Synthesis of Novel Cationic Poly(Ethylene Glycol) Containing Lipids

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°In this paper, the synthesis of novel divalent cationic lipids with poly(ethylene glycol) segments is described. The lipids consist of an unsaturated double-chain hydrophobic moiety based on 3,4-dihydroxy benzoic acid, attached to a hydrophilic poly(ethylene glycol) spacer which contains a divalent cationic end group. As poly(ethylene glycol) spacers monodisperse triethylene glycol and telechelic poly(ethylene glycol)s with an average degree of polymerization of 9, 23, and 45 were used. The divalent cationic end group was attached by coupling a protected dibasic amino acid to the PEG spacer and following cleavage of the protecting groups. These novel class of cationic lipids is of particular interest for nonviral gene delivery applications.

INTRODUCTION

In the past few years, a very large research effort has been devoted in developing new compounds which are carriers of DNA[1] into human cells. Especially complexes of DNA and cationic liposomes, which are mixtures of neutral and cationic lipids, so-called CL−DNA complexes, seem to be very promising vectors for nonviral gene therapy applications (*1*−*6*). The advantages of these complexes compared to viral methods are lower toxicity, simpler preparation, the lack of immune response from the body and their ability to carry large pieces of DNA.

There are two major arguments for the utilization of lipids with cationic headgroups as DNA carriers. First, the ionic interactions between the positively charged headgroup of the lipid and the negatively charged phosphate groups of the DNA molecule lead to efficient complexation and compaction of DNA mimicking highly compact viral DNA particles. In fact, it has been found that CL−DNA complexes are comprised of highly ordered self-assembled structures, either of the lamellar type (*7*−*10*), or of the inverted hexagonal type (*11*). Second, a positively charged CL−DNA complex is able to interact with the negatively charged cell membrane. This enhances the chances for the complex to enter the cytoplasm of the cell by endocytosis or fusion (*11*, *12*). Liposomes for gene therapy applications normally consist of mixtures of neutral lipids and cationic lipids. This enables the independent variation of two important parameters: the charge density of lipid membrane at different neutral lipid/cationic lipid ratios and the overall charge of the CL−DNA complex at different total lipid/DNA ratios (*7*, *8*, *11*). Commonly used neutral lipids are 1,2-dioleyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleyl-*sn*-glycero-3-phosphoethanolamine (DOPE). Scheme 1 shows three of the most commonly used cationic lipids in gene therapy applications (*13*).

In contrast to the cationic lipids shown above, our research goal was to develop a new class of cationic lipids which have a hydrophilic flexible spacer between the hydrophobic alkyl chains and the cationic headgroup. We decided to use poly(ethylene glycol) as spacer unit. PEG is a nonionic water soluble polymer of extremely low immunogenicity which has been studied and applied in a broad range of scientifically and technologically important problems in colloidal science, complex fluids (*14*), drug delivery, and gene therapy (*15*) as well as in biology and biotechnology (*16*−*18*). PEG-containing lipids have also been used in Stealth liposomes (*19*−*22*) as in vivo drug carrier systems. These liposomes consist of closed bilayer shells of phospholipids covered with PEG-based lipids anchored to the membrane. The concept of a poly(ethylene glycol) containing spacer between hydrophobic lipid segments and biologically relevant ligands such as peptides or oligosaccharide-linked glycoproteines is described in studies of Zalipsky et al. (*23*, *24*) and Frisch et al. (*25*). Zalipsky reported also on long circulating liposomes containing a monovalent cationic amino-PEG-phosphatidylethanolamine with an average degree of polymerization of the PEG chain of 45 (*26*).

The properties of cationic lipids and their applications in the area of gene therapy may potentially be strongly influenced by the variation of the chain length of the poly(ethylene glycol) spacer and the number of positive charges on the headgroup. To fully explore and elucidate the role of the cationic lipid, we synthesized new types of multivalent cationic lipids. In these lipids, the PEG spacer increases the area per charged lipid. This allows us to directly vary the electrostatic interaction by significantly changing the lipid membrane charge density. In addition, an outer shell of poly(ethylene glycol) seg-

