Ca-NIR: A Ratiometric Near-Infrared Calcium Probe Based on Dihydroxanthene-hemicyanine Fluorophore

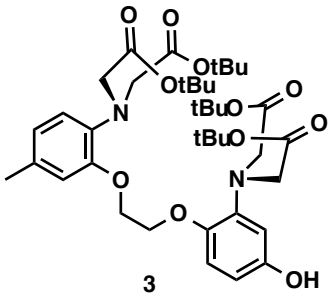
Supporting information

1. Synthesis of Ca-NIR

NMR spectra were recorded on a Bruker Avance III 400 MHz spectrometer and a Bruker Avance III - 500 MHz. Mass spectra were obtained by Electrospray ionization (ESI) using an Agilent Q-TOF 6520 mass spectrometer.

1 was synthesized according to a published protocol.[1]

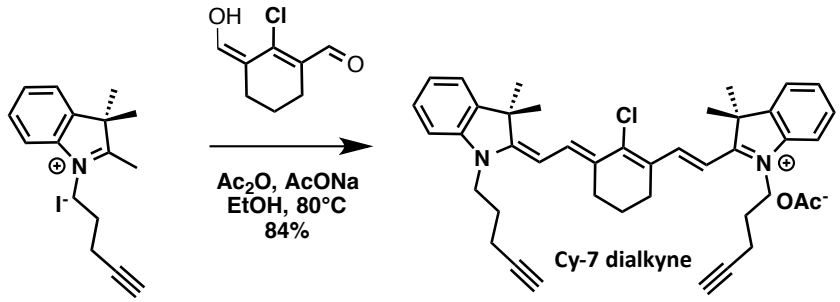
Synthesis of 3:



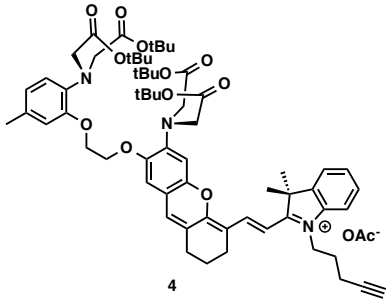
3

To a solution of **1** (1.590 g, 4.367 mmol) in acetonitrile (15 mL) was added sodium iodide (650 mg, 4.368 mmol, 1 eq.), tert-butyl bromoacetate (3.87 mL, 26.20 mmol, 6 eq.) and DIEA (6.1 mL, 35.00 mmol, 8 eq.). The solution was heated at 100°C for 12 hrs. The solution was then cooled to room temperature and aqueous HCl (1M) was added. The product was extracted with DCM and the organic phase was neutralised with a saturated solution of $NaHCO_3$ before being dried over anhydrous $MgSO_4$. The solution was filtered and evaporated. The crude was purified by column chromatography on silica gel (Heptane/EtOAc : 9/1 to 7/3) to obtain 2.94 g of **2** (Yield: 82%) as a yellowish syrup. Rf=0.48 (Heptane /EtOAc, 7/3). The product quickly turned black due to its oxidation therefore it was directly involved in the next step: To a solution of **2** (2,94 g, 3.580 mmol) in EtOAc (40 mL) and MeOH (40 mL) was added Pd/C 10% (300 mg) the solution was placed under an atmosphere of hydrogen and was allowed to stir at room temperature overnight. The solution was filtered off celite and evaporated to obtain 2,53 g of **3** (Yield: 97%) as a yellowish syrup. Rf=0.37 (Heptane /EtOAc, 6/4). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 6.80-6.69 (m, 4H, H Ar), 6.40 (d, *J* = 2.6 Hz, 1H, H Ar), 6.32 (dd, *J* = 8.6, 2.7 Hz, 1H, H Ar), 5.38 (s, 1H, OH), 4.29 (s, 4H, CH_2-O), 4.06 (s, 8H, N-CH_2), 2.26 (s, 3H, Me), 1.43 (2s, 36H, 4 *t*Bu). ^{13}C-NMR (101 MHz; $CDCl_3$): δ 170.6 (CO), 170.4 (CO), 151.0 (C Ar), 150.5 (C Ar), 144.2 (C Ar), 141.1 (C Ar), 137.0 (C Ar), 131.9 (C Ar), 121.9 (C Ar), 119.8 (C Ar), 117.8 (C Ar), 115.7 (C Ar), 107.9 (C Ar), 107.1 (C Ar), 81.2 (Cq *t*Bu), 81.0 (Cq *t*Bu), 68.8 (CH_2O), 67.5 (CH_2O), 54.59 (NCH_2), 54.4 (NCH_2), 28.1 (CH_3 *t*Bu), 22.7 (CH_3). HRMS (ESI^+), calcd for $C_{39}H_{58}N_2O_{11}Na$ $[M+Na]^+$ 753.3933, found 753.3951.

Synthesis of Cy-7 dialkyne



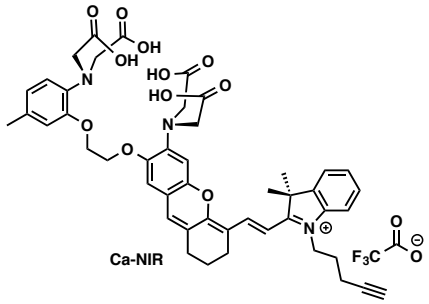
To a solution of pentynyl-indoleninium[2] (700 mg, 1.980 mmol) and 2-chloro-1-formyl-3-(hydroxymethylene)-1-cyclohexene (170 mg, 0.991 mmol, 0.5 eq) in ethanol (3 mL) was added acetic anhydride (250 μL) followed by sodium acetate (81 mg, 0.0991 mmol, 0.5 eq). The solution was allowed to stir at 80°C for 3 h before being evaporated. The crude was purified by column chromatography on silica gel (DCM/MeOH : 95/5 to 9/1) to obtain the 540 mg of Cy-7 dialkyne alkyne (84%) as a golden dark green solid. Rf=0.50 (DCM/MeOH : 95/5). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 8.37 (d, *J* = 14.1 Hz, 2H), 7.45-7.25 (m, 8H+ solvent pick), 6.37 (d, *J* = 14.1 Hz, 2H), 4.39 (t, *J* = 7.2 Hz, 4H, 2 N-CH_2), 2.78 (t, *J* = 5.9 Hz, 4H, $2CH_2$), 2.47 (td, *J* = 6.4, 2.3 Hz, 4H, $2CH_2$), 2.19 (s, 3H, OAc^-), 2.16 (t, *J* = 2.5 Hz, 2H, CH alkyne), 2.10 (t, *J* = 6.8 Hz, 4H, 2 CH_2), 1.98 (t, *J* = 5.8 Hz, 2H, CH_2), 1.75 (s, 12H, 4 CH_3). ^{13}C NMR (75 MHz; $CDCl_3$): δ 172.5 (CqN^+), 150.7 (C Ar), 144.6 (C Ar), 142.1 (C Ar), 141.0 (C Ar), 129.0 (C Ar), 127.9 (C Ar), 125.4 (C Ar), 122.3 (C Ar), 111.1 (C Ar), 101.6 (C Ar), 83.0 (C≡CH), 70.3 (C≡CH), 49.4 (CH_2-N^+), 43.5 (Cq), 31.0, 28.3 (CH_3 indolenine), 26.9, 26.1, 20.7, 16.3. HRMS (ESI+), calcd for $C_{40}H_{44}ClN_2$ $[M]^+$ 587.3188, found 587.3179.



