Pyridinium-Based Cationic Lipids as Gene-Transfer Agents

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Cationic lipids are a promising alternative to viral vectors for gene therapy, allowing the delivery of larger plasmids without immunogenicity, despite their lower transfection efficiency. Among them, heterocyclic systems with imidazolium or pyridinium polar head groups have definite advantages such as the excellent transfection profiles and low cytotoxicity. Our approach for synthesizing heterocyclic cationic lipids differs from those previously described because we synthesize a pyridinium ring from simple starting materials. First a pyrylium salt is formed via diacylation of alkenes. The pyrylium salt is then converted by primary amines into pyridinium salts. Appropriate choice of the primary amine allows the attachment of two hydrophobic chains yielding compounds **21A** and **25A** (with various chain lengths derived from palmitic, stearic and oleic acids). The same strategy allowed the preparation of lipophilic derivatives **21B**, **25B** useful as strongly fluorescent markers for the study of the properties of biological membranes. Preliminary tests with some of the compounds **21A** and **25A**, on several cell lines, showed comparable transfection efficiencies and lower cytotoxicity than those obtained with standard commercial transfection agents.

Introduction

In the last decade gene therapy has emerged as a revolutionary approach to treat diseases at the level where they are generated: the living cell.[1−3] When the cellular machinery is impaired due to a deficient gene, a functional gene incorporated into an appropriate vector is delivered to the affected cells/tissues. After internalization, the DNA is transferred to the nucleus, where the gene is integrated into the host genome. After transcription, it is translated into the proteins needed to correct the cellular imbalance. From this viewpoint gene therapy can be considered as a new way to deliver proteins into living cells.

The efficiency of the overall process is critical for achieving a therapeutic effect.[4] Viral vectors have been most frequently employed because they have a high efficiency,[5,6] but they possess several major disadvantages, such as immunogenicity, permanent integration of the foreign plasmid into the host's DNA, difficulties associated with good manufacturing practice (GMP) production or storage, and a limited size of the plasmid that can be inserted into the virion.[1−3]

Cationic lipids are a promising non-viral alternative, having low immunogenicity and cytotoxicity; they can involve plasmids with practically unlimited size, and they can be easily manufactured and stored in bulk quantities under GMP-compliant norms.[7−11] They are amphiphilic molecules that contain a polar (cationic) head linked by a spacer to a hydrophobic tail. When a certain concentration is reached, they can self-assemble, by co-operative hydrophobic intermolecular binding, forming cationic liposomes. In this form, cationic lipids can efficiently bind and compact DNA molecules by electrostatic association between the positively charged polar heads of the lipids and the negatively charged phosphate groups of the DNA, forming cationic lipid−DNA complexes (lipoplexes[12]).[13−15] The genetic material is protected from the action of nucleases and is thus able to reach the desired target cells. Similarly, cationic polymers can also associate and compact DNA, forming another type of chemical transfection systems — polyplexes.[12,16]

The main problem associated with the therapeutic use of non-viral transfection systems is their lower efficiency,

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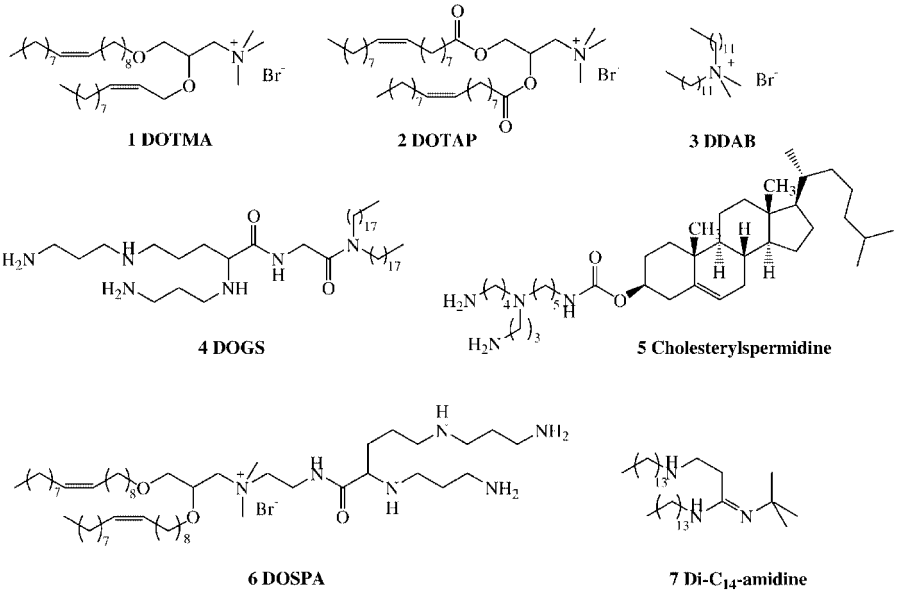
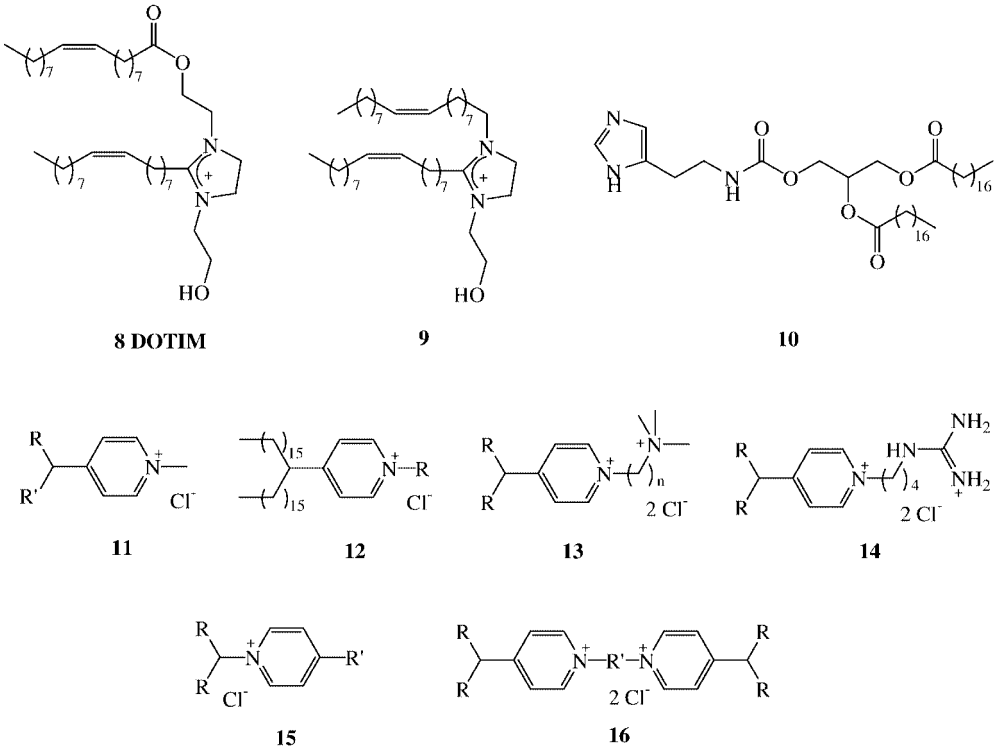


which amounts to a few percent of that achieved by viral vectors. Consequently, substantial efforts have been devoted to understanding these physiological barriers, their correlation with the chemical structure and physico-chemical properties of the lipoplexes, and the ways to overcome them.[7,10] Despite the fact that a fairly large variety of commercial cationic lipid-based transfection systems are available nowadays (formulas **1**−**7**), basic characteristics, such as in vivo efficiency and intrinsic cytotoxicity, remain to be improved.

Only very few cytofectins with the positive charge on an aromatic heterocyclic ring have been described so far,[7,8,17−19] and practically all of them are either pro-cationic (i.e. amines that become protonated at physiological pH), or are prepared by the quaternization of a preformed neutral nitrogen-containing aromatic ring, often followed by exchange of the anion (formulas **8**−**16**). It is known that alkyl-trimethylammonium cations can be cleaved because they may undergo a Hofmann degradation yielding an alkene and trimethylamine, but such a reaction is unlikely when the positively charged nitrogen atom is in a heterocyclic ring. Furthermore, positively charged aromatic heterocycles have planar rings with delocalized charge, a fact that can have a major impact on physico-chemical properties such as lipid hydration, supramolecular assembly, interaction with nucleosomes, etc.[7,8] In this context, Engbert's group reported recently the cationic lipids **11**−**16**, based on pyridinium polar heads.[18−20] The best results were obtained with oleoyl and stearoyl hydrophobic chains: the corresponding amphiphiles exhibited higher transfection ef-



