Electronic Supplementary Material (ESI) for ChemComm

Tracking Mitochondrial $^1O_2$-ROS Production Through a Differen-tial Mitochondria-Nucleoli Fluorescent Probe**

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Experimental Procedures

Materials and Methods

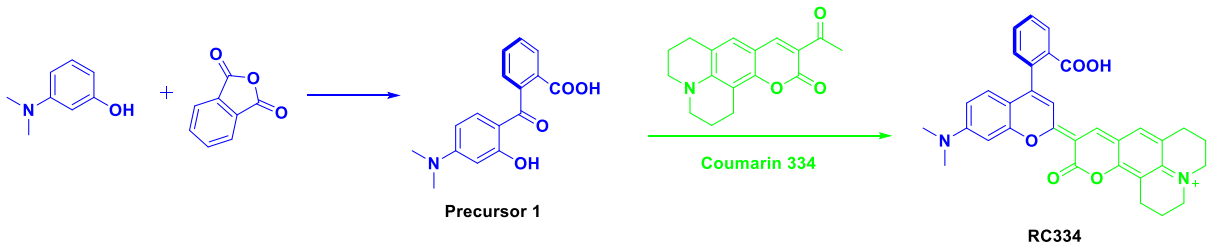
Cell Culture

Live HeLa cells were cultured in Minimum Essential Medium Alpha (MEM alpha, Gibco, Gaithersburg MD) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad CA) at 37°C with 5% CO_2.

Live Cell Measurements

Live HeLa cells were seeded in three different 8-well μ-slides at a density of 10 000 cells per well for 12 hours prior experiments using RPMI medium supplemented with 10 FBS. Then, specific concentrations of **RC334**, 5-amino-levulinic acid (ALA), oligomycin A, antimycin A or specific organelle trackers in RPMI media were added on each slide 45 minutes for the fluorophores or 8 hours for ALA before imaging experiments. For mitochondrial imaging, cell cultures were washed two times with RPMI. During confocal imaging the microscope parameters were maintained constant using a 63x oil immersion objective.

General Probe Synthesis

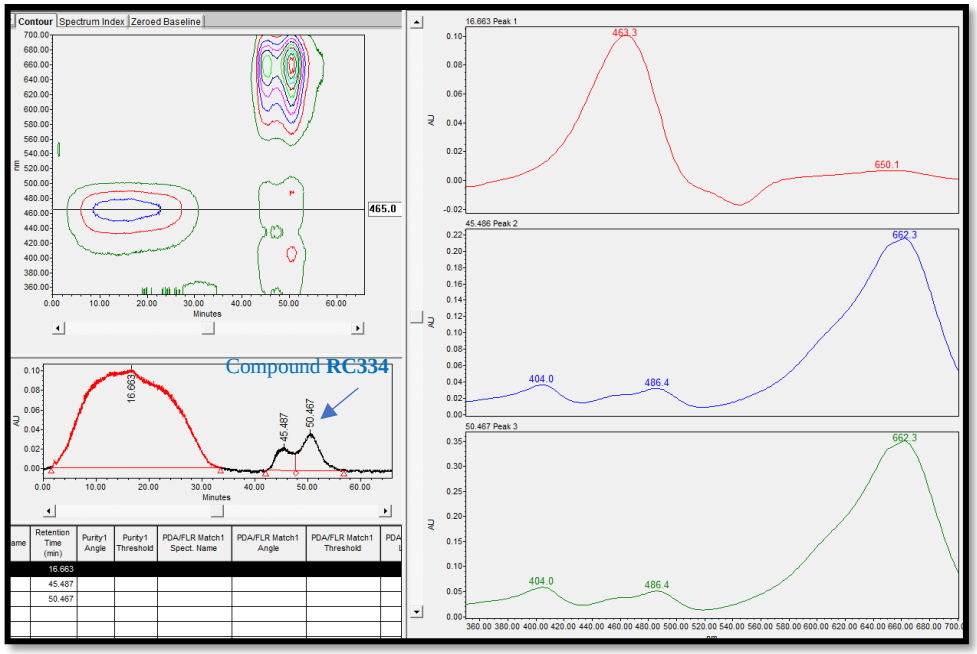


Scheme S1. Synthetic methodology for probe **RC334**.

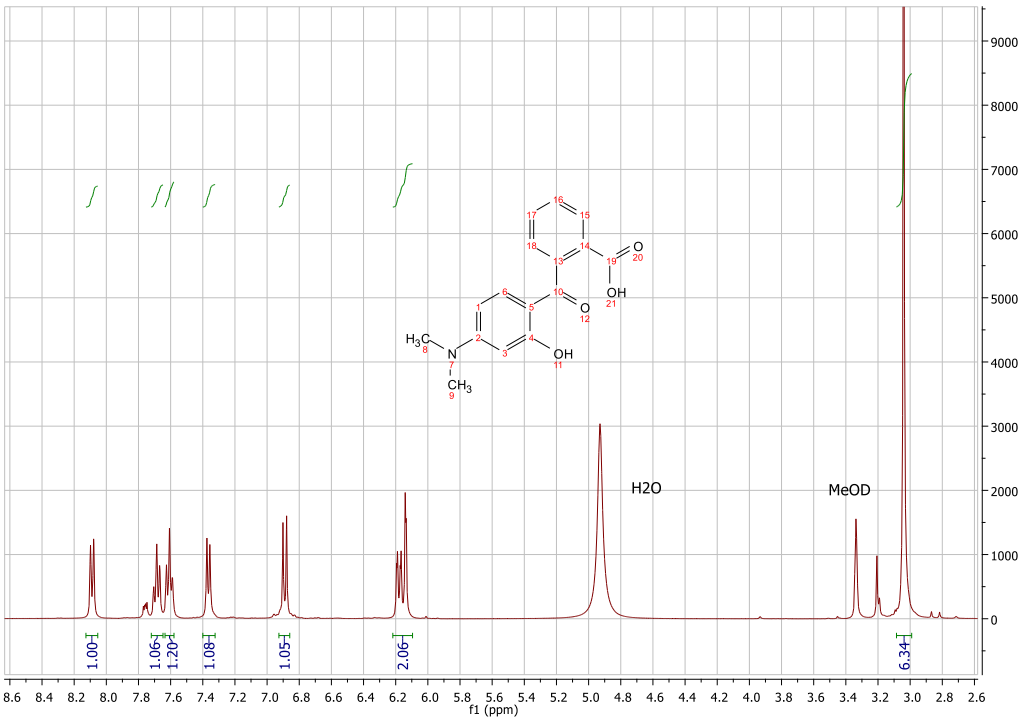
A solution of 3-(dimethylamino)phenol (2.00 g, 15 mmol, Sigma-Aldrich, Mexico) and phthalic anhydride (2.60 g, 18 mmol, Sigma-Aldrich, Mexico) were refluxed in toluene for 24 hours, then the solvent was removed by evaporation at reduced pressure and 100 mL of 35% NaOHaq solution was added and further stirred for 12 hours. Then, after acidification with 1M HCl the precipitate was filtered and recrystallized from MeOH : H_2O obtaining 3.60 g of a pale brown crystalline powder of precursor **1** (85% yield).

