

Supporting information

Near Infrared Light Mediated Release of Doxorubicin Using Upconversion Nanoparticles

Authors M. Michael Dcona^{a,b}, Qing Yu^c, John A. Capobianco^{c*}, and Matthew C.T. Hartman^{a,b*}

^a Department of Chemistry, Virginia Commonwealth University, 1001 West Main Street,
P. O. Box 842006, Richmond, Virginia 23284

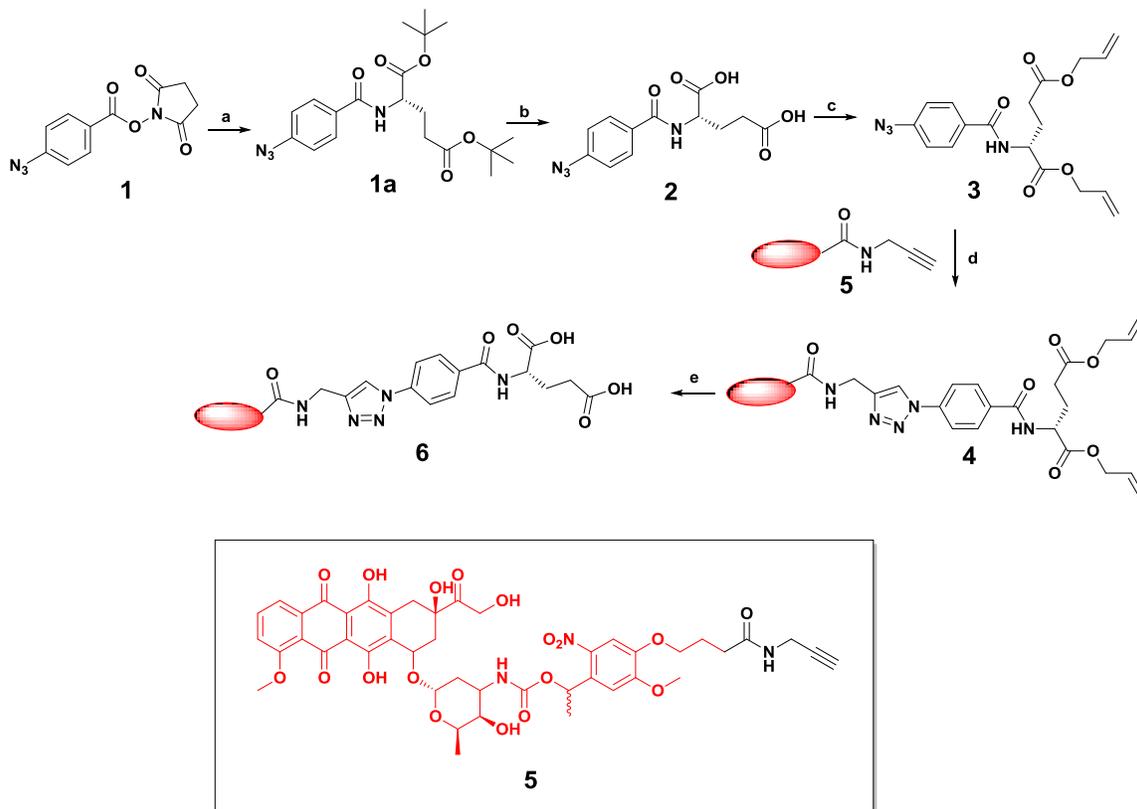
^b Massey Cancer Center, VCU, 401 College Street, Richmond, Virginia 23298

Email: mchartman@vcu.edu

^c Department of Chemistry and Biochemistry, Center for NanoScience Research,
Concordia University, 7141 Sherbrooke Street, Montreal, QC, Canada, H4B 1R6

E-mail: john.capobianco@concordia.ca

1) Synthesis of Compound Dox-(COOH)₂ (6)

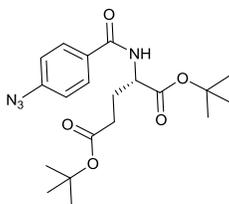


Supporting Scheme 1 Reagents and conditions: a) $\text{Glu}(\text{OtBu})_2$, $\text{N}(\text{Et})_3$, DMF, 12 h, rt. b) 20% TFA in DCM, 10h, rt. c) Allyl Bromide, K_2CO_3 , DMF, 5h, rt. d) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, sodium ascorbate, TBTA, (1:1) DMSO: Water, 24h, rt. e) $\text{Pd}(\text{PPh}_3)_4$, morpholine, DMF, 1h, rt.

1a) General methods: All reagents were purchased from Sigma-Aldrich, Fisher Scientific or TCI America unless otherwise specified and were used as received. Dimethyl Formamide (DMF) used as solvent for chemical synthesis was dried by vacuum distillation. All reactions were carried out in oven-dried glassware and under Ar or N₂ atmosphere. The reactions were carried out in foil-wrapped flasks, protected from light. Flash chromatography was performed using Flash Silica Gel (32-63 μ). ¹H NMR/ ¹³C spectra were recorded on 400 MHz Bruker AVANCE instrument. HPLC purification was carried out using a Shimadzu Prominence system using Vydac (218TP C18 5 μ) column using 0.1% TFA in acetonitrile and water as eluents and was monitored at $\lambda_{\text{max}} = 480$ nm. M/S data was collected using MALDI/TOF (positive and negative modes) or ESI negative modes. Compound **5** was synthesized as described¹.

1b) Chemical Synthesis of Intermediates

Synthesis of di-tert-butyl 2-(4-azidobenzamido)pentanedioate (1a):



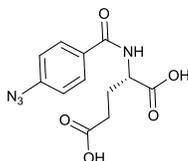
To a stirred solution of 6-azidobenzoic-OSu¹ (**1**) (261 mg, 1 mmol, 1 eq.) in DMF (10 mL) was added L-glutamate di-*tert*-butyl ester hydrochloride (338 mg, 1.3 mmol, 1.3 eq.) and triethylamine (278 μ L, 2 mmol, 2 eq.). The reaction was allowed to stir in the dark for 12 h. The reaction was evaporated *in vacuo* and the crude reaction mixture was diluted with 15 mL CH₂Cl₂ and washed with 10 mL of 1 N aq. HCl and then with brine.

The organic layer was dried over anhydrous Na₂SO₄, filtered, and concentrated under reduced pressure to give a pale white solid, which was further purified via flash chromatography using a mixture of ethyl acetate and hexanes (1:1) to yield the title d compound as a pale solid **1a** (320 mg, 85%).

¹H NMR (400 MHz, CDCl₃) δ = 7.83 (2H, m, Ar-H), 7.07 (2H, m, Ar-H), 7.00 (1H, m,), 4.65 (1h, , -CH-), 2.01-2.26 (2H, m, -CH₂-), 2.28-2.47 (2H, m -CH₂-), 1.49 (9H, s, Boc), 1.42 (9H, s, Boc).

¹³C NMR (100 MHz, CDCl₃) δ = 172.73, 171.32, 166.06, 143.51, 130.44, 128.96, 118.96, 82.47, 80.96, 53.03, 31.71, 28.07, 28.04, 27.34.

Synthesis of 2-(4-azidobenzamido) pentanedioic acid (**2**):



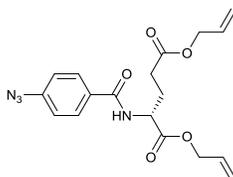
100 mg (0.25 mmoles) of compound **1a** was solubilized in a solution of 20% TFA in DCM (3 mL) and stirred for ~10 h in a foil-wrapped flask. The solvents in the reaction mixture were removed *in vacuo*. To the pale colored sticky solid, cold ether (25 mL) was added. The suspension was vortexed for 5 min and then centrifuged in a 50 mL of falcon tube. This procedure was repeated to yield product as a brown goeey solid 70% yield (50 mg).

¹H NMR (400 MHz, CDCl₃) 12.38 (2H, brs,-COOHx2), 8.62 (1H, d, *J* = 7.6 Hz, CONH), 7.95 (2H, m, Ar-H), 7.22 (2H, m, Ar-H), 4.93 (m, 1H,-CH-), 2.36 (2H, 7.2 Hz, -CH₂-), 2.09 (1H, m, -CH₂-), 1.96 (1H, m, -CH₂-).