4

To a solution of **3** (110 mg, 0.150 mmol) and **Cy-7 dialkyne** (100 mg, 0.165 mmol, 1.1 eq) in DMF (3 mL) was added triethylamine (200 μL, 1.500 mmol, 10 eq). The solution was allowed to stir at 100°C for 20 min before being cooled down to 50°C. The solution was then allowed to stir overnight at 50°C. The solvents were evaporated and the product was extracted with DCM, washed with an aqueous solution of HCl (1M) and dried over anhydrous $MgSO_4$. The solution was filtered and evaporated. The crude was purified by reverse phase chromatography (C-18 column, Water, 0.1% TFA / ACN, 0.1% TFA, 80/20 to 0/100 within 30 min) to obtain 50 mg of **4** (Yield: 28%) as a dark blue syrup. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.54 (d, *J* = 14.3 Hz, 1H, CH alkene), 7.47-7.42 (m, 3H, H Ar), 7.36-7.31 (m, 2H, H Ar), 7.07 (s, 1H, H Ar), 6.85 (d, *J* = 8.4 Hz, 2H, H Ar), 6.76 (d, *J* = 6.7 Hz, 2H, H Ar), 6.70 (s, 1H, H Ar), 6.33 (d, *J* = 14.3 Hz, 1H, CH alkene), 4.44-4.41 (m, 4H, OCH_2), 4.34-4.31 (m, 2H, CH_2-N^+), 4.25 (s, 4H, NCH_2), 4.03 (s, 4H, NCH_2), 2.81-2.78 (m, 2H, CH_2), 2.72-2.69 (m, 2H, CH_2), 2.43-2.39 (m, 2H,

CH_2), 2.28 (s, 3H, CH_3 BAPTA), 2.22 (t, 2H, CH_2), 2.17 (t, *J* = 2.2 Hz, 1H, C≡CH), 2.12-2.08 (m, 2H, CH_2), 1.98-1.92 (m, 2H, CH_2), 1.80-1.74 (m, 6H, 2 CH_3 indolenine), 1.51 (s, 18H, 2 *t*Bu), 1.43 (s, 18H, 2 *t*Bu).^{13}C NMR (126 MHz; $CDCl_3$): δ 174.5 (C=N^+), 170.3 (CO ester), 169.4 (CO ester), 163.1 (C Ar), 150.3 (C Ar), 150.2 (C Ar), 148.4 (C Ar), 145.5 (C Ar), 143.4 (C Ar), 141.8 (C Ar), 140.8 (C Ar), 137.2 (C Ar), 136.7 (C Ar), 132.5 (C Ar), 129.2 (C Ar), 126.9 (C Ar), 126.1 (C Ar), 122.7 (C Ar), 122.2 (C Ar), 120.2 (C Ar), 116.6 (C Ar), 116.1 (C Ar), 115.5 (C Ar), 114.8 (C Ar), 111.4 (C Ar), 110.6 (C Ar), 103.2 (C Ar), 101.0 (C Ar), 82.6 (C≡CH), 82.2 (Cq *t*Bu), 81.2 (Cq *t*Bu), 70.4 (C≡CH), 68.6 (CH_2O), 67.4 (CH_2O), 55.4 (CH_2N), 54.6 (CH_2N), 49.66 (Cq indolenine), 43.3(CH_2-N^+), 28.5 (CH_3 indolenine), 28.14 (6 CH_3 *t*Bu), 28.12 (6 CH_3 *t*Bu), 25.9(CH_2), 20.8 (CH_3 BAPTA), 20.3 (CH_2), 16.0 (CH_2). HRMS (ESI^+), calcd for $C_{63}H_{82}N_3O_{11}$ $[M]^+$ 1056.5944, found 1056.5937.



To a solution of **4** (40 mg, 0.037 mmol) in DCM (4 mL) was added 4 mL of TFA. The solution was allowed to stir at 60°C for 1h30. The solvents were evaporated and the crude was purified reverse phase chromatography (C-18 column, Water, 0.1% TFA / ACN, 0.1% TFA, 80/20 to 0/100 within 30 min) to obtain 22 mg of **4** (Yield: 71%) as a dark blue fluffy solid after lyophilisation. ^{1}H-NMR (500 MHz, MeOD): δ 8.69 (d, *J* = 14.5 Hz, 1H, H alkene), 7.58 (d, *J* = 7.0 Hz, 2H, H Ar), 7.50-7.44 (m, 2H, H Ar), 7.38-7.35 (m, 1H, H Ar), 7.18 (s, 1H, H Ar), 6.86-6.80 (m, 3H, H Ar), 6.71 (dd, *J* = 8.1, 0.8 Hz, 1H, H Ar), 6.41 (d, *J* = 14.4 Hz, 1H, H alkene), 4.38-4.35 (m, 10H, 2 CH_2-N,2 OCH_2, N^+CH_2), 4.07 (s, 4H, 2 CH_2-N), 2.83-2.82 (m, 2H, CH_2), 2.74 (d, *J* = 5.6 Hz, 2H, CH_2), 2.53 (t, *J* = 2.5 Hz, 1H, C≡CH), 2.41 (dd, *J* = 6.5, 2.4 Hz, 2H, CH_2), 2.28 (s, 3H, CH_3 BAPTA), 2.07 (d, *J* = 6.7 Hz, 2H, CH_2), 1.96-1.96 (m, 2H, CH_2), 1.82 (s, 6H, 2 CH_3 indolenine). ^{13}C NMR (126 MHz; MeOD): δ 174.9 (CO), 174.5 (CO), 173.2 (CO), 163.0 (CO), 150.4 (Cq Ar), 150.2 (Cq Ar), 148.1 (Cq Ar), 145.2 (Cq Ar), 143.3 (CH alkene), 141.9 (Cq Ar), 141.3 (Cq Ar), 136.7 (Cq Ar), 136.4 (CH Ar), 132.5 (Cq Ar), 128.6 (CH Ar), 126.2 (Cq Ar), 125.6 (CH Ar), 122.2 (Cq Ar), 121.4 (CH Ar), 119.1 (CH Ar), 115.7 (Cq Ar), 114.7 (Cq Ar), 114.3 (CH Ar), 111.1 (CH Ar), 109.5 (CH Ar), 102.0 (CH Ar), 100.3 (CH alkene), 82.4 (C≡CH), 70.2 (C≡CH), 68.1 (CH_2O), 66.73 (CH_2O), 66.64, 55.0 (CH_2N), 54.5 (CH_2N), 49.7 (Cq indolenine), 48.4, 42.8 (CH_2-N^+), 29.5 (CH_2), 28.4 (CH_2), 27.3 (CH_3 indolenine), 25.8 (CH_2), 23.9 (CH_2), 20.3 (CH_2), 19.6 (CH_3 BAPTA), 15.1 (CH_2). HRMS (ESI^+), calcd for $C_{47}H_{50}N_3O_{11}$ $[M]^+$ 832.3440, found 832.3426.

Picture of **Ca-NIR**

2. Spectroscopy

Absorption spectra were recorded on a Cary 4000 spectrophotometer (Varian) and fluorescence spectra on a Fluoromax 4 (Jobin Yvon, Horiba) spectrofluorometer. Fluorescence emission spectra and absorption spectra were systematically recorded at room temperature, unless indicated. Fluorescence spectra were obtained with an excitation wavelength of 600 nm.

Table S1. Spectroscopic properties of **Ca-NIR** in absence and in the presence of calcium. Quantum yields were obtained using Rhodamine-800 as a reference (25% in EtOH).[3]

	$\lambda_{Abs\ max}$ (nm), ε ($M^{-1}.cm^{-1}$)	$\lambda_{Em\ max}$ (nm)	QY (%)
Ca-NIR (EGTA 1mM)	709, 92500	733	1.7
Ca-NIR (Ca^{2+}, 1mM)	616, 52000 666, 56300	684	0.6

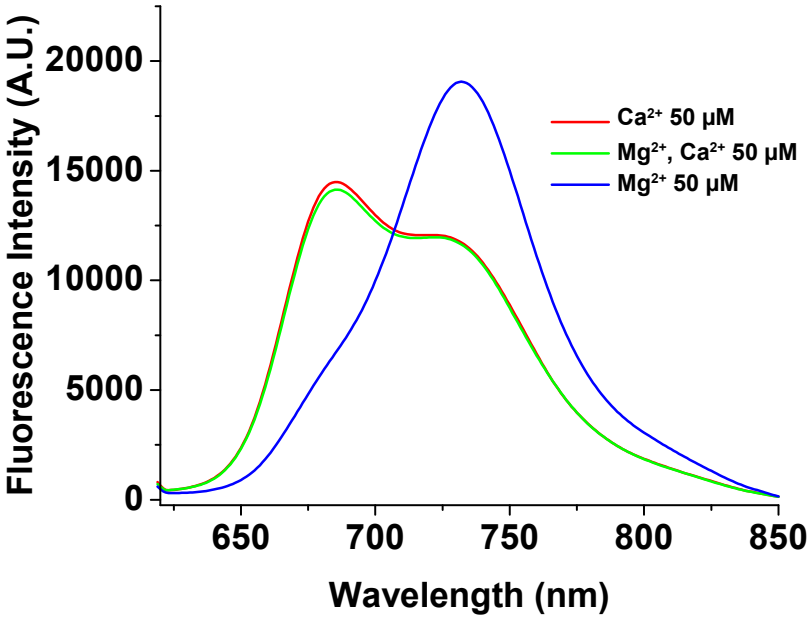


Figure S1. Emission spectra of **Ca-NIR** (1 µM) in the presence of Ca^{2+}, Mg^{2+} and an equimolar mixture of Ca^{2+} and Mg^{2+}. Experiments were performed in 30 mM MOPS buffer, 100 mM KCl, pH 7.2.

