Charge-Reversal Amphiphiles for Gene Delivery

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All solvents were dried and freshly distilled prior to use. All chemicals were purchased from Aldrich or Acros and used without further purification. All reactions were performed under nitrogen atmosphere. NMR spectra were recorded on a Varian INOVA spectrometer operating at 400 MHz (for ^{1}H). Elemental analysis was obtained from Atlantic Microlab, Inc. A TA Instruments DSC 2920 Modulated DSC was used to collect T_m data. Calf thymus DNA and EtBr were purchased from Sigma. Fluorescence studies were carried out with a Fluorolog 3, ISA Jobin Yvon-Spex, Horiba group.

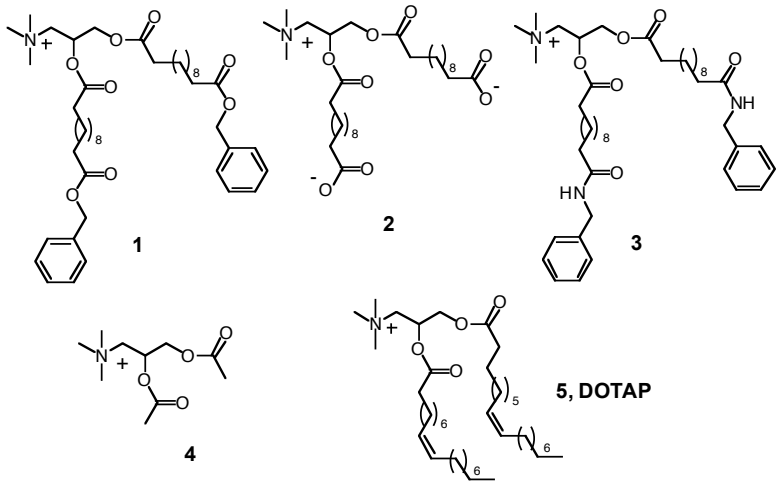
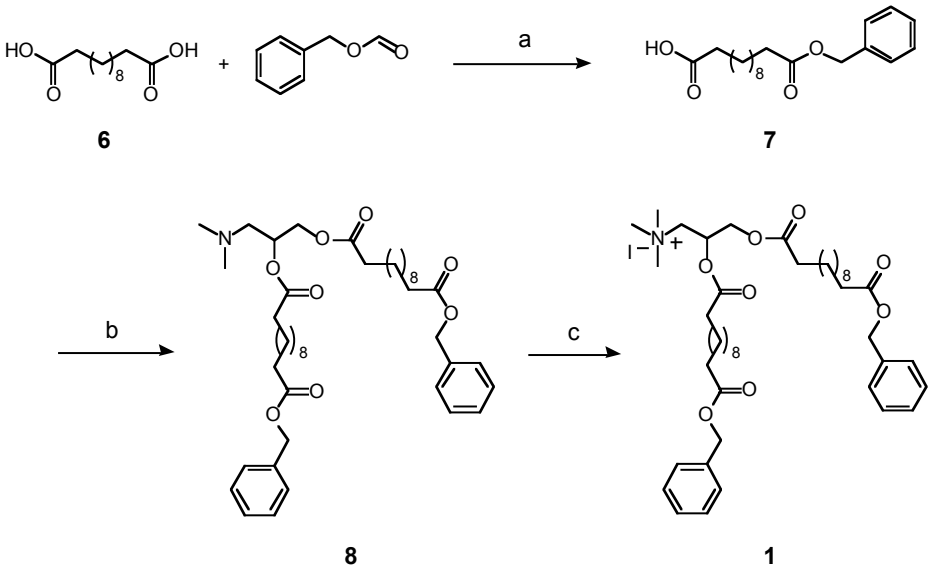


Figure S1. Structures of amphiphiles under study.

