Enzymatic Synthesis of α-D-Pentofuranose -1- Phosphate
as a universal glycosyl donor for modified nucleosides synthesis
using thermostable nucleoside phosphorylase

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Abstract

α-D-pentofuranose-1-phosphate is a key molecule in the pentose phosphate pathway as well as in the nucleoside/nucleotide metabolic pathway. It is physiologically synthesized from D-pentoses by phosphopentomutase or from nucleosides by nucleoside phosphorylase. It is further used as a carbon and energy source via its utilization in glycolysis and/or re-entering the pentose phosphate pathway. Pentofuranose-1-phosphates are interesting precursors for the synthesis of natural and modified nucleosides. Additionally, for some pentofuranose-1-phosphates, it was shown that they inhibit the action of some nucleoside phosphorylases.

Industrially, α-D-pentofuranose-1-phosphates are synthesized chemically by multi-step and laborious processes that result in a low final yield which is an anomeric mixture (α- and β-). This mixture is difficult to separate. Their use as a universal glycosylating agent for the synthesis of nucleosides and analogues thereof has been limited by their scarce availability that resulted from their tedious synthesis. Nucleosides and their analogues are clinically and biotechnologically important molecules that are used in diverse applications including chemotherapy, cell visualization and protein detection.

The enzymatic synthesis of the α-D-pentofuranose-1-phosphates is an interesting alternative synthesis route. Five natural and modified α-D-pentofuranose-1-phosphates; α-D-ribofuranose-1-phosphate, 2-deoxy-α-D-ribofuranose-1-phosphate, α-D-arabinofuranose-1-phosphate, 2'-deoxy-2'-fluoro-α-D-arabinofuranose-1-phosphate, and 2'-deoxy-2'-fluoro-α-D-ribofuranose-1-phosphate, were enzymatically synthesized by thermostable pyrimidine nucleoside phosphorylases as a

pure α-anomer. These pentose-1-phosphates were purified as their barium salts in gram scale with a purity > 95% and were applied for the enzymatic synthesis of eniluracil nucleoside derivatives.

Using both; transglycosylation and direct glycosylation approaches, the synthesis of 5-ethynyl-deoxyuridine, (2′S)-2′-deoxy-2′-fluoro-5-ethynyluridine, and 2’-deoxy-2′-fluoro-5-ethynyluridine was evaluated and their yields were compared. The direct glycosylation reaction showed a significantly higher yield for the synthesis of all the tested compounds. Thus, our results showed the selective enzymatic synthesis of the α-anomer of pentofuranose-1-phosphates with high purity and their superior use as precursors for the synthesis of modified by direct glycosylation reaction.

Zusammenfassung

α-D-Pentofuranose-1-phosphat ist ein Schlüsselmolekül im Pentose-Phosphat-Weg sowie im Nukleosid / Nukleotid-Stoffwechselweg. Es wird physiologisch synthetisiert aus D-Pentosen durch Phosphopentomutase oder aus Nukleosiden durch Nukleosidphosphorylasen. Es wird als Kohlenstoff- und Energieträger verwendet entweder direkt in der Glykolyse oder über den Pentose-Phosphat-Weg. Pentofuranose-1-phosphate sind interessante Vorläufermoleküle für die Synthese von natürlichen und modifizierten Nukleosiden. Darüber hinaus wurde für einige Pentofuranose-1-phosphate gezeigt, dass sie die Wirkung einiger Nukleosid-Phosphorylasen hemmen.

Industriell werden α-D-Pentofuranose-1-phosphate durch mehrstufige und langwierige chemische Verfahren synthetisiert. Dies führt zu einer geringen Endausbeute, da eine Mischung aus beiden anomeren Formen (α- und β-) entsteht, die nur schwer zu trennen sind. Ihre Verwendung als universelles Startmolekül für die Synthese von Nukleosiden bzw. Nukleosidanaloga war aufgrund der komplizierten Herstellung nur eingeschränkt möglich. Natürliche und modifizierte Nukleoside sind klinisch und biotechnologisch wichtige Moleküle, die in verschiedenen Anwendungen eingesetzt werden, u.a. in der Chemotherapie, bei der Zellvisualisierung und beim Proteinnachweis.

Die enzymatische Synthese dieser potentiell wichtigen Moleküle ist daher zunehmend von großem Interesse. Fünf α-D-Pentofuranose-1-phosphate (α-D-Ribofuranose-1-phosphat, 2-Desoxy-α-D-ribofuranose-1-phosphat, α-D-arabinofuranose-1-phosphat, 2-desoxy-2-fluoro-α-D-arabinofuranose-1-phosphat, 2-Desoxy-2-fluoro-α-D-ribofuranose-1-phosphat) wurden in dieser Arbeit durch thermostabile

Pyrimidinnukleosidphosphorylasen enzymatisch synthetisiert. Diese Pentose-1-phosphate wurden als ihre Bariumsalze im Grammmaßstab gereinigt und wurden für die enzymatische Synthese von Enyluracil-Nukleosid-Derivaten verwendet. Unter Nutzung der Transglykosylierungsreaktion oder der direkten Glycosylierungsmethode wurden 5-Ethinyldeoxyuridin, (2'S)-2'-Desoxy-2'-fluoro-5-ethinyluridin und 2'-Desoxy-2'-fluoro-5-ethinyluridin hergestellt. Die direkte Glycosylierungsreaktion zeigte für alle getesteten Verbindungen eine signifikant höhere Ausbeute. Es zeigte sich, dass die enzymatische Synthese modifizierter Pentofuranose-1-phosphate mit sehr hoher Reinheit (>95%, nur α-anomer) möglich war. Die produzierten Verbindungen waren sehr gut geeignet für die Synthese modifizierter Nukleoside in Glykosylierungsreaktionen.

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Declaration of Originality

I hereby confirm that this thesis is my own work and that I have not sought or used any inadmissible help of third parties to produce this work and that I have clearly referenced all sources used in the work.

Berlin, 2019

Sarah Kamel

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List of Abbreviations

2A6MP:	2-amino-6-methoxypurine
2CA:	2-chloroadenine
2FA:	2-fluoroadenine
^{2F}Ara-1P:	2'-deoxy-2'-fluoro-α-D-arabinofuranose-1-phosphate
^{2F}Ara-EdU:	(2′S)-2′-deoxy-2′-fluoro-5-ethynyluridine
^{2F}Ara-U:	2'-deoxy-2'-fluoro-β-D-arabinofuranosyl-uracil
$_{2F}$Rib-1P:	2'-deoxy-2'-fluoro-α-D-ribofuranose-1-phosphate
$_{2F}$Rib-EdU:	2'-deoxy-2′-fluoro-5-ethynyluridine
$_{2F}$Rib-U:	2'-deoxy-2'-fluoro-β-D-ribofuranosyl-uracil
5-EU:	5-ethynyluracil
Ade:	Adenine
Ah:	*Aeromonas hydrophilia*
AhTP:	*Aeromonas hydrophilia* thymidine phosphorylase
Ara-1P:	α-D-arabinofuranose-1-phosphate
Ara-A:	9-(β-D-arabinofuranosyl)adenine
Ara-U:	β-D-arabinofuranosyl-uracil
Arg:	Arginine
Asn:	Asparagine
B. cereus:	*Bacillus cereus*
Bs:	*Bacillus subtilis*
BsPyNP:	*Bacillus subtilis* pyrimidine nucleoside phosphorylase
CV:	Column volume
Da:	Dalton
dAdo:	2'-deoxyadenosine
DERA:	D-2-deoxyribose-5-phosphate aldolase
dGuo:	2'-deoxyguanosine
dRib-1P:	2-deoxy-α-D-ribofuranose-1-phosphate

E. coli:	*Escherichia coli*
EC:	Enzyme commission
EcTP:	*Escherichia coli* thymidine phosphorylase
EcUP:	*Escherichia coli* uridine phosphorylase
EdU:	5-ethynyl-deoxyuridine
Gln:	Glutamine
Glu:	Glutamate
Gly-3P:	Glyceraldehyde-3-phospahte
Gs:	*Geobacillus stearothermophilus*
GsPNP:	*Geobacillus stearothermophilus* purine nucleoside phosphorylase
GsPyNP:	*Geobacillus stearothermophilus* pyrimidine nucleoside phosphorylase
Gt:	*Geobacillus thermoglucosidasius*
GtPyNP:	*Geobacillus thermoglucosidasius* pyrimidine nucleoside phosphorylase
HBV:	Hepatits B virus
HCV:	Hepatits C virus
HIV:	Human immunodeficiency virus
HPLC:	High performance liquid chromatography
HPRT:	Hypoxanthine-guanine phosphoribosyl transferase
HSV:	Herpes simplex virus
Ile:	Isoleucine
Ka:	*Klebsiella aerogenes*
KaPyNP:	*Klebsiella aerogenes* pyrimidine nucleoside phosphorylase
K_{cat}:	Catalytic constant
K_m:	Michaelis-Menten's constant
KP:	Potassium phosphate
L. lactis:	*Lactobacillus lactis*
LBc:	*Lactobacillus casei*

LBcTP:	*Lactobacillus casei* thymidine phosphorylase
LBcUP:	*Lactobacillus casei* uridine phosphorylase
Lys:	Lysine
MCA:	Monochloroacetic acid
Mh:	*Mycoplasma hyorhinis*
MhPyNP:	*Mycoplasma hyorhinis* pyrimidine nucleoside phosphorylase
MTAP:	S-methylthioadenosine phosphorylase
NA:	Nucleoside analogue
NP:	Nucleoside phosphorylase
Pentose-1P-Ba:	Barium salt of Pentose-1P
Pentose-1P:	α-D-pentofuranose-1-phosphate
Pentose-5P:	D-pentose-5-phosphate.
Pi:	Inorganic phosphate
PNP:	Purine nucleoside phosphorylase
PPM:	Phosphopentomutase
PPP:	Pentose phosphate pathway
PRPP:	5-phosphoribosyl-1-pyrophosphate
PyNP:	Pyrimidine nucleoside phosphorylases
R_f:	Retention factor
Rib-1P:	α-D-ribofuranose-1-phosphate
RK:	Ribokinase enzyme
R_T:	Retention time
RT:	Room temperature
$T_{1/2}$:	Half-life
Thd:	Thymidine
Thy:	Thymine
TLC:	Thin layer chromatography
TP:	Thymidine phosphorylase
TPI:	Triose phosphate isomerase enzyme
Tt:	*Thermus thermophilus*

TtPyNP:	*Thermus thermophilus* pyrimidine nucleoside phosphorylase
UP:	Uridine phosphorylase
Ura:	Uracil
Urd:	Uridine
Val:	Valine
V_{max}:	Maximum velocity
V_{VDW}:	Van der Waal

List of Publications

This work is based on the following publications:

Paper I (Published version)

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Paper IV

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higher yields of nucleoside analogues compared to transglycosylation reactions. [Manuscript in preparation] ***Equal contribution**

Other Publications not included in the thesis:

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Authors Contributions to the Papers

Paper	Co-author	Contribution
I	Heba Yehia	Wrote section 4 and prepared the figures and tables.
	Sarah Kamel	Wrote section 4 and prepared the figures and tables.
	Katharina Paulick	Assisted in figures and tables preparation.
	Peter Neubauer	Designed the general concept and paper outlook.
	Anke Wagner	Designed the general concept and paper outlook, wrote sections 1-3 and 5 and prepared the figures and the tables.
II	Sarah Kamel	Wrote sections 1-3 and prepared the figures and tables.
	Heba Yehia	Wrote sections 4 and 5 and prepared the figures and tables.
	Peter Neubauer	Designed the general concept and outlook.
	Anke Wagner	Designed the general concept and paper outlook and wrote sections 6 and 7.
III	Sarah Kamel	Wrote the paper, carried out the experimental work, and paper conceptualization.
	Max Weiß	Assisted with experimental work.
	Hendrik F.T. Klare	NMR spectroscopic analysis.
	Igor A. Mikhailopulo	critical discussion of the results and critical reading of the paper
	Peter Neubauer and Anke Wagner	Supervision and project conceptualization.

	Heba Yehia	Wrote the paper, carried out the experimental work, and paper conceptualization
IV	Sarah Kamel	Wrote the paper, carried out the experimental work, and paper conceptualization
	Peter Neubauer and Anke Wagner	Supervision and project conceptualization

1 Introduction

The increasing interest in green chemistry approaches and sustainable processes has drawn much attention to biocatalysis. The use of naturally or engineered enzymes will allow a revolutionized synthesis of different pharmaceutically and technologically important compounds. With an increased application of sustainable enzymatic synthesis routes, the search for new enzymes and their implementation to produce different compounds is in continuous development.

Among these compounds are the clinically important nucleosides and their core building blocks; the activated pentose moieties (pentofuranose-1-phosphates). Both compounds are mainly synthesized chemically. The chemical synthesis has some critical disadvantages including the long and tedious multi-step procedure that require several intermediate purification processes and result in an anomeric mixture that is hard to separate. Hence, the processes are mainly inefficient, costly and lead to low yields. Therefore, the wide application for functional, metabolic and therapeutic studies is hindered.

The discovery of nucleoside phosphorylases and their later implementation for the synthesis of nucleoside analogues paved the way for a sustainable and environmentally-friendly process. These enzymes catalyze the reversible phosphorolytic cleavage of nucleosides to α-D-pentofuranose-1-phosphate and nucleobase. The use of mesophilic enzymes has shown a huge potential for the synthesis of nucleosides, however with the need to synthesize heavily modified nucleoside analogues and by looking forward to process-upscaling, more durable enzymes were needed. Thus, searching for and employing extremophilic enzymes came in focus.

Nucleosides and their analogues are well studied for their potential therapeutic and biotechnological applications. They are proven to be efficient anti-viral, anti-bacterial and chemotherapeutic agents. Additionally, their mechanisms of action are well understood and explained. Therefore, several approaches have been described for their enzymatic synthesis. On the other hand, pentoses and their phosphate derivatives were only poorly studied for their potential therapeutic, biotechnological and industrial application, although they are key molecules in many physiological pathways. This might be related to the difficulty of their chemical synthesis and purification especially their modified analogues.

Therefore, in this work, five (natural and modified) α-D-pentofuranose-1-phosphates were enzymatically synthesized in gram scale using thermostable nucleoside phosphorylases. To this end, three new nucleoside phosphorylase enzymes derived from three distinctive thermophilic organisms were characterized for their kinetic parameters. Additionally, their use in the enzymatic synthesis of Pentose-1P was evaluated. Moreover, the chemical and chromatographic purification of these five α-D-pentofuranose-1-phosphates are shown. Finally, their essential use in the synthesis of some biotechnologically important nucleoside analogues is shown as a track of their application and as an advancement in the enzymatic synthesis of nucleoside analogues.

2 Literature Review

2.1 Pentose-1-phosphates in metabolism; synthesis and utilization

Pentofuranose-1-phosphate (Pentose-1P) forms a family of five-membered heterocyclic oxygen containing sugars with a phosphate group at C1 position. α-D-ribofuranose-1-phosphate (Rib-1P) and 2-deoxy-α-D-ribofuranose-1-phosphate (dRib-1P) are the two physiologically existing Pentose-1Ps (Giorgelli *et al.*, 1997; Giannecchini *et al.*, 2005; Tozzi *et al.*, 2006). In connection to Pentose-1P metabolism, these molecules are synthesized from the phosphorylation of nucleosides by nucleoside phosphorylases (NPs) and/or by the interconversion of D-pentose-5-phosphate (Pentose-5P) by phosphopentomutase (PPM, EC 5.4.2.8) (**Figure 1**) (Giorgelli *et al.*, 1997; Tozzi *et al.*, 2006).

The synthesized Pentose-1P is further used for down-stream cellular processes (**Figure 1**): i. They are used as carbon and energy source through their utilization in the pentose phosphate pathway (PPP) or in glycolysis, thus, leading to the production of ATP (Giannecchini *et al.*, 2005; Tozzi *et al.*, 2006). ii. They are involved in de-novo and salvage nucleotide synthesis (Berg *et al.*, 2015). iii. Rib-1P is recycled for the synthesis of xanthosine and inosine monophosphate (Giorgelli *et al.*, 1997; Kilstrup *et al.*, 2005). iv. Rib-1P is used for the direct ribosylation of uracil (Ura) to uridine (Urd) by uridine phosphorylase (UP) (EC 2.4.2.3) through a process called Rib-1P-dependent pyrimidine salvage, which was found to be predominant in normal rat tissues (Mascia *et al.*, 1999; Tozzi *et al.*, 2006). The formed uridine undergoes phosphorylation by kinases to produce the respective nucleotide (Mascia *et al.*, 2000). In addition to their physiological role, Rib-1P is involved in the metabolism of

some drugs such as fluoro-uracil, (E)-5-(2-bromovinyl)uracil and others, thus activating and improving their therapeutic effect (Cao *et al.*, 2004; Mikhailopulo *et al.*, 2011). Although involved in different crucial cellular functions, dRib-1P and its dephosphorylated form 2-deoxyribose were reported to be involved in cancer development by promoting angiogenesis (Nakajima *et al.*, 2009; Elamin *et al.*, 2016).

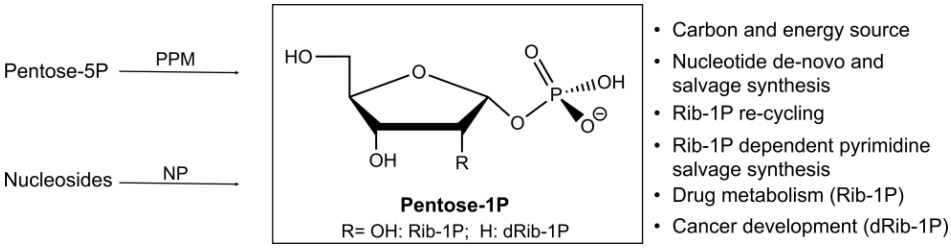


Figure 1. An overview of the Pentose-1P metabolic synthesis and utilization. PPM: phosphopentomutase; NP: nucleoside phosphorylase; Rib-1P: α-D-ribofuranose-1-phosphate; dRib-1P: 2-deoxy-α-D-ribofuranose-1-phosphate.

Pentose-1Ps are central molecules that stand in the middle of three principal physiological pathways, the PPP and glycolysis on one hand and the nucleosides/nucleotides metabolism and interconversion pathway on the other hand (**Figure 2**). The complexity of the nucleoside metabolism pathway and its diversity among different organisms and even between different tissues within the same organism renders it difficult to have a generalized mechanism that considers all the possibilities. However, these differences might be a strong base for drug development and targeted therapy (Mascia *et al.*, 2000). For example, some enzymes are only present in microorganisms compared to mammals as inosine and guanosine kinase and adenosine phosphorylase. PPP is a well-studied pathway since the 1930's (Stincone *et al.*, 2015), it's an essential pathway that is involved in many regulatory and biochemical processes, within this pathway Pentose-5P is a pivotal molecule.

Pentose-5P, a substrate for Pentose-1P synthesis, is synthesized by different pathways (**Figure 2**) (Tozzi *et al.*, 2006; Stincone *et al.*, 2015). Mainly, it is produced in the PPP particularly in its oxidative branch which link the PPP to the glycolysis. Furthermore, it is formed as an intermediate in the non-oxidative branch of PPP by the interconversion of sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate (Gly-3P) by the enzyme transketolase (EC 2.2.1.1). This reaction again links the PPP to glycolysis as glyceraldehyde-3-phosphate is supplied to and from the glycolysis. Pentose-5Ps can also be synthesized by the phosphorylation of D-pentoses by ribokinases (RK, EC 2.7.1.15). Pentose-5P serves as a precursor for the enzymatic synthesis of 5-phosphoribosyl-1-pyrophosphate (PRPP), the latter is the building block for nucleotide de-novo and salvage synthesis pathways (Kilstrup *et al.*, 2005; Jiménez *et al.*, 2008; Berg *et al.*, 2015).

Most of the reactions in the Pentose-1P metabolism are reversible. Only due to several intracellular factors, reactions' equilibrium is shifted either toward the anabolic or the catabolic utilization of the Pentose-1P. As mentioned earlier and elaborated in **Figure 2**, Rib-1P and dRib-1P are synthesized through the reversible phosphorolytic cleavage of ribo- and deoxyribo-nucleosides by NPs, respectively. Nucleosides are supplied externally or by the degradation of nucleic acids. There are two different kinds of nucleosides: purines and pyrimidines. Purine nucleosides' metabolism is mediated by purine nucleoside phosphorylase (PNP, EC 2.4.2.1), while pyrimidine nucleosides' metabolism is mediated by pyrimidine nucleoside phosphorylase (PyNP, EC 2.4.2.2), UP or thymidine phosphorylase (TP, EC 2.4.2.4).

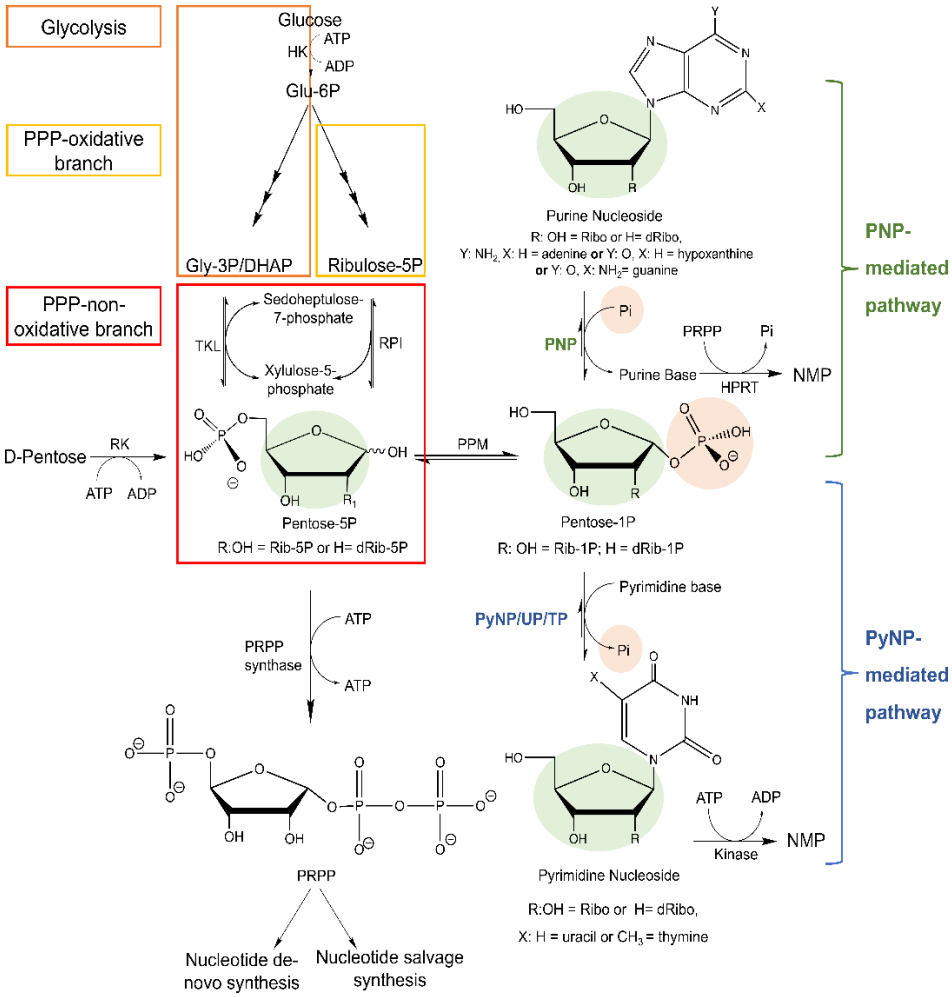


Figure 2. Detailed illustration of the synthesis and utilization of Pentose-1P as a central molecule between pentose phosphate pathway and nucleotide/nucleoside metabolism. HK: Hexose kinase; Gly-3P: glyceraldehyde-3-phosphate; DHAP: dihydroxyacetone phosphate; TKL: transketolase; RPI: Ribose-5-Phosphate isomerase; RK: ribose kinase; PRPP: phosphoribosyl pyrophosphate; PPM: phosphopentomutase; PNP: purine nucleoside phosphorylase; HPRT: hypoxanthine-guanine phosphoribosyl transferase; NMP: nucleoside monophosphate; PyNP: pyrimidine nucleoside phosphorylase; UP: uridine phosphorylase; TP; thymidine phosphorylase.

PNP mediated pathway favors the catabolic reaction i.e. the synthesis of the Pentose-1P, this could be explained by several reasons (Tozzi *et al.*, 2006). First, the high intracellular phosphate concentration compared to that of the nucleoside. Additionally, the absence of inosine and guanosine kinase in mammals thus both nucleosides are not consumed in another pathway (Mascia *et al.*, 2000). Moreover, particularly with inosine and guanosine metabolism, the formed bases (guanine and hypoxanthine) are irreversibly attached to PRPP for the salvage synthesis of inosine and guanosine monophosphate by the enzyme hypoxanthine-guanine phosphoribosyl transferase (HPRT) (EC 2.4.2.8). Furthermore, the formed Pentose-1P is used as a carbon and energy source (Giannecchini *et al.*, 2005). Finally, the formed Pentose-1P is used for pyrimidine salvage synthesis.

On contrary, UP, TP or PyNP mediated metabolic pathway favor the anabolic reaction (Mascia *et al.*, 2000; Tozzi *et al.*, 2006; Mikhailopulo *et al.*, 2011), meaning, the utilization of Pentose-1P and the synthesis of nucleoside as demonstrated in rat brain. This equilibrium shift is supported by the continuous phosphorylation of the pyrimidine nucleoside to nucleoside mono-, di- and tri-phosphate. Additionally, the absence of uracil phosphoribosyl transferase in mammals and the absence of uracil degradation to β-alanine particularly in the CNS. The latter two reasons together with the continuous supply of Pentose-1P from purine nucleoside metabolism lead to the accumulation of the reactant thus shifting the reaction towards the nucleoside formation. It is established that the Rib-1P links the purine and the pyrimidine salvage pathways, and that the catalytic activity of both enzymes (PNP and UP) results in the transfer of ribose from a purine to a pyrimidine nucleoside (Cappiello *et al.*, 1998).

One possibility of the pyrimidine salvage synthesis is the direct ribosylation of uracil and thymine using Rib-1P and dRib-1P, respectively. On contrary, it has been reported that purine salvage is entirely dependent

on the formation PRPP (Mascia *et al.*, 2000). However, evidences of the utilization of Rib-1P for the synthesis of a purine nucleoside from another purine nucleoside (Rib-1P recycling or the Rib-1P shuttle) using nucleoside phosphorylases have been reported too (Giorgelli *et al.*, 1997; Mascia *et al.*, 2000). In this case, the phosphorylase is coupled with an additional deaminating enzyme (**Figure 3**). It is believed that Rib-1P recycling is the biological way to overcome the lack of some enzymes in certain organisms or tissues. This mechanism has been reported for mammals as well as bacteria.

The synthesis of xanthosine from guanosine by PNP and guanase (EC 3.5.4.3) was reported in rat liver. Guanosine is phosphorolytically cleaved by PNP to produce Rib-1P and guanine base. The produced guanine is deaminated by the enzyme guanase to xanthine. The latter is then reattached to Rib-1P produced in the first reaction giving xanthosine (**Figure 3A**). This cascade reaction is necessary to overcome the absence of guanosine deaminase (EC 3.5.4.15) in mammals which is responsible for the direct conversion of guanosine to xanthosine (Giorgelli *et al.*, 1997). The same mechanism was observed in *B. cereus, L. lactis and E. coli,* to overcome the absence of adenosine deaminase (EC 3.5.4.4). In this case, the cell resort to Rib-1P recycling to convert adenine to hypoxanthine by the consecutive action of adenosine phosphorylase, adenine deaminase (EC 3.5.4.2) and PNP (**Figure 3B**). Additionally, *L. lactis and E.coli* utilize the formed hypoxanthine for the subsequent synthesis of inosine- and guanosine- monophosphate (Giorgelli *et al.*, 1997; Kilstrup *et al.*, 2005; Tozzi *et al.*, 2006).

Pentose-1Ps are key molecules in different biological processes like nucleotides synthesis which are the building blocks for the molecules of life (DNA and RNA). They are as well consumed in PPP for energy and carbon expenditure. Thus, the availability of these Pentose-1P is valuable for metabolic and therapeutic studies.

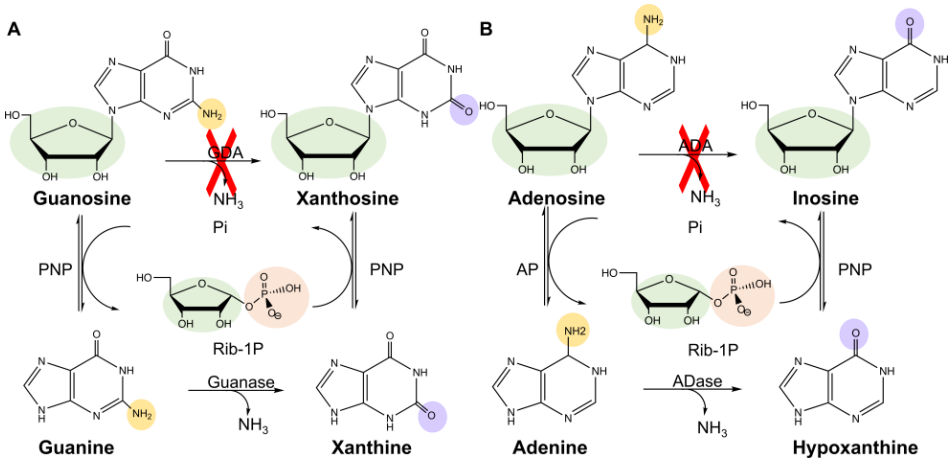


Figure 3. Rib-1P recycling for the synthesis of purine nucleosides. GDA: guanosine deaminase; PNP: purine nucleoside phosphorylase; ADA: adenine deaminase; AP: adenosine phosphorylase; ADase: adenine deaminase. Absent reactions due to the lack of some enzymes are demonstrated by red crosses.

2.2 Nucleoside phosphorylases as valuable biocatalysts

2.2.1 Nucleoside phosphorylases

Understanding the natural physiological pathways provides innovative solutions for in-vitro applications. By looking into the above discussed pathway(s), NPs are shown to be interesting enzymes in the metabolism of both Pentose-1P and nucleoside. Thus, they have been studied for more than 100 years form different aspects; including physiological, clinical and catalytical properties (Levene *et al.*, 1911; Jensen *et al.*, 1975; Pugmire *et al.*, 1998; Bzowska *et al.*, 2000; Larson *et al.*, 2010). Several nucleoside phosphorylases have been identified, isolated and

characterized from many organisms and were applied as efficient biocatalysts. Crystallographic studies were conducted on nucleoside phosphorylases to understand their 3D structures and correlate them to their substrate spectrum (Pugmire *et al.*, 1998, 2002; Norman *et al.*, 2004; Larson *et al.*, 2010; Prokofev *et al.*, 2018). A comprehensive description on nucleoside phosphorylases' classification, mechanism of action, active site residues, substrate spectrum, as well as their applications in producing pharmaceutically active compounds, are discussed in detail **in Paper I and Sections 1.2 and 1.7 in Paper II.**

As mentioned earlier, NPs catalyze the reversible phosphorolysis of nucleosides into their respective nucleobase and Pentose-1P in the presence of inorganic phosphate (Pi). They are classified based on their structure into two families; NP-I and NP-II. NPs are also classified according to their substrate spectrum into a different category and are accordingly, given different enzyme commission (EC) numbers (Bzowska *et al.*, 2000; Pugmire *et al.*, 2002). NP-I family members catalyze reactions with purine nucleosides/bases and uridine/uracil and includes PNP, S-methylthioadenosine phosphorylase (MTAP) and UP. NP-II family members are specific for pyrimidine nucleosides/bases and includes TP and PyNP. From the structural point of view, UP is categorized as NP-I member, however, from the catalytic point of view it is well accepted and generally described with other PyNPs (Del Arco *et al.*, 2018).

2.2.1.1 Thermophilic nucleoside phosphorylases

NPs were primarily identified and characterized from mesophilic organisms as *E. coli* (Panova *et al.*, 2007), *S. typhimurium* (Blank *et al.*, 1975), *B. subtilis* (Gao *et al.*, 2006), *M. hyorhinis* (Voorde *et al.*, 2012) and others. However, mesophilic enzymes interfered with the wide spread of the enzymatic synthesis approaches and their industrial application (Del Arco *et al.*, 2018). This is because mesophilic enzymes have short

lifespan, as well as being unstable in organic solvents. These are sometimes necessary for the solubility of the nucleoside or the nucleobase. Moreover, their purification costs are high. Finally, they have a limited substrate spectrum. These limitations derived the attention to exploring extremophilic environments. Extremophiles are those organisms living in environment with extreme conditions like high temperature or high pressure, as well as high concentrations of salts, heavy metals, and/or organic solvents. Thus, enzymes from these organisms are considered valuable biocatalysts (Hussein *et al.*, 2011; Del Arco *et al.*, 2018) as they have a wider substrate spectrum. They are, additionally, highly stereo- and regioselective and have minimal or no side reactions. Moreover, they are stable under harsh reaction conditions as high temperature, high pressure, long reaction time and/or organic solvents. Furthermore, they could be easily purified. A huge focus was given to thermophilic enzymes that are stable at high temperatures ranging from 45°C to 100°C. Some NPs were identified, expressed and characterized from thermophilic microorganisms, including *Geobacillus stearothermophilus, Thermus thermophilus and Geobacillus thermoglucosidasius* (Carteni'-Farina *et al.*, 1979; Hamamoto *et al.*, 1996; Almendros *et al.*, 2012; Szeker *et al.*, 2012; Zhou *et al.*, 2013; Bagarolo *et al.*, 2015).

2.2.2 Nucleoside phosphorylases in the synthesis of Pentose-1Ps

The utilization of NPs for the enzymatic synthesis of Pentose-1Ps was first introduced during the earlier attempts to identify and characterize NPs from different organisms (Kalckar, 1947; Friedkin, 1950). Using NPs from rat and calf liver, Rib-1P and dRib-1P were synthesized in a mg-scale. More details on the enzymatic synthesis of Pentose-1P are found in **section 2.4.2**.

2.2.3 Nucleoside phosphorylases in the synthesis of nucleosides and their analogues

2.2.3.1 Nucleosides and nucleoside analogues

NPs are employed in the synthesis of nucleosides and their analogues. Nucleosides are biochemical molecules composed of two main parts; a nucleobase attached to a pentofuranose moiety by an N-glycosidic bond (**Figure 4**). Nucleosides can be classified into naturally occurring and synthetic one. Naturally occurring are either unmodified nucleosides (canonical nucleosides) or modified (non-canonical). Canonical nucleosides are uridine, cytidine and thymidine (Thd) (pyrimidines) or guanosine and adenosine (purines). They are the structural subunits of DNA and RNA and are involved in several cellular and metabolic processes (Savić *et al.*, 2015). Non-canonical nucleosides are parts of tRNA and are important for RNA processing (Agris, 2015). Synthetic nucleosides are those with different modifications on the pentose, the nucleobase or both moieties (**Figure 4**). They are used for clinical and biotechnological applications (Galmarini *et al.*, 2002; Neef *et al.*, 2011; Jordheim *et al.*, 2013). Detailed descriptions of nucleosides and their analogues are mentioned in **Paper II-Section 1.1.**

Nucleoside analogues (NAs) have been of increasing interest for more than 50 years due to their therapeutic and biotechnological applications. Because of their ability to integrate in DNA and RNA strands, they are labelled and used for cellular visualization (Neef *et al.*, 2011). Therapeutically, nucleoside analogues are used for the treatment of infections with different viruses like Hepatitis C and B viruses (HCV, HBV), herpes simplex virus (HSV), and human immune deficiency virus (HIV). They are, additionally, used as chemotherapeutic agents for the treatment of different types of cancers as kidney, breast, bladder and pancreatic cancer or hematological malignancies (Galmarini *et al.*, 2002; Lapponi *et*

al., 2016). Due to their structural similarities with natural nucleosides, they can be integrated into the DNA or RNA strands and stall their further elongation. Another mechanism of action is the inhibition of essential cellular enzymes as DNA or RNA polymerases (Galmarini *et al.*, 2002; Jordheim *et al.*, 2013).

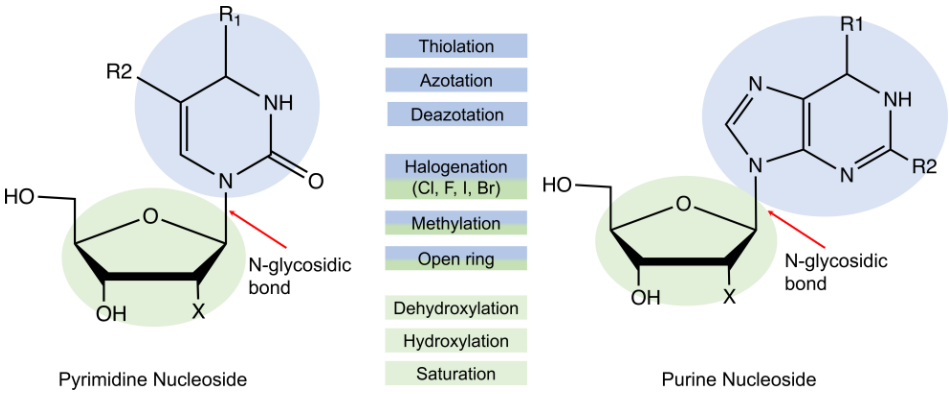


Figure 4. Structure of pyrimidine and purine nucleosides. Possible modifications of the sugar and the base moieties are shown. Marked in blue and green are the possible modifications on both the base and the sugar moieties. respectively.

