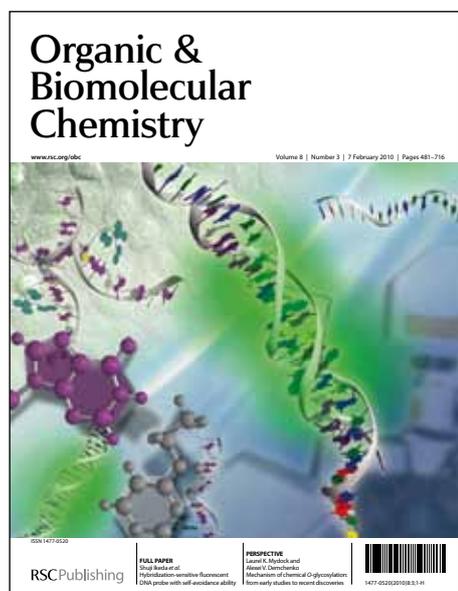


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ARTICLE

Cationic lipophosphoramidates with two different lipid chains: synthesis and evaluation as gene carriers.

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Cationic lipids constitute a family of synthetic vectors commonly used for nucleic acids delivery. We herein report the results of a systematic study that aimed to compare the transfection efficacies of cationic lipophosphoramidates possessing either two identical lipid chains (termed symmetric cationic lipids) or two different lipid chains (non-symmetric cationic lipids). In addition, we also compared the transfection results of such a ‘molecular approach’ (the two different lipid chains being included in the same molecule) with those of a ‘supramolecular approach’ in which two types of symmetrical cationic lipids were mixed in one liposomal formulation. Thus, the present work allowed us first to optimize the methods used to synthesize non-symmetric cationic lipophosphoramidates. In addition, we could also identify two non-symmetric cationic lipids exhibiting high transfection efficiencies with a series of mammalian cell lines, both vectors being characterized by a single phytanyl chain and either an oleyl or a lauryl lipid chain.

Introduction

Cationic lipids constitute a family of vectors for nucleic acid delivery that have been widely studied since the pioneering work of Felgner,¹ Behr² and Wu³. This class of vectors presents the uniqueness, when compared to viral or polymer-based vectors, to possess a fully defined structure at its molecular level. Moreover, fine-tuning of the physico-chemical properties of the supramolecular aggregates resulting from the self-assembly of a liposomal solution and plasmid DNA (pDNA) can be achieved by the incorporation of a helper lipid.⁴ Many structural modifications were performed to produce such new synthetic vectors with either an original polar head, a new hydrophobic domain or a reactive functional group that renders these vectors sensitive to different stimuli (pH⁵, red/ox⁶, enzyme⁷, light⁸, ultrasound⁹). All these studies led to the conclusion that the hydrophobic domain¹⁰ and the cationic polar head group¹¹ constitute two important structural features that have an impact on both *in vitro* and *in vivo* transfection efficiency. As regards the lipid part, we have previously reported the high *in vivo* transfection efficacies of vectors belonging to a class of bio-inspired cationic lipids (lipophosphoramidates)¹² and characterized by polyunsaturated lipid chains¹³. Moreover, the use of phytanyl chains (methylated alkyl chains) improved even further these results.¹⁴ The presence of two phytanyl chains likely explains these

interesting results. Indeed, such a vector adopts a non-lamellar supramolecular organisation according to ³¹P NMR studies. In all those recent studies and in many others recently reviewed,¹⁰ the cationic lipids are composed of a hydrophobic domain consisting of two identical lipid chains (this type of structure will be identified herein as a ‘symmetric cationic lipid’ in opposition to cationic lipids with two different lipid chains termed ‘non-symmetric cationic lipids’); this may actually be explained by chemical synthesis reasons (symmetric lipid derivatives being indeed easier to produce than non-symmetric ones as their synthesis usually involves protection/deprotection steps¹⁵). It has however been reported that non-symmetric cationic lipids can have remarkable efficacies in *in vitro*¹⁶ and *in vivo* transfection assays¹⁷. An example is the cationic lipid MLRI (a glycerolipid with a non-symmetric lipid domain - C12:0 and C14:0 – Figure 1).¹⁸ It should however be stressed here that the comparison of the transfection efficacies of symmetric and non-symmetric cationic lipids is an issue only rarely addressed in previous works. Furthermore, the few studies addressing this point led to somewhat contradictory conclusions. For instance, by using a series of cationic lipids characterized by a cyclic ammonium polar head, Chaudhuri *et al.* couldn’t reach a firm conclusion, as, depending on the experimental parameters, the best vector was either the symmetric or the non-symmetric cationic lipid.¹⁹ Interestingly,

Woude *et al.*, who were the first to compare the influence of non-symmetrical lipid chains on gene transfection activity, have reported that the cationic lipid Saint-8 (a pyridinium derivative possessing an oleyl - C18:1- and a stearyl - C18:0 - lipid chain – Figure 1) was more efficient than its bis-stearyl derivative but less efficient than its bis-oleyl analogue (*in vitro* assays).²⁰ Springer *et al.* have shown that a cationic lipid with a C12:0 and a C14:0 lipid chain was more efficient than the corresponding symmetric cationic lipids.²¹ Mac Donald *et al.* who compared several symmetric and non-symmetric cationic phosphoglycerolipids (formulated with DOPE) found that the highest transfection activity was observed with a symmetric cationic lipid.²² Finally, Nantz *et al.* have shown that the non-symmetric cationic lipid MLRI was more efficient than the symmetric cationic lipid tested (*in vivo* assays).²³ All those contradictory results strongly invited us to further investigate this question in a manner permitting to evaluate more systematically the use of non-symmetric cationic lipids for gene transfection. Thus, in the present study, we report the synthesis of non-symmetric cationic lipophosphoramidates in which the lipid chains exhibit different features: saturated, unsaturated or branched alkyl chains. Moreover, the inclusion of a cholesteryl unit was also considered since it may be an interesting lipid domain as suggested by biological results.²⁴ For obvious comparative purposes, the corresponding symmetric phosphoramidates were also synthesized and studied. In the present work, we also investigated the influence of the lipid domain (symmetric *versus* non-symmetric) on gene transfection activity not only at the molecular level (presence of two different chains in the structure of a given cationic lipid) but also at the supramolecular level (mixture of two symmetric cationic lipids). Finally, it is noteworthy that the ‘molecular approach’ required first to develop a robust synthetic method to produce non-symmetric cationic lipids. Moreover, we also aimed to develop a synthetic pathway allowing the production of large quantities of materials (> 1g) since large-scale production is an important issue when considering *in vivo* transfection experiments.

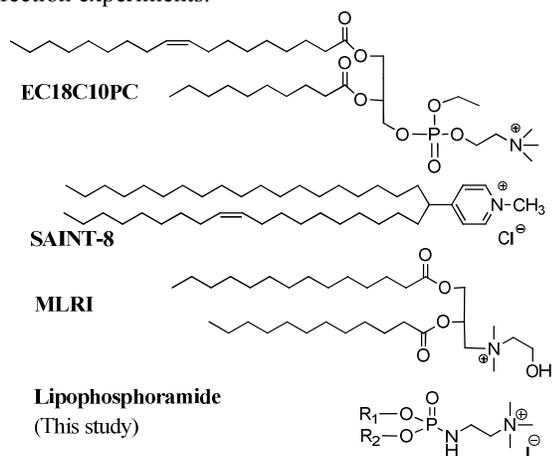


Fig. 1 Chemical structures of non-symmetrical cationic lipids (EC18C10PC, SAINT-8, MLRI) and structure of the targeting non-symmetrical lipophosphoramidates.

Results and discussion

In our previous work we have reported the synthesis of symmetric cationic lipophosphonates and lipophosphoramidates and their use for gene delivery. These bio-inspired phospholipids²⁵ were synthesised by following versatile procedures allowing different structural modifications, including the modification of the nature of the lipid chains,¹⁴ the phosphorus functional group²⁶ and the structure of the cationic polar headgroup.^{11a} However, we have never studied so far the synthesis and use of cationic lipophosphoramidates bearing two different lipid chains, although that previous work had indicated that other non-symmetric cationic lipids could exhibit high transfection efficacies.^{18-20,24} In addition, our goal wasn't only to identify novel efficient transfection agents, but we also wondered whether replacing a phytanyl chain by another lipid alkyl chain might facilitate the preparation of liposomal solutions while keeping the very high transfection efficiency observed with the bis-phytanyl phosphoramidate BSV18 (whose formulation as a liposomal solution was found to be quite difficult).¹⁵

The synthesis of non-symmetric cationic lipophosphoramidates was first attempted following the synthetic scheme previously reported for the synthesis of symmetric lipophosphoramidates.¹⁵ This approach required first the preparation of a dialkylphosphite bearing, in the present case, two different alkyl chains. For this purpose, a transesterification reaction involving diphenylphosphite and two different lipid alcohols (myristyl alcohol and oleyl alcohol, figure SI1) was investigated. This method, initially reported by Hoffman *et al.*²⁷ for the synthesis of a non-symmetric bis lipid-phosphite, produced in our hands the expected non-symmetric phosphite but with a purity that never exceeding 90% (see supporting materials SI1). Modification of the temperature of the reaction media (0°C or 20°C) as well as regulation of the addition rate (rapid or very slow addition rate controlled by a syringe pump) did never improve this result.

We thus attempted a second method in order to obtain non symmetrical phosphites with a better purity. Here, the first step was the mono-hydrolysis of symmetrical dialaurylphosphite with ammonium hydroxide in ethanol by adapting a reported method (Figure 2).²⁸ The monoalkylphosphite was obtained as a white solid after a tedious step of concentration under vacuum (vacuum must be carefully controlled to prevent bumping). Next, by adapting a reported procedure,²⁹ this mono-alkylphosphite, isolated as a salt, was then alkylated with oleyl

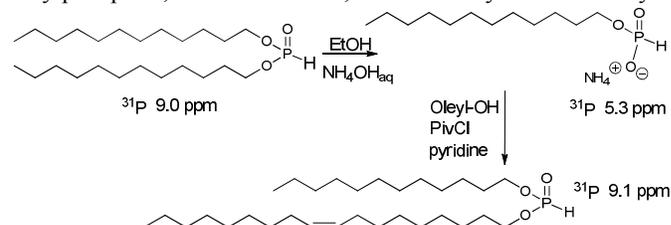


Fig. 2 Two steps sequence for the synthesis of non-symmetric-dialkylphosphite from symmetric dialkylphosphite.

