Supporting Information

Dark Dynamic Therapy: Photosensitization without Light Excitation using Chemiluminescence Resonance Energy Transfer in a Dioxetane-Erythrosin B Conjugate

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1. Materials and General Methods

All chemicals and instruments were obtained from commercial suppliers. NMR data was acquired on a 400 MHz Bruker Avance III NMR Spectrometer or 500 MHz Agilent DD2 NMR Spectrometer at the University of Toronto. HRMS data was acquired on an Agilent 6538 UHD at the University of Toronto and at the Shimadzu Center for Advanced Analytical Chemistry based at the University of Texas, Arlington. For compounds, **CL-E1a**, **CL-E1**, **CL-E2**, and **NTR-CL-E1**, ammonium acetate was used as the mobile phase for HRMS to minimize deiodination[1]. *In vitro* assays were performed in PBS pH 7.4 (1X) containing no calcium chloride or magnesium chloride and DMSO (purchased from Fisher Scientific). UV-Vis absorption spectra were recorded on a Shimadzu UV-1800 spectrometer and fluorescence experiments performed on a Shimadzu RF-6000 spectrometer (purchased from Mandel Scientific Company Inc.). A 1.0 cm path length quartz cuvette (purchased from Starna Scientific Ltd.) was used for both instruments. Luminescence and absorbance measurements were performed on Tecan Spark 20M (purchased from Tecan Austria GmbH). Statistical analyses performed using GraphPad Prism. Dioxetane formation reactions were conducted using a mounted 625 nm LED (17 nm bandwidth, 920 mW LED Output Power; 21.9 μW mm^{-2} maximum irradiance; M625L4) purchased from ThorLabs. After obtaining NMRs of compounds containing a dioxetane, samples were repurified by RP-HPLC to ensure all experiments were conducted with 100% intact dioxetane. General ROS sensor abbreviated as: 2',7'-dichlorodihydrofluorescin diacetate ($DCFH_2$-DA) (purchased from Sigma Aldrich), 2',7'-dichlorodihydrofluorescin ($DCFH_2$) (prepared in 0.1 M $NaOH_{(aq)}$), and 2',7'-dichlorofluorescein (DCF) (purchased from Sigma Aldrich). MCF7 and A549 cells were purchased from American Type Culture Collection (ATCC) and MDA-MB231 triple-negative breast cancer cells without and with stable NTR expression were provided from Dr. Ramasamy Paulmurugan's Lab from Stanford University School of Medicine[2].

2. Synthetic Procedures

CL-OMe: Synthesized from previously reported procedures[3,4]. ^{1}H NMR (400 MHz, chloroform-*d*) $\boldsymbol{\delta}$ 7.94 (d, 16.1 Hz, 1H), 7.43 (d, 8.0 Hz, 1H), 6.88 (d, 1.5 Hz, 1H), 6.81 (s, 1H), 6.61 (d, 16.1 Hz, 1H), 5.78 (s, 1H), 3.81 (s, 3H), 3.31 (s, 3H), 3.24 (s, 3H) 2.70 (s, 1H), 1.97-1.94 (m, 12H). ^{13}C NMR (400 MHz, chloroform-*d*) $\boldsymbol{\delta}$ 169.06, 156.08, 142.87, 140.87, 139.19, 134.38, 129.30, 122.39, 121.38, 118.31, 117.07, 60.97, 58.47, 52.20, 39.63, 37.51, 32.80, 28.65, 21.51, 14.63. HRMS (ESI+) m/z calculated for $C_{22}H_{26}O_4$: 354.18, $[M+H]^+$ found at 355.1905. See Scheme S1 for structure and Figure S27, S28, and S29 for characterization.

CL-COOH: CL-OMe (1 eq., 0.080 g, 0.22 mmol) was dissolved in 1:1 THF/H_2O (3.7 mL) containing NaOH (2 eq., 0.011 g, 0.44 mmol). The solution turned to bright yellow following addition of base. The reaction mixture was then stirred at 80 °C and hydrolysis of the methyl ester was monitored by TLC (approx. 4 h for completion). The crude mixture was warmed to r.t., diluted with EtOAc, and washed with 0.1 M $HCl_{(aq)}$ and brine. The organic layer was dried with sodium sulfate and the solvent was removed under reduced pressure. **CL-COOH** was purified by silica chromatography in 50:50 hexanes/EtOAc to give 0.046 g (0.14 mmol) an off-white solid (62% yield). ^{1}H NMR (400 MHz, d_6-DMSO) $\boldsymbol{\delta}$ 12.21 (s, 1H), 10.22 (s, 1H), 7.82 (d, 16.3 Hz, 1H), 7.55 (d, 7.8 Hz, 1H), 6.85 (s, 1H), 6.74 (d, 8.1 Hz, 1H), 6.49 (d, 16.1 Hz, 1H), 3.12 (s, 3H), 3.15 (s, 1H), 2.64 (s, 1H) 1.94-1.65 (m, 12H). ^{13}C NMR (400 MHz, d_6-DMSO) $\boldsymbol{\delta}$ 168.30, 156.58, 143.05, 139.37, 138.16, 131.86, 128.65, 120.48, 120.29, 118.42, 116.47, 59.97, 57.64, 36.77, 32.01, 29.95, 27.84, 20.96, 14.29. HRMS (ESI+) m/z calculated for $C_{21}H_{24}O_4$: 340.17, $[M+H]^+$ found at 341.1747. See Scheme S1 and Figure S30, S31, and S32 for characterization.

EryB-Linker: Erythrosin B (purchased from Sigma Aldrich) (1 eq., 1.00 g, 1.20 mmol) was added to a round-bottom flask and purged under an argon environment. Anhydrous DMF (8 mL) was added, followed by

the addition of potassium carbonate (3 eq., 0.498 g, 3.60 mmol) and stirred for 5 min. at r.t. The mixture was then cooled to 0 °C and EDC (1.2 eq., 0.223 g, 1.44 mmol) was added and the mixture was stirred for 10 min. with the temperature maintained at 0 °C. *N,N'*-dimethylethylenediamine (3 eq., 0.39 mL, 3.60 mmol) was added dropwise and the reaction mixture was stirred at r.t. overnight. After solvent removal under reduced pressure, the crude was purified by silica gel chromatography (column equilibrated with 2% acetic acid) using a gradient of DCM to 20% MeOH. The product eluted in 20% MeOH (bright red band) to give 0.421 g (0.47 mmol) compound **EryB-Linker** (39% yield) as a bright pink solid. ^{1}H NMR (400 MHz, *d*$_6$-DMSO) $\boldsymbol{\delta}$ 7.68 (m, 3H), 7.52 (m, 1H) 7.30 (s, 2H), 3.56 (s, 3H), 3.24 (t, 2H), 2.99 (s, 3H), 2.88 (s, 2H).^{13}C NMR (400 MHz, *d*$_6$-DMSO) $\boldsymbol{\delta}$ 171.97, 159.29, 157.48, 155.52, 146.54, 138.34, 130.08, 129.07, 128.38, 111.29, 96.22, 76.28, 53.81, 49.51, 44.06, 35.54, 34.96, 24.25, 16.13, 14.49. HRMS (ESI+) m/z calculated for $C_{24}H_{18}I_4N_2O_4$: 905.74, $[M+H]^+$ found at 906.7514. See Scheme S2 and Figure S33, S34, and S35 for characterization.

CL-E1a: CL-COOH (1 eq., 0.070 g, 0.21 mmol) was added to a round-bottom flask and purged under an argon environment and then dissolved in anhydrous DMF (0.25 mL). DIPEA (3 eq., 0.11 mL, 0.63 mmol) was added and the reaction turned yellow and stirred for 2 min. at r.t. Next, DCC (1.1 eq., 0.048 g, 0.23 mmol) and DMAP (10 mol-%, 0.003 g, 0.021 mmol) were added to the mixture and stirred at 0 °C for 10 min., where it turned from yellow to orange. After activation of the carboxylic acid, **EryB-Linker** (2 eq., 0.380 g, 0.42 mmol) in anhydrous DMF (0.25 mL) was added dropwise to the reaction mixture. The mixture turned bright pink and was left to stir overnight at 50 °C and protected from light. After removing the DMF under reduced pressure, the crude product was purified by silica chromatography using a gradient of $CHCl_3$ to 10% MeOH (product elutes in 5% MeOH) in a red-pink band to yield 0.030 g (0.024 mmol) of **CL-E1a** (12% yield) as a bright pink oil. ^{1}H NMR (500 MHz, *d*$_4$*-methanol*) $\boldsymbol{\delta}$ 7.96 (d, 17.6 Hz, 1H), 7.76 (m, 1H), 7.68 (m, 1H), 7.59 (m, 2H), 7.49 (d, 8.0 Hz, 1H), 7.23 (d, 8.0 Hz, 1H), 7.07 (s, 1H), 6.82 (s, 2H), 6.56 (d, 17.6 Hz, 1H), 3.30 (s, 3H), 3.21 (m, 3H), 3.15 (s, 1H), 2.99 (d, 10.1 Hz, 2H), 2.69 (s, 1H), 1.97-1.86 (m, 12H). ^{13}C NMR (500 MHz, *d*$_4$*-methanol*) $\boldsymbol{\delta}$ 171.02, 159.74, 158.19, 150.80, 144.78, 143.99, 142.06, 140.15, 135.22, 133.85, 129.88, 128.46, 125.05, 122.27, 122.04, 117.78, 66.51, 59.71, 58.57, 58.34, 40.39, 38.39, 35.89, 33.95, 33.25, 31.82, 30.95, 30.92, 30.64, 29.93, 29.90, 27.74, 27.19, 24.96, 23.91, 20.90, 14.60, 14.10. HRMS (ESI+) m/z calculated for $C_{45}H_{40}I_4N_2O_7$: 1227.90, [M+] found at 1227.0897. See Scheme S3 and Figure S36, S37 and S38 for characterization.

CL-E1: CL-E1a was dissolved in DCM (2 mL) and transferred to a 2-neck round-bottom flask. A catalytic amount (1 mg approx.) of methylene blue (purchased from Sigma Aldrich) was added and the mixture cooled to 0 °C on ice. Oxygen gas was then bubbled into the reaction mixture while being irradiated with a 625 nm LED for 2 hours, while maintaining the temperature at 0 °C. The solvent from the crude reaction mixture was removed under reduced pressure and purified first by silica chromatography in 90:10 EtOAc/MeOH and then using RP-HPLC in ACN (0.1% formic acid). **CL-E1** elutes at 20 min. in 100% ACN (45 min. method, 10 min. gradient from 5-100% ACN and then hold 100% ACN for 15 min.). **CL-E1** was obtained in 69% yield (97% purity). ^{1}H NMR (500 MHz, chloroform-*d*) $\boldsymbol{\delta}$ 7.80 (d, 15.38 Hz, 1H), 7.72 (m, 1H), 7.67 (m, 1H), 7.63 (m, 1H), 7.59 (m, 1H), 7.55 (t, 1H), 7.52 (s, 1H), 7.44 (m, 2H), 7.42 (m, 1H), 7.22 (d, 15.38 Hz, 1H), 3.38 (d, 2H), 3.28 (s, 6H), 3.13 (s, 1H), 3.00 (s, 3H), 1.85-2.24 (m, 16H); We note that during the NMR acquisition period of 1 hour we observed the appearance of the benzoate ester peak at 4.02 ppm, corresponding to the expected Schaaps CL breakdown product. ^{13}C NMR (500 MHz, chloroform-*d*) $\boldsymbol{\delta}$ 173.68, 172.90, 170.19, 157.77, 143.69, 143.00, 141.03, 138.43, 131.82, 128.75, 128.39, 126.34, 124.56, 121.29, 120.39, 118.88, 118.34, 117.54, 110.65, 103.53, 95.97, 58.69, 57.87, 52.87, 48.26, 37.46, 36.11, 32.49, 30.42, 29.91, 28.59, 28.23, 21.57. HRMS (ESI+) m/z calculated for $C_{45}H_{40}I_4N_2O_9$: 1259.89, [M+] found at 1258.8930. We also observe the expected methyl-ester breakdown product commonly produced in MS

for unprotected dioxetanes. Calculated for $C_{35}H_{26}I_4N_2O_8$: 1109.79, $[M-H]^-$ found at 1108.78. See Scheme S3 and Figure S39, S40, S41, S42 and S43 for characterization.

CL-Aa: CL-COOH (1 eq., 0.100 g, 0.29 mmol) was added to a round-bottom flask and purged under an argon environment and then dissolved in anhydrous DMF (0.5 mL). TEA (3 eq., 0.12 mL, 0.88 mmol) was added and the reaction turned yellow and stirred for 2 min. at r.t. Next, PyBOP (2 eq., 0.302 g, 0.58 mmol) was added to the mixture and stirred at r.t. for 10 min., where it turned from yellow to red. After activation of the carboxylic acid, dimethylamine in 2.0 M THF (2 eq., 0.29 mL, 0.58 mmol) was added dropwise to the reaction mixture. The mixture was left to stir overnight at 40 °C and protected from light. The mixture turned light yellow after an overnight reaction. After removing the DMF under reduced pressure, **CL-Aa** was purified first by silica chromatography in EtOAc and then via RP-HPLC in ACN (0.1% formic acid). **CL-Aa** elutes at 39 min. peak in 100% ACN (45 min. method, 10 min. gradient from 5-100% ACN and then hold 100% ACN for 15 min.). to yield 0.021 g (0.06 mmol) of **CL-Aa** (20% yield) as an off white solid. ^{1}H NMR (500 MHz, *d*$_6$-DMSO) $\boldsymbol{\delta}$ 10.10 (s, 1H), 7.69 (d, 15.5 Hz, 1H), 7.64 (d, 18.0 Hz, H), 7.15 (d, 15.5 Hz, H), 6.82 (s, 1H), 6.71 (dd, 7.9 Hz, 1H), 3.22 (s, 3H), 3.16 (s, 1H), 3.12 (s, 3H), 2.92 (s, 3H), 2.63 (s, 1H), 1.95-1.69 (m, 12H). ^{13}C NMR (500 MHz, *d*$_6$-DMSO) $\boldsymbol{\delta}$ 166.19, 156.11, 143.11, 137.21, 136.20, 131.33, 128.08, 121.47, 120.06, 117.60, 116.37, 57.52, 47.47, 36.97, 36.74, 35.46, 31.95, 29.85, 27.78, 25.83. HRMS (ESI-) m/z calculated for $C_{23}H_{29}NO_3$: 367.21, $[M-H]^-$ found at 366.2077. See Scheme S4 and Figure S44, S45, S46 and S47 for characterization.

CL-A: CL-Aa was dissolved in DCM (2 mL) and transferred to a 2-neck round-bottom flask. A catalytic amount (1 mg approx.) of methylene blue (purchased from Sigma Aldrich) was added and the mixture cooled to 0 °C on ice. Oxygen gas was then bubbled into the reaction mixture while being irradiated with a 625 nm LED for 2 hours, while maintaining the temperature at 0 °C. The solvent from the crude reaction mixture was removed under reduced pressure and purified first by silica chromatography in 50:50 EtOAc/hexanes and then using RP-HPLC in ACN (0.1% formic acid). **CL-A** elutes at 26 min. peak in 100% ACN (45 min. method, 10 min. gradient from 5-100% ACN and then hold 100% ACN for 15 min.). **CL-A** was obtained in 92% yield (99% purity). ^{1}H NMR (500 MHz, *d*$_6$-DMSO) $\boldsymbol{\delta}$ 10.28 (s, 1H), 7.76 (d, 1H), 7.69 (d, 1H), 7.49 (d, 1.7 Hz, 1H), 7.38 (dd, 8.1 Hz, 1H), 7.19 (d, 15.6 Hz, 1H), 3.14 (s, 3H), 3.12 (s, 3H), 2.92 (s, 3H), 2.89 (s, 1H), 2.37 (s, 1H), 2.10-1.38 (m, 12H). We note that during the NMR acquisition period, some of the dioxetane has broken down to produce the corresponding benzoate: $\boldsymbol{\delta}$ 10.45 (s, 1H), 7.79 (d, 8.2 Hz, 1H), 7.73 (d, 1H), 7.49 (d, 1.7 Hz, 1H), 7.39 (dd, 8.1 Hz, 1.7 Hz, 1H), 7.25 (d, 15.6 Hz, 1H), 3.83 (s, 3H), 3.14 (s, 3H) and the 2-adamantanone with $\boldsymbol{\delta}$ 2.89 (s, 1H), 2.37 (s, 1H), 2.10-1.38 (m, 12H). ^{13}C NMR (500 MHz, *d*$_6$-DMSO) $\boldsymbol{\delta}$ 165.97, 165.76, 156.11, 136.34, 131.05, 129.78, 128.54, 126.72, 120.30, 119.97, 119.00, 116.49, 111.49, 94.72, 52.33, 46.47, 36.98, 35.82, 31.92, 28.82, 26.97, 25.52. HRMS (ESI+) m/z calculated for $C_{23}H_{29}NO_5$: 399.20, $[M+H]^+$ found at 400.2114. See Scheme S4 and Figure S48, S49, S50 and S51 for characterization.

CL Benzoate: synthesized from previously reported procedures[3]. ^{1}H NMR (400 MHz, chloroform-*d*) $\boldsymbol{\delta}$ 7.74 (d, 8.3 Hz, 1H), 7.63 (s, 1H), 7.32 (dd, 8.2, 2.0 Hz, 1H), 5.71 (s, 1H), 3.91 (s, 3H). ^{13}C NMR (400 MHz, chloroform-*d*) $\boldsymbol{\delta}$ 166.90, 155.55, 147.95, 139.02, 132.69, 123.48, 116.25, 92.16, 53.80, 52.89. HRMS (ESI-) m/z calculated for $C_8H_7IO_3$: 277.94, $[M-H]^-$ found at 276.9376. See Scheme S8 for structure and Figure S52, S53, and S54 for characterization.

CL-A Benzoate: CL Benzoate (1 eq., 0.150 g, 0.54 mmol) was dried down into a round-bottom flask and purged under an argon environment. Anhydrous ACN (1 mL) was added, followed by the addition of TEA (4.2 eq., 0.32 mL, 2.3 mmol). *N,N*-dimethylacrylamide (3 eq., 0.17 mL, 1.6 mmol) was then added to the reaction mixture dropwise. Palladium (II) acetate (0.05 eq., 0.007 g, 0.03 mmol) and tri(*o*-tolyl)-phosphine

(0.01 eq., 0.002 g, 0.005 mmol) were then added to the flask, where the colour changed from yellow to orange brown. The reaction mixture was transferred to a high-pressure tube using a needle and syringe, and then stirred overnight at 120 ºC under an argon environment. The crude reaction mixture was diluted with EtOAc, washed with brine, dried using sodium sulphate and then had the solvent removed under reduced pressure. **CL-A Benzoate** was purified using silica column chromatography using a gradient of hexanes to EtOAc (product elutes in 100% EtOAc in pale yellow band). **CL-A Benzoate** was obtained as an off-white solid in 32% yield (0.042 g, 0.17 mmol). ^{1}H NMR (400 MHz, d_6-DMSO) $\boldsymbol{\delta}$ 10.45 (s, 1H), 7.78 (dd, 8.1, 1.6 Hz, 1H), 7.71 (d, 15.6 Hz, 1H), 7.50 (s, 1H), 7.40 (dd, 8.1, 1.6 Hz, 1H), 7.25 (d, 15.6 Hz, 1H), 3.83 (s, 3H), 3.14 (s, 3H), 2.93 (s, 3H). ^{13}C NMR (400 MHz, d_6-DMSO) $\boldsymbol{\delta}$ 166.03, 165.83, 156.18, 135.24, 131.12, 128.56, 126.79, 120.33, 120.00, 116.57, 52.38, 37.04, 35.54. HRMS (ESI+) m/z calculated for $C_{13}H_{15}NO_4$: 249.10, $[M+H]^+$ found at 250.1078. See Scheme S8 and Figure S55, S56, and S57 for characterization.

CL-benzyl 1: CL-OMe (1 eq., 0.140 g, 0.40 mmol) was added to a round bottom flask and purged under an argon environment. Anhydrous DMF (5.2 mL) was added, followed by the addition of potassium carbonate (1.5 eq., 0.083 g, 0.60 mmol) and stirred for 30 min. at r.t. where the mixture turned bright yellow. Next, benzyl bromide (3 eq., 0.14 mL, 1.2 mmol) was added dropwise to the reaction mixture and then stirred overnight at r.t. The crude mixture was diluted in water and extracted 3 times in EtOAc, dried with sodium sulfate, and the solvent removed under reduced pressure. **CL-benzyl 1** was purified with silica column chromatography using a gradient of hexanes to 10% EtOAc (product elutes in pale yellow band in 10:90 EtOAc/hexanes). **CL-benzyl 1** was obtained as 0.073 g (0.16 mmol) of a yellow oil in 41% yield. ^{1}H NMR (400 MHz, chloroform-*d*) $\boldsymbol{\delta}$ 8.05 (d, 16.1 Hz, 1H), 7.49 (d, 8.2 Hz, 1H), 7.44 (d, 7.2 Hz, 2H), 7.38 (d, 2H), 7.30 (d, 1H), 6.93 (s, 1H), 6.92 (d, 1H), 6.54 (d, 16.1 Hz, 1H), 5.18 (d, 3.9 Hz, 2H), 3.80 (s, 3H), 3.24 (s, 4H), 2.62 (s, 1H), 1.97-1.71 (m, 12H). ^{13}C NMR (400 MHz, chloroform-*d*) $\boldsymbol{\delta}$ 168.41, 157.50, 143.38, 140.32, 139.35, 136.95, 133.81, 129.07, 128.84, 128.76, 128.40, 127.64, 123.29, 122.60, 118.57, 113.90, 70.66, 70.16, 58.33, 52.01, 39.50, 37.55, 32.77, 32.03, 30.84, 28.68, 23.10, 14.56. HRMS (ESI+) m/z calculated for $C_{29}H_{32}O_4$: 444.23, $[M+H]^+$ found at 445.2317. See Scheme S5 and Figure S58, S59, and S60 for characterization.

CL-benzyl 2: CL-benzyl 1 (0.051 g, 0.11 mmol) was dissolved in DCM (2 mL) and transferred to a 2-neck round-bottom flask. A catalytic amount (1 mg approx.) of methylene blue (purchased from Sigma Aldrich) was added and the mixture cooled to 0 ^{o}C on ice. Oxygen gas was then bubbled into the reaction mixture while being irradiated with a 625 nm LED for 2 hours, while maintaining the temperature at 0 ^{o}C. The crude mixture was then diluted in water and extracted 3 times with EtOAc. The organic layer was dried with sodium sulfate and the solvent was removed under reduced pressure. **CL-benzyl 2** was purified by silica column chromatography in 20:80 EtOAc/hexanes (pale yellow band) to yield 0.035 g (0.073 mmol) of product (67% yield). ^{1}H NMR (400 MHz, chloroform-*d*) $\boldsymbol{\delta}$ 8.05 (d, 16.2 Hz, 1H), 7.55 (d, 7.9 Hz, 1H), 7.43 (m, 6H), 6.58 (d, 16.2 Hz, 1H), 5.27 (s, 2H), 3.80 (s, 3H), 3.17 (s, 3H), 2.98 (s, 1H), 1.87-1.37 (m, 12H). ^{13}C NMR (400 MHz, chloroform-*d*) $\boldsymbol{\delta}$ 171.57, 168.13, 139.81, 138.47, 136.71, 129.15, 128.48, 127.58, 119.92, 112.21, 96.06, 70.73, 60.83, 52.13, 50.38, 39.70, 36.78, 35.16, 33.51, 32.76, 31.94, 26.42, 26.24, 21.48, 14.64. HRMS (ESI+) m/z calculated for $C_{29}H_{32}O_6$: 476.22, $[M+Na]^+$ found at 499.2087. See Scheme S5 and Figure S61, S62, and S63 for characterization.

CL-benzyl 3: CL-benzyl 2 (1 eq., 0.114 g, 0.24 mmol) was dissolved in 1:1 THF/H_2O (4 mL) containing NaOH (2eq., 0.019 g, 0.48 mmol). The mixture was stirred at 80 ^{o}C and reaction progress monitored by TLC (4 h reaction time approx.). The crude was warmed to r.t., diluted with EtOAc, and washed with 0.1 M $HCl_{(aq)}$ and brine. After drying the organic layer with sodium sulfate and removing solvent under reduced pressure, **CL-benzyl 3** was purified by silica column chromatography in 50:50 EtOAc/hexanes (pale yellow band). 0.050 g (0.11 mmol) of **CL-benzyl 3** was obtained in 45% yield. ^{1}H NMR (400 MHz, chloroform-*d*) $\boldsymbol{\delta}$

8.15 (d, 16.1 Hz, 1H), 7.60 (d, 8.0 Hz, 1H), 7.44 (m, 6H), 7.29 (d, 1H), 6.64 (d, 18.1 Hz, 1H), 5.28 (s, 2H), 3.18 (s, 3H), 2.98 (s, 1H), 1.88-1.38 (m, 13H). ^{13}C NMR (400 MHz, chloroform-*d*) $\boldsymbol{\delta}$ 172.43, 171.65, 141.86, 136.64, 129.19, 128.54, 112.19, 96.10, 70.80, 60.87, 54.23, 50.41, 36.77, 35.17, 33.53, 32.10, 31.95, 29.70, 26.42, 26.25, 21.50, 14.65. HRMS (ESI+) m/z calculated for $C_{28}H_{30}O_6$: 462.20, $[M+Na]^+$ found at 485.1941. See Scheme S5 and Figure S64, S65, and S66 for characterization.

CL-E2: To a round-bottom flask, **CL-benzyl 3** (1 eq., 0.050 g, 0.11 mmol) was dried down and purged under argon. Anhydrous DMF (0.2 mL) and TEA (3 eq., 0.05mL, 0.33 mmol) were added and stirred for 2 min. at r.t. where the reaction turned slightly more yellow. PyBOP (2 eq., 0.114 g, 0.22 mmol) was added and stirred for 10 min. at r.t. where it turned orange. Next, **EryB-Linker** (2 eq., 0.199 g, 0.22 mmol) in anhydrous DMF (0.3 mL) was added dropwise and the bright pink reaction mixture was left to stir overnight at 40 °C. After removing the DMF under reduced pressure, the mixture was purified by silica chromatography using a gradient of $CHCl_3$ to 10% MeOH (product elutes in 5% MeOH) in red-pink band. **CL-E2** was further purified by RP-HPLC in ACN (0.1% formic acid). **CL-E2** elutes at 30 min. peak in 100% ACN (50 min. method, 15 min. gradient from 5-100% ACN and then hold 100% ACN for 15 min.) to yield 0.015 g (0.011 mmol) of **CL-E2** (10% yield) as a bright pink oil (93% purity). ^{1}H NMR (500 MHz, chloroform-*d*) $\boldsymbol{\delta}$ 7.84 (d, 15.6 Hz, 1H), 7.65 (s, 1H), 7.60 (d, 1H), 7.52-7.46 (m, 8 H), 7.38 (t, 27.3 Hz, 2H), 7.33 (m, 1H), 7.29 (m, 1H), 7.11 (d, 15.5 Hz, 1H), 5.17 (s, 2H), 3.93 (s, 3H), 3.49 (m, 3H), 3.20 (s, 3H), 2.98 (s, 1H), 2.78 (s, 2H), 2.62 (s, 2H), 2.01 (s, 1H), 1.83-1.59 (m, 12H). ^{13}C NMR (500 MHz, chloroform-*d*) $\boldsymbol{\delta}$ 168.52, 167.68, 157.76, 146.71, 138.78, 138.12, 136.30, 132.08, 131.04, 130.12, 129.15, 128.86, 128.56, 122.56, 121.68, 113.31, 71.21, 52.73, 44.79, 41.39, 38.52, 35.81, 30.06, 29.70, 27.58. HRMS (ESI+) m/z calculated for $C_{52}H_{46}I_4N_2O_9$: 1349.94, [M+] found at 1349.9474. See Scheme S5 and Figure S67, S68, S69, and S70 for characterization.

Amino-PN: synthesized from previously reported procedure[5]. ^{1}H NMR (400 MHz, chloroform-*d*) $\boldsymbol{\delta}$ 8.67 (d, 1H), 8.24 (d, 1H), 8.02 (d, 1H), 7.79 (m, 3H), 7.63 (d, 1H), 4.49 (d, 2H). ^{13}C NMR (400 MHz, methanol-d_4) $\boldsymbol{\delta}$ 186.18, 144.71, 137.80, 135.21, 134.95, 133.93, 132.43, 130.03, 128.98, 128.78, 128.15, 74.49, 71.36, 64.71, 62.44, 41.00. HRMS (ESI+) m/z calculated for $C_{14}H_{11}NO$: 209.08, $[M+H]^+$ found at 210.0908. See Scheme S6 and Figure S71, S72, S73, and S74 for characterization.

CL-PNa: CL-COOH (1 eq., 0.50 g, 0.14 mmol) was added to a round-bottom flask and purged under an argon environment and then dissolved in anhydrous DMF (0.2 mL). TEA (3 eq., 0.1 mL, 0.42 mmol) was added and the reaction turned yellow and stirred for 2 min. at r.t. Next, PyBOP (2 eq., 0.146 g, 0.28 mmol) was added to the mixture and stirred at r.t. for 10 min. where it turned from yellow to red. After activation of the carboxylic acid, **Amino-PN** (1 eq., 0.030 g mL, 0.14 mmol) in anhydrous DMF (0.2 mL) was added dropwise to the reaction mixture. The mixture was left to stir overnight at 40 °C and protected from light. The mixture turned yellow brown after overnight reaction. After removing the DMF under reduced pressure, **CL-PNa** was purified using RP-HPLC in ACN (0.1% formic acid). **CL-PNa** elutes at 40 min. peak in 100% ACN (60 min. method, 25 min. gradient from 5-100% ACN and then hold 100% ACN for 15 min.). to yield 0.007 g (0.014 mmol) of **CL-PNa** (10% yield) as a yellow solid. ^{1}H NMR (500 MHz, chloroform-*d*) $\boldsymbol{\delta}$ 8.67 (d, 6.9 Hz, 1H), 8.22 (d, 8.1 Hz, 1H), 8.04 (d, 8.3 Hz, 1H), 7.92 (s, 1H), 7.83 (m, 3H), 7.61 (t, 1H), 7.38 (d, 7.9 Hz, 1H), 6.87 (d, 1H), 6.66 (d, 1H), 6.60 (d, 1H), 4.58 (d, 6.3 Hz, 2H), 3.29 (s, 3H), 3.23 (s, 1H), 2.69 (s, 1H), 2.00-1.78 (m, 12H). ^{13}C NMR (500 MHz, chloroform-*d*) $\boldsymbol{\delta}$ 168.84, 166.12, 157.76, 143.71, 141.53, 139.99, 139.12, 138.25, 135.54, 132.48, 130.91, 128.91, 128.06, 127.47, 127.32, 127.26, 121.56, 121.14, 120.93, 117.70, 117.36, 58.12, 51.77, 48.55, 46.71, 39.63, 39.42, 37.63, 32.67, 30.66, 28.87, 26.84, 26.52. HRMS (ESI+) m/z calculated for $C_{35}H_{33}NO_4$: 531.24, $[M+H]^+$ found at 532.2507. See Scheme S6 and Figure S75, S76, S77, and S78 for characterization.

CL-PN: CL-PNa was dissolved in DCM (2 mL) and transferred to a 2-neck round-bottom flask. A catalytic amount (1 mg approx.) of methylene blue (purchased from Sigma Aldrich) was added and the mixture cooled to 0 °C on ice. Oxygen gas was then bubbled into the reaction mixture while being irradiated with a 625 nm LED for 2 hours, while maintaining the temperature at 0 °C. The solvent from the crude reaction mixture was removed under reduced pressure and purified first by silica chromatography in EtOAc and then using RP-HPLC in ACN (0.1% formic acid). **CL-PN** elutes at 23 min. in 100% ACN (45 min. method, 10 min. gradient from 5-100% ACN and then hold 100% ACN for 15 min.). **CL-PN** was obtained in 45% yield (94% purity). ^{1}H NMR (500 MHz, chloroform-*d*) $\boldsymbol{\delta}$ 8.68 (d, 7.34 Hz, 1H), 8.25 (d, 7.96 Hz, 1H), 8.05 (d, 8.30 Hz, 1H), 7.98 (d, 6H – expected 1H), 7.90 (d, 1H), 7.84 (m, 1H), 7.52 (m, 7.51 Hz, 6H – expected 3H), 7.43 (m, 1H), 6.81 (m, 15.8 Hz, 1H), 6.64 (m, 6H – expected 1H), 4.59 (d, 2H), 3.23 (s, 12 H – expected 3H), 3.02 (s, 1H), 2.55 (s, 1H), 1.66-2.19 (m, 64H – expected 12H); We note that during the NMR acquisition period of 1 hour we observed the appearance of new peaks at 3.91, 3.82 and 2.61 ppm, as well as peaks intensifying at 7.98, 7.52, 6.64, 3.23 and 1.66 – 3.02 ppm, which led to higher than expected integrations for intact **CL-PN**. ^{13}C NMR (500 MHz, chloroform-*d*) $\boldsymbol{\delta}$ 167.94, 139.52, 137.99, 128.93, 119.16, 111.69, 111.56, 95.60, 51.67, 49.92, 46.87, 40.73, 39.17, 36.25, 34.65. 33.09, 33.03, 32.17, 31.60, 31.45, 27.34, 25.90, 25.77. HRMS (ESI+) m/z calculated for $C_{35}H_{33}NO_6$: 563.23 was not found. However, we do observe the expected methyl-ester breakdown product commonly produced in MS for unprotected dioxetanes. Calculated for $C_{25}H_{19}NO_5$: 413.13, $[M+H]^+$ found at 414.2072. See Scheme S6 and Figure S79, S80, S81 and S82 for characterization.

CL-OMe dioxetane: CL-OMe was dissolved in DCM (2 mL) and transferred to a 2-neck round-bottom flask. A catalytic amount (1 mg approx.) of methylene blue (purchased from Sigma Aldrich) was added and the mixture cooled to 0 °C on ice. Oxygen gas was then bubbled into the reaction mixture while being irradiated with a 625 nm LED for 2 hours, while maintaining the temperature at 0 °C. The solvent from the crude reaction mixture was removed under reduced pressure and purified first by silica chromatography in 20:80 EtOAc/hexanes and then using RP-HPLC in ACN (0.1% formic acid). **CL-OMe dioxetane** elutes at 25 min. peak in 100% ACN (45 min. method, 10 min. gradient from 5-100% ACN and then hold 100% ACN for 15 min.). **CL-OMe dioxetane** was obtained in 63% yield (99% purity). ^{1}H NMR (500 MHz, chloroform-*d*) $\boldsymbol{\delta}$ 7.99 (d, 16.1 Hz, 1H), 7.50 (m, 2H), 7.26 (s, 1H), 6.63 (d, 16.7 Hz, 1H), 3.82 (s, 3H), 3.22 (s, 3H), 3.02 (s, 1H), 2.18 (s, 1H), 1.91-1.46 (m, 12H). ^{13}C NMR (500 MHz, chloroform-*d*) $\boldsymbol{\delta}$ 168.33, 155.59, 139.88, 138.47, 129.40, 122.97, 121.98, 120.93, 119.72, 112.01, 96.09, 60.80, 52.12, 50.39, 36.71, 35.12, 33.56, 32.63, 32.07, 31.91, 26.24, 21.42, 14.55. HRMS (ESI+) m/z calculated for $C_{22}H_{29}O_6$: 386.17, [M+] not found. However, we do observe the expected methyl-ester breakdown product commonly produced in MS for unprotected dioxetanes. Calculated for $C_{12}H_{12}O_5$: 236.07, $[M+H]^+$ found at 237.08 and $[M+Na]^+$ found at 259.06. See Scheme S7 and Figure S83, S84, S85 and S86 for characterization.

