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Synthesis of cationic cardiolipin analogues

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Abstract

An approach was developed to synthesize a new class of cationic cardiolipin analogues containing two quaternary ammonium groups with tetra alkyl groups retaining "glycerol" moiety, the central core of the molecule. Cationic cardiolipin analogues were modified via introduction of either two or four oxyethylene groups to enhance the solubility in polar solvents. These newly synthesized cationic cardiolipin analogues can be applied to a broad range of drug delivery systems such as transfection reagents.

Keywords: Cationic lipids; Cardiolipin; Transfection; Gene delivery; Lipid-based formulations

1. Introduction

In recent years, a significant research effort has been devoted in developing new cationic lipids to assist delivery of DNA [1], mRNA [2], siRNA [3], antisense oligonucleotide [4], proteins [5], and antiviral [6,7] into human cells. Cationic liposomes are recognized as an important carrier to deliver anionic species, such as genes or other

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nucleic acids, to cells. Cationic liposomes are thought to interact electrostatically with negatively charged nucleic acid sequences to form complexes that facilitate penetration of these agents into cells. Thus, cationic lipids could play a role in delivering anionic agents into target cells and tissues in the treatment of disease. Consequently, a need has arisen for the development of a new class of cationic lipids that are less toxic and synthetic methods that are simple and scalable. Since the first description by Felgner et al. [8] and their discovery of a potential cationic lipid such as *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) for transfection, an increasing number of new cationic lipids of different structures have been synthesized [9] and their respective transfection activities in wide variety of cell types have been reported.

Cationic lipids are composed of three parts (Fig. 1), a cationic head group, a lipophilic tail group, and a linker that tethers the hydrophilic head group and hydrophobic tail group. Cationic head groups include quaternary ammonium salt lipids, lipoamines (primary, secondary, and tertiary amine lipids), and combinations of both lipoamines and quaternary amines. Tail groups usually consist of saturated or unsaturated alkyl chains (12–18 carbons in length) or cholesteryl groups.

Commercially available transfection reagents include, DOTMA [8], 1,2-dioleyloxy-3-(trimethylammonio)propane chloride (DOTAP) [10], *N*,*N*-dimethyl-*N*-[2-(sperminecarboxamido)ethyl]-2,3-bis(dioleyloxy)-1-propanaminium pentahydrochloride (DOSPA) [11], 1,3-dioleoyloxy-2-(6-carboxyspermyl)propyl amide (DOSPER) [12], dimethyldioctadecylammonium bromide (DDAB) [13], *N*,*N*-dioleyl-*N*,*N*-dimethylammonium chloride (DODAC) (Fig. 2), and cationic cardiolipin analogue **3** [14] (PCL-2) (Fig. 4). These cationic lipids, with combination of helper lipid like 1,2-dioleoyl phosphatidylethanolamine (DOPE) or 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), showed transfection activity.

The structure of cationic lipids is an important determinant for their transfection activity. By comparing the different structures and their transfection activities in the same family or different families of lipids, some common conclusions can be made. The hydrocarbon chain length has effects on transfection activity in cell culture. The transfection activity of cationic lipids of varying chain length is usually in the order C14:0 (myristyl) > C16:0 (palmityl) > C18:0 (stearyl) [15]. It is possible that shorter hydrocarbon chains decrease the rigidity of bilayer and favor a higher intermembrane transfer rate and lipid mixing, resulting in potential disruption of the endosome and consequent DNA escape from endosomal degradation [16]. Structural variations at the linker region such as length, the specific type of chemical bonds, and the relative position of the hydrocarbon chains can affect the transfection efficiency,

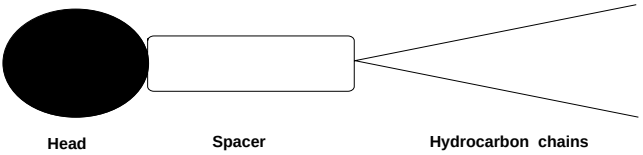


Fig. 1. Basic structure of cationic lipids.

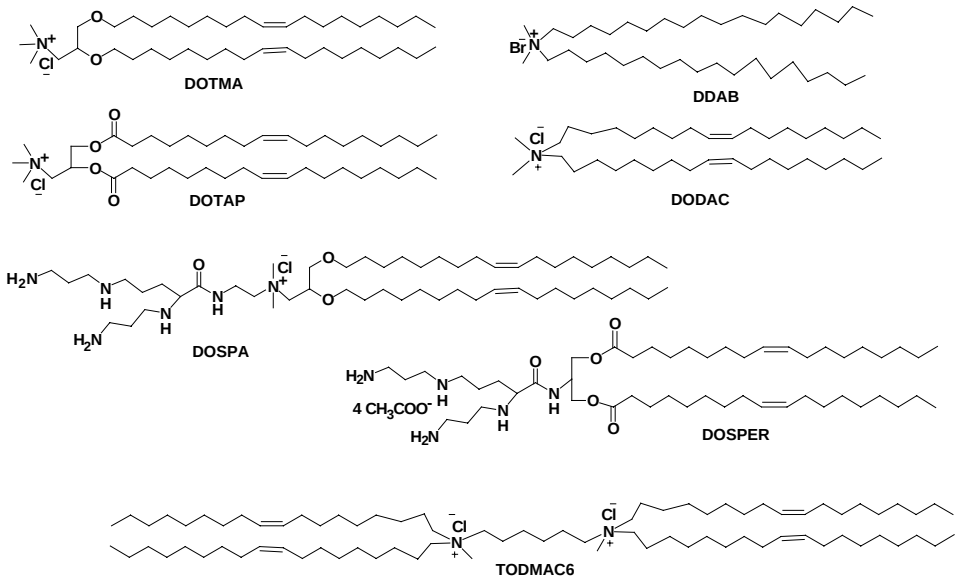


Fig. 2. Chemical structures of cationic lipids.

biodegradability, and stability of the cationic lipids. Cationic lipid such as DOTMA, which contains an ether linkage, was shown to have much greater in vivo transfection efficiency [17] than the corresponding ester analogue DOTAP. Notwithstanding the lower transfection efficiency of DOTAP, it was used for in vivo gene delivery on human subjects due to its low cytotoxicity while DOTMA is used only for in vitro studies. The quaternary ammonium lipids (DOTMA or DOTAP) of this family were modified by replacing the methyl group with the alkylene alcohol or alkylene tail group with alkyl group to give 1,2-dioleyloxypropyl-3-dimethylhydroxyethyl ammonium [18] (DORIE) or 1,2-dimyristyloxy propyl-3-dimethylhydroxyethyl ammonium (DMRIE), respectively. The hydroxyl group in DORIE or DMRIE may increase the interaction of DNA with lipids or improve the interaction of the DNA/liposome complexes with cellular membranes, leading to greater in vitro and in vivo activity than DOTMA or DOTAP. *N*,*N*,*N*′,*N*′-Tetraoleyl-*N*,*N*-dimethyl-1,6-hexanediammonium chloride [19] (TODMAC6, divalent and tetra alkyl) was shown to possess better transfection properties compared to DODAC (monovalent and dialkyl).

