Cationic Nucleoside Lipids Derived from Universal Bases: A Rational Approach for siRNA Transfection

Claire Ceballos,[†] Salim Khiati,[‡] Carla A. H. Prata,[§] Xiao-Xiang Zhang,[§] Suzanne Giorgio,[†] Philippe Marsal,[†] Mark W. Grinstaff,[§] Philippe Barthélémy,*[,‡] and Michel Camplo*[,†]

Centre Interdisciplinaire de Nanoscience de Marseille CINaM, Upr-Cnrs 3118, Université Aix-Marseille II, Luminy, case 913, 13288 Marseille cedex 09, France, Inserm, U869, Université de Bordeaux, F-33076, Bordeaux, France, and Departments of Chemistry and Biomedical Engineering, Metcalf Center for Science and Engineering, Boston University, Boston, Massachusetts 02215. Received January 6, 2010; Revised Manuscript Received April 27, 2010

Cationic nucleoside lipids (CNLs) derived from 5-nitroindole and 4-nitroimidazole bases were prepared from D-ribose by using a straightforward chemical synthesis. TEM experiments indicate that these amphiphilic molecules self-assemble to form supramolecular organizations in aqueous solutions. Electrophoresis and standard ethidium bromide (EB) fluorescence displacement assay shows that CNLs are able to bind siRNA. We demonstrated that both the nature of the universal bases and the stereochemistry of the anomeric position (α, β) have an impact on the CNLs-siRNA complex formation. Correlations among chemical structure, stereochemistry, siRNA knockdown effect, and binding affinities for all the compounds were shown and analyzed with a simple molecular modeling study. The best binding affinities for siRNA were found for the β anomer of the 5-nitroindole CNL which exhibits protein knockdown activity similar to the standard siPORT NeoFX positive control. It is noteworthy that no significant cytotoxicity at the tested concentration was observed for the novel CNLs.

INTRODUCTION

Currently, there is substantial interest in the design of bioinspired molecules having a double functionality based on the combination of nucleic acids and lipid characteristics (*1*−*4*). Nucleoside lipids are emerging as tools for constructing both supramolecular assemblies and nucleic acid delivery applications. The 2′-deoxyribosyl derivatives of 3-nitropyrrole and 5-nitroindole represent a new class of compounds known as universal bases. When incorporated into oligonucleotides, they show little discrimination in their base-pairing properties, as they have no hydrogen bonding capability. Recently (*5*), we have described the synthesis and siRNA transfection activities of cationic nucleoside lipids (CNLs) **A** and **B** based on a 3-nitropyrrole universal base (Figure 1). The encouraging biological results with such derivatives prompt us to further investigate the potential of new CNL analogues containing the 5-nitroindole and 4-nitroimidazole universal bases (Figure 2). We hypothesized that the chemical structure of CNLs could have an impact on the siRNA/CNL stability and siRNA delivery. Indeed, puzzling questions are emerging from the siRNA/CNL complexes, for example, how can the structure and/or the stereochemistry of the universal base affect the formation siRNA/CNL or the transfection properties? To address these issues, the following two structural parameters were modulated: (i) the nature of the universal bases (5-nitroindole and 4-nitroimidazole) and (ii) the stereochemistry of the anomeric position.

The target CNLs shown in Figure 2 possess three key components: a quaternized amine at the 5 position, two oleic acid chains linked at the 2 and 3 positions, and a 5-nitroindole or a 4-nitroimidazole attached at the anomeric position. As for the 3-nitropyrrole derivatives **A** and **B** shown in Figure 1, the cationic charge of these new analogues is responsible for electrostatic binding to the nucleic acids, whereas the oleyl chains

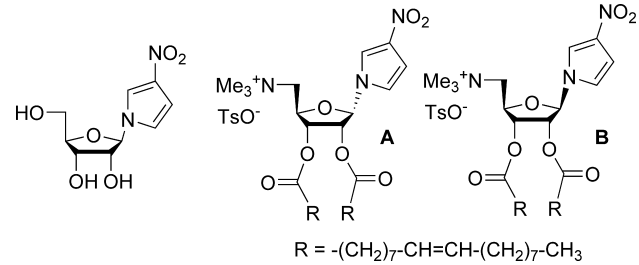


Figure 1. Chemical structures of the (β) universal nucleobase (left) and both CNLs (tosylate salts of 1′-(2′,3′-dioleyl-5′-trimethylammonium-α-D-ribofuranosyl)-3-nitropyrrole **A** and 1′-(2′,3′-dioleyl-5′-trimethylammonium-β-D-ribofuranosyl)-3-nitropyrrole **B** (*2*).

contribute to stabilization of the resulting nucleic acid−nucleolipid assembly through hydrophobic chain−chain interactions.

The 5-nitroindole universal base was selected because 1-(2′-deoxyribofuranosyl)-5-nitroindole has been shown to be generally less destabilizing than 1-(2′-deoxyribofuranosyl)-3-nitropyrrole analogue, with only a 2 °C decrease in melting temperature (T_m) value when incorporated toward the end of a 17mer duplex and 5 °C in the middle (*6*). It was suggested that this stabilization occurs because this nucleoside is sufficiently large to be able to intercalate into the opposite strand. The crystal structure of 1-(2′-deoxyribofuranosyl)-5-nitroindole shows that the base residues stack in columns, with overlap between each aromatic base (*7*). Also, 1-(2′-deoxyribofuranosyl)-5-nitroindole is nondiscriminating toward the natural DNA bases, with a T_m range of 3 °C, similar to that observed with the 3-nitropyrrole analogue.

Herein, we report the synthesis, physicochemical properties, and siRNA transfection efficiency of the universal-base nucleolipids, 2′,3′-dioleyl-5′-*O*-(α,β)-D-ribofuranosyl-5-nitroindole and 2′,3′-dioleyl-5′-*O*-(α-D-ribofuranosyl-4-nitroimidazole.

MATERIAL AND METHODS

Unless noted otherwise, all starting materials were purchased from Sigma-Aldrich, Acros, or Alfa Aesar and used as received.

* Corresponding author. camplo@univmed.fr.
[†] Upr-Cnrs 3118.
[‡] Inserm, U869.
[§] Boston University.

10.1021/bc100005k
Published on Web 05/19/2010

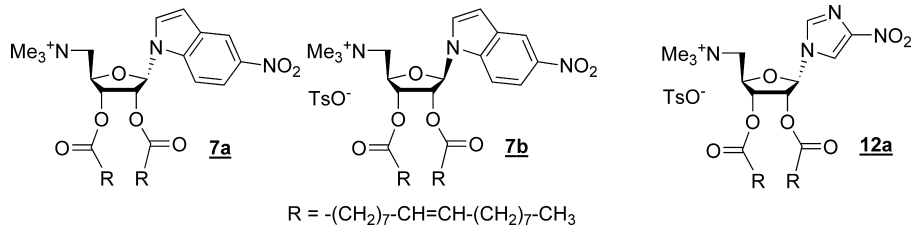


Figure 2. Chemical structures of the universal cationic nucleoside lipids (CNLs) used in this study. Tosylate salts of 1-(2,3-dioleyl-5-trimethylammonium-α-D-ribofuranosyl)-5-nitroindole and 1-(2,3-dioleyl-5-trimethylammonium-*β*-D-ribofuranosyl)-5-nitroindole, **7a** and **7b**, respectively. 1-(2,3-dioleyl-5-trimethylammonium-*β*-D-ribofuranosyl)-4-nitroimidazole) **12a**.

Some solvents were distilled under argon over appropriate drying agent: tetrahydrofuran and diethylether over sodium/benzophenone, dichloromethane over $CaCl_2$. Unless otherwise stated, the other solvents were used as commercially available. All compounds were characterized by ^1H NMR, ^{13}C NMR, and mass spectroscopy. 250 and 62.5 MHz ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AC 250. The spectra were taken at 20 °C and referenced against residual proton-solvent signals (^1H, $CDCl_3$, 7.26 ppm; and ^{13}C, 77 ppm). The ^1H NMR coupling constants (*J*) are reported in hertz. ESI-MS and elemental analyses were performed at the spectropole of the Faculté des Sciences et Techniques de Saint Jerôme, Marseille, France. Electrospray ionization mass spectra (ESI-MS) were recorded with an API///Plus sciex triple quadripole spectrophotometer in the positive mode. Elemental analyses were recorded with a Thermo Finnigan EA 1112 elemental analysis apparatus driven by the Eager 300 software. TEM microscopy experiments were performed on a Philipps CM10 (negative staining with ammonium molybdate 1% in water, Cu/Pd carbon coated grids). Analytical thin layer chromatography (TLC) was performed on precoated glass plates with Kieselgel 60F254 neutral with aluminum support plate (0.25 mm layer thickness), SDS. The developed plates were air-dried and exposed to UV light and/or sprayed with a solution of cerium sulfate (1%) and molybdic acid (1.5%) in 10% aqueous sulfuric acid and heated at 150 °C. Preparative thin layer chromatography was performed on glass plates with Merck silica gel 60F-254 as the adsorbent (layer thickness 2 mm or 1 mm). Column chromatography was performed on Merck silica gel 60 (0.063−0.200 mm). Melting point (mp) were measured with an Electrothermal 9100. The synthesis of compounds **1**, **2**, and **2′** is described in the Supporting Information.

1-(5-*O*-*tert*-Butyldimethylsilyl-2,3-*O*-isopropylidene-D-ribofuranosyl)-5-nitroindole, 3. *Step 1.* Carbon tetrachloride (433 μL, 4.49 mmol) was added to a stirring solution of **2** (872 mg; 2.87 mmol) in anhydrous THF (10 mL). The solution was cooled to −70 °C and hexamethylphosphorous triamide (700 μL, 3.82 mmol) was added dropwise for 5 min, and the mixture was allowed to stir at the same temperature for 5 min. The resulting chewy compound was allowed to warm to room temperature and further stirred for 2 h. The solvent was evaporated and the residue dissolved in diethyl ether. The resulting insoluble material was removed by decantation and the organic layer was evaporated to give the crude compound **3** as an orange oil.