Then, precursor **1** (417 mg, 1.48 mmol) was immediately added to solution of coumarin 334 (500 mg, 1.75 mmol) in 8mL H_2SO_4 at 0°C and stirred at 90°C for 12 hours. Then after reaching room temperature, 5 grams of ice were added to the crude product following with 800 μL $HClO_4$ addition. The crude product precipitated and was filtered, extracted (dicholoromethane : brine, 4X) and dried under anhydrous $NaSO_4$. Then, the product was purified by RP-HPLC using an isocratic method, with a mixture of MeOH : H_2O (70:30 v/v) as eluent. The column was a Luna 5u C18 (2) 100 Å, 50 x 21 mm, 5 microns. A flow of 10 mL/min was used and 500 μL of **RC334** solution was injected (50 mg /2 mL of MeOH). The main impurity comes out at 5 min and ends at 10 min while **RC334** has a retention time of 15 min. The product was recovered, and the solvents were evaporated. *Right*: is shown a representative RP-HPLC chromatogram. A dark-green powder was obtained (149 mg, 20% yield). ^{1}H NMR (700 MHz, $MeCD_3$) δ/ppm 8.51 (s, 1H), 8.09 (s, 1H), 7.88 (s, 1H), 7.61 (dt, J = 7.7 Hz, 2H), 7.31 (s, 1H), 7.18 (s, 1H), 6.88 – 6.73 (m, 3H), 3.42 (m, 4H), 3.05 (s, 6H), 2.67 (m, 2H), 2.45 (m, 2H), 1.93 (m, 4H). ^{13}C{^{1}H} NMR (175 MHz, $MeCD_3$) δ/ppm 171.5, 161.6, 161.2, 157.8, 156.8, 155.4, 152.1, 150.4, 144.2, 134.3, 129.1, 128.9, 128.7, 128.5, 128.2, 128.1, 127.9, 120.8, 114.5, 114.4, 111.3, 109.6, 104.7, 102.7, 95.9, 49.8, 49.3, 38.8, 28.7, 26.0, 19.7, 18.7, 18.6. UV-Vis in methanol λ/nm (ε/dm^3 mol^{-1} cm^{-1}) 650 (180760). The measured fluorescence quantum yield of **RC334** in methanol was 0.096 using coumarin 343 as standard. ESI HRMS-TOF: *m/z* 533.2077 $[M\text{-}H]^+$ found, 533.21 calculated.

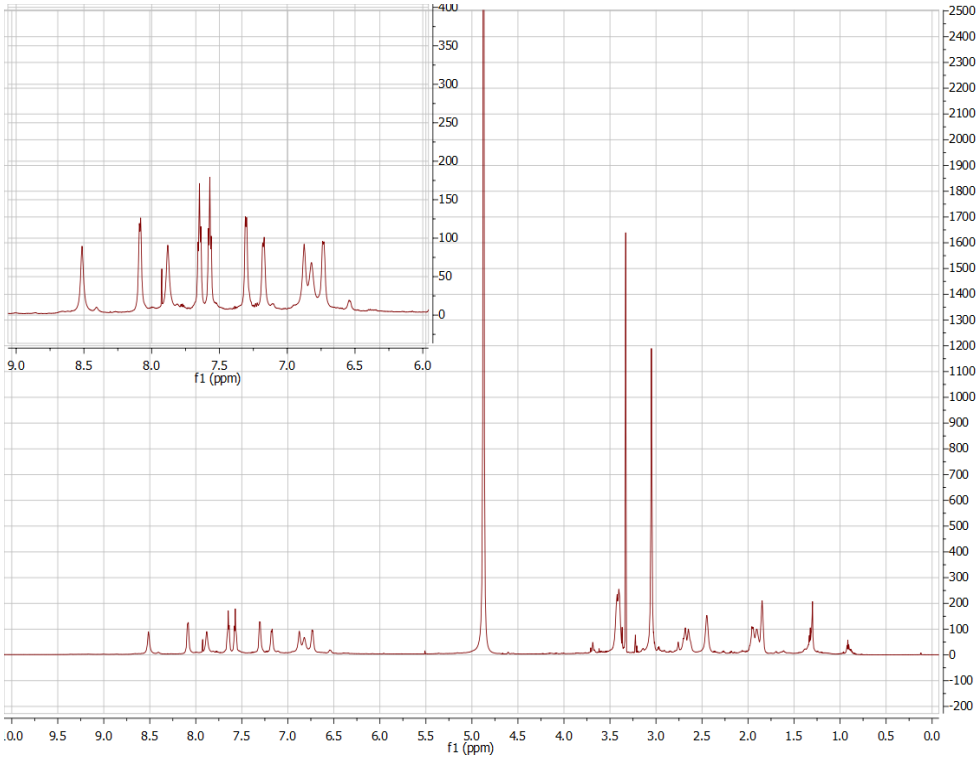
HPLC purification analysis

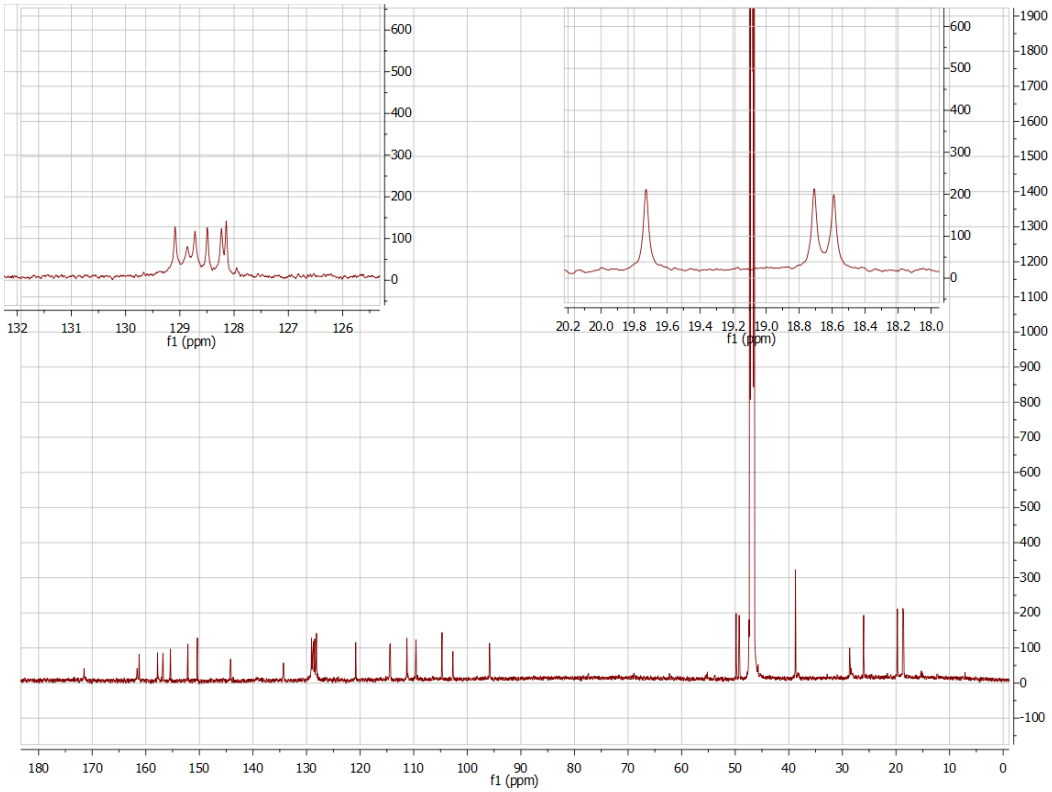


^{1}H NMR spectrum of precursor **1** in CD_3OD.

