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

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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 by-product 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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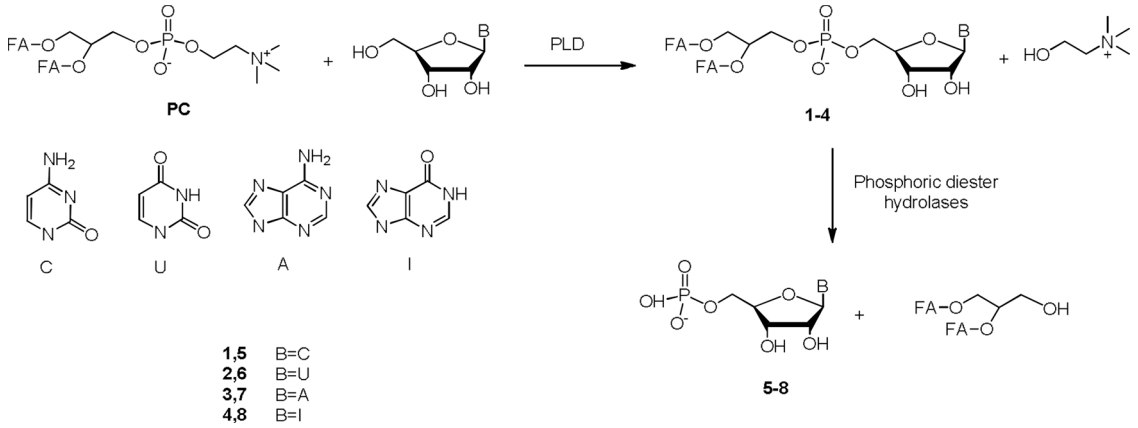

Scheme 1. Proposed enzymatic strategy for nucleoside 5′-monophosphates (NMPs, **5-8**) synthesis. FA: fatty acyl moiety; PLD: phospholipase D; PC: phosphatidylcholine.

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, $C\underline{H}_2-CH_2-COO$); 2,39–2,44 (m, 4H, $CH_2-C\underline{H}_2-COO$); 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