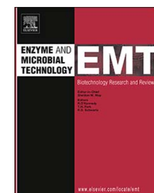
Enzyme and Microbial Technology 111 (2018) 1–6

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/enzmictec



An enzymatic alternative for the synthesis of nucleoside 5′-monophosphates

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ARTICLE INFO

Keywords:
Biocatalysis
Nucleosides
Nucleoside 5′-monophosphates
Phosphodiesterases
Phospholipases
Phosphorylation

ABSTRACT

A new procedure was carried out for the synthesis of nucleoside 5′-monophosphates, involving the use of two enzymes. The first step applied phospholipase D from *Streptomyces netropsis* and phosphatidylcholine as phosphatidyl donor, to give 5′-(3-*sn*-phosphatidyl) nucleosides (C, U, A, I). These were selectively hydrolysed in the second step by the action of phospholipase C from *Bacillus cereus* to produce the respective 5′-nucleotides. Application of this methodology on a preparative scale conducted to 5′-adenosine monophosphate in 63% overall yield from adenosine. The regioselectivity of these enzymes avoids protection steps, the overall synthesis is performed under mild reaction conditions and product isolation is easily achieved.

1. Introduction

Nucleoside analogues have numerous applications in medicine as antiviral and antitumor drugs, acting mainly as replication inhibitors [1,2]. Although these compounds are rapidly catabolised to inactive derivatives and have poor bioavailability, their pharmacokinetic behavior can be improved by the introduction of polar or ionic functions at the 5′-position [3,4]. Therefore preparation of nucleoside 5′-monophosphates (NMPs) has been extensively studied [5]. Some of these derivatives are also often used as food additives: inosine and guanosine 5′-monophosphate salts are flavour potentiators [6], and disodium salts of uridine, cytidine, adenosine and guanosine 5′-monophosphates are used in the preparation of pediatric formulas providing such high levels of nucleotides as in breast milk, reinforcing thus the baby immunity [7].

Owing to the importance of NMPs, numerous strategies for their preparation have been developed. The chemical syntheses involve the use of a phosphite ester obtained by the reaction of a phosphoramidite with a nucleoside, followed by oxidation [8], or the direct phosphorylation by chlorophosphates [3]. These procedures, which make use of harmful solvents and reagents, afford limited results due to either the need of protection steps or partial regioselectivity.

Natural nucleotides have also been obtained through the hydrolysis of nucleic acids by P_1 nuclease of *Penicillium citrinum* or β nuclease of *Ustilago maydis,* producing a mixture of the four natural NMPs that hampers their purification [9]. Another method of nucleotide production is fermentation: in this way inosine and uridine 5′-monophosphates can be obtained using *Corynebacterium ammoniagenes*. However, the principal disadvantages of this procedure are the low secretion of the nucleotides to the extracellular medium and the laborious and costly purification of the products [10–12]. NMPs can also be prepared by the action of nucleoside phosphotransferases, which catalyse the phosphorylation of nucleosides by low energy phosphate esters [13], and kinases [14], of limited application due to their high substrate specificity and the requirement of ATP as phosphate donor. Bacterial acid phosphatases have also been used in the synthesis of NMPs, from the corresponding nucleoside and tetrasodium pyrophosphate as the phosphate donor [15–17]. Besides, the phosphotransferase activity of these enzymes has been improved by molecular biology techniques, obtaining higher molar yields of inosine 5′-monophosphate (5′-IMP) [18]; in particular, 79% yield in 5′-IMP was obtained by using a mutant of *Escherichia blattae* acid phosphatase [19]. However, the purification step is still labourious due the presence of phosphoric acid, the byproduct of the reaction.

The synthesis of organic phosphomonoesters has been explored by D'Arrigo et al. [20], who proposed an indirect method including the use of two enzymes: phospholipase D (PLD) and phospholipase C (PLC). In addition to its natural hydrolytic activity, PLD has an outstanding position due to its transphosphatidylation potential (Scheme 1): in the presence of a small alcohol it can catalyse the exchange of the choline moiety of its natural substrate, phosphatidylcholine, affording a transphosphatidylation product. PLDs from bacterial sources, especially from *Streptomyces,* have been applied to the synthesis of such phosphatidyl derivatives including secondary and more complex alcohols [20–22]. On the other hand, PLC catalyses the hydrolysis of phosphatidylcholine producing a diglyceride and choline phosphate [23]. Thus, the sequential use of both phospholipases can produce several

