Electronic Supplementary Information for

Design of Photo-cleavable Lipids and their Applications in Liposomal "Uncorking"

Binita Chandra, Sanku Mallik* and D. K. Srivastava

Department of Chemistry, Biochemistry and Molecular Biology,
North Dakota State University,
Fargo, ND 58105.

Mallik: Fax: 701-231-8831; Tel: 701-231-8829; E-mail: sanku.mallik@ndsu.edu.

Srivastava: Fax: 701-231-8324, Tel: 701-231-7831. E-mail: dk.srivastava@ndsu.edu.

Synthesis of lipids 1 and 2:

Materials:

Commercial reagents were purchased from either Aldrich or Acros Chemical Co. The protected amino-acids were purchased from Nova Biochem. The Di-Boc protected ornithine was prepared in the lab following standard Boc-protection protocol. Nitric acid (90%) was from Alfa Aesar. All solvents used for reactions were analytical grade and were used without further purification.

Melting points were determined on a micro melting point apparatus. ^{1}H and ^{13}C NMR spectra were recorded using 300, 400 or 500 MHz spectrometers using the Varian software. Solvents used for NMR were one of the following: $CDCl_3$, CD_3OD and DMSO-d_6 with TMS as the internal standard. Elemental analyses were obtained from facilities at Desert Analytics (Tucson, AZ). TLC was performed with Adsorbosil plus IP, 20 x 20 cm plate, 0.25 mm (Altech Associates, Inc.). Chromatography plates were visuialized by either UV light or in an iodine chamber. For drying water-wet compounds, lyophilization (Freeze Dry system/ Freezone 4.5; Labconco) was used. Reactions were performed either under an atmosphere of N_2 or using a guard tube. For extractive workups, the organic layer was dried over anhydrous Na_2SO_4, and concentrated *in vacuo*

4-(Boc-aminomethyl)-3-nitrobenzoic acid (3):

Trifluoroacetic anhydride (5.9 mL, 41.34 mmol) was added in small portions to solid 4-(aminomethyl) benzoic acid (2.5 g, 16.54 mmol), while applying external cooling in an ice-bath. Upon completion of addition, the reaction mixture was homogeneous. Stirring was continued at 25 ^{0}C for 2 h, and then ice water was added to precipitate the product. The white solid was collected by filtration, washed with water and dried. Yield: 3.63 g (88%), mp: 199-203 ^{o}C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.91 (d, J = 7.8 Hz, 2H), 7.37 (d, J = 7.8 Hz, 2H), 4.44 (d, J = 5.7 Hz, 2H).

The above compound (3.63 g, 14.68 mmol) was added portion wise over 1 h to 90% nitric acid (20 mL) at -5 ^{o}C. The mixture was stirred further for 1.5 h at 0 ^{o}C and then poured onto ice to precipitate the product. The precipitated solid was filtered, washed with plenty of water to neutral pH, and lyophilized to provide an off-white solid (3.95 g, 92%). mp: 210 ^{0}C; ^{1}H NMR ($CDCl_3$; 300 MHz): δ 8.61 (d, J = 1.6 Hz, 1H), 8.17 (dd, J = 1.6, 8.1 Hz, 1H), 7.507 (d, J = 8.1 Hz, 1H), 4.74 (d, J = 6.0 Hz, 2H).

