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Research article

Synthesis, self-assembly and lipoplex formulation of two novel cyclic phosphonate lipids

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ABSTRACT

Background: Synthetic cationic lipids hold much potential as gene packaging and delivery agents for the treatment of inherited and acquired life threatening diseases, such as cancer, AIDS, cardiovascular diseases, and certain autoimmune disorders.
Methods: We report the synthesis, self-assembly as characterized by critical micelle concentrations and plasmid DNA gel retardation using two novel cyclic, phosphonate cationic lipids **2a** and **2b**, which were synthesized by derivatizing two diastereomeric macrocyclic phosphonates **1a** and **1b** with a 2-carbon hydroxylamine linker, *N*, *N*-dimethylethanolamine (**3**).
Results: The production of cyclic phosphonate lipids **2a** and **2b** in 73% and 60% yields, respectively, was achieved using classical synthetic methods involving nucleophilic substitution at the phosphorus centre. The characterization of these lipids with Mass Spectrometry, ^{1}H, ^{13}C and ^{31}P Nuclear Magnetic Resonance supported the proposed stereochemistry and molecular structure of $C_{17}H_{36}NO_3P$ for these lipids. Critical micelle concentrations (CMC) for **2a** and **2b** were found to be 1.2 mM and 1.4 mM, respectively, at 25°C, providing evidence for the self-assembling ability of these novel cyclic lipids. Finally, lipid-DNA complexes (lipoplexes) formulated at various N/P (+/−) molar charge ratios and containing the co-lipid, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) were shown to retard DNA in an agarose gel retardation assay.
Conclusions: The synthesis, aggregation and DNA binding properties of these novel cyclic phosphonate lipids suggest that they may have utility serving as gene packaging and delivery agents.

Keywords: Synthetic lipids, cyclic phosphonates, 14-membered, critical micelle concentration, Lipid-DNA lipoplex

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INTRODUCTION

Gene transfer is an important advancement in treating genetic and acquired diseases. This form of therapy introduces exogeneous sequences of DNA (genes) into cells to correct defective genes. Cationic lipids are promising non-viral systems for gene transfer. The central concept of cationic lipid transfection is the conversion of negatively charged DNA into a neutral lipid-like entity through ion-pairing complexation with the cationic lipid.

Despite the advantages that cationic lipids have over viral vectors, including non-immunogenicity and ease of production, there are still several limitations that need to be overcome. The major limitation of cationic lipids is their unsatisfactory transfection efficiency when compared to viral vectors. Further limitations include their limited shelf-life, lack of specificity, inactivation of the transfection complex and reduction of cellular uptake by serum. Over the past several years, there has been substantial effort focused on synthesizing novel cationic lipids that surmount these challenges.[1,2]

Many of the known cationic lipids employed as transfection reagents have structural features in common with the naturally occurring, zwitterionic phosphatidylcholine(PC) lipid (Fig. 1). For example,

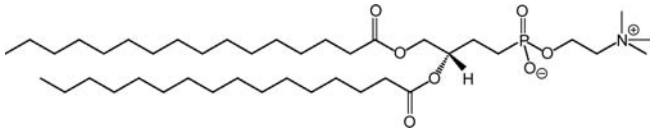


Figure 1. An example of a phosphatidylcholine variant, 1,2-dihexadecanoyl-sn-glycero-3-phosphocholine.

MacDonald and co-workers[3] have studied numerous cationic PC derivatives, where they have O-alkylated related PC lipids to produce cationic analogues, and demonstrated modulated transfection efficiencies by varying the hydrophobic domain length and/or degree of unsaturation. Common features associated with these and similar cationic lipid vectors, aside from the cationic head group, are the hydrophobic "tails" that consist of one or two saturated or mono-unsaturated hydrocarbon chains containing 16 to 18 carbon atoms. The purpose of our study was to synthesize and characterize two novel phosphonate lipids as potential DNA transfection reagents. Our recent research efforts towards the development of novel cationic lipid vectors have focused on structural modifications to the hydrophobic domain, specifically, modifications that offer varying degrees of flexibility within the rigidity-range (Fig. 2) associated with the hydrophobic core of lipid

