Supplementary Information

A silicon rhodamine 1,2-dioxetane chemiluminophore for *in vivo* near infrared imaging

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1. Experimental Methods

1.1 Synthetic Procedures

1.1.1 General Synthetic Materials and Methods

Reagents were purchased from Sigma-Aldrich (St. Louis, MO), Thermo Fisher Scientific (Waltham, MA), TCI America (Portland, OR), Alfa Aesar (Ward Hill, MA), EMD Millipore (Billerica, MA), Oakwood Chemical (West Columbia, SC), or Cayman Chemical (Ann Arbor, MI) and used without further purification. ^{1}H NMR and ^{13}C NMR were obtained on a JEOL 500 MHz spectrometer in $CDCl_3$ or CD_3OD (Cambridge Isotope Laboratories, Cambridge, MA). All chemical shifts are reported in the standard notation of parts per million. Splitting patterns are indicated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, doublet of doublets; bs, broad singlet. Low resolution mass spectroscopy was performed on a Advion LCMS (ESI source).

***N*-(10-(5-(4-(tert-butoxycarboyl)piperazine-1-carbonyl)-2-methylphenyl)-7-(dimethylamino)-5,5-dimethyldibenzo[b,e]silin-3(5H)-ylidene)-*N*-methylmethanaminium (1).** In a 100 mL round bottom flask equipped with a stir bar under N_2 atmosphere, **SiR**[1,2] (150 mg, 0.338 mmol, 1.0 equiv) was dissolved in 20 mL DCM. The reaction was cooled to 0 °C. *N,N*-Diisopropylethylamine (DIPEA) (120 µL, 0.676 mmol, 2.0 equiv) and *N,N,N′,N′*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl) uronium hexafluorophosphate (HBTU) (260 mg, 0.676 mmol, 2.0 equiv) were then added and stirred for 10 min, at which point the HBTU activated ester was visible by TLC. 1-Boc-piperazine (130 mg, 0.406 mmol, 1.2 equiv) was then added, and the reaction was allowed to stir at room temperature overnight. The reaction was diluted in DCM and washed with sat. aq. NH_4Cl, and brine, then dried over Na_2SO_4, filtered, and concentrated under reduced pressure. Column chromatography (7% MeOH:DCM) yielded the *t*-butyl protected SiR-piperazine **1** (147 mg, 0.24 mmol, 71%) as a blue solid. ^{1}H NMR (500 MHz, CD_3OD): δ 7.52 – 7.53 (m, 2H), 7.37 (d, 2H, *J* = 2.8 Hz), 7.20 (s, 1H), 7.08 (d, 2H, *J* = 9.7 Hz), 6.79 (dd, 2H, *J1* = 2.8, *J2* = 9.7 Hz), 3.42-3.70 (m, 8H), 3.35 (s, 12H), 2.06 (s, 3H), 1.43 (s, 9H), 0.59 (s, 3H), 0.58 (s, 3H); ^{13}C NMR (125 MHz, CD_3OD): δ 170.56, 167.50, 154.88, 154.49, 148.21, 140.72, 139.29, 138.19, 132.94, 130.50, 127.52, 127.35, 127.01, 121.05, 114.10, 80.39, 39.65, 27.28, 18.13, -2.35, -2.64.

***N*-(7-(dimethylamino)-5,5-dimethyl-10-(2-methyl-5-(piperazine-1-carbonyl)phenyl)dibenzo[b,e]silin-3(5H)-ylidene)-*N*-methylmethanaminium (2).**

In a 20 mL vial, the *t*-butyl protected SiR-piperazine (53 mg, 0.088 mmol) was dissolved in 1.5 mL DCM. TFA (0.5 mL) was added and stirred for 2.5 hr at RT. After this time, TLC and mass spectroscopy indicated complete conversion of the starting material to the product. The volatiles were evaporated off to obtain compound **2** (45 mg, 0.088 mmol, quantitative) as a blue solid which was carried forward without further purification. ^{1}H NMR (500 MHz, CD_3OD): δ 7.52-7.59 (m, 2H), 7.36 (d, 2H, *J* = 2.8 Hz), 7.27 (s, 1H), 7.07 (d, 2H, *J* = 9.7 Hz), 6.77 (dd, 2H, *J1* = 2.8, *J*2 = 9.7 Hz), 3.60-3.88 (m, 8H), 3.34 (s, 12H), 2.08 (s, 3H), 0.59 (s, 3H), 0.58 (s, 3H). ^{13}C NMR (125 MHz, CD_3OD): δ 170.54, 167.37, 154.48, 148.22, 140.71, 139.42, 138.72, 131.97, 130.59, 127.67, 127.43, 126.99, 121.04, 114.08, 42.93, 39.62, 18.13, -2.41, -2.66. HRMS calcd for $C_{31}H_{39}N_4OSi^+$ $[M+H]^+$ 511.2888, found 511.2876.

***N*-(10-(5-(4-((E)-3-(2-acetoxy-4-(((1r,3r)-adamantan-2-ylidene)(methoxy)methyl)-3-chlorophenyl)acryloyl)piperazine-1-carbonyl)-2-methylphenyl)-7-(dimethylamino)-5,5-dimethyldibenzo[b,e]silin-3(5H)-ylidene)-*N*-methylmethanaminium (4).** In a 25 mL round bottom flask equipped with a stir bar under N_2 atmosphere, Compound **3**[1] (41 mg, 0.098 mmol, 1.0 equiv) was dissolved in 3 mL dry DCM. The reaction was cooled to 0 °C. DIPEA (51 µL, 0.29 mmol, 3.0 equiv) and HBTU (110 mg, 0.29 mmol, 3.0 equiv) were then added and stirred for 30 min, at which time the HBTU activated ester was visible by TLC. Compound **2** (50 mg, 0.098 mmol, 1.0 equiv) was then added, and the reaction was allowed to stir overnight. Upon completion, the reaction was neutralized with sat. aq. NH_4Cl, extracted with DCM, dried with Na_2SO_4, filtered, and concentrated under reduced pressure. Column chromatography (7% MeOH/DCM) yielded compound **4** (70 mg, 0.077 mmol, 78%) as a blue solid. ^{1}H NMR (500 MHz, CD_3OD): δ 7.81 (d, 1H, *J* = 6.3 Hz), 7.52-7.62 (m, 3H), 7.35 (d, 2H, *J* = 2.8 Hz), 7.21-7.24 (m, 3H), 7.08 (d, 2H, *J* = 9.7 Hz), 6.80 (dd, 2H, *J1* = 2.9 Hz, *J2* = 9.7), 3.59-3.78 (m, 8H), 3.33 (s, 12H), 3.27 (s, 3H), 3.24 (s, 1H), 2.39 (s, 3H), 2.08 (s, 3H), 1.75-2.08 (m, 12H), 0.59 (s, 3H), 0.58 (s, 3H); ^{13}C NMR (125 MHz, CD_3OD): δ 170.60, 168.47, 167.49, 165.90, 154.48, 148.21, 146.11, 140.71, 139.34, 139.30, 138.29, 137.12, 132.79, 132.30, 130.56, 129.74, 129.39, 128.53, 127.54, 127.40, 127.00, 124.68, 121.04, 114.13, 56.14, 39.63, 38.83, 38.69, 38.25, 36.75, 33.01, 29.77, 28.46, 28.31, 18.97, 18.12, -2.37, -2.68. HRMS calcd for $C_{54}H_{62}ClN_4O_5Si^+$ $[M+H]^+$ 909.4173, found 909.4145.