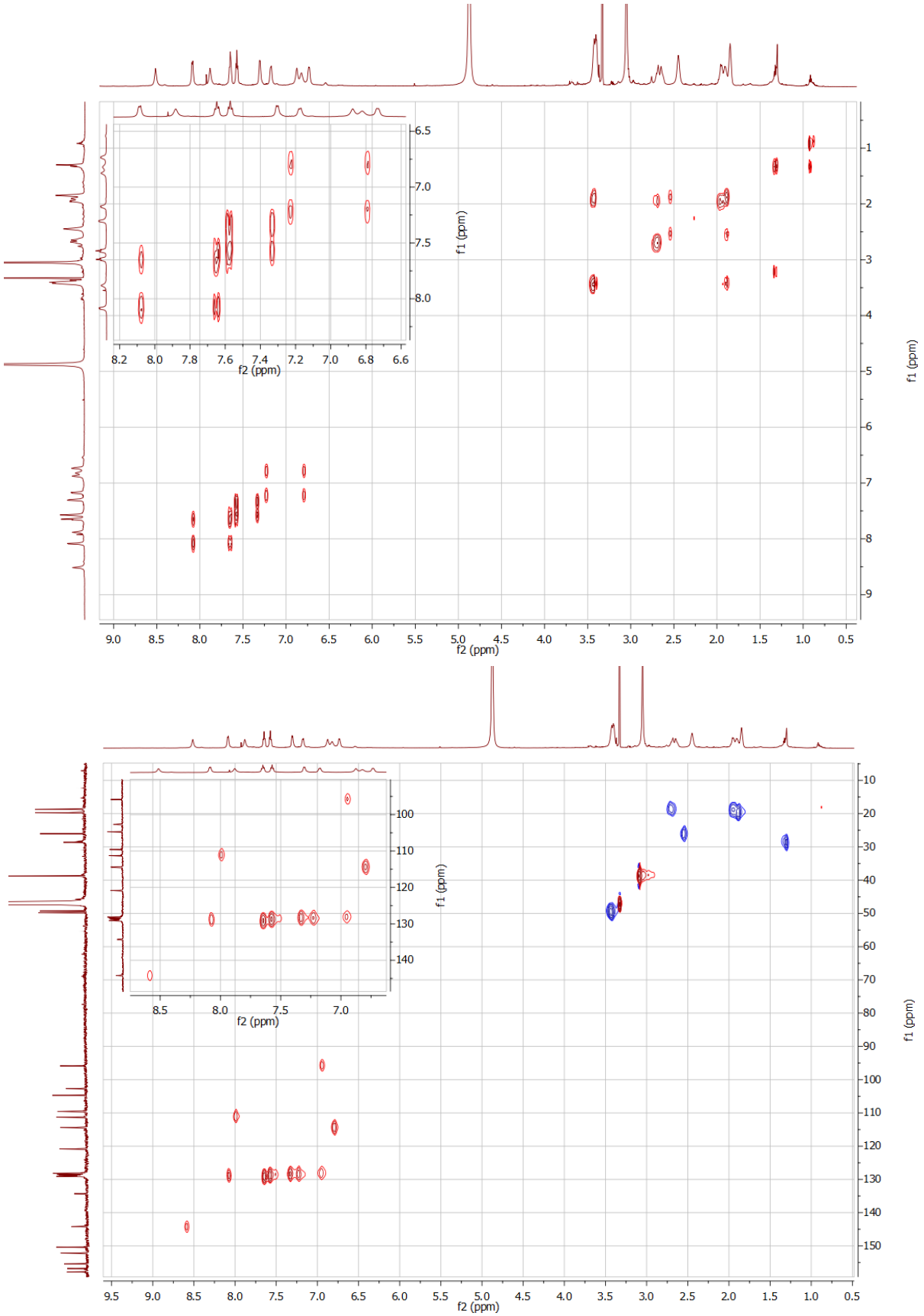


^{1}H and ^{13}C NMR spectra of probe **RC334** in CD_3OD.

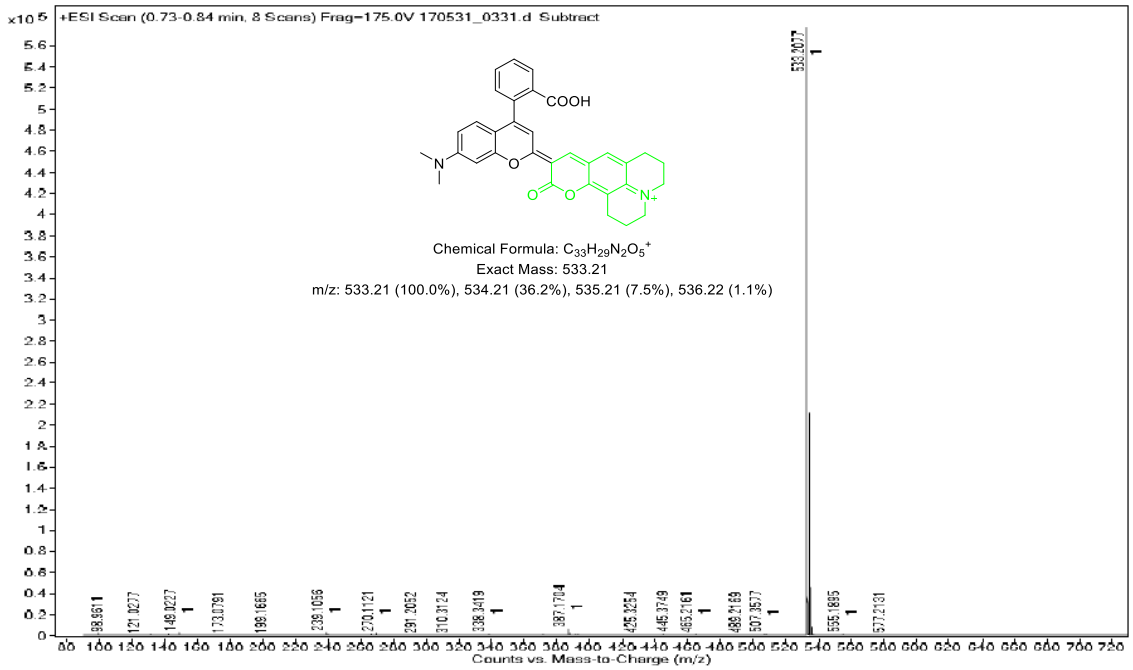




Gradient COSY and HSQC NMR spectra of probe **RC334** in CD_3OD.



HRMS ESI[+] spectrum of probe **RC334**.



Materials and measurements.

Commercially available starting materials, components of buffer solutions (CHES, MOPS, MES from Sigma, Mexico) and solvents were used as supplied. ^{1}H and ^{13}C NMR spectra were recorded at room temperature on a 700 MHz Bruker unity spectrometer. Chemical shifts (ppm) are relative to $(CH_3)_4Si$. High resolution mass spectrometry (ESI-TOF) was obtained by using an Agilent Technologies 6530 Accurate-Mass Q-TOF LC/MS equipment. Fluorescence experiments were measured either on a FluoroMax spectrofluorometer from HORIBA Scientific or in a Cary Eclipse fluorimeter, UV-Vis absorption spectra were taken on a Thermo Scientific Evolution diode array UV-Vis spectrophotometer.

Preparation of ROS agents:

1) Hydrogen peroxide (H2O2), Sodium hypochlorite (NaClO), , Iron(III) chloride ($FeCl_3$), Sodium bicarbonate ($NaCO_3$), Sodium nitrate ($NaNO_3$) and Sodium nitrite ($NaNO_2$) were obtained from Sigma-Aldrich and used as provided. Sodium peroxynitrite ($NaNOO_2$) was purchased from Merk Millipore (US1516620-1SET) and used as provided.
2) Singlet oxigen ($^{1}O_2$) was prepared as follows: The singlet oxygen concentration was determined by the following reaction: $NaClO + H_2O_2 \rightarrow NaCl + {}^{1}O_2 + H_2O$. The following was mixed: NaClO = [14%] (1 mL) with H_2O_2 = [30%] (2 mL) where the limiting reagent is sodium hypochlorite. So, 1mL NaClO (14%) is equiv. to 0.14 g of pure NaClO, giving 0.6266 M of NaClO. Then, a dilution was made 1 mL in 40 mL to obtain a 1.6 µM NaClO solution. After that a linear regression analysis was performed using the commercially available Singlet Oxygen Sensor Green®, as described in literature (Singlet Oxygen Production in Water: Aggregation and Charge-Transfer Effects. The Journal of Physical Chemistry, 1996, 100(16), 6555–6560).
3) Superoxide ion ($O_2^{\bullet-}$): was prepared by reaction of commercially available potassium dioxide (KO_2, Sigma-Aldrich, 278904) and DMSO using supporting electrolyte and tetrabutylammonium, as previously reported [M. Hayyan, M. A. Hashim, I. M. AlNashef, *Chem. Rev*. 2016, **116**, 3029–3085.].
4) Hydroxyl radical ($OH^{\bullet}$) were produced by Fenton reaction using 10 equiv. H_2O_2 + 1 equiv. $FeCl_2$.

