The effect of charge-reversal amphiphile spacer composition on DNA and siRNA delivery

Xiao-Xiang Zhang,[1] Carla A. H. Prata,[1] Dan Luo,[2] Thomas J. McIntosh,[3] Philippe Barthelemy,[4] and Mark W. Grinstaff[1]*

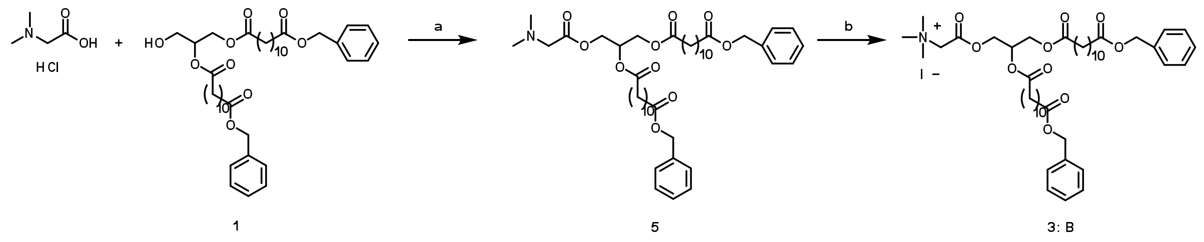
[1]*Departments of Biomedical Engineering and Chemistry, Boston University, Boston MA 02215.* [2]*Department of Biological and Environmental Engineering, Cornell University, Ithaca, NY, 14853.* [3]*Department of Cell Biology, Duke University Medical Center, Durham, NC, 27710.* [4]*Departement de Chimie, Université Victor Segalen Bordeaux 2 Bordeaux, France.*

Correspondence should be addresses to MWG; author E-mail: mgrin@bu.edu

Supporting Information:

Instrumentation:

All solvents were dried and freshly distilled prior to use. All chemicals were purchased from Aldrich 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 and ^{13}C at 300 and 80 Mhz, respectively). Elemental analysis was obtained from Atlantic Microlab, Inc. A TA Instruments DSC 2920 Modulated DSC was used to collect thermal data. Calf thymus DNA and EtBr were purchased from Sigma. Fluorescence studies were carried out with a PTI emission instrument. Dynamic light scattering was performed with a 90 Plus particle size analyzer from Brookhaven Instruments.



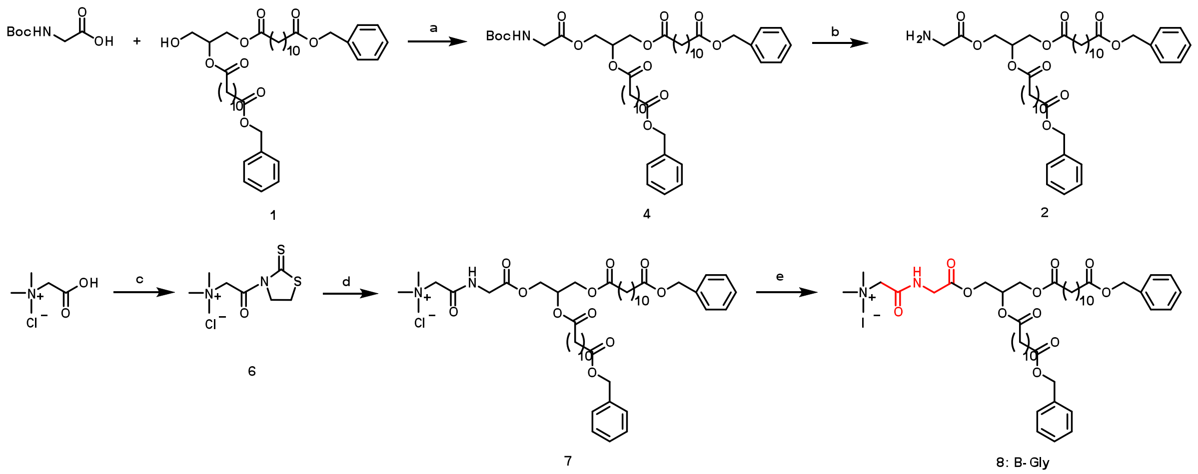
a) EDCI, DMAP in THF, 71%; b) MeI in DCM. 96%

12-benzyl '1, 1-3-hydroxypropane-1,2-diyl didodecanedioate (1) was synthesized as previously described (Prata, Carla A. H.; Li, Yougen; Luo, Dan; McIntosh, Thomas J.; Barthelemy, Philippe; Grinstaff, Mark W. (2008) A new helper phospholipid for gene delivery. *Chem. Commun.* 13, 1566-1568.)

12-benzyl '1, 1-3-(2-(dimethylamino)acetoxy)propane-1,2-diyl didodecanedioate (5): To a THF (5 mL) solution of **1** (2.24 g, 3.2 mmol), N,N-dimethylglycine hydrochloride (0.89 g, 6.4 mmol), EDCI (0.67 g, 3.6 mmol) and DMAP (0.43 g, 3.6 mmol) were added. It was stirred at RT for 18h before filtered. The filtrate was purified through silical gel chromatography (30% EtOAc/Hexane to 50% EtOAc/Hexane) to afford the product as colorless oil (71%). ^{1}H NMR (CDCl$_3$) δ ppm 1.23 (d, 24H), 1.54-1.65 (m, 8H), 2.26-2.34 (m, 14H), 3.18 (s, 2H), 4.10-4.15 (m, 2H), 4.28-4.32 (m, 2H), 5.09 (s, 4H), 5.29-5.32 (m, 1H), 7.28-7.36 (m, 10H); ^{13}C NMR (CDCl$_3$) δ ppm 24.77, 24.89, 28.87, 29.04, 29.05, 29.17, 29.33, 29.66, 33.96, 34.27, 45.08, 60.08, 61.99, 66.02, 69.25, 128.13, 128.49, 136.07, 173.23, 173.67; HRMS calcd. for [C$_{45}$H$_{67}$NO$_{10}$+H]$^+$ 782.4843, found 782.4774.

2-(2,3-bis(12-(benzyloxy)-12-oxododecanoyloxy)propoxy)-N,N,N-trimethyl-2-oxoethanaminium iodide (3): **5** (0.82 g, 1.0 mmol) was dissolved in CH$_2$Cl$_2$ and excess MeI was added. The solution was stirred at RT for 8h and the solvent was removed under reduced pressure. The residue was precipitated in cold ether twice. The product was a powder (96%). ^{1}H NMR (CDCl$_3$) δ ppm 1.23 (d, 24H), 1.54-1.65 (m, 8H), 2.26-2.34 (m, 14H), 3.18 (s, 2H), 4.10-4.15 (m, 2H), 4.28-4.32 (m, 2H), 5.09 (s, 4H), 5.29-5.32 (m, 1H), 7.28-7.36 (m, 10H); ^{13}C NMR (CDCl$_3$) δ

ppm 24.74, 24.86, 29.01, 29.04, 29.15, 29.30, 29.65, 33.97, 34.25, 54.57, 61.13, 63.26, 66.02, 72.38, 128.08, 128.11, 128.49, 136.04, 163.75, 173.23, 173.67; HRMS calcd. for $[C_{46}H_{70}INO_{10}\text{-}I]^+$ 796.5000, found 796.4999.



a) Boc-Gly-OH, DCC, DMAP in DCM, 96%; b) 50% TFA in DCM, 94%; c) thiazolidine-2-thione, DCC in DMF, 34%; d) TEA in DMF, 67%; e) KI in EtOH, reflux, 62%.