Step 2. To a cooled solution (0 °C) of 5-nitroindole (906 mg, 5.59 mmol) dissolved in anhydrous acetonitrile (9 mL) was added NaH (60%, 223 mg, 5.61 mmol); the solution was stirred at room temperature for 30 min. To the resulting bright yellow solution, the chlorosugar obtained from the first step was dissolved in anhydrous acetonitrile and transferred via canula. The reaction mixture was then warmed to room temperature and stirred for 40 h. The mixture was poured into H_2O (50 mL) and extracted with CH_2Cl_2 (3 × 50 mL). The organic layers were combined and successively washed with saturated aqueous NH_4Cl and saturated aqueous NaCl. The organic layer was dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by flash column chromatography (90/10: cyclohexane/EtOAc) to give **3** (502 mg, 39%) as a yellow oil (anomeric mixture). ^1H NMR ($CDCl_3$): δ 8.57 (1H, d, J = 2.14 Hz, H_4); 8.11 (1H, dd, J = 2.14 Hz, J = 9.12 Hz, H_6); 7.60 (1H, d, J = 3.43 Hz, H_3); 7.30 (1H, d, J = 9.12 Hz, H_9); 6.71 (1H, d, J = 3.43 Hz, H_8); 6.48 (1Hα, d, J = 4.11 Hz, $H_{1'\alpha}$); 6.03 (1H*β*, d, J = 3.38 Hz, $H_{1'\beta}$); 4.96 (1H, d, J = 6.06 Hz, $H_{2'}$); 4.88 (1H, dd, J = 4.15 Hz, J = 6.06 Hz, $H_{3'}$); 4.45 (1H, m, $H_{4'}$); 3.95 (1H, dd, J = 2.14 Hz, J = 11.05 Hz, $H_{5'}$); 3.83 (1H, dd, J = 2.14 Hz, J = 11.05 Hz, $H_{5''}$); 1.43 (3H, s, CH_3−C); 1.30 (3H, s, CH_3−C); 1.00 (9H, s, $(CH_3)_3$−C); 0.15 (6H, s, $Si(CH_3)_2$). ^{13}C NMR ($CDCl_3$) δ: 138.34 (C_2); 129.81 (C_5); 128.14 (C_7); 118.08 (C_9); 117.30 (C_6); 113.29 (C_4); 109.24 (C_3); 104.16 (C−$(CH_3)_2$); 100.57 (C_8); 88.33 ($C_{1'}$); 82.76 ($C_{2'}$); 82.74 ($C_{4'}$); 80.53 ($C_{3'}$); 66.06 ($C_{5'}$); 29.69 (CH_3−C); 25.89 (CH_3−C); 25.71 ($(CH_3)_3$−C); 18.13 ($(CH_3)_3$−C); −5.46 ($Si(CH_3)_2$). MS (GT, FAB$^+$): 449 $[M+H]^+$. Rf (C_6H_{12}/EtOAc: 90/10): 0.54.

1-(2,3-*O*-Isopropylidene-D-ribofuranosyl)-5-nitroindole, 4a and 4b. To a solution of **3** (596 mg, 1.49 mmol) in dry THF (5 mL) was added TBAF (1 M in THF) (3 mL, 2.98 mmol) at room temperature, and the mixture was stirred for 30 min. The solution was evaporated under reduced pressure, diluted with CH_2Cl_2, and washed with brine. The organic layer was dried on $MgSO_4$ and evaporated under reduced pressure. The crude product was purified by flash column chromatography (cyclohexane/EtOAc: 70/30) to give the anomer α **4a** (198 mg, 54%) as a yellow oil and the anomer *β* **4b** (43 mg, 11%) as a yellow oil.

1-(2,3-*O*-Isopropylidene-α-D-ribofuranosyl)-5-nitroindole, 4a. ^1H NMR ($CDCl_3$): δ 8.57 (1H, d, J = 2.16 Hz, H_4); 8.10 (1H, dd, J = 2.16 Hz, J = 9.10 Hz, H_6); 7.60 (1H, d, J = 3.43 Hz, H_3); 7.41 (1H, d, J = 9.10 Hz, H_9); 6.72 (1H, d, J = 3.43 Hz, H_8); 6.45 (1H, d, J = 3.93 Hz, $H_{1'}$); 4.97 (2H, m, $H_{3'}$ + $H_{2'}$); 4.50 (1H, t, J = 2.75 Hz, $H_{4'}$); 3.99 (1H, dd, J = 2.75 Hz, J = 11.33 Hz, $H_{5'}$); 3.88 (1H, dd, J = 2.75 Hz, J = 11.33 Hz, $H_{5''}$); 1.66 (1H, sl, O<u>H</u>); 1.40 (3H, s, CH_3−C); 1.30 (3H, s, CH_3−C). ^{13}C NMR ($CDCl_3$) δ: 141.79 (C_2); 138.39 (C_5); 129.78 (C_7); 128.08 (C_9); 118.04 (C_6); 117.23 (C_4); 113.59 (C_3); 109.59 ($(CH_3)_2$−C); 104.27 (C_8); 87.92 ($C_{1'}$); 82.75 ($C_{2'}$); 82.00 ($C_{4'}$); 80.45 ($C_{3'}$); 64.57 ($C_{5'}$); 25.67 (CH_3−C); 24.13 (CH_3−C). MS (GT, FAB$^+$): 335 $[M+H]^+$. HRMS (FAB$^+$): calculated for $[M+H]^+$ 335.1237, found 335.1243. Rf (C_6H_{12}/EtOAc: 50/50): 0.22.

1-(2,3-*O*-Isopropylidene-*β*-D-ribofuranosyl)-5-nitroindole, 4b. ^1H NMR ($CDCl_3$): δ 8.53 (1H, d, J = 2.13 Hz, H_4); 8.10 (1H, dd, J = 2.13 Hz, J = 9.10 Hz, H_6); 7.52 (1H, d, J = 9.10 Hz, H_3); 7.47 (1H, d, J = 3.36 Hz, H_9); 6.70 (1H, d, J = 3.36 Hz, H_8); 6.05 (1H, d, J = 3.13 Hz, $H_{1'}$); 4.94 (2H, m, $H_{3'}$ + $H_{2'}$); 4.31 (1H, q, J = 3.29 Hz, $H_{4'}$); 3.92 (1H, dd, J = 3.29 Hz, J = 12.00 Hz, $H_{5'}$); 3.81 (1H, dd, J = 3.29 Hz, J = 12.00 Hz, $H_{5''}$); 2.32 (1H, sl, O<u>H</u>); 1.64 (3H, s, CH_3−C); 1.37 (3H, s, CH_3−C). ^{13}C NMR ($CDCl_3$) δ: 142.09 (C_2); 138.32 (C_5); 128.43 (C_7); 127.76 (C_9); 118.05 (C_6); 117.71 (C_4); 114.96 (C_3); 110.11 ($(CH_3)_2$−C); 105.42 (C_8); 91.76 ($C_{1'}$); 85.03 ($C_{2'}$); 84.42 ($C_{4'}$); 80.29 ($C_{3'}$); 62.29 ($C_{5'}$); 27.23 (CH_3−C); 25.22 (CH_3−C). SM

(GT, FAB$^+$): 335 [M+H]$^+$. HRMS (FAB$^+$): calculated for [M+H]$^+$ 335.1237, found 335.1244; Rf (C_6H_{12}/EtOAc: 50/50): 0.38.

1-(2,3-*O*-Isopropylidene-5-*O*-tosyl-α-D-ribofuranosyl)-5-nitroindole, 5a. Compound **4a** (100 mg, 0.30 mmol) was dissolved in dry dichloromethane (4 mL) and the solution was cooled to 0 °C. NEt_3 (168 μL, 1.20 mmol) was added, as well as tosyl chloride (171 mg, 0.90 mmol), in small portions. The solution was then allowed to warm to room temperature and stirred overnight. Dichloromethane was added and the solution was successively washed with brine and aqueous $NaHCO_3$ 5% w/v. The organic layer was dried on $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by preparative layer chromatography (cyclohexane/EtOAc: 60/40) to give **5a** (137 mg, 94%) as a yellow oil. ^{1}H NMR ($CDCl_3$) δ: 8.55 (1H, d, J = 2.21 Hz, H_4); 8.10 (1H, dd, J = 2.21 Hz, J = 9.00 Hz, H_6); 7.83 (2H, d, J = 9.00 Hz, H_{Ar}); 7.54 (1H, d, J = 3.47 Hz, H_3); 7.35 (3H, m, H_{Ar} + H_9); 6.70 (1H, d, J = 3.47 Hz, H_8); 6.26 (1H, d, J = 2.37 Hz, $H_{1'}$); 4.93 (2H, m, $H_{2'}$ + $H_{3'}$); 4.55 (1H, t, J = 2.37 Hz, $H_{4'}$); 4.27 (2H, d, J = 2.64 Hz, $H_{5'}$ + $H_{5''}$); 2.44 (3H, s, CH_3−C_{Ar}); 1.41 (3H, s, CH_3−C); 1.25 (3H, s, CH_3−C). ^{13}C NMR ($CDCl_3$) δ: 145.76 (C_{Ar}); 141.97 (C_2); 138.41 (C_5); 132.06 (C_{Ar}); 130.20 (2 × $\underline{C}H_{Ar}$); 129.64 (C_7); 128.06 (2 × $\underline{C}H_{Ar}$); 127.86 (C_9); 117.98(C_6); 117.36(C_4); 114.04 (C_3); 109.54 ((CH_3)$_2$−$\underline{C}$); 104.52 (C_8); 87.84 ($C_{1'}$); 81.67 ($C_{2'}$); 80.09 ($C_{3'}$); 79.81($C_{4'}$); 71.22 ($C_{5'}$); 25.65 ($\underline{C}H_3$−C); 24.09 ($\underline{C}H_3$−C); 21.67 ($\underline{C}H_3$−C_{Ar}). MS (GT, FAB$^+$): 489 [M+H]$^+$; 506 [M+NH_4]$^+$. Rf (C_6H_{12}/EtOAc: 70/30): 0.22.