Quantum Chemical Calculations

Quantum Chemical Calculations were obtained by using DFT and TD-DFT with Polarizable Continuum Model[1] as performed in the Gaussian 09 code,[2] using a PBE0/6-31+G(d,p)/IEF-PCM (water) level of theory to determine the optimized molecular geometry of **RC334**. Then, a frequency analysis corroborates that the geometry corresponds to an energy minimum, finding no imaginary frequencies. As a first step in the analysis of the electron charge distribution in the molecules, the electrostatic potentials were computed to compare the local charge distribution between these molecules. Finally, Natural Transition Orbital (NTO)[3] analysis was computed at the same level of theory to further understand the optical properties for probe **RC334**.

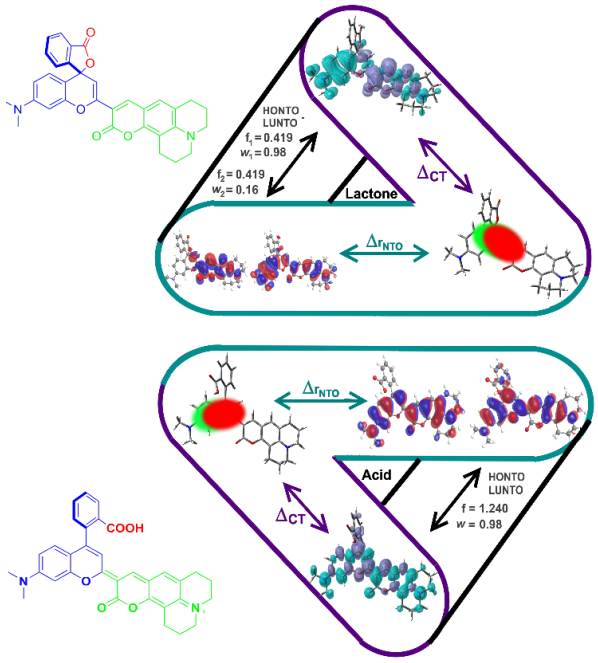
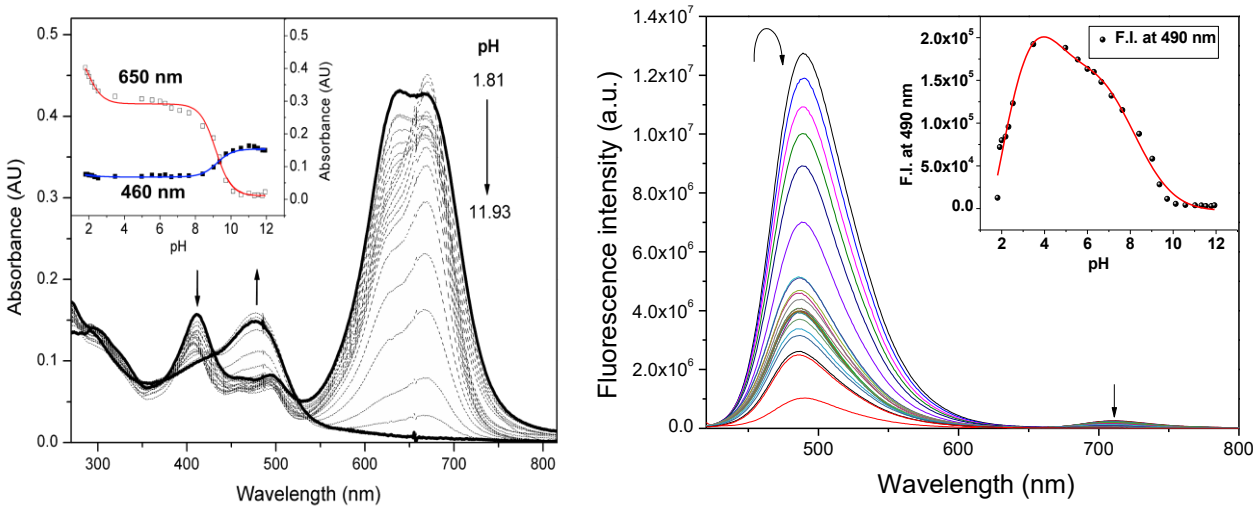


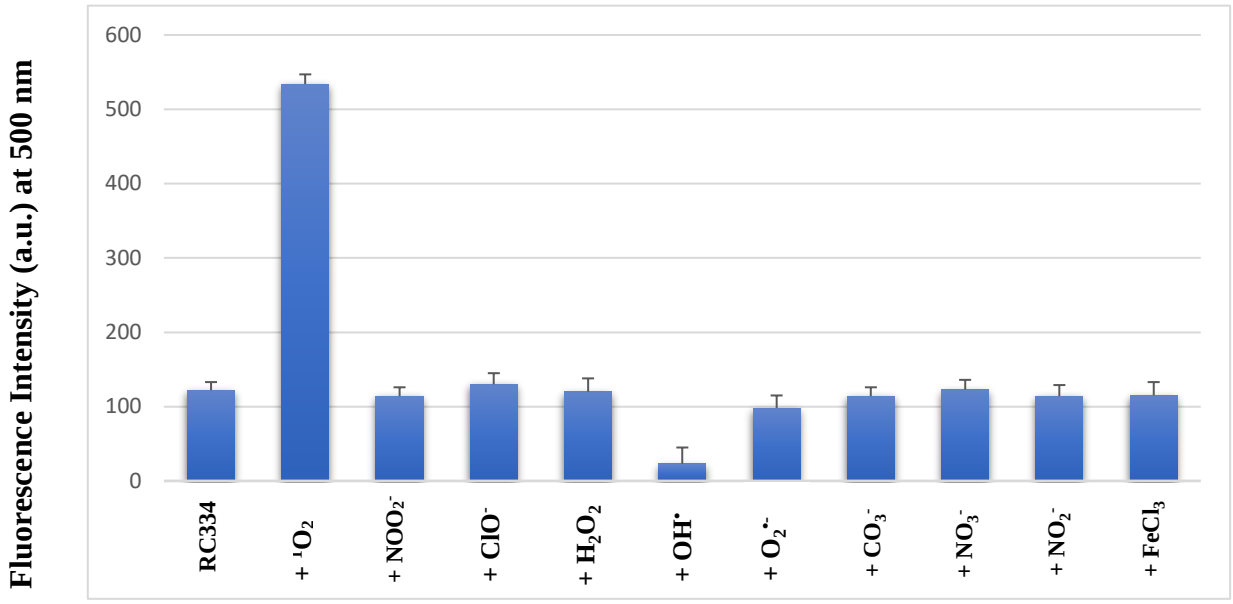
Figure S1. (Left) Molecular structure drawing of the synthesized **RC334** probe in the open-acid and closed-*spiro*lactone forms. (Right) PBE TDDFT state energies for the ground-state optimized geometry of **RC334** in the closed-*spiro*lactone (above) and open-acid (below) forms. Arrows highlight the complementarity of the electron density difference distribution (green-purple structures), charge transfer index upon photoexcitation (in green and red) and natural transition orbital pairs (in blue and red).



