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Lipophosphoramidates as Lipidic Part of Lipospermines for Gene Delivery

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The DNA compacting properties of polyamines (and especially spermine) are well-known, hence the use of spermine as the cationic part in several synthetic DNA carriers. Here, we describe the synthesis of modified spermines, with a "lipophosphoramidate" as the lipidic part, and their use for *in vitro* transfection efficacy. Physicochemical measurements (particle size, zeta potentials, pK_a determination) and gel retardation assays were also performed. Theoretical membrane-disrupting ability was established by FRET. The set of results indicates that, on the whole, lipophosphoramidates constitute an interesting alternative to "classical" lipidic parts of cationic lipid used as DNA carriers.

INTRODUCTION

Together with polymers, cationic lipids hold the most important place among synthetic carriers for gene delivery. In these vectors, the lipidic part generally consists of a cholesteryl group or two $C_{12}-C_{20}$ saturated or unsaturated hydrocarbon chains. To the first category belong, for instance, the commercial DC-Chol (*1*), BGTC (a bis-guanidinium cholesterol derivative) (*2*), and Lipid 67 (a spermine cholesterol derivative) (*3*) (Scheme 1).

Chronologically, DOTMA (*4*) is the first representative of the second category, and since 1987, several dozen cationic lipids including two hydrocarbon chains, with various spacers and polar heads, have been published (*5*, *6*). Some representative examples are given in Scheme 2.

Because of its compacting properties, the naturally occurring spermine was used as the polar head in several carriers, and among them, apart from Lipid 67 already quoted, appear DOGS (*7*) and DOSPA, the cationic part of Lipofectamine (*8*). Since lipophosphoramidates proved to be efficient for *in vivo* gene delivery when associated with ammonium, phosphonium, or arsonium cations (*9*), we wondered about their efficiency when associated to spermine as the cationic part.

EXPERIMENTAL PROCEDURES

Syntheses of Lipidic Spermines. Solvents were freshly distilled on appropriate driers and reactions run under nitrogen atmosphere. All compounds were fully characterized by ^{1}H (400 MHz), ^{13}C (100 MHz), and ^{31}P (121.49 MHz) NMR spectroscopy (Bruker AC 300 and Advance DRX 400 spectrometers). Coupling constants *J* are given in hertz. The following abbreviations were used: s for singlet, d doublet, t triplet, q quadruplet, qt quintuplet, and m for multiplet. When needed, ^{13}C heteronuclear HMQC and HMBC were used to unambiguously establish structures.

N^4-(3-Phtalimidopropyl)-N^1,N^8,N^{12}-tris-tert-butyloxycarbonyl−Spermine ***6***. SpermineBoc$_3$ (1 g, 2 mmol, 1 equiv) and *N*-(3-bromopropyl)phthalimide (666 mg, 2.48 mmol, 1.25 equiv) were dissolved in dry acetonitrile (30 mL) with an excess of K_2CO_3 (1.37 g, 9.95 mmol, 5 equiv). The mixture was stirred for 18 h under reflux. Then, acetonitrile was evaporated under vacuum, and the crude product was dissolved in methylene chloride (50 mL). The organic layer was washed with $NaHCO_3$ (2 × 50 mL) and water (50 mL), dried with $MgSO_4$, and evaporated to give after purification by column chromatography ($CHCl_3$/EtOH 100:0 to 80:20) 910 mg of compound **6** (66%). R_f = 0.50 ($CHCl_3$/EtOH 90:10). ^{1}H NMR (DMSO-d_6): 7.84 (m, 2H), 7.82 (m, 2H), 6.70 (m, 2H), 3.57 (t, *J* = 7.2, 2H), 3.07 (m, 4H), 2.89 (m, 2H), 2.87 (m, 2H), 2.37 (t, *J* = 6.6, 2H), 2.30 (t, *J* = 6.8, 2H), 2.29 (t, *J* = 6.8, 2H), 1.68 (qt, *J* = 7.2, *J* = 14.1, 2H), 1.53 (m, 2H), 1.44 (qt, *J* = 6.7, *J* = 13.2, 2H), 1.37 (m, 2H), 1.34 (m, 27H), 1.27 (qt, *J* = 6.7, *J* = 13.9, 2H). ^{13}C NMR (DMSO-d_6): 167.9, 155.5, 154.6, 134.3, 131.6, 122.9, 78.2, 77.4, 77.2, 52.9, 51.1, 50.9, 44.3, 38.3, 37.6, 36.0, 28.4, 28.2, 28.1, 28.0, 26.8, 25.7, 23,7.

N^4-(3-Aminopropyl)-N^1,N^8,N^{12}-tris-tert-butyloxycarbonyl−Spermine ***7***. Compound **6** (880 mg, 1.28 mmol, 1 equiv) and an excess of hydrazine monohydrate (1.98 mL, 64 mmol, 50 equiv) were dissolved in THF/EtOH (80:20; 20 mL). The mixture was stirred 5 h at 80 °C and then 18 h at 50 °C. After evaporation under reduced pressure, the residue was dissolved in $CHCl_3$ (50 mL) and washed with 5% sodium hydroxide solution (2 × 50 mL). The organic phase was separated, dried over anhydrous magnesium sulfate, filtered, concentrated, and subjected to flash chromatography using 3% NH_4OH/MeOH to give the desired amine **7** (573 mg, 80%). R_f = 0.42 (MeOH + 5% NH_4OH). ^{1}H NMR (DMSO-d_6): 6.70 (m, 2H), 3.08 (m, 4H), 2.90 (m, 2H), 2.87 (m, 2H), 2.53 (m, 2H), 2.35 (m, 2H), 2.30 (m, 2H), 2.29 (m, 2H), 1.58 (m, 2H), 1.51 (m, 2H), 1.44 (m, 2H), 1.37 (m, 2H), 1.35 (m, 27H), 1.29 (m, 2H). ^{13}C NMR (DMSO-d_6): 155.5, 154.6, 78.2, 77.4, 77.2, 53.1, 51.5, 51.1, 48.6, 48.6, 39.8, 38.4, 37.6, 28.9, 28.7, 28.2, 28.0, 27.0, 26.2, 23.8.