Despite numerous investigations of cationic lipids in gene therapy, most of the known cationic lipids possess certain toxicities. Recognizing the need for development of new cationic lipids to improve gene therapy and drug delivery, we undertook a program to design and synthesize a new class of dimeric cationic lipids (Fig. 3). It has been suggested that the ability of cationic lipids to facilitate intracellular delivery is related to their ability to induce non-bilayer hexagonal H_{II} phase in combination with anionic lipids at endosomal stage, and that the most potent cationic lipids are those that are the most effective inducers of H_{II} phase organization [20]. It is known that the ability of lipids to adopt the H_{II} phase is related to their dynamic molecular shapes. In general, a

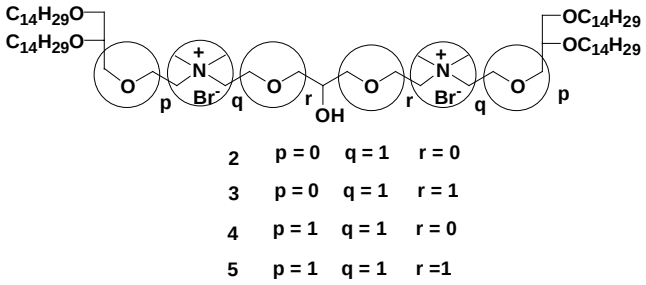


Fig. 3. General structure of cationic cardiolipin analogue.

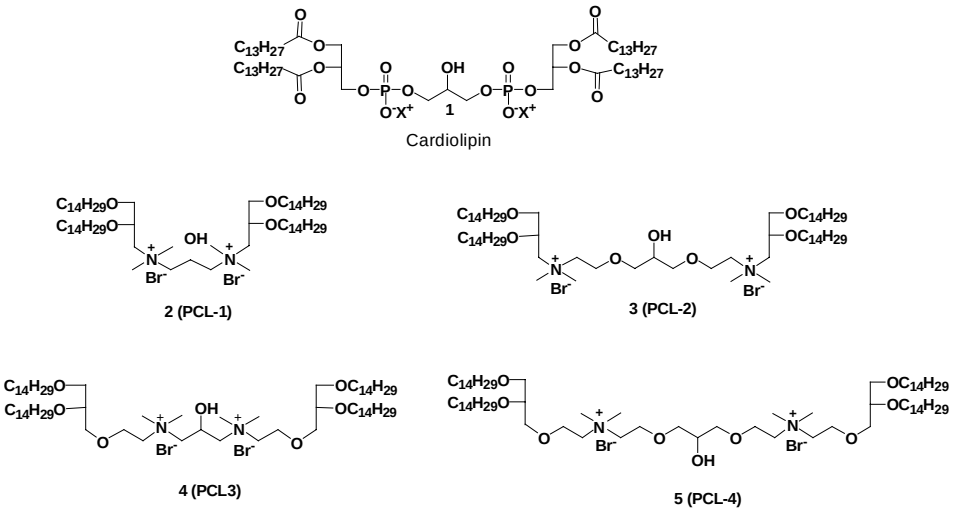


Fig. 4. Chemical structures of cardiolipin and cationic cardiolipin analogues.

lipid with a small cross-sectional area in the headgroup region and a larger acyl chain cross-sectional area exhibits a "cone" shape compatible with H_{II} phase organization and are known to enhance the transfection efficiency [21]. One method to increase the "cone" shape character is to create a dimer of cationic lipids joined together in the headgroup region by a spacer with reasonable length. Recently, Cullis [19] utilized this strategy to synthesize a novel dimer cationic lipid and achieved improved transfection activity as compared with monomer lipid. In the present study, we adopted similar strategy to design novel cationic cardiolipin (dimeric cationic lipid) analogues. We developed synthetic methods for cationic cardiolipin (dimeric cationic lipid) analogues. Cardiolipin **1** (glycerol-bridged dimeric phosphatidic acid) [22] constitutes a class of complex phospholipids that occur mainly in the heart and skeletal muscles, showing high metabolic activity. Cardiolipin has two negatively charged phosphate groups, which were replaced with quaternary ammonium groups to provide cationic cardiolipin analogue **2** [14] (Fig. 4). Cationic cardiolipin analogue **2** was further modified with the

addition of oxyethylene group on both sides of the central glycerol moiety to obtain cationic cardiolipin analogue **3** [14]. Exchanging the position of oxa group and the quaternary ammonium group will provide cationic cardiolipin analogue **4**. The cationic cardiolipin analogue **3** was modified with the addition of oxyethylene group on both sides of the quaternary ammonium group to yield spacer cationic cardiolipin analogue **5** (Fig. 4). The cationic cardiolipin analogues were designed accommodating some key features from known cationic lipids such as the presence of two positive charges and four saturated hydrocarbon chains with ether linkages (C14:0, myristyl). The central glycerol units in all these cationic lipids were retained to mimic the naturally occurring cardiolipin. We anticipated that these chemical modifications would be helpful for an efficient transfection.

2. Materials and methods

2.1. General

Melting points were determined at atmospheric pressure and uncorrected. ^{1}H NMR spectra were recorded on Varian Inova NMR spectrometer at 300 and 500 MHz. ^{13}C NMR spectra were recorded on Varian Inova (500 MHz) NMR spectrometer at 125 MHz. ^{1}H chemical shifts are reported in ppm from internal tetramethylsilane. ^{13}C chemical shifts are reported in ppm relative to $CDCl_3$ (77.0 ppm). Mass spectral analyses [electron spray ionization (ESI)] were carried out on Triple Quadruple LC/MS/MS mass spectrometer API 4000 (Applied Biosystems, Foster City, CA). Infrared (IR) spectra were recorded on a Nicolet Nexus 470 FT-IR. Samples were prepared by ATR method. Thin-layer chromatography (TLC) was carried out on Merck silica gel 60 F_{254} plates (250 μm) and developed with the appropriate solvents. The TLC spots were visualized either by UV light or by heating plates sprayed with a solution of phosphomolybdic acid (5% ethanolic solution). Flash column chromatography was carried out on silica gel (230–400 mesh). All chemicals and anhydrous solvents were purchased from Aldrich Chemical (Milwaukee, WI). All of the extracts were dried over anhydrous Na_2SO_4.

2.2. Synthesis of bromo intermediates

2.2.1. 1,3-Dibromo-2-*O*-benzyl glycerol (7)

To a solution of 2-*O*-benzyl glycerol (**6**) (20 g, 109 mmol) in anhydrous dichloromethane (275 mL) under argon atmosphere at 0 °C was added triphenylphosphine (64.4 g, 230 mmol) followed by carbon tetrabromide (76.3 g, 230 mmol). The reaction mixture was stirred at 0 °C for 2 h. The reaction mixture was diluted with water (300 mL) and the organic layer was separated, dried over sodium sulfate. The organic layer was concentrated under reduced pressure and crude product was purified by column chromatography over a silica gel (230–400 mesh) with 1–5% ethyl acetate in hexane to obtain 1,3-dibromo-2-*O*-benzyl glycerol (**7**) (26 g, 77%) as colorless oil. TLC (SiO_2) hexane/ethyl acetate (9:1) $R_f \sim 0.80$. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.52–3.62 (m, 4H), 3.78–3.82 (m, 1H), 4.65 (s, 2H), 7.30–7.38 (m, 5H).

*2.2.2. 1,3-Dibromopropane-2-ol (**8**)*

1,3-Dibromo-2-*O*-benzyl glycerol (**7**) (23 g, 74.6 mmol) was dissolved in ethanol (100 mL) and hydrogenated with 10% palladium on carbon (0.5 g) for 4 h at 50 psi. After filtration of the catalyst, the solution was evaporated under reduced pressure. The crude material was subjected to silica-gel column chromatography (230–400 mesh) and eluted with 1–20% ethyl acetate in hexane to obtain 1,3-dibromopropane-2-ol (**8**) (15 g, 93%) as colorless oil. TLC (SiO_2) hexane/ethyl acetate (3.5:1) $R_f \sim 0.5$. ^1H NMR ($CDCl_3$, 500 MHz): δ 2.66 (br s, 1H, OH), 3.58–3.59 (m, 4H), 4.01–4.03 (m, 1H).

