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Short communication

Synthesis and evaluation of new phosphonolipid compounds for gene delivery

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Abstract

The preparation of a series of novel water soluble cationic lipid derivatives possessing phosphonate ester groups linked to the *para*-position of *N*-methyl pyridinium moieties and bearing either identical or different alkyl chains is reported. The obtained phospholipids were tested for transfection efficiency into three different mammalian cell lines alone and in conjunction with diphytanoylphosphatidylethanolamine (DiPPE) or dioleylphosphatidylethanolamine (DOPE), using an assay adapted for 96-well microplates based on the detection of a colorimetric change caused by the production of a chromogen induced by expressed secreted human placental alkaline phosphatase. In our conditions, the highest transfection activities of cells HEK293 and hard-to-transfect cell lines B16 and CHO were achieved with a 4-phosphonobutylpyridinium compound used at 1:5, 1:10 or 3:6 DNA/lipid ratio bearing two myristyl chains in the presence of the fusogenic helper lipid DiPPE.

Keywords: Transfection; Cationic lipid; Non-viral gene transfer

1. Introduction

Gene therapy constitutes a mode of treatment of genetic disorders in which genes are inserted into eukaryotic cells [1], and to date over 1340 gene therapy clinical trials have been completed or approved in different countries, using over 100 genes [2]. One of the major problems of gene therapy is the efficiency of introduction and stabilization of nucleic acids in the target cells, which are currently ensured by gene transfer systems based on viral [3] or synthetic non-viral vectors [4]. Delivery systems based on non-viral vectors, such as cationic lipids or cationic polymers, are presently less efficient than viral systems, which have been used in the two-thirds of the therapy clinical trials performed to date [2]. Nevertheless, non-viral systems have proved to be generally less toxic or immunogenic, more easily produced, and to exhibit a greater stability. Cationic lipids, like other non-viral gene transfer vectors, interact with the negatively charged phosphate backbone of nucleic acids to form a compact structure able to enter a cell. A number of cationic lipids have been synthesized, including monovalent and polyvalent cationic lipids, cholesterol derivatives or guanidine-containing compounds, some of them being now commercially available, such as DOTMA

Abbreviations: DMEM, Dulbecco's modified eagle's medium; FCS, Foetal calf serum; DOTAP, 1,2-Dioleoyl-3-trimethylammonium propane; DOTMA, *N*-(2,3-Dioleoyloxy)propyl-*N*,*N*,*N*-trimethylammoinum chloride; DC-Chol, 3β[*N*-(*N*',*N*'-Dimethylaminoethane)carbamoyl]cholesterol; DOGS, 5-Carboxyspermylglycine dioctadecylamide; DOSPA, 2,3-Dioleoyloxy-*N*-{2-[1,4-bis(3-aminopropymino)-2-butylcarboxamido]ethyl}-*N*,*N*-dimethyl-1-propylammonium bromide; CHO, Chinese hamster ovarian adenocarcinoma; B16, Mouse melanoma; C2C12, Mouse muscle myoblasts; HepG2, Human hepatoblastoma; GFP, Green fluorescent protein; DOPE, 1,2-Dimyristoyl-*sn*-glycero-3-phosphoethanolamine; DiPPE, 1,2-Diphytanoyl-*sn*-glycero-3-phosphoethanolamine; SEAP, Secreted embryonic alkaline phosphatase.

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(Lipofectin®) [5], DOSPA (LipofectAMINE®) [6], DOTAP [7], DC-Chol [8] or DOGS (Transfectam®) [9]. All these cationic lipids carry a DNA-interacting head group with a net positive charge in physiological conditions or at the lower pHs found in the endosome environment, a hydrophobic lipid anchor group such as cholesterol or fatty acid chains of various lengths and unsaturation states, and a linker group that binds the polar group to the lipidic moiety. Spacer groups are commonly ester [7], amide [9] or urethane [10] groups, but other groups such as phosphate [9] or phosphonate [11] have also been employed. The positively charged polar groups commonly used are also of various natures, including quaternary ammonium ions, polycations such as spermine, or cationic polymeric compounds such as polyethylenimine, poly-lysine or their derivatives. In 1997, a series of double-chained pyridinium compounds were described by van der Woude et al. that displayed interesting transfection properties, and were found to be essentially non-toxic toward cells [12]. In this work, we report the synthesis and the evaluation of new pyridinium-based phosphonolipids, bearing either two identical or two different alkyl chains, as transfection agents. The lipid chains C_{14}-saturated (myristyl), C_{18}-unsaturated (oleyl) and phytanyl moieties were examined (Fig. 1).

2. Results

2.1. Chemistry

2.1.1. Synthesis of dialkylphosphite compounds

Phosphite derivatives bearing two identical chains (homodialkylphosphites) were obtained by direct esterification of phosphorus trichloride by the corresponding alcohol, as previously described [11]. On the other hand, the unsymmetrical introduction of two different alkyl chains leading to heterodialkylphosphites has not been reported. Consequently, a procedure for their preparation was developed, based on two successive transesterification reactions of commercially available diphenylphosphite (Fig. 2) [13].

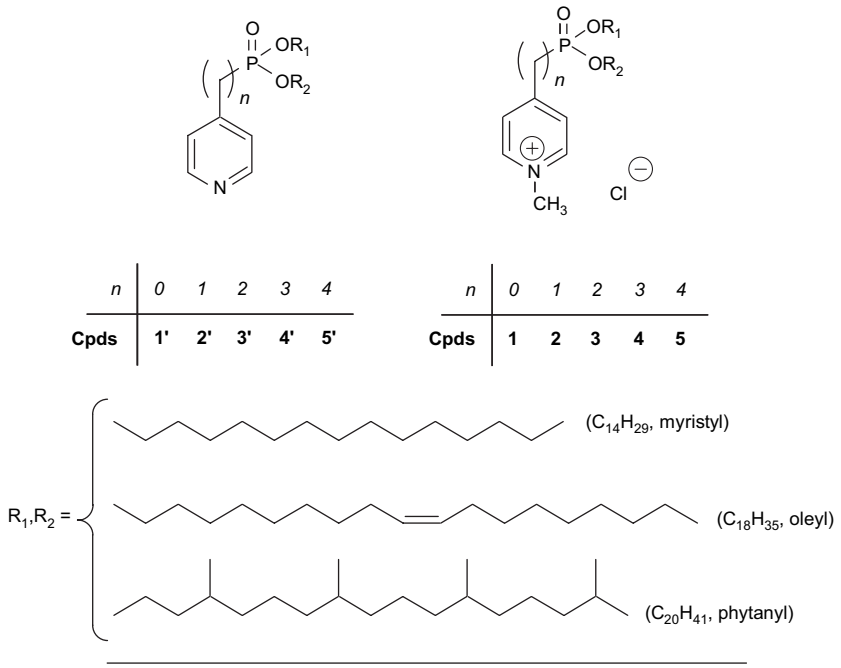
2.1.2. Synthesis of pyridine (*1′–5′*) and N-methyl-pyridinium compounds (*1–5*)