¹³C NMR (100 MHz, CDCl₃)

δ = 173.78, 173.30, 165.59, 142.46, 130.44, 129.34, 118.82, 64.86, 51.95, 30.37, 25.90, 15.10.

Synthesis of diallyl (4-azidobenzoyl) glutamate (3):



100 mg (0.34 mmol, 1 eq.) of compound **2** was dissolved in 10 mL of anhydrous DMF. To this solution, allyl bromide (136 μL, 0.51 mmol., 1.5 eq.) was added and K₂CO₃ (62 mg, 0.51 mmol., 1.5 eq.). The reaction was stirred for 1 h at 0°C and then at room temperature for 4h. The crude product was dissolved in 100% ethyl acetate and washed with H₂O at neutral pH. The organic phase was dried with anhydrous MgSO₄, filtered and evaporated to give 117 mg (92%) of the title compound white solid **3**.

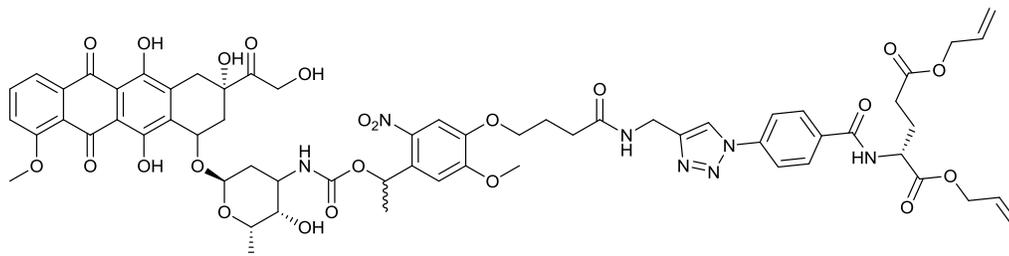
¹H NMR (400 MHz, CDCl₃)

δ = 7.83 (2H, d, *J* = 8.8 Hz, Ar-H), 7.069-7.09 (3H, m, CONH, Ar-H), 5.2-5.376 (5H, m, -CH-COO-), 4.791-4.841 (1H, m, -CH-), 4.66-4.685 (2H, m, -CH-O-), 4.55-4.57 (2H, m, -CH-O-),

¹³C NMR (100 MHz, CDCl₃)

δ = 172.98, 171.65, 166.15, 143.71, 131.89, 131.46, 130.13, 129, 119.09, 119.03, 118.54, 66.25, 65.55, 52.52, 30.49, 27.07.

Synthesis of Photocaged Dox-Glu (COOallyl)₂ conjugate (4):



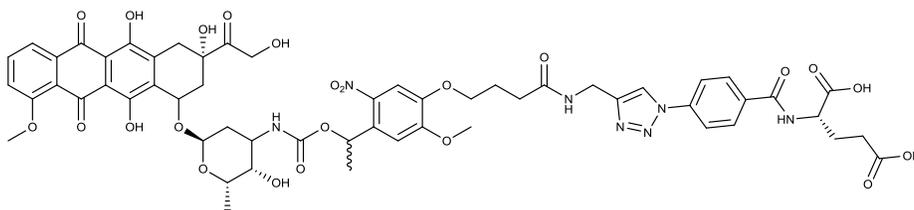
Photocaged Doxorubicin alkyne (**5**)¹ (50 mg, 0.055 mmol, 1eq.) and compound **3** (31 mg, 0.082 mmol, 1.5 eq.) were stirred in 1:1 mixture of water and DMSO (2 mL). 50 μ L (0.5 mg, 0.0055 mmol, 0.1 eq.) of a freshly prepared aqueous solution of sodium ascorbate (10 mg/mL) was added to the reaction, followed by 5 μ L (0.05 mg, 0.01 eq., 0.0002 mmol) of freshly prepared aqueous solution of copper (II) sulfate pentahydrate (12.5 mg/mL), and finally 5.5 mg (0.1 eq., 0.002 mmol) of TBTA (Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine) was added. The red colored heterogenous mixture was stirred vigorously until the reaction was completed (~24h) as judged by TLC (1: 4 CH₃OH/CHCl₃). The reaction mixture was concentrated *in vacuo*, filtered, and the resulting solution was purified on RP-HPLC using isocratic elution of 20 % organic eluent (0.1 % TFA in acetonitrile) for 5 minutes and then increasing the acetonitrile fraction in gradient fashion from 20 % to 100% over 30 minutes at 10 mL/min (see Fig. **6**). The fractions (~10 mL each) were collected into tubes containing 200 μ L of ammonium bicarbonate (6 mg/ mL) solution to quench the TFA. The two diastereomers eluted between 26 and 28.5 min. The fractions containing diastereomers were mixed and the solvent was evaporated which left behind red solid with total mass of 55 mg, which was a mixture of the title compound and ammonium bicarbonate. This solid was taken to next step without further purification, after necessary data analysis.

¹H NMR (DMSO-d₆, 400 MHz): (2x means both diastereomeric products had indistinguishable ppm values for those protons)

δ = 13.94 (s, 1H), 13.99 (s, 2H), 13.19 (m, 2x1H), 8.86-8.88 (m, 2x1H), 8.64-8.67 (m, 2x1H), 8.37-8.41 (m, 2x1H), 7.95-8.03 (m, 2x4H, Ar-H), 7.57-7.61 (m, 2x1H), 7.47 (s, 1H, Ar-H), 7.42 (s, 1H, Ar-H), 7.08 (s, 1H, Ar-H), 7.05 (s, 1H, Ar-H), 6.06-6.07 (m, 2x1H), 5.97-5.99 (m, 2x1H), 5.34-3.35(m, 2x1H), 5.20-5.27(m, 2x4H), 5.12-5.16(m, 2x4H), 4.872 (m, 2x1H), 4.762 (m, 2x2H), 4.60 (m, 2x1H), 4.54-4.55 (m, 2x2H), 4.47-4.48 (m, 2x4H), 4.30-4.34 (m, 2x2H), 3.90-4.03 (m, 2x11H), 3.85 (s, 2H), 3.80(s, 2H), 2.26 (m, 6H), 1.40 (m, 3x2H), 1.03 (m, 3x2H)

MALDI-TOF: Chemical Formula: C₆₂H₆₇N₇O₂₃Na⁺: (M + Na)⁺ Calc. 1300.42
Obsd. 1300.32

Synthesis of Photocaged drug-Glu(COOH)₂ conjugate (6):



50 mg (1 eq., 0.039mmol) of allyl derivative of photocaged doxorubicin (**6**) was stirred under argon in 10 mL of DMF along with Pd(PPh₃)₄ (7.5 mg, 0.0065 mmol, 0.167 eq.) and morpholine (332 μ L, 3.82 mmol) at 0° C. Later, the reaction temperature was raised to room temperature and stirred for 1 h and then the solvents in the reaction mixture were removed *in vacuo*. The crude material was washed with ether to get rid of any residual doxorubicin or other soluble reactants. Further, this reaction mixture was air dried and later purified on RP-HPLC using isocratic elution of 20 % organic eluent (0.1 % TFA in acetonitrile) for 5 minutes and then increasing the acetonitrile fraction in gradient fashion

from 20 % to 100% over 30 minutes at 10 ml/min. The fractions (~10 mL each) were collected into tubes containing 200 μ L of ammonium bicarbonate (6 mg/ mL) solution to quench the TFA. The two diastereomers eluted between 20 and 22 min. The fractions containing the diastereomers were evaporated leaving 30 mg of a red solid containing the title compound and a small amount of Pd(PPh₃)₄.