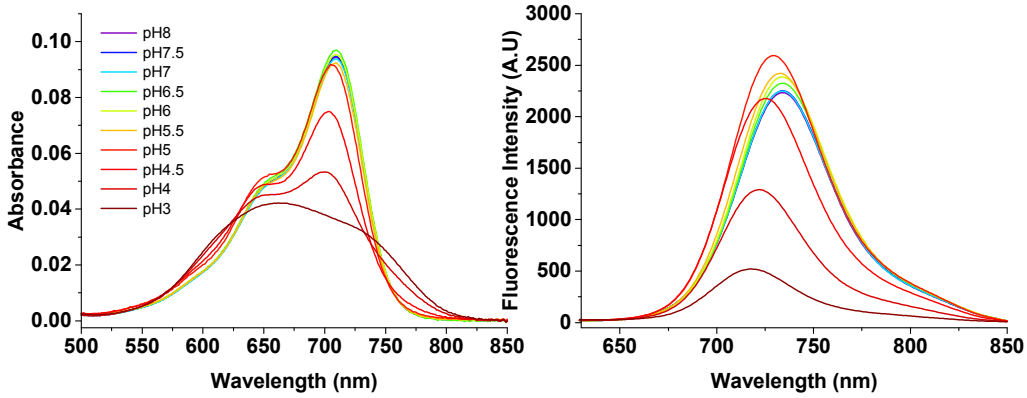


Figure S2. Absorption (left) and emission spectra (right) of **Ca-NIR** (1 μM) upon acidification from pH 8 to pH 3 in PBS citrate (10 mM) buffer.

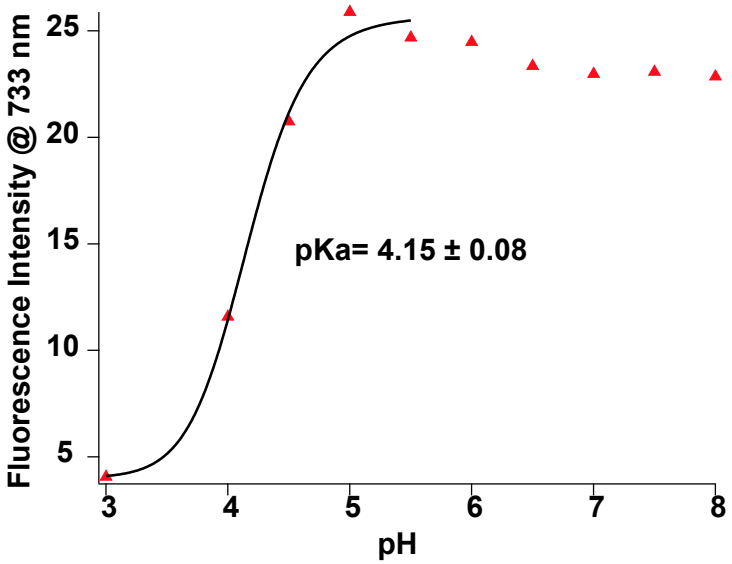


Figure S3. Plot of the fluorescence intensity of **Ca-NIR** (1 μM) at 733 nm against pH. The fit curve, according to Hill's equation, yielded the pKa.

3. Cellular studies

KB cells (ATCC® CCL-17) were grown in minimum essential medium (MEM, Gibco-Invitrogen) with 10% fetal bovine serum (FBS, Lonza), 1% non-essential amino acids (Gibco-Invitrogen), 1% MEM vitamin solution (Gibco-Invitrogen), 1% L-Glutamine (Sigma Aldrich) and 0.1% antibiotic solution (gentamicin, Sigma-Aldrich) at 37° C in humidified atmosphere containing 5% CO_2. Cells were seeded onto a chambered coverglass (IBiDi®) at a density of 1×10^5 cells/well 24h before the microscopy measurement. For imaging, the medium was removed and the attached cells were washed with HBSS (Gibco-Invitrogen) three times. Then, a freshly prepared solution of Ca-NIR (5 μM in MOPS 30 mM, KCl 100 mM, pH 7.2, digitonin: 100 $\mu g.mL^{-1}$ was used to help the penetration of calcium and EGTA in the cells) in the presence of calcium (1 mM) or in the presence of EGTA (1mM) was added to the cells without any washing step and the cells were images. Confocal microscopy experiments were performed by using a Leica TCS SPE-II with HXC PL APO 63x/1.40 OIL CS objective. The microscope settings were: excitation with a 634 nm laser, emission was collected in

two distinct channels. Channel A: 650-700 nm, channel B: 700-800 nm. The images were processed with icy[4] and ImageJ softwares. The ratio images were obtained with the help of the imageJ plugin: Ratio Intensity Color Version 2.5.

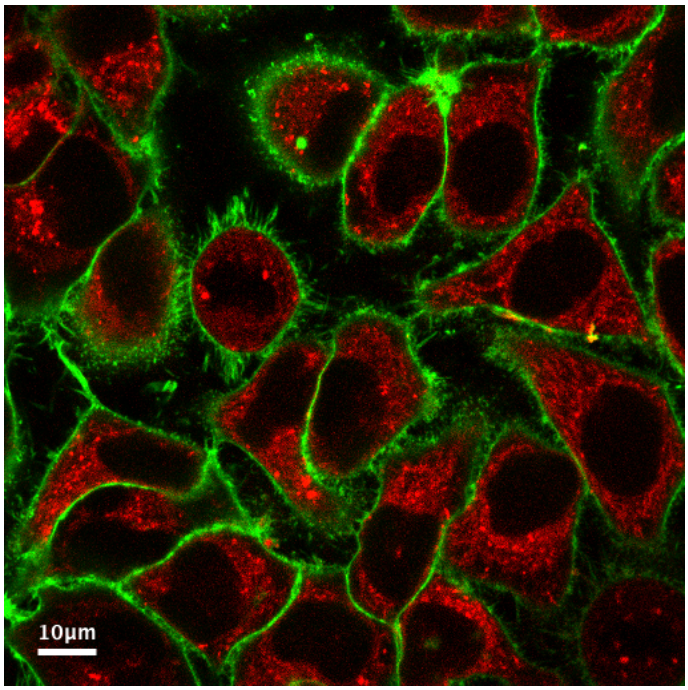


Figure S4. Laser scanning confocal microscopy of KB cells incubated for 1 h in the presence of **Ca-NIR** (1 µM in Opti-MEM). The excess of **Ca-NIR** was washed 3 times with HBSS and the cells were visualized in HBSS. Green color: the plasma membrane was stained with MemBright®-488 (excitation 488 nm, channel 498-550 nm) 5 min before imaging. Red color: **Ca-NIR** was excited at 634 nm and emission was collected from 650 to 800 nm.

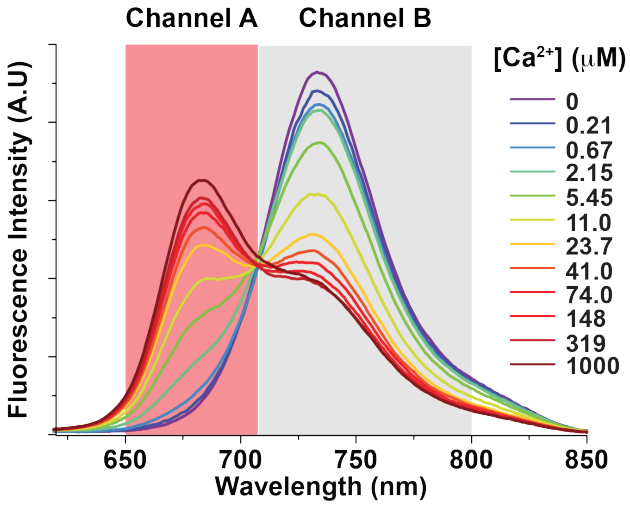


Figure S5. Emission spectra of **Ca-NIR** upon titration with calcium and spectral windows used for channels A and B for ratiometric imaging.

