Protein aggregation monitoring in cells under oxidative stress: a novel fluorescent probe based in a 7-azaindole-BODIPY derivative

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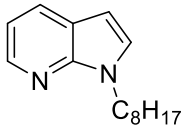
Supplementary information

Content:

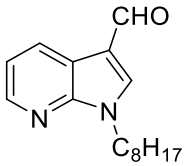
1. Synthesis

1-octyl-1H-pyrrolo[2,3-b]pyridine (**7AI-5**)



A mixture of 7-azaindole (7AI) (1.00 g, 8.47 mmol), KOH (722 mg, 12.9 mmol), $[CH_3(CH_2)_3]_4N(HSO_4)$ (PTC) (151 mg, 0.446 mmol), 1-iodooctane (1.98 mL, 11.0 mmol) in acetone (95 mL) was refluxed for 24 h. After cooling, the solvent was removed under reduced pressure. Then, the reaction mixture was extracted in CH_2Cl_2/H_2O and the organic layer was dried over anhydrous magnesium sulfate and filtered. The solvent was removed and the residue was purified by silica gel column chromatography using Hexane:CH_2Cl_2 (1:1) as eluent to afford **7AI-5** as a brown oil. Yield: 1.260 g (65%).

1-octyl-1H-pyrrolo[2,3-b]pyridine-3-carbaldehyde (**7AI-6**)

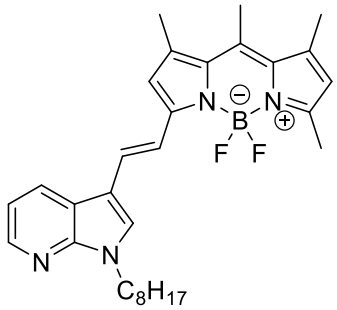


To a solution of *N*,*N*-dimethylformamide (DMF) (20 mL) at 0 ºC was added $POCl_3$ (6.16 mL, 65.9 mmol) under nitrogen. After 20 min at 0ºC the compound **7AI-5** (1.17 g, 5.09 mmol) in 5 mL of DMF was added to the reaction and was stirred at 0 ºC for 50 min. Afterwards heated for 3 h at 80 ºC. The reaction mixture was cooled and then extracted with CH_2Cl_2/H_2O. The organic layer was dried over anhydrous magnesium sulfate and filtered. The solvent was evaporated under reduced pressure; the residue was purified by column chromatography on silica gel with dichloromethane as eluent to obtain **7AI-6** as a brown oil. Yield: 0.84 g (64 %).

^{1}H-RMN (300 MHz, $CDCl_3$) δ (ppm): 9.97 (s, 1H), 8.55 (dd, J =1.54 Hz, J = 7.84 Hz, 1H), 8.42 (dd, J = 1.54 Hz, J = 4.80 Hz, 1H), 7.86 (s, 1H), 7.26 (dd, J = 4.80 Hz, J = 7.84 Hz, 1H), 4.35 (t, J = 7.31 Hz, 2H), 1.97-1.87 (m, 2H), 1.34-1.24 (m, 2H), 0.85 (t, J = 6.70 Hz, 3H).^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 184.5, 148.2, 144.8, 138.0, 130.7, 118.8,

117.9, 116.3, 45.6, 31.7, 30.0, 29.1, 26.7, 22.5, 14.0. APCI$^+$ MS m/z 259.2 $[M+H]^+$ HRMS (APCI) calcd. for $C_{16}H_{23}N_2O$ 259.1801, found 259.1805.

(E)-5,5-difluoro-1,3,9,10-tetramethyl-7-(2-(1-octyl-1H-pyrrolo[2,3-b]pyridin-3-yl)vinyl)-5H-4λ^4,5λ^4-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinine (**1**)



In a round bottom of 25 mL were added **7AI-6** (141 mg, 0.546 mmol), **BODYPY** (143 mg, 0.546 mmol), piperidine (0.125 mL, 1.27 mmol), acetic acid (0.125 mL, 2.19 mmol) in toluene (17.5 mL). The reaction was refluxed using Dean–Stark apparatus for 48 h at 130ºC. The reaction mixture was cooled and then extracted with CH_2Cl_2/H_2O. The organic layer was dried over anhydrous magnesium sulfate and filtered. After removing the solvent under reduced pressure, the residue was purified by column chromatography on silica gel with dichloromethane as eluent to obtain **1** as a pink solid. Yield: 15 mg (5 %).

^{1}H-RMN (300 MHz, $CDCl_3$) δ (ppm): 8.40 (m, 2H), 7.67-7.36 (m, 4H), 6.69 (s, 1H), 6.07 (s, 1H), 4.32 (m, 2H), 2.61 (s, 3H), 2.58 (s, 3H), 2.49 (s, 3H), 2.44 (s, 3H), 1.9 (m, 2H), 1.29 (m, 10H), 0.86 (m, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 153.1, 151.8, 148.5, 143.6, 141.1, 139.2, 138.6, 133.5, 132.0, 129.5, 129.0, 129.0, 120.7, 118.6, 117.2, 116.9, 115.6, 112.7, 44.9, 31.8, 30.3, 29.2, 29.1, 26.9, 22.6, 17.6, 17.2, 16.3, 14.5, 14.1. MALDI MS m/z 502.3 $[M]^+$ calcd. for $C_{30}H_{37}F_2N_4B$ 502.3079, found 502.3095.

