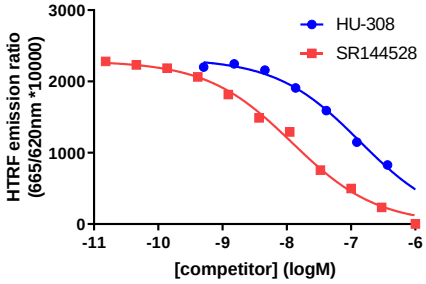
Cold ligand replacement experiment			
Concentration (uM)	**Comparison**	**p adj**	
		8-SiR	**7-AF488**
0	WT DMSO vs. hCB_2R DMSO	>0.9999	>0.9999
	hCB_2R DMSO vs. hCB_2R JWH133	>0.9999	>0.9999
	WT JWH133 vs. hCB_2R JWH133	>0.9999	>0.9999
0.005	WT DMSO vs. hCB_2R DMSO	>0.9999	<0.0001
	hCB_2R DMSO vs. hCB_2R JWH133	>0.9999	<0.0001
	WT JWH133 vs. hCB_2R JWH133	>0.9999	>0.9999
0.014	WT DMSO vs. hCB_2R DMSO	0.1363	<0.0001
	hCB_2R DMSO vs. hCB_2R JWH133	0.4272	<0.0001
	WT JWH133 vs. hCB_2R JWH133	>0.9999	>0.9999
0.04	WT DMSO vs. hCB_2R DMSO	0.0002	<0.0001
	hCB2R DMSO vs. hCB_2R JWH133	0.0076	<0.0001
	WT JWH133 vs. hCB_2R JWH133	>0.9999	0.4945
0.12	WT DMSO vs. hCB_2R DMSO	<0.0001	<0.0001
	hCB_2R DMSO vs. hCB_2R JWH133	0.0077	<0.0001
	WT JWH133 vs. hCB_2R JWH133	>0.9999	0.0843
0.37	WT DMSO vs. hCB_2R DMSO	<0.0001	<0.0001
	hCB_2R DMSO vs. hCB_2R JWH133	0.0287	<0.0001
	WT JWH133 vs. hCB_2R JWH133	>0.9999	0.0002

TR-FRET

Supplementary table S-9. TR-FRET determination of 8-SiR binding parameters.

Probe	Dye	k_{on} (M^{-1} min^{-1})	k_{off} (min^{-1})	Kinetic K_d (nM)	Saturation K_d(nM)
8	**Silicon-Rhodamine**	5.0 ± 1.0 x10^6	0.27 ± 0.08	52 ± 9	30 ± 6

Supplementary table S-10. CB_2R ligand competition affinity binding determination by TR-FRET using 8-SiR.



Compound	K_i (nM)	
	TR-FRET[a]	Radioligand binding[b]
HU-308	49 ±16	$23^{9}/36^{4}$
SR144528	5 ± 3	$0.6^{10}/13^{4}$

[a] Competition binding studies between **8** and increasing concentrations of CB_2R specific ligands HU-308 and SR144528 for hCB_2R. Data were used to calculate K_i values HU-308 and SR144528. [b] Literature data obtained from radioligand binding assay.

Cell culture: Cells were maintained in a humidified environment at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) containing blasticidin (5 µg/mL; Invitrogen) and (Zeocin; 20 µg/mL; Invitrogen). For inducible expression, SNAP-tagged human CB2 receptor cDNAs, in pcDNA4/TO were introduced through transfection, using PEI into HEK293TR cells (Invitrogen, which express Tet repressor protein to allow inducible expression). A mixed population stable line was selected by resistance to blasticidin (TR vector, 5 µg/mL) and Zeocin; (receptor plasmid, 20 µg/mL). For receptor-inducible expression, cells were seeded into t175 cm^2 flasks, grown to 70% confluence and DMEM containing 1 µg/ml tetracycline added. 24h later cells were labelled with SNAP-Lumi4-Tb (CisBio) and membranes prepared as described in detail below.

Terbium labeling of SNAP-tagged CB_2R HEK293-TR cells: Cell culture medium was removed from the t175 cm^2 flasks containing confluent adherent CB2 HEK293-TR cells. Cells were washed 1× in PBS (GIBCO Carlsbad, CA) followed by 1x Tag-lite labeling medium (LABMED, CisBio) to remove the excess cell culture media, then ten millilitre of LABMED containing 100 nM of SNAP-Lumi4-Tb was added to the flask and incubated for 1 h at 37 °C under 5% CO_2. Cells were washed 1× in PBS (GIBCO Carlsbad, CA) to remove the excess of SNAP-Lumi4-Tb then detached using 5 mL of GIBCO enzyme-free Hank's-based cell dissociation buffer (GIBCO, Carlsbad, CA) and collected in a vial containing 5 ml of DMEM (Sigma-Aldrich) supplemented with 10% fetal calf serum. Cells were pelleted by centrifugation (5 min at 1500 rpm) and the pellets were frozen to −80 °C. To prepare membranes, homogenization steps were conducted at 4 °C (to avoid receptor degradation) as described in Herenbrink et al., 2016.

Fluorescent ligand-binding assays: All fluorescent ligand binding experiments were conducted in white 384-well Optiplate plates, in assay binding buffer, either Hanks Balanced Salt Solution (HBSS), 5mM HEPES, 0.5% BSA, 0.02% pluronic F-127 pH 7.4, and 100 µM GppNHp. GppNHp was included to remove the G protein-coupled population of receptors that can result in two distinct populations of binding sites in membrane preparations, since the Motulsky-Mahan model is only appropriate for ligands competing at a single site. In all cases, nonspecific binding was determined by the presence of 1 µM SR144528. Data are presented in mean ± SEM from a representative of 3-8 experiments.

Determination of fluorescent ligand binding kinetics and equilibrium affinity: To accurately determine association rate (k_{on}) and dissociation rate (k_{off}) values, the observed rate of association (k_{ob}) was calculated using at least five different concentrations **8-SiR**. The appropriate concentration of fluorescent ligand binding was incubated with human CB_2R HEK293-TR cell membranes (4 µg per well) in assay binding buffer (final assay volume, 40 µL). The degree of fluorescent ligand bound to the receptor was assessed at multiple time points by HTRF detection to allow construction of association kinetic curves. The resulting data were globally fitted to the association kinetic model (Eq. 1, *see signal detection and data analysis section below*) to derive a single best-fit estimate for k_{on} and k_{off} as described under data analysis. Saturation analysis was performed at equilibrium, by simultaneously fitting total and Nonspecific (NSB) binding data (Eq. 2, *see signal detection and data analysis section below*) allowed the determination of fluorescent ligand binding affinity.

Competition binding: To determine the affinity of CB_2R-specific ligands, we used a simple competition kinetic binding assay. This approach involves the simultaneous addition of both fluorescent ligand and competitor to the CB_2R preparation. 62.5 nM **8-SiR**, concentration which avoid ligand depletion in this assay volume, were added simultaneously with increasing concentrations of the unlabeled compound to CB_2R cell membranes (4 µg per well) in 40 µL of assay buffer in a 384-well

plate incubated at room temperature with orbital mixing. The degree of fluorescent ligand bound to the receptor was assessed at equilibrium by HTRF detection. Nonspecific binding was determined as the amount of HTRF signal detected in the presence of SR144528 (1 µM) and was subtracted from total binding, to calculate specific binding for construction of IC_{50} curves.

Signal detection and data analysis: Signal detection was performed on a Pherastar FSX (BMG Labtech, Offenburg, Germany). The terbium donor was always excited with eight laser flashes at a wavelength of 337 nm. TR-FRET signals were collected at 665 (acceptor) and 620 nm (donor) when using the red acceptor fluorescent ligand **8-SiR**. HTRF ratios were obtained by dividing the acceptor signal by the donor signal and multiplying this value by 10'000. All experiments were analyzed by non-regression using Prism 8.0 (GraphPad Software, San Diego, USA).

Fluorescent ligand association data were fitted as follows to a global fitting model using GraphPad Prism 8.0 to simultaneously calculate k_{on} and k_{off} using the following equation,

$k_{ob} = [L]^*k_{on} + k_{off}$ (Eq. 1)

$Y = Y_{max}*(1-\exp(-1*k_{ob}*X))$

Where, k_{ob} equals the observed rate of ligand association and k_{on} and k_{off} are the association and dissociation-rate constants respectively of the fluorescent ligand. In this globally fitted model of tracer binding, tracer concentrations [L] are fixed, k_{on} and k_{off} are share parameters whilst k_{obs} is allowed to vary. Here, Y is the level of receptor-bound tracer, Y_{max} is the level of tracer binding at equilibrium, X is in units of time (eg. min) and k_{obs} is the rate in which equilibrium is approached (eg.min^{-1}).

Saturation binding data were analyzed by non-linear regression according to a one-site equation by globally fitting total and NSB. Individual estimates for the fluorescent ligand dissociation constant (K_d) were calculated using the following equations where L is the fluorescent ligand concentration:

$$\text{Total binding} = \text{Specific} + NSB = \frac{\text{Bmax} * [\text{L}]}{Kd + [L]} + \text{slope} * [\text{L}] + Background \qquad \text{NSB} = \text{slope} * [\text{L}] + Background \qquad \text{(Eq. 2)}$$

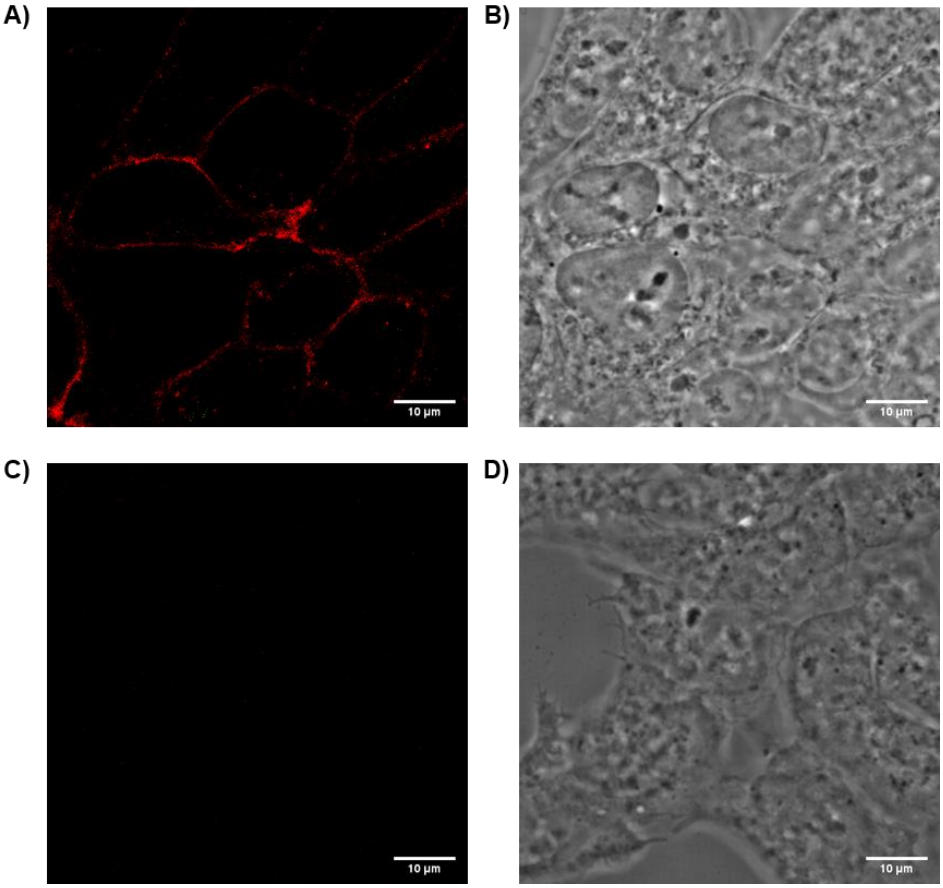
Fitting the total and NSB data sets globally (simultaneously), sharing the value of slope, provides one best-fit value for both the K_d and the B_{max}.

Competition displacement binding data were fitted to sigmoidal (variable slope) curves using a 'four-parameter logistic equation':

$Y = \text{Bottom} + (\text{Top-Bottom})/(1+10^{(\log IC50-X).\text{Hill coefficient}})$ (Eq. 3)

IC_{50} values obtained from the inhibition curves were converted to K_i values using the method of Cheng and Prusoff.[11]

$K_i = IC_{50}/(1+[\text{fluorescent tracer concertation}]/K_d)$ (Eq. 4)



Supplementary figure S-5. A) Confocal and B) widefield images of SNAP-CB$_2$R expressing T-REx 293 cells labelled with SNAP surface (red) and SNAP cell (green). C) Confocal and D) widefield images of non-expressing T-Rex 293 cells depicted processed in the same way as A and B.

Methodology

Six-well Costar cell culture plates (Corning) containing 18 mm square no 1.0 coverslips was poly-D-lysine coated. T-REx 293 cells, expressing SNAP-tagged hCB$_2$R on a pcDNA4/TO vector, were seeded at a density of 600 000/well in growth medium (DMEM high glucose (Sigma-Aldrich) with 10% FBS, 20 ug/mL Zeocin, 15 ug/mL Blasticidin). After 7h, CB$_2$R expression was induced using 1 ug/mL tetracycline. The cells were stained with SNAP dyes 24 h after induction: First, cells were incubated for 30 min with 200 nM SNAP-surface 647 (New England Biolabs), washed 3x 5 min in growth medium to remove non-reacted dye. Secondly, the cells were stained with 200 nM SNAP-cell Oregon Green (New England Biolabs) for 30 min and washed 3x 5 min in growth medium to remove non-reacted dye. The cells were incubated for 30 min in growth medium to enable non-reacted dye to diffuse out of the cells. Lastly, they were washed for 3x 5 min washes in PBS and fixed with 3% PFA. The coverslip was mounted in vecta shield anti-fade mounting medium (Vector Laboratories).

Cells were imaged using a Zeiss LSM 710 laser scanning confocal microscope fitted with a Zeiss Plan-Apochromat 63x/1.40 NA oil immersion objective. A Helium Neon 633 nm laser and Argon laser at 488 were used to excite the Alexa fluor 647 and Alexa fluor 488 fluorophores respectively and emission collected using a 488/ 561/ 633 multi beam splitting filter. Images were taken at 512 × 512 pixels per frame with line average of 4 and a slice of 53.36 um for confocal images. Laser power and gain were kept constant between experiments.

pcDNA4TO_SNAP-CB2-TwinStrep-1D4 plasmid DNA sequence:

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CMV promotorserotonin 5HT3A receptor signal peptideSNAP-tagCB2TwinStrep purification tag1D4 purification tag

SNAP-CB2-TwinStrep-1D4 protein sequence:

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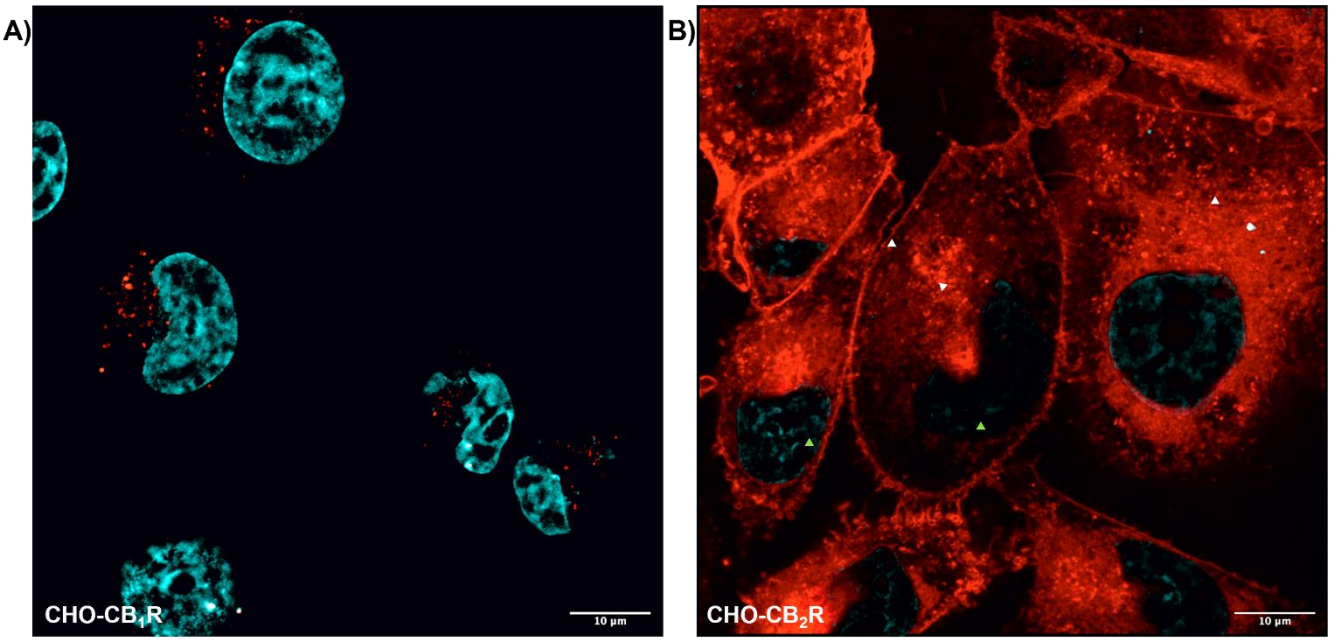
serotonin 5HT3A receptor signal peptideSNAP-tagCB2TwinStrep purification tag1D4 purification tag

Time-lapse confocal microscopy and airyscan high-resolution imaging



Supplementary figure S-6. Airyscan high-resolution imaging of hCB_1R- and hCB_2R-overexpressing CHO cells incubated either for 10 min with 0.4 µM **8** (red) and counterstained with Hoechst 33342 (cyan). A) hCB_1R cells displayed only negligible staining resulting as sparse dot structures near to the cell nucleus; B) In hCB_2R cells, **8** was readily incorporated into plasma membranes, and reached nearly all internal membranes within 10 minutes, particularly those located in the perinuclear zone, resembling endoplasmic reticulum and/or Golgi complex (green triangles). Small vesicles, reminiscent of early endosomes (white triangles), appeared below the plasma membrane and within the cytosol. *See also Supplementary Videos S-1 and S-2.*

Cell extraction and culturing

Mouse primary spleenocytes: Animals were handled according to ethical regulations on the use and welfare of experimental animals of the European Union (EU Directive 2010/63/EU) and the Italian Ministry of Health (art. 31, D.lgs. 26/2014), and the procedures were approved by the bioethical committee of Fondazione Santa Lucia of Rome (protocol n. 421/2019-PR). C57BL/6J wild-type mice were sacrificed by decapitation according to institutional guidelines, spleens were surgically removed and washed in complete medium (RPMI 1640 medium without Ca^{2+} and Mg^{2+}, supplemented with 10% heat inactivated FBS (HyClone, Logan, UT, USA), 10 U/mL penicillin/streptomycin and 2 mM L-glutamine). The spleens were cut in small pieces of approximately 1 µL volume by using a sterile scalpel. Spleen samples were then placed in a 27 mm Nunc Glass Base dish (Thermo Fisher Scientific, Monza, Italy) with 5 mL of complete medium and incubated for 1 hour at 37 °C and 5% CO_2. Splenocytes were separated by pressing the minced spleens serially through pipette tips of decreasing size. The cells were counted using a Neubauer chamber (NanoEntek, Seoul, South Korea). Finally, $5x10^5$ cells were seeded in each well of a 8-well sterile Lab-Tek chambered 1.0 borosilicate cover glass (Thermo Fisher Scientific) with 400 µL complete medium and incubated at 37°C and 5% CO_2 until imaging experiments.

Human primary macrophages: To obtain human macrophages, peripheral blood mononuclear cells from healthy donors were isolated by Ficoll density gradient centrifugation. Monocytes were enriched from the leukocytes by plastic adherence in 24-well plates, and differentiated into monocyte-derived macrophages in 1640 RPMI medium containing 10% FBS, 5% human serum, 100 U/mL penicillin/streptomycin and supplemented with 25 ng/ml macrophage colony-stimulating factor for 6-7 days at 37 °C in a humidified 5% CO_2 atmosphere. All donors gave informed consent, and the study protocol was approved the bioethical committee of Fondazione Santa Lucia of Rome (CE/Prog. 589).

Imaging methodology

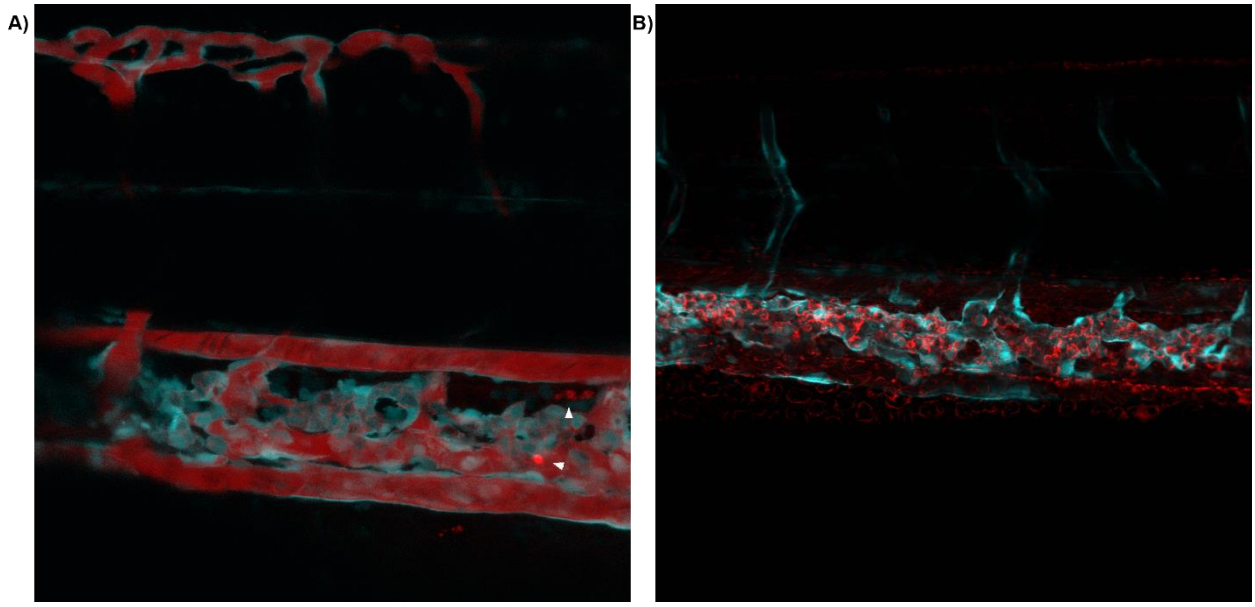
To minimize receptor internalization and thus to maximize the signal on the plasma membrane, confocal imaging experiments were performed at 22 °C. For real-time labelling studies, cells were plated onto 8-well chamber slides (Ibidi, Milan, Italy), at a density of 20'000 cells/well and cultured for 24 h. For a nuclear staining, the medium was replaced by 1 µg/mL Hoechst 33342 in RPMI (Sigma-Aldrich, Milan, Italy) and the cells were incubated for 10 min at 37 °C, then washed with PBS twice. A small volume of **8-SiR**, dissolved at 10 mM in DMSO, was mixed with the 20% (w/v) Pluronic F-127 stock solution in DMSO (Sigma-Aldrich) at a ratio of 1:1 immediately before use. Prior to imaging, the solution of **8** and Pluronic F-127 was diluted at 0.4 µM in HEPES-buffered RPMI and quickly added to the cells without any washing step. **8-SiR** was excited using a 640 nm laser line and the corresponding fluorescence emission was detected using a 655 nm long-pass filter, whereas Hoechst 33342 was excited with a dedicated 405 nm UV diode, and the corresponding fluorescence emission was detected using a 490/40 nm band-pass filter. Images within each experiment were collected by using identical laser-power, offset, and gain setting that was adjusted to minimize the level of auto-fluorescence under 640 nm. Live imaging was performed at 22 ± 2 °C by recording one frame every 15 sec for 10 min. At the end of recording session, living cells were imaged using the Airyscan mode. Each image was taken at the equatorial plan of the cells, using the ZEN Blue 2.3 software (Zeiss). Super-resolution image processing was performed using the Airyscan processing toolbox within the ZEN software package. The data were exported as TIFF files and analyzed using the Fiji software (National Institutes of Health; https://imagej.net/Fiji). A Gaussian kernel filter was applied to the images using a standard deviation of 0.8 pixels. All intensity profiles were background subtracted and normalized to the frame taken at the end of registration. For presentation purposes, images were exported in Art studio Pro version 2.0.13 (Lucky Clan, Lodz, Poland; http://www.luckyclan.com) for adjustments of brightness and contrast.

Video files

Time-lapse image sequences were converted from the czi file format to audio-video interleave (AVI) movies using Fiji software (National Institutes of Health; https://imagej.net/Fiji). Cells were incubated with Hoechst 33342 (cyan) to counter stain the nuclei.

Video S-1. Time-lapse video of CHO-hCB_1R cells labeled with 0.4 µM **8-SiR** (red) for 10 min.
Video S-2. Time-lapse video of CHO-hCB_2R cells labeled with 0.4 µM **8-SiR** (red) for 10 min.
Video S-3. Time-lapse video of murine splenocytes incubated with 0.05% DMSO (vehicle) for 10 min to assess video background.
Video S-4. Time-lapse video of murine splenocytes labeled with 0.4 µM **8-SiR** (red) for 10 min.
Video S-5. Time-lapse video of murine splenocytes labeled with 0.4 µM **8-SiR** (red) in the presence of 4 µM JWH133 for 10 min.
Video S-6. Time-lapse video of human macrophages labeled with 0.6 µM **8-SiR** (red) for 10 min.
Video S-7. Time-lapse video of human macrophages labeled with 0.6 µM **8-SiR** (red) in the presence of 4 µM JWH133 for 10 min.

Zebrafish Experiments



Supplementary figure S-7. A) **SI-1-Cy5.5** (red) injected in 3dpf Tg(*fli1*:EGFP) zebrafish larvae (cyan). B) Cy5.5 fluorophore without the recognition element (red) injected in 3dpf Tg(fli1:EGFP) zebrafish larvae (cyan) shows unspecific binding to all cell types.

Methodology

Zebrafish were handled in compliance with the local animal welfare directives of Leiden University (License number 10612) and following standard zebrafish rearing protocols (https://zfin.org), thus adhering to the international guidelines from the EU Animal Protection Direction 2010/63/EU. All experiments were performed on zebrafish larvae up to three days post fertilization (dpf), which have not yet reached the free-feeding stage. Embryos were collected in egg water (60 µg/mL Instant Ocean sea salts) and maintained in E3-buffered medium at 28.5°C during the duration of the experiments. We used the Tg(*kdrl*:mCherry) and Tg(*fli1*:EGFP) lines, which produce the fluorescent proteins mCherry and EGFP in blood vessels during embryogenesis, respectively, and the Tg(*mpeg1.1*:mCherry-F)line, in which the membrane of macrophages is labelled by mCherry-F. To prevent pigmentation, the Tg(*fli1*:EGFP) line was raised in a Casper background (genotype mitfa$^{w2/w2}$; mpv17$^{a9/a9}$), while for the Tg(*kdrl*:mCherry) and the Tg(*mpeg1.1*:mCherry-F) lines 0.2mM N-phenylthiourea (PTU; Sigma-Aldrich) was added to the E3 medium at 1dpf.

At 3dpf, larvae were anaesthetized with 0.02% buffered 3-aminobenzoic acid ethyl ester (Tricaine, Sigma) and 1nL of CB_2R probe (**7**, **8**, or **SI-1**, 0.5 µM in PBS) or unspecific Cy5.5 fluorophore (control, 0.5 µM in PBS) was microinjected directly into the bloodstream via the Duct of Cuvier using glass microcapillaries. Larvae were then mounted in 2% low melting agarose (140727, SERVA) in E3 medium containing 0.2 mM PTU and 0.02% Tricaine shortly after microinjection and imaged for 3 hours using a Leica TCS SP8 confocal microscope with a 40x water immersion objective (NA = 0.8), equipped with 488 nm, 532 nm, and 638 nm laser lines. For visualization purposes, a maximum intensity Z-projection was generated from the Raw images of the CHT tissue, pseudo colored and processed into time lapse videos with Fiji (National Institutes of Health; https://imagej.net/Fiji).

Video files

Time-lapse image sequences were converted from the lif file format to audio-video interleave (AVI) movies using Fiji software (National Institutes of Health; https://imagej.net/Fiji).

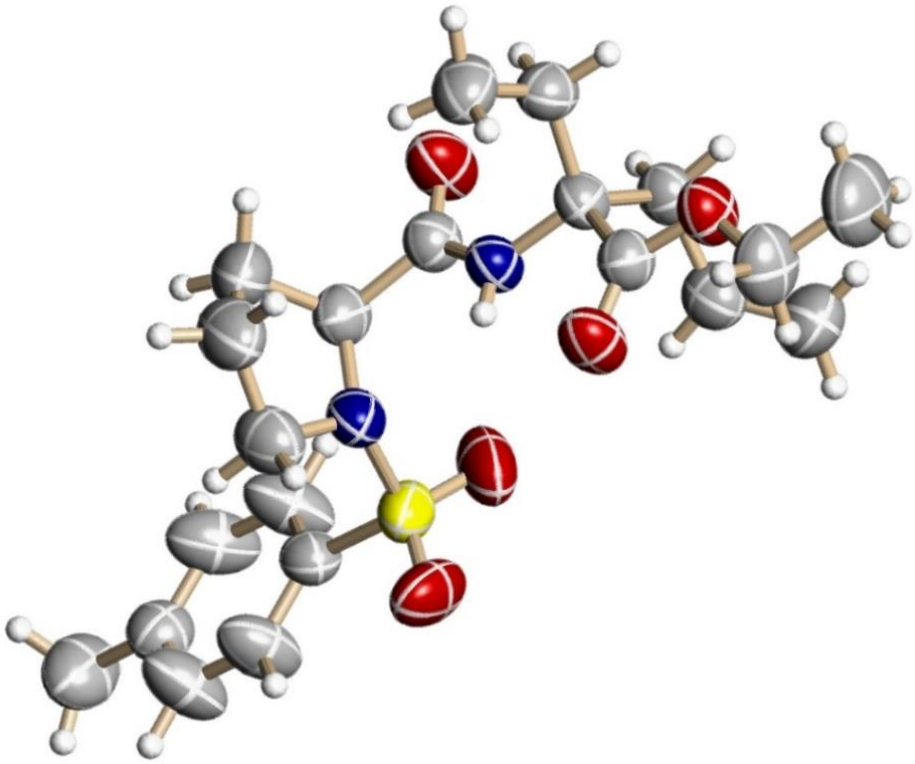
Video S-8. Z-stack video CHT region of 0.5 µM **8-SiR** (red, top) injected in Tg(*mpeg1.1*:mCherry-F) zebrafish (macrophages, bottom). Arrows indicate probe labelling in motile macrophages.
Video S-9. Time-lapse video of the CHT region of 3dpf Tg(*kdrl*:mCherry) zebrafish larvae (cyan) during 15 min shortly after injecting 0.5 µM **7-Alexa 488** (red). Arrows indicate labelled motile cells in the CHT or crawling in the adjacent fin.
Video S-10. Time-lapse video of the CHT region of 3dpf Tg(*fli1*:EGFP) zebrafish larvae (cyan) during 15 min shortly after injecting 0.5 µM **SI-1-Cy5.5** (red). Arrows indicate labelled motile cells.

X-RAY CRYSTAL STRUCTURE AND DETERMINATION OF (*R*)-2 ABSOLUTE CONFIGURATION USING PROLINE DERIVATIVE SI-34

Crystals were grown by vapor diffusion using chloroform. A suitable single crystal was mounted in a loop. Data were collected at room temperature on a Synergy-S diffractometer (Rigaku) with Cu-K-alpha-radiation (1.54184Å) and processed with the Crysalis-package. Structure solution and refinement was performed using the ShelXTL software (Bruker AXS, Karlsruhe). Detailed crystallographic information on data collection and structure refinement are shown in Supplementary table S-11 including the CCDC deposition number. CCDC 1923120 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/structures.

Supplementary table S-11. Crystallographic information on data collection and structure refinement.

Formula	$C_{21}H_{30}N_2O_5S$
Crystal class	Orthorhombic
Space group	P2(1)2(1)2(1)
a (Å)	11.83040(10)
b (Å)	12.83900(10)
c (Å)	15.08690(10)
α (°)	90
β (°)	90
γ (°)	90
Volume (A^3)	2291.56
Z	4
Crystal size (mm)	0.30 x 0.25 x 0.10
Radiation type	Copper
Wavelength (Å)	1.54184
Density (mg/cm^{-3})	1.225
Temperature (K)	293(2)
Reflections measured	51264
Independent reflections	4179
R_1 [I>2sigma(I)]	0.0305
wR_2 [I>2sigma(I)]	0.0874
R_1 (all data)	0.0314
wR_2 (all data)	0.0882
Numbers of parameters	270
Goodness of fit	1.035
CCDC	1923120
Absolut structure parameter	- 0.014 ± 0.007



Supplementary figure S-8. Molecular structure of compound **SI-34** determined by X-ray crystal structure analysis with thermal ellipsoids (drawn at the 50% probability level) for non-hydrogen atoms (heteroatoms S, N, O, are colored in yellow, blue, red, respectively); detailed crystallographic information on data collection and structure refinement are shown in Supplementary table S-11. The absolute configuration of the quaternary carbon atom was determined as (*R*).

EXPERIMENTAL PROCEDURES

General remarks

All chemicals were purchased from commercial suppliers and used as received unless otherwise specified. The following starting materials were synthesized according to previously described methods: RO6852763 (**1**),[12] ethyl 2-aminobutanoate (**SI-28**),[13] ethyl 2-(benzylideneamino)butanoate (**SI-29**),[14] ethyl (L)-2-amino-3-(4-methoxyphenyl)propanoate,[15] ethyl S-methyl-L-cysteinate,[16] 3-cyclopropyl-2-methylpyridine,[17] ethyl 2-(6-bromo-5-(3,3-difluoroazetidin-1-yl)pyrazine-2-carboxamido)-2-ethylbutanoate,[18] 2-(2-((*tert*-butoxycarbonyl)amino)ethoxy)ethyl 4-methylbenzenesulfonate,[19] 2,2-dimethyl-4-oxo-3,8,11-trioxa-5-azatridecan-13-yl 4-methylbenzenesulfonate,[20] 2,2-dimethyl-4-oxo-3,8,11,14-tetraoxa-5-azahexadecan-16-yl 4-methylbenzenesulfonate,[21] 2,2-dimethyl-4-oxo-3,8,11,14,17-pentaoxa-5-azanonadecan-19-yl 4-methylbenzenesulfonate,[22] 6-((*tert*-butoxycarbonyl)amino)hexyl 4-methylbenzenesulfonate,[23] 2-(2-(2-azidoethoxy)ethoxy)ethyl 4-methylbenzenesulfonate.[24] Non-aqueous reactions were carried out under an inert atmosphere of argon or nitrogen. Microwave heating of reactions was carried out on a Biotage Initiator apparatus. Yields refer to isolated compounds, estimated to be >95% pure as determined by LC-MS, LC-HRMS and/ or ^{1}H-NMR. TLC: Merck, TLC Silica gel 60 F254. Chromatographic separations were carried out using Biotage Isolera One apparatus with RediSep® Rf columns from Teledyne Isco. High performance liquid chromatography (HPLC) separations were carried out using Waters LC150-System with a Macherey-Nagel VP 250/21 Nucleodur 100-7 C18Ec column, eluted with a gradient system of 10:90 to 90:10 acetonitrile:water with 0.1% TFA as acidic modifier. Chiral separation was performed on Waters equipment using a 250 x 50 mm Reprosil Chiral NR column, eluted under isocratic mode with 90:10 nHep:(EtOH+0.01 mol NH_4OAc) at a 35 mL/min flow.

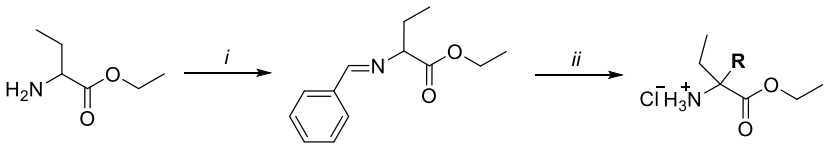
Mass and UV spectra were obtained with two different spectrometers using the same column. LCMS (method 1): Agilent Technologies 6220 Accurate Mass TOF LC/MS linked to Agilent Technologies HPLC 1200 Series instrument using a Thermo Accuore RP-MS 2.6 µm, 30 x 2.1 mm column at 25 °C (Eluent A = H_2O with 0.1% TFA; Eluent B = Acetonitrile with 0.1% TFA), at a flow of 0.8 mL/min with the following gradient: 0.0 min to 0.2 min, 95% A; 1.1 min, 1% A; 2.5 min, stop time; 1.3 min, post time; UV-detection: 220 nm, 254 nm, 300 nm. LCMS (method 2): Agilent Technologies 6120 Quadrupole LC/MS instrument linked to Agilent Technologies HPLC 1290 Infinity using a Thermo Accuore RP-MS 2.6 µm, 30 × 2.1 mm column at 25 °C (Eluent A = H_2O with 0.1% TFA; Eluent B = Acetonitrile with 0.1% TFA), at a flow of 0.8 mL/min with the following gradient: 0.0 min to 0.2 min, 95% A; 1.1 min, 1% A; 2.5 min, stop time; 1.3 min, post time; UV-detection: 220 nm, 254 nm, 300 nm. Alternatively, LC−high-resolution MS spectra were recorded with an Agilent LC system consisting of an Agilent 1290 high-pressure system, a CTC PAL autosampler, and an Agilent 6520 QTOF. The separation was achieved on a Zorbax Eclipse Plus C18 1.7 µm, 2.1 mm × 50 mm column at 55 °C (Eluent A = H_2O with 0.01% formic acid; Eluent B = acetonitrile with 0.01% formic acid) at a flow of 1 mL/min with the following gradient: 0.0 min, 5% B; 0.3 min, 5% B; 4.5 min, 99% B; 5 min, 99% B.

NMR spectra were recorded at either 295 K (300 MHz) or 300 K (600 MHz) at either Bruker AV 300 (300 MHz, 75 MHz) or Bruker AV 600 (600 MHz, 151 MHz) spectrometers for ^{1}H-NMR, ^{13}C-NMR (151 MHz), [^{1}H-^{13}C]-HSQC, [^{1}H-^{13}C]-HSQC-dept, [^{1}H-^{1}H]-NOESY and [^{1}H-^{13}C]-HMBC. Spin multiplicities are described as singlet (s), duplet (d), triplet (t), quartet (q), and mulitplet (m). Coupling constant (^{n}J, whereby n equals the number of bonds between the coupled nuclei) are recorded in Hz. NMR data were analyzed with MestReNova or ACD-Spectrus software. All ^{13}C-NMR-spectra were recorded with ^{1}H-broad-band decoupling. All chemical shifts for experiments done in $CDCl_3$ are reported in ppm (δ) relative to tetramethylsilane (δ = 0.00 ppm) and were calibrated with respect to their deuterated solvents. For experiments done in DMSO-d_6 the deuterated DMSO-d_6 solvent signal was used as the reference with 2.50 ppm.

FACS analysis was performed on BD Fortessa. Confocal imaging was performed with a confocal laser scanning microscope ZEISS LSM 800 equipped with an Airyscan detection unit (Zeiss, Oberkochen, Germany). To maximize the resolution enhancement, a high NA oil immersion alpha Plan-Apochromat 63X/1.40 oil Corr M27 objective was used.

Synthesis of amino ester building blocks

Synthesis of α,α-disubstituted α-aminoesters

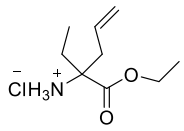


Supplementary scheme S-1. Synthesis of α,α-disubstituted α-amino esters. Reactants and Conditions: (*i*) Benzaldehyde, Et_3N, DCM, $MgSO_4$, r.t., 30 h;[14] (*ii*) a. Allyl bromide or 1-bromo-3-methoxypropane, LDA, THF, -78 °C to r.t., 24 h, b. HCl conc., H_2O, Et_2O, 0 °C to r.t., 15 h (91%).

General procedure for the synthesis of racemic α,α-disubstituted α-aminoesters

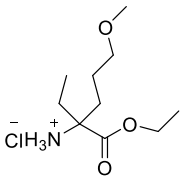
To a solution of lithium diisopropylamide (LDA, 1.5 equiv.) in THF (4 mL) cooled to –78 °C was added **SI-29** (1.0 equiv.) in THF (4 mL), followed by the dropwise addition of allyl bromide or 1-bromo-3-methoxypropane (1.5 equiv.). The mixture was stirred at room temperature for 24 h, and then concentrated under reduced pressure. The residue was then partitioned between EtOAc and water. The organic layer was separated, and the aqueous phase was extracted with EtOAc (4x). The combined organic extracts were washed with brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude residue was dissolved in diethyl ether (15 mL) and treated with 1 M HCl (3.5 equiv.) at 0 °C. The reaction mixture was allowed to warm to room temperature, and stirred for additional 15 h. The ether layer was separated, and the water phase washed with DCM (2x). The combined DCM washes were extracted with 0.2 M HCl (2x). The aqueous layers were combined and lyophilized to yield the corresponding amino ester hydrochloric salt, without the need of further purification steps.

Ethyl 2-amino-2-ethylpent-4-enoate (*SI-30*)



Following the general procedure above, **SI-30** was obtained in 96% yield, over 2 steps (1.1 g) starting from allyl bromide (0.73 mL, 8.41 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 5.74 – 5.60 (m, 1H), 5.21 – 5.05 (m, 2H), 4.15 (q, *J* = 7.1 Hz, 2H), 2.53 (dd, *J* = 13.5, 6.4 Hz, 1H), 2.21 (dd, *J* = 13.5, 8.4 Hz, 1H), 1.84 – 1.66 (m, 3H), 1.60 – 1.48 (m, 1H), 1.25 (t, *J* = 7.1 Hz, 3H), 0.83 (t, *J* = 7.5 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 176.8, 132.9, 119.5, 119.4, 61.0, 44.0, 32.9, 14.4, 8.3. HRMS (ESI) *m/z* [*M*+H]$^+$ calcd. for $C_9H_{17}NO_2$: 172.1259, found 172.1337.

Ethyl 2-amino-2-ethyl-5-methoxypentanoate *(SI-31)*



Following the general procedure above, **SI-31** was obtained in 92% yield, over 2 steps (158 mg) starting from 1-bromo-3-methoxypropane (0.15 mL, 1.40 mmol). ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm 8.86 (bs, 2H), 4.30 – 4.26 (m, 2H), 3.44 – 3.29 (m, 2H), 3.29 (s, 3H), 2.15 – 1.95 (m, 5H), 1.65 – 1.58 (m, 1H), 1.32 (t, *J* = 7.1 Hz, 3H), 1.09 (t, *J* = 7.5 Hz, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ ppm 170.2, 72.0, 64.9, 62.6, 58.5, 33.3, 30.0, 24.1, 14.3, 8.5. HRMS (ESI) *m/z* [*M*+H]$^+$ calcd. for $C_{10}H_{21}NO_3$: 204.1588, found 204.1588.

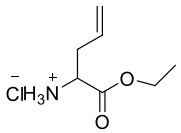
General procedure for the synthesis of racemic α-monosubstituted α-amino esters

To a solution of lithium diisopropylamide (1.05 equiv.) in anhydrous THF (3 mL) cooled to –78 °C was added *N*-(diphenylmethylene)glycine ethyl ester (1.00 equiv.). The reaction mixture was stirred for 1 h at this temperature. Afterwards, allyl bromide or 1-bromo-3-methoxypropane (1.05 equiv.) was slowly added. The mixture was stirred at –78 °C for additional 1 h, and at room temperature for additional 12 h. The reaction was concentrated under reduced pressure, and the remained residue partitioned between EtOAc and water. The organic layer was separated, and the aqueous phase was extracted with EtOAc (4x). The combined organic extracts were washed with brine (5 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure

The crude diphenylmethylene ester analogue was dissolved in diethyl ether (3 mL) and treated with 1 M HCl (3.5 equiv.) at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred for additional 15 h. The ether layer was separated, and the water phase was extracted with DCM (2x). The DCM extracts were extracted with HCl (0.2 M, 2x). The

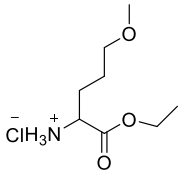
aqueous layers were combined and lyophilized to yield the corresponding amino ester hydrochloric salt, without the need of further purification.

Ethyl 2-aminopent-4-enoate *(SI-32)*



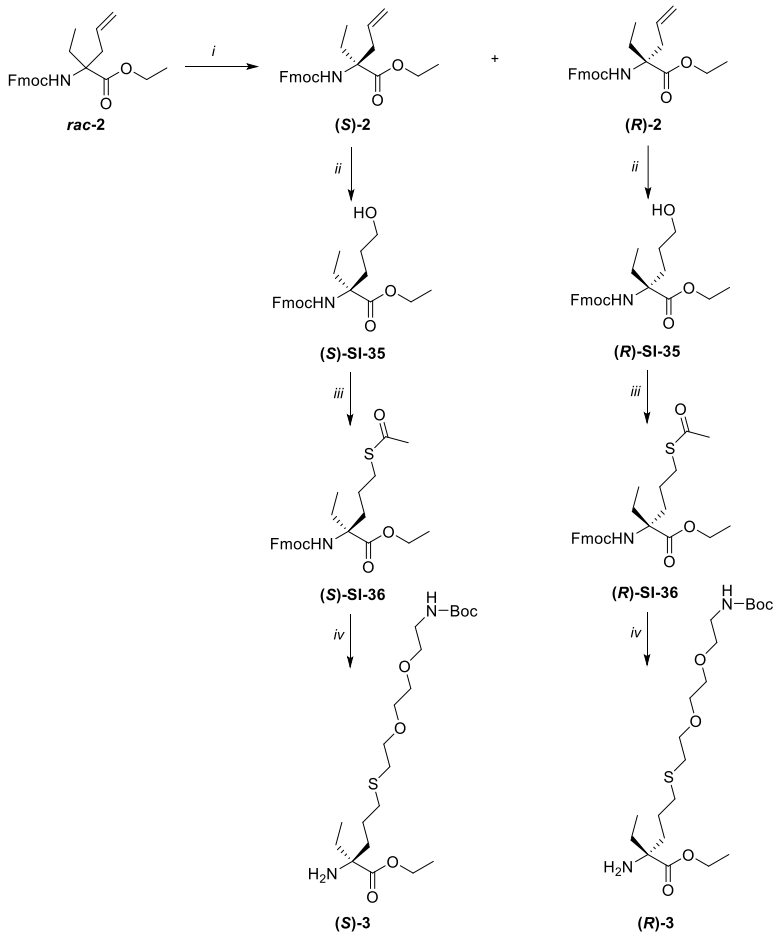
Following the general procedure above, **SI-32** was obtained in 91% yield, over 2 steps (277 mg) starting from allyl bromide (0.17 mL, 1.96 mmol). ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm 8.68 (bs, 2H), 5.88 – 5.81 (m, 1H), 5.29 (d, *J* = 16.8 Hz, 1H), 5.22 (d, *J* = 9.7 Hz, 1H), 4.31 – 4.15 (m, 3H), 2.86 – 2.79 (m, 2H), 1.27 (t, *J* = 6.6 Hz, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ ppm 168.7, 130.3, 121.3, 62.6, 52.9, 34.5, 14.1. HRMS (ESI) *m/z* $[M+H]^+$ calcd. for $C_7H_{13}NO_2$: 144.1020, found 144.1020.

