Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Double Click Macrocyclization with Sondheimer Diyne of Aza-Dipyrrins for B-F_{ree} Bioorthogonal Imaging

Dan Wu, Gonzalo Durán-Sampedro, Sheila Fitzgerald, Massimiliano Garre and Donal F. O'Shea*

Department of Chemistry, RCSI, 123 St Stephen's Green, Dublin 2, Ireland

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Author Contributions

DOS: Conceptualization, funding acquisition, supervision, writing original draft.

Investigation and Methodology

GDS, DW: Developed the synthetic route,

GDS, DW, SF: Contributed to compound synthesis and analytical characterisations,

DW: Carried out in water and in MeOH macrocyclization studies,

DW: Conducted the photophysical studies,

DW, MG: Carried out cell imaging experiments,

DW, MG: Carried out the cell imaging data analysis.

General Experimental

All reactions involving air-sensitive reagents were performed under nitrogen in oven-dried glassware using syringe-septum cap techniques. All solvents were purified and degassed before use. Chromatographic separation was carried out under pressure 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) using UV light (254 nm) as visualizing agent. 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. ESI (HRMS) mass spectra were acquired using a Bruker micrOTOF-Q III spectrometer interfaced to a Dionex UltiMate 3000 LC in positive and negative modes as required. Masses were recorded over the range 100-1800 m/z. Operating conditions were as follows: end-plate offset 500V capillary 4500V, nebulizer 2.0Bar, dry gas 8.0 L/min, and dry temperature 180 °C. MicroTof control 3.2 and HyStar 3.2 software were used to carry out the analysis. APCI (HRMS) experiments were carried out on a Bruker micrOTOF-Q III spectrometer interfaced to a Dionex UltiMate 3000 LC. The instrument was operated in positive or negative mode as required. 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: Capillary voltage 4000 V, corona 4000 nA, nebulizer gas 2.0 Bar, dry gas 3.0 L/min, dry gas temperature

200 °C, vap. temperature 400 °C. MicroTof control and HyStar software were used to carry out the analysis. All absorbance spectra were recorded with a Varian Cary 50 scan UV-visible spectrometer and fluorescence spectra were recorded with a Varian Cary eclipse fluorescence spectrometer. Data was plotted in SigmaPlot 8 software. Confocal, STED and FLIM images acquired using Leica Stellaris 8 (Leica objective 100X / 1.49 HC PL APO CS2) and were processed using lightning deconvolution mode. Analysis of raw files using ImageJ 1.53q. MDA-MB 231 cells obtained from Merck Life Science Limited.

Synthesis

1-(4-Methoxyphenyl)-3-(nitromethyl)-6-((tetrahydro-2*H*-pyran-2-yl)oxy)hexan-1-one **4** was synthesised following the literature procedure.[1]

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

A solution of **4** (4 g, 11 mmol) and *p*-TsOH (4.2 g, 22 mmol, 2 equiv.) in EtOH (100 mL) was heated under reflux for 4 h. After cooling to room temperature, the solvent was removed *in vacuo* and the residue obtained dissolved in water (50 mL), neutralized with saturated aqueous $NaHCO_3$ and extracted with EtOAc (3 x 100 mL). The combined organic layers were washed with H_2O (3 x 50 mL), dried over Na_2SO_4, filtered and the solvent was evaporated under reduced pressure. The residue was then purified by flash chromatography on silica gel (1:1 petroleum ether/EtOAc) to give **5** (2.8 g, 91%) as thick yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.92 (d, *J* = 8.9 Hz, 2H), 6.93 (d, *J* = 8.9 Hz, 2 H), 4.58-4.49 (m, 2 H), 3.86 (s, 3 H), 3.69-3.65 (m, 2 H), 3.16-3.00 (m, 2 H), 2.90-2.86 (m, 1 H), 1.67-1.52 (m, 4 H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 196.8, 163.8, 130.3, 129.6, 113.9, 78.7, 62.0, 55.5, 39.5, 33.1, 29.5, 28.0. MS (ESI^-) *m/*z $[M-H]^-$ calc. for $C_{14}H_{18}NO_5$ 280.31; found 280.39.

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

A solution of **5** (2.8 g, 10 mmol) and triethylamine (3.5 mL, 25 mmol, 2.5 equiv) in CH_2Cl_2 (50 mL) was stirred for 15 min at 0 ºC, treated dropwise with methanesulfonyl chloride (1.9 mL, 2.5 mmol, 2.5 equiv) and 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 brine (100 mL), dried over Na_2SO_4, filtered and reduced to dryness *in vacuo* to give thick yellow oil. Without further purification, the oil was dissolved in anhydrous DMF (50 mL) and treated with NaN_3 (1.6 g, 25 mmol, 2.5 equiv) and heated under reflux for 4 h. The reaction mixture was allowed to reach room temperature, diluted with EtOAc (100 mL) and washed with water (3 x 100 mL). The combined organic layers were dried over Na_2SO_4, filtered and the solvent was evaporated under reduced pressure. The residue was then purified by flash chromatography on silica gel (9:1 petroleum ether/EtOAc) to give **6** (2.6 g, 85%) as yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.91 (d, *J* = 8.9 Hz, 2 H), 6.93 (d, *J* = 8.9 Hz), 4.58-4.47 (m, 2 H), 3.86 (s, 3 H), 3.30 (t, *J* = 6.6 Hz, 2 H), 3.15-2.97 (m, 2 H), 2.86 (m, 2 H, 1 H), 1.68-1.60 (m, 2 H), 1.57-1.51 (m, 2 H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 196.2, 163.9, 130.4, 129.6, 113.9, 78.6, 55.6, 51.2, 39.4, 33.1, 28.9, 26.3. MS (ESI^+) *m/z*: $[M+Na]^+$ calc. $C_{14}H_{18}N_4O_4Na$ 329.32; found 329.15.

Synthesis of 3-(3-azidopropyl)-*N*-(3-(3-azidopropyl)-5-(4-methoxyphenyl)-1H-pyrrol-2-yl)-5-(4-methoxyphenyl)-2*H*-pyrrol-2-imine **7**.

A solution of **6** (3 g, 9.8 mmol) and ammonium acetate (30 g, 390 mmol) in MeOH (50 mL) was heated under reflux for 9 h. The reaction mixture was cooled to rt, water (50 mL) added, extracted with CH_2Cl_2 (3 x 100 mL). The combined organic extracts were washed with saturated $NaHCO_3$ (2 x 50 mL), water (2 x 50 mL), dried over Na_2SO_4, filtered and the filtrate concentrated under reduced pressure. The residue was purified by column chromatography on silica gel using cyclohexane and EtOAc (70/30) to give **7** as a purple solid (472 mg, 18%), m.p. 102-103 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.80 (d, *J* = 8.6 Hz, 4H), 7.02 (d, *J* = 8.6 Hz, 4H), 6.68 (s, 2H), 3.90 (s, 6H), 3.37 (t, *J* = 6.9 Hz, 4H), 2.84 (t, *J* = 7.3 Hz, 4H), 2.04-2.01 (m, 4H), NH not observed. ^{13}C NMR (101 MHz, $CDCl_3$) δ: 161.1, 154.3, 149.4, 143.8, 128.0, 125.2, 115.7, 114.6, 55.5, 51.0, 29.4, 23.3 ppm. IR (DCM): 2095 cm^{-1}. HRMS (ESI$^+$) *m/z*: $[M+H]^+$ calc. $C_{28}H_{30}N_9O_2$ 524.2517; found 524.2521.

Synthesis of (3^2Z,7^{3a}Z,7^{7b}Z)-1^5,3^5-bis(4-methoxyphenyl)-7^1,7^{10}-dihydro-1^1*H*,3^2*H*-2-aza-7(1,10)-dibenzo[3,4:7,8]cycloocta[1,2-*d*:5,6-*d'*]bis([1,2,3]triazole)a-1,3(2,3)-dipyrrolacyclodecaphane **8** and (3^2Z,7^{3a}Z,7^{7b}Z)-1^5,3^5-bis(4-methoxyphenyl)-7^1,7^8-dihydro-1^1*H*,3^2*H*-2-aza-7(1,8)-dibenzo[3,4:7,8]cycloocta[1,2-*d*:5,6-*d'*]bis([1,2,3]triazole)a-1,3(2,3)-dipyrrolacyclodecaphane **9**.

A solution of **7** (45 mg, 0.086 mmol) in solvent as per table 1 (10 mL) was treated with **1** (17 mg, 0.086 mmol) and stirred at room temperature until reaction was judged to have reached completion by TLC or HPLC. The reaction mixture was concentrated under reduced pressure with isomer separation by silica gel column chromatography eluting with CH_2Cl_2 / EtOAc (6:4) to give **8** and **9** as purple solids in 95% yield.

8 m.p. >250 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.83 (d, *J* = 8.8 Hz, 4H), 7.60 (dd, *J* = 5.8, 3.4 Hz, 2H), 7.42 (dd, *J* = 5.8, 3.4 Hz, 2H), 7.13 (dd, *J* = 5.8, 3.3 Hz, 2H), 7.08 (d, *J* = 8.8 Hz, 4H), 7.02 (dd, *J* = 5.7, 3.4 Hz, 2H), 6.69 (s, 2H), 4.60 (td, *J* = 13.2, 5.5 Hz, 2H), 4.36 (td, *J* = 13.1, 4.4 Hz, 2H), 3.92 (s, 6H), 2.89 (dt, *J* = 13.6, 4.8 Hz, 2H), 2.60 – 2.43 (m, 2 H), 2.15 (td, *J* = 12.5, 6.3 Hz, 2H), 2.02 (dt, *J* = 18.0, 8.3 Hz, 2H), NH not observed. ^{13}C NMR (100 MHz, $CDCl_3$) δ: 161. 5, 153.9, 148.6, 146.3, 142.6, 132.8, 131.1, 130.6, 129.9, 129.7, 129.1, 128.0, 127.9, 125.1, 117.2, 115.0, 55.7, 47.9, 30.8, 24.1. HRMS (APCI$^+$) *m/z*: $[M+H]^+$ calc $C_{44}H_{38}N_9O_2$ 724.3143; found 724.3149.