2.1.2.1. General strategy. The appropriate pyridine derivatives **1′**, **2′** and **4′** (Schemes 1, 2 and 4, respectively) were obtained using a Michaelis–Becker type coupling reaction between the *H*-phosphonate anion of a homo- or heterodialkylphosphite, and pyridine or a halogenated pyridine. Due to the instability of 4-(chloropropyl)pyridine, compounds **4′** were obtained in poor yields, and only compound **4a** was assessed as a vector for transfection. For **3′**, a similar strategy was used with 4-vinylpyridine as electrophile instead of a halogenated pyridine (Scheme 3). However, as for compounds **4′**, they were obtained in poor yields and only compound **3a** was assessed. The butylated pyridine compounds **5′** were synthesized by addition of the carbanion of 4-methyl pyridine to dialkyl-3-bromopropylphosphonates (Scheme 5). Treatment of all pyridylphosphonates **1′–5′** by methyl iodide, followed by anion exchange using Dowex (Cl^-) gave the *N*-methyl pyridinium chloride compounds **1–5**, respectively. All spectrophotometric and spectroscopic data were consistent with the assigned structures.

*2.1.2.2. Synthesis of 4-(dialkylphosphono)-1-methyl pyridinium compounds (**1**, n = 0, Fig. 1)*. Several methods for the synthesis of diethyl-4-pyridylphosphonate compounds have been reported such as the reaction of 4-bromopyridine hydrochloride with diethylphosphite in the presence of triethylamine and tetrakis(triphenylphosphine)palladium as catalyst [14]. Here, compounds **1** were obtained in two steps, as depicted in Scheme 1, with overall yields ranging from 19–33%. 4-Pyridylphosphonate intermediates (**1′**) were first prepared with a one-pot procedure following a modified method described by Kers and Stawinski [15]. This method involves the transient *in situ* formation of stable *N*-protected dihydropyridine intermediates by means of trityl chloride, which allow a regiospecific nucleophilic attack on the 4-position of the pyridine ring by the *H*-phosphonate anion of the corresponding dialkylphosphite, followed by a spontaneous rearomatization of the pyridine ring with loss of the trityl moiety. In our experimental conditions, DBU was used as base to form the phosphanion. In a second step, quaternarization by methyl iodide afforded the *N*-methyl pyridinium chloride compounds **1**.

*2.1.2.3. Synthesis of 4-(1-(dialkylphosphono)methyl)-1-methyl pyridinium compounds (**2**, n = 1, Fig. 1)*. Compounds **2** were synthesized in two steps from commercially available 4-(chloromethyl)pyridine hydrochloride (Scheme 2). Pyridine intermediates **2′** were first prepared using a protocol previously described for the synthesis of the diethylphosphono derivative: 4-(chloromethyl)pyridine hydrochloride was reacted with 1.5 eq. of the sodium salt of dialkylphosphite in THF to afford the corresponding (dialkylphosphono)methyl pyridine intermediates **2′**, with yields ranging from 22 to 38%. These intermediates **2′** were then N-alkylated by methyl iodide to yield the *N*-methyl pyridinium chloride compounds **2**.

*2.1.2.4. Synthesis of 4-(2-(dialkylphosphono)ethyl)-1-methyl pyridinium compounds (**3**, n = 2, Fig. 1)*. Attempts to synthesize the esters of 2-(pyridyl)ethylphosphonic acids **3′** either from 4-picoline in the presence of halogenomethyl dialkylphosphonate or from the reaction of 4-(halogenomethyl)pyridine with a methyl dialkylphosphonate derivative failed, leading to a complex mixture in the former case, and affording the autocondensation product of the methylphosphonate derivative as major product in the latter case. Nevertheless, one member of the series was obtained with a satisfactory yield (compound **3′a**, $R_1 = R_2 = C_{14}H_{29}$) using a method described by Maruszewska-Wieczorkowska and Michalski [16] based on nucleophilic addition of the corresponding dialkylphosphite on 4-vinylpyridine, known to behave as a typical α,β-unsaturated compound, in the presence of sodium ethanoate (Scheme 3). However, this compound was found to readily undergo retroaddition, and thus to be rather instable in our experimental



Compounds	*n*	R_1	R_2
1'a, 1a	*0*	$C_{14}H_{29}$	$C_{14}H_{29}$
1'b, 1b		$C_{18}H_{35}$	$C_{18}H_{35}$
1'c, 1c		$C_{20}H_{41}$	$C_{20}H_{41}$
1'd, 1d		$C_{18}H_{35}$	$C_{14}H_{29}$
1'e, 1e		$C_{20}H_{41}$	$C_{14}H_{29}$
1'f, 1f		$C_{20}H_{41}$	$C_{18}H_{29}$
2'a, 2a	*1*	$C_{14}H_{29}$	$C_{14}H_{29}$
2'b, 2b		$C_{18}H_{35}$	$C_{18}H_{35}$
2'c, 2c		$C_{20}H_{41}$	$C_{20}H_{41}$
2'd, 2d		$C_{18}H_{35}$	$C_{14}H_{29}$
2'e, 2e		$C_{20}H_{41}$	$C_{14}H_{29}$
2'f, 2f		$C_{20}H_{41}$	$C_{18}H_{35}$
3'a, 3a	*2*	$C_{14}H_{29}$	$C_{14}H_{29}$
4'a, 4a	*3*	$C_{14}H_{29}$	$C_{14}H_{29}$
5'a, 5a	*4*	$C_{14}H_{29}$	$C_{14}H_{29}$
5'b, 5b		$C_{18}H_{35}$	$C_{18}H_{35}$
5'c, 5c		$C_{20}H_{41}$	$C_{20}H_{41}$
5'd, 5d		$C_{18}H_{35}$	$C_{14}H_{29}$
5'e, 5e		$C_{20}H_{41}$	$C_{14}H_{29}$
5'f, 5f		$C_{20}H_{41}$	$C_{18}H_{35}$

Fig. 1. Structures of pyridine and pyridinium chloride phosphonolipid compounds.

conditions. Alkylation of **3′a** with methyl iodide gave the corresponding methyl pyridinium.