¹H NMR (DMSO-d₆, 400 MHz): (2x means both diastereomeric products had indistinguishable ppm values for those protons)

δ = 14.06 (s, 1H), 14.01 (s, 1H), 12.31 (br. s, 4H, COOH), 8.77-8.78 (m, 2x1H), 8.72-8.74 (m, 2x1H), 8.42-8.46 (m, 2x1H), 8.09-8.11 (m, 2x2H, Ar-H), 8.02-8.05 (m, 2x2H, Ar-H), 7.36 (m, 2x1H, Ar-H), 7.17 (s, 1H), 7.13 (s, 1H), 6.02-6.07 (m, 2x1H), 5.40-5.42 (m, 2x1H), 5.21-5.23 (m, 2x1H), 4.95 (m, 2x1H), 4.79 (m, 2x1H), 4.65(m, 2x1H), 4.56 (m, 2x2H), 4.38-4.42 (m, 2x4H), 3.98-4.01 (m, 2x4H), 3.93(m, 2x1H), 3.87(s, 2x1H) 3.76-3.78 (m, 2x2H), 3.52 (m, 2x1H), 3.10-3.13 (m, 2x1H), 3.0 (s, 1H), 2.72 (s, 1H) 2.27 (m, 2x3H), 1.99 (m, 2x4H), 1.63-1.66 (m, 2x4H), 1.46-4.50(m, 2x3H), 1.11-1.13 (m, 2x3H).

HRMS: Chemical Formula: C₅₆H₅₈N₇O₂₃⁻: Calculated Mass = 1196.358

Observed Mass = 1196.355

2) Synthesis of UCNPs and conjugates

2a) Synthesis of LiYF₄: Tm³⁺/Yb³⁺ (UCNPs).

The colloidal upconverting LiYF₄: Tm³⁺ 0.5 mol%/Yb³⁺ 25 mol% nanoparticles were synthesized *via* a thermal decomposition method established by our group.² All the

chemicals utilized during the synthesis were purchased from Alfa Aesar and were used without further purification. Two steps are involved in the synthesis; the preparation of the trifluoroacetate lanthanide precursors and the formation of ligand-capped nanoparticles. The lanthanide precursors were prepared by dissolving 210.3 mg (0.931 mmol) of yttrium oxide, 123.2 mg (0.313 mmol) of ytterbium oxide and 2.4 mg (0.00625 mmol) of thulium oxide in a mixture of 5 mL of trifluoroacetic acid and 5 mL of water. The reaction mixture was heated to 80 °C under reflux for 12 hours and then slowly evaporated to dryness at 60 °C. A second solution (solution A) containing 12.5 mL of oleic acid and 12.5 mL of 1-octadecene was degassed at 150 °C for 30 min. 299.9 mg (2.5 mmol) of CF_3COOLi was added to the lanthanide precursor and then dissolved in 7.5 mL of oleic acid and 7.5 mL of 1-octadecene (solution B). Solution B was slowly heated to 125 °C under vacuum and solution A was heated to 315 °C under a gentle flow of argon gas. Solution B was transferred into solution A via a syringe and pump system at a rate of 1.5 mL/min. The combined solution was heated under argon at 315 °C for 90 min with continuous stirring. The solution was allowed to cool to room temperature. Ethanol was used to precipitate out the nanoparticles. Nanoparticles were isolated by centrifugation at 4000 rpm (equal to relative centrifugal force of 1350 g) for 15 min and then washed with ethanol/hexane (4:1) mixture twice.

The nanoparticles prepared are capped with oleate ligand on the surface and are hydrophobic. To render the nanoparticles hydrophilic and water dispersible, the capping oleate was removed *via* an HCl treatment. An HCl solution of pH 4 was added to the nanoparticles and stirred vigorously for 2 hours. During this process the oleate was protonated producing oleic acid, the hydrophobic oleic acid was extracted using ethyl

ether and removed together with organic layer. The resulting hydrophilic nanoparticles were precipitated using acetone and isolated by centrifugation.

2b) Surface modification of UCNPs with Dox

Typically, 10 mg of oleate-free UCNPs were dispersed in 4 mL of water followed by the addition of 1 mg of compound **7** (saturated by MnCl_2 to form Dox- Mn^{2+} complex) in 1 mL of DMSO. The mixture was adjusted to a neutral pH to deprotonate the carboxylic group on the Dox and stirred for 3 days at room temperature. The modified UCNPs were precipitated in acetone and then isolated by centrifugation at 4000 rpm for 15 minutes. Dox dissolves in acetone and was removed with the supernatant.

2c) Determination of number of DOX on the surface of nanoparticles

The concentration of Dox on the surface of the $\text{LiYF}_4:\text{Tm}^{3+}/\text{Yb}^{3+}$ -UCNPs was determined by measuring the absorbance of Dox- $\text{LiYF}_4:\text{Tm}^{3+}/\text{Yb}^{3+}$ -UCNPs at 540 nm. Using this absorbance value and its absorption coefficient obtained from the calibration curve of Dox, which was calculated to be $5.7 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ at 540 nm, we found approximately 1.5×10^3 molecules of Dox on the surface of the UCNPs (Figure S4).

2d) Determination of the energy transfer efficiency.

The energy transfer efficiency was calculated using the following equation:

$$E = 1 - I_1/I_2,$$

where E is the energy transfer efficiency I_1 is the intensity of Dox-UCNPs and I_2 is the intensity of oleate-free UCNPs at ~365 nm.

2e) UCNP Characterization

Fourier Transform Infrared (FTIR) Spectroscopy

FTIR spectra of the as-synthesized oleate-capped $\text{LiYF}_4: \text{Tm}^{3+}/\text{Yb}^{3+}$ -UCNPs, and the oleate-free $\text{LiYF}_4: \text{Tm}^{3+}/\text{Yb}^{3+}$ -UCNPs were measured on a Nicolet 6700 FTIR spectrometer using the KBr pellet.

Transmission Electron Microscopy (TEM)

TEM analysis of the colloidal dispersion of $\text{LiYF}_4: \text{Tm}^{3+}/\text{Yb}^{3+}$ -UCNPs was performed using a Philips CM200 microscope operating at 200 kV equipped with a charge-coupled device (CCD) camera (Gatan). Prior to analysis, the sample was dispersed in toluene to yield an approximate 0.5 wt% solution. A few drops of the resulting solution were evaporated on a formvar/carbon film supported on a 300 mesh copper grid (3 mm in diameter).

Upconversion Luminescence Spectroscopy

The upconversion visible emission spectra of the oleate-free $\text{LiYF}_4: \text{Tm}^{3+}/\text{Yb}^{3+}$ -UCNPs and the Dox- Ln^{3+} -UCNP were obtained upon 980 nm excitation, using a USB4000 Fiber Optic Spectrometer from Ocean Optics. For the upconversion studies, the samples (0.2 wt% in DMSO) were placed in 1 cm path-length quartz cuvettes (Hellma, QS). The emission was detected using a Toshiba TCD1304AP (3648-element linear silicon CCD array) detector with a range from 200 - 1100 nm.

UV/Vis absorption measurement

UV/Vis absorption spectra of $\text{Dox}(\text{COOH})_2$, $\text{Dox}(\text{COOH})_2\text{-Y}^{3+}$ complex, $\text{Dox}(\text{COOH})_2\text{-Mn}^{2+}$ complex and $\text{Dox}(\text{COOH})_2\text{-Ln}^{3+}$ -UCNP were all measured in solvent DMSO and were recorded using Varian (Mulgrave, Victoria, Australia) Cary 5 and 5000 spectrophotometers.

3) Supporting figures

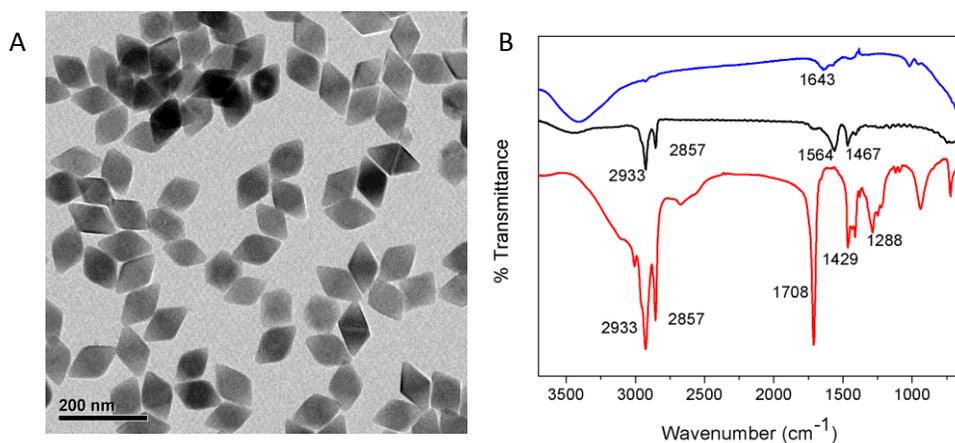


Figure S1. (A) Transmission Electron Microscopy image of LiYF₄: Tm³⁺/Yb³⁺-UCNPs. (B) FT-IR spectra of oleate-free UCNPs (blue), oleate-capped UCNPs (black), and oleic acid (red).