General Procedure for the Synthesis of Phosphoramidate−Spermines ***3*** *and* ***9***. Fatty phosphites (1 equiv) and 1 equiv of triprotected spermine **1** or 3-aminopropylspermine **7** were dissolved in anhydrous CH_2Cl_2. Then, 10 equiv of CCl_4 and 1.5 equiv of DIPEA were added. The reaction was carried out under a nitrogen atmosphere and stirred for 1 h at 0 °C and

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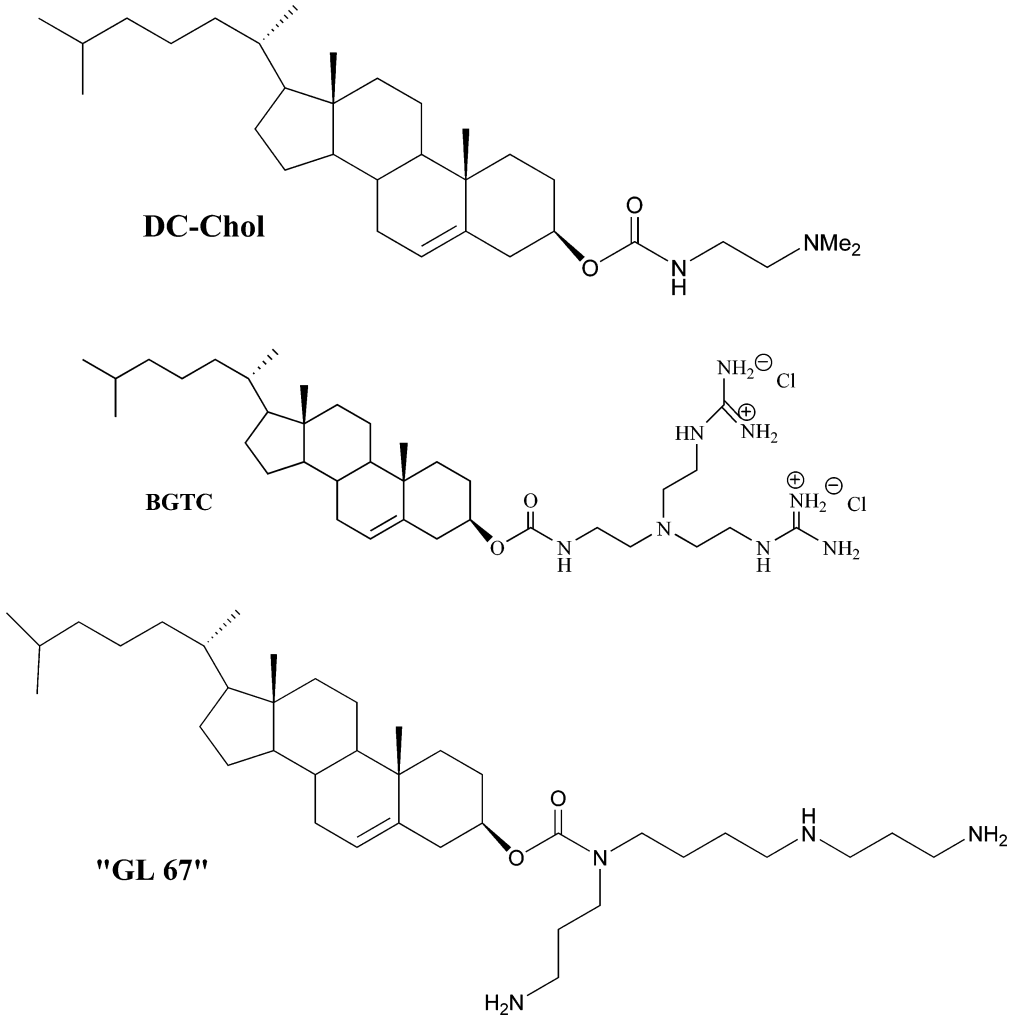
Scheme 1. Some Structures of Cationic Lipids with a Cholesteryl Lipidic Moiety



then for 18 h at room temperature. After evaporation under reduced pressure, the residue was dissolved in $CHCl_3$ (50 mL) and washed with water (2 × 50 mL). The organic phase was separated, dried over anhydrous magnesium sulfate, filtered, and evaporated to give after purification by column chromatography ($CHCl_3$/EtOH 100:0 to 50:50) the desired compounds **2** (**2a**, R = $C_{14}H_{29}$; **2b**, R = $C_{18}H_{35}$) and **8** (**8a**, R = $C_{14}H_{29}$; **8b**, R = $C_{18}H_{35}$).

NMR data for **2a**: ^{1}H ($CDCl_3$) 5.28 (s, 2H), 3.95 (m, 4H), 3.22−2.94 (m, 12H), 1.80−1.25 (m, 12H), 1.44 (m, 27H), 1.30−1.25 (m, 44H), 0.80 (t, J = 6.7, 6H). ^{31}P ($CDCl_3$) 11.25.

NMR data for **2b**: ^{1}H ($CDCl_3$) 5.32 (s, 4H), 4.05 (m, 4H), 3.25−2.93(m, 12H), 1.99 (m, 8H), 1.57−1.64 (m, 12H), 1.44 (m, 27H), 1.29−1.20 (m, 44H), 0.88 (t, J = 6.7 Hz, 6H). ^{31}P ($CDCl_3$) 11.85.

NMR data for **8a**: ^{1}H ($CDCl_3$) 5.28 (s, 2H), 3.95 (m, 4H) 3.42−2.94 (m, 10H), 2.42 (m, 6H), 1.80−1.64 (m, 14H) 1.44 (m, 27H), 1.30−1.25 (m, 44H) 0.80 (t, J = 6.7, 6H). ^{31}P ($CDCl_3$) 9.71.

NMR data for **8b**: ^{1}H ($CDCl_3$) 5.34 (s, 4H), 3.98 (m, 4H), 3.15−2.95 (m, 10H), 2.43 (m, 6H), 1.99 (m, 8H), 1.80−1.64 (m, 14H), 1.43 (m, 27H), 1.29−1.20 (m, 44H), 0.89 (t, J = 6.7, 6H). ^{31}P ($CDCl_3$) 9.70.

Protected phosphoramidates (**2a**,**b** or **8a**,**b**) were then deprotected into a CH_2Cl_2−CF_3CO_2H (1/1 v/v) mixture during 2 h at room temperature (RT). After washing with water, then drying ($MgSO_4$), solvent was split off. Phosphoramidates−spermines **3** (**3a**, R = $C_{14}H_{29}$; **3b**, R = $C_{18}H_{35}$) and **9** (**9a**, R = $C_{14}H_{29}$; **9b**, R = $C_{18}H_{35}$) were obtained quantitatively as their trifluoroacetic salts.