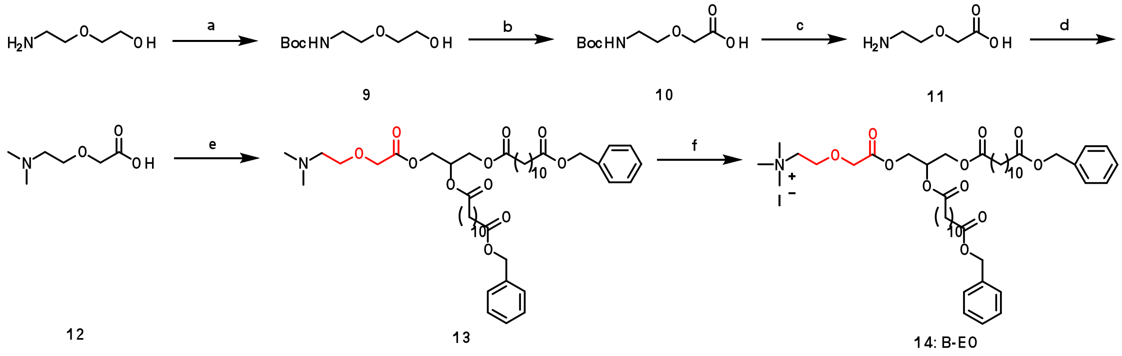
12-benzyl '1, 1-3-(2-(tert-butoxycarbonylamino)acetoxy)propane-1,2-diyl didodecanedioate (4): To a 15 mL CH_2Cl_2 solution of **1** (5.05 g, 7.0 mmol), BocGlyOH (1.14 g, 7.9 mmol), DCC (1.63 g, 7.9 mmol) and DMAP (cat.) were added. It was stirred at RT for 18h and filtered to remove insoluble DCU. A silica gel chromatography (20% EtOAc/Hexanes) was used to obtain the product as an oil (72%). ^{1}H NMR ($CDCl_3$) δ ppm 1.23 (d, 24H), 1.45 (s, 9H), 1.54-1.65 (m, 8H), 2.28-2.37 (m, 8H), 3.92-3.94 (d, 2H), 4.13-4.17 (m, 2H), 4.31-4.35 (m, 2H), 5.02(s, 1H), 5.11 (s, 4H), 5.29-5.32 (m, 1H), 7.28-7.36 (m, 10H); ^{13}C NMR ($CDCl_3$) δ ppm 24.73, 24.88, 28.23, 29.01, 29.05, 29.16, 29.31, 33.90, 34.06, 34.26, 42.28, 61.76, 66.01, 70.22, 80.01, 128.11, 128.48, 136.05, 155.53, 169.66, 173.26, 173.65; HRMS calcd. for $[C_{48}H_{71}NO_{12}+Na]^+$ 876.4874, found 876.4851.

12-benzyl '1, 1-3-(2-aminoacetoxy)propane-1,2-diyl didodecanedioate (2): **4** (4.43 g, 5.2 mmol) was dissolved in 6 ml of CH_2Cl_2, and 6 mL TFA was added slowly to this ice cold solution. The reaction was allowed to runat RT for 1h. The solvent and TFA were then removed under reduced pressure. The residue was precipitates in cold ether to afford the product as a powder (97%). ^{1}H NMR (CDCl$_3$) δ ppm 1.23 (d, 24H), 1.54-1.65 (m, 8H), 2.28-2.37 (m, 8H), 3.92-3.94 (d, 2H), 4.13-4.17 (m, 2H), 4.31-4.35 (m, 2H), 5.02(s, 1H), 5.11 (s, 4H), 5.29-5.32 (m, 1H), 7.28-7.36 (m, 10H); ^{13}C NMR (CDCl$_3$) δ ppm 24.73, 24.88, 29.01, 29.05, 29.16, 29.31, 33.90, 34.06, 34.26, 42.28, 61.76, 66.01, 70.22, 128.11, 128.48, 136.05, 155.53, 169.66, 173.26, 173.65; HRMS calcd. for $[C_{43}H_{63}NO_{10}+H]^+$ 754.5430, found 754.4554.

N,N,N-trimethyl-2-oxo-2-(2-thioxothiazolidin-3-yl)ethanaminium chloride (6): To a 5 mL DMF solution of 1-carboxy-N,N,N-trimethylmethanaminium chloride (2 g, 13.0 mmol), thiazolidine-2-thione (1.55 g, 13.0 mmol), DCC (2.69 g, 14.3 mmol) and DMAP (cat.) were added, and the solution was stirred for 24h. The solution was filtered to remove insoluble DCU. DMF was removed under reduced pressure. CH$_2$Cl$_2$ was added and filtered. The residue was dissolved in methanol and filtered. The filtrate was dried to afford a powder (34%). ^{1}H NMR (CD$_3$OD) δ ppm 3.38 (s, 9H), 3.47-3.51 (t, 2H), 4.64-4.68 (t, 2H), 5.29 (s, 2H); ^{13}C NMR (CD$_3$OD) δ ppm 30.11, 54.71, 56.91, 69.07, 166.44, 204.33.

17-(12-(benzyloxy)-12-oxododecanoyloxy)-N,N,N-trimethyl-3,14,20,23-tetraoxo-1-phenyl-2,15,19-trioxa-22-azatetracosan-24-aminium chloride (7): To a DMF solution of **2** (0.65 g, 2.5 mmol), **6** (1.55 g, 1.7 mmol) and TEA (0.26 g, 2.5 mmol) were added, and the reaction was stirred at RT for 18h. Next, the DMF was removed and the residue was dissolved in 10 mL of EtOAc and washed with 0.1N HCl (2mL) twice. The product was purified by silica gel chromatography (EtOAc to MeOH) to give an oil (67%). ^{1}H NMR (CDCl$_3$) δ ppm 1.26 (d, 24H), 1.58-1.65 (m, 8H), 2.28-2.37 (m, 8H), 3.39 (s, 9H), 4.03-4.05 (d, 2H), 4.13-4.17 (m, 2H), 4.31-4.35 (m, 2H), 4.41 (s, 2H), 5.11 (s, 4H), 5.29-5.32 (m, 1H), 7.28-7.36 (m, 10H), 9.67 (s, 1H); ^{13}C NMR (CDCl$_3$) δ ppm 30.11, 54.71, 56.91, 69.07, 166.44, 204.33; HRMS calcd. for $[C_{48}H_{73}ClN_2O_{11}-Cl]^+$ 853.5214, found 853.5202.

17-(12-(benzyloxy)-12-oxododecanoyloxy)-N,N,N-trimethyl-3,14,20,23-tetraoxo-1-phenyl-2, 15,19-trioxa-22-azatetracosan-24-aminium iodide (8): Compound **7** (0.4 g, 0.45 mmol) was dissolved in ethanol and excess KI was added. The reaction was refluxed for 24h before ethanol was removed. The residue was dissolved in CH_2Cl_2 and filtered. The filtrate was precipitated in cold ether to afford the product as a powder (75%); ^{1}H NMR ($CDCl_3$) δ ppm 1.26 (d, 24H), 1.58-1.65 (m, 8H), 2.28-2.37 (m, 8H), 3.39 (s, 9H), 4.03-4.05 (d, 2H), 4.13-4.17 (m, 2H), 4.31-4.35 (m, 2H), 4.41 (s, 2H), 5.11 (s, 4H), 5.29-5.32 (m, 1H), 7.28-7.36 (m, 10H), 9.67 (s, 1H); ^{13}C NMR ($CDCl_3$) δ ppm 24.74, 24.77, 24.89, 29.02, 29.05, 29.16, 29.32, 33.88, 33.95, 34.07, 34.28, 41.26, 54.83, 61.65, 61.91, 65.21, 65.32, 66.03, 68.44, 70.44, 128.08, 128.13, 128.51, 136.08, 168.22, 168.51, 173.31, 173.58, 173.73; HRMS calcd. for $[C_{48}H_{73}IN_2O_{11}\text{-}I]^+$ 853.5293, found 853.5304.



a) Boc_2O in EtAc, 98%; b) TEMPO, BAIB in $MeCN/H_2O$, 23%; c) TFA in DCM, 72%;
d) formaldehyde, Pd(II), H_2 in MeOH, 65%; e) 1, EDCI, DMAP in DCM, 70%; f) MeI in DCM, 65%.

tert-butyl 2-(2-hydroxyethoxy)ethylcarbamate (9): Di-tert-butyl dicarbonate (26.3 g, 0.12 mol) was added to a 30 mL of EtOAc containing 2-(2-aminoethoxy) ethanol (10.55g, 0.1mol) and stirred at RT for 18h. Following silica gel chromatography the product was isolated as an oil (98%); ^{1}H NMR ($CDCl_3$) δ ppm 1.40 (s, 9H), 2.64 (s, 1H), 3.28-3.31 (m, 2H), 3.50-3.54 (m, 4H), 3.68-3.71 (t, 2H), 5.08 (s, 1H); ^{13}C NMR ($CDCl_3$) δ ppm 28.03, 28.23, 28.45, 28.66, 40.28, 61.38, 61.57, 61.62, 61.85, 70.04, 70.26, 70.48, 71.95, 72.17, 72.14, 79.31, 156.08; HRMS calcd. for $[C_9H_{19}NO_4+Na]^+$ 228.1212, found 228.1205.