2. Spectroscopic characterization

Table S1. Photophysical characterization of 1 in different solvents: absorption maximum wavelength (λ_{ab}^{max}), fluorescence emission maximum wavelength (λ_{em}^{max}) and fluorescence quantum yield (Φ_F).

Solvent	λ_{ab}^{max} (nm)	λ_{em}^{max} (nm) [a]	Φ_F (λ_{ex} = 560 nm) [b]	Φ_F (λ_{ex} = 465 nm) [c]
Toluene	584	598 nm	67 %	10 %
THF	580	598 nm	58 %	7 %
Ethanol	573	598 nm	76 %	4 %
DMSO	581	611 nm	75 %	5 %

[a] The sample was excited at 560 nm. [b] Conditions: 10 µM, λ_{ex} = 560 nm ($\Delta\lambda_{ex}$ = 10 nm), λ_{em} = 540-750 nm ($\Delta\lambda_{em}$ = 0.15 nm). [c] Conditions: 10 µM, λ_{ex} = 465 nm ($\Delta\lambda_{ex}$ = 10 nm), λ_{em} = 445-750 nm ($\Delta\lambda_{em}$ = 0.12 nm).

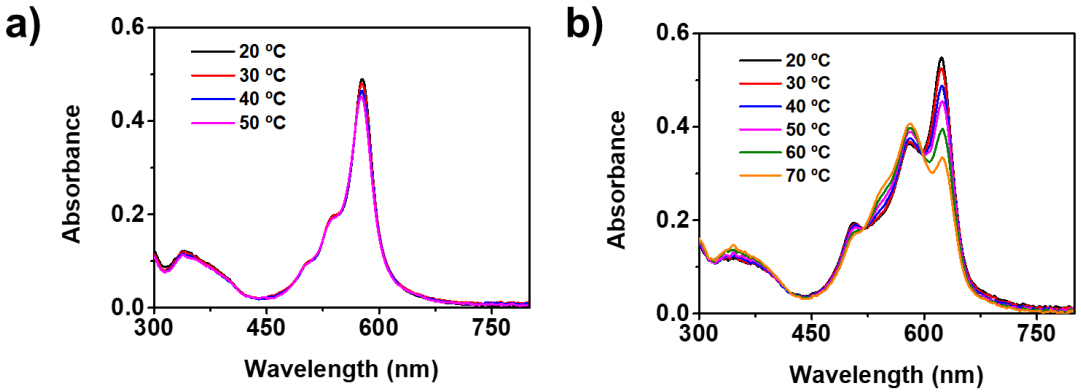


Fig. S1. UV-Vis absorption spectra of **1** (70 µM) in (a) THF solution and (b) aqueous solution acquired at different temperatures.

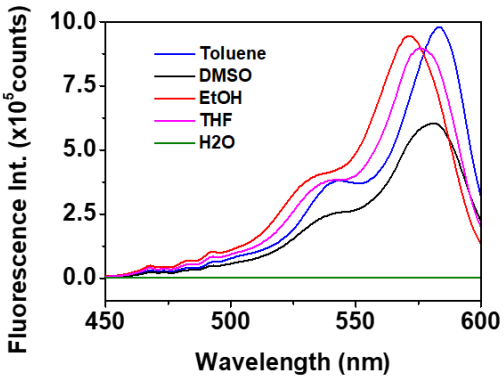


Fig. S2. Fluorescence excitation spectrum recorded for **1** (10 µM) in different solvents (λ_{em} = 620 nm).

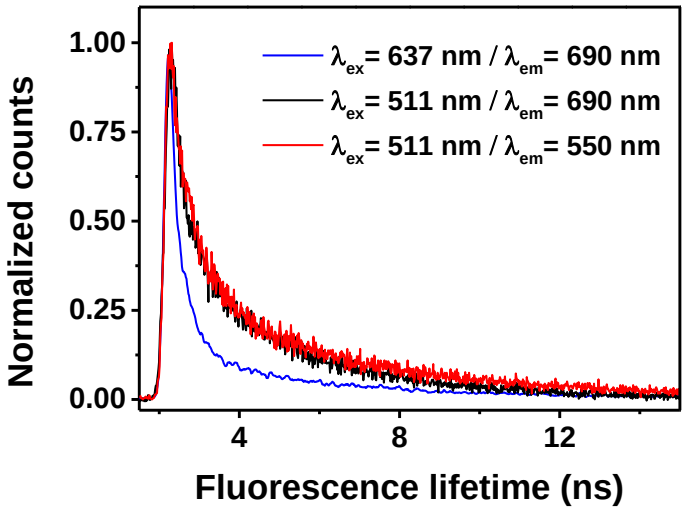


Fig. S3. Fluorescence emission decay profiles recorded for compound **1** (10 μM) in THF solution at different experimental conditions (λ_{ex} is the excitation wavelength; λ_{em} is the collecting wavelength).

Table S2. Results of the fitting of the fluorescence decay profiles recorded for **1** in different experimental conditions: fluorescence lifetimes (τ_i), amplitudes (A_i), Chi-squared (χ^2) and average fluorescence lifetime (τ_{av}).