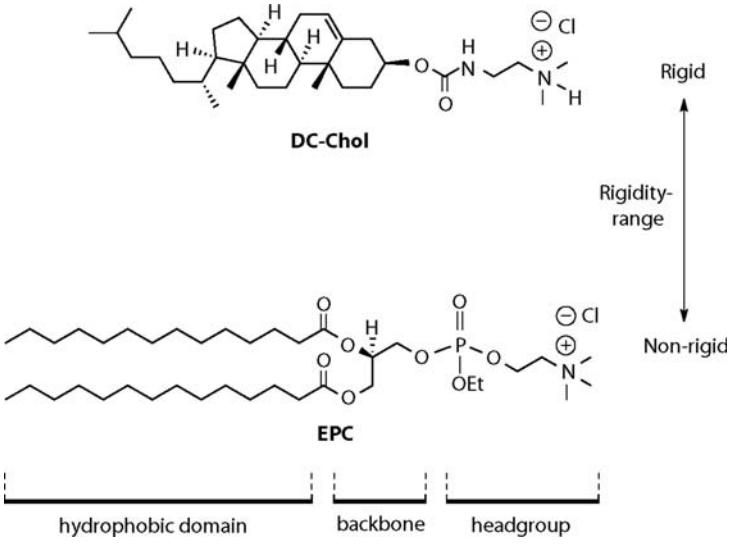


Figure 2. Lipid structural features and the rigidity range associated with the hydrophobic domain of common cationic lipids DC-Chol and EPC (taken from Ref.[6]).

vectors,[4–6] where the known cationic lipids 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine (EPC) and 3β-[*N*(*N*′,*N*′-dimethylaminoethyl)-carbamoyl]cholesterol(DC-Chol) represent two extremes along the rigidity-range. Our lipids described within are unique in that they contain a macrocyclic (phosphonate) hydrophobic region. Studies by Arakawa et al. indicate that macrocyclic lipids can aggregate to more tightly packed structures than their acyclic counterparts.[7] We hypothesize that the rigid lipids **2a** and **2b** will compact DNA (or antisense molecules) sufficiently different from acyclic cationic lipids (such as EPC), and may ultimately facilitate increased cellular uptake of the lipoplexes. The recent report by Goldring et al.[6] supports our hypothesis that macrocyclic lipids possess interesting properties as non-viral gene delivery vectors, including enhanced transfection efficiency.

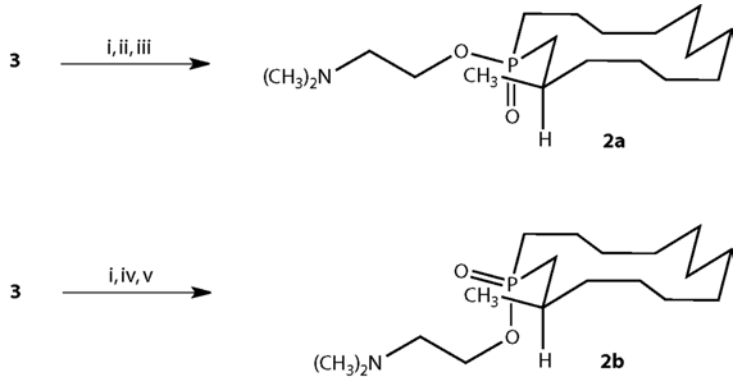
RESULTS AND DISCUSSION

Synthesis of 14-membered ring phosphonates 1a and 1b

Two diastereomeric macrocyclic phosphonates, **1a** and **1b**, were synthesized in nine steps as reported previously by Pungente et al.[8] The reactions afforded 107 mg (45.3%) of **1a**, 37.3 mg (15.8%) of **1b**, and 24.7 mg (5.20%) of acyclic dimer, as confirmed by complete chemical characterization.

Synthesis of O-(*N*, *N*-dimethylethanolamino)-phosphonates 2a and 2b

Displacement of the phenoxy moiety of **1a** by the alkoxide anion of linker *N*, *N*-dimethylethanolamine (**3**) in refluxing tetrahydrofuran (THF) under an inert atmosphere (N_2) resulted in the production of **2b** in 60% yield. By the same method, compound **2a** was generated from phosphonate **1b** in a yield of 73%. LC-MS analyses of compounds **2a** and **2b** were consistent with the molecular formula of $C_{17}H_{36}NO_3P$ (see Scheme 1).



Scheme 1. Reagents and conditions: i) 1.6 M *n*-BuLi, THF, −78°C; ii) 1b, THF, −78°C – RT; iii) reflux, 73%; iv) 1a, THF, −78°C – rt; v) reflux, 60%.

