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Mitochondria-Localized *In Situ* Generation of Rhodamine Photocage with Fluorescence Turn-On Enabling Cancer Cell-Specific Drug Delivery Triggered by Green Light

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Experimental Procedures

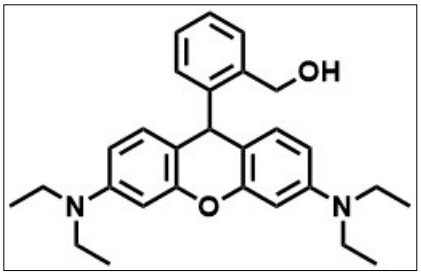
1. General Information

All reagents were purchased from Sigma Aldrich and were used without further purification. Dimethyl sulfoxide and dichloromethane were distilled from CaH_2 before use. ^{1}H NMR spectra were recorded on a BRUKER-AC 400-MHz spectrophotometer. Chemical shifts are reported in ppm from tetramethylsilane with the solvent resonance as the internal standard ($CDCl_3$: 7.26 ppm). Data are reported as follows: chemical shifts, multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet), coupling constant (Hz). ^{13}C NMR (100 MHz) spectra were recorded on a BRUKER– AC 400 MHz spectrometer with complete proton decoupling. Chemical shifts are reported in ppm from tetramethylsilane with the solvent resonance as the internal standard ($CDCl_3$: 77.0 ppm). UV/Vis absorption spectra were recorded on a Shimadzu UV–2450 UV/ Vis spectrophotometer; fluorescence emission spectra were recorded on a Hitachi F–7000 fluorescence spectrophotometer HRMS spectra were recorded on a JEOL–AccuTOF JMS–T100L mass spectrometer. Photolysis of the caged compounds was carried out using 125 W medium-pressure Hg lamp supplied by SAIC (India). Chromatographic purification was done with 60–120-mesh silica gel (Merck). For reaction monitoring, precoated silica gel 60 F254 TLC sheets (Merck) were used. RP–HPLC was recorded using acetonitrile and water in the mobile phase, at a flow rate of 1 mL/min.

2. Synthesis of drug delivery system based on rhodamine photocage (Rhod-cbl, 4)

Synthesis of (2-(3,6-bis(diethylamino)-9H-xanthen-9-yl)phenyl)methanol (2)

A solution of the rhodamine B (**1**) (1 g, 2.1 mmol) in THF (20 mL) was slowly added to a suspension of $NaBH_4$ (0.095 g, 2.5 mmol) at 0 °C over a period of 10 min. The mixture was stirred until the evolution of gas ceases. Iodine (0.266 g, 1.0 mmol) was added slowly (10 min) at 0 °C. Additional hydrogen evolved. The reaction mixture was further stirred for 1 h at room temperature. Methanol was added carefully in cold condition after the completion of the reaction and the mixture extracted with ethyl acetate. The combined ethyl acetate extract was washed with brine and dried over Na_2SO_4 filtered and evaporated. The residue was purified by column chromatography on silica gel (PET ether : EtOAc = 8:2) to give a violet oily product (**2**) with 82 % yield, (0.74 g, 1.7 mmol).

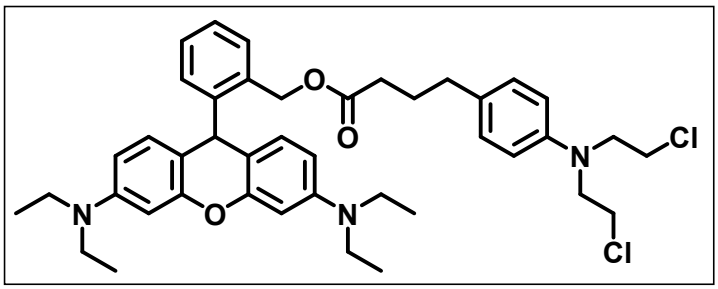


^{1}H NMR (400 MHz, CDCl3) δ 7.45 – 7.38 (m, 1H), 7.26 – 7.23 (m, 3H), 6.68 (d, *J* = 8.6 Hz, 2H), 6.39 (s, 2H), 6.28 (d, *J* = 7.1 Hz, 2H), 5.35 (s, 1H), 4.56 (s, 2H), 3.32 (q, *J* = 7.1 Hz, 8H), 1.15 (t, *J* = 7.0 Hz, 12H). ^{13}C NMR (101 MHz, CDCl3) δ 151.7, 147.8, 144.7, 138.6,

131.4, 129.9, 129.6, 128.0, 126.9, 111.4, 107.5, 98.8, 63.0, 44.4, 40.1, 12.6.

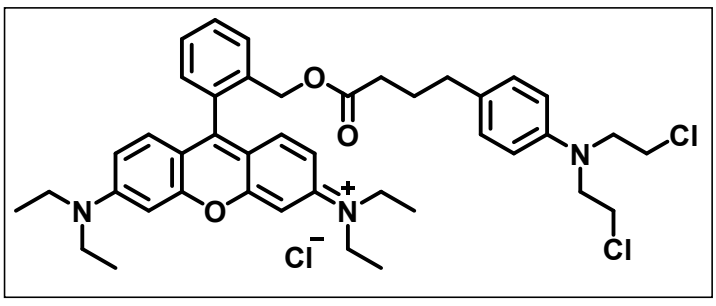
2-(3,6-Bis(diethylamino)-9H-xanthen-9-yl)benzyl-4-(4-(bis(2-chloroethyl) amino)phenyl)butanoate (3)

The chlorambucil (0.05 g, 0.16 mmol) was dissolved in dry DCM and cooled to 0 °C. To the solution, EDC (0.047 g, 0.24 mmol) was added. After 5 min compound **2** (0.069 g, 0.16 mmol) and DMAP (0.019 g, 0.16 mmol) was added to the reaction mixture. The reaction was allowed to stir at room temperature for overnight. After the completion of the reaction, water was added and the mixture was extracted with DCM. The combined DCM extract was washed with brine and dried over Na_2SO_4 filtered and evaporated. The residue was purified by column chromatography on silica gel column (PET ether : EtOAc = 9:1) to give violet coloured oily product (**3**) in 76 % yield (0.084 g, 0.12 mmol).



^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33 (d, *J* = 7.1 Hz, 1H), 7.24 (d, *J* = 7.3 Hz, 1H), 7.18 (t, *J* = 6.7 Hz, 2H), 7.02 (d, *J* = 8.6 Hz, 2H), 6.66 (d, *J* = 8.7 Hz, 2H), 6.58 (d, *J* = 8.7 Hz, 2H), 6.38 (d, *J* = 2.5 Hz, 2H), 6.27 (d, *J* = 2.6 Hz, 1H), 6.25 (d, *J* = 2.6 Hz, 1H), 5.37 (s, 1H), 5.26 (s, 2H), 3.70 – 3.65 (m, 4H), 3.60 (dd, *J* = 10.0, 4.1 Hz, 4H), 3.32 (q, *J* = 7.0 Hz, 8H), 2.52 (t, *J* = 7.6 Hz, 2H), 2.32 (t, *J* = 7.4 Hz, 2H), 1.92 – 1.85 (m, 2H), 1.15 (t, *J* = 7.0 Hz, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.2, 151.9, 147.7, 146.9, 144.3, 133.2, 131.5, 130.7, 130.2, 129.9, 129.7, 129.3, 126.3, 112.3, 111.7, 107.5, 98.8, 65.4, 53.7, 44.4, 42.0, 40.5, 34.0, 33.7, 26.6, 12.7. HR-MS calc for $C_{42}H_{52}Cl_2N_3O_3$ $[M+H]^+$: 716.3380, found: 716.3371.

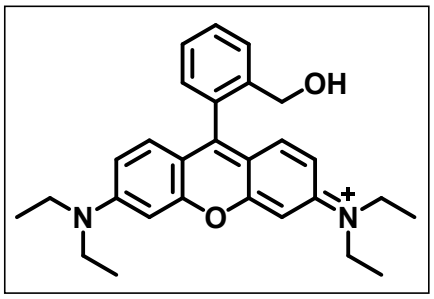
N-(9-(2-(((4-(4-(bis(2-chloroethyl)amino)phenyl)butanoyl)oxy)methyl)phenyl)-6- (diethylamino)-3H-xanthen-3-ylidene)-N-ethylethanaminium chloride (4)



The compound **3** (0.05 g, 0.07 mmol) was taken up into DCM (10 mL), and chloranil (0.027 g, 0.11 mmol) was added. The mixture was stirred at room temperature for 30 min, then washed with water and brine, dried over Na_2SO_4, filtered, and evaporated. Then the residue was purified by column chromatography on silica gel column (DCM : MeOH = 85:15) to give a violet semi-solid product (**4**) with 91 % yield (0.047 g, 0.64 mmol). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.63 (d, *J* = 4.3 Hz, 2H), 7.60 – 7.55 (m, 1H), 7.24 (d, *J* = 7.3 Hz, 1H), 7.11 (d, *J* = 9.3 Hz, 2H), 6.95 (d, *J* = 8.3 Hz, 2H), 6.90 (d, *J* = 9.4 Hz, 2H), 6.85 (s, 2H), 6.59 (d, *J* = 8.3 Hz, 2H), 4.80 (s, 2H), 3.69-3.58 (m,

16H), 2.38 (t, J = 7.4 Hz, 2H), 2.08 (t, J = 7.4 Hz, 2H), 1.70 (quintet, J = 7.5 Hz, 2H), 1.32 (t, J = 5.9 Hz, 12H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.7, 157.8, 155.8, 155.6, 144.4, 134.3, 131.7, 131.2, 130.5, 129.5, 129.5, 129.4, 128.8, 114.5, 113.7, 112.4, 100.0, 96. 7, 63.4, 53.6, 46.3, 40.60, 33.9, 33.2, 26.4, 12.7. HR-MS calc for $C_{42}H_{50}Cl_2N_3O_3^+$ $[M]^+$: 714.3224, found: 714.3227.

