Electronic Supplementary Material (ESI) for ChemComm.

Supplementary Information

Selective detection of carboxylesterase 2 activity in cancer cells using an activity-based chemiluminescent probe

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1. General

All reagents were commercially available and used without further purification unless otherwise noted. Human CES2 (expressed in HEK293 cells, C-terminal His tag) was purchased from Sino Biological Incorporated (10380-H08H) and human CES1 (expressed in baculovirus infected BTI insect cells, isoform B) was purchased from Sigma (E0287). Anhydrous solvents were obtained through 4 Å molecular sieve, anhydrous THF was obtained through the Mbraun MBSPS5 solvent drying system. The inert atmosphere was obtained by argon. ^{1}H NMR and ^{13}C NMR spectra were recorded at room temperature on Bruker (400 or 500 MHz) in $CDCl_3$ with tetramethylsilane (TMS) as an internal standard. Coupling constants *(J values*) are given in Hz and chemical shifts are reported as parts per million (ppm). Splitting patterns are designated as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and p (pentet). NMR spectra were processed with MestReNova program. Column chromatography was performed by using thick-walled glass columns and silica Gel 60 (Merck 230 – 400 mesh). Thin layer chromatography (TLC Merck Silica Gel 60 F254) was performed by using commercially prepared 0.25 mm silica gel plates and visualization was provided by UV lamp. Mass analyses were performed with Vion Waters LC/MS UHPLC. The relative proportions of solvents in chromatography solvent mixtures refer to the volume:volume ratio. Absorbance spectra were obtained on a Shimadzu UV-1800 UV Spectrophotometer. Fluorescence emission spectra were obtained on a Shimadzu RF-6000 Spectro Fluorophotometer. Luminescence and viability measurements were read by a Tecan Spark plate reader.

2. Synthesis

Compounds **1**[1] and **3**[2] were synthesized according to literature.

Compound **2**:

Triphenyl phosphine (0.710 g, 2.71 mmol) and iodine (0.690 g, 2.71 mmol) were dissolved in anhydrous DCM and stirred for 10 minutes under argon atmosphere at room temperature. Imidazole (0.074 g, 1.20 mmol) was added to the stirred solution and after 10 minutes stirring compound **1** (0.400 g, 1.77 mmol) was added to the solution. The reaction mixture was stirred overnight under argon atmosphere and monitored by TLC. Upon completion, the reaction mixture was concentrated under reduced pressure and the product was obtained without further purification as white solid (0.200 g, 65% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.88 – 7.85 (m, 2H), 7.80 (s, 1H), 7.60 – 7.58 (m, 2H), 7.57 – 7.54 (m, 1H), 7.52 – 7.48 (m, 2H), 7.42 – 7.39

(m, 2H), 4.48 (s, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.8, 137.6, 135.6, 132.2, 129.8, 129.0, 127.1, 120.5, 77.4; HRMS: m/z calculated for $C_{14}H_{12}INO$: 337.9964; found: 338.0037 $[M+H]^+$.

Compound **4**:

3 (0.0950 g, 0.260 mmol) and potassium carbonate (0.370 g, 2.67 mmol) were dissolved in anhydrous ACN and stirred for 30 minutes under argon atmosphere at room temperature. **2** (0.110 g, 0.330 mmol) was added to the stirred solution and the reaction mixture was further stirred overnight and monitored by TLC. Upon completion, reaction mixture was diluted with EtOAc (50 mL) and washed with brine (25 mL x 3). Organic phase was dried over Na_2SO_4 and concentrated under reduced pressure. The compound **4** was obtained through purification with column chromatography (Hex: EtOAc 95:5) as yellow solid (0.0710 g, 48% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.14 (s, 1H), 7.88 (dd, *J* = 7.2, 1.8 Hz, 2H), 7.69 (d, *J* = 8.1 Hz, 2H), 7.54 (m, 1H), 7.50 – 7.45 (m, 2H), 7.43 (d, *J* = 16.9 Hz, 1H), 7.39 – 7.34 (m, 2H), 7.29 (d, *J* = 8.0 Hz, 1H), 7.08 (d, *J* = 8.0 Hz, 1H), 5.83 (d, *J* = 16.9 Hz, 1H), 5.02 (q, *J* = 10.8 Hz, 2H), 3.33 (s, 3H), 3.28 (s, 1H), 2.06 (s, 1H), 1.99 – 1.71 (m, 12H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 171.2, 166.0, 153.2, 144.9, 139.3, 139.2, 138.8, 134.8, 132.9, 131.9, 131.4, 130.0, 129.9, 128.7, 127.9, 127.2, 124.4, 120.4, 118.1, 98.0, 75.9, 60.4, 57.4, 39.2, 39.0, 38.6, 37.0, 33.0, 29.7, 28.3, 28.2, 21.1, 14.1; MS: m/z calculated for $C_{35}H_{33}ClN_2O_3$: 564.22; found: 587.27 $[M+Na]^+$.

CL-CES2:

Compound **4** (0.0100 g, 0.0170 mmol) and few milligrams of methylene blue were dissolved in DCM. The reaction mixture was irradiated with red light (625 nm LED, purchased from ThorLabs, M625L3) and stirred for 1.5 hours at 0 ºC, with oxygen gas bubbled in the solution. Upon completion, the reaction mixture was concentrated under reduced pressure. **CL-CES2** was obtained through purification with column chromatography using (EtOAc) as white solid (0.00850 g, 80% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.96 – 7.87 (m, 4H), 7.70 (d, *J* = 8.5 Hz, 2H), 7.59 – 7.55 (m, 1H), 7.51 (dd, *J* = 8.2, 6.6 Hz, 2H), 7.45 – 7.41 (m, 1H), 7.41 – 7.28 (m, 3H), 5.85 (d, *J* = 16.8 Hz, 1H), 5.01 – 4.91 (m, 2H), 3.24 (s, 3H), 3.04 (s, 1H), 2.32 (d, *J* = 12.0 Hz, 1H), 1.99 – 1.59 (m, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 176.5, 166.2, 144.7, 139.3, 136.8, 135.4, 132.5, 131.5, 130.6, 130.3, 129.3, 127.5, 124.9, 120.8, 119.7, 118.2, 112.1, 99.6, 96.8, 77.6, 76.6, 50.2, 37.0, 34.1, 32.6, 32.0, 30.2, 26.6, 26.3; HRMS: m/z calculated for $C_{35}H_{33}ClN_2O_5$: 596.2078: found: 619.1974 $[M+Na]^+$.

3. Photophysical Measurements

CL-CES2 was aliquoted and stored dry at -20 ºC until needed. Stock solutions of CL-CES2 were prepared at a concentration of 2 mM in DMSO (Molecular Probes™ DMSO, Anhydrous).

Enzyme UV-Vis Assays:

Enzyme assays were performed in a 1 cm path length, 50 µL quartz cuvette. CL-CES2 was diluted to a final concentration of 20 µM in DPBS containing magnesium chloride and calcium chloride, pH 7.4. CES1 or CES2 was added to a final concentration of 55 nM. The samples were incubated for 15 h at 37 ºC. UV-Vis spectra were collected before and after the incubation.

p-NPA CES1 Activity Test:

To confirm activity of CES1, 55 nM CES1 was added to 3 mM p-NPA in DPBS at 37 ºC and UV-Vis spectra were measured in 30 s intervals.

