**General Experimental Procedures.** $^1$H and $^{13}$C spectra were recorded at 300 and 75 MHz respectively, using CDCl$_3$ (unless noted otherwise) as solvent. Chemical shifts are given in δ units with respect to residual CHCl$_3$ (7.26 ppm for $^1$H) or CDCl$_3$ (77.0 ppm center line in $^{13}$C). Infrared (IR) spectra were obtained on a Mattson FTIR 3000 infrared spectrophotometer using solutions in CHCl$_3$ unless otherwise noted. Melting points are uncorrected. High-resolution mass spectra determinations were conducted at University of Minnesota, Mass spectrum facility (Minneapolis, MN) and University of Colorado at Boulder, Center Analytical Laboratory (Boulder, CO). Elemental analyses were performed by Midwest Mirolabs of Indianapolis, IN. Column chromatography was carried out on 230-400 mesh silica gel, slurry packed in glass columns, eluting with the solvents indicated. Thin layer chromatography was performed on Merck Kieselgel 60 F254 plates, staining with an ethanolic phosphomolybdic acid and sulfuric acid solution.

**(1-(hydroxymethyl)-3-oxetanyl)methyl tetradecanoate (2a).** Oxetane 1 (5.0 g, 41 mmol) was dried in vacuo at 100 °C for 1h and then dissolved in THF (120 mL). To the solution at room temperature was added freshly distilled triethylamine (10 mL, 72 mmol) and N,N-(dimethylamino)pyridine (0.40g, 3.3 mmol) and the reaction was cooled to 0 °C. Myristoyl chloride (8.9 mL, 33 mmol) was added via cannula to the reaction mixture, and the resulting yellowish solution was stirred at 0 °C for 4h and then warmed to room temperature and stirred an additional 4h. The reaction was quenched by addition of diethyl ether and washing with sat'd aq. NaHCO$_3$, followed by washing with brine. The organic layer was separated and dried (Na$_2$SO$_4$). The solvents were removed by rotary evaporation, and the crude product was purified by silica gel column chromatography to yield 8.0g (74%) of the ester 2a as white powder; mp = 49.5 - 50.5 °C; TLC (1:1, HOAc:Hex) $R_f$ = 0.25; IR (CHCl$_3$) 3371, 2914, 2850, 1736 cm$^{-1}$; $^1$H NMR (CDCl$_3$) δ 4.51 (s, 4H), 4.45 (s, 2H), 3.83 (d, $J = 7$Hz, 2H), 2.38 (t, $J = 7$Hz, 2H), 2.20 (t, $J = 7$Hz, 1H), 1.65 (t, $J = 7$Hz, 2H), 1.35 (m, 20H), 0.88 (t, $J = 7$Hz, 3H); $^{13}$C NMR δ 174.4, 75.9, 64.9, 64.1, 44.2, 34.2, 31.9, 29.6 (m), 25.0, 22.7, 14.1.
(1-Formyl-3-oxetanyl)methyl tetradecanoate (3a). To a solution of oxalyl chloride (1.3 mL, 15 mmol) in CH₂Cl₂ (25 mL) at -78 °C was added dropwise dry DMSO (2.3 mL, 30 mmol). The resulting mixture was stirred at -78 °C for 15 minutes whereupon a solution of ester 2a (3.0 g, 9.2 mmol) in CH₂Cl₂ (20 mL) was added to slowly via cannula. After stirring at -78 °C for 1.5 hours, dry DIPEA (8.0 mL, 46 mmol) was add via syringe and the reaction was stirred at -78 °C an additional 30 mins. before warming to 0 °C and stirring for 10 mins. The reaction was diluted with CH₂Cl₂ and washed with 3% ammonium chloride (3x200 mL). The organic layer was separated dried (Na₂SO₄). After removal of the solvents, the crude solid was purified by silica gel column chromatography, eluting with a gradient of 25 to 50% ethyl acetate in hexane, to afford 2.4g (80%) of aldehyde 3a as white solid; mp = 40.2 - 41.8 °C; TLC (1:1, HOAc:Hex) Rf = 0.40; IR (CHCl₃) 2925, 2854, 1720, 1465 cm⁻¹; ¹H NMR (CDCl₃) δ 9.95 (s, 1H), 4.88 (d, J = 6Hz,2H), 4.56 (d, J = 6Hz, 4H), 2.25 (t, J = 6Hz, 2H), 1.65 (m, 2H), 1.30 (m, 20H), 0.85 (t, J = 6Hz, 3H); ¹³C NMR (CDCl₃) δ 198.1, 173.5, 72.7, 63.1, 52.4, 34.0, 31.1, 29.6(m), 24.8, 22.7, 14.1.

