Design and synthesis of nucleolipids as possible activated precursors for oligomer formation via intramolecular catalysis: stability study and supramolecular organization

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Abstract

Background: Fatty acid vesicles are an important part of protocell models currently studied. As protocells can be considered as pre-biological precursors of cells, the models try to contribute to a better understanding of the (cellular) origin of life and emphasize on 2 major aspects: compartmentalization and replication. It has been demonstrated that lipid-based membranes are amenable to growth and division (shell replication). Furthermore compartmentalization creates a unique micro-environment in which biomolecules can accumulate and reactions can occur. Pioneering research by Sugawara, Deamer, Luisi, Szostak and Rasmussen gave more insight in obtaining autocatalytic, self-replicating vesicles capable of containing and reproducing nucleic acid sequences (core replication). Linking both core and shell replication is a challenging feat requiring thorough understanding of membrane dynamics and (auto)catalytic systems. A possible solution may lie in a class of compounds called nucleolipids, who combine a nucleoside, nucleotide or nucleobase with a lipophilic moiety. Early contributions by the group of Yanagawa mentions the prebiotic significance (as a primitive helical template) arising from the supramolecular organization of these compounds. Further contributions, exploring the supramolecular scope regarding phospholiponucleosides (e.g. S-diolelyphosphatidyl derivatives of adenosine, uridine and cytidine) can be accounted to Baglioni, Luisi and Berti. This emerging field of amphiphiles is being investigated for surface behavior, supramolecular assembly and even drug ability.

Results: A series of α/β-hydroxy fatty acids and α-amino fatty acids, covalently bound to nucleoside-5′-monophosphates via a hydroxyl or amino group on the fatty acid was examined for spontaneous self-assembly in spherical aggregates and their stability towards intramolecular cleavage. Staining the resulting hydrophobic aggregates with BODIPY-dyes followed by fluorescent microscopy gave several distinct images of vesicles varying from small, isolated spheres to higher order aggregates and large, micromicron sized particles. Other observations include rod-like vesicle precursors. NMR was used to assess the stability of a representative sample of nucleolipids. 1D 31P NMR revealed that β-hydroxy fatty acids containing nucleotides were pH-stable while the α-analogs are acid labile. Degradation products identified by [1H-31P] heteroTOCSY revealed that phosphoesters are cleaved between sugar and phosphate, while phosphoramidates are also cleaved at the lipid-phosphate bond. For the latter compounds, the ratio between both degradation pathways is influenced by the nucleobase moiety. However no oligomerization of nucleotides was observed; nor the formation of 3′-5′-cyclic nucleotides, possible intermediates for oligonucleotide synthesis.

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**Conclusions:** The nucleolipids with a deoxyribose sugar moiety form small or large vesicles, rod-like structures, vesicle aggregates or large vesicles. Some of these aggregates can be considered as intermediate forms in vesicle formation or division. However, we could not observe nucleotide polymerization or cyclic nucleotide function of these nucleolipids, regardless of the sugar moiety that is investigated (deoxyribose, ribose, xylose). To unravel this observation, the chemical stability of the constructs was studied. While the nucleolipids containing β-hydroxy fatty acids are stable as well in base as in acid circumstances, others degraded in acidic conditions. Phosphoramidate nucleolipids hydrolyzed by P-N as well as P-O bond cleavage where the ratio between both pathways depends on the nucleobase. Diester constructs with an α-hydroxy stearic acid degraded exclusively by hydrolysis of the 5’-O-nucleoside ester bond. As the compounds are too stable and harsh conditions would destroy the material itself, more reactive species such as lipid imidazolates of nucleotides need to be synthesized to further analyze the potential polymerization process.

**Keywords:** Nucleolipid, Vesicles, Hydroxy fatty acids, Protocell, Chemical stability, Supramolecular assembly, Intramolecular catalysis, Fluorescence microscopy, BODIPY, NMR stability study

**Background**

Fatty acid vesicles are an important part of protocell models currently studied [1,2]. As protocells can be considered as pre-biological precursors of cells [3], the models try to contribute to a better understanding of the (cellular) origin of life and emphasize on 2 major aspects: compartmentalization and replication [2,4-6]. It has been demonstrated that lipid-based membranes are amenable to growth and division [1,7]. Small unilamellar vesicles divide after micelle addition [8]. Autocatalytic self-replicating micelles are formed from amphiphiles generated from the alkaline hydrolysis of ethyl caprylate (shell replication) [4]. Furthermore compartmentalization creates a unique micro-environment in which biomolecules can accumulate [9,10] and reactions can occur [11]. Pioneering research by Sugawara [12], Deamer [13], Luisi [4], Szostak [7,14,15] and Rasmussen [16] gave more insight in obtaining autocatalytic, self-replicating vesicles capable of containing and reproducing nucleic acid sequences (core replication).

Linking both core and shell replication is a challenging feat requiring thorough understanding of membrane dynamics [7] and (auto)catalytic systems [4,17]. A possible solution may lie in a class of compounds called nucleolipids, who combine a nucleoside, nucleotide or nucleobase with a lipophilic moiety. Early contributions by the group of Yanagawa [18] mentions the prebiotic significance (as a primitive helical template) arising from the supramolecular organization of these compounds. Further contributions, exploring the supramolecular scope regarding phospholiponucleosides (e.g. 5′-diolylphosphatidyl derivatives of adenosine, uridine and cytidine) can be accounted to Baglioni, Luisi and Berti. This emerging field of amphiphiles is being investigated for surface behavior, supramolecular assembly and even drug ability [19,20]. Besides improving permeability, modifying medically active nucleosides or nucleotides with long alkyl chains has proven (also by our group) to be an adequate prodrug tactic [10,21].

Now we designed a series of nucleolipids as possible activated precursors for obtaining oligonucleotides. Besides its role as supramolecular recognition element ensuring the vicinity of the nucleophilic 2′- and 3′-hydroxyl groups and the electrophilic (activated) phosphate, it is necessary that the lipid part of the conjugate is also a good leaving group. This may be achieved by intramolecular catalysis; as we have recently demonstrated that a carboxylic acid functionality introduced in α-position of a phosphoramidate or phosphodiester group may help in catalyzing the cleavage of the phosphoramidate or phosphodiester bonds. This occurs by means of a cyclic intermediate that forms under (mild) acidic conditions (Scheme 1b). One must also consider the competing acidic hydrolysis (Scheme 1a) and cleavage of the ester bond between nucleoside and phosphate (not depicted). Previous results have shown that the cleavage ratio of Nucleoside-O-P and P-X-leaving group depends on the nature of the latter bond, the leaving group (with or without nearby carboxyl group), nucleobase and pH (a more detailed discussion of these factors can be found in the work of Maiti et al [22]).

Depending on the cleaved bond, this might lead to oligonucleotide formation due to the leaving group properties of the lipid moiety or through the formation of cyclic nucleotides, (e.g. 3′-5′ cyclic GMP) which are able to polymerize in water to give short RNA fragments [23]. As the properties of the phospholipids with and without nucleoside are different, the potential of obtaining a dynamic system is present.

Here, we have investigated the potential of α/β-hydroxy fatty acids and α-amino fatty acids, covalently bound to nucleoside-5′-monophosphates via a hydroxyl or an amino group on the fatty acid (Figure 1) to spontaneous self-assemble in spherical aggregates. Their stability towards intramolecular cleavage was examined and thus their
ability to function as (activated) monomer for oligonucleotide synthesis was assessed.

Results and discussion

Chemistry

Several types of phospholipid conjugates of nucleotides were synthesized as represented by structure A and B (Figure 1). A stearic acid scaffold should provide an optimal balance between membrane fluidity and sufficient permeability [2]. A nucleoside moiety consists of either a (deoxy)ribofuranose or a xylofuranose linked to thymine or adenine; thus creating amphiphiles with large, polar head groups.

The (−)-α-hydroxy stearic acid 3 is prepared in two steps from stearic acid 1 by bromination [24] using Hell-Volhard-Zelinsky condition giving compound 2 followed by hydrolysis in 88% overall yield (Scheme 2). The (−)-β-hydroxy stearic acid 8 is synthesized by the procedure described by Masamune [25,26], which involves homologation of palmitic acid 5 [27] using in situ generated magnesium monomethylmalonate to a preformed acyl imidazole to produce the β-keto stearic acid methyl ester, which is reduced [28] with sodium borohydride in ethanol providing 7. Saponification with 1 N NaOH resulted in the formation of (−)-β-hydroxy stearic acid 8 in 77% overall yield (Scheme 3).

The benzyl esters of α-hydroxy stearate 4 was synthesized [29] by heating α-hydroxystearic acid 3, with benzyl bromide in presence of triethylamine, and catalytic amount of TBAI in toluene for 12 h in a yield of 56%. The benzyl ester of β-hydroxy stearate 9 is prepared in a similar way.

The phosphoramidite approach is used for the synthesis of the lipid-nucleotide conjugates. The phosphoramidites of (−)-α 12 and (−)-β-hydroxy benzyl stearate 13 (Scheme 4) were prepared by reaction of 4 and 9, respectively, with the phosphoramidite reagent 11 (Scheme 4), which was obtained by reaction of bis(diisopropylamino)chlorophosphine [30] 10 with benzyl alcohol.

The protected 2′,3′-O-protected xylofuranose derivative 27 with an adenine base moiety (Scheme 6) was prepared, starting from commercially available diacetone-D-glucose 17.

Benzoylation of 17 was carried out using sodium hydride and benzyl bromide in dry dimethylformamide.

Figure 1 General structure of nucleolipids studied in this manuscript: a: α-hydroxystearic acid (X = O), α-aminostearic acid (X = NH) and b: β-hydroxystearic acid.
Scheme 2 Synthesis of (−)-α-hydroxy stearate. a) PBr₃, Br₂, 95°C, 6 h; b) 2 M NaOH, 85°C, 2 h; c) Et₃N, BnBr, TBAI, toluene, reflux 12 h.

Scheme 3 Synthesis of (−)-β-hydroxy stearate. a) 1,1′-carbonyldiimidazole, magnesium methylmalonate, THF, rt 24 h; b) NaBH₄, EtOH, rt, 15 min; c) 1 M NaOH, 15 min rt; d) Et₃N, BnBr, TBAI, toluene, reflux 12 h.