1-(2,3-*O*-Isopropylidene-5-*O*-tosyl-β-D-ribofuranosyl)-3-nitroindole, 5b. Compound **5b** was obtained in 53% yield from compound **4b** by the procedure described above. Purification by preparative layer chromatography (cyclohexane/EtOAc: 60/40) gave a yellow oil. ^{1}H NMR ($CDCl_3$): δ 8.56 (1H, d, J = 2.13 Hz, H_4); 8.05 (1H, dd, J = 2.13 Hz, J = 9.10 Hz, H_6); 7.52 (2H, d, J = 7.96 Hz, H_{Ar}); 7.44 (1H, d, J = 9.10 Hz, H_3); 7.37 (1H, d, J = 3.45 Hz, H_9); 7.27 (2H, d, J = 7.96 Hz, H_{Ar}); 6.71 (1H, d, J = 3.45 Hz, H_8); 6.02 (1H, d, J = 2.84 Hz, $H_{1'}$); 4.91 (2H, m, $H_{3'}$ + $H_{2'}$); 4.41 (1H, q, J = 2.84 Hz, $H_{4'}$); 4.30 (1H, dd, J = 2.84 Hz, J = 10.90 Hz, $H_{5'}$); 4.19 (1H, dd, J = 2.84 Hz, J = 10.90 Hz, $H_{5''}$); 2.41 (3H, s, CH_3−C_{Ar}); 1.63 (3H, s, CH_3−C); 1.36 (3H, s, CH_3−C). ^{13}C NMR ($CDCl_3$) δ: 154.49 (C_{Ar}); 142.22 C_2); 138.24(C_5); 131.97 (C_{Ar}); 129.98 (2 × $\underline{C}H_{Ar}$); 128.51 (C_7); 127.89 (2× $\underline{C}H_{Ar}$); 127.72 (C_9); 118.04 (C_6); 117.74 (C_4); 115.30 (C_3); 110.15 ((CH_3)$_2$−$\underline{C}$); 105.54 (C_8); 91.99 ($C_{1'}$); 84.08 ($C_{2'}$); 81.96 ($C_{3'}$); 80.12 ($C_{4'}$); 68.42 ($C_{5'}$); 27.17 ($\underline{C}H_3$−C); 25.19 ($\underline{C}H_3$−C); 21.62 ($\underline{C}H_3$−C_{Ar}). MS (GT, FAB$^+$): 489 [M+H]$^+$, 511 [M+Na]$^+$. Rf (C_6H_{12}/EtOAc: 70/30): 0.60.

1-(2,3-dioleyl-5-*O*-tosyl-α-D-ribofuranosyl)-5-nitroindole, 6a. At 0 °C, compound **5a** (40 mg, 0.08 mmol) was dissolved in 1 mL of an aqueous trifluoroacetic acid/H_2O (6/1) solution and stirred for 1 h at 0 °C. The solvents were coevaporated with methanol under reduced pressure, and the residue was dried in vacuo. The crude product was dissolved in dry CH_2Cl_2 (4 mL) and the solution was cooled to 0 °C. Dicyclohexylcarbodiimide (68 mg, 0.33 mmol), (dimethylamino)pyridine (40 mg, 0.33 mmol), and oleic acid (93 mg, 0.33 mmol) were added, and the mixture was stirred at room temperature overnight. DCU was then filtered, and the filtrate was successively washed with aqueous 2 N HCl and aqueous saturated $NaHCO_3$ solutions. The organic layer was dried on $MgSO_4$, filtered, and evaporated under reduced pressure. The crude product purified by flash column chromatography (cyclohexane/EtOAc: 70/30) gave **6a** (51 mg, 65%) as a yellow oil. ^{1}H NMR ($CDCl_3$): δ 8.55 (1H, d, J = 2.21 Hz, H_4); 8.11 (1H, dd, J = 2.21 Hz and J = 9.16 Hz, H_6); 7.84 (2H, d, J = 8.27 Hz, H_{Ar}); 7.46 (1H, d, J = 2.21 Hz, H_3); 7.43 (3H, m, H_{Ar} + H_9); 6.68 (1H, d, J = 2.84 Hz, $H_{1'}$); 6.33 (1H, d, J = 5.37 Hz, H_8); 5.62 (1H, t, J = 5.69 Hz, $H_{2'}$); 5.46 (1H, m, $H_{3'}$); 5.33 (4H, m, 2 × C$\underline{H}$=C$\underline{H}$); 4.56 (1H, q, J = 2.84 Hz, $H_{4'}$); 4.30 (2H, dd, J = 2.84 Hz, J = 11.21 Hz, $H_{5'}$ + $H_{5''}$); 2.43 (3H, s, CH_3−C_{Ar}); 2.34 (2H, t, J = 7.66 Hz, C$\underline{H}_2$−CO); 2.25 (2H, t, J = 7.33 Hz, C$\underline{H}_2$−CO); 2.00 (8H, m, 2 × C$\underline{H}_2$−CH=CH−C$\underline{H}_2$); 1.60 (4H, m, 2 × C$\underline{H}_2$−CH_2−CO); 1.26 (40H, m, 2 × 10 CH_2); 0.87 (6H, t, J = 6.48 Hz, 2 × CH_3). ^{13}C NMR ($CDCl_3$) δ: 172.15 (CO); 171.53 (CO); 145.50 (C_{Ar}); 142.12 (C_2); 138.70 (C_5); 132.06 (C_{Ar}); 130.01 (2 × $\underline{C}H$=$\underline{C}H$); 129.53 (2 × CH_{Ar}); 128.96 (C_7); 128.49 (2 × CH_{Ar}); 127.97 (C_9); 125.85 (C_6); 125.19 (C_4); 104.31 (C_3); 100.78 (C_8); 79.82 ($C_{1'}$); 74.43 ($C_{4'}$); 73.80 ($C_{3'}$); 73.59 ($C_{2'}$); 65.57 ($C_{5'}$); 31.87 (2 × $\underline{C}H_2$−CO); 29.78−28.67 (2 × 9 CH_2); 27.18 (2 × $\underline{C}H_2$−CH=CH−$\underline{C}H_2$); 24.39 (2 × $\underline{C}H_2$−CH_2−CO); 22.65 ($\underline{C}H_3$−C_{Ar}); 21.66 (2 × CH_3−$\underline{C}H_2$); 14.09 (2 × CH_3). MS (GT, FAB$^+$): 978 [M+H]$^+$; 1000 [M+23]$^+$. Rf (C_6H_{12}/EtOAc: 80/20): 0.39.

1-(2,3-Dioleyl-5-*O*-tosyl-β-D-ribofuranosyl)-5-nitroindole, 6b. Compound **6b** was obtained from **5b** with the same procedure described above. Purification by flash column chromatography (cyclohexane/EtOAc: 60/40) gave **6b** as a yellow oil in 73% yield. ^{1}H NMR ($CDCl_3$): δ 8.56 (1H, d, J = 2.21 Hz, H_4); 8.08 (1H, dd, J = 2.21 Hz, J = 9.11 Hz, H_6); 7.81 (2H, d, J = 8.37 Hz, H_{Ar}); 7.46 (2H, m, H_3 + H_9); 7.32 (2H, d, J = 8.37 Hz, H_{Ar}); 6.74 (1H, d, J = 3.47 Hz, $H_{1'}$); 6.13 (1H, d, J = 6.63 Hz, H_8); 5.45 (1H, t, J = 6.00 Hz, $H_{2'}$); 5.34 (6H, m, 2 × C$\underline{H}$=C$\underline{H}$ + $H_{3'}$ + $H_{4'}$); 4.34 (2H, m, $H_{5'}$ + $H_{5''}$); 2.43 (3H, s, CH_3−C_{Ar}); 2.37 (2H, t, J = 7.26 Hz, CH_2−CO); 2.25 (2H, t, J = 7.26 Hz, CH_2−CO); 1.99 (8H, m, 2 × C$\underline{H}_2$−CH=CH−C$\underline{H}_2$); 1.63 (4H, m, 2 × C$\underline{H}_2$−CH_2−CO); 1.26 (40H, m, 2 × 10 CH_2); 0.87 (6H, t, J = 6.27 Hz, 2 × CH_3). ^{13}C NMR ($CDCl_3$) δ: 172.44 (CO); 171.86 (CO); 145.50 (C_{Ar}); 142.34 (C_2); 138.91 (C_5); 132.04 (C_{Ar}); 130.07 (2 × $\underline{C}H$=$\underline{C}H$); 130.08 (2 × CH_{Ar}); 129.53 (C_7); 127.99 (2 × CH_{Ar}); 127.25 (C_9); 118.12 (C_6); 117.82 (C_4); 109.62 (C_3); 99.69 (C_8); 86.90 ($C_{1'}$); 73.63 ($C_{4'}$); 73.02 ($C_{3'}$); 70.46 ($C_{2'}$); 64.07 ($C_{5'}$); 31.87 (2 × $\underline{C}H_2$−CO); 29.72−28.89 (2 × 9 CH_2); 27.19 ($\underline{C}H_2$−CH=CH−$\underline{C}H_2$); 27.12 ($\underline{C}H_2$−CH=CH−$\underline{C}H_2$); 24.76 ($\underline{C}H_2$−CH_2−CO); 24.62 ($\underline{C}H_2$−CH_2−CO); 22.65 ($\underline{C}H_3$−C_{Ar}); 21.65 (2 × CH_3−$\underline{C}H_2$); 14.09 (2 × CH_3). MS (GT, FAB$^+$): 977 [M+H]$^+$; 999 [M+23]$^+$. Rf (C_6H_{12}/EtOAc: 60/40): 0.45.