*2.2.3. 1,3-Bis[(2-ethoxy tetrahydro-2H-pyran)]-2-O-benzyl glycerol (**9**)*

To a stirred suspension of sodium hydride (59.3 g, 1.48 mol, 60% in oil) in anhydrous dimethylformamide (300 mL) under argon atmosphere at 0 °C, a solution of 2-*O*-benzyl glycerol (**6**) (90 g, 0.49 mol) in dimethylformamide (700 mL) was added over a period of 2 h maintaining the temperature below 15 °C. After stirring at room temperature for 2 h, 2-(2-bromoethoxy)tetrahydro-2*H*-pyran (310 g, 1.48 mol) was added at 0 °C over a period of 3 h maintaining the temperature below 10 °C. The reaction mixture was stirred at room temperature for 12 h, then cooled to 0 °C, and ice water was added slowly to quench the excess sodium hydride. The reaction mixture was concentrated under reduced pressure to remove maximum DMF and the crude solution was diluted with water (1 L) and extracted with ethyl acetate (2 × 500 mL). The organic layer was washed with aqueous saturated sodium chloride (500 mL) and dried over sodium sulfate. The solvent was concentrated under reduced pressure. The crude product was purified by column chromatography over a silica gel (230–400 mesh) with 10–30% ethyl acetate in hexane to obtain 1,3-bis-[(2-ethoxy tetrahydro-2*H*-pyran)]-2-*O*-benzyl glycerol (**9**) (154 g, 71%) as colorless oil. TLC (SiO_2) hexane/ethyl acetate (3:2) $R_f \sim 0.40$. ^1H NMR ($CDCl_3$, 300 MHz): δ 1.41–1.82 (m, 12H), 3.41–3.98 (m, 17H), 4.61 (br s, 2H), 4.78 (s, 2H, OCH_2Ph), 7.24–7.45 (m, 5H, Ph-H).

*2.2.4. 3,7-Dioxa-5-O-benzyl-1,9-nonanediol (**10**)*

To a solution of 1,3-bis[(2-ethoxy tetrahydro-2*H*-pyran)]-2-*O*-benzyl glycerol (**9**) (50 g, 0.11 mol) in methanol (500 mL) was added 1 M HCl in ether (5 mL) and stirred at room temperature for 2 h. The reaction mixture was neutralized with solid sodium bicarbonate until the solution became neutral. The reaction mixture was filtered and concentrated under reduced pressure. The crude product was dissolved in ethyl acetate (1 L), washed with water (100 mL), and dried over sodium sulfate. The organic layer was concentrated under reduced pressure and the crude product was purified by column chromatography over a silica gel (70–230 mesh) eluting with ethyl acetate, followed by 5% methanol in ethyl acetate to obtain 3,7-dioxa-5-*O*-benzyl-1,9-nonanediol (**10**) (27 g, 88%) as colorless oil. TLC (SiO_2) ethyl acetate $R_f \sim 0.10$. ^1H NMR ($CDCl_3$, 300 MHz): δ 2.58 (br s, 2H, OH), 3.50–3.81 (m, 13H), 4.68 (s, 2H, OCH_2Ph), 7.21–7.42 (m, 5H, Ph-H).

*2.2.5. 1,9-Dibromo-3,7-dioxa-5-O-benzylnonane (**11**)*

To a solution of 3,7-dioxa-5-*O*-benzyl-1,9-nonanediol (**10**) (27 g, 0.1 mol) in anhydrous dichloromethane (400 mL) under argon atmosphere at 0 °C, was added triphe-

nylphosphine (65.5 g, 0.25 mol) followed by carbon tetrabromide (79.4 g, 0.24 mol). The reaction mixture was stirred at 0 °C for 2 h. The reaction mixture was diluted with water (300 mL) and the organic layer was separated, and dried over sodium sulfate. The organic layer was concentrated under reduced pressure and crude product was purified by column chromatography over a silica gel (70–230 mesh) with 20% ethyl acetate in hexane to obtain 1,9-dibromo-3,7-dioxa-5-*O*-benzylnonane (**11**) (36 g, 91%) as colorless oil. TLC (SiO_2) hexane/ethyl acetate (3:2) $R_f \sim 0.60$. ^1H NMR ($CDCl_3$, 500 MHz): δ 3.45 (t, $J = 6.5$ Hz, 4H, CH_2Br), 3.60–3.67 (m, 4H, OCH_2), 3.72–3.82 (m, 5H), 4.70 (s, 2H, OCH_2Ph), 7.28–7.38 (m, 5H, Ph-H).

*2.2.6. 1,3-bis-(2-Bromoethoxy)propane-2-ol (**12**)*

1,9-Dibromo-3,7-dioxa-5-*O*-benzylnonane (**11**) (36 g, 90.90 mmol) was dissolved in ethanol (110 mL) and hydrogenated with 10% palladium on carbon (3.6 g) for 2 h at 50 psi. After filtration of the catalyst, the solution was evaporated under reduced pressure. The crude material was subjected to silica-gel column chromatography (70–230 mesh) and eluted with 60% ethyl acetate in hexane to obtain 1,3-bis-(2-bromoethoxy)propane-2-ol (**12**) (26 g, 94%) as colorless oil. TLC (SiO_2) hexane/ethyl acetate (1:1) $R_f \sim 0.30$. ^1H NMR ($CDCl_3$, 500 MHz): δ 2.08 (s, 1H, OH), 3.45 (t, $J = 6.0$ Hz, 4H, CH_2Br), 3.54–3.64 (m, 4H, OCH_2), 3.63–3.85 (m, 4H, OCH_2), 3.96–4.02 (m, 1H).

2.3. Synthesis of tertiary amine intermediates

*2.3.1. (S)-1,2-bis-Tetradecyloxy-3-O-benzylpropane (**14**)*

To a stirred suspension of sodium hydride (54.5 g, 1.36 mol, 60% in oil) in anhydrous dimethylformamide (220 mL) under argon atmosphere at 0 °C, a solution of (*S*)-1-*O*-benzyl glycerol (**13**) (62 g, 0.34 mol) in dimethylformamide (400 mL) was added over a period of 1 h maintaining the internal temperature below 20 °C. After stirring at room temperature for 2 h, tetradecyl bromide (377.4 g, 1.36 mol) was added at 0 °C over a period of 2 h. After complete addition, the reaction mixture was stirred for 2 h at room temperature and the temperature was gradually increased to 70 °C, and then stirred for 5 h. The reaction mixture was cooled to 0 °C and quenched with few drops of cold water. The mixture was diluted with saturated ammonium chloride (500 mL). The aqueous layer was extracted with ethyl acetate (1 L) and washed with water (3 × 1 L), and dried over sodium sulfate. The organic layer was concentrated under reduced pressure and the crude product was purified by column chromatography over a silica gel (70–230 mesh) eluting with 2–10% ethyl acetate in hexane to obtain (*S*)-1,2-bis-tetradecyloxy-3-*O*-benzylpropane (**14**) (146 g, 75%) as colorless oil. TLC (SiO_2) hexane/ethyl acetate (1:9) $R_f \sim 0.53$. ^1H NMR ($CDCl_3$, 300 MHz): δ 0.88 (t, $J = 6.7$ Hz, 6H), 1.25 (br s, 44H), 1.49–1.58 (m, 4H), 3.41 (t, $J = 6.5$ Hz, 2H), 3.42–3.61 (m, 7H), 4.54 (m, 2H, OCH_2Ph), 7.23–7.32 (m, 5H, Ph-H).

