Electronic Supplementary Information

Electronic tuning of self-healing fluorophores for live-cell and single-molecule imaging

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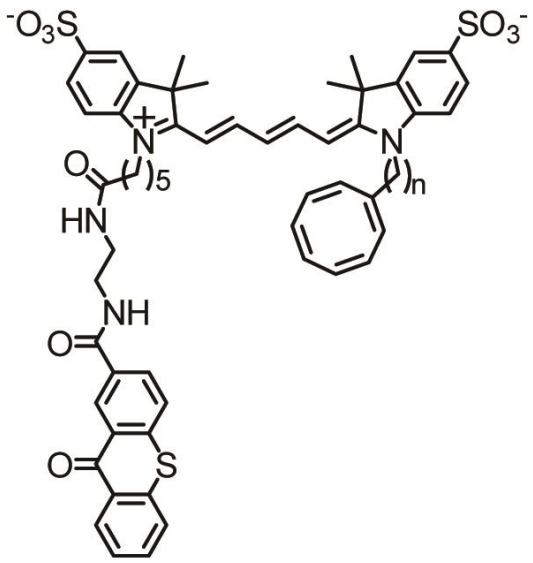


TX-Cy5-COT(n)
n=1, 2, 3, 4, 5, 10

Chart S1. Structures of OTX-Cy5-COT(n) used to determine triplet state lifetimes of Cy5-COT(n).

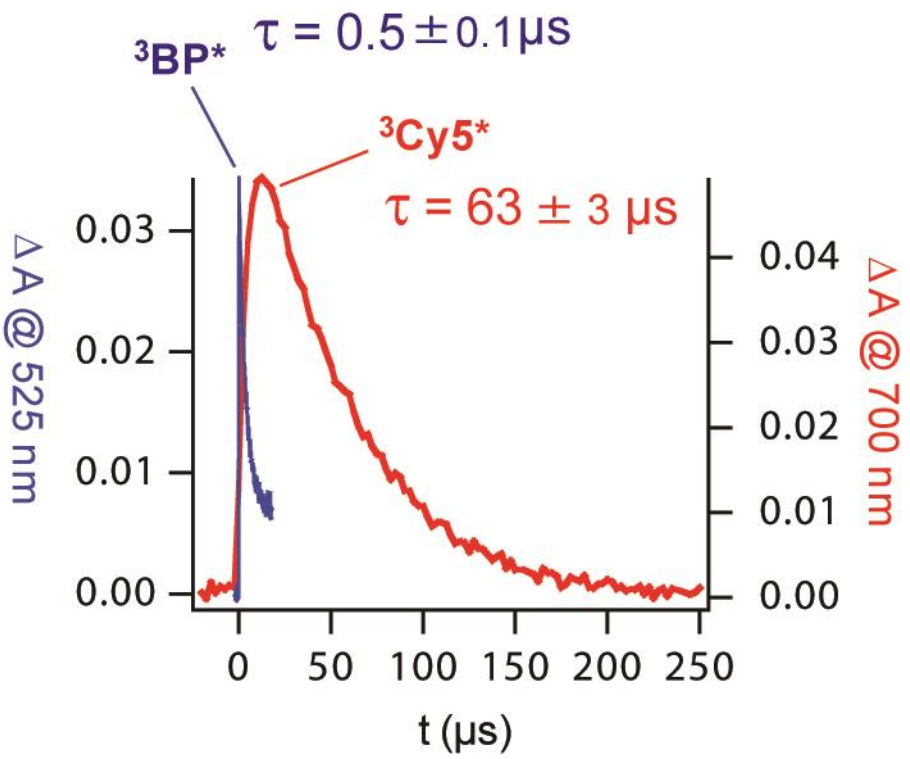


Figure S1. Cy5 triplet absorption traces recorded at 700 nm and BP triplet absorption traces recorded at 525 nm after pulsed laser excitation (355 nm, 5 ns pulse width) of deoxygenated acetonitrile solutions of BP (3 mM) and Cy5 (10 µM). The triplet lifetimes (τ) are derived from a kinetic fitting model considering the growth kinetics due to energy transfer from ^{3}BP* to Cy5.[1]

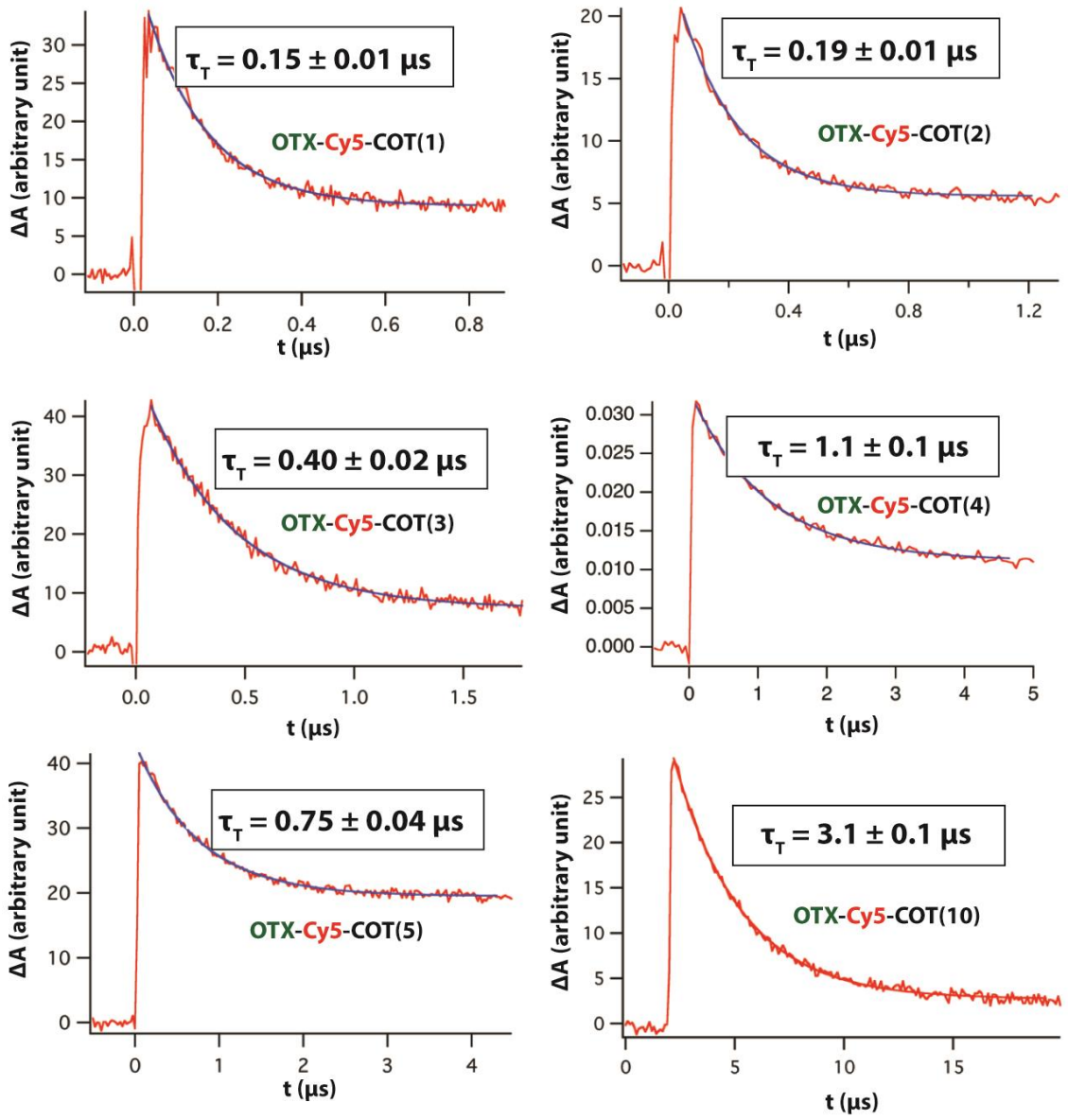


Figure S2. Transient absorption traces for OTX-Cy5-COT(n), n = 1, 2, 3, 4, 5, 10. Transient absorption traces at 700 nm after pulsed laser excitation (355 nm, 5 ns pulse width) of deoxygenated acetonitrile solutions of OTX-Cy5-COT(n) (~10 µM). The optical path length is 1 cm. The transient absorptions at 700 nm were fit (blue line) to single-exponential functions, accounting for the decay of Cy5 triplets.

$$\frac{d(\Delta A)}{dt} = -a_1 e^{(-k_1 t)} + a_2 e^{(-k_2 t)}$$

$$k_1 = 1.1 \times 10^6\ s$$

$$k_2 = 0.31 \times 10^6\ s$$

$$\tau^{^3Cy5^*} = 1/k_2 = 3.2 \pm 0.3\ \mu s$$

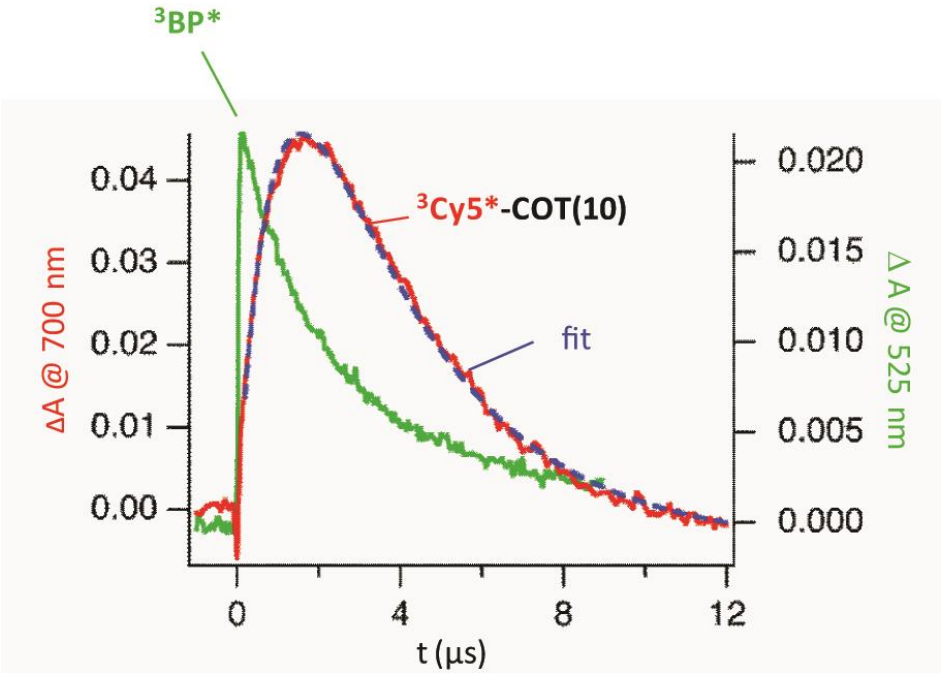


Figure S3. Transient absorption traces at 525 nm and 700 nm after pulsed laser excitation (355 nm, 5 ns pulse width) of deoxygenated acetonitrile solutions of BP (~20 mM) and Cy5-COT(10) (~39 µM). The optical path length is 2 mm. The trace at 700 nm was fit (blue line) to a double-exponential function, which accounts for the growth kinetics (k_1) and decay (k_2) of Cy5 triplets.

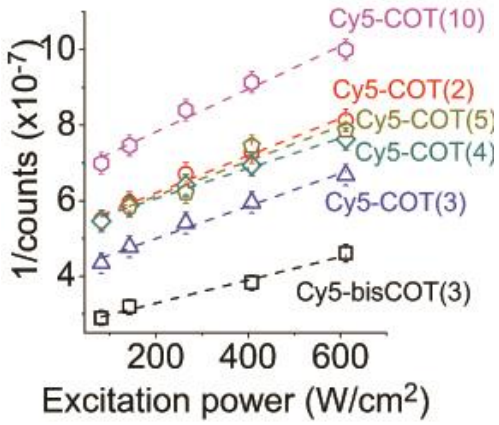


Figure S4. Inverse of the photon counts observed for Cy5-COT(n) and Cy5-bisCOT(3) in deoxygenated solution as a function of excitation power.

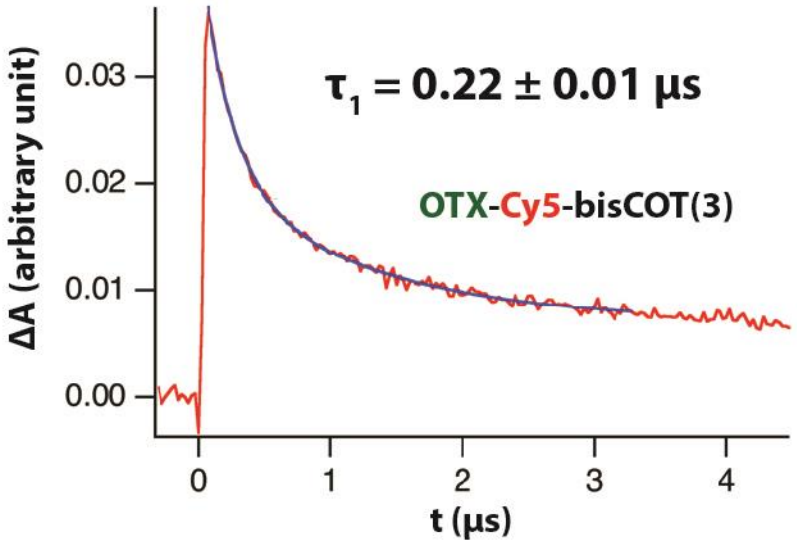


Figure S5. Transient absorption of OTX-Cy5-bisCOT(3). Transient absorption traces at 700 nm after pulsed laser excitation (355 nm, 5 ns pulse width) of deoxygenated acetonitrile solutions of OTX-Cy5-bisCOT(3) (~10 μM). The optical path length is 1 cm. The transient absorption at 700 nm was fit (blue line) to a double-exponential function, accounting for the decay of Cy5 triplets.

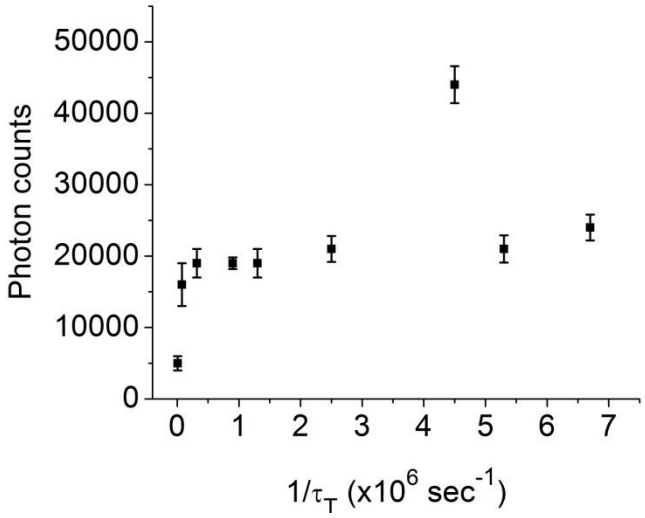


Figure S6. The photon counts for Cy5, Cy5-COT(n), and Cy5-bisCOT(3) in ambient oxygen conditions as a function of the inverse of triplet state lifetime ($1/\tau_T$).

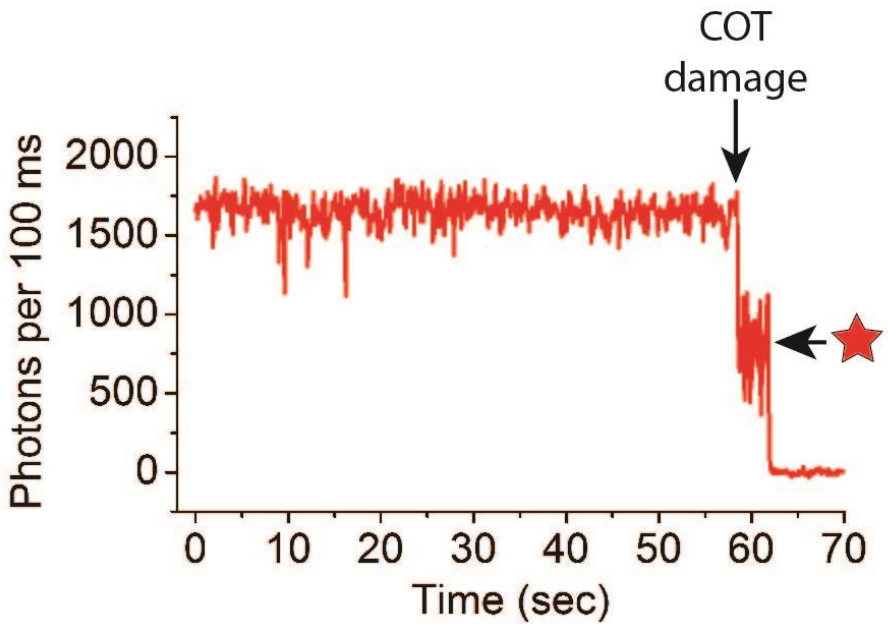


Figure S7. In deoxygenated conditions, the photobleaching of Cy5-COT(3) infrequently proceeds *via* a short-lived intermediate (red star) that exhibits the characteristics of the parent Cy5 fluorophore.

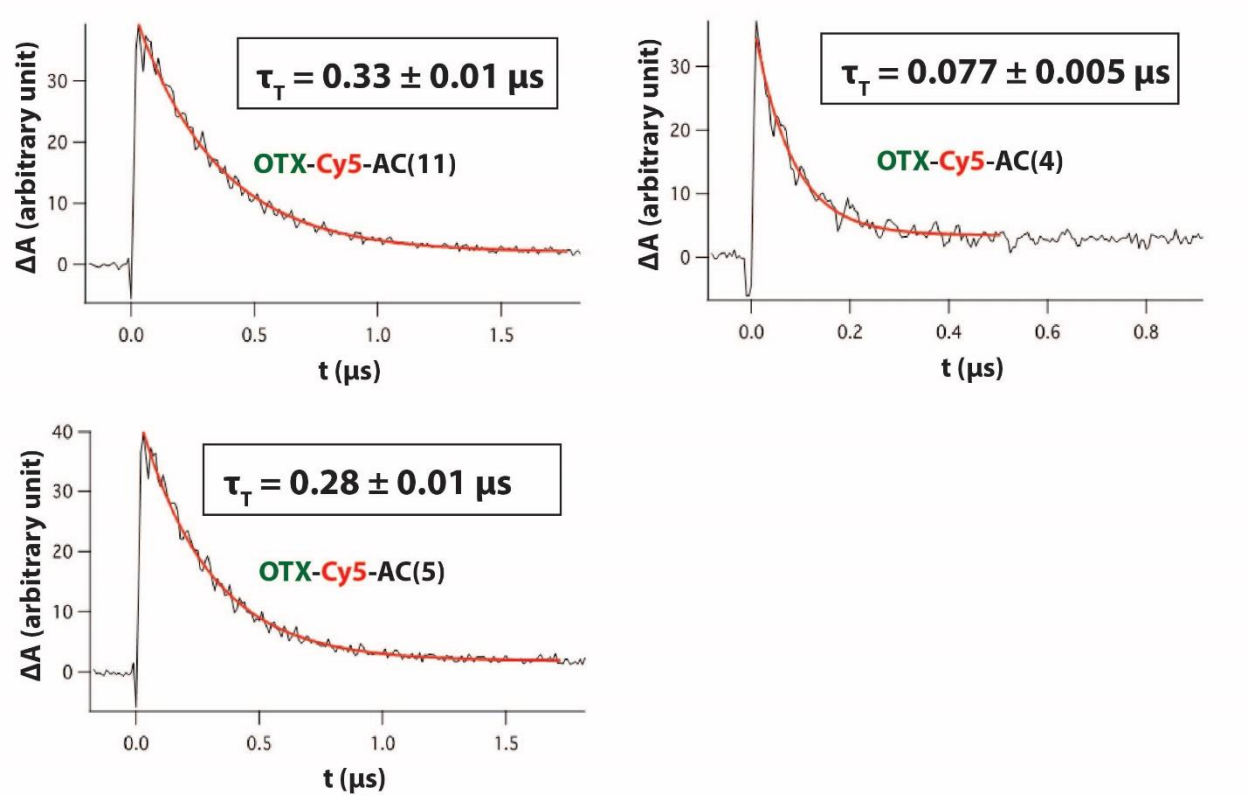


Figure S8. Transient absorption of OTX-Cy5-AC(n), n = 4, 5, 11. Transient absorption traces at 700 nm after pulsed laser excitation (355 nm, 5 ns pulse width) of deoxygenated acetonitrile solutions of OTX-Cy5-AC(n) (~10 µM). The optical path length is 1 cm. The transient absorption at 700 nm was fit (red line) to a single-exponential function, accounting for the decay of Cy5 triplets.

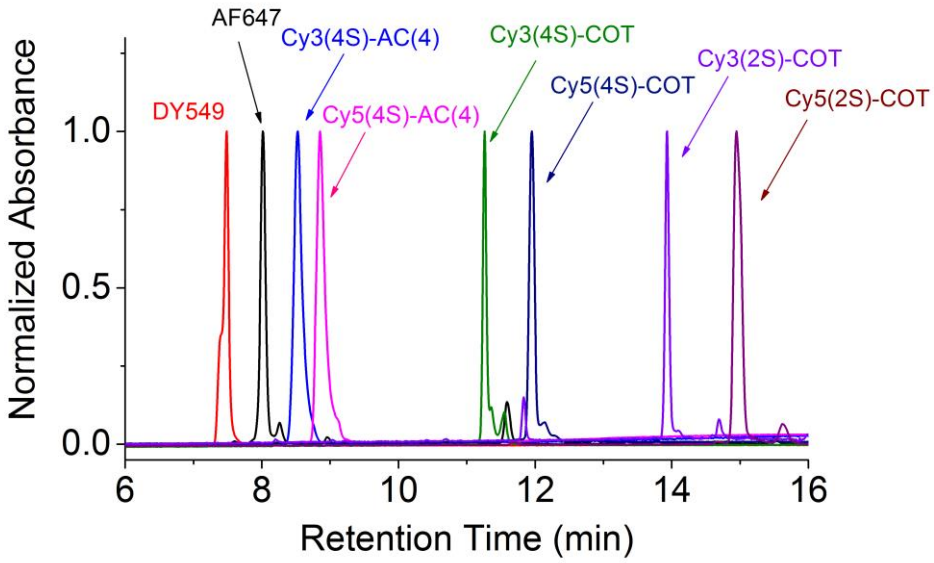


Figure S9. Relative retention times observed for benzylguanine-activated forms of Dy549, AF647, Cy3(4S)-AC(4), Cy5(4S)-AC(4), Cy3(2S)-COT, Cy5(2S)-COT, Cy3(4S)-COT and Cy5(4S)-COT dyes using a linear acetonitrile gradient on a reverse-phase, analytical C18 column.

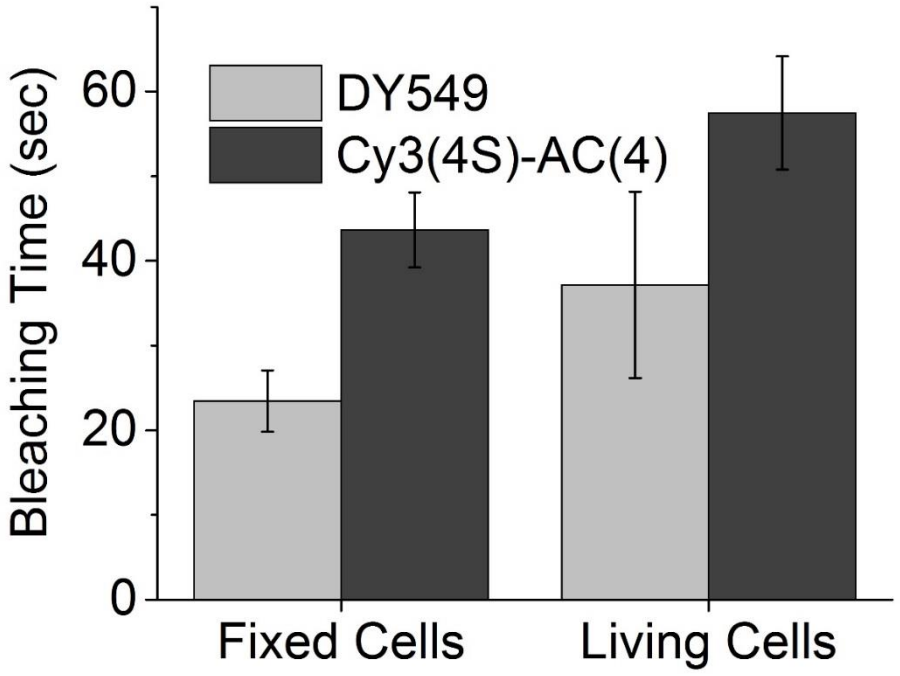


Figure S10. Photobleaching times for DY549-BG and Cy3(4S)-AC(4)-BG labeled SNAPf-D2s expressed in CHO cells prior to, and after, glutaraldehyde fixation (Methods).

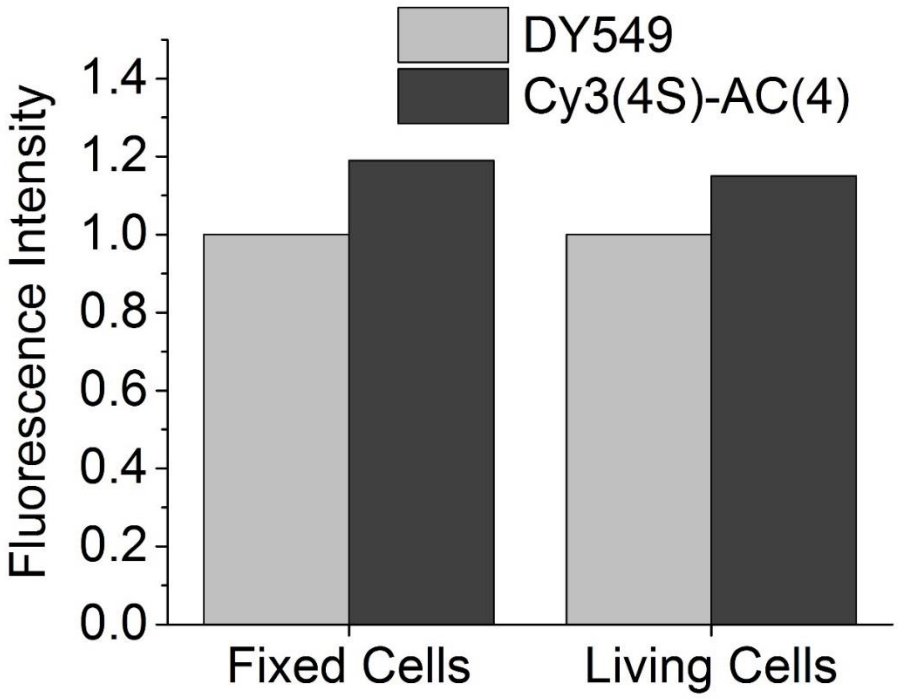


Figure S11. Average fluorescence intensities (normalized to Dy549) observed for DY549-BG and Cy3(4S)-AC(4)-BG labeled SNAPf-D2s expressed in CHO cells prior to, and after, glutaraldehyde fixation (Methods).

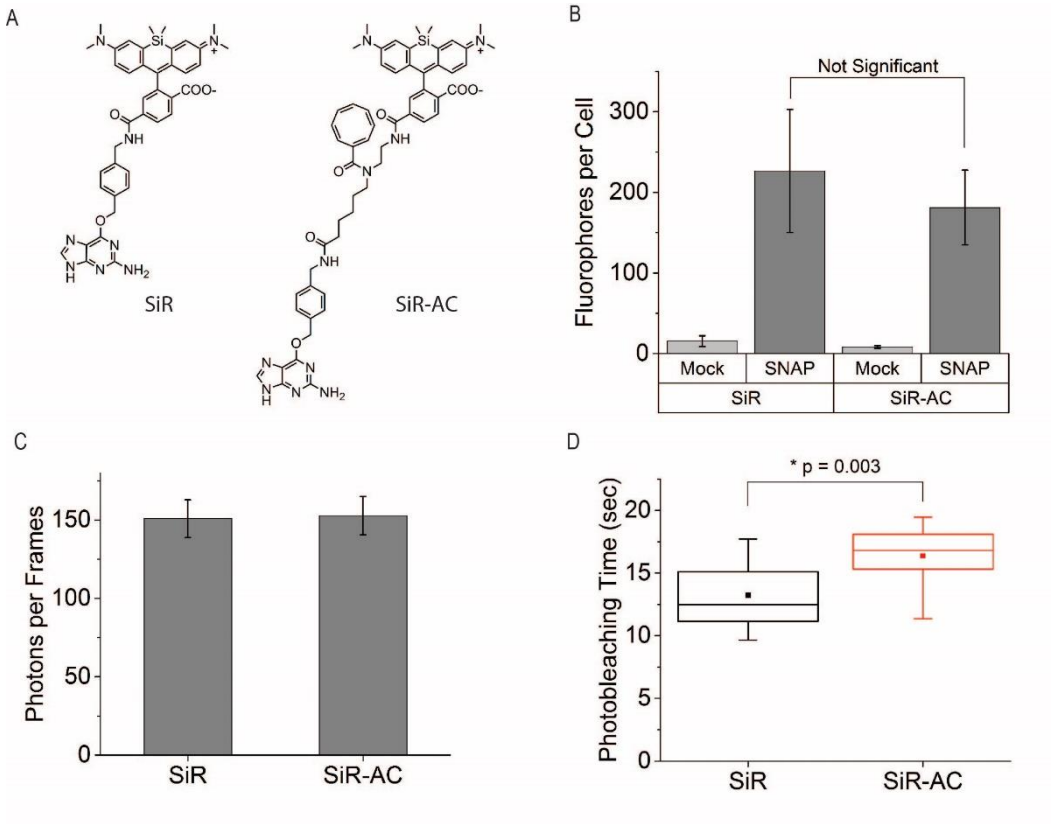


Figure S12. The conjugation of AC in proximity to SiR improves its performance *in vitro* and in cells. (A) Structures of benzylguanine-derivatives of SiR and SiR-AC. (B) Number of SiR and SiR-AC fluorophores per cell for cells expressing $G\alpha_{i1}$-SNAPf (SNAP) compared to cells that are not (Mock). (C) Fluorescence intensity observed for SiR and SiR-AC in fixed cells. (D) Whisker plot comparing photobleaching time of SiR and SiR-AC labeled in fixed cells; squares indicate mean; boxes span 25 to 75 percentile; whiskers span from min. to max.

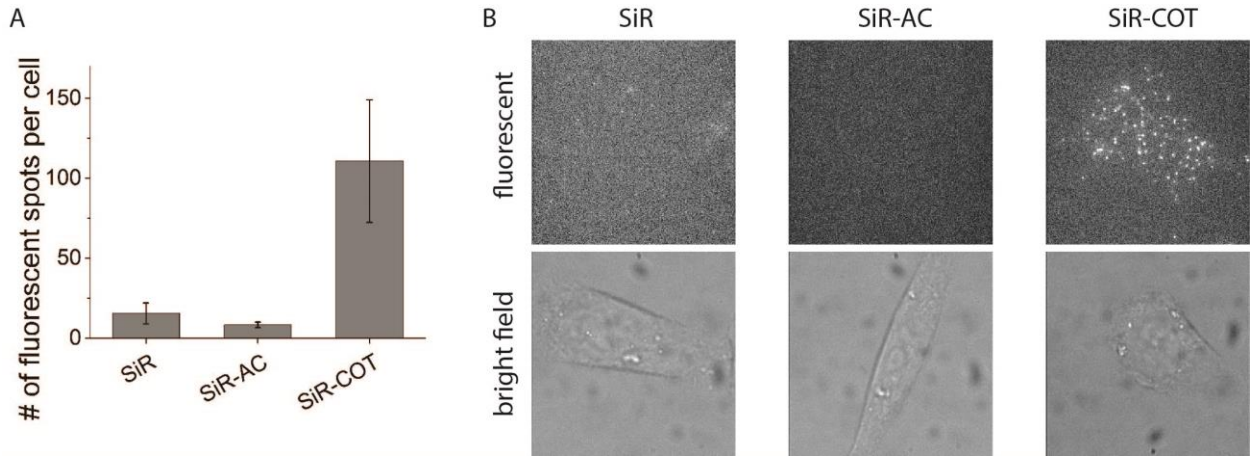


Figure S13. SiR-COT shows high non-specific binding and background fluorescence in cellular imaging. (A) Number of fluorescent spots per cell for cells that are not transfected with SNAP (Mock cells). (B) Representative bright-field and fluorescent images of mock cells after incubation with SiR, SiR-AC, or SiR-COT.

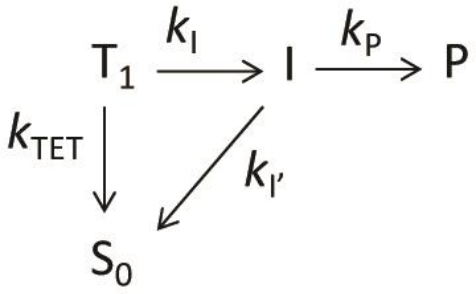
Table S1. Spectral properties of Cy5 and its self-healing derivatives

	Abs max (nm)[a]	Em max (nm)[b]
Cy5	647	662
Cy5-AC(4)	651	666
Cy5-AC(5)	650	666
Cy5-bisCOT(3)	655	671

(a) Absorbance spectra were measured in phosphate buffered saline (PBS) at 2 μM. (b) Emission spectra were measured in PBS at 500 nM.