2-Bromoethyl 3-(1-(tetradecanoyloxymethyl)-3-oxetanyl)prop-2-enolate (5a). To a solution of aldehyde 3a (2.0 g, 6.1 mmol) in THF (50 mL) at 0 °C was added triethylamine (8.5 mL, 61 mmol) and lithium bromide (2.4 g, 25 mmol). To the reaction mixture was then added a solution of the phosphonate reagent 4 (1.9 g, 6.1 mmol) in THF (20 mL) via cannula. The mixture was stirred at 0 °C for 0.5h and then warmed to room temperature and stirred an additional 4 h. The reaction was diluted with diethyl ether and washed with brine, and the resulting organic layer was separated and dried (Na₂SO₄). The solvents were removed by rotary evaporation and the residue was purified by column chromatography, eluting with 20% ethyl acetate in hexane containing 1% triethylamine, to obtain 2.3 g (79%) of the vinyl ester 5a as a colorless oil; TLC (35:65, HOAc:Hex) Rf = 0.60; IR (CHCl₃) 2924,2854,1734,1654,1467 cm⁻¹; ¹H NMR (CDCl₃) δ 7.15 (d, J = 16Hz, 1H), 6.00 (d, J = 16Hz, 1H), 4.66-4.56 (m, 4H), 4.53 (t, J=6Hz, 2H), 4.40(s, 2H), 3.54(t, J=6Hz, 2H), 2.32(t, J = 7Hz, 2H), 1.58 (m, 2H), 1.24 (m, 20H), 0.88 (t, J
2-Bromoethyl 3-(3,5,8-trioxa-4-tridecylbicyclo[2.2.2]octyl)prop-2-enoate (6a).
To a solution of the vinyl ester 5a (2.3 g, 4.8 mmol) in CH₂Cl₂ (50 mL) at 0 °C was added boron trifluoride etherate (92 µL, 0.7 mmol). The reaction solution was gradually warmed to room temperature and stirred 12h. The reaction was cooled to 0 °C and quenched by addition of triethylamine (0.67 mL, 4.8 mmol) and stirred 0.5h. The mixture was diluted with Et₂O and filtered through a pad of Celite to remove the boron trifluoride-triethylamine complex. The filtrate was concentrated by rotary evaporation to yield the crude product which was purified by column chromatography (NOTE: the SiO₂ must be pretreated by storing as a slurry in hexane containing 1% triethylamine), eluting with a gradient of 25%-50% ethyl acetate in a hexane solution containing 1% triethylamine, to obtain 1.6g (67%) of ortho ester 6a as white solid; mp = 58.5 - 61.0 °C; TLC (35:65, HOAc:Hex) Rf = 0.60; IR (CHCl₃) 2921, 2850, 1718, 1654,1469 cm⁻¹; ¹H NMR (CDCl₃) δ 6.63 (d, J = 16Hz, 1H), 5.77 (d, J=16Hz, 1H), 4.45 (t, J = 6Hz, 2H), 4.06 (s, 6Hz), 3.53 (t, J = 6Hz, 2H), 1.66 (t, J=6Hz, 2H), 1.44-1.40 (m, 2H), 1.24 (m, 20Hz), 0.88 (t, J = 6Hz, 3H); ¹³C NMR (CDCl₃) δ 164.7, 142.1, 122.7, 110.0, 69.9, 64.2, 36.3, 36.2, 31.9, 29.5(m), 28.3, 23.0, 22.8, 14.1; Anal. calcd for C₂₃H₉₉O₅Br C, 58.10; H, 8.23; Found C, 57.83; H, 8.41.

2-(Methyltetradecylamino)ethyl 3-(3,5,8-trioxa-4-tridecylbicyclo[2.2.2]octyl)prop-2-enoate. To a solution of amine 7 (0.43g, 1.6 mmol) in THF (15 mL) at 0 °C was added n-butyl lithium (0.83 mL of a 2.2 M solution in hexane, 1.8 mmol). The resulting solution was stirred at 0 °C for 15 minutes whereupon a solution of the bromoethyl ortho ester 6a (0.65g, 14 mmol) in THF (10 mL) at 0 °C was added via cannula. The reaction mixture was briefly stirred at 0 °C and then slowly allowed to warm to room temperature and stirred for 6 h. The reaction solvents were concentrated to one fourth the volume by rotary evaporation and the concentrate was diluted by addition of CH₂Cl₂. The mixture was washed with saturated NaHCO₃ and the organic
layer was dried (Na₂SO₄). After removal of solvents, the residue was purified by silica gel column chromatography (NOTE: the SiO₂ must be pretreated by storing as a slurry in hexane containing 1% triethylamine), eluting with a gradient of 1-2.5% methanol in CH₂Cl₂ containing 1% triethylamine, to yield 0.55g (64%) of the unsaturated amino ester as white solid; mp = 55.0-56.8 °C; TLC (7.5% MeOH in CH₂Cl₂) Rₜ = 0.50; IR (CHCl₃) 2917, 2849, 1710, 1684, 1469 cm⁻¹; ¹H NMR (CDCl₃) δ 6.55 (d, J = 16Hz, 1H), 5.70 (d, J = 16Hz, 1H), 4.22 (t, J = 6Hz, 2H), 4.04 (s, 6H), 2.64 (t, J = 6Hz, 2H), 2.37 (t, J=6Hz, 2H), 2.26-2.34 (m, 5H), 1.68 (m, 2H), 1.40 (m, 2H), 1.25 (m, 44H), 0.88 (t, J = 6Hz, 6H); ¹³C NMR (CDCl₃) δ 165.3, 141.1, 123.5, 109.9, 70.1, 62.8, 58.1, 55.6, 42.7, 36.5, 36.1, 31.9, 29.6(m), 27.3, 27.2, 22.7, 14.0; HRMS (MALDI) Calc'd for C₃₈H₇₁NO₅ (M+H)+ 622.5405; found 622.5412.