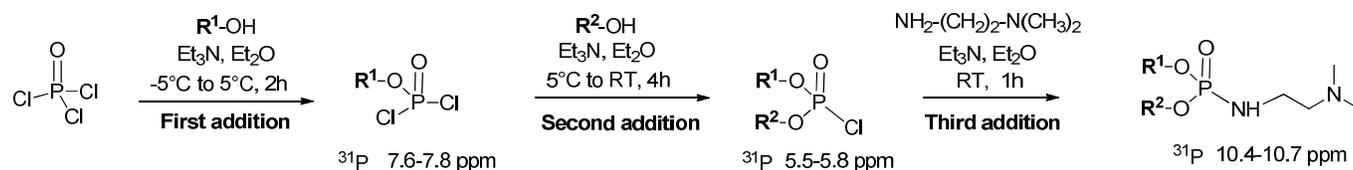


Fig. 3 One-pot procedure for the synthesis of non-symmetric lipophosphoramidate ($R^1 \neq R^2$).

alcohol *via* the formation of a mixed anhydride, which was formed *in situ* by the addition of pivaloyl chloride. This method produced the expected non-symmetrical phosphite but, unfortunately, the next step (Atherton-Todd reaction) was unsuccessful, likely because of some remaining traces of acid derivatives. Moreover, the experimental difficulties probably due to the tensioactive properties of the intermediate monoalkylphosphite, led us to investigate a third synthetic strategy. For the third method (Figure 3) POCl_3 was used as starting compound. Three successive additions of nucleophilic reagents (two lipid alcohols and one amine) were achieved in diethylether used as solvent and in presence of triethylamine acting as a base. After each nucleophile addition, ^{31}P NMR was used to check the completeness of the reaction. Moreover, the temperature of the reaction medium was very carefully controlled. The addition of the first alcohol was achieved at a starting temperature of -5°C and then the temperature was slowly increased up to 5°C in 2h. Next, the second alcohol was added at 5°C and the reaction medium was stirred for 4h while the temperature of the reaction medium was slowly increased up to 20°C . Then, after checking by ^{31}P NMR the completeness of this step, *N,N*-dimethylethylenediamine was added at 20°C and the reaction medium was stirred further for 1h. In this procedure, the shortest lipid alcohol was first added, followed by the second lipid alcohol and, at the end, the more reactive nucleophilic reagent which was, herein, the primary amine derivative. This procedure was very efficient and, as observed by ^{31}P NMR and other spectroscopic methods, the non-symmetric lipophosphoramidate was the sole compound detected (See SI2). This one pot procedure allowed the production of up to 5 g of non-symmetric lipophosphoramidate and it is likely that larger quantities can be obtained.

Following this procedure, a series of seven non-symmetric lipophosphoramidates was synthesised as shown in table 1 (NMR data, SI3). This series include saturated lipid chains (C12:0 ; C14:0 ; C18:0), substituted alkyl chains (phytanyl), unsaturated alkyl chains (oleyl) and a cholesteryl moiety. These lipophosphoramidates were transformed into cationic lipids by the alkylation of the tertiary amine with methyl iodide to produce the cationic non-symmetric lipophosphoramidates shown in Table 1. With the aim to study the consequences arising from the presence of two different lipid chains in the molecular structure of a given vector on gene transfection efficiency, we next undertook to compare the efficiencies of such non-symmetric vectors with those of symmetric lipids.

These symmetric compounds (see Table 1) were synthesized according to reported procedures^{13b} starting from a Atherton-Todd coupling between dialkylphosphite and *N,N*-dimethylethylenediamine followed by the quaternarization of the amine with methyl iodide. For the cholesteryl-based derivatives (compounds **13** and **14**; Table 1), the cholesteryl fragment was first functionalized via a carbamate functional group with the aim to incorporate a primary alcohol function which was subsequently used to produce cholesterol-based amphiphiles (**13** and **14**). It is worth noticing that the synthesis of the bis-cholesteryl derivative was not successful since it was isolated as a mixture of compounds and it was consequently not included in the biological tests.

For the transfection experiments, the cationic lipids were formulated as liposomal solutions by hydration of a lipid film followed by a sonication period (60 min.). The liposomal solutions (1.5 mmol.L^{-1}) were characterized by DLS to determine both the size of the supramolecular objects formed and their zeta potentials. As shown in supporting material (SI4), the sizes of the liposomes mainly ranged from 150 to 250 nm with, in some cases, smaller aggregates (70-130 nm). As expected, the zeta potentials were clearly positives (form +30 to +66 mV).

We next evaluated the pDNA compaction ability and the *in vitro* gene transfection efficiency of these liposomal preparations. Obviously, both non-symmetric lipids and symmetric ones were studied here. Accordingly, for each non-symmetric cationic lipid, bearing the lipid chains R^1 and R^2 , the two symmetric cationic lipids (R^1, R^1 and R^2, R^2) were also tested. Moreover, an additional formulation, based on a mixture of the two symmetric cationic lipids in a 1/1 molar ratio, was also considered. This last experiment aimed to study the influence of two types of lipid chains present at a supramolecular level (mixture of symmetric compounds) while the formulations based on non-symmetric lipids addressed the question of the influence of the structure of the lipid domain at the molecular level.

As indicated above, to evaluate the pDNA condensation ability, DNA retardation assays on agarose gel electrophoresis were performed for each type of lipoplex at different charge ratios (CR) (see SI-5). In these experiments, a full condensation of pDNA was observed at CR equal to or higher than 4. No significant differences were observed when symmetric and non-symmetric derivatives were compared.

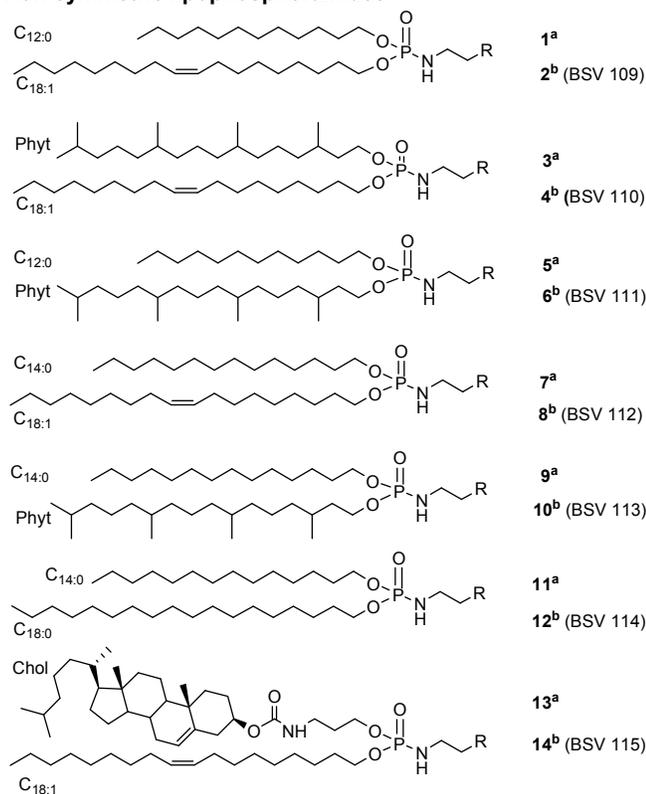
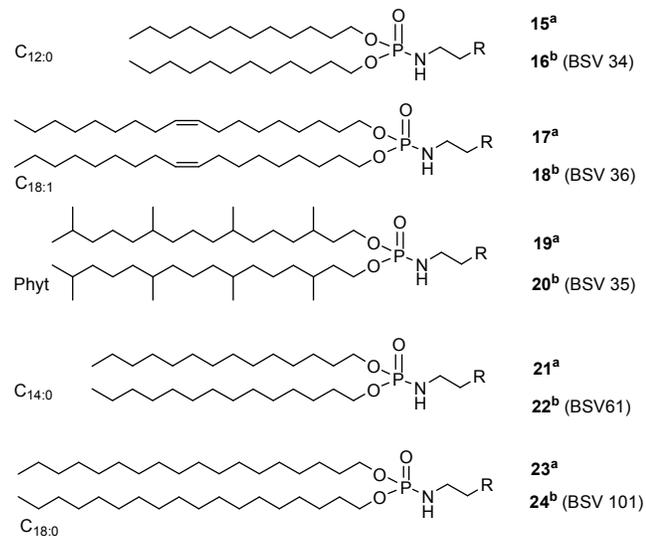
For comparison of the transfection efficacies four cell lines were considered (A549, 16HBE, CFBE and SKMel28). The A549, 16HBE and CFBE cell lines are derived from human airway epithelial cells (lung carcinoma, normal bronchial epithelium and cystic fibrosis (CF) bronchial epithelium, respectively); these cell lines were chosen with a view to evaluate novel vectors for nonviral lung gene therapy for CF, the most common hereditary genetic disease in the Caucasian population. In addition, SKMel 28 (human melanoma cells) was selected to study the transfection of another cancer cell line in connection with our works aiming to develop new therapeutic approaches.³⁰ The luciferase-expressing pCMV-*Luc* plasmid was used in these *in vitro* transfection assays. This plasmid allowed the quantification of the transfection activity by bioluminescence (activity expressed as Relative Light Units (RLU)/mg protein). Each formulation was tested at different cationic lipid/pDNA CR (1, 2, 4 and 8) in triplicate and a total number of 26 formulations were studied. As the amount of experimental data obtained was hence very high, the full dataset for these transfection experiments are shown in Supporting Material (see SI6). Thus, we only present here a concise analysis of all those transfection results with the view to get a better insight into the influence of non-symmetric vectors on transfection efficacy and thus also to select the most promising formulation. Practically, we have selected some data and presented them, as detailed below, in a manner that we found likely to facilitate the comparison between the different vectors as shown in Table 2. First, for our analysis, we only took into account the transfection activity values obtained at a lipoplex CR of 4. Although other CRs were tested (see supporting information), we have indeed previously reported that a CR of 4 (a clearly positive but not too high CR) is generally not only efficient for *in vitro* transfection but also for *in vivo* gene transfection. **Error! Bookmark not defined.** Second, the transfection activity values observed (expressed as RLU/mg protein) were divided by the activity of the commercially-available control cationic lipid Lipofectamine (LFM) used at its best CR (CR=8), a procedure yielding relative transfection efficacy values. These values are shown in Table 2. In order to allow a quick identification of the best vectors in table 2, those displaying a transfection efficacy 10-fold (A549), 100-fold (16HBE and CFBE) or 15-fold (SKMel28) above the efficacy of the Lipofectamine control are shown shaded. Of note, Table 2 shows the relative transfection values of the various formulations grouped into classes (series), each series being composed (except for series 7) of the non-symmetric compound, its two corresponding symmetric compounds and the formulation consisting in the mixture of the two symmetric compounds.

The first interesting information is that most of the formulations tested were more efficient (relative value > 1) than the control Lipofectamine and frequently a 10-times higher efficacy was observed, except for cationic lipids bearing saturated linear alkyl chains (series 6). Indeed, the cationic lipid **24**, which possesses two C18:0 lipid chains, was inefficient while the decrease of the chain length (C14:0 ; compound **22**) resulted in

slightly improved efficacies but at a level still close to those observed with LFM (values close to 1). In this series, mixing of the two lipid chains at a molecular level (non-symmetric cationic lipid **12**) produced a cationic lipid with low transfection efficacy. Second, it is also noteworthy here that two cell lines (16HBE and CFBE) were easily transfected with the lipophosphoramidates while the two others (A549 and SKMel28) were more difficult to transfect. This is especially interesting when considering lung gene therapy for Cystic Fibrosis, where the bronchial epithelium is the target issue; Among the symmetric cationic lipids, two (bis-oleyl C18:1 **18** and bis-phytanyl **20**) were clearly identified as the best vectors tested. This is consistent with our previous results showing that these two types of lipid chains generated very efficient formulations for gene delivery when associated with different cationic polar headgroups. Interestingly, the cationic lipophosphoramidate **4** (series 2), in which an oleyl chain is combined with a phytanyl chain, exhibited good transfection efficacies with all four cell lines tested, these efficacy values remaining nevertheless lower, in some cases, than those observed with the symmetric bis-phytanyl **20**. It should however be stressed here that the liposomal formulation of cationic lipid **4** was readily obtained after one night of hydration, while a hydration period of at least 7 days was required for compound **20**. The formulation of a mixture of **18** and **20** in a 1/1 ratio was also readily achieved by hydration (1 night) but this formulation was slightly less efficient than those based on the non-symmetric compound **4**.

