Supplementary Information

Observing bioorthogonal macrocyclizations in the nuclear envelope of live cells using on/on fluorescence lifetime microscopy

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SI Movie 3: 3D nuclear topography representation from Fig. 8F showing nuclear invaginations following incubation with **1** in HeLa Kyoto cells. Video width 37 µm.

SI Movie 4: Phasor plot over time for the bioorthogonal reaction in MDA-MB 231 cells shown in Fig. 10.

SI Movie 5: Phasor plot over time for the bioorthogonal reaction in HeLa Kyoto cells shown in Fig. 10.

SI Movie 6: 3D FLIM imaging of bioorthogonal reaction from T_{zero} and $T_{45\ min}$ in the MDA-MB 231 cells shown in Fig. 12. Video width 29 µm.

General Experimental

All reactions involving air-sensitive reagents were performed under nitrogen in oven-dried glassware using syringe-septum cap techniques. Chromatographic separation was carried out on Merck silica gel 60 using flash-column techniques. Reactions were monitored by thin-layer chromatography (TLC) carried out on 0.25 mm silica gel coated aluminium plates (60 Merck F254). Unless specified, all reagents were used as received without further purifications. ^{1}H NMR, ^{13}C NMR, COSY, NOESY spectra were recorded at rt at 400 MHz and 101 MHz and calibrated using residual non-deuterated solvent as an internal reference. Products analysed by reverse phase chromatography on a HPLC (Shimadzu) equipped with analytical (YMC-triart phenyl, 4.6 × 150 mm I.D. S-5 µm or Phenomenex, 110 Å, 5 µm, C18, 100 mmd/250 mmL, 12 nm) columns, eluent with acetonitrile / water. APCI (HRMS) experiments were carried out on a Bruker microTOF-Q III spectrometer interfaced to a Dionex UltiMate 3000 LC. Agilent tuning mix APCI-TOF was used to calibrate the system. Masses were recorded over a range of 100-1800 m/z. Operating conditions were as follows: All absorbance spectra were recorded with a Varian Cary 50 scan UV-visible spectrometer and fluorescence spectra were recorded with a Horiba Scientific FluoroMax spectra fluorometer. Data were plotted in SigmaPlot 8 software. Confocal and FLIM images were acquired using a Leica Stellaris 8 (Leica objective 100X / 1.49 HC PL APO CS2) and were processed using Leica LasX software. Analysis of raw files was performed using ImageJ 1.53q. MDA-MB 231 cells obtained from Merck Life Science Limited.

Synthesis

Synthesis of 6-azido-1-(4-methoxyphenyl)-3-(nitromethyl)hexan-1-one **5**.[1]

A solution of 6-hydroxy-1-(4-methoxyphenyl)-3-(nitromethyl)hexan-1-one **4**[2] (2.1 g, 7.5 mmol) and triethylamine (2.6 mL, 18.8 mmol) in CH_2Cl_2 (40 mL) was stirred for 15 min at 0 ^{o}C, then treated dropwise with methanesulfonyl chloride (1.5 mL, 18.8 mmol), stirred under N_2 for 30 min. The reaction was quenched with NH_4Cl (50 mL), extracted with EtOAc (3 x 50 mL), washed with water (50 mL) and brine (50 mL), dried over Na_2SO_4 and filtered. The solvent was removed *in vacuo* and the resulting yellow oil was dissolved in anhydrous DMF (40 mL), treated with NaN_3 (1.18 g, 18 mmol) and stirred under N_2 at rt for 16 h. The reaction mixture was diluted with EtOAc (100 mL), washed with water (4 x 100 mL) and brine (2 x 50 mL). the combined organic layers were dried over Na_2SO_4, filtered and the solvent was removed in *vacuo*. The residue was purified by flash chromatography on silica gel (3:1 cyclohexane/EtOAc) to give **5** (2.2 g, 95%) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.80 (d, *J* = 8.8 Hz, 4H), 7.02 (d, *J* = 8.8 Hz, 4H), 6.69 (s, 2H), 3.9 (s, 6H), 3.37 (t, *J* = 6.9 Hz, 4H), 2.84 (t, *J* = 7.4 Hz, 4H), 2.05 – 2.00 (m, 4H).

Synthesis of bis-azido functionalized BF_2 azadipyrromethene **1**.

A solution of **5** (1.52 g, 4.96 mmol) and ammonium acetate (15.3 g, 198.4 mmol) in MeOH (30 mL) was heated under reflux for 9 h. The reaction mixture was cooled to rt, water (50 mL) added, extracted with DCM (3 x 50 mL). The combined organic extracts were washed with saturated $NaHCO_3$ (2 x 50 mL), water (50 mL) and brine (50 mL), dried over Na_2SO_4, filtered and the solvent was removed in *vacuo*. The solid was dried under reduced pressure overnight to give the azadipyrrin as a purple solid (260 mg, 24%) and used without further purification. The isolated solid (260 mg, 0.50 mmol) and DIPEA (0.86 mL, 4.98 mmol) were added to a round bottomed flask with DCM (20 mL). The system was flushed with N_2, $BF_3.Et_2O$ (0.86 mL, 6.98 mmol) was added and the solution was stirred at rt for 3 h. The solvent was removed in *vacuo* and the residue obtained was dissolved in DCM (15 mL) and an aqueous acid solution was added. Following separation of the organic phase, it was washed with H_2O (2 x 20 mL) and brine (1 x 20 mL). The organic layer was separated, dried over Na_2SO_4, filtered and evaporated to dryness. The resulting solid was purified by flash column chromatography on silica gel (DCM eluent) yielding **1** as a dark green solid (242 mg, 85%). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.02 (d, *J* =

9.0 Hz, 4H), 6.98 (d, *J* = 9.0 Hz, 4H), 6.64 (s, 2H), 3.87 (s, 6H), 3.39 (t, *J* = 6.8 Hz, 4H), 2.86 (t, *J* = 7.5 Hz, 4H), 2.02 (dt, J = 14.1, 6.8 Hz, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 162.0, 158.4, 146.0, 145.9, 131.7, 124.1, 120.9, 114.4, 55.6, 50.9, 29.3, 23.2. ^{19}F NMR (376 MHz, $CDCl_3$) δ: -133.2. λ_{max} Abs/nm (EtOH): 657 nm, λ_{max} Em/nm (EtOH): 679 nm, 0.21 QY. MS (APCI) m/z $[M-H]^-$ calc. for $C_{28}H_{28}N_9O_2BF_2$ 571.2438; found 571.2443.

Synthesis of macrocycles **3** and **6**.

Compound **1** (102mg, 0.18 mmol) and Sondheimer diyne **2** (40 mg, 0.2 mmol) were added to a round bottom flask with DCM (20 mL). The flask was flushed with nitrogen and the solution was stirred for at rt for 3 h. The solvent was removed in *vacuo* and the products were purified via flash column Chromatography on silica gel (DCM eluent increasing to 9:1 DCM:ethyl acetate) to give **3** (92 mg, 66.3%) and **6** (27 mg, 19.4%). **3** ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.08 (d, *J* = 9.0 Hz, 4H), 7.61 (dd, *J* = 5.7, 3.4 Hz, 2H), 7.46 (dd, *J* = 5.7, 3.4 Hz, 2H), 7.23 (dd, *J* = 5.7, 3.4 Hz, 2H), 7.07 (dd, *J* = 5.7, 3.4 Hz, 2H), 7.03 (d, *J* = 9.0 Hz, 4H), 6.70 (s, 2H), 4.58 (td, *J* = 13.2, 5.3 Hz, 2H), 4.36 (td, *J* = 13.2, 4.4 Hz, 2H), 3.90 (s, 6H), 2.89 (m, 2H), 2.61 (m, 2H), 2.18 (m, 2H), 2.02 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 162.4, 158.1, 146.2, 145.0, 144.4, 133.1, 131.8, 131.1, 130.5, 130.4, 129.9, 129.2, 127.8, 123.8, 122.1, 114.6, 55.6, 47.5, 31.0, 23.8. ^{19}F NMR (101 MHz, $CDCl_3$) δ: -131.2, -133.8. λ_{max} (EtOH): 665 nm, 0.17 QY. MS ($APCI^-$) m/z $[M-H]^-$ calc. for $C_{44}H_{36}N_9O_2BF_2$ 771.3066; found 771.3062. **6** ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.94 (d, *J* = 8.9 Hz, 4H), 7.79 (d, *J* = 7.5 Hz, 2H), 7.55 (dt, *J* = 20.7, 7.0 Hz, 2H), 7.32 (d, *J* = 7.5 Hz, 2H), 6.93 (d, *J* = 8.9 Hz, 4H), 6.49 (s, 2H), 4.72 (m, 2H), 4.36 (m, 2H), 3.84 (s, 6H), 2.13 (m, 4H), 1.90 (m, 2H), 1.73 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 161.7, 158.5, 145.8, 145.2, 144.8, 134.9, 133.0, 131.6, 130.3, 129.4, 129.2, 127.5, 124.4, 119.9, 114.2, 55.5, 48.0, 29.5, 20.7. ^{19}F NMR (101 MHz, $CDCl_3$) δ: -133.3. λ_{max} (EtOH): 656 nm, 0.19 QY. MS ($APCI^-$) m/z $[M-H]^-$ calc. for $C_{44}H_{36}N_9O_2BF_2$ 771.3066; found 771.3065.

Bioorthogonal reaction of **1** and **2** in aqueous PS20.

