

University of Groningen

## Novel biodegradable pyridinium amphiphiles for gene delivery

Pijper, D; Bulten, E; Smisterova, J; Wagenaar, A; Hoekstra, Dirk; Engberts, Jan; Hulst, R; Šmisterová, Jarmila

*Published in:*  
European Journal of Organic Chemistry

*DOI:*  
[10.1002/ejoc.200300361](https://doi.org/10.1002/ejoc.200300361)

**IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.**

*Document Version*  
Publisher's PDF, also known as Version of record

*Publication date:*  
2003

[Link to publication in University of Groningen/UMCG research database](#)

*Citation for published version (APA):*

Pijper, D., Bulten, E., Smisterova, J., Wagenaar, A., Hoekstra, D., Engberts, J. B. F. N., ... Šmisterová, J. (2003). Novel biodegradable pyridinium amphiphiles for gene delivery. *European Journal of Organic Chemistry*, (22), 4406-4412. DOI: 10.1002/ejoc.200300361

**Copyright**

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

**Take-down policy**

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

*Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.*

## Novel Biodegradable Pyridinium Amphiphiles for Gene Delivery

Dirk Pijper,<sup>[a]</sup> Erna Bulten,<sup>[c]</sup> Jarmila Šmisterová,<sup>[c]</sup> Anno Wagenaar,<sup>[a]</sup> Dick Hoekstra,<sup>[d]</sup>  
Jan B. F. N. Engberts,<sup>\*[a]</sup> and Ron Hulst<sup>\*[a,b]</sup>

**Keywords:** Amphiphiles / Biodegradable / Gene technology / Vesicles

Biodegradable synthetic cationic pyridinium-based amphiphiles (SAINTs) prove to be promising non-viral carrier systems for delivery of DNA into eukaryotic cells. Six novel SAINTs were synthesised from 3,5-pyridinedicarboxylic acid as starting material, with two ester groups as linkers between the cationic headgroup and the hydrophobic tails. The vesicle-forming properties of the amphiphiles were studied by

differential scanning calorimetry and transmission electron microscopy, whereas the hydrolysis of the diesters in water was investigated by NMR spectroscopy. Finally, the transfection potential and cytotoxicity were determined on COS-7 and HepG-2 cells in culture.

(© Wiley-VCH Verlag GmbH & Co. KGaA, 69451 Weinheim, Germany, 2003)

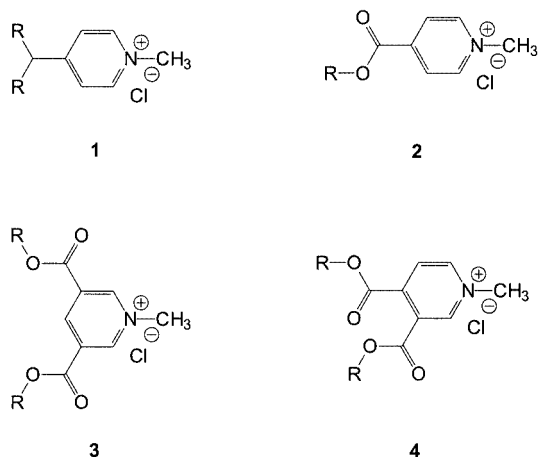
### Introduction

Gene therapy is widely regarded as a promising method in the treatment of diseases caused by genetic disorders (e.g., cystic fibrosis) and, more importantly, can also be applied for the delivery of any type of therapeutic protein, opening up possibilities for treatment of a wide range of diseases, including infectious (e.g., AIDS), acquired (e.g., Parkinson's, arthritis) or cardiovascular (e.g., arteriosclerosis) diseases, besides several types of cancer.<sup>[1–3]</sup> The concept is straightforward, and involves the induction of expression of certain proteins in the diseased target cells through the introduction of foreign DNA.

The most efficient carrier systems for the transfer of DNA into cells (transfection) at present are viral vectors.<sup>[4]</sup> These, however, are associated with risks regarding uncontrolled recombination of viral DNA with host DNA and, in particular, immunogenicity.<sup>[5–7]</sup> These problems can (partly) be circumvented by the use of nonviral synthetic vector systems. Cationic amphiphiles have established a promising position as reliable transfection agents, and numerous studies have reported a number of cationic amphiphiles promoting the delivery and expression of foreign

DNA in eukaryotic cells.<sup>[8–14]</sup> Although promising, great improvements in the transfection efficiency are still necessary and their use is often accompanied by undesired cytotoxic complications.

Our group has previously reported on a class of cationic pyridinium amphiphiles (SAINTs = Synthetic Amphiphiles Interdisciplinary) showing high transfection efficiency combined with low cytotoxicity.<sup>[15–17]</sup> Thanks to the synthetic ease with which structural modifications can be introduced in the original SAINT concept, this class is very suitable for studies regarding the relationship between chemical structure, transfection efficiency and cytotoxicity.



R = C<sub>12:0</sub>, C<sub>14:0</sub>, C<sub>14:1</sub>, C<sub>16:0</sub>, C<sub>16:1</sub>, C<sub>18:0</sub>, C<sub>18:1</sub>

Figure 1. Generalised structures **1** and **2** of SAINT amphiphiles; diester SAINTs **3** represent the amphiphiles used in the current study; diesters **4** represent possible structural variations<sup>[18]</sup>

<sup>[a]</sup> Physical Organic Chemistry Unit, Stratingh Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands  
Fax: (internat.) +31-(0)50-3634269  
E-mail: j.b.f.n.engberts@chem.rug.nl

<sup>[b]</sup> Kiadis BV, Niels Bohrweg 11–13, 2333 CA Leiden, The Netherlands

<sup>[c]</sup> BiOMaDe Technology Foundation, Nijenborgh 4, 9747 AG Groningen, The Netherlands.

<sup>[d]</sup> Department of Membrane Cell Biology, University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands

In a recent study,<sup>[18]</sup> an ester moiety was introduced as a linker between the cationic headgroup and the hydrophobic tails in order to increase the transfection potential of the cytofectin for enhanced (controlled) release.<sup>[19,20]</sup> Recent findings clearly point in the direction of an endocytotic pathway by which the lipoplexes are taken up by the cell.<sup>[21]</sup> It was initially anticipated that, at the level of endosomal escape, the lipoplex would be destabilised through acid-induced hydrolysis of the amphiphiles, facilitating the escape of DNA.<sup>[22,23]</sup> Incorporation of biodegradable ester groups into the amphiphiles might also enhance intracellular metabolism, thus avoiding long-term persistence of the unnatural cationic lipids in cellular membranes and corresponding undesirable consequences for cellular functions.<sup>[24–26]</sup>

Here we describe the synthesis and some characteristics of six new pyridinium amphiphiles in which the two hydrocarbon tails are connected to the headgroup by *two* ester linkages. In these diester amphiphiles, hydrolysis of the first ester is significantly enhanced by the electron-withdrawing cationic pyridine unit, in combination with the second ester group. The hydrolysis of the second ester moiety is strongly retarded through deactivation by the zwitterionic nature of the headgroup after hydrolysis of the first ester moiety.