9 m.p. > 250°C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.78 (d, *J* = 7.4 Hz, 2H), 7.72 (d, *J* = 8.6 Hz, 4H), 7.54 (dt, *J* = 20.1, 7.4 Hz, 4H), 7.31 (d, *J* = 7.4 Hz, 2H), 6.97 (d, *J* = 8.7 Hz, 4H), 6.54 (s, 2H), 4.76-4.63 (m, 2H), 4.42-4.29 (m, 2H), 3.87 (s, 6H), 2.17-1.98 (m, 4H), 1.93-1.82 (m, 2H), 1.70 – 1.58 (m, 2H), NH not observed. ^{13}C NMR (100 MHz, $CDCl_3$) δ: 160.9, 154.4, 149.1, 145.3, 142.8, 134.7, 133.0, 131.5, 130.2, 129.3, 129.1, 128.0, 127.7, 125.6, 114.8, 114.6, 55.6, 48.2, 29.8, 29.5, 20.8. HRMS (ESI^+) *m/z*: $[M+H]^+$ calc $C_{44}H_{38}N_9O_2$ 724.3143; found 724.3148.

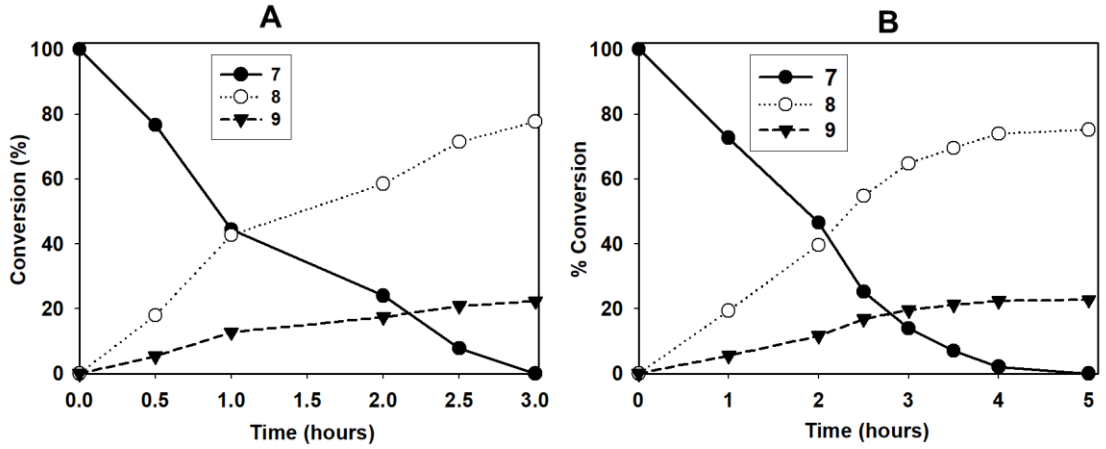


Fig. S1. Conversion plots for the reaction of **7** with **1** producing **8** and **9** in MeOH at (A) 4 mM concentration and (B) 5 μM concentration.

Fig. S2A. ^{1}H NMR (400 MHz, $CDCl_3$) of **5**.

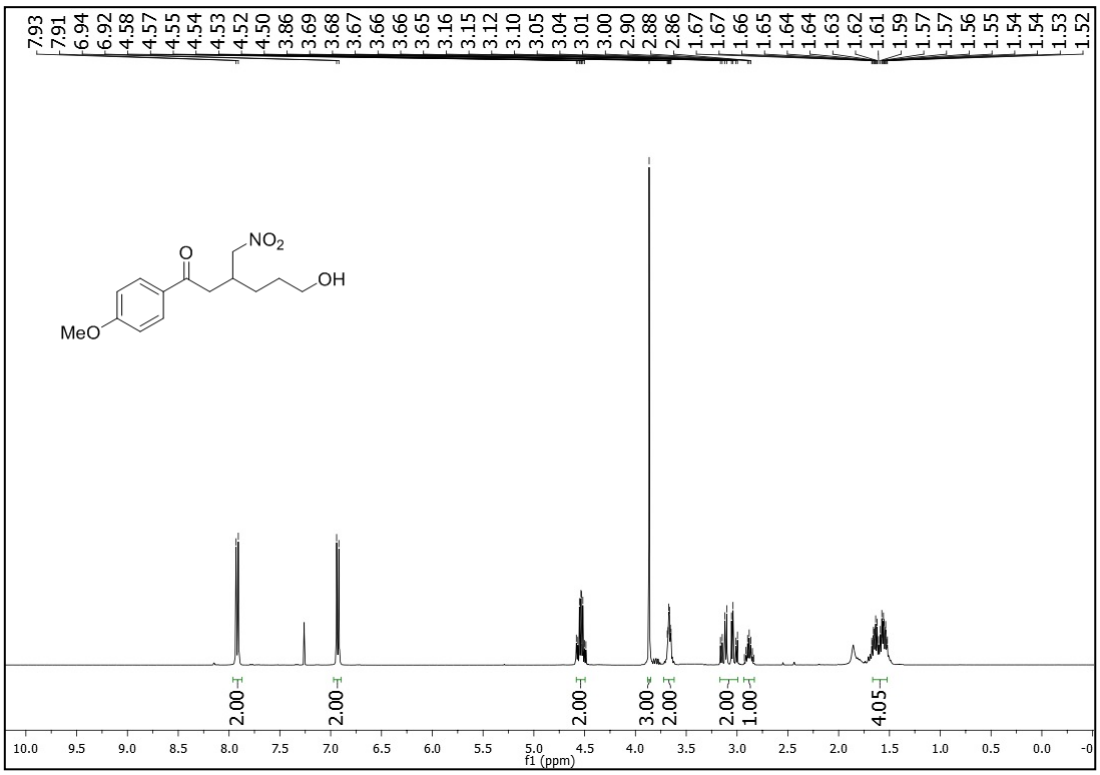


Fig. S2B. ^{13}C NMR (101 MHz, $CDCl_3$) of **5**.

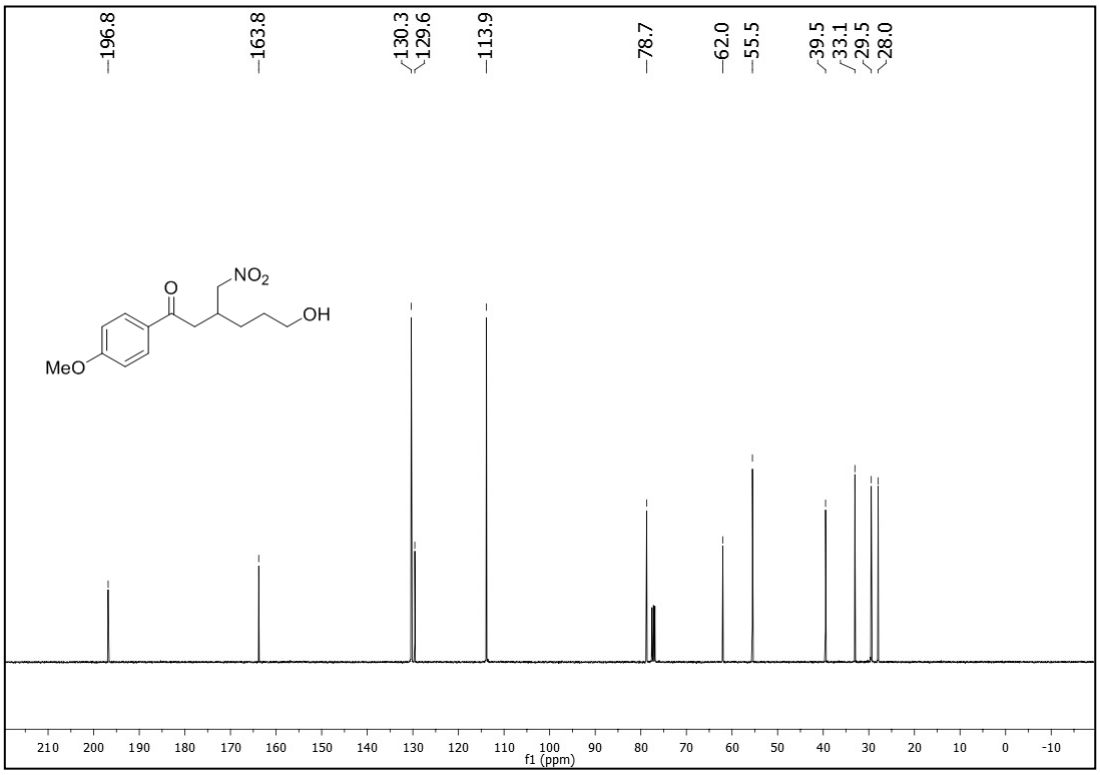


Fig. S3A. ^{1}H NMR (400 MHz, $CDCl_3$) of **6**.

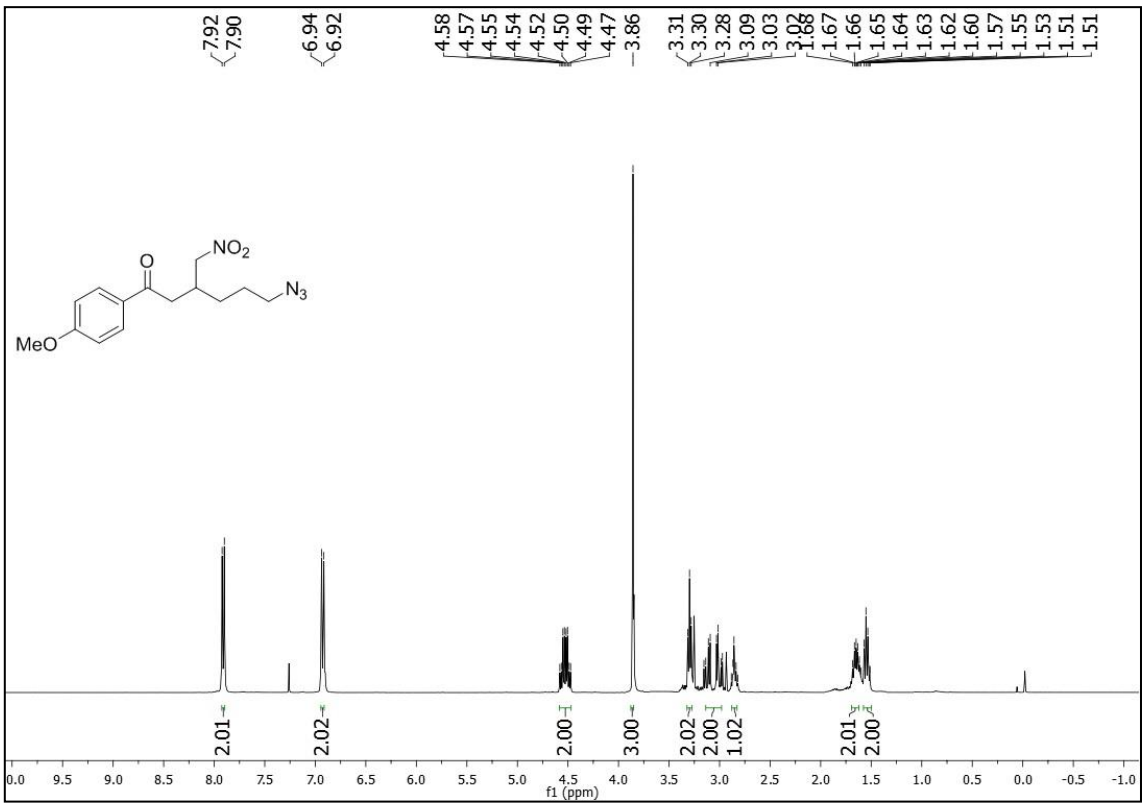


Fig. S3B. ^{13}C NMR (101 MHz, $CDCl_3$) of **6**.

