Supporting Information

Sulfonated red and far-red rhodamines to visualize SNAP- and Halo-tagged cell surface proteins

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1. General

All chemical reagents and anhydrous solvents for synthesis were purchased from commercial suppliers (AAT Bioquest, Roth, Sigma-Aldrich, Fluka, Acros, Fluorochem, TCI) and were used without further purification or distillation.

BG-NH$_2$ and **CA-NH$_2$** were prepared and obtained as described previously.[1]

NMR spectra were recorded in deuterated solvents on a Bruker AVANCE III 600 and calibrated to residual solvent peaks (^{1}H in ppm): MeOD-d$_4$ (3.31). Multiplicities are abbreviated as follows: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, br = broad, m = multiplet. Coupling constants *J* are reported in Hz. Spectra are reported based on appearance, not on theoretical multiplicities derived from structural information.

LC-MS was performed on an Agilent 1260 Infinity II LC System equipped with Agilent SB-C18 column (1.8 µm, 2.1 × 50 mm). Buffer A: 0.1% FA in H_2O Buffer B: 0.1% FA acetonitrile. The typical gradient was from 10% B for 0.5 min → gradient to 95% B over 5 min → 95% B for 0.5 min → gradient to 99% B over 1 min with 0.8 mL/min flow.

High resolution mass spectrometry was performed using a Bruker maXis II ETD hyphenated with a Shimadzu Nexera system. The instruments were controlled via Brukers otofControl 4.1 and Hystar 4.1 SR2 (4.1.31.1) software. The acquisition rate was set to 3 Hz and the following source parameters were used for positive mode electrospray ionization: End plate offset = 500 V; capillary voltage = 3800 V; nebulizer gas pressure = 45 psi; dry gas flow = 10 L/min; dry temperature = 250 °C. Transfer, quadrupole and collision cell settings are mass range dependent and were fine-adjusted with consideration of the respective analyte's molecular weight. For internal calibration sodium format clusters were used. Samples were desalted via fast liquid chromatography. A Supelco Titan^{TM} C18 UHPLC Column, 1.9 µm, 80 Å pore size, 20 × 2.1 mm and a 2 min gradient from 10 to 98% aqueous MeCN with 0.1% FA (H_2O: Carl Roth GmbH + Co. KG ROTISOLV® Ultra LC-MS; MeCN: Merck KGaA LiChrosolv® Acetonitrile hypergrade for LC-MS; FA - Merck KGaA LiChropur® Formic acid 98%-100% for LC-MS) was used for separation. Sample dilution in 10% aqueous ACN (hyper grade) and injection volumes were chosen dependent of the analyte's ionization efficiency. Hence, on-column loadings resulted between 0.25–5.0 ng. Automated internal re-calibration and data analysis of the recorded spectra were performed with Bruker's DataAnalysis 4.4 SR1 software.

Preparative or semi-preparative HPLC was performed an Agilent 1260 Infinity II LC System equipped with columns as followed: preparative column –Reprospher 100 C18 columns (10 µm: 50 x 30 mm at 20 mL/min flow rate; semi-preparative column – 5 µm: 250 x 10 mm at 4 mL/min flow rate. Eluents A (0.1% TFA in H_2O) and B (0.1% TFA in MeCN) were applied as a linear gradient. Peak detection was performed at maximal absorbance wavelength.

Abbreviations: Boc: *tert*-butoxycarbonyl; DIPEA: *N,N*-diisopropylethylamine; DMSO: dimethylsulfoxide; FA: formic acid; HBTU: (2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; TFA: trifluoroacetic acid; TSTU: *N,N,N′,N′*-Tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate.

2. Synthesis

2.1. General procedure A for Buchwald-Hartwig coupling and deprotection

A flame-dried Schlenk flask was charged with bistriflate **1** (AAT Bioquest, 1.0 equiv.), $Pd_2(dba)_3$ (0.2 equiv.), XPhos (0.3 equiv.), Cs_2CO_3 (4.8 equiv.), and dissolved in dry 1,4-dioxanes (345 equiv.) under an argon atmosphere. Methyl azetidine 3-carboxylate hydrochloride (3.0 equiv.) was added and the reaction mixture was heated to 80 °C for 4 h. After cooling, MeOH (345 equiv.) and 1 M LiOH (345 equiv.) were added and the reaction was allowed to stir for 1 h at r.t. before it was quenched with 1 mL HOAc. All volatiles were removed in vacuo, the residue taken up in DMSO:MeCN:H_2O:HOAc (2:1:1:0.02), filtered using a 0.45 μm syringe filter and subjected to RP-HPLC purification.

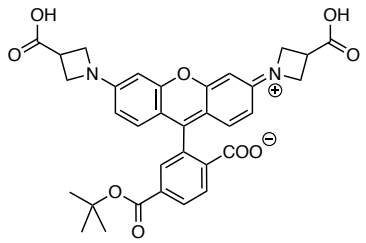
2.2. General procedure B for taurine coupling and deprotection

A 4 mL dram vial was charged with bisacid **2** (1.0 equiv.) dissolved in DMF (2 mg/1 mL) and DIPEA (8.0 equiv.) and TSTU (2.8 equiv.) were combined before taurine (3.0 equiv.) was added in one portion. The reaction mixture was allowed to incubate for 1 h before it was quenched by addition of HOAc (10.0 equiv.) and 10 vol% water and subjected to RP-HPLC purification. The product containing fractions were pooled and lyophilized, and upon dryness redissolved in neat TFA and incubated for 3 h at r.t. before all volatiles were removed under a gentle stream of nitrogen, before subjecting to RP-HPLC purification. The product containing fractions were pooled and lyophilized.

2.3. General procedure C for peptide coupling with TSTU

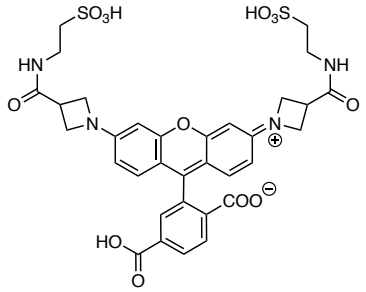
A 4 mL dram vial was charged with acid **3** (1.0 equiv.) dissolved in DMF (2 mg/1 mL) and DIPEA (8.0 equiv.) and TSTU (1.4 equiv.) were combined before either **BG-NH_2** or **CA-NH_2** (1.5 equiv.) was added in one portion. The reaction mixture was allowed to incubate for 1 h before it was quenched by addition of HOAc (10.0 equiv.) and 10 vol% water and subjected to RP-HPLC purification. The product containing fractions were pooled and lyophilized to obtain the desired product.

2.4. 4-(*tert*-Butoxycarbonyl)-2-(3-(3-carboxyazetidin-1-ium-1-ylidene)-6-(3-carboxy-azetidin-1-yl)-3*H*-xanthen-9-yl)benzoate (2a)



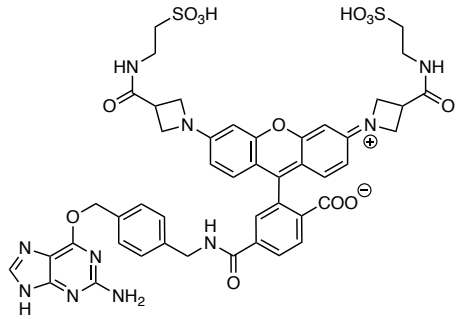
2a was prepared according to general procedure A.
bistriflate: **1a** (150 mg, 215 µmol), yield: 95 mg (74%). Red powder.
^{1}H NMR (600 MHz, MeOD-d_4): δ [ppm] = 8.40 (d, J = 8.2 Hz, 1H), 8.33 (dd, J = 8.2, 1.7 Hz, 1H), 7.89 (d, J = 1.7 Hz, 1H), 7.11 (m, 2H), 6.77-6.56 (m, 4H), 4.50 (dd, J = 10.4, 8.6 Hz, 4H), 4.42 (dd, J = 10.6, 5.9 Hz, 4H), 3.78-3.54 (m, 2H), 1.59 (s, 9H).
HRMS (ESI): calc. for $C_{33}H_{31}N_2O_9$ $[M+H]^+$: 599.2024, found: 599.2021.

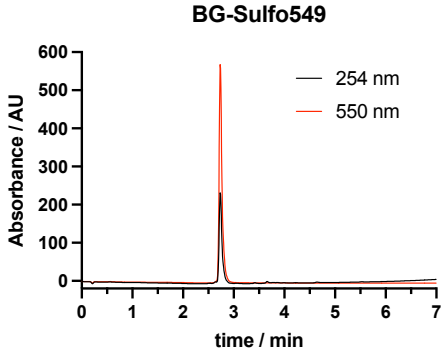
2.5. 4-Carboxy-2-(3-(3-((2-sulfoethyl)carbamoyl)azetidin-1-ium-1-ylidene)-6-(3-((2-sulfoethyl)carbamoyl)azetidin-1-yl)-3*H*-xanthen-9-yl)benzoate (3a)



3a was prepared according to general procedure B.
acid: **2a** (20 mg, 33.4 µmol), yield: 12.5 mg (49%). Red powder.
^{1}H NMR (600 MHz, MeOD-d_4): δ [ppm] = 8.60-8.19 (m, 2H), 7.98 (d, J = 1.7 Hz, 1H), 7.10 (d, J = 9.1 Hz, 2H), 6.80-6.34 (m, 4H), 4.42 (m, 4H), 4.37 (m, 4H), 3.67-3.60 (m, 6H), 3.00 (t, J = 6.6 Hz, 4H).
HRMS (ESI): calc. for $C_{33}H_{33}N_4O_{13}S_2$ $[M+H]^+$: 757.1480, found: 757.1476.

