Cholesterol Phosphate Derivatives: Synthesis and Incorporation into a Phosphatase and Calcium-Sensitive Triggered Release Liposome

S. C. Davis and F. C. Szoka Jr.*

Department of Biopharmaceutical Sciences and Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, California 94143-0446. Received May 6, 1998;
Revised Manuscript Received August 27, 1998

A series of cholesterol derivatives that position a phosphate monoester at increasing distance from the sterol ring system was synthesized, and their utility as a triggered release liposome tested. Stable anionic liposomes consisting of the novel cholesterol phosphate derivatives and dioleoylphosphatidylethanolamine (DOPE) can be induced to collapse upon phosphatase-catalyzed removal of the phosphate group. Control liposomes containing DOPE and cholesterol phosphate or phosphatidic acid, which are not phosphatase substrates, do not undergo phosphatase-mediated collapse. The phosphatase-sensitive liposomes also collapse in the presence of calcium. The precise concentration of calcium that induces the collapse is controlled by the structure of the cholesterol phosphate derivative. Plasmid DNA encoding luciferase, encapsulated in the cholesterol derivative/DOPE liposomes, transfected cells in vitro. The level of transfection is dependent upon the cholesterol derivative and is mediated by both a calcium-independent and a calcium-dependent pathway; however, the involvement of phosphatase in the latter mechanism is not yet resolved. The transfection efficiency is between 10^6 and 10^7 of luciferase activity in relative light units per milligram of protein, which is similar to transfection values reported using other triggered release liposomes.

INTRODUCTION

One approach to the delivery of drugs and DNA to the cytoplasm of cells is to use triggered release liposomes (*1*, *2*). Such liposomes are stable under normal physiological conditions but release their contents upon receipt of a defined stimulus (*1*). This allows the controlled delivery of the liposome contents to the site of the stimulus, either intracellularly or at defined sites in the body.

Triggered release liposomes rely on the ability of lipids to interconvert among different phases in aqueous media. The two phases most commonly utilized in triggered release liposomes are the lamellar and the inverted hexagonal phase. When the lipids prefer the lamellar phase, liposomes are formed which are capable of encapsulating exogenous material. When the lipids form an inverted hexagonal phase, no vesicle structure is possible.

A commonly employed lipid for triggered release compositions is dioleoylphosphatidylethanolamine (DOPE)[1] which prefers the inverted hexagonal phase under physiological conditions (*3*) but can be stabilized in the lamellar phase by the addition of at least 25 mole percent of a charged colipid. Removal of the charge from the colipid is sufficient to induce the phase change. Several different strategies have been used for the controlled removal of charge from the surface of a phosphatidylethanolamine/colipid bilayer, including pH change [e.g., cholesterol hemisuccinate, CHEMS (*4*) and oleic acid (*5*)], enzyme catalysis [e.g., phospholipase C or acetylcholine esterase (*6*)], and light [cleavage of plasmalogen vinyl ethers (*7*)].

We hypothesized that lipids with a doubly negatively charged phosphate monoester headgroup (similar to phosphatidic acids) would stabilize phosphatidylethanolamine bilayers. Two mechanisms for the triggered release of the liposomes could then be envisaged, enzymatic and ionic. Phosphatase enzymes which catalyze the hydrolysis of phosphate monoesters could remove the phosphate and its corresponding charge from the bilayer surface, thus allowing collapse to the hexagonal phase and release of the liposome contents. Two examples of this class of enzymes are alkaline phosphatase (*8*, *9*), which is a widely specific, highly active enzyme found in serum and on the cell membrane, and acid phosphatase, which is found intracellularly in endosomes and lysosomes (*10*). Alkaline phosphatase has long been utilized in the area of pro-drugs to convert inactive phosphate derivatives into the active compounds (*11*). Recently, this enzyme has been shown to inactivate endotoxin by removing the phosphate from this lipopolysaccharide (*12*). This suggested that alkaline phosphatase would be capable of hydrolyzing phosphate from a lipid derivative. Second, divalent cations such as calcium are well-known to cause aggregation and fusion (*13*) of anionic liposomes, and in this phosphate containing system, the calcium effect may trigger the collapse to the hexagonal phase (*14*).

* Address correspondence to this author. Fax: (415) 476 0688. Telephone: (415) 476 3895. E-mail: szoka@cgl.ucsf.edu.

[1] Abbreviations: DOPE, dioleoylphosphatidylethanolamine; PE, phosphatidylethanolamine; HEPES, (hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HBS, HEPES buffered saline; ANTS, 8-aminonaphthalene-1,2,3-trisulfonic acid; DPX, *p*-xylenebis(pyridinium)bromide; CHEMS, cholesterol hemisuccinate; REV, reversed-phase evaporation vesicles; RLU, relative light units; I. U., international unit; sat, saturated aqueous solution; POPG, palmitoyloleoylphosphatidylglycerol.

10.1021/bc980047y CCC: $15.00
Published on Web 10/08/1998

We show here that novel cholesterol phosphate derivatives can be incorporated into PE liposomes, confer calcium and phosphatase sensitivity, and can be used to trigger the release of liposome contents.

EXPERIMENTAL SECTION

General. All purchased chemicals were of ACS grade or better and were used without further purification. Solvents for HPLC analysis were obtained as HPLC grade and were filtered (0.2 μM) and degassed before use. NMR spectra were recorded on a GE 300 MHz instrument and chemical shifts are expressed as parts per million using as internal standard, tetramethylsilane (δ = 0.0 ppm) for ^{1}H NMR and $CDCl_3$ (δ = 77.0 ppm) for ^{13}C NMR. Optical rotation measurements were taken on a Perkin-Elmer 241 Polarimeter using the sodium line. IR measurements were taken on a Nicolet Impact 400 as a film on a 3M Type 62 disposable IR card. Calf intestinal alkaline phosphatase and DNase 1 were purchased from Boehringer Mannheim. Acid phosphatase (Sweet potato) was purchased from Sigma (St. Louis, MO).

Cholest-5-en-3β-tosylate (1). To a solution of cholesterol (5 g, 12.9 mmol) in anhydrous pyridine (70 mL) was added *p*-toluenesulfonyl chloride (5 g, 26 mmol), and the mixture was stirred for 16 h under an inert atmosphere at room temperature. The solvent was filtered off, and the white mass washed with pyridine (50 mL) and water (100 mL). Recrystallization from chloroform/methanol gave the product as white crystals (6.5 g, 12 mmol, 93%). R_f = 0.8 ($CHCl_3$). ^{1}H (300 MHz, $CDCl_3$): δ 0.65 (3H, s); 0.85−1.57 (33H, m); 1.78−2.01 (6H, m); 2.23−2.29 (2H, m); 2.44 (3H, s); 4.28−4.33 (1H, m); 5.29 (1H, d, J = 4.5 Hz); 7.32 (2H, d, J = 8 Hz); 7.79 (2H, d, J = 8 Hz). ^{13}C (75 MHz, $CDCl_3$): δ 11.77, 18.66, 19.11, 21.59, 22.52, 22.78, 23.77, 24.20, 27.96, 28.16, 28.58, 31.70, 31.81, 35.71, 36.12, 36.30, 36.83, 38.84, 39.46, 39.61, 42.25, 49.86, 56.05, 56.59, 82.15, 123.68, 127.58, 129.69, 134.65, 138.79, 144.34. LSIMS: *m/z* 369 (M − tosylate, 100%).

Cholest-5-en-3β-oxyethan-2-ol (2a). To a suspension of cholest-5-en-3β-tosylate (**1**) (500 mg, 0.9 mmol) in anhydrous dioxane (9 mL) was added ethylene glycol (1 g, 23.4 mmol), and the mixture was stirred under reflux for 4 h in an inert atmosphere. The solution was cooled, and the solvent removed in vacuo. The white residue was partitioned between diethyl ether (20 mL) and water (20 mL), and the organic layer was washed sequentially with sat $NaHCO_3$ (2 × 10 mL), water (10 mL) and sat brine (10 mL). The solution was dried over magnesium sulfate, and the solvent removed in vacuo. Recrystallization of the residue from chloroform/methanol gave the product as white crystals (350 mg, 0.81 mmol, 88%). Mp 97−98 °C. Anal ($C_{29}H_{50}O_2$) C, H. R_f = 0.3 ($CHCl_3$). α_D^{20} (c = 0.55, $CHCl_3$) −31.6° [lit. −30.95° (*15*)]. IR (film): 3369 (m, br, O−H), 2930 (st, C−H), 2866 (st, C−H), 1466 (m, C=C), 1380 (m). ^{1}H (300 MHz, $CDCl_3$): δ 0.69 (3H, s); 0.86−1.57 (33H, m); 1.78−2.04 (6H, m); 2.19−2.22 (2H, m); 2.35−2.37 (1H, m); 3.17−3.21 (1H, m); 3.60 (2H, t, J = 4.5 Hz); 3.73 (2H, t, J = 4.5 Hz); 5.36 (1H, d, J = 4.5 Hz). ^{13}C (75 MHz, $CDCl_3$): δ 11.84, 18.70, 19.35, 21.06, 22.54, 22.79, 23.82, 24.27, 28.00 28.21, 28.41, 31.57, 31.90, 35.77, 36.18, 36.85, 37.17, 39.10, 39.51, 39.78, 42.31, 50.18, 56.16, 56.76, 62.08, 68.92, 121.71, 140.71. EI$^+$: *m/z* 429 (M − H+, 12%); 369 (100%). EI−HRMS $C_{29}H_{50}O_2$: Calcd, 430.3811; found, 430.3800.