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https://doi.org/10.1016/j.enzmictec.2017.12.004
Received 11 July 2017; Received in revised form 16 November 2017; Accepted 18 December 2017
Available online 19 December 2017
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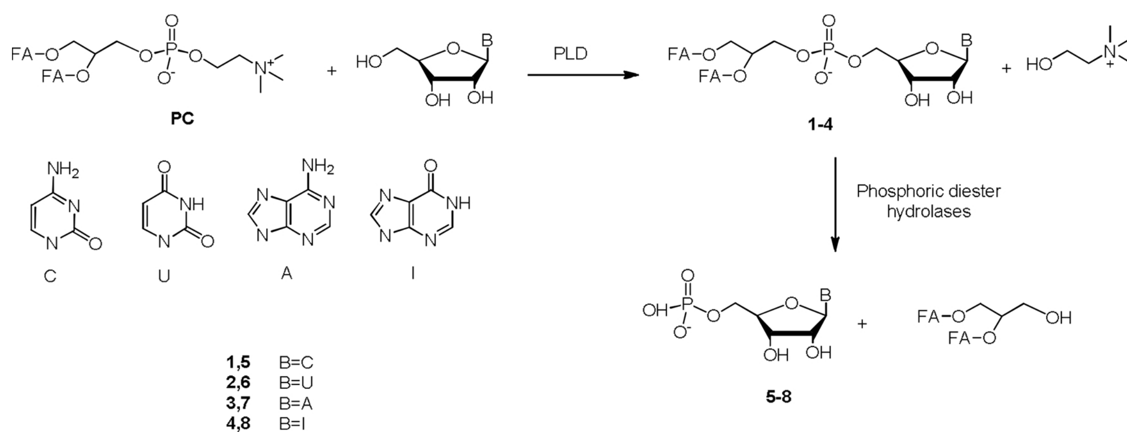

Scheme 1. Proposed enzymatic strategy for nucleoside 5′-monophosphates (NMPs, **5-8**) synthesis. FA: fatty acyl moiety; PLD: phospholipase D; PC: phosphatidylcholine.

phosphorylated organic compounds like glycerophosphate, dihydroxyacetone phosphate and synthetic lysophospholipids [24–26], but as far as we know, this strategy has not been applied to the synthesis of MNPs. Regarding the use of PLD in the field of nucleosides, the synthesis of diverse phosphatidylnucleosides has been reported [27,28]. In this paper, we report a procedure based on the utilization of PLD in the first step and PLC in the second hydrolytic step to get the respective nucleoside 5′-monophosphates (Scheme 1). Moreover, other enzymes with phosphodiesterase activity were evaluated as biocatalysts for the hydrolytic step of the synthesis.

2. Materials and methods

2.1. General

Phospholipase C from *Bacillus cereus* (type V, ≥200 U mg^{-1} solid), phospholipase C from *Clostridium perfringens* (type I, lyophilized, 10–15 U mg^{-1} protein), phosphodiesterase I from *Crotalus adamentus* venom (type VI, crude dried venom, ≥0.01 U mg^{-1} solid), phosphodiesterase I from *Crotalus atrox* venom (type IV, crude dried venom, ≥0.01 U mg^{-1} solid), phosphodiesterase I from *Bothrops atrox* (type V, crude dried venom, ≥0.01 U mg^{-1} solid), phosphodiesterase 3′,5′-cyclic-nucleotide-specific from bovine brain (lyophilized, 15–30 U mg^{-1} protein), Benzonase nuclease (≥ 250 U μl^{-1} buffered aqueous glycerol solution) and Nuclease S_1 from *Aspergillus oryzae* (50 KU mg^{-1} solid) were purchased from Sigma-Aldrich. The enzymes were used without any further treatment or purification. Phosphatidylcholine (95%) was purchased in Avanti Polar Lipids and soybean lecithin (Verolec F-62 TOP, Emulgrain) was provided by Gelfix (Argentina). Phospholipid fraction of lecithin used for reported preparative experiments was obtained by precipitation of phospholipids with acetone, followed by purification through alumina column chromatography, eluting the phospholipid fraction with chloroform/methanol 9:1.

All employed reagents and solvents were of analytical grade and obtained from commercial sources.

TLC was performed on Silica gel 60 F_{254} plates (Merck) and flash column chromatography was carried out using silica gel Merck 60.

For transphosphatidylation reactions, HPLC analyses (Gilson, 321 Pump, UV/VIS-156) were carried out by using a C-8 column (Grace; length: 150 mm; internal diameter: 4.6 mm; particle size: 5 μm). A gradient from ammonium acetate (0.2 M, pH 7)/methanol (60/40 v/v) to methanol was employed as the mobile phase, at a flow rate of 1 mL min^{-1} and detection at 260 nm. In the hydrolytic reactions, HPLC analyses were carried out using a C-18 column, the mobile phase consisted of triethylammonium acetate (0.1 M, pH 8,8) and acetonitrile (99/1 v/v); at a flow rate of 1 mL min^{-1} and detection at 260 nm.

NMR spectra were recorded on a Bruker AC-500 spectrometer, at 500 MHz for ^{1}H and 125 MHz for ^{13}C using DMSO-d_6 as solvent and TMS as internal standard or at 202 MHz for ^{31}P using D_2O as solvent and phosphoric acid as external standard.

2.2. Preparation of crude PLD from Streptomyces netropsis

The biocatalyst was obtained from culture broth filtrate of *Streptomyces netropsis*. The microorganism was grown at 30 °C for 72 h in a 500 mL Erlenmeyer flask containing 100 mL of a growth medium composed of yeast extract (2% w/v), glucose (0.5% w/v) and sulfate magnesium heptahydrate (0.1% w/v), at pH = 7.2. The mycelium was separated by filtration and the enzymatic crude was obtained by precipitation from the supernatant by addition of previously cooled acetone (–20 °C) until 60% of final volume. The mixture was maintained at 4 °C for 1 h and then centrifuged at 3500 rpm for 5 min at 4 °C to obtain the final enzymatic crude, which was dried at room temperature under reduced pressure.