The chemical synthesis of NAs has been extensively studied and many approaches have been developed over the past decades. Several chemical methods were reported (Liu *et al.*, 2008; Peng, 2013). The most effective method require the initial presence of glycosyl-1-phosphates which are usually quite expensive and rarely available commercially (Zamyatina *et al.*, 2009). Despite the enormous advances made in this field it still holds several disadvantages. The chemical synthesis requires several protection and deprotection steps. It's final yield is a mixture of both α- and β- anomers which render the purification process very difficult. Moreover, the harsh reaction conditions including low pH, lots of harmful organic solvents, long reaction duration and high temperatures contributed to the notion of exploring alternative synthesis approaches.

With the uprising of green chemistry and the new advancements of enzyme technologies; new trends to enzymatically synthesize nucleoside analogues have been investigated and reported in several reviews (Mikhailopulo *et al.*, 2013; Lapponi *et al.*, 2016; Pérez *et al.*, 2018). The application of NPs in the synthesis of nucleoside analogues has spread vastly because of their high regio- and sterio-selectivity, their wide range of substrates acceptance, and their abundance in almost all living organisms. These factors render nucleoside phosphorylases a valuable biocatalyst for the synthesis of nucleoside analogues (Cattaneo *et al.*, 2017). Advances in nucleoside analogues synthesis and upscaling production approaches are presented in detail in **Paper I and Section 1.3 of Paper II.**

2.2.3.2 Transglycosylation reaction as an enzymatic synthesis approach of nucleoside analogues

One of the most well studied and reported approach employing NPs as a biocatalyst is the one-pot two steps transglycosylation reaction. Transglycosylation reactions offer an easy one step synthesis where an exchange of nucleobase over a pentose-sugar moiety takes place in the presence of inorganic phosphate. The reaction is catalyzed by nucleoside phosphorylases forming Pentose-1P as an intermediate (**Figure 5**). It is more common that NPs in one-pot two-steps transglycosylation reaction catalyze the reversible transfer of the sugar moiety from a pyrimidine nucleoside (sugar donor) to a purine base (sugar acceptor). This is attributed to relative uncomplicated chemical synthesis routes available for pyrimidine nucleosides (Mikhailopulo *et al.*, 2011). Using mesophilic and thermophilic NPs, transglycosylation reactions were used for the synthesis of natural and modified purine nucleosides which are reviewed in (Mikhailopulo, 2007; Mikhailopulo *et al.*, 2010; Lapponi *et al.*, 2016) and summarized in **Table 1**.

As mentioned earlier, transglycosylation reactions have several advantages, however, there are still some challenges in the synthesis of some highly modified nucleoside analogues, especially those with C2' modifications and/or large group modifications on the base moiety (Mikhailopulo *et al.*, 2011). This was manifested during the synthesis of 6-amino-9-(2-deoxy-2-fluoro-β-D-ribofuranosyl)purine and 2,6-diamino-9-(2-deoxy-2-fluoro-β-D-ribofuranosyl)purine by transglycosylation. 2'-deoxy-2'-fluoro-β-D-ribofuranosyluracil ($_{2F}$Ribo-U) was used as a sugar donor and the corresponding base as the sugar acceptor. While, $_{2F}$Ribo-U was not phosphorolyzed by *E. coli* UP, E. *coli* TP showed very low activity. Enormous amounts of *E. coli* TP (2400 U - 42000 U) and PNP (3900U – 84000U) were used and the reaction took place over a course of 2- 17 days (Tuttle *et al.*, 1993).

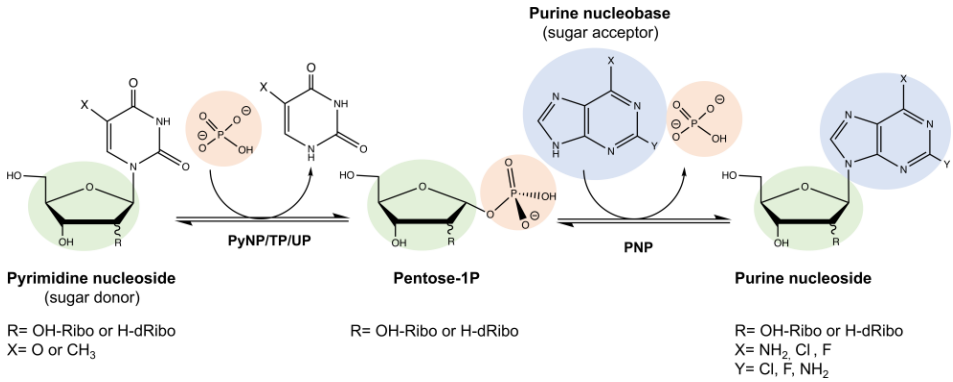


Figure 5. Transglycosylation reaction. PyNP: pyrimidine nucleoside phosphorylase; TP: thymidine phosphorylase; UP: uridine phosphorylase; PNP: Purine nucleoside phosphorylase.

Limitations of the transglycosylation reaction could be correlated to the strengthened N-glycosidic bond by the action of C2' modifications. Thus, the nucleoside cleavage to form the intermediate Pentose-1P is harder; hence, longer reaction duration is needed. Additional limitation is the

decreased affinity of the employed enzyme to some substrates due to the bulkiness of the modifications. Thus, stalling the formation of the second nucleoside. Moreover, the differences in the reaction dynamics between the nucleoside cleavage and the nucleoside formation reactions would as well stop the reaction from proceeding forward. Transglycosylation reaction comprises two steps; the first step is the phosphorolytic cleavage of a nucleoside (sugar donor). This is driven by high concentrations of the phosphate, whereas, the second step, the glycosylation of the second nucleobase (sugar acceptor), is hindered by the presence of phosphate. In a given reaction where the nucleoside cleavage and the nucleoside formation take place simultaneously, the reaction proceeds smoothly, and up to 90% product formation is achieved as shown in **Table 1**. However, for the above-mentioned cases (C2' and/or bulky base modifications), the product formation might be minimal to none (Mikhailopulo *et al.*, 2011; Serra *et al.*, 2013). Accordingly, to overcome difficulties in the synthesis of NAs by transglycosylation reactions, several approaches were explored and developed as a practical alternative and others were developed to manipulate the transglycosylation reaction itself.

Among the recent approaches to modulate the transglycosylation reaction is creating a dynamic mathematical model that was developed for both steps of the transglycosylation reaction (Wilms *et al.*, 2017). By defining most of the reaction variables, a mathematical model that consider the different kinetic parameters (K_m and K_{cat}), enzyme optimum temperature and the solubility of the reactants and the products was built. This model would latter serve as a valuable tool to choose the best reaction conditions to maximize the product yield with the minimum possible production costs.

Suitable enzymatic approaches, that can serve as alternative, are further elaborated in **Paper I in Section 1.3.** In short, these approaches are, the direct glycosylation of nucleobases by Pentose-1P using NP enzyme. Another approach is the one pot transformation of D-pentoses to

nucleosides using RK, PPM and NP enzymes sequentially. The last approach is the transformation of D-glyceraldyde-3-phosphate to nucleoside using D-2-deoxyribose-5-phosphate aldolase (DERA)/triose phosphate isomerase (TPI), PPM and NP enzymes successively (**Figure 6**). This work is focused on the first enzymatic approach, the application of Pentose-1Ps as a universal glycosylating agent.

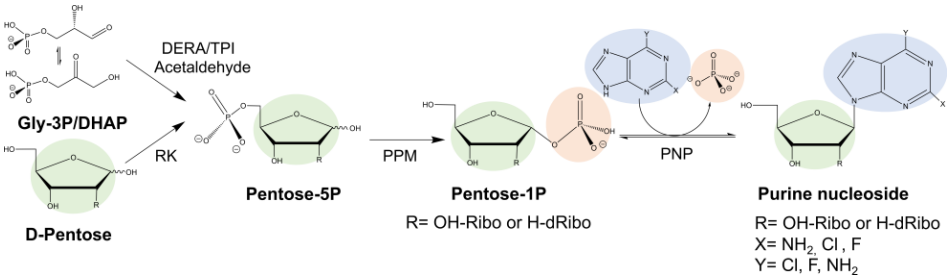


Figure 6. Alternative enzymatic synthesis way to transglycosylation reaction. Gly-3P: glyceraldhyde-3-phosphate; DHAP: dihydroxyacetone phosphate; DERA: deoxyribose phosphate aldolase; TRI: triose phosphate isomerase; PPM: phosphopentomutase; PNP: purine nucleoside phosphorylase

2.3 Pentose-1Ps as precursors in nucleoside synthesis

The use of Pentose-1P as a universal glycosylating agent for nucleoside synthesis has been of increasing interest. It provides a practical alternative for the transglycosylation reaction. The direct condensation of the Pentose-1P moiety with a nucleobase in the presence of nucleoside phosphorylases was first introduced upon the discovery of nucleoside phosphorylases, when Friedkin and co-workers (Friedkin, 1950; Friedkin *et al.*, 1950, 1954) studied the catalysis of deoxyribonucleoside by nucleoside phosphorylases isolated from calf liver. dRib-1P was isolated as cyclohexylammonium salt and applied for the glycosylation of several pyrimidine nucleosides. Since then, this approach has been successfully

Table 1. Nucleoside analogues that were synthesized by transglycosylation reactions. The reaction conditions and final yields are summarized

Nucleoside	Biocatalyst	Sugar donor: sugar acceptor	Reaction medium	Reaction time [h] /reaction temperature [°C]	Yield [a] [%]	Reference
2-chlorodeoxy-adenosine [Cladribine]	*E. coli* [whole cell]	dGuo: 2CA [3:1]	10 mM Pi [pH 7.2], 10% DMSO	21h/ 60°C	65%	Mikhailopulo *et al.*, 1993
	Recombinant TtPyNP[b] and GtPNP[b]	Thd: 2CA [2:0.6]	2mM Pi [pH 7]	1h/ 70°C	55%	Zhou *et al.*, 2015
	E. coli [whole cell] producing Recombinant GstPNP[b]	dGuo: 2CA [3:1]	5 – 10 mM Pi [pH 8.0]	4h/ 65°C-70°C	95%	Taran *et al.*, 2008
		dAdo: 2CA [3:1]			75%	
	E. coli [whole cell] producing Recombinant GsPNP[b]/ Exogenous *E. coli* TP	Thd: 2CA [3:1]			75%	
9-β-d-arabinosyl-2-fluoroadenine [Fludarabine]	Immobilized *Citrobacter koseri*	Ara-U: 2FA [2:1]	30 mM Pi [pH 7]	14 h/ 60°C	58%	Nóbile *et al.*, 2012
2-Amino-9-(b-D-arabinofuranosyl) -6-methoxypurine [Nelarabine]	Recombinant *E. coli* UP and PNP	Ara-U: 2A6MP [not given]	5 mM Pi [pH 7]	Not given/ 45°C	53%	Konstantinova *et al.*, 2011
9-β-D -arabinofuranosyl -adenine [Vidarabine]	Immobilized *Citrobacter koseri*	Ara-U: Ade [2:1]	20 mM Pi [pH 7]	26 h/ 60°C	71%	Nóbile *et al.*, 2012

[a] Yields are Based on the bioconversion.[b] Thermophilic enzymes.

TtPyNP: *Thermus thermophilus* pyrimidine nucleoside phosphorylase. **GtPyNP:** *Geobacillus thermoglucosidasius* pyrimidine nucleoside phosphorylase. **GsPNP:** *Geobacillus stearothermophilus* purine nucleoside phosphorylase.

dGuo: deoxyguanosine. **2CA:** 2-chloroadenine. **2FA:** 2-fluoroadenine. **Ade:** adenine. **2A6MP:** 2-amino-6methoxypurine

used for the synthesis of several clinically important nucleosides like Cladribine and Vidarabine (**Table 2,** Lapponi *et al.*, 2016). This approach amends some of the above-mentioned limitations of the transglycosylation reaction. It permits simpler optimization for the reaction-conditions including enzyme choice, reaction temperature and concentration of the reactants. First, the choice of the applied enzyme is very crucial for the synthesis. Using the direct glycosylation approach, the differences between the sugar-donor's and the sugar-acceptor's solubility and stability will not affect the enzyme choice. Additionally, the reaction temperature will only depend on the stability of one reactant and one product. Which is in contrary to transglycosylation, where the stability of the four reaction's components (sugar donor, sugar acceptor, intermediate Pentose-1P and the product) must be taken into consideration. Furthermore, the optimization of the substrate and the phosphate concentration. As mentioned earlier the high phosphate concentration shifts the reaction towards the cleavage of the nucleoside; thus, increasing the phosphate concentration might be needed for the cleavage reaction and the formation of the intermediate. However, for the transglycosylation reaction the high phosphate concentration hinders the second reaction (nucleoside formation). Additional advantage of the direct glycosylation approach, Pentose-1P acts as a substrate for both PyNPs and PNPs with no discrimination. This is different from the transglycosylation reaction where the choice of the sugar donor is dependent on the enzyme activity. Nonetheless, the unavailability and tedious synthesis and purification of the Pentose-1P remains the bottleneck of this approach. Although, different chemical and enzymatic synthesis approaches have been reported, yet, only few are of practical application (Mikhailopulo, 2007; Mikhailopulo *et al.*, 2010, 2011).

2.4 α-D-Pentofuranose-1-phosphate synthesis

With the discovery of different physiological sugar phosphates and the crucial role they play in several metabolic pathways, both chemical and enzymatic approaches have been developed. However, the lack of proper biotechnological tools back then hindered the wide spread application of the enzymatic synthesis. Thus, up to now the vast majority of the sugar phosphates are synthesized chemically.

2.4.1 Chemical synthesis of α-D-pentofuranose-1-phosphate

Two major categories of the chemical synthesis are described so far (Florkin *et al.*, 1963; Zamyatina *et al.*, 2009). In the first one, the phosphorylating agent is phosphoric acid and it takes place when the electron rich nucleophile (phosphoric acid) attack the electrophilic anomeric carbon of the sugar. Glucose-6-phosphate is an example of sugar phosphates synthesized using this strategy. In the second category the anomeric hydroxyl group act as a nucleophile and attack a phosphite group as in phosphoramidite. In approaches of the second category phosphoric acid cannot be used because of the high electron density. Thus, other phosphate containing molecules as phosphorylchloride, tetrabenzylpyrophosphate or imidazoylphosphites are used. There have been several described methods for the chemical synthesis of sugar phosphates as well as their halogenated analogues (Cori *et al.*, 1937; Colowick, 1938; MacDonald, 1962; Chittenden, 1972; Penglis, 1981; Tsuchiya, 1990), almost all of them were described earlier during the 20th century (Mikhailopulo, 2007; Mikhailopulo *et al.*, 2010). Despite the several chemical methods described for the synthesis of sugar phosphates, only few were specific for the synthesis of pentose-1Ps (**Table 3).**

Table 2. Clinically important nucleosides analogues synthesized by direct glycosylation. Reaction conditions and obtained yields are summarized

Nucleoside	Biocatalyst	Pentose-1P [a] [Pentose-1P: Base]	Reaction medium	Reaction time/ reaction temperature [°C]	Yield [%]	Reference
2-chlorodeoxy-adenosine [Cladribine]	*E. coli* PNP	dRib-1P: 2CA*	H_2O	45°C	98%[b] / 88%[c]	Komatsu *et al.*, 2005
(9-(2-deoxy-2-fluoro-α-d-arabinofuranosyl)-2-chloroadenine [Clofarabine]	Recombinant *E. coli* PNP	^{2F}Ara-1P: 2CA [0.67 μmol: 0.2 μmol]	H_2O	7 days/ 52°C	67%[b]	Fateev *et al.*, 2014
9-β-d-arabinosyl-2-fluoroadenine [Fludarabine]	Recombinant *E. coli* PNP	Ara-1P: 2FA [2.85 mmol: 0.3 mmol]	H_2O	1h/ 55°C	98%[b] / 77%[c]	Konstantinova *et al.*, 2011
9-β-D-arabinofuranosyl-guanine [Nelarabine]	Recombinant *E. coli* PNP	Ara-1P: 2A6MP [1 mmol: 0.1 mmol]	H_2O	36h/ 45°C	40%[c]	Konstantinova *et al.*, 2011

[a] Pentose-1P were synthesized in a chemical process.
[b] Yield is based on bioconversion.
[c] Yield is based on isolated product.
* molar ratio is not mentioned.

In 1957, Tener and co-workers (Tener *et al.*, 1957) described the chemical synthesis of ribofuranose-1-phosphate from 5-O-acetyl-D-ribofuranosyl bromide 2,3-cyclic carbonate or 3,5-di-O-benzoyl-D-ribofuranosyl halides with triethylammonium dibenzyl phosphate. The reaction was performed in benzene solution at room temperature, an anomeric mixture of both the α- and β- form was obtained with a ratio of 3:1. The final yield of the purified barium salt of ribofuranose-1-phosphate was 55-60%. Not so long after, MacDonald and Fletcher (MacDonald *et al.*, 1962) described the chemical synthesis of deoxyribose-1-phosphate and its purification as cyclohexylammonium salt with a final yield of 26% of an anomeric mixture. The reaction between 2-deoxy-3,5-di-O-*p*-toluoyl-D-ribosyl chloride and di-silver phosphate took place in benzene solution at room temperature. The most efficient stereoselective synthesis of 2-deoxy-α-D-ribofuranose-1-phosphate as a biscyclohexylamine salt was first reported in 2002, using the asymmetric transformation approach. A ratio of the desired α- anomer to the β- anomer of 98.8:1.2 was observed (Komatsu *et al.*, 2002).

The procedure from Macdonald and coworkers (MacDonald *et al.*, 1962; MacDonald, 1986) was applied later for the synthesis and purification of arabinose-1-phosphate and 2-deoxy-2-fluoro-arabinofuranose-1-phosphate (Konstantinova *et al.*, 2011; Fateev *et al.*, 2014). D-arabinofuranose tetraacetate, 1-O-methyl-2,3,5-tri-O-benzoyl-a-D-arabinofuranose and 1-O-Acetyl-2,3,5-tri-O-benzoyl-α-D-arabinofuranose were used as a substrate for the synthesis of Ara-1P. The reaction between the substrates and phosphoric acid took place at 70°C in the presence of dioxane or THF as a solvent, followed by the addition of lithium hydroxide. The reaction was then treated with several successive steps of methanol and acetone addition and filtration for 2 days. The final yield was 40% and a mixture of the furanose and the pyranose form of arabinose-1-phosphate was obtained with a ratio of 2:1 or 1:2 depending on the substrate and the solvent used. The chemical synthesis of

arabinose-1-phospahte from 1-O-Acetyl-2,3,5-tri-O-benzoyl-arabinofuranose, 1-bromide- 2,3,5-tri-O-benzoyl-arabinofuranose was as well reported earlier. A yield of 5.4% was obtained (Maryanoff *et al.*, 1988).

2-deoxy-2-fluoro-D-arabinofuranosyl-1-phosphate was chemically synthesized from 2-deoxy-2-fluoro-1,3,5-tri-O-benzoyl-α-D-arabinofuranose (Yamada *et al.*, 2004, 2009; Fateev *et al.*, 2014). Two routes were discussed. In the first approach the substrate 2-deoxy-2-fluoro-1,3,5-tri-O-benzoyl-α-D-arabinofuranose was converted to its 1-bromide derivative. The 1-bromide was hydrolyzed producing α-1-hydroxyl derivative which was then phosphorylated yielding the desired 1-phosphate. The other route is the direct phosphorolysis of the 1-bromide to 2-deoxy-2-fluoro-D-arabinofuranosyl-1-phosphate.

Despite the great advances made and the numerous processes developed, all the reported methods are tedious, laborious and required several deprotection steps and multiple purifications of intermediates. Additionally, most of the used solvents are hazardous and environmentally harmful. Among the major limitations of the chemical synthesis is the formation of an anomeric mixture, from which it is challanging to separate the desired α-anomer. Accordingly, developing and implementing enzymatic synthesis approaches is of huge advantage.

2.4.2 Enzymatic synthesis of α-D-pentofuranose-1-phosphate

As mentioned earlier, the first enzymatic synthesis and purification of Rib-1P and dRib-1P was reported during the earlier attempts to identify and characterize NPs from different organisms (Kalckar, 1947; Friedkin, 1950). Later, NPs were used for the synthesis of Ara-1P (Utagawa, Morisawa, Yamanaka, Yamazaki, Yoshinaga and Hir'osh, 1985), Rib-1P and dRib-1P (Fateev *et al.*, 2015). However, up until now no reports

showed the possibility of synthesizing C2 modified Pentose-1Ps. This might be attributed to limited substrate spectra of the applied mesophilic enzymes. Although the yield of Ara-1P obtained by Utagawa and co-workers was quite satisfying, yet the reported purification method was long and laborious compared to the one reported by Fateev and co-workers.

Other potential enzymatic approaches are the one pot transformation of D-pentoses to Pentose-1P using RK and PPM successively, and the transformation of Gly-3P using DERA/TPI and PPM (Mikhailopulo *et al.*, 2010). These approaches, as previously stated, were reported for the synthesis of nucleosides rather than Pentose-1Ps, and no attempts were reported for the purification of Pentose-1Ps from these reactions. As elaborated in **Figure 6**, both approaches employed at least two enzymes and at least one of these enzymes catalyzes a reversible reaction which complicates the synthesis as lot of optimizations might be necessary. It, as well, complicates the purification process of the Pentose-1Ps from the substrate and other products as Pentose-5P.

Table 3. Chemical synthesis of Pentose-1Ps. Reaction conditions and obtained yield are summarized

Pentose-1P	Number of steps	Duration of synthesis [a]	Reactants	Reagents	Yield [b]	Reference
Ribose-1-phosphate (Rib-1P)	4 main steps	1 day	5-O-acetyl-D-ribofuranosyl bromide 2,3-cyclic carbonate, Triethylammonium di-benzyl phosphate.	Benzene, methanol, lithium hydroxide, palladium (as a catalyst), barium hydroxide, ethanol, acetone.	55 %, [3:1]	Tener *et al.*, 1957
	4 main steps	1 day	3,5 di-benzoyl-D-ribofuranosyl halides, Triethylammonium di-benzyl phosphate		44%	
2-deoxyribose-1-phosphate (dRib-1P)	3 main steps	2 days	2-deoxy-3,5-di-O-p-toluoyl-D-ribosyl chloride, disilver phosphate.	Benzene, lithium hydroxide, ethanol, cyclohexylamine, ether, magnesium acetate, ammonium hydroxide, ammonia, acetone.	26%	MacDonald *et al.*, 1962
	4 main steps	5 days	3',5'-O-bis (4-chlorobenzyl)-2 deoxy-α-D-ribosyl-chloride, orthophosphoric acid, N-butylamine.	Acetonitrile, 4-methyl-2-pentanone, cyclohexylamine, methanol, ethanol.	92%, [98.8: 1.2]	Komatsu *et al.*, 2002
Arabinose-1-phosphate (Ara-1P)	4 main steps	-	1-O-Acetyl-2,3,5-tri-O-benzoyl-arabinofuranose, 1-bromide- 2,3,5-tri-O-benzoyl-arabinofuranose, dibenzyl phosphate.	Lithium-ammonia solution, cyclohexylamine.	5.4%, [1:5]	Maryanoff *et al.*, 1988
	3 main steps	1 day	Tetraacetate of D-arabinofuranose, Phosphoric acid.	Dioxane, lithium hydroxide, ethanol.	40%, [1:2][c]	Konstantinova *et al.*, 2011
	3 main steps	1 day	1-O-Acetyl-2,3,5-tri-O-benzoyl-a-D-arabinofuranose, phosphoric acid.		40%, [2:1][c]	
	4 steps	3 days	α-D-Arabinofuranosyl chloride, tris(4-chlorobenzoate), Phosphoric acid.	1-Butanamine, N,N-dibutyl 4-Methyl-2-pentanone, cyclohexamine, methanol, ammonia.	57.4%[d]	Komatsu *et al.*, 2001

Pentose-1P	Number of steps	Duration of synthesis [a]	Reactants	Reagents	Yield [b]	Reference
^{2F}Ara-1P	6 steps	24h	2-deoxy-2-fluoro-1,3,5 -tri-O-benzoyl-α-D-arabinofuranose.	Hydrogen bromide, acetic acid, dichloromethane, triethylamine, dimethylformamide, acetonitrile, 1H-tetrazole, 2-cyanoethanol, tert-butyl hydroperoxide, ammonium hydroxide, methanol, tetrahydrofuran.	43%[d]	Yamada *et al.*, 2004, 2009
	4 steps	4 days	2-deoxy-2-fluoro-1,3,5 -tri-O-benzoyl-α-D-arabinofuranose.	Phosphoric acid, acetyl bromide, dioxane, tri-N-butylamine, lithium hydroxide, chloroform, acetone, methanol, diethyl ether.	42.7%, [1:1]	Fateev *et al.*, 2014

[a] all the synthesis durations are interrupted with several purification and washing steps.
[b] yield is based on the final synthesis steps with the ratio of α- and β- anomers.
[c] yield is a mixture of furanose to pyranose.
[d] yield is in reference to the starting material.

3 Aims and Structure of the Current Work

Pentose-1Ps are of high physiological importance, additionally, they might have a big potential in industrial applications as precursors for the synthesis of nucleoside analogues. Due to the drawbacks of the chemical synthesis for Pentose-1Ps and because transglycosylation reactions for the synthesis of nucleoside analogues cannot be universally used; two main research questions were formulated:

1- **Is the enzymatic synthesis and purification of modified Pentose-1Ps (Ara-1P, $_{2F}$Rib-1P, ^{2F}Ara-1P) feasible using thermostable nucleoside phosphorylases? Is the gram-scale synthesis with sufficient product yield and high product purity possible?**

Three Pentose-1Ps were of interest; α-D-arabinofuranose-1-phosphate (Ara-1P), 2-deoxy-2-fluoro-α-D-arabinofuranose-1-phosphate (^{2F}Ara-1P) and 2-deoxy-2-fluoro-α-D-ribofuranose-1-phosphate ($_{2F}$Rib-1P). This is because of their potential application in the synthesis of nucleoside analogues. Rib-1P and dRib-1P were used to set-up the technology as the precursor nucleosides are cheap substrates and phosphorolytic cleavage of natural nucleosides are highly efficient. To answer this question a set of sub research questions were raised:

a. **Which thermophilic enzymes can be employed for the synthesis of Pentose-1Ps?**

Three PyNPs from different thermophilic organisms were characterized in accordance with their kinetic parameters, temperature profile and substrate affinity.

b. Which reaction and purification conditions should be used to obtain the highest bioconversion and final yield of Pentose-1Ps?

To answer this question, different reaction conditions were examined including enzyme, nucleoside and phosphate concentrations and reaction temperature. Additionally, two purification protocols were tested.

c. What is the stability of Pentose-1Ps at different temperatures and pH values?

To use the Pentose-1Ps in downstream processes like nucleoside synthesis, the $t_{1/2}$ life of each sugar was tested at different temperatures and in buffers with different pH values.

d. Would the chromatographic purification be an efficient alternative for the precipitation method?

Three chromatographic approaches were compared and their efficiency in the separation of the Pentose-1P and phosphate were evaluated.

2- Does the availability of these Pentose-1Ps improve the enzymatic synthesis of some challenging nucleoside analogues?

The synthesis of the biotechnologically important 5-EU nucleoside analogues; EdU, $_{2F}$Rib-EdU and ^{2F}Ara-EdU, was of interest because of their significant application in RNA/DNA labelling, hence, cellular visualization. To address this interest, the following sub-questions were raised:

a. Is the synthesis of these nucleoside analogues possible using transglycosylation reaction?

The synthesis of 5-EU nucleoside derivatives from a sugar donor nucleoside was examined using the transglycosylation approach.

Nucleoside-1 phosphorolytic cleavage and nucleoside-2 formation was closely monitored and evaluated.

b. What conditions should be used for the glycosylation reaction?

Different ratios of Pentose-1P and nucleobase as well as different enzyme concentrations were tested for the synthesis of EdU. The best working conditions were latter used for the synthesis of $_{2F}$Rib-EdU and ^{2F}Ara-EdU.

c. Would the direct glycosylation approach improve the biosynthetic yields of the nucleosides in interest compared to the transglycosylation approach?

The three nucleoside analogues of interest were synthesized using both approaches and the bioconversion yields were compared.

4 Materials and Methods

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, United States) reversed phase C18 column (150 × 4.6 mm). Thermostable NPs, PyNP-Y01, PyNP-Y02, PyNP-Y04, PyNP-Y05, PNP-N01, and PNP-N03 were obtained from BioNukleo (Berlin, Germany) and were used as recommended by the manufacturer.

4.1 Enzyme characterization

Thermophilic pyrimidine nucleosides phosphorylases; PyNP-Y01, PyNP-Y04 and PyNP-Y05 derived from three different thermophilic bacteria were characterized for their temperature optimum, kinetic parameters and substrate specificity.

Unless otherwise specified, all enzymatic reactions were performed at pH 7 and the general reaction conditions were 20 mM nucleoside in 100 mM potassium phosphate (KP) buffer and a final enzyme concentration of 0.2 $mg.ml^{-1}$. Enzymatic reactions were stopped with equal volume of methanol. The nucleoside phosphorolytic cleavage was determined by HPLC using method A (**section 4.5**).

4.1.1 Thermal characterization

To find the optimum temperature for the enzyme activity; the phosphorolytic cleavage of Urd to Ura and Rib-1P was tested at different temperatures. Reaction mixture (Urd in the KP buffer) was pre-heated at the respective temperatures (20 °C – 100 °C) for 10 mins, 2 µl of the enzyme was added to a final concentration of 50 $\mu g.ml^{-1}$. Reactions were stopped after 10 mins with equal volume of methanol. The phosphorolysis of Urd to Ura was measured on HPLC (method A, **section 4.5**) and the highest conversion rate was set as 100% activity.

4.1.2 Kinetic parameters

Activity tests were performed in triplicates unless otherwise specified. Five different substrate concentrations (0.4 mM – 5 mM) were used in 10 mM KP buffer, the reaction mixture was pre-heated for 5 min. A final enzyme concentration of 2 $ug.ml^{-1}$ was added. Samples at different time points were taken and the enzymatic reaction was stopped with equal volumes of methanol. Phosphorolytic cleavage of nucleosides was measured on HPLC using method A (**section 4.5**). Initial reaction rates were plotted against the substrate concentrations. The resulting slopes were plotted and fitted using a non-linear regression. The Michaelis-Menten's constant (K_m) and the maximum velocity (V_{max}) were determined (Sigma-plot 11.0) and the catalytic constant (K_{cat}) was calculated.

4.1.3 Substrate specificity

PyNP-Y01, PyNP-Y04, PyNP-Y05 were tested for their activity towards Urd, Thd, 2'-deoxy-2'-fluorouridine ($_{2F}$Rib-U), 1-(β-D-arabinofuranosyl)uracil (Ara-U) and 1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)uracil (^{2F}Ara-U) at 60°C. Substrate to phosphate

concentration was 1:10. Reaction conditions were as follows unless otherwise specified, reaction mixture of 1 mM substrate in 10 mM KP buffer was pre-heated for 5 mins at the corresponding optimum temperature for each enzyme. A final enzyme concentration of 0.2 $mg.ml^{-1}$ was added. Samples were taken at different time points and the reaction was stopped with equal volume of methanol. The nucleoside phosphorolytic cleavage was determined by HPLC (**section 4.5**).

4.2 Enzymatic synthesis and purification of Pentose-1Ps

4.2.1 Effect of varying phosphate concentration on the enzymatic reaction

To examine the effect of phosphate concentration on the reaction speed and the nucleoside phosphorolytic cleavage [%], enzymatic reactions were conducted in KP buffer pH 7 with different concentrations (100 mM -750 mM). All used substrates (Urd, Thd, Ara-U, $_{2F}$Rib-U, and ^{2F}Ara-U) were tested with concentrations varying between 50 mM and 200 mM.

4.2.2 Enzymatic synthesis of Pentose-1Ps

Detailed method for the enzymatic synthesis of Pentose-1P are described in **Paper III**. Urd, Thd, Ara-U, $_{2F}$Rib-U, or ^{2F}Ara-U were used as substrates for the enzymatic synthesis of Rib-1P, dRib-1P, Ara-1P, $_{2F}$Rib-1P, and ^{2F}Ara-1P, respectively. 200 mM substrate was used in 500 mM KP buffer. Enzyme concentrations, reaction temperature and duration were dependent on each Pentose-1P.

4.2.3 Purification of Pentose-1Ps as Barium salt by precipitation

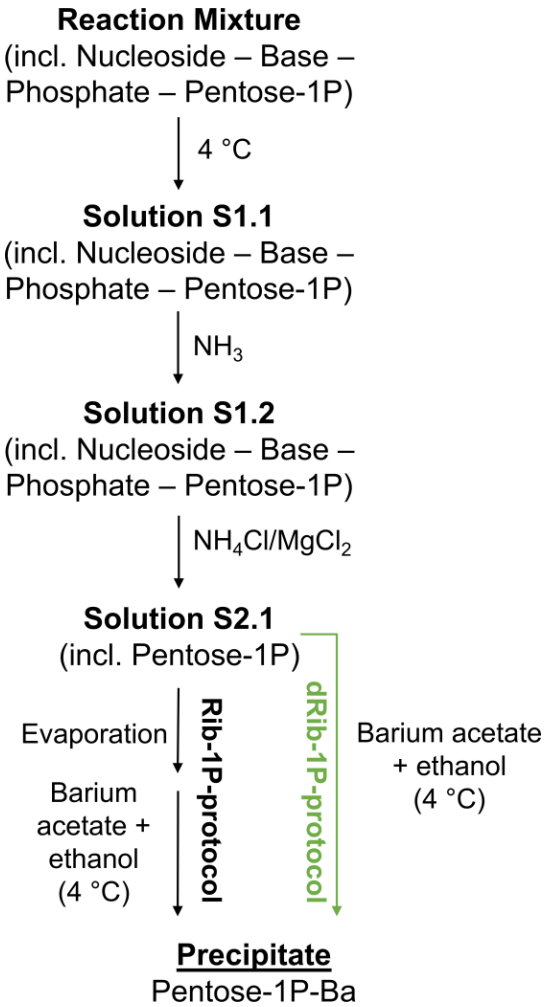
Detailed purification protocols are mentioned in **Paper III** and illustrated in **scheme 1**. After the enzymatic synthesis, the reaction mixture was incubated at 4°C overnight and the precipitated nucleobase/nucleoside were removed. 1/3 of the reaction volume of aqueous ammonia solution (25% w/v) was added. Phosphate was then precipitated as NH_4MgPO_4, and the solution was filtered.

For Rib-1P, $_{2F}$Rib-1P and ^{2F}Ara-1P, the filtrate was evaporated to the original reaction volume. Ammonia (25% w/v) was again added in 1/10 of the concentrated volume followed by the addition of barium acetate in an equimolar concentration to the starting nucleoside. Respective barium salts of Rib-1P, $_{2F}$Rib-1P and ^{2F}Ara-1P were precipitated at 4°C by the addition of equal an equal volume of ethanol to the reaction volume. For dRib-1P and Ara-1P the filtrate was used directly for barium precipitation at 4°C.

The precipitated salts were collected and washed 2 - 3 times with absolute ethanol and dried at 40 - 50°C depending on the Pentose-1P. The relative yield of Pentose-1P-Ba compared to the starting concentration of the substrate was calculated according to **formula 1**. Standard deviations were calculated from at least 3 independent experiments unless otherwise specified.

Formula 1.

$$\text{Yield } \% = \frac{\text{Amount of the produced barium salt of pentose}-1\text{P [mmol]}}{\text{Amount of the substrate [mmol]}} \times 100$$



Scheme 1. **Schematic presentation of the purification protocol**. Showing the steps of Pentose-1Ps purification by precipitation using ribose-1-phosphate and deoxyribose-1-phospphate protocols.

4.2.4 Chromatographic purification

Based on the applied method suitable amount of the enzymatic reaction was applied to the column. All purifications were conducted at room temperature using a peristaltic pump or at 4 °C using an Äkta™ Avant system (GE Healthcare, Munich, Germany). Mobile phase, column

volume (CV) and flowrate were dependent on each method and shown in **Table 4**. Samples, taken from all collected fractions, were applied to silica plates (**section 4.6)**. Bands visualized on the silica plates were densitometrically analyzed (**section 4.7**).