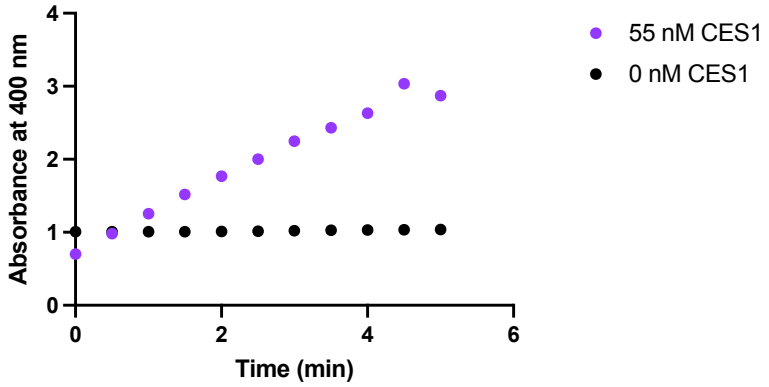


Figure S1. Time dependent absorption signal of p-NPA (3 mM) at 400 nm before and after addition of CES1 in DPBS (pH 7.4).

Enzyme Fluorescence Assays:

Enzyme assays were performed in a 1 cm path length, 50 µL quartz cuvette. CL-CES2 was diluted to a final concentration of 20 µM in DPBS containing magnesium chloride and calcium chloride, pH 7.4. CES1 or CES2 was added to a final concentration of 55 nM and the samples were left to incubate 15 h at 37 ºC. Emission spectra were collected before and after the incubation at excitation wavelength of 390 nm.

Enzyme Luminescence Assays:

Enzyme assays were performed in 96-well plates purchased from Thermo Fisher Scientific (Nunc™ MicroWell™ 96-Well, Nunclon Delta-Treated, Flat-Bottom Microplate) and

luminescence readings were acquired on a Tecan Speck plate reader from 398-653 nm with 1 s integration time. Stock solution of probe was diluted to a final concentration of 20 µM in PBS and enzyme was added to a final concentration of 55 nM, unless indicated otherwise, and immediately read at 37°C. Inhibitor assays were performed with 10 min preincubation with the inhibitor at 37 °C, with inhibitor diluted in to DPBS solution at 1% v/v DMSO.

Limit of Detection:

Detection limit was calculated by following a literature example.[3]

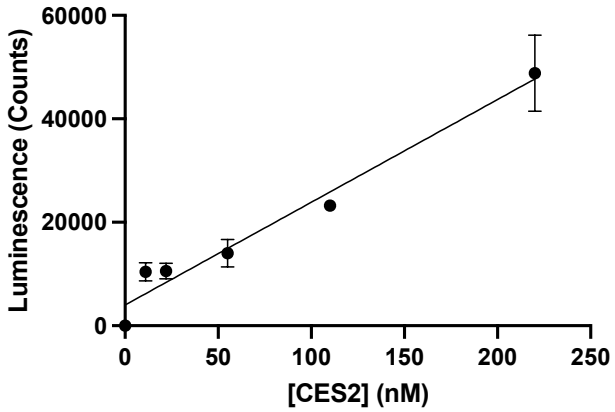


Figure S2. Luminescence signal of **CL-CES2** (20 µM) at 37 °C in DPBS, pH 7.4 (1% v/v DMSO) upon addition of increasing concentrations of CES2 enzyme. Points are replicates of n=3, error bars represent standard deviation.

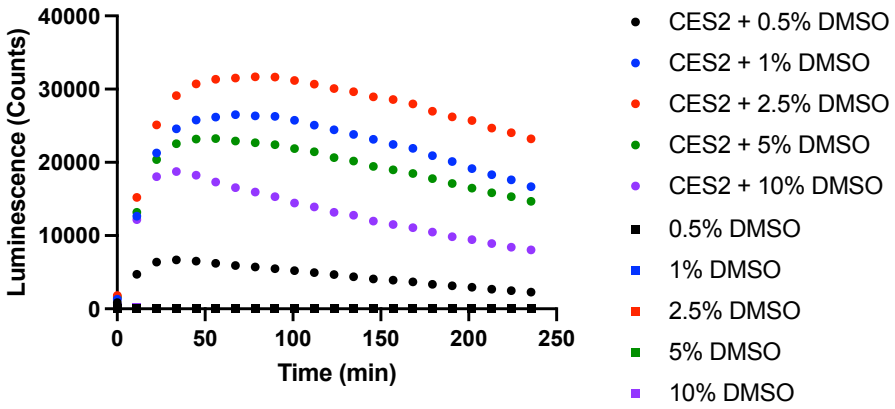


Figure S3. Luminescence time course of **CL-CES2** (20 µM) with 55 nM carboxylesterase in DPBS (pH 7.4) having different DMSO contents (0.5% - 10%) at 37 °C.

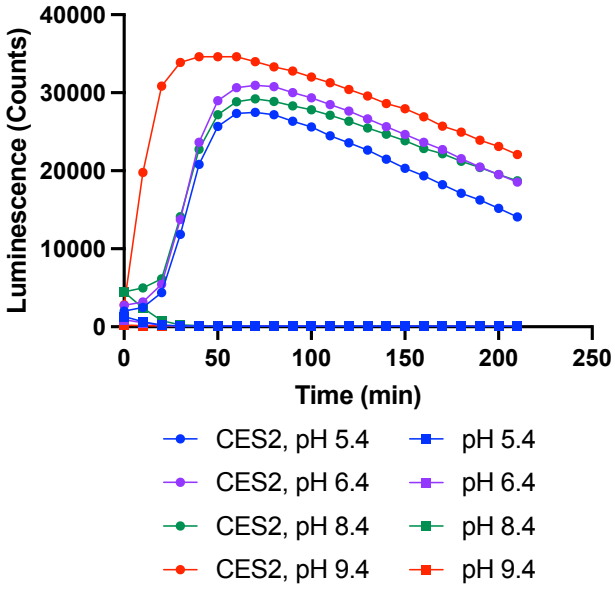
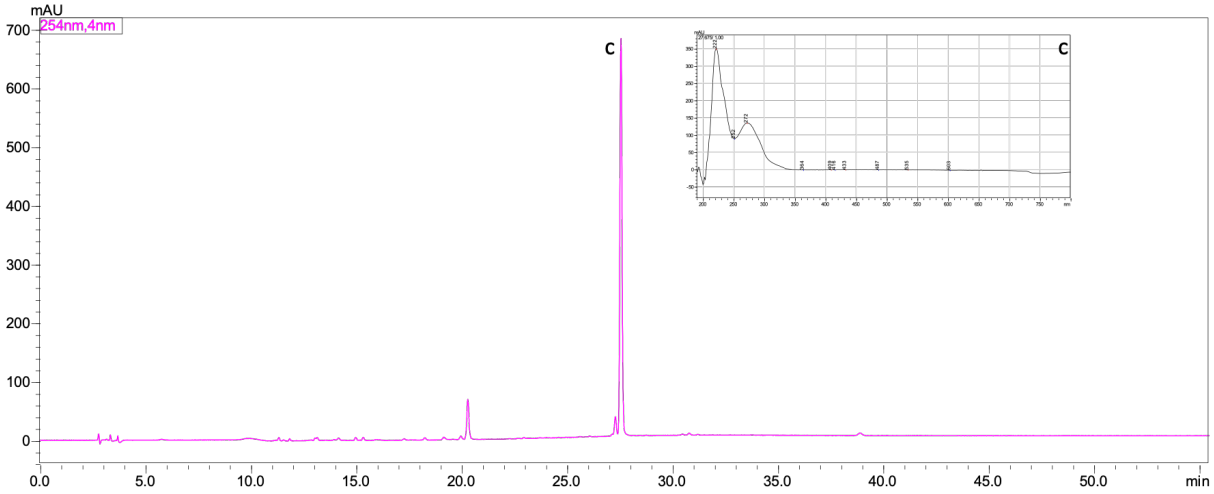


Figure S4. Luminescence time course of **CL-CES2** (20 µM) with 55 nM carboxylesterase in aqueous solutions buffered at different pHs at 37 ºC.