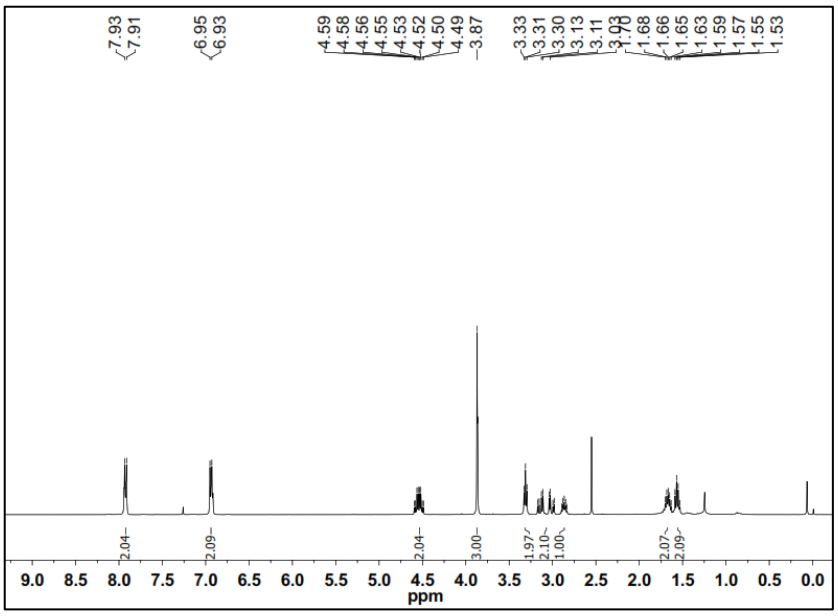
Stock solutions of **1** (10 µM), benzyl azide (20 µM) and **2** (12 µM) in aqueous (HPLC grade) PS20 (1mM) were prepared. 5 mL of either the **1** or the benzyl azide solution was combined with 5 mL of the **2** solution in a round bottom flask and stirred at 37 °C. Reaction progress was monitored using reverse-phase chromatography on a HPLC (Shimadzu) equipped with analytical (YMC-triart phenyl, 4.6 × 150 mm I.D. S-5 µm or Phenomenex, 110 Å, 5 µm, C18, 100 mmd/250 mmL, 12 nm) columns, using an eluent of 4:1 CH_3CN:H_2O.

Table S1: ASIS values (proton and fluorine) for **3**

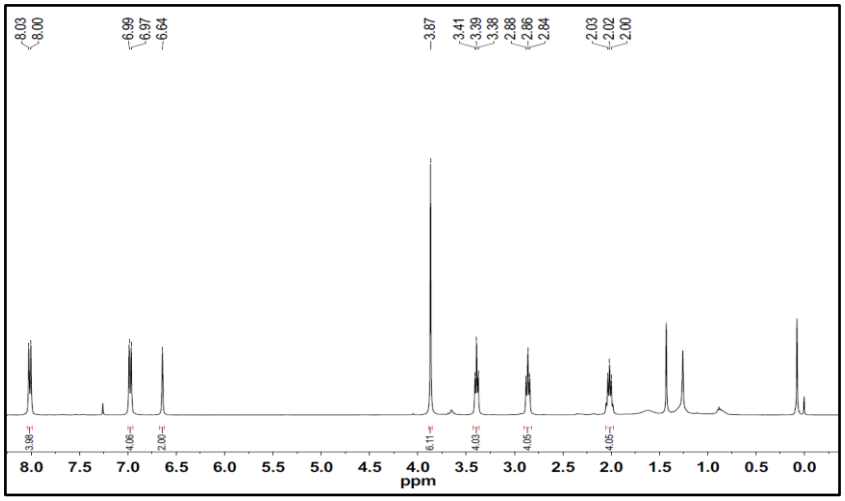
Chemical shift (ppm) $CDCl_3$	Chemical shift (ppm) $CDCl_3/C_6D_6$	ASIS = $\delta_{C_6D_6} - \delta_{CHCl_3}$
8.08	8.09	+0.01
7.61	7.73	+0.12
7.46	7.32	-0.14
7.23	7.04	-0.19
7.07	6.69	-0.38
7.03	6.89	-0.14
6.70	6.89	-0.31
4.58	4.39	-0.19
4.36	4.12	-0.24
3.90	3.56	-0.34
2.89	2.63	-0.26
2.61	2.28	-0.33
2.18	1.96	-0.22
2.02	1.84	-0.18

Figure S1: Analytical spectra for **5**, **1**, **3** and **6**

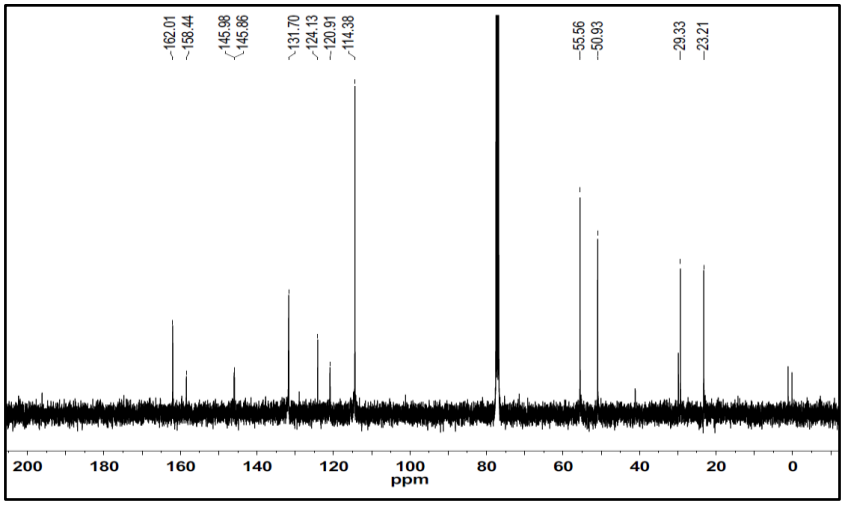
^{1}H NMR (400 MHz, $CDCl_3$) of **5**[1]