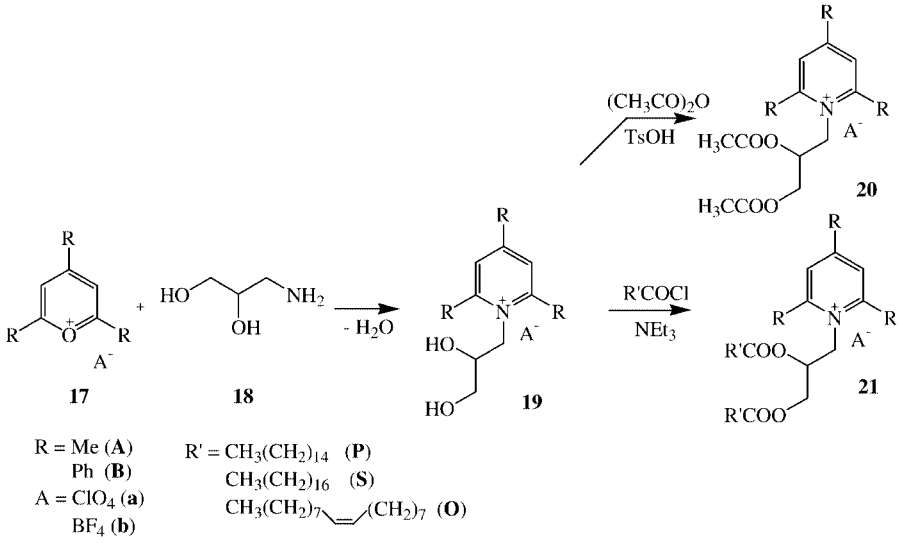
ficiencies and a reduced cytotoxicity when compared with classical transfection systems such as 2,3-dioleoyloxypropyl-1-trimethylammonium bromide (DOTMA, **1**). Insights regarding the mechanism of action of this class of compounds as well as a structure-activity relationship study have been recently disclosed.[21]

We report here new pyridinium cationic lipids obtained following a novel approach. Instead of starting from a neutral nitrogen-containing heterocyclic ring and quaternizing it with an alkylating agent, we have used a high-yield procedure to generate simultaneously the heterocyclic ring and the positively charged nitrogen atom, via pyrylium salts (**17**). A simple approach for obtaining pyrylium salts consists of the reaction of isobutene (generated in situ for *tert*-butanol) with an excess of acylating agent (the diacylation of alkenes has been called the Balaban−Nenitzescu−Praill reaction).[22] Pyrylium salts react with primary amines (**18** or **22**) yielding pyridinium salts in high yields.[23,24] Compounds **21a−c** and **25a−c** were obtained,[25] using different hydrophobic anchors connected by ester linkages, as shown below. This structure has therefore a polar head and a forked hydrophobic tail. Additionally, the pyridinium propanediols **19** and **23**, as well as their acetate derivatives (compounds **20** and **24**) were extensively studied with respect to their NMR spectra.

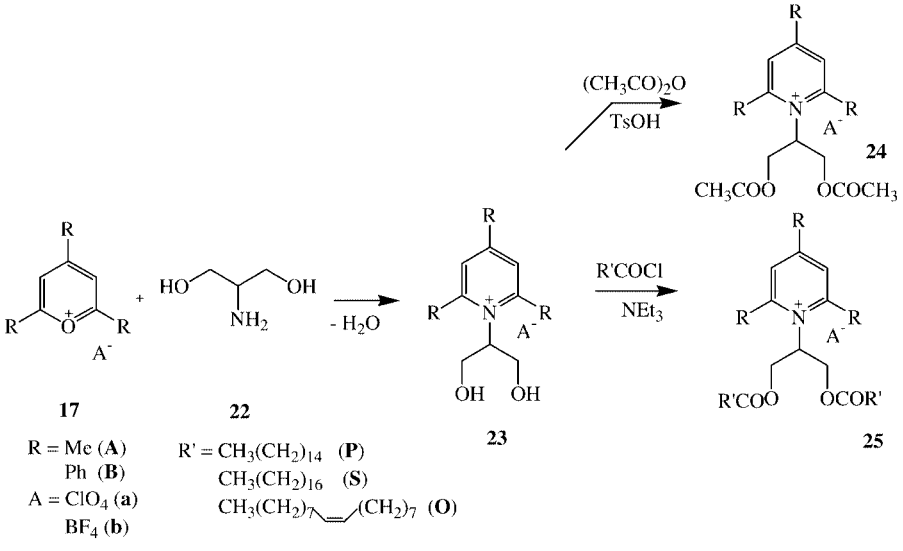
Results and Discussion

Synthesis and NMR Structural Assignments

In the first step the key diol intermediates **19** and **23** were synthesized from the corresponding aminopropanediols



Scheme 1



Scheme 2

and trisubstituted pyrylium salts (Schemes 1 and 2). 2,4,6-Trimethylpyrylium and triphenylpyrylium salts (perchlorate or fluoroborate)[23,24,26] were prepared according to the literature.[27,28]

Despite different attempts, not all these intermediates could be obtained in crystalline form, with some remaining as viscous liquids. Interestingly, the behavior of the two types of pyridinium propanediols was different: in the series derived from 1-amino-2,3-propanediol only the trimethylpyridinium salts **19A** crystallized, while in the series derived from 2-amino-1,3-propanediol (serinol) only the triphenyl derivatives **23B** were obtained in a crystalline form. The influence of the pyridinium rotor on the dihydroxypropyl backbone, as well as the association of the two hydroxymethylene groups by intramolecular and intermolecular hydrogen bonds was investigated in detail by means of NMR spectroscopy (Tables 1−4).

The data from Table 2 and 4 show that rotation of the substituted pyridinium ring around the C1−N bond is partially restricted: both the ^{13}C NMR signals of the α-quaternary carbon atoms and those of the α-methyl groups are broadened, and this feature is maintained in different deuterated solvents with different polarities. The existence of different "frozen" conformations can also be seen in the

Table 1. Chemical shifts (δ, ppm) and coupling constants (*J*, Hz,) in ^1H NMR spectra (300 MHz) in different solvents of 1-(2,3-dihydroxypropyl)-2,4,6-trimethylpyridinium perchlorate **(19Aa)**; assignments are given by means of signal integration, selective decoupling and HETCOR (^1H-^{13}C)

Solvent	Temperature	H-β	HO-2	HO-3	H-1	H-1	H-2	H-3	H-3	CH_3-α	CH_3-γ
DMSO-d_6	20°C	7.73 s	5.42 d [5.0]	5.09 t [5.6]	4.56 dd [3.6, 14.9]	4.48 dd [9.5, 14.9]	3.92 m	3.56 m [4.6, 5.1, 11.1]	3.46 m [5.2, 6.7, 11.1]	2.81 s	2.49 s
DMSO-d_6	60°C	7.70 s	5.30 bs	4.90 bs	4.56 dd [3.6, 14.9]	4.49 dd [9.3, 14.9]	3.92 m	3.57 dd [4.6, 11.1]	3.48 dd [6.6, 11.1]	2.81 s	2.49 s
DMSO-d_6 + TFA	20°C	7.22 s	-	-	4.62 dd [3.4, 14.8]	4.53 dd [9.5, 14.8]	3.98 m	3.62 dd [4.6, 11.1]	3.53 dd [6.6, 11.1]	2.85 s	2.51 s
DMSO-d_6 + TFA	45°C	7.70 s	-	-	4.62 dd [3.6, 14.8]	4.54 dd [9.5, 14.8]	3.98 m	3.63 dd [4.6, 11.1]	3.54 dd [6.5, 11.1]	2.85 s	2.51 s
DMSO-d_6 +TFA	70°C	7.68 s	-	-	4.63 dd [3.6, 14.8]	4.54 dd [9.3, 14.8]	3.99 dddd [3.6, 4.6, 6.5, 9.3]	3.63 dd [4.6, 11.1]	3.54 dd [6.5, 11.1]	2.84 s	2.51 s
DMSO-d_6 + TFA	90°C	7.66 s	-	-	4.63 dd [3.8, 15.0]	4.54 dd [9.1, 15.0]	3.99 m	3.64 dd [4.7, 11.2]	3.55 dd [6.4, 11.2]	2.84 s	2.51 s
CD_3NO_2	20°C	7.60 s	3.57 d [4.9]	3.16 t [5.5]	4.72 dd [9.9, 15.0]	4.61 dd [3.3, 15.0]	4.24 dqd* [3.3, 5.1, 9.9*]	3.79 dt [11.5, 4.7]	3.73 dt [11.5, 5.1]	2.88 s	2.56 s
C_5D_5N	20°C	7.39 s	-	-	4.98 m [9.1, 14.7]	4.98 m [9.1, 14.7]	4.60 m	4.19 dd [4.6, 11.1]	4.12 dd [6.3, 11.1]	2.96 s	2.27 s
D_2O	20°C	7.42 s	-	-	4.48 dd [9.5, 15.1]	4.40 dd [3.9, 15.1]	4.03 m	3.64 dd [4.8, 11.8]	3.58 dd [5.3, 11.8]	2.64 s	2.34 s
DMFA -d_7	20°C	7.82 s	5.67 d [4.9]	5.18 t [5.7]	4.80 dd [3.8, 15.0]	4.72 dd [9.3, 14.7]	4.19 m [4.6]	3.76 dt [4.7, 11.5]	3.66 dt [6.5, 11.0]	2.95 s	2.56 s

[a] dqd represents the multiplicity of a doublet of quadruplets of doublets. The small doublet of 3.3 Hz is the coupling with one H-1, the quadruplet comes from almost equal couplings of H-2 with OH (4.9 Hz) and with two H-3′s (4.7 and 5.1 Hz respectively), while the large doublet of 9.9 Hz is the coupling with the other H-1.