Tosylate Salt of 1-(2,3-Dioleyl-5-trimethylammonium-α-D-ribofuranosyl)-5-nitroindole, 7a. Anhydrous triethylamine (1 mL) was transferred to a pressure tube cooled at −50 °C via canula. Next, anhydrous acetonitrile (2 mL) and a solution of **6a** (51 mg, 0.05 mmol) in dry THF (1 mL) were added. The tube was sealed and heated in an oil bath at 50 °C during 48 h, then cooled to −20 °C and opened. The solvents were evaporated under reduced pressure to give **7a** (54 mg, quantitative) as a yellow oil. ^{1}H NMR ($CDCl_3$): δ 8.48 (1H, d, J = 2.05 Hz, H_4); 7.88 (1H, dd, J = 2.05 Hz, J = 9.07 Hz, H_6); 7.74 (2H, d, J = 8.05 Hz, H_{Ar}); 7.49 (1H, d, J = 2.05 Hz, H_3); 7.35 (1H, d, J = 6.48 Hz, H_9); 7.11 (2H, d, J = 8.05 Hz, H_{Ar}); 6.66 (1H, d, J = 3.50 Hz, $H_{1'}$); 6.21 (1H, d, J = 6.48 Hz, H_8); 5.33 (4H, m, 2 × C$\underline{H}$=C$\underline{H}$); 4.70 (2H, m, $H_{2'}$ + $H_{3'}$); 4.28 (1H, m, $H_{4'}$); 3.41 (9H, s, NCH_3^+); 3.33 (2H, m, $H_{5'}$ + $H_{5''}$); 2.31 (3H, s, CH_3−C_{Ar}); 2.24 (4H, t, J = 7.26 Hz, 2 × CH_2−CO); 1.98 (8H, m, 2 × C$\underline{H}_2$−CH=CH−C$\underline{H}_2$); 1.53 (4H, m, 2 × C$\underline{H}_2$−CH_2−CO); 1.25 (40H, m, 2 × 10 CH_2); 0.87 (6H, t, J = 6.65 Hz, 2 × CH_3). ^{13}C NMR ($CDCl_3$) δ: 172.65 (CO); 171.82 (CO); 145.50 (C_2); 143.39 (C_{Ar}); 142.29 (C_5); 139.63 (C_{Ar}); 130.03 (2 × $\underline{C}H$=$\underline{C}H$); 130.00 (2 × CH_{Ar}); 129.49 (C_7); 128.76 (2 × CH_{Ar}); 127.16 (C_9); 125.91 (C_6); 125.73 (C_4); 101.20 (C_3); 96.05 (C_8); 83.17 ($C_{1'}$); 71.98 ($C_{3'}$); 66.55 ($C_{4'}$); 59.44 ($C_{2'}$); 66.93 ($C_{5'}$); 54.34 (NCH_3^+); 31.84 (2 × $\underline{C}H_2$−CO); 30.23−28.90 (2 × 10 CH_2); 27.07 (2 × $\underline{C}H_2$−CH=CH−$\underline{C}H_2$); 22.62 (2 × $\underline{C}H_2$−CH_2−CO); 21.22 (CH_3−C_{Ar}); 21.22 (2 × CH_3−$\underline{C}H_2$);

14.09 (2 × CH_3). MS (GT, FAB^+): 864 $[M-TsO^-]^+$. Anal. Calcd. C, 68.12; H, 9.00; N, 4.00; found C, 68.13; H, 8.97; N, 4.11.

Tosylate Salt of 1-(2,3-Dioleyl-5-trimethylammonium-β-D-ribofuranosyl)-5-nitroindole, 7b. This compound was prepared from **6b** with the same procedure described above to yield **7b** as a brown oil with quantitative yield. ^1H NMR ($CDCl_3$): δ 8.48 (1H, d, $J = 2.21$ Hz, H_6); 8.05 (1H, dd, $J = 2.21$ Hz, $J = 9.16$ Hz, H_4); 7.73 (3H, m, H_3 + H_{Ar}); 7.47 (1H, d, $J = 6.16$ Hz, H_9); 7.12 (2H, d, $J = 7.90$ Hz, H_{Ar}); 6.57 (1H, d, $J = 3.43$ Hz, $H_{1'}$); 6.21 (1H, d, $J = 6.16$ Hz, H_8); 5.32 (4H, m, 2 × C<u>H</u>═C<u>H</u>); 4.74 (1H, m, $H_{3'}$); 4.64 (1H, m, $H_{2'}$); 4.22 (1H, m, $H_{4'}$); 3.34 (9H, s, $N(CH_3)_3^+$); 3.27 (2H, m, $H_{5'}$ + $H_{5''}$); 2.32 (3H, s, CH_3-C_{Ar}); 2.26 (4H, t, $J = 7.42$ Hz, 2 × CH_2−CO); 1.98 (8H, m, 2 × C<u>H</u>$_2$−CH═CH−C<u>H</u>$_2$); 1.54 (4H, m, 2 × C<u>H</u>$_2$−CH_2−CO); 1.24 (40H, m, 2 × 10 CH_2); 0.86 (6H, t, $J = 6.32$ Hz, 2 × CH_3). ^{13}C NMR ($CDCl_3$) δ: 172.15 (CO); 171.86 (CO); 145.50 (C_2); 142.12 (2 × C_{Ar}); 138.70 (C_5); 132.06 (2 × C_{Ar}); 130.01 (2 × C<u>H</u>═C<u>H</u>); 129.53 (2 × CH_{Ar}); 128.96 (C_7); 128.49 (2 × CH_{Ar}); 127.97 (C_9); 125.85 (C_6); 125.19 (C_4); 104.31 (C_3); 100.78 (C_8); 79.82 ($C_{1'}$); 74.43 ($C_{3'}$); 73.80 ($C_{4'}$); 73.59 ($C_{2'}$); 65.57 ($C_{5'}$); 55.45 ($N(\underline{C}H_3)_3^+$); 31.87 (2 × <u>C</u>H_2−CO); 29.78−28.67 (2 × 9 CH_2); 27.18 (2 × <u>C</u>H_2−CH═CH−<u>C</u>H_2); 22.65 (2 × <u>C</u>H_2−CH_2−CO); 21.66 (<u>C</u>H_3−C_{Ar}); 21.65 (2 × CH_3−<u>C</u>H_2); 14.09 (2 × CH_3). MS (GT, FAB^+): 864 $[M-TsO^-]^+$. HMRS (FAB^+): calculated for $[M-TsO^-]^+$ 864.6460; found 864.6443. Anal. Calcd. C, 68.13; H, 9.01; N, 3.80; found C, 68.13; H, 8.97; N, 4.11.

1-(5-*O*-*tert*-Butyldiphenylsilyl-2,3-*O*-isopropylidene-D-ribofuranosyl)-4-nitroimidazole, 8. Compound **2′** (1.22 g, 2.85 mmol) was converted to **8** according to the procedure of glycosylation described for **3** and purified by flash column chromatography (cyclohexane/EtOAc: 90/10→80/20) to give **8a** (324 mg, 22%) and **8b** (170 mg, 11%).

1-(5-*O*-*tert*-Butyldiphenylsilyl-2,3-*O*-isopropylidene-α-D-ribofuranosyl)-4-nitroimidazole, 8a. ^1H NMR ($CDCl_3$) δ: 8.00 (1H, d, $J = 1.13$ Hz, H_5); 7.96 (1H, m, H_2); 7.66 (4H, m, H_{Ar}); 7.43 (6H, m, H_{Ar}); 6.69 (1H, d, $J = 4.99$ Hz, $H_{1'}$); 5.22 (1H, t, $J = 5.53$ Hz, $H_{2'}$); 4.78 (1H, d, $J = 5.53$ Hz, $H_{3'}$); 4.56 (1H, m, $H_{4'}$); 3.95 (1H, dd, $J = 2.10$ Hz, $J = 11.39$ Hz, $H_{5'}$); 3.73 (1H, dd, $J = 2.10$ Hz, $J = 11.39$ Hz, $H_{5''}$); 1.25 (3H, s, CH_3−C); 1.12 (9H, s, $(CH_3)_3$−C); 1.01 (3H, s, CH_3−C). ^{13}C NMR ($CDCl_3$): δ 138.55 (C_4); 135.52 (C_2); 133.06 (2 × C_{Ar}); 132.31 (2 × CH_{Ar}); 132.04 (2 × CH_{Ar}); 130.17 (2 × CH_{Ar}); 130.09 (4 × CH_{Ar}); 127.99 (C_5); 113.19 ($(CH_3)_2-\underline{C}$); 91.12 ($C_{1'}$); 84.11 ($C_{2'}$); 82.03 ($C_{4'}$); 81.11 ($C_{3'}$); 65.60 ($C_{5'}$); 26.81 (<u>C</u>H_3−C); 25.15 (<u>C</u>H_3−C); 24.37 ($(\underline{C}H_3)_3$−C); 19.04 ($(CH_3)_3-\underline{C}$). MS (GT, FAB^+): 524 $[M+H]^+$. Rf (C_6H_{12}/EtOAc: 90/10): 0.23.

1-(5-*O*-*tert*-Butyldiphenylsilyl-2,3-*O*-isopropylidene-β-D-ribofuranosyl)-4-nitroimidazole, 8b. ^1H NMR ($CDCl_3$) δ: 7.85 (1H, d, $J = 1.44$ Hz, H_5); 7.64 (4H, m, H_{Ar}); 7.52 (1H, d, $J = 1.46$ Hz, H_2); 7.44 (6H, m, H_{Ar}); 6.10 (1H, d, $J = 2.91$ Hz, $H_{1'}$); 4.91 (2H, m, $H_{2'}$ + $H_{3'}$); 4.44 (1H, t, $J = 2.10$ Hz, $H_{4'}$); 3.99 (1H, dd, $J = 2.10$ Hz, $J = 11.44$ Hz, $H_{5'}$); 3.79 (1H, dd, $J = 2.10$ Hz, $J = 11.44$ Hz, $H_{5''}$); 1.37 (3H, s, CH_3−C); 1.32 (3H, s, CH_3−C); 1.11 (9H, s, $(CH_3)_3$−C). ^{13}C NMR ($CDCl_3$) δ: 135.49 (C_4); 135.33 (C_2); 134.85 (2 × C_{Ar}); 132.11 (4 × CH_{Ar}); 130.34 (2 × CH_{Ar}); 130.26 (4 × CH_{Ar}); 128.07 (C_5); 113.98 ($(CH_3)_2-\underline{C}$); 89.41 ($C_{1'}$); 83.87 ($C_{2'}$); 82.32 ($C_{4'}$); 80.41 ($C_{3'}$); 66.13 ($C_{5'}$); 26.89 (2 × <u>C</u>H_3−C); 25.51 ($(\underline{C}H_3)_3$−C); 19.09 ($(CH_3)_3-\underline{C}$). MS (GT, FAB^+): 524 $[M+H]^+$. mp = 40 °C. Rf (C_6H_{12}/EtOAc: 90/10): 0.18.