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[1]Abbreviations: CL, cationic liposome; COSY, correlation spectroscopy; DCC, *N*,*N*′-dicyclohexyl carbodiimide; DMRIE, 1,2-dimyristoyloxypropyl-3-dimethylhydroxyethylammonium bromide; DMAP, 4-dimethylamino pyridine; DNA, deoxyribonucleic acid; DOPC, 1,2-dioleyl-*sn*-glycero-3-phosphocholine; DOPE, 1,2-dioleyl-*sn*-glycero-3-phosphoethanolamine; DOSPA, 2,3-dioleyloxy-*N*-[2-(sperminecarboxamido)ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluoroacetate; DOTAP, 1,2-dioleyl-3-trimethylammoniumpropane chloride; GPC, gel permeation chromatography; PEG, poly(ethylene glycol); TBAH, tetrabutylammonium hydrogensulfate; tBOC, *tert*-butyloxycarbonyl; TEG, triethylene glycol; THF, tetrahydrofuran; TsCl, *p*-Toluenesulfonyl chloride.

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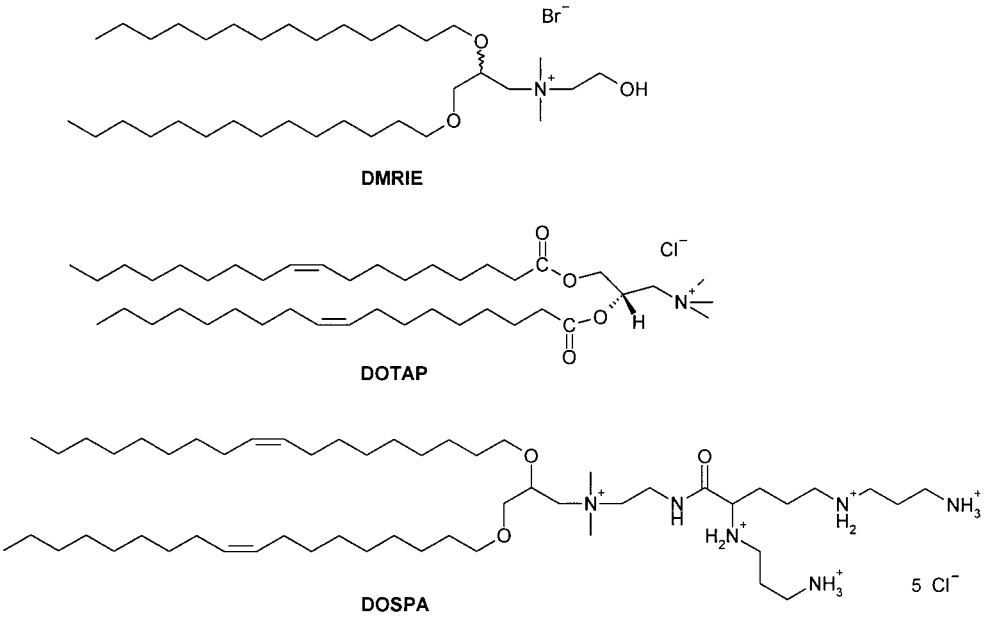
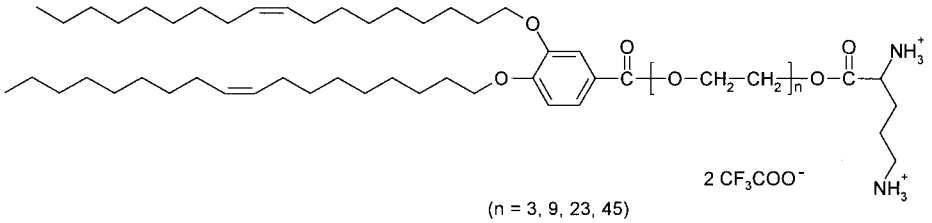
Scheme 1. Cationic Lipids Used in Gene Therapy Applications



Scheme 2. Cationic Lipids with Poly(ethylene glycol) Spacer Segments



ments protects the complexes from the immune response of the body (*19*−*22*). This paper describes the synthesis of divalent cationic lipids with poly(ethylene glycol) spacers. The lipids consist of an unsaturated double-chain hydrophobic moiety based on 3,4-dihydroxy benzoic acid, attached to a hydrophilic poly(ethylene glycol) spacer which contains a divalent cationic end group. As poly(ethylene glycol) spacers monodisperse triethylene glycol and telechelic poly(ethylene glycol)s with an average degree of polymerization of 9, 23, and 45 were used. Scheme 2 shows the chemical structure of the cationic lipids presented in this paper.