Next, we explored the transfection efficiencies of the association of oleyl chains (C18:1) with other alkyl chains. Its association with a lauryl chain (C12:0) produced compound **2** (series 1, Table 2). This non-symmetric cationic lipid **2** exhibited good to excellent transfection efficacies. When compared to the bis-oleyl derivative **18**, its efficiency was almost similar or even better in the case of CFBE cells. However, the transfection efficacies of this non-symmetric and of the best symmetric derivative of this series (compound **18**) were not very different. In this series, the mixture of the two symmetric compounds **16** and **18** resulted in a formulation exhibiting relative transfection efficacy values similar to those observed for compound **18** alone (symmetric C18:1) or the non-symmetric compound **2**.

The association of a C18:1 with a C14:0 lipid chain (compound **8**, series 4) led to similar conclusions which can be summarised as follows: the best vector in this series was the symmetric bis-oleyl derivative **18** while the non-symmetric compound **8** and the mixture of symmetric cationic lipids (bis-myristyl **22** and bis-oleyl **18**) produced slightly less efficient formulations. The last combination including an oleyl chain consisted to combine it with a cholesteryl moiety. In that case, due to the difficulties to produce the bis-cholesteryl derivatives, only two formulations were tested as indicated in Table 2. Here, the replacement of one oleyl chain by a cholesteryl moiety clearly decreased the transfection efficacies. Finally, we believe it

Table 1 : Molecular structure of the symmetric and non-symmetric neutral and cationic phospholipid synthesized in this study.**Non-symmetric lipophosphoramides****Symmetric lipophosphoramides**

^a R = N(CH₃)₂; ^b R = N(CH₃)₃⁺

relevant to further focus our attention on cationic lipids with a phytanyl chain since our previous worked demonstrated that a cationic lipophosphoramidate with two phytanyl chains was very efficient for both *in vitro* and *in vivo* experiments.¹⁴ These previous results were attributed to the tendency of this type of

Table 2 : Relative transfection efficacies of non-symmetric and symmetric lipophosphoramidates on four cell lines. For each cell line, data were normalized by assigning a value of 1 to LFM luciferase expression.

Series	Number - structure ^a	A549 ^b	16HBE ^b	CFBE ^b	SKMe128 ^b
1	2 C12:0/C18:1	2	27	318	1
	16 C12:0/C12:0	1	1	3	1
	18 C18:1/C18:1	1	39	167	8
	16+18 (1/1 ratio)	2	34	222	< 1
2	4 Phyt/C18:1	13	105	101	19
	18 C18:1/C18:1	1	39	167	8
	20 Phyt/Phyt	38	683	159	13
	18+20 (1/1 ratio)	2	122	40	13
3	6 C12:0/Phyt	39	381	280	18
	16 C12:0/C12:0	1	1	3	1
	20 Phyt/Phyt	38	683	159	13
	16+20 (1/1 ratio)	2	128	32	1
4	8 C14:0/C18:1	2	24	10	1
	18 C18:1/C18:1	1	39	167	8
	22 C14:0/C14:0	1	5	5	1
	18+22 (1/1 ratio)	2	28	6	1
5	10 C14:0/Phyt	13	79	40	16
	20 Phyt/Phyt	38	683	159	13
	22 C14:0/C14:0	1	5	5	1
	20+22 (1/1 ratio)	8	88	33	32
6	12 C14:0/C18:0	< 1	< 1	< 1	< 1
	24 C18:0/C18:0	< 1	< 1	< 1	< 1
	22 C14:0/C14:0	1	5	5	1
	24+22 (1/1 ratio)	< 1	< 1	< 1	< 1
7	14 Chol/C18:1	< 1	2	3	< 1
	18 C18:1/C18:1	1	39	167	8
	Lipofectamine	1	1	1	1

a) Label code: Lauryl lipid chain (C12:0); Oleyl lipid chain (C18:1); Phytanyl lipid chain (Phyt); Myristyl lipid chain (C14:0); Stearyl lipid chain (C18:0); cholesteryl lipid chain (Chol). b) Relative transfection efficacies calculated as follow: [RLU/mg of a selected formulation at CR = 4] / [RLU/mg prot. of lipofectamine (LFM) at its best CR (CR=8)]

cationic lipid to adopt an inverted hexagonal phase. Here, as discussed above, the association of a phytanyl and an oleyl chain was an interesting combination not only because high transfection efficacies were obtained but also because the duration of the formulation protocol was strongly shortened when compared to the bis-phytanyl compound **20**. The association of one phytanyl and one lauryl chain (C12:0/Phyt compound **6** - series 3, Table 2) produced one of the best vector among those tested in this study. Indeed, the transfection efficacies were even improved when compared to the non-symmetric compound **4** (Phytanyl/oleyl). The comparison of the transfection efficacies obtained with compound **6** and with a mixture of the symmetric compounds **16** (bis-lauryl) and **20** (bis-phytanyl) was clearly in favour of the non-symmetric cationic lipid **6**. It is noteworthy that this vector **6** exhibited excellent transfection efficacies on the four cell lines tested. Interestingly, the non-symmetric compound **6** was readily formulated as a liposomal solution after only one night of hydration. Finally, the last series of compounds (series 5, Table

2) was characterized by the association of a phytanyl and a myristyl chain (C14:0). In that case, the best vector was clearly the bis-phytanyl derivative **20** while the non-symmetric

compound **10** (C14:0/phyt) and the mixture of the two symmetric derivatives **22** (bis-myristyl) and **20** (bis-phytanyl) exhibited almost similar transfection efficacies.

All together, these transfection results indicate that it isn't possible to edit a general rule stipulating that non-symmetric derivatives would be more or less efficient than symmetric cationic lipids. However, the present work allowed us to identify two promising non-symmetric cationic lipids: compound **4** (with oleyl and phytanyl chains) and compound **6** (with lauryl and phytanyl chains). Indeed, these two non-symmetric cationic lipids exhibited very high transfection efficacies with all 4 cell lines tested. Moreover, their formulation as liposomal solution was significantly simpler when compared to the bis-phytanyl-based cationic lipid **20**. Indeed, liposomal solutions of compounds **4** or **6** can be readily obtained with a hydration period of one night instead of, at least, 7 days for compound **20**.

The toxicity of lipoplexes produced from both symmetrical and non-symmetrical cationic lipids were evaluated. Most of the formulations were not toxic and presented similar results to the untransfected cells. (S17)

Conclusion

Different methods were tested for the synthesis of cationic lipophosphoramidates possessing two different lipid chains. Among these methods, the sequential addition of the two different lipid alcohols on phosphorus oxychloride followed by the addition of the amine, in a one pot sequence, was the most efficient. This synthetic scheme allowed the production of a series of 7 non-symmetric cationic lipids and, more widely, it appears to be the method of choice for the synthesis of amphiphilic derivatives characterized by two different hydrophobic domains. In this study, we systematically compared the transfection efficacy of these non-symmetric cationic lipophosphoramidates with that of their symmetric counterparts (characterized by two identical hydrophobic lipid chains). Four cell lines were used for this purpose. The transfection results indicate that it is not possible to reach a general conclusion stipulating that the non-symmetric cationic lipids are always better or worse than the symmetric vectors. Indeed, the choice of the two lipid chains combined in the structure of a non-symmetric cationic lipid appears to have a great influence on the final transfection efficiency. Our study once again indicates that the presence of two identical phytanyl chains (compound **20**) leads to an efficient vector, whose formulation in liposomal solution requires a prolonged hydration time. Indeed, this result is consistent with our previous study reporting the interesting transfection results obtained with the vector BSV18 (a bis-phytanyl lipophosphoramidate).¹⁴ Most interestingly, the two non-symmetric cationic lipids **4** (BSV110) and **6** (BSV11) exhibited

high transfection efficacies with all four mammalian cell lines tested and it is noteworthy that liposomal formulations of compounds **4** or **6** could be obtained more easily than in the case of the symmetric cationic lipid **20**. This study warrants further evaluations of these cationic lipids, especially under *in vivo* experimental settings. Moreover, as we have recently evidenced that good transfection reagents in that family of vectors can also display potent antibacterial activities,³¹ the effects of such compounds with regard to different bacterial strains should be also determined. Finally, our results suggest that the incorporation of a phytanyl chain may constitute a valuable strategy to obtain cationic lipids exhibiting high transfection efficacies.

Experimental section

Solvents were dried with a solvent purification system MBraun-SPS (THF, CH₂Cl₂). All compounds were fully characterized by ¹H (400.133 MHz), ¹³C (125.773 MHz) and ³¹P (161.970 MHz) NMR spectroscopy (Avance DRX 400 and Avance DRX 500 spectrometers). The lipids were purified by flash chromatography. Coupling constants J are given in Hertz. The following abbreviations were used: s for singlet, d doublet, t triplet, q quadruplet, m for multiplet. When needed, ¹³C and ³¹P heteronuclear HMQC and HMBC were used to unambiguously establish molecular structures. Mass spectroscopy analyses were performed by Brest (service commun de spectrometrie de masse) on a Bruker Autoflex MALDI TOF-TOF III LRF200 CID. Commercial compounds [Oleyl alcohol 85 %, 2-bromoethylamine hydrobromide] were used as received except DIPEA which was distilled over KOH. The mean particle diameter and zeta potential (ξ) of the liposomes and lipoplexes were measured using a 3000 Zetasizer (Malvern Instruments) at 25 °C (see supplementary material).

General protocol for the synthesis of cationic lipophosphoramidates with two distinct alkyl chains.

Step 1 : Synthesis of neutral lipophosphoramidates

Synthesis of *O*-lauryl-*O*-oleyl-*N*-(2-dimethylaminoethyl)phosphoramidate **1**
Triethylamine (2.27 mL; 16.3 mmol) was added to a cooled (0°C) solution of POCl₃ (2.50 g ; 16.3 mmol) in diethylether (20 mL) and placed under argon atmosphere. Dodecanol (3.04 g ; 16.3 mmol) was then added dropwise. The solution was stirred for 1h30 at 0°C. Then, a solution of oleyl alcohol (4.38 g ; 16.3 mmol) and triethylamine (2.49 mL ; 17.9 mmol) placed in diethylether (10 mL) was slowly added at 0°C. This solution was further stirred for 4h while the temperature was slowly increased up to 20°C. Then, a solution of *N,N*-dimethylethylenediamine (1.44 g ; 16.3 mmol) and triethylamine (2.27 mL ; 16.3 mmol) in diethylether (5 mL) was added and the solution was stirred for 1h. Finally, diethylether was added (50 mL) and the solution was washed with brine. The organic layer was dried over MgSO₄, filtered and concentrated under reduced pressure to produce the desired

compound as an oil that was engaged in the next step without any further purification (77% yield; 7.39 g).

