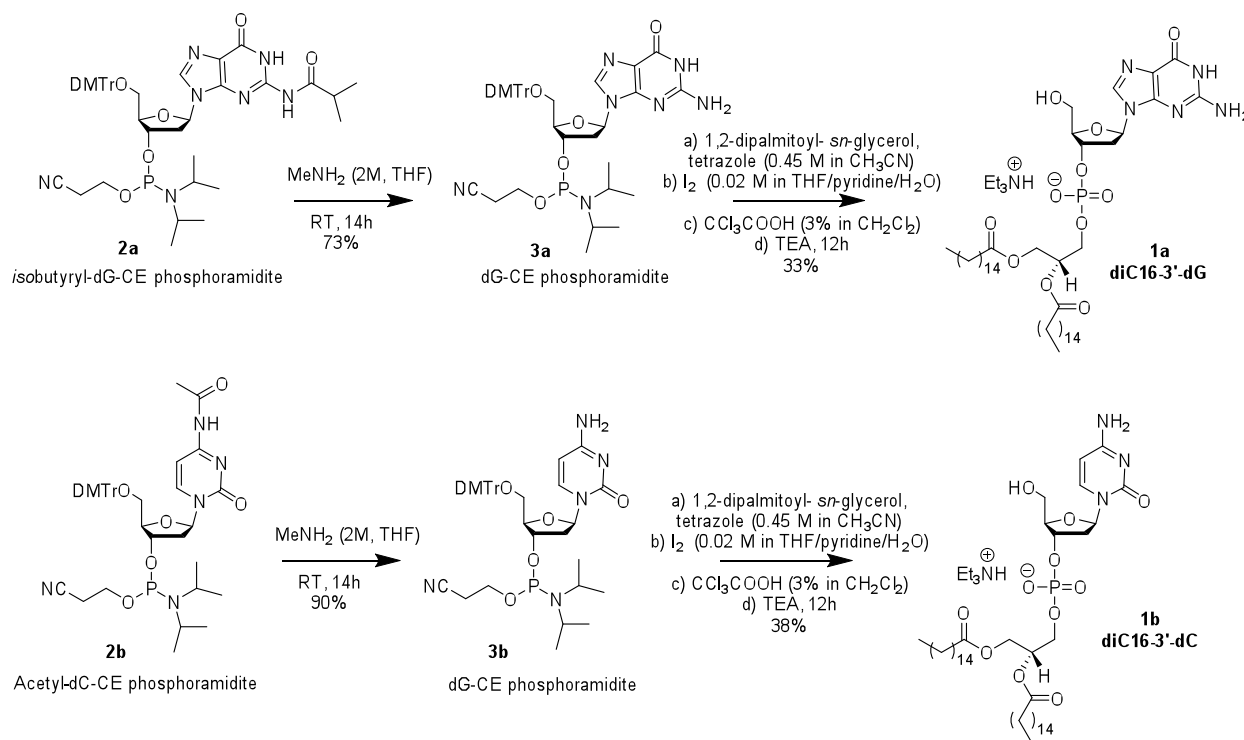


Cytidine- and guanosine-based nucleotide-lipids

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Supporting Information

I. Synthesis



Scheme S1: Synthesis of nucleotide-lipids diC16-3'-dG **1a** and diC16-3'-dC **1b**.

Synthesis of triethylammonium (2*R*,3*S*,5*R*)-5-(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)-2-(hydroxymethyl)tetrahydrofuran-3-yl ((*R*)-2,3-bis(palmitoyloxy)propyl) phosphate (diC16-3'-dG)

1a:

Step 1 (deprotection of isobutyryl group):

(2*R*,3*S*,5*R*)-2-((bis (4-methoxyphenyl)(phenyl)methoxy)methyl)-5-(2-isobutyramido-6-oxo-1,6-dihydro-9*H*-purin-9-yl)tetrahydrofuran-3-yl (2-cyanoethyl) diisopropylphosphoramidite (isobutyryl-dG-CE phosphoramidite) **2a** (2 g, 2.38 mmol, 1 equiv.) was dissolved in MeNH₂ (2 M in THF, 23.81 mL, 47.6

mmol, 20 equiv.) at room temperature under argon. After stirring for 14 h, reaction went to completion (TLC control), the reaction medium was evaporated under reduced pressure. The crude was purified by flash chromatography on silica gel with the following eluent: 1% MeOH/DCM (v/v) containing 1% TEA and then 3% MeOH/DCM (v/v) containing 1% TEA to provide (2*R*,3*S*,5*R*)-5-(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)tetrahydrofuran-3-yl (2-cyanoethyl) diisopropylphosphoramidite **3a** (1.33 g, 73 %). **3a** was submitted to next reaction step without further characterization.