(A) **(B)**

Figure S2. (**A**) UV-Visible and (**B**) fluorescnece spectra of 10 µM **RC334** at variable pH (25 °C and 50 mM NaCl). Insets show absorbance *vs.* pH profiles at selected wavelengths, solid lines are the theoretical fittings.

A)



B)

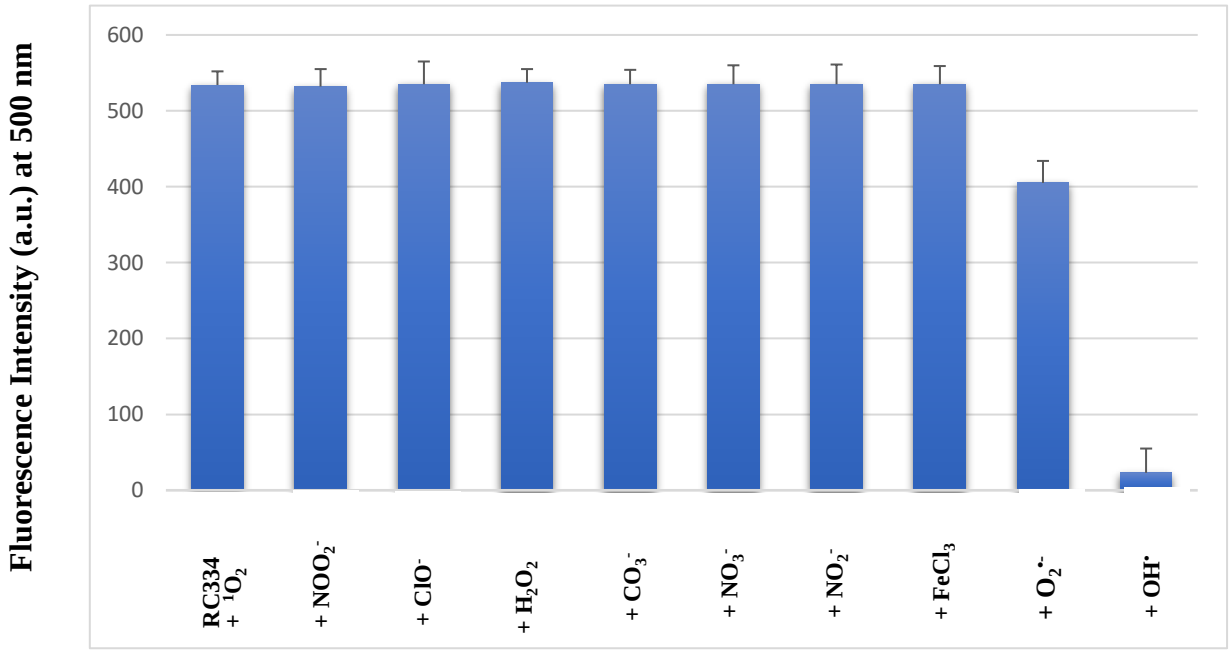
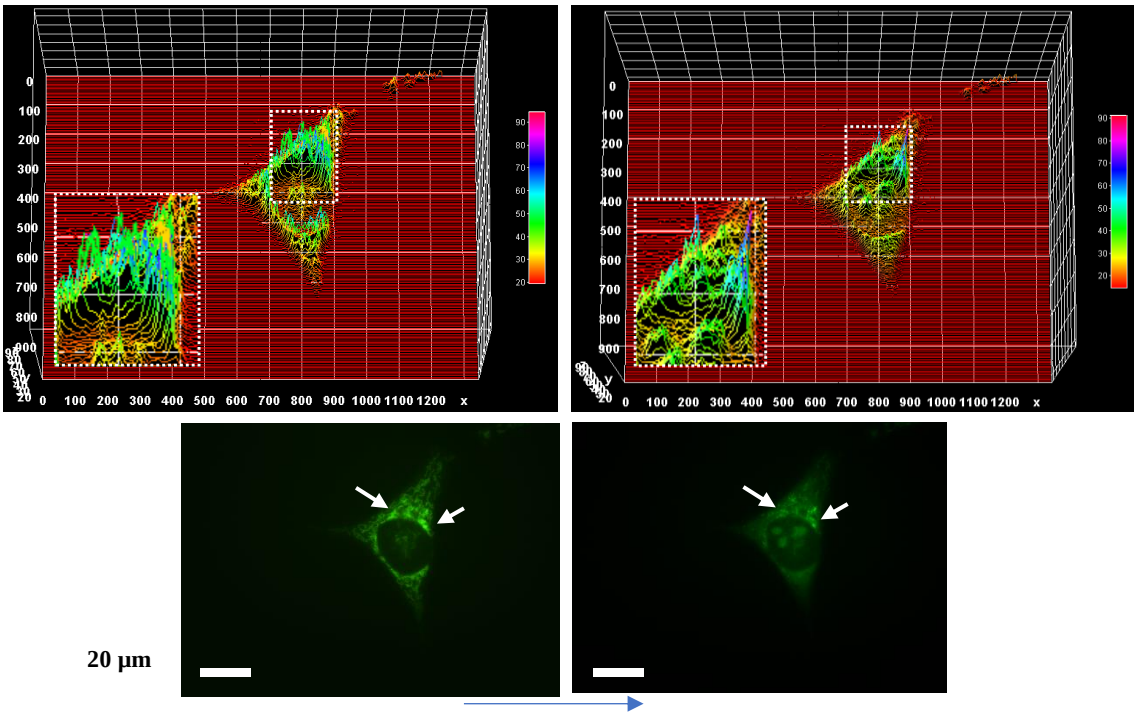
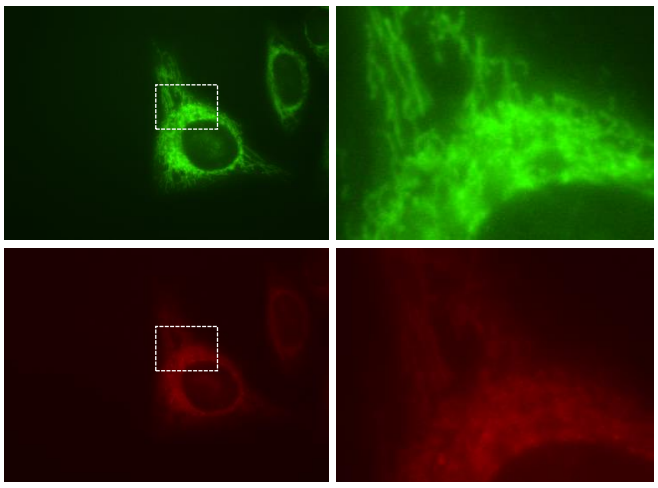


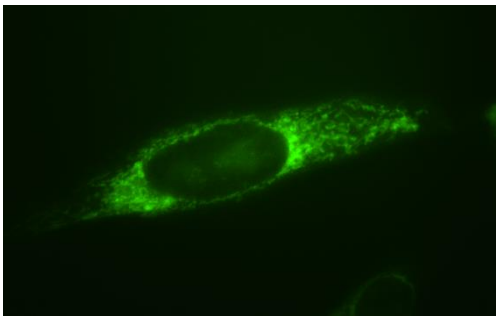
Figure S3. Selectivity and competition graphs. A) Fluorescence intensity bars at λ_{em} = 500 nm for **RC334** under different oxidants. B) Competition experiments for RC334+$^1O_2$ with different oxidants. NOO_2 = Peroxynitrite, ClO = Hypochlorite, H_2O_2 = Hydrogen peroxide, CO_3^- = Bicarbonate, NO_3^- = nitrate, NO_2^- = nitrite, $FeCl_3$ = Ferric chloride, $O_2^{•-}$ = Superoxide, $OH^•$ = Hydroxyl radical. All ROS species concentrations are in 1000% molar excess (0.8 M) with respect to the **RC334**.