Scheme 4 Synthesis of the phosphoramidite reagents. a) BnOH, Et₃N, Et₂O; b) 4, dry DCM, 1H-Tetrazole at 0°C, 15 min at rt; c) 9, dry DCM, 1H-Tetrazole at 0°C, 15 min at rt.
Selective hydrolysis of the terminal isopropylidene group followed by oxidation with sodium periodate, and borohydride reduction, afforded the 1,2-acetonide 20, in 88% yield over three steps. After benzoylation of 20 [33,34] and treatment of 21 with Ac₂O/H₂SO₄, a mixture of the anomers of 1,2-di-O-acetyl-3-O-benzyl-5-O-benzoyl-D- xylofuranose 22 was obtained. Condensation of 22 with N⁶-benzoyladenine [35,36], provided the desired β-anomer 23 in 86% yield.

Deprotection of 23 was carried out by using saturated methanolic ammonia to give the nucleoside 24 in 68% yield. The selective silylation of the 5'-hydroxyl group of compound 24 was carried out with TBDMSI, imidazole in dry DMF to obtain nucleoside 25. The 2'-hydroxyl group of compound 25 was protected with a carbobenzyloxy group. Finally, the 5'-O-TBDMS group is removed with 1 M TBAF in dry THF to obtain compound 27.

The synthesis of the phosphotriesters was accomplished in a one-pot method by reacting 12 and 13 with the protected nucleosides 14, 15, 16 and 27 in the presence of 1H-tetrazole followed by oxidation with hydrogen peroxide at -78°C, resulting in the phosphotriesters 29, 32, 35, 38, 41, 44, 46, and 48 (Scheme 7).

Deprotection of the benzyl and the carbobenzyloxy group of phosphotriesters 32 and 41 was performed by...
hydrogenolysis in the presence of Pd/C in THF/MeOH. Hydrogenolysis in THF/H2O as solvent in the presence of K2CO3, was carried out for the deprotection of the phosphotriesters of the adenine derivatives (29, 35, 38, 44, 46, and 48) to obtain phosphodiesters 30, 36, 39, 45, 47, 49.

α-(?)-Aminostearic acid 50 was synthesized based on the method reported by Toth [37] and Porter [38], starting from 1-bromohexadecane and diethylacetamido malonate. The crude material was directly used for esterification to obtain compound 50 (Schemes 8 and 9).

The synthetic protocol, used for the synthesis of the phosphoramidates of dAMP 51 and dTMP 53 was based on literature prescription [39,40] using dicyclohexylcarbodiimide (DCC) as coupling agent for the conjugation of nucleotides and amino acid. The phosphoramidates 51, 53 were obtained by refluxing the nucleoside monophosphates and the amino acid methyl ester in t-BuOH and water (5:1) in the presence of DCC (dicyclohexylcarbodiimide). Deprotection of methyl ester was performed using 0.5 N NaOH in MeOH/H2O-5:1 at room temperature for 4 h result in the phosphoramidates 52 and 54.

The self-assembling properties of these nucleolipids were analyzed by fluorescent microscopy using water soluble, and organic soluble (chloroform) fluorescent dyes 55 and 56 respectively (Figure 2). Synthesis of the new, water-soluble BODIPY dye 55 was carried out by conjugating 8-S-Methyl BODIPY [41] with taurine in presence of sodium hydrogen carbonate in DMSO:DCM (1:1) at room temperature. BODIPY 56 was prepared according to the procedure previously reported by Dehaen [42,43] at rt.

The nucleolipids, which have been analyzed, consist of an α-hydroxy fatty acid (30, 33, 36, 47), a β-hydroxy fatty acid (39, 42, 45, 49) or a α-amino fatty acid (52, 54). The polar head group is a nucleoside monophosphate (dAMP or dTMP) connected to the lipid by a phosphodiester bond (30, 33, 36, 39, 42, 45, 47, 49) or by a phosphoramidate bond (52, 54). The sugar is either a deoxyribose (30, 33, 39, 42, 52, 54), or a ribofuranose (36, 45) or a
Scheme 8 Synthesis of xylose nucleolipids. a) 12, dry DCM, 1H-tetrazole, H$_2$O$_2$, rt, 4 h.; b) 13, dry DCM, 1H-tetrazole, H$_2$O$_2$, rt, 4 h.; c) H$_2$, Pd/C, THF, rt, 48 h. Compounds 47 and 48 were isolated as 2Na$^+$ salt.

Scheme 9 Synthesis of the phosphoramidate nucleolipids. a) dAMP, DCC, t-BuOH, H$_2$O, reflux, 2 h; b) TMP, DCC, t-BuOH, H$_2$O, reflux, 2 h; c) 0.5 N NaOH, MeOH, rt. Compounds 52 and 54 were isolated as 2Na$^+$ salt.
xylofuranose (47, 49). The reason for this selection is that we would like to evaluate a) if the sugar moiety may influence the self-aggregation process, b) if oligomerization may lead to DNA and/or RNA sequences, c) if it would be possible to form 3′-O, 5′-O-cyclic phosphates in solution, d) if the properties of the leaving group may influence oligomerization or cyclic phosphate formation. For example, an α-hydroxy acid may lead to a 5-membered intermediate and a β-hydroxy acid to a 6-membered intermediate during activation of the phosphodiester bond (Scheme 1). A phosphoramidate may be activated as leaving group by acidification of the medium. A xylofuranose

![Figure 2 Structures of fluorescent dyes used for the visualization of vesicles.](image)

**Figure 2** Structures of fluorescent dyes used for the visualization of vesicles.

![Figure 3 Representative fluorescent images of compound 30 with dye 55.](image)

**Figure 3** Representative fluorescent images of compound 30 with dye 55. Final concentrations of products for 3a-b, d = 1.5 mM 30, 5.7 mM 55, 0.15 mM HCl, 7% DMSO in H2O; for 3c = 1.1 mM 30, 4.4 mM 55, 0.11 mM HCl, 5% DMSO in H2O. (Scale bar 10 μm).
Figure 4 Fluorescent microscopic images of the compound 33 with dye 56. Final concentrations of products for 4a-b = 0.4 mM 33, 0.013 mM 56 in H2O/dioxane/THF (3: 47:50); for 4c-d = 0.8 mM 33, 0.025 mM 56, 6% THF in H2O. (Scale bar 10 μm).

Figure 5 Fluorescent microscopic images of the compound 42 with dye 56. Final concentrations of products for 5a-c = 0.3 mM 42, 0.009 mM 56, 5% THF in H2O. (Scale bar 10 μm).

Figure 6 Fluorescent microscopic images of the compound 52 with dye 56. Final concentrations of products for 6a-b = 0.39 mM 52, 0.013 mM 56 in H2O/dioxane/THF (3: 47:50); for 6c = 0.66 mM 52, 0.022 mM 56, 5% THF in H2O. (Scale bar 10 μm).

Figure 7 Fluorescent microscopic images of the compound 54 with dye 56. Final concentrations of products for 7a-c = 0.3 mM 54, 0.009 mM 56, 2% THF in H2O. (Scale bar 10 μm).
may lead easier to cyclic nucleoside formation than a ribofuranose. The availability of as well A as T nucleolipids would allow us to study mixed vesicles, in which aggregation may be influenced by base pairing.

For studying the aggregation of the compounds, following 2'-deoxynucleolipids have been used: 30, 33, 39, 42, 52, and 54. The fluorescent Bodipy dyes (55, 56) were used to monitor self-assembly of the nucleolipids by visualization under fluorescent microscopy using a spin coat method on the surface of a microscopic glass plate. Vesicle formation of nucleolipids in water was facilitated by adding small amounts of organic solvents to solubilize respectively the dye (THF) or the nucleolipid (DMSO). Soon after dissolving the nucleolipid by vortexing, a structural transition towards thermodynamically more stable spherical structures is observed. Vesicular aggregations are formed ranging from about a few to 10 μm (large vesicles), depending on the dilution and solvent conditions. Also irregular (small and large tubular structures) aggregates are formed in some cases. A series of representative examples (most frequently occurring aggregates) for the phospholipids 30, 33, 42, 52, 54 are shown.

Figure 3 shows microphotographs of compound 30 in H2O using dye 55 for visualization. Single vesicles are formed (Figure 3a) as well as several vesicle aggregates in which three (Figure 3b) or four (Figure 3c, d) water compartments are present. This could be intermediate stages in vesicle association or vesicle dissociation. Some of them (Figure 3d) are similar in morphology to the thread-like vesicles [7], which are formed before division in daughter vesicles. Figure 4 gives the images of compound 33 using dye 56. Here, single, spherical vesicles are formed using water-miscible organic solvents (Figure 4a, b), in water vesicles tend to associate (Figure 4c, d).

Figure 5 is representative for the images observed using compound 42 and dye 56. As well the vesicles (5a, b, c) are observed as rod-like structures (5a, c) which may be precursor structures for vesicle formation. Finally, the aggregates formed by the phosphoramidate conjugates with an adenine (52) and thymine (54) base moiety were visualized using dye 56. The pictures of 52 (Figure 6 in THF/dioxane) and 54 (Figure 7b, c in water) shows the start of the formation of vesicle colonies [44].

Figure 8 NMR study of 30 in acid conditions (pH-5.22 in DMSO). (a) time dependent degradation in acidic conditions monitored by 1D 31P NMR spectra. (b) degradation reaction of compound 30 in acidic conditions based on characterization of hydrolysis products by NMR. Arrows indicate cross peaks observed in the 2D spectrum depicted in C. (c) 1H-31P-heteroTOCSY after 1 day in acidic conditions.
Figure 9 NMR study of 52. (a) time dependent degradation in D$_2$O. (b) HP-heteroTOCSY after 1 day in D$_2$O. (c) HP-heteroTOCSY after 1 day in DMSO. (d) degradation reaction of nucleolipid 52 in acidic conditions. Arrows indicate cross peaks observed in the 2D spectra depicted in (b) and (c).
For studying the potential of the nucleolipids to di- (poly)merize and/or to form cyclic nucleotides, compounds with a deoxyribose (30, 33, 39, 42, 52 and 54), a ribose (36, 45) or a xyllose (47, 49) sugar moiety were envisaged. Both basic and acid circumstances were considered. Oligomerization could occur by an intermolecular reaction in which the 2'-OH or 3'-OH group of the sugar moiety attacks the 5'-O-phosphoester function, using the hydroxy (amino) lipid as leaving group. The carboxylate group in the α- or β-position may catalyze this reaction. Alternatively, a 3'-O, 5'-O-cyclic nucleotide may be formed (by intramolecular reaction) which could oligomerize in solution. During the synthesis of the compounds, we already observed that some of them are not stable in acidic medium. Therefore, all compounds were treated in acid and in base medium (pH 4 and pH 12) for a period of 48 h. However in none of the cases, polymerization products were detected using NMR spectroscopy. These negative results could be explained by the high chemical stability of the nucleolipids (only starting material present) or by hydrolysis of the compounds in acidic or basic medium. Therefore, we have evaluated this stability for representative examples (30, 33, 52, 54).