Step 2 (coupling with lipid):

(2*R*,3*S*,5*R*)-5-(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)-2-((bis(4methoxyphenyl)(phenyl)methoxy)methyl)tetrahydrofuran-3-yl(2-cyanoethyl)diisopropylphosphoramidite **3a** (1.2 g, 1.64 mmol, 1 equiv.) and 1,2-dipalmitoyl-*sn*-glycerol (1.21 g, 2.14 mmol, 1.3 equiv.) were dissolved in anhydrous THF (25 mL). A tetrazole solution in acetonitrile (0.45 M, 6.2 mL, 2.8 mmol, 1.7 equiv.) was added, and the reaction mixture was stirred overnight at room temperature followed by oxidation step with I₂ solution (0.02 M in THF/Pyridine/H₂O, 140 mL, 2.8 mmol, 1.7 equiv.). After stirring 6 h at room temperature, the solvent was removed under high vacuum and the crude was dissolved in ethyl acetate (100 mL) and then washed with saturated Na₂S₂O₃ solution (2 x 25 mL). The organic layers were dried over anhydrous sodium sulfate, and the solvent was then evaporated. The residue was dissolved in DCM (20 mL) and trichloroacetic acid (TCA, 3% in DCM) solution (15 mL) was added under argon atmosphere. After stirring 4 h at room temperature, 5 mL of methanol and 25 mL of sodium hydrogen carbonate solution were added. The aqueous phase was extracted with DCM (2 x 30 mL), and then the organic fractions were collected, dried over anhydrous sodium sulfate, and the solvent evaporated. The crude was lastly dissolved in 33% triethylamine (TEA) in DCM (50 mL DCM + 25 mL TEA) and stirred overnight at room temperature (to ensure the complete deprotection of cyanoethyl chain). The solvent was then removed under high vacuum. The product **1a** was isolated as a white solid after purification on silica gel (DCM:methanol:TEA from 98:1:1 to 90:9:1) to provide the expected product **1a** (852 mg, 33 %).

$R_f = 0.3$ (DCM:methanol:TEA 90:9:1). ^1H NMR (300 MHz, CDCl_3): δ in ppm 0.85 (t, 6H, $J=6.5$ Hz, 2CH_3 of palmitoyl chain), 1.22 (s, 48H, 24CH_2 (palmitoyl chain)), 1.27 (t, 9H, $J=7.4$ Hz, 3CH_3 (triethylammonium)), 1.55 (bs, 4H, $2\text{CH}_2\text{-CH}_2\text{-CO}$), 2.00-2.34 (m, 5H, $2\text{CH}_2\text{-CO}$, H_2' (sugar)), 2.51 (bs, 1H, H_2'' (sugar)), 3.02 (q, $J=7.2$, 3CH_2 (triethylammonium)), 3.25-4.91 (m, 8H, 2CH_2 (glycerol), H_3' (sugar), H_4' (sugar), $2\text{H}_5'$ (sugar)), 5.21 (bs, 1H, $-\text{CH}-$ glycerol), 6.02 (d, 1H, $J=6.5$ Hz, H-5(base)), 6.12 (t, 1H, $J=5.3$ Hz, H_1'), 8.05 (bs, 1H, H-6 (base)). ^{13}C NMR (125 MHz, CDCl_3): δ ppm 7.7 (CH_3 , triethylammonium), 13.1, 13.2 (CH_3 palmitoyl chain), 21.7, 21.7 ($\text{CH}_2\text{-CH}_3$ palmitoyl chain), 23.8-24.1 (m, $\text{CH}_2\text{-CH}_2\text{-C=O}$), 28.3-28.8 (CH_2 palmitoyl chain), 30.9, 31.0 ($\text{CH}_2\text{-CH}_2\text{-CH}_3$ palmitoyl chain), 33.1-33.4 (m, $\text{CH}_2\text{-C=O}$ palmitoyl chain), 36.5 (m, C_2' -sugar), 44.9 (CH_2 triethylammonium), 61.7 ($\text{CH}_2\text{-O-C=O}$ glycerol), 61.8 ($\text{CH}_2\text{-O-P=O}$ glycerol), 62.5 (m, C_5' -sugar), 69.3 (CH, C_3' -sugar), 69.4 (CH glycerol), 85.7 (C, C_1' -sugar), 86.3 (CH, C_4' -sugar), 113.6 (CO-C=C , base), 138.4 (CH=N , base), 151.7 (NNC=C), 153.0 (Cguanidine, base), 159.3 (C=O , base), 172.1 (C=O palmitoyl chain), 172.4 (C=O palmitoyl chain).

^{13}C NMR being performed using a spectrometer with a cryoprobe, the presence of several signals for most of the peaks is certainly due to the presence of conformers. ^{31}P NMR (121 MHz, CDCl_3): δ in ppm 1.86. HRMS (ESI) A^- , theoretical $m/z=896.55192$, measured $m/z=896.5525$ (1 ppm).

Synthesis of triethylammonium (2*R*,3*S*,5*R*)-5-(4-amino-2-oxopyrimidin-1(2*H*)-yl)-2-(hydroxymethyl)tetrahydrofuran-3-yl ((*R*)-2,3-bis(tetradecanoyloxy)propyl) phosphate (diC16-3'-dC) 1b:

Step 1 (deprotection of isobutyryl group):

(2*R*,3*S*,5*R*)-5-(4-acetamido-2-oxopyrimidin-1(2*H*)-yl)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)tetrahydrofuran-3-yl(2-cyanoethyl) diisopropylphosphoramidite (Ac-dC-CE phosphoramidite) **2b** (2 g, 2.6 mmol, 1 equiv.) was dissolved in MeNH_2 (2 M in THF, 25.9 mL, 51.8 mmol, 20 equiv.) at room temperature under argon atmosphere and stirred during 2 h to reaction completion (TLC control). The reaction medium was then evaporated under reduced pressure. The crude was purified by flash chromatography on silica gel with the following eluent: 1% MeOH/DCM (v/v)