Table S2. Photostability of SiR and SiR-AC observed *in vitro* conjugated to His-tagged SNAPf protein and imaged by TIRF microscopy (Methods).

	Photon counts in deoxygenated buffer ($\times 10^4$)	Photon counts in ambient oxygen ($\times 10^4$)
SiR	3.0 ± 0.1	1.9 ± 0.1
SiR-AC	48 ± 2	3.6 ± 0.1



$$\Phi_{B,T} = \frac{\frac{k_P}{k_{I'}} k_I}{k_I + k_{TET}}$$

$$\frac{1}{\Phi_{B,T}} = \frac{k_{I'}}{k_P k_I} k_{TET} + \frac{k_{I'}}{k_P} = \frac{k_{I'}}{k_P k_I} \frac{1}{\tau_T} + \frac{k_{I'}}{k_P}$$

Scheme S1. Photobleaching pathways from the triplet state in the absence of oxygen. T_1: triplet excited state; S_0: ground state; I: intermediate state to photobleaching; P: photobleached product; $\Phi_{B,T}$: Quantum yield of photobleaching from triplet state; k_{TET}: rate of intramolecular triplet energy transfer; τ_T: triplet state lifetime.

Calculating the effective concentration of protective agents

For intramolecular reactions, the effective concentration of a reactant is defined as the ratio between the first-order rate constant of the intra-molecular reaction (k_{intra}) and the second-order rate constant of the corresponding inter-molecular reaction (k_{inter}).[2] For self-healing fluorophores, k_{intra} is defined as the rate of triplet energy transfer, which is equal to $1/\tau_T$. For the Cy5-COT(3) fluorophore our triplet lifetime measurements suggest that k_{intra} is ~10^6 s^{-1}. The bimolecular rate constant of triplet energy transfer between COT and small organic molecules, k_{inter}, has been reported to be ~10^7 - 10^9 M^{-1} s^{-1}.[3-5] Given the assumption that k_{inter} between COT and the Cy5 fluorophore is also in the range of 10^7 - 10^9 M^{-1} s^{-1}, we can therefore estimate that the effective concentration of COT in the context of the Cy5-COT(3) molecule is 10^6 s^{-1}/ 10^7 - 10^9 M^{-1} s^{-1} (k_{intra}/k_{inter}) = 1 – 100 mM.

Cell labeling and preparation for TIRF imaging.

Generation of Stably Expressing SNAPf-D2s Cell Line

T-Rex Chinese hamster ovary (CHO) cells (Thermo Fisher Scientific) were stably transected with pFRT/lacZeo (Thermo Fisher Scientific), and single clones were selected by treating with 15 μg mL^{-1} blasticidin and 50 μg mL^{-1} zeocin. Stable integration of a previously described pcDNA5/FRT/TO-IRES construct encoding the amino-terminal SNAPfast-tagged human dopamine D2 receptor short isoform (D2s) [6] into each of the newly generated T-Rex Flp-In CHO clones was achieved by Flp recombinase-mediated DNA recombination using standard protocols and selected in 500 μg mL^{-1} hygromycin in the continued presence of 15 μg mL^{-1} blasticidin. Each stably expressing SNAPf-D2s CHO clone was labeled with saturating concentrations of BG-DY549 and imaged using objective-based TIRF to select a clone with a level of receptor expression optimal for single molecule imaging. The resulting clone was maintained as previously described [6] in the presence of 500 μg mL^{-1} hygromycin and15 μg mL^{-1} blasticidin.

Transfection and Expression of $G\alpha_{i1}$-SNAPf in Cells

Flp-in CHO cells (Thermo Fisher Scientific) were plated in six-well tissue culture dishes and cultured in Hams F12 (Cellgro) containing 10% fetal bovine serum and 1% glutamine prior to transfection. Cells at ~70% confluence were transfected for 6 h with a pcDNA3.1 construct encoding $G\alpha_{i1}$-SNAPfast at 333 ng mL^{-1} medium using Lipofectamine LTX (Thermo Fisher Scientific) as directed by the manufacturer's protocol. SNAPfast was inserted into $G\alpha_{i1}$ at amino acid position 91 in the same position where luciferase was introduced previously [7]. The construct was verified by sequencing and shown not to significantly alter function as compared to wild-type. Cells were labeled and used for experiments 16-18 h after transfection.

Cell Labeling and Preparation for TIRF Microscopy

To label SNAPf-D2s, cells were grown to ~70% confluence, washed with Dulbecco's phosphate-buffered saline (DPBS), resuspended in enzyme-free dissociation buffer (Millipore), and incubated in suspension with 500 nM of either BG-DY549 (NEB), BG-AF647 (NEB), BG-Cy3-AC, or BG-Cy5-AC in DPBS supplemented with 0.1% BSA for 30 min at 37 °C. After labeling, the cells were washed in suspension 5 times with DPBS supplemented with 0.1% BSA to remove excess fluorophore. Cells were then seeded on fibronectin-coated (0.1 μg μl^{-1}) (Sigma-Aldrich) glass

coverslips, (dimensions 22 x 22 mm, thickness 0.17 nm, SCHOTT Nexterion) and incubated in FluoroBrite DMEM medium (Thermo Fisher Scientific) for 1.5-2.0 h at 37 °C in 5% CO_2. The coverslips were cleaned before cell attachment using a procedure described previously [6]. Prior to imaging, coverslips with seeded cells were washed 7 times in DPBS, assembled into an imaging chamber [6], and either imaged for live cell experiments or fixed in a 5% paraformaldehyde solution overnight at 4 °C for next day imaging. To reduce approximately 50% of ambient oxygen collecting data, protocatechuate 3,4-dioxygenase from *Pseudomonas sp*. (Sigma-Aldrich) (PCD) and protocatechuic acid (Sigma-Aldrich) (PCA) were diluted in DPBS to a final concentration of 4 nM and 2 mM, respectively. After 30 minutes, half of the PCD/PCA mixture was added to the imaging chamber and half to a separate chamber with an oxygen meter to monitor oxygen depletion.

For labeling of intracellular $G\alpha_{i1}$-SNAPf, cells were labeled with 500 nM of SIR dye in Hams F12 (Cellgro) containing 10% fetal bovine serum and 1% glutamine for 30 min at 37 °C in 5% CO_2, and subsequently incubated in fresh media for 45 min before being resuspended in enzyme-free cell dissociation buffer (Millipore). After labeling, the cells were washed 5 times in DPBS supplemented with 0.1% BSA, seeded on coverslips, and prepared for microscopy as described above for SNAPf-D2s expressing cells.

Objective-Based TIRF Microscopy

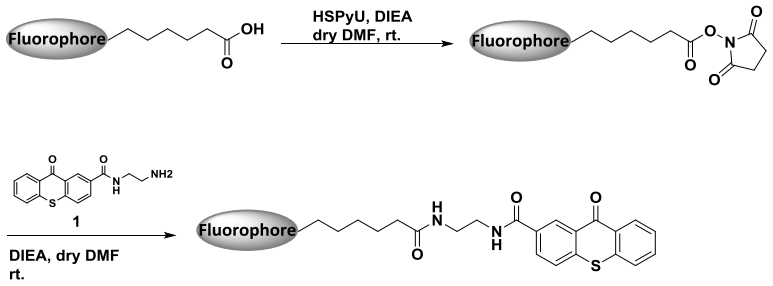
Image sequences were collected as described previously [6] using an objective-based TIRF microscope (IX81 with CellTIRF illuminator, Olympus) equipped with a 100× oil-immersion objective (100xUAPON NA 1.49, Olympus).BG-DY549 and BG-Cy3(4S)-AC(4) fluorophores were excited by a 532 nm laser line (Torus 150 mW, Laser Quantum). BG-AF647, BG-Cy5(4S)-AC(4), BG-SiR, and BG-SiR-AC were excited by a 640 nm laser line (100 mW, Olympus). Fluorescence emission was separated from excitation light using a dual band filter set suited for Cy3 and Cy5 fluorophores (ZET532/640x, ZET532/640m, ZT532/640rcp, Croma). Emission was further passed through a dual emission image splitter (OptoSplit-II, CAIRN) using an Optosplit filter cube for separating Cy3 and Cy5 emission (ET585/65m, ET655lp, zt640rdc) and projecting the two wavelengths onto an EMCCD camera (Evolve 512, Photometrics). Image sequences were collected at a time resolution of 40 ms for labeled SNAPf-D2s and or 25 ms for labeled G□i1-SNAPfast.

Synthesis and characterization

General Procedures

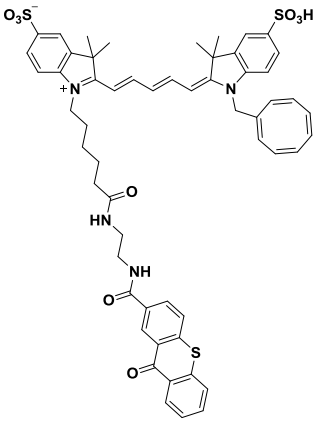
Unless otherwise stated, all commercially available materials were purchased from Aldrich, TCI, or Alfa Aesar and were used without any further purification. When necessary, solvents and reagents were dried prior to use, using standard protocols. All non-aqueous reactions were carried out in oven-dried glassware under an atmosphere of Argon. Analytical thin layer chromatography (TLC) was performed on silica gel 60, F254 plates (0.25 mm thickness) from SiliCycle. Visualization was accomplished by either irradiation under a 254 nm UV lamp or by staining with an aqueous solution of ceric ammonium molybdate (CAM). Flash chromatography was performed on silica gel 60 (230- 400 mesh). All LC-based separations involved a mobile phase of 10 mM TEAA pH 7.0 buffer (solvent A) or 0.1% formic acid aq. (solvent A) and pure acetonitrile (solvent B). HPLC separations were performed using a Varian PrepStar SD-1 solvent delivery system equipped with a Varian ProStar 335 diode array detector and a Waters Atlantis®Prep T3 column (5 µm, 19 x 150 mm), with a similarly packed guard column. LCMS separations were performed using a Waters ACQUITY UPLC system equipped with ACQUITY PDA (diode array) and FLR (fluorescence) detectors, a Waters Micromass SQD 2000 spectrometer, and a Waters ACQUITY HSS T3 column (1.8 µm, 2.1 x 100 mm). 1 H and 13C NMR spectra were acquired on a Bruker DRX-500 spectrometer at 500 MHz and 125 MHz respectively. Chemical shifts are expressed in parts per million downfield from tetramethylsilane (TMS), using either TMS or the solvent resonance as an internal standard (TMS, 1 H: 0 ppm; chloroform, 13C: 77.0 ppm). Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad), coupling constant, and integration.

Chemical Synthesis of 9-Oxothioxanthene-2-carboxylic Acid (OTX) Coupled Fluorophore



Fluorophore carboxylic acid 150 nmol was dissolved in 200 µL dry DMF, and then 50 µL DIEA was added to this DMF solution followed by addition of 300 nmol of Dipyrrolidino(N-succinimidyloxy)carbenium hexafluorophosphate (HSPyU). The mixture was vortexed and then incubated in the dark at room temperature. The reaction was monitored by LCMS until it was completed. Once completed, the reaction solution was poured into 15 mL of ethyl acetate (EtOAc), centrifuged. The residue was dissolved in 2 mL of 5% formic acid aq. solution, and purified using a semi-preparative HPLC C18 T3 column (Waters) with a 10 mM TEAA pH7.0 buffer mobile phase in a gradient of 10-90% acetonitrile. After evaporation of acetonitrile, the product was concentrated, buffer exchanged over a Sep-Pak C18 column and eluted with methanol followed by evaporation in a speed vac. to give the dry fluorophore-NHS ester product.

Fluorophore NHS ester 100 nmol was dissolved in 200 µL dry DMF, and then 50 µL DIEA was added to this DMF solution followed by addition of 200 nmol of amine **1** in 50 µL of dry DMF. The mixture was vortexed and then incubated in the dark at room temperature. The reaction was monitored by LCMS until it was completed. Once completed, the reaction was diluted to 3 mL with distilled, deionized water (ddH2O). Desired product was purified using a semi-preparative HPLC C18 T3 column (Waters) with a 10 mM TEAA pH7.0 buffer mobile phase in a gradient of 10-90% acetonitrile. After evaporation of acetonitrile, the product was concentrated, buffer exchanged over a Sep-Pak C18 column and eluted with methanol followed by evaporation in a speed vac. to give the final product.



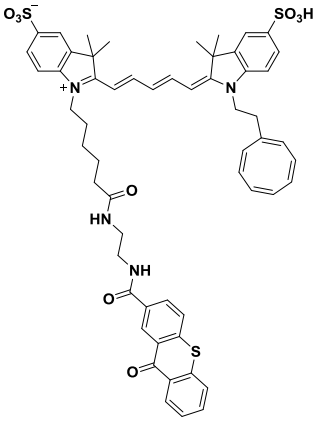
2

Cy5-COT(1)-OTX (2) 2-((1E,3E)-5-((E)-1-(((1E,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraen-1-yl)methyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-1-(6-oxo-6-((2-(9-oxo-9H-thioxanthene-2-carboxamido)ethyl)amino)hexyl)-3H-indol-1-ium-5-sulfonate

ESI-MS: *m/z* calculated for $C_{56}H_{56}N_4O_9S_3$ $[M+H]^+$ 1025.3, found 1025.1

LCMS: 15-85% B over 2.5 min, rt = 1.36 min

HPLC: 15-80% B over 20 min, rt = 10.99 min



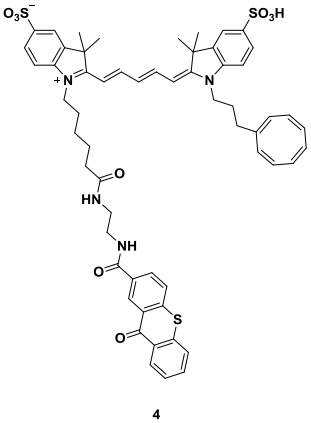
3

Cy5-COT(2)-OTX (3) 2-((1E,3E)-5-((E)-1-(2-((1Z,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraen-1-yl)ethyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-1-(6-oxo-6-((2-(9-oxo-9H-thioxanthene-2-carboxamido)ethyl)amino)hexyl)-3H-indol-1-ium-5-sulfonate

ESI-MS: *m/z* calculated for $C_{57}H_{58}N_4O_9S_3$ $[M+H]^+$ 1039.3, found 1039.2

LCMS: 15-85% B over 2.5 min, rt = 1.39 min

HPLC: 15-80% B over 20 min, rt = 11.50 min



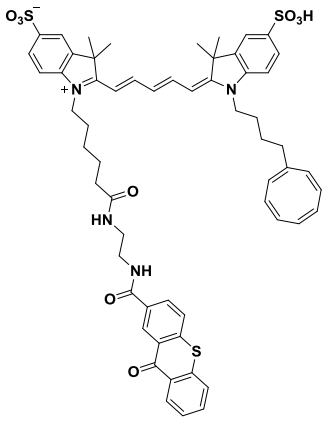
4

Cy5-COT(3)-OTX (4) 2-((1E,3E)-5-((E)-1-(3-((1Z,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraen-1-yl)propyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-1-(6-oxo-6-((2-(9-oxo-9H-thioxanthene-2-carboxamido)ethyl)amino)hexyl)-3H-indol-1-ium-5-sulfonate

ESI-MS: *m/z* calculated for $C_{58}H_{60}N_4O_9S_3$ $[M+H]^+$ 1053.3, found 1053.1

LCMS: 15-85% B over 2.5 min, rt = 1.46 min

HPLC: 15-80% B over 20 min, rt = 11.86 min



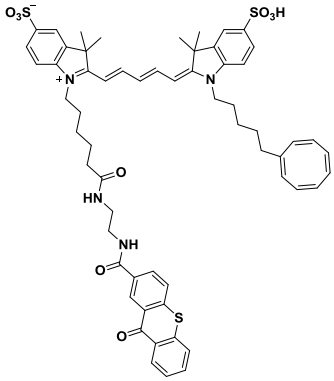
5

Cy5-COT(4)-OTX (5) 2-((1E,3E)-5-((E)-1-(4-((1Z,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraen-1-yl)butyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-1-(6-oxo-6-((2-(9-oxo-9H-thioxanthene-2-carboxamido)ethyl)amino)hexyl)-3H-indol-1-ium-5-sulfonate

ESI-MS: *m/z* calculated for $C_{59}H_{62}N_4O_9S_3$ $[M+H]^+$ 1067.4, found 1067.3

LCMS: 15-85% B over 2.5 min, rt = 1.55 min

HPLC: 15-80% B over 20 min, rt = 12.00min



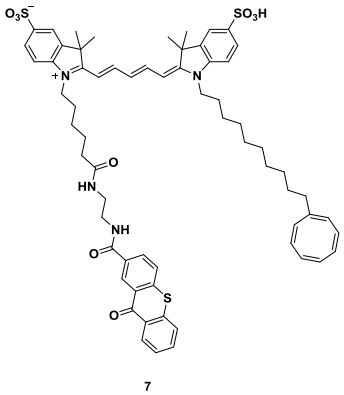
6

Cy5-COT(5)-OTX (6) 2-((1E,3E)-5-((E)-1-(5-((1Z,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraen-1-yl)pentyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-1-(6-oxo-6-((2-(9-oxo-9H-thioxanthene-2-carboxamido)ethyl)amino)hexyl)-3H-indol-1-ium-5-sulfonate

ESI-MS: *m/z* calculated for $C_{60}H_{64}N_4O_9S_3$ $[M+H]^+$ 1081.4, found 1081.2

LCMS: 15-85% B over 2.5 min, rt = 1.57 min

HPLC: 15-80% B over 20 min, rt = 12.60 min



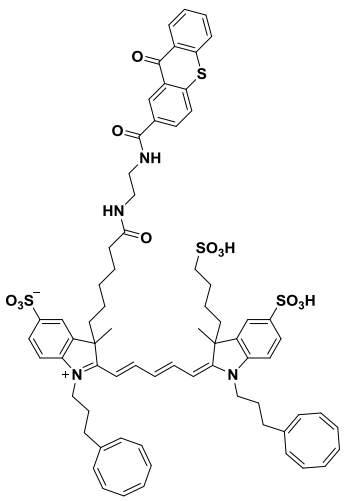
7

Cy5-COT(10)-OTX (7) 2-((1E,3E)-5-((E)-1-(10-((1Z,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraen-1-yl)decyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-1-(6-oxo-6-((2-(9-oxo-9H-thioxanthene-2-carboxamido)ethyl)amino)hexyl)-3H-indol-1-ium-5-sulfonate

ESI-MS: *m/z* calculated for $C_{65}H_{74}N_4O_9S_3$ $[M+H]^+$ 1151.5, found 1051.2

LCMS: 25-95% B over 2.0 min, rt = 1.67 min

HPLC: 25-95% B over 20 min, rt = 15.34 min



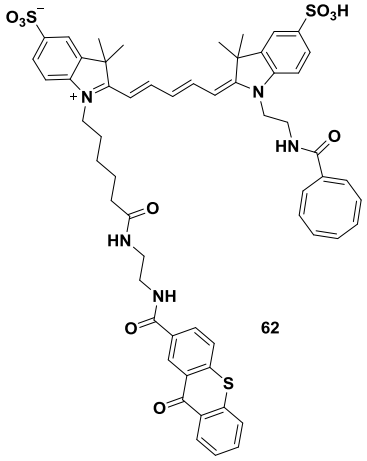
8

Cy5-bisCOT(3)-OTX (8) 1-(3-((1Z,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraen-1-yl)propyl)-2-((1E,3E)-5-((E)-1-(3-((1Z,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraen-1-yl)propyl)-3-methyl-5-sulfo-3-(4-sulfobutyl)indolin-2-ylidene)penta-1,3-dien-1-yl)-3-methyl-3-(6-oxo-6-((2-(9-oxo-9H-thioxanthene-2-carboxamido)ethyl)amino)hexyl)-3H-indol-1-ium-5-sulfonate

ESI-MS: *m/z* calculated for $C_{71}H_{76}N_4O_{12}S_4$ $[M- H]^-$ 1303.4, found 1303.2

LCMS: 15-85% B over 2.5 min, rt = 1.58 min

HPLC: 15-80% B over 20 min, rt = 12.70 min



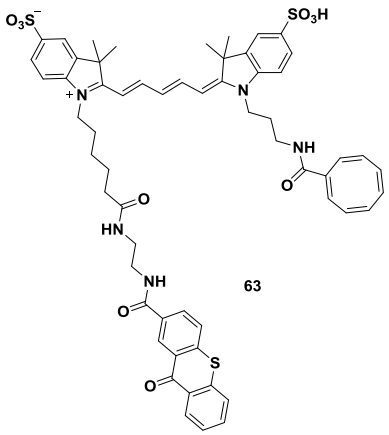
62

Cy5-AC(2)-OTX (62) 2-((1E,3E)-5-((E)-1-(2-((1E,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraene-1-carboxamido)ethyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-1-(6-oxo-6-((2-(9-oxo-9H-thioxanthene-2-carboxamido)ethyl)amino)hexyl)-3H-indol-1-ium-5-sulfonate

ESI-MS: *m/z* calculated for $C_{58}H_{59}N_5O_{10}S_3$ $[M+ H]^+$ 1082.3, found 1082.6

LCMS: 5-70% B over 2.5 min, rt = 1.81 min

HPLC: 15-80% B over 20 min, rt = 10.42min

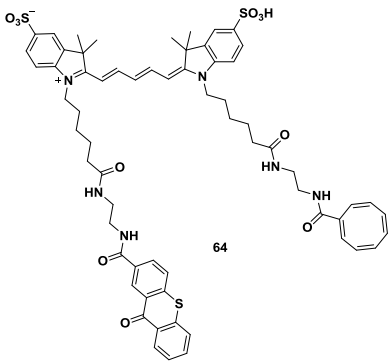


Cy5-AC(3)-OTX (63) 2-((1E,3E)-5-((E)-1-(3-((1E,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraene-1-carboxamido)propyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-1-(6-oxo-6-((2-(9-oxo-9H-thioxanthene-2-carboxamido)ethyl)amino)hexyl)-3H-indol-1-ium-5-sulfonate

ESI-MS: *m/z* calculated for $C_{59}H_{61}N_5O_{10}S_3$ $[M+H]^+$ 1096.4, found 1096.5

LCMS: 5-70% B over 2.5 min, rt = 2.04 min

HPLC: 15-80% B over 20 min, rt = 10.80 min



Cy5-AC(11)-OTX (64) 2-((1E,3E)-5-((E)-1-(6-((2-((1E,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraene-1-carboxamido)ethyl)amino)-6-oxohexyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-1-(6-oxo-6-((2-(9-oxo-9H-thioxanthene-2-carboxamido)ethyl)amino)hexyl)-3H-indol-1-ium-5-sulfonate

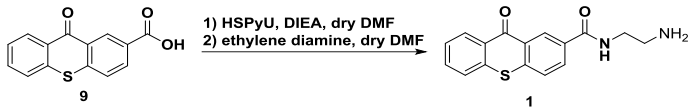
ESI-MS: *m/z* calculated for $C_{64}H_{70}N_6O_{11}S_3$ $[M+H]^+$ 1195.4, found 1195.5

LCMS: 5-70% B over 2.5 min, rt = 2.37 min

HPLC: 15-80% B over 20 min, rt = 10.88 min

Chemical Synthesis

1 Preparation of OTX-NH_2 (1)



9 1

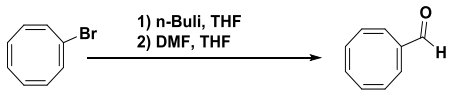
N-(2-aminoethyl)-9-oxo-9H-thioxanthene-2-carboxamide(1): 9-oxothioxanthene-2-carboxylic acid (**9**) (8.0 mg, 0.031 mmol, 1.0 equiv) was dissolved in 1 mL dry DMF. Diisopropylethylamine (DIEA) (100 µL, 2.9mmol, 18.5 equiv) was added to the DMF solution followed by addition of Dipyrrolidino(N-succinimidyloxy)carbenium hexafluorophosphate (HSPyU) (26 mg, 0.062 mmol, 2.0 equiv). The mixture was vortexed and then incubated at room temperature. The reaction was monitored by LCMS and completed in 15 mins. This solution was then added to 100 µL ethylene diamine in 500 µL dry DMF and stirred at rt. for 15 mins. Next, the reaction was diluted by 3 mL of H_2O and purified using a semi-preparative HPLC C18 T3 column (Waters) with a 0.1% formic acid mobile phase in a gradient of 10-90% acetonitrile. After evaporation of acetonitrile, the product was concentrated, buffer exchanged over a Sep-Pak C18 column and eluted with methanol followed by evaporation in a speed vac. to yielding the amine product **1** as a white solid (5.6 mg, 51.9%).

^{1}H NMR (D_2O): δ 8.37(s, 1H), 8.03(d, 1H, J=2.0Hz), 7.83(d, 1H, 8.0Hz), 7.35(m, 2H), 7.18(t, 2H, J= 8.0Hz), 7.11(d, 1H, J= 8.4Hz), 3.54(t, 2H, J= 6.2Hz), 3.17(t, 2H, J= 6.2Hz);

^{13}C NMR (D_2O): δ 180.0, 168.0, 141.5, 136.8, 133.2, 129.8, 129.4, 128.7, 127.3, 127.0, 126.9, 126.7, 126.5, 126.1, 39.2, 37.5;

ESI-MS: *m/z* calculated for $C_{16}H_{14}N_2O_2S$ $[M+H]^+$ 299.1, found 299.1

2 Preparation of Cy5-COT (1) (17)



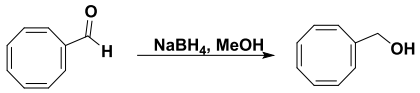
10 11

(1E,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraene-1-carbaldehyde(11): *n*-BuLi (2.5 M in hexanes) (2.2 mL, 5.46 mmol, 1.2 equiv) was added slowly at -78^o C to a solution of COT-Br (**10**)[6] (1.0 g, 5.46 mmol, 1 equiv) in 20 mL THF. The resulting mixture was stirred for 30 mins at -78^o C. To this solution was

added DMF (398 mg, 5.46 mmol, 1 equiv) in 2 mL dry THF. The reaction mixture was allowed to warm up to room temperature slowly and stirred overnight. The resulting solution was extracted by EtOAc, and dried over Na_2SO_4. This organic solution was then filtered, and the filtrate was concentrated by evaporation under reduced pressure. The remaining crude product was purified by silica gel column using 1:5 EtOAc: Hexanes. Product aldehyde **11**[8] (245 mg 34%) was obtained as a light yellow oil.

^{1}H NMR ($CDCl_3$): δ 9.48(s, 1H), 6.01-5.78(m, 7H)

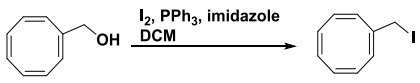
^{13}C NMR ($CDCl_3$): δ 192.6, 152.2, 144.1, 135.2, 134.7, 132.6, 131.1, 129.7, 127.4



11 12

((1E,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraen-1-yl)methanol(12): To the solution of aldehyde **11** (88.0 mg, 0.67 mmol, 1.0 equiv) in 7 mL MeOH was added $NaBH_4$ (80mg, 2.1mmol, 3.0 equiv) at 0^o C in 3 portions. The resulting mixture was stirred at 0^o C for 2 hrs. The reaction was then warmed up to room temperature, quenched by addition of $NaHCO_3$ sat. aq. solution, diluted with 100 mL EtOAc. The bi-layer solution was partitioned and the aqueous layer was removed. The organic solution was washed with H_2O, then brine, dried over Na_2SO_4, and filtered. The filtrate was concentrated by evaporation under reduced pressure. The crude product was purified by silica gel column using 1:3 EtOAc: Hexanes. Product alcohol **12** [9] (75mg, 85%) was obtained as light yellow oil.

^{1}H NMR ($CDCl_3$): δ5.94-5.82(m, 7H), 4.05(d, 2H, J=5.7Hz), 1.60(t, 1H, J=5.7Hz)

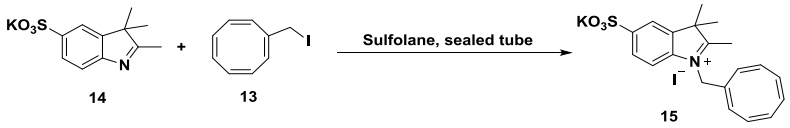


12 13

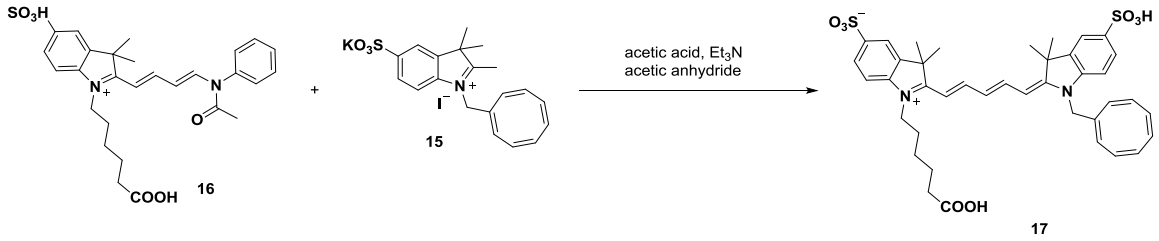
(1E,3Z,5Z,7Z)-1-(iodomethyl)cycloocta-1,3,5,7-tetraene(13): Alcohol **12** (36 mg, 0.27 mmol, 1.0 equiv) was dissolved in 10 mL dry DCM. Triphenolphosphine (77 mg, 0.29 mmol, 1.1 equiv) was added to this solution followed by addition of imidazole (73 mg, 1.07 mmol, 4.0 equiv) and I_2 flakes (75 mg, 0.29 mmol, 1.1 equiv) at room temperature. The resulting solution was stirred at room temperature for 2 hours before it was diluted with EtOAc, washed by H_2O and brine, dried over Na_2SO_4, and filtered. The filtrate was concentrated by evaporation under reduced pressure.

The crude product was purified by silica gel column using 1:3 EtOAc: Hexanes. Product **13** (48 mg, 75%) was obtained as light yellow oil.

^{1}H NMR ($CDCl_3$): δ6.01-5.72(m, 7H), 3.91(s, 2H)



1-(((1E,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraen-1-yl)methyl)-2,3,3-trimethyl-3H-indol-1-ium-5-sulfonate, potassium salt(15): To a sealed tube were added indole potassium salt **14** [6] (10 mg, 0.036 mmol, 1.0 equiv), (iodo-methyl) - COT **13** (10.5 mg, 0.043 mmol, 1.2 equiv) and 0.5 mL sulfolane. The mixture was heated to 100^o C and stirred overnight. The resulting purple solution was poured into 15 mL of EtOAc to precipitate the crude product **15** (15 mg, recovery 79%) as a purple solid. The precipitate was dried in vacuum and carried onto the next step without further purification. ESI-MS: *m/z* calculated for $C_{20}H_{21}IKNO_3S$ $[M+H-I-K]^+$ 356.1, found 356.3



1-(5-carboxypentyl)-2-((1E,3E)-5-((E)-1-(((1E,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraen-1-yl)methyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-3H-indol-1-ium-5-sulfonate(17): Indolelium **15** (15 mg, 0.031 mmol, 1.0 equiv) and indolelium **16**[10] (16 mg, 0.031 mmol, 1.0 equiv) were dissolved in 2 mL of glacial acetic acid and 0.6 mL of acetic anhydride. To this solution was added 0.3 mL of trimethylamine slowly while stirring. The reaction solution was heated to 110^o C and stirred for 3 hrs. The resulting dark blue solution was then cooled to room temperature and poured into 45 mL of EtOAc to precipitate the crude product. The precipitate was dissolved in 10 mL of water and purified using a semi-preparative HPLC C18 T3 column (Waters) with a 0.1% formic acid mobile phase in a gradient of 10-90% acetonitrile. After evaporation of acetonitrile, the product was concentrated, buffer exchanged over a Sep-Pak C18

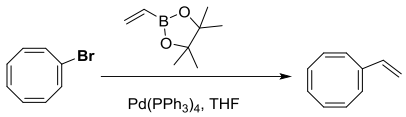
column and eluted with methanol followed by evaporation in a speed vac. to give the fluorophore product **17** (5.8 mg, 25.7%) as a dark blue solid.