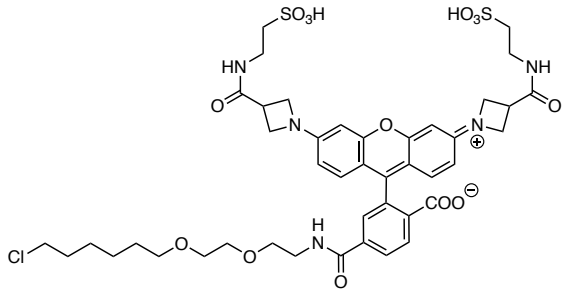
2.6. 4-((4-(((2-Amino-9*H*-purin-6-yl)oxy)methyl)benzyl)carbamoyl)-2-(3-(3-((2-sulfoethyl)carbamoyl)azetidin-1-ium-1-ylidene)-6-(3-((2-sulfoethyl)carbamoyl)-azetidin-1-yl)-3*H*-xanthen-9-yl)benzoate (BG-Sulfo549)

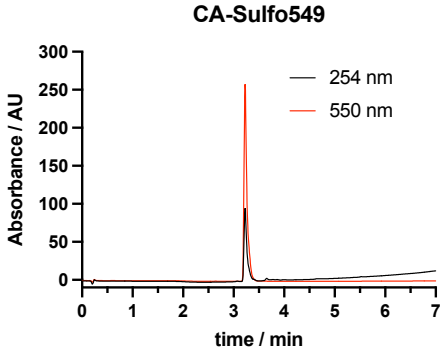




BG-Sulfo549 was prepared according to general procedure C.
amine: **BG-NH$_2$**
acid: **3a** (6.0 mg, 7.9 µmol), yield: 3.0 mg (38%). Red powder.
^{1}H NMR (600 MHz, DMSO-d$_6$): δ [ppm] = 9.29 (s, 1H), 8.65 (s, 1H), 8.30 (d, J = 8.3 Hz, 1H), 8.25 (d, J = 9.3 Hz, 1H), 8.09 (s, 2H), 7.89 (s, 1H), 7.50 (d, J = 8.0 Hz, 2H), 7.36 (d, J = 8.0 Hz, 2H), 7.00 (d, J = 9.0 Hz, 2H), 6.65 (d, J = 9.2 Hz, 2H), 6.63 (s, 1H), 5.55 (s, 2H), 4.50-4.46 (m, 2H), 4.40-4.32 (m, 4H), 4.28-4.20 (m, 4H), 2.60 (t, J = 7.2 Hz, 4H). (some protons masked under water peak).
HRMS (ESI): calc. for $C_{46}H_{46}N_{10}O_{13}S_2$ $[M+2H]^{2+}$: 505.1338, found: 505.1336.

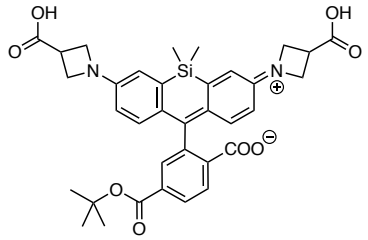
2.7. 4-((2-(2-((6-Chlorohexyl)oxy)ethoxy)ethyl)carbamoyl)-2-(3-(3-((2-sulfoethyl)-carbamoyl)azetidin-1-ium-1-ylidene)-6-(3-((2-sulfoethyl)carbamoyl)azetidin-1-yl)-3*H*-xanthen-9-yl)benzoate (CA-Sulfo549)





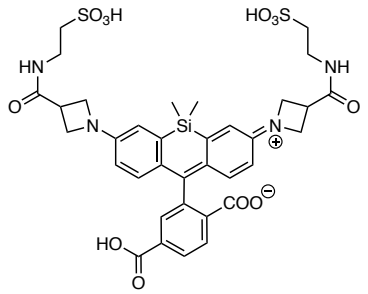
CA-Sulfo549 was prepared according to general procedure C.
amine: **Halo-NH$_2$**
acid: **3a** (6.0 mg, 7.9 µmol), yield: 4.5 mg (59%). Red powder.
^{1}H NMR (600 MHz, MeOD-d$_4$): δ [ppm] = 8.42 (d, J = 8.2 Hz, 1H), 8.23 (dd, J = 8.3, 1.8 Hz, 1H), 7.85 (s, 1H), 7.12 (s, 1H), 7.10 (s, 1H), 6.67 (dd, J = 9.2, 2.2 Hz, 2H), 6.57 (d, J = 2.1 Hz, 2H), 4.42 (q, J = 8.5 Hz, 4H), 4.37 (q, J = 4.8 Hz, 4H), 3.69-3.62 (m, 10H), 3.62-3.56 (m, 4H), 3.55 (t, J = 6.6 Hz, 2H), 3.45 (t, J = 6.5 Hz, 2H), 3.03 (t, J = 6.6 Hz, 4H), 1.74 (p, J = 7.1 Hz, 2H), 1.53 (p, J = 7.0 Hz, 2H), 1.42 (p, J = 5.0 Hz, 2H), 1.38-1.30 (m, 2H).
HRMS (ESI): calc. for $C_{43}H_{53}ClN_5O_{14}S_2$ $[M+H]^+$: 962.2713, found: 962.2721.

2.8. 4-(*tert*-Butoxycarbonyl)-2-(3-(3-carboxyazetidin-1-ium-1-ylidene)-7-(3-carboxy-azetidin-1-yl)-5,5-dimethyl-3,5-dihydrodibenzo[*b*,*e*]silin-10-yl)benzoate (2b)



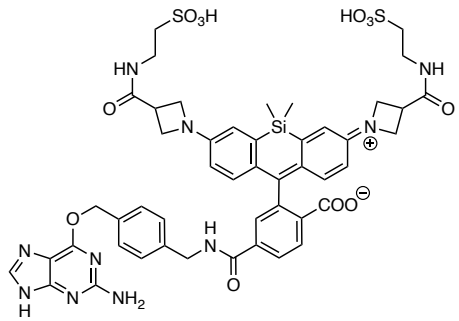
2b was prepared according to general procedure A.
bistriflate: **1b** (139 mg, 203 µmol), yield: 20.0 mg (37%). Blue powder.
^{1}H NMR (600 MHz, MeOD-d_4): δ [ppm] = 8.35-8.01 (m, 2H), 7.71 (d, J = 1.1 Hz, 1H), 7.01–6.71 (m, 4H), 6.40 (dd, J = 9.1, 2.6 Hz, 2H), 4.44-4.09 (m, 8H), 3.74-3.48 (m, 2H), 1.57 (s, 9H), 0.64 (s, 3H), 0.55 (s, 3H).
HRMS (ESI): calc. for $C_{35}H_{37}N_2O_8Si$ $[M+H]^+$: 641.2314, found: 641.2314.

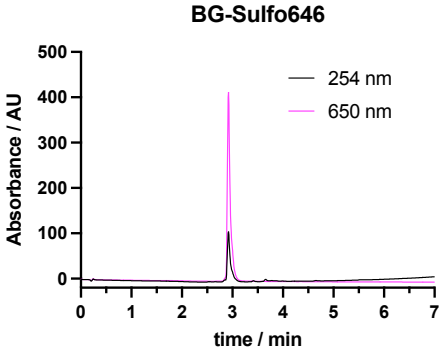
2.9. 4-Carboxy-2-(5,5-dimethyl-3-(3-((2-sulfoethyl)carbamoyl)azetidin-1-ium-1-ylidene)-7-(3-((2-sulfoethyl)carbamoyl)azetidin-1-yl)-3,5-dihydrodibenzo[*b*,*e*]silin-10-yl)benzoate (3b)



3b was prepared according to general procedure B.
acid: **2b** (20.0 mg, 31.3 µmol), yield: 12.5 mg (50%). Blue powder.
^{1}H NMR (600 MHz, MeOD-d_4): δ [ppm] = 8.36-8.14 (m, 2H), 7.81 (d, J = 1.7 Hz, 1H), 7.05-6.70 (m, 4H), 6.43-6.19 (m, 2H), 4.59-4.22 (m, 8H), 3.64 (t, J = 6.6 Hz, 4H), 3.00 (t, J = 6.6 Hz, 4H), 0.61 (s, 3H), 0.54 (s, 3H).
HRMS (ESI): calc. for $C_{35}H_{39}N_4O_{12}S_2Si$ $[M+H]^+$: 799.1770, found: 799.1767.

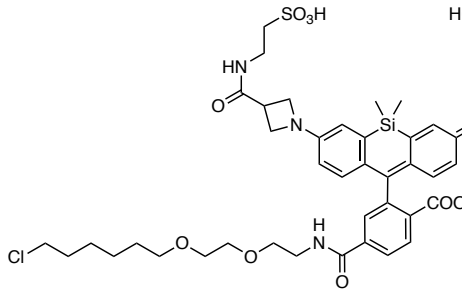
2.10. 4-((4-(2-(2-Amino-9*H*-purin-6-yl)ethyl)benzyl)carbamoyl)-2-(5,5-dimethyl-3-(3-((2-sulfoethyl)carbamoyl)azetidin-1-ium-1-ylidene)-7-(3-((2-sulfoethyl)-carbamoyl)azetidin-1-yl)-3,5-dihydrodibenzo[*b*,*e*]silin-10-yl)benzoate (BG-Sulfo646)

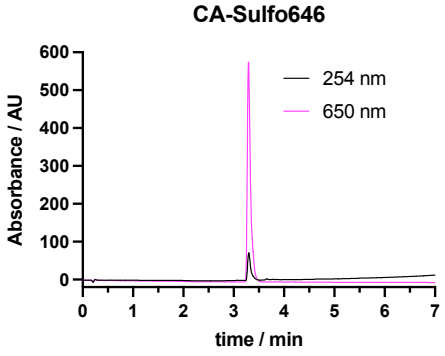




BG-Sulfo646 was prepared according to general procedure C.
amine: **BG-NH$_2$**
acid: **3b** (6.0 mg, 7.5 µmol), yield: 2.5 mg (32%). Blue powder.
^{1}H NMR (600 MHz, DMSO-d$_6$): δ [ppm] = 9.30 (t, J = 5.8 Hz 1H), 8.12 (d, J = 7.9 Hz, 1H), 8.04 (d, J = 7.8 Hz, 1H), 7.94 (s, 2H), 7.70 (s, 1H), 7.50 (d, J = 7.9 Hz, 2H), 7.34 (d, J = 7.9 Hz, 2H), 6.75 (s, 2H), 6.63 (d, J = 8.7 Hz, 2H), 6.63 (d, J = 8.7 Hz, 2H), 6.36 (d, J = 8.7 Hz, 2H), 5.58 (s, 2H), 4.44 (d, J = 5.7 Hz, 2H), 4.06-3.93 (m, 4H), 3.88-3.77 (m, 4H), 2.61-2.56 (m, 6H), 2.09 (s, 1H), 2.08 (s, 1H), 1.92 (s, 1H), 1.24 (s, 4H), 0.60 (s, 3H), 0.49 (s, 3H).
HRMS (ESI): calc. for $C_{48}H_{52}N_{10}O_{12}S_2Si$ $[M+2H]^{2+}$: 526.1483, found: 526.1481.