Table 2. ^{13}C NMR chemical shifts (δ, ppm; 75 MHz) in different solvents of 1-(2,3-dihydroxypropyl)-2,4,6-trimethylpyridinium perchlorate **(19Aa)**; assignments are given by means of signal integration, APT and HETCOR (^1H-^{13}C)

Solvent	Temperature	C-γ	C-α	C-β			C-2	C-3	C-1	CH_3-α	CH_3-γ
DMSO-d_6	20°C	157.05	155.20 (broad)	128.00	127.95 t: 127.95	127.90	69.79	63.62	54.74	21.20 (broad)	20.81
DMSO-d_6	60°C	156.79	154.97 (broad)	127.75	127.71 t: 127.71	127.67	69.55	63.36	54.59	20.89 (sharp)	20.48
DMSO-d_6 + TFA	20°C	157.70	155.83 (broad)	128.58	128.53 t: 128.53	128.48	70.47	64.18	55.38	21.68 (broad)	21.11
DMSO-d_6 + TFA	45°C	157.54	155.69 (sharp)		128.38 (broad)*		70.35	64.07	55.30	21.47 (sharp)	20.91
DMSO-d_6 + TFA	70°C	157.40	155.56		128.23 (sharp)		70.24	63.69	55.21	21.28	20.79
CD_3NO_2	20°C	159.92	156.90 (broad)		129.70		71.64	65.17	55.37	22.19	21.62
C_5D_5N	20°C	157.67	155.46 (broad)		128.52		71.21	64.79	55.56	21.58 (broad)	20.91
D_2O	20°C	161.01	157.52 (broad)		130.89 (broad)		72.13	65.49	56.33	23.49 (broad)	23.08
DMFA -d_7	20°C	158.26	156.32 (broad)		128.82 (broad)		71.10	64.79	55.70	21.61 (broad)	21.11

[a] At 318 K (45 °C) coalescence occurred for the three separate signals of C-β.

Table 3. Chemical shifts (δ, ppm) and coupling constants (*J*, Hz) in ^{1}H NMR spectra (300 MHz) of compounds of type **19**, **20**, **23**, and **24**; assignments are given by means of signal integration, selective decoupling and HETCOR (^{1}H-^{13}C)

Comp.	Solvent	Hβ		Ph	OH		CH_2 (1)		CH	CH_2 (3)		CH_3 (Py+)			CH_3CO	
		($H\beta_A$)	($H\beta_B$)		(2-OH)	(3-OH)	H_A	H_B		H_A	H_B	α	(α`)	γ	(2-)	(3-)
19Aa	DMSO-d_6	7.73 s		-	5.42 d [5.0]	5.09 t [5.6]	4.56 dd [3.6; 14.9]	4.48 dd [9.5; 14.9]	3.92 m [4.6; 6.5; 9.3]	3.56 m [5.1; 4.6; 11.1]	3.46 m [5.2; 6.7; 11.1]	2.81 s		2.49 s	-	-
20Aa	$CDCl_3$	7.54 s		-	-	-	4.89 dd [9.7; 15.7]	4.82 dd [4.7; 15.7]	5.49 m [4.7]	4.46 dd [4.0; 11.2]	4.29 dd [4.6; 11.2]	2.88 s		2.54 s	2.13 s	1.93 s
19Ab	DMSO-d_6	7.70 s		-	not seen		4.55 dd [3.6; 14.9]	4.48 dd [9.5; 14.9]	3.92 m	3.57 dd [4.6; 11.0]	3.48 dd [6.7; 11.0]	2.80 s		2.49 s	-	-
20Ab	$CDCl_3$	7.49 s		-	-	-	4.87 dd [9.7; 15.8]	4.80 dd [4.8; 15.8]	5.48 m [4.1;4.8]	4.45 dd [4.1; 12.3]	4.27 dd [4.9; 12.3]	2.86 s		2.53 t [0.7]	2.12 s	1.92 s
19Ba	DMSO-d_6	8.39 s		8.32 d [6.6]; 7.84-7.90 m; 7.60-7.70 m	not seen		4.76 dd [3.8; 14.1]	4.43 dd [9.8; 14.1]	3.28 m	2.94 dd [5.0; 11.1]	2.80 dd [6.3; 11.1]	-		-	-	-
20Ba	$CDCl_3$	7.92 s		7.49-7.85 m	-	-	5.08 dd [3.1; 14.4]	5.01 dd [8.8; 14.5]	4.93 m	3.74 dd [4.6; 12.1]	3.46 dd [4.1; 12.1]	-		-	1.92 s	1.76 s
19Bb	DMSO-d_6	8.39 s		8.23 d [6.5]; 7.87 m; 7.66 m	not seen		4.77 dd [3.7; 14.1]	4.43 dd [9.8; 14.1]	3.28 m	2.94 dd [4.9; 11.1]	2.80 dd [6.2; 11.1]	-		-	-	-
20Bb	$CDCl_3$	7.91 s		7.81 d [6.4]; 7.52-7.69 m	-	-	5.21 dd [2.7; 15.1]	5.03 dd [9.9; 15.1]	4.90 m	3.75 dd [4.7; 12.1]	3.48 dd [4.5; 12.1]	-		-	1.91 s	1.76 s
23Aa	DMSO-d_6	7.75 d [2.0]	7.71 d [2.0]	-	5.36 bs		4.01 dd [8.4; 12.2]		5.27 m	3.92 dd [5.6; 12.3]		2.84 s	2.81 s	2.48 s	-	
24Aa	$CDCl_3$	7.62 s		-	-		4.80 dd [7.9; 12.3]		5.61 m	4.65 dd [5.6; 12.3]		2.94 s	2.90 s	2.58 s	2.02 s	
23Ab	DMSO-d_6	7.76 d [2.0]	7.72 d [2.0]	-	5.62 bs		3.99 dd [8.5; 12.2]	3.90 dd [5.6; 12.2]	5.31 m	3.07 dd [4.8; 14.6]	3.02 dd [4.8; 14.6]	2.91 s	2.82 s	2.46 s	-	
	DMSO-d_6 + TFA	7.68 d [1.8]	7.65 d [1.8]	-	-		3.99 dd [8.4; 12.2]	3.90 dd [5.6; 12.2]	5.29 m [2.7; 7.0]	3.05 dd [4.9; 14.5]	3.02 dd [4.7; 14.5]	2.85 s	2.79 s	2.43 s	-	
24Ab	$CDCl_3$	7.63 d [2.2]	7.62 d [2.2]	-	-		4.80 dd [8.0; 12.4]		5.61 m [0.7; 6.6]	4.63 dd [5.5; 12.4]		2.92 s	2.88 s	2.57 s	2.00 s	
23Ba	DMSO-d_6	8.39 bs		8.24 d [6.8]; 7.55-7.83 m	5.36 t [5.2]		3.60 dd [5.0; 8.0]	3.56 dd [5.0; 8.0]	5.08 m [6.9]	3.35 dd [5.4; 11.5]	3.31 dd [5.5; 11.5]	-	-	-	-	
	DMSO-d_6 (75°C)	8.31 s		8.18 d [6.7]; 7.55-7.83 m	5.19 bs		3.56 dd [8.2; 11.6]		5.14 m	3.36 dd [6.5; 11.6]		-	-	-	-	
24Ba	$CDCl_3$	7.79 -7.86 bs		7.38-7.78 m	-		4.38 dd [7.3; 12.2]		5.35 m [6.9]	4.00 dd [6.4; 12.2]		-	-	-	1.97 s	
23Bb	DMSO-d_6	8.38 bs		8.24 d [6.9]; 7.55-7.85 m	5.48 t [5.2]		3.58 dd [5.1; 8.0]	3.54 dd [5.1; 8.0]	5.27 m [6.9]	3.31 m		-	-	-	-	
	DMSO-d_6 + TFA	8.39 bs		8.24 d [6.8]; 7.55-7.86 m	not seen		3.60 dd [8.4; 11.6]		5.15 m	3.36 dd [6.2; 11.6]		-	-	-	-	
24Bb	$CDCl_3$	7.47-8.03 m			-		4.78 dd [6.9; 12.3]		5.34 m [6.7]	4.04 dd [6.5; 12.2]		-	-	-	1.94 s	

pattern of the pyridinium β-carbon signal, which appears with different multiplicities. Moreover, dynamic NMR spectrometry on the 1-pyridinium-2,3-propanediol **19Aa** showed that when the temperature is raised all these signals sharpen; the coalescence temperature was found to be about 318 K (45 °C). This temperature corresponds to a rotational barrier of about 17−20 kcal/mol, in agreement with previous findings.[29,30] The dynamic ^{1}H NMR and the use of trifluoroacetic acid as catalyst for accelerating proton exchange also evidenced the existence of an intramolecular hydrogen bond in this case.