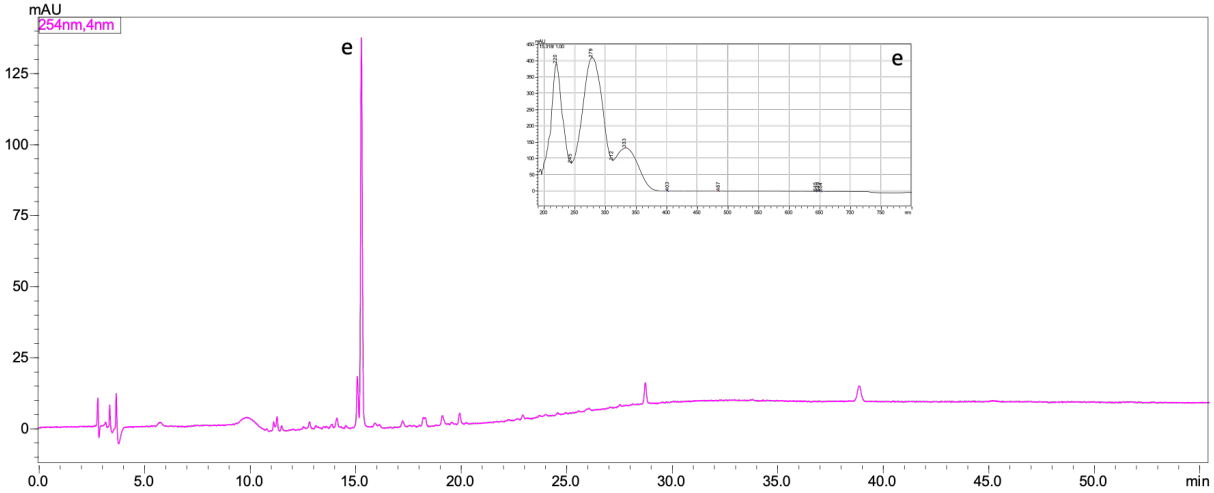
4. HPLC

Standards and reactions with CES2 were analyzed by HPLC. Samples of control compounds were prepared to 200 µM in DPBS pH 7.4 with maximum 1% (v/v) DMSO. CES2 reactions were prepared by incubating 200 µM substrate with 2 µM CES2 in DPBS pH 7.4. All samples were incubated at 37 C for 15 h. The solutions were injected for HPLC analysis. HPLC was performed on a LC-20AT array detector, a CBM-20A VP system controller, and an ACE Equivalence 5 C18 column (250 3 mm ID). The mobile phase consisted of gradient as follows with a flow rate of 0.4 mL/min consisted of: i) 5 min of 55% ACN/0.1% TFA + 45% MilliQ water/0.1% TFA, ii) 13 min gradient from i) to 100% ACN/0.1% TFA, iii) 27 min at 100% ACN/0.1% TFA, iv) 10 min gradient from iii) to 55% ACN/0.1% TFA + 45% MilliQ water/0.1% TFA.

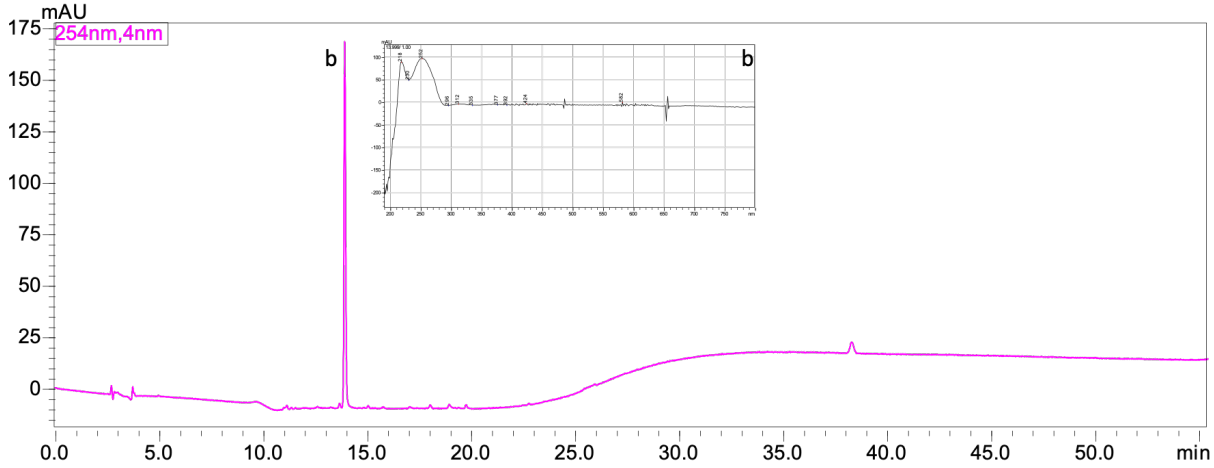
CL-CES2



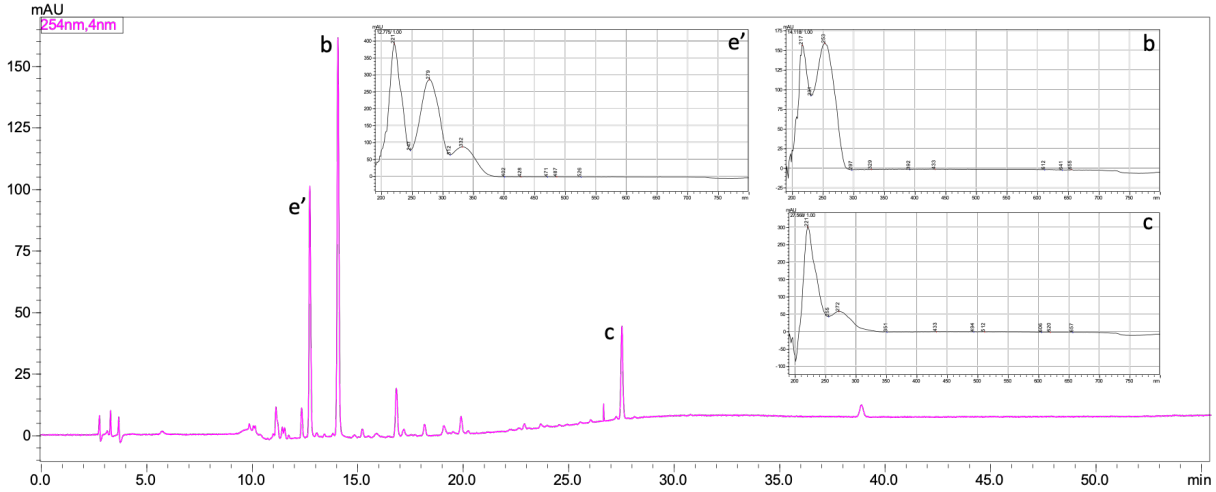
Benzoate



Enzyme (CES2)