$^3$P NMR was used to study the stability of the nucleolipids in acidic (pH 4) and in base (pH 12) environment. One-dimensional $^3$P spectra were used as a fast screening experiment to monitor degradation of the conjugates. Two-dimensional $^1$H-$^3$P correlation spectra were used to characterize the $^3$P containing products formed by degradation of the nucleolipids. Correlations were established using a heteroTOCSY experiment with a DIPSI spinlock of 50 ms, allowing correlations of $^3$P resonances with several $^1$H resonances of adjacent spin systems.

The β-hydroxystearic acid containing nucleolipids (39, 42, 45, 49) are stable in both acidic and basic conditions with no difference in the $^3$P and $^1$H NMR signals over time at different pHs. All other compounds are stable in basic conditions (pH = 12 in D$_2$O) while degradation occurs in acidic conditions. For phosphate diesters, a gel is formed instantly upon lowering pH in water. Although (hydro)gelation is an interesting property and promising application of nucleolipids, this was not further investigated [45]. Due to hampered NMR measurements in aqueous conditions, sample degradation in acid medium was monitored in DMSO.

An example NMR study on an nucleolipid diester with α-hydroxy stearic acid is given in Figures 8 and 9 for compound 30. Original $^3$P signals appear in 1D $^3$P spectra between 0.1 and 0.0 ppm corresponding to Cα in R and S enantiomers in nucleolipid 30. Due to degradation in acidic conditions, a new $^3$P signal rises slightly downfield (0.4 ppm) while the original signals decrease. In a 2D-heteroTOCSY the original signals close to 0 ppm correlate with protons in the spin systems of the ribose ring (5'/5''/4') as well as the lipid (α, β, γ). The new signal at 0.4 ppm only correlates with protons of the lipid (α, β, γ), indicating that the covalent bond between lipid and phosphorus still exists after degradation. In acidic medium (pH 4-5), the thymidine congener 33 is degraded in the same way as the adenine congener 30, which shows that the cleavage mechanism is not dependent on the nucleobase (Figure 10).

Decomposition of the phosphoramidate 52 was first studied in aqueous, acidic conditions (Figure 10). We observed decrease of the original $^3$P signal (7 ppm) in D$_2$O while to signals rose at 2 and 1 ppm. The latter were assigned to dAMP and inorganic phosphate respectively using [$^1$H,$^3$P]-heteroTOCSY. Since the ratio of both new signals is constant over time, we suggest that initial cleavage occurs at both amide and ester bonds in the P linkage yielding dAMP and dA respectively. While dAMP is stable in the reaction medium, the phosphorylated lipid rapidly undergoes hydrolysis releasing inorganic phosphate. In DMSO, stability of the phosphorylated lipid is increased, making it observable as an intermediate in 1D and 2D NMR spectra ($^3$P signal at 8.8 ppm). The H8 signals from the nucleobases in 52, dAMP and dA are nicely resolved and allowed to determine a ratio of 1/4 for dAMP and dA formation: 20% of adenosine monophosphate and lipid are formed via pathway A and 80% of deoxyadenosine, inorganic phosphate and lipid are formed in pathway B. The thymine containing congener of 52 (54) also follows both degradation pathways in acidic conditions: 53% pathway A with formation of dTMP and lipid and 47% pathway B with formation of thymidine, inorganic phosphate and the lipid. Indicating that the nucleobase influences the ratio between P-O and P-N bond cleavage.

![Figure 10 Time dependent changes in 1D $^3$P NMR spectrum of nucleolipid 33 in DMSO at acidic conditions. Emergence of 2 degradation products coming from phosphate (1) and Lip-OPO$_3$ (2).](http://www.jsystchem.com/content/5/1/5)
To summarize, among all investigated systems, only \( \alpha \)-amino compounds have shown the desired, however nucleobase dependent, bond breakage upon acidifying: the only problem being that the nucleophile is water and not the 2 - or 3 - hydroxyl groups. The \( \beta \)-hydroxystearic acid containing nucleolipids are stable in both acidic and basic conditions. This difference between \( \alpha \) and \( \beta \) derivatives is analogues to previous calculations (done on a model in which the nucleoside had been replaced by a methyl group), showing that the formation of a six-membered intermediate by the attack of the \( \beta \)-carboxyl group is higher in energy (5 kcalmol\(^{-1}\)) than the five-membered ring formed by an \( \alpha \)-carboxyl group [22]. The proposed mechanism, predicting acidic instability, in Scheme 1b is supported by the fact that only amino derivatives cleaved at the desired bond, due to the preferred protonation of a phosphoramidate over a phosphodiester.

**Experimental**

**Benzy1 2-hydroxyoctadecanoate (4)**

To a solution of \( \alpha \)-hydroxy stearic acid 3 (10 g, 33.28 mmol), triethylamine (6.73 g, 9.25 ml, 66.56 mmol), TBAI (1.23 g, 3.33 mmol) in toluene (150 mL), benzyl bromide (5.7 g, 5.03 mL, 33.3 mmol) is added, and held at 90°C overnight. After the completion of the reaction, the reaction mixture is triturated with diethyl ether, the precipitate formed was washed with ether and dried to obtain the desired compound 4 as white solid (7.25 g, 55.7%). 1H NMR (300 MHz, CDCl\(_3\), ppm): \( \delta \) 7.37 (m, 5H, Ar), 5.25 (q, 2H, CH\(_2\)), 4.23 (m, 1H), 2.79 (br, 1H, -OH), 1.82-1.73 (m, 1H), 1.69 (m, 1H), 1.25 (s, 28H, -CH\(_2\)), 0.90 (t, -CH\(_3\)). 13C NMR (300 MHz, CDCl\(_3\): 172.50 (C = O), 135.37(-C-), 128.75 (-C-Ar), 128.65 (-C-Ar), 128.44 (-C-Ar), 70.64 (-C-H), 67.36 (Ar-CH\(_2\)), 34.52 (-CH\(_2\)), 32.05 (C, CH\(_2\)). HRMS (ESI+) m/z Calculated for C\(_{25}\)H\(_{43}\)O\(_3\) (MH+): 391.3206, found 391.3199.

**Benzyl, (benzyl stearate)-2-yl, N, N-diisopropyl phosphoramidite ([\( \beta \)]-12)**

To a stirring mixture of benzylxy bis(N, N-diisopropylamino)phosphine (10 g, 29.55 mmol) and alpha-hydroxy benzyl stearate (5.77 g, 14.77 mmol) in dry DCM (100 mL), was added 0.5 M solution of 1H-Tetrazole (29.6 mL, 14.77 mmol) in ACN dropwise at 0°C. After the addition, cooling bath was removed and the reaction mixture was allowed to stir at rt for 15 min. The reaction mixture was diluted with DCM (200 mL), and the organic layer was washed with 1 M TEAB solution. The extracts were dried over Na\(_2\)SO\(_4\) and concentrated by flash column chromatography using eluent 400:50:10-Hexane:EtOAc:TEA (R\(_f\)= 0.5) to obtain the phosphoramidite 12 (7.10 g, 71%). P\(^{31}\) NMR (CDCl\(_3\)): \( \delta \) 149.10, 148.69.

**Benzyl, (benzyl stearate)-3-yl, N, N-diisopropyl phosphoramidite ([\( \beta \)]-13)**

To a stirring mixture of benzyloxy bis(N, N-diisopropylamino)phosphate (11, 1.74 g, 5.12 mmol) and \( \beta \)-hydroxy stearic benzyl ester (9, 1 g, 2.56 mmol) in dry DCM (10 mL) was added 0.5 M solution of 1H-tetrazole (5.12 mL, 2.56 mmol) in ACN dropwise at 0°C. After the addition, cooling bath was removed and the reaction mixture was allowed to stir at room temperature for 15 min. The reaction mixture was diluted with DCM (150 mL), and was washed with 1 M TEAB (100 mL) solution. Dichloromethane layer was dried over Na\(_2\)SO\(_4\) and concentrated in vacuo, and the obtained oil was purified by silica gel flash column chromatography using the eluent 400:50:10-Hexane:EtOAc:TEA (R\(_f\)= 0.5) to get 13 as an pale yellow oil (0.985 g, 61%). P\(^{31}\) NMR (300 MHz, CDCl\(_3\), ppm): \( \delta \) 147.63, 147.07.

**3-O-benzyl-1,2,5,6-Di-O-isopropylidene-\( \alpha \)-D-glucofuranose (17)**

To a stirring solution of diacetone glucose (10 g, 38.42 mmol) in anhydrous DMF at 0°C, was added sodium hydride (60% in mineral oil (w/w), total 2.3 g, 25.14 mmol) portion wise. Stirring was continued for 1 h at 0°C, and then benzyl bromide (5.51 g, 46.10 mmol) was added drop wise. After addition, ice bath was removed, and stirring continued overnight at room temperature. After completion of the reaction, excess of sodium hydride
in the reaction mixture was quenched by the addition of ice cold water (25 mL). The reaction mixture was extracted with EtOAc (4 150 mL) and the combined organic phase was dried (Na2SO4) and concentrated under vacuum, and the residue was purified by silica gel column chromatography using eluents hexane:ethylacetate-8:2 to give the title compound 17 (13 g, 96%) as oil. 

1H NMR (300 MHz, CDCl3, ppm): 7.37-7.28 (m, 5H, Ar), 5.99 (d, 1H, 1′-H), 4.73 (dd, CH2-Ar), 4.64 (s, 1H, 4′-H), 4.30 (dd, 1H, 2′-H), 3.92 (d, 1H, 3′-H), 3.91-3.1 (m, 2H, 5′-H, 5′′-H), 2.24 (dd, 1H), 1.48 (s, 3H, CH3), 1.33 (s, 3H, CH3). 