*2.3.2. (R)-1,2-bis-Tetradecyloxypropan-3-ol (**15**)*

A solution of (*S*)-1,2-bis-tetradecyloxy-3-*O*-benzylpropane (**14**) (70 g, 0.12 mmol) was dissolved in ethyl acetate (280 mL) and hydrogenated with 10% palladium on carbon (3 g) for 12 h at 50 psi. After filtration of the catalyst, the solution was

evaporated under reduced pressure. The residue was dissolved in hot ethanol (500 mL) and kept at −20 °C overnight. The precipitated solid was filtered and dried under vacuum to obtain (*R*)-1,2-bis-tetradecyloxypropan-3-ol (**15**) (54 g, 92%) as a white solid. Mp 44–45 °C. TLC (SiO_2) hexane/ethyl acetate (1:9) $R_f \sim 0.17$. ^1H NMR ($CDCl_3$, 300 MHz): δ 0.88 (t, $J = 6.7$ Hz, 6H), 1.25 (br s, 44H), 1.59–1.57 (m, 4H), 2.2 (t, $J = 5.7$ Hz, 1H, OH), 3.37–3.73 (m, 9H).

*2.3.3. (S)-1,2-bis-Tetradecyloxy-3-bromopropane (**16**)*

To a solution of (*R*)-1,2-bis-tetradecyloxypropan-3-ol (**15**) (52 g, 0.1 mol) in anhydrous dichloromethane (280 mL) under argon atmosphere at 0 °C triphenylphosphine (35.1 g, 0.13 mol) was added. A solution of carbon tetrabromide (46.2 g, 0.13 mol) in dichloromethane (240 mL) was added to the reaction mixture dropwise in a period of 1 h and further stirred at 0 °C for 3 h. The reaction mixture was diluted with water (500 mL) and the organic layer was separated, and dried over sodium sulfate. The organic layer was concentrated under reduced pressure and the crude product was purified by column chromatography over a silica gel (230–400 mesh) with 1–5% ethyl acetate in hexane to obtain (*S*)-1,2-bis-tetradecyloxy-3-bromopropane (**16**) (53 g, 90%) as colorless oil. TLC (SiO_2) hexane/ethyl acetate (1:9) $R_f \sim 0.72$. ^1H NMR ($CDCl_3$, 300 MHz): δ 0.88 (t, $J = 6.7$ Hz, 6H), 1.25 (br s, 44H), 1.51–1.61 (m, 4H), 3.39–3.61 (m, 9H).

*2.3.4. (R)-1,2-bis-Tetradecyloxy-3-dimethylamino propane (**17**)*

(*S*)-1,2-Bis-tetradecyloxy-3-bromopropane (**16**) (50 g, 0.09 mol) was dissolved in a 2 M methanolic solution of dimethylamine (400 mL) in a screw-top pressure bottle. The pressure bottle was sealed and heated in an oil bath at 88–90 °C for 60 h while stirring. The pressure bottle was cooled to room temperature before it was opened. The solution was concentrated under reduced pressure. The crude residue was dissolved in ethyl acetate (500 mL) and washed with water (500 mL). The organic layer was concentrated under reduced pressure and purified by column chromatography over a silica gel (230–400 mesh) with 5–20% ethyl acetate in hexane as eluent to obtain (*R*)-1,2-bis-tetradecyloxy-3-dimethylamino propane (**17**) (41 g, 88%) as light colored oil. TLC (SiO_2) methanol/chloroform (1:9) $R_f \sim 0.51$. ^1H NMR ($CDCl_3$, 500 MHz): δ 0.88 (t, $J = 6.8$ Hz, 6H), 1.25 (s, 44 H), 1.51–1.58 (m, 4 H), 2.35 (s, 6H, N–CH_3), 2.41–2.58 (m, 2H, N–CH_2), 3.39–3.64 (m, 7H).

*2.3.5. (R)-1,2-bis-Tetradecyloxy-3-O-benzylpropane (**19**)*

To a stirred suspension of sodium hydride (76.4 g, 1.91 mol, 60% in oil) in anhydrous dimethylformamide (400 mL) under argon atmosphere at 0 °C, a solution of (*R*)-1-*O*-benzyl glycerol (**18**) (87 g, 0.47 mol) was added in dimethylformamide (470 mL) over a period of 1 h, while maintaining the internal temperature below 20 °C. After stirring at room temperature for 2 h, tetradecyl bromide (530.2 g, 1.91 mol) was added at 0 °C over a period of 2 h. After complete addition, the reaction mixture was stirred at room temperature for 2 h and then temperature was gradually increased to 60 °C, and stirred for 14 h. The reaction mixture was cooled to 0 °C, added few drops of ice water and diluted with water (3 L) and added 50 mL conc.

HCl solution. The aqueous layer was extracted with ethyl acetate (2 × 1 L). The organic layer was washed with brine (500 mL) and dried over sodium sulfate. The organic layer was concentrated under reduced pressure and the crude product was purified by column chromatography over a silica gel (230–400 mesh) eluting with 2–10% ethyl acetate in hexane to obtain (*R*)-1,2-bis-tetradecyloxy-3-*O*-benzylpropane (**19**) (217 g, 79%) as colorless oil. TLC (SiO_2) hexane/ethyl acetate (1:9) $R_f \sim 0.53$. ^1H NMR ($CDCl_3$, 500 MHz): δ 0.88 (t, $J = 6.6$ Hz, 6H), 1.25 (br s, 44H), 1.49–1.58 (m, 4H), 3.39–3.60 (m, 9H), 4.54 (s, 2H, OCH_2Ph), 7.23–7.32 (m, 5H, Ph-H).

2.3.6. (*S*)-1,2-bis-Tetradecyloxypropan-3-ol (**20**)

(*R*)-1,2-Bis-tetradecyloxy-3-*O*-benzylpropane (**19**) (103 g, 0.17 mol) was dissolved in ethyl acetate (350 mL) and hydrogenated with 10% palladium on carbon (2.4 g) for 4 h at 50 psi. After filtration of the catalyst, the solution was evaporated under reduced pressure. The residue was dissolved in hot hexane (700 mL) and kept at −20 °C overnight. The separated solid was filtered and dried to afford (*S*)-1,2-bis-tetradecyloxypropan-3-ol (**20**) (77 g, 89%) as a white solid. Mp 44–45 °C. TLC (SiO_2) hexane/ethyl acetate (1:9) $R_f \sim 0.17$. ^1H NMR ($CDCl_3$, 500 MHz): δ 0.88 (t, $J = 6.7$ Hz, 6H), 1.25 (br s, 44H), 1.51–1.57 (m, 4H), 2.22 (br s, 1H, OH), 3.40–3.73 (m, 9H).

2.3.7. (*R*)-2-[2-(2,3-bis-Tetradecyloxypropoxy)ethoxy]tetrahydro-2*H*-pyran (**21**)

To a stirred suspension of (*S*)-1,2-bis-(tetradecyloxy)-propane-3-ol (**20**) (135 g, 0.278 mol) in anhydrous dimethylformamide (540 mL) under argon atmosphere at 0 °C, sodium hydride (39 g, 0.97 mol, 60% in oil) was added over a period of 1 h maintaining the temperature below 15 °C. After stirring at room temperature for 2 h, 2-(2-bromoethoxy)tetrahydro-2*H*-pyran (204 g, 0.976 mol) was added at 0 °C over a period of 3 h maintaining the temperature below 10 °C. The reaction mixture was stirred at room temperature for 48 h. The reaction mixture was cooled to 0 °C and ice water was added slowly to quench excess sodium hydride. The reaction mixture was concentrated under reduced pressure to remove most of the dimethylformamide and the crude solution was diluted with water (3 L) and extracted with ethyl acetate (1 L). The organic layer was washed with aqueous saturated sodium chloride (500 mL) and dried over sodium sulfate. The solvent was concentrated under reduced pressure. The crude product was purified by column chromatography over silica gel (230–400 mesh) with 1–8% ethyl acetate in hexane to obtain (*R*)-2-[2-(2,3-bis-tetradecyloxypropoxy)ethoxy]tetrahydro-2*H*-pyran (**21**) (138 g, 81%) as colorless oil. TLC (SiO_2) hexane/ethyl acetate (1:4) $R_f \sim 0.30$; ^1H NMR ($CDCl_3$, 500 MHz): δ 0.88 (t, $J = 6.7$ Hz, 6H), 1.25 (s, 44H), 1.53–1.87 (m, 10H), 3.40–3.65 (m, 13H), 4.81–4.56 (m, 2H), 4.62–4.67 (m, 1H).