Step 2: Synthesis of the cationic lipophosphoramidates by alkylation.

Synthesis of 2-(*O*-lauryl-*O*-oleylphosphoramidoyl)-ethyltrimethylammonium iodide 2

Methyl iodide (1.00 mL) was added to a solution of *O*-lauryl-*O*-oleyl-*N*-(2-dimethylaminoethyl)phosphoramidate (200 mg; 0.34 mmol; 1 eq) in 5 mL of CH₂Cl₂. The mixture was stirred at R.T. for one night. The excess of methyl iodide and CH₂Cl₂ were removed under vacuum. The compound was purified by chromatography on silica gel to give the compound.

***O*-lauryl-*O*-oleyl-*N*-(2-dimethylaminoethyl)phosphoramidate 1**

Isolated as a yellow oil (7.39 g; 77 % yield). ¹H-NMR (400.04 MHz, CDCl₃): 0.87 (t, ³J_{H_{HH}} = 8 Hz, 6H, CH₃); 1.25 (s, 40H, CH₂); 1.65 (m, 4H, CH₂ β); 1.99 (m, 4H, CH₂-CH=CH); 2.23 (s, 6H, N-(CH₃)₂); 2.41 (t, ³J_{H_{HH}} = 4 Hz, 2H, CH₂-N-(CH₃)₂); 2.97 (m, 2H, NH-CH₂); 3.30 (m, 1H, NH); 3.97 (m, 4H, CH₂ α); 5.34 (m, 2H, CH=CH). ¹³C-NMR (125.80 MHz, CDCl₃): 13.99 (s, 2 x CH₃); 22.58 (s, CH₂); 25.51 (s, CH₂); 27.10 (s, CH=CH-CH₂); 29-30 (s, CH₂); 30.22 (d, ³J_{CP} = 6 Hz, CH₂ β); 30.76 (s, CH₂); 31.81 (s, CH₂); 38.44 (s, CH₂-NH); 44.89 (s, N(CH₃)₂); 59.50 (d, ³J_{CP} = 6 Hz, CH₂-N(CH₃)₂); 66.16 (d, ²J_{CP} = 5 Hz, CH₂ α); 129.68 (s, CH=CH); 129.81 (s, CH=CH). ³¹P-NMR (161.94 MHz, CDCl₃): 10.7. IR (cm⁻¹): 3200 (ν_{NH}); 1231 (ν_{P=O}); 1005 (ν_{P-O-C}). MS (MALDI-TOF): m/z calcd for C₃₄H₇₂N₂O₃P (M+H): 587.528; found 587.485.

2-(*O*-lauryl-*O*-oleylphosphoramidoyl)-ethyltrimethylammonium iodide 2

Isolated as a pale yellow wax (200 mg; 81 % yield). ¹H-NMR (400.04 MHz, CDCl₃): 0.87 (t, ³J_{H_{HH}} = 8 Hz, 6H, CH₃); 1.25 (s, 40H, CH₂); 1.66 (m, 4H, CH₂ β); 2.00 (m, 4H, CH₂-CH=CH); 3.48 (s, 9H, N-(CH₃)₃); 3.60 (m, 2H, NH-CH₂); 3.87 (t, ³J_{H_{HH}} = 8 Hz, 2H, CH₂-N-(CH₃)₃); 3.98 (q, ³J_{H_{HH}} = 8 Hz, 4H, CH₂ α); 4.40 (m, 1H, NH); 5.34 (m, 2H, CH=CH). ¹³C-NMR (125.80 MHz, CDCl₃): 14.11 (s, 2 x CH₃); 22.68 (s, CH₂); 25.58 (s, CH₂); 27.22 (s, CH=CH-CH₂); 29-30 (s, CH₂); 30.43 (d, ³J_{CP} = 6 Hz, CH₂ β); 31.91 (s, CH₂); 36.19 (NH-CH₂); 54.87 (s, N(CH₃)₃); 66.77 (s, CH₂-N(CH₃)₃); 67.15 (d, ²J_{CP} = 5 Hz, CH₂ α); 129.74 (s, CH=CH); 129.97 (s, CH=CH). ³¹P-NMR (161.94 MHz, CDCl₃): 9.3. IR (cm⁻¹): 3300 (ν_{NH}); 1223 (ν_{P=O}); 990 (ν_{P-O-C}). MS (MALDI-TOF): m/z calcd for C₃₅H₇₄N₂O₃P (M⁺): 601.544; found 601.545.

***O*-oleyl-*O*-phytanyl-*N*-(2-dimethylaminoethyl)phosphoramidate 3**

Isolated as a yellow oil (3.98 g; 85 % yield). ¹H-NMR (400.04 MHz, CDCl₃): 0.80-0.90 (m, 18H, CH₃ phytanyl and CH₃ oleyl); 1.00-1.80 (m, 48H, CH₂, CH phytanyl and CH₂ oleyl); 1.99 (m, 4H, CH₂-CH=CH); 2.22 (s, 9H, N-(CH₃)₂); 2.38 (t, ³J_{H_{HH}} = 8 Hz, 2H, CH₂-N-(CH₃)₂); 2.95 (m, 2H, NH-CH₂); 3.30 (m, 1H, NH); 3.98 (m, 4H, CH₂ α); 5.34 (m, 2H, CH=CH). ¹³C-NMR (125.80 MHz, CDCl₃): 13.99 (s, 2 x CH₃); 19.40 to

19.80 (s, CH₃ phytanyl); 22.60 to 22.80 (s, CH₃ phytanyl); 22.72 (s, CH₃-CH₂ oleyl); 24.30 to 24.80 (s, CH₂ phytanyl); 27.19 (s, CH=CH-CH₂); 27.96 (s, CH phytanyl); 29.28 (d, ³J_{CP} = Hz, CH₂ β); 29.35 (s, CH phytanyl); 30.47 (d, ³J_{CP} = 6 Hz, CH₂ β); 32.78 (s, CH phytanyl); 38.56 (s, NH-CH₂); 37.30 to 37.50 (s, CH₂ phytanyl); 39.41 (s, CH₂ phytanyl); 45.03 (s, N(CH₃)₂); 59.63 (s, CH₂-N(CH₃)₂); 62.81 (d, ²J_{CP} = 5 Hz, CH₂ α); 64.73 (d, ²J_{CP} = 5 Hz, CH₂ α); 129.74 (s, CH=CH); 129.92 (s, CH=CH). ³¹P-NMR (161.94 MHz, CDCl₃): 10.7. IR (cm⁻¹): 1228 (ν_{P=O}); 983 (ν_{P-O-C}). MS (MALDI-TOF): m/z calcd for C₄₂H₈₈N₂O₃P (M+H): 699.653; found 699.614.

2-(*O*-oleyl-*O*-phytanylphosphoramidoyl)-ethyltrimethylammonium iodide 4

Isolated as a yellow oil (130 mg; 54 % yield). ¹H-NMR (400.04 MHz, CDCl₃): 0.80-0.90 (m, 18H, CH₃ phytanyl and CH₃ oleyl); 1.00-1.80 (m, 48H, CH₂, CH phytanyl and CH₂ oleyl); 2.00 (m, 4H, CH₂-CH=CH); 3.47 (s, 9H, N-(CH₃)₃); 3.55 (m, 2H, NH-CH₂); 3.87 (t, ³J_{H_{HH}} = 8 Hz, 2H, CH₂-N-(CH₃)₃); 4.00 (m, 4H, CH₂ α); 4.41 (m, 1H, NH); 5.33 (m, 2H, CH=CH). ¹³C-NMR (125.80 MHz, CDCl₃): 14.16 (s, 2 x CH₃); 19.50 to 19.80 (s, CH₃ phytanyl); 22.70 to 22.80 (s, CH₃ phytanyl); 22.72 (s, CH₃-CH₂ oleyl); 24.40 to 24.90 (s, CH₂ phytanyl); 27.20 (s, CH=CH-CH₂); 28.01 (s, CH phytanyl); 29.28 (d, ³J_{CP} = Hz, CH₂ β); 29.35 (s, CH phytanyl); 30.47 (d, ³J_{CP} = 6 Hz, CH₂ β); 32.66 (s, CH phytanyl); 36.22 (s, NH-CH₂); 37.30 to 37.50 (s, CH₂ phytanyl); 39.41 (s, CH₂ phytanyl); 54.99 (s, N(CH₃)₃); 65.74 (d, ²J_{CP} = Hz, CH₂ α); 66.80 (s, CH₂-N(CH₃)₃); 67.29 (d, ²J_{CP} = 5 Hz, CH₂ α); 129.79 (s, CH=CH); 130.01 (s, CH=CH). ³¹P-NMR (161.94 MHz, CDCl₃): 8.9. IR (cm⁻¹): 1224 (ν_{P=O}); 972 (ν_{P-O-C}). MS (MALDI-TOF): m/z calcd for C₄₃H₉₀N₂O₃P (M⁺): 713.669; found 713.652.

***O*-lauryl-*O*-phytanyl-*N*-(2-dimethylaminoethyl)phosphoramidate 5**

Isolated as a yellow wax (2.01 g; 100 % yield). ¹H-NMR (400.04 MHz, CDCl₃): 0.87 (m, H, CH₂); 1.00-1.60 (m, H, CH₂); 2.37 (s, 6H, N-(CH₃)₂); 2.56 (m, 2H, CH₂-N-(CH₃)₂); 3.08 (m, 2H, NH-CH₂); 3.50 (m, 1H, NH); 3.98 (m, 4H, CH₂ α). ¹³C-NMR (125.80 MHz, CDCl₃): 14.10 (s, 2 x CH₃); 19.40 to 19.80 (s, CH₃ phytanyl); 22.60 to 22.80 (s, CH₃ phytanyl); 22.65 (s, CH₃-CH₂ lauryl); 24.30 to 24.80 (s, CH₂ phytanyl); 25.59 (s, CH₂ lauryl); 28.02 (s, CH phytanyl); 29.00 to 30.00 (s, CH₂ phytanyl); 31.89 (s, CH phytanyl); 37.20 to 37.60 (s, CH₂ phytanyl); 38.36 (s, NH-CH₂); 39.34 (s, CH₂ phytanyl); 45.00 (s, N(CH₃)₂); 59.55 (s, CH₂-N(CH₃)₂); 64.77 (d, ²J_{CP} = 5 Hz, CH₂ α); 66.39 (d, ²J_{CP} = 6 Hz, CH₂ α). ³¹P-NMR (161.94 MHz, CDCl₃): 10.4. IR (cm⁻¹): 1229 (ν_{P=O}); 971 (ν_{P-O-C}). MS (MALDI-TOF): m/z calcd for C₃₆H₇₈N₂O₃P (M): 617.575; found 617.531.

