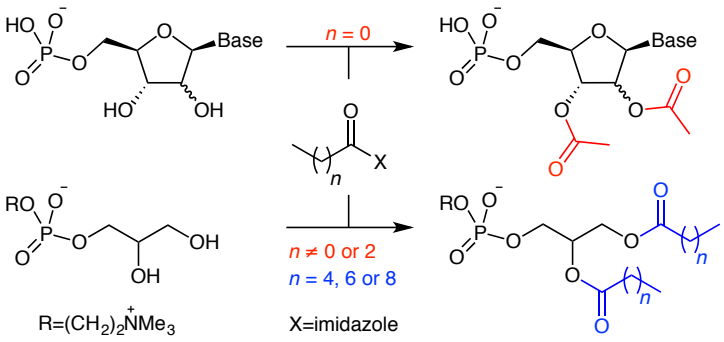
Selective Acylation of Nucleosides, Nucleotides and Glycerol-3-phosphocholine in Water

Christian Fernandez-Garcia
Matthew W. Powner*

University College London, 20 Gordon Street, London WC1H 0AJ, UK

matthew.powner@ucl.ac.uk



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Abstract: A convenient selective synthesis of 2',3'-di-*O*-acetyl-nucleotide-5'-phosphates, 2',3'-di-*O*-acetyl-nucleotide-5'-triphosphates and 2',3',5'-tri-*O*-acetyl-nucleosides in water has been developed. Furthermore, a long-chain selective glycerol-3-phosphocholine diacylation is elucidated. These reactions are environmentally benign, rapid, high yielding and the products are readily purified. Importantly, this reaction may indicate a prebiotically plausible reaction pathway for the selective acylation of key metabolites to facilitate their incorporation into protometabolism.

Key words: prebiotic chemistry, nucleotides, lipids, water, acylation

Life is the quintessential example of a complex chemical system; the molecular choreography involved in sustained cellular evolution is not only one of the most remarkable phenomena in nature, but is also astonishingly a process that must have self-initiated under geochemically plausible constraints. Although clues to the chemical origins of life are built into the universally conserved metabolites of biochemistry, understanding the origins of these metabolites remains an unmet challenge.[1] Historical evidence from the Hadean and early Archean eons (>3.5 billon years ago)—the time life arose on Earth—is severely limited,[2] therefore it is widely recognised that the origins of life must be reinvented rather than discovered.[3,4] Accordingly, the role synthetic chemistry must play in elucidating the origins of life cannot be overestimated, and we have set out to expand our knowledge of chemistry in relation to this question.

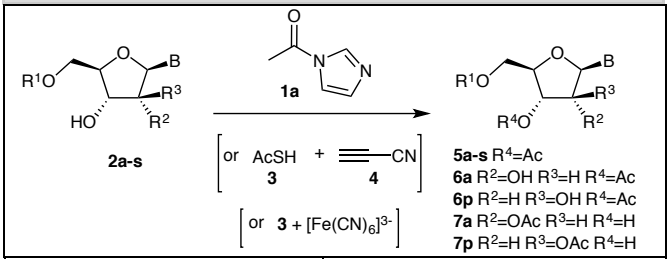
Protecting-group strategies are ubiquitous for the control of multistep organic syntheses,[5] and although such strategies remain almost unexplored in prebiotic chemistry, there is no fundamental reason that temporary modification of reactive functionalities to direct chemical reactivity towards advantageous pathways should not apply at the origins of life.

Acetylation is one of the most-widely exploited strategies for hydroxyl modification; protocols often employ acetic anhydride or acetyl chloride in (toxic) solvents such as pyridine,[5] and afford peracetylated products without discriminating between functional groups. To improve the selectivity and environmental impact, a wide range of additives (principally under solvent-free conditions) have been explored[6] and numerous *N*-deacetylation methods reported to address the deviation from ester selectivity.[7] However, these methods are incompatible with certain carbohydrates, nucleotides and nucleosides. To avoid these harsh conditions, Schwartz's reagent[8] or superheated methanol[9] have been exploited but cost, time and tedious reaction protocols are drawbacks to their application. Furthermore, biologically important highly polar or charged groups, such as (poly)phosphates or sulfates, result in low solubility in the organic solvents predominately used for acetylation, which adversely affects product yields and reaction times. Formation of *n*-alkylammonium salts[10] or ionic liquids[11] have been reported to improve solubility. However, groups such as triphosphates do not tolerate harsh conditions and can be difficult to separate from ionic liquids. Aqueous acetylation protocols have received limited attention because the typical acetylating agents acetic anhydride and acetyl chloride hydrolyse quickly in water.[12] However, acylation is an essential reaction motif in living systems,[13-16] though biochemical acylation processes are enzyme (or ribosome) catalysed, before the advent of sophisticated enzymatic control, biologically essential acyl-transfer reactions must have been controlled by predisposed reactivity.[1] Accordingly, the role of prebiotic acyl-group transfer warrants further investigation.