Dodecanedioic acid monobenzyl ester (7): Dodecanoic diacid (1 mmol) and Dowex 50W-X2 (50-100 mesh) (1.0 g) were stirred in benzyl formate/ octane (2:8, 10 mL) at 80 ºC. The reaction was stirred for 12 h. The solution was then filtrated and the filtrate evaporated. The crude product was purified by column chromatography to afford the compound as a white powder (79 % yield). ^{1}H NMR ($CDCl_3$) δ ppm 1.34 (m, 10 H, $(CH_2)_n$), 1.61 (m, 4H, CH_2), 2.32 (m, 4H, CH_2), 5.08 (s, 2H, CH_2), 7.34 (m, 5H, Ph); ^{13}C NMR ($CDCl_3$) δ ppm 25.16 ($\underline{C}H_2CH_2CO_2Ph$), 25.25 ($\underline{C}H_2CH_2CO_2H$), 29.40 $(CH_2)_n$, 29.57 $(CH_2)_n$, 29.71 $(CH_2)_n$, 34.40 ($\underline{C}H_2CO_2Ph$), 34.62 ($\underline{C}H_2CO_2H$), 66.55 ($CO_2\underline{C}H_2Ph$), 127.22(Ph), 127.86(Ph), 128.38(Ph), 128.76(Ph), 136.29(Ph), 174.04 ($\underline{C}O_2Ph$), 179.73 ($\underline{C}O_2H$). MH^+ (GC-CIMS) = 321. The data agreed with the literature values (Saitoh, M., Fujisaki, S., Ishii, Y., Nishiguchi, T., *Tetrahedron Letters*, **1996**, *37*, 6733-6736).



Reagents and conditions: a) octane, Dowex 50W-X2, 80 °C, 12 hrs, yield 79%; b) 3-(dimethylamino)-1,2-propanediol, DCC, DMAP, DCM, 25 °C, 48 hrs, yield 80%; d) MeI / DCM, 25 °C, 4 hrs, yield 90%.

Scheme S1. Synthesis of amphiphile 1.

Dodecanedioic acid benzyl ester 2-(11-benzyloxycarbonyl-undecanoyloxy)-3-dimethylamino -propyl ester (8): To solution of **7** (1.78 g, 5.5 mmol), 3-dimethylamino-propane-1,2-diol (0.3 mL, 2.5 mmol) and DMAP (catalytic amount) in DCM (15 mL), a solution of DCC (1.1 g, 5.5 mmol) in DCM (5 mL) was added. After the addition, the solution was stirred for 2 days. The reaction mixture was then filtered to remove the insoluble DCU. Concentration of the filtrate followed by chromatography (50% EtOAc/DCM to 100% EtOAc) afforded the product as colorless oil (80% yield). ^{1}H NMR

($CDCl_3$) δ ppm 1.23 (d, J = 4.8 Hz, 24H), 1.32 – 1.63 (m, 8H), 2.23 – 2.33 (m, 14H), 2.37 – 2.47 (m, 2H), 4.03 – 4.08 (m, 1H), 4.03 – 4.34 (m, 1H), 5.07 (s, 4H), 5.13 – 5.19 (m, 1H), 7.25 – 7.35 (m, 10H); ^{13}C NMR ($CDCl_3$) δ ppm 25.13, 29.30, 29.42, 29.58, 34.17, 34.34, 34.51, 34.59, 46.22, 59.55, 64.10, 66.10, 66.24, 69.36, 128.35, 128.73, 136.34, 173.38, 173.67, 173.84; HRMS calcd. for $[C_{43}H_{65}NO_8 + H]^+$ 724.4788, found 724.4785.

[2,3-Bis-(11-benzyloxycarbonyl-undecanoyloxy)-propyl]-trimethyl-ammonium; iodide (1): MeI (1 mL, 15 mmol) was added to a solution of **3** (0.9 g, 1.2 mmol) in DCM (5 mL). The solution was stirred for 4 hours and then concentrated. The residue was washed with ethyl ether to afford the product (90 % yield) as a white powder. ^1H NMR ($CDCl_3$) δ ppm 1.22 (d, J = 3.6 Hz, 24H), 1.55 – 1.61 (m, 8H), 2.30 (t, J = 7.2 Hz, 8H), 3.49 (s, 9H), 3.82 – 3.87 (m, 1H), 4.08 – 4.13 (m, 1H), 4.45 – 4.49 (m, 2H), 5.07 (s, 4H), 5.57 (s, 1H), 7.27 – 7.34 (m, 10H); ^{13}C NMR ($CDCl_3$) δ ppm 24.78, 24.92, 25.11, 29.28, 29.38, 29.52, 29.56, 34.16, 34.38, 34.50, 54.95, 63.08, 65.89, 66.25, 66.38, 128.32, 128.35, 128.74, 136.32, 172.92, 173.37, 173.88; HRMS calcd. for $[C_{44}H_{68}NO_8I - I]^+$ 738.4945, found 738.4951. Elemental analysis: C, 61.28%; H, 8.10%; N, 2.33% (theory: C, 61.03%; H, 7.92%; N, 1.62%).