3. Characterization of photoproduct (5)



^{1}H NMR (400 MHz, $CDCl_3$) δ 7.30-7.41 (m, 2H), 7.27 (d, J = 6.4 Hz, 1H), 6.98 (d, J = 7.5 Hz, 1H), 6.72 (d, J = 8.5 Hz, 2H), 6.42 (s, 2H), 6.35 (d, J = 7.2 Hz, 2H), 5.21 (s, 2H), 3.38–3.30 (m, 8H), 1.16 (t, J = 5.0 Hz, 12H). HR-MS calc for $C_{28}H_{33}N_2O_2^+$ $[M\text{-}Cl^-]$: 429.2537, found: 429.2540.

4. Measurement of fluorescence quantum yield

The fluorescence quantum yield (QY) of Rhod-cbl (**4**) were determined by the reference point method.[1] The aqueous solution of rhodamine B (Φ_f = 0.31 in water)[2] was used as the standard for the fluorescence quantum yield determinations of compounds with solvent refractive index correction. An error of 10 % is estimated for the fluorescence quantum yields. The absorbance values of the solutions at the excitation wavelength were measured with UV–Vis spectrophotometer. Photoluminescence (PL) emission spectra of all the sample solutions were recorded by Hitachi F-7000 fluorescence spectrophotometer at the excitation wavelength of **4**. Further, the fluorescence quantum yield of **4** were calculated using equation (1).

$$\Phi_S / \Phi_R = A_S / A_R \times (Abs)_R / (Abs)_S \times \eta_S^2 / \eta_R^2 \qquad (1)$$

Where **Φ** represents quantum yield, **Abs** represents absorbance, **A** represents the area under the fluorescence curve, and **η** is the refractive index of the medium. The subscripts **S** and **R** denote the corresponding parameters for the sample and reference, respectively.

5. Quantum yield of deprotection of photocage Rhod-cbl (4)

The solutions of rhodamine based Rhod-cbl (**4**) in water (10^{-4} M) were prepared individually. Half of the solution was kept in dark and the remaining half was irradiated with light (λ ~ 546 nm), using a medium-pressure mercury lamp (125 W), incident intensity (I_0) = 2.886×10^{16} quanta s^{-1} cm^{-2} with a UV cut-off

filter (1 M $NaNO_2$ solution). At regular intervals of time, 20 μL of the aliquots was taken and analyzed by RP–HPLC using acetonitrile/water (9:1) as the mobile phase at a constant flow rate (1 mL min^{-1}), (detection: UV 254 nm). Peak areas were determined by RP–HPLC, which indicated a gradual decrease of the caged compounds with time, with an average of three runs. The reaction was followed until the consumption of the caged compound is less than 5 % of the initial area. Based on HPLC data for each caged compounds, we plotted normalized [A] (HPLC peak area) versus irradiation time. An exponential correlation for the disappearance of the caged compounds was observed, which suggested a first order reaction. Further, the quantum yield for the photolysis of caged compounds was calculated using the following equation (2)

$$(\Phi)_{CG} = (\Phi)_{act} \times [(k_p)_{CG}/(k_p)_{act}] \times [F_{act}/F_{CG}] \quad (2)$$

Where, the subscript 'CG' and 'act' denote caged compound and actinometer, respectively. Φ is the relative photochemical quantum yield, k_p is the photolysis rate constant and F is the fraction of light absorbed. Freshly prepared aq ferrioxalate solution (0.15 M in 0.05 M H_2SO_4) was used as an actinometer (Φ_{act} = 0.1512) at 546 nm. [3]

6. Monitoring the course of photolysis of caged compound rhodamine-phenylacetic acid (Rhod-Phac) conjugate by the means of ^{1}H NMR spectroscopy

The (Rhod-Phac) (1×10^{-4} M) in water with light (λ ~ 546 nm) was irradiated for 60 min. After photolysis, the aliquot was evaporated under vacuum and the residue was dissolved in $CDCl_3$ and the ^{1}H NMR was recorded. The ^{1}H NMR spectrum (see **Figure S)** at zero time showed characteristic signals at δ 4.83 corresponding to α-CH_2 protons of ester moiety of (Rhod-Phac). In addition, it was also noticed the characteristic benzylic proton at δ 3.38 of caged phenylacetic acid. Interestingly after 60 min of irradiation, it was observed that the intensity of the signals at δ 4.83 corresponding to α-CH_2 protons of ester moieties of (Rhod-Phac) decreases indicating the photodecomposition of (Rhod-Phac). At the same time, a new signal at δ 5.21 was observed which corresponds to α-CH_2 protons of the photoproduct N-(6-(diethylamino)-9-(2-(hydroxymethyl)phenyl)-3H-xanthen-3-ylidene)-N-ethylethanaminium (**5**). Further, a significant shift in the δ value of benzylic proton of phenylacetic acid from 3.38 to 3.64 ppm was observed indicating the release of caged phenylacetic acid.

7. Preparative photolysis

Rhod-cbl (**4**) (0.03 g, 0.05 mmol) was dissolved in 50 mL water/acetonitrile 1:1 (v/v) mixture and photolysed under light (λ ~ 546 nm) isolated from a medium-pressure mercury lamp (125 W). The completion of the photolysis was determined by TLC. Finally, after completion of the photolysis, the solvent was removed. The residue was purified by preparative layer chromatography (PLC) on a silica gel with (DCM : MeOH = 8:2) to give a violet product (**5**) with 66 % yield (0.014 g, 0.033 mmol). The purity of the photoproduct was confirmed with RP-HPLC analysis with acetonitrile/water (9:1) as the mobile phase at a constant flow rate (1 mL min^{-1}) which gave a peak at t_R = 2.77 min (**Figure S5**).

8. Experimental procedure for biological application study

8.1. *In vitro* cellular uptake studies of Rhod-cbl in the cancerous and non-cancerous cell line

The degrees of cellular uptake for the Rhod-cbl were studied qualitatively in cancerous cell lines B16F10 melanoma and the non-cancerous cell line CHO (Chinese Hamster Ovary) cells by confocal microscopy. B16F10 and CHO cells were seeded (5×10^4) in 35 mm cover glass with 1 mL of culture medium (0.02 % DMSO in cell culture medium) for 12 h, then the cells were washed with 1X PBS (3 × 1 mL) and treated with Rhod-cbl (2 μM) for 4 h. Cells were washed with 1X PBS (3 × 1 mL) then fixed with 4% paraformaldehyde and cell nuclei were stained with DAPI. Mount on coverslip onto glass slide and images were recorded by confocal microscopy (Nikon Ti Eclipse).

8.2. Intracellular distribution of HRhod-cbl in B16F10 cell line

Intracellular localization of the HRhod-cbl by cancerous B16F10 cell line was monitored by confocal laser scanning microscopy (CLSM). The cells were cultured following the standard protocols and were incubated with the HRhod-cbl (2 μM) under 5 % CO_2 at humified conditions at 37 °C for 4 h. Cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) and the lysosome and mitochondria were stained separately with lysotracker green and mytotracker green respectively. The live imaging was done by Nikon confocal microscope (Eclipse Ti-E) using the respective filter.

8.3. Cellular Uptake study with Flow Cytometry

The degree of cellular uptake was studied quantitatively using flow cytometry. Cancerous B16F10 and noncancerous CHO cells were cultured separately in 6 well plates at a density of 1×10^5 cells per well for 8 h. The cells were then incubated with HRhod-cbl (2 μM) for three different time intervals (0 min, 30 min, 1 h, 2 h, 3 h and 4 h respectively). Cells were harvested by trypsinization, washed with PBS (3 × 1 mL) and were analysed using flow cytometer (BD FACS canto II) under the PE channel. The shift of the fluorescently labelled cells was compared with untreated cells using FCS software.

8.4. *In Vitro* cell viability assay of rhodamine based DDS

The *in vitro* cytotoxic studies of HRhod-cbl, Rhod-OH and free drug (cbl) have been carried out using the MTT (3-(4,5- dimethylthiazole-2-yl)-2,5–diphenyltetrazolium bromide) assay. Briefly, cancerous cell line B16F10 as well as normal cell line CHO were grown separately in their log phase. Cells were seeded in 96-well plates in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal bovine serum (FBS) for 8 h. The cells were incubated with different concentrations (5-40 μM) of HRhod-cbl in HEPES buffer, at 37 °C in 5 % CO_2 for 72 h. Then cytotoxicity was measured using the MTT assay before and after light irradiation. It was evident from the MTT assay data that the HRhod-cbl showed no toxicity to the corresponding cell lines before irradiation, however, an increase in cytotoxicity was observed after irradiation specifically in the cancer cells.