1-(2,3-*O*-Isopropylidene-α-D-ribofuranosyl)-4-nitroimidazole, 9a. Compound **8a** (325 mg, 0.62 mmol) was converted to **9a** according to the procedure described for **4a** and purified by flash column chromatography (cyclohexane/EtOAc: 90/10→EtOAc) to give **9a** (80 mg, 46%). ^1H NMR ($CDCl_3$) δ: 7.99 (1H, d, $J = 1.13$ Hz, H_5); 7.93 (1H, m, H_2); 6.62 (1H, d, $J = 2.78$ Hz, $H_{1'}$); 5.15 (1H, t, $J = 5.93$ Hz, $H_{3'}$); 4.87 (1H, dd, $J = 2.78$ Hz, $J = 5.93$ Hz, $H_{2'}$); 4.63 (1H, t, $J = 2.78$ Hz, $H_{4'}$); 3.95 (1H, dd, $J = 2.78$ Hz, $J = 11.59$ Hz, $H_{5'}$); 3.81 (1H, dd, $J = 2.78$ Hz, $J = 11.59$ Hz, $H_{5''}$); 2.41 (1H, sl, O<u>H</u>); 1.27 (3H, s, CH_3−C); 0.98 (3H, s, CH_3−C). ^{13}C NMR ($CDCl_3$) δ: 139.31 (C_4); 135.00 (C_2); 118.72 (C_5); 113.96 ($(CH_3)_2-\underline{C}$); 89.30 ($C_{1'}$); 83.73 ($C_{2'}$); 82.13 ($C_{4'}$); 80.35 ($C_{3'}$); 64.39 ($C_{5'}$); 25.50 (<u>C</u>H_3−C); 24.15 (<u>C</u>H_3−C). MS (GT, FAB^+): 286 $[M+H]^+$; 308 $[M+Na]^+$. Rf (EtOAc): 0.43.

1-(2,3-*O*-Isopropylidene-β-D-ribofuranosyl)-4-nitroimidazole, 9b. Compound **8b** (166 mg, 0.32 mmol) was converted to **9b** according to the procedure described for **4b** and purified by flash column chromatography (cyclohexane/EtOAc: 70/30) to give **9b** (58 mg, 64%) as a white solid. mp = 142−144 °C. ^1H NMR ($CDCl_3$) δ: 7.91 (1H, d, $J = 1.37$ Hz, H_5); 7.61 (1H, d, $J = 1.37$ Hz, H_2); 6.11 (1H, d, $J = 2.51$ Hz, $H_{1'}$); 4.97 (1H, dd, $J = 2.51$ Hz, $J = 4.48$ Hz, $H_{3'}$); 4.90 (1H, dd, $J = 1.58$ Hz, $J = 4.48$ Hz, $H_{2'}$); 4.49 (1H, t, $J = 2.51$ Hz, $H_{4'}$); 3.98 (1H, dd, $J = 2.51$ Hz, $J = 11.47$ Hz, $H_{5'}$); 3.85 (1H, dd, $J = 2.51$ Hz, $J = 11.47$ Hz, $H_{5''}$); 1.38 (3H, s, CH_3−C); 1.33 (3H, s, CH_3−C). ^{13}C NMR ($CDCl_3$) δ: 138.32 (C_4); 137.85 (C_2); 132.85 (C_5); 113.49 ($(CH_3)_2-\underline{C}$); 90.93 ($C_{1'}$); 84.28 ($C_{2'}$); 81.73 ($C_{4'}$); 81.12 ($C_{3'}$); 63.74 ($C_{5'}$); 25.03 (<u>C</u>H_3−C); 24.40 (<u>C</u>H_3−C). MS (GT, FAB^+): 286 $[M+H]^+$; 308 $[M+Na]^+$. Rf (EtOAc): 0.72.

1-(2,3-*O*-Isopropylidene-5-*O*-tosyl-α-D-ribofuranosyl)-4-nitroimidazole, 10a. Compound **9a** (80 mg, 0.28 mmol) was converted to **10a** according to the procedure described for **5a** and purified by flash column chromatography (cyclohexane/EtOAc: 70/30) to give **10a** (50 mg, 46%) as an orange oil. ^1H NMR ($CDCl_3$) δ: 7.86 (1H, d, $J = 1.46$ Hz, H_5); 7.80 (2H, d, $J = 7.90$ Hz, H_{Ar}); 7.56 (1H, d, $J = 1.46$ Hz, H_2); 7.40 (2H, d, $J = 7.90$ Hz, H_{Ar}); 5.93 (1H, d, $J = 4.11$ Hz, $H_{1'}$); 4.89 (2H, m, $H_{4'}$ + $H_{2'}$); 4.56 (1H, t, $J = 1.74$ Hz, $H_{3'}$); 4.22 (2H, d, $J = 2.37$ Hz, $H_{5'}$ + $H_{5''}$); 2.47 (3H, s, CH_3-C_{Ar}); 1.36 (3H, s, CH_3−C); 1.30 (3H, s, CH_3−C). ^{13}C NMR ($CDCl_3$) δ: 145.93 (C_4); 138.81 (C_2); 134.85 (C_{Ar}); 131.83 (C_{Ar}); 130.25 (2 × CH_{Ar}); 127.87 (2 × CH_{Ar}); 118.43 (C_5); 114.59 ($(CH_3)_2-\underline{C}$); 89.12 ($C_{1'}$); 81.66 ($C_{2'}$); 81.07 ($C_{3'}$); 79.96 ($C_{4'}$); 70.90 ($C_{5'}$); 25.45 (<u>C</u>H_3−C); 24.15 (<u>C</u>H_3−C); 21.69 (<u>C</u>H_3-C_{Ar}). MS (GT, FAB^+): 440 $[M+H]^+$. Rf (C_6H_{12}/EtOAc: 70/30): 0.36.

1-(2,3-*O*-isopropylidene-5-*O*-tosyl-β-D-ribofuranosyl)-4-nitroimidazole, 10b. Compound **9b** (140 mg, 0.49 mmol) was converted to **10b** according to the procedure described for **5b** and purified by preparative layer chromatography (cyclohexane/EtOAc: 30/70) to give **10a** (90 mg, 42%) as a pale yellow oil. ^1H NMR ($CDCl_3$) δ: 7.98 (1H, d, $J = 1.43$ Hz, H_5); 7.82 (3H, d + m, $J = 8.53$ Hz, H_{Ar} + H_2); 7.38 (2H, d, $J = 8.53$ Hz, H_{Ar}); 6.34 (1H, d, $J = 2.84$ Hz, $H_{1'}$); 5.05 (1H, t, $J = 4.84$ Hz, $H_{4'}$); 4.77 (1H, d, $J = 2.84$ Hz, $H_{2'}$); 4.70 (1H, t, $J = 2.84$ Hz, $H_{3'}$); 4.26 (2H, m, $H_{5'}$ + $H_{5''}$); 2.46 (3H, s, CH_3-C_{Ar}); 1.23 (3H, s, CH_3−C); 0.96 (3H, s, CH_3−C). ^{13}C NMR ($CDCl_3$) δ: 145.77 (C_4); 134.02 (C_2); 132.37 (C_{Ar}); 131.31 (C_{Ar}); 130.19 (2 × CH_{Ar}); 127.84 (2 × CH_{Ar}); 121.95 (C_5); 114.07 ($(CH_3)_2-\underline{C}$); 81.35 ($C_{1'}$); 81.22 ($C_{2'}$); 80.73 ($C_{3'}$); 70.75 ($C_{4'}$); 69.87 ($C_{5'}$); 22.98 (<u>C</u>H_3−C); 24.36 (<u>C</u>H_3−C); 21.70 (<u>C</u>H_3-C_{Ar}). MS (GT, FAB^+): 440 $[M+H]^+$. Rf (C_6H_{12}/EtOAc: 90/10): 0.74.

1-(2,3-dioleyl-5-*O*-tosyl-α-D-ribofuranosyl)-4-nitroimidazole, 11a. Compound **10a** (80 mg, 0.28 mmol) was converted to **11a** according to the procedure described for **6a** and purified by flash column chromatography (cyclohexane/EtOAc: 70/30) to give **11a** (41 mg, 32%) as a yellow oil. ^1H NMR ($CDCl_3$): δ 7.88 (1H, d, $J = 1.46$ Hz, H_5); 7.82 (2H, d, $J = 8.06$ Hz, H_{Ar}); 7.51 (1H, d, $J = 1.46$ Hz, H_2); 7.39 (2H, d, $J = 8.06$ Hz, H_{Ar}); 6.09 (1H, d, $J = 5.49$ Hz, $H_{1'}$); 5.48 (2H, m, $H_{2'}$ + $H_{3'}$); 5.34 (4H, m, 2 × CH_2−C<u>H</u>═C<u>H</u>−CH_2); 4.58 (1H, m, $H_{4'}$); 4.34 (1H, dd, $J = 2.21$ Hz, $J = 11.26$ Hz, $H_{5'}$); 4.21 (1H, dd, $J = 2.21$

Hz, $J = 11.26$ Hz, $H_{5''}$); 2.47 (3H, s, CH_3-C_{Ar}); 2.32 (4H, m, $2 \times CH_2-CO$); 1.99 (8H, m, $2 \times CH_2-CH=CH-CH_2$); 1.56 (4H, m, $2 \times CH_2-CH_2-CO$); 1.26 (40H, m, 2×10 CH_2); 0.87 (6H, t, $J = 6.64$ Hz, $2 \times CH_3$). ^{13}C NMR ($CDCl_3$) δ: 173.01 (CO); 172.59 (CO); 149.54 (C_{Ar}); 147.69 (C_2); 143.42 (C_{Ar}); 140.11 (C_{Ar}); 133.65 ($2 \times CH=CH$); 131.27 ($2 \times CH_{Ar}$); 130.96 ($2 \times CH_{Ar}$); 118.88 (C_5); 87.71 ($C_{1'}$); 78.45 ($C_{4'}$); 76.23 ($C_{3'}$); 71.36 ($C_{2'}$); 65.67 ($C_{5'}$); 32.36 ($2 \times CH_2-CO$); 30.78 ($CH_2-CH=CH-CH_2$); 30.66 ($CH_2-CH=CH-CH_2$); 29.88−29.19 (2×9 CH_2); 26.76 (CH_2-CH_2-CO); 25.84 (CH_2-CH_2-CO); 23.05 (CH_3-C_{Ar}); 21.52 ($2 \times CH_3-CH_2$); 14.09 ($2 \times CH_3$). MS (GT, FAB^+): 929 $[M+H]^+$.