CL-CES2 + CES2



Benzoate + CES2

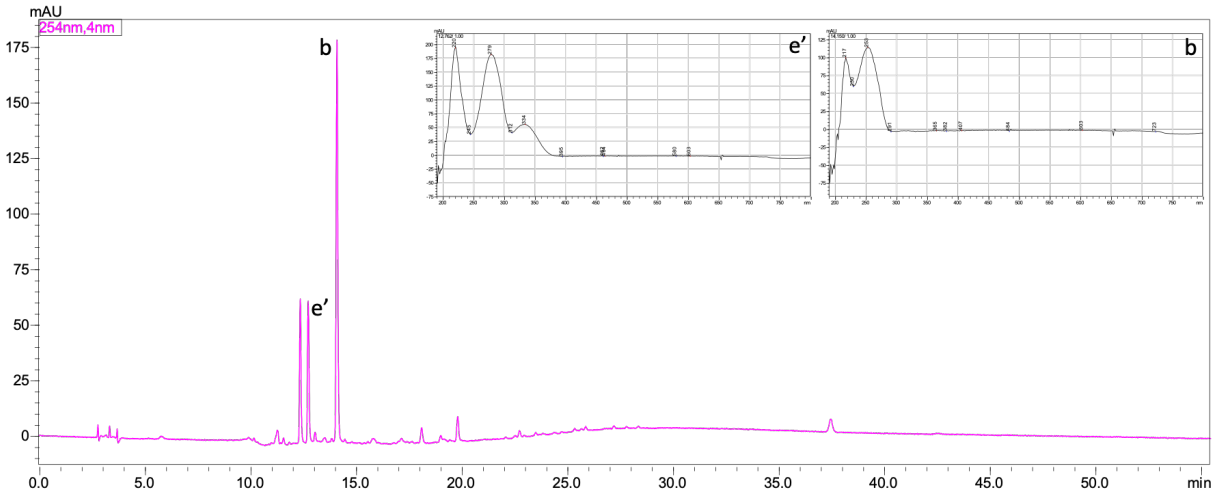


Figure S5. HPLC chromatograms for CL-CES2, benzoate, CES2, CES2 treated probe and CES2 treated benzoate. c: CL-CES2; e: benzoate; b: enzyme; e': shifted benzoate.

5. Cell Culture Studies

Cell Cultures:

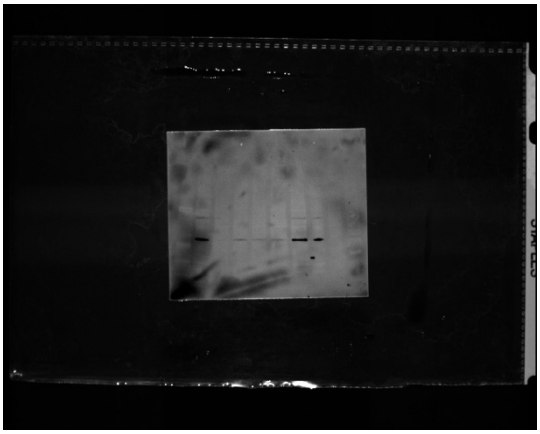
MIA PaCa2 cells were maintained in DMEM with sodium pyruvate, CFPAC-1 cells were grown in RPMI, and HepG2 were maintained in EMEM, with all media supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) antibiotics/antimycotics and grown at 37 °C in 5% CO_2 environment.

Western Blotting:

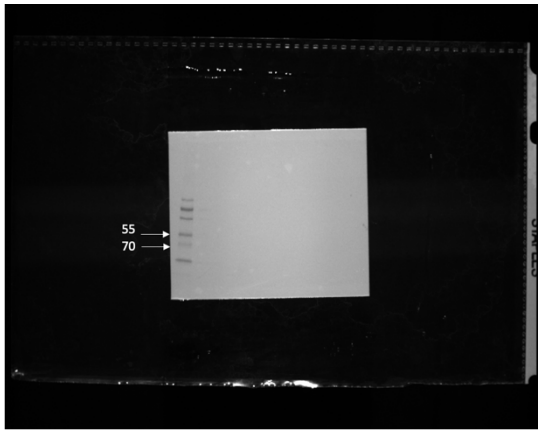
Cells were grown to ~90% confluency and were subjected to lysis in RIPA buffer. The protein content of the lysates was determined by BCA assay (Pierce™ Rapid Gold BCA Protein Assay Kit). The lysates were denatured and reduced in Laemmli buffer with 25 mM DTT and loaded

onto a precast Mini-PROTEAN TGX Gel (4-20%; Bio-Rad Cat. #4561093) with PageRuler™ Plus Prestained Protein Ladder (Sigma 94964) used as a reference. Proteins were separated by SDS-PAGE (120 V) using Tris-glycine running buffer. Proteins were transferred to a PVDF membrane via ThermoFisher Scientific Power Blotter System. Following transfer, the membrane was blocked for 1 h at room temperature with 5% (w/v) skim milk in TBST buffer. Blocking buffer was discarded, the membrane was quickly rinsed with TBST, and fresh blocking buffer with rabbit anti-CES2 antibody (Sigma-Aldrich HPA018897; 3:5000 dilution) was added and incubated overnight under agitation at 4 °C. The membrane was then washed with TBST three times (10 min intervals) and incubated with secondary antibody goat anti-rabbit IgG (H&L) HRP Conjugate (ImmunoReagents Inc. GtxRb-003-DHRPX; 11:2500 dilution) in 5% (w/v) skim milk in TBST buffer for 1.5 h at room temperature. The membrane was washed three times with TBST (10 min intervals) and ECL Substrate (Bio-Rad Clarity Western ECL Substrate) was added for detection. The membrane was imaged on a iBright™ FL1500 Imaging System. The membrane was washed one time with TBST (10 min) and incubated with mouse anti-β-actin antibody-loading control (HRP) (mAbcam 8226; 1:5000 dilution) with agitation overnight at 4 °C. The membrane was washed three times with TBST, ECL substrate was added, and the membrane was imaged again. Blots were analysed using iBright Analysis software.

a)



b)



c)

d)

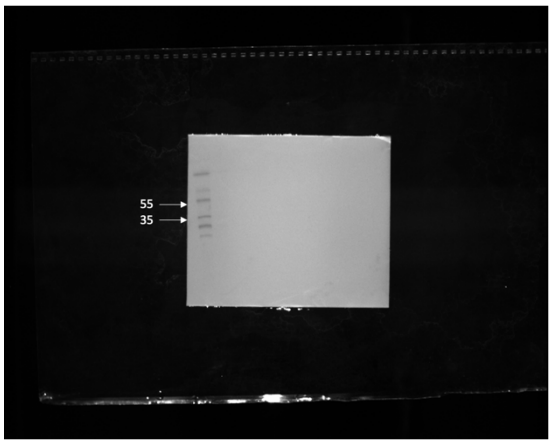


Figure S6. Complete uncropped Western blot images of cancer cells for CES2 and β-actin proteins. (a) CES2 levels. Lane 1: Ladder, Lanes 2-4: (not for this paper), Lanes 5-7: MIA PaCa2, CFPAC-1, HepG2, Lanes 8-10: (not for this paper). (b) Ladder for CES2 Levels. (c) β-actin levels. Lane 1: Ladder, Lanes 2-4: (not for this paper), Lanes 5-7: MIA PaCa2, CFPAC-1, HepG2, Lanes 8-10: (not for this paper). (d) Ladder for β-actin levels.