Figures

9. ^{1}H and ^{13}C NMR spectra

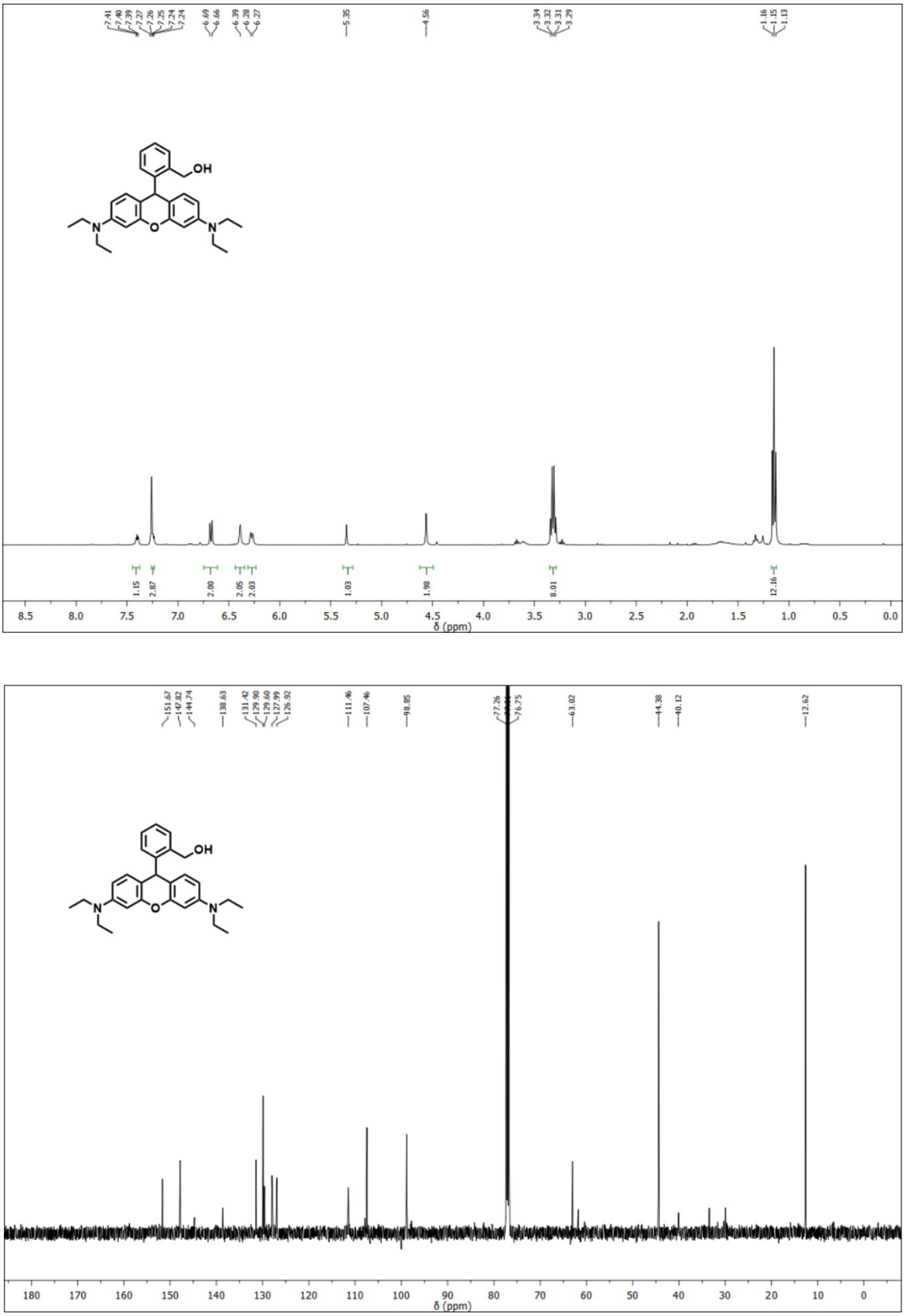


Fig. S1. ^{1}H and ^{13}C NMR spectra of **2** in $CDCl_3$.

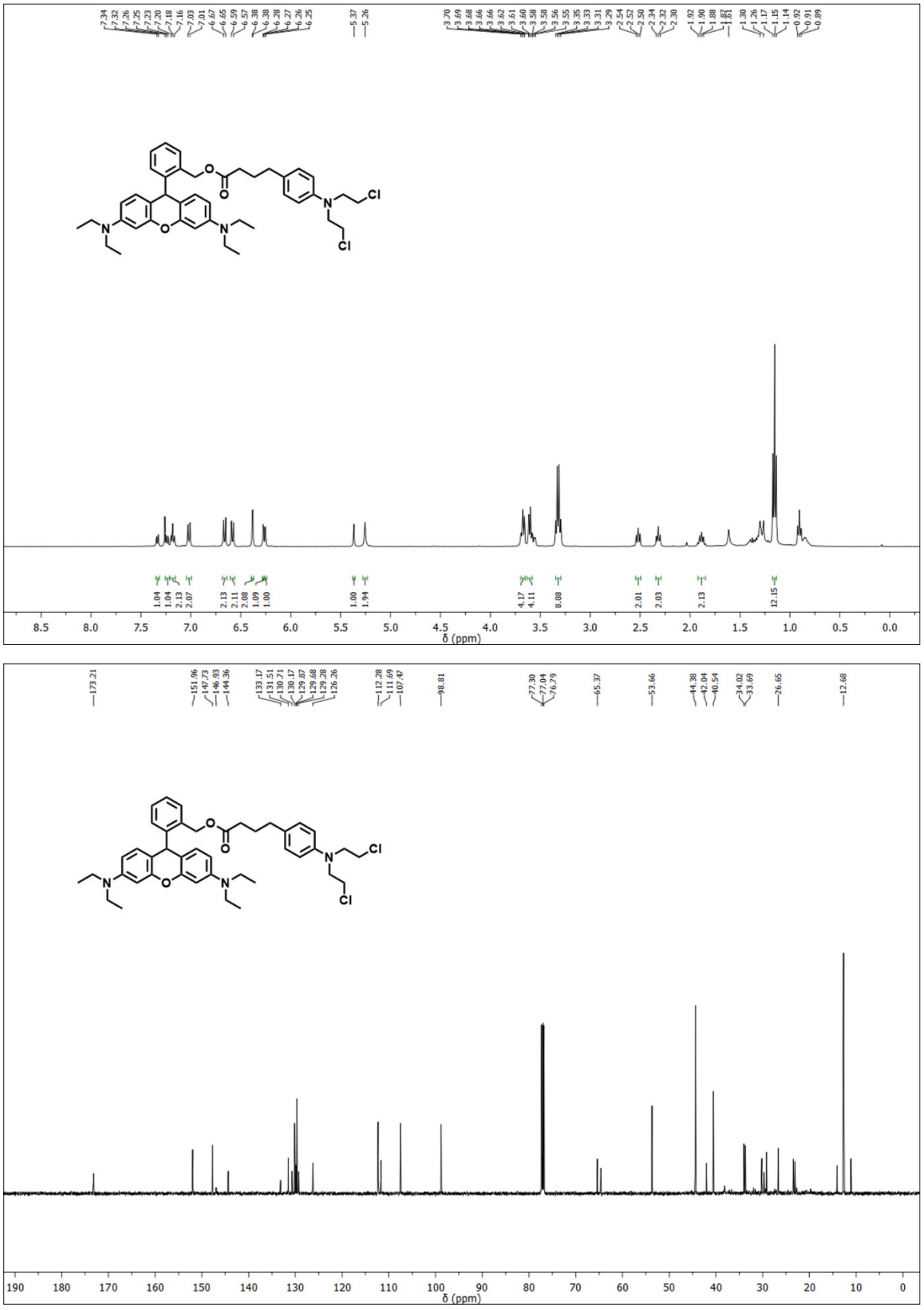


Fig. S2. ^{1}H and ^{13}C NMR spectra of **3** in $CDCl_3$.

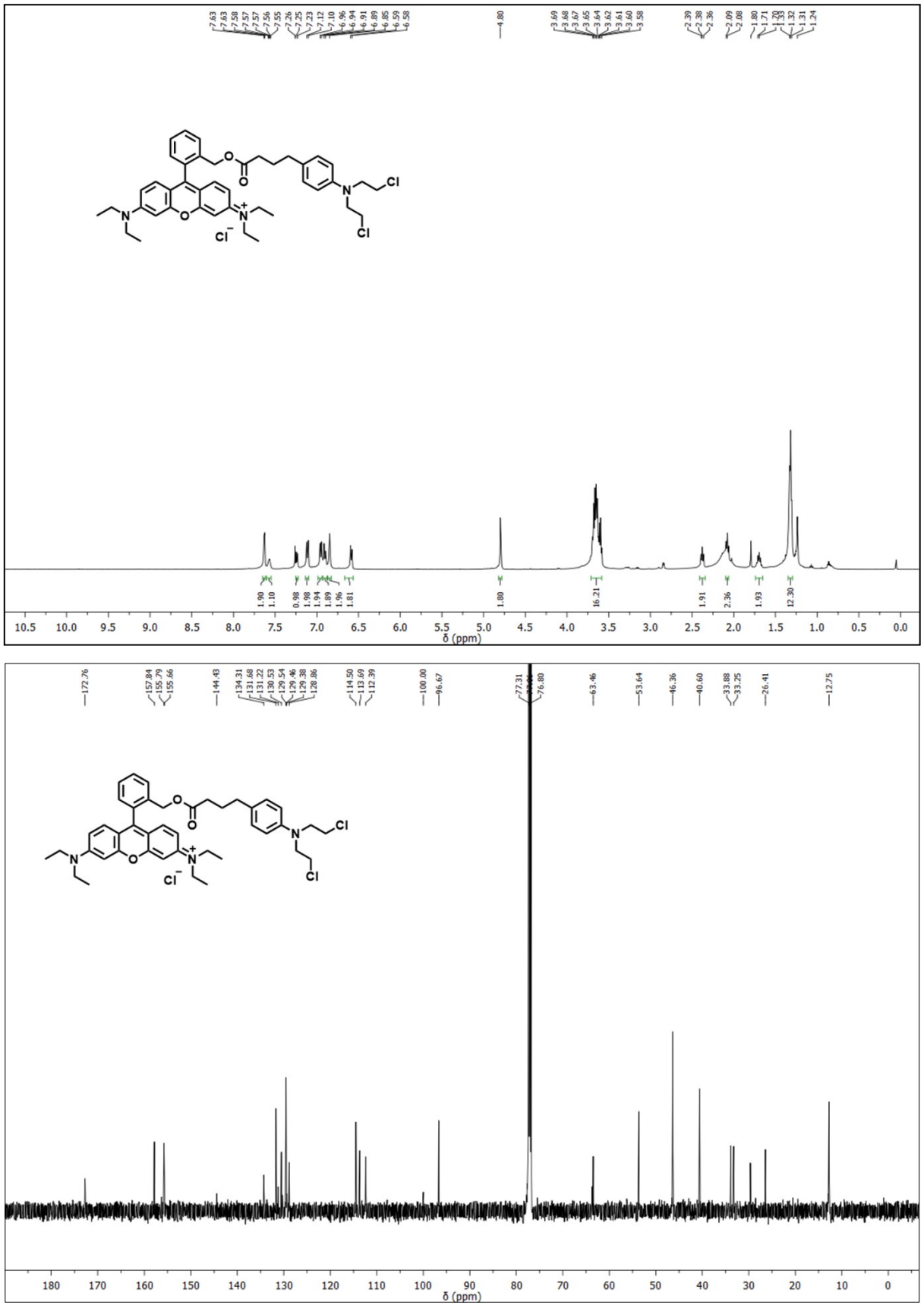


Fig. S3. ^{1}H and ^{13}C NMR spectra of **4** in $CDCl_3$.