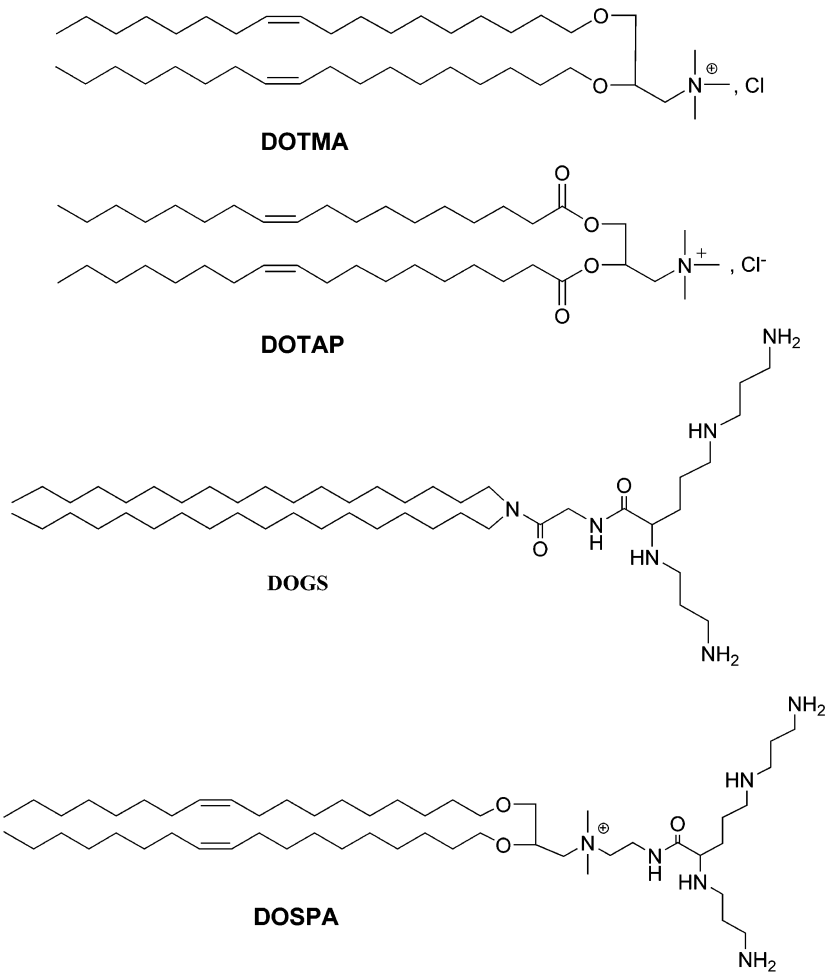
NMR data for **3a**: ^{1}H (DMSO-d_6) 3.83 (m, 4H), 2.99 (m, 2H), 2.96 (m, 2H), 2.89 (m, 2H), 2.87 (m, 2H), 2.86 (m, 2H), 2.76 (t, J = 7.5, 2H), 1.98 (q, J = 7.3, 2H), 1.75 (q, J = 6.9, 2H), 1.59 (m, 2H), 1.55 (m, 4H), 1.53 (m, 2H), 1.22 (m, 44H), 0.80 (t, J = 6,7, 6H). ^{13}C (DMSO-d_6) 65.9, 47.1, 45.5, 44.3, 43.7, 37.1, 36.7, 26.9, 24, 23.3, 30.5, 30−20, 13.9. ^{31}P (DMSO-d6) 11.21.

NMR data for **3b**: ^{1}H (DMSO-d_6) 5.3 (m, 4H), 3.81 (m, 4H), 2.98 (m, 2H), 2.85 (m, 2H), 2.83 (m, 2H), 2.75 (m, 2H), 2.63 (m, 2H), 2.60 (m, 2H), 1.96 (m, 8H), 1.72 (m, 2H), 1.70 (m, 2H), 1.55 (m, 4H), 1.46 (m, 2H), 1.38 (m, 2H), 1.22 (m, 44H), 0.84 (t, J = 6.9, 6H). ^{13}C (DMSO-d_6) 129.6, 65.5, 48.2, 46.2, 45.1, 42.2, 39.7, 36.7, 29.7, 27.5, 27.1, 26.5, 25.1, 24.7, 30−20, 13.8. ^{31}P (DMSO-d_6) 10.87.

NMR data for **9a**: ^{1}H (DMSO-d_6) 5.74 (m, 1H), 3.81 (m, 4H), 2.85 (t, J = 7.2, 2H), 2.77 (t, J = 7.4, 2H), 2.74 (m, 2H), 2.72 (m, 2H), 2.63 (m, 2H), 2.38 (t, J = 6.5, 2H), 2.36 (t, J = 6.5, 2H), 2.33 (t, J = 6.8, 2H), 1.70 (m, 2H), 1.61 (m, 2H), 1.53 (m, 2H), 1.48 (m, 2H), 1.40 (m, 2H), 1.37 (m, 2H), 1.22 (m, 44H), 0.84 (t, J = 6.6, 6H). ^{13}C (DMSO-d_6) 65.8, 52.7, 50.5, 50.2, 48.5, 45.7, 39.5, 37.5, 37.2, 29.7, 28.9, 28.8, 28.7, 26.1, 24.7, 30−20, 14. ^{31}P (DMSO-d_6) 10.20.

NMR data for **9b**: ^{1}H (DMSO-d_6) 5.3 (m, 4H), 4.81 (m, 1H), 3.80 (m, 4H), 2.85 (m, 2H), 2.82 (m, 2H), 2.76 (m, 2H), 2.74 (m, 2H), 2.72 (m, 2H), 2.40 (m, 2H), 2.38 (m, 2H), 2.33 (m, 2H), 1.96 (m, 8H), 1.78 (m, 2H), 1.62 (m, 2H), 1.55 (m, 2H), 1.49(m, 2H), 1.48 (m, 2H), 1.39 (m, 2H), 1.22 (m, 44H), 0.84 (t, J = 6.6, 6H). ^{13}C (DMSO-d_6) 129.6, 65, 52.6, 50.7, 47.5, 44.9, 39, 37.3, 37, 29.9, 28.9, 28.8, 28.7, 26.5, 25, 24.4, 30−20, 13.8. ^{31}P (DMSO-d_6) 10.72.