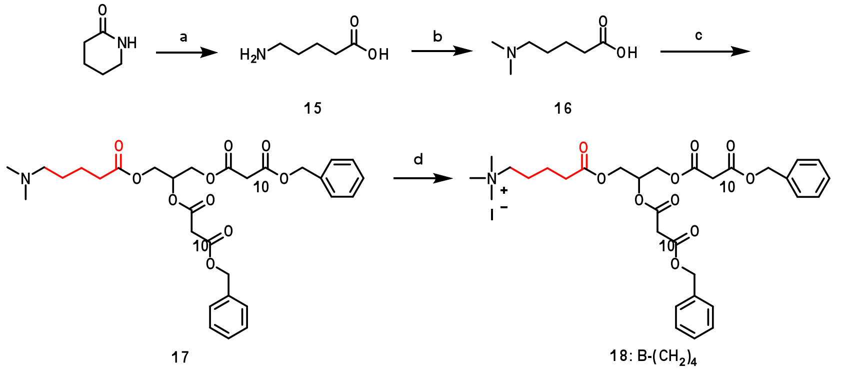
2-(2-(tert-butoxycarbonylamino)ethoxy)acetic acid (10): Compound **9** (6.7 g, 32.7 mmol) was dissolved in MeCN/H_2O (5:1) and BAIB (12.6 g, 39.2 mmol) and TEMPO (0.32 g, 6.5 mmol) were added. The reaction was stirred at RT for 24h before being extracted with CH_2Cl_2 (20mL X3). The product was purified by silica gel chromatography (12% MeOH/EtOAc with a few drops of acetic acid) and obtained as an oil (23%); ^{1}H NMR ($CDCl_3$) δ ppm 1.46 (s, 9H), 3.32-3.34 (t, 2H), 3.57-3.59 (t, 2H), 4.07 (s, 2H), 5.36 (s, 1H); ^{13}C NMR ($CDCl_3$) δ ppm 27.73, 28.32, 29.12, 40.55, 65.83, 69.92, 78.87, 156.19, 177.48; HRMS calcd. for $[C_9H_{17}NO_5+Na]^+$ 242.1004, found 242.1032.

2-(2-aminoethoxy) acetic acid (11): Compound **10** (0.62 g, 2. 8mmol) was dissolved in 2mL of CH_2Cl_2, and 2 mL of TFA was added slowly to this ice cold solution. It was allowed to react at RT for 1h. Solvent and TFA were then removed under reduced pressure. The residue was precipitated in cold ether to obtain the product as a powder (72%). ^{1}H NMR (CD_3OD) δ ppm 3.11-3.14 (t, 2H), 3.73-3.75 (t, 2H), 4.14 (s, 2H); ^{13}C NMR (CD_3OD) δ ppm 40.63, 68.13, 68.58, 174.36; HRMS calcd. for $[C_4H_9NO_3+Na]^+$ 142.0480, found 142.0501.

2-(2-(dimethylamino)ethoxy)acetic acid (12): 1N NaOH was added to MeOH solution of **11** (0.45 g, 1.9 mmol) till pH=12. Excess formaldehyde and a catalytic amount of Pd(II) on carbon was added. The reaction was performed at RT under 50 psi H_2 for 16h. Next the solution was filtered through celite, the solvent was removed and then re-dissolved in MeOH. The solution was filtered and 1N HCl was added till pH=4. The product was precipitated in cold ether to afford an oil (65%). ^{1}H NMR (CD_3OD) δ ppm 2.94 (s, 6H), 3.33-3.36 (t, 2H), 3.84-3.86 (t, 2H), 4.18 (s, 2H); ^{13}C NMR (CD_3OD) δ ppm 43.49, 58.15, 65.35, 68.85, 174.36; HRMS calcd. for $[C_6H_{13}NO_3+H]^+$ 148.0974, found 148.0988.

12-benzyl '1,1-3-(2-(2-(dimethylamino)ethoxy)acetoxy)propane-1,2-diyl didodecanedioate (13): Compound **1** (2.7 g, 6.8 mmol) and **12** (0.5 g, 3.4 mmol) were reacted in the presence of EDCI (1.29 g, 6.8 mmol) and DMAP (cat.) at RT for 16h. The reaction mixture was purified via silica gel chromatography (25% EtOAc/Hexane to 15% MeOH/EtOAc) and an oil was obtained (70%). ^{1}H NMR ($CDCl_3$) δ ppm 1.24 (s, 24H), 1.55-1.63 (m, 8H), 2.26-2.35 (m, 8H), 2.40 (s, 6H), 2.68-2.71 (t, 2H), 3.67-3.71 (t, 2H), 4.09-4.15 (m, 4H), 4.25-4.34 (m, 2H), 5.09 (s, 4H), 5.27-5.30 (m, 1H), 7.27-7.35 (m, 10H); ^{13}C NMR ($CDCl_3$) δ ppm 24.77, 24.90, 29.04, 29.07, 29.18, 29.33, 33.94, 34.05, 34.11, 34.28, 45.16, 61.84, 66.02, 68.04, 68.07, 128.12, 128.50, 136.10, 169.59, 173.21, 173.64; HRMS calcd. for $[C_{47}H_{71}NO_{11}+Na]^+$ 848.4925, found 848.4941.

17-(12-(benzyloxy)-12-oxododecanoyloxy)-N,N,N-trimethyl-3,14,20-trioxo-1-phenyl-2,15,19,22-tetraoxatetracosan-24-aminium iodide (14): Compound **13** (1.68 g, 2.0 mmol) was dissolved in CH_2Cl_2 and excess MeI was added. The solution was stirred at RT for 6h and the solvent was removed under reduced pressure. The residue was dissolved in CH_2Cl_2 and precipitated in cold ether twice to obtain the product as a solid (65%). ^{1}H NMR ($CDCl_3$) δ ppm 1.23 (s, 24H), 1.54-1.63 (m, 8H), 2.26-2.33 (m, 8H), 3.25-3.27 (t, 2H), 3.51 (s, 9H), 4.04 (s, 2H), 4.09-4.15 (m, 2H), 4.32-4.36 (m, 2H), 5.07 (s, 4H), 5.21-5.25 (m, 1H), 6.83-6.93 (m, 1H), 7.27-7.35 (m, 10H), 8.16-8.31 (m, 1H); ^{13}C NMR ($CDCl_3$) δ ppm 24.76, 24.87, 29.01, 29.04, 29.15, 29.30, 33.91, 34.03, 34.25, 52.13, 54.94, 61.50, 65.28, 65.80, 66.00, 67.54, 67.64, 70.61, 128.08, 128.11, 128.49, 136.05, 169.58, 170.63, 173.27, 173.66; HRMS calcd. for $[C_{48}H_{74}INO_{11}-I]^+$ 840.5262, found 840.5244.