2-(Methyltetradecylamino)ethyl 3-(3,5,8-trioxa-4-tridecylbicyclo[2.2.2]octyl)propanoate (8). To a solution of the unsaturated amino ester (0.55 g, 0.88 mmol) in dry benzene (25 mL) at room temperature was added successively triethylamine (1 mL), 4Å molecular sieves (ca. 0.10g) and 10% palladium on carbon (0.14g). The reaction was then fitted with a balloon containing hydrogen gas and stirred at room temperature for 3h. The reaction mixture was filtered through a pad of Celite and the filtrate was concentrated to afford the crude product. Purification by silica gel column chromatography (NOTE: the SiO₂ must be pretreated by storing as a slurry in hexane containing 1% triethylamine), eluting with a gradient of 1-2.5% methanol in CH₂Cl₂ containing 1% triethylamine, afforded 0.50 g (91%) of the ortho ester lipid 8 as a colorless wax; TLC (7.5% MeOH in CH₂Cl₂) Rₜ = 0.50; IR (CHCl₃) 2950,2852,1730,1460 cm⁻¹; ¹H NMR (CDCl₃) δ 4.15 (t, J = 6Hz, 2H), 3.89 (s, 6H), 2.58 (t, J = 6Hz, 2H), 2.45 (t, J = 6Hz, 2H), 2.24 (M, 5H), 1.61(m, 2H), 1.40 (m, 2H), 1.25 (m, 44H), 0.88 (t, J = 6Hz, 6H); ¹³C NMR (CDCl₃) δ 172.9, 109.7, 71.8, 71.1, 70.9, 63.0, 58.6, 56.0, 43.1, 37.0, 33.0, 32.2, 30.1(m), 28.5, 27.6, 25.1, 23.1, 14.5; HRMS(FAB) calc'd for C₃₈H₇₃NO₅ (M + H⁺) 624.5566; found 624.5591.
Ammonium Iodide 9. In a sealed tube, the hydrogenated ortho ester amine (0.070 g, 0.11 mmol) was dissolved in a large excess of iodomethane (1.5 mL, 24 mmol, pre-purified by passing through a short plug of basic alumina). The solution was purged with argon and stirred at room temperature for 3 hrs. The iodomethane was evaporated (NOTE: use a fume hood) and the residue was dissolved in CH₂Cl₂. Rotary evaporation of the solvent was performed to remove residual iodomethane and gave 0.086 g (100%) of the ammonium iodide 9 as white powder; mp = 162-165 °C; IR (CHCl₃) 2950, 2852, 1739, 1468 cm⁻¹; ¹H NMR (CDCl₃) δ 4.57 (t, J = 6Hz, 2H), 4.07 (t, J = 6Hz, 2H), 3.88 (s, 6H), 3.60 (t, J = 6Hz 2H), 3.43 (s, 6H), 2.32 (t, J = 6Hz, 2H), 1.75 (m, 2H), 1.60 (m, 4H), 1.25 (m, 44H), 0.86 (t, J = 6Hz, 6H); ¹³C NMR (CDCl₃) δ 171.5, 109.1, 70.2, 65.7, 62.5, 57.8, 52.0, 36.5, 32.4, 31.8, 29.2, 28.0, 26.1, 24.2, 22.8, 22.5, 14.0; Anal. calcd for C₃₉H₇₆NO₅I C, 61.16; H, 10.00, N, 1.83. Found C, 61.23; H 10.09; N, 1.82.