Since the pyridinium ring constitutes the polar head in the structure of the new cationic lipids, we investigated its steric requirements further by preparing the corresponding acetates, which are compounds with structures related to common designs of transfection vectors. The acetates were readily available by acetylation of diols **19** and **23** with acetic anhydride. The NMR spectroscopic data for all these compounds are summarized in Table 3 and 4. For acetates **20** or **24** the rotation of the pyridinium ring was found to be less restricted than for the parent diols.

A general feature in the ^{1}H NMR spectra of compounds **19** and **20** (derived from a 2,3-disubstituted backbone) is the larger geminal coupling constant ^2J between the diastereotopic N−CH_2 protons (14.1−15.8 Hz) than between the diastereotopic O−CH_2 protons (11.0−12.3 Hz). This effect is better explained by the vicinity of π-electrons for the N−CH_2 protons, rather than by differences in bond angles or electronegativities of adjacent groups. Also, the large difference in the vicinal coupling constants between H−C2 and the two N−CH_2 protons (one ^3J value of 2.7−4.8 Hz, and another of 8.8−9.8 Hz) is also due to the proximity of the aromatic ring. By contrast, the largest ^3J values between H−C2 and the two O−CH_2 protons (4.0−6.7 Hz) are practically equal. Raising the temperature from 20 °C to 70 °C did not change these coupling constants; therefore one can exclude any effect of restricted rotations.

In the case of compounds **23** and **24** (derived from a 1,3-disubstituted backbone) the restricted rotation of the pyridinium ring generates the magnetic non-equivalence of the two constitutionally equivalent CH_2 groups. The differences between the geminal coupling constants of the enantiotopic CH_2 protons are reduced; these values appear in the range of 11.5−14.6 Hz. The vicinal coupling constants between H−C2 and the two groups of CH_2 protons are also differ-

Table 4. ^{13}C NMR chemical shifts (δ, ppm; 75 MHz), in different solvents, of compounds of type **19**, **20**, **23**, and **24**; assignments by signal integration, APT and HETCOR (^1H-^{13}C)

Comp.	Solvent	C=O (Acetates)		Cγ(Py+)	Cα(Py+)		Ph - Substituents of Py+								Cβ(Py+)		CH	CH_2		Me-substituents of Py+			$\underline{CH_3}CO$ (acetates)	
		C=O (2)	C=O (3)		(Cα)	(Cα')	CqPhγ	CqPhα	CγPhγ	CβPhγ	CαPhγ	CγPhα	CβPhα	CαPhα	Cβ	Cβ'	(C2)	3-CH_2	1-CH_2	$CH_3\alpha$	$CH_3\alpha'$	$CH_3\gamma$	2-Ac	3-Ac
19Aa	DMSO-d_6	-	-	157.05	155.20 broad		-	-	-	-	-	-	-	-	127.95		69.72	63.49	54.71	21.20		20.80	-	-
20Aa	$CDCl_3$	170.28	169.48	158.96	155.31		-	-	-	-	-	-	-	-	128.74		68.37	62.44	51.50	21.46		21.51	20.56	20.31
19Ab	DMSO-d_6	-	-	155.46	157.54		-	-	-	-	-	-	-	-	128.26		69.01	63.70	54.88	21.46		21.10	-	-
20Ab	$CDCl_3$	170.35	169.54	158.83	155.52		-	-	-	-	-	-	-	-	128.62		68.55	62.49	51.58	21.40		21.53	20.55	20.32
19Ba	DMSO-d_6	-	-	157.49	154.53		133.44	133.81	132.77	131.19	128.91	129.80	129.32	130.02	126.08		68.84	63.61	58.24	-		-	-	-
20Ba	$CDCl_3$	169.67	169.65	157.50	156.55		133.47	132.59	132.51	131.36	129.57 broad	129.57 broad	128.17	129.78	126.58		69.09	61.67	54.33	-		-	20.73	20.28
19Bb	DMSO-d_6	-	-	157.40	154.52		133.44	133.82	132.76	131.18	128.91	129.88 broad	129.32	130.01	126.96		68.83	63.60	58.26	-		-	-	-
20Bb	$CDCl_3$	169.61		157.58	156.10		133.32	132.63	132.47	131.22	129.43	129.72	128.03	129.72	126.23		69.11	61.62	54.55	-		-	20.62	20.23
23Aa	DMSO-d_6	-		157.38	156.72	155.26	-	-	-	-	-	-	-	-	130.05	128.36	70.57	59.28		23.05	21.70	20.62	-	
24Aa	$CDCl_3$	170.32		159.64	156.18	154.78	-	-	-	-	-	-	-	-	131.12	129.46	63.51	61.75		23.07	22.18	21.48	20.42	
23Ab	DMSO-d_6	-		157.07	156.79	155.11	-	-	-	-	-	-	-	-	129.83	128.16	70.43	58.98		23.09	21.67	20.57	-	
24Ab	$CDCl_3$	170.32		159.65	156.22	154.84	-	-	-	-	-	-	-	-	130.99	129.35	63.50	61.62		22.84	21.93	21.32	20.30	
23Ba	DMSO-d_6	-		?*	153.45		132.79	133.76 broad	132.44	130.78	138.76	129.55	128.52 broad	129.84	?		73.27	60.26		-		-	-	
	DMSO-d_6 (75°C)	-		157.57	153.29		132.54	133.41	131.98	130.37	128.29	129.14	128.08	129.47	126.57		73.01	60.04		-		-	-	
24Ba	$CDCl_3$	169.64		?	156.51		133.53	132.74 broad	132.32	131.45	129.10 broad	129.61 broad	128.52	129.53	?		65.84	62.67		-		-	20.42	
23Bb	DMSO-d_6	-		?	153.33		133.79	132.81	132.40	130.73	128.76	129.54	128.46 split into 3 signals: 128.60, 128.46, 128.25	129.91	126.86 broad		73.41	60.19		-		-	-	
24Bb	$CDCl_3$	169.42		157.85	155.35		133.46	132.71	132.45	131.32	128.16	129.70	128.88 broad	129.51	128.88		66.30	62.58		-		-	20.29	

[a] "?" signifies that the signal is so broad that it disappeared in the baseline noise.

Table 5. Chemical shifts (δ, ppm) and coupling constants (*J*, Hz,) in ^1H NMR spectra (300 MHz) of pyridinium lipids of type **21** and **25** (in $CDCl_3$); assignments are given by means of signal intensity, selective decoupling and HETCOR (^1H-^{13}C)

Comp	Hβ		CH_2 (1)	CH	CH_2 (3)		CH_3 (Py+)			Fatty acid chain							
										CO-$\underline{CH_2}$			CO-CH_2-$\underline{CH_2}$-		CH=CH	$(CH_2)n$	CH_3
										3-$CO\underline{CH_2}$		2-$CO\underline{CH_2}$	3-$COCH_2\underline{CH_2}$	2-$COCH_2\underline{CH_2}$			
	($H\beta_A$)	($H\beta_B$)			H_A	H_B	α	α'	γ	H_a	H_b						
21AaP	7.45 s		4.87 d [7.3]	5.52 m [4.0; 5.2;]	4.49 dd [4.0; 12.3]	4.24 dd [5.2; 12.3]	2.88 s		2.52 s	2.22 dt [7.4; 7.4; 16.1]	2.08 dt [7.4; 7.4; 16.1]	2.36 t [7.5]	1.41 qnt [7.4]	1.62 qnt [7.4]	-	1.10-1.38 m	0.88 t [6.7]
21AaS	7.46 s		4.89 d [7.5]	5.52 m	4.50 dd [4.0; 12.4]	4.25 dd [5.2; 12.3]	2.89 s		2.52 s	2.22 dt [7.5; 7.5; 16.0]	2.08 dt [7.4; 7.4; 16.1]	2.36 t [7.5]	1.41 qnt [7.5]	1.62 qnt [7.5]	-	1.00-1.36 m	0.88 t [6.3]
21AaO	7.45 s		4.88 d [7.4]	5.52 m [4.2; 5.4]	4.49 dd [4.0; 12.3]	4.25 dd [5.2; 12.3]	2.88 s		2.53 s	2.22 dt	2.08 dt	2.36 t [7.5]	1.41 qnt [7.4]	1.62 m	5.26-5.41 m	1.03-1.38 m	0.88 t [6.7]
25AaP	7.62 d [2.1]	7.58 d [2.1]	4.83 dd [7.6; 12.4]	5.58 m [6.6]	4.65 dd [5.8; 12.4]		2.95 s	2.88 s	2.58 s	2.27 t [7.5]			1.50 qnt [7.0]		-	1.00-1.40 m	0.88 t [6.7]
25AaS	7.62 brs	7.59 brs	4.85 dd [7.6; 12.3]	5.55 m	4.66 dd [5.7; 12.3]		2.97 s	2.90 s	2.58 s	2.27 t [7.5]			1.50 qnt		-	1.00-1.40 m	0.88 t [6.7]
25AaO	7.59 d [2.2]	7.55 d [2.2]	4.85 dd [7.4; 12.3]	5.55 m [6.7]	4.66 dd [6.0; 12.3]		2.95 s	2.89 s	2.58 s	2.27 t [7.5]			1.50 m		5.27-5.44 m	1.20-1.40 m	0.88 t [6.7]
21AbP	7.44 s		4.83 d [7.4]	5.51 m [3.9; 5.2]	4.48 dd [3.9; 12.2]	4.24 dd [5.3; 12.2]	2.86 s		2.51 s	2.21 dt [7.4; 7.4; 16.1]	2.07 dt [7.4; 7.4; 16.1]	2.35 t [7.5]	1.40 qnt [7.2]	1.62 qnt [7.2]	-	1.08-1.34 m	0.88 t [6.8]
21AbS	7.45 s		4.87 m	5.52 m	4.48 dd [2.6; 12.4]	4.23 dd [5.0; 12.2]	2.88 s		2.53 s	2.21 dt [7.4; 7.4; 16.1]	2.07 dt [7.4; 7.4; 16.1]	2.37 t [7.4]	1.40 qnt [7.2]	1.62 qnt [7.2]	-	1.00-1.36 m	0.88 t [6.4]
21AbO	7.38 s		4.88-4.94 m	5.51 m [4.6; 5.0]	4.48 dd [4.2; 12.4]	4.23 dd [5.4; 12.3]	2.89 s		2.52 s	2.23 dt [7.4; 7.4; 16.1]	2.07 dt [7.4; 7.4; 16.1]	2.37 t [7.4]	1.42 qnt [7.4]	1.63 qnt [7.4]	5.28-5.42 m	1.20-1.38 m	0.88 t [6.7]
25AbP	7.60 d [1.9]	7.56 d [1.9]	4.82 dd [7.5; 12.4]	5.54 m [5.8; 7.5]	4.64 dd [5.8; 12.4]		2.93 s	2.86 s	2.57 s	2.26 t [7.4]			1.50 m		-	1.00-1.40 m	0.88 t [6.7]
25AbS	7.66 s	7.63 s	4.97 dd [7.4; 12.3]	5.61 m [5.6; 6.6]	4.75 dd [5.8; 12.3]		3.04 s	2.95 s	2.59 s	2.26 t [7.4]			1.50 m		-	1.00-1.40 m	0.88 t [6.3]
25AbO	7.70 s	7.67 s	4.95 dd [7.6; 12.4]	5.61 m	4.73 dd [5.8; 12.4]		3.04 s	2.94 s	2.60 s	2.26 dt [1.5; 7.4]			1.50 m		5.27-5.42 m	1.03-1.42 m	0.88 t [6.7]