NTR-CL: synthesized from previously reported procedures[6]. ^{1}H NMR (400 MHz, chloroform-*d*) $\boldsymbol{\delta}$ 8.26 (d, 8.4 Hz, 2H), 7.93 (d, 16.3 Hz, 1H) 7.69 (d, 8.4 Hz, 2H), 7.63 (d, 1.5 Hz, 1H), 6.49 (d, 16.3 Hz, 1H), 5.04 (s, 1H), 3.23 (s, 3H), 3.03 (s, 1H), 2.11-1.39 (m, 12H). ^{13}C NMR (400 MHz, chloroform-*d*) $\boldsymbol{\delta}$ 177.42, 171.46, 154.49, 148.44, 143.22, 140.29, 136.43, 131.44, 129.97, 129.20, 126.08, 124.35, 121.55, 112.06, 96.81, 75.16, 67.51, 60.90, 50.20, 36.99, 34.35, 34.07, 33.17, 32.64, 32.01, 30.15, 26.58, 26.27, 21.49, 21.16, 14.64, 1.46. HRMS (ESI-) m/z calculated for $C_{28}H_{28}ClNO_8$: 541.15, $[M-H]^-$ found at 540.1425. See Scheme S9 for structure and Figure S87, S88, and S89 for characterization.

NTR-CL-E1: NTR-CL (1 eq., 0.050 g, 0.09 mmol) was added to a round-bottom flask and purged under an argon environment and then dissolved in anhydrous DMF (0.2 mL). TEA (3 eq., 0.04 mL, 0.27 mmol) was added and the reaction turned yellow and stirred for 2 min. at r.t. Next, PyBOP (1.1 eq., 0.053 g, 0.10 mmol) was added to the mixture and stirred for 10 min. at r.t.. After activation of the carboxylic acid, **EryB-**

Linker (2 eq., 0.168 g, 0.18 mmol) in anhydrous DMF (0.4 mL) was added dropwise to the reaction mixture. The mixture turned bright pink and was left to stir overnight at 40 ^{o}C and protected from light. The crude mixture was diluted in chloroform and washed once with brine, dried using sodium sulfate, and then solvent removed under reduced pressure. The extracted product was purified by silica chromatography using a EtOAc, followed by a gradient of $CHCl_3$ to 10% MeOH (product elutes in 5% MeOH) in a red-pink band. The columned fraction was further purified by RP-HPLC in a in ACN (0.1% trifluoroacetic acid). **NTR-CL-E1** elutes at 30 min. in 100% ACN (50 min. method, 10 min. gradient from 5-100% ACN and then hold 100% ACN for 20 min.) to yield 0.017 g (0.012 mmol) of **NTR-CL-E1** (13% yield) as a bright pink/red solid (99% purity). ^{1}H NMR (500 MHz, chloroform-*d*) $\boldsymbol{\delta}$ 8.22 (d, 8.2 Hz, 2H), 7.88 (d, 15.6 Hz, 1H), 7.71 (d, 8.2 Hz, 2H), 7.61 (m, 2H), 7.56 (s, 2H), 7.46 (d, 8.1 Hz, 1H), 7.44 (m, 1H), 7.32 (d, 1H), 7.18 (m, 1H), 6.93 (d, 15.6 Hz, 1H), 5.08 (d, 13.7 Hz, 2H), 3.33 (s, 3H), 3.29 (s, 1H), 3.22 (d, 2.2 Hz, 3H), 3.18 (s, 2H), 2.99 (d, 4.5 Hz, 3H), 2.74 (s, 2H), 2.30 (s, 1H), 2.01-1.59 (m, 12H). ^{13}C NMR (500 MHz, chloroform-*d*) $\boldsymbol{\delta}$ 205.80, 201.82, 168.64, 146.47, 143.57, 138.81, 137.38, 136.72, 132.43, 131.32, 130.83, 130.24, 129.14, 129.10, 128.34, 127.01, 124.19, 121.63, 120.49, 74.51, 57.76, 56.34, 47.53, 45.25, 40.81, 38.84, 38.61, 37.54, 36.29, 33.63, 32.33, 30.14, 29.70, 28.05, 27.84, 23.10, 14.53, 1.43. HRMS (ESI-) m/z calculated for $C_{52}H_{44}ClI_4N_3O_{11}$: 1428.88, $[M-H]^-$ found at 1427.8816. See Scheme S9 and Figure S90, S91, S92, and S93 for characterization.

4NB-Reso: Resorufin sodium salt (0.047 g, 0.2 mmol) was first dissolved in DCM (5 mL). 4-nitrobenzyl bromide (0.086 g, 0.4 mmol) and tetrabutylammonium bromide (0.129 g, 0.4 mmol) was then added. K_2CO_3 (0.055 mg, 0.4 mmol) was dissolved in distilled H_2O (5 mL) and added dropwise to the above solution. The reaction mixture was stirred vigorously overnight at r.t. and protected from light. The crude reaction mixture was extracted with DCM and brine. The organic layer was dried with sodium sulfate and solvent removed under reduced pressure. The extracted product was first purified by silica chromatography using a gradient of DCM and MeOH (up to 5%), where **4NB-Reso** elutes in 5% MeOH in the orange-yellow coloured band. The columned product was further purified using RP-HPLC in a gradient of milli-q water and MeOH (0.1% formic acid), where **4NB-Reso** elutes in 34 min. peak in 100% MeOH (45 min. method, 25 min. gradient from 5-100% MeOH and then hold 100% MeOH for 5 min.) to yield 0.045 g (1.3 mmol) as an orange solid (65% yield) (95% purity). ^{1}H NMR (500 MHz, chloroform-*d*) $\boldsymbol{\delta}$ 8.29 (d, 8.7 Hz, 2H), 7.76 (d, 8.9 Hz, 1H), 7.64 (d, 8.6 Hz, 2H), 7.43 (d, 9.8 Hz, 1H), 7.01 (dd, 1H), 6.88 (d, 2.7 Hz, 1H), 6.83 (dd, 9.8 Hz, 2.0 Hz, 1H), 6.32 (d, 2.0 Hz, 1H), 5.29 (s, 2H). ^{13}C NMR (500 MHz, chloroform-*d*) $\boldsymbol{\delta}$ 186.27, 161.69, 146.25, 145.56, 142.69, 134.73, 131.81, 128.78, 127.72, 124.08, 113.85, 106.95, 101.19, 69.39. HRMS (ESI+) m/z calculated for $C_{19}H_{12}N_2O_5$: 348.07, $[M-H]^+$ found at 349.0817. See Scheme S10 and Figure S94, S95, S96, and S97 for characterization.

3. Characterization Data

Concentration Measurements and Sample Preparation: Absorbance spectra of compounds were measured in PBS pH 7.4 or EtOH (i.e., for all Erythrosin B containing compounds) in a 1.0 cm path length quartz cuvette.

The molar extinction coefficient of the methyl ester of **CL-A** (i.e., **CL-A Benzoate**) in PBS pH 7.4 was measured to be 430 ± 33 M^{-1} cm^{-1} at 400 nm. A ^{1}H NMR of **CL-A Benzoate** (unknown concentration) was acquired in d_6-DMSO, containing 1,4-dioxane as a standard (final concentration 10 mM). After integrating the NMR peaks and measuring the concentration of **CL-A Benzoate** in the NMR sample, the absorbance of the NMR sample was measured within a concentration range of 0-100 µM. The Beer Lambert Law (A = εcl, where A = absorbance, ε = molar extinction coefficient, c = concentration, l = path length of the cuvette) was then used to calculate the molar extinction coefficient based on the slope of the A vs. c plot. We also measured the molar extinction coefficient of **4NB-Reso** via quantitative NMR in d_6-DMSO

containing 1,4-dioxane as a standard (final concentration 10 mM). Using the Beer Lambert Law, the molar extinction coefficient of **4NB-Reso** was measured to be 19 000 ± 1000 M^{-1} cm^{-1} at 458 nm in DMSO.

The reported extinction coefficient for Erythrosin B in EtOH (107 000 M^{-1} cm^{-1} at 532 nm)[7] was used for **CL-E1**, **CL-E1a**, **CL-E2**, and **NTR-CL-E1** and the reported extinction coefficient of phenalenone (**PN**) in PBS pH 7.4 (10 000 M^{-1} cm^{-1} at 400 nm [8]) was added to the measured extinction coefficient of **CL-A Benzoate** (since both the CL scaffold and PN have absorbance at 400 nm), such that $\varepsilon_{400\,nm}$ 10 430 M^{-1} cm^{-1} was used for **CL-PN.** The concentration of DCF was measured in PBS pH 7.4 using the molar extinction coefficient of 59 000 M^{-1} cm^{-1} [9]. Stock solutions of dioxetane compounds were prepared fresh in DMSO for each experiment and then dried under vacuum after use.

Stock solutions of all non-dioxetane compounds were prepared in DMSO. A stock solution of sodium azide (NaN_3) was prepared in HyPure ™ Cell Culture Grade Water (purchased from GE Life Sciences). A stock solution of 10 mM nicotinamide adenine dinucleotide, reduced disodium salt (NADH; Sigma-Aldrich) was made in PBS pH 7.4 (1X) solution containing no calcium chloride or magnesium chloride (Thermo Fisher Scientific). Stock solutions of Nitroreductase (NTR) from *Escherichia coli* (N9284; Sigma Aldrich) were made in PBS pH 7.4 (1X) solution and stored at -20 ºC.

Luminescence Spectra and Dioxetane Half Lives: Luminescence emission for compounds **CL-E1**, **CL-E1a**, **CL-E2**, **CL-A**, and **CL-PN** were measured in PBS pH 7.4 containing 5% DMSO. Immediately before measurements were taken, each compound was added for a final concentration of 20 µM into a 96-well plate (cat.# 167008; purchased from ThermoFisher Scientific) containing PBS pH 7.4 and DMSO, mixed thoroughly, and put into the plate reader (no lid). Complete luminescence spectra (398–653 nm) were recorded on a kinetic loop every 2 min. for a total of 1 hour at 37 °C. The luminescence intensity was normalized to 1 at the wavelength of maximum emission and plotted as a function of time to determine the half-lives of dioxetane breakdown. Each experiment was performed in triplicate using independent samples.

Chemiluminescence Quantum Yield Measurements: Luminescence emission for compounds **CL-OMe dioxetane** (i.e., CL standard), **CL-E1**, and **CL-A**, and **CL-PN** were measured in PBS pH 7.4 containing 5% DMSO. Immediately before measurements were taken, each compound was added for a final concentration of 10 µM into a 96-well plate (cat.# 167008; purchased from ThermoFisher Scientific) containing PBS pH 7.4 and DMSO, mixed thoroughly, and put into the plate reader (no lid). Complete luminescence spectra (398–653 nm) were recorded on a kinetic loop every 2 min. for a total of 40 min. at 37 °C so that the maximum CL emission could be recorded for each compound. For each compound, the CL spectrum with the highest luminescence intensity during the time course was baseline corrected and then the sum of the intensities for the curve were added together to obtain the area under the CL spectrum. Equation 1 was then used to calculate the CL quantum yield (Φ_{CL}) using $\Phi_{CL,\,water}$ = 2.3% for **CL-OMe dioxetane**[3] as the standard. Each experiment was performed in triplicate using independent samples.

Equation 1. adapted from [10,11] $$\boldsymbol{\Phi_{CL}} = \frac{\boldsymbol{\Sigma_{compound}+\Sigma_{standard}}}{\boldsymbol{\Phi_{standard}}}$$

ROS Sensing *in vitro* using $DCFH_2$ Sensor: ROS production was measured for compounds **CL-E1**, **EryB-Linker**, **CL-E1a**, **CL-E2**, **CL-A**, and **CL-PN** in PBS pH 7.4 containing 5% DMSO (60 µL total volume). $DCFH_2$ (5 µM from a 500 µM stock in 0.1 M $NaOH_{(aq)}$) was added to a cuvette containing PBS pH 7.4 (57 µL). Immediately before each measurement, the sample was added for a final concentration of 10 µM (DMSO

volume was raised for the final volume of DMSO to be 3 µL), added to the cuvette, mixed thoroughly, and placed directly into the fluorometer. Fluorescence scans were recorded initially (0 min.) and then at 5 min. time intervals for a total of 40 min. at 37 °C. The excitation light was blocked in between measurements with aluminum foil (i.e., cuvette left in the dark in between scans). The production of ROS was monitored by the oxidation of non-fluorescent $DCFH_2$ to fluorescent DCF[12] (λ_{em} = 525 nm) and plotted as a function of time, with background fluorescence from the sensor alone subtracted from all fluorescence data. To determine if singlet oxygen was the primary type of ROS being produced, the experiment with **CL-E1** was repeated in the presence of excess NaN_3 (10 mM). Experiments were also repeated with 5, 10, and 20 µM **CL-E1** to determine if there was a dose dependent increase in ROS production.

ROS production from **NTR-CL-E1** or **CL-E2** (10 µM) was measured with $DCFH_2$ (5 µM) in PBS pH 7.4 (80 µL total volume) with the addition of NADH (200 µM), and with or without NTR (1.5 µM). The fluorescence of DCF was measured before the addition of NTR and after 24 h incubation at 37 ºC and then plotted as a ratio of the final (24 h): initial (0 h) fluorescence at 525 nm. The ROS from control compound **4NB-Reso** was measured under similar conditions, except for using 100 µM NADH and 0.5 µM NTR and incubating the samples for 40 min. total (time it takes for Resorufin to be released from **4NB-Reso**). Background fluorescence from the sensor alone was subtracted from all data.

Fluorometer method: λ_{ex} 490 nm (1.5 nm slit), λ_{em} 500-545 nm (3.0 nm slit), 600 nm min^{-1}, high sensitivity. Each experiment was performed in triplicate using independent samples.

Singlet Oxygen Sensing *in vitro* using ABDA: A 1 mM stock solution of 9,10-anthracenediyl-bis-(methylene)dimalonic acid (ABDA) (purchased from Cayman Chemicals) was made in DMSO. To a solution of ABDA (10 µM) in PBS pH 7.4 containing 5% DMSO (60 µL total volume), **CL-E1** (10 µM final concentration) was added, mixed thoroughly, and placed immediately into UV-Vis maintained at 37 °C. Absorbance scans were taken every 5 min., for a total of 40 min., with the excitation light blocked between measurements with aluminum foil. Singlet oxygen production was measured by monitoring the formation of the oxidized endoperoxide product with the corresponding decrease in the absorbance of ABDA at 400 nm[13,14]. Experiment performed in triplicate using independent samples.

Singlet Oxygen Sensing *in vitro* using DPBF: A 50 mM stock solution of 1,3-diphenylisobenzofuran (DPBF) (purchased from Sigma Aldrich) was prepared in ethanol. To a solution of DPBF (50 µM) in 1:1 D_2O/ethanol containing 5% DMSO (60 µL total volume), **CL-E1** (10 or 20 µM final concentration) was added, mixed thoroughly, and placed immediately into UV-VIs maintained at 37 °C. Absorbance scans were taken every 5 min., for a total of 40 min., with the excitation light blocked between measurements with aluminum foil. Singlet oxygen production was measured by monitoring the formation of the photo-dimer product with the corresponding decrease in the absorbance of DPBF at 400 nm[14]. Experiment performed in triplicate using independent samples.

Singlet Oxygen Sensing *in vitro* using SOSG: A 500 µM stock solution of Singlet Oxygen Sensor Green (SOSG) (purchased from Thermo Fischer Scientific) was prepared in methanol. To a solution of SOSG (5 µM) in PBS pH 7.4 containing 5% DMSO (60 µL total volume), **CL-E1** (10 µM final concentration) was added, mixed thoroughly, and placed immediately into fluorometer maintained at 37 °C. Fluorescence scans were taken every 10 min., for a total of 40 min., with the excitation light blocked between measurements with aluminum foil. Singlet oxygen production was measured by monitoring the formation of the endoperoxide product with the corresponding increase in the fluorescence of SOSG at 530 nm. Fluorometer method: λ_{ex} 490 nm (1.5 nm slit), λ_{em} 500-650 nm (3.0 nm slit), 600 nm min^{-1}, high sensitivity. Experiment performed in triplicate using independent samples.

Singlet Oxygen Sensing from Erythrosin B with Irradiation *in vitro* using SOSG and $DCFH_2$: Stock solutions (100 µM each) of $DCFH_2$ and SOSG were prepared as previously described above. A solution containing 1 µM each of the sensor and Erythrosin B were prepared in PBS pH 7.4 (60 µL total volume) and irradiated using a 530 nm LED (60.46 mW cm^{-2}) for 9 min. total (33 J cm^{-2}). Samples containing each sensor alone (1 µM) were also prepared and irradiated under the same conditions. The fluorescence of DCF or SOSG was measured every 3 min. using the appropriate fluorometer method. DCF fluorometer method: λ_{ex} 490 nm (1.5 nm slit), λ_{em} 500-545 nm (3.0 nm slit), 600 nm min^{-1}, high sensitivity. SOSG fluorometer method: λ_{ex} 490 nm (1.5 nm slit), λ_{em} 500-650 nm (3.0 nm slit), 600 nm min^{-1}, high sensitivity. Experiment performed in triplicate using independent samples.

DCF Calibration Curve for ROS Quantification and CL-E1 Singlet Oxygen Efficiency: We estimated the amount of singlet oxygen produced by **CL-E1** by relating the fluorescence intensity of DCF to its concentration, using commercially available DCF and its known molar extinction coefficient, to construct a calibration curve. 2',7'-dichlorofluorescein, the oxidized product of the ROS sensor $DCFH_2$, was purchased from Sigma Aldrich. A stock solution of DCF was prepared in DMSO and concentration measured in PBS pH 7.4, using its molar extinction coefficient of 59 500 M^{-1} cm^{-1} at 505 nm[9]. The green fluorescence of DCF (0.05–5 µM) was measured in PBS pH 7.4 containing 5% DMSO (60 µL total volume) to obtain a linear curve relating DCF (nmols) with expected fluorescence intensity from $DCFH_2$ sensor after oxidation. Fluorometer method: λ_{ex} 490 nm (1.5 nm slit), λ_{em} = 500–545 nm (3.0 nm slit), 600 nm min^{-1}, high sensitivity. Experiment performed in triplicate using independent samples.

Experimental singlet oxygen efficiency: Using the DCF intensities produced by **CL-E1** (Figure 3B), we calculated the amount of singlet oxygen produced (as nmols) using the line of best fit from the calibration curve in Figure S11. Dividing the amount of singlet oxygen calculated by each given amount **CL-E1** then taking the average yields the singlet oxygen efficiency.

Theoretical singlet oxygen efficiency:

Efficiency = Chemiexcitation Yield * Energy Transfer Efficiency * Singlet O_2 Quantum Yield of Erythrosin B

Chemiexcitation yield = Fraction of dioxetane that is excited. We can use the CL yield we measured for **CL-A** (i.e., missing the Erythrosin B) ($\Phi_{CL\text{-}A}$).
Energy transfer efficiency = Fraction of excited dioxetane that transfers energy to form an excited Erythrosin B in **CL-E1**. Since the chemiluminescence from **CL-E1** is quenched compared to **CL-A**, we can subtract **CL-A** yield from the **CL-E1** yield divided by **CL-A** yield to estimate fraction of excited dioxetane that undergoes resonance energy transfer. This assumes the chemiluminescence emission lost from **CL-A** having attached Erythrosin B is solely due to energy transfer.
Singlet O_2 Quantum Yield = Fraction of excited Erythrosin B in **CL-E1** that will produce singlet O_2. We can use the singlet O_2 quantum yield of Erythrosin B.($\Phi_{\Delta(EryB)}$)

CL-E1 theoretical singlet oxygen efficiency = $\Phi_{CL-A} \times \left(\frac{\Phi_{CL-A}-\Phi_{CL-E1}}{\Phi_{CL-A}}\right) \times \Phi_{\triangle(EryB)} = 0.016 \times \left(\frac{0.016-0.0002}{0.016}\right) \times 0.60 = 0.0095 \times 100 = 0.95\%$

NTR Activation towards NTR-CL-E1 Monitored by Analytical RP-HPLC: Samples containing **NTR-CL-E1** (50 µM) and NADH (200 µM) in PBS pH 7.4 (25 µL total volume) were incubated overnight with or without NTR (1.5 µM) in a 37 ºC water bath. Activation of **NTR-CL-E1** was monitored with analytical RP-HPLC using a gradient of milli-q water and ACN (with 0.1% formic acid); 55 min. method, 10 min. gradient from 5-

100% ACN and then hold 100% ACN for 20 min. The peak corresponding to the expected CL benzoate ester breakdown product at 41 min. was collected and analyzed by HRMS to confirm its identity. HRMS (ESI+) m/z calculated for $C_{35}H_{25}ClI_4N_2O_8$: 1143.75, $[M+H]^+$ found at 1144.7537.

NTR Activation of 4NB-Reso Monitored by Fluorescence: 4NB-Reso (2.5 μM) was added to a solution of PBS pH 7.4 (60 μL total volume) containing NADH (100 μM). 0.5 μM NTR was added to the sample, incubated at 37 ºC, while the fluorescence at 585 nm was measured every second until it plateaued. Both an initial and final fluorescence and absorbance spectrum were measured to compare to free Resorufin for confirmation of its release. Fluorometer method: λ_{ex} 572 nm (3.0 nm slit), λ_{em} = 580–800 nm (3.0 nm slit), 600 nm min^{-1}, high sensitivity. Time course fluorometer method: λ_{ex} 572 nm (3.0 nm slit), λ_{em} = 585 nm (3.0 nm slit), reading every 1 sec., high sensitivity. Experiment performed in triplicate using independent samples.

4. Cell Studies

Cell Culture: MCF7 and A549 cell lines (purchased from American Type Culture Collection; ATCC) were cultured in Dulbecco's Modification Eagle's Medium (DMEM) without sodium pyruvate and with sodium pyruvate, respectively. Both media contains 4.5 g L^{-1} glucose and L-glutamine supplemented with 10% FBS and 1% antibiotic-antimycotic solution. MDA-MB231 triple-negative breast cancer cells +/- stable NTR expression were provided from Dr. Ramasamy Paulmurugan's Lab from Stanford University School of Medicine[2]. Both NTR+ and NTR- cells were cultured in DMEM with sodium pyruvate, containing 4.5 g L^{-1} glucose and L-glutamine supplemented with 10% FBS and 1% antibiotic-antimycotic solution, with NTR+ cell media also containing puromycin antibiotic selection (100 ng mL^{-1}). Cells were cultured in 75 cm^2 culture flasks at 37ºC under 5% CO_2 in a humidified incubator. Incubation of compounds in cells were performed in serum-free DMEM (0% FBS) and washes were done with Dulbecco's Phosphate Buffered Saline (D-PBS) 1X without calcium and magnesium. Cell culture media and D-PBS (purchased from WINSET Inc.) were both warmed to 37 °C before their addition to cells.

Imaging of ROS Production *in Cellulo* using $DCFH_2$-DA Fluorescent Sensor (General Procedure): Cells were seeded at a concentration of 40 000 cells $well^{-1}$ in DMEM without sodium pyruvate (250 μL) in an 8-well chamber (cat.# 155409; purchased from ThermoFisher Scientific) and cultured overnight at 37 °C with 5% CO_2. The DMEM media was then replaced with serum-free DMEM (250 μL) and incubated for 1 hour at 37 °C with 5% CO_2. After this time, the media was removed, and the cells were treated with 10 μM $DCFH_2$-DA (0.2% DMSO) in D-PBS (250 μL total volume) for 30 min. at 37 °C and washed once with D-PBS.

Time course with no wash: MCF7 cells were treated with **CL-E1** (5 μM, 1% DMSO) in OPTI-MEM (purchased from WINSET Inc.) and incubated at 37 °C with 5% CO_2 and imaged after 0, 10, 20, and 40 min. of incubation. No washes were done in between imaging.

Time course with independent samples: MCF7 cells were treated with **CL-E1** (5 μM, 1% DMSO) in serum-free DMEM without sodium pyruvate and incubated at 37 °C with 5% CO_2. After 0, 10, 20 or 40 min. of incubation with compound, the media was removed, and cells were washed two times with D-PBS. Individual samples were used for each time point.

ROS Production after 10 min. Incubation with **CL-E1** and Control Compounds: MCF7 cells were treated with 5 μM **CL-E1** or control compounds (**EryB-Linker**, **CL-E1a**, **CL-E2**, **CL-A**, and **CL-PN**) (1% DMSO) in serum-free DMEM and incubated at 37 °C with 5% CO_2 for 10 min. The media was removed, and cells were

washed two times with D-PBS. To measure ROS production of **CL-E1** in the presence of a singlet oxygen scavenger, NaN_3 (10 mM) was added to cells with **CL-E1**. To measure ROS from broken down **CL-E1**, **CL-E1** was added to serum-free media and incubated outside of cells in a 37 °C water bath for 10 min. and 40 min., and then added to cells culture in the same media. These experiments were repeated in A549 cells.

ROS Production after Incubation with **NTR-CL-E1**: MDA-MB231 cells (+/- NTR) were treated with 10 µM **NTR-CL-E1** (1% DMSO) in OPTI-MEM and incubated at 37 ºC (5% CO_2) for 45 min. or 90 min. Before the incubation with **NTR-CL-E1** was finished, $DCFH_2$-DA was added directly to the wells (10 µM final concentration) and incubated for 30 min. at 37 ºC (5% CO_2). The media was then removed, and cells washed once with D-PBS.

D-PBS (250 µL) was added to wells for fluorescence imaging. Fluorescence microscopy images were acquired using an Olympus IX73 inverted microscope and cellSens software was used to control the camera and capture the images (500 ms integration time, 1% excitation power, 20x magnification). The fluorescence of DCF (the compound produced upon oxidation of $DCFH_2$ by ROS[12]) was visualized using an EGFP filter set (λ_{ex} = 440–495 nm, λ_{em} = 500–550 nm). Quantification of DCF fluorescence from compounds all have background from sensor alone subtracted out. Each experiment was performed in triplicate using independent samples.

Cellular Localization of CL-E1: MCF7 cells were seeded at a concentration of 40 000 cells well^{-1} in DMEM without sodium pyruvate (250 µL) in an 8-well chamber (cat.# 155409; purchased from ThermoFisher Scientific) and cultured overnight at 37 °C with 5% CO_2. The DMEM media was then replaced with serum-free DMEM (250 µL) and incubated for 1 hour at 37 °C with 5% CO_2. The old media was removed, and replaced with **CL-E1** (10 µM, 1% DMSO) in D-PBS (200 µL total volume). To image the time dependent production of the CL benzoate[15], cells were placed on a heated microscope stage (at 37 °C) and imaged every 10 min. for 60 min. total in the same field of view (no washes) using both filter set A and B (see below for specs). To determine if there is good overlap between the fluorescence of CL benzoate and Erythrosin B, MCF7 cells were incubated with **CL-E1** (10 µM, 1% DMSO) in D-PBS and imaged after 20 min. using filter sets A and B. Next, DAPI was added (5 µM final concentration) to determine if there were any nuclear signals from **CL-E1** indicating nuclear permeability, After incubating cells with DAPI for 10 min. on a heated microscope stage (at 37 °C), the media was removed, cells washed once with D-PBS, and then D-PBS was added for imaging. The cyan fluorescence from DAPI was imaged using a DAPI filter set (λ_{ex} = 377/50-25 nm, λ_{em} = 417–477 nm) and **CL-E1** was imaged using the fluorescence from Erythrosin B with filter set B. Fluorescence microscopy images were acquired using an Olympus IX73 inverted microscope and cellSens software was used to control the camera and capture the images (500 ms integration time, 10% excitation power, 40x magnification). The yellow fluorescence of CL benzoate was visualized using a custom filter set A (λ_{ex} = 400/40-25 nm, λ_{em} = 560/94-25 nm). The green fluorescence from Erythrosin B was visualized using filter set B (i.e., YFP filter set) (λ_{ex} = 509/22-25 nm, λ_{em} = 544/24-25 nm). Each experiment was performed in triplicate using independent samples.

Cell Viability: MCF7 cells were seeded at a concentration of 10 000 cells well^{-1} in DMEM without sodium pyruvate (100 µL) in a 96-well plate (cat.# 167008; purchased from ThermoFisher Scientific) and cultured overnight at 37 °C with 5% CO_2. The DMEM media was then replaced with serum-free DMEM (100 µL) and incubated for 3 h at 37 °C with 5% CO_2. The media was removed, cells treated with **CL-E1** (0.5–64 µM, 1% DMSO), in serum-free DMEM and incubated overnight at 37 °C with 5% CO_2. Cell viability was quantified using a standard MTT assay (2 h incubation at 37 °C with 5% CO_2 with 0.5 mg mL^{-1} thiazolyl blue

tetrazolium tribromide in DMEM without sodium pyruvate)[16]. Each concentration was performed in triplicate using independent samples.

Cytotoxicity of **CL-E1** was compared to control compounds (**CL-A**, **CL-PN**, **CL-E1a**, **CL-E2**, and **EryB-Linker**) within the same concentration range. Additional **CL-E1** controls were the addition of the break down product (40 min. pre-incubated **CL-E1**) to cells and co-incubation of **CL-E1** with NaN_3 (10 mM). Each experiment was performed in triplicate using independent samples.

Cytotoxicity of NaN_3 towards MCF7 cells was measured using a concentration range of 0.5 - 10 mM, under the same overnight incubation at 37 °C with 5% CO_2 in serum-free DMEM. Each concentration was performed in triplicate using independent samples.

Cytotoxicity of **NTR-CL-E1** and **CL-E2** were measured in MDA-MB231 cells +/- NTR. Cells were incubated with **NTR-CL-E1** (0.1-32 μM, 1% DMSO) or **CL-E2** (8-32 μM; 1% DMSO) in serum-free DMEM, and incubated for 24 h or 72 h at 37 °C with 5% CO_2.

Mechanism of Cell Death: MCF7 cells were seeded at a concentration of 50 000 cells $well^{-1}$ in DMEM without sodium pyruvate (250 μL) in an 8-well chamber (cat.# 155409; purchased from ThermoFisher Scientific) and cultured overnight at 37 °C with 5% CO_2. The DMEM media was removed and replaced with serum-free DMEM without sodium pyruvate (250 μL) and incubated for 1 h at 37 °C with 5% DMSO. The media was removed, and cells were treated with **CL-E1** (32 μM) in serum-free DMEM without sodium pyruvate and incubated at 37 °C with 5% DMSO for 1 h. DMEM media was then removed, and cells were washed once with D-PBS. Next, Annexin V-FITC and propidium iodide were added as green and red fluorescence indicators of apoptosis and necrosis, respectively[17]. Annexin V-FITC (10 μL) and propidium iodide (10 μL) (kit purchased from Invitrogen) were added to cells in 1X Annexin V Binding Buffer and incubated for 15 min. at 37 °C. Cells were washed once with D-PBS and then D-PBS was added to the cells for imaging 1 h, 2 h, and 4 h after treatment with **CL-E1**. Fluorescence microscopy images were acquired using an Olympus IX73 inverted microscope and cellSens software was used to control the camera and capture the images (500 ms integration time, 3% excitation power, 20x magnification). The green fluorescence from Annexin V-FITC was visualized using an EGFP filter set (λ_{ex} = 440–495 nm, λ_{em} = 500–550 nm) and the red fluorescence from PI using a custom filter set (λ_{ex} 530/55-25 nm, λ_{em} 650/100-25 nm). Each experiment was performed in triplicate using independent samples.

5. Supporting Figures

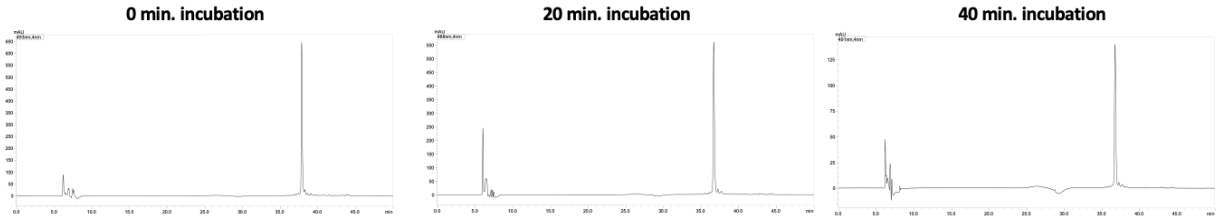


Figure S1. Stability of **CL-E1** (50 µM) in DMSO at 37 °C. After incubation for 0, 20, and 40 minutes in DMSO, the **CL-E1** sample integrity was monitored via analytical RP-HPLC in a gradient of ACN (with 0.1% TFA). No change in elution time (37 min.) was observed, confirming the integrity of the dioxetane in DMSO (λ_{abs} 490 nm). Measurements were performed in duplicate using independent samples.

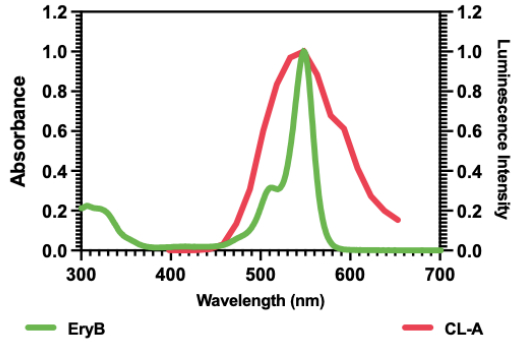


Figure S2. Overlay of the normalized spectra of the absorbance of **EryB-Linker** and luminescence of **CL-A** in PBS pH 7.4 containing 5% DMSO.

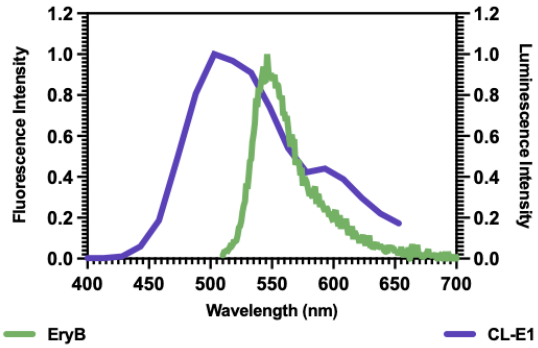


Figure S3. Overlay of the normalized spectra of the fluorescence of **EryB-Linker** and luminescence of **CL-E1** in PBS pH 7.4 containing 5% DMSO.

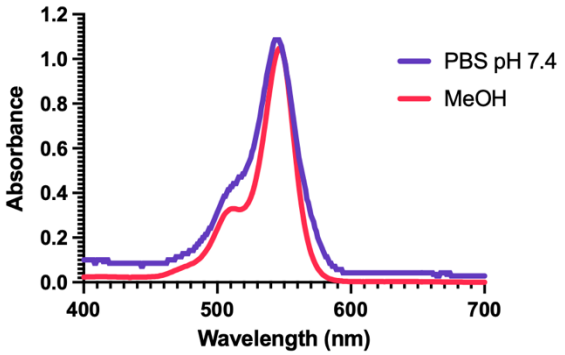


Figure S4. Normalized absorption spectrum of **CL-E1** in water (PBS pH 7.4) and in an organic solvent (MeOH). Measurements were performed in triplicate.