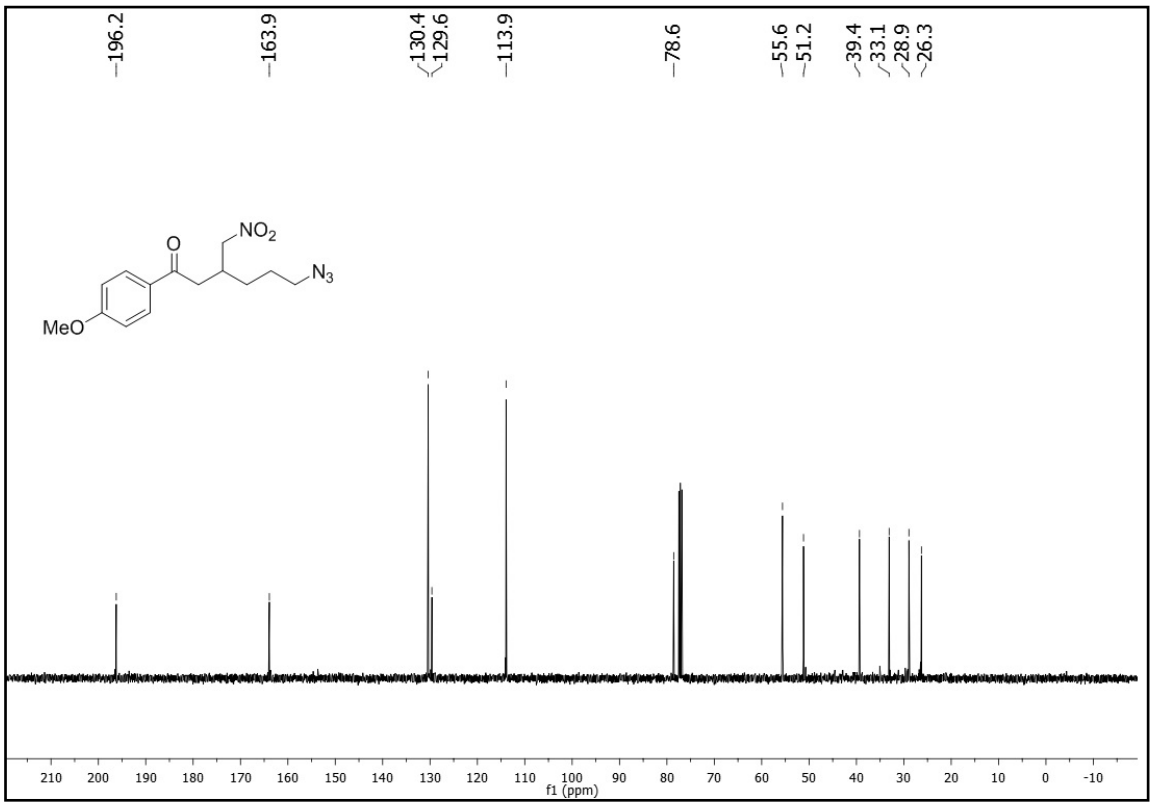


Fig. S4A. ^{1}H NMR (400 MHz, $CDCl_3$) of **7.**

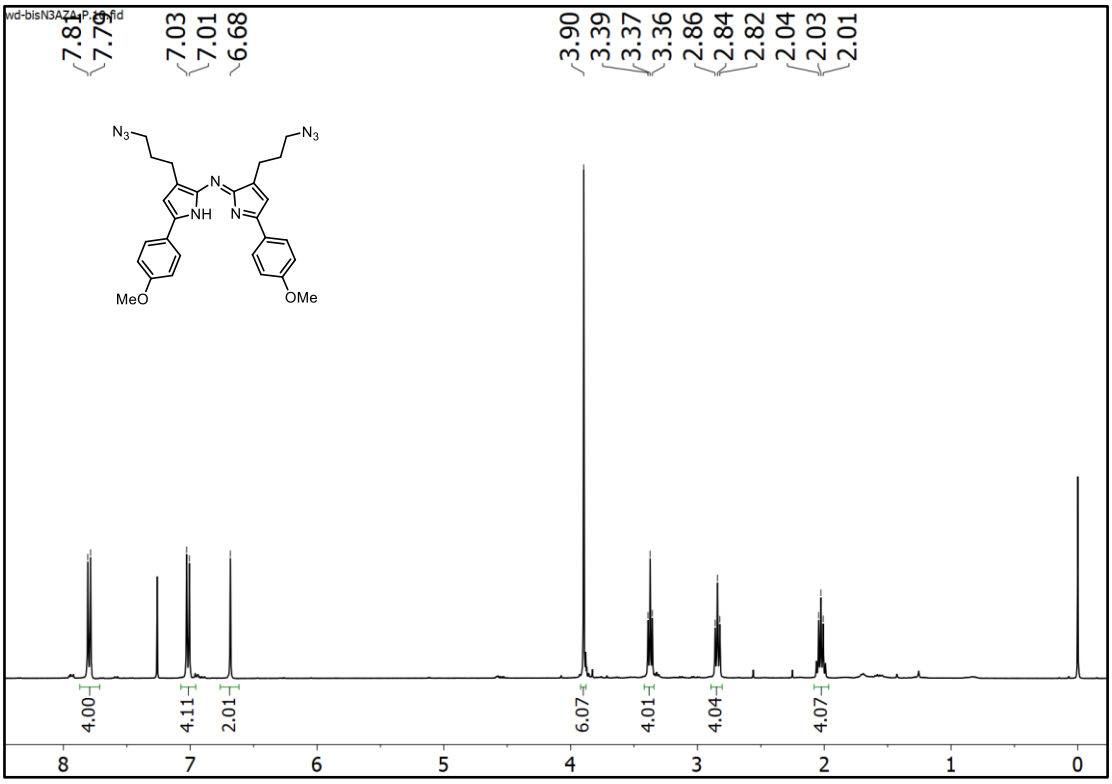


Fig. S4B. ^{13}C NMR (101 MHz, $CDCl_3$) of **7**.

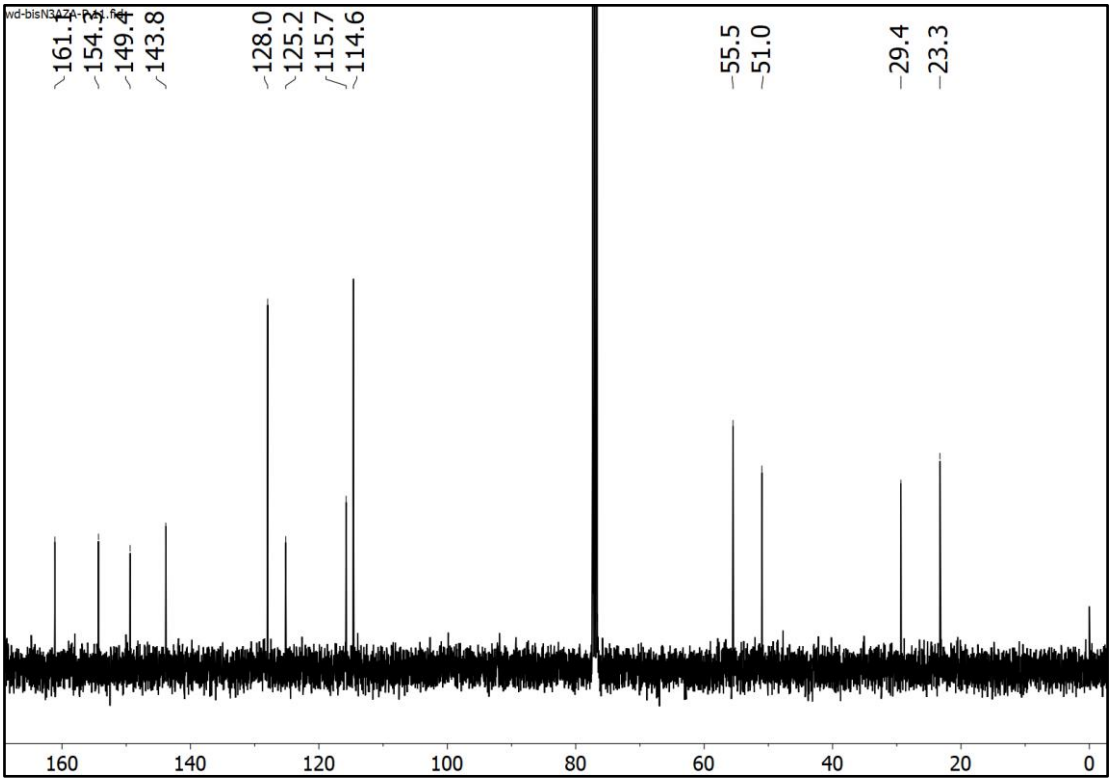
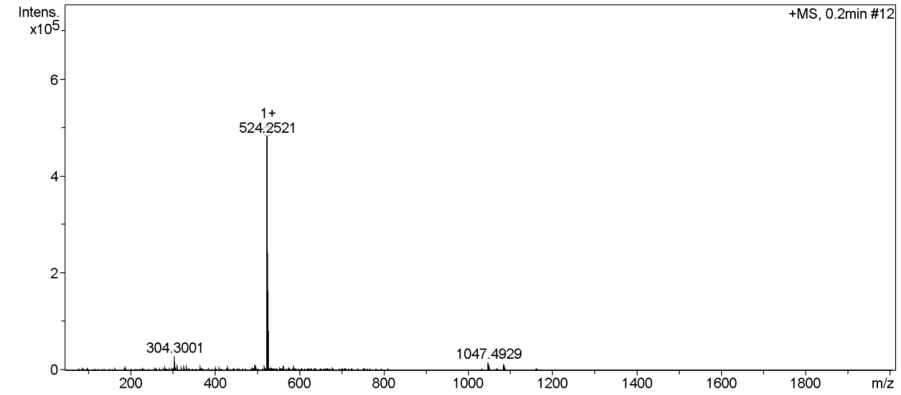


Fig. S4C. ESI Positive Mode HRMS of **7**.