4.3 Stability of Pentose-1Ps at different temperatures and pH values

Half-life ($t_{1/2}$) of Pentose-1P-Ba salts were determined at different temperatures and pH values. 25 mM solutions of the Pentose-1P-Ba salts were prepared in 10 mM KP buffer (pH 7). To determine temperature stability, samples were incubated at different temperatures (room temperature (RT), 40°C, 60°C and 80°C). To evaluate stability at different pH values, samples were incubated at room temperature in buffers with varying pH values of 1, 3, 5, 7, 9, 11 or 13. Daily samples were taken over a period of 14 days and analyzed by TLC. Signal intensities on the TLC plates were quantified using the ChemiDocMP imaging system (Biorad, Germany). Percentage of the residual amounts of Pentose-1P were calculated in relation to amounts calculated at day 0. Data points were fitted with a nonlinear regression model using GraphPad Prism 7.0 (GraphPad Software Inc, San Diego, CA, http://www.graphpad.com/) and the $t_{1/2}$ life was extrapolated. More details are mentioned in **Paper III**.

Table 4. Tested chromatographic methods and conditions of purification. Size exclusion and adsorption chromatography were performed with a pump at RT and ion exchange chromatography at the Äkta system.

Principle of separation	Material used	Column volume [ml]	Column dimensions [height × width cm]	Mobile phase	Elution	Flow rate [ml/min]
Size exclusion	Sephadex G10	211	43 ×2.5	Ultrapure water	Isocratic	6
		248	119 × 1.6			
Ion exchange	Quaternary ammonia [-$CH_2N^+(CH_3)a_3$][a]	6	------	20 mM bisTris, 1M NaCl [pH 6.2]	Gradient (20 CV, end conc. 1M NaCl)	6
Adsorption normal phase	Silica gel	16	9 × 1.5	n-propanol: H_2O: NH_3	Isocratic	5
		88	18 × 2.5			

[a] Resource Q column (GE-Healthcare life sciences).

4.4 Synthesis of nucleoside analogues

4.4.1 Synthesis of nucleoside analogues by transglycosylation reaction

EdU, (2′S)-2′-deoxy-2′-fluoro-5-ethynyluridine (^{2F}Ara-EdU) and 2'-deoxy-2′-fluoro-5-ethynyluridine ($_{2F}$Rib-EdU) were synthesized in a one-pot two-steps transglycosylation reaction by using 5-ethynyluracil (5-EU) nucleobase as the sugar acceptor, whereas, sugar donors were Thd and deoxyadenosine (dAdo) for the synthesis of EdU and ^{2F}Ara-U and $_{2F}$Rib-U for the synthesis of ^{2F}Ara-EdU and $_{2F}$Rib-EdU, respectively. PyNP and PNP were used to catalyze the enzymatic cleavage and the subsequent nucleoside synthesis. The ratio of the sugar donor to sugar acceptor was 10:1. Using Thd, $_{2F}$Rib-U, ^{2F}Ara-U as sugar donors, 2 ml reaction mixtures of 10 mM sugar donor, 1 mM sugar acceptor and 5U of PyNP-Y04 in 2mM potassium phosphate (KP) buffer (pH 7) were prepared. The reaction mixture was incubated at 40°C for 30 h. With dAdo as a sugar donor, a final reaction volume of 2 mL was used. A reaction mixture of 10 mM sugar donor, 1 mM sugar acceptor, 5U of PyNP-Y04 and 5U PNP-N02 in 2mM KP buffer (pH 7) was prepared. The reaction was incubated at 40°C for 30 h.

4.4.2 Synthesis of nucleoside analogues by glycosylation reaction

The three eniluracil containing nucleoside analogues (EdU, ^{2F}Ara-EdU, $_{2F}$Rib-EdU) were synthesized by the direct glycosylation of the nucleobase with the respective Pentose-1P in the presence of PyNP as a biocatalyst. Pentose-1P and sugar acceptor were used in a ratio of 2:1 and PyNP-Y04 was used at a concentration of 0.5 mg/ml. EdU was synthesized in a final

volume of 2 mL with 8 mM 5-ethynyluracil, 16 mM dRib-1P-Ba salt and 0.1 or 0.5 mg/mL PyNP-Y04 at 40°C for 5h. ^{2F}Ara-EdU and $_{2F}$Rib-EdU were enzymatically-synthesized at 50°C in a total reaction volume of 2 mL with 6 mM 5-ethynyluracil and 12 mM ^{2F}Ara-1P-Ba salt or $_{2F}$Rib-1P-Ba salt at 50°C for 24h and PyNP-Y04 concentration of 0.5 mg/mL.

4.5 High performance liquid chromatography (HPLC)

Nucleosides and nucleobases were separated using a reversed phase C18 column (Kinetex 150x 4.6 mm, Phenomenex). HPLC analysis was performed as previously reported (method A) (Szeker *et al.*, 2012). The conversion percentage of nucleosides was calculated as described previously (**Formula 2**) (Zhou *et al.*, 2013). For the synthesis of 5-EU nucleoside analogues HPLC analysis was performed with the following gradient: from 97% 20 mM ammonium acetate and 3% acetonitrile to 72% 20 mM ammonium acetate and 28% acetonitrile in 11 min (method B). Retention times (R_T) according to both methods are described in **Table 5**. Standard deviations were calculated from at least 3 independent experiments unless otherwise specified.

Table 5. Retention times (R_T) of nucleosides and nucleobases using both HPLC methods.

Compound	R_T [min]	
	Method A	Method B
Uridine	3.2	—
Uracil	2.7	2.9
Thymidine	4.7	5.6
Thymine	3.7	4.3
Ara-U	3.5	—
^{2F}Ara-U	4.5	5.3
$_{2F}$Rib-U	4.3	5.0
dAdo	—	6.2
Ade	—	4.6
5-EU	—	4.4
EdU	—	4.1
^{2F}Ara-EdU	—	4.1
$_{2F}$Rib-EdU	—	4.1

Formula 2.

$$\text{Conversion } \% = \frac{\text{Conc. of the product [mM]}}{\text{Conc. of the product [mM]} + \text{ Conc. of the substrate [mM]}} \times 100$$

4.6 Thin layer chromatography (TLC)

Pentose-1Ps, nucleosides and nucleobases were analyzed by TLC on silica plates (Merck). 2 µl of standard mixture and samples from different time points were loaded on the silica plates. 10 mM standard solution was used unless otherwise specified. A solvent mixture of n-propanol, ammonia and H_2O was used in a ratio of 11 : 2 : 7 (Halmann *et al.*, 1969) for Rib-1P and dRib-1P reactions (**mobile phase A**), and in a ratio of 11 : 2 : 5 for Ara-1P, $_{2F}$Rib-1P and ^{2F}Ara-1P (**mobile phase B**).

Nucleoside and nucleobase signals were detected by UV light at 254 nm (Merck). Rib-1P, dRib-1P, Ara-1P were detected colorimetrically using p-anisaldehyde-sulphuric acid solution (Stahl *et al.*, 1961). Inorganic phosphate as well as $_{2F}$Rib-1P and ^{2F}Ara-1P were detected using Hanes reagent (Stanley, 1964). Silica plates were sprayed with the respective solutions and incubated in an oven at 110°C for 5 - 15 min. Retention factor (R_f) values were calculated (**Table 6**).

Table 6. Retention factor (R_f) values of tested compounds on TLC.

Compound	R_f Value (Mixture A)	Compound	R_f Value (Mixture B)
Uridine	0.6	**Ara-U**	0.7
Uracil	0.7	**Uracil**	0.7
Rib-1P	0.4	**Ara-1P**	0.3
Ribose	0.5	**Arabinose**	0.4
Thymidine	0.7	**^{2F}Ara-U**	0.7
Thymine	0.7	**^{2F}Ara-1P**	0.3
dRib-1P	0.3	**$_{2F}$Rib-U**	0.7
Deoxyribose	0.6	**$_{2F}$Rib-1P**	0.3

4.7 Densitometric analysis using ImageJ

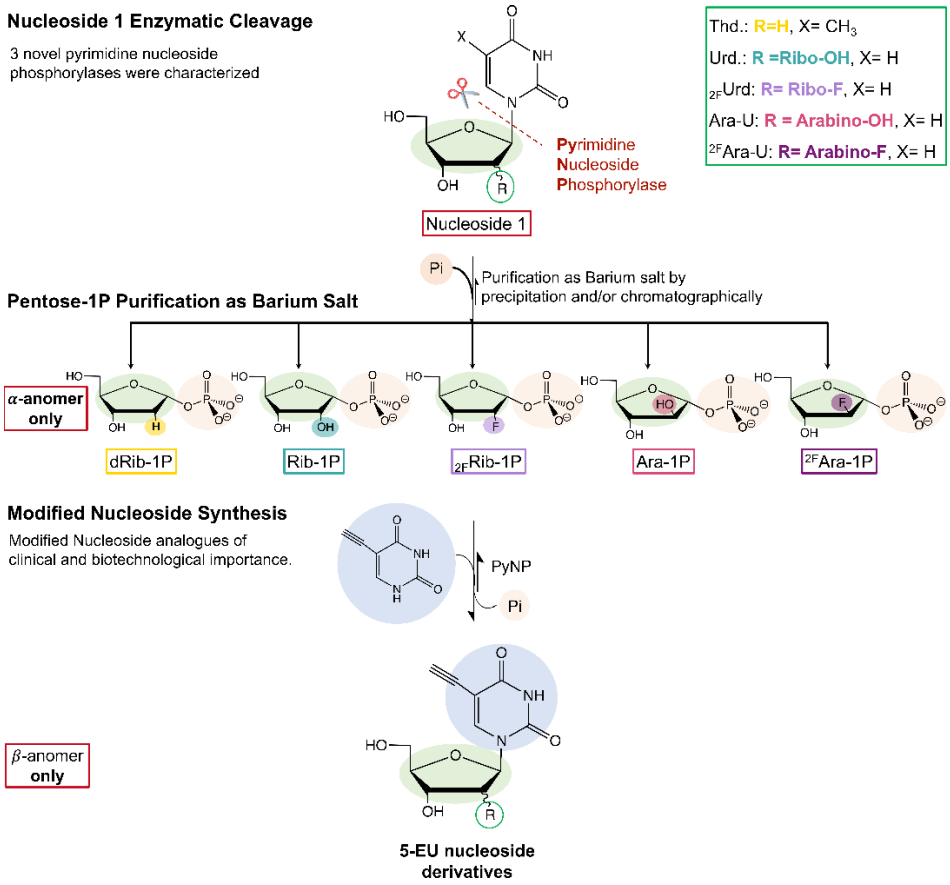
Densitometric analysis of the TLC was performed using ImageJ software (National Institute of Health, USA, http:// www.imagej.nih.gov/ij). The TLC silica plates were treated as described in **section 4.6**. The intensities of the bands on the TLC silica plates were measured and plotted against the fractions using Sigma-plot 11.0.

5 Results

With the aim of the synthesis and purification of Pentose-1Ps and their further application in nucleoside analogues synthesis, the structure of this work was built and illustrated in **scheme 2**. To establish the enzymatic reaction, three new thermostable PyNPs were characterized for their temperature optima, enzyme affinity and catalytic efficiency. Their activity towards the sugar-donor nucleosides was evaluated. Accordingly, the suitable enzymes were chosen for the synthesis of Pentose-1Ps.

To synthesize the modified Pentose-1Ps, first, reaction conditions were evaluated and optimized for the natural Pentose-1Ps; Rib-1P and dRib-1P. Evaluated conditions were the phosphate and the substrate concentrations. Moreover, for each modified Pentose-1P, reaction temperature and duration were evaluated (**Paper-III**). The five Pentose-1Ps were purified by precipitation as Barium salts. Additionally, chromatographic purification was examined as an alternative purification approach.

Finally, three of the synthesized Pentose-1Ps; dRib-1P, $_{2F}$Rib-1P and ^{2F}Ara-1P, were employed in the synthesis of 5-EU nucleoside derivatives by direct glycosylation reaction. Yields from the direct glycosylation reaction were compared with yields obtained from the traditional transglycosylation reaction.



Scheme 2. Schematic presentation showing the structure of the current work.

5.1 Characterization of thermostable NPs

5.1.1 Thermal characterization of PyNPs

Three new thermostable pyrimidine nucleoside phosphorylases; PyNP-Y01, PyNP-Y04 and PyNP-Y05, were characterized for their temperature profile (**Figure 7**). The highest activity for PyNP-Y01 was measured at 80°C and was set to 100%. There was no significant loss of activity at lower temperatures. The relative calculated activity of

PyNP-Y01 at 20°C was 81%. However, upon increasing the temperature to 90°C and 100°C, a drastic loss of activity was observed and was calculated to be 26% and 2%, respectively.

The activity of PyNP-Y04 increased with increasing the temperature to the highest tested temperature (100°C). The activity was slightly increasing between 20°C and 70°C followed by a significant rise of activity starting from 80°C.

The activity of PyNP-Y05 showed a uniform bell-shaped curve with the maximum activity at 50°C and almost complete loss of activity (6%) at 70°C. At temperatures below 50°C, the activity was gradually decreasing with temperature.

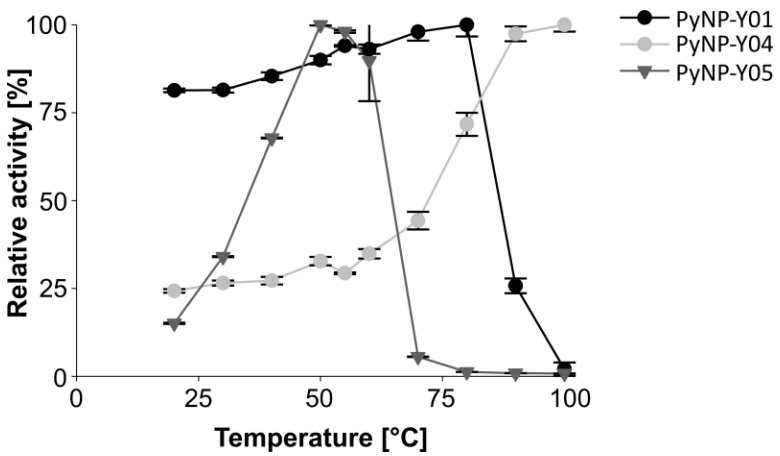


Figure 7. Relative activity of the tested PyNPs at different temperatures. The temperature optima were determined using uridine as a substrate and enzyme concentration of 50 µg.ml^{-1}. The reaction was stopped after 10 minutes and the highest activity was set to 100% for each enzyme. Error bars show the standard deviation of three independent samples. If error bars are not seen the difference is very small.

5.1.2 Kinetic parameters of PyNPs

The kinetic parameters of the thermostable PyNPs, PyNP-Y01, PyNP-Y04, PyNP-Y05 were determined in 10 mM KP buffer. The results and the reaction conditions are summarized in **Table 7**. The three tested enzymes showed higher affinity (low K_m values) to Urd compared to Thd. PyNP-Y04 showed the better substrate affinity among the three enzymes which was reflected by the lowest K_m values for both Urd and Thd. However, the K_{cat}/K_m ratio which best describe the enzyme catalytic efficiency showed different pattern. The K_{cat}/K_m ratio of PyNP-Y01 is the best (highest values) compared to all tested PyNPs. PyNP-Y01 has better affinity (K_m value) and better catalytic efficiency (K_{cat}/K_m value) towards Urd compared to Thd. The results showed that all three tested enzymes are more specific to Urd than Thd with at least 1.5 folds as was the case in PyNP-Y04. PyNP-Y05 showed a more prominent specificity to Urd than Thd (9 folds).

5.1.3 Substrate specificity of PyNPs

The specific activity of the four nucleoside phosphorylases were tested against the substrates of interest that would later be used for the Pentose-1P synthesis. Our results (**Table 8**) showed both enzymes (PyNP-Y01 and -Y04) have comparable activities towards all tested substrates. PyNP-Y05 showed very low or no activity towards the modified substrates $_{2F}$Rib-U and ^{2F}Ara-U.

Table 7. Kinetic parameters of the studied PyNPs on uridine and thymidine compared to some published PyNPs, TPs and UPs.

Enzyme	K_m [μM]		K_{cat} [s^{-1}]		K_{cat}/K_m [s^{-1} μM^{-1}]		Reaction conditions	Ref.
	Urd	Thd	Urd	Thd	Urd	Thd		
PyNP-Y01	914	2900	243.3	389	0.266	0.134	50°C, pH 7	This study
PyNP-Y04	338.9	804.2	30.2	48.41	0.089	0.060	80°C, pH 7	This study
PyNP-Y05	810.6	51800	33.4	242.5	0.041	0.0046	60°C, pH 7	This study
GtPyNP	2342	1282	275	83	0.12	0.06	60°C, pH 7	Szeker *et al.*, 2012
GsPyNP	190	160	NA	NA	NA	NA	60°C, pH 7	Hori *et al.*, 1989
TtPyNP	145	435	279	679	1.92	1.56	80°C, pH 7	Szeker *et al.*, 2012
MhPyNP	92	473	8.5	20.5	0.092	0.046	37°C, pH 7.6	Voorde *et al.*, 2012
EcTP	NA	190	NA	130	NA	0.68	37°C, pH 7.4	Petaccia *et al.*, 2016
	60	300	$<1x10^{-4}$	198	$<1.7x10^{-6}$	0.66	26°C, pH 6.5	Panova *et al.*, 2004, 2007
EcUP	80	270	98	5	1.22	0.02	26°C, pH 7.5	Panova *et al.*, 2004; Szeker *et al.*, 2012
LBcTP	NA	13200	NA	NA	NA	NA	37°C, pH 6	Avraham *et al.*, 1990
LBcUP	50000	NA	NA	NA	NA	NA	37°C, pH 7	

NA: data not available.
Gt: *Geobacillus thermoglucosidasius*; Tt: *Thermus thermophilus;* Gs: *Geobacillus stearothermophillus;* Mh: *Mycoplasma hyorhinis*; Ec: *Escherichia coli*; LBc: *Lactobacillus casei.*

Table 8. Activity of PyNPs towards natural and modified pyrimidine nucleosides.

Enzyme	Enzyme activity [conversion %]				
	Urd	Thd	Ara-U	$_{2F}$Rib-U	^{2F}Ara-U
PyNP-Y01	90% [a]	27%	82%	74%	28%
PyNP-Y04	91% [a]	32%[a]	65%	65%	45%
PyNP-Y05	40%	2%	NT[b]	5%[b]	ND[b]

NT: Not tested
ND: No detected product peak
Reactions were done at 1mM substrate in 10 mM KP buffer pH 7 unless otherwise specified.
[a] 1mM substrate in 50 mM KP buffer pH 7.
[b] 20 mM substrate in 100 mM KP buffer pH 7

5.2 Pentose-1Ps synthesis and purification

5.2.1 Optimization of reaction and purification conditions for the synthesis of Pentose-1Ps

5.2.1.1 Impact of phosphate on the final bioconversion yield of PyNP catalyzed phosphorolysis reactions

The impact of varying phosphate concentrations on the final product yields of the enzymatic reaction was examined (**Figure 8**). Two sets of experiments were conducted, in one set the phosphate concentration was kept constant at 500 mM whereas, the substrate concentration was increased from 50 mM to 100 mM and 200 mM (**Figure 8A**). In the second set of experiments, the phosphate concentration was increased from 100 mM to 750 mM while maintaining the substrate concentration constant at 100 mM for Thd or 50 mM for Ara-U and $_{2F}$Rib-U (**Figure 8B**).

Using PyNP-Y02 as a biocatalyst, the percentage of phosphorolysis of Thd after 1h increased from 30% to 50%, 55% and 60% at 100 mM, 250 mM, 500mM and 750 mM KP, respectively (**Figure 8B**). The amount of Ara-U phosphorolyzed increased from 55% to 70% with increasing the phosphate concentration from 100 mM to 750 mM. While the percentage of phosphorolyzed $_{2F}$Rib-U was increased from 55% to 70%, 75% and 78% upon increasing the KP concentration from 100 mM to 250 mM, 500 mM and 750 mM, the equilibrium was not reached after 24h reaction. PyNP-Y04 was used for the phosphorolysis of both substrates, Ara-U and $_{2F}$Rib-U.

The higher the ratio between the substrate and the phosphate concentration the better was the percentage of phosphorolysis. The highest examined ratio of substrate to phosphate was 1:15 (50 mM substrate: 750 mM KP). This showed the highest phosphorolysis percentage compared to the other tested reaction conditions for both substrates; Ara-U and $_{2F}$Rib-U (**Figure 8B**). For the phosphorolysis of Thd, the highest examined ratio was 1:10 (50 mM Thd: 500 mM KP) (**Figure 8A**). 65% of Thd was phosphorolyzed under these conditions, whereas the lowest tested ratio was 1:1 (100 mM Thd: 100 mM KP) and the percentage of phosphorolysis was 36% (**Figure 8B**). The same pattern was as well observed for Urd phosphorolysis. The percentage of phosphorolysis was decreased from 70% to 30% upon lowering the ratio of 1:10 (50 mM Urd: 500 mM KP) to 1: 2.5 (200 mM Urd: 500 mM KP) (**Figure 8A**).

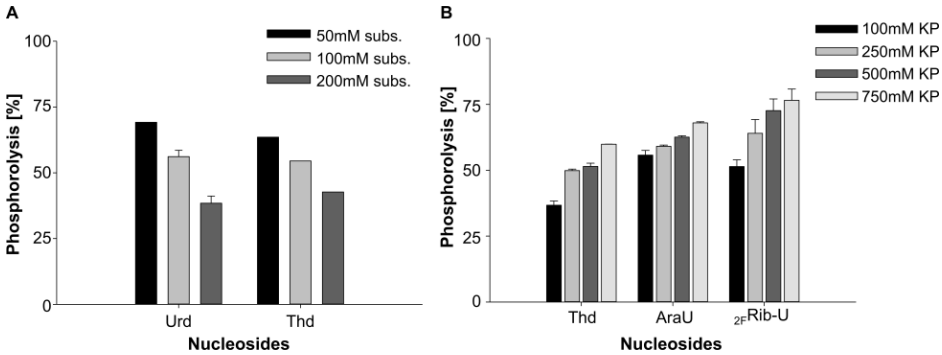


Figure 8. **Effect of phosphate concentration on the phosphorolysis of nucleosides.** (**A**) Phosphorolysis [%] of different concentrations of Urd and Thd at a constant KP concentration (500 mM, pH 7) after 6h. 0.1 mg/ml PyNP-Y02 was used in a concentration of 0.1mg/ml at 50°C. (**B**) Phosphorolysis [%] of Thd (100 mM), Ara-U (50 mM) and $_{2F}$Rib-U (50 mM) after 24h in different concentrations of KP buffer pH 7. For Thd phosphorolysis, 0.1 mg ml^{-1} PyNP-Y02 was used at 50°C. For the phosphorolysis of AraU and $_{2F}$Rib-U, 0.5mg ml^{-1} PyNP-Y04 was used at 80°C. Error bars show the standard deviation of three independent samples. If error bars are not seen the difference is very small.

5.2.2 Enzymatic synthesis and chemical purification of Pentose-1Ps

Rib-1P, dRib-1P, Ara-1P, ^{2F}Ara-1P and $_{2F}$Rib-1P were enzymatically synthesized. The details of the synthesis and purification are shown and discussed in detail in **paper III**.

First, Rib-1P and dRib-1P were synthesized and purified as per the published protocol (Fateev *et al.*, 2015). Details of the initial synthesis conditions are described in **Paper III**, enzymatic bioconversion and yields after barium precipitation are shown in **Paper III, Figure 1A and 1B**. Afterwards, the protocol was optimized. The ratio that showed the highest bioconversion (50 mM substrate and 500 mM KP) was used and

Pentose-1P purification as barium salt was carried out. Surprisingly, no Pentose-1P-Ba salt was obtained after purification. Thus, different substrate to phosphate ratios were examined, 1:5 and 1:2.5. Although by decreasing the ratio of substrate to phosphate, the bioconversion is as well decreased as discussed above in section 5.2.1, yet the purified Pentose-1P-Ba salt yield increased as shown in **Paper III, Figure 2**. The highest yield of Pentose-1P-Ba salt was obtained with 200 mM substrate and 500 mM KP (**Figure 9A**).

Accordingly, conditions for Ara-1P, $_{2F}$Rib-1P and ^{2F}Ara-1P were chosen to be 200 mM substrate and 500 mM KP. Using these conditions and PyNP-Y04 as biocatalyst, the obtained bioconversion of Ara-1P, $_{2F}$Rib-1P and ^{2F}Ara-1P was 37% after 24h, 34% and 27% after 72h respectively. The yield of the barium salts of these three sugars were 13% for Ara-1P-Ba salt and 23% for both $_{2F}$Rib-1P-Ba and ^{2F}Ara-1P-Ba salts (**Figure 9B**).

The structure of all synthesized Pentose-1P-Ba salts were confirmed by NMR spectroscopy and their stability was evaluated at different pH values and different temperatures. Detailed results are presented in **paper III**.

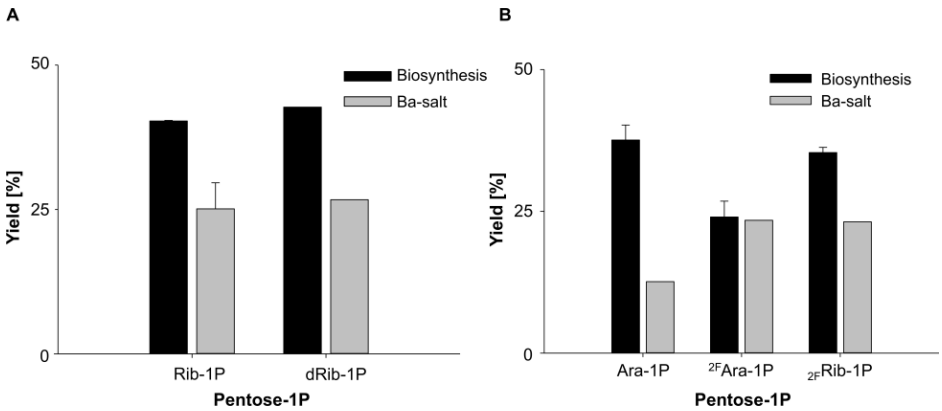


Figure 9. Yields of Pentose-1Ps after biosynthesis and purification as Barium salt. (A) Results shown for Rib-1P and dRib-1P are from the final optimized conditions (200mM nucleoside and 500 mM KP buffer, pH 7) PyNP-y02 was use at concentrations of 0.1 mg ml^{-1} and 0.5 mg ml^{-1} for Rib-1P and dRib-1P, respectively, and reaction temperature of 50°C and 40°C, respectively. (B) Modified Pentoses-1P were synthesized from their corresponding nucleosides (200 mM) in the presence of 500 mM KP and 0.5 mg ml^{-1} PyNP-Y04. Reaction temperatures were 80°C for the synthesis of Ara-P and ^{2F}Ara-1P and 65°C for the synthesis of $_{2F}$Rib-1P. Error bars show the standard deviation of three independent samples. If error bars are not seen the difference is very small.

5.2.3 Chromatographic purification of Pentose-1Ps

The co-precipitation of Pentose-1Ps from Pi was widely described in literature (Florkin *et al.*, 1963). In an attempt to improve the purification process to separate Pentose-1Ps from Pi, different chromatographic methods were tested. The separation of Ara-1P from the reaction mixture of Ara-U, Ura and Pi was used to test the different chromatographic methods. The tested methods were size exclusion (sephadex G-10), ion exchange (quaternary ammonia [-CH2N+(CH3)3]) and adsorption (silica gel) chromatography.

5.2.3.1 Size exclusion chromatography

Sephadex-G10 was used for the separation of Ara-1P from the reaction mixture. Two trials were conducted and the difference between both was the column length. It was 43 cm in the first trial and 119 cm in the second. The separation of the Ara-1P and Pi was improved upon increasing the column length, however a complete separation was not achieved.

In the first trial, the total number of fractions collected were 82. Ara-1P was the first to be eluted and it was detected in fractions 2 – 38. Pi was co-eluting with Ara-1P starting from fraction 7. It was detected until fraction 38. Thus, only 5 out of 37 fractions contained pure Ara-1P free from Pi (**Figure 10A**). Ara-U and Ura were eluted later with no interference with Ara-1P fractions.

To improve the separation of the Ara-1P and Pi, a longer column (119 cm) was used. A total of 94 fractions were collected and Ara-1P was eluted in

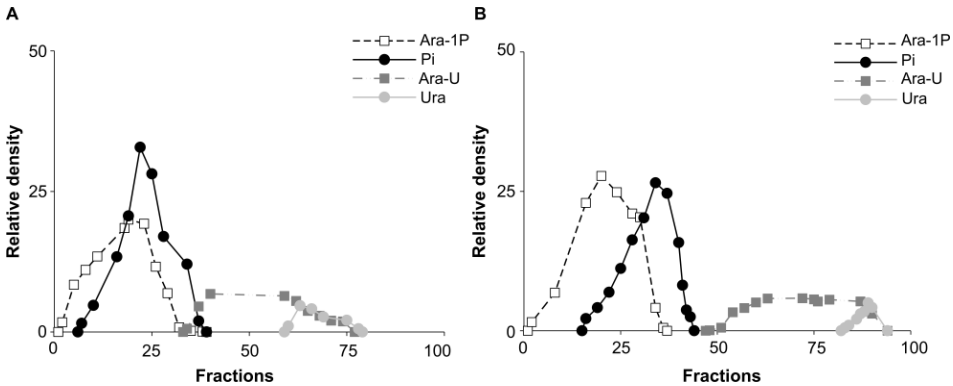


Figure 10. Application of size exclusion chromatography for the purification of Ara-1P using sephadex G-10. (**A**) The first separation experiment was performed with a column length of 43 cm. In total 82 fractions were collected. (**B**) Improved separation of Ara-1P from Pi, with a column length of 119 cm. The relative density of the TLC was measured using imageJ software.

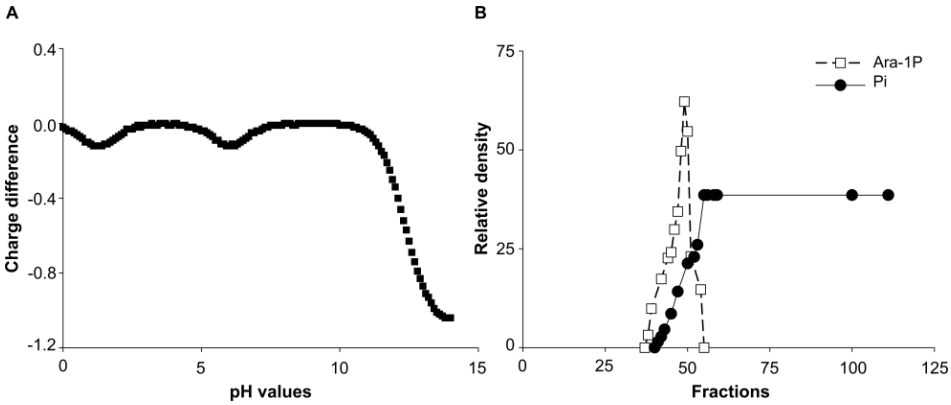
fractions 2– 36. Again, Pi was co-eluted with Ara-1P starting from fraction 16. Pi was detected until fraction 43. Thus, the elution of Pi was shifted to later retention times with increasing the column length. The number of fractions containing Ara-1P free from Pi increased to 14 out of 35 fractions (**Figure 10B**)

5.2.3.2 Ion exchange chromatography

For the ion exchange chromatography, a Resource-Q column was used at an Äkta™ avant system. Creating a charge difference between Ara-1P and Pi was important to achieve a good separation. Analyzing the ionization pattern of Ara-1P and Pi, it was concluded that the maximum charge difference was at pH 1, 6.2 and 14 (**Figure 11A**). Thus, different experimental trials were conducted at pH 6.2.

The reaction mixture was applied to the column in a concentration of 37 µmol and 162 µmol of Ara-1P and Pi, respectively. The elution was done by gradually increasing the concentration of NaCl to a final concentration of 1 M.

In total, 245 fractions were collected; 15 were collected during the column wash and 230 were collected during elution. Ara-U and Ura were detected in the washing fractions 3 – 7. No traces of Ara-U or Ura were detected in any of the elution fractions. For the remaining 230 fractions, only 111 were analyzed as Ara-1P was totally eluted in fractions 38 – 54. Pi was eluted in fractions 41 until the last analyzed fraction 111 (**Figure 11B**).



difference between Ara-1P and Pi at different pH values**.** Ionization pattern of both molecules was predicted using Chemicalize, a product of ChemAxon (https://chemicalize.com). (**B**) Separation pattern of Ara-1P and Pi. The relative density of the bands on the TLC plates was calculated using imageJ software.

5.2.3.3 Adsorption normal phase chromatography

Normal phase chromatography using silica gel beads was applied for the separation of Ara-1P and Pi. Pre-experimental trials were initially conducted on TLC. Seven different mobile phases were investigated to test the separation of the reaction mixture's components. Preliminary results showed that the best separation of Ara-1P and Pi was obtained upon using Dioxane: n-propanol: NH_3: H_2O (4:2:3:4), however, due to the toxicity of the Dioxane, the second-best mobile phase, n-propanol: H_2O: NH_3 (11:5:2), was used for the column chromatography.

Two experiments were conducted having a difference in the column volume (CV). The separation was significantly improved upon increasing the CV from 16 ml to 88 ml as well as doubling the column height. In the first experiment, a total of 45 fractions were collected (**Figure 12A**). Ara-U and Ura were eluted in the first 5 and 8 fractions, respectively. Ara-1P was co-eluted with them starting from fraction 2. Ara-1P was detected until fraction 30. Pi on the other hand was eluted in fractions 13 to 45. Accordingly, fractions containing Ara-1P free from Pi were 11 fractions (fractions 2 – 12).

In the second experiment, Ara-1P elution started in fraction 18 right after the complete elution of Aa-U and Ura that were eluted in fractions 1 – 9. Ara-1P was detected until fraction 78 with a total of 60 fractions. Pi was eluted in fraction 64 to 180. Hence, the number of fractions where Pi and Ara-1P were co-eluted were only 15 (**Figure 12B**). 45 fractions contained pure Ara-1P free from Pi.

To test the feasibility of using the chromatographically purified Ara-1P for the synthesis of nucleoside. All the pure fractions containing Ara-1P were collected. After evaporation of the mobile phase, Ara-1P was precipitated as Barium salt in the presence of ethanol and was then used for the synthesis of 9-(β-D-arabinofuranosyl)adenine (Ara-A). The biosynthesis

of Ara-A was 48% while it was 94% upon using the chemically purified Ara-1P-Ba. The decrease in the yield of Ara-A might be due to the interference of the residual amounts of the mobile phase with the enzymatic synthesis. Prolonged washing of Ara-1P before Barium precipitation might prevent such interference.

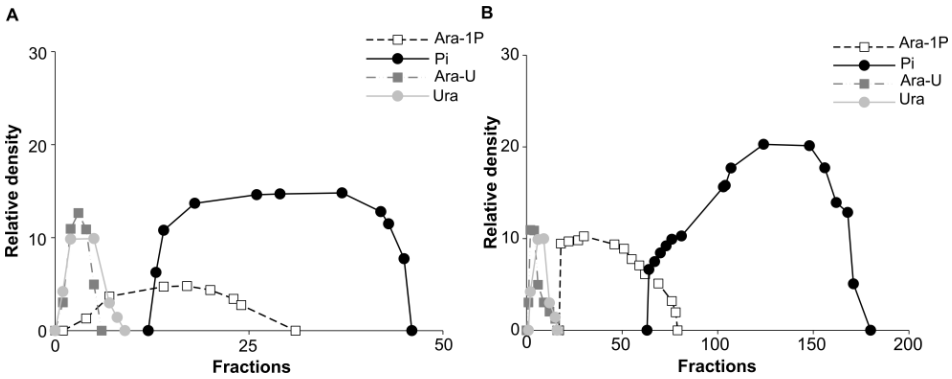


Figure 12. Application of adsorption normal phase chromatogram for the purification of Ara-1P using silica gel. (**A**) First experimental trial where only few fractions contained pure Ara-1P with no contamination of other reaction components. The column volume was 16 ml and the total number of fractions obtained were 45 fractions. (**B**) Improved separation of Ara-1P from Pi by increasing the column volume to 88 ml, the total number of fractions containing pure Ara-1P were 45 fractions. The relative density of the bands on TLC plates was measured using imageJ software.

5.3 Synthesis of nucleoside analogues by transglycosylation and glycosylation approaches

EdU, $_{2F}$Rib-EdU and ^{2F}Ara-EdU nucleosides were enzymatically synthesized by NPs through the transglycosylation and the direct glycosylation approaches. The obtained biosynthesis yields were compared.