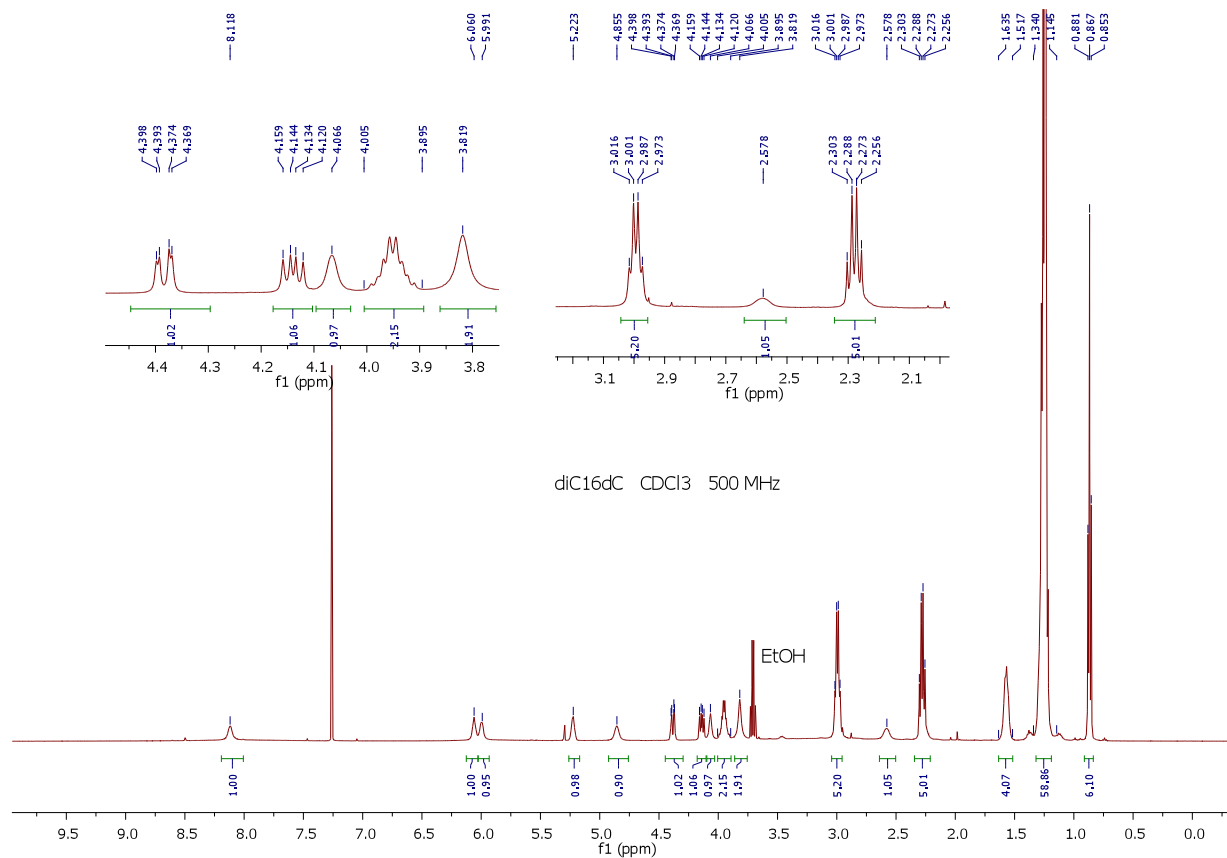
containing 1% TEA and then 3% MeOH/DCM (v/v) containing 1% TEA to provide (2*R*,3*S*,5*R*)-5-(4-amino-2-oxopyrimidin-1(2*H*)-yl)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)tetrahydrofuran-3-yl(2-cyanoethyl) diisopropylphosphoramidite **3b**. (1.68 g, 90 %). **3b** was submitted to next reaction step without further characterization.