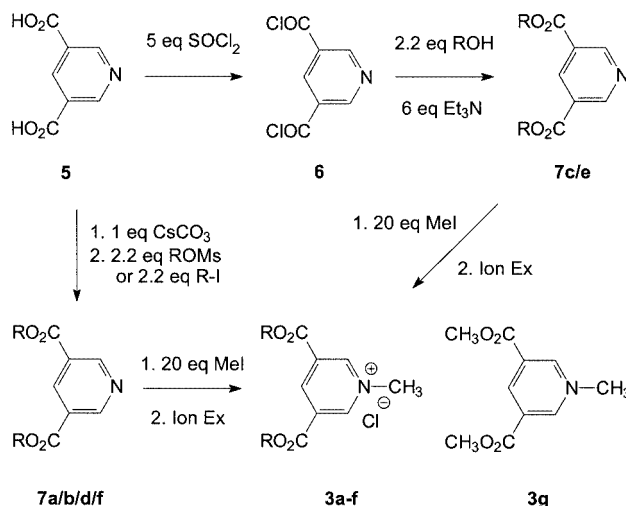
The selective hydrolysis of only one of the two esters offers unprecedented potential to direct the morphology as a function of the conditions of the surroundings or the endosomal stage. Hypothetically, selective hydrolysis of the lipoplex in the endosome could result in a transition from the “relatively stable” lamellar bilayer vesicular to an “aggressive and fusogenic” micellar/hexagonal H<sub>II</sub> phase.<sup>[27]</sup> The latter process might give rise to destabilisation and rupture of the endosomal membrane, enhancing the release of DNA into the cytoplasm and thus increasing the transfection efficiency.<sup>[28,29]</sup> Some of the physicochemical properties of the new diester amphiphiles and the pH-dependent hydrolytic behaviour of the esters are reported, together with the transfection potentials.

## Results and Discussion

### Synthesis

The diesters **7c** and **7e** (Scheme 1) were prepared by treatment of 3,5-pyridinedicarboxylic acid (**5**) with excess thionyl chloride to yield the acid chloride **6** (which was not isolated), followed by addition of 2.2 equivalents of hexadecyl alcohol or octadecyl alcohol and 6 equivalents of triethylamine. The crude products were purified by column chromatography, affording **7c** and **7e** as white solid materials in reasonable yields.

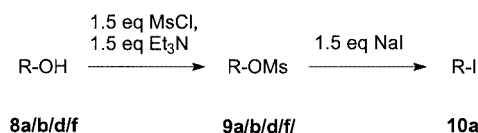
Unfortunately, the esterification of tetradecyl alcohol and the unsaturated alcohols by this methodology gave very low yields. The diesters **7a**, **7b**, **7d** and **7f** were therefore prepared by treatment of the caesium salt of 3,5-pyridinedicarboxylic acid (**5**) with 2.2 equivalents of the appropriate iodide or, alternatively, the mesylate, yielding the diester products in excellent yields<sup>[30,31]</sup> (Scheme 1).



R = **a** C<sub>14:0</sub>, **b** C<sub>14:1</sub>, **c** C<sub>16:0</sub>, **d** C<sub>16:1</sub>, **e** C<sub>18:0</sub>, **f** C<sub>18:1</sub>

Scheme 1. Synthesis of diester SAINTs **3**; see text for explanation<sup>[18]</sup>

For this sequence, the alcohols **8a/b/d/f** were converted into the corresponding mesylates **9a/b/d/f** by straightforward treatment with methanesulfonyl chloride in the presence of triethylamine as a base (Scheme 2). Treatment of mesylate **9a** with NaI in dry acetone afforded iodide **10a**.



Scheme 2. Synthesis of mesylates **9** and iodide **10a**<sup>[18]</sup>

Subsequently, the diesters **7a–g** were methylated on the nitrogen atom by treatment with a 20-fold excess of methyl iodide in dry acetone, resulting in complete conversion into the *N*-methylated products as the relatively unstable iodide salts (for a discussion on the degradation pathways of the iodide products, see ref.<sup>[36]</sup>). Ion exchange carried out with the very mild ion-exchange material Sephadex (DEAE A25, chloride form) yielded the desired amphiphiles **3a–g** as the more stable chloride salts.

In addition to the long-tailed pyridinium amphiphiles, the methyl ester **3g** was prepared as a model compound in order to investigate the hydrolytic behaviour of the diester system without possible interference from the long alkyl tails.

### Characteristics of the Amphiphilic Aggregates

All the amphiphiles used in this study form bilayer vesicles after hydration and sonication, as verified by transmission electron microscopy (TEM). Figure 2 shows a transmission electron micrograph of **3f**, negatively stained

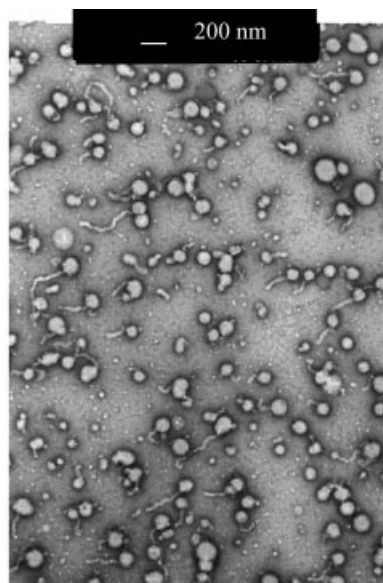


Figure 2. Transmission electron micrograph (TEM) of vesicles of **3f**, negatively stained with UAc; vesicle size varies between 40 and 200 nm; bar represents 200 nm

with uranyl acetate (UAc). The vesicle size typically varied between 40 and 200 nm.

The fluidity of the lipid bilayer is generally believed to play an important role in the interactions between the lipids and the DNA in the lipid-DNA complex (lipoplex).<sup>[32,33]</sup> It also appears that a high fluidity has a beneficial effect on the transfection efficiency. The main phase transition temperatures for the bilayer vesicles formed by amphiphiles **3a–f** were therefore determined by differential scanning calorimetry (DSC); the results are summarized in Table 1.