Dodecanedioic acid mono-tert-butyl ester To an ice-cold solution of dodecanedioic acid **6** (15 g, 65 mmol), *tert*-butyl alcohol (64 mL, 650 mmol) and DMAP (catalytic amount) in THF (80 mL), a solution of DCC (16 g, 78 mmol) in THF (20 mL) was slowly added. After the addition, the solution was warmed to room temperature and stirred for 24 hours. The reaction mixture was then filtered to remove the insoluble DCU. Concentration of the filtrate followed by chromatography (20% EtOAc / Hexane to 40% EtOAC / Hexane) afforded 9 g (50% yield) of the product. ^1H NMR ($CDCl_3$) δ ppm 1.23 (s, 12H), 1.40 (s, 9H), 1.51 – 1.60 (m, 4H), 2.16 (t, J = 7.2 Hz, 2H), 2.30 (t, J = 7.6 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ ppm 24.85, 25.28, 28.30, 29.24, 29.37, 29.43, 29.55, 34.23, 35.81, 80.13, 173.62, 180.08. The data agreed with the literature values (Ogawa, Y., Kodaka, M. and Okuno, H. *Chemistry and Physics of Lipids* **2002,** *119*, 51 – 68).

Dodecanedioic acid 2-(11-tert-butoxycarbonyl-undecanoyloxy)-3-dimethylamino-propyl ester tert-butyl ester: To a solution of dodecanedioic acid mono-tert-butyl ester (4.6 g, 16 mmol), 3-dimethylamino-propane-1,2-diol (0.9 mL, 7.6 mmol) and DMAP (catalytic amount) in DCM (40 mL), a solution of DCC (4 g, 20 mmol) in DCM (10 mL) was slowly added. After the addition, the solution

was stirred for 2 days. The reaction mixture was then filtered to remove the insoluble DCU. Concentration of the filtrate followed by chromatography (50% EtOAc / DCM to 100% EtOAc) afforded 2.3 g (46% yield) of product as a colorless oil. ^{1}H NMR ($CDCl_3$) δ = 1.23 (d, 24H), 1.42 (s, 18H), 1.58 (m, 8H), 2.23 (m, 8H), 2.47 (m, 4H), 2.62 (m, 2H), 4.03 – 4.34 (m, 2H), 5.19 (m, 1H); ^{13}C NMR ($CDCl_3$) δ = 25.33, 29.30, 29.40, 29.52,30.0, 30.3, 34.27, 34.32, 46.22, 64.10, 66.10, 69.36, 173.28, 173.47;

[2,3-Bis-(11-tert-butoxycarbonyl-undecanoyloxy)-propyl]-trimethyl-ammonium; iodide: MeI (1 mL, 15 mmol) was added to a solution of dodecanedioic acid 2-(11-tert-butoxycarbonyl-undecanoyloxy)-3-dimethylamino-propyl ester tert-butyl ester (1.1 g, 1.7 mmol) in DCM (5 mL). The solution was stirred for 4 hours and then concentrated. The residue was recrystallized in DCM/Ethyl ether to afford the product (75 % yield) as a white powder. ^{1}H NMR ($CDCl_3$) δ ppm 1.23 (s, 24H), 1.39 (s, 9H), 1.52 – 1.56 (m, 8H), 2.15 (t, J = 7.6 Hz, 4H), 2.29 – 2.33 (m, 4H), 3.50 (s, 9H), 3.82 – 3.88 (m, 1H), 4.08 – 4.12 (m, 1H), 4.45 – 4.49 (m, 2H), 5.07 (s, 4H); ^{13}C NMR ($CDCl_3$) δ ppm 24.78, 24.93, 25.28, 28.31, 29.26, 29.37, 29.45, 29.55, 29.60, 34.16, 34.38, 35.79, 54.95, 63.05, 65.88, 66.34, 80.09, 172.91, 173.37, 173.53; HRMS calcd for $[C_{38}H_{72}NO_8I - I]^+$ 670.5258, found 670.5268.