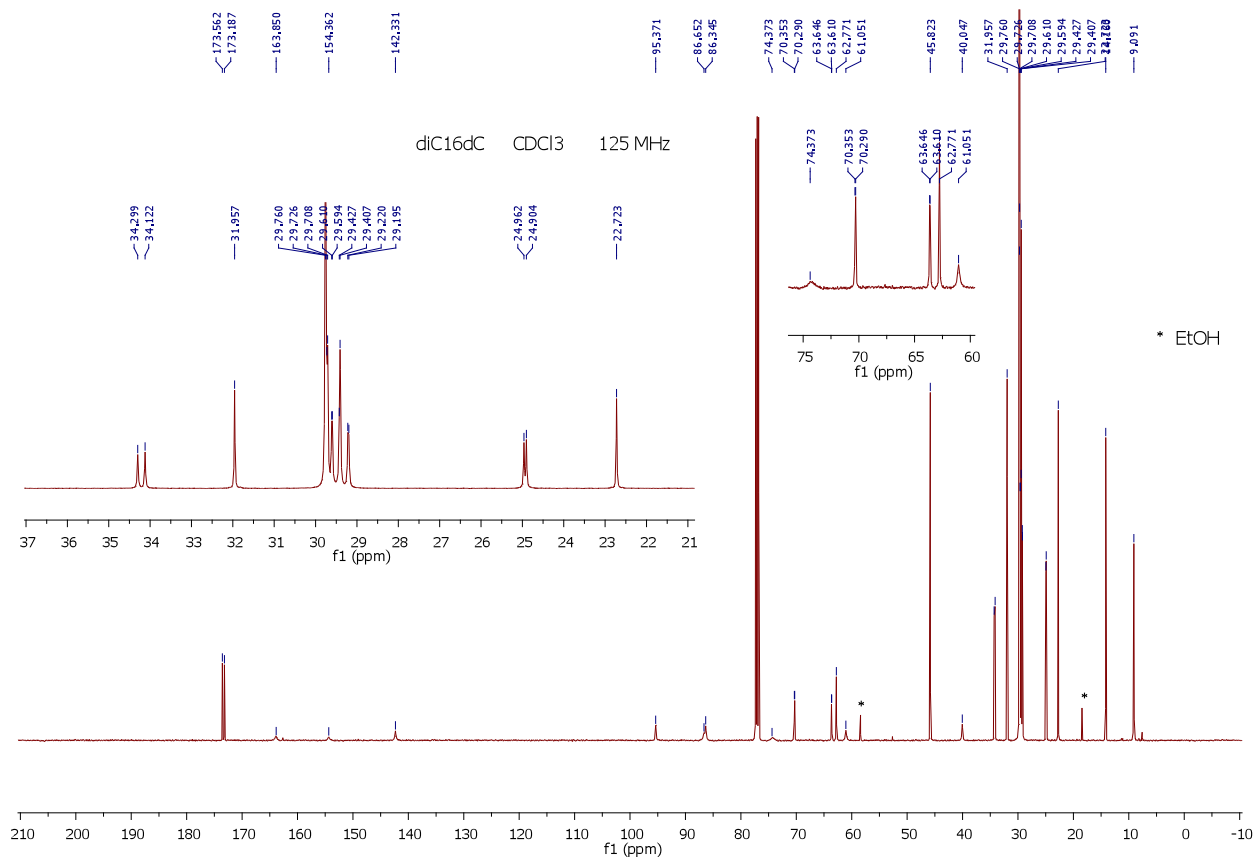
Step 2 (coupling with lipid): (2*R*,3*S*,5*R*)-5-(4-amino-2-oxopyrimidin-1(2*H*)-yl)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)tetrahydrofuran-3-yl(2-cyanoethyl) diisopropylphosphoramidite **3b** (product of step 1) (1.4 g, 1.81 mmol, 1 equiv.) and 1,2-dipalmitoyl-*sn*-glycerol (1.34 g, 2.36 mmol, 1.3 equiv.) were dissolved in anhydrous THF (25 mL). A tetrazole solution in acetonitrile (0.45 M, 6.9 mL, 3.1 mmol, 1.7 equiv.) was added, and the reaction mixture was stirred overnight at room temperature followed by oxidation step with I₂ solution (0.02 M in THF/Pyridine/H₂O, 154 mL, 3.1 mmol, 1.7 equiv.). After 6 h at room temperature, the solvent was evaporated under high vacuum and the intermediate products were dissolved in ethyl acetate (100 mL) and then washed with saturated Na₂S₂O₃ solution (2 x 25 mL). The organic layers were dried over anhydrous sodium sulfate, and the solvent was removed under vacuum. The resulting crude product was dissolved in DCM (20 mL) and trichloroacetic acid (TCA, 3% in DCM) solution (15 mL) under argon atmosphere, after 4 h at room temperature, 5 mL of methanol and 25 mL of sodium hydrogen carbonate solution were added. The aqueous phase was extracted with DCM (2 x 30 mL), and then the organic layers were collected, dried over anhydrous sodium sulfate, and the solvent evaporated. The residue was lastly dissolved in 33% trimethylamine (TEA) in DCM (50 mL DCM + 25 mL TEA) and stirred overnight at room temperature (to ensure the complete deprotection of cyanoethyl chain). The solvents were evaporated under high vacuum. The product **1b** (653 mg, 38%). was isolated as a white solid. (DCM:methanol:TEA from 98:1:1 to 90:9:1).

$R_f = 0.3$ (DCM:methanol:TEA 90:9:1). ¹H NMR (500 MHz, CDCl₃): δ ppm 0.86 (t, 6H, $J=7$ Hz, 2CH₃ of palmitoyl chain), 1.14-1.34 (m, 57H, 24CH₂ (palmitoyl chain) + 9H, 3CH₃ (triethylammonium)), 1.51-1.63 (m, 4H, 2CH₂-CH₂-CO), 2.28 (dd, 4H, $J=7.5, 15$ Hz, 2CH₂-CO + H2'(sugar)), 2.57 (brs, 1H, H2''(sugar)), 2.99 (q, 6H, $J=7.2$, 3CH₂ (triethylammonium)), 3.82 (brs, 2H, CH₂ (glycerol)), 3.89-4.00 (m, 2H, CH₂ (glycerol)), 4.06 (brs, 1H, H4'(sugar)), 4.13 (dd, 1H, $J=7.5, 12.5$ Hz, H5'(sugar)), 4.38 (dd, 1H,