Table 1. Phase transition temperatures of diester amphiphiles **3** as determined by DSC

Compound (R)	PTT (°C)
<b>3a</b> (C <sub>14:0</sub> )	46
<b>3b</b> (C <sub>14:1</sub> )	<0
<b>3c</b> (C <sub>16:0</sub> )	64
<b>3d</b> (C <sub>16:1</sub> )	<0
<b>3e</b> (C <sub>18:0</sub> )	72
<b>3f</b> (C <sub>18:1</sub> )	<0

The amphiphiles with saturated hydrocarbon chains all show clear phase transitions, with an increase of about 15° for each two additional CH<sub>2</sub> groups, consistently with earlier findings.<sup>[17]</sup> The signal shifted slightly towards lower temperatures in successive temperature scans, probably as a result of partial hydrolysis of the amphiphiles due to the elevated temperatures during the measurements. This hypothesis was confirmed by <sup>1</sup>H NMR (results not shown), which clearly showed that approximately 30% of the ester moieties in the amphiphiles had been hydrolysed after the DSC experiment. The amphiphiles with unsaturated tails showed no phase transitions in the temperature range measured (0–100 °C); it is assumed that the bilayers formed by these amphiphiles have phase transitions below 0 °C. Dur-

ing a transfection experiment carried out at 37 °C, these bilayers adopt a fluid liquid-crystalline phase, whereas bilayers of the “saturated” amphiphiles adopt an ordered, gel-like phase. From these results it may be expected that the amphiphiles with the unsaturated tails should show higher transfection efficiencies than their counterparts with saturated tails.<sup>[32,33]</sup> As can be seen below, this expectation was partly fulfilled.

## Hydrolysis

To investigate the hydrolytic behaviour of the diester amphiphiles, time-resolved pH-dependent <sup>1</sup>H NMR spectroscopy was used to follow the hydrolysis of model compound **3g**. By recording of <sup>1</sup>H NMR spectra at subsequent time intervals and by measurement of the integrals of the three protons on the pyridine ring, the concentrations of the starting diester, the singly hydrolysed monoester and the doubly hydrolysed diacid were monitored. The concentrations of the diester and the monoester are plotted as a function of time and pH<sup>[34]</sup> in Figure 3.

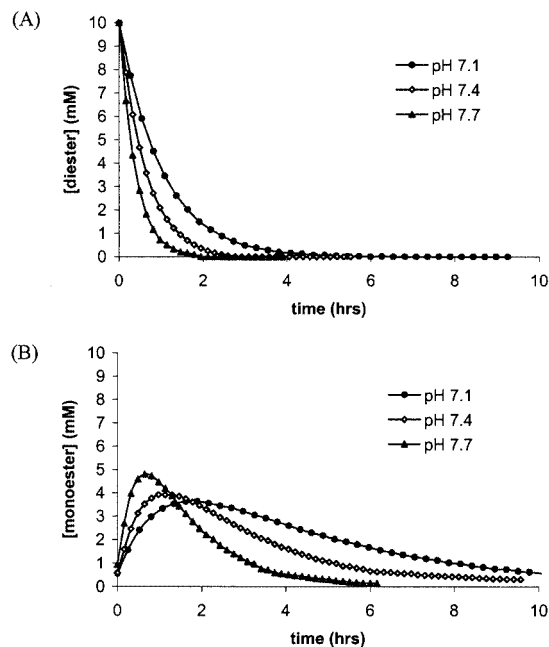


Figure 3. Hydrolysis of the methyl ester **3g** is monitored by following the concentration changes of **3g** (A) and singly hydrolysed monoester (B) with time at different pHs; measurements were performed at 37 °C in HEPES buffer (1 M), initial concentration of **3g** ca. 10 mM

Pseudo-first order rate constants ( $k_{\text{obsd.}}$ ) were determined from a fit to the curve in Figure 3 (A) and the second half of the curve in Figure 3 (B) (starting from the moment that the concentration **3g** is zero) for the first and the second ester hydrolysis, respectively. The results are listed in Table 2.

Table 2. Pseudo-first-order ( $k_{\text{obsd.}}$ ) rate constants for the hydrolysis of the methyl ester **3g** at different pHs

pH	$k_{\text{obsd.}}$ ( $\text{min}^{-1}$ ) 1st hydrolysis	$k_{\text{obsd.}}$ ( $\text{min}^{-1}$ ) 2nd hydrolysis
7.1	0.017	0.0038
7.4	0.023	0.004
7.7	0.038	0.0098

Both hydrolytic reactions are significantly accelerated at higher pH, indicating that the observed rate is mainly being determined by hydroxide ion-catalysed hydrolysis, whereas hydrolysis at much lower pH values did not proceed at significant rates. From plots of  $k_{\text{obsd.}}$  against the hydroxide ion concentration,  $k_{\text{OH}^-}$  could be determined as  $8.2 \text{ l}\cdot\text{mol}^{-1}\cdot\text{min}^{-1}$  for the first ester hydrolysis and  $1.9 \text{ l}\cdot\text{mol}^{-1}\cdot\text{min}^{-1}$  for the second. At endosomal pH ( $\approx 5.0$ ),  $k_{\text{obsd.}}$  would be  $8.19\cdot 10^{-5} \text{ min}^{-1}$  for the first hydrolysis, corresponding with a half-life of 141 h, while at physiological pH ( $\approx 7.4$ )  $k_{\text{obsd.}}$  would be  $2.3\cdot 10^{-3} \text{ min}^{-1}$ , with an expected half-life of 30 min. The endosomal pH drop strongly *retards* the hydrolytic efficiency, instead of the expected acceleration (*vide supra*).

It is also clear that the hydrolysis of the first ester moiety is faster than the hydrolysis of the second ester group. At physiological pH,  $k_{\text{obsd.}}$  of the second hydrolysis is estimated as  $4.8\cdot 10^{-3} \text{ min}^{-1}$ , corresponding to a half-life of 143 h, in comparison with 30 min for the first ester group hydrolysis. This can be explained by the fact that the first ester is activated towards hydrolysis by the neighbouring electron-withdrawing cationic headgroup as well as by the second ester moiety, whereas after hydrolysis of the first ester the remaining ester is adjacent only to a less strongly electron-withdrawing zwitterionic headgroup. In this system, controlled hydrolysis of one of the two ester linkages is possible, offering the potential to direct the morphology as a function of the pH conditions of the surroundings (or stage of the transfection event).

Unfortunately it was not possible to use NMR to perform hydrolysis measurements on the amphiphiles in the vesicular state, due to the high concentrations of substrate and, in particular, the buffers needed, which caused the vesicles to aggregate and precipitate from the solution. It also proved impossible to monitor the hydrolysis by other techniques, such as by mapping the UV absorptions. We expect the amphiphiles to show the same hydrolytic behaviour as the model compound **3g**; that is, base-catalysed hydrolysis and faster hydrolysis of the first ester moiety than of the second group.