qnt indicates quintet

[a] qnt indicates quintet.

ent. The two α-methyl groups as well as the pyridinium β-protons are also magnetically non-equivalent; these facts are confirmed by the ^{13}C NMR spectra.

In the final step of the synthesis of the novel cationic lipids the diols **19** and **23** were condensed with palmitoyl, stearoyl, or oleoyl chloride in acetonitrile in the presence of a stoichiometric amount of triethylamine, similarly to the synthesis of *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium (DOTAP, **2**).[31] The crude products were purified by flash chromatography to yield the final products **21** and **25** in pure form. The cationic lipids were extensively characterized by standard analytical and spectroscopic methods that confirmed the structures (see Table 5 and 6 and the Exp. Sect.). Mention must be made that owing to the perchlorate and tetrafluoroborate counterions, known to affect the process of combustion, the elemental analyses

Table 6. ^{13}C NMR chemical shifts (δ, ppm; 75 MHz) in different solvents for pyridinium lipids of type **21** and **25** (in $CDCl_3$); assignments are given by means of signal intensity, APT and HETCOR (^1H-^{13}C)

Comp	C=O		C(Py+)					CH	CH2		OCOCH2			OCOCH2CH2			CH=CH	(CH2)n	CH2CH3	CH3	CH3 from Py+		
	2-CO	3-CO	γ	α	(α')	β	(β')		3-CH2	1-CH2	2-OCOCH2		3-OCOCH2	2-OCOCH2CH2		3-OCOCH2CH2					α	(α')	γ
											HA	HB		HA	HB								
21AaP	173.06	172.38	158.58	155.55		128.61		68.39	62.36	51.97	33.86		33.65	22.74		24.52	-	31.88, 29.68, 29.66, 29.63, 29.58, 29.48, 29.43, 29.33, 29.28, 29.18, 29.10, 28.89	22.65	14.08	21.63		21.75
21AaS	173.05	172.36	158.59	155.53		128.62		68.38	62.36	51.93	33.85		33.63	24.73		24.51	-	31.87, 29.65-29.60m, 29.46, 29.42, 29.31, 29.27, 29.17, 29.08, 28.86	22.64	14.06	21.60		21.73
21AaO	173.07	172.38	158.60	155.54		128.62		68.42	62.39	51.99	33.87	33.83	33.63	24.72	24.65	24.50	130.11, 129.98, 129.68, 129.48	31.87, 29.72, 29.66, 29.64, 29.48, 29.42, 29.28, 29.18, 29.10, 29.03, 28.94	22.64	14.08	21.64		21.73
25AaP	173.16		159.60	155.94	155.00	131.18	129.34	63.63	61.60		33.62			24.59				31.88, 29.66, 29.62, 29.59, 29.44, 29.32, 29.22, 28.99	22.65	14.08	23.10	22.33	21.54
25AaS	173.14		159.63	155.94	154.97	131.11	129.33	63.64	61.65		33.59			24.55				31.84, 29.68-29.38m, 29.28, 29.16, 29.10, 29.01, 28.94, 28.77	22.61	14.03	23.13	22.38	21.54
25AaO	173.20		159.59	155.93	154.96	131.14	129.34	63.64	61.61		34.07	33.95	33.63	24.91	24.70	24.58	130.04, 129.96, 129.69, 129.59	31.86, 29.73, 29.65, 29.56, 29.48, 29.42, 29.28, 29.18, 29.12, 29.05, 28.95	22.64	14.08	23.14	22.39	21.59
21AbP	173.08	172.38	158.55	155.59		128.51		68.42	62.35	51.76	33.85		33.64	24.75		24.53		31.89, 29.68, 29.66, 29.63, 29.58, 29.48, 29.43, 29.33, 29.28, 29.19, 29.11, 28.88	22.65	14.08	21.54		
21AbS	173.07	172.38	158.58	155.58		128.53		68.40	62.35	51.73	33.85		33.63	24.74		24.52		31.88, 29.68-29.58m, 29.47, 29.42, 29.31, 29.27, 29.17, 29.09, 28.87	22.64	14.07	21.54		
21AbO	173.07	172.38	158.48	155.62		128.49		68.47	62.38	51.89	34.05	33.81	33.61	24.72	24.49	24.49	130.09, 129.95, 129.68, 129.47	31.85, 29.78-29.50m, 29.47m, 29.26, 29.18, 29.12, 29.04, 28.94	22.63	14.06	21.55		21.61
25AbP	173.16		159.60	155.96	155.05	131.08	129.25	63.63	61.53		33.60			24.59				31.88, 29.66, 29.63, 29.59, 29.44, 29.32, 29.21, 28.99	22.65	14.07	22.94	22.16	21.45
25AbS	173.21		159.40	156.03	155.28	131.11	129.26	63.80	61.70		33.66			24.59				31.86, 29.63m, 29.41, 29.30, 29.18, 29.14, 28.98	22.62	14.05	23.29		21.61
25AbO	173.18		159.36	156.09	155.28	131.13	129.30	63.84	61.66		33.64			24.58			130.04, 129.90, 129.70, 129.55	31.85, 29.70, 29.64, 29.47, 29.40, 29.27, 29.13, 29.08, 29.02, 28.94	22.63	14.06	23.34		21.62

were done for nitrogen only. The transition temperatures (T_c) of the lipids were determined by differential scanning calorimetry (DSC).

The NMR spectra of cationic lipids are similar to the NMR spectra of corresponding acetates. From the ^1H NMR spectra of the oleoyl derivatives, where no *trans* coupling constant was observed in the δ = 5.5 ppm alkenic proton range, we infer that no *cis-trans* isomerization has taken place during the reaction.

Along with the 2,4,6-trimethylpyridinium derivatives, we obtained two 2,4,6-triphenyl-substituted pyridinium lipids by the same synthetic strategy (see Exp. Sect. for characterization). The presence of the 2,4,6-triphenylpyridinium moiety, which has a strong UV fluorescence and a more pronounced lipophilic character, raises the possibility of using these compounds as markers for bio-membrane studies.

Biological Assays

Transfection experiments were carried out in vitro on HeLa cells (human carcinoma cell line ATCC No. CCL-2), using a pGL3-Control plasmid (Promega, Madison, WI), which encodes the firefly luciferase reporter. The structurally related and commercially available DOTAP (**2**) mentioned earlier was chosen as reference.