Scheme 2. Some Cationic Lipids with a Double-Tailed Hydrocarbon Chain



*N^4-Chlolesteryl−Spermine **5***. To a mixture of 0.688 g of triBocSpermine **1** (1.37 mmol) and 0.360 mL (2.06 mmol) of diisopropylethylamine (DIPEA) into CH_2Cl_2 (10 mL) at 0 °C is added a solution of 0.614 g of cholesterylchloroformate (1.37 mmol) into CH_2Cl_2 (10 mL). The mixture is kept 1 h at 0 °C, then 18 h at RT under nitrogen atmosphere. After washing (H_2O) and drying ($MgSO_4$), the solvent is split off. Purification was accomplished by column chromatography (silica gel), eluting with a gradient of 5−20% EtOH in CH_2Cl_2, and led to 0.8 g (64%) of the triprotected compound **4** as white crystals. ^{1}H NMR ($CDCl_3$) 5.37 (s, 1H), 5.29 (s, 2H), 4.50 (m, 1H), 3.40−3.05 (m, 12H), 2.38−2.20 (m, 2H), 2.10−1.05 (m, 61H), 1.01 (s, 3H), 0.91 (m, 3H), 0.86 (d, J = 6.5, 6H), 0.68 (s, 3H).

Compound **4** was then deprotected as described above, and the cholesterylspermine **5** was thus obtained quantitatively. ^{1}H NMR ($CDCl_3$) 5.37 (s, 1H), 4.50 (m, 1H), 3.40−3.05 (m, 12H), 2.32−2.15 (m, 2H), 2.10−0.94 (m, 34H) 0.99 (s, 3H), 0.91 (m, 3H) 0.86 (d, J = 7, 6H), 0.67 (s, 3H).

*N^4-Chlolesteryl-(4-amidopropyl)−Spermine **11***. From 0.874 g of cholesterylchloroformate (1.95 mmol), 1.09 g of triprotected 4-aminopropylspermine **7** (1.95 mmol), and 0.5 mL of DIPEA (2.92 mmol) into 30 mL of CH_2Cl_2, with the same conditions as for the synthesis of **5**, 1.23 g (1.26 mmol, 65%) of the triprotected derivative **10** is obtained as white crystals after purification by column chromatography (silica gel, eluting with a gradient of 5−50% EtOH in CH_2Cl_2). ^{1}H NMR ($CDCl_3$) 5.37 (s, 1H), 5.29 (s, 2H), 4.48 (m, 1H), 3.22−2.95 (m, 10H), 2.43−2.20 (m, 8H), 2.05−1.01 (m, 63H), 1.01 (s, 3H), 0.91 (m, 3H), 0.86 (d, J = 6.5, 6H), 0.68 (s, 3H).

Deprotection in a CF_3CO_2H/CH_2Cl_2 mixture as described above led quantitatively to cholesteryl derivative **11**. ^{1}H NMR ($CDCl_3$) 5.37 (s, 1H), 4.48 (m, 1H), 3.22−2.90 (m, 10H), 2.43−2.15 (m, 8H), 2.05−1.01 (m, 36H), 1.01 (s, 3H), 0.91 (m, 3H), 0.86 (d, J = 6.5, 6H), 0.68 (s, 3H).

***In Vitro* Experiments.** *Cell Culture.* Two different cell lines (A549 and HeLa) were used. The alveolar type II epithelial cell line A549 (*19*) and the human adenocarcinoma epithelial cell line HeLa were obtained from the American Type Culture Collection (respectively, cat. no. CCL-185 and ccl-2, ATCC, Rockville, MD). These cells were grown in D-MEM (Gibco-BRL, UK) and supplemented with 10% fetal calf serum (FCS) (Gibco-BRL, UK) and 100 U/mL of penicillin/streptomycin. All the cells were maintained in a humidified incubator with 5% CO_2 at 37 °C.

Complex Formulation. Each of cationic lipids was prepared alone or in combination with the neutral colipid DOPE (w/w = 1:1). Lipids were kept and formulated in chloroform solutions; then, chloroform was removed by rotary evaporation to produce dried lipid films. A total of 1 mL of sterile pyrogen-free DI water per milligram of lipid was added, and the vials were sealed and stored at 4 °C overnight. Vesicles were then prepared by sonicating the aqueous suspension for 10 min in a bath sonicator.

Cationic lipid/DNA complexes were formed as follows: plasmid DNA (1 μg) was diluted with sterile pyrogen-free deionized water and added to the lipid solution in a polystyrene tube. Lipoplexes were kept at room temperature for 30 min before being used for transfection.

Determination of the Lipoplex Charge Ratio. The charge ratio was calculated theoretically as the molar ratio of the cationic lipids to DNA. In order to study the impact of the formulation on gene expression, various charge ratios (±) were prepared with a constant amount of pDNA (1 μg). As a control, 2 μL of

Lipofectamine (Invitrogen) was used, accordingly to manufacturer's instructions, and in these conditions, for 1 μg of delivered DNA, the N/P ratio is equal to 2.

Transfection Protocol. 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. Transfection was performed as described by Felgner et al. (*4*) with the following modifications. Appropriate amounts of the cationic lipids and the plasmid vector in OptiMem were complexed, and about 200 μL was added to each well. After 2 h 30 min incubation at 37 °C, the medium was removed, and fresh medium was added. Following a further 48 h at 37 °C, the cells were assayed for luciferase expression using a chemiluminescent assay (Promega). Assays were carried out as described by the manufacturer. The total protein concentration of the cell culture was determined using the BC assay kit (Uptima). Luciferase activity of each condition was expressed to total relative light units (Total RLU) per mg of total protein. Results are reported as means ± SEM.

DNA Binding Ability. To 1 μg of plasmid DNA in Optimem (Gibco) were added cationic lipids at concentrations corresponding to an N/P charge ratio ranging from 0.5 to 8. The mixture was incubated for 30 min at room temperature. The complexes were subjected to electrophoresis in 1% agarose gel at 100 V, 90 mA. The gel was stained with ethidium bromide (10 mg/mL) and visualized on an UV illuminator (Fischer Bioblock).

FRET Experiments. The FRET method described by Pagano et al. (*10*) was used for assaying membrane fusion. In this method, membranes labeled with a combination of fluorescence energy transfer donor and acceptor lipid probes, respectively, NBD-PE and Rhod-PE (both from Molecular Probes), are mixed with unlabeled cationic lipids.