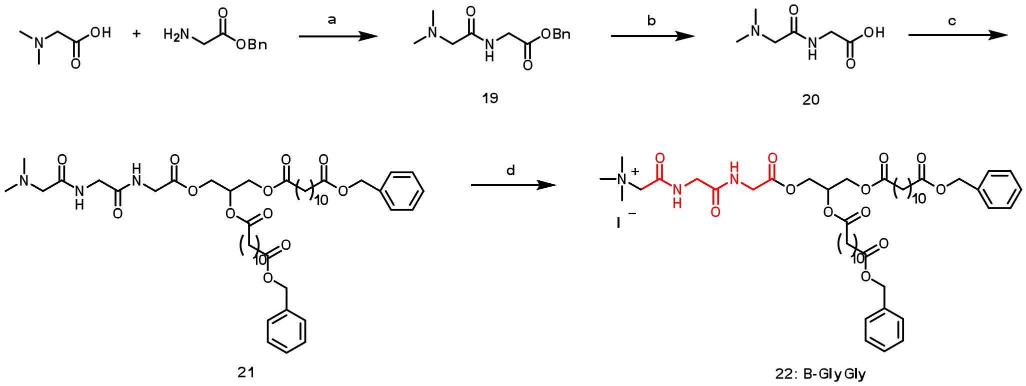
a)5N HCl, 80C, 48hr, 92%; b)formaldehyde, Pd(II), H_2 in MeOH, 54%; c)1, EDCI, DMAP in DCM, 39%; d) MeI in DCM, 91%

5-aminopentanoic acid (15): Piperidin-2-one (4.12 g, 41.2 mmol) was added to 5N HCl solution and heated at 80°C for 48h. Then 1N NaOH was added to the solution until pH=10. The solvent was removed and the residue was dissolved in acetone and filtered. The product was dissolved in methanol, and KOH in methanol was added till pH=10. The solution was filtered to remove insoluble salt. Finally, the filtrate was dried to afford a solid (92%). ^{1}H NMR (CD_3OD) δ ppm 1.66-1.69 (m, 2H), 2.34-2.37 (t, 2H), 2.90-2.93 (t, 2H); ^{13}C NMR (CD_3OD) δ ppm 22.71, 27.99, 34.02, 40.43, 176.81; HRMS calcd. for $[C_5H_{12}NO_2+H]^+$ 119.0946, found 119.0934.

5-(dimethylamino)pentanoic acid (16): Excess formaldehyde and catalytic amount of Pd(II) on carbon was added to a methanol solution of **15** (0.46 g, 3.0 mmol). The reaction was performed at RT under 50 psi H_2 for 16h. Then solution was filtered through celite, the solvent was removed and the residue was dissolved in methanol. The solution was filtered and 1N HCl was added till pH=4 and the product was precipitated in cold ether to afford an oil (84%). ^{1}H NMR (CD_3OD) δ ppm 1.55-1.62 (m, 2H), 1.65-1.72 (m, 2H), 2.17-2.21 (t, 2H), 2.76 (s, 6H), 2.98-3.02 (t, 2H); ^{13}C NMR (CD_3OD) δ ppm 23.83, 25.51, 37.27, 43.06, 43.30, 58.53, 181.18; HRMS calcd. for $[C_7H_{15}NO_2+H]^+$ 146.1181, found 146.1183.

5-(2,3-bis(12-(benzyloxy)-12-oxododecanoyloxy)propoxy)-N,N,N-trimethyl-5-oxopentan-1-aminium (17): Compound **1** (0.7 g, 1 mmol) and **12** (0.3 g, 2 mmol) were reacted in the presence of EDCI (0.21 g, 1.1 mmol) and DMAP (cat.) at RT for 16h. The reaction mixture was purified through silica gel chromatography (EtOAc to 15% MeOH/EtOAc) to afford an oil (39%). ^{1}H NMR ($CDCl_3$) δ ppm 1.23-1.25 (m, 26H), 1.56-1.65 (m, 10H), 2.27-2.36 (m, 16H), (4.09-4.14 (m, 2H), 4.27-4.31 (m, 2H), 5.09 (s, 4H), 5.21-5.25 (m, 1H), 7.30-7.34 (m, 10H); ^{13}C NMR ($CDCl_3$) δ ppm 24.81, 24.92, 29.07, 29.09, 29.21, 29.37, 34.00, 34.30, 62.00, 66.05, 128.16, 128.53, 136.10, 165.38, 173.30, 173.64; HRMS calcd. for $[C_{48}H_{73}NO_{10}+H]^+$ 824.5313, found 824.5300.

5-(2,3-bis(12-(benzyloxy)-12-oxododecanoyloxy)propoxy)-N,N,N-trimethyl-5-oxopentan-1-aminium iodide (18): Compound **17** (0.4 g, 0.5 mmol) was dissolved in CH_2Cl_2 and excess MeI was added. The solution was stirred at RT for 6h and the solvent was removed under reduced pressure. The residue was dissolved in CH_2Cl_2 and precipitated in cold ether twice to obtain the product as a solid (91%). ^{1}H NMR ($CDCl_3$) δ ppm 1.24 (s, 24H), 1.56-1.63 (m, 12H), 2.27-2.35 (m, 10H), 3.26 (s, 9H), 3.45-3.49 (t, 2H), 4.06-4.10 (m, 2H), 4.37-4.11 (m, 2H), 5.09 (s, 1H), 5.14-5.16 (m, 1H), 7.28-7.36 (m, 10H); ^{13}C NMR ($CDCl_3$) δ ppm 24.86, 24.92, 28.97, 29.08, 29.20, 29.36, 34.03, 34.29, 53.36, 54.58, 61.66, 66.05, 69.73, 128.13, 128.16, 128.54, 171.95, 173.40, 173.70; HRMS calcd. for $[C_{49}H_{76}INO_{10}-I]^+$ 838.5469, found 838.5432.



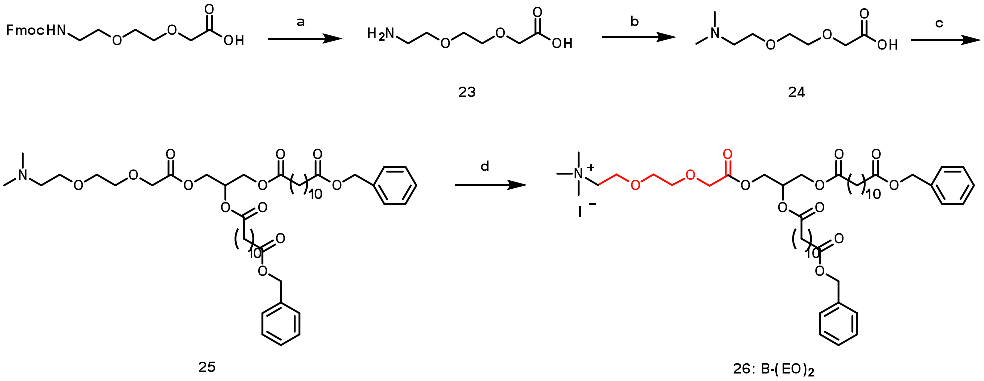
a) DCC, DMAP in DMF, 76%; b) Pd(II), H_2 in THF, 95%; c) 2, DCC, DMAP in DCM, 96%; d) MeI in DCM, 80%.

benzyl 2-(2-(dimethylamino)acetamido)acetate (19): N,N-dimethylglycine hydrochloride (1.20 g, 8.6 mmol) and glycine benzyl ester hydrochloride (1.74 g, 8.6 mmol) were reacted in the presence of DCC (1.95 g, 9.5 mmol) and DMAP (2.21 g, 18.1 mmol) in CH_2Cl_2 at RT for 16h. The reaction mixture was purified through silica gel chromatography (50% to 67% EtOAc /hexane) to afforded an oil (76%). ^1H NMR ($CDCl_3$) δ ppm 2.32 (s, 6H), 3.00 (s, 2H), 4.13-4.14 (d, 2H), 5.19 (s, 2H), 7.28-7.40 (m, 5H), 7.65 (s, 1H); ^{13}C NMR ($CDCl_3$) δ ppm 40.65, 45.92, 62.97, 67.06, 128.35, 128.46, 128.56, 135.13, 169.75, 171.12; HRMS calcd. for $[C_{12}H_{18}N_2O_3+Na]^+$ 273.1215, found 273.1213.