Good transfection efficiencies were obtained in the case of tetrafluoroborates **21Ab** and **25Ab** (Figure 1). The perchlorates were practically devoid of any detectable biological effect in these specific assays. This fact might be due to

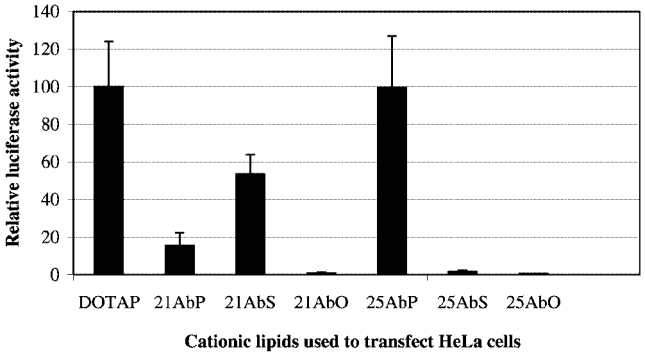


Figure 1. Relative luciferase activity for pyridinium tetrafluoroborate lipids **21Ab** and **25Ab**

inhibition of sulfation of proteoglycans as reported in the literature for the chlorate anion.[32] Compound **25AbP** showed a transfection efficiency similar to that of DOTAP.

A very important parameter of the transfection assays is the cytotoxicity of the cationic vectors. Interestingly, the new cationic lipids exhibit a lower cytotoxicity (20−50%) than the DOTAP reference (60−75%). This finding agrees with the results of Engbert's group[18,19] regarding the reduced cytotoxicity of pyridinium cationic lipids.

Conclusions

Two series of substituted pyridinium cationic lipids were synthesized via pyrylium precursors and long-chain fatty acid derivatives, and extensively characterized: 1-(2,3-diacy-

loxypropane-1-yl)-2,4,6-trimethylpyridinium and 1-(1,3-diacyloxypropane-2-yl)-2,4,6-trimethylpyridinium salts. The related acetates, derived from the same key diol intermediates, were also synthesized. A detailed NMR study, including dynamic NMR spectroscopy, was performed on all these compounds, for elucidating their structural particularities.

Biological assessment of the novel lipids showed transfection efficiencies that reached the level of commercial lipidic vectors but were less cytotoxic. Also, this strategy will allow the preparation of fluorescent lipids with possible application as membrane markers, as will be shown in a future paper.

Experimental Section

Materials and Methods: Melting points for the diols, acetates and ketals were determined on a Boetius heating plate microscope and are uncorrected. For the cationic lipids **21** and **25** the phase transition temperature T_c was determined by differential scanning calorimetry (DSC), using a TA-Instruments Q100 DSC, and a heating rate of 5 °C/minute. The IR spectra were recorded on a Nicolet Avatar 360 FTIR spectrophotometer, in the range 650−4000 cm^{-1}, using a ZnSe-attenuated total reflectance (ATR) accessory. The compounds were dissolved in a small amount of solvent (MeOH for the diols, $CHCl_3$ for the esters), the resultant solutions being left to evaporate to dryness on the surface of the ZnSe crystals of the ATR accessory. This technique was imposed by the poor results obtained in transmission mode for some of the compounds, due to the lack of crystallization, hygroscopicity and other problems related with working with KBr pellets. The NMR spectra were recorded at about 303 K with a Varian Gemini 300BB spectrometer operating at 300 MHz for ^1H and at 75 MHz for ^{13}C. Chemical shifts are reported as δ values, using TMS as internal standard for proton spectra and the solvent resonance for carbon spectra: δ = 77.00 ppm in $CDCl_3$, δ = 39.50 ppm in [D_6]DMSO, the line from δ = 123.50 ppm in [D_5]pyridine, δ = 62.80 ppm in [D_3]nitromethane, δ = 30.10 ppm in [D_7]DMF. Assignments were made based on signal intensity, selective decoupling and COSY (^1H-^1H) and HETCOR (^1H-^{13}C) sequences. Nitrogen elemental analysis was performed by combustion, mixing the samples with small amounts of quartz sand.

Racemic 3-amino-1,2-propanediol was purchased from Fluka, and racemic 2-amino-1,3-propanediol hydrochlorid from Aldrich; triethylamine, acetic anhydride, acetic acid, acyl chlorides and other solvents were obtained from Acros, Aldrich, Fluka and/or Merck. TLC was performed on silica gel 60-F_{254} plates (from Merck), eluting with MeOH/$CHCl_3$ (20:80, v/v). The pyrylium salts were prepared according to the literature.[23,24]
CAUTION: Since perchlorates may explode when they are heated or hit in dry form, they should be stored moist with water.

The HeLa cell line was obtained from ATCC, MD. The plasmid pGL3-control, encoding a firefly luciferase reporter, was from Promega (Madison, WI). As transfection reference we used *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate (DOTAP), obtained from Roche Molecular Biochemicals (Indianapolis, IN). The BCA protein assay kit was from Pierce (Rockford, IL).

General Procedure for the Preparation of the Pyridinium Diols 19 and 23: Aminopropanediol (10 mmol) was dissolved whilst stirring in 30 mL of anhydrous ethanol. When using the aminopropanediol hydrochloride, 10 mmol of freshly cut sodium was first stirred with the ethanol to yield sodium ethoxide, and the hydrochloride (10 mmol) was added to the resultant ethoxide solution, which was stirred for 15 minutes and then filtered to separate sodium chloride. Next, the corresponding pyrylium salt (12 mmol of perchlorate or tetrafluoroborate) was added, followed immediately by the addition of 12 mmol of triethylamine. The resultant homogeneous mixture was heated to reflux for 15 minutes, then 25 mmol of glacial acetic acid was added and the reflux was continued for 1−3 hours (TLC control). After this period, concentrated aqueous ammonia (2 mL) was added and the mixture was heated for 5 min in order to convert any unchanged pyrylium salt into the corresponding pyridine, which is soluble in diethyl ether. The final solution was cooled and poured, whilst stirring, into anhydrous diethyl ether (200−300 mL). The resulting insoluble heavier oily layer was separated and washed with two additional portions (20 mL each) of diethyl ether. After a final separation, the oily layer was taken up in a few milliliters of hot isopropyl alcohol, treated with charcoal, filtered and allowed to cool slowly, whereupon crystallization occurred; if not, the resultant viscous oil was separated from the mother liquor, redissolved in the minimum amount of hot methanol or 2-propanol, and the concentrated solution was allowed to cool. Sometimes, crystallization occurred only after a longer time, even in case of very pure compounds (some of them could never be obtained in crystalline form). Yields were in the range of 50−80%. The products were recrystallized from methanol or 2-propanol.

General Procedure for the Preparation of the Pyridinium Diacetates 20 and 24: The *N*-(propanediol)pyridinium salt **19** or **23** (5 mmol) was treated with 15 mL of acetic anhydride and a small amount of solid 4-toluenesulfonic acid. The mixture was heated to reflux for 10−30 minutes (TLC control), then allowed to cool and poured into 100 mL of anhydrous diethyl ether, whereupon the desired product crystallized. If not, the oily product was washed with two additional portions (10 mL each) of diethyl ether, decanted, dissolved in the minimum amount of hot methanol or 2-propanol, treated with charcoal, filtered and allowed to cool slowly, whereupon crystallization occurred. Yields varied from 55 to 88%. The products were recrystallized from methanol or 2-propanol.

General Procedure for the Preparation of the Pyridinium Lipids 21 and 25: The *N*-(propanediol)pyridinium salt **19** or **23** (2 mmol) was dissolved whilst stirring in 15 mL of anhydrous acetonitrile. Triethylamine (0.56 mL, 4 mmol) was added, followed by dropwise addition of acid chloride R′COCl (4.4 mmol), whereupon the color became yellow, and triethylamine hydrochloride started to precipitate. After stirring at room temperature for 3 hours, the solution was refluxed for another 2 hours. The solvent was evaporated (rotavapor) under reduced pressure, and the residue was extracted with 15 mL of distilled water and 15 mL of chloroform. The aqueous layer was separated, extracted with 15 mL of chloroform, and discarded. The combined chloroform extracts were shaken with 15 mL of distilled water, dried over sodium sulfate, and the solvents evaporated under reduced pressure. Final purification was effected by flash chromatography on silica gel 60 (40−60 μ^m) with a solvent mixture of chloroform and methanol (80:20 v/v), followed by recrystallization from acetonitrile for perchlorates and hexafluorophosphates, or from *n*-hexane for tetrafluoroborates.

1-(2,3-Dihydroxypropyl)-2,4,6-trimethylpyridinium Perchlorate (19Aa): M.p. 121−122 °C; NMR: see Table 1−4. IR (thin film on ZnSe ATR crystal): $\tilde{\nu}$ = 1080 cm^{-1} (ClO_4^-), 1480, 1577, 1640, 2927, 3484. $C_{11}H_{18}NO_2^+$ ClO_4^- (295.72): calcd. N 4.74; found N 4.80.

1-(2,3-Dihydroxypropyl)-2,4,6-trimethylpyridinium Tetrafluoroborate (19Ab): M.p. 96−97 °C; NMR: see Table 3 and 4. IR: $\tilde{\nu}$ = 998 cm^{-1}, 1041, 1083, 1481, 1577, 1640, 2936, 3533. $C_{11}H_{18}NO_2^+$ BF_4^- (283.07): calcd. N 4.95; found N 5.05.

1-(2,3-Dihydroxypropyl)-2,4,6-triphenylpyridinium Perchlorate (19Ba): NMR: see Table 3 and 4. IR: $\tilde{\nu}$ = 702 cm^{-1}, 767, 891, 1061 (ClO_4^-), 1562, 1599, 1618, 2936, 3476. $C_{26}H_{24}NO_2^+$ ClO_4^- (481.92): calcd. N 2.91; found N 3.25.