***N*-(10-(5-(4-((E)-3-(4-(((1r,3r)-adamantan-2-ylidene)(methoxy)methyl)-3-chloro-2-hydroxyphenyl)acryloyl)piperazine-1-carbonyl)-2-methylphenyl)-7-(dimethylamino)-5,5-dimethyldibenzo[b,e]silin-3(5H)-ylidene)-*N*-methylmethanaminium (5).** Compound **4** (15 mg, 0.016 mmol) was dissolved in 0.5 mL MeOH in a 20 mL vial. NH_4OAc (93 mg, 1.2 mmol, 75 equiv) was dissolved in 0.5 mL water then added and allowed to stir under N_2 atmosphere at RT for 3 days. Upon completion as determined by TLC and mass spectrometry, the reaction was washed with water and extracted with DCM, dried with Na_2SO_4, and concentrated under reduced pressure. Column chromatography (5-10% MeOH/DCM) yielded Compound **5** (10 mg, 0.012 mmol,

70%) as a blue solid. ^{1}H NMR (500 MHz, CD_3OD): δ 7.93 (d, 1H, *J* = 15.4 Hz), 7.52-7.58 (m, 3H), 7.36 (d, 2H, *J* = 2.8 Hz), 7.20-7.24 (m, 2H), 7.09 (d, 2H, *J* = 9.7 Hz), 6.79-6.81 (m, 3H), 3.59-3.81 (m, 8H), 3.34 (s, 12H), 3.26 (s, 3H), 3.21 (s, 1 H), 2.08 (s, 3H), 2.05 (s, 1H), 1.69-1.97 (m, 12H), 0.60 (s, 3H), 0.59 (s, 3H); ^{13}C NMR (125 MHz, CD_3OD): δ 170.58, 167.50, 166.95, 154.49, 151.99, 148.22, 140.73, 140.14, 139.30, 138.29, 136.37, 132.82, 131.14, 130.54, 127.58, 127.42, 127.02, 125.82, 123.77, 122.88, 121.99, 121.04, 114.11, 55.94, 39.64, 38.82, 38.70, 38.40, 38.25, 36.83, 33.03, 29.69, 28.51, 28.37, 18.13, -2.36, -2.68. HRMS calcd for $C_{52}H_{60}ClN_4O_4Si^+$ $[M+H]^+$ 867.4067, found 867.4045.

***N*-(10-(5-(4-((E)-3-(3-chloro-2-hydroxy-4-((1R,2r,3S,5r,7s)-4'-methoxyspiro[adamantane-2,3'-[1,2]dioxetane]-4'-yl)phenyl)acryloyl)piperazine-1-carbonyl)-2-methylphenyl)-7-(dimethylamino)-5,5-dimethyldibenzo[b,e]silin-3(5H)-ylidene)-*N*-methylmethanaminium (SiRCL-1).** In a round bottom flask, compound **5** (30 mg, 0.035 mmol), was dissolved in 2 mL DCM at 0 °C. 1 mg of methylene blue was added. The reaction was irradiated under yellow light for 3 hr while stirring and monitored by ESI-MS. Upon completion, the crude was evaporated onto silica gel and loaded for column chromatography in 5-10% MeOH/DCM, yielding **SiRCL-1** (18 mg, 57%) as a blue solid. ^{1}H NMR (500 MHz, CD_3OD): δ 7.93 (d, 1H, *J* = 15.4 Hz), 7.70 (br s, 1 H), 7.53-7.70 (m, 4H), 7.36 (d, 2H, *J* = 2.8 Hz), 7.24-7.29 (m, 2H), 7.09 (d, 2H, *J* = 9.1), 6.79 (dd, 2H, *J1* = 2.3, *J2* = 9.2), 3.60-3.82 (m, 8H), 3.34 (s, 12H), 3.16 (s, 3H), 2.93 (s, 1 H), 2.09 (s, 3H), 1.70-2.06 (m, 13H), 0.59 (s, 3H), 0.58 (s, 3H) ; ^{13}C NMR (125 MHz, CD_3OD): δ 170.59, 167.59, 166.11, 154.49, 152.58, 148.22, 140.72, 139.36, 138.29, 133.72, 132.78, 130.53, 127.62, 127.42, 126.93, 123.66, 121.34, 121.03, 120.40, 118.99, 114.09, 111.62, 95.92, 51.67,, 39.61,, 33.65, 31.85, 31.73, 31.56, 31.32, 29.41, 29.13, 26.35, 26.00, 18.11, -2.38, -2.70. HRMS calcd for $C_{52}H_{60}ClN_4O_6Si^+$ $[M+H]^+$ 899.3965, found 899.3924.

1.2 *In vitro* spectroscopic measurements

1.2.1 General Spectroscopic Methods.

Fluorescence, chemiluminescence, and absorbance spectra were acquired on a HORIBA QM-8075-11 spectrophotometer in a 1.6 mL quartz cuvette (Starna Cells, Atascadero, CA). Each experiment was independently conducted in triplicate at ambient temperature. Background readings were acquired and subtracted from sample spectra. Acquisition of spectra was started approximately 5–10 sec after mixing of reagents.

1.2.2 SiRCL-1 pH dependence.

A solution of 20 µM **SiRCL-1** in 30% DMSO and pH 3 buffer (100 mM sodium citrate, pH = 2.98) was prepared in a cuvette, which was gently shaken to ensure mixing. Emission was then collected from 400–800 nm with excitation slits at 0 nm, emission slits at 15 nm, and integration time of 0.1 s. The process was repeated with buffers at pH 4 (100 mM sodium citrate, pH = 4.00), pH 5 (100 mM sodium citrate, pH = 4.97), pH 6 (100 mM PBS, pH = 5.85), pH 7.4 (100 mM PBS, pH = 7.45), pH 8 (50 mM Tris and 10 mM NaCl, pH = 7.93), pH 9 (100 mM Tris, pH = 8.98), and pH 12 (100 mM NaOH, pH = 12).

1.2.3 SiRCL-1 kinetic decay and half-life determination.

A solution of 20 µM **SiRCL-1** in 30% DMSO and pH 7.4 buffer (100 mM PBS, pH = 7.45) was prepared in a cuvette, which was gently shaken to ensure mixing. Emission from 400–800 nm was then collected at time points of 0, 1, 2, 3, 4, 5, 10, 20 and 30 min after mixing with excitation slits at 0 nm, emission slits at 15 nm, and integration time of 0.1 s. The intensities at 540 nm and 680 nm were plotted against time to generate decay curves and the decay constant *k* was determined by mathematically fitting the experimental data to a model for exponential decay (Eq 1). Half-life was calculated using Eq 2.

$$I_t = I_0\, e^{-kt} + B \qquad \text{(Eq 1)}$$
$$t_{1/2} = \ln(2)/k \qquad \text{(Eq 2)}$$