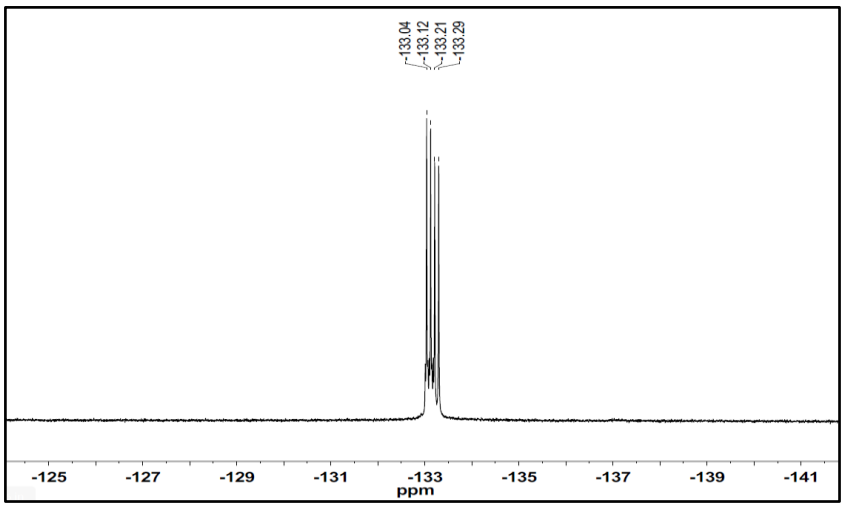
^{1}H NMR (400 MHz, $CDCl_3$) of **1**



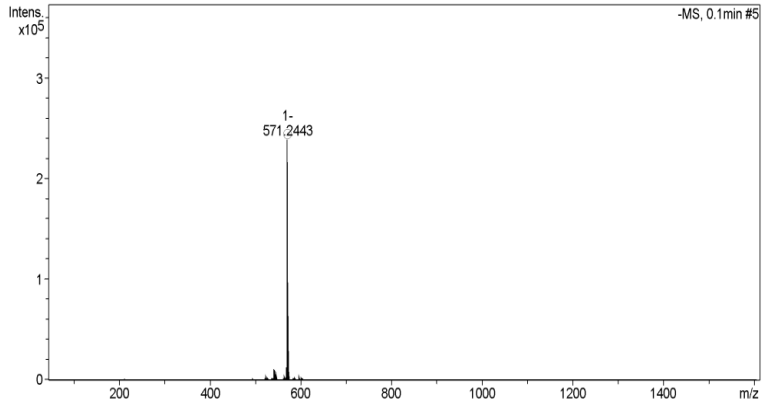
^{13}C NMR (101 MHz, $CDCl_3$) of **1**



^{19}F NMR (376 MHz, $CDCl_3$) of **1**

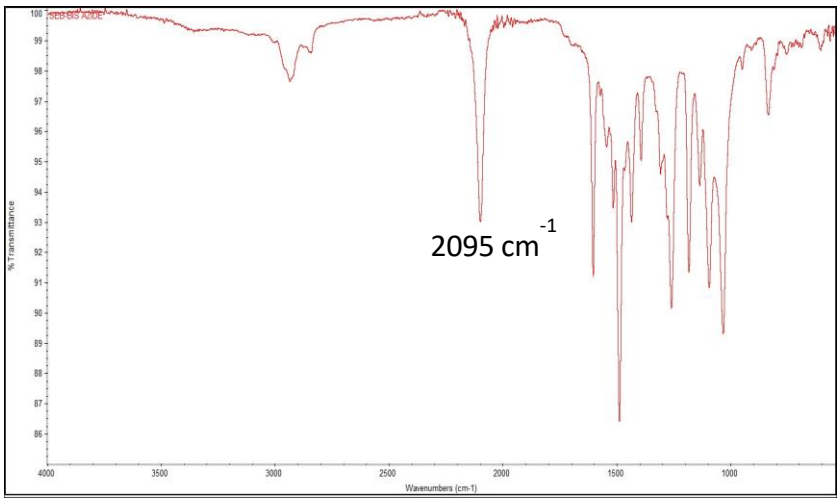


APCI Negative Mode HRMS of **1**

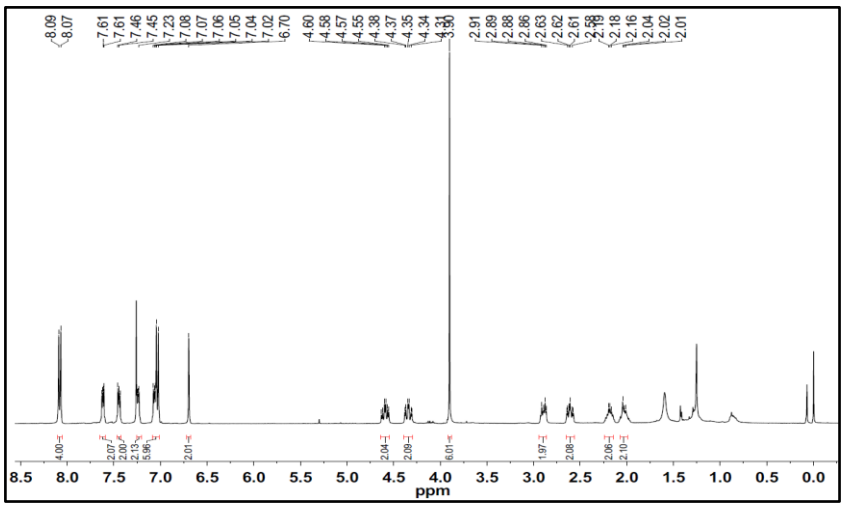


Meas. m/z	#	Ion Formula	m/z	err [mDa]	err [ppm]	rdb	N-Rule	e¯ Conf	mSigma
571.244306	1	C28H28BF2N9O2	571.243755	0.6	1.0	19.0	ok	odd	58.5