1-(2,3-Dihydroxypropyl)-2,4,6-triphenylpyridinium Tetrafluoroborate (19Bb): NMR: see Table 3 and 4. IR: $\tilde{\nu}$ = 701 cm^{-1}, 767, 891, 1046, 1495, 1562, 1599, 1619, 2929, 3528. $C_{26}H_{24}NO_2^+$ BF_4^- (469.28): calcd. N 2.98; found N 3.32.

1-(2,3-Diacetoxypropyl)-2,4,6-trimethylpyridinium Perchlorate (20Aa): M.p. 128−129 °C; NMR: see Table 3 and 4. IR: $\tilde{\nu}$ = 1084 cm^{-1} (ClO_4^-), 1371, 1577, 1640, 1743, 2948. $C_{15}H_{22}NO_4^+$ ClO_4^- (379.79): calcd. N 3.69; found N 3.74.

1-(2,3-Diacetoxypropyl)-2,4,6-trimethylpyridinium Tetrafluoroborate (20Ab): M.p. 119−120 °C; NMR: see Table 3 and 4. IR: $\tilde{\nu}$ = 1026 cm^{-1}, 1035, 1061, 1216, 1577, 1641, 1743, 2993. $C_{15}H_{22}NO_4^+$ BF_4^- (367.14): calcd. N 3.81; found N 3.80.

1-(2,3-Diacetoxypropyl)-2,4,6-triphenylpyridinium Perchlorate (20Ba): NMR: see Table 3 and 4. IR: $\tilde{\nu}$ = 704 cm^{-1}, 749, 892, 1090 (ClO_4^-), 1215, 1561, 1599, 1620, 1745. $C_{30}H_{28}NO_4^+$ ClO_4^- (566.00): calcd. N 2.47; found N 2.82.

1-(2,3-Diacetoxypropyl)-2,4,6-triphenylpyridinium Tetrafluoroborate (20Bb): NMR: see Table 3 and 4. IR: $\tilde{\nu}$ = 700 cm^{-1}, 766, 891, 1042, 1224, 1495, 1561, 1599, 1620, 1737. $C_{30}H_{28}NO_4^+$ BF_4^- (553.35): calcd. N 2.53; found N 2.78.

1-(2,3-Dipamitoyloxypropyl)-2,4,6-trimethylpyridinium Perchlorate (21AaP): T_c: 106 °C; NMR: see Table 5 and 6. IR: $\tilde{\nu}$ = 1085 cm^{-1} (ClO_4^-), 1370, 1577, 1643, 1745, 2860, 2930. $C_{43}H_{78}NO_4^+$ ClO_4^- (772.53): calcd. N 1.81; found N 1.68.

1-(2,3-Distearoyloxypropyl)-2,4,6-trimethylpyridinium Perchlorate (21AaS): T_c: 110 °C; NMR: see Table 5 and 6. IR: $\tilde{\nu}$ = 1084 cm^{-1} (ClO_4^-), 1371, 1578, 1642, 1744, 2861, 2938. $C_{47}H_{86}NO_4^+$ ClO_4^- (828.64): calcd. N 1.69; found N 1.50.

1-(2,3-Dioleoyloxypropyl)-2,4,6-trimethylpyridinium Perchlorate (21AaO): T_c: 57 °C; NMR: see Table 5 and 6. IR: $\tilde{\nu}$ = 1084 cm^{-1} (ClO_4^-), 1370, 1578, 1640, 1664, 1743, 2850, 2938, 3024. $C_{47}H_{82}NO_4^+$ ClO_4^- (824.61): calcd. N 1.70; found N 1.38.

1-(2,3-Dipamitoyloxypropyl)-2,4,6-trimethylpyridinium Tetrafluoroborate (21AbP): T_c: 100 °C; NMR: see Table 5 and 6. IR: $\tilde{\nu}$ = 1026 cm^{-1}, 1035, 1061, 1216, 1578, 1643, 1743, 2860, 2940. $C_{43}H_{78}NO_4^+$ BF_4^- (759.89): calcd. N 1.84; found N 1.75.

1-(2,3-Distearoyloxypropyl)-2,4,6-trimethylpyridinium Tetrafluoroborate (21AbS): T_c: 103 °C; NMR: see Table 5 and 6. IR: $\tilde{\nu}$ = 1025 cm^{-1}, 1034, 1064, 1216, 1577, 1643, 1743, 2860, 2943. $C_{47}H_{86}NO_4^+$ BF_4^- (815.99): calcd. N 1.72; found N 1.58.

1-(2,3-Dioleoyloxypropyl)-2,4,6-trimethylpyridinium Tetrafluoroborate (21AbO): T_c: 52 °C; NMR: see Table 5 and 6. IR: $\tilde{\nu}$ = 1084 cm^{-1}, 1371, 1577, 1640, 1666, 1743, 2864, 2948; 3024. $C_{47}H_{82}NO_4^+$ BF_4^- (811.96): calcd. N 1.73; found N 1.50.

1-(2,3-Dipalmitoyloxypropyl)-2,4,6-triphenylpyridinium Perchlorate (21BaP): T_c: 134 °C. ^1H NMR ($CDCl_3$): δ = 7.91 (s, 2 H, Hβ pyridinium) 7.79 (m, J = 1.5, 8.1 Hz, 2 H, Hα from phenylα), 7.5−7.7 (m, 13 H, H from phenyl groups), 5.03 (m, J = 4.7, 12.2 Hz, 3 H, CH_2-N + CH), 3.77 (dd, J = 3.9, 12.2 Hz, 1 H, H_A from CH_2-O), 3.45 (dd, J = 3.9, 12.2 Hz, 1 H, H_B from CH_2-O), 2.30 (t, J = 7.5 Hz, 1 H, H_A from 2-COCH_2), 2.22 (dt, J = 7.4, 7.4, 16.1 Hz, 1 H, H_A from 3-COCH_2), 2.08 (dt, J = 7.4, 7.4, 16.1 Hz, 1 H, H_B from 3-COCH_2), 1.63 (qnt, J = 7.4 Hz, 2 H, 2-$COCH_2CH_2$), 1.42 (qnt, J = 7.4 Hz, 2 H, 3-$COCH_2CH_2$), 1.10−1.38 (m, 48 H, 24 CH_2 from fatty chains), 0.88 (t, J = 6.7, 6 H, 2 CH_3 from fatty chains) ppm. ^{13}C NMR ($CDCl_3$): δ = 172.52 (2-CO), 172.47 (3-CO), 157.63 (Cγ pyridinium), 156.54 (2Cα pyridinium), 133.61, 132.67, 132.51, 131.36, 129.80, 129.58, 129.49, 128.12, 127.40 (all from phenylic substituents of pyridinium ring) 126.61 (2Cβ pyridinium), 68.88 (CH), 61.62 (O-CH_2), 54.45 (N-CH_2), 34.10, 33.93, 33.53, 31.90, 29.66, 29.57, 29.48, 29.42, 29.34, 29.23, 29.13, 29.03, 24.95, 24.62, 24.52, 22.67 (all from fatty chains), 14.09 (2CH_3 from fatty chains) ppm. IR: $\tilde{\nu}$ = 722 cm^{-1}, 856, 930, 1098 (ClO_4^-), 1170, 1239, 1378, 1413, 1440, 1574, 1637, 1695, 2853, 2930, 2980, 3001. $C_{58}H_{(84}NO_)_4^+$ ClO_4^- (958.74): calcd. N 1.46; found N 1.60.

1-(1,3-Dihydroxypropane-2-yl)-2,4,6-trimethylpyridinium Perchlorate (23Aa): NMR: see Table 3 and 4. IR: $\tilde{\nu}$ = 1090 cm^{-1} (ClO_4^-), 1481, 1574, 1640, 3490. $C_{11}H_{18}NO_2^+$ ClO_4^- (295.72): calcd. N 4.74; found N 5.02.

1-(1,3-Dihydroxypropane-2-yl)-2,4,6-trimethylpyridinium Tetrafluoroborate (23Ab): NMR: see Table 3 and 4. IR: $\tilde{\nu}$ = 1036 cm^{-1}, 1055, 1224, 1572, 1635, 3483. $C_{11}H_{18}NO_2^+$ BF_4^- (283.07): calcd. N 4.95; found N 5.27.

1-(1,3-Dihydroxypropane-2-yl)-2,4,6-triphenylpyridinium Perchlorate (23Ba): M.p. 197−198 °C; NMR: see Table 3 and 4. IR: $\tilde{\nu}$ = 702 cm^{-1}, 764, 891, 1100 (ClO_4^-), 1494, 1561, 1598, 1618, 3473. $C_{26}H_{24}NO_2^+$ ClO_4^- (481.92): calcd. N 2.91; found N 3.14.