1.3 Quantum yield determination.

The CL quantum yields (F_{CL}) were determined using a variation of the methodology previously reported using a luminol calibration standard.[3,4] Eq 3 summarizes calculation of F_{SiRCL}, the chemiluminescent quantum yield of SiRCL-1 (Eq 3). Q_{SiRCL} is the total light emission from **SiRCL-1**, obtained by integrating the kinetic profile of 20 µM **SiRCL-1** chemiluminescent decay in PBS (pH = 7.4) with 30% DMSO over 3600 s at 680 nm. n_{SiRCL} is the number of moles of **SiRCL-1**. F_{lum} is the calibration factor to convert the total light emission of the sample from arbitrary units to Einstein units, calculated using the chemiluminescent standard luminol and Equation 4, where F_{lum} is the reference chemiluminescence quantum yield of luminol (0.0114 ± 0.0006 E mol-1), Q_{lum} is the integrated emission intensity under a kinetic emission profile of luminol in PBS at pH 11.6 with consecutive additions of hemin,

and n_{lum} is the number of moles of luminol used. F_{spect} is the spectral correction factor, calculated as the light that passes through the wavelength selective monochromator at a particular wavelength (integrated intensity from [λ − ½ slits] to [λ + ½ slits]) as a fraction of the total light emission of the sample across all wavelengths (integrated intensity from 400 to 800 nm) (Equation 5). This accounts for the differences in spectral shape between luminol emission and **SiRCL-1** emission. $F_{spect(lum)}$ was calculated using luminol emission spectrum, and $F_{spect(SiRCL)}$ with **SiRCL-1** emission spectrum. With incorporation of F_{spect}, only the chemiluminescence emission decay at a single wavelength needs to be considered for quantum yield calculation despite there being dual emission peaks in the spectrum of **SiRCL-1**. To account for the sensitivity of the photomultiplier tube detector at different wavelengths, we enabled real-time corrections on the instrument which automatically correct the raw data based on the manufacturer provided correction factors at each wavelength. These corrected traces were used in all calculations.

$$\Phi_{\mathrm{SiRCL}} = \frac{Q_{SiRCL} f_{lum} F_{spect(lum)}}{n_{SiRCL} F_{spect(SiRCL)}} \quad \text{(Eq 3)}$$

$$f_{lum} = \frac{\phi_{lum} n_{lum}}{Q_{lum}} \quad \text{(Eq 4)}$$

$$F_{spect} = \frac{\int_{\lambda - slit/2}^{\lambda + slit/2} S(\lambda) d\lambda}{\int_{\lambda_i}^{\lambda_f} S(\lambda) d\lambda} \quad \text{(Eq 5)}$$

1.4 Tissue depth penetration.

20 µL of a solution of 100 µM **SiRCL-1** in PBS buffer (100 mM PBS, pH = 7.45) with 30% DMSO were poured in 96-well white opaque plates and observed using IVIS Spectrum (Perkin-Elmer, Waltham, MA) in 'Luminescent' and 'Photograph' modes. The exposure time was set to 10 s, binning was set to medium, FOV was set to C (12.9 cm), excitation was blocked. The chemiluminescence light intensity of **SiRCL-1** was measured using 540 and 680 nm bandpass filters. Layers of commercially processed avian tissue (turkey- Walmart, Dallas, TX) were used to mimic animal tissue. The layers of turkey (0 to 20 layers, each being about 1.54 mm thick measured using Vernier caliper) were placed over selected wells. Regions of interest (ROIs) on the images were marked and measured to give the total flux for each wavelength. Experiments were replicated thrice.

1.5 *In-vivo imaging.*

The kinetics were investigated using IVIS Spectrum (Perkin-Elmer, Waltham, MA) in emission mode with capture set to autoexposure and FOV set to C (12.9 cm). Nude mice (3-month-old, strain 69 athymic nude, NCl Grantee, Envigo) were anesthetized and injected in the intraperitoneal cavity with 20 µL of 100 µM **SiRCL-1** (100 mM PBS, pH 7.45, 10% DMSO) and then immediately imaged. A total of n = 6 mice were injected and imaged in two independent experiments with n = 3 mice each. Raw images are shown in Figures S3 and S4. The investigation was approved by the UT Southwestern Institutional Animal Care and Use Committee under Animal Protocol Number APN 2017-102329.

2. Figures

Figure S1. Molecular structure of the model energy donor species, methyl acrylate dioxetane, used to estimate the spectrum of the energy transfer donor.

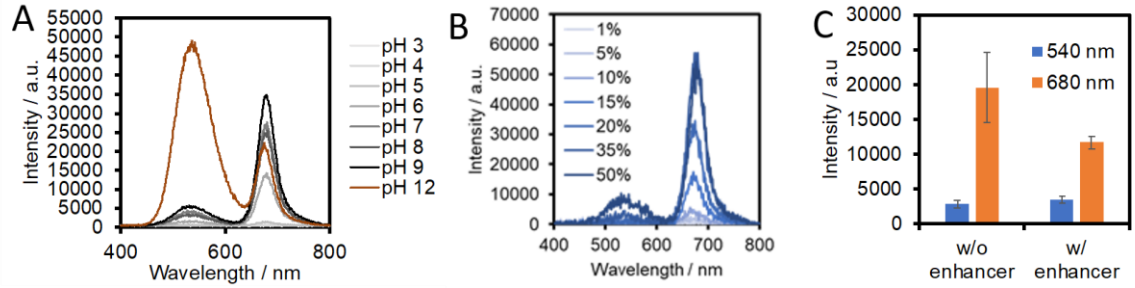
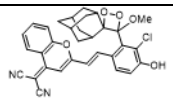
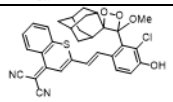
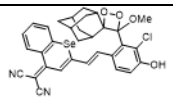
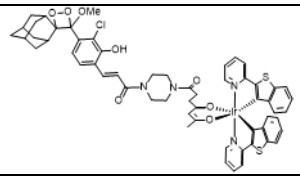


Figure S2. (A) Chemiluminescence emission traces of 20 µM **SiRCL-1** in 30% DMSO and pH buffers between pH 3 and 12. (B) Chemiluminescence emission traces of 20 µM **SiRCL-1** in varying DMSO concentration at pH 7.4 (C) Chemiluminescence emission of **SiRCL-1** at 540 nm and 680 nm without and with the addition of 5% Emerald II enhancer in PBS 7.4 with 30% DMSO.

Table S1. Summary of photophysical properties of some reported NIR 1,2-dioxetane-based chemiluminescent probes (>600 nm) along with **SiRCL-1**

Probe name	Probe Structure	λ max $_{CL}$ (nm)	Ø $_{CL}$ x 10^{-3} (Einsteins/mol)	Ref.
DPD-O		660	8.2	J. Am. Chem. Soc. **2017**,139, 13243-13248.
DPD-S		760	2.3	Angew. Chem. Int. Ed. **2021**, 60, 3999-4003.
DPD-Se		780	1.2	
IrCL-1		615	6.6±0.1 (air) 10.2±0.1 (N_2)	Angew. Chem. Int. Ed. **2022**, 61, e202115704.