*2.1.2.5. Synthesis of 4-(3-(dialkylphosphono)propyl)-1-methyl pyridinium compounds (**4**, n = 3, Fig. 1).* Recently, a convenient synthesis for phosphonate compounds from dialkylphosphites and alkyl halides has been reported using cesium carbonate [17]. Using these reported conditions, we successfully synthesized, but in poor yields, the ester of 2-(pyridyl)propylphosphonic acid **4′a** starting from 4-(chloropropyl)pyridine,

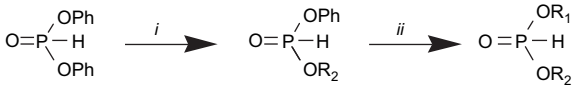


Fig. 2. Synthesis of dialkylphosphite compounds: (*i*) pyridine (0.8 eq.), R_1OH (0.8 eq.), 2 h; (*ii*) pyridine (0.8 eq.), R_2OH (0.8 eq.), 3 h.

prepared from 4-pyridinepropanol, and the corresponding dimyristyl phosphite (Scheme 4). Alkylation using methyl iodide of intermediate **4′a** gave the desired pyridinium compound **4a**.

*2.1.2.6. Synthesis of 4-(4-(dialkylphosphono)butyl)-1-methyl pyridinium compounds (**5**, n = 4, Fig. 1).* Esters of 2-(pyridyl)-butylphosphonic acids (**5′**) were synthesized as outlined in Scheme 5 according to an established procedure based on the nucleophilic attack of the carbanion of 4-methyl pyridine, generated with lithium diisopropylamide, to the appropriately dialkyl-3-bromopropylphosphonate derivatives, readily obtained in three steps from commercially available 1,3-dibromopropane. The pyridyl intermediates were then alkylated by methyl iodide to yield the corresponding methyl pyridinium compounds **5**.

2.2. Transfection properties of phosphonolipids

To evaluate transfection activities of the phosphonolipids, a rapid colorimetric assay adapted for a 96-well microplate was developed that greatly decreases the analysis time over traditional transfection assays. This spectrophotometric assay, based on the quantification of SEAP activity in supernatants of cell cultures by means of a colorimetric enzyme assay (QUANTI-Blue™, InvivoGen), was validated by comparison to a classical method based on SEAP-catalyzed *p*-nitrophenyl phosphate hydrolysis.[1] At the same time, the efficacy of transfection was also assessed visually by looking at GFP-expressing cells with a fluorescence microscope (data not shown), and the toxicity of each lipid/DNA complex was roughly estimated by examination of monolayer confluency. Transfection was examined in hard-to-transfect Chinese hamster ovary (CHO) cells, and transfected DNA consisted of a plasmid (pVITRO14, InvivoGen) encoding green fluorescent protein (GFP) and secreted human placental alkaline phosphatase (SEAP). Phosphonolipids were assayed at various DNA/lipid ratios (1:5, 1:10 and 3:6, w:w) either alone or in the presence of neutral lipid helpers dioleylphosphatidylethanolamine (DOPE) or diphytanoylphosphatidylethanolamine (DiPPE). As control, FuGENE® 6 (Roche) was used.[2] Fig. 3 depicts SEAP activity of cells CHO treated with selected phosphonolipids at different DNA/lipid ratios in the presence or the absence of DiPPE or DOPE. All other compounds (**1a–d**, **2b**, **2d–f**, **3a**, **4a** and **5c–f**) were found to be inactive or poorly active as vectors of transfection. As shown in Fig. 3, except for compounds **1e** and **2c**, the absence of lipid helpers dramatically reduced the transfection activity. Overall, phosphonolipids that comprise two identical lipid chains were found to induce transfection of CHO cells more efficiently than those bearing two different chains (not shown). Unlike the other members of the series, compound **2f**, bearing two different alkyl chains (phytanyl and oleyl), was found to display suitable transfection activities in the presence of DOPE, comparable to that of FuGENE® 6, but only at a DNA/lipid ratio of 1/10. The most effective compound on CHO cells was 4-(4-(ditetradecanylphosphono)butyl)-1-methyl pyridinium chloride (**5a**) in combination with lipid helper DiPPE, and to a lesser extent with DOPE, the best results being obtained for a DNA/lipid ratio of 3/6 (Fig. 3), and no toxicity for this compound was evidenced in our experimental conditions. The mixture **5a**/DIPPE has also been tested and proved to be effective on human kidney HEK 293 cells and other hard-to-transfect cell lines, such as mouse melanoma B16 cells and mouse myoblast C2C12 cells.[3] Surprinsingly, compound **5b** with two oleyl chains exhibited satisfactory activities in the presence of lipid helpers at DNA/lipid ratios of 1/5 and 3/6, but totally lost its transfection capacity at a ratio of 1/10.

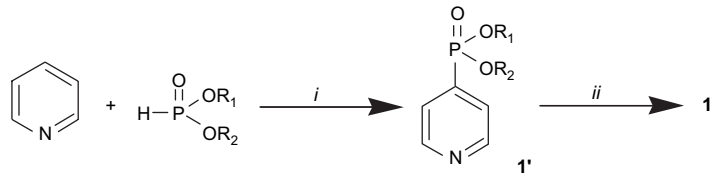
3. Discussion

Cationic lipids have been widely used as a safe non-viral method of delivery of nucleic acids both in vitro and in vivo, but the mechanism by which DNA is transfected is complex and not well understood. For this purpose, a number of cationic lipids have been synthesized, some of them have already been used in the clinical setting, having advantage of safety and non-immunogenesity over viral vectors, but the problems of low efficiency and cytoxicity have not been solved yet. The aim of this study was to extend previous work on cationic lipid compounds as non-viral vectors for gene delivery by investigating a series of new pyridinium-based phosphonolipids, including for the first time compounds bearing two different alkyl chains that would enhance the understanding of the structure-function relationship in designing such compounds. Multiple factors influence the delivery process including the physical structure and stability of the lipid/DNA complex and its interaction with the cell membrane. Moreover, transfection activities of the complexes are cell type dependent since the cellular membrane varies with

[1] For the detection of SEAP, QUANTI-Blue™ (InvivoGen) solution has been compared to a classical method based on *p*NPP hydrolysis: purified human alkaline phosphatase (Calbiochem) has been serially diluted in DNEM and measured spectrophotometrically with SEAP Reporter Assay Kit (InvivoGen) at 405 nm or QUANTI-Blue™ (InvivoGen) at 655 nm (see Supplementary data associated with this article).

[2] FuGENE® 6 transfection reagent is a proprietary secret mixture of lipids and other components supplied in 80% ethanol by Roche that has been shown to form a complex with DNA and to transport it into animal cells, and to transfect many common cell types, including HeLa, NIH 3T3, COS-1, COS-7, and CHO-K1.