Ethyl 2-amino-5-methoxypentanoate *(SI-33)*



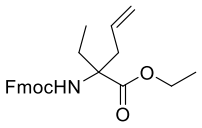
Following the general procedure above, **SI-33** was obtained in 82% yield, over 2 steps (106 mg) starting from1-bromo-3-methoxypropane (0.09 mL, 0.79 mmol). ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm 8.65 (bs, 2H), 4.22 (d, *J* = 54.0 Hz, 3H), 3.44 (bs, 2H), 3.33 (s, 3H), 2.17 (bs, 2H), 1.80 (bs, 2H), 1.30 (s, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ ppm 169.4, 71.9, 62.8, 58.8, 53.2, 27.8, 25.4, 14.3. HRMS (ESI) *m/z* $[M+H]^+$ calcd. for $C_8H_{17}NO_3$: 176.1209, found 176.1281.

Synthesis of chiral amino ester building blocks

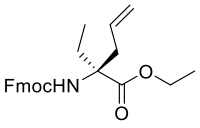


Supplementary scheme S-2. Synthesis of amino ester building block **3**. Reactants and conditions: (*i*) a. Fmoc-Cl, 1,4-dioxane, Na_2CO_3 sat. sol., 0 °C to r.t., 3 h (70%), b. chiral HPLC separation; (*ii*) a. 9-BBN, THF, r.t., 20 h, b. EtOH, 30 min, r.t., then 2 M NaOH, H_2O_2, 0 °C, 1 h (75%); (*iii*) Thioacetic acid, DIAD, PPh_3, THF, 0 °C to r.t., 2 h (77%); (*iv*) *O*-tosyl *N*-Boc PEG2, EtONa, KI, EtOH, -25 °C to r.t. (44%).

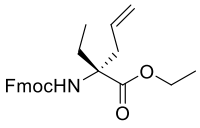
Ethyl 2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-2-ethylpent-4-enoate *(2)*



***rac*-2**



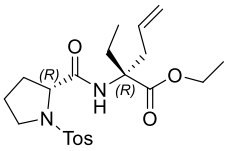
(*S*)-2



(*R*)-2

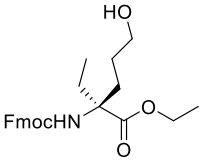
In a 100 mL round bottom flask ethyl 2-amino-2-ethylpent-4-enoate hydrochloride **SI-30** (700 mg, 4.09 mmol) was dissolved in a 1 M Na_2CO_3 aq. sol. (20 mL) and cooled to 0 °C. (9H-fluoren-9-yl)methylcarbonochloridate (1.1 g, 4.09 mmol) in 1,4-dioxane (20 mL) was added at 0 °C. The reaction mixture was stirred for 3 h at ambient temperature, poured onto water (30 mL) and extracted with EtOAc (3x 50 mL). The combined organic layers were dried over Na_2SO_4 and concentrated in vacuo. The crude material was purified by column chromatography (Silica gel, 80 g, nHex:EtOAc, 10 to 50% EtOAc) to give *rac*-**2** (1.12 g, 3.06 mmol, 70%) as a colorless oil. ^{1}H-NMR (300 MHz, $CDCl_3$): δ ppm 7.77 (dt, *J* = 7.5, 0.9 Hz, 2 H), 7.61 (d, *J* = 7.4 Hz, 2 H), 7.41 (td, *J* = 7.5, 1.4 Hz, 2 H), 7.32 (td, *J* = 7.5, 1.2 Hz, 2 H), 5.85 (s, 1 H), 5.52 – 5.70 (m, 1 H), 5.12 – 5.01 (m, 2 H), 4.47 – 4.17 (m, 5 H), 3.12 (dd, *J* = 14.0, 7.2 Hz, 1 H), 2.52 (dd, *J* = 14.0, 7.5 Hz, 1 H), 2.37 (dq, *J* = 14.6, 7.4 Hz, 1 H), 1.82 (dq, *J* = 14.6, 7.3 Hz, 1 H), 1.31 (t, *J* = 7.1 Hz, 3 H), 0.79 (t, *J* = 7.4 Hz, 3 H).^{13}C-NMR (75 MHz, $CDCl_3$): δ ppm 173.4, 154.1, 144.1, 144.0, 141.4, 132.5, 127.8, 127.8, 127.2, 125.2, 125.2, 120.1, 119.0, 77.2, 66.3, 64.5, 62.0, 47.4, 39.7, 28.4, 14.4, 8.4. HRMS (ESI) m/z $[M+H]^+$ calcd. for $C_{24}H_{27}NO$: 394.1941; found 394.2016.
The racemic mixture was separated into the two stereoisomers by reversed-phase chiral HPLC (Nucleosil-Si, 40 g, nHep:EtOAc, 0 to 100% EtOAc); $[\alpha]^{20}_d$ = - 3.16° for (*S*)-**2**; $[\alpha]^{20}_d$ = + 4.63° for (*R*)-*17* (*c* = 1.0, MeOH).

Ethyl (*S*)-2-ethyl-2-((*R*)-1-tosylpyrrolidine-2-carboxamido)pent-4-enoate *(SI-34)*



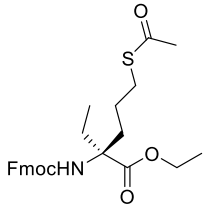
To a stirring solution of (*R*)-**2** (199 mg, 0.51 mmol) in DCM (10 mL) at room temperature piperidine (250 µL, 2.53 mmol) was added. After 4 h stirring, the reaction mixture was concentrated in vacuo to give the corresponding amine as colorless oil, which was used crude for the next reaction.
N-Toluenesulfonyl-(*S*)-proline (53 mg, 0.20 mmol) was dissolved in acetonitrile (5 mL). DIPEA (70 µL, 0.40 mmol) and HATU (76 mg, 0.20 mmol) were subsequently added, and the solution was stirred for 10 min at room temperature. Crude amine (ethyl (*R*)-2-amino-2-ethylpent-4-enoate, 34 mg, 0.20 mmol) was then added and the reaction was stirred at room temperature for 12 h. The solvents were evaporated and the crude was purified by column chromatography (Silica gel, 4 g, cHex:EtOAc 20 to 30% EtOAc) to provide 42 mg of **SI-34** (50%) as crystalline solid. ^{1}H-NMR (300 MHz, $CDCl_3$): δ ppm 7.79 – 7.69 (m, 2 H), 7.65 (s, 1 H), 7.38 – 7.27 (m, 2 H), 5.83 – 5.64 (m, 1 H), 5.13 – 5.08 (m, 1 H), 5.08 – 5.02 (m, 1 H), 4.22 (qd, *J* = 7.1, 1.6 Hz, 2 H), 4.07 – 4.02 (m, 1 H), 3.59 - 3.49 (m, 1 H), 3.23 – 3.13 (m, 1 H), 3.15 - 3.05 (m, 1 H), 2.50 (dd, *J* = 13.9, 8.2 Hz, 1 H), 2.43 (s, 3 H), 2.42 – 2.31 (m, 1 H), 2.13 - 2.05 (m, 1 H), 1.91 (dt, *J* = 14.1, 7.3 Hz, 1 H), 1.86 – 1.54 (m, 3 H, 3-H), 1.28 (t, *J* = 7.1 Hz, 3 H), 0.76 (t, *J* = 7.4 Hz, 3 H). ^{13}C-NMR (75 MHz, $CDCl_3$): δ ppm 172.9, 170.3, 144.3, 133.3, 132.6, 130.0, 128.0, 118.9, 64.8, 62.9, 61.8, 49.8, 39.5, 30.4, 27.7, 24.6, 21.7, 14.4, 8.4. HRMS (ESI) m/z $[M+H]^+$ calcd. for $C_{21}H_{31}N_2O_5S$: 423.1948, found: 423.1950.

Ethyl 2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-2-ethyl-5-hydroxypentanoate *(SI-35)*



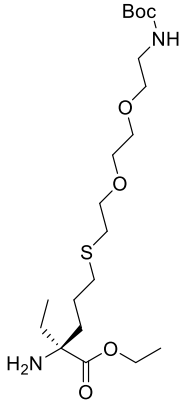
(S)-**2** (484 mg, 1.23 mmol) was dissolved in THF (20 mL) and 9-BBN (0.5 M in THF, 6 mL, 3 mmol) was added. The reaction was stirred for 20 h at room temperature, then 2 M NaOH aq. sol. (5.54 mL, 11.1 mmol) and H_2O_2 aq. sol. (35%, 4.3 mL, 44.3 mmol) were added and the reaction was stirred for further 45 min. Water (10 mL) was added and the mixture was extracted with EtOAc (2x). The combined organic layers were washed with brine (1x), dried over Na_2SO_4 and filtered. Purification of the crude by column chromatography (Silica gel, 40 g, nHep:EtOAc, 10 to 50% EtOAc) yielded **SI-35** (379 mg, 75% yield) as colorless oil. ^{1}H NMR (600 MHz, DMSO-d_6): δ ppm 7.89 (d, *J* = 7.6 Hz, 2 H), 7.71 (br d, *J*=7.4 Hz, 2 H), 7.45 – 7.37 (m, 3 H), 7.35 – 7.29 (m, 2 H), 4.26 (br d, *J* = 7.2 Hz, 2 H), 4.23 – 4.17 (m, 1 H), 4.05 (q, *J* = 7.0 Hz, 2 H), 3.34 (br t, *J* = 6.0 Hz, 5 H), 1.77 – 1.70 (m, 1 H), 1.88 – 1.66 (m, 3 H), 1.29 – 1.21 (m, 1 H), 1.30 – 1.20 (m, 1 H), 1.12 (t, *J* = 7.1 Hz, 3 H), 0.71 (br t, *J* = 7.4 Hz, 3 H). ^{13}C NMR (151 MHz, DMSO-d_6): δ ppm 173.0, 154.4, 143.8, 140.7, 127.6, 127.0, 125.2, 120.1, 65.2, 60.8, 60.2, 46.7, 29.4, 26.5, 26.0, 14.8, 7.5. HRMS (ESI) *m/z* [*M*+H]$^+$ calcd. for $C_{24}H_{29}NO_5$: 412.2046; found 412.2119.
The same procedure was applied for the synthesis of **(*R*)-SI-35**.

Ethyl 2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-5-(acetylthio)-2-ethylpentanoate *(SI-36)*



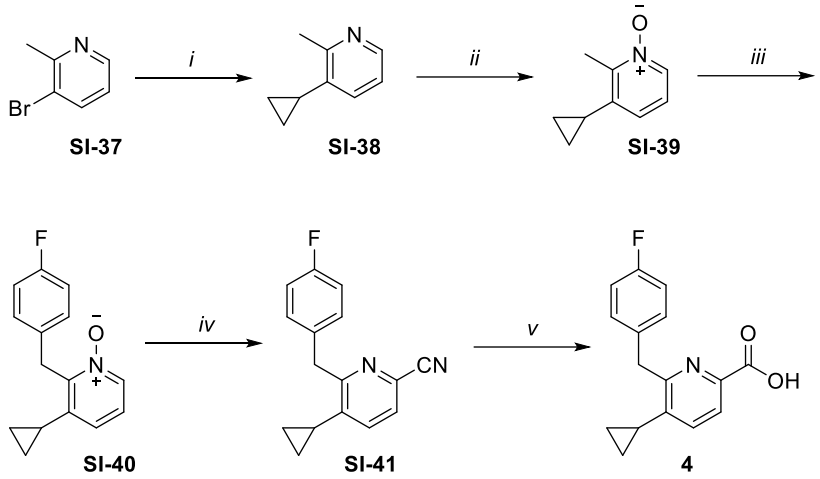
Triphenylphosphine (546 mg, 2.08 mmol) was dissolved in THF (6 mL), the solution was cooled to 0° C and DIAD (409 µL, 2.08 mmol) was added dropwise. After 10 min the Mitsunobu betaine formed as a precipitate and **(*S*)-SI-35** (428 mg, 1.04 mmol) and thioacetic acid (149 µL, 2.08 mmol) dissolved in THF (6 mL) were added. The mixture was stirred for 45 min at 0 °C, then the cooling was removed, and the reaction was stirred for 1 additional hour. The solvent was evaporated and the residue was purified by column chromatography (Silica gel, 40 g, nHep:EtOAc, 0 to 20% EtOAc) to obtain the title product **SI-36** (380 mg, 78% yield) as colorless oil. ^{1}H NMR (600 MHz, $CDCl_3$): δ ppm 7.77 (d, *J* = 7.5 Hz, 2H), 7.60 (d, *J* = 7.5 Hz, 2H), 7.42 – 7.38 (m, 2H), 7.34 – 7.30 (m, 2H), 5.83 (s, 1H), 4.43 – 4.34 (m, 2H), 4.24 (dq, *J* = 19.1, 6.3 Hz, 3H), 2.85 – 2.76 (m, 2H), 2.42 – 2.31 (m, 2H), 2.28 (s, 3H), 1.79 – 1.73 (m, 1H), 1.33 – 1.25 (m, 5H), 0.74 (t, J = 7.4 Hz, 3H). ^{13}C NMR (151 MHz, $CDCl_3$): δ ppm 195.7, 173.7, 168.8, 154.0, 144.1, 141.5, 127.8, 127.2, 125.2, 120.1, 72.5, 66.3, 64.5, 62.1, 47.5, 34.6, 30.7, 29.0, 28.7, 27.1, 25.1, 24.6, 21.7, 14.4, 8.4. HRMS (ESI) *m/z* [*M*+H]$^+$calcd. for $C_{26}H_{31}NO_5S$: 470.1923; found 470.1996. The same procedure was applied for the synthesis of **(*R*)-SI-36**.

E**thyl 18-amino-18-ethyl-2,2-dimethyl-4-oxo-3,8,11-trioxa-14-thia-5-azanonadecan-19-oate *(3)***



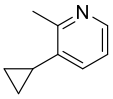
(*S*)-SI-36 (100 mg, 0.21 mmol) and linker 2,2-dimethyl-4-oxo-3,8,11-trioxa-5-azatridecan-13-yl 4-methylbenzenesulfonate (155 mg, 0.38 mmol) were dissolved in abs. ethanol (8 mL) and the solution was deoxygenated by a stream of argon. After, the reaction mixture was cooled to -25 °C and potassium iodide (11 mg, 64 µmol) and sodium ethanolate (44 mg, 0.64 mmol) were added. The solution was slowly allowed to warm up to room temperature and stirred for 2 h at the same temperature. Water was added and the aqueous phase was extracted with EtOAc (3x). The combined organic layers were dried over Na_2SO_4, filtered, and the crude was purified by column chromatography (Silica gel, 10 g, DCM:MeOH, 3 to 5% MeOH, $KMnO_4$ stain) to yield the title product (41 mg, 44%) as colorless oil. ^{1}H-NMR (600 MHz, DMSO-d_6): δ ppm 6.74 (m, 1 H), 4.09 (q, *J* = 7.1, 2 H), 3.55 – 3.45 (m, 6 H), 3.37 (t, *J* = 6.1 Hz, 2 H), 3.07 – 3.03 (m, 2 H), 2.62 – 2.61 (m, 2 H), 2.49 – 2.44 (m, 2 H), 1.71 – 1.41 (m, 5 H), 1.37 (s, 9 H), 1.35 - 1.22 (m, 1 H), 1.18 (t, *J* = 7.1 Hz, 3 H), 0.77 (t, *J* = 7.5 Hz, 3 H). ^{13}C-NMR (151 MHz, DMSO-d_6): δ ppm 175.8, 155.6, 77.6, 70.2, 69.5, 69.1, 60.7, 60.2, 39.8, 38.2, 32.3, 31.7, 30.4, 28.2, 23.8, 14.2, 8.1. HRMS (ESI) *m/z* [*M*+H]$^+$ calcd. for $C_{20}H_{41}N_2O_6S$: 437.2680, found: 437.2690.$[\alpha]^{20}_{d}$ = - 1.08°(*c* = 1.0, MeOH). The same procedure was applied for the synthesis of **(*R*)-3**, $[\alpha]^{20}_{d}$ = + 1.90°(*c* = 1.0, MeOH).

Synthesis of picolinic acid



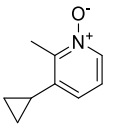
Supplementary scheme S-4. Synthetic pathway of key building block **4**. Reactants and conditions: (*i*) Cyclopropylboronic acid, $Pd(OAc)_2$, PCy_3, K_3PO_4, toluene, 120 °C, 18 h (76%); (*ii*) *m*-CPBA, DCM, 60 °C, 12 h (73%);[17] (*iii*) *p*-Fluorophenylboronic acid, $Pd_2(dba)$, XPhos, NaO*t*Bu, Toluene, 110 °C (MW), 45 min (56%); (*iv*) Benzoyl chloride, TMSCN, DCM, 40 °C, 18 h (48%); (*v*) NaOH, H_2O, methanol, 100 °C, 48 h (80%).[12]

3-Cyclopropyl-2-methylpyridine *(SI-38)*



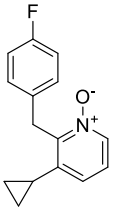
In a screw-cap vial, **SI-37** (3-bromo-2-methylpyridine, 2 g, 11.6 mmol), cyclopropylboronic acid (1.3 g, 15.1 mmol) and tripotassium phosphate (K_3PO_4, 7.4 g, 34.9 mmol) were dissolved in toluene (116 mL) and the mixture was degassed by bubbling argon gas through the solution. Palladium(II) acetate (Pd(OAc)2, 131 mg, 0.58 mmol) and tricyclohexylphosphine (PCy_3, 326 mg, 1.16 mmol) were added and the reaction was heated to 120 °C for 18 h. The mixture was allowed to cool down to room temperature, poured into water and filtered over celite. EtOAc was added and phases were separated. The aqueous phase was extracted with EtOAc (1x) and the combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. Purification of the crude by column chromatography (Silica gel, 20 g, nHep:EtOAc, 10 to 30% EtOAc) yielded **SI-38** (1.17 g, 76%) as yellow oil. ^{1}H-NMR (600 MHz, DMSO-d_6): δ ppm 8.23 (dd, *J* = 4.8, 1.7 Hz, 1 H), 7.31 (dd, *J* = 7.8, 1.7 Hz, 1 H), 7.16 – 7.02 (m, 1 H), 2.56 (s, 3 H), 1.93 (tt, *J* = 8.4, 5.3 Hz, 1 H), 1.04 – 0.84 (m, 2 H), 0.64 – 0.60 (m, 2 H). The analytical data are in accordance with those reported in the literature.[17]

3-Cyclopropyl-2-methylpyridine 1-oxide *(SI-39)*



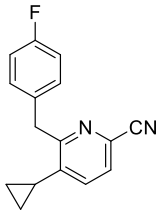
Compound **SI-38** (1.5 g, 11.3 mmol) was dissolved in DCM (80 mL) and 3-chlorobenzoperoxoic acid (6.4 g, 26.1 mmol) was added. The solution was stirred at 50 °C for 18 h and was allowed to cool to room temperature. Afterwards, 2 M $KHCO_3$ aq. sol. (30 mL) and EtOAc (50 mL) were added and the phases were separated. The organic layer was again washed with 2 M $KHCO_3$ aq. sol. (2x) and the combined aqueous layers were extracted with EtOAc (2x). The combined organic layers were dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude compound was purified by column chromatography (Silica gel, 80 g, DCM:MeOH, 0 to 5% MeOH) to yield **SI-39** (1.24 g, 70%) as brown resin. ^{1}H-NMR (600 MHz, DMSO-d_6): δ ppm 8.18 – 8.09 (m, 1 H), 7.15 (dd, J = 8.0, 6.5 Hz, 1 H), 6.96 (d, J = 8.0 Hz, 1 H), 2.48 (s, 3 H), 2.05 – 1.88 (m, 1 H), 1.06 – 0.88 (m, 2 H), 0.72 – 0.61 (m, 2 H). ^{13}C-NMR (151 MHz, DMSO-d_6): δ ppm 148.1, 140.0, 136.4, 122.7, 122.2, 13.3, 13.9, 7.2. HRMS (ESI) m/z $[M+H]^+$ calcd. for $C_9H_{12}NO$: 150.0913, found: 150.0931.

3-Cyclopropyl-2-(4-fluorobenzyl)pyridine 1-oxide *(SI-40)*



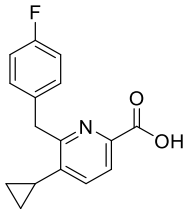
In a microwave vial (5 mL), 1-bromo-4-fluorobenzene (363 mg, 2.07 mmol), **SI-39** (464 mg, 3.11 mmol) and sodium *tert*-butylate (124 mg, 1.29 mmol) were dissolved in toluene (15 mL) and the solution was deoxygenated with a stream of argon. Tris(dibenzylideneacetone)dipalladium(0) (48 mg, 51.9 µmol) and dicyclohexyl(2',4',6'-triisopropyl-[1,1'-biphenyl]-2-yl)phosphane (49 mg, 104 µmol) were added and the vial was put into the microwave for 45 min at 110 °C. The mixture was diluted with DCM and water and the phases were separated. The aqueous phase was extracted with DCM and the combined organic phases were dried over Na_2SO_4, filtered and the resulting crude was purified by column chromatography (Silica gel, 10 g, DCM:MeOH, 0 to 5% MeOH) to yield **SI-40** (280 mg, 56% yield) as orange resin, together with recovered 98 mg **SI-39** (21%). ^{1}H-NMR (600 MHz, DMSO-d_6): δ ppm 8.15 (dd, *J* = 6.4, 0.9 Hz, 1 H), 7.30 – 7.28 (m, 2 H), 7.23 (dd, *J* = 8.1, 6.4 Hz, 1 H), 7.10 – 7.06 (m, 2 H), 6.96 (d, *J* = 8.1 Hz, 1 H), 4.45 (s, 2 H), 1.99 (tt, *J* = 8.4, 5.2 Hz, 1 H), 1.00 – 0.90 (m, 2 H), 0.72 – 0.63 (m, 2 H). ^{13}C-NMR (151 MHz, DMSO-d_6): δ ppm 161.3 (d, $^1J_{CF}$= 241.5 Hz), 150.2, 141.2, 137.1, 134.3 (d, $^4J_{CF}$= 3.0 Hz), 130.5 (d, $^3J_{CF}$ = 9.1 Hz), 124.3, 122.5, 115.46 (d, $^2J_{CF}$= 21.2 Hz), 31.3, 12.9, 8.4.HRMS (ESI) *m/z* [*M*+H]$^+$ calcd. for $C_{15}H_{15}FNO$: 244.1132, found: 244.1156.

5-Cyclopropyl-6-(4-fluorobenzyl)picolinonitrile *(SI-41)*



Compound **SI-40** (50 mg, 206 µmol) was dissolved in dry DCM (8 mL) and the solution was cooled with a water/ice bath. Subsequently trimethylsilane carbonitrile (33 µL, 267 µmol) and benzoyl chloride (25 µL, 216 µmol) were added and the reaction was stirred for 18 h while warming up to room temperature. The solution was diluted with EtOAc, washed with 2 M $KHCO_3$ aq. sol., dried over Na_2SO_4 and filtered. Purification by column chromatography (Silica gel, 4 g, Hept:EtOAc, 0 to 25% EtOAc) yielded **SI-41** (39 mg, 75% yield) as colorless oil. ^{1}H NMR (250 MHz, $CDCl_3$): δ ppm 8.01 (d, ^3J = 8 Hz, 1 H), 7.47 (d, ^3J = 8 Hz, 1 H), 7.14 (dd, ^3J = 8.8 Hz, ^4J = 6.1 Hz, 2 H), 6.99 (dd, ^3J = 8 Hz, 2 H), 4.38 (s, 2 H), 1.98 – 1.89 (m, 1 H), 1.08 – 1.04 (m, 2 H), 0.80 – 0.71 (m, 2 H). The analytical data are in accordance with those reported in the literature.[12]

5-Cyclopropyl-6-(4-fluorobenzyl)picolinic acid *(4)*



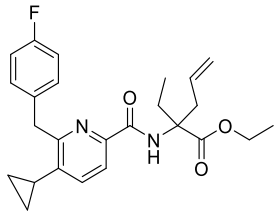
In a 25 mL round bottom flask **SI-41** (300 mg, 1.19 mmol) was dissolved in water (10 mL) and MeOH (3 mL). NaOH (190 mg, 4.76 mmol) was added, and the reaction mixture was heated to 80 °C upon stirring for 24 h. After, the reaction mixture was poured onto 0.5 M HCl aq. sol. (25 mL) and extracted with EtOAc (3x). The combined organic layers were dried over Na_2SO_4 and concentrated *in vacuo*. The crude material was purified by HPLC (10 to 90% acetonitrile in water with 0.1% TFA) to give the title compound **4** (257 mg, 0.95 mmol, 80%) as a white solid.^{1}H NMR (600 MHz, $CDCl_3$): δ ppm 8.06 – 7.98 (d, ^3J = 7.9 Hz, 1 H), 7.51 (d, ^3J = 7.9 Hz, 1 H), 7.15 (dd, ^3J = 9.0 Hz, ^4J = 6.3 Hz, 2 H), 7.02 – 6.98 (dd, ^3J = 9.1 Hz, ^3J = 9.0 Hz, 2 H), 4.42 – 4. 34 (s, 2 H), 1.98 – 1.91 (m, 1 H), 1.10 – 1.03 (m, 2 H), 0.75 – 0.68 (m, 2 H). The analytical data are in accordance with those reported in the literature.[12]

Synthesis of SAR compounds

General peptide coupling procedure

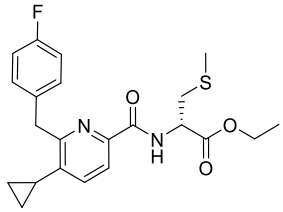
To 1.0 equiv. of 5-cyclopropyl-6-(4-fluorobenzyl)picolinic acid (**4**) in DCM (2 mL) at room temperature were added DIPEA (5.0 equiv.) and DMTMM or BOP-Cl (1.1 equiv.). The mixture was stirred at room temperature for 1 h before the desired amino ester (1.0 equiv.) in DCM (1 mL) was added. The mixture was stirred at room temperature for 24 h, diluted with DCM (5 mL) and washed with 0.2 M HCl (3x) and brine (1x). The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. Purification was performed either by reversed-phase preparative HPLC or MPLC methodologies as described for each example. For reversed-phase preparative HPLC purifications, the crude residues were dissolved in acetonitrile and water (1:1) mixture. Fractions containing the product were combined and either lyophilized (HPLC) or concentrated under reduced pressure (MPLC) to yield the desired non-labeled compound.

Ethyl 2-(5-cyclopropyl-6-(4-fluorobenzyl)picolinamido)-2-ethylpent-4-enoate *(SI-2)*



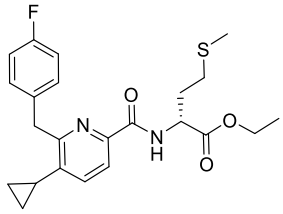
Following the general procedure above, **SI-2** was obtained in 56% yield (239 mg) starting from picolinic acid **4** (300 mg, 1.01 mmol) and ethyl 2-aminopent-4-enoate **SI-30** (173 mg, 1.01 mmol). Purification was performed by column chromatography (Silica gel, 4 g, 10% EtOAc in cHex). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 8.95 (s, 1H), 7.92 (d, *J* = 7.9 Hz, 1H), 7.40 (d, *J* = 8.0 Hz, 1H), 7.31 – 7.20 (m, 2H), 6.97 (t, *J* = 8.6 Hz, 2H), 5.72 – 5.58 (m, 1H), 5.16 – 4.96 (m, 2H), 4.43 – 4.22 (m, 4H), 3.30 (dd, *J* = 14.0, 6.9 Hz, 1H), 2.69 – 2.49 (m, 2H), 2.00 – 1.88 (m, 2H), 1.33 (t, *J* = 7.1 Hz, 3H), 1.05 – 0.96 (m, 2H), 0.81 (t, *J* = 7.4 Hz, 3H), 0.83 – 0.65 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 173.3, 1643.6, 163.2, 160.0, 158.5, 146.9, 139.9, 134.8, 134.8, 134.8, 132.8, 130.6, 130.5, 119.8, 118.6, 115.3, 115.1, 65.0, 61.8, 40.8, 39.4, 28.3, 14.4, 12.8, 8.5, 7.9. HRMS (ESI) *m/z* [*M*+H]$^+$ calcd. for $C_{25}H_{29}FN_2O_3$: 447.2162, found 447.2083.

Ethyl *N*-(5-cyclopropyl-6-(4-fluorobenzyl)picolinoyl)-(*S*)-methyl-(*L*)-cysteinate *(SI-3)*



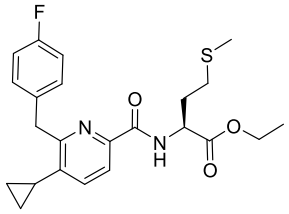
Following the general procedure above, **SI-3** was obtained in 36% yield (27 mg) starting from picolinic acid **4** (49 mg, 0.18 mmol) and ethyl*(S)*-methyl-*(L)*-cysteinate (30 mg, 0.18 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 8.70 (d, *J* = 8.2 Hz, 1H), 7.94 (d, *J* = 7.9 Hz, 1H), 7.39 (d, *J* = 8.0 Hz, 1H), 7.22 (dd, *J* = 8.4, 5.4 Hz, 2H), 6.97 (t, *J* = 8.6 Hz, 2H), 4.97 (dt, *J* = 8.3, 5.4 Hz, 1H), 4.36 (s, 2H), 4.26 (q, *J* = 7.1 Hz, 2H), 3.05 (dd, *J* = 5.3, 2.6 Hz, 2H), 2.11 (s, 3H), 1.96 – 1.86 (m, 1H), 1.31 (t, *J* = 7.1 Hz, 3H), 1.09 – 0.91 (m, 2H), 0.67 (t, *J* = 5.2 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 170.7, 164.2, 163.0, 159.8, 158.5, 145.7, 140.3, 134.5, 134.4, 130.4, 130.3, 120.2, 115.2, 114.9, 61.7, 51.9, 40.6, 36.5, 16.2, 14.1, 12.6, 7.8. HRMS (ESI) *m/z* [*M*+H]$^+$ calcd. for $C_{22}H_{25}FN_2O_3S$: 417.1581, found 417.1658. $[\alpha]^{20}_d$ = - 0.04° (*c* = 1.0, $CHCl_3$).

Ethyl (5-cyclopropyl-6-(4-fluorobenzyl)picolinoyl)-(*L*)-methioninate *(SI-4)*



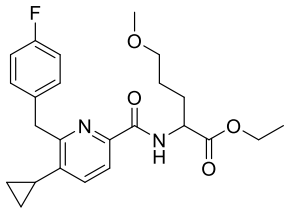
Following the general procedure above, **SI-4** was obtained in 48% yield (70 mg) starting from picolinic acid **4** (92 mg, 0.34 mmol) and ethyl*(L)*-methioninate (60 mg, 0.34 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 8.56 (d, *J* = 8.4 Hz, 1H), 7.94 (d, *J* = 7.9 Hz, 1H), 7.40 (d, *J* = 8.0 Hz, 1H), 7.21 (dd, *J* = 8.4, 5.4 Hz, 2H), 6.98 (t, J = 8.7 Hz, 2H), 4.86 (td, *J* = 7.9, 5.0 Hz, 1H), 4.36 (s, 2H), 4.24 (q, *J* = 7.1 Hz, 2H), 2.55 (t, *J* = 7.6 Hz, 2H), 2.35 – 2.20 (m, 1H), 2.09 (s, 4H), 1.95 – 1.86 (m, 1H), 1.30 (t, *J* = 7.1 Hz, 3H), 1.08 – 0.89 (m, 2H), 0.78 – 0.56 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 171.8, 164.5, 163.2, 159.9, 158.5, 145.8, 140.6, 134.6, 134.5, 130.5, 130.4, 120.4, 115.4, 115.1, 61.7, 51.6, 40.7, 32.2, 30.1, 15.5, 14.2, 12.7, 8.0, 8.0. HRMS (ESI) *m/z* [*M*+H]$^+$ calcd. for $C_{23}H_{27}FN_2O_3S$: 431.1723, found 431.1787. $[\alpha]^{20}_d$ = + 0.04° (*c* = 1.0, $CHCl_3$).

Ethyl (5-cyclopropyl-6-(4-fluorobenzyl)picolinoyl)-(*D*)-methioninate *(SI-5)*



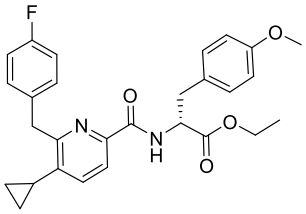
Following the general procedure above, **SI-5** was obtained in 41% yield (12 mg) starting from picolinic acid **4** (20 mg, 0.07 mmol) and ethyl (*D*)-methioninate (13 mg, 0.07 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 8.52 (d, *J* = 8.5 Hz, 1H), 7.94 (d, *J* = 7.9 Hz, 1H), 7.39 (d, *J* = 8.0 Hz, 1H), 7.25 – 7.15 (m, 2H), 7.03 – 6.93 (m, 2H), 4.86 (td, *J* = 7.9, 5.0 Hz, 1H), 4.36 (s, 2H), 4.24 (q, *J* = 7.1 Hz, 2H), 2.54 (t, *J* = 8.0 Hz, 2H), 2.32 – 2.20 (m, 1H), 2.14 – 2.02 (m, 1H), 2.09 (s, 4H), 1.94 – 1.85 (m, 1H), 1.30 (t, *J* = 7.1 Hz, 3H), 1.03 – 0.97 (m, 2H), 0.69 – 0.64 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 171.9, 164.5, 163.2, 160.0, 158.5, 145.9, 140.6, 134.7, 130.6, 130.5, 120.5, 115.4, 115.2, 61.7, 51.7, 40.8, 32.3, 30.2, 15.6, 14.3, 12.8, 8.0, 8.0. HRMS (ESI) *m/z* [*M*+H]$^+$ calcd. for $C_{23}H_{27}FN_2O_3S$: 453.1777, found 453.1594.$[\alpha]^{20}_d$ = - 0.06° (*c* = 1.0, $CHCl_3$).

Ethyl 2-(5-cyclopropyl-6-(4-fluorobenzyl)picolinamido)-5-methoxypentanoate *(SI-7)*



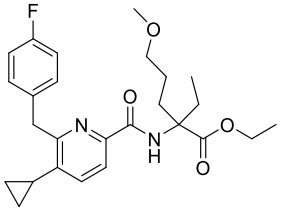
Following the general procedure above, **SI-7** was obtained in 23% yield (27 mg) starting from picolinic acid **4** (76 mg, 0.28 mmol) and ethyl 2-amino-5-methoxypentanoate **SI-33** (50 mg, 0.28 mmol). Purification was performed by reversed-phase preparative HPLC (30 to 90% acetonitrile in water with 0.1% TFA). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 8.44 (d, *J* = 8.4 Hz, 1H), 7.94 (d, *J* = 7.9 Hz, 1H), 7.38 (d, *J* = 7.9 Hz, 1H), 7.23 – 7.14 (m, 2H), 7.03 – 6.93 (m, 2H), 4.78 – 4.71 (m, 1H), 4.35 (s, 2H), 4.22 (q, *J* = 7.1 Hz, 2H), 3.39 (t, *J* = 6.2 Hz, 2H), 3.31 (s, 3H), 2.09 – 1.97 (m, 1H), 1.94 – 1.79 (m, 2H), 1.69 – 1.59 (m, 2H), 1.29 (t, *J* = 7.1 Hz, 3H), 1.02 – 0.96 (m, 2H), 0.69 – 0.63 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 172.3, 164.5, 162.5, 160.8, 158.5, 146.1, 140.4, 134.6, 130.5, 120.5, 115.4, 115.2, 72.1, 61.5, 58.7, 52.2, 40.8, 29.6, 25.7, 14.4, 12.8, 8.0, 8.0. HRMS (ESI) *m/z* [*M*+H]$^+$ calcd. for $C_{24}H_{29}FN_2O_4$: 429.2119, found 4298.2190.

Ethyl (*L*)-2-(5-cyclopropyl-6-(4-fluorobenzyl)picolinamido)-3-(4-methoxyphenyl) propanoate *(SI-8)*



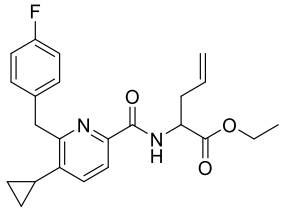
Following the general procedure above, **SI-8** was obtained in 38% yield (37 mg) starting from picolinic acid **4** (54 mg, 0.20 mmol) and ethyl *(L)*-2-amino-3-(4-methoxyphenyl)propanoate (45 mg, 0.20 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 8.58 (d, *J* = 8.2 Hz, 1H), 7.94 (d, *J* = 8.0 Hz, 1H), 7.41 (d, *J* = 7.9 Hz, 1H), 7.20 – 7.12 (m, 2H), 7.11 – 7.04 (m, 2H), 7.01 – 6.91 (m, 2H), 6.88 – 6.71 (m, 2H), 4.97 (dt, *J* = 8.3, 6.0 Hz, 1H), 4.33 (d, *J* = 5.0 Hz, 2H), 4.21 (q, *J* = 7.1 Hz, 2H), 3.76 (s, 3H), 3.33 – 3.03 (m, 2H), 2.02 – 1.81 (m, 1H), 1.27 (t, *J* = 7.1 Hz, 3H), 1.10 – 0.95 (m, 2H), 0.68 (h, *J* = 3.8 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 171.3, 164.7, 163.2, 160.0, 158.8, 158.6, 145.4, 140.9, 134.8, 134.4, 130.5, 130.4, 127.9, 120.6, 115.4, 115.1, 114.0, 61.7, 55.3, 53.7, 40.7, 37.3, 14.3, 12.8, 8.1, 8.1. HRMS (ESI) *m/z* [*M*+H]$^+$ calcd. for $C_{28}H_{29}FN_2O_4$: 477.2118, found 477.2188. $[\alpha]^{20}_d$ = + 0.54° (*c* = 1.0, $CHCl_3$).

Ethyl 2-(5-cyclopropyl-6-(4-fluorobenzyl)picolinamido)-2-ethyl-5-methoxypentanoate *(SI-9)*



Following the general procedure above, **SI-9** was obtained in 25% yield (50 mg) starting from picolinic acid **4** (117 mg, 0.43 mmol) and ethyl 2-amino-2-ethyl-5-methoxypentanoate **SI-31** (88 mg, 0.43 mmol). Purification was performed by reversed-phase preparative HPLC (30 to 90% acetonitrile in water with 0.1% TFA). ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm 9.00 (s, 1H), 7.90 (d, *J* = 7.9 Hz, 1H), 7.38 (d, *J* = 7.9 Hz, 1H), 7.27 – 7.24 (m, 2H), 6.96 (t, *J* = 8.7 Hz, 2H), 4.36 (s, 2H), 4.32 – 4.25 (m, 2H), 3.35 – 3.27 (m, 2H), 3.26 (d, *J* = 1.1 Hz, 3H), 2.60 – 2.50 (m, 2H), 2.00 – 1.86 (m, 3H), 1.59 – 1.48 (m, 1H), 1.41 – 1.34 (m, 1H), 1.32 (t, *J* = 7.1 Hz, 3H), 1.02 – 0.96 (m, 2H), 0.77 (t, *J* = 7.4 Hz, 3H), 0.67 – 0.64 (m, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ ppm 173.8, 163.4, 162.4, 160.8, 158.5, 147.0, 139.9, 134.8, 134.8, 130.6, 130.5, 119.8, 115.3, 115.2, 72.6, 65.1, 61.8, 58.5, 40.8, 31.9, 28.6, 24.7, 14.4, 12.8, 8.6, 7.9. HRMS (ESI) *m/z* [*M*+H]$^+$ calcd. for $C_{26}H_{33}FN_2O_4$: 457.2428, found 457.2500.

Ethyl 2-(5-cyclopropyl-6-(4-fluorobenzyl)picolinamido)pent-4-enoate *(SI-42)*

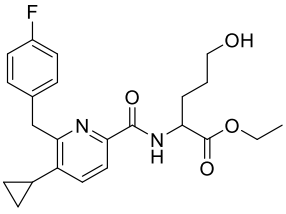


Following the general procedure above, **SI-42** was obtained in 28% yield (83 mg) starting from picolinic acid **4** (200 mg, 0.74 mmol) and ethyl 2-aminopent-4-enoate **SI-32** (133 mg, 0.74 mmol). Purification was performed by column chromatography (Silica gel, 4 g, 10% EtOAc in cHex). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 8.46 (d, *J* = 8.3 Hz, 1H)v, 7.93 (d, *J* = 8.0 Hz, 1H), 7.38 (d, *J* = 8.0 Hz, 1H), 7.24 – 7.15 (m, 2H), 7.03 – 6.91 (m, 2H), 5.78 – 5.67 (m, 1H), 5.16 – 5.09 (m, 2H), 4.84 – 4.78 (m, 1H), 4.34 (d, *J* = 3.1 Hz, 2H), 4.30 – 4.17 (m, 2H), 2.75 – 2.54 (m, 2H), 1.95 – 1.86 (m, 1H), 1.29 (t, *J* = 7.1 Hz, 3H), 1.03 – 0.96 (m, 2H), 0.69 – 0.63 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 171.5, 164.3, 163.2, 160.0, 158.5, 146.0, 140.4, 134.7, 132.6, 130.6, 130.5, 120.4, 119.2, 115.4, 115.1, 61.5, 51.8, 40.8, 36.9, 14.4, 12.8, 8.0, 7.9. HRMS (ESI) *m/z* [*M*+H]$^+$ calcd. for $C_{23}H_{25}FN_2O_3$: 419.1918, found 419.1736.

General hydroboration-oxidation procedure

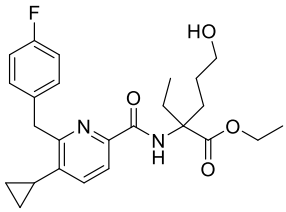
To a solution of the corresponding alkene (1.0 equiv.) in THF (2 mL) at room temperature and nitrogen atmosphere were added 9-BBN (0.5 M in THF, 1.5 equiv.). The mixture was stirred at room temperature for 36 h, after which time the excess of 9-BBN was quenched with ethanol (3.5 equiv.). The mixture was stirred at room temperature for 30 min, followed by the concurrent dropwise addition of 2 M NaOH aq. sol. (4 mL/mmol) and H_2O_2 aq. sol. (30%, 4 mL/mmol) at 0 °C. After complete addition, stirring was continued at the same temperature for additional 1 h. The solution was extracted with diethyl ether (3x), dried over Mg_2SO_4 and filtered. Subsequent purification by column chromatography (Silica gel, 4 g or 15 g, cHex:EtOAc, 0 to 50% EtOAc) was performed to yield the desired product.

Ethyl 2-(5-cyclopropyl-6-(4-fluorobenzyl)picolinamido)-5-hydroxypentanoate *(SI-43)*



Following the general procedure above, **SI-43** was obtained in 34% yield (29 mg) starting from alkene **SI-42** (83 mg, 0.21 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 8.52 (d, *J* = 8.3 Hz, 1H), 7.93 (d, *J* = 7.9 Hz, 1H), 7.38 (d, *J* = 8.0 Hz, 1H), 7.24 – 7.14 (m, 2H), 7.04 – 6.90 (m, 2H), 4.79 (td, *J* = 7.9, 5.2 Hz, 1H), 4.35 (s, 2H), 4.23 (q, *J* = 7.1 Hz, 2H), 3.69 (t, *J* = 6.2 Hz, 2H), 2.12 – 1.98 (m, 2H), 1.95 – 1.82 (m, 3H), 1.70 – 1.57 (m, 2H), 1.29 (t, *J* = 7.1 Hz, 3H), 1.04 – 0.95 (m, 2H), 0.71 – 0.61 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 172.3, 164.6, 163.2, 160.0, 158.5, 145.9, 140.5, 134.7, 130.6, 130.5, 120.5, 115.4, 115.1, 62.3, 61.6, 52.0, 40.8, 29.7, 28.4, 14.3, 12.8, 8.0, 8.0. HRMS (ESI) *m/z* [*M*+H]$^+$ calcd. for $C_{23}H_{27}FN_2O_4$: 437.1949, found 437.1843.