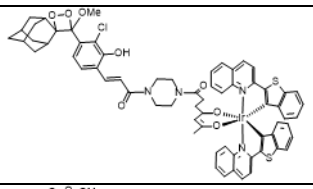
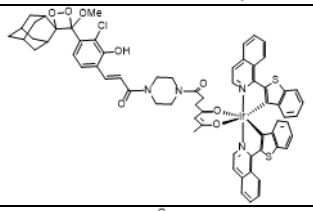
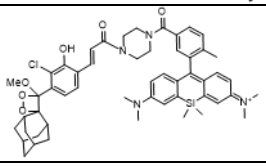
IrCL-2		668	7.3±0.7 (air) 12.4±0.4 (N_2)	Angew. Chem. Int. Ed. **2022**, 61, e202115704.
IrCL-3		705	7.2±0.2 (air) 7.5±0.1 (N_2)	
SiRCL-1		680	3.9 ± 0.81	This work

Figure S3. Raw chemiluminescent images for the first independent experiment in Figure 4B (n = 3 mice). Sequence of chemiluminescence images of living female mice acquired after intraperitoneal cavity injection of **SiRCL-1** (100 µM in 10% DMSO-PBS buffer solution (10 mM, pH=7.4), total volume = 20 µL) at 680 nm in an IVIS Spectrum. Each frame is 30 seconds apart. Odd frames use a 540 nm filter and even frames use a 680 nm filter.

Figure S4. Raw chemiluminescent images for the second independent experiment in Figure 4B (n = 3 mice). Sequence of chemiluminescence images of living female mice acquired after intraperitoneal cavity injection of **SiRCL-1** (100 µM in 10% DMSO-PBS buffer solution (10 mM, pH=7.4), total volume = 20 µL) at 680 nm in an IVIS Spectrum. Each frame is 30 seconds apart. Odd frames use a 540 nm filter and even frames use a 680 nm filter.