Luminescence Assays in Cancer Cells:

Cells were seeded at a density of 20,000 cells/well in white 96 well plates purchased from Thermo Fisher Scientific (96 Well White/Clear Bottom Plate, TC Surface) and allowed to adhere overnight. Following this, media was discarded, cells were washed once with DPBS, and were given Opti-MEM. LPA or BNPP inhibitor was diluted in accordingly to a final concentration of 5 µM (1% v/v DMSO) and left to incubate for 30 min. Following this, the cells were washed once with DPBS, given fresh DPBS and CL-CES2 was diluted in at a final concentration of 20 µM and immediately read at 37°C.

Cell Viability:

Cells were seeded at a density of 20,000 cells/well in 96-well plates purchased from Thermo Fisher Scientific (Nunc™ MicroWell™ 96-Well, Nunclon Delta-Treated, Flat-Bottom Microplate) and left to adhere overnight. CL-CES2 was diluted in, with total DMSO concentration not exceeding 1% v/v and was left to incubate for 24 h under 5% CO_2 and 37 °C conditions. Following this, MTT was diluted in at a final concentration of 0.5 mg/mL and left to incubate for 2 h. The solution was removed and replaced with 100 µL DMSO per well. The absorbance values at 560 nm and 630 nm (reference) were read by a Tecan Spark plate reader. Cell viability was determined by normalizing each experimental sample (treated with CL-CES2) to the control group (untreated) upon subtracting background absorbance at 630 nm

subtracted from the 560 nm absorbance value, with the cell viability of the control group adjusted to 100%.

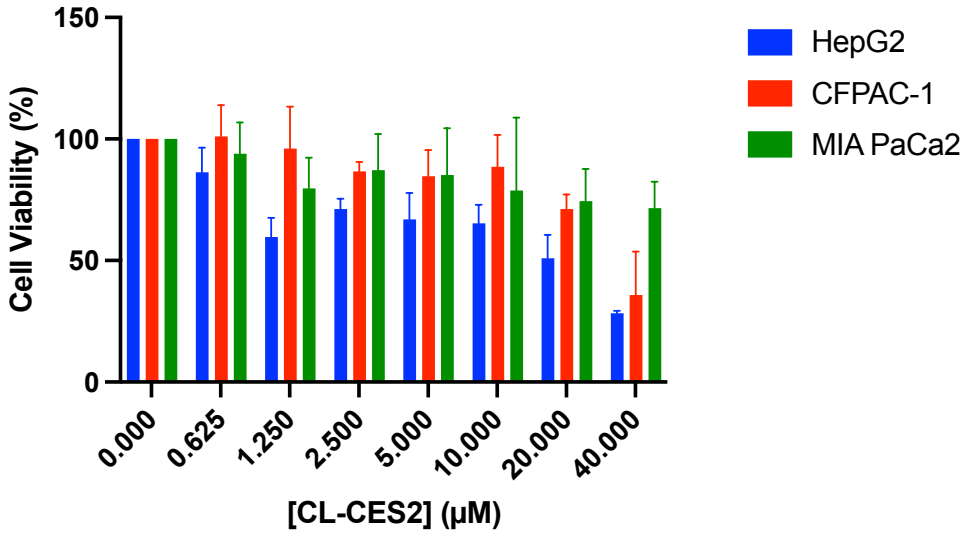


Figure S7. Cell viability assay of HepG2, CFPAC-1, and MIA PaCa2 cells following 3h incubation with CL-CES2. Data are shown as mean ± standard deviation of triplicate experiments.

6. NMR Spectra

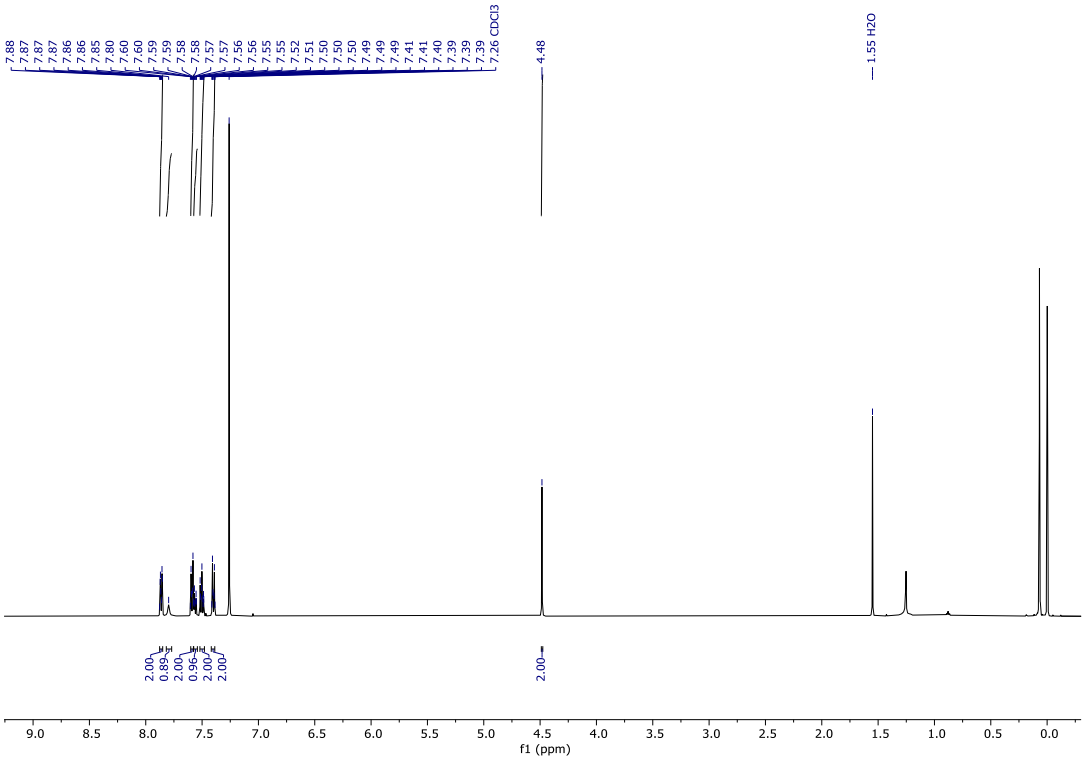


Figure S8. ^{1}H NMR spectrum of **2**.