A solution of compound **2** (0.68 g, 2.33 mmol) and K_2CO_3 (0.81 g, 5.88 mmol) in MeOH-H_2O (1:1, v/v; 16 mL) was maintained at 25 ^{0}C for 10 h. The dark yellow solution was concentrated, and DMF (3 x 10 mL) was added and each time removed *in vacuo.* The resultant solid was dissolved in dioxane-H_2O (1:1, v/v; 10 mL) to form a solution**.** Di-*tert*-butyl dicarbonate (0.77 g, 3.54 mmol) was added, and after 2.5 h, the reaction mixture was concentrated *in vacuo.* Ether and water were added, and the aqueous phase was washed with ether, brought to pH 3.0 with 10% aqueous citric acid, and extracted with ethyl acetate. The combined organic phases were washed with brine, dried over Na_2SO_4, and concentrated in vacuo to give the title product as a yellow solid (0.67 g, 97%), m.p. 124-126 ^{0}C; ^{1}H NMR ($CDCl_3$; 300 MHz): δ 8.74 (d, J = 1.6 Hz, 1H), 8.30 (dd, J = 1.6, 8.0 Hz, 1H), 7.77 (d, J = 8.0 Hz, 1H), 4.65 (d, J = 6.3 Hz, 2H), 1.44 (s, 9H).

Compounds 4 and 5:

Compound **3** (0.8 g, 2.7 mmol) was dissolved in $CHCl_3$ (20 mL) and stearic acid (0.71 g, 2.7 mmol), HOBt (0.364 g, 2.7 mmol), HBTU (1.024 g, 2.7 mmol) and Et_3N (0.75 mL, 5.4 mmol) were added to the solution. The mixture was stirred at room temperature for 10 h. The reaction mixture was then washed with water, the organic phase dried and solvent was removed *in vacuo.* The residue was purified by column chromatography (eluant: 5% methanol in chloroform, R_f = 0.3) to obtain the pure product as a yellow solid (1.19 g, 81%), mp: 84-86 ^{0}C; ^{1}H NMR ($CDCl_3$; 300 MHz): δ 8.41 (d, J = 1.8 Hz, 1H), 8.00 (dd, J = 1.8, 8.1 Hz, 1H), 7.68 (d, J = 8.1 Hz, 1H), 6.41 (br, s, NH, 1H), 5.30 (broad s, NH, 1H), 4.59 (d, J = 6.6 Hz, 2H), 3.45 (q, J = 6.9 Hz, 2H), 1.57-1.65 (m, 2H), 1.42 (s, 9H), 1.24-1.33 (m, 30H), 0.87 (t, J = 6.9 Hz, 3H).

To the above compound (1.16 g, 2.12 mmol), was added, 4 N HCl in dioxane (8 mL) and the reaction mixture stirred at room temperature for 3 h. The solvent was then removed under vacuum and water added to the residue. The insoluble white solid was filtered, washed with plenty of water and dried to give the deprotected compound (0.89 g, 94%) as a yellow solid. The compound was carried on to the next step without further purification. ^{1}H NMR ($CDCl_3$; 300 MHz): δ 8.54 (d, J = 1.8 Hz, 1H), 8.03 (dd, J = 1.8, 7.5 Hz, 1H), 7.75 (d, J = 7.5 Hz, 1H), 4.31 (s, 2H), 3.31 (q, J = 6.9 Hz, 2H), 1.53 (m, 2H), 1.1-1.4 (m, 30H), 0.78 (t, J = 7 Hz, 3H).

The deprotected compound mentioned in the previous step (0.3 g, 0.67 mmol), Boc-Asp(O^{t}Bu)-OH.DCHA salt (0.316 g, 0.67 mmol), HOBT (0.091 g, 0.67 mmol) and HBTU (0.25 g, 0.67 mmol) were taken in DMF (15 mL) and N-methylmorpholine (0.15 mL, 1.34 mmol) was added. The reaction mixture was stirred at room temperature overnight. The solvent was removed *in vacuo*. Water was added to the residue and extracted with ethyl acetate. The combined organic phases were dried and solvent was removed by rotary evaporation. The crude product was purified by silica gel chromatography (eluant: $CHCl_3$, R_f = 0.2) to yield compound **4** as a white solid (0.480 g, 99%), mp: 90-92 ^{0}C; ^{1}H NMR ($CDCl_3$; 500 MHz): δ 8.41 (d, J = 1.6 Hz, 1H), 7.98 (dd, J = 1.6, 8.0 Hz, 1H), 7.70 (d, J = 8.0 Hz, 1H), 4.82-4.70 (m, 2H), 4.51-4.44 (m, 1H), 3.47 (q, J = 7 Hz, 2H), 2.96-2.88 (m, 1H), 2.62-2.58 (m, 1H), 1.66-1.58 (m, 2H), 1.45 (s, 9H), 1.42 (s, 9H), 1.40-1.20 (m, 30H), 0.88 (t, J = 7.0 Hz, 3H).