3. Spectra

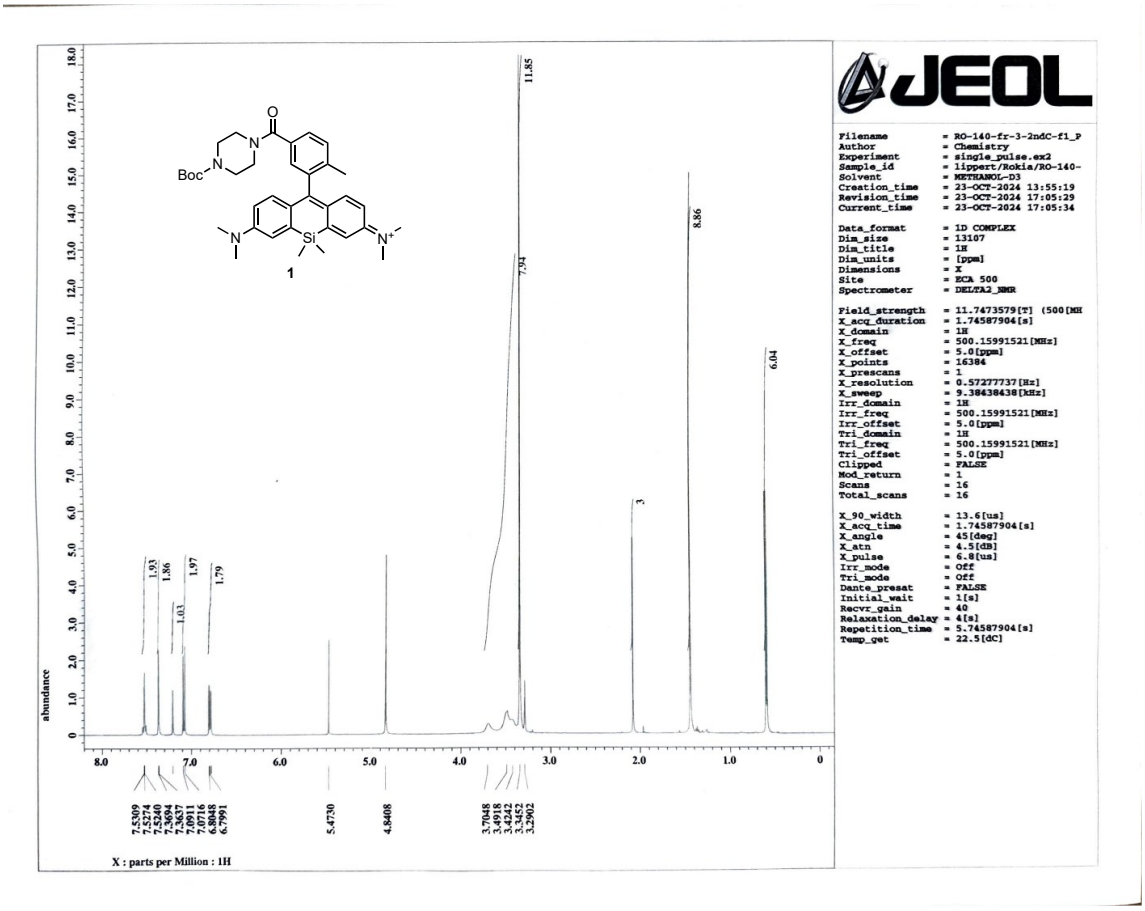


Figure S5. 1H-NMR spectrum (500 MHz, CD_3OD) of compound 1

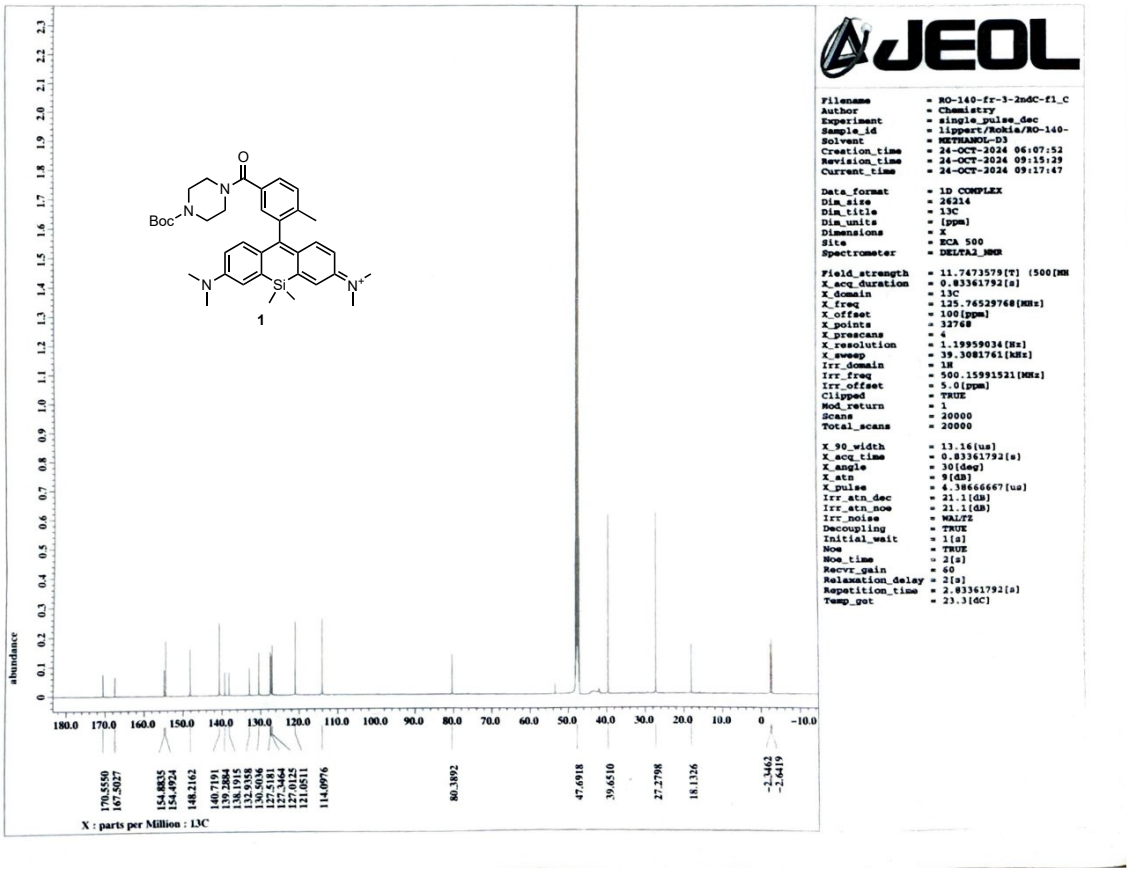


Figure S6. 13C-NMR spectrum (125 MHz, CD_3OD) of compound 1

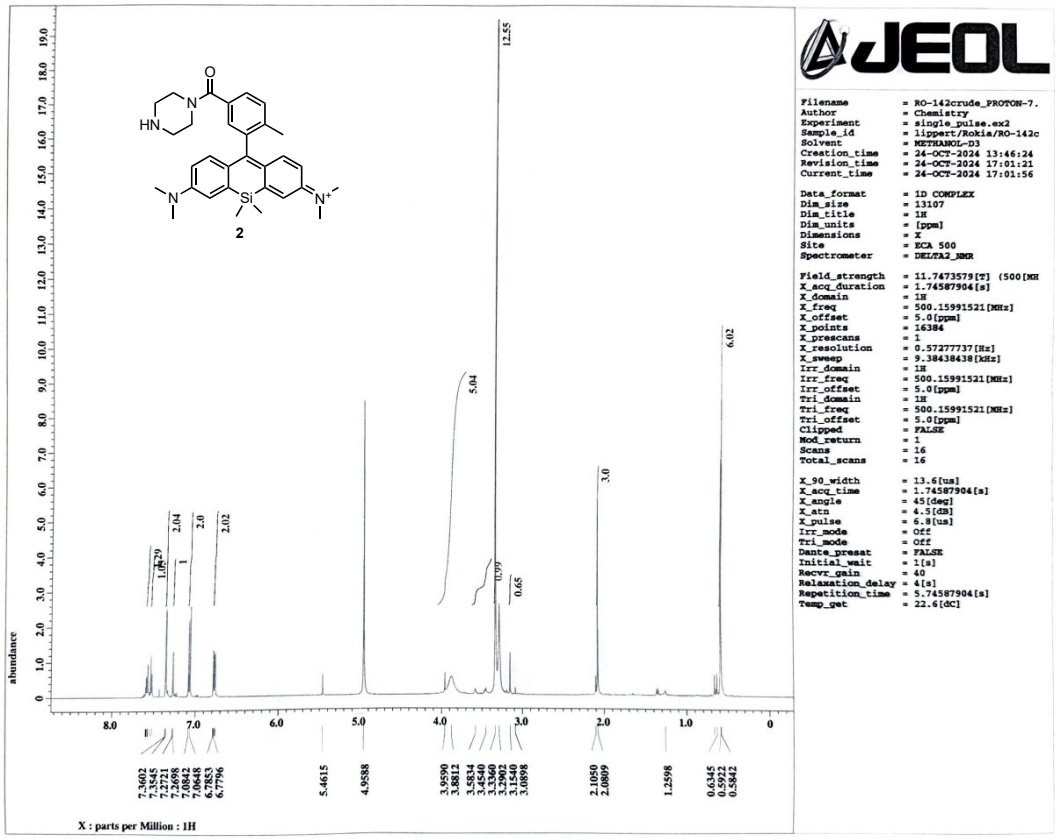


Figure S7. 1H-NMR spectrum (500 MHz, CD3OD) of compound 2