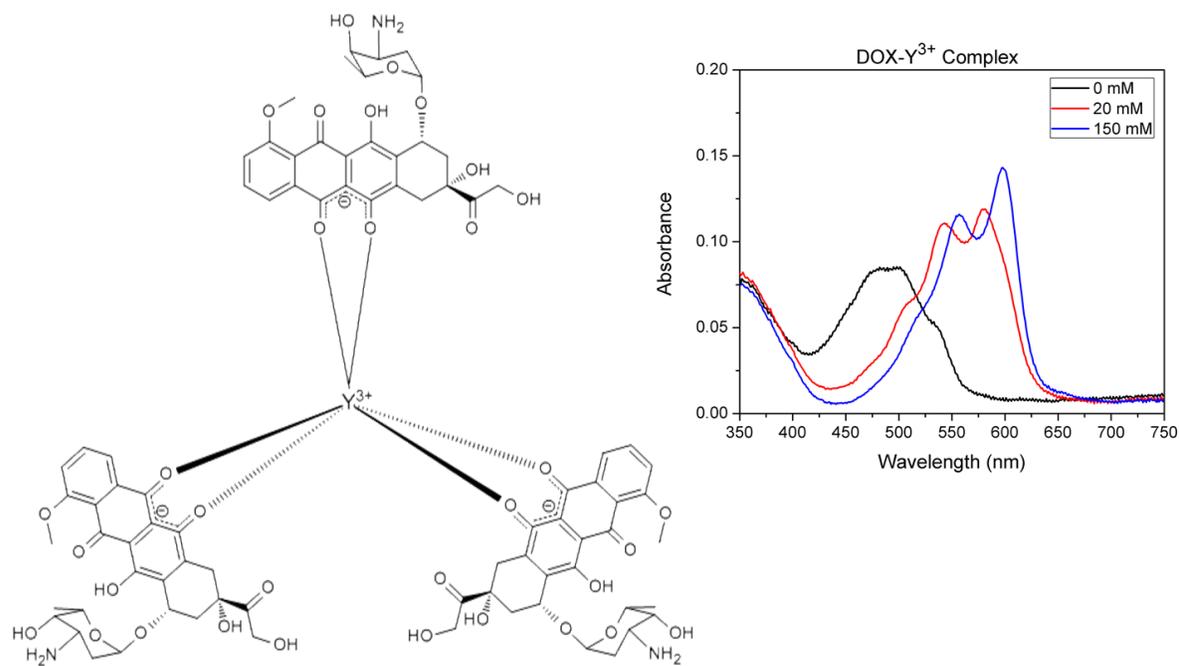


Figure S2. Structure of Dox-Y³⁺ complex and the absorption spectra of the complex with different [Y³⁺] concentrations. The concentration of Dox-(COOH)₂ was kept at a constant of 10 μg/mL and all the measurements used DMSO as the solvent.

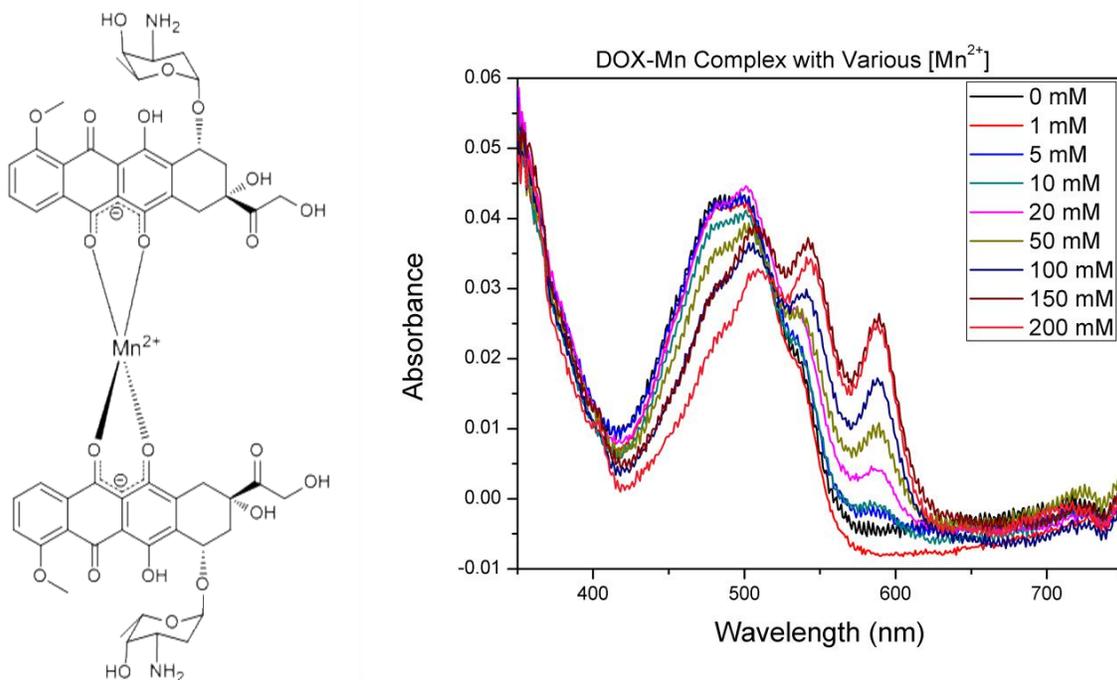


Figure S3. Proposed structure of the $Dox(COOH)_2 \cdot Mn^{2+}$ complex and the absorption spectra of the complex at different Mn^{2+} concentrations. The concentration of $Dox(COOH)_2$ was kept at a constant of $5 \mu\text{g/mL}$ and all the measurements used DMSO as the solvent.

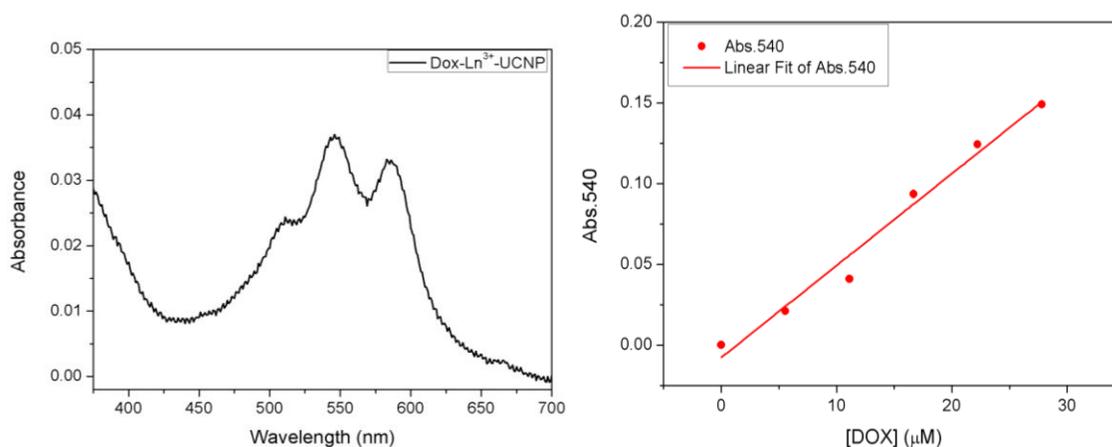


Figure S4. UV/Vis spectrum of 1 mg of Dox-UCNPs in 1.5 mL of DMSO (left). Calibration curve of the $Dox-(COOH)_2$ and Mn^{2+} complex used to obtain its absorption coefficient at 540 nm (Right). The absorbance at 540 nm was used to calculate the concentration and number of Dox on the surface of UCNPs.