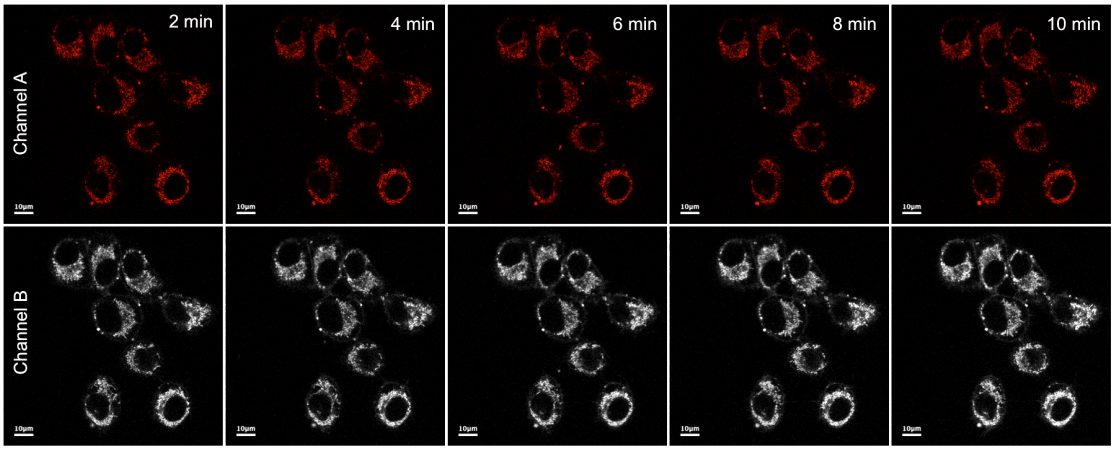


Figure S6. Laser scanning confocal microscopy of KB cells incubated for 1 h in the presence of **Ca-NIR** (1 μM in Opti-MEM). The excess of **Ca-NIR** was washed 3 times with HBSS and the cells were visualized in HBSS. Time laps over 10 min, Channels A and B were scanned with a frequency of 8 frames (2 scans per frame) per minute for 10 minutes (total scan: 160). Excitation wavelength was 635 nm (50% power for A: 27 μW, 70% for B: 63 μW).

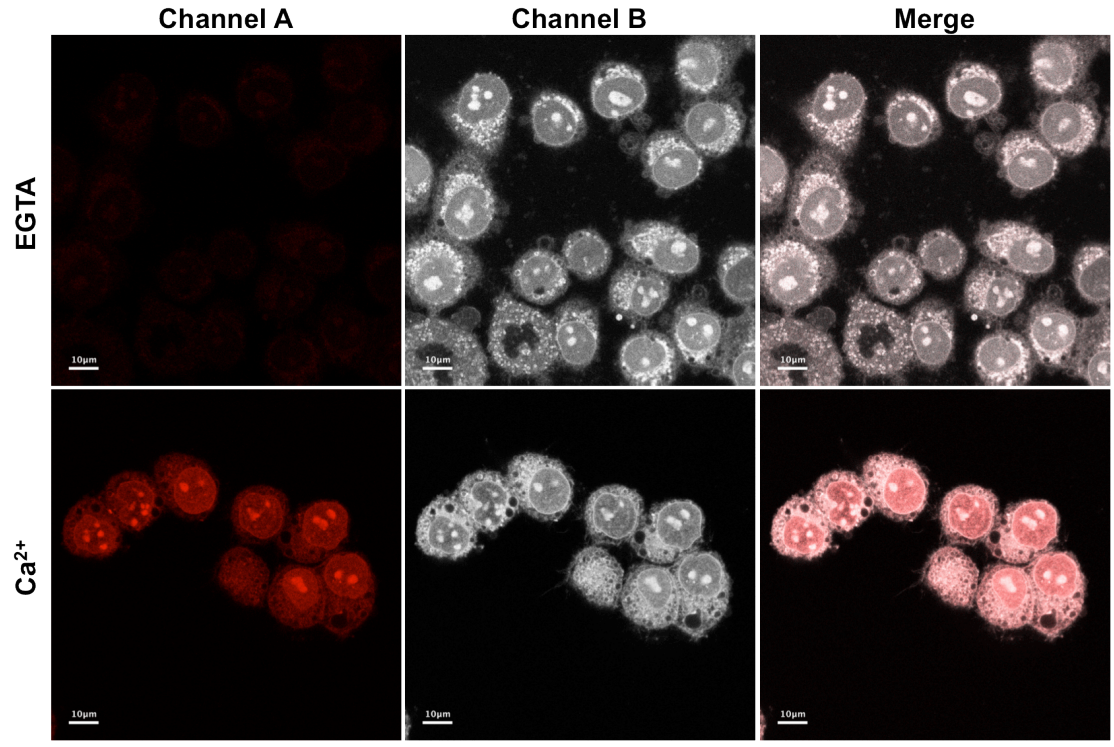


Figure S7. Laser scanning confocal microscopy of KB cells in the presence of **Ca-NIR** (5 μM in MOPS 30 mM, KCl 100 mM, pH 7.2, digitonin: 100 $\mu g.mL^{-1}$). Top line: cells were in the presence of EGTA (1 mM). Bottom line: cells were in the presence of Ca^{2+} (1 mM). The right images are the merge of channel A and channel B. Images were taken 10 minutes after addition of **Ca-NIR**.

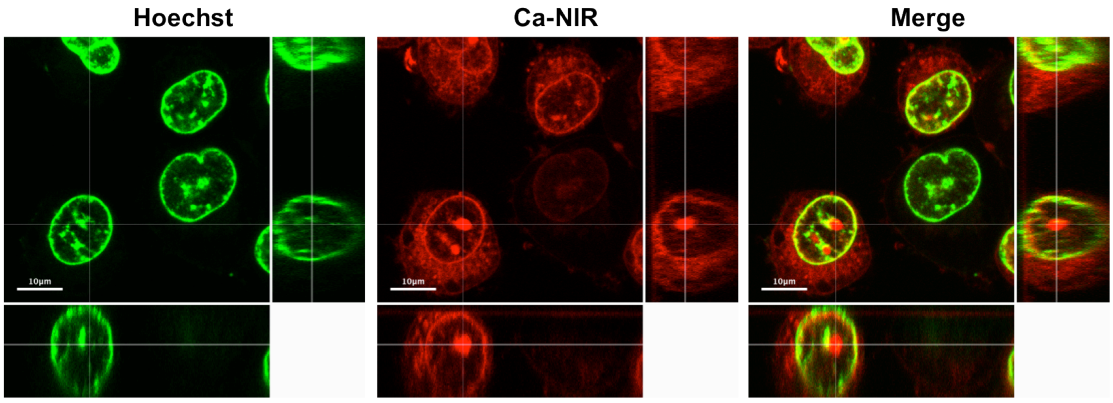


Figure S8. Laser scanning confocal microscopy images with orthogonal projection of KB cells in the presence of **Ca-NIR** (5 µM in HBSS, digitonin: 20 µg/mL). The Nuclei were stained with Hoechst (5 µg/mL), and the signal of **Ca-NIR** was collected in channel A and B. The merge shows the colocalization of **Ca-NIR** and Hoechst (yellow colour) in the nuclear envelop whereas in the nucleoli two distinct signals can be observed.

