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# Chemo-enzymatic synthesis of α-D-pentofuranose-1-phosphates using thermostable pyrimidine nucleoside phosphorylases

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ABSTRACT

α-D-pentofuranose-1-phosphates (Pentose-1Ps) are key intermediates in nucleoside metabolism and important precursors for the enzymatic synthesis of modified nucleosides. To date, Pentose-1Ps are mainly produced by chemical approaches which have numerous disadvantages. Therefore, several enzymatic methods employing mesophilic enzymes have been developed but are not widely applied due to their limited substrate spectrum. Here we report the use of thermostable nucleoside phosphorylases for the chemo-enzymatic synthesis of modified Pentose-1Ps (2-deoxy-2-fluoro-α-D-ribofuranose-1-phosphate, α-D-arabinofuranose-1-phosphate, and 2-deoxy-2-fluoro-α-D-arabinofuranose-1-phosphate), which are interesting building blocks for the synthesis of modified nucleosides. After optimizing the synthesis protocol using the natural substrates uridine and thymidine, grams of modified Pentose-1Ps were purified as their Ba-salts with over 95% purity. Their structures were confirmed by NMR spectroscopy and the temperature and pH stability of natural and modified Pentose-1Ps in aqueous solution was -evaluated. Four of the Pentose-1P-Ba salts were stable with no visible degradation up to 60 °C and pH above 5, while 2-deoxy-α-D-ribofuranose-1-phosphate was less stable. The presented protocol provides an easy, fast, and environmentally-friendly method to produce grams of modified Pentose-1P-Ba salts of high purity.

## 1. Introduction

α-D-pentofuranose-1-phosphates (Pentose-1Ps) are essential building blocks of larger biomolecules and are known to be involved in many physiological pathways, including energy storage and nucleoside metabolism. They can be processed further and incorporated in pathways for histidine or riboflavin synthesis [1]. Among them, 2-deoxy-α-D-ribofuranose-1-phosphate (dRib-1P) is synthesized from thymidine by phosphorolytic cleavage by thymidine phosphorylase (TP), an enzyme involved in nucleoside metabolism, angiogenesis and the degradation of antiviral agents [2]. TP was shown to be highly expressed in some cancer cells, leading to increased levels of dRib-1P and its corresponding sugar (2-deoxy-D-ribose) [3,4], which promotes resistance to hypoxia-induced apoptosis.

Several analogues of 2-deoxyribose-1-phosphate were tested for their biological activity against HIV or cancer cell proliferation [5,6]. While 2-deoxyribose-1-phosphate derivatives showed no or only low activity, the free carbosugars revealed micromolar activity against HIV.

*Abbreviations:* Pentose-1Ps, α-D-pentofuranose-1-phosphates; NPs, nucleoside phosphorylases; Rib-1P, α-D-ribofuranose-1-phosphate; dRib-1P, 2-deoxy-α-D-ribofuranose-1-phosphate; Rib-5P, D-ribose-5-phosphate; Ara-1P, α-D-arabinofuranose-1-phosphate; $_{2F}$Rib-1P, 2-deoxy-2-fluoro-α-D-ribofuranose-1-phosphate; $^{2F}$Ara-1P, 2-deoxy-2-fluoro-α-D-arabinofuranose-1-phosphate; Urd, uridine; Ura, uracil; Thd, thymidine; Ara-U, 1-(β-d-arabinofuranosyl)uracil; $^{2F}$Ara-U, 1(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)uracil; $_{2F}$Ribo-U, 2′-deoxy-2′-fluorouridine; O-2,2′-anhydro-D-uridine, Anhydro-U; KP, potassium phosphate buffer; Pentose-1P-Ba, barium salt of α-D-pentofuranose-1-phosphates; Rib-1P-Ba, barium salt of α-D-ribofuranose-1-phosphate; dRib-1P-Ba, barium salt of 2-deoxy-α-D-ribofuranose-1-phosphate; Ara-1P-Ba, barium salt of α-D-arabinofuranose-1-phosphate; $_{2F}$Rib-1P-Ba, barium salt of 2-deoxy-2-fluoro-α-D-ribofuranose-1-phosphate; $^{2F}$Ara-1P-Ba, barium salt of 2-deoxy-2-fluoro-α-D-arabinofuranose-1-phosphate

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3,5-Dichlorobenzoyl-substituted 2-deoxy-D-ribose-1-phosphate was found to inhibit a variety of pyrimidine and purine nucleoside phosphorylases (with preference to uridine and inosine hydrolyzing enzymes), leading to increased half-life of pyrimidine analogues drugs used for the treatment of viral infections and cancer [5].