^{1}H NMR (MeOD-d4): δ 8.36(m, 2H), 7.90(m, 4H), 7.42(d, 1H, J=8.4Hz), 7.33(d, 1H, J=8.4Hz), 6.72(t, 1H, 12.4Hz), 6.45(d, 1H, J= 13.8Hz), 6.38(d, 1H, J=13.8Hz), 5.82- 5.67(m, 7H), 4.78(br, 2H), 4.17(t, 2H, J=7.6Hz), 2.25(t, 2H, J=7.3Hz), 1.86(t, 2H, J=7.6Hz), 1.79(s, 6H), 1.77(s, 6H), 1.72(t, 2H, J=7.6Hz), 1.54(m, 2H),

^{13}C NMR (MeOD-d4): δ 208.7, 174.9, 173.2, 155.5, 154.3, 143.9, 143.3, 142.4, 141.6, 141.5, 140.8, 140.3, 139.2, 136.4, 134.0, 131.6, 131.0, 129.9, 129.7, 126.7, 126.6, 126.4, 120.0, 119.9, 114.7, 110.7, 110.4, 104.8, 104.6, 29.3, 26.9, 26.6, 26.3, 25.7

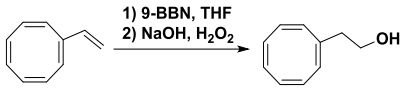
ESI-MS: *m/z* calculated for $C_{40}H_{44}N_2O_8S_2$ $[M+H]^+$ 745.3, found 745.6

3 Cy5-COT (2) (22)

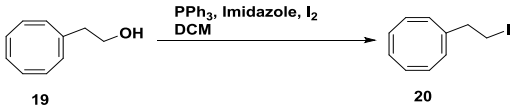


10 18

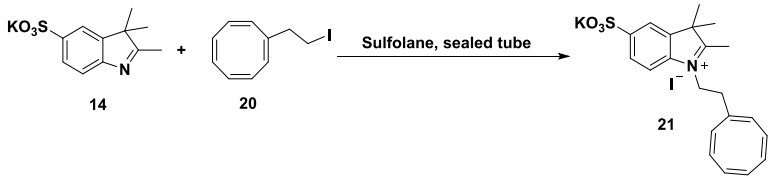
(1Z,3Z,5Z,7Z)-1-vinylcycloocta-1,3,5,7-tetraene(18): COT-Br (**10**) (473 mg, 2.58 mmol, 1.0 equiv) was dissolved in 10 mL THF. To this solution was added vinylboronic acid pinacol ester (397 mg, 2.58 mmol, 1equiv), followed by 2.58 mL of 3N NaOH and tetrakis(triphenylphosphine)palladium(0) (60 mg, 0.052 mmol, 0.02 equiv). The reaction mixture was heated to reflux and stirred overnight. After cooled to room temperature, the reaction solution was diluted with diethyl ether (Et_2O), washed by H_2O and brine, dried over Na_2SO_4, and filtered. The filtrate was concentrated under reduced pressure. The residue was purified by silica gel column using 1:3 EtOAc: Hexanes. Product **18** (178 mg, 53%) was obtained as a light yellow oil. ^{1}H NMR ($CDCl_3$): δ 6.45(dd, 1H, J1=17.5Hz, J2=10.5Hz), 6.00-5.78(m, 7H), 5.08(d, 1H, J=17.5Hz), 4.98(d, 1H, J=10.5Hz)



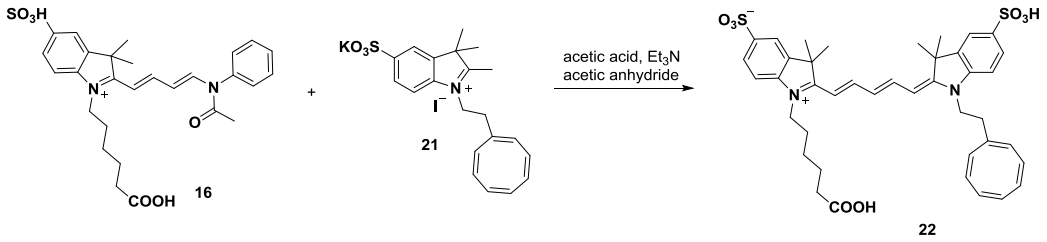
2-((1Z,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraen-1-yl)ethan-1-ol(19): Compound **18** (160 mg, 1.23 mmol, 1 equiv) was dissolved in 2 mL dry THF and, and cooled to 0° C. To this solution was slowly added 9-BBN 0.5 M THF solution (3 mL, 1.5 mmol, 1.2 equiv). The reaction solution was stirred for 3 hrs while slowly warming to room temperature. The solution was then cooled to 0° C again, and 3 mL of 2N NaOH aq. solution was added, followed by 3 mL H_2O_2 (35%). The resulting solution was stirred for another 3 hrs while slowly warming to room temperature. The reaction was extracted by EtOAC, washed by H_2O and brine, dried over Na_2SO_4, and filtered. The filtrate was concentrated by evaporation under reduced pressure. Residue was purified by silica gel column using 1:3 EtOAc: Hexanes. Product **19** [8] (51 mg, 25%) was obtained as light yellow oil. ^{1}H NMR ($CDCl_3$): δ 6.01-5.76(m, 7H), 3.67(d, 2H, J=6.2Hz), 2.38(br, 2H)



(1Z,3Z,5Z,7Z)-1-(2-iodoethyl)cycloocta-1,3,5,7-tetraene(20): Alcohol **19** (45 mg, 0.31 mmol, 1.0 equiv) was dissolved in 10 mL dry DCM. To this solution was added triphenolphosphine (88 mg, 0.34 mmol, 1.1 equiv), followed by addition of imidazole (83 mg, 1.24 mmol, 4.0 equiv), and I_2 flakes (78 mg, 0.34 mmol, 1.1 equiv) at room temperature. The resulting solution was stirred at room temperature for 2 hours, diluted by EtOAc, washed by H_2O and brine, dried over Na_2SO_4, and filtered. The filtrate was concentrated by evaporation under reduced pressure. Residue was purified by silica gel column using 1:3 EtOAc: Hexanes. Product **20** (42 mg, 54%) was obtained as light yellow oil. ^{1}H NMR ($CDCl_3$): δ5.95-5.58(m, 7H), 3.23(br, 2H), 2.56(t, 2H, J=6.2Hz)



1-(2-((1Z,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraen-1-yl)ethyl)-2,3,3-trimethyl-3H-indol-1-ium-5-sulfonate, potassium salt(21): To a sealed tube were added indole **14** potassium salt (10 mg, 0.036 mmol, 1.0 equiv), (iodoethyl) - COT **20** (11.1 mg, 0.043mmol, 1.2 equiv) and 0.5 mL sulfolane. The reaction was heated to 100^{o} C and stirred overnight. The resulting purple solution was poured into 15 mL of EtOAc to precipitate the crude product **21** (17 mg, recovery 89%) as a purple solid. The precipitate was dried and carried onto the next step without further purification. ESI-MS: *m/z* calculated for $C_{20}H_{21}IKNO_3S$ $[M+H-I-K]^+$ 370.2, found 370.2



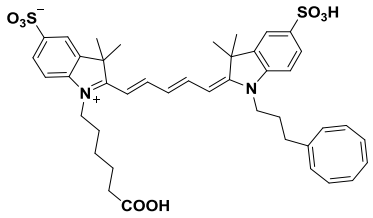
1-(5-carboxypentyl)-2-((1E,3E)-5-((E)-1-(2-((1Z,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraen-1-yl)ethyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-3H-indol-1-ium-5-sulfonate(22): Indolelium **21** (17 mg, 0.032 mmol, 1.0 equiv) and indolelium **16** (16.7 mg, 0.032mmol, 1.0 equiv) were dissolved in 2 mL of glacial acetic acid and 0.6 mL of acetic anhydride. To this solution was added 0.3 mL of trimethylamine slowly while stirring. The reaction solution was heated to 110^{o} C and stirred for 3 hrs. The resulting dark blue solution was then cooled to room temperature and poured into 45 mL of EtOAc to precipitate the crude product. The precipitate was then dissolved in 10 mL of water and purified using a semi-preparative HPLC C18 T3 column (Waters) with a 0.1% formic acid mobile phase in a gradient of 10-90% acetonitrile. After evaporation of acetonitrile, the product was concentrated, buffer exchanged over a Sep-Pak C18 column and eluted with methanol followed by evaporation in a speed vac yielding the fluorophore product **22** (4.3mg, 17.9%) as a dark blue solid.

^{1}H NMR (MeOD-d4): δ 8.35(m, 2H), 7.90(m, 4H), 7.39(d, 1H, J=8.6Hz), 7.34(d, 1H, J=8.6Hz), 6.72(t, 1H, 12.4Hz), 6.40(d, 1H, J= 13.8Hz), 6.35(d, 1H, J=13.8Hz), 5.87- 5.52(m, 7H), 4.22(t, 2H, J=6.7Hz), 4.16(t, 2H, J=7.6Hz), 2.61(br, 2H), 2.28(t, 2H, J=7.3Hz), 1.86(t, 2H, J=7.6Hz), 1.79(s, 6H), 1.77(s, 6H), 1.72(t, 2H, J=7.6Hz), 1.54(m, 2H)

^{13}C NMR (MeOD-d4): δ 174.2, 173.8, 155.0, 154.6, 143.6, 143.5, 142.1, 141.9, 141.3, 141.2, 139.3, 133.0, 132.8, 131.8, 131.7, 131.5, 131.1, 130.3, 128.4, 126.7, 126.5, 126.4, 120.0, 119.9, 110.7, 104.5, 104.1, 49.3, 49.2, 43.8, 42.8, 34.6, 29.4, 26.8, 26.5, 26.4, 26.2, 25.1

ESI-MS: *m/z* calculated for $C_{41}H_{46}N_2O_8S_2$ $[M+H]^+$ 759.3, found 759.3

4 Cy5-COT (3) (23)



23

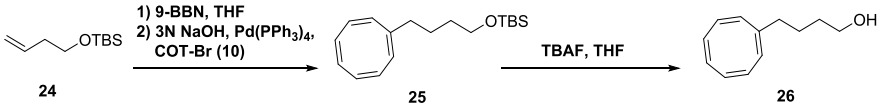
1-(5-carboxypentyl)-2-((1E,3E)-5-((E)-1-(3-((1Z,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraen-1-yl)propyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-3H-indol-1-ium-5-sulfonate(23): Synthesized by following previously published procedures.[11]

^{1}H NMR (MeOD-d4): δ 8.35(m, 2H), 7.92(m, 4H), 7.39(d, 1H, J=8.9Hz), 7.36(d, 1H, J=8.3Hz), 6.70(t, 1H, 12.4Hz), 6.40(d, 1H, J= 13.8Hz), 6.36(d, 1H, J=13.8Hz), 5.97- 5.68(m, 7H), 4.16(t, 4H, J=7.4Hz), 2.26(m, 4H), 1.93(t, 2HJ=7.6Hz), 1.86(t, 2H, J=7.6Hz), 1.79(s, 12H), 1.72(t, 2H, J=7.6Hz), 1.54(m, 2H)

^{13}C NMR (MeOD-d4): δ 208.7, 174.3, 173.7, 155.1, 154.6, 143.5, 143.4, 142.3, 142.2, 141.9, 141.3, 141.2, 133.6, 132.3, 131.8, 131.7, 131.5, 130.9, 128.0, 126.7, 126.5, 126.2, 120.0, 110.4, 110.2, 104.1, 49.32, 49.30, 49.1, 43.8, 43.3, 35.9, 34.1, 29.3, 26.8, 26.4, 26.3, 26.2, 25.3

ESI-MS: *m/z* calculated for $C_{42}H_{48}N_2O_8S_2$ $[M+H]^+$ 773.3, found 773.2

5 Cy5-COT (4) (29)



24 25 26

4-((1Z,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraen-1-yl)butan-1-ol(26): TBS ether **24**[12] (680 mg, 3.66 mmol, 1 equiv) was dissolved in 10 mL dry THF. 9-BBN(0.5M in THF) (8.8 mL, 4.4 mmol, 1.2 equiv) was added slowly to the TBS ether solution at 0^{o} C. The solution was warmed to room temperature and stirred for 3 more hrs. To this solution was then added 4 mL 3N NaOH aq.

solution, followed by tetrakis(triphenylphosphine)palladium(0) (84 mg, 0.072nmol, 0.02 equiv) and COT-Br (**10**) (680, 3.66mmol, 1 equiv) . The resulting mixture was refluxed overnight, cooled to room temperature, diluted by EtOAc, washed by H_2O and brine, dried over Na_2SO_4, and filtered. The filtrate was concentrated under reduced pressure. Residue was filtered through a short silica gel plug using 1:5 EtOAc: Hexanes and concentrated. The product **25** (1.0 g, recovery 92%) was obtained as a light yellow oil.

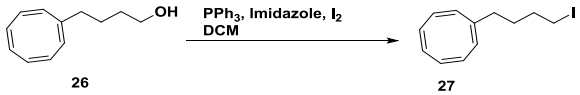
^{1}H NMR ($CDCl_3$): δ 5.81-5.76(m, 6H), 5.58(s, 1H), 3.64(t, 2H, J=6.5Hz), 2.01(t, 2H, J=7.5Hz), 1.59(br, 2H), 1.48(br, 2H), 0.93(s, 9H), -0.08(s, 6H)

^{13}C NMR ($CDCl_3$): δ 144.5, 134.4, 132.5, 131.9, 131.8, 131.1, 130.9, 126.2, 63.1, 37.4, 32.3, 26.0, 27.4, 18.4, -5.2

To the TBS ether product **25** solution in 20mL dry THF was added TBAF (1 M in THF) (7.3 mL, 7.3 mmol, 2 equiv). The reaction solution was stirred at room temperature for 2 hrs. The reaction was then diluted by EtOAC, washed by H_2O and brine, dried over Na_2SO_4, and filtered. The filtrate was concentrated by evaporation under reduced pressure. Residue was purified by silica gel column using 1:5 EtOAC: Hexanes. Product alcohol **26** (470 mg, 73% over 2 steps) was obtained as a light yellow oil.

^{1}H NMR ($CDCl_3$): δ 5.82-5.78(m, 6H), 5.57(s, 1H), 3.68(t, 2H, J=6.5Hz), 2.10(t, 2H, J=7.5Hz), 1.65(br, 2H), 1.51(br, 2H), 1.35(s, 1H)

^{13}C NMR ($CDCl_3$): δ 144.2, 134.2, 132.4, 132.0, 131.9, 131.3, 130.9, 126.5, 62.9, 37.4, 32.2, 24.6



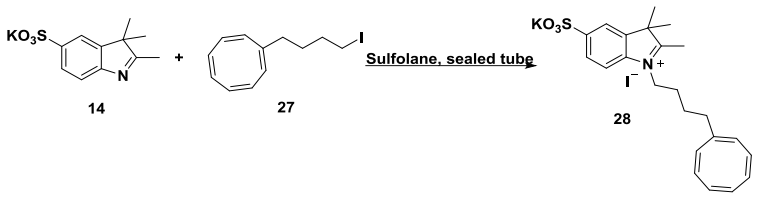
26 27

(1Z,3Z,5Z,7Z)-1-(4-iodobutyl)cycloocta-1,3,5,7-tetraene(27): Alcohol **26** (240 mg, 1.36 mmol, 1.0 equiv) was dissolved in 10 mL dry DCM. To this solution were added triphenolphosphine (392 mg, 1.50 mmol, 1.1 equiv), imidazole (370 mg, 5.44 mmol, 4.0 equiv), followed by the addition of I_2 flakes (380 mg, 1.50 mmol, 1.1 equiv) at room temperature. The resulting solution was stirred at room temperature for 2 hours, diluted by EtOAc, washed by H_2O and brine, dried over Na_2SO_4, and filtered. The filtrate was concentrated by evaporation under reduced pressure. Residue was

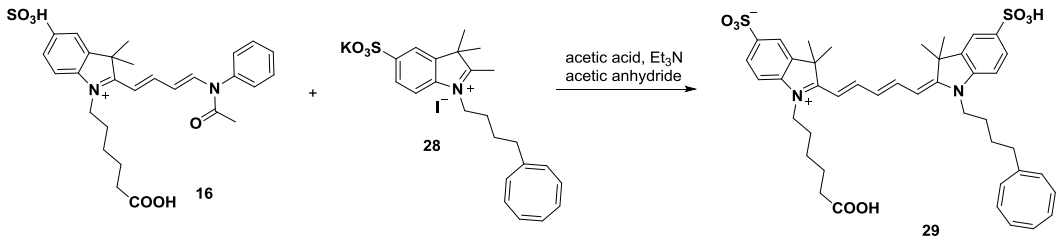
purified by silica gel column using 1:3 EtOAc: Hexanes. Product **27** (261 mg, 67%) was obtained as light yellow oil.

^{1}H NMR ($CDCl_3$): δ 5.83-5.77(m, 6H), 5.58(s, 1H), 3.23(t, 2H, J=7.0Hz), 2.10(t, 2H, J=7.5Hz), 1.58(br, 2H), 1.55(t, 2H, J=7.4Hz)

^{13}C NMR ($CDCl_3$): δ 143.7, 134.0, 132.3, 132.0, 131.9, 131.5, 131.1, 126.8, 36.5, 32.7, 29.1, 7.1



1-(4-((1Z,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraen-1-yl)butyl)-2,3,3-trimethyl-3H-indol-1-ium-5-sulfonate, potassium salt(28): To a sealed tube were added indole **14** potassium salt (10 mg, 0.036 mmol, 1.0 equiv), (iodobutyl) - COT **27** (12.3 mg, 0.043 mmol, 1.2 equiv) and 0.5 mL sulfolane. The reaction slurry was heated to 100^o C and stirred overnight. The resulting purple solution was poured into 15 mL of EtOAc to precipitate the crude product **28** (16 mg, recovery 79%) as a purple solid. The precipitate was dried under vacuum and carried onto the next step without further purification. ESI-MS: *m/z* calculated for $C_{23}H_{27}IKNO_3S$ $[M+H-I-K]^+$ 398.2, found 398.4



1-(5-carboxypentyl)-2-((1E,3E)-5-((E)-1-(4-((1Z,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraen-1-yl)butyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-3H-indol-1-ium-5-sulfonate(29): Indolelium **28** (16 mg, 0.030 mmol, 1.0 equiv) and indolelium **16** (15.8 mg, 0.030 mmol, 1.0 equiv) were dissolved in 2 mL of glacial acetic acid and 0.6 mL of acetic anhydride. To this solution was added 0.3 mL of trimethylamine slowly while stirring. The reaction solution was heated to 110^o C for 3 hrs. The resulting dark blue solution was then cooled to room temperature

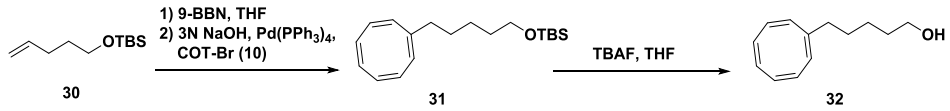
and poured into 45 mL of EtOAc to precipitate the crude product. The precipitate was dissolved in 10 mL of water and purified using a semi-preparative HPLC C18 T3 column (Waters) with a 0.1% formic acid mobile phase in a gradient of 10-90% acetonitrile. After evaporation of acetonitrile, the product was concentrated, buffer exchanged over a Sep-Pak C18 column and eluted with methanol followed by evaporation in a speed vac to give the fluorophore product **29** (3.8 mg, 16%) as a dark blue solid.

^{1}H NMR (MeOD-d4): δ 8.34(t, 2H, J=13.8Hz), 7.92(m, 4H), 7.38(m, 2H), 6.73(t, 1H, 12.4Hz), 6.39(d, 1H, J= 13.7Hz), 6.38(d, 1H, J=13.7Hz), 5.77- 5.58(m, 7H), 4.16(m, 4H), 2.26(t, 2H, J=7.3Hz), 2.19(t, 2H, J=6.9Hz), 1.86(t, 2H, J=7.6Hz), 1.78(s, 12H), 1.74-1.71(m, 4H), 1.57-1.51(m, 4H)

^{13}C NMR (MeOD-d4): δ 180.2, 174.0, 173.8, 154.9, 154.7, 143.6, 143.5, 143.2, 142.0, 141.9, 141.3, 141.2, 133.5, 131.8, 131.4, 130.7, 127.2, 126.7, 126.6, 126.4, 119.9, 110.5, 110.3, 104.0, 103.9, 49.2, 29.1, 43.8, 43.6, 36.5, 29.4, 26.8, 26.4, 26.39, 26.33, 26.1, 25.6, 25.0

ESI-MS: *m/z* calculated for $C_{43}H_{50}N_2O_8S_2$ $[M+H]^+$ 787.3, found 787.5

6 Preparation of Cy5-COT (5) (35)



5-((1Z,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraen-1-yl)pentan-1-ol(32): TBS ether **30**[13] (470 mg, 2.35 mmol, 1 equiv) was dissolved in 10 mL dry THF. 9-BBN (0.5M in THF) (5.64 mL, 2.8 mmol, 1.2 equiv) was added slowly to the TBS ether solution at 0^{o} C. The solution was warmed to room temperature and stirred for 3 more hrs. Next, 4 mL 3N NaOH aq. solution, tetrakis(triphenylphosphine)palladium(0) (54 mg, 0.047 nmol, 0.02 equiv) and COT-Br (**10**) (430, 2.35 mmol, 1 equiv) were added. The reaction was refluxed overnight, cooled to room temperature, diluted by EtOAc, washed by H_2O and brine, dried over Na_2SO_4, and filtered. The filtrate was concentrated by evaporation under reduced pressure. Residue was filtered through a short silica gel plug using 1:5 EtOAc: Hexanes and concentrated. The product **31** (688 mg, recovery 88%) was carried onto the next step without further purification.

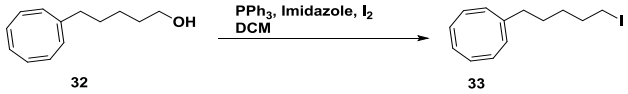
^{1}H NMR ($CDCl_3$): δ 5.85-5.76(m, 6H), 5.58(s, 1H), 3.63(t, 2H, J=6.7Hz), 2.07(t, 2H, J=7.1Hz), 1.55(m, 2H), 1.43(br, 4H), 0.92(s, 9H), 0.08(s, 6H)

^{13}C NMR ($CDCl_3$): δ 144.6, 134.4, 132.5, 131.9, 131.8, 131.0, 130.8, 126.1, 63.3, 37.7, 32.7, 28.3, 26.0, 25.4, 18.4, -5.2

To the TBS ether product **31** solution in 20 mL dry THF was added TBAF (1M in THF) (4.7mL, 4.7mmol, 2 equiv). The reaction solution was stirred at room temperature for 2 hrs. The reaction was then diluted by EtOAC, washed by H_2O and brine, dried over Na_2SO_4, and filtered. The filtrate was concentrated by evaporation under reduced pressure. Residue was purified by silica gel column using 1:5 EtOAC: Hexanes. Product alcohol **32** (362mg, 81% over 2 steps) was obtained as a light yellow oil.

^{1}H NMR ($CDCl_3$): δ 5.85-5.76(m, 6H), 5.58(s, 1H), 3.67(t, 2H, J=6.5Hz), 2.08(t, 2H, J=6.6Hz), 1.60(t, 2H, J=7.0Hz), 1.43(br, 4H), 1.31(s, 1H)

^{13}C NMR ($CDCl_3$): δ 144.5, 134.4, 132.4, 131.91, 131.90, 131.2, 130.9, 126.3, 63.0, 37.6, 32.6, 28.1, 25.1

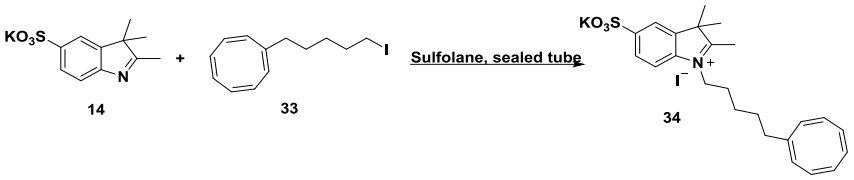


32 33

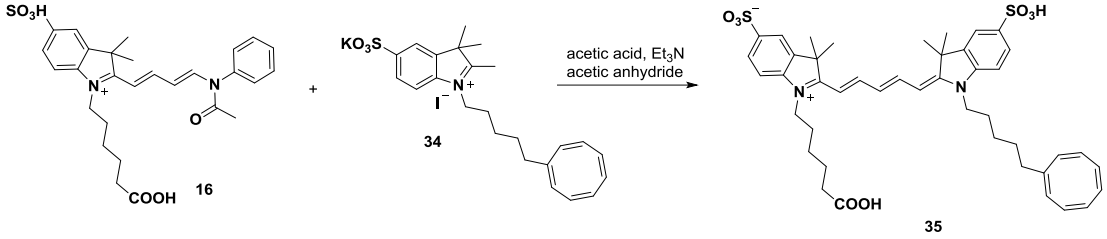
(1Z,3Z,5Z,7Z)-1-(5-iodopentyl)cycloocta-1,3,5,7-tetraene(33): Alcohol **32** (360mg, 1.89mmol, 1.0 equiv) was dissolved in 10 mL dry DCM. To this solution were added triphenolphosphine (546 mg, 2.08 mmol, 1.1 equiv), imidazole (514 mg, 7.56 mmol, 4.0 equiv), followed by the addition of I_2 flakes (528 mg, 2.08 mmol, 1.1 equiv) at room temperature. The resulting solution was stirred at room temperature for 2 hours, diluted by EtOAc, washed by H_2O and brine, dried over Na_2SO_4, and filtered. The filtrate was concentrated by evaporation under reduced pressure. Residue was purified by silica gel column using 1:3 EtOAc: Hexanes. Product **33** (506 mg, 89%) was obtained as a light yellow oil.

^{1}H NMR ($CDCl_3$): δ 5.85-5.75(m, 6H), 5.58(s, 1H), 3.22(t, 2H, J=7.1Hz), 2.08(t, 2H, J=7.0Hz), 1.87(t, 2H, J=7.0Hz), 1.46(br, 4H)

^{13}C NMR ($CDCl_3$): δ 144.2, 134.2, 132.4, 131.94, 131.92, 131.3, 131.0, 126.4, 37.4, 33.5, 29.9, 27.3, 7.1



1-(5-((1Z,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraen-1-yl)pentyl)-2,3,3-trimethyl-3H-indol-1-ium-5-sulfonate, potassium salt(34): To a sealed tube were added indole **14** potassium salt (10 mg, 0.036 mmol, 1.0 equiv), (iodopentyl) - COT **33** (13 mg, 0.043 mmol, 1.2 equiv) and 0.5 mL sulfolane. The reaction slurry was heated to 100^{o} C and stirred overnight. The resulting purple solution was poured into 15 mL of EtOAc to precipitate the crude product **34** (18 mg, recovery 86%) as a purple solid. The precipitate was dried and carried onto the next step without further purification. ESI-MS: *m/z* calculated for $C_{24}H_{29}IKNO_3S$ $[M+H-I-K]^+$ 412.2, found 412.5



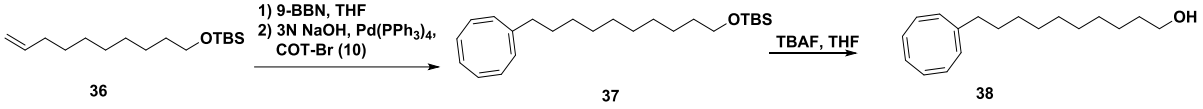
1-(5-carboxypentyl)-2-((1E,3E)-5-((E)-1-(5-((1Z,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraen-1-yl)pentyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-3H-indol-1-ium-5-sulfonate(35): Indolelium **34** (18 mg, 0.031 mmol, 1.0 equiv) and indolelium **16** (16.4 mg, 0.031 mmol, 1.0 equiv) were dissolved in 2 mL of glacial acetic acid and 0.6 mL of acetic anhydride. To this solution was added 0.3 mL of trimethylamine slowly while stirring. The reaction solution was heated to 110^{o} C and stirred for 3 hrs. The resulting dark blue solution was then cooled to room temperature and poured into 45 mL of EtOAc to precipitate the crude product. The precipitate was then dissolved in 10mL of water and purified using a semi-preparative HPLC C18 T3 column (Waters) with a 0.1% formic acid mobile phase in a gradient of 10-90% acetonitrile. After evaporation of acetonitrile, the product was concentrated, buffer exchanged over a Sep-Pak C18 column and eluted with methanol followed by evaporation in a speed vac. to give the fluorophore product **35** (5.5 mg, 22%) as a dark blue solid.

^{1}H NMR (MeOD-d4): δ 8.34(t, 2H, J=13.0Hz), 7.92(m, 4H), 7.38(m, 2H), 6.71(t, 1H, 12.4Hz), 6.39(d, 1H, J= 13.7Hz), 6.38(d, 1H, J=13.7Hz), 5.77- 5.51(m, 7H), 4.16(m, 4H), 2.29(t, 2H, J=7.1Hz), 2.08(t, 2H, J=6.4Hz), 1.86(m, 4H), 1.78(s, 12H), 1.73-1.70(m, 4H), 1.54-1.51(m, 4H)

^{13}C NMR (MeOD-d4): δ 174.0, 154.8, 143.6, 143.5, 142.0, 141.2, 136.2, 134.5, 133.7, 131.9, 131.7, 131.4, 131.1, 130.5, 129.4, 127.9, 127.4, 126.7, 126.6, 126.5, 126.3, 125.9, 125.3, 120.0, 110.3, 110.2, 103.9, 103.8, 29.2, 49.1, 438.8, 43.7, 36.8, 29.4, 27.4, 26.8, 26.7, 26.5, 26.4, 26.2, 25.6, 25.1

ESI-MS: *m/z* calculated for $C_{44}H_{52}N_2O_8S_2$ $[M+H]^+$ 801.3, found 801.6

7 Preparation of Cy5-COT (10) (41)



10-((1Z,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraen-1-yl)decan-1-ol(38): TBS ether **36**[14] (664 mg, 2.45 mmol, 1 equiv) was dissolved in 10 mL dry THF. 9-BBN (0.5M in THF) (5.9 mL, 2.95 mmol, 1.2 equiv) was added slowly to the TBS ether solution at 0^o C. The solution was warmed to room temperature and stirred for 3 more hrs. To this solution were then added 4 mL 3N NaOH aq. solution, tetrakis(triphenylphosphine)palladium(0) (56 mg, 0.05 nmol, 0.02 equiv) and COT-Br (**10**) (448 mg, 2.45 mmol, 1 equiv). The mixture was refluxed overnight, cooled to room temperature, diluted by EtOAc, washed by H_2O and brine, dried over Na_2SO_4, and filtered. The filtrate was concentrated by evaporation under reduced pressure. Residue was purified by silica gel column using 1:20 EtOAC: Hexanes. Product **37** (649mg, 70.7%) was obtained as a light yellow oil.

^{1}H NMR ($CDCl_3$): δ 5.77-5.74(m, 6H), 5.52(s, 1H), 3.57(t, 2H, J= 5.6Hz), 2.00(t, 2H, J= 6.1Hz), 1.52-1.25 (m, 16H), 0.87(s, 9H), 0.03(s, 6H)

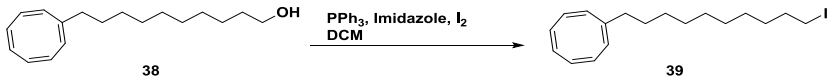
^{13}C NMR ($CDCl_3$): δ144.8, 134.5, 132.5, 131.9, 131.8, 130.9, 128.4, 125.9, 63.3, 37.7, 32.9, 29.6, 29.5, 29.4, 29.1, 26.0, 25.8, 18.4, 0.0,-5.2

To the crude TBS ether product **37** (649 mg, 1.73 mmol, 1 equiv) solution in 20mL dry THF was added TBAF (1M in THF) (5.2 mL, 5.2 mmol, 3 equiv). The reaction solution was stirred at room temperature for 2 hrs. The reaction was then diluted by EtOAC, washed by H_2O and brine, dried

over Na_2SO_4, and filtered. The filtrate was concentrated by evaporation under reduced pressure. Residue was purified by silica gel column using 1:5 EtOAC: Hexanes. Product alcohol **38** (391 mg, 87%) was obtained as a light yellow oil.

^{1}H NMR ($CDCl_3$): δ 5.77-5.71(m, 6H), 5.52(s, 1H), 3.61(t, 2H, J= 5.5Hz), 2.00(t, 2H, J=6.1Hz), 1.58-1.26(m, 16H);

^{13}C NMR ($CDCl_3$): δ 144.8, 134.5, 132.5, 131.9, 131.8, 131.2, 129.7, 128.4, 63.1, 37.7, 33.0, 32.8, 29.6, 29.5, 29.4, 29.0, 28.4, 25.7

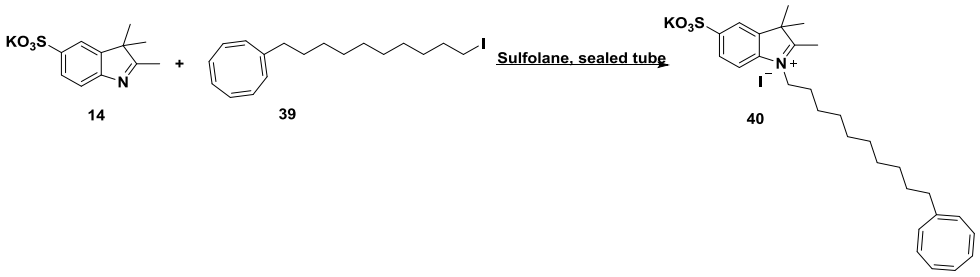


38 39

(1Z,3Z,5Z,7Z)-1-(10-iododecyl)cycloocta-1,3,5,7-tetraene(39): Alcohol **38** (192 mg, 0.74 mmol, 1.0 equiv) was dissolved in 10 mL dry DCM. To this solution were added triphenolphosphine (232 mg, 0.89 mmol, 1.2 equiv), and imidazole (200 mg, 2.95 mmol, 4.0 equiv), followed by the addition of I_2 flakes (225 mg, 0.89 mmol, 1.2 equiv) at room temperature. The resulting solution was stirred at room temperature for 2 hours, diluted by EtOAc, washed by H_2O and brine, dried over Na_2SO_4, and filtered. The filtrate was concentrated by evaporation under reduced pressure. Residue was purified by silica gel column using 1:3 EtOAc: Hexanes. Product **39** (211 mg, 77%) was obtained as a light yellow oil.