2.3. PLD-catalysed transphosphatidylation reaction of nucleosides

Typically, the reaction media consisted of phosphatidylcholine (5 mg, PM = 775.09) dissolved in chloroform (670 μL, 9.63 mM) and 10 mM of the respective nucleoside in acetate buffer 50 mM, pH = 6 (330 μL). The reactions were initiated by the addition of 2 mg of *Streptomyces* PLD obtained as above described, and shaken at 37 °C or 45 °C and 200 pm. Aliquots were taken at different times and analyzed by HPLC. For preparative purposes phospholipid fraction isolated from commercial lecithin was employed; for the scale up of phosphatidyladenosine, the quantities above reported were scaled 50 times (reaction volume: 50 mL). When times for maximal phosphatidylnucleoside conversion were reached, the aqueous phase was extracted with CH_2Cl_2 (2 × 10 mL) and the organic phase evaporated at reduced pressure. The phosphatidylnucleosides were purified by silicagel column chromatography using a gradient of CH_2Cl_2/MeOH (from 95:5 to 70:30 v/v). By applying this procedure, the following previously reported products [29,30] were obtained:

5′-(3-sn-phosphatidyl)cytidine (**1**)

NMR-^{1}H (DMSO-d_6, 500 MHz): δ 0,95–0.97 (m, 6H, $-CH_3$s); 1,28–1,32 (m, aliphatic CH_2s); 1,58–1,62 (m, 4H, $\underline{CH_2}-CH_2-COO$); 2,39–2,44 (m, 4H, $CH_2-\underline{CH_2}-COO$); 3,82–3,87 (m, 4H, *sn*-3-CH_2, H5′$_a$, H5′$_b$); 3,92–3,96 (m, 1H, H2′, H3′); 3,99 (t, 1H, *J* = 4,6 Hz, H2′); 4,22 (dd, 1H, J_1 = 11,7, J_2 = 5,7 Hz, *sn*-1-CH_2); 4,49 (dd, 1H, J_1 = 4,1, J_2 = 1,1 Hz, *sn*-1-CH_2); 5,22–5,25 (m, 1H, *sn*-2-CH); 5,74 (d, 1H, *J* = 8,2 Hz, H5); 5,88 (d, 1H, *J* = 5,5, H1′); 8.00 (d, 1H, *J* = 8,2 Hz, H6).

NMR-^{13}C (DMSO-d_6, 125 MHz): δ 14,21 (CH_3); 25,32 ($\underline{C}H_2-CH_2-COO$); 27,56 ($\underline{C}H_2-C{=}C$); 29,58–32,36 (aliphatic CH_2s); 34,48; 34,62 ($\underline{C}H_2-COO$); 63,02 (*sn*-1-CH_2); 64,06 (*sn*-3-CH_2); 64,14

(C-5′); 69,30 (C-3′); 70,91 (*sn*-2-CH); 75,67 (C-2′); 83,49 (C-4′); 91,03 (C-1′); 95,58 (C-5); 130,12 (C=C); 143,69 (C-6); 155,86 (C-2); 165,24 (C-4);172,02; 174,44 (COs).

5′-(3-sn-phosphatidyl) adenosine (**2**)

RMN-^{1}H (DMSO-d_6, 500 MHz): δ 0,83–0.92 (m, CH_3s); 1,27–1,34 (m, aliphatic CH_2s); 3,80–3,82 (m, 2H, H5′$_a$, H5′$_b$); 3,88–3,91 (m, 2H, *sn*-3-CH_2); 4.06–4,09 (m, 1H, H4′); 4.19 (dd, 1H, J_1 = 11,9 J_2 = 7,1 Hz, *sn*-1-CH_2); 4.26 (t, 1H, J = 5,7 Hz, H3′); 4.35 (dd, 1H, J_1 = 11,9, J_2 = 2,7 Hz, *sn*-1-CH_2); 4.65 (t, 1H, J = 5,7 Hz, H2′); 5,09–5,12 (m, 1H, *sn*-2-CH); 5.98 (d, 1H, J = 5,7 Hz, H1′); 7.31 (s, 2H, −NH_2); 8.20 (s, 1H, H2); 8.50 (s, 1H, H8).

NMR-^{13}C (DMSO-d_6, 125 MHz): δ 13,89 (CH_3); 24,38; 24,45 ($\underline{C}H_2$−CH_2−COO); 26,62; 26,65 ($\underline{C}H_2$−C=C); 28,42–31,32 (aliphatic CH_2s); 33,37; 33,58 ($\underline{C}H_2$−COO); 62,20 (*sn*-1-CH_2); 62,37 (C-5′); 64,45 (*sn*-3-CH_2); 70,45 (*sn*-2-CH); 70,92 (C-3′); 73,99 (C-2′); 83,94 (C-4′); 86,84 (C-1′); 118,83 (C5$_a$); 127,72 (C=C); 139,30 (C-8); 149,66 (C-4); 152,59 (C-2); 155,97 (C-6);172,51; 172,26 (COs).