2-(*O*-lauryl-*O*-phytanylphosphoramidoyl)-ethyltrimethylammonium iodide 6

Isolated as a yellow oil (140 mg; 57 % yield). ¹H-NMR (400.04 MHz, CDCl₃): 0.85-1.70 (m, 62H, CH₂ lauryl and CH₂

phytanyl); 3.45 (s, 9H, N-(CH₃)₃); 3.50 (m, 2H, NH-CH₂); 3.81 (t, ³J_{HH} = 8 Hz, 2H, CH₂-N-(CH₃)₃); 3.99 (m, 4H, CH₂ α); 4.50 (m, 1H, NH). ¹³C-NMR (125.80 MHz, CDCl₃): 14.16 (s, CH₃ lauryl); 19.40 to 19.80 (s, CH₃ phytanyl); 22.70 to 22.80 (s, CH₃ phytanyl); 24.40 to 24.90 (s, CH₂ phytanyl); 25.62 (s, CH₂ lauryl); 28.02 (s, CH phytanyl); 29-30 (s, CH phytanyl and CH lauryl); 30.43 (d, ³J_{CP} = 6 Hz, CH₂ β lauryl); 32.85 (s, CH phytanyl); 36.21 (s, NH-CH₂); 37.36 to 37.48 (s, CH₂ phytanyl); 39.40 (s, CH₂ phytanyl); 55.01 (s, N(CH₃)₃); 65.74 (s, CH₂ α); 66.77 (s, CH₂-N(CH₃)₃); 67.33 (d, ²J_{CP} = 6 Hz, CH₂ α). ³¹P-NMR (161.94 MHz, CDCl₃): 9.3. IR (cm⁻¹): 1223 (ν_{P=O}); 973 (ν_{P-O-C}). MS (MALDI-TOF): m/z calcd for C₃₇H₈₀N₂O₃P (M⁺): 631.591; found 631.540.

***O*-myristyl-*O*-oleyl-*N*-(2-dimethylaminoethyl)phosphoramidate 7**

Isolated as a yellow wax (2.00 g; 100 % yield). ¹H-NMR (400.04 MHz, CDCl₃): 0.87 (t, ³J_{HH} = 8 Hz, 6H, CH₃); 1.24 (s, 44H, CH₂); 1.65 (m, 4H, CH₂ β); 1.99 (m, 4H, CH₂-CH=CH); 2.28 (s, 6H, N-(CH₃)₂); 2.45 (t, ³J_{HH} = 4 Hz, 2H, CH₂-N-(CH₃)₂); 3.00 (m, 2H, NH-CH₂); 3.35 (m, 1H, NH); 3.97 (m, 4H, CH₂ α); 5.34 (m, 2H, CH=CH). ¹³C-NMR (125.80 MHz, CDCl₃): 14.10 (s, 2 x CH₃); 22.67 (s, CH₂); 25.60 (s, CH₂); 27.20 (s, CH=CH-CH₂); 29-30 (s, CH₂); 30.22 (d, ³J_{CP} = 6 Hz, CH₂ β); 30.76 (s, CH₂); 31.81 (s, CH₂); 38.38 (s, CH₂-NH); 44.90 (s, N(CH₃)₂); 59.56 (d, ³J_{CP} = 6 Hz, CH₂-N(CH₃)₂); 66.41 (d, ²J_{CP} = 5 Hz, CH₂ α); 129.76 (s, CH=CH); 129.94 (s, CH=CH). ³¹P-NMR (161.94 MHz, CDCl₃): 10.6. IR (cm⁻¹): 1231 (ν_{P=O}); 990 (ν_{P-O-C}). MS (MALDI-TOF): m/z calcd for C₃₆H₇₆N₂O₃P (M+H): 615.559; found 615.518.

***2*-(*O*-myristyl-*O*-oleylphosphoramidoyl)-ethyltrimethylammonium iodide 8**

Isolated as a yellow oil (228 mg; 93 % yield). ¹H-NMR (400.04 MHz, CDCl₃): 0.88 (t, ³J_{HH} = 8 Hz, 6H, CH₃); 1.25 (s, 44H, CH₂); 1.66 (m, 4H, CH₂ β); 2.00 (m, 4H, CH₂-CH=CH); 3.44 (s, 9H, N-(CH₃)₃); 3.58 (m, 2H, NH-CH₂); 3.84 (t, ³J_{HH} = 8 Hz, 2H, CH₂-N-(CH₃)₃); 3.99 (m, 4H, CH₂ α); 4.50 (m, 1H, NH); 5.34 (m, 2H, CH=CH). ¹³C-NMR (125.80 MHz, CDCl₃): 14.16 (s, 2 x CH₃); 22.73 (s, CH₂); 25.63 (s, CH₂); 27.27 (s, CH=CH-CH₂); 29-30 (s, CH₂); 30.47 (d, ³J_{CP} = 6 Hz, CH₂ β); 30.76 (s, CH₂); 31.97 (s, CH₂); 36.61 (s, CH₂-NH); 55.18 (s, N(CH₃)₃); 67.31 (d, ³J_{CP} = 6 Hz, CH₂-N(CH₃)₃); 67.35 (d, ²J_{CP} = 5 Hz, CH₂ α); 129.81 (s, CH=CH); 130.02 (s, CH=CH). ³¹P-NMR (161.94 MHz, CDCl₃): 9.4. IR (cm⁻¹): 1240 (ν_{P=O}); 988 (ν_{P-O-C}). MS (MALDI-TOF): m/z calcd for C₃₇H₇₈N₂O₃P (M⁺): 629.575; found 629.561.

***O*-myristyl-*O*-phytanyl-*N*-(2-dimethylaminoethyl)phosphoramidate 9**

Isolated as a yellow oil (7.89 g; 94 % yield). ¹H-NMR (400.04 MHz, CDCl₃): 0.79-1.64 (m, 66H, CH₂); 2.22 (s, 6H, N-(CH₃)₂); 2.39 (m, 2H, CH₂-N-(CH₃)₂); 2.96 (m, 2H, NH-CH₂); 3.40 (m, 1H, NH); 3.94 (m, 4H, CH₂ α). ¹³C-NMR (125.80 MHz, CDCl₃): 14.10 (s, 2 x CH₃); 19.50 to 19.60 (s, CH₃ phytanyl); 22.52 (s, CH₃ phytanyl); 22.58 (s, CH₂); 22.61 (s,

CH₃ phytanyl); 24.20 to 24.70 (s, CH₂ phytanyl); 25.38 (s, CH₂ lauryl); 27.86 (s, CH phytanyl); 29.00 to 30.00 (s, CH₂ phytanyl); 31.83 (s, CH phytanyl); 32.68 (s, CH₂); 37.20 to 37.60 (s, CH₂ phytanyl); 38.41 (s, NH-CH₂); 39.27 (s, CH₂ phytanyl); 44.87 (s, N(CH₃)₂); 59.55 (s, CH₂-N(CH₃)₂); 64.62 (d, ²J_{CP} = 6 Hz, CH₂ α); 66.23 (d, ²J_{CP} = 6 Hz, CH₂ α). ³¹P-NMR (161.94 MHz, CDCl₃): 10.7. IR (cm⁻¹): 1229 (ν_{P=O}); 982 (ν_{P-O-C}). MS (MALDI-TOF): m/z calcd for C₃₈H₈₂N₂O₃P (M+H): 645.606; found 645.571.

***2*-(*O*-myristyl-*O*-phytanylphosphoramidoyl)-ethyltrimethylammonium iodide 10**

Isolated as a yellow oil (0.49 g; 100 % yield). ¹H-NMR (400.04 MHz, CDCl₃): 0.85-1.70 (m, 66H, CH₂ myristyl and CH₂ phytanyl); 3.46 (s, 9H, N-(CH₃)₃); 3.56 (m, 2H, NH-CH₂); 3.87 (t, ³J_{HH} = 8 Hz, 2H, CH₂-N-(CH₃)₃); 4.01 (m, 4H, CH₂ α); 4.39 (m, 1H, NH). ¹³C-NMR (125.80 MHz, CDCl₃): 14.20 (s, CH₃ myristyl); 19.47 to 19.75 (s, CH₃ phytanyl); 22.63 to 22.73 (s, CH₃ phytanyl); 24.40 to 24.90 (s, CH₂ phytanyl); 25.62 (s, CH₂ lauryl); 29-30 (s, CH phytanyl and CH lauryl); 30.43 (d, ³J_{CP} = 6 Hz, CH₂ β myristyl); 32.85 (s, CH phytanyl); 36.21 (s, NH-CH₂); 37.36 to 37.48 (s, CH₂ phytanyl); 40.06 (s, CH₂ phytanyl); 54.88 (s, N(CH₃)₃); 65.67 (s, CH₂ α); 66.67 (s, CH₂-N(CH₃)₃); 67.25 (d, ²J_{CP} = 6 Hz, CH₂ α). ³¹P-NMR (161.94 MHz, CDCl₃): 9.3. IR (cm⁻¹): 3431 (ν_{NH}); 1226 (ν_{P=O}); 973 (ν_{P-O-C}). MS (MALDI-TOF): m/z calcd for C₃₉H₈₄N₂O₃P (M⁺): 659.621; found 659.592.

***O*-myristyl-*O*-stearyl-*N*-(2-dimethylaminoethyl)phosphoramidate 11**

Isolated as a yellow oil (5.35 g; 66 % yield). ¹H-NMR (400.04 MHz, CDCl₃): 0.87 (t, ³J_{HH} = 8 Hz, 6H, CH₃); 1.25 (s, 52H, CH₂); 1.64 (m, 4H, CH₂ β); 2.24 (s, 6H, N-(CH₃)₂); 2.41 (t, ³J_{HH} = 4 Hz, 2H, CH₂-N-(CH₃)₂); 2.84 (m, 2H, NH-CH₂); 3.35 (m, 1H, NH); 3.97 (m, 4H, CH₂ α). ¹³C-NMR (125.80 MHz, CDCl₃): 14.13 (s, 2 x CH₃); 22.71 (s, CH₂); 25.49 (s, CH₂); 29-30 (s, CH₂); 30.43 (d, ³J_{CP} = 7 Hz, CH₂ β); 30.87 (s, CH₂); 31.94 (s, CH₂); 38.49 (s, CH₂-NH); 44.99 (s, N(CH₃)₂); 59.62 (d, ³J_{CP} = 7 Hz, CH₂-N(CH₃)₂); 66.39 (d, ²J_{CP} = 6 Hz, CH₂ α). ³¹P-NMR (161.94 MHz, CDCl₃): 10.7. IR (cm⁻¹): 3190 (ν_{NH}); 1235 (ν_{P=O}); 920 (ν_{P-O-C}). MS (MALDI-TOF): m/z calcd for C₃₆H₇₈N₂O₃P (M+H): 617.575; found 617.576.