The chemoselective *O*2'-acetylation of nucleotide-3'-phosphates in water, via intermolecular mixed anhydride synthesis followed by intramolecular acyl transfer (employing thioesters, *N*-carboxyanhydrides and *N*-acetyl imidazole (**1a**) as prebiotic acetylating agents) has been reported.[17,18] This strategy for temporary acetylation has been exploited to control regioselective ligation of (natural) 5'-3'-RNA under prebiotically

plausible conditions.[17] Imidazole **1a** has also been reported to regioselectively acetylate carbohydrates and simple diols in aqueous tetramethylammonium hydroxide.[19] Here, we further outline the utility of acylation as a methodology for the robust and reversible modification of nucleotide hydroxyl moieties. Specifically, we report an aqueous acetylation protocol and the chemoselective synthesis of 2',3'-di-*O*-acetyl-nucleotides and 2',3',5'-tri-*O*-acetyl-nucleosides by using activated thioesters or imidazole **1a** in water. We also demonstrate a remarkable long-chain effect that promotes the long-chain (≥C_6) diacylation of the zwitterion glycerol-3-phosphocholine (**10**).

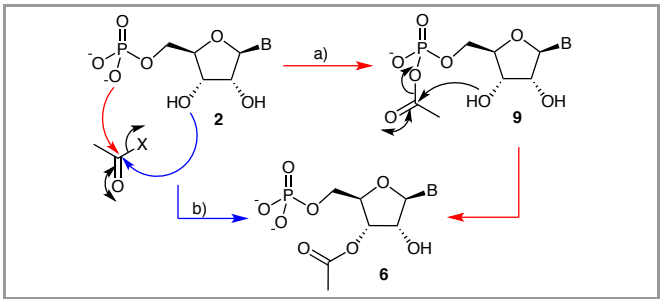
Table 1 Selective *O*-acetylation of nucleotides (**2**) in water.



	Nucleoside/nucleotide (**2**)				*O*-Acetyl-nucleoside/nucleotide (**5**)				
	B	R^1	R^2	R^3	R^1	R^2	R^3	%[a]	%[b]
a	Cyt	PO_3^{2-}	OH	H	PO_3^{2-}	OAc	H	85[c]	98[c]
a	Cyt	PO_3^{2-}	OH	H	PO_3^{2-}	OAc	H	-	33[d]
a	Cyt	PO_3^{2-}	OH	H	PO_3^{2-}	OAc	H	-	80[e]
b	Ura	PO_3^{2-}	OH	H	PO_3^{2-}	OAc	H	67[c]	95[c]
c	Ade	PO_3^{2-}	OH	H	PO_3^{2-}	OAc	H	85[c]	87[c]
d	Gua	PO_3^{2-}	OH	H	PO_3^{2-}	OAc	H	70[c]	83[c]
e	Ino	PO_3^{2-}	OH	H	PO_3^{2-}	OAc	H	71[c]	86[c]
f	Ade	$P_3O_9^{4-}$	OH	H	$P_3O_9^{4-}$	OAc	H	73[c]	82[c]
g	Cyt	$P_3O_9^{4-}$	OH	H	$P_3O_9^{4-}$	OAc	H	76[c]	90[c]
h	Ura	$P_3O_9^{4-}$	OH	H	$P_3O_9^{4-}$	OAc	H	76[c]	80[c]
i	Cyt	H	OH	H	OAc	OAc	H	77[c]	80[c]
j	Ura	H	OH	H	OAc	OAc	H	74[c]	75[c]
k	Ade	H	OH	H	OAc	OAc	H	74[c]	75[c]
l	Gua	H	OH	H	OAc	OAc	H	53[c,f]	56[c,f]
m	Ino	H	OH	H	OAc	OAc	H	70[c]	78[c]
n	Xan	H	OH	H	OAc	OAc	H	63[c]	63[c]
o	Ade	PO_3^{2-}	H	OH	PO_3^{2-}	H	OAc	91[c]	92[c]
p	Cyt	PO_3^{2-}	H	OH	PO_3^{2-}	H	OAc	66[c]	80[c]
q	Uri	H	H	OH	OAc	H	OAc	78[c]	85[c]
r	Ade	H	H	OH	OAc	H	OAc	47[c,f]	-
s	Xan	H	H	OH	OAc	H	OAc	74[c]	75[c]