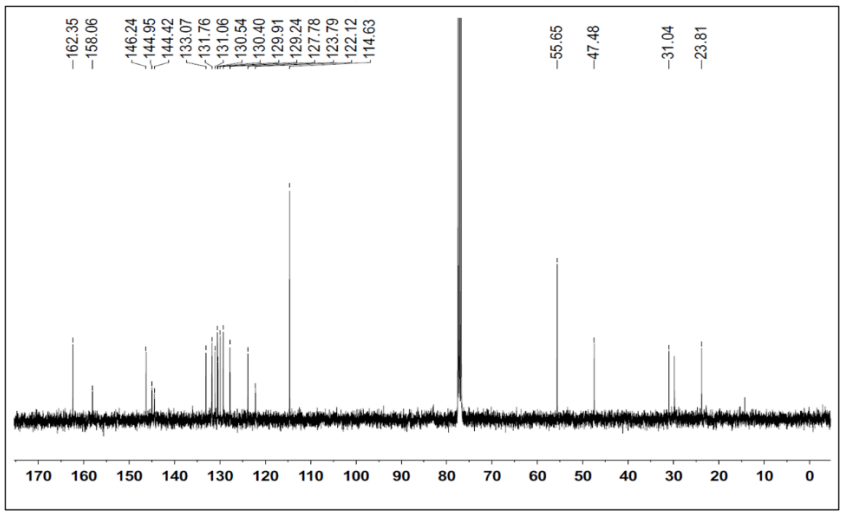
Infra-red spectrum of **1**



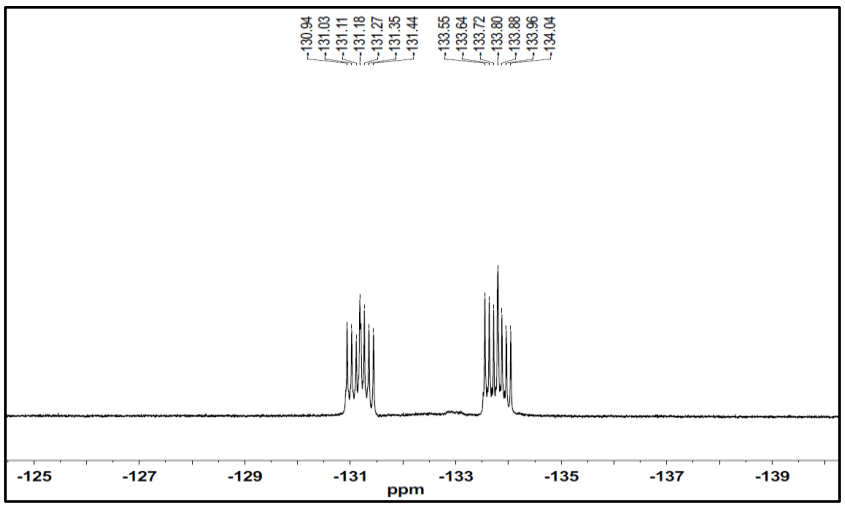
^{1}H NMR (400 MHz, $CDCl_3$) of **3**



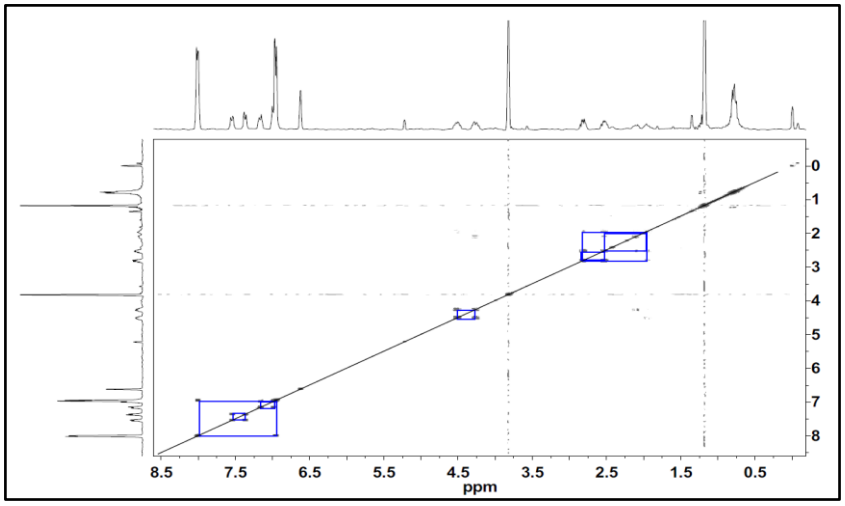
^{13}C NMR (101 MHz, $CDCl_3$) of **3**



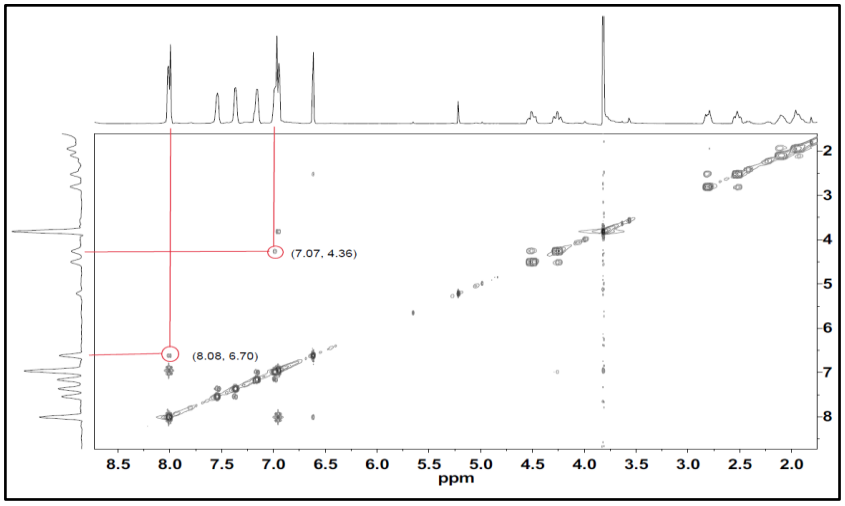
^{19}F NMR (376 MHz, $CDCl_3$) of **3**



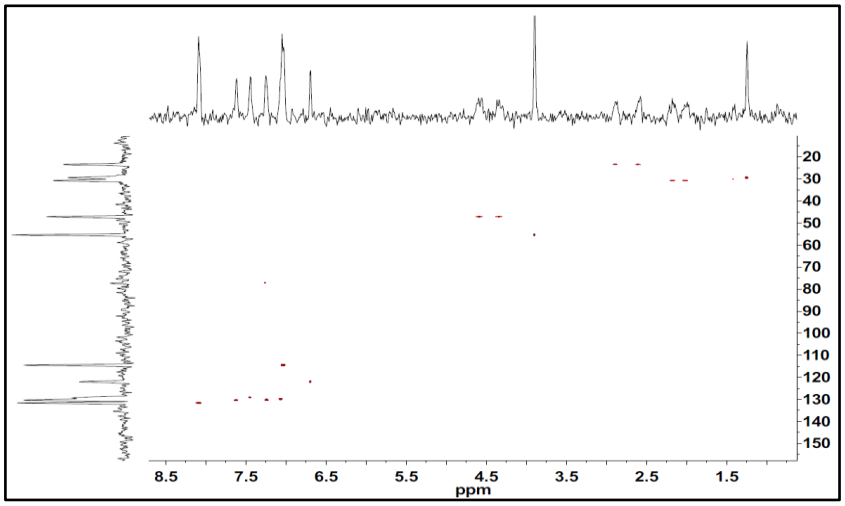
2D COSY of **3**



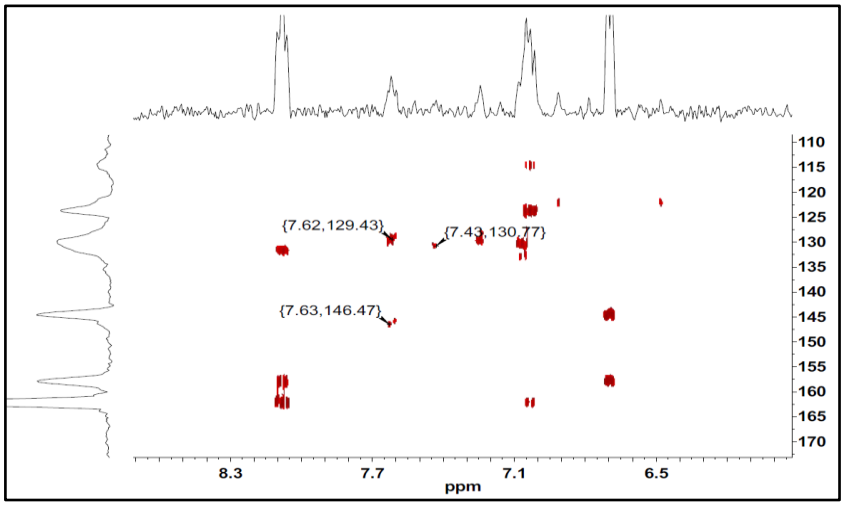
NOESY of **3**



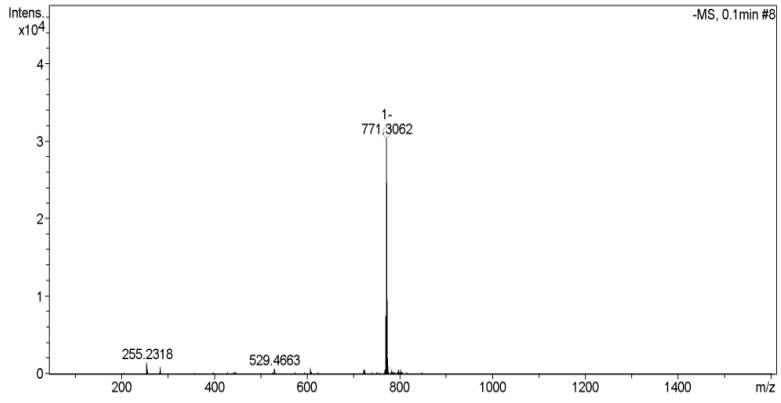
HSQC of **3**



HMBC of **3**

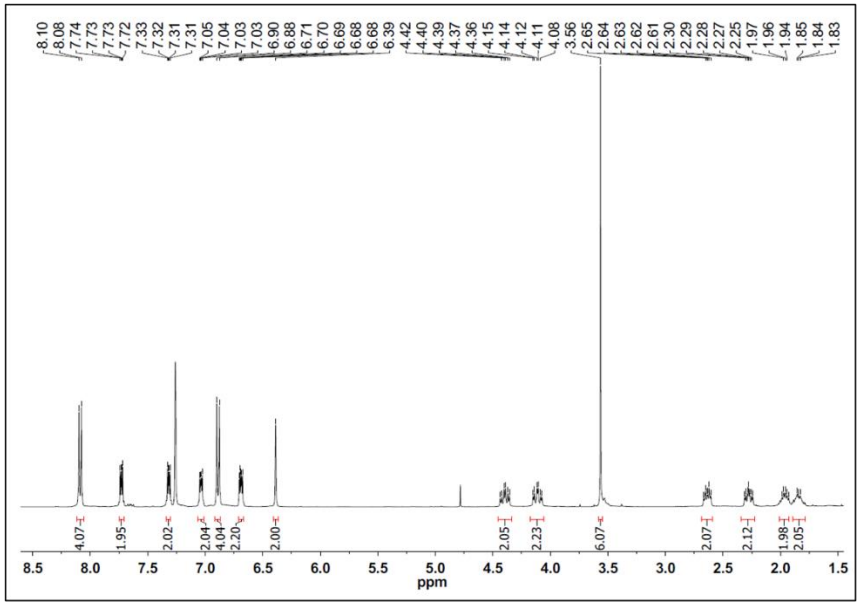


APCI Negative Mode HRMS of **3**

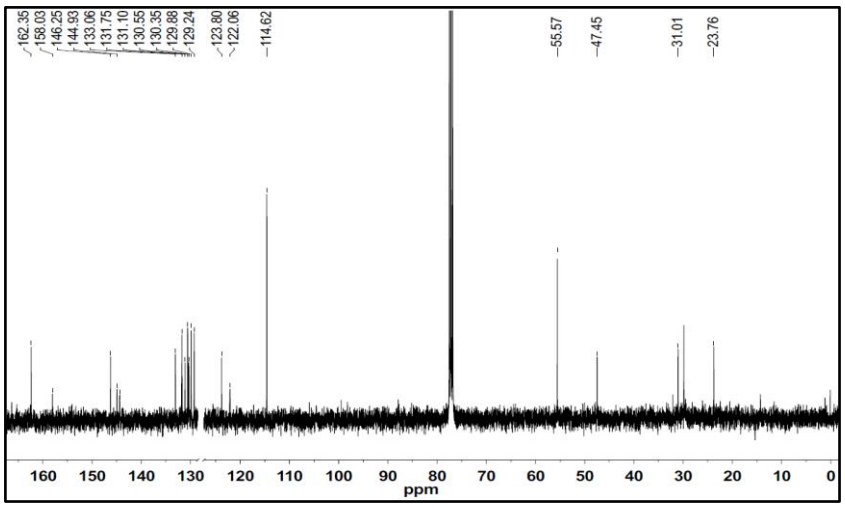


Meas. m/z	#	Ion Formula	m/z	err [mDa]	err [ppm]	rdb	N-Rule	e⁻ Conf	mSigma
771.306233	1	C44H36BF2N9O2	771.306605	0.4	0.5	31.0	ok	odd	51.4

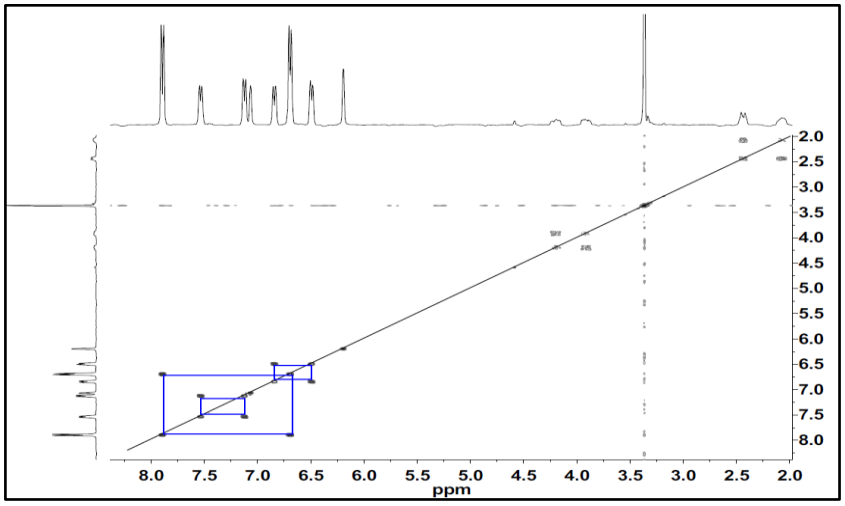
^{1}H NMR (400 MHz, C_6D_6 / $CDCl_3$) of **3**



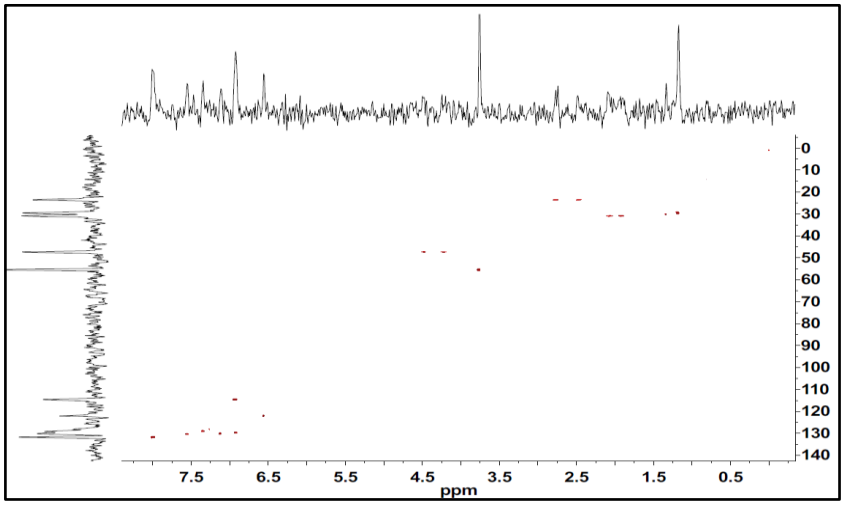
^{13}C NMR (101 MHz, C_6D_6 / $CDCl_3$) of **3**