Meas. m/z	#	Ion Formula	m/z	err [mDa]	err [ppm]	rdb	N-Rule	e⁻ Conf	mSigma
524.252071	1	C28H30N9O2	524.251698	0.4	0.7	18.5	ok	even	1.8

Fig. S4D. Infra-red spectrum of **7**.

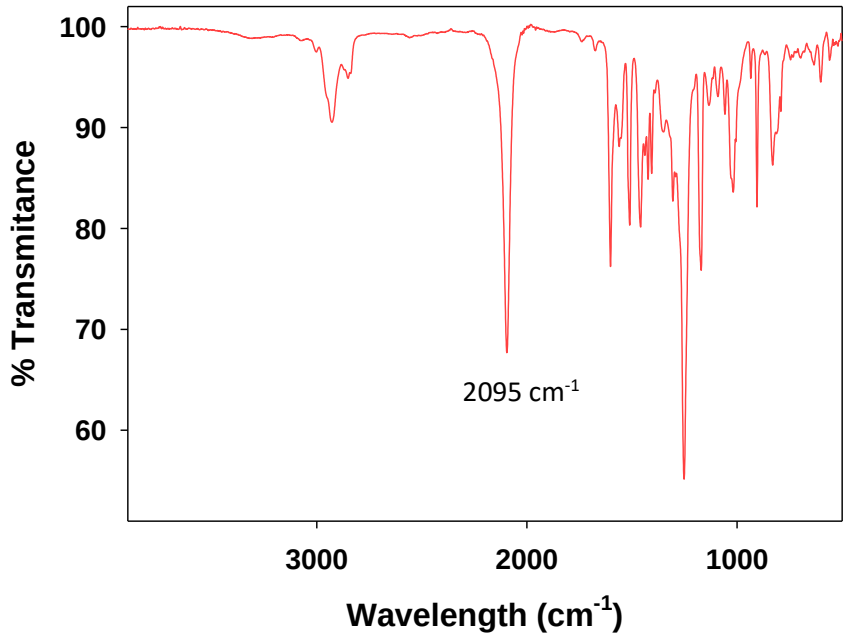


Fig. S5A. ^{1}H NMR (400 MHz, $CDCl_3$) of **8**.

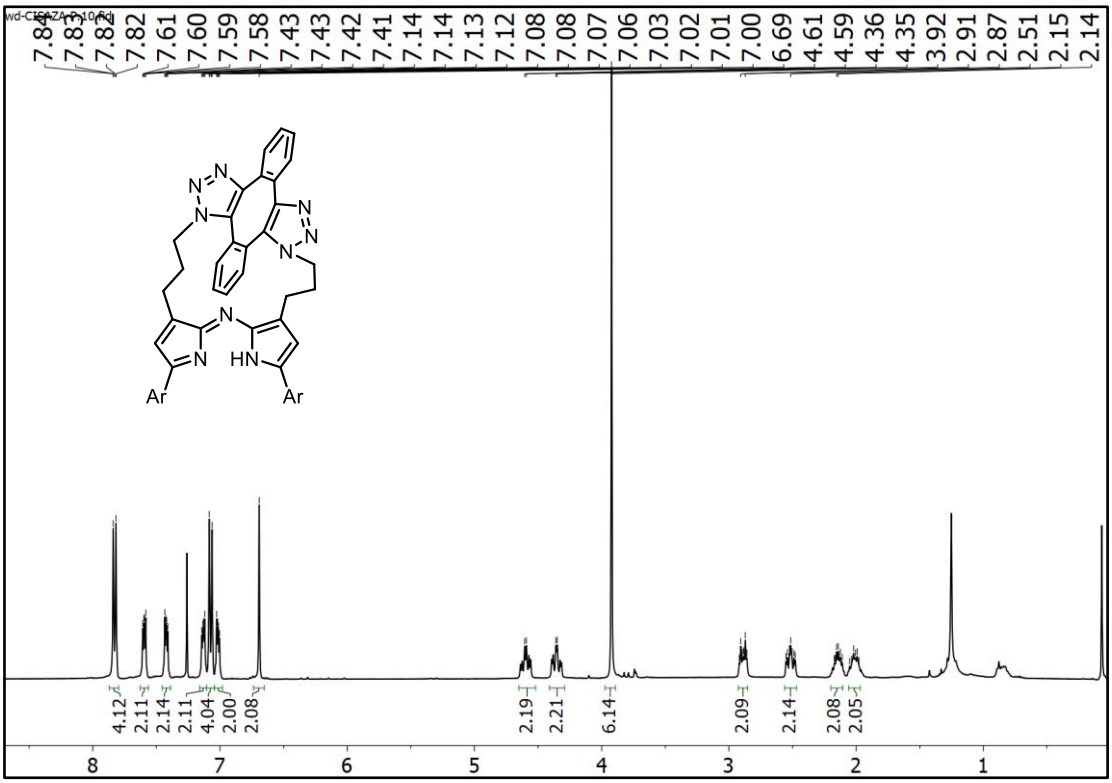


Fig. S5B. ^{13}C NMR (101 MHz, $CDCl_3$) of **8**.

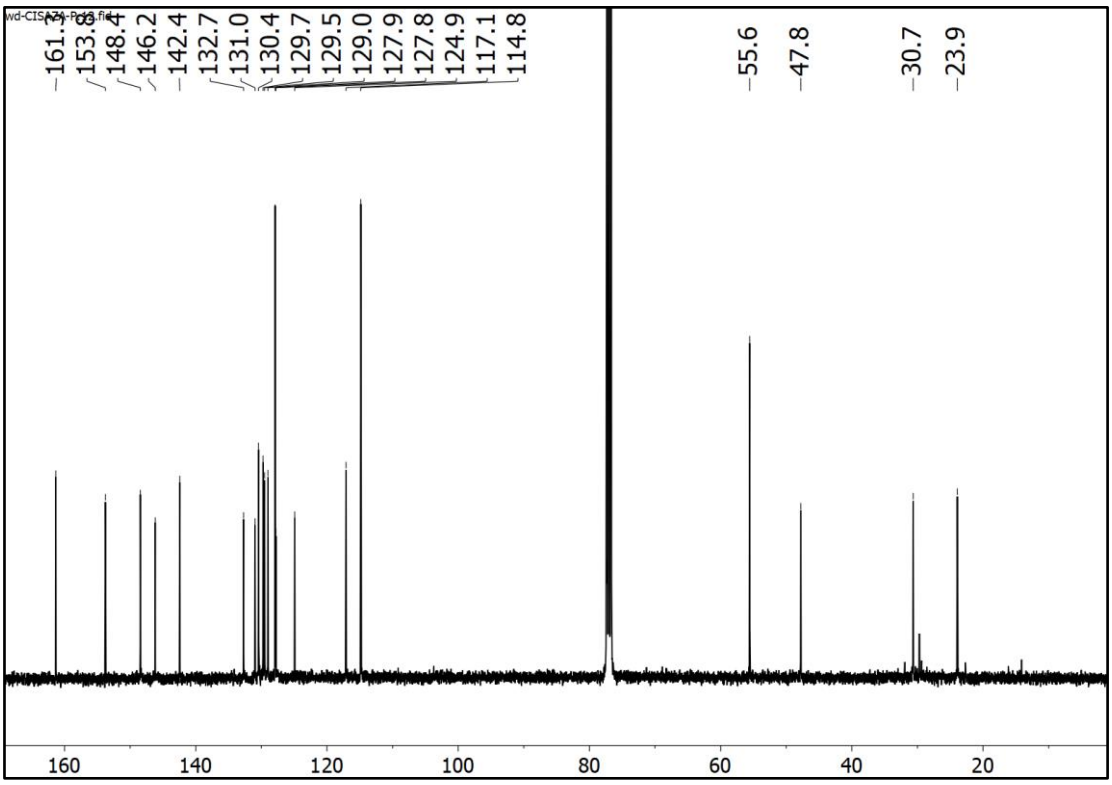


Fig. S5C. 2D COSY of **8**.