13C NMR (300 MHz, CDCl3, ppm): δ 137.66, 128.33, 127.76, 127.58, 111.65, 108.86, 108.86, 105.27, 82.64, 81.68, 81.31, 72.50, 72.29, 67.35, 26.81, 26.75, 26.20, 25.40.

3-O-benzyl-1,2-O-isopropylidene-α-D-glucosanone (18) In a 250 mL round bottom flask 3-O-benzyl-1,2,5,6-di-O-isopropylidene-α-D-glucosanone (7.00 g, 19.97 mmol) was dissolved in 1:1 methanol/1% aqueous sulfuric acid (110 mL), and the resulting solution was stirred at rt. After completion of the reaction (approximately 5 h, monitored by TLC), the reaction mixture was quenched with triethylamine (pH7). The residue was concentrated in vacuo to afford the crude residue as syrup, which was purified by silica gel flash column chromatography using eluents hexane:ethylacetate (8:2) to obtain the compound 19 (110 mL) and Ac2O (15 mL) was added dropwise sulfuric acid (1.5 mL). Stirring was continued until consumption of starting material. The reaction mixture was diluted with diethyl ether. The organic solvents were dried on Na2SO4 and concentrated under reduced pressure. The residue is purified by silica gel column chromatography using eluents hexane:ethylacetate (8:2) to obtain the compound 20 (33.47, 4 g, 88.5%). 

1H NMR (300 MHz, CDCl3, ppm): δ 7.37-7.28 (m, 5H, Ar-H), 5.99 (d, 1H, 1′-H), 4.73 (dd, CH2-Ar), 4.64 (s, 1H, 4′-H), 4.30 (dd, 1H, 2′-H), 3.92 (d, 1H, 3′-H), 3.91-3.1 (m, 2H, 5′-H, 5′′-H), 2.24 (dd, 1H), 1.48 (s, 3H, CH3), 1.33 (s, 3H, CH3). 

13C NMR (300 MHz, CDCl3, ppm): δ 137.19 (-C-, Ar), 128.78(CH, Ar), 128.31 (CH, Ar), 111.90(C), 105.20(C-1′), 88.27 (C-4′), 86.20 (C-2′), 80.22 (C-3′), 72.04 (CH2-Ar), 61.09 (C-5′), 26.93 (CH3), 26.43(CH3). HRMS (ESI+) m/z Calculated for C15H20O5 [M + Na]+ : 303.1203, found 303.1207.

3-O-benzyl-1,2-O-isopropylidene-β-D-xylofuranosanone (21) To a suspension of 3-O-Benzyl-1,2-O-isopropylidene-β-D-xylofuranosanone (4 g, 14.26 mmol) in benzoyl chloride (1.64 g, 2 mL,17.12 mmol), was added sulfamic acid (0.55 g, 5.70 mmol). The reaction mixture was stirred at 60°C for 3 h, allowed to come to room temperature and the reaction mixture was poured into ice cold saturated NaHCO3 solution, and extracted with minimum amount of diethyl ether. The organic solvents were dried on Na2SO4 and concentrated under reduced pressure. The residue was purified by silica gel column chromatography using eluents hexane:ethylacetate (8:2) to obtain the compound 21 (36) (4.8 g, 91%). 

1H NMR (300 MHz, CDCl3, ppm): δ 8.01 (d, 2H), 7.54-7.51 (m, 1H), 7.40-7.37 (m, 2H), 7.30-7.26 (m, 4H), 7.24 (d, 1H), 5.99 (d, 1H), 4.70-4.60 (m, 3H), 3.67-0.48 (m, 3H), 4.04 (br s, 1H), 1.49 (s, 3H, CH3), 1.32 (s, 3H, CH3). 

13C NMR (300 MHz, CDCl3, ppm): δ 142.21, 137.16, 133.03, 129.88, 129.75, 128.53, 128.31, 128.07, 127.77, 111.82, 105.32, 82.13, 81.52, 78.14, 71.88, 26.83, 26.25.

(9-2′-O-acetyl-3′-O-benzyl-5′-O-benzoyl) β-D-xylofuranosyl) 6-N-benzoyladenine (23) To a cooled solution of 5-O-benzoyl-3-O-benzyl-1,2-O-isopropylidene-β-D-xylofuranosanone (4 g, 10.40 mmol) in AcOH (40 mL) and Ac2O (15 mL) was added dropwise sulfuric acid (1.5 mL). Stirring was continued at room temperature until TLC analysis shows disappearance of the starting material, the mixture was poured into ice-water, extracted with CHCl3 (3 100mL), washed with saturated NaHCO3 solution, and dried over Na2SO4. The solvent was removed under reduced pressure and the residue is used directly in the next step. TLC (7:3 hexanes EtOAc-Rf = 0.4).

To a suspension of 22 (5 g, 11.67 mmol) and N6-benzoyladenine (4.2 g, 17.50 mmol) in anhydrous acetonitrile (60 mL) was added drop wise 1 M SnCl4 in dichloromethane (23.5 mL, 23.34 mmol) under argon. The resulting mixture was allowed to stir for 4 h at room temperature. After completion of the reaction, saturated aq NaHCO3 was added slowly until the evolution of carbon dioxide ceased. Then the mixture was filtered through a pad of
Celite 545, that was subsequently was washed with CHCl₃ (3x100 mL). The combined filtrate was washed successively with saturated aq NaHCO₃ (3 100 mL) and brine (2x100 mL), dried (Na₂SO₄). The filtrates were concentrated under reduced pressure. The residue obtained was purified by silica gel column chromatography using 3% MeOH in CHCl₃ as eluent (Rf-0.5), affording nucleoside 23 [36] (6.12 g, 86%) as a colorless solid. ¹H NMR (500 MHz, DMSO-d₆, δ ppm): δ 9.36 (br s, 1H, -NH), 8.77 (s, 1H, H-2), 8.42 (s, 1H, H-8), 8.03 (dd, 5H, Ar), 7.59 (q, 2H, Ar), 7.50 (t, 2H, Ar), 7.43 (t, 2H, Ar), 7.27 (m, 5H, Ar), 6.64 (s, 1H, H-1’), 5.54 (s, 1H, H-2’), 4.76 (dd, 2H), 4.74 (dd, 1H), 4.65 (m, 1H), 4.16 (d, 1H), 2.18 (s, 3H). ¹³C NMR (500 MHz, DMSO-d₆, δ ppm): δ 169.69 (C = O, CH₃), 166.38 (C = O, Ar), 164.63 (C = O, Ar-NH), 153.03 (C-2), 151.57 (C-6), 149.58 (C-4), 141.81 (C-8), 136.24 (CH=Ar), 133.45 (CH=Ar), 132.92 (CH=Ar), 129.88 (CH=Ar), 129.68 (CH=Ar), 129.02 (CH=Ar), 128.84 (CH=Ar), 128.59 (CH=Ar), 128.39 (CH=Ar), 127.99 (CH=Ar), 122.92 (CH=Ar), 87.82 (‘-C), 81.18 (‘-C), 79.78 (‘-C), 79.75 (‘-C), 72.36 (CH₂=Ar), 62.43 (C-5’), 20.99 (CH₃). HRMS (ESI+) m/z Calculated for C₃₃H₃₉N₅O₇ (MH⁺): 608.2139, found 608.2135.

9-(3’-O-benzyl) β-D-xylfofuranoysyl)-adenine (24) To a solution of nucleoside 23 (2 g, 3.29 mmol) in MeOH (20 mL) was added saturated methanolic ammonia (100 mL) in a sealed tube and the mixture was stirred at 85°C for 3 h. After completion of the reaction, the reaction mixture was concentrated to dryness under reduced pressure and the residue was coevaporated with toluene (5x10 mL). The residue obtained was purified by column chromatography using 5% MeOH in CHCl₃ to afford nucleoside 24 (0.8 g, 68%). ¹H NMR (500 MHz, DMSO-d₆, δ ppm): δ 8.17 (s, 1H, H-2), 8.14 (s, 1H, H-8), 7.35-7.27 (m, 7H, Ar, -NH₂), 6.04 (d, 1H, 2’-OH), 5.95 (s, 1H, 1’-H), 4.94 (br, 2H, 5’,-OH), 4.68 (s, 1H, 2’-H), 4.67 (dd, 2H, CH₂), 4.34 (m, 1H, 4’-H), 4.05 (br s, 1H, 3’-H), 3.81-3.73 (m, 5’,-H, 5’’-H). ¹³C NMR (500 MHz, DMSO-d₆, δ ppm): δ 156.04 (C-6), 152.67 (C-2), 149.21 (C-4), 139.01 (C-8), 138.04(C-3, Ar), 128.29(CH=Ar), 127.56 (-CH=Ar), 127.40 (-CH=Ar), 118.67(C-5), 88.74 (‘-C), 82.68 (‘-C), 77.26 (2’-C), 71.29 (CH₂=Ar), 59.44 (5’-C). HRMS (ESI+) m/z Calculated for C₂₅H₂₉N₅O₇Si (MH⁺): 538.1509, found 538.1510.