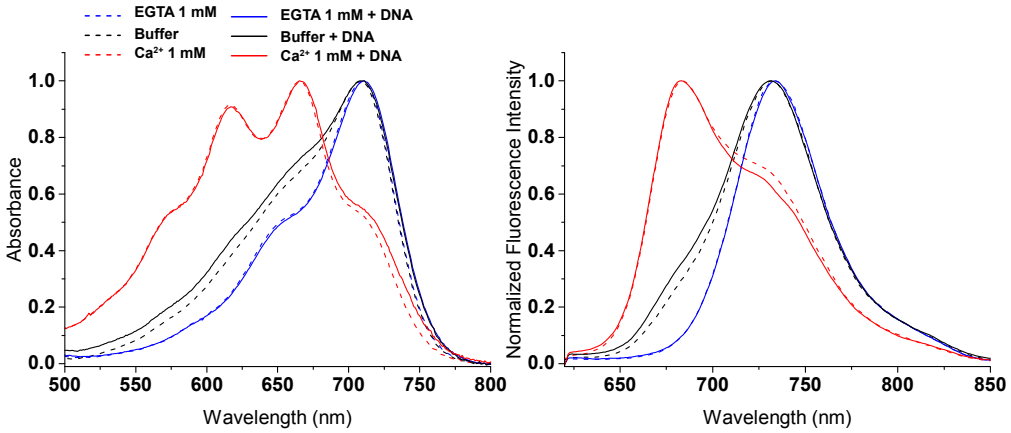


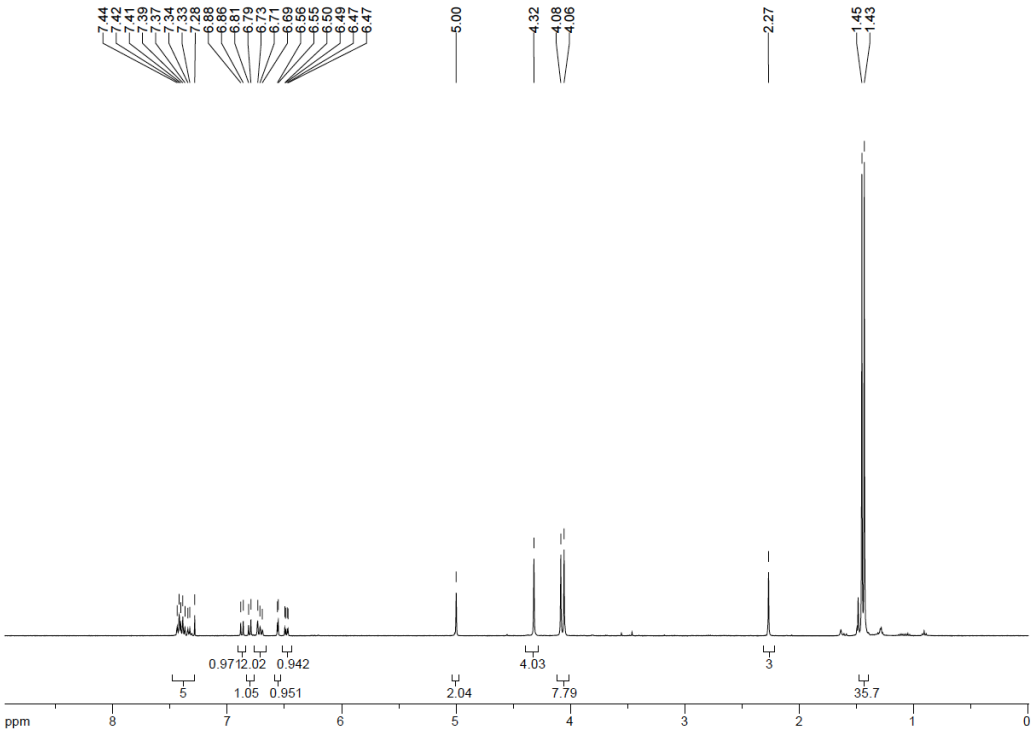
Figure S9. Normalized Absorbance (left) and emission (right) spectra of **Ca-NIR** (1 µM) in the presence or absence of calcium (MOPS 30 mM, KCl 100 mM, pH 7.2) without DNA (dashed lines) and in the presence of 150 µM nucleotide bases calf thymus DNA (solid lines). The concentration of nucleotide bases was determined by absorption spectroscopy at 260 nm with an average molar extinction coefficient of 6600 M^{-1} cm^{-1}.[5]

	EGTA (1 mM)[a]	Calcium (1 mM)[a]	Only buffer[a]
Quantum yield decrease	11%	7%	21%

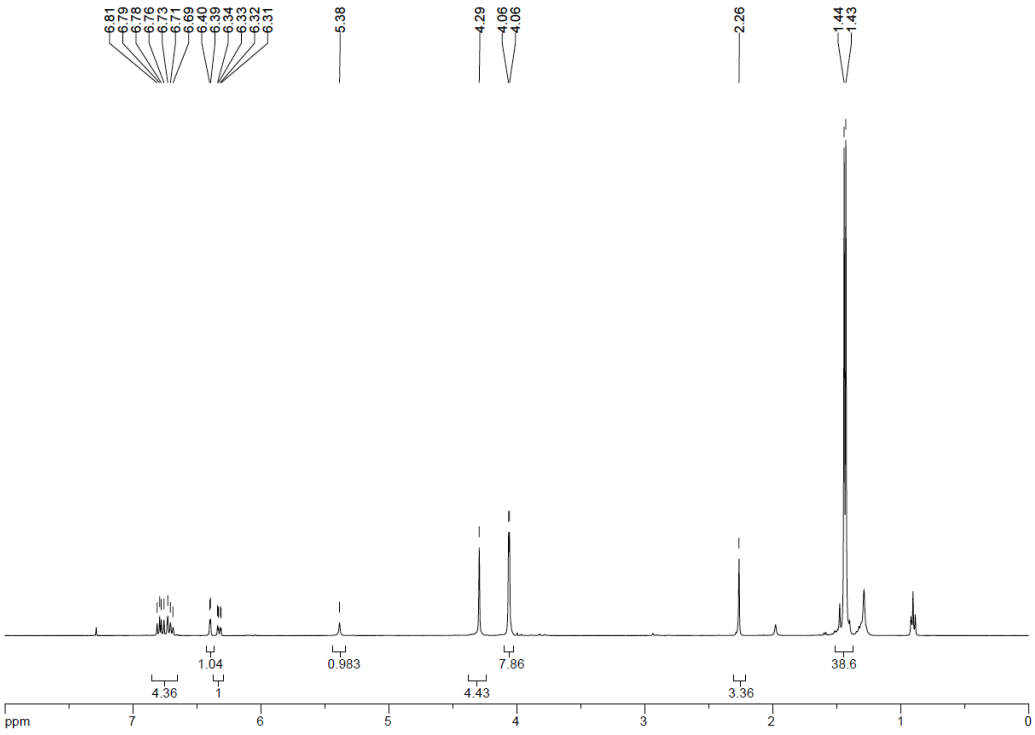
[a] MOPS 30 mM, KCl 100 mM, pH 7.2

Table S1. Decrease of the quantum yield of **Ca-NIR** (1 µM) in various conditions in the presence of excess calf-thymus DNA (150 µM).