Solvent (Conditions)	Assigned Transition	τ_1 (ns)	A_1	τ_2 (ns)	A_2	χ^2	τ_{av} (ns)
THF (λ_{ex}=637nm/λ_{em}=690nm)	$S_1 \rightarrow S_0$	3.17	0.245	—	—	1.15	3.2
THF (λ_{ex}=511nm/λ_{em}=550nm)	$S_2 \rightarrow S_0$	1.80	0.276	4.60	1.000	0.96	4.3
THF (λ_{ex}=511nm/λ_{em}=690nm)	$S_2/S_1 \rightarrow S_0$	3.87	0.135	—	—	1.01	3.9
water (λ_{ex}=637nm/λ_{em}=690nm)	$S_1 \rightarrow S_0$	0.145	2.16	1.35	0.041	1.02	0.32
water (λ_{ex}=511nm/λ_{em}=550nm)	$S_2 \rightarrow S_0$	3.00	0.156	5.78	0.241	1.01	5.0
water (λ_{ex}=511nm/λ_{em}=690nm)	$S_2/S_1 \rightarrow S_0$	0.314	0.507	4.07	0.0407	1.04	2.2
water ([BSA]:[**1**] = 5:1) (λ_{ex}=637nm/λ_{em}=690nm)	$S_1 \rightarrow S_0$	0.405	0.268	3.65	0.0952	1.07	2.8
water ([BSA]:[**1**] = 5:1) (λ_{ex}=511nm/λ_{em}=550nm)	$S_2 \rightarrow S_0$	2.32	0.298	5.99	0.369	1.01	5.1
water ([BSA]:[**1**] = 5:1) (λ_{ex}=511nm/λ_{em}=690nm)	$S_2/S_1 \rightarrow S_0$	0.557	0.208	4.48	0.0652	1.02	3.4

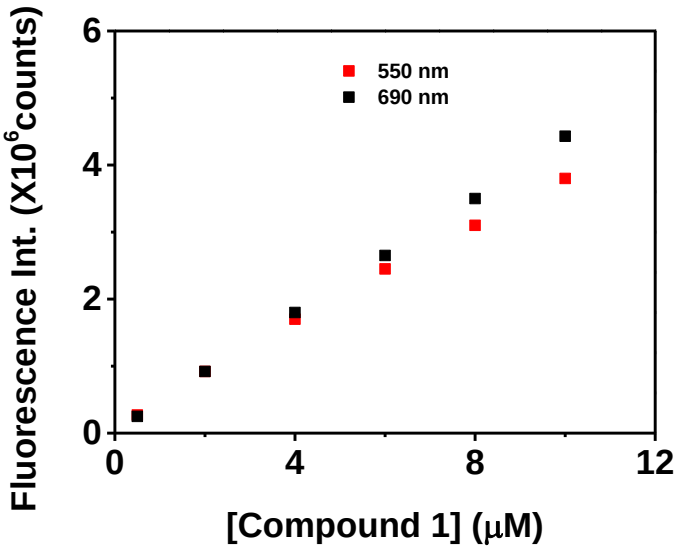


Fig. S4. Fluorescence emission intensity recorded at 550 nm (λ_{ex} = 465 nm) and 690 nm (λ_{ex} = 560 nm) for different concentrations of compound **1** in THF solution.

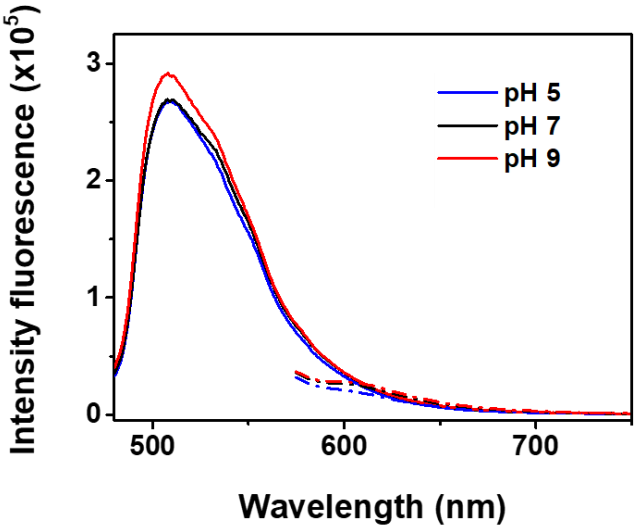


Fig. S5. Fluorescence spectrum of compound **1** (20 μM) in aqueous solution at different pH values exciting at 465 nm (solid lines) and 560 nm (dashed lines).