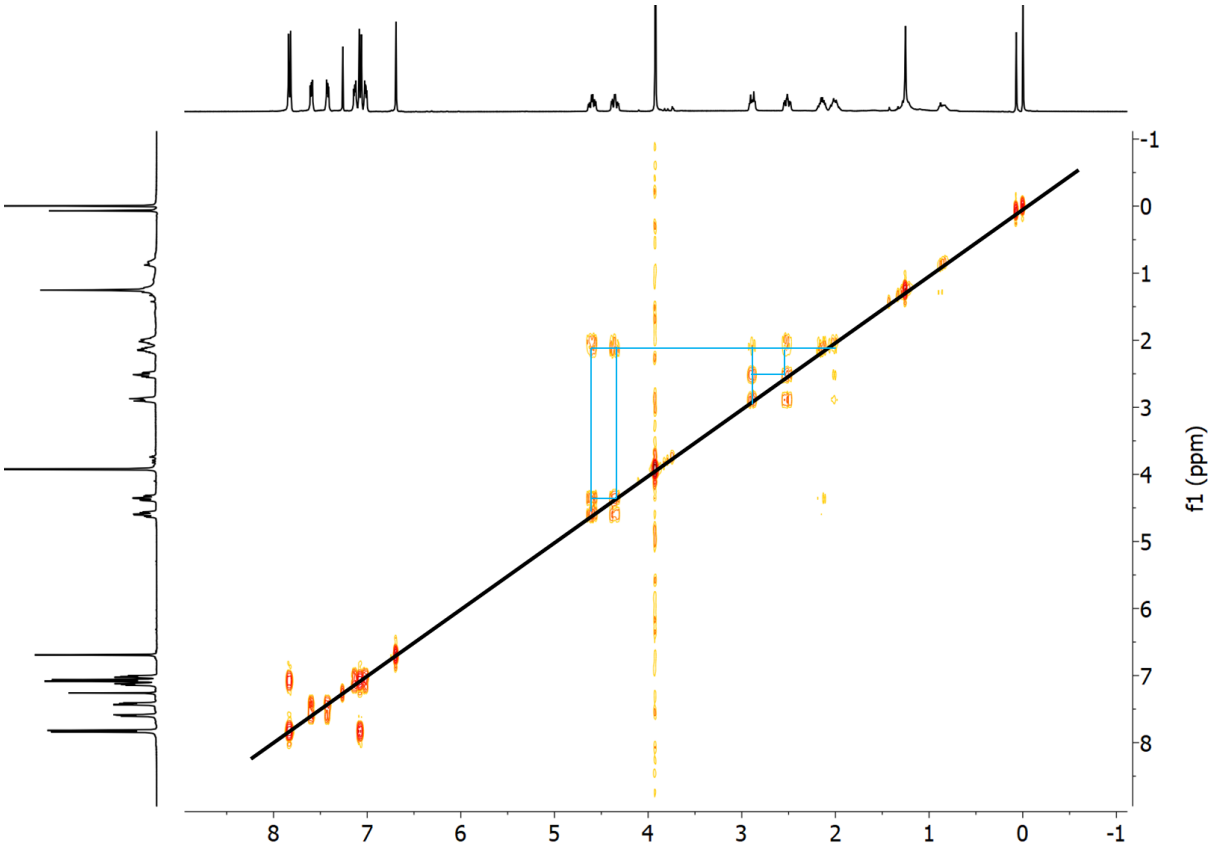


Fig. S5D. NOESY of **8**.

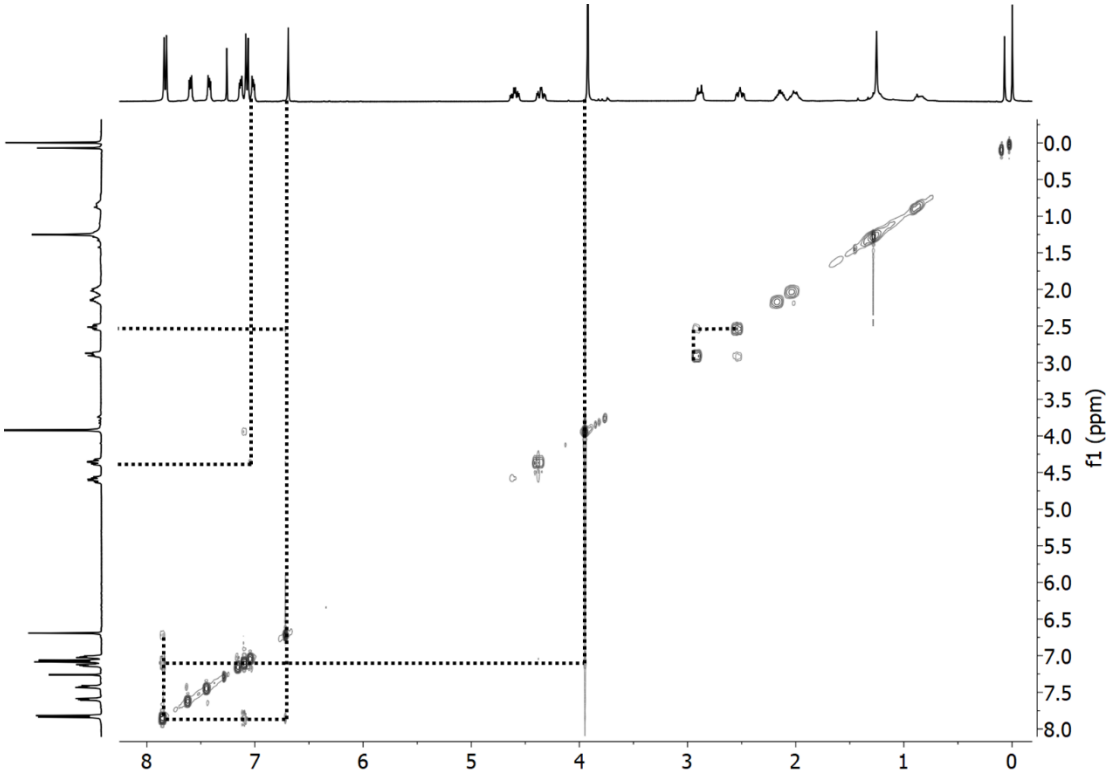
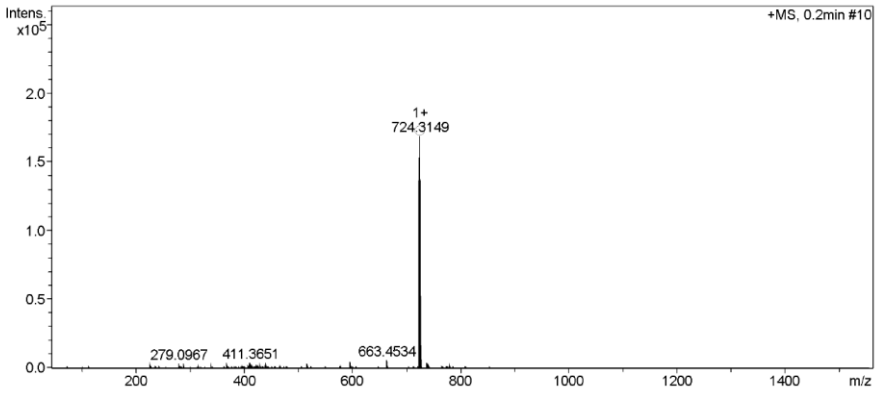


Fig. S5E. APCI Positive Mode HRMS of **8**.



Meas. m/z	#	Ion Formula	m/z	err [mDa]	err [ppm]	rdb	N-Rule	e¯ Conf	mSigma
724.314897	1	C44H38N9O2	724.314298	0.6	0.8	30.5	ok	even	12.0

Fig. S6A. ^{1}H NMR (400 MHz, $CDCl_3$) of **9**.

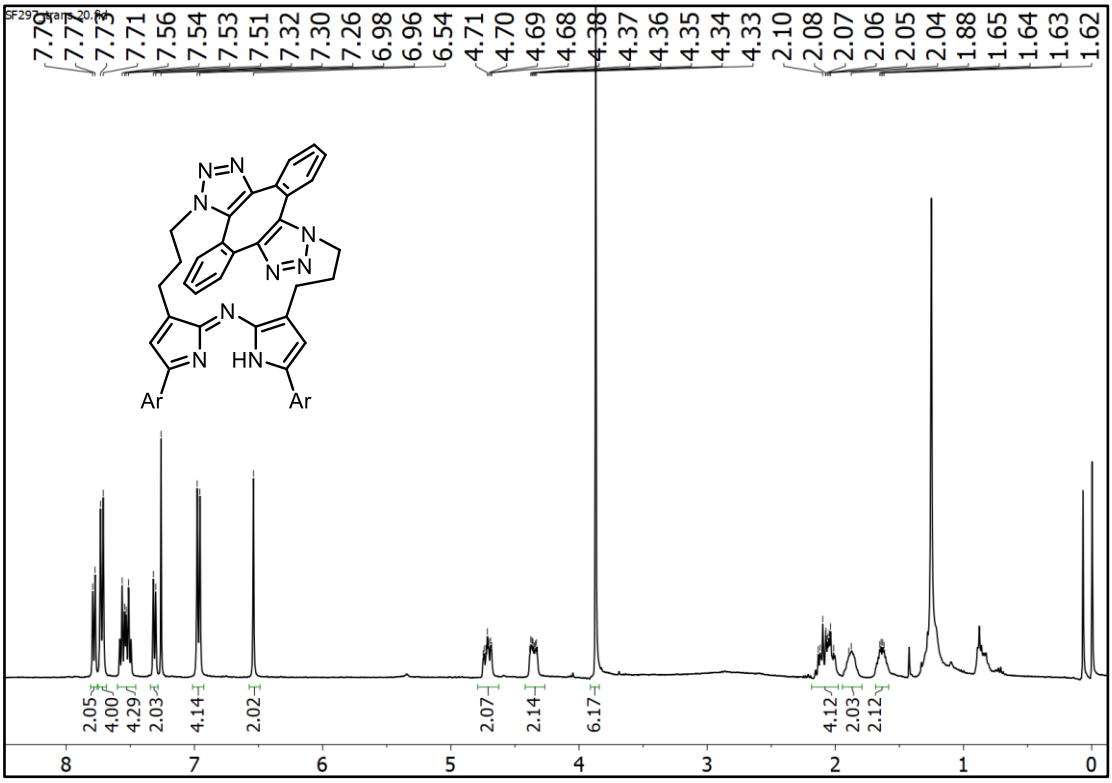


Fig. S6B. ^{13}C NMR (101 MHz, $CDCl_3$) of **9**.