2.11. 4-((2-(2-((6-Chlorohexyl)oxy)ethoxy)ethyl)carbamoyl)-2-(5,5-dimethyl-3-(3-((2-sulfoethyl)carbamoyl)azetidin-1-ium-1-ylidene)-7-(3-((2-sulfoethyl)-carbamoyl)azetidin-1-yl)-3,5-dihydrodibenzo[*b*,*e*]silin-10-yl)benzoate (CA-Sulfo646)

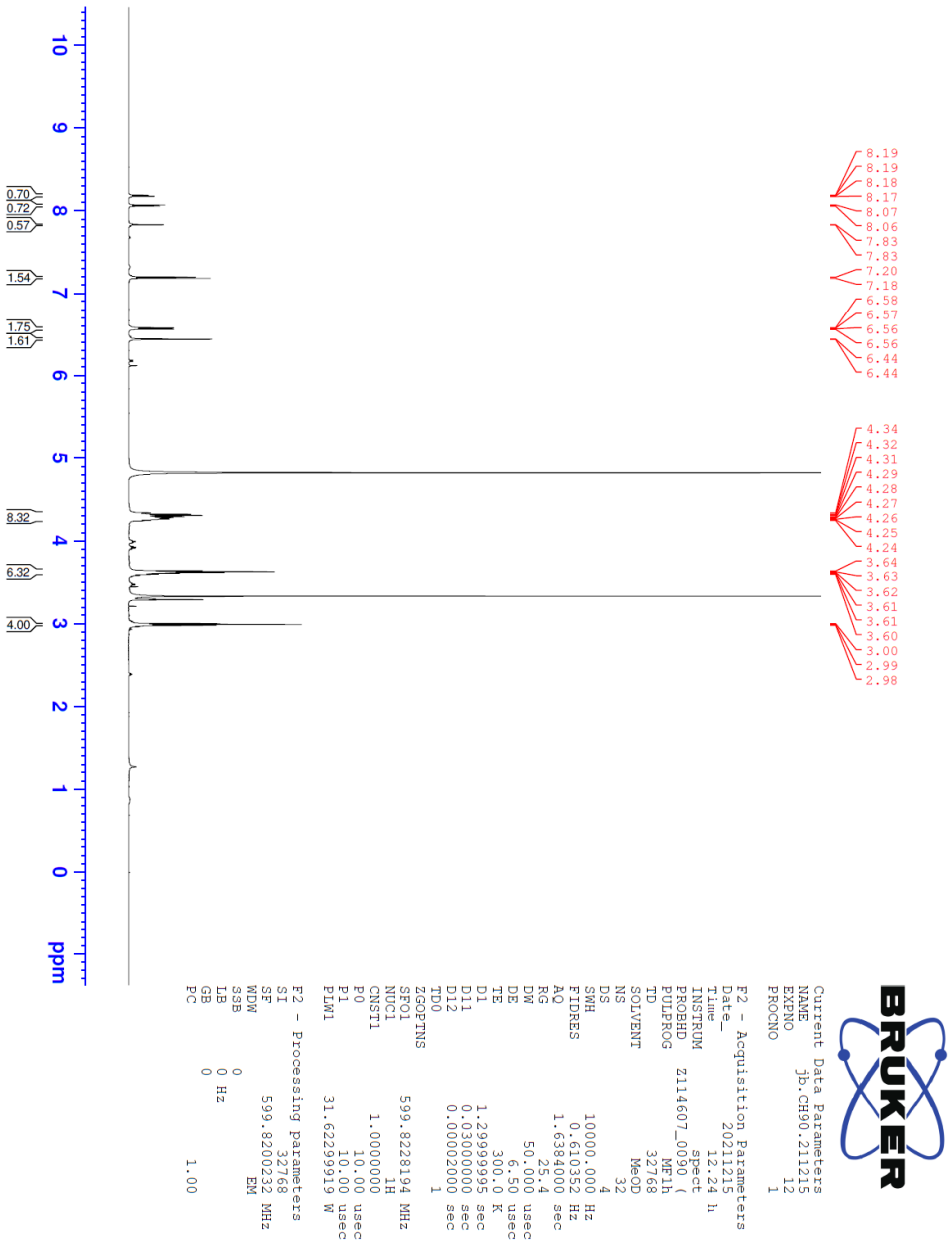




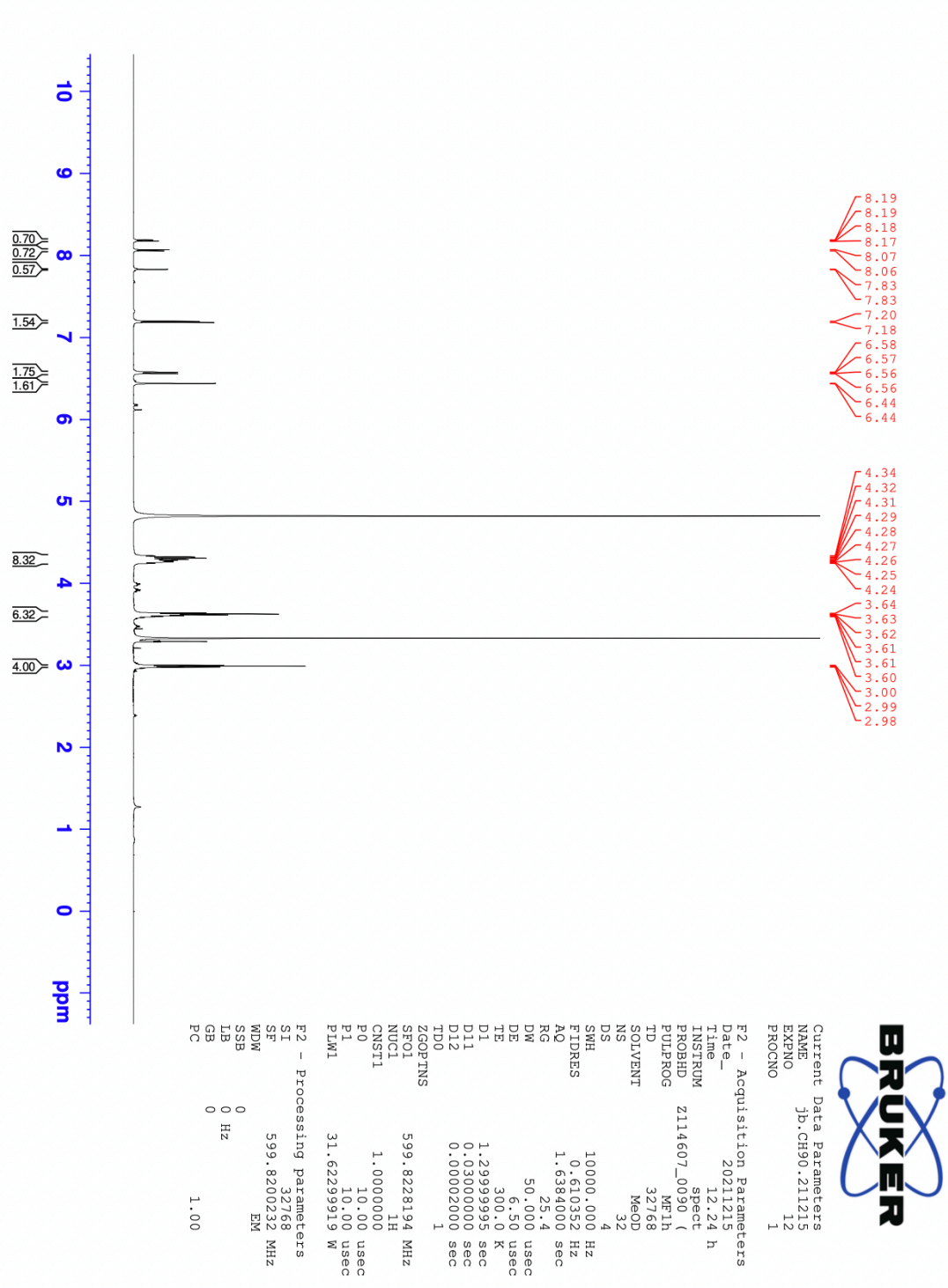
CA-Sulfo646 was prepared according to general procedure C.
amine: **Halo-NH$_2$**
acid: **3b** (6.0 mg, 7.5 µmol), yield: 5.2 mg (69%). Blue powder.
^{1}H NMR (600 MHz, MeOD-d$_4$): δ [ppm] = 8.42 (d, J = 8.2 Hz, 1H), 8.23 (dd, J = 8.3, 1.8 Hz, 1H), 7.85 (s, 1H), 7.12 (s, 1H), 7.10 (s, 1H), 6.67 (dd, J = 9.2, 2.2 Hz, 2H), 6.57 (d, J = 2.1 Hz, 2H), 4.42 (q, J = 8.5 Hz, 4H), 4.37 (q, J = 4.8 Hz, 4H), 3.69-3.62 (m, 10H), 3.62-3.56 (m, 4H), 3.55 (t, J = 6.6 Hz, 2H), 3.45 (t, J = 6.5 Hz, 2H), 3.03 (t, J = 6.6 Hz, 4H), 1.74 (p, J = 7.1 Hz, 2H), 1.53 (p, J = 7.0 Hz, 2H), 1.42 (p, J = 5.0 Hz, 2H), 1.38-1.30 (m, 2H).
HRMS (ESI): calc. for $C_{45}H_{56}ClN_5O_{13}S_2Si$ $[M-2H]^{2-}$: 500.6392, found: 500.6390.