5′-(3-sn-phosphatidyl)uridine (**3**)

NMR-^{1}H (DMSO-d_6, 500 MHz): δ 0,96-0.98 (m, −CH_3s); 1,29–1,36 (m, aliphatic CH_2s); 1,60–1,64 (m, 4H, $\underline{C}\underline{H}_2$−$CH_2$−COO); 2,34–2,38 (m, 4H, CH_2−$C\underline{H}_2$−COO); 3,95–3,99 (m, 4H, *sn*-3-CH_2, H5′$_a$, H5′$_b$); 4,04–4,07 (m, 1H, H2′); 4,10–4,12 (m, 1H, H3′); 4,21 (dd, 1H, J_1 = 11,8, J_2 = 7,2 Hz, *sn*-1-CH_2); 4,24–4,26 (m, 2H, H4′, *sn*-1-CH_2); 5.21 (m, 1H, *sn*-2-CH); 5.66 (d, 1H, J = 8,0, H5); 6.12 (d, 1H, J = 4,6 Hz, H1′); 8.05 (d, 1H, J = 8,0 Hz, H6); 11,37 (s, 1H, −NH).

NMR-^{13}C (DMSO-d_6, 125 MHz): δ 13,89 (CH_3); 24,40; 24,48 ($\underline{C}H_2$−CH_2−COO); 26,62; 26,65 ($\underline{C}H_2$−C=C); 28,37–31,31 (aliphatic CH_2s); 33,39; 33,58 ($\underline{C}H_2$−COO); 62,41 (*sn*-1-CH_2); 62,56 (*sn*-3-CH_2); 64,22 (C-5′); 67,41 (*sn*-2-CH); 70,46 (C-3′); 74,24 (C-2′); 83,28 (C-4′); 85,18 (C-1′); 99,93 (C-5); 129,62; 129,69 (C=C); 142,34 (C-6); 150,43 (C-2); 163,30 (C-4);172,20; 172,26 (COs).

5′-(3-sn-phosphatidyl)inosine (**4**)

NMR-^{1}H (DMSO-d_6, 500 MHz): δ 0,82-085 (m, −CH_3s); 1,23–1,30 (m, aliphatic CH_2s); 1,45–1,48 (m, 4H, $C\underline{H}_2$−CH_2−COO); 2,20–2,24 (m, 4H, CH_2−$C\underline{H}_2$−COO); 3,75–3,77 (m, 2H, H5′$_a$, H5′$_b$); 3,80–3,82 (m, 2H, *sn*-3-CH_2); 4,00–4,04 (m, 2H, H4′ *sn*-1-CH_2); 4.17 (q, 1H, J = 5,6 Hz, H3′); 4.28 (dd, 1H, J_1 = 12,0, J_2 = 3,1 Hz, *sn*-1-CH_2); 4.53 (q, 1H, J = 5,6 Hz, H2′); 5,03–5,06 (m, 1H, *sn*-2-CH); 5.89 (d, 1H, J = 5,6 Hz, H1′); 8.05 (s, 1H, H2); 8.37 (s, 1H, H8).

NMR-^{13}C (DMSO-d_6, 125 MHz): δ 13,60 (CH_3); 27,31–31,58 (aliphatic CH_2s); 32,31; 33,21 ($\underline{C}H_2$COO); 65,37 (C-5′); 63,98 (*sn*-3-CH_2); 69,35 (*sn*-2-CH); 70,30 (C-3′); 73,42 (C-2′); 86,74 (C-4′); 96,84 (C-1′); 115,83 (C5); 140,30 (C-8); 151,66 (C-4); 152,40 (C2); 158.78 (C-6);171,21; 172,50 (COs).

2.4. PLD-catalysed transphosphatidylation reaction of nucleosides with surfactants

In this case, the reaction protocol was similar to that above described for biphasic transphosphatidylation, the only modification being the addition of surfactant to the aqueous solution, which also contained Triton X-100 or Tween 20. The resulting mixtures were shaken at 200 rpm and 45 °C. Aliquots were taken at different times and analysed by HPLC.

2.5. Enzymatic hydrolysis of 5′-(3-sn-phosphatidyl)-nucleosides

2.5.1. CHCl3/buffer reaction system

Typically, the phosphatidylnucleoside (6 mg, 21.7-22.3 mM, depending on the assayed phosphatidylnucleoside) was dissolved in chloroform (300 µL) and the enzyme (1U) in the corresponding buffer (600 µL) was added: PLC from *Bacillus cereus* in 100 mM HEPES, 50 mM, pH 7.5; Nuclease S_1, in 50 mM sodium acetate, 300 mM NaCl, 5 mM $ZnSO_4$, pH 4.5; for the other assayed enzymes the buffer was 10 mM tris-HCl, 10 mM $MgCl_2$, pH 8.9. The different mixtures were incubated in an orbital shaker at 200 rpm and 37 °C, aliquots were taken at different times and analysed by HPLC using a C-18 column. For analytical purposes, TLC was developed employing *n*-propanol/ammonia/H_2O (15:11:2.25, v/v/v) as the mobile phase and plates visualised under UV.