Pentose-1Ps have drawn attention as precursors in the chemoenzymatic synthesis of nucleosides and their analogues [7,8]. So far, enzymatic transglycosylation is used as a standard technique for the synthesis of purine nucleoside analogues and has been extensively studied and reviewed [8–12]. Transglycosylation reactions were shown to be successful for the production of riboside and deoxyriboside analogues, however, the synthesis of some arabinosides or C2′-fluoro-modified purine nucleosides was more challenging [9–11]. C2′-modified nucleosides are highly relevant both in molecular biology and pharmaceutical industry. Several C2′-modified drugs, such as fludarabine, 2′-deoxy-2′-fluorocytidine or clofarabine, are approved and used for the treatment of cancer [13,14]. Therefore, the direct synthesis of nucleoside analogues, starting form Pentose-1Ps, is an interesting approach to reach high product yields for base and sugar modified nucleoside analogues.

The chemical synthesis of natural Pentose-1Ps and their halogenated analogues has been investigated and several methods were reported during the 20$^{th}$ century [9,10,15–20]. However, the described protocols are laborious and result in unsatisfactory yields. Furthermore, the chemical synthesis mostly results in a mixture of both anomeric forms (α- and β-anomers), which complicates purification.

Due to challenges observed with chemical synthesis routes, different chemo-enzymatic approaches were developed for the synthesis of Pentose-1Ps [8]: i) A retro-synthesis starting from glyceraldehyde-3-phosphate (Gla-3P)/ dihydroxyacetone phosphate (DHAP) using D-2-deoxyribose-5-phosphate aldolase (DERA), triose phosphate isomerase (TRI) and phosphopentomutase (PPM) [21,22]. ii) A one-pot enzymatic transformation of D-pentoses to Pentose-1Ps by ribokinase (RK) and PPM [7]. iii) An enzymatic transformation of furanoses by a lipase from *Candida antartica* followed by several acetylation and deacetylation steps. The intermediate D-pentofuranose-5-phosphate was converted to Pentose-1P by PPM [23]. iv) Enzymatic phosphorolysis of nucleosides using mesophilic nucleoside phosphorylases (NPs) to produce Rib-1P, dRib-1P [24–26] and Ara-1P [27] as barium or cyclohexylamine salts. As the latter is a one-step reaction, conditions can be adjusted to achieve high product yields much easier than for approaches i. to iii.

A more widespread application of enzymatic phosphorolysis is hampered by the substrate spectrum of mesophilic enzymes. Within the last few years thermostable NPs have drawn more and more attention in the production of modified nucleosides. These enzymes, from thermophilic or hyperthermophilic microorganisms, catalyze reactions with the same stereoselectivity as mesophilic NPs, but they show a broader substrate spectrum [28–32]. While mesophilic enzymes show only a low activity against C2′-sugar-modified compounds such as 1-(β-D-arabinofuranosyl)uracil (Ara-U), 2′-deoxy-2′-fluorouridine ($_{2F}$Ribo-U) or 1(2′-deoxy2′-fluoro-β-D-arabinofuranosyl)uracil ($^{2F}$Ara-U), thermophilic enzymes are highly active on these substrates. Therefore, NPs of thermophilic organisms are interesting enzymes to produce a wide variety of modified Pentose-1Ps.

The present study describes the enzymatic synthesis and purification of three Pentose-1Ps (2-deoxy-2-fluoro-α-D-ribofuranose-1-phosphate, α-D-arabinofuranose-1-phosphate, and 2-deoxy-2-fluoro-α-D-arabinofuranose-1-phosphate) as α-anomers. These are precursors of nucleoside-based drugs (e.g. ribavirin or cytarabine) of utmost importance in the treatment of cancer or viral infections. Good product yields were obtained for Pentose-1Ps synthesis using the corresponding uracil nucleosides as substrates and a thermostable pyrimidine nucleoside phosphorylase as a biocatalyst. Pentose-1Ps were purified as barium salts in g-scale by three consecutive precipitation steps. Purity was above 95% and NMR spectroscopy revealed the exclusive formation of the α-anomer. To improve downstream processing of the Pentose-1Ps, i.e. their purification or their use in enzymatic reactions, temperature and pH stability of Pentose-1Ps was determined. Both natural and modified Pentose-1Ps showed increased stability at pH values above 9. New possibilities to study the roles of Pentose-1Ps in metabolism and cancer development are enabled by the availability of modified Pentose-1Ps. Additionally, they can be used to produce base and sugar modified nucleosides through a straightforward and environmentally-friendly enzymatic reaction.