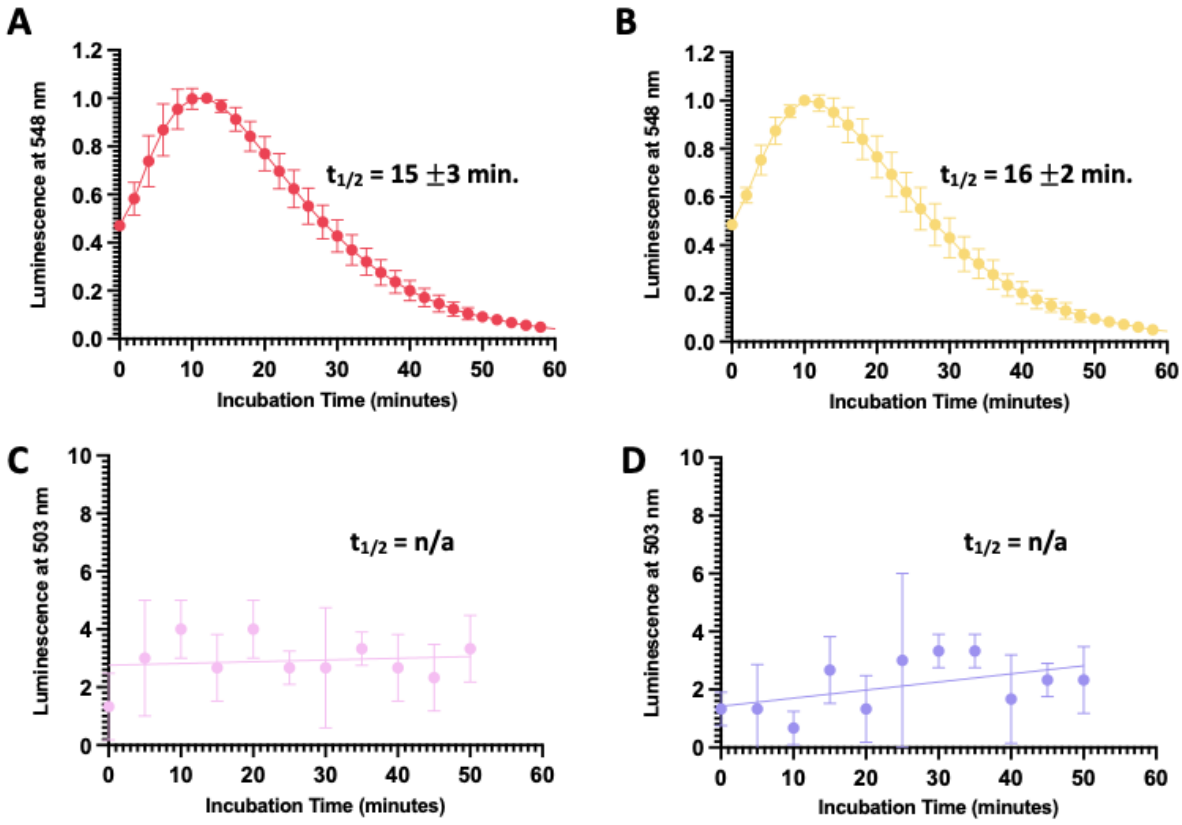


Figure S5. Chemiluminescence time course of A) **CL-A** and B) **CL-PN** plotted at their luminescence wavelength maximum and normalized to 1 at the time of maximum intensity. Half-life of dioxetanes were measured to be 15 ±3 min. and 16 ±2 min., respectively in PBS pH 7.4 containing 5% DMSO. Control compounds C) **CL-E1a** and D) **CL-E2** do not show any chemiluminescence under the same conditions. Measurements were performed in triplicate using independent samples.

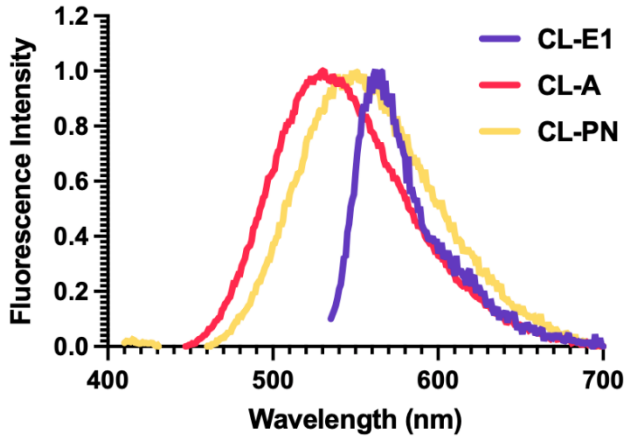


Figure S6. Normalized fluorescence emission spectrum of key compounds in PBS pH 7.4. **CL-E1** (λ_{ex} 525 nm), **CL-A** (λ_{ex} 400 nm), and **CL-E2** (λ_{ex} 400 nm).

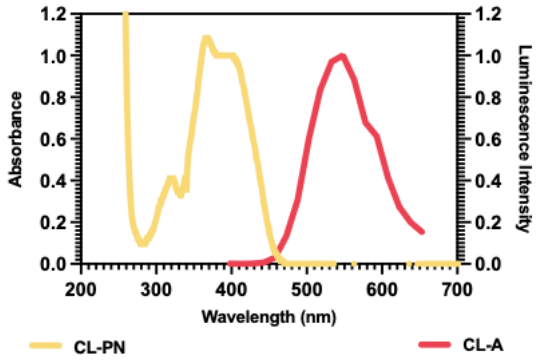


Figure S7. Overlay of the normalized spectra of the absorbance of **PN** and luminescence of **CL-A** in PBS pH 7.4 containing 5% DMSO.

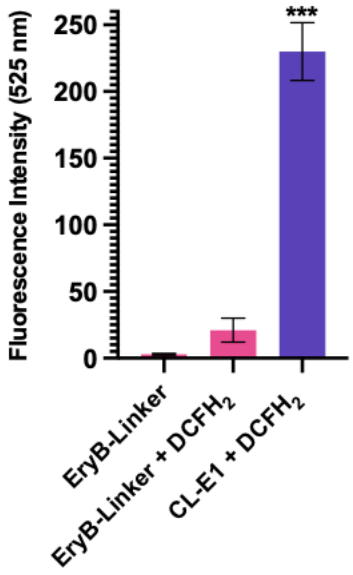


Figure S8. A solution containing **EryB-Linker** (10 µM) only under the fluorometer method used for $DCFH_2$ (λ_{ex} 490 nm), where minimal fluorescence at 525 nm (i.e., DCF emission max) was observed. ROS experiments were then repeated by incubating **EryB-Linker** (10 µM) with $DCFH_2$ (5 µM) and recorded the fluorescence over time. Minimal increase in DCF fluorescence in the presence of **EryB-Linker** was observed. Analyzed by two-tailed *t*-test, *p*-value = 0.0001 indicated by ***. Conditions: PBS pH 7.4 containing 5% DMSO at 37°C. λ_{ex} 490 nm. Measurements were performed in triplicate using independent samples.

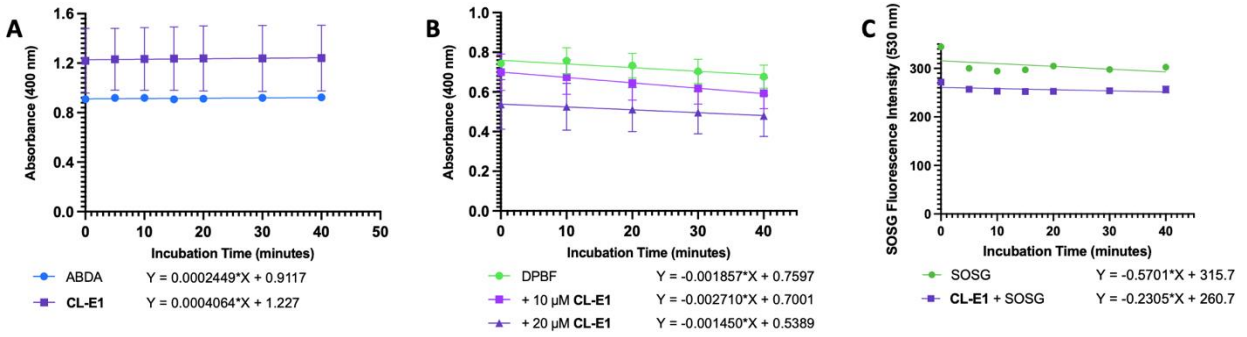


Figure S9. Singlet oxygen specific sensors A) ABDA (10 µM) was incubated with **CL-E1** (10 µM), B) DPBF (50 µM) with **CL-E1** (10 and 20 µM), and C) SOSG (5 µM) with **CL-E1** (10 µM), to measure singlet oxygen produced by **CL-E1** in PBS pH 7.4 (5% DMSO) (A and C) or 1:2 D_2O/PBS pH 7.4 (B), at 37°C. No decrease in the absorbance of ABDA or DPBF at 400 nm or increase in fluorescence of SOSG at 530 nm, which would be indicative of sensor oxidation by singlet oxygen, was observed in the presence of **CL-E1**. For SOSG: λ_{ex} 490 nm. Measurements were performed in triplicate.

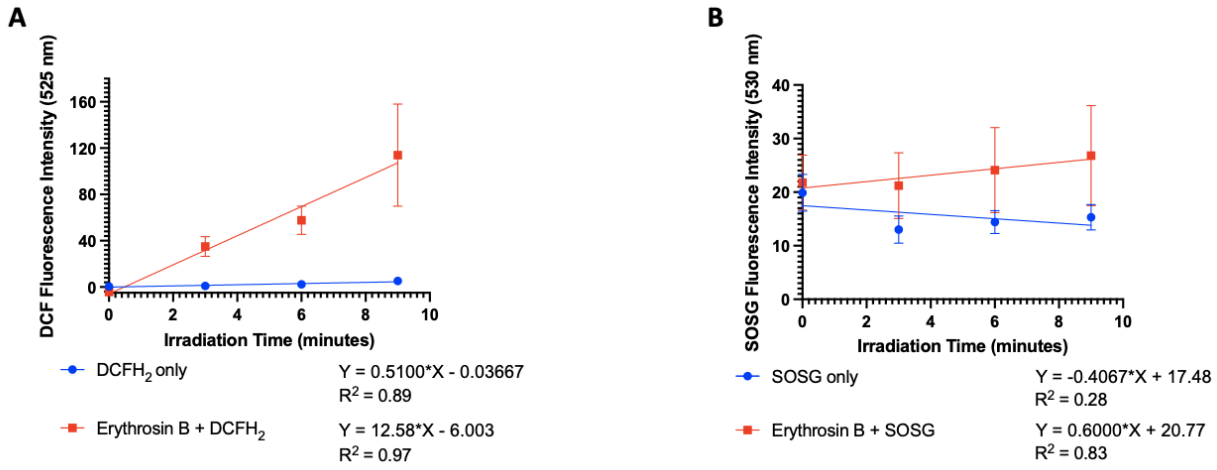
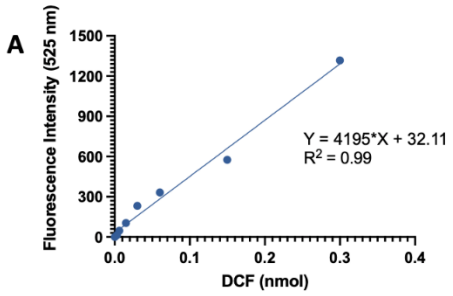


Figure S10. ROS produced by Erythrosin B (1 µM) during irradiation with a 530 nm LED in the presence of 1 µM of A) general ROS sensor, $DCFH_2$ or B) singlet oxygen specific sensor, SOSG. The fluorescence of DCF for the sample containing Erythrosin B is higher than background from $DCFH_2$ sensor alone suggesting response of the general ROS sensor to singlet oxygen produced by Erythrosin B. In contrast, Erythrosin B irradiated in the presence of SOSG does not show a large increase in the fluorescence of oxidized SOSG demonstrating SOSG's general lack of response to singlet oxygen. Conditions: PBS pH 7.4 (1% DMSO). λ_{ex} 490 nm. Measurements were performed in triplicate.



B

[CL-E1] (µM)	CL-E1 (nmol)	Singlet Oxygen (nmol)	Singlet Oxygen Efficiency (%)
5	0.3	0.009 ±0.003	3.1 ±0.3
10	0.6	0.022 ±0.002	4.1 ±0.9
20	1.2	0.044 ±0.002	3.7 ±0.7

Figure S11. ROS calibration curve relating the fluorescence of DCF at 525 nm with the amount of DCF (i.e., representative of ROS amount). Using the equation of the line (Y = 4195X + 32.11, where Y represents fluorescence of DCF at 525 nm and X represents the amount of ROS), the quantity of singlet oxygen produced by **CL-E1** could be calculated. The y-intercept (32.11) can be disregarded because background fluorescence from the $DCFH_2$ sensor alone was already subtracted from **CL-E1** ROS data. B) Singlet oxygen produced by **CL-E1** at increasing concentrations was calculated using the line of best fit from A to quantify the amount. A linear increase in the amount of singlet oxygen produced is consistent with the 2-fold increases in **CL-E1** concentrations tested with $DCFH_2$. The average singlet oxygen efficiency was calculated to be 3.6 ±0.9 % Conditions: PBS pH 7.4 (5% DMSO) at 37 °C. λ_{ex} 490 nm. Measurements were performed in using independent samples.

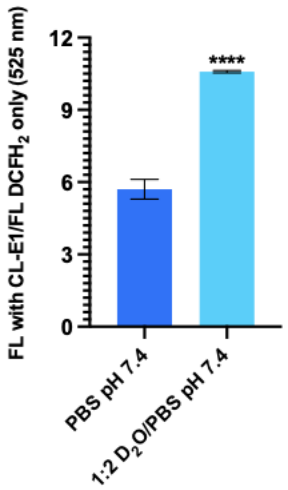


Figure S12. ROS produced by **CL-E1** (10 µM) monitored by the fluorescence of the ROS sensor, $DCFH_2$ (5 µM) in either PBS pH 7.4 or 1:2 D_2O/PBS pH 7.4. A 10.58 ±0.04-fold change in the fluorescence of DCF in the presence of **CL-E1** compared to ROS sensor alone was observed in D_2O solution, while only and 5.7 ± 0.4-fold in PBS. The larger fluorescence fold change in the presence of D_2O is consistent with the extended lifetime of singlet oxygen under these conditions. Analyzed by two-tailed *t*-test, *p*-value < 0.0001 indicated by ****. Conditions: PBS with or without D_2O (pH maintained at 7.4) (5% DMSO) at 37 ^oC. λ_{ex} 490 nm. Measurements were performed in triplicate using independent samples.

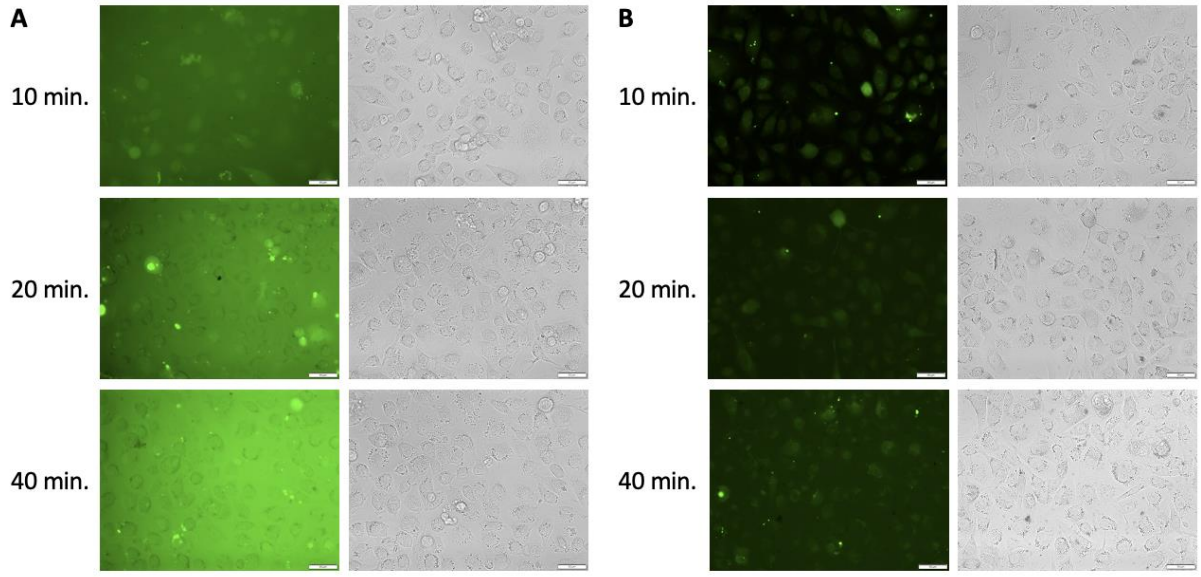


Figure S13. ROS production by **CL-E1** in MCF7 breast cancer cells. Cells were incubated with the general ROS sensor, $DCFH_2$-DA (10 µM) for 30 min., followed by addition of 5 µM **CL-E1**. The increase in the green fluorescence from the ROS sensor in the presence of **CL-E1** was observed for A) in a no wash experiment where maximum fluorescence signals were observed after 40 min. of incubation and B) where individual samples were washed prior to imaging. The highest intracellular fluorescence signals were observed after 10 min. of incubation with **CL-E1**, and after 10 min. the oxidized DCF product started to diffuse out of the cells. 20x, scale bar = 50 µm. ROS sensor imaged by: λ_{ex} = 470–490 nm, λ_{em} = 500–550 nm. Experiments were performed in triplicate using independent samples.

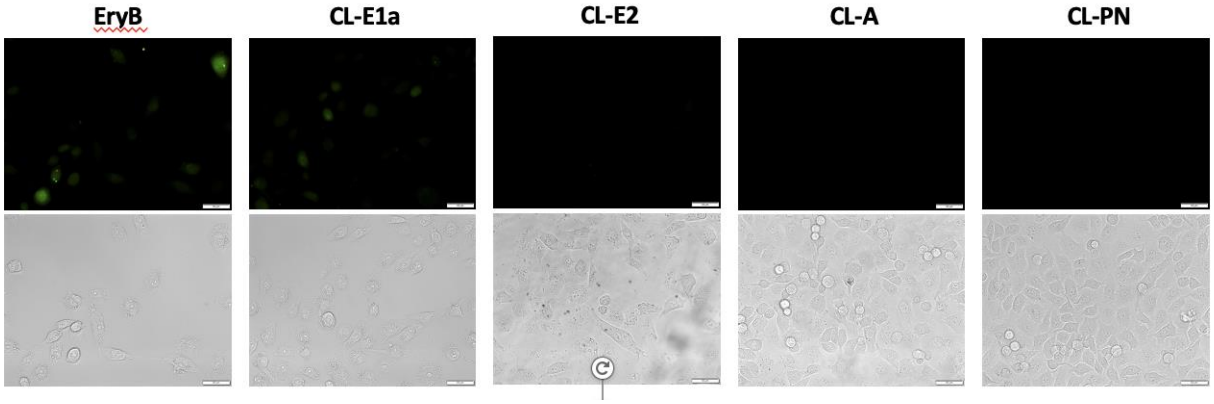


Figure S14. ROS production by control compounds in MCF7 breast cancer cells. Cells were incubated with the general ROS sensor, $DCFH_2$-DA (10 µM) for 30 min., followed by addition of 5 µM compound. Minimal green fluorescence from the ROS sensor in the presence of all control compounds was observed after 40 min. of incubation. 20x, scale bar = 50 µm. ROS sensor imaged by: λ_{ex} = 470–490 nm, λ_{em} = 500–550 nm. Experiments were performed in triplicate using independent samples.

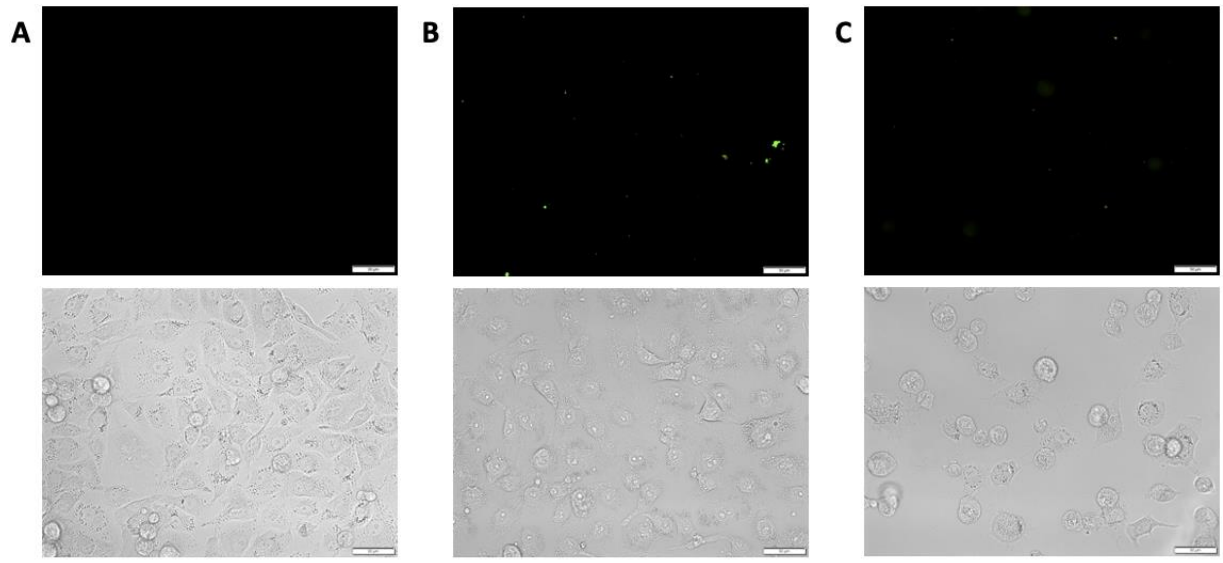


Figure S15. Controls for $DCFH_2$ sensor experiments in MCF7 breast cancer cells. Cells were incubated A) without or B) with the general ROS sensor, $DCFH_2$-DA (10 µM), or C) with $DCFH_2$-DA and NaN_3 (10 mM) for 30 min. Minimal background fluorescence from the cells and the sensor were observed. 20x, scale bar = 50 µm. ROS sensor imaged by: λ_{ex} = 470–490 nm, λ_{em} = 500–550 nm. Experiments were performed in triplicate using independent samples.

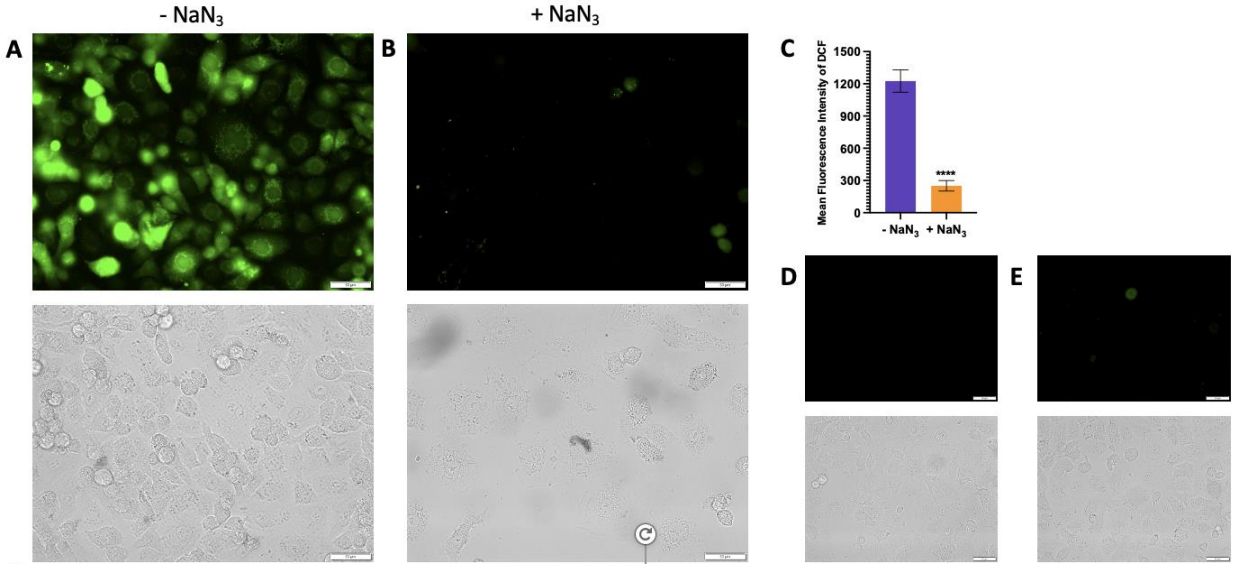


Figure S16. ROS production by **CL-E1** in A549 lung cancer cells. Cells were incubated with the general ROS sensor, $DCFH_2$-DA (10 µM) for 30 min., followed by 10 min. incubation with 5 µM A) **CL-E1** or B) **CL-E1** and singlet oxygen scavenger NaN_3 (10 mM). An increase in the green fluorescence from the ROS sensor in the presence of **CL-E1** only is indicative of ROS produced from **CL-E1** without light excitation demonstrating **CL-E1** could be useful in treating more than one type of cancer. C) Quantification of the mean fluorescence intensity of DCF demonstrates a statistically significant decrease of ROS production by **CL-E1** in the presence of singlet oxygen scavenger NaN_3. Quantification of the mean fluorescence intensity of DCF was analyzed by two-tailed *t*-test, *p*-value < 0.0001 indicated by ****. Controls for $DCFH_2$ sensor in A549 are shown in D) no compound or sensor and E) $DCFH_2$ sensor only, where minimal green fluorescence is present. 20x, scale bar = 50 µm. ROS sensor imaged by: λ_{ex} = 470–490 nm, λ_{em} = 500–550 nm. Experiments were performed in triplicate using independent samples.

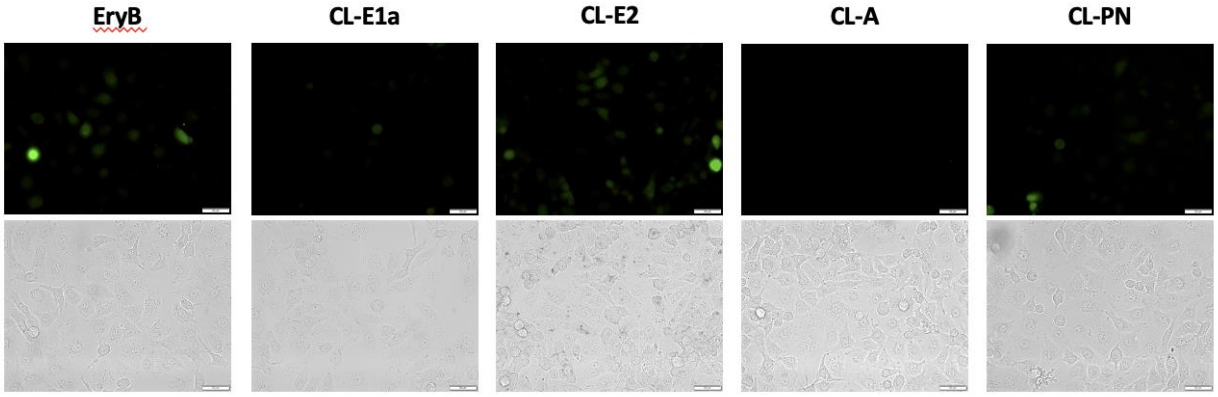


Figure S17. ROS production by control compounds in A549 lung cancer cells. Cells were incubated with the general ROS sensor, $DCFH_2$-DA (10 µM) for 30 min., followed by addition of 5 µM compound. Minimal green fluorescence from the ROS sensor in the presence of all control compounds was observed after 40 min. of incubation. 20x, scale bar = 50 µm. ROS sensor imaged by: λ_{ex} = 470–490 nm, λ_{em} = 500–550 nm. Experiments were performed in triplicate using independent samples.

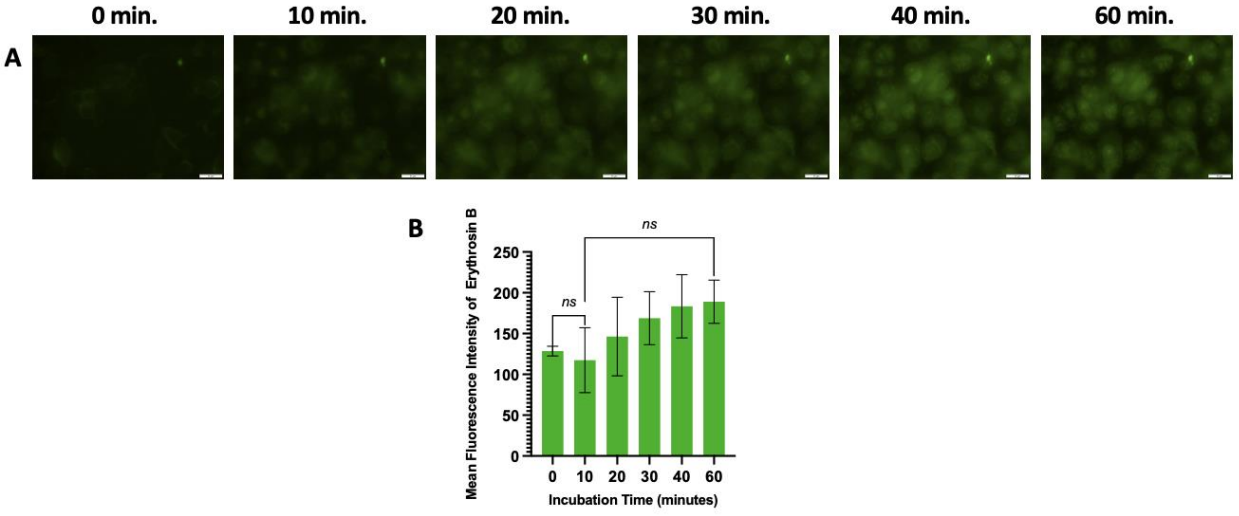


Figure S18. Fluorescence time course of MCF7 breast cancer cells incubated with 10 µM **CL-E1** for a total of 60 min. A) The attached photosensitizer Erythrosin B to the CL scaffold shows green fluorescence (λ_{ex} 509 nm), consistent with its linkage to the CL scaffold before and after dioxetane breakdown with minimal change to the fluorescence once it has entered cells. 40x, scale bar = 25 µm. B) Quantification of the mean fluorescence intensity Erythrosin B portion of **CL-E1** confirms the expected fluorescence trends. Data for 0 min. incubation represents cells imaged immediately after addition of **CL-E1**. Analyzed by two-tailed *t*-test, *p*-value > 0.05 is *ns*. Experiments were performed in triplicate using independent samples.

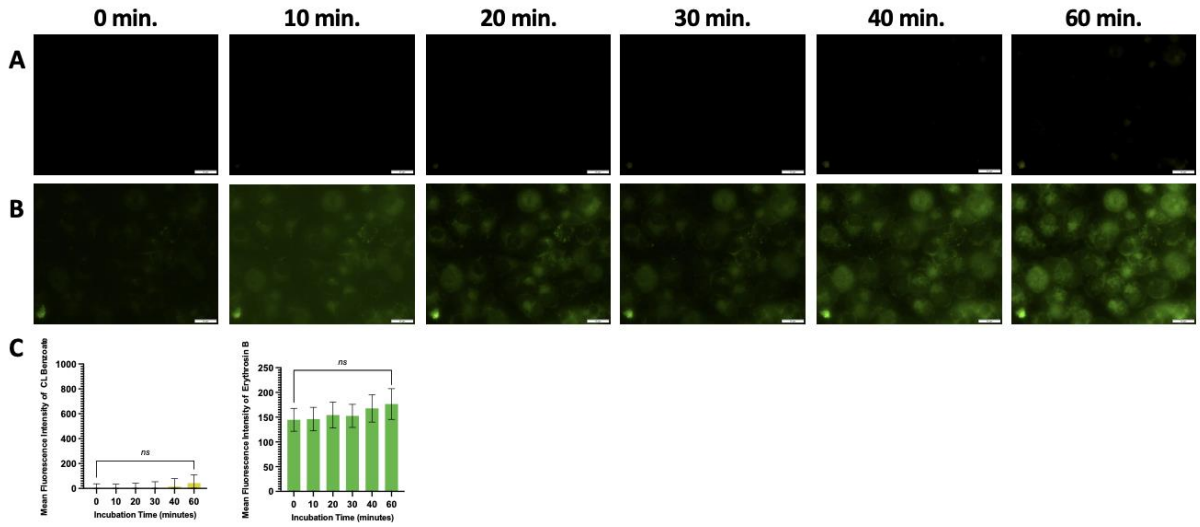


Figure S19. Fluorescence time course of MCF7 breast cancer cells incubated with 10 µM **CL-E2** for total of 60 min. A) Yellow fluorescence (λ_{ex} = 400 nm), which would represent the production of a CL benzoate is not observed since no dioxetane breakdown occurs with the benzyl protecting group on the phenol of the CL scaffold. B) The attached photosensitizer Erythrosin B to the CL scaffold shows green fluorescence (λ_{ex} = 509 nm), consistent with its linkage to the CL scaffold with minimal change to the fluorescence. 40x, scale bar = 25 µm. C) Quantification of the mean fluorescence intensity of the CL benzoate and Erythrosin B portion of **CL-E2** confirms the expected fluorescence trends. Data for 0 min. incubation represents cells imaged immediately after addition of **CL-E2**. Analyzed by two-tailed *t*-test, *p*-value > 0.05 is *ns*. Experiments were performed in triplicate using independent samples.

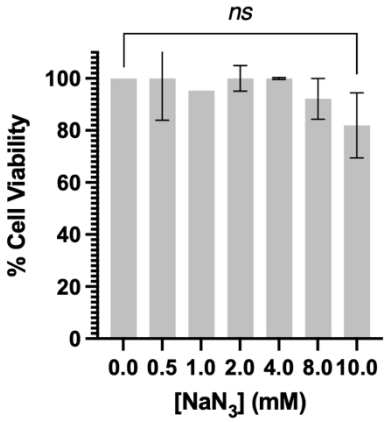


Figure S20. Cell viability of MCF7 cells incubated with NaN_3 overnight, then assayed for cell viability using an MTT assay. Analyzed by two-tailed *t*-test, *p*-value > 0.05 is non-significant (ns). Experiments were performed in triplicate.

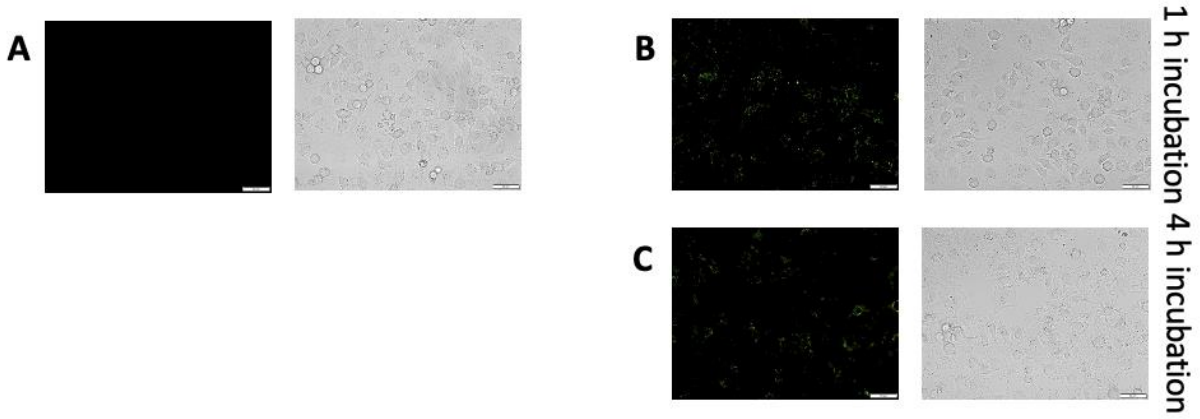


Figure S21. MCF7 cells incubated with A) no compound or cell death indicators, and cell death indicators only (i.e., Annexin V-FITC and PI overlay) after B) 1 hour and C) 4 hours, demonstrates that red fluorescence observed is due to toxicity of **CL-E1**. 20x, scale bar = 50 µm. Annexin V-FITC imaged by: λ_{ex} = 470–490 nm, λ_{em} = 500–550 nm and PI imaged by: λ_{ex} = 505–555 nm, λ_{em} = 600–700 nm. Experiments were performed in triplicate using independent samples.