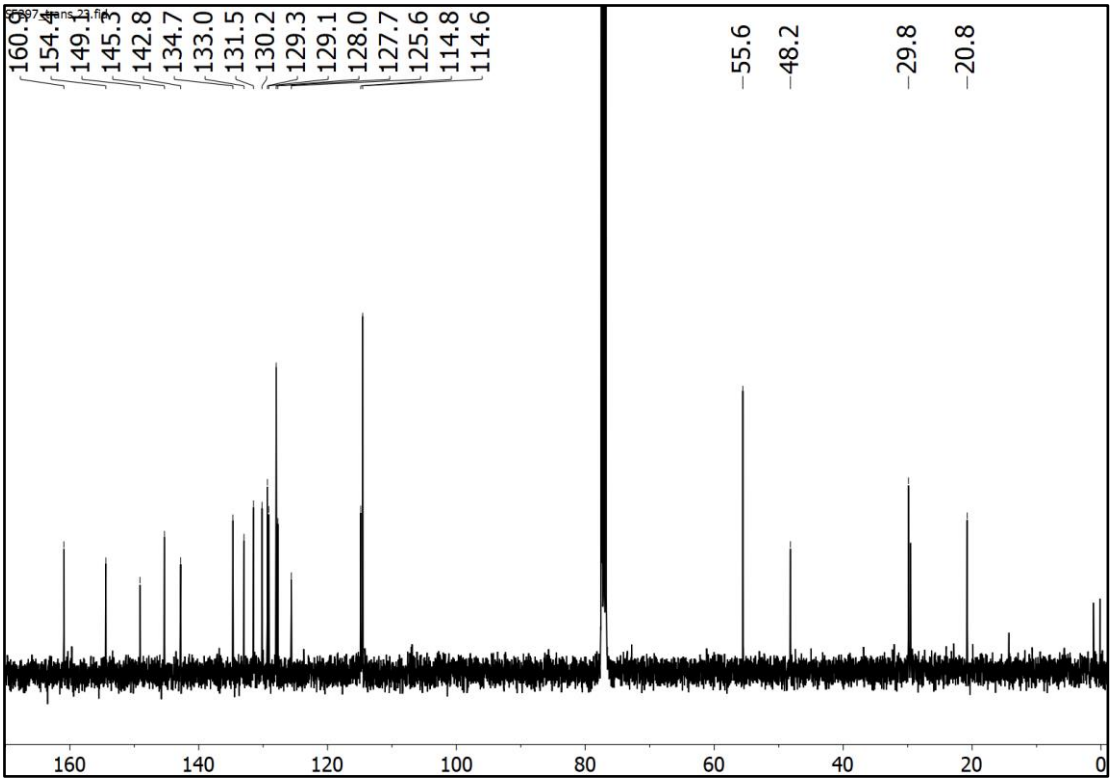


Fig. S6C. 2D COSY (400 MHz, $CDCl_3$) of **9**.

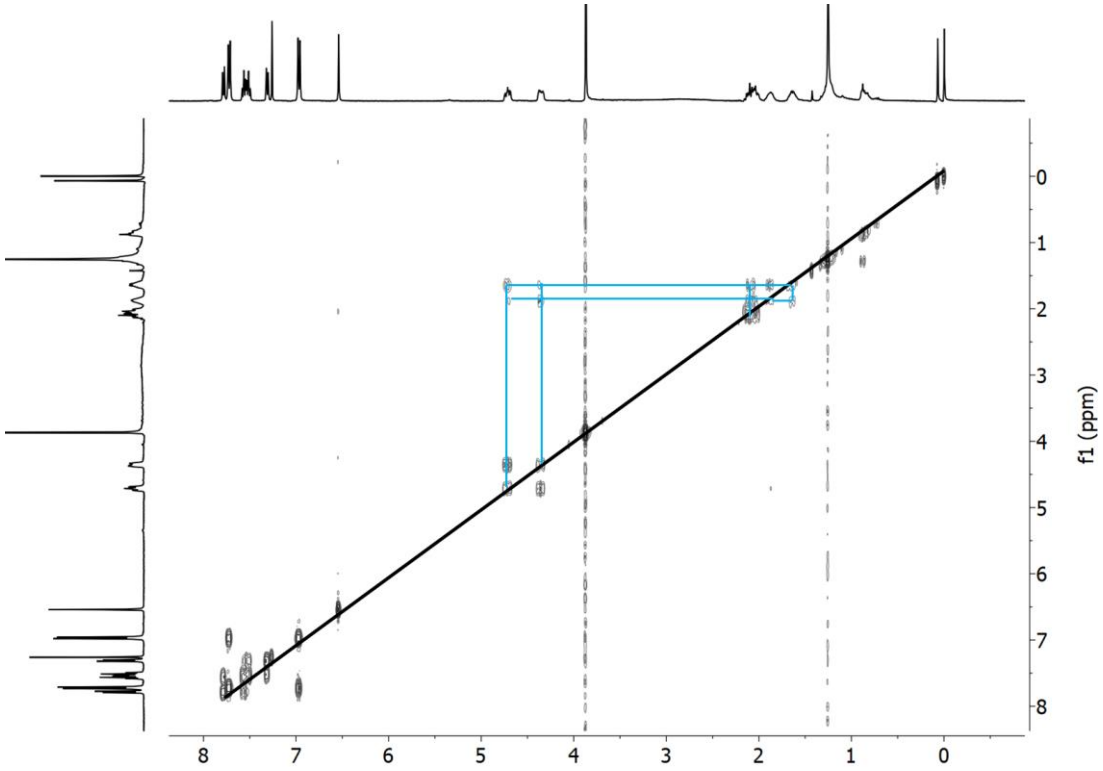
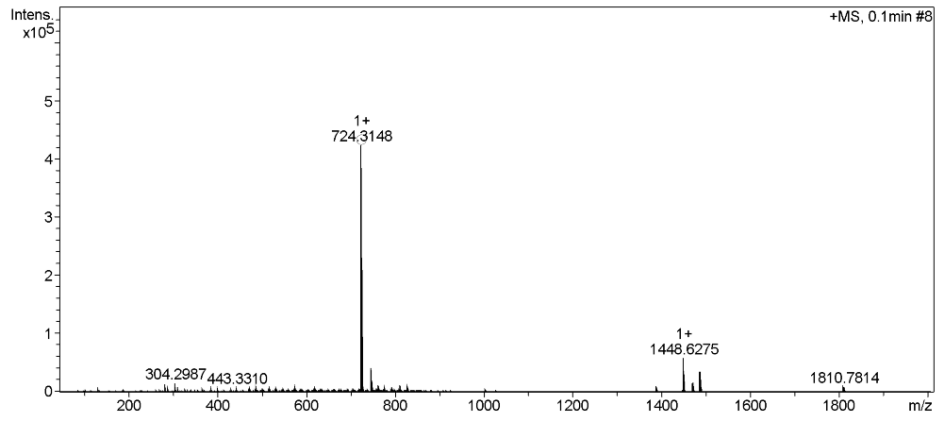


Fig. S6D. ESI Positive Mode HRMS of **9**.



Meas. m/z	#	Ion Formula	m/z	err [mDa]	err [ppm]	rdb	N-Rule	e⁻ Conf	mSigma
724.314812	1	C44H38N9O2	724.314298	0.5	0.7	30.5	ok	even	17.4

Fig. S7. Normalised absorption and Fluorescence (excitation 590 nm, slit width 5 nm) Spectra of **7** (3 μM) in water/PS20.

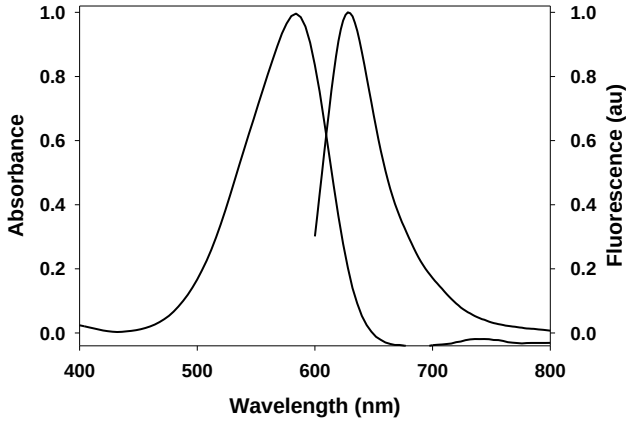


Fig. S8. Normalised absorption and Fluorescence (excitation at 590 nm, slit width 5 nm) Spectra of **8** (3 μM) in water/PS20.

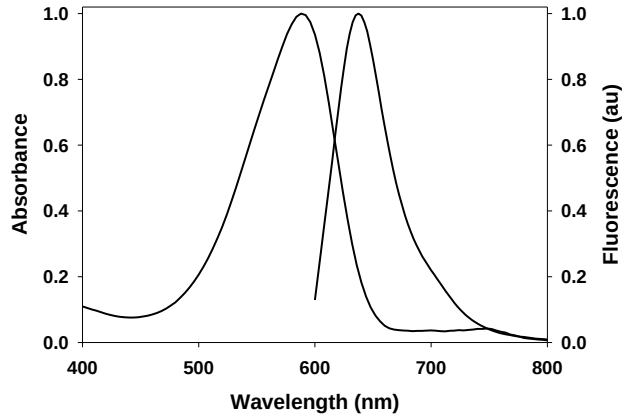


Fig. S9. Normalised absorption and Fluorescence (excitation at 590 nm, slit width 5 nm) Spectra of **9** (3 μM) in water/PS20.