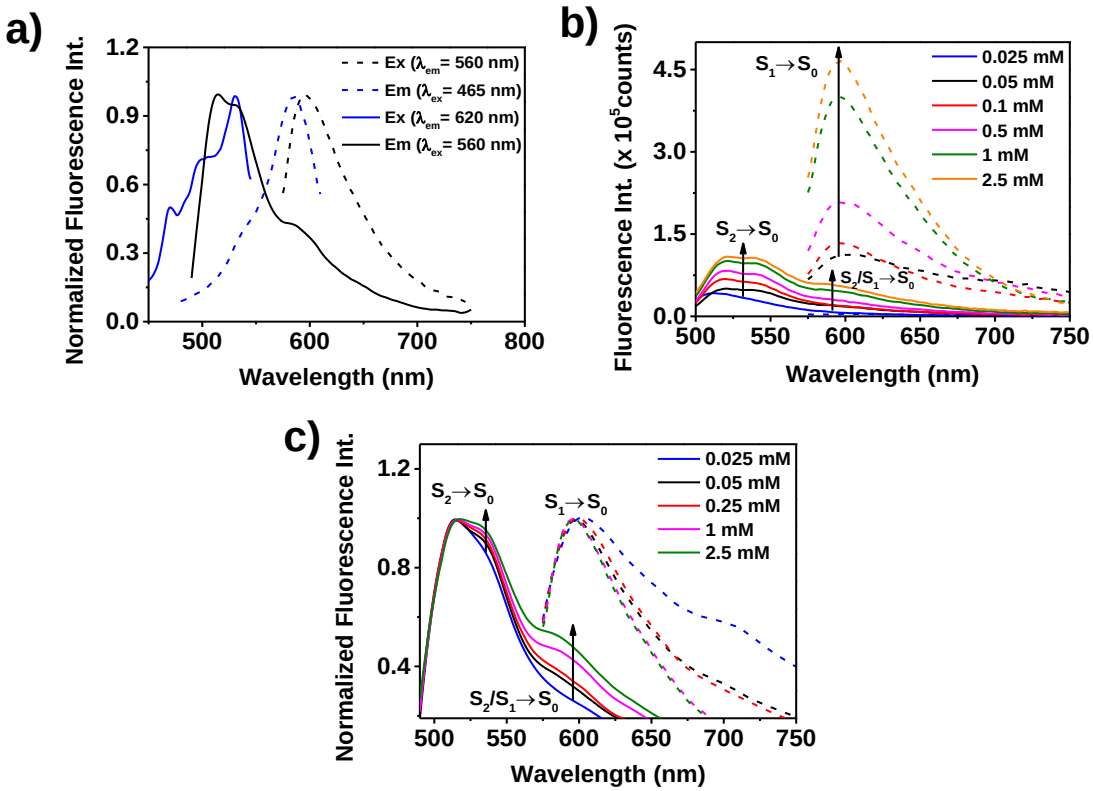


Fig. S6. Fluorescence spectra of compound **1** (50 µM) in aqueous solution and presence of BSA: (a) Different fluorescence excitation and emission bands found for **1** (and 250 µM of BSA) (excitation and emission wavelengths are shown in the Fig. as λ_{ex} and λ_{em}, respectively). (b) Fluorescence emission spectra of **1** (50 µM) exciting at 465 nm (solid lines) and 560 nm (dashed lines) in presence of increasing concentrations of BSA (also shown in Fig. 4b). (c) Normalized fluorescence emission spectra of (b).

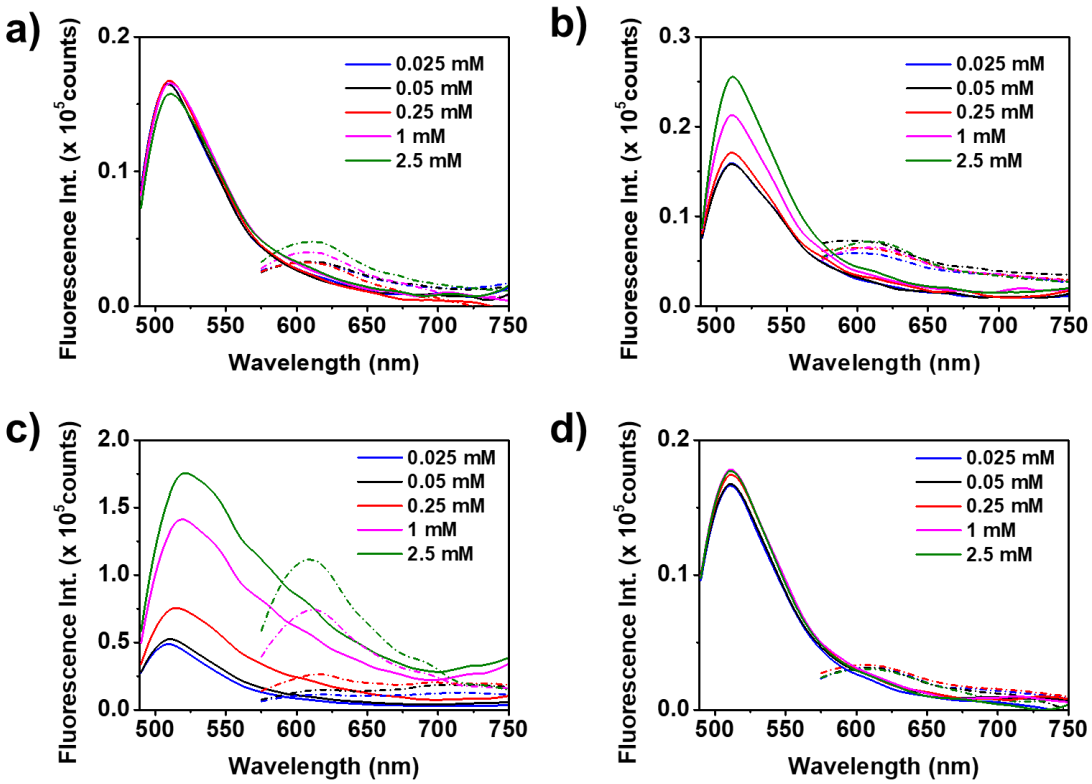


Fig. S7. Fluorescence emission spectrum of **1** in aqueous solution adding different quantities of (a) coconut oil, (b) Ficoll 400, (c) pepsin and (d) DNA. Concentration of **1** was 50 µM. Solid line (λ_{ex} = 465 nm) and dashed line (λ_{ex} = 560 nm).