(1-(Hydroxymethyl)-3-oxetanyl)methyl octanoate (2b). Oxetane 1 (3.0 g, 25 mmol) was dried in vacuo at 100 °C for 1h and then dissolved in THF (50 mL). To the solution at room temperature was added freshly distilled triethylamine (4.2 mL, 30 mmol) and N,N-(dimethylamino)pyridine (0.25g, 2.0 mmol) and the reaction was cooled to 0 °C. Octanoyl chloride (3.5 mL, 20 mmol) was added via cannula to the reaction mixture, and the resulting yellowish solution was stirred at 0 °C for 4h and then warmed to room temperature and stirred an additional 4h. The reaction was quenched by addition of diethyl ether and washing with saturated aq. NaHCO₃, followed by washing with brine. The organic layer was separated and dried (Na₂SO₄). The solvents were removed by rotary evaporation, and the crude product was purified by silica gel column chromatography to yield 3.2 g (65%) of ester 2b as an oil; IR 3437, 1748, 1466 cm⁻¹; ¹H NMR δ 4.41 - 4.46 (m, 4H), 4.33 (s, 2H), 3.83 (d, J = 5.7 Hz, 2H), 2.77 (t, J = 5.7 Hz, 1H), 2.31 (t, J = 7.4 Hz, 1H), 1.56 - 1.61 (m, 2H), 1.24 (m, 8H), 0.88 (t, J = 4.5 Hz, 3H); ¹³C NMR δ 174.4, 75.8, 64.8, 63.8, 44.0, 34.1, 31.6, 29.0, 28.8, 24.9, 22.5, 14.0.
(1-Formyl-3-oxetanyl)methyl octanoate (3b). To a solution of oxalyl chloride (1.8 mL, 20 mmol) in CH₂Cl₂ (50 mL) at -78 °C was added dropwise dry DMSO (3.2 mL, 42 mmol). The resulting mixture was stirred at -78 °C for 15 minutes whereupon a solution of ester 2b (3.1 g, 13 mmol) in CH₂Cl₂ (10 mL) was added to slowly via cannula. After stirring at -78 °C for 1.5 hours, dry DIPEA (11 mL, 64 mmol) was add via syringe and the reaction was stirred at -78 °C an additional 30 mins. before warming to 0 °C and stirring for 10 mins. The reaction was diluted with CH₂Cl₂ and washed with 3% ammonium chloride (3 x 200 mL). The organic layer was separated dried (Na₂SO₄). After removal of the solvents, the crude solid was purified by silica gel column chromatography, eluting with a gradient of 25 to 50% ethyl acetate in hexane, to afford 2.4 g (78 %) of aldehyde 3b as an oil; IR 2958, 2931, 2856, 1732, 1466 cm⁻¹; ¹H NMR δ 9.89 (s, 1H), 4.83 (d, J = 4.5 Hz, 2H), 4.56 (d, J = 7.8 Hz, 4H), 2.29 (t, J = 7.8 Hz, 2H), 1.59 (m, 2H), 1.30 (m, 8H), 0.85 (t, J = 6.2 Hz, 3H); ¹³C NMR δ 198.1, 173.5, 72.7, 63.0, 50.3, 34.0, 31.6, 29.0, 28.8, 24.8, 22.5, 14.0; HRMS(FAB) calc'd for C₁₃H₂₂O₄ (M + Na⁺) 265.1410; found 265.1415.

2-Bromoethyl 3-(1-(octanoyloxymethyl)-3-oxetanyl)prop-2-enoate (5b). To a solution of aldehyde 3b (2.0 g, 8.3 mmol) in THF (40 mL) at 0 °C was added triethylamine (12 mL, 83 mmol) and lithium bromide (2.9 g, 33 mmol). To the reaction mixture was then added a solution of the phosphonate reagent 4 (3.1 g, 10 mmol) in THF (10 mL) via cannula. The mixture was stirred at 0 °C for 0.5h and then warmed to room temperature and stirred an additional 4 h. The reaction was diluted with diethyl ether and washed with brine, and the resulting organic layer was separated and dried (Na₂SO₄). The solvents were removed by rotary evaporation and the residue was purified by column chromatography, eluting with 20% ethyl acetate in hexane containing 1%triethyl amine, to obtain 2.4 g (74 %) of the vinyl ester 5b as a colorless oil; IR 2924, 2854, 1734, 1654, 1467 cm⁻¹; ¹H NMR δ 7.10 (d, J = 16.1 Hz, 1H), 6.00 (d, J = 16.1
Hz, 1H), 4.66-4.56 (m, 4H), 4.53 (t, J = 6.1 Hz, 2H), 4.40 (s, 2H), 3.54 (t, J = 6.1 Hz, 2H), 2.34 (t, J = 7.4 Hz, 2H), 1.58 (m, 2H), 1.24 (m, 8H), 0.88 (t, J = 5.5 Hz, 3H); $^{13}$C NMR δ 173.1, 165.0, 147.4, 121.1, 65.9, 63.7, 44.3, 33.7, 31.2, 28.7-28.1(m), 24.5, 22.2, 13.6; HRMS(FAB) calc'd for C$_{17}$H$_{27}$BrO$_5$ (M + Na$^+$) 413.0934; found 413.0947.