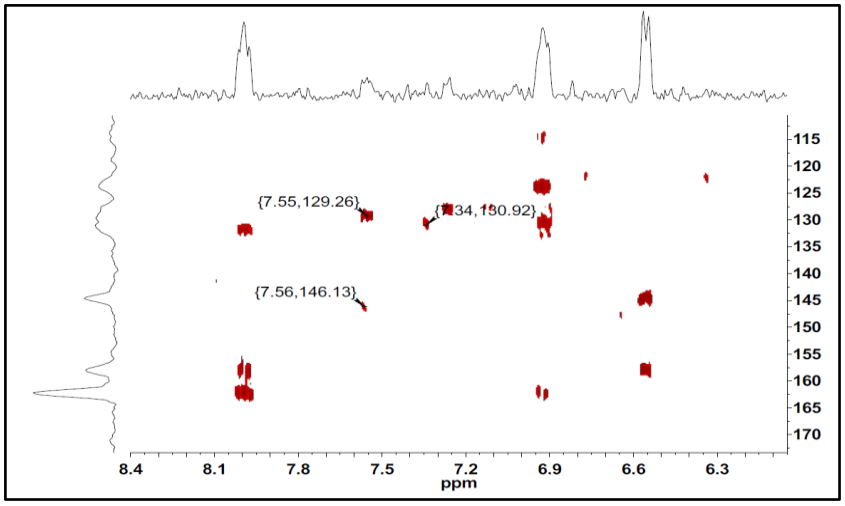
2D COSY of **3** in C_6D_6 / $CDCl_3$



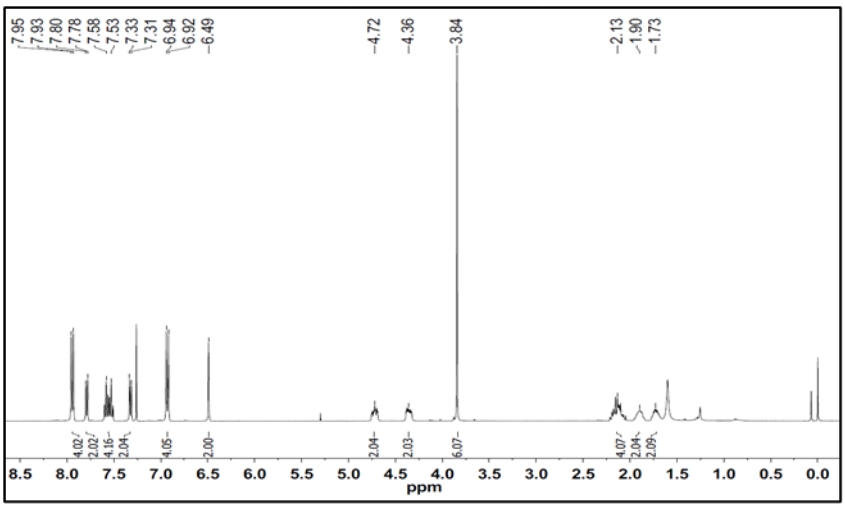
HSQC of **3** in C_6D_6 / $CDCl_3$



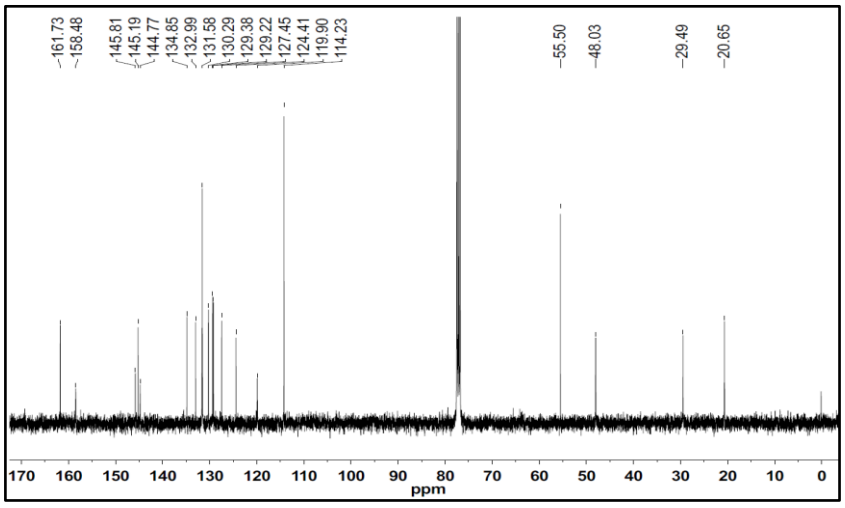
HMBC of **3** in $CDCl_3$ / C_6D_6



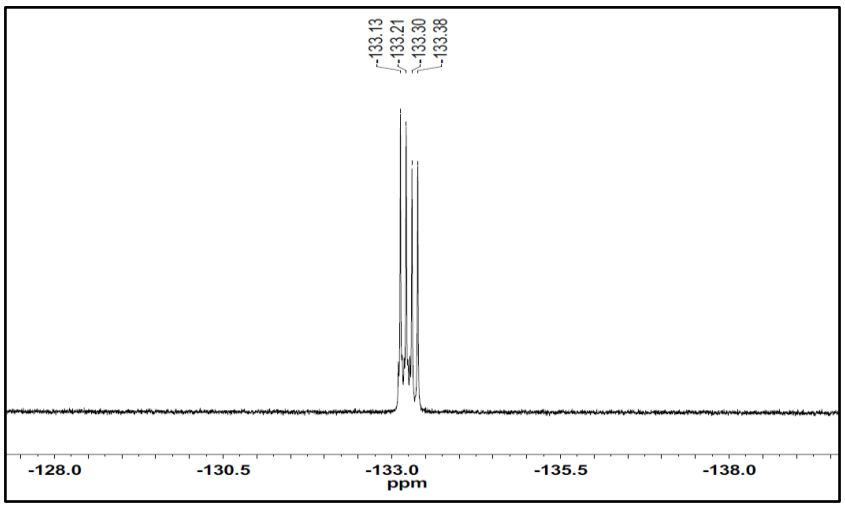
^{1}H NMR (400 MHz, $CDCl_3$) of **6**



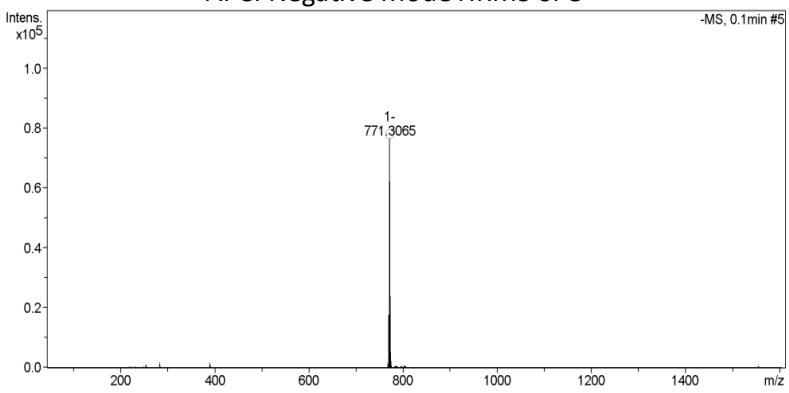
^{13}C NMR (101 MHz, $CDCl_3$) of **6**



^{19}F NMR (376 MHz, $CDCl_3$) of **6**



APCI Negative Mode HRMS of **3**



Meas. m/z	#	Ion Formula	m/z	err [mDa]	err [ppm]	rdb	N-Rule	e⁻ Conf	mSigma
771.306452	1	C44H36BF2N9O2	771.306605	-0.2	-0.2	31.0	ok	odd	25.4

Figure S2: X-Ray Structure of **3** (CCDC 2338184)