3. DFT calculations

Table S3. Calculated vertical transitions for compound **1**: wavelength (λ_{vert}^{calc}), oscillator strength (*f*) and main components of the transitions (% Contribution). Calculations were performed at the TD-BLYP/6-31G* level of theory including CPCM solvation effects (tetrahydrofuran and water).

Solvent	Transition	λ_{vert}^{calc} (nm [eV])	*f*	% Contr.
THF	$S_0 \rightarrow S_1$	587 [2.11]	0.931	HOMO→LUMO (96%)
	$S_0 \rightarrow S_2$	466 [2.66]	0.086	HOMO→LUMO+1 (57%)
				HOMO-1→LUMO (35%)
Water	$S_0 \rightarrow S_1$	582 [2.13]	0.919	HOMO→LUMO (96%)
	$S_0 \rightarrow S_2$	465 [2.67]	0.086	HOMO→LUMO+1 (59%)
				HOMO-1→LUMO (34%)

4. Cell viability and ROS level assays

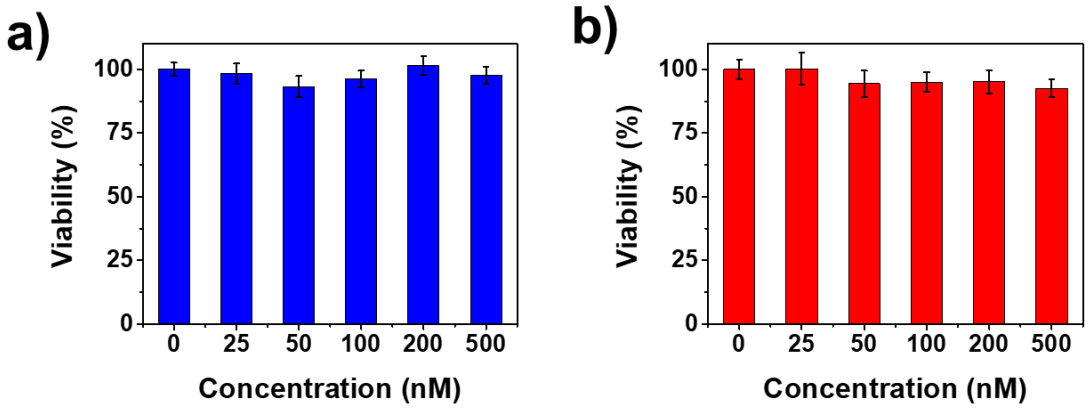


Fig. S8. Cell viability of MCF-7 cells treated with compound **1** for (a) 2 hours or (b) 24 hours.

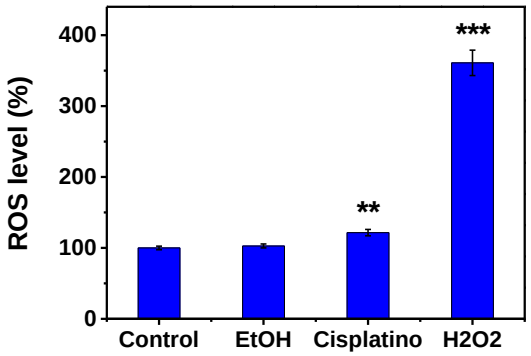


Fig. S9. Average ROS level in MCF-7 cells treated with ethanol (1M), cisplatin (10 µM) and H_2O_2 (0.5 mM) for 3 hours. The results are normalized with respect to the control and are shown as the mean (±SEM) of at least three independent experiments. * $p < 0.05$, ** $p < 0.005$ and *** $p < 0.0005$.

5. FLIM experiments on live cells

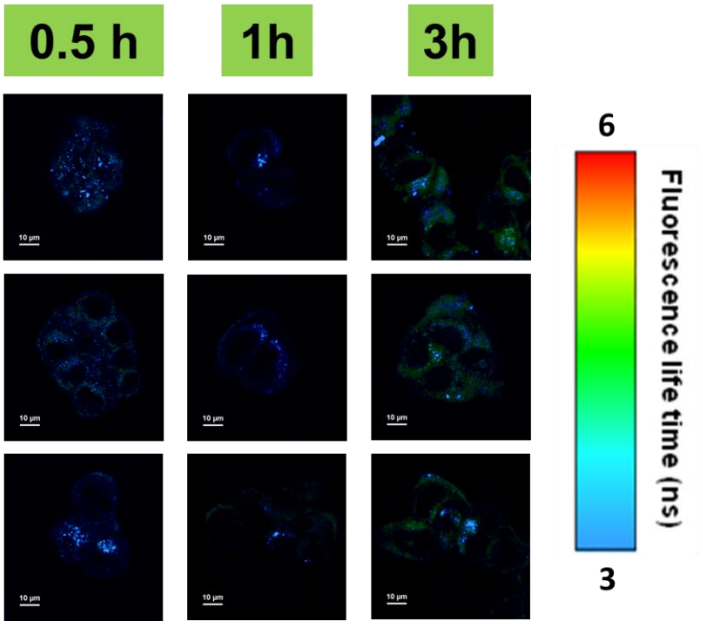


Fig. S10. Additional FLIM images acquired for MCF-7 cells in similar conditions that control cells of Fig. 6. Cells were only treated with compound **1** (0.1 μM; λ_{ex} = 511 nm; a 690/70 nm bandpass filter was used to collect the fluorescence emission). The incubation times were 0.5, 1 and 3 hours.