### Transfection of Cells in Culture

Information on the number of transfected cells was obtained by the use of plasmid DNA containing Green Fluorescent Protein (GFP). Vesicles composed of cationic lipid/DOPE in 1:1 ratio were mixed with an equal volume of plasmid DNA in a molar charge ratio of 2.5:1. After

10–15 min mixing at room temp. the resulting complexes were added to culture cells (COS-7 or HepG-2) and incubated at 37 °C for 48 h. The percentage of GFP-positive cells was then identified by Fluorescent Activated Cell Sorting (FACS). The transfection results are summarized in Figure 4. Transfection efficiencies are compared with that of SAINT-2, an amphiphile showing excellent transfection efficiency and a generally remarkable low toxicity towards different cell types.<sup>[15]</sup>

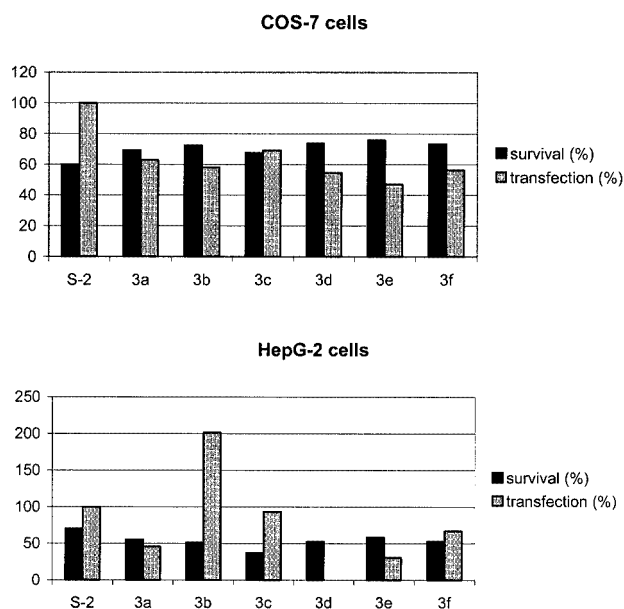


Figure 4. Transfection efficiencies of SAINT diesters **3**; transfection efficiencies were evaluated by use of FACS, GFP was used as reporter protein; SAINT-2 transfection efficiency is arbitrarily set at 100%; cell survival is expressed as the percentage of surviving cells after the transfection event

The transfection efficiencies of the SAINT diesters with COS-7 cells ranged from 47 to 69% relative to SAINT-2, in combination with a generally somewhat lower toxicity. Previous work on non-ester SAINT amphiphiles had shown that the introduction of a double bond in the hydrophobic tail resulted in a significant increase in transfection efficiency,<sup>[17]</sup> whereas in monoester SAINTs the transfection efficiency was considerably reduced.<sup>[18]</sup> For the SAINT diesters, a trend similar to that of the monoester adducts, though less pronounced, is observed. It can be speculated that this behaviour may arise from a different morphology of the lipid bilayer formed by the diesters relative to the non-ester SAINTs, caused by the significant differences in headgroup structure and the fact that the hydrophobic tails are connected to the pyridinium headgroup in a 3,5 orientation and not to the same carbon atom as in the non-ester SAINTs.

Generally, lower transfection efficiencies were obtained with HepG2 cells than with the COS-7 cell line. The clearest differences between the two cell lines were observed for **3b** ( $\text{C}_{14:0}$ ), **3c** ( $\text{C}_{16:0}$ ) and **3d** ( $\text{C}_{16:1}$ ). On HepG2 cells, **3b**

reached approximately 60% of the transfection efficiency with COS-7 and, surprisingly, a two times higher efficiency with the HepG2 cell line than seen with SAINT-2, whereas **3d** showed no transfection at all. In relation to SAINT-2, toxicity was generally somewhat higher, although on average high survival rates were observed.

## Conclusions

Six novel cationic pyridinium-based amphiphiles were prepared by employment of two hydrolytically active ester moieties as linking entities between the headgroup and the hydrocarbon tails. Although it was initially envisaged that a drop in pH (from physiological to endosomal pH) should trigger localised rapid hydrolysis of the esters, resulting in destabilisation of the lipoplex in the endosome, the esters appeared to hydrolyse by base catalysis, producing fast hydrolysis at physiological pH and higher stability in the endosomal pH range. In this respect, the use of an acid-labile or "activated" ketal functional moiety, which *does* hydrolyse by acid catalysis, would be an interesting option for further research. Furthermore, it should be emphasised that under physiological conditions hydrolysis might be dependent not only on pH, but also, and probably more importantly, on enzymatic pathways, so an amide linker that would hydrolyse solely by enzyme catalysis might also be employed.

Despite the observed pH behaviour, diester amphiphiles **3** showed moderate to good transfection efficiencies in relation to SAINT-2 on the two cell lines studied. Moreover, the observed toxicity was generally very low, though strongly dependent upon the cell lines under study.

## Experimental Section

**General:** All reactions were carried out in anhydrous solvents under nitrogen in oven-dried glassware. For column chromatography, Al<sub>2</sub>O<sub>3</sub> (activity II-III) was used, prepared by addition of the indicated amount of water to Merck Al<sub>2</sub>O<sub>3</sub> 90 active neutral (activity I). Melting points were determined with a Kofler melting point microscope. Several compounds displayed liquid crystalline behaviour; in these cases no melting points are reported. <sup>1</sup>H NMR spectra were recorded with Varian Gemini 200 and Varian VXR 300 spectrometers operating at 200 and 300 MHz for the proton channels, respectively. Elemental analyses were carried out at the Analytical Department of the University of Groningen. (*Z*)-11-Tetradecen-1-ol, (*Z*)-11-hexadecen-1-ol, oleyl alcohol, 1-tetradecanol, 1-hexadecanol and 1-octadecanol were obtained from Aldrich, whereas 3,5-pyridinedicarboxylic acid was purchased from Acros Organics.

**General Procedure for the Synthesis of Mesylates 9a/b/d/f:** Triethylamine (30 mmol) and then mesyl chloride (30 mmol) were added dropwise to a solution of alcohol (20 mmol) in dichloromethane (50 mL), cooled to 0 °C. After stirring at 0 °C for 40 min, the reaction mixture was quenched with water (15 mL). The organic layer was washed with 1 M HCl, a saturated Na<sub>2</sub>CO<sub>3</sub> solution and brine, after which the combined aqueous layers were extracted with dichloromethane. The combined organic layers were dried with

MgSO<sub>4</sub>. Evaporation of the solvents yielded slightly viscous, yellowish oils.<sup>[18,31,35]</sup>

**Tetradecyl Methanesulfonate (9a):**<sup>[18,35]</sup> Yield 5.42 g, 98%. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ = 0.86 (t, 3 H), 1.23 (m, 22 H), 1.79 (m, 2 H), 2.98 (s, 3 H), 4.19 (t, 2 H) ppm.