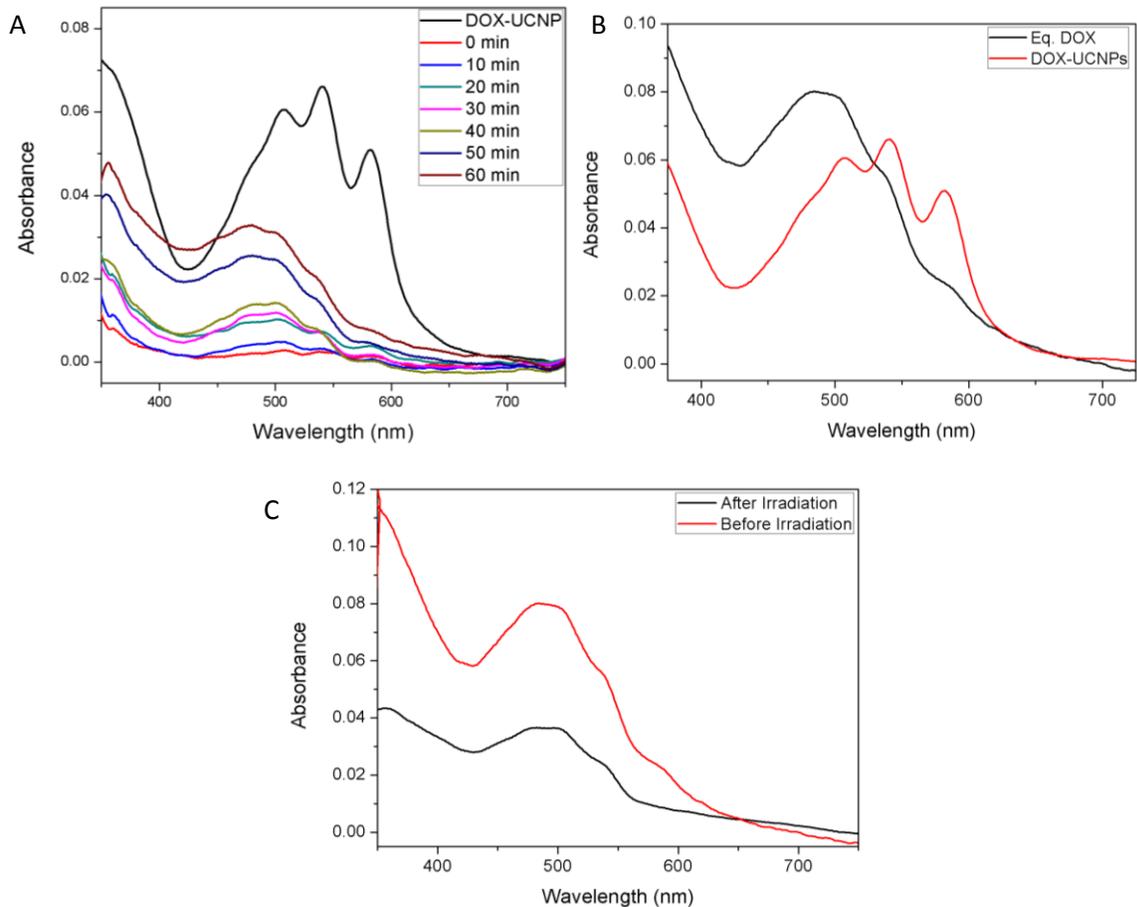


Figure S5. Evidence of Doxorubicin release by absorbance (A) Absorption spectra of the supernatant from Dox-Ln-UCNPs solution upon 980 nm irradiation at different times were measured at the time shown. At each time point the sample was sonicated and centrifuged prior to measuring absorbance. The absorbance of the DOX-UCNPs at $t = 0$ was also measured to highlight the expected shift in absorbance spectra upon release. (B) The absorption spectrum of free Dox-(COOH)₂ was also measured using the same concentration as Dox-UCNPs under the same condition to facilitate the calculation of % release. (C) The absorption spectra of an equivalent amount of Dox-(COOH)₂ compared with the spectrum of Dox released from the UCNPs. The red line gives the theoretical maximum of Dox release from the UCNPs