^{1}H NMR ($CDCl_3$): δ 5.77-5.71(m, 6H), 5.51(s, 1H), 3.16(t, 2H, J=5.5Hz), 2.00(t, 2H, J=6.1Hz), 1.80(q, 2H, J=6.1Hz), 1.36-1.26(m, 14H);

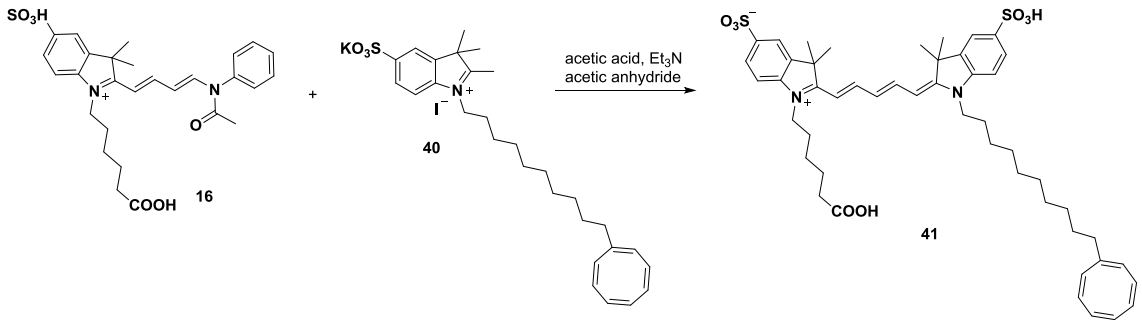
^{13}C NMR ($CDCl_3$): δ 144.8, 134.9, 132.5, 131.9, 131.8, 130.8, 129.8, 128.4, 37.7, 33.6, 30.5, 29.56, 29.4, 29.2, 29.1, 28.5, 28.4, 7.26



14 39 40

1-(10-((1Z,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraen-1-yl)decyl)-2,3,3-trimethyl-3H-indol-1-ium-5-sulfonate, potassium salt(40): To a sealed tube were added indole **14** potassium salt (10 mg,

0.036 mmol, 1.0 equiv), (iodo decyl) - COT **39** (16 mg, 0.043 mmol, 1.2 equiv) and 0.5 mL sulfolane. The reaction slurry was heated to 100^{o} C and stirred overnight. The resulting purple solution was poured into 15 mL of EtOAc to precipitate the crude product **40** (21 mg, recovery 91%) as a purple solid. The precipitate was dried under vacuum and carried onto the next step without further purification. ESI-MS: *m/z* calculated for $C_{29}H_{39}IKNO_3S$ $[M+H-I-K]^+$ 482.3, found 482.3



1-(5-carboxypentyl)-2-((1E,3E)-5-((E)-1-(10-((1Z,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraen-1-yl)decyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-3H-indol-1-ium-5-sulfonate(41): Indolelium **40** (21 mg, 0.032 mmol, 1.0 equiv) and indolelium **16** (16.8 mg, 0.032 mmol, 1.0 equiv) were dissolved in 2 mL of glacial acetic acid and 0.6mL of acetic anhydride. To this solution was added 0.3 mL of trimethylamine slowly while stirring. The reaction solution was heated to 110^{o} C and stirred for 3 hrs. The resulting dark blue solution was then cooled to room temperature and poured into 45 mL of EtOAc to precipitate the crude product. The precipitate was then dissolved in 10mL of water and purified using a semi-preparative HPLC C18 T3 column (Waters) with a 0.1% formic acid mobile phase in a gradient of 10-90% acetonitrile. After evaporation of acetonitrile, the product was concentrated, buffer exchanged over a Sep-Pak C18 column and eluted with methanol followed by evaporation in a speed vac. to give the fluorophore product **41** (3.6mg, 13%) as a dark blue solid.

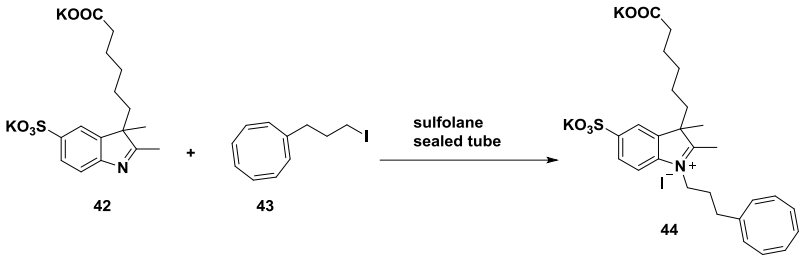
^{1}H NMR (MeOD-d4): δ 8.34(t, 2H, J=13.1Hz), 7.92(m, 4H), 7.38(m, 2H), 6.72(t, 1H, 12.4Hz), 6.39(d, 2H, J= 13.7Hz), 5.76- 5.51(m, 7H), 4.16(m, 4H), 3.67(m, 4H), 2.26(t, 2H, J=7.3Hz), 2.02(t, 2H, J=7.0Hz), 1.86(m, 4H), 1.78(s, 12H), 1.74(m, 2H), , 1.53(m, 2H), 1.47-1.31(m, 10H)

^{13}C NMR (MeOD-d4): δ 174.0, 173.9, 154.9, 154.7, 144.2, 143.6, 143.5, 142.0, 141.9, 141.3, 141.2, 134.0, 131.9, 131.5, 131.4, 130.8, 130.4, 128.1, 126.7, 126.6, 126.4, 126.0, 125.5, 120.0, 110.3,

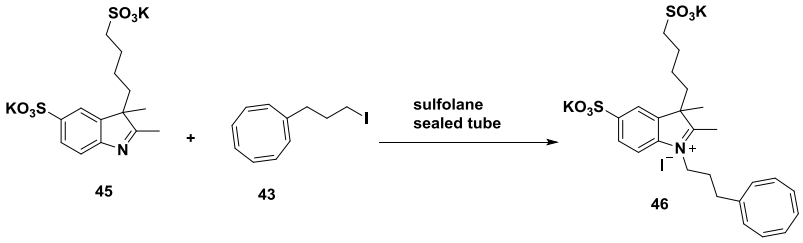
110.2, 103.9, 103.8, 49.2, 49.1, 43.8, 43.7, 37.3, 36.3, 32.7, 31.7, 29.4, 29.2, 29.1, 29.0, 28.9, 28.5, 28.1, 27.0, 26.8, 26.4, 26.3, 25.4, 22.3

ESI-MS: *m/z* calculated for $C_{49}H_{62}N_2O_8S$ $[M+H]^+$ 871.4, found 871.5

8 Preparation of Cy5-bisCOT (3) (48)

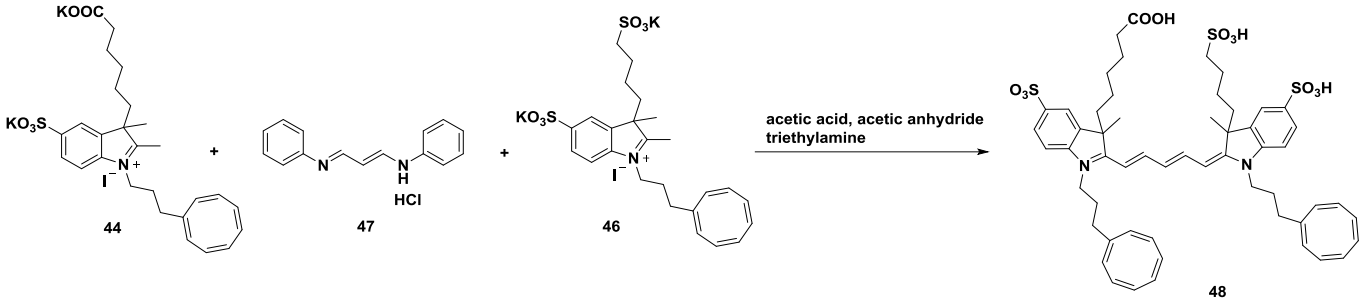


Dipotassium mono(6-(1-(3-((1Z,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraen-1-yl)propyl)-2,3-dimethyl-5-sulfonato-3H-indol-1-ium-3-yl)hexanoate)(44): To a sealed tube were added indole **42**[15] potassium salt (30 mg, 0.07 1mmol, 1.0 equiv), (iodopropyl) - COT **43** (30 mg, 0.11 mmol, 1.6 equiv) and 0.5 mL sulfolane. The reaction slurry was heated to 100°C and stirred overnight. The resulting purple solution was poured into 15mL of EtOAc to precipitate the crude product **44** (42 mg, recovery 86%) as a purple solid. The precipitate was dried under vacuum and carried onto the next step without further purification. ESI-MS: *m/z* calculated for $C_{27}H_{32}IK_2NO_5S$ $[M+2H-I-2K]^+$ 484.2, found 484.4



Dipotassium mono(1-(3-((1Z,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraen-1-yl)propyl)-2,3-dimethyl-3-(4-sulfonatobutyl)-3H-indol-1-ium-5-sulfonate)(46): To a sealed tube were added indole **42**[10] potassium salt (30 mg, 0.069 mmol, 1.0 equiv), (iodopropyl) - COT **43** (30 mg, 0.11 mmol, 1.6 equiv) and 0.5 mL sulfolane. The reaction slurry was heated to 100° C and stirred overnight. The resulting purple solution was poured into 15 mL of EtOAc to precipitate the crude product **46** (45 mg, recovery 92%) as a purple solid. The precipitate was dried in vacuum and carried onto the

next step without further purification. ESI-MS: *m/z* calculated for $C_{25}H_{30}IK_2NO_6S_2$ $[M+2H-I-2K]^+$ 506.2, found 506.3



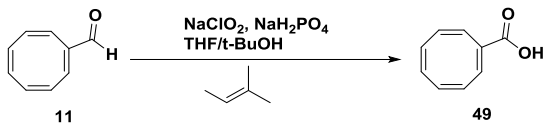
6-(5-((λ^1-oxidanyl)dioxo-λ^6-sulfanyl)-1-(3-((1Z,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraen-1-yl)propyl)-2-((1E,3E)-5-((E)-1-(3-((1Z,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraen-1-yl)propyl)-3-methyl-5-sulfo-3-(4-sulfobutyl)indolin-2-ylidene)penta-1,3-dien-1-yl)-3-methyl-3H-1λ^4-indol-3-yl)hexanoic acid(48): Indolelium **44** (30 mg, 0.042 mmol, 1 equiv) and malonaldehyde dianilide hydrochloride (10.7 mg, 0.042 mmol, 1 equiv) were dissolved in 2mL of glacial acetic acid and 0.6 mL of acetic anhydride. The reaction solution was heated to 110^o C and stirred for 3 hrs and then poured into 45mL of EtOAc to precipitate the intermediate product. The precipitate was then taken into another flask. To this flask were added indoleline **46** (30 mg, 0.042 mmol 1 equiv), 2 mL glacial acetic acid and 0.6 mL acetic anhydride followed by 0.3 mL of trimethylamine slowly while stirring. The reaction solution was heated to 110^o C and stirred for 3 hrs. The resulting dark blue solution was then cooled to room temperature and poured into 45 mL of EtOAc to precipitate the crude product. The precipitate was then dissolved in 10 mL of water and purified using a semi-preparative HPLC C18 T3 column (Waters) with a 10 mM TEAA pH7.0 mobile phase in a gradient of 10-90% acetonitrile. After evaporation of acetonitrile, the product was concentrated, buffer exchanged over a Sep-Pak C18 column and eluted with methanol followed by evaporation in a speed vac. to give the fluorophore product **48** (6.9 mg, 16%) as a dark blue solid.

^{1}H NMR (MeOD-d4): δ 8.35(t, 2H, J=12.8Hz), 7.92(m, 4H), 7.38(m, 2H), 6.64(t, 1H, 12.3Hz), 6.39(m, 2H), 5.99- 5.70(m, 14H), 4.20(br, 4H), 2.65(m, 2H), 2.49(m, 2H), 2.27(m, 6H), 2.09(m, 4H), 1.93(m, 4H), 1.78(s, 3H), 1.77(s, 3H), 1.74(m, 2H), , 1.45(m, 2H), 0.94-0.63(m, 4H)

^{13}C NMR (MeOD-d4): δ 172.9, 172.5, 154.4, 154.2, 144.4, 142.3, 142.1, 142.0, 139.6, 139.4, 133.6, 132.4, 131.8, 131.5, 130.9, 128.0, 126.7, 126.6, 120.1, 120.0, 110.2, 104.5, 53.9, 53.7, 50.9, 43.5, 41.0, 40.8, 36.3, 34.2, 33.5, 29.4, 29.0, 26.3, 25.7, 25.6, 25.5, 24.5, 24.4, 23.7

ESI-MS: *m/z* calculated for $C_{55}H_{64}N_2O_{11}S_3$ $[M-H]^-$ 1023.4, found 1023.7, $[M-2H]^{2-}/2$ 511.1, found 511.3

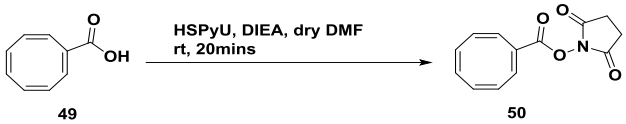
9 Preparation of Cy5-AC(4) (54)



11 49

Aldehyde **11** (70 mg, 0.53 mmol) was dissolved in a mixture of THF (0.45mL), t-BuOH (0.45mL), 2-methyl-2-butene (0.45mL), and H_2O (0.15mL). To this solution was added NaH_2PO_4 (126mg, 1.06mmol) at 0^o C, followed by the addition of a solution of $NaClO_2$ (78 mg, 0.689 mmol) in 0.5 mL H_2O. The reaction was stirred at 0^o C for 80min, warmed to RT, and stirred overnight. The reaction was then extracted with EtOAc, dried with Na_2SO_4, and filtered. The filtrate was concentrated under reduced pressure. The residue was purified by silica gel column using 1:3 EtOAc: Hexanes. Product acid **49** (60 mg, 76.5%) was obtained as a yellow oil.

^{1}H NMR ($CDCl_3$): δ 7.29 (s, 1H), 6.01-5.81(m, 6H)

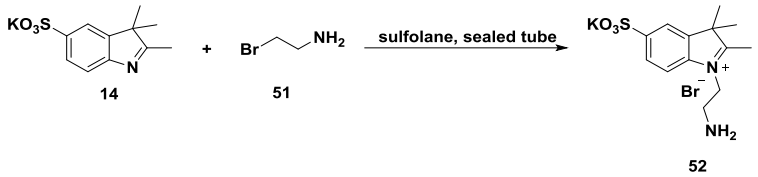


49 50

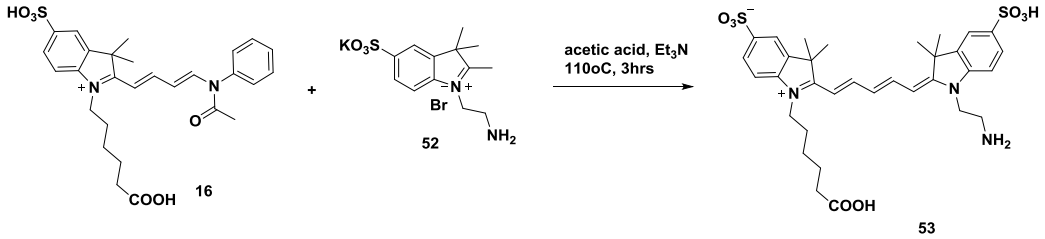
Acid **49** (60 mg, 0.405 mmol) was dissolved in a mixture of 2 mL dry DMF and 0.3 mL DIEA. HSPyU (333 mg, 0.81 mmol) was added to this solution. The reaction solution was stirred at RT for 20mins. The resulting mixture was purified by silica gel column using 1:3 EtOAc: Hexanes. Product NHS ester **50** (66 mg, 66.7%) was obtained as a yellow oil.

^{1}H NMR ($CDCl_3$): δ 7.29 (s, 1H), 6.04-5.85(m, 6H), 2.82(s, 4H)

^{13}C NMR ($CDCl_3$): δ 169.2, 160.6, 147.6, 135.5, 135.0, 132.3, 131.5, 129.5, 129.2, 127.5, 25.6

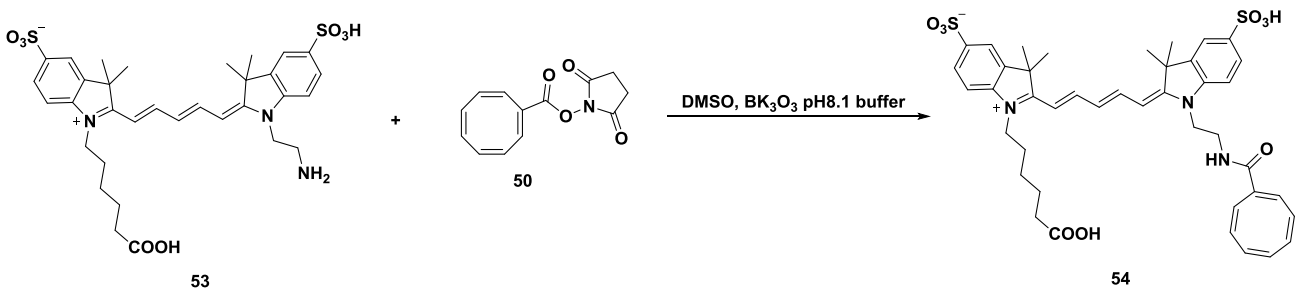


1-(2-aminoethyl)-2,3,3-trimethyl-3H-indol-1-ium-5-sulfonate, potassium salt (52) : To a sealed tube were added indole 14 potassium salt (100 mg, 0.36 mmol, 1.0 equiv), 2-bromoethyl amine HBr salt (**51**) (221 mg, 1.08 mmol, 3.0 equiv) and 2 mL sulfolane. The reaction slurry was heated to 120^{o} C and stirred overnight. The resulting purple solution was poured into 45 mL of EtOAc to precipitate the crude product **52** (126 mg, recovery 87%) as a purple solid. The precipitate was dried under vacuum and carried onto the next step without further purification. ESI-MS: *m/z* calculated for $C_{13}H_{18}BrKN_2O_3S$ $[M+H-Br-K]^+$ 283.1 ,found 283.2



2-((1E,3E)-5-((E)-1-(2-aminoethyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-1-(5-carboxypentyl)-3,3-dimethyl-3H-indol-1-ium-5-sulfonate (53): Indolelium **52** (163 mg, 0.31 mmol, 1.0 equiv) and indolelium **16** (126 mg, 0.31 mmol, 1 equiv) were dissolved in 2 mL of glacial acetic acid. To this solution was added 0.3 mL of trimethylamine slowly while stirring. The reaction solution was heated to 110^{o}C and stirred for 2 hrs. The resulting dark blue solution was then cooled to room temperature and poured into 45 mL of EtOAc to precipitate the crude product. The precipitate was then dissolved in 10 mL of water and purified using a semi-preparative HPLC C18 T3 column (Waters) with a 0.1% formic acid mobile phase in a gradient of 10-90% acetonitrile. After evaporation of acetonitrile, the product was concentrated, buffer exchanged over a Sep-Pak C18 column and eluted with methanol followed by evaporation in a speed vac. to give the fluorophore product **53** (51 mg, 24.5%) as a dark blue solid.

ESI-MS: *m/z* calculated for $C_{33}H_{41}N_3O_8S_2$ $[M+H]^+$ 672.2 ,found 672.2

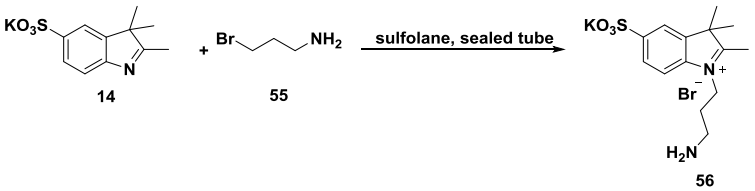


53 50 54

1-(5-carboxypentyl)-2-((1E,3E)-5-((E)-1-(2-((1E,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraene-1-carboxamido)ethyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-3H-indol-1-ium-5-sulfonate (54): Fluorophore **53** 1 µmol was dissolved in a mixture of 200 µL DMSO, 20 µL 0.5M potassium borate pH8.1 buffer, and 150 µL ddH_2O. NHS ester **50** (1 mg, 4 µmol, 4 equiv) in 100 µL DMSO was then added to the fluorophore solution. The reaction was monitored by LCMS until completion. Once completed, the reaction was purified using a semi-preparative HPLC C18 T3 column (Waters) with a 10 mM TEAA pH7.0 buffer mobile phase in a gradient of 10-90% acetonitrile. After evaporation of acetonitrile, the product was concentrated, buffer exchanged over a Sep-Pak C18 column and eluted with methanol followed by evaporation in a speed vac. to give the dry product **54** (730 nmol, 73%).

ESI-MS: *m/z* calculated for $C_{42}H_{47}N_3O_9S_2$ $[M+H]^+$: 802.3 found 802.3

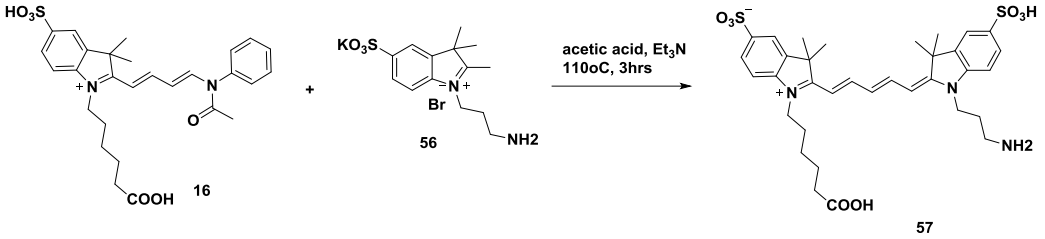
10 Preparation of Cy5-AC(5) (58)



14 55 56

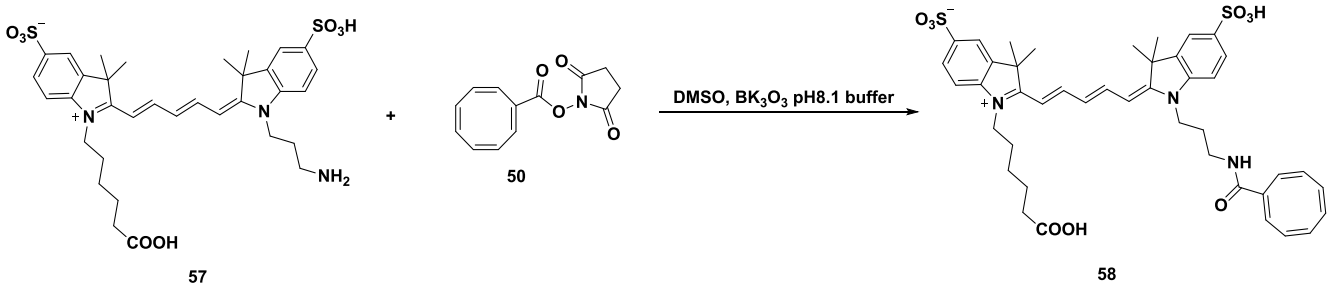
1-(3-aminopropyl)-2,3,3-trimethyl-3H-indol-1-ium-5-sulfonate, potassium salt (56) : To a sealed tube were added indole **14** potassium salt (100 mg, 0.3 6mmol, 1.0 equiv), 3-bromopropyl amine HBr salt (**55**) (236 mg, 1.08 mmol, 3 equiv) and 2 mL sulfolane. The reaction slurry was heated to 120^o C and stirred overnight. The resulting purple solution was poured into 45 mL of EtOAc to precipitate the crude product **56** (112 mg, recovery 75%) as a purple solid. The precipitate was

dried under vacuum and carried onto the next step without further purification. ESI-MS: *m/z* calculated for $C_{14}H_{20}BrKN_2O_3S$ $[M+H-Br-K]^+$ 297.1 ,found 297.2



2-((1E,3E)-5-((E)-1-(3-aminopropyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-1-(5-carboxypentyl)-3,3-dimethyl-3H-indol-1-ium-5-sulfonate (57): Indolelium **56** (112 mg, 0.27 mmol, 1 equiv) and indolelium **16** (141 mg, 0.27 mmol, 1 equiv) were dissolved in 2 mL of glacial acetic acid. To this solution was added 0.3 mL of trimethylamine slowly while stirring. The reaction solution was heated to 110^o C and stirred for 3 hrs. The resulting dark blue solution was then cooled to room temperature and poured into 45 mL of EtOAc to precipitate the crude product. The precipitate was then dissolved in 10mL of water and purified using a semi-preparative HPLC C18 T3 column (Waters) with a 0.1% formic acid mobile phase in a gradient of 10-90% acetonitrile. After evaporation of acetonitrile, the product was concentrated, buffer exchanged over a Sep-Pak C18 column and eluted with methanol followed by evaporation in a speed vac. to give the fluorophore product **57** (38 mg, 19.9%) as a dark blue solid.

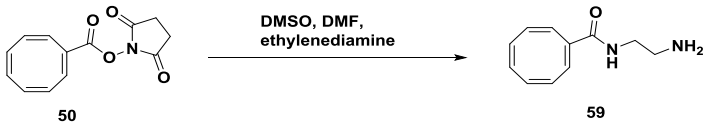
ESI-MS: *m/z* calculated for $C_{34}H_{43}N_3O_8S_2$ $[M+H]^+$ 686.3 ,found 686.3



1-(5-carboxypentyl)-2-((1E,3E)-5-((E)-1-(3-((1E,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraene-1-carboxamido)propyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-3H-indol-1-ium-5-sulfonate (58): Fluorophore **57** 1 µmol was dissolved in a mixture of

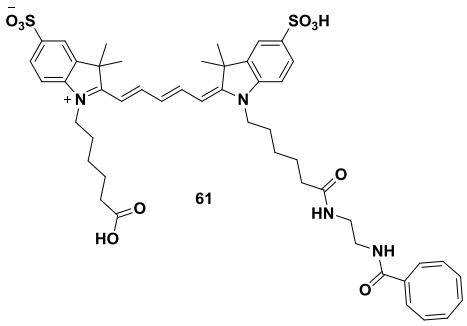
200 µL DMSO, 20 µL 0.5M potassium borate pH 8.1 buffer, and 150 µL ddH_2O. NHS ester **50** (1 mg, 4 µmol, 4 equiv) in 100 µL DMSO was then added to the fluorophore solution. The reaction was monitored by LCMS until completion. Once completed, the reaction was purified using a semi-preparative HPLC C18 T3 column (Waters) with a 10 mM TEAA pH7.0 buffer mobile phase in a gradient of 10-90% acetonitrile. After evaporation of acetonitrile, the product was concentrated, buffer exchanged over a Sep-Pak C18 column and eluted with methanol followed by evaporation in a speed vac. to give the dry product **58** (820 nmol, 82%). ESI-MS: *m/z* calculated for $C_{43}H_{49}N_3O_9S_2$ $[M+H]^+$: 816.3 found 816.4

11. Preparation of Cy5-AC(11) (61)



50 59

To a solution of 50 mg ethylenediamine in 0.3 mL DMF was slowly added NHS ester **50** (24.5 mg, 0.1 mmol) in 0.2 mL DMSO. The reaction solution was stirred at RT for 10mins. The crude product was purified using a semi-preparative HPLC C18 T3 column (Waters) with a 0.1% formic acid mobile phase in a gradient of 10-90% acetonitrile. The product was concentrated by rot-vap to give the dry product **59** (11 mg, 57.9%). ESI-MS: *m/z* calculated for $C_{11}H_{14}N_2O$ $[M+H]^+$: 191.1 found 191.1

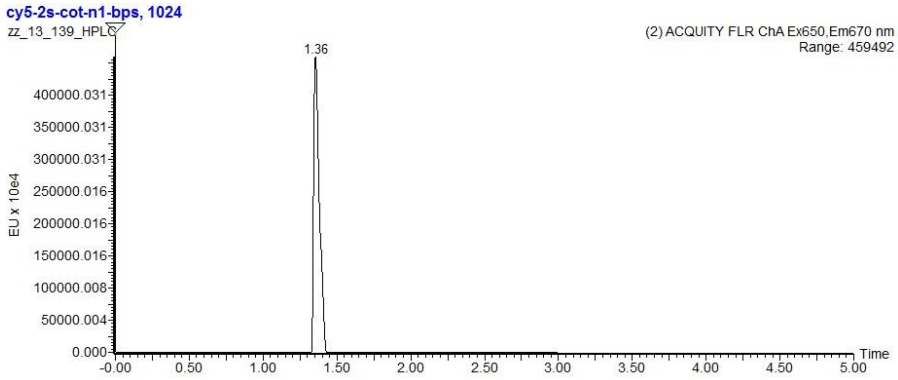


61

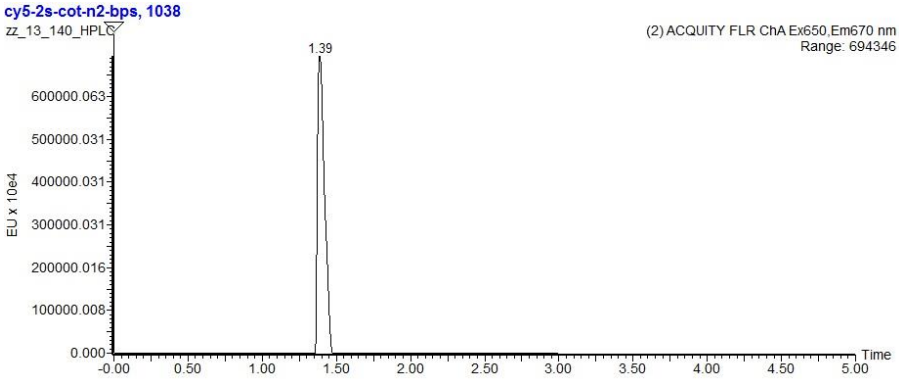
2-((1E,3E)-5-((E)-1-(5-carboxypentyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-1-(6-((2-((1E,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraene-1-carboxamido)ethyl)amino)-6-oxohexyl)-3,3-dimethyl-3H-indol-1-ium-5-sulfonate (61): GE Bis-NHS-Cy5(**60**) 1 µmol was dissolved in a mixture of 200 µL DMSO, 20 µL 0.5 M potassium borate pH8.1 buffer, and 150 µL ddH_2O. Amine **59** (0.19 mg, 1 µmol, 1 equiv) in 100 µL DMSO was then added to the fluorophore solution. The reaction was monitored by LCMS until completion. Once completed, the reaction was purified using a semi-preparative HPLC C18 T3 column (Waters) with a 10 mM TEAA pH 7.0 buffer mobile phase in a gradient of 10-90% acetonitrile. After evaporation of acetonitrile, the product was concentrated, buffer exchanged over a Sep-Pak C18 column and eluted with methanol followed by evaporation in a speed vac. to give the dry product **61** (245nmol, 24.5%). ESI-MS: *m/z* calculated for $C_{48}H_{58}N_4O_{10}S_2$ $[M+H]^+$: 915.4 found 915.7

LC/MS characterization of OTX-coupled fluorophores

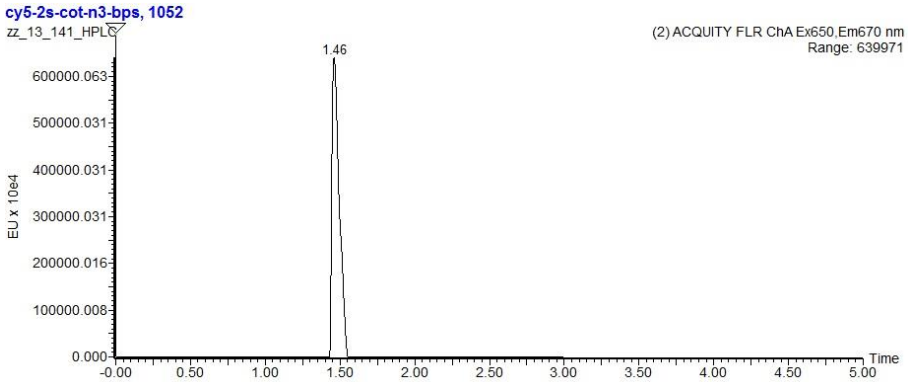
Cy5-COT(1)-OTX (2)



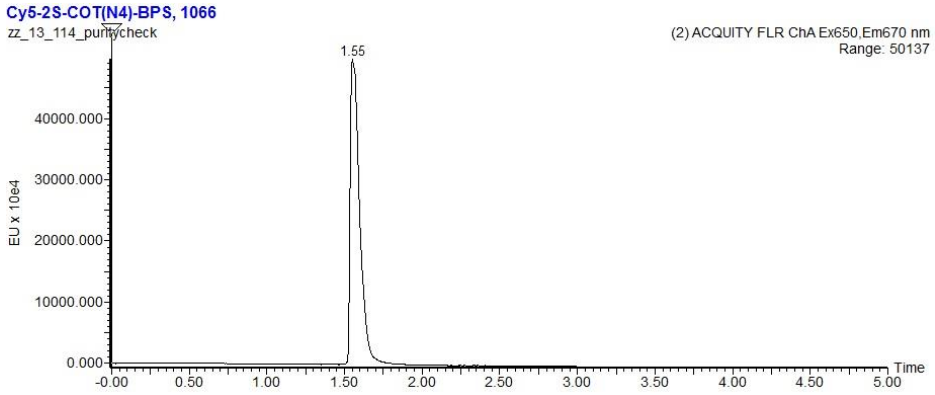
Cy5-COT(2)-OTX (3)