**2-Bromoethyl 3-(3,5,8-trioxa-4-heptylcyclo[2.2.2]octyl)prop-2-enoate (6b).** To a solution of the vinyl ester 5b (1.5 g, 3.8 mmol) in CH$_2$Cl$_2$ (20 mL) at 0 °C was added boron trifluoride etherate (97 μL, 0.77 mmol). The reaction solution was gradually warmed to room temperature and stirred 12h. The reaction was cooled to 0 °C and quenched by addition of triethylamine (0.53 mL, 3.8 mmol) and stirred 0.5h. The mixture was diluted with Et$_2$O and filtered through a pad of Celite to remove the boron trifluoride•triethylamine complex. The filtrate was concentrated by rotary evaporation to yield the crude product which was purified by column chromatography (NOTE: the SiO$_2$ must be pretreated by storing as a slurry in hexane containing 1% triethylamine), eluting with a gradient of 25%-50% ethyl acetate in a hexane solution containing 1% triethylamine, to obtain 0.98 g (65 %) of ortho ester 6b as white solid, mp = 54.2-55.6 °C; IR 2921, 2850, 1718, 1654, 1469 cm$^{-1}$; $^1$H NMR δ 6.65 (d, J = 16.3 Hz, 1H), 5.75 (d, J = 16.3 Hz, 1H), 4.45 (t, J = 6.1 Hz, 2H), 4.06 (s, 6Hz), 3.53 (t, J = 6.1 Hz, 2H), 1.63-1.70 (m, 2H), 1.44-1.40 (m, 2H), 1.24 (m, 8Hz), 0.88 (t, J = 6.7 Hz, 3H); $^{13}$C NMR δ 164.7, 142.1, 122.7, 110.0, 69.9, 64.2, 36.3, 36.2, 31.9, 29.5(m), 28.3, 23.0, 22.8, 14.1.

**2-(N-Methyl-N-(2-hydroxyethyl)amino)ethyl 3-(3,5,8-trioxa-4-heptylcyclo[2.2.2]octyl)prop-2-enoate.** To a solution N-methylidethanolamine (10) (0.11 mL, 0.96 mmol) in THF (5 mL) at 0 °C was added n-butyl lithium (0.23 mL of a 2.5 M solution in hexane, 0.58 mmol). The resulting solution was stirred at 0 °C for 15 minutes whereupon a solution of the ortho ester 6b (0.15 g, 0.38 mmol) in THF (5 mL) at 0 °C was added via cannula. The reaction mixture was briefly stirred at 0 °C and then slowly allowed to warm to room temperature and
stirred for 6 h. The reaction solvents were concentrated to one fourth the volume by rotary evaporation and the concentrate was diluted by addition of CH₂Cl₂. The mixture was washed with saturated NaHCO₃ and the organic layer was dried (Na₂SO₄). After removal of solvents, the residue was purified by silica gel column chromatography (NOTE: the SiO₂ must be pretreated by storing as a slurry in hexane containing 1% triethylamine), eluting with a gradient of 2-5% methanol in CH₂Cl₂ containing 1% triethylamine, to yield 0.095 g (64 %) of the unsaturated amino ester as colorless oil; TLC: 10% MeOH in CH₂Cl₂, Rf = 0.3; IR (CHCl₃) 3469 (br), 2954, 2856, 1722, 1658, 1468 cm⁻¹; ¹H NMR δ 6.55 (d, J = 16.4 Hz, 1H), 5.75 (d, J = 16.4 Hz, 1H), 4.20 (t, J = 5.6 Hz, 2H), 4.05 (s, 6H), 3.52 (t, J = 5.6 Hz, 2H), 2.70 (t, J = 5.5 Hz, 2H), 2.55 (t, J = 5.5 Hz, 2H), 2.33 (s, 3H), 1.65 (m, 2H), 1.57 (m, 2H), 1.22 (m, 8H), 0.82 (t, J = 4.3 Hz, 3H); ¹³C NMR δ 165.7, 141.4, 123.0, 109.7, 69.6, 62.3, 59.1, 58.3, 55.7, 42.0, 36.4, 36.0, 31.6, 29.3, 29.1, 22.9, 22.5, 14.0; HRMS(MALDI) calc’d for C₂₀H₃₅NO₆ (M + H⁺) 386.2537; found 386.2533.

2-(N-methyl-N-(2-tetradecanoyloxyethyl)amino)ethyl 3-(3,5,8-trioxa-4-heptyl bicyclo[2.2.2]octyl)prop-2-enolate. To a solution of the unsaturated amino ester (0.15g, 0.39 mmol) in CH₂Cl₂ (3.9 mL) at 0 °C was added freshly distilled triethylamine (0.17 mL, 1.2 mmol) and myristoyl chloride (0.12 mL, 0.43 mmol). The mixture was stirred 1h at 0 °C and then warmed to room temperature overnight. The mixture was then diluted with CH₂Cl₂ and washed with saturated NaHCO₃. The organic layer was separated and dried (Na₂SO₄). After removal of solvents by rotary evaporation, the residue was purified by column chromatograph (NOTE: the silica gel must be pretreated by storing as slurry in hexane containing 1% triethyl amine), eluting with a gradient of 0-2% methanol in CH₂Cl₂ containing 1% triethylamine, to yield 0.21 g (88 %) of the corresponding unsaturated amino diester as yellowish oil; TLC, 10% MeOH in CH₂Cl₂, Rf = 0.65; IR 1732, 1657, 1468 cm⁻¹; ¹H NMR δ 6.55 (d, J = 16.4 Hz, 1H), 5.75 (d, J = 16.4 Hz, 1H), 4.18 (t, J = 5.6 Hz, 2H), 4.12 (t, J = 5.9 Hz) 4.01 (s, 6H), 2.66 (m, 4H), 2.31 (s, 3H), 2.25 (t, J = 7.6 Hz, 2H), 1.61-1.66 (m, 2H), 1.40-1.57 (m,4H), 1.22 (m, 24H), 0.82 (t, J = 3.8
Hz, 6H); $^{13}$C NMR δ 173.1, 164.7, 140.8, 122.9, 109.5, 69.6, 62.22, 61.5, 55.6, 55.5, 42.5, 36.1, 36.0, 33.9, 31.4, 29.2(m), 24.5, 22.6, 22.4, 13.5; HRMS(MALDI) calc'd for C$_{34}$H$_{61}$NO$_{7}$ (M + H$^+$) 596.4521; found 596.4505.