Ethyl 2-(5-cyclopropyl-6-(4-fluorobenzyl)picolinamido)-2-ethyl-5-hydroxypentanoate *(SI-44)*

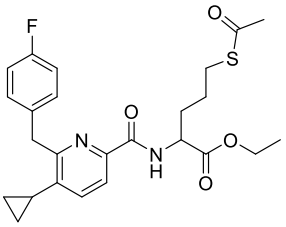


Following the general procedure above, **SI-44** was obtained in 99% yield (246 mg) starting from alkene **SI-2** (239 mg, 0.56 mmol). ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm 9.02 (s, 1H), 7.89 (d, *J* = 8.0 Hz, 1H), 7.27 – 7.24 (m, 3H), 6.99 – 6.94 (m, 2H), 4.41 (s, 2H), 4.36 (s, 2H), 4.33 – 4.27 (m, 2H), 3.62 – 3.55 (m, 1H), 2.65 – 2.60 (m, 1H), 2.56 – 2.52 (m, 1H), 2.05 – 1.95 (m, 1H), 1.95 – 1.85 (m, 2H), 1.55 – 1.49 (m, 1H), 1.43 – 1.35 (m, 1H), 1.33 (td, *J* = 7.1, 1.8 Hz, 3H), 1.02 – 0.98 (m, 2H), 0.77 (td, *J* = 7.4, 2.6 Hz, 3H), 0.67 – 0.65 (m, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ ppm 173.8, 163.6, 160.9, 158.6, 146.9, 140.0, 134.8, 130.5, 130.5, 119.9, 115.3, 115.2, 65.1, 62.8, 61.9, 40.8, 31.5, 28.9, 27.8, 14.4, 14.4, 12.9, 8.6, 7.9. HRMS (ESI) *m/z* [*M*+H]$^+$ calcd. for $C_{25}H_{31}FN_2O_4$: 443.2268, found 443.2337.

General thio-Mitsunobu procedure

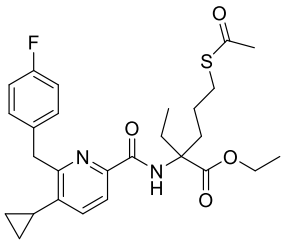
DIAD (2.0 equiv.) in anhydrous THF (0.2 mL) was added dropwise to a stirred solution of Ph_3P (2.0 equiv.) in anhydrous THF (1 mL) at 0 °C under nitrogen atmosphere. The mixture was stirred at this temperature for 30 min, until formation of a white precipitate of Mitsunobu betaine, and a solution of thioacetic acid (2.0 equiv.) and the corresponding alcohol (2.0 equiv.) in anhydrous THF (1 mL) was added slowly. The reaction was stirred at 0 °C for 1 h and then allowed to reach room temperature and stirred for additional 1 h. The reaction mixture was concentrated, and the residue taken up into a mixture of diethyl ether and cyclohexane (1:1) and triturated at 0 °C. The resulting white solid was filtered off and washed with diethyl ether and cyclohexane (1:1) mixture. The filtrate was evaporated under reduced pressure, and the residue purified by column chromatography (Silica gel, 4 g, cHex:EtOAc, 5 to 15% EtOAc).

Ethyl 5-(acetylthio)-2-(5-cyclopropyl-6-(4-fluorobenzyl)picolinamido)pentanoate *(SI-45)*



Following the general procedure above, **SI-45** synthesized starting from compound **SI-43** (29 mg, 0.07 mmol). After filtration work-up, intermediate **SI-45** was obtained, along with DIAD by-products, and used crude for further reaction steps. HRMS (ESI) *m/z* [*M*+H]$^+$ calcd. for $C_{25}H_{29}FN_2O_4S$: 495.1827, found 495.1718.

Ethyl 5-(acetylthio)-2-(5-cyclopropyl-6-(4-fluorobenzyl)picolinamido)-2-ethylpentanoate *(SI-46)*

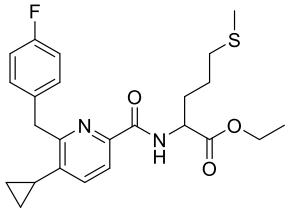


Following the general procedure above, **SI-46** was obtained in 55% yield (24 mg) starting from compound **SI-44** (39 mg, 0.09 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 9.01 (s, 1H), 7.92 (d, *J* = 7.9 Hz, 1H), 7.41 (d, *J* = 8.0 Hz, 1H), 7.32 – 7.21 (m, 3H), 7.05 – 6.93 (m, 2H), 4.42 – 4.25 (m, 4H), 2.90 – 2.78 (m, 2H), 2.71 – 2.48 (m, 2H), 2.30 (s, 3H), 2.04 – 1.84 (m, 3H), 1.66 – 1.51 (m, 1H), 1.45 – 1.25 (m, 3H), 1.07 – 0.97 (m, 2H), 0.78 (t, *J* = 7.4 Hz, 3H), 0.73 – 0.64 (m, 2H). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.99 (s, 1H), 7.90 (d, J = 7.9 Hz, 1H), 7.38 (d, J = 8.0 Hz, 1H), 7.30 – 7.19 (m, 3H), 7.02 – 6.91 (m, 2H), 4.39 – 4.23 (m, 4H), 2.88 – 2.76 (m, 2H), 2.69 – 2.45 (m, 2H), 2.28 (s, 3H), 2.01 – 1.81 (m, 3H), 1.64 – 1.49 (m, 1H), 1.43 – 1.23 (m, 5H), 1.04 – 0.95 (m, 2H), 0.76 (t, J = 7.4 Hz, 3H), 0.70 – 0.62 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 195.8, 173.6, 163.5, 160.0, 158.5, 146.8, 140.0, 134.8, 134.8, 130.6, 130.5, 119.8, 115.4, 115.1, 65.0, 61.9, 40.8, 34.4, 30.7, 29.1, 28.6, 24.7, 24.7, 14.4, 12.8, 8.5, 7.9, 7.9. HRMS (ESI) *m/z* [*M*+H]$^+$ calcd. for $C_{27}H_{33}FN_2O_4S$: 523.2058, found 523.2169.

General acetylthio cleavage procedure using iodomethane

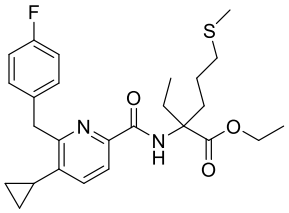
Thioether derivative (1.0 equiv.) and iodomethane (1.0 equiv.) were added to oxygen-free absol. ethanol (2 mL) under nitrogen atmosphere. The suspension was cooled to – 20 °C, sodium ethoxide (2.2 equiv.) was added, and the mixture was allowed to warm up slowly to room temperature. The resulting pale-yellow solution was stirred for 20 h. The reaction mixture was concentrated, and the resulting residue was dissolved in an acetonitrile and water (1:1) solution and purified by reversed-phase preparative HPLC (30 to 90% acetonitrile in water with 0.1% TFA). The fractions containing the product were combined and lyophilized to yield the desired compound.

Ethyl 2-(5-cyclopropyl-6-(4-fluorobenzyl)picolinamido)-5-(methylthio)pentanoate *(SI-6)*



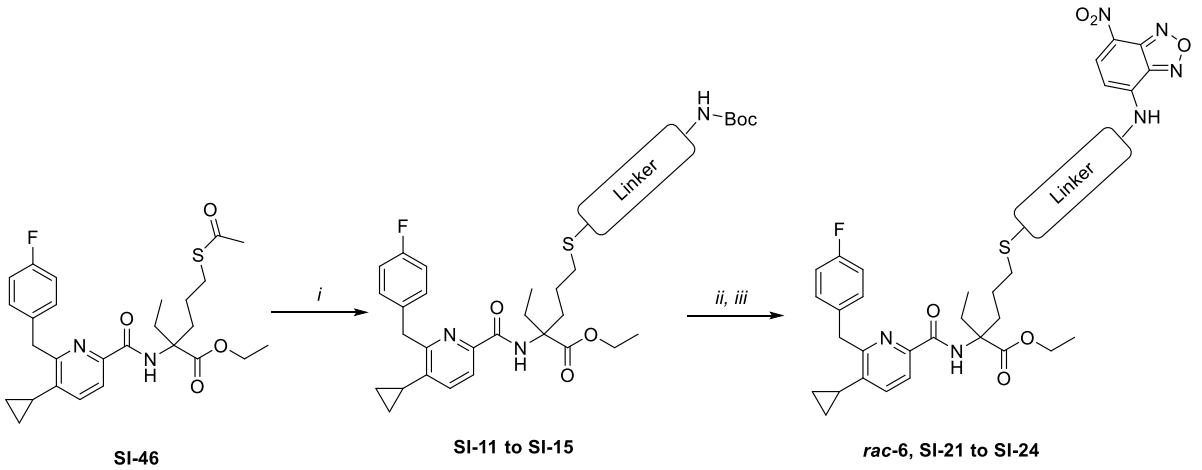
Following the general procedure above, **SI-6** was obtained in 16% yield (4 mg), over 2 steps starting from crude compound **SI-45**. ^{1}H NMR (600 MHz, DMSO-d_6) δ ppm 8.58 (d, *J* = 8.1 Hz, 1H), 7.78 (d, *J* = 8.0 Hz, 1H), 7.52 (d, *J* = 8.0 Hz, 1H), 7.38 – 7.29 (m, 2H), 7.11 (tt, *J* = 9.8, 3.0 Hz, 2H), 4.51 – 4.47 (m, 1H), 4.41 – 4.34 (m, 2H), 4.19 – 4.09 (m, 2H), 2.53 – 2.51 (m, 1H), 2.48 – 2.44 (m, 1H), 2.10 – 2.03 (m, 1H), 2.00 – 1.84 (m, 5H), 1.57 – 1.52 (m, 2H), 1.20 (t, *J* = 7.1 Hz, 3H), 1.03 – 0.95 (m, 2H), 0.70 – 0.68 (m, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ ppm 171.6, 163.7, 161.6, 160.0, 158.1, 146.5, 140.4, 135.0, 134.1, 130.7, 130.6, 120.0, 115.0, 114.8, 60.7, 52.0, 32.5, 30.0, 24.7, 14.4, 14.0, 12.1, 8.3, 8.2. HRMS (ESI) *m/z* [*M*+H]$^+$ calcd. for $C_{24}H_{29}FN_2O_3S$: 445.1901, found 445.1930.

Ethyl 2-(5-cyclopropyl-6-(4-fluorobenzyl)picolinamido)-2-ethyl-5-(methylthio)pentanoate *(SI-10)*



Following the general procedure above, **SI-10** was obtained in 46% yield (9 mg) starting from compound **SI-46** (20 mg, 0.04 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 9.06 (s, 1H), 7.91 (d, *J* = 8.0 Hz, 1H), 7.40 (d, *J* = 8.0 Hz, 1H), 7.31 – 7.21 (m, 2H), 7.04 – 6.91 (m, 2H), 4.38 (s, 2H), 4.29 (q, *J* = 7.1 Hz, 2H), 2.67 – 2.36 (m, 4H), 2.09 – 1.83 (m, 6H), 1.67 – 1.52 (m, 1H), 1.42 – 1.31 (m, 4H), 1.03 – 0.97 (m, 2H), 0.77 (t, *J* = 7.4 Hz, 3H), 0.71 – 0.64 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 173.8, 163.3, 160.0, 158.5, 146.7, 140.2, 135.0, 134.7, 130.6, 130.5, 120.0, 115.4, 115.1, 65.2, 61.9, 40.7, 34.4, 34.1, 28.6, 24.2, 15.5, 14.5, 12.8, 8.6, 8.0. HRMS (ESI) *m/z* [*M*+H]$^+$ calcd. for $C_{26}H_{33}FN_2O_3S$: 473.2296, found 473.2283.

Synthesis of racemic NBD-labeled probes



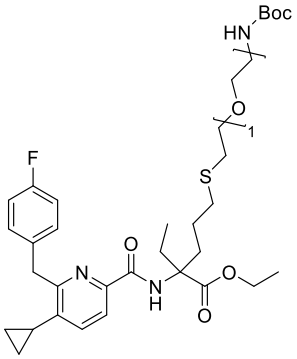
SI-46 **SI-11 to SI-15** ***rac*-6, SI-21 to SI-24**

Supplementary scheme S-3. General approach to racemic NBD-labeled probes for the pyridine scaffold. Reactants and conditions: (*i*) *O*-tosyl*N*-Boc-PEG1-4 or hexyl linker, EtONa, KI, ethanol, -20 °C to r.t., 18 h (46 to 56%); (*ii*) DCM:TFA (9:1), 0 °C to r.t., 2 h (100%); (*iii*) NBD-Cl, Cs_2CO_3, DMF, 0 °C to r.t., 24 h (18 to 49%).

General procedure for linker attachment

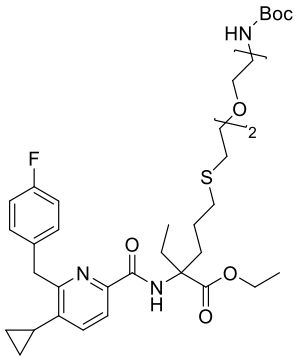
Thioderivative **SI-46** (1.0 equiv.) and the corresponding tosyl linker (1.8 equiv.) were added to absolute ethanol previously degassed (2 mL) under nitrogen atmosphere. The suspension was cooled to – 20 °C, sodium ethoxide (3.0 equiv.) was added along with catalytic amounts of potassium iodide (0.3 equiv.), and the mixture was allowed to warm up slowly to room temperature. The resulting solution was stirred for 20 h. The reaction mixture was concentrated under reduced pressure. The resulting residue was dissolved in acetonitrile and water (1:1) and purified by reversed-phase preparative HPLC (30 to 90% acetonitrile in water with 0.1% TFA).

Ethyl 15-(5-cyclopropyl-6-(4-fluorobenzyl)picolinamido)-15-ethyl-2,2-dimethyl-4-oxo-3,8-dioxa-11-thia-5-aza-hexadecan-16-oate *(SI-11)*



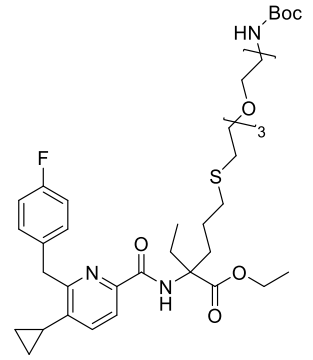
Following the general procedure above, **SI-11** was obtained in 50% yield (16 mg) starting from linker 2-(2-((*tert*-butoxycarbonyl)amino)ethoxy)ethyl 4-methylbenzenesulfonate[19] (32 mg, 0.09 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 9.02 (s, 1H), 7.89 (d, *J* = 7.9 Hz, 1H), 7.39 (d, *J* = 8.0 Hz, 1H), 7.30 – 7.21 (m, 2H), 6.97 (t, *J* = 8.6 Hz, 2H), 4.95 (bs, 1H), 4.46 – 4.19 (m, 4H), 3.56 – 3.43 (m, 4H), 3.29 – 3.24 (m, 2H), 2.67 – 2.47 (m, 6H), 2.06 – 1.82 (m, 4H), 1.64 – 1.56 (m, 1H), 1.43 (s, 9H), 1.33 (t, *J* = 7.1 Hz, 3H), 1.05 – 0.94 (m, 2H), 0.76 (t, *J* = 7.4 Hz, 3H), 0.79 – 0.64 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 173.7, 163.5, 163.2, 160.0, 158.5, 156.1, 146.8, 140.1, 134.8, 130.6, 130.5, 119.8, 115.4, 115.1, 79.4, 70.7, 70.0, 65.1, 61.9, 40.8, 40.5, 34.5, 32.5, 31.6, 28.6, 28.6, 24.8, 14.5, 12.8, 8.6, 7.9. HRMS (ESI) *m/z* [*M*+Na]$^+$ calcd. for $C_{34}H_{48}FN_3O_6S$: 668.3278, found 668.3154.

Ethyl 18-(5-cyclopropyl-6-(4-fluorobenzyl)picolinamido)-18-ethyl-2,2-dimethyl-4-oxo-3,8,11-trioxa-14-thia-5-aza-nonadecan-19-oate *(SI-12)*



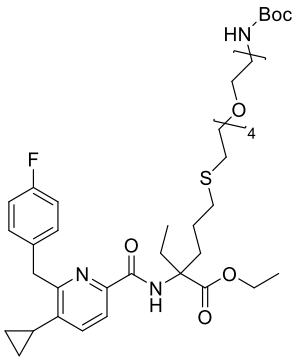
Following the general procedure above, **SI-12** was obtained in 49% yield (13 mg) starting from linker 2,2-dimethyl-4-oxo-3,8,11-trioxa-5-azatridecan-13-yl 4-methylbenzenesulfonate[20] (29 mg, 0.07 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 9.01 (s, 1H), 7.89 (d, *J* = 7.9 Hz, 1H), 7.38 (d, *J* = 8.0 Hz, 1H), 7.30 – 7.20 (m, 2H), 7.03 – 6.92 (m, 2H), 5.02 (bs, 1H), 4.40 – 4.23 (m, 4H), 3.62 – 3.45 (m, 8H), 3.34 – 3.30 (m, 2H), 2.68 – 2.44 (m, 6H), 2.05 – 1.82 (m, 3H), 1.64 – 1.55 (m, 1H), 1.43 (s, 9H), 1.35 – 1.18 (m, 4H), 1.03 – 0.97 (m, 2H), 0.76 (t, *J* = 7.4 Hz, 3H), 0.72 – 0.62 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 173.7, 163.5, 163.2, 158.5, 156.1, 146.8, 140.0, 134.8, 130.6, 130.5, 119.8, 115.4, 115.1, 79.3, 71.0, 70.4, 70.3, 65.1, 61.9, 40.9, 40.4, 34.5, 32.5, 31.5, 28.6, 24.9, 14.5, 12.9, 8.6, 7.9. HRMS (ESI) *m/z* [*M*+Na]$^+$ calcd. for $C_{36}H_{52}FN_3O_7S$: 712.3462, found 712.3346.

Ethyl 21-(5-cyclopropyl-6-(4-fluorobenzyl)picolinamido)-21-ethyl-2,2-dimethyl-4-oxo-3,8,11,14-tetraoxa-17-thia-5-azadocosan-22-oate *(SI-13)*



Following the general procedure above, **SI-13** was obtained in 52% yield (19 mg) starting from linker 2,2-dimethyl-4-oxo-3,8,11,14-tetraoxa-5-azahexadecan-16-yl 4-methylbenzenesulfonate[21] (40 mg, 0.09 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 9.01 (s, 1H), 7.89 (d, *J* = 7.9 Hz, 1H), 7.38 (d, *J* = 8.0 Hz, 1H), 7.30 – 7.21 (m, 2H), 6.97 (t, *J* = 8.6 Hz, 2H), 5.03 (bs, 1H), 4.43 – 4.21 (m, 4H), 3.66 – 3.68 (m, 12H), 3.33 – 3.27 (m, 2H), 2.69 – 2.44 (m, 6H), 2.08 – 1.76 (m, 4H), 1.64 – 1.55 (m, 1H), 1.43 (s, 9H), 1.33 (t, *J* = 7.1 Hz, 3H), 1.03 – 0.96 (m, 2H), 0.76 (t, *J* = 7.4 Hz, 3H), 0.78 – 0.64 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 173.7, 163.5, 163.2, 160.0, 158.5, 156.1, 146.8, 134.8, 130.6, 130.5, 119.8, 115.4, 115.1, 79.3, 71.0, 70.7, 70.4, 70.4, 65.1, 61.9, 40.8, 34.5, 32.5, 31.5, 28.6, 24.9, 14.5, 12.8, 8.6, 7.9. HRMS (ESI) *m/z* [*M*+Na]$^+$ calcd. for $C_{38}H_{56}FN_3O_8S$: 756.3792, found 756.3682.

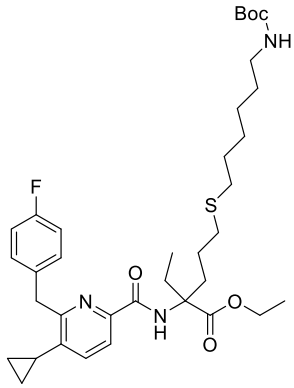
Ethyl 24-(5-cyclopropyl-6-(4-fluorobenzyl)picolinamido)-24-ethyl-2,2-dimethyl-4-oxo-3,8,11,14,17-pentaoxa-20-thia-5-azapentacosan-25-oate *(SI-14)*



Following the general procedure above, **SI-14** was obtained in 56% yield (22 mg) starting from linker 2,2-dimethyl-4-oxo-3,8,11,14,17-pentaoxa-5-azanonadecan-19-yl 4-methylbenzenesulfonate[22] (44 mg, 0.09 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 9.01 (s, 1H), 7.89 (d, *J* = 7.9 Hz, 1H), 7.38 (d, *J* = 8.0 Hz, 1H), 7.29 – 7.21 (m, 2H), 6.97 (t, *J* = 8.6 Hz, 2H), 5.04 (bs, 1H), 4.39 – 4.24 (m, 4H), 3.68 – 3.47 (m, 16H), 3.35 – 3.30 (m, 2H), 2.69 – 2.42 (m, 6H), 2.08 – 1.80 (m, 4H), 1.65 – 1.51 (m, 1H), 1.43 (s, 9H), 1.33 (t, *J* = 7.1 Hz, 3H), 1.04 – 0.95 (m, 2H), 0.76 (t, *J* = 7.4 Hz, 3H), 0.78 – 0.64 (m, 2H). ^{13}C NMR

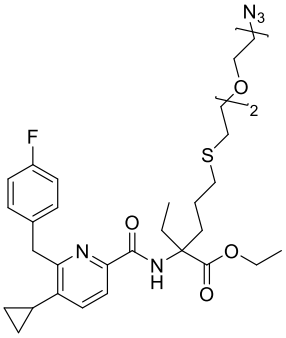
(75 MHz, $CDCl_3$) δ ppm 173.7, 163.4, 160.0, 158.5, 156.1, 146.8, 140.1, 134.9, 130.6, 130.5, 119.9, 115.4, 115.1, 79.3, 70.7, 70.7, 70.7, 70.4, 70.3, 65.1, 61.9, 40.8, 40.5, 34.5, 32.5, 31.5, 28.6, 24.8, 14.5, 12.8, 8.6, 7.9. HRMS (ESI) *m/z* [*M*+Na]$^+$ calcd. For $C_{40}H_{60}FN_3O_9S$: 800.4078, found 800.3942.

Ethyl 5-((6-((tert-butoxycarbonyl)amino)hexyl)thio)-2-(5-cyclopropyl-6-(4-fluorobenzyl) picolinamido)-2-ethyl-pentanoate *(SI-15)*



Following the general procedure above, **SI-15** was obtained in 51% yield (17 mg) starting from linker 6-((*tert*-butoxycarbonyl)amino)hexyl 4-methylbenzenesulfonate[23] (33 mg, 0.09 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 9.02 (s, 1H), 7.89 (d, *J* = 7.9 Hz, 1H), 7.38 (d, *J* = 8.0 Hz, 1H), 7.32 – 7.19 (m, 2H), 6.96 (t, *J* = 8.6 Hz, 2H), 4.53 (s, 1H), 4.38 – 4.24 (m, 4H), 3.11 – 3.04 (m, 2H), 2.67 – 2.37 (m, 6H), 2.07 – 1.84 (m, 3H), 1.60 – 1.24 (m, 22H), 1.06 – 0.94 (m, 2H), 0.76 (t, *J* = 7.4 Hz, 3H), 0.79 – 0.63 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 173.8, 163.5, 160.0, 158.3, 156.1, 146.8, 140.0, 134.8, 130.6, 130.5, 119.8, 115.4, 115.1, 79.2, 65.1, 61.9, 40.8, 34.6, 32.1, 32.0, 30.1, 29.6, 28.6, 28.6, 26.5, 24.7, 14.4, 12.8, 8.6, 7.9, 7.9. HRMS (ESI) *m/z* [*M*+Na]$^+$ calcd. for $C_{36}H_{52}FN_3O_5S$: 680.3664, found 680.3516.

Ethyl 5-((2-(2-(2-azidoethoxy)ethoxy)ethyl)thio)-2-(5-cyclopropyl-6-(4-fluorobenzyl)picolinamido)-2-ethyl-pentanoate *(SI-20)*

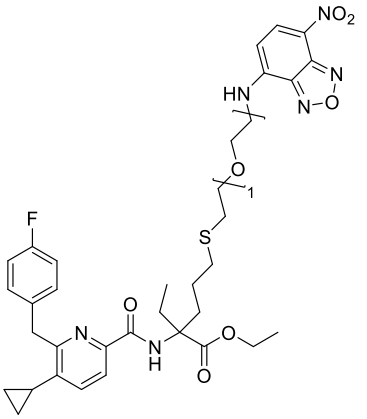


Following the general procedure above, **SI-20** was obtained in 46% yield (7 mg) starting from linker 2-(2-(2-azidoethoxy)ethoxy)ethyl 4-methylbenzenesulfonate[24] (10 mg, 0.04 mmol).This example was synthesized without addition of potassium iodide. ^{1}H NMR (600 MHz, DMSO-d_6) δ ppm 8.71 (s, 1H), 7.76 (d, *J* = 8.0 Hz, 1H), 7.53 (d, *J* = 8.0 Hz, 1H), 7.36 – 7.30 (m, 2H), 7.14 – 7.06 (m, 2H), 4.36 (s, 2H), 4.22 (q, *J* = 7.1 Hz, 2H), 3.58 – 3.55 (m, 2H), 3.52 – 3.42 (m, 6H), 3.38 – 3.34 (m, 2H), 2.54 (t, *J* = 6.8 Hz, 2H), 2.50 – 2.44 (m, 2H), 2.40 – 2.26 (m, 2H), 2.10 – 2.06 (m, 1H), 1.92 – 1.88 (m, 1H), 1.87 – 1.80 (m, 1H), 1.41 – 1.35 (m, 1H), 1.30 – 1.21 (m, 4H), 1.01 – 0.98 (m, 2H), 0.71 – 0.66 (m, 5H). ^{13}C NMR (151 MHz, DMSO-d_6) δ ppm 173.8, 162.3, 161.6, 160.0, 158.0, 145.8, 140.2, 135.0, 134.4, 130.7, 119.3, 115.0, 114.9, 70.2, 69.6, 69.4, 69.2, 63.6, 61.34, 50.0, 33.4, 31.3, 30.4, 27.6, 23.9, 14.0, 12.1, 8.1, 8.0. HRMS (ESI) *m/z* [*M*+Na]$^+$ calcd. for $C_{31}H_{42}FN_5O_5S$: 638.2801, found 638.2927.

General Boc-deprotection and NBD-labeling procedure

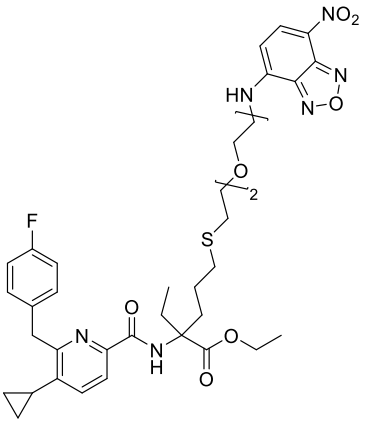
TFA (0.25 mL) was added dropwise to a stirring solution of the corresponding *N*-Boc-protected compounds **SI-11** to **SI-15** (1.0 equiv.) in DCM (2.25 mL) at 0 °C. The reaction mixture was stirred at room temperature for 2 h. Afterwards, the mixture was concentrated under reduced pressure, and the residue re-suspended in EtOAc (5 mL). The solvent was removed under reduced pressure. This process was repeated 3 times to remove TFA traces. The removal of the *tert*-butyloxycarbonyl group was quantitative as observed by TLC (50% EtOAc in cHex). Intermediates ***rac*-5** and **SI-16** to **SI-19** were obtained as the corresponding TFA salts, without the need of further purification.
To a solution of ***rac*-5** or **SI-16** to **SI-19** (1.0 equiv.) and Cs_2CO_3 (5.0 equiv.) in DMF (2 mL) at room temperature 7-chloro-4-nitrobenzofurazan (1.0 equiv.) was added. The reaction was stirred at room temperature for 24 h. The reaction mixture was concentrated under reduced pressure, and the obtained residue was diluted with a mixture of acetonitrile and water (1:1) and purified by reversed-phase preparative HPLC (30 to 90% acetonitrile in water with 0.1% TFA, 32 min run). The fractions containing the product were combined and lyophilized to yield the desired product.

Ethyl 2-(5-cyclopropyl-6-(4-fluorobenzyl)picolinamido)-2-ethyl-5-((2-(2-((7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)-amino)ethoxy)ethyl)thio)pentanoate *(SI-21)*



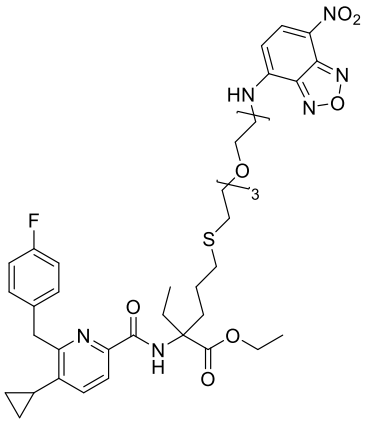
Following the general procedure above, **SI-21** was obtained in 49% yield (5 mg) starting from compound **SI-11** (10 mg, 0.02 mmol). ^{1}H NMR (*cryo* 600 MHz, DMSO-d_6) δ ppm 9.41 (s, 1H), 8.69 (s, 1H), 8.50 (d, *J* = 8.9 Hz, 1H), 7.74 (d, *J* = 8.0 Hz, 1H), 7.51 (d, *J* = 8.1 Hz, 1H), 7.34 – 7.29 (m, 2H), 7.11 – 7.06 (m, 2H), 6.44 (d, *J* = 9.0 Hz, 1H), 4.34 (s, 2H), 4.20 (q, *J* = 7.1 Hz, 2H), 3.70 – 3.63 (m, 4H), 3.51 (t, *J* = 6.7 Hz, 2H), 2.54 (t, *J* = 6.7 Hz, 2H), 2.46 – 2.41 (m, 2H), 2.34 – 2.24 (m, 2H), 2.09 – 2.05 (m, 1H), 1.88 – 1.75 (m, 2H), 1.38 – 1.32 (m, 2H), 1.20 (t, *J* = 7.1 Hz, 4H), 1.10 – 0.96 (m, 2H), 0.70 – 0.64 (m, 4H). ^{13}C NMR (*cryo* 151 MHz, DMSO-d_6) δ ppm 172.8, 162.3, 161.6, 160.0, 158.2, 158.0, 146.1, 145.3, 144.4, 144.1, 140.3, 137.8, 135.0, 134.4, 130.7, 120.9, 119.3, 114.9, 99.5, 70.2, 67.6, 63.6, 61.4, 43.3, 33.4, 31.3, 30.5, 26.6, 23.9, 14.4, 12.1, 8.2, 8.1, 8.0. HRMS (ESI) *m/z* [*M*+Na]$^{+}$ calcd. for $C_{35}H_{41}FN_6O_7S$: 731.2760, found 731.2646.

Ethyl 2-(5-cyclopropyl-6-(4-fluorobenzyl)picolinamido)-2-ethyl-5-((2-(2-(2-((7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)-amino)ethoxy)ethoxy)ethyl)thio)pentanoate *(rac-6)*



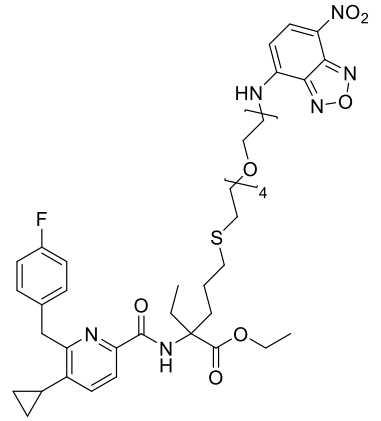
Following the general procedure above, ***rac*-6** was obtained in 26% yield (3 mg) starting from compound **SI-12** (10 mg, 0.01 mmol). ^{1}H NMR (*cryo* 600 MHz, DMSO-d_6) δ ppm 9.43 (s, 1H), 8.70 (s, 1H), 8.49 (d, *J* = 9.0 Hz, 1H), 7.74 (d, *J* = 8.0 Hz, 1H), 7.51 (d, *J* = 8.0 Hz, 1H), 7.34 – 7.30 (m, 2H), 7.11 – 7.06 (m, 2H), 6.54 (d, *J* = 9.0 Hz, 1H), 4.34 (s, 2H), 4.31 (q, *J* = 7.1 Hz, 2H), 3.69 (t, *J* = 5.3 Hz, 2H), 3.49 – 3.51 (m, 3H), 3.43 – 3.39 (m, 3H), 2.48 (t, *J* = 6.7 Hz, 2H), 2.40 – 2.45 (m, 2H), 2.35 – 2.26 (m, 3H), 2.10 – 2.04 (m, 2H), 1.92 – 1.77 (m, 3H), 1.38 – 1.33 (m, 2H), 1.21 (t, *J* = 7.1 Hz, 3H), 1.01 – 0.96 (m, 2H), 0.70 – 0.65 (m, 4H). ^{13}C NMR (*cryo* 151 MHz, DMSO-d_6) δ ppm 172.8, 162.3, 161.6, 160.0, 158.0, 145.8, 145.3, 144.4, 144.1, 140.3, 137.8, 135.0, 134.4, 130.7, 130.6, 120.8, 119.3, 115.1, 114.9, 99.5, 70.2, 69.8, 69.4, 63.6, 61.4, 43.4, 33.4, 31.3, 30.4, 27.6, 23.9, 14.1, 12.1, 8.1, 8.0. HRMS (ESI) *m/z* [*M*+Na]$^{+}$ calcd. for $C_{37}H_{45}FN_6O_8S$: 775.3027, found 775.2911.

Ethyl 16-(5-cyclopropyl-6-(4-fluorobenzyl)picolinamido)-16-ethyl-1-((7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)a-mino)-3,6,9-trioxa-12-thiaheptadecan-17-oate *(SI-22)*



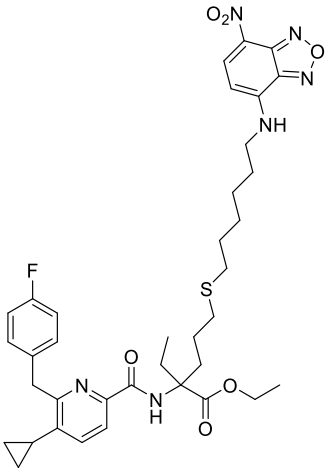
Following the general procedure above, **SI-22** was obtained in 49% yield (3 mg) starting from compound **SI-13** (10 mg, 0.01 mmol). ^{1}H NMR (*cryo* 600 MHz, DMSO-d_6) δ ppm 9.44 (s, 1H), 8.70 (s, 1H), 8.50 (d, *J* = 8.8 Hz, 1H), 7.75 (d, *J* = 8.0 Hz, 1H), 7.51 (d, *J* = 8.0 Hz, 1H), 7.34 – 7.30 (m, 2H), 7.12 – 7.06 (m, 2H), 6.47 (d, *J* = 9.0 Hz, 1H), 4.34 (s, 2H), 4.21 (q, *J* = 7.1 Hz, 2H), 3.70 (t, *J* = 5.3 Hz, 2H), 3.70 – 3.62 (m, 2H), 3.55 – 3.53 (m, 2H), 3.49 – 3.47 (m, 3H), 3.41 – 3.39 (m, 5H), 3.37 – 3.34 (m, 2H), 2.45 (td, *J* = 7.1, 3.0 Hz, 2H), 2.37 – 2.26 (m, 2H), 2.10 – 2.05 (m, 2H), 1.92 – 1.87 (m, 1H), 1.84 – 1.79 (m, 1H), 1.41 – 1.33 (m, 2H), 1.21 (t, *J* = 7.1 Hz, 3H), 1.01 – 0.97 (m, 2H), 0.70 – 0.65 (m, 4H). ^{13}C NMR (*cryo* 151 MHz, DMSO-d_6) δ ppm 172.8, 162.3, 161.6, 160.0, 158.0, 145.8, 145.3, 144.1, 140.3, 137.8, 135.0, 134.4, 130.7, 130.6, 120.8, 119.3, 115.1, 114.9, 99.5, 70.1, 69.8, 69.7, 69.4, 68.0, 63.6, 61.4, 43.4, 33.4, 31.3, 30.4, 27.6, 23.9, 14.1, 12.1, 8.1, 8.0. HRMS (ESI) *m/z* [*M*+Na]$^{+}$ calcd. for $C_{39}H_{49}FN_6O_9S$: 819.3277, found 819.3161.

Ethyl 19-(5-cyclopropyl-6-(4-fluorobenzyl)picolinamido)-19-ethyl-1-((7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)a-mino)-3,6,9,12-tetraoxa-15-thiaicosan-20-oate *(SI-23)*



Following the general procedure above, **SI-23** was obtained in 23% yield (3 mg) starting from compound **SI-14** (14 mg, 0.02 mmol). ^{1}H NMR (*cryo* 600 MHz, DMSO-d_6) δ ppm 9.45 (s, 1H), 8.71 (s, 1H), 8.50 (d, *J* = 9.0 Hz, 1H), 7.75 (d, *J* = 8.0 Hz, 1H), 7.52 (d, *J* = 7.9 Hz, 1H), 7.35 – 7.30 (m, 2H), 7.12 – 7.07 (m, 2H), 6.47 (d, *J* = 9.0 Hz, 1H), 4.34 (s, 2H), 4.21 (q, *J* = 7.1 Hz, 2H), 3.70 (t, *J* = 5.3 Hz, 1H), 3.56 – 3.54 (m, 2H), 3.50 – 3.48 (m, 2H), 3.46 – 3.40 (m, 16H), 3.38 – 3.36 (m, 2H), 2.47 – 2.44 (m, 1H), 2.37 – 2.27 (m, 2H), 2.10 – 2.06 (m, 1H), 1.93 – 1.97 (m, 1H), 1.85 – 1.79 (m, 1H), 1.41 – 1.33 (m, 1H), 122 (t, *J* = 7.1 Hz, 3H), 1.03 – 0.95 (m, 2H), 0.72 – 0.64 (m, 4H). ^{13}C NMR (*cryo* 151 MHz, DMSO-d_6) δ ppm 172.8, 162.3, 161.6, 160.0, 158.0, 145.8, 145.3, 144.4, 144.1, 140.3, 137.8, 135.0, 134.4, 130.7, 130.6, 120.8, 119.3, 115.0, 114.9, 99.5, 70.2, 69.8, 69.7, 69.7, 69.4, 68.0, 63.6, 61.4, 60.2, 43.4, 33.4, 31.3, 30.4, 27.6, 23.9, 14.1, 12.1, 8.2, 8.0. HRMS (ESI) *m/z* [*M*+Na]$^{+}$ calcd. for $C_{41}H_{53}FN_6O_{10}S$: 841.3595, found 841.3658.

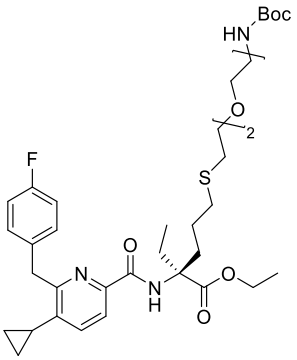
Ethyl 2-(5-cyclopropyl-6-(4-fluorobenzyl)picolinamido)-2-ethyl-5-((6-((7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino)hexyl)thio)pentanoate *(SI-24)*



Following the general procedure above, **SI-24** was obtained in 18% yield (2 mg) starting from compound **SI-15** (11 mg, 0.02 mmol). ^{1}H NMR (*cryo* 600 MHz, DMSO-d_6) δ ppm 9.52 (t, *J* = 5.7 Hz, 1H), 8.72 (s, 1H), 8.50 (d, *J* = 8.9 Hz, 1H), 7.75 (d, *J* = 8.0 Hz, 1H), 7.51 (d, *J* = 8.0 Hz, 1H), 7.35 – 7.31 (m, 2H), 7.11 – 7.06 (m, 2H), 6.38 (d, *J* = 9.0 Hz, 1H), 4.34 (d, *J* = 3.5 Hz, 2H), 4.21 (q, *J* = 7.1 Hz, 2H), 3.42 (q, *J* = 6.8, 6.3 Hz, 2H), 2.40 (t, *J* = 7.1 Hz, 2H), 2.37 – 2.27 (m, 4H), 2.10 – 2.06 (m, 1H), 1.93 – 1.78 (m, 3H), 1.59 – 1.64 (m, 2H), 1.44 – 1.20 (m, 11H), 1.02 – 0.96 (m, 2H), 0.70 – 0.65 (m, 4H). ^{13}C NMR (*cryo* 151 MHz, DMSO-d_6) δ ppm 172.8, 162.3, 161.6, 160.0, 158.0, 145.8, 145.2, 144.4, 144.1, 140.3, 138.0, 135.0, 134.4, 130.7, 130.6, 120.5, 119.3, 115.0, 114.9, 99.1, 63.6, 61.4, 43.3, 33.4, 30.8, 30.7, 28.9, 27.8, 27.7, 27.5, 25.9, 23.7, 14.1, 12.1, 8.2, 8.1. HRMS (ESI) *m/z* [*M*+Na]$^{+}$ calcd. for $C_{37}H_{45}FN_6O_6S$: 743.3244, found 743.3021.

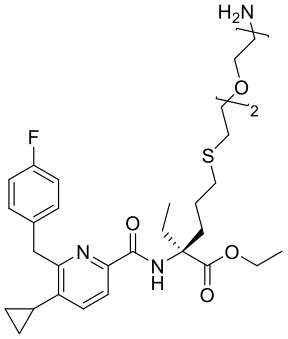
Synthesis of enantiomerically pure probes

Ethyl (*S*)-18-(5-cyclopropyl-6-(4-fluorobenzyl)picolinamido)-18-ethyl-2,2-dimethyl-4-oxo-3,8,11-trioxa-14-thia-5-azanonadecan-19-oate *((S)-SI-12)*



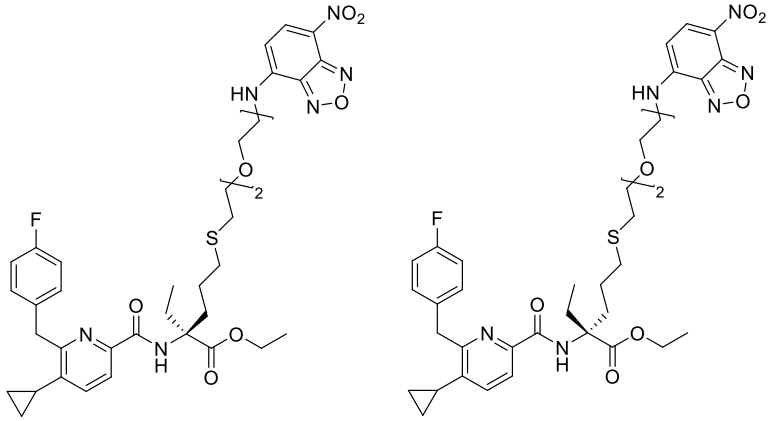
Picolinic acid **4** (50 mg, 184 µmol) was dissolved in DCM/acetonitrile (1 mL, 1:1). DIPEA (48 µL, 276 µmol) and HATU (70 mg, 184 µmol) were added, and the reaction mixture was stirred at room temperature for 10 min. Afterwards, **(*S*)-3** (89 mg, 203 µmol) dissolved in DCM/acetonitrile (1 mL, 1:1) was added and the reaction was stirred at room temperature for 18 h. The solvents were evaporated and the residue was purified by column chromatography (Silica gel, 4 g, nHep:EtOAc, 10 to 90% EtOAc) to yield **(*S*)-SI-12** (105 mg, 82%) as a colorless oil. ^{1}H-NMR (600 MHz, $CDCl_3$): δ ppm 9.00 (s, 1 H), 7.89 (d, *J* = 7.9 Hz, 1 H), 7.39 (m, 1 H), 7.26 (m, 2 H), 6.97 (m, 2 H), 5.00 (bs, 1 H), 4.37 (s, 2 H), 4.33 – 4.23 (m, 2 H), 3.64 – 3.47 (m, 8 H), 3.36 – 3.23 (m, 2 H), 2.67 – 2.62 (m, 2 H), 2.62 – 2.44 (m, 4 H), 2.07 – 1.97 (m, 1 H), 1.95 – 1.83 (m, 2 H), 1.58 (m, 1 H), 1.43 (s, 9 H), 1.33 (t, *J* = 7.1 Hz, 3 H), 1.32 (m, 1 H), 1.03 – 0.97 (m, 2 H), 0.76 (t, *J* = 7.4 Hz, 3 H), 0.69 – 0.60 (m, 2 H). ^{13}C-NMR (151 MHz, $CDCl_3$): δ ppm 173.7, 163.5, 161.5 (d, $^{1}J_{CF}$= 243.7 Hz), 158.6, 146.9, 140.0, 134.8, 134.8, 130.6 (d, $^{3}J_{CF}$ = 7.6 Hz), 119.8, 115.4 (d, $^{2}J_{CF}$ = 21.1 Hz), 79.3, 71.0, 70.4, 65.1, 61.9, 40.9, 40.5, 34.6, 32.5, 31.5, 28.6, 28.6, 24.9, 14.5, 12.9, 8.6, 7.9, 7.9. HRMS (ESI) *m/z* [*M*+H]$^{+}$ calcd. for $C_{36}H_{53}FN_3O_7S$: 690.3583, found: 690.3586.$[\alpha]^{20}_{d}$ = + 6.34° (*c* = 1.0, $CHCl_3$).
The same procedure was applied for the synthesis of **(*R*)-SI-12**, $[\alpha]^{20}_{d}$ = - 6.17° (*c* = 1.0, $CHCl_3$).

Ethyl (*S*)-5-((2-(2-(2-aminoethoxy)ethoxy)ethyl)thio)-2-(5-cyclopropyl-6-(4-fluorobenzyl)picolinamido)-2-ethyl-pentanoate *((S)-5)*



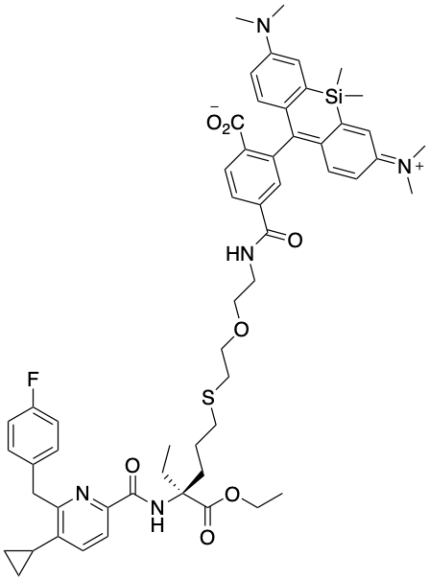
Compound **(*S*)-SI-12** (27 mg, 39 µmol) was dissolved in DCM (2 mL) and trifluoroacetic acid (2 mL) was added. The solution was stirred at room temperature for 30 min, then poured into 2 M aq. $KHCO_3$ (30 mL). The mixture was extracted with EtOAc (2x). The combined organic layers were washed with 1 M NaOH aq. sol., dried over Na_2SO_4 and filtered. Evaporation of the solvent yielded deprotected picolinamide **(*S*)-5** (28 mg, 97%) as a colorless oil. The product was used crude for dye conjugation, and characterization was conducted at the next step.
The same procedure was applied for the synthesis of **(*R*)-5**.