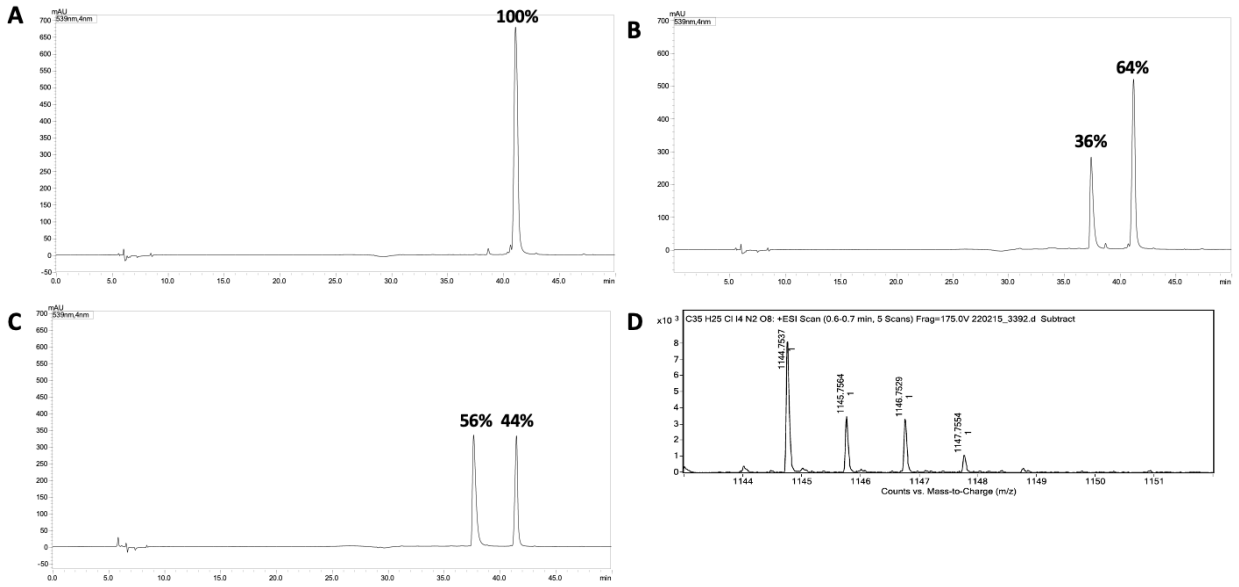


Figure S22. Overnight incubation of **NTR-CL-E1** (50 µM) with NADH (200 µM) in A) absence of NTR; or presence of B) 1.5 µM NTR; or C) 10 µM NTR. Removal of the NTR trigger group to produce the CL benzoate ester breakdown product was monitored by analytical HPLC, where the presence of a new peak at 37 min, relative to intact **NTR-CL-E1** (41 min. elution time) appears only with NTR incubation (relative percentages

by peak area reported above each peak). D) The peak at 37 min was confirmed to be the CL benzoate ester by HRMS. Expected mass 1143.75. Observed is $[M+H]^+$ = 1144.7537. Incubation conditions: PBS pH 7.4 (1% DMSO) at 37°C. HPLC conditions: milli-q water + ACN (0.1% formic acid). λ_{abs} 540 nm. Measurements were performed in duplicate.

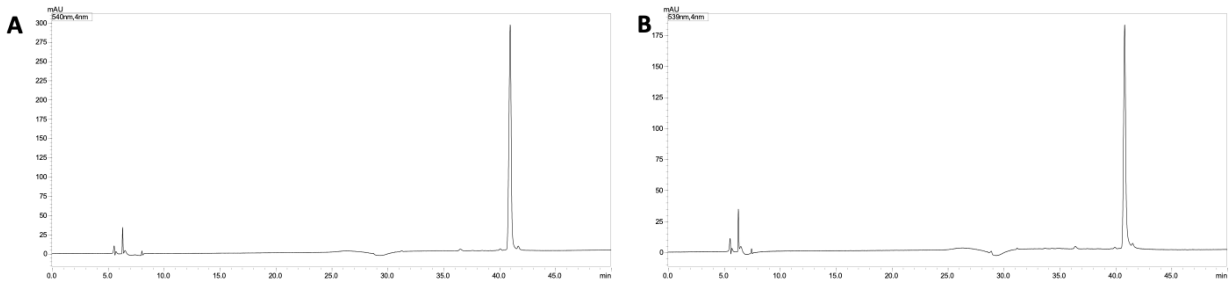


Figure S23. Overnight incubation of **CL-E2** (50 µM) with NADH (200 µM) in the A) presence or B) absence of 1.5 µM NTR. Stability of **CL-E2** monitored by analytical HPLC analysis, where NTR has no effect on **CL-E2** based on the elution time of 41 min remaining the same. Conditions: PBS pH 7.4 (1% DMSO) at 37°C. λ_{abs} 540 nm. Measurements were performed in duplicate.

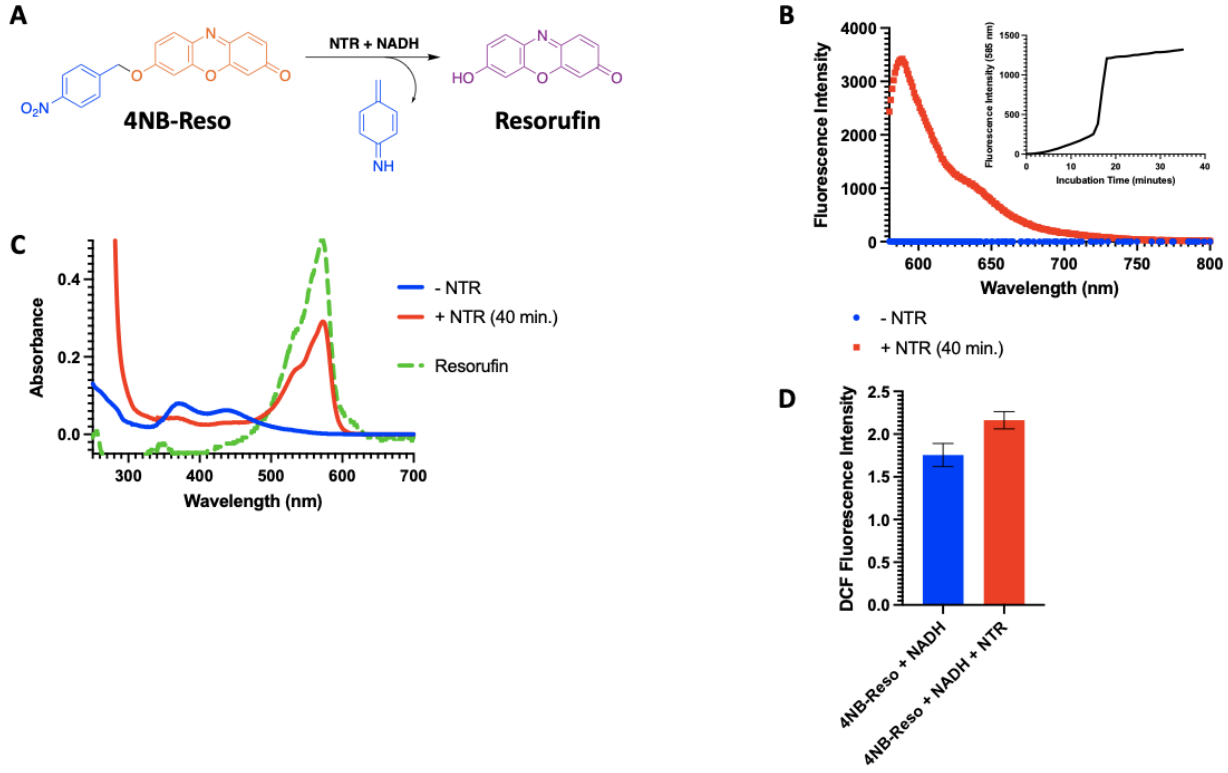


Figure S24. A) Proposed activation mechanism of **4NB-Reso** by NTR to release free Resorufin. B) Initially, the fluorescence of Resorufin is quenched when attached to the 4-nitrobenzyl group (blue). Incubation of **4NB-Reso** (2.5 µM) with 0.5 µM NTR and NADH (100 µM) for 40 min., results in an increase in fluorescence (red) at 585 nm, accompanied by C) a change in absorbance spectrum (blue to red) that matches that of free Resorufin (green dashed). D) Incubation of **4NB-Reso** (10 µM) with the ROS sensor $DCFH_2$ (5 µM) and NADH (100 µM) in the absence vs. presence of NTR (0.5 µM) for 40 min., results in minimal DCF

fluorescence demonstrating that no significant ROS is produced during the reduction of the nitro group by NTR. Conditions: PBS pH 7.4 (5% DMSO) at 37°C. λ_{ex} 490 nm. Measurements were performed in triplicate.

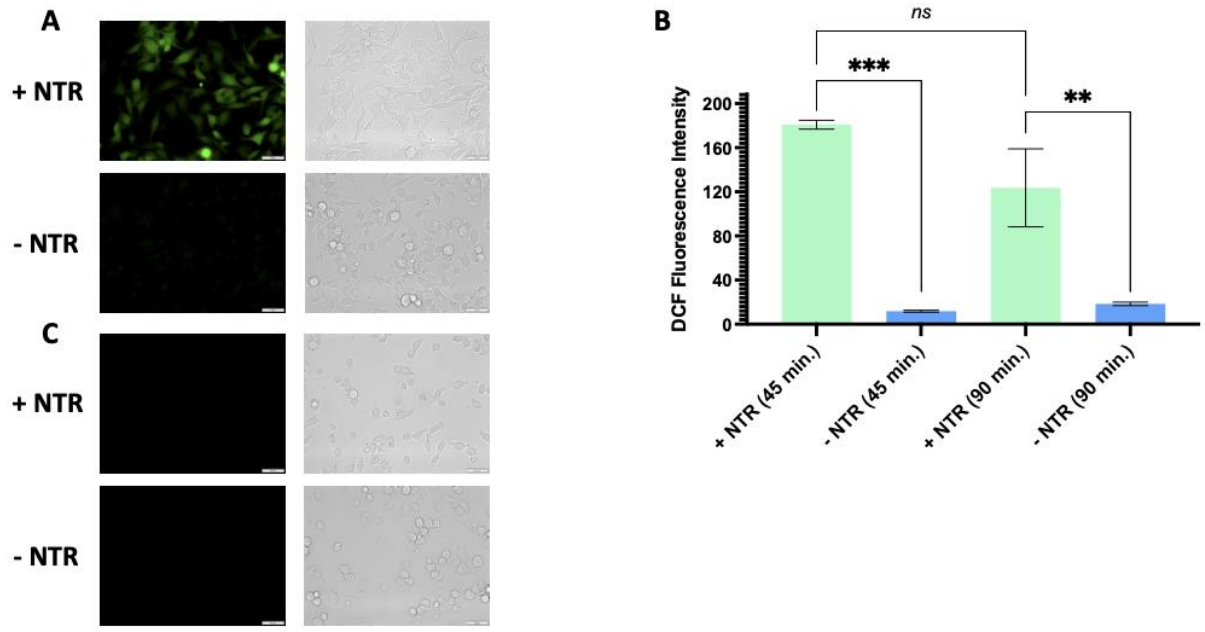


Figure S25. ROS production by **NTR-CL-E1** in MDA-MB231 triple negative breast cancer cells +/- NTR expression. A) Cells were incubated with **NTR-CL-E1** (10 µM) for 60 min., followed by the addition of the general ROS sensor, $DCFH_2$-DA (10 µM) for an additional 30 min. (90 min. total incubation with **NTR-CL-E1**). Increase in the green fluorescence from the oxidized ROS sensor (DCF) in the presence of **NTR-CL-E1** was only present in cells expressing NTR, demonstrating selective activation and ROS production. B) Quantification of the mean fluorescence intensity of DCF for both 45 min and 90 min incubation show a statistically significant increase in ROS production by **NTR-CL-E1** in the NTR-expressing MDA-MB231 cells compared to those without NTR. Quantification of the mean fluorescence intensity of DCF was analyzed by two-tailed *t*-test, *p*-value < 0.001 indicated by *. C) Background fluorescence from $DCFH_2$ only (10 µM) in both MDA-MB-231 cells (+/- NTR). 20x, scale bar = 50 µm. ROS sensor imaged by: λ_{ex} 470-490 nm, λ_{em} 500-550 nm. Experiments were performed in triplicate.

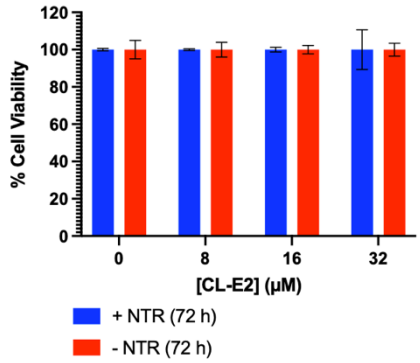
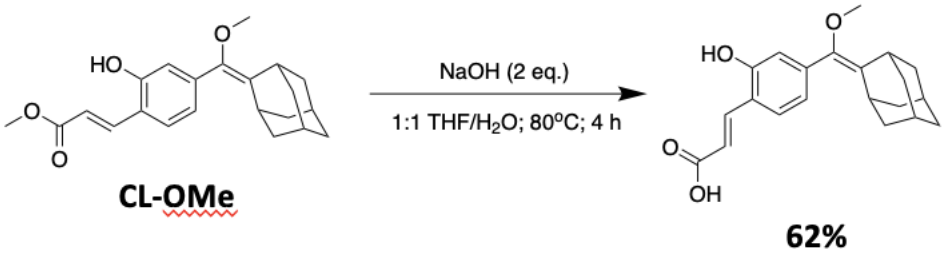
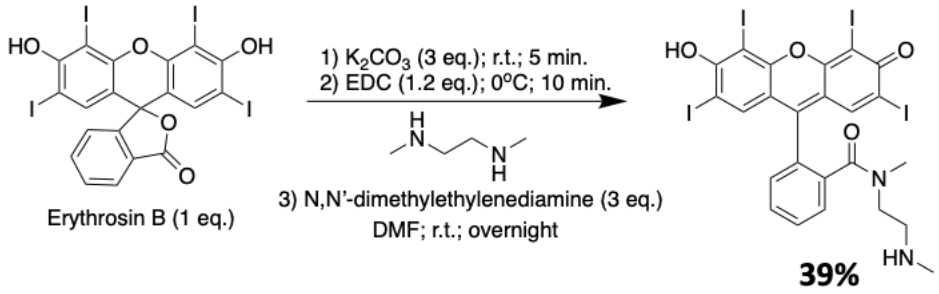


Figure S26. Incubation of **CL-E2** in MDA-MB231 triple negative breast cancer cells +/- NTR expression for 72 hours shows minimal cytotoxicity in cells expressing NTR (blue), and without NTR expression (red), highlighting the requirement for an NTR-responsive trigger group to initiate chemiexcitation and singlet oxygen production to exert cytotoxicity. Measurements were performed in triplicate.

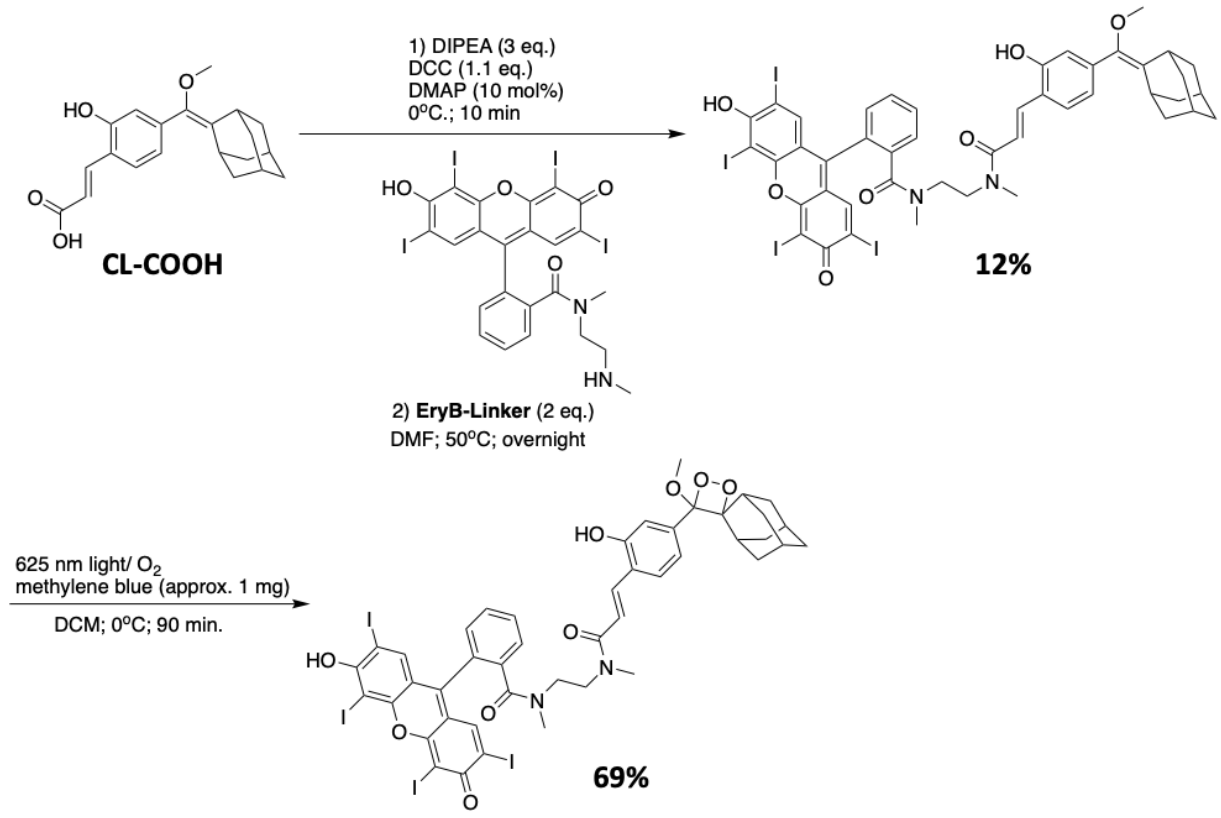
Synthetic Schemes



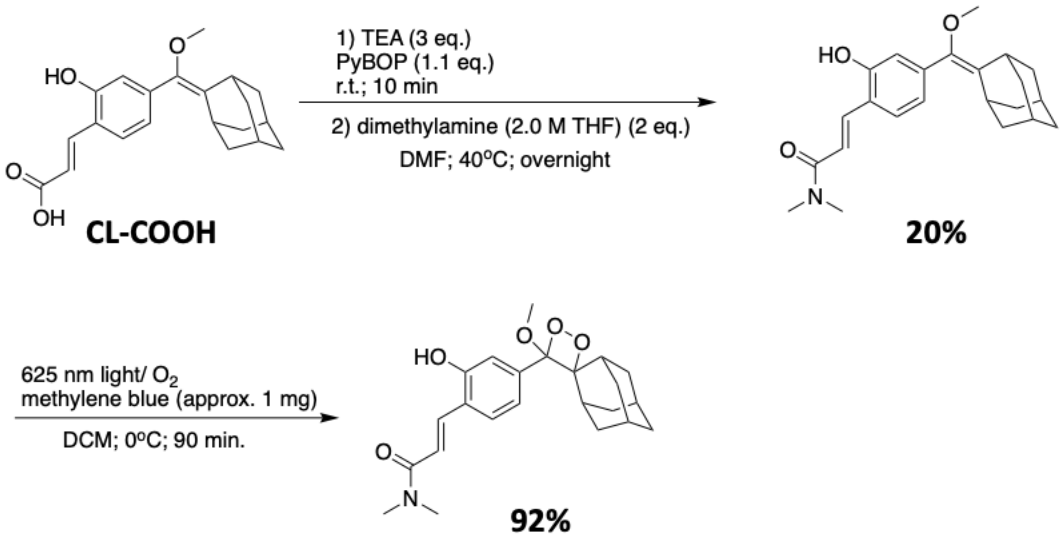
Scheme S1. Synthesis of compound **CL-COOH**.



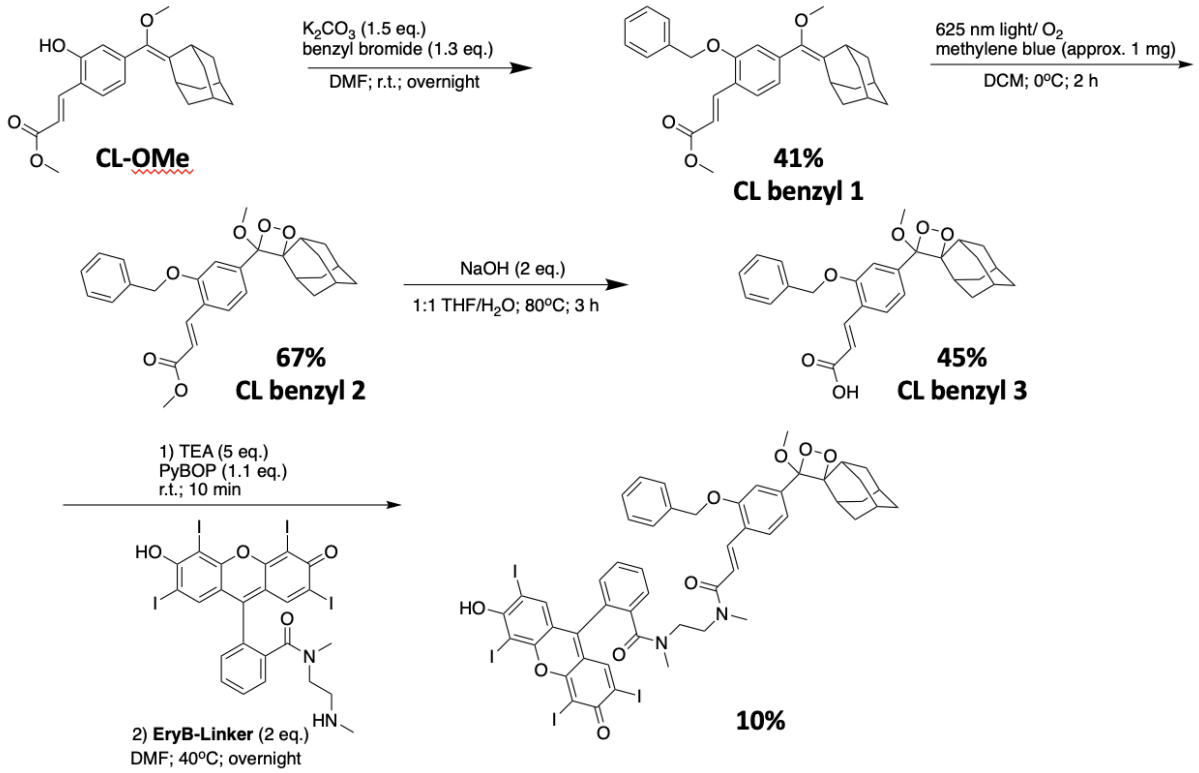
Scheme S2. Synthesis of compound **EryB-Linker**.



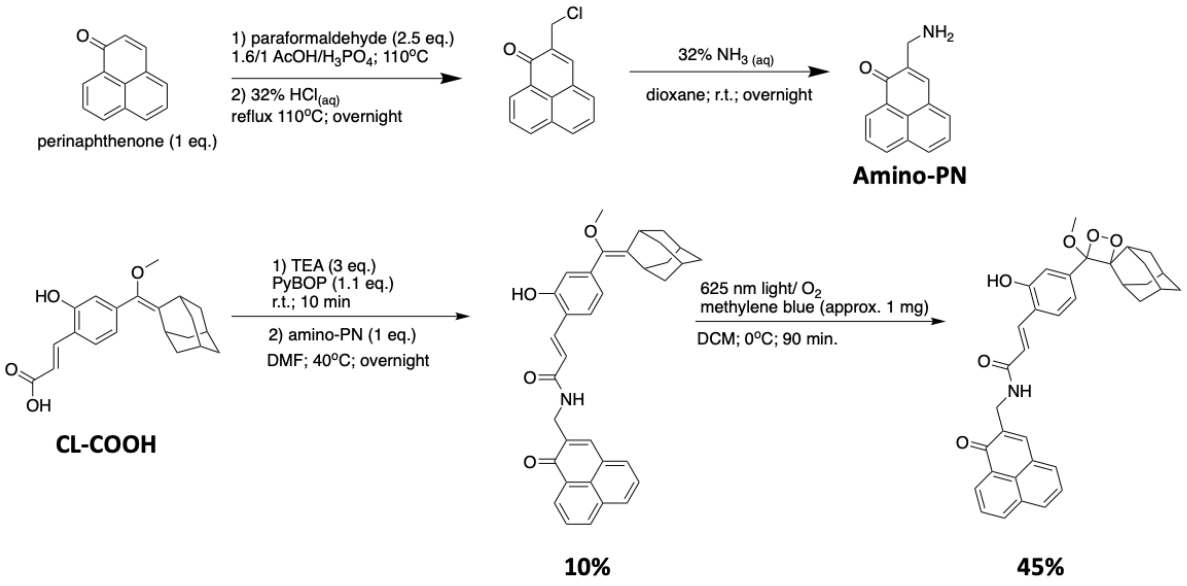
Scheme S3. Synthesis of compound **CL-E1**.



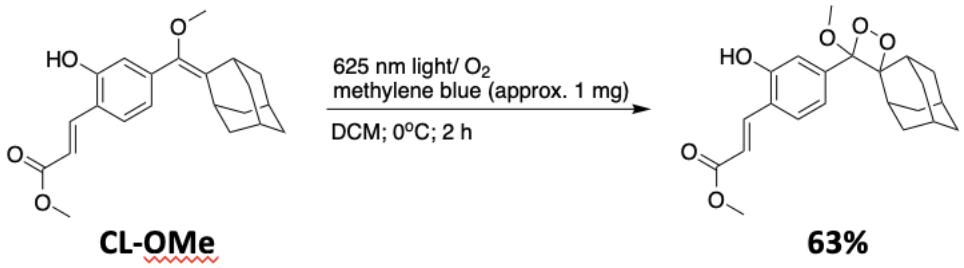
Scheme S4. Synthesis of compound **CL-A**.



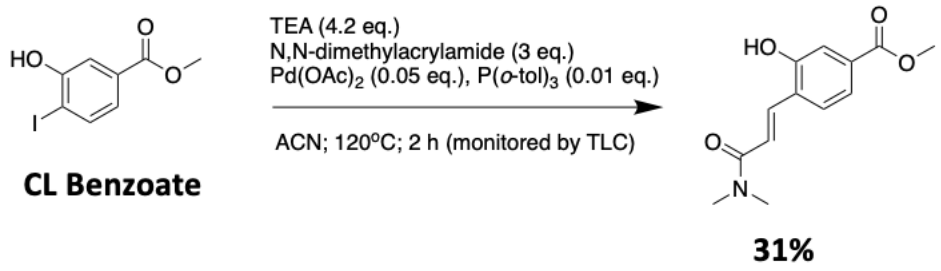
Scheme S5. Synthesis of compound **CL-E2**.



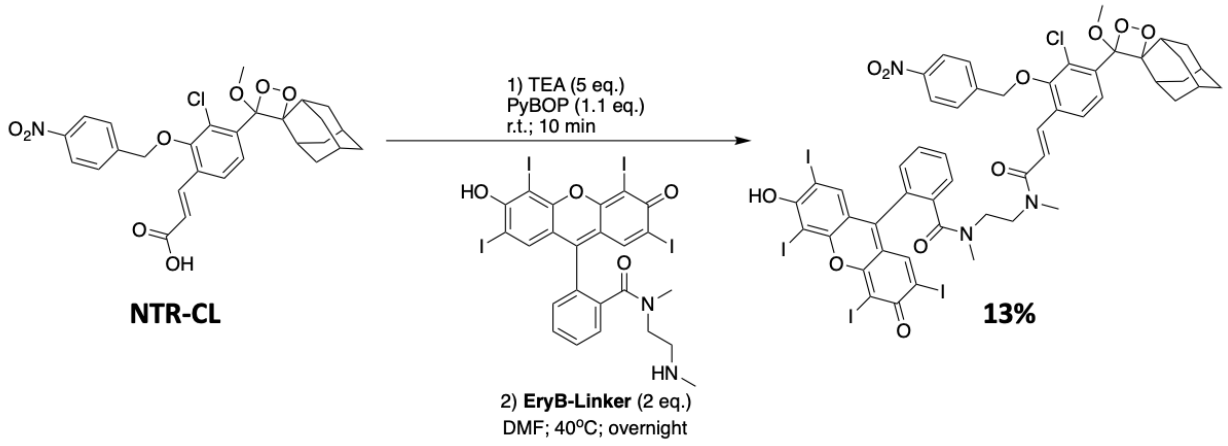
Scheme S6. Synthesis of compound **CL-PN**.



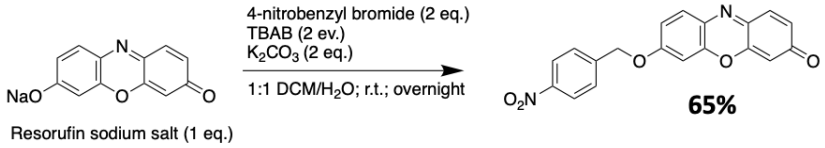
Scheme S7. Synthesis of compound **CL-OMe dioxetane**.



Scheme S8. Synthesis of compound **CL-A Benzoate**.



Scheme S9. Synthesis of **NTR-CL-E1**.



Scheme S10. Synthesis of **4NB-Reso**.

Compound Characterization

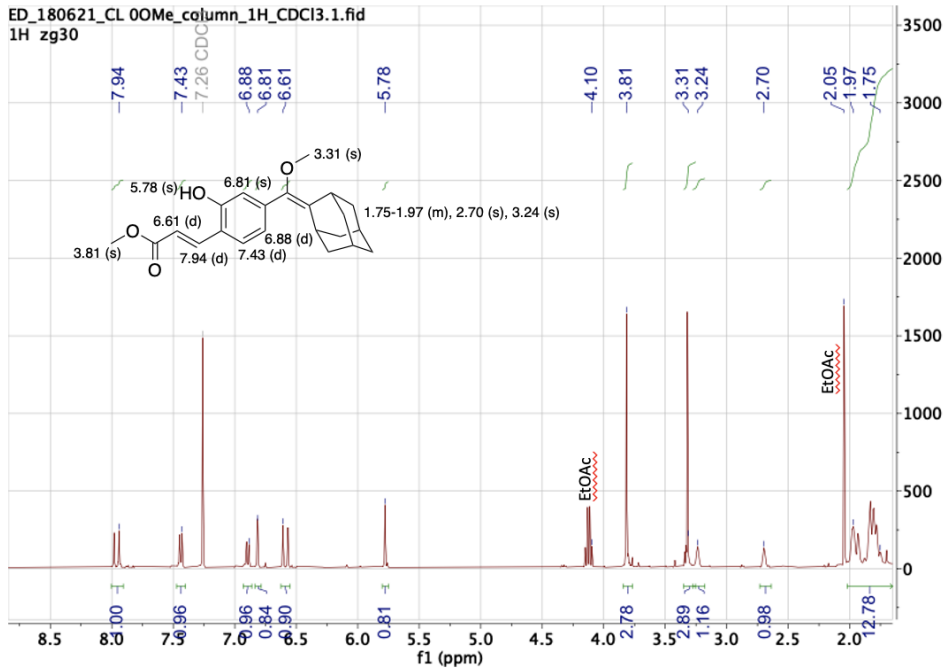


Figure S26. ^{1}H NMR of **CL-OMe** in chloroform-*d*.

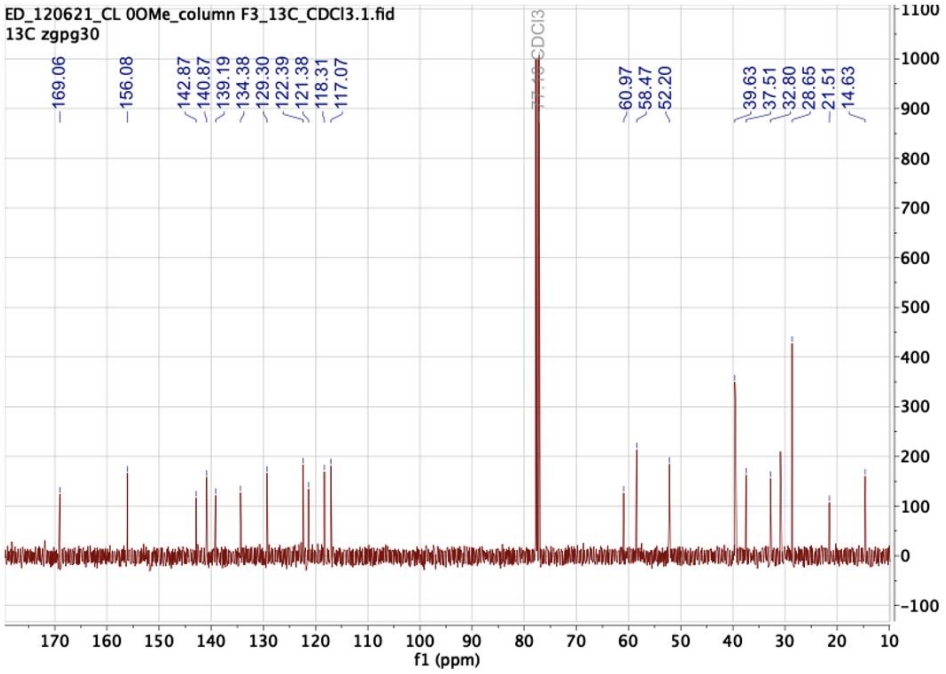


Figure S27. ^{13}C NMR of **CL-OMe** in chloroform-*d*.

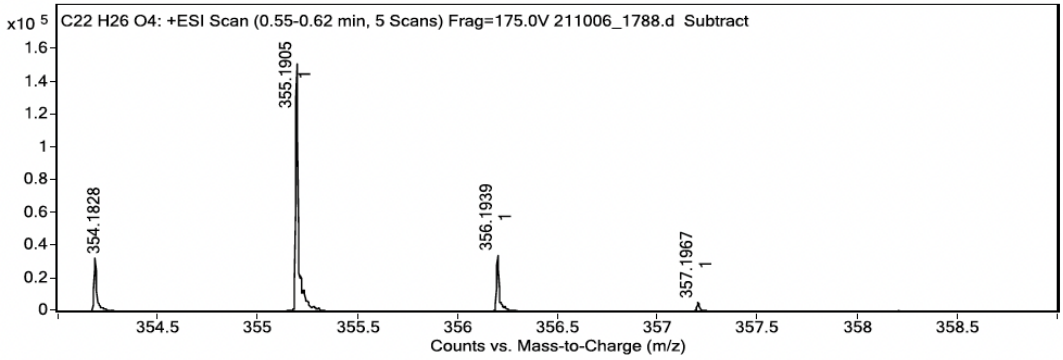


Figure S28. HRMS of **CL-OMe**. Expected mass 354.18. Observed is $[M+H]^+$ = 355.1905.

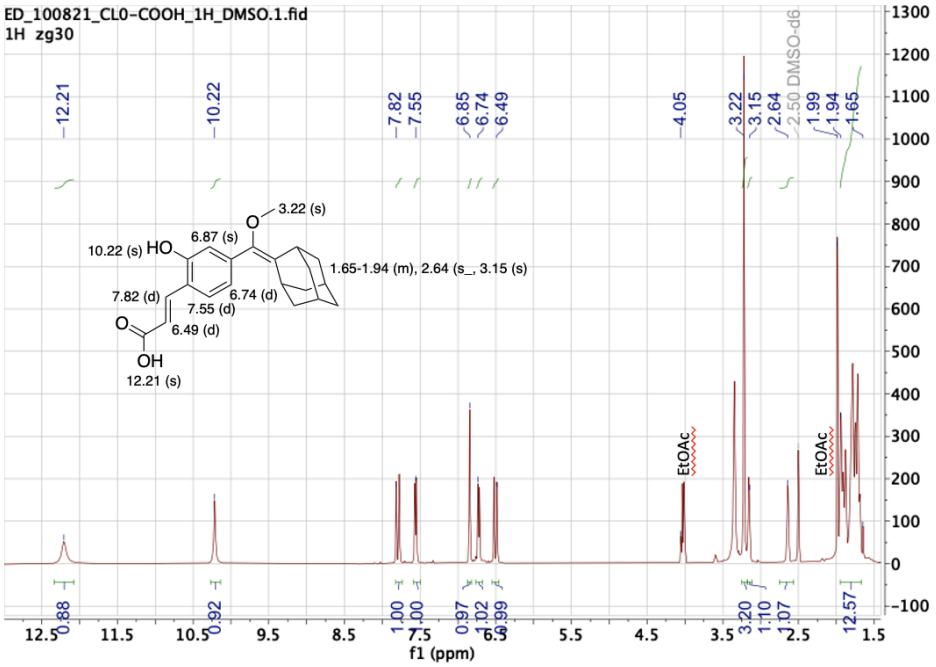


Figure S29. ^{1}H NMR of **CL-COOH** in d_6-DMSO.