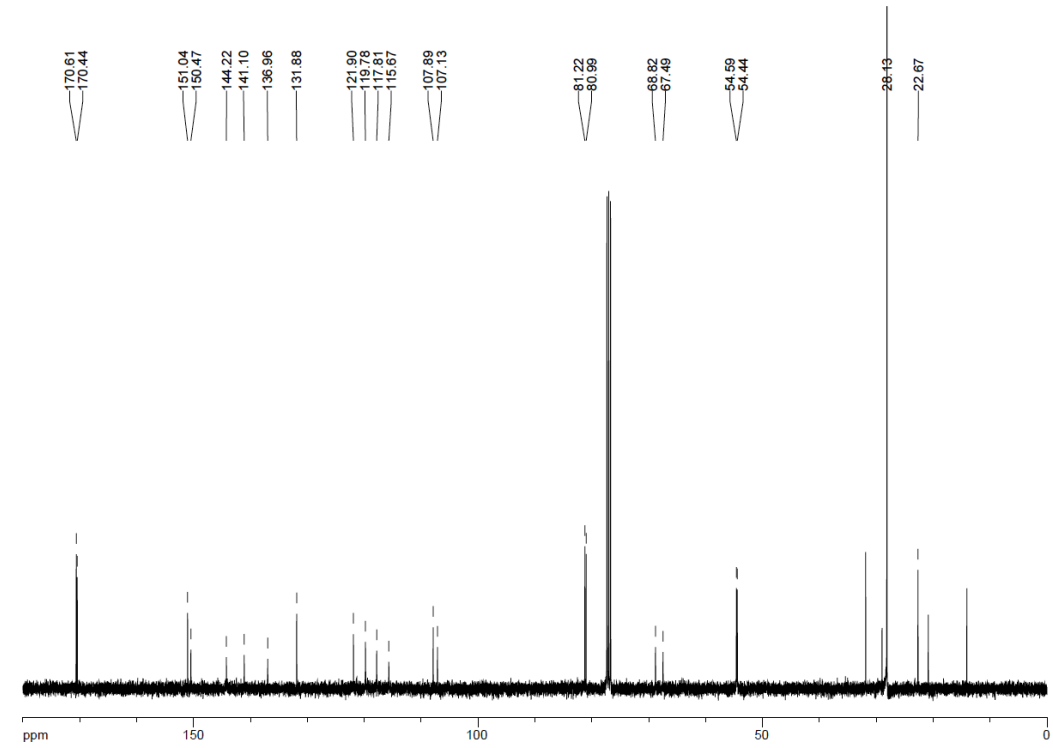
4. NMR and mass spectra



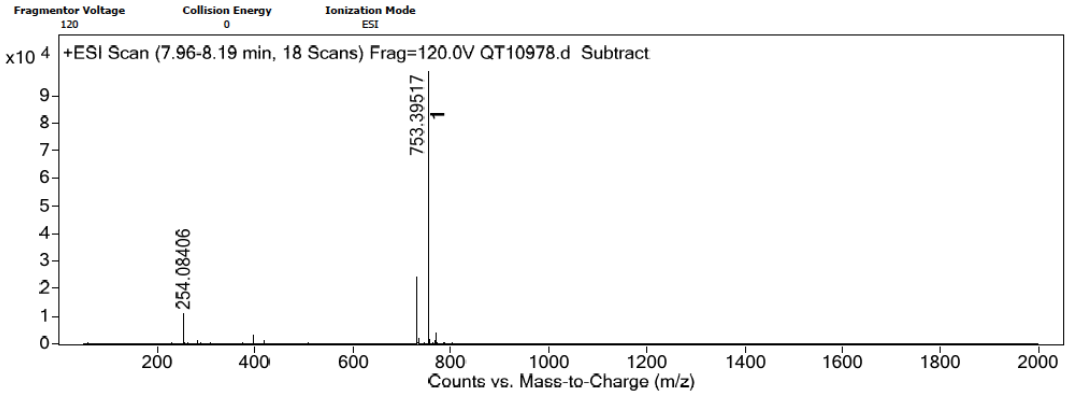
Control ^{1}H-NMR of **2** ($CDCl_3$)



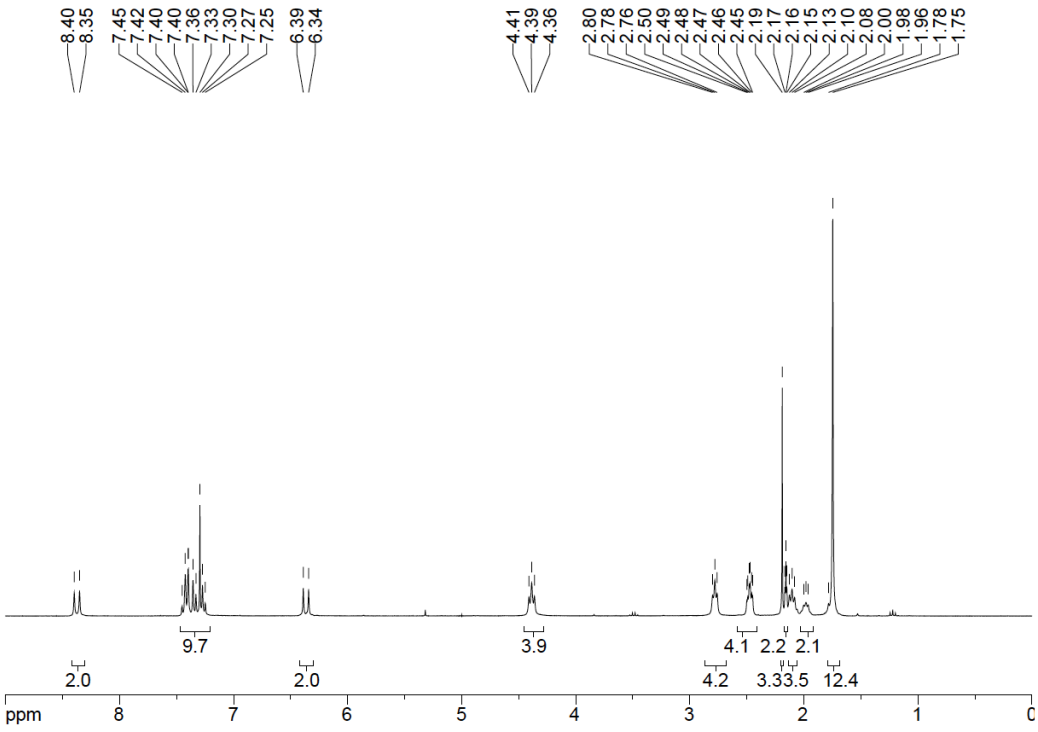
^{1}H-NMR spectrum of **3** ($CDCl_3$)



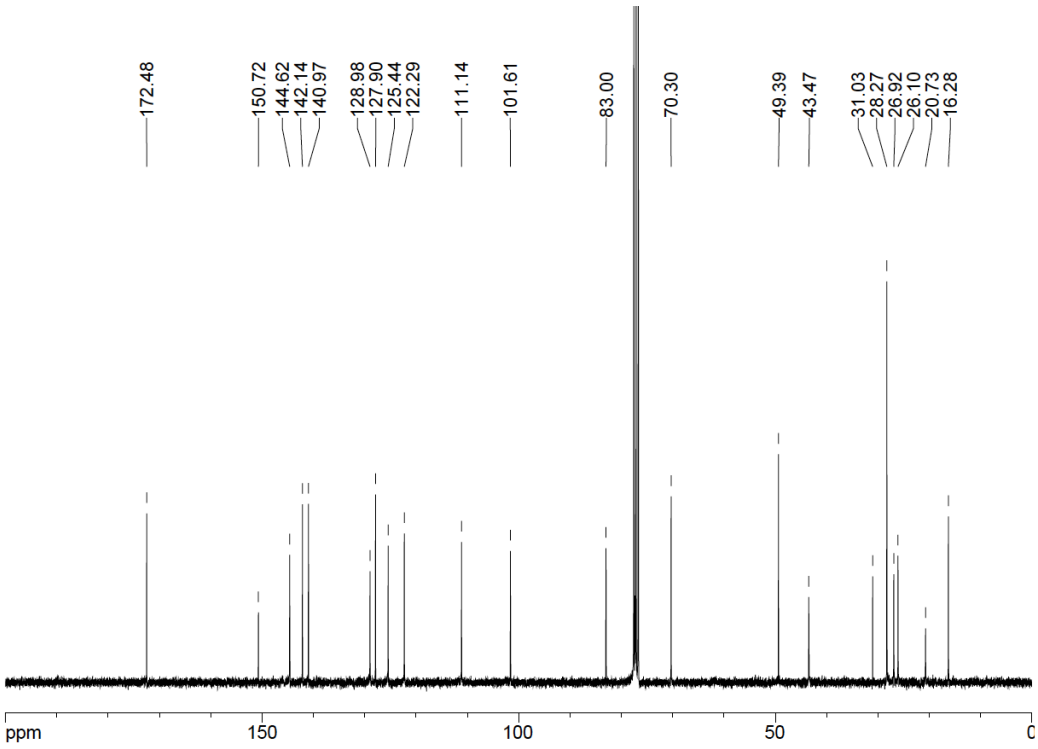
^{13}C-NMR spectrum of **3** ($CDCl_3$)