2-(N-Methyl-N-(2-tetradecanoyloxyethyl)amino)ethyl 3-(3,5,8-trioxa-4-heptyl bicyclo[2.2.2]octyl) propanoate (11). To a solution of the unsaturated amino diester intermediate (0.10g, 0.17 mmol) in dry benzene (3 mL) at room temperature was added successively triethylamine (0.1 mL), 4Å molecular sieves (ca. 0.050 g) and 10% palladium on carbon (0.020 g). The reaction was then fitted with a balloon containing hydrogen gas and stirred at room temperature for 3h. The reaction mixture was filtered through a pad of Celite and the filtrate was concentrated to afford the crude product. Purification by silica gel column chromatography (NOTE: the SiO$_2$ must be pretreated by storing as a slurry in hexane containing 1% triethylamine), eluting with a gradient of 0-2% methanol in CH$_2$Cl$_2$ containing 1% triethylamine, afforded 0.092 g (92 %) of amino diester 11 as a colorless oil; TLC, 7.5% MeOH in CH$_2$Cl$_2$, R$_f$ = 0.60; IR 1734, 1468 cm$^{-1}$; $^1$H NMR δ 4.10-4.14 (m, 4H), 3.87 (s, 6H), 2.65 (m, 4H), 2.18-2.30 (m, 7H), 1.49-1.64 (m, 6H), 1.37-1.40 (m, 2H), 1.22 (m, 24H), 0.82 (m, 6H); $^{13}$C NMR δ 173.7, 172.3, 109.2, 72.3, 72.4, 61.8, 55.9, 55.8, 42.8, 36.5, 34.2, 32.5, 31.8, 31.7, 29.5(m), 28.0, 24.6, 24.5, 23.0, 22.6, 14.0; HRMS (NALDI) calc'd for C$_{34}$H$_{63}$NO$_{7}$ (M$^+$) 597.4605; found 597.4596.

N,N-Dimethyl-N-(2-[3-(3,5,8-trioxa-4-heptylbicyclo[2.2.2]octyl)propanoyloxy] ethyl)-1-(2-tetradecanoyloxy)ethyl ammonium iodide (12). In a sealed tube, the previous amino diester intermediate (0.050 g, 0.083 mmol) was dissolved in large excess of iodomethane (1.5 mL, 24 mmol, pre-purified by passing through a short column of basic alumina). The solution was purged with argon and stirred at 0 °C for 3 h. The iodomethane then was evaporated (NOTE: use a well-ventilated fume hood) and the residue was dissolved in CH$_2$Cl$_2$. Rotary evaporation of the solvent was conducted to remove any residual iodomethane.
In this manner, 0.061 g (100%) of 12 was obtained as white powder, mp = 92.0 °C (dec); IR 1738, 1468 cm\(^{-1}\); \(^1\)H NMR \(\delta\) 4.59 (m, 4H), 4.12 (m, 4H), 3.89 (s, 6H), 3.53 (s, 6H), 2.32-2.38 (m, 4H), 1.63-1.53 (m,6H), 1.30-1.22 (m, 26H), 0.82 (m, 6H); \(^{13}\)C NMR \(\delta\) 172.6, 171.6, 109.2, 70.6, 63.9, 63.8, 57.9, 57.4, 52.9, 36.5, 34.0, 32.4, 31.8, 31.6, 29.0-29.5(m), 28.1, 24.8, 24.6, 24.2, 23.0, 22.6, 22.5, 14.0; HRMS calc'd for C\(_{35}\)H\(_{66}\)INO\(_7\) (M+D\(^+\)) 612.4839; found 612.4834.

**Procedure for Hydrolysis of Ortho Ester 8.** In a flask, ortho ester amine 8 (0.090 g, 0.14 mmol) was added to a mixture of 6 mL dioxane, 4 mL potassium biphthalate buffer (pH = 4.5) and 15 mL deionized water. The acidity was adjusted by addition of glacial acetic acid to pH = 4.5. The mixture was stirred at 38 °C and the hydrolysis progress was monitored by thin layer chromatography. After 12h, the reaction solution was neutralized by addition of solid NaHCO\(_3\) and then diluted with CH\(_2\)Cl\(_2\). The organic layer was concentrated and the residue was separated by silica gel column chromatography, eluting with a gradient of 1-5% methanol in CH\(_2\)Cl\(_2\) containing 1% triethylamine, to obtain 0.050 g of lactone 14 and 0.035 g of amine 7.