Cholest-5-en-3β-oxyhexan-6-ol (2b). This compound was prepared similarly to cholest-5-en-3β-oxyethan-2-ol (**2a**) with 1,6-hexanediol to give a white powder that was recrystallized from chloroform/methanol (810 mg, 1.66 mmol, 83%). Anal. ($C_{33}H_{58}O_2$) C, H. R_f = 0.3 ($CHCl_3$). α_D^{20} (c = 1.25, $CHCl_3$) −26.9°. IR (film): 3300 (m, br, O−H), 2928 (st, C−H), 2860 (st, C−H), 1463 (m, C=C), 1371 (m). ^{1}H (300 MHz, $CDCl_3$): δ 0.65 (3H, s); 0.82−1.53 (41H, m); 1.74−2.00 (6H, m); 2.10−2.19 (1H, m); 2.29−2.33 (1H, m); 3.05−3.10 (1H, m); 3.42 (2H, t, J = 6.5 Hz); 3.57 (2H, t, J = 6.5 Hz); 5.30 (1H, d, J = 4.5 Hz). ^{13}C (75 MHz, $CDCl_3$); δ 11.94, 18.71, 19.39, 21.04, 22.61, 22.78, 23.94, 24.56, 25.60, 26.04, 27.93, 28.27, 28.60, 28.70, 30.13, 32.11, 32.71, 35.62, 35.76, 36.90, 37.66, 39.38, 39.50, 42.31, 50.18, 56.14, 56.78, 62.96, 67.97, 120.64, 141.12. $^+$LSIMS: *m/z* 486 (9%, M$^+$); 369 (100%). EI−HRMS $C_{33}H_{58}O_2$: calcd, 486.4437; found, 486.4423.

Cholest-5-en-3β-oxypent-3-oxa-an-5-ol (2c). This compound was prepared similarly to cholest-5-en-3β-oxyethan-2-ol (**2a**) with diethylene glycol to give an oil that was purified on silica eluting with chloroform to give a gum (830 mg, 1.75 mmol, 87%). Anal. ($C_{31}H_{54}O_3$) C, H. R_f = 0.15 ($CHCl_3$). α_D^{20} (c = 1.06, $CHCl_3$) −28.5°. IR (film): 3407 (m, br, O−H), 2929 (st, C−H), 2861 (st, C−H), 1462 (m, C=C), 1366 (m). ^{1}H (300 MHz, $CDCl_3$): δ 0.63 (3H, s); 0.81−1.55 (32H, m); 1.76−1.99 (6H, m); 2.17−2.21 (1H, m); 2.30−2.37 (1H, m); 3.15−3.28 (2H, m); 3.53−3.69 (8H, m); 5.29 (1H, d, J = 4.5 Hz). ^{13}C (75 MHz, $CDCl_3$): δ 11.77, 18.64, 19.29, 20.99, 22.48, 22.74, 23.77, 24.20, 27.91, 28.15, 28.23, 31.83, 35.70, 36.11, 36.76, 37.12, 38.89, 39.43, 39.70, 42.25, 50.09, 56.10, 56.68, 51.70, 67.32, 70.67, 72.51, 79.52, 121.60, 140.63. $^+$LSIMS: *m/z* 473 (10%, M − H); 369 (100%). EI−HRMS $C_{31}H_{54}O_3$: calcd, 474.4073; found, 474.4059.

Cholest-5-en-3β-oxy-oct-3,6-oxa-an-8-ol (2d). This compound was prepared similarly to cholest-5-en-3β-oxyethan-2-ol (**2a**) with triethylene glycol to give an oil that was purified on silica eluting with chloroform to give a gum (450 mg, 0.87 mmol, 94%). R_f = 0.15 ($CHCl_3$). α_D^{20} (c = 0.69, $CHCl_3$) −29.3° [lit. −25.38° (*15*)]. IR (film): 3425 (m, br, O−H), 2932 (st, C−H), 2859 (st, C−H), 1460 (m, C=C), 1376 (m). ^{1}H (300 MHz, $CDCl_3$); δ 0.85−1.60 (33H, m); 1.79−2.06 (6H, m); 2.18−2.29 (1H, m); 2.37−2.41 (1H, m); 3.16−3.23 (1H, m); 3.60−3.78 (12H, m); 5.37 (1H, d, J = 4.5 Hz). ^{13}C (75 MHz, $CDCl_3$); δ 11.76, 18.63, 19.27, 20.97, 22.47, 22.72, 23.74, 24.19, 27.90, 28.14, 28.18, 31.79, 31.84, 35.69, 36.10, 36.75, 37.11, 38.87, 39.42, 39.69, 42.21, 50.08, 56.07, 56.67, 61.57, 66.96, 67.08, 70.18, 70.47, 70.71, 72.58, 79.47, 121.52, 140.71. EI$^+$: *m/z* 517 (M − H$^+$, 7%); 383 (21%); 369 (100%). EI−HRMS $C_{33}H_{58}O_4$: calcd, 518.4335; found, 518.4315.

Cholest-5-en-3β-oxyethane-2-phosphate (3a). To a stirred solution of cholest-5-en-3β-oxyethan-2-ol (**2a**) (86 mg, 0.2 mmol) in anhydrous chloroform (0.5 mL) was added a solution of tetra-*n*-butylammonium phosphate (340 mg, 1 mmol) in chloroform (0.5 mL) immediately followed by addition of trichloroacetonitrile (120 μL, 1.2 mmol). The solution was stirred under an inert atmosphere for 15 min and the solvent was removed in vacuo. The residue was dissolved in water saturated *n*-butanol (5 mL), and the solution washed sequentially with *n*-butanol-saturated water (2 × 2 mL), *n*-butanol-saturated 1 M HCl (2 × 2 mL), and sat brine (2 mL). The organic phase was dried over magnesium sulfate and filtered, and the solvent removed in vacuo. The residue was dissolved in chloroform (2 mL), and sodium iodide (150 mg, 1 mmol) in acetone (2 mL) was added. After 15 min, the precipitate was separated by centrifugation, the solvent decanted, and the precipitate washed with chloroform (2 × 2 mL) to yield the product as the sodium

salt (80 mg, 0.14 mmol, 72%). (Cholesterol C_2 phosphate) R_f = 0.35 ($CHCl_3$/MeOH/H_2O 65/25/4). IR (film): 2924 (st, C−H), 2844 (st, C−H), 2365 (st, PO−H), 2340 (st, PO−H), 1650 (m), 1457 (m, C═C). ^{1}H (300 MHz, $CDCl_3$/MeOD/d_5-pyridine/DCl 10/2/1/1) (*16*): δ 0.70 (3H, s), 0.83−1.60 (33H, m); 1.83−2.05 (6H, m); 2.16−2.18 (1H, m); 2.33−2.38 (1H, m); 3.20−3.33 (1H, m); 3.74 (2H, t, J = 4.5 Hz); 4.17 (2H, t, J = 4.5 Hz), 5.36−5.38 (1H, m). ^{13}C (75 MHz, $CDCl_3$/MeOD/d_5-pyridine/DCl 10/2/1/1): δ 11.17, 18.03, 18.61, 20.46, 21.80, 22.04, 23.19, 23.65, 27.35, 27.59, 31.31, 35.16, 35.58, 36.18, 36.51, 38.26, 38.90, 39.19, 41.74, 49.62, 55.60, 56.18, 65.81, 66.52, 79.19, 121.39, 139.77. $^-$LSIMS: m/z 509 (M − H$^-$, 100%).

Cholest-5-en-3β-oxyhexane-6-phosphate (3b). This compound was prepared similarly to cholest-5-en-3β-oxyethane-2-phosphate (**3a**) to yield a white powder (75 mg, 0.13 mmol, 66%). R_f = 0.35 ($CHCl_3$/MeOH/H_2O 65/25/4). IR (film): 2930 (st, C−H), 2856 (st, C−H), 2359 (st, PO−H), 2327 (st, PO−H), 1656 (m), 1451 (m, C═C). ^{1}H (300 MHz, $CDCl_3$/MeOD/d_5-pyridine/DCl 10/2/1/1): δ 0.72 (3H, s), 0.78−1.69 (33H, m); 1.84−2.07 (6H, m); 2.19−2.24 (1H, m); 2.34−2.37 (1H, m); 3.17−3.19 (1H, m); 3.46−3.52 (2H, m); 3.99−4.09 (2H, m), 5.38 (1H, d, J = 4 Hz). ^{13}C (75 MHz, $CDCl_3$/MeOD/d_5-pyridine/DCl 10/2/1/1): δ 10.77, 17.66, 18.25, 20.01, 21.41, 21.67, 22.66, 23.21, 23.90, 24.29, 24.70, 26.85, 27.14, 30.86, 34.66, 35.08, 35.78, 36.18, 38.08, 38.40, 38.72, 41.26, 49.14, 55.08, 55.70, 66.90, 78.05, 120.57. $^-$ESI: m/z 565 (M − H$^-$, 26%).