[2,3-Bis-(11-carboxy-undecanoyloxy)-propyl]-trimethyl-ammonium; iodide (2) 0.70 g of [2,3-bis-(11-tert-butoxycarbonyl-undecanoyloxy)-propyl]-trimethyl-ammonium iodide and TFA (6 mL) in DCM (24 mL) were stirred at room temperature for 4 hours. The solution was then concentrated and the residue was recrystallized in MeOH/ethyl ether to afford 0.54 g (90 % yield) of product. ^{1}H NMR (DMSO – d_6) δ ppm 1.19 (s, 24H), 1.41 – 1.48 (m, 8H), 2.13 (t, J = 7.6 Hz, 4H), 2.24 – 2.34 (m, 4H), 3.05 (s, 9H), 3.64 – 3.74 (m, 2H), 4.00 – 4.04 (m, 1H), 4.31 – 4.34 (m, 1H), 5.44 (d, J = 3.2 Hz, 1H), 11.95 (s, 2H); ^{13}C NMR (DMSO – d_6) δ ppm 24.76, 24.93, 25.14, 29.04, 29.21, 29.35, 29.40, 29.52, 33.89, 34.18, 34.31, 53.80, 63.69, 65.47, 66.60, 172.75, 173.14, 175.11; HRMS calcd for $[C_{30}H_{56}NO_8I - I]^+$ 558.4006, found 558.4001. Elemental analysis: C, 52.22%; H, 8.20%; N, 2.01% (theory: C, 52.55%; H, 8.23%; N, 2.04%).

11-Benzylcarbamoyl-undecanoic acid

To a solution of dodecanedioic acid **6** (15 g, 65 mmol), benzylamine 9 (64 mL, 650 mmol) in THF (80 mL), a solution of DCC (16 g, 78 mmol) in THF (20 mL) was added. The reaction mixture was stirred for 24 hours and then filtered to remove the insoluble DCU. Concentration of the filtrate followed by

chromatography (20% EtOAc/hexane) afforded 9 g (50% yield) of the product as white solid. ^{1}H NMR (DMSO– d_6) δ ppm 1.18 (m, 12H), 1.44 (m, 4H), 2.11 (m, 4H), 4.20 (s, 2H), 7.23 (m, 5H), 8.21 (m, 1H); ^{13}C NMR (DMSO– d_6) δ ppm 24.86, 25.89, 29.09, 29.35, 29.48, 29.67, 29.86, 33.91, 34.28, 35.78, 46.34, 127.99, 128.75, 140.07, 173.01, 178.12; HRMS calcd for $[C_{19}H_{29}NO_3H]^+$ 319.2147, found 320.2241.

11-Benzylcarbamoyl-undecanoic acid 2-(11-benzylcarbamoyl-undecanoyloxy)-3-dimethylamino-propyl ester

To a solution of 11-benzylcarbamoyl-undecanoic acid (0.5 g, 1.56 mmol), 3-dimethylamino-propane-1,2-diol (0.08 mL, 0.78 mmol), and DMAP (catalytic amount) in DCM (20 mL), a solution of DCC (0.4 g, 20 mmol) in DCM (5 mL) was added. After the addition, the solution was stirred for 2 days. The reaction mixture was then filtered to remove the insoluble DCU. Concentration of the filtrate followed by chromatography (EtOAc) afforded 0.56 g (46% yield) of product as a colorless oil. ^{1}H NMR ($CDCl_3$) δ ppm = 1.20 (m, 24H), 1.59 (m, 8H), 2.25 (m, 14H), 2.37 (m, 2H), 2.60 (m, 4H), 4.02-4.34 (m, 2H), 5.04 (s, 4H), 5.17 (m, 1H), 7.22 (m, 10H); ^{13}C NMR ($CDCl_3$) δ ppm = 25.13, 28.79, 29.31, 29.43, 29.58, 34.33, 34.53, 46.24, 59.57, 64.10, 66.22, 69.38, 128.23, 128.59, 133.55, 144.52, 173.38, 173.66, 173.89; HRMS calcd for $[C_{43}H_{67}IN_3O_6H]^+$ 722.5108, found 722.5095.

[2,3-Bis-(11-benzylcarbamoyl-undecanoyloxy)-propyl]-trimethyl-ammonium; iodide (3)

MeI (1 mL, 15 mmol) was added to a solution of 11-benzylcarbamoyl-undecanoic acid 2-(11-benzylcarbamoyl-undecanoyloxy)-3-dimethylamino-propyl ester (0.5 g, 0.7 mmol) in DCM (5 mL). The solution was stirred for 4 hours and then concentrated. The residue was washed with ethyl ether to afford 0.57 g of product (95 % yield) as white powder. ^{1}H NMR (DMSO) δ ppm 1.18 (s, 24H), 1.45 (m, 8H), 2.30 (m, 8H), 3.27 (s, 9H), 3.36 (dd, 2H), 4.08 (m, 2H), 4.37 (m, 4H), 5.44 (s, 1H), 7.19 – 7.30 (m, 10H); ^{13}C NMR (DMSO) δ ppm 24.78, 24.98, 26.37, 29.05, 29.40, 29.56, 29.64, 29.86, 33.91, 34.20, 53.85, 70.24, 72.42, 72.44, 127.98, 128.83, 139.38, 172.74, 173.12. HRMS calcd for $[C_{44}H_{70}IN_3O_6^+ - I]^+$ 736.5265, found 736.5260. Elemental analysis: C, 61.22%; H, 8.17%; N, 4.86% (theory: C, 61.17%; H, 8.05%; N, 4.81%).