2.3.8. (*R*)-2-(2,3-bis-Tetradecyloxypropoxy)ethanol (**22**)

To a solution of (*R*)-2-[2-(2,3-bis-tetradecyloxypropoxy)ethoxy]tetrahydro-2*H*-pyran (**21**) (105 g, 0.17 mol) in methanol (1 L) was added 1 M HCl in ether (5 mL) and stirred at room temperature for 2 h. The reaction mixture was neutralized with solid sodium bicarbonate until the solution became neutral. The reaction mixture was filtered and concentrated under reduced pressure. The residue was dissolved in ethyl

acetate (1 L) and washed with water (200 mL) and dried over sodium sulfate. The organic layer was concentrated under reduced pressure and crude product was purified by column chromatography over a silica gel (230–400 mesh) eluting with 5–15% ethyl acetate in hexane to obtain (*R*)-2-(2,3-bis-tetradecyloxypropoxy)ethanol (**22**) (72 g, 79%) as colorless oil. TLC (SiO_2) hexane/ethyl acetate (1:4) $R_f \sim 0.18$. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.88 (t, $J = 6.7$ Hz, 6H), 1.25 (s, 44H), 1.51–161 (m, 4H), 2.58 (br s, 1H, OH), 3.41–3.73 (m, 13H).

*2.3.9. (R)-1-[1-(2-Bromoethoxymethyl)-2-tetradecyloxyethoxy]tetradecane (**23**)*

To a solution of (*R*)-2-(2,3-bis-tetradecyloxypropoxy)ethanol (**22**) (54 g, 102.1 mmol) in anhydrous dichloromethane (540 mL) under argon atmosphere at 0 °C triphenylphosphine (34.7 g, 132.7 mmol) was added followed by carbon tetrabromide (43.9 g, 132.7 mmol). The reaction mixture was stirred at 0 °C for 2 h and diluted with water (1.5 L). The organic layer was separated, dried over sodium sulfate, and concentrated under reduced pressure. The crude product was purified by column chromatography over a silica gel (230–400 mesh) with 5% ethyl acetate in hexane to obtain (*R*)-1-[1-(2-bromoethoxymethyl)-2-tetradecyloxyethoxy]tetradecane (**23**) (57 g, 94%) as colorless oil. TLC (SiO_2) hexane/ethyl acetate (1:5) $R_f \sim 0.65$. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.88 (t, $J = 6.7$ Hz, 6H), 1.25 (s, 44H), 1.51–157 (m, 4H), 3.40–3.61 (m, 11H), 3.79 (t, $J = 6$ Hz, 2H).

*2.3.10. (R)-[2-(2,3-bis-Tetradecyloxypropoxy)ethyl]dimethylamine (**24**)*

(*R*)-1-[1-(2-Bromoethoxymethyl)-2-tetradecyloxyethoxy]tetradecane (**23**) (54 g, 91.3 mmol) was dissolved in 2 M methanolic solution of dimethylamine (810 mL) in a screw-top pressure bottle. The pressure bottle was sealed and heated in an oil bath while stirring at 88–90 °C for 60 h. The pressure bottle was cooled to room temperature before it was opened. The solution was concentrated under reduced pressure. The crude product was dissolved in ethyl acetate (1 L) and washed with water (1 L). The organic layer was concentrated under reduced pressure and purified by column chromatography over a silica gel (230–400 mesh) with 5–50% ethyl acetate in hexane as eluent to obtain (*R*)-[2-(2,3-bis-tetradecyloxypropoxy)ethyl]dimethylamine (**24**) (29 g, 57%) as light colored oil. TLC (SiO_2) methanol/chloroform (1:9) $R_f \sim 0.46$. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.88 (t, $J = 6.7$ Hz, 6H), 1.25 (s, 44H), 1.52–1.56 (m, 4H), 2.26 (s, 6H, N-CH_3), 2.52 (t, $J = 5.8$ Hz, 2H), 3.39–4.61 (m, 11H). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 14.07, 22.65, 26.06, 26.11, 29.33, 29.47, 29.61, 29.63, 29.64, 29.66, 29.67, 30.08, 31.89, 45.91, 58.82, 69.79, 70.56, 71.29, 71.61, 77.83.

2.4. Synthesis of cationic cardiolipin analogues

*2.4.1. 1,3-bis-(1,2-Ditetradecyloxypropyl-3-N,N-dimethyl ammonium bromide) propane-2-ol (**2**) [PCL-1]*

A solution of (*R*)-1,2-bis-tetradecyloxy-3-dimethylamino propane (**17**) (3.51 g, 6.88 mmol) and 1,3-dibromopropan-2-ol (**8**) (0.3 g, 1.37 mmol) in anhydrous ethanol (30 mL) was refluxed for a period of 7 days. The reaction mixture was

cooled and the solvent was evaporated to give a crude waxy solid. The crude compound was dissolved in hot hexane (50 mL) and stirred at room temperature for 6 h. The separated solid was filtered and washed with cold hexane (3 × 10 mL) to remove the starting material (*R*)-1,2-bis-tetradecyloxy-3-dimethylamino propane. The solid was dissolved in dichloromethane and acetone was added (ratio 1:10). The flask was sealed and stored at 0 °C overnight. The white solid was filtered and washed with cold acetone (20 mL). The recrystallization procedure was repeated two times. The compound was dried for 24 h under high vacuum and then over P_2O_5 for 36 h to obtain cationic cardiolipin analogue (**2**) (1.3 g, 76%) as a white solid. Mp 106–108 °C. TLC (SiO_2) methanol/chloroform (1:9) single spot $R_f \sim 0.11$. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.88 (t, $J = 6.8$ Hz, 12H), 1.24–1.31 (m, 88H), 1.53–1.57 (m, 8H), 2.08 (s, 1H, OH), 3.42–3.86 (m, 28H), 3.97 (d, $J = 13.5$ Hz, 1H), 4.20 (t, $J = 5.0$ Hz, 1H), 4.37 (t, $J = 4.5$ Hz, 1H), 4.55 (t, $J = 12.5$ Hz, 2H), 5.18–5.24 (m, 1H), 6.57 (d, $J = 10.5$ Hz, 1H). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 14.03, 22.60, 25.94, 25.96, 26.16, 29.28, 29.37, 29.39, 29.52, 29.55, 29.56, 29.58, 29.61, 29.63, 29.99, 30.00, 31.84, 52.78, 53.16, 54.89, 62.30, 64.99, 66.36, 68.52, 68.61, 68.94, 69.28, 69.40, 69.89, 72.01, 72.02, 72.80, 72.85; IR 3407 (br), 3223 (br), 2956 (s), 2920 (s), 1633, 1467, 1377, 1116, 971, 893, 720 cm^{-1}; ESI-MS 1159.4 [M+1-Br], 1079.9 [M+1-2Br], 542.2 [M+1–2Br/2]; Anal. Calcd for $C_{69}H_{144}Br_2N_2O_5$: C, 66.74; H, 11.69; N, 2.26; Br, 12.87. Found: C, 66.32; H, 11.62; N, 2.31; Br, 13.27.

