Supplementary Information (SI) for Chemical Science.
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Supporting Information

Visualization of membrane localization and functional state of CB_2R pools by matched agonist and inverse agonist probe pairs

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Abstract

The diversity of physiological roles of the endocannabinoid system has turned it into an attractive yet elusive therapeutic target. However, chemical probes with various functionalities could pave the way for a better understanding of the endocannabinoid system at the cellular level. Notably, inverse agonists of CB_2R – a key receptor of the endocannabinoid system - lagged behind despite the evidence regarding the therapeutic potential of its antagonism. Herein, we report a matched fluorescent probe pair based on a common chemotype to address and visualize both the active and inactive states of CB_2R, selectively. Alongside with extensive cross-validation by flow cytometry and confocal microscopy, we successfully visualize the intracellular localization of CB_2R pools in live cells. The synthetic simplicity together with the high CB_2R-selectivity and specificity of our probes, turn them into valuable tools in chemical biology and drug development that can benefit the clinical translatability of CB_2R-based drug.

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Tert-butyl (1-(5-cyclopropyl-6-(4-fluorobenzyl)pyridin-2-yl)-3,3-diethyl-1,4-dioxo-8,11-dioxa-2,5-diazatridecan-13-yl)carbamate (10b). 31

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Molecular docking

The previously reported cryo-EM structure for CB_2R complexed with the CB_2R agonist, AM12033 (PDB: 6KPF)[1] was used as a template to dock CB_2R agonists. As template for the inactive state of CB_2R the PDB structure 5ZTY[2] was used. Docking experiments were performed interactively using MOE software (Chemical Computing Group) with default settings (Molecular Operating Environment (MOE), 2022.02; Chemical Computing Group ULC, 1010 Sherbrooke St. West, Suite #910, Montreal, QC, Canada, H3A 2R7, 2022.). The most reasonable docking pose with respect to molecular interactions and internal conformational strain was energy-minimized within the binding pocket. Adjacent amino acid side chains were energy-minimized without restraints. The resulting docking pose was checked for consistency with the available structure-activity relationship (SAR) information. Visualization was performed using the PyMOL Molecular Graphics System, v.2.0 (Schrödinger, LLC).

Selectivity panel

Supplementary table S- 1. In vitro pharmacology data of TAMRA-probes **12** and **16** for a representative set of common off-targets.

Assay name[a]	Readout	Value (inhibition%)	
		12	**16**
α_{2A} (h) (antagonist radioligand)	Specific binding	11	-4
β_1 (h) (agonist radioligand)	Specific binding	6	-16
β_2 (h) (antagonist radioligand)	Specific binding	-5	-9
AT1 (h) (antagonist radioligand)	Specific binding	4	-2
BZD (central) (agonist radioligand)	Specific binding	-12	-8
CCK1 (CCKA) (h) (agonist radioligand)	Specific binding	1	-17
D_1 (h) (antagonist radioligand)	Specific binding	2	0
M_1 (h) (antagonist radioligand)	Specific binding	24	1
M_2 (h) (antagonist radioligand)	Specific binding	57	1
μ (MOP) (h) (agonist radioligand)	Specific binding	45	-10
PCP (antagonist radioligand)	Specific binding	-21	-5
5-HT1A (h) (agonist radioligand)	Specific binding	-4	-19
Ca^{2+} channel (L, diltiazem site) (benzothiazepines) (antagonist radioligand)	Specific binding	-1	8
xanthine oxidase/ superoxide O2- scavenging	Enzymatic activity	42	4
HIV-1 protease	Enzymatic activity	22	14
norepinephrine transporter (h) (antagonist radioligand)	Specific binding	11	-5
acetylcholinesterase (h)	Enzymatic activity	4	-13
5-HT3 (h) (antagonist radioligand)	Specific binding	-1	-18

5-HT transporter (h) (antagonist radioligand)	Specific binding	5	-10
A_1 (h) (agonist radioligand)	Specific binding	5	0
A_3 (h) (agonist radioligand)	Specific binding	69	-3
GR (h) (agonist radioligand)	Specific binding	1	-14
5-HT2A (h) (agonist radioligand)	Specific binding	-10	-3
PPARgamma (h) (agonist radioligand)	Specific binding	-3	1
ZAP70 kinase (h)	Enzymatic activity	16	16
H_1 (h) (antagonist radioligand)	Specific binding	-3	-4
AR(h) (agonist radioligand)	Specific binding	0	4
N muscle-type (h) (antagonist radioligand)	Specific binding	5	-9
H_2 (h) (antagonist radioligand)	Specific binding	-25	-3
D_{2S} (h) (agonist radioligand)	Specific binding	26	-13
H_3 (h) (agonist radioligand)	Specific binding	17	-31
5-HT2B (h) (agonist radioligand)	Specific binding	-28	-2
FP (h) (agonist radioligand)	Specific binding	15	8
α_{1A} (h) (antagonist radioligand)	Specific binding	16	1
GSK3alpha (h)	Enzymatic activity	-25	7
GSK3beta (h)	Enzymatic activity	-21	9
CDK2 (h) (cycA)	Enzymatic activity	-16	-4
N neuronal alpha4beta2 (h) (agonist radioligand)	Specific binding	11	2
Abl kinase (h)	Enzymatic activity	-114	0
ACE (h)	Enzymatic activity	-27	-37
PDE3B (h)	Enzymatic activity	0	-10

PDE4D2 (h)	Enzymatic activity	12	0
COX2(h)	Enzymatic activity	15	23
κ (h) (KOP) (agonist radioligand)	Specific binding	87	-4
CB_1 (h) (agonist radioligand)	Specific binding	22	-9
MAO-A Human monoamine oxydase A	Enzymatic activity	-5	4
glycine (strychnine-insensitive)	Specific binding	-2	0
MMP-9 (h)	Enzymatic activity	10	0
Estrogen ER alpha (h) (agonist radioligand)	Specific binding	-14	-19

[a] 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).

In vitro pharmacology

Radioligand binding assay

Cell culture and membrane preparation

CHOK1hCB$_1$_bgal and CHOK1hCB$_2$_bgal cells (DiscoverRx, Fremont, CA, USA) were cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham supplemented with 10% fetal calf serum, 1 mM glutamine, 50 µg/mL penicillin, 50 µg/mL streptomycin, 300 mg/mL hygromycin and 800 µg/mL geneticin in a humidified atmosphere at 37°C and 5% CO_2. Cells were subcultured twice a week at a ratio of 1:20 on 10-cm diameter plates by trypsinization. For membrane preparation the cells were subcultured with a ratio of 1:10 and transferred to 15 cm ø plates. The cells were collected by scraping in 5 mL phosphate-buffered saline (PBS) and centrifuged at 1,000 x g for 5 min. Pellets derived from 30 plates were combined and resuspended in 20 mL cold Tris-HCl, $MgCl_2$ buffer (50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$). The cell suspension was homogenized using an UltraTurrax homogenizer (Heidolph Instruments Schwabach, Germany). Membranes and cytosolic fractions were separated by centrifugation in a Beckman Optima LE-80K ultracentrifuge (Beckman Coulter Inc., Fullerton, CA, USA) at 100,000 x g for 20 min at 4°C. The supernatant was discarded. The pellet was resuspended in 10 mL cold Tris-HCl, $MgCl_2$ buffer and homogenization and centrifugation steps were repeated. The membranes were resuspended in 10 mL cold Tris-HCl, $MgCl_2$ buffer. Aliquots of 100 µL were stored at -80°C until further use. The protein concentration was determined using the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA, USA).

[^{3}H]CP55940 Displacement assay

[^{3}H]CP55940 displacement assays on 96-well plates were performed in 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.1% BSA assay buffer. Membrane aliquots of either CHOK1hCB$_1$_bgal or CHOK1CB$_2$_bgal containing 1 or 2.5 µg membrane protein, respectively, were incubated at 25°C for 2 h in the presence of ~1.5 nM [^{3}H]CP55940 (specific activity 106.5 Ci/mmol; PerkinElmer, Waltham, MA). At first, all compounds were tested at a final concentration of 10 µM compound. When radioligand displacement was greater than 50%, full curves were recorded to determine

the affinity (pK_i) values of the compounds. To determine the total binding, a control without test compound was included. Nonspecific binding was determined in the presence of 10 µM Rimonabant (CHOK1hCB$_1$_bgal) or AM630 (CHOK1hCB$_2$_bgal). The total assay volume was 100 µL. The final concentration of DMSO was 0.25%. The incubation was terminated by rapid vacuum filtration through GF/C 96-well filter plates, to separate the bound and free radioligand, using a PerkinElmer Filtermate-harvester (Revvity, Waltham, Ma, USA). Filters were subsequently washed twenty times with ice-cold assay buffer. The filter-bound radioactivity was determined by scintillation spectrometry using a Microbeta2® 2450 microplate counter (Revvity, Waltham, Ma, USA), after addition of 25 µL MicroScint-O (Revvity, Waltham, Ma, USA) and 3h incubation.

Data Analysis

All experimental data were analyzed using GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA). The data were normalized to % specific radioligand binding, where total binding is 100% and nonspecific binding is 0%. Nonlinear regression for one-site was used to determine the IC_{50} values from the full curve [^{3}H]CP55940 displacement assays. The pK_i values were obtained using the Cheng-Prusoff equation[4]: $K_i = \frac{IC_{50}}{1 + \frac{[L]}{K_D}}$, where [L] is the exact concentration [^{3}H]CP55940 determined per experiment and the K_D is the dissociation constant of [^{3}H]CP55940, which is 0.84 and 0.48 nM for CB$_1$R and CB$_2$R, respectively (data not shown). All data were obtained from at least three separate experiments performed in duplicate.

Supplementary table S-2. Binding affinities of probes **12-18**

Cmpd	**Dye**	**pK_i ± SEM (K_i, nM)^a or displacement at 10 µM (%)± SEM ^{b}**		**Selectivity**
		hCB$_1$R	**hCB$_2$R**	
12	TAMRA	24 ± 6	6.91 ± 0.08 (124)	>80
13	SiR	6.34 ± 0.22 (462)	6.17 ± 0.16 (684)	0.7
14	Alexa488	36 ± 6	6.85 ± 0.12 (141)	>70
15	Alexa647	5 ± 2	6.07 ± 0.05 (852)	>11
16	TAMRA	7 ± 7	6.39 ± 0.06 (409)	>24
17	SiR	21 ± 4	5.79 ± 0.02 (1622)	>6
18	Alexa488	6 ± 7	17 ± 6	n.a.

[a] pK_i and K_i (nM) values obtained from [^{3}H]CP55,940 displacement assays on CHO membranes stably expressing hCB_1R or hCB_2R. [b] Percentage of [^{3}H]CP55,940 displacement by 10 µM compound. Values are means ± standard error of the mean (SEM) of at least three independent experiments performed in duplicate. [c] Selectivity was determined by calculating the ratio of K_i (CB_1R)/K_i (CB_2R). n.a. is not applicable.

The Homogeneous Time Resolved Fluorescence (HTRF) assay

The HTRF cAMP assay was conducted using the cAMP-Gs Dynamic kit (62AM4PEC, Cisbio). Briefly, the CHO cell lines that stably overexpressed the CB_2 receptor were cultured in Ham's F12 medium supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/Ml streptomycin, and 400 µg/mL of the selective antibiotic G418. To assess the probes, the cells were harvested using Gibco™ versene solution and resuspended in F-12 medium at a concentration of 4.0×10^5 cells/mL. Subsequently, the cells were dispensed into 384-well low volume plates at a volume of 5 µL per well. The cells were then stimulated with compounds that had been diluted in Stimulation Buffer (2.5 µL per well). After 15 min incubation at room temperature, 2.5 µL of 25µM forskolin was added to each well, followed by another 15 min incubation. To halt the reactions, 5 µL of cAMP-d2 conjugate working solution was added to each well, followed by 5 µL of anti-cAMP cryptate working solution. The plates were then incubated for 1 h at room temperature. Following incubation, the plates were analyzed using a PerkinElmer Envision reader to measure the fluorescence ratio at 620 nm and 665 nm.

<u>Data analysis</u>

The HTRF ratio was plotted against compound concentration using Prism 8.1 software (GraphPad). The HTRF ratio was calculated as follows:

HTRF Ratio = (Signal at 665 nm / Signal at 620 nm) × 10^4

All HTRF ratio data sets of test compounds were normalized to the E_{max} of APD371 (100%) and obtained the means ± standard error of the mean (SEM) of four independent experiments performed in technical replicates.

Absorption-emission maxima and quantum yield

Absorption-emission maxima of the fluorescent probes were determined using a Tecan Safire II UV-Vis fluorescence and absorbance plate reader. All measurements were performed at room temperature. For this purpose, 50 µL of 10 µM solution of probes **12-18** in PBS (pH=7.4) in presence of 0.1 % (v/v) DMSO were placed in a Corning™ 384-well Polystyrene Microplates and the UV/Vis absorbance spectra were first recorded in wavelength range of 300-800 nm (scan step 5 nm) to determine the wavelength with the maximal absorbance signal which later used for excitation of the corresponding compound to measure the fluorescent emission signal. The absolute quantum yield was determined using a HAMAMATSU PHOTONICS K.K Absolute PL Quantum Yield Spectrometer with Xenon lamp bulb L11562. For this purpose, 3 mL of 100 nM solution of probes **12-18** in PBS (pH=7.4) in presence of 0.1 % (v/v) DMSO were placed into a quartz cuvette with a rod (Size: 12.5*12.5*140 mm) and after excitation, the quantum yields was recorded with the suppliers software version 4.6.0 CD-ROM and reported as percentage.

Supplementary table S-3. Photophysical properties of probes **12-18**

Cmpd	Dye	Φ (%)[a]	$\lambda_{ex}/\lambda_{em}$ (nm)[b]
12	TAMRA	52.7	555/585
13	SiR	30.3	652/672
14	Alexa488	77.1	498/523
15	Alexa647	40.1	651/674
16	TAMRA	9	560/585
17	SiR	10.5	651/670
18	Alexa488	17.5	496/520

[a] Fluorescence quantum yield. The values are means of at least two independent experiments performed in duplicate. [b] Wavelengths at the maxima of the excitation and emission spectra.

Quantum yield of TAMRA-probe pair **12** and **16** were further measured in more lipophilic mediums. For this purpose, 10 µM solution of probes in PBS (pH=7.4) were diluted to 100 nM using a mixture of 1:1 and 1:2 PBS/dioxane. The measurements performed in duplicates.