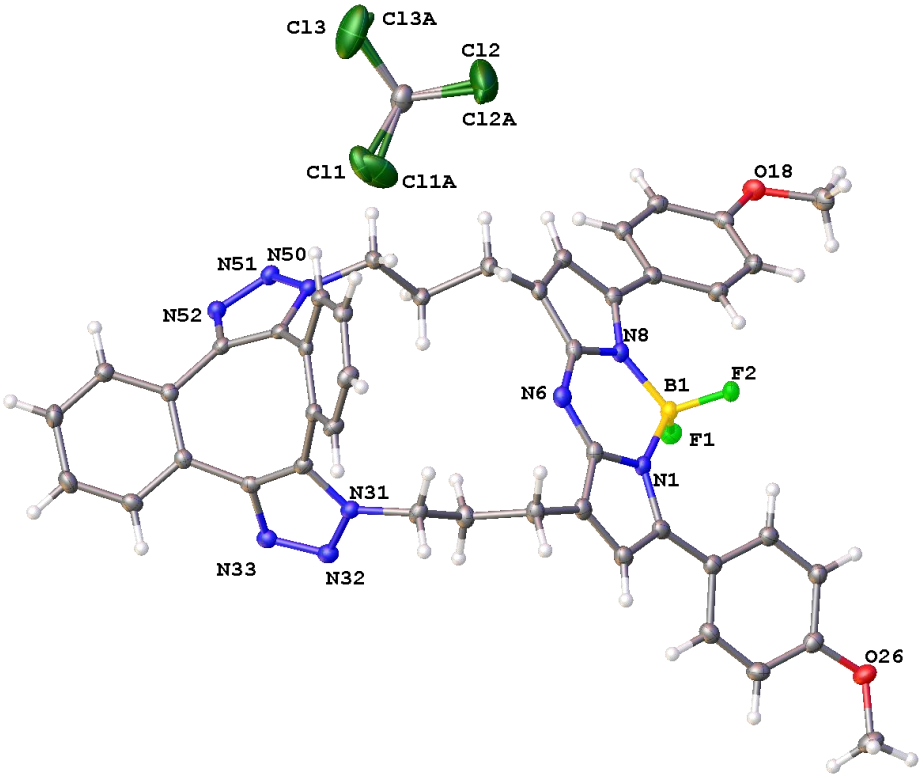
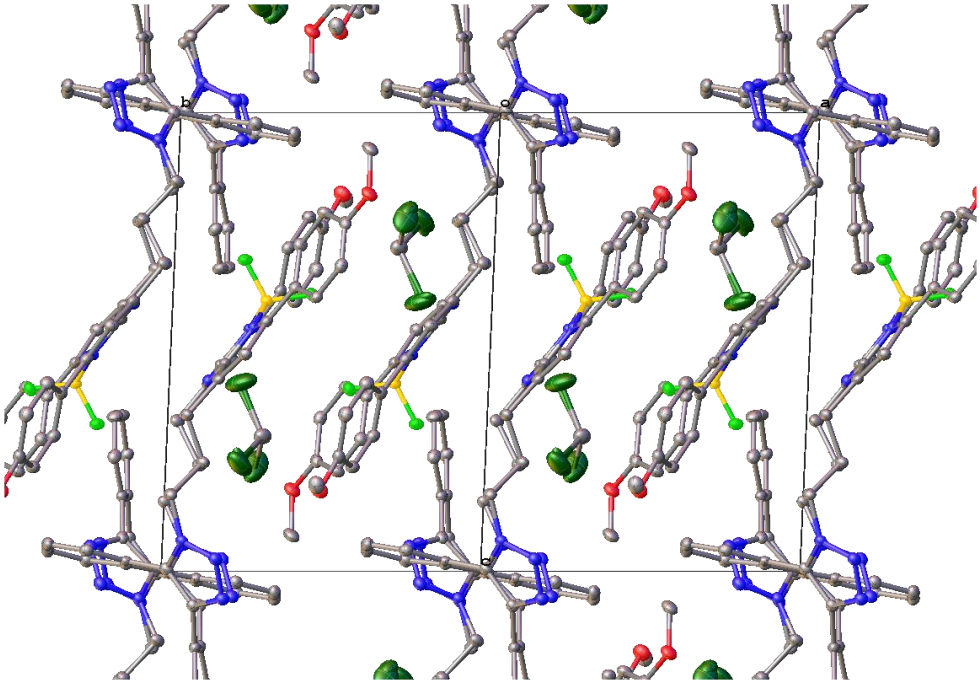
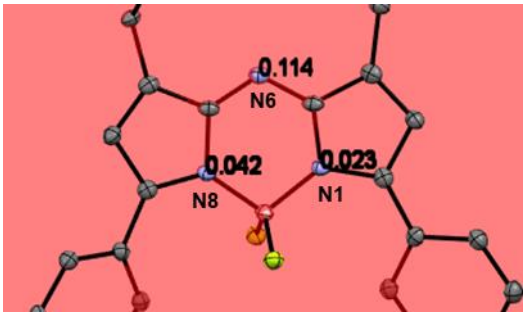


Illustration of the molecular structure of **3** with selected atom labelling. Disorder in $CHCl_3$ shown.



Packing diagram of 3 viewed normal to (110). Hydrogen atoms omitted for clarity



Planarity of the central 5-6-5-fused heterocyclic rings, with the largest deviation from this 12-atom plane being 0.114 Å for the N6 atom.

Table S2. Crystal data and structure refinement for **3** (data deposited as CCDC 2338184).

Empirical formula	$C_{45}H_{37}BCl_3F_2N_9O_2$	
Formula weight	890.99	
Temperature	100(2) K	
Wavelength	0.71073 Å	
Crystal system	Triclinic	
Space group	$P\bar{1}$	
Unit cell dimensions	a = 11.0839(13) Å	α= 106.314(5)°.
	b = 13.719(2) Å	β= 104.337(4)°.
	c = 15.383(3) Å	γ = 105.219(3)°.
Volume	2031.6(6) $Å^3$	
Z	2	
Density (calculated)	1.456 Mg/m^3	
Absorption coefficient	0.288 mm^{-1}	
F(000)	920	
Crystal size	0.35 x 0.2 x 0.17 mm^3	
Theta range for data collection	2.892 to 26.547°.	
Index ranges	-13≤h≤13, -17≤k≤17, -19≤l≤19	
Reflections collected	16496	
Independent reflections	16496 [R(int) = 0.0570]	
Completeness to theta = 25.242°	99.6 %	
Absorption correction	Semi-empirical from equivalents	
Max. and min. transmission	0.745373 and 0.708248	
Refinement method	Full-matrix least-squares on F^2	
Data / restraints / parameters	16496 / 33 / 591	
Goodness-of-fit on F^2	1.029	
Final R indices [I>2σ(I)]	R1 = 0.0433, wR2 = 0.1079	
R indices (all data)	R1 = 0.0560, wR2 = 0.1150	
Largest diff. peak and hole	0.319 and -0.357 e.$Å^{-3}$	

Figure S3: Representative HPLC trace from the reactions of **1** and benzyl azide with Sondheimer diyne **2**

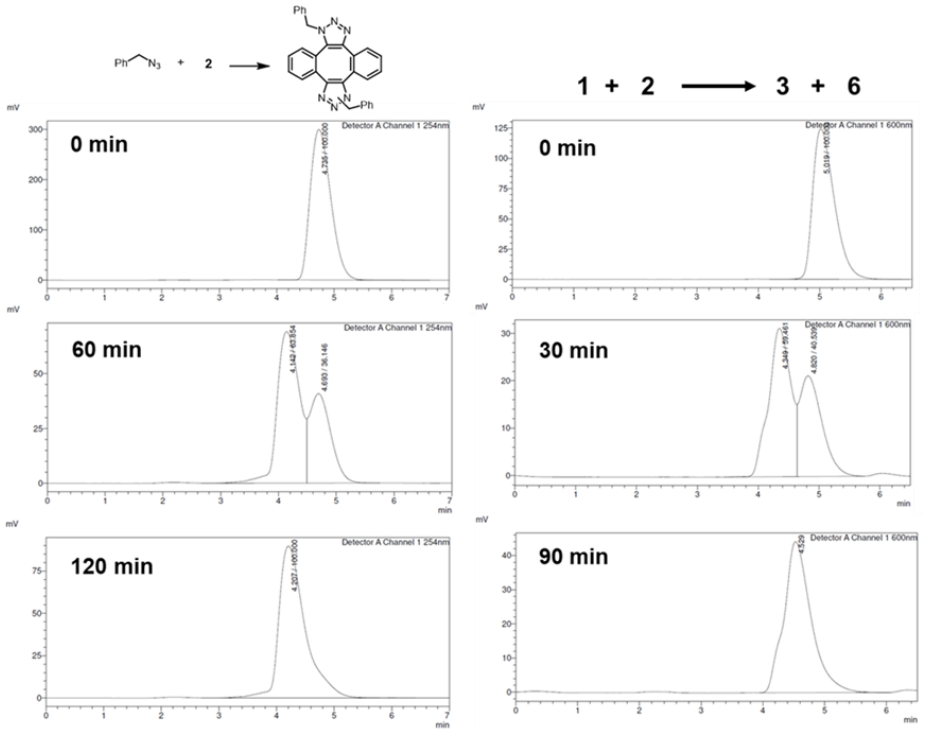
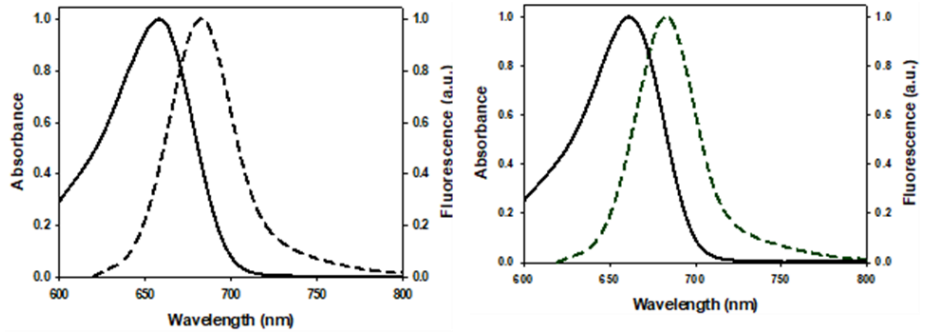
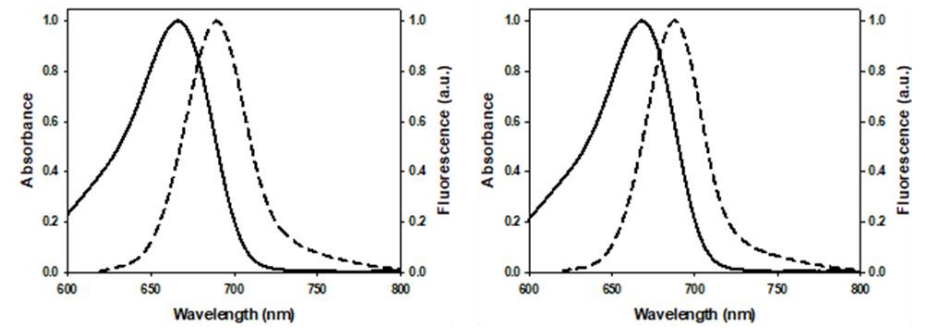