EXPERIMENTAL PROCEDURES

Materials and Reagents. Oleyl bromide, 3,4-dihydroxy benzoic acid ethyl ester (protocatechuic acid ethyl ester), L-ornithine hydrochloride, di-*tert*-butyl pyrocarbonate, and *N*,*N*′-dicyclohexyl carbodiimide (DCC) were purchased from Sigma. 4-Dimethylamino pyridine (DMAP), *p*-toluenesulfonyl chloride (TsCl), tetrabutylammonium hydrogensulfate (TBAH), acrylonitrile, triethylene glycol (TEG), and poly(ethylene glycol)s (PEG) were obtained from Aldrich. Triethylene glycol and poly(ethylene glycol)s were dried in a vacuum prior to use. All other reagents and solvents were at least analytical grade and used as received.

General Methods. Infrared spectra were recorded on a Perkin-Elmer 1605 FTIR spectrophotometer calibrated using polystyrene film. ^{1}H and ^{13}C NMR spectroscopies were carried out on a Bruker Avance 200 MHz spectrometer. Molecular weight distributions were determined on a gel permeation chromatography system equipped with an Acuflow Series II pump (eluent, THF; flow rate, 1 mL/min), four Phenomenex Phenogel 5 columns (10^5, 10^4, 10^3, and 500 Å pore size), and a VDS Optilab RI-detector. The GPC was calibrated with polystyrene standards. Elemental analysis was carried out on a Control Equipment Corp. organic elemental analyzer model CEC 440HA.

3,4-Di(oleyloxy) Benzoic Acid (1). A total of 5.0 g (15.0 mmol) of oleyl bromide, 1.24 g (6.8 mmol) of protocatechuic acid ethyl ester, 2.8 g (20.4 mmol) of potassium carbonate, and 0.13 g (0.8 mmol) of potassium iodide were suspended in 40 mL of cyclohexanone and stirred at 100 °C for 18 h under nitrogen. Because of the light sensitivity of oleyl bromide, the flask was covered with aluminum foil. The hot reaction mixture was filtered and the solvent was removed in a vacuum. The residue was dissolved in 30 mL of ethanol containing 1.14 g (20 mmol) potassium hydroxide and refluxed for 4 h. The hot reaction mixture was added to 80 mL of water. Acidifying with concentrated hydrochloric acid to pH 1 resulted in the precipitation of a white solid. The solid was filtered off and washed several times with water. The crude acid was recrystallized from 20 mL of ethanol and dried in a vacuum.

Yield: 3.2 g (72%). mp: 60.5 °C. TLC (silica gel) chloroform/methanol (9:1): R_f 0.42. ^{1}H NMR ($CDCl_3$): δ 0.88 (t, 6H), 1.20−1.58 (m, 44H), 1.78−1.96 (m, 4H), 1.96−2.12 (dt, 8H), 4.08 (t, 4H), 5.37 (t, 4H), 6.90 (d, 1H), 7.60 (d, 1H), 7.74 (dd, 1H) ppm.

C18-PEG-OH (2a−d): General Procedure. A total of 1.30 g (2.0 mmol) of 3,4-di(oleyloxy) benzoic acid (**1**), 20 mmol of poly(ethylene glycol), 0.39 g (2.1 mmol) of *p*-toluenesulfonyl chloride, 0.13 g (1.1 mmol) of 4-dimethylamino pyridine, 0.13 g (0.5 mmol) of tetrabutylammonium hydrogensulfate, and 1.9 g (13.6 mmol) of potassium carbonate were suspended in 60 mL of tetrahydrofuran. The reaction mixture was stirred for 24 h

at 45 °C. After cooling to room temperature, the mixture was poured into 700 mL of water and 100 mL of methylene chloride were added. The solution was slowly acidified with dilute hydrochloric acid till pH 1 was reached. The organic layer was separated, washed four times with 400 mL of water and dried over magnesium sulfate. The solvent was removed in a vacuum and the residue dissolved in 40 mL of ethanol. The colorless solution was kept overnight at 4 °C, which resulted in the formation of a white precipitate. After removal of the precipitate by filtration, the solvent was removed in a vacuum. Traces of byproducts could be removed by filtration over an aluminum oxide (basic) containing column (extraction with ethyl acetate and ethanol). The product was dried in a vacuum.