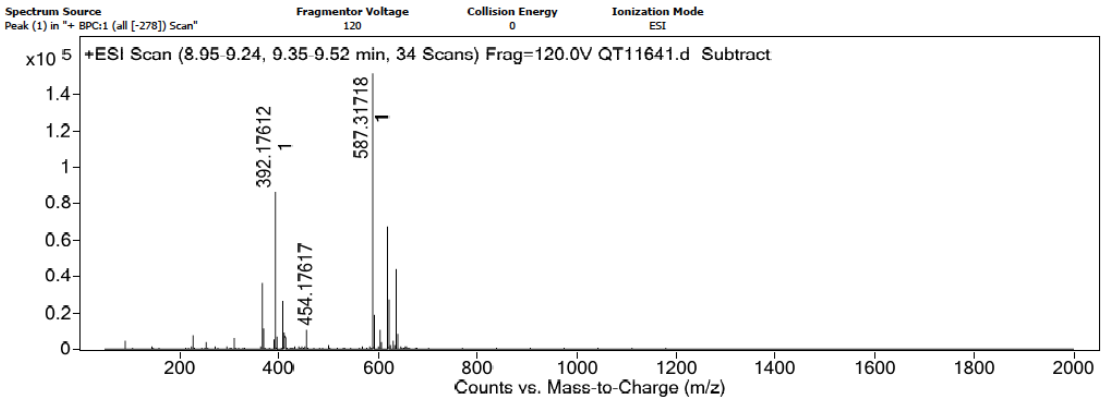
HRMS Spectrum of **3**



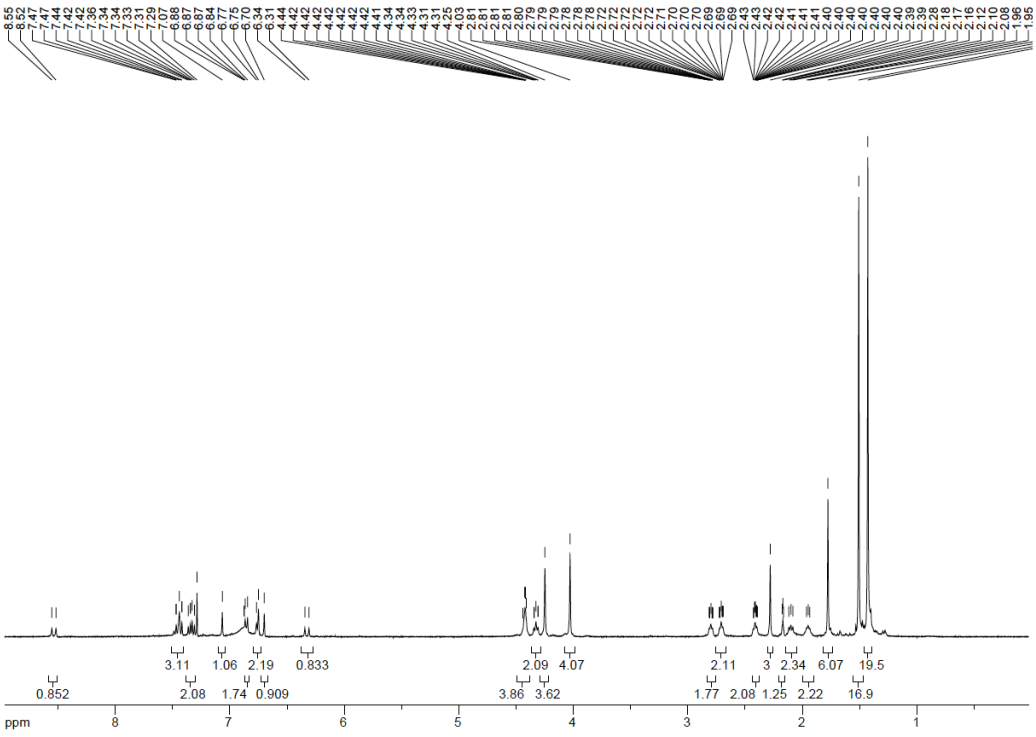
^{1}H-NMR spectrum of **Cy-dialkyne** ($CDCl_3$)



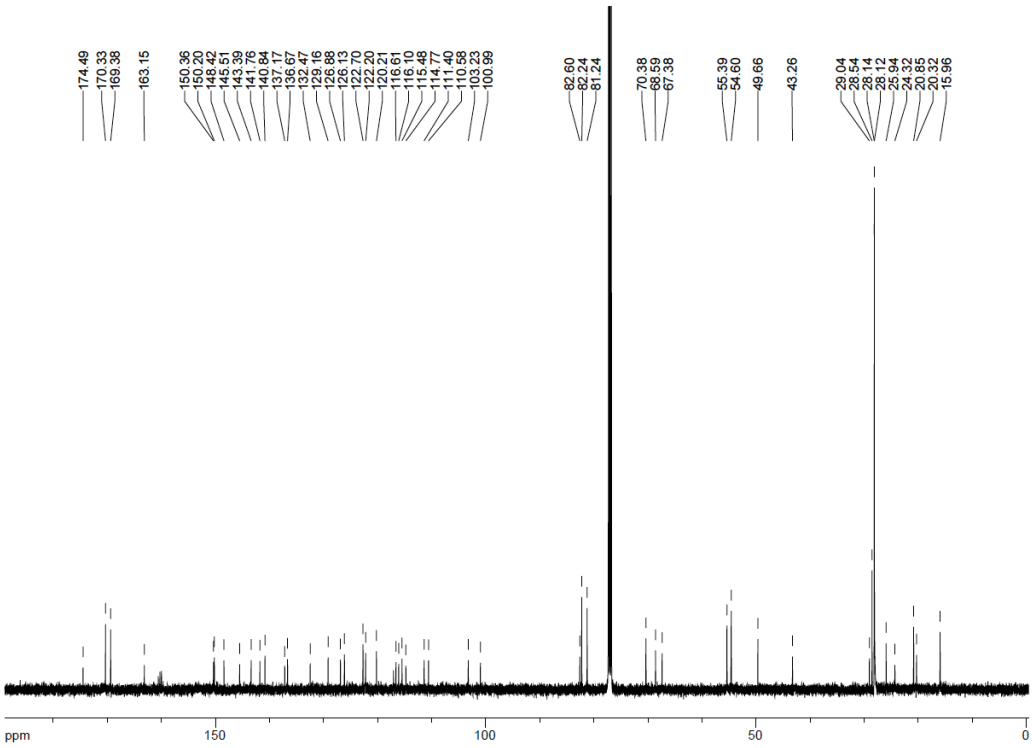
^{13}C-NMR spectrum of **Cy-dialkyne** ($CDCl_3$)