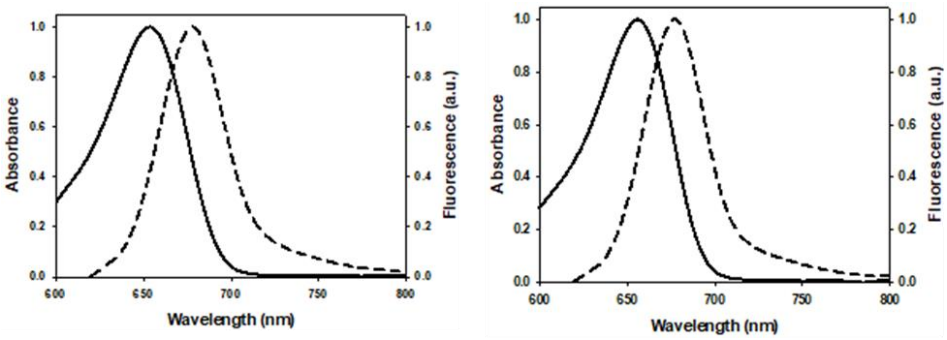
Figure S4: Absorption and emission spectra of **1**, **3** and **6**



Normalised absorption (solid trace) and emission spectra (excit. 610 nm, slit width 5 nm) (dashed trace) of **1** in chloroform (left) and toluene (right).



Normalised absorption (solid trace) and emission spectra (excit. 610 nm, slit width 5 nm) (dashed trace) of **3** in chloroform (left) and toluene (right).



Normalised absorption (solid trace) and emission spectra (excit. 610 nm, slit width 5 nm) (dashed trace) of **6** in chloroform (left) and toluene (right).

Table S3. Radiative (k_r) and non-radiative (k_{nr}) decay constants for **1**, **3** and **6**.[a]

entry	comp.	solvent	k_r (ns^{-1})	k_{nr} (ns^{-1})
1	**1**	$CHCl_3$	0.055	0.166
2	**1**	toluene	0.091	0.136
3	**1**	EtOH	0.058	0.219
4	**3**	$CHCl_3$	0.045	0.151
5	**3**	toluene	0.042	0.158
6	**3**	EtOH	0.038	0.184
7	**6**	$CHCl_3$	0.048	0.144
8	**6**	toluene	0.048	0.152
9	**6**	EtOH	0.040	0.172

Calculated as described in reference 3.

Cell Culture

MDA-MB 231 human breast cancer cells were cultured in Dulbecco's Modified Eagles Medium supplemented (DMEM) with 10% fetal bovine serum (FBS), 1% L Glutamine, and Penicillin-Streptomycin (1000 U/mL)(DMEM+++), and incubated at 37 °C and 5% CO_2. Cells were seeded on to an eight well chamber slide (Ibidi) at a density of 1 × 104 cells per well for 24 h before being switched to the same prewarmed medium depleted of FBS (DMEM-++). About 30 h later, cells were ready for staining and imaging.
Cells were washed 2 times with 250 µL of prewarmed PBS and stained for 90 min at 37 °C and 5% CO_2 with the **1**, **3** or **6** to a final concentration of 0.4 µM in 250 µL of prewarmed DMEM-++. Cells were ready for imaging after being washed twice with PBS and once with DMEM-++. Bioorthogonal reactions were carried out directly under the microscope by gently adding **2** in H_2O/PS20 to reach a final concentration of 0.4 µM in the well. A bigger pipette was immediately used to mix by pipetting up and down a few times. A control stimulation was performed by adding the same volume of the buffer H_2O/PS20. The acquisition of lifetime imaging was started shortly after, within a minute. For HeLa cells, the same protocol was followed except that the cells were seeded for 48 h before imaging and kept in DMM+++.

Microscopy

CLSM and FLIM images were acquired on a Leica Stellaris 8 Falcon microscope fitted with NKT Photonics White Light Laser (440nm-790nm) and controlled by LAS X (version. 4.4.0.24861). Live cell imaging experiments were carried out under Okolab incubation system to maintain the temperature at 37 °C and CO_2 at 5%. Images were acquired using a Leica HC PL APO CS2 100X/1.40 oil immersion objective.
Excitation wavelength was obtained tuning the White Light Laser (WLL) tuned at 660 nm. A HyD detector was used to collect emission in the range 675nm-750nm for CLSM and 3D stacks. The FLIM function was used to acquire the real time fluorescence lifetime imaging in solution or in the cells. A mono-exponential/1 component fit was used to give the best fitting curve and to extract the lifetimes at different time points. Either the full image or a small ROI corresponding to the nuclear invaginations were fitted after removing background counts. Phasor plots analysis were processed by LAS X Falcon software, for a better visualization of the changes in the lifetime. Spectral emission scans were performed in solution and in cells with an excitation at 660 nm and emission collected from 665 to 780 nm with a step size of 3 nm and a bandwidth of 5 nm. A HyD detector was used for the collection.

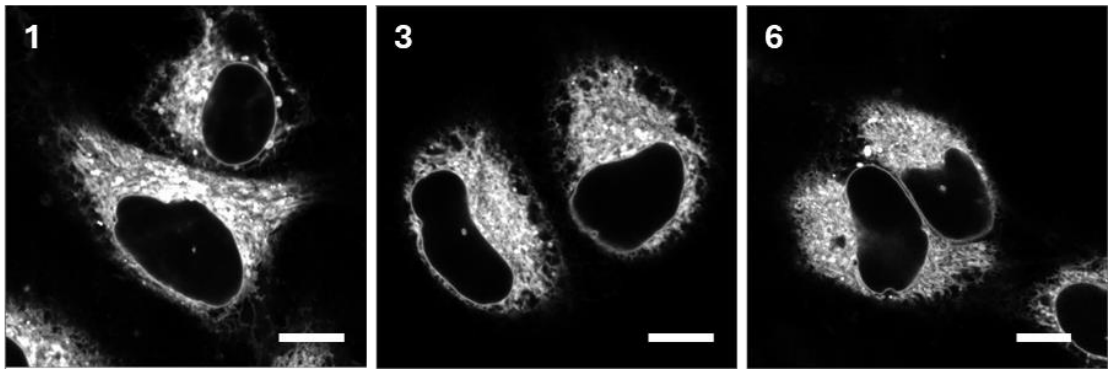
In solution fluorescence lifetime measurement of 1, 3 and 6

In solution fluorescence lifetimes measurements of **1**, **3** and **6** in 10 µM solutions were acquired using the parameters described above. Experiments were conducted in 8-well chamber slide (Ibidi) except for toluene solutions that were measured using concave glass slide and a glass coverslip.

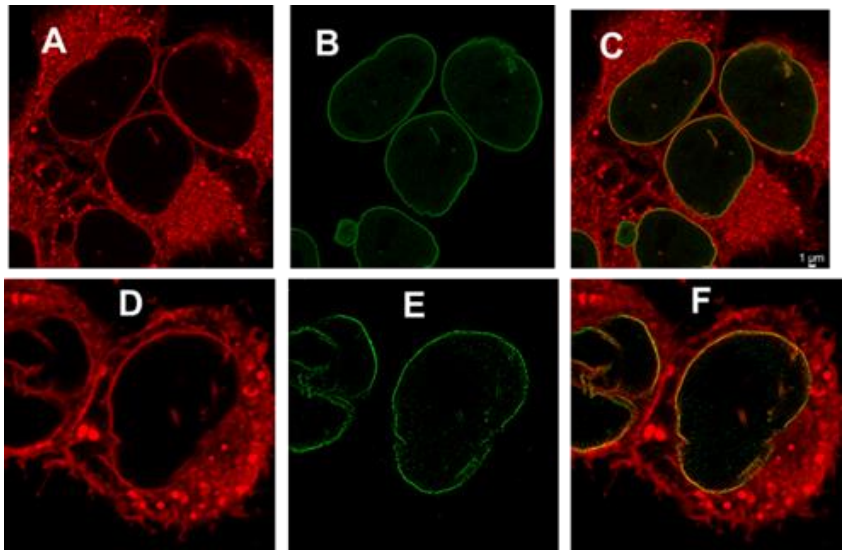
In solution fluorescence lifetime measurement of the reaction of 1 and 2

To monitor the bioorthogonal reaction of **1** (10 µM) and **2** (10 µM) in solution, fluorescence lifetime timelapses were conducted in H_2O/PS20 at 37 °C. Fluorescence lifetimes (fitted to a mono-exponential decay) were measured of a solution of **1** alone every 30 s for 10 min and then treated with **2** and measurements recoded for a further 45 min.