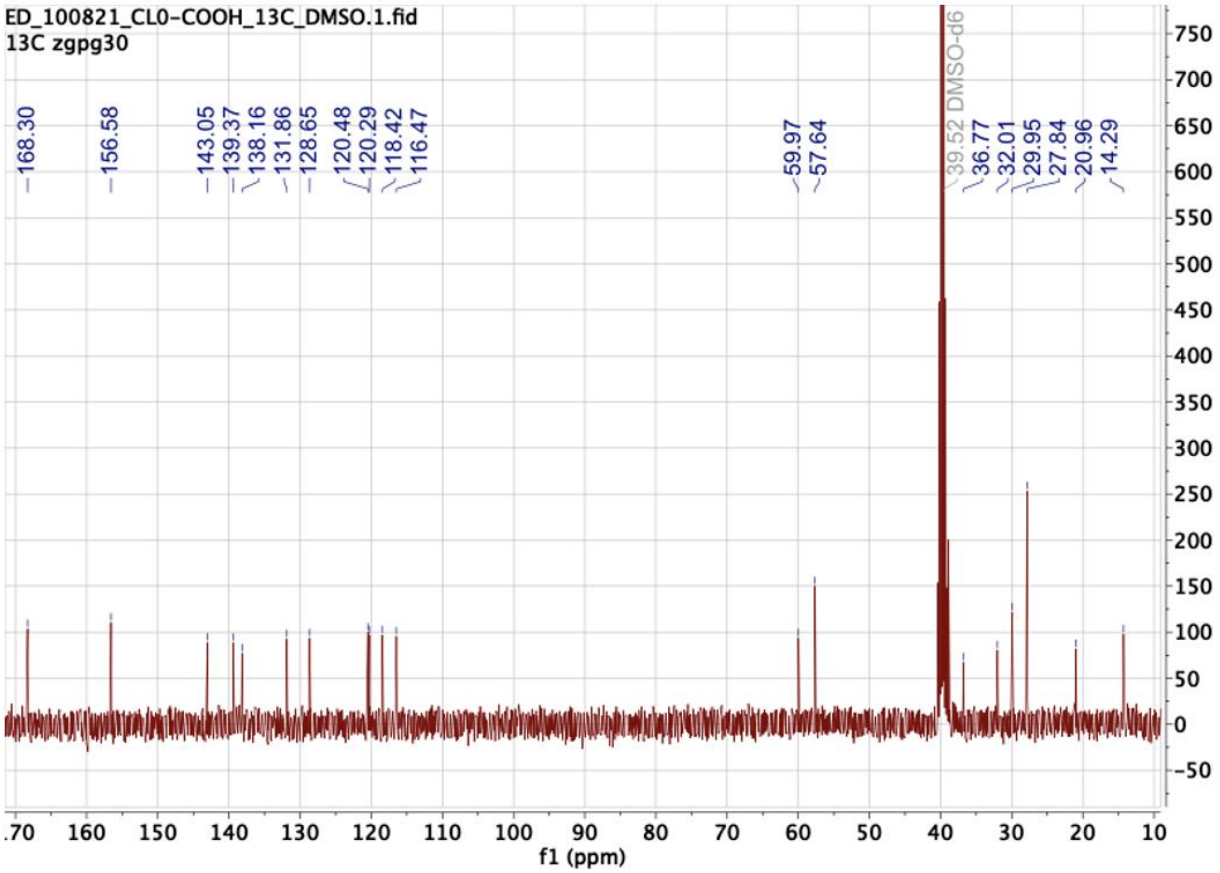


Figure S30. ^{13}C NMR of **CL-COOH** in d_6-DMSO.

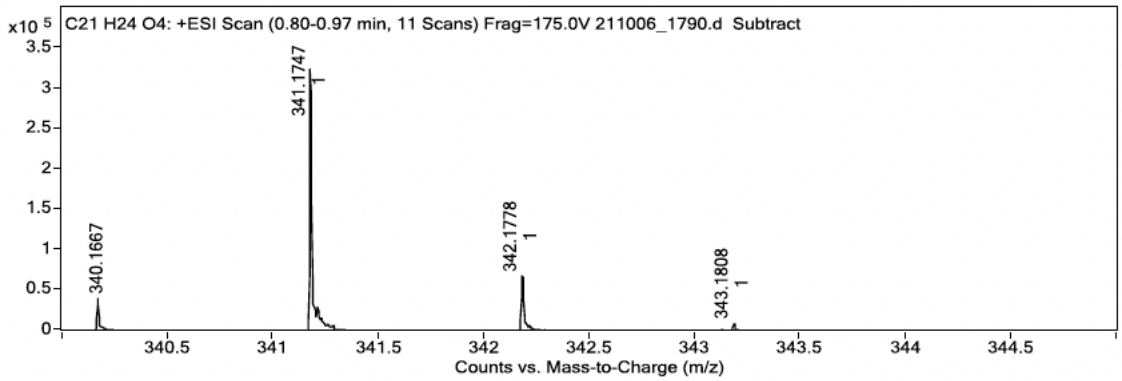


Figure S31. HRMS of **CL-COOH**. Expected mass 340.17. Observed is $[M+H]^+$ = 341.1747.

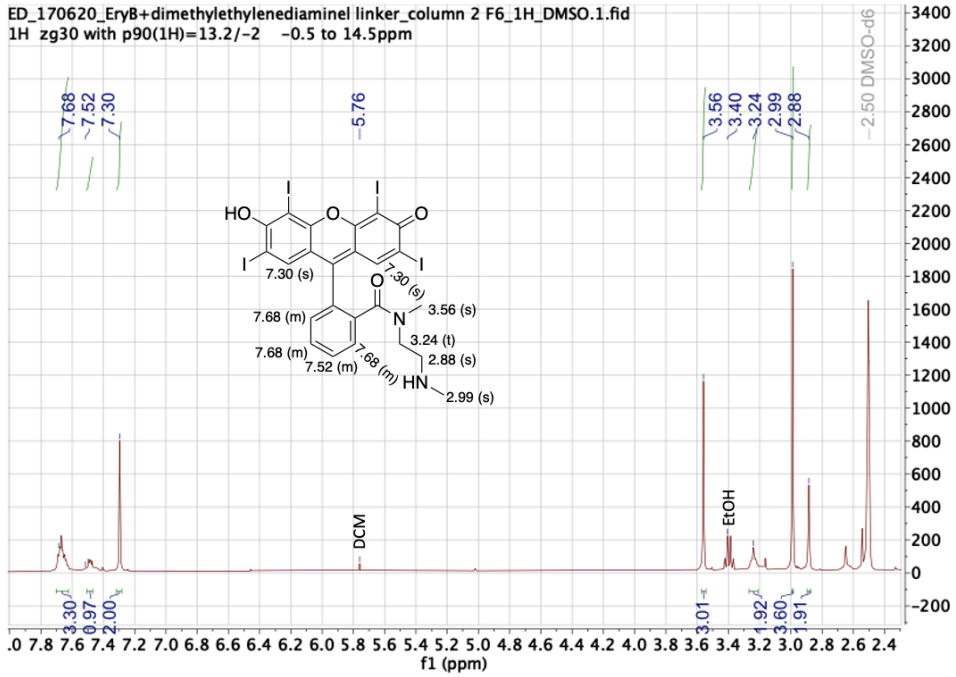


Figure S32. ^{1}H NMR of **EryB-Linker** in d_6-DMSO.

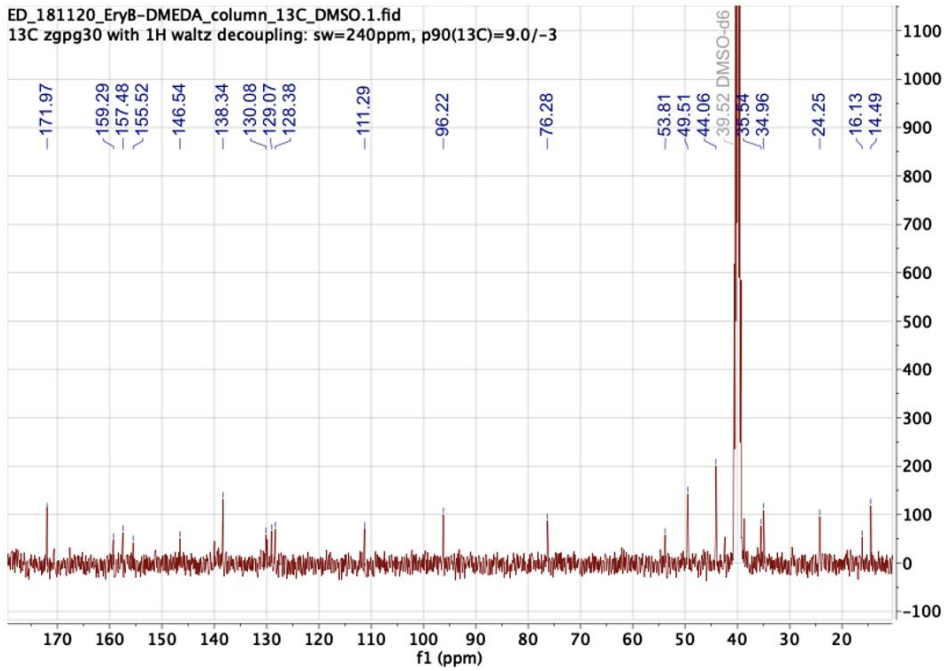


Figure S33. ^{13}C NMR of **EryB-Linker** in d_6-DMSO.

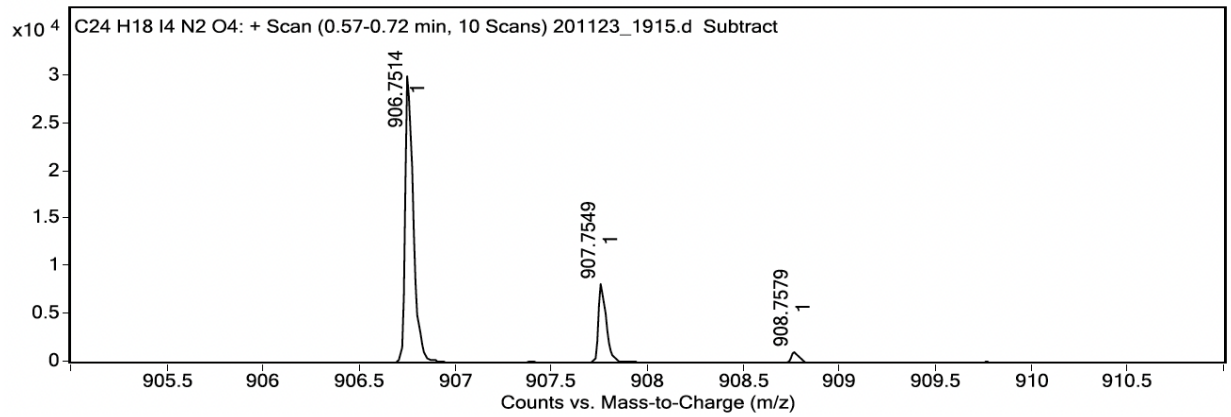


Figure S34. HRMS of **EryB-Linker**. Expected mass 905.74. Observed is $[M+H]^+$ = 906.7514.

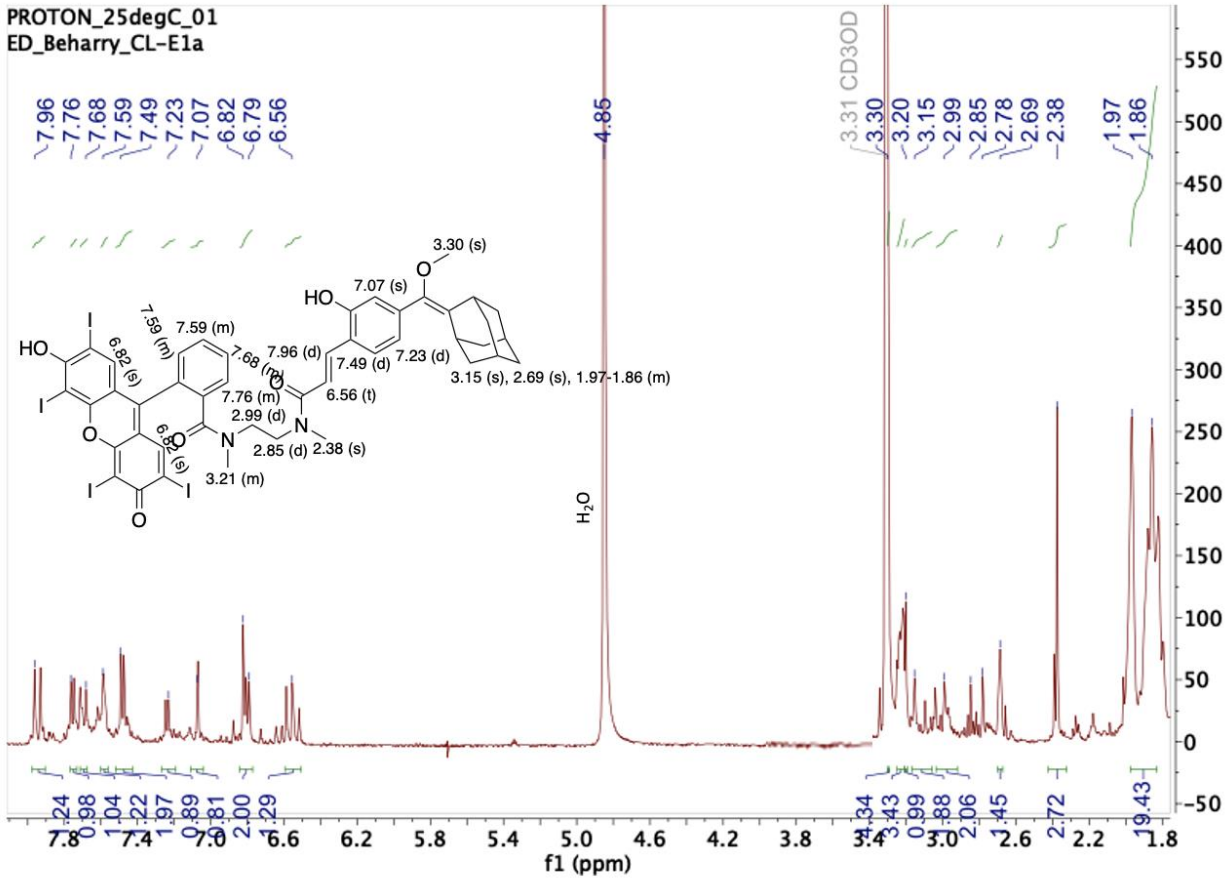


Figure S35. ^{1}H NMR of **CL-E1a** in *d*$_4$-methanol.

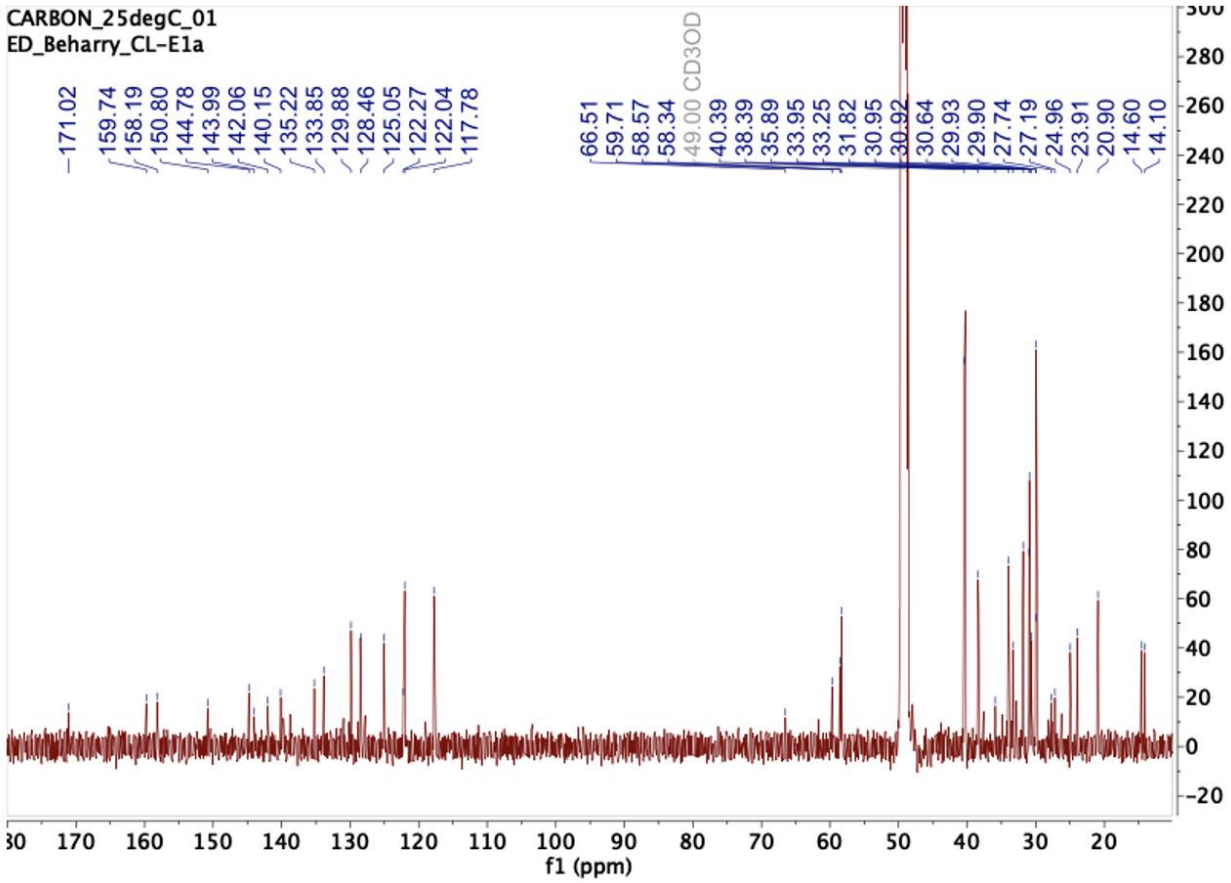


Figure S36. ^{13}C NMR of **CL-E1a** in d_4-methanol.

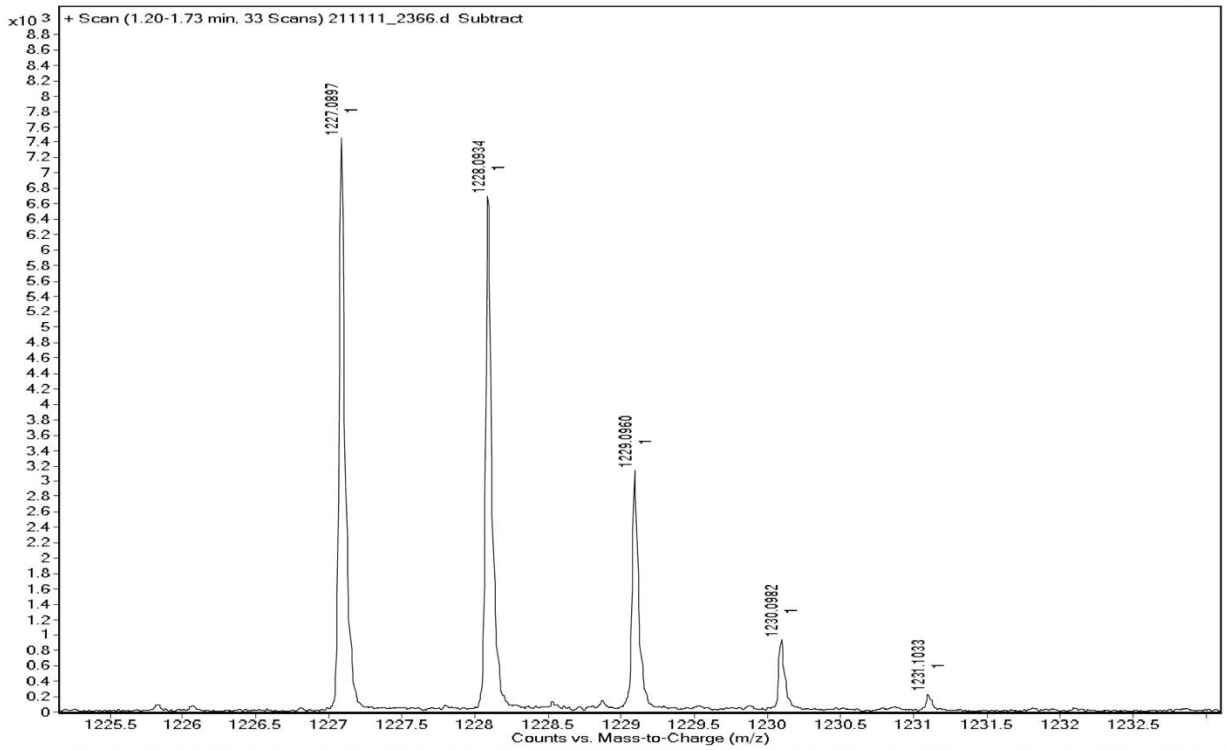


Figure S37. HRMS of **CL-E1a**. Expected mass 1227.89. Observed is [M] = 1227.0897.

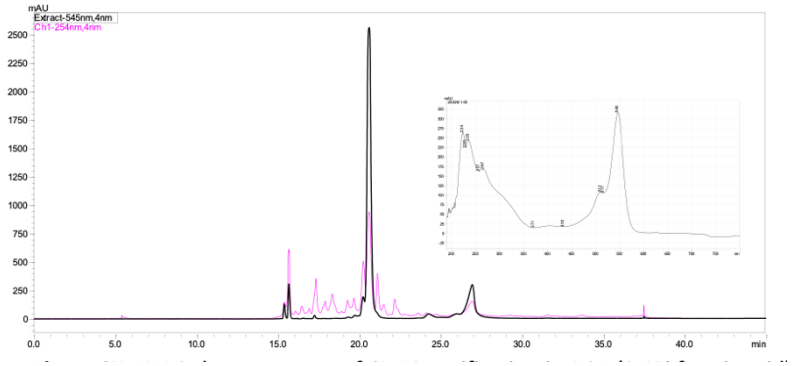


Figure S38. HPLC chromatogram of **CL-E1**, purification in ACN (0.1% formic acid). Product elutes at 20 min.

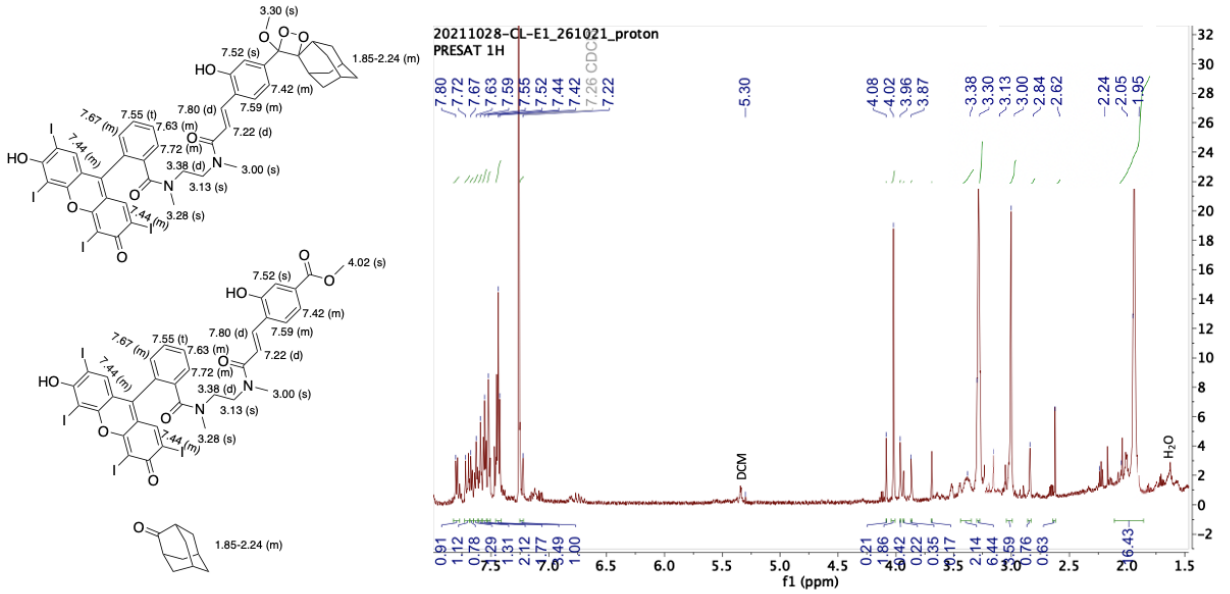


Figure S39. ^{1}H NMR of **CL-E1** in chloroform-*d*.

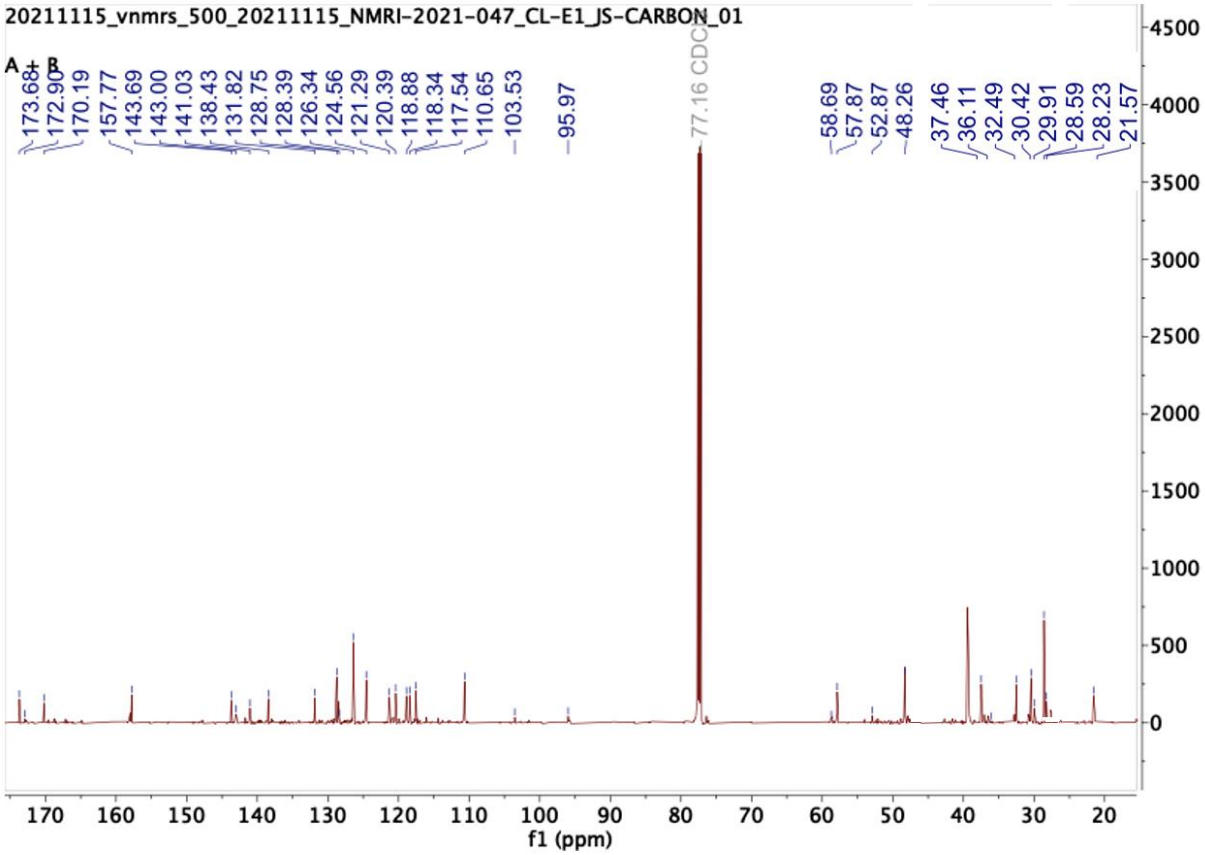


Figure S40. ^{13}C NMR of **CL-E1** in chloroform-*d*.

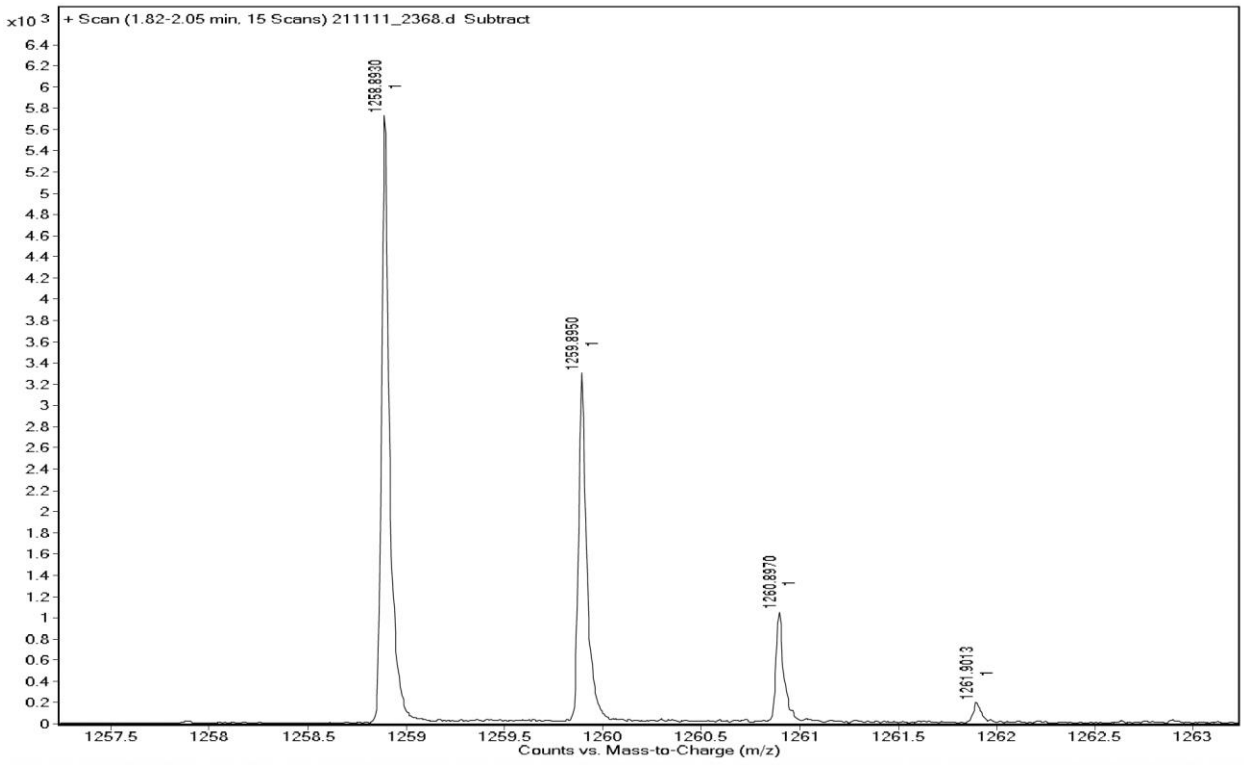


Figure S41. HRMS of **CL-E1**. Expected mass 1258.90. Observed is [M] = 1258.8930.

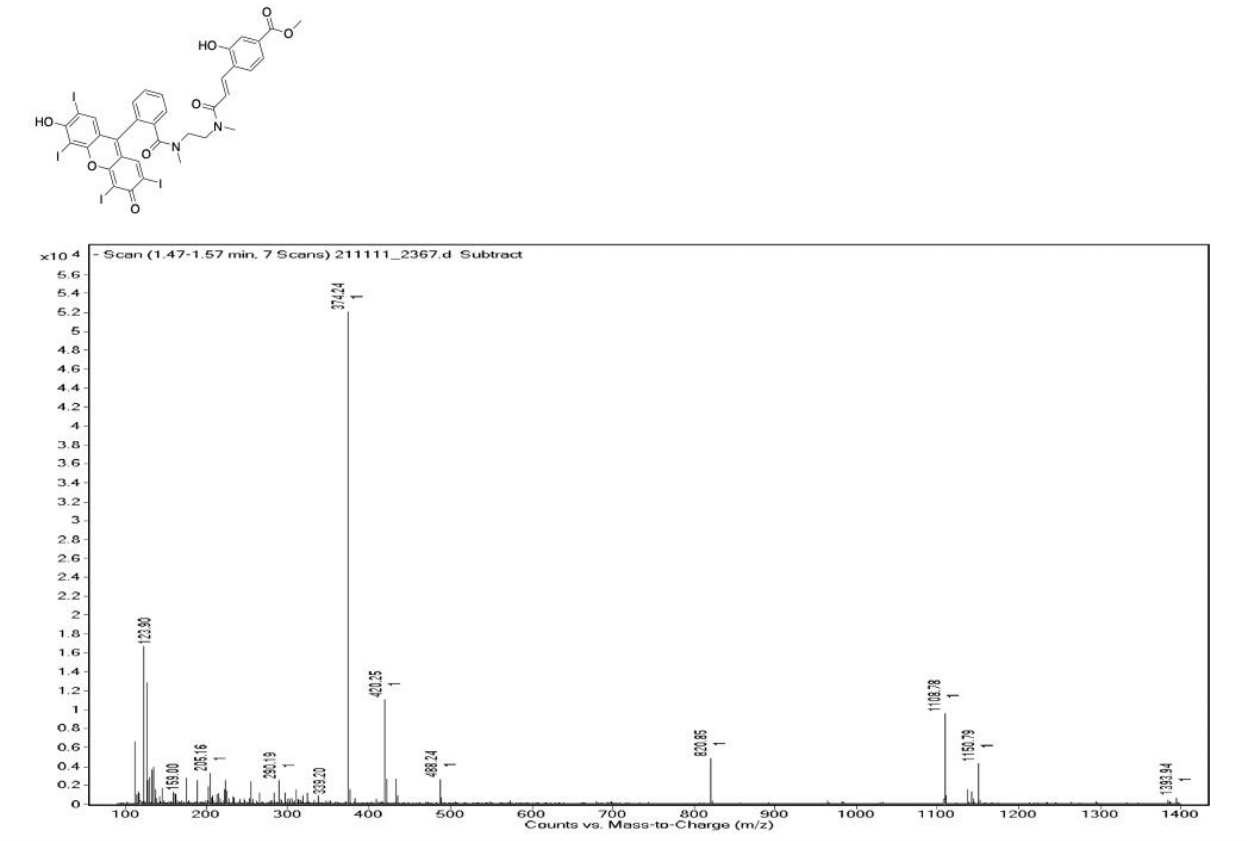


Figure S42. MS of **CL-E1** breakdown product. Expected mass 1109.79. Observed is $[M-H]^-$ = 1108.78.

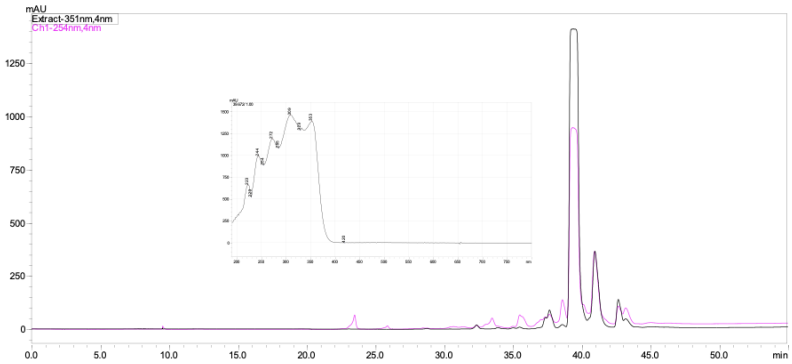


Figure S43. HPLC chromatogram of **CL-Aa** purification in ACN (0.1% formic acid). Product elutes at 39 min.