2-(2-(dimethylamino)acetamido)acetic acid (20): A catalytic amount of Pd(II) on carbon was added to a THF solution of **19** (0.46 g, 3.0 mmol) with a few drops of TEA. The reaction was performed at RT under 50 psi H_2 for 16h. Next, the solution was filtered through celite and the solvent was removed to afford the product as a powder (84%). ^1H NMR (CD_3OD) δ ppm 2.52 (s, 6H), 3.31 (s, 2H), 3.78 (s, 2H); ^{13}C NMR (CD_3OD) δ ppm 43.10, 44.57, 61.27, 169.31, 174.96; HRMS calcd. for $[C_6H_{12}N_2O_3+Na]^+$ 183.0746, found 183.0736.

12-benzyl '1,1-2-methyl-4,7,10-trioxo-11-oxa-2,5,8-triazatetradecane-13,14-diyl didodecanedioate (21): To a DMF solution of **20** (0.20 g), **2** (1.18 g, 1.6 mmol) was added along with DCC (0.35 g, 1.7 mmol) and DMAP (cat.). The reaction was stirred at RT for 16h. Next the reaction mixture was filtered to remove DCU. The product was purified via silica gel chromatography (EtOAc to 15% MeOH/EtOAc) to afford an oil (50%). ^1H NMR ($CDCl_3$) δ ppm 1.26 (s, 24H), 1.58-1.65 (m, 8H), 2.29-2.37 (m, 14H), 3.05 (s, 2H), 4.02-4.06 (m, 4H), 4.12-4.19 (m, 2H), 4.31-4.35 (m, 2H), 5.11 (s, 4H), 5.26-5.29 (m, 1H), 6.97 (s, 1H), 7.29-7.38 (m, 10H), 7.81 (s, 1H); ^{13}C NMR ($CDCl_3$) δ ppm 24.66, 24.80, 28.96, 29.08, 29.23, 33.81, 33.99, 34.17, 41.00, 42.53, 45.90, 61.58, 62.69, 65.92, 70.39, 128.02, 128.40, 135.97, 168.84, 169.17, 171.58, 173.18, 173.56; HRMS calcd. for $[C_{49}H_{73}N_3O_{12}+H]^+$ 896.5273, found 896.5174.

12-benzyl '1,1-2-methyl-4,7,10-trioxo-11-oxa-2,5,8-triazatetradecane-13,14-diyl didodecanedioate iodide (22): Compound **21** (0.4 g, 0.5 mmol) was dissolved in CH_2Cl_2 and excess MeI was added. The solution was stirred at RT for 6h and the solvent was removed under reduced pressure. The residue was dissolved in CH_2Cl_2 and precipitated in cold ether twice to obtain the product as an oil (80%). ^1H NMR ($CDCl_3$) δ ppm 1.27 (s, 24H), 1.58-1.65 (m, 8H), 2.29-2.38 (m, 8H), 3.45 (s, 9H), 4.03-4.31(m, 8H), 4.80 (s, 2H), 5.11 (s, 4H), 5.19-5.21 (m, 1H), 7.30-7.39 (m, 10H), 7.59-7.62 (m, 1H), 8.42-8.54 (m, 1H); ^{13}C NMR ($CDCl_3$) δ ppm 25.03, 25.15, 29.31, 29.44, 29.59, 34.22, 34.39, 34.53, 40.99, 41.10, 43.09, 55.30, 61.73, 62.36, 63.30, 65.16, 66.28, 68.74, 70.30, 128.35, 128.38, 128.59, 128.76, 136.30, 143.15, 163.87, 168.99, 169.08, 169.49, 169.80, 173.28, 173.61, 173.66, 173.96; HRMS calcd. for $[C_{50}H_{76}IN_3O_{12}+Na]^+$ 1060.4371, found 1060.4301.



a) 5% piperidine in DMF, 86%; b) formaldehyde, Pd(II), H_2 in MeOH, 76%; c) 1, EDCI, DMAP in DCM, 45%; d) MeI in DCM, 86%.

2-(2-(2-aminoethoxy)ethoxy)acetic acid (23): Pipperidine (0.1 mL) was added to a 2mL DMF solution of 2-(2-(2-Fmoc-aminoethoxy)ethoxy)acetic acid (0.39 g, 1 mmol) and reacted at RT for 4h. Next, the DMF was then removed under reduced pressure. The residue was washed 3 times with toluene and dried to afford product as an oil (89%). ^1H NMR (CD_3OD) δ ppm 3.05-3.08 (t, 2H), 3.58-3.60 (t, 2H), 3.64-3.66 (t, 2H), 3.69-3.71 (t, 2H), 3.85 (s, 2H); ^{13}C NMR

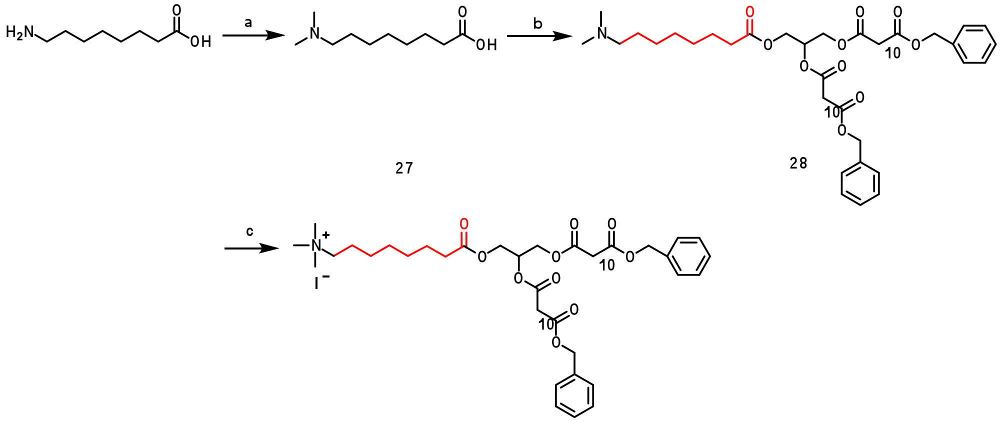
(CD_3OD) δ ppm 40.46, 68.06, 71.06, 71.28, 71.54, 178.05; HRMS calcd. for $[C_6H_{13}NO_4+Na]^+$ 186.0742, found 186.0755.

2-(2-(2-(dimethylamino)ethoxy)ethoxy)acetic acid (24): Excess formaldehyde and a catalytic amount of Pd(II) on carbon were added to a methanol solution of **23** (0.12 g, 0.74 mmol). The reaction was performed at RT under 50 psi H_2 for 16h. Next, the solution was filtered through celite, the solvent was removed and the residue was re-dissolved in methanol. Finally, the solution was filtered to afford the product as an oil (79%). ^{1}H NMR (CD_3OD) δ ppm 2.86 (s, 6H), 3.26-3.29 (t, 2H), 3.57-3.59 (t, 2H), 3.69-3.71 (t, 2H), 3.81-3.84 (t, 2H), 3.88 (s, 2H); ^{13}C NMR (CD_3OD) δ ppm 43.52, 57.69, 65.59, 71.23, 71.32, 71.66, 178.23; HRMS calcd. for $[C_8H_{17}NO_4+Na]^+$ 214.1055, found 214.1051.

12-benzyl '1,1-2-methyl-10-oxo-5,8,11-trioxa-2-azatetradecane-13,14-diyl didodecanedioate (25): Compound **1** (0.4 g, 0.57 mmol) and **24** (0.12 g, 0.64 mmol) were reacted in the presence of EDCI (0.12 g, 0.64 mmol) and DMAP (cat.) at RT for 16h. The reaction mixture was purified via silica gel chromatography (EtOAc to 10% MeOH/EtOAc) to afford an oil (45%). ^{1}H NMR ($CDCl_3$) δ ppm 1.24 (s, 24H), 1.56-1.63 (m, 8H), 2.26-2.34 (m, 14H), 2.57-2.60 (t, 2H), 3.59-3.64 (m, 4H), 3.69-3.72 (t, 2H), 4.10-4.14 (m, 4H), 4.28-4.32 (m, 2H), 5.09 (s, 4H), 5.28-5.30 (m, 1H), 7.28-7.36 (m, 10H); ^{13}C NMR ($CDCl_3$) δ ppm 24.77, 24.91, 29.05, 29.08, 29.18, 29.34, 29.41, 33.94, 34.29, 45.34, 58.25, 61.87, 66.03, 68.29, 68.84, 69.67, 70.31, 70.87, 128.13, 128.51, 136.08, 169.64, 173.24, 173.67; HRMS calcd. for $[C_{49}H_{75}NO_{12}+Na]^+$ 892.5187, found 892.5209.