1-(1,3-Dihydroxypropane-2-yl)-2,4,6-triphenylpyridinium Tetrafluoroborate (23Bb): M.p. 232−234 °C; NMR: see Table 3 and 4. IR: $\tilde{\nu}$ = 702 cm^{-1}, 764, 890, 1493, 1561, 1598, 1618, 3232. $C_{26}H_{24}NO_2^+$ BF_4^- (469.28): calcd. N 2.98; found N 3.15.

1-(1,3-Diacetoxypropane-2-yl)-2,4,6-trimethylpyridinium Perchlorate (24Aa): M.p. 111−112 °C; NMR: see Table 3 and 4. IR: $\tilde{\nu}$ = 1091 cm^{-1} (ClO_4^-), 1223, 1572, 1638, 1743. $C_{15}H_{22}NO_4^+$ ClO_4^- (379.79): calcd. N 3.69; found N 3.65.

1-(1,3-Diacetoxypropane-2-yl)-2,4,6-trimethylpyridinium Tetrafluoroborate (24Ab): M.p. 110−111 °C; NMR: see Table 3 and 4. IR: $\tilde{\nu}$ = 1034 cm^{-1}, 1055, 1222, 1573, 1637, 1743. $C_{15}H_{22}NO_4^+$ BF_4^- (367.14): calcd. N 3.81; found N 3.86.

1-(2,3-Diacetoxypropane-2-yl)-2,4,6-triphenylpyridinium Perchlorate (24Ba): M.p. 201−202 °C; NMR: see Table 3 and 4. IR: $\tilde{\nu}$ = 704 cm^{-1}, 766, 892, 1045, 1092 (ClO_4^-), 1220, 1494, 1562, 1598, 1620, 1746. $C_{30}H_{28}NO_4^+$ ClO_4^- (566.00): calcd. N 2.47; found N 2.43.

1-(2,3-Diacetoxypropane-2-yl)-2,4,6-triphenylpyridinium Tetrafluoroborate (24Bb): NMR: see Table 3 and 4. IR: $\tilde{\nu}$ = 704 cm^{-1}, 765, 891, 1042, 1220, 1561, 1598, 1618, 1742. $C_{30}H_{28}NO_4^+$ BF_4^- (553.35): calcd. N 2.53; found N 2.84.

1-(1,3-Dipamitoyloxypropane-2-yl)-2,4,6-trimethylpyridinium Perchlorate (25AaP): T_c: 66 °C; NMR: see Table 5 and 6. IR: $\tilde{\nu}$ = 1089 cm^{-1} (ClO_4^-),, 1374, 1580, 1640, 1747, 2863, 2931. $C_{43}H_{78}NO_4^+$ ClO_4^- (772.53): calcd. N 1.81; found N 1.73.

1-(1,3-Distearoyloxypropane-2-yl)-2,4,6-trimethylpyridinium Perchlorate (25AaS): T_c: 70 °C; NMR: see Table 5 and 6. IR: $\tilde{\nu}$ = 1084 cm^{-1} (ClO_4^-), 1375, 1580, 1640, 1747, 2860, 2940. $C_{47}H_{86}NO_4^+$ ClO_4^- (828.64): calcd. N 1.69; found N 1.43.

1-(1,3-Dioleoyloxypropane-2-yl)-2,4,6-trimethylpyridinium Perchlorate (25AaO): T_c: 48 °C; NMR: see Table 5 and 6. IR: $\tilde{\nu}$ = 1088 cm^{-1} (ClO_4^-), 1375, 1580, 1640, 1675, 1743, 2855, 2935, 3021. $C_{47}H_{82}NO_4^+$ ClO_4^- (824.61): calcd. N 1.70; found N 1.41.

1-(1,3-Dipamitoyloxypropane-2-yl)-2,4,6-trimethylpyridinium Tetrafluoroborate (25AbP): T_c: 70 °C; NMR: see Table 5 and 6. IR: $\tilde{\nu}$ = 1025 cm^{-1}, 1039, 1061, 1217, 1578, 1640, 1745, 2863, 2940. $C_{43}H_{78}NO_4^+$ BF_4^- (759.89): calcd. N 1.84; found N 1.71.

1-(1,3-Distearoyloxypropane-2yl)-2,4,6-trimethylpyridinium Tetrafluoroborate (25AbS): T_c: 73 °C; NMR: see Table 5 and 6. IR: $\tilde{\nu}$ = 1022 cm^{-1}, 1035, 1063, 1217, 1575, 1641, 1742, 2863, 2943. $C_{47}H_{86}NO_4^+$ BF_4^- (815.99): calcd. N 1.72; found N 1.42.

1-(1,3-Dioleoyloxypropane-2-yl)-2,4,6-trimethylpyridinium Tetrafluoroborate (25AbO): T_c: 43 °C; NMR: see Table 5 and 6. IR: $\tilde{\nu}$ = 1082 cm^{-1}, 1370, 1574, 1640, 1665, 1740, 2862, 2948; 3025. $C_{47}H_{82}NO_4^+$ BF_4^- (811.96): calcd. N 1.73; found N 1.43.

1-(1,3-Dipalmitoyloxypropane-2-yl)-2,4,6-triphenylpyridinium Perchlorate (25BaP): T_c: 125 °C. ^1H NMR ($CDCl_3$): δ = 7.90 (vbs, 2 H, Hβ pyridinium); 7.78 (m, J = 1.5, 8.1 Hz, 2 H, Hα phenyls-α), 7.43−7.7 (m, 13H from phenyls), 5.30 (t, J = 6.7 Hz, 1 H, CH), 4.45 (dd, J = 6.8, 12.2, 2 H, 1-CH_2-O), 4.03 (dd, J = 6.6, 12.2 Hz, 2 H, 3-CH_2-O), 2.18 (dt, J = 7.5 Hz, 2 H, 1-COCH_2), 2.17 (t, J = 7.5 Hz, 2 H, 3-COCH_2), 1.46 (qnt, J = 7.4 Hz, 4 H, 2 $COCH_2CH_2$), 1.15−1.36 (m, 48 H, 24 CH_2 from fatty chains), 0.88 (t, J = 6.7 Hz, 6 H, 2 CH_3 from fatty chains) ppm. ^{13}C NMR ($CDCl_3$): δ = 172.30 (2CO), 156.50 (broad, Cγ pyridinium), 156.32 (2Cα pyridinium), 133.47, 132.21, 131.34, 129.64, 129.44, 128.97, 128.93, 128.80, 128.39 (all from Ph substituents of pyridinium), 127.0 (very broad, Cβ pyridinium), 66.09 (CH), 62.59 (2 CH_2 propane), 42.64, 33.84, 33.49, 31.77, 29.54, 29.49, 29.45, 29.27, 29.21, 29.11, 29.03, 28.96, 28.86, 27.33, 24.66, 24.47, 23.74, 22.53 (all from CH_2 from fatty chains), 13.96 ($2CH_3$ from fatty chains) ppm. IR (thin film on ZnSe ATR crystal): $\tilde{\nu}$ = 724 cm^{-1}, 874, 932, 1098 (ClO_4^-), 1173, 1242, 1384, 1415, 1443, 1567, 1642, 1690, 2850, 2932, 2980, 3010. $C_{58}H_{84}NO_4^+$ ClO_4^- (958.74): calcd. N 1.46; found N 1.58.

Transfection Experiments

Assay Designed to Measure Transfection Efficiency

All of the pyridinium cationic lipids, along with the DOTAP reference, were tested in triplicate in two independent assays. The pyridinium cationic lipids containing tetrafluoroborate (BF_4^-) or perchlorate (ClO_4^-) anion were dissolved in ethanol/chloroform. The lipid solution was evaporated to dryness using a Speedvac rotary evaporating device, under vacuum. Traces of solvents were removed from the lipid film by keeping the vials under vacuum for another 3 hours. Ultrapure water was added to the dry lipid film, so that a final 1 μg/μL concentration could be reached, and the lipid film was allowed to hydrate overnight. The next day it was sonicated in an ultrasonic bath for 15 minutes in order to generate the liposomes. A solution containing 1.5 μg of DNA was prepared by mixing 0.8 μg of pGL3-Control plasmid DNA and 0.7 μg of sonicated salmon sperm DNA in cell culture medium. In a separate tube, 9 μL of liposome solution was diluted in 21 μL of cell culture medium and was added to the vial containing the DNA. After incubation for 20 minutes at room temperature, the lipoplex mixture was diluted in the cell culture medium to a final volume of 0.5 mL then added directly to a single 16 mm tissue culture dish containing approximately 7000 HeLa cells. After a 4 hours incubation at 37 °C in 5% CO_2, the lipid/DNA mixture was removed from the cells and replaced with fresh cell culture medium. Cells were harvested 28 hours post-transfection.

Procedure used to measure luciferase activity

Luciferase assays were conducted using reagents manufactured by Promega (Madison, WI). Cell extracts were prepared by adding cell lysis buffer to each dish of HeLa cells. A dish of HeLa cells represented a sample. An aliquot from each sample was mixed with luciferin to measure luciferase activity in an Automat luminometer (EG&G Company, Gaithersburg, MD). The total amount of protein present in each sample was used to normalize the luciferase activity.

Cytotoxicity Determinations

The determination of cytotoxicity was effected by counting under the microscope the number of dead cells post-transfection in three sets of experiments.

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