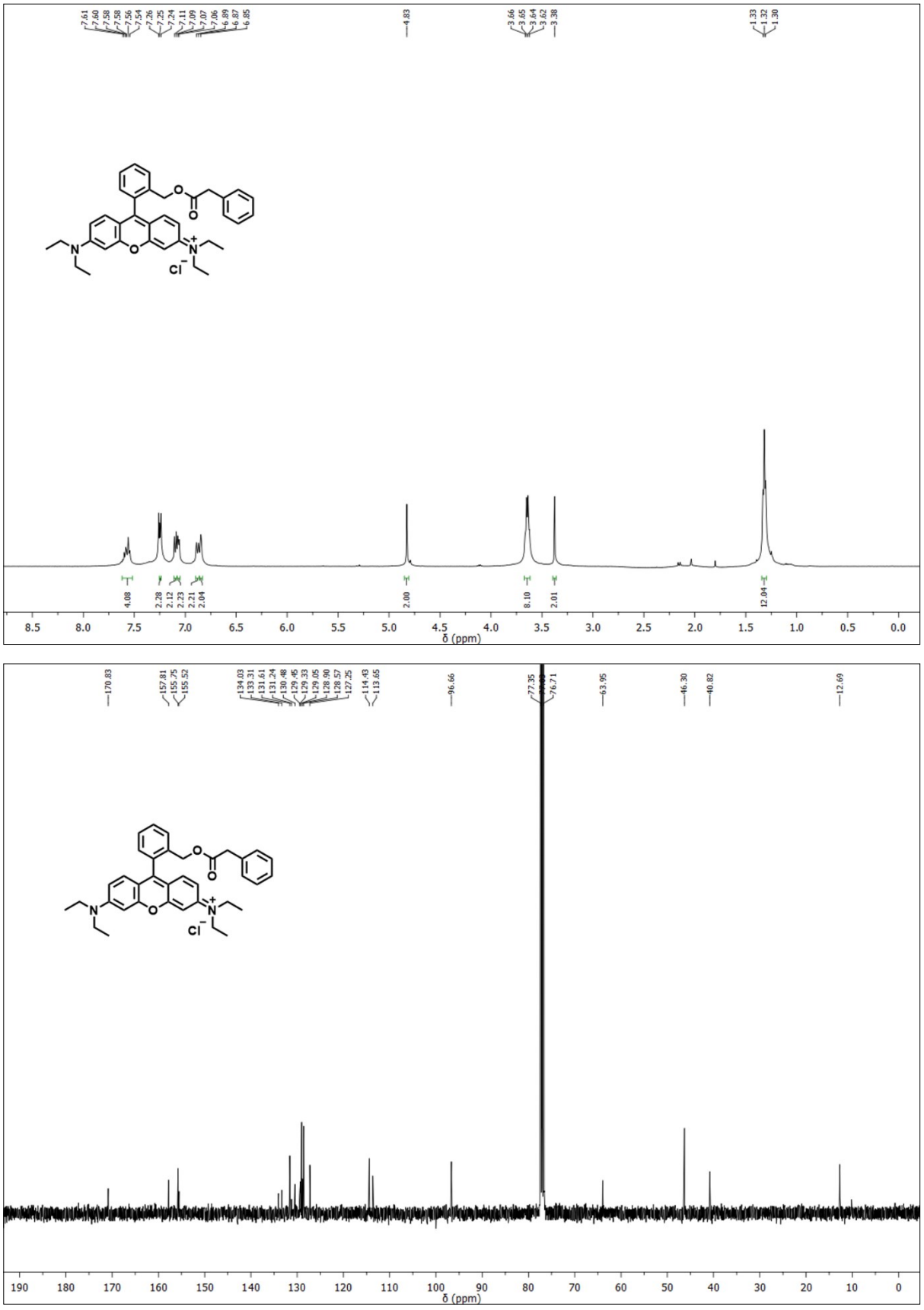


Fig. S4. ^{1}H and ^{13}C NMR spectra of Rhod-Phac in $CDCl_3$.

10. HRMS spectra

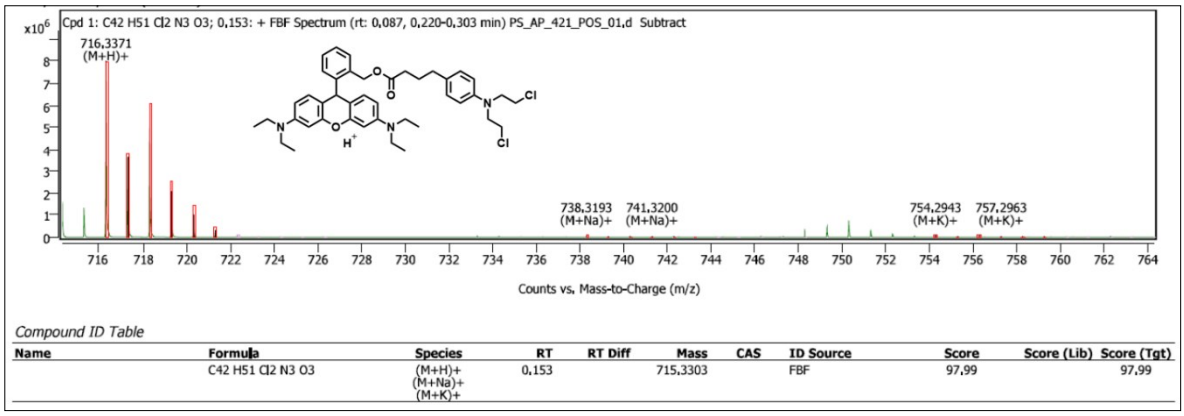


Fig. S5. HRMS spectrum of **3.**

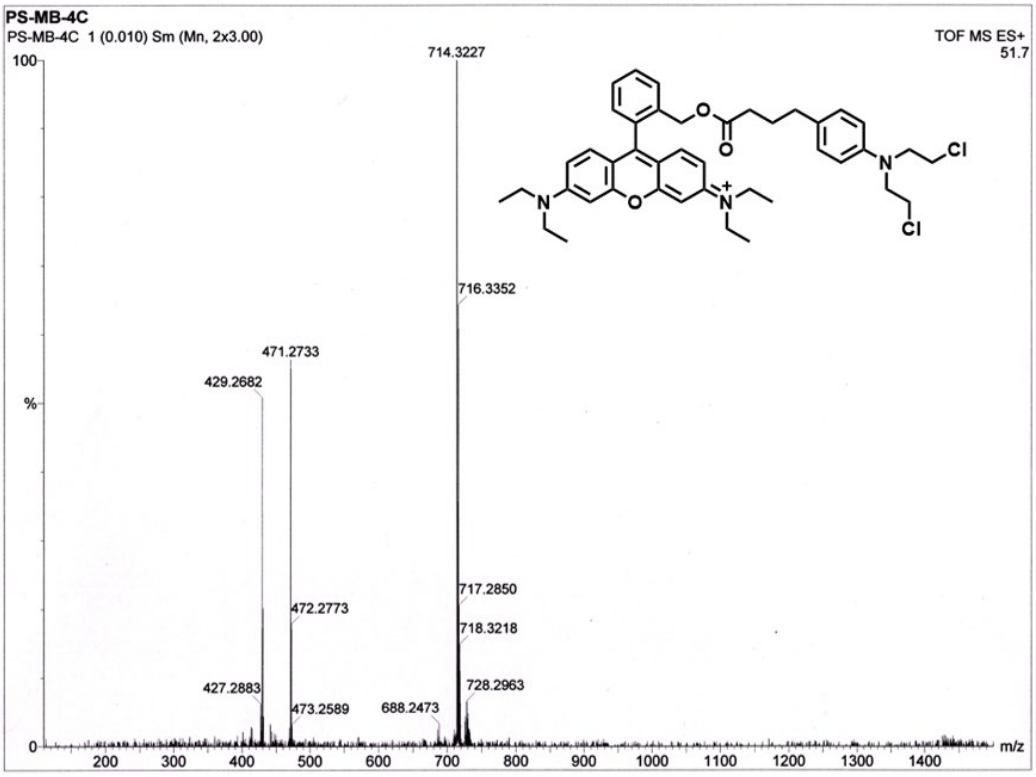


Fig. S6. HRMS spectrum of **4.**

11. Absorption of HRhod-OH and HRhod-cbl

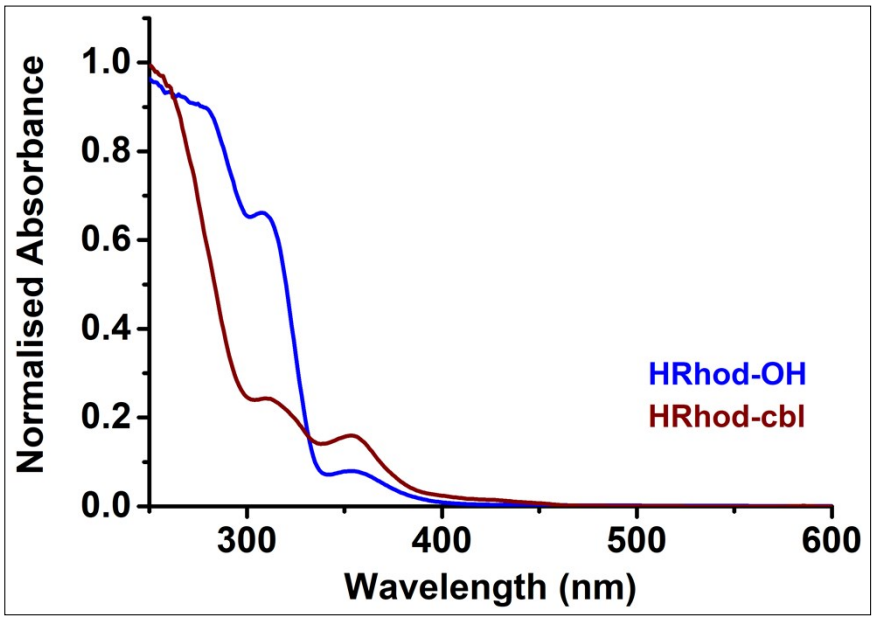


Fig. S7. Normalised absorption spectra of HRhod-OH and HRhod-cbl in acetonitrile/HEPES buffer (1:19). (C = 10^{-4} M).