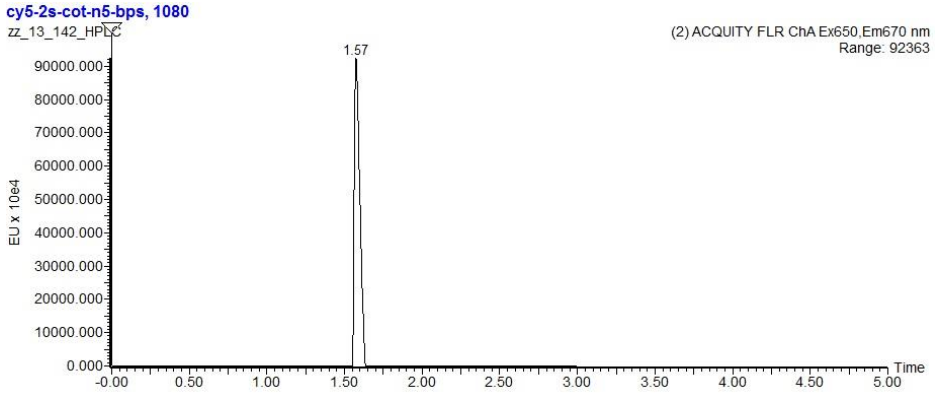
Cy5-COT(3)-OTX (4)



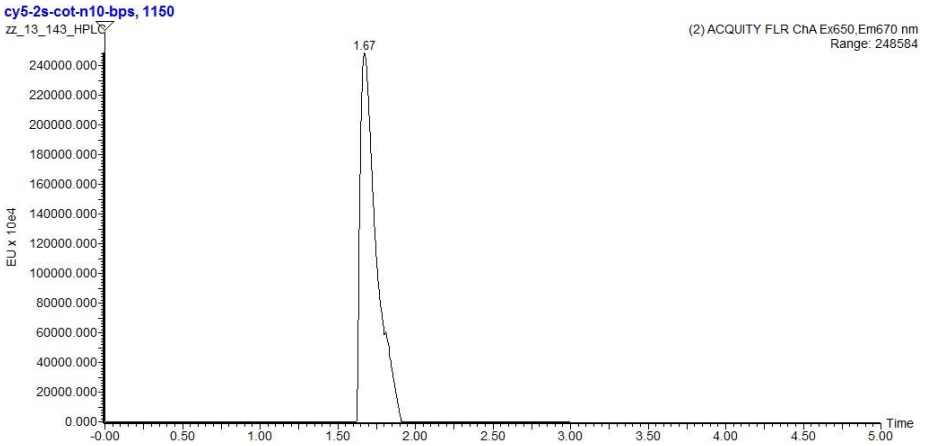
Cy5-COT(4)-OTX (5)



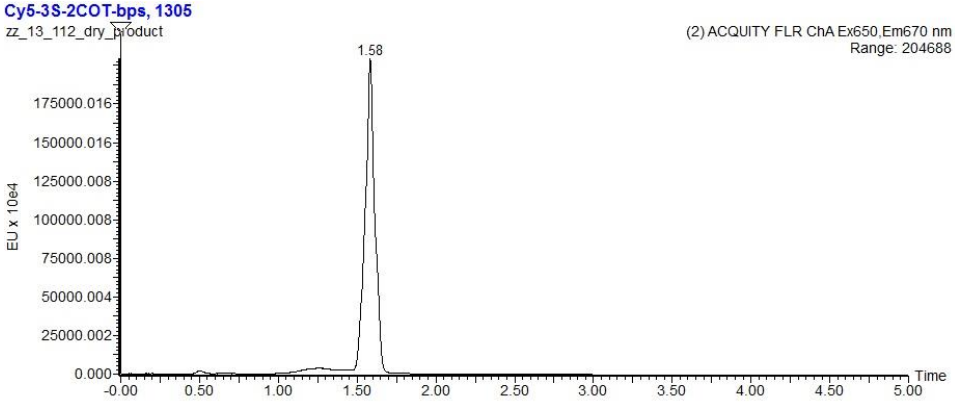
Cy5-COT(5)-OTX (6)



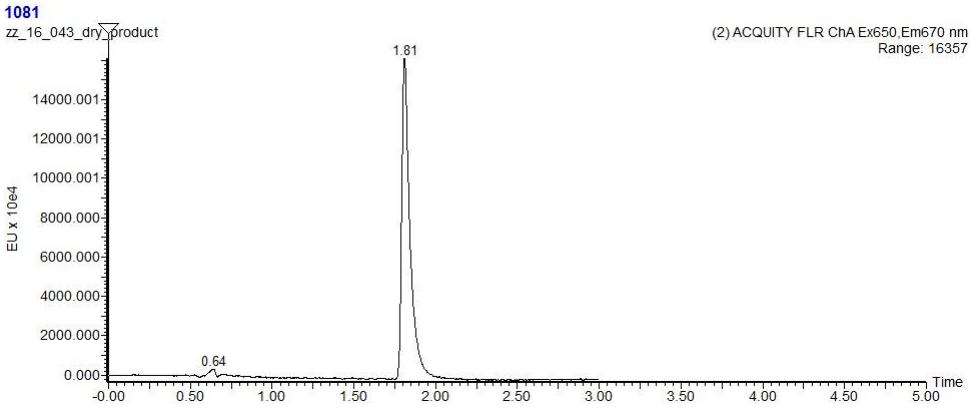
Cy5-COT(10)-OTX (7)



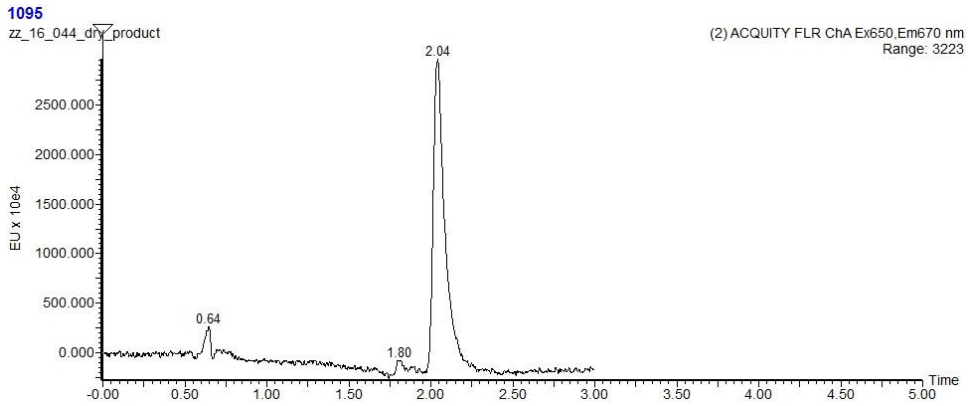
Cy5-bisCOT(3)-OTX (8)



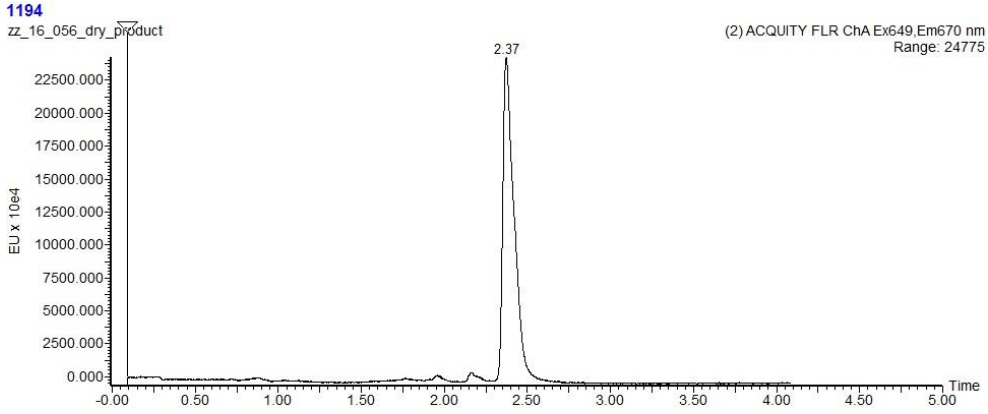
Cy5-AC(4)-OTX(62)



Cy5-AC(5)-OTX(63)

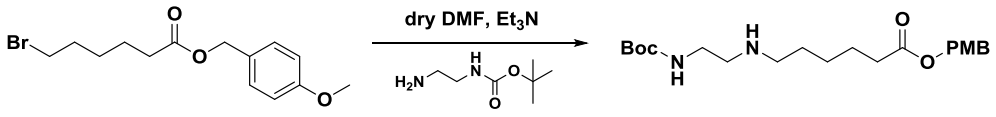


Cy5-AC(11)-OTX(64)



Chemical Synthesis of 6-((4-(aminomethyl)benzyl)oxy)-7H-purin-2-amine (BG-NH_2) Coupled Fluorophore

1. Preparation of linker compound (62)



61 62

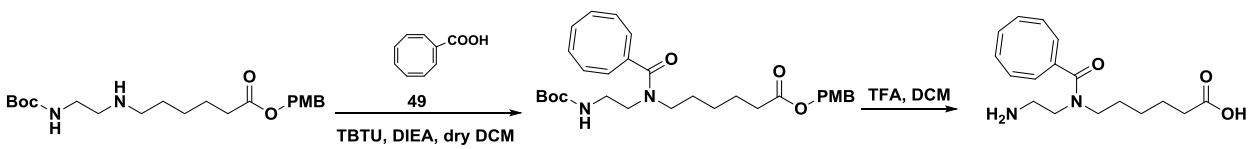
4-methoxybenzyl 6-((2-((tert-butoxycarbonyl)amino)ethyl)amino)hexanoate (62): Bromo-PMB ester **61** 236 mg (1 eq. 0.75 mmol) and Boc protected ethylene diamine 240 mg (2 eq. 1.50 mmol) were dissolved in 5mL dry DMF, 0.21 mL (2 eq., 1.50 mmol) was added. The reaction solution was stirred at RT. Overnight. DMF was then removed bin vacuum, residue was diluted with 50 mL DCM, washed with H_2O (50 mL x 2), dried with Na_2SO_4, filtered, concentrated and purified by silica gel chromatography using 1:10 MeOH/DCM. Product **62** 182 mg was obtained as light yellow oil (46.0%)

^{1}H NMR ($CDCl_3$): δ 7.28(d, 2H, J=8.6 Hz), 6.87(d, 2H, J=8.6 Hz), 5.43(b, 1H), 5.03(s, 2H), 4.45(b, 1H), 3.79(s, 3H), 2.96(m, 2H), 2.83(m, 2H), 2.69(t, 2H, J=7.5 Hz), 2.32 (t, 2H, J=7.5 Hz), 1.60 (m, 4H), 1.43(s, 9H), 1.34(m, 2H);

^{13}C NMR ($CDCl_3$): δ 173.4, 159.6, 156.3, 130.1, 128.1, 113.9, 65.9, 55.3, 48.8, 39.3, 34.1, 28.4, 28.3, 26.5, 24.5;

ESI-MS: *m/z* calculated for $C_{21}H_{34}N_2O_5$ $[M+H]^+$ 395.3, found 395.4

2. Preparation of linker-AC-Carboxylic Acid (64)



62 63 64

6-((1E,3Z,5Z,7Z)-N-(2-aminoethyl)cycloocta-1,3,5,7-tetraene-1-carboxamido)hexanoic acid (64): Compound **62** 60 mg (1 eq., 0.152 mmol) and COT-COOH (49) 22.5 mg (1 eq., 0.152 mmol) were dissolved in 5 mL of dry DCM, 0.05 mL DIEA was added to the solution, followed by 68 mg of TBTU (1.4 eq., 0.213 mmol). The reaction was stirred overnight at RT, concentrated, residue was purified by silica gel column using 1:3 EtOAc/Hexanes. Compund **63** 61 mg was obtained as light yellow powder (75.2%).

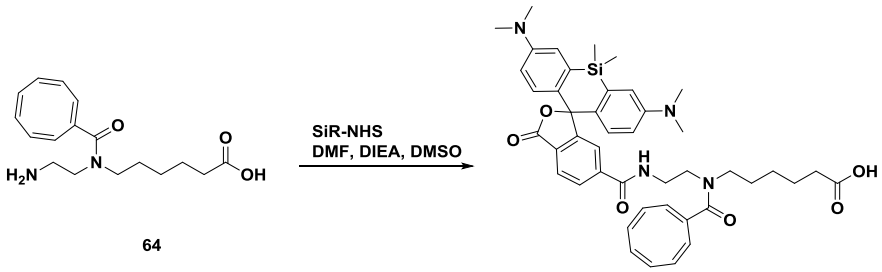
^{1}H NMR ($CDCl_3$): δ 7.28(d, 2H, J=8.6 Hz), 6.87(d, 2H, J=8.6 Hz), 5.92-5.81(m, 7H), 5.04(s, 2H), 3.80(s, 3H), 3.56(b, 2H), 3.45(b, 2H), 3.30(b, 2H), 2.32 (t, 2H, J=7.5 Hz), 1.65 (m, 4H), 1.42(s, 9H), 1.30(m, 2H);

^{13}C NMR ($CDCl_3$): δ 173.3, 171.4, 159.6, 156.2, 140.8, 133.6, 133.1, 132.7, 131.9, 131.4, 130.7, 130.2, 128.1, 113.9, 66.0, 55.3, 49.4, 44.3, 39.0, 34.2, 28.7, 28.4, 26.3, 24.6;

The resulting 61 mg compound **63** was dissolved in 1.5 mL DCM, cooled to 0^{o}C. TFA 1 mL was added slowly at this temperature. The reaction was warmed up to RT, and stirred for 1 hr. The solvent was removed by vacuum, the crude material was purified using a semi-preparative HPLC C18 T3 column (Waters) with a 0.1% formic acid aq. mobile phase in a gradient of 10-90% acetonitrile. The product fractions were concentrated by rot-vap to give the final product **64** 23 mg as a yellow oil (67%).

ESI-MS: *m/z* calculated for $C_{17}H_{24}N_2O_3$ $[M+H]^+$ 305.2, found 305.2

3. **Preparation of SiR-Linker-AC-Carboxylic Acid**



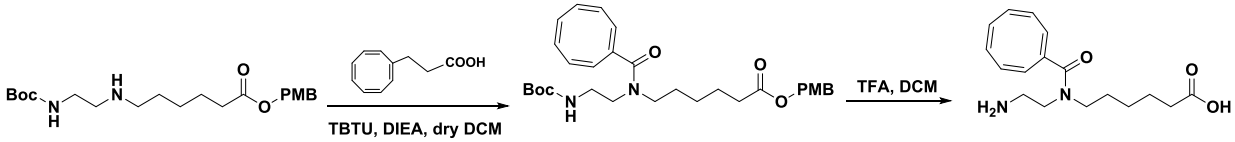
64

65

Compound 64 1mg was dissolved in 400 uL DMF, 100 uL DIEA. To this solution was added SiR-NHS ester 1mg in 400 uL DMSO. The reaction solution was kept at RT for 15 mins, then injected directly into HPLC for purification. The crude material was purified using a semi-preparative HPLC C18 T3 column (Waters) with a 0.1% formic acid aq. mobile phase in a gradient of 10-90% acetonitrile. The product fractions were concentrated by rot-vap to give the product **65.**

ESI-MS: *m/z* calculated for $C_{44}H_{50}N_4O_6Si$ $[M+H]^+$ 759.4, found 759.4

4. Preparation of linker-COT-Carboxylic Acid (67)



62 66 67

6-((1E,3Z,5Z,7Z)-N-(2-aminoethyl)cycloocta-1,3,5,7-tetraene-1-carboxamido)hexanoic acid (67): Compound **62** 60 mg (1 eq., 0.152 mmol) and COT-propanoic acid 26.8 mg (1 eq., 0.152 mmol) were dissolved in 5 mL of dry DCM, 0.05 mL DIEA was added to the solution, followed by 68 mg of TBTU (1.4 eq., 0.213 mmol). The reaction was stirred overnight at RT, concentrated, residue was purified by silica gel column using 1:3 EtOAc/Hexanes. Compund **66** 70 mg was obtained as light yellow powder (83.3%).

^{1}H NMR ($CDCl_3$): δ 7.28(d, 2H, J=8.6 Hz), 6.87(d, 2H, J=8.6 Hz), 5.83-5.60(m, 7H), 5.04(s, 2H), 3.80(s, 3H), 3.43 (m, 2H), 3.22(m, 4H), 2.31(m, 6H), 1.65(m, 2H), 1.57(m, 2H), 1.44(s, 9H), 1.29(m, 2H);

The resulting 70 mg compound **66** was dissolved in 1.5 mL DCM, cooled to 0^{o}C. TFA 1 mL was added slowly at this temperature. The reaction was warmed up to RT, and stirred for 1 hr. The solvent was removed by vacuum, the crude material was purified using a semi-preparative HPLC C18 T3 column (Waters) with a 0.1% formic acid aq. mobile phase in a gradient of 10-90% acetonitrile. The product fractions were concentrated by rot-vap to give the final product **67** 36 mg as a yellow oil (86%).

ESI-MS: *m/z* calculated for $C_{19}H_{28}N_2O_3$ $[M+H]^+$ 333.2, found 333.2

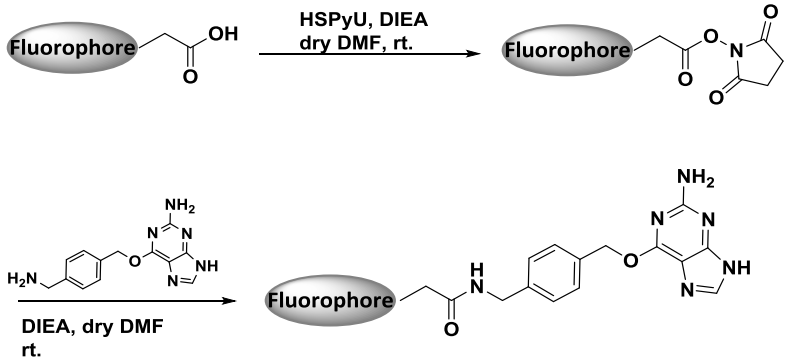
5. **Preparation of SiR-Linker-COT-Carboxylic Acid**

67 68

Compound **67** 1mg was dissolved in 400 uL DMF, 100 uL DIEA. To this solution was added SiR-NHS ester 1mg in 400 uL DMSO. The reaction solution was kept at RT for 15 mins, then injected directly into HPLC for purification. The crude material was purified using a semi-preparative HPLC C18 T3 column (Waters) with a 0.1% formic acid aq. mobile phase in a gradient of 10-90% acetonitrile. The product fractions were concentrated by rot-vap to give the product **68.**

ESI-MS: *m/z* calculated for $C_{46}H_{54}N_4O_6Si$ $[M+H]^+$ 787.4, found 787.3

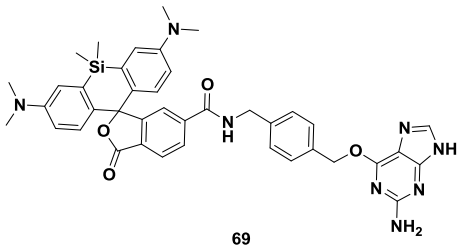
6. Preparation of Fluorophore -BG



Fluorophore carboxylic acid 150 nmol was dissolved in 200 µL dry DMF, and then 50 µL DIEA was added to this DMF solution followed by addition of 300 nmol of Dipyrrolidino(N-succinimidyloxy)carbenium hexafluorophosphate (HSPyU). The mixture was vortexed and then

incubated in the dark at room temperature. The reaction was monitored by LCMS until it was completed. Once completed, the reaction solution was poured into 15 mL of ethyl acetate (EtOAc), centrifuged. The residue was dissolved in 2 mL of 5% formic acid aq. solution, and purified using a semi-preparative HPLC C18 T3 column (Waters) with a 10 mM TEAA pH7.0 buffer mobile phase in a gradient of 10-90% acetonitrile. After evaporation of acetonitrile, the product was concentrated, buffer exchanged over a Sep-Pak C18 column and eluted with methanol followed by evaporation in a speed vac. to give the dry fluorophore-NHS ester product.

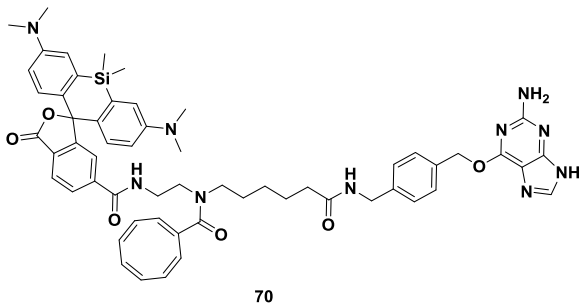
Fluorophore NHS ester 100 nmol was dissolved in 200 µL dry DMSO, and then 50 µL DIEA was added to this DMF solution followed by addition of 200 nmol of BG-NH_2 in 50 µL of dry DMF. The mixture was vortexed and then incubated in the dark at room temperature. The reaction was monitored by LCMS until it was completed. Once completed, the reaction was diluted to 3 mL with distilled, deionized water (ddH2O). Desired product was purified using a semi-preparative HPLC C18 T3 column (Waters) with a 10 mM TEAA pH7.0 buffer mobile phase in a gradient of 10-90% acetonitrile. After evaporation of acetonitrile, the product was concentrated, buffer exchanged over a Sep-Pak C18 column and eluted with methanol followed by evaporation in a speed vac. to give the final product.



69

SiR-BG (69) N-(4-(((2-amino-9H-purin-6-yl)oxy)methyl)benzyl)-3,7-bis(dimethylamino)-5,5-dimethyl-3'-oxo-3'H,5H-spiro[dibenzo[b,e]siline-10,1'-isobenzofuran]-6'-carboxamide

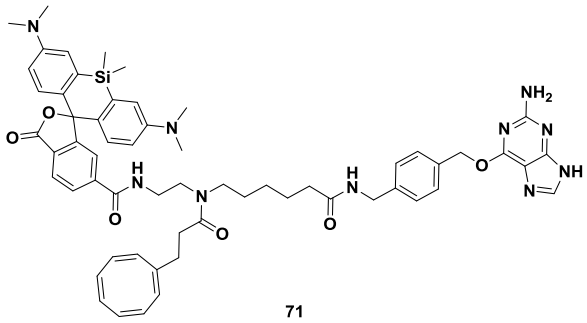
ESI-MS: *m/z* calculated for $C_{40}H_{40}N_8O_4Si$ $[M+H]^+$ 725.3, found 725.3



70

SiR-Linker-AC-BG (70) N-(2-((1E,3Z,5Z,7Z)-N-(6-((4-(((2-amino-9H-purin-6-yl)oxy)methyl)benzyl)amino)-6-oxohexyl)cycloocta-1,3,5,7-tetraene-1-carboxamido)ethyl)-3,7-bis(dimethylamino)-5,5-dimethyl-3'-oxo-3'H,5H-spiro[dibenzo[b,e]siline-10,1'-isobenzofuran]-6'-carboxamide

ESI-MS: *m/z* calculated for $C_{57}H_{62}N_{10}O_6Si$ $[M+H]^+$ 1011.5, found 1011.2

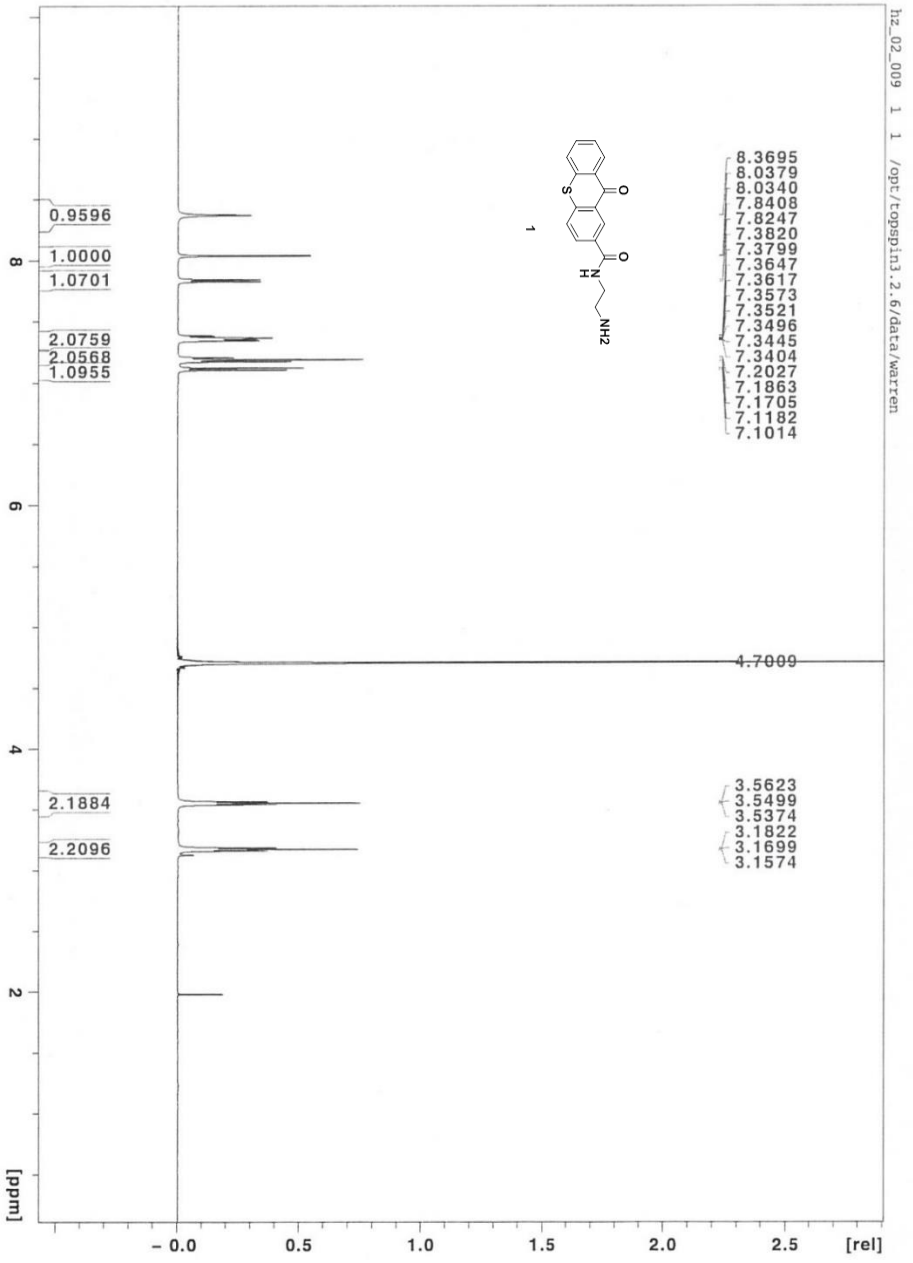


71

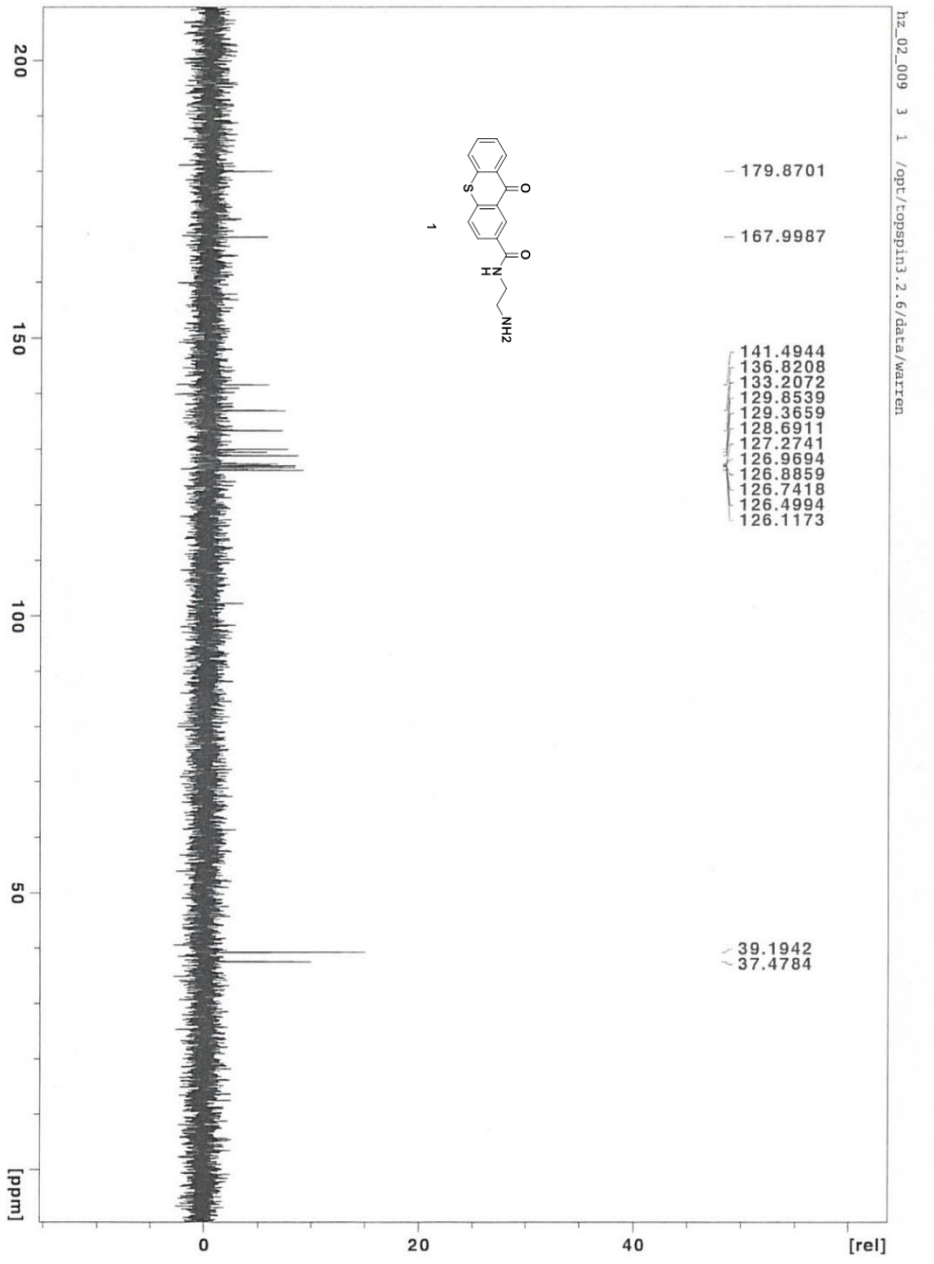
SiR-Linker-COT-BG (71) N-(2-(N-(6-((4-(((2-amino-9H-purin-6-yl)oxy)methyl)benzyl)amino)-6-oxohexyl)-3-((1Z,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraen-1-yl)propanamido)ethyl)-3,7-bis(dimethylamino)-5,5-dimethyl-3'-oxo-3'H,5H-spiro[dibenzo[b,e]siline-10,1'-isobenzofuran]-6'-carboxamide

ESI-MS: *m/z* calculated for $C_{59}H_{66}N_{10}O_6Si$ $[M+H]^+$ 1039.5, found 1039.4