Cholest-5-en-3β-oxypent-3-oxa-ane-5-phosphate (3c). This compound was prepared similarly to cholest-5-en-3β-oxyethane-2-phosphate (**3a**) to yield a white powder (55 mg, 0.14 mmol, 50%). R_f = 0.35 ($CHCl_3$/MeOH/H_2O 65/25/4). IR (film): 2929 (st, C−H), 2856 (st, C−H), 2359 (st, PO−H), 2342 (st, PO−H), 1662 (m), 1463 (m, C═C). ^{1}H (300 MHz, $CDCl_3$/MeOD/d_5-pyridine/DCl 10/2/1/1): δ 0.68 (3H, s); 0.68−1.57 (33H, m); 1.77−2.06 (6H, m); 2.16−2.24 (1H, m); 2.31−2.37 (1H, m); 3.16−3.20 (1H, m); 3.64−3.71 (8H, m); 4.08−4.12 (2H, m); 5.33 (1H, d, J = 4 Hz). ^{13}C (75 MHz, $CDCl_3$/MeOD/d_5-pyridine/DCl 10/2/1/1): δ 11.43, 18.29, 18.41, 18.89, 20.64, 22.09, 22.33, 23.40, 23.85, 27.54, 27.79, 31.48, 35.25, 35.76, 36.38, 36.72, 38.24, 38.52, 39.07, 41.90, 49.56, 49.75, 55.76, 56.33, 64.60, 66.64, 70.16, 79.21, 121.43, 140.11. $^-$LSIMS: m/z 554 (M − H$^-$, 28%).

Cholest-5-en-3β-oxy-oct-3,6-oxa-ane-8-phosphate (3d). This compound was prepared similarly to cholest-5-en-3β-oxyethane-2-phosphate (**3a**) to yield a white powder (65 mg, 0.11 mmol, 55%). Cholesterol triethylene phosphase R_f = 0.35 ($CHCl_3$/MeOH/H_2O 65/25/4). IR (film): 2932 (st, C−H), 2861 (st, C−H), 2361 (st, PO−H), 2338 (st, PO−H), 1647 (m), 1456 (m, C═C). ^{1}H (300 MHz, $CDCl_3$/MeOD/d_5-pyridine/DCl 10/2/1/1): δ 0.68 (3H, s), 0.86−1.60 (33H, m); 1.77−2.04 (6H, m); 2.12−2.19 (1H, m); 2.31−2.36 (1H, m); 3.14−3.19 (1H, m); 3.61−3.70 (12H, m); 4.12−4.15 (2H, m), 5.33 (1H, d, J = 5 Hz). ^{13}C (75 MHz, $CDCl_3$/MeOD/d_5-pyridine/DCl 10/2/1/1): δ 11.48, 18.33, 18.96, 20.68, 22.17, 22.43, 23.45, 23.91, 27.63, 27.86, 31.50, 35.41, 35.81, 36.44, 36.75, 38.56, 39.13, 39.37, 41.93, 49.33, 49.77, 55.77, 56.36, 65.35, 66.67, 69.35, 70.27, 76.22, 79.02, 79.44, 121.39. $^-$LSIMS: m/z 597 (M − H$^-$, 38%).

Leakage Studies. A premixed solution of cholesterol and DOPE (5/4 molar ratio) was evaporated to a lipid film, and the film was exposed to high vacuum (<150 mTorr) for at least 2 h before use. The lipids were hydrated in 20 mM HEPES, 12.5 mM ANTS, and 45 mM DPX at pH 7.0 with vortexing and brief sonication (*17*). The liposomes thus formed were then extruded through a hand held extrusion device (Avestin, Ottawa, Canada) with a 0.2 μm polycarbonate membrane (Nucleopore Corp., Pleasanton, CA). The liposomes were applied to a Sephadex G50 gel filtration column (1 × 20 cm) to remove any unencapsulated dye. The liposome concentration was quantified using the phosphorus assay of Bartlett (*18*). The dequenching of ANTS upon liposome leakage was measured according to Ellens et al. (*17*). The fluorescence arising from excitation at 360 nm was measured through a Schott GG 435 nm cutoff filter (50% transmittance at 435 nm). Leakage experiments were performed at room temperature in triplicate for each condition, and each cuvette (Polymethacrylate Fluorimeter cuvette, Sigma, St. Louis, MO) was stirred and analyzed separately. The initial fluorescence of the intact liposomes at time 0 was measured and set as F_0, or 0% leakage. The fluorescence for each cuvette was measured at the times indicated after addition of the enzyme. At the end of the time course F_{100}, 100% leakage was defined by the fluorescence arising from addition of the detergent, dodecyloctaethylene glycol monoether (C12E8) (Calbiochem, La Jolla, CA). The percent leakage at time t was determined for each cuvette using the formula ($F_t - F_0$/$F_{100} - F_0$)100, and then the results were combined. Leakage experiments with calcium were performed sequentially with timepoints measured every second, and the percentage of F_{100} observed at the plateau point was calculated using the same formula as before. Lipid-mixing experiments were performed according to the method of Struck et al. (*19*). NBD-PE (0.5 mol %) and 0.5 mol % Rhodamine-PE were incorporated into cholesterol C2 phosphate (**3a**)/DOPE liposomes, and these were mixed with unlabeled liposomes (9-fold excess). Lipid mixing was followed using the increase of fluorescence at 535 nm upon addition of calcium. Values in the figures are the mean ± standard deviation from three determinations.

Identification of the Products of Reaction. One micromole of cholesterol C2 phosphate (**3a**)/DOPE (4/5) liposomes in HEPES buffered saline (10 mM HEPES and 150 mM NaCl, pH 7.4) (10 mL) was incubated with 0, 20, and 100 I. U. of alkaline phosphatase in triplicate. The samples were placed in sealed tubes and mixed by slow rotation (∼50 rpm) for 24 h at room temperature and the incubation stopped by freezing and lyophilization. The residue was redissolved in water (1 mL), and the lipids were extracted using the Bligh and Dyer protocol (*20*) and dried down separately for HPLC analysis. The aqueous phase was relyophilized, taken up in water (300 μL) and the phosphate concentration measured using a modification of the method of Drueckes et al. (*21*). Thirty microliters of this solution was mixed with the assay mixture (125 μL) and incubated at 30 °C for 10 min, and the absorbance measured at 630 nm. To form the assay mixture, ammonium molybdate (10 mM final) was dissolved in a solution of zinc acetate (15 mM) at pH 5.0. This was mixed with a 10% ascorbate solution, pH 5.0, and a solution of sodium dodecyl sulfate (200 mg/mL) in a 40:10:1 ratio. The assay mixture was used immediately.

The lipids isolated in the Bligh and Dyer extraction were dissolved in chloroform/methanol (2/1, 200 μL) and separated on a Hewlett-Packard 1090 HPLC system with a Partisil ODS3 column (5 μm, 4.5 mm × 250 mm) using methanol (1 mL/min) as the mobile phase and UV detection at 215 nm. The cholesterol C2 alcohol (**2a**) was indentified by coinjection with synthetic material and quantified via a standard curve of **2a**.

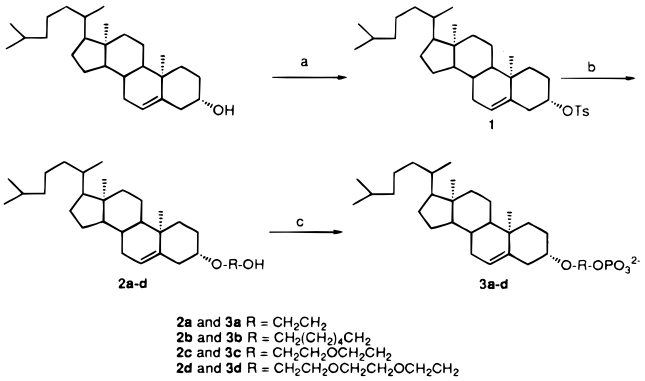
Encapsulation of Plasmid DNA in Liposomes. A cholesterol phosphate derivative/DOPE (4/5) lipid film (20 μmol) was dissolved in a mixture of diethyl ether and 1,1,2-trichloro-2,2,1-trifluoroethane (57:43, 3 mL), and 10 mM HEPES, 5% glucose buffer at pH 7.4 (0.5 mL) was added. The mixture was vigorously sonicated to form an emulsion to which plasmid DNA (375 μg) (pLC0785.112, a generous gift of GeneMedicine Inc.) in 10 mM HEPES and 5% glucose, pH 7.4 (0.5 mL), was added followed by further brief sonication. The emulsion was then dried down to the gel phase, which was broken under reduced pressure to yield liposomes, of 300 nm z-average diameter as determined by dynamic light scattering (Coulter Electronics Inc., Hialeah, FL). Using the multimodel algorithms, these liposomes were purified on a discontinuous Ficoll gradient to remove excess DNA (*22*). DNA encapsulation efficiency was measured after determining the amount of lipid phosphate and DNA in the fraction containing the liposomes. The DNA concentration was measured using the Hoescht dye 33258 (Polysciences, Inc., Warrington, PA) and a DNA fluorometer (TKO100, Hoeffer Scientific Instruments, San Francisco, CA). The liposomal phosphate was quantified using the assay of Bartlett (*18*) for the total phosphate and subtracting the amount of phosphate contributed by the associated DNA.

DNase Protection Assay. Plasmid DNA (1 μg), either encapsulated in liposomes or free, was dissolved in HBS (10 μL) and DNase was added and the mixture incubated at room temperature for 16 h. The samples were then purified by a Bligh and Dyer extraction and the DNA separated by electrophoresis on a 0.8% agarose gel with ethidium bromide staining.