***2*-(*O*-myristyl-*O*-stearylphosphoramidoyl)-ethyltrimethylammonium iodide 12**

Isolated as a yellow powder (0.69 g; 79 % yield). ¹H-NMR (400.04 MHz, CDCl₃): 0.87 (t, ³J_{HH} = 8 Hz, 6H, CH₃); 1.24 (s, 52H, CH₂); 1.65 (m, 4H, CH₂ β); 3.47 (s, 9H, N-(CH₃)₃); 3.54 (m, 2H, NH-CH₂); 3.85 (t, ³J_{HH} = 8 Hz, 2H, CH₂-N-(CH₃)₃); 3.99 (m, 4H, CH₂ α); 4.40 (m, 1H, NH). ¹³C-NMR (125.80 MHz, CDCl₃): 14.16 (s, 2 x CH₃); 22.68 (s, CH₂); 25.58 (s, CH₂); 29-30 (s, CH₂); 30.44 (d, ³J_{CP} = 10 Hz, CH₂ β); 31.92 (s, CH₂); 36.21 (s, NH-CH₂); 54.92 (s, N(CH₃)₃); 66.76 (s, CH₂-N(CH₃)₃); 67.19 (d, ²J_{CP} = 12 Hz, CH₂ α). ³¹P-NMR (161.94 MHz, CDCl₃): 9.4. IR (cm⁻¹): 3312 (ν_{NH}); 1240 (ν_{P=O}); 989

($\nu_{\text{P-O-C}}$). MS (MALDI-TOF) : m/z calcd for $\text{C}_{37}\text{H}_{80}\text{N}_2\text{O}_3\text{P}$ (M^+) : 631.590 ; found 631.549.

***O*-oleyl-*O*-[3-({cholest-5-en-3-yloxy}carbonyl)amino propyl]-*N*-(2-dimethylaminoethyl)phosphoramidate 13**

Isolated as a yellow oil (0.80 g ; 28 % yield). $^1\text{H-NMR}$ (400.04 MHz, CDCl_3) : 0.6-2.0 (m, 41H, CH_2 cholesteryl, $\text{CH}_2\text{-CH=CH}$ and CH_2 oleyl) ; 2.10-2.40 (m, 8H, CH_2 cholesteryl and $\text{N-(CH}_3)_2$) ; 2.54 (m, 2H, $\text{CH}_2\text{-N-(CH}_3)_2$) ; 3.05 (m, 2H, NH-CH_2) ; 3.90-4.10 (m, 4H, CH_2 α) ; 4.45 (m, 1H, CH cholesteryl) ; 5.30-5.40 (m, 3H, CH=CH and CH cholesteryl). $^{13}\text{C-NMR}$ (125.80 MHz, CDCl_3) : 11.86 (s, CH_3) ; 14.13 (s, CH_3 oleyl) ; 18.72 (s, CH_3) ; 19.34 (s, CH_3) ; 21.05 (s, CH_2) ; 22.56 (s) ; 22.69 (s) ; 22.82 (s) ; 23.84 (s) ; 24.29 (s) ; 25.61 ; 27.22 ; 28.00 ; 28.20 ; 29-31 (s, CH_2) ; 31.87 (s, CH) ; 31.91 (s, CH_2) ; 35.80 (s, CH) ; 36.19 ; 36.56 ; 37.01 ; 37.16 ; 38.34 ; 38.60 ; 39.52 ; 39.75 ; 42.32 ; 44.87 (s, $\text{N(CH}_3)_2$) ; 50.01 ; 56.14 ; 56.70 ; 59.60 (d, $^3\text{J}_{\text{CP}} = 7$ Hz, $\text{CH}_2\text{-N(CH}_3)_2$) ; 63.69 (d, $^2\text{J}_{\text{CP}} = 5$ Hz, CH_2 α) ; 66.63 (d, $^2\text{J}_{\text{CP}} = 6$ Hz, CH_2 α) ; 74.18 ; 122.40 ; 129.77 (s, CH=CH) ; 129.98 (s, CH=CH) ; 139.90 ; 156.30. $^{31}\text{P-NMR}$ (161.94 MHz, CDCl_3) : 10.6. IR (cm^{-1}) : 3331 (ν_{NH}) ; 1249 ($\nu_{\text{P=O}}$) ; 1034 ($\nu_{\text{P-O-C}}$). MS (MALDI-TOF) : m/z calcd for $\text{C}_{53}\text{H}_{98}\text{N}_3\text{O}_5\text{P}$ (M) : 888.724 ; found 888.703.

2-(*O*-oleyl-*O*-[3-({cholest-5-en-3-yloxy}carbonyl)amino propyl]phosphoramidoyl)-ethyltrimethylammonium iodide 14

Isolated as a yellow oil (0.37 g ; 100 % yield). $^1\text{H-NMR}$ (400.04 MHz, CDCl_3) : 0.6-2.0 (m, 41H, CH_2 cholesteryl, $\text{CH}_2\text{-CH=CH}$ and CH_2 oleyl) ; 2.10-2.40 (m, 2H, CH_2 cholesteryl) ; 3.44 (s, 9H, $\text{N-(CH}_3)_3$) ; 3.60 (m, 2H, NH-CH_2) ; 3.80 (m, 2H, $\text{CH}_2\text{-N-(CH}_3)_3$) ; 3.90-4.10 (m, 4H, CH_2 α) ; 4.45 (m, 1H, CH cholesteryl) ; 5.30-5.40 (m, 3H, CH=CH and CH cholesteryl). $^{13}\text{C-NMR}$ (125.80 MHz, CDCl_3) : 11.88 (s, CH_3) ; 14.15 (s, CH_3 oleyl) ; 18.74 (s, CH_3) ; 19.37 (s, CH_3) ; 21.07 (s, CH_2) ; 22.58 (s) ; 22.71 (s) ; 22.84 (s) ; 23.88 (s) ; 24.31 (s) ; 25.62 ; 27.26 ; 28.02 ; 28.25 ; 29-31 (s, CH_2) ; 31.88 (s, CH) ; 31.93 (s, CH_2) ; 35.83 (s, CH) ; 36.21 ; 36.60 ; 37.02 ; 38.64 ; 39.53 ; 39.76 ; 42.34 ; 50.02 ; 54.81 ; 56.18 ; 56.72 ; 74.43 ; 122.51 ; 129.79 (s, CH=CH) ; 129.99 (s, CH=CH) ; 139.88 ; 156.45. $^{31}\text{P-NMR}$ (161.94 MHz, CDCl_3) : 9.6. IR (cm^{-1}) : 3348 (ν_{NH}) ; 1246 ($\nu_{\text{P=O}}$) ; 1011 ($\nu_{\text{P-O-C}}$). MS (MALDI-TOF) : m/z calcd for $\text{C}_{54}\text{H}_{101}\text{N}_3\text{O}_5\text{P}$ (M^+) : 902.748 ; found 902.717.

General protocol for the synthesis of cationic lipophosphoramidates with two identical alkyl chains.

Synthesis of *O,O*-phytanyl-*N*-(2-dimethylaminoethyl)phosphoramidate 19

Method 1 (Atherton-Todd reaction): To a solution of *N,N*-Dimethylethylenediamine (0.38 g ; 4.27 mmol) in CH_2Cl_2 (20 mL) with molecular sieves was added DIPEA (1.10 g ; 8.55 mmol). The reaction mixture is stirred at room temperature for 5 min and then cooled down with an ice water bath. Diphtanylphosphite (2.50 g ; 3.89 mmol) and bromotrichloromethane (8.48 g ; 42.76 mmol) were then added

successively. The reaction mixture was stirred at 0-5°C for 1h, then at room temperature for 5h. Evaporation of the solvent produced a residue that was dissolved in diethyl ether. Filtration and evaporation of the solvent. The compound was purified by flash chromatography using dichloromethane/methanol as eluent to produce a yellow oil (22% yield ; 0.62 g).

Synthesis of *O,O*-dioleyl-*N*-(2-dimethylaminoethyl)phosphoramidate 17

Method 2: Triethylamine (5.71 mL ; 41.1 mmol) was added to a cooled (0°C) solution of POCl_3 (3.00 g ; 19.6 mmol) in diethylether (20 mL) and placed under argon atmosphere. Oleyl alcohol (10.50 g ; 39.1 mmol) was slowly added at 0°C. This solution was further stirred for 5h while the temperature was slowly increased up to 20°C. Then, a solution of *N,N*-dimethylethylenediamine (1.72 g ; 19.6 mmol) and triethylamine (2.27 mL ; 16.3 mmol) in diethylether (5 mL) was added and the solution was stirred for 1h. Finally, diethylether was added (50 mL) and the solution was washed with brine. The organic layer was dried over MgSO_4 , filtered and concentrated under reduced pressure to produce the desired compound as an oil that was engaged in the next step without any further purification (73% yield ; 9.58 g).

Synthesis of 2-(*O,O*-dioleylphosphoramidoyl)-ethyltrimethylammonium iodide 18

Methyl iodide (5.00 mL) was added to a solution of *O,O*-dioleyl-*N*-(2-dimethylaminoethyl)phosphoramidate 17 (1.0 g ; 1.49 mmol ; 1 eq) in 10 mL of CH_2Cl_2 . The mixture was stirred at R.T. for one night. The excess of methyl iodide and CH_2Cl_2 were removed under vacuum. The compound was purified by chromatography on silica gel to give the compound (68% yield ; 0.82 g).

***O,O*-dilauryl-*N*-(2-dimethylaminoethyl)phosphoramidate 15**

Isolated as a yellow oil (2.01 g ; 55 % yield) with method 1. $^1\text{H-NMR}$ (400.04 MHz, CDCl_3) : 0.87 (t, $^3\text{J}_{\text{HH}} = 8$ Hz, 6H, CH_3) ; 1.25 (s, 40H, CH_2) ; 1.66 (m, 4H, CH_2 β) ; 2.26 (s, 6H, $\text{N-(CH}_3)_2$) ; 2.45 (m, 2H, $\text{CH}_2\text{-N-(CH}_3)_2$) ; 3.05 (m, 2H, NH-CH_2) ; 3.97 (m, 4H, CH_2 α). $^{13}\text{C-NMR}$ (125.80 MHz, CDCl_3) : 14.00 (s, 2 x CH_3) ; 22.58 (s, CH_2) ; 25.51 (s, CH_2) ; 29-30 (s, CH_2) ; 30.31 (d, $^3\text{J}_{\text{CP}} = 8$ Hz, CH_2 β) ; 31.82 (s, CH_2) ; 38.35 (s, $\text{CH}_2\text{-NH}$) ; 44.84 (s, $\text{N(CH}_3)_2$) ; 59.49 (d, $^3\text{J}_{\text{CP}} = 7$ Hz, $\text{CH}_2\text{-N(CH}_3)_2$) ; 66.26 (d, $^2\text{J}_{\text{CP}} = 6$ Hz, CH_2 α). $^{31}\text{P-NMR}$ (161.94 MHz, CDCl_3) : 9.8. IR (cm^{-1}) : 3210 (ν_{NH}) ; 1230 ($\nu_{\text{P=O}}$) ; 987 ($\nu_{\text{P-O-C}}$). MS (MALDI-TOF) : m/z calcd for $\text{C}_{28}\text{H}_{62}\text{N}_2\text{O}_3\text{P}$ (M+H) : 505.450 ; found 505.378.

2-(*O,O*-dilaurylphosphoramidoyl)-ethyltrimethylammonium iodide 16

Isolated as a yellow oil (0.20 g ; 78 % yield) by following the same experimental protocol than the one used for the synthesis of 18. $^1\text{H-NMR}$ (400.04 MHz, CDCl_3) : 0.87 (t, $^3\text{J}_{\text{HH}} = 8$ Hz, 6H, CH_3) ; 1.25 (s, 36H, CH_2) ; 1.66 (m, 4H, CH_2 β) ; 3.45 (s, 9H, $\text{N-(CH}_3)_3$) ; 3.60 (m, 2H, NH-CH_2) ; 3.87 (t, $^3\text{J}_{\text{HH}} = 8$ Hz, 2H, $\text{CH}_2\text{-N-(CH}_3)_3$) ; 3.98 (m, 4H, CH_2 α) ; 4.45 (m, 1H, NH).