Supplementary table S-4. Quantum yields of TAMRA-probe pair **12** and **16** in lipophilic mediums

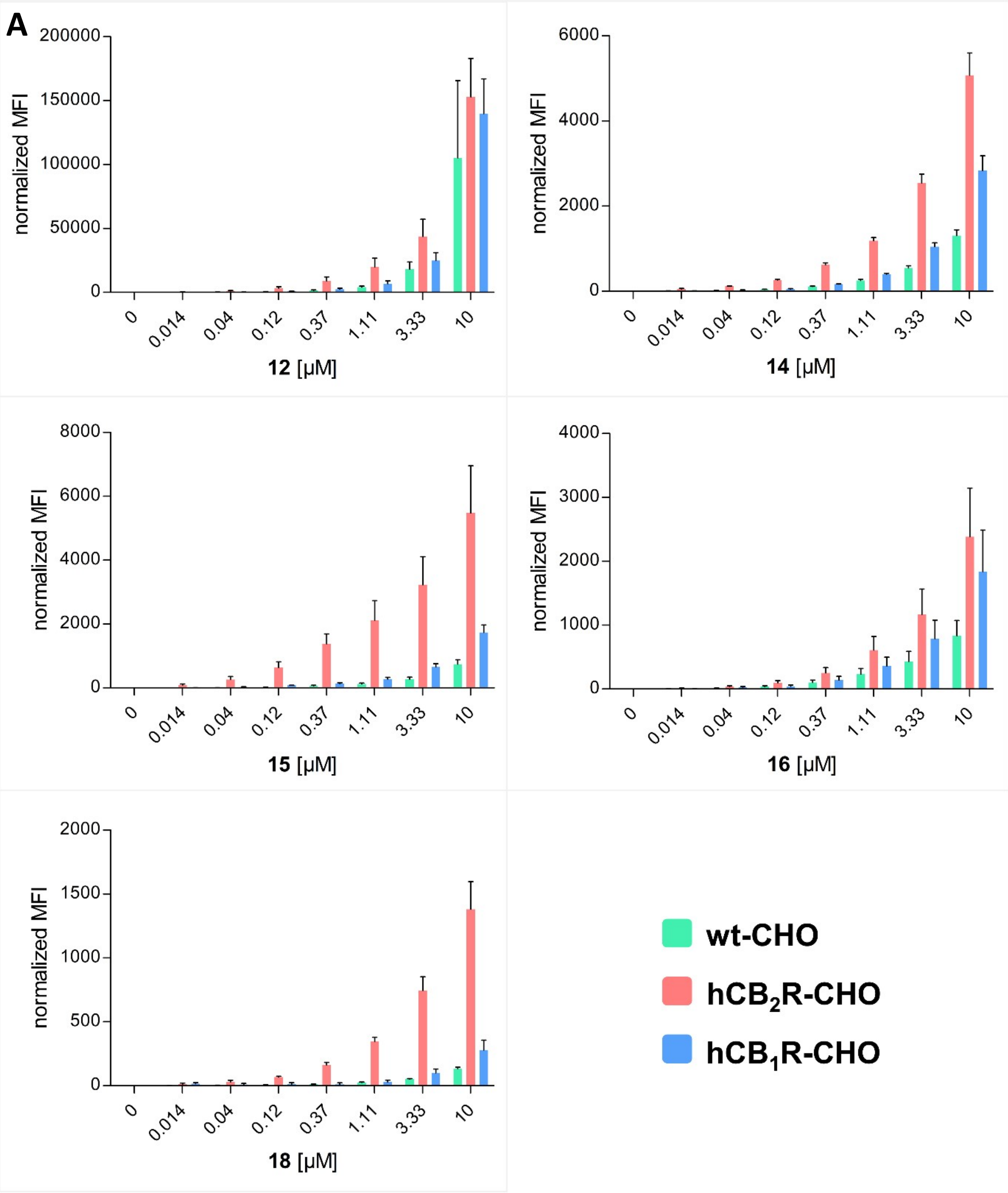
Compound	λ_{ex}	Radio of PBS/dioxane	Φ (%)
12	555	1:1	55.4
		1:2	57.9
16	560	1:1	34.9
		1:2	44.1

Fluorescence assays

Flow cytometry assay

Chinese Hamster Ovary (CHO) cells overexpressing either hCB_2R or hCB_1R were cultured in DMEM/F-12 (Gibco) supplemented with 10% fetal bovine serum (FBS, Sigma), Hypoxanthine / Thymidine Supplement (Gibco), 0.4 mg/mL Geneticin (Gibco) and 0.2 mg/mL Hygromycin B. For each experiment, fresh cells were thawed and expanded for 48 hours in a humidified incubator at 37°C with 5% CO_2. To prevent loss of receptor expression, cells were not passaged before analysis.

For the validation of CB_2R-fluoroprobes via flow cytometry, cells were harvested using Versene Solution (Gibco, USA), washed with cold FACS buffer (PBS / 0.5% BSA / 2 mM EDTA) and resuspended in FACS buffer at $5x10^5$ cells/mL. 50,000 CHO WT (wildtype control) or CHO cells overexpressing hCB_2R or hCB_1R were incubated with the probes at concentrations ranging from 0.014 – 10 µM for 30 minutes at 4°C in the dark. After incubation, the cells were washed twice with FACS buffer and a minimum of 10,000 events were acquired on a BD LSRFortessa cell analyzer (BD Biosciences, USA). The mean fluorescence intensity (MFI) was calculated for each sample using the FlowJo software (BD Biosciences, USA) (Supplementary figure S-1). All experiments were performed three times. MFI of each cell line from each experiment has been normalized by subtracting the autofluorescence at concentration = 0 µM from other concentrations.


A
normalized MFI
200000
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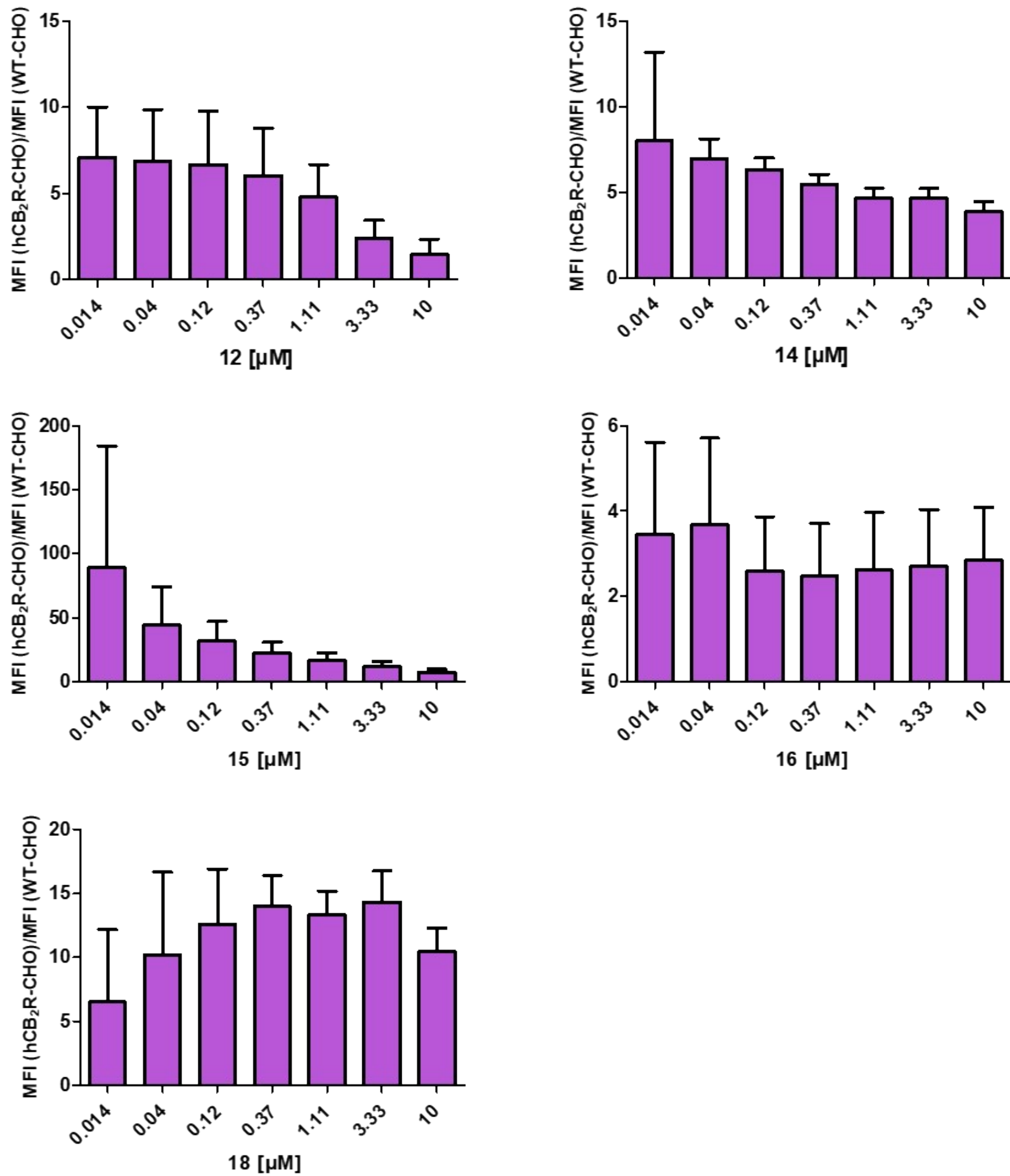
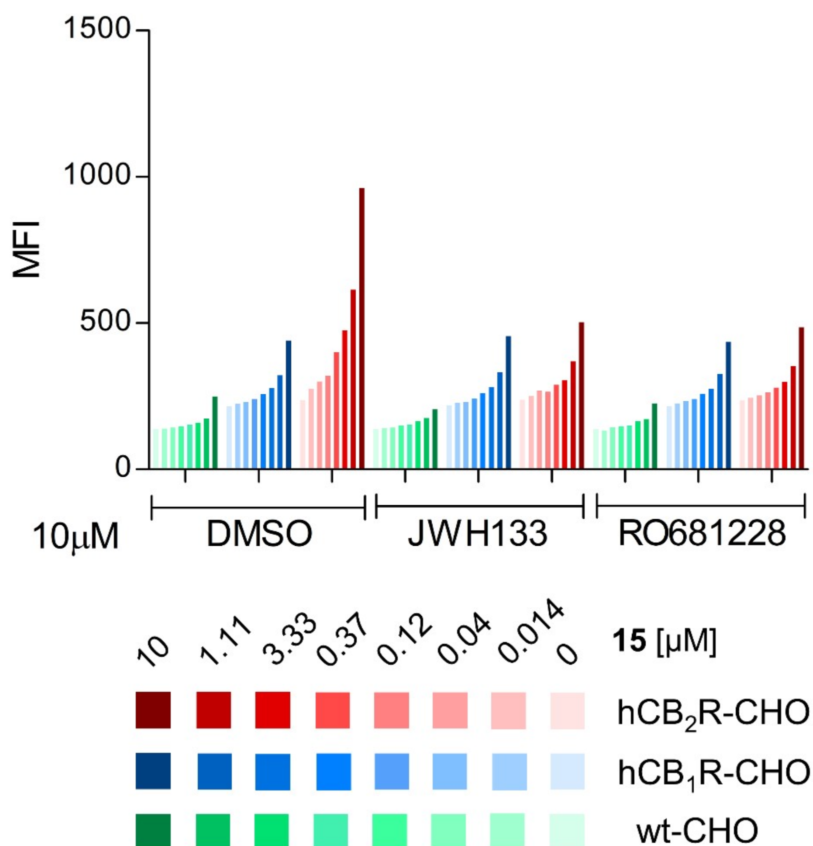
wt-CHO
hCB$_2$R-CHO
hCB$_1$R-CHO



Supplementary figure S-1. A) FACS analysis of the normalized mean fluorescent intensity (MFI) of WT, hCB_2R, hCB_1R overexpressing CHO cells at different concentrations of probes **12,14, 15, 16** and **18;** B) Ratio of normalized MFI (hCB_2R-CHO) and MFI (WT-CHO) as a specificity coefficient.

Cold ligand displacement experiment

Cold ligand displacement experiment using probe **15** in presence of CB_2R agonist **JWH133** and inverse agonist **RO6851228** was measured by flow cytometry (Supplementary figure S- 2): Cells were harvested using Versene Solution (Gibco, USA), washed with cold FACS buffer (PBS / 0.5% BSA / 2 mM EDTA) and resuspended in FACS buffer at $5x10^5$ cells/mL. 50,000 CHO WT (wildtype control) or CHO cells overexpressing hCB_2R or hCB_1R were pre-incubated with 10 µM agonist **JWH133** and inverse agonist **RO6851228** for 30 minutes at 4°C. After pre-incubation, probe **15** was added at concentrations ranging from 0.014 – 10 µM for 30 minutes at 4°C in the dark. Cells were washed twice with FACS buffer and a minimum of 10,000 events were acquired on a BD LSRFortessa cell analyzer (BD Biosciences, USA). The mean fluorescence intensity (MFI) was



calculated for each sample using the FlowJo software (BD Biosciences, USA).

Supplementary figure S- 2. Cold ligand displacement experiment of probe **15**. Flow cytometry analysis of WT, hCB_2R, hCB_1R overexpressing CHO cells pre-incubated with 10 µM of competitor ligands **JWH133** (agonist) and **RO6851228** (inverse agonist) and subsequently stained with varying concentrations of probe **15**.

Time-lapse confocal microscopy

Cell culture

CHO cells: The cell lines CHO-DUKX-CRE Luci, CHO-DUKX-CRE Luci-hCB_1R and CHO-DUKX-CRE Luci-hCB_2R were provided by Roche. The cells were cultured in DMEM-F12 medium (Gibco) with 10% heat inactivated FBS, 1x HT supplement (Gibco) and Hygromycin B (0.2 µg/mL, Roche). The hCB_1R and hCB_2R expressing cells were additionally selected using Geneticin (0.4 µg/mL, Gibco).

HEK293 cells: The human receptor-inducible HEK293 cell system with the cell lines HEK293TR, HEK293TR-SNAP-hCB1R, HEK293TR-SNAP-hCB1R were provided by Dmitry B. Veprintsev, University of Nottingham.
The 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).

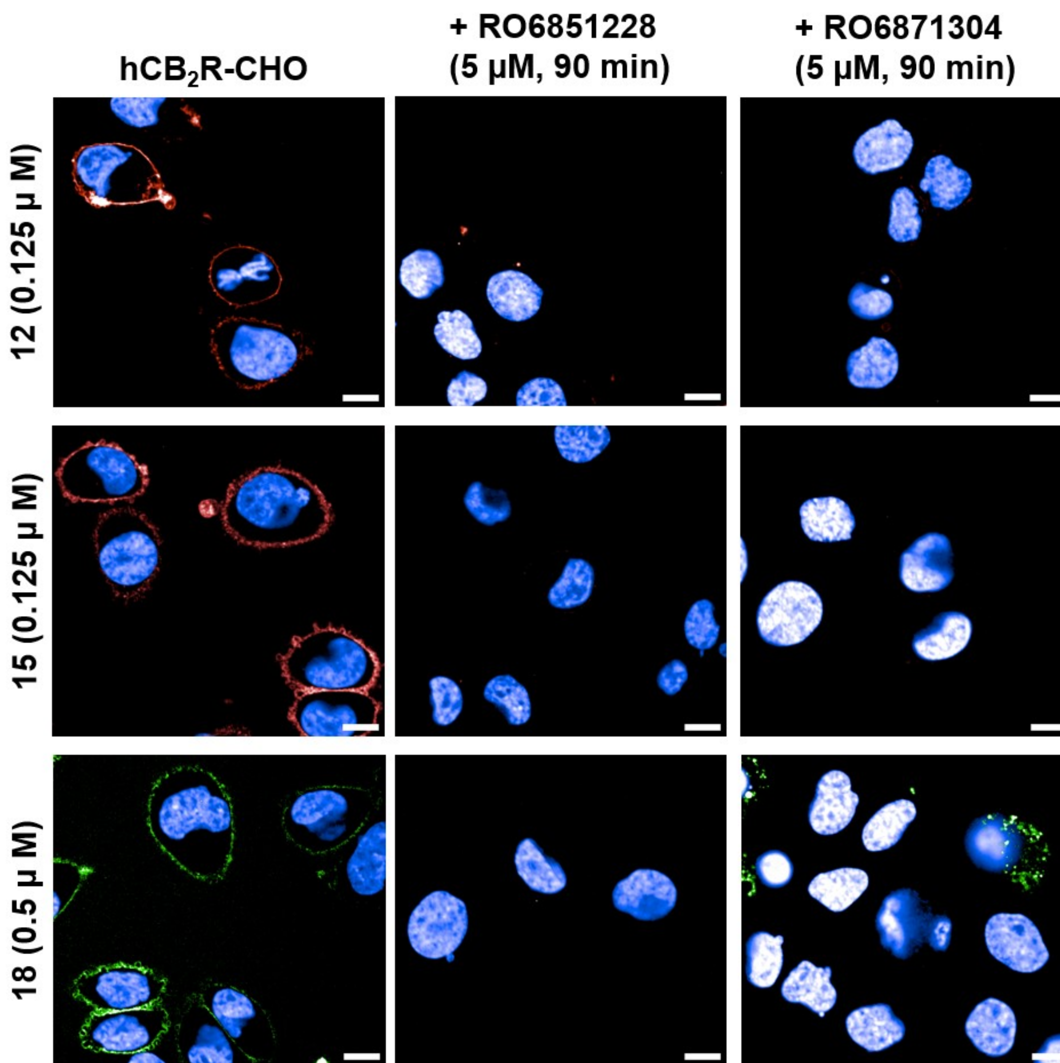
Methodology

The CHO cells were plated onto a 384-well microplate (PhenoPlate, Revvity), at a density of 3,500 cells/well (40 µL) and incubated for 20 h. The cell nuclei were stained using 0.9 µM Hoechst 33342 for 1 h incubation. After replacement of medium to serum free conditions without Phenol red (20 µL), fluorescent probes were added (10 µL), and tested at different concentrations (125 nM, 250 nM, 500 nM). In case of blocking experiments, nuclei stained cells were incubated with inhibitors (5 µM) for 90 min under serum free conditions before probe administration.