*2.4.2. 1,3-bis-(1,2-Ditetradecyloxypropyl-3-N,N-dimethyl-3-ethoxy ammonium bromide)propane-2-ol (**3**) [PCL-2]*

A solution of (*R*)-1,2-bis-tetradecyloxy-3-dimethylamino propane (**17**) (35.6 g, 69.8 mmol) and 1,3-bis-(2-bromoethoxy)propane-2-ol (**12**) (7.1 g, 23.2 mmol) in anhydrous ethanol (430 mL) was refluxed at 78–80 °C over a period of 5 days. The hot reaction mixture was transferred to Erlenmeyer flask and acetone (4.3 L) was added dropwise while stirring over a period of 2 h. The mixture was kept at −20 °C overnight. The solid was filtered and washed with cold acetone (500 mL) to obtain a colorless white solid (28 g). The crude solid was purified by recrystallization in the mixture of warm methanol and acetone (ratio 1:10) and then stored at 0 °C overnight. The solid was separated, filtered, and washed with cold acetone (300 mL). The recrystallization was repeated two times to yield a pure product. The compound was desiccated for 24 h under high vacuum and then over P_2O_5 for 36 h to obtain cationic cardiolipin analogue (**3**) (24 g, 78%) as a white solid. Mp 159–160 °C; TLC (SiO_2) methanol/chloroform (1:9) single spot $R_f \sim 0.13$. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.88 (t, $J = 6.7$ Hz, 12H), 1.25 (s, 88H), 1.52–1.71 (m, 8H), 3.41–3.68 (m, 29H), 3.95–4.19 (m, 14H), 4.63 (br s, 1H, OH). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 13.88, 22.45, 25.84, 25.98, 29.14, 29.23, 29.27, 29.38, 29.44, 29.46, 29.49, 29.82, 31.70, 52.93, 53.00, 53.42, 64.95, 66.48, 68.63, 68.77, 69.12, 71.75, 72.58, 73.24; IR 3395 (br), 2917 (s), 2872 (s), 1467 (s), 1126 (br) cm^{-1}; ESI-MS 1248.5 [M+1-Br], 584.2 [M+1–2Br/2] Anal. Calcd for $C_{73}H_{152}Br_2N_2O_7$: C, 65.93; H, 11.52; N, 2.11; Br, 12.02. Found: C, 65.65; H, 11.49; N, 2.13; Br, 12.17.

*2.4.3. 1,3-bis-(1,2-Ditetradecyloxy-4-oxa-hexyl-6-N,N-dimethyl ammonium bromide)propane-2-ol (**4**) [PCL-3]*

A solution of (*R*)-[2-(2,3-bis-tetradecyloxypropoxy)ethyl]dimethylamine (**24**) (39 g, 70.2 mmol) and 1,3-dibromopropan-2-ol (**8**) (5.1 g, 23.4 mmol) in anhydrous ethanol (440 mL) was refluxed at 78–80 °C over a period of 7 days. The reaction mixture was cooled and the solvent was evaporated to give a crude waxy solid. The crude compound was dissolved in hot hexane (200 mL), stirred at room temperature for 60 min, and kept at −20 °C for 2 h. The separated solid was filtered and washed with cold hexane (3 × 50 mL) to remove excess starting material (*R*)-[2-(2,3-bis-tetradecyloxypropoxy)ethyl]dimethylamine. The solid was dissolved in warm methanol and acetone (ratio 1:10). The flask was stored at 0 °C overnight. The white solid was filtered and washed with cold acetone (100 mL). The recrystallization procedure was repeated two times. The compound was desiccated for 24 h under high vacuum and then over P_2O_5 for 36 h to obtain cationic cardiolipin analogue (**4**) (23 g, 74%) as a white solid. Mp 190–193 °C. TLC (SiO_2) methanol/chloroform (1:9) single spot $R_f \sim 0.12$. ^{1}H NMR δ ($CDCl_3$, 500 MHz): δ 0.88 (t, $J = 6.8$ Hz, 12H), 1.25 (s, 88H), 1.49–1.58 (m, 8H), 3.39–3.72 (m, 32H), 3.81–4.01 (m, 8H), 4.49 (d, $J = 13.0$ Hz, 2H), 5.24–5.30 (m, 1H), 6.48 (d, $J = 9.0$ Hz, 1H). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 13.98, 22.56, 25.97, 26.00, 29.23, 29.37, 29.38, 29.52, 29.54, 29.57, 29.58, 30.02, 31.79, 52.95, 52.98, 54.13, 61.71, 65.12, 65.66, 65.70, 66.56, 69.82, 69.83, 70.43, 70.44, 71.53, 71.56, 71.72, 77.37; IR 1117 (br s), 1467, 2917 (s), 2872 (s), 3398 (br s) cm^{-1}; ESI-MS: 1248.7 [M+1-Br$^-$], 584.6 [M+1–2Br$^-$/2]; Anal. Calcd for $C_{73}H_{152}Br_2N_2O_7$: C, 65.93; H, 11.52; N, 2.11; Br, 12.02. Found: C, 65.57; H, 11.36; N, 2.12; Br, 12.22.

*2.4.4. 1,3-bis-(1,2-Ditetradecyloxy-4,10-dioxa-decyl-7-N,N-dimethyl ammonium bromide)propane-2-ol (**5**) [PCL-4]*

A solution of (*R*)-[2-(2,3-bis-tetradecyloxypropoxy)ethyl]dimethylamine (**24**) (21 g, 37.88 mmol) and 1,3-bis-(2-bromoethoxy)propane-2-ol (**12**) (3.84 g, 12.6 mmol) in anhydrous ethanol (250 mL) was refluxed at 78–80 °C over a period of 7 days. The hot reaction mixture was transferred to an Erlenmeyer flask and acetone (2 L) was added dropwise, while stirring over a period of 3 h and kept at −20 °C overnight. The solid was filtered and washed with cold acetone (100 mL) to obtain a colorless white solid (18 g). The crude solid was purified by recrystallization in warm methanol and acetone (ratio of 1:10) and then stored at −20 °C overnight. The solid was separated, filtered, and washed with cold acetone (50 mL). The recrystallization was repeated two times to yield pure compound. The compound was desiccated for 24 h and then over P_2O_5 for 36 h to obtain cationic cardiolipin analogue (**5**) (14 g, 78%) as a white solid. Mp 198–200 °C. TLC (SiO_2) methanol/chloroform (1:9) single spot $R_f \sim 0.13$. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.88 (t, $J = 6.9$ Hz, 12H), 1.25 (s, 88H), 1.48–1.59 (m, 8H), 3.38–3.65 (m, 36H), 3.89–4.13 (m, 16H). ^{13}C NMR ($CDCl_3$, 125 MHz): 13.89, 22.46, 25.87, 25.90, 29.14, 29.28, 29.30, 29.43, 29.45, 29.48, 29.92, 31.70, 52.77, 52.83, 64.37, 64.87, 64.98, 65.36, 68.77, 69.99, 70.32, 71.35, 71.59, 72.62, 77.27. IR 1131, 1470, 2917, 2956, 3339 cm^{-1}; ESI-MS: 1336.4 [M+1-Br$^-$], 628.4 [M+1–2Br$^-$/2]. Anal. Calcd for $C_{77}H_{160}Br_2N_2O_9$: C, 65.22; H, 11.37; N, 1.98; Br, 11.27. Found: C, 64.91; H, 11.27; N, 1.98; Br, 11.18.

2.5. Preparation of liposomes

Liposomes were prepared from newly synthesized cationic lipids and helper lipids using thin-film hydration method [23]. In brief, cationic lipid and DOPE were dissolved in chloroform in a round-bottomed flask. The solvent was removed under reduced pressure using rotary evaporator to form a lipid film and further dried under vacuum overnight. The lipid film was hydrated with MilliQ water. The bulk cationic liposomes were extruded through 0.2 μm pore size polycarbonate filters (Whatman) three times and 0.1 μm pore size polycarbonate filters five times. The extruded liposomes were sterilized by filtering through 0.22 μm sterile filter unit (Millipak 20 positively charged). The prepared liposomes had a mean particle size of <130 nm. The size of the liposomes was characterized using a light scattering particle sizer (Nicomp Model 380, Santa Barbara, CA).