In each case, the nucleophilic substitution of the phenoxy moieties of **1a** and **1b** by the alkoxide of *N*, *N*-dimethylethanolamine to give **2b** and **2a**, respectively, resulted in inversion of stereochemistry at the phosphorus centre, as illustrated in Fig. 3. Inversion of stereochemistry at the phosphorus centre upon nucleophilic substitution of cyclic phosphonates has been previously reported.[8] Furthermore, the relative stereochemical assignments for **2a** and **2b** (Fig. 3), made on the basis of correlations between the ^{1}H NMR chemical shift data between the C-1 methyl and C-1 methine protons, as well as on the ^{31}P NMR chemical shift data (summarized in Table 1), support the inversion of stereochemistry at the phosphorus centres of **1a** and **1b**. The stereochemical assignments of macrocyclic phosphonates, **1a** and **1b**, elucidated and reported elsewhere,[8] provided confirmation of the stereochemical assignments for **2a** and **2b**, reported herein. It has been observed that the ^{31}P NMR signal for the equatorial P-OR isomer is lower field than the corresponding axial isomer in the cyclic phosphonates of hexopyranoses[9] and in other six-membered ring phosphonates.[10] This trend supports our stereochemical assignments for

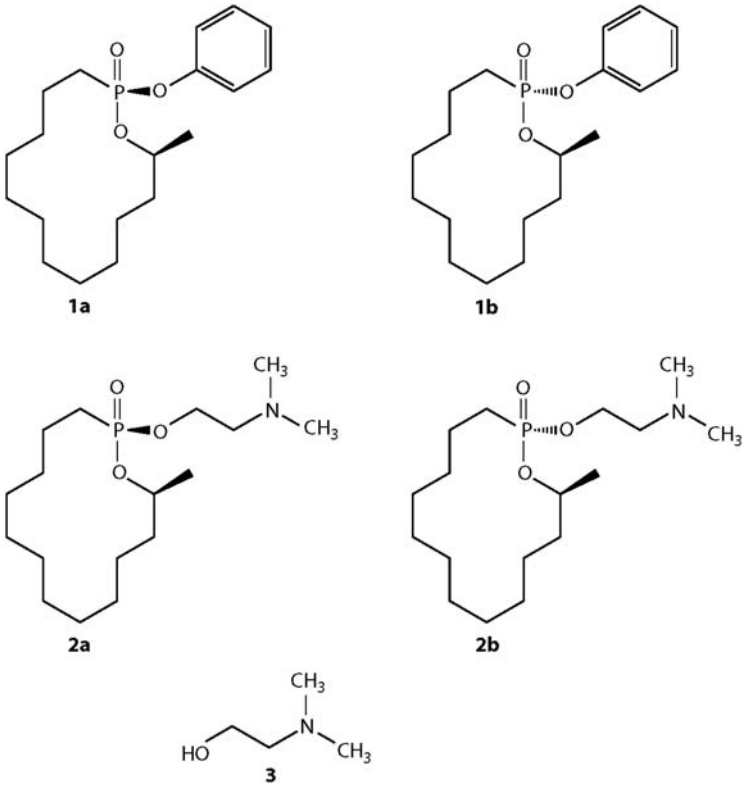


Figure 3. Structures and stereochemistry of compounds 1a, 1b, 2a, 2b and 3.

compounds **2a** and **2b**, since the ^{31}P NMR shift for isomer **2a** is downfield from that of isomer **2b**. In addition to these ^{31}P NMR correlations, ^{1}H NMR chemical shift data between the C-1 methyl protons and the C-1 methine proton of **2a** and **2b**, observed in Fig. 4 and summarized in Table 1, further confirmed the relative stereochemical assignments for these macrocyclic phosphonates. As is the case with the ^{1}H NMR assignments observed for macrocycle **1a**, the chemical shift separation between the methine multiplet at 4.64 ppm and the methyl doublet at 1.34 ppm for compound **2a** is greater than those same signals for **2b**, found to be at 4.50 ppm and 1.39 ppm, respectively, which is consistent with **1b** (Table 1).

Further support for the inversion at the phosphorus centers of **1a** and **1b** on displacement with **3** is suggested by thin layer chromatography. Based on the relative R_f-values for isomers **1a** and **1b** in Table 1 (where isomer **1a** has the OPh group in a pseudoequatorial orientation), this trend suggests that macrocyclic phosphonates with pseudoequatorial OR groups are less polar than the pseudoaxial isomer. Derivatization of the less polar macrocycle **1a** with linker **3** produced the polar *N*, *N*-dimethylethanolamino-derivative **2b**, while the reaction of the less polar isomer **1b** with **3** generated the polar isomer **2a** (as seen in Table 1).