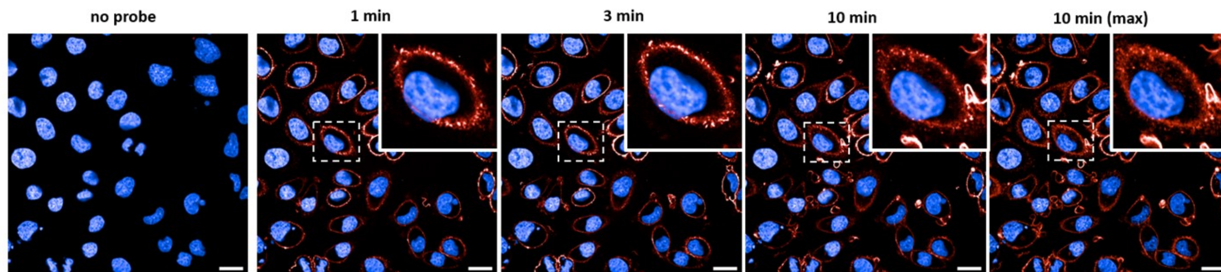
Confocal live cell imaging was performed using the Opera Phenix™ High Content Screening System (Revvity) at 22°C. The probe fluorescence was monitored by kinetic measurements of 10 min with a break for probe administration. The fluorescence of one image per sample was captured using a water immersion objective (63x, NA 1.15, field of view 0.21x0.21mm) at each time point. Probe detection was realized using the appropriate laser for excitation and filter for

fluorescence emission. Image acquisition parameters, including laser power, offset, and gain settings, were kept constant.

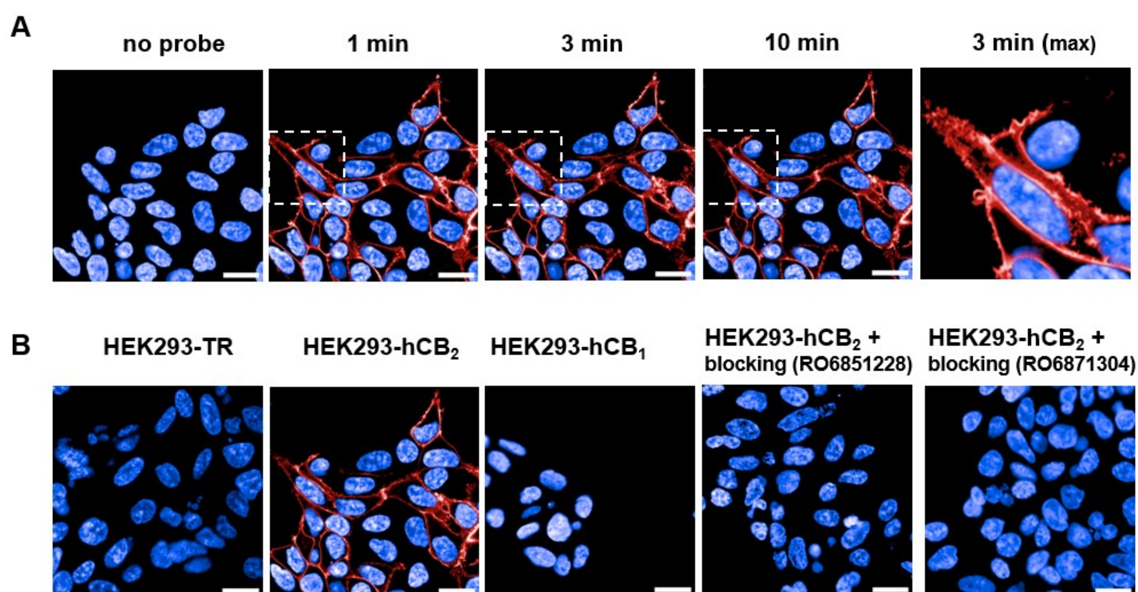
The HEK293 cells were plated onto a 384-well microplate (PhenoPlate, Revvity), at a density of 3500 cells/well (40 µL) with 1 µg/ml tetracycline for receptor-inducible expression and incubated for 48 h. The rest of the protocol is identical to the described above for CHO cell system.



Supplementary figure S- 3. Blocking experiments with competitive non-labeled ligands **RO6851228** (CB_2R-inverse agonist) and **RO6871304** (CB_2R-agonist) on overexpressing hCB_2R CHO cells using time-lapse confocal microscopy technique. Prior to probe administrations, cells were preincubated with 5 µM of inhibitors for 90 min. The overexpressing hCB_2R CHO cells were co-stained with probes **12, 15, 18.** and Hoechst 33342 (blue, nucleus counter stain). The images were recorded 10 min after incubation and are representative of two independent experiments. Scale bars, 10 µm.



Supplementary figure S-4. Time-lapse confocal microscopy in CHO cells overexpressed with hCB_2R. The figure represents images recorded at 1, 3 and 10 min after incubation with probe **12** and are representative of two independent experiments. Scale bars, 20 µm.

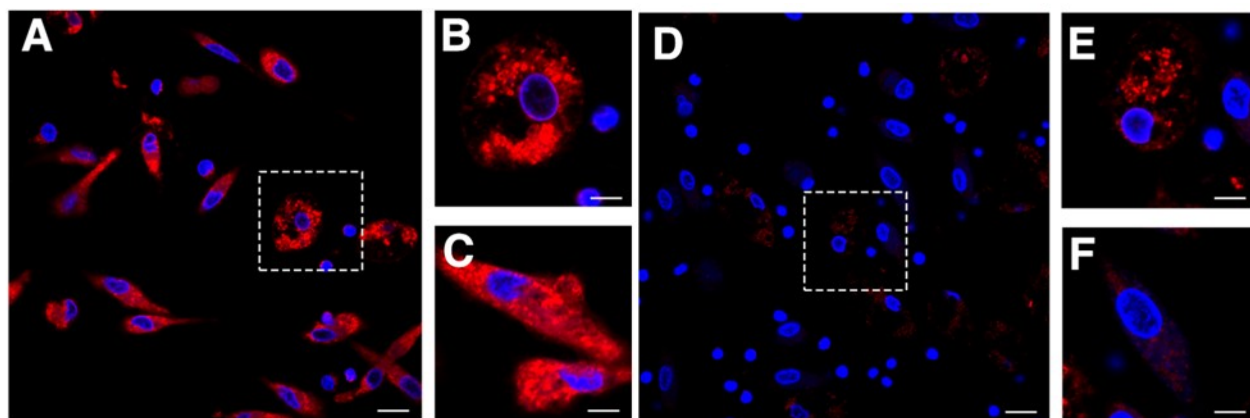


Supplementary figure S-5. Time-lapse confocal microscopy in HEK cells overexpressed with hCB_2R. A) Images recorded at 1, 3 and 10 min after incubation with probe **12** and are representative of two independent experiments. B) Specificity of the probes producing fluorescent signal in HEK293-CB2 cells, but not in HEK293-CB1 cells. Fluorescent signal is also absent when receptor is occupied by non-fluorescent ligands RO6851228 and RO6871304. Scale bars, 20 µm.

Primary human macrophages

For the primary macrophage culture, peripheral blood mononuclear cells (PBMCs) were thawed and allowed to adhere for 2 hours in complete RPMI 1640 medium on 8-well chamber slides (Ibidi, Milan, Italy) at a density of 150,000 cells per well. After the adhesion period, non-adherent cells were removed, and the adherent monocytes were gently rinsed with PBS. These cells were

then cultured in fresh complete medium supplemented with 50 ng/mL of macrophage colony-stimulating factor (M-CSF; Miltenyi Biotec, Bergisch Gladbach, Germany) to induce M0 (homeostatic macrophage) polarization over a period of 6 days. The medium was replenished with fresh supplemented medium on days 2 and 4, following the protocol described by Chiurchiù et al. (2021, DOI:10.3390/biom11040502). For nuclear visualization, the culture medium was replaced with RPMI (Sigma-Aldrich, Milan, Italy) containing 1 µg/mL of Hoechst 33342, followed by a 10-minute incubation at 37 °C and two subsequent PBS washes. Fluorescent probes were prepared at a concentration of 10 mM in DMSO and mixed with a 20% (w/v) Pluronic F-127 solution in DMSO (Sigma-Aldrich) at a 1:1 ratio immediately before use. Imaging was performed using a Nikon A1R+ laser scanning confocal microscope (Nikon Instruments, Tokyo, Japan) equipped with a 60× NA1.0 objective. Probes **12** and **16** were excited using a 549 nm laser, their fluorescence emission was captured with a 576 nm filter. Image acquisition parameters, including laser power, offset, and gain settings, were kept constant to minimize autofluorescence variation within each experiment. Data were exported as TIFF files and subsequently analyzed using Fiji software (National Institutes of Health; https://imagej.net/Fiji). Finally, for presentation purposes, images were exported to Affinity Designer version 1.10.6 (https://affinity.serif.com/it/) for adjustments in brightness and contrast.

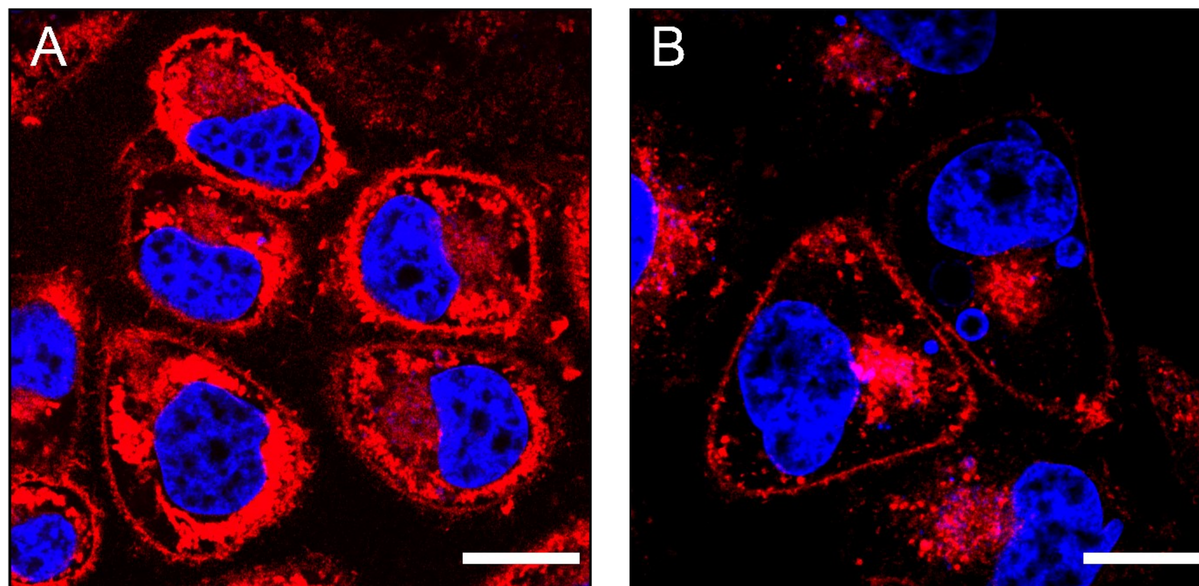


Supplementary figure S-6. Confocal microscopy of primary human macrophages. Confocal imaging of primary human macrophages. (A-C) Cells were stained for 15 min with compound **12** (0.8 µM, red). (D-F) Cells were stained for 15 min with compound **16** (0.8 µM, red). Cells were co-incubated with Hoechst 33342 for nuclear counterstaining (nuclei, blue). Imaging was performed using a confocal microscope with a 60x objective. Panels (A) and (D) show the full field of view, while the remaining panels (B, C, E, F) were obtained using a 1.5x digital zoom for enhanced detail. The images presented are representative of two independent experiments, and in each case five fields were examined. Scale bars in all images represent 10 µm.

Super-resolution confocal imaging

For the high-resolution imaging experiments, CHO cells expressing hCB_2R were seeded in 8-well chamber slides (Ibidi, Milan, Italy) at a density of 50,000 cells per well and incubated for 24 hours. To visualize cell nuclei, the culture medium was replaced with RPMI (Sigma-Aldrich, Milan, Italy) containing 1 µg/mL of Hoechst 33342, followed by a 10-minute incubation at 37 °C, and two subsequent washes with PBS. Fluorescent probes were prepared at a concentration of 10 mM in DMSO and then were mixed with a 20% (w/v) Pluronic F-127 solution in DMSO (Sigma-Aldrich) at a 1:1 ratio immediately before use. For Lysotracker (Lyfe Technologies) staining, cells were incubated with 30 nM of the dye for 10 minutes. For endocytosis-mediated receptor internalization experiment cells were pretreated with 0.4 M sucrose and 5 µg/mL filipin for 30 minutes (R. C. Sarott, et al 2020). Imaging was conducted using a ZEISS LSM 800 confocal laser scanning microscope equipped with an Airyscan detection unit (Zeiss, Oberkochen, Germany). To optimize resolution, a high numerical aperture oil immersion alpha Plan-Apochromat 63X/1.40 oil DIC M27 objective was employed. 12 and 16 probes were excited using a 549 nm laser, their fluorescence emission was captured with a 576 nm filter. Hoechst 33342 was excited with a dedicated 405 nm UV diode, and its fluorescence emission was detected through a 490/40 nm band-pass filter. Image acquisition parameters, including laser power, offset, and gain settings, were kept constant to minimize autofluorescence variation within each experiment. LysoTracker was excited using 488 nm laser, the fluorescence emission was captured with 530/30 nm filter. Super-resolution image processing was carried out using the Airyscan processing toolbox within the ZEN software. Data were exported as TIFF files and subsequently analyzed using Fiji software (National Institutes of Health; https://imagej.net/Fiji). (ImageJ2, version: 2.14.0/1.54f[5]). This included background subtraction, noise reduction, and normalization to enhance data quality. Regions of interest (ROIs) were selected for both the plasma membrane and internal membranes, and the mean fluorescence intensity within those ROIs was measured. The resulting intensity values were analyzed further using Prism 10 for macOS (version 10.1.1, GraphPad Software) for statistical analysis.

Finally, for presentation purposes, images were exported to Affinity Designer version 1.10.6 (https://affinity.serif.com/it/) for adjustments in brightness and contrast.



Supplementary figure S- 7. Super-resolution confocal imaging of overexpressing hCB_2R CHO cells using different concentrations of TAMRA-probe pair **12** and **16** to counterbalance their binding affinities. The cells were stained for 15 min with (A) **12** (0.2 µM, red) or (B) **16** (0.8 µM, red) and Hoechst 33342 (blue, nucleus counter stain). The mean fluorescence intensity (MFI) of **12**-labeled CB_2R showed a plasma membrane labelling intensity of the CB_2R approximately ten-fold higher than that of **16**-labeled CB_2R (**12**-labeled CB_2R = 170 ± 40 MFI; **16**-labeled CB_2R = 18 ± 2 MFI; unpaired *t*-test, t = 22.283, df = 4, p-value < 0.0001). Cells were optically sectioned using confocal laser-scanning microscopy equipped with an Airyscan detector. Images are representative of three independent experiments. Scale bars, 10 µm.