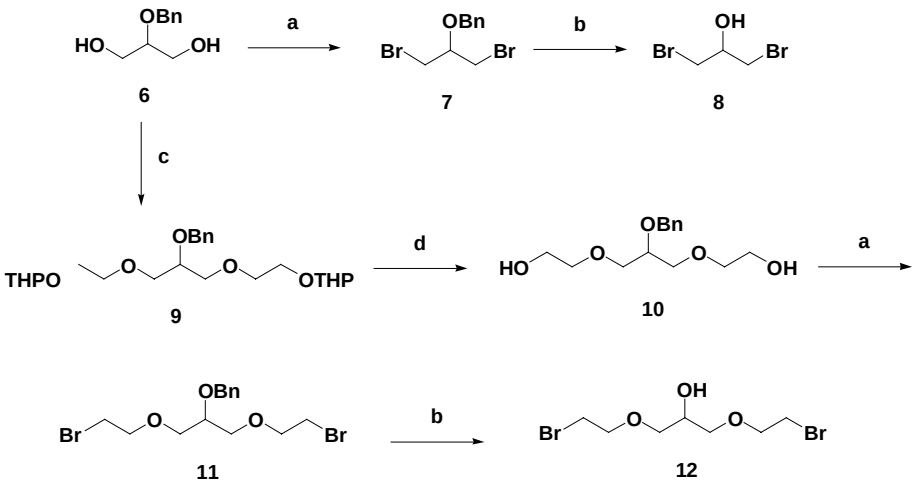
2.6. Cell culture and in vitro transfection

Chinese hampster ovary (CHO) cells were purchased from American Type Culture Collection (Manassas, VA) and were maintained in Kaighn's modification of Ham's F12 medium (F12K) with 10% HI-FBS. At one day prior to transfection, CHO cells (10,000 cells per well) were seeded in 96-well plates and then cultured overnight in a 5% CO_2 incubator. The cells were then washed with phosphate-buffered saline to remove the residual serum before adding the transfection mixture.

The transfection efficiency of prepared liposomes was determined using β-galactosidase (β-gal) reporter gene assay (Promega, Madison, WI) as previously described [24]. The optical density of the sample was read at 414 nm using a plate reader (Thermo Electron, Franklin, MA). The amount of β-gal expression was calculated based on exogenous β-gal standard.

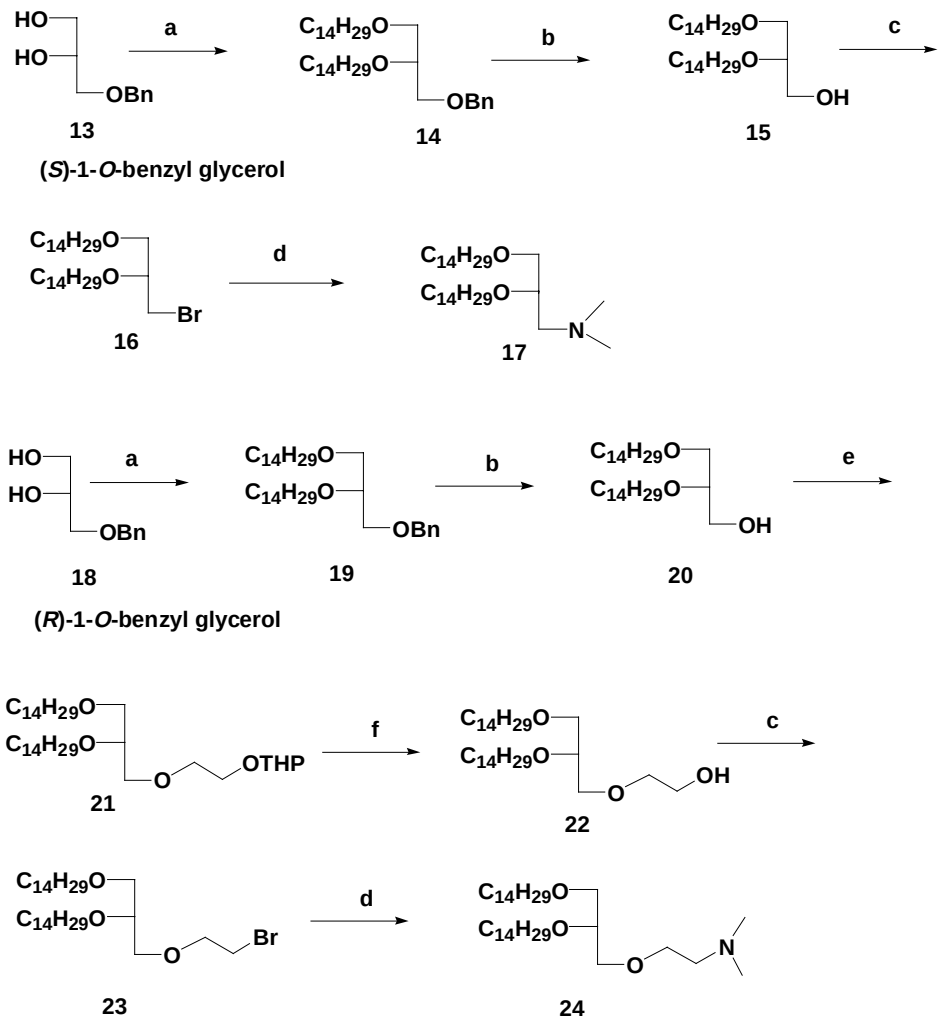
3. Results and discussions

The synthesis of bromo intermediates **8** and **12** is outlined in Scheme 1. Commercially available 2-*O*-benzyl glycerol **6** was brominated with triphenylphosphine and carbon tetrabromide in dichloromethane to afford 1,3-dibromo-2-*O*-benzyl glycerol **7** in yield of 87%. Debenzylation of **7** via hydrogenation over 10% Pd/C catalyst in ethanol gave 1,3-dibromopropan-2-ol **8** in 94% yield. Alkylation of 2-*O*-benzyl glycerol **6** with 2-(2-bromoethoxy)tetrahydro-2*H*-pyran in the presence of sodium hydride (60% in oil) in dimethylformamide afforded 1,3-bis-[(2-ethoxy tetrahydro-2*H*-pyran)]-2-*O*-benzyl glycerol **9** in 71% yield. The THP derivative **9** on deprotection in methanol with catalytic amount of 1 M HCl solution in diethyl ether at room temperature for 2 h gave the corresponding diol derivative 3,7-dioxa-5-*O*-benzyl-1,9-nonanediol **10** in 88% yield. The diol **10** on bromination using Ph_3P/CBr_4 in CH_2Cl_2 yielded 1,9-dibromo-3,7-dioxa-5-*O*-benzyl nonane **11** in 91% yield. Debenzylation of **11** via hydrogenation provided 1,3-bis-(2-bromoethoxy)propane-2-ol **12** in 94% yield.