[3] On HEK293 cells, compound **5a** was found to display transfection activities in the absence or presence of lipid helpers DiPPE or DOPPE comparable to that of our reference FuGENE® 6 at both 1/6 and 1/12 DNA/lipid ratios. Comparable transfection activities were also observed on B16 cells and C2C12 cells in the presence of lipid helpers DiPPE or DOPPE, but only for a 1/12 DNA/lipid ratio, and strongly decreased for a 1/6 DNA/lipid ratio. In the absence of lipid helper, a significantly (three-fold) higher activity than that of FuGENE® 6 for B16 cells transfection was observed.



Scheme 1. Synthesis of 4-(dialkylphosphono)-1-methyl pyridinium chloride: (*i*) dialkylphosphite (1 eq.), trityl chloride (1.2 eq.), DBU (2.4 eq.), pyridine, 6 h (RT); (*ii*) methyl iodide (4 eq.), acetone, 1 h (RT); Dowex® ion exchange (Cl^-) resin.

cell type and cycle. It is therefore important to understand the factors governing the relationship between the lipid structure and the activity of transfection. The alkyl chains (length, degree of saturation) and the linker are, for instance, important elements influencing the transfection reagent structure, as well as the nature of the head group that can also dramatically alter the charge interactions with DNA and consequently the transfection capability of the final DNA/lipid complex. Independently of the chemical structure, other parameters like the DNA/lipid ratio influence the transfection activity of the lipid compounds, as observed with compound **5b**. Likewise, the presence of lipid helper plays also a crucial role for transfection. In this work, we prepared a series of new pyridinium-based phosphonolipids, bearing two identical or different alkyl chains, and some of them were found to exhibit good transfection activities. However, the results of the current study show that no significant correlation can be observed between structure and transfection properties, and thus support the notion that transfection activity strongly depends on complex multifactorial components. Consequently further work will be needed to understand the structure–function relationships of these compounds. One of the compounds (**5a**) was found to display a higher transfection efficiency of mammalian cells in the presence of lipid helpers than the commercially available gene delivery lipid FuGENE® 6, and thus have a great potency as vector for gene delivery. Moreover, considering the importance of cationic lipids for gene delivery, compounds bearing two lipid chains of different nature have received surprisingly little attention. Here, we showed that such compounds also display significant transfection activities, and would seem to merit further investigation.

4. Experimental protocols

4.1. Chemistry

All chemicals were obtained from Sigma–Aldrich and used without further purification. Silica gel 60 (200–400 mesh, Merck) was used for column flash chromatography, and silica gel plates 60F-254 nm (0.2-mm thickness, Merck) were used for thin layer chromatography. Nuclear magnetic resonance spectra were recorded on a Brucker AC 300. Mass spectrometry was performed on a quadripolar Nermag R10-10C spectrometer.

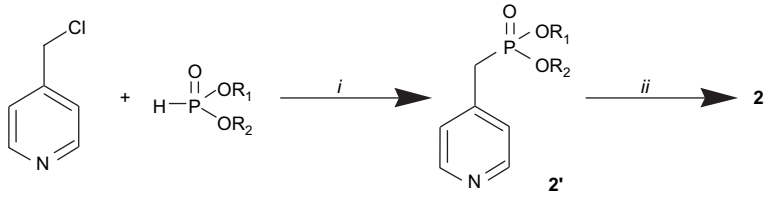
4.2. General procedure for the preparation and characterization of selected compounds (*1e*, *1f*, *2a*, *2c*, *2f*, *5a*, *5b*)

4.2.1. Synthesis of compounds *1*

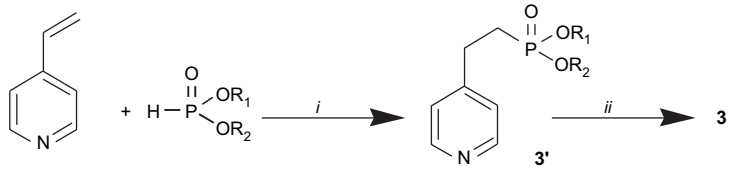
To a solution of phosphite (1 mmol) in pyridine (10 mL) were added trityl chloride (1.2 mmol) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (2.4 mmol), successively. After 6 h under magnetic stirring at room temperature, the solvent was evaporated under reduced pressure. The residue was dissolved in dichloromethane, and washed (3×) with a 5% aqueous solution of sodium hydrogenocarbonate. The organic layer was dried on magnesium sulfate, and evaporated to dryness under reduced pressure. The residue was then column-purified on silica gel using a mixture of heptane and diethyl ether (1:1) then ethyl ether as eluent to obtain pyridine compounds **1′**.

*4.2.1.1. Compound **1′e***. Yield, 50% (yellow oil). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 0.80–0.87 (m, 24 H), 0.90 (d, 6H, $^3J_{H-H} = 6.0$ Hz), 0.98–1.78 (m, 48H), 3.96–4.10 (m, 4H), 7.58 (dd, 2H, $^3J_{H-H} = 6.0$ Hz, $^3J_{H-P} = 15.0$ Hz), 8.70 (t, 2H, $^3J_{H-H} = 6.0$ Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 14.0, 19.5, 19.7, 22.6, 22.7, 22.8, 24.4, 24.5, 24.8, 26.1, 28.0, 29.1–29.6, 30.6 (d, $^3J_{C-P} = 6.4$ Hz), 31.9, 32.8, 37.0–37.8, 39.4, 65.2 (d, $^2J_{C-P} = 6.3$ Hz), 66.8 (d, $^2J_{C-P} = 6.2$ Hz), 125.5 (d, $^2J_{C-P} = 7.5$ Hz) 137.5 (d, $^1J_{C-P} = 186.4$ Hz), 150.1 (d, $^3J_{C-P} = 12.8$ Hz). ^{31}P NMR (81 MHz, $CDCl_3$) δ (ppm) 14.8. ES-MS (*m/z*): 658 (MNa^+).

*4.2.1.2. Compound **1′f***. Yield, 40% (yellow oil). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 0.80–0.87 (m, 15H), 0.90 (d, 3H, $^3J_{H-H} = 6.1$ Hz), 1.00–1.77 (m, 48H), 1.89–2.08 (m, 4H), 3.11 (d, 2H, $^2J_{H-P} = 22.3$ Hz), 3.89–4.09 (m, 4H),



Scheme 2. Synthesis of 4-(1-(dialkylphosphono)methyl)-1-methyl pyridinium chloride: (*i*) a – NaH (2.5 eq.), dialkylphosphite (1.5 eq.), THF, 30 min (RT); b – 4-(chloromethyl)pyridine hydochloride (1 eq.), 18 h (reflux); (*ii*) methyl iodide (4 eq.), acetone, 1 h (RT); Dowex® ion exchange (Cl^-) resin.