TR-FRET methodology

Cell culture: SNAP-tagged CB2R T-Rex^{TM}-293 cells were maintained in a humidified environment at 37 °C and 5% CO2 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 T-Rex^{TM}-293 cells (Invitrogen, which express the 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 cm2 flasks, grown to 80-90% confluence and DMEM

containing 1 µg/ml tetracycline added. 48h later cells were labelled with SNAP-Lumi4-Tb (CisBio) and membranes prepared as described in detail below.

Terbium labeling of SNAP-tagged CB2R* T-Rex^{TM}-293 *cells: Cell culture medium was removed from the t175 cm^2 flasks containing confluent adherent CB2 T-Rex^{TM}-293 cells. Cells were washed 1× in PBS (GIBCO Carlsbad, CA) followed by 1x Tag-lite labelling medium (LABMED, CisBio) to remove the excess cell culture media, then ten millilitres of LABMED containing 100 nM of SNAP-Lumi4-Tb was added to the flask and incubated for 1 h at 37 °C under 5% CO2. 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 350g) and the pellets were frozen to −80 °C.

Membrane preparation: All steps were conducted at 4 °C to avoid tissue degradation. Cell pellets were thawed and resuspended using ice-cold buffer containing 10 mM HEPES and 10 mM EDTA, pH 7.4. The suspension was homogenized using an electrical homogenizer Ultra-Turrax (Ika-Werk GmbH & Co. KG, Staufen, Germany) and subsequently centrifuged at 1200 × *g* for 5 min. The pellet obtained then, containing cell nucleus and other heavy organelle was discarded and supernatant was centrifuged for 30 min at 48,000 *x g* at 4 °C (Beckman Avanti J-251 Ultra-centrifuge; Beckman Coulter, Fullerton, CA). The supernatant was discarded, and the pellet was resuspended using the same buffer (10 mM HEPES and 10 mM EDTA, pH 7.4) and centrifuged a second time for 30 min as described above. Finally, the supernatant was discarded, and the pellet resuspended using ice-cold 10 mM HEPES and 0.1 mM EDTA, pH 7.4. Protein concentration determination was carried out using the bicinchoninic acid assay kit (Sigma-Aldrich) and using BSA as a standard. The final membrane suspension was aliquoted and maintained at -80 °C until required for the assays.

Determination of fluorescent ligand binding affinity: Fluorescent ligand binding experiments were conducted in white 384-well Optiplate plates, in assay binding buffer, Hanks Balanced Salt Solution (HBSS, Sigma-Aldrich) containing 5mM HEPES, 0.5% BSA, 0.02% pluronic acid pH 7.4, and

100 µM GppNHp. Association binding curves were constructed by incubating appropriate concentrations of fluorescent ligand with human CB2R T-Rex^{TM}-293 cell membranes (1 µg per well) in assay binding buffer (final assay volume, 40 µL). 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. In all cases, nonspecific binding was determined by the presence of 10 µM (SR144,528). 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_d, 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) allowing the determination of fluorescent ligand binding affinity.

Signal detection and data analysis: Signal detection was performed on a Pherastar FSX (BMG Labtech, Offenburg, Germany). The terbium donor was always excited with four laser flashes at a wavelength of 337 nm. TR-FRET signals were collected at 570 (acceptor) and 490 nm (donor) when using the TAMRA based fluorescent ligands 12 and 16. 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 9.0 (GraphPad Software, San Diego, USA).

Association data was fitted as follows to a global fitting model to simultaneously calculate k_{on} and k_{off} using the following equation where k_{ob} equals the observed rate of association and L is the concentration of fluorescent ligand:

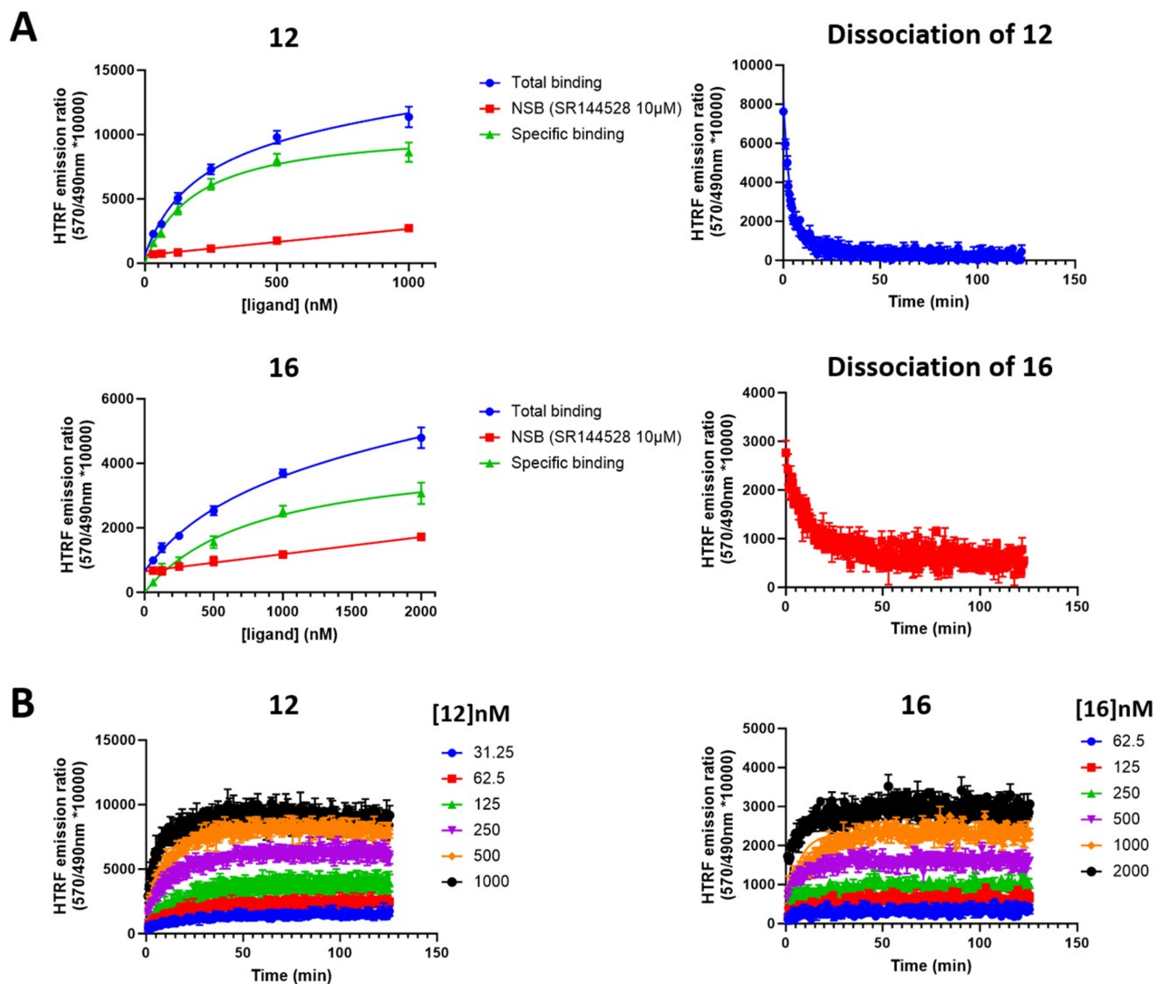
$$k_{ob}=[L] \times k_{on}+ k_{off} \qquad \text{(Eq. 1)}$$

Saturation binding data were analysed 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:

$$Total\ binding\ =\ Specific + NSB = \frac{Bmax * [L]}{Kd + [L]} + slope * [L] + Background$$

$$NSB = slope * [L] + Background \qquad \text{(Eq. 2)}$$

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}.



Probe	k_{on} (M^{-1} min^{-1})	k_{off} (min^{-1})	Kinetic K_d (nM)	Saturation K_d (nM)	Direct dissociation k_{off} (min^{-1})
12	1.9 ± 0.2 x 10^5 (3)	0.039 ± 0.004 (3)	211 ± 18 (3)	215 ± 18 (4)	0.25 ± 0.01 (3)
16	9.6 ± 0.6 x 10^4 (3)	0.073 ± 0.006 (3)	758 ± 20 (3)	666 ± 57 (4)	0.11 ± 0.01 (3)

Supplementary figure S- 8. HTRF characterization of agonist 12 and inverse agonist 16 binding (A) saturation analysis and direct dissociation induced by excess of SR144528 (B) kinetic association experiments using HEK293-hCB2R cell membranes.

Chemical synthesis

Experimental procedures

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: 5-cyclopropyl-6-(4-fluorobenzyl)picolinic acid (**8**)[6], 6-(((1*S*,2*S*)-2-(hydroxymethyl)cyclopropyl)methoxy)-5-(3-methoxyazetidin-1-yl)picolinic acid (**9**)[7]. Reactions with air or moisture-sensitive substances were carried out under an inert atmosphere of nitrogen with the help of the Schlenk technique, if not otherwise indicated. TLC analyses were performed using Merck 60 F254 aluminum sheets and the spots were visualized under UV light (254 nm) and with reagents such as $KMnO_4$ or ninhydrin solutions. Purification with flash chromatography was carried out using Biotage Isolera One apparatus or Combiflash NextGen 300+ apparatus equipped with ELSD detector using RediSep®Rf columns from Teledyne Isco. Mobile phases were used as binary mixtures with the volume ratios indicated in respective procedures. The temperature was 25 °C and UV detection was performed at 220 or 254 nm. Peparative HPLC was carried out using a Gilson PLC 2050 system, a Gilson PLC 2250 or a Shimadzu system with the following components: CBM20A, LC20AP, SPD, 20A, FC200Al. The temperature was 25°C and UV detection was performed at 220, 254 or 550 nm. As stationary phase a Macherey-Nagel VP 250/21 Nucleodur 100-7 C18Ec column (flow rate of 30 mL/min) or a Macherey-Nagel VP 250/10 Nucleodur 100-5 C18Ec column (flow rate of 5 mL/min) was used. As mobile phase acetonitrile:water with 0.1% TFA as acidic modifier or acetonitrile:water without TFA was used. Mobile phases were used as binary mixtures with the volume ratios indicated in respective procedures. NMR spectra were recorded at either Bruker AV 300 (T = 295K, ^{1}H at 300 MHz, ^{13}C at 75 MHz), Bruker AV 600 (T =300K, ^{1}H at 600 MHz, ^{13}C at 151 MHz) or Bruker AV 750cryo (T =300K, ^{1}H at 750 MHz, ^{13}C at 189 MHz) spectrometers using solvents as indicated. All chemical shifts (δ) are given as parts per million (ppm) relative to the residual solvent peak. Spin multiplicities are described as singlet (s), duplet (d), triplet (t), quartet (q), doublet of doublet (dd), doublet of triplet (dt), mulitplet (m) and br s (broad-singlet). Coupling constant are recorded in Hz. NMR data were analyzed with MestReNova or Bruker TopSpin software. Analytical HPLC-MS and purity

analyses were performed with Agilent 1260 series HPLC system employing a DAD detector (at 300, 254 and 220 nm) equipped with Agilent Technologies 6120 Quadrupole LC/MS in electrospray positive and negative ionization modes (ESI-MS). A Thermo Accuore RP-MS (30 × 2.1 mm, 2.6 µm) column was used with a flow rate 0.8 mL/min in combination with the following separation conditions: 0.1% formic acid in water (solvent A); 0.1% formic acid in ACN (solvent B); System (1) 5% B for 0.5 min, from 5 to 95% B in 6.5 min, 95% B for 1 min (stop point at 8 min); System (2) 5% B for 0.2 min, from 5 to 95% B in 0.9 min, 95% B for 1.4 min (stop point at 2.5 min). Data analysis was performed with ChemStation software. The purity of all test compounds was determined to be >95%. High-resolution mass spectrometry (HRMS) analyses were carried out on Agilent Technologies 6530 Accurate Mass Q-ToF LC/MS linked to Agilent Technologies HPLC 1260 Infinity II and HRMS results are reported in m/z. Compound names are derived from Chemdraw and are not necessarily identical with the IUPAC nomenclature.

General procedure A for the synthesis of linkers (7a-d)

To a solution of commercially available 2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-2-ethylbutanoic acid (**5**) (1.0 equiv.) in anhydrous DMF (10 mL/mmol) were added anhydrous DIPEA (1.5 eqiuv.) and HATU (1.0 equiv.) and stirred for 20 min. Then a solution of corresponding **6a-d** commercially available (1.1 equiv.) in anhydrous DMF (3 mL/mmol) was added. The reaction was stirred for 1 h and the solvent was removed under reduced pressure. The crude was dissolved in EtOAc and washed with $NaHCO_3$ solution (1M) and brine, dried over anhydrous Na_2SO_4, filtered, and concentrated to yield the desired product as white powder. The crude mixture was used for the next step without further purification.

(9*H*-Fluoren-9-yl)methyl (13-ethyl-2,2-dimethyl-4,12-dioxo-3,8-dioxa-5,11-diazapentadecan-13-yl)carbamate (7a). 7a was prepared from **6a** (127.1 mg, 0.62 mmol) following the general procedure A (255 mg, 83%). ESI-MS (*m/z*) 562.3 ([M+Na]$^+$). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 7.76 (d, *J* = 7.6 Hz, 1H), 7.61 (d, *J* = 7.8 Hz, 2H), 7.39 (td, *J* = 7.6, 0.8 Hz, 2H), 7.31 (td, *J* = 7.4, 1.2 Hz, 2H), 6.27 (br s, 1H), 6.16 (br s, 1H), 4.90 (br s, 1H), 4.38 (d, *J* = 7.0 Hz, 2H), 4.22 (t, *J* = 6.8 Hz, 1H), 3.58 – 3.42 (m, 6H), 3.29 (s, 1H), 2.55 – 2.31 (m, 2H), 1.68 – 1.49 (m, 2H), 1.44 (s, 9H), 0.78 (t, *J* = 7.0 Hz, 7H).^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 172.98, 156.18, 154.31, 144.11, 141.45, 127.77, 127.18, 125.20, 120.09, 79.75, 70.29, 69.65, 66.25, 64.33, 47.45, 40.62, 39.75, 29.17, 28.52, 8.20. HRMS (ESI): *m/z* calculated for $C_{30}H_{41}N_3O_6$ + H$^+$ [M+ H$^+$]: 540.3068. Found: 540.3057.

(9*H*-Fluoren-9-yl)methyl (16-ethyl-2,2-dimethyl-4,15-dioxo-3,8,11-trioxa-5,14-diazaoctadecan-16-yl)carbamate (7b). 7b was prepared from **6b** (155 mg, 0.62 mmol) following the general procedure A (315 mg, 95%). ESI-MS (*m/z*) 606.3 ([M+Na]$^+$). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 7.75 (dd, *J* = 7.6, 1.1 Hz, 2H), 7.61 (d, *J* = 7.4 Hz, 2H), 7.38 (td, *J* = 7.5, 1.2 Hz, 2H), 7.30 (td, *J* = 7.4, 1.2 Hz, 2H), 6.65 – 6.13 (m, 1H), 4.34 (d, *J* = 7.2 Hz, 2H), 4.21 (t, *J* = 6.9 Hz, 1H), 3.75 – 3.43 (m, 10H), 3.30 (t, *J* = 5.2 Hz, 1H), 2.58 – 2.31 (m, 2H), 1.75 – 1.52 (m, 2H), 1.44 (s, 9H), 0.77 (t, *J* = 7.4 Hz, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 173.11, 156.30, 154.14, 144.16, 141.41, 127.71, 127.15, 125.24, 120.03, 79.79, 70.56, 70.33, 69.91, 66.18, 64.35, 47.43, 39.93, 39.11, 29.02, 28.51, 8.23. (One carbon was not resolved). HRMS (ESI): *m/z* calculated for $C_{32}H_{45}N_3O_7$ + H$^+$ [M+ H$^+$]: 584.3330. Found: 584.3310.