Transfection of Monkey Kidney Fibroblast Cells. CV-1 cells were plated in a 96 well plate at 2×10^4 cells/well in 100 μL of growth medium consisting of DME-H21 with 10% fetal calf serum and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin). The cells were incubated for 19 h at 37 °C in a 95% air, 5% CO_2 atmosphere. The growth medium was then removed, the cells were washed with serum free medium, and serum free medium was added (150 μL). The transfection system was then added in 50 μL of 10 mM HEPES and 5% glucose, pH 7.4, buffer, and the cells were incubated in this medium for 3 h. Subsequently, the medium was replaced with complete growth medium, and the cells were incubated for a further 36 h. The medium was removed, and the cells were washed with phosphate-buffered saline and assayed for luciferase activity and total cell protein (Bio-Rad BCA protein assay). For the phosphatase inhibitor experiments, inorganic phosphate (10 mM final) and L-*p*-bromotetramisole (1 mM final) (Sigma, St. Louis, MO) were added to the serum-free medium before addition of the transfection system. Nonencapsulated DNA/liposomes were prepared by mixing 100 nmol of cholesterol phosphate derivative/DOPE (4/5) liposomes with 1 μg of pLC 0785.112 (GeneMedicine Inc.) in 50 μL of buffer before addition to the cells.

Transfections in low-calcium media were performed utilizing calcium/magnesium-free Hanks-buffered salt solution, supplemented with nonessential amino acids, antibiotics, and glutamine (2 mM) in place of serum free media. The calcium concentration noted was added to the media from a stock solution of $CaCl_2$ immediately before use. Where noted, the salt wash was performed, immediately after aspiration of the transfection medium, with 1 M NaCl and 20 mM HEPES buffer, pH 7.4, followed by a media wash and incubation in growth medium for a further 36 h.

Scheme 1. Synthesis of Cholesterol Phosphate Derivatives[a]



[a] (a) *p*-Toluene sulfonyl chloride, pyridine, ambient, 16 h, quant. (b) HO-R-OH, 1,4-dioxane, reflux, 4 h, 88%. (c) (i) Tetra-n-butylammonium phosphate, trichloroacetonitrile, 15 min. (ii) NaI, 15 min, 50−70% (two steps).

RESULTS

Synthesis. Our approach to the synthesis of a phosphatase-sensitive lipid employed cholesterol as the lipophilic anchor due to the ability of cholesterol to promote the lamellar to hexagonal transition in PE membranes (*23*) and for synthetic ease. Since the structural requirements of phosphatase enzymes using lipids as substrates are not known, we sought a versatile synthesis amenable to the production of a series of lipids (Scheme 1). S_N1 displacement of tosylate from the 3-β position of cholesterol with ethylene glycol had been shown to be an efficient method for derivatizing cholesterol (*15*), and this displacement proceeds smoothly for a wide variety of diols. Phosphorylation of the prepared diols was performed via the method of Danilov et al. (*24*) and the tetra-*n*-butylammonium salt was smoothly transformed into the sodium salt with sodium iodide.

Liposome Formation. To determine which lipids would be most appropriate to use in a triggered release system a variety of cholesterol alcohol derivatives (eight in all) (*25*) were synthesized. These were then tested for the ability to stabilize a DOPE bilayer. At pH 7.4, none of the derivatives formed liposomes with DOPE in a 4/5 cholesterol alcohol/DOPE ratio. At pH 9.0, DOPE is sufficiently charged to support a bilayer in the presence of cholesterol. Inclusion of the cholesterol alcohol derivatives in place of cholesterol showed that only those lipids with hydrophilic linkers or hydrophobic linkers of six carbons or less formed liposomes with DOPE. All the formulations collapsed upon reduction of the pH to 7.4, after protonation of the DOPE (*25*). This suggested that phosphatase-mediated removal of the phosphate groups from the cholesterol phosphate derivative/DOPE liposomes would initiate collapse of the liposomes with concomitant release of the contents. This initial screen of linker structure against liposome stability allowed us to identify a number of lipids to study in greater detail. The selected lipids were structurally distinguished by a short linker (**3a**), the longest stable hydrophobic linker (**3b**), a hydrophilic linker (**3c**), and the longest linker tested (**3d**), respectively (Scheme 1).

Sensitivity to Phosphatase. Liposomes were formed from mixtures of PE with three cholesterol phosphate derivatives (**3a, b, d**). The sensitivity of the phosphate derivatives to alkaline phosphatase was first measured by the leakage of encapsulated ANTS/DPX (*17*). Exposure of the liposomes to alkaline phosphatase resulted in the leakage of contents from all three compositions

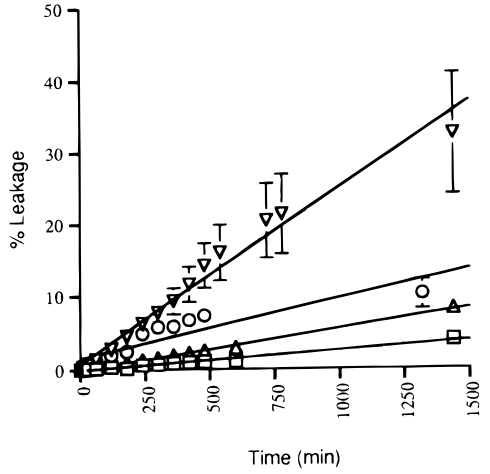


Figure 1. Effect of alkaline phosphatase upon the stability of liposomes encapsulating ANTS/DPX. (□) Cholesterol triethylene phosphate (**3d**)/DOPE in HBS (cholesterol C2 phosphate (**3a**)/DOPE and cholesterol C6 phosphate (**3b**)/DOPE showed the same leakage profile); (▽) cholesterol C2 phosphate (**3a**)/DOPE with 10 I. U. alkaline phosphatase; (○) cholesterol C6 phosphate (**3b**)/DOPE with 10 I. U. alkaline phosphatase; (△) cholesterol triethylene phosphate (**3d**)/DOPE with 10 I. U. alkaline phosphatase.

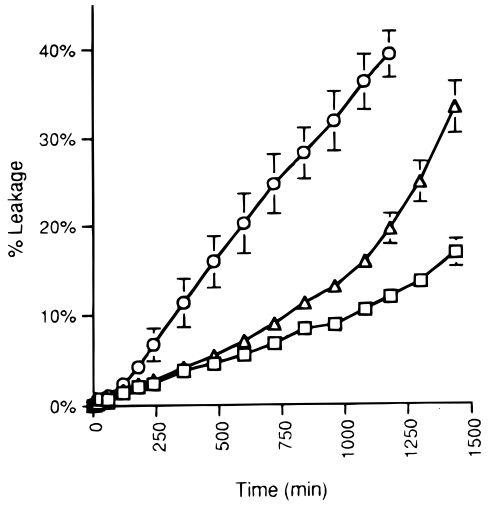


Figure 2. Dose response of cholesterol C2 phosphate (**3a**)/DOPE liposomes to alkaline phosphatase. (□) Cholesterol C2 phosphate(**3a**)/DOPE in HBS; (△) cholesterol C2 phosphate (**3a**)/DOPE with 1 I. U. alkaline phosphatase; (○) cholesterol C2 phosphate (**3a**)/DOPE with 20 I. U. alkaline phosphatase.

(Figure 1). In contrast, liposomes formulated with cholesterol phosphate or egg phosphatidate with DOPE showed no leakage under these conditions, showing that they were not phosphatase sensitive. The cholesterol C2 phosphate (**3a**)/DOPE liposomes were most sensitive to alkaline phosphatase, and further experiments were performed with this system.

The leakage of dye from the liposomes in the presence of alkaline phosphatase exhibited two phases, a lag phase followed by a more rapid phase. Exposure of cholesterol C2 phosphate (**3a**)/DOPE liposomes to increasing amounts of enzyme lead to an increased rate of release from the liposome (Figure 2). The lag phase before liposome leakage was also observed to be sensitive to the amount of enzyme activity present in the assay. Inactivation of the enzyme by heat treatment completely blocked the enzyme-mediated leakage of dye from the liposome (Figure 3). The leakage of dye from the liposomes was

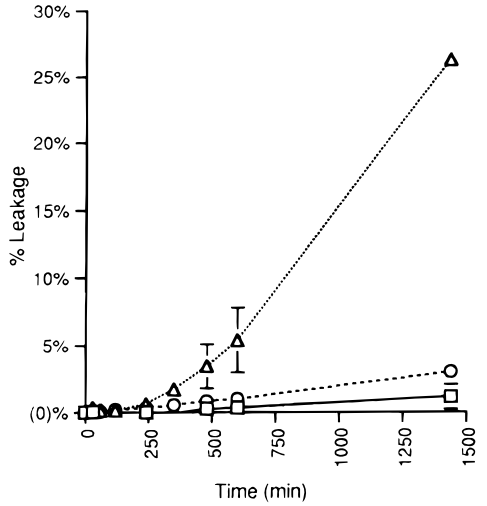


Figure 3. Effect of heat inactivation upon the alkaline phosphatase mediated leakage. (□) Cholesterol C2 phosphate (**3a**)/DOPE in HBS; (△) cholesterol C2 phosphate (**3a**)/DOPE with 10 I. U. alkaline phosphatase; (○) cholesterol C2 phosphate (**3a**)/DOPE with heat inactivated alkaline phosphatase.

Table 1. Stoichiometry of Alkaline Phosphatase Catalyzed Liposome Destruction

incubation conditions	cholesterol C2 alcohol (nmol)	inorganic phosphate (nmol)
no alkaline phosphatase	not detected	30 ± 15
20 units of alkaline phosphatase	190 ± 10	190 ± 40
100 units of alkaline phosphatase	180 ± 15	160 ± 30

thus shown to be dependent upon and sensitive to the presence of active alkaline phosphatase.