As the labeled membrane, we used liposomes made with L-α-phosphatidylcholine (PC) and fluorescent probes, or liposomes made with PC, L-α-phosphatidylethanolamine (PE), L-α-phosphatidyl-L-serine (PS), cholesterol (Chol), and fluorescent probes, (PC/PE/PS/Chol/NBD-PE/Rhod-PE approximately 44/25/10/20/0.8/0.2 m/m), a lipid composition close to that of the plasma membrane. All lipids were from Sigma. Stock solutions of each lipid were prepared in chloroform and stored in the dark, at 4 °C. Aliquots of these solutions were mixed and dried under reduced pressure. The dried lipid film formed was hydrated with a 5 mM Hepes buffer (pH 7 or pH 5.5) containing NaCl (150 mM). Suspensions were vigorously vortexed for 5 min and sonicated at 40 °C for 10 min.

Stock solutions of each cationic lipid were prepared in chloroform (5 $g.L^{-1}$). An aliquot was dried under reduced pressure, and liposome suspensions (1 $g.L^{-1}$) were prepared as described before.

Labeled and unlabeled liposomes were added in a buffer solution and vortexed. The final Rhod-PE and NBD-PE concentrations are 6.10^{-8} $mol.L^{-1}$ and 3.10^{-7} $mol.L^{-1}$. The labeled membrane final concentration is 15 $mg.L^{-1}$, corresponding approximately to 2.10^{-5} M for PC.

The Rhod-PE/lipid ratio was chosen after determination of the FRET efficiency versus Rhod-PE/PC molar ratio. A ratio closed from 0.003 was chosen so that lipid fusion undergoes a significant decrease of FRET efficiency. NBD-PE concentrations did not affect the FRET efficiency as described before (*10*).

Fluorescence measurements were performed 1 h later, at room temperature, by exciting the sample at 450 nm. A Varian Cary Eclipse Fluorescence spectrophotometer was used. Following each measurement, liposomes were disrupted with Triton X-100 (1% final concentration) in order to eliminate energy transfer and to allow the determination of the concentration of NBD-PE from its emission intensity.

Table 1. Potentiometric Determination of the pK_a of Phosphoramidate−Spermines

	3b	9b	spermine [18]
pK_1	10.60	11.10	10.8
pK_2	9.80	10.78	10.0
pK_3	7.00	8.62	8.8
pK_4	< 2	6.78	7.90

The efficiency of energy transfer was calculated from the fluorescence emission intensity of NBD-PE at 530 nm by using eq 1

$$E = 1 - F/F_0 \quad (1)$$

Fluorescence intensities were recorded in the presence (F_0) and absence (F) of Triton X-100. Values obtained in the presence of Triton X-100 were corrected for sample dilution and for the effect of Triton-X on the quantum yield of NBD-PE.

The relative fluorescence intensity, E_R (in %), is calculated using relation 2

$$E_R = (E_{mix}/E_{lab}) \times 100 \quad (2)$$

where E_{mix} and E_{lab} are the FRET efficiency calculated in the presence (E_{mix}) or absence (E_{lab}) of unlabeled liposomes for a same labeled liposome concentration.

pK_a Determinations. Potentiometric measurements were performed in a jacketed cell thermostated at 25.0 °C, kept under inert atmosphere of purified argon, using an automatic titrator (Metrohm, DMS Titrino 716) connected to a microcomputer. The free hydrogen concentrations were measured with a glass−Ag/AgCl combined electrode (Metrohm) filled with 0.1 M NaCl. The electrode was calibrated in order to read $-\log[H^+]$, designated as p[H], by titration of a small quantity of diluted HCl by standardized NaOH at 0.10 M ionic strength and 25 °C (and determining the equivalent point by the Gran's method) followed by adjustment of the meter by using the program GLEE so as to minimize the calculated p[H] vs observed values. log K_w for the system, defined in terms of $\log([H^+][OH^-])$, was found to be −13.78 at the ionic strength employed and was maintained fixed during refinements (*11*, *12*). NaCl was employed as the supporting electrolyte to maintain the ionic strength at 0.10 M.

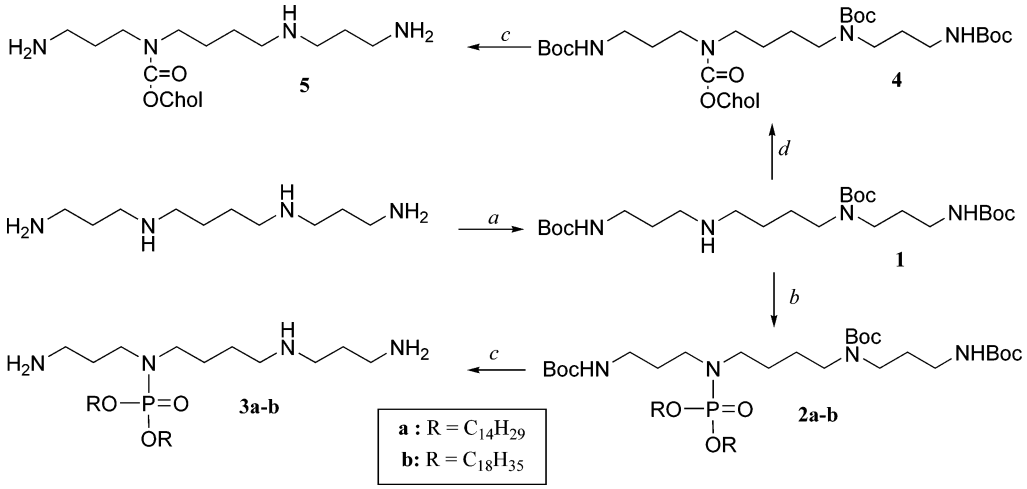
Potentiometric measurements obtained from NaOH titration of protonated polyamines were made at about 1 mM in concentration and ionic strength μ = 0.10 M (NaCl). Each titration makes use of at least 10 points per neutralization of a hydrogen ion equivalent, and titrations were repeated until a satisfactory agreement was reached. A minimum of three sets of data was used in each case to calculate the overall stability constants and their standard deviations. The standard deviations obtained for the different recognition constants were ±0.02 of the last significant digit reported in Table 1. The range of accurate p[H] measurements was considered to be 2−12. Equilibrium constants and species distribution diagrams were calculated by using the program *HYPERQUAD 2003* (*13*).

The stability constants K_{lh} were noted with respect to ternary species L_lH_h where l and h are, respectively, the stoichiometric number of L (ligand) and H (proton).