3. NMR spectra

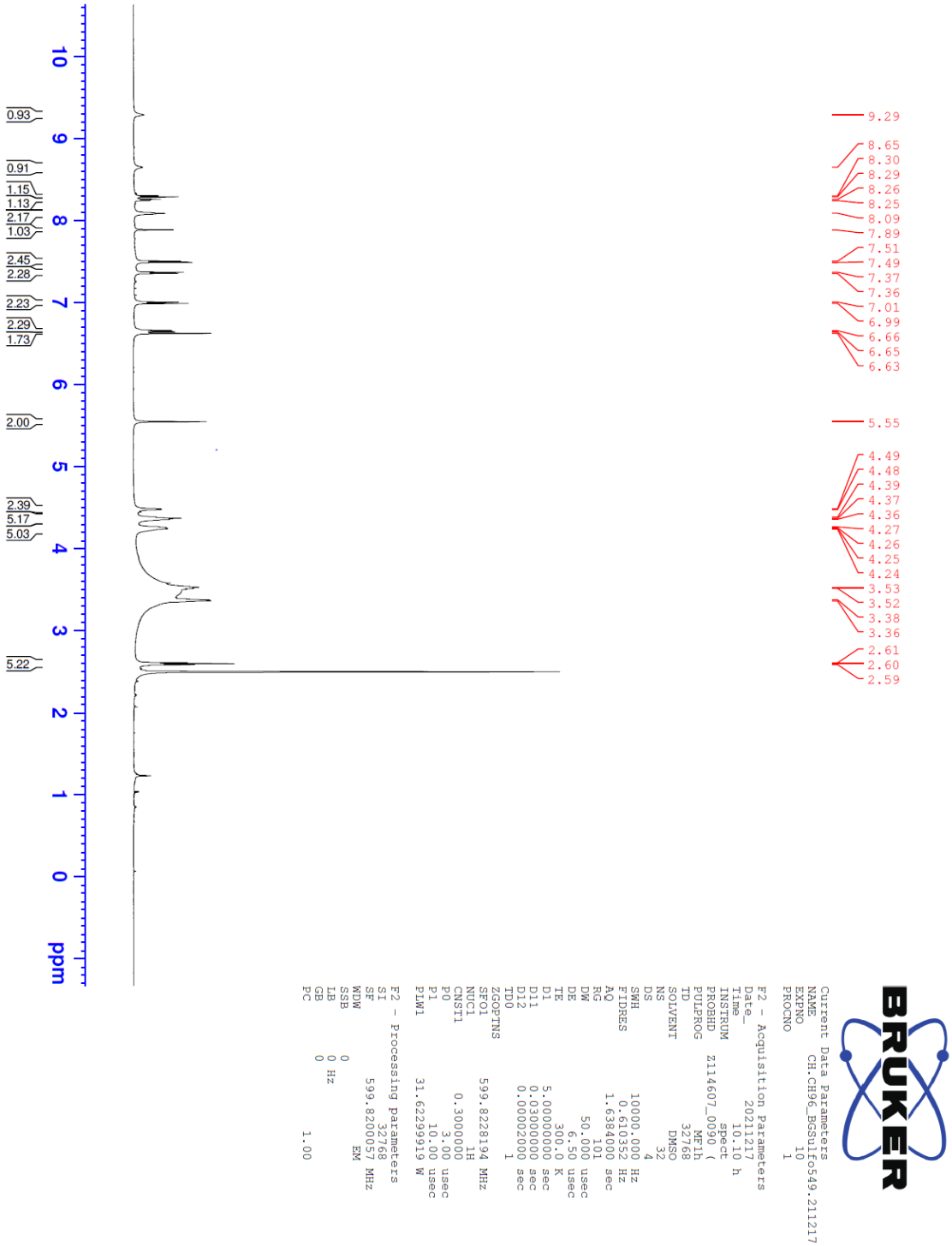
3.1. 4-(*tert*-Butoxycarbonyl)-2-(3-(3-carboxyazetidin-1-ium-1-ylidene)-6-(3-carboxy-azetidin-1-yl)-3*H*-xanthen-9-yl)benzoate (2a)



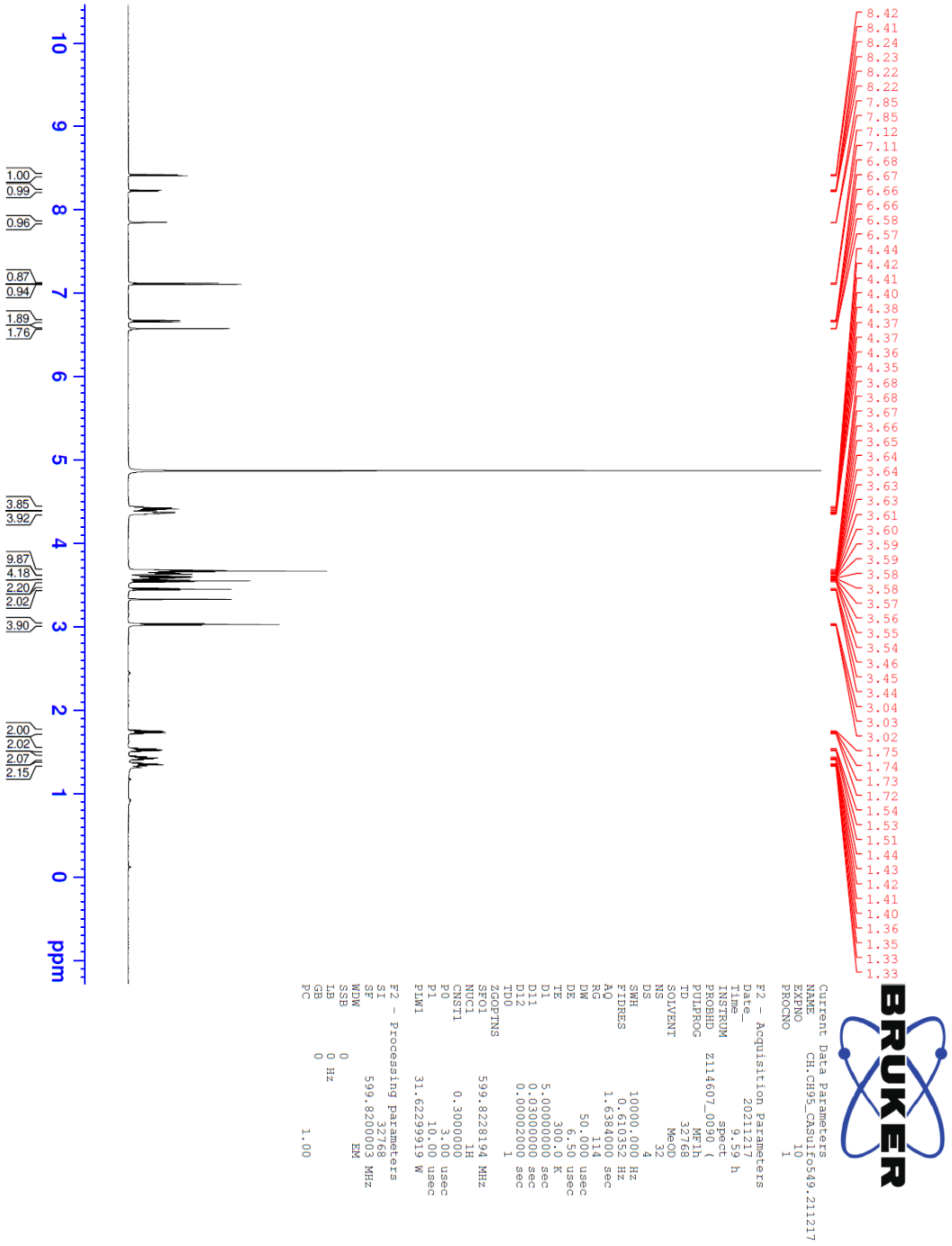
3.2. 4-Carboxy-2-(3-(3-((2-sulfoethyl)carbamoyl)azetidin-1-ium-1-ylidene)-6-(3-((2-sulfoethyl)carbamoyl)azetidin-1-yl)-3*H*-xanthen-9-yl)benzoate (3a)



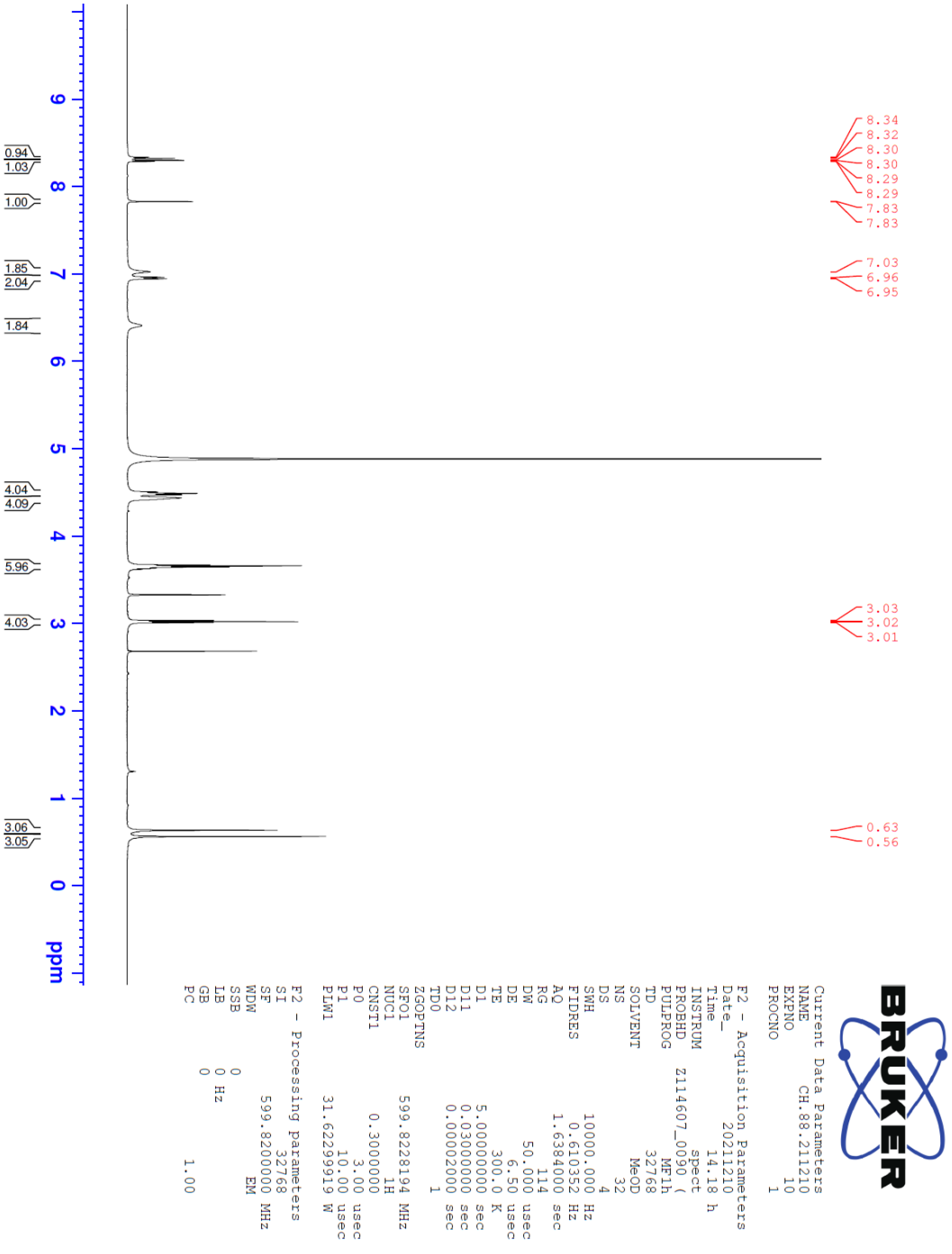
3.3. 4-Carboxy-2-(3-(3-((2-sulfoethyl)carbamoyl)azetidin-1-ium-1-ylidene)-6-(3-((2-sulfoethyl)carbamoyl)azetidin-1-yl)-3*H*-xanthen-9-yl)benzoate (3a)4-((4-(((2-Amino-9*H*-purin-6-yl)oxy)methyl)benzyl)carbamoyl)-2-(3-(3-((2-sulfoethyl)carbamoyl)azetidin-1-ium-1-ylidene)-6-(3-((2-sulfoethyl)carbamoyl)-azetidin-1-yl)-3*H*-xanthen-9-yl)benzoate (BG-Sulfo549)