12. Emission spectra of HRhod-cbl recorded in the presence of various reactive oxygen species

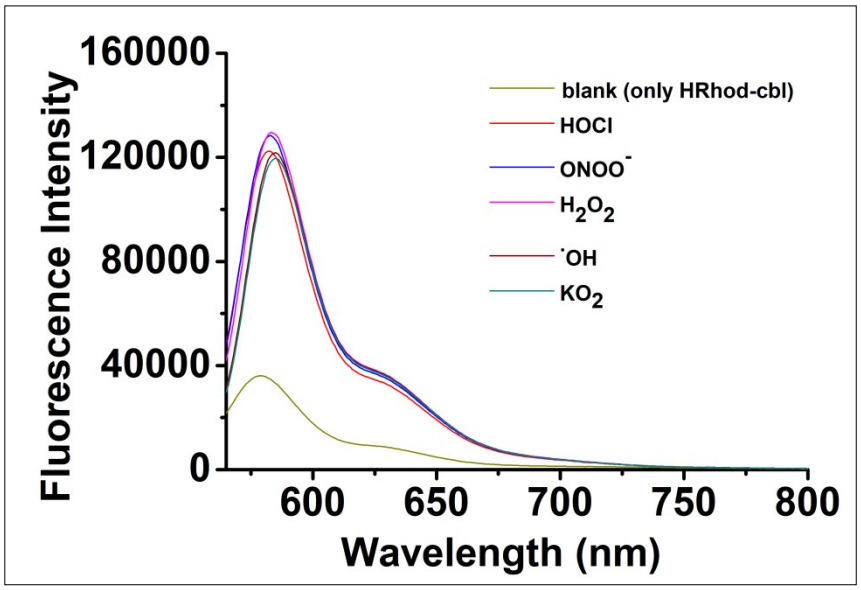


Fig. S8. Emission spectra of HRhod-cbl recorded in the presence of various reactive oxygen species at 100 μM, after 30 min of addition of the ROS. λ_{ex} = 560 nm.

13. Reverse-phase HPLC chromatogram of reaction of HRhod-cbl with five kinds of distinct ROS

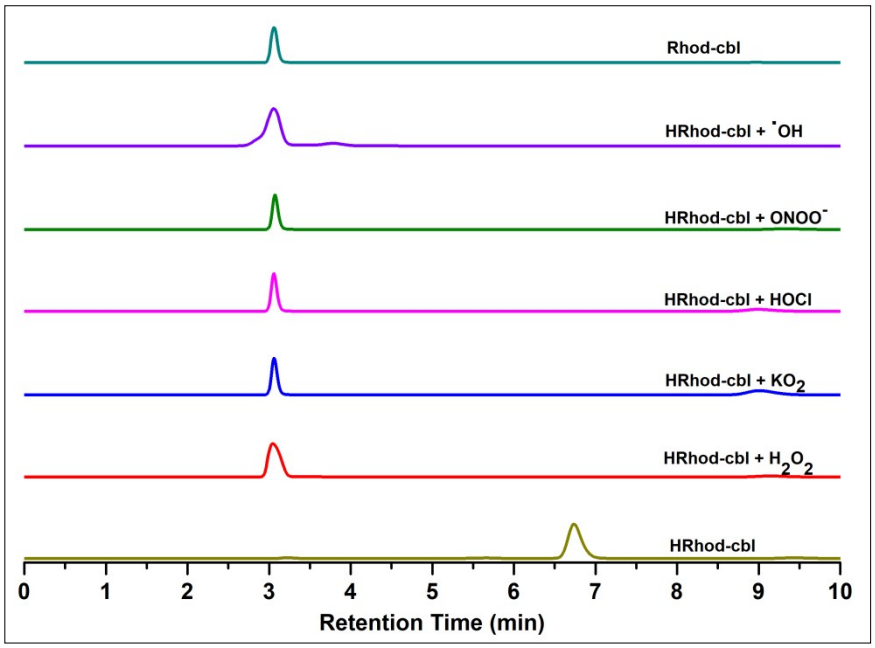


Fig. S9. Reverse-phase HPLC chromatogram of HRhod-cbl and reaction of HRhod-cbl with five kinds of distinct ROS including potassium superoxide (KO_2), H_2O_2, hydroxyl radical (·OH), hypochlorous acid (HOCl) and peroxynitrite ($ONOO^-$) respectively at 100 μM, for 30 min each, using acetonitrile/water (9:1) as the mobile phase at a constant flow rate (1 mL min^{-1}).

14. Stability of HRhod-cbl

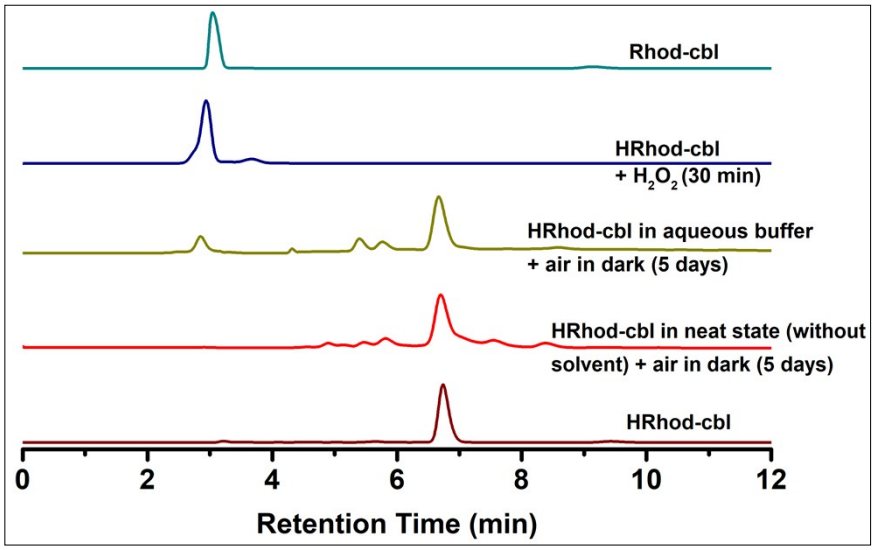


Fig. S10. Reverse-phase HPLC chromatogram for the study of the stability of HRhod-cbl in the presence of air and aqueous buffer using acetonitrile/water (9:1) as the mobile phase at a constant flow rate (1 mL min^{-1}).

15. Percentage of the release of the caged anticancer drug (cbl) from Rhod-cbl (4)

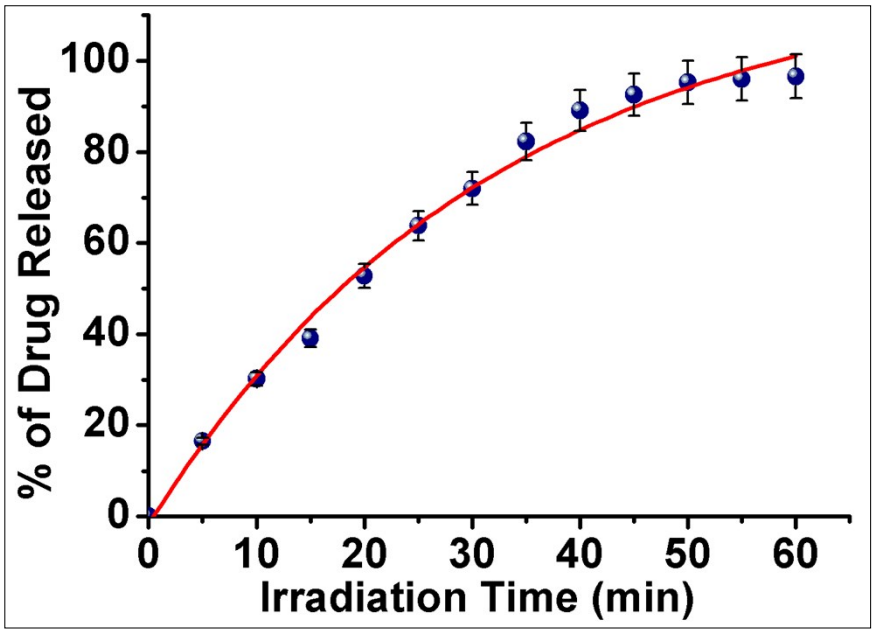


Fig. S11. Percentage of the release of the caged anticancer drug (cbl) from Rhod-cbl (**4**) with respect to irradiation time.