Ethyl 2-(5-cyclopropyl-6-(4-fluorobenzyl)picolinamido)-2-ethyl-5-((2-(2-(2-((7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino)ethoxy)ethoxy)ethyl)thio)pentanoate *(6-NBD)*



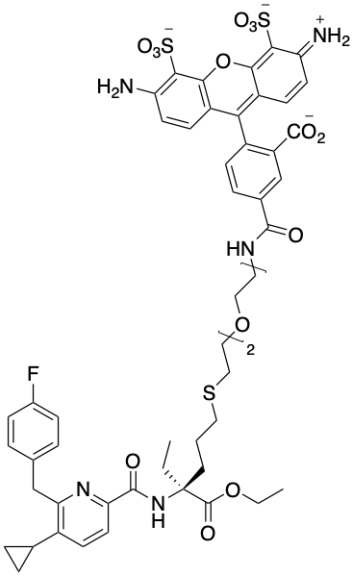
Following the general NBD-labeling procedure described for racemic compound ***rac*-6**, 2.8 mg of (*S*)-NBD conjugate **6** were obtained (73%) starting from amine **(*S*)-5** (3 mg, 5.1 µmol).
Following the general NBD-labeling procedure described for racemic compound ***rac*-6**, 4.5 mg of (*R*)-NBD conjugate **6** were obtained (54%) starting from amine **(*R*)-5** (6.2 mg, 11.0 µmol).

Ethyl (*S*)-1-(3,7-bis(dimethylamino)-5,5-dimethyl-3'-oxo-3'H,5H-spiro[dibenzo[b,e]siline-10,1'-isobenzofuran]-6'-yl)-15-(5-cyclopropyl-6-(4-fluorobenzyl)picolinamido)-15-ethyl-1-oxo-5,8-dioxa-11-thia-2-azahexadecan-16-oate *(8-SiR)*



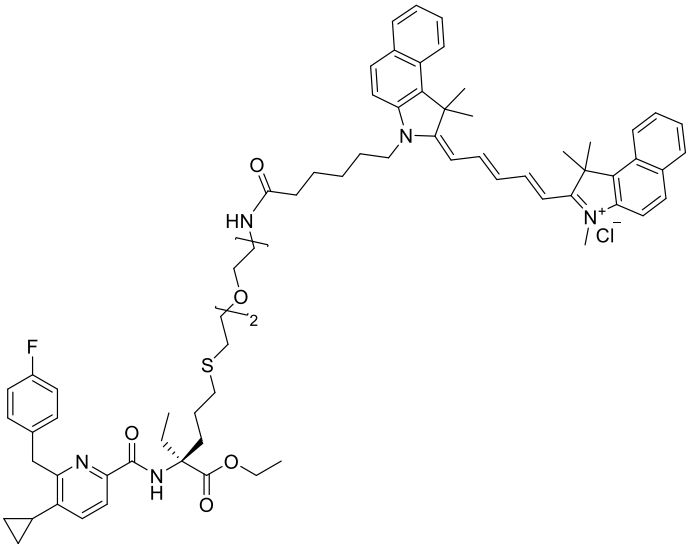
Silicon Rhodamine carboxylic acid (5 mg, 10.6 µmol) was dissolved in acetonitrile (2 mL) and DIPEA (4 µL, 21.2 µmol) and HATU (4 mg, 10.6 µmol) were added successively. The solution was stirred for 5 min, then **(*S*)-5** (7 mg, 11.7 µmol) was added and the reaction was stirred at room temperature overnight. The mixture was directly purified by HPLC (30 to 90% acetonitrile in water with 0.1% TFA, 32 min run) to yield 8 mg (73%) **8-SiR** as blue powder after lyophilization. ^{1}H-NMR (600 MHz, $CDCl_3$): δ ppm 9.03 (s, 1 H), 8.02 (d, *J* = 8.0 Hz, 1 H), 7.94 (dd, *J* = 8.0, 1.4 Hz, 1 H), 7.86 (d, *J* = 8.0 Hz, 1 H), 7.75 (d, *J* = 1.4 Hz, 1 H), 7.37 (d, *J* = 8.0 Hz, 1H), 7.35 (d, *J* = 2.7 Hz, 2 H), 7.26 – 7.23 (m, 2 H), 7.10 (bs, 1 H), 7.00 – 6.94 (m, 4 H), 6.90 (m, 2 H), 4.36 (s, 2 H), 4.28 (m, 2 H), 3.65 – 3.51 (m, 10 H), 3.08 (s, 12 H), 2.56 (t, *J* = 6.7 Hz, 2 H), 2.62 – 2.55 (m, 1 H), 2.52 – 2.40 (m, 3 H), 2.02– 1.96 (m, 1 H), 1.95 – 1.83 (m, 2 H), 1.61 – 1.49 (m, 1 H), 1.32 (t, *J* = 7.1 Hz, 3 H), 1.35 – 1.27 (m, 1 H), 1.01 (m_c, 2 H), 0.75 (t, *J* = 7.4 Hz, 3 H), 0.70 (s, 3 H), 0.66 (m, 2 H), 0.62 (s, 3 H). ^{13}C-NMR (151 MHz, $CDCl_3$): δ ppm 173.6, 169.1, 166.3, 163.7, 161.7 (d, $^{1}J_{CF}$ = 244 Hz), 158.7, 152.7, 147.5, 146.6, 140.3, 140.0, 139.0, 136.5, 134.8, 134.7, 130.6 (d, $^{3}J_{CF}$ = 7.8 Hz), 129.9, 129.0, 127.9, 127.0, 124.4, 121.0, 119.8, 116.9, 115.3 (d, $^{2}J_{CF}$ = 21.3 Hz), 69.6, 70.2, 70.3, 70.9, 65.1, 62.0, 42.8, 40.8, 40.3, 34.5, 32.5, 31.6, 28.7, 24.8, 14.4, 12.9, 8.5, 8.0, 8.0, 1.2, 0.1.HRMS (ESI) *m/z* [*M*+H]$^+$ calcd. for $C_{58}H_{71}FN_5O_8SSi$: 1044.4771, found: 1044.4814.

Ethyl (*S*)-1-(3,7-bis(dimethylamino)-5,5-dimethyl-3'-oxo-3'H,5H-spiro[dibenzo[b,e]siline-10,1'-isobenzofuran]-6'-yl)-15-(5-cyclopropyl-6-(4-fluorobenzyl)picolinamido)-15-ethyl-1-oxo-5,8-dioxa-11-thia-2-azahexadecan-16-oate *(7-Alexa 488)*



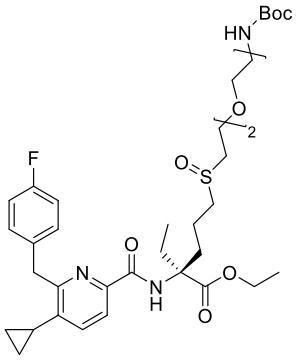
Alexa 488 carboxylic acid tris-triethylammonium salt (5 mg, 6.0 µmol) was dissolved in DMF (2 mL) and DIPEA (2 µL, 12.0 µmol) was added. HATU (2.3 mg, 6.0 µmol) was added and the solution wasstirredatr.t. for 10 min, then **(*S*)-5** (4.1 mg, 7.0 µmol) was added and stirring was continued for 5 h atr.t. The mixture was directly purified by HPLC (5-75% ACN in 22 min, 6 min to 100%) to yield Alexa 488 conjugate **7** (3 mg, 37%) as purple powder after lyophilization. PAMPA assay: P_{eff}: 0 cm/s*10^{-6}, Acceptor: 0%; Membrane: 99.2%; %Donor: 0.7%. Due to the low amount of compound, NMR spectra of probe **7** could not be recorded. HRMS (ESI) m/z [M+H]$^+$ calcd. for $C_{52}H_{57}FN_5O_{15}S_3$: 1106.2992, found: 1106.2986.

2-((1E,3E,5E)-5-(3-((*S*)-5-(5-cyclopropyl-6-(4-fluorobenzyl)picolinamido)-5-ethyl-4,19-dioxo-3,12,15-trioxa-9-thia-18-azatetracosan-24-yl)-1,1-dimethyl-1,3-dihydro-2H-benzo[e]indol-2-ylidene)penta-1,3-dien-1-yl)-1,1,3-trimethyl-1H-benzo[e]indol-3-ium *(SI-1)*



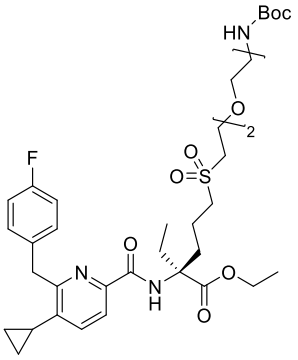
Cyanine5.5 carboxylic acid (5 mg, 8.1 µmol) was dissolved in acetonitrile (2 mL) and DIPEA (5.6 µL, 32.3 µmol) was added. HATU (3.1 mg, 8.1 µmol) was added and the solution was stirred at room temperature for 10 min, then **(*S*)-5** (5.7 mg, 9.7 µmol) was added and stirring was continued for 18 h at room temperature The mixture was directly purified by HPLC (30 to 90% acetonitrile in water with 0.1% TFA, 32 min run) to yield picolinamide-Cy5.5 conjugate **SI-1** (7.7 mg, 64%) as blue powder after lyophilization. ^{1}H-NMR (600 MHz, $CDCl_3$): δ ppm 9.00 (s, 1 H), 8.17 – 8.05 (m, 4 H), 7.95 – 7.93 (m, 4 H,), 7.88 (d, *J* = 7.9 Hz, 1 H), 7.62 (t, *J* = 7.5 Hz, 2 H), 7.48 (dddd, *J* = 8.1, 6.9, 3.6, 1.0 Hz, 2 H), 7.39 – 7.34 (m, 3 H), 7.25 - 7.22 (m, 2 H), 6.96 (m, 2 H), 6.75 (t, *J* = 12.4 Hz, 1 H), 6.35 (d, *J* = 13.7 Hz, 1 H), 6.26 (d, *J* = 13.5 Hz, 1 H), 4.35 (s, 2 H), 4.32 – 4.25 (m, 2 H), 4.18 – 4.11 (m, 2 H), 3.74 (s, 3 H), 3.60 – 3.53 (m, 8 H), 3.45 (q, *J* = 5.6 Hz, 2 H), 2.66 – 2.59 (m, 2 H), 2.61 – 2.42 (m, 4 H), 2.32 (t, *J* = 7.3 Hz, 2 H), 2.02, 2.00 (2 s, 12 H), 2.03 – 1.98 (m, 1 H), 1.94 – 1.84 (m, 4 H), 1.77 (quin, *J* = 7.5 Hz, 2 H), 1.60 - 1.52 (m, 1 H), 1.61 – 1.51 (m, 2 H), 1.32 (t, *J* = 7.1 Hz, 3 H), 1.35 – 1.28 (m, 1 H), 1.01 – 0.97 (m, 2 H), 0.76 (t, *J* = 7.4 Hz, 3 H), 0.67 – 0.64 (m, 2 H). ^{13}C-NMR (151 MHz, $CDCl_3$): δ ppm 174.4, 174.3, 173.7, 173.5, 163.4, 161.5 (d, $^{1}J_{CF}$= 243.7 Hz, 158.4, 152.7, 152.3, 146.7, 140.1, 139.9, 139.3, 134.6, 134.0, 133.4, 131.9, 131.8, 130.7, 130.6, 130.4 (d, $^{3}J_{CF}$ = 7.6 Hz), 130.1, 128.2, 127.8, 126.2, 125.2, 125.0, 122.2, 122.1, 119.7, 115.1 (d, *J* = 21.2 Hz), 110.5, 110.3, 103.8, 103.2, 70.7, 70.1, 69.6, 64.9, 61.8, 51.2, 50.9, 44.4, 40.7, 39.2, 35.8, 34.4, 32.3, 31.9, 31.4, 28.5, 27.6, 27.6, 27.3, 26.3, 25.1, 24.7, 14.3, 12.7, 8.4, 7.8. HRMS (ESI) *m/z* [*M*]$^{+}$ calcd. for $C_{71}H_{85}FN_5O_6S$: 1154.6199, found: 1154.6216.

Ethyl (2*S*)-2-(5-cyclopropyl-6-(4-fluorobenzyl)picolinamido)-5-((2,2-dimethyl-4-oxo-3,8,11-trioxa-5-azatridecan-13-yl)sulfinyl)-2-ethylpentanoate *(SI-25)*



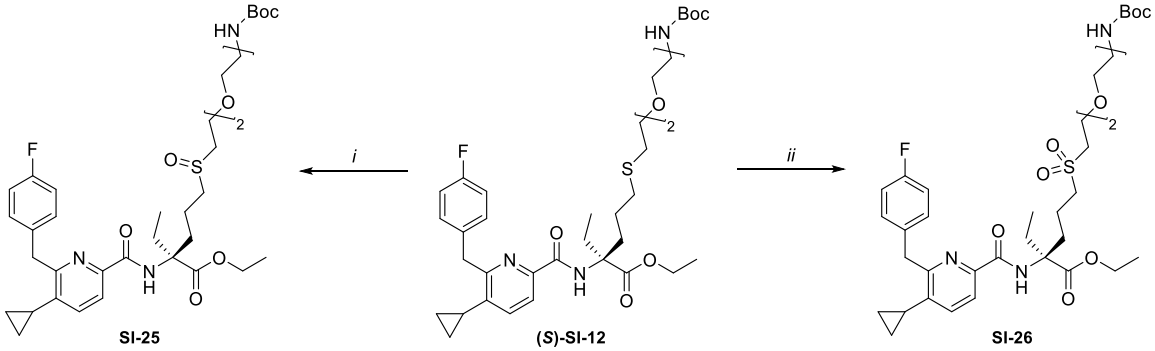
Compound **(*S*)-SI-12** (10 mg, 14.5 µmol) was dissolved in DCM/hexafluoroisopropanol (2 mL, 1:1) and H_2O_2 aq. sol (35%, 1.86 mL, 21.7 µmol) was added. The solution was stirred at room temperature for 2 h, and then was diluted with EtOAc (5 mL). The organic layer was successively washed with $Na_2S_2O_3$ sat. sol. (1x) and $NaHCO_3$ sat. sol. (1x). The organic phase was dried over $MgSO_4$, filtered and the solvents were evaporated to yield 10 mg (quant.) sulfoxide **SI-25** as colorless oil.^{1}H-NMR (300 MHz, $CDCl_3$): δ ppm 9.03 (s, 1 H), 7.88 (d, J = 8.0 Hz, 1 H), 7.39 (d, J = 8.0 Hz, 1 H), 7.25 (m, 2 H), 6.97 (m, 2 H), 5.12 (bs, 1 H), 4.37 (s, 2 H), 4.30 (2 q, *J* = 7.1 Hz, 2 H), 3.90 – 3.48 (m, 8 H), 3.29 (m, 2 H), 2.97 – 2.60 (m, 5 H), 2.52 (m, 1 H), 2.19 – 2.01 (m, 1 H), 1.97 – 1.53 (m, 4 H), 1.42 (s, 9 H), 1.33 (2 t, *J* = 7.1 Hz, 3 H), 1.00 (mc, 2 H), 0.77 (t, *J* = 7.4 Hz, 3 H), 0.67 (m, 2 H). ^{13}C-NMR (75 MHz, $CDCl_3$): δ ppm 173.2, 173.2, 163.5, 163.6, 161.5 (d, $^{1}J_{CF}$ = 244 Hz), 158.5, 156.0, 146.5, 146.5, 140.1, 140.1, 134.7, 134.6 (d, $^{4}J_{CF}$ = 3.1 Hz), 130.4 (d, $^{3}J_{CF}$ = 7.8 Hz), 119.7, 115.2 (d, $^{2}J_{CF}$ = 21.2 Hz), 79.2, 70.1, 70.3, 70.5, 64.8, 64.9, 63.5, 63.6, 62.0, 62.0, 52.5, 52.6, 52.7, 52.8, 40.7, 40.3, 34.0, 34.2, 28.7, 17.8, 18.5, 14.3, 12.7, 8.4, 7.8 (two diastereomers). HRMS (ESI) *m/z* [*M*+H]$^{+}$calcd. for $C_{36}H_{53}FN_3O_8S$: 706.3532, found: 706.3533.

Ethyl (S)-2-(5-cyclopropyl-6-(4-fluorobenzyl)picolinamido)-5-((2,2-dimethyl-4-oxo-3,8,11-trioxa-5-azatridecan-13-yl)sulfonyl)-2-ethylpentanoate *(SI-26)*



Compound **(*S*)-SI-12** (10 mg, 14.5 µmol) was dissolved in DCM (2 mL) and the solution was cooled to 0 °C. 3-Chloroperoxybenzoic acid (8 mg, 43.5 µmol) was added and the reaction was stirred at 0 °C for 2 h. After, the solution was diluted with EtOAc (3 mL) and the organic layer was successively washed with $Na_2S_2O_3$ sat. sol. (1x) and 1 M NaOH aq. sol. (2x). The organic phase was dried over $MgSO_4$, filtered and the solvents were evaporated to yield 10 mg (quant.) sulfone **SI-26** as colorless oil. ^{1}H-NMR (300 MHz, $CDCl_3$): δ ppm 9.03 (s, 1 H), 7.88 (d, *J* = 8.0 Hz, 1 H), 7.39 (d, *J* = 8.0 Hz, 1 H), 7.25 (m, 2 H), 6.98 (m, 2 H), 5.21 (bs, 1 H), 4.37 (s, 2 H), 4.31 (q, *J* = 7.1 Hz, 2 H), 3.85 (m, 2 H), 3.55 (m, 4 H), 3.51 (t, *J* = 5.3 Hz, 2 H), 3.29 (m, 2 H), 3.16 – 2.96 (m, 4 H), 2.66 (m, 1 H), 2.51 (m, 1 H), 2.07 (m, 1 H), 1.98 – 1.52 (m, 4 H), 1.43 (s, 9 H), 1.33 (t, *J* = 7.1 Hz, 3 H), 1.00 (m, 2 H), 0.76 (t, *J* = 7.4 Hz, 3 H), 0.67 (m, 2 H).^{13}C-NMR (75 MHz, $CDCl_3$): δ ppm 173.2, 163.8, 161.7 (d, $^1J_{CF}$ = 244 Hz), 158.7, 156.3, 146.5, 140.3, 134.8, 134.7 (d, $^4J_{CF}$ = 3.3 Hz), 130.6 (d, $^3J_{CF}$= 7.9 Hz), 119.9, 115.3 (d, $^2J_{CF}$ = 21.2 Hz), 79.4, 70.0, 70.3, 70.5, 64.9, 64.8, 62.2, 54.6, 53.6, 40.9, 40.4, 33.8, 28.8, 28.6, 17.1, 14.4, 12.9, 8.5, 8.0. HRMS (ESI) *m/z* [*M*+H]$^+$calcd. for $C_{36}H_{53}FN_3O_9S$: 722.3481, found: 722.3496.

Linker oxidation studies



Supplementary scheme S-6. Oxidation of compound **(*S*)-SI-12** to the corresponding sulfoxide **SI-25** and sulfone **SI-26** derivatives. Reagents and conditions: (*i*) H_2O_2, DCM:HFIP (1:1), r.t., 2 h (100%); (*ii*) *m*-CPBA, DCM, 0 °C to r.t., 2 h (100%).

At the final synthetic step oxidation of the thio-linker to its sulfoxide analog **SI-25** was observed to a minor extend. To further investigate the influence of this oxidative process on binding affinity, *N*-Boc-protected compound **(*S*)-SI-12** was synthesized and subsequently converted to the corresponding sulfoxide **SI-25** and sulfone **SI-26** (Supplementary scheme S-6). Sulfoxide **SI-25** is equally potent for human CB_2R than thioether **(*S*)-SI-12** (hCB_2R K_i: 28 nM for **(*S*)-SI-12** and hCB_2R K_i: 29 nM for **SI-25**; Supplementary table S-5), while sulfone **SI-26** exhibits a slightly lower binding affinity (hCB_2R K_i: 45 nM; Supplementary table S-5). Due to its higher polarity the sulfone linker holds potential for next generation probes since high polarity often comes along with low unspecific binding.[25] All three ligands **(*S*)-SI-3**, **SI-25** and **SI-26** are potent full agonists for hCB_2R with minimum 577-fold selectivity over hCB_1R.

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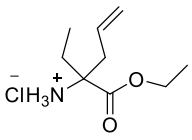
NMR SPECTRA



SI-30

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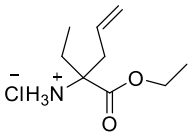

SI-30

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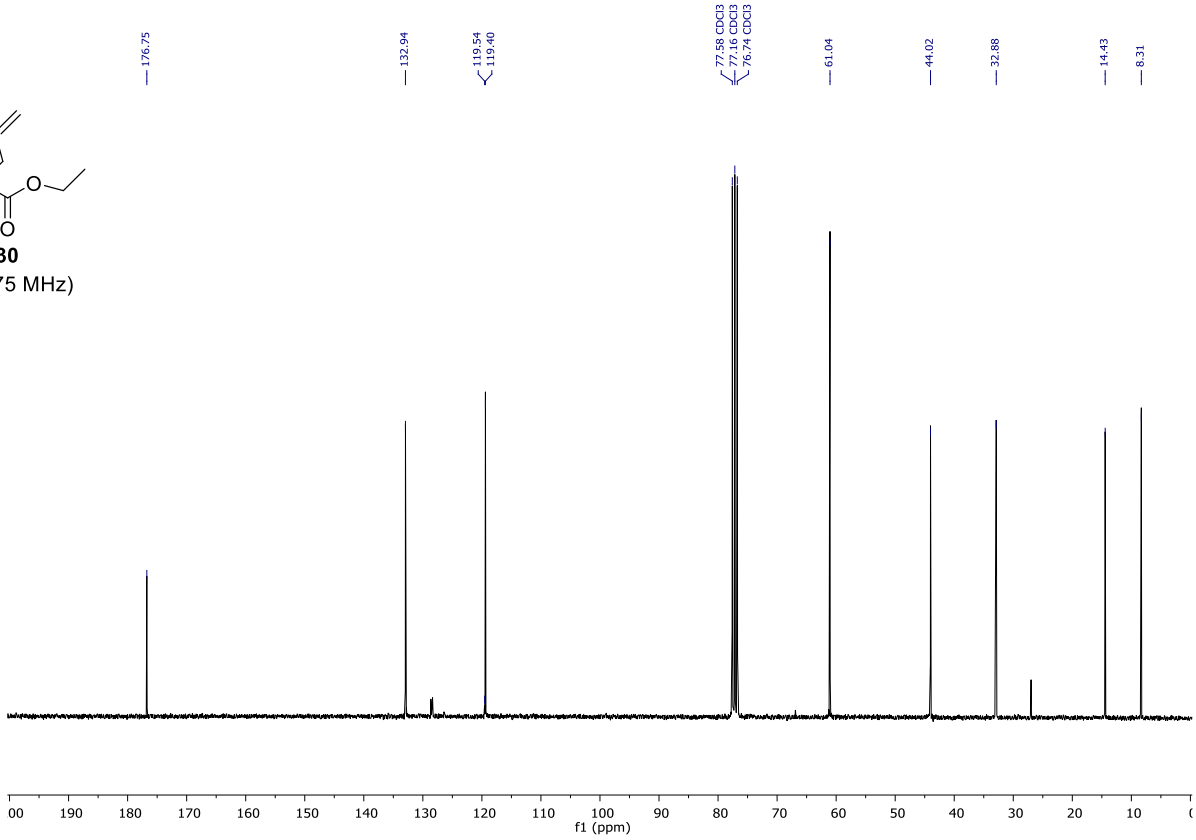

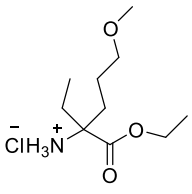

SI-31

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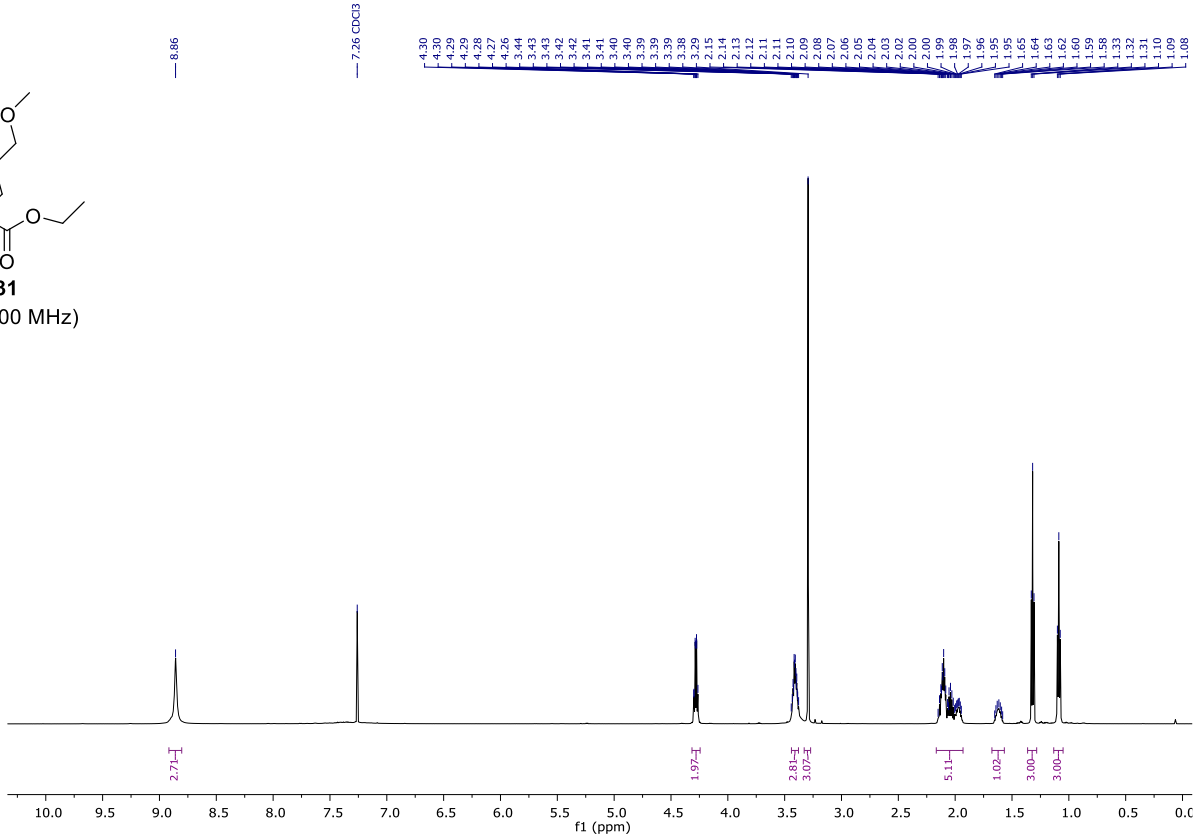

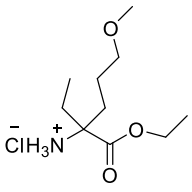

SI-31

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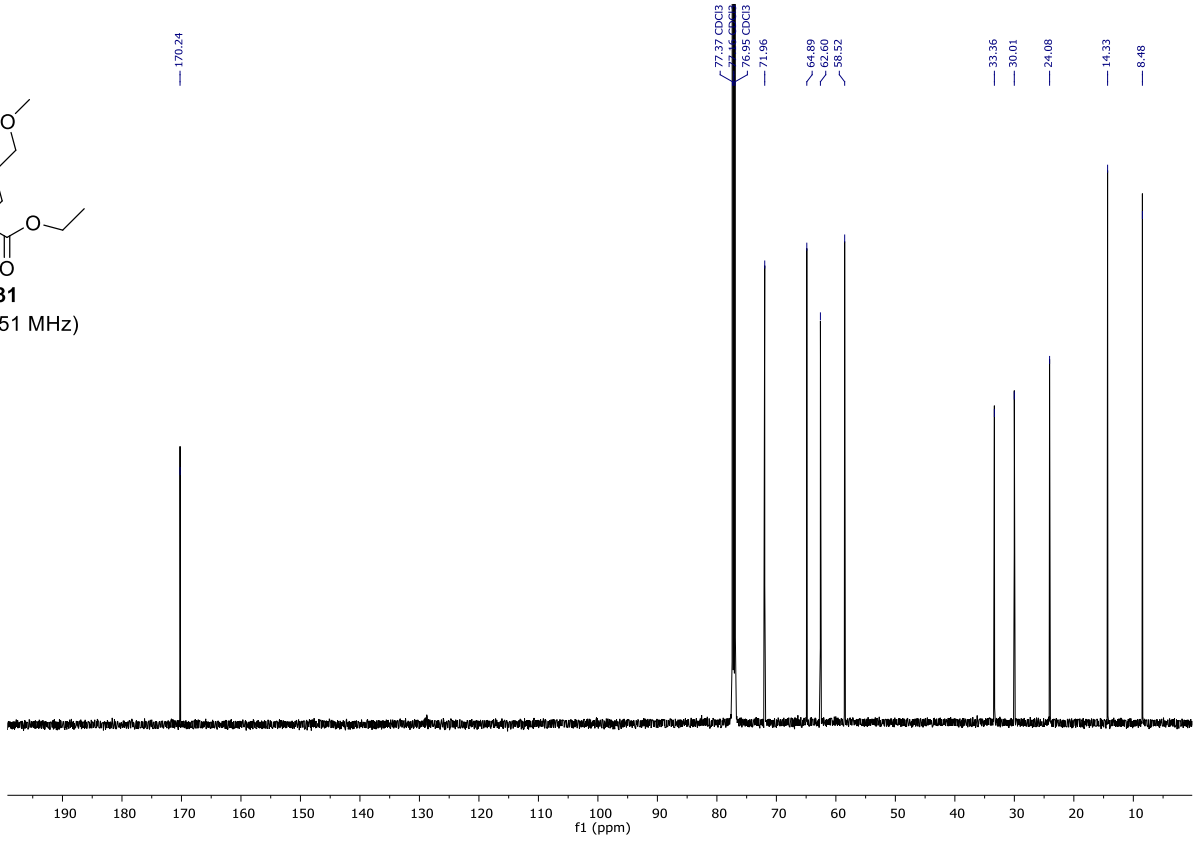

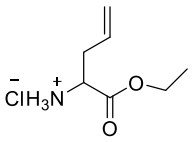

SI-32

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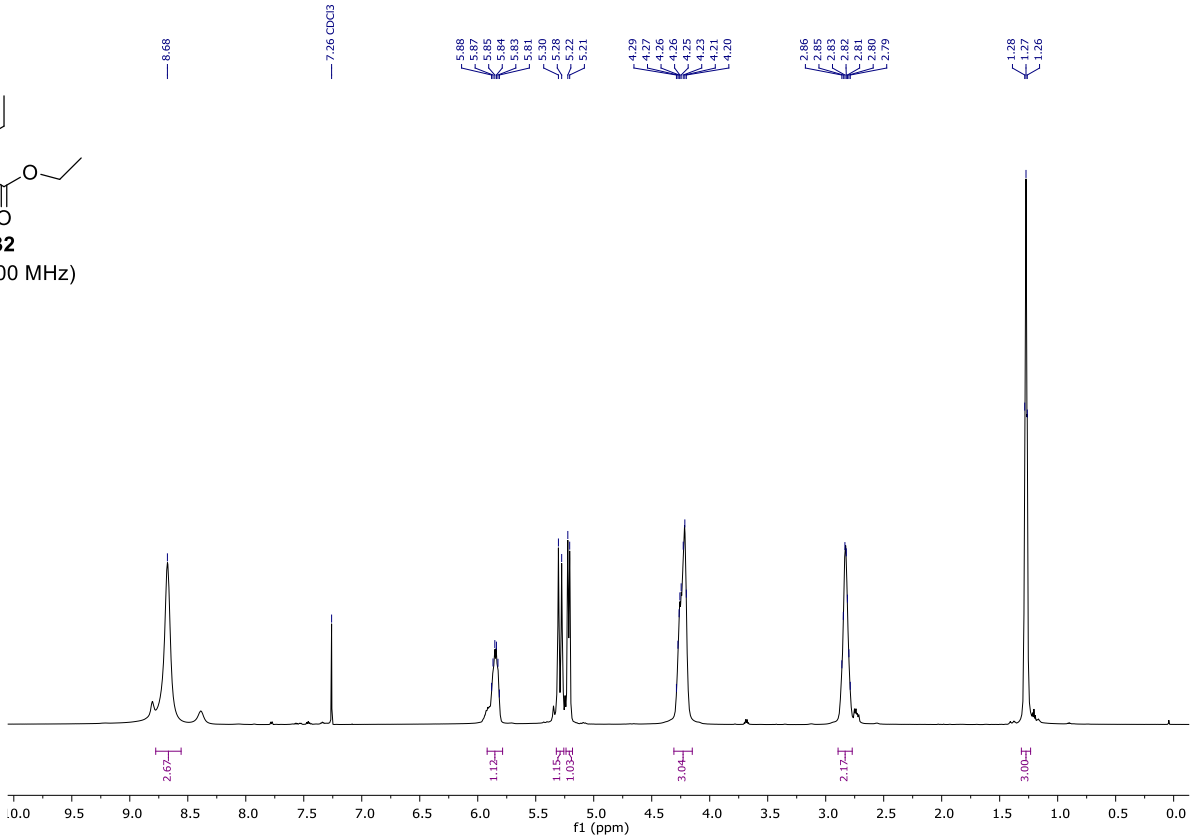

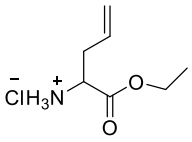

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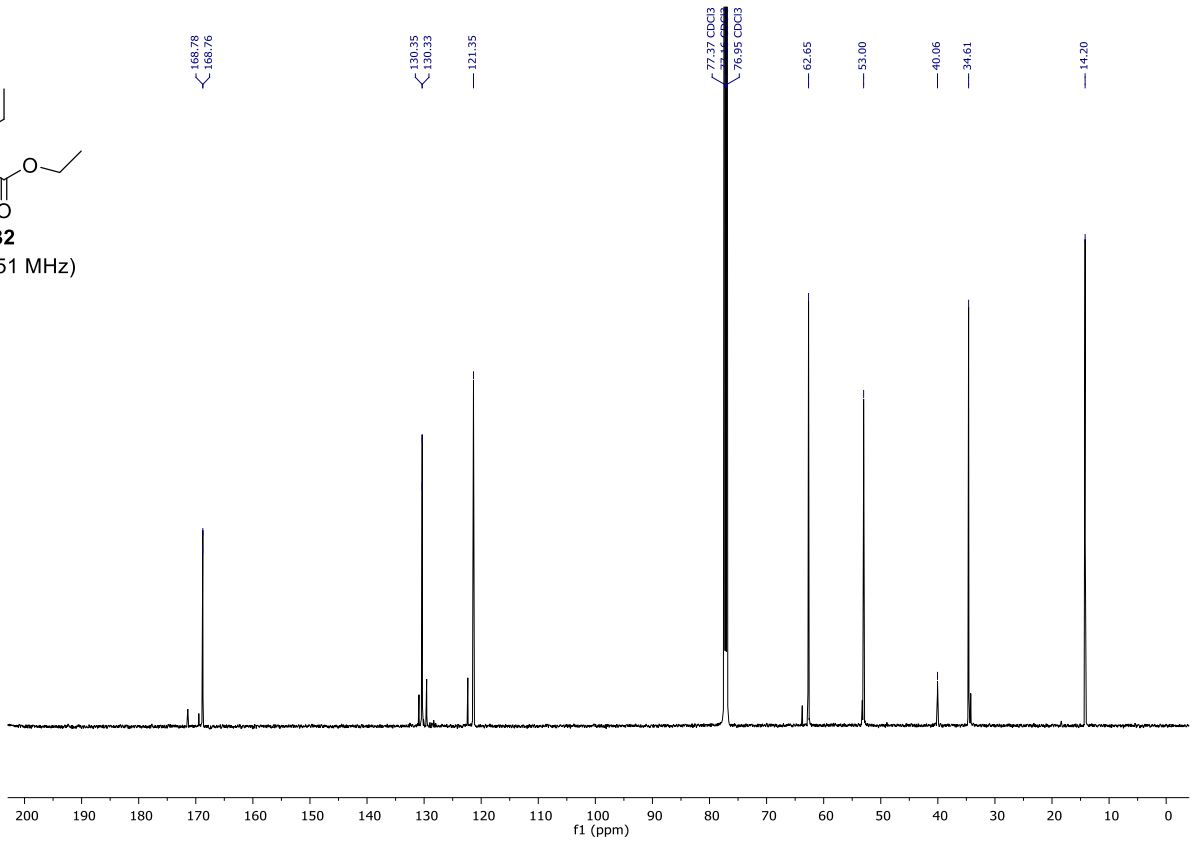

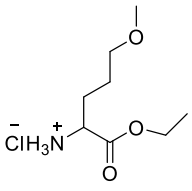

SI-33

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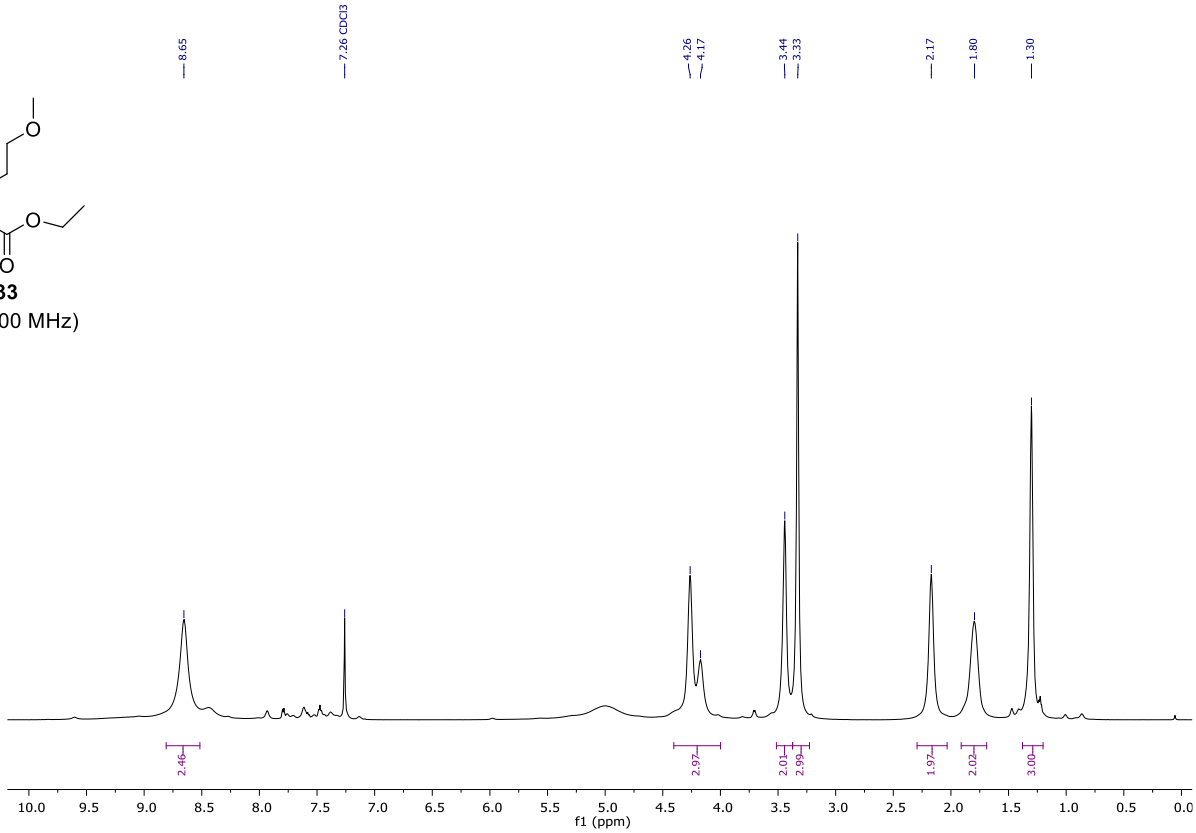

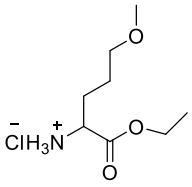

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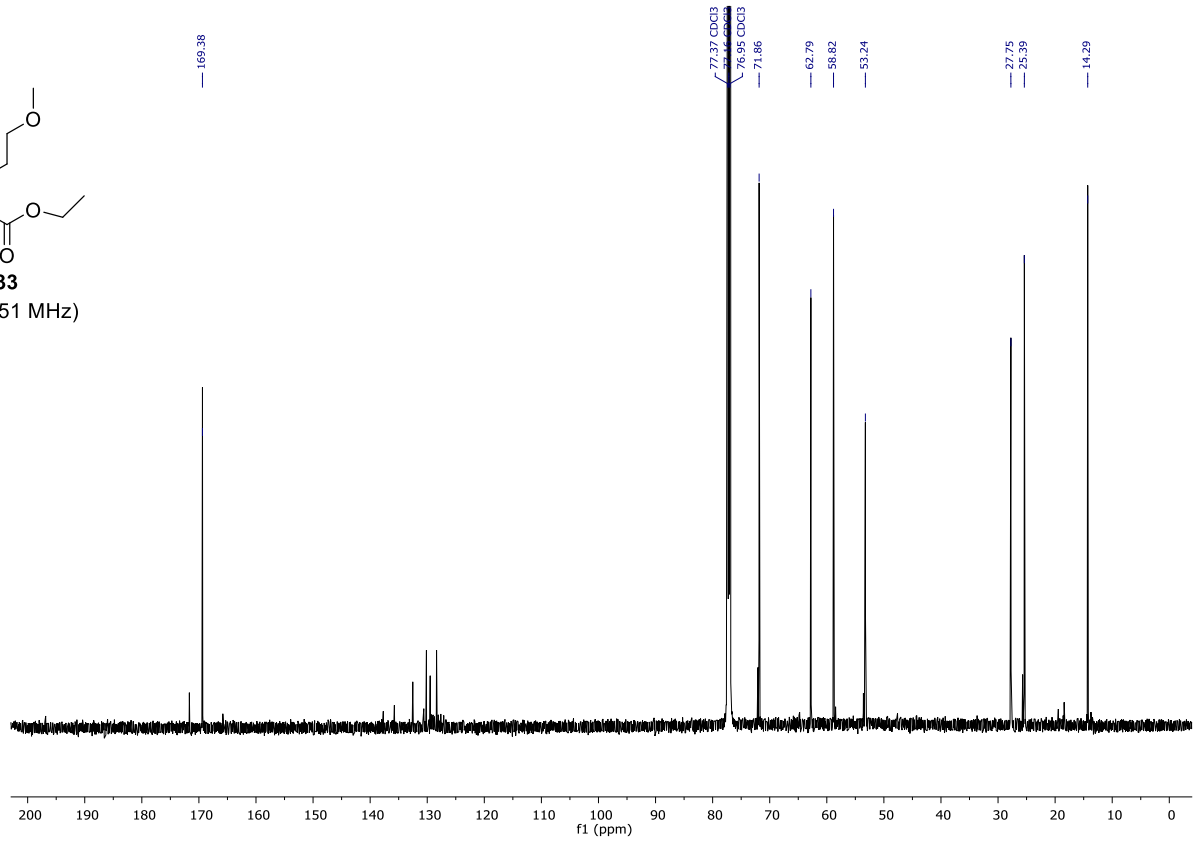