For the transglycosylation, Thd, $_{2F}$Rib-U or ^{2F}Ara-U, were used as the sugar donors (nucleoside 1-sugar donor) and 5-EU nucleobase as the sugar acceptor, thermostable NPs were used as the biocatalyst. On the other hand, for the direct glycosylation reaction, the enzymatically synthesized dRib-1P, $_{2F}$Rib-1P, and ^{2F}Ara-1P were used. Upon applying the transglycosylation approach, the biosynthesis yield of EdU, $_{2F}$Rib-EdU and ^{2F}Ara-EdU after 30h was 41%, 18% and 0% respectively, whereas that from the direct glycosylation approach was 60% for EdU after 4h and 47% and 51% for $_{2F}$Rib-EdU and ^{2F}Ara-EdU, respectively, after 24h (**Figure 13**). Detailed results of both synthesis approaches are presented in **paper IV.**

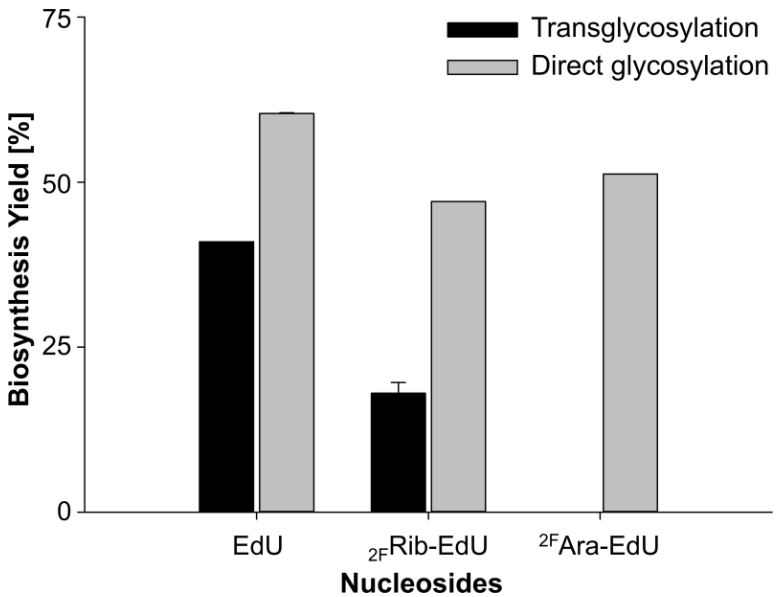


Figure 13. Nucleoside synthesis using transglycosylation and glycosylation approach. Transglycosylation reactions were performed at 40°C for 30 h. 10 mM sugar donor, 1 mM sugar acceptor and 5U of PyNP-Y04 in 2 mM potassium phosphate (KP) buffer (pH 7) were used for the synthesis of modified nucleoside analogues. Pentose-1P and nucleobase (sugar acceptor) were used in a ratio of 2:1 and PyNP-Y04 was used at a concentration of 0.5 mg ml^{-1} Error bars show the standard deviation of three independent samples. If error bars are not seen the difference is verv small.

6 Discussion

Five Pentose-1Ps were enzymatically synthesized and purified as their barium salts by precipitation or chromatographic purification methods. Rib-1P, dRib-1P, Ara-1P, $_{2F}$Rib-1P and ^{2F}Ara-1P were produced in gram scale from their corresponding nucleosides using thermostable PyNPs. Up to our knowledge, this is the first report on the enzymatic synthesis of $_{2F}$Rib-1P, and ^{2F}Ara-1P using thermostable nucleoside phosphorylases. Additionally, the enzymatic synthesis and enhanced chemical purification approach of Ara-1P is reported as well. The presented study is a basis for the application of Pentose-1P as a universal glycosylating agent. Furthermore, natural and modified Pentose-1Ps can be used for extensive metabolic studies.

To be able to produce a wide spectrum of Pentose-1Ps and nucleoside analogues, thermostable nucleoside phosphorylases; PyNP-Y01, PyNP-Y04 and PyNP-Y05, were characterized for their temperature optima and substrate affinity. Reaction temperature is a critical factor in the synthesis of Pentose-1Ps as higher reaction temperatures can facilitate enzymatic reactions or on the opposite lead to the degradation of substrate or product. PyNP-Y01, PyNP-Y05, and PyNP-Y04 were obtained from organism having an optimum growth temperature of 50°C for the first two and 80°C for the latter. The activity of PyNP-Y01 and PyNP-Y05 was drastically lost at temperatures above the optimum (**Figure 7**). This might be due to the breaking of hydrogen bonds which are responsible for protein stabilization (Pace *et al.*, 2014). Hence, protein unfolding and loss of activity. Moreover, the noticed low activities of PyNP-Y04 and PyNP-Y05 at suboptimal temperatures could be explained by the conformational rigidity at low temperatures (Eisenmesser *et al.*, 2002).

The thermal stability of enzymes is attributed to several factors including higher content of hydrogen bonds and/or salt bridges, increased compactness and hydrophobicity (Das *et al.*, 2000; Kumar *et al.*, 2000; Razvi *et al.*, 2006). Salt bridges arise from the interactions between negatively charged carboxylate ion ($RCOO^-$) of glutamate (E) or aspartic acid (D) and the positively charged ammonium or guanidium groups (RNH_3^+ or $RNHC(NH_2)_2^+$ groups) of lysine (K) and arginine (R), respectively. Hence, a higher content of E, K, R, valine (V) and isoleucine (I) residues was observed in thermostable enzymes. In contrast, the number of glutamine (Q) and asparagine (N) residues is low since they are more labile to deamination which decrease the stability.

The sequences of different PyNPs and TPs from diverse organisms were analyzed and compared to the three PyNPs studied in this work (**Table 9**). Analyzed enzymes had temperature optima ranging from 37°C - 100°C. Among all analyzed PyNPs and TPs, TtPyNP had the best parameters for the thermophilic signature. It showed the highest amounts of E and R (10.2% and 8.5%, respectively) and the lowest amounts of Q and N (0.9% and 0.5% respectively). Additionally, it has the highest number of salt bridges among all analyzed sequences. These features are well correlate with the thermal stability of TtPyNP at 80°C (Szeker *et al.*, 2012). Enzyme half-life as reported at this temperature was more than 24h.

Although PyNP-Y04 and TtPyNP have very close temperature optima, yet PyNP-Y04 showed decreased amounts of E (9.3%), R (2.9%), V (7.3%) compared to TtPyNP (E=10.2, R=8.5 and V=8%). PyNP-Y04, however, had higher content of residues K (7.3%) and I (7%) compared to TtPyNP's (K=5.2%, I=2.8%). Despite the differences in the individual residues, the overall percentages of the amino acids that determine the thermophilic signature is very close. The percentages are 33.8% and 34.7% for PyNP-Y04 and TtPyNP, respectively.

Compared to *E. coli* TP (EcTP), PyNP-Y04 has a higher amount of amino acid residues required for the enzyme thermostability which is 33.8% compared to 29.8%. Additionally, lower amounts of amino acid residues that contribute to enzyme destabilization were observed with values of 3.5% and 5.2% for PyNP-Y04 and EcTP, respectively (**Table 9**). The number of salt bridges of PyNP-Y04 is elevated compared to the three analyzed mesophilic enzymes.

PyNP-Y01 was among the thermophilic enzymes with the highest number of salt bridges (172) which might have contributed to the sustained activity over a wide range of temperatures (**Figure 7**). In contrast, the amino acid residues' analysis showed the lowest total content of amino acids required for thermophilic signature (27.4%) compared to all analyzed sequences. *Mycoplasma hyorhinis* pyrimidine nucleoside phosphorylase (MhPyNP) showed the highest total content of the same residues (38.3%) among all analyzed sequences, despite being a mesophilic enzyme (Voorde *et al.*, 2012). Nonetheless, it showed the highest content of the amino acid residues as well that are susceptible to deamination (8.4%), and thus protein destabilization.

A decreased protein volume has been reported to increase the stability of proteins under pressure (Chen *et al.*, 2015). Some reports also attributed the reduced protein volume to thermal stability (Kumar *et al.*, 2000; Razvi *et al.*, 2006). Compared to EcTP, both PyNP-Y01 and PyNP-Y05 showed a decreased Van der Waal volume (intrinsic volume of the protein) and packing density. This is in good accordance with the suggested hypothesis. In contrast, the packaging density was reduced for PyNP-Y04. The Van der Waal volume, however, was higher compared to EcTP. This observation might correlates with the assumption that protein packing is not an essential factor for thermal stability (Karshikoff *et al.*, 1998).

Table 9. Analysis of the thermal stability contributing factors.

PyNP	Temp. Optima [°C]	Amino Acid Content [%]									Salt Bridges	Protein Volume	
		Glu (E)	Lys (K)	Arg (R)	Val (V)	Ile (I)	Total	Gln (Q)	Asn (N)	Total		V_{VDW} (Å^3)	Packing Density
PyNP-Y04	>100[a]	9.3	7.3	2.9	7.3	7	33.8	2	1.5	3.5	159	42543.0	0.729
TtPyNP	>95[b]	10.2	5.2	8.5	8	2.8	34.7	0.9	0.5	1.4	244	40953.644	0.734
PyNP-Y01	80[a]	7.2	3.6	6.7	7.2	2.7	27.4	3.1	0.4	3.5	172	41001.5	0.723
GsPyNP	70[c]	6.7	8.3	3	8.5	7.4	33.9	2.5	2.5	5	168	41721.945	0.717
GtPyNP	60[b]	7.7	8.4	3.2	8.6	7	34.9	1.9	1.9	3.8	122	41438.3	0.725
PyNP-Y05	50[a]	6.1	8	2.9	7.8	7.5	32.3	2.2	1.7	3.9	158	41403.0	0.721
KaPyNP	50[d]	5.5	5	5.5	8.2	6.1	30.3	2	2.3	4.3	156	42039.289	0.742
BsPyNP	45[e]	7.4	8.5	3	8.8	7.6	35.3	2.5	3.2	5.7	169	41701.502	0.727
Nucleoside phosphorylases from mesophilic organisms													
EcTP	37[f]	5.5	5	5.5	7.7	6.1	29.8	2.5	2.7	5.2	149	42242.6	0.74
MhPyNPs	37[g]	8.4	13.2	1.4	6.5	8.8	38.3	3.5	4.9	8.4	140	43429.887	0.723
LBcTP	37[x]	4.4	7.4	3.7	7.6	7.4	30.5	3.5	2.5	6	142	41386.260	0.734
AhTP	30[z]	5.4	4.1	5.6	8.6	5.6	29.3	3.2	2.5	5.7	127	42022.265	0.731

Highest Lowest

[a] Experimentally determined optimum temperature (this study).[b] Experimentally determined optimum temperature (Szeker et al., 2012); Tt: Thermus thermophilus; Gt: Geobacillus thermoglucosidasius. [c] Experimentally determined optimum temperature (Hori et al., 1989); Gs: Geobacillus stearothermophillus. [d] Reported used temperature for enzymatic reaction (Tang et al., 2010); Ka: Klebsiella aerogenes. [e] Reported used temperature of immobilized enzyme at pH 10 in enzymatic reactions (Rocchietti et al., 2004); Bs: Bacillus subtilis. [f] Experimentally determined optimum temperature (Schwartz, 1971); Ec: Escherichia coli. [g] Reported better temperature for the enzymatic reaction compared to the 20°C (Voorde et al., 2012); Mh: Mycoplasma hyorhinis. [x] Reported used temperature for the enzymatic reaction (Avraham et al., 1990); LBc: Lactobacillus casei. [z] Reported optimum temperature for nucleoside synthesis using whole cell biocatalyst (Lewkowicz et al., 2006). Ah: Aeromonas hydrophilia. Amino acid content was analyzed using ExPASy server (Gasteiger et al., 2005). Salt bridges were analyzed using ESBRI online tool (Costantini et al., 2008). Protein volume is analyzed using ProteinVolume 1.3 online tool (Chen et al., 2015); V_{VDW}: Van der Waal Volume.

The kinetic parameters of PyNP-Y01, PyNP-Y04 and PyNP-Y05 as well as their substrate affinities were evaluated. All tested enzymes showed higher affinity to Urd compared to Thd which was reflected by lower K_m values. Based on the substrate spectrum, NPs are differentiated into PyNP (accepting both Urd and Thd), UP (specific for Urd) and TP (specific for Thd) (Pugmire *et al.*, 2002). Kinetic parameters of PyNPs from different organisms with temperature optima between 37°C - 80°C were evaluated (**Table 7**). Most of the studied enzymes (including PyNP-01, -04 and -05) showed a higher affinity to Urd compared to Thd. Exceptions were GtPyNP and GsPyNP (Hori *et al.*, 1989; Szeker *et al.*, 2012). Both showed a higher affinity to Thd. The lowest described K_m value for Urd was 92 µM observed with MhPyNP (Voorde *et al.*, 2012, **Table 7**). The highest affinity for Thd was described for GsPyNP (**Table 7**), a K_m value of 160 µM was determined (Hori *et al.*, 1989). Compared to PyNPs, UP and TP were described to be specific for Urd and Thd, respectively. Hence, a higher affinity towards their specific substrates is expected. The assumption got confirmed for EcUP (**Table 7**). With a K_m value of 80 µM (Panova *et al.*, 2004; Szeker *et al.*, 2012), it was the lowest compared to values observed for PyNP. For EcTP, however, K_m values of 190 to 300 µM were described in literature (Panova *et al.*, 2004, 2007), which reflect a lower substrate affinity to Thd compared to GsPyNP as an example (**Table 7**).

Phosphorolytic cleavage of Ara-U, $_{2F}$Ribo-U and ^{2F}Ara-U is challenging due to strengthening of the N-glycosidic bond especially in the presence of a fluoro-group at the C2' position (Szeker *et al.*, 2012; Fateev *et al.*, 2014). Of the studied enzymes, PyNP-Y04 showed the highest activity against these three substrates. Up to our knowledge, only few reports showed the enzymatic cleavage of the above-mentioned substrates using thermophilic or mesophilic nucleoside phosphorylases.

UPs from *Enterobacter aerogenes* (Ea) (Utagawa, Morisawa, Yamanaka, Yamazaki, Yoshinaga and Hirose, 1985) and *Lactobacillus casei*

(Avraham *et al.*, 1990) and PyNP from *Thermus thermophilus* (Zhou, 2014) were reported to accept Ara-U as a substrate and catalyze its phosphorolytic cleavage. EaUP catalyzed the reaction at 60°C with only 10% of its activity toward the natural substrate Urd (K_m value 1800µM). On contrary, LBcUP has almost the same activity (94.6%) as that of uridine at 37°C, however, LBcUP affinity towards uridine is low as demonstrated by the high K_m value 38000 µM (**Table 7**). TtPyNP cleaved 28% of Ara-U, at 80°C after 21h, to its respective base and Pentose-1P. This was achieved using a substrate to phosphate ratio of 1:10. Even though, a lower substrate to phosphate ratio (1:2.5) was used, the phosphorolytic cleavage of Ara-U using PyNP-Y04 was shown to 37% at 80°C after 24h (**Figure 9B**). *Citrobacter koseri* (whole cell catalysis) was reported to use Ara-U as a sugar donor in transglycosylation reactions for the synthesis of different arabino-purine nucleosides. Reactions yielded in high percentage of final nucleosides, yet, no data on the cleavage percentage of Ara-U was reported (Nóbile *et al.*, 2012).

The phosphorolytic cleavage of $_{2F}$Ribo-U using EcUP at 37°C was not possible. However, the reaction proceeded very slowly using EcTP at 24000U and reaction periods up to 2 weeks (Tuttle *et al.*, 1993). The same substrate was cleaved using GtPyNP at 60°C and the obtained cleavage was 0.45%. A better cleavage (65%) was reported with TtPyNP at 80°C after 17h using a ratio of substrate to phosphate of 1:10 (Szeker *et al.*, 2012). A cleavage of 72% with PyNP-Y04 was achieved after 24h using the same ratio and temperature (**Figure 8B**). TtPyNP was as well reported to catalyze the cleavage of ^{2F}Ara-U at 80°C. A conversion of 46% was achieved after 17h using a ratio of substrate to phosphate of 1:10 (Szeker *et al.*, 2012). GtPyNP on the other hand was not able to utilize ^{2F}Ara-U as a substrate, the same was observed for PyNP-Y05 (**Table 8**). Whereas, PyNP-Y04 cleaved 27% of ^{2F}Ara-U at 80°C using a substrate to phosphate ratio of 1:2.5 (**Figure 9B**). The decreased phosphorolysis with PyNP-Y04

compared to TtPyNP is explained by the lower substrate to phosphate ratio.

Therefore, the obtained results together with the previously published data support the hypothesis that thermostable PyNPs have a wider substrate spectrum. Thus, they would be better employed as biocatalysts in reactions with modified nucleosides.

The implementation of the enzymatic synthesis approach was motivated by the drawbacks of chemical synthesis routes for both Pentose-1Ps and nucleoside analogues. One crucial advantage of the enzymatic synthesis is the exclusive formation of the α-anomer of Pentose-1Ps. In contrast, using chemical methods both anomers are formed as reported in the chemical synthesis of several Pentose-1Ps and summarized in **Table 3**. Elaborated discussion on the enzymatic synthesis and the purification of the Pentose-1P analogues is presented in **paper III**.

Pentose-1Ps were successfully produced in a chemo-enzymatic process with purities > 95% in gram-scale. The enzymatic reactions are highly influenced by the ratio of phosphate to substrate. The higher the ratio, the higher the bioconversion. It was shown additionally that the starting concentration of the nucleoside is very crucial for final yield of the Pentose-1P-Barium salt. High nucleoside concentrations showed increased final Pentose-1P-Barium salt yields despite the decreased bioconversion percentages (**Paper III – Figure 2**). These results are attributed to the similar ionization pattern of Pentose-1Ps and phosphate leading to their co-precipitation in the presence of NH_4Cl and $MgCl_2$ (Florkin *et al.*, 1963).

Additional factors that influence the enzymatic reaction is the temperature. The higher the temperature, the faster is the reaction. However, the choice of the reaction temperature is affected by different factors. One important consideration is the stability of the substrate nucleoside and the produced Pentose-1P. For instance, $_{2F}$Rib-U (substrate) is unstable at 80°C (enzyme optimum temperature) due to the release of HF group at the C2'-position leading to the formation of Anhydro-U and subsequent formation of Ara-U (Szeker *et al.*, 2012). Thus, a reaction temperature of 65°C was used instead. Another example is the instability of dRib-1P (product) at 60°C (enzyme optimum temperature), thus reaction was performed at 40°C. Therefore, the above considerations should be taken into account when preforming the reaction and additionally for the proceeding purification process.

Chemical and chromatographic methods have been reported for the purification of sugar-phosphates. Despite the advancements made in both approaches most methods hold some disadvantages. One critical disadvantage of the chemical method is the co-precipitation of Pentose-1P with the Pi in the presence of $MgCl_2$ and NH_4Cl_2 (Florkin *et al.*, 1963). Thus, investigating chromatographic purification approach was of interest. Most reported chromatographic methods focus on sugar-phosphates generally (Groth *et al.*, 1952; Lerner *et al.*, 1968; Smrcka *et al.*, 1988; Jeong *et al.*, 2007) with only few focusing on Pentose-1P (Khym *et al.*, 1954; Jones *et al.*, 1960). Three main separation techniques seemed promising, none of which was reported to separate the Pentose-1P from the same reaction mixture of nucleoside, base and phosphate. Investigated methods were size-exclusion, ion-exchange and adsorption chromatography.

Sephadex G-10 was previously reported for the separation of nucleosides, nucleotides and bases (Braun, 1967; Lerner *et al.*, 1969). Braun showed the successful separation of nucleosides from their respective

mono-phosphate. It was as well reported for the separation of nucleobase from their corresponding nucleoside mono-phosphate derivatives (Braun, 1967). The minimum size difference between the separated molecules was less than 100 Da. Nucleoside and nucleoside monophosphate showed a molecular weight of 244 Da and 322 Da, respectively. Using phosphate buffer, elution was in accordance to the molecular size, where larger molecules were eluted first (Braun, 1967). However, our results showed the earlier elution of Ara-1P (229 Da) and Pi (97 Da) and delayed elution of Ara-U (244 Da) (**Figure 10A, B**). This could be explained by the tight association of water molecules (mobile phase) to the phosphate group which increased the molecular mass of Ara-1P and Pi hence the earlier elution (Lerner *et al.*, 1969). An Additional reason for the earlier elution of Pi, is the repulsion between the negatively charged Pi and carboxylate groups (gel matrix) which reduces the retention of Pi in the matrix's pores. Although our results showed that an increased column length (119 cm) improved the separation of Pi and Ara-1P, complete separation, however, was not achieved (**Figure 10B**).

Ion-exchange chromatography using Dowex-I have been reported to separate different sugar phosphates from each other or from a mixture of nucleotides and inorganic phosphate (Groth *et al.*, 1952; Jones *et al.*, 1960; Lerner *et al.*, 1968). Therefore, a Resource-Q column was used which has the same functional group comparable to that of Dowex-I. A very poor separation of the Ara-1P and Pi was obtained (**Figure 11B**). This might be due to the similar ionization pattern of both molecules. The difference in the charge pattern was best seen at pH values of 1.5, 6.2 and above 11 (**Figure 11A**). Due to acid liability of all Pentose-1Ps as shown in **Paper III**, and the column material upper pH limit (pH 12), experiments were conducted at pH 6.2. Jones and Burt (Jones *et al.*, 1960) demonstrated the separation of different phosphates of pentoses and hexoses from fish tissue extract at pH 8 - 2. The extract mixture

included glucose-1-phosphate, glucose-6-phosphate, ribose-5-phosphate, ribose-1-phosphate and others. Additionally, their results showed that the physiological Pi interferes with the binding of the sugar-phosphates to the column but not the elution. Using the same material, Dowex-I, the separation of ribose-5-phosphate from inorganic phosphate was reported using a gradient of monochloroacetate (Groth *et al.*, 1952). A purity of 90 - 100% was achieved. The same result was not obtained in this study for the separation of Ara-1P and Pi using the described protocol.

The most promising results for the chromatographic purification was obtained with adsorption chromatography using silica beads, which were in accordance with the results obtained with the TLC. Up to our knowledge, the use of normal-phase silica column chromatography is not described in literature for the separation of sugar phosphates and inorganic phosphate. Silica gel was, however, extensively reported for analytical studies of sugar phosphates, nucleosides and or nucleobases using TLC (Chittenden, 1972; Okumura, 1979; Choi *et al.*, 1986; Timmons *et al.*, 2008; Liu *et al.*, 2015; Wen *et al.*, 2016). Oukumura showed the separation of 1- or 6- monophosphate hexoses and 1,6-diphosphate hexoses on silica plates using a mixture of n-butanol - acetic acid - water as mobile phase (Okumura, 1979). Additionally, the separation of both anomeric forms of (α- and β-) of galactofuranose-1-phosphate was as well reported using silica gel plates (Chittenden, 1972). Although 100% separation between the Ara-1P and Pi was not achieved yet increasing the column volume resulted in a significant improvement of separation. This shows the potential of achieving a complete separation of Ara-1P and Pi by further optimization.

Therefore, chromatographic purification is viable and valuable alternative to chemical purification approach. On one hand, it could be automated; thus, facilitate the purification process. On the other hand, the co-precipitation of the Pentose-1P with the phosphate could be overcome.

In literature, major attention was given to the synthesis of C2-fluoro modified Pentose-1Ps. They are considered the building blocks for the corresponding purine and pyrimidine nucleoside analogues with clinical and biotechnological value (Mikhailopulo *et al.*, 2011, 2013; Lapponi *et al.*, 2016; Seley-Radtke *et al.*, 2018). Their broad spectrum of biological activities is due to the increased strength of the N-glycosidic bond caused by the addition of the fluorine group to the C2 position of the sugar moiety (Fateev *et al.*, 2014; Elzagheid, 2016). Moreover, 5-ethynyluracil nucleoside derivatives, including ^{2F}Ara-EdU, were recently shown to be valuable tools for DNA/RNA labelling and cellular visualization (Neef *et al.*, 2011, 2012, 2014). Like most of the nucleosides, 5-EU containing nucleoside derivatives are synthesized by tedious and laborious, multi-steps chemical approaches (Shealy *et al.*, 1986; Qu *et al.*, 2011; Neef *et al.*, 2012). Thus, up to our knowledge, the enzymatic synthesis of 5-EU nucleoside analogues by thermostable NPs is reported in the present study for the first time. EdU, ^{2F}Ara-EdU and $_{2F}$Rib-EdU were of interest. Their enzymatic synthesis by two enzymatic synthesis approaches were compared. Transglycosylation reaction and direct glycosylation using the enzymatically synthesized Pentose-1Ps were analyzed. Both reactions were catalyzed by thermostable NPs. Using the transglycosylation approach, EdU was synthesized from Thd (sugar donor) with a maximum yield of 55% after 30 min. The percentage of formed EdU declined to 41% at the end of the reaction's duration (30h) (**Paper IV-Figure 1B**). Differently, the percentage of formation of $_{2F}$Rib-EdU and ^{2F}Ara-EdU was 18% and 0% after 30h. However, using the direct glycosylation approach, the formation of these nucleosides increased to 60%, 47% and 51% respectively.

During the transglycosylation reaction, the formation of the nucleoside 2 (sugar acceptor) is influenced by the cleavage of nucleoside 1 (sugar donor). Thd, $_{2F}$Rib-U and ^{2F}Ara-U were cleaved to their corresponding

Pentose-1P with 30%, 3% and 0.3%, respectively (**Paper IV-Figure 1A**). These results show that cleavage of $_{2F}$Rib-U and ^{2F}Ara-U is not favorable under the transglycosylation conditions due to the low phosphate concentrations. Therefore, the availability of Pentose-1Ps and their use in the direct glycosylation of the nucleobase improved the synthesis of the three investigated nucleoside analogues. Detailed discussion on 5-EU nucleoside analogues is presented in **paper IV**.

7 Conclusion

For modified nucleoside synthesis, the separation of both the phosphorolysis reaction and the glycosylation reaction offers the possibility of optimizing the conditions of each reaction separately. Which is considered as an advantage to maximize the final product yield. Meaning, for the phosphorolysis reaction, increasing the phosphate concentration to shift the equilibrium towards the synthesis of the Pentose-1P. This leads to a higher bioconversion percentages and higher yields of Pentose-1P. On contrary, for the glycosylation reaction, no phosphate should be added, in order to minimize the backward reaction. Hence, higher yield of the nucleoside of interest. One disadvantage of this approach is the extra needed purification step for the Pentose-1Ps. However, Pentose-1Ps are in fact interesting molecules to have in a pure form. Their availability will open doors for many metabolic and drug development studies.

This work showed the possibility of synthesizing Pentose-1Ps with different modifications, and the presented protocol could be applied for the synthesis of differently modified Pentose-1P.

Another important consideration for the NP catalyzed reactions, is the biocatalyst employed in the reaction. The use of thermophilic enzymes is shown to have advantages over the mesophilic ones. They are easily purified, durable, can well tolerate different modification thus are active against wider substrate spectrum. Accordingly, identifying and characterizing novel thermostable NPs will enable the synthesis of more diverse nucleoside analogues and will as well as facilitate the upscaling of the synthesis process.

8 Outlook

With an increase of available NPs (both natural and engineered) an increased spectrum of nucleoside analogues can be produced by enzymatic synthesis. Thus, exploring different environments with diverse characteristics as hydrothermal vents, the ocean's brine pools or oil reservoirs will lead to the identification of interesting enzymes candidates. We showed the feasibility of applying NPs to produce natural and modified Pentose-1P which are of increasing interest in the synthesis of nucleosides analogues and highly valuable compounds. A method was developed which can be easily transferred to production of other Pentose-1P analogues like C2-amino modified or C2- and C3-dideoxy-modified compounds. The availability of diverse library of Pentose-1P will undoubtedly improve the enzymatic synthesis of many nucleoside analogues. Additionally, it would enhance our understanding of the mechanism of action and the mechanisms of binding of NPs to the different substrates and their accommodation to different modifications on the Pentose-1P by co-crystallization. Consequently, more insight into specific site mutations to engineer novel NPs.

Improving the purification process from the traditional chemical way to the chromatographic purification will increase final product yields of Pentose-1Ps. Upscaling will be simplified, and cost-efficient industrial process could be developed.

Modified Pentose-1Ps can be studied as drug molecules which could act as inhibitors for different enzymes involved in their metabolic process. Having these Pentose-1Ps and others in hand with high purity and as the physiological anomer (α- anomer) would encourage such metabolic studies. In time, this would drive us to better understanding of the physiological process and potentially a novel class of medication.

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10 Theses

- Three novel thermostable pyrimidine nucleoside phosphorylases (PyNP-Y01, PyNP-Y04, PyNP-Y05) were characterized. Kinetic parameters were determined, and substrate spectra were compared. Their activity was confirmed at temperatures above 60°C.

- C2-fluoro modified Pentose-1P ($_{2F}$Rib-1P and ^{2F}Ara-1P) and Ara-1P were enzymatically synthesized using thermostable pyrimidine nucleoside phosphorylases and were purified as barium salt in gram scale with a purity higher than 95% as determined by both HPLC and TLC.

- All Pentose-1Ps were exclusively synthesized as an α-anomer and the structures of the purified Pentose-1Ps were confirmed by NMR spectroscopy.

- Modified Pentose-1Ps and Rib-1P were stable at high temperatures up to 60°C and at pH values between 5-13. dRib-1P was the least stable among the tested Pentose-1P.

- Direct glycosylation reaction for the synthesis of nucleoside analogues with Pentose-1Ps as substrates allows for a more controlled reaction optimization compared to transglycosylation.
    - Phosphate concentration can be adjusted. In the synthesis reaction low phosphate concentration are a precaution for high product yields.
    - With Pentose-1Ps as substrate optimum enzymes can be used for the synthesis reaction.
    - The temperature of the reaction can be modulated based on the stability and the solubility of less molecules. Nucleoside 1 (sugar donor) stability doesn’t play a role.

- The enzymatic yield of C2'-fluoro-modified-5-EU nucleoside derivatives (2FRib-EdU and 2FAra-EdU) using the direct glycosylation approach was significantly higher compared to the transglycosylation approach.
- The availability of the Pentose-1Ps in high purity (only α-anomer) will allow for a better understanding of nucleoside phosphorylases kinetics as well as more detailed metabolic, therapeutic and diagnostic studies.
- Chromatographic purification of Pentose-1Ps is a possible alternative to purification by precipitation. The adsorption-normal-phase chromatography using silica beads showed a huge potential for developing an efficient purification method and was superior to size-exclusion and ion-exchange chromatography.

Publications

Paper I

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REVIEW ARTICLE

Substrate Spectra of Nucleoside Phosphorylases and their Potential in the Production of Pharmaceutically Active Compounds

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Abstract: ***Background***: Nucleoside phosphorylases catalyze the reversible phosphorolysis of pyrimidine and purine nucleosides in the presence of phosphate. They are relevant to the appropriate function of the immune system in mammals and interesting drug targets for cancer treatment. Next to their role as drug targets nucleoside phosphorylases are used as catalysts in the synthesis of nucleosides and their analogs that are widely applied as pharmaceuticals.

Methods: Based on their substrates nucleoside phosphorylases are classified as pyrimidine and purine nucleoside phosphorylases. This article describes the substrate spectra of nucleoside phosphorylases and structural properties that influence their activity. Substrate ranges are summarized and relations between members of pyrimidine or purine nucleoside phosphorylases are elucidated.

Results: Nucleoside phosphorylases accept a broad spectrum of substrates: they accept both base and sugar modified nucleosides. The most widely studied nucleoside phosphorylases are those of *Escherichia coli,* mammals and pathogens. However, recently the attention has been shifted to thermophilic nucleoside phosphorylases due to several advantages. Nucleoside phosphorylases have been applied to produce drugs like ribavirin or fludarabine. However, limitations were observed when drugs show an open ring structure. Site-directed mutagenesis approaches were shown to alter the substrate specificity of nucleoside phosphorylases.

Conclusion: Nucleoside phosphorylases are valuable tools to produce modified nucleosides with therapeutic or diagnostic potential with high affinity and specificity. A wide variety of nucleoside phosphorylases are available in nature which differ in their protein sequence and show varying substrate spectra. To overcome limitations of the naturally occurring enzymes site-directed mutagenesis approaches can be used.

Keywords: Nucleoside phosphorylase, pyrimidine analog, purine analog, catalytic mechanism, structure, function, mutagensesis.

1. INTRODUCTION

The reversible phosphorolysis of purine and pyrimidine nucleosides is a biochemical reaction of high importance in the nucleoside metabolism [1]. The reversible phosphorolytic cleavage of nucleosides by nucleoside phosphorylases (NP) was confirmed in 1947 by Kalckar [2].

The physiological role of NPs comprises the reversible phosphorolysis of ribo- and deoxyribonucleosides to nucleobase and α-D-pentofuranose-1-phosphate in the presence of inorganic phosphate. *In vitro* studies revealed that the equilibrium of the reaction catalyzed by nucleoside phosphorylases is shifted towards the reverse (synthetic) reaction [3]. It is more pronounced in purine nucleoside phosphorylases (PNP) than in pyrimidine nucleoside phosphorylases (PyNP) [4, 5]. Equilibrium constants for the phosphorolysis reaction of uridine between 0.51 and 0.61 were obtained for *E. coli* PyNP [4], whereas a value of 0.0175 was determined for *E. coli* PNP with inosine as a substrate. Nevertheless, *in vivo* phosphorolysis of nucleosides is favored due to the following arguments [6]: (i) The intracellular concentration of inorganic phosphate (Pi) is higher than that of nucleosides. (ii) By using the released bases hypoxanthine and guanine with hypoxanthine-guanine phosphoribosyl transferase (HPRT) and, in certain tissues, xanthine oxidase or guanine deaminase (guanase), respectively, the equilibrium of the PNP reaction is shifted towards α-D-pentofuranose-1-phosphate accumulation. (iii) The absence of any kinase in mammals acting on inosine and guanosine favors the channeling of purine nucleosides towards phosphorolysis. (iv) Liberated α-D-pentofuranose-1-phosphate is catabolized as energy source [7]. (v) Nucleoside phosphorylases often belong to the same regulon as other nucleoside catabolizing enzymes [8, 9].

However, in some specific cases nucleoside phosphorylases are involved in the salvage of nucleobases, and, therefore, represent an opportunity to the organisms that cannot perform *de novo* synthesis of nucleosides. For example, parasites like trypanosomes and *Leishmania* in general lack a *de novo* purine biosynthetic pathway [10]. Starvation of purines causes purine-less death in cultured cells. Enzymes of the purine salvage pathway, including PNPs, were detected in *P. falciparum*, suggesting a recovery of purine bases from the mammalian host by the parasite to survive. Due to its need for purine bases *Plasmodium* is dependent on intact pathways for purine salvage. As differences exist in the active sites between human and parasite enzymes, PNPs of the pathogens are interesting targets for the development of specific inhibitors, which would kill the parasite but only slightly influence the human host.

Mutations in the PNP locus in humans cause a gradual decrease in T-cell mediated-immunity. B-cells, however, remain unaffected [11]. T-cells are capable of transporting and phosphorylating deoxyguanosine (dGuo) to deoxyguanosine triphosphate (dGTP), which accumulates compared to unmutated cells. In humans two enzymes can metabolize dGuo, PNP and deoxycytidine kinase (dCyK). dGuo is phosphorolyzed by PNP to guanine and 2'-deoxyribose-1-phosphate. The function of dCyK in dividing T-cells is the salvage of deoxycytidine to form deoxycytidine triphosphate

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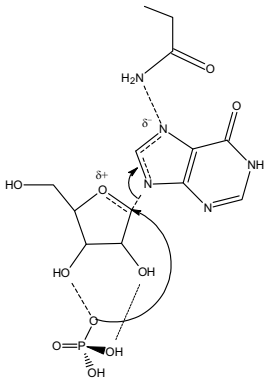
Current Pharmaceutical Design



Scheme 1. Proposed catalytic mechanism of human PNP. Figure adapted from Pugmire and Ealick [1].

(dCTP). The affinity of dGuo to PNP is higher than to dCyK. Therefore, it predominantly undergoes phosphorolysis by PNP. When dGuo accumulates beyond normal levels, *e.g.* in case of inactive human PNP, dGuo is converted to deoxyguanosine monophosphate (dGMP) by dCyK. Deoxyguanosine monophsophate is metabolized to dGTP, which results in an accumulation of dGTP. This leads to an inhibition of ribonucleotide reductase, thus preventing the conversion of ribonucleoside diphosphates to corresponding deoxyribonucleoside diphosphates. An inhibition of DNA synthesis and cell replication is observed when the level of deoxyribonucleotides is reduced. The suppression of proliferation of immature T-cells is the result. Therefore, the development of PNP inhibitors is of great importance due to their potential as immunosuppressive drugs for organ transplantation or the treatment of T-cell leukemia and T-cell related autoimmune diseases [3, 11]. The interest in thymidine phosphorylase (TP) strongly increased as it was shown to be an angiogenic factor and to be equal to the platelet-derived endothelial cell growth factor [12, 13].