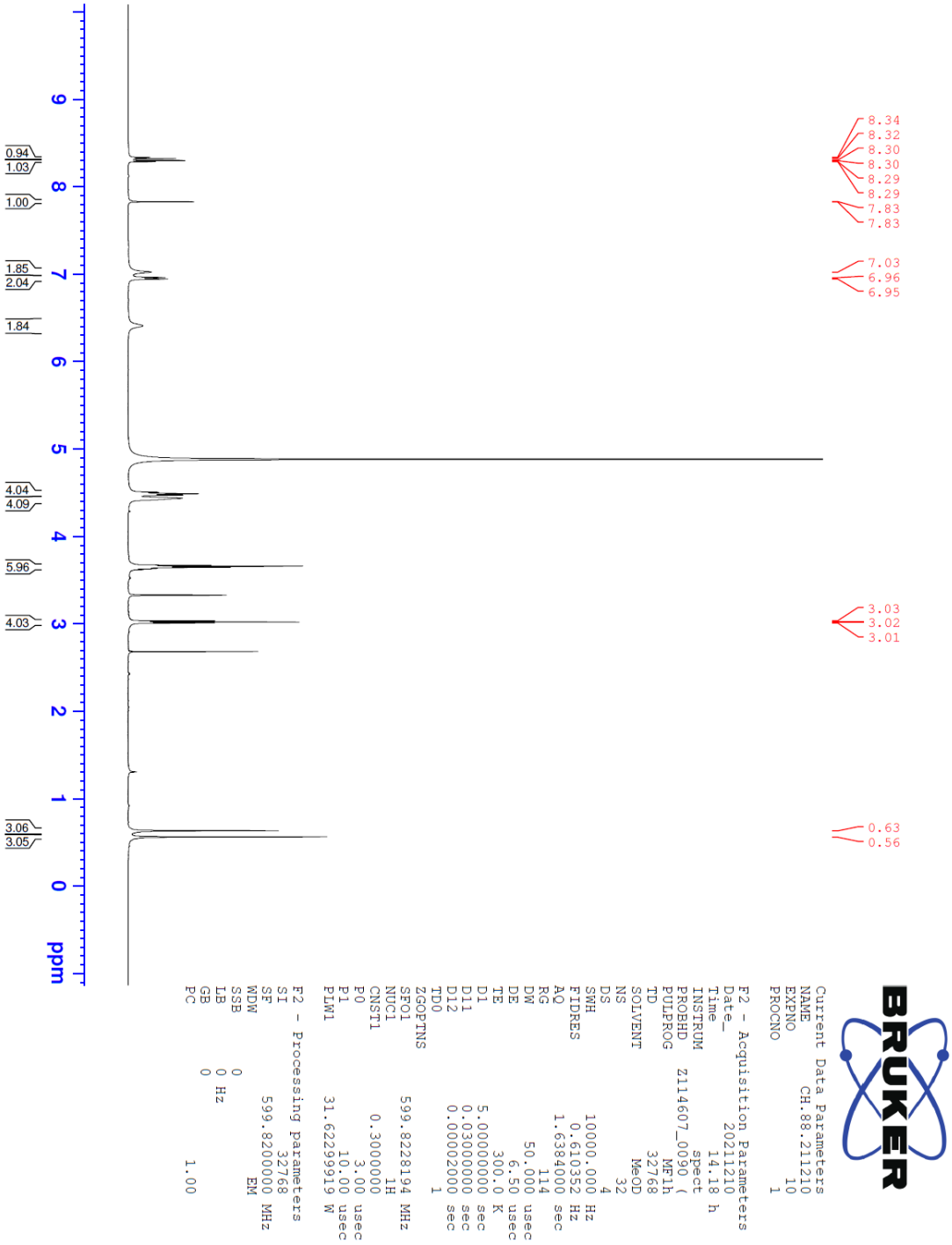
3.4. 4-((2-(2-((6-Chlorohexyl)oxy)ethoxy)ethyl)carbamoyl)-2-(3-(3-((2-sulfoethyl)-carbamoyl)azetidin-1-ium-1-ylidene)-6-(3-((2-sulfoethyl)carbamoyl)azetidin-1-yl)-3*H*-xanthen-9-yl)benzoate (CA-Sulfo549)



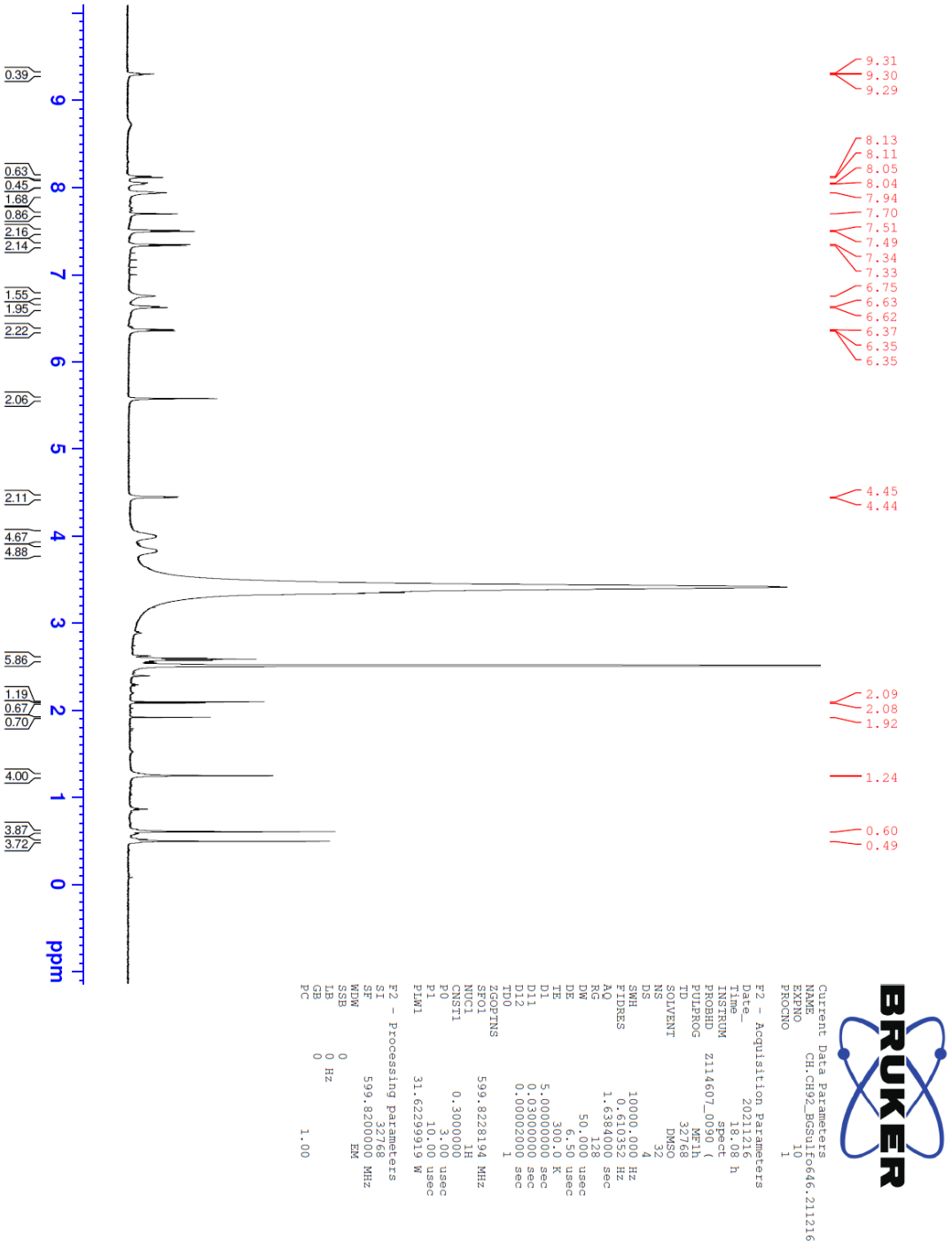
3.5. 4-(*tert*-Butoxycarbonyl)-2-(3-(3-carboxyazetidin-1-ium-1-ylidene)-7-(3-carboxy-azetidin-1-yl)-5,5-dimethyl-3,5-dihydrodibenzo[*b*,*e*]silin-10-yl)benzoate (2b)