Scheme 3. Synthesis of 4-(2-(dimyristylphosphono)ethyl)-1-methyl pyridinium chloride (**3a**, $R_1 = R_2 = C_{14}H_{29}$): (*i*) Na (0.15 eq.), ethanol, 18 h (50 °C); (*ii*) methyl iodide (4 eq.), acetone, 1 h (RT); Dowex® ion exchange (Cl^-) resin.

5.29–5.43 (m, 4H), 7.58 (dd, 2H, $^3J_{H-H} = 6.8$ Hz, $^3J_{H-P} = 15.7$ Hz), 8.72 (t, 2H, $^3J_{H-H} = 6.8$ Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 14.0, 19.5, 19.7, 22.6, 22.7, 22.8, 24.4, 24.5, 24.8, 25.6, 27.2, 28.0, 29.1–29.6, 29.4, 30.7 (d, $^3J_{C-P} = 5.9$ Hz), 31.9, 32.8, 33.2 (d, $^1J_{C-P} = 146.0$ Hz), 37.0–37.8, 39.5, 65.2 (d, $^2J_{C-P} = 6.1$ Hz), 66.8 (d, $^2J_{C-P} = 6.7$ Hz), 125.3 (d, $^2J_{C-P} = 7.5$ Hz), 129.9, 129.8, 137.6 (d, $^1J_{C-P} = 186.4$ Hz), 159.9 (d, $^3J_{C-P} = 12.8$ Hz). ^{31}P NMR (81 MHz, $CDCl_3$) δ (ppm) 14.6. ES-MS (*m/z*): 712 (MNa^+).

Methyl iodide (1.2 mmol) was added to a solution of **1′** (0.4 mmol) in dry acetone (5 mL) under magnetic stirring at room temperature. After 1 h, the solvent was removed under reduced pressure and the residue was purified by chromatography on alumine (act IV) (dichloromethane/methanol, 98:2), and on ion exchange resin (Dowex 1X8-200 Cl^-) with methanol as eluent. Recrystallization from a mixture of acetonitrile and acetone gave pure pyridinium chloride compounds **1**.

*4.2.1.3. Compound **1e***. Yield, 60% (pale yellow solid). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 0.80–0.87 (m, 24H), 0.90 (d, 6H, $^3J_{H-H} = 6.1$ Hz), 0.98–1.78 (m, 48H), 4.09–4.30 (m, 4H), 4.90 (s, 3H), 8.21–8.32 (m, 2H), 9.70–9.87 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 14.0, 19.5, 19.7, 22.6, 22.7, 22.8, 24.4, 24.5, 24.8, 26.1, 28.0, 29.1–29.6, 30.6 (d, $^3J_{C-P} = 6.4$ Hz), 31.9, 32.8, 37.0–37.8, 39.4, 49.7, 65.2 (d, $^2J_{C-P} = 6.3$ Hz), 66.8 (d, $^2J_{C-P} = 6.7$ Hz), 129.9 (d, $^2J_{C-P} = 5.7$ Hz), 146.2 (d, $^1J_{C-P} = 186.0$ Hz), 146.1 (d, $^3J_{C-P} = 10.3$ Hz). ^{31}P NMR (81 MHz, $CDCl_3$) δ (ppm) 8.1. ES-MS (*m/z*): 650 (M^+-Cl^-).

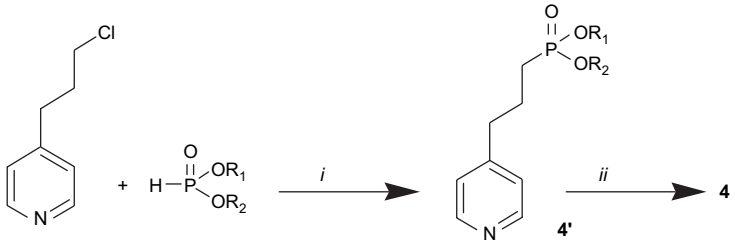
*4.2.1.4. Compound **1f***. Yield, 55% (pale yellow solid). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 0.80–0.87 (m, 15H), 0.90 (d, 3H, $^3J_{H-H} = 6.1$ Hz), 1.00–1.77 (m, 48H), 1.89–2.08 (m, 4H), 4.07–4.29 (m, 4H), 4.91 (s, 3H), 5.29–5.43 (m, 4H), 8.26–8.35 (m, 2H), 9.71–9.86 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 14.0, 19.5, 19.7, 22.6, 22.7, 22.8, 24.4, 24.5, 24.8, 25.6, 27.2, 28.0, 29.1–29.6, 29.4, 30.7 (d, $^3J_{C-P} = 5.9$ Hz), 31.9, 32.8, 33.2 (d, $^1J_{C-P} = 146.0$ Hz), 37.0–37.8, 39.5, 49.6, 65.2 (d, $^2J_{C-P} = 6.3$ Hz), 66.8 (d, $^2J_{C-P} = 6.8$ Hz), 129.9 (d, $^3J_{C-P} = 5.8$ Hz), 146.2 (d, $^1J_{C-P} = 186.3$ Hz), 146.1 (d, $^3J_{C-P} = 10.8$ Hz). ^{31}P NMR (81 MHz, $CDCl_3$) δ (ppm) 8.3. ES-MS (*m/z*): 704 (M^+-Cl^-).

*4.2.2. Synthesis of compounds **2***

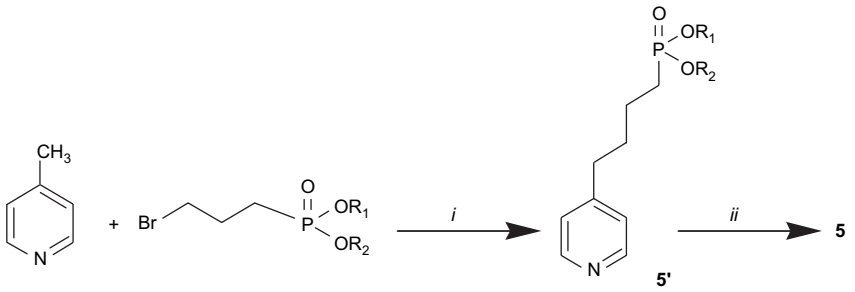
The adequate phosphite (0.8 mmol) compound was slowly added to a suspension of sodium hydride (1.33 mmol) in dry THF (8 mL), and the solution was stirred at room temperature for 1 h. 4-(Chloromethyl)pyridine (0.53 mmol) was then added to this solution, and the mixture was heated at reflux for 18 h. After cooling, water was added, and the solution was extracted with ethyl acetate (3×). The organic layer was then dried over magnesium sulfate, and evaporated to dryness. The crude product was purified by chromatography on silica gel (ethyl ether, then ethyl acetate as eluent) to afford pyridine compounds **2′**.