RESULTS AND DISCUSSION

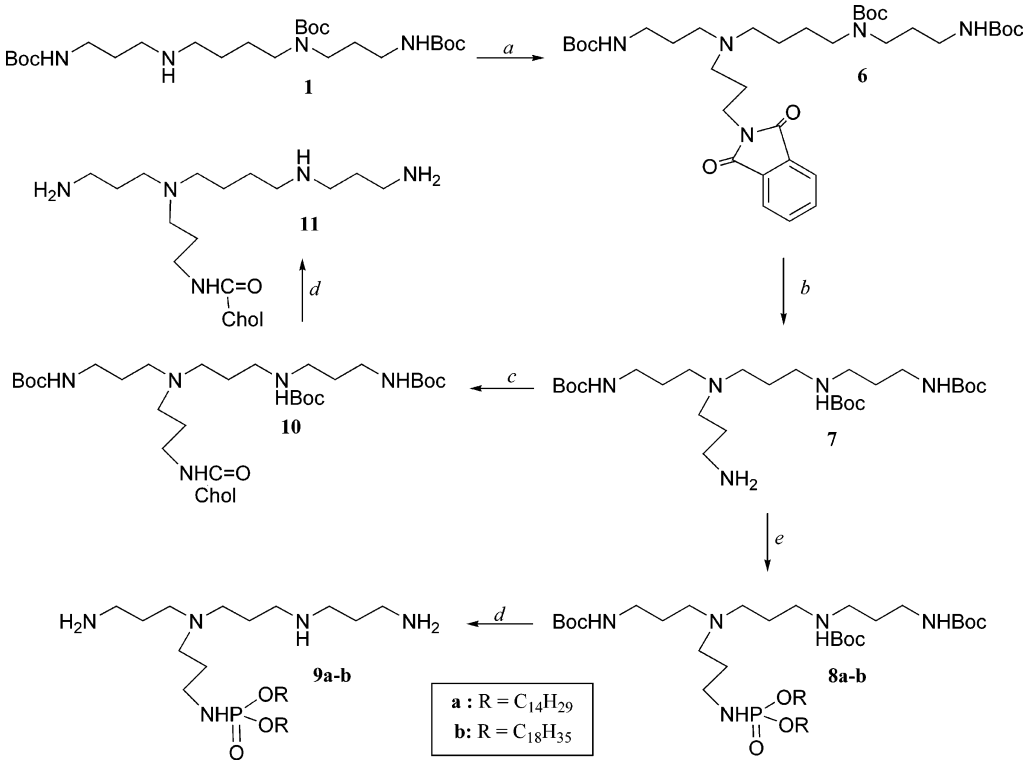
Syntheses of Lipidic Spermines. One of the most popular routes to phosphoramidates is the reaction of a hydrogen phosphite with an amine, in the presence of carbon tetrachloride (*14*). We adapted this procedure for reacting lipidic phosphites with spermine, first triprotected with Boc-ON (*15*), thus leading to lipophosphorylated spermines **3a** (R = $C_{14:0}$) or **3b** (R = $C_{18:1}$), after TFA deprotection (lipidic phosphites were obtained

Scheme 3. Syntheses of Lipophosphoramidate−Spermines 3 and Cholesteryl Spermine 5[a]



[a] (a) Boc-ON (3 equiv)/THF, 18 h, RT, 72%; (b) $(RO)_2P(O)H/CCl_4$ (10 equiv)/DIPEA (1.5 equiv), RT, 18 h; (c) TFA/CH_2Cl_2 (1:1), RT, 3 h; (d) CholOC(O)Cl, DIPEA (1.5 equiv), RT, 18 h.

Scheme 4. Syntheses of Lipophosphoramidate−Propylspermines 9 and Cholesteryl−Propylspermine 11[a]



[a] (a) 3-Bromopropylphthalimide (1.25 equiv), K_2CO_3 (5 equiv), CH_3CN, reflux, 18 h, 66%; (b) hydrazine monohydrate (50 equiv), THF/EtOH (80:20), 80 °C, 5 h; then 50 °C, 18 h, 80%; (c) cholOC(O)Cl, DIPEA (1.5 equiv), RT, 18 h; (d) TFA/CH_2Cl_2 (1:1), RT, 3 h; (e) $(RO)_2P(O)H/CCl_4$ (10 equiv)/DIPEA (1.5 equiv), RT, 18 h.

by reaction of fatty alcohols with diphenyl phosphite). For the purpose of comparison with other lipidic parts, the cholesteryl derivative of spermine **5** was synthesized and obtained unambiguously by reaction of cholesteryl chloroformate with Boc-triprotected spermine **1** and TFA deprotection (Scheme 3).

During our previous works about phosphonolipids as DNA carriers, we noticed that, when an ammonium cation was replaced by a phosphonium or arsonium (i.e., a more bulky polar head), a good transfection efficiency required a longer spacer between the cationic part and the phosphoryl group (*16*, *17*). So, we also designed and synthesized analogues of lipids **3** and **5** including a longer spacer between spermine and the lipidic part. For that purpose, the Boc-triprotected spermine with a pendant aminopropyl arm was first synthesized from Boc-triprotected spermine **1** and 3-bromopropylphtalimide, then hydrazinolysis of the phtalimide protecting group. Subsequent reaction with fatty phosphites or cholesteryl chloroformate led to phosphoramidates **9a** and **9b** or cholesteryl derivative **11,** after deprotection (Scheme 4).

***In Vitro* Biological Tests.** *In Vitro Transfection Efficiency.* Two different cell lines (A549 and HeLa) were used to compare the efficiency of the different lipidic parts, without (**3b**, **5**) or with a spacer (**9b**, **11**), and with (+D) or without DOPE added. The efficacy of each lipid (trough luciferase activity) was established for an increasing N/P charge ratio from 0.5 to 8, according to routinely used and previously described procedures (*4*, *9*, *16*). As an example, Chart 1 shows the values of total RLU/mg of protein for HeLa cells, according to increasing N/P

Chart 1. Comparison of Charge Ratio and DOPE Influence on the Transfection Efficiency for HeLa Cell Line