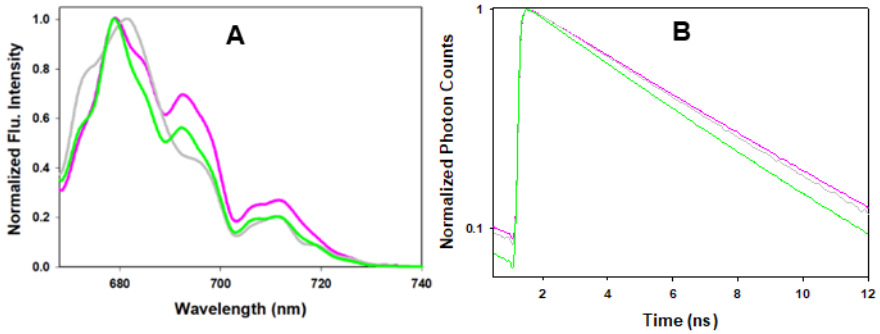
SI Figure S5: HeLa Kyoto cell CLSM images showing subcellular localization of **1**, **3** and **6 (**0.4 µM concentration) following a 90 min incubation. Scale bar 10 µm.



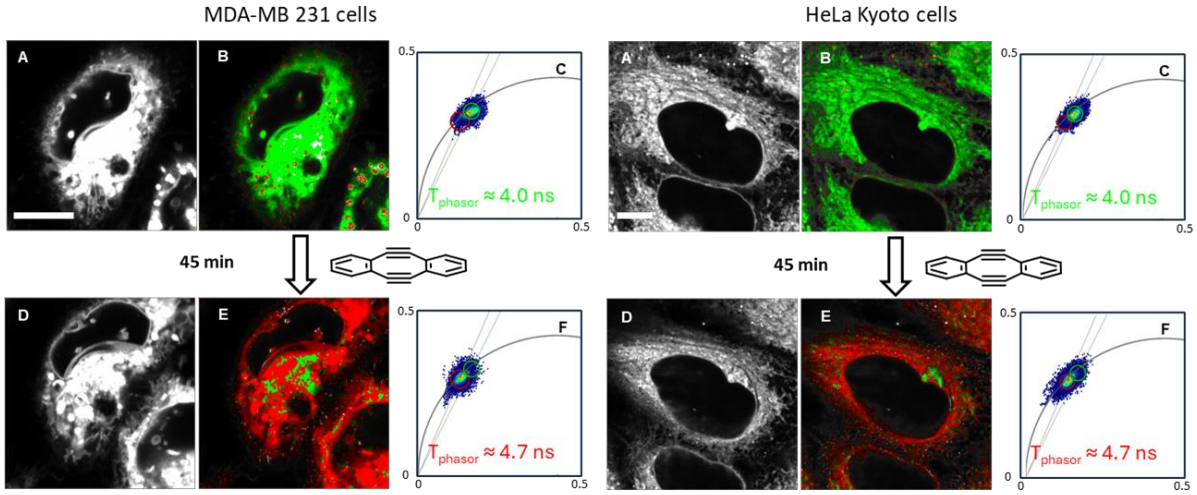
SI Figure S6. Colocalization study of **1** (red) with CF 594 dye antibody against nuclear lamin B1 (green) in HeLa Kyoto (top panel) and MDA-MB 231 cells (bottom panel). A/D CLSM images of **1** (0.4 µM concentration) in the nuclear membrane, nuclear invaginations and endoplasmic reticulum. B/E antibody CF594 nuclear lamina B stain showing nuclear membrane and nuclear invaginations. C/F Overlay of images showing colocalizations.



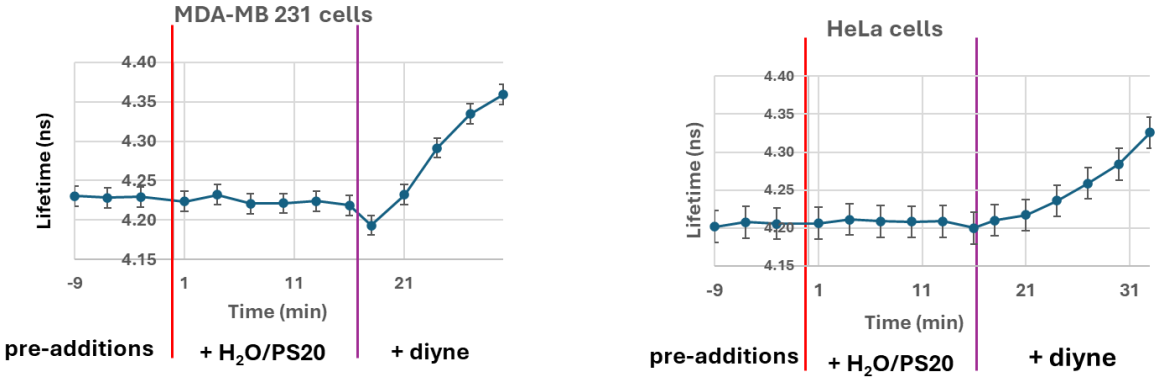
SI Figure S7: (A) Intracellular emission spectra and (B) lifetimes decay curves of **1** (green trace), **3** (pink trace) and **6** (grey trace) in HeLa Kyoto cells.



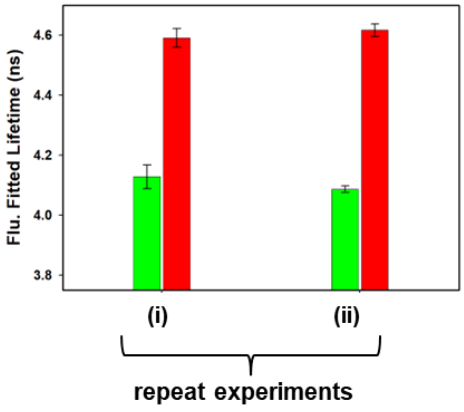
SI Figure S8: Representative images from repeat experiments of Figure 10. Bioorthogonal macrocyclization of **1** and **2** in MDA-MB 231 (left) and HeLa Kyoto (right) cells. (A) Intensity image at $T_{zero.}$ (B) FLIM image at $T_{zero.}$ (C) Expanded region of the phasor plot at $T_{zero.}$ (D) Intensity image at $T_{45\ min.}$ (E) FLIM image at $T_{45\ min.}$ (F) Expanded region of the phasor plot at $T_{45\ min.}$. B and E corresponds to A and D respectively with the pixels located inside the green or red circle on the phasor plot coloured accordantly. Same intensity scale is applied. Scale bar 10 µm.



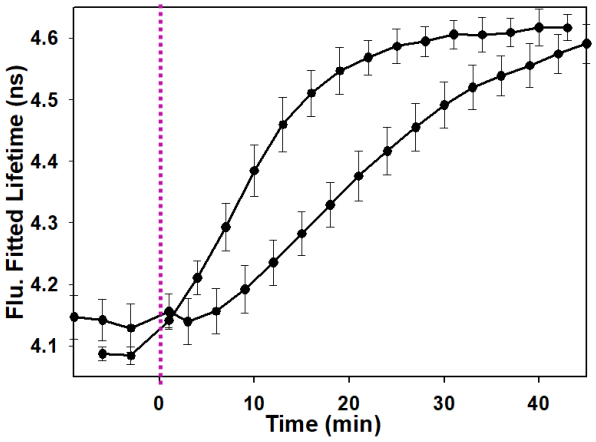
SI Figure S9. Bioorthogonal FLIM control experiments. Average fitted lifetime of 4 different regions. Same volume of buffer (H_2O/PS20) and the buffer supplemented with diyne was added to the cells.



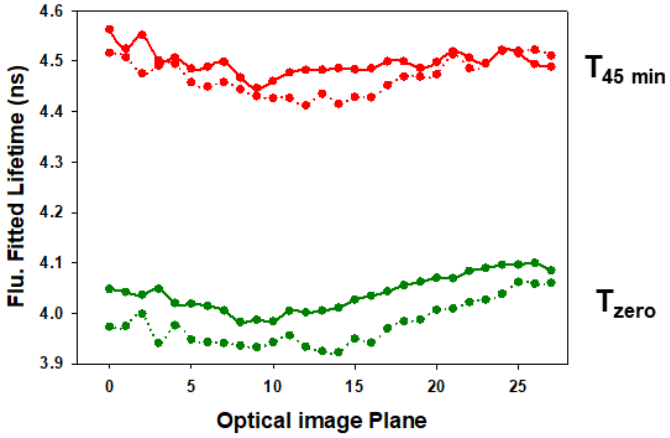
SI Figure S10: Bar graph of measured lifetime data at T_{zero} (green bars) and $T_{45\ min}$ (red bars) accumulated from multiple entire cell fields of view (FOV) in HeLa Kyoto cells.



SI Figure S11: Plot of FLIM tracking of bioorthogonal macrocyclization of **1** and **2** in HeLa Kyoto cells (pink dotted line indicates the addition of **2**).



SI Figure S12: Repeat experiments showing the measured fitted lifetimes for each optical slice at T_{zero} (green trace) and $T_{45\ min}$ (red trace) in MDA-MB 231 cells.



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1. Wu, D.; Durán-Sampedro, G.; Fitzgerald, S.; Garre, M.; O'Shea, D. F., *Chem. Comm.* **2023,** *59*, 1951-1954.
2. Wu, D.; Durán-Sampedro, G.; O'Shea, D. F.,. *Front. Chem. Sci. Eng.* **2019,** *14*, 97-104.
3. Dhar, S.; Bhattacharya, S.; Rana, D.K.; Roy, S.S.; Roy, S.; Bhattacharya, S.C. *J. Luminescence* **2012**, 132 957.