*4.2.2.1. Compound **2′a***. Yield, 38% (pale yellow solid). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 0.87 (t, 6H, $^3J_{H-H} = 6.6$ Hz), 1.20–1.45 (m, 44H), 1.60–1.70 (m, 4H), 3.07 (d, 2H, $^2J_{H-P} = 21.0$ Hz), 3.93 (q(td), 4H, $^3J_{H-H} = {}^3J_{H-P} = 6.0$ Hz), 7.18–7.20 (m, 2H), 8.46–8.52 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 14.0, 22.6, 25.5, 29.1–29.6, 30.6 (d, $^3J_{C-P} = 6.4$ Hz), 31.8, 33.4 (d, $^1J_{C-P} = 146.3$ Hz), 66.4 (d, $^2J_{C-P} = 6.8$ Hz), 125.0 (d, $^3J_{C-P} = 6.0$ Hz), 141.4 (d, $^2J_{C-P} = 8.3$ Hz), 149.7 (d, $^4J_{C-P} = 3.0$ Hz). ^{31}P NMR (81 MHz, $CDCl_3$) δ (ppm) 24.2. ES-MS (*m/z*): 566 (MH^+).

*4.2.2.2. Compound **2′c***. Yield, 27% (yellow oil). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 0.81–0.89 (m, 24H), 0.90 (d, 6H, $^3J_{H-H} = 6.1$ Hz), 0.98–1.78 (m, 48H), 3.06 (d, 2H, $^2J_{H-P} = 22.0$ Hz), 3.82–4.03 (m, 4H), 7.15–7.28 (m, 2H), 8.42–8.61



Scheme 4. Synthesis of 4-(3-(dimyristylphosphono)propyl)-1-methyl pyridinium chloride (**4a**, $R_1 = R_2 = C_{14}H_{29}$): (*i*) a – Cs_2CO_3 (3 eq.), dialkylphosphite (1 eq.), TBAI (3 eq.), DMF, 1 h (RT); b – 4-(chloropropyl)pyridine (3 eq.), 48 h (RT); (*ii*) methyl iodide (4 eq.), acetone, 1 h (RT); Dowex® ion exchange (Cl^-) resin.



Scheme 5. Synthesis of 4-(4-(dialkylphosphono)butyl)-1-methyl pyridinium compounds: (*i*) a − BuLi (1 eq.), diisopropylamine (1 eq.), THF, 30 min (−15 °C); b − picoline (1 eq.), 15 min (−15 °C). c − R−Br (1 eq.), 18 h (RT); (*ii*) methyl iodide (4 eq.), acetone, 1 h (RT); Dowex® ion exchange (Cl^-) resin.

(m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 19.5, 19.7, 22.6, 22.7, 24.4, 24.5, 24.8, 28.0, 29.4, 32.8, 33.4 (d, $^1J_{C-P}$ = 137.3 Hz), 37.0−37.8, 39.4, 64.9 (d, $^2J_{C-P}$ = 6.0 Hz), 125.3 (d, $^3J_{C-P}$ = 6.7 Hz), 141.2 (d, $^2J_{C-P}$ = 9.0 Hz), 149.7 (d, $^4J_{C-P}$ = 2.9 Hz). ^{31}P NMR (81 MHz, $CDCl_3$) δ (ppm) 24.2. ES-MS (*m/z*): 756 (MNa^+).

*4.2.2.3. Compound **2′f***. Yield, 28% (yellow oil). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 0.80−0.87 (m, 15H), 0.90 (d, 3H, $^3J_{H-H}$ = 6.1 Hz), 1.00−1.77 (m, 48H), 1.89−2.08 (m, 4H), 3.11 (d, 2H, $^2J_{H-P}$ = 22.3 Hz), 3.94−4.05 (m, 4H), 5.29−5.43 (m, 4H), 7.15−7.30 (m, 2H), 8.46−8.63 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 14.0, 19.5, 19.7, 22.6, 22.7, 22.8, 24.4, 24.5, 24.8, 25.6, 27.2, 28.0, 29.1−29.6, 29.4, 30.7 (d, $^3J_{C-P}$ = 5.9 Hz), 31.9, 32.8, 33.2 (d, $^1J_{C-P}$ = 146.0 Hz), 37.0−37.8, 39.5, 64.9 (d, $^2J_{C-P}$ = 6.3 Hz), 66.6 (d, $^2J_{C-P}$ = 6.8 Hz), 125.1 (d, $^3J_{C-P}$ = 8.9 Hz), 129.9, 129.8, 141.5 (d, $^2J_{C-P}$ = 8.3 Hz), 149.8 (d, $^4J_{C-P}$ = 2.7 Hz). ^{31}P NMR (81 MHz, $CDCl_3$) δ (ppm) 24.2. ES-MS (*m/z*): 727 (MNa^+).

The pyridinium compounds **2** were obtained from compounds **2′** by the same procedure used for the preparation of compounds **1**.

*4.2.2.4. Compound **2a***. Yield, 60% (white solid). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 0.87 (t, 6H, $^3J_{H-H}$ = 6.6 Hz), 1.20−1.45 (m, 44H), 1.60−1.70 (m, 4H), 3.07 (d, 2H, $^2J_{H-P}$ = 21.0 Hz), 3.62 (d, 2H, $^2J_{H-P}$ = 24.0 Hz), 3.94−4.10 (m, 4H), 4.58 (s, 3H), 7.93−8.00 (m, 2H), 9.10−9.18 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 14.0, 22.6, 25.5, 29.1−29.6, 30.6 (d, $^3J_{C-P}$ = 6.4 Hz), 31.8, 33.8 (d, $^1J_{C-P}$ = 133.9 Hz), 48.6, 67.4 (d, $^2J_{C-P}$ = 6.8 Hz), 129.1 (d, $^3J_{C-P}$ = 5.8 Hz), 145.2, 152.6 (d, $^2J_{C-P}$ = 8.2 Hz). ^{31}P NMR (81 MHz, $CDCl_3$) δ (ppm) 21.4. ES-MS (*m/z*): 580 (M^+-Cl^-).