Yield: 60−80%. GPC (THF): M_w/M_n 1.01−1.03. ^{1}H NMR ($CDCl_3$): δ 0.88 (t, 6H), 1.20−1.58 (m, 44H), 1.78−1.96 (m, 4H), 1.96−2.12 (dt, 8H), 3.56−3.90 (m, 4n-2H), 4.08 (t, 4H), 4.45 (t, 2H), 5.37 (t, 4H), 6.90 (d, 1H), 7.60 (d, 1H), 7.74 (dd, 1H) ppm.

Di-*tert*-butyloxycarbonyl-L-ornithine (3). A total of 2.53 g (15 mmol) L-ornithine hydrochloride were dissolved in 12 mL of 2 N sodium hydroxide solution and cooled with an ice−water bath. Within 1 h, 9.8 g (45 mmol) of di-*tert*-butyl pyrocarbonate dissolved in a small amount of tetrahydrofuran and 19 mL of 4 N sodium hydroxide was added in portions to the reaction mixture. The colorless solution was stirred overnight. The solution was acidified with dilute hydrochloric acid and the product extracted twice with 200 mL of ether. The combined ether phases were washed several times with water and dried over magnesium sulfate. The solvent was removed in a vacuum and the product dried in high vacuum at room temperature.

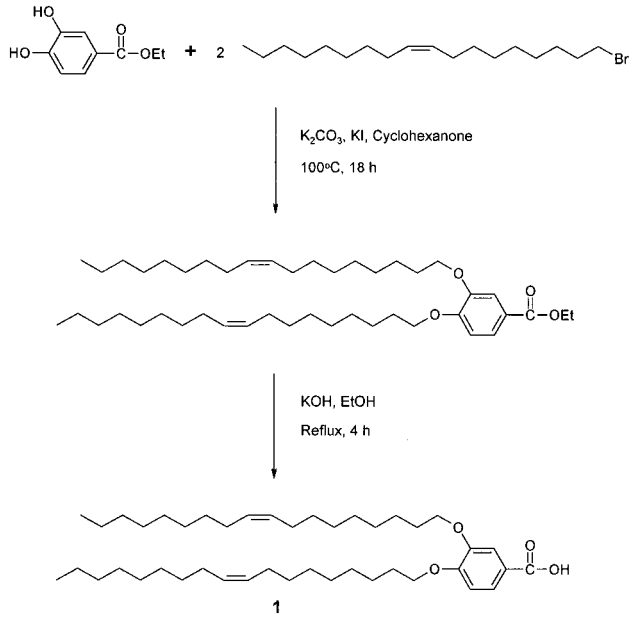
Yield: 4.8 g (97%). ^{1}H NMR ($CDCl_3$): δ 1.45 (s, 18H), 1.65−2.05 (m, 4H), 3.05−3.25 (m, 2H), 4.32 (m, 1H), 4.80 (b, 1H), 5.28 (b, 1H) ppm.

C18-PEG-Orn(BOC) (4a−d): General Procedure. A total of 1.4 mmol of C18-PEG-OH (**2a**−**d**), 0.51 g (1.6 mmol) of di-*tert*-butyloxy-L-ornithine (**3**), and 42 mg (0.35 mmol) of 4-dimethylamino pyridine were dissolved in 40 mL of methylene chloride. A total of 0.35 g (1.7 mmol) of *N*,*N*′-dicyclohexyl carbodiimide was dissolved in a minimal amount of methylene chloride and added to the reaction mixture. The solution was stirred for 3 h at room temperature. The dicyclohexyl urea was filtered off, and the solvent was removed in a vacuum. The crude product was dissolved in 30 mL of acetone and kept at 4 °C overnight. Dicyclohexyl urea was filtered off again, and the solvent was removed in a vacuum. Traces of unreacted di-*tert*-butyloxy-L-ornithine could be removed by filtration over an aluminum oxide (basic) containing column (elution with acetone). The product was dried in a vacuum.

Yield: 50−95%. GPC (THF): M_w/M_n 1.02−1.05. ^{1}H NMR ($CDCl_3$): δ 0.85 (t, 6H), 1.20−1.58 (m, 44H), 1.40 (s, 18H), 1.65−1.92 (m, 8H), 1.92−2.12 (dt, 8H), 3.02−3.22 (m, 2H), 3.56−3.88 (m, 4n−4H), 4.02 (t, 4H), 4.15 (m, 1H), 4.29 (t, 2H), 4.42 (t, 2H), 5.35 (t, 4H), 6.83 (d, 1H), 7.50 (d, 1H), 7.63 (dd, 1H) ppm.