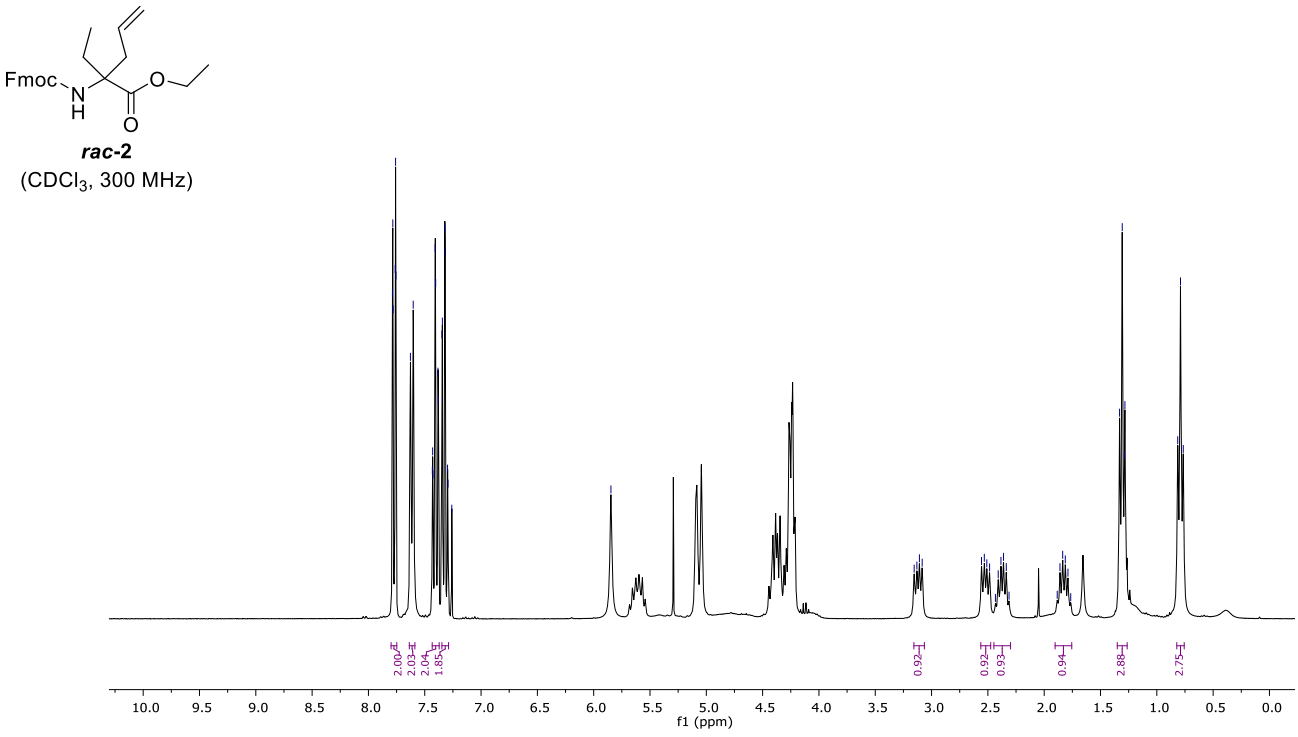

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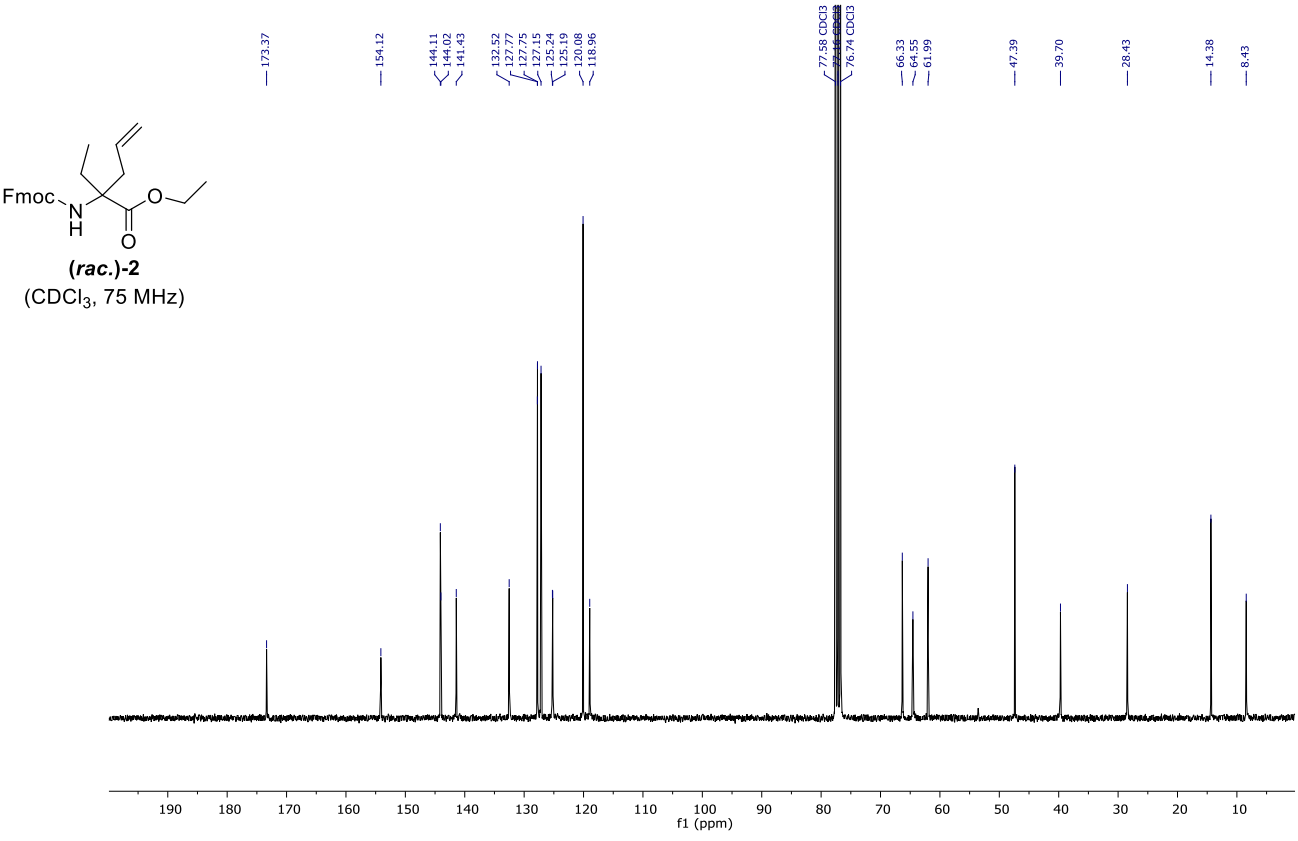

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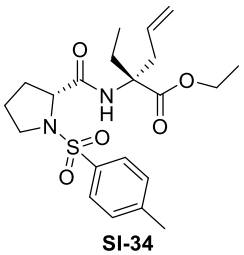

SI-34

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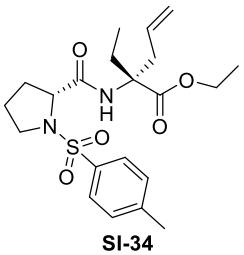
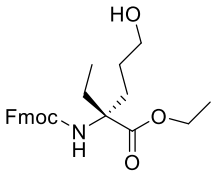


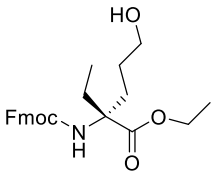
SI-34

($CDCl_3$, 75 MHz)



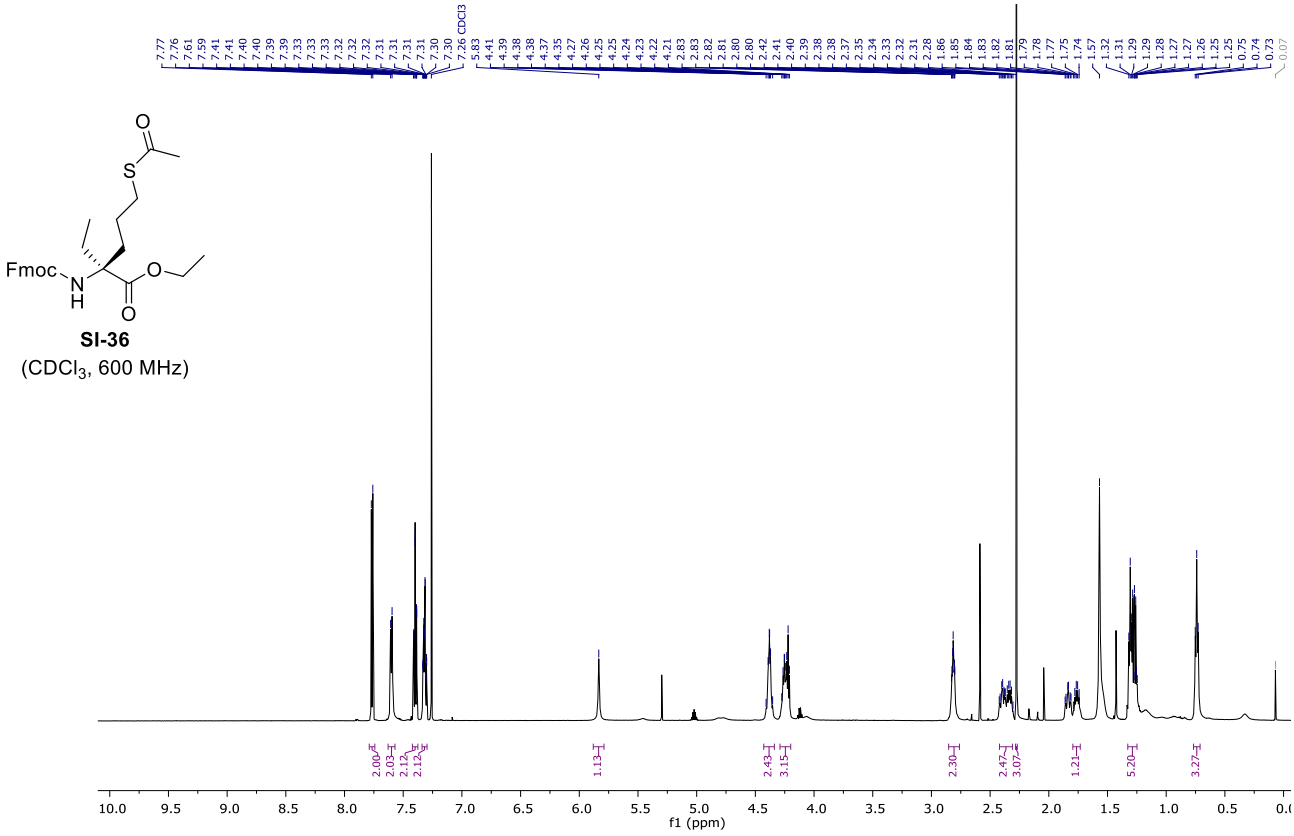
SI-35

(DMSO-d_6, 600 MHz)



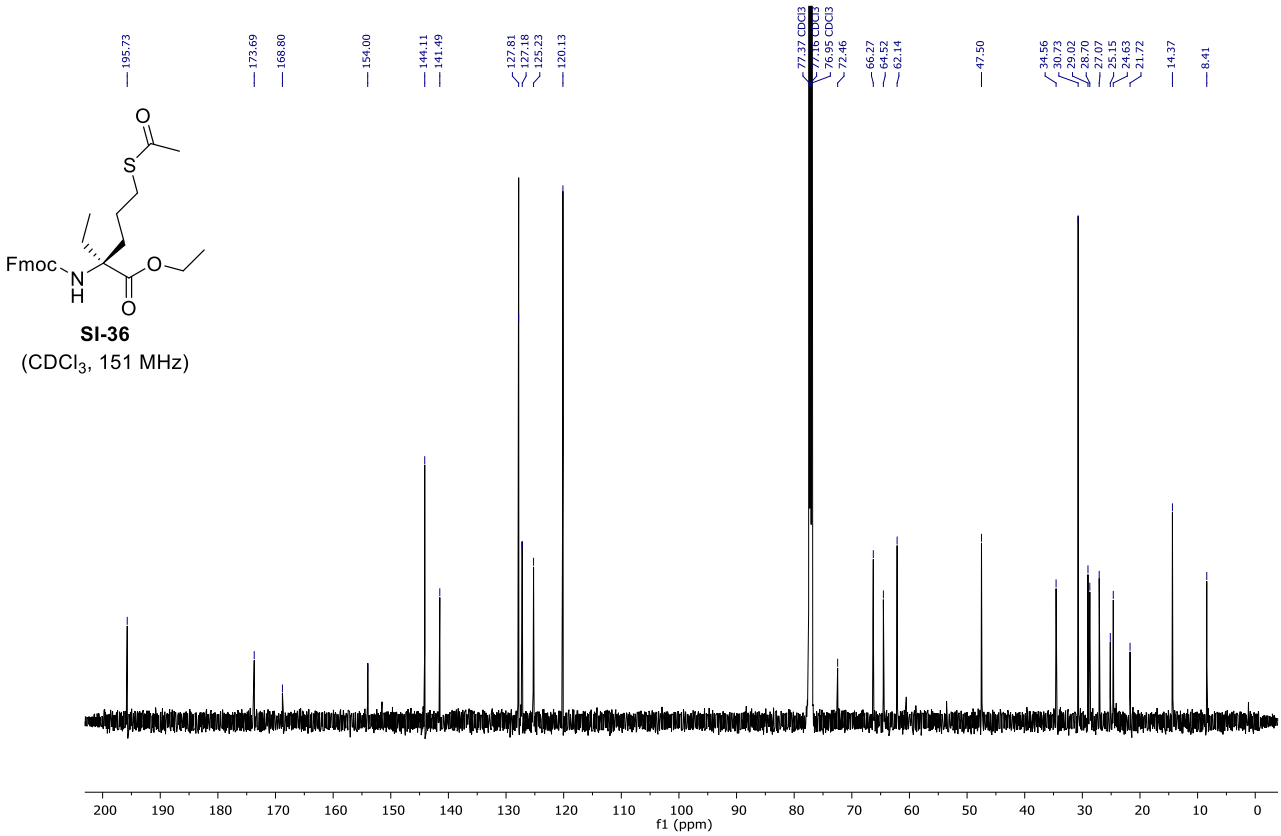
SI-35

(DMSO-d_6, 151 MHz)



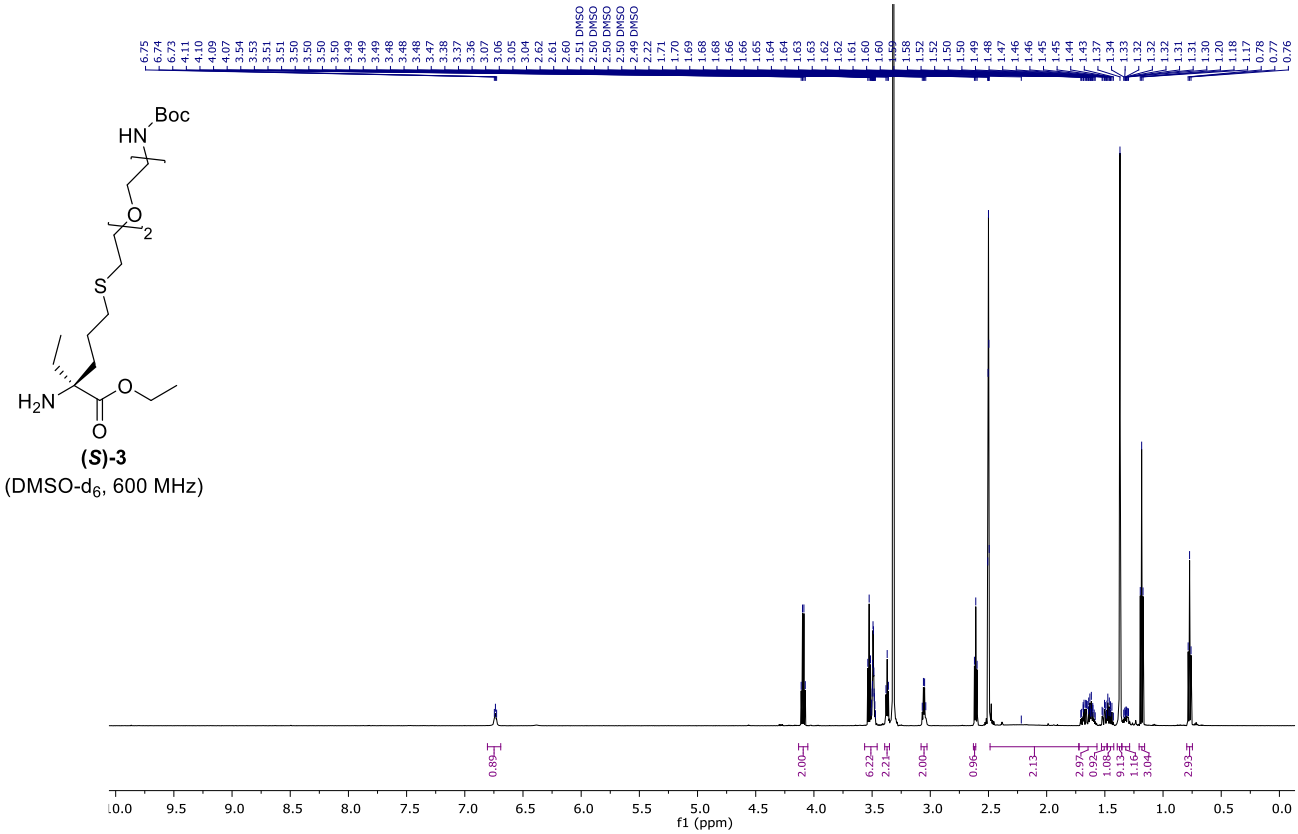
SI-36

($CDCl_3$, 600 MHz)

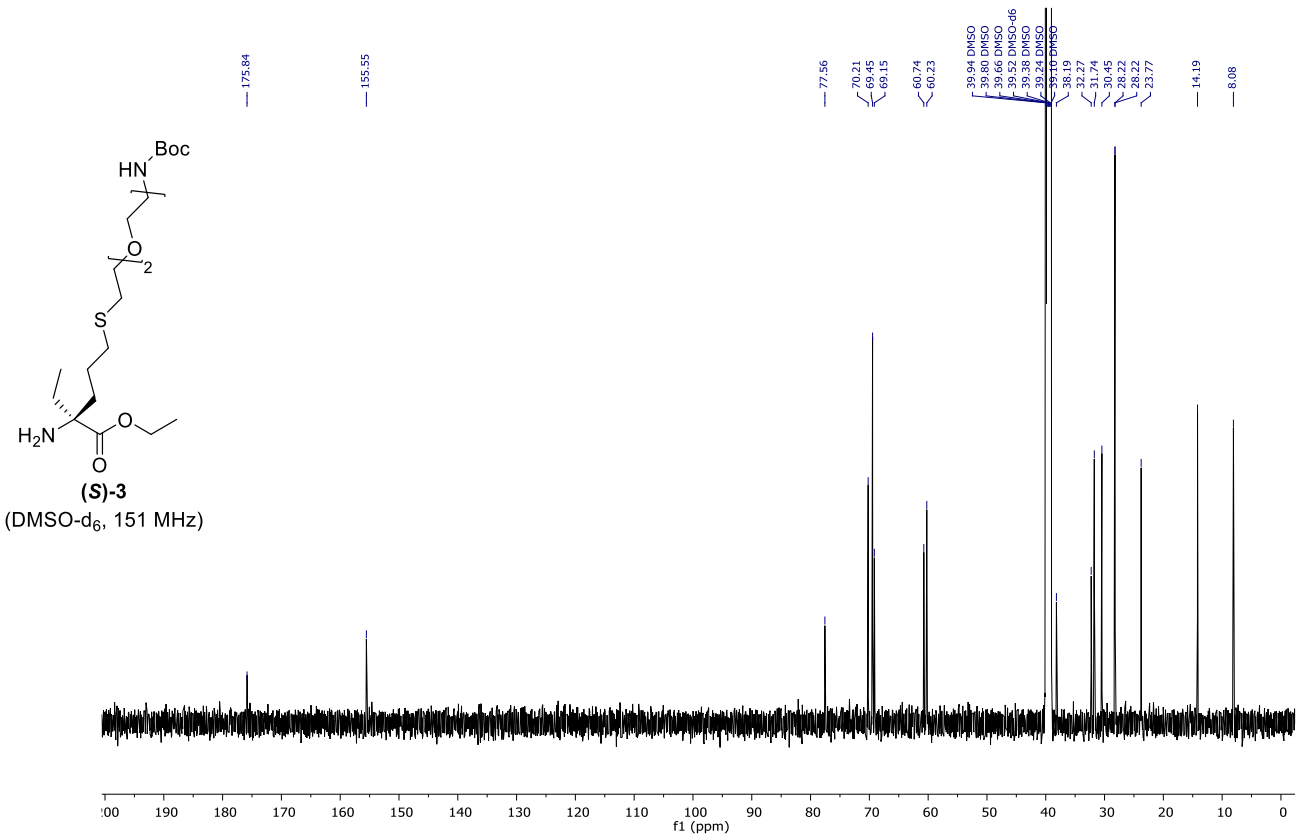


SI-36

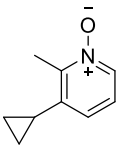
($CDCl_3$, 151 MHz)



(*S*)-3
(DMSO-d_6, 600 MHz)

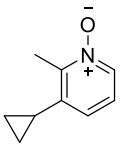


(*S*)-3
(DMSO-d_6, 151 MHz)



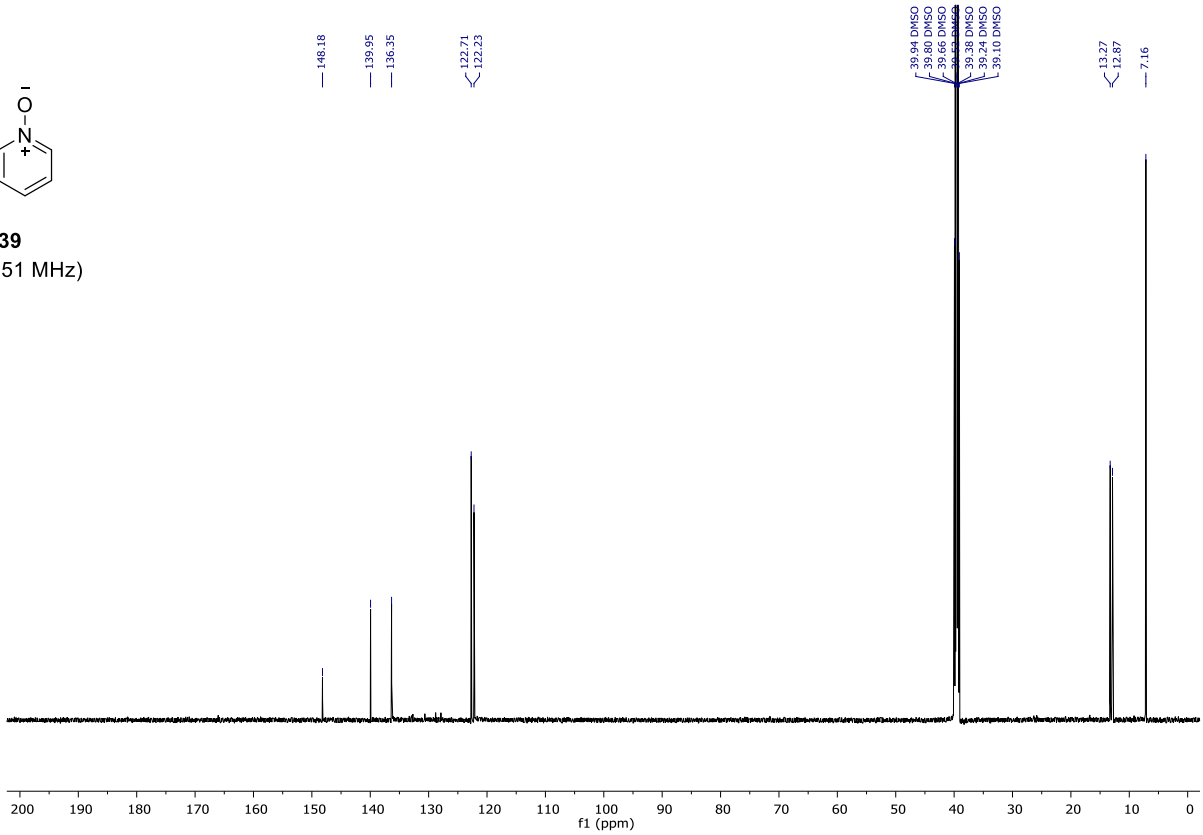
SI-39

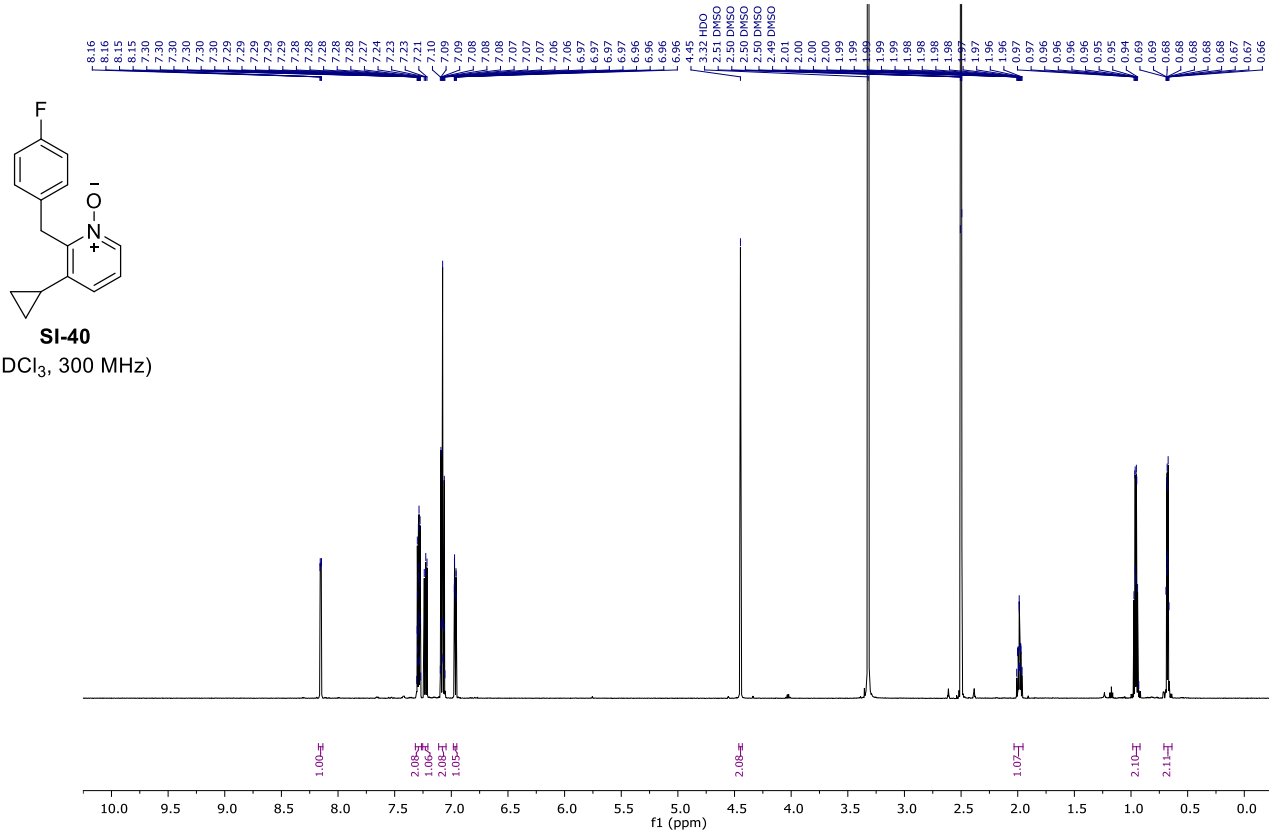
($CDCl_3$, 300 MHz)



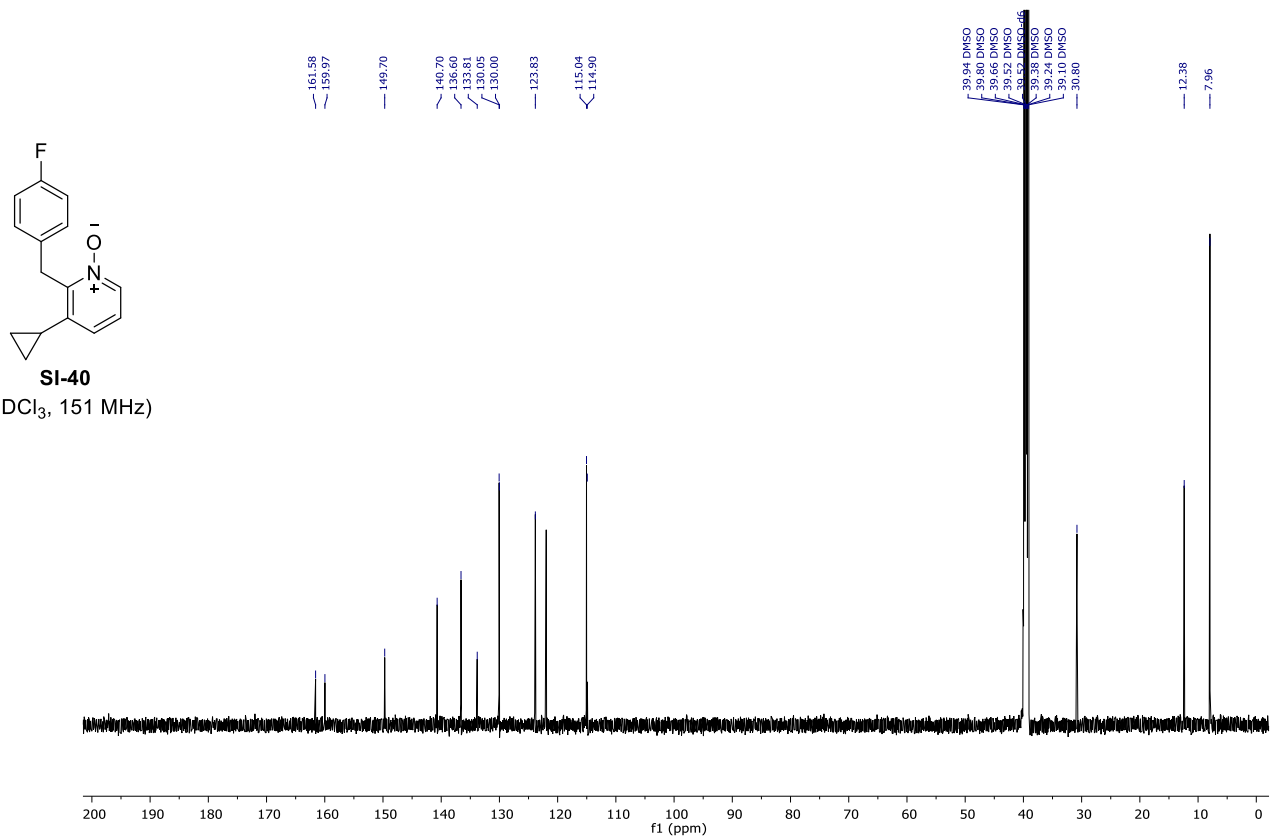
SI-39

($CDCl_3$, 151 MHz)

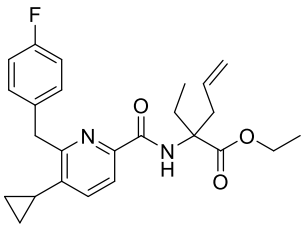




SI-40
($CDCl_3$, 300 MHz)

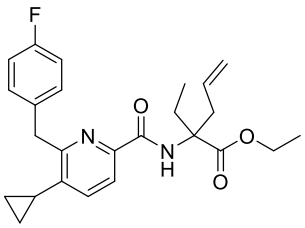


SI-40
($CDCl_3$, 151 MHz)



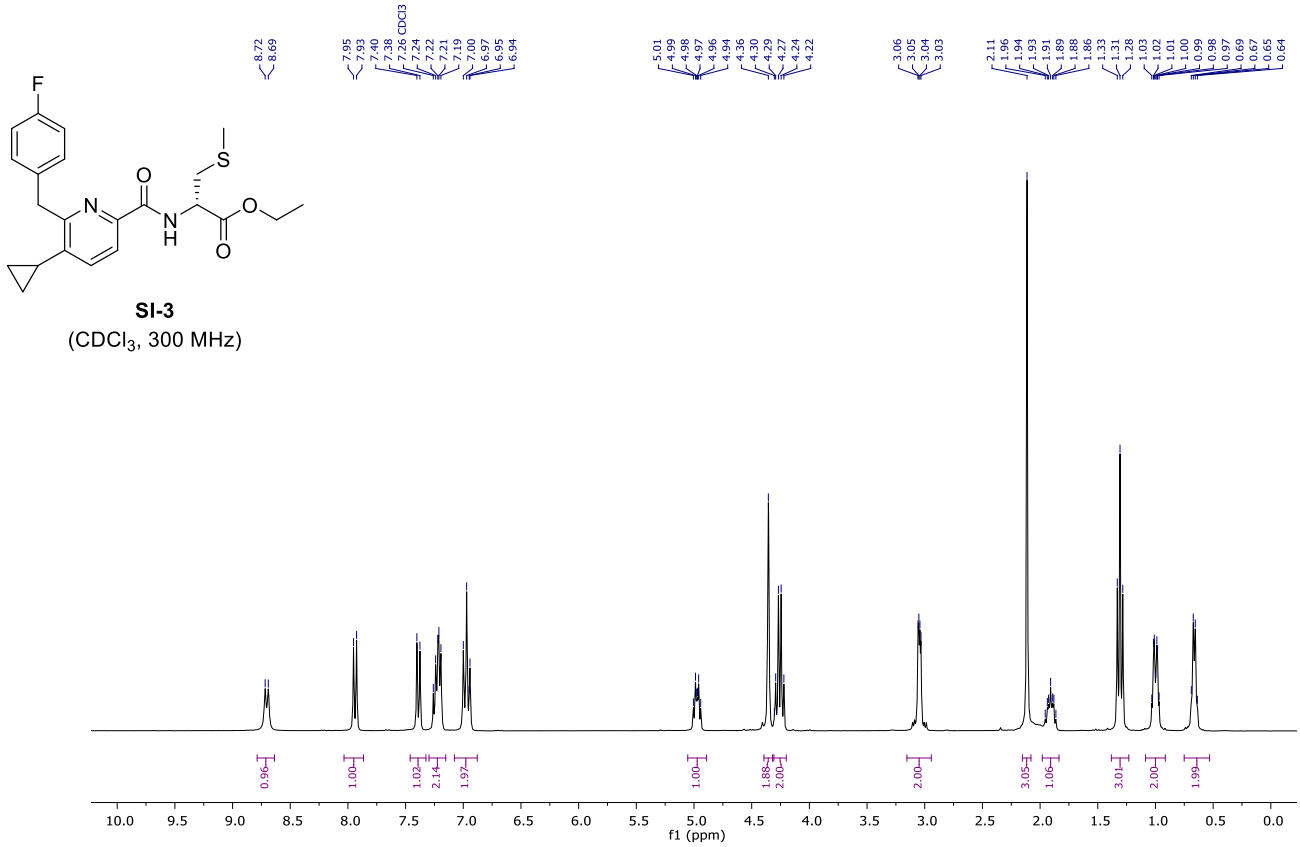
SI-2

($CDCl_3$, 300 MHz)

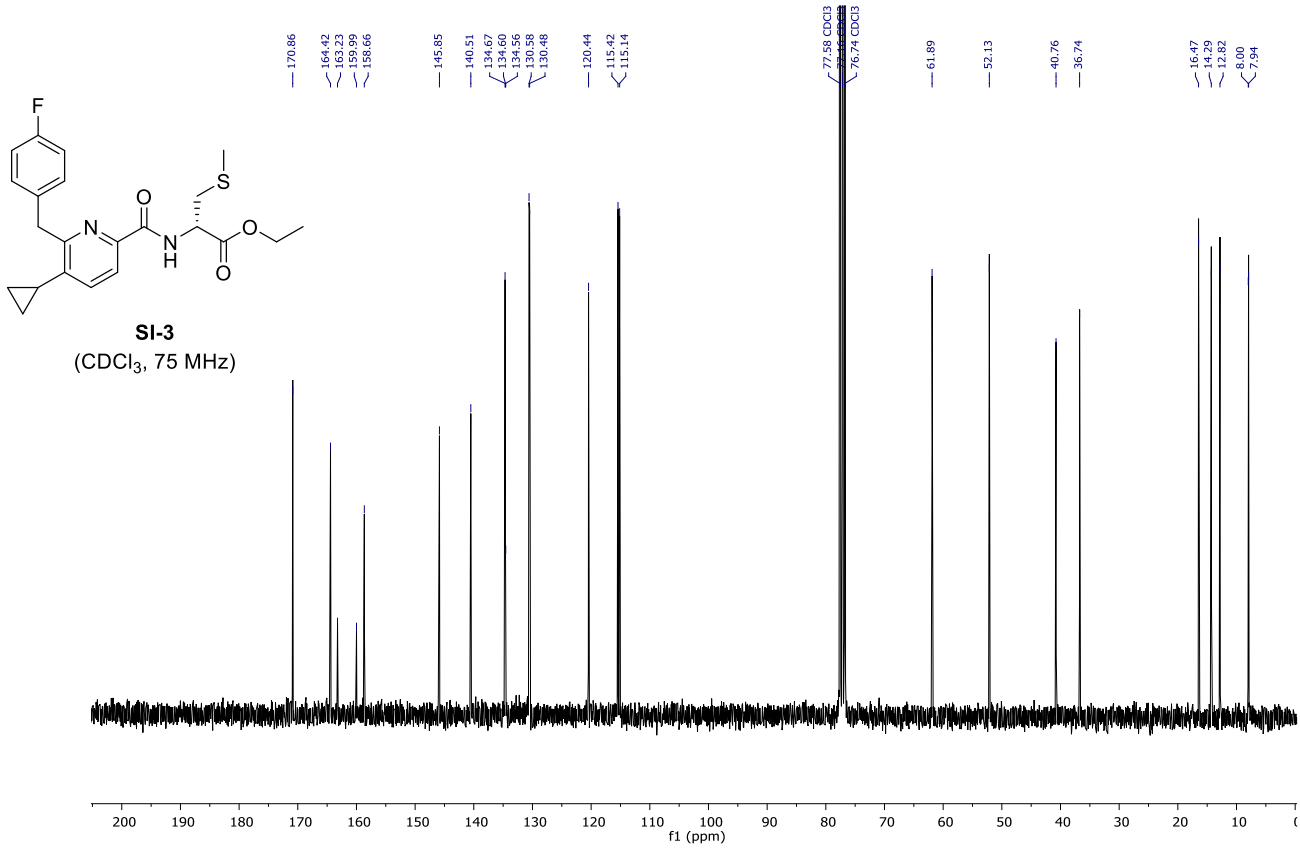


SI-2

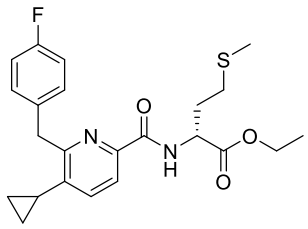
($CDCl_3$, 75 MHz)



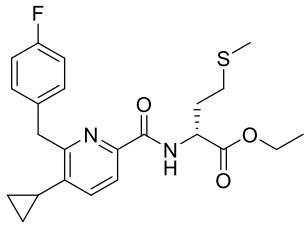
SI-3
($CDCl_3$, 300 MHz)



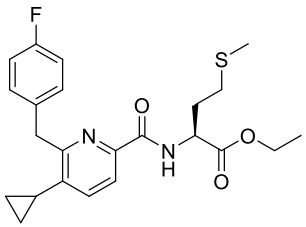
SI-3
($CDCl_3$, 75 MHz)



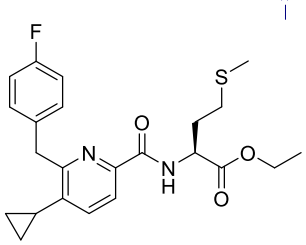
SI-4
($CDCl_3$, 300 MHz)



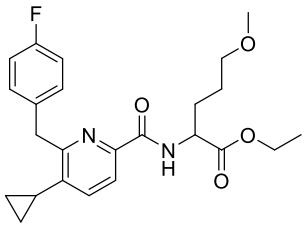
SI-4
($CDCl_3$, 75 MHz)



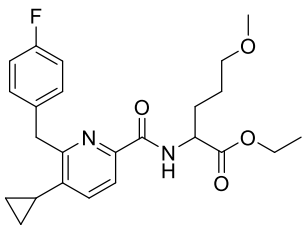
SI-5
($CDCl_3$, 300 MHz)



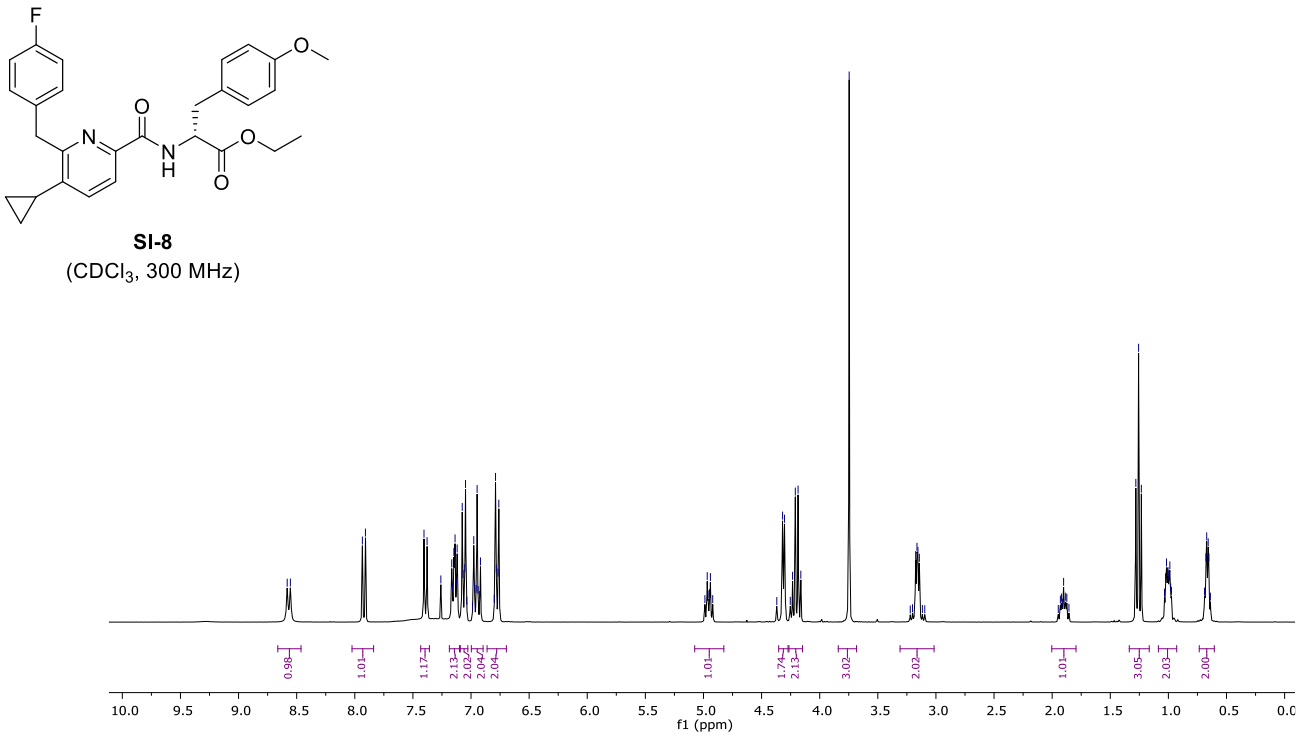
SI-5
($CDCl_3$, 75 MHz)



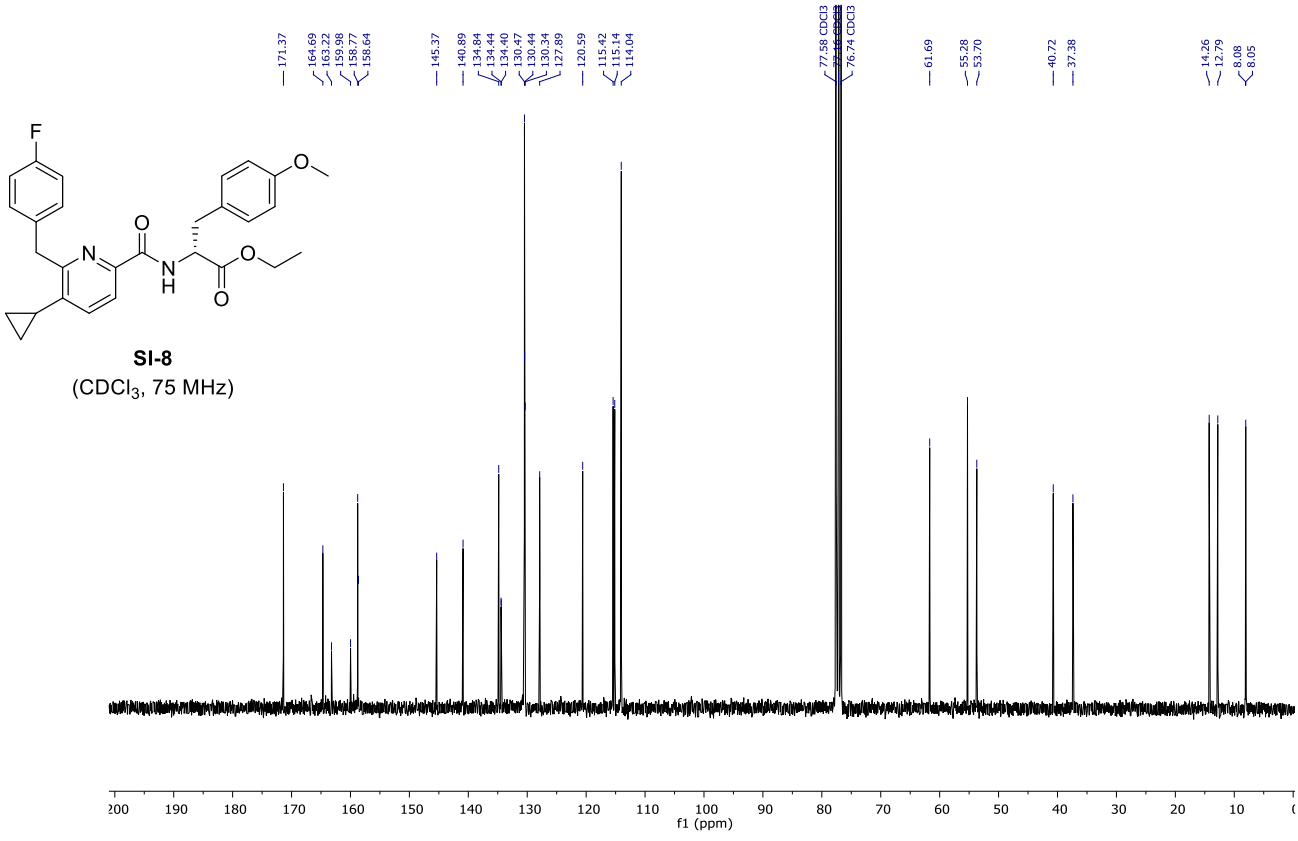
SI-7
($CDCl_3$, 300 MHz)