2. CLASSIFICATION OF NUCLEOSIDE PHOSPHORYLASES.

Based on their specific substrates, nucleoside phosphorylases were classified, and enzyme names and enzyme commission numbers (EC) were ascribed (Table **1**). In 2002, Pugmire and Ealick published an extensive study on the classification of NPs [1]. Structural studies revealed that only two families of NPs exist. The first family (NP-I family) consists of NPs that show a homo-trimeric (mammalian PNP) or a homo-hexameric (bacterial PNP) quaternary structure, in addition to uridine phosphorylase (UP) and 5'-methylthioadenosine phosphorylase (MTAP). Both purine and pyrimidine nucleosides are used as substrates. Despite the diversity in substrate spectra, amino acid sequence and quaternary structure is observed, all members of this family show a characteristic subunit topology. A two-domain subunit fold and a dimeric quaternary structure was described for members of the NP-II family. A significant level of sequence identity (>30%) was shown for NPs of the second family. Members of this family are specific for pyrimidine nucleosides [1]. They catalyze the phosphorolysis of both thymidine and uridine substrates in lower organisms, but are specific for thymidine in mammals and other higher organisms.

The classification by Pugmire and Ealick [1] is up to now a valid tool for the classification of NPs. However, not all NPs from different species fit the described model. As an example *E. coli* PNP-II is similar to trimeric low molecular mass PNPs in sequence, promoter structure and specificity. However, it forms a high molecular mass hexamer by dimerization of two trimers [14]. Crystallization and gel-filtration data gave hints for the existence of hexameric species of PNP-II. In 2000, Bzowska and co-workers reviewed a number of other PNPs with dimeric or tetrameric quaternary structures [3]. Similar results were obtained for UP. Both human UP [15] and trypanosomal UP [16] were shown to form homodimers although the classification described by Pugmire an Ealick [1] indicates that UPs are hexamers.

Other deviations from the classification proposed by Pugmire and Ealick [1] concern the substrate specificity. The nucleoside phosphorylase of *Klebsiella* was described to use both pyrimidines and purine nucleosides as substrates [17]. Next to purine nucleosides like adenosine the enzyme also catalyzed the phosphorolysis of pyrimidine nucleosides like uridine, 2'-deoxyuridine, and thymidine. The formation of adenine arabinoside, an antiviral drug, from uridine arabinoside and adenine was shown. Additionally, it was described that nucleoside phosphorylases show activity with cytidine [18]. Additionally, a novel activity for NPs was described: in *Pseudomonas aeruginosa* where a NP was found that converts 5´-deoxy-5´-methylinosine but not 5´-deoxy-5´-methyladenosine [19].

3. CATALYTIC MECHANISM

The catalytic mechanism is analogous in all nucleoside phosphorylases [1]. As shown for human PNP nucleoside binding occurs in a high-energy conformation, which leads to a steric strain favoring the cleavage of the glycosidic bond (Scheme **1**). Electrons flow from O4´ of the sugar moiety to the purine ring and an oxocarbenium ion is formed. It is stabilized by the negatively charged phosphate ion. Interactions of amino acids of the active site with N7 position of the nucleoside are likely to improve the electron flow from the glycosidic bond to the purine ring. Further information on the catalytic mechanism of selected NPs is given in the specific chapters.

Table 1. Nucleoside phosphorylases described in the presented review.

Enzyme name	Acronym	EC number
Thymidine phosphorylase	TP	2.4.2.4
Uridine phosphorylase	UP	2.4.2.3
Pyrimidine nucleoside phosphorylase	PyNP	2.4.2.2
Purine nucleoside phosphorylase	PNP	2.4.2.1
5´-Methylthioadenosine phosphorylase	MTAP	2.4.2.28
Guanosine phosphorylase	GP	2.4.2.15

4. SUBSTRATE SPECTRA OF NUCLEOSIDE PHOSPHORYLASES

NPs can be classified according to several parameters: (i) Based on their quaternary structure and substrate specificity, they can be divided into NP-I family and NP-II family as stated before. (ii) Functionally, this class can be subdivided into PNP (EC 2.4.2.1), PyNP (EC 2.4.2.2), UP (EC 2.4.2.3), TP (EC 2.4.2.4), guanosine phosphorylase (GP, EC 2.4.2.15), S-methyl-5'-thioadenosine phosphorylase (MTAP, EC 2.4.2.28), adenosine nucleoside phosphorylase (ANP) and inosine-guanosine phosphorylase. As neither adenosine nucleoside phosphorylase, guanosine phos-phorylase or inosine-guanosine phosphorylases were investigated in view of their substrate specificity or their structure according to our knowledge, they are only shortly described in this review.

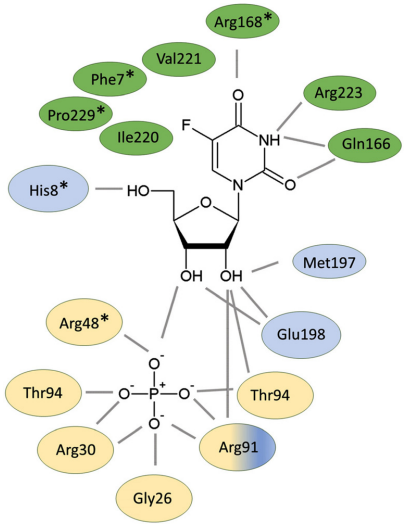


Fig. (1). Schematic drawing of the active site of *E. coli* uridine phosphorylase. Residues binding the base, the sugar-moiety and phosphate are shown in dark grey, light grey and white, respectively. Residues from the adjacent subunit are marked with *.

ANP has been identified in two species of the genus *Mycobacterium* and in 13 out of 16 tested mycoplasmatales, whose PNPs are strictly specific for 6-oxopurines. ANP from *Mycobacterium smegmatis* was shown to also catalyze inosine cleavage and is not inhibited by 9-benzyl-9-deazaguanine, a potent PNP inhibitor, which ascertains the fact that it belongs to another subgroup [20, 21]. Another member of the ANP class was partially purified from *Schistosoma mansoni* [22]. It catalyzed the cleavage of substrates which are unrecognized by the organism's adenosine kinase or deaminase namely: 2-fluoroadenosine, 5'-deoxy-5'-iodo-2-fluoroadenosine. This explains why infections with these parasites are treated with *e.g.* 2-fluoroadenosine. After cleavage by ANP the toxic 2-fluoroadenine accumulates in the cells [23].

There are only a few publications on GPs, isolated from *Oryctolagus cuniculus* and *Trichomonas vaginalis*. The available data show that GPs are strictly specific for guanosine and deoxyguanosine as substrates [24 ,25].

4.1. Substrate Recognition and Substrate Spectra of Members of the NP-I Family

PNP enzymes are most widely studied within the two families of nucleoside phosphorylases due to their importance as drug targets. PNP enzymes are subdivided into hexameric PNP (bacterial) and trimeric PNP (mammalian). MTAP and UP also belong to the NP-I family. Both PNP (*Escherichia coli* and bovine) and MTAP (human) have been extensively described by Pugmire and Ealick [1]. However, the UP active site was not well described. Therefore, the active site of uridine phosphorylase will be described in more detail in the following chapter. A comparison will show similarities and differences for members of the NP-I family. Active site residues which determine substrate specificity will be described.

4.1.1. Active Site of Uridine Phosphorylase

UP belongs to the NP-I family. Structure comparison revealed that UP form a heterogenous group. Both hexameric (*E. coli* and *S. typhimurium UP*) and homodimeric (*H. sapiens* and *T. brucei*) structures were observed [15, 16, 26, 27]. A sequence alignment of uridine phosphorylases suggests that UPs from all eukaryotic parasites are active in a homodimeric form [16]. While for *E. coli, S. typhimurium* and *T. brucei* UPs metal ion binding sites are described [15, 16, 26, 27], they are absent in both human UPs.

Between the phosphate and uracil binding sites the ribose binding site is placed in *E. coli* UP. Either a ribose moiety of a nucleoside or that of ribose 1-phosphate is bound [26]. A number of hydrogen bonds (primarily to the side-chains of His8 and Glu198) position the ribose moiety in the active site of the enzyme (Fig. **1**). His8 belongs to the adjacent monomer and forms together with a bridging water molecule hydrogen bonds to the 5′ hydroxyl group of ribose. Hydrogen bonds to the 2′ and 3′ hydroxyl groups of the ribose moiety are formed by Glu198 (Fig. **1**), which positions the ribose on top of the phosphate ion. In this setting the 3′ hydroxyl group of the ribose forms a hydrogen bond to O3 of the phosphate ion. Hydrogen bond interactions are also observed between the 2′ hydroxyl of the ribose moiety and Met197 and Arg91, respectively [26]. Met197 is also involved in forming a part of the ribose binding pocket. The van der Waals surface of its sulphur atom point in the ribose binding pocket, which might be responsible for the stabilization of the largely hydrophobic part of the upper face area of ribose and the correct positioning of the ribose in the active site. The importance of the methionine residue is shown by its conservation across all members of the NP-I family.

The uracil binding pocket of an occupied UP active site forms a rectangular slot [26]. Residues Thr95, Gly96, Tyr195 and Glu196 form the lower area, while the upper area consists primarily of Phe162. Phe162 is located vertical to the base and is forming an edge-to-face π-stack arrangement with the uracil moiety. The area around C5 of the base consists of Ile220, Val221, Pro229 and Phe7, which are non-polar amino acids [26, 28]. Pro229 and Phe7 belong to the neighboring monomer. These residues form a hydrophobic pocket at the 5 position of uracil (Fig. **1**). Arg168, Arg223 and Gln166 are important residues in the binding pocket of uracil (Figure **1**) [26, 28]. Gln166 is conserved among all UPs and forms the discriminating interaction with uracil (Fig. **1**) [26, 28]. Gln166 forms two hydrogen bonds to uracil: I. An interaction between the amide nitrogen atom with O2 of uracil is observed. II. A hydrogen bond is formed between the carbonyl oxygen atom and N3 of uracil. Side-chains of Gln166 positions the amide nitrogen atom so that it is able to set up a second hydrogen bond to the main-chain carbonyl of Thr161.

In *E. coli* either orthophosphate or the phosphate moiety of ribose 1-phosphate is bound to the phosphate binding site of UP [26]. A binding pocket is formed by the three arginine residues Arg30, Arg48 and Arg91. Each residue forms two hydrogen bonds to the phosphate's oxygen atoms (Fig. **1**). Arg48 is located in the adjacent monomer. Thr94 and Gly26 are also interacting with the phosphate ion. If ribose-1-phosphate is located in the active site, a hydrogen bond is formed between 3'-OH of the ribose and O3 of its phosphate moiety.

A similar mechanism of substrate binding between UP and PNP was assumed involving a random sequential binding. The UP of *E. coli* showed remarkable changes during substrate binding: a mixture of open, intermediate and fully closed active sites were observed [26]. Parts of β5, β6, β7, β9 and the N-terminal half of α7 showed conformational changes after substrate binding. While in the open active site the side-chain of Arg30 in the phosphate binding site is poorly ordered. It showed significantly stronger electron density in the closed phosphate binding site. This is probably responsible for a stabilization of both hydrogen interactions made to phosphate oxygen atoms. By this change Glu238 comes into a position where it can form a hydrogen bond to Arg30 and Ile92. In the presence of phosphate also Arg91 undergoes a conformational change and is forming hydrogen bonds to the phosphate oxygen atoms and Glu196. In the absence of phosphate Arg91 moves away from the phosphate pocket and hydrogen bond to Val24 and Thr67

are formed. In the closed conformation a water molecule takes the psoition of Arg91. It interacts by hydrogen bonds to Glu196 and Arg91. The movements lead to a sealing of the uracil pocket in the closed conformation.

The uridine phosphorylase of *S. typhimurium* was shown to possess a potassium ion binding site. It was found in the intermonomeric region of each homodimer [29]. The coordinating atoms were Glu49, Ile69, Ser73, Glu49 (neighboring monomer), Ile69 (neighboring monomer) and Ser73 (neighboring monomer). They are arranged as distorted octahedron or triangular prism. Close to each K^+ a water molecule is bound to Ile69 of the adjacent subunits in all homodimers. K^+ enhances the enzymatic activity of *E. coli* UP [26]. A structure comparison of *S. typhimurium* UP with and without K^+ revealed that K^+ stabilizes the structure of the unliganded substrate binding sites in an open or an intermediate conformation of the active site [28]. Because of the high homology between uridine phosphorylases of *E. coli* and *S. typhimurium*, K^+ might indirectly alter the enzymatic activity by stabilizing the L2 loop in the open conformation [28, 29]. No K^+ ion was detected in structures of human uridine phosphorylase with BAU as substrate [15].

The UP of *T. brucei* is stabilized by an intermolecularly coordinated calcium (Ca^{2+}) ion [16]. The Ca^{2+} ion is coordinated by four oxygen atoms from each monomer for a total of eight ligands: the carbonyl oxygen of Met87, Oδ1 and Oδ2 of Asp90, and Oδ1 of Asn91. A slightly distorted square antiprism is formed by the eight oxygen ligands around the metal. One square face is formed by interactions of the two Asp90 carboxylate side chains, while the other square face is formed by Met87 and Asn91. As the UP of *T. brucei* only forms a dimer it is more dependent on the stabilizing effect of an intermonomer metal compared to *E. coli* or *S. typhimurim* UPs which form hexamers. Although the human UP also exists in a homodimeric structure, it does not bind metal ions and, hence, uses an alternate strategy for dimer stabilization. A structure-based sequence alignment revealed that the primary coordinating residue (Glu49 in *E. coli* UP) is not observed in the human protein [15]. Interestingly, it has little impact on the surrounding structure. Human UP reached a greatly increased size of the interface of the dimer by changing two structural elements: I. An elongated N-terminus of the protein is present which forms a strand-turn-strand structure interrupted by two short helices. II. α-helix α3 of the bacterial enzymes is substituted by an strand-turn-strand motif [15]. These modifications together increase the interface area between subunits from 2791 sq. Å in *E. coli* UP to 3292 sq. Å in human UP-I (~18%).

4.1.2. Comparison of the Active Sites within the NP-I Family

E. coli UP and *E. coli* PNP show an almost identical arrangement of residues [26]. Three arginine residues, the main-chain nitrogen atoms of a glycine (Gly20 of PNP and Gly26 in UP) and the hydroxyl groups of Ser90 (PNP) and Thr94 (UP) form the phosphate binding site. His8, Arg91, Glu198 and Met197 that form interactions with ribose are conserved in all UPs and have equivalents in PNP.

Residues involved in base binding are Phe200 of bovine PNP, Phe177 of human MTAP, Phe159 of *E. coli* PNP and Phe162 of *E. coli* UP [1]. They form herringbone stacking interaction. Only residues of bovine PNP and human MTAP, which show a trimeric form, are structurally conserved. Residues are located in a loop between ß-strand 3b and helix H5 in bovine PNP. While Glu201 of bovine PNP interacts with N1 of the purine base in human MTAP a water molecule interacts with the purine base [1]. Structures of hexameric PNPs don´t show interactions at this position.

In PNP, Ser203, Asp204 and some water molecules form the base binding pocket [26]. In UP Ile220 and Val221 set up the hydrophobic base binding pocket, which results in a reversal of the orientation of the hydrogen bonding interactions compared to PNP. The π-stack interaction between a phenylalanine residue (Phe159 in PNP, Phe162 in UP) and the base are conserved between PNP and UP. In the NP-I family this phenylalanine residue is conserved.

A comparison of *E. coli* UP and PNP revealed the existence of both open and closed forms. However, the closed forms when tightly bound to a substrate differ remarkly and result from contrasting movements. In the closed form of PNP, a conformational change at the C-terminal α7-helix (UP numbering) leads to a formation of a γ turn at residues 220-222 due to a displacement of residues 214-219 of α7 in the active site. This movement results in approximation of Arg217 and Asp204. While region 224-230 of UP undergoes large changes when forming the closed formation, the loop region 208-211 of PNP does not show significant movement. In the active site of PNP in vicinity to Glu210 (Glu227 in UP) no residues are observed that could act as interaction partners. Additionally, the PNP loop region (6 residues) is shorter than in UP (10 residues). Moreover, during substrate binding PNP monomers rotate as a whole relative to each other, no equivalent movement is observed in UP.

The K_m values for the affinity of human PNP for guanosine and inosine have been determined to be 12 mM and 45 mM, respectively [3]. A correlation between observed hydrogen bonds and K_m values has been detected; a higher number of intermolecular hydrogen bonds (eight) between human PNP and guanosine is correlated with the lower K_m value compared with inosine, which presented six intermolecular hydrogen bonds [30].

4.1.3. Differences Between Hexameric and Trimeric PNP Enzymes

In hexameric PNP the active site is formed of amino acids belonging to two subunits (Fig. **1**C). In *E. coli,* His4 and Arg43 are belonging to the adjacent subunit. Both interact directly with the ligand via hydrogen bonds [31]. In contrast, in human PNP Phe159, which is the only residue from the adjacent subunit does not directly interact with ligands. However, it is involved in forming the hydrophobic environment around the sugar.

In *E. coli* PNP, the base binding site is more exposed and accessible compared to the counterpart in human PNP [3]. While N1-H of the purine base interacts *via* hydrogen bond with Glu201 in human PNP, N1-H of *E. coli* PNP is linked to a water molecule (Fig. **1**A,C) [3].

The phosphate binding site of *E. coli* PNP is more positively charged than in human PNP as it is formed out of three Arg residues (Arg24, Arg87, Arg43) compared to only one Arg residue (Arg84), respectively.

4.1.4. Amino Acid Residues Defining Substrate Specificity in Members of the NP-I Family

C6-position of the base is strongly responsible for substrate specificity. Residues involved in base binding are located in a structurally conserved position in ß-strand 5b of all four members of the NP-I family [1]. The aspartic acid side chains in human MTAP (Asp220) and *E. coli* PNP (Asp204) allow for hydrogen bonding with the 6-amino and 6-oxo purine nucleosides, while Asn243 of human and bovine PNP specifies 6-oxo purine nucleosides as their substrates. It was shown that specificity of trimeric PNP could be altered to that of bacterial type PNPs by changing Asn243 to Asp [3].

In *E. coli* UP and human UP amino acid residues Gln166, Arg168 and Arg223 are conserved, suggesting that both form similar interactions with uracil [26]. Gln166 and Arg168 (Gln246 and Arg248 *T. brucei*) are described to be the key residues for UP specificity and are located in the 163-185 region of *E. coli* UP (residues 243-264 in *T. brucei*). Therefore, this region is called UP specific region. The absence of this region (and therefore Gln166) in PNP results in a larger base binding pocket and less specificity.

4.1.5. Substrate Spectrum of PNPs

PNPs with trimeric quaternary structure were described to specifically use 6-oxopurines and their nucleosides, but not 6-aminopurine nucleosides like adenosine as substrate [1, 3]. In contrast, hexameric PNPs are known to accept both 6-aminopurine and 6-oxopurine nucleosides. However, in some cases they were described to prefer adenosine compared to 6-oxopurine nucleosides [32-34]. The following paragraphs will show that PNPs use a large number of modified nucleosides next to the natural substrates.

Natural nucleosides as well as their analogs recognized by PNP, have the glycosidic bond between N9 of the purine base and C1' of the sugar moiety. However, studies show that few exceptions can also be recognized by this class of enzymes. Bovine and *E. coli* PNP phosphorolyze both inosine and adenosine analogs with an N3-C1´ glycosidic bond. Although the enzymes' affinity for these substrates is very poor, as demonstrated by high K_m values, the overall reaction kinetics is balanced by high V_{max}. Another exception is the reported cleavage of 7-(ß-D-ribofuranosyl) hypoxanthine and 7-(ß-D-ribofuranosyl) adenine by bovine, *E. coli* and human PNP [56, 67, 100].

An unusual finding was that also pyrimidine and open purine rings could be cleaved by some PNPs. Thermostable PNPs from *Deinococcus geothermalis* and *Geobacillus thermoglucosidasius* have the ability to cleave the glycosidic bond in cytidine and 2'-deoxycytidine. Open purine rings that bear structure similarity to inosine are cleaved by some PNPs; Calf and *E. coli* PNP tolerate nicotinamide-1-ribose as a substrate. Ribavirin, which is another inosine-mimicking substrate, is cleaved by *Bacillus subtilis* and *Brevibacterium acetylicum* PNPs to give 1,2,4-triazole-3-carboxamide and D-ribose-1-phosphate. It is as well a substrate for human PNP [52, 56, 62].

Generally, small alkyl group substitutions at C7 of the nucleobase are well accepted in most cases and phosphorolysis of the respective compounds was successful. However, the synthetic reaction did not work. Using 7-methylguanine and 7-methylhypoxanthine as substrates, for example, the reaction catalyzed by bovine PNP can be monitored by the phosphorolysis of the fluorescent nucleoside substrate over reaction time [61, 65, 101].

Electronegative groups at C6 seem to be a prerequisite to provide the electron density needed for protonation according to the postulated mechanism of action. Therefore, purine nucleosides with thioalkyl, alkyl, thiol and halogen modifications at C6 were successfully synthesized by many bacterial and mammalian PNPs. Details are shown in Table **2** [31, 54, 57, 58, 63, 102].

PNPs exhibit optimal activity when ribose is the sugar moiety of the investigated nucleoside. Enzyme structure studies demonstrate that there is not much room for change, especially at the 2' and 3' positions. Thus major changes in the original sugar structure are not reported as acceptable substrates. For example, human PNP loses 50% of its reaction velocity when the OH group is removed from the 2' position of inosine, although K_m is in the same range. A more detrimental effect is demonstrated using 3'-deoxyadenosine as substrate, more than 90% of the activity is lost and K_m increased 50 times. When replacing the 3'-OH by 3'-NH_2, some activity is retained as the electronegative character of C1´ is maintained [103]. Nevertheless, studies have proven that arabinose and arabinose derivatives are substrates of *E. coli* PNP and various thermostable PNPs [37, 44, 55]. Omission of the hydroxyl group at the 3' position is also accepted in some cases [35, 39, 104, 105]. Modifications on the 5' position are less studied, except for few examples that provide evidence that nucleosides with halogen substitution in C5´ are used as substrate for PNPs [23, 42, 106] and some can also recognize S-methyl-5-thioribose (MTR), the natural substrate of the closely related MTAP enzyme [36, 40, 43].

As mentioned before, bacterial hexameric PNPs were shown to have a broader substrate spectrum than mammalian trimeric PNPs. The available experimental data demonstrate that the hexameric enzyme from *Salmonella enterica* has a molecular weight of 130 kDa (purified through gel filtration). It shows activity for adenosine, 2'-deoxyadenosine, 2'-deoxyinosine, guanosine and inosine. None of these substrates showed less than 75% activity compared to that of 2'-deoxyguanosine, which was considered as the best substrate [5]. Hexameric PNP of *E. coli* showed a lower reaction rate, yet it was able to phosphorolyze both, purine and pyrimidine nucleosides. In addition, *E. coli* PNP can cleave many purine nucleoside analogs, as demonstrated in Table **2** [5, 31]. In difference, bovine PNP is a trimeric PNP which was reported not to cleave adenosine [107], but contradictory data from Moyer and Fischer indicate that it can use adenosine as substrate but the K_m value is almost 6 times higher (*i.e.* lower affinity) than that of guanosine and deoxyguanosine [108]. Buckoreelall and coworkers analyzed the substrate spectrum of two trimeric PNPs from *M. smegmatis*: the first one was not able to utilize adenosine or 2'-deoxyadenosine as substrates while the other could phosphorolyze them, together with guanosine, inosine and adenosine analogs [20].

4.1.6. Substrate Spectrum of 5'-Methylthioadenosine Phosphorylase

MTAP was first described in rats [109]. It cleaves the glycosidic bond of 5'-deoxy-5'-methylthioadenosine. Another surprising observation was the activity of the enzyme from the thermophilic archaeon *Aeropyrum pernix* against pyrimidine molecules, *e.g.* cytidine and deoxycytidine. This is in accordance with observations made for *E. coli* recombinant PNP that was able to use cytidine, 2'-deoxycytidine and 2'-deoxyuridine as substrates [37, 110, 111].

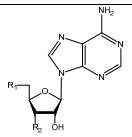
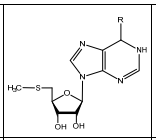
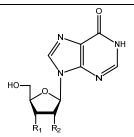
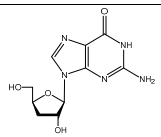
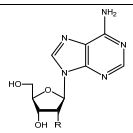
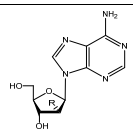
MTAPs from different sources exhibit a range of substrate tolerance that does not always comply with the empirical strict specificity. For example, 6-oxopurine guanosine and inosine are accepted by some organisms. *Pyrococcus furiosus* can use both nucleosides. A slightly lower substrate affinity was observed for these compounds (K_m values of 0.916 and 0.963 mM, respectively) in comparison to the natural substrate MTA (K_m of 0.147 mM). The hexameric enzyme from the thermoacidophilic archaeon *Sulfolobus solfataricus* also has the capacity to use both substrates even more efficiently than MTA. However, it is reported that this enzyme shows more sequence similarity to PNPs than to some known MTAPs [75, 77, 112]. *Aeropyrum pernix* produces another thermophilic enzyme that can cleave inosine, 2'-deoxyinosine and 2'-2´-amino-2´-deoxyinosine [37]. Additionally, small functional group additions to the purine ring, usually at C2, are accepted in many cases. In contrast to PNPs, halogen substitutions seem to be more favored than amino groups [54, 70, 71, 78, 80]. Human MTAP showed a wider acceptance towards modifications of the purine base (*e.g*: 1-deaza or 8-azaadenine) (Table **3**) [70].

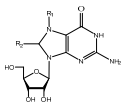
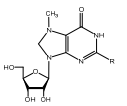
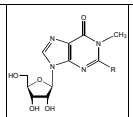
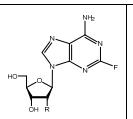
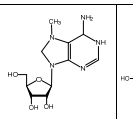
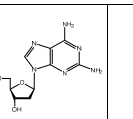
The sugar binding site of MTAP lacks H-bond donating residues except for M196 in case of human MTAP (Fig. **1**) which accounts for its recognition of some substitutions like halogens, cyclic and aryl groups or normal and branched alkyl chains or halogens at the 5' position [68-70, 72, 76, 113]. Changes at the 2' position are tolerated by some members of this enzyme group: *Leishmania* spec., *Trypanosoma* spec. and human MTAP have the capacity to use 2'-deoxyadenosine. The parasite MTAPs were also able to accept amino or halogen substituted compounds [70, 73, 79, 114]. Diastereomeric arabinose analogs were not accepted by most of the MTAP enzymes except for MTAP from *Aeropyrum pernix,* which was able to phosphorolyze 2'-fluoro-2'-arabinoadenosine [37].

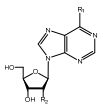
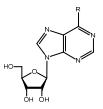
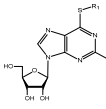
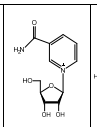
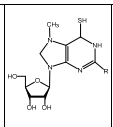
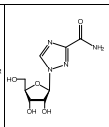
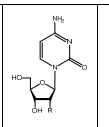
4.1.7. Substrate Spectrum of Uridine Phosphorylase

Uridine phosphorylases have been found and characterized in many prokaryotic organisms including, *E. coli, Lactobacillus casei, Enterobacter aerogenes, Salmonella typhimurium* [38, 81, 83, 114, 115] as well as eukaryotic organisms including *Mus musculus, Rattus norvegicus, Saccharomyces cerevisiae, Schistosoma mansoni* and *Homo sapiens* [86, 87, 89, 92, 93, 96, 97]. The latter are widely studied as potential drug targets.

Table 2. Substrate spectrum of purine nucleoside phosphorylases. Thermophilic organisms are marked by asterisk.

Sugar-modified nucleosides						
						
1: R_1= F, R_2= OH, 2: R_1= OH, R_2= H	3: R= O, 4: R= NH_2	5: R_1, R_2= H		6: R= NH_2, 7: R= F	8: R= F (Ara), 9: R= OH	
		5 [35]				*B. acetylicum*
	3 [36]					*B. taurus*
				7,8 [37]		*D. geothermalis**
2 [38]						*E. aerogenes*
					8 [31]	*E. coli*
				7,8 [37]		*G. thermoglucosidasius**
		5 [39]				*H. sapiens*
2 [35]		5 [35]	[35]			*P. carotovorum*
	3 [40]					*P. falciparum*
	4 [41]					*P. yoelii*
1 [42]						*S. cattleya*
	4 [43]					*S. solfataricus**
	3,4 [40]					*T. gondii*
					9 [44]	*T. thermophilus**

6-Oxopurine and 6-aminopurine base-modified nucleosides							
							
1: R_1= CH_3CH_2, R_2= H 2: R_1= $CH_3(CH_2)_2$, R_2= H 3: R_1= $CH_3(CH_2)_3$, R_2= H	4: R_1= CH_3, R_2= CH_3 5: R_1= $CH(CH_3)_2$, R_2= H 6: R_1= H, R_2= CH_3	7: R= H 8: R= NH_2	9: R= H 10: R= NH_2				
1-6 [45]		7 [46], 8 [3,45,46]					*B. taurus*
		7 [3,45,47], 8 [45,48]	9, 10 [45]	1,2 [31]	[48]		*E. coli*
		7 [3], 8 [3,49]			[3]		*H. sapiens*
		7,8 [50]					*M. tuberculosis*
						[44]	*T. thermophilus**
				1 [51]			*T. vaginalis*

Base-modified nucleosides							
							
1: R_1= CH_3, R_2= H 2: R_1= CH_3, R_2= OH 3: R_1= CH_3CH_2, R_2= OH 4: R_1= n-propyl, R_2= OH 5: R_1= n-butyl, R_2= OH 6: R_1= isobutyl, R_2= OH	7: R= cyclopropyl 8: R= cyclobutyl 9: R= cyclopentyl 10: R= phenyl	11: R_1= H, R_2= H, R_3= OH 12: R_1= H, R_2= NH_2, R_3= OH 13: R_1= CH_3, R_2= H, R_3= H 14: R_1= CH_3, R_2= H, R_3= OH		14: R= H 15: R= NH_2		16: R= H 17: R= OH	

(Table 2) Contd....

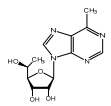
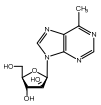
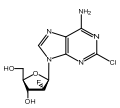
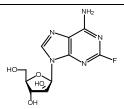
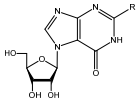
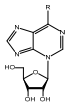
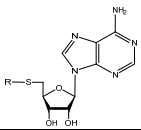
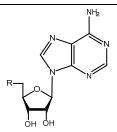
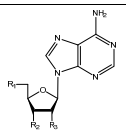
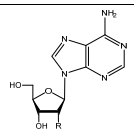
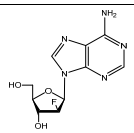
Base-modified nucleosides							
							B. acetylicum
					[52]		*B. subtilis*
			[3]	15 [53]			*B. taurus*
						16,17 [37]	*D. geothermalis**
1, 2 [54,55] 3-6 [55]	7-10 [55]	13,14 [31]	[3,56]	15 [3]			*E. coli*
1,2 [54]							*F. tularensis*
						16,17 [37]	*G. thermoglucosidasius**
		11[57], 12 [58]		14 [59] 15 [60,61]	[62]		*H. sapiens*
				15 [50]			*M. tuberculosis*
		11 [63]		15 [64]			*R. norvegicus*
				15 [65]			*S. scrofa*
1,2 [54]							*T. vaginalis*
Sugar- and base-modified nucleosides and nucleosides with non-typical base binding							
							
				1: R= NH_2, 2: R= H	3: R= NH_2, 4: R= O		
[54]	[55]	[66]	[55]	2 [3]	3,4 [3,67]		*E. coli*
[54]							*T. vaginalis*
				1,2[3]	3,4 [3,67]		*B. taurus*
				1,2 [3]			*H. sapiens*

Table 3. Substrate spectrum of 5´-methylthioadenosine phosphorylases. Thermophilic organisms are marked by asterisk.

Sugar-modified nucleosides					
					
1: R= CH_3CH_2 2: R= $CH_3(CH_2)_3$ 3: R= $(CH_3)_2CHCH_2$ 4: R= $CH_3(CH_2)_2$ 5: R= $OH(CH_2)_2$	7: R= OH 8: R= Cl 9: R= I 10: R= H 11: R= $SeCH_3$	13: R_1, R_2= OH, R_3= H 14: R_1= OH, R_2, R_3= H 15: R_1, R_3= H, R_2= OH 16: R_1, R_3= OH, R_2= H	17: R= F 18: R= Cl 19: R= NH_2		
	7 [37]		17,18,19 [37]	[37]	*A. pernix**
2,3[68]					*C. acidophila**
1 [69]	7 [70,71] 8,10 [70] 9 [71] 10 [70-72] 11 [69]	13 [71] 14,15 [70]			*H. sapiens*
	10 [73]	13[73]			*L. donovani*
	10 [74]				*M. musculus*
	7 [75]				*P. furiosus**
1,4 [76]					*R. norvegicus*

(Table 3) Contd....

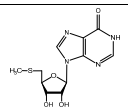
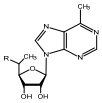
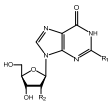
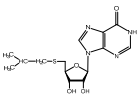
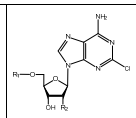
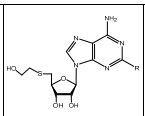
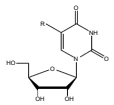
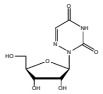
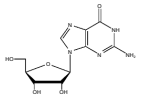
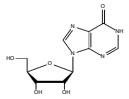
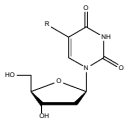
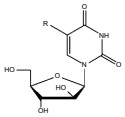
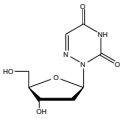
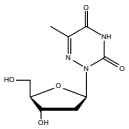
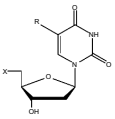
	7 [54,77]					*S. solfataricus**
5 [78]	7[79]	13 [79,80] 14, 15, 16 [80]	17,18 [79]			*T. brucei*
Base-modified nucleosides						
						
[68]						*C. acidophila**
[69]						*H. sapiens*
Sugar- and base-modified nucleosides						
							
1: R= OH (allo) 2: R= OH (tallo)	3: R_1= H, R_2= OH 4: R_1= NH_2, R_2= OH 5: R_1, R_2= H	6: R= H 7: R= OH		8: R_1= H, R_2= H 9: R_1= H, R_2= OH 10: R_1= CH_3, R_2= H	11: R= NH_2 12: R= F	13: R= H 14: R= OH	
	3,5 [37]					13,14 [37]	*A. pernix**
			[68]				*C. acidophila**
				8-10 [71]			*H. sapiens*
	3,4 [75]						*P. furiosus**
1,2 [26]	3 [54,77] 4 [75]	6,7[54]					*S. solfataricus**
		6 [80]			11,12 [78]		*T. brucei*

Table 4. Substrate spectrum of uridine phosphorylases.

Sugar-modified nucleosides: 2´-moiety, ring-modifications					
					
[38]	[38]				*E. aerogenes*
[81]					*E. coli*
	[82]				*L. casei*
[83]					*S. typhimurium*
[84]					*S. oneidensis*
[85]					*G. intestinalis*
[86]					*H. sapiens*
[86,87]					*M. musculus*
[88]					*R. norvegicus*
[89]					*S. mansoni*

(Table 4) Contd....

Base-modified nucleosides					
					
1: R = Br, 2: R= F, 3: R= CH_3					
1, 3 [81]					*E. coli*
3 [82]					*L. casei*
1, 2 [83]					*S. typhimurium*
3 [84]					*S. oneidensis*
3 [90]					*A. pernix*
2 [91-93]					*H. sapiens*
2, 3 [87,93,94]	[87]				*M. musculus*
				[95]	*P. falciparum*
2 [94]					*R. norvegicus*
		[96]			*S. cerevisiae*
			[16]		*T. brucei*
Sugar- and base-modified nucleosides					
					
4: R = Br 5: R = F	6: R= CH_2-CH_3			7: R = F, X = H	
4, 5 [81]					*E. coli*
5 [97]				7 [91]	*H. sapiens*
4, 5 [87,93]		[87]	[87]	7 [93,98]	*M. musculus*
	6 [99]				*R. norvegicus*

Although UP substrate affinity differs from one organism to another, almost all UPs share some major characteristics with few exceptions: (i) most of the characterized UPs have their maximum activity against uridine except for UP of *Lactobacillus casei* which has a higher activity towards 5-methyluridine (117%) [82]. (ii) They have lower relative activity towards thymidine. In case of *Escherichia coli* and *Lactobacillus casei* UPs relative activity for thymidine was 2% [81] and 6% [82], respectively, compared to the activity towards uridine. Human UP showed the same pattern, however, with lower activity [81]. (iii) They have no activity towards cytidine, with the exception of the enzyme from *Saccharomyces cerevisiae* [96]. Mitterbauer and coworkers showed that the ORF YDR400w of *Saccharomyces cerevisiae* encodes for a UP expressing gene and the produced protein accepts both uridine and cytidine as substrates but not inosine, thymidine, guanosine or adenosine. (iv) 5-modified nucleosides are well accepted by UP enzymes with the exception of *Lactobacillus casei* UP which has no activity towards 5-bromouridine [82]. *Salmonella typhimurium* UP is able to phosphorylate 5-bromo and 5-fluorouridine [83], *Escherichia coli* UP accepts C5-modified pyrimidine nucleosides as well [81].