C18-PEG-Orn (5a−d). General Procedure. A total of 1.0 mmol of C18-PEG-Orn(BOC) (**4a**−**d**) was dissolved in 5 mL of trifluoroacetic acid and cooled with an ice−water bath. After complete dissolution, the solution was stirred for additional 5 min. Longer reaction times may result in an increase of byproducts. The solvent was removed in a vacuum and the residue was dried in high vacuum. The crude product was purified by column chromatography on silica gel and elution with chloroform/methanol (4:1).

Scheme 3. Synthesis of 3,4-Di(oleyloxy) Benzoic Acid (1)



Yield: 30−70%. TLC (silica gel) chloroform/methanol (4:1): R_f 0.20−0.23. ^{1}H NMR ($CDCl_3$): δ 0.84 (t, 6H), 1.20−1.58 (m, 44H), 1.65−1.92 (m, 8H), 1.92−2.09 (dt, 8H), 2.98−3.12 (m, 2H), 3.56−3.90 (m, 4n-4H), 4.04 (t, 4H), 4.12 (m, 1H), 4.24 (t, 2H), 4.45 (t, 2H), 5.34 (t, 4H), 6.84 (d, 1H), 7.51 (d, 1H), 7.61 (dd, 1H), 7.75−8.35 (b, 6H) ppm. ^{13}C NMR ($CDCl_3$): d 14.3, 22.9, 26.3, 27.4, 30.0, 32.1, 69.3, 69.5, 70.0, 111.9, 114.1, 122.2, 130.0, 130.2, 148.1, 153.9, 167.0, 169.2 ppm. Elemental analysis (**5a**) $C_{58}H_{98}N_2O_{12}F_6$: calcd: C, 61.68; H, 8.75; N, 2.48. Found: C, 61.43; H, 9.12; N, 2.41.

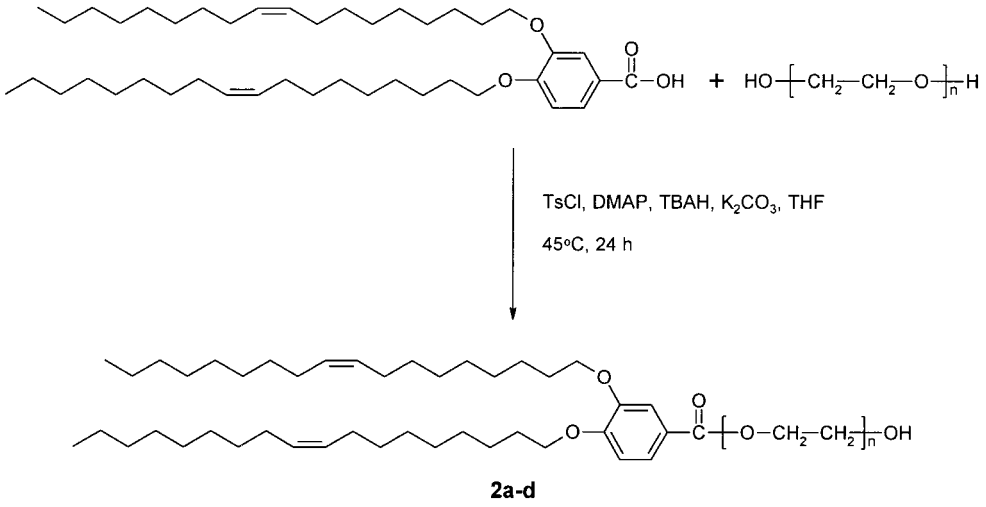
RESULTS AND DISCUSSION

The cationic PEG-containing lipids were synthesized following a route which consists of three main stages: (a) synthesis of the hydrophobic moiety of the lipid; (b) coupling of the hydrophilic PEG spacer segment; (c) attachment of the cationic headgroup to the lipid.