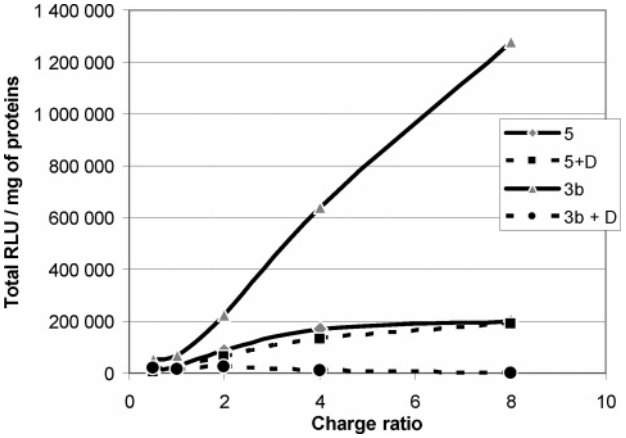


Chart 2. Luciferase Activity for HeLa Cells[a]

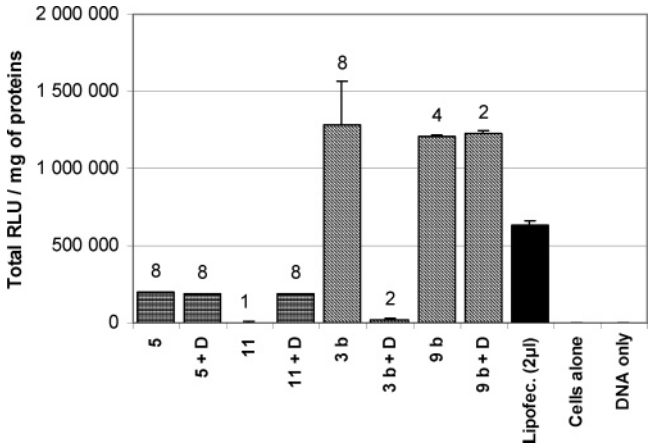
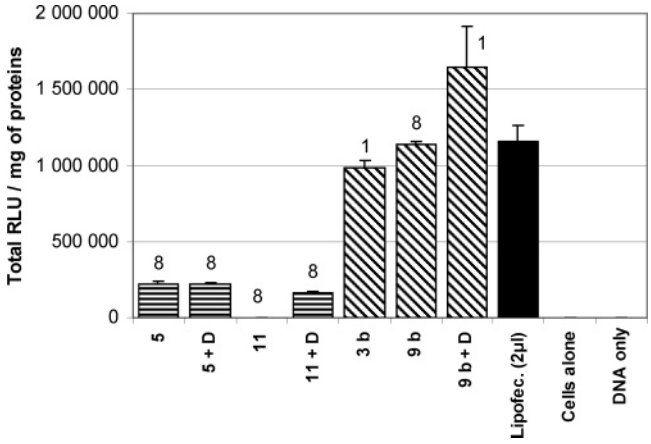


Chart 3. Luciferase Activity for A549 Cells[a]



ratios. Charts 2 and 3 indicate the average efficiencies of each lipid at its better N/P ratio (marked on top of each bar). Using the above-mentioned protocols and formulations, the efficacy of the phosphoramidates has the same magnitude as lipofectamine or slightly higher, whereas the cholesteryl derivatives were about ten times lower. But, insofar as the lipid/DOPE ratio used was the same (1:1) in all our experiments, these results have not a comparative but only an indicative value, since formulations were not optimized for each lipid. Compound **5**, for instance, is presented as most efficient at a 1:2 lipid/DOPE ratio (*3*).

DNA Binding Ability. Electrophoretic mobility of plasmid DNA was examined with increasing N/P ratios (0.5−8) using 1% agarose gel (DNA was visualized by ethidium bromide staining). Complete binding of DNA was observed at an N/P ratio lower than 2 for phosphoramidate **9b**, for instance, whereas binding was not complete at a N/P ratio of 8 for cholesteryl spermine **5** (Figure 1).

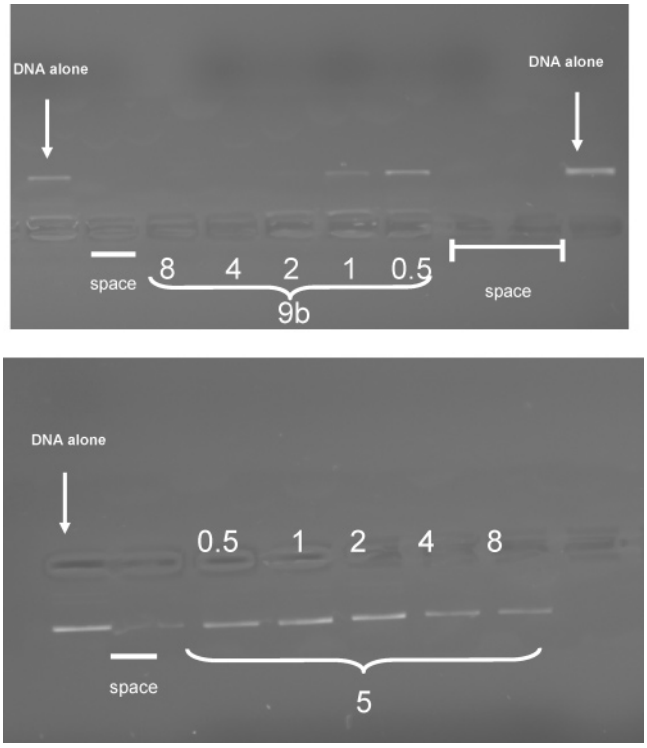


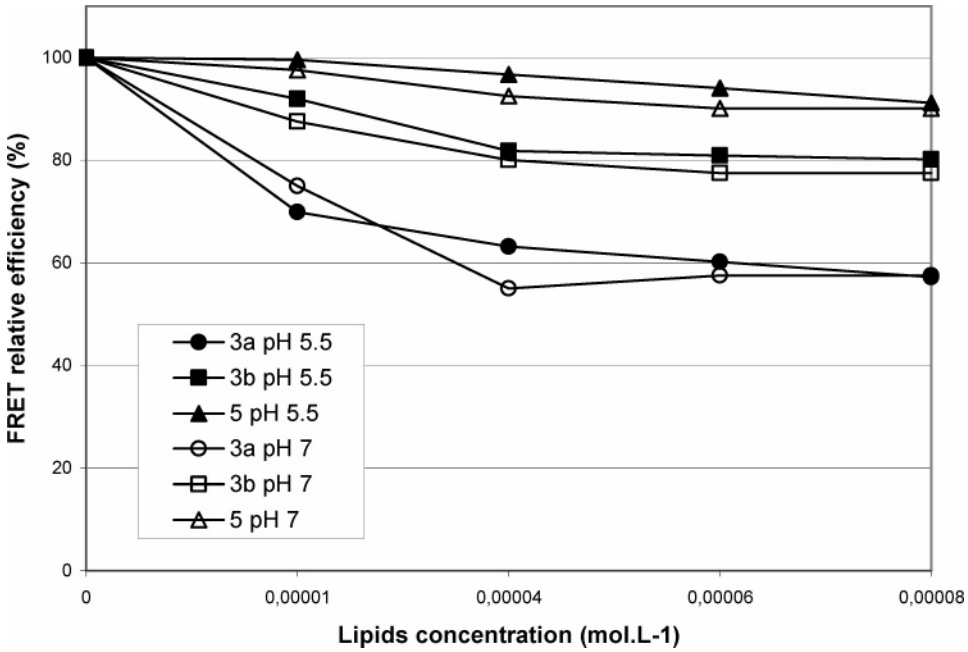
Figure 1. DNA binding ability of phosphoramidate **9b** and cholesteryl **5**.