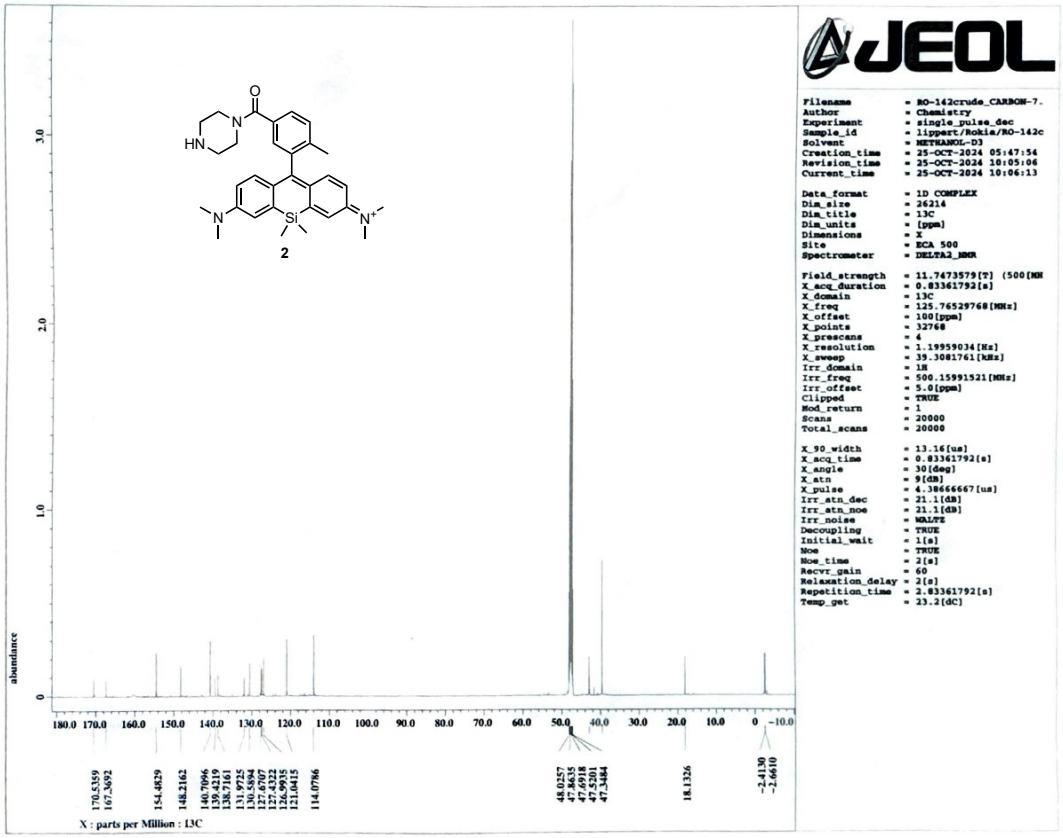


Figure S8. 13C-NMR spectrum (125 MHz, CD_3OD) of compound 2

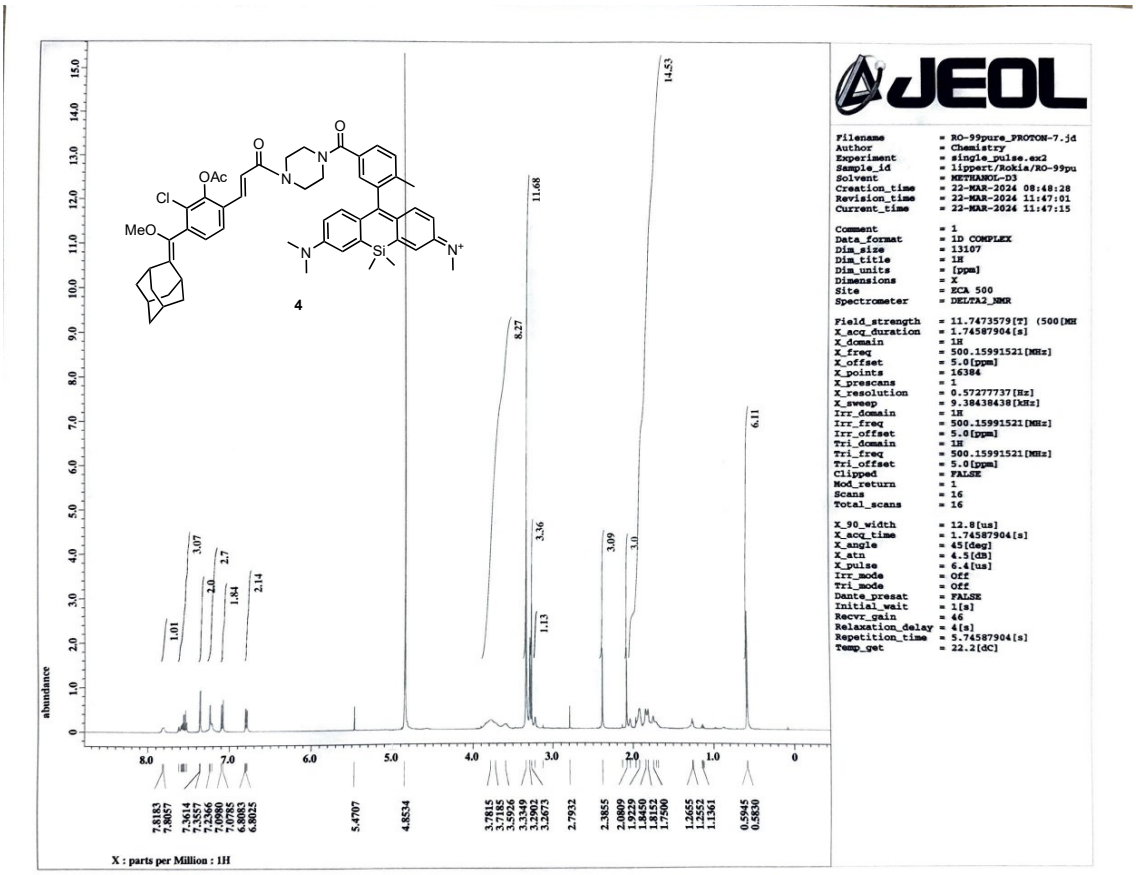


Figure S9. 1H-NMR spectrum (500 MHz, CD_3OD) of compound 4

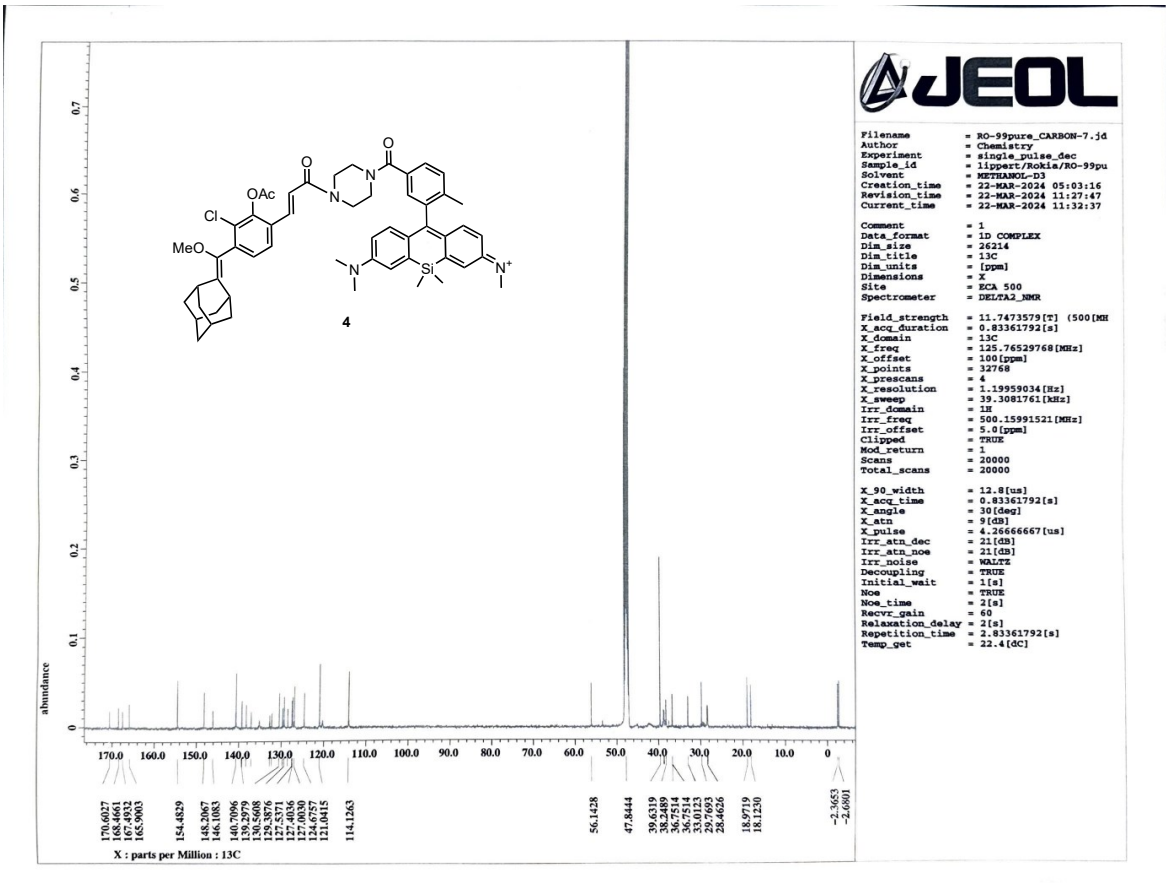


Figure S10. 13C-NMR spectrum (125 MHz, CD_3OD) of compound 4

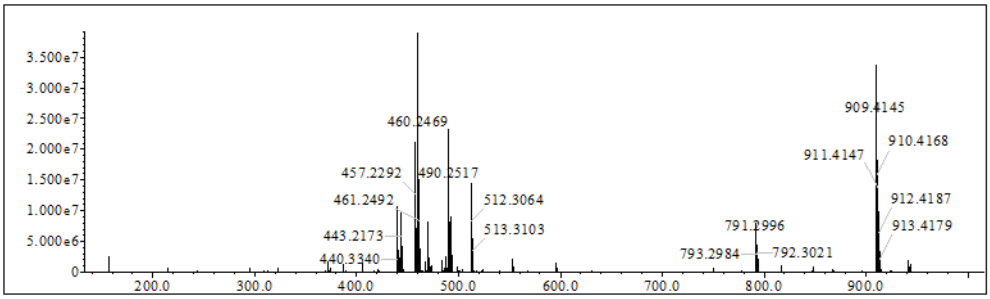