2.5.2. Buffer reaction system

The preparation of phosphatidylnucleoside liposomes was done by the lipid hydration method [31]. Typically, a dry phosphatidylnucleoside film (6 mg, 21.7–22.3 mM, depending on the assayed phosphatidylnucleoside) was dispersed in each of the previously mentioned buffers. Phosphatidylnucleosides spontaneously form polydisperse vesicular suspensions by simply shaking. Then, the samples were sonicated in a bath type sonicator for 30 min at room temperature. The reactions were initiated by addition of 1 U of each enzyme and maintained at 37 °C and 200 rpm. Aliquots were taken at different times and after addition of dichloromethane, the resulting aqueous phase was separated and analyzed by TLC and HPLC using the same previously mentioned method.

In all cases, HPLC retention times for the obtained nucleoside 5′-monophosphates were compared with those of the commercially available samples.

2.6. Preparative synthesis of adenosine 5′-monophosphate (7)

The transphosphatidylation medium reaction consisted of 260 mg of phosphatidylcholine dissolved in 33 mL of $CHCl_3$ and 17 mL of acetate buffer (50 mM, pH = 6) containing 45 mg of adenosine (9.9 mM). The reaction was initiated with the addition of 102 mg of PLD and kept at 37 °C and 200 rpm for 24 h. The product was purified by silicagel column chromatography as above described for **1** obtaining 124 mg of 5′-(3-*sn*-phosphatidyl)adenosine.

The hydrolysis of the intermediate was performed in 10 mL of TRIS buffer, 50 mM, pH = 8 and was initiated with the addition of 20 U of PLC from *Bacillus cereus*. The reaction was kept at 37 °C for 6 days and extracted with dichloromethane (2 × 5 mL). The produced AMP was precipitated by the addition of 3.6 mL of 0.5 M $CaCl_2$ and cold ethanol (final concentration 75% v/v) to the resulting aqueous phase. After drying at reduced pressure over P_2O_5, 33 mg of a white solid was obtained (97% of recovery, determined by HPLC), corresponding to 63% yield based on initial adenosine.

Adenosine 5′-monophosphate (**7**): NMR-^{31}P (D_2O, 202 MHz): δ 3.84.

3. Results and discussion

3.1. PLD-catalysed transphosphatidylation of nucleosides

The PLD source was selected through a screening of different *Streptomyces* strains; the reaction medium consisted of a biphasic system: phosphatidylcholine dissolved in chloroform and the concentrated extracellular medium of each strain containing cytidine, chosen as model of nucleoside. By this way, *Streptomyces netropsis* was selected as PLD source and the crude of this enzyme was obtained by acetone precipitation from the extracellular medium [13].

Given that the catalytic mechanism of PLD involves the formation of an enzyme-substrate complex, which can be attacked either by an alcohol producing the transphosphatidylation product or by water yielding phosphatidic acid, the concentration of the phospholipid and the nucleophile influences the final quantity of the synthetic product. This effect was evaluated by varying the nucleoside/phosphatidylcholine (N/P) molar ratio, the obtained results are shown in Table 1. Taking into account that an excess of the more expensive nucleoside is not desirable, the N/P = 0.5 was chosen for preparative purposes. Moreover, decrease of phosphatidylnucleoside conversion at 48 h (26%) compared with 26 h (39%) is to be explained by hydrolysis and shows that reaction time must be controlled to obtain the maximal conversion of the desired product.

Table 1
Effect of the nucleoside/phosphatidylcholine (N/P) molar ratio on the conversion of PLD-catalysed reaction of cytidine.

N/P	Conversion into 5′-(3-*sn*-phosphatidyl)cytidine (**1**) (%)[a]
10	2 (20[b])
5	13 (65[b])
1	5
0.5	39
0.5	26[c]
0.1	27

[a] Determined at 26 h by HPLC on the basis of nucleoside concentration.
[b] Determined on the basis of the limiting reagent (phosphatidylcholine).
[c] Determined at 48 h by HPLC on the basis of nucleoside concentration.

Table 2
Transphosphatidylation of nucleosides catalysed by PLD from *Streptomyces netropsis* at 37 °C (Scheme 1).

Phosphatidyl acceptor	Product	Conversion (%)[a]	t (h)
Cytidine	**1**	39	26
		26	48
Uridine	**2**	56	20
		50	48
Adenosine	**3**	84	6
		55	24
		28	48
Inosine	**4**	25	24
		20	48

[a] Determined by HPLC.

In addition to cytidine, other representative nucleosides were also assayed in the PLD- catalysed transphosphatidylation, as intermediates in the NMPs synthetic route (Table 2).

PLD catalysed regioselectively the transfer reaction of the phosphatidyl residue to the primary hydroxyl group of the tested nucleosides. The maximum conversions were obtained at 6–26 h (Table 2), adenosine affording the highest production in the shortest time. Then the phosphatidylnucleoside concentration decreased slightly for three of the tested phosphatidylnucleosides and sharply for the adenosine derivative. This behaviour indicates again that phosphatidylnucleoside reaches a maximal conversion and undergoes hydrolysis afterwards. For those nucleosides that afforded lower conversions at 37 °C, the effect of raising the temperature to 45 °C (Table 3) was assessed. As observed, the reaction velocity was accelerated and the best results were obtained within 2 h for the tested nucleosides. In general, the obtained conversions were higher than 50%, but at longer times the product concentration decreased to 20–30% and then no further reaction was observed. Various factors such as phosphatidylnucleoside hydrolysis, nucleophile recognition, balancing of the competitive reactions and nucleoside solubility may be responsible for the observed results.