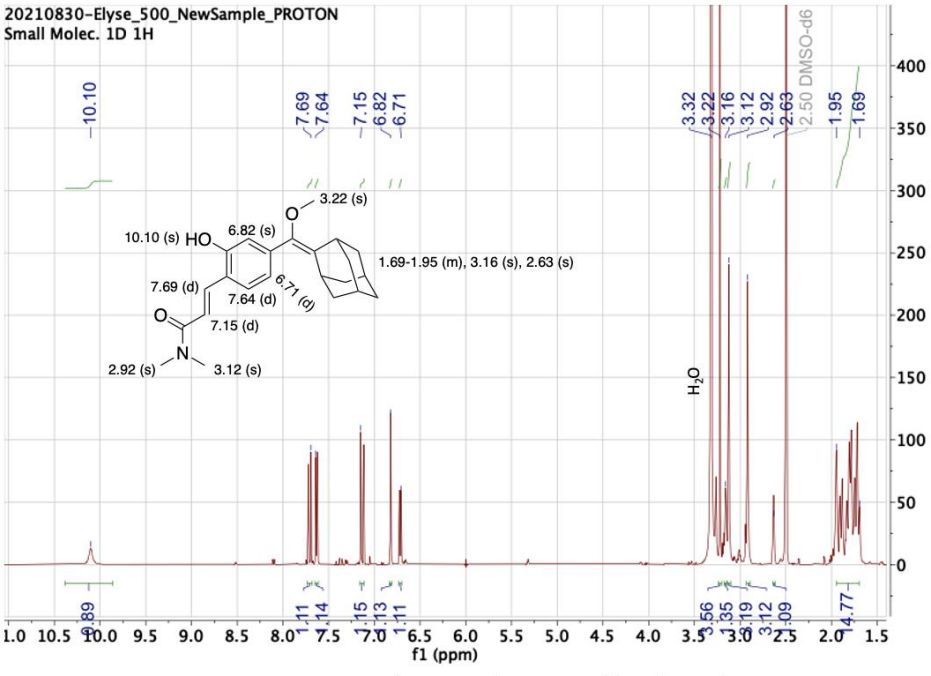


Figure S44. ^{1}H NMR of **CL-Aa** in chloroform-*d*.

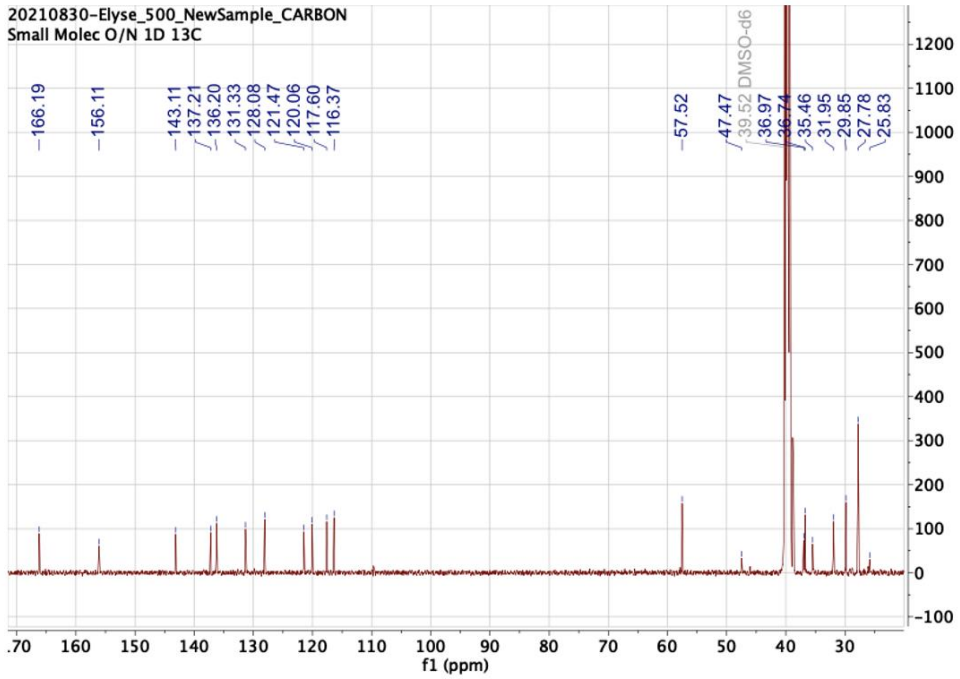


Figure S45. ^{13}C NMR of **CL-Aa** in chloroform-*d*.

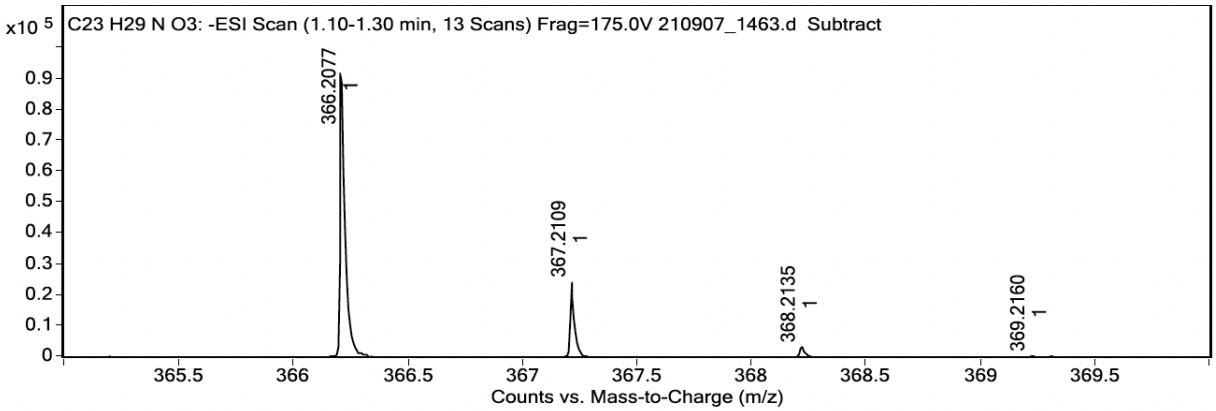


Figure S46. HRMS of **CL-Aa**. Expected mass 367.21. Observed is $[M-H]^-$ = 366.2077.

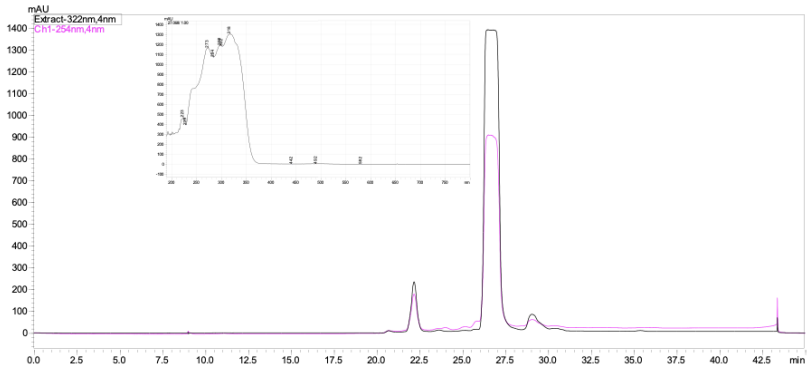


Figure S47. HPLC chromatogram of **CL-A** purification in ACN (0.1% formic acid). Product elutes at 26 min.

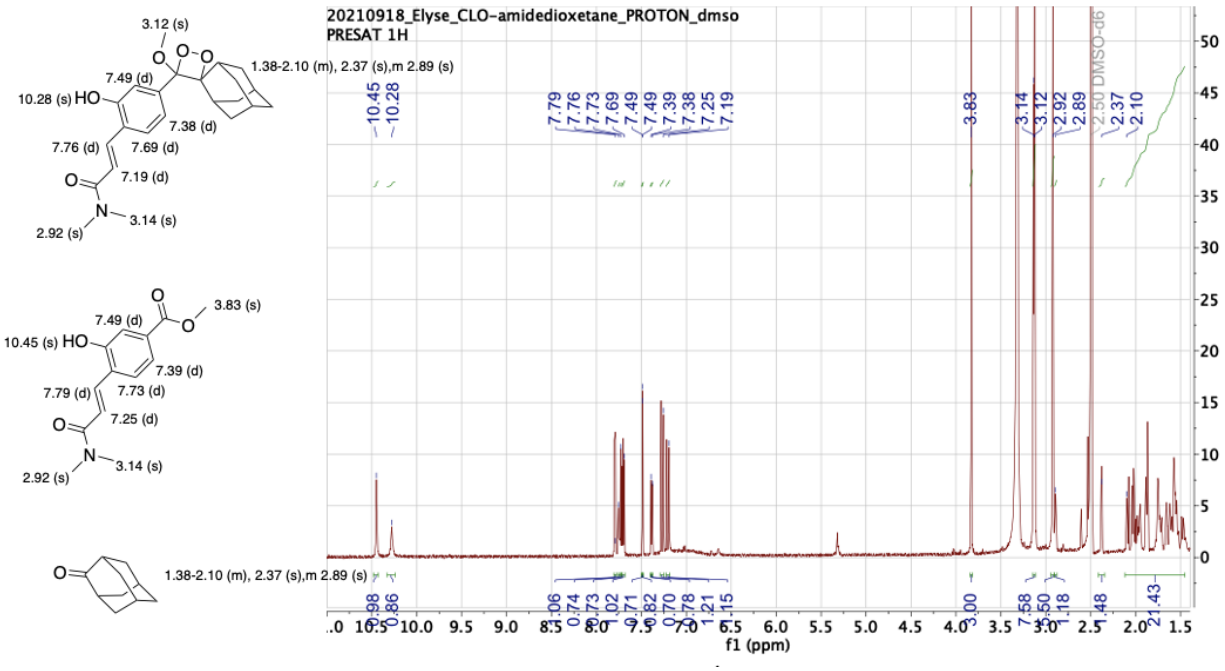


Figure S48. ^{1}H NMR of **CL-A** in chloroform-*d*.

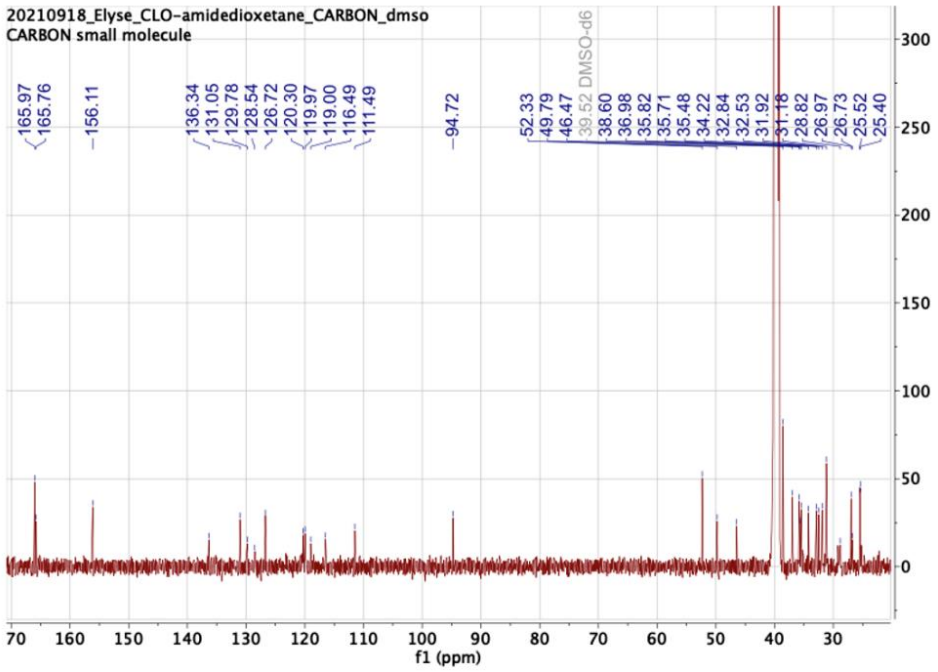


Figure S49. ^{13}C NMR of **CL-A** in chloroform-*d*.

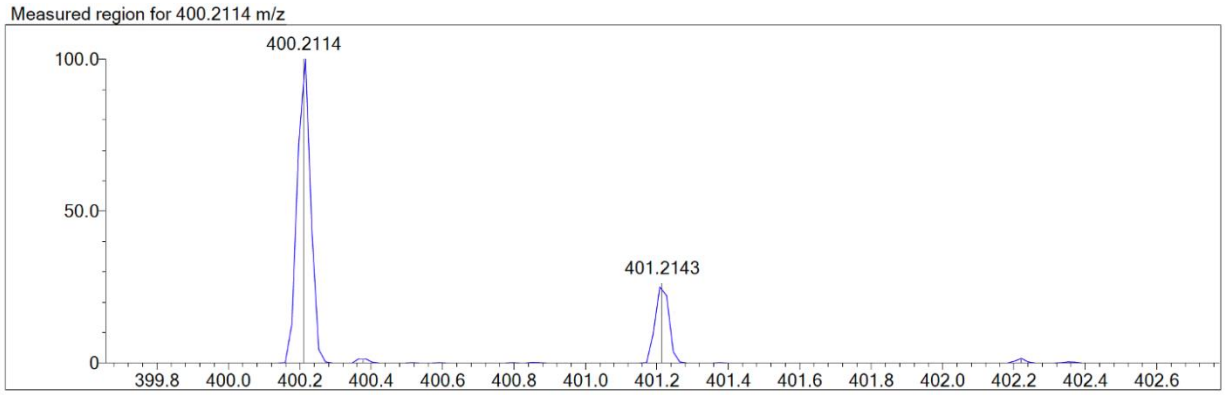


Figure S50. HRMS of **CL-A**. Expected mass 399.20. Observed is $[M+H]^+$ = 400.2114.

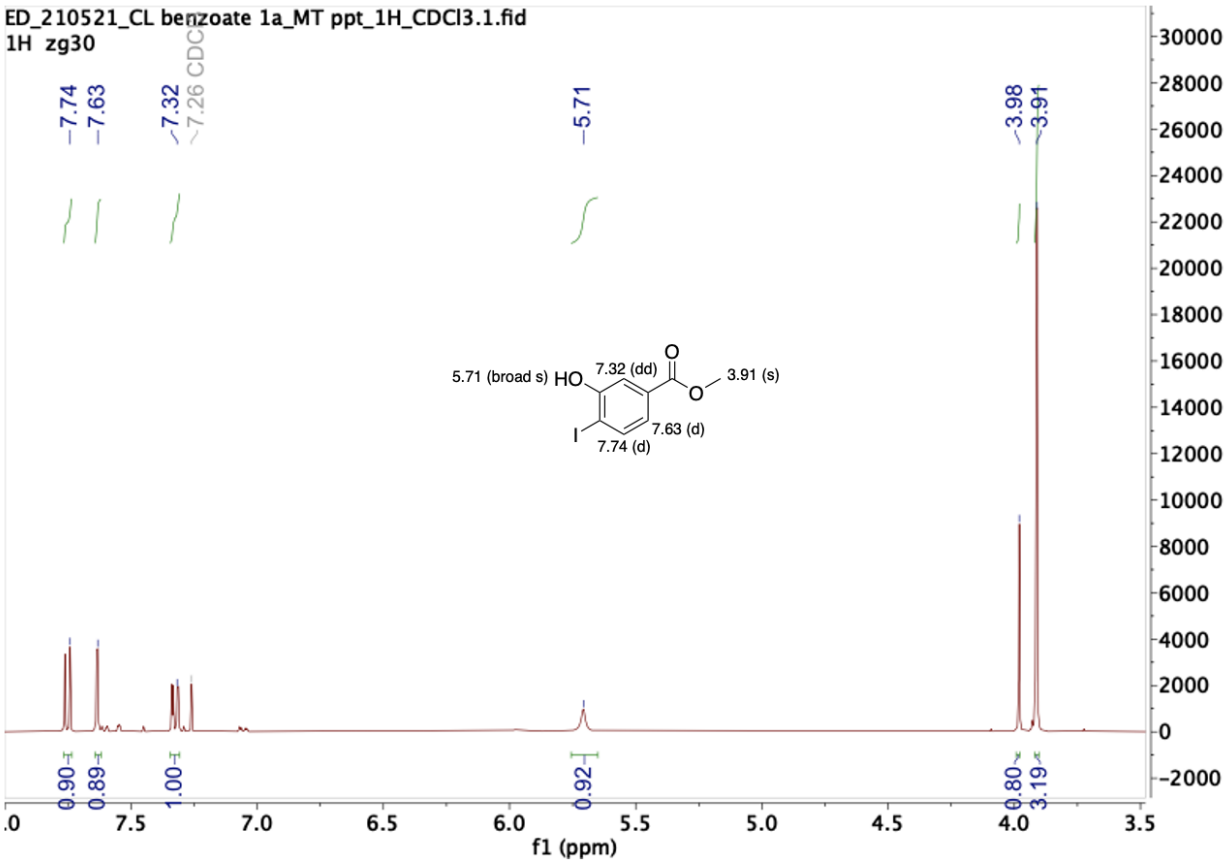


Figure S51. ^{1}H NMR of **CL Benzoate** in chloroform-*d*.

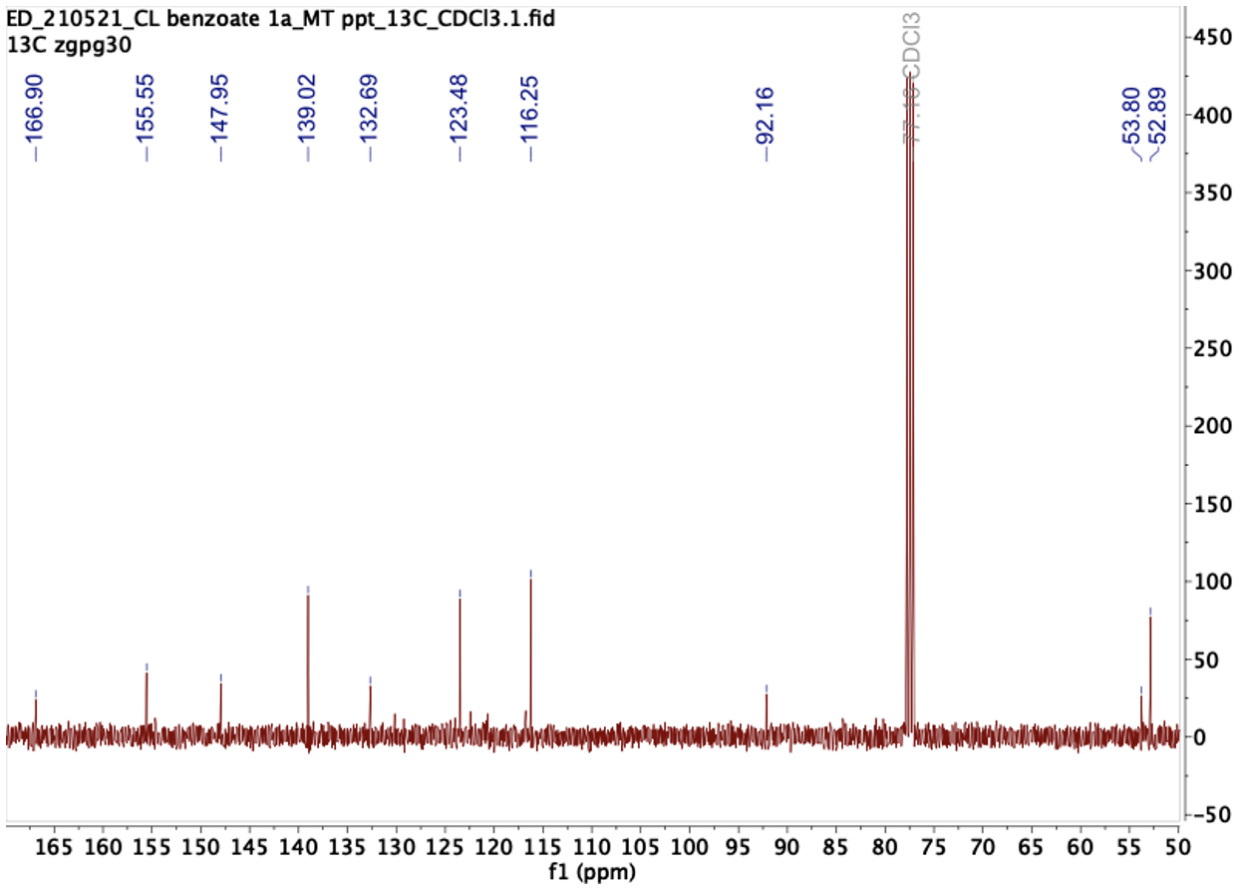


Figure S52. ^{13}C NMR of **CL Benzoate** in chloroform-*d*.

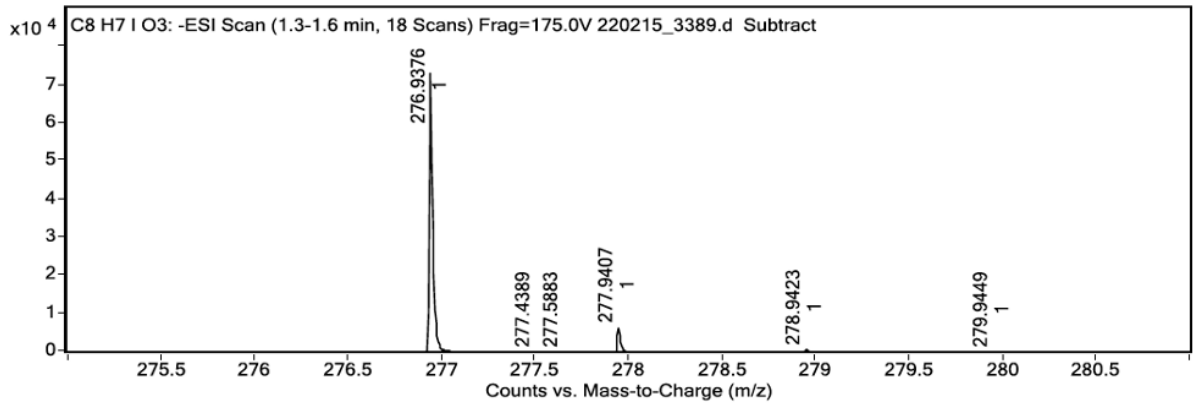


Figure S53. HRMS of **CL Benzoate**. Expected mass 277.94. Observed is $[M-H]^-$ = 276.9376.

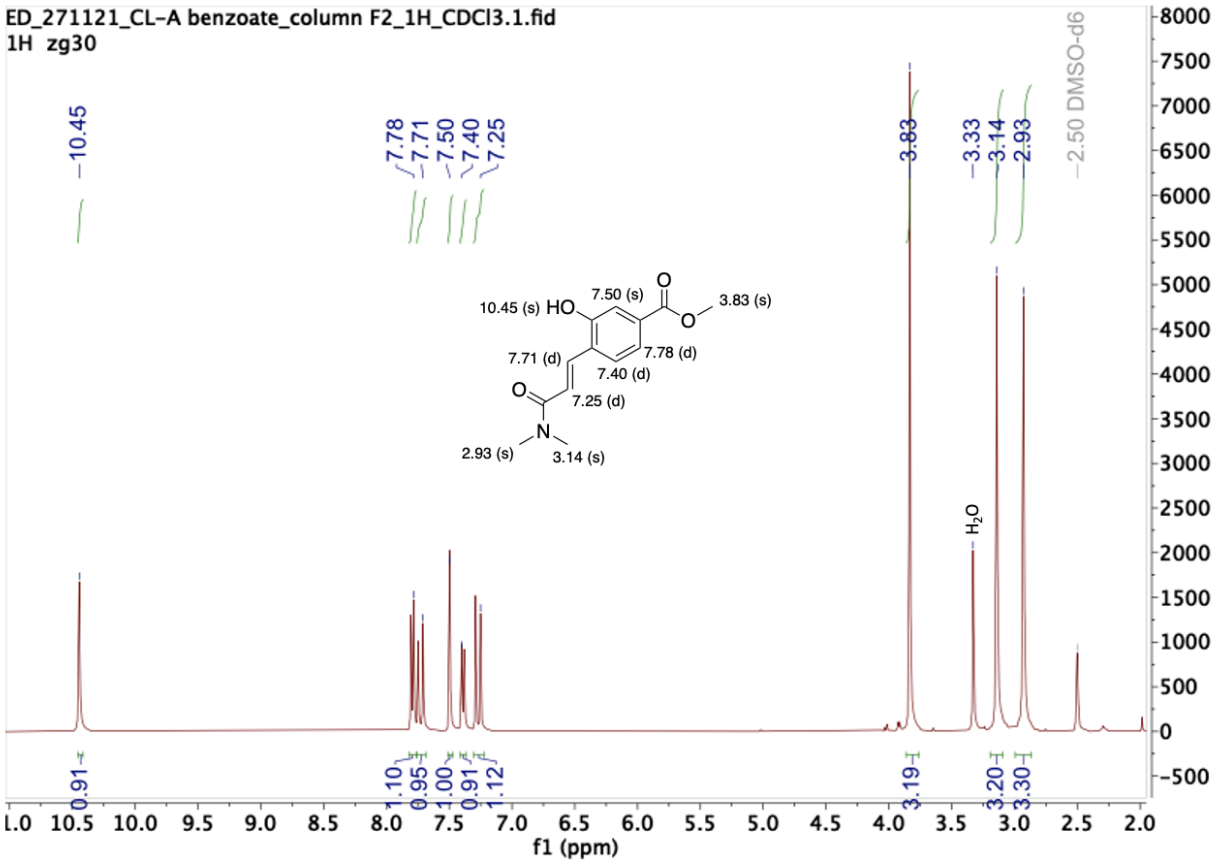


Figure S54. ^{1}H NMR of **CL-A Benzoate** in d_6-DMSO.

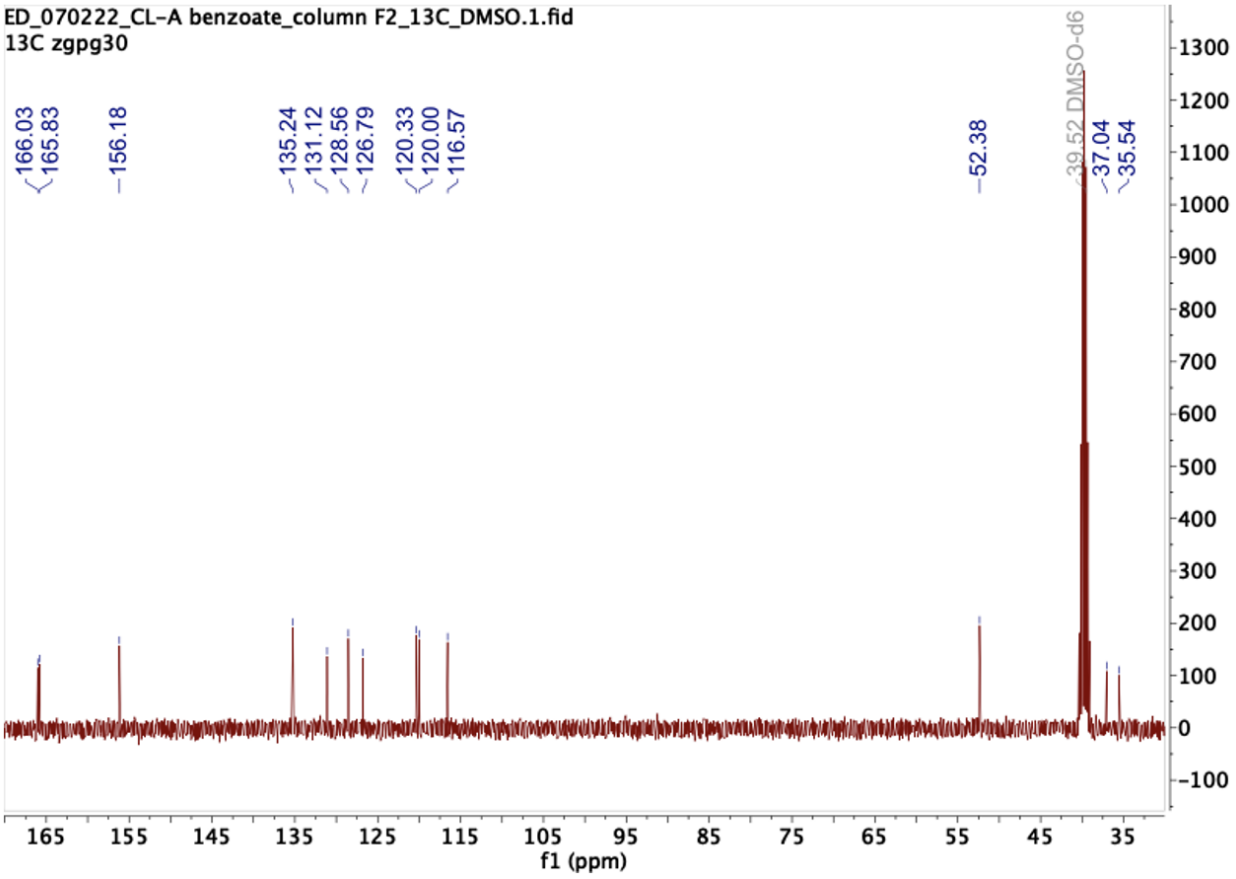


Figure S55. ^{13}C NMR of **CL-A Benzoate** in d_6-DMSO.

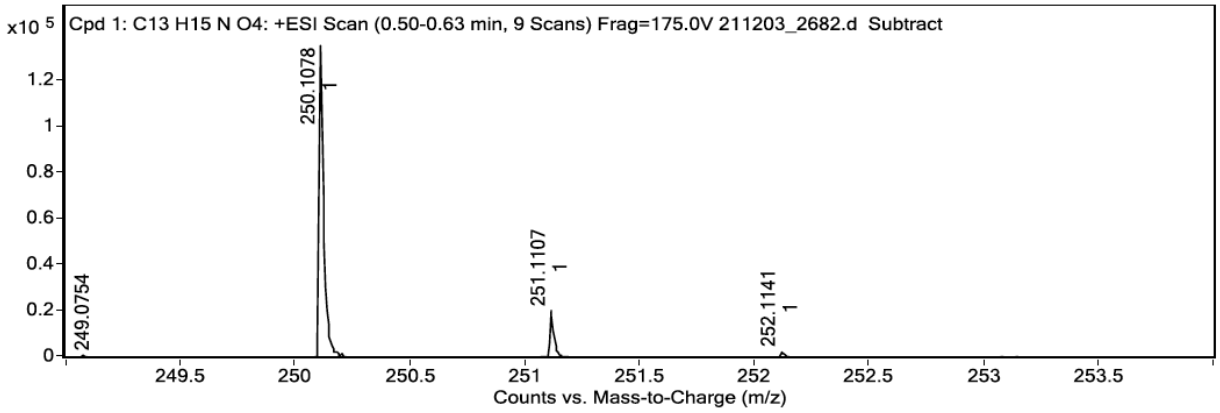


Figure S56. HRMS of **CL-A Benzoate**. Expected mass 249.10. Observed is $[M+H]^+$ = 250.1078.

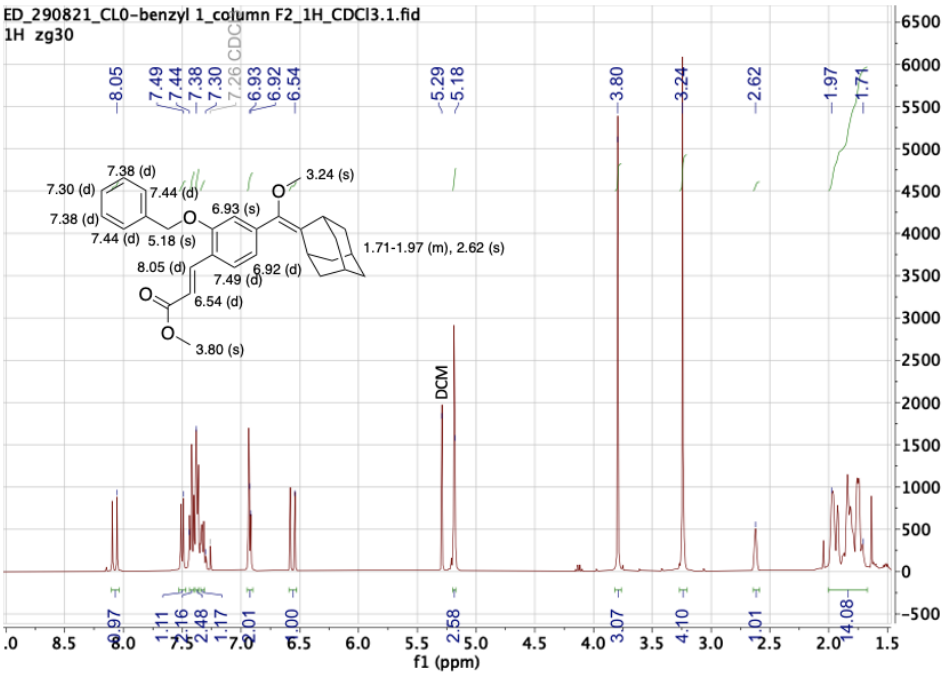


Figure S57. ^{1}H NMR of **CL-benzyl 1** in chloroform-*d*.

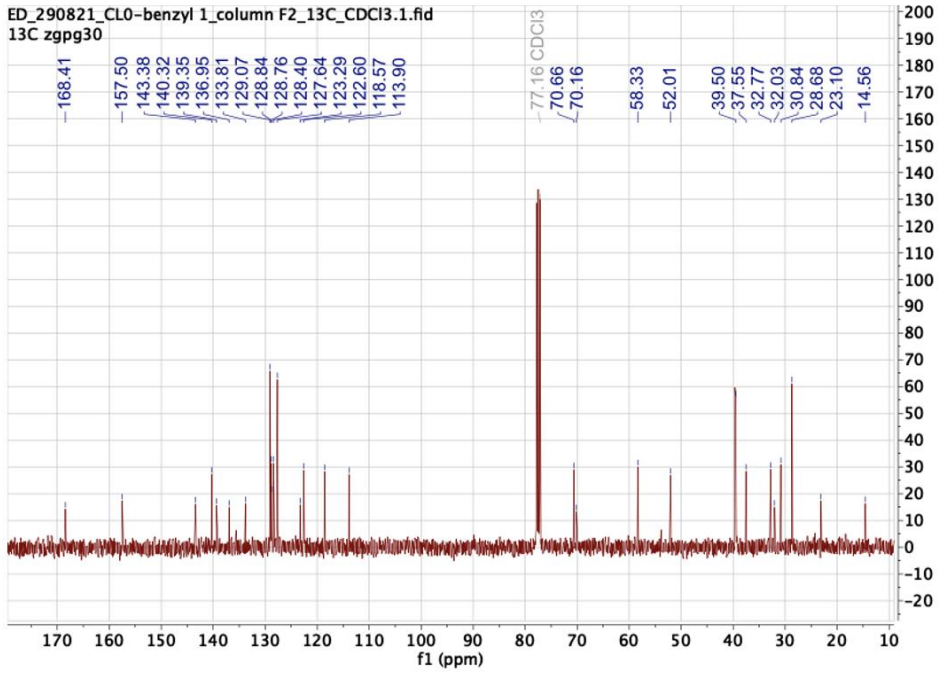


Figure S58. ^{13}C NMR of **CL-benzyl 1** in chloroform-*d*.

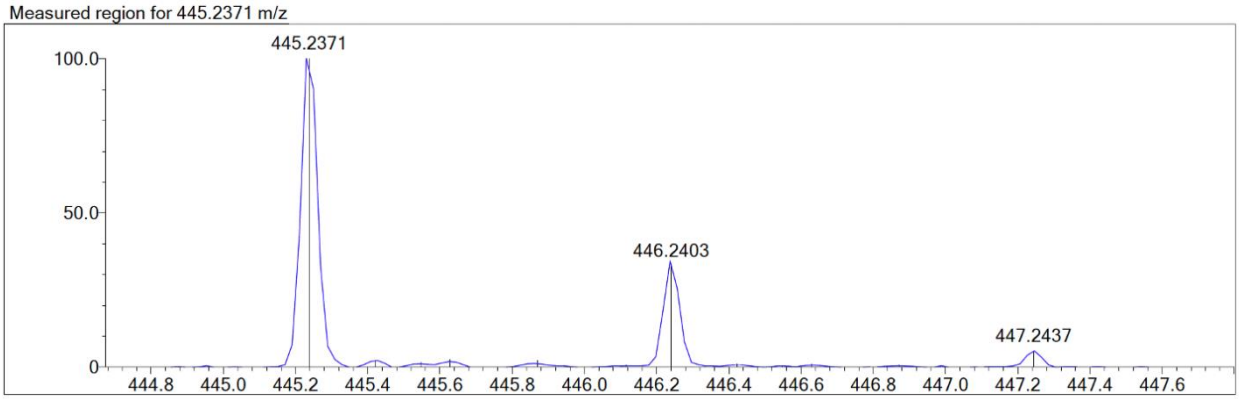


Figure S59. HRMS of **CL-benzyl 1**. Expected mass 444.23. Observed is $[M+H]^+$ = 445.2371.