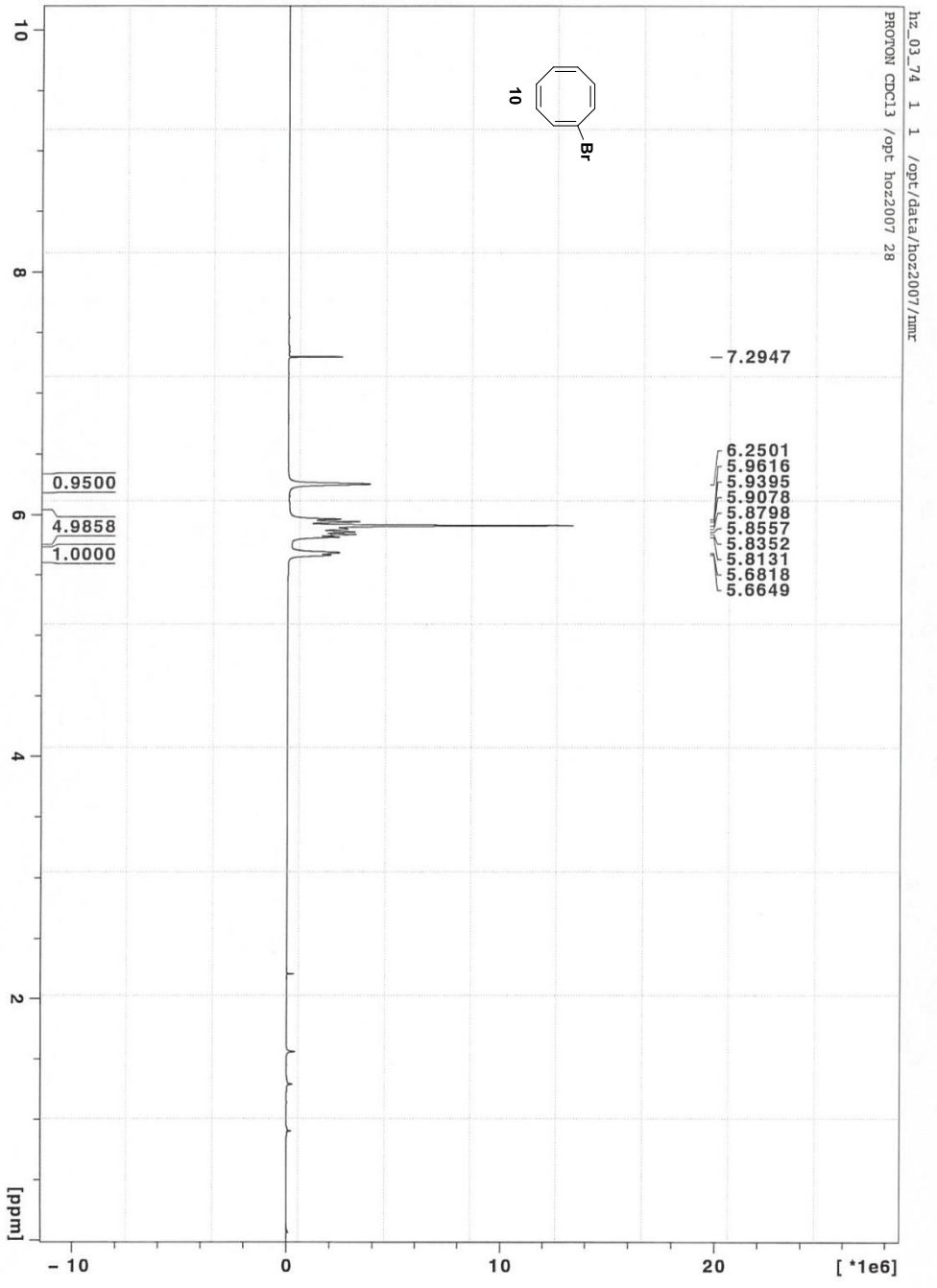
NMR Spectra



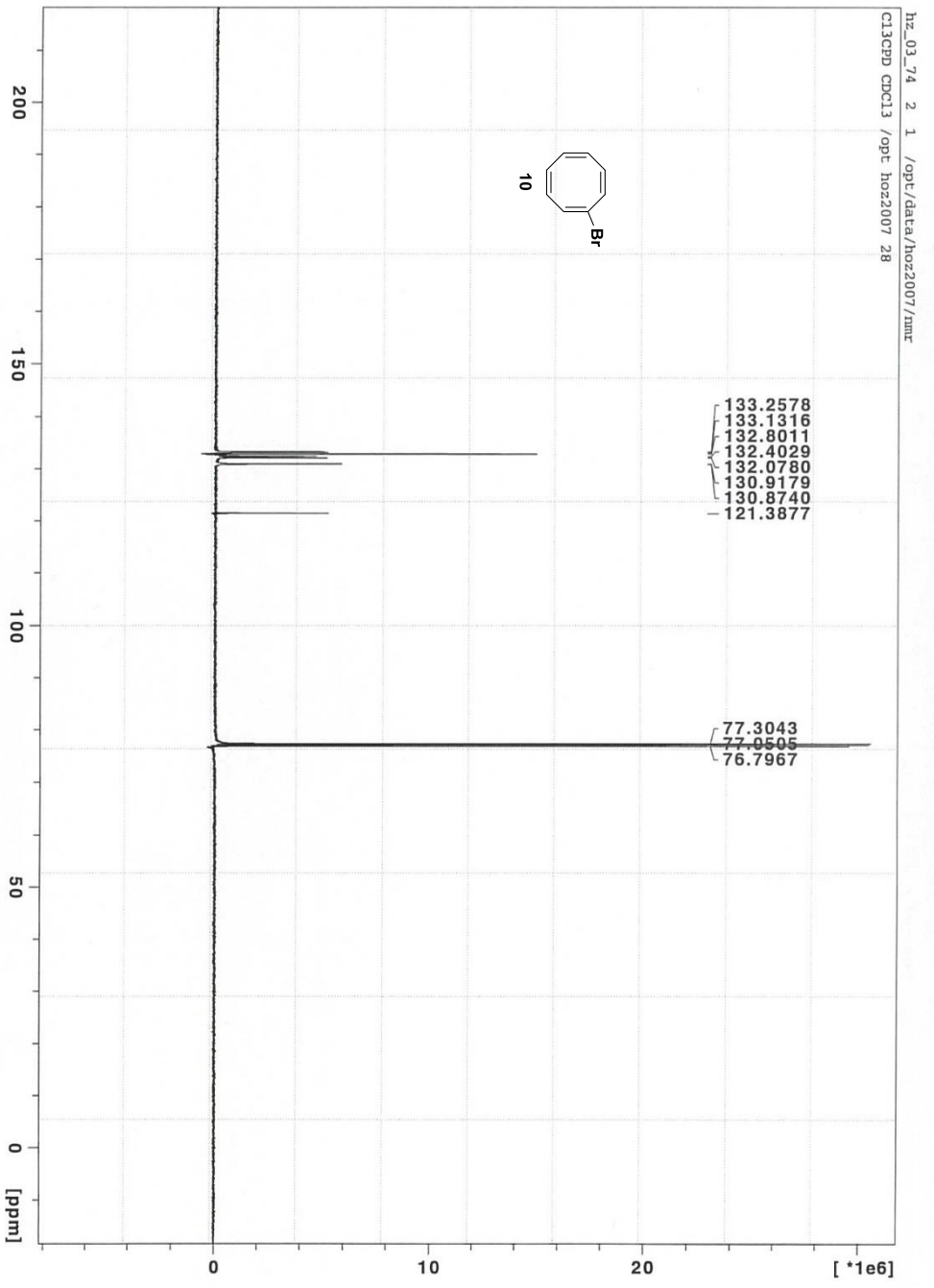
Compound 1 (^{1}H, D_2O, 500 MHz)



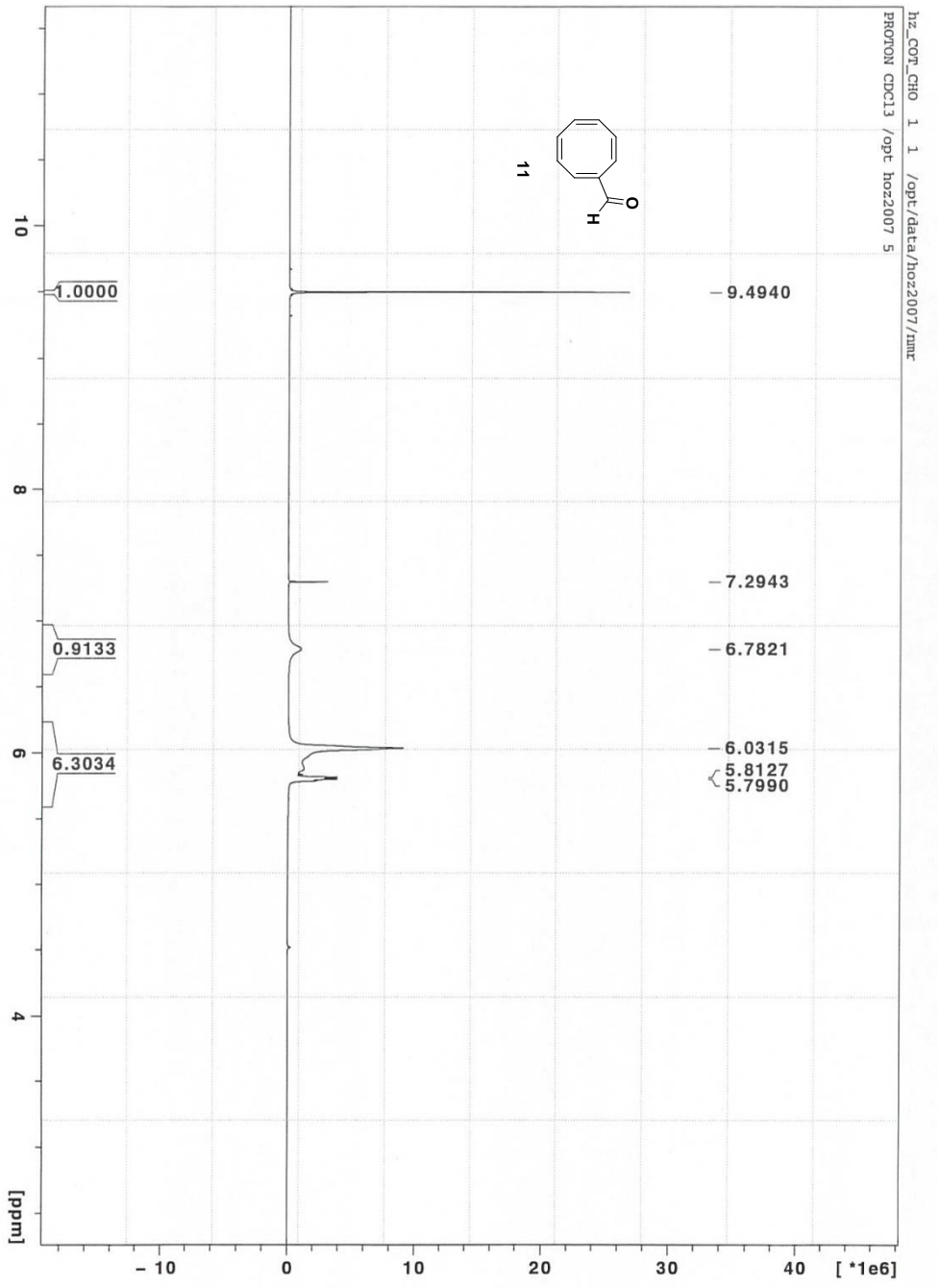
Compound 1 (^{13}C, D_2O, 125 MHz)



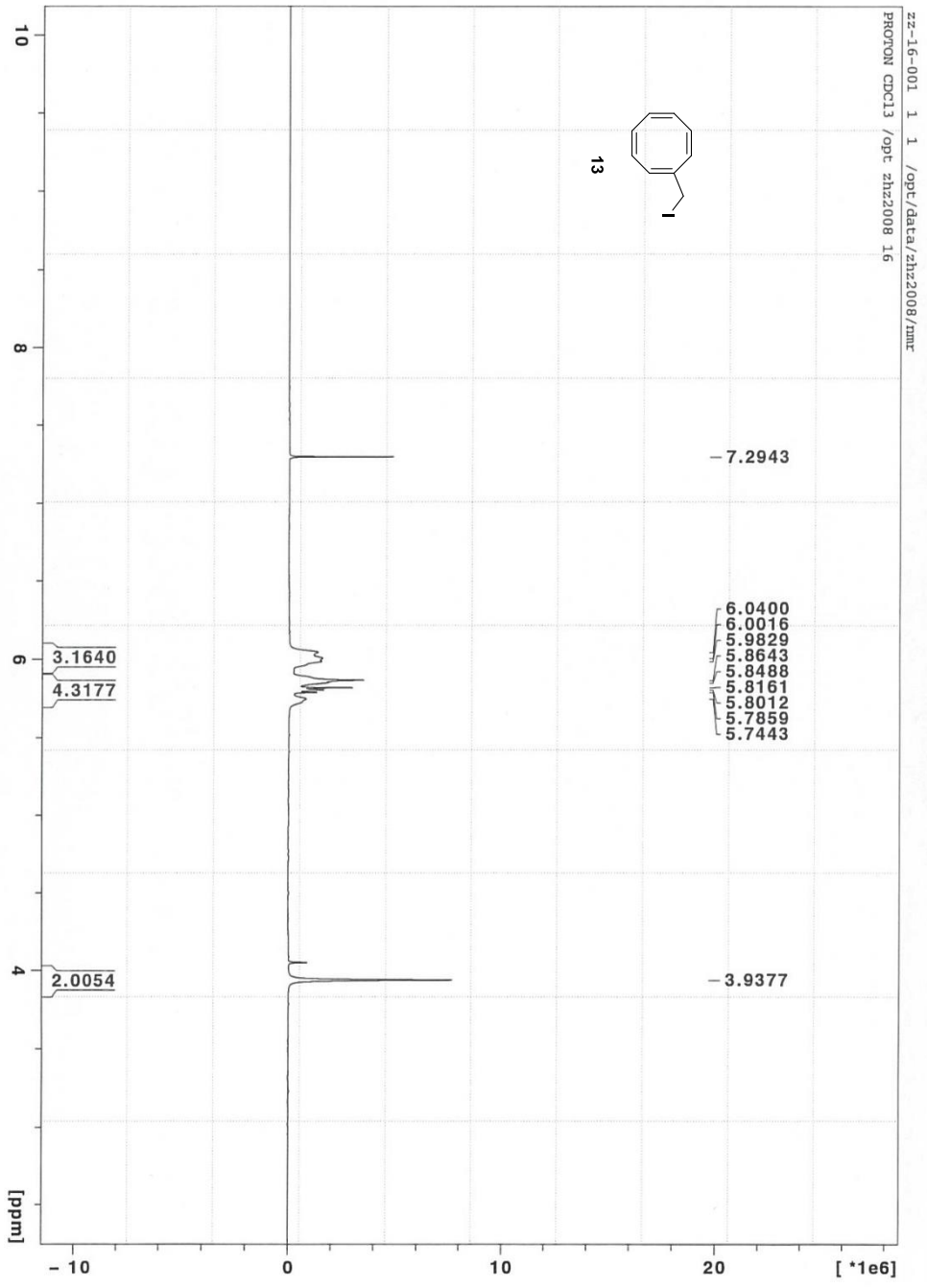
Compound 10 (^{1}H, $CDCl_3$, 500 MHz)



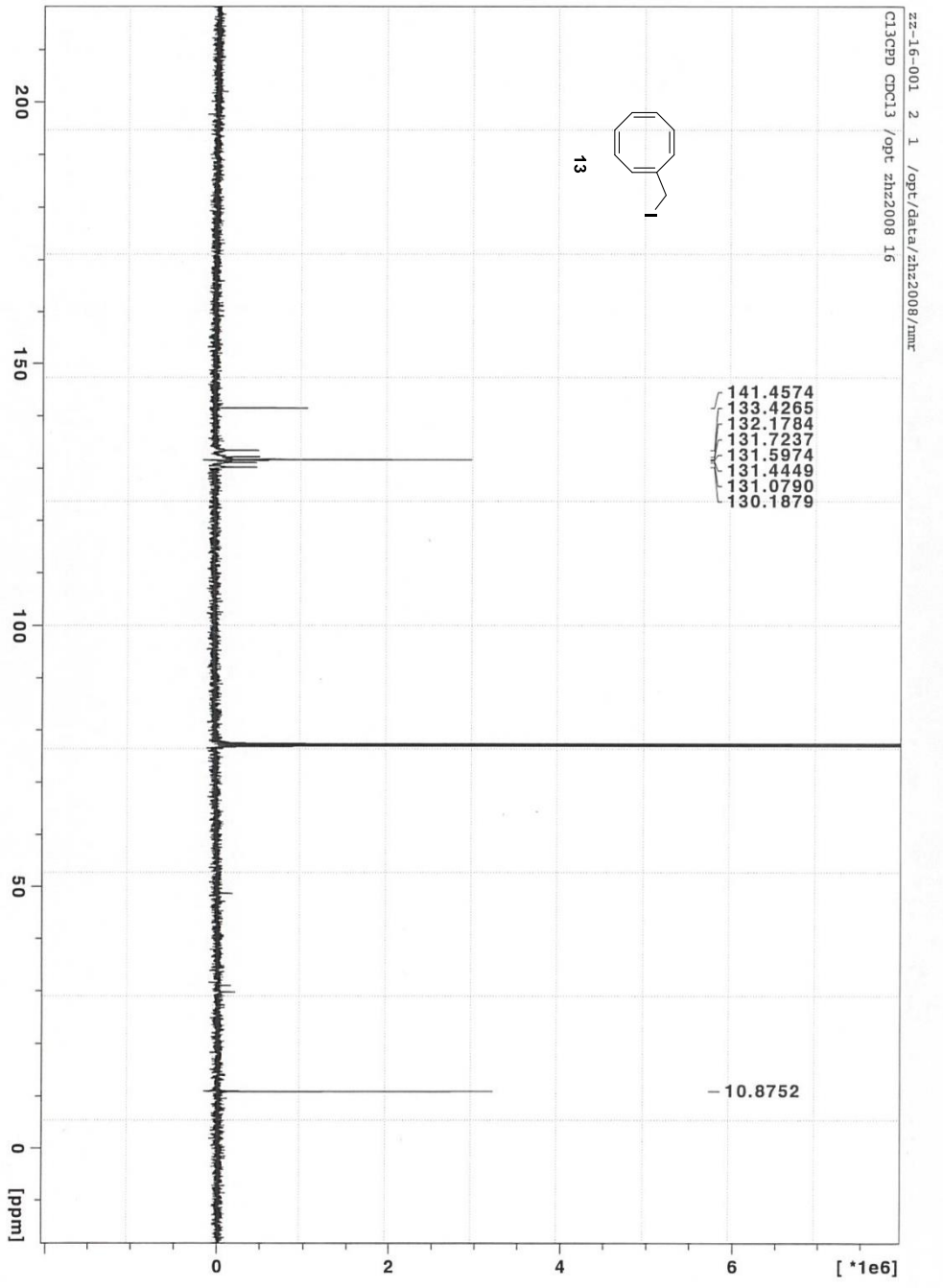
Compound 10 (^{13}C, $CDCl_3$, 125 MHz)



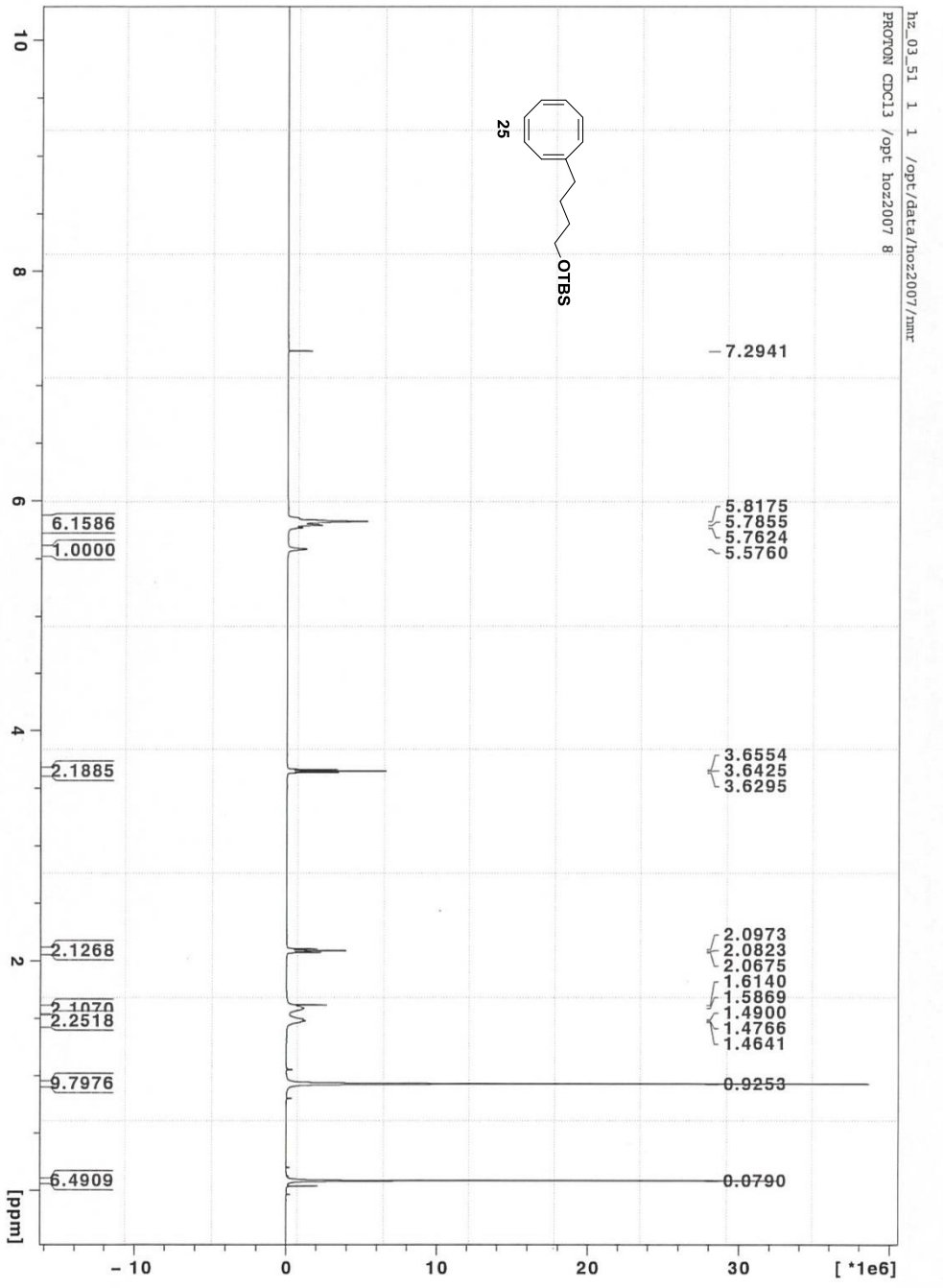
Compound 11 (^{1}H, $CDCl_3$, 500 MHz)



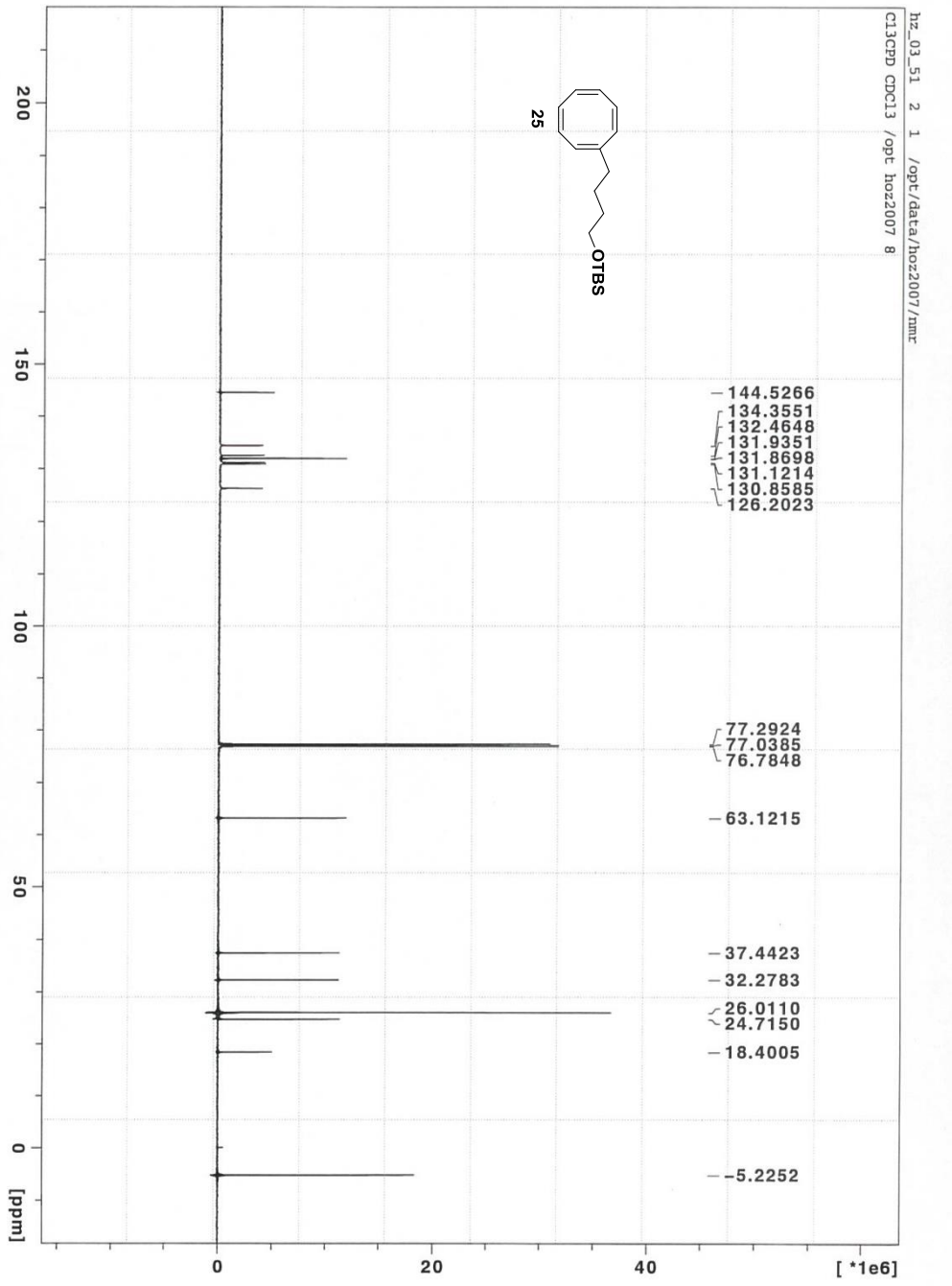
Compound 13 (^{1}H, $CDCl_3$, 500 MHz)



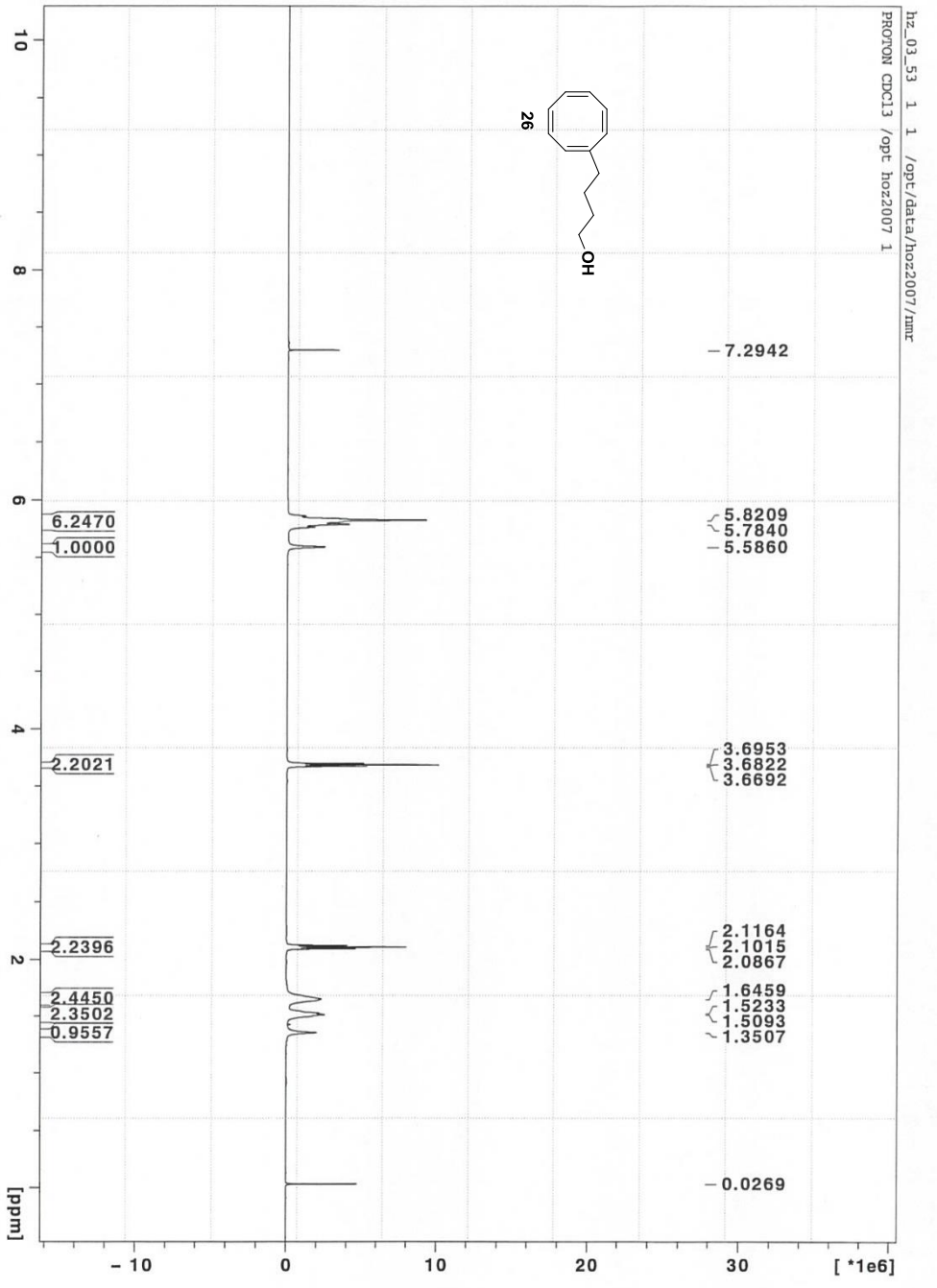
Compound 13 (^{13}C, $CDCl_3$, 125 MHz)



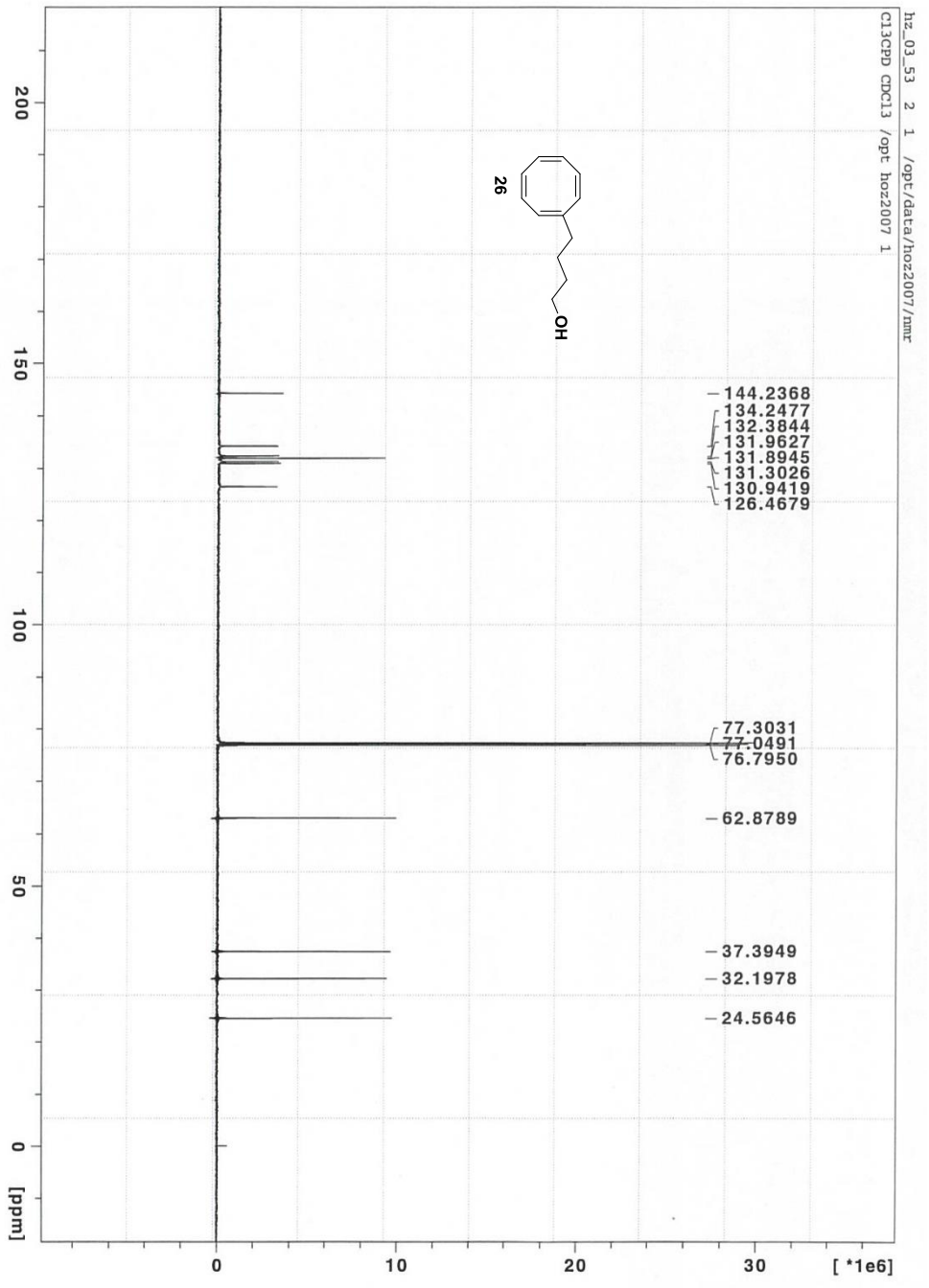
Compound 25 (^{1}H, $CDCl_3$, 500 MHz)