Tosylate Salt of 1-(2,3-Dioleyl-5-trimethylammonium-α-D-ribofuranosyl)-4-nitroimidazole, 12a. Compound **11a** (80 mg, 0.28 mmol) was converted to **12a** according to the procedure described for **7a** to give **11a** as a yellow oil with quantitative yield. ^1H NMR ($CDCl_3$): δ 7.95 (1H, d, $J = 1.46$ Hz, H_5); 7.68 (3H, d+m, $J = 7.90$ Hz, $H_{Ar} + H_2$); 7.15 (2H, d, $J = 7.90$ Hz, H_{Ar}); 5.76 (1H, d, $J = 5.50$ Hz, $H_{1'}$); 5.33 (4H, m, $2 \times CH_2-CH=CH-CH_2$); 4.91 (3H, m, $H_{2'} + H_{3'} + H_{4'}$); 3.37 (9H, s, NCH_3^+); 3.30 (2H, m, $H_{5'} + H_{5''}$); 2.33 (3H, s, CH_3-C_{Ar}); 2.15 (4H, m, CH_2-CO); 1.99 (8H, m, $2 \times CH_2-CH=CH-CH_2$); 1.50 (4H, m, $2 \times CH_2-CH_2-CO$); 1.26 (40H, m, 2×10 CH_2); 0.87 (6H, t, $J = 6.16$ Hz, $2 \times CH_3$). MS (GT, FAB^+): 815 $[M-TsO^-]^+$. Anal. Calcd. 65.63; H, 9.11; N, 5.62; Found C, 65.69; H, 9.19; N, 5.67.

Vesicle Preparation. 1 mg/mL of compounds **7a** or **7b** was dissolved in chloroform. The solution was evaporated and dried under vacuum for 2 h. 1 mL of DI water was added, and the solution was hydrated overnight at 4 °C. The resulting solution was sonicated for 20 min prior to any measurements.

Electrophoresis Studies. Electrophoresis studies were conducted in 2% agarose gels containing ethidium bromide in a 0.5 Tris-Borate-EDTA buffer. Cationic lipoplexes were prepared 10 min before use. 250 ng of siRNA (21 bp, Eurogentec SR-CL010−005) was mixed with lipids at different ratios (final volume is 20 μL). Then, 20 μL of each lipoplexes (samples) were mixed with 4 μL of loading buffer (glycerol 30%, v/v), bromophenol blue 0.25% (w/v), and xylene cyanol 0.25 (w/v) and subjected to agarose gel electrophoresis for 20 min at 100 V. The electrophoresis gel was visualized and digitally photographed using a G.BOX camera.

Exclusion Assay. Ten milliliters of EB (10 mg/mL) was diluted in 580 mL in deionized water. Ten microliters of 90 mg/mL solution of siRNA was added. A varying amount of lipid (depending on the siRNA/lipid ratio required) was added to the EB solution finally. The solutions were mixed on a benchtop vortex, and the fluorescence was measured ($\lambda_{exc} = 470$ nm, $\lambda_{em} = 600$ nm; 0.5 cm path length glass cuvette). The fluorescence was expressed as the percentage of the maximum fluorescence signal when EB was bound to the siRNA in the absence of lipid.

ζ Potential and Size Distribution of 7b CNLs (with and without siRNA). Experiments were realized with N/P ratio of 10 (CNLs/siRNA).

Transmission Electronic Microscopy (TEM). TEM experiments were performed on vesicle samples **7a**, **7b**, and **12a** using a Philips CM 10 (negative staining with ammonium molybdate 1% water, Cu/Pd carbon coated grids). Both (α) and (β) anomer nucleolipids formed small vesicles with diameters between 40 and 60 nm, respectively. Cationic lipoplexes were prepared as described above and were visualized by negative staining microscopy. Ten microliter aliquots of samples (either liposomes or lipoplexes) were transferred to a carbon-coated grid for 10 min. Then, the samples were dried and stained with 2.5% (w/w) of uranyl acetate for 30 s. The specimens were observed with a Hitachi H 7650 electron microscope. Both (α) and (β) anomer nucleolipids formed small vesicles with diameters between 40 and 60 nm, respectively.

siRNA Transfection. Trypsine adherent cells were diluted in normal growth medium to 1×10^5 cells per milliliter. The transfectant reagent was diluted in serum free medium and incubated at room temperature for 10 min before use. Next, siRNA (GAPDH siRNA or negative control siRNA, Ambion) was diluted in serum free medium. The siRNA and the tranfection reagent were mixed, incubated for 10 min at room temperature, and dispensed into a culture plate. Depending on the experimental design, the ratio of lipid, siRNA, and incubation time was varied. Cell suspension was overlayed onto the transfection mixture, then incubated at 37 °C and 5% CO_2. Assays for gene knockdown were assessed after 48 h depending on the experimental protocol.

The gene knockdown assay performed was KDalert GAPDH Assay (Ambion) following the manufacturer's protocol. Briefly, 48 h after siRNA transfection, the culture medium was aspirated from transfected cells; 200 μL KDalert Lysis Buffer was added to each sample well and the mixture incubated at 4 °C for 20 min to lyse the cells. The cell lysate was pipeted up and down 4−5 times to homogenize the lysate. 10 μL aliquots of each lysate or GAPDH enzyme dilution (including the GAPDH working stock) were transfered to the wells of a clean 96 well plate. 90 μL aliquots of KDalert master mix were added to each sample using a multichannel pipettor to dispense the KDalert master mix quickly. The increase in fluorescence of the samples was measured at room temperature.

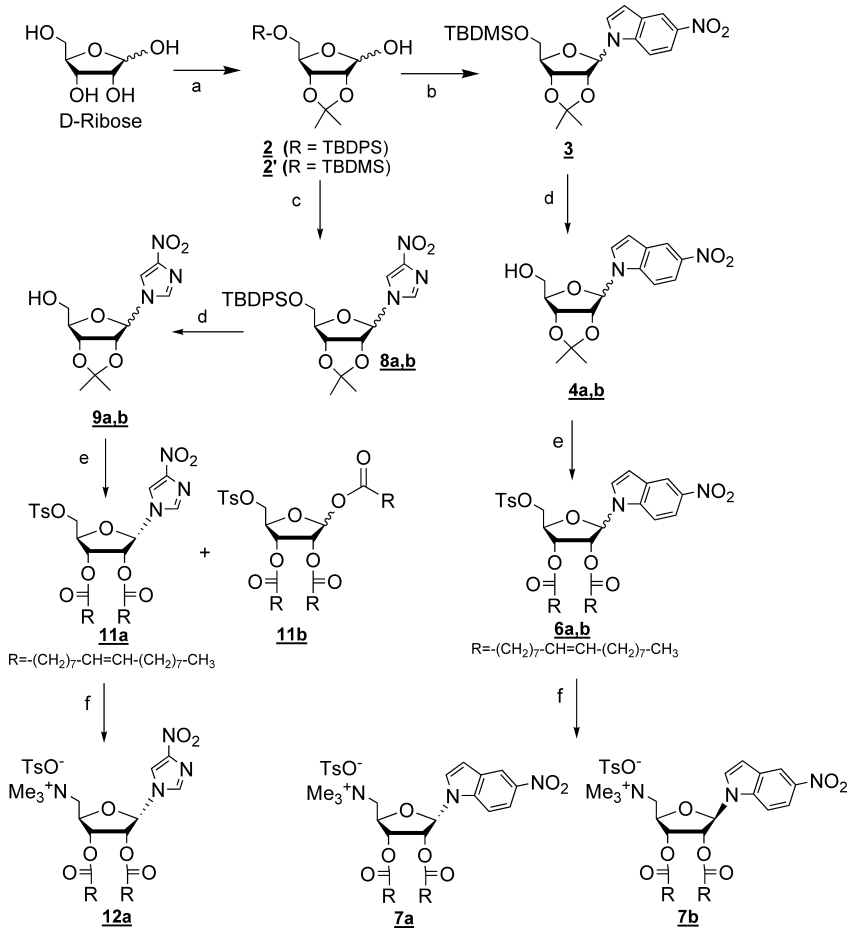
Cytotoxicity. Cytotoxicity was assessed using both a formazan-based proliferation assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay kit, Promega) and a total protein-based assay (Pierce). Briefly, CHO cells were seeded onto a 24 well plate with an appropriate density of 1×10^6 cells per well. After 48 h, the MTS substrate was added to each well, and the plate was incubated for 4 h at 37 °C in a humidified, 5% CO_2 incubator. The amount of soluble formazan produced by cellular reduction of the substrate MTS was recorded at 490 nm using a multiwell plate reader. For the total protein based proliferation assay, cells were lysed at the same time as transfection efficiency was assayed. 10 μL aliquots of lysates were transferred to a separate multiwell plate. Total protein contents were assessed using the Coomassie Blue protein kit (Pierce, Rockford, IL) following the manufacturer protocol. Negative and positive controls were nontreated cells and commercial lipids treated cells, respectively. The proliferation results were expressed as percentages of nontreated cells.

Molecular Modeling. In order to obtain some information about the structure−activity of these universal base analogue nucleolipids, modelization of the five tested compounds was performed at the density functional theory (DFT) level (B3LYP) with a 6-31G* split-valence basis set (*8, 9*). Cavity surface area (CSA) and (polarized solute)-solvent (PSS) are determined within the same methodology in a solvent, water, using a PCM model (*10*). Briefly, cavity surface area (CSA) and (polarized solute)-solvent (PSS) were calculated for all the compounds as well as the quotient MC = |PSS/CSA|.