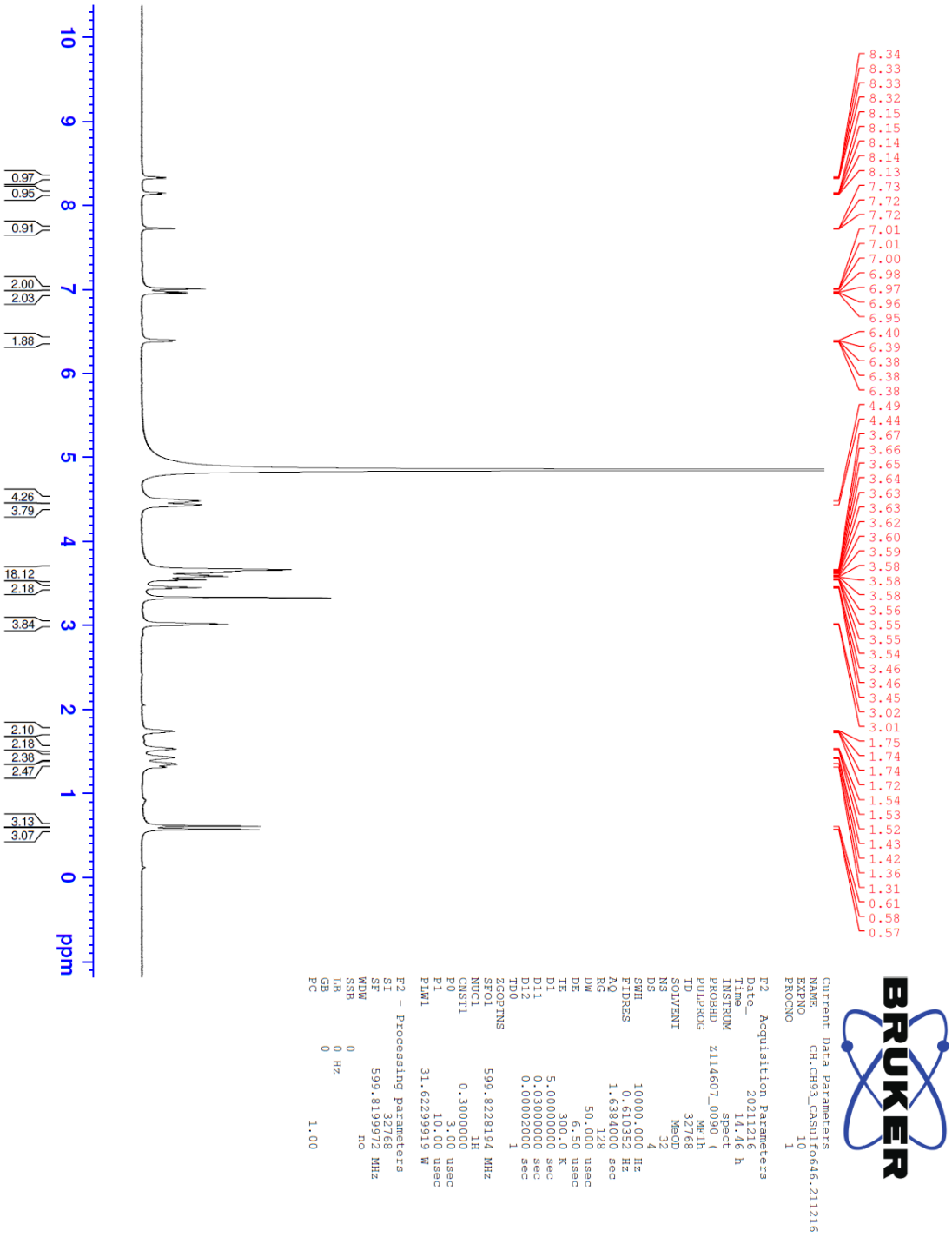
3.6. 4-Carboxy-2-(5,5-dimethyl-3-(3-((2-sulfoethyl)carbamoyl)azetidin-1-ium-1-ylidene)-7-(3-((2-sulfoethyl)carbamoyl)azetidin-1-yl)-3,5-dihydrodibenzo[*b*,*e*]silin-10-yl)benzoate (3b)



3.7. 4-((4-(2-(2-Amino-9*H*-purin-6-yl)ethyl)benzyl)carbamoyl)-2-(5,5-dimethyl-3-(3-((2-sulfoethyl)carbamoyl)azetidin-1-ium-1-ylidene)-7-(3-((2-sulfoethyl)-carbamoyl)azetidin-1-yl)-3,5-dihydrodibenzo[*b*,*e*]silin-10-yl)benzoate (BG-Sulfo646)



3.8. 4-((2-(2-((6-Chlorohexyl)oxy)ethoxy)ethyl)carbamoyl)-2-(5,5-dimethyl-3-(3-((2-sulfoethyl)carbamoyl)azetidin-1-ium-1-ylidene)-7-(3-((2-sulfoethyl)-carbamoyl)azetidin-1-yl)-3,5-dihydrodibenzo[*b*,*e*]silin-10-yl)benzoate (CA-Sulfo646)



4. Protein mass spectrometry

$SNAP_f$ sequence:
MASWSHPQFEKGADDDDKVPHMDKDCEMKRTTLDSPLGKLELSGCEQGLHRIIFLGKGTSAADAVEVPAP
AAVLGGPEPLMQATAWLNAYFHQPEAIEEFPVPALHHPVFQQESFTRQVLWKLLKVVKFGEVISYSHLAA
LAGNPAATAAVKTALSGNPVPILIPCHRVVQGDLDVGGYEGGLAVKEWLLAHEGHRLGKPGLGAPGFSSI
SAHHHHHHHHHH

Strep-Tag II, Enterokinase-site, SNAPf, His-Tag

After His-tag purification, two posttranslational $SNAP_f$ constructs were observed: one with removed start codon $SNAP_f$ (2–222) and one without the N-terminal Strep-Tag II and Enterokinase-site $SNAP_f$ (22–222):

	calc.	found	Δ ppm
$SNAP_f$ (2–222)	23865.101	23865.141	1.67
$SNAP_f$ (22–222)	21618.103	21618.142	1.80
$SNAP_f$ (2–222):JF_{549}	24420.317	24420.357	1.65
$SNAP_f$ (22–222):JF_{549}	22173.319	22173.349	1.36
$SNAP_f$ (2–222):JF_{646}	24462.386	24462.386	0.00
$SNAP_f$ (22–222):JF_{646}	22215.358	22215.386	1.72
$SNAP_f$ (2–222):Sulfo549	24722.305	24722.340	1.43
$SNAP_f$ (22–222):Sulfo549	22475.307	22475.337	1.35
$SNAP_f$ (2–222):Sulfo646	24764.334	24764.387	2.16
$SNAP_f$ (22–222):Sulfo646	22517.336	22517.387	2.28

Halo sequence:
MHHHHHHHHHHENLYFQGIGTGFPFDPHYVEVLGERMHYVDVGPRDGTPVLFLHGNPTSSYVWRNIIPHV
APTHRCIAPDLIGMGKSDKPDLGYFFDDHVRFMDAFIEALGLEEVVLVIHDWGSALGFHWAKRNPERVKG
IAFMEFIRPIPTWDEWPEFARETFQAFRTTDVGRKLIIDQNVFIEGTLPMGVVRPLTEVEMDHYREPFLN
PVDREPLWRFPNELPIAGEPANIVALVEEYMDWLHQSPVPKLLFWGTPGVLIPPAEAARLAKSLPNCKAV
DIGPGLNLLQEDNPDLIGSEIARWLSTLEI

Halo, His-Tag

	calc.	found	Δ ppm
Halo	35465.901	35465.985	2.37
Halo:JF_{549}	36089.251	36089.293	1.16
Halo:JF_{646}	36131.230	36131.297	1.85
Halo:Sulfo549	36391.188	36391.291	2.83
Halo:Sulfo646	36433.217	36433.317	2.74

5. Protein constructs

SNAP–TM–Halo sequence:

METDTLLLWVLLLWVPGSTGDYPYDVPDYAGAQPARSMDKDCEMKRTTLDSPLGKLELSGCEQGLHEIIF
LGKGTSAADAVEVPAPAAVLGGPEPLMQATAWLNAYFHQPEAIEEFPVPALHHPVFQQESFTRQVLWKLL
KVVKFGEVISYSHLAALAGNPAATAAVKTALSGNPVPILIPCHRVVQGDLDVGGYEGGLAVKEWLLAHEG
HRLGKPGLGGRLEVLFQGVDEQKLISEEDLNAVGQDTQEVIVVPHSLPFKVVVISAILALVVLTIISLII
LIMLWQKKPRGAQPARSGSEIGTGFPFDPHYVEVLGERMHYVDVGPRDGTPVLFLHGNPTSSYVWRNIIP
HVAPTHRCIAPDLIGMGKSDKPDLGYFFDDHVRFMDAFIEALGLEEVVLVIHDWGSALGFHWAKRNPERV
KGIAFMEFIRPIPTWDEWPEFARETFQAFRTTDVGRKLIIDQNVFIEGTLPMGVVRPLTEVEMDHYREPF
LNPVDREPLWRFPNELPIAGEPANIVALVEEYMDWLHQSPVPKLLFWGTPGVLIPPAEAARLAKSLPNCK
AVDIGPGLNLLQEDNPDLIGSEIARWLSTLEISGVD*

IgK signal peptide, HA-tag, SNAP, myc, transmembrane domain, Halo-Tag

Halo–TM–SNAP sequence:

METDTLLLWVLLLWVPGSTGDYPYDVPDYAGAQPARSGSEIGTGFPFDPHYVEVLGERMHYVDVGPRDGT
PVLFLHGNPTSSYVWRNIIPHVAPTHRCIAPDLIGMGKSDKPDLGYFFDDHVRFMDAFIEALGLEEVVLV
IHDWGSALGFHWAKRNPERVKGIAFMEFIRPIPTWDEWPEFARETFQAFRTTDVGRKLIIDQNVFIEGTL
PMGVVRPLTEVEMDHYREPFLNPVDREPLWRFPNELPIAGEPANIVALVEEYMDWLHQSPVPKLLFWGTP
GVLIPPAEAARLAKSLPNCKAVDIGPGLNLLQEDNPDLIGSEIARWLSTLEISGVDEQKLISEEDLNAVG
QDTQEVIVVPHSLPFKVVVISAILALVVLTIISLIILIMLWQKKPRGAQPARSMDKDCEMKRTTLDSPLG
KLELSGCEQGLHEIIFLGKGTSAADAVEVPAPAAVLGGPEPLMQATAWLNAYFHQPEAIEEFPVPALHHP
VFQQESFTRQVLWKLLKVVKFGEVISYSHLAALAGNPAATAAVKTALSGNPVPILIPCHRVVQGDLDVGG
YEGGLAVKEWLLAHEGHRLGKPGLGGRLEVLFQGVD*