*4.2.2.5. Compound **2c***. Yield, 61% (yellow solid). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 0.81−0.89 (m, 24H), 0.90 (d, 6H, $^3J_{H-H}$ = 6.1 Hz), 0.98−1.78 (m, 48H), 3.49 (d, 2H, $^2J_{H-P}$ = 23.1 Hz), 3.96−4.18 (m, 4H), 4.68 (s, 3H), 7.88−8.09 (m, 2H), 9.19−9.30 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 19.5, 19.7, 22.6, 22.7, 24.4, 24.5, 24.8, 28.0, 29.4, 32.8, 34.3 (d, $^1J_{C-P}$ = 133.5 Hz), 37.0−37.8, 39.4, 48.5, 64.9 (d, $^2J_{C-P}$ = 6.2 Hz), 125.4 (d, $^3J_{C-P}$ = 5.7 Hz), 145.3, 152.4 (d, $^2J_{C-P}$ = 8.1 Hz). ^{31}P NMR (81 MHz, $CDCl_3$) δ (ppm) 21.2. ES-MS (*m/z*): 748 (M^+-Cl^-).

*4.2.2.6. Compound **2f***. Yield, 55% (yellow solid). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 0.80−0.87 (m, 15H), 0.90 (d, 3H, $^3J_{H-H}$ = 6.1 Hz), 1.00−1.77 (m, 48H), 1.89−2.08 (m, 4H), 3.11 (d, 2H, $^2J_{H-P}$ = 22.3 Hz), 3.49 (d, 2H, $^2J_{H-P}$ = 23.1 Hz), 3.96−4.18 (m, 4H), 4.68 (s, 3H), 5.29−5.43 (m, 4H), 7.88−8.07 (m, 2H), 9.19−9.30 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 14.0, 19.5, 19.7, 22.6, 22.7, 22.8, 24.4, 24.5, 24.8, 25.6, 27.2, 28.0, 29.1−29.6, 29.4, 30.7 (d, $^3J_{C-P}$ = 5.9 Hz), 31.9, 32.8, 34.4 (d, $^1J_{C-P}$ = 133.1 Hz), 37.0−37.8, 39.5, 48.7, 65.8 (d, $^2J_{C-P}$ = 6.2 Hz), 67.3 (d, $^2J_{C-P}$ = 6.7 Hz), 129.2 (d, $^3J_{C-P}$ = 7.5 Hz), 129.9, 129.8, 145.3, 152.4 (d, $^2J_{C-P}$ = 8.1 Hz). ^{31}P NMR (81 MHz, $CDCl_3$) δ (ppm) 21.2. ES-MS (*m/z*): 718 (M^+-Cl^-).

*4.2.3. Synthesis of compounds **5***

A 1.6 M butyllithium solution in hexane (0.43 mL) was added at −15 °C to a 0.3 M solution of diisopropylamine in dry THF (2.4 mL) and magnetically stirred for 30 min. To the LDA solution were added dropwise at −15 °C freshly distilled 4-picoline (70 μL), and after 15 min, a solution of the corresponding dialkyl-3-bromopropylphosphonate (0.7 mmol) in dry THF (5 mL). The solution was stirred overnight at room temperature, and the solvent was removed under reduced pressure. The residue was dissolved in ethyl acetate, and washed with a saturated aqueous ammonium chloride solution. The organic layer was then dried over magnesium sulfate and evaporated to dryness. Compounds **5′** were then purified by chromatography on silica gel (eluent: petroleum ether/ethyl acetate 3:2).

*4.2.3.1. Compound **5′a***. Yield, 58% (pale yellow solid). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 0.87 (t, 6H, $^3J_{H-H}$ = 6.6 Hz), 1.20−1.45 (m, 44H), 1.57−1.79 (m, 14H), 2.60 (t, 2H, $^3J_{H-H}$ = 6.7 Hz), 3.90−4.00 (m, 4H), 7.06 (d, 2H, $^3J_{H-H}$ = 5.8 Hz), 8.44 (d, 2H, $^3J_{H-H}$ = 5.8 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 14.0, 22.1 (d, $^2J_{C-P}$ = 5.3 Hz), 22.6, 25.3 (d, $^1J_{C-P}$ = 141.9 Hz), 29.1−29.6, 30.6 (d, $^3J_{C-P}$ = 6.4 Hz), 31.0 (d, $^3J_{C-P}$ = 16.6 Hz), 31.8, 34.7 (d, $^4J_{C-P}$ = 0.8

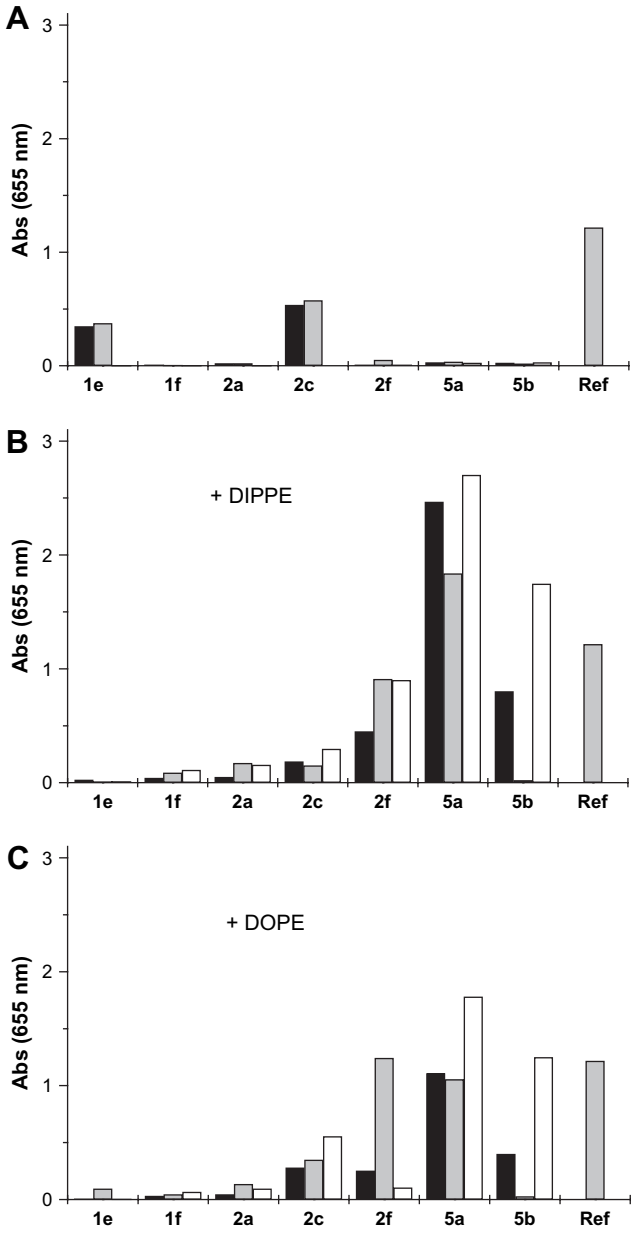


Fig. 3. CHO cells transfection activities of selected phosphonolipids without lipid helpers (**A**), and in the presence of DiPPE (**B**) or DOPE (**C**) at different lipid/DNA ratios (▪ 1/5, ▩ /10, ▫ 3/6). As control (Ref), FuGENE® 6 transfection reagent (Roche) was used. In vitro transfection experiments were performed as described in the Section 4. Each bar represents the mean of three independent measurements. For clarity, error bars are not included, but are typically about ± 0.2 Abs in size for values absorbance-values superior to 0.5.