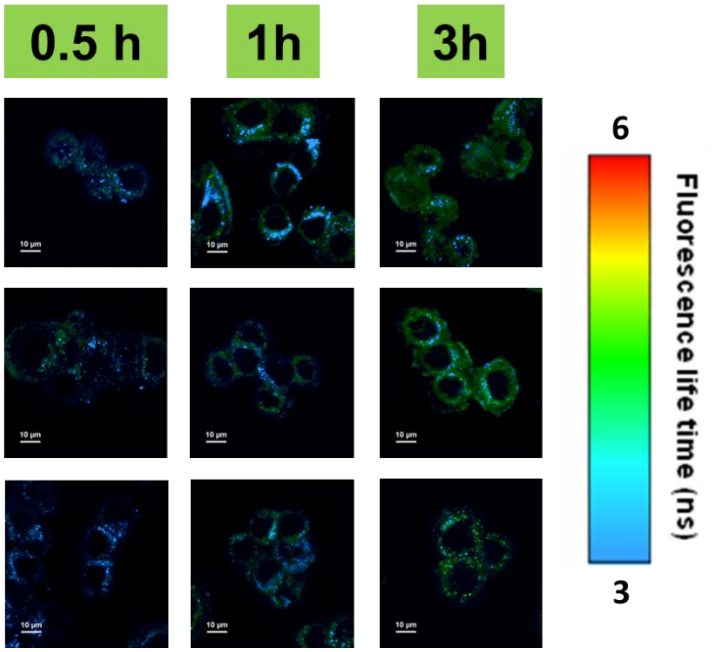


Fig. S11. Additional FLIM images acquired for MCF-7 cells in similar conditions that cells treated with ethanol in Fig. 6. Cells were treated with compound **1** (0.1 μM) and ethanol (1 M). The incubation times were 0.5, 1 and 3 hours (λ_{ex} = 511 nm; a 690/70 nm bandpass filter was used to collect the fluorescence emission).

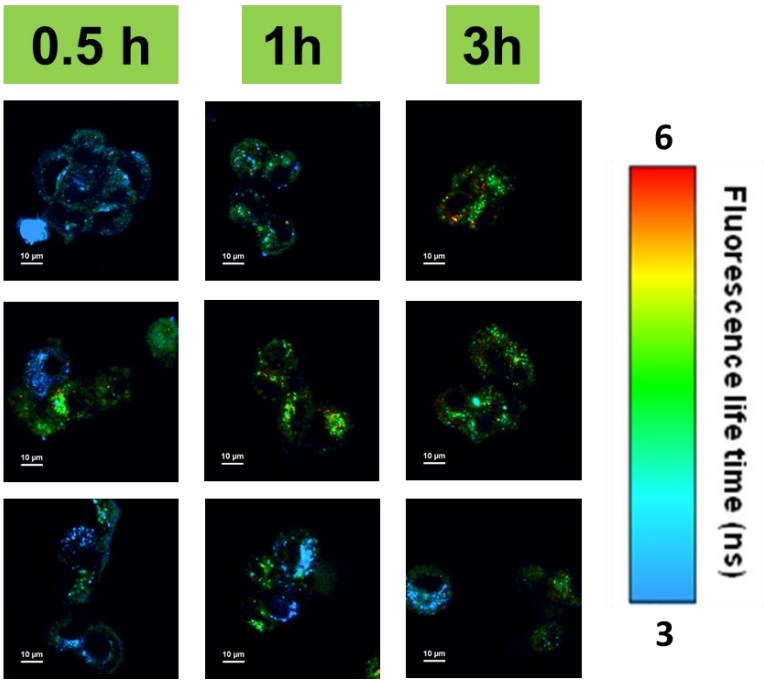


Fig. S12. Additional FLIM images acquired for MCF-7 cells in similar conditions that cells treated with hydrogen peroxide in Fig. 6. Cells were treated with compound **1** (0.1 μM) and hydrogen peroxide (0.5 mM). The incubation times were 0.5, 1 and 3 hours (λ_{ex} = 511 nm; a 690/70 nm bandpass filter was used to collect the fluorescence emission).