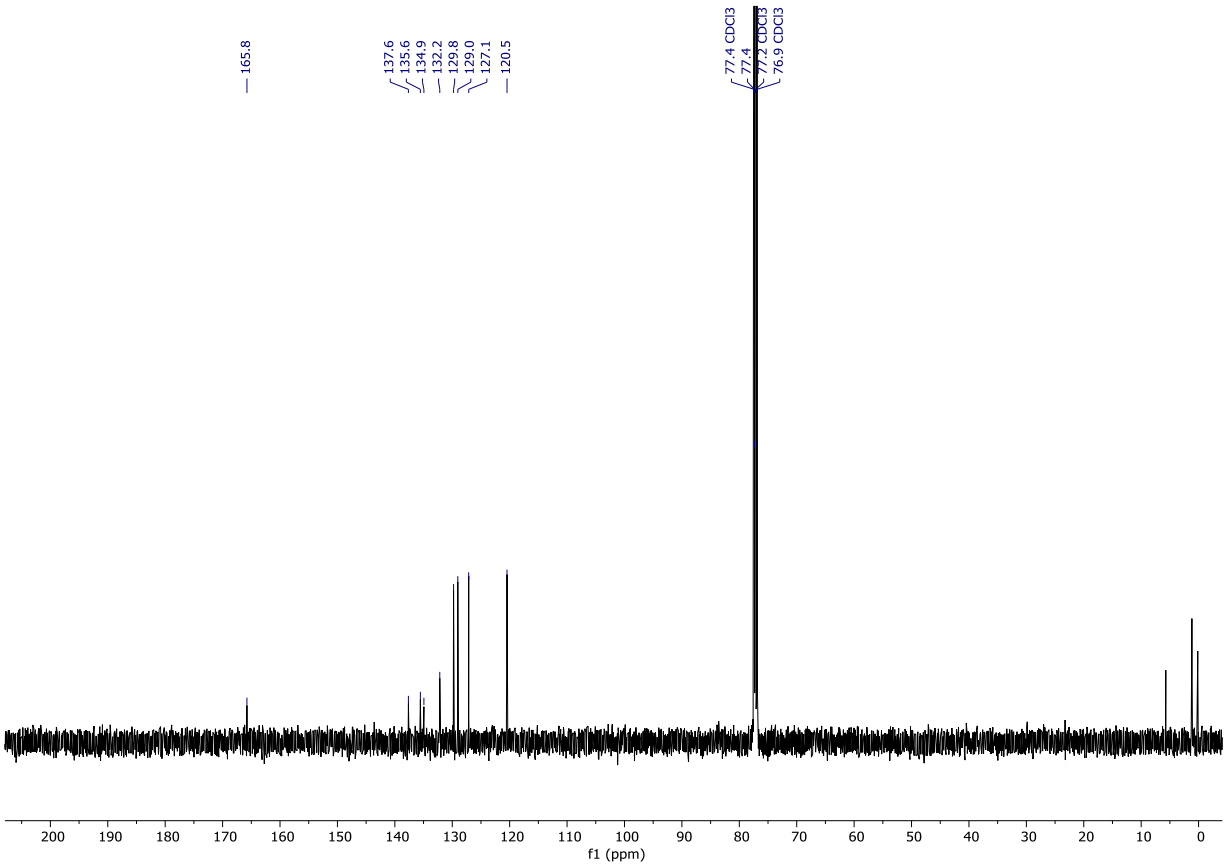


Figure S9. ^{13}C NMR spectrum of **2**.

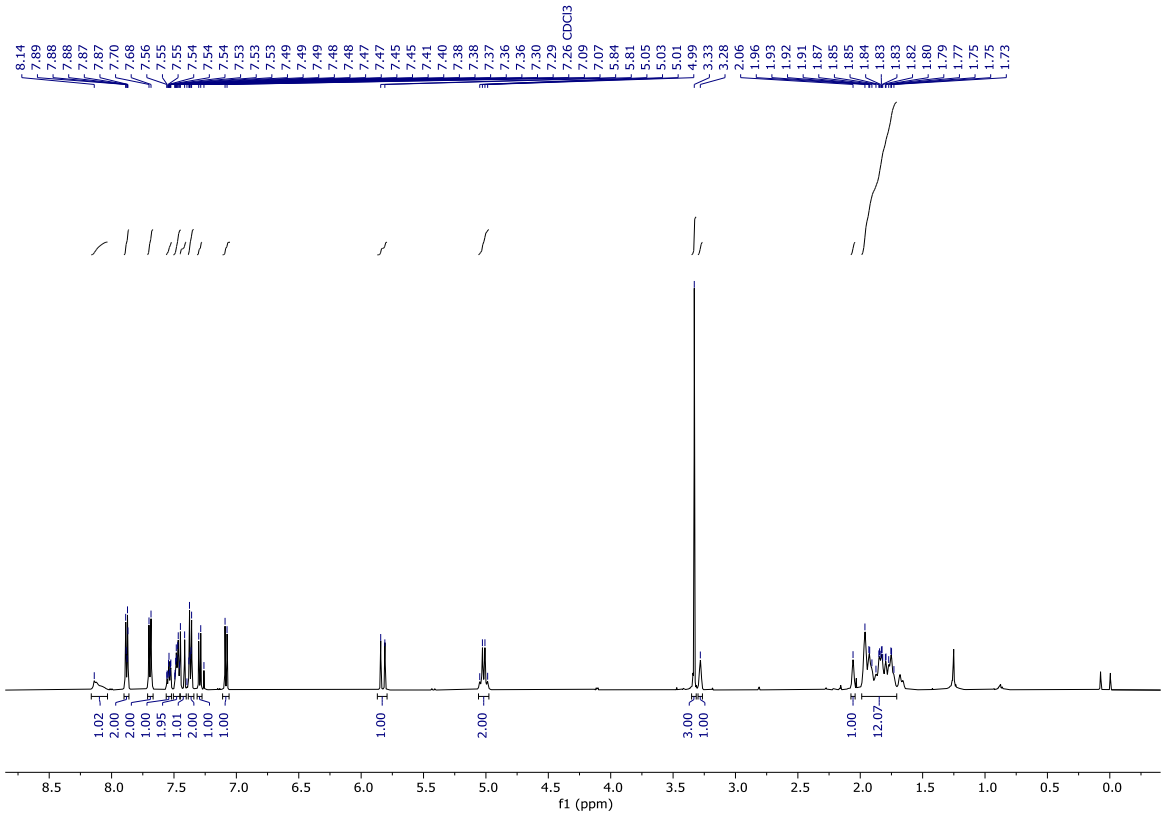


Figure S10. ^{1}H NMR spectrum of **4**.