(2,3-Diacetoxy-propyl)-trimethyl-ammonium; iodide (4): A mixture of 3-dimethyl -amino-propane-1,2-diol (0.30 mL, 2.5 mmol), acetyl anhydride (2 mL, excess) and DCM (5mL) was stirred overnight at room temperature. MeI (2 mL, excess) was then added to the solution and the reaction was stirred for an additional 4 hours. Next, the solution was concentrated and ethyl ether (20 mL) was added to the residue to precipitate the product (0.73 g, 85 % yield). ^{1}H NMR (DMSO – d_6) δ ppm 2.02 (d, J = 10.8 Hz, 6H), 3.07 (s, 9H), 3.68 – 3.73 (m, 2H), 4.04 – 4.08 (m, 1H), 4.25 – 4.29 (m, 1H), 5.40 (d, J = 4 Hz, 1H); ^{13}C NMR (DMSO – d_6) δ ppm 21.22, 21.59, 53.89, 63.76, 65.38, 66.72, 170.33, 170.70; HRMS calcd for $[C_{10}H_{20}NO_4I - I]^+$ 218.1392, found 218.1398. Elemental analysis: C, 34.76%; H, 5.77%; N, 4.03% (theory: C, 34.80%; H, 5.84%; N, 4.06%).

Liposome preparation

Chloroform solutions of the amphiphiles were mixed and dried to a thin film by rotary evaporation. The residual solvent was removed under vacuum for 4h. Dried lipids were dispersed with 100 mM Tris buffer, 100 mM NaCl, pH 7.4, and sonicated (bath) for 10 min. The concentration of each lipid was 3 mM.

Modulated differential scanning calorimetry.

0.5 mg of amphiphile in 5μL of water was hermetically sealed in an aluminum pan. The modulation was set to ±1.00 ºC every 40 s, and the pan was equilibrated at –15 ºC. The temperature was increased at 0.5 ºC/min to 70 ºC where it has held for 2 min. The temperature was then reduced to –10 ºC and held at this temperature for 2 min. This heating-cooling cycle was repeated two more times before the sample was held isothermal at –10 ºC for 20 min. The data was colleted on the third cycle.

DNA binding affinities

DNA binding studies were carried out by competitive displacement fluorometric assay using ethidium bromide. This assay involves the addition of aliquots of the compound to a 3 mL solution of EthBr (1.3 μM) and calf thymus DNA (3 μM) in buffer (100 mM NaCl, 100 mM Tris, pH 7.4) with the decrease of fluorescence (λ_{exc}=546 nm, λ_{em}=600 nm; 1 cm path length glass cuvette, slit width 3 nm) recorded after 5 minutes of equilibrium time following each addition. This procedure is a slight modification of that previously reported (Geall, A.J.; Blasgbrough, I. S., *J. Pharm. Biomed*. **2000**, *22*, 849-859).

X-ray diffraction

The liposomes were centrifugated to give a hydrated pellet that was sealed in a quartz-glass X-ray capillary and mounted in a point-collimation X-ray camera on a stationary anode generator (McIntosh, T. J., *Biophys. J.*, **1980**, *29*, 237-246) Diffraction patterns were obtained using a flat plate film cassette loaded with Kodak DEF X-ray film. The specimen to film distance was 10 cm with exposure times of 2-6 hours. The low angle reflections were determined in accordance with Bragg's law $2d \sin\theta = h\lambda$, where λ is the wavelength (1.54 Å), d is the repeat period, h is the number of the diffraction order, and θ is the Bragg angle.

TEM

TEM microscopy experiments were performed on a Philips CM 10 (negative staining with ammonium molybdate 1% in water, Cu/Pd carbon coated grids).