^{13}C -NMR (125.80 MHz, CDCl_3) : 14.16 (s, 2 x CH_3) ; 22.73 (s, CH_2) ; 25.63 (s, CH_2) ; 29-30 (s, CH_2) ; 30.47 (d, $^3J_{\text{CP}} = 6$ Hz, CH_2 β) ; 31.96 (s, CH_2) ; 36.58 (NH- CH_2) ; 54.91 (s, N(CH_3) $_3$) ; 67.06 (s, CH_2 -N(CH_3) $_3$) ; 67.25 (d, $^2J_{\text{CP}} = 5$ Hz, CH_2 α). ^{31}P -NMR (161.94 MHz, CDCl_3) : 9.3. IR (cm^{-1}) : 3260 (ν_{NH}) ; 1225 ($\nu_{\text{P=O}}$) ; 999 ($\nu_{\text{P-O-C}}$). MS (MALDI-TOF) : m/z calcd for $\text{C}_{29}\text{H}_{64}\text{N}_2\text{O}_3\text{P}$ (M^+) : 519.465 ; found 519.455.

***O,O*-dioleyl-*N*-(2-dimethylaminoethyl)phosphoramidate 17**

Isolated as a yellow oil (9.58 g ; 73 % yield) with method 2. ^1H -NMR (400.04 MHz, CDCl_3) : 0.86 (t, $^3J_{\text{HH}} = 8$ Hz, 6H, CH_3) ; 1.25 (s, 44H, CH_2) ; 1.63 (m, 4H, CH_2 β) ; 1.99 (m, 8H, CH_2 -CH=CH) ; 2.34 (s, 6H, N-(CH_3) $_2$) ; 2.55 (t, $^3J_{\text{HH}} = 4$ Hz, 2H, CH_2 -N-(CH_3) $_2$) ; 3.05 (m, 2H, NH- CH_2) ; 3.60 (m, 1H, NH) ; 3.96 (m, 4H, CH_2 α) ; 5.34 (m, 4H, CH=CH). ^{13}C -NMR (125.80 MHz, CDCl_3) : 14.06 (s, 2 x CH_3) ; 22.64 (s, CH_2) ; 25.44 (s, CH_2) ; 27.17 (s, CH=CH- CH_2) ; 29-30 (s, CH_2) ; 30.40 (d, $^3J_{\text{CP}} = 6$ Hz, CH_2 β) ; 30.76 (s, CH_2) ; 31.87 (s, CH_2) ; 38.13 (s, CH_2 -NH) ; 44.72 (s, N(CH_3) $_2$) ; 59.62 (d, $^3J_{\text{CP}} = 6$ Hz, CH_2 -N(CH_3) $_2$) ; 66.44 (d, $^2J_{\text{CP}} = 5$ Hz, CH_2 α) ; 129.72 (s, CH=CH) ; 129.91 (s, CH=CH). ^{31}P -NMR (161.94 MHz, CDCl_3) : 10.5. IR (cm^{-1}) : 1231 ($\nu_{\text{P=O}}$) ; 965 ($\nu_{\text{P-O-C}}$). MS (MALDI-TOF) : m/z calcd for $\text{C}_{40}\text{H}_{82}\text{N}_2\text{O}_3\text{P}$ ($\text{M}+\text{H}$) : 669.606 ; found 669.590.

***2-(O,O*-dioleylphosphoramidoyl)-ethyltrimethylammonium iodide 18**

Isolated as a yellow oil (0.82 g ; 68 % yield) by following the same experimental protocol than the one used for the synthesis of **18**. ^1H -NMR (400.04 MHz, CDCl_3) : 0.87 (t, $^3J_{\text{HH}} = 8$ Hz, 6H, CH_3) ; 1.25 (s, 44H, CH_2) ; 1.65 (m, 4H, CH_2 β) ; 1.99 (m, 8H, CH_2 -CH=CH) ; 3.47 (s, 9H, N-(CH_3) $_3$) ; 3.55 (m, 2H, NH- CH_2) ; 3.86 (t, $^3J_{\text{HH}} = 4$ Hz, 2H, CH_2 -N-(CH_3) $_3$) ; 3.99 (m, 4H, CH_2 α) ; 4.45 (m, 1H, NH) ; 5.34 (m, 4H, CH=CH). ^{13}C -NMR (125.80 MHz, CDCl_3) : 14.15 (s, 2 x CH_3) ; 22.71 (s, CH_2) ; 25.39 (s, CH_2) ; 27.26 (s, CH=CH- CH_2) ; 29-30 (s, CH_2) ; 30.41 (d, $^3J_{\text{CP}} = 6$ Hz, CH_2 β) ; 31.93 (s, CH_2) ; 36.20 (s, CH_2 -NH) ; 55.01 (s, N(CH_3) $_3$) ; 66.76 (d, $^3J_{\text{CP}} = 6$ Hz, CH_2 -N(CH_3) $_3$) ; 67.44 (d, $^2J_{\text{CP}} = 5$ Hz, CH_2 α) ; 129.77 (s, CH=CH) ; 130.11 (s, CH=CH). ^{31}P -NMR (161.94 MHz, CDCl_3) : 8.9. IR (cm^{-1}) : 1236 ($\nu_{\text{P=O}}$) ; 970 ($\nu_{\text{P-O-C}}$). MS (MALDI-TOF) : m/z calcd for $\text{C}_{41}\text{H}_{84}\text{N}_2\text{O}_3\text{P}$ (M^+) : 683.622 ; found 683.607.

***O,O*-phytanyl-*N*-(2-dimethylaminoethyl)phosphoramidate 19**

Isolated as a yellow oil (0.62 g ; 22 % yield) with method 1. ^1H -NMR (400.04 MHz, CDCl_3) : 0.87 (m, 30H) ; 1.00-1.70 (m, 48H, CH_2 phytanyl) ; 2.59 (s, 6H, N-(CH_3) $_2$) ; 2.82 (m, 2H, CH_2 -N-(CH_3) $_2$) ; 3.24 (m, 2H, NH- CH_2) ; 4.05 (m, 4H, CH_2 α). ^{13}C -NMR (125.80 MHz, CDCl_3) : 19.40 to 19.80 (s, CH_3 phytanyl) ; 22.64 (s, CH_3 phytanyl) ; 22.73 (s, CH_3 phytanyl) ; 24.36 (s, CH_2 phytanyl) ; 24.50 (s, CH_2 phytanyl) ; 24.81 (s, CH_2 phytanyl) ; 28.12 (s, CH phytanyl) ; 29.50 (s, CH phytanyl) ; 32.80 (s, CH phytanyl) ; 37.30 to 37.50 (s, CH_2 phytanyl) ; 38.20 (s, NH- CH_2) ; 39.38 (s, CH_2 phytanyl) ; 44.77 (s, N(CH_3) $_2$) ; 59.58 (s, CH_2 -N(CH_3) $_2$) ; 64.86 (d, $^2J_{\text{CP}} = 5$ Hz, CH_2 α). ^{31}P -NMR (161.94 MHz, CDCl_3) : 10.2. IR (cm^{-1}) : 3219

(ν_{NH}) ; 1229 ($\nu_{\text{P=O}}$) ; 980 ($\nu_{\text{P-O-C}}$). MS (MALDI-TOF) : m/z calcd for $\text{C}_{44}\text{H}_{94}\text{N}_2\text{O}_3\text{P}$ ($\text{M}+\text{H}$) : 729.700 ; found 729.677.

***2-(O,O*-diphytanylphosphoramidoyl)-ethyltrimethylammonium iodide 20**

Isolated as a yellow oil (0.12 g ; 100 % yield) by following the same experimental protocol than the one used for the synthesis of **18**. ^1H -NMR (400.04 MHz, CDCl_3) : 0.87 (m, 30H) ; 1.00-1.70 (m, 48H, CH_2 phytanyl) ; 3.46 (s, 9H, N-(CH_3) $_3$) ; 3.55 (m, 2H, NH- CH_2) ; 3.87 (t, $^3J_{\text{HH}} = 8$ Hz, 2H, CH_2 -N-(CH_3) $_3$) ; 4.02 (m, 4H, CH_2 α) ; 4.41 (m, 1H, NH). ^{13}C -NMR (125.80 MHz, CDCl_3) : 19.40 to 19.80 (s, CH_3 phytanyl) ; 22.70 to 22.80 (s, CH_3 phytanyl) ; 24.40 to 24.90 (s, CH_2 phytanyl) ; 28.02 (s, CH phytanyl) ; 29.39 (s, CH phytanyl) ; 32.85 (s, CH phytanyl) ; 36.57 (s, NH- CH_2) ; 37.20 to 37.60 (s, CH_2 phytanyl) ; 39.41 (s, CH_2 phytanyl) ; 55.01 (s, N(CH_3) $_3$) ; 65.74 (d, $^2J_{\text{CP}} = 5$ Hz, CH_2 α) ; 66.80 (s, CH_2 -N(CH_3) $_3$). ^{31}P -NMR (161.94 MHz, CDCl_3) : 9.3. IR (cm^{-1}) : 3237 (ν_{NH}) ; 1228 ($\nu_{\text{P=O}}$) ; 973 ($\nu_{\text{P-O-C}}$). MS (MALDI-TOF) : m/z calcd for $\text{C}_{45}\text{H}_{96}\text{N}_2\text{O}_3\text{P}$ (M^+) : 743.716 ; found 743.693.

***O,O*-dimyristyl-*N*-(2-dimethylaminoethyl)phosphoramidate 21**

Isolated as a yellow oil (6.65 g ; 91 % yield) with method 2. ^1H -NMR (400.04 MHz, CDCl_3) : 0.87 (t, $^3J_{\text{HH}} = 8$ Hz, 6H, CH_3) ; 1.24 (s, 44H, CH_2) ; 1.66 (m, 4H, CH_2 β) ; 2.31 (s, 6H, N-(CH_3) $_2$) ; 2.50 (m, 2H, CH_2 -N-(CH_3) $_2$) ; 3.05 (m, 2H, NH- CH_2) ; 3.97 (m, 4H, CH_2 α). ^{13}C -NMR (125.80 MHz, CDCl_3) : 14.06 (s, 2 x CH_3) ; 22.64 (s, CH_2) ; 25.57 (s, CH_2) ; 29-30 (s, CH_2) ; 30.36 (d, $^3J_{\text{CP}} = 6$ Hz, CH_2 β) ; 31.89 (s, CH_2) ; 32.83 (s, CH_2) ; 38.47 (s, CH_2 -NH) ; 44.95 (s, N(CH_3) $_2$) ; 59.60 (d, $^3J_{\text{CP}} = 6$ Hz, CH_2 -N(CH_3) $_2$) ; 66.30 (d, $^2J_{\text{CP}} = 5$ Hz, CH_2 α). ^{31}P -NMR (161.94 MHz, CDCl_3) : 10.5. IR (cm^{-1}) : 1232 ($\nu_{\text{P=O}}$) ; 990 ($\nu_{\text{P-O-C}}$). MS (MALDI-TOF) : m/z calcd for $\text{C}_{32}\text{H}_{70}\text{N}_2\text{O}_3\text{P}$ ($\text{M}+\text{H}$) : 561.512 ; found 561.512.