(9*H*-Fluoren-9-yl)methyl (19-ethyl-2,2-dimethyl-4,18-dioxo-3,8,11,14-tetraoxa-5,17-diazahenicosan-19-yl)carbamate (7c). 7c was prepared from **6c** (182 mg, 0.62mmol) following the general procedure A (339 mg, 95%). ESI-MS (*m/z*) 650.4 ([M+Na]$^+$). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 7.76 (d, *J* = 7.3 Hz, 2H), 7.62 (d, *J* = 7.4 Hz, 2H), 7.39 (td, *J* = 7.8, 1.2 Hz, 2H), 7.30 (td, *J* = 7.4, 1.3 Hz, 2H), 6.67 – 6.13 (m, 2H), 5.07 (br s, 1H), 4.35 (d, *J* = 7.0 Hz, 2H), 4.22 (t, *J* = 6.9 Hz, 1H), 3.68 – 3.46 (m, 12H), 3.30 (t, *J* = 5.2 Hz, 2H), 2.57 – 2.31 (m, 2H), 1.70 – 1.50 (m, 2H), 1.44 (s, 9H), 0.93 – 0.62 (m, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 172.98, 156.19, 154.16, 144.17, 141.43, 127.73, 127.16, 125.25, 120.05, 79.58, 70.57, 70.55, 70.37, 70.33, 70.24, 69.88, 66.19, 64.35, 47.45, 40.72, 39.77, 29.13, 28.54, 8.25. HRMS (ESI): *m/z* calculated for $C_{34}H_{49}N_3O_8$ + H^+ [M+ H^+]: 628.3592. Found: 628.3589.

(9*H*-Fluoren-9-yl)methyl (22-ethyl-2,2-dimethyl-4,21-dioxo-3,8,11,14,17-pentaoxa-5,20-diazatetracosan-22-yl)carbamate (7d). 7d was prepared from **6d** (209.3 mg, 0.62 mmol) following the general procedure A (372 mg, 97%). ESI-MS (*m/z*) 694.3 ([M+Na]$^+$). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 7.76 (d, *J* = 7.4 Hz, 2H), 7.62 (d, *J* = 7.4 Hz, 2H), 7.39 (td, *J* = 7.5, 1.1 Hz, 2H), 7.30 (td, *J* = 7.4, 1.3 Hz, 2H), 6.51 (br s, 1H), 6.31 (br s, 1H), 5.12 (br s, 1H), 4.44 – 4.28 (m, 2H), 4.22 (t, *J* = 6.9 Hz, 1H), 3.72 – 3.47 (m, 16H), 3.36 – 3.25 (m, 2H), 2.60 – 2.28 (m, 2H), 1.78 – 1.49 (m, 2H), 1.43 (s, 9H), 0.77 (t, *J* = 7.5 Hz, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 172.92, 156.15, 154.17, 144.17, 141.43, 127.73, 127.16, 125.25, 120.05, 79.37, 70.70, 70.64, 70.62, 70.58, 70.44, 70.35, 70.30, 69.86, 66.19, 64.35, 47.45, 40.57, 39.76, 29.19, 28.55, 8.27. HRMS (ESI): *m/z* calculated for $C_{36}H_{53}N_3O_9$ + H^+ [M+ H^+]: 672.3855. Found: 672.3890.

General procedure B for the synthesis of precursors (10a-d and 11a-d)

Mixture A: To a solution of **7a-d** (1.0 equiv.) in anhydrous DMF (27 mL/mmol), DBU (1.5 equiv.) was added. After 30 min, HOAt (1.6 equiv.) was added and the resulting mixture was allowed to stir for another 10 min.

Mixture B: To a solution of **8** for compounds **10a-d** or **9** for compounds **11a-d** (1.5 equiv.) in anhydrous DMF (18 mL/mmol) was added DIPEA (1.5 equiv.) and HATU (1.5 equiv.) and stirred for 20 min. Then mixtures A and B were combined and the progress of the reaction was judged

by LCMS. After removal of the solvent under reduced pressure, the residue was purified by either preparative HPLC or by automated flash chromatography on silica.

***Tert*-butyl (2-(2-(2-(5-cyclopropyl-6-(4-fluorobenzyl)picolinamido)-2-ethylbutanamido)ethoxy)ethyl)carbamate (10a).** **10a** was prepared from **7a** (52.2 mg, 0.096 mmol) following the general procedure B. The crude mixture was purified by purified by automated flash chromatography on silica (cyclohexane:EtOAc 0 to 60%) to yield the desired product as yellow viscous oil (46 mg, 83%). ESI-MS (*m/z*) 571.3[M+H]$^+$.^{1}H NMR (600 MHz, $CDCl_3$) δ ppm: 8.78 (s, 1H), 7.94 (d, *J* = 8.0 Hz, 1H), 7.39 (d, *J* = 7.9 Hz, 1H), 7.24 – 7.20 (m, 2H), 6.99 – 6.95 (m, 2H), 6.91 (t, *J* = 5.4 Hz, 1H), 5.26 (br s, 1H), 4.36 (s, 2H), 3.55 – 3.52 (m, 2H), 3.51 – 3.48 (m, 4H), 3.29 – 3.27 (m, 2H), 2.37 (dd, *J* = 14.4, 7.3 Hz, 2H), 1.95 – 1.90 (m, 1H), 1.85 (dd, *J* = 14.3, 7.3 Hz, 2H), 1.43 (s, 9H), 1.03 – 0.99 (m, 2H), 0.81 (t, *J* = 7.4 Hz, 6H), 0.69 – 0.66 (m, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ ppm: 173.36, 164.27, 161.67 (d, $^1J_{C\text{-}F}$ = 244.1 Hz), 158.55, 156.25, 146.74, 140.20, 134.76, 134.68, 130.66 (d, $^3J_{C\text{-}F}$ = 7.9 Hz, 2C), 119.97, 115.27 (d, $^2J_{C\text{-}F}$ = 21.2 Hz, 2C), 79.37, 70.34, 69.72, 65.06, 40.80, 40.61, 39.63, 28.58 (3C), 28.32 (2C), 12.82, 8.22 (2C), 7.93 (2C). HRMS (ESI): *m/z* calculated for $C_{31}H_{43}FN_4O_5 + H^+$ [M+ H$^+$]: 571.3290. Found: 571.3312. HPLC (System 2): t_R = 1.37 min at λ = 254 nm, Purity > 99%.

***Tert*-butyl (1-(5-cyclopropyl-6-(4-fluorobenzyl)pyridin-2-yl)-3,3-diethyl-1,4-dioxo-8,11-dioxa-2,5-diazatridecan-13-yl)carbamate (10b).** **10b** was prepared from **7b** (184.1 mg, 0.315 mmol) following the general procedure B. The crude mixture was purified by automated flash chromatography on silica (cyclohexane:EtOAc 0 to 60%) to yield the desired product as yellow viscous oil (147.5 mg, 76%). ESI-MS (*m/z*) 615:3 [M+H]$^+$. ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm: 8.94 (s, 1H), 7.90 (d, *J* = 7.9 Hz, 1H), 7.37 (d, *J* = 8.0 Hz, 1H), 7.24 – 7.22 (m 2H), 6.96 – 6.93 (m, 2H), 6.67 (br s, 1H), 5.02 (br s, 1H), 4.36 (s, 2H), 3.68 – 3.48 (m, 10H), 3.33 – 3.21 (m, 2H), 2.46 (dd, *J* = 14.6, 7.4 Hz, 2H), 1.94 – 1.87 (m, 1H), 1.79 (dq, *J* = 14.6, 7.3 Hz, 2H), 1.42 (s, 9H), 1.02 – 0.93 (m, 2H), 0.79 (t, *J* = 7.4 Hz, 6H), 0.69 – 0.61 (m, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ ppm: 173.32, 163.89, 161.60 (d, $^1J_{C\text{-}F}$ = 243.8 Hz), 158.4, 156.16, 146.93, 140.03, 134.78, 134.66, 130.59(d, $^3J_{C\text{-}F}$ = 7.9 Hz, 2C), 119.80, 115.19 (d, $^2J_{C\text{-}F}$ = 21.2 Hz, 2C), 79.46, 70.39 (2C), 70.26, 70.06, 64.96, 40.85, 40.45, 39.66, 28.53 (3C), 28.48 (2C), 12.80, 8.26 (2C), 7.90 (2C). HRMS (ESI): *m/z* calculated for

$C_{33}H_{47}FN_4O_6$ + H$^+$ [M+ H$^+$]: 615.3552. Found: 615.3594. HPLC (System 2): t_R = 1.36 min at λ = 254 nm, Purity = 99.6%.

***Tert*-butyl (1-(5-cyclopropyl-6-(4-fluorobenzyl)pyridin-2-yl)-3,3-diethyl-1,4-dioxo-8,11,14-trioxa-2,5-diazahexadecan-16-yl)carbamate (10c). 10c** was prepared from **7c** (181.5 mg, 0.291 mmol) following the general procedure B. The crude mixture was purified by automated flash chromatography on silica (cyclohexane:EtOAc 0 to 60%) to yield the desired product as yellow viscous oil (142 mg, 74%). ESI-MS (*m/z*) 659.4 [M+H]$^+$. ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm: 8.95 (br s, 1H), 7.89 (d, *J* = 8.0 Hz, 1H), 7.37 (d, *J* = 8.0 Hz, 1H), 7.24 – 7.22 (m, 2H), 6.96 – 6.93 (m, 2H), 6.68 (br s, 1H), 5.06 (br s, 1H), 4.36 (s, 2H), 3.64 – 3.56 (m, 10H), 3.55 – 3.49 (m, 4H), 3.32 – 3.25 (m, 2H), 2.49 – 2.38 (m, 2H), 1.94 – 1.86 (m, 1H), 1.85 – 1.74 (m, 2H), 1.42 (s, 9H), 1.01 – 0.92 (m, 2H), 0.78 (t, *J* = 7.4 Hz, 6H), 0.68 – 0.62 (m, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ ppm: 173.41, 163.88, 161.59 (d, $^1J_{C\text{-}F}$ = 243.88 Hz), 158.54, 156.24, 146.84, 140.04, 134.76, 134.65, 130.59 (d, $^3J_{C\text{-}F}$ = 7.8 Hz, 2C), 119.78, 115.17 (d, $^2J_{C\text{-}F}$ = 21.0 Hz, 2C), 79.39, 70.57, 70.48, 70.44, 70.22 (2C), 70.12, 64.91, 40.83, 40.45, 39.58, 28.51 (3C), 28.39 (2C), 12.77, 8.24 (2C), 7.89 (2C). HRMS (ESI): *m*/*z* calculated for $C_{35}H_{51}FN_4O_7$ + H$^+$ [M+ H$^+$]: 659.3815. Found: 659.3838.

***Tert*-butyl (1-(5-cyclopropyl-6-(4-fluorobenzyl)pyridin-2-yl)-3,3-diethyl-1,4-dioxo-8,11,14,17-tetraoxa-2,5-diazanonadecan-19-yl)carbamate (10d). 10d** was prepared from **7d** (57.68 mg, 0.086 mmol) following the general procedure B. The crude mixture was purified by automated flash chromatography on silica (cyclohexane:EtOAc 0 to 60%) to yield the desired product as yellow viscous oil (40.1 mg, 66%). ESI-MS (*m/z*) 703.4 [M+H]$^+$. ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm: 9.11 (br s, 1H), 7.90 (d, *J* = 7.9 Hz, 1H), 7.37 (d, *J* = 7.9 Hz, 1H), 7.28 – 7.20 (m, 2H), 6.98 – 6.90 (m, 2H), 6.74 (br s, 1H), 5.15 (br s, 1H), 4.37 (s, 2H), 3.65 – 3.56 (m, 14H), 3.56 – 3.50 (m, 4H), 3.33 – 3.27 (m, 2H), 2.59 – 2.50 (m, 2H), 1.92 – 1.85 (m, 1H), 1.80 – 1.71 (m, 2H), 1.43 (s, 9H), 1.00 – 0.96 (m, 2H), 0.79 (t, *J* = 7.4 Hz, 7H), 0.67 – 0.64 (m, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ ppm: 173.25, 163.73, 161.61 (d, $^1J_{C\text{-}F}$ = 243.9 Hz), 158.56, 156.17, 147.14, 139.91, 134.84, 134.67, 130.57 (d, $^3J_{C\text{-}F}$ = 7.9 Hz, 2C), 119.80, 115.20 (d, $^2J_{C\text{-}F}$ = 21.3 Hz, 2C), 79.27, 70.72, 70.66 (2C), 70.61, 70.47, 70.36 (2C), 70.05, 65.12, 40.91, 40.51, 39.70, 28.68 (2C), 28.57 (3C), 12.81, 8.35 (2C), 7.90 (2C). HRMS (ESI): *m/z* calculated for $C_{37}H_{55}FN_4O_8$ + H$^+$ [M+ H$^+$]: 703.4077. Found: 703.4107. HPLC (System 2): t_R = 1.37 min at λ = 254 nm, Purity > 99%.

***Tert*-butyl (2-(2-(2-ethyl-2-(6-(((1*S*,2*S*)-2-(hydroxymethyl)cyclopropyl)methoxy)-5-(3-methoxyazetidin-1-yl)picolinamido)butanamido)ethoxy)ethyl)carbamate (11a).** **11a** was prepared from **7a** (10 mg, 0.018 mmol) following the general procedure B. The crude mixture was purified by preparative HPLC (30 to 75% acetonitrile in water) to yield the desired product as yellow viscous oil (5 mg, 38%). ESI-MS (*m/z*) 608.4 $[M+H]^+$. ^1H NMR (300 MHz, $CDCl_3$) δ ppm: 9.19 (s, 1H), 7.63 (d, *J* = 7.9 Hz, 1H), 6.61 (d, *J* = 7.8 Hz, 1H), 6.41 (br s, 1H), 5.08 (dd, *J* = 11.6, 4.6 Hz, 1H), 5.04 (br s, 1H), 4.31 – 4.23 (m, 3H), 3.90 – 3.83 (m, 3H), 3.56 – 3.40 (m, 7H), 3.33 (s, 3H), 3.32 – 3.27 (m, 2H), 2.98 (dd, *J* = 11.8, 9.3 Hz, 1H), 2.85 – 2.70 (m, 2H), 1.77 – 1.59 (m, 3H), 1.44 (s, 9H), 1.20 – 1.12 (m, 1H), 0.84 (t, *J* = 7.3 Hz, 3H), 0.77 (t, *J* = 7.3 Hz, 3H), 0.67 – 0.56 (m, 1H), 0.50 – 0.44 (m, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm:174.25, 163.84, 156.18, 151.63, 138.14, 136.42, 118.22, 116.21, 79.56, 70.47, 70.40, 70.35, 69.54, 66.51, 65.19, 60.33, 60.23, 56.32, 40.52, 39.95, 29.53 (2C), 28.55 (3C), 20.09, 17.08, 8.51, 8.48, 8.37. HRMS (ESI): *m*/*z* calculated for $C_{30}H_{49}N_5O_8 + H^+$ $[M+ H^+]$: 608.3654. Found: 608.3662. HPLC (System 2): t_R = 1.17 min at λ = 254 nm, Purity > 99%.

***Tert*-butyl (3,3-diethyl-1-(6-(((1*S*,2*S*)-2-(hydroxymethyl)cyclopropyl)methoxy)-5-(3-methoxyazetidin-1-yl)pyridin-2-yl)-1,4-dioxo-8,11-dioxa-2,5-diazatridecan-13-yl)carbamate (11b).** **11b** was prepared from **7b** (24 mg, 0.041 mmol) following the general procedure B. The crude mixture was purified by preparative HPLC (40 to 80% acetonitrile in water) to yield the desired product as yellow viscous oil (20 mg, 74%). ESI-MS (*m/z*) 652.4 $[M+H]^+$. ^1H NMR (300 MHz, Methanol-d_4) δ ppm: 7.52 (d, *J* = 7.9 Hz, 1H), 6.70 (d, *J* = 7.9 Hz, 1H), 4.47 (dd, *J* = 11.4, 6.6 Hz, 1H), 4.35 – 4.25 (m, 3H), 4.18 (dd, *J* = 11.4, 7.7 Hz, 1H), 3.90 – 3.85 (m, 2H), 3.66 – 3.43 (m, 12H), 3.34 (s, 3H), 3.21 (t, *J* = 5.7 Hz, 2H), 2.66 – 2.54 (m, 2H), 1.87 – 1.74(m, 2H), 1.43 (s, 9H), 1.39 – 1.30 (m, 1H), 1.22 – 1.12 (m, 1H), 0.79 – 0.72 (m, 6H), 0.70 - 0.66 (m, 1H), 0.60 – 0.54 (m, 1H). ^{13}C NMR (151 MHz, Methanol-d_4) δ ppm: 175.34, 165.58, 158.42, 153.08, 139.96, 136.58, 118.68, 117.37, 80.10, 71.63, 71.30 (2C), 71.07, 70.62, 70.58, 66.46, 66.40, 61.32 (2C), 56.37, 41.24, 40.75, 29.51 (2C), 28.77 (3C), 20.59, 17.18, 9.16, 8.60 (2C). HRMS (ESI): *m*/*z* calculated for $C_{32}H_{53}N_5O_9 + H^+$ $[M+ H^+]$: 652.3916. Found: 652.3950. HPLC (System 2): t_R = 1.18 min at λ = 254 nm, Purity = 99.6%.