**(*Z*)-Tetradec-11-enyl Methanesulfonate (9b):**<sup>[18,35]</sup> Yield 5.33 g, 97%. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ = 0.83 (t, 3 H), 1.30 (m, 14 H), 1.72 (m, 2 H), 2.00 (m, 4 H), 2.99 (s, 3 H), 4.20 (t, 2 H), 5.31 (m, 2 H) ppm.

**(*Z*)-Hexadec-11-enyl Methanesulfonate (9d):**<sup>[18,35]</sup> Yield 5.45 g, 90%. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ = 0.82 (t, 3 H), 1.24 (m, 18 H), 1.71 (m, 2 H), 1.98 (m, 4 H), 2.98 (s, 3 H), 4.19 (t, 2 H), 5.31 (m, 2 H) ppm.

**(*Z*)-Octadec-11-enyl Methanesulfonate (9f):**<sup>[18,35]</sup> Yield 6.28 g, 95%. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ = 0.82 (t, 3 H), 1.24 (m, 22 H), 1.71 (m, 4 H), 1.98 (m, 4 H), 2.98 (s, 3 H), 4.19 (t, 2 H), 5.31 (m, 2 H) ppm.

**1-Iodotetradecane (10a):**<sup>[18,36]</sup> NaI (27.3 mmol) was added to a solution of tetradecyl mesylate (17.5 mmol) in acetone (50 mL), and the mixture was heated at reflux for 2 h. After the mixture had been cooled, the salts were filtered off. Low-temperature crystallisation from ethanol yielded colourless crystals, which turned into colourless oil at room temperature; yield 3.97 g, 70%. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ = 0.84 (t, 3 H), 1.24 (m, 22 H), 1.81 (m, 2 H), 3.19 (t, 2 H) ppm.

**General Procedure for the Synthesis of the Pyridinium Diesters 7c/e:**<sup>[18,31]</sup> Thionyl chloride (30 mmol) was added dropwise to a suspension of 3,5-pyridinedicarboxylic acid (6 mmol) in toluene (20 mL). After the mixture had been heated at reflux for one hour, solvents and excess thionyl chloride were evaporated off. This yielded the carboxylic chloride as a brown solid, which was used in the esterification reaction without further purification. The diacid dichloride (6 mmol) was added in one portion to a solution of alcohol (13.2 mmol) in dichloromethane (50 mL). Triethylamine (36 mmol) was added dropwise to the refluxing solution, and the reaction mixture was heated at reflux overnight. After evaporation of the solvents, the yellow mixture was suspended in *n*-hexane and the salts were filtered off. The diester was obtained pure after column chromatography over Al<sub>2</sub>O<sub>3</sub> (act. III), with dichloromethane/*n*-hexane (1:1) as eluent. The products were obtained as colourless solids.

**Dihexadecyl Pyridine-3,5-dicarboxylate (7c):**<sup>[18,31]</sup> Yield 1.48 g, 40%. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ = 0.87 (t, 6 H), 1.22 (m, 52 H), 1.79 (m, 4 H), 4.38 (t, 4 H), 8.82 (s, 1 H), 9.38 (s, 2 H) ppm.

**Diocetadecyl Pyridine-3,5-dicarboxylate (7e):**<sup>[18,31]</sup> Yield 1.61 g, 40%. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ = 0.83 (t, 6 H), 1.22 (m, 60 H), 1.74 (m, 4 H), 4.37 (t, 4 H), 8.81 (s, 1 H), 9.33 (s, 2 H) ppm.

**Procedure for the Synthesis of the Pyridinium Diesters 7b/d/f:**<sup>[31]</sup> Caesium carbonate (6 mmol) was added to a suspension of 3,5-pyridinedicarboxylic acid (6 mmol) in DMF (40 mL). The mixture was stirred for 1 h. Mesylate (13.2 mmol) was added dropwise, and the mixture was stirred overnight at 75 °C. After evaporation of the solvents, the yellow mixture was suspended in *n*-hexane and the salts were filtered off. The pure diester was obtained as a colourless oil after column chromatography over Al<sub>2</sub>O<sub>3</sub> (act. III), with dichloromethane/*n*-hexane (1:1) as eluent.

**(*Z*)-Di(tetradec-11-enyl) Pyridine-3,5-dicarboxylate (7b):**<sup>[31]</sup> Colourless oil, yield 0.67 g, 20%. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ = 0.93 (t, 6 H), 1.23 (m, 28 H), 1.75 (m, 4 H), 1.95 (m, 8 H), 4.35 (t, 4 H), 5.30 (m, 4 H), 8.81 (s, 1 H), 9.35 (s, 2 H) ppm.

**(Z)-Di(hexadec-11-enyl) Pyridine-3,5-dicarboxylate (7d):**<sup>[31]</sup> Colourless oil, yield 1.84 g, 50%. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ = 0.85 (t, 6 H), 1.22 (m, 36 H), 1.72 (m, 4 H), 1.95 (m, 8 H), 4.33 (t, 4 H), 5.28 (m, 4 H), 8.79 (s, 1 H), 9.30 (s, 2 H).

**(Z)-(Di)octadec-11-enyl) Pyridine-3,5-dicarboxylate (7f):**<sup>[31]</sup> Colourless oil, yield 2.32 g, 58%. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ = 0.85 (t, 6 H), 1.23 (m, 44 H), 1.78 (m, 4 H), 1.99 (m, 8 H), 4.38 (t, 4 H), 5.37 (m, 4 H), 8.82 (s, 1 H), 9.38 (s, 2 H) ppm.

**Ditetradecyl Pyridine-3,5-dicarboxylate (7a):**<sup>[31]</sup> Caesium carbonate (6 mmol) was added to a suspension of 3,5-pyridinedicarboxylic acid (6 mmol) in DMF (40 mL). The mixture was stirred for 1 h. 1-Iodotetradecane (13.2 mmol) was added and the mixture was stirred overnight at 75 °C. After evaporation of the solvents, the yellow mixture was suspended in *n*-hexane and the salts were filtered off. The pure diester was obtained as a white solid after column chromatography over Al<sub>2</sub>O<sub>3</sub> (act. III), with dichloromethane/*n*-hexane (1:1) as eluent. Colourless solid, yield 3.17 g, 88%. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ = 0.87 (t, 6 H), 1.22 (m, 44 H), 1.78 (m, 4 H), 4.38 (t, 4 H), 8.82 (s, 1 H), 9.36 (s, 2 H) ppm.