12-benzyl '1,1-2-methyl-10-oxo-5,8,11-trioxa-2-azatetradecane-13,14-diyl didodecanedioate iodide (26): Compound **25** (0.4 g, 0.5 mmol) was dissolved in CH_2Cl_2 and excess MeI was added. The solution was stirred at RT for 6h and solvent was removed under reduced pressure.

The residue was dissolved in CH_2Cl_2 and precipitated in cold hexane twice to obtain the product as an oil (95%). ^1H NMR ($CDCl_3$) δ ppm 1.24 (s, 24H), 1.58-1.61 (m, 8H), 2.28-2.32 (m, 8H), 3.44 (s, 9H), 3.64-3.70 (m, 4H), 3.94-4.14 (m, 8H), 4.32-4.36 (m, 2H), 5.09 (s, 4H), 5.22-5.28 (m, 1H), 7.30-7.38 (m, 10H); ^{13}C NMR ($CDCl_3$) δ ppm 24.69, 24.89, 28.89, 28.97, 29.04, 29.06, 29.12, 29.18, 29.26, 29.33, 33.96, 34.27, 54.99, 61.56, 65.09, 65.90, 66.04, 67.88, 70.04, 70.27, 70.61, 128.11, 128.14, 128.51, 136.06, 169.52, 173.27, 173.69; HRMS calcd. for $[C_{50}H_{78}INO_{12}\text{-}I]^+$ 885.5602, found 885.5607.



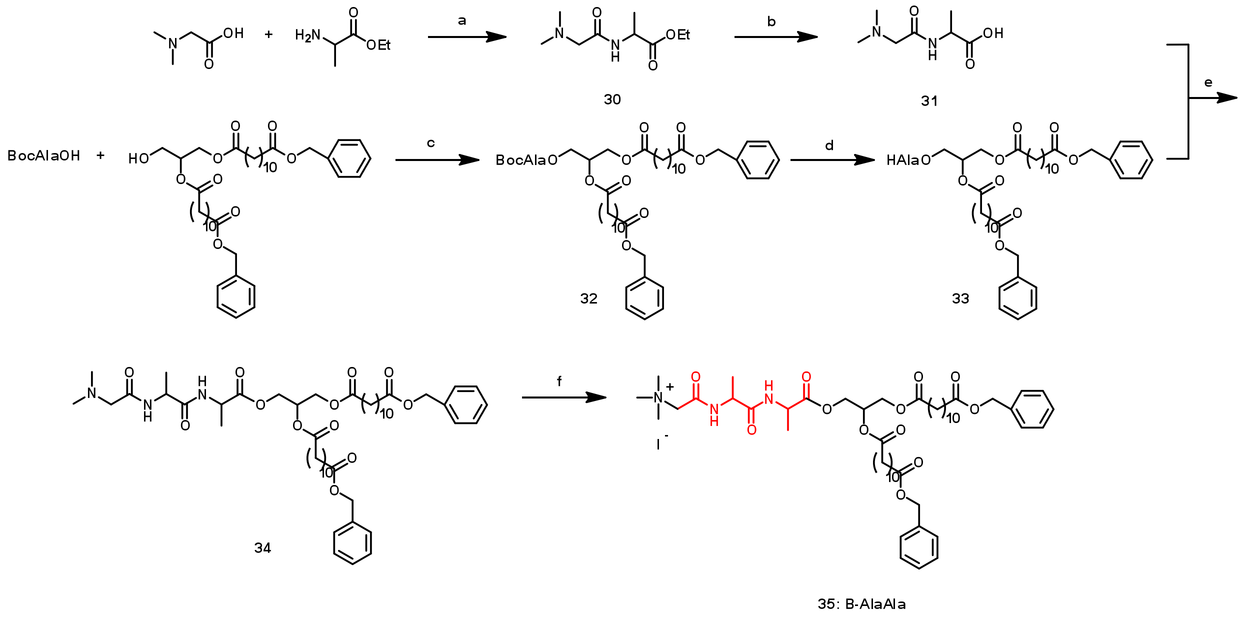
29: B-$(CH_2)_7$

a) formaldehyde, Pd(II), H_2 in MeOH, 82%; b) 1, EDCI, DMAP in DCM, 40%; c) MeI in DCM, 88%.

8-(dimethylamino)octanoic acid (27): Excess formaldehyde and a catalytic amount of Pd(II) on carbon was added to a methanol solution of 8-aminooctanoic acid (1.6 g, 1 mmol). The reaction was performed at RT under 50psi H_2 for 16h. Next, the solution was filtered through celite and the solvent was removed. The residue was dissolved in methanol and filtered to afford the product as an oil (82%). ^1H NMR (CD_3OD) δ ppm 1.33-1.37 (m, 8H), 1.57-1.60 (t, 2H), 2.16-2.20 (t, 2H), 2.79 (s, 6H), 2.99-3.03 (t, 2H); ^{13}C NMR (CD_3OD) δ ppm 25.59, 26.80, 27.20, 29.73, 30.07, 37.56, 43.37, 58.97, 181.15; HRMS calcd. for $[C_{10}H_{21}NO_2+H]^+$ 188.1651, found 188.1644.

12-benzyl '1,1-3-(8-(dimethylamino)octanoyloxy)propane-1,2-diyl didodecanedioate (28): Compund **1** (2.73 g, 3.9 mmol) and **27** (1.1 g, 5.8 mmol) were reacted in the presence of EDCI (0.83 g, 4.3 mmol) and DMAP (cat.) at RT for 16h. The reaction mixture was purified through silica gel chromatography (EtOAc to 10% MeOH/EtOAc) to afford an oil (40%). ^{1}H NMR ($CDCl_3$) δ ppm 1.21-1.29 (m, 32H), 1.57-1.63 (m, 10H), 2.26-2.34 (m, 10H), 2.45 (s, 6H), 2.56-2.60 (t, 2H), 4.09-4.14 (m, 2H), 4.25-4.29 (m, 2H), 5.09 (s, 4H), 5.21-5.24 (m, 1H), 7.28-7.34 (m, 10H); ^{13}C NMR ($CDCl_3$) δ ppm 24.66, 24.81, 24.85, 24.91, 25.24, 25.30, 25.53, 26.90, 28.86, 28.88, 28.93, 29.07, 29.09, 29.21, 29.36, 29.41, 33.99, 34.03, 34.16, 34.29, 43.28, 58.07, 62.02, 66.05, 68.89, 128.12, 128.15, 128.52, 136.09, 172.73, 173.27, 173.68, 173.72; HRMS calcd. for $[C_{51}H_{79}NO_{10}+H]^+$ 866.5782, found 866.5755.