[a] Isolated yield. [b] NMR yield. [c] Nucleoside/nucleotide **2** (100mM) and **1a** (10 eq.) at pH 8 and RT after 4 h. [d] Nucleotide **2a** (100mM), thioacid **3** (10 eq.) and cyanoacetylene (**4**; 10 eq.) at pH 8 and RT after 4 h. A mixture of 2'/3'-mono-*O*-acetyl-cytidines **6a**/**7a** (33%) and 2',3'-di-*O*-acetyl-cytidine **5a** (33%) was observed. [e] Nucleotide **2a** (100mM), thioacid **3** (10 eq.) and ferricyanide (10 eq.) at pH 8 and RT after 4 h. Pyrophosphate derivative **8** was observed in 15% yield.[23] [f] Unoptimised; insoluble starting material.

Protecting-group strategies have received limited attention in prebiotic synthesis.[17] Therefore, we were intrigued to investigate the acetylation of (natural) nucleotide 5'-phosphates (**2a-e**; Table 1). The reaction of cytidine-5'-phosphate (**2a**) with thioacid **3**[20] and cyanoacetylene (**4**)[17] proceeds at wide range of pH values (pH 6 – 12), but acetylation occurred solely at the 2' and 3'-hydroxyl groups. Nucleobase acetylation was not observed. A mixture of cytidine-5'-phosphate **2a** (33%), mono-*O*-acetyl-cytidine-5'-phosphates **6a**/**7a** (33%) and diacetyl-cytidine-5'-phosphate **5a** (33%) was obtained 30 min after the addition of **3** and **4** (10 eq. each) to **2a** at pH 8 (Supplementary Information, Fig. S1).[21] We next investigated the acetylation of **2a** with **3** and ferricyanide (10 eq. each),[22] which afforded diacetyl-cytidine-5'-phosphate **5a** in good yield (80%), alongside mono-*O*-acetyl-cytidine-5'-phosphates **6a** and **7a** (5% combined yield) and, intriguingly, pyrophosphate derivative **8** (15%).[23] Increasing the amounts of **3** and ferricyanide to 20 equivalents further promoted phosphate activation and synthesis of pyrophosphate **8** (40%; Supplementary Information, Fig. S2). Finally, we investigated the reaction of imidazole **1a**. Little acetylation of **2a** was observed at pH 5 or 6, even with excess **1a** (10 eq.), likely due rapid hydrolysis at acidic pH.[24] Conversely, 2',3'-di-*O*-acetyl cytidine-5'-phosphate **5a** (98%) was the major product at pH 7 – 8 (Supplementary Information, Fig. S3). The reaction was complete after <10 min, then only slow hydrolysis of excess **1a** was observed and **5a** was stable to the reaction conditions for >1 d. Lower stoichiometries of **1a** (6 or 8 eq.) afforded **5a** in 77 and 85% yield, respectively. We next investigated the reaction of uridine-5'-phosphate (**2b**) with imidazole **1a** (1 – 10 eq.) at pH 8. Acetylation was observed at all stoichiometries, but diacetylation became efficient with **1a** (4 eq.) to furnish diacetyl-uridine-5'-phosphate **5b** in 77% yield, alongside two monoacetyl-uridine-5'-phosphates **6b** and **7b** (23%), after 4 h. Higher stoichiometries of **1a** (6 - 10 eq.) furnished diacetyl-uridine-5'-phosphate **5b** in 95% yield (Supplementary Information, Fig. S5). We next investigated the acetylation of nucleotides **2c** – **2h** (100mM; Table 1).[25] All nucleotides were effectively acetylated to furnish **5c-h** (80-95%), and no nucleobase or phosphate derivatives were observed.[26]



Scheme 1 Potential pathways for nucleotide acetylation: a) intramolecular acyl-transfer from mixed anhydride **9**; b) direct hydroxyl acylation. B=nucleobase; X=nucleofuge.