***2-(O,O*-dimyristylphosphoramidoyl)-ethyltrimethylammonium iodide 22**

Isolated as a white powder (0.32 g ; 81 % yield) by following the same experimental protocol than the one used for the synthesis of **18**. ^1H -NMR (400.04 MHz, CDCl_3) : 0.88 (t, $^3J_{\text{HH}} = 8$ Hz, 6H, CH_3) ; 1.25 (s, 44H, CH_2) ; 1.65 (m, 4H, CH_2 β) ; 3.45 (s, 9H, N-(CH_3) $_3$) ; 3.58 (m, 2H, NH- CH_2) ; 3.84 (t, $^3J_{\text{HH}} = 8$ Hz, 2H, CH_2 -N-(CH_3) $_3$) ; 3.98 (m, 4H, CH_2 α). ^{13}C -NMR (125.80 MHz, CDCl_3) : 14.14 (s, 2 x CH_3) ; 22.71 (s, CH_2) ; 25.63 (s, CH_2) ; 29-30 (s, CH_2) ; 30.47 (d, $^3J_{\text{CP}} = 6$ Hz, CH_2 β) ; 31.95 (s, CH_2) ; 36.38 (s, CH_2 -NH) ; 54.94 (s, N(CH_3) $_3$) ; 66.95 (d, $^3J_{\text{CP}} = 6$ Hz, CH_2 -N(CH_3) $_3$) ; 67.23 (d, $^2J_{\text{CP}} = 5$ Hz, CH_2 α). ^{31}P -NMR (161.94 MHz, CDCl_3) : 9.4. IR (cm^{-1}) : 1240 ($\nu_{\text{P=O}}$) ; 988 ($\nu_{\text{P-O-C}}$). MS (MALDI-TOF) : m/z calcd for $\text{C}_{33}\text{H}_{72}\text{N}_2\text{O}_3\text{P}$ (M^+) : 575.528 ; found 575.484.

***O,O*-stearyl-*N*-(2-dimethylaminoethyl)phosphoramidate 23**

Isolated as a yellow wax (6.86 g ; 78 % yield) with method 2. ^1H -NMR (400.04 MHz, CDCl_3) : 0.87 (t, $^3J_{\text{HH}} = 8$ Hz, 6H, CH_3) ; 1.25 (s, 60H, CH_2) ; 1.65 (m, 4H, CH_2 β) ; 2.26 (s, 6H, N-(CH_3) $_2$) ; 2.45 (m, 2H, CH_2 -N-(CH_3) $_2$) ; 3.05 (m, 2H, NH- CH_2)

; 3.97 (m, 4H, $\underline{\text{CH}_2}$ α). ^{13}C -NMR (125.80 MHz, CDCl_3) : 14.07 (s, 2 x $\underline{\text{CH}_3}$) ; 22.66 (s, $\underline{\text{CH}_2}$) ; 25.59 (s, $\underline{\text{CH}_2}$) ; 29-30 (s, $\underline{\text{CH}_2}$) ; 30.38 (d, $^3\text{J}_{\text{CP}} = 7$ Hz, $\underline{\text{CH}_2}$ β) ; 30.87 (s, $\underline{\text{CH}_2}$) ; 31.90 (s, $\underline{\text{CH}_2}$) ; 38.27 (s, $\underline{\text{CH}_2}$ -NH) ; 44.78 (s, N($\underline{\text{CH}_3}$) $_2$) ; 59.52 (d, $^3\text{J}_{\text{CP}} = 6$ Hz, $\underline{\text{CH}_2}$ -N($\underline{\text{CH}_3}$) $_2$) ; 66.35 (d, $^2\text{J}_{\text{CP}} = 5$ Hz, $\underline{\text{CH}_2}$ α). ^{31}P -NMR (161.94 MHz, CDCl_3) : 10.7. IR (cm^{-1}) : 3300 (ν_{NH}) ; 1220 ($\nu_{\text{P=O}}$) ; 994 ($\nu_{\text{P-O-C}}$). MS (MALDI-TOF) : m/z calcd for $\text{C}_{40}\text{H}_{86}\text{N}_2\text{O}_3\text{P}$ (M+H) : 673.638 ; found 673.652.

2-(*O,O*-distearylphosphoramidoyl)-ethyltrimethylammonium iodide 24

Isolated as a pale yellow wax (0.45 g ; 72 % yield) by following the same experimental protocol than the one used for the synthesis of **18**. ^1H -NMR (400.04 MHz, CDCl_3) : 0.87 (t, $^3\text{J}_{\text{HH}} = 8$ Hz, 6H, $\underline{\text{CH}_3}$) ; 1.25 (s, 60H, $\underline{\text{CH}_2}$) ; 1.65 (m, 4H, $\underline{\text{CH}_2}$ β) ; 3.46 (s, 9H, N-($\underline{\text{CH}_3}$) $_3$) ; 3.56 (m, 2H, NH- $\underline{\text{CH}_2}$) ; 3.87 (t, $^3\text{J}_{\text{HH}} = 4$ Hz, 2H, $\underline{\text{CH}_2}$ -N-($\underline{\text{CH}_3}$) $_3$) ; 3.99 (m, 4H, $\underline{\text{CH}_2}$ α) ; 4.45 (m, 1H, NH). ^{13}C -NMR (125.80 MHz, CDCl_3) : 14.14 (s, 2 x $\underline{\text{CH}_3}$) ; 22.71 (s, $\underline{\text{CH}_2}$) ; 25.60 (s, $\underline{\text{CH}_2}$) ; 29-30 (s, $\underline{\text{CH}_2}$) ; 30.42 (d, $^3\text{J}_{\text{CP}} = 7$ Hz, $\underline{\text{CH}_2}$ β) ; 31.95 (s, $\underline{\text{CH}_2}$) ; 36.23 (NH- $\underline{\text{CH}_2}$) ; 54.92 (s, N($\underline{\text{CH}_3}$) $_3$) ; 66.78 (s, $\underline{\text{CH}_2}$ -N($\underline{\text{CH}_3}$) $_3$) ; 67.25 (d, $^2\text{J}_{\text{CP}} = 6$ Hz, $\underline{\text{CH}_2}$ α). ^{31}P -NMR (161.94 MHz, CDCl_3) : 9.3. IR (cm^{-1}) : 3308 (ν_{NH}) ; 1240 ($\nu_{\text{P=O}}$) ; 988 ($\nu_{\text{P-O-C}}$). MS (MALDI-TOF) : m/z calcd for $\text{C}_{41}\text{H}_{88}\text{N}_2\text{O}_3\text{P}$ (M^+) : 687.653 ; found 687.622.

Liposomes formulation

The cationic lipids were formulated as liposomal solution by using the hydration of a lipid film. First, an aliquot of a concentrated solution of a desired lipid in chloroform (e.g. **2**, 22 mg, 1.0 mL) was placed in a glass tube and the solvent was evaporated to produce a thin lipid film. Then 1 mL of water was added on this film. After a hydration period (12 h except for compound **20** : 2 days) at 4°C the solution was vortexed (1 min) and sonicated (6 x 10 min) at 45 kHz using a VWR ultrasonic bath. The size and the zeta potential of these formulation were determined for each liposomal solution.

DNA Condensation

Liposomal solutions were mixed with pDNA (3,7kb, pCMV-*Luc*) as follows : to 1 μg of pDNA in Optimem (Gibco) were added cationic lipids at concentrations corresponding to +/- charge ratios (CR) ranging from 1 to 8. These mixtures were incubated for 30 min at RT. The complexes were subjected to electrophoresis in 0.8% agarose gel at 100 V, 90 mA. The gel was stained with ethidium bromide (10 mg/mL) in order to be thereafter visualized using an UV transilluminator (Fischer Bioblock).

Notes and references

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In vitro reporter gene measurements: luciferase assays.

Cells were grown in MEM (16HBE, CFBE and SKMel28), or DMEM (A549), supplemented with 10% bovine fetal serum, 1% antibiotic or 0.4% for the SKMel28, and 1% L-Glutamine, in a humidified incubator with 5% CO_2 at 37°C.

The protocol of the experiments was similar to those previously reported.^{26a} Shortly, cells were seeded 24 h before transfection onto a 24-well plate at a density of 100,000 cells per well and incubated overnight in a humidified 5% CO_2 atmosphere at 37 °C. About 200 μL of a solution of lipoplex was added to each well. Lipofectamine (LFM) was used as a positive control. After 2h30 of incubation at 37 °C, 20 μL of the surrounding was picked up to evaluate the toxicity of each formulation (see below) The culture medium was removed to be replaced with fresh. Following a further 48 h incubation at 37 °C, the cells were lysed in order to be assayed for luciferase expression using a chemiluminescent assay (Luciferase Assay System, Promega). The total protein content of the cell lysate was determined using the BC assay kit (Uptima). Data are expressed as relative light units (RLU) per milligram of total proteins (mean \pm SD with n = 3).

Evaluation of early toxicity

The toxicity of the different lipid/DNA complexes was determined by using a chemiluminescent assay (Toxilight - Cambrex, Liège, Belgium). Briefly, this test consists of the measurements of the release of a normally cytoplasmic enzyme (adenylate kinase, AK) from damaged cells into the surrounding medium. The reaction involves two steps. The first one requires the addition of ADP as a substrate for AK. In the presence of AK, the ADP is converted to ATP for assay by bioluminescence. Then, the bioluminescence method utilizes an enzyme Luciferase, which catalyzes the emission of photons from ATP and luciferin. By combining both reactions, the emitted light intensity is directly related to the AK concentration. The relative light units (RLU) are conversely proportional to the intensity of damages induced to the cells consequently of their exposure with the complexes studied. Untransfected cells were used to evaluate the AK background activity indicative of an absence of toxicity.

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† Footnotes should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

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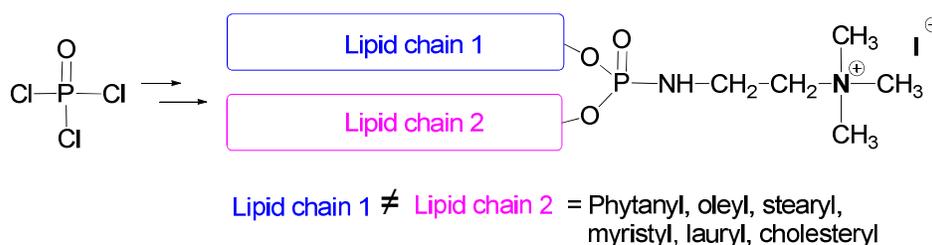
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Graphical abstract

Cationic lipophosphoramidates with two different lipid chains: synthesis and evaluation as gene carriers.

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The synthesis of cationic lipids possessing two different lipid chains is reported. Their capacity to carry DNA was systematically compared with cationic lipid possessing two identical lipid domains. The association of a phytanyl chain with a lauryl or oleyl chain produced very efficient vectors for gene delivery.