**N-(2-Hydroxyethyl)-N-methyltetradecylamine (7).** IR (CHCl\(_3\)) 3400 (br), 1466 cm\(^{-1}\); \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 3.55 (t, \(J = 5.0\) Hz, 2H), 2.95 (br s, 1H), 2.55 (t, \(J = 5.0\) Hz, 2H), 2.42 (t, \(J = 7.7\) Hz, 2H), 2.25 (s, 3H), 1.45 (m, 2H), 1.23 (m, 22H), 0.80 (m, 3H); \(^{13}\)C NMR (CDCl\(_3\)) \(\delta\) 58.7, 58.0, 57.5, 41.3, 31.4, 29.1 (m), 26.9, 26.5, 22.1, 13.5; HRMS (MALDI): calc'd for C\(_{17}\)H\(_{37}\)NO (M+H\(^+\)) 272.2953, found 272.2948.

**4-Hydroxymethyl-4-myristoyloxymethyl-5-hydroxypentanoic acid lactone (14)**
mp = 52.5 - 54.5 °C; IR (CHCl\(_3\)) 3436 (broad), 2900, 2850, 1743, 1467 cm\(^{-1}\); \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 4.17 (d, \(J = 4\)Hz, 2H), 4.10 (d, \(J = 3\)Hz, 2H), 3.48 (s, 2H), 2.55 (t, \(J = 7\)Hz, 2H), 2.33 (t, \(J = 7\)Hz, 2H), 1.74 (m, 2H), 1.61 (m, 2H), 1.25 (m, 20H), 0.87 (t, \(J = 6\)Hz, 3H); \(^{13}\)C NMR (CDCl\(_3\)) \(\delta\) 174.0, 172.3, 69.9, 67.1, 64.6, 63.4, 38.9, 34.1, 31.9, 29.2-29.1(m), 24.9, 23.7, 22.6, 14.0; HRMS (FAB): calc'd for C\(_{21}\)H\(_{38}\)O\(_5\) (M + H\(^+\)) 371.2797, found 371.2781
Liposome Formulation and Characterization Using TEM

**Liposome Formulation.** Liposomes were prepared by mixing ammonium salt 9 (1 \( \mu \)mol) with various amounts of 2,3-dioleoylphosphatidylethanolamine (DOPE) in chloroform in a vial to obtain formulations with molar ratios ranging from 1:3 to 3:1 lipid:DOPE. The chloroform was removed by rotary evaporation, and the resultant lipid mixtures were dried in vacuo overnight to obtain lipid thin films. The films were hydrated with 1 mL PBS buffer (pH = 7.4) to give 1 mM suspensions. The lipid suspensions were sonicated at 25 \(^{\circ}\)C for 10 minutes using a bath-type sonicator (Laboratory Supplies Co., INC, Hicksville, NY) to complete the liposome formulation.

**Negative Staining Electron Microscopy.** An ortho ester liposome sample was prepared for analysis by transmission electron microscopy as follows: 10 \( \mu \)L of the liposome formulation (described above) was placed on formvar and carbon coated 400 mesh copper grids (Ted Pella, Inc. Redwood, CA). After 3-5 minutes, the grids were gently blotted near to dry with Whatman \#1 filter paper and then 10 \( \mu \)L of 1% aqueous phosphotungstic acid (pH = 5.8) was added and allowed to stand for 3-5 minutes. After the aqueous phosphotungstic acid was blotted, the grids were allowed to dry at room temperature. The grids were viewed and photographed using a Philips EM 400 transmission electron microscope operated at 100 KV.

**Figure.** TEM (1% phosphotungstic acid) of vesicles formed from a 1:3 mixture of 9 and DOPE; A. magnification = 28,000 times, B. magnification = 200,000 times.
Liposome Formulation, Entrapment and pH-Induced Release of the Fluorescence Probe Calcein

Encapsulation of Calcein. A thin film of ortho ester lipid 9 and DOPE, prepared by mixing 2 μmol of the lipid 9 and 6 μmol DOPE, was dried in vacuo overnight. The dried film was then suspended in 1mL PBS solution containing 50 mM calcein to give a suspension with a lipid concentration of 2 mM. The pH of the suspension was adjusted to pH=7.4 by addition of 1N NaOH. The suspension was sonicated for 20 minutes at room temperature using a bath sonicator (Laboratory supplies, Hicksville, NY). Untrapped 'free' calcein was removed by gel filtration of the liposome suspension, accomplished by passing the suspension through Sephadex G-100 (Pharmacia Fine Chemical Inc, Piscataway, N.J) while eluting with PBS solution. The liposome fractions (early fractions) were collected and used directly in the release study.