Next to base modifications, sugar modifications are also accepted by UPs. The UPs of *Lactobacillus casei* and the *Enterobacter aerogenes* have 94.6 % and 10 % relative activity towards uracil-arabinoside, respectively [38, 82].

Depending on the organism, the activity towards uridine nucleoside analogs differs. Human and murine UP were studied in detail [93]. UP is found in almost all human tissues as well as in tumors, while TP is decreased or absent in most of the tumors. The specificity of the UP and TP is different between different mammalian species and even within the same species. The UPs identified from the murine liver and from the human placenta have a wider substrate range compared to that identified from the human liver. The human liver UP is highly specific for uridine, with lower cleavage capacity towards 5-fluorouridine (15%) compared to the murine's liver UP activity (85%). Interestingly, recombinant human UP cloned from liver does not share any sequence similarity with known UPs except for 15 amino acids. Murine UP has additional activity towards 5'-fluorouridine, and 5'-fluoro-2'-deoxyuridine [93, 98], which are not accepted by the human liver UP. On the contrary, TP isolated and characterized from the human liver has a broader specificity compared to its counterpart from the murine's liver [93].

4.2. Substrate Recognition and Substrate Spectra of Members of the NP-II Family

Members of the NP-II family can be differentiated into TPs and PyNPs. Both members catalyze the reversible phosphorolytic cleavage of the glycosidic bond of pyrimidine nucleosides or analogs thereof in the presence of phosphate [1, 116]. TP and PyNP share a common two-domain subunit fold and a high level of sequence and structure identity [1]. Although uridine phosphorylase catalyzes a similar reaction like TP and PyNP, it does not belong to the NP-II family due to distinct structural characteristics. Compared to UP, TP has a high specificity for the 2´-deoxyribofuranose moiety of pyrimidine nucleosides [1]. PyNP does not discriminate between uridine and thymidine and therefore, phosphorolyzes both compounds [117]. PyNPs are very interesting biocatalysts suitable for certain synthetic applications, *e.g.* for the enzymatic synthesis of valuable nucleoside analogs. PyNPs were described for *Bacillus stearothermophillus* [117], *Bacillus subtilis* [118], *Hameophilus influenza* [81], *Geobacillus thermoglucosidasius, Thermus thermophilus* [119], *Mycoplasma hyorhinis* [120].

4.2.1. Active Site of Members of the NP-II Family

The active site of PyNPs was well described by Pugmire and Ealick [1]. However, data were missing for the active site of TP. Detailed data are given in the following paragraphs.

In human TP, the transition state is stabilized by both polar and apolar active-site residues [121]. The pyrimidine ring is positioned in the vicinity of Val241 by the side chains of Tyr199 and Leu148, on one side, and that of Ile218 on the other side. The O2 and O4 of thymine interact *via* hydrogen bonds with Lys221 and Arg202, respectively (Fig. **2**). Whereas, the hydroxyl group of Ser217 accepts a hydrogen bond from N3, the imidazole ring of His116 makes two hydrogen bonds with N1 and O2 of the base [121] (Fig. **2**). The O5′ of the deoxyribose interacts *via* Hydrogen bonds with Thr151 and Gly119. Thr154 hydrogen bonds to O3′ of the deoxyribose and phosphate. O3′ of the deoxyribose donates another Hydrogen bonds to the phosphate. The phosphate oxygen 3 accepts two hydrogen bonds, one from Lys115 and another from Ser117 (Fig. **2**). This elaborated hydrogen-bonding pattern facilitates the nucleophilic attack of the phosphate oxygen 2 onto the sugar ring atom C1′. Asp203 hydrogen bonds to Gly149 and therefore plays a crucial role in facilitating the correct placement of Leu148 and loop stabilization required for efficient catalysis [121].

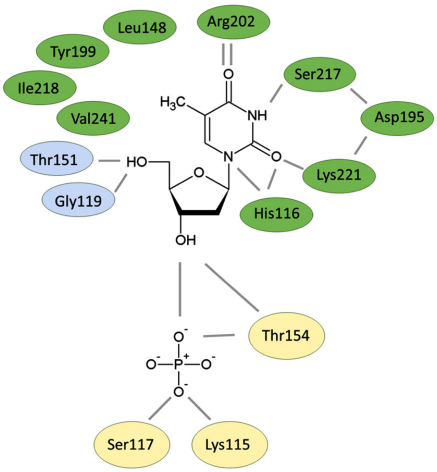


Fig. (2). Schematic drawing of the active site of human thymidine phosphorylase. Residues binding the base, the sugar-moiety and phosphate are shown in dark grey, light grey and white, respectively.

El Omari and coworkers were able to build a loop (AS 405-415) in human TP, which was missing in earlier structure [122]. The loop appears to be not essential for domain closure, however, it might be involved in the stabilization of the closed form (by α and α/β domain interaction across the active-site cleft) and the TP dimer.

Although inhibitors for nucleoside phosphorylases are not discussed in the recent review, inhibitor studies revealed an interesting result for thymidine phosphorylases. Liekens and coworkers described that 5'-O-trityl-inosine (KIN59) inhibits recombinant bacterial (*E. coli*) and human thymidine phosphorylase (TPase) with an IC50 of 44 μM and 67 μM, respectively [123]. However, KIN59 neither bound to the pyrimidine nucleoside-binding site nor the phosphate-binding site of the enzyme, which indicates the existence of an allosteric binding site at the enzyme regulating its biological activity.

4.2.2. Comparison of the Active Sites of Thymidine Phosphorylases and Pyrimidine Phosphorylases

Thyimidine phosphorylases of *H. sapiens* and *E. coli* and PyNP of *B. stearothermophilus* share an overall sequence similarity of ~42% [124] and comprise an α-domain and a mixed α/β-domain connected by three polypeptide loops [116, 125, 126]. The α-domain consists of six α-helices (α1-α4 and α8-α9), and the α/β domain consists of a central mixed β-sheet (β1-β5, β13) surrounded by α helices (α5-α7 and α10-α16) and two small antiparallel β-sheets (consisting of strands β6, β7, β9, and β11, and β8, β10, and β12 respectively) flanked by two α-helices (α17 and α18) [125]. The loops act as a hinge which allows the two domains to switch between the open (inactive) and closed (active) conformations. The movement is responsible for bringing the active site residues together [126]. The main structural differences between NPs of *H. sapiens*, *E. coli* and *B. stearothermophilus* are in the α/β domain where human TP has an additional helix (α15), an extra turn in α16, and a more extended C-terminal region [125]. The small antiparallel β-sheet (β8, β10, and β12) is present in human TP and PyNP of *B. stearothermophilus* but not in *E. coli* TP. A glycine rich loop described first in *E. coli* TP is also present in human TP and PyNP of *B. stearothermophilus* (residues 144 to 154 in human TP) [124]. The loop was described to be important for the binding of the catalytic phosphate.

The structure of *B. stearothermophilus* PyNP revealed that a metal binding site is in close vicinity to the phosphate binding site [126]. This unidentified penta-coordinate metal interacts with the backbone carbonyl oxygen atoms of residues Leu243, Ala246, and Gly88, and the side chains of Glu255 and Thr90 (numbering of *B. stearothermophilus* PyNP). In the human TP structure, no metal ion was detected, however, it was suggested that the Nζ atom of Lys124 (which corresponds to Thr90 of *B. stearothermophilus* PyNP) takes the function of the metal ion of *B. stearothermophilus* PyNP and interacts with the equivalent residues [125].

4.2.3. Factors Determining Substrate Specificity within NP-II Family

The active-site residues involved in binding the substrate are highly conserved among all sequences of the NP-II family [1]. The only exceptions are Val241 in human TP (which corresponds to Phe207 in *B. stearothermophilus* PyNP and Phe210 in *E. coli* TP) and a methionine residue which replaces Lys108 of PyNP [124, 126]. The replacement of methionine with lysine in PyNP it might result in a changed hydrogen bonding pattern with the phosphate oxygen that binds to the 2´-hydroxyl group of the ribose moiety. This might be responsible for difference in substrate specificity of TP and PyNP [1]. Balaev and coworkers described a different explanation for the specificity of TP for thymidine and not uridine [127]. The location of the furanose component in the TP active site might influence substrate specificity. In the presence of thymidine TP subunit closure was not observed although cleavage reaction

was shown to occur. In accordance with previous studies it was supposed that phosphate is stabilizing the transition state.

4.2.4. Substrate Spectrum of Thymdine Phosphorylase

Studies on the substrate range of thymidine phosphorylases have shown that the absence of the hydroxyl group (-OH) at the 2' position of the nucleoside is important for the reactions catalyzed by TP enzymes. It was described for both *E. coli* TP and a TP extracted from liver cancer cells. Uridine was converted with only very low yields by *E. coli* TP, while it was not used as substrate by human TP at all [128, 129]. Both uridine and 2´-methyluridine served as inhibitors of *E. coli* TP which supports the importance of the absence of the 2'-hydroxyl group for the phosphorolysis to take place. More details on the K_m and k_{cat} or conversion rates of these substrates can be found in [130, 131].

The 3´-hydroxyl group plays a crucial role for substrate recognition by TP [130, 131]. The absence of 3′-hydroxyl group or inverted configuration at C3′ atom revealed that those compounds were not or only very weak substrates. 3′-Amino-3′-deoxythymidine was not a TP substrate at pH 6.5, but it undergoes phosphorolysis when the pH reaches 8.0 [130]. This was associated with the presence of the 3′-amino group in the vicinity of the α-amino group of an amino acid residue in the active site; thus, the two protonated groups are repelled at pH 6.5. At pH above 8.0, the positive charges on nucleoside and protein amino groups disappear and phosphorolysis could take place.

Substitution at 5´-OH group show only little impact on substrate recognition of TP, which shows that 5´-OH group is not interacting with the TP active site (Table **4**). Among the tested 5' substitutions of thymidine, 5'-deoxy-, 5'-chloro-, 5'-amino- and 5´-thio modifications were described as substrates for *E. coli* TP [130-132]. The azide (-N3) and 5'-iodo modified thymidine analogs were not used as substrates by this enzyme.

Studies on modifications at the base moiety of thymidine analogs revealed that it accepts a large number of substituents at the C5 position of the thymine base [115, 131, 133]. Next to deoxyuridine, 5-bromo-2´-deoxyuridine was substrate for *H. sapiens*, *E. coli*, *L. casei* and *S. thyphimurium* TP (Table **5**, [82, 130, 134-137]). 5-Iodo-2´-deoxyuridine was substrate for *H. sapiens*, *E. coli* and *L. casei* TP [82, 135, 137, 138]. 5-Fluoro- and 5-nitro-2´-deoxyuridine were substrates for human and *E. coli* TP [133, 137, 138]. However, substitution at the C6 position of the base has strong impact on enzyme activity [131]. Hence, decreased levels of conversion were observed with a higher aromaticity of the pyrimidine ring. The reason seems to be difficulties in polarizing the base by amino-acid residues in the active site. Only 6-azathymidine was described as substrate with low conversion by *E. coli* TP [131].

Kinetic parameters and inhibition studies were performed to determine substrate specificity of human TP from liver and placenta compared to TP from mouse liver [156]. The substrate spectrum of TP in human liver was different to that of human placenta or mouse liver as a broader substrate spectrum was observed. Human hepatic TP phosphorolyzed all the tested nucleosides including uridine, thymidine, deoxyuridine, 5-fluorouridine (FU), 5-fluoro-2'-deoxyuridine and 5'-deoxy-5-fluorouridine. Approximately 15% of uridine and 85% of FU phosphorolysis in human liver is performed by human TP. Phosphorolysis of uridine is a surprising result as it contradicts literature showing the importance of the 2´-OH group for thymidine phosphorylases of higher organisms [1]. Murine hepatic TP is, in coherence with literature, more specific to deoxyribosides, and is not involved in the phosphorolysis of uridine. Additionally, only 15% of FU is cleaved by murine hepatic TP. TP phosphorolyzed 5'-deoxy-5-fluorouridine in both human and murine livers. Compared to TP form human liver, higher similarity was observed between TP from human placenta und murine liver. It appears that the specificities of human hepatic TP is distinct from those from extrahepatic tissues. As the same was observed for human uridine phosphorylase, it was suggested that tissue-specific isozymes of pyrimidine nucleoside phosphorylases exist in humans. The inter- and intra-species differences in substrate specificities and activities between human and murine pyrimidine nucleoside phosphorylases may have an important impact on the validity of attempts to introduce inhibitors of these enzymes into the clinic or on drawing conclusions about the metabolism and the chemotherapeutic use of pyrimidine analogs in humans based on studies in mice.

4.2.5. Substrate Spectrum of Pyrimidine Nucleoside Phosphorylases

Pyrimidine nucleoside phosphorylases (PyNP) are homodimeric enzymes found in many lower organisms [119, 151] as *Geobacillus stearothermophillus* [117, 157], *M. hyorhinis* [120, 154] , *H. influenza* [115] and others. Unlike TP and UP, these enzymes catalyze the reversible phosphorolysis of both uridine and thymidine as natural substrate [10, 26]. PyNPs share almost 40 % of structure similarities with TP and less than 20 % with UP [151], this could be correlated to the differences in NP families, where TPs and PyNPs comprise NP-II family whereas UP belongs to NP-I family [119]. Due to its affinity to both uridine and thymidine nucleoside analogs, PyNPs are expected to have a wide range of substrates due to its high tolerability to some modifications that are not accepted by UP or TP. For instance, it has been reported that the 2' position does not play a crucial role in the catalytic activity of *B.* subtilis towards pyrimidine nucleoside analogs which contrasts with *E.coli* and *Lactobacillus casei* TP, as mentioned earlier [82, 130, 131, 138].

Despite this, in 2013, Serra *et al*. compared the PyNP of *B. subtilis* with the *E. coli* TP [138]. A very similar behavior of both enzymes towards the tested substrates was described. Both enzymes used 5-halogenated-2'-deoxynucleoside analogs with almost similar rates and conversion percentages, except for the fluoro-modified one. The PyNP from *B. subtilis* showed a slightly higher conversion percentage than the *E. coli* TP. Additionally, they were both able to cleave the glycosidic bond of the (E)-5-(2-bromovinyl)-2'-deoxyuridine (brivudin), however, with lesser extent than all the other examined substrates and this could be due to the steric hindrance of the bulky bromovinyl (CH=CHBr) group. It is worth mentioning that even though both enzymes could phosphorolyze brivudin yet the PyNP of *B. subtilis* showed a higher conversion rate (33%) compared to the *E. coli TP* (25%).

Generally, most PyNPs isolated from different organisms have been shown to be active towards uridine, thymidine and 2'-deoxyuridine [115, 117, 138, 152]. Additionally, *H. influenza* and *G. stearothermophilus* are highly tolerable to 5-bromo and 5-methyl modified uridine and 5-bromodeoxyuridine [115, 117, 152]. A difference in the activity towards arabino nucleoside analogs was identified for different PyNP. PyNP of *G. stearothermophillus* does not accept arabinouracil and is not able to cleave its glycosidic bond [138]. Whereas, PyNP isolated from *Thermus thermophilus* was able to utilize 1-(2-deoxy-2-fluoro- ß-D-arabinofuranosyl) uracil with a conversion of 46%. The same enzyme was also active towards 2'-deoxy- 2'-fluorouridine with even higher conversion percentage (65%) [119].

4.3. Comparison of Substrate Spectra Between Bacterial and Mammalian PNP Enzymes and MTAP

After comparing the substrate spectra of bacterial and mammalian PNPs and MTAP we can show that they share some, mainly natural, substrates (Fig. **3**A). Amongst these are guanosine, inosine, 5´-methylthioadenosine, but also adenosine. Bacterial and mammalian PNPs also share some substrates which are 7-methyl-modified inosine, guanosine and adenosine. Additionally, Ribavirin, which is a known anti-viral drug is converted by both classes of PNPs. While bacterial PNP and MTAP have overlapping substrate spectra, it was not observed for mammalian PNP and MTAP. Both bacterial PNP and MTAP use some 6-methyl-, 2´-amino- and

Table 5. Substrate spectrum of thymidine phosphorylases. Phosphorolysis of the given nucleoside analogs was analyzed except for those studied by Serra *et al*. (2013), which are marked by*.

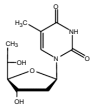
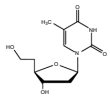
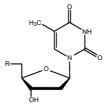
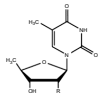
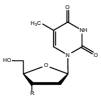
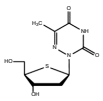
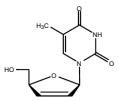
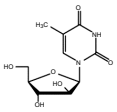
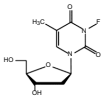
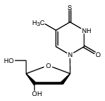
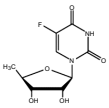
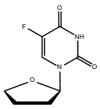
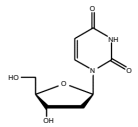
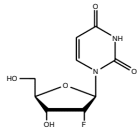
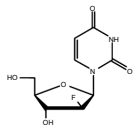
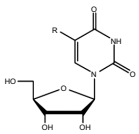
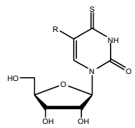
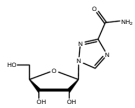
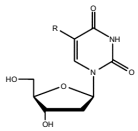
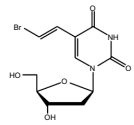
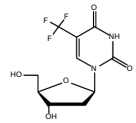
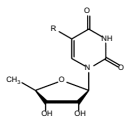
sugar-modified nucleosides: 5´-moiety					
					
			1: R = NH2 2: R = SH 3: R = Cl 4: R = F	5: R = H 6: R = OH	
			4 [139,140]		*H. sapiens*
[130]	[130]	[130]	1-3 [130,131]	5, 6 [130,131,138]	*E. coli*
Sugar-modified nucleosides: 3´-moiety, ring-modifications					
					
7: R = NH_2 8: R= SH					
7, 8 [130]		[130,131]			*E. coli*
			[82]		*L. casei*
Base-modified nucleosides					
					
9: R = Br 10: R= I 11: R = F 12: NO2					
9, 10, 11, 12 [133,137,141-143]	[140]				*H. sapiens*
12 [133,144]		[130,145]*	[131]		*E. coli*
9, 10, 11 [82]					*L. casei*
9 [136]					*S. thyphi.*
Sugar- and base-modified nucleosides					
					
[146]	[147,148]				*H. sapiens*
	[82]				*L. casei*

Table 6. Substrate spectrum of pyrimidine nucleoside phosphorylases. Thermophilic organisms are marked by asterisk.

Sugar-modified nucleosides: 2´-moiety, ring-modifications				
				
[118,138]				*B. subtilis*
[117,149]				*G. stearothermophilus**
	[119]			*G. thermoglucosidasius**
[150]				*S. pyogenes*
	[119]	[119]		*T. thermophilus**
Base-modified nucleosides				
				
1: R = Br, 2: R = F 3: R= I, 4: R = CH_3	5: R = CH_3 6: R= H			
1, 4 [117,126,149,151]				*G. stearothermophilus**
4 [119]				*G. thermoglucosidasius**
1, 4 [115,152]				*H. influenzae*
2, 3 [120,153,154]				*M. hyorhins*
		[155]		*P.carotovorum*
Sugar- and base-modified nucleosides				
				
7: R = Br, 8: R = F 9: R = I, 10: R = Cl 11: R=H, 12: R = CH_3			13: R = H 14: R = F	
7, 8, 9, 11, 12 [118,138]	[118,138]	[118]	[118]	*B. subtilis*
7 [149]				*G. stearothermophilus**
7 [115]				*H. influenzae*
7, 8, 9, 10 [120,153,154]		[120,153,154]		*M. hyorhins*

2´-fluoro-modified substrates. Interestingly, they also phosphorolyze cytidine and 2´-deoxycytidine which shows that purine nucleoside phosphorylases are not strictly bound to purine nucleosides and their analogs.

Bacterial PNPs are able to convert nucleoside analogs which possess halogen substituents at the sugar-moiety (Fig. **3A**). 2'-fluoroadenosine, 2'-fluoroarabinoadenosine, 5'-deoxy-5'-fluoroadenosine or 2´-fluoro-2'-deoxyadenosine have been used as substrate. Mammalian PNP are able to convert a number of nucleosides which show base modifications with amino- or alkyl-groups. Additionally, 5-aza-7-deazaguanine and 8-azahypoxanthine were accepted substrates. MTAPs have a preference for nucleosides which harbor halogen (fluoro, iodo, chloro)-modifications at the base. Furthermore, 5´-alkyl- groups were well tolerated. MTAPs also used 1-deaza-5'-methylthioadenosine riboside and 8-aza-5'methylthioadenosine riboside as substrates.

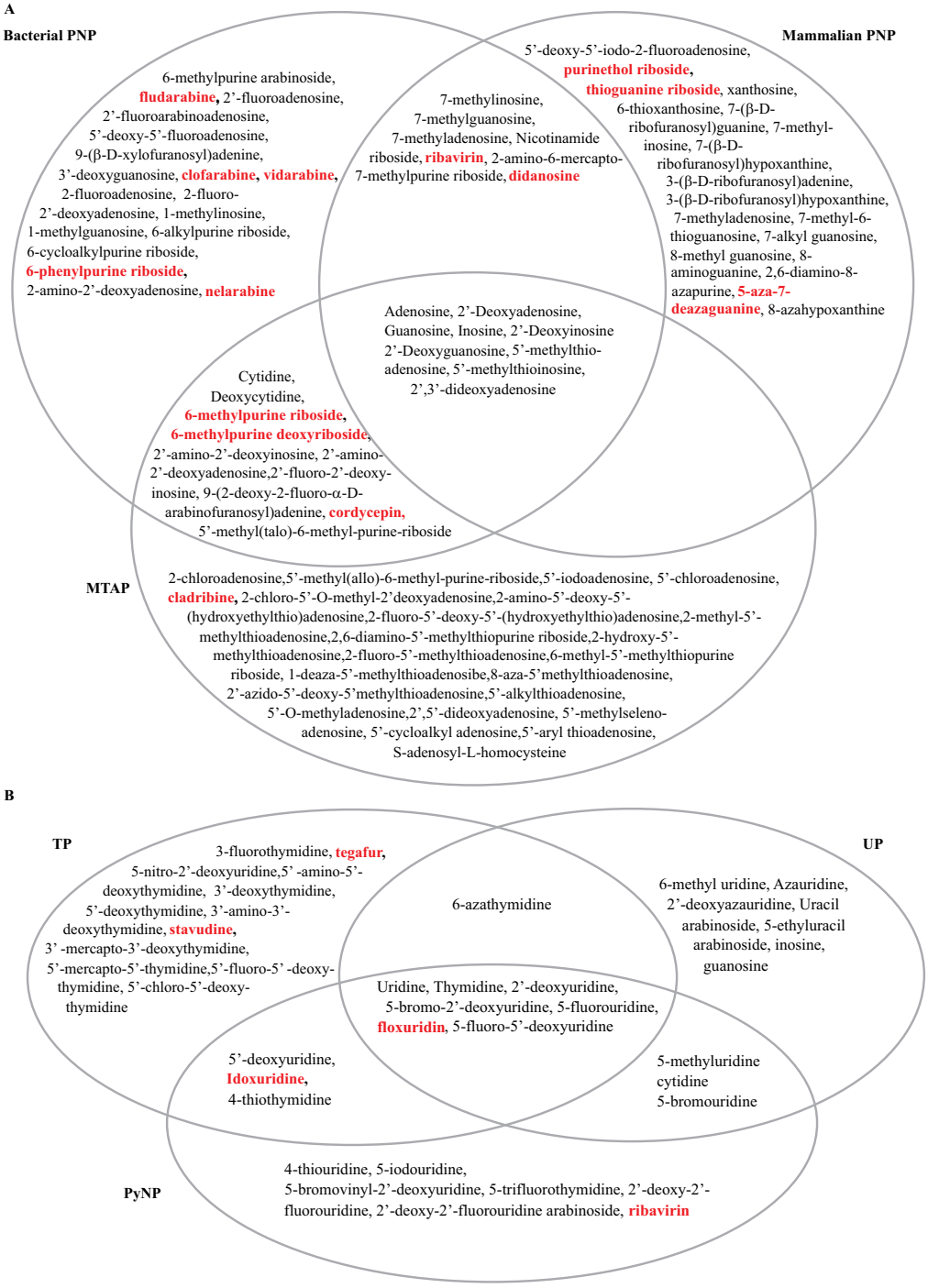


Fig. (3). Comparison of substrates phosphorolyzed by members of NP subfamilies. A) hexameric PNP, trimeric PNP and MTAP, B) UP, TP and PyNP. Drugs or compounds with therapeutic activity are written in bold and are underlined.

4.4. Comparison of Substrate Spectra Between TP, UP and PyNP

TP, UP and PyNP are described to use pyrimidine nucleosides as their substrates. However, for UP the phosphorolysis of guanosine and inosine was described (Fig. **3B**). TP, UP and PyNP have a few overlapping substrates which are uridine, 2'-deoxyuridine, 5-bromo-2'-deoxyuridine, 5-fluorouridine, 5-fluoro-2'-deoxyuridine, 5-fluoro-5'-deoxyuridine, but also thymidine. TP and PyNP share the following substrates: 5'-deoxyuridine, 5-iodo-2'-deoxyuridine and 4-thiothymidine. UP and PyNP both phosphorolyze 5-methyl uridine, cytidine and 5-bromouridine. TP and UP do not have common substrates which are not used by PyNP.

TP uniquely catalyzes the conversion of 3´- and 5´-modified nucleoside analogs like 3'-deoxythymidine, 5'-deoxythymidine, 3'-amino-3'-deoxythymidine, 3'-deoxy-2',3'-didehydrothymidine, 3'-mercaptothymidine or 5'-amino-5'-deoxythymidine (Fig. **3B**). UPs are able to use aza-nucleosides like azathymidine or azauridine. Additionally, arabinosides are well accepted substrates. PyNPs were described to use nucleosides with different halogen substituents in C5 of the base. Examples are 5-iodouridine, 5-bromovinyl-2'-deoxyuridine, 5-trifluorothymidine or 5-trifluoro-2'-dexyuridine. Interestingly, they convert 2´-deoxy-2´-fluoro-mucleosides like 2'-deoxy-2'-fluorouridine and 2'-deoxy-2'-fluorouridine arabinoside, which are interesting precursors for modified oligonucleotides.

4.5. Production of Pharmaceutically Active Compounds by Nucleoside Phosphorylases

NPs are frequently applied for the production of modified nucleosides [18, 110] since they have a fairly broad substrate specificity as shown in the previous chapters. For recent reviews see [18, 158, 159]. Transglycosylation reactions are most widely used to produce nucleoside analogs. The transgylcosylation of nucleosides, catalyzed by nucleoside phosphorylases, is a two-step reaction. In the first step, a nucleoside that is the pentofuranosyl donor is phosphorolytically cleaved into the corresponding heterocyclic base and α-D-pentofuranosyl-1-phosphate. In a second step, this activated carbohydrate moiety is reacting with the heterocyclic base which is used as pentofuranosyl acceptor. This process was very efficient for the synthesis of modified ribosides and deoxyribosides [158, 159]. However, difficulties can result from the difference in the dynamics of the two consecutive reactions. In the first step of the transglycosylation reaction inorganic phosphate is required, while in the second reaction high phosphate concentration inhibit product formation.

An alternative to the transglycosylation reaction is the use of α-D-pentofuranosyl-1-phosphate as substrate for the production of modified nucleosides. The transfer of a pentofuranosyl moiety prepared by chemical or enzymatic methods to purine or pyrimidine bases catalyzed by NPs was shown to be a very efficient method for the production of nucleoside analogs [18, 158, 159]. The widespread application of this reaction, however, is hampered by the fact that α-D-pentofuranosyl-1-phosphate is not very stable and expensive.

For technical application, the least laborious way to use NPs for the production of nucleoside analogs is the application of whole cells as biocatalysts. Effort for downstream processing is low which leads to cost-effective biocatalysts preparation. Moreover, whole cells are a kind of immobilization vehicle which increases stability of the nucleoside phosphorylases. A drawback is the complexity of whole cells. They possess not only NP activity but various other activities which can lead to decreased product yields as shown by Lewkowicz and coworkers [160]. Adenosine was produced from uridine and adenine catalyzed by *E. coli* whole cells. While 94% product yield was observed after 1 h of reaction, they observed that after prolonged reaction times product yields decreased. After 24 h only uracil and adenine were detected.

The application of purified enzyme has the advantage that side-reactions can be avoided. For industrial applications, it is highly desirable to use either immobilized enzymes or continuous membrane reactors to be able to recover the enzyme from the reaction mixture. As a result downstream processing is simplified and biocatalyst recycling becomes possible. As immobilization also increases the stability of nucleoside phosphorylases diverse methods have been exploited [138, 161-163].

To date, NPs from different mesophilic bacteria, such as *Escherichia coli* [164-166] or *Bacillus subtilis* [167], have been applied for the production of pharmacologically active compounds [164, 165]. Thermostable NPs from thermophilic or hyperthermophilic microorganisms show the same regio- and stereo-selectivity as mesophilic NPs, but have further advantages for industrial applications: I. Thermophilic enzymes can be easily purified with high protein recovery which drastically reduces production costs of the enzyme [44]. II. They tolerate a wide range of pH which reduces the need to strictly regulate the pH of the reaction. III. Three purine nucleoside phosphorylases of *Thermus thermophilus* were shown to have a remarkable resistance to aggressive organic solvents such as DMFA, DMSO, or 2-MeTHF, which can be used to increase the water solubility of substrates. IV. Using thermophilic enzymes as catalyst, reactions can be performed at higher temperatures, which reduces the viscosity of the medium or increase the solubility and concentration of some substrates [168]. V. With an increased thermal stability, a broader substrate spectrum was also observed for nucleoside phosphorylases [37, 44, 104, 119]. Thermal stability is not a result of a unique phenomenon. Instead different strategies were found in nature to increase enzyme stabilization: increased electrostatic interaction, greater hydrophobicity and better atom packing, deletion and shortening of loops and disulfide bonds are just some examples [44].

To make NPs a valuable tool in industrial application, upscaling of the production process in necessary. A scale-up from 0.05 mL to 150 mL in a stirring reactor was performed by Nóbile and coworkers to produce fludarabine, vidarabine and 2,6-diaminopurine arabinsoide with product yields of 58%, 71% and 77%, respectively [173]. Immobilized whole cells of *Citrobacter koseri* were used as biocatalyst. The production of 5-methyl uridine in a scale of 1 L was performed in a stirring reactor [174]. PNP of *Bacillus halodurans* and UP of *E. coli* were applied and product yields of 85% with a productivity of 10.6 g L^{-1} h^{-1} were achieved. Challenging parameters were identified: within a large volume in a batch process the mixing of the reaction is a critical parameter. With increased stirring speed, denaturation of the biocatalysts was observed.

The review of Mikhailopulo describes well the chemo-enzymatic production of nucleosides with biological relevance [158]. It shows clearly that nucleoside phosphorylases can be used to produce drugs like cladribine or mizoribine which are applied for the treatment of cancer (Table **7**). In 2003, Mikhailopulo and Miroshnikov described the enzymatic production of fludarabine, clofarabine or vidarabine with *E. coli* ribokinase, phosphopentomutase and PNP [169]. Phosphorolytic cleavage was determined for a number of other approved drugs like ribavirin or didanosin [35]. Purified PNP of *Brevibacterium acetylicum* was used as biocatalyst. Compared to inosine the relative activity was 22% for both compounds (Table **8**). Recently, we tested the phosphorolytic cleavage of nucleoside analog drugs listed in European Pharmacopoeia. PyNPs from thermophilic microorganisms used both cytarabine and zidovudine as substrate, while gemcitabine and lamivudine were not cleaved. PNP or MTAP of thermophilic microorganisms showed phosphorolytic activity with ribavirin and didanosin (our study). Nucleoside analogs with open sugar ring structures like aciclovir, ganciclovir and tenofovir were not accepted as substrate. Aciclovir was tested as substrate for three PNPs of

Table 7. Drugs produced in a (chemo)enzymatic process involving nucleoside phosphorylases.

Drug	Applied Nucleoside Phosphorylase(s)	Product Yield	References
Fludarabine	*E. coli* PNP (starting from α-D-pentofuranose-1-P)	Not given	[169]
	E. coli PNP	1.300 nmoles/mg/hr	[55]
Clofarabine	*E. coli* PNP (starting from α-D-pentofuranose-1-P)	Not given	[169]
Vidarabine	*E. coli* PNP (starting from α-D-pentofuranose-1-P)	Not given	[169]
Cladribine	*E. coli* whole cells	56-95%	[158]
Nelarabine	*E. coli* UP, PNP	53% (with high excess of enzyme)	[158]
Mizoribine	*E. coli* UP, PNP	83%	[158]
6-Methylpurine riboside	*E. coli* PNP	84.000 nmoles/mg/hr	[55]
		96.000 nmoles/mg/hr	[54]
	*Tv*PNP	155.000 nmoles/mg/hr	[54]
	*Ss*MTAP	4.000 nmoles/mg/hr	[54]
	*Ft*PNP	4800 nmoles/mg/hr	[54]
	Human PNP	12 nmoles/mg/hr	[54]
6-Methylpurine deoxy-riboside	*E. coli* PNP	461.000 nmoles/mg/hr	[55]
		528.000 nmoles/mg/hr	[54]
	*Tv*PNP	484.000 nmoles/mg/hr	[54]
	*Ss*MTAP	12.000 nmoles/mg/hr	[54]
	*Ft*PNP	3900 nmoles/mg/hr	[54]

Table 8. Phosphorolytic cleavage of approved nucleoside analog drugs by nucleoside phsophorylases

Drug	Applied Nucleoside Phosphorylase	Product Yield	References
Ribavirin	*Brevibacterium acetylicum* PNP	15%	[35]
Didanosine	*Brevibacterium acetylicum* PNP	Not given (22% residual activity compared to inosine)	[35]
Purinethol	*Rn*PNP: riboside	14%	[10]
	*Ec*PNP: deoxyriboside	*Km*= 126 μM	[11]
Thioguanin	PNP from human sarcoma	Not given	[12]
5-Aza-7-deazaguanine	*E. coli* PNP	*Km*= 0.15 mM	[13]
Tegafur	Human TP	Not given	[147]
Stavudine	*E. coli* TP	Not given	[132]
Idoxuridine	*Salmonella typhimurum* TP	Not given	[135,136]
Floxuridine	Human TP	Not given	[135,136]

Thermus thermophilus and *Brevibacterium acetylicum* before. However, no phosphorolytic activity was observed [44, 105].

In addition to their potential to produce available drugs in industrial scale, nucleoside phosphorylases can also be used to synthesize new bioactive compounds. 6-phenyl purine riboside was produced by bacterial PNP starting with pentose-1-phosphate. It was tested, along with other related compounds that bear aromatic substitution on the purine ring, against three different cancer cell lines where it showed good cytostatic activity against L1210 (lymphocytic leukemia), HeLa (cervical cancer) and CCRF-CEM (acute lymphoblastic leukemia) cells [110]. 6-methyl purine riboside which is used as substrate by both bacterial PNP and MTAP had antifungal (against *Ascochyta pisi*, *Nematospora coryli*, *Neurospora crassa*), antiviral (against vesicular stomatitis virus) and antitumor activity [175]. Marasco and coworkers successfully tested this compound against five human tumor cell lines (ovarian carcinoma, non-small cell lung carcinoma, colon adenocarcinoma, breast adenocarcinoma and adriamycin resistant breast adenocarcinoma) [161].

Table 9. Mutations made in nucleoside phosphorylases that change substrate specificity.