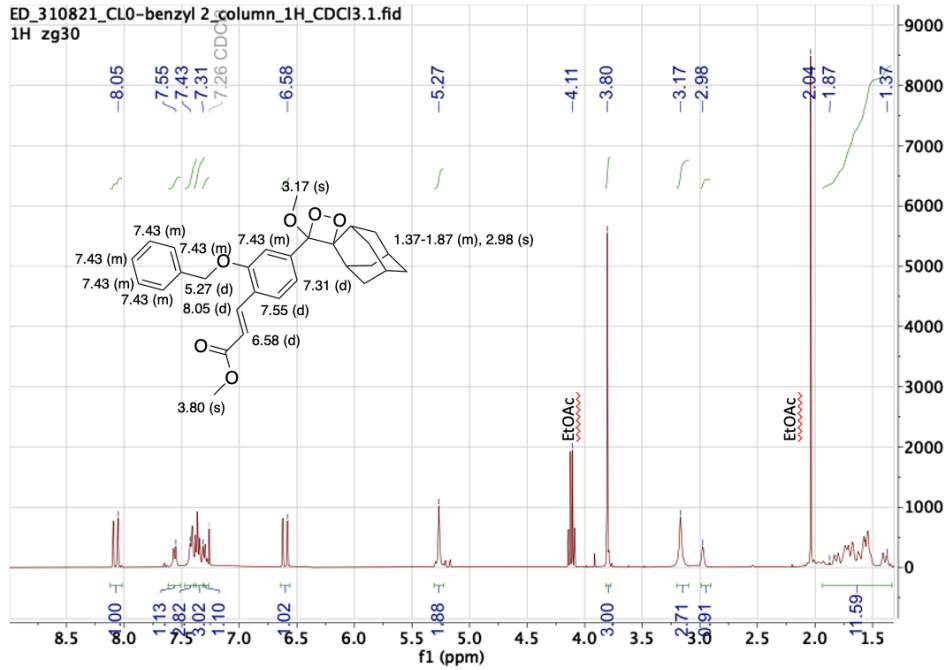


Figure S60. ^{1}H NMR of **CL-benzyl 2** in chloroform-*d*.

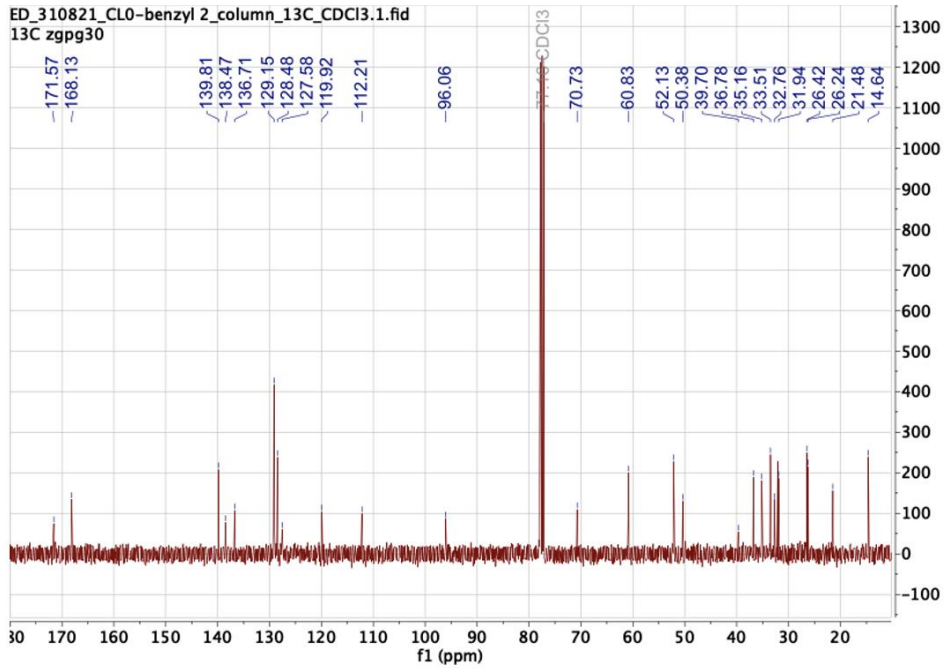


Figure S61. ^{13}C NMR of **CL-benzyl 2** in chloroform-*d*.

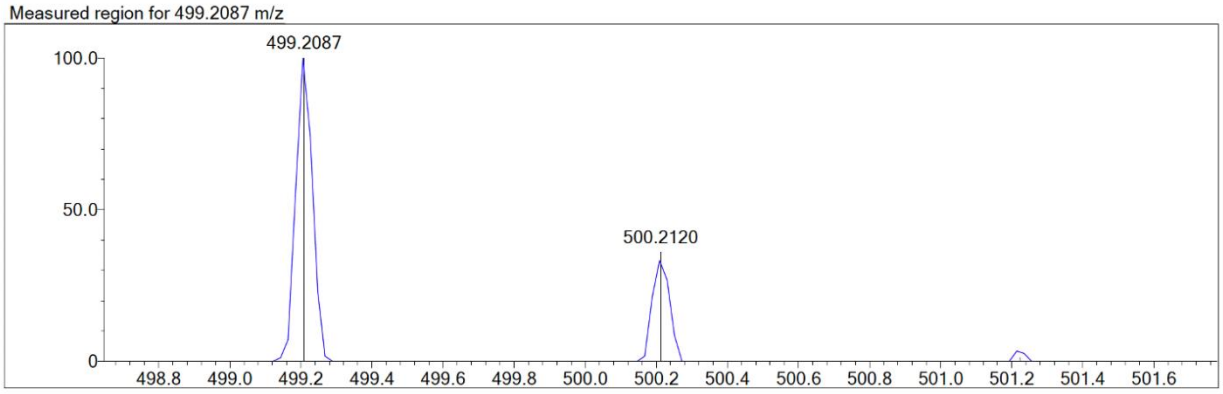


Figure S62. HRMS of **CL-benzyl 2**. Expected mass 476.22. Observed is $[M+Na]^+$ = 499.2087.

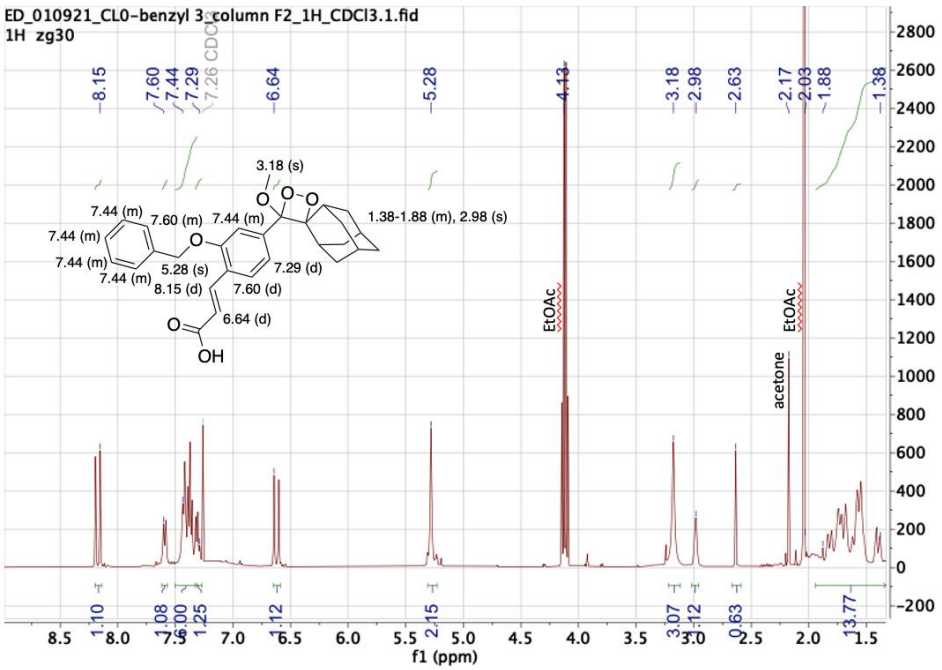


Figure S63. ^{1}H NMR of **CL-benzyl 3** in chloroform-*d*.

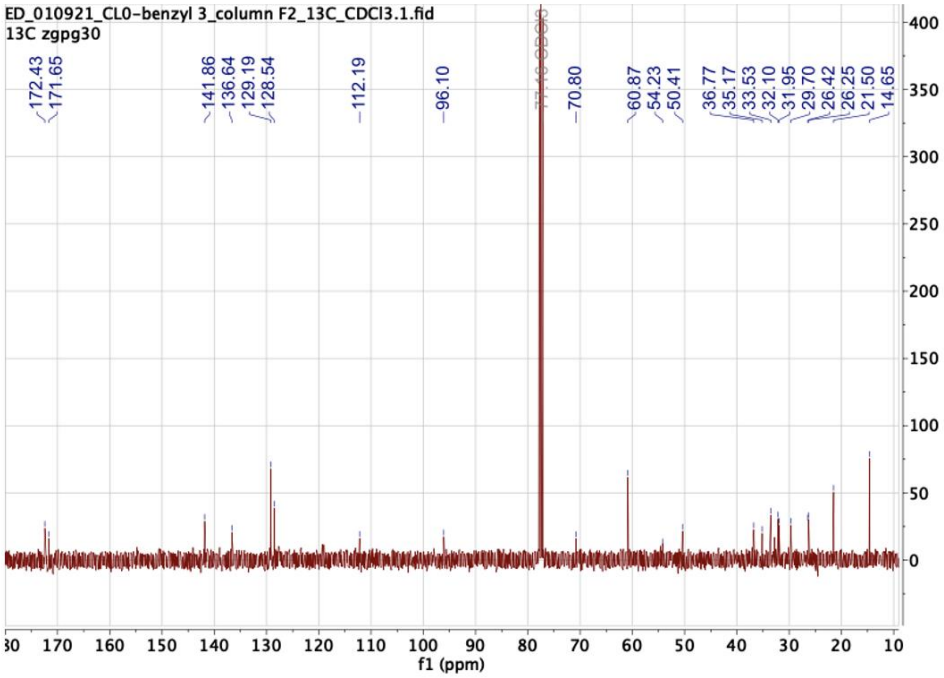


Figure S64. ^{13}C NMR of **CL-benzyl 3** in chloroform-*d*.

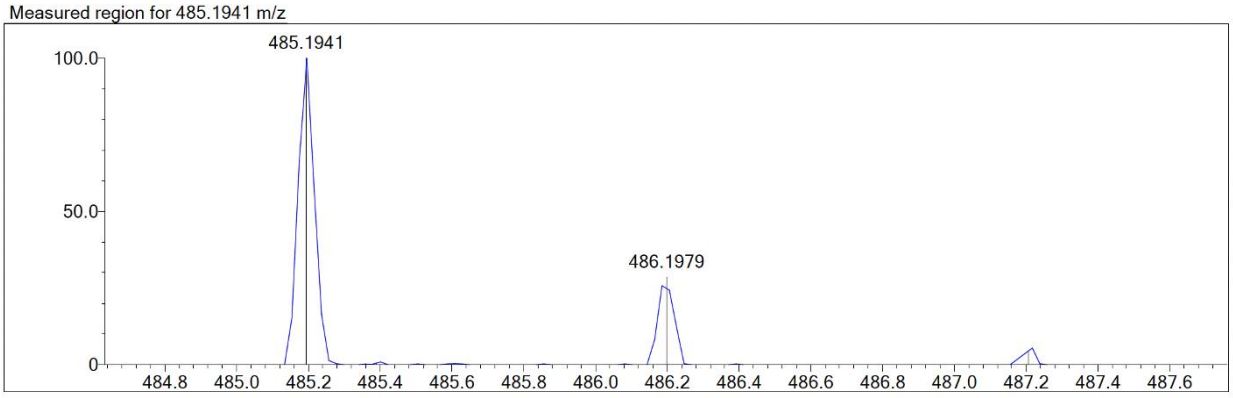


Figure S65. HRMS of **CL-benzyl 3**. Expected mass 462.20. Observed is $[M+Na]^+$ = 485.1941.

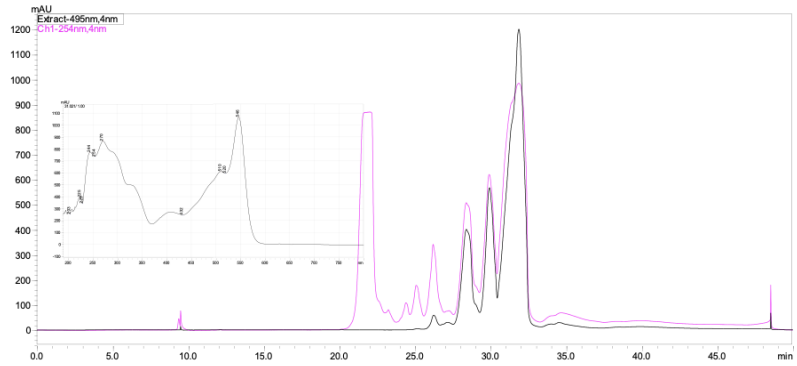


Figure S66. HPLC chromatogram of **CL-E2** purification in ACN (0.1% formic acid). Product elutes at 30 min.

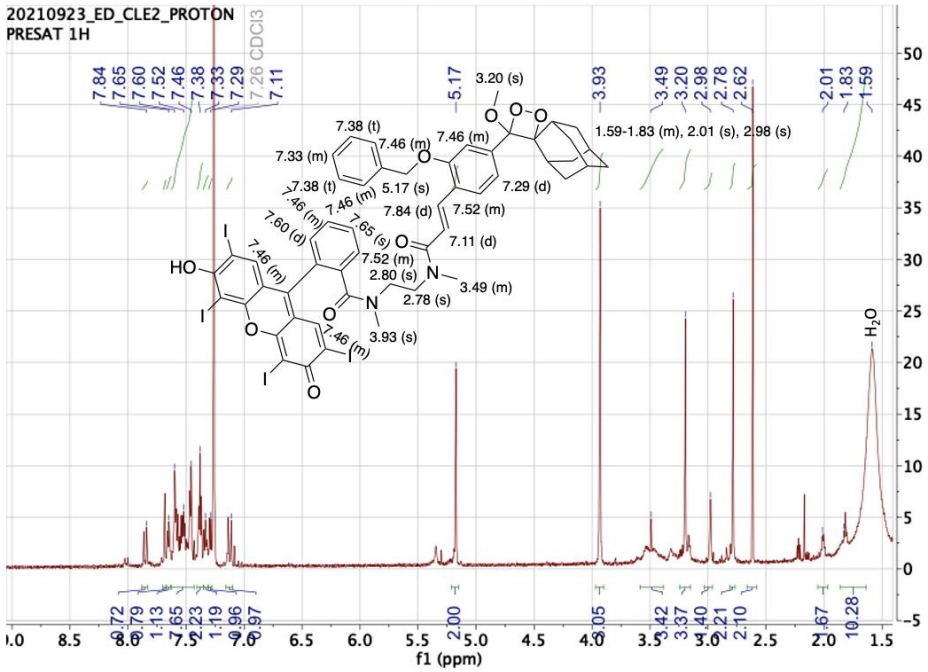


Figure S67. ^{1}H NMR of **CL-E2** in chloroform-*d*.

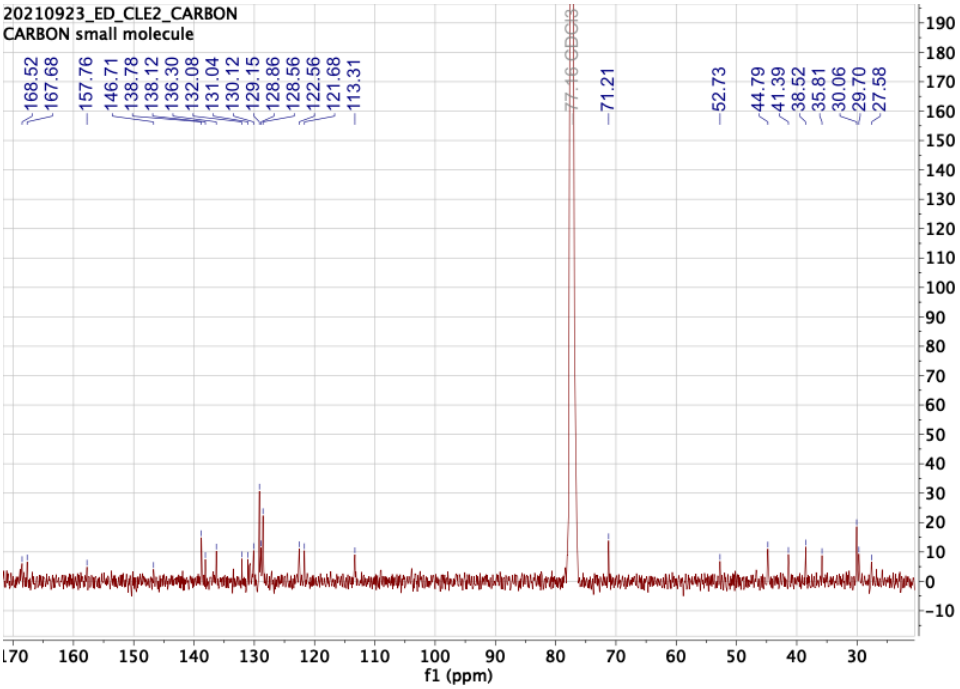


Figure S68. ^{13}C NMR of **CL-E2** in chloroform-*d*.

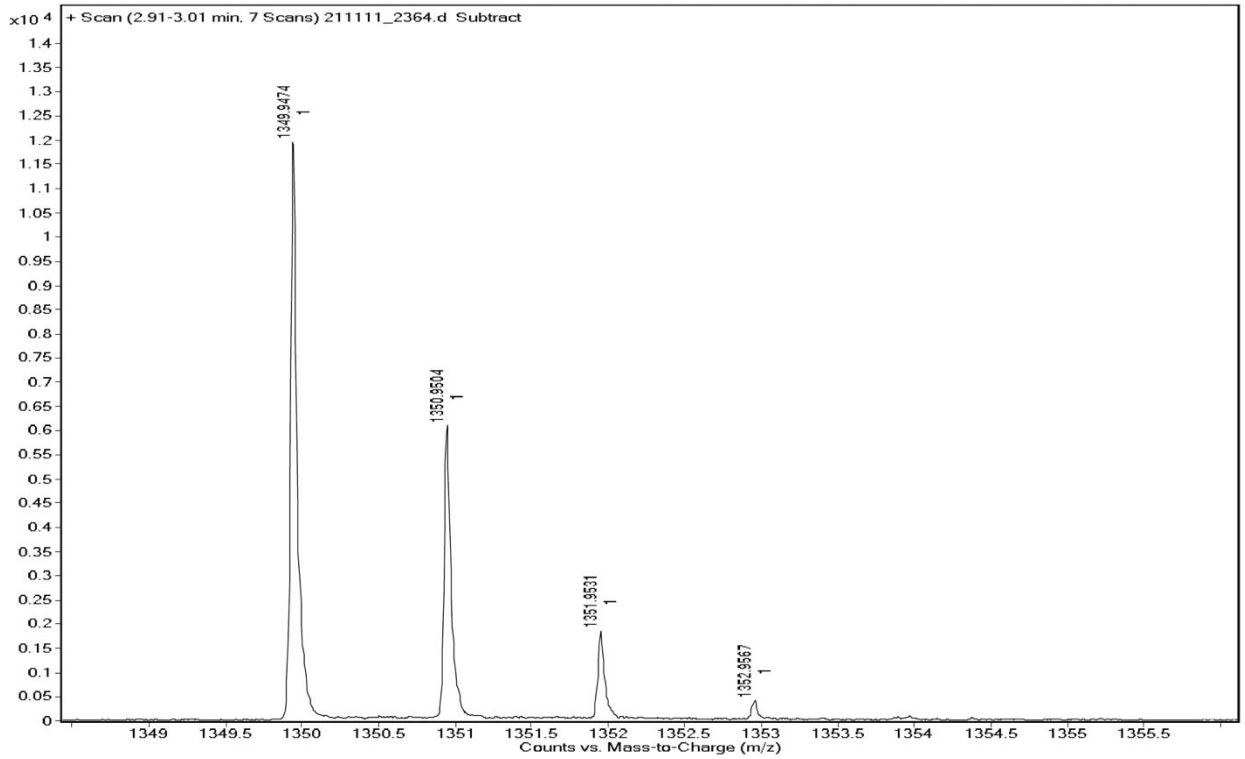


Figure S69. HRMS of **CL-E2**. Expected mass 1349.95. Observed is [M] = 1349.9474.

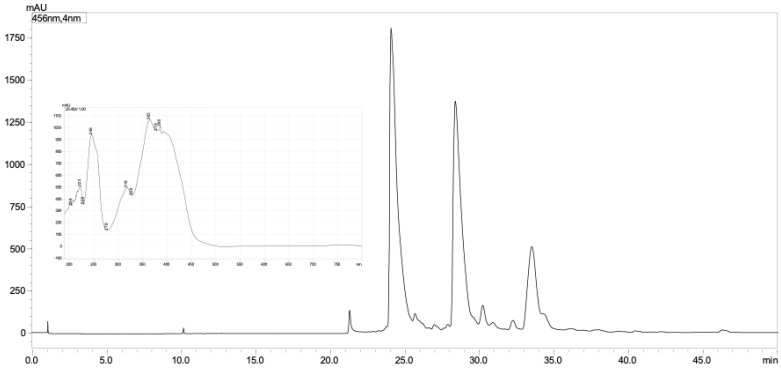


Figure S70. HPLC chromatogram of **Amino-PN** purification in ACN (0.1% formic acid). Product elutes at 24 min.

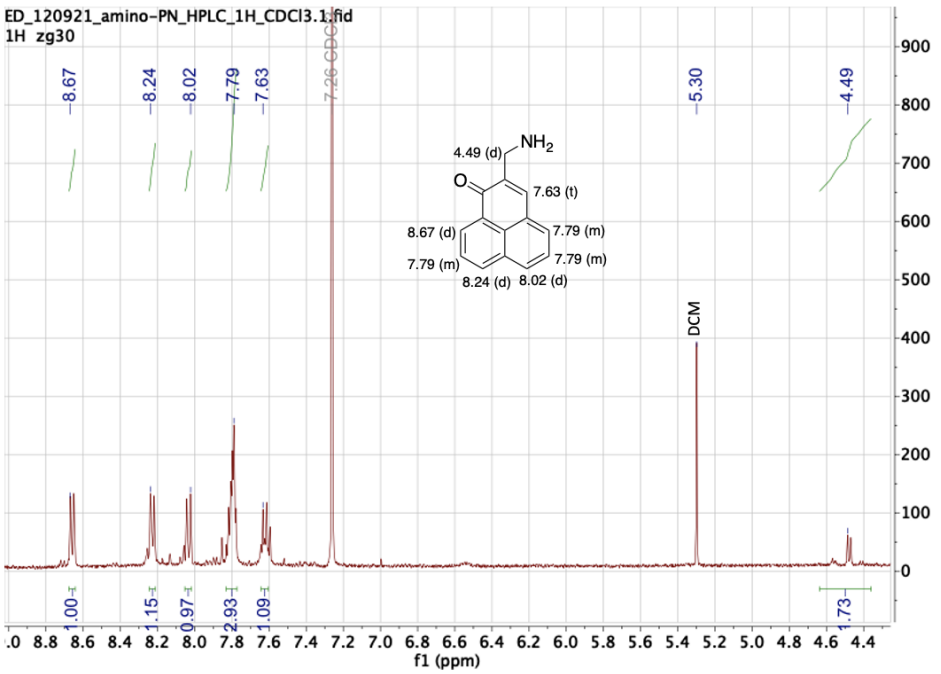


Figure S71. ^{1}H NMR of **Amino-PN** in chloroform-*d*.

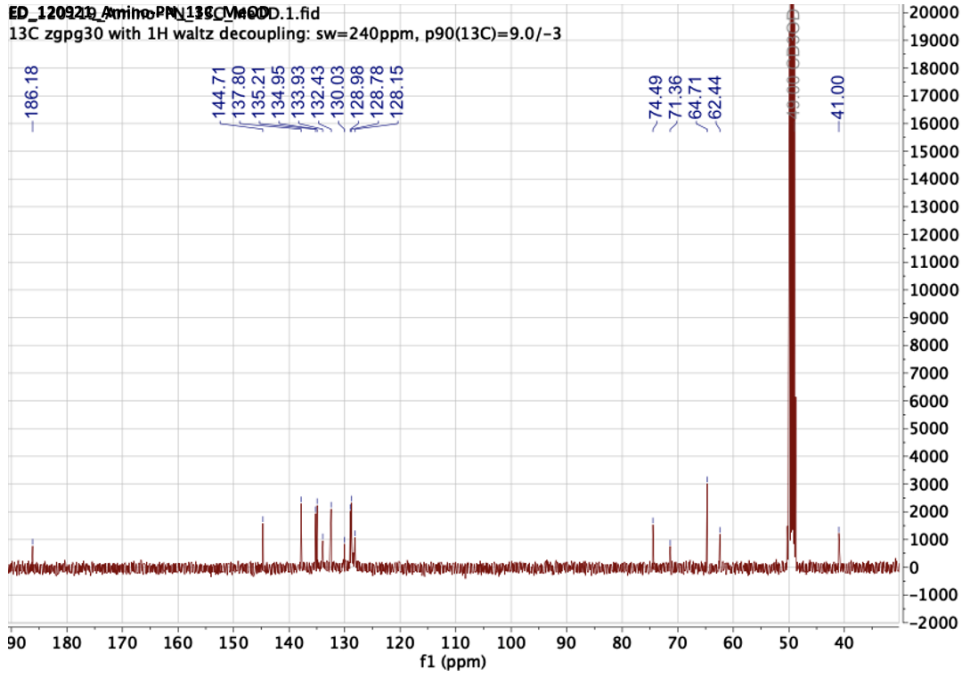


Figure S72. ^{13}C NMR of **Amino-PN** in methanol-d_6.

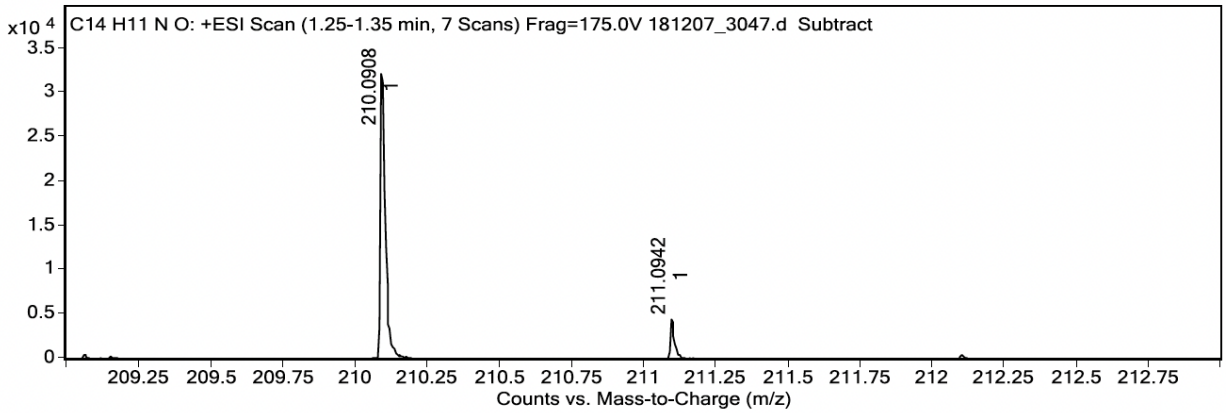


Figure S73. HRMS of **Amino-PN**. Expected mass 209.08. Observed is $[M+H]^+$ = 210.0908.

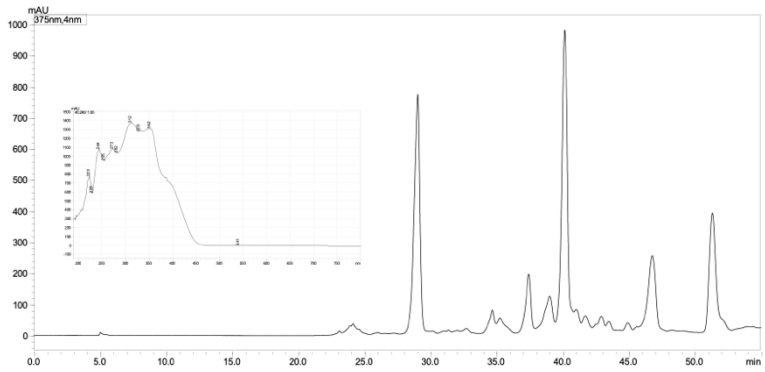


Figure S74. HPLC chromatogram of **CL-PNa** purification in ACN (0.1% formic acid). Product elutes at 41 min.

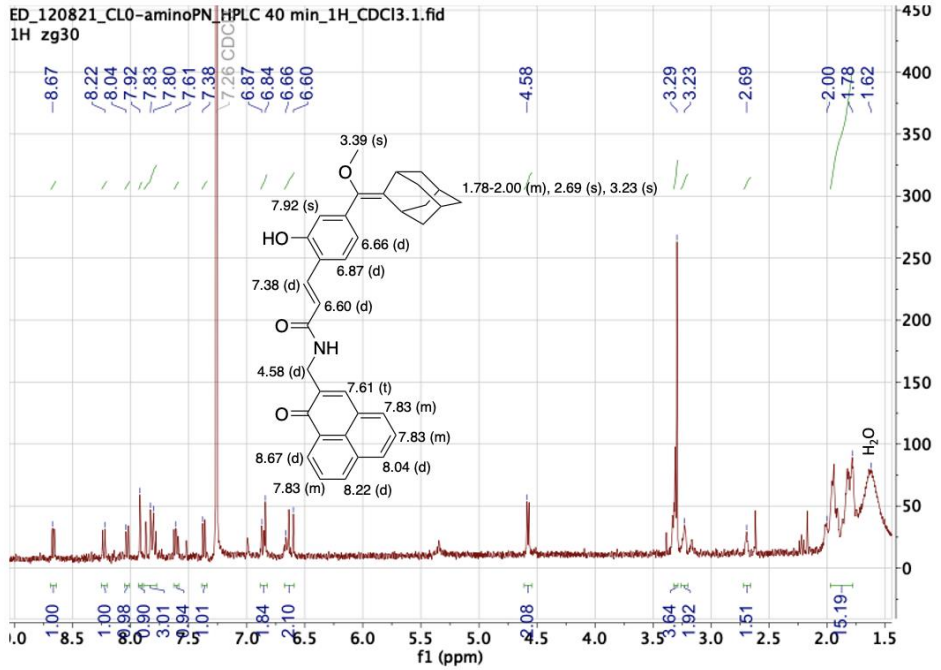


Figure S75. ^{1}H NMR of **CL-PNa** in chloroform-*d*.

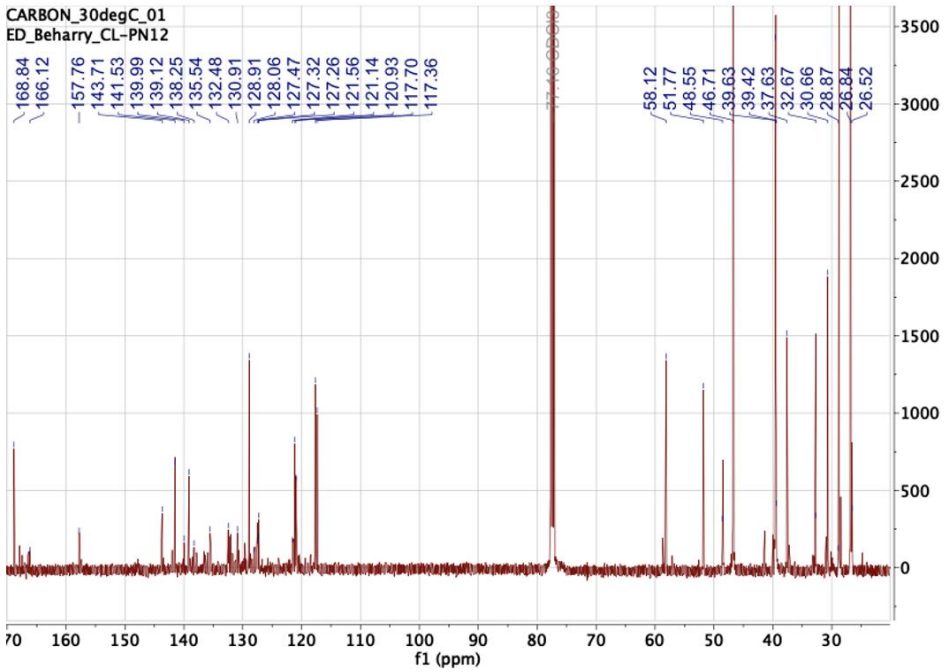


Figure S76. ^{13}C NMR of **CL-PNa** in chloroform-*d*.

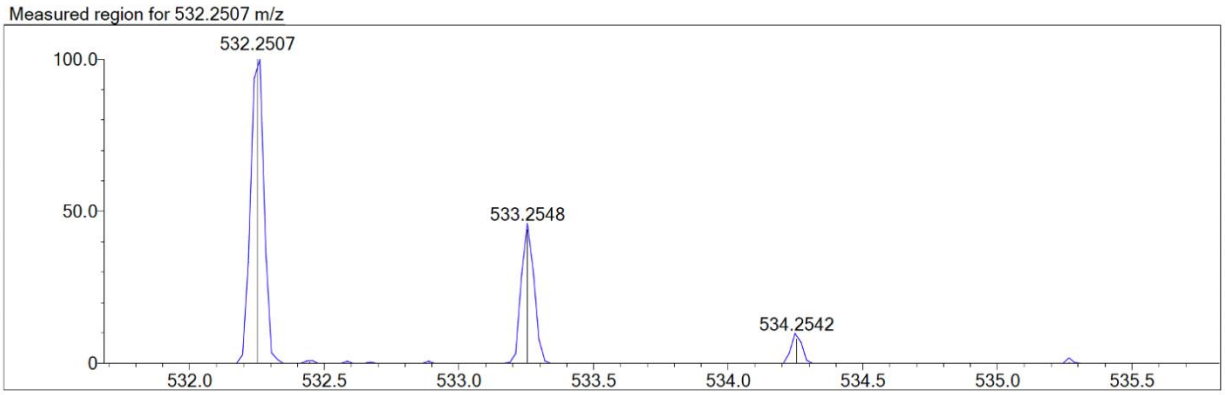


Figure S77. HRMS of **CL-PNa**. Expected mass 531.24. Observed is $[M+H]^+$ = 532.2507.

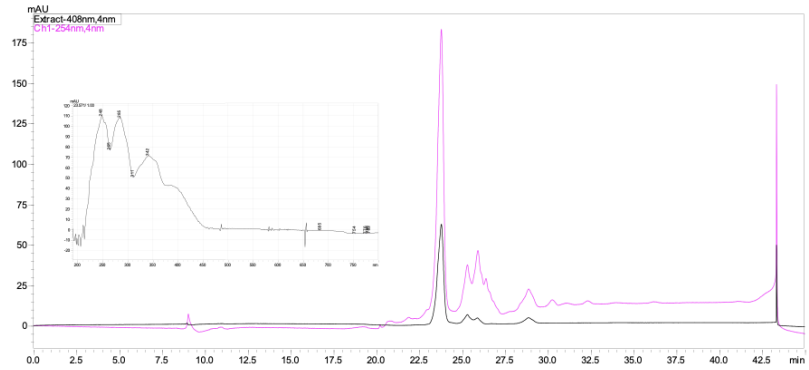


Figure S78. HPLC chromatogram of **CL-PN** purification in ACN (0.1% formic acid). Product elutes at 23 min.