Table 3
Effect of the addition of nonionic detergents (Triton X-100 and Tween 20) on the maximum conversions obtained in *Streptomyces netropsis* PLD-catalysed transphosphatidylation of ribonucleosides at 45 °C.

Phosphatidyl acceptor	Product	(%)[a]	Triton X-100		Tween 20	
			conversion (%)[b]	t(h)	conversion (%)[b]	t(h)
Cytidine	**1**	0	61	2	61	2
		0.1	52	4	34	1
		0.5	35	4	68	2
Uridine	**2**	0	56	1	56	1
		0.1	55	4	24	1
		0.5	24	2	66	2
Inosine	**4**	0	67	2	67	2
		0.1	39	4	42	2
		0.5	36	6	71	2

[a] Percentage of the surfactant in the reaction mixture (v/v).
[b] Determined by HPLC.

The addition of surfactants was also evaluated at 45 °C, since they can promote the formation of emulsions that could increase the effective catalytic interface. Given that the presence of ionic detergents can negatively affect the enzyme activity [32], two nonionic detergents, Triton X-100 and Tween 20, were assessed in concentrations below 1% (Table 3).

The presence of each detergent had different effects on the reaction profile, however, regardless of the nucleoside acting as nucleophile, at 24 h no further conversion was observed. This was probably due to a deleterious effect exerted by the surfactant on the protein structure. Triton X-100 had a negative effect on the formation of phosphatidylnucleosides but the addition of Tween 20 (0.5 v/v) increased the conversion of the tested nucleosides.

3.2. Enzymatic hydrolysis of 5′-(3-sn-phosphatidyl)-nucleosides to nucleoside 5′-monophosphates (5–8)

For the final step of the proposed synthetic strategy (Scheme 1), the regioselective cleavage of the phosphodiester bonds of the intermediate phosphatidylnucleosides was assayed using several phosphoric diester hydrolases in order to obtain the corresponding NMPs. Benzonase, 3′,5′-cyclic-nucleotide-specific phos-phodiesterase from bovine brain, phospholipase C from *Clostridium perfringens* (PLC_{CP}) and phosphodiesterase I from *Bothrops atrox* did not catalyse the hydrolytic reaction (data not shown). Although PLC_{CP} is able to hydrolyze different amphiphilic compounds such as phosphatidylcholine, lysophosphatidylcholine, sphingomyelin and phosphatidylserine [19], the results obtained with this enzyme may be attributed to differences in structure and polarity between natural substrates and the tested compounds. The phosphodiesterase I from *Crotalus adamentus* catalysed the phosphodiester bond hydrolysis of two substrates, 5′-(3-*sn*-phosphatidyl)uridine (**2**) and 5′-(3-*sn*-phosphatidyl)cytidine (**1**), but for the first intermediate the reaction was not selective, giving rise also to the formation of uridine, whereas with the cytidine derivative only free nucleoside was formed, in all cases with low conversions. With phosphodiesterase I from *Crotalus atrox*, the same phosphatidylnucleosides were hydrolysed, producing only 32% and 3% of uridine and cytidine respectively; while with Nuclease S_1 only **2** reacted, giving 3% of uridine.

On the other hand, phospholipase C from *Bacillus cereus* (PLC_{BC}) was able to selectively hydrolyse the phosphodiester bond to form the NMPs from the tested derivatives; maximum conversions obtained after reaching a stationaty stage are shown in Table 4.

In addition to chloroform, the use of other organic solvents as phosphatidylnucleoside reservoir was also evaluated, the options being

Table 4
Hydrolysis of 5′-(3-*sn*-phosphatidyl)nucleosides (**1-4**) catalysed by PLC_{BC} (Scheme 1).

Substrate	5′-Phosphorylated nucleoside (product)	Reaction system[a]	Conversion (%)[b]
1	Cytidine (**5**)	$CHCl_3$/buffer	23 (26)[c]
1	Cytidine (**5**)	Buffer[d]	72 (144)
2	Uridine (**6**)	$CHCl_3$/buffer	35 (31)
2	Uridine (**6**)	Buffer[d]	82 (120)
3	Adenosine (**7**)	$CHCl_3$/buffer	41 (48)
3	Adenosine (**7**)	Buffer[d]	75 (140)
4	Inosine (**8**)	$CHCl_3$/buffer	0
4	Inosine (**8**)	Buffer[d]	73 (144)

[a] Buffer: 100 mM HEPES, 50 mM $CaCl_2$, pH = 7.5.
[b] Determined by HPLC.
[c] In parenthesis, the time of maximum conversion in hours.
[d] The phosphatidylnucleosides were employed as liposomes.

Table 5
Results obtained in the synthesis of nucleoside 5′-monophosphates (**5-8**) through the consecutive reactions catalysed by PLD and PLC.

Nucleoside	Product	PLD[a]	PLC[a]	Overall conv (%)
Cytidine	**5**	68	72	49
Uridine	**6**	66	82	54
Adenosine	**7**	84	75	63
Inosine	**8**	71	73	52

[a] Conversion determined by HPLC.

limited due to the low solubility of these derivatives. Ethers are commonly used to dissolve phospholipids, thus methyl *tert*-butyl ether (MTBE) was assayed. The obtained results (data not shown) differ from those observed with chloroform, showing a marked decrease in conversions of adenosine and uridine 5′-monophosphates. These results suggest that chloroform was more convenient as phospholipid reservoir than MTBE.