**Dimethyl Pyridine-3,5-dicarboxylate (7g):**<sup>[37]</sup> Sulfuric acid (15 mL) was added dropwise to a suspension of 3,5-pyridinedicarboxylic acid (6 mmol) in methanol (150 mL), while the mixture was cooled with ice, after which the mixture was heated at reflux overnight. The pH of the mixture was neutralised by addition of solid Na<sub>2</sub>CO<sub>3</sub>, and the mixture was extracted several times with diethyl ether. After drying with MgSO<sub>4</sub>, evaporation of the diethyl ether yielded a colourless solid, yield 0.82 g, 70%. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ = 3.98 (s, 6 H), 8.87 (t, 1 H), 9.37 (d, 2 H) ppm.

**General Procedure for the Synthesis of the Methylated Pyridinium Diesters 3a–g:** A solution of pyridine diester **7** (1 mmol) and methyl iodide (20 mmol) in dry acetone (30 mL) was heated at reflux overnight. After evaporation of the solvent and excess methyl iodide, the crude iodide salts were used in ion-exchange chromatography without further purification. Ion-exchange chromatography over a Sephadex column (chloride form, DEAE, A25), with methanol as eluent, afforded the products as colourless/waxy solids. These were crystallised from acetonitrile (the waxy solids at low temperature).

**1-Methyl-3,5-bis(tetradecyloxycarbonyl)pyridinium Chloride (3a):** Colourless solid, m.p. 115–117 °C, yield 0.30 g, 50%.

**Iodide Salt:** <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ = 0.80 (t, 6 H), 1.22 (m, 44 H), 1.78 (m, 4 H), 4.39 (t, 4 H), 4.86 (s, 3 H), 9.21 (s, 1 H), 9.69 (s, 2 H) ppm.

**Chloride Salt:** <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ = 0.82 (t, 6 H), 1.23 (m, 44 H), 1.80 (m, 4 H), 4.41 (t, 4 H), 5.05 (s, 3 H), 9.33 (s, 1 H), 9.91 (s, 2 H) ppm. <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ = 12.61, 21.18, 24.29, 26.90, 27.72, 27.85, 28.01, 28.15, 30.41, 48.87, 66.20, 129.00, 142.81, 148.45, 159.21 ppm. C<sub>36</sub>H<sub>64</sub>ClNO<sub>4</sub>: calcd. C 70.84, H 10.57, N 2.29; found C 70.83, H 10.58, N 2.30. Mass calcd. for C<sub>36</sub>H<sub>64</sub>ClNO<sub>4</sub> 610.361; found 559.4 [– HCl and CH<sub>3</sub>].

**(Z)-1-Methyl-3,5-bis(tetradec-11-enyloxycarbonyl)pyridinium Chloride (3b):** Colourless, waxy solid, yield 0.27 g, 45%.

**Iodide Salt:** <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ = 0.90 (t, 6 H), 1.27 (m, 28 H), 1.79 (m, 4 H), 1.98 (m, 8 H), 4.41 (t, 4 H), 4.82 (s, 3 H), 5.30 (m, 4 H), 9.25 (s, 1 H), 9.67 (s, 2 H) ppm.

**Chloride Salt:** <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ = 0.91 (t, 6 H), 1.27 (m, 28 H), 1.79 (m, 4 H), 1.99 (m, 8 H), 4.41 (t, 4 H), 4.95 (s, 3 H), 5.30 (s, 3 H), 9.26 (s, 1 H), 9.80 (s, 2 H) ppm. <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ =

12.88, 18.98, 24.27, 25.57, 26.90, 27.70, 27.76, 28.00, 28.25, 48.89, 66.18, 127.76, 129.00, 129.99, 142.79, 148.47, 159.23 ppm. C<sub>36</sub>H<sub>60</sub>ClNO<sub>4</sub>·1H<sub>2</sub>O: calcd. C 69.26, H 10.01, N 2.24; found C 69.34, H 10.25, N 2.26. Mass calcd. for C<sub>36</sub>H<sub>60</sub>ClNO<sub>4</sub>: 606.330; found 555.4 [– HCl and CH<sub>3</sub>].

**1-Methyl-3,5-bis(hexadecyloxycarbonyl)pyridinium Chloride (3c):**<sup>[30]</sup> Colourless solid, m.p. 113–115 °C, yield 0.43 g, 65%.

**Iodide Salt:** <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ = 0.82 (t, 6 H), 1.25 (m, 52 H), 1.80 (m, 4 H), 4.42 (t, 4 H), 4.90 (s, 3 H), 9.25 (s, 1 H), 9.72 (s, 2 H) ppm.

**Chloride Salt:** <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ = 0.82 (t, 6 H), 1.27 (m, 52 H), 1.81 (m, 4 H), 4.43 (t, 4 H), 5.05 (s, 3 H), 9.35 (s, 1 H), 9.91 (s, 2 H) ppm. <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ = 12.62, 21.18, 24.29, 26.91, 27.72, 27.86, 28.03, 28.20, 30.42, 48.92, 66.24, 129.08, 142.88, 148.40, 159.20 ppm. C<sub>40</sub>H<sub>72</sub>ClNO<sub>4</sub>: calcd. C 72.09, H 10.89, N 2.10; found C 71.83, H 10.85, N 2.10. Mass calcd. for C<sub>40</sub>H<sub>72</sub>ClNO<sub>4</sub>: 666.469; found 615.5 [– HCl and CH<sub>3</sub>].

**(Z)-1-Methyl-3,5-bis(hexadec-11-enyloxycarbonyl)pyridinium Chloride (3d):** Colourless, waxy solid, yield 0.32 g, 48%.

**Iodide Salt:** <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ = 0.86 (t, 6 H), 1.25 (m, 36 H), 1.81 (m, 4 H), 1.99 (m, 8 H), 4.41 (t, 4 H), 4.90 (s, 3 H), 5.33 (m, 4 H), 9.23 (s, 1 H), 9.73 (s, 2 H) ppm.

**Chloride Salt:** <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ = 0.91 (t, 6 H), 1.25 (m, 36 H), 1.80 (m, 4 H), 1.99 (m, 8 H), 4.41 (t, 4 H), 4.99 (s, 3 H), 5.33 (s, 3 H), 9.30 (s, 1 H), 9.85 (s, 2 H) ppm. <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ = 12.49, 20.82, 24.27, 25.39, 25.67, 26.90, 27.72, 27.78, 28.01, 28.25, 30.44, 48.90, 66.18, 128.30, 128.32, 129.02, 142.80, 148.46, 159.23 ppm. C<sub>40</sub>H<sub>68</sub>ClNO<sub>4</sub>·1H<sub>2</sub>O: calcd. C 70.61, H 10.37, N 2.06; found C 70.26, H 10.50, N 2.06. Mass calcd. for C<sub>40</sub>H<sub>68</sub>ClNO<sub>4</sub>: 662.437; found 611.4 [– HCl and CH<sub>3</sub>].

**1-Methyl-3,5-bis(octadecyloxycarbonyl)pyridinium Chloride (3e):** Colourless solid, m.p. 116–118 °C, yield 0.33 g, 46%.