Cell culture and Transfection experiment

Chinese hamster ovarian cells (CHO, ATCC, Manassas, VA) were cultured in complete F12K media (ATCC) containing 10% fetal calf serum (Sigma) and 1% penicillin and streptomycin (500 IU/mL and 5000 μg/ml, respectively, Mediatech, Herndon, VA) at 37 ^{o}C in 5% CO_2 with humidity. When the CHO cells reached about 90% confluency, they were split into 48-well plates with a 1:4 ratio using a standard trypsin-based technique. Transfections were performed 24 hours later by modification of previously published methods (Luo, D., Haverstick, K., Belcheva, N., Han, E. & Saltzman, W. M. *Macromolecules* **35**, 3456-3462 (2002) & Luo, D., Woodrow-Mumford, K., Belcheva, N. & Saltzman, W. M. *Pharm. Res.* **16**, 1300-1308 (1999). Briefly, plasmid DNA coding for a reporter gene, β-galactosidase (β-gal, pVax-LacZ1, Invitrogen) was first mixed with lipids in potassium phosphate buffer (PBS) at room temperature. The reporter gene was first mixed with the cationic amphiphile, at a 5:1 amphiphile/DNA ratio, in potassium phosphate buffer (PBS) at room temperature. The mixture was incubated for 15 minutes at room temperature before adding to the cells. The amount of DNA used was the same as used in naked DNA control and positive control (commercially available transfection reagents). After incubation at 37 °C and 5% CO_2 for 2 hours, medium containing the mixtures was gently removed and fresh growth medium was added. Transfection efficiencies were assessed at 48 h post transfection depending on the experimental design. Negative controls were constructed with 1.0 mL of serum-free F12 K medium and naked DNA controls were using 1.0 mL of serum-free F12 K medium with 10.0 μL (1 μg) of reporter gene. Positive controls were performed

according to the manufacturer's protocol. Briefly stated, 2.0 μL of TransFast® transfection reagent (1mg/mL) (Promega, Madison, WI) was mixed with 10.0 μL (1 μg) of reporter gene in 1.0 mL of serum free F12 K medium for 15 minutes at room temperature before transfecting cells.

Reporter gene transfection efficiency assay

Reporter gene (β-gal) assay was performed with a β-galactosidase enzyme assay system (Promega, Madison, WI) following the manufacturer protocol. Briefly, cells were first lysed using M-PER buffer (Pierce, Rockford, Illinois) and enzyme activities were determined. A standard curve was constructed for each experiment using dilutions of purified β-gal protein. The β-gal activities from experimental samples were determined by comparison to the standard curve (enzyme activity vs. enzyme concentration). Efficiency of each transfection was calculated as β-gal activity normalized to total protein.

Cytotoxity

Cytotoxity was assessed using both a formazan-based proliferation assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay kit, Promega) and a total protein-based assay (Pierce). Briefly cells were seeded onto a 96-well microtiter plate with an appropriate density of 1 x 10^4 cells per well. The test chemical was added onto the cells 24 hours later. After 24h MTS (substrates) were added to each well and the plate was incubated for 4 h at 37 ^oC in a humidified, 5% CO_2 incubator. The amount of soluble formazan produced by cellular reduction of the substrates MTS was recorded at 490 nm using a multi-well plate reader. For the total protein-based proliferation assay, cells were lysed at the same time when transfection efficiency was assayed. A 5 uL of lysates were transferred to a separate multi-well plate. Total protein contents were assessed using the Coomassie Blue protein kit (Pierce, Rockford, IL) following the manufacturer protocol. Negative and positive controls were non-treated cells and commercial lipids treated cells, respectively. The proliferation results were expressed as percentages of non-treated cells.

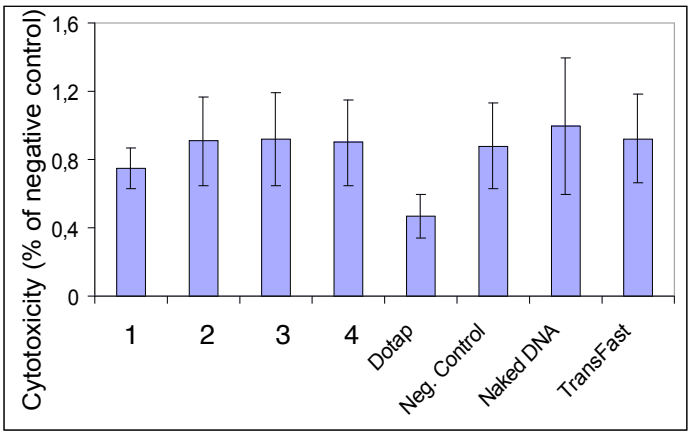


Figure S2. Cytotoxicity results with the synthesized amphiphiles