***Tert*-butyl (3,3-diethyl-1-(6-(((1*S*,2*S*)-2-(hydroxymethyl)cyclopropyl)methoxy)-5-(3-methoxyazetidin-1-yl)pyridin-2-yl)-1,4-dioxo-8,11,14-trioxa-2,5-diazahexadecan-16-yl)carbamate (11c).** **11c** was prepared from **7c** (12 mg, 0.019 mmol) following the general procedure B. The crude mixture was purified by preparative HPLC (5 to 75% acetonitrile in water) to yield the desired product as yellow viscous oil (6.9 mg, 52%). ESI-MS (*m/z*) 696.4 $[M+H]^+$. ^1H NMR (300 MHz, Methanol-d_4) δ ppm: 9.24 (s, 1H), 7.52 (d, *J* = 7.9 Hz, 1H), 6.69 (d, *J* = 7.9 Hz, 1H), 4.47 (dd, *J* = 11.4, 6.6 Hz, 1H), 4.35 – 4.24 (m, 3H), 4.18 (dd, *J* = 11.4, 7.7 Hz, 1H), 3.89 – 3.85 (m, 2H), 3.68 – 3.34 (m, 16H), 3.34 (s, 3H), 3.25 – 3.19 (m, 2H), 2.66 – 2.54 (m, 2H), 1.87 – 1.76 (m, 2H), 1.43 (s, 9H), 1.41 – 1.27 (m, 1H), 1.25 – 1.12 (m, 1H), 0.79 – 0.72 (m, 6H), 0.72 – 0.66 (m, 1H), 0.60 – 0.54 (m, 1H). ^{13}C NMR (75 MHz, Methanol-d_4) δ ppm: 153.07, 139.98, 136.58, 118.69, 117.37, 71.61, 71.57, 71.23, 71.21 (2C), 71.08, 70.64, 70.55, 66.46, 66.42, 61.32 (2C), 56.38, 41.28, 40.80, 29.52 (2C), 28.77 (3C), 20.58, 17.18, 9.16, 8.63 (2C). Due to low concentration, four quaternary carbons were not resolved. HRMS (ESI): *m/z* calculated for $C_{34}H_{57}N_5O_{10} + H^+$ $[M+ H^+]$: 696.4178. Found: 696.4174. HPLC (System 2): t_R = 1.16 min at λ = 254 nm, Purity > 99%.

***Tert*-butyl (3,3-diethyl-1-(6-(((1*S*,2*S*)-2-(hydroxymethyl)cyclopropyl)methoxy)-5-(3-methoxyazetidin-1-yl)pyridin-2-yl)-1,4-dioxo-8,11,14,17-tetraoxa-2,5-diazanonadecan-19-yl)carbamate (11d).** **11d** was prepared from **7d** (20 mg, 0.03 mmol) following the general procedure B. The crude mixture was purified by preparative HPLC (30 to 75% acetonitrile in water) to yield the desired product as yellow viscous oil (10 mg, 50%). ESI-MS (*m/z*) 740.4 $[M+H]^+$. ^1H NMR (600 MHz, $CDCl_3$) δ ppm: 9.28 (s, 1H), 7.62 (d, *J* = 7.8 Hz, 1H), 6.67 (br s, 1H), 6.61 (d, *J* = 7.8 Hz, 1H), 5.16 – 5.13 (m, 2H), 4.31 – 4.21 (m, 3H), 3.89 (dd, *J* = 11.9, 4.5 Hz, 1H), 3.84 (td, *J* = 8.3, 3.9 Hz, 2H), 3.65 – 3.60 (m, 12H), 3.60 – 3.56 (m, 2H), 3.55 – 3.50 (M, 4H), 3.47 – 3.43 (M, 1H), 3.33 (s, 3H), 3.31 (br s, 2H), 2.95 (dd, *J* = 11.8, 9.4 Hz, 1H), 2.83 – 2.74 (m, 2H), 1.74 – 1.70 (m, 1H), 1.67 – 1.60 (m, 2H), 1.43 (s, 9H), 1.15 (tq, *J* = 9.1, 4.5 Hz, 1H), 0.81 (t, *J* = 7.4 Hz, 3H), 0.74 (t, *J* = 7.3 Hz, 3H), 0.62 (dt, *J* = 8.6, 5.0 Hz, 1H), 0.45 (dt, *J* = 9.1, 5.0 Hz, 1H). ^{13}C NMR (151 MHz, $CDCl_3$) δ ppm: 174.22, 163.72, 156.21, 151.64, 138.18, 136.56, 118.14, 116.12, 79.31, 70.74, 70.69 (2C), 70.62, 70.54, 70.50, 70.40, 70.35 (2C), 69.74, 66.51, 65.25, 60.32, 60.22, 56.28, 40.51, 39.97, 29.45 (2C), 28.59 (3C), 20.12, 17.17, 8.54, 8.50, 8.41. HRMS (ESI): *m/z* calculated for

$C_{36}H_{61}N_5O_{11}$ + Na^+ [M+ Na^+]:. 762.4260 Found: 762.4304. HPLC (System 2): t_R = 1.18 min at λ = 254 nm, Purity > 99%.

***N*-(1-(3',6'-Bis(dimethylamino)-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-5/6-yl)-13-ethyl-1,12-dioxo-5,8-dioxa-2,11-diazapentadecan-13-yl)-5-cyclopropyl-6-(4-fluorobenzyl)picolinamide (12).** A solution of **10b** (19.5 mg, 0.03 mmol) in DCM (2 mL) was placed in an ice bath and treated with TFA (21 µL, 0.27 mmol, 9 equiv.). The reaction was allowed to slowly reach to rt and stirred for 3h. The solvent was co-evaporated with toluene (3×) and the crude material was used for the next step without further purification. ESI-MS (*m/z*) 515.3 $[M+H]^+$. The residue (13.6 mg) was dissolved in anhydrous DMF (1 mL) and was added to a mixture of 5- and 6-TAMRA-COOH (mixed isomers) (17 mg, 0.04 mmol), EDC.HCl (8 mg, 0.042 mmol), HOAT (5.8 mg, 0.042 mmol) and DIPEA (19 µL, 0.105 mmol) in anhydrous DMF (2 mL). The reaction mixture was allowed to stir at rt for overnight. After removal of solvent under reduced pressure, the residue was purified by preparative HPLC (5 to 50% acetonitrile in water with 0.1% TFA) to yield **12** as pink-purple powder (13.7 mg, 56%). ESI-MS (*m/z*) 927.4 $[M+H]^+$. ^1H NMR (*cryo* 600 MHz, Methanol-d_4) δ ppm: 8.79 (d, *J* = 1.8 Hz, 1H), 8.39 (d, *J* = 8.3 Hz, 1H), 8.27 (dd, *J* = 7.9, 1.9 Hz, 1H), 8.21 (dd, *J* = 8.3, 1.8 Hz, 1H), 7.84 (d, *J* = 1.8 Hz, 1H), 7.80 (d, *J* = 7.96 Hz, 1H), 7.78 (d, *J* = 7.96 Hz, 1H), 7.51-7.47 (m, 3H), 7.31 – 7.27 (m, 4H), 7.14-7.12 (m, 4H), 7.06 – 7.03 (m, 4H), 6.99 – 6.92 (m, 8H), 4.37 (s, 2H), 4.35 (s, 2H), 3.73 - 3.52 (m, 20H), 3.47 (t, *J* = 5.6 Hz, 2H), 3.37 (t, *J* = 5.6 Hz, 2H), 3.29 (m, 24H), 2.50-2.44 (m, 4H), 2.02 (m, 2H), 1.85-1.78 (m, 4H), 1.03-1.00 (m, 4H), 0.75 – 0.69 (m, 12H), 0.69-0.67 (m, 4H). To avoid complications, the integrations were reported for 1:1 mixture. The ratio of 5-/6- isomers is 2:1. HRMS (ESI): *m*/*z* calculated for $C_{53}H_{59}FN_6O_8$ + H^+ [M+ H^+]: 927.4451. Found: 927.4459. HPLC (System 1): t_R = 4.15 min at λ = 254 nm, Purity> 99%.

***N*-(1-(3,7-Bis(dimethylamino)-5,5-dimethyl-3'-oxo-3'*H*,5*H*-spiro[dibenzo[*b*,*e*]siline-10,1'-isobenzofuran]-6'-yl)-13-ethyl-1,12-dioxo-5,8-dioxa-2,11-diazapentadecan-13-yl)-5-cyclopropyl-6-(4-fluorobenzyl)picolinamide (13)**

To a solution of **10b** (10 mg, 16.3 µmol, 1 equiv.) in anhydrous DCM (2 mL) was added TFA (11 µL, 0.146 mmol, 9 equiv.) dropwise at 0 °C and stirred for 3 h. The solvent was co-evaporated

with toluene (3×) and the crude material was used for the next step without further purification. ESI-MS (*m/z*) 515.3 $[M+H]^+$. The residue (6 mg, 11.6 µmol, 1.1 equiv.) was dissolved in anhydrous DMF (0.2 mL) and was added to a mixture of SiR-COOH (5 mg, 10.6 µmol, 1 equiv.), HATU (4 mg, 10.6 µmol, 1 equiv.), and DIPEA (5.5 µL, 31.7 µmol, 3 equiv.) in anhydrous DMF (0.2 mL). The reaction mixture was allowed to stir at rt for overnight. After removal of solvent under reduced pressure, the residue was purified by preparative HPLC (30 to 95% acetonitrile in water with 0.1% TFA) to yield **11** as blue powder (8.8 mg, 86%). ESI-MS (*m/z*) 970.4 $[M+H]^+$. ^{1}H NMR (600 MHz, Methanol-d_4) δ ppm: 9.36 (br s, 1H, CONH), 8.22 (d, *J* = 8.2 Hz, 1H), 8.10 (dd, *J* = 8.2, 1.7 Hz, 1H), 7.98 (t, *J* = 5.7 Hz, 1H, CONH), 7.81 (d, *J* = 7.8 Hz, 1H), 7.72 (d, *J* = 1.6 Hz, 1H), 7.49 (d, *J* = 8.0 Hz, 1H), 7.30 – 7.27 (m, 2H), 7.25 (d, *J* = 2.9 Hz, 2H), 6.94 - 6.97 (m, 2H), 6.90 (d, *J* = 9.4 Hz, 2H), 6.70 (dd, *J* = 9.4, 2.8 Hz, 2H), 4.37 (s, 2H), 3.63 (t, *J* = 5.5 Hz, 2H), 3.61 – 3.58 (m, 4H), 3.54 (t, *J* = 5.4 Hz, 2H), 3.52 (t, *J* = 5.5 Hz, 2H), 3.36 (t, *J* = 5.5 Hz, 2H), 3.20 (s, 12H), 2.45 (dq, *J* = 14.8, 7.4 Hz, 2H), 2.03 – 1.99 (m, 1H), 1.81 (dq, *J* = 14.6, 7.3 Hz, 2H), 1.03 – 0.99 (m, 2H), 0.71 (t, *J* = 7.4 Hz, 6H), 0.69 – 0.67 (m, 2H), 0.65 z(s, 3H), 0.57 (s, 3H). ^{13}C NMR (151 MHz, Methanol-d_4) δ 175.04, 168.89, 165.15, 162.91 (d, $^1J_{C\text{-}F}$ = 242.6 Hz), 160.11, 147.77, 142.06, 139.47ZZ, 136.36 (d, $^4J_{CF}$ = 3.0 Hz), 135.65, 131.70 (d, $^3J_{C\text{-}F}$ = 7.8 Hz, 2C), 130.20, 129.02, 120.74, 120.61 (2C), 115.97 (d, $^2J_{C\text{-}F}$ = 21.4 Hz, 2C), 115.11 (2C), 71.31 (2C), 70.62, 70.35, 66.32, 41.47, 41.07, 40.84 (4C), 40.64, 29.11 (2C), 13.43, 8.64 (2C), 8.53 (2C), -0.58, -1.57. Due to low concentration, four quaternary carbons were not resolved. HRMS (ESI): *m/z* calculated for $C_{55}H_{65}FN_6O_7Si + H^+$ $[M+ H^+]$: 969.4746. Found: 969.4778. HPLC (System 2): t_R = 1.42 min at λ = 254 nm, Purity = 99.6%.

3',6'-Diamino-5-((1-(5-cyclopropyl-6-(4-fluorobenzyl)pyridin-2-yl)-3,3-diethyl-1,4-dioxo-8,11-dioxa-2,5-diazatridecan-13-yl)carbamoyl)-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthene]-4',5'-disulfonic acid (14)

To a solution of **10b** (20 mg, 32.5 µmol, 1 equiv.) in anhydrous DCM (2 mL) was added TFA (25 µL, 0.325 mmol, 10 equiv.) dropwise at 0 °C and stirred for 3 h. The solvent was co-evaporated with toluene (3×) and the crude material was used for the next step without further purification. ESI-MS (*m/z*) 515.3 $[M+H]^+$. The obtained product (1.47 mg, 2.9 µmol, 1.18 equiv.) was dissolved in anhydrous DMF (0.2 mL) and was added to a mixture of Alexa Fluor 488 5-SDP ester (Invitrogen) (2 mg, 2.4 µmol, 1 equiv.) and DIPEA (1.3 µL, 7.3 µmol, 3 equiv.). The reaction mixture