9-(5’-O-tert-butylidimethylsilyl-3’-O-benzyl)-β-D-xylfofuranoysyl)-adenine (25) To a cooled suspension of 9-(5’-O-tert-butylidimethylsilyl-3-O-Benzyl-β-D-xylfofuranoysyl)-adenine (6 g, 16.78 mmol) and imidazole (2.85 g, 41.97 mmol) in anhydrous N,N-dimethylformamide was added tert-butylidimethylchloro silane (3.03 g, 16.78 mmol) in anhydrous DMF under argon. The reaction mixture was allowed to stir for 20 h at room temperature. After completion of the reaction, the organic solvent was removed under high vacuum. The residue was dissolved in 150 mL of ethyl acetate, the solution was washed with two times 80 mL of water, and one time with 30 mL of brine and the extract was dried on Na₂SO₄ and the organic solvent was concentrated under high vacuum. The residue obtained was purified by silica gel column chromatography using 2% MeOH in DCM to obtain the compound 25 as colorless solid (7.56 g, 95%). ¹H NMR (500 MHz, CDCl₃, δ ppm): δ 8.16 (s, 1H, H-2), 8.11 (s, 1H, H-8), 7.33 (m, 7H, Ar, -NH₂), 6.05 (d, 1H, 2’-OH), 5.96 (d, 1H, 1’-H), 4.69 (s, 2H, 2’-H), 4.66-4.53 (m, Ar-CH₂), 4.32 (m, 1H, 4’-H), 4.06 (m, 1H, 3’-H), 4.00-3.86 (m, 2H, 5’,-H, 5’’-H), 0.85 (s, 9H, -(CH₃)₃). ¹³C NMR (500 MHz, DMSO-d₆, δ ppm): δ 155.85(C-6), 153.79 (C = O, Cbz) 153.16(C-2), 149.52 (C-4), 139.04(C-8), 136.70 (‘-C), 134.41 (‘-C), 128.83 (CH=Ar), 128.68 (CH=Ar), 128.53 (-CH=Ar), 128.50 (‘-CH=Ar), 128.17 (‘-CH=Ar), 119.30(C-5), 88.69(C-4’), 83.31(C-4’), 82.91 (C-3’), 79.92 (C-2’), 72.45 (CH₂=H), 70.57 (-CH₂=C), 60.37 (C-5’), 25.88 (‘-CH₃), 18.29 (‘-C), -5.31 (CH₃), -5.42 (CH₃). HRMS (ESI+) m/z Calculated for C₃₉H₃₉N₅O₆Si (MH⁺): 547.2374, found: 547.2379.
To a solution of 26 (2.3 g, 33 mmol) in 30 mL of anhydrous THF was added 1 M TBAF in THF (8.63 g, 330 mmol) at 0°C under N₂ atmosphere. The solution was stirred for 12 h at room temperature, and all volatiles were removed using a rotary evaporator. The residue was dissolved in EtOAc (100 mL) and washed with cold saturated NaHCO₃ solution (30 mLx2), and brine (30 mL). The organic solvent was dried over Na₂SO₄ and filtered. The filtrate was concentrated in vacuo to obtain the potassium salt of 27 (1.41 g) as white solid in 86% yield. ¹H NMR (500 MHz, CDCl₃ ppm): δ 8.15 (s, 1H, H-2), 8.14 (s, 1H, H-8), 7.37-7.28 (m, 10H, 2xAr), 6.20 (s, 1H, 1H-Tetrazole), 5.71 (t, 1H, 2¹H), 5.17 (s, 2H, -NH2), 5.03 (t, 1H, 5¹O-H), 4.75 (dd, 2H, CH₂- Ar), 4.38 (m, 1H, 4¹H), 4.30 (m, 1H, 3¹H), 3.78 (m, 2H, 5¹H, 5²H). ¹³C NMR (500 MHz, CDCl₃ ppm): 156.05, 172.98, 172.89, 156.12, 152.02, 152.53, 152.39, 149.22, 149.17, 148.91, 139.57, 139.24, 139.11, 119.28, 119.00, 118.94, 88.02, 85.97, 83.96, 83.17, 73.49, 71.17, 71.14, 71.00, 65.44, 65.18, 61.92, 50.01, 31.31, 29.06, 28.91, 28.85, 28.83, 28.72, 24.65, 22.12, 13.99. ³¹P NMR (500 MHz, CDCl₃ ppm): 1.31, 1.21. HRMS (ESI-) m/z Calculated for C₂₈H₄₇N₅O₈P₁ (MH⁻): 612.3167, found 612.3171.

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To the stirring solution of 14 (1 g, 25.94 mmol) and 12 (2.45 g, 38.92 mmol) in 5 mL dry DCM was added 0.5 M solution of 1H-Tetrazole (25.94 mL, 129.74 mmol) drop wise at 0°C. The reaction mixture was allowed to stir at room temperature for 4 h. Then the reaction mixture was cooled down to -78°C and hydrogen peroxide 35% (W/V, 10 mL) was added. After stirring for 5 min at -78°C, cooling bath was removed and the reaction mixture was allowed to stir at room temperature for further 30 min. The reaction mixture is diluted with DCM (150 mL) and washed with 1 M phosphoric acid (70 mL), 5%aq. sodium bicarbonate (70 mL) and with brine (60 mL), dried over sodium sulfate, filtered and concentrated in vacuo. The obtained residue was purified by column chromatography using EtOAc to obtain the title compound 29 as oil. ¹H NMR (300 MHz, DMSO-d₆ ppm): δ 8.33 (m, 1H, H-2), 8.18 (m, 1H, H-8), 7.38 -7.26 (m, 15H, Ar-H), 6.54 (br s, 1H), 5.34-5.04 (m, 6H, 3xCH₂), 4.90-4.82 (m, 1H), 4.32 (m, 2H), 2.17 (s, 1H), 1.78 (m, 2H), 2.58 (m, 28H, CH₂), 0.89 (t, 3H, CH₃). ¹³C NMR (500 MHz, DMSO-d₆ ppm): δ 170.16, 155.59, 154.37, 153.28, 139.08, 138.78, 138.83, 134.83, 128.85, 128.72, 128.55, 128.18, 128.13, 128.01, 127.97, 120.04, 84.20, 83.03, 82.92, 70.31, 67.40, 37.55, 33.09, 32.06, 29.84, 29.79, 29.65, 29.49, 19.16, 24.66, 22.82, 14.24. HRMS (ESI+) m/z Calculated for C₅₀H₉₁N₅O₁₈P₁ (MH+): 928.4619, found 928.4582.

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To the stirring solution of 29 (0.2 g, 0.21 mmol) in THF, K₂CO₃ (60 mg, 4.31 mmol) and water (2 mL) is added, followed by Palladium (10%) on charcoal, and stirring continued at room temperature under hydrogen for 72 h. After completion of the reaction, the mixture is filtered on celite 545, and the celite pad was washed with THF: Water-1:1, and the filtrate was evaporated and purified by column chromatography using eluent DCM:MeOH: H₂O-17:7:1 to obtain the potassium salt of 30 as white solid. ¹H NMR (500 MHz, DMSO-d₆ ppm): δ 8.37 (d, 1H, H-2), 8.12 (d, 1H, H-8), 7.25 (t, 2H), 6.37 (m, 1H), 5.43 (br s, 1H), 4.44 (br, 1H) 4.29 (m, 1H), 3.96 (t, 1H), 3.90-3.85 (m, 1H), 3.78 (m, 1H), 2.74 (m, 1H), 2.28 (m, 1H), 1.66 (m, 1H), 1.51 (m, 1H), 1.23 (m, 28H), 0.86 (t, 3H). ¹³C NMR (500 MHz, DMSO-d₆ ppm): 172.89, 172.82, 156.12, 152.02, 152.53, 152.39, 149.22, 149.17, 148.91, 139.57, 139.24, 139.11, 119.28, 119.00, 118.94, 88.02, 85.97, 83.96, 83.17, 73.49, 71.17, 71.14, 71.00, 65.44, 65.18, 61.92, 50.01, 31.31, 29.06, 28.91, 28.85, 28.83, 28.72, 24.65, 22.12, 13.99. ³¹P NMR (500 MHz, DMSO-d₆ ppm): 1.31, 1.21. HRMS (ESI-) m/z Calculated for C₂₈H₄₇N₅O₁₈P₁ (M⁻): 612.3167, found 612.3171.
in anhydrous dichloromethane, was added 0.5 M solution of 1H-Tetrazole (46.7 mL, 23.34 mmol) to the stirring solution of 32 (2 g, 2.17 mmol) in THF: MeOH-1:1 (30 mL) was added Palladium on Charcoal (10%), and kept for stirring under hydrogen for 6 h at room temperature. After completion of the reaction, the reaction mixture was passed through celite pad and the celite pad is washed with THF-MeOH mixture (200 mL). The organic solvents were removed under vacuum, and the obtained white residue was purified by silica gel chromatography using DCM:MeOH:H2O-17:7:1. The organic solvents were removed with a rotavapor and the aqueous filtrate was concentrated in vacuo. The filtrate was concentrated in vacuo and purified by silica gel column chromatography (DCM:MeOH:H2O-17:7:1) to obtain the potassium salt of 33 as white solid (0.81 g, 61%). 1H NMR (300 MHz, D2O, ppm): δ 6.97, 7.15, 7.27 (m, 15H), 6.45 (m, 2H), 5.31 (d, 1H), 5.13 (d, 1H), 4.29-4.40 (m, 1H), 2.78-2.89 (m, 1H), 2.68-2.54 (m, 1H), 1.65 (m, 2H, CH2), 1.25 (m, 26H, 13xCH2), 0.89 (t, 3H, CH3). 13C NMR (300 MHz, D2O, ppm): δ 173.02, 165.43, 150.90, 136.63, 110.88, 110.77, 85.53, 74.38, 70.84, 70.60, 64.69, 39.23, 38.98, 33.09, 31.58, 29.53, 29.40, 29.27, 29.09, 24.74, 22.27, 15.33, 11.69, 11.61. 31P NMR (300 MHz, D2O, ppm): δ -0.80, -0.95. HRMS (ESI+) m/z Calculated for C50H67N2O12P1 (MH-): 919.4504, found 919.4512.

Thymidine-5′-(O-stearic acid-2-yl) phosphate (33)

To a stirring solutions of 32 (2 g, 2.17 mmol) in THF: MeOH-1:1 (30 mL) was added Palladium on Charcoal (10%), and kept for stirring under hydrogen for 6 h at room temperature. After completion of the reaction, the reaction mixture is stirred at rt for 12 h. Then the reaction mixture is diluted with DCM:MeOH:H2O-17:7:1. The organic solvents were removed with a rotavapor and the aqueous filtrate was concentrated in vacuo. The filtrate was concentrated in vacuo and purified by silica gel column chromatography (DCM:MeOH:H2O-17:7:1) to obtain the potassium salt of 33 as white solid (0.81 g, 61%). 1H NMR (300 MHz, D2O, ppm): δ 6.97, 7.15, 7.27 (m, 15H), 6.45 (m, 2H), 5.31 (d, 1H), 5.13 (d, 1H), 4.29-4.40 (m, 1H), 2.78-2.89 (m, 1H), 2.68-2.54 (m, 1H), 1.65 (m, 2H, CH2), 1.25 (m, 26H, 13xCH2), 0.89 (t, 3H, CH3). 13C NMR (300 MHz, D2O, ppm): δ 173.02, 165.43, 150.90, 136.63, 110.88, 110.77, 85.53, 74.38, 70.84, 70.60, 64.69, 39.23, 38.98, 33.09, 31.58, 29.53, 29.40, 29.27, 29.09, 24.74, 22.27, 15.33, 11.69, 11.61. 31P NMR (300 MHz, D2O, ppm): δ -0.80, -0.95. HRMS (ESI+) m/z Calculated for C50H67N2O12P1 (MH-): 919.4504, found 919.4512.