It is of particular note that the mild conditions of our protocol avoids cleavage of the triphosphate moiety whilst achieving completely selective *O*-acetylation. It has previously been proposed that the predominant pathway for nucleotide acetylation in water occurs via mixed anhydride **9** (Scheme 1),[17,27] however our observation that triphosphates are readily acetylated suggests direct hydroxyl group acetylation.[28] To evaluate these proposed mechanisms, we next investigated the acetylation of cytidine **2i** (100mM), which lacks a phosphate moiety. Upon incubation of **2i** with imidazole **1a** (10 eq.) in D_2O/H_2O (1:1) at room temperature and pH 5 – 8, a remarkably similar pH profile to that of nucleotides **2a-h** was observed, with

little acetylation at pH 5 and an excellent 80% yield of tri-*O*-acetyl-cytidine **5i** at pH 8 (Supplementary Information, Fig. S6).

Pleasingly, we again found our acetylation protocol was general to all the nucleosides investigated (**2i** – **2n**). Although guanosine **2l** (100mM) was insoluble in water, incubation of a suspension of **2l** at room temperature and pH 8 with imidazole **1a** furnished a homogenous solution after 30 min, and continued incubation led to the direct precipitation of guanosine **5l** (53%, unoptimised) after 4 h. Incubation of inosine **2m** and imidazole **1a** in water also furnished inosine **5m** (70%) as a white precipitate after 1 h. Finally, to investigate the effect of diol stereochemistry on nucleoside acetylation, we next examined the reaction of *arabino*-nucleosides (**2o** – **2s**) with imidazole **1a**. Good yields (75-92%) were obtained for all arabinosides, except insoluble *arabino*-adenine **1r** (47%, unoptimised), and the trans-2',3'-stereochemistry was not observed to hinder or decrease the rate of acetylation. However, upon exploring lower stoichiometries of imidazole **1a** (1 – 4 eq.) we interestingly observed that *arabino*-cytidine **2p** exhibited significantly greater 3'-OH acetylation than 2'-OH acetylation (**1a** (2 eq.): **6p**/**7p**=87:13; **1a** (4 eq.): **6p**/**7p**=78:22; Supplementary Information, Fig. S7).

We have a long-standing interest in the generational relationship between nucleotides and lipids,[29] two classes of metabolite thought to be essential to the origins of life,[3,29,30] and having investigated the acetylation of (natural) nucleotide 5'-phosphates (**2a-e**) we were intrigued to investigate the synthesis of diacyl phospholipids. Modern cell membranes are composed primarily of diacyl (or dialkyl) glycerol phospholipids, and double-chain amphiphiles exhibit increased hydrophobicity relative to single-chain amphiphiles,[31] which promotes self-assembly at lower concentrations and (competitive) growth in lipid mixtures.[32] Furthermore, the increased volume of the alkyl chains of the double-chain amphiphiles, which are cylindrical to a first approximation, favours bilayer packing over micelles.[33] We were also attracted to zwitterionic head groups, which are not cross-linked by Mg^{2+} or Ca^{2+} and are much-more stable to the ionic conditions necessary for RNA folding, catalysis and ligation with respect to acidic head groups.[34] Accordingly, we chose to investigate the acylation of glycerol-3-phosphocholine **10** as a model zwitterionic head group. Upon incubation of glycerol **10** with imidazole **1a** (10 eq.) we observed remarkably a low yield of diacetylation at neutral pH: only 15 and 40% at pH 7 and 8, respectively (Supplementary Information, Fig. S8). It is of note that glycerol **10** diacetylation is considerably less efficient than nucleotide **2** acetylation, even 20 equivalents of imidazole **1a** only furnished partial diacetylation (53%). Therefore, to investigate this apparent differentiation in nucleotide versus glycerol acetylation, we next incubated uridine-5'-phosphate **2b**/glycerol **10** (1:1) with imidazole **1a** (10 eq.) at pH 7. Intriguingly, we observed near-quantitative diacetylation of **2b** to afford diacyl-uridine-5'-phosphate **5b** (>90%; Supplementary Information, Fig. S11), and minimal diacetylation of **10** (9%), which demonstrates an efficient differentiation of RNA and lipid precursors by aqueous acetylation. We next turned our attention to long-chain acyl imidazoles (**1b-e**) that could potentially promote esterification and retard hydrolysis by aggregation.[35] Accordingly, we investigated the reaction of glycerol **10** with *N*-butyryl (**1b**), *N*-hexanoyl (**1c**), *N*-octanoyl (**1d**) and *N*-decanoyl imidazoles (**1e**).[36] Unsurprisingly, no reaction was observed in pure water due to insolubility of the long-chain acyl imidazole reagents. However, when water/acetonitrile (20:1 – 1:1) was used, to promote a biphasic assembly of *N*-hexanoyl, *N*-octanoyl or *N*-decanoyl imidazole (**1c-e**), vigorous stirring with **10** at pH 7.0 resulted in remarkably efficient long-chain diacylation (Fig. 1).