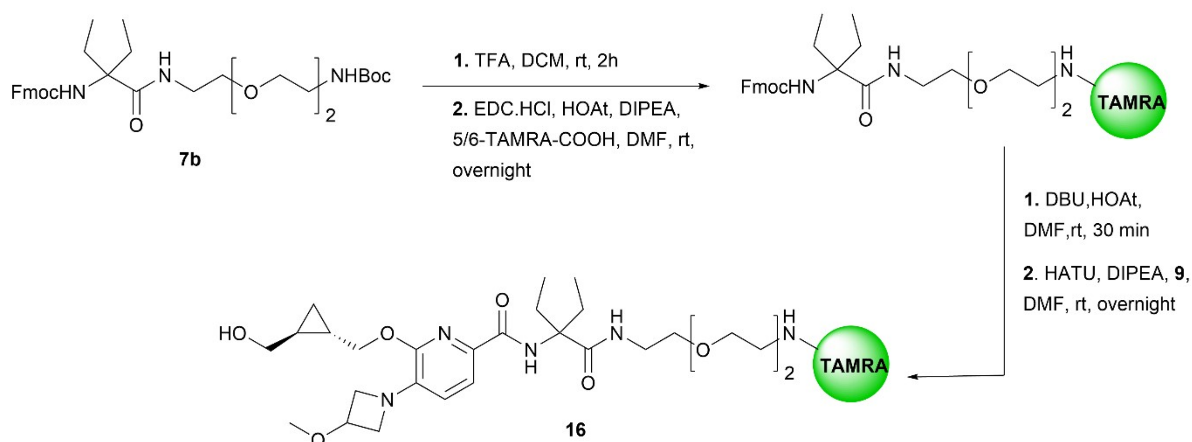
was allowed to stir at rt for overnight. After removal of solvent under reduced pressure, the residue was purified by preparative HPLC (5 to 95% acetonitrile in water with 0.1% TFA) to yield **14** as blue powder (2.6 mg, 42%). ESI-MS (*m/z*) 1031.3 $[M+H]^+$.^{1}H NMR (600 MHz, Methanol-d_4) δ 8.77 (d, *J* = 1.7 Hz, 1H), 8.26 (dd, *J* = 7.8, 1.8 Hz, 1H), 7.82 (d, *J* = 8.0 Hz, 1H), 7.52 (d, *J* = 8.0 Hz, 1H), 7.43 (d, *J* = 7.9 Hz, 1H), 7.34 – 7.23 (m, 2H), 7.02 (d, *J* = 9.3 Hz, 2H), 6.94 (m, 4H), 4.37 (s, 2H), 3.72 (t, *J* = 5.4 Hz, 2H), 3.69 – 3.63 (m, 6H), 3.61 (t, *J* = 5.6 Hz, 2H), 3.47 (t, *J* = 5.6 Hz, 2H), 2.44 (m, 2H), 2.01 (m, 1H), 1.84 (m, 2H), 1.05 – 0.98 (m, 2H), 0.74 (t, *J* = 7.4 Hz, 5H), 0.70 – 0.65 (m, 2H). ^{13}C NMR (*cryo*, 189 MHz, Methanol-d_4) δ ppm: 175.11, 168.21, 167.10, 164.84, 162.92 (d, $^1J_{C\text{-}F}$ = 242.7 Hz), 161.71, 159.94, 157.41, 157.16, 147.42, 142.29, 137.81 (d, $^4J_{CF}$ = 5.0 Hz), 136.16, 132.71, 132.50 (2C), 132.43, 131.88, 131.76 (d, $^3J_{C\text{-}F}$ = 7.8 Hz, 2C), 131.50, 120.87, 120.74 (2C), 115.98 (d, $^2J_{C\text{-}F}$ = 21.4 Hz, 2C), 114.54, 113.05, 71.47, 71.36, 70.65, 70.49, 66.38, 41.19 (2C), 40.66, 30.76, 29.04 (2C), 13.45, 8.74 (2C), 8.52 (2C). Due to low concentration, four quaternary carbons were not resolved. HRMS (ESI): *m*/*z* calculated for $C_{49}H_{51}FN_6O_{14}S_2$ + H^+ $[M+ H^+]$:1031.2961 Found:.1031.2938. HPLC (System 1): t_R = 3.39 min at λ = 254 nm, Purity = 99%.

3-(1-(5-Cyclopropyl-6-(4-fluorobenzyl)pyridin-2-yl)-3,3-diethyl-1,4,15-trioxo-8,11-dioxa-2,5,14-triazaicosan-20-yl)-2-((1*E*,3*E*)-5-((*E*)-3,3-dimethyl-5-sulfonato-1-(3-sulfonatopropyl)indolin-2-ylidene)penta-1,3-dien-1-yl)-3-methyl-1-(3-sulfonatopropyl)-3*H*-indol-1-ium-5-sulfonate (15)

To a solution of **10b** (4 mg, 6.5 µmol, 1 equiv.) in anhydrous DCM (2 mL) was added TFA (5 µL, 65 µmol, 10 equiv.) dropwise at 0 °C and stirred for 3 h. The solvent was co-evaporated with toluene (3×) and the crude material was used for the next step without further purification. ESI-MS (*m/z*) 515.3 $[M+H]^+$. The obtained product (2.5 mg, 4.9 µmol, 1.2 equiv.) was dissolved in anhydrous DMF (0.5 mL) and was added to a mixture of Alexa Fluor™ 647 carboxylic acid, tris(triethylammonium) salt, ThermoFisher) (5 mg, 4.1 µmol, 1 equiv.) HATU (2.3 mg, 6.2 µmol, 1.5 equiv.), and DIPEA (2.2 µL, 12.3 µmol, 3 equiv.) in anhydrous DMF (0.5mL). The reaction mixture was allowed to stir at rt for overnight. After removal of solvent under reduced pressure, the residue was purified by preparative HPLC (5 to 95% acetonitrile in water with 0.1% TFA) to yield **15** as dark blue powder (4.7 mg, 85%). ESI-MS (*m/z*) 678.4 $[M/2+H]^+$. HRMS (ESI): *m/z*

calculated for $C_{64}H_{84}FN_6O_{17}S_4 + H^+$ [M+ H^+]: 1355.4760 Found: 1355.4759. HPLC (System 1): t_R = 2.74 min at λ = 254 nm, Purity = 99%.

***N*-(1-(3',6'-Bis(dimethylamino)-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-5/6-yl)-13-ethyl-1,12-dioxo-5,8-dioxa-2,11-diazapentadecan-13-yl)-6-(((1*S*,2*S*)-2-(hydroxymethyl)cyclopropyl)methoxy)-5-(3-methoxyazetidin-1-yl)picolinamide (16).**



To a solution of **7b** (77.4 mg, 132.6 µmol) in DCM (4.5 mL) was added TFA (0.5 mL). The reaction was stirred for 2 h at rt. The solvent was co-evaporated with Toluene (ca. 1mL) under reduced pressure. This procedure was repeated two more times to remove residual TFA. The residue was used for the next step without further purification. ESI-MS (*m/z*) 484.2 $[M+H]^+$. A portion of the crude material (56.3 mg, 94.2 µmol) was dissolved in anhydrous DMF (1 mL) and added to a solution of 5- and 6-TAMRA-COOH (66.7 mg, 155.0 µmol), EDC.HCl (31.7 mg, 165.4 µmol), HOAt (22.3 mg, 165.0 µmol) and anhydrous DIPEA (70.0 µL, 410.9 µmol) anhydrous in DMF (1 mL). The reaction mixture was allowed to stir at rt for overnight. After removal of solvent under reduced pressure, the residue was purified by preparative HPLC (5 to 75% acetonitrile in water with 0.1% TFA) to yield a mixture of (9*H*-fluoren-9-yl)methyl (1-(3',6'-bis(dimethylamino)-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-5/6-yl)-13-ethyl-1,12-dioxo-5,8-dioxa-2,11-diazapentadecan-13-yl)carbamate as purple powder (14.8 mg, 16%) ESI-MS (*m/z*) 896.4 $[M+H]^+$. ^1H NMR (600 MHz, Methanol-d_4) δ ppm: 8.79 (d, *J* = 1.8 Hz, 1H), 8.40 (d, *J* = 8.2 Hz, 1H), 8.27 (dd, *J* = 7.9, 1.8 Hz, 1H), 8.21 (dd, *J* = 8.3, 1.8 Hz, 1H), 7.83 (d, *J* = 1.8 Hz, 1H), 7.76 (dd, *J* = 7.6, 2.3 Hz, 4H), 7.58 (dd, *J* = 14.0, 7.5 Hz, 4H), 7.48 (d, *J* = 7.9 Hz, 1H), 7.35 (td, *J* = 7.5, 3.2 Hz, 4H), 7.26 (q, *J* = 6.8 Hz, 4H), 7.08

(d, J = 9.5 Hz, 4H), 6.98 (dt, J = 9.5, 2.7 Hz, 4H), 6.92 – 6.88 (m, 4H), 4.4 - 4.25 (m, 4H), 4.14 – 4.03 (m, 2H), 3.71 (t, J = 5.2 Hz, 2H), 3.69 – 3.36 (m, 22H), 3.26 (s, 24H), 2.05 – 1.91 (m, 2H), 1.77 (s, 2H), 0.72 (s, 12H). To avoid complications, the integrations were reported for 1:1 mixture. The ratio of 5-/6- isomers is 1:1. Mixture A: To a solution of obtained intermediate (8 mg, 8.9 µmol) in anhydrous DMF (1 mL) was treated with DBU (2 µL, 10.7 µmol). After 30 min, HOAt (2 mg, 14.3 µmol) was added and the resulting mixture was allowed to stir for another 10 min. Mixture B: To a solution of **9**(4.1 mg, 13.4 µmol) in anhydrous DMF (1 mL) was added DIPEA (8 µL, 45 µmol) and HATU (3.7 mg, 9.8 µmol) and stirred for 20 min. Then mixtures A and B were combined and the progress of the reaction was judged by LCMS. After removal of the solvent under reduced pressure, the residue was purified by preparative HPLC (5 to 50% acetonitrile in water) to yield **16** as purple powder (8.3 mg, 96 %). ESI-MS (*m/z*) 964.3[M+H]$^+$. ^{1}H NMR (*cryo* 750 MHz, Methanol-d_4) δ ppm: 9.20 (s, 1H), 9.15 (s, 1H), 8.54 (d, J = 1.9 Hz, 1H), 8.15 (d, J = 8.2 Hz, 1H), 8.10 (dd, J = 8.2, 1.8 Hz, 1H), 8.07 (dd, J = 7.8, 1.9 Hz, 1H), 7.72 (d, J = 1.7 Hz, 1H), 7.50 (d, J = 7.8 Hz, 1H), 7.46 (d, J = 7.8 Hz, 1H), 7.36 (d, J = 7.8 Hz, 1H), 7.26 – 7.22 (m, 4H), 7.01 – 6.98 (m, 4H), 6.90 (d, J = 2.3 Hz, 4H), 6.67 (d, J = 7.8 Hz, 1H), 6.65 (d, J = 7.8 Hz, 1H),4.43 – 4.39 (m, 2H), 4.30 – 4.28 (m, 6H), 4.17 – 4.12 (m, 2H), 3.87 – 3.83 (m, 4H), 3.73 – 3.68 (m, 4H), 3.67 – 3.59 (m,12), 3.58 – 3.53 (m, 4H), 3.50 – 3.46 (m, 4H), 3.39 - .3.35 (m, 4H), 3.33 (s, 6H), 3.26 (s, 24H), 2.57– 2.52 (m, 4H), 1.81 – 1.75 (m, 4H), 1.31 – 1.29 (m, 2H), 1.14 – 1.11 (m, 2H), 0.74 – 0.69 (m, 12H), 0.67 – 0.64 (m, 2H), 0.55 – 0.52 (m, 2H). To avoid complications, the integrations were reported for 1:1 mixture. The ratio of 5-/6- isomers is 1:1. HRMS (ESI): *m/z* calculated for $C_{52}H_{65}FN_7O_{11}$ + H^+ [M+ H^+]: 964.4815. Found: 964.4824. HPLC (System 1): t_R = 3.14 min at λ = 254 nm, Purity = 98.4%.

***N*-(1-(3,7-Bis(dimethylamino)-5,5-dimethyl-3'-oxo-3'*H*,5*H*-spiro[dibenzo[*b*,*e*]siline-10,1'-isobenzofuran]-6'-yl)-13-ethyl-1,12-dioxo-5,8-dioxa-2,11-diazapentadecan-13-yl)-6-(((1*S*,2*S*)-2-(hydroxymethyl)cyclopropyl)methoxy)-5-(3-methoxyazetidin-1-yl)picolinamide (17)**

To a solution of **11b** (10 mg, 15.3 µmol, 1 equiv.) in anhydrous DCM (1.5 mL) was added TFA (11 µL, 0.14 mmol, 9 equiv.) dropwise at 0 °C and stirred for 3 h. The mixture was immediately transferred to a separatory funnel diluted and partitioned with DCM (20 mL) and sat. solution of Na_2CO_3. The organic phase was separated and the aqueous phase was extracted with DCM (x2). The combined organic phases were washed with brine and dried over Na_2SO_4, filtered and

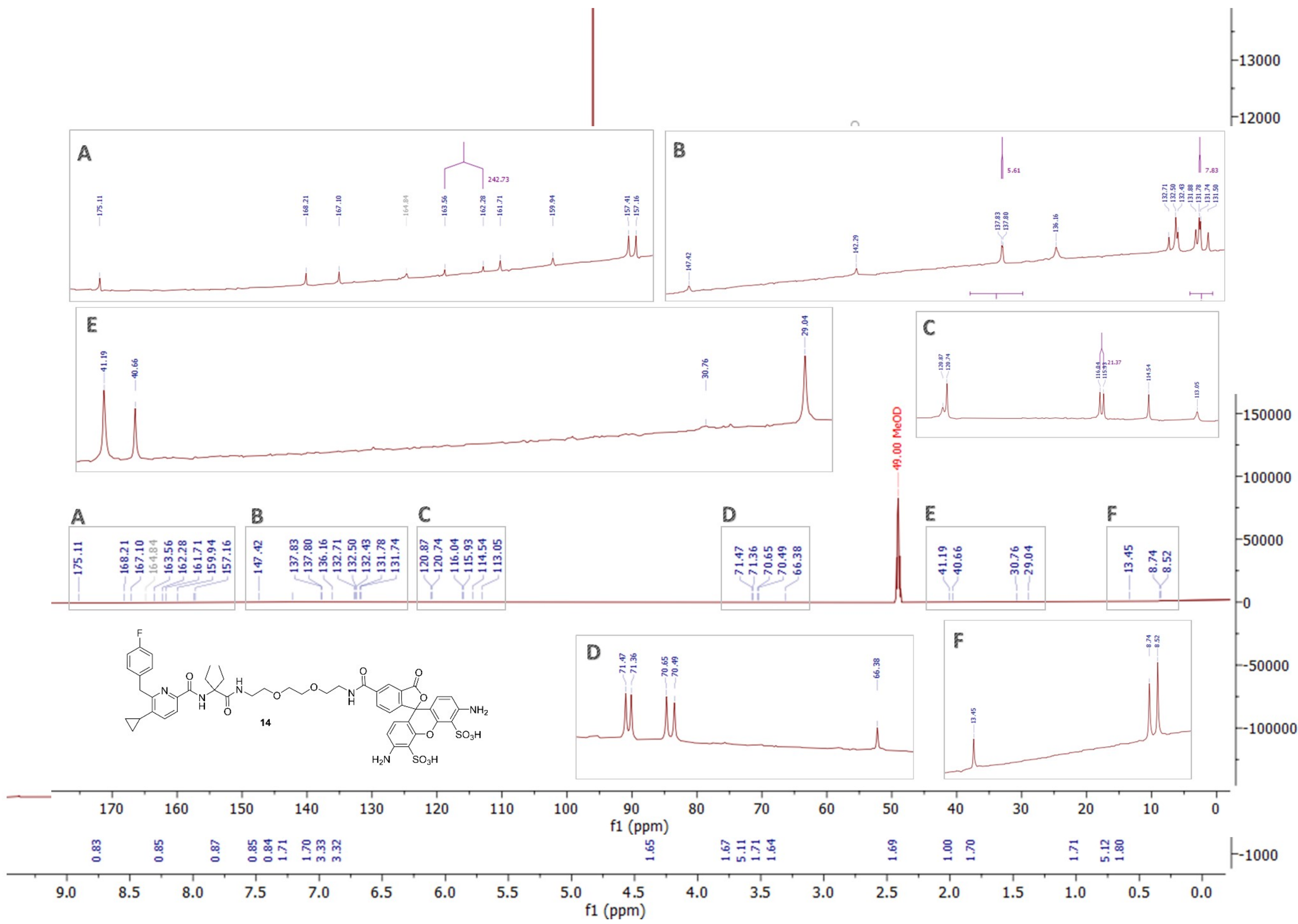
concentrated. ESI-MS (*m/z*) 651.9 $[M+H]^+$. The residue (6.4 mg, 11.6 µmol, 1.1 equiv.) was dissolved in anhydrous DMF (0.2 mL) and was added to a mixture of SiR-COOH (5 mg, 10.6 µmol, 1 equiv.), HATU (4 mg, 10.6 µmol, 1 equiv.), and DIPEA (5.5 µL, 31.7 µmol, 3 equiv.) in anhydrous DMF (0.2 mL). The reaction mixture was allowed to stir at rt for overnight. After removal of solvent under reduced pressure, the residue was purified by preparative HPLC (5 to 75% acetonitrile in water) to yield **17** as blue powder (5.2 mg, 49%). ESI-MS (*m/z*) 503.5 $[M/2+H]^+$. ^1H NMR (600 MHz, Methanol-d_4) δ ppm: 9.15 (br s, 1H, CONH), 8.91 (br s, 1H, CONH), 8.24 (d, J = 8.2 Hz, 1H), 8.11 (dd, J = 8.2, 1.7 Hz, 1H), 7.71 (d, J = 1.7 Hz, 1H), 7.59 (br s, 1H, CONH), 7.51 (d, J = 7.9 Hz, 1H), 7.26 (d, J = 2.8 Hz, 2H), 6.91 (d, J = 9.4 Hz, 2H), 6.72 (dd, J = 9.5, 2.8 Hz, 2H), 6.68 (d, J = 7.8 Hz, 1H), 4.42 (dd, J = 11.3, 6.6 Hz, 1H), 4.31 – 4.27 (m, 3H), 4.16 (dd, J = 11.3, 7.6 Hz, 1H), 3.87 – 3.85 (m, 2H), 3.65 (t, J = 5.5 Hz, 2H), 3.63 – 3.58 (m, 4H), 3.56 (t, J = 5.5 Hz, 2H), 3.53 (t, J = 5.5 Hz, 2H), 3.49 (dd, J = 11.4, 6.3 Hz, 1H), 3.39 – 3.35 (m, 3H), 3.33 (s, 3H), 3.22 (s, 12H), 2.57 - 2.50 (m, 2H), 1.80 – 1.73 (m, 2H), 1.34 – 1.30 (m, 1H), 1.15 - 1.12 (m, 1H), 0.73 – 0.69 (m, 6H), 0.67 – 0.64 (m, 1H), 0.63 (s, 3H), 0.58 (s, 3H), 0.55 – 0.52 (m, 1H). ^{13}C NMR (151 MHz, Methanol-d_4) δ ppm: 175.33, 168.30, 165.57, 153.07, 139.98, 136.55, 128.98, 120.86 118.69, 117.42, 115.13, 71.64, 71.33 (2C), 71.61 (2C), 70.39, 61.33 (2C), 56.39, 41.07, 40.84, 40.72, 29.45 (2C), 20.59, 17.16, 9.17, 8.62 (2C) -0.59, -1.57. Due to low concentration, some quaternary carbons were not resolved. HRMS (ESI): *m/z* calculated for $C_{54}H_{71}N_7O_{10}Si + H^+$ $[M+ H^+]$: 1006.5104. Found: 1006.5155. HPLC (System 1): t_R = 3.86 min at λ = 220 nm, Purity = 97.6%.