The expected products of the reaction of a phosphatase with cholesterol C2 phosphate (**3a**) are the cholesterol C2 alcohol (**2a**) and inorganic phosphate. To detect the products of any reaction between the liposomes and alkaline phosphatase, a large scale (1 μmol) incubation of the cholesterol C2 phosphate (**3a**)/DOPE liposomes with the enzyme was performed. Due to problems with product inhibition of the enzyme, it was necessary to perform the incubation at the same concentration as the leakage experiments (0.1 mM). After 24 h, the reaction was frozen and the mixture lyophilized. The residue was rehydrated in a small volume of water, and the lipids were separated by a Bligh and Dyer extraction (*20*).

HPLC separation of the lipids isolated in the extraction allowed identification and quantification of the lipids formed in the reaction. The lipids were isolated utilizing the method described by New (*26*) for the quantification of α-tocopherol with cholesterol as a standard. Under these conditions, cholesterol C2 alcohol (**2a**) eluted at 15 min, 2 min after cholesterol eluted. Analysis of the lipids isolated from the extraction showed the presence of a peak eluting at 15 min only in the reactions containing alkaline phosphatase. Coinjection of the reaction products with cholesterol C2 alcohol (**2a**) resulted in enhancement of this peak. To determine the amount of cholesterol C2 alcohol (**2a**) produced, a standard curve of **2a** was set up and a linear relationship between the area under the peak and the amount of material injected was shown. From this curve, 190 ± 10 nmol of **2a** was formed in the reaction with alkaline phosphatase (Table 1).

Identification and quantification of inorganic phosphate in the aqueous phase was performed at pH 5.0 to avoid any contamination from lipids that remained in the aqueous phase. The presence of zinc in the assay mixture

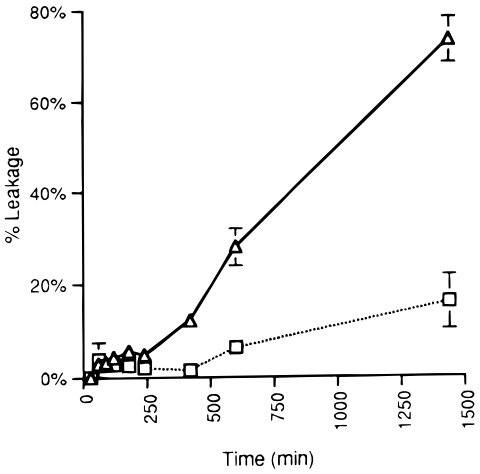


Figure 4. Effect of acid phosphatase upon cholesterol C2 phosphate (**3a**)/DOPE liposomes. (□) Cholesterol C2 phosphate (**3a**)/DOPE in acetate buffered saline, pH 5.0; (△) cholesterol C2 phosphate (**3a**)/DOPE with 2 I. U. acid phosphatase.

allows the formation of the reduced phosphomolybdate complex under mild conditions (*21*). Inorganic phosphate formation was shown to be dependent upon the presence of alkaline phosphatase. In the presence of alkaline phosphatase, 190 ± 40 nmol of inorganic phosphate are formed after 24 h (Table 1). In the absence of enzyme only, background levels of phosphate were detected in the assay. Under these conditions, only 40% of the initial cholesterol C2 phosphate (**3a**) was hydrolyzed by the enzyme. This was ascribed to product inhibition by inorganic phosphate, which at 20 μM concentration was severalfold higher than its K_i for the enzyme.

To test the general nature of the reaction observed with alkaline phosphatase, a second physiologically relevant isozyme, an acid phosphatase (*10*), was tested. Incubation of the cholesterol C2 phosphate (**3a**)/DOPE liposomes at pH 5, the optimal pH for this enzyme, highlighted the stability of this liposome composition. In the presence of acid phosphatase, the leakage of dye from the liposomes exhibited two phases, a lag phase followed by a more rapid phase (Figure 4). The extent of leakage observed for this isozyme was significantly higher than that observed with the alkaline phosphatase.

Sensitivity to Calcium. The second mechanism for triggering a phase change in an anionic liposome is the use of divalent cations. Addition of 0.4 mM calcium to cholesterol C2 phosphate (**3a**)/DOPE liposomes causes complete leakage of the liposome contents and the formation of large flocculate aggregates. The lipid mixing assay of Struck et al. (*19*) gave a transition threshold for this effect of 150 μM calcium (data not shown).

The ability of calcium to aggregate and fuse liposomes is thought to be mediated by the dehydration of the bilayer surface (*27*). Accordingly, a number of cholesterol phosphate derivatives (**3b**−**d**) in which the linker moiety was longer and more hydrophilic than the cholesterol C2 phosphate (**3a**) was formed into liposomes with DOPE and tested for their calcium sensitivity. The sensitivity to Ca^{2+} was reduced by increasing the hydrophilicity and length of the linker between the cholesterol moiety and the phosphate group (Table 2). The triethylene glycol phosphate (**3d**) is stable up to 1 mM Ca^{2+}, which is greater than physiological levels in some tissues in vivo (*28*). Intriguingly, the cholesterol C2 phosphate (**3a**)/DOPE liposomes are much more sensitive than the parent molecule in the series, cholesterol phosphate, and then the diacyl glycerol lipid, egg phosphatidate.

Table 2. Effect of Calcium upon the Stability of Cholesterol Phosphate Derivative/DOPE Liposomes

liposome formulation	200 μM Ca^{2+}	500 μM Ca^{2+}	1 mM Ca^{2+}	2 mM Ca^{2+}
cholesterol C2 phosphate (**3a**)/DOPE	+[a]	++	++	++
cholesterol C6 phosphate (**3b**)/DOPE	−	+	++	++
cholesterol diethylene phosphate (**3c**)/DOPE	−	−	+	++
cholesterol triethylene phosphate (**3d**)/DOPE	−	−	+	++
cholesterol phosphate/DOPE	−	+	++	++
egg phosphatidate/DOPE	−	−	−	+

[a] The liposomes were exposed to the indicated calcium concentration and the rate of dye leakage and increase of turbidity was measured. (−) No detectable effect. (+) Leakage of <20%. (++) leakage and aggregation of >60%.

Encapsulation of DNA. Encapsulation of DNA in such negatively charged liposomes was potentially a problem but was readily achieved utilizing a modification of the REV methodology (*29*). The liposomes were purified on a discontinuous Ficoll gradient to remove excess DNA. In a typical procedure, the final liposome concentration was 18 nmol of lipid and 0.2 μg of DNA/μL of solution. This corresponds to a 29% encapsulation efficiency for DNA.

The DNA was shown to be intact and encapsulated within the liposomes using a DNase protection assay. Liposomes encapsulating DNA were exposed to DNase for 16 h at room temperature and the lipids were then extracted. Plasmid DNA was completely degraded under these conditions (lane 7), whereas the encapsulated DNA was not degraded (lane 2) even in the presence of 10% serum (lane 4, the material at the wells for lanes 3 and 4 is serum derived) (Figure 5). The DNA also gave the same mobility profile as the initial plasmid DNA, indicating that it had not been sheared in the encapsulation process. Indeed, the encapsulated DNA can be extracted via the Bligh and Dyer protocol (*20*) and complexed with the heat-activated dendrimer (*30*). This complex is completely transfection competent (data not shown), confirming the agarose gel data that the DNA structure was not damaged in the encapsulation protocol.

Transfection Studies. The ability of the cholesterol C2 phosphate (**3a**)/DOPE liposomes to transfect CV-1 cells in vitro is shown in Figure 6. The transfection efficiency observed was intermediate in level between the degraded cationic polyamidamine dendrimer (*29*), which was used as a positive control, and naked DNA as shown.

Transfection experiments in the presence of phosphatase inhibitors such as inorganic phosphate (*31*) and L-*p*-bromotetramisole (*32*) showed that the ability of the liposome system to transfect CV-1 cells was not affected by inhibition of phosphatase. At the levels of inhibitor included in the transfection experiments, no phosphatase activity was observed. At 1 mM, L-*p*-bromotetramisole mediated some cellular toxicity which reduced the transfection levels for all transfecting agents. The relative transfection levels were unaffected by the treatments, however, indicating that another mechanism is at work (Figure 6).