Table 1. Key ^{1}H and ^{31}P NMR chemical shifts(δ values) and TLC R_f-values for the phenyl as well as the *N*, *N*-dimethylaminoethanol cyclic phosphonate derivatives.

Compound	δ ^{1}H for CH_3 protons (ppm)	δ ^{1}H for methiane protons (ppm)	δ ^{31}P (ppm)	TLC R_f values
1a	1.22	4.75	29.6	0.71[a]
1b	1.43	4.65	27.8	0.57[a]
2a	1.34	4.64	31.0	0.30[b]
2b	1.39	4.50	29.8	0.18[b]

[a] Obtained using a 1:1 mixture of petroleum ether and ethyl acetate.
[b] Obtained using a mixture of 5% MeOH in CH_2Cl_2 with 3 drops or approximately 1.5%, of ammonium hydroxide.

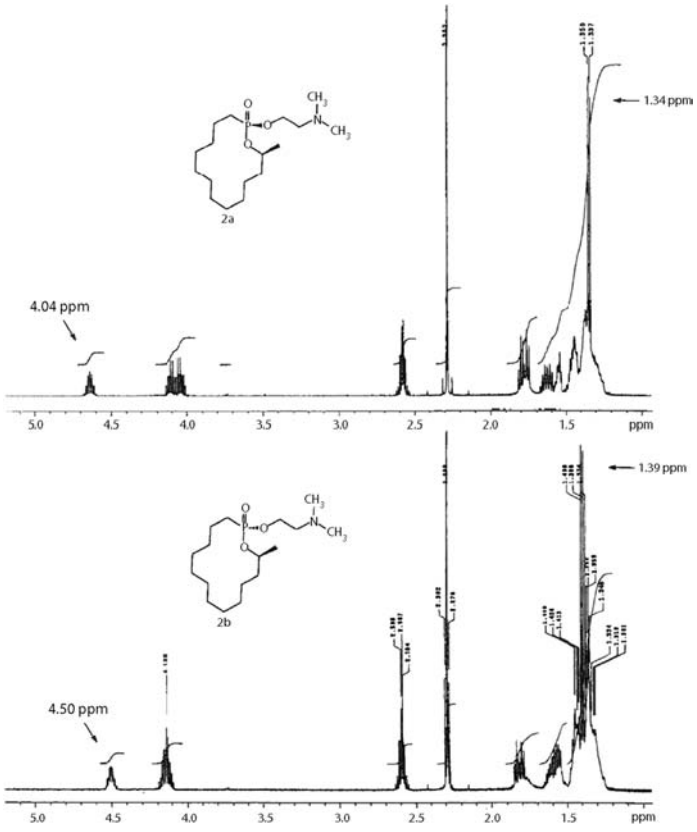


Figure 4. ^{1}H NMR Spectra for compounds 2a and 2b highlighting the key chemical shifts (δ values).

Determination of CMC for liposome formulations

Critical micelle concentrations of the novel macrocyclic lipids were determined using the fluorescence anisotropy method.[11] To validate the accuracy of this method, the CMC of sodium dodecyl sulfate (SDS)was determined, and was found to be consistent with that reported in the literature.[11] The CMC values for macrocyclic lipids **2a** and **2b** were found to be 1.2 mM and 1.4 mM, respectively at 25°C (Fig. 5), illustrating that these novel phosphonate lipids have the ability to self-assemble.

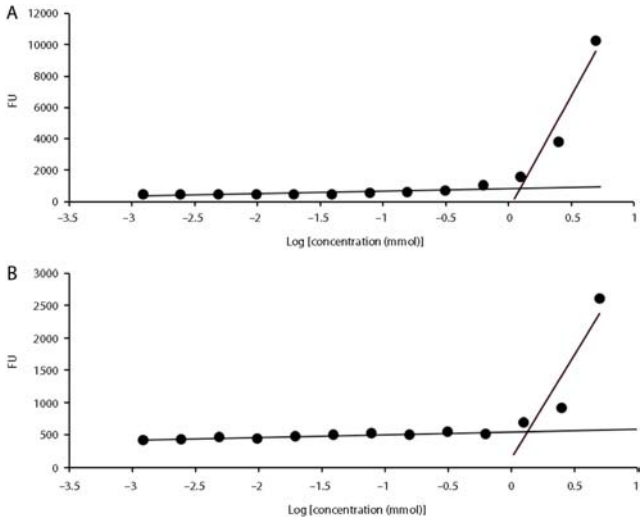


Figure 5. Dependence of fluorescence intensity on concentration of (A) 2a and (B) 2b at 25°C.