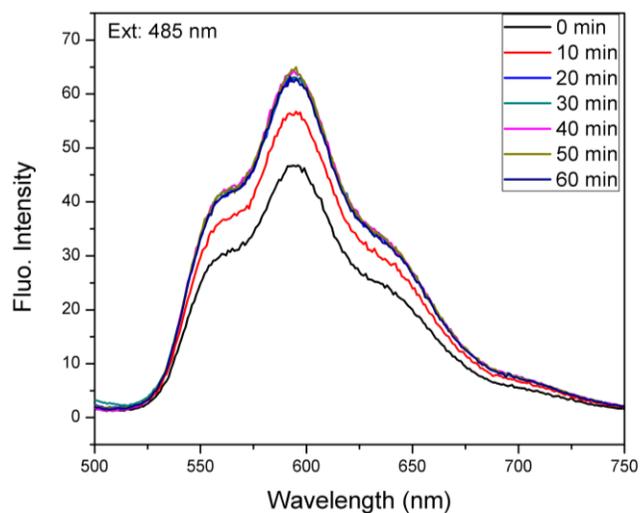
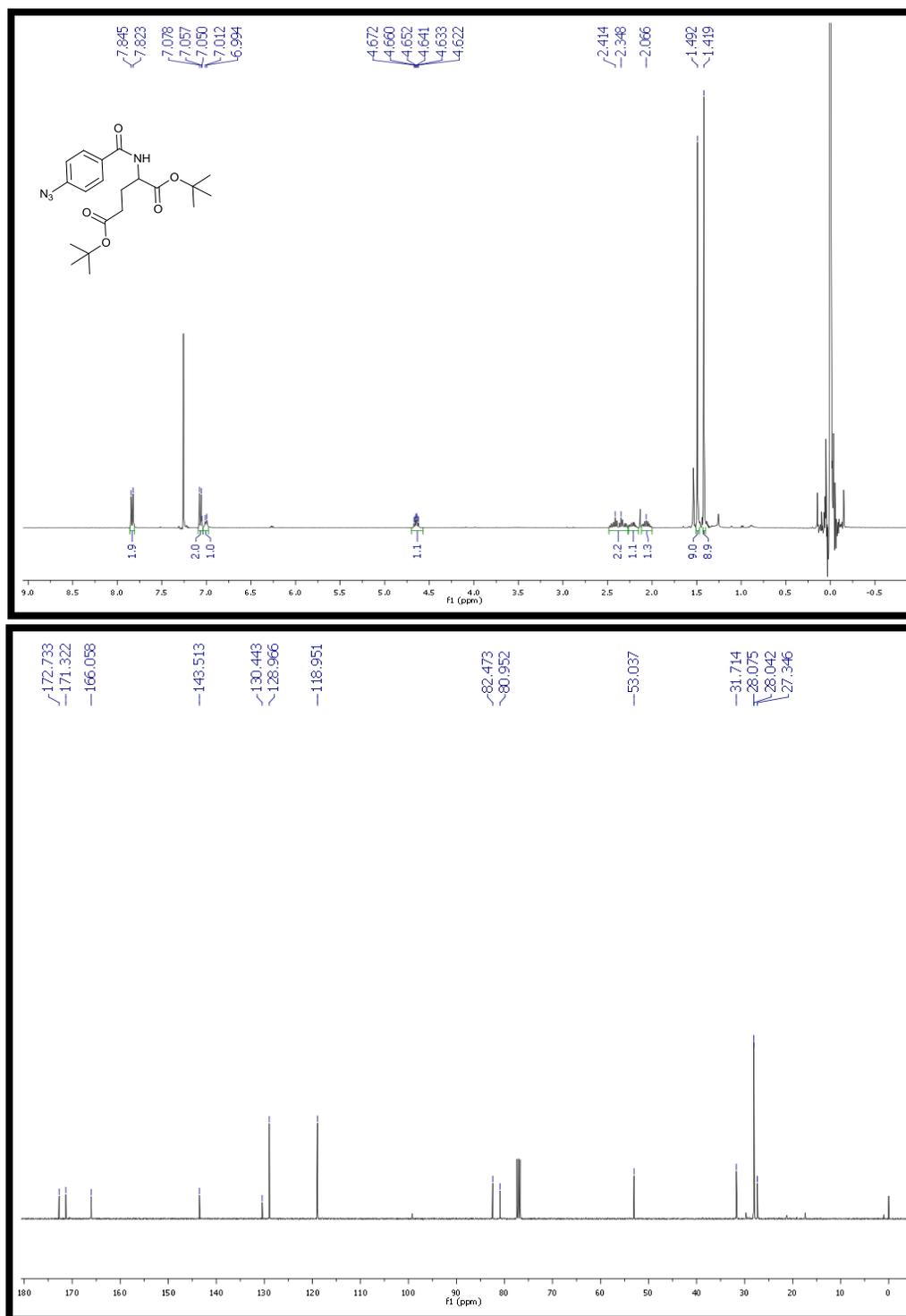
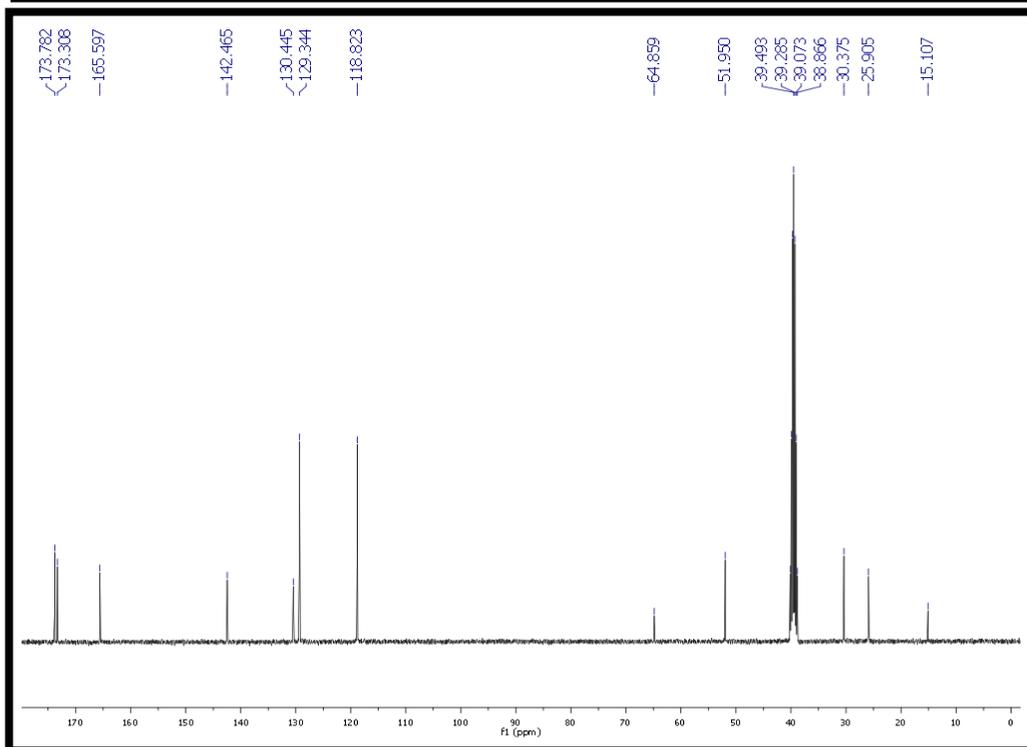
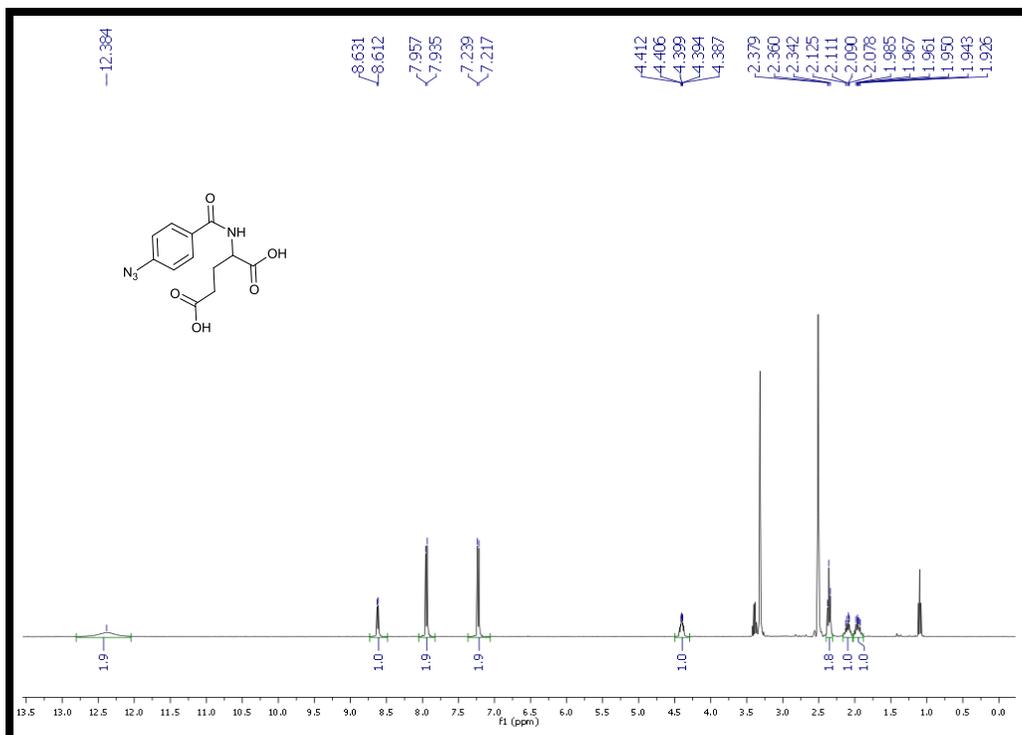


Figure S6. Evidence of Doxorubicin release through enhancement of fluorescence. Fluorescent spectra of Dox-UCNPs obtained under 980 nm irradiation at different times. The samples were excited at 485 nm and emitted at 590 nm.