Hz), 65.5 (d, $^{2}J_{C-P} = 6.8$ Hz), 123.8, 149.7, 150.8. ^{31}P NMR (81 MHz, $CDCl_3$) δ (ppm) 31.9. ES-MS (*m/z*): 608 (MH^+).

*4.2.3.2. Compound **5′b***. Yield, 50% (colorless oil). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 0.87 (t, 6H, $^{3}J_{H-H} = 6.6$ Hz), 1.13–1.45 (m, 44H), 1.56–1.81 (m, 14H), 2.61 (t, 2H, $^{3}J_{H-H} =$ 6.7 Hz), 3.90–4.00 (m, 4H), 5.29–5.43 (m, 4H), 7.08 (d, 2H, $^{3}J_{H-H} = 5.8$ Hz), 8.45 (d, 2H, $^{3}J_{H-H} = 5.8$ Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 14.1, 22.3 (d, $^{2}J_{C-P} =$ 5.3 Hz), 22.7, 25.5 (d, $^{1}J_{C-P} = 140.3$ Hz), 27.2, 29.1–29.6, 30.4 (d, $^{3}J_{C-P} = 5.8$ Hz), 31.0 (d, $^{3}J_{C-P} = 16.6$ Hz), 31.8, 34.7 (d, $^{4}J_{C-P} = 0.8$ Hz), 65.7 (d, $^{2}J_{C-P} = 6.7$ Hz), 123.8, 129.9, 129.8, 149.5, 150.8. ^{31}P NMR (81 MHz, $CDCl_3$) δ (ppm) 31.9. ES-MS (*m/z*): 716 (MH^+).

The pyridinium compounds **5** were obtained from compounds **5′** by the same procedure used for the preparation of compounds **1**.

*4.2.3.3. Compound **5a***. Yield, 96% (yellow solid). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 0.87 (t, 6H, $^{3}J_{H-H} = 6.6$ Hz), 1.20–1.45 (m, 44H), 1.50–1.82 (m, 14H), 2.89 (t, 2H, $^{3}J_{H-H} =$ 7.2 Hz), 3.98 (q(td), 4H, $^{3}J_{H-H} = {}^{3}J_{H-P} = 7.0$ Hz), 4.65 (s, 3H), 7.09 (d, 2H, $^{3}J_{H-H} = 6.1$ Hz), 9.10 (d, 2H, $^{3}J_{H-H} =$ 6.1 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 14.0, 22.6 (d, $^{2}J_{C-P} = 4.6$ Hz), 24.7 (d, $^{1}J_{C-P} = 140.1$ Hz), 29.1–29.6, 30.3 (d, $^{3}J_{C-P} = 18.0$ Hz), 31.8, 35.7, 48.9, 66.4 (d, $^{2}J_{C-P} =$ 6.8 Hz), 130.7, 145.0, 161.4. ^{31}P NMR (81 MHz, $CDCl_3$) δ (ppm) 31.2. ES-MS (*m/z*): 662 (M^+-Cl^-).

*4.2.3.4. Compound **5b***. Yield, 30% (colorless oil). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 0.87 (t, 6H, $^{3}J_{H-H} = 6.6$ Hz), 1.30–1.45 (m, 44H), 1.50–1.82 (m, 14H), 2.62 (t, 2H, $^{3}J_{H-H} =$ 6.8 Hz), 3.91–4.07 (m, 4H), 4.65 (s, 3H), 5.29–5.43 (m, 4H), 7.09 (d, 2H, $^{3}J_{H-H} = 6.1$ Hz), 8.48 (d, 2H, $^{3}J_{H-H} =$ 6.1 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 14.1, 22.6 (d, $^{2}J_{C-P} = 4.6$ Hz), 22.7, 25.1 (d, $^{1}J_{C-P} = 139.7$ Hz), 25.5, 27.2, 29.0–29.6, 30.4 (d, $^{3}J_{C-P} = 5.8$ Hz), 30.6 (d, $^{3}J_{C-P} =$ 6.4 Hz), 30.7 (d, $^{3}J_{C-P} = 16.5$ Hz), 31.8, 35.7, 48.9, 66.4 (d, $^{2}J_{C-P} = 6.8$ Hz), 129.9, 129.8, 130.7, 145.0, 161.4. ^{31}P NMR (81 MHz, $CDCl_3$) δ (ppm) 31.2. ES-MS (*m/z*): 730 (M^+-Cl^-).

4.3. Transfection evaluation

Transfection assay were performed as follows: phosphonolipids (5 mg/mL in ethanol) were tested alone or mixed (vol/vol) with DOPE or DIPPE (5 mg/mL in ethanol). Lipid/DNA complexes were prepared as following: lipid was first diluted in DMEM and incubated for 5 min at room temperature. Diluted lipid was added to plasmid DNA (pVITRO14 LGFP hSEAP; 0.5 mg/ml in water), at various DNA/lipid ratio (w/w, 1:5, 1:10 and 3:6) and incubated for 15 min at room temperature. FuGENE® 6 (Roche) was used as control according to manufacturer's instructions. Cells (HEK 293, B16 and CHO) were obtained from ATCC and MEF cells were purified from mouse embryos; all were grown in the recommended medium. For the transfection assay, cells were washed and resuspended at 1.25×10^5 cells/ml in DMEM containing 10% heat-inactivated FCS, and 200 μL (25 000 cells) of cell suspension was added to each well of a flat-bottomed 96-well plate. Lipid/DNA complexes (10 μL/well) were then dropped onto the cells and the plates were incubated at 37 °C in a CO_2 incubator for 48 h (for a more detailed protocol on transfection, see Supplementary data associated with this article). Toxicity was evaluated by examination of the monolayer confluency. The efficacy of transfection was assessed visually by looking at GFP-expressing cells with a fluorescence microscope or spectrophotometrically by quantifying the amount of

SEAP released by transfected cells using QUANTI-Blue™ (InvivoGen): 20 µl of supernatant were mixed with 180 µl of QUANTI-Blue™ solution followed by 3 h incubation at 37 °C and the absorbance was measured at 655 nm.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ejmech.2007.11.002.

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