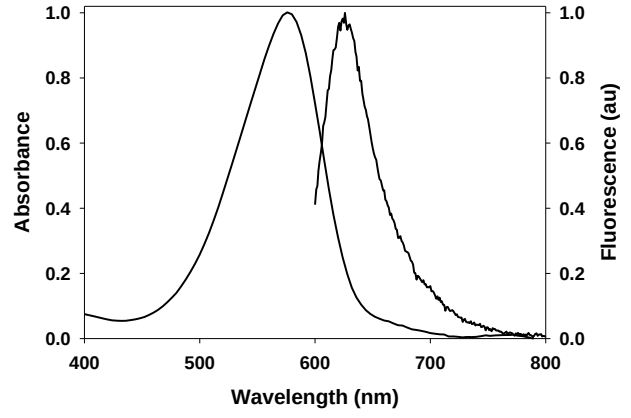
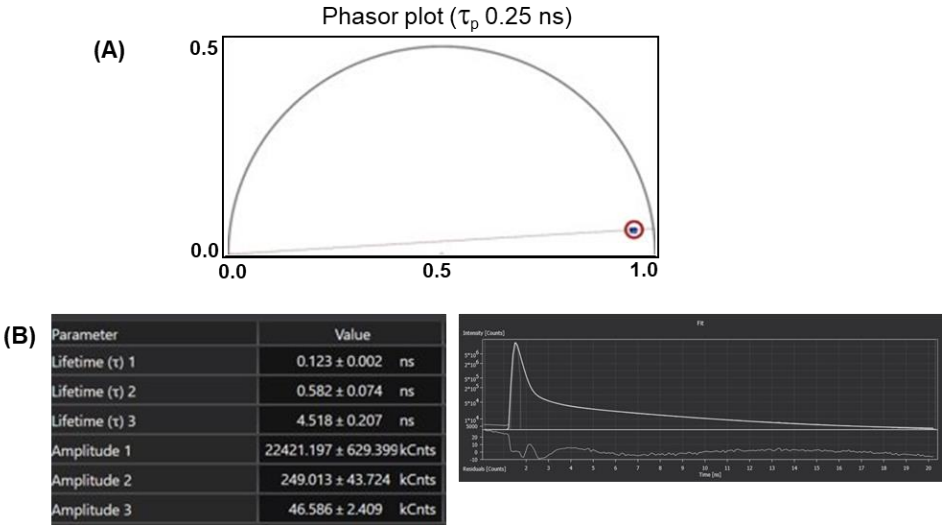
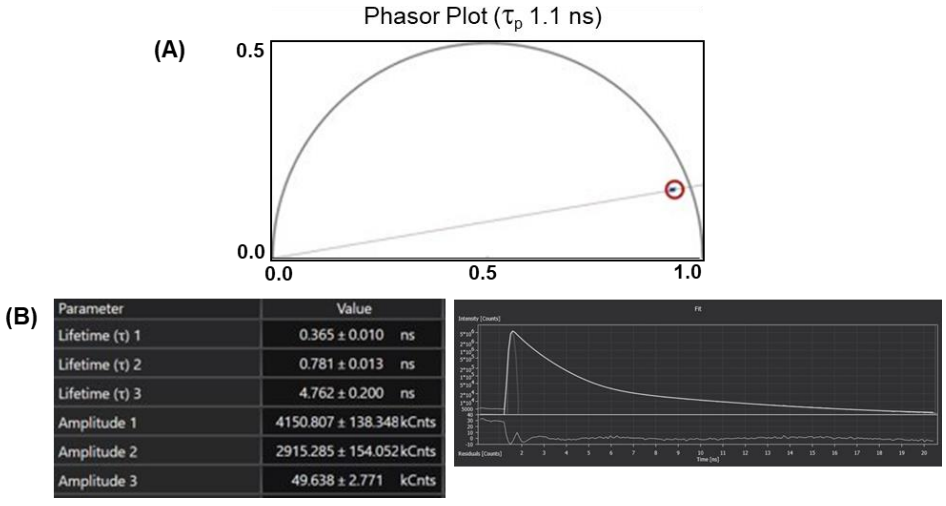


Fig. S10. Phasor Plot Analysis and Lifetime Data for **7** (10 μM) in water/PS20.



Parameter	Value
Lifetime (τ) 1	0.123 ± 0.002 ns
Lifetime (τ) 2	0.582 ± 0.074 ns
Lifetime (τ) 3	4.518 ± 0.207 ns
Amplitude 1	22421.197 ± 629.399 kCnts
Amplitude 2	249.013 ± 43.724 kCnts
Amplitude 3	46.586 ± 2.409 kCnts

Fig. S11. Phasor Plot Analysis and Lifetime data for **8** (10 μM) in water/PS20.



Parameter	Value
Lifetime (τ) 1	0.365 ± 0.010 ns
Lifetime (τ) 2	0.781 ± 0.013 ns
Lifetime (τ) 3	4.762 ± 0.200 ns
Amplitude 1	4150.807 ± 138.348 kCnts
Amplitude 2	2915.285 ± 154.052 kCnts
Amplitude 3	49.638 ± 2.771 kCnts

Cell Culture

MDA-MB 231 human breast cancer cells were seeded on to an eight well chamber slide (Ibidi) at a density of 1×10^4 cells per well 24 h before imaging. Cells were cultured in Dulbecco's Modified Eagles Media supplemented (DMEM) with 10% fetal bovine serum (FBS), 1% L-Glutamine, and Penicillin-Streptomycin (1000 U/mL), and incubated at 37 °C and 5% CO_2. The slide was place on the microscope stage surrounded by an incubator to maintain the temperature at 37 °C and CO_2 at 5%. Bright field imaging was used to choose a field of view and focus on a group of cells.

Microscopy

CLSM, bright field, FLIM and STED 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. Excitation wavelength was using White Light Laser (WLL) tuned at 594 nm. Detection was using HyD S2 detector with collection range 608nm-698nm, analogue mode, gain value 10. Images were acquired using a Leica HC PL APO CS2 100X/1.40 oil immersion objective. The FLIM function was used to acquire the real time fluorescence life time imaging in the cells. The three components were chosen to give the best fitting curve. The separate lifetime shows the different region with various lifetime in cells. Phasor plots analysis were processed by LAS X Falcon software, the raw data were collected from FLIM acquisition. STED images were acquired with the depletion laser at 775 nm with intensity of 20%. Image processing was completed by using software ImageJ 1.53f51 (National Institutes of Health, USA).

Bioorthogonal reactions in MDA-MB-231 cells

The MDA-MB-231 cells were seeded on an eight well chamber slide (Ibidi) incubated for 24 hours in 300 μL DMEM medium. The cell media was replaced by 285 μL fresh prewarmed DMEM medium. The 15 μL of compound **7** (stock solution of 100 μM, 0.3 % PS20) in PBS solution was added to one well to make the **7** with the final concentration of 5 μM, the cells are allowed to incubate for 1 hour. The medium was poured off, and the cells were washed two times with 200 μL of prewarmed PBS solution. The cells then were treated with a solution of diyne **1**, diluted from a 300 μM stock solution in PS20/H_2O solution, in DMEM for 60 minutes at 10 μM. The images were taken under live condition at 37 °C.

Fig. S12. CLSM imaging of **7**. (A) Brightfield image (B) CLSM image (C) Overlay of brightfield and CLSM images; scale bars 5 μm.

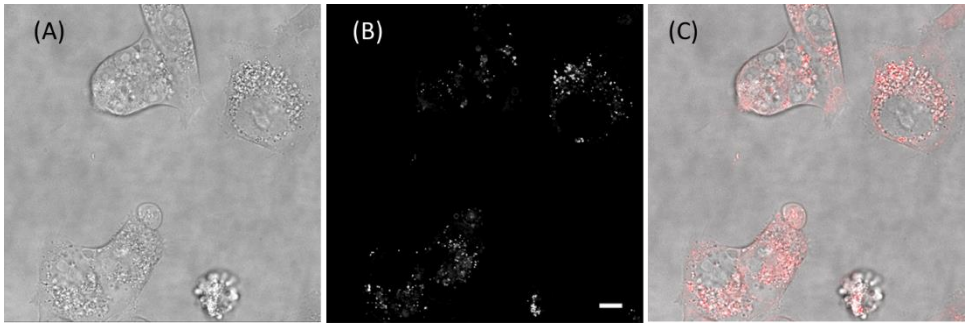


Fig. S13. Replicate experiments for CLSM imaging of **8**. (A) Brightfield image (B) CLSM image (C) Overlay of brightfield and CLSM images, scale bars 5 μm.

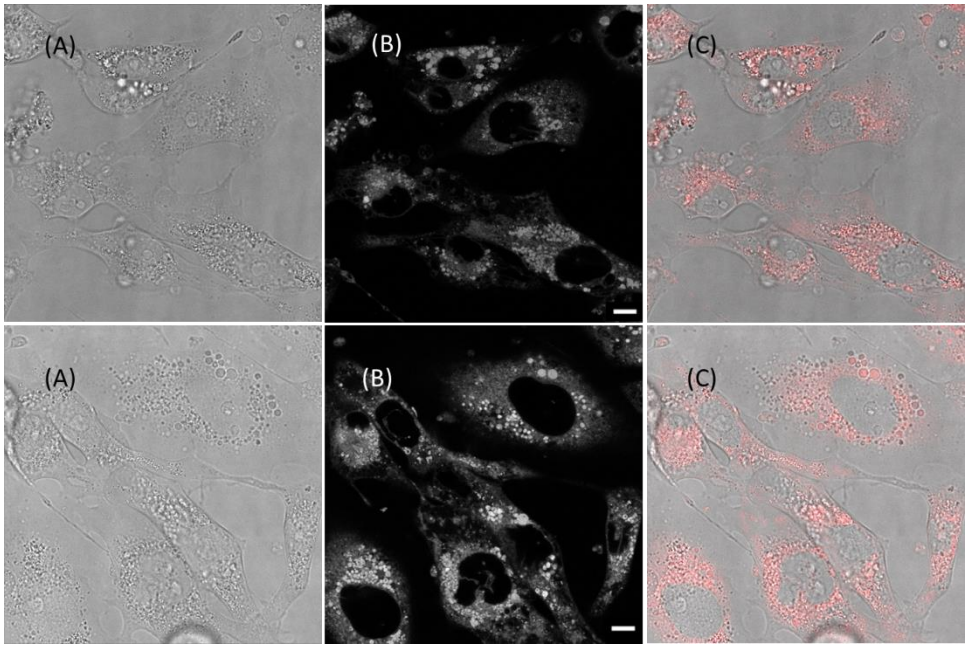


Fig. S14. Replicated experiments of STED imaging of MDA-MB 231 cells following incubation with **8** (5 μM) for 1 h, scale bars 5 μm. (A) CLSM image. (B) STED image of the same FOV. (C) Expansion of box inset from panel (B).

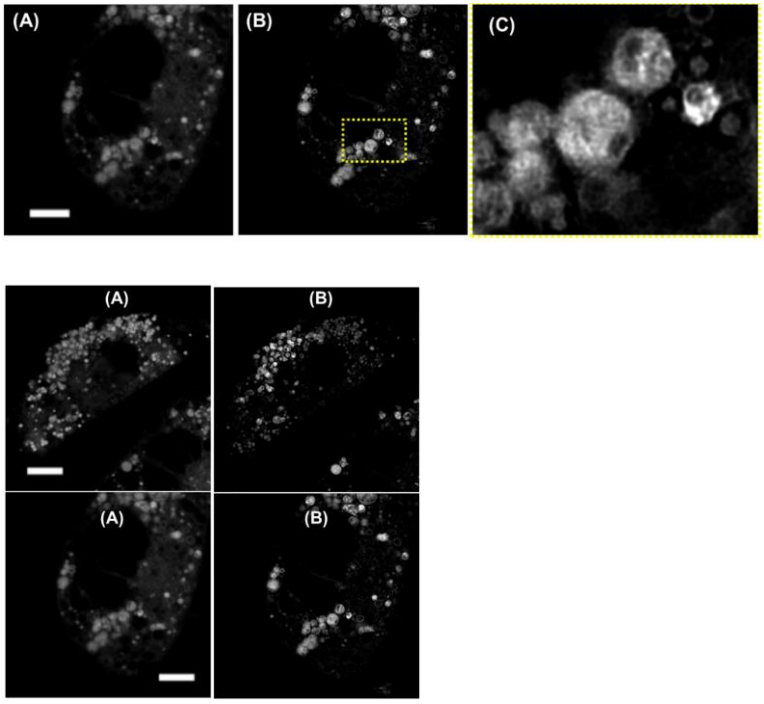


Fig. S15. FLIM images of MDA-MB 231 cells following 1 h incubation with **7** shown in Fig. 4 panels A and B, scale bars 5 μm. (A) Panels subdivided by the three intracellular regions with differing lifetimes. (B) FLIM data and fitted decay curve. (C) Phasor mapped FLIM image and phasor plot.