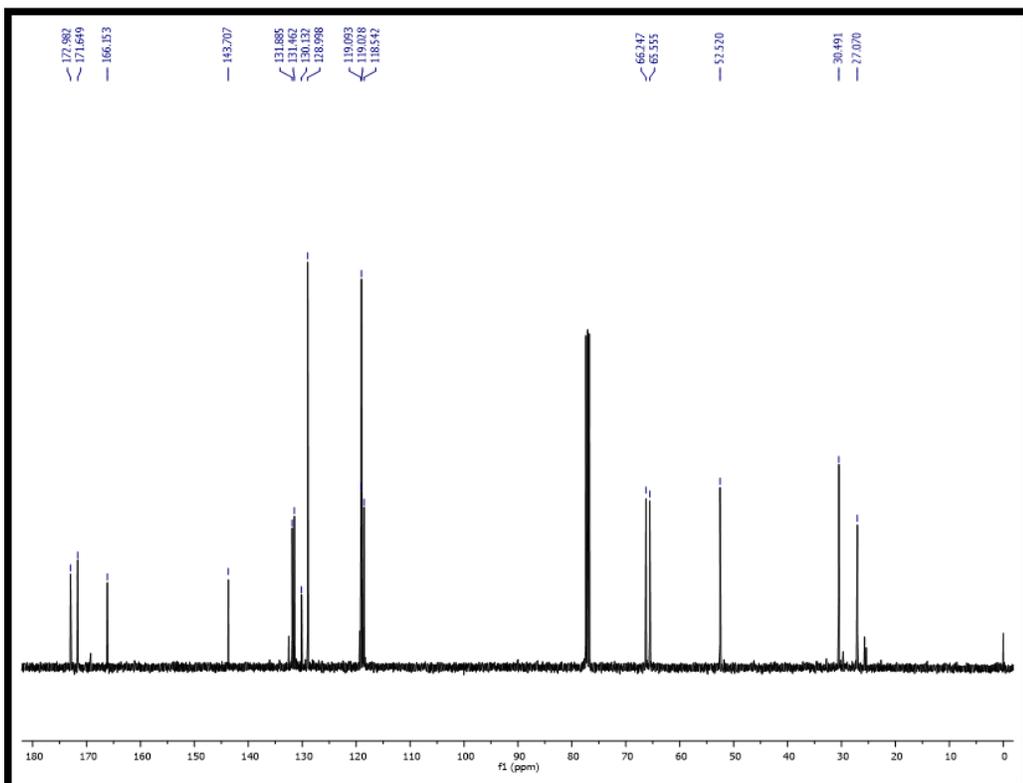
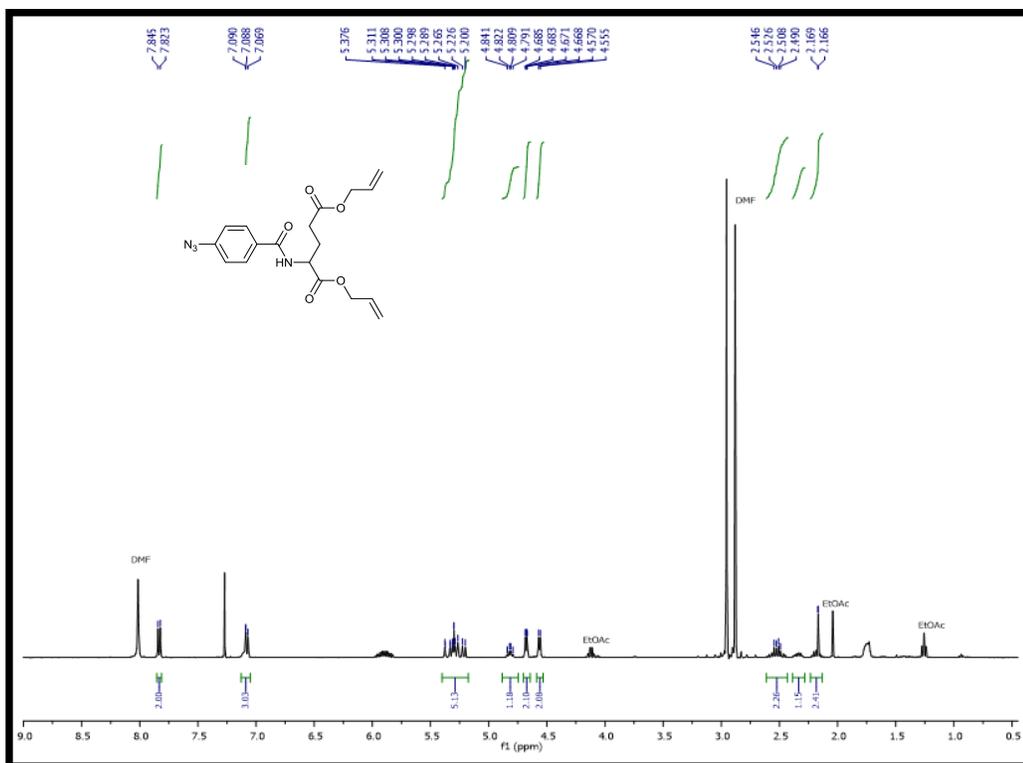
8) HPLC chromatogram, HRMS and NMR spectra of the intermediates



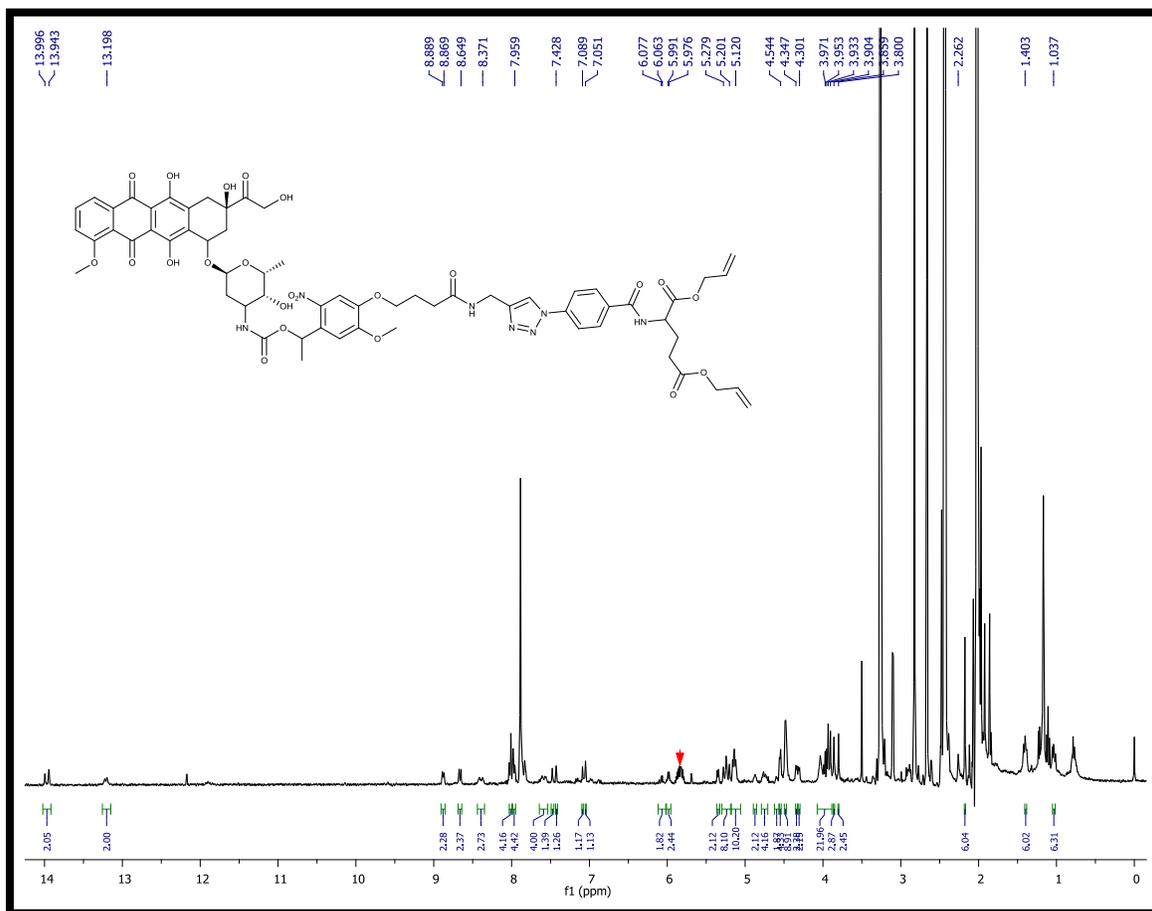
^1H NMR and ^{13}C NMR of compound **1a**