The amphiphilic nature of compounds (**5-8**) makes them able to form micellar structures in aqueous media. These structural arrangements are natural targets for PLC_{BC} and the use of these systems could be more suitable than organic solvent solutions since negative effects of a liquid–liquid interface on the enzyme conformation can be avoided. Therefore, liposomes of these derivatives were prepared through the lipid hydration method [32] and hydrolysis was assayed. All the enzymes with phosphodiesterase activity were again evaluated as biocatalysts and once more, only the use of PLC_{BC} afforded the desired compounds (Table 4), giving better conversions compared with the $CHCl_3$/buffer reaction systems. These results were obtained at longer periods of time; for example 5′-(3-*sn*-phosphatidyl)cytidine showed the maximum production of cytidine 5′-monophosphate in 6 days. In none of the tested cases the reaction was complete, a fact that may be attributed to the accumulation in the reaction medium of 1,2-diacyglycerol, a byproduct of the hydrolysis. Bangham et al. [31] have shown that this compound is a potent PLC inhibitor and observed that the lecithin hydrolysis catalysed by this enzyme stops before reaching total consumption of the substrate.

Taking into account the thermodynamic instability of the involved supramolecular arrangements, temperatures higher than 37 °C were not assayed.

Table 5 summarizes the whole results considering both stages of the proposed synthetic strategy.

For preparative purposes, adenosine 5′-monophosphate has been synthesised in a larger scale. Only one chromatographic purification was necessary, in the first step of the overall reaction. In the present methodology, the final reaction mixture consists of unreacted phosphatidylnucleoside, the byproduct 1,2-diacylglycerol and of the corresponding 5′-nucleoside monophosphate, the latter being the only species that remains in the aqueous phase. This contrasts with usual procedures for nucleoside phosphorylation, either chemical or biocatalysed, in which high amounts of inorganic phosphate and unreacted nucleoside are present making isolation and purification cumbersome. Therefore, the phosphorylated target product could be easily precipitated from the buffer solution with calcium chloride, Tris buffer was employed for the hydrolytic step to avoid buffer salt co-precipitation. By this way, it was possible to recover 97% of AMP present in the aqueous solution, obtaining the calcium salt of AMP in 58% overall yield from adenosine.

4. Conclusions

An alternative enzymatic procedure for the preparation of nucleoside 5′-monophosphates was developed. The method consists of two enzymes acting consecutively: phospholipase D transfers a phosphatidyl residue to the 5′-hydroxyl of nucleosides and then phospholipase C catalyses a selective hydrolysis of the phosphodiester bond producing nucleoside 5′-monophosphates. The regioselectivity of these enzymes avoids the requirement of protection steps and allows an easy final purification by precipitation of the nucleoside 5′-monophosphate.

Acknowledgements

We thank UNQ and ANPCyT for partial financial support. LEI and AMI are members of the scientific research career of CONICET. JYS is a CONICET fellow.