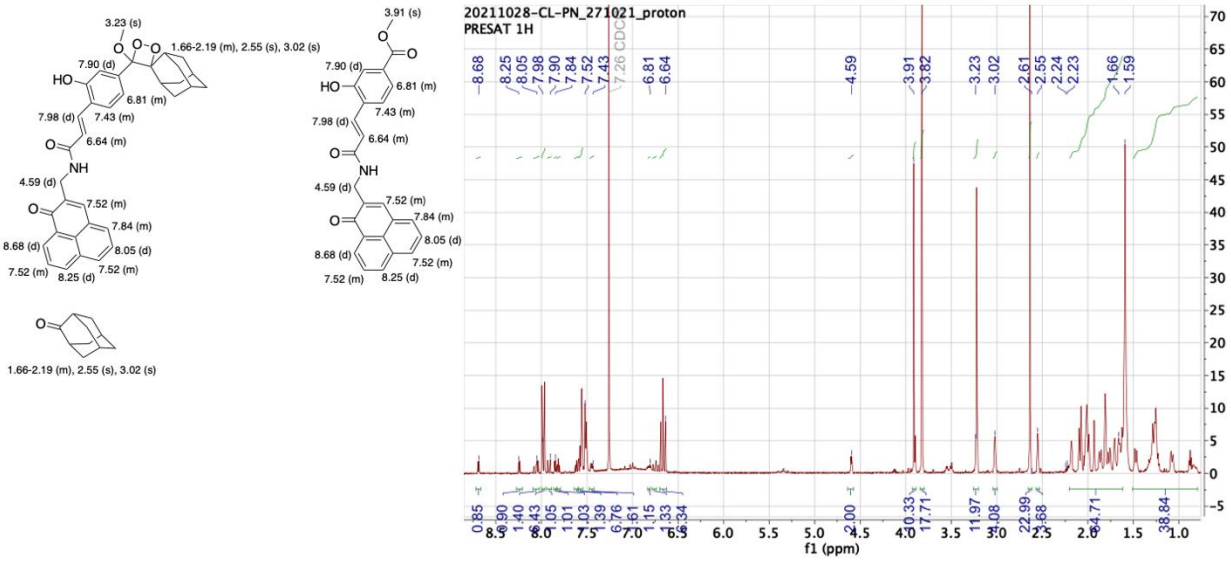


Figure S79. ^{1}H NMR of **CL-PN** in chloroform-*d*.

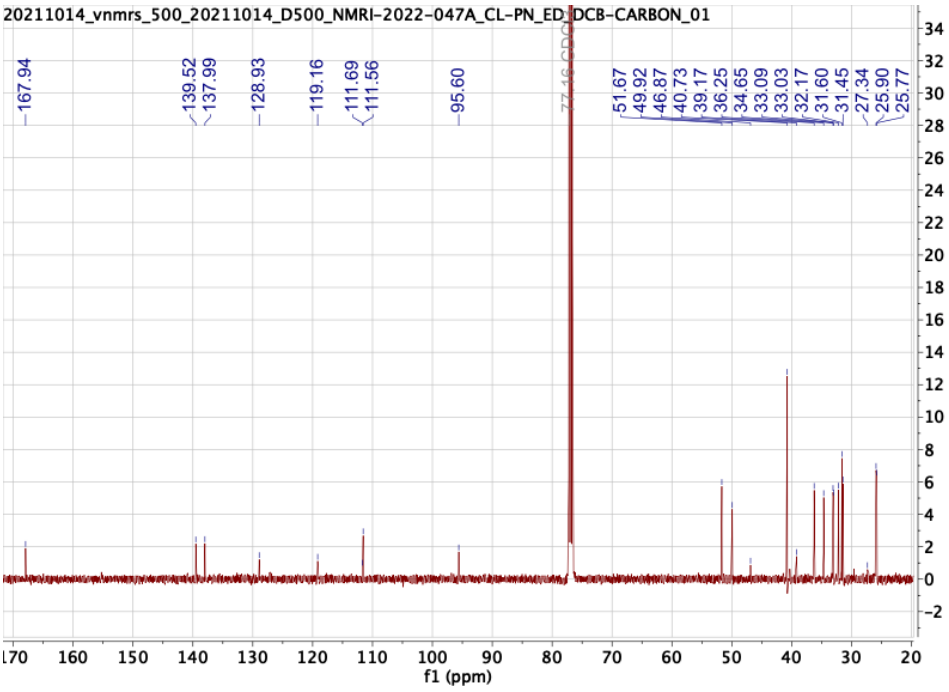


Figure S80. ^{13}C NMR of **CL-PN** in chloroform-*d*.

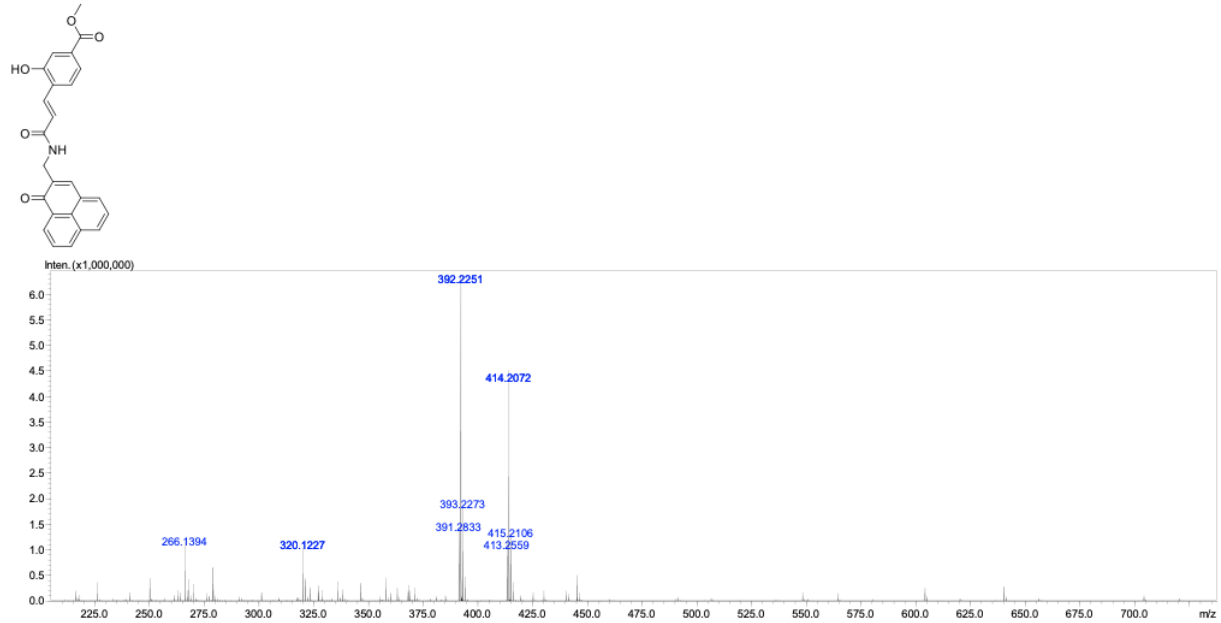


Figure S81. HRMS of **CL-PN** breakdown product. Expected breakdown product mass 413.13. Observed is $[M+H]^+$ = 414.2072.

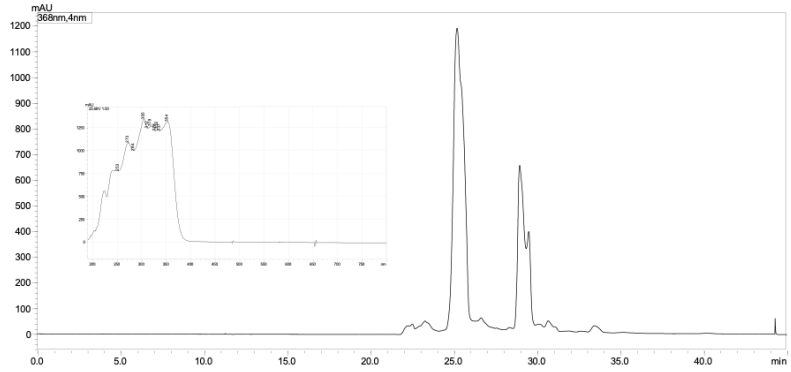


Figure S82. HPLC chromatogram of **CL-OMe dioxetane** purification in ACN (0.1% formic acid). Product elutes at 24 min.

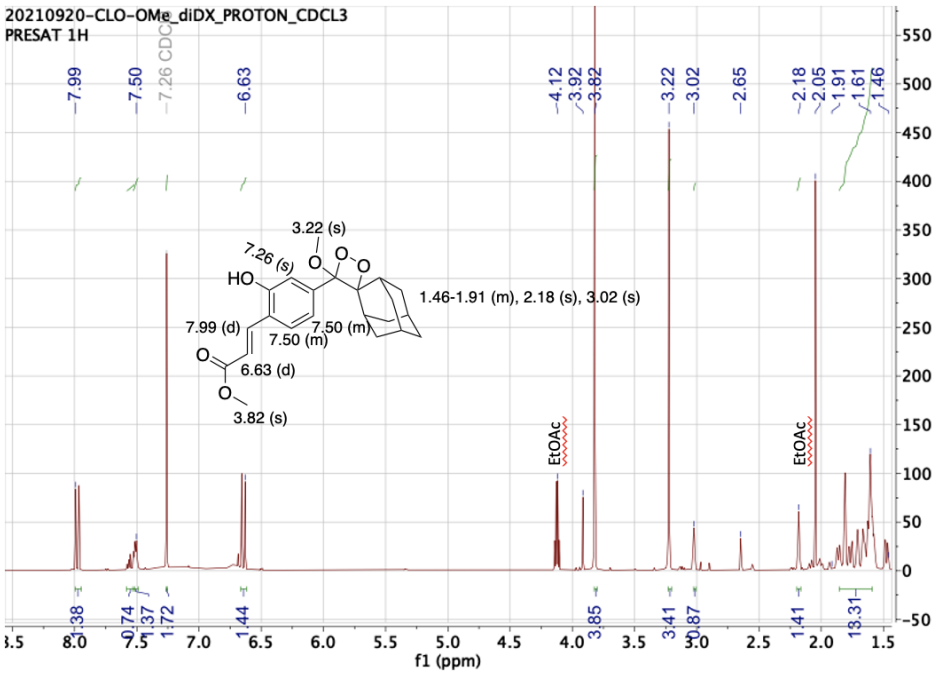


Figure S83. ^{1}H NMR of **CL-OMe dioxetane** in chloroform-*d*.

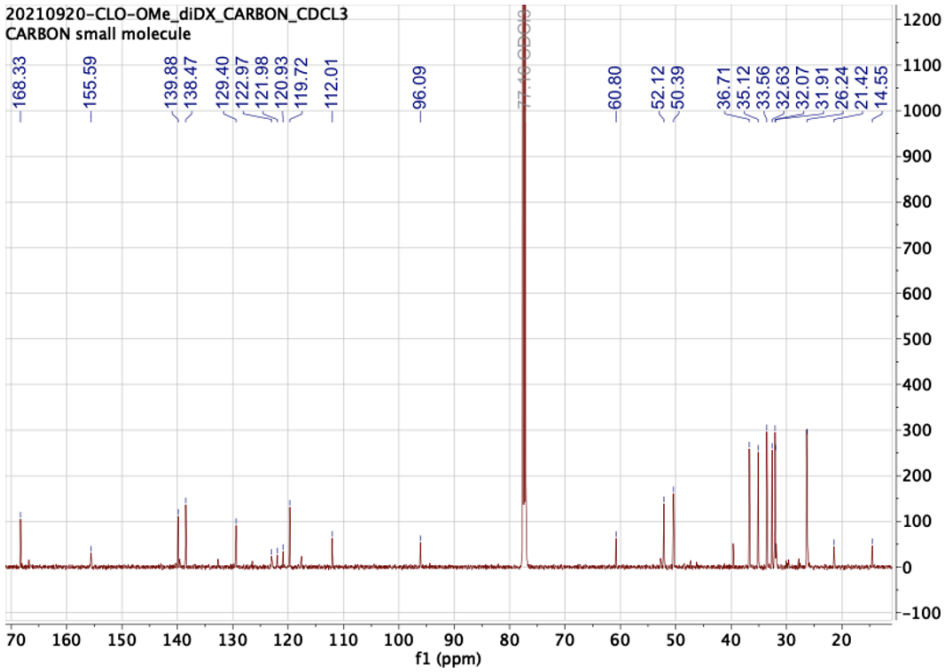


Figure S84. ^{13}C NMR of **CL-OMe dioxetane** in chloroform-*d*.

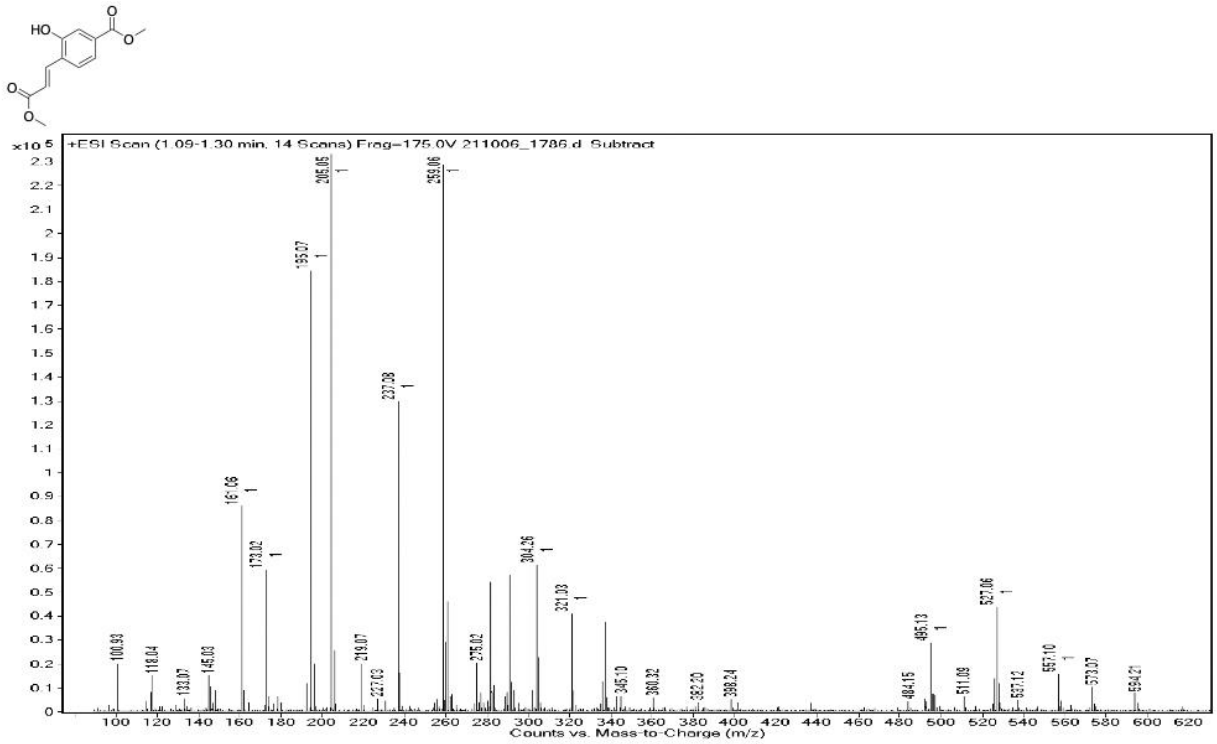


Figure S85. MS of **CL-OMe** breakdown product. Expected breakdown product mass 236.07. Observed is $[M+H]^+$ = 237.08.

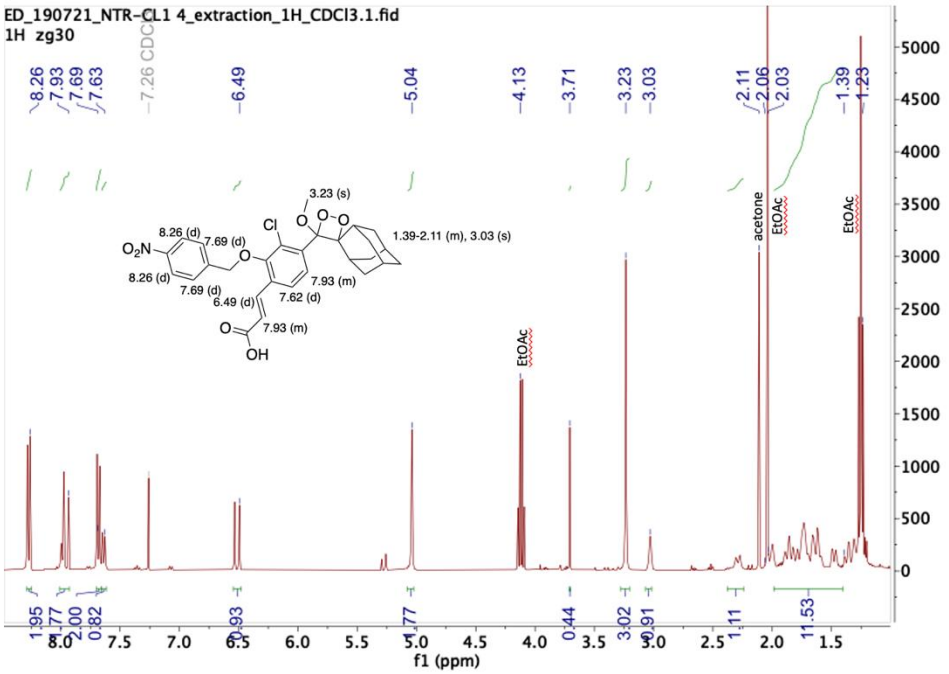


Figure S86. ^{1}H NMR of **NTR-CL** in chloroform-*d*.

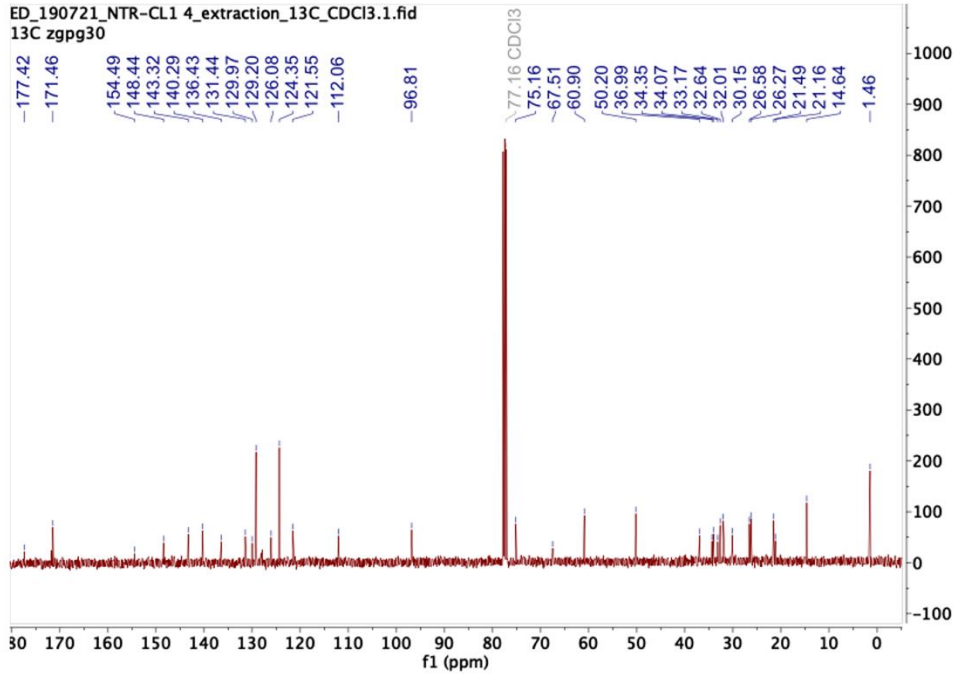


Figure S87. ^{13}C NMR of **NTR-CL** in chloroform-*d*.

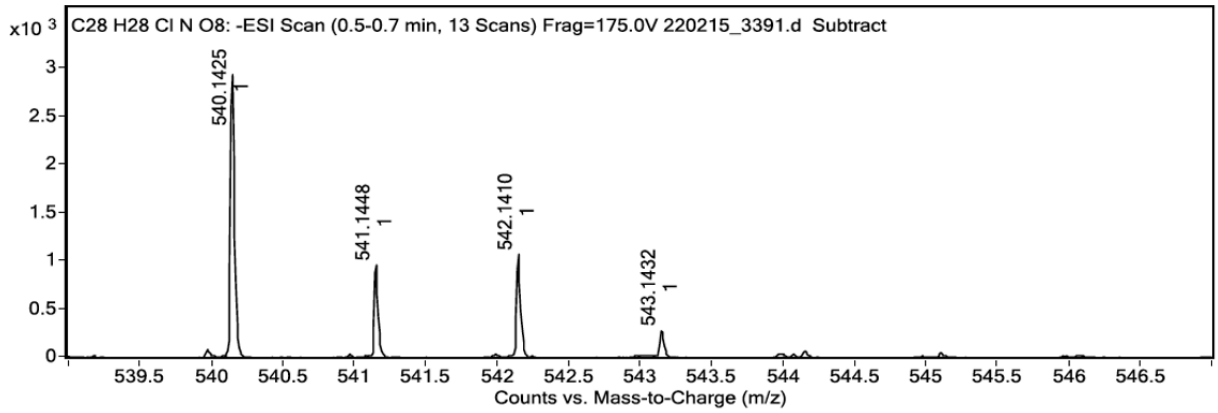


Figure S88. HRMS of **NTR-CL**. Expected mass 541.15. Observed is $[M-H]^-$ = 540.1425.

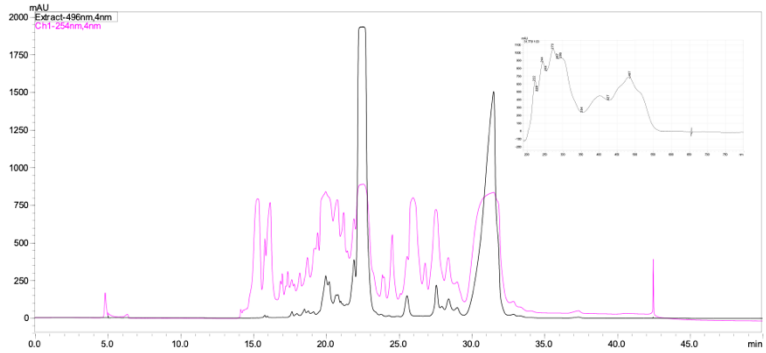


Figure S89. HPLC chromatogram of **NTR-CL-E1** purification in ACN (0.1% trifluoroacetic acid). Product elutes at 30 min.

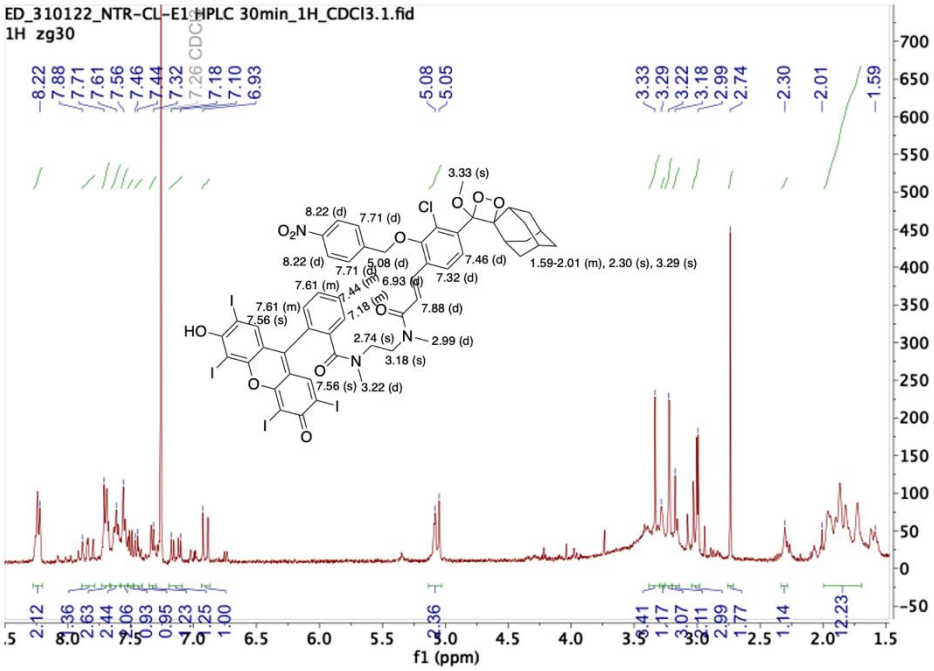


Figure S90. ^{1}H NMR of **NTR-CL-E1** in chloroform-*d*.

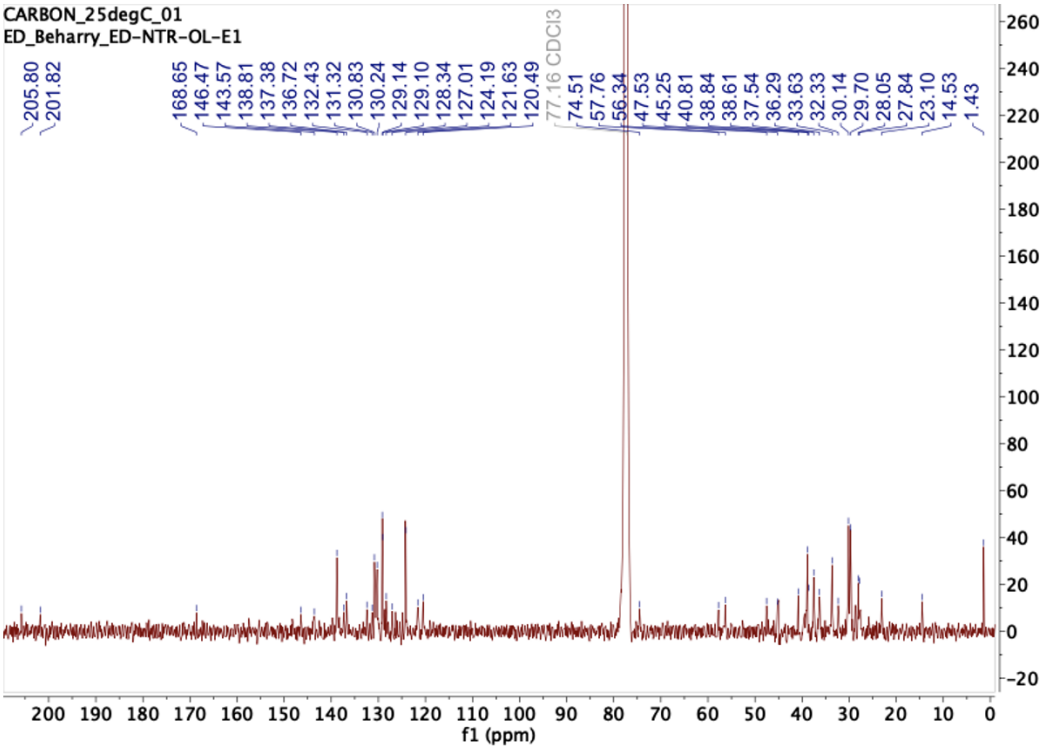


Figure S91. ^{13}C NMR of **NTR-CL-E1** in chloroform-*d*.

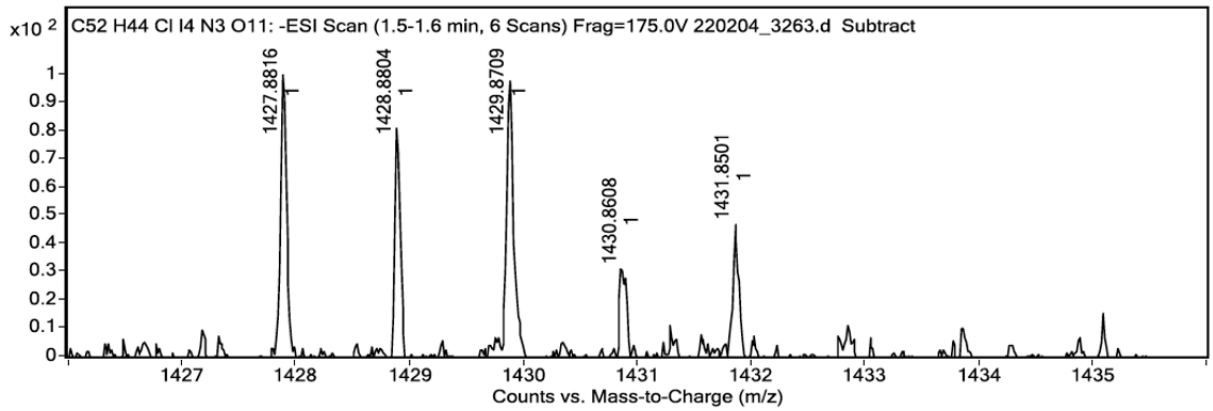


Figure S92. HRMS of **NTR-CL-E1**. Expected mass 1428.88. Observed is $[M-H]^-$ = 1427.8816.

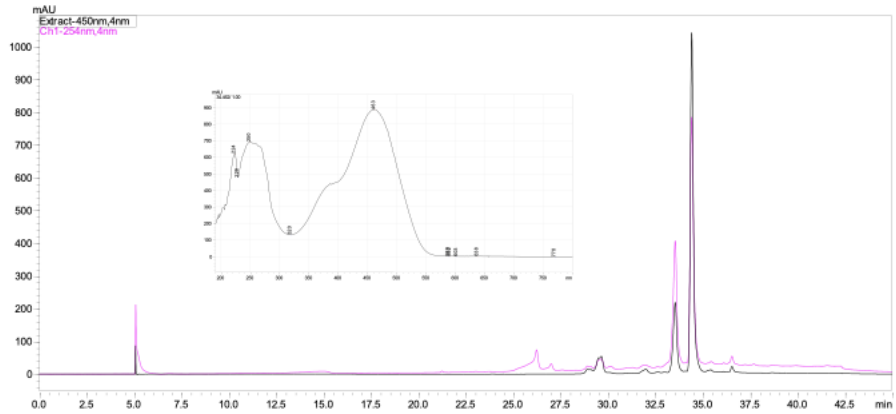


Figure S93. HPLC chromatogram of **4NB-Reso** purification in MeOH (0.1% formic acid). Product elutes at 34 min.

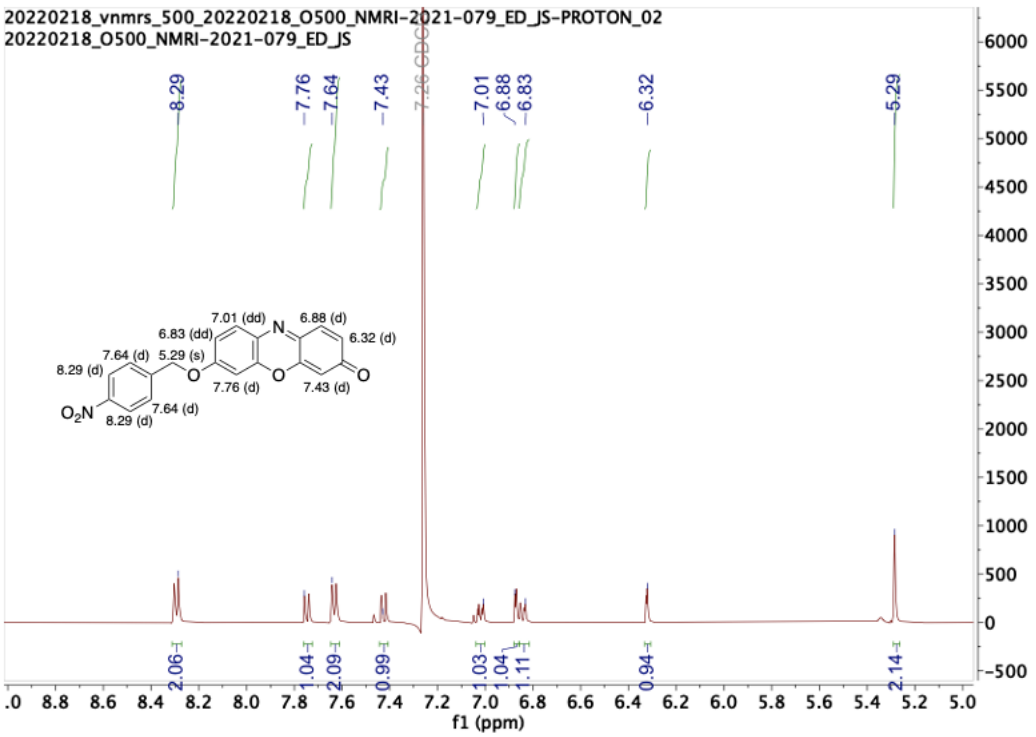


Figure S94. ^{1}H NMR of **4NB-Reso** in chloroform-*d*.

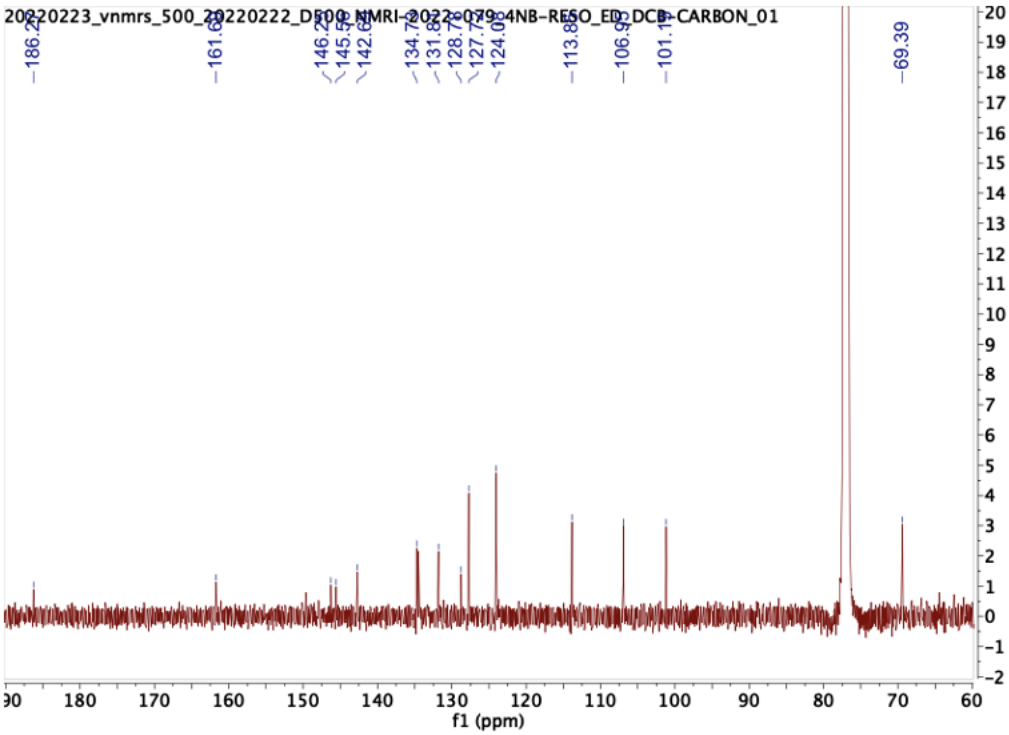


Figure S95. ^{13}C NMR of **4NB-Reso** in chloroform-*d*.

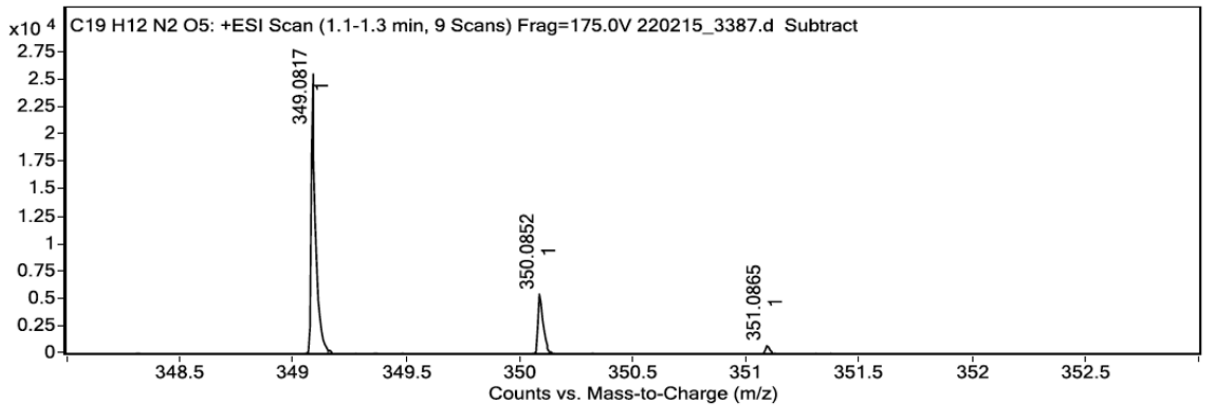


Figure S96. HRMS of **4NB-Reso**. Expected mass 348.07. Observed is $[M+H]^+$ = 349.0817.

6. References

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