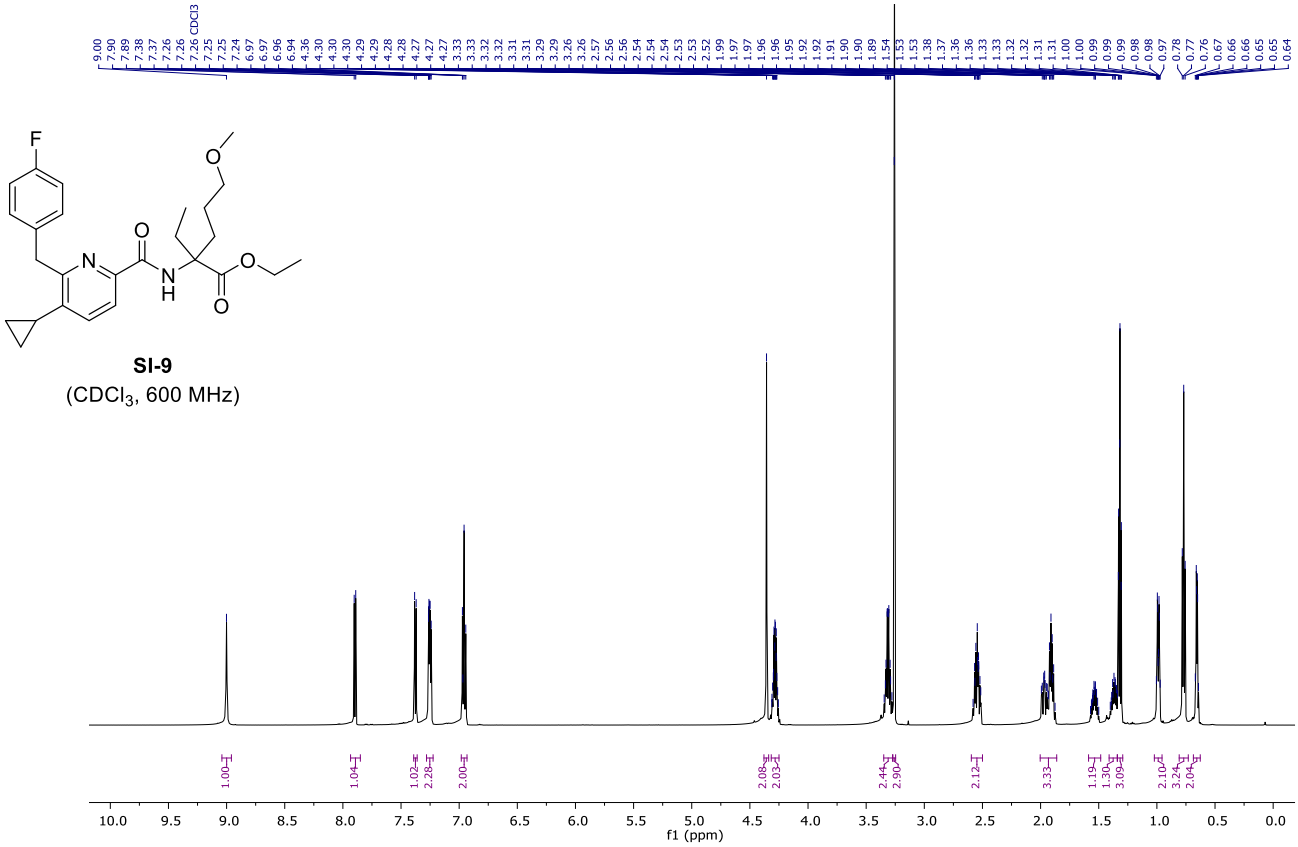
SI-7
($CDCl_3$, 75 MHz)



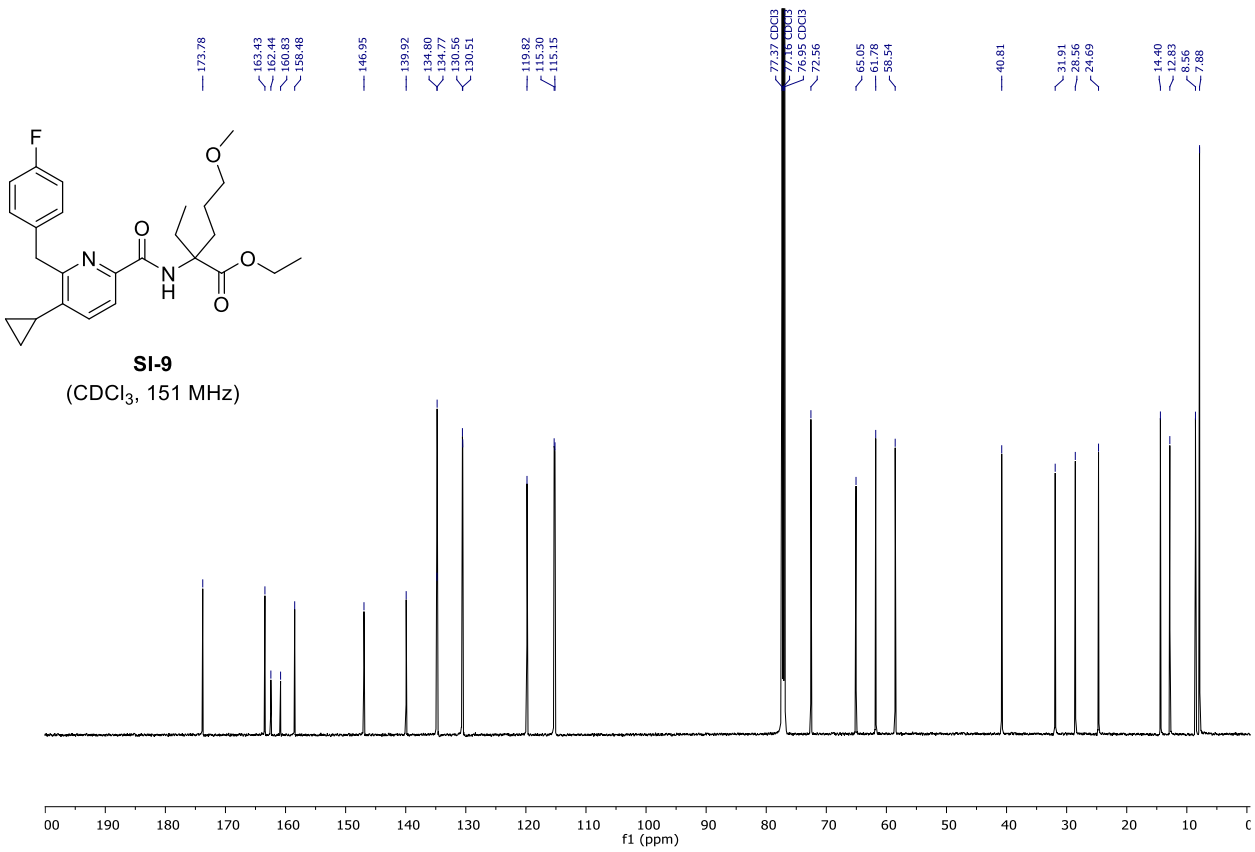
SI-8
($CDCl_3$, 300 MHz)



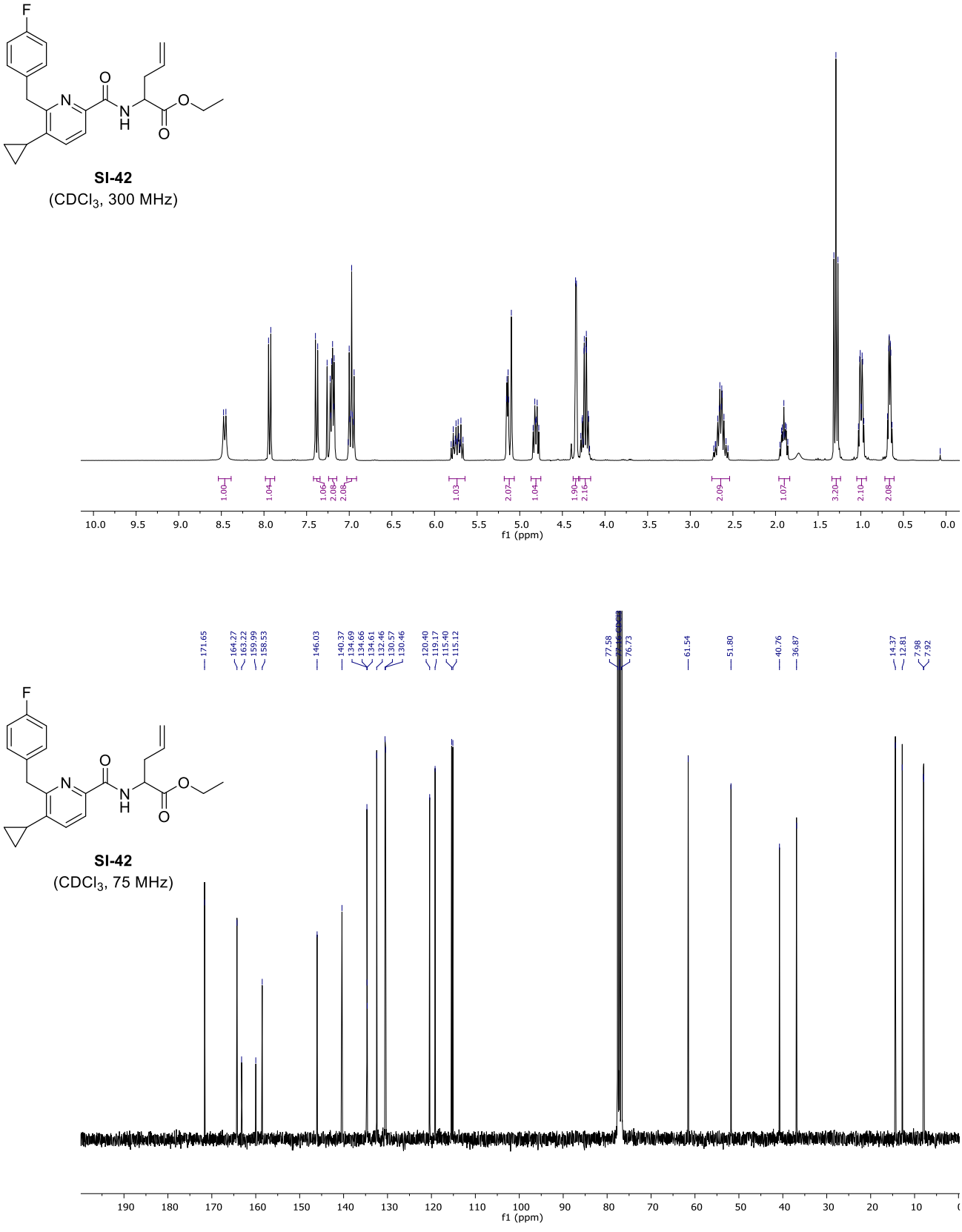
SI-8
($CDCl_3$, 75 MHz)

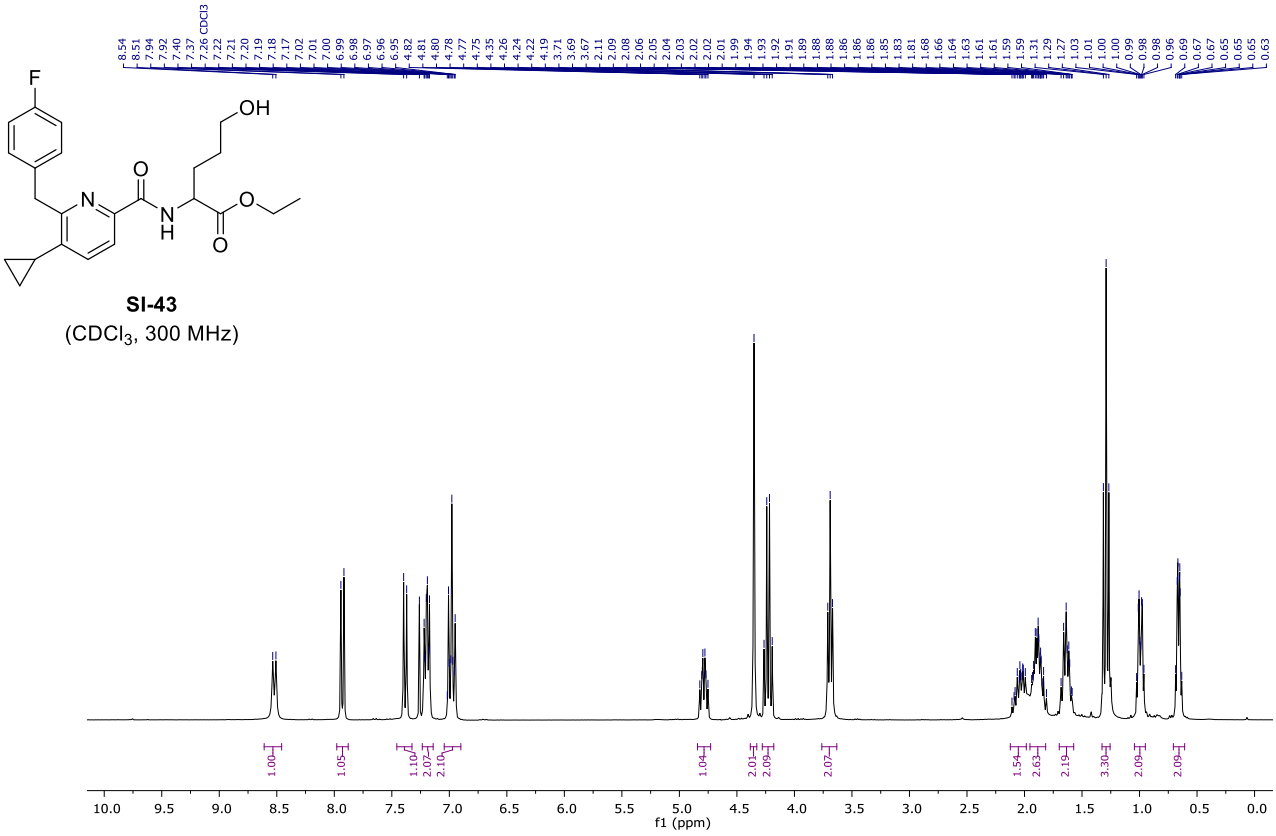


SI-9
($CDCl_3$, 600 MHz)



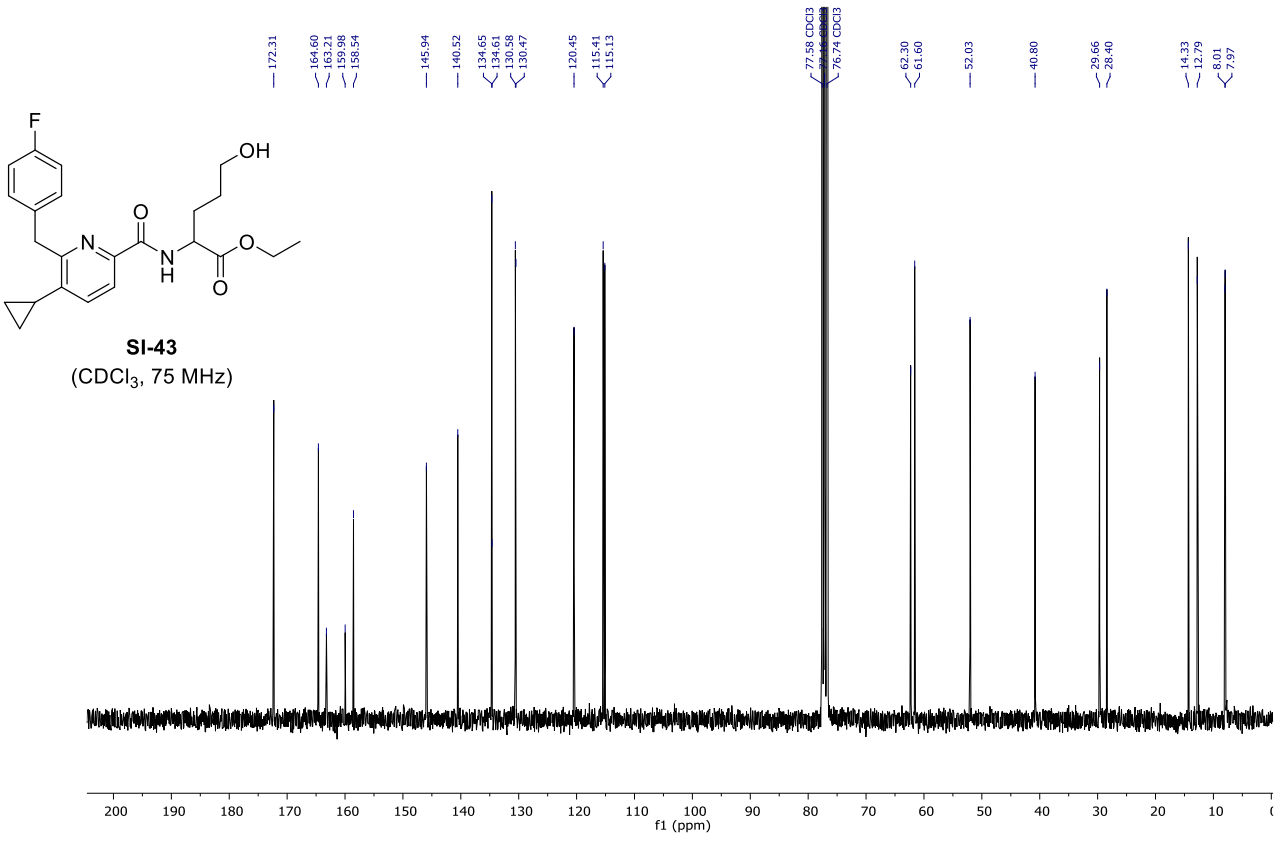
SI-9
($CDCl_3$, 151 MHz)


F
O
N
N
H
O
O
SI-42
(CDCl3, 300 MHz)
f1 (ppm)
F
O
N
N
H
O
O
SI-42
(CDCl3, 75 MHz)
f1 (ppm)



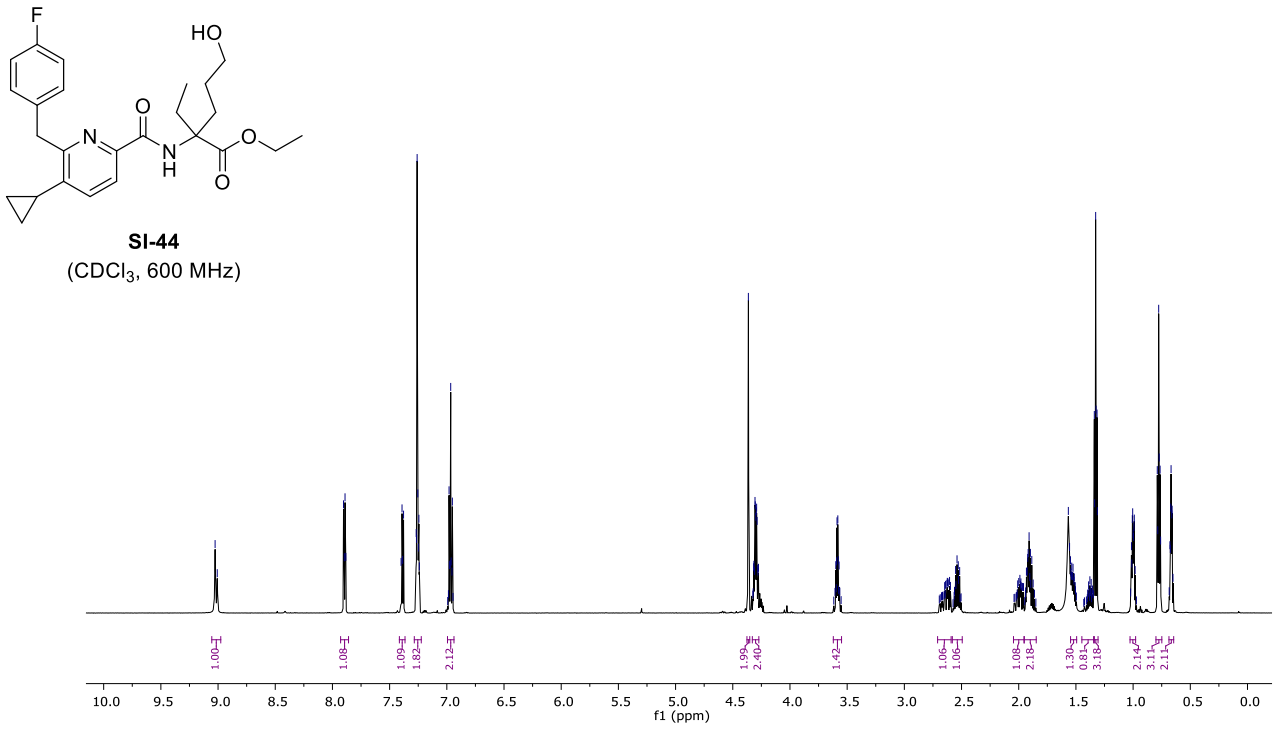
SI-43

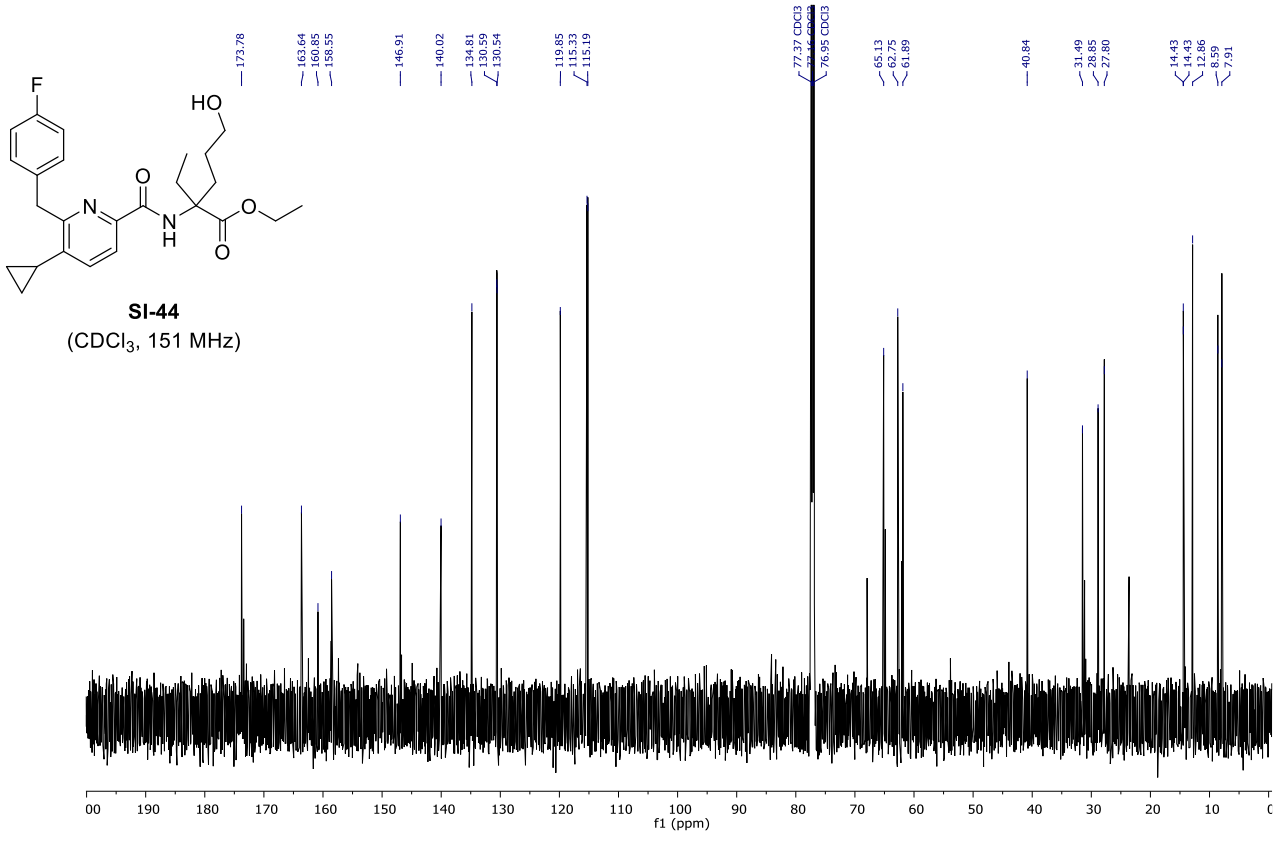
($CDCl_3$, 300 MHz)

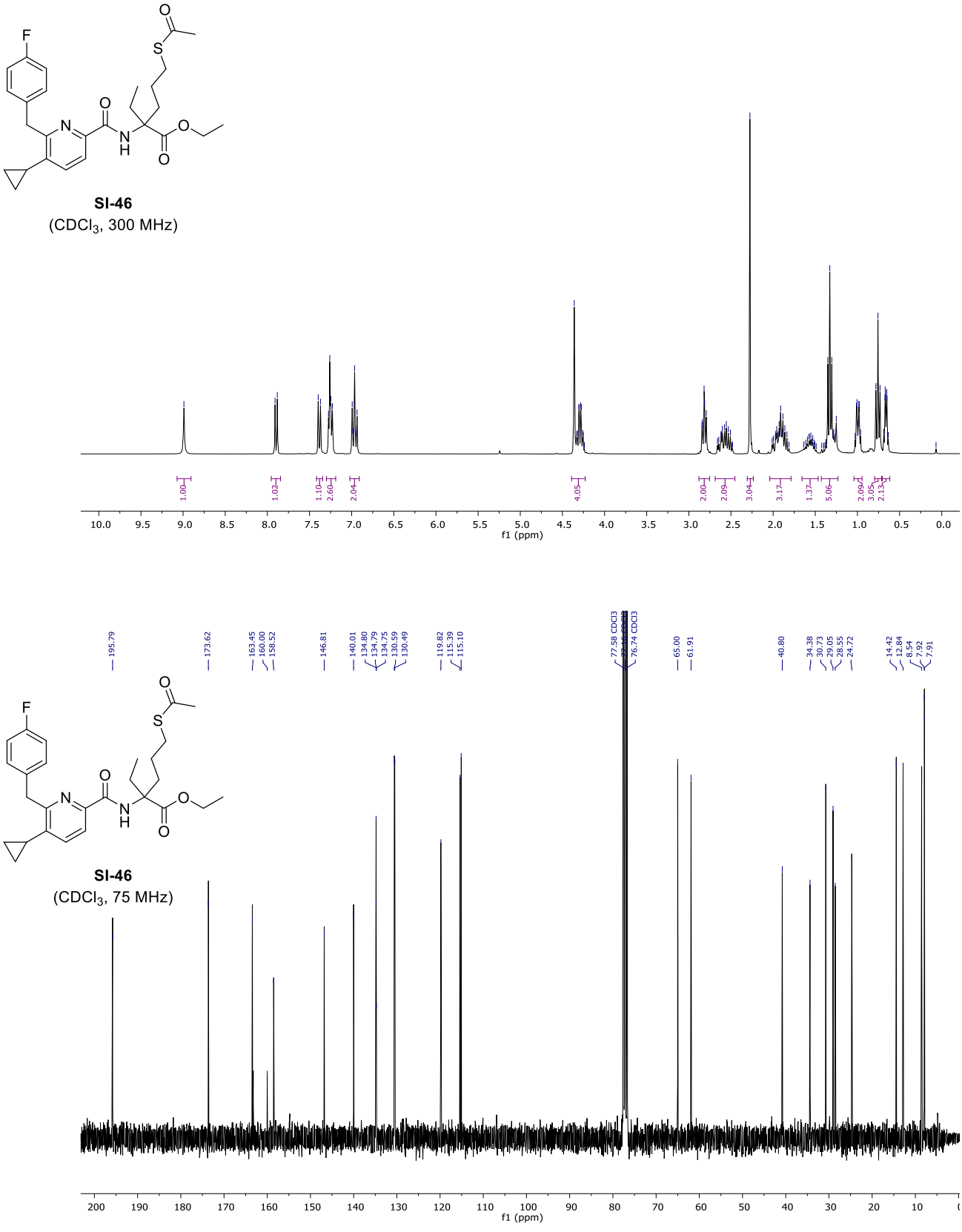


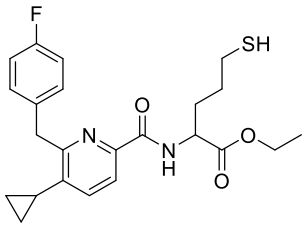
SI-43

($CDCl_3$, 75 MHz)

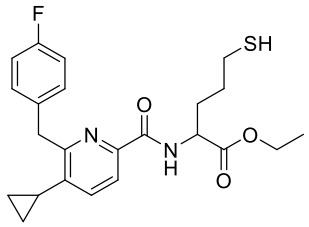

F
HO
O
N
N
H
O
O
SI-44
(CDCl3, 600 MHz)
f1 (ppm)


F
HO
O
N
N
H
O
O
SI-44
(CDCl3, 151 MHz)
f1 (ppm)

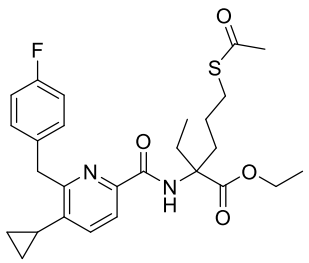

F
O
S
O
N
N
H
O
O
O
SI-46
(CDCl3, 300 MHz)
f1 (ppm)
SI-46
(CDCl3, 75 MHz)
f1 (ppm)



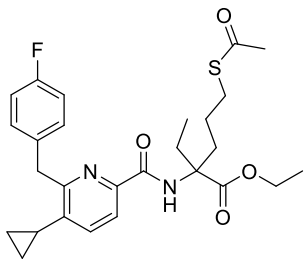
SI-6
(DMSO-d_6, 600 MHz)



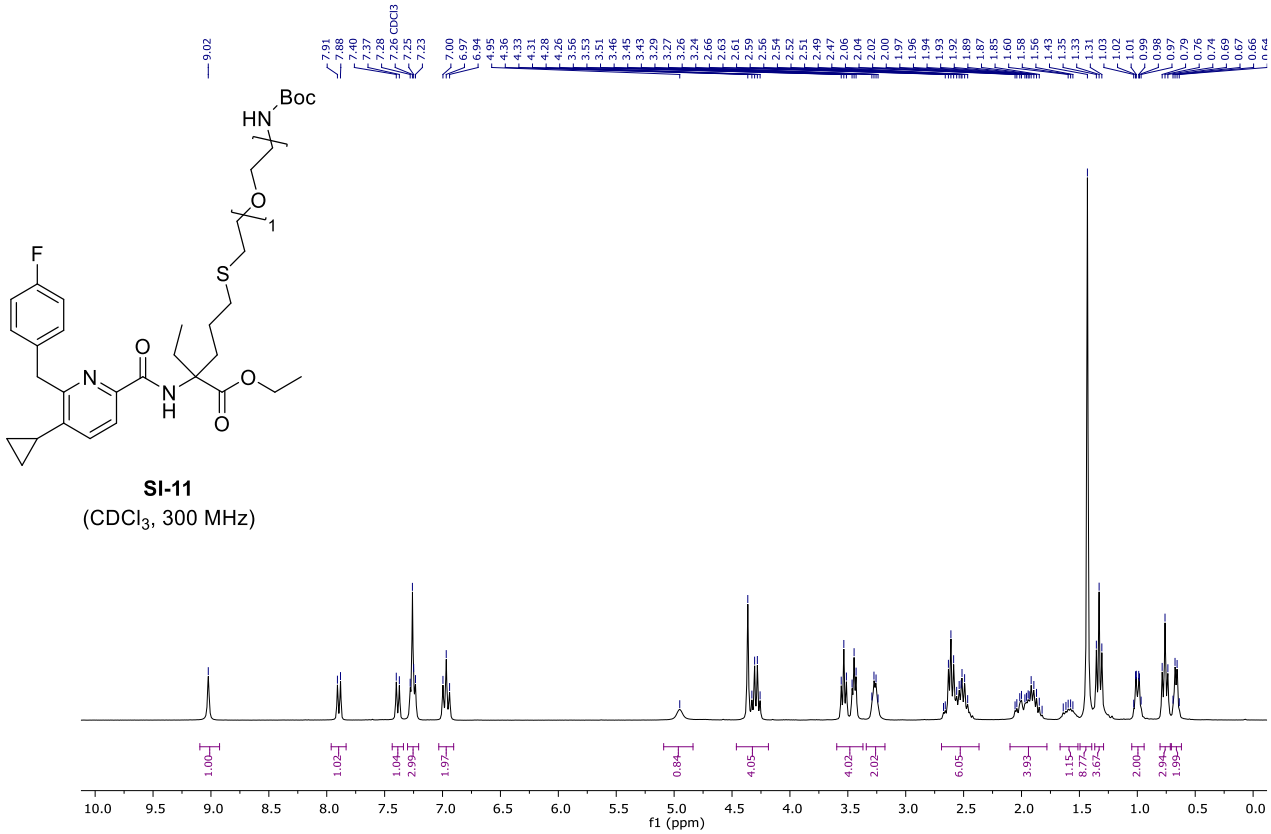
SI-6
(DMSO-d_6, 151 MHz)

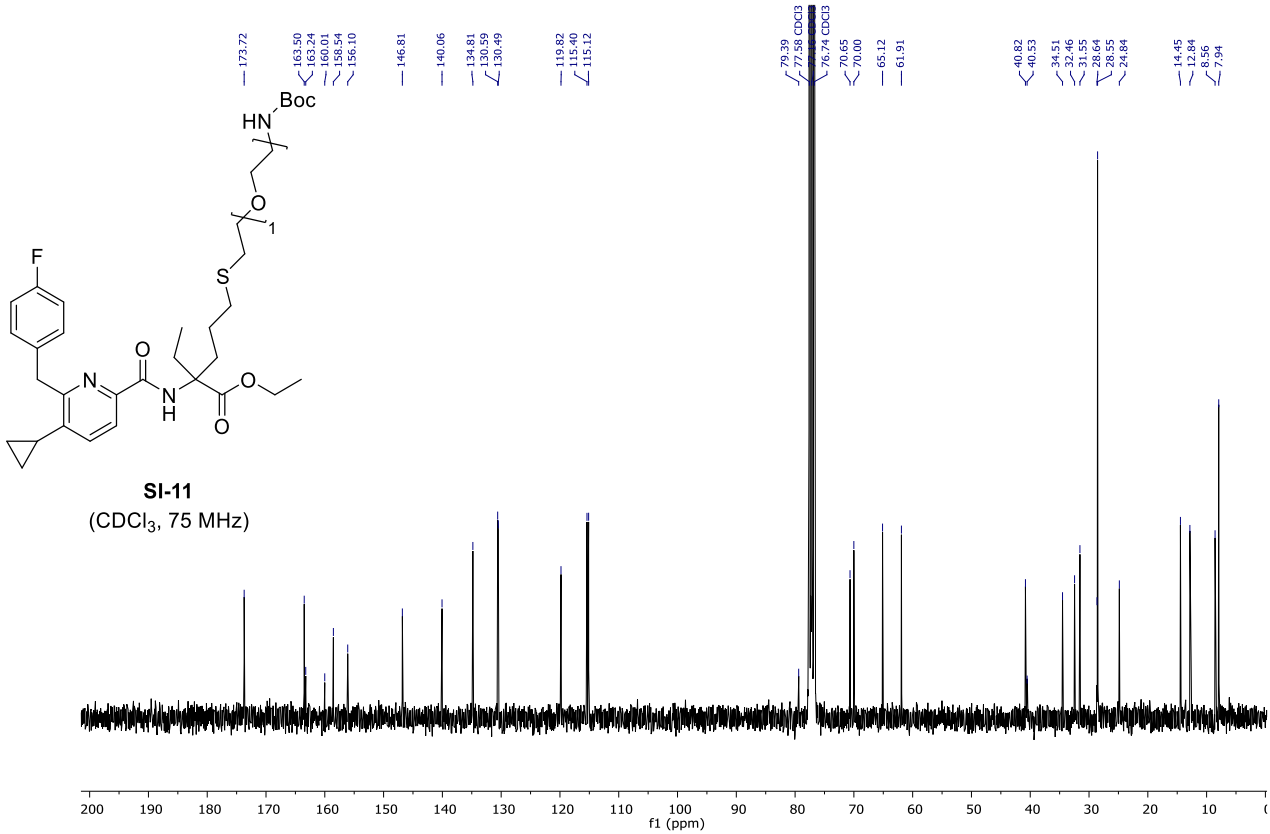


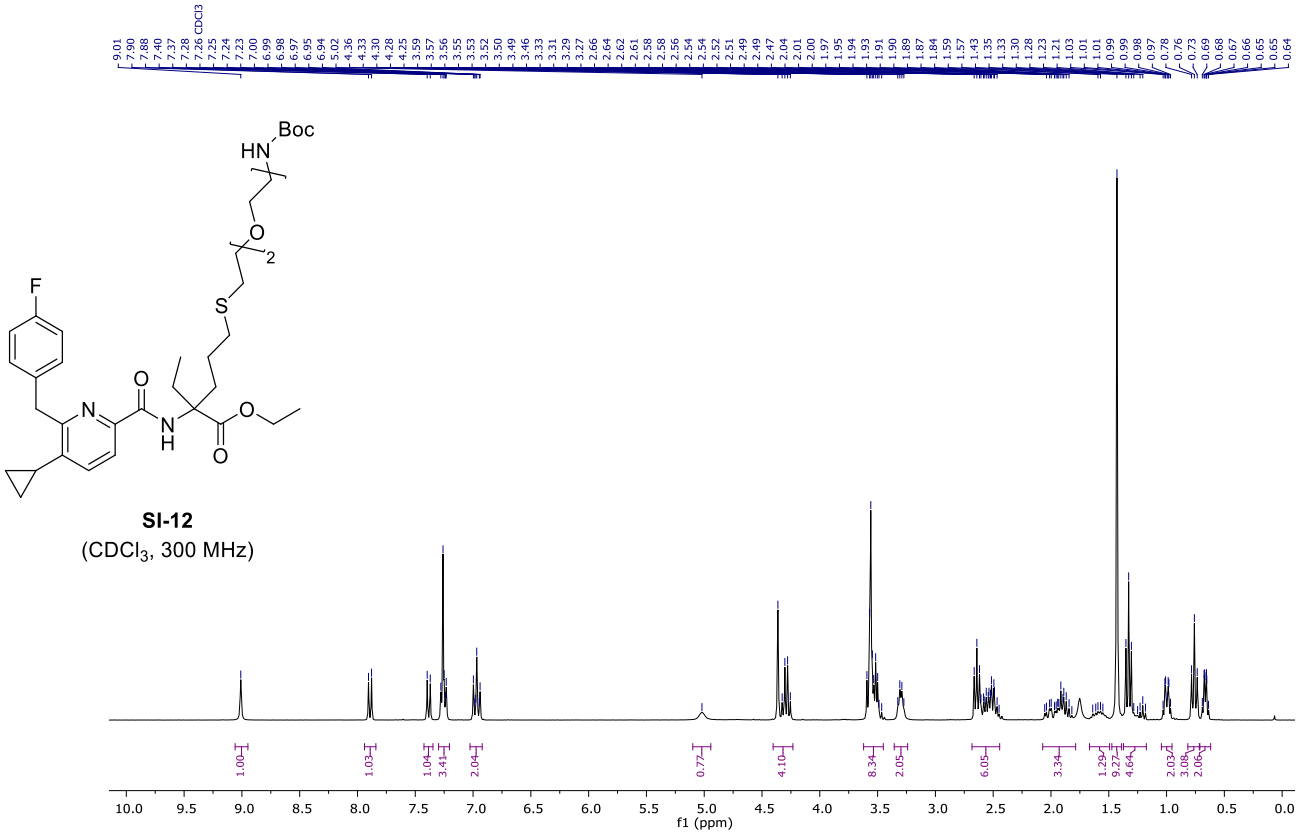
SI-10
($CDCl_3$, 300 MHz)

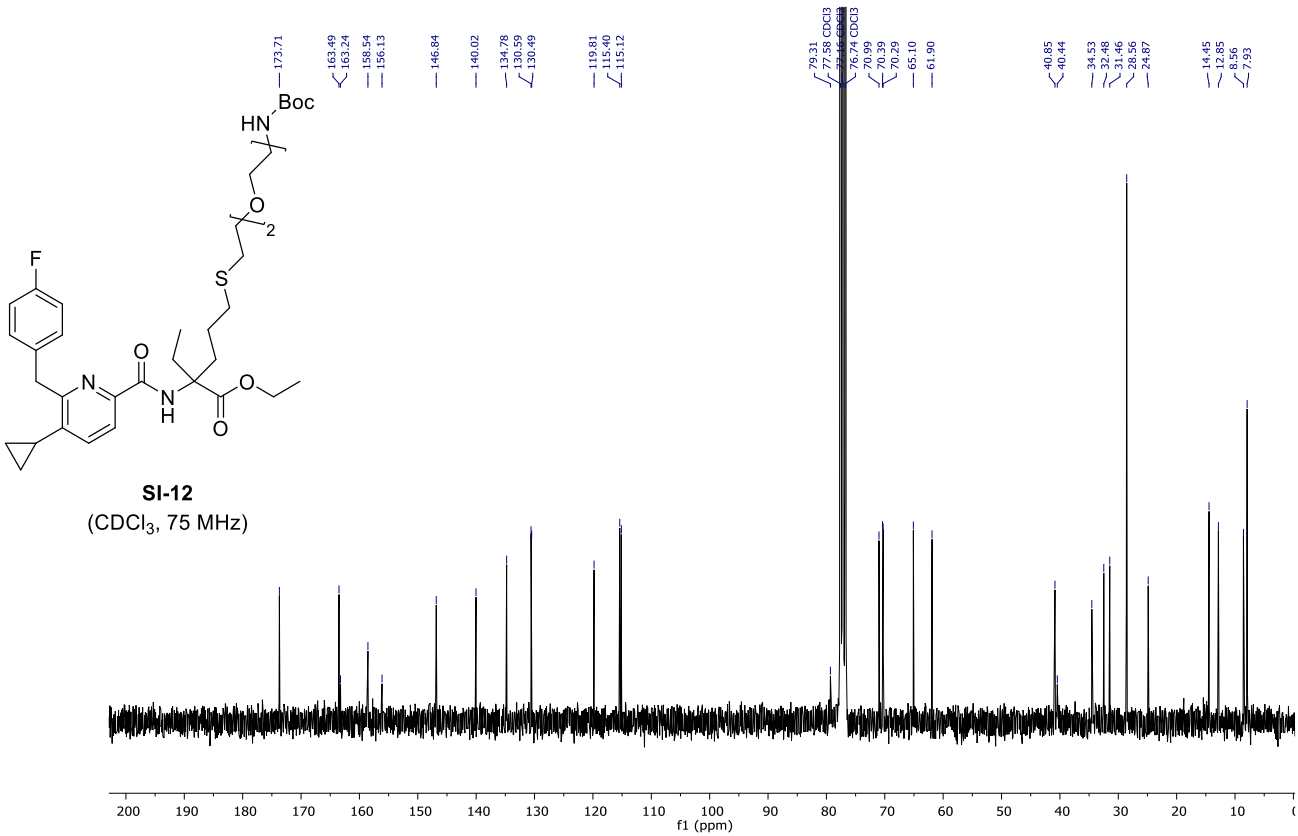


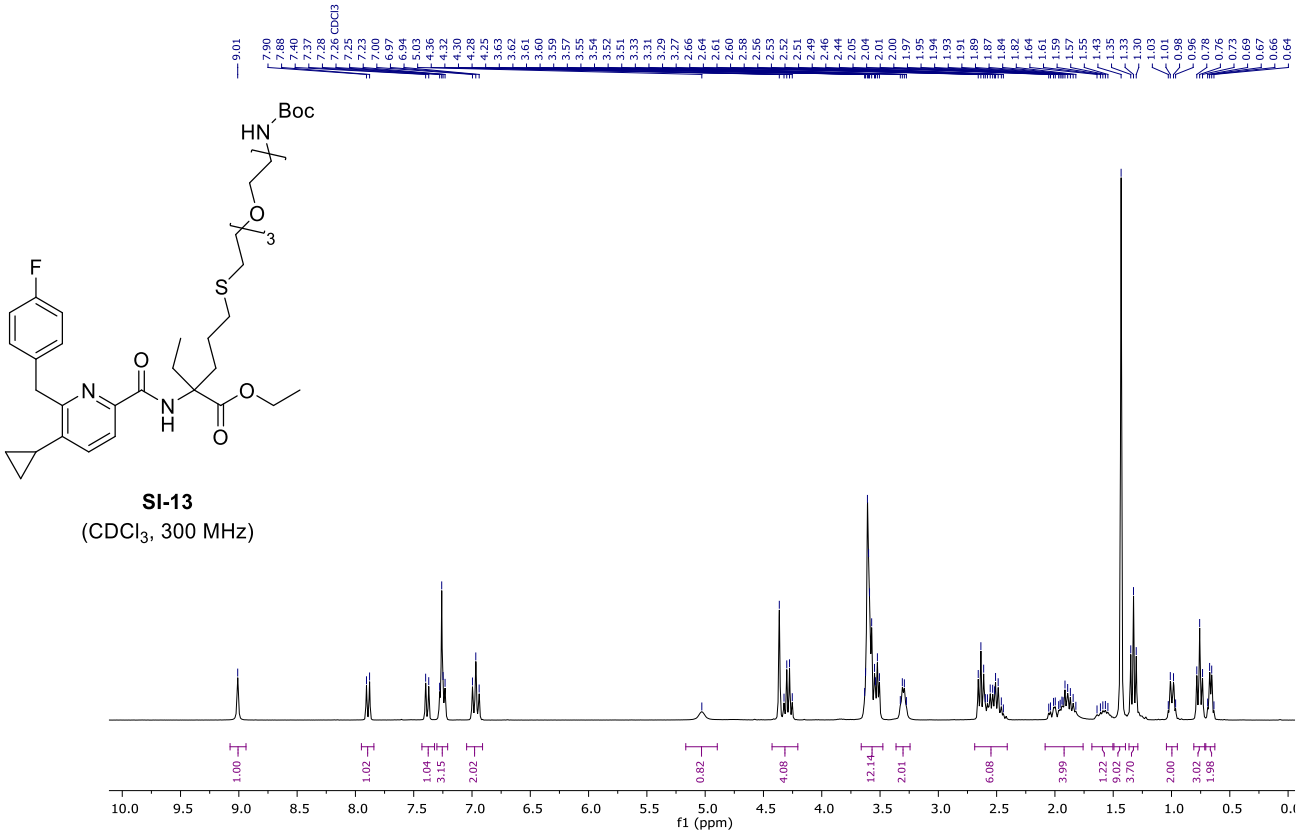
SI-10
($CDCl_3$, 75 MHz)


Boc
HN
O
1
S
F
O
N
N
H
O
O
O
SI-11
(CDCl3, 300 MHz)
f1 (ppm)