In an analogous way, the deprotected compound (0.3 g, 0.67 mmol), Boc-Glu(O^{t}Bu)-OH (0.2 g, 0.67 mmol), HOBT (0.09 g, 0.67 mmol) and HBTU (0.25 g, 0.67 mmol) were taken in DMF (15 mL) and N-methylmorpholine (0.15 mL, 1.34 mmol) was added. The reaction mixture was stirred at room temperature overnight. The work-up procedure was the same as described for compound **4**. The crude product was then purified by silica gel chromatography (eluant: $CHCl_3$, R_f = 0.3) to provide the glutamic acid derivative **5** as a yellow solid. Yield: 0.34 g (70%) ^{1}H NMR ($CDCl_3$; 500 MHz): δ 8.42 (d, J = 1.6 Hz, 1H), 8.98 (dd, J = 1.6 Hz, 8.0 Hz, 1H), 7.71 (d, J = 8.0 Hz, 1H), 4.75 (d, J = 6 Hz, 2H), 4.15-4.08 (m, 1H), 3.47 (q, J = 7 Hz, 2H), 2.43-2.37 (m, 1H), 2.31-2.25 (m, 1H), 2.11-2.02 (m, 1H), 1.95-1.87 (m, 1H), 1.65-1.6 (m, 2H), 1.45 (s, 9H), 1.42 (s, 9H), 1.40-1.20 (m, 30H), 0.88 (t, J = 7.0 Hz, 3H).

Lipid 1:

To the Boc-Asp(O^{t}Bu) derivative (0.40 g, 0.56 mmol), was added 4 mL of trifluoroacetic acid and a drop of anisole. The reaction mixture was stirred at room temperature for two hours. It was then slowly added to water and aqueous NaOH solution was slowly added to neutralize the TFA. The precipitate was collected by filtration, washed with plenty of water and dried to give lipid **1** as a off-white solid (0.27 g, 85%) ; mp: 154-157 ^{0}C; ^{1}H NMR (DMSO-*d*$_6$; 400 MHz) (without exchangeable protons): δ 8.43 (d, J = 1.6 Hz, 1H), 8.10 (dd, J = 1.6, 8.0 Hz, 1H), 7.62 (d, J = 8.0 Hz, 1H), 4.66-4.56 (m, 2H), 3.96-3.92 (m, 1H), 3.24 (q, J = 7 Hz, 2H), 2.75-2.58 (m, 2H), 1.95 (m, 2H) 1.60-1.40 (m, 2H), 1.35-1.17 (m, 30H), 0.82 (t, J = 7.0 Hz, 3H); ^{13}CNMR (DMSO-*d*$_6$; 400 MHz) δ 176.05, 171.99, 64.42, 148.52, 136.77, 135.50, 132.54, 130.66, 123.81, 50.71, 37.19, 31.87, 30.38, 29.58-29.23, 27.10, 22.64, 14.45. Anal. Calcd. for $C_{30}H_{50}N_4O_6.3CF_3COONa.4H_2O$: C, 41.46; H, 5.61; N, 5.37. Found: C, 41.25; H, 5.92; N, 5.43.