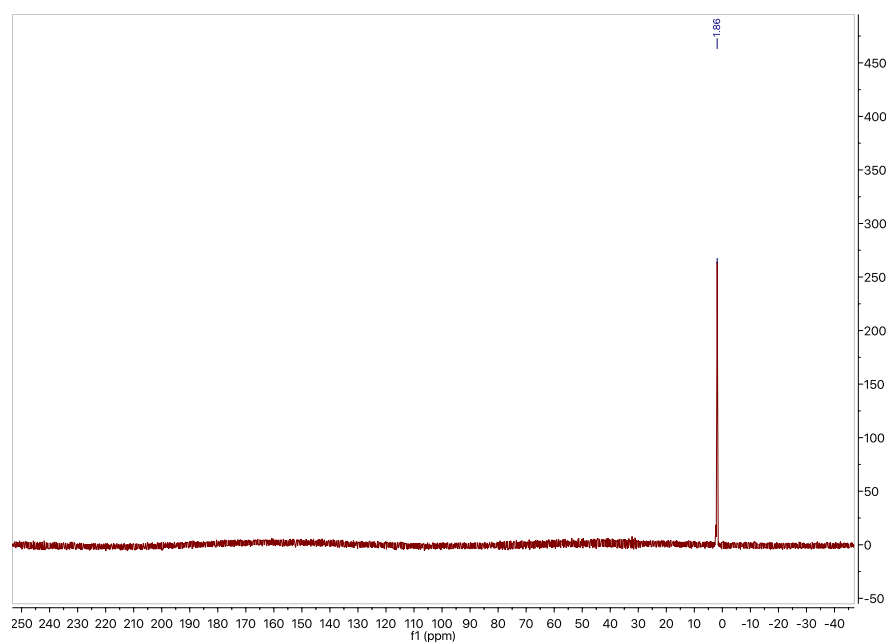
$J=2.5, 12$ Hz, H5'(sugar)), 4.85 (brs, 1H, H3' (sugar), 5.22 (brs, 1H, -CH- glycerol), 5.99 (brs, 1H, H-5(base)), 6.06 (brs, 1H, H1'), 8.11 (brs, 1H, H-6 (base)). ^{13}C NMR (125 MHz, CDCl_3): δ ppm 9.1 (CH_3 , triethylammonium), 14.2 (CH_3 palmitoyl chain), 22.7 (CH_2 palmitoyl chain), 24.9 ($\text{CH}_2\text{-CH}_2\text{C=O}$), 25.0 ($\text{CH}_2\text{-CH}_2\text{C=O}$), 29.2, 29.2, 29.4, 29.4, 29.6, 29.6, 29.7, 29.7, 29.8 (CH_2 palmitoyl chain), 32.0 ($\text{CH}_2\text{-CH}_2\text{-CH}_3$ chain), 34.1 ($\text{CH}_2\text{-C=O}$ chain), 34.3 ($\text{CH}_2\text{-C=O}$ chain), 40.0 (C2' -sugar), 45.8 (CH_2 triethylammonium), 61.1 ($\text{CH}_2\text{-O-C=O}$ glycerol), 62.8 (C5' -sugar), 63.6 (d, $J_{\text{C-P}}=18$ Hz, $\text{CH}_2\text{-O-P=O}$ glycerol), 70.3 (d, $J_{\text{C-P}}=31.5$ Hz, CH glycerol), 74.4 (brs, C3' -sugar), 86.3 (CH, C1' -sugar), 86.7 (CH, C4' -sugar), 95.4 (CH, C5 -base), 142.3 (CH, C6 -base), 154.4 (C=O , C2 -base), 163.9 (C, C4 -base), 173.2 (palmitoyl C=O chain), 173.6 (palmitoyl C=O chain). ^{31}P NMR (121 MHz, CDCl_3): δ in ppm 1.90. HRMS (ESI) MS [A], theoretical $m/z=856.54577$, measured $m/z=856.5459$ (0 ppm).

¹H NMR spectrum of triethylammonium (2R,3S,5R)-5-(4-amino-2-oxopyrimidin-1(2H)-yl)-2-(hydroxymethyl)tetrahydrofuran-3-yl ((R)-2,3-bis(tetradecanoyloxy)propyl) phosphate (diC16-3'-dC) **1b** at 500 MHz