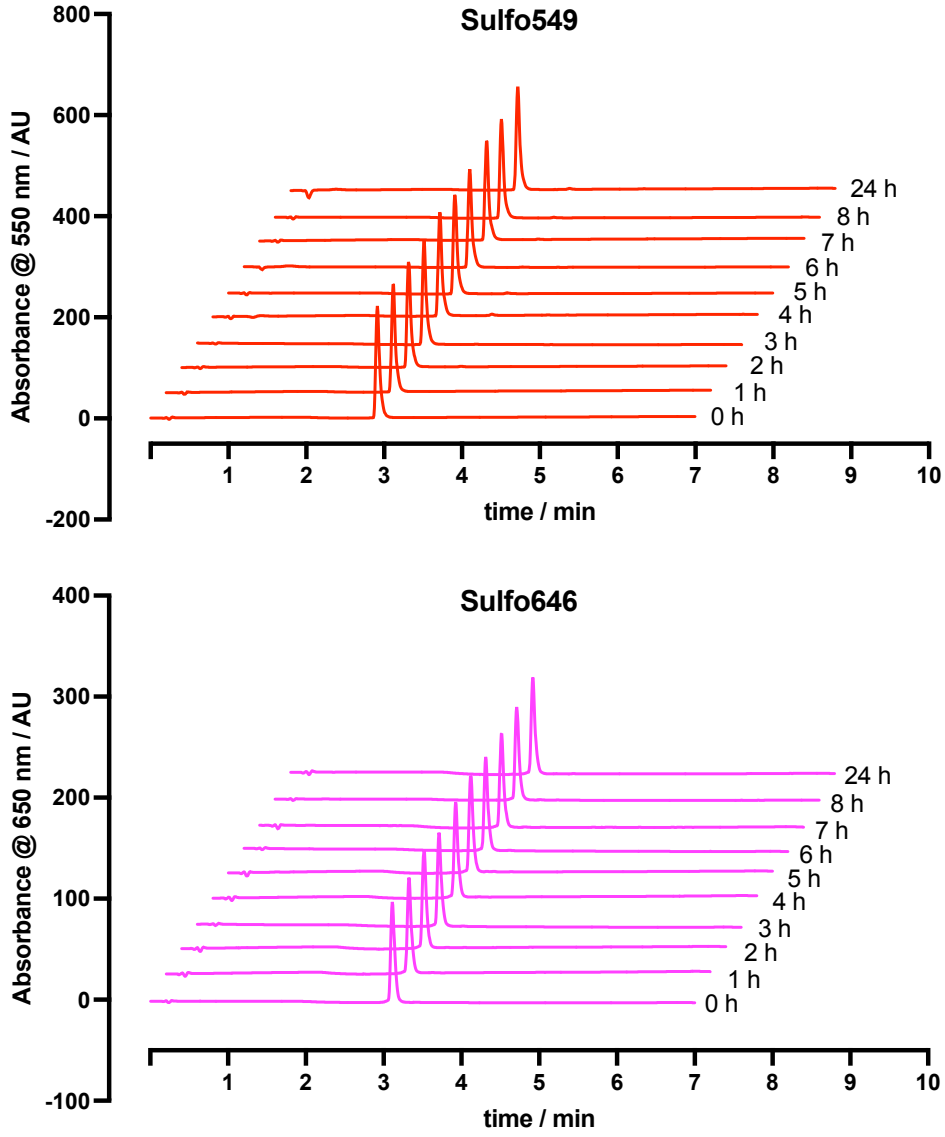
IgK signal peptide, HA-tag, Halo-Tag, myc, transmembrane domain, SNAP

Halo-GLP1R sequence:

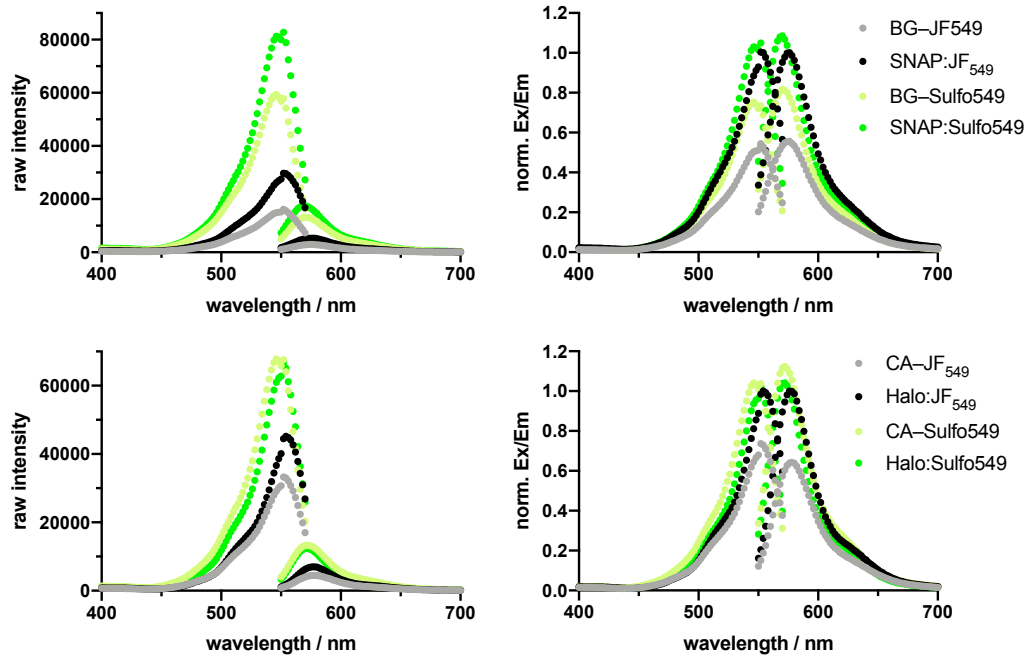
MALPVTALLLPLALLLHAARPAAASGIDYKDDDDKAGIDAIGSEIGTGFPFDPHYVEVLGERMHYVDVGP
RDGTPVLFLHGNPTSSYVWRNIIPHVAPTHRCIAPDLIGMGKSDKPDLGYFFDDHVRFMDAFIEALGLEE
VVLVIHDWGSALGFHWAKRNPERVKGIAFMEFIRPIPTWDEWPEFARETFQAFRTTDVGRKLIIDQNVFI
EGTLPMGVVRPLTEVEMDHYREPFLNPVDREPLWRFPNELPIAGEPANIVALVEEYMDWLHQSPVPKLLF
WGTPGVLIPPAEAARLAKSLPNCKAVDIGPGLNLLQEDNPDLIGSEIARWLSTLEISGDIRPQGATVSLW
ETVQKWREYRRQCQRSLTEDPPPATDLFCNRTFDEYACWPDGEPGSFVNVSCPWYLPWASSVPQGHVYRF
CTAEGLWLQKDNSSLPWRDLSECEESKRGERSSPEEQLLFLYIIYTVGYALSFSALVIASAILLGFRHLH
CTRNYIHLNLFASFILRALSVFIKDAALKWMYSTAAQQHQWDGLLSYQDSLSCRLVFLLMQYCVAANYYW
LLVEGVYLYTLLAFSVLSEQWIFRLYVSIGWGVPLLFVVPWGIVKYLYEDEGCWTRNSNMNYWLIIRLPI
LFAIGVNFLIFVRVICIVVSKLKANLMCKTDIKCRLAKSTLTLIPLLGTHEVIFAFVMDEHARGTLRFIK
LFTELSFTSFQGLMVAILYCFVNNEVQLEFRKSWERWRLEHLHIQRDSSMKPLKCPTSSLSSGATAGSSM
YTATCQASCS*

CD8 signal peptide, FLAG-tag, Halo-tag, GLP1R

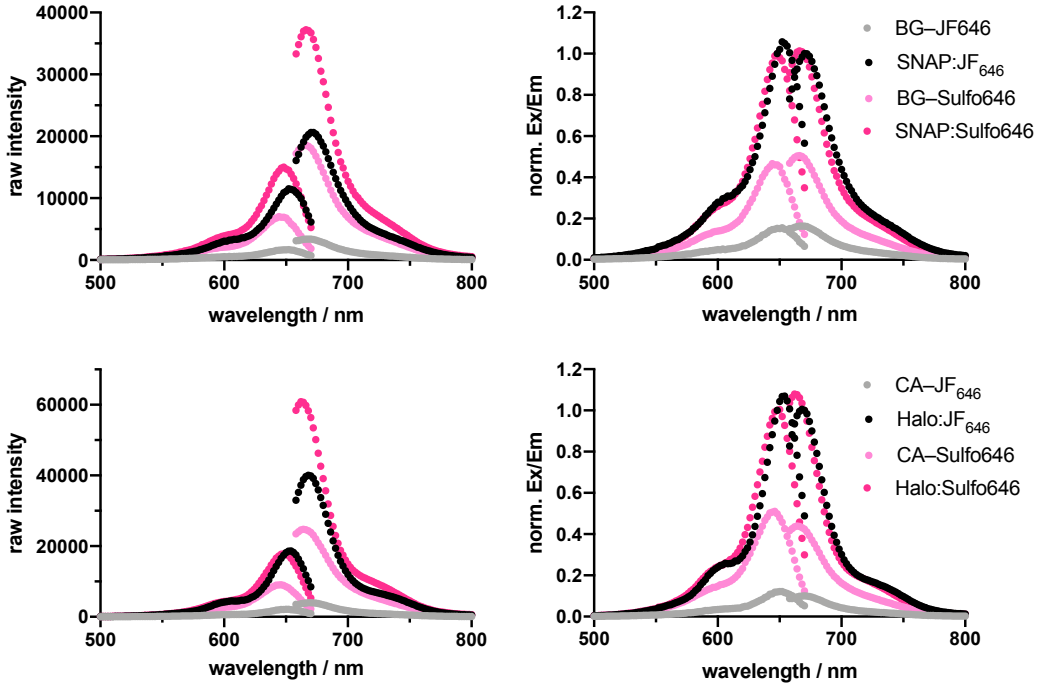
6. Supplemental Figures



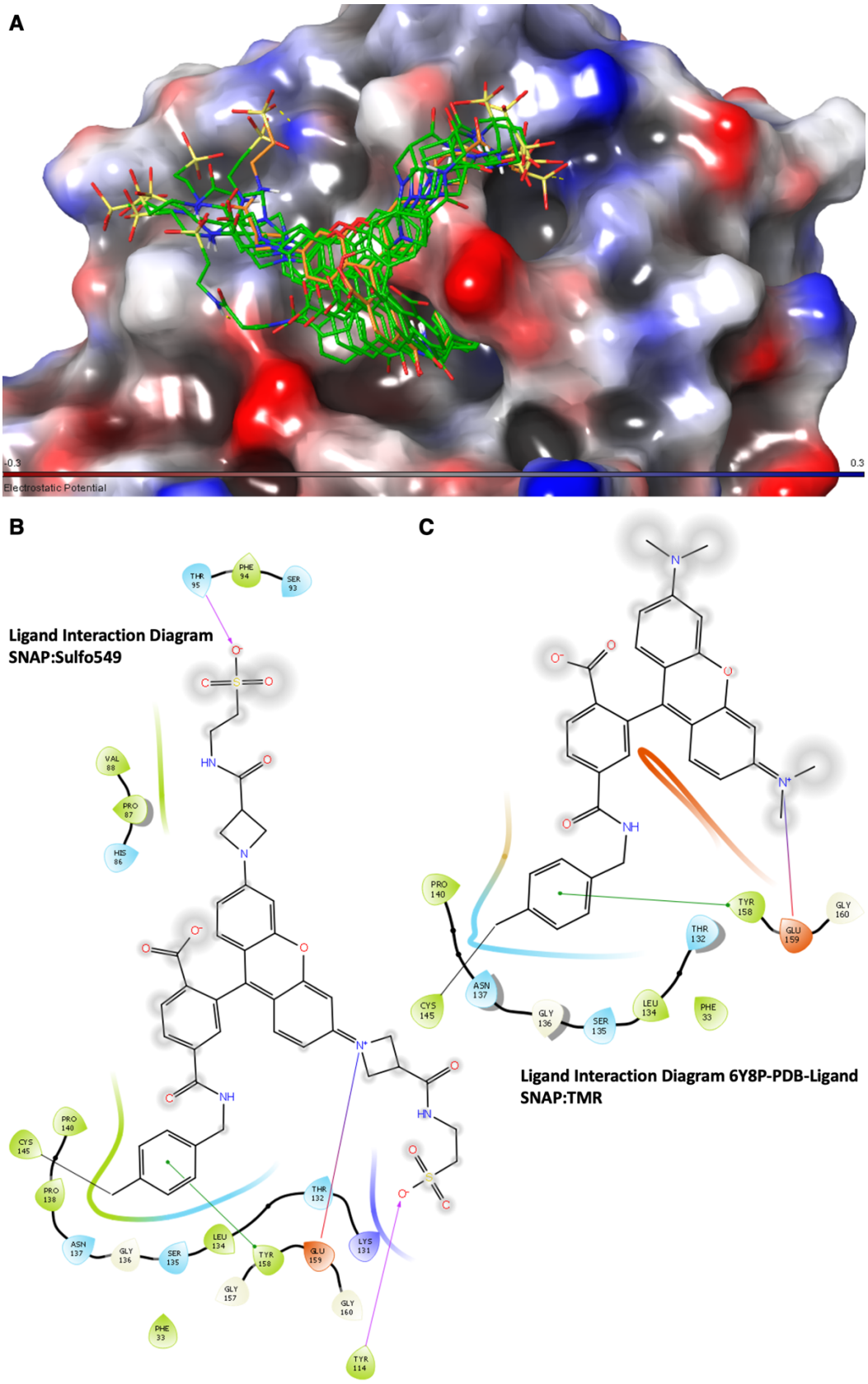
Supplemental Figure S1: Stability of Sulfo549 and Sulfo646 in PBS measured by LCMS over 24 hours.