Lipid 2:

To the Boc-Glu(O^{t}Bu) derivative (0.26 g, 0.36 mmol), was added 4 mL of trifluoroacetic acid and a drop of anisole. The reaction mixture was stirred at room temperature for 2 h. The work-up procedure was the same as described for lipid **1**. Lipid **2** was isolated as a white solid (0.2 g, 99%) ; ^{1}H NMR (DMSO-*d*$_6$) (exchangeable protons not reported): δ 8.49 (d, J = 1.6 Hz, 1H), 8.14 (dd, J= 1.6, 8.0 Hz, 1H), 7.64 (d, J = 8.0 Hz, 1H), 4.70-4.62 (m, 2H), 3.92-3.84 (m, 1H), 3.24 (q, J = 7 Hz, 2H), 2.36-2.22 (m, 2H), 1.99-1.90 (m, 2H), 1.58-1.40 (m, 2H), 1.35-1.10 (m, 30H), 0.82 (t, J = 7.0 Hz, 3H); ^{13}CNMR ($CDCl_3$-CD_3OD; 400 MHz): δ 176.02,

169.43, 165.68, 147.94, 135.73, 135.38, 132.15, 130.50, 124.08, 52.69, 40.97, 40.47, 31.96, 30.38, 29.73-29.38, 27.13, 26.72, 22.70, 14.03. Anal. Calcd. for $C_{31}H_{52}N_4O_6.CF_3COONa.H_2O$: C, 55.61; H, 7.85; N, 7.86. Found: C, 55.37; H, 8.07; N, 8.02.

***Preparation of dye-encapsulated small liposomes*:**

The photocleavable lipid (0.45 μmoles, 5 mol%) and solid 1,2-distearoyl-sn-glycero-3-phosphocholine (6.716 mg, 8.55 μmoles, 85 mol%) were dissolved in 5 mL of anhydrous chloroform and a very small amount (0.5 mL) of anhydrous methanol in a 25 mL clean, oven-dried round bottomed flask. The organic solvents were then removed in a rotary evaporator under reduced pressure maintaining the bath temperature at 40 ^{0}C until a thin and uniform lipid film was formed on the walls of the round bottomed flask. The flask was left on the rotary evaporator for an additional 15 minutes and then allowed to dry *in vacuo* for at least 20 hours.

In another clean dry glass vial, 56 mg (150 μmoles) of 6-carboxyfluorescein was taken in 3 mL of HEPES buffer (25 mM, pH = 8.0). The dye was dissolved by first bath-sonicating (to reduce the particle size of the solid granules of the dye) to form a dark brown transparent solution. The thin dry lipid film was then hydrated with the dye solution (3 mL) by rotating slowly in the rotary evaporator bath at 60 ^{0}C for 1 hour. The resulting suspension was then subjected to probe sonication (power: 50 W) at 60 ^{0}C for 1 hour with constant nitrogen bubbling, to get a clear dark red liposome solution. The total lipid concentration was 9 mM. The osmolarity of the liposome solution was measured with a standard micro osmometer.

Sephadex G-50 resin (particle size 50-150μ) was mixed with excess of water to form a gel and the gel was hydrated overnight at 40 ^{0}C in the water bath of a regular rotary evaporator. A chromatography column was packed with the gel after cooling to room temperature and equilibriated with 200 mL of water whose osmolarity was made equal to that of the liposome solution by the addition of solid sodium chloride. The liposome solution was then loaded on top of the column and slowly eluted. The liposomes came out first as a yellow non-fluorescent solution and were collected.

***Transmission electron microscopy of small liposomes*:**

Poly-L-lysine (0.5%) was placed on formvar film carbon coated 300 mesh grid for 30 seconds and wicked off with torn filter paper and allowed to dry. Liposome sample was placed on the same grid for 30 seconds and wicked off. The grid was then negatively stained with 0.5% phosphotungstic acid pH adjusted to 7-8 for 1.5 min and wicked off. After allowing the sample to dry, images were obtained using a JEOL 100CX II Transmission Electron Microscope at 80 KeV.