3',6'-Diamino-5/6-((3,3-diethyl-1-(6-(((1*S*,2*S*)-2-(hydroxymethyl)cyclopropyl)methoxy)-5-(3-methoxyazetidin-1-yl)pyridin-2-yl)-1,4-dioxo-8,11-dioxa-2,5-diazatridecan-13-yl)carbamoyl)-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthene]-4',5'-disulfonic acid (18)

To a solution of **11b** (6 mg, 9.2 µ mol) in anhydrous DCM (1 mL) was added TFA (6 µL, 0.83 mmol, 9 equiv.) dropwise at 0 °C and stirred for 3 h. The mixture was immediately transferred to a separatory funnel diluted and partitioned with DCM (20 mL) and sat. solution of Na_2CO_3. The organic phase was separated and the aqueous phase was extracted with DCM (x2). The combined organic phases were washed with brine and dried over Na_2SO_4, filtered and concentrated. ESI-MS (*m/z*) 651.9 $[M+H]^+$. The residue (4 mg, 7.2 µmol, 1.2 equiv.) was dissolved in anhydrous DMF (0.5 mL) and was added to a mixture of Alexa Fluor® 488 carboxylic acid, tris(triethylammonium)

salt (ThermoFisher) as mixed isomers (5 mg, 6 μmol, 1 equiv.), HATU (2 mg, 6.6 μmol, 1.1 equiv.), and DIPEA (2.6 μL, 15 μmol,2.5 equiv.) in anhydrous DMF (0.5 mL). The reaction mixture was allowed to stir at rt for overnight. After removal of solvent under reduced pressure, the residue was purified by preparative HPLC (5 to 75% acetonitrile in water) to yield **18** as yellow powder (1.7 mg, 26%). ESI-MS (*m/z*) 1068.0 $[M+H]^+$. HRMS (ESI): *m/z* calculated for $C_{48}H_{57}N_7O_{17}S_2 + H^+$ $[M+ H^+]$: 1068.3325. Found: 1068.3329. HPLC (System 1): t_R = 2.45 min at λ = 254 nm, Purity = 98%.

NMR Spectra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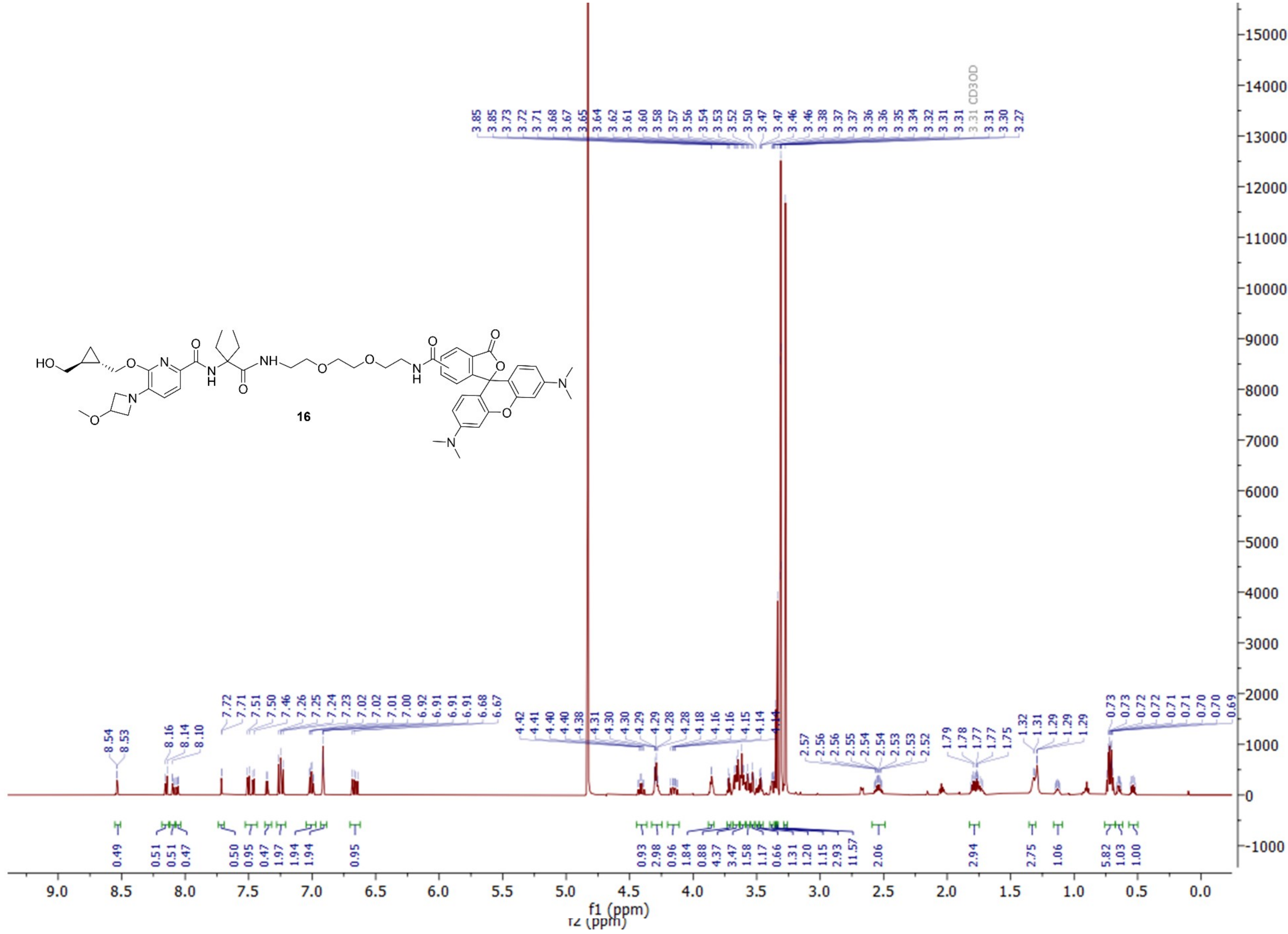
f1 (ppm)
f1 (ppm)



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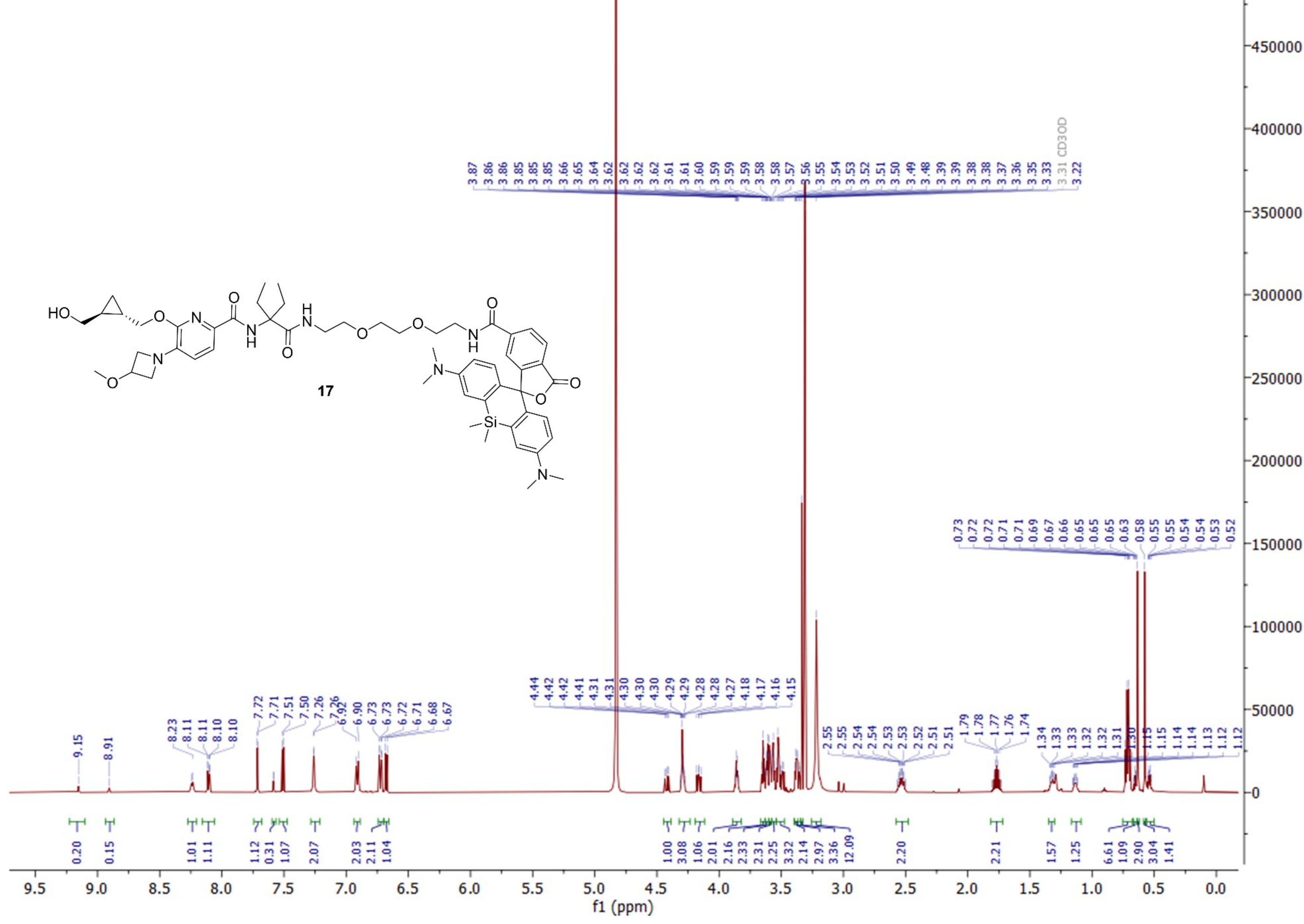
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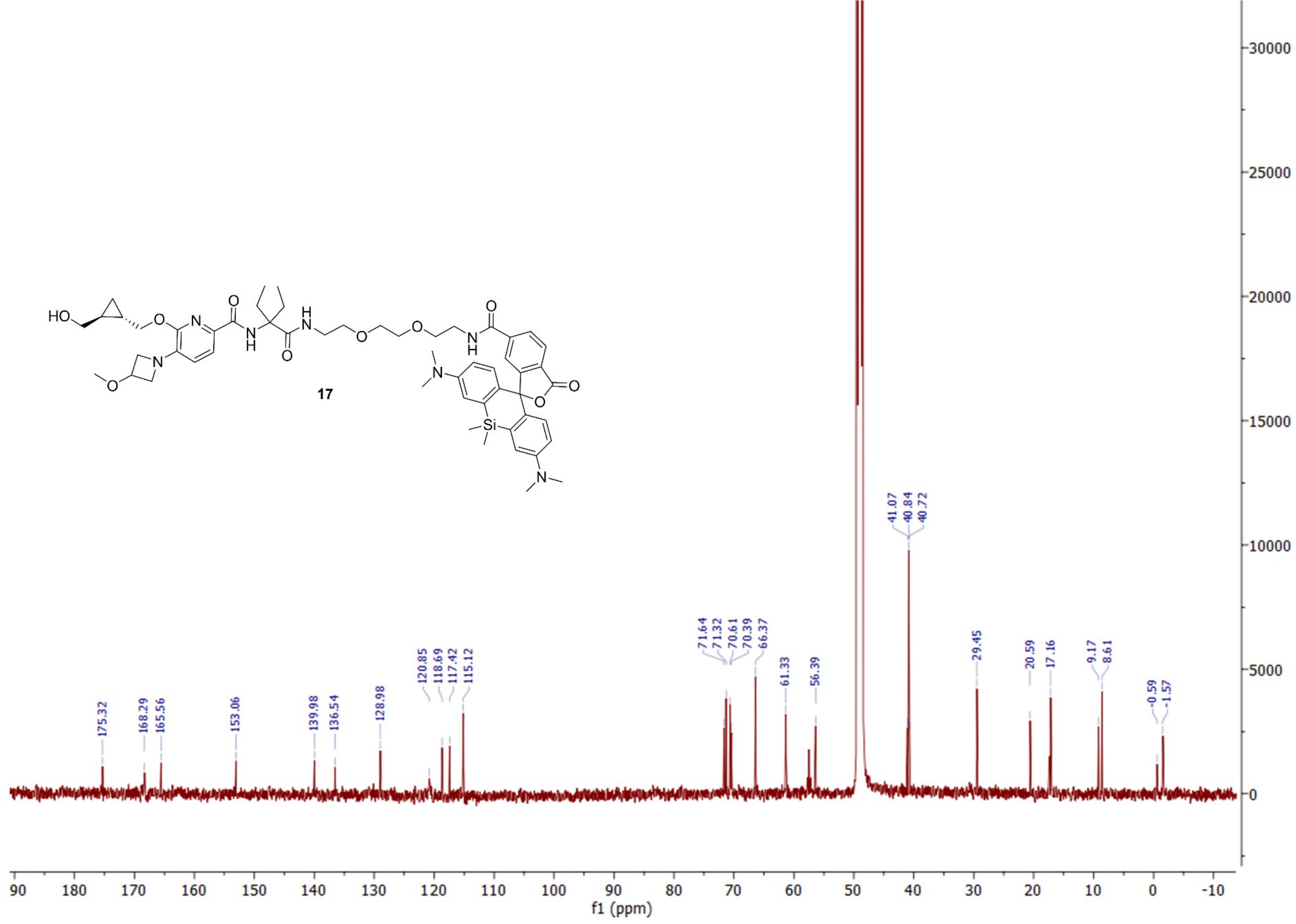
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-1.57
f1 (ppm)
17

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