Organism	Mutation	Description	References
E. coli-PNP II	N239D	Mutant enzyme shows no activity with the wild-type substrates inosine, xanthosine and guanosine. Unlike the wild-type enzyme, the mutant enzyme shows activity with adenosine.	[14]
	Y191L	Specificity change to conventional trimeric PNPs. Mutant enzyme shows no activity with the wild-type PNP-II substrate xanthosine. K_m for inosine decreased. K_m of guanosine was increased.	
E. coli PNP (natural mutation)	M64V	The mutant is able to cleave numerous 5'-modified nucleoside analogs (9-[6-deoxy-alpha-L-talofuranosyl]-6-methylpurine (methyl(talo)-MeP-R), 9-[6-deoxy-alpha-L-talofuranosyl]-2-F-adenine, and 9-[alpha-L-lyxofuranosyl]-2-F-adenine) with much greater efficiency than the wild type enzyme. No activity with adenosine or inosine was observed.	[54,170]
Homo sapiens PNP	N243D	The mutation leads to changed substrate specificity, making 6-amino- and 6-oxopurines equally good as substrates and clearly favoring adenosine over inosine and guanosine.	[171]
	Y88A, Y88V, Y88I, Y88S, Y88T, Y88N, Y88D, Y88E	Increase in K_m for dideoxyinosine.	[39]
	Y88F, Y88H, Y88L, Y88M, Y88C, Y88Q	Increase in K_m for dideoxyinosine. Y88F displayed the highest overall $k_{cat}/K_m$and the highest turnover rate.	
	Y88F/M170T	Strong increase in k_{cat}/K_m for dideoxyinosine.	
	Y88F/ M170T/ G4E/ Q172L/ T177AC	Q172L and T177AC counteract the positive effect of M170T on k_{cat}/K_m for dideoxyinosine.	
Mus musculus PNP	K244Q	Ration of turnover-number/K_m is 83% of that for wild-type enzyme, no activity with adenosine.	[172]
	N243D	8-Fold increase in K_m-value for inosine and a 100-fold decrease in the ratio of turnover-number/K_m. Phosphorolysis of adenosine with a K_m-value of 0.045 mM and ratio of turnover-number/K_m 8-fold that with inosine.	
	N243D/K244Q	14-Fold increase in K_m-value for inosine and 7-fold decrease in the ratio of turnover-number/K_m as compared to the wild-type enzyme. Phosphorolysis of adenosine with a K_m-value of 0.042 mM and a ratio of turnover-number/K_m twice that of the single D243D substitution.	
	N243T	Mutant enzyme shows no activity with adenosine.	

4.6. Mutagenesis Approaches to Alter Substrate Specificity

Mutagenesis approaches have been widely used to confirm the relevance of amino acid residues for structure and catalytic activity in nucleoside phosphorylases. For detailed information see Supplementary Table **1**.

Additionally, mutagenesis approaches have been used to change the substrate specificity of purine nucleoside phosphorylases (Table **9**). Already in 1999 Maynes and coworkers showed the important role of Asn243 for substrate specificity of murine PNP [172]. By changing asparagine to aspartic acid the substrate specificity was changed to that of bacterial PNP and adenosine was recognized as substrate. Similar results were obtained for human PNP: The mutation Asn243Asp strongly influences substrate specificity, making 6-amino- and 6-oxopurines equally good as substrates and clearly favoring adenosine over inosine and guanosine [176]. In *E. coli* PNP-II, which is similar to mammalian PNPs, the site-directed mutagenesis of Asn239Asp also changed substrate specificity [14]. Mutant enzyme shows no activity with the wild-type substrates inosine, xanthosine and guanosine. Unlike the wild-type enzyme, the mutant enzyme shows activity with adenosine.

In human PNP, a single amino acid (Tyr88) was identified to be involved in ribose selectivity [39]. A tyrosine to phenylalanine mutant (Tyr88Phe) was shown to have improved catalytic activity with respect to 2′,3′-dideoxyinosine. Kinetic characterization of this mutant determined a 9-fold improvement in k_{cat} and greater than 2-fold reduction in K_m.

Point mutations in the active site of calf PNP led to the formation of non-typical ribosides [177]. While with wild-type calf PNP N7- and N8-ribosides are observed as products, the Asn243Asp mutant produced ribosyl substitution at positions N9 and N7. Similar results were observed with a mutated form of *E. coli* PNP (Asp204Asn). It was used to produce non-typical ribosides of 8-azaadenine and 2,6-diamino-8-azapurine.

Interestingly, a natural occurring mutant of *E. coli* PNP (Met64Val) shows a modified substrate spectrum [54, 170]. The mutant is able to cleave numerous 5'-modified nucleoside analogs like (9-[6-deoxy-α-L-talofuranosyl]-6-methylpurine (methyl(talo)-MeP-R), 9-[6-deoxy-α-L-talofuranosyl]-2-F-adenine, and 9-[α-L-lyxofuranosyl]-2-F-adenine) with higher efficiency than the wild type enzyme. No activity with adenosine or inosine was observed.

CONCLUSION

Nucleoside phosphorylases are an interesting family of enzymes which play a crucial role in nucleoside metabolism. Additionally, they are very interesting targets for the treatment of viral and carcinogenic diseases. Many studies are published that focus on the medical importance of nucleoside phosphorylases and the de-

velopment of inhibitors thereof. Substrate spectra of NPs were studied to understand the mechanism of catalysis during phosphorolytic cleavage of nucleosides. This knowledge is valuable for the application of nucleoside phosphorylases in the production of nucleoside based drugs or precursors for modified oligonucleotides like antisense oligonucleotides or aptamers.

LIST OF ABBREVIATIONS

NP	=	Nucleoside Phosphorylases
PNP	=	Purine Nucleoside Phosphorylase
PyNP	=	Pyrimidine Nucleoside Phosphorylase
E. coli	=	*Escherichia coli*
Pi	=	Inorganic Phosphate
P. falciparum	=	*Plasmodium falciparum*
dGuo	=	2'-Deoxyguanosine
dGTP	=	Deoxyguanosine Triphosphate
dCyK	=	Deoxycytidine Kinase
dCTP	=	2'-Deoxycytidine Triphosphate
dGMP	=	Deoxyguanosine Monophosphate
TP	=	Thymidine Phosphorylase
UP	=	Uridine Phosphorylase
ANP	=	Adenosine Nucleoside Phosphorylase
GP	=	Guanosine Phosphorylase
MTAP	=	S-Methyl-5'-thioadenosine phosphorylase
S. typhimurium	=	*Salmonella typhimurium*
H. sapiens	=	*Homo sapiens*
T. brucei	=	*Trypanosoma brucei*
bPNP	=	Bovine Purine Nucleoside Phosphorylase
K_m	=	Michaelis-Menten Constant
MTR	=	S-methyl-5-thioribose
kDa	=	Kilodalton
M. smegmtis	=	*Mycobacterium smegmatis*
MTA	=	5'-Deoxy-5'-(methylthio)adenosine
ORF	=	Open Reading Frame
Thd	=	Thymidine
B. stearothermophilus	=	*Bacillus stearothermophilus*
K*cat*	=	Turnover Number
L. casei	=	*Lactobacillus casei*
FU	=	5-Fluorouridine
5'-dFU	=	5'-Deoxy-5'-fluorouridine
M. hyorhinis	=	*Mycoplasma hyorhinis*
B. subtilis	=	*Bacillus subtilis*
H. influenza	=	*Haemophilus influenza*
E. aerogenes	=	*Enterobacter aerogenes*
S. oneidensis	=	*Shewanella oneidensis*
G. intestinalis	=	*Giardia intestinalis*
S. mansoni	=	*Schistosoma mansoni*

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

A.W. and K.P. are CEO of the biotech startup BioNukleo GmbH. The authors have no other relevant affiliations or financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No conflict of interest is known to the other authors.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

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Paper II

1

Enzymatic Synthesis of Nucleoside Analogues by Nucleoside Phosphorylases

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1.1 Introduction

1.1.1 Nucleosides and Nucleoside Analogues

Nucleosides primarily consist of a nitrogenous base (nucleobase), which is either a purine base or a pyrimidine base and a five-carbon sugar (pentose). The base and sugar are covalently linked via an *N*-glycosidic bond (Figure 1.1). The pentose sugar moiety of naturally occurring canonical nucleosides is either ribose or deoxy-ribose whereas the nucleobase might be either a purine (adenine, guanine) or a pyrimidine (cytosine, uracil, thymine). These nucleosides are structural subunits of nucleic acids and are involved in several cellular processes including enzyme regulation and metabolism, DNA and RNA synthesis, and cell signaling [1, 2].

Naturally occurring nucleoside analogues (non-canonical nucleosides) are found in almost all types of RNA especially in tRNAs and they are crucial for RNA processing. Non-canonical analogues are nucleosides with different modifications on the pentose and/or the base [3] (Figure 1.1). There are more than 109 known post-transcriptional modifications in the three phylogenetic domains [4]. Pseudouridine is the most ubiquitous analogue and is sometimes considered as the fifth RNA-related nucleoside [5].

Non-natural nucleoside analogues are synthetic molecules that structurally mimic their physiological counterparts and also act as antimetabolites [2]. Nucleoside analogues access cells through specific nucleoside transporters. Within the cells, they are phosphorylated by nucleoside kinases, which leads to increased levels of di- and tri-phosphorylated nucleoside analogues in virus-infected or cancer cells. The first and the second phosphorylation step can also be catalyzed by viral kinases in cells infected by some DNA viruses. Owing to differences in the substrate spectrum of human and viral kinases, virus-specific drugs can be

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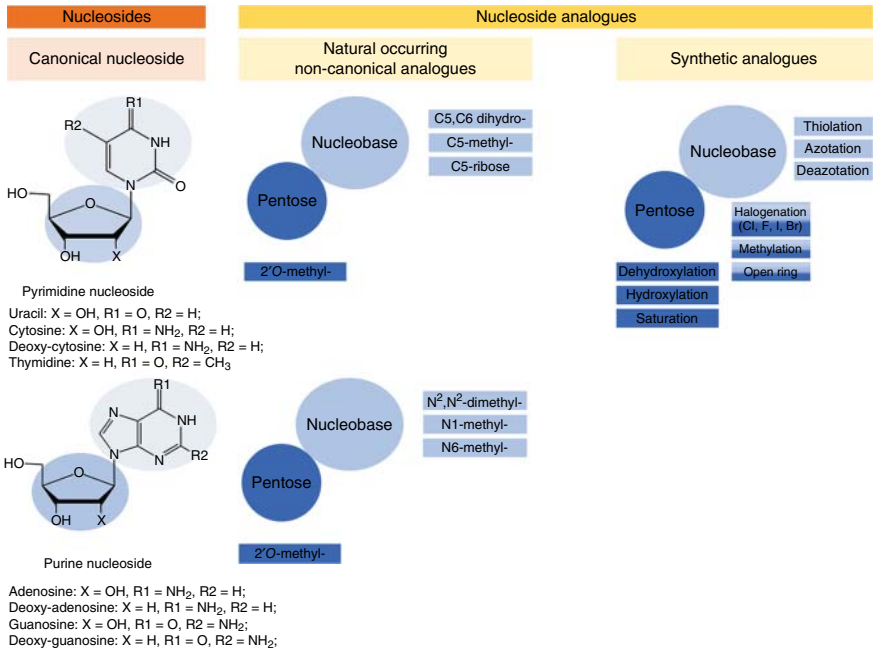

Figure 1.1 Classification of nucleosides and nucleoside analogues. Canonical (unmodified) nucleosides are the building blocks of DNA and RNA. Non-canonical (naturally modified on pentose moiety, base moiety or both) are mainly occurring in RNA. Synthetic nucleosides are used in the treatment of viral and bacterial infections as well as in cancer treatment.

developed. The active forms of nucleoside analogues interfere with intracellular enzymes such as human and viral polymerases, kinases, DNA methyl transferase, ribonucleotide reductase, nucleoside phosphorylases (NPs) or thymidylate synthase [2, 6]. Furthermore, they can be incorporated into newly synthesized DNA and RNA, which may induce termination of the polymerization process, accumulation of mutations in viral progeny, or induction of apoptosis.

For more than 50 years, nucleosides and their analogues have been used as small molecule drugs for the treatment of several viral infections as well as for hematological malignancies and solid tumors. The first FDA approved antiviral nucleoside analogue was idoxuridine, which is used for the treatment of HSV-1 (herpes simplex virus) [7]. In 1969, cytarabine was approved for the treatment of acute myeloid leukemia [2]. Since then, the interest in nucleoside analogues based drugs has tremendously grown. Currently, more than 39 approved nucleoside analogue drugs or drug combinations are approved for the treatment of seven human viral infections, which include HSV, varicella zoster virus (VZV), hepatitis-B virus (HBV), hepatitis-C virus (HCV), human immunodeficiency virus (HIV), respiratory syncytial virus (RSV), and human cytomegalovirus (HCMV) [7]. For treatment of cancer and viral infections, 50% and 20%, respectively, of all approved drugs belong to the class of nucleoside analogues [8]. Additional clinical indications for nucleoside analogues application include chronic hyperuricemia, immune suppression in organ transplant

surgeries, and autoimmune disease as well as chronic obstructive pulmonary disease and asthma [2].

Emerging from the significance of nucleoside analogues, there have been continuous attempts to improve and simplify their synthesis processes. With the world moving toward green chemistry approaches, the enzymatic synthesis of nucleoside analogues offers several advantages over chemical methods, which include higher total yields, a higher regio- and stereo-selectivity, and higher product purity. This allows for more biological and clinical trials [9]. Accordingly, enzymatic strategies are considered as a step forward to a more efficient synthesis of nucleosides and their analogues.

1.1.2 Enzymes Involved in the Enzymatic Synthesis of Nucleoside Analogues

Two main classes are employed in the enzymatic synthesis of nucleosides and their analogues: NPs and *N*-deoxyribosyltransferases (NDTs). In this chapter, the focus is on enzymatic approaches using NPs. NPs are of high interest as biocatalysts because of their wide substrate spectrum and abundance in almost all living organisms.

1.2 Nucleoside Phosphorylases

NPs are enzymes belonging to the transferases family (EC 2.4 and EC 2.7.7). NPs catalyze the reversible phosphorolysis of nucleosides into their respective nucleobase and pentofuranose-1-phosphate (Pentose-1P). NPs have been extensively studied since 1911 when Levene and Medigrecenau [10, 11], and Johnes [10–13] observed the enzymatic hydrolysis of nucleosides. Later, Levene et al. isolated an enzyme (nucleosidase) from cattle's spleen, kidney, and pancreas, which catalyzed the hydrolysis of both inosine and adenosine in phosphate buffer, yielding a base and a ribose moiety [14–16]. In 1947, Kalckar demonstrated that the formed ribose was in fact ribose-1-phosphate and that the isolated enzyme was a purine nucleoside phosphorylase (PNP) [17]. Later, it was proven that *Escherichia coli* cells and cell extracts thereof contained enzymes that could phosphorolyze thymidine to thymine and deoxyribose-1-phosphate [18].

1.2.1 Classification and Substrate Spectra of Nucleoside Phosphorylases

Nucleoside phosphorylases are classified based either on their substrate specificity/affinity (Table 1.1) or on their structure [19]. In 2002, Pugmire and Ealick described a structure-based classification of NPs in two distinct families [19, 20]: NP-I and NP-II (Figure 1.2). They demonstrated that members of an NP-I family share the following characteristics [20]: (i) they have a single domain subunit, (ii) they share a common α/β-subunit fold, (iii) their quaternary structure is either trimeric (mammals/higher organisms) or hexameric (bacteria/lower organisms), (iv) they accept both purine nucleosides (bacterial and mammalian PNPs) and

Table 1.1 Nucleoside phosphorylases with acronyms and EC numbers.

Enzyme name	Acronym	EC number
Thymidine phosphorylase	TP	2.4.2.4
Uridine phosphorylase	UP	2.4.2.3
Pyrimidine nucleoside phosphorylase	PyNP	2.4.2.2
Purine nucleoside phosphorylase	PNP	2.4.2.1
5′-Methylthioadenosine phosphorylase	MTAP	2.4.2.28
Guanosine phosphorylase	GP	2.4.2.15

pyrimidine nucleosides (uridine phosphorylase, UP) as substrates, and (v) their substrate-binding sites are similarly arranged. Nevertheless, they are quite different in their quaternary structures, amino acid sequence, and substrate specificity. Additionally, active sites of the hexameric family members are significantly different from those of the trimeric enzymes of higher eukaryotes, which makes them attractive targets for the specific treatment of bacterial or parasitic infections.

Members of the NP-II family are characterized by (i) having two domain subunits: a small α-domain and a large α/β-domain separated by a large cleft, (ii) a dimeric quaternary structure, (iii) being specific to pyrimidine nucleosides, (iv) accepting both thymidine and uridine as substrates in case of lower organisms, and thymidine in higher organisms' members of the family, (v) having more than 30% sequence similarity, and (vi) undergoing high conformational changes and domain movements during catalysis.

1.2.1.1 Nucleoside Phosphorylase-I Family

Members of the NP-I family are subdivided into PNP, uridine nucleoside phosphorylase and 5′-deoxy-5′-methythioadenoside phosphorylase (MTAP) (Figure 1.2). They catalyze reversible phosphorolysis of the *N*-glycosidic bond of purines, uridine, and 5′-deoxy-5′-methythioadenoside (MTA), respectively. PNPs are the most widely studied group of nucleoside phosphorylases due to their importance as drug targets.

Purine Nucleoside Phosphorylases (PNPs) PNPs are further classified into two main groups, trimeric PNPs and hexameric PNPs (Figure 1.2). Trimeric PNPs also named as low molecular mass PNPs (approx. 31 kDa per subunit) are specific for 6-oxopurines (guanine and hypoxanthine) and their nucleosides. The hexameric PNPs (also known as high molecular mass PNPs) consist of subunits with a size of approximately 26 kDa [20] and have a broader substrate spectrum. They accept both, 6-oxopurines and 6-aminopurines (adenine) and their nucleosides. Initially, trimeric forms were described to be specific for mammals and hexameric forms for bacteria. However, later it was shown that *E. coli*, *Bacillus subtilis*, and *Bacillus stearothermophilus* possess both forms [20–24].

Several reports showed that PNPs accept a wide range of substrate, thereby showing the wide substrate spectrum of these enzymes. Generally, the substrate

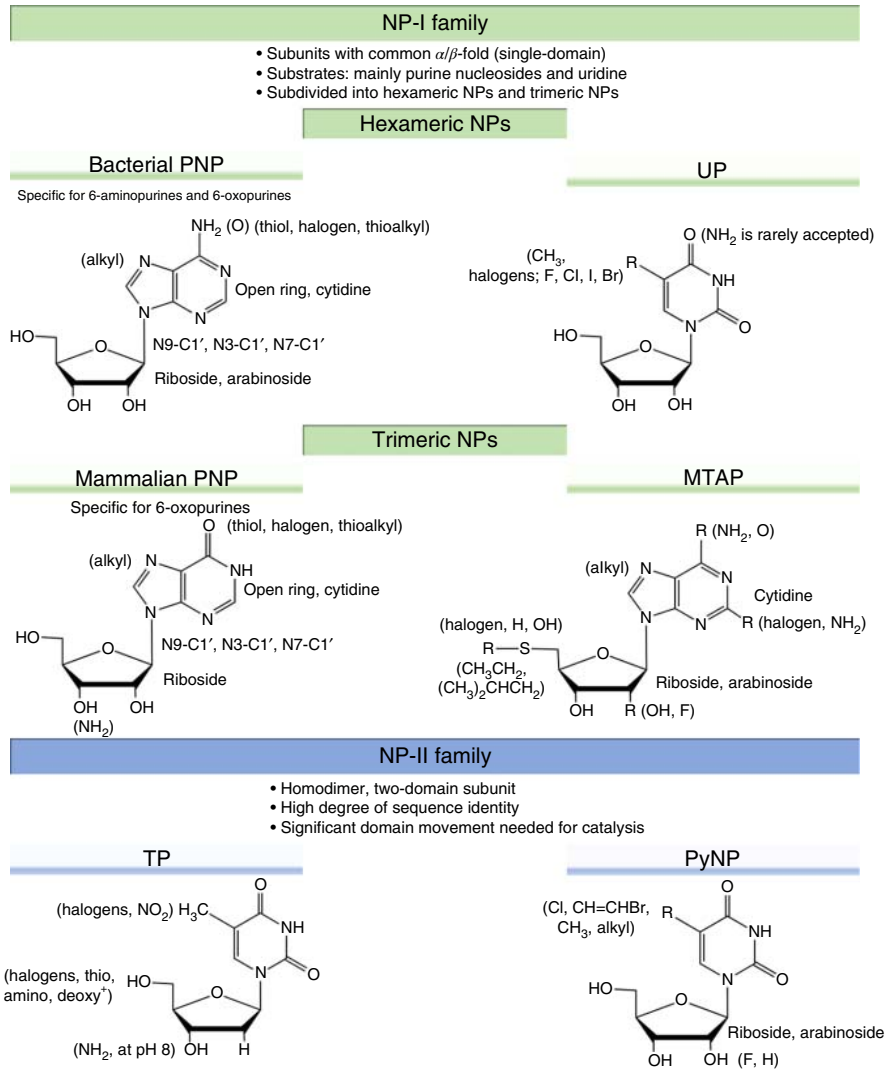

Figure 1.2 Classification of nucleoside phosphorylases (NPs) and their substrate affinities. NPs are classified into two main families: NP-I and NP-II. NP-I family is further subdivided into hexameric and Trimeric NPs. NP-II family is subdivided into two main classes: TP and PyNP. Residues labeled in red are crucial for enzyme–substrate interaction; green and blue labeled substitutions are those accepted by enzymes.

spectrum of hexameric PNPs is larger than that of the trimeric ones [19]. As it will be mentioned in detail later, modifications on the nucleobase and the sugar moiety highly influence the substrate recognition by the enzyme, and both the binding to and releasing from the active sites (Figure 1.2).

PNPs mostly recognize and cleave the N9-C1′ glycosidic bond of purines. However, bonds between N3-C1′ and N7-C1′ are cleaved as well by PNPs from different origins including *E. coli* and bovine and human PNPs [25–27]. Base substitutions with electronegative groups (thiol, halogen, and thioalkyl) at C6

and small alkyl substitution at N7 are well accepted by some members of the PNP family (Figure 1.2). Unexpectedly, open ring purine nucleoside analogues (as ribavirin) bearing structure similarities to inosine were found to be cleaved by some PNPs from various organisms including *E. coli*, *B. subtilis*, *Brevibacterium acetylicum*, calf, and human [27–29].

Maintaining the electronegativity at C1′ is crucial for substrate recognition by PNPs. Hence, ribo- and arabinonucleosides are well accepted by PNPs of *E. coli* and a number of thermostable PNPs [30–32]. The loss of 2′-OH and or 3′-OH reduced the phosphorylation efficiency of human PNP. The replacement of 3′-OH by an amino group improved the activity compared to 3′-deoxy-nucleosides due to the retained electronegativity [33]. Nonetheless, there are some exceptions that also accept 3′-deoxynucleosides, such as PNPs isolated from *B. acetylicum, Plasmodium carotovorum*, and *Geobacillus thermoglucosidasius* [30, 34, 35].

An interesting feature of PNPs is their ability to accept pyrimidine nucleosides as substrate. Thus, they may be interesting catalysts for the synthesis of cytidine and deoxycytidine that are not utilized by many pyrimidine nucleoside phosphorylases (PyNPs) [30].

5′-Deoxy-5′-methylthioadenoside Phosphorylase (MTAP) An interesting member of the NP-I family is MTAP, which was primarily characterized in rats to work on MTA. MTAPs show a wide substrate spectrum. In addition to MTA some MTAPs as those isolated from *Pyrococcus furiosus* and *Sulfolobus solfataricus* accept 6-oxopurine nucleosides (guanosine and inosine). For these enzymes, higher sequence similarities to PNPs compared to the known MTAPs were shown [36–38]. Substitutions with halogen or amino groups at the C2 position of the purine ring are very well tolerated [39–43] (Figure 1.2). MTAP isolated from *Aeropyrum pernix* has some unique characteristics: on one hand, it is active toward some pyrimidines including cytidine and deoxycytidine, and on the other hand, unlike most reported MTAPs, it phosphorolyzes 2′-fluoro-modified arabinoside [30], which is an interesting activity when considering the enzymatic synthesis of nucleoside analogues that can be used as drugs.

Uridine Nucleoside Phosphorylase Different prokaryotic and eukaryotic organisms including *E. coli*, *Lactobacillus casei*, *Enterobacter aerogenes*, *Salmonella typhimurium*, *Saccharomyces cerevisiae*, *Schistosoma mansoni*, *Mus musculus*, *Rattus norvegicus*, and *Homo sapiens* were reported to possess a UP [19].

Although UPs of different origins vary in their substrate spectrum, they show some common features: none of the reported UPs catalyze cytidine phosphorolysis except the UP from *S. cerevisiae*. However, all accept 2′-deoxy nucleosides, although with much lower affinity compared to uridine. Nucleosides that are halogenated or methylated at the C5 position are generally well accepted and tolerated [19] (Figure 1.2).

1.2.1.2 Nucleoside Phosphorylase-II Family

The NP-II family comprises phosphorylases that catalyze the reversible cleavage of the glycosidic bond of thymidine (thymidine phosphorylase [TP]) and pyrimidines (PyNPs) in the presence of phosphate. Although UP catalyzes a similar reaction, it has been classified under the NP-I family due to its distinct structural

features. As mentioned earlier, both TP and PyNP share two-domain subunits with a high percentage (≈40%) of sequence and structural similarities [19, 20, 44]. However, one major catalytic difference between TP and PyNP exists: TP has a high specificity toward C2′ deoxy nucleosides compared to PyNPs [45]. Hence, PyNPs possess a wider substrate spectrum than TP.

Thymidine Phosphorylase Structure–function relationship studies have provided some insight into the acceptance of base and sugar modifications by TP. Regarding the sugar moiety, the absence of a hydroxyl group (—OH) at the C2′ position of the nucleoside is crucial for TP binding. Both, *E. coli* and human TP, do not accept uridine as a substrate. In fact, uridine and 2′-methyl uridine were shown to inhibit the catalytic action of *E. coli* TP [46–49]. On the other hand, the presence of the 3′-OH group of the nucleoside is crucial for interactions between the substrate and the enzyme. This is reinforced by the fact that 3′-amino-3′-deoxythymidine was phosphorolyzed by TP only at pH 8 and not at pH 6.5, because at pH 6.5 both the 3′-amino group of the nucleoside and the amino group of the enzyme are positively charged [48]. The 5′-position of the nucleoside does not strongly influence the catalytic reaction. Various 5′ modifications were tolerated by *E. coli* TP including halogens, thio, amino, and deoxy substitutions [48–50] (Figure 1.2). Modifications in the C5 position of the base are generally well tolerated by TP, while substitutions at C6 have a big impact on the catalytic potential. Substitutions that increase the aromaticity of the nucleobase hinder the base to leave the catalytic pocket of the enzyme [49].

Pyrimidine Nucleoside Phosphorylases PyNPs have been isolated and characterized from several organisms including *B. stearothermophilus*, *B. subtilis*, *G. thermoglucosidasius*, *Thermus thermophilus*, *Hameophilus influenza*, and *Mycoplasma hyorhinis* [45, 51–55]. PyNPs are of increasing interest due to their broader substrate affinity and tolerability compared to those of TP or UP. Thus, a wider application of these enzymes to produce synthetic nucleoside analogues is envisioned.

PyNPs well tolerate modification at the C5 position of the base. PyNPs from various organisms are able to catalyze the glyosidic bond cleavage of 2′-deoxy-5-halogenated nucleoside analogues (Figure 1.2). Additionally, a bromovinyl (CH=CHBr) group at C5 was tolerated by *E. coli* TP and *B. subtilis* PyNP. Several other modifications at C5 including alkyl groups are also well accepted by PyNPs from various sources [45, 56–58] (Figure 1.2). Interestingly, compounds with modifications at the C2′ position of the nucleoside are also accepted as substrates by PyNPs. Arabinosides as well as 2′-deoxy-2′-fluororibosides and 2′-deoxy-2′-fluoroarabinosides are substrates for some PyNPs, predominantly for those isolated from thermophilic organisms such as *T. thermophilus* [54] (Figure 1.2).

1.2.2 Limitations in the Current Classification

Although the classification of PNPs into two main categories as proposed by Pugmire and Ealick [20] is applicable for most of the known PNPs, there are still some exceptions that do not fit into this categories.

The low molecular mass inosine-guanosine phosphorylase extracted from the vegetative state and the spores of *Bacillus cereus* was shown to either have a tetrameric quaternary structure or to equilibrate between the dimeric and the tetrameric form [24]. *B. stearothermophilus* (JTS-859 strain) as mentioned earlier contains both forms of PNPs: a low molecular mass and a high molecular mass PNP. It was reported that the low molecular mass enzyme forms a dimer rather than a trimer whereas the high molecular mass form is a tetramer rather than a hexamer [24].

E. coli hexameric PNP-II (product of *xapA* gene) has an amino acid sequence, promotor structure, and substrate specificity similar to the low molecular mass PNPs. As observed for low molecular mass PNPs, *E. coli* PNP-II does not accept adenosine or adenosine derivatives as substrates [19, 24, 59].

1.2.3 Reaction Mechanism

Nucleoside phosphorylases reversibly cleave *N*-glycosidic bonds of β-nucleosides with configurational inversion producing α-pentofuranose-1-phosphate and a free nucleobase. The catalytic mechanism of the phosphorolytic cleavage has been reported for several members of the NP-I family. Most of the reported NPs were shown to have a similar catalytic mechanism (Figure 1.3). NP-II members are expected to follow the same pattern.

The mechanism described for human PNP illustrated that the general reaction follows an S_N1 mechanism [20, 60]. The nucleoside binds to the enzyme in a high energy conformation producing a steric strain on the glycosidic bond, thus favoring bond cleavage. An oxocarbenium ion is formed during the transition state by the flow of an electron from O4′ to the purine base rendering the base with a negative charge. The formed oxocarbenium ion is stabilized by phosphate ions. Additionally, negative charges on the base moiety are stabilized by interactions of the enzymes active site to the N-atom of the base, thus facilitating glycosidic bond cleavage [19, 20, 60, 61].

The mechanism of phosphorolytic cleavage became clear by the availability of crystal structures; however, the exact mechanism of nucleoside synthesis is still not clear and difficult to elaborate due to the difficulty of crystallizing pentose-1-phospahte alone with PNP. Yet, it is suggested that the presence of the

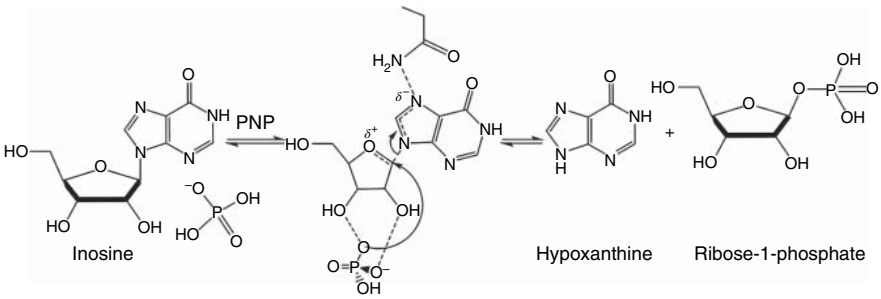

Figure 1.3 Catalytic mechanism described for PNP.

base favors the binding of pentose-1-phosphate to the catalytic site. Otherwise, the latter would cause steric hindrance of base binding [24, 60, 61].

PNP reactions follow non-Michaelis Menten kinetics. However, an exact kinetic mechanistic model was not agreed upon so far [24, 60]. Crystal structure data suggest the formation of ternary complexes of enzyme/nucleoside/phosphate and enzyme/base/pentose-1-phosphate. Whether these complexes are formed in a sequentially defined order or by random binding was not yet confirmed. Nevertheless, while there is no evidence for the occurrence of a ping–pong mechanism, a sequential order of the reaction is assumed by most authors [24].

1.2.4 Domain Structure and Active Site Residues of Nucleoside Phosphorylases

1.2.4.1 NP-I Family Members

As mentioned earlier, members of the NP-I family share a single domain structure that is highly conserved [62]. Nevertheless, amino acid residues in the active sites are not necessarily conserved among different members of the NP-I family.

One interesting difference between the four subgroups (trimeric PNP, MTAP, hexameric PNP, and UP) of the NP-I family is the observed sequence motifs. Although sequence motifs do not necessarily reflect functional differences, they help to show differences and similarities between subgroups of enzymes. There are nine identified sequence motifs in NP-I family (Figure 1.4). These structural regions are denoted with letters A–I. Their exact position in the primary amino acid sequence was reviewed by Pugmire and Ealick in 2002 [20]. Numbering codes were assigned based on structure similarities, for example B_1, and B_2 are similar regarding their quaternary structure's position; however, their amino acid sequence differs significantly. Not all members of the subclasses have the same number of motifs, but all of them encompass motif A_1. Noteworthy, MTAP (E_3, H_2) and UP (H_3, I_1) have two unique motifs that are involved in the active site and inter-subunit interactions [20].

From nine motifs identified in the members of the NP-I family, seven are involved in the formation of the active site and six are involved in subunit interaction. The observed structural similarities between trimeric PNP and MTAP as well as between hexameric PNP and UP are also reflected in the motif structure (Figure 1.4). Amino acid residues involved in binding of phosphate,

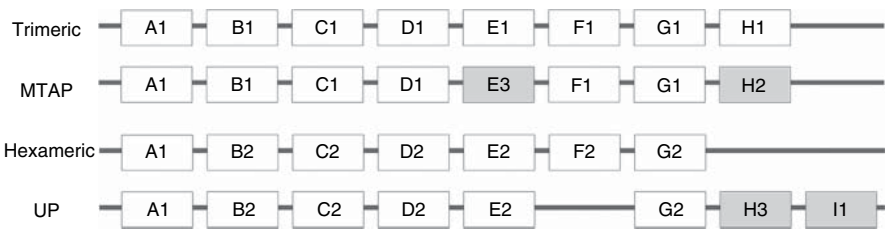

Figure 1.4 Structural motifs described for members of the NP-I family. Highlighted in grey are domains that are unique for either MTAP or UP. Trimeric- trimeric PNP, Hexameric-hexameric PNP.

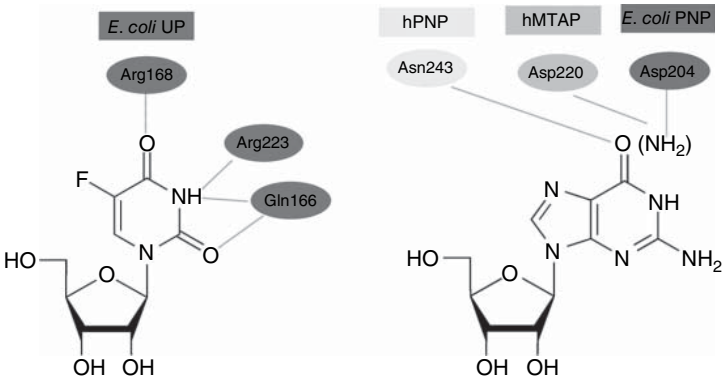

Figure 1.5 Schematic presentation of amino acid residues that define substrate specificity of different nucleoside phosphorylases.

nucleobase, and pentose sugar differ between hexameric and trimeric members of the NP-I family [19, 20]. Detailed information on amino acids involved in substrate binding has been recently reviewed [19].

Functionally, the four subclasses of the NP-I family showed different specificities toward substrates. Trimeric PNPs are specific to 6-oxopurines and MTAPs are described to be specific for 5′deoxy-5′-methylthioadenosinde, whereas hexameric PNPs accept both 6-oxopurines and 6-aminopurines (adenosine). UP is strictly specific to pyrimidine derivatives with no distinct specificity toward the C2′ position. These functional divergences are also reflected by differences in the amino acid residues found in the active sites. hMTAP (Asp220) and *E. coli* PNP (Asp204) form hydrogen bonds with the 6-amino group of the purine base. The replacement of an aspartic acid residue (Asp) with asparagine (Asn) as in the case of human and bovine PNP (Asn243) restricted the activity of human and bovine PNP toward 6-oxopurines (Figure 1.5). As another example, *E. coli* and human UP are conserved in residues Gln166, Arg168, and Arg223, which are described to be responsible for the specificity of UP [19].

1.2.4.2 NP-II Family Members

TP and PyNP are the main two enzyme groups within the NP-II family. Enzymes from both classes share a high degree of sequence similarity (33–67%). Furthermore, they share two main domain subunits, a large α/β domain and a smaller α domain, that are separated from each other by a large cleft. The amino acid residues of the α domain are more conserved. Detailed information on amino acids involved in substrate binding has been recently summarized [19].

Active site residues are highly conserved among TP and PyNP with few exceptions that might be responsible for differences in substrate acceptance. While TP is highly specific for 2′-deoxy nucleosides, PyNP catalyzes the cleavage of a number of 2′-modified nucleosides. A possible explanation that is revealed by structural studies is the substitution of lysine (Lys108) of PyNP with methionine in both *E. coli* and human TP [19]. This replacement alters the

hydrogen bonding organization between the 2′-hydroxyl group of the nucleoside and the oxygen atom of the phosphate. An alternative explanation is a different positioning of 2′-modified nucleosides in the active site of TP compared to 2′-deoxy nucleosides [63].

1.3 Enzymatic Approaches to Produce Nucleoside Analogues Using Nucleoside Phosphorylases

Despite the huge advances achieved in the chemical synthesis of nucleosides and their analogues, the existing drawbacks led to a continuous interest in developing enzymatic approaches. In 1954, M. Friedkin and D. Roberts [64, 65] reported the first attempts to enzymatically synthesize pyrimidine nucleoside analogues using TP isolated from horse liver. Since then several studies have been conducted on enzymes to be used in the synthesis of nucleoside analogues.