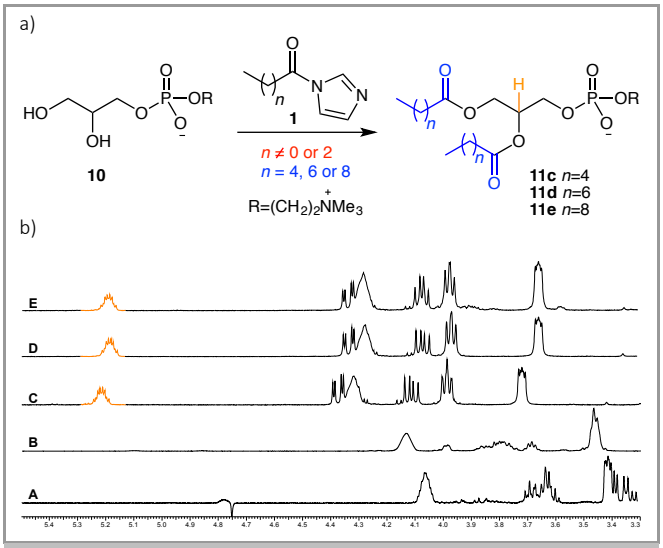


Figure 1 a) Long-chain selective diacylation of glycerol **10** with *N*-acyl imidazoles (**1c-e**). b) ^{1}H NMR spectra (400 MHz, 3.5 – 5.5 ppm) showing the reaction of glycerol **10 (100mM)** with: **A**) *N*-acetyl imidazole (**1a** *n*=0); **B**) *N*-butyryl imidazole (**1b** *n*=2); **C**) *N*-hexanoyl imidazole (**1c** *n*=4); **D**) *N*-octanoyl imidazole (**1d** *n*=6); **E**) *N*-decanoyl imidazole (**1e** *n*=8) in H_2O/MeCN (4:1), pH 7 and RT after 4 h.

We were also intrigued to observe a clear switch in reactivity between the short-chain (*n*=0 and 2) and long-chain (*n*=4, 6, and 8) acylation. Minimal short-chain diacylation (<5%) was observed at any concentration or water/acetonitrile ratio (20:1 – 4:1), whereas highly efficient synthesis of long-chain diacyl-glycerols **11c-e** (>95% NMR yield) was observed (Fig. 1).[37] Accordingly, we suggest that this reactivity switch, and apparent long-chain effect, may have been important during the selective assembly of prebiotic amphiphiles and vesicles. Further investigation into the mechanism of amphiphile self-assembly, which likely promotes long-chain diacylation through reagent/reactant co-localization, and the application of these model reactions into prebiotic amphiphile synthesis and vesicle assembly, nucleotide activation and photo-protection[38] are currently underway in our laboratory.

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Supporting Information

YES (this text will be updated with links prior to publication)

Primary Data

NO

References and Note

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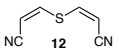
(21) Alongside the cytidine products precipitation of (2*Z*,2'*Z*)-3,3'-thiodiacrylonitrile (**12**) was observed.[17]



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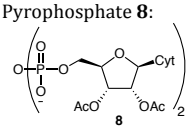
(23) Pyrophosphate **8**:



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(25) **General nucleoside acetylation protocol:** Nucleoside/nucleotide (**2**; 100mM) and *N*-acetyl imidazole (**1a**; 10 eq.) were dissolved in water (pH 8; adjusted with 4M NaOH). The solution was incubated at RT for 4 h and NMR spectra were periodically acquired. The product was purified by reverse-phase (C18) flash coumn chromatography (eluted at pH 4 with 100mM NH_4HCO_2/MeCN 98:2 to 80:20). The fractions containing **5** were lyophilised to yield a white powder.