12-benzyl '1,1-3-(8-(dimethylamino)octanoyloxy)propane-1,2-diyl didodecanedioate iodide (29): Compound **28** (0.43 g, 0.5 mmol) was dissolved in CH_2Cl_2 and excess MeI was added. The solution was stirred at RT for 6h and the solvent was removed under reduced pressure. The residue was dissolved in CH_2Cl_2 and precipitated in cold ether twice to obtain the product as an oil (88%). ^{1}H NMR ($CDCl_3$) δ ppm 1.23-1.37 (m, 30H), 1.57-1.62 (m, 8H), 1.72-1.75 (m, 2H), 2.26-2.34 (m, 10H), 3.39 (s, 9H), 3.53-3.57 (t, 2H), 4.09-4.14 (m, 2H), 4.25-4.29 (m, 2H), 5.09 (s, 4H), 5.19-5.22 (m, 1H), 7.27-7.36 (m, 10H); ^{13}C NMR ($CDCl_3$) δ ppm 22.93, 24.42, 24.79, 24.83, 24.89, 25.73, 28.53, 28.65, 29.02, 29.05, 29.17, 29.33, 33.84, 33.98, 34.26, 53.57, 61.88, 66.01, 67.01, 68.91, 128.08, 128.12, 128.50, 136.05, 172.64, 173.27, 173.67; HRMS calcd. for $[C_{52}H_{82}INO_{10}-I]^+$ 880.5939, found 880.5869.



a) DCC, DMAP in CH_2Cl_2, 86%; b) KOH in MeOH, 90%; c) DCC, DMAP in CH_2Cl_2, 91%; d) TFA in CH_2Cl_2, 92%; e) EDCI, DMAP in CH_2Cl_2, 68%; f) MeI in CH_2Cl_2, 79%

Ethyl 2-(2-(dimethylamino)acetamido)propanoate (30): N,N-dimethylglycine hydrochloride (5.00 g, 35.8 mmol) and alanine ethyl ester hydrochloride (5.50 g, 35.8 mmol) were reacted in the presence of DCC (8.13 g, 39.5 mmol) and DMAP (2.21 g, 75.2 mmol) in CH_2Cl_2 at RT for 16h. The reaction mixture was filtered and purified through silica gel chromatography (EtOAc to 5% MeOH/EtOAc) to afforded a white powder (86%). ^1H NMR ($CDCl_3$) δ ppm 1.21 (t, 3H), 1.34 (d, 3H), 2.25 (s, 6H), 2.83 (q, 2H), 4.10-4.16 (q, 2H), 4.51-4.59 (m, 1H), 7.50-7.52 (d, 1H); ^{13}C NMR ($CDCl_3$) δ ppm 13.96, 18.23, 45.76, 47.20, 61.15, 62.83, 170.15, 172.74; HRMS calcd. for $[C_9H_{18}N_2O_3+H]^+$ 203.1396, found 203.1390.

2-(2-(dimethylamino)acetamido)propanoic acid (31): KOH was added to a methanol solution of **30** (5.24g, 25.9mmol) until pH=12. Reaction was finished after stirring at RT for 6h showed on TLC. Next, IRC-50 resin was added and the flask was shaked for 2h. The resin was removed by filtration to afford the product as a powder (94%). ^1H NMR (CD_3OD) δ ppm 1.29 (d, 3H), 2.38 (s, 6H), 3.09-3.22 (m, 2H), 4.15-4.22 (m, 1H), 8.07 (d, 1H), 8.18 (s, 1H); ^{13}C NMR

(CD_3OD) δ ppm 18.33, 45.18, 50.29, 61.77, 169.09, 178.42; HRMS calcd. for $[C_7H_{14}N_2O_3+H]^+$ 175.1083, found 175.1090.

12-dibenzyl O'1,O1-(3-((2-((tert-butoxycarbonyl)amino)propanoyl)oxy)propane-1,2-diyl) didodecanedioate (32): To a CH_2Cl_2 solution of Boc-Alanine-OH (0.18g, 0.9mmol), **1** (0.54g, 0.8mmol) was added along with DCC (0.19g, 0.9mmol) and DMAP (cat.). The reaction was stirred at RT for 16h. Next the reaction mixture was filtered and purified via silica gel chromatography (20 to 33% EtOAc/Hexanes) to afford an oil (91%). ^{1}H NMR ($CDCl_3$) δ ppm 1.26 (s, 24H), 1.36 (d, 3H), 1.44 (s, 9H), 1.59-1.68 (m, 8H), 2.27-2.36 (m, 8H), 4.12-4.19 (m, 2H), 4.28-4.34 (m, 3H), 5.02 (d, 1H), 5.11 (s, 4H), 5.28-5.31 (m, 1H), 7.29-7.38 (m, 10H); ^{13}C NMR ($CDCl_3$) δ ppm 18.54, 24.71, 24.73, 28.25, 29.03, 29.04, 29.15, 29.31, 33.89, 33.91, 34.26, 49.15, 61.83, 66.00, 79.82, 128.11, 128.31, 128.48, 136.08, 154.91, 172.55, 173.13, 173.25, 173.62; HRMS calcd. for $[C_{49}H_{73}N_3O_{12}+Na]^+$ 890.5030, found 890.5047.

O'1,O1-(3-((2-aminopropanoyl)oxy)propane-1,2-diyl) 12-dibenzyl didodecanedioate (33): **32** (0.66g, 0.8mmol) was dissolved in 2mL of CH_2Cl_2, and 2mL TFA was added slowly to this ice cold solution. The reaction was allowed to run at RT for 1h. The solvent and TFA were then removed under reduced pressure. The residue was precipitates in cold ether to afford the product as a oil (97%). ^{1}H NMR ($CDCl_3$) δ ppm 1.26 (s, 24H), 1.57-1.65 (m, 11H), 2.28-2.37 (m, 8H), 4.13-4.20 (m, 3H), 4.32-4.36 (m, 2H), 5.11 (s, 4H), 5.27 (s, 1H), 7.30-7.40 (m, 10H); ^{13}C NMR ($CDCl_3$) δ ppm 15.76, 24.71, 24.89, 28.98, 29.04, 29.12, 29.15, 29.29, 33.82, 34.31, 49.22, 61.28, 61.39, 66.17, 72.16, 128.14, 128.18, 128.53, 135.99, 169.31, 173.31, 173.73, 174.05; HRMS calcd. for $[C_{44}H_{65}NO_{10}+H]^+$ 768.4687, found 768.4691.

12-dibenzylO'1,O1-(2,6,9-trimethyl-4,7,10-trioxo-11-oxa-2,5,8-triazatetradecane-13,14-diyl) didodecanedioate (34): To a CH_2Cl_2 solution of **33** (0.43g, 0.4mmol), **31** (0.09g, 0.5mmol) was added along with EDCI (0.09g, 0.5mmol) and DMAP (cat.). The reaction was stirred at RT for 16h. The product was purified via silica gel chromatography (EtOAc to 10% MeOH/EtOAc) to afford an oil (68%). ^{1}H NMR (CDCl$_3$) δ ppm 1.26 (s, 24H), 1.38-1.40 (m, 6H), 1.58-1.67 (m, 8H), 2.27-2.36 (m, 14H), 2.99 (s, 2H), 4.12-4.21 (m, 2H), 4.26-4.33 (m, 2H), 4.49-4.55 (m, 2H), 5.11 (s, 4H), 5.26-5.29 (m, 1H), 6.73 (d, 1H), 7.29-7.38 (m, 10H), 7.60 (d, 1H); ^{13}C NMR (CDCl$_3$) δ ppm 17.73, 18.02, 24.73, 24.75, 24.90, 29.04, 29.06, 29.16, 29.32, 33.92, 34.28, 45.82, 47.98, 48.04, 48.12, 48.17, 61.77, 61.80, 66.01, 70.34, 128.12, 128.49, 136.09, 170.59, 171.60, 171.84, 173.16, 173.26, 173.65; HRMS calcd. for $[C_{51}H_{77}N_3O_{12}+H]^+$ 924.5586, found 924.5587.

12-dibenzylO'1,O1-(2,6,9-trimethyl-4,7,10-trioxo-11-oxa-2,5,8-triazatetradecane-13,14-diyl) didodecanedioate iodide (35): Compound **34** (0.23 g, 0.2mmol) was dissolved in CH$_2$Cl$_2$ and excess MeI was added. The solution was stirred at RT for 6h and the solvent was removed under reduced pressure. The residue was dissolved in CH$_2$Cl$_2$ and precipitated in cold ether twice to obtain the product as an oil (79%). ^{1}H NMR (CDCl$_3$) δ ppm 1.26 (s, 24H), 1.42-1.49 (m, 3H), 1.53-1.58 (m, 3H), 1.61-1.65 (m, 8H), 2.29-2.37 (m, 8H), 3.49 (s, 9H), 4.12-4.62 (m, 8H), 5.11 (s, 4H), 5.18-5.28 (m, 2H), 6.90-7.07 (m, 1H), 7.30-7.35 (m, 10H), 8.61-8.74 (m, 1H); ^{13}C NMR (CDCl$_3$) δ ppm 17.54, 17.98, 24.79, 24.92, 29.06, 29.08, 29.19, 29.34, 33.92, 34.28, 48.21, 50.83, 55.36, 61.69, 64.74, 66.04, 70.10, 71.39, 128.11, 128.15, 128.53, 136.11, 162.22, 171.33, 172.23, 173.30, 173.38, 173.71, 173.73; HRMS calcd. for $[C_{52}H_{80}IN_3O_{12}-I]^+$ 938.5742, found 938.5743.