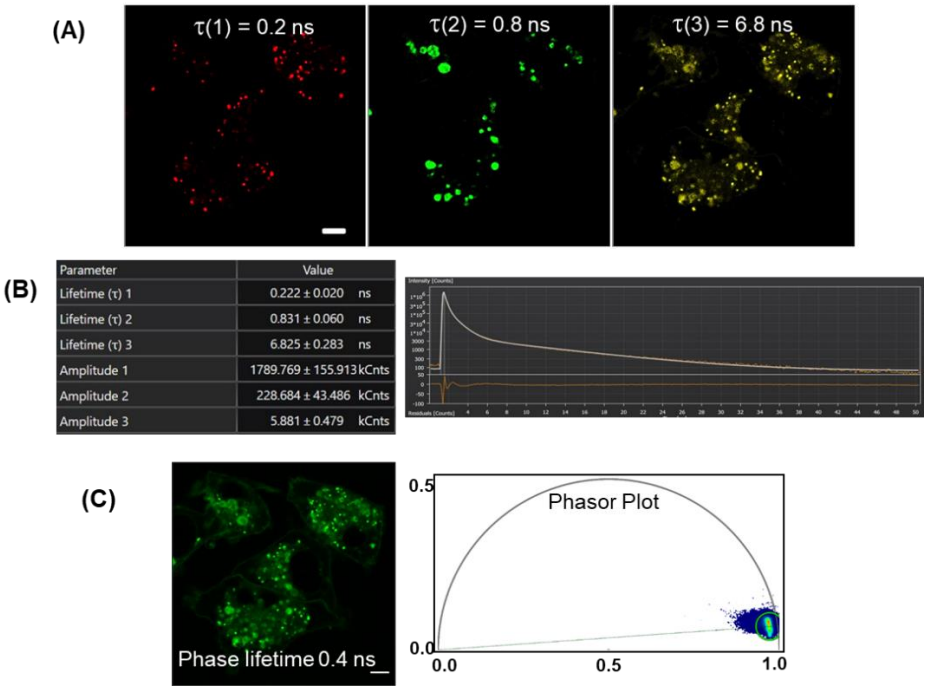


Fig. S16. FLIM images of MDA-MB 231 cells following the macrocyclization reaction of **7** (5 μM) with **1** (10 μM) to produce **8**, shown in Fig. 4, panels C and D, scale bars 5 μm. (A) Panels subdivided by the three intracellular regions with differing lifetimes. (B) FLIM data and fitted decay curve. (C) Phasor mapped FLIM image and phasor plot.

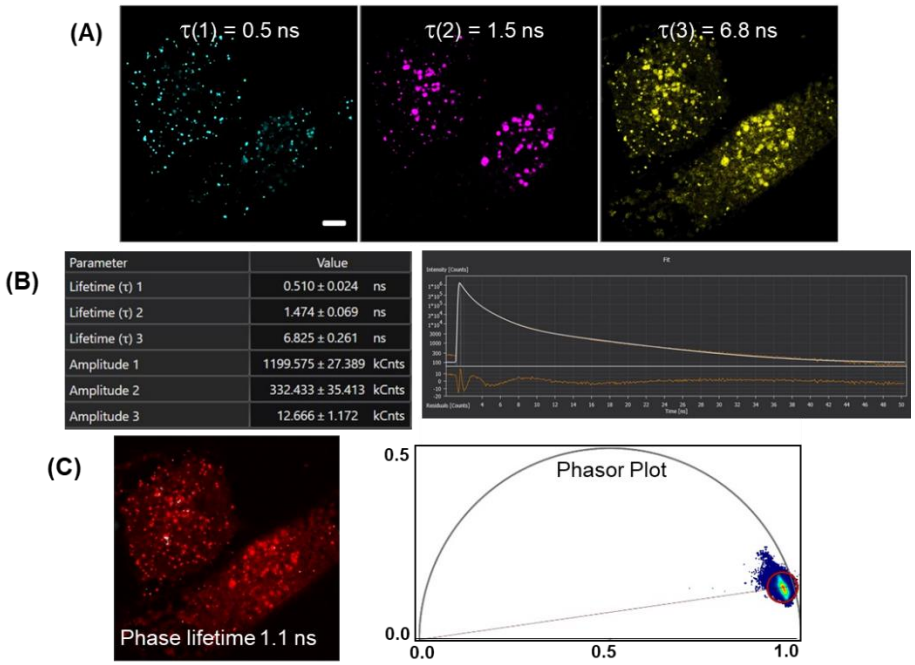


Fig. S17. FLIM images of MDA-MB 231 cells following 1 h incubation with **8**, scale bars 5 μm. (A) Panels subdivided by the three intracellular regions with differing lifetimes. (B) FLIM data and fitted decay curve. (C) Phasor mapped FLIM image and phasor plot.

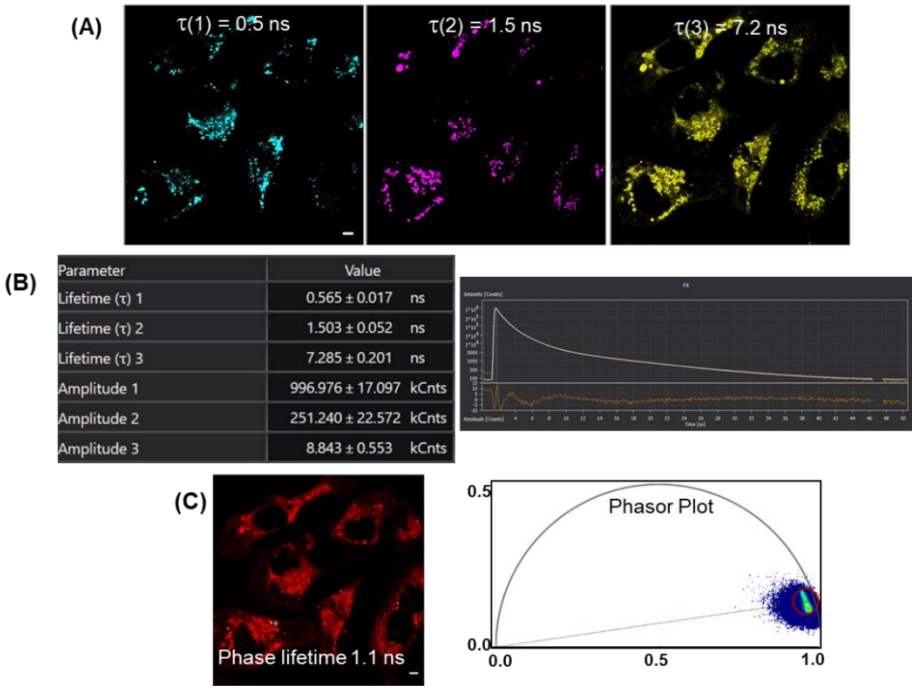
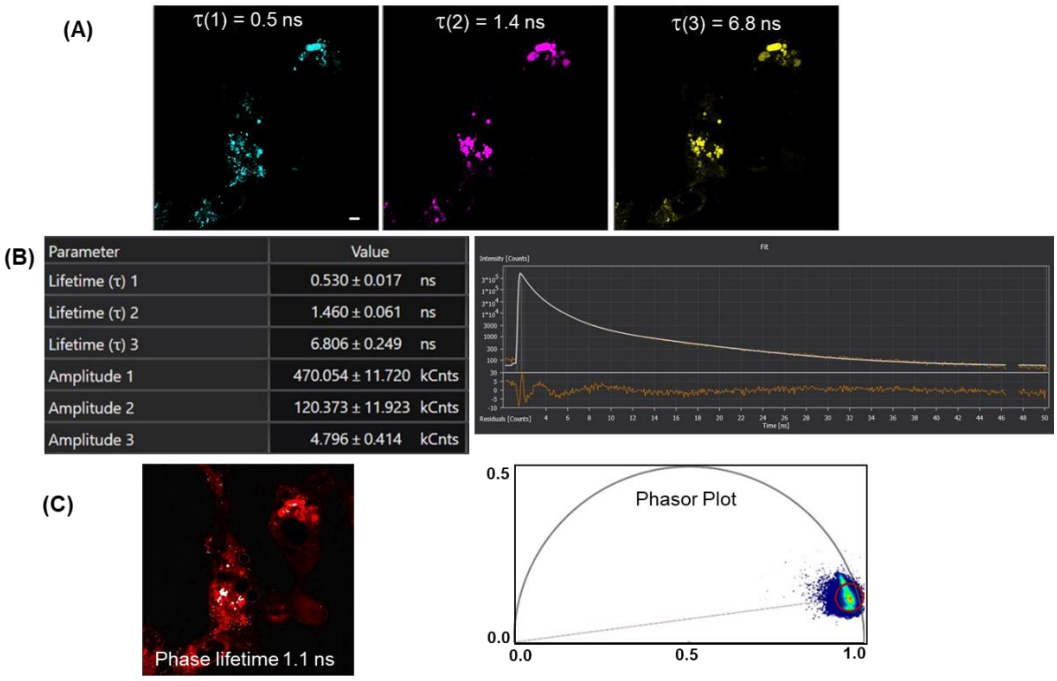


Fig. S18. Control experiment showing FLIM images of MDA-MB 231 cells following the incubation with **8** (5 μM) for 1 h followed by the incubation with **1** (10 μM) for 1 h showing no change in lifetime, scale bars 5 μm. (A) Panels subdivided by the three intracellular regions with differing lifetimes. (B) FLIM data and fitted decay curve. (C) Phasor mapped FLIM image and phasor plot.



References

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