FRET Experiments. In an attempt to explain these results, we undertook a comparative study toward the ability of these lipidic spermines to disrupt an artificial membrane with a lipid composition close to that of the plasma membrane. We exploited the widely used fluorescence resonance energy transfer (FRET) technique (*10*). Briefly, this technique depends upon the interactions that occur between two fluorophores when the emission band of one (the energy donor) overlaps with the excitation band of the second (the energy acceptor) when the probes are in close proximity. If a couple of two lipidic fluorophores, one energy donor (NBD-PE, for instance) and one energy acceptor (Rho-PE, for instance) is introduced into a liposome; any fusion event of such a doubly labeled liposome with a second liposome (devoid of any fluorophore) will decrease the efficiency of resonance energy transfer. Thus, any decrease in FRET efficiency provides evidence for membrane fusion.

The model membrane was made with PC, L-α-phosphatidylethanolamine (PE), L-α-phosphatidyl-L-serine (PS), cholesterol (Chol), and fluorescent probes (PC/PE/PS/Chol/NBD-PE/Rhod-PE approximately 44/25/10/20/0.8/0.2 m/m). To that membrane were added the different lipidic spermines at increasing concentrations from 10^{-6} to 10^{-4} mol/L, with DOPE added (1:1 m/m) or not, in each case. Chart 4 (no DOPE added) is a representative example of the observed differences between cholesteryl spermine **5** (90% average efficacy) and phosphoramidates **3a** and **3b** (60% and 80% average efficacy, respectively).

From this example, a correlation between FRET and *in vitro* efficacies seems to appear, since phosphoramidates **3a** and **3b** exhibited lower FRET values than cholesteryl derivative **5**, thus indicating a theoretical greater membrane-disrupting ability, at a pH of 7 (extracellular) or 5.5 (endosomal). However, as the curves were practically merged for the two pH values, there is

Chart 4. Comparative Fret Efficiency of Phosphoramidates 3 and Cholesteryl 5 at pH 5.5 and 7



no obvious endosomal acidity-induced advantage for lipophosphoramidates. The same experiments conducted with compounds **9a**, **9b**, and **11** gave rather similar results, but the lack of reproducibility to get homogeneous vesicles, notably with cholesteryl derivative **11**, made those results (and therefore a comparison) unconfirmed (data not shown).

Sizes and pK_a. Another difference lies in the behavior of these lipids in water: after 5 min in an ultrasonic bath, the average sizes of the particles of each suspended lipid (1 mg/mL) were 2800 and 640 nm for cholesteryl derivatives **5** and **11**, compared to 290 and 770 nm for phosphoramidates **3b** and **9**.

The pK_a values of the two phosphoramidates **3b** and **9b** were determined by potentiometric titration (see Experimental Procedures for details) and quoted in Table 1. If compared to the pK_a of spermine, the presence of the lipidic phosphoramidate obviously leads to a decrease of the basicity of the third amine in the case of **3b** (7.0 vs 8.8 for spermine) and of the fourth for **9b** (6.78 vs 7.9 for spermine).

CONCLUSION

As previously observed in the case of monocationic lipids (*9*), the use of a lipophosphoramidate group, as linker between the lipidic part and the cationic head, is a very attractive approach to design synthetic vectors for DNA delivery. In this study, the lipophosphoramidate lipidic group has been attached either directly or *via* a short spacer on a spermine unit. In a will of comparison, the spermine has been also functionalized with a cholesterol unit, acting as the lipidic part, *via* a carbamate group. We observed that the influence of the lipid part on the transfection efficiency is important. Notably, the spermine−lipid vectors having two oleoyl chains attached on a phosphoramidate group are the most efficient. Also, the adjunction of DOPE as an helper neutral lipid in the formulation has no effect in the case of compound **9b** or a dramatically negative effect in the case of compound **3b**. When the spermine−phospholipid is formulated alone, the presence or absence of a spacer between the spermine unit and the phosphoramidate group has only a low influence on the transfection results. As in the case of DOGS (*7*), the introduction of a lipidic group close to the third amine of spermine lowers the value of its pK_a, thus widening the field of basicity, hence, probably, an increased "buffer effect". This explanation could be one of the reasons for the efficiency of compounds **3b** and **9b**.

However, the standard formulations used were not optimized, with regard to the optimal cationic lipid/DOPE ratios (which, of course, are not necessarily the same for each compound) or concerning the sizes of lipidic vesicles in water. Since liposomes have sizes from 290 nm for lipid **3b** to 2800 nm for cholesteryl **5** (and no attempt was made to reduce these sizes), comparisons must be taken cautiously and have only an indicative value.

Since the last pK_a values of **3b** and **9b** are 7.0 and 6.78, respectively, these tri- and tetraamines can be considered polycationic species at the physiological pH despite the fact that a local variation of pH could modify the cationic state of these vectors. In another work (*20*), we examined the transfection efficacies of lipophosphoramidates bearing a couple of cations (N^+/P^+ or N^+/As^+), permanent regardless of the pH. Transfection assays of these dicationic compounds, performed on the same cell lines (HeLa and A549), with the same experimental conditions, systematically expressed luciferase levels twice as high.

Nevertheless, the lipophosphoramidate group constitutes an interesting alternative to the "classical" cholesteryl or double aliphatic chains generally used as the lipidic part in cationic lipids. As lipophosphoramidates were found especially efficient for *in vivo* experiments when associated to an ammonium, a phosphonium, or an arsonium cation, the behavior of lipids **3** and **9** will soon be considered to that effect.

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