**Iodide Salt:** <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ = 0.82 (t, 6 H), 1.25 (m, 60 H), 1.78 (m, 4 H), 4.40 (t, 4 H), 4.81 (s, 3 H), 9.24 (s, 1 H), 9.63 (s, 2 H) ppm.

**Chloride Salt:** <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ = 0.83 (t, 6 H), 1.26 (m, 60 H), 1.81 (m, 4 H), 4.41 (t, 4 H), 5.00 (s, 3 H), 9.35 (s, 1 H), 9.83 (s, 2 H) ppm. <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ = 12.63, 21.19, 24.29, 26.92, 27.72, 27.87, 28.03, 28.21, 30.42, 48.90, 66.25, 129.07, 142.90, 148.37, 159.16 ppm. C<sub>44</sub>H<sub>80</sub>ClNO<sub>4</sub>: calcd. C 73.14, H 11.16, N 1.94; found C 72.86, H 11.08, N 1.95. Mass calcd. for C<sub>44</sub>H<sub>80</sub>ClNO<sub>4</sub>: 722.576; found 671.5 [– HCl and CH<sub>3</sub>].

**(Z)-1-Methyl-3,5-bis(octadec-11-enyloxycarbonyl)pyridinium Chloride (3f):** Colourless waxy solid, yield 0.36 g, 50%.

**Iodide Salt:** <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ = 0.81 (t, 6 H), 1.25 (m, 44 H), 1.77 (m, 4 H), 1.95 (m, 8 H), 4.38 (t, 4 H), 4.82 (s, 3 H), 5.29 (m, 4 H), 9.19 (s, 1 H), 9.70 (s, 2 H) ppm.

**Chloride Salt:** <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ = 0.81 (t, 6 H), 1.25 (m, 44 H), 1.77 (m, 4 H), 1.97 (m, 8 H), 4.40 (t, 4 H), 4.98 (s, 3 H), 5.30 (s, 3 H), 9.25 (s, 1 H), 9.83 (s, 2 H) ppm. <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ = 12.58, 21.13, 24.25, 25.66, 26.87, 27.70, 27.77, 27.89, 27.97, 28.11, 28.21, 30.36, 31.06, 48.78, 66.08, 128.16, 128.42, 128.82, 142.66, 148.56, 159.21 ppm. No conclusive elemental analysis could be obtained, probably due to small remainders of solvents. C<sub>44</sub>H<sub>76</sub>ClNO<sub>4</sub>: calcd. C 73.55, H 10.66, N 1.95; found C 72.76, H 11.34, N 1.95. Mass calcd. for C<sub>44</sub>H<sub>76</sub>ClNO<sub>4</sub>: 718.545; found 667.4 [– HCl and CH<sub>3</sub>].

**1-Methyl-3,5-bis(methyloxycarbonyl)pyridinium Chloride (3g):** Colourless solid, decomposes above 150 °C, yield 0.20 g, 80%.

**Iodide Salt:**  $^1\text{H}$  NMR ( $\text{D}_2\text{O}$ ):  $\delta = 4.12$  (s, 6 H), 4.59 (s, 3 H), 9.51 (s, 1 H), 9.66 (s, 2 H) ppm.

**Chloride Salt:**  $^1\text{H}$  NMR ( $\text{D}_2\text{O}$ ):  $\delta = 4.12$  (s, 6 H), 4.59 (s, 3 H), 9.54 (s, 1 H), 9.69 (s, 2 H) ppm.  $^{13}\text{C}$  NMR ( $\text{D}_2\text{O}$ ):  $\delta = 47.52, 52.56, 129.25, 143.66, 148.00, 161.07$  ppm.  $\text{C}_{10}\text{H}_{12}\text{ClNO}_4 \cdot 1/2\text{H}_2\text{O}$ : calcd. C 47.16, H 5.15, N 5.50; found C 47.22, H 5.10, N 5.56. Mass calcd. for  $\text{C}_{10}\text{H}_{12}\text{ClNO}_4$ : 245.663; found 210.0 [– HCl].

**Preparation of the Vesicle Solutions:** A solution of the amphiphile in a small amount of dichloromethane was concentrated under a stream of nitrogen. The resulting lipid film was subjected to high vacuum for several hours in order to remove all the residual solvent. The lipid was then hydrated in water to a final lipid concentration of 2 mM and sonicated for several minutes at a temperature above the phase transition temperature of the particular cationic amphiphile by use of a Branson B15 sonication tip.

**EM, DSC, and NMR Measurements:** EM experiments (negative staining) were carried out with a Jeol JEM 1200 EX electron microscope operating at 80 kV. Samples were prepared on carbon-coated collodion grids and stained with uranyl acetate (UAc). Differential scanning calorimetry measurements were performed with a Microcal VP-DSC apparatus. Samples used were 2 mM, and measurements were carried out with heating rates of 1 °C/min. Time-resolved  $^1\text{H}$  NMR experiments were performed on a Varian UnityPlus 500 MHz spectrometer. Stock solutions of **3g** (20 mM in  $\text{D}_2\text{O}$  at pH 5.5) were mixed 1:1 with HEPES buffer (2 M in  $\text{D}_2\text{O}$  at pH 7.1/7.4/7.7) prior to measurement.

**Transfection Experiments:** Cells (COS-7 and HepG-2) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, The Netherlands) containing 7% fetal calf serum, 2 mM L-glutamine, 100 units/mL penicillin and 100 mg/mL streptomycin at 37 °C and under 5%  $\text{CO}_2$ . EGFP-N1 plasmid DNA (Clontech, Palo Alto, CA) was isolated from *Escherichia coli* by use of a Qiagen plasmid kit (Qiagen, Chatsworth, CA) by the manufacturer's instructions. Cells (COS-7 and HepG-2) were seeded into twelve-well plates at  $1.5 \cdot 10^5$  (HepG2)/ $0.8 \cdot 10^5$  (COS-7) cells per well and allowed to grow overnight. Lipoplexes were prepared in cell culture medium (DMEM): 0.5 mL of medium, containing 1  $\mu\text{g}$  of pEGFP-N1 (Clontech) was added to an equal volume of medium containing cationic lipid, at a charge ratio (cationic lipid/DNA) of 2.5:1. After the incubation with lipoplexes for 4 h at 37 °C, the transfection medium was replaced by complete cell culture medium. After 24 h, the medium was refreshed, and after another 24 h the cells expressing reporter protein (GFP) were quantified by FACS analysis (Elite, Coulter, Miami, FL;  $\lambda_{\text{ex}} = 488$  nm,  $\lambda_{\text{em}} = 530$  nm; 5000 events).

[1] R. C. Crystal, *Science* **1995**, *270*, 404–410.