16. RP-HPLC chromatogram of the isolated photoproduct 5

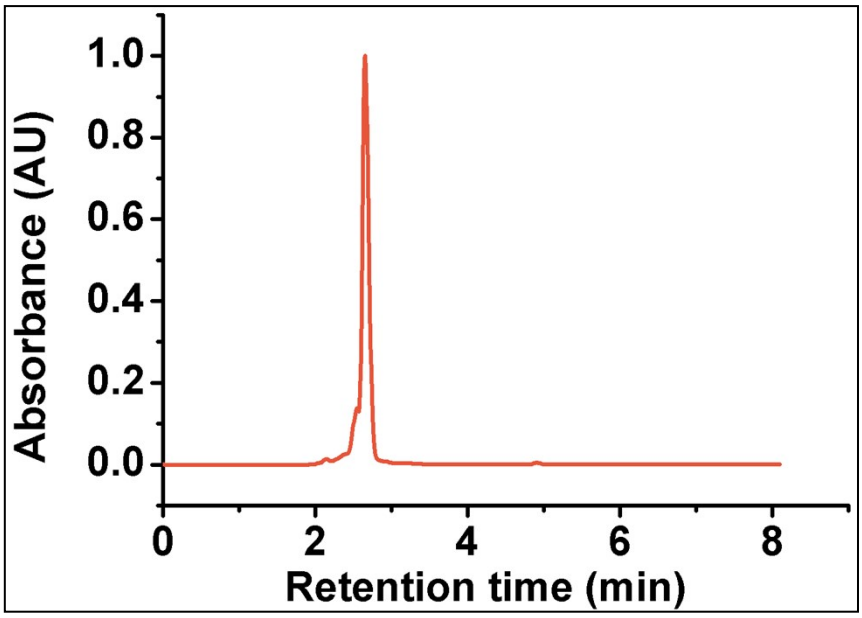


Fig. S12. RP-HPLC chromatogram of the isolated photoproduct **5**.

17. ^{1}H NMR and HRMS spectra of the photoproduct (5)

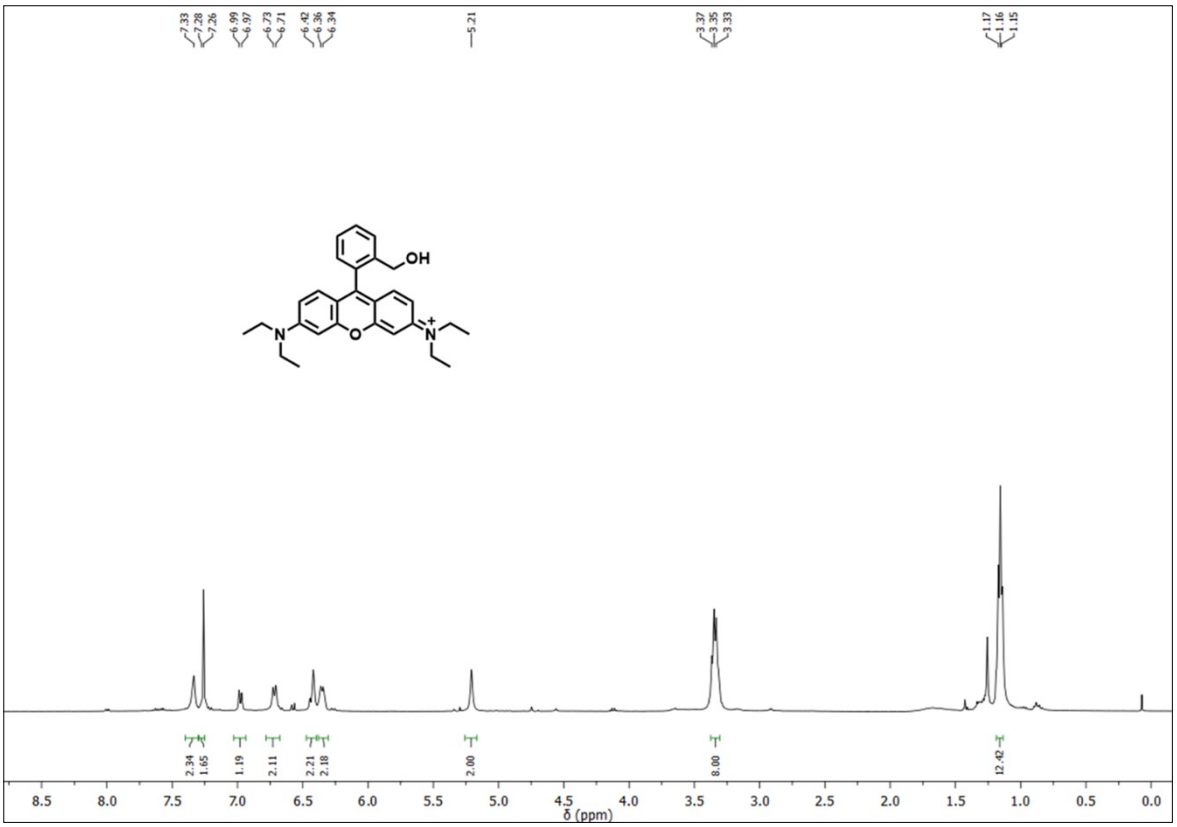


Fig. S13. ^{1}H NMR spectrum of isolated photoproduct (**5**) (400 MHz, $CDCl_3$).

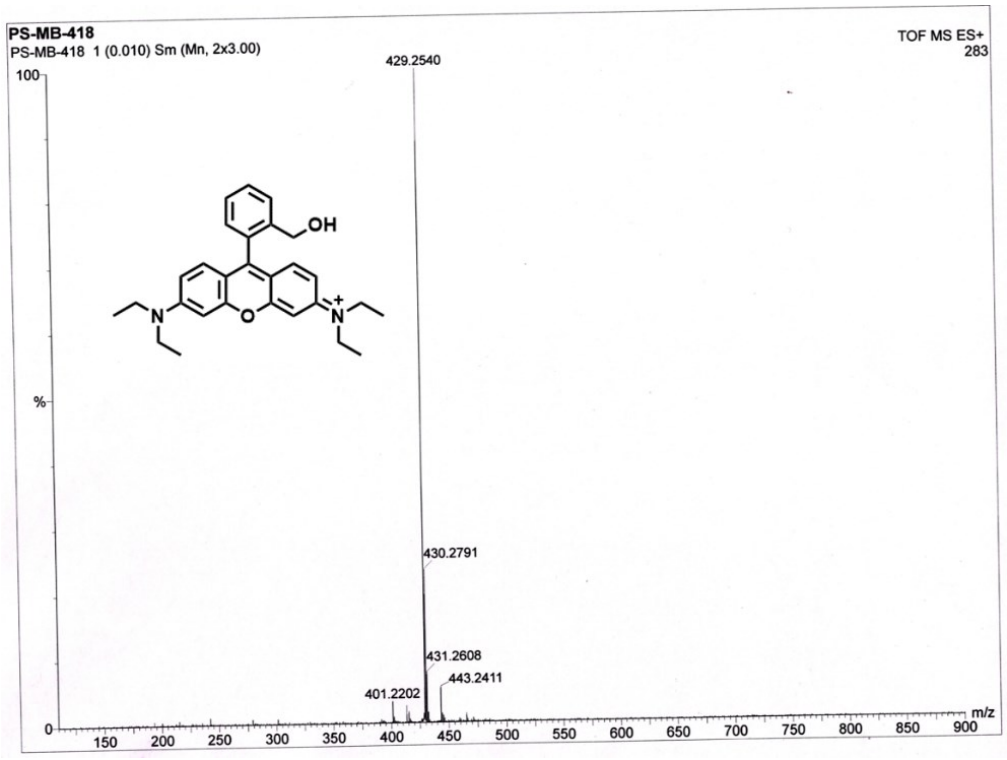


Fig. S14. HRMS spectrum of photoproduct **5.**

18. ^{1}H NMR study of Rhod-Phac in $CDCl_3$ during photolysis

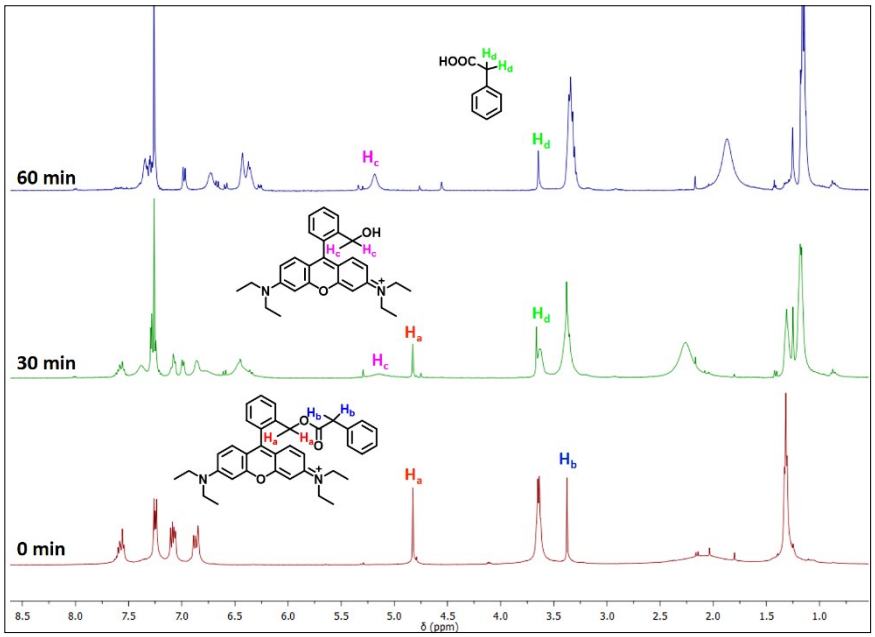


Fig. S15. ^{1}H NMR study of Rhod-Phac in $CDCl_3$ during photolysis.