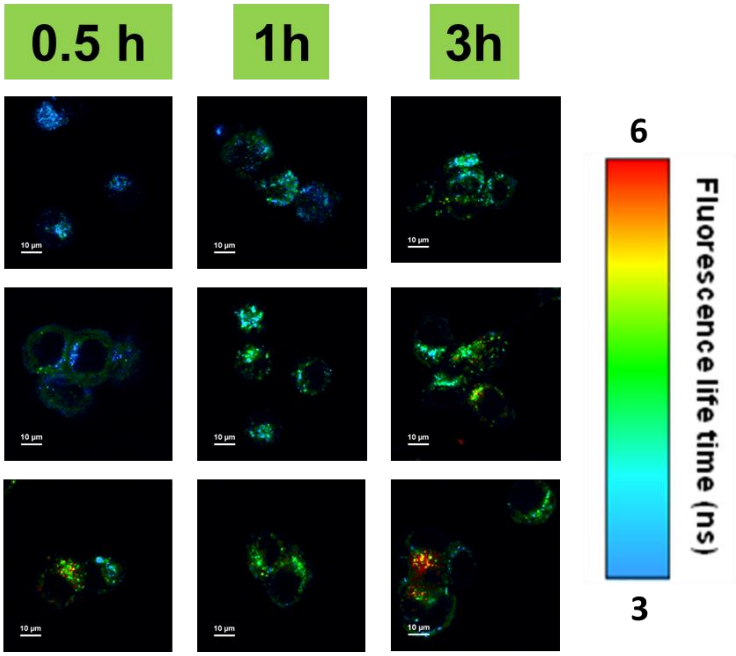


Fig. S13. Additional FLIM images acquired for MCF-7 cells in similar conditions that cells treated with cisplatin in Fig. 6d. Cells were treated with compound **1** (0.1 μM) and cisplatin (10 μM). The incubation times were 0.5, 1 and 3 hours (λ_{ex} = 511 nm; a 690/70 nm bandpass filter was used to collect the fluorescence emission).

Table S4. Raw data of Fig. 7: average fluorescence lifetimes (τ_{AV}) determined by FLIM for single MCF-7 cells treated with ethanol (EtOH), hydrogen peroxide (H_2O_2) and cisplatin, along with control MCF-7 cells. The incubation times were 0.5, 1 and 3 hours (λ_{ex} = 511 nm; a 690/70 nm bandpass filter was used to collect the fluorescence emission).

0.5 hours				**1 hour**				**3 hours**			
Control	EtOH	H_2O_2	Cisplatin	Control	EtOH	H_2O_2	Cisplatin	Control	EtOH	H_2O_2	Cisplatin
3.21	3.51	4.11	4.31	3.01	4.01	4.81	3.01	3.51	3.51	2.71	3.21
2.69	2.79	3.89	4.29	2.42	3.69	4.49	2.99	3.79	3.99	2.79	5.79
2.59	2.99	3.39	3.99	2.37	3.59	3.99	3.79	3.39	2.99	2.99	3.19
3.09	2.99	4.39	3.89	2.53	3.59	4.39	4.99	3.59	3.59	2.99	3.79
2.59	2.79	3.19	3.79	3.07	3.89	3.99	3.99	3.69	3.39	3.59	3.99
2.91	3.01	3.21	4.61	2.68	3.61	4.21	4.01	3.31	3.61	3.21	2.81
2.41	3.41	3.51	4.01	2.69	3.41	4.61	2.41	3.81	3.51	3.01	4.61
2.19	3.39	3.19	3.99	2.41	2.79	4.99	2.99	3.89	3.99	2.39	3.99
2.39	3.19	3.19	2.99	2.19	2.79	2.69	2.99	3.59	3.09	3.59	5.69
2.49	2.79	3.49	2.79	2.58	2.99	2.49	2.59	3.69	3.79	4.99	3.19
3.43	3.83	3.53	3.63	2.98	2.73	2.63	2.53	3.63	4.33	4.03	3.83
2.83	3.63	3.23	3.63	2.85	3.43	2.23	4.03	3.23	3.23	3.53	4.03
2.83	3.63	4.03	2.63	2.83	2.83	2.63	3.63	3.03	3.93	5.53	4.43
3.23	3.53	2.93	2.23	2.58	2.53	2.53	3.03	3.53	3.83	3.03	5.63
3.61	2.81	3.41	2.61	2.22	3.01	3.01	3.21	3.61	4.01	3.01	2.81
2.61	3.01	3.61	2.51	2.59	3.21	3.01	2.81	3.21	4.11	3.01	3.41
2.51	3.01	3.41	2.71	3.09	3.81	3.01	3.01	3.41	3.21	3.01	3.41
2.51	2.81	3.61	2.51	3.61	3.71	3.01	5.41	3.01	3.81	3.61	5.81
2.37	2.97	4.37	2.67	3.43	3.77	3.57	3.37	3.17	3.47	3.57	3.87
2.37	3.37	3.57	2.47	3.08	3.57	3.57	3.37	3.17	3.87	2.77	2.77
3.07	3.37	2.57	3.07	2.51	4.17	2.77	2.97	3.37	3.87	3.27	2.77
3.17	3.17	3.97	3.12	2.37	3.57	3.27	2.77	3.57	3.77	3.17	2.97
2.68	2.78	3.98	3.18	2.51	4.18	3.18	4.38	3.38	3.68	2.98	3.78
2.78	3.78	3.58	3.03	3.21	3.18	2.98	3.78	3.28	3.58	2.98	4.78
3.08	3.58	2.78	3.08	3.23	4.18	2.98	4.48	3.48	3.98	3.18	3.18
2.58	3.58	2.58	2.78	2.51	4.08	3.18	3.18	3.48	3.98	3.18	2.68
2.88	2.98	2.68	2.88	3.17	3.18	3.18	4.18	2.98	3.48	2.98	3.38
2.42	3.12	2.52	2.87	2.39	3.42	3.02	2.82	3.82	4.02	2.82	3.12
2.22	3.02	2.22	3.02	2.59	3.62	2.82	4.62	3.42	3.02	5.62	4.02
2.42	3.22	2.22	3.32	2.49	3.22	3.62	3.72	2.72	3.62	4.82	3.02
2.51	3.61	2.81	3.21	2.61	3.21	3.51	3.31	2.61	3.41	4.51	6.01
3.41	3.61	2.81	3.11	2.42	3.61	3.51	4.61	3.81	3.61	5.51	3.81
2.51	3.61	2.61	3.11	2.51	3.91	3.41	2.81	3.51	3.51	4.01	2.51
2.85	3.25	2.75	3.20	3.41	4.15	3.65	4.05	3.85	4.05	4.45	3.45