Figure S11. HRMS of compound 4

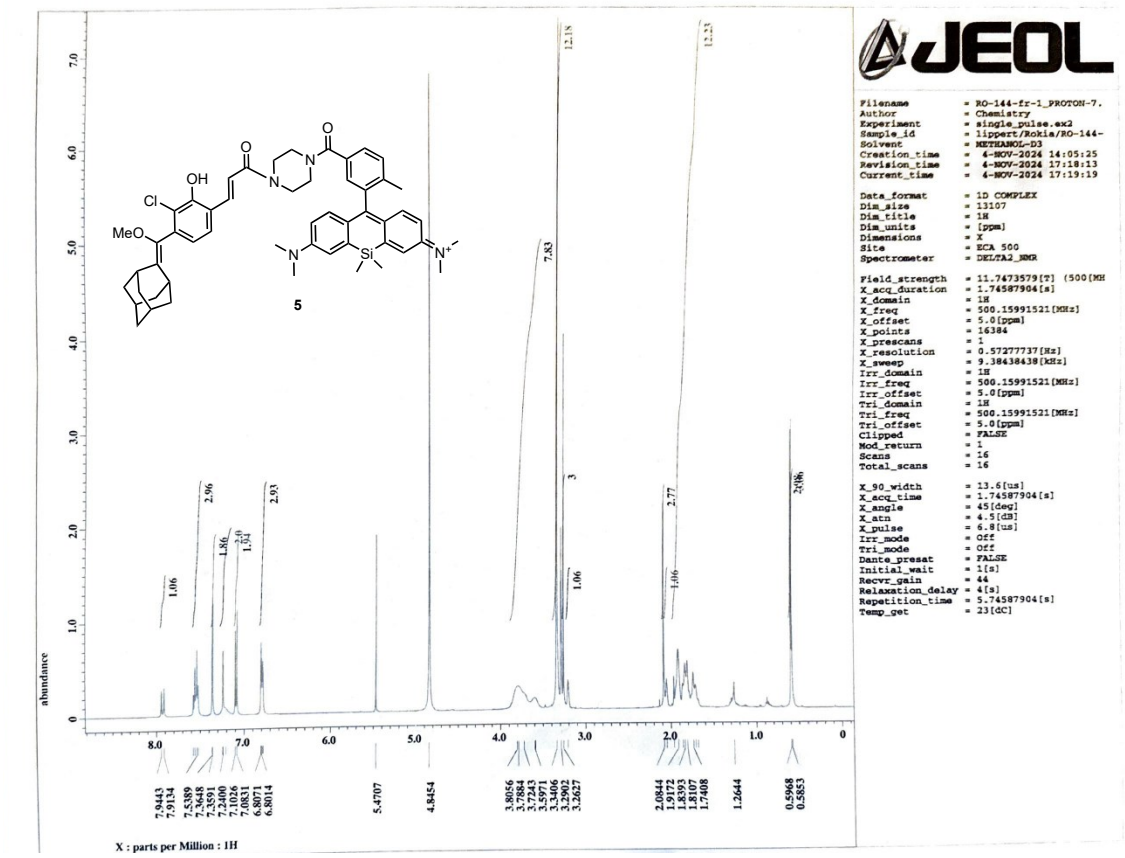


Figure S12. 1H-NMR spectrum (500 MHz, CD_3OD) of compound 5

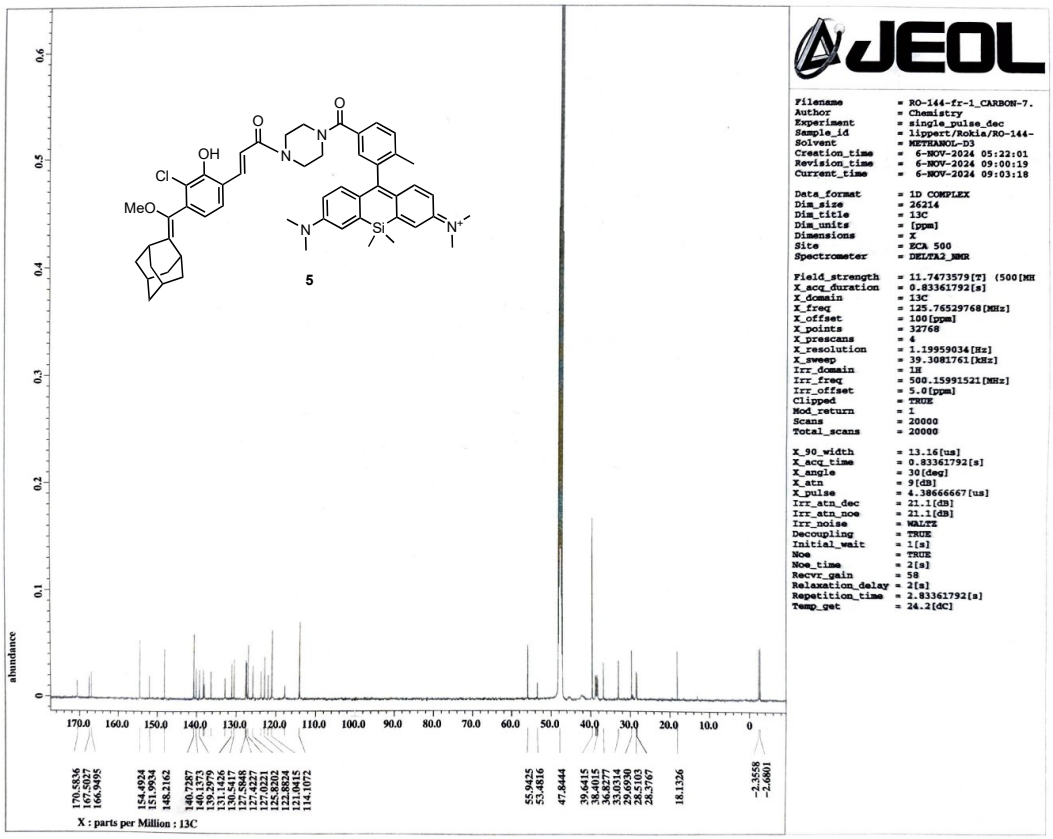


Figure S13. 13C-NMR spectrum (125 MHz, CD_3OD) of compound 5

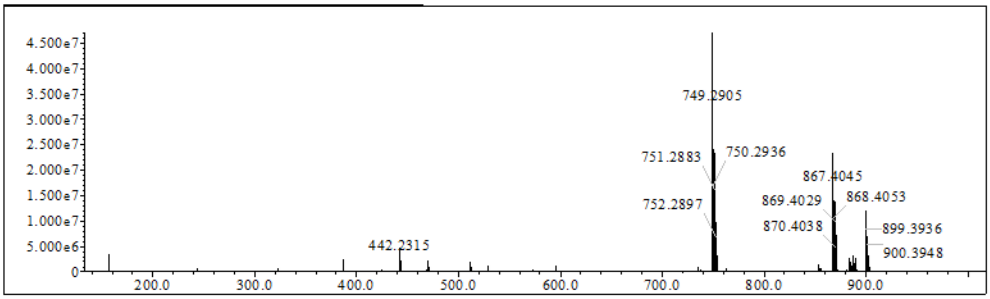


Figure S14. HRMS of compound 5