Adenosine-5′-(O-stearic acid-2-yl) phosphate (36)

To a solution of 35 (2 g, 1.85 mmol) in THF, K2CO3 (0.52 g, 37 mmol) and water (2 mL) was added. To this, Pd (10%) on Charcoal is added and kept for stirring at room temperature under hydrogen for 72 h. After the completion of the reaction, the mixture is filtered on celite 545, filtrate was washed with THF:Water-1:1. The filtrate was concentrated to obtain the potassium salt of 36 as a white solid (0.734 g, 1.16 mmol) in 5 mL dry DCM was added 0.5 M solution of 1H-tetrazole (12.5 mL, 6.22 mmol) drop wise at 0°C and the reaction mixture was stirred at rt for 30 min. The reaction mixture is diluted with DCM and washed with 1 M phosphoric acid, 5%aq sodium bicarbonate and with brine, dried over sodium sulfate, filtered and concentrated on vacuo, and purified by column chromatography (EtOAc) which gives 38 as an oil. 1H NMR (300 MHz, CDCl3, ppm): δ 8.4 (br s, 1H, H-2), 7.13 (br s, 2H, -NH2), 5.92 (d, 1H, H-1’), 5.32 (m, 1H), 4.61 (br, 1H), 4.21 (br s, 1H), 4.04 (s, 1H), 3.90 (br s, 1H), 2.08 (s, 1H), 1.68 (br, 1H), 1.52 (br, 1H), 1.22 (m, 28H), 0.86 (t, 3H, CH3).

3′-O-benzylxoycarbonyl, deoxyadenosine-5′-(O-benzyl, O-(benzyl stearate)-2-yl) phosphate (38)

To the stirring solution of 14 (0.3 g, 0.77 mmol) and 13 (0.734 g, 1.16 mmol) in 5 mL dry DCM was added 0.5 M solution of 1H-tetrazole (12.5 mL, 6.22 mmol) drop wise at 0°C, and the reaction mixture was stirred at room temperature for 12 h. Then the reaction mixture was cooled down to -78C and hydrogen peroxide 35% (W/V) was added. After stirring for 5 min at -78C, cooling bath was removed and the reaction mixture was allowed to stir at rt for 30 min. The reaction mixture is diluted with DCM and washed with 1 M phosphoric acid, 5%aq sodium bicarbonate and with brine, dried over sodium sulfate, filtered and concentrated on vacuo, and purified by column chromatography (EtOAc) which gives 38 as an oil. 1H NMR (300 MHz, CDCl3, ppm): δ 8.4 (br s, 1H, H-2), 7.13 (br s, 2H, -NH2), 5.92 (d, 1H, H-1’), 5.32 (m, 1H), 4.61 (br, 1H), 4.21 (br s, 1H), 4.04 (s, 1H), 3.90 (br s, 1H), 2.08 (s, 1H), 1.68 (br, 1H), 1.52 (br, 1H), 1.22 (m, 28H), 0.86 (t, 3H, CH3)
2′-deoxadenosine-5′-(O-stearic acid-3-yl) phosphate (39)

To a stirring solution of 38 (0.2 g, 0.21 mmol) in THF, K₂CO₃ (60 mg, 4.31 mmol) and water (2 mL) is added. This reaction mixture was filtered on celite-545 and the celite pad is washed with THF-MeOH mixture (200 mL). The organic solvents were removed under vacuum, and the obtained white residue was purified by silica gel chromatography using DCM:MeOH:H₂O-17:7:1. The organic solvents were removed in the rotavapor and the azeotropic solvent was removed by lyophilization to obtain the desired product 42 as white solid (0.94 g, 71%).

1H NMR (500 MHz, D₂O, ppm): δ 7.84 (br m, 1H, 6.32 (br m, 1H), 4.60 (br m, 2H), 4.16 (br m, 2H), 2.61 (br m, 2H), 2.39 (br m, 2H), 1.94 (s, 3H), 1.72 (s, 2H), 1.21 (s, 2H, CH₂), 0.83 (s, 3H, CH₃).

31P NMR (500 MHz, D₂O, ppm): δ -1.04, -1.12. HRMS (ESI-) m/z Calculated for C₂₈H₄₈N₅O₈P₁ (MH⁻): 612.3167, found: 612.3173.

Thymidine-5′-(O-stearic acid-3-yl)-phosphate (42)

To the stirring solution of 41 (2 g, 2.17 mmol) in THF: MeOH-1:1 (30 mL) was added Pd (10%) on charcoal, and kept for stirring under hydrogen for 6 h at room temperature. After completion of the reaction (monitored by TLC), the reaction mixture was passed through celite pad and the celite pad is washed with THF-MeOH mixture (200 mL). The organic solvents were removed under vacuum, and the obtained white residue was purified by column chromatography (DCM:MeOH:H₂O-17:7:1) to obtain the desired product 42 as white solid (0.94 g, 71%).

Thymidine-5′-(O-stearic acid-3-yl)-phosphate (42)

To the stirring solution of 41 (2 g, 2.17 mmol) in THF: MeOH-1:1 (30 mL) was added Pd (10%) on charcoal, and kept for stirring under hydrogen for 6 h at room temperature. After completion of the reaction (monitored by TLC), the reaction mixture was passed through celite pad and the celite pad is washed with THF-MeOH mixture (200 mL). The organic solvents were removed under vacuum, and the obtained white residue was purified by silica gel chromatography using DCM:MeOH:H₂O-17:7:1. The organic solvents were removed in the rotavapor and the azeotropic solvent was removed by lyophilization to obtain the desired product 42 as white solid (0.94 g, 71%).

1H NMR (500 MHz, D₂O, ppm): δ 7.84 (br m, 1H, 6.32 (br m, 1H), 4.60 (br m, 2H), 4.16 (br m, 2H), 2.61 (br m, 2H), 2.39 (br m, 2H), 1.94 (s, 3H), 1.72 (s, 2H), 1.21 (s, 2H, CH₂), 0.83 (s, 3H, CH₃).

31P NMR (500 MHz, D₂O, ppm): δ -1.04, -1.12. HRMS (ESI-) m/z Calculated for C₂₈H₄₈N₅O₁₀P₁ (MH⁻): 603.3051, found: 603.3048.

2′,3′-O-dibenzyloxy carbonyl, adenosine-5′-(O-benzyl stearate)-3-yl, O-benzyl phosphate (44)

To a stirring solution of 16 (2 g, 3.73 mmol) and 13 (2.81 g, 4.48 mmol) in dry DCM was added 0.5 M solution of 1H-tetrazole (0.5 M in dry acetonitrile (75 ml, 37.34 mmol) dropwise at 0°C and the reaction mixture was stirred at rt for 30 min. The reaction mixture is diluted with DCM and washed with 1 M phosphoric acid, 5% sodium bicarbonate and with brine, dried over sodium sulfate, filtered and concentrated in vacuo, purified by column chromatography and 1H NMR which gives 44 as an oil.

1H NMR (500 MHz, CDCl₃, ppm): δ 9.27 (br s, 1H, NH), 7.43-7.31 (m, 15H, 3 Ar-H), 6.37 (m, 1H), 5.16 (s, 2H, Ar-Ch₂), 5.12-5.03 (m, 4H, Ar-Ch₂), 4.86 (m, 1H), 4.18 (m, 2H), 2.74-2.64 (m, 2H), 2.36 (m, 1H), 2.03 (m, 1H), 1.89 (d, 2H), 1.69 (m, 2H), 1.25 (m, 2H, -CH₂), 0.89 (t, 3H, CH₃).

1H NMR (300 MHz, CDCl₃, ppm): δ 169.87, 163.75, 154.36, 154.00, 135.63, 135.59, 135.11, 135.02, 134.76, 134.73, 128.85, 128.76, 128.68, 128.63, 128.50, 128.46, 128.36, 128.28, 128.09, 128.01, 127.95, 111.81, 111.73, 84.82, 84.44, 82.59, 82.48, 78.07, 78.02, 70.22, 70.20, 69.87, 69.80, 69.70, 69.63, 67.13, 67.06, 67.00, 66.72, 66.68, 60.05, 40.18, 40.12, 37.28, 37.20, 35.39, 31.97, 29.75, 29.71, 29.69, 29.60, 29.51, 29.41, 29.38, 24.91, 24.85, 22.71, 14.18, 12.44. ᵃ₋¹¹³C NMR (300 MHz, CDCl₃): δ -1.68, -1.86, -2.06, -2.25. HRMS (ES⁺) m/z Calculated for C₅₀H₄₀N₂O₁₂P₁ (MH⁺): 919.4504, found 919.4498.
Adenosine-5'-O-stearic acid-3-yl-phosphate (45)

To the solution of 44 (0.3 g, 0.27 mmol) in THF, K₂CO₃ (77 mg, 0.55 mmol) and water 2 mL was added. To this Pd (10%) on charcoal is added and held for stirring at rt under hydrogen for 12 h. After completion of the reaction, the reaction mixture is filtered on celite and the solvent is evaporated and purified by column chromatography (DCM: MeOH: H₂O-17:7:1) to obtain the potassium salt of 45 as white solid. ¹H NMR (500 MHz, D₂O, ppm): δ 8.42 (d, 1H, H-2), 8.12 (s, 1H, H-8), 7.24 (br s, 2H, -NH₂), 5.91 (d, 1H, H-8), 4.53 (m, 1H, 1H, 4.19 (m, 2H), 4.01 (br s, 1H), 3.93 (m, 2H), 2.68 (m, 1H), 2.40 (ddd, 1H), 1.91 (m, 1H), 1.60 (m, 1H), 1.45 (m, 1H), 1.23 (m, 26H, CH₂), 0.86 (t, 3H, CH3). ¹³C NMR (500 MHz, D₂O, ppm): δ 172.50, 165.45, 155.98, 152.55, 149.58, 139.32, 139.19, 127.96, 126.94, 118.83, 86.93, 83.74, 74.01, 73.88, 71.17, 70.82, 70.64, 67.48, 64.73, 64.41, 60.48, 53.30, 47.85, 43.37, 42.56, 35.52, 31.28, 29.04, 28.69, 24.89, 22.08, 17.27, 16.36, 14.22. ³¹P NMR (300 MHz, CDCl₃, ppm): δ -0.69. HRMS(ES⁻) m/z Calculated for C₂₈H₄₈N₅O₉P₁ (MH⁻): 628.3116, found: 628.3123.