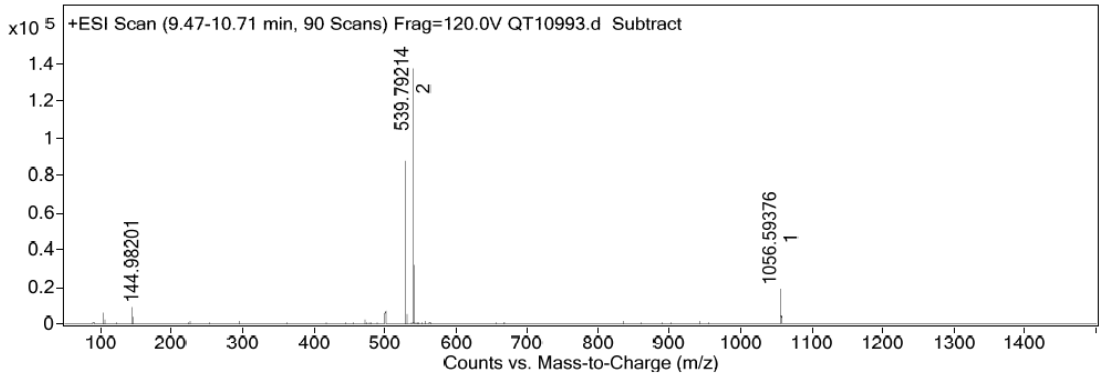
HRMS Spectrum of **Cy7-dialkyne**



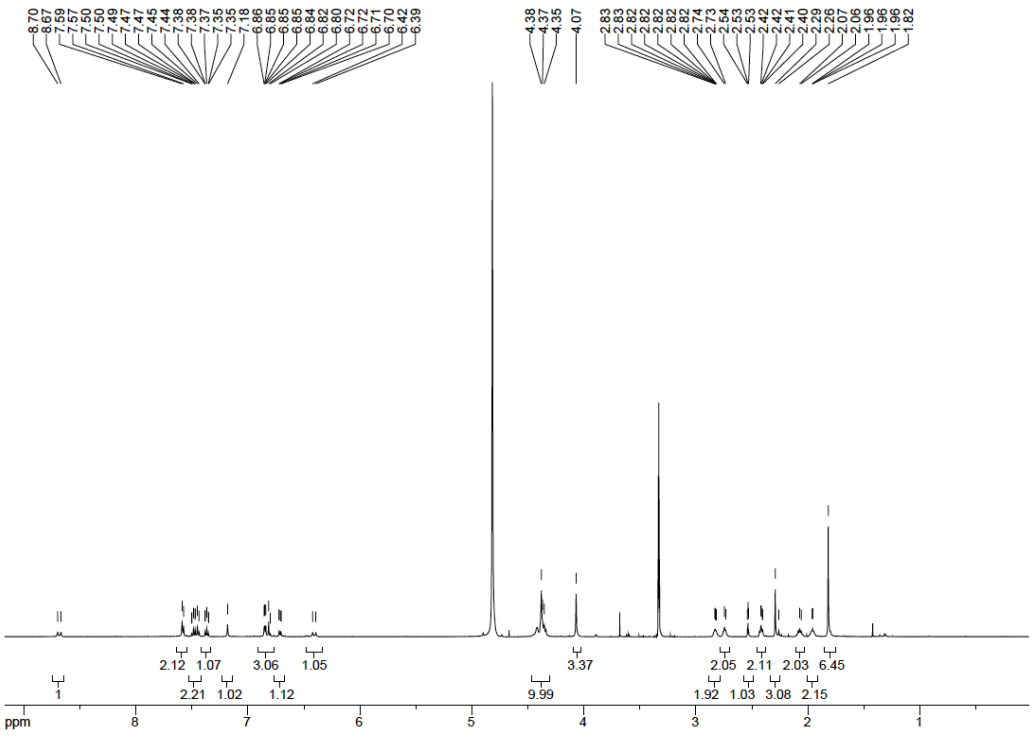
^{1}H-NMR spectrum of **4** ($CDCl_3$)



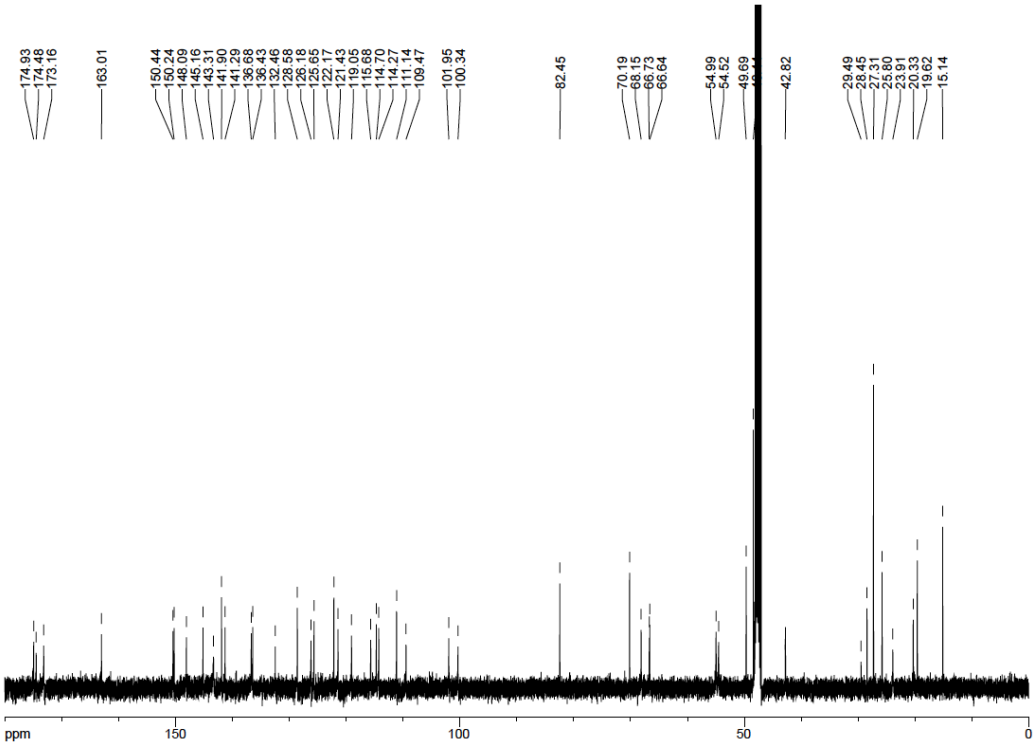
^{13}C-NMR spectrum of **4** ($CDCl_3$)



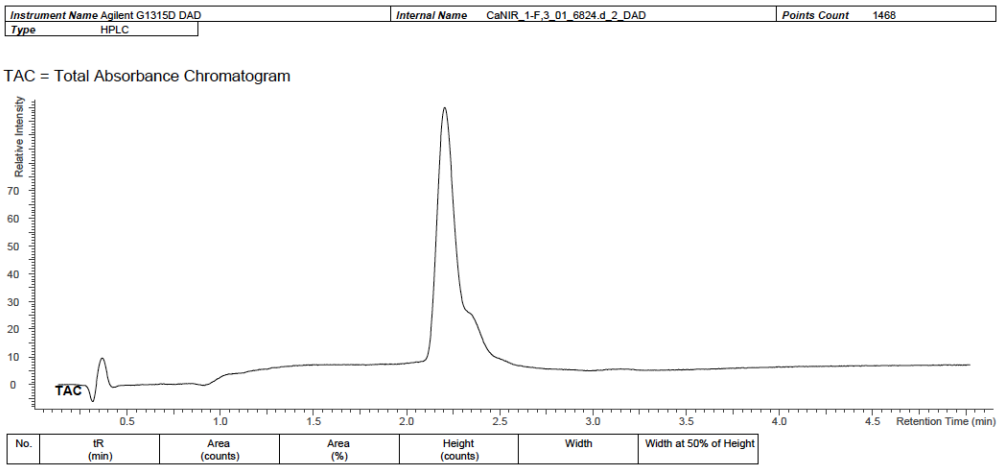
HRMS Spectrum of **4**



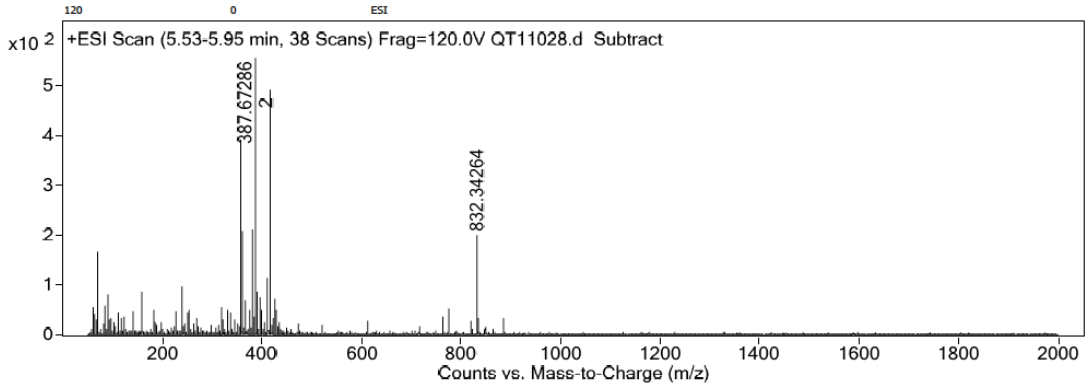
^{1}H-NMR spectrum of **Ca-NIR** (MeOD, 500 Mhz)



^{13}C-NMR spectrum of **Ca-NIR** (MeOD, 126 Mhz)



HPLC of **Ca-NIR**, C-18 column, eluted with ACN (0.05% formic acid) and water (0.05% formic acid), total absorbance detection.



HRMS Spectrum of **Ca-NIR**

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