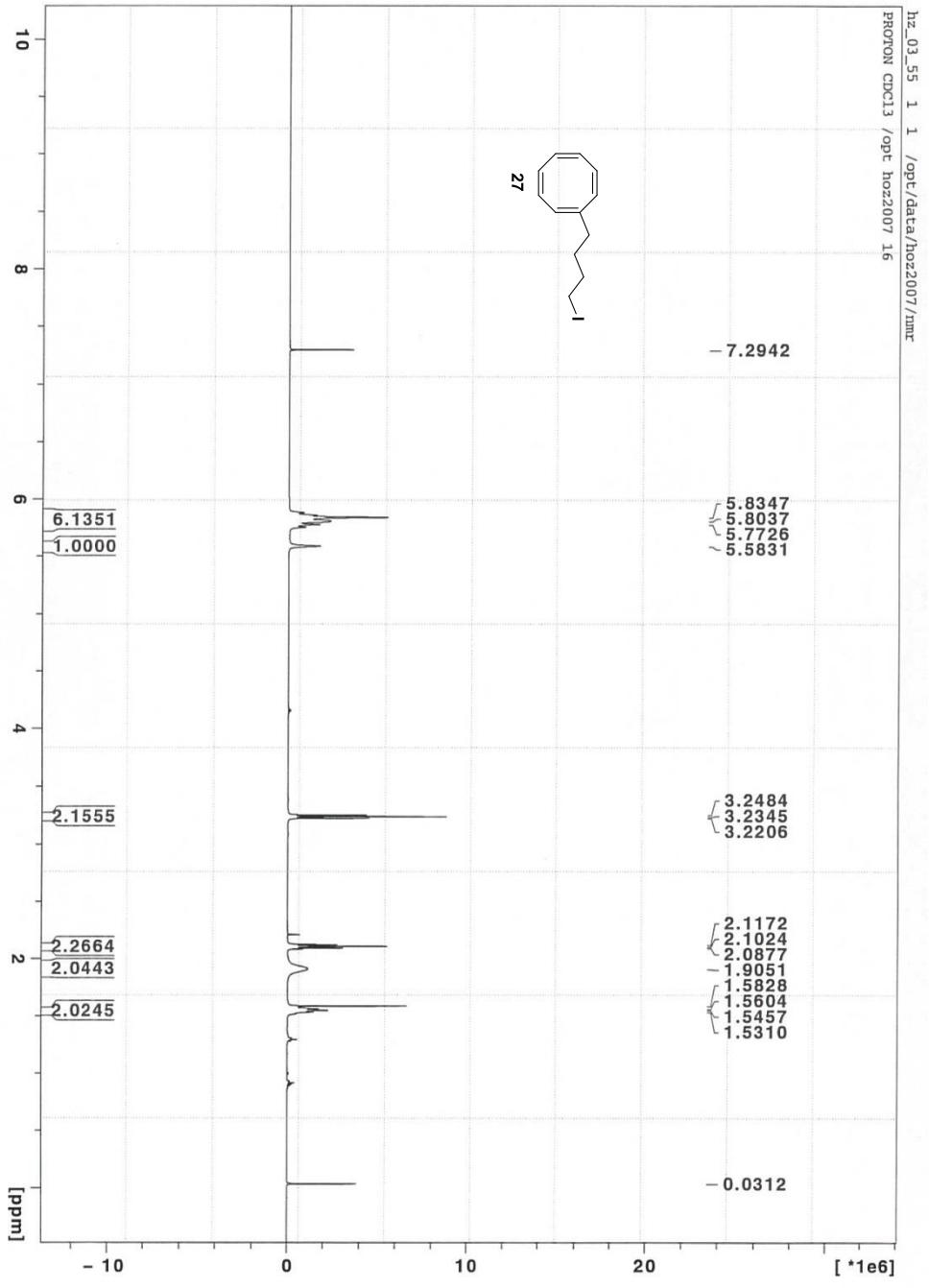
Compound 25 (^{13}C, $CDCl_3$, 125 MHz)



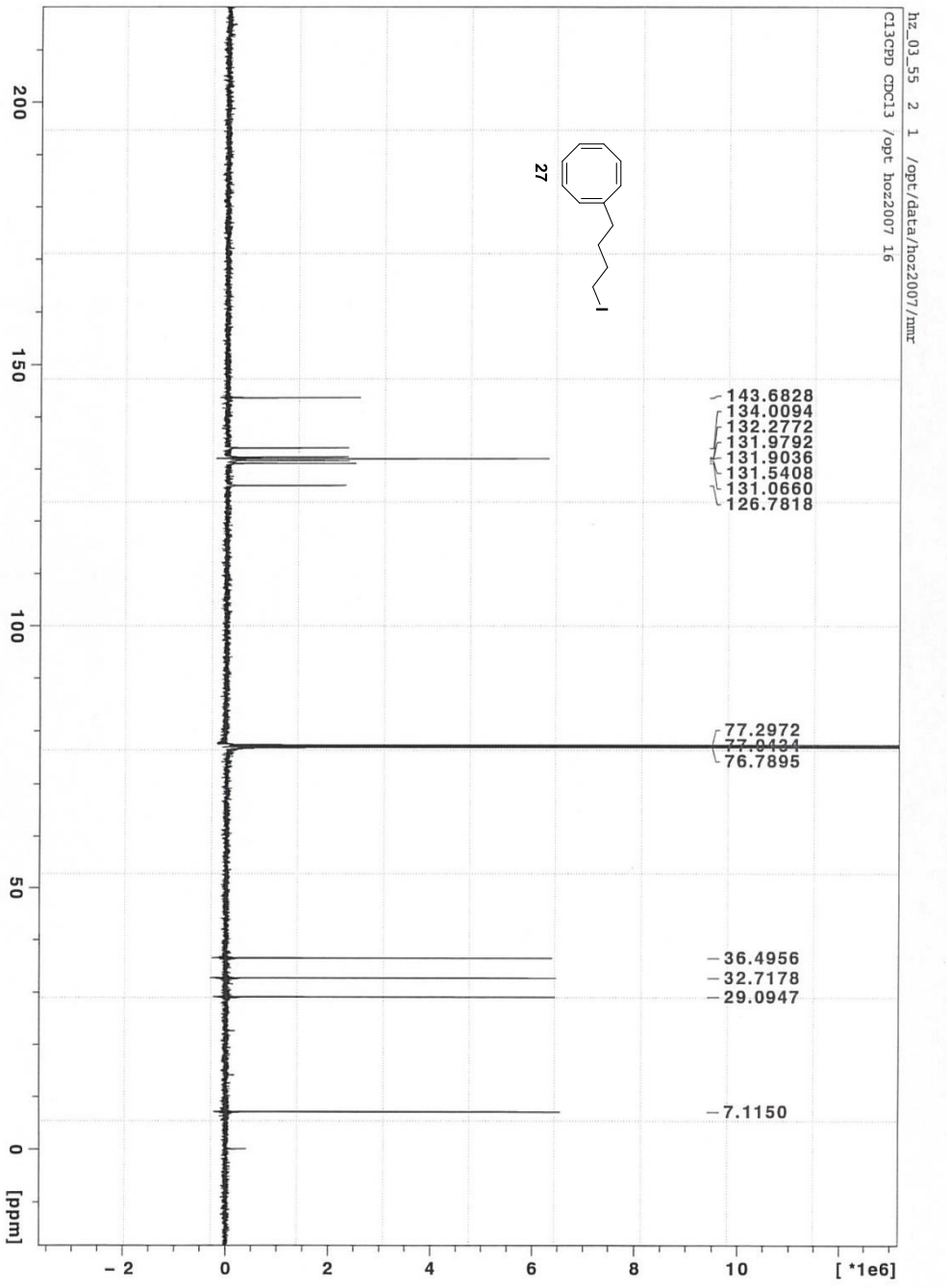
Compound 26 (^{1}H, $CDCl_3$, 500 MHz)



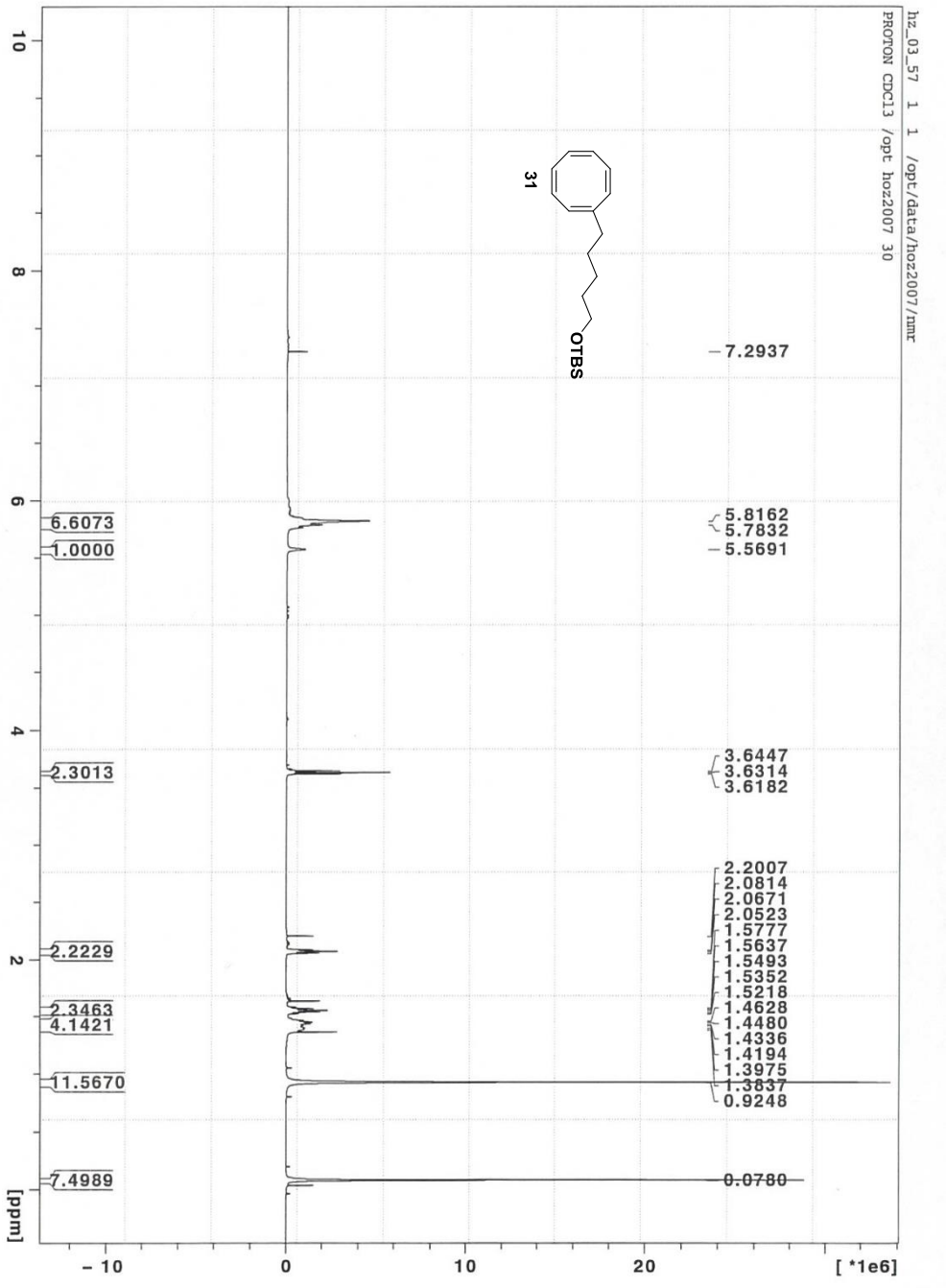
Compound 26 (^{13}C, $CDCl_3$, 125 MHz)



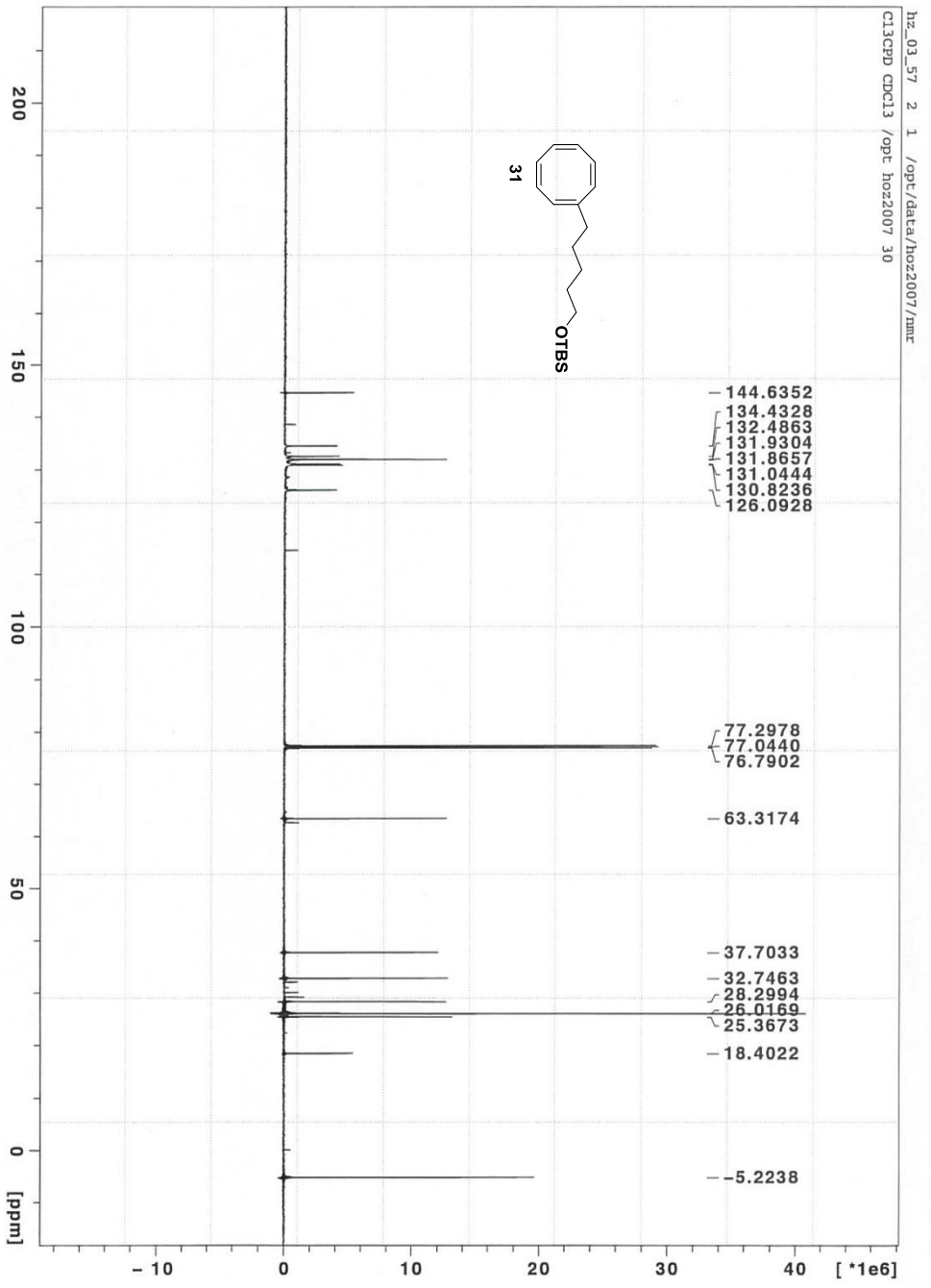
Compound 27 (^{1}H, $CDCl_3$, 500 MHz)



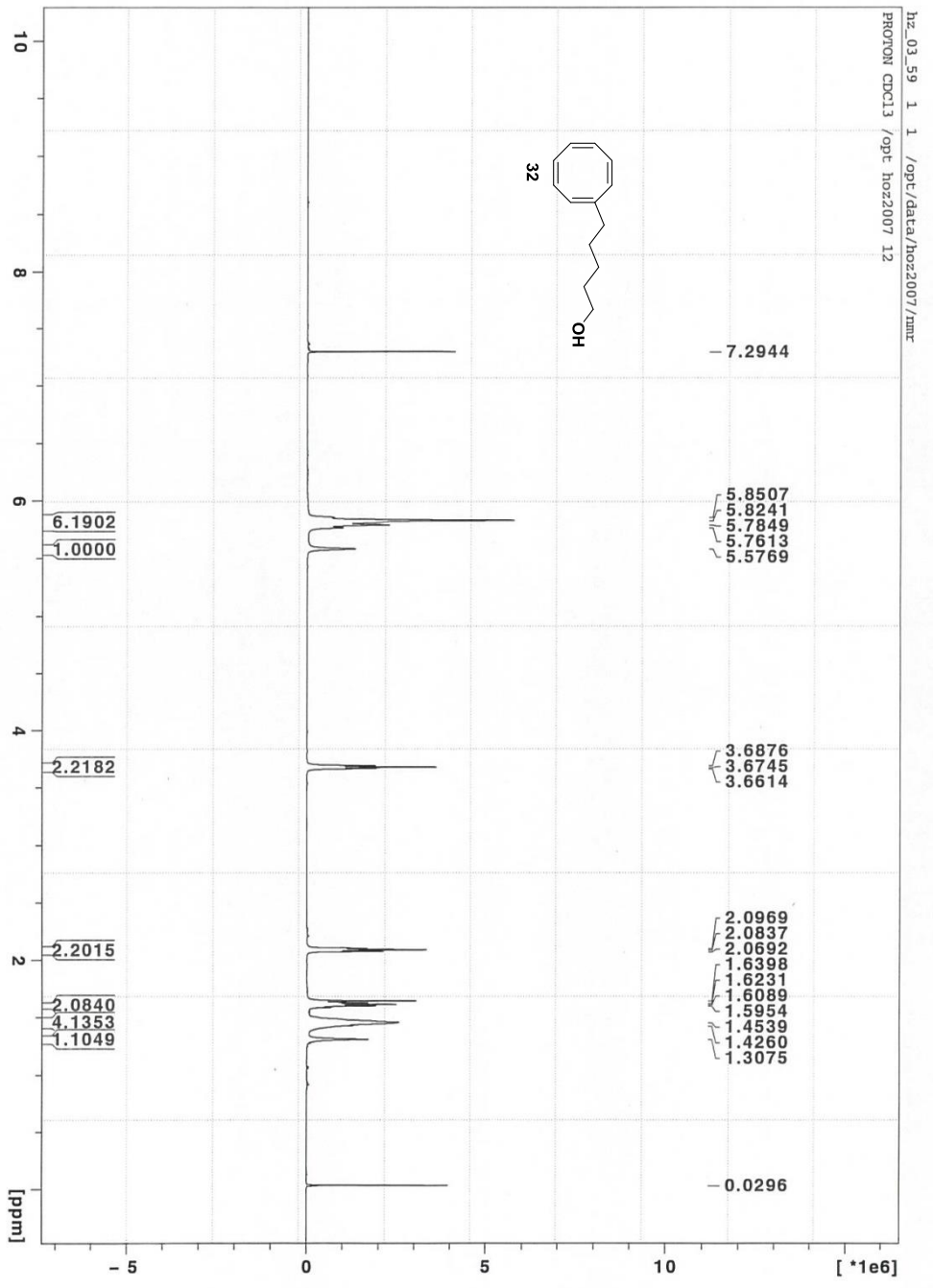
Compound 27 (^{13}C, $CDCl_3$, 125 MHz)



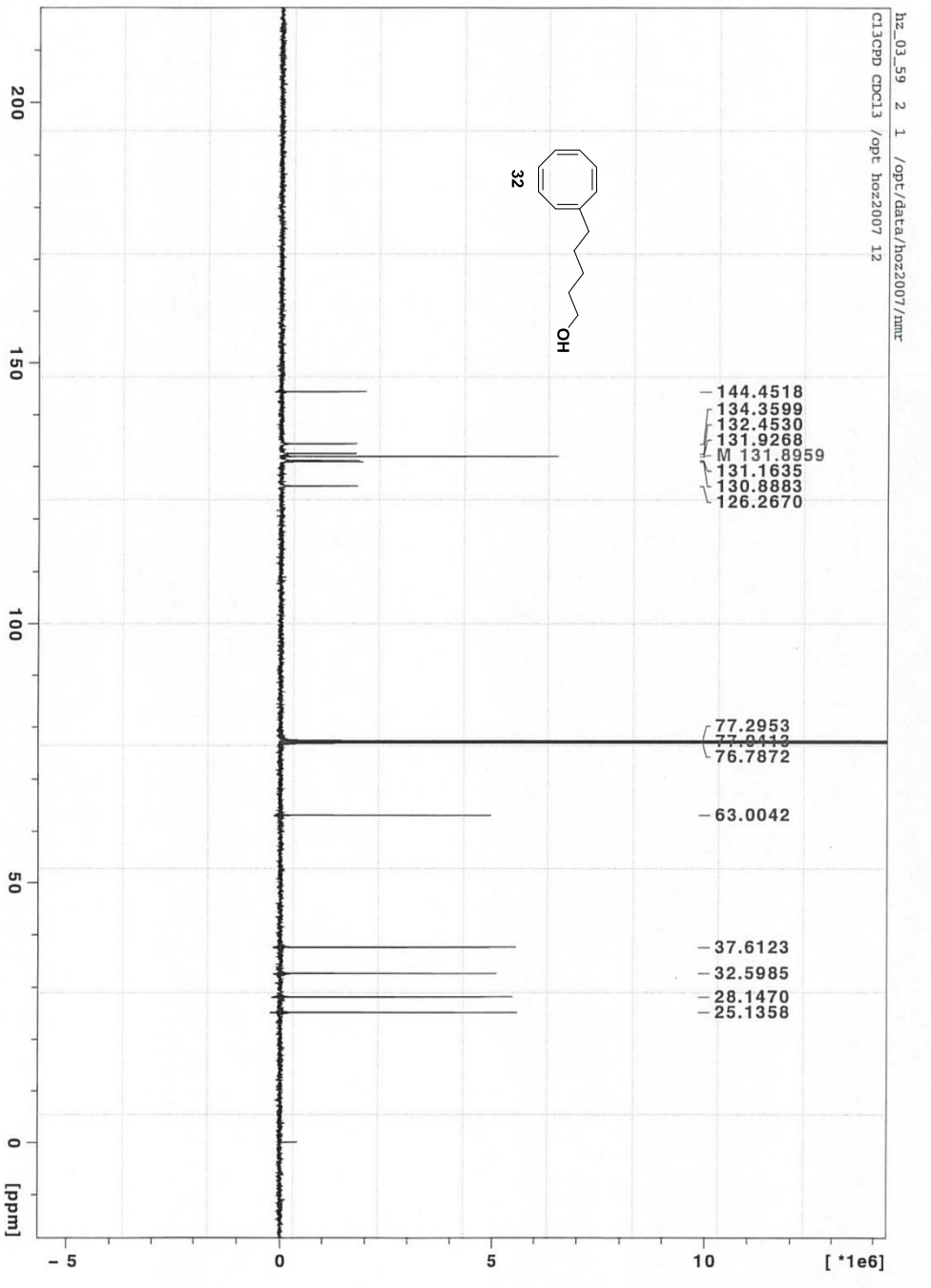
Compound 31 (^{1}H, $CDCl_3$, 500 MHz)



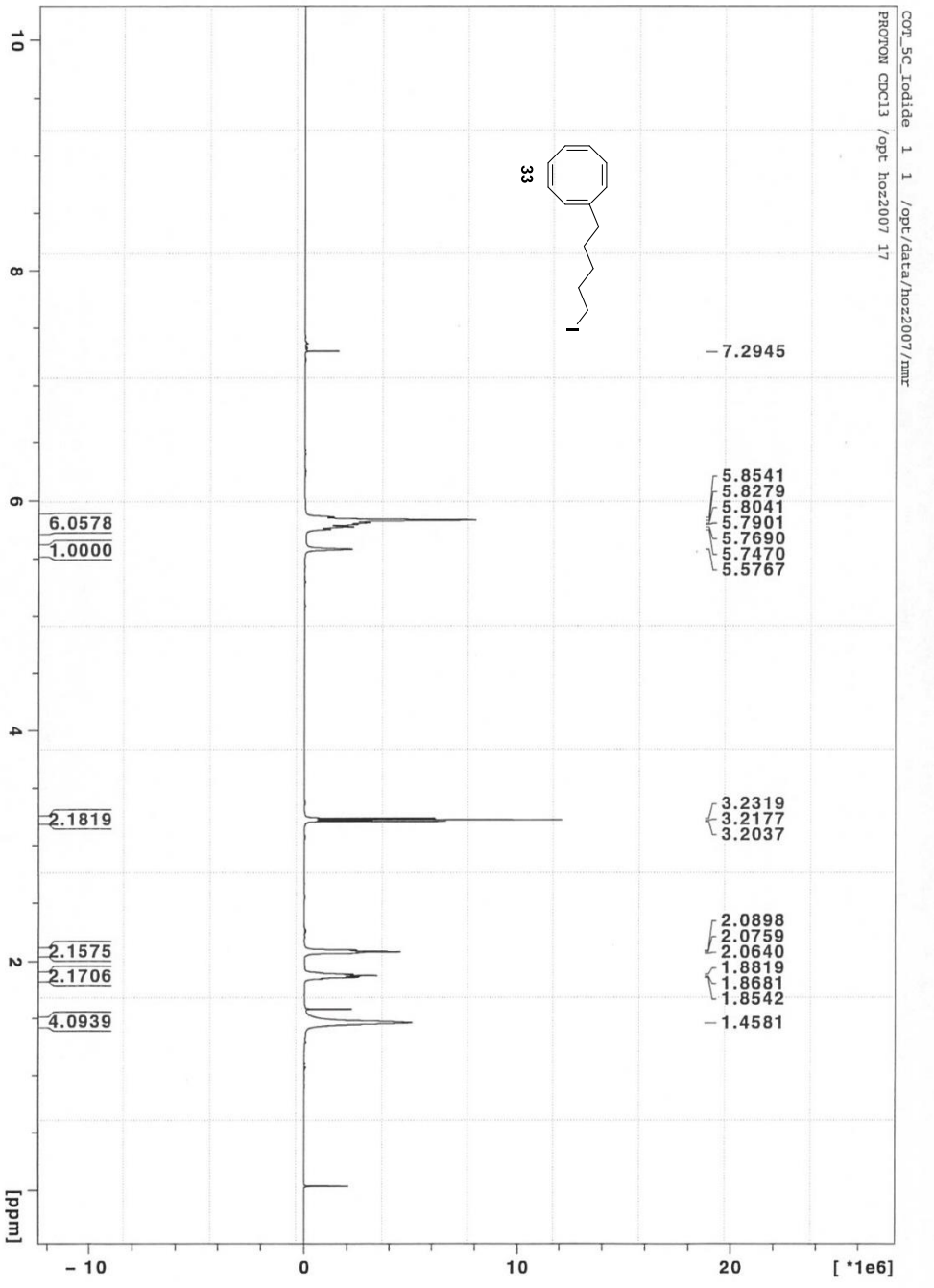
Compound 31 (^{13}C, $CDCl_3$, 125 MHz)



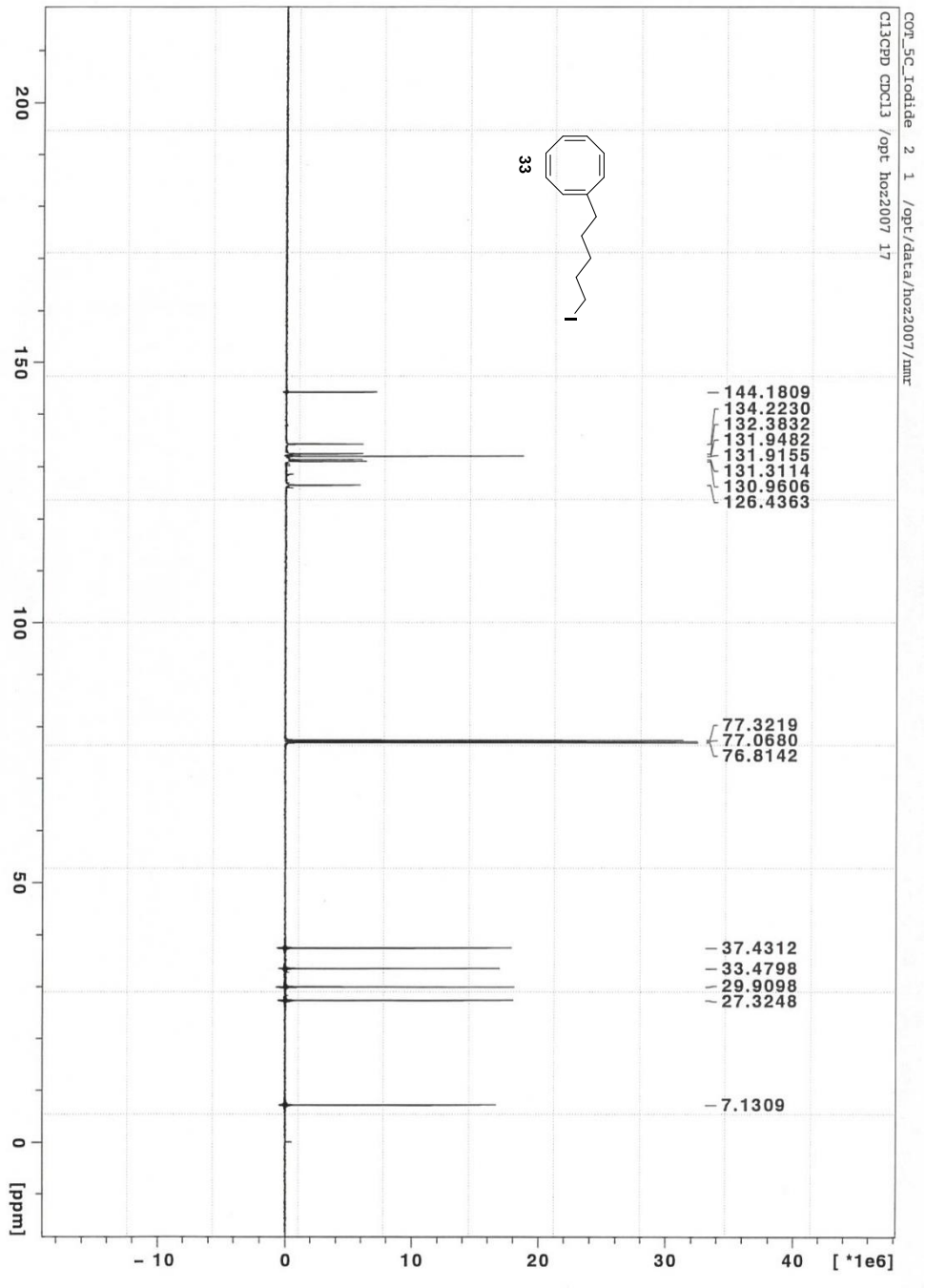
Compound 32 (^{1}H, $CDCl_3$, 500 MHz)



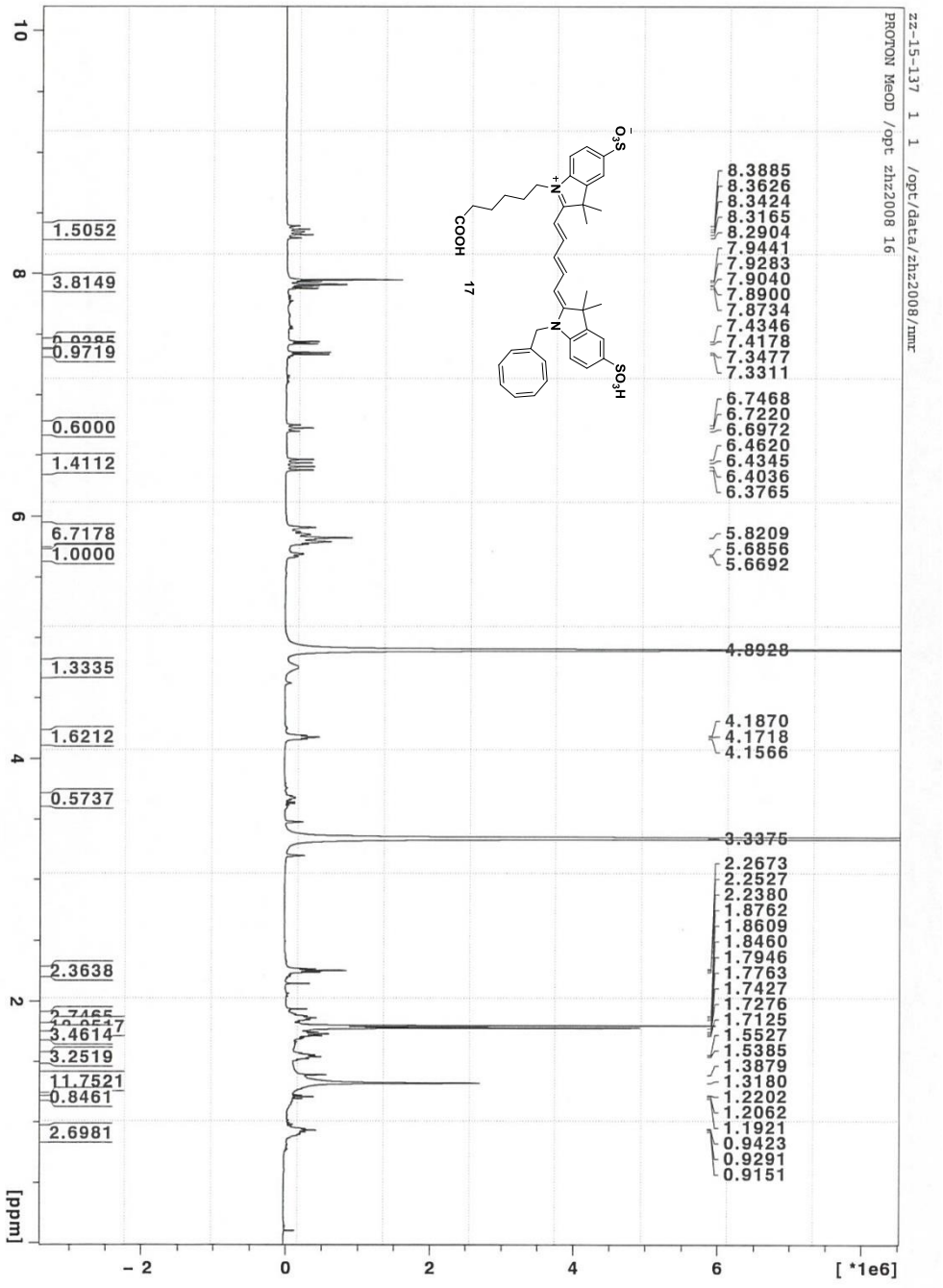
Compound 32 (^{13}C, $CDCl_3$, 125 MHz)