19. Time resolved fluorescence decay curve of 4

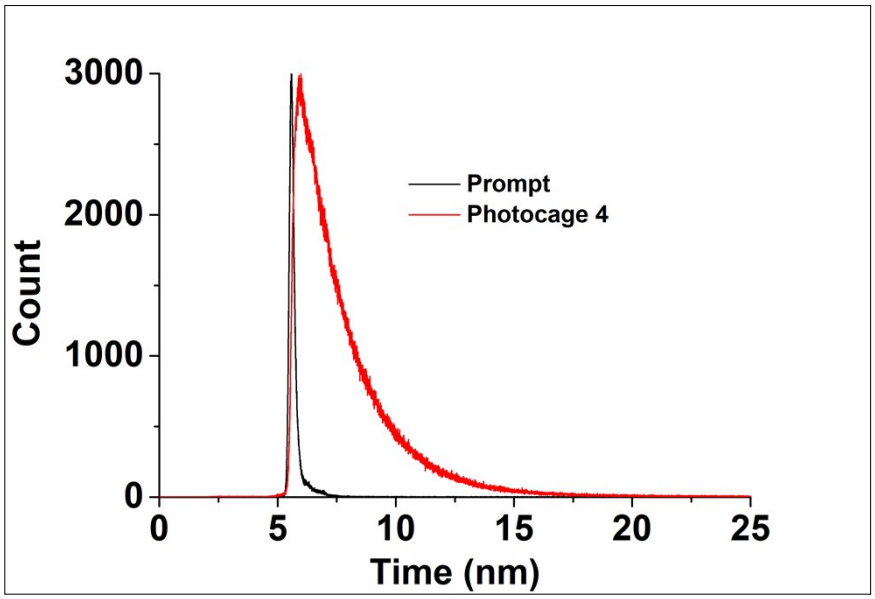


Fig. S16. Time resolved fluorescence decay curve of **4** at 590 nm.

20. Quenching study

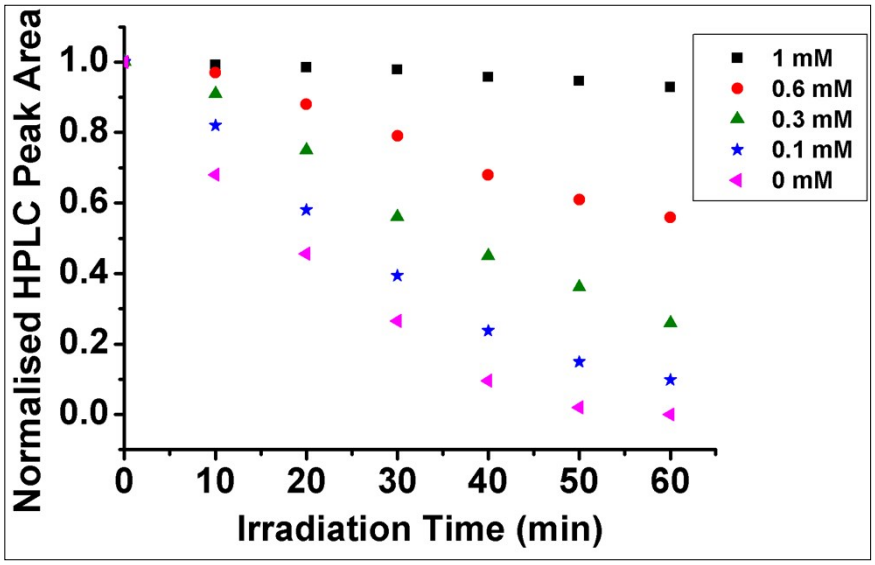


Fig. S17. Time course of photolysis for the **4** in the presence of the different amounts of singlet quencher azulene (0 mM-1 mM).

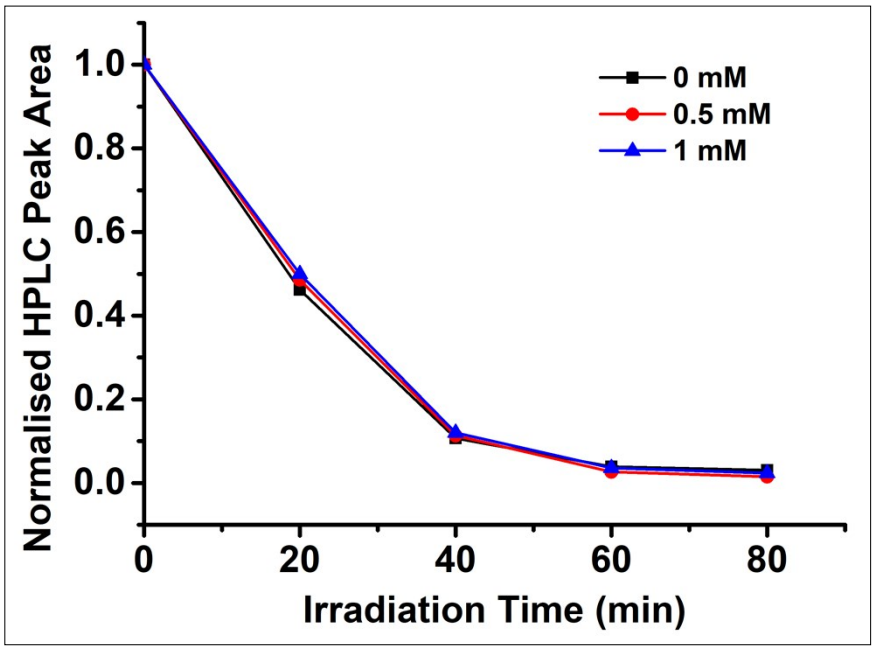


Fig. S18. Time course of photolysis for the **4** in the presence of the different amounts of triplet quencher potassium sorbate (0 mM -1 mM).

21. Photochemical rate constant determination

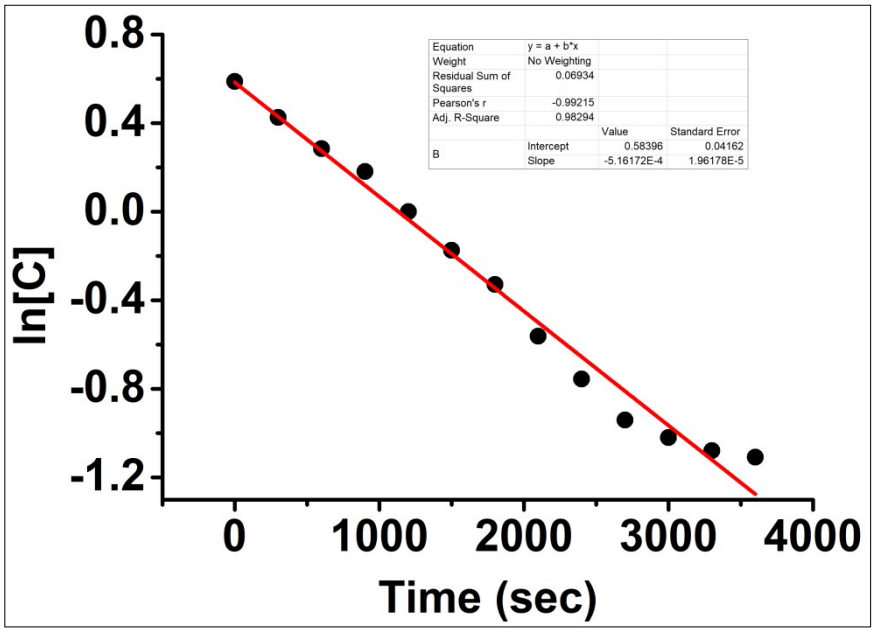


Fig. S19. ln[C] versus irradiation time for the photolysis of **4** in water. [C] = concentration of **4**, it was determined by HPLC and is the average of 3 runs. ([C] = normalized peak area for **4**).

22. Temporal control of light over the drug release

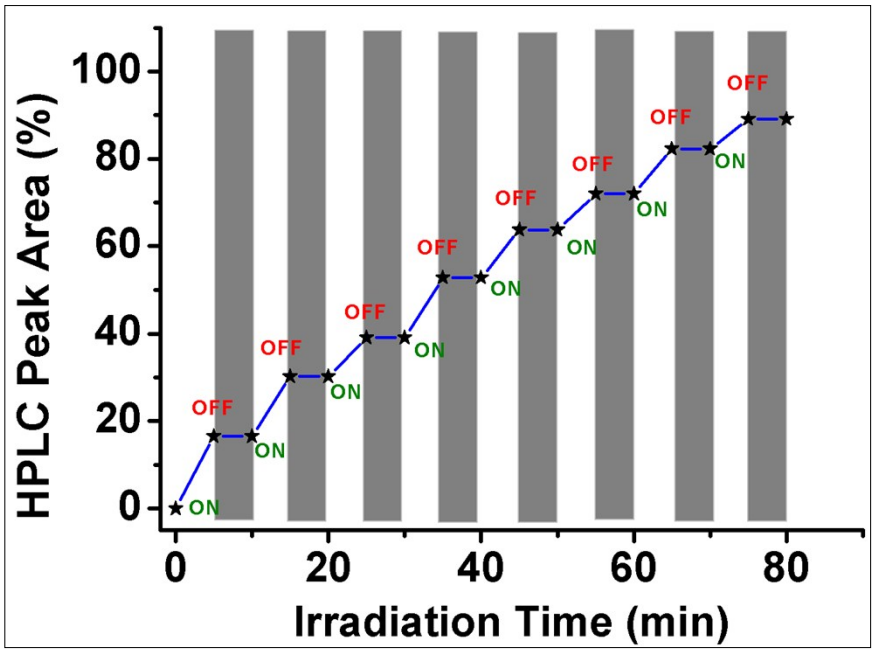


Fig. S20. Release of cbl from **4** under bright and dark conditions. "ON" and "OFF" implies the switching on and off of the light source, respectively. Dark areas represent the period of no light irradiation.