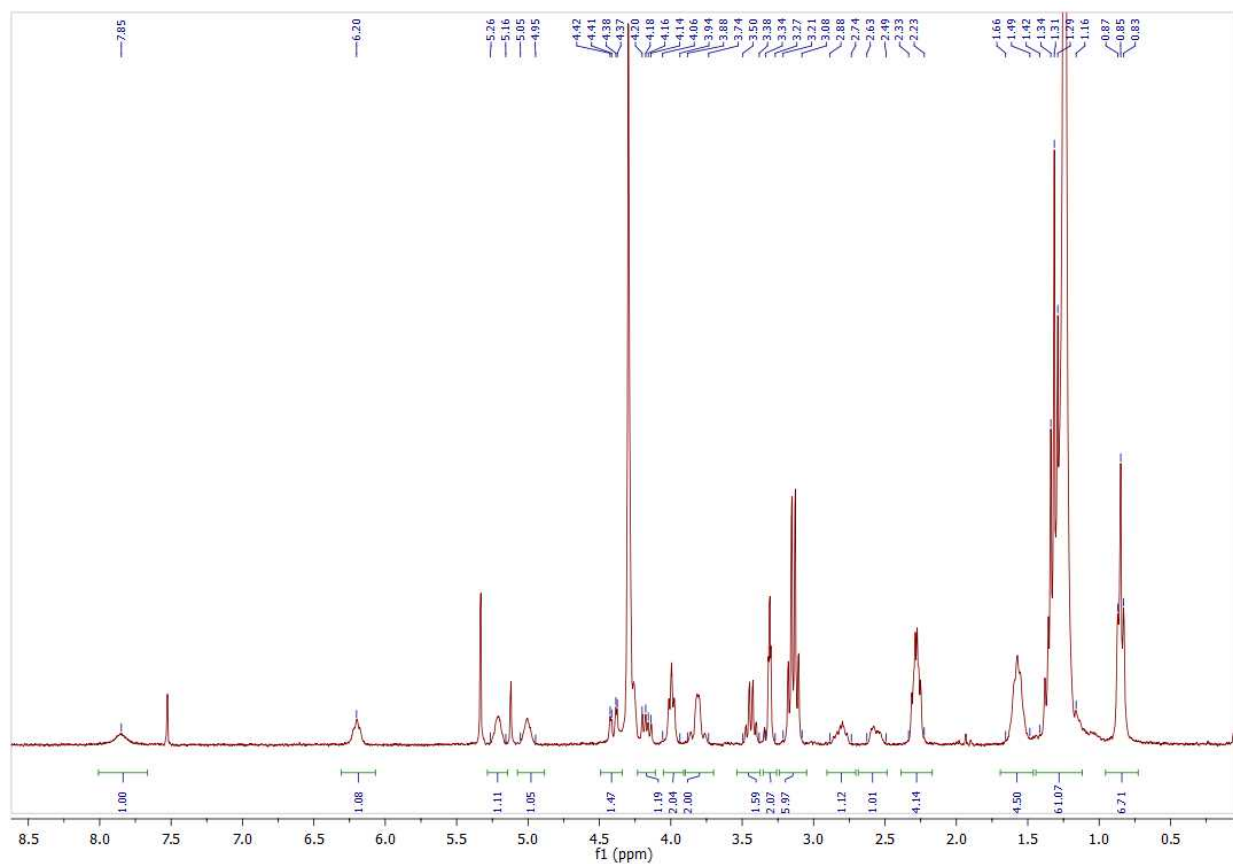


¹³C NMR spectrum of triethylammonium (2*R*,3*S*,5*R*)-5-(4-amino-2-oxopyrimidin-1(2*H*)-yl)-2-(hydroxymethyl)tetrahydrofuran-3-yl ((*R*)-2,3-bis(tetradecanoyloxy)propyl) phosphate (diC16-3'-dC) **1b** at 125MHz

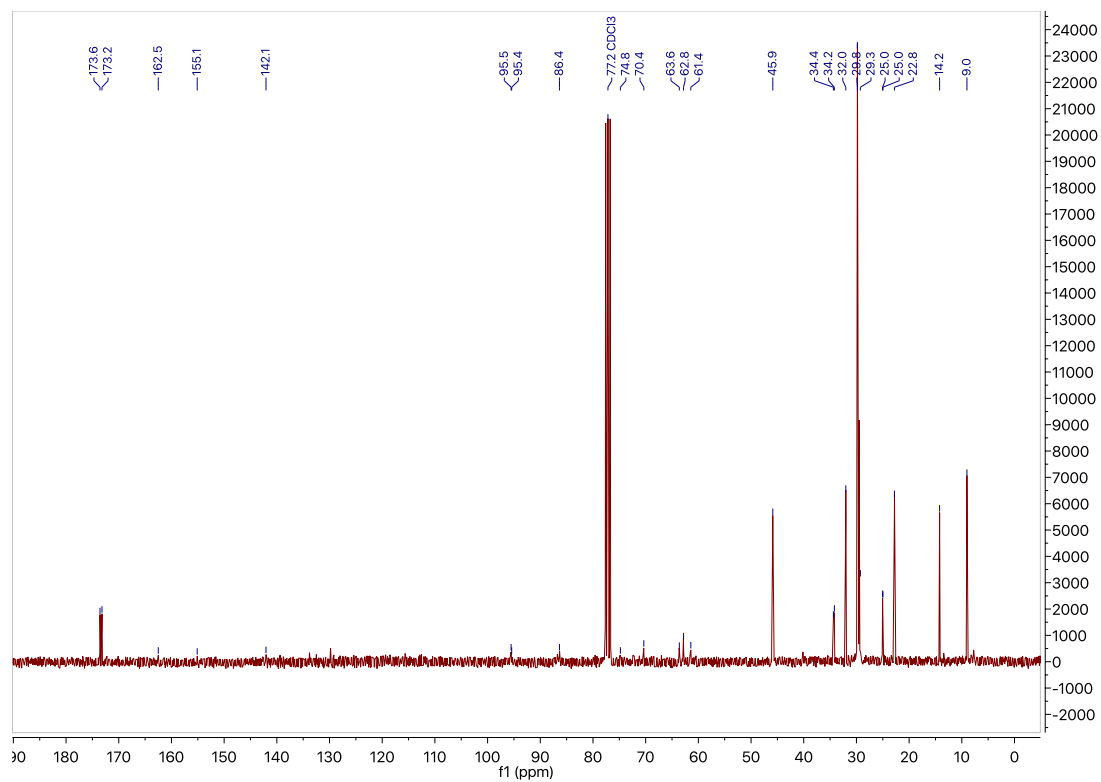


³¹P NMR spectrum of triethylammonium (2*R*,3*S*,5*R*)-5-(4-amino-2-oxopyrimidin-1(2*H*)-yl)-2-(hydroxymethyl)tetrahydrofuran-3-yl ((*R*)-2,3-bis(tetradecanoyloxy)propyl) phosphate (diC16-3'-dC) **1b** at 125MHz