Liposome preparation

A chloroform solution of the lipid (1.15 mM) was added to a pear shaped flask and the solution was evaporated under vacuum leaving a thin film deposited onto the flask wall. A total of 1 mL Tris buffer (100mM Tris, 100 mM NaCl, pH 7.4) was then added and the film was peeled off by vortexing and hydrating by sonicating at 60°C for 15 minutes. The milky aqueous suspension was extruded through a polycarbonate membrane (100 nm) using an Aventi polar lipids mini-extruder until a homogeneous liposome solution was obtained (typically 21 extrusions). The lipoplexes were prepared by addition of the DNA to the liposomes solution previously prepared, in a ratio lipid/DNA 8:1.

Dynamic light scattering

The diameter of the liposomes and lipid/DNA assemblies was measured at a 90° angle using a Brookhaven Instruments Model 90 Plus. All solutions used in the study including the lipid and DNA solutions of desired concentrations were prepared and filtered through 0.2 μm pore size filter (Whatman) into the scattering cell. The diameter reported corresponds to the average of three experiments in aqueous 100 mM Tris buffer, NaCl 100 mM (pH7.4). The 90 Plus particle sizing software was used for data acquisition and analysis.

Modulated differential scanning calorimetry

The thermal properties of the lipid were measured on a DSC (TA Q100). 0.5 mg of the lipid in 5 μL 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 –50 ºC. The temperature was increased at 0.5 ºC/min to 90 ºC where it has held for 2 min. The temperature was then reduced to –50 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 colleted on the third cycle were analyzed to obtain the Tm..

DNA binding affinities

A competitive displacement fluorescence quenching assay using ethidium bromide was used for the DNA binding studies. 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.

X-ray diffraction

X-ray diffraction was performed on both fully hydrated liposomes and oriented multilayers by methods detailed in previous publications (references 4 and 5). In the case of the fully hydrated preparations, the liposomes were pelleted by centrifugation, the pellet was then transferred to a sealed quartz-glass X-ray capillary and analyzed with a point collimated X-ray beam. For the oriented multilayers, liposomes were partially dried onto a curved glass surface under a controlled relative humidity atmosphere. These oriented multilayers were transferred to a humidity chamber and X-rayed with a line-focussed beam oriented at a grazing angle to the glass surface. Typically, for both unoriented and oriented preparations, the distances of specimen to film were 10 cm and the exposure times were 2-6 hours. A Rigaku rotating anode X-ray generator was used to produce Cu Kα X-radiation and diffraction patterns were obtained using a flat plate film cassette loaded with Kodak DEF X-ray film.

A stationary anode Jerrel-Ash generator (Jerrel-Ash Div., Fisher Scientific Co., Waltham, MA) was used to produce Cu Kα X-radiation. 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 (1.54Å), d is the repeat period, h is the number of the diffraction order, and θ is the Bragg angle.

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 °C in 5% CO_2 with humidity. When the CHO cells reached about 90% confluency, the cells were split into 24-well plates with a 1:4 ratio using standard trypsin-based technique. Transfections were performed 24 hours later by modification of previously published methods.[2] 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. Depending on the experimental design, the ratio of DNA and amphiphile was varied. 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. 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.

Reporter gene transfection efficiency assay

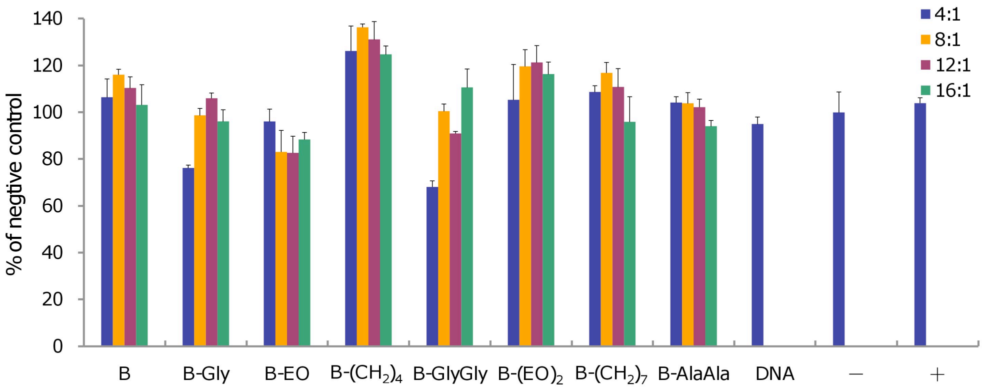
Reporter gene (β-gal) assay was performed with a β-galactosidase enzyme assay system (Promega, Madison, WI) following the manufacturer protocol. Briefly cells were first lysed using M-PER buffer (Pierce, Rockford, Illinois) and enzyme activities were determined. A standard curve was constructed for each 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.

Cytotoxicity

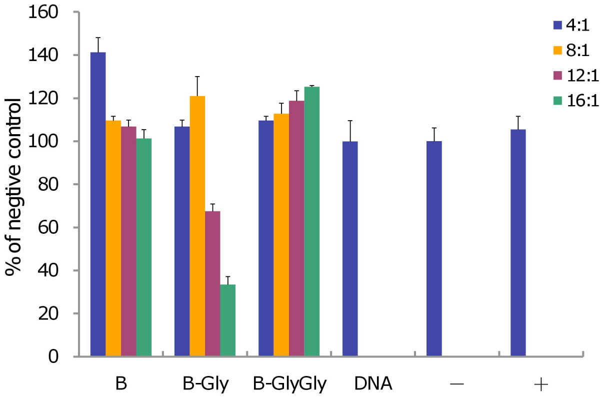
Cytotoxicity was assessed using both a formazan-based proliferation assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay kit, Promega) and a total protein-based assay

(Pierce). Briefly CHO (or NIH 3T3) cells were seeded onto a 24-well microliter plate with an appropriate density of 1 x 10^5 cells per well. After 48h the MTS substrate was added to each well and the plate was incubated for 1 h at 37 °C 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 μL 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

CHO cells



NIH 3T3 cells



siRNA transfection assay

The adherent cells were trypsinized and diluted in normal growth medium to 1X10^5 cells per mL. The transfection reagent (i.e., amphiphile) was diluted in serum free medium and incubated at room temperature for 10 min. RNA was also diluted in serum free medium. Next, both solutions were combined, incubated for 10 min at room temperature and then dispensed into a 96-well culture plate. Depending on the experimental design, the ratio of lipid to siRNA was varied. The cell suspension was then overlayed onto the transfection mixture and incubated at 37 °C and 5% CO_2. Gene knockdown was assessed after 48 h.

Specifically, the gene knockdown assay performed was the KDalert™ GAPDH Assay (Ambion) following the manufacturer's protocol. Briefly, 48 h after siRNA transfection, the culture medium was aspirated from transfected cells. The KDalert Lysis Buffer (200 μL) was added to each sample well. The cells were then incubated at 4°C for 20 min to lyse the cells. The cell lysate was pipetted up and down 4-5 times to homogenize the lysate. Next, 10 μL of each lysate or GAPDH Enzyme dilution (including the GAPDH Working Stock; control soluiton) was transferred to another 96 well plate. Finally, 90 μL of KDalert Master Mix was added to each sample using a multi-channel pipettor and the increase of fluorescence at room temp in a four-minute time period was measured.