A)



B)





RC334 *vs.* Endoplasmic reticulum tracer

Figure S4. A) 20 µM **RC334** 30 min *then* 100 µM **ALA** PDT treatment for 8 hours and then **ERTracker®** for 30 min. B) are source images with arrows showing endoplasmic reticulum localization. Green and red colors represent the green and red confocal channels using GFP and TxR setup, respectively.

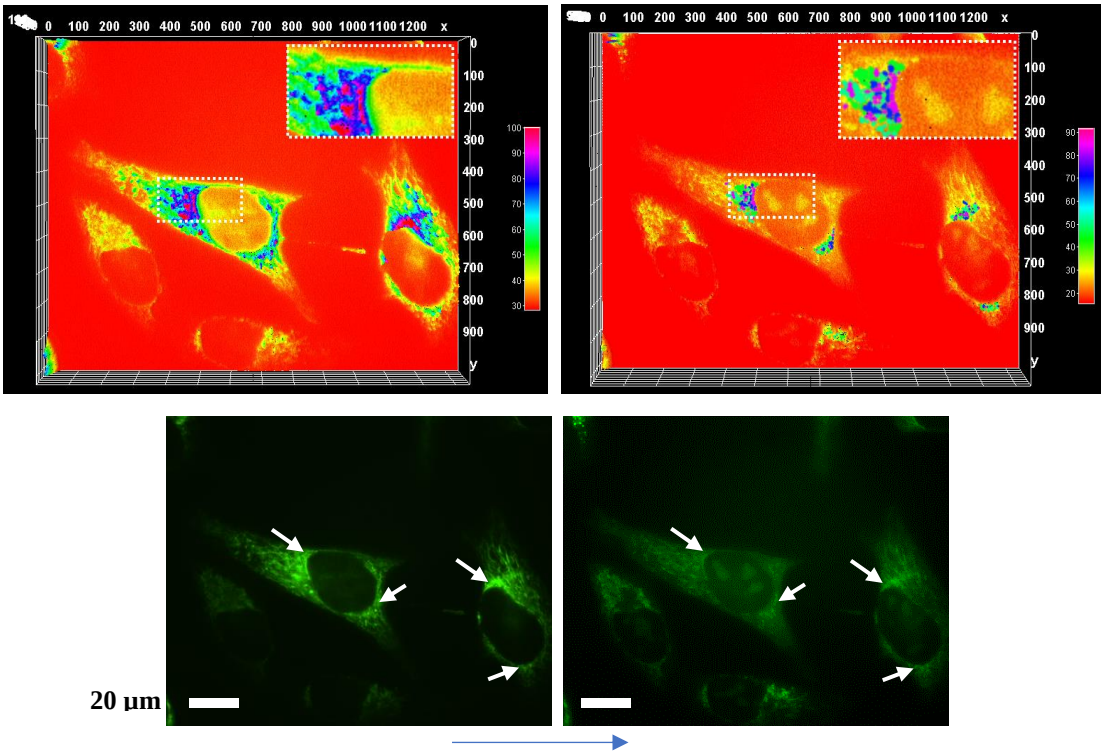


Figure S5. 20 µM **RC334** 30 min *then* 100 µM **ALA** PDT treatment for 8 hours and then **GolgiTracker®** for 30 min. Below are source images with arrows showing slight Golgi apparatus localization. Below: 25 µM **RC334** incubation. Green color represents the green confocal channel using the GFP setup.

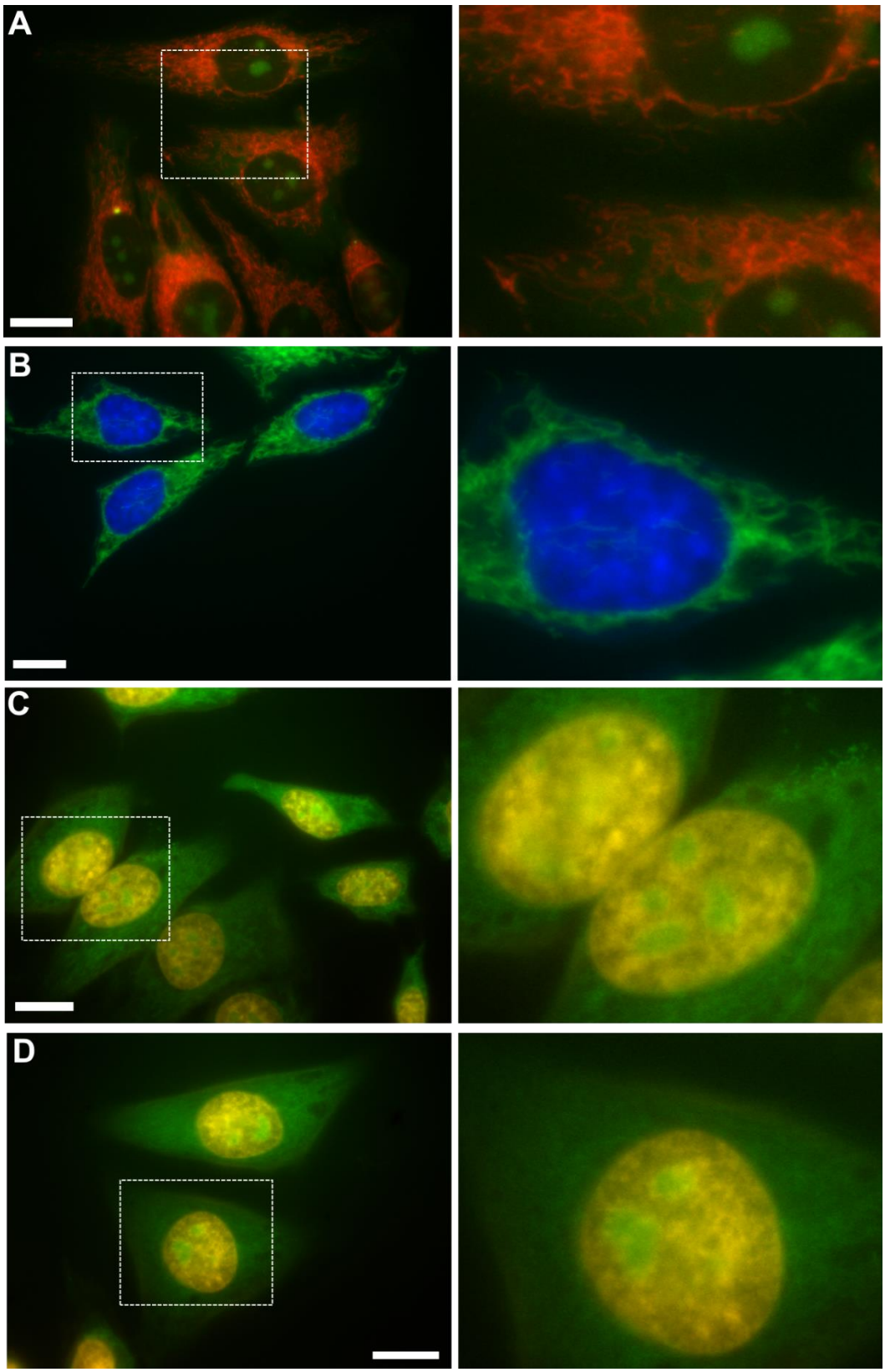
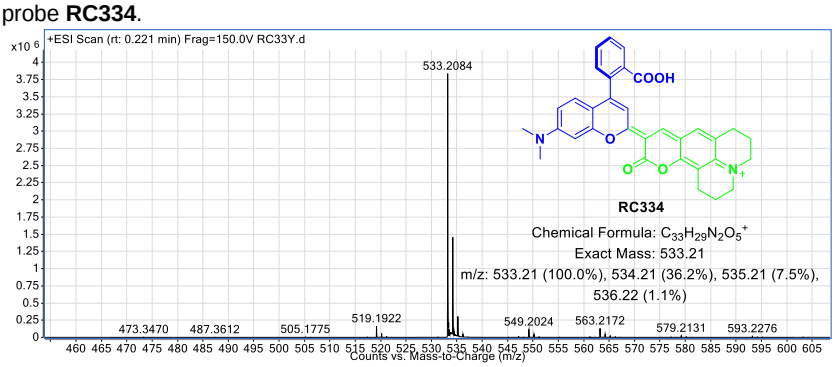
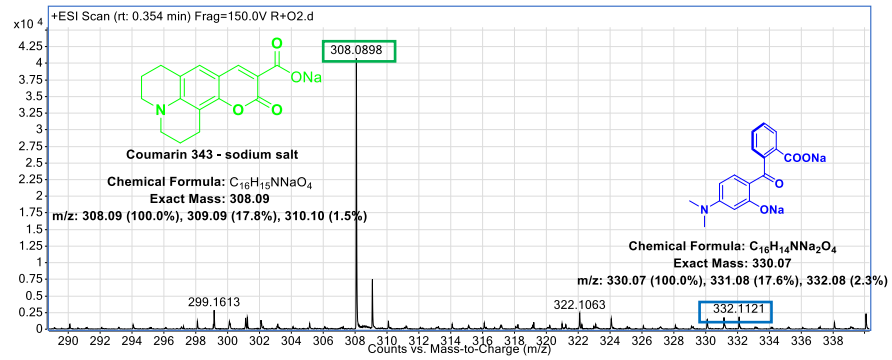