The ability of the liposomes to mediate transfection in the absence of encapsulation is shown in Figure 7. The cells were exposed to DNA and cholesterol C2 phosphate (**3a**)/DOPE liposomes with the DNA either inside or outside of the liposome. The transfection efficiency was the same in both cases, whereas naked DNA did not transfect in the absence of lipid. The flocculate lipid/DNA aggregate formed upon exposure to the calcium in the

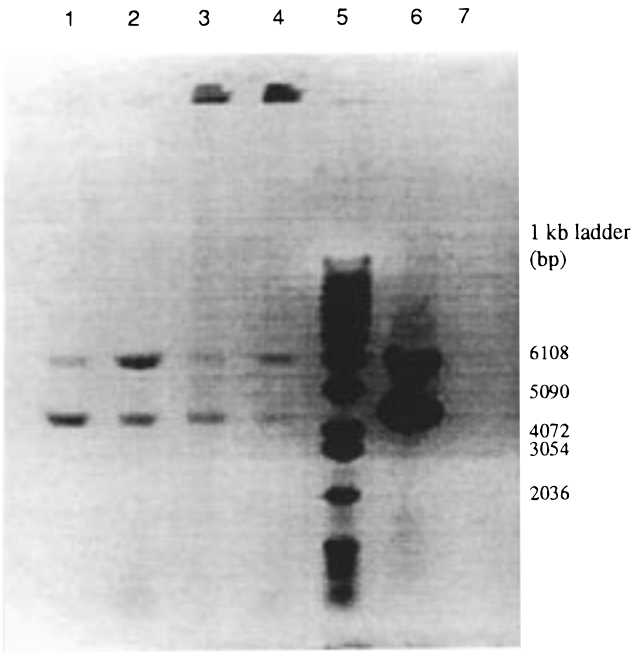


Figure 5. DNase protection assay with cholesterol C2 phosphate (**3a**)/DOPE liposomes: The plasmid DNA was extracted from the liposome after incubation and electrophoresed on an 0.8% agarose gel with ethidium bromide staining. Lane 1; cholesterol C2 phosphate (**3a**)/DOPE liposomes in HBS. Lane 2; cholesterol C2 phosphate (**3a**)/DOPE liposomes in HBS + 10 I. U. DNase 1. Lane 3; cholesterol C2 phosphate (**3a**)/DOPE liposomes in 10% serum in HBS. Lane 4; cholesterol C2 phosphate (**3a**)/DOPE liposomes in 10% serum + 10 I. U. DNAse 1. Lane 5; 1 kb DNA ladder. Lane 6; plasmid DNA in HBS. Lane 7; plasmid DNA in HBS + 10 I. U. DNAse 1.

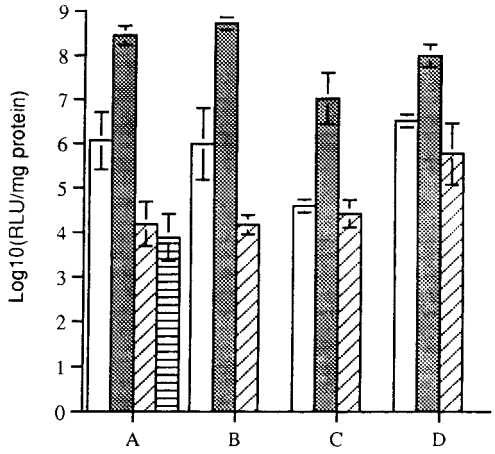


Figure 6. Effect of phosphatase inhibitors upon the transfection of CV-1 cells with a luciferase encoding plasmid. Transfection with cholesterol C2 phosphate (**3a**)/DOPE liposomes (0.5 μg of encapsulated DNA/well) (open bars); cationic dendrimer (0.6 μg DNA/well) (dark bars); plasmid DNA (0.5 μg of DNA/well) (angled stripes); untreated cells (horizontal stripes). Condition A; No additions to serum free medium. Condition B; 50 nmol of inorganic phosphate added to medium. Condition C; 1 mM L-*p*-bromotetramisole added to medium. Condition D; 10 mM inorganic phosphate added to medium.

culture medium must therefore mediate the transfection. DNA encapsulated by the more calcium stable cholesterol triethylene phosphate (**3d**)/DOPE liposomes caused efficient transfection, whereas the mixed liposome/DNA suspension resulted in significantly reduced levels of transfection. These liposomes do not flocculate on addition to media but are active in transfection, indicating that a different mechanism of transfection is occurring

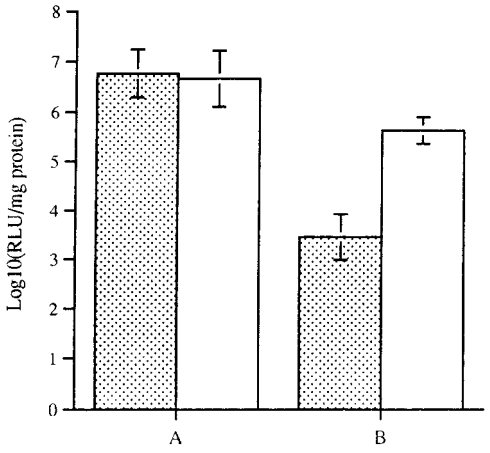


Figure 7. Effect of encapsulation of the plasmid on transfection efficiency. Transfection with liposomes premixed with plasmid DNA (1 μg of DNA/well) (dotted bars). Transfection with liposomes encapsulating plasmid (1 μg of DNA/well) (open bars). Condition A; cholesterol C2 phosphate (**3a**)/DOPE liposomes. Condition B; cholesterol triethylene phosphate (**3d**)/DOPE liposomes.

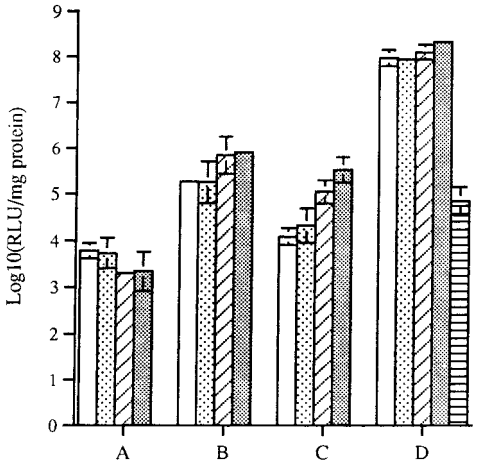


Figure 8. Effect of calcium upon the transfection of CV-1 cells with a luciferase encoding plasmid: Transfection in the presence of 0 mM Ca^{2+} (open bars); 0.1 mM Ca^{2+} (dotted bars); 0.5 mM Ca^{2+} (angled stripes); 1.36 mM Ca^{2+} (dark bars); 0 mM Ca^{2+} with salt wash (horizontal stripes). Condition A; naked DNA (0.5 μg of DNA/well). Condition B; cholesterol triethylene phosphate (**3d**)/DOPE liposomes (0.5 μg of encapsulated DNA/well). Condition C; cholesterol C2 phosphate (**3a**)/DOPE liposomes (0.5 μg of encapsulated DNA/well). Condition D; cationic dendrimer (0.6 μg DNA/well).

in this case, from that observed with the cholesterol C2 phosphate (**3a**)/DOPE system. We have tested anionic liposome formulations prepared from naturally occurring lipids, such as POPG/DOPE, cholesterol phosphate/DOPE or egg phosphatidate/DOPE, and these *do not* transfect when they are simply mixed with DNA.

Transfection of cells in vitro with reduced levels of calcium in the media was performed using calcium/magnesium-free Hanks-buffered salt solutions supplemented with an energy source (glutamine) and the required level of calcium. Cell viability was unaffected by this treatment in untransfected controls. The cholesterol C2 phosphate (**3a**)/DOPE liposomes were shown to be sensitive to the calcium concentration (Figure 8), showing only background levels of transfection in the absence of added calcium. Cholesterol triethylene phosphate (**3d**)/DOPE liposomes were less affected by the calcium concentration and were still active in transfection in the absence of any

added divalent cations. The cationic dendrimer was unaffected by the calcium concentration unless the surface-bound polyplex was removed by salt wash before addition of calcium-containing media.

DISCUSSION

Nonviral gene therapy remains a relatively inefficient process that could benefit from improved approaches for gene delivery. We hypothesized that a phosphatase-triggered release lipid composition could sequester DNA to prevent degradation until the target site is reached. The DNA would be released when triggered by the relatively high concentration of phosphatase isozymes associated with the cell surface and lysozymes (*8*, *28*). A triggered release liposome sensitive to this activity may therefore provide an efficient method for the transfection of cells or delivery of drugs.

To test this hypothesis, we synthesized a series of novel phosphate monoester derivatives of cholesterol. The synthesis allowed a large number of different lipids to be constructed from cholesterol tosylate (Scheme 1), a stable crystalline solid which has been used for over 60 years as an intermediate for the derivatization of cholesterol at the 3-β position (*33*). The stereochemistry of the incoming nucleophile is defined by the cholesterol geometry, and in the absence of ionic promoters, the 3-β isomer is formed exclusively. Phosphorylation of the alcohol and counterion exchange provided the desired cholesterol phosphate derivatives. The cholesterol phosphate derivatives (**3a**−**d**) support a bilayer with DOPE at neutral pH whereas the expected products of phosphatase action, the cholesterol alcohol derivatives (**2a**−**d**), do not (*25*).

Alkaline phosphatase, a triggering enzyme, is associated with the extracellular matrix of cells and is known to be elevated in many disease states such as cancer (*8*). This enzyme has a pH optimum in vitro of 10.5, but acts upon polyanionic substrates at physiological pH (*12*). Moreover, the enzyme dephosphorylates and inactivates lipopolysaccharide (bacterial endotoxin) at the lipid−water interface of the micelle surface and should be capable of hydrolyzing the appropriate cholesterol phosphate derivatives in a bilayer. Liposomes encapsulating a fluorophore were prepared from a number of cholesterol phosphate derivatives formulated with DOPE and used to examine liposome leakage in the presence of the triggering enzyme. Exposure of these liposomes to physiologically relevant levels (*28*) of alkaline phosphatase led to a time-dependent leakage of the aqueous contents (Figure 1). Importantly, the naturally occurring lipids, cholesterol phosphate, and egg phosphatidate, were resistant to phosphatase-mediated leakage under these conditions. As the cholesterol C2 phosphate (**3a**)/DOPE system was significantly more sensitive to the presence of the enzyme than the other cholesterol derivatives, it was decided to test the activity of alkaline phosphatase more rigorously upon this system.