9-(O-benzyl, O-(benzyl stearate)-2-yl-5'-phospho), 3'-O-benzyl, 2'-O-benzylxoycarbonyl xylofuranosyl) adenine (46)

To a stirred solution of 27 (1.2 g, 24.41 mmol) and 12 (2.3 g, 36.62 mmol) in 12 mL dry DCM was added 0.5 M solution of 1H-Tetrazole (24.41 mL, 122.07 mmol) drop wise at 0°C, and the reaction mixture was stirred at room temperature for 12 h. Then the reaction mixture was cooled down to -78°C and hydrogen peroxide 35% (W/V) was added. After stirring for 5 min at -78°C, cooling bath was removed and the reaction mixture was allowed to stir at room temperature for further 30 min. The reaction mixture is diluted with DCM (150 mL) and washed with 1 M phosphoric acid (100 mL), 5%aq. sodium bicarbonate (100 mL) and with brine (80 mL), dried over sodium sulfate, filtered and concentrated in vacuo. The obtained residue was purified by column chromatography (EtOAc) giving 46 (100 mg, 4%) as an oil. ¹H NMR (300 MHz, CDCl₃, ppm): δ 8.35 (s, 1H, H-2), 8.13 (s, 1H, H-8), 7.31 (d, 2H), 5.91 (br s, 1H), 4.31 (m, 2H), 4.12 (m, 2H), 4.05 (m, 1H), 3.87 (m, 1H), 3.80-3.61 (m, 1H), 1.63 (m, 2H), 1.19 (m, 2H, CH₂), 0.85 (t, CH₃). ¹³C NMR (300 MHz, CDCl₃, ppm): δ 156.06, 155.96, 152.43, 149.02, 148.78, 139.79, 118.44, 89.42, 83.62, 82.01, 80.93, 75.05, 74.87, 60.64, 59.27, 54.96, 31.34, 29.20, 28.78, 28.28, 22.12, 13.92. ³¹P NMR (300 MHz, CDCl₃, ppm): δ 1.67, 1.36. HRMS (ESI⁻) m/z Calculated for C₂₈H₄₈N₅O₉P₁ (MH⁻): 628.3116, found: 628.3123.

9-(O-benzyl, O-(benzyl stearate)-3-yl-5'-phospho), 3'-O-benzyl, 2'-O-benzylxoycarbonyl xylofuranosyl) adenine (48)

To a stirred solution of 27 (1.2 g, 24.41 mmol) and 13 (2.3 g, 3.66 mmol) in 20 mL dry DCM was added 0.5 M solution of 1H-Tetrazole (54 mL, 24.44 mmol) drop wise at 0°C, and the reaction mixture was stirred at room temperature for 12 h. Then the reaction mixture was cooled down to -78°C and hydrogen peroxide 35% (W/V) was added. After stirring for 5 min at -78°C, cooling bath was removed and the reaction mixture was allowed to stir at rt for 30 min. The reaction mixture is diluted with DCM and washed with 1 M phosphoric acid, 5%aq. sodium bicarbonate and with brine, dried over sodium sulfate, filtered and concentrated in vacuo. The obtained residue was purified by column chromatography (EtOAc) which gives 48 as an oil. ¹H NMR (500 MHz, CDCl₃, ppm): δ 8.33 (s, 1H, H-2), 8.10 (m, 1H, H-8), 7.38-7.19 (m, 20H, 4° Ar), 6.33 (d, 1H, H-1°), 5.82 (br s, 2H, -NH₂), 5.42 (br s, 2H), 5.19 (m, 2H), 5.08-5.00 (m, 4H), 4.83 (m, 1H), 4.66 (dd, 1H), 4.54 (dd, 1H), 4.38 (m, 1H), 4.35 (m, 2H), 4.10 (m, 1H), 3.55-3.46 (m, 1H), 2.77-2.72 (m, 1H, H-5°), 2.63-2.58 (m, 1H, H-5°), 1.66 (m, 2H, CH₂), 1.25 (m, 28H, CH₂), 0.89 (t, 3H, CH₃). ¹³C NMR (500 MHz, CDCl₃, ppm): δ 169.91, 155.68, 153.75, 153.22, 149.53, 139.02, 136.24, 135.82, 135.62, 134.84, 133.87, 132.92, 132.56, 131.72, 131.34, 129.77, 128.87, 128.67, 128.57, 128.47, 128.42, 128.19, 128.12, 128.06, 127.92, 119.54, 87.38, 87.33, 82.60, 82.52, 81.34, 81.23, 81.16, 80.12, 79.94, 76.04, 75.97, 72.40, 70.80, 69.87, 69.82, 69.72, 69.65, 67.25, 65.37, 65.30, 65.21, 65.10, 65.03, 33.06, 32.97, 32.02, 29.80, 29.75, 29.71, 29.60, 29.45, 29.41, 29.11, 24.60, 22.78, 14.22. ³¹P NMR (300 MHz, CDCl₃, ppm): δ -1.58, -1.68, -1.82, -1.91. HRMS (ESI+) m/z Calculated for C₇₅H₇₂N₅O₁₉P₁ (MH+): 1034.5038, found: 1034.5048.
The solution of 48 (0.2 g, 0.21 mmol) in THF, K2CO3 (60 mg, 0.43 mmol) and water (2 mL) is added. To this PD (10%) on charcoal is added and held for stirring at rt under hydrogen for 12 h. After the completion of the reaction, the mixture is filtered on celite-545 and the solvent is evaporated and the residue is purified on column chromatography (DCM:MeOH:H2O-17:7:1) to obtain the product (0.28 g, 71%) as white solid.1H NMR (500 MHz, DMSO-d6, ppm): δ 8.26 (m, 1H), 1.81 (d, 1H, H-8), 5.98 (br s, 1H), 4.63 (m, 2H), 4.46 (m, 2H), 4.25 (m, 2H), 3.60 (t, 1H), 2.44 (m, 2H), 1.78 (t, 1H), 1.55 (m, 2H), 1.06 (m, 26H, CH2), 0.74 (t, 3H, CH3). 13C NMR (500 MHz, DMSO, ppm): δ 179.31, 162.09, 154.98, 152.10, 152.03, 148.07, 147.75, 139.95, 139.86, 118.24, 118.09, 89.68, 88.46, 81.43, 81.39, 80.54, 80.11, 79.59, 75.66, 75.53, 74.57, 62.98, 62.75, 61.24, 48.57, 44.01, 35.17, 33.73, 31.45, 29.33, 29.24, 28.93, 28.12, 24.69, 24.63, 22.97, 22.17. 31P NMR (500 MHz, D2O, ppm): -0.94, -1.09. HRMS (ES-) m/z Calculated for C29H50N6O7P1 (MH+): 625.3483 found 625.3453.

2′-deoxyadenosine-5′-(N-stearic acid-2-yl)-phosphoramidate (51)
In a 100 mL two neck flask, 2′-deoxyadenosine 5′-O-monophosphate (0.3 g, 0.9 mmol) and methyl α-aminostearate (2 g, 6.20 mmol) were dissolved in a mixture of t-butanol and water (5:1). Triethylamine (0.5 mL), and a freshly prepared solution of N,N-dicyclohexylcarbodiimide (DCC, 1.6 g, 7.75 mmol) in t-butanol (0.5 g/mL) was added to the reaction mixture under argon atmosphere, and the reaction was allowed to reflux for 2 h. Another 5 eq of DCC was added to the reaction mixture which was refluxed for 1 h. Upon completion, the reaction mixture was cooled down to room temperature and the reaction mixture was concentrated in vacuo. The residue obtained was purified by silica gel column chromatography (DCM:MeOH:H2O-17:7:1) to obtain 51 (0.44 g, 77%) as white solid. 1H NMR (500 MHz, DMSO, ppm): δ 8.38 (d, 1H, H-2), 8.12 (s, 1H, H-8), 7.22 (br s, 2H), 6.35 (t, 1H), 4.41 (s, 1H), 3.92 (br s, 1H), 3.78-3.74 (m, 2H), 3.67 (m, 1H), 3.55 (s, 2H), 3.52 (s, 1H), 3.02 (dd, 3H), 2.69-2.63 (m, 1H), 1.47 (m, 2H), 1.22-1.14 (m, 28H, 14xCH2), 0.85 (t, 3H, CH3). 13C NMR (500 MHz, DMSO, ppm): δ 175.11, 155.95, 152.50, 149.18, 139.20, 118.86, 117.45, 82.97, 71.34, 64.14, 54.47, 51.28, 45.20, 31.26, 29.01, 28.87, 28.73, 28.67, 24.94, 22.07, 13.93, 8.46. 31P NMR (500 MHz, DMSO): δ 4.51. HRMS (ES+) m/z Calculated for C29H50N6O7P1 (MH+): 625.3483 found 625.3453.

2′-deoxyadenosine-5′-(N-stearic acid-2-yl)-phosphoramidate (52)
A solution of 51 (0.4 g, 0.63 mmol) in MeOH/H2O (4:1 v/v, 3 mL, containing 0.4 M of NaOH (0.051 g, 1.27 mmol) was stirred at room temperature under nitrogen for 2 h. After completion of the reaction, the solvents were removed under reduced pressure. The crude reaction mixture was purified by silica gel chromatography (DCM:MeOH:H2O-17:7:1) to obtain 52 (0.28 g, 71%) as white solid. 1H NMR (500 MHz, DMSO, ppm): δ 8.42 (d, 1H, H-2), 8.11 (d, 1H, H-8), 7.23 (br s, 2H, -NH2), 6.37 (m, 1H), 4.44 (d, 1H), 3.97 (m, 1H), 3.82-3.71 (m, 3H), 2.69 (m, 1H), 2.27 (m, 1H), 1.65 (br s, 1H), 1.21 (m, 28H), 0.85 (t, 3H). 13C NMR (500 MHz, DMSO, ppm): δ 176.18, 156.08, 152.59, 149.31, 139.31, 119.01, 86.45, 83.21, 71.62, 64.31, 64.09, 55.13, 54.78, 31.87, 31.39, 29.14, 25.90, 25.87, 22.20, 14.06. 31P NMR (500 MHz, DMSO): δ 7.08, 6.96. HRMS (ES-) m/z Calculated for C29H50N6O7P1 (MH+): 611.3327, found: 611.3323.