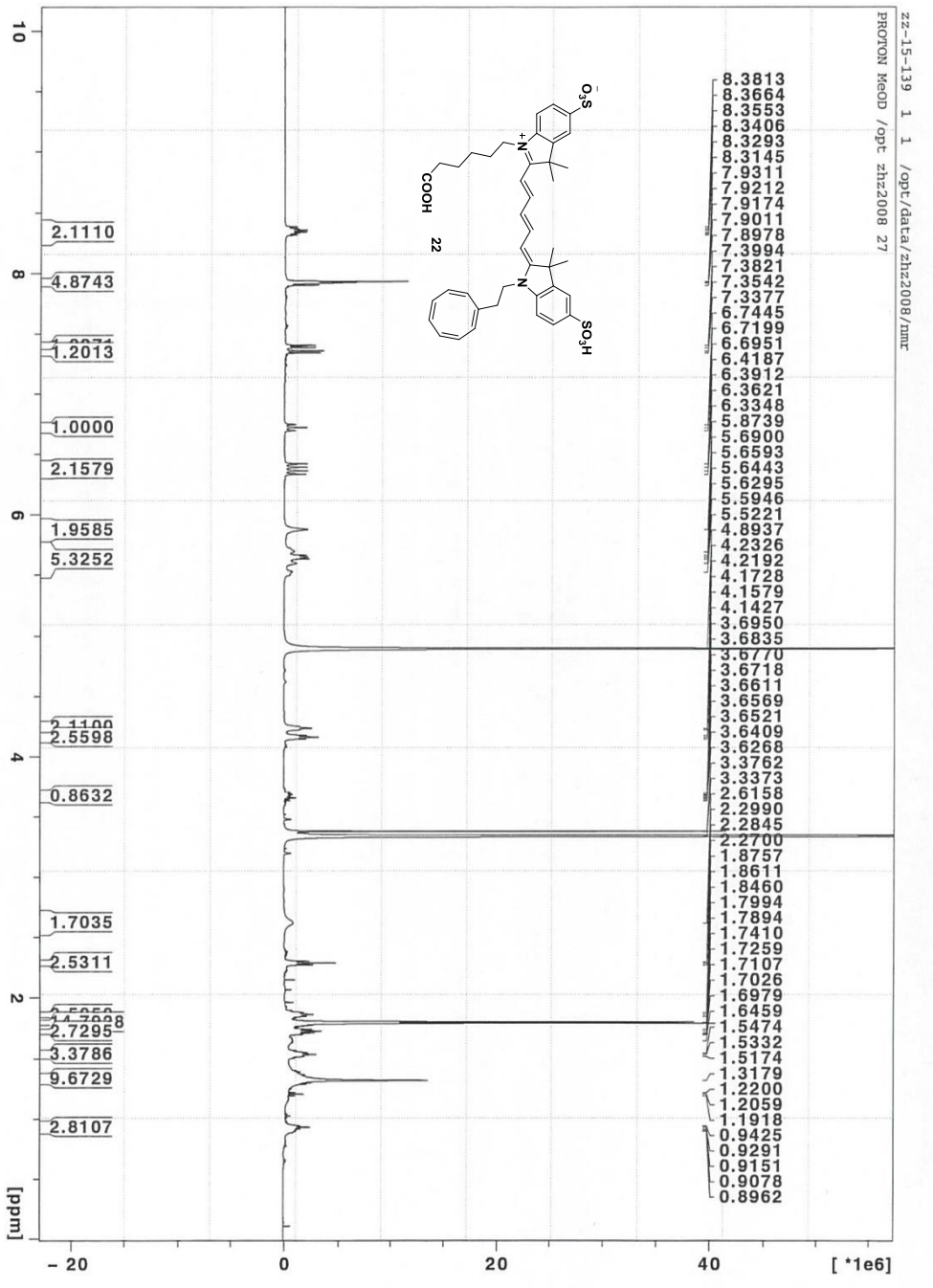
Compound 33 (^{1}H, $CDCl_3$, 500 MHz)



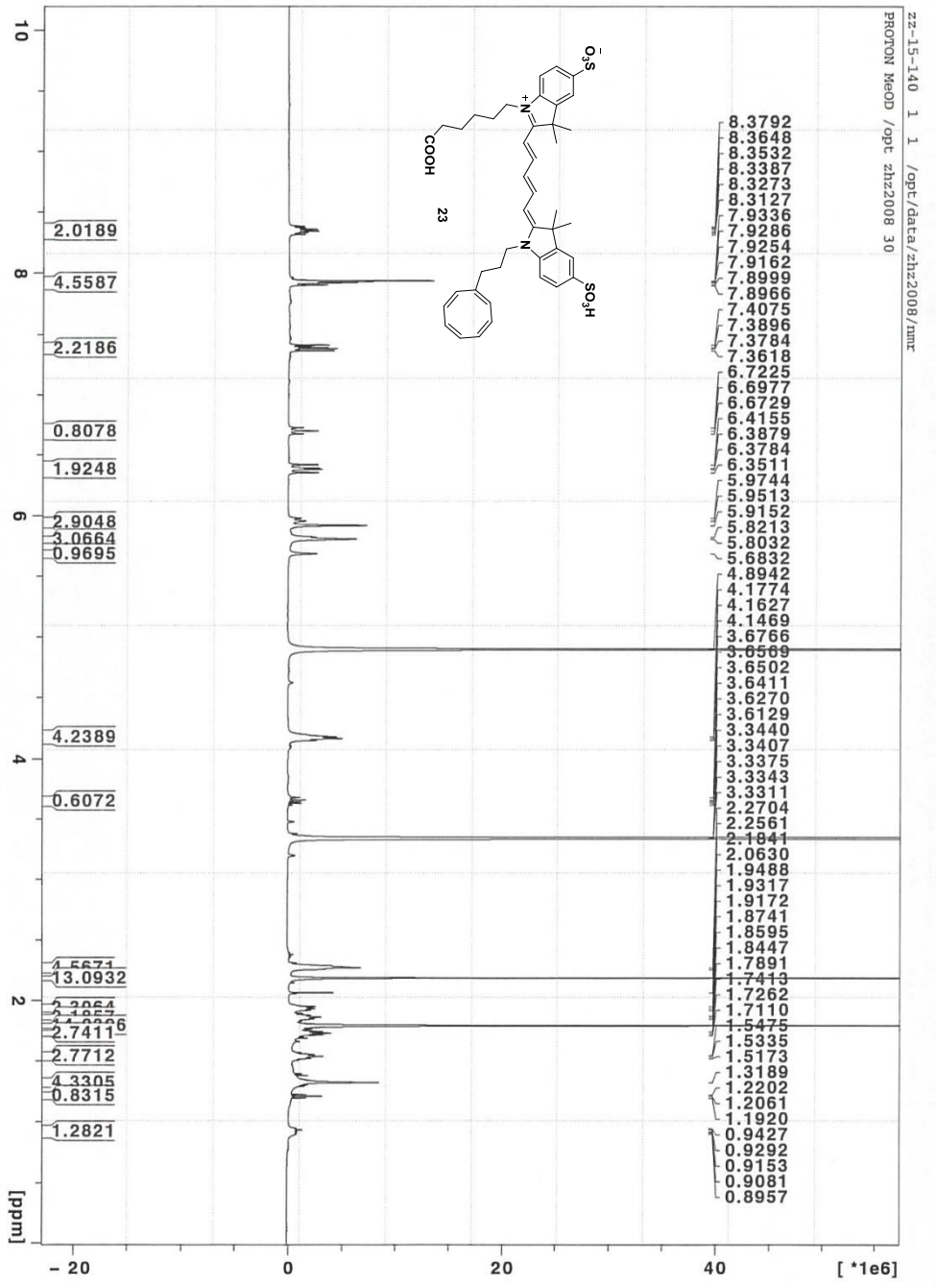
Compound 33 (^{13}C, $CDCl_3$, 125 MHz)



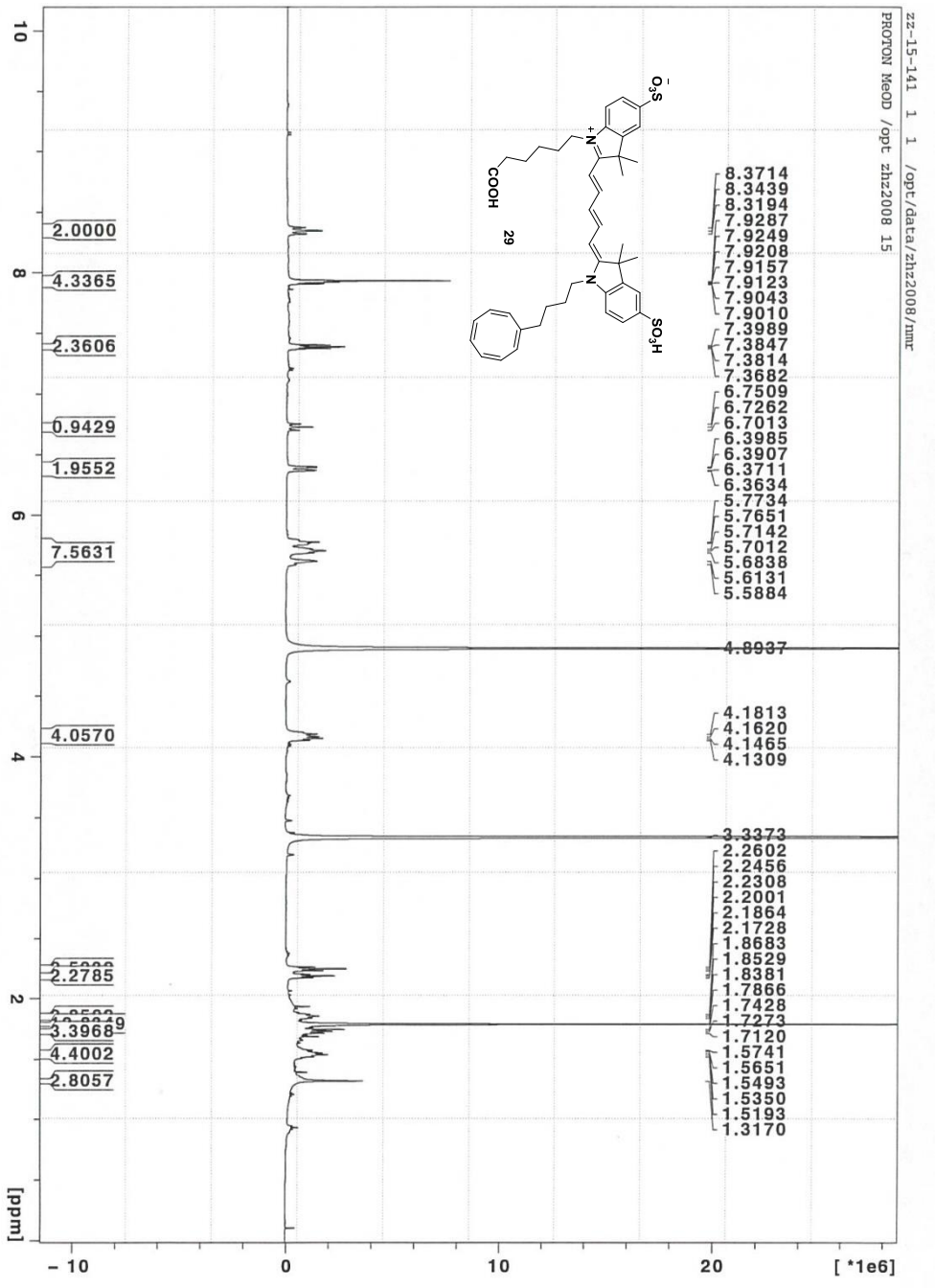
Compound 17 (^{1}H, MeOD, 500 MHz)



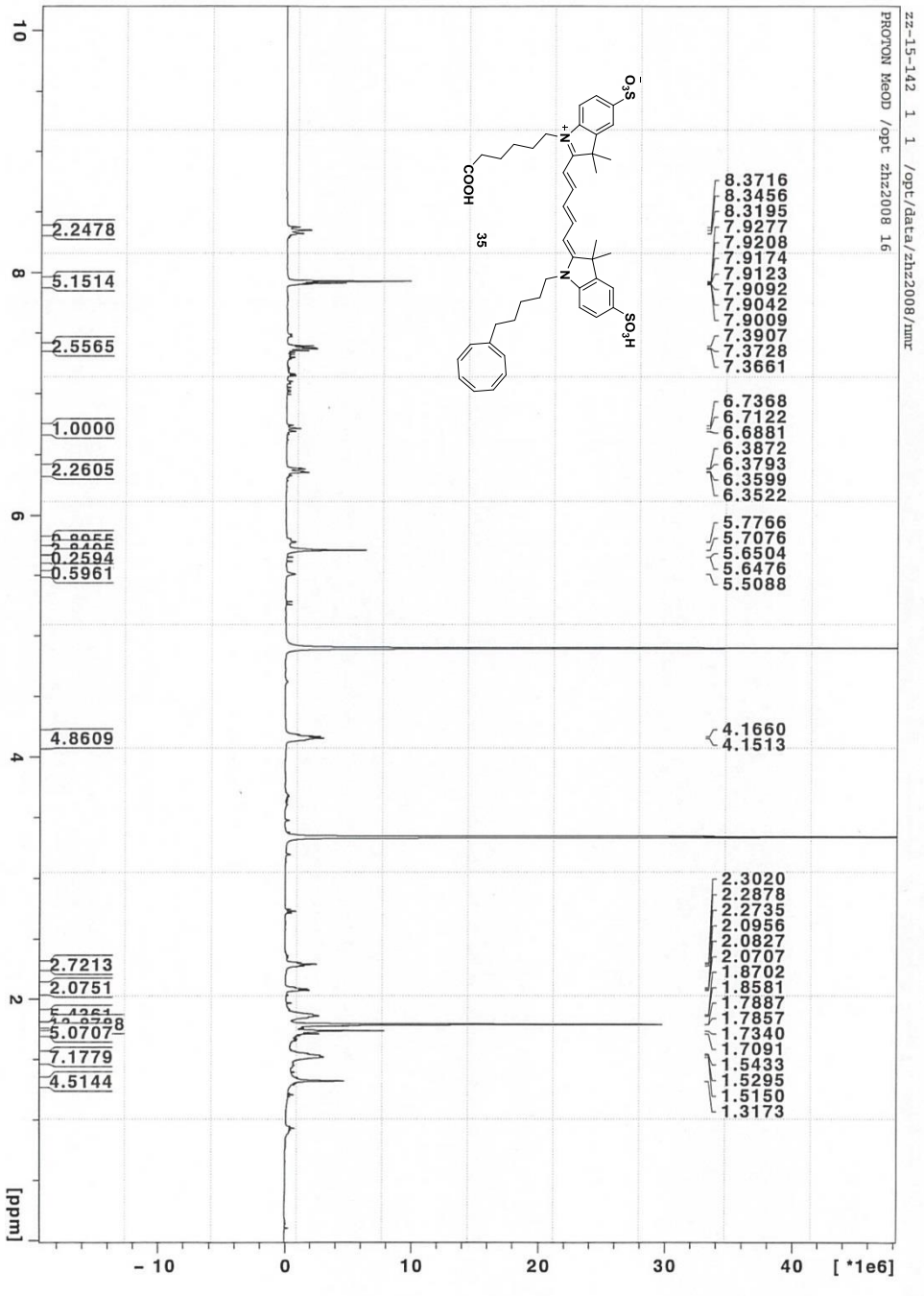
Compound 22 (^{1}H, MeOD, 500 MHz)



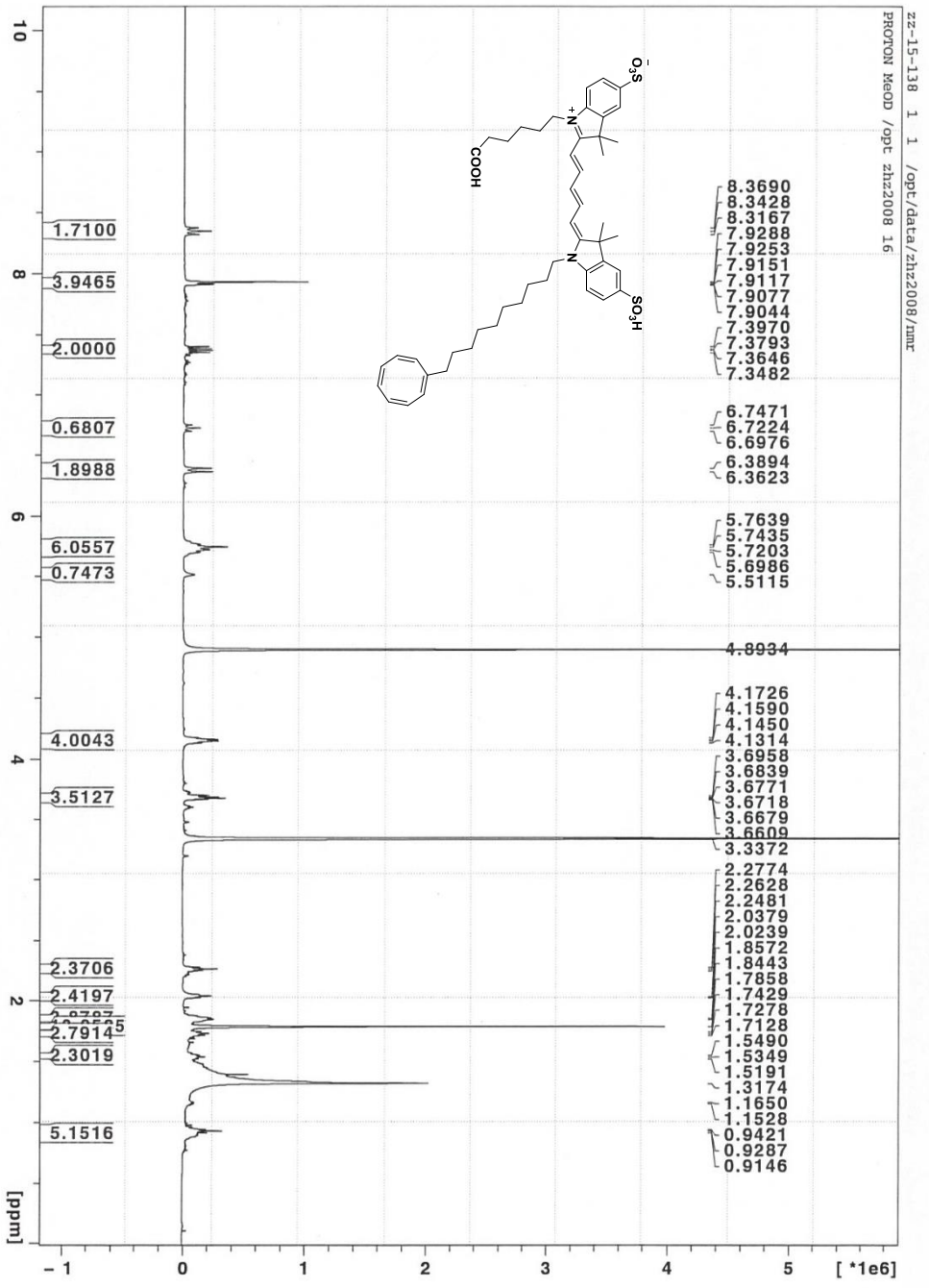
Compound 23 (^{1}H, MeOD, 500 MHz)



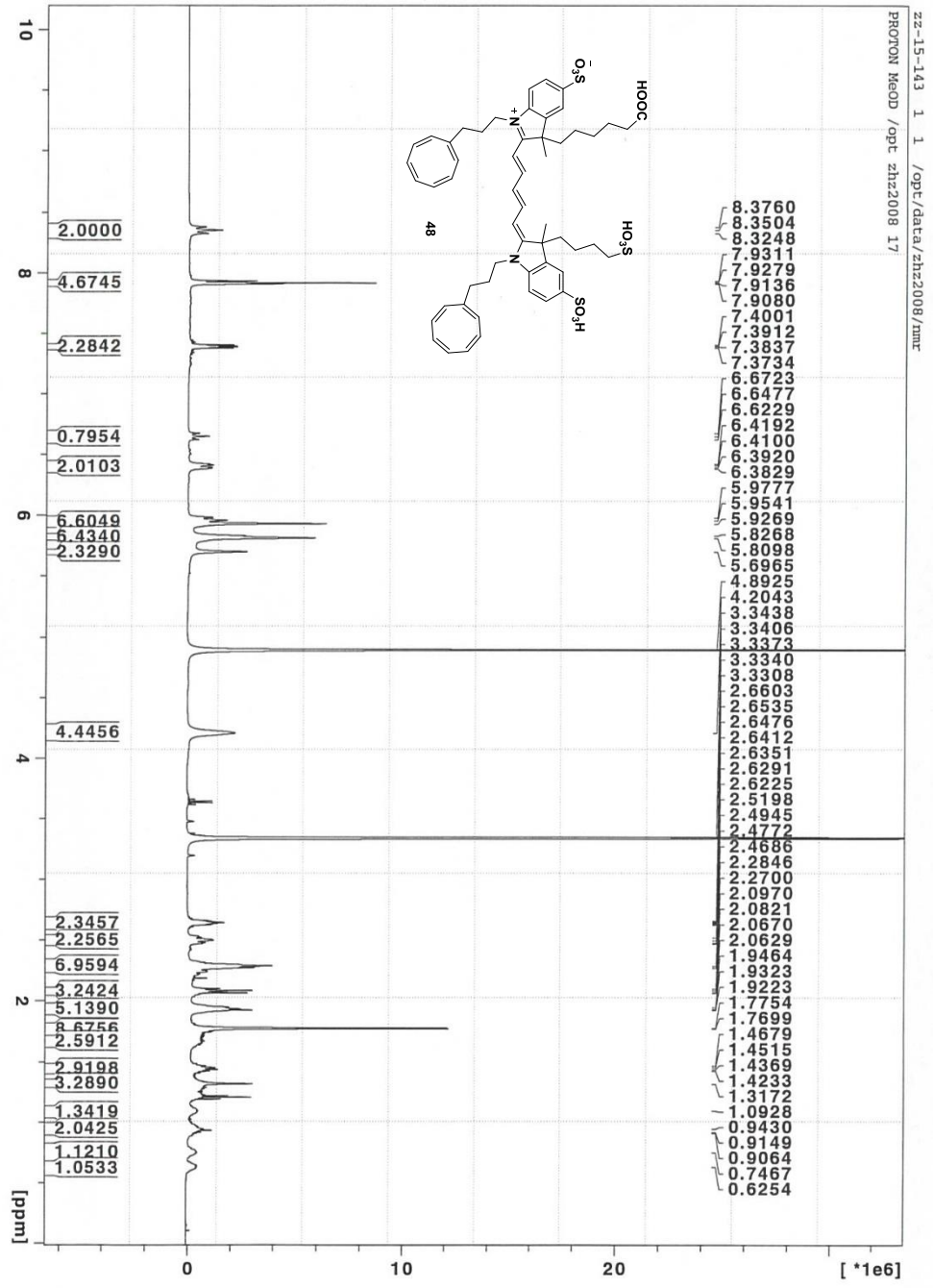
Compound 29 (^{1}H, MeOD, 500 MHz)



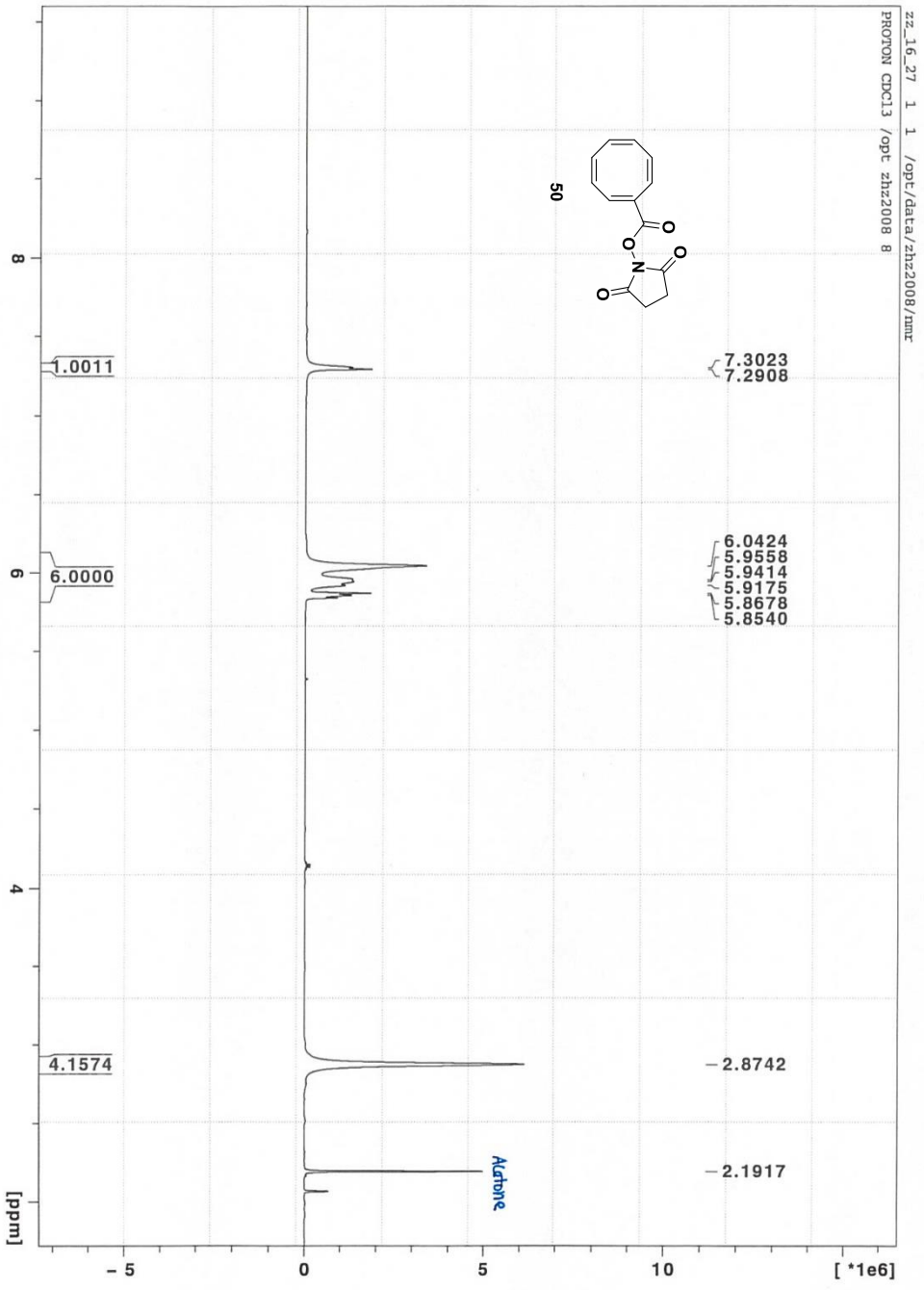
Compound 35 (^{1}H, MeOD, 500 MHz)



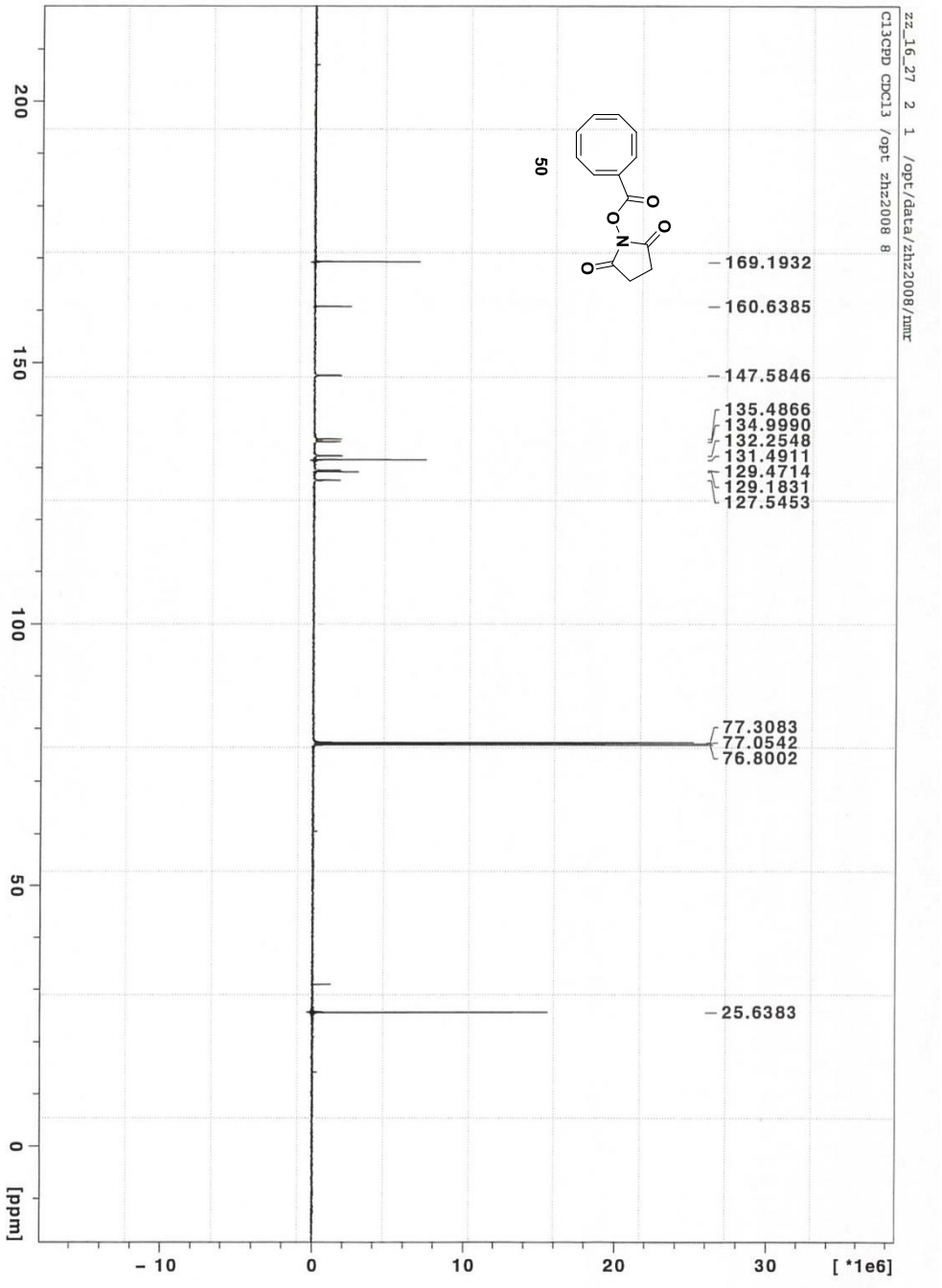
Compound 41 (^{1}H, MeOD, 500 MHz)



Compound 48 (^{1}H, MeOD, 500 MHz)



Compound 50 (^{1}H, $CDCl_3$, 500 MHz)



Compound 50 (^{13}C, $CDCl_3$, 125 MHz)

References

(1) Zheng, Q.; Jockusch, S.; Zhou, Z.; Altman, R. B.; Warren, J. D.; Turro, N. J.; Blanchard, S. C. *J. Phys. Chem. Lett.* **2012**, *3*, 2200.

(2) Jencks, W. P. *Catalysis in Chemistry and Enzymology*; Dover, 1987.

(3) Forward, P. J.; Gorman, A. A.; Hamblett, I. *J. Chem. Soc., Chem. Commun.* **1993**, 250.

(4) Das, T. N.; Priyadarshini, K. I. *J. Chem. Soc., Faraday Trans.* **1994**, *90*, 963.

(5) Widengren, J.; Chmyrov, A.; Eggeling, C.; Lofdahl, P. A.; Seidel, C. A. *J. Phys. Chem. A* **2007**, *111*, 429.

(6) Altman, R. B.; Terry, D. S.; Zhou, Z.; Zheng, Q.; Geggier, P.; Kolster, R. A.; Zhao, Y.; Javitch, J. A.; Warren, J. D.; Blanchard, S. C. *Nat. Methods* **2012**, *9*, 68.

(7) Gales, C.; Van Durm, J. J.; Schaak, S.; Pontier, S.; Percherancier, Y.; Audet, M.; Paris, H.; Bouvier, M. *Nat Struct Mol Biol* **2006**, *13*, 778.

(8) Paquette, L. A.; Henzel, K. A. *J. Am. Chem. Soc.* **1975**, *97*, 4649.

(9) Elbert, R.; Folda, T.; Ringsdorf, H. *J. Am. Chem. Soc.* **1984**, *106*, 7687.

(10) Markova, L. I.; Fedyunyayeva, I. A.; Povrozin, Y. A.; Semenova, O. M.; Khabuseva, S. U.; Terpetschnig, E. A.; Patsenker, L. D. *Dyes and Pigments* **2013**, *96*, 535.

(11) Blanchard, S. C.; Altman, R.; Warren, J. D.; Zhou, Z.; Google Patents: 2013.

(12) Hartmann, O.; Kalesse, M. *Organic Letters* **2012**, *14*, 3064.

(13) Yan, W.; Li, Z.; Kishi, Y. *J. Am. Chem. Soc.* **2015**, *137*, 6219.

(14) Brimble, M. A.; Flowers, C. L.; Hutchinson, J. K.; Robinson, J. E.; Sidford, M. *Tetrahedron* **2005**, *61*, 10036.

(15) Patsenker, L.; Terpetschnig, E.; Fedyunyaeva, I.; Kolosova, O.; Klochko, A.; Google Patents: 2007.