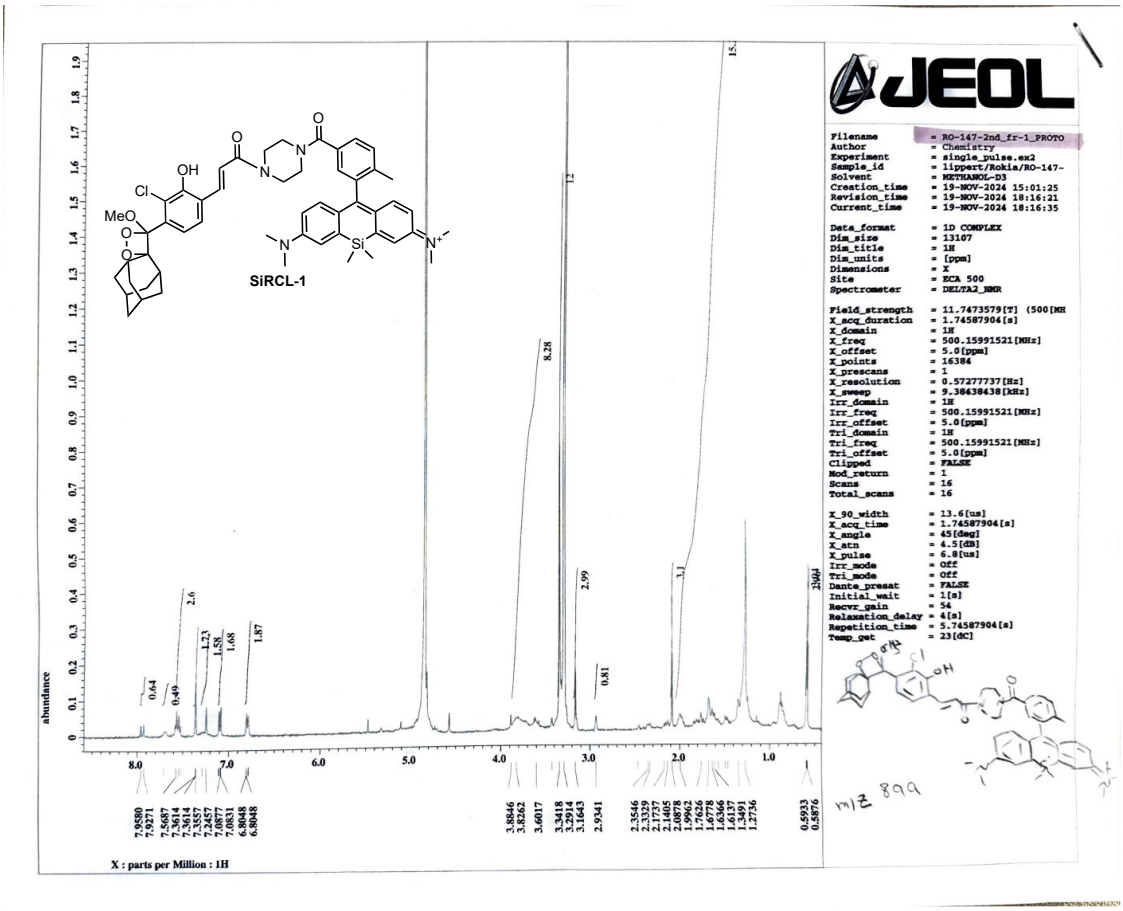


Figure S15. 1H-NMR spectrum (500 MHz, CD_3OD) of SiRCL-1

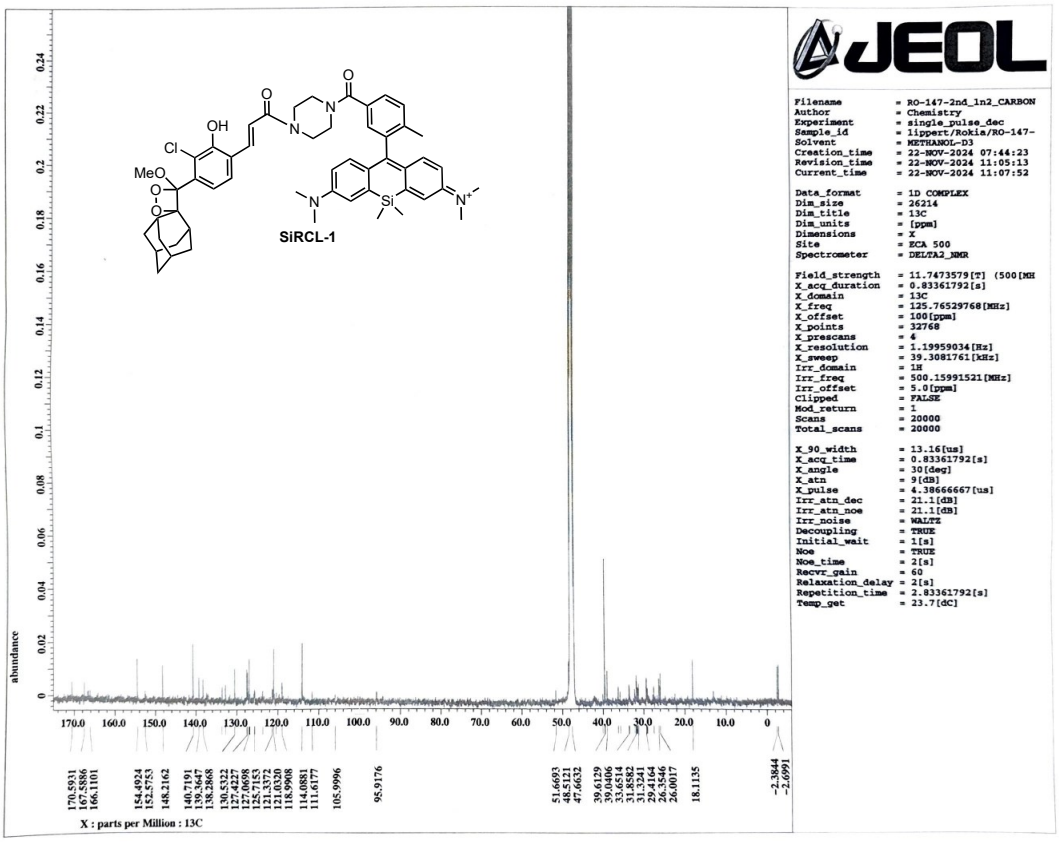


Figure S16. 13C-NMR spectrum (125 MHz, CD_3OD) of SiRCL-1

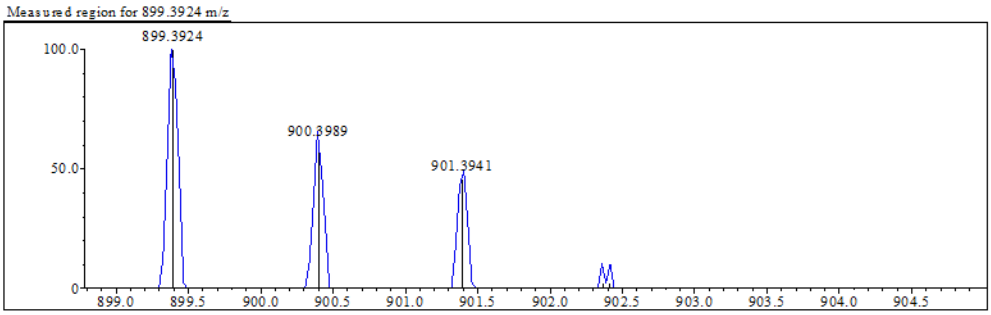


Figure S17. HRMS of SiRCL-1

4. References

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