Thymidine-5′-(N-(methyl stearate)-2-yl)-phosphoramidate (53)
In a 100 mL two neck flask, 2′-deoxythymidine 5′-O-monophosphate (0.5 g, 1.55 mmol) and methyl α-aminostearate 50 (2 g, 6.20 mmol) were dissolved in a mixture of 5:1. Triethylamine (0.5 mL), and a freshly prepared solution of N,N-dicyclohexylcarbodiimide (DCC, 1.6 g, 7.75 mmol) in t-butanol (0.5 g/mL) was added to the reaction mixture under argon atmosphere, and the reaction was allowed to reflux for 2 h. Another 5 eq of DCC was added to the reaction mixture which was refluxed for 1 h. Upon completion, the reaction mixture was cooled down to room temperature and the reaction mixture was concentrated in vacuo. The residue obtained was purified by silica gel column chromatography (DCM:MeOH:H2O-17:7:1) to obtain 53 (0.69 g, 72%) as white solid. 1H NMR (500 MHz, DMSO, ppm): δ 10.82 (br s, 1H, -NH), 7.28 (d, 1H), 5.70 (m, 1H), 5.24 (s, 1H), 3.76 (br s, 1H), 2.67 (s, 2H), 1.56 (m, 2H), 1.32 (br s, 2H), 0.71 (m, 28H, 14xCH2), 0.34 (m, 3H, CH3). 13C NMR (300 MHz, DMSO, ppm): δ 175.26, 163.79, 150.50, 136.26, 109.62, 86.03, 85.92, 83.74, 70.97, 63.82, 54.54, 54.44, 51.19, 49.19, 45.10, 34.30, 31.28, 30.21, 29.03, 28.75, 28.70, 25.01, 24.62, 23.83, 22.07, 13.87, 12.06, 8.36. 31P NMR (300 MHz, DMSO, ppm): δ 3.70. HRMS (ES-) m/z Calculated for C29H52N6O7P1 (MH+): 616.3368, found: 616.3371.
Thymidine-5′-(N-stearic acid-2-yl)-phosphoramidate (54)
A solution of 53 (0.5 g, 0.8 mmol) in MeOH/H$_2$O (4:1 v/v), was added 0.4 M NaOH (0.065 g, 1.61 mmol), and the mixture was stirred at room temperature under nitrogen for 2 h. The solvent was removed under reduced pressure. The resulting crude material was purified by chromatography (DCM:MeOH:H$_2$O=17:7:1) to obtain 54 (0.34 g, 69%) as white solid. $^1$H NMR (500 MHz, DMSO): δ 11.24 (s, 1H), 7.84 (s, 1H), 6.22 (q, 1H), 5.36 (br s, 1H), 4.31 (d, 1H), 3.86-3.72 (m, 1H), 2.12-2.01 (m, 2H), 1.80 (m, 1H), 1.76 (t, 1H), 1.23 (m, 28H), 0.86 (t, 3H, CH$_3$). $^{13}$C NMR (500 MHz, DMSO): 176.13, 163.89, 150.58, 136.39, 109.79, 86.12, 83.84, 71.35, 71.00, 31.38, 29.12, 26.03, 25.85, 22.18, 14.05, 12.16, 12.12. $^{31}$P NMR (500 MHz, DMSO): δ 7.29, 7.00. HRMS (ES-) m/z Calculated for C$_{28}$H$_{49}$N$_3$O$_9$P$_1$ (MH-): 602.3211, found 602.3210.

Bodipy (55)
8-S-Methyl Bodipy (120 mg, 0.5 mmol, prepared according to ref. [41]) is dissolved in DMSO/DCM (1/1; v/v, 5 mL) and mixed with taurine (60 mg, 0.5 mmol) and NaHCO$_3$ (42 mg, 0.5 mmol). The resulting mixture is stirred at room temperature until TLC indicates complete consumption of the starting material, and the formation of a highly polar compound displaying blue fluorescence. The reaction mixture is diluted with water (10 mL) and di-chloromethane (10 mL) and extracted. The aqueous layer is collected and lyophilized to yield the desired product 55 as a pale yellow solid. $^1$H NMR(300 MHz, DMSO, ppm): δ 7.47 (br s, 1H, -CH), 7.36 (d, 1H, -CH), 7.24 (br s, 1H, -CH), 7.11 (d, 1H, -CH), 6.49-6.47 (m, 1H, -CH), 6.31-6.29 (m, 1H, -CH), 3.99 (t, 2H, -CH$_2$), 2.92 (t, 2H, -CH$_2$). $^{13}$C NMR (500 MHz, DMSO, ppm): δ 147.79, 132.15, 129.05, 122.45, 122.18, 114.57, 113.77, 112.42, 40.42.

Vesicles preparation and visualization
Nucleolipid aggregate-vesicle formation was performed by dissolving the nucleolipids in water or DMSO and dilute with water or THF/dioxane (1:1) in a glass vial. To this solution Bodipy fluorescent dye either in water, THF or chloroform was added, vortexed for 10 seconds to stimulate vesicle formation and set aside for 5 min. The pH of the nucleolipids emulsion is found to be 6.82. If the pH was lowered further, the emulsion appeared opalescent. An aliquot of the mixture (100 μL) was pipetted out from this reaction mixture and spin coated at 3000 rpm on a microscope glass plate for 2 min, resulting in a thin layer adequate for optical microscopy.

Vesicles formed in presence of Bodipy fluorescent dye were monitored under fluorescent microscopy and the images were recorded with Olympus Fluoview FV1000 by carrying out excitation wavelength readings at 532 nm with 100 zoom. Two fluorescent dyes were used for the vesicle encapsulation: aqueous soluble dye 55 (soluble in both water) and organic soluble dye 56 (chloroform/THF soluble), which were used at a concentration of 0.5 μM.

Stability study by NMR
Samples were prepared in D$_2$O or DMSO and the pH of the sample was adjusted by the addition of a small volume (a few μL) of HCl or NaOH solutions in D$_2$O (0.1 M). $^{31}$P NMR was used to study the stability of the nucleolipids in acidic (pH4) and in base (pH12) environment. One-dimensional $^{31}$P spectra were used as a fast screening experiment to monitor degradation of the conjugates. Two-dimensional $^1$H-$^{31}$P correlation spectra were used to characterize the $^{31}$P containing products formed by degradation of the nucleolipids. Correlations were established using a Proton-detected hetero-TOCSY experiment [24] with a DIPSI spinlock of 50 ms, allowing correlations of $^{31}$P resonances with several $^1$H resonances of adjacent spin systems.

Conclusions
We have synthesized a series of amphiphiles in which the polar group consists of an adenine or thymine nucleotide and the lipid moiety is based on stearic acid. The nucleolipids are constructed from a phosphodiester or phosphoramidate bond formed between α- or β-hydroxyl group or the α-amino group present on the lipid moiety and the 5′-phosphate group of the nucleotide. These molecules have been analyzed for their potential to form vesicles in water. This can be considered as a model of a protocell with a shell containing covalently bond nucleotides, which may be used to establish an information system in the vesicle by polymerization. The functionalized lipid may function as leaving group for the polymerization reaction and the α (or β) carboxylic acid may catalyze phosphodiester cleavage.

The nucleolipids with a deoxyribose sugar moiety may form small or large vesicles, rod-like structures or vesicle aggregates. Some of these aggregates can be considered as intermediate forms in vesicle formation or division. It seems that a diversity of communication systems, by diffusion (in/out the vesicle) or exchange of material between vesicles (via vesicle fusion), between such vesicles exist. Suggesting that a protocell could stay out of equilibrium by diverse forms of material exchange.

However, we could not observe nucleotide polymerization or cyclic nucleotide formation of these nucleolipids, regardless of the sugar moiety that is investigated (deoxyribose, ribose, xylose). To unravel this observation, the chemical stability of the constructs was studied. While the nucleolipids containing β-hydroxy fatty acids are stable as well in base as in acid circumstances, others degraded in acidic conditions. Phosphoramidate
nucleolipids hydrolyzed by P-N as well as P-O bond cleavage where the ratio between both pathways depends on the nucleobase. Diester constructs with a α-hydroxy stearic acid degraded exclusively by hydrolysis of the phosphorus to 5'-O-nucleoside ester. To summarize, among all investigated systems, only α-amino compounds have shown the desired bond breaking, the only problem being that the nucleophile is water and not the 2- or 3-hydroxyl groups. Favoring the intramolecular mechanism in order to promote polymerization, acyclic sugar moieties such as in GNA could be considered. Also their prebiotic relevance makes them ideal candidates to explore protocells capable of simultaneous core and shell replication [48]. As the compounds are too stable and harsh conditions would destruct the material itself, more reactive species (such as lipid imidazolates of nucleotides) need to be synthesized to further analyze the potential polymerization process. This research could be based on the original work of L. Orgel [49], in which he used phosphorimidazolate for nucleotide polymerization. Furthermore, quantitative investigation is in order to address the interesting (hydro) gelating properties of these new phosphodiester nucleolipids in acidic aqueous environment.

Methods
All reactions were performed in an inert atmosphere (argon). Chemicals and solvents were purchased from Sigma-Aldrich, TCI Europe, and Alfa Aesar, and were used without further purification. Unless otherwise mentioned, each reaction vessel was oven dried prior to use. Column chromatography was performed using silica gel (63-200 mesh) obtained from the Sigma-Aldrich Company. Analytical thin-layer chromatography (TLC) was performed on Merck pre-coated aluminum plates (silica gel 60, F254) and visualized under 254-nm UV light. 1H, 31P and 13C NMR spectra were obtained on a Bruker 300 MHz and Bruker 500 MHz instrument at ambient temperature. Data were processed using the software Topspin 2.1. 

Competing interests
The authors declare that they have no competing interests.

Authors contributions
KLG performed synthesis and experiments for vesicle formation and assistance in fluorescence microscopy. PS assisted with organic synthesis and JR performed mass spectrometry determination; HPM wrote the introduction and revised the manuscript. VL and WD performed the synthesis of bodipy dyes. JH performed fluorescence microscopy. EL did the NMR experiments and wrote the NMR section. PH was responsible for the idea; follow up of the research groups, coordination and writing the manuscript. All authors read and approved the final manuscript.

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