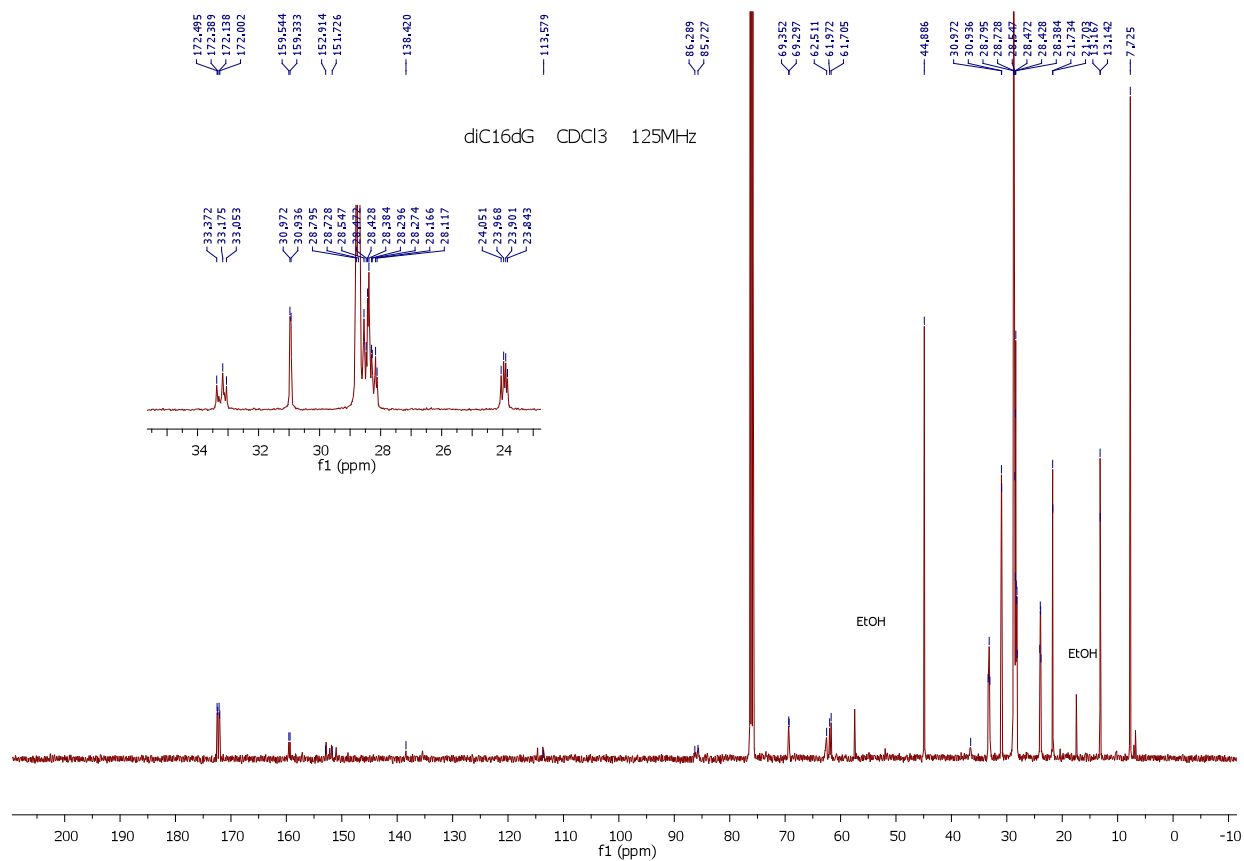
(hydroxymethyl)tetrahydrofuran-3-yl ((*R*)-2,3-bis(tetradecanoyloxy)propyl) phosphate (diC16-3'-dC) **1b**



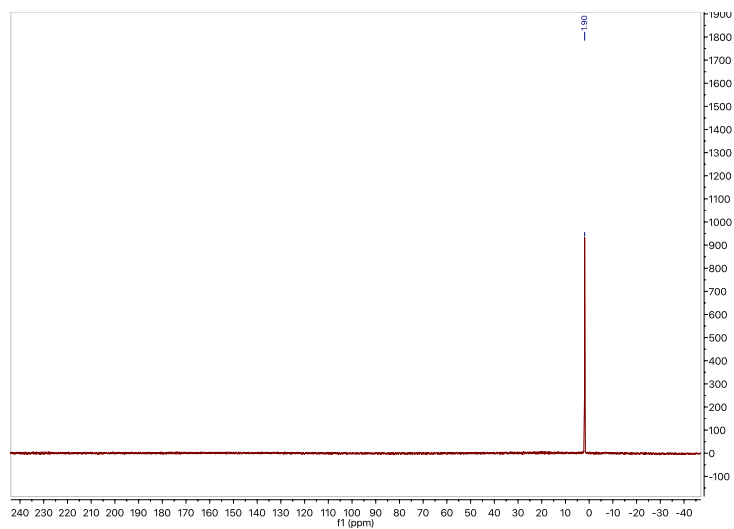
¹H NMR (CDCl₃/MeOD 1:1, 323K) spectrum of triethylammonium (2*R*,3*S*,5*R*)-5-(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)-2-(hydroxymethyl)tetrahydrofuran-3-yl ((*R*)-2,3-bis(palmitoyloxy)propyl) phosphate (diC16-3'-dG) **1a** at 300 MHz