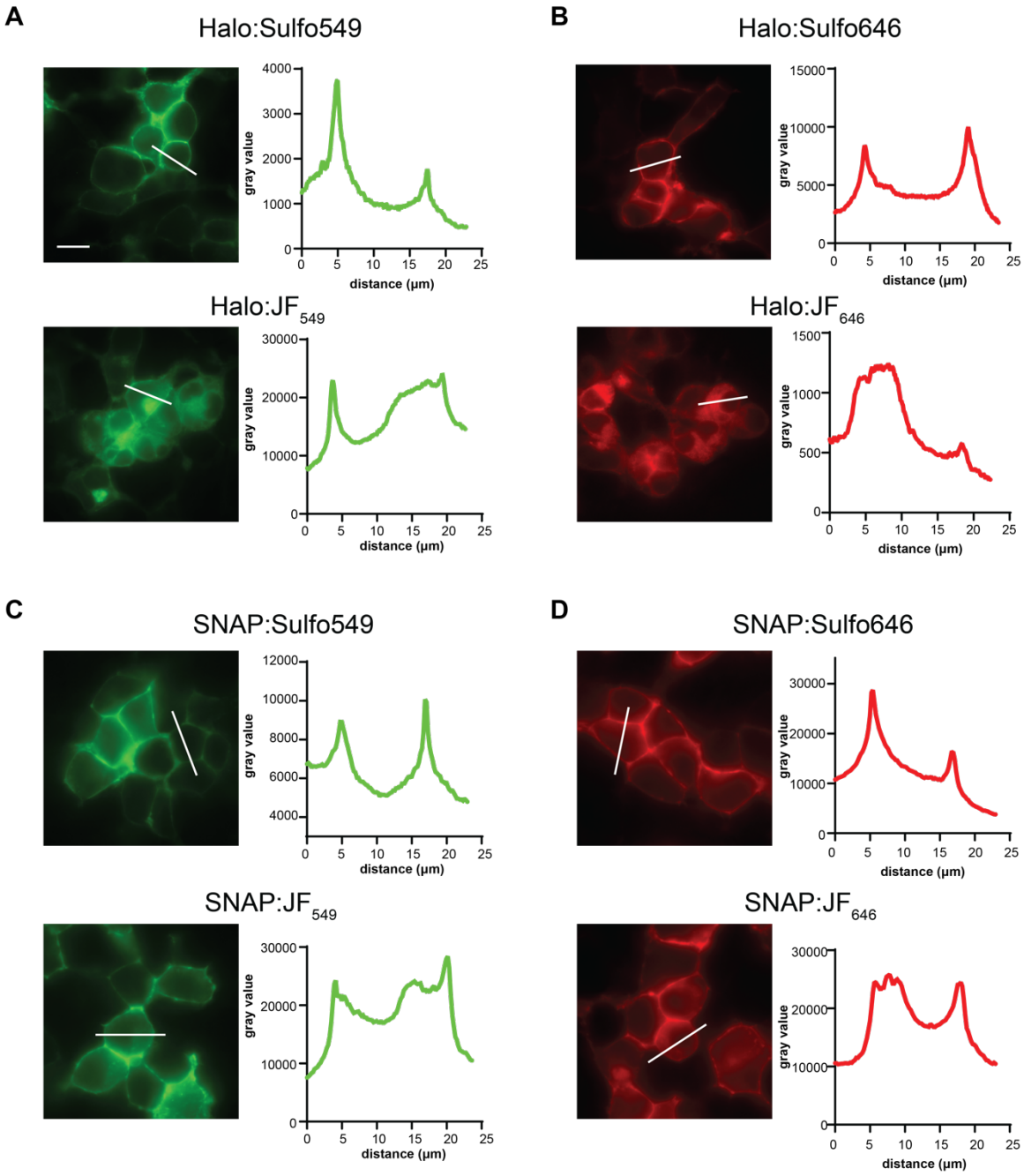
Supplemental Figure S2: Excitation and emission spectra of bound and unbound 549 probes with raw intensity (left) and normalized to JF dye excitation and emission (right, cf. Figure 2).



Supplemental Figure S3: Excitation and emission spectra of bound and unbound 646 probes with raw intensity (left) and normalized to JF dye excitation and emission (right, cf. Figure 2).

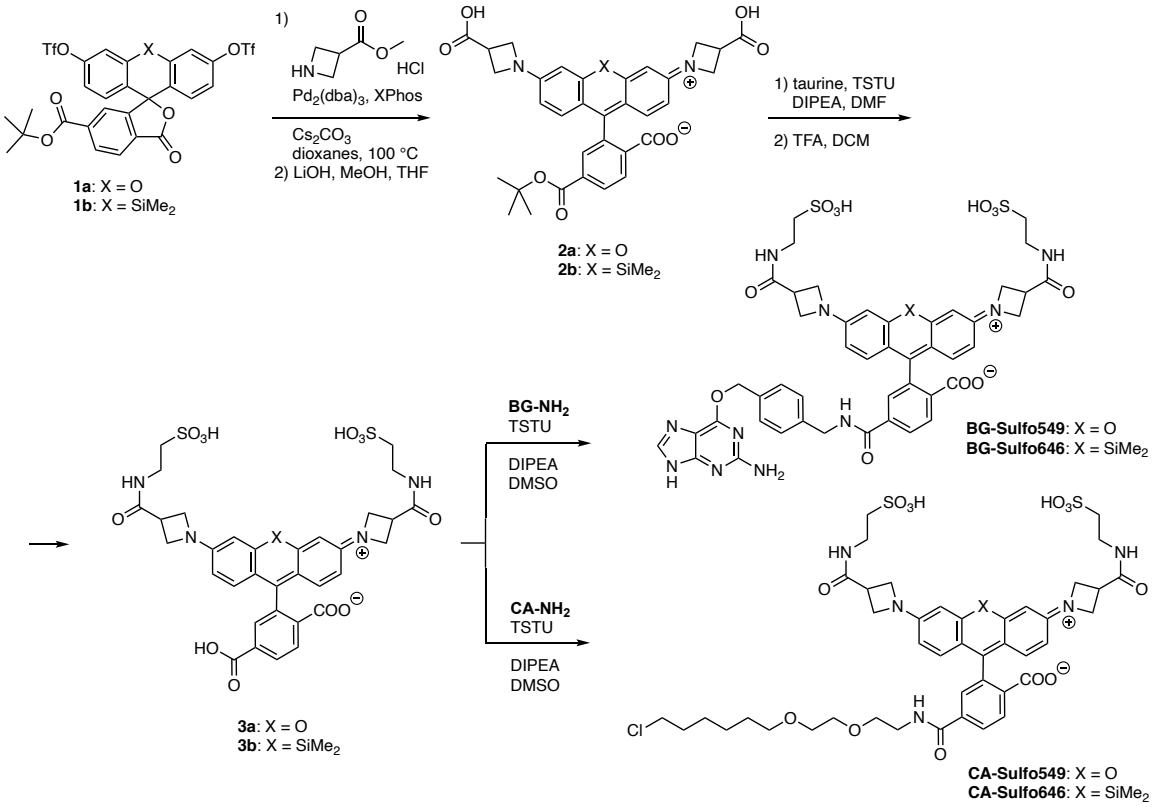

A
-0.3
0.3
Electrostatic Potential
B
Ligand Interaction Diagram
SNAP:Sulfo549
C
Ligand Interaction Diagram 6Y8P-PDB-Ligand
SNAP:TMR

Supplemental Figure S4: Molecular docking of Sulfo549. A) Electrostatic potential surface show 8 out of 10 poses with good overlap. Pose 1: orange C atoms; hydrogen bonds to the sulfonates from Thr95 and Tyr114 are dashed yellow. **B)** Several new interactions predicted between Sulfo549 ligand with the SNAP protein surface that TMR could not make and is not observed in the X-ray structure pdb: 6y8p.



Supplemental Figure S5: HEK293 cells transfected with SNAP- or Halo-β2AR and labelled with JaneliaFluor or Sulfo Dyes. A) Halo-β2AR transfected cells stained with CA-Sulfo549 vs. CA-JF_{549}. **B)** Halo-β2AR transfected cells stained with CA-Sulfo646 vs. CA-JF_{646}. **C)** SNAP-β2AR transfected cells stained with BG-Sulfo549 vs. BG-JF_{549}. **D)** SNAP-β2AR transfected cells stained with BG-Sulfo646 vs. BG-JF_{646}. Scale bar = 10 µm.

7. Supplemental Schemes



Supplemental Scheme 1: Synthesis of Sulfo dyes.

8. References

[1] A. Acosta-Ruiz, V. A. Gutzeit, M. J. Skelly, S. Meadows, J. Lee, P. Parekh, A. G. Orr, C. Liston, K. E. Pleil, J. Broichhagen, J. Levitz, *Neuron* **2020**, *105*, 446-463.