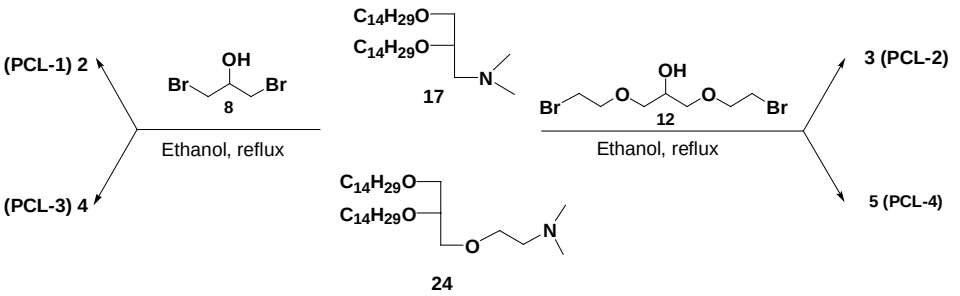
Scheme 1. Preparation of bromo intermediate. (a) Ph_3P, CBr_4, 0 °C, (b) 10% Pd-C, H_2, 50 psi, ethanol, (c) NaH, $BrCH_2CH_2OTHP$, DMF, 0 °C to rt, (d) 1 M HCl in diethyl ether, MeOH, rt.

The synthesis of tertiary amine intermediates **17** and **24** is outlined in Scheme 2. Williamson etherification of commercially available (*S*)-1-*O*-benzyl glycerol **13** with tetradecyl bromide in the presence of sodium hydride (60% in oil) in dimethylformamide afforded (*S*)-1,2-bis-tetradecyloxy-3-*O*-benzylpropane **14** in 75% yield. Debenzylation of **14** via hydrogenation over 10% Pd/C catalysts in ethyl acetate gave (*R*)-1,2-bis-tetradecyloxy propane-3-ol **15** in 92% yield. The alcohol **15** on brominating using Ph_3P/CBr_4 in dichloromethane afforded (*S*)-1,2-bis-tetradecyloxy-3-bromopropane **16** in 90% yield. The bromo compound **16** on heating with 10-fold excess of 2 M methanolic dimethylamine solution in pressure bottle gave (*R*)-1,2-bis-tetradecyloxy-3-*N*,*N* dimethylamine propane **17** (88% yield). Similarly, commercially available (*R*)-1-*O*-benzyl glycerol **18** was converted to (*S*)-1,2-bis-tetradecyloxy propane-3-ol **20** in two steps. Alkylation of (*R*)-1-*O*-benzyl glycerol **18** with tetradecyl bromide in the presence of sodium hydride (60% in oil) in dimethylformamide afforded (*S*)-1,2-bis-tetradecyloxy-3-*O*-benzylpropane **19** in 79% yield. Debenzylation of **19** via hydrogenation over 10% Pd/C catalysts in ethyl acetate gave (*S*)-1,2-bis-tetradecyloxy propane-3-ol **20** in 89% yield. Alkylation of **20** with 2-(2-bromoethoxy)tetrahydro-2*H*-pyran in the presence of sodium hydride in dimethylformamide afforded (*R*)-2-[2-(2,3-bis-tetradecyloxypropoxy)ethoxy]tetrahydropyran **21** in 81% yield. The THP derivative **21** was deprotected in methanol with catalytic amount of 1 M HCl solution in diethyl ether at room temperature for 2 h to yield 79% of the corresponding alcohol, (*R*)-2-(2,3-bis-tetradecyloxypropoxy)ethanol **22**. The alcohol **22** on bromination using Ph_3P/CBr_4 in dichloromethane yielded 94% of (*R*)-1-[1-(2-bromoethoxymethyl)-2-tetradecyloxy-ethoxy]tetradecane **23**. The bromo compound **23** on heating with 10-fold excess of 2 M methanolic dimethylamine solution in pressure bottle gave (*R*)-[2-(2,3-bis-tetradecyloxypropoxy)ethyl]dimethylamine **24** in 57% yield.



Scheme 2. Preparation of tertiary amine intermediate. (a) NaH, $C_{14}H_{29}Br$, DMF, 60–70 °C, (b) 10% Pd-C, H_2, 50 psi, ethyl acetate, (c) Ph_3P, CBr_4, CH_2Cl_2, 0 °C, (d) 2 M dimethylamine in methanol, pressure bottle, 90 °C, (e) NaH, $BrCH_2CH_2CH_2OTHP$, DMF, 0 °C to rt, (f) 1 M HCl in diethyl ether, MeOH, rt.

The synthesis of cationic cardiolipin analogues **2**, **3**, **4**, and **5** is outlined in Scheme 3. The bromo compounds **8** and **12** were reacted with **17** in ethanol at reflux temperature for 5–7 days to yield cationic cardiolipin analogues 1,3-bis-(1,2-ditetradecyloxypropyl-3-*N*,*N*-dimethyl ammonium bromide)propane-2-ol **2** (76%) and 1,3-bis-(1,2-ditetradecyloxypropyl-3-*N*,*N*-dimethyl-3-ethoxy ammonium bromide) propane-2-ol **3** (78%), respectively. The bromo compounds **8** and **12** were reacted with **24** in ethanol under reflux temperature for 5–7 days to yield cationic cardiolipin analogues, 1,3-bis-(1,2-ditetradecyloxy-4-oxa-hexyl-6-*N*,*N*-dimethyl ammonium



Scheme 3. Synthesis of cationic cardiolipin analogues.

bromide)propane-2-ol **4** (74%) and 1,3-bis-(1,2-ditetradecyloxy-4,10-dioxa-decyl-7-*N*,*N*-dimethyl ammonium bromide)propane-2-ol **5** (78%), respectively.

The transfection efficiencies of new cationic lipids **4** (PCL3) and **5** (PCL4) were evaluated and compared with PCL2-based transfection reagent, NeoPhectin at a charge ratio of 1:1 cationic lipid/DNA (+/−) (Fig. 5). NeoPhectin is a currently marketed transfection reagent composed of cationic lipid **3** (PCL2): DOPE at a molar ratio of 1:2 and showed high transfection among the cell lines including CHO cells [24]. Further, liposome containing compound **3** showed lower toxicity in mice than the DOTAP-based In Vivo GeneSHUTTLE [24]. In this study, the new cationic lipids (**4** and **5**) were formulated having cationic lipid:DOPE ratio 1:2 similar to NeoPhectin. The results showed that the transfection efficiency of formulation containing compound **4** is similar to NeoPhectin while compound **5** exhibit lower transfection. The presence of additional two oxyethylene groups in compound **5** may have resulted in a shape other than cone and hence showed low transfection. We anticipate cationic lipids **4** and **5** to exhibit lower toxicity similar to compound **3.**

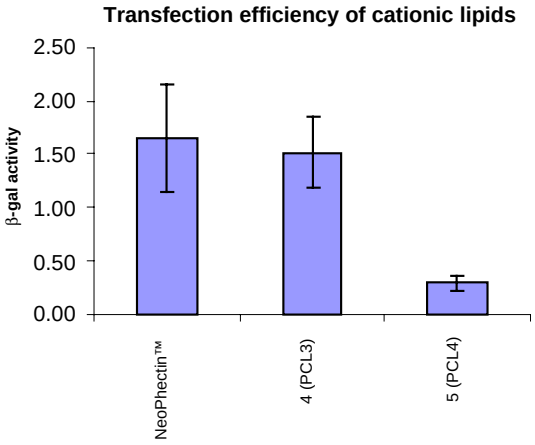


Fig. 5. Transfection of liposomes containing cationic lipids **3**, **4**, **5** in vitro. The experiment was repeated four times with triplicate wells for each set.

4. Conclusion

We have prepared a series of new cationic cardiolipin analogues. The process is scalable from gram to kilogram scale. Formulations containing **3** (PCL-2, NeoPhectin) have been shown to transfect [24,25] different cell lines with up to five times more efficiency compared to commonly used Lipofectin. Formulations containing compound **5** exhibited similar transfection properties when compared to NeoPhectin. These cationic cardiolipin analogues could be used to formulate a broad range of therapeutic agents, including antisense oligonucleotide as well as gene transfection agents. Application of these cationic cardiolipin analogues to deliver antisense oligonucleotide (AON) [26,27] and siRNA [28] into the targeted cells in vitro and in vivo is under investigation and will be published in future.

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