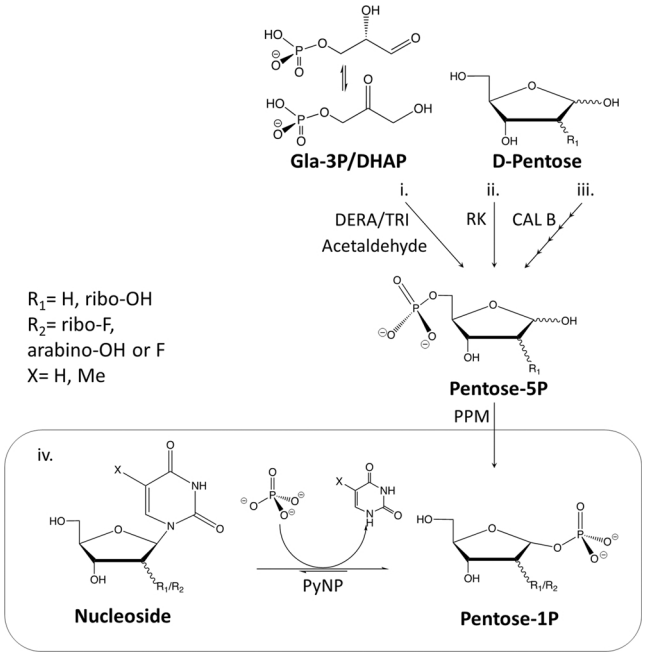
## 2. Methods

### 2.1. General information

All chemicals and solvents were of analytical grade or higher and purchased, if not stated otherwise, from Sigma-Aldrich (Steinheim, Germany), Carl Roth (Karlsruhe, Germany), TCI Deutschland (Eschborn, Germany), Carbosynth (Berkshire, UK) or VWR (Darmstadt, Germany). HPLC analyses were carried out with an Agilent 1200 series system equipped with an Agilent DAD detector using a Phenomenex (Torrance, CA, United States) reversed phase C18 column (150 × 4.6 mm). Thermostable NPs: PyNP-Y02 (E-PyNP-0002) and PyNP-Y04 (E-PyNP-0004) were obtained from BioNukleo (Berlin, Germany) and used as recommended by the manufacturer.

### 2.2. Enzymatic synthesis of Pentose-1Ps

For the production of Pentose-1Ps in gram scale, Urd, Thd, Ara-U, 2′-deoxy-2′-fluorouridine ($_{2F}$Ribo-U) and $^{2F}$Ara-U were phosphorolyzed using thermostable pyrimidine nucleoside phosphorylases (PyNPs) PyNP-Y02 or PyNP-Y04 to Rib-1P, dRib-1P, Ara-1P, $_{2F}$Rib-1P and $^{2F}$Ara-1P (Scheme 1 ). PyNPs were heterologously expressed in *E. coli* and originate from thermophilic bacteria with temperature optima of 60 °C




**Scheme 1.** Schematic representation of approaches used for the enzymatic synthesis of Pentose-1Ps. The enzymatic reaction catalyzed by thermostable nucleoside phosphorylases PyNP-Y02 or PyNP-Y04 is framed (iv). Different nucleosides with modifications at the C2-position of the sugar are applied. Urd: $R_1$ = ribo-OH, X = H, Thd: $R_1$ = H, X = Me, Ara-U: $R_2$ = arabino-OH, X = H, $_{2F}$Ribo-U: $R_2$ = ribo-F, X= H, $^{2F}$Ara-U: $R_2$ = arabino-F, X = H. **Gla-3P**: glyceraldehyde-3-phosphate, **DHAP**: dihydroxyacetone phosphate, **DERA**: D-2-deoxyribose-5-phosphate aldolase, **TRI**: triose phosphate isomerase, **PPM**: phosphopentomutase, **CAL B**: *Candida antartica* lipase, **RK**: ribose kinase.

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