pH-Mediated Release of Entrapped Calcein. A Perkin Elmer LS 50B luminescence spectrometer was used for the fluorescence assay. The excitation and emission wavelength were 470 and 510 nm, respectively, and the excitation and emission slit width were 5 nm and 3 nm, respectively. A 25 μL liposome sample was aliquoted from a liposome fraction collected after Sephadex gel filtration and added to a cuvette containing 3 mL PBS. The relative fluorescence intensity was measured using the fluorescence spectrometer (value measured = F0 = 171). An appropriate amount of 1N HCl was then added to a portion of the same collected liposome fraction to achieve a pH = 3.5. After stirring 10 mins at room temperature, a 25 μL aliquot was removed to measure the relative fluorescence intensity by addition to a cuvette containing 3 mL PBS (value measured = F1 = 205). As a control experiment, the liposomes were completely disrupted by adding 0.2% Triton X-100 (Aldrich chemical Co, Milwaukee, WI) to the original liposome fraction after gel filtration. Measurement of the relative fluorescence on a 25 μL aliquot was performed in similar manner (value measured = F2 = 245). The percentage of liposome leakage was calculated using the following formula: Leakage percentage = (F1- F0)/(F2- F0) = 45.9%.
Protocol for Transfection of NIH 3T3 Cells

**Liposome formulation.** The cationic lipid (1.0 μmol of either 9, 12, DOTAP or DC-Chol) and DOPE (1.0 μmol) were added as chloroform solutions to a 1.9 mL sample vial. The chloroform was evaporated using a stream of dry argon at room temperature. The resulting thin lipid films were placed under vacuum for 2-3 h to ensure that all traces of solvent were removed. Sterile water (1.0 mL) was then added to hydrate the lipid thin films, and the resultant suspension was vigorously mixed (vortex) at room temperature. The resultant 1.0 mM lipid suspension was used within 2 h of hydration.

**Cell culture.** NIH 3T3 cells were obtained from ATCC (CRL 1658) and grown in Dulbecco’s Modified Eagle’s Medium (DMEM; GIBCO) with 10% fetal calf serum (GIBCO) in a humidified 10% CO₂ incubator at 37°C.

**Transfection experiments.** The NIH 3T3 cells were plated at 50,000 cells per well on a standard 24 well plate (Corning, Corning, NY) 24 h prior to transfection. Cells were approximately 80% confluent at the time of transfection. The growth media was removed via aspiration and each well was washed once with 0.5 mL buffered saline and overlaid with only MEM. Cationic lipid-DNA complexes were prepared 15-40 mins prior to transfection. The pGL3-control vector encoding for firefly luciferase (Promega, Madison, WI) was slowly added to a diluted (DMEM or MEM) quantity of the cationic lipid:DOPE suspension in a polystyrene tube (Falcon #2058), and the lipid-DNA complex was diluted to a final volume of 800 μL. Typically (e.g. using DOTAP), 24 μL of the lipid suspension was used to complex 4.0 μg of the plasmid DNA, yielding a 2:1 cationic lipid:DNA phosphate molar ratio. Immediately on DNA addition, the suspension was vortexed and allowed to incubate for 15 min at room temperature. A 200 μL aliquot of the resultant lipid-DNA suspension was added to each well (1.0 μg DNA/well, n = 4). The treated cells were then incubated for 4 h at 37 °C. At this time, 500 μL of the appropriate growth media including 10% FCS was added to all wells and the cells cultured for 48 h prior to lysis and analysis.
Luciferase assay. Relative luciferase activity was determined by using the Enhanced Luciferase Assay Kit and a Moonlight 2010 luminometer (Analytical Luminescence Laboratories, Sparks, MD). Concentrated luciferase lysis buffer (233.3 μL) was applied to each well. Removal of growth media was not necessary prior to application of the lysis buffer. This technique enhances reproducibility by avoiding the possibility of cell loss during media removal. Luciferase light emissions from 31.1 μL of the lysate were measured over a 10 second period, and results were expressed as a function of assumed total lysate volume of 933.3 μL. Activity has been expressed as relative light units, which are a function of assay conditions, luciferase concentration, luminometer photomultiplier tube sensitivity and background. Results are summarized as the mean (n = 4) and standard deviation of total luciferase light units (RLU) obtained from cells lysed after transfection of 1.0 μg of DNA.

Results

![Graph showing luciferase activity](image)

Transfection of NIH 3T3 cells. All cationic lipids were formulated with equimolar DOPE, and the resultant liposome suspensions were used to deliver 1 ug DNA/well at a cationic lipid:DNA phosphate charge ratio of 2:1. The results are expressed as total relative light units (RLU) of luciferase luminescence. Each data point reflects the mean value from four transfections.
Figure 1. TEM (1% phosphotungstic acid) of vesicles formed from a 1:3 mixture of lipid 9 and DOPE; A = 28,000 times, B = 200,000 times.