Figure S6. Colocalization images of (A) MitoTracker Deep Red® and **RC334** probe after $^1O_2$ – ROS formation, the image shows a very poor colocalization with Pearson's coefficient (PC) = 0.019. The excitation wavelengths are 488 nm and 596 nm for the green and red images, respectively. (B) Colocalization image of Hoechst dye in blue (λ_{exc} = 404 nm) and **RC334** probe (λ_{exc} = 488 nm) before ROS formation to show the absence of nuclear distribution of **RC334**. (C) and (D) Colocalization images between Hoechst dye *in false-red color* (λ_{exc} = 404 nm) and **RC334** probe (λ_{exc} = 488 nm) after $^1O_2$ – ROS formation to show fluorescence intensity overlap in yellow, PC = 0.87. This PC values is due to the lack of nucleolar staining of Hoechst dye.

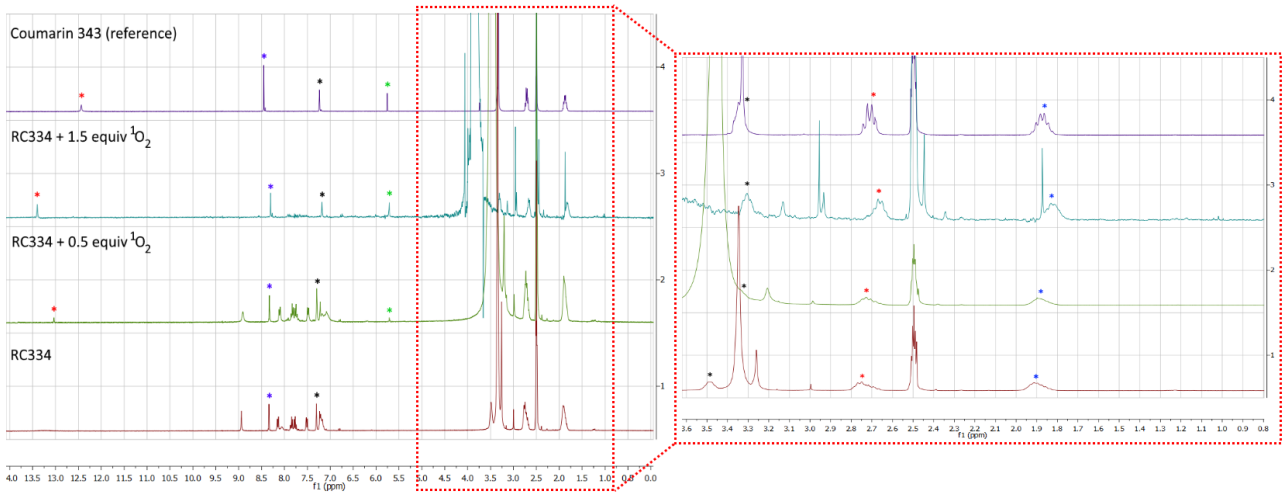
A) ESI-TOF scan for probe **RC334**.



B) ESI-TOF^{+} scan for probe **RC334** after 1equivalent addition of singlet oxygen.



C) ^{1}H-NMR spectra for the $^{1}O_2$ titration of probe **RC334**. Coumarin 343 spectrum is show as reference control.



D)

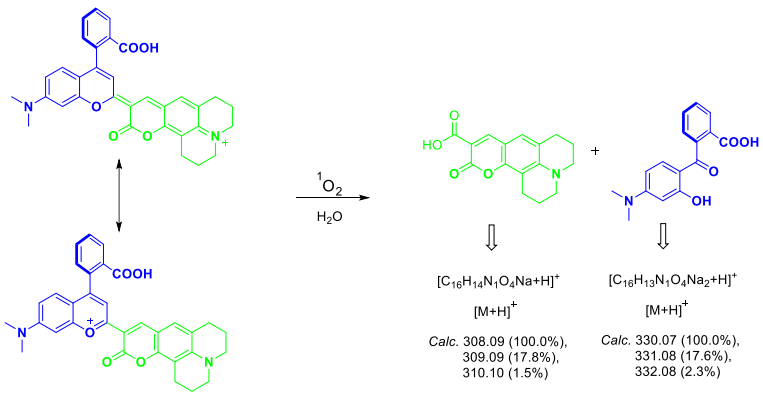


Figure S7. A) and B) High-Resolution Mass Spectrometry (ESI-TOF technique) and, C) ^{1}H-NMR titrations for **RC334** with $^{1}O_2$. D) Schematic representation for the oxidation mechanism.

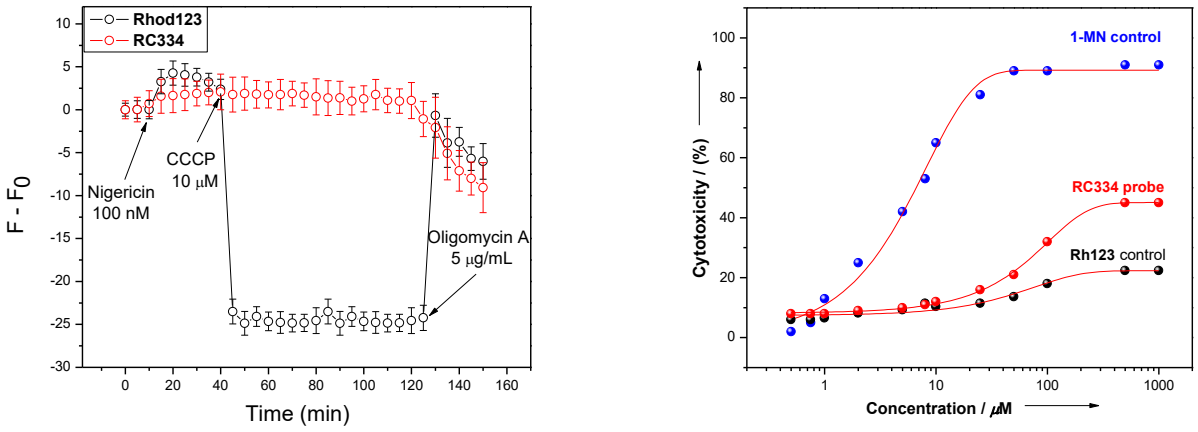


Figure S8. (left) Effect of nigericin during charge gradient depolarization with CCCP proton uncoupler on the **RC334** and Rhodamine 123 fluorescence signals. (Right) Cytotoxicity of different fluorescent probes in HeLa cells. The traces are exhibited from a representative trial. The LC_{50} >100 μM was determined for **RC334**. The controls are Rhodamine 123 (Rh123) and 1-Methylnaphthalene (1-MN).