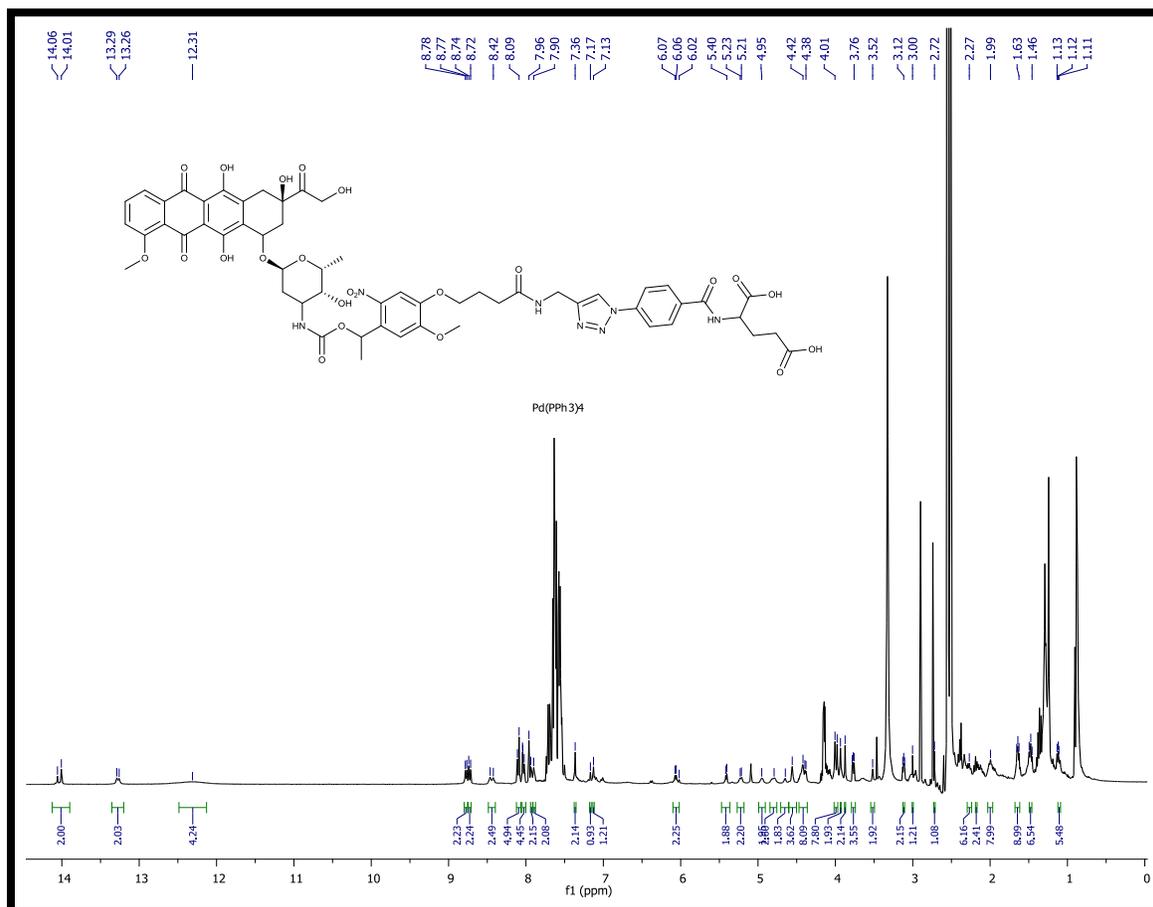
¹H NMR and ¹³C NMR of compound 2



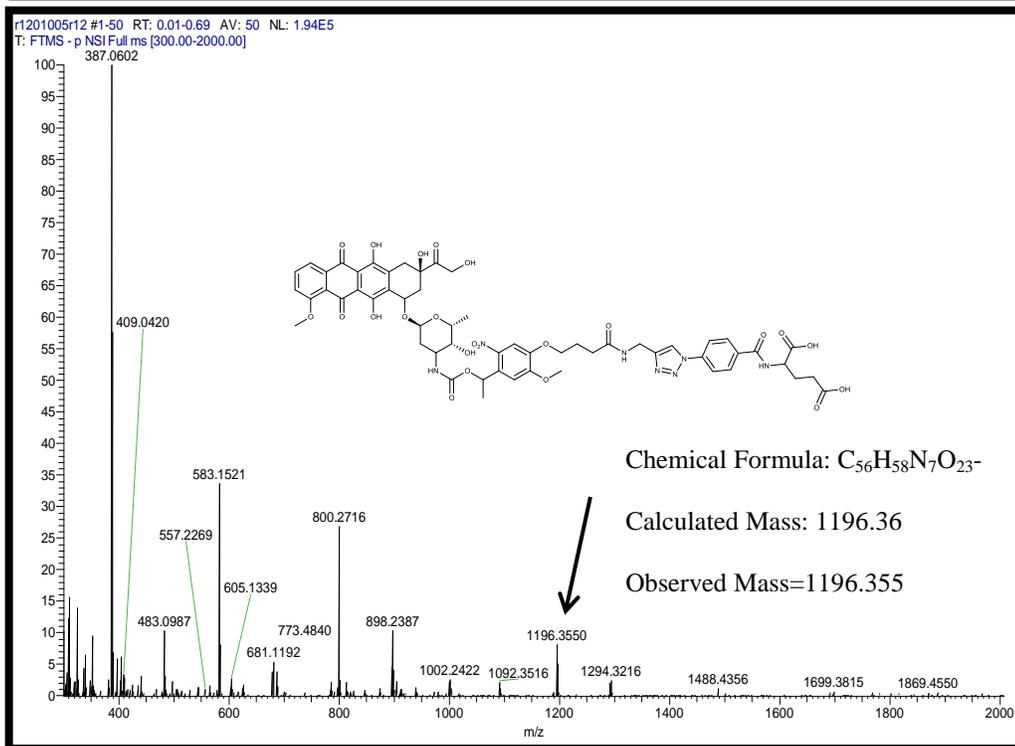
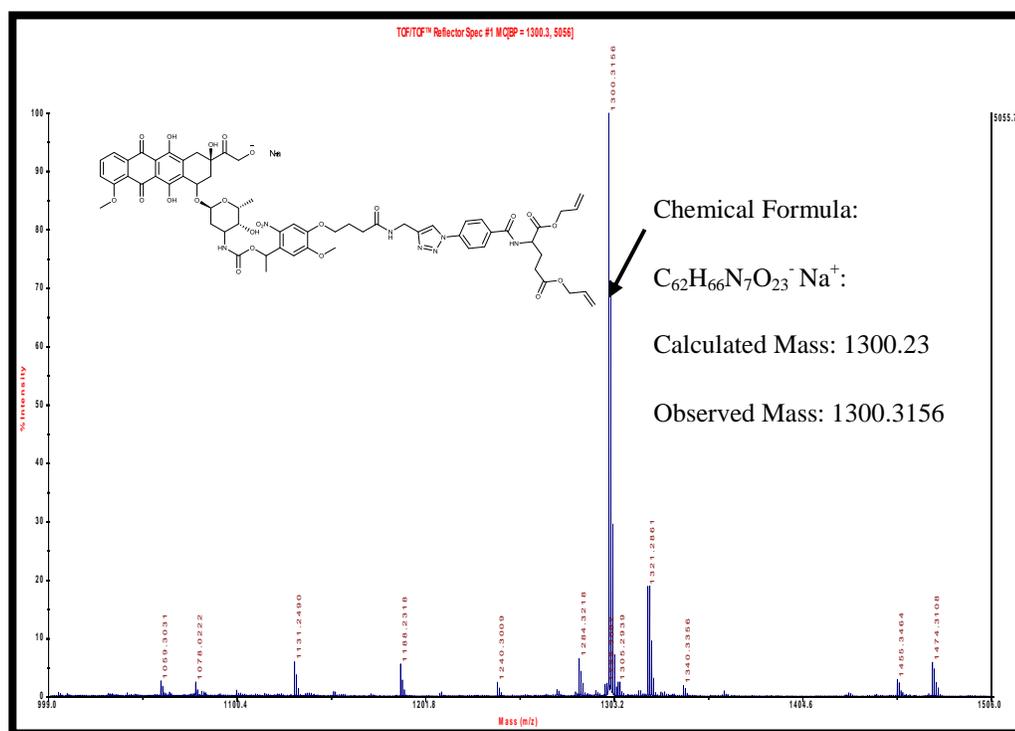
¹H NMR / ¹³C NMR of compound 3 (red pointer indicates impurity found in subsequent NMR data);



¹H NMR of compound 4



¹H NMR of compound 6



HRMS of compound 4 and compound 6

References

1. Dcona, M. M.; Mitra, D.; Goehle, R. W.; Gewirtz, D. A.; Lebman, D. A.; Hartman, M. C. Photocaged permeability: a new strategy for controlled drug release. *Chem. Commun. (Camb)* **2012**, *48*, 4755-4757.
2. V. Mahalingam, F. Vetrone, R. Naccache, A. Speghini and J. A. Capobianco, *Adv. Funct. Mater.* 2009, **21**, 4025.