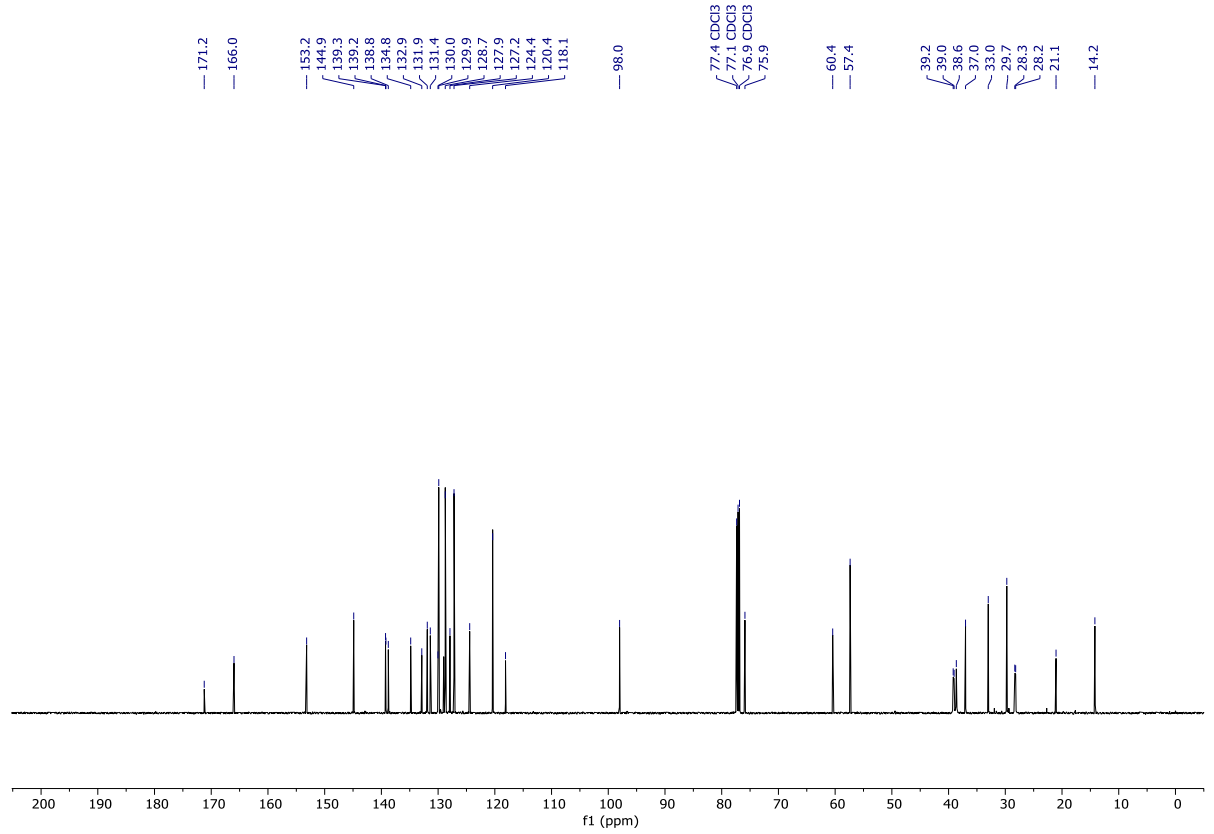


Figure S11. ^{13}C NMR spectrum of **4**.

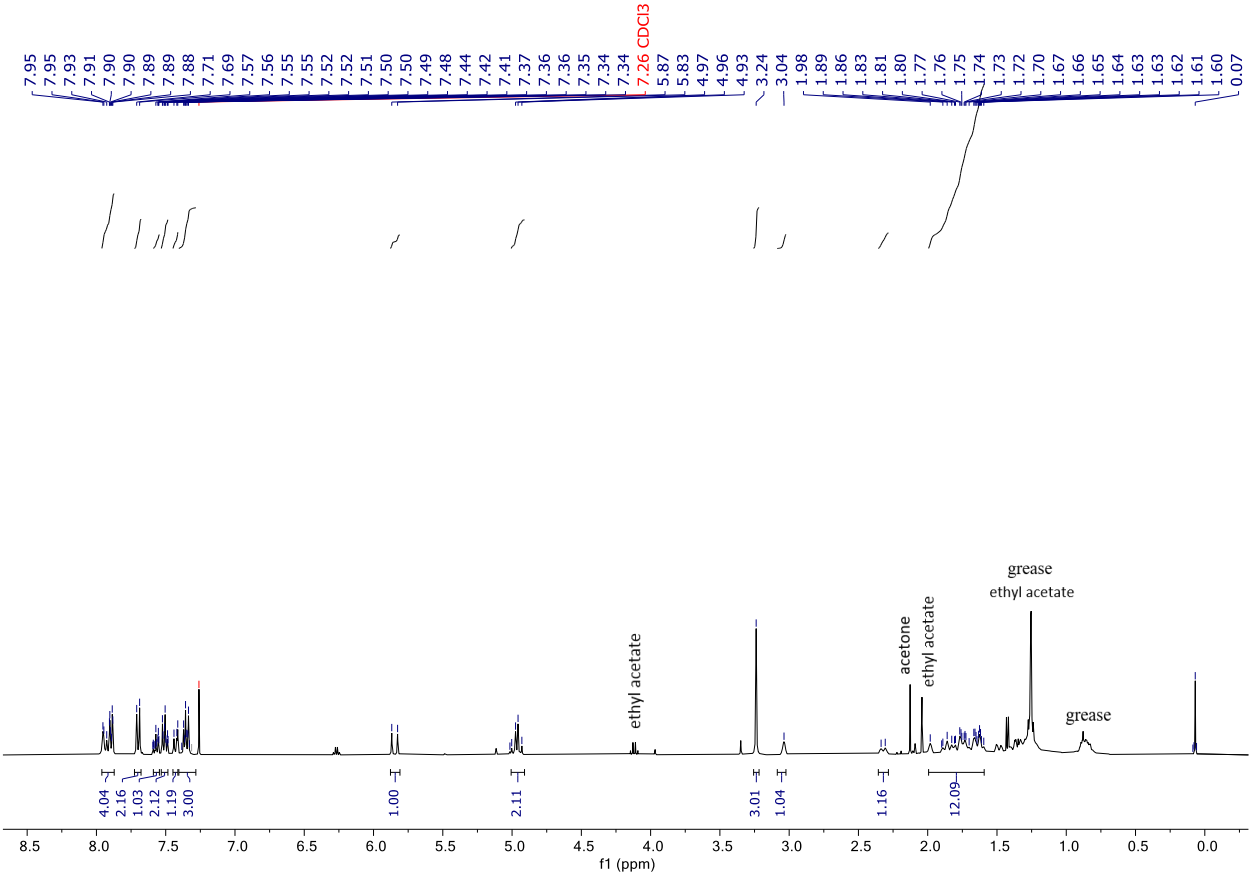


Figure S12. ^{1}H NMR spectrum of **CL-CES2**.

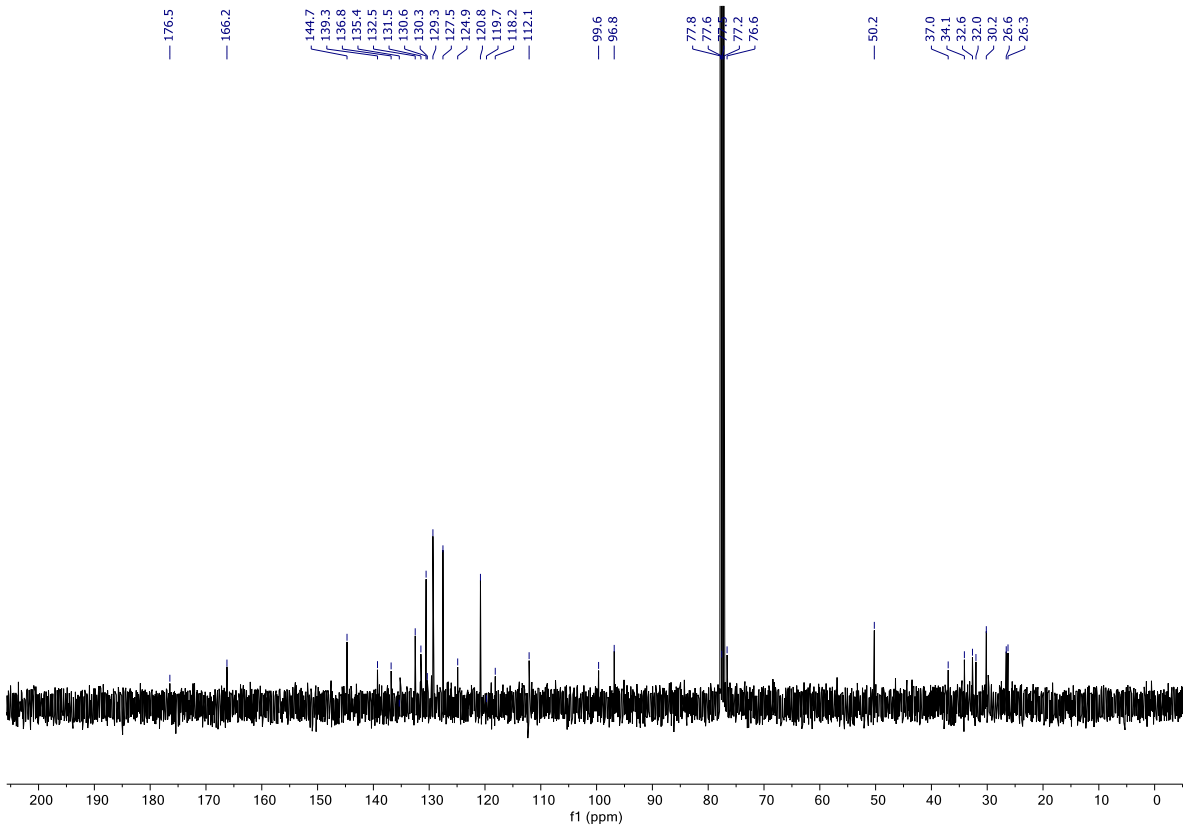


Figure S13. ^{13}C NMR spectrum of **CL-CES2**.