For the synthesis of the hydrophobic moiety of the lipid, we used a dihydroxy- and monocarboxy-substituted benzene ring as a central unit. This substitution pattern enables working with different linkage groups for the aliphatic alkyl chains (ether linkage) and the poly(ethylene glycol) segment (ester linkage). In this paper, we focus on the use of 3,4-dihydroxy benzoic acid which leads to two-chain lipids. Two-chain lipids are also the ones which are commonly found in natural membranes. Altough the benzene ring is an unusual unit in natural lipids there should be pointed out that ether and ester derivatives of the 3,4-dihydroxy benzoic acid (protocatechuic acid) are found in many fruits (*27*). As alkyl chains unsaturated C18 chains were used which will provide strong anchoring of the lipid in the bilayer membrane and chain flexibility due to the cis-double bond. 3,4-Di(oleyloxy) benzoic acid (**1**) was synthesized by etherification of 3,4-dihydroxy benzoic acid ethyl ester with oleyl bromide and following cleavage of the ethyl ester with potassium hydroxide (Scheme 3) (*28*−*30*).

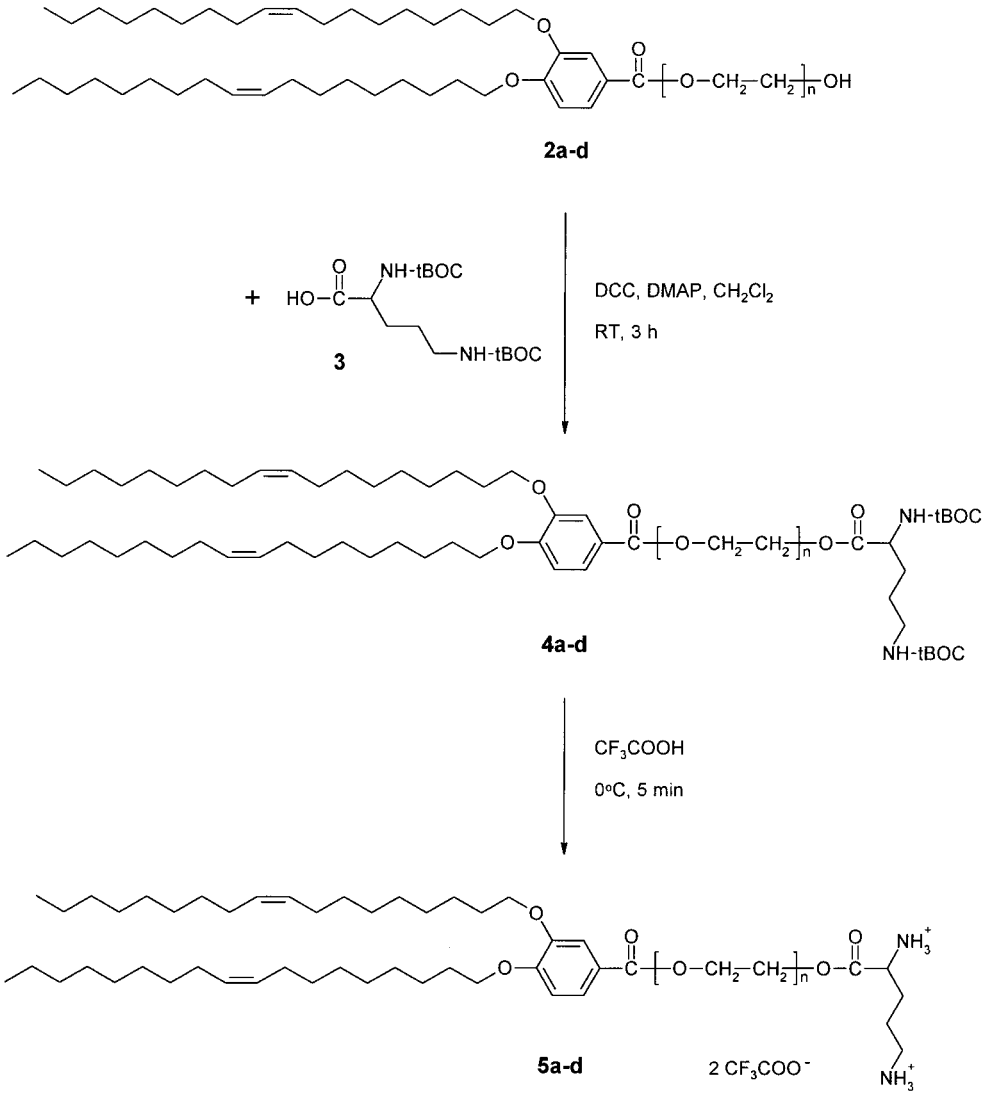
The next synthetic stage is the coupling reaction of 3,4-di(oleyloxy) benzoic acid (**1**) with α,ω-dihydroxy poly(ethylene glycol) (Scheme 4) based on a procedure of

Scheme 4. Synthesis of Hydroxy Functionalized PEG Containing Lipids (2a−d)[a]



[a] a, $n = 3$; b, $n = 9$; c, $n = 23$; d, $n = 45$.