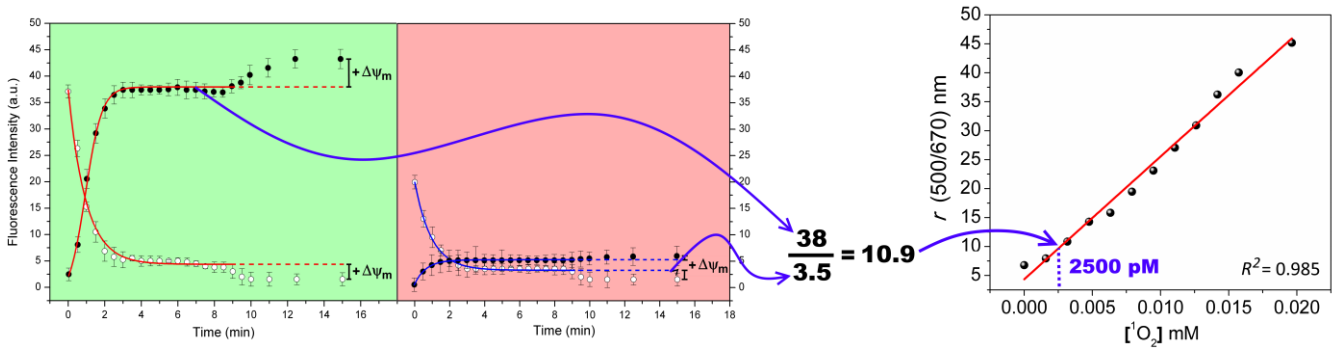


Figure S9. $^1O_2$ quantification using the calibration plot obtained under *in vitro* conditions. The λ-ratiometric method was used by taking the red signal (670 nm) as internal reference. Then, the following standard procedure was used:

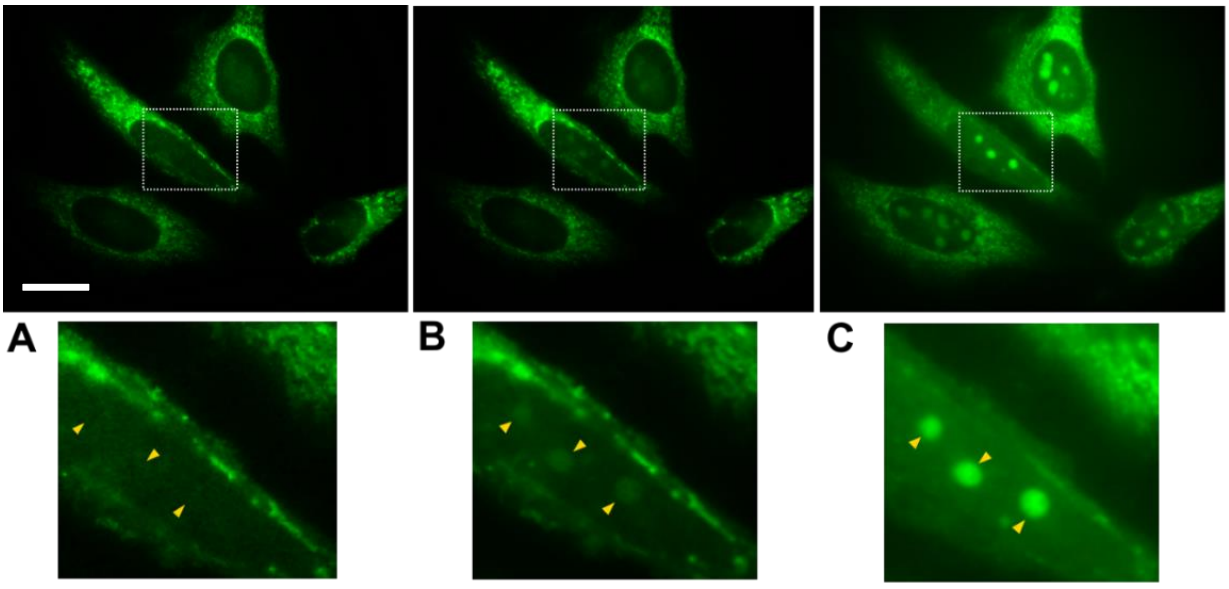


Figure S10. Effect of eight-hours ALA incubation and 0.1 J/cm^2 irradiation. A) Before treatment and, B) after 4 hours and, C) 8 hours treatment (C). The image was manually focused, and excitation light was fully shielded between recordings to prevent artefacts and photobleaching. Scale bar represents 20 μm. Green color represents the green confocal channel using the GFP setup.

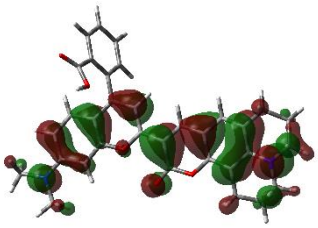
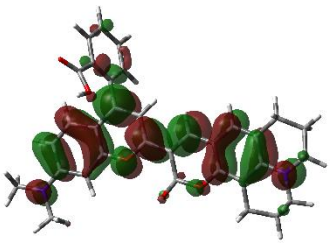
Table S1. Summary of charge-transfer (CT) indexes parameters upon photoexcitation.

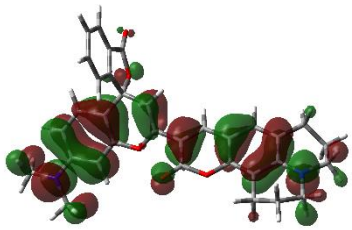
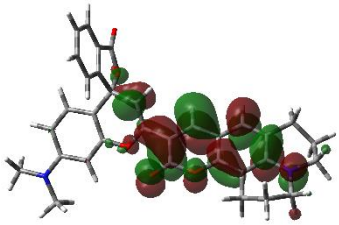
Charge transfer results - Open Form					
			Electron goes from		
			X	Y	Z
CT charge (e)	0.45	Center (Ang)	1.10228	-0.75578	-0.00666
CT distance (Ang	1.16	± spread (Ang	4.35938	1.75641	0.33853
CT dipole (Debye	2.67				
			to		
H index (Ang)	3.39		X	Y	Z
		Center (Ang)	-0.81719	0.21325	-0.18859
t index (Ang)	-2.23	± spread (Ang	2.41312	1.14829	0.33570

Charge transfer results - Closed Form					
			Electron goes from		
			X	Y	Z
CT charge (e)	0.74	Center (Ang)	2.61654	2.01107	-0.44836
CT distance (Ang	5.07	± spread (Ang	4.14527	1.92793	0.47065
CT dipole (Debye	18.00				
			to		
H index (Ang)	2.92		X	Y	Z
		Center (Ang)	-2.00060	-0.06436	-0.11190
t index (Ang)	2.15	± spread (Ang	1.69772	0.87280	0.77235

Table S2. Dominant natural transition orbital (NTO) pairs for the first excited singlet state of **RC334**. The left panels quote in sequence transition energies in eV, oscillator strength (f), NTO eigenvalues (w) and associated MO levels.

RC334 absorption	Hole	Particle

2.362 eV;
f = 1.240;
w = 0.98;
HOMO – LUMO
		

3.399 eV;
f = 0.419;
w = 0.98;
HOMO – LUMO
		

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