7. Mass Spectra

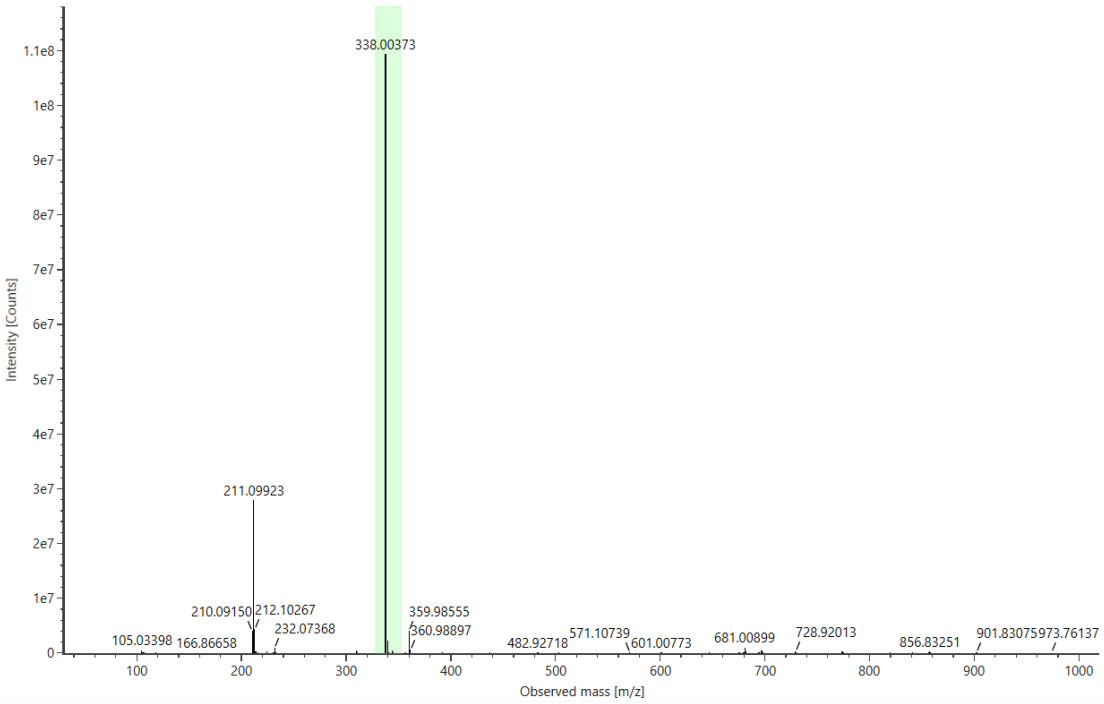


Figure S14. HRMS spectrum of **2**.

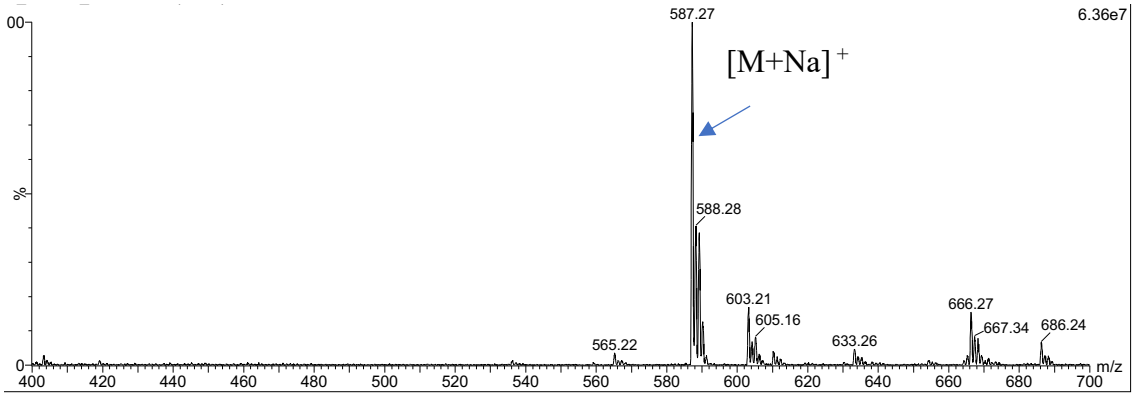


Figure S15. MS spectrum of **4**.

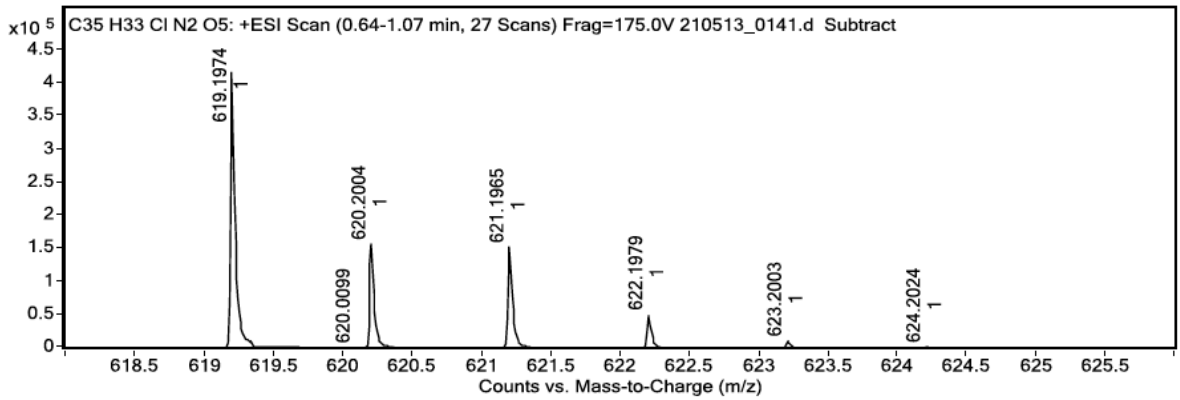


Figure S16. HRMS spectrum of **CL-CES2**.

8. References

1. L. Pignataro, C. Bovio, M. Civera, U. Piarulli and C. Gennari, *Chem. Eur. J*. 2012, **18**, 10368 – 10381.
2. O. Green, T. Eilon, N. Hananya, S. Gutkin, C. R. Bauer and D. Shabat, *ACS Cent. Sci.* 2017, **3**, 349−358.
3. S. J. Park, Y. J. Kim, J. S. Kang, I. Y. Kim, K. S. Choi, H. M. Kim, *Anal. Chem.*, 2018, **90**, 9465-9471.