Scheme 5. Synthesis of Cationic PEG Containing Lipids (5a−d)[a]



[a] a, $n = 3$; b, $n = 9$; c, $n = 23$; d, $n = 45$.

Percec et al. (*31*). Poly(ethylene glycol) was used in a 10-fold excess to avoid esterification of both hydroxyl groups. After reaction was completed, the excess PEG was removed by extraction with water followed by filtration of the product over an aluminum oxide (basic) containing column. The absence of traces of excess PEG was checked by gel permeation chromatography. The resulting hydroxy functionalized PEG containing lipids (**2a**−**d**) are useful intermediates for the attachment of a variety of biologically relevant end groups.

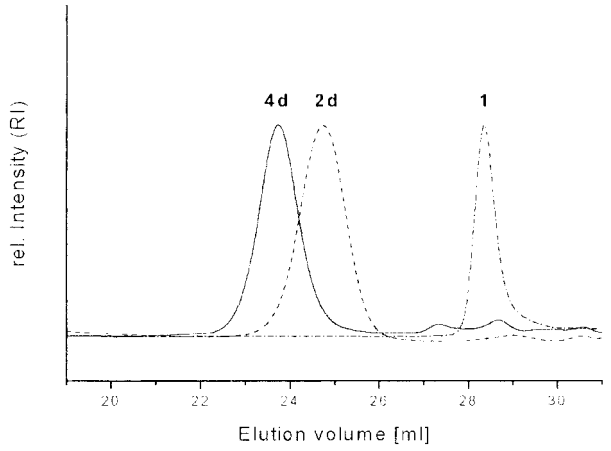


Figure 1. GPC elution diagrams of 3,4-di(oleyloxy) benzoic acid (**1**), the hydroxy functionalized PEG containing lipid (**2d**), and the corresponding PEG containing lipid with the tBOC protected end group (**4d**); eluent, THF; flow rate, 1 mL/min.

The carriers of the positive charge in cationic lipids are amino groups. In this work, we attached a *tert*-butyloxycarbonyl (tBOC) protected dibasic amino acid (L-ornithine) to the free hydroxyl group of the PEG segment (Scheme 5). The use of dicyclohexyl carbodiimide as esterification reagent allows us to run the reaction under mild conditions at room temperature (*32*). The use of a small excess of the protected L-ornithine ensures the complete conversion of the hydroxy functionalized PEG containing lipid. Excess L-ornithine could be removed by filtration over an aluminum oxide (basic) containing column. The purity of the product was checked by GPC and NMR spectroscopy. Figure 1 compares the GPC curves of 3,4-di(oleyloxy) benzoic acid (**1**), the hydroxy functionalized PEG containing lipid (**2d**), and the corresponding PEG containing lipid with the tBOC-protected end group (**4d**). The shift to higher molecular weight (lower elution volume) between the different coupling stages can be clearly observed. In addition, all peaks possess a monomodal distribution.

Cleavage of the *tert*-butyloxycarbonyl protecting groups with trifluoroacetic acid and purification of the crude product by column chromatography on silica gel lead to the cationic polymer lipids (**5a**−**d**) (Scheme 5). The structure was verified by ^{1}H NMR, ^{13}C NMR, COSY-NMR spectroscopy, and elemental analysis.

In conclusion, the successful synthesis of cationic PEG-containing lipids was demonstrated. These lipids have structural advantages compared to other cationic lipids. The divalent cationic headgroup of the lipid may potentially condense neighboring DNA strands, which further compacts DNA inside the complex. These compact structures are thought to enhance the transport of DNA into cells. Of particular interest in this series is the variability of the length of the poly(ethylene glycol) spacer. This spacer is expected to make the DNA strand−strand complexation more efficient and to avoid the immune response from the body by using the Stealth effect.

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