RESULTS AND DISCUSSION

Synthesis. The (α) and (β) anomers of 2′,3′-dioleyl-5′-*O*-D-ribofuranosyl-3-nitroindole, **7a** and **7b**, respectively, and the (α) anomer 2′,3′-dioleyl-5′-*O*-D-ribofuranosyl-4-nitroimidazole, **12a**, were synthesized from D-ribose as shown in Scheme 1. Accordingly, 5-*O*-*tert*-butyldimethylsilyl-2,3-*O*-isopropylidene-D-ribose **2** and 5-*O*-*tert*-butyldimethylsilyl-2,3-*O*-isopropylidene-D-ribose **2′** were prepared in two steps (see Supporting Information) from D-ribose following a published procedure (*11*). Next, the stereoselective chlorination of the protected

Scheme 1[a]



[a] Reaction conditions: (a) (i) acetone, $CuSO_4$, **1** (94%), (ii) TBDMSCl or TBDPSCl, DMAP, TEA; **2** (35%) and **2′** (46%); (b) (i) CCl_4, HMPT, (ii) 5-nitroindole, NaH, CH_3CN; **3** (40%); (c) (i) CCl_4, HMPT, (ii) 4-nitroimidazole, NaH, CH_3CN; **8a** (22%) and **8b** (11%); (d) TBAF dry THF; **4a/4b** (5/1; 65%); **9a** (46%), **9b** (64%); (e) (i) TsCl, TEA, DCM; **5a** (94%), **5b** (56%) and **10a** (42%), **10b** (46%), (ii) TFA/H_2O, quant., (iii) Oleic acid, DCC/DMAP; **6a** (65%), **6b** (73%), and **11a** (32%); (f) NMe_3, CH_3CN 60°C, 40 h, quantitative.

sugars **2** and **2′** with CCl_4 and hexamethylphosphorus triamide (HMPT) (*12*) was followed by coupling with the anions of 3-nitroindole pyrrole or 4-nitroimidazole. Deprotection of the resulting derivative **3** and **8** afforded the anomeric mixture of (α) **4a** (53%), (*β*) **4b** (18%), and (α) **9a** (53%), (*β*) **9b** (18%). As described previously for nitropyrrole derivatives, the (α) forms were found to be the major isolated polar isomers. The relative stereochemistry of these compounds was assigned by ^{1}H NMR when compared to the chemical shifts of the acetonide methyl group as well as those of the H-1′ (*13*). The anomeric pairs of compounds **4** and **9** were then separately tosylated with TsCl/TEA, deprotected with TFA/H_2O, and coupled with oleic acid using DCC/DMAP to afford the nucleolipids **6a**, **6b**, **11a**, and the side product **11b**. The latter was isolated and characterized by mass spectra. Unfortunately, we were unable to isolate the *β* anomer of **11a**. Indeed, the instability of the sulfonated intermediates has been demonstrated for adenosine analogues with intramolecular cyclization of the 5′-carbon and N3-position of the adenine ring (*14, 15*). It is likely that such an intermolecular cyclization occurs in the case of the nitroindole intermediate **11a**.

Finally, compounds **6a**,**b**, and **11a** were reacted with trimethylamine for two days in a sealed tube to afford the target nucleolipids **7a**, **7b**, and **12a**.

Complexation Studies. In order to determine whether the CNLs **7a**, **7b**, and **12a** bind nucleic acids, we performed (i) siRNA (21 bp) gel electrophoresis and (ii) standard ethidium bromide (EB) fluorescence displacement assays with siRNA (21 bp) as a model system (*16*). The siRNA gel electrophoresis was performed on compounds **7a** and **7b**; as shown in Figure 3, a full complexation occurs for a charge ratio **7b**/siRNA (*β* anomer) of 10/1 (Figure 3 column 6), whereas in the case of the **7a**/siRNA (α anomer), free siRNA is observed for all the ratios investigated (Figure 3 columns 8−12).

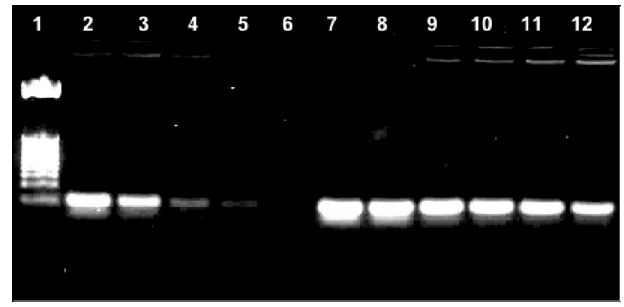


Figure 3. Electrophoretic mobility of **7b** (*β*) and **7a** (α). The lines represent the following (*1*): DNA stepladder 25 pb (*2*−*6*), siRNA with **7b** at different ratios N/P (2, 4, 6, 8, 10 respectively (*7*)), siRNA (*8*−*12*), siRNA with **7a** at different ratios N/P (2, 4, 6, 8, 10, respectively).

The binding affinity of siRNA by CNLs was also investigated by the fluorescence titration of EB with a premixed solution of siRNA and various concentrations of CNLs. In the presence of siRNA, the fluorescence emission of EB is enhanced relative to that in water as a result of EB intercalation between the RNA base pairs. The subsequent addition of CNLs to the siRNA−EB solution results in CNL binding to RNA and displacement of

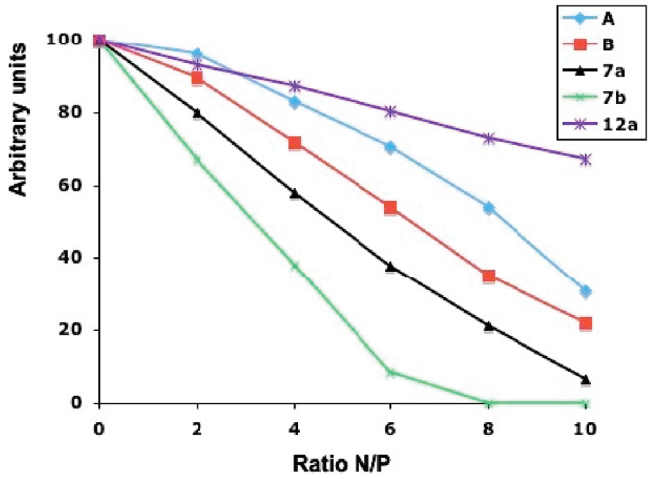


Figure 4. EB exclusion assays with **A**, **B**, **7a**, **7b**, and **12a** in the presence of siRNA (fluorescence intensity arbitrary units versus N/P charge ratio).

EB from the helix to water, which induces a decrease in fluorescence intensity. As shown in Figure 4, the fluorescence intensity decreases rapidly for all the CNLs investigated, including both 5-nitroindole derivatives **7b** (β), **7a** (α), the 4-nitroimidazole **12a**, and nitropyrrole CNLs previously reported (α,β anomers, compounds **A** and **B**, respectively). Interestingly, with a total decrease in fluorescence for N/P ratios higher than 8, the β anomer 5-nitroindole derivative **7b** exhibits the best binding affinity of the CNL series. The α anomer **7a**, which forms **7a**/siRNA complex at higher N/P ratios (higher than 10) compared to β analogue **7b**, shows a lower binding affinity indicating that stereochemistry of the anomeric position has an impact on the complexation with siRNA double helix. Likewise, a higher binding affinity is observed for nitropyrrole CNL **B** (β) compared to CNL **A** (α) confirming the importance of anomeric configuration in the complex formation. As expected, the nature of the CNL universal base has an impact on the formation of CNLs/siRNA complexes. With no residual EB fluorescence for ratio of 10/1, the 5-nitroindole derivatives **7b** (β) and **7a** (α) feature the best binding affinity, whereas 4-nitroimidazole **12a** is the least efficient of the CNLs. It is well-known that intermolecular overlapping of p-orbitals in π-conjugated systems causes $\pi-\pi$ interactions. Hence, these interactions become stronger as the number of π-electrons increases. Accordingly, the best binding affinity observed for the 5-nitroindole universal base can be explained by the 10 π electrons, whereas the 6 π electrons of both nitroindole and nitropyrrole induce fewer $\pi-\pi$ interactions.

Electronic Microscopy Studies, ζ Potential, and Size Distribution. TEM experiments were performed on vesicle samples **7a**, **7b**, and **12a** using a Hitachi H 7650 electron microscope (negative staining with 2.5% (w/w) of uranyl acetate). Both (α) and (β) anomer nucleolipids formed small vesicles with diameters between 40 and 60 nm, respectively (Supporting Information Figure S1). TEM experiments were also performed on samples prepared with siRNA. For these experiments, the CNL vesicles were incubated for 15 min in the presence of siRNA (21 bp). TEM micrographs (Figure 5) of the CNL-siRNA systems exhibited multilamellar vesicles (which are not observed in the case of CNL vesicles; see Supporting Information Figure S2) suggesting that the siRNA molecules are entrapped within the vesicular structures. ζ-potential and size distribution were determined for the CNL vesicles of **7b** with and without siRNA. As expected, the results show a significative decrease of ζ-potential (from 74 to 36.7 mV), as well as an increase of size distribution (from 108 to 360 nm) in the presence of siRNA suggesting its complexation with the CNL vesicles of **7b**.

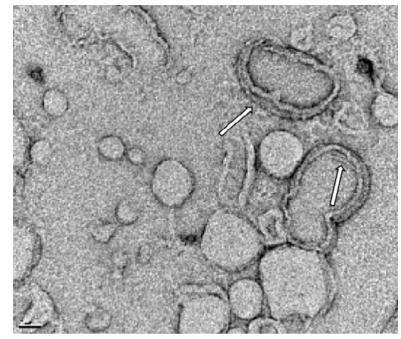


Figure 5. TEM image of CNL-siRNA systems obtained with **7b** (bar = 50 nm).

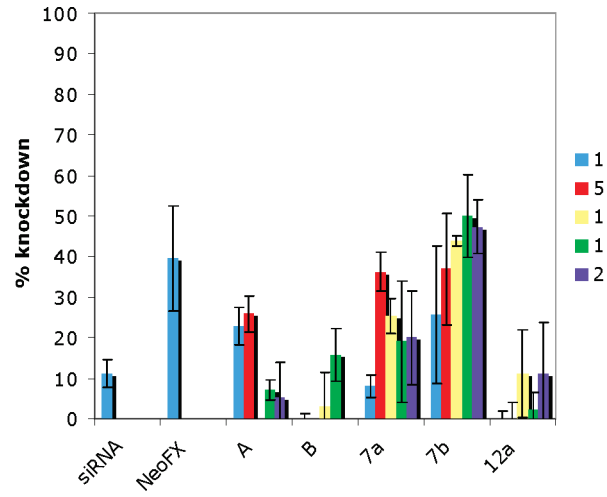


Figure 6. siRNA transfection results after 48 h in HepG2 as a function of nucleolipid (**A**, **B**, **7a**,**7b**, and **12a**) and N/P mole ratio. $n = 3$.