Selected data: 2',3'-Di-*O*-acetyl-β-cytidine-5'-phosphate (5a) Starting from **1a** (160 mg, 0.50 mmol), **5a** (172 mg, 85%) was obtained as a white powder. ^{1}H NMR (600 MHz, D_2O) δ 8.08 (d, *J* = 7.9 Hz, 1 H, **H**-(C6)), 6.22 (d, *J* = 7.9 Hz, 1 H, **H**-(C5)), 6.11 (d, *J* = 5.1 Hz, 1 H, **H**-(C1')), 5.41 (dd, *J* = 5.4, 5.1 Hz, 1 H, **H**-(C2')), 5.38 (dd, *J* = 5.4, 4.4 Hz, 1 H, **H**-(C3')), 4.48 (ddd, *J* = 4.9, 4.4, 2.4 Hz, 1 H, **H**-(C4')), 4.13 (ABXY, *J* = 11.9, 4.4, 2.4 Hz, 1 H, **H**-(C5')), 4.02 (ABXY, *J* = 11.9, 4.9, 2.4 Hz, 1 H, **H**-(C5'')), 2.09 (s, 3 H, **Ac**-(C3')), 2.05 (s, 3 H, **Ac**-(C2')). ^{13}C NMR (151 MHz, D_2O) δ 173.4 (3'-OAc), 173.1 (2'-OAc), 160.7 (C4), 150.1 (C2), 144.3 (C6), 96.5 (C5), 88.2 (C1'), 82.5 (d, C4'), 74.5 (C2'), 71.5 (C3'), 64.4 (d, C5'), 20.6 (3'-OAc), 20.5 (2'-OAc). ^{31}P NMR (162 MHz, D_2O, ^{1}H-decoupled) δ 0.30. IR (neat, cm^{-1}) 1746, 1660, 1489, 1462, 1429, 1375, 1075. HRMS (ESI) (*m/z*): [M+H$^+$] calcd for $C_{13}H_{19}N_3O_{10}P$, 408.0803; found, 408.0810.

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(37) **Example procedure for glycerol-3-phosphocholine acylation (11d):**
Glycerol **10** (130 mg, 100mM) and *N*-octanoyl imidazole (**1d**; 10 eq.) were suspended in H_2O/MeCN (4:1). The resulting biphasic solution was stirred vigorously for 4 h and lyophilised. The residue was purified by SiO_2 flash coumn chromatography (CH_2Cl_2/MeOH 90:10 to 40:60 and then CH_2Cl_2/MeOH/H_2O (10%) 40:54:6 to 10:81:9). The fractions containing **11d** were concentrated in vacuo and purified by reverse-phase (C18) flash column chromatography (H_2O/MeOH 9:1 to 0:10). The fractions containing **11d** were lyophilised to yield 173 mg (67%) of 1,2-di-*O*-octanoyl-sn-glycero-3-phosphocholine (**11d**) as a white powder.
^{1}H NMR (600 MHz, CD_3OD) δ 5.09 - 5.16 (m, 1 H, **H**-(C2)), 4.32 (dd, *J* = 12.0, 3.3 Hz, 1 H, **H**-(C1)), 4.12 - 4.22 (m, 2 H, **H**-(C1′)), 4.07 (dd, *J* = 12.0, 6.8 Hz, 1 H, **H**-(C1)), 3.86 - 3.93 (m, 2 H, **H**-(C3)), 3.49 - 3.57 (m, 2 H, **H**-(C2′)), 3.12 (s, 9 H, $N(CH_3)_3$), 2.18 - 2.27 (m, 4 H, $COC\underline{H}_2$), 1.44 - 1.56 (m, 4 H, $C\underline{H}_2$), 1.15 - 1.28 (m, 16 H, $(C\underline{H}_2)_4$), 0.76 - 0.84 (m, 6 H, $CH_2C\underline{H}_3$). ^{13}C NMR (151 MHz, CD_3OD) δ 175.1 ($\underline{C}OCH_2$), 174.8 ($\underline{C}OCH_2$), 72.0 (C2), 67.6 (C2′), 65.0 (C3), 63.8 (C1), 60.6 (C1′), 54.8 ($N(CH_3)_3$), 35.2, 35.0 ($CO\underline{C}H_2$), 33.0, 30.3, 30.2, 26.2, 26.1, 23.8 ($(\underline{C}H_2)_6$), 14.57 ($CH_2\underline{C}H_3$). ^{31}P NMR (162 MHz, D_2O, ^{1}H-decoupled) δ -0.57. HRMS (ESI) (*m/z*): [M+H$^+$] calcd for $C_{24}H_{49}NO_8P$, 510.3190; found, 510.3193.

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