References

[1] E. De Clercq, Antiviral drugs in current clinical use, J. Clin. Virol. 30 (2004) 115–133.
[2] E. De Clercq, H.J. Field, Antiviral prodrugs –the development of successful prodrug strategies for antiviral chemotherapy, Br. J. Pharmacol. 147 (2006) 1–11.
[3] R.J. Jones, N. Bischoefberger, Nucleotide prodrugs, Antivir. Res. 27 (1995) 1–17.
[4] L.E. Iglesias, E.S. Lewkowicz, R. Medici, P. Bianchi, A.M. Iribarren, Biocatalytic approaches applied to the synthesis of nucleoside prodrugs, Biotech. Adv. 33 (2015) 412–434.
[5] E. De Clercq, Antiviral drug discovery and development: where chemistry meets with biomedicine, Antivir. Res. 67 (2005) 56–75.
[6] S. Yamaguchi, K. Ninomiya, Unami and food palatability, J. Nutr. 130 (2000) 921–926.
[7] B.D. Gill, H.E. Indyk, Determination of nucleotides and nucleosides in milks and pediatric formulas: a review, J. AOAC Int. 90 (2007) 1354–1364.
[8] E.E. Nifantiev, M.K. Grachev, S.Y. Burmistrov, Amides of trivalent phosphorus acids as phosphorylating reagents for proton-donating nucleophiles, Chem. Rev. 100 (2000) 3755–3800.
[9] S.U. Gite, V. Shankar, Single-strand-specific nucleases, Crit. Rev. Microbiol. 21 (1995) 101–122.
[10] X. Wang, M. Yin, Z. Xiao, C. Ma, Z. Lin, P.G. Wang, P. Xu, Production of uridine 5'-monophosphate by *Corynebacterium ammoniagenes* ATCC 6872 using a statistically improved biocatalytic process, Appl. Microbiol. Biotechnol. 76 (2007) 321–328.
[11] S. Teshiba, A. Furuya, Agric. Biol. Chem. 46 (1982) 2257–2263.
[12] B. Abbouni, H.M. Elhariry, G. Auling, Overproduction of NAD+ and 5′-inosine monophosphate in the presence of 10 (M Mn^{2+} by a mutant of *Corynebacterium ammoniagenes* with thermosensitive nucleotide reduction (nrdts) after temperature shift, Arch. Microbiol. 182 (2004) 119–125.
[13] L.L. Birichevskaya, S.V. Kvach, G.G. Sivets, E.N. Kalinichenko, A.I. Zinchenko, I. Mikhailopoulo, A comparison of enzymatic phosphorylation and phosphatidylation of beta-L- and beta-D-nucleosides, Biotechnol. Lett. 29 (2007) 585–5891.
[14] H. Mori, A. Iida, T. Fujio, S. Teshiba, A novel process of inosine 5'-monophosphate production using overexpressed guanosine/inosine kinase, Appl. Microbiol. Biotechnol. 48 (1997) 693–698.
[15] Y. Asano, Y. Mihara, H. Yamada, A novel selective nucleoside phosphorylating enzyme from *Morganella morganii*, J. Biosci. Bioeng. 87 (1999) 732–738.
[16] L. Babich, A.F. Hartog, M. van der Horst, R. Wever, Continuous-flow reactor-based enzymatic synthesis of phosphorylated compounds on large scale, Chem. Eur. J. 18 (2012) 6604–6609.
[17] R. Médici, J.I. Garaycoechea, A.L. Valino, C.A. Pereira, E.S. Lewkowicz, A.M. Iribarren, A comparative study on phosphotransferase activity of acid phosphatases from *Raoultella planticola* and *Enterobacter aerogenes* on nucleosides, sugars and related compounds, Appl. Microb. Biotechnol. 98 (2014) 3013–3022.
[18] K. Ishikawa, Y. Mihara, N. Shimba, N. Ohtsu, H. Kawasaki, E. Suzuki, Y. Asano, Enhancement of nucleoside phosphorylation activity in an acid phosphatase, Protein Eng. 15 (2002) 539–543.
[19] Y. Mihara, K. Ishikawa, E. Suzuki, Y. Asano, Improving the pyrophosphate-inosine phosphotransferase activity of *Escherichia blattae* acid phosphatase by sequential site-directed mutagenesis, Biosci. Biotechnol. Biochem. 68 (2004) 1046–1050.
[20] P. D'Arrigo, L. De Ferra, V. Piergianni, A. Selva, S. Servi, A. Strini, Preparative transformation of natural phospholipids catalysed by phospholipase D from *Streptomyces*, J. Chem. Soc. Perkin Trans. 1 (1996) 2651–2656.
[21] R. Ulbrich-Hofmann, Enzyme-catalysed transphosphatidylation, Eur. J. Lipid Sci. Technol. 105 (2003) 305–308.
[22] M. Waite, The PLD superfamily: insights into catalysis, Biochim. Biophys. Acta 1439 (1999) 187–197.
[23] R.W. Titball, Bacterial phospholipases C, Microbiol. Rev. 57 (1993) 347–366.
[24] P. D'Arrigo, V. Piergianni, G. Pedrocchi-Fantoni, S. Servi, Indirect enzymatic phosphorylation: preparation of dihydroxyacetone phosphate, J. Chem. Soc. Chem. Commun. 250 (6) (1995) 2505–2506.
[25] M. Shinitzky, P. Friedman, R. Haimovitz, Formation of 1,3-cyclic glycerophosphate by the action of phospholipase C on phosphatidylglycerol, J. Biol. Chem. 268 (1993) 14109–14115.
[26] C. Virto, P. Adlercreutz, Two-enzyme system for the synthesis for 1-lauroyl-rac-glycerophosphate (lysophosphatidic acid) and 1-lauroyl-dihydroxyacetonephosphate, Chem. Phys. Lipids 104 (2000) 175–184.
[27] S. Shuto, H.A. Itoh, Sakai, K. Nakagami, S. Imamura, A. Matsuda, Nucleosides and nucleotides—CXXXVII. Antitumor phospholipids with 5-fluorouridine as a cytotoxic polar-head: synthesis of 5-phosphatidyl-5-fluorouridines by phospholipase D-catalyzed transphosphatidylation, Bioorg. Med. Chem. 3 (1995) 235–243.

[28] S. Shuto, H. Itoh, S. Ueda, S. Imamura, K. Fukukawa, M. Tsujino, A. Matsuda, T. Ueda, A facile enzymatic synthesis of 5'-(3-sn-phosphatidyl)nucleosides and their antileukemic activities, Chem. Pharm. Bull. 36 (1988) 209–217.
[29] D. Berti, P. Baglioni, S. Bonaccio, G. Barsacchi-Bo, P.L. Luisi, Base complementarity and nucleoside recognition in phosphatidylnucleoside vesicles, J. Phys. Chem. B 102 (1998) 303–308.
[30] D.N. Rubingh, The influence of surfactants on enzyme activity, Curr. Opin. Colloid Interface Sci. 1 (1996) 598–603.
[31] A. Bangham, R. Dawson, Electrokinetic requirements for the reaction between *Cl. perfringens* α-toxin (phospholipase C) and phospholipid substrates, Biochim. Biophys. Acta 59 (1962) 103–115.
[32] V. Torchilin, V. Weissig, Liposomes A Practical Approach, 2nd ed., Oxford University Press, USA, 2003.