23. Confocal Images

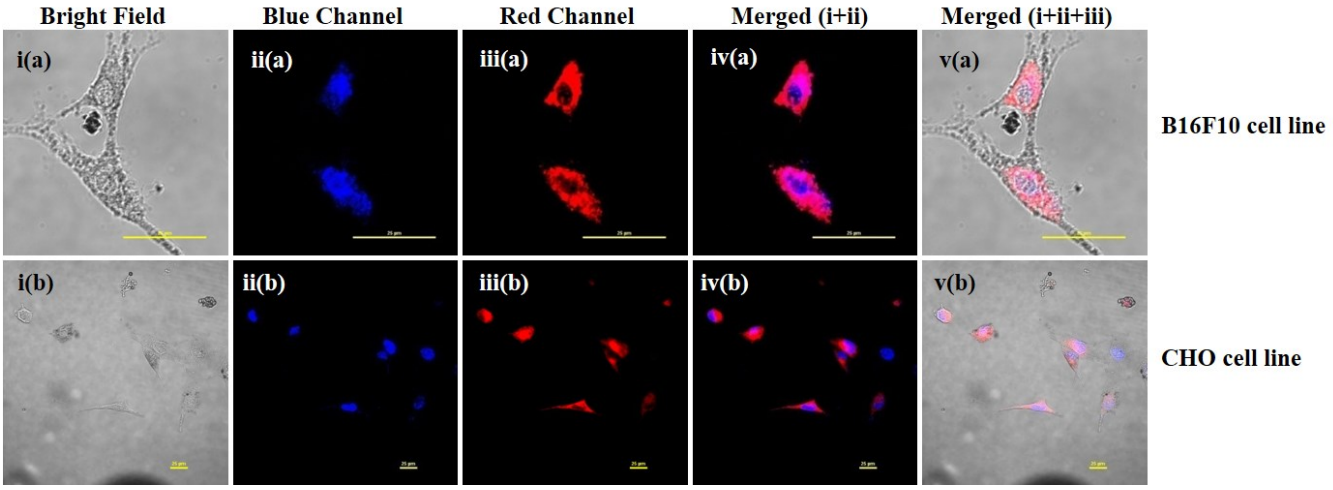


Fig. S21. CLSM images of (a) B16F10, (b) CHO cell lines incubated with Rhod-cbl: (i) bright field image, (ii) blue channel, (iii) red channel and (iv) merged images (red and blue channels). Scale bar: 25 µm.

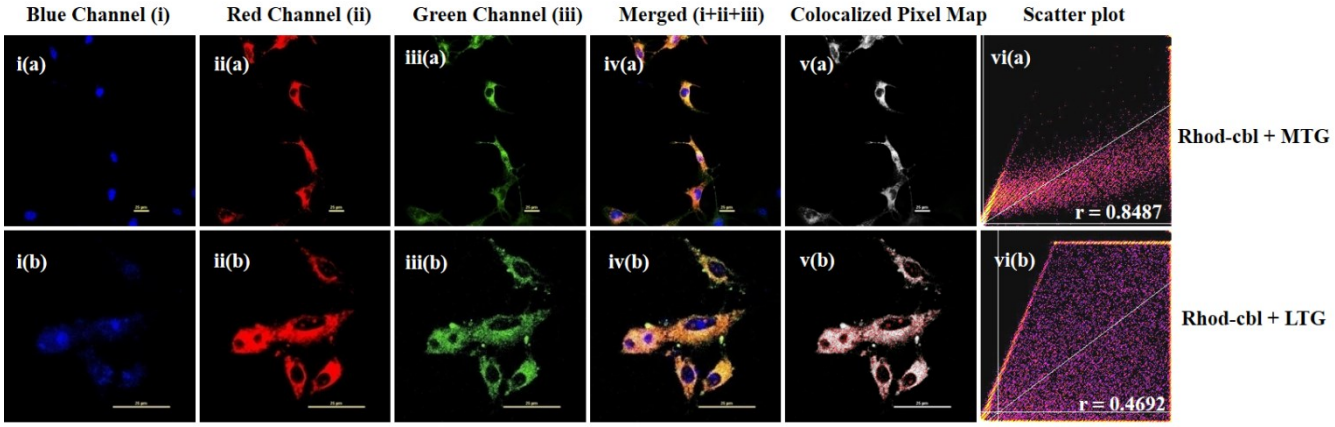


Fig. S22. CLSM images of the B16F10 cell lines incubated with Rhod-cbl, DAPI, LTG and MTG: (i) blue channel, (ii) red channel, (iii) green channel, (iv) merged images and (v) a colocalization pixel map of the corresponding blue, red and green channel, (vi) corresponding scatter plots. (via) Concentrated pixels along the diagonals indicate a high degree of colocalization of Rhod-cbl and MTG; (ivb): scattered pixels along the diagonals indicate a lower degree of colocalization of Rhod-cbl and LTG. Scale bar: 25 µm.

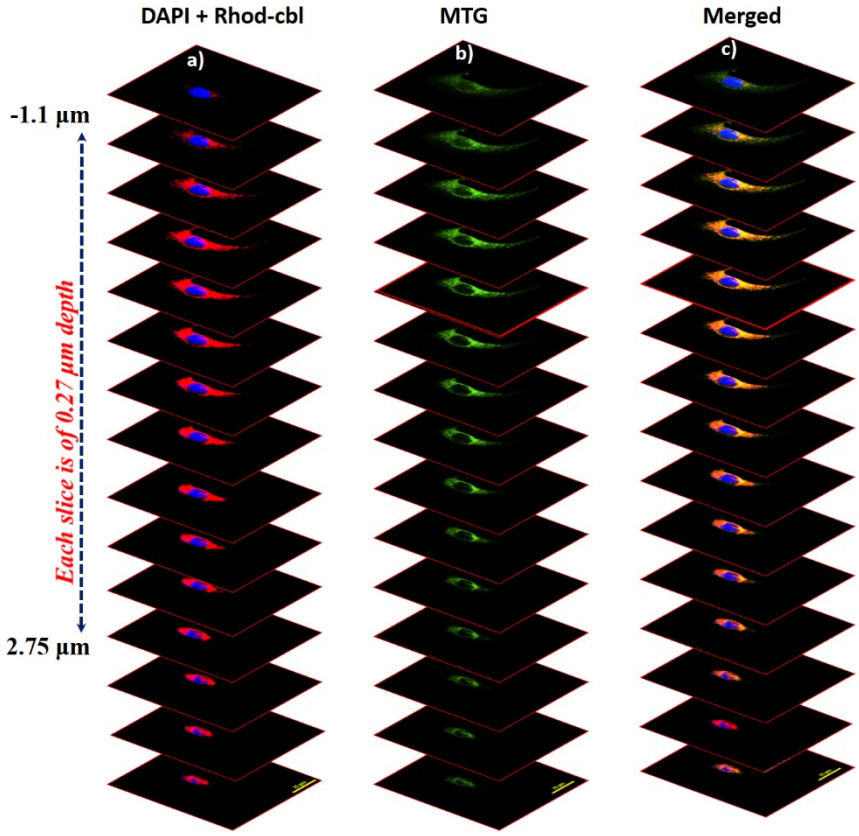


Fig. S23. CLSM images of the B16F10 cell lines incubated with Rhod-cbl, MTG and DAPI: (a) DAPI + Rhod-cbl, (b) MGT, and (c) Merged (all 3D Z stack images).

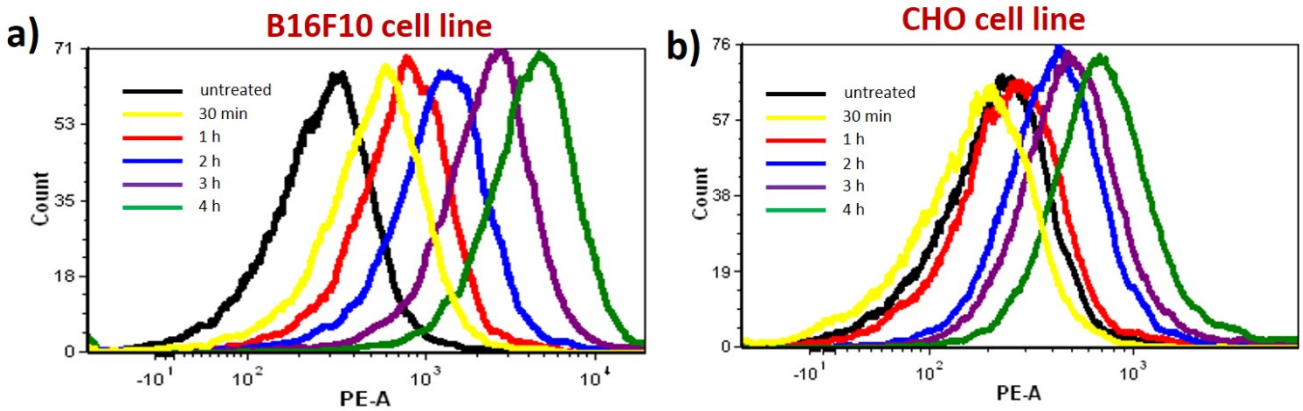


Fig. S24. Flow cytometry analysis indicating the time-dependent fluorescence enhancement after incubation of HRhod-cbl (2 µM) with a) B16F10 and b) CHO cells for different time intervals.

24. References

1. S. Mandal, C. Ghatak, V. G. Rao, S. Ghosh, N. Sarkar. *J. Phys. Chem. C* **2012**, *116*, 5585−5597.
2. D. Magde, G. E. Rojas, P. Seybold, *Photochem. Photobiol.* **1999**, *70*, 737–744.
3. C. G. Hatchard, C. A. Parker, *Proc. R. Soc. London*, *A* **1956**, *235*, 518–536.