[2] W. F. Anderson, *Nature* **1998**, *392* (Suppl.), 25–30.

[3] K. K. Hunt, S. A. Vorburgen, *Science* **2002**, *297*, 415–416.

[4] I. MacLachlan, P. Cullis, R. W. Graham, *Curr. Opin. Mol. Ther.* **1999**, *1*, 252–259.

[5] Y. Ilan, G. Droguett, N. R. Chowdhury, Y. Li, K. Sengupta, N. R. Thummala, A. Davidson, J. R. Chowdhury, MS Horwitz, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 2587–2592.

[6] G. M. Rubanyi, *Mol. Asp. Med.* **2001**, *22*, 113–142.

[7] D. Ferber, *Science* **2001**, *294*, 1638–1642.

[8] A. D. Miller, *Angew. Chem. Int. Ed.* **1998**, *37*, 1768–1785.

[9] J. P. Behr, *Bioconj. Chem.* **1994**, *5*, 382–389.

[10] P. L. Felgner, T. R. Gadek, M. Holm, R. Roman, H. W. Chan, M. Wenz, J. P. Northrop, G. M. Ringold, M. Danielsen, *Proc. Natl. Acad. Sci. USA* **1987**, *84*, 7413–7417.

[11] B. Abdallah, L. Sachs, B. A. Demeneix, *Biol. Cell* **1995**, *85*, 1–7.

[12] G. Byk, C. Dubertret, V. Escriou, M. Frederic, G. Jaslin, R. Rangara, B. Pitard, J. Crouzet, P. Wils, B. Schwartz, D. Scherman, *J. Med. Chem.* **1998**, *41*, 229–235.

[13] J. Marshall, J. B. Nietupski, E. R. Lee, C. S. Siegel, P. W. Rafter, S. A. Rudginsky, C. D. Chang, S. J. Eastman, D. J. Harris, R. K. Scheule, S. H. Cheng, *J. Drug Target.* **2000**, *7*, 453–469.

[14] M. J. Bennett, A. M. Aberle, R. P. Balasubramaniam, J. G. Malone, R. W. Malone, M. H. Nantz, *J. Med. Chem.* **1997**, *40*, 4069–4078.

[15] I. van der Woude, H. W. Visser, M. B. A. ter Beest, A. Wagenaar, M. H. J. Ruiters, J. B. F. N. Engberts, D. Hoekstra, *Biochim. Biophys. Acta* **1995**, *1240*, 34–40.

[16] I. van der Woude, A. Wagenaar, A. A. P. Meekel, M. B. A. ter Beest, M. H. J. Ruiters, J. B. F. N. Engberts, D. Hoekstra, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 1160–1165.

[17] A. A. P. Meekel, A. Wagenaar, J. Šmistrová, J. E. Kroeze, P. Haadisma, B. Bosgraaf, M. C. A. Stuart, A. Brisson, M. H. J. Ruiters, J. B. F. N. Engberts, D. Hoekstra, *Eur. J. Org. Chem.* **2000**, *4*, 665–673.

[18] A. Roosjen, J. Šmistrová, C. Driessen, J. T. Anders, A. Wagenaar, D. Hoekstra, R. Hulst, J. B. F. N. Engberts, *Eur. J. Org. Chem.* **2002**, *7*, 1271–1277.

[19] J. A. Boomer, D. H. Thompson, S. M. Sullivan, *Pharm. Res.* **2002**, *19*, 1292–1301.

[20] S. Obika, W. Yu, A. Shimoyama, T. Uneda, T. Minami, K. Miyashita, T. Doi, T. Imanishi, *Biol. Pharm. Bull.* **1999**, *22*, 187–190.

[21] I. S. Zuhorn, R. Kalicharan, D. Hoekstra, *J. Biol. Chem.* **2002**, *277*, 18021–18028.

[22] B. Tycko, F. R. Maxfield, *Cell* **1982**, *28*, 643–651.

[23] I. Mellman, R. Fuchs, A. Helenius, *Annu. Rev. Biochem.* **1986**, *55*, 663–700.

[24] H. Farhood, R. Bottega, R. M. Epand, L. Huang, *Biochim. Biophys. Acta* **1992**, *1111*, 239–346.

[25] R. Leventis, J. R. Silvius, *Biochim. Biophys. Acta* **1990**, *1023*, 124–132.

[26] A. M. Aberle, F. Tablin, J. Zhu, N. J. Walker, D. C. Gruenert, M. H. Nantz, *Biochemistry* **1998**, *37*, 6533–6540.

[27] For an example of the morphological behaviour of SAINT materials see: J. Šmistrová, A. Wagenaar, M. C. A. Stuart, E. Polushkin, E. ten Brinke, R. Hulst, J. B. F. N. Engberts, D. Hoekstra, *J. Biol. Chem.* **2001**, *276*, 47615–47622.

[28] S. Audouy, D. Hoekstra, *Mol. Membr. Biol.* **2001**, *18*, 129–143.

[29] P. C. Bell, M. Bergsma, I. P. Dolbnya, W. Bras, M. C. A. Stuart, A. E. Rowan, M. C. Feiters, J. B. F. N. Engberts, *J. Am. Chem. Soc.* **2003**, *125*, 1551–1558.

[30] E. J. R. Südholter, J. B. F. N. Engberts, *J. Am. Chem. Soc.* **1980**, *102*, 2467.

[31] E. J. R. Südholter, J. B. F. N. Engberts, W. H. de Jeu, *J. Phys. Chem.* **1982**, *86*, 1908–1913.

[32] J. H. Felgner, R. Kumar, C. N. Sridhar, C. J. Wheeler, Y. J. Tjai, R. Border, P. Ramsey, M. Martin, P. L. Felgner, *J. Biol. Chem.* **1994**, *269*, 2550–2561.

[33] I. S. Zuhorn, V. Oberle, W. H. Visser, J. B. F. N. Engberts, U. Bakowsky, E. Polushkin, D. Hoekstra, *Biophys. J.* **2002**, *83*, 2096–2108.

[34] Formally the term pD instead of pH should be used, as the measurements were performed in  $\text{D}_2\text{O}$ .

[35] W. J. Baumann, H. K. Mangold, *J. Org. Chem.* **1964**, *29*, 3055–3069.

[36] I. Muizebelt, P. Oosting, C. van der Pol, A. Wagenaar, J. Šmistrová, E. Bulten, C. Driessen, I. S. Zuhorn, D. Hoekstra, R. Hulst, J. B. F. N. Engberts, *Eur. J. Org. Chem.*, manuscript accepted.

[37] M. Kristiansen, J. Eriksen, K. A. Joergensen, *J. Chem. Soc., Perkin Trans. 1* **1990**, *1*, 101–103.

Received June 18, 2003