The proposed catalytic route of liposome destruction is based on the sequential removal of phosphate from individual lipid molecules. Little dye release is expected until a critical point of cholesterol hydrolysis is reached. Once there is insufficient cholesterol phosphate remaining in the bilayer to stabilize the lamellar phase, the liposomes will collapse to form the hexagonal phase with concomitant release of their contents. This observed lag phase is also sensitive to the enzyme concentration (Figure 2)—the greater the enzyme concentration the shorter the lag phase. Inactivation of the enzyme by heat treatment (Figure 3) clearly indicates that an active enzyme, rather than a nonspecific protein−liposome interaction, must catalyze the transformation. This release profile may provide an advantage for in vivo delivery applications; the slow release of the lag phase might counteract the fast release observed in the burst phase with most controlled release systems (*34*).

The catalytic destruction of the liposomes is dependent upon the hydrolysis of the cholesterol C2 phosphate (**3a**) to the cholesterol C2 alcohol (**2a**) and inorganic phosphate. The expected products were isolated, identified, and quantified; inorganic phosphate and cholesterol C2 alcohol are produced in a 1:1 molar ratio (Table 1). This is consistent with the stoichiometry of the reaction of alkaline phosphatase with its substrate. Acid phosphatase also mediated the leakage contents from the cholesterol C2 phosphate (**3a**)/DOPE liposomes at pH 5.0 and was significantly more effective than alkaline phosphatase. The leakage profile induced by acid phosphatase was very similar to that observed with alkaline phosphatase supporting a catalytic mechanism of liposome collapse.

Divalent cations have been extensively used to trigger a phase change in anionic liposomes. Calcium, and to a lesser extent, magnesium and zinc are known to interact with anionic lipids such as phosphatidic acid (*35*), phosphatidylserine (*36*), and CHEMS (*14*), leading to phase separation and aggregation of liposomes. Depending upon the lipid composition, the calcium concentrations found in cell culture and in vivo are sufficient to mediate this transition.

Calcium is a very efficient trigger for the cholesterol C2 phosphate (**3a**)/DOPE liposomes and caused complete leakage of liposome contents at the comparatively low concentration of 0.4 mM. The threshold value at which calcium begins to show effects is 150 μM for this liposome composition. In vivo administration would expose the liposomes to higher calcium concentrations, so we examined if calcium sensitivity was dependent upon the cholesterol phosphate derivative (**3a**−**d**). The structure of the linker has a pronounced effect upon the calcium sensitivity as shown in Table 2. Adding a linker initially slightly increases the sensitivity of DOPE liposomes to the effects of calcium [compare cholesterol phosphate to cholesterol C2 phosphate (**3a**)] then decreases the sensitivity, presumably because the phosphate group is positioned further from the bilayer surface. Incorporation of oxygens into the linker region improves the calcium stability still further, most likely because the linker is more hydrophilic. Liposomes formulated with the most hydrophilic derivative (**3d**) were largely unperturbed by physiological levels of calcium (*28*). Thus, the cholesterol phosphate derivative/DOPE liposomes form a triggered release system which is sensitive to two separate triggering mechanisms: phosphatase isozymes and calcium.

The utility of this system for delivery purposes was evaluated using gene transfer. Transfection with DNA encapsulated in the cholesterol C2 phosphate (**3a**)/DOPE liposome was tested using kidney fibroblast cells (CV-1) and the luciferase reporter gene. The levels of luciferase activity detected after transfection compare favorably with the CHEMS/DOPE pH-triggered release system (*22*) at 10^7 RLU/mg of protein. This is 3−4 orders of magnitude above background, although lower than the cationic heat activated dendrimer (*30*).

To test whether the transfection was entirely or partially mediated by phosphatase activity, transfections were performed in the presence of known phosphatase

inhibitors. Inorganic phosphate competitively inhibits phosphatase isozymes with a micromolar K_i (*31*). With 10 mM phosphate in the transfection medium, no phosphatase activity was observed with the cells using *p*-nitrophenyl phosphate as the marker. The second inhibitor L-*p*-bromotetramisole (*32*) is a specific inhibitor of alkaline phosphatases, and complete inhibition of phosphatase activity is observed at the concentration used. The ability of the C2 system to transfect in the absence of alkaline phosphatase activity means that this isozyme is not the dominant mechanism in the transfection process but does not rule out the involvement of acid phosphatase.

Calcium was known (vide supra) to cause the aggregation of the liposomes at the concentration found in cell culture media (1.36 mM), and flocculate particles were observed in the transfection medium. The interaction of calcium with the negatively charged liposomes, causing aggregation and fusion of the liposomes, could mediate the transfection. Moreover, calcium phosphate precipitation is a widely used method for the transfection of cells in culture, though at much higher concentrations of calcium and phosphate (*37*). Addition of the same amount of inorganic phosphate as was found in the liposome experiments (50 nmol of P_i) to naked DNA or to the cholesterol C2 phosphate (**3a**)/DOPE liposomes had no effect upon the transfection efficiency (Figure 6). Therefore, a direct effect of released phosphate via a DNA/calcium phosphate precipitation does not mediate the transfection. However, the liposomes may provide a multivalent surface that could form a coprecipitate with DNA in the presence of calcium to mediate the transfection. Formation of this coprecipitate would not require the DNA to be encapsulated in the liposome before addition of the calcium as long as it could be recruited into the aggregate. Indeed, if cholesterol C2 phosphate (**3a**)/DOPE liposomes are mixed with DNA at the same concentrations used in the encapsulated transfection experiments, the transfection efficiency is identical.

The ability of anionic liposomes to form cochleates in the presence of divalent cations has long been recognized (*38*). More recently, this has been used to encapsulate macromolecules such as proteins and DNA inside the cochleate (*39*). These cochleates have been proposed for use as an oral DNA vaccine which is absorbed by the lower intestine before slowly releasing the trapped immunogen, thus, initiating a strong immune response (*40*). The cholesterol C2 phosphate (**3a**)/DOPE liposomes readily formed cochleate-like structures upon the addition of calcium to the suspension. Fluorescent DNA was incorporated into the precipitate even without preencapsulation, and addition of EDTA lead to partial release and partial encapsulation of the DNA in giant liposomes (data not shown). In the case of the cholesterol C2 phosphate (**3a**)/DOPE system, the coprecipitate appears to mediate the transfection of cells in culture.

The more calcium stable cholesterol triethylene phosphate (**3d**)/DOPE liposomes do not form flocculates in the culture medium. DNA encapsulated in the liposomes, however, is capable of transfecting cells in culture (Figure 7) which implies that the formation of visible aggregates is not essential for transfection with this liposome composition. Plasmid DNA mixed with empty liposomes of this composition is incapable of transfection. Indeed, only the cholesterol C2 phosphate (**3a**)/DOPE liposomes have shown this remarkable activity to date.

Removal of calcium from the transfection medium prevents endocytosis and will stop the calcium-mediated transfection of cells observed with CV-1 cells. The ability of cholesterol C2 phosphate (**3a**)/DOPE liposomes to mediate transfection is dependent upon the presence of calcium in the medium (Figure 8). In the absence of the calcium required to form the flocculate, the cholesterol C2 phosphate (**3a**)/DOPE liposome did not transfer DNA into cells. The cationic dendrimer-mediated transfection is also unaffected by the calcium concentration in the culture medium. The cationic polyplex, as opposed to the anionic liposomes, would be expected to bind to the cell surface electrostatically and undergo endocytosis once calcium was returned to the system. Removal of surface-bound polyplex with a salt wash prior to the readministration of calcium containing media significantly reduced transfection.

Transfection mediated by the cholesterol triethylene phosphate (**3d**)/DOPE liposomes are less affected by the absence of calcium in the media. Thus, the phosphatase-sensitive cholesterol triethylene phosphate (**3d**)/DOPE liposomes do not mediate transfection exclusively through a calcium mediated mechanism.

The ability to modulate the calcium sensitivity of the triggered release liposomes and to utilize other release mechanisms should allow liposome formulations to be designed for specific tasks based upon the site of action and desired route of administration. For example, the extremely high levels (~50 units/mL) of acid phosphatase present in the prostate gland (*28*) suggests that this system may be an efficient agent for disease states of this gland. Alternatively, by utilizing the antibody-directed enzyme prodrug therapy (ADEPT) approach (*41*), very high concentrations of the activating enzyme, such as alkaline phosphatase (*42*), can be directed to tumors. The passively circulating cholesterol phosphate derivative/DOPE liposomes would then be specifically degraded at the site of the tumor, targeting the transfection or drug delivery to the desired site of action.

CONCLUSION

We have proposed and synthesized a novel class of triggered release liposomes. These conditionally stable liposomes collapse in the presence of phosphatase isozymes or calcium which results in the release of the liposome contents. The sensitivity of the liposomes to each triggering mechanism can be modulated by changing the structure of the cholesterol phosphate derivative. This allows the triggered release liposome to be tailored to give the desired balance of sensitivity for a particular purpose and site of action. DNA encapsulated in these liposomes is capable of transfecting CV-1 cells in vitro at levels competitive with other triggered release systems. The transfection is mediated by calcium for certain formulations, but other transfection mechanisms start to play a role in the less calcium-sensitive cholesterol triethylene phosphate (**3d**)/DOPE formulations.