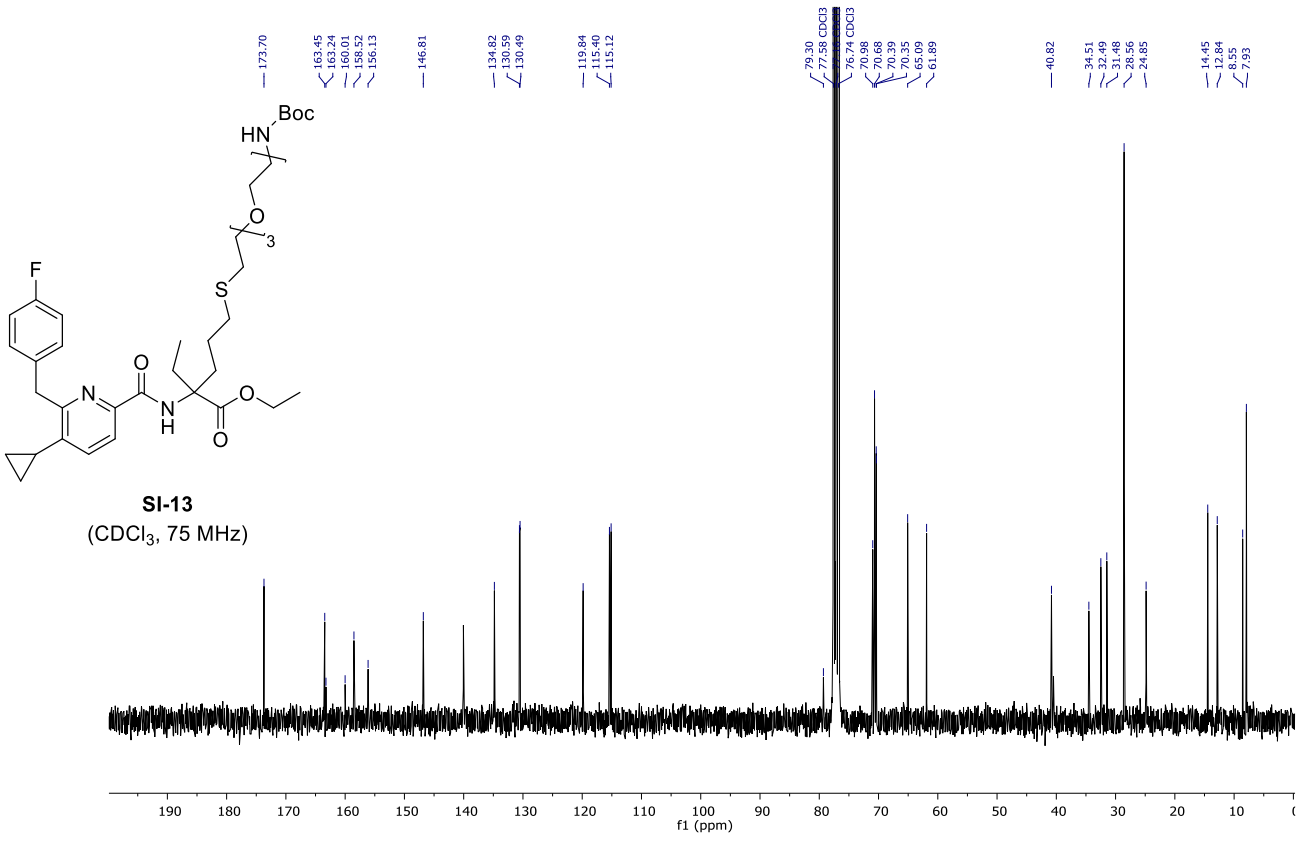

Boc
HN
O
1
S
F
O
N
N
H
O
O
O
SI-11
(CDCl3, 75 MHz)
f1 (ppm)


Boc
HN
O
2
S
F
O
N
N
H
O
O
O
SI-12
(CDCl3, 300 MHz)
f1 (ppm)

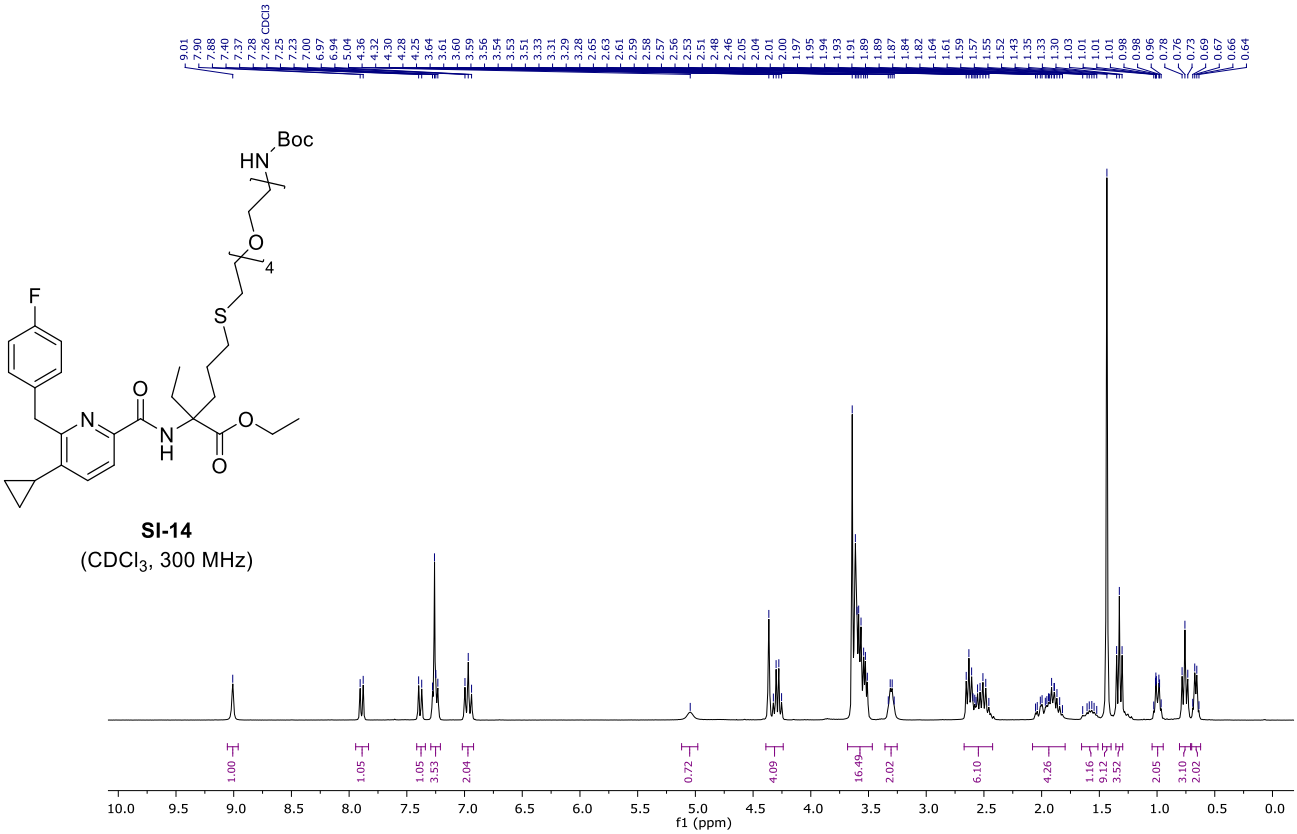

Boc
HN
O
2
S
F
O
N
N
H
O
O
O
SI-12
(CDCl3, 75 MHz)
f1 (ppm)

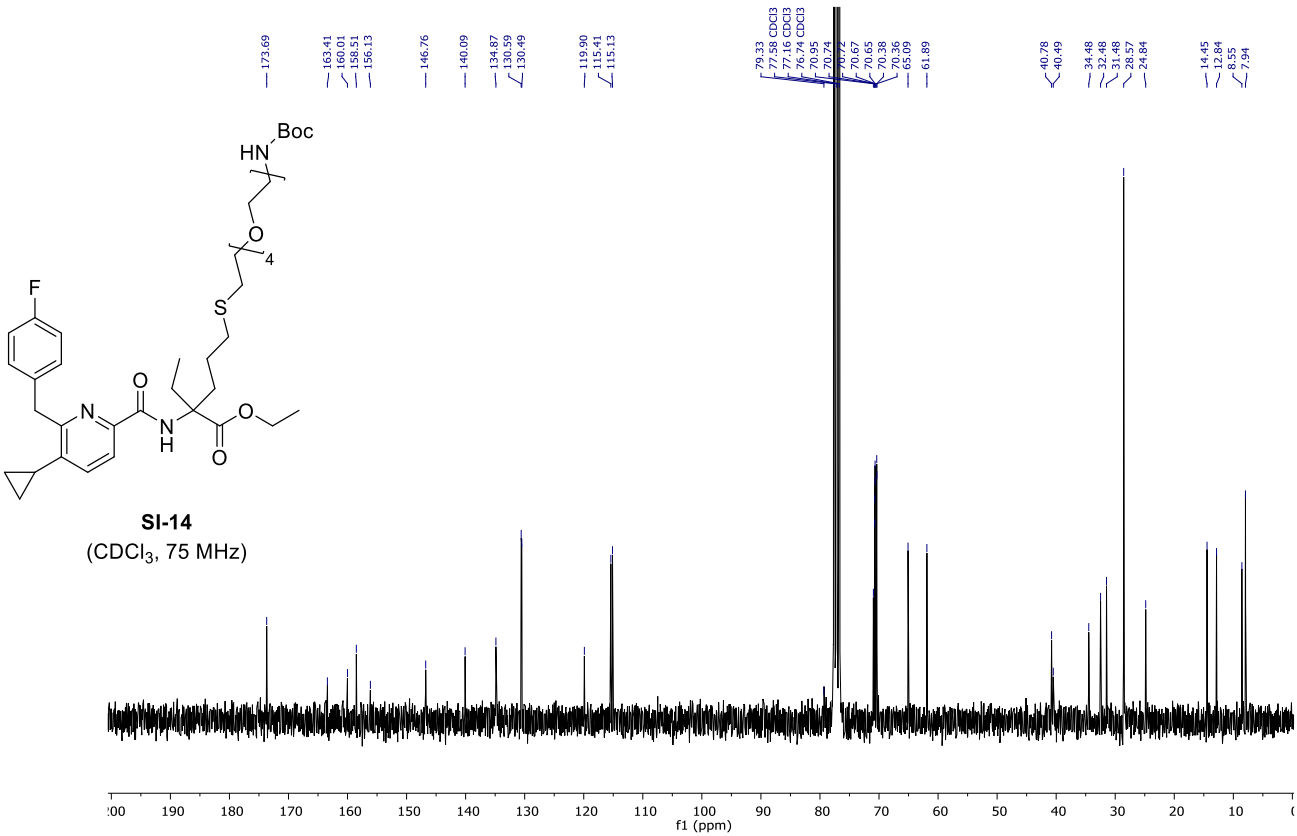


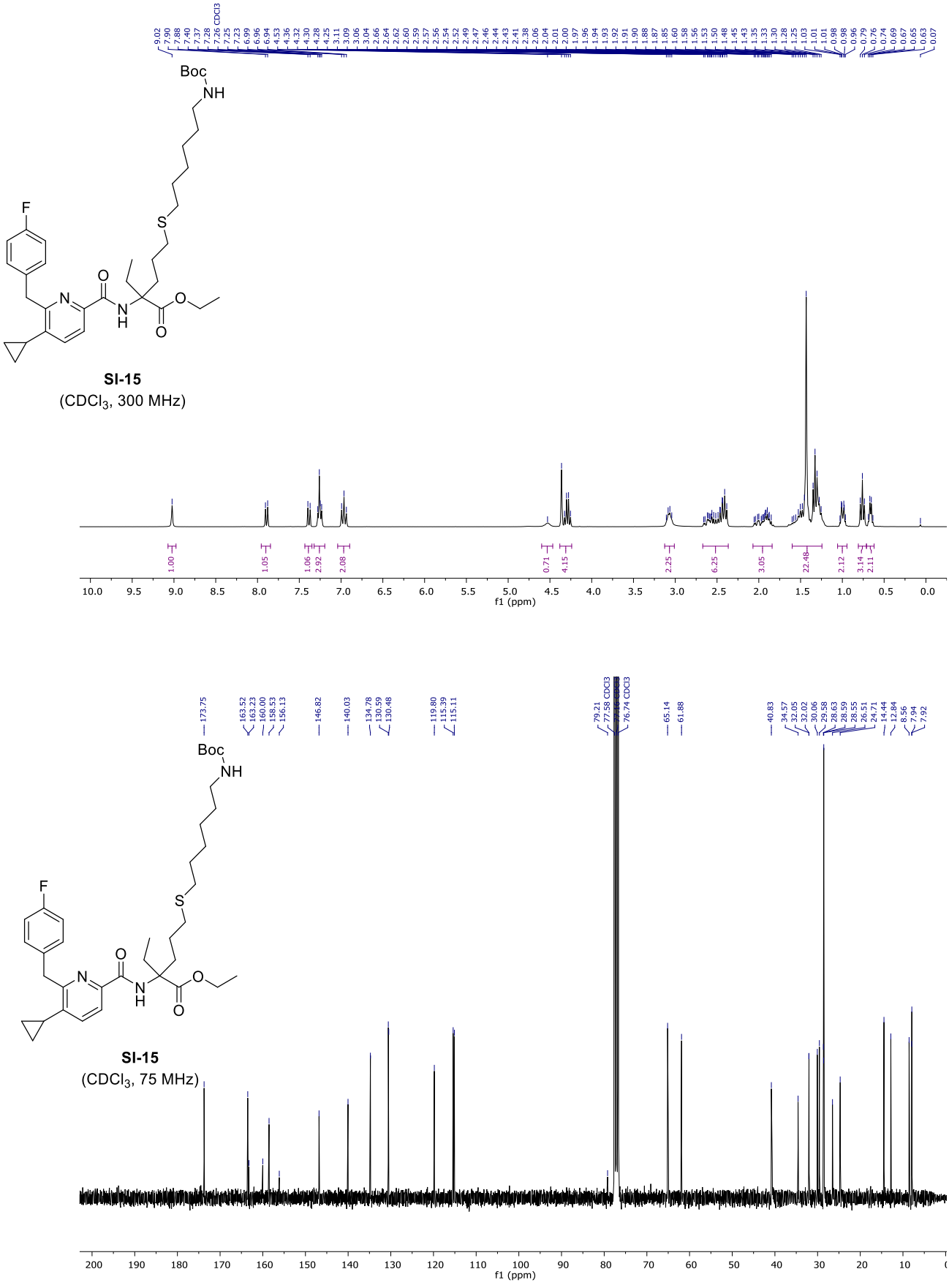
SI-13
($CDCl_3$, 300 MHz)

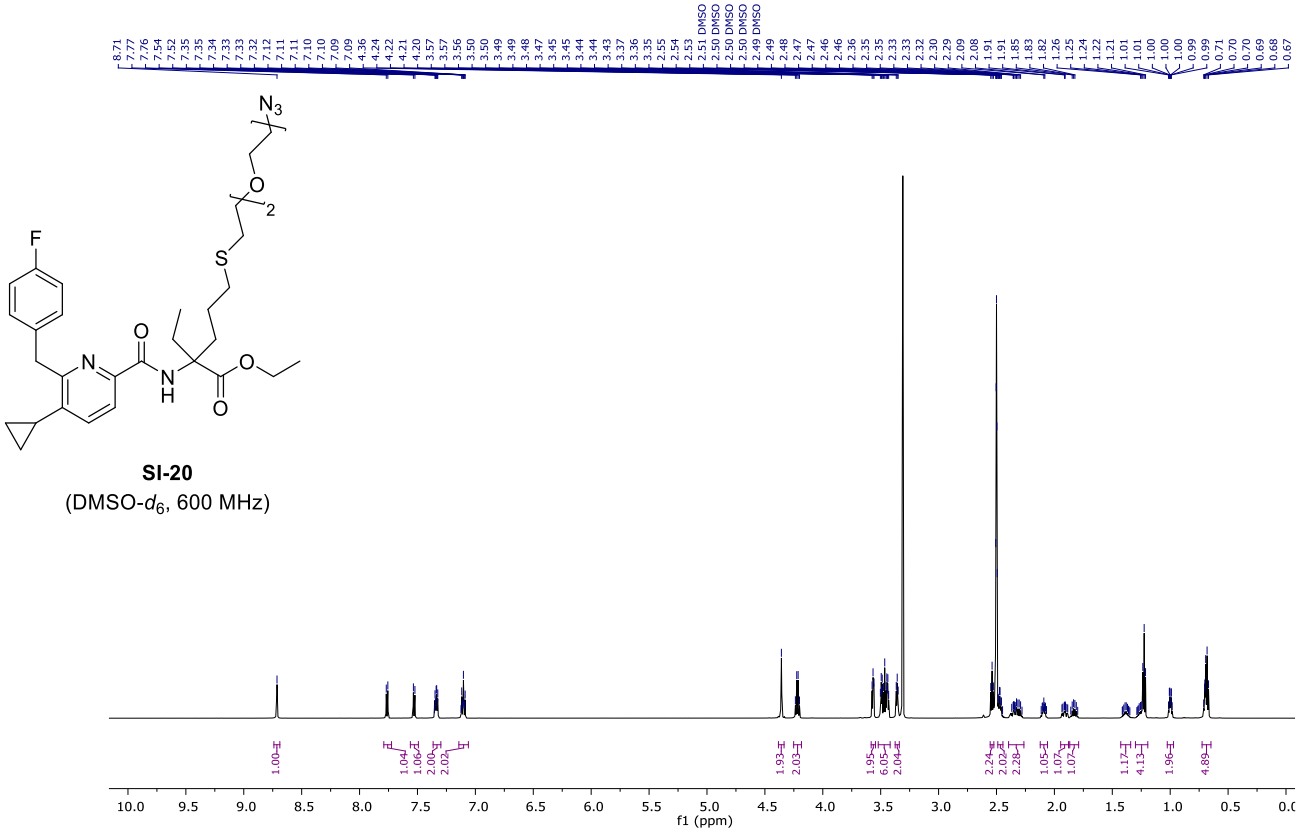


SI-13
($CDCl_3$, 75 MHz)

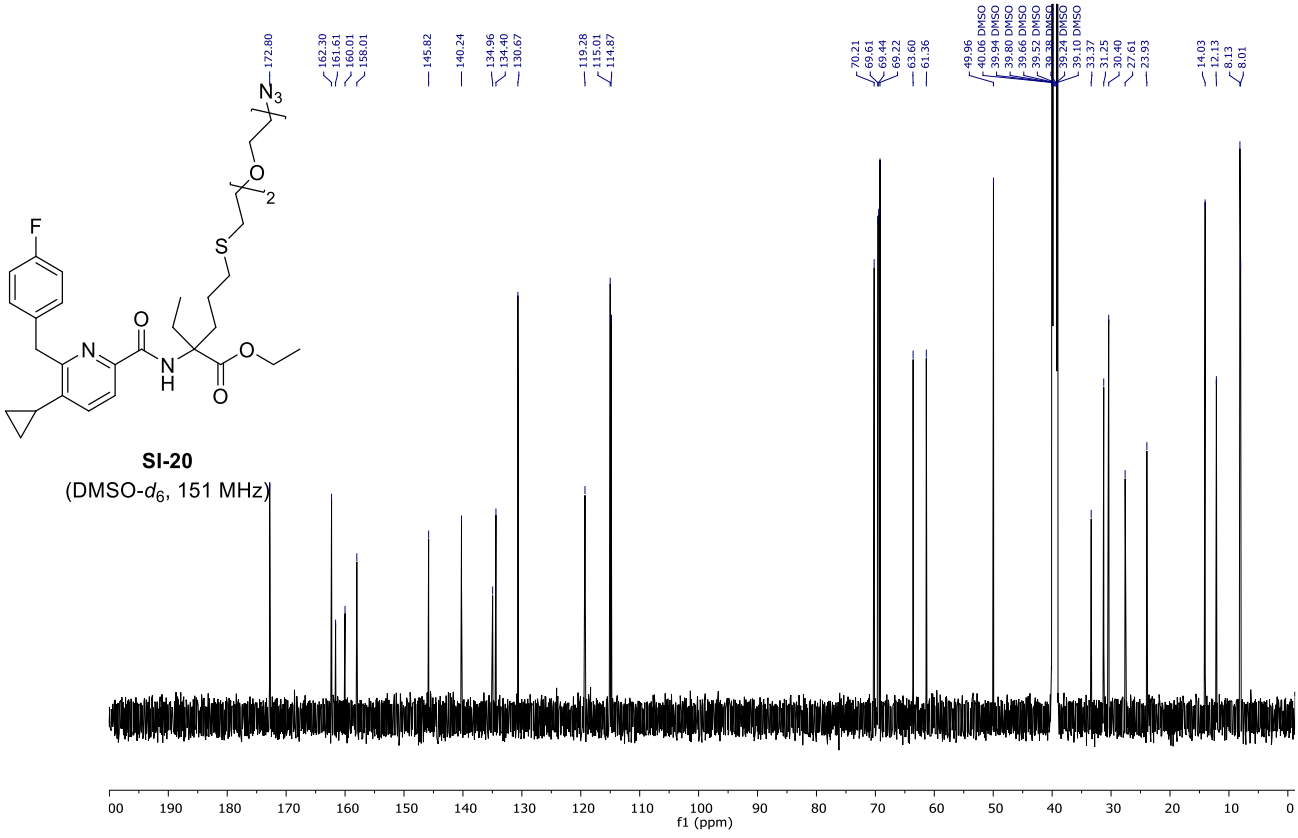

Boc
HN
O
4
S
F
O
N
N
H
O
O
O
SI-14
(CDCl3, 300 MHz)
f1 (ppm)


Boc
HN
O
4
S
F
O
N
N
H
O
O
O
SI-14
(CDCl3, 75 MHz)
f1 (ppm)

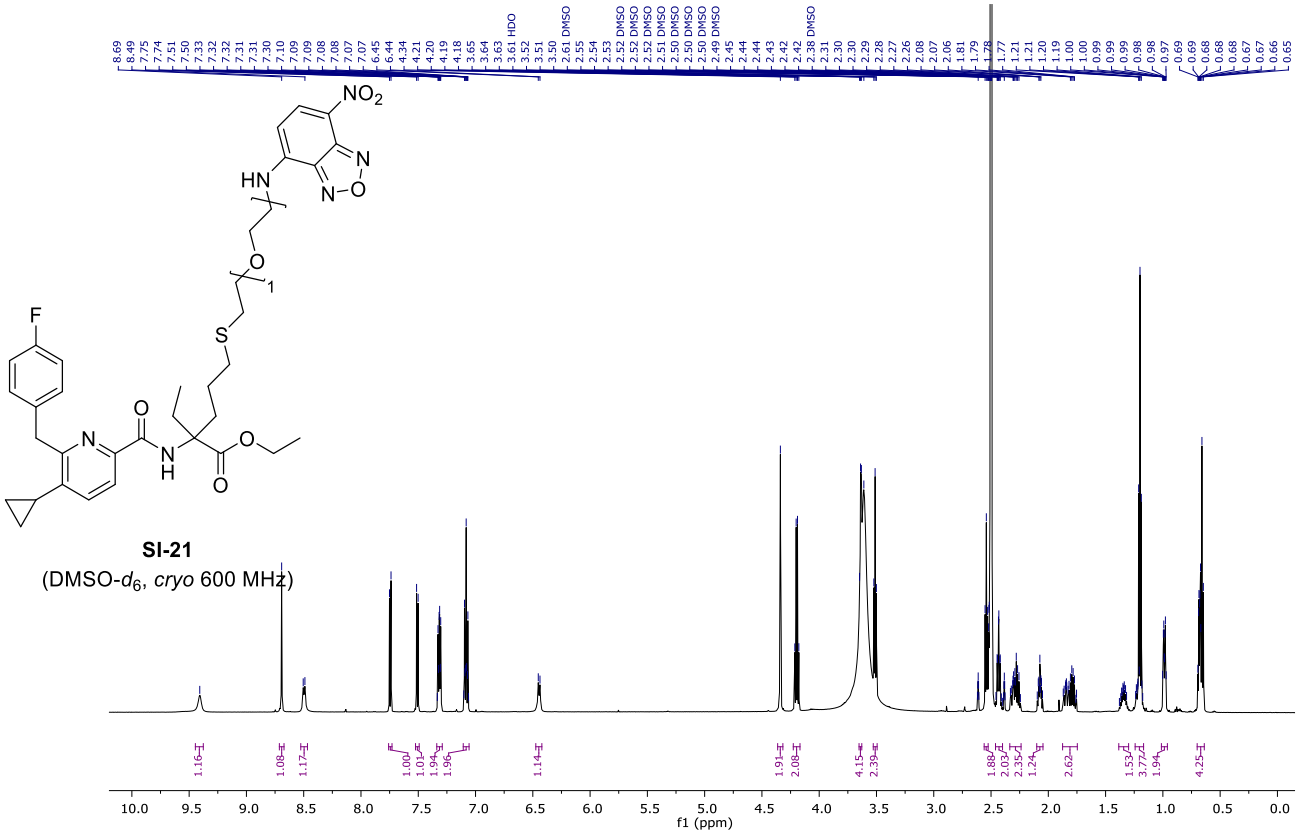

Boc
NH
F
S
O
N
N
H
O
O
O
SI-15
(CDCl3, 300 MHz)
f1 (ppm)
SI-15
(CDCl3, 75 MHz)
f1 (ppm)

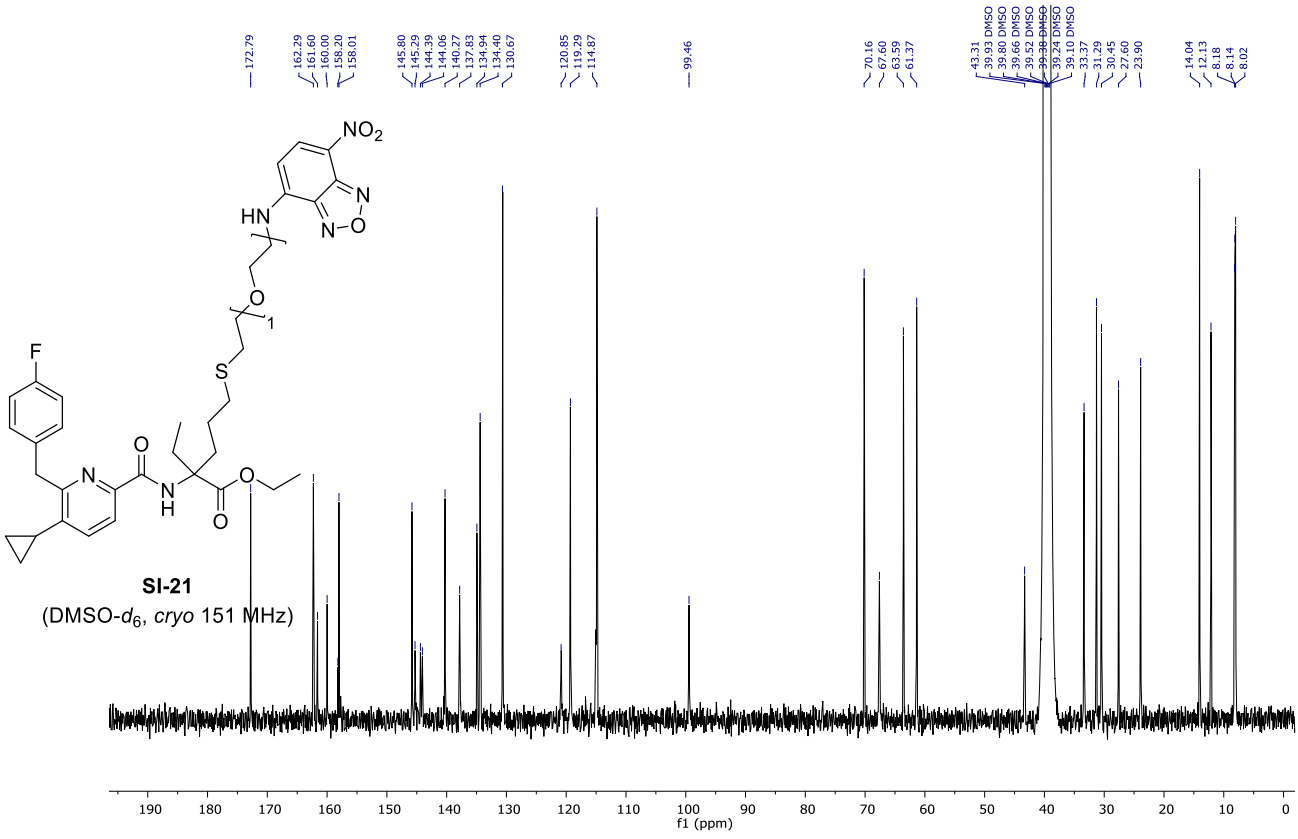


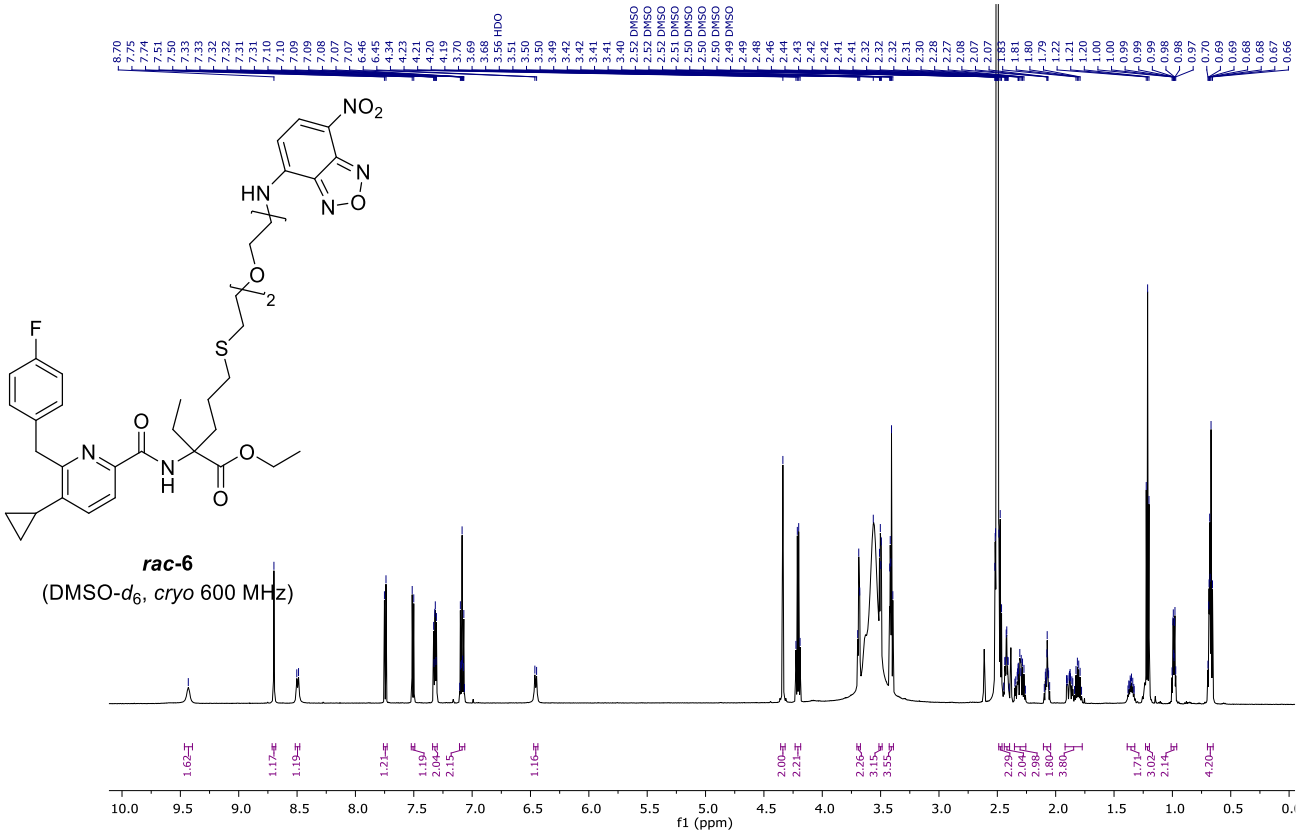
SI-20
(DMSO-d_6, 600 MHz)

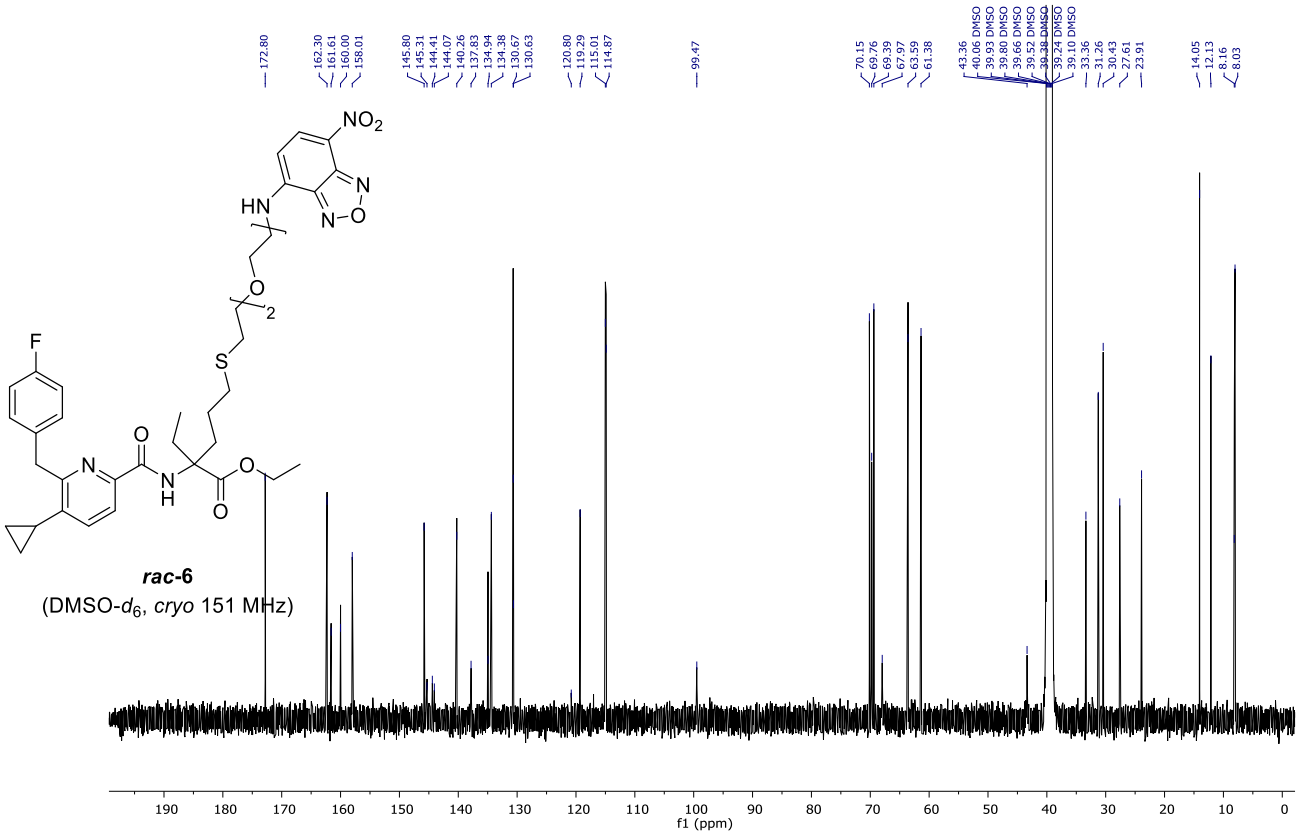


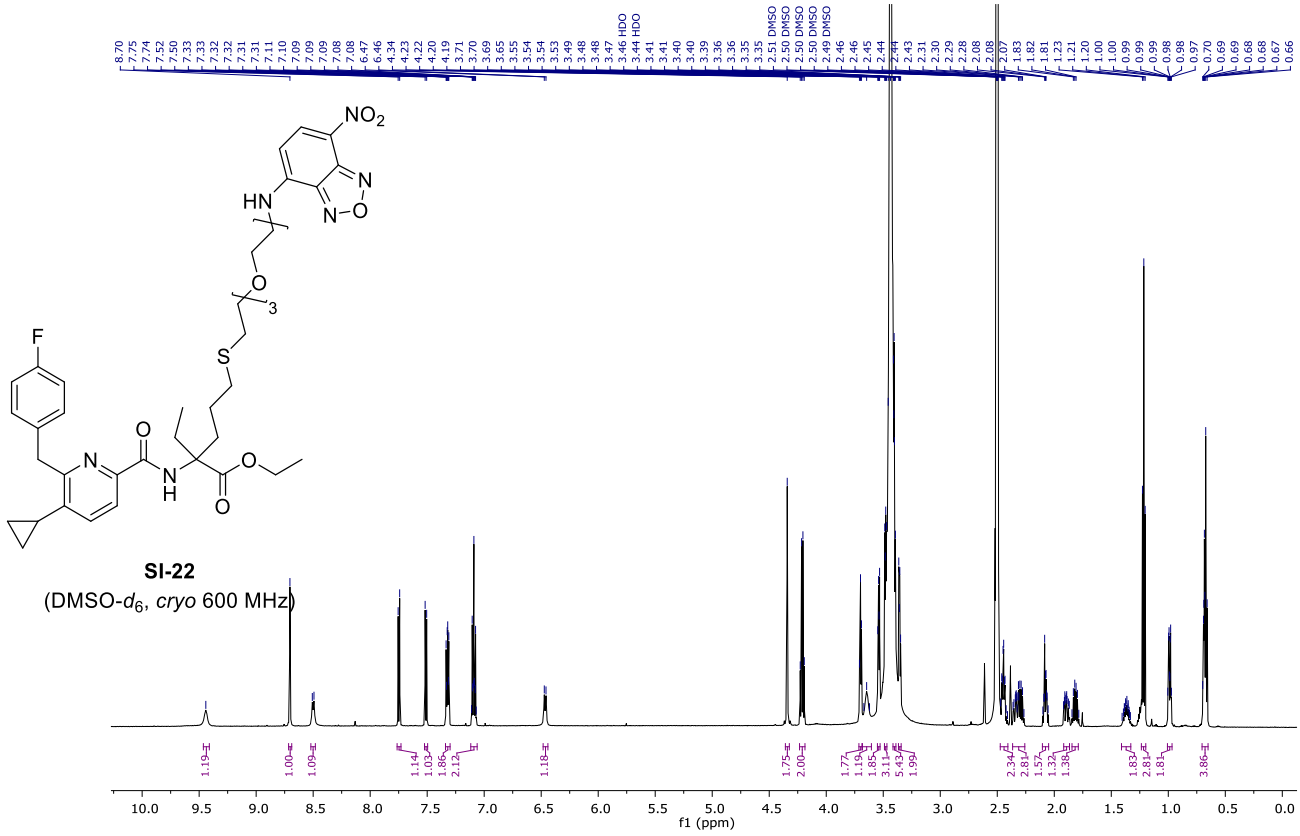
SI-20
(DMSO-d_6, 151 MHz)

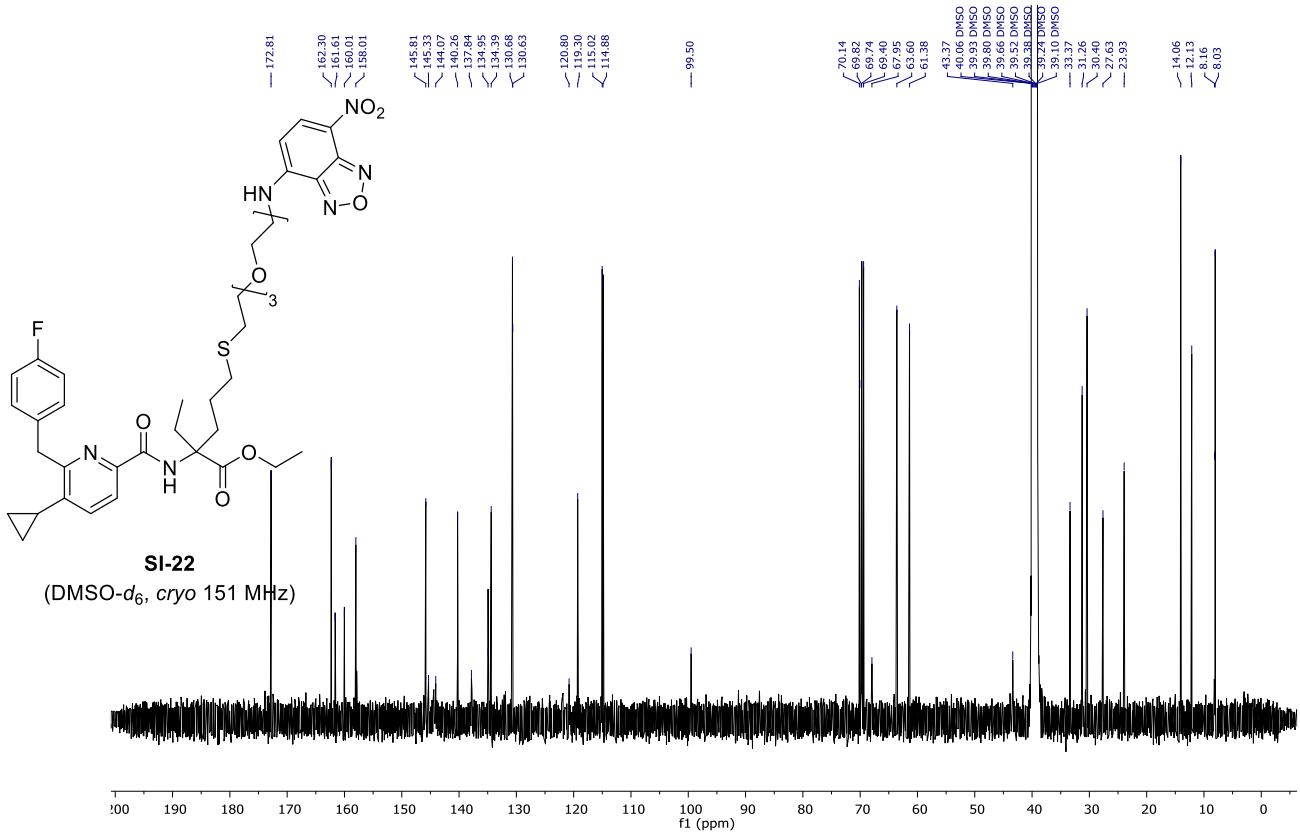

SI-21
(DMSO-d_6, *cryo* 600 MHz)
f1 (ppm)

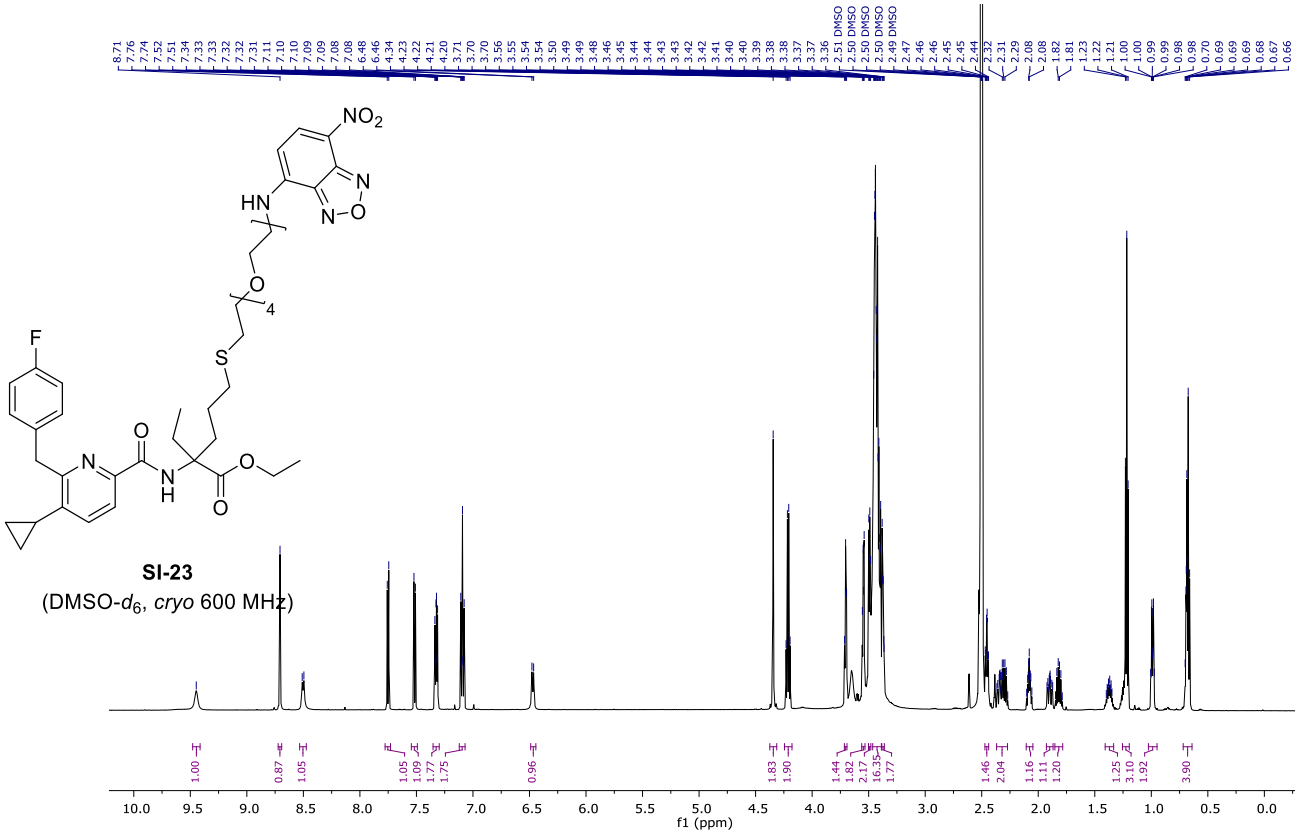

SI-21
(DMSO-d_6, *cryo* 151 MHz)
f1 (ppm)