Finally, we prepared and investigated lipoplex formulations of lipids **2a** and **2b**. Combining the positively charged liposomes with negatively charged DNA resulted in the formation of lipoplexes. Each formulation contained a 1:1 molar ratio of novel cationic lipid: DOPE (a neutral co-lipid). Next, the lipoplexes were assessed for binding to pDNA through a gel retardation assay. A neutral or net positively charged lipoplex particle would prevent DNA migration through the agarose gel, thus demonstrating lipoplex formation. From the gel retardation assay there was evidence that both macrocyclic lipid formulations showed near complete retention, particularly at the higher molar charge ratios, and both lipoplexes exhibited some degree of retention from a N:P molar charge ratio of 3:1 (see Fig. 6).

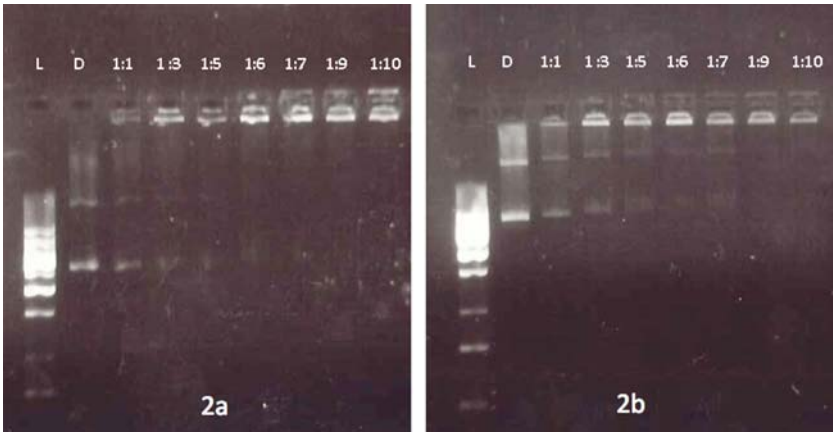


Figure 6. Gel retardation assays of liposomes formulated with a cationic lipid, 2a (A) or 2b (B) and DOPE as co-lipid, combined with pDNA at various N:P (+/−) molar charge ratios, ranging from 0.5:1 to 10:1, and run through a 1% agarose gel impregnated with the pDNA gel stain, ethidium bromide. Lanes L and D denote the 1kb DNA ladder and pDNA, respectively. The gels were visualized using a Geliance transilluminator.

MATERIALS AND METHODS

Materials

The macrocyclic phosphonate lipids **2a** and **2b** were synthesized as described within the experimental section below. *N*, *N*-dimethylethanolamine(**3**) was generously provided by Professor Alan Storr, Department of Chemistry, University of British Columbia (UBC). Unless otherwise stated, all other chemicals were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Anhydrous reagents and solvents were purified and prepared according to literature procedures.[12] Chromatographic solvents and reagents were used as received unless noted otherwise. The low boiling fraction (350–60°C) of petroleum ether was used. *n*-Butyl lithium (*n*-BuLi) was standardized by titration against 2,2-diphenylacetic acid in THF at room temperature to the appearance of a faint yellow color. 1,6-Diphenyl-1,3,5-hexatriene(DPH) was obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). SDS was obtained from Bio-Rad (Mississauga, ON, Canada). THF was obtained from BDH (Toronto, ON, Canada). DOPE was obtained from Avanti Polar Lipids (Alabaster, AL, USA). Plasmid DNA containing the β-galactosidase gene, pCMVβ*lacZnls12co* was obtained from Marker Gene Technologies, Inc. (Eugene, Oregon, USA).

General methods

Unless otherwise stated, all reactions were performed under a N_2 atmosphere using flame-dried glassware. Cold temperature baths were prepared as follows: −78°C (dry ice-acetone) and 0°C (ice-water).