ACKNOWLEDGMENT

This work was supported by NIH grants GM 26691, DK 46052, and DK 47766. Dr. Szoka has a financial interest in and serves as a consultant to GeneMedicine, Inc., a biotechnology company developing gene medicines. We thank A. Vlassov for the translation of ref *24*, X. Guo for proofreading the manuscript, and L. S. Uyechi for the generous gift of the fluorescent DNA.

LITERATURE CITED

(1) Gerasimov, O. V., Rui, Y., and Thompson, D. H. (1996) Triggered release from liposomes mediated by physically and chemically induced phase transitions. *Vesicles* (Rosoff M., Ed.) pp 681−740, Marcel Dekker, Inc., New York.

(2) Chu, C.-J., and Szoka, F. C. (1994) pH-Sensitive Liposomes. *J. Liposome Res. 4*, 361−395.
(3) Marsh, D. (Ed.) (1990) *CRC Handbook of Lipid Bilayers*, pp 128−130, 266−267, CRC Press, Boca Raton, FL.
(4) Chu, C.-J., Dijkstra, J., Lai, M.-Z., Hong, K., and Szoka, F. C. (1990) Efficiency of cytoplasmic delivery by pH-sensitive liposomes to cells in culture. *Pharm. Res. 7*, 824−834.
(5) Düzgünes, N., Straubinger, R. M., Baldwin, P. A., Friend, D. S., and Papahadjopoulos, D. (1985) Proton-induced membrane fusion of oleic acid-phosphaphatidylethanolamine liposomes. *Biochemistry 24*, 3091−3098.
(6) Menger, F. M., and Johnston, D. E. (1991) Specific enzyme-induced decapsulation. *J. Am. Chem. Soc. 113*, 5467−5468.
(7) Anderson, V. C., and Thompson, D. H. (1992) Triggered release of hydrophilic agents from plasmalogen liposomes using visible light or acid. *Biochim. Biophys. Acta 1109*, 33−42.
(8) Millàn, J. L., and Fishman, W. H. (1995) Biology of human alkaline phosphatases with special reference to cancer. *Crit. Rev. Clin. Lab. Sci. 32*, 1−39.
(9) Simopoulos, T. T., and Jencks, W. P. (1994) Alkaline phosphatase is an almost perfect enzyme. *Biochemistry 33*, 10375−10380.
(10) deDuve, C., Pressman, B. C., Gianetto, R., Wattiaux, R., and Appelmans, F. (1955) Tissue fractionation studies 6. Intracellular distribution patterns of enzymes in rat-liver tissue. *Biochem. J. 60*, 605−617.
(11) Banerjee, P. K., and Amidon, G. L. (1985) Design of drugs based on enzyme−substrate specificity. *Design of prodrugs* (Bundgard H., Ed.) pp 93−135, Elsevier, Amsterdam.
(12) Poelstra, K., Bakker, W. W., Klok, P. A., Hardonk, M. J., and Meijer, D. K. F. (1997) A physiologic function for alkaline phosphatase: Endotoxin detoxification. *Lab. Invest. 76*, 319−327.
(13) Sundler, R., and Papahadjopoulos, D. (1981) Control of membrane fusion by phospholipid headgroups I. Phosphatidate/phosphatidylinositol specificity. *Biochim. Biophys. Acta 649*, 743−750.
(14) Lai, M.-Z., Vail, W. J., and Szoka, F. C. (1985) Acid- and calcium-induced structural changes in phosphatidylethanolamine membranes stabilized by cholesteryl hemisuccinate. *Biochemistry 24*, 1654−1661.
(15) Patel, K. R., Li, M. P., Schuh, J. R., and Baldeschweiler, J. D. (1985) Modification of vesicle surfaces with amphiphilic sterols. Effect on permeability and in vivo tissue distribution. *Biochim. Biophys. Acta 814*, 256−264.
(16) Wang, Y., and Hollingsworth, R. I. (1995) A solvent system for the high-resolution proton nuclear magnetic resonance spectroscopy of membrane lipids. *Anal. Biochem. 225*, 242−251.
(17) Ellens, H., Bentz, J., and Szoka, F. C. (1985) H^+- and Ca^{2+}-induced fusion and destabilization of liposomes. *Biochemistry 24*, 3099−3106.
(18) Bartlett, G. R. (1959) Phosphorus assay in column chromatography. *J. Biol. Chem. 234*, 466−468.
(19) Struck, D. K., Hoekstra, D., and Pagano, R. E. (1981) Use of resonance energy transfer to monitor membrane fusion. *Biochemistry 20*, 4093−4099.
(20) Bligh, E. G., and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol. 37*, 911−917.
(21) Drueckes, P., Schinzel, R., and Palm, D. (1995) Photometric microtitre assay of inorganic phosphate in the presence of acid-labile organic phosphates. *Anal. Biochem. 230*, 173−177.
(22) Legendre, J.-Y., and Szoka, F. C. (1992) Delivery of plasmid DNA into mammalian cell lines using pH-sensitive liposomes: comparison with cationic liposomes. *Pharm. Res. 9*, 1235−1242.
(23) Cullis, P. R., and De Kruijff, B. (1979) Lipid polymorphism and the functional roles of lipids in biological membranes. *Biochim. Biophys. Acta 559*, 399−420.
(24) Danilov, L. L., Maltsev, S. D., and Shibaev, V. N. (1990) Phosphorylation of nonacosanol and cholesterol with tetra-*n*-butylammonium dihydrogen phosphate and trichloroacetonitrile. *Bioorg. Khim. 16*, 1002−1003.
(25) Davis S. C., and Szoka F. C. (1998) Conditionally Stable Liposomes for Gene Delivery. *Intelligent materials and novel concepts for controlled release technologies* (Dinh S. M., and DeNuzzio J. Eds.) ACS Symposium Series, American Chemical Society, Washington, DC (in press).
(26) New, R. R. C. (Ed.) (1990) *Liposomes a practical approach*, pp 124−125 IRL Press, Oxford.
(27) Sundler, R., Düzgünes, N., and Paphadjopoulos, D. (1981) Control of membrane fusion by phospholipid headgroups II. The role of phosphatidylethanolamine in mixtures with phosphatidate and phosphatidylinositol. *Biochim. Biophys. Acta 649*, 751−758.
(28) Tietz, N. W. (Ed.) (1995) *Clinical Guide to Laboratory Tests*, W. B. Saunders and Co., Philadelphia PA.
(29) Szoka, F., and Papahadjopoulos, D. (1978) Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. *Proc. Natl. Acad. Sci. U.S.A. 75*, 4194−4198.
(30) Tang, M. X., Redemann, C. T., and Szoka, F. C. (1996) In vitro gene delivery by degraded polyamidoamine dendrimers. *Bioconjugate Chem. 7*, 703−714.
(31) Snyder, S. L., and Wilson, I. B. (1972) Phosphoramidic acids. A new class of nonspecific substrates for alkaline phosphatase from *Escherichia coli*. *Biochemistry 11*, 1616−1623.
(32) Metaye, T., Mettey, Y., Lehuede, J., Vierfond, J. M., and Lalegerie, P. (1998) Comparative inhibition of human alkaline phosphatase and diamine oxidase by bromo-levamisole, cimetidine and various derivatives. *Biochem. Pharmacol. 37*, 4263−4268.
(33) Wallis, E. S., Fernholz, E., and Gephart, F. T. (1937) Molecular rearrangements in the sterols. I. The action of anhydrous potassium acetate on cholesteryl *p*-toluenesulfonate in acetic acid solution. *J. Am. Chem. Soc. 59*, 137−140.
(34) Langer, R. S., and Peppas, N. A. (1981) Present and future applications of biomaterials in controlled drug delivery systems. *Biomaterials 2*, 201−214.
(35) Leventis, R., Gagné, J., Fuller, N., Rand, R. P., and Silvius, J. R. (1986) Divalent cation induced fusion and lipid lateral segregation in phosphatidylcholine-phosphatidic acid vesicles. *Biochemistry 25*, 6978−6987.
(36) Brock, T. G., Nagaprakash, K., Margolis, D. I., and Smolen, J. E. (1994) Modeling degranulation with liposomes: effect of lipid composition on membrane fusion. *J. Membr. Biol. 141*, 139−148.
(37) Jordan, M., Schallhorn, A., and Wurm, F. M. (1996) Transfecting mammalian cells: optimization of critical parameters affecting calcium-phosphate precipitate formation. *Nucleic Acids Res. 24*, 596−601.
(38) Papahadjopoulos, D., Vail, W. J., Jacobson, K., and Poste, G. (1975) Cochleate lipid cylinders: formation by fusion of unilamellar lipid vesicles. *Biochim. Biophys. Acta 394*, 483−491.
(39) Gould-Fogerite, S., Mazurkiewicz, J. E., Raska, K., Voelkerding, K., Lehman, J. M., and Mannino, R. J. (1989) Chimerasome-mediated gene transfer in vitro and in vivo. *Gene 84*, 429−438.
(40) Gould-Fogerite, S., and Mannino, R. J. (1996) Mucosal and systemic immunization using cochleate and liposome vaccines. *J. Liposome Res. 6*, 357−379.
(41) Niculescu-Duvaz, I., and Springer, C. J. (1997) Antibody-directed enzyme prodrug therapy (ADEPT): A review. *Adv. Drug Del. Rev. 26*, 151−172.
(42) Wallace, P. M., and Senter, P. D. (1991) In vitro and in vivo activities of monoclonal antibody-alkaline phosphatase conjugates in combination with phenol mustard phosphate. *Bioconjugate Chem. 2*, 349−352.

BC980047Y