Transfection. The transfection of siRNA with **A**, **B**, **7a**, **7b**, and **12a** was next performed to evaluate the reduction in protein synthesis. Experiments used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) siRNA (Ambion) on human liver (HepG2) cell line, and the complete details can be found in the Supporting Information. siRNA transfection results were accessed after 48 h as a function of both anomer and cation/anion (N/P) mole ratio. In each experiment, controls using GAPDH siRNA and negative control siRNA were used at 30 mM. siPORT NeoFX was used as the positive control, and the previously evaluated 3-nitropyrrole nucleolipids A and B (*2*) (Figure 1) were tested again in the experiment. As shown in Figure 6, siRNA knockdown was observed for most nucleolipids, with values similar to the positive control for compounds **7a** and **7b**. The transfection activity of **7a** was significant vs siRNA with N/P ratios of 5 and 10, whereas for **7b**, it was significant with N/P ratios of 5, 10, 15, and 20. Interestingly, the 5-nitroindole CNL **7b** (β), which features the best binding affinity for siRNA, is also globally the most efficient in term of transfection. In addition, none of the CNLs showed a significant cytotoxicity at these concentrations, similar to that to the negative control (Supporting Information Figure S3).

Molecular Modeling. A basic molecular modeling method described in the section above was used to calculate the MC quotient (MC = |PSS/CSA|). This quotient represents the stabilization in kilojoules per mole per surface unit; it reflects the system's capacity to maintain its cohesion, i.e., the smaller MC values reflect lower membrane cohesion. As shown in Table 1 with N/P ratios of >5, it can be seen that low MC values are correlated both to better transfection activity (Table 1, line 4)

Table 1. Relationship among MC Quotient, % Knockdown, and Fluorescence Intensity for Compounds A, B, 7a−b, and 12a−b[a]

compounds	**12a**	**A**	**B**	**7a**	**7b**
PSS (kJ.mol^{-1})	309.57	287.79	270.78	279.81	269.07
CSA (Å^2)	389.02	396.10	390.84	438.56	432.64
MC = |PSS/CSA| (kJ.mol^{-1} Å^{-2})	0.80	0.73	0.69	0.64	0.62
% knockdown (N/P ratios of 15)	3	8	15	18	50
fluorescence intensity (N/P ratios of 10, Figure 4)	70	30	20	5	0

[a] CSA: Cavity Surface Area. PSS: (Polarized Solute)-Solvent.

and to binding affinity (Figure 4). These results confirm that to a certain extent (N/P ratio >5) the lowest membrane cohesion of these series of CNLs vesicles are beneficial for the release and the activities of siRNA in the cytoplasm.

It is known that subtle change such as headgroup nature, headgroup charges, counterion nature, and so forth can dramatically affect the transfection properties (*17, 18*). However, for the first time to our knowledge, the results highlight the impact of both stereochemistry and base nature of these nucleoside amphiphiles, i.e., β anomeric configuration, as well as highly π conjugated bases, is likely to improve the transfection efficacy. These findings could provide a rationale for the design of universal base nucleoside amphiphiles for siRNA delivery.

CONCLUSION

In summary, we have synthesized and characterized several new universal base CNLs for siRNA delivery. The results obtained by gel electrophoresis and ethidium bromide exclusion assays clearly indicate that (i) the nature of the universal base (5-nitroindole and 4-nitroimidazole) and (ii) the stereochemistry of the anomeric position (α, β) have an impact on the CNLs-siRNA complex formation. We observed that the CNL featuring the nitroindole as universal base exhibits the best binding affinity for siRNA. We also demonstrated that higher binding affinities for siRNA are observed for β anomers of both nitroindole and nitropyrrole CNLs. Molecular modeling has shown that calculation of simple values (MC) reflecting the system's capacity to maintain its cohesion can be correlated to both binding affinities and transfection efficiencies. On the basis of this rational approach, work is in progress to synthesize and improve the efficacy of new, original cationic nucleoside amphiphiles for siRNA delivery.

ACKNOWLEDGMENT

The authors thank the CNRS and the "Ministère de l'enseignement supérieur et de la recherche" for financial support.

Supporting Information Available: Synthesis of compounds **1**, **2**, and **2′**; ζ potential and size distribution of **7b** CNLs; TEM images of **7a** and **12a**; cytotoxicities of CNLs on HepG2 cell; EB exclusion assays with **7a** and **7b**. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Chabaud, P., Camplo, M., Payet, D., Serin, G., Moreau, L., Barthélémy, P., and Grinstaff, M. (2006) Cationic nucleoside lipids derived for gene delivery. *Bioconjugate Chem. 17*, 466–472.

(2) Gissot, A., Camplo, M., Grinstaff, M. W., and Barthélémy, P. (2008) Nucleoside based amphiphiles: A Successful Marriage of nucleic acids with lipids. *Org. Biomol. Chem. Rev. 6*, 1324–1333.

(3) Moreau, L., Camplo, M., Wathier, M., Taib, N., Laguerre, M., Bestel, I., Grinstaff, M. W., and Barthélémy, P. (2008) Real time imaging of supramolecular assembly formation via programmed nucleolipid recognition. *J. Am. Chem. Soc. 130*, 14454–14455.

(4) Barthélémy, P. (2009) Nucleoside-based lipids at work: From supramolecular assemblies to biological applications. *C. R. Chim. 12*, 171–179.

(5) Ceballos, C., Prata, C. H., Giorgio, S., Payet, D., Barthélémy, P., Grinstaff, M. W., and Camplo, M. (2009) Cationic nucleoside lipids possessing a 3-nitropyrrole universal base for siRNA delivery. *Bioconjugate Chem. 20*, 193–196.

(6) Loakes, D. (2001) The applications of universal DNA base analogues. *Nucleic Acids Res. 29*, 2437–2447.

(7) Loakes, D., Hill, F., Brown, D. M., and Salisbury, S. A. (1997) Stability and structure of DNA oligonucleotides containing non-specific base analogues. *J. Mol. Biol. 270*, 426–435.

(8) Lee, C. T., Wang, W. T., and Parr, R. G. (1988) Development of the the colle-salvettu correlation-energy formula into a functional of the electron density. *Phys. Rev. B 37*, 785–789.

(9) Frisch, M. J., Trucks, G. W., Schlegel, H. B., Scuseria, G. E., Robb, M. A., Cheeseman, J. R., Montgomery, Jr., J. A. ,Vreven, T., Kudin, K. N., Burant, J. C., Millam, J. M., Iyengar, S. S., Tomasi, J., Barone, V., Mennucci, B., Cossi, M., Scalmani, G., Rega, N.,Petersson, G. A., Nakatsuji, H., Hada, M., Ehara, M., Toyota, K., Fukuda,R., Hasegawa, J., Ishida, M., Nakajima, T., Honda, Y., Kitao, O., Nakai, H., Klene, M., Li, X., Knox, J. E., Hratchian, H. P., Cross, J. B., Bakken, V., Adamo, C., Jaramillo, J., Gomperts, R., Stratmann, R. E., Yazyev, O., Austin, A. J., Cammi, R., Pomelli, C., Ochterski, J. W., Ayala, P. Y., Morokuma, K., Voth, G. A., Salvador, P., Dannenberg, J. J., Zakrzewski, V. G., Dapprich, S., Daniels, A. D., Strain, M. C., Farkas, O., Malick, D. K., Rabuck, A. D., Raghavachari, K., Foresman, J. B., Ortiz, J. V., Cui, Q., Baboul, A. G., Clifford, S., Cioslowski, J., Stefanov, B. B., Liu, G., Liashenko, A., Piskorz, P., Komaromi, I., Martin, R. L., Fox, D. J., Keith, T., Al-Laham, M. A., Peng, C. Y., Nanayakkara, A., Challacombe, M., Gill, P. M. W., Johnson, B., Chen, W., Wong, M. W., Gonzalez, C., Pople, J. A. (2004) *Gaussian 03, rev C.02*, Gaussian, Inc., Wallingford, CT.

(10) Cossi, M., Scalmani, G., Rega, N., and Barone, V. (2002) New developments in the polarizable continuum model for quantum mechanical and classical calculations on molecules in solution. *J. Chem. Phys. 117*, 43–54.

(11) Stewart, A. O., and Williams, R. M. (1985) C-glycosidation of pyridyl thioglycosides. *J. Am. Chem. Soc. 107*, 4289–4296.

(12) Harusawa, S., Imazu, T., Takashima, S., Araki, L., Ohishi, H., Kurihara, T., Sakamoto, Y., Yamamoto, Y., and Yamatodani, A. J. (1999) Synthesis of 4(5)-[5-(aminomethyl)tetrahydrofuran-2-yl- or 5-(aminomethyl)-2,5-dihydrofuran-2-yl]imidazoles by efficient use of a PHSe group: Application to novel histamine H3-ligands. *J. Org. Chem. 64*, 8608–8615.

(13) Imbach, J. L., Barascut, J. L., Kam, B. L., Rayner, B., Tamby, C., and Tapiero, C. (1973) Synthesis of nucleosides. III. New criterion determination of the anomer configuration of ribosidic nucleosides. *J. Heterocycl. Chem. 10* (6), 1069–70.

(14) Bakthavachalam, V., Lin, L. G., Cherian, X. M., and Czarnik, A. W. (1987) Synthesis, stereochemistry, intramolecular cyclization, and rates of hydrolysis of adenosine 2′,3′-acetals. *Carbohydr. Res. 170*, 124–135.

(15) Clark, V. M., Todd, A. R., and Zussman, J. (1951) Nucleotides. Part VIII cycloNucleoside salts. A novel rearrangement of some toluene-p-sulphonylnucleosides. *J. Chem. Soc.* 2952–2958.

(16) Geall, A. J., and Blasgbrough, I. S. (2000) Rapid and sensitive ethidium bromide fluorescence quenching assay of polyamine conjugate-DNA interactions for the analysis of lipoplex formation in gene therapy. *J. Pharm. Biomed. Anal. 22*, 849–859.

(17) Karmali, P. P., and Chaudhuri, A. (2006) Cationic liposomes as non-viral carriers of gene medicines: resolved issues, open questions, and future promises. *Med. Chem. Res. 27* (5), 696–722.

(18) Alberle, A. M., Bennet, M. J., Malone, R. W., and Nantz, M. H. (1996) The counterion influence of cationic lipid-mediated transfection of plasmid DNA. *Biochim. Biophys. Acta 1299*, 281–283.

BC100005K