All reactions were monitored by thin layer chromatography (TLC) and judged to be complete when the starting material was consumed as determined by TLC. In the description of reaction work-ups, washing with brine refers to a saturated solution of NaCl; drying of the organic phase with $MgSO_4$; and removal of solvent *in vacuo*, or concentration of solvent with the use of a rotary evaporator using a water aspirator and heating using a water bath.

Preparative flash chromatography[13] was performed using 230–400 mesh ASTM silica gel supplied by E. Merck Co (Germany). As an indicator of purity, all compounds were purified such that a single spot was evident by TLC.

Except where noted, proton nuclear magnetic resonance(^{1}H NMR) spectra were recorded in deuteriochloroform solutions on a Bruker AC-200 (200 MHz) or Bruker WH-400 (400 MHz) spectrometer. Chemical shifts are reported in parts per million (ppm) on the δ scale versus chloroform (δ 7.24 ppm) as an internal standard. Signal multiplicity, spin–spin coupling constants (where possible), and integration ratios are indicated in parentheses. Carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded in deuteriochloroform solutions on a Bruker WH-400 (100 MHz) spectrometer. Chemical shifts are reported in parts per million (ppm) on the δ scale versus chloroform (δ 77.0 ppm) as an internal standard. Phosphorus nuclear magnetic resonance (^{31}P NMR) spectra with proton decoupling were recorded in deuteriochloroform solutions on a Bruker AC-200 (81 MHz) spectrometer. Chemical shifts are given in parts per million (ppm) on the δ scale versus 85% phosphoric acid as an external standard.

LC-MS experiments were carried out on a Micromass (Montreal, Quebec, Canada) Quattro triple-quadrupole mass spectrometer interfaced to a HP 1090Series II HPLC system equipped with a Phenomenex (Torrance, CA, USA) C_8 column (100 mm × 2.1 mm i.d., 5 μm).

Thin-layer chromatography was performed on Merck silica gel 60 F254 pre-coated aluminum sheets. Visualization was achieved by irradiation with ultraviolet light at 254 nm and/or by spraying with anisaldehyde reagent (a solution of 1 mL anisaldehyde, 5 mL conc. H_2SO_4 and 10 mL glacial acetic acid in 90 mL MeOH) followed by heating.

Determination of CMC for lipids

CMC determinations were carried out on a CytoFluor 4000 spectrofluorophotometer with a polarization accessory to measure DPH fluorescence, as described elsewhere.[11] Briefly, a 10 mM stock solution of DPH in THF was prepared, and 1 μL was added to aqueous solutions of the novel lipids of various concentrations prepared in polypropylene Eppendorf Safe-Lock Tubes™. The samples were then allowed to equilibrate overnight at 25°C for 24 h in a dark chamber. Each sample was then split into 3 aliquots in a 96-well plate and analyzed using a CytoFluor 4000 Multi-Well Plate Reader, with the wavelengths for excitation and emission set to 340 nm and 440 nm, respectively. The average of three fluorescent readings for each sample was plotted against concentration, and the CMC value was identified in the plot as the concentration at which an abrupt change in the depolarization of DPH fluorescence occurred.

Preparation of liposome formulations

Hydrated liposomal (cationic lipid/co-lipid) formulations consisting of cationic lipid/DOPE (1:1 molar ratio) were generated from stock solutions with thin films by combining the required amounts of each chloroform solution of lipid and co-lipid, and removing the chloroform under reduced pressure. The thin films were then dissolved in a known amount of sterile water to a final lipid concentration of 1 mM, incubated overnight at 4°C followed by sonication for 30 minutes at 60°C.

Preparation of liposome-DNA (lipoplex) formulations

Combining the positively charged liposomes with negatively charged plasmid DNA in various defined molar charge ratios formed lipoplexes with N:P (+/−)charge ratios of 1:1, 3:1, 5:1, 6:1, 7:1, 9:1 and 10:1, respectively. Briefly, liposome concentrations were derived from 1 mM hydrated stock solutions. The DNA and HEPES buffer were mixed together first, then an equal volume of liposome diluted with HEPES was added and mixed, and the mixture was incubated at room temperature for 30 minutes to allow complexes to form.

Gel retardation assays of lipoplex formulations

A gel retardation assay was used to study the interaction between cationic lipids and DNA. Lipoplexes were formed as described above with 0.5 μg of DNA in a total volume of 10 μl. Samples were loaded onto a 1% agarose gel impregnated with ethidium bromide and run at 105 V for 120 minutes in TE buffer. Plasmids included in lipoplexes exhibited impeded migration in the electric field.