6. Two-photon fluorescence microscopy experiments

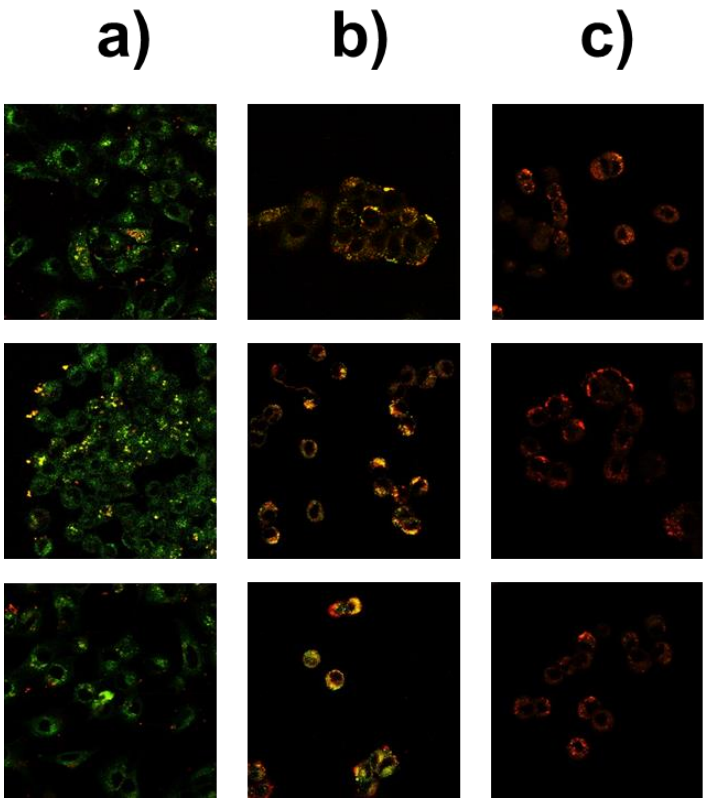


Fig. S14. Additional two-photon fluorescence microscopy images recorded for A549 cells treated with compound **1** (0.1 μM) and ethanol (1 M) cells in similar conditions that in Fig. 8. Cell were excited at 1022 nm and the emission was collected in two wavelength windows: 515 - 575 nm (green channel) and 650 - 750 nm (red channel). Only the merge image of both channels is shown. (a) Control cells (not treated with ethanol). (b) Incubation time of 3 hours. (c) Incubation time of 6 hours.

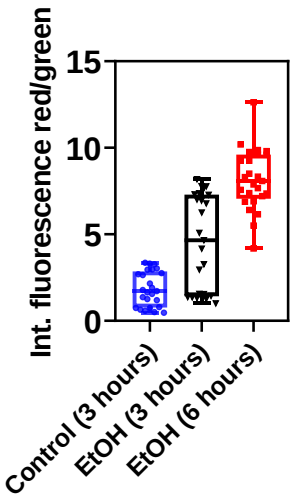


Fig. S15. Box plot of the ratio of fluorescence intensities of the red and green channels from two-photon fluorescence microscopy images. A549 cells were treated with compound **1** (0.1 μM) and ethanol (1 M) cells for 3 and 6 hours. The excitation wavelength was 1022 nm and the fluorescence emission was collected in two wavelength windows: 515 - 575 nm (green channel) and 650 - 750 nm (red channel).

Table S5. Raw data of Fig. S15: ratio of fluorescence intensities of the red and green channels from two-photon fluorescence microscopy images. A549 cells were treated with compound **1** (0.1 μM) and ethanol (1 M) cells for 3 and 6 hours. The excitation wavelength was 1022 nm and the fluorescence emission was collected in two wavelength windows: 515 - 575 nm (green channel) and 650 - 750 nm (red channel).

Control (3 hours)	**EtOH** (3 hours)	**EtOH** (6 hours)
1.19	1.37	4.18
0.74	1.45	5.50
0.64	1.35	12.63
0.74	1.56	6.40
2.16	4.17	8.51
1.91	7.37	7.36
1.78	2.96	9.25
1.64	4.65	8.33
1.72	5.08	10.20
1.56	3.27	9.61
0.79	6.94	9.76
1.37	6.78	8.30
0.58	7.78	9.80
0.50	7.28	9.25
1.25	7.01	9.60
0.47	7.67	9.87
2.65	7.77	6.88
2.69	7.31	7.88
2.75	6.25	7.38
3.36	8.18	6.91
2.95	1.32	7.57
3.31	1.38	7.67
3.27	1.01	7.21
3.03	1.25	6.15
2.97	1.54	8.08