^{13}C NMR spectrum of triethylammonium (2*R*,3*S*,5*R*)-5-(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)-2-(hydroxymethyl)tetrahydrofuran-3-yl ((*R*)-2,3-bis(palmitoyloxy)propyl) phosphate (diC16-3'-dG) **1a** at 75MHz



¹³C NMR spectrum of triethylammonium (2*R*,3*S*,5*R*)-5-(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)-2-(hydroxymethyl)tetrahydrofuran-3-yl ((*R*)-2,3-bis(palmitoyloxy)propyl) phosphate (diC16-3'-dG) **1a** at 125 MHz



³¹P NMR spectrum of triethylammonium (2*R*,3*S*,5*R*)-5-(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)-2-(hydroxymethyl)tetrahydrofuran-3-yl ((*R*)-2,3-bis(palmitoyloxy)propyl) phosphate (diC16-3'-dG) **1a**

III. Physico-Chemistry Experiments

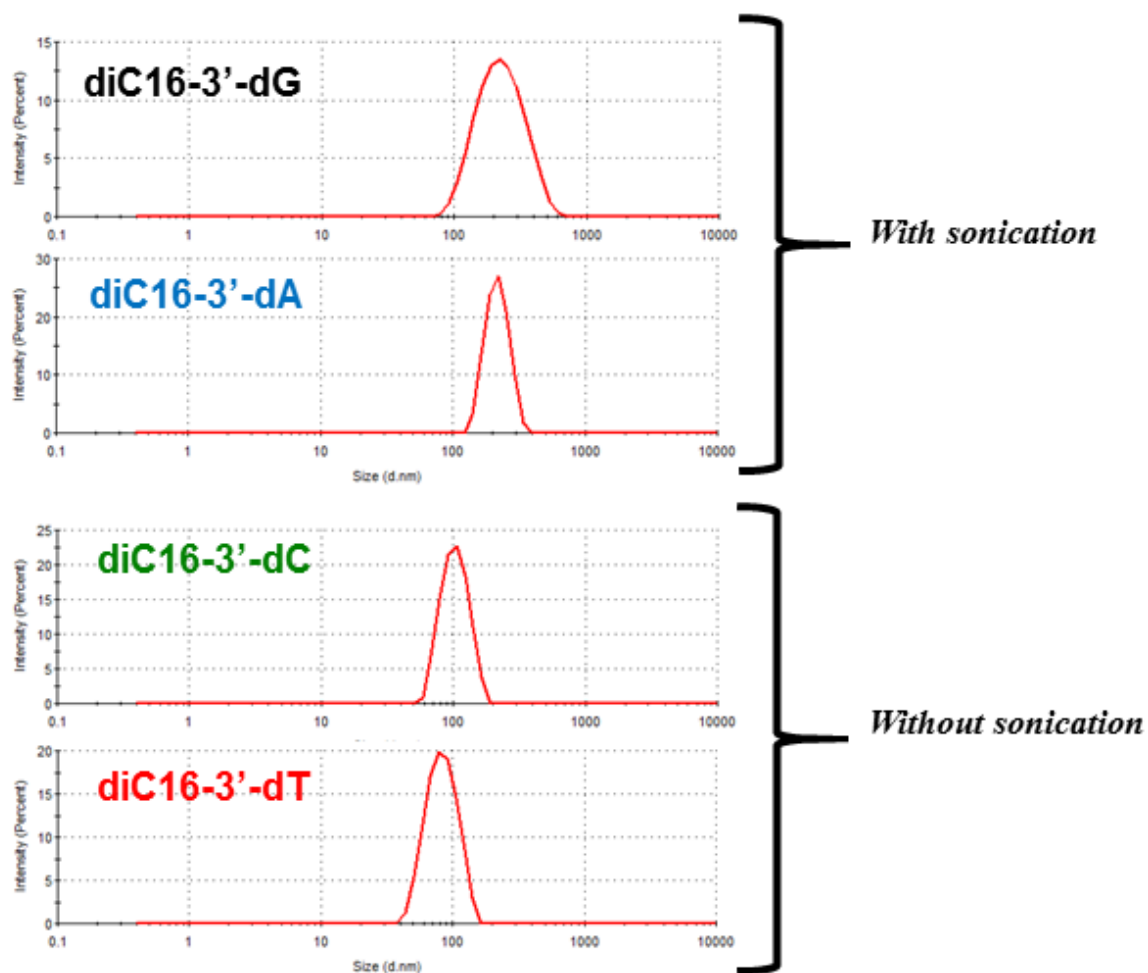


Figure S1: Intensity Size distribution of NLs at 100 μM by DLS