EXPERIMENTAL

1-Phenyl-1-oxo-1-phospho-13-tetradecanolide (1a and 1b). Macrocyclic phosphonates **1a** and **1b** were synthesized as outlined previously by Pungente et al.[8] The products were purified by flash column chromatography using 3:1 cyclohexane and ethyl acetate to afford 107 mg (45.3%) of **1a** and 37.3 mg (15.8%) of **1b**. In addition, 24.7 mg of a dimeric side product was isolated.

1-O-(*N*, *N*-Dimethylethanolamino)-1-oxo-1-phospho-13-tetradecanolide(2b). To 5.0 mL of THF in a 10 mL round bottom flask, was added *N*, *N*-dimethylethanolamine (**3**) (30 μL, 0.30 mmol) via syringe at −78°C under a N_2atmosphere. The alcohol was treated with 1.6 M *n*-BuLi (140 μL, 0.23 mmol) added via glass syringe. The alkoxide was allowed to form for 5 min before **1a** dissolved in 5 mL of THF was cannulated into the reaction vessel via a stainless steel cannula. This mixture was allowed to warm slowly to room temperature. The reaction vessel was then fitted with a condenser and allowed to reflux for 5 h. The THF was then removed under reduced pressure and the crude was purified by flash column chromatography (5% MeOH in CH_2Cl_2) to afford 30 mg (60%) of **2b** as a colourless oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.50 (*m*, 1H), 4.14 (*m*, 2H), 2.59 (*m*, 2H), 2.29 (*s*, 6H), 1.81 (*m*, 2H), 1.22−1.68 (*m*, 20H), 1.39 (*d*, $J = 6.2$ Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 73.93, 63.75, 59.53, 45.80, 36.87, 27.24, 26.53, 26.17, 26.08, 25.81, 25.06, 24.96, 24.72, 22.51, 22.40, 22.54; ^{31}P NMR (81 MHz, $CDCl_3$) δ: 29.8; LC-MS calcd. for $C_{17}H_{36}NO_3P$: 333.2, found 334.2 ($M^+ + 1$).

1-O-(*N*, *N*-Dimethylethanolamino)-1-oxo-1-phospho-13-tetradecanolide(2a). Alcohol **3** (14 μL, 0.14 mmol) was added via syringe to 4 mL of THF in a 10 mL round bottom flask at −78°C under a N_2 atmosphere. The alcohol was treated with 1.6 M *n* -BuLi (64 μL, 0.10 mmol) added to the reaction vessel via glass syringe. The alkoxide was allowed to form for 5 min before **1b** dissolved in 4 mL of THF and was cannulated into the reaction vessel via a stainless steel cannula. This mixture was allowed to warm slowly to room temperature. The reaction vessel was then fitted with a condenser and allowed to reflux for 5 h. The THF was then removed under reduced pressure and the crude was purified by flash column chromatography (5% MeOH in CH_2Cl_2) to afford 17 mg (73%) of **2a** as a colourless oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.64 (*m*, 1H), 4.10(*m*, 1H), 4.05 (*m*, 1H), 2.58 (*m*, 2H), 2.28 (*s*, 6H), 1.80 (*t*, 1H), 1.77 (*t*, 1H), 1.20−1.70 (*m*, 20H), 1.34 (*d*, $J = 6.2$ Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 73.45, 62.82, 59.34, 45.81, 37.20, 27.84, 26.52, 26.17, 26.00, 25.84, 25.80, 25.31, 25.02, 23.14, 22.59, 21.45; ^{31}P NMR (81 MHz, $CDCl_3$) δ: 31.0; LC-MS calcd. for $C_{17}H_{36}NO_3P$: 333.2, found 334.2 ($M^+ + 1$).

CONCLUSIONS

The synthesis and derivatization of two diastereomeric macrocyclic phosphonates resulted in the production of two novel phosphonate lipids, **2a** and **2b**. The characterization of **2a** and **2b** by Mass Spectrometry, ^{1}H and ^{31}P NMR supports the proposed structure and stereochemistry of these lipids. Critical micelle concentration experiments demonstrated the ability of these novel phosphonate lipids to self-assemble, while the gel retardation assays reveal that the two novel macrocyclic lipids, **2a** and **2b**, achieved efficient plasmid DNA complexation.

COMPETING INTERESTS:

The authors of this work have no competing interests.

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