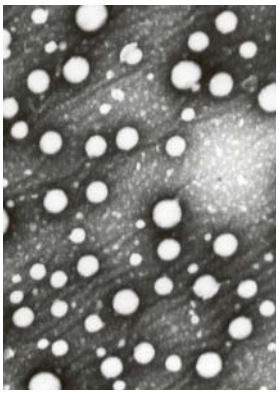

Figure S1. Transmission electron microscopic picture of liposomes incorporating 5% of lipid **1** is shown (magnification: 46,000).

***Leakage experiments from the liposomes*:**

The fluorescence emission spectrum of the dye-encapsulated liposomes was recorded with excitation at 580 nm. The quartz cuvet was then placed under a UV lamp (100 W lamp for the 365 nm irradiation). Every 5 minutes, the cuvet was transferred to the fluorimeter and the emission spectrum was recorded. The intensity of the emission maximum (520 nm) was plotted as a function of time to generate the release curves for the dye-encapsulated liposomes.

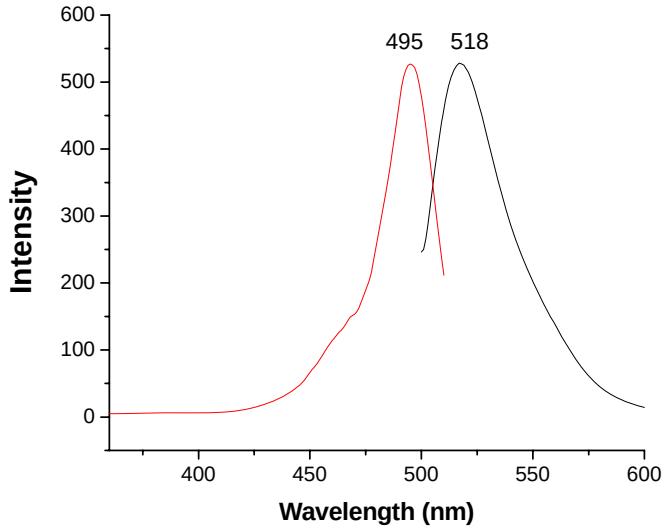

Figure S2. Excitation and emission spectra of a solution of 6-carboxyfluorescein in HEPES (25 mM, pH = 8.0) buffer is shown. For this spectrum, the parameters are: [dye] = 50 μM; slit widths for excitation and emission monochromators: 5 nm. The excitation spectrum was recorded with emission monochromator at 518 nm; for the emission spectrum, the excitation wavelength was 480 nm.

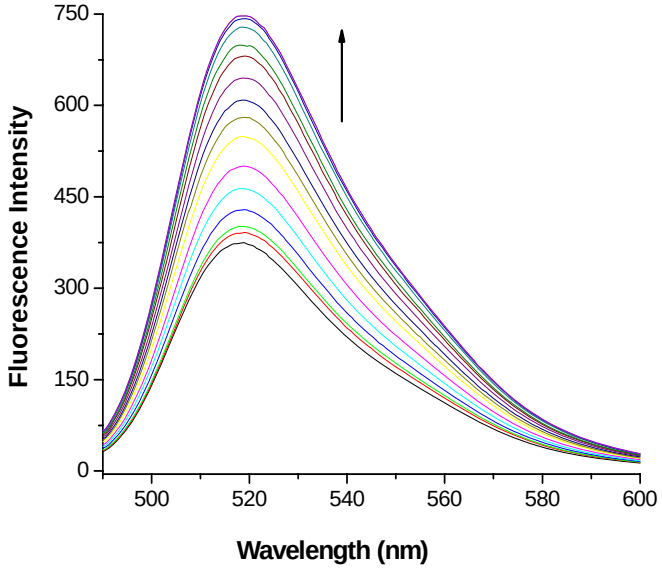

Figure S3. Time dependent increase in fluorescence emission intensity at 518 nm (λ_{ex} = 495 nm) upon irradiation of 6-carboxyfluosceine encapsulated liposomes at 365 nm. The spectra were recorded during 180 minutes of irradiation, in 10 minute intervals.