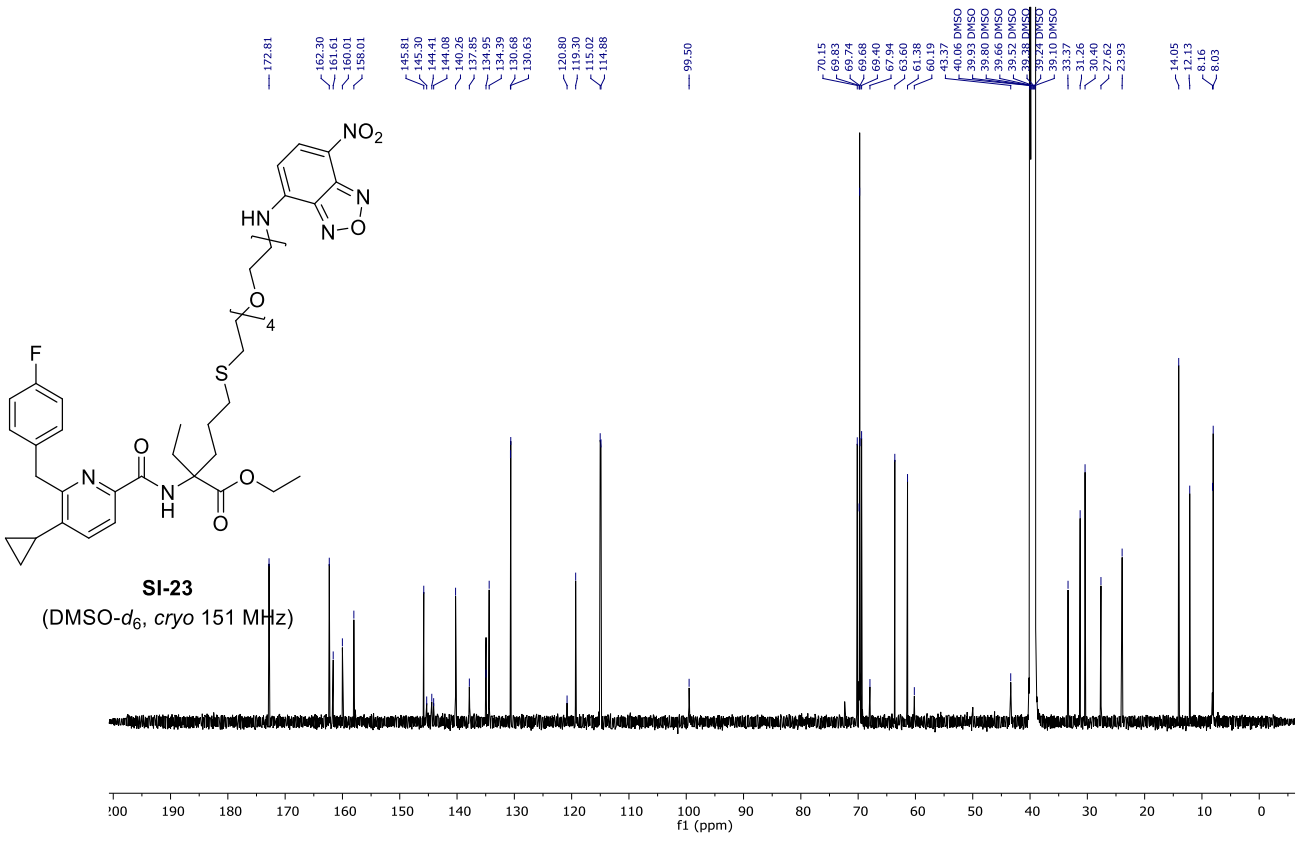

NO2
HN
N
N–O
O
2
S
F
O
N
N
H
O
O
rac-6
(DMSO-d6, cryo 600 MHz)
f1 (ppm)

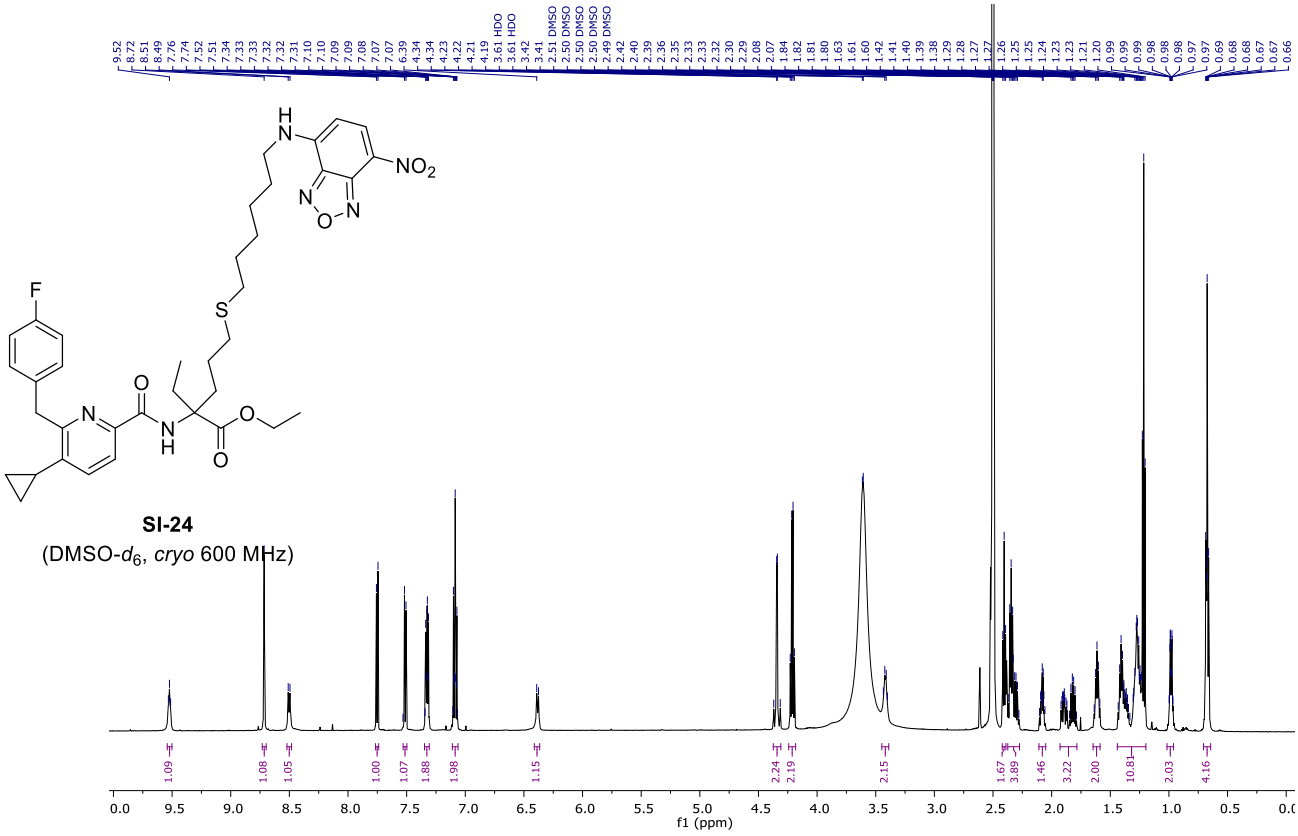

NO2
HN
N
N–O
O
2
S
F
O
N
N
H
O
O
rac-6
(DMSO-d6, cryo 151 MHz)
f1 (ppm)

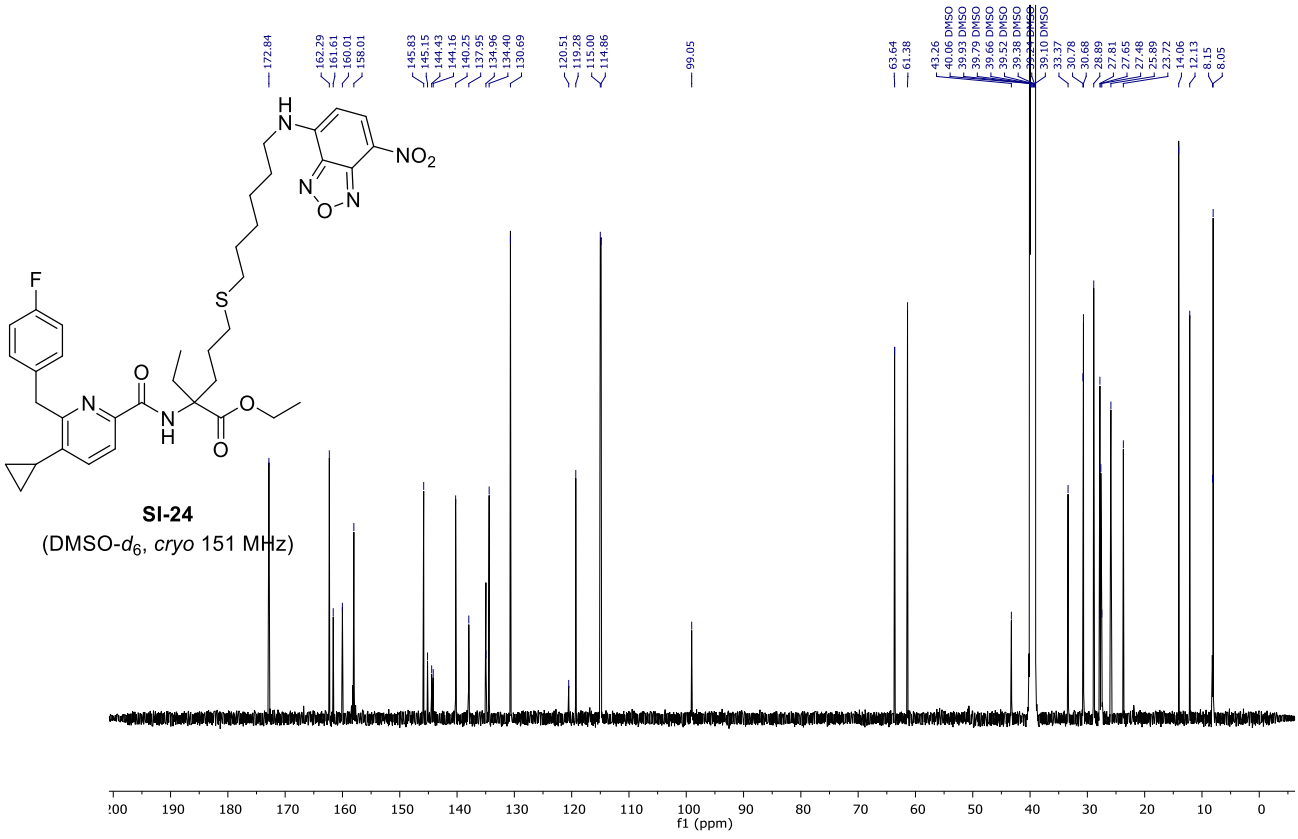

NO2
HN
N
N–O
O
3
S
F
O
N
N
H
O
O
SI-22
(DMSO-d6, cryo 600 MHz)
f1 (ppm)

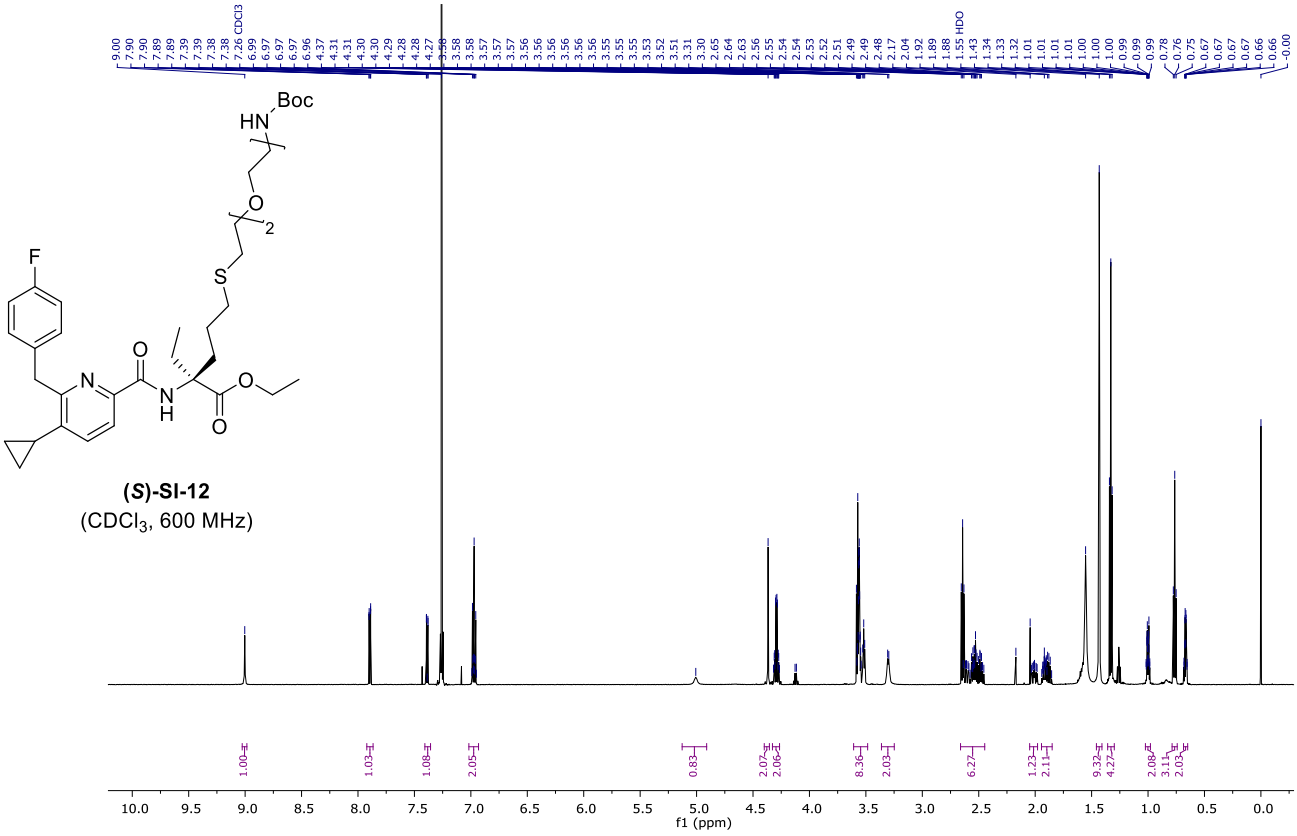

NO2
HN
N
N–O
O
3
S
F
O
N
N
H
O
O
SI-22
(DMSO-d6, cryo 151 MHz)
f1 (ppm)


SI-23
(DMSO-d_6, *cryo* 600 MHz)
f1 (ppm)

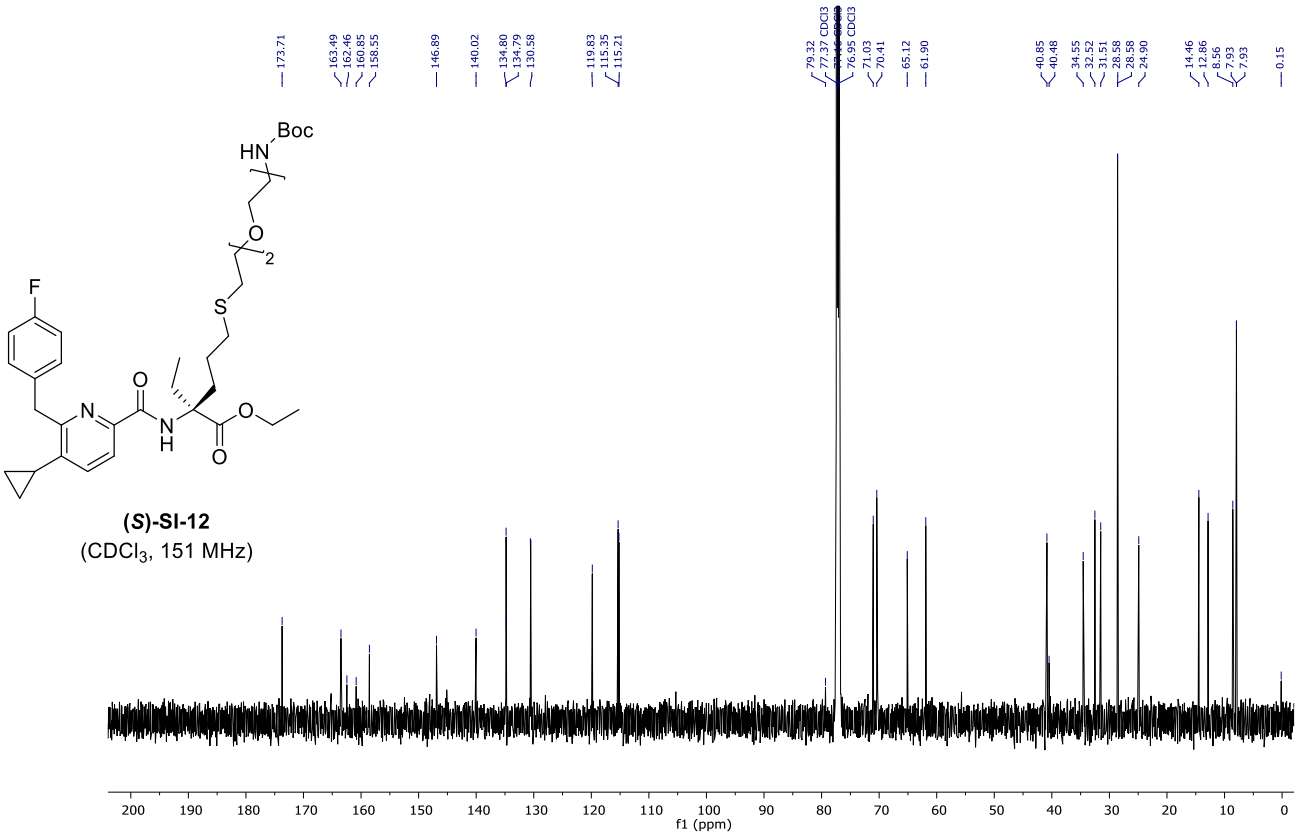

SI-23
(DMSO-d_6, *cryo* 151 MHz)
f1 (ppm)


SI-24
(DMSO-d_6, *cryo* 600 MHz)
f1 (ppm)

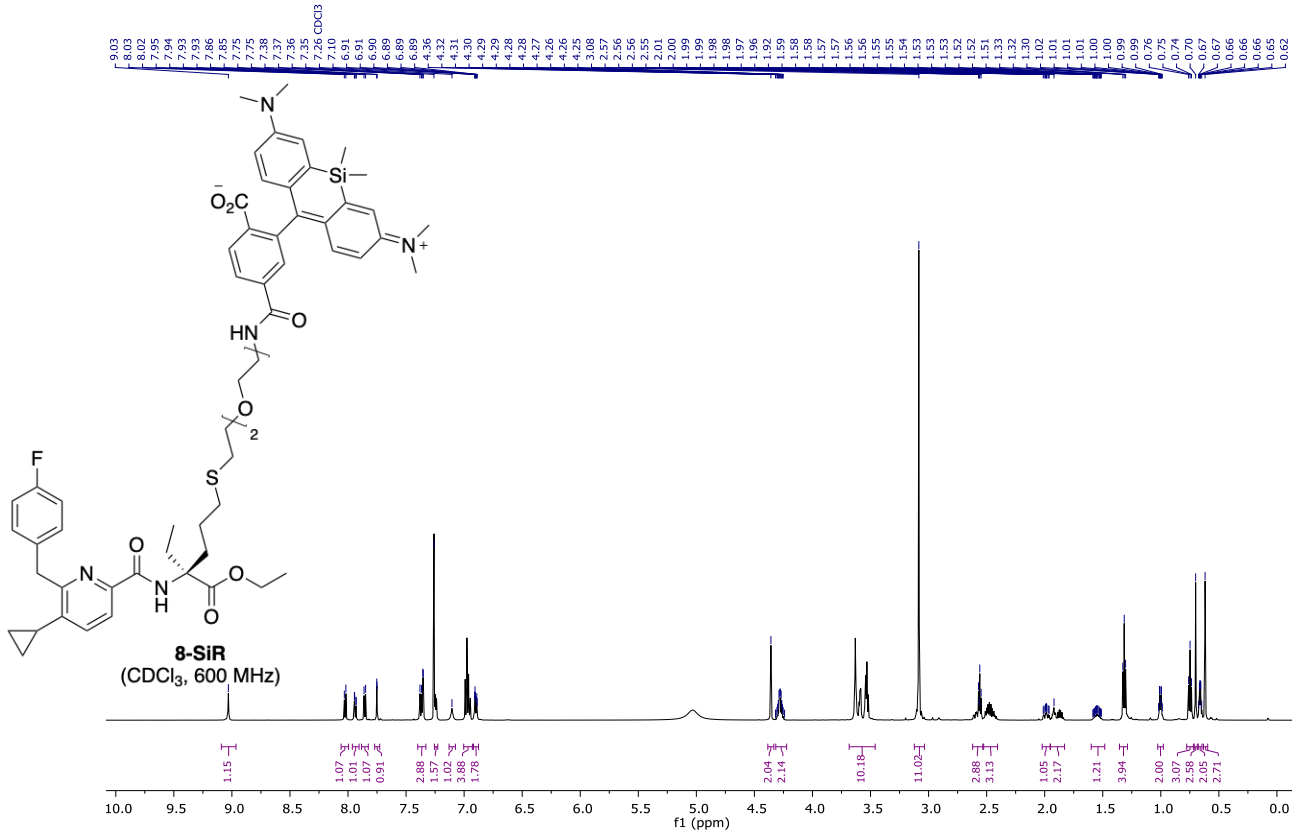

SI-24
(DMSO-d_6, *cryo* 151 MHz)
f1 (ppm)

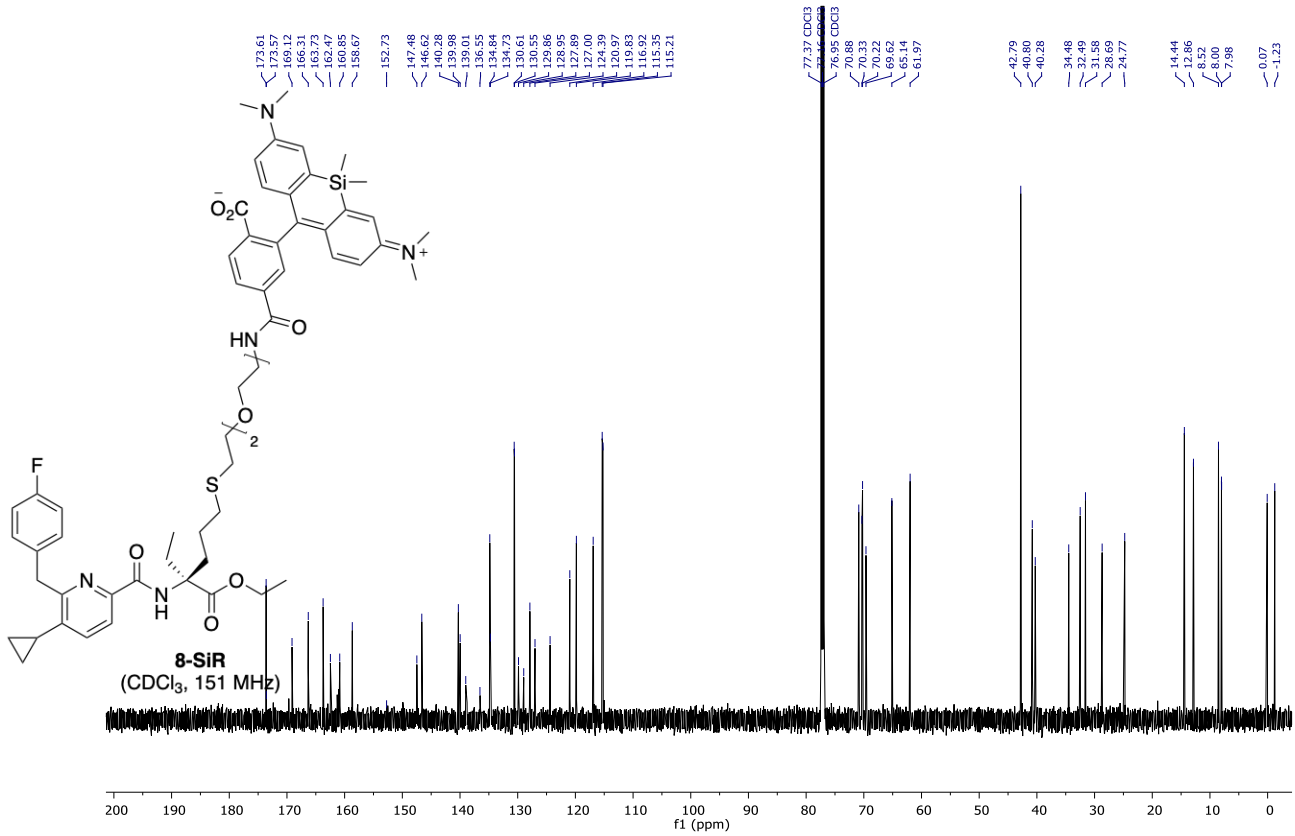


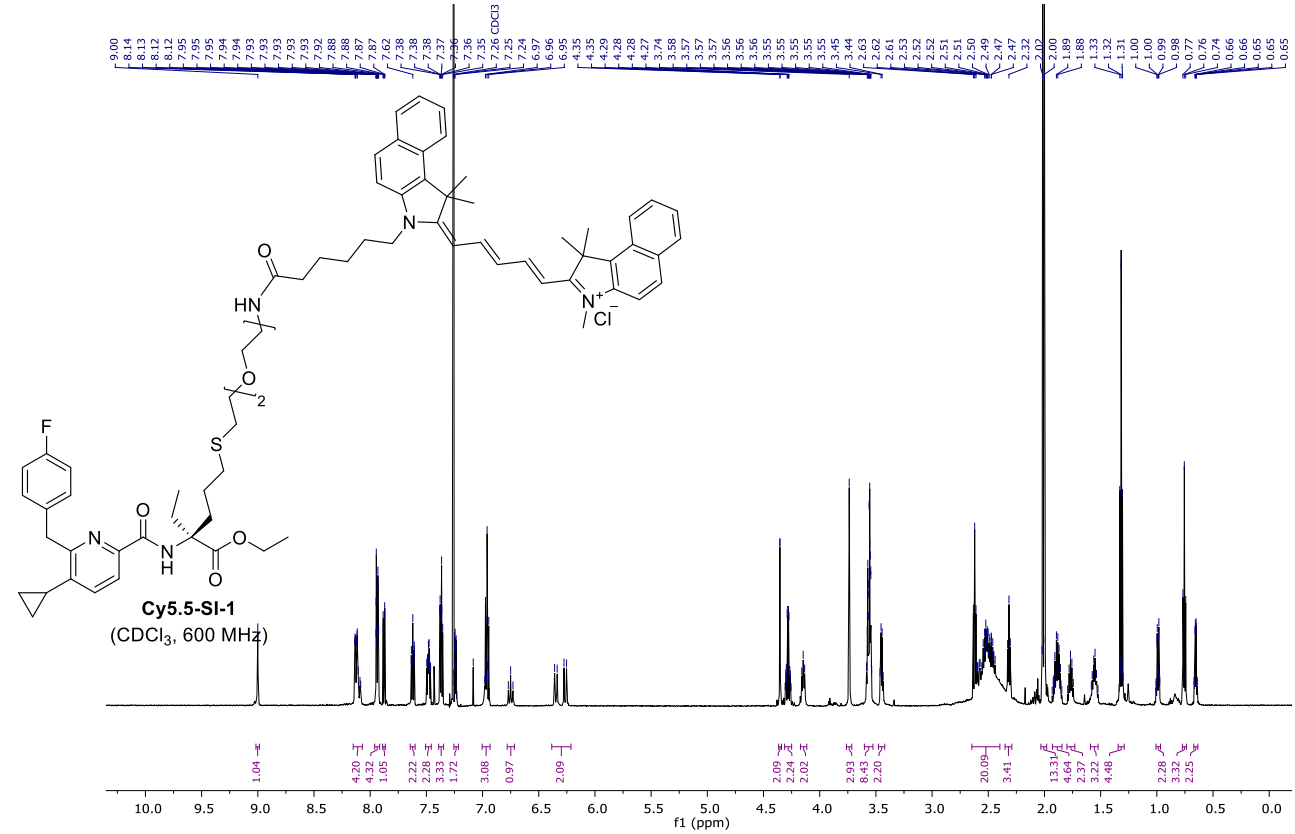
(*S*)-SI-12
($CDCl_3$, 600 MHz)

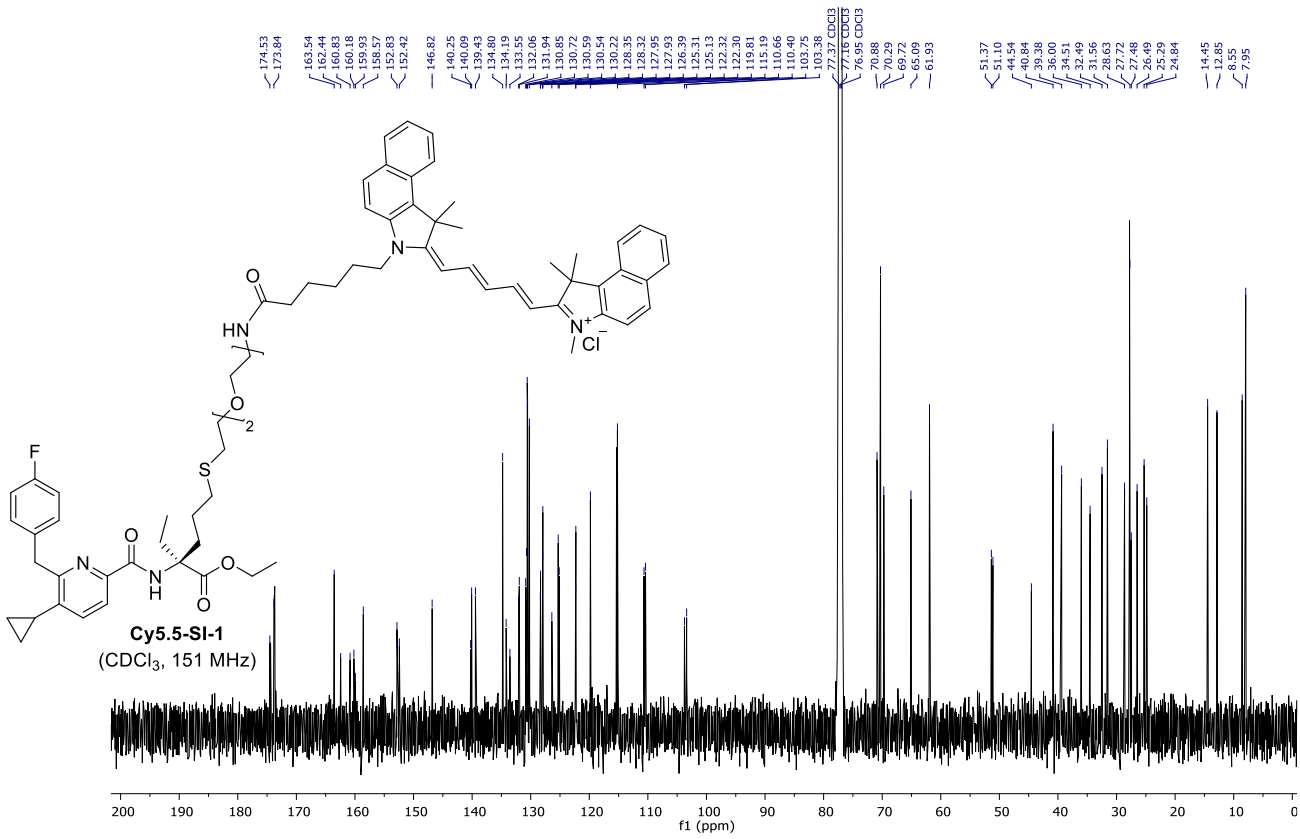


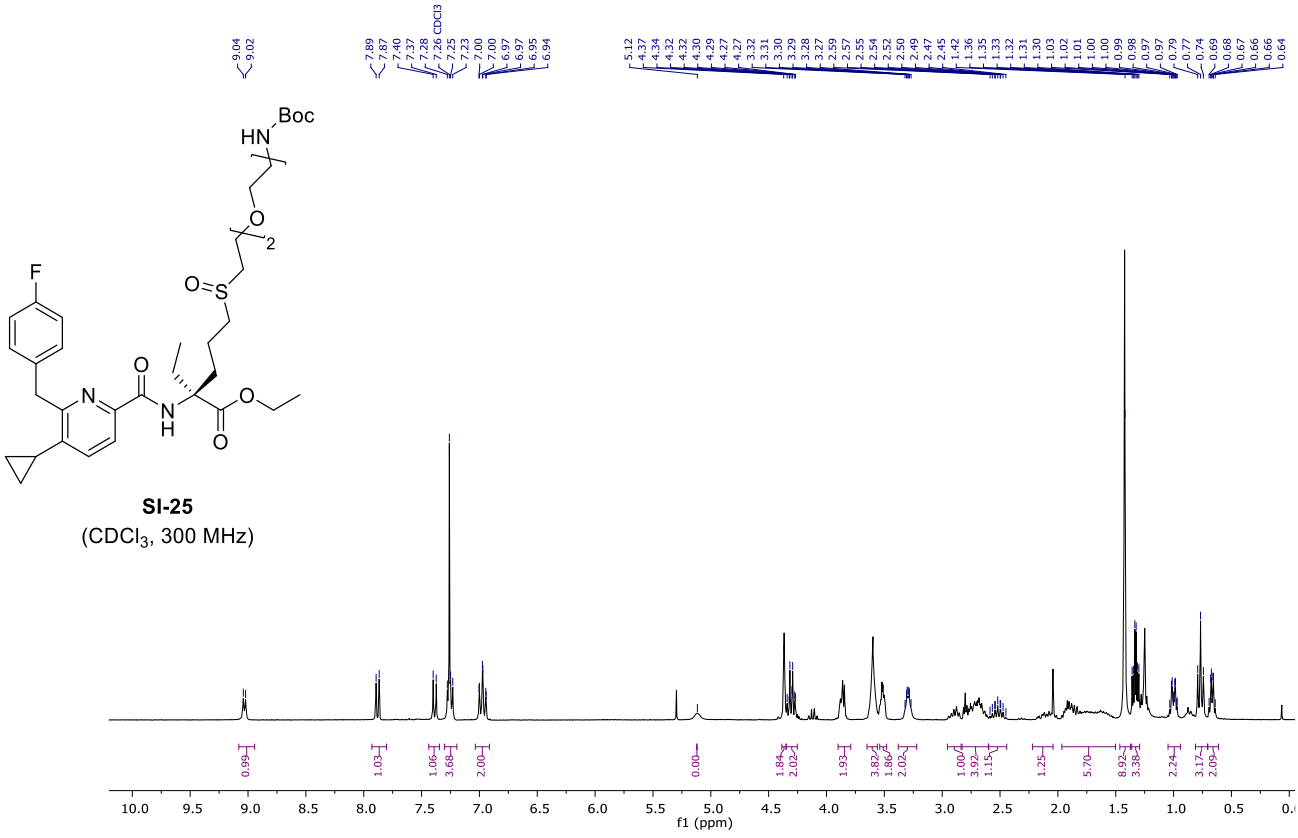
(*S*)-SI-12
($CDCl_3$, 151 MHz)


8-SiR
(CDCl3, 600 MHz)
f1 (ppm)

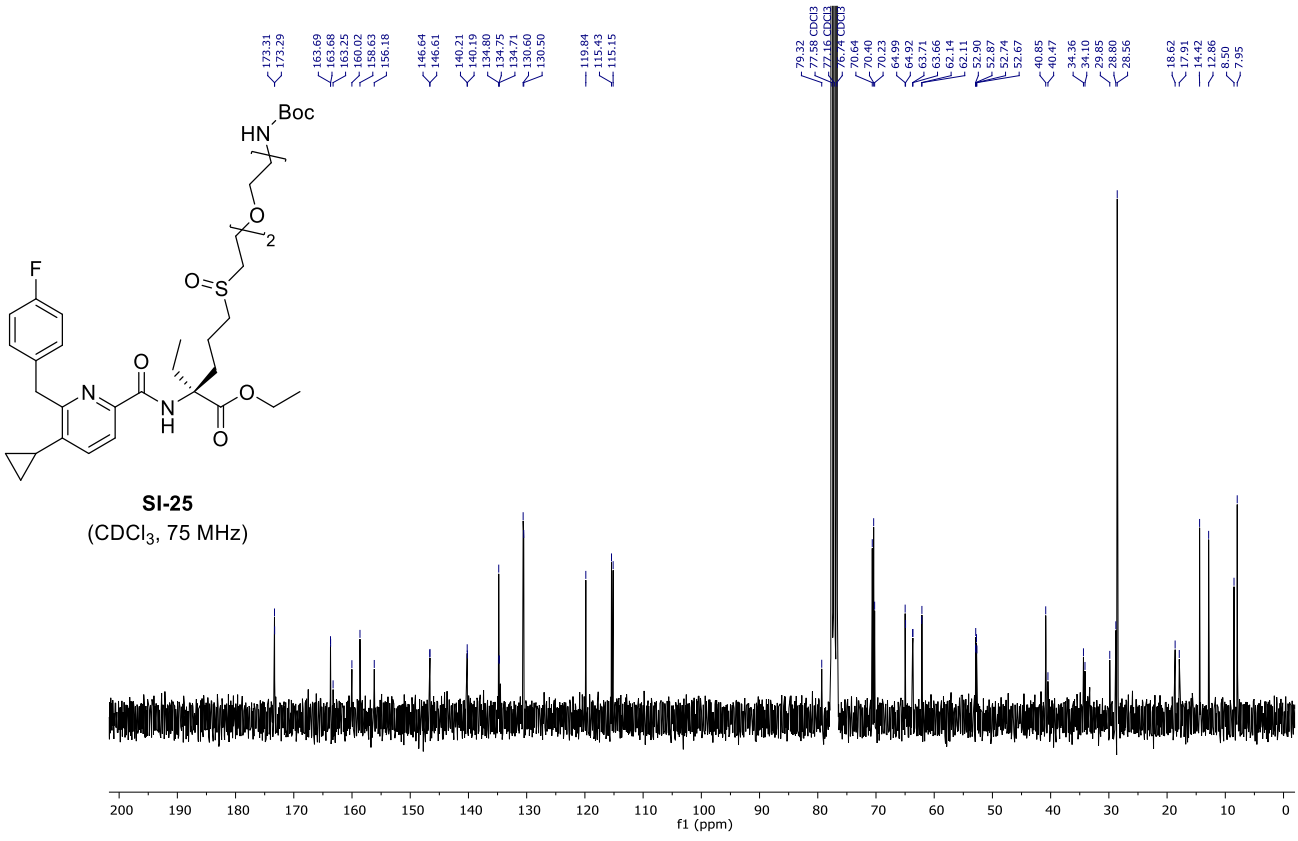

8-SiR
(CDCl3, 151 MHz)
f1 (ppm)


Cy5.5-SI-1
(CDCl3, 600 MHz)
f1 (ppm)

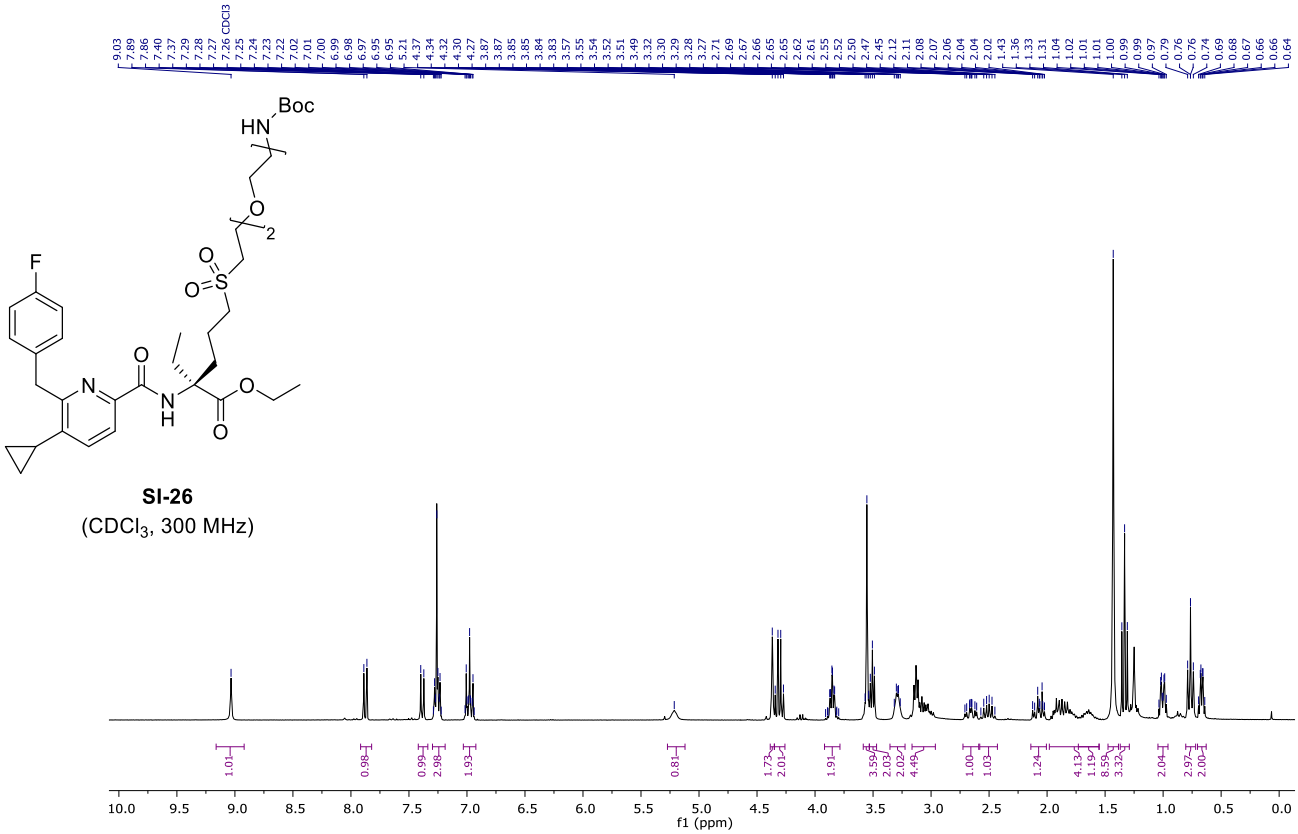

Cy5.5-SI-1
(CDCl3, 151 MHz)
f1 (ppm)

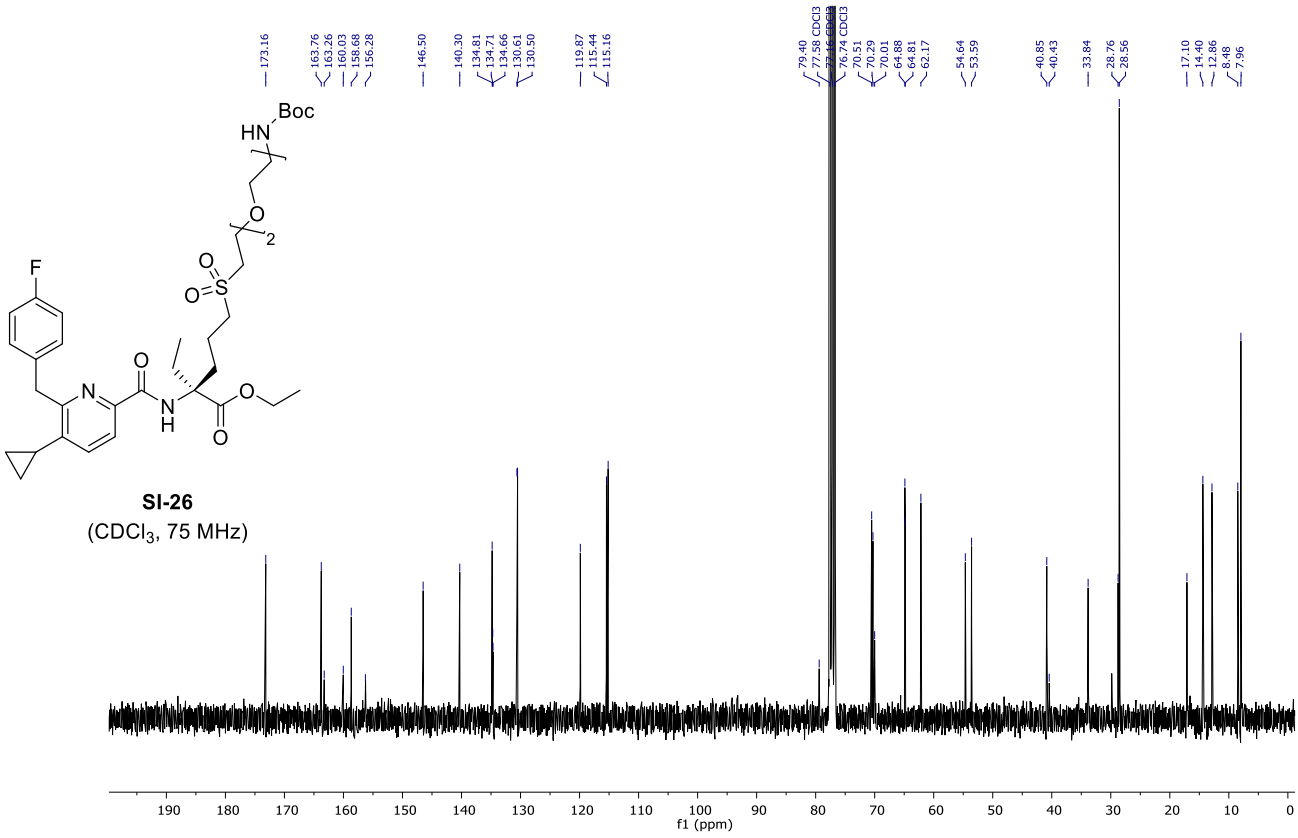


SI-25
($CDCl_3$, 300 MHz)



SI-25
($CDCl_3$, 75 MHz)


Boc
HN
O
2
O
O
S
F
N
O
N
H
O
O
O
SI-26
(CDCl3, 300 MHz)
f1 (ppm)


Boc
HN
O
2
O
O
S
F
N
O
N
H
O
O
O
SI-26
(CDCl3, 75 MHz)
f1 (ppm)