As mentioned earlier, nucleoside phosphorylases catalyze the reversible cleavage of *N*-glycosidic bond of nucleosides. Thus, the condensation of a nucleobase and pentose-1-phosphate can be used for the synthesis of nucleosides or their analogues. Different synthesis strategies were developed based on this mechanism of action.

1.3.1 One-pot Two-Step Transglycosylation Reaction

Enzymatic transglycosylation has been extensively studied [9, 66, 67]. It is the transfer of a pentofuranose moiety from a chemically synthesized or a naturally occurring nucleoside to a base of interest. Pentose-1P is formed as an intermediate in the reaction (Figure 1.6a). Sugar donor is a pyrimidine nucleoside (cleavage reaction) and sugar acceptor is a purine base (synthesis reaction) or vice versa. This reaction takes place in the presence of phosphate. As noted earlier, reactions catalyzed by NPs are reversible, thus both cleavage and synthesis take place simultaneously and the reaction applies to equilibrium rules and dynamics that might hinder high product yields.

In case of producing a purine nucleoside via transglycosylation, it is generally assumed that the reaction equilibrium is on the side of the starting nucleoside and that the reaction of the PyNP is faster compared to the PNP due to the modifications in the purine base. In other words, in a transglycosylation reaction the formed intermediate (Pentose-1P) may easily undergo the backward reaction to the starting nucleoside than to the final product. High phosphate concentration enhances nucleoside cleavage [19], but hinders the synthesis of the nucleoside of interest. Therefore, adjusting the phosphate concentration in transglycosylation reactions is a critical factor. Although the transglycosylation reaction was proven to be an efficient way for the synthesis of mainly riboside and deoxy-ribosides nucleoside analogues, the synthesis of some arabinosides and fluoro-modified-ribosides/arabinosides is more challenging [66–68]. Hence, further enzymatic approaches were investigated.

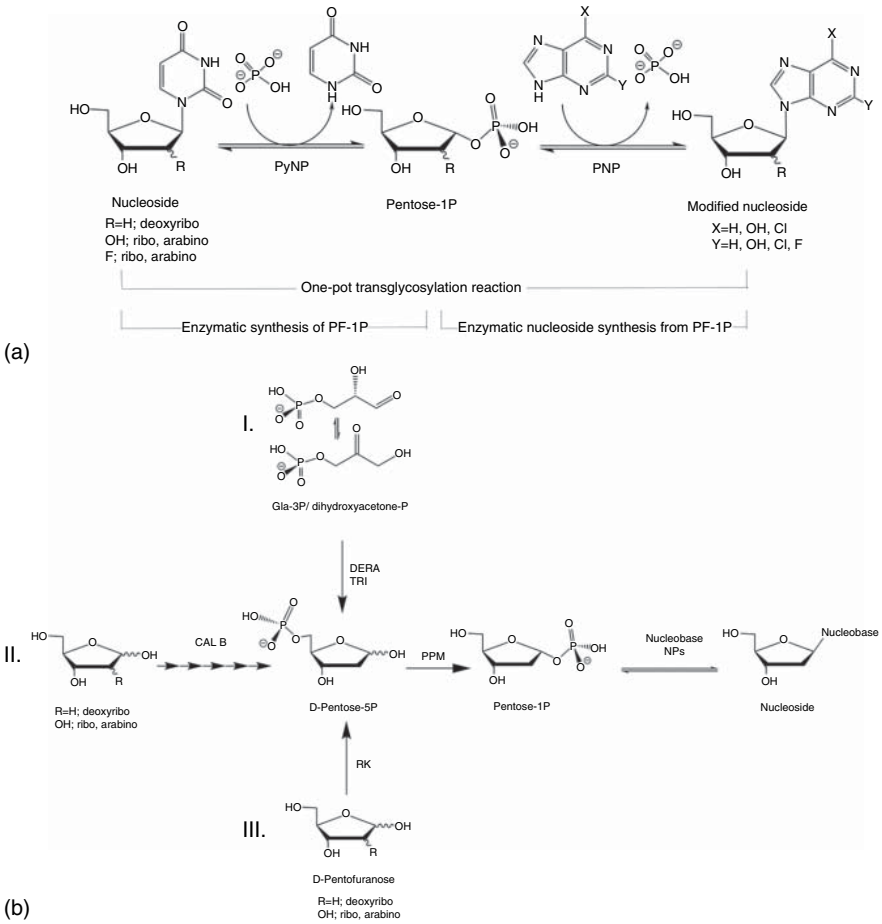

Figure 1.6 Approaches for the synthesis of nucleosides and nucleoside analogues. (a) One-pot transglycosylation reaction. (b(I)) Enzymatic synthesis starting from glyceraldehyde-3-phosphate. (b(II)) Chemo-enzymatic synthesis starting from deoxyribose, ribose, or arabinose. (b(III)) Enzymatic synthesis starting from D-pentoses (deoxyribose, ribose, or arabinose). PF-1P: Pentose-1P

1.3.2 Pentofuranose-1-phosphate as Universal Glycosylating Substrate for Nucleoside Phosphorylase (NP)

1.3.2.1 Nucleoside Synthesis from Chemically Synthesized Pentose-1P

The chemical synthesis of glycosyl phosphates has a long history and has been thoroughly studied and reported [69–73]. Although details of the chemical synthesis will not be discussed in this chapter, it is worth mentioning that the most efficient and currently used method for the synthesis of pyranose-1-phosphate is that proposed by MacDonald in 1962 [70]. Later, this method was also used for the production of α-D-pentofuranose-1-phosphate (Pentose-1P) [68, 74]. Tediousness of the chemical synthesis of Pentose-1P as well as low yields, and the difficulty of purifying the wanted α-anomeric isoform have so far restricted the use of Pentose-1P as a substrate for NPs; generally, the availability of Pentose-1P

is a serious bottleneck for the synthesis of modified nucleoside analogues. Nevertheless, the chemical synthesis of Pentose-1P followed by the enzymatic condensation with a nucleobase using NPs is still considered as a valuable chemo-enzymatic route for nucleoside synthesis.

1.3.2.2 Nucleosides Synthesis from D-Glyceraldehyde-3-phosphate

2′-deoxyribonucleoside synthesis from glyceraldehyde-3-phosphate was first reported as a potential enzymatic approach for nucleoside synthesis in the beginning of this century [75, 76]. The authors described the transformation of D-glyceraldehyde-3-phosphate or dihydroxyacetone monophosphate to nucleoside analogues through three main consecutive steps. The transformation was performed as a one-pot reaction without purification of the intermediates. In the first step, a condensation of D-glyceraldehyde-3-phosphate or dihydroxyacetone-phosphate with acetaldehyde is catalyzed by D-2-deoxyribose-5-phosphate aldolase (DERA) and triose phosphate isomerase (TRI). The observed product is D-ribofuranose-5-phosphate. In the second step the stereospecific transformation of D-ribofuranose-5-phosphate into α-D-ribofuranose-1-phosphate (Rib-1P) by phosphopentomutase (PPM) is performed. Finally, the enzymatic condensation of Rib-1P with a heterocyclic nucleobase is catalyzed by NPs (Figure 1.6b(I)).

1.3.2.3 Nucleoside Synthesis from D-Pentose

In 2008 [77], the chemo-enzymatic synthesis of β-D-ribofuranosyl, β-D-deoxyribofuranosyl and β-D-arabinofuranosyl nucleosides from 5-phosphate of D-ribose, D-arabinose and 2-deoxy-D-ribose was reported. The pentose-5-phosphate was chemo-enzymatically prepared through different selective steps of acetylation and deacetylation in the presence of lipase B from *Candida antartica*. The formed pentofuranose-5-phosphate (Pentose-5P) was transformed into Pentose-1P using PPM. Pentose-1P was then used by nucleoside phosphorylase for the enzymatic condensation reaction with nucleobase yielding nucleosides and their analogues (Figure 1.6b(II)). Inosine, 6-mercaptopurine riboside and ribavirin were successfully synthesized by the above method [77].

More recently [78], a different and more efficient strategy was proposed based on transforming D-pentose to Pentose-5P using ribokinase (RK). Pentose-5P was later converted to Pentose-1P by PPM. Pentose-1P was used as a substrate for NP to synthesize nucleoside analogues in the presence of heterocyclic nucleobases like uracil, thymine, and hypoxanthine (Figure 1.6b(III)).

1.3.2.4 Nucleoside Synthesis from Enzymatically Produced Pentose-1P

We have recently proposed a two-pot enzymatic approach to produce nucleoside analogues as an alternative to the one-pot transglycosylation [79]. This approach is based on the ability of nucleoside phosphorylase to cleave the glycosidic bond of nucleoside yielding Pentose-1P, which is then purified as barium salt. It is applied in a separate step as a substrate for the synthesis of modified nucleosides using NPs (Figure 1.6a). This approach allows for easier optimization of each of the reactions, which leads to increased yields of the desired nucleoside analogue.

1.4 Approaches to Produce Nucleoside Analogues

Since nucleoside phosphorylases were first described by Kalckar [80], many research projects were conducted to test whether definite nucleoside analogues are used as substrates for nucleoside phosphorylases. Different forms of the enzyme (e.g. free or immobilized) were applied for the production of known drugs. One common objective of various studies was the optimization of already existing methods for large-scale industrial production with the aim to overcome some drawbacks and limitations such as (i) product inhibition, (ii) poor solubility of substrates, and/or (iii) consumption of large amounts of enzymes that might be hard to express. Such scale-up activities focused mainly on compounds with biological activity or of commercial value. In the coming sections, we try to summarize these approaches.

1.4.1 Whole Cell Catalysis

The application of whole cells can be regarded as an easy, time-saving, and economic option to produce nucleoside analogues. Expensive and time-consuming steps like cells lysis, enzyme extraction, and purification are avoided. Additionally, the retrieval and reuse of cells via centrifugation or ultrafiltration is possible. Best yields and conversion rates were achieved by cells harvested at the stationary phase where the salvage pathway for nucleoside production is strongly activated, which correlate with large amounts of nucleoside phosphorylases [81]. Ribavirin, dideoxyinosine, adenine arabinoside, and 2′-amino-2′-deoxyadenosine were synthesized using whole cells and different pentofuranosyl donors [81–86]. One requirement for the successful application of whole cell biocatalysts is that both the sugar donor and acceptor are well soluble. Using whole cell catalysts, side reactions that are catalyzed by other enzymes acting on the same class of substrates have to be considered, for example deaminases or lipases [87, 88].

Complex media were proven to be better than defined media for producing NPs in *Enterobacter* species [89]. Many nucleobases, nucleosides, Rib-1P, and nucleotide monophosphates as inducing agents for potentiating the expression of NPs were tested. Conversion by some eukaryotic cancer cell lines was also reported [90, 91].

Another biologically important molecule is benzimidazole, whose nucleoside exhibits antitumor, antifungal, antiparasitic, analgesics, antiviral, and antihistamine activities, and is being used in the treatment of cardiovascular diseases and in therapies related to neurology, endocrinology, and ophthalmology. Bentancor and coworkers [92] published a screening study for producing benzimidazole riboside and 2′-deoxyriboside using whole cells catalysts. Uridine was applied as a ribose donor and thymidine as the 2′-deoxyribose donor, respectively. Optimal temperature for the reaction was 60 °C for ribosides and 45 °C for deoxyribosides, respectively, suggesting that the higher temperature inhibits the action of thymidine phosphorylase [93, 94]. However, recent data show that α-deoxyribose-1-phosphate is unstable and degrades at higher temperature [95].

Mainly, free cells were applied to produce nucleoside analogues, but immobilized cells were also used. Immobilized whole cells can be either alive or dead

depending on (i) whether the enzyme is intra- or extracellular, (ii) the molecular weight and the size of the enzyme, (iii) substrate and product, and (iv) the cell's permeability to them. As an example, *Aeromonas hydrophila* cells were entrapped in agarose beads and afforded the same kinetics and yields as those by the free cells. The entrapped cells, however, were used three times before any decrease in activity was detected [81]. A mutant strain of *E. coli* was cross-linked to glutaraldehyde and successfully used to produce ribavirin and the anticancer drug cladribine [96].

The widespread application of whole cell extracts is restricted because broadly used bacterial whole cells that are used as biocatalysts for the production of modified nucleosides co-express a number of enzymes that can (i) consume substrates; (ii) catalyze undesired transformations of substrates or formed nucleosides; and (iii) secrete desired products in the medium, which makes purification challenging [97].

1.4.2 Crude Enzyme Extract

Compared to purified enzyme, the application of crude cell extract is a less laborious and a more economical approach. It is suitable to carry out preliminary screening assays to determine the catalytic potential of enzymes especially in the cases of overexpression where the target enzyme is available in high concentration. A drawback of crude enzyme extracts is a shorter half-life compared to that of purified enzymes [53, 56, 82, 98–100].

The application of crude enzyme extracts is principally advantageous in case of thermophilic enzymes. If the enzymes are heterologously expressed in a recombinant host, proteolysis and interference from the host proteins can be inhibited through an additional heat shock step [101]. This was implemented in the preliminary tests conducted for the characterization of nucleoside phosphorylases of *T. thermophilus*. The extract was heated at 80 °C for one hour and then centrifuged. Afterward, the supernatant was successfully tested for its phosphorolysis activity with a variety of nucleosides [31].

As compared to whole cell catalysis, cell lysate or enzyme extracts result in a more reproducible and robust reaction as the Pentose-1P intermediate is not consumed as an energy source, which might happen under certain conditions using bacterial cells. It can finally end up with the reaction being totally reserved in the backward direction [102].

1.4.3 Application of Purified Enzymes

The use of purified enzyme preparations has the advantage that side-reactions are avoided. To date, NPs from different mesophiles, like *E. coli* [103–105] or *B. subtilis* [106], were applied in the synthesis of pharmacologically active compounds [103, 104].

Thermostable NPs from thermophilic or hyperthermophilic microorganisms catalyze enzymatic reactions with the same high regio- and stereo-selectivity compared to mesophilic NPs, but have several advantages for large-scale applications: (i) thermophilic enzymes can be easily purified with high protein recovery

after heterologous production and costs are low as a heat treatment usually is sufficient as a purification step [31]; (ii) a high pH tolerance is observed, which reduces the need to finely regulate the pH of the reaction; (iii) it was shown that PNPs of *T. thermophilus* show a remarkable resistance to aggressive organic solvents such as dimethylformamide or dimethyl sulfoxide, which are required in the synthesis of compounds with low water solubility; (iv) using thermophilic catalysts, reactions can be performed at elevated temperatures, which are frequently required to reduce the viscosity of the medium or to increase the solubility and concentration of some substrates [107]; (v) it was observed that an increased thermal stability correlates with a broader substrate spectrum [30, 31, 54, 108].

For industrial applications of purified enzymes, it is highly desirable to use either immobilized catalysts or continuous reactors to be able to recover the enzyme from the reaction mixture. This simplifies downstream processing and facilitates biocatalyst recycling. As immobilization might also increase the stability of nucleoside phosphorylases, diverse methods have been exploited [57, 101, 109, 110]. Approaches for both immobilized enzymes and continuous reactors are described in more detail in the following paragraphs.

1.4.3.1 Immobilized Enzymes

Biocatalyst immobilization denotes binding an enzyme to an inert support (natural, synthetic, or inorganic in nature) where it maintains its activity without blocking or disrupting the active sites. The ideal support has to be stable, endures sheering force of the reaction, can be regenerated, and has no effect on the type of reaction [111, 112]. Usually, enzyme immobilization has a positive effect regarding thermal stability, pH resistance, and longevity (e.g. storage time and number of operational cycles).

Immobilization of purified enzymes is an important tool for large-scale commercial application of biocatalysts. Considering the cost load of the enzyme in the reaction, one economically favorable feature is the ability to reuse the enzyme as long as it retains its activity. The general immobilization principles for enzymes are (i) adsorption to the support's surface, (ii) cross-linking (enzyme molecules bind to each other) in the presence of spacers (e.g. polyethylene glycol) to reduce steric hindrance, (iii) covalent binding to a support, (iv) entrapment within a polymer, (v) encapsulation within a surrounding matrix, and (vi) affinity binding [113, 114] (Figure 1.7). The most described immobilization method for NPs is entrapment in different matrices like agar, agarose, and alginates [81, 86, 116].

Immobilization of pure enzyme to produce nucleoside analogues in a transglycosylation reaction might be a complicated process as it involves the co-binding of a PNP and PyNP. Multimeric enzymes are known to be quite difficult to immobilize as it is necessary to consider the correct assembly and multisubunit attachment, least desorption, and minimal deactivation [117–119]. However, feasibility was proven as immobilized PyNPs from *B. subtilis* and TP from *E. coli* were applied for the synthesis of 5-halogenated-pyrimidine-2′-deoxyribonucleosides by transglycosylation in fully aqueous medium [57]. For both enzymes, immobilization was shown to strongly enhance the stability upon incubation in acetonitrile. Both PyNP and TP could be re-used for at least five times with no loss of productivity. In another approach, immobilized UP of *Clostridium perfringens*

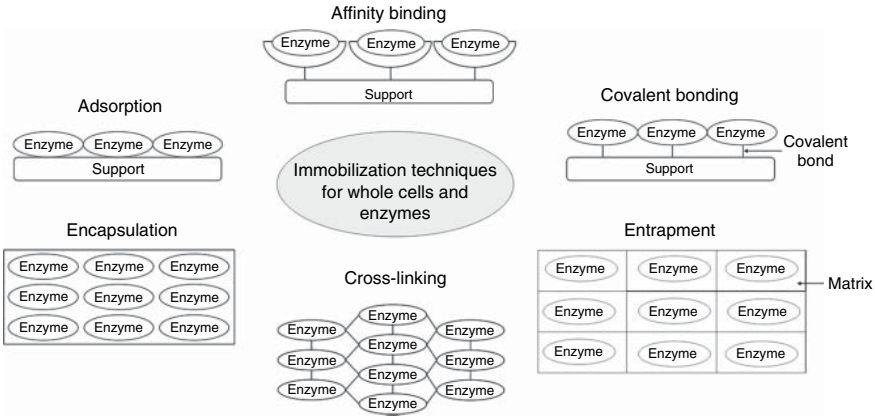

Figure 1.7 Immobilization techniques applied for whole cells and purified enzymes. Source: Adapted from Sirisha et al. 2016 [111] and Zhao et al. 2015 [115].

and PNP of *A. hydrophila* were used as biocatalysts for the production of vidarabine in a transglycosylation reaction [120].

Different matrices and techniques were compared by Rocchietti and coworkers for UP and PNP from *B. subtilis* [117]. While covalent bonding distorted the protein structure and resulted in decreased activity and stability, UP was best immobilized via ionic bonding to Sepabeads and post-immobilization treatment with polyaldehyde was necessary to cross-link the support and protein. PNP was best immobilized on glycoxyl-agarose using the surfactant Triton X-100 that prevents agglomeration of the protein and hence, allows diffusion into the support. The combined use of these two enzymes allowed the reaction to take place at a higher optimal temperature (45 °C) and pH (pH = 10) compared to the free enzymes. Using these conditions, guanine conversion proceeded, which is usually limited due to low solubility.

1.4.3.2 Enzyme Reactors

Few trials were recorded in literature using different NPs in enzyme reactors for the production of nucleoside analogues. Preparative scale nucleoside synthesis was conducted in column reactors by some research groups. Hori and colleagues operated their experiment for 17 days at 60 °C to produce 5-methyl uridine from a thymine and inosine mixture [101]. Immobilized crude enzyme extract from the thermophilic bacterium *B. stearothermophilus* was bound to an ion-exchange column. The reaction mixture was fed in a pre-optimized rate with the aim to overcome the equilibrium constant and to guarantee that the hypoxanthine solubility limit is not exceeded [101].

In two other approaches an enzyme column reactor was connected to a HPLC [121, 122]. The purified PNP from *A. hydrophila* was immobilized on silica particles of definite particle size. The system could run over the analytical column or bypass it via a six-port switching valve and it was used to test the substrate affinity of the enzyme and compare the reaction kinetics of free and immobilized enzyme [121]. Later it was shown that the system was stable for 10 months [122].

The described system was used for a faster routine activity screening. Based on a "design of experiment" optimized study, they produced different modified nucleosides including a number of halogenated analogues.

1.5 Upscaling Approaches for the Production of Nucleoside Analogues

Despite the high cost of soluble enzymes that limited the wide-spread application of nucleoside phosphorylases in industrial processes, some successful upscaling approaches were reported. These are almost all dedicated to the synthesis of molecules with known biological activity. For example, many research studies were conducted to execute large-scale industrial enzymatic or chemo-enzymatic processes to produce the antiviral drug ribavirin from 1,2,4-triazole-3-carboxamide (TCA) using purified enzymes or bacterial cell cultures.

Chen et al. performed a fed-batch fermentation process for a recombinant *Bacillus* strain using glucose as the sole carbon source in an attempt to produce ribavirin in a preparative scale [123]. The fermentation was carried out in 3 l volume and TCA was added in the middle of the log phase. Tween 80 was added to increase the permeability of the cell membrane for the enzyme and thereby increase TCA conversion to ribavirin.

A method carried out in 7.5 l volume was described using a genetically engineered *Bacillus* strain that secretes PNP extracellularly. TCA was used as the substrate for the production of ribavirin [124]. In another upscaling experiment, the chemo-enzymatic synthesis of ribavirin in a 20 l reaction volume was described [125]. A conversion rate of 97% was observed in the enzymatic reaction using TCA and guanosine as substrates and a heterologously produced *E. coli* PNP as biocatalyst.

1.6 Production of Pharmaceutically Active Compounds by Nucleoside Phosphorylases

The chemo-enzymatic synthesis of nucleosides with biological relevance was well described in a review by Mikhailopulo in 2007 [67]. The application of nucleoside phosphorylases in the production of drugs like cladribine or mizoribine was proven (Table 1.2). Both are useful drugs for the treatment of cancer. In 2003, the enzymatic synthesis of clofarabine, fludarabine, or vidarabine with *E. coli* RK, PPM, and PNP [136] was shown.

The phosphorolytic cleavage of a number of other approved drugs like ribavirin or didanosin was also shown [34] (Table 1.2). Using PNP of *B. acetylicum* the relative activity was 22% for both compounds compared to inosine. Recently, the phosphorolytic cleavage of nucleoside analogue drugs listed in the European Pharmacopoeia by thermophilic PyNPs, PNPs, and MTAPs was tested [19]. Both cytarabine and zidovudine were accepted as substrates by PyNP, while

Table 1.2 Drugs synthesized by different nucleoside phosphorylases.

Purine-based drugs			Pyrimidine-based drugs		
Purine nucleoside phosphorylase (PNP)		*S*-methylthio-adenine phosphorylase (MTAP)	Pyrimidine phosphorylase (PyNP)	Thymidine phosphorylase (TP)	Uridine phosphorylase (UP)
6-Methyl purine [32, 39] Cordycepin [34, 108]			Floxuridine [57, 126, 127]		
Fludarabine [32]	Clofarabine [128]				
Vidarabine [31, 120]	6-Phenyl purine riboside [32]		Idoxuridine [129, 130]		
Nelarabine [131]	Ribavirin [28, 29]	Cladribine [41]		Tegafur [132]	
Didanosine [34, 35]	Purinethol riboside [116]			Stavudine [133]	
Thioguanine riboside [134]	5-Aza-7-deazaguanine [9, 135]				

gemcitabine and lamivudine were not cleaved. PNPs and MTAPs of thermophilic microorganisms phosphorolyzed ribavirin and didanosin. Nucleoside analogues with open sugar ring structures like aciclovir, ganciclovir, and tenofovir were not accepted as substrate. This is in accordance with studies performed with three PNPs of *T. thermophilus* or *B. acetylicum* and aciclovir as substrate. No phosphorolytic activity was observed [31, 137]. For ganciclovir it was even shown that it is an inhibitor for PNP [24].

1.7 Outlook for the Application of Nucleoside Phosphorylase in the Production of Nucleoside Analogues

Nucleoside phosphorylases play a crucial role in nucleoside metabolism. The substrate spectra of naturally available NPs have been studied intensively during the past decades. This knowledge was the basis for the application of nucleoside phosphorylases in the production of nucleoside based drugs or precursors for modified oligonucleotides (e.g. aptamers). A number of different approaches have been developed to increase product yields and drastically reduce costs, which opens the way to use NPs for industrial-scale production. The limitations of available nucleoside phosphorylases will be overcome in the near future by optimizing the biocatalysts using genetic engineering.

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Paper III

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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 20th 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.

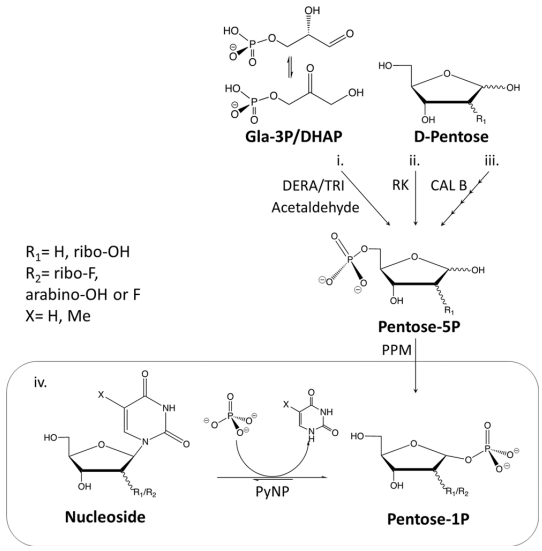
2. Methods

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.

Table 1
Conditions used for the synthesis of Pentose-1Ps.

	Rib-1P	dRib-1P	$_{2F}$Rib-1P	Ara-1P	^{2F}Ara-1P
Substrate concentration [mM]	200 mM	350 mM	200 mM	200 mM	200 mM
Phosphate concentration [mM]	500 mM	500 mM	500 mM	500 mM	500 mM
Enzyme/ final enzyme conc. [mg/ml]	PyNP-Y02/ 0.1	PyNP-Y02/ 0.5	PyNP-Y04/ 1	PyNP-Y04/ 1	PyNP-Y04/ 1
Reaction temperature [°C]	50 °C	40 °C	65 °C	80 °C	80 °C
Protocol to produce the Ba-salt	Rib-1P-Protocol	dRib-1P-Protocol	Rib-1P-Protocol	dRib-1P-Protocol	Rib-1P-Protocol

(PyNP-Y02) and 80 °C (PyNP-Y04). Enzymes were purified by affinity chromatography. Substrate concentrations were 200 mM for Urd, Ara-U, $_{2F}$Ribo-U and ^{2F}Ara-U. 350 mM of Thd was used to produce dRib-1P (Table 1). Enzyme concentrations between 0.1 and 1 mg/ml were used. Enzymatic reactions were performed in 500 mM potassium phosphate (KP) buffer at pH 7. Reaction temperatures between 40 °C and 80 °C were applied. Regular samples were taken to monitor enzymatic cleavage of nucleoside and Pentose-1Ps formation by high performance liquid chromatography (HPLC) and thin layer chromatography (TLC), respectively.

To determine the optimum substrate to phosphate ratio, enzymatic reactions were performed in 500 mM KP buffer using Urd and Thd concentrations ranging from 50 mM to 200 mM. Thermostable PyNP-Y02 was applied at concentrations of 0.1 or 0.5 mg/ml. For Urd and Thd reactions' temperatures of 50 °C and 40 °C were used, respectively.

2.3. High performance liquid chromatography (HPLC)

HPLC was performed as previously reported [29]. Nucleoside depletion and base formation were quantified at 260 nm using pure compound standards. Retention times were as follows: Urd (3.2 min), Thd (4.7 min), Ara-U (3.5 min), $_{2F}$Ribo-U (4.3 min), ^{2F}Ara-U (4.5 min), O-2,2′-anhydro-D-uridine (Anhydro-U, 2.5 min), uracil (Ura, 2.7 min), thymine (3.7 min). The % of conversion of nucleosides was calculated as described previously (Formula (1)) [28]. Standard deviations were calculated from at least 3 independent experiments unless otherwise specified. Average standard deviation of all enzymatic reactions is calculated to be 5% to 15%.

$$Conversion\ \% = \frac{Conc.\ of\ the\ product\ [mM]}{Conc.\ of\ the\ product\ [mM] + Conc.\ of\ the\ substrate\ [mM]} \times 100 \quad (1)$$

2.4. Thin layer chromatography (TLC)

2 µl of standard mixture and samples from different time points were loaded on silica plates (Merck). 10 mM standard solution was used unless otherwise specified. A solvent mixture of n-propanol, ammonia and H_2O was used in a ratio of 11 : 2 : 7 [33] for Rib-1P and dRib-1P reactions, whereas the three solvents were applied in a ratio of 11 : 2 : 5 for Ara-1P, $_{2F}$Rib-1P and ^{2F}Ara-1P.

Nucleoside and base signals were detected by UV light at 254 nm (Merck). Pentose-1Ps were detected colorimetrically by heating the TLC plates at 110 °C for 5–15 min after treatment with p-anisaldehyde-sulphuric acid solution (p-anisaldehyde, concentrated H_2SO_4 and 96% ethanol at a ratio of 1 : 1 : 18 [34]). Inorganic phosphate as well as fluoro-modified Pentose-1Ps were detected using Hanes reagent [35].

2.5. Purification of Pentose-1Ps as barium salts

The previously described protocols [24–26] were modified (Fig. 1C). After the enzymatic synthesis, an equilibrium was reached for Urd and Thd phosphorolysis, and the reaction mixture was incubated at 4 °C overnight and filtered through a polyvinylidene difluoride (PVDF) filter (0.8 µm) to remove the precipitated nucleobase/nucleosides. An amount of aqueous ammonia solution (25% w/v) equal to 1/3 of the reaction mixture's volume was added. Phosphate was precipitated with a solution of $MgCl_2$ and NH_4Cl. $MgCl_2$ was applied in an equimolar concentration to the phosphate and NH_4Cl was 10x the phosphate concentration. The solution was incubated for 3 h at 4 °C and subsequently filtered.

For Rib-1P (**Rib-1P-protocol**), the filtrate was evaporated down to the original reaction volume. Ammonia (25% w/v) was again added equal to 1/10 of the concentrated volume, followed by the addition of barium acetate. For dRib-1P (**dRib-1P-protocol**) the filtrate was used directly for barium precipitation.

Barium acetate was added to the filtrate at an equimolar concentration compared to the nucleoside substrate, followed by addition of absolute ethanol. The barium salts of Pentose-1Ps were precipitated at 4 °C over 24 h. The precipitated salts were collected by centrifugation at 10,000xg for 10 min. The collected pellets were washed 2–3 times with absolute ethanol and dried at 40 °C to 50 °C depending on and optimized for the specific product. The relative yield of Pentose-1P-Ba salts compared to the starting concentration of the substrate was calculated as the ratio of Pentose-1Ps concentration (mmol) to substrate concentration (mmol) (Formula (2)). Standard deviations were calculated from at least 3 independent experiments unless otherwise specified.

$$Yield\ \% = \frac{Amount\ of\ the\ produced\ barium\ salt\ of\ pentose-1P\ [mmol]}{Amount\ of\ the\ substrate\ [mmol]} \times 100 \quad (2)$$

2.6. NMR spectroscopic analysis

^1H, ^{13}C, ^{19}F, and ^{31}P NMR spectra were recorded in D_2O (purchased from Euriso-Top) on a Bruker Avence III 700 MHz or Bruker Avence III 500 MHz instrument. Chemical shifts are reported in parts per million (ppm) and are referenced to the residual solvent resonance as the internal standard (δ = 4.79 ppm for HDO). ^{13}C, ^{19}F and ^{31}P NMR spectra are referenced in compliance with the unified scale for NMR chemical shifts as recommended by the IUPAC stating the chemical shift relative to Me_4Si, CCl_3F and H_3PO_4. Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, m = multiplet, m_c = centrosymmetric multiplet), coupling constants (Hz) and integration.

2.7. Pentose-1Ps half-life and residual [%] determination at different temperature and different pH

To determine the half-life ($t_{1/2}$) of Pentose-1P-Ba salts at different temperatures and pH values, 25 mM solutions of the Pentose-1P-Ba salts were prepared in 10 mM KP buffer (pH 7). For the temperature stability, samples were incubated at different temperatures (room temperature (RT), 40 °C, 60 °C, 80 °C). To evaluate stability at different pH values, samples were incubated at room temperature in buffers of pH values of 1, 3, 5, 7, 9, 11 or 13 (Suppl. Table 1). Daily samples were taken over a period of 14 days and analyzed by TLC. The concentration was determined by TLC with reference to the standard solutions

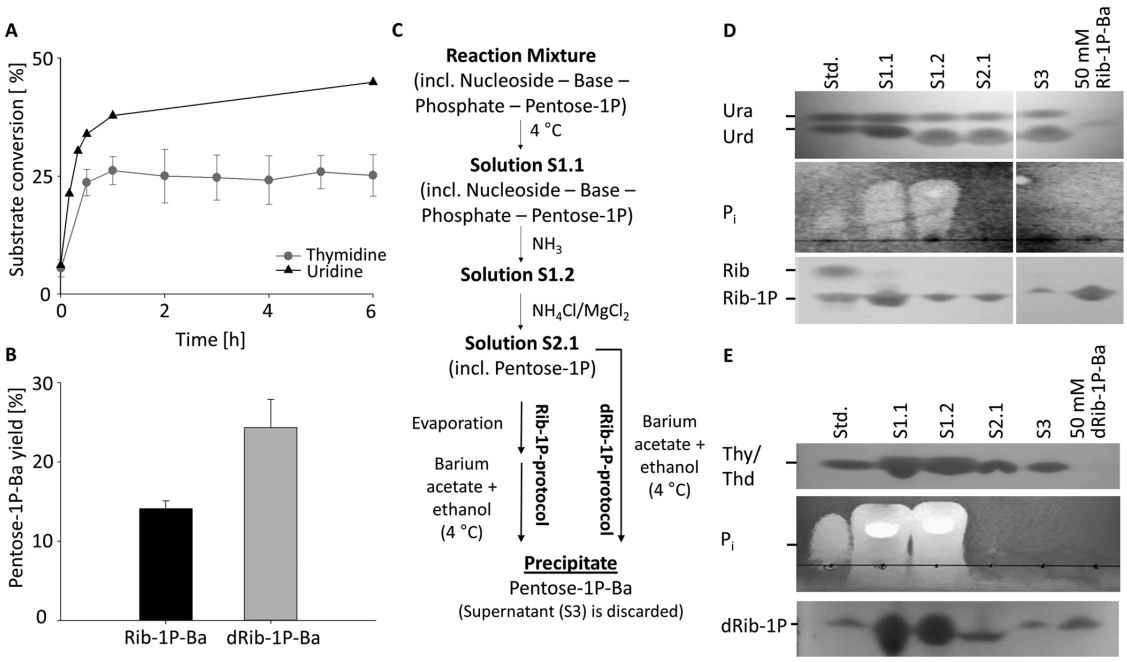


Fig. 1. Chemoenzymatic synthesis of the barium salts of Rib-1P and dRib-1P using thermostable PyNP-Y02. (A) Relative conversion of uridine and thymidine to Rib-1P and dRib-1P, respectively. (B) Relative yields of Rib-1P-Ba and dRib-1P-Ba compared to the starting concentration of uridine and thymidine, respectively. (C) Schematic presentation of the steps used for the purification of Rib-1P (Rib-1P-protocol) and dRib-1P (dRib-1P-protocol). (D and E) Samples taken during the purification of Rib-1P-Ba and dRib-1P-Ba were analyzed by TLC. Nucleosides and nucleobases were detected by UV. Phosphate was visualized colorimetrically using Hanes reagent. Rib-1P and dRib-1P were detected colorimetrically using p-anisaldehyde stain. S1.1: Solution after precipitation of base/nucleoside; S1.2: Solution after addition of NH_3; S2.1: Solution after precipitation of phosphate with $NH_4Cl/MgCl_2$; S3: Solution after precipitation of Pentose-1P as barium salt.

(25 mM) of Pentose-1P-Ba salts and the corresponding pentoses. For ^{2F}Ara-1P and $_{2F}$Rib-1P the corresponding sugars were not available. Images of the TLC plates were taken, and signal intensity was used for quantification using the ChemiDocMP imaging system (Biorad, Germany). Percentage of the residual amounts of Pentose-1P-Ba salts were calculated relatively to day 0. The $t_{1/2}$ was extrapolated after fitting the data points with a nonlinear regression model using GraphPad Prism 7.0 (GraphPad Software Inc, San Diego, CA, http://www.graphpad.com/).

3. Results

3.1. Developing a protocol to produce Pentose-1Ps using thermostable NPs

To identify optimum synthesis conditions for Pentose-1Ps using thermostable NPs, methods as described in literature for Rib-1P and dRib-1P were used [26]. For the synthesis of Rib-1P, 50 mM Urd was cleaved in the presence of 100 mM phosphate. Urd conversion reached 40% after 6 h of incubation at 50 °C (Fig. 1A). dRib-1P was enzymatically synthesized using 350 mM Thd in 500 mM potassium phosphate buffer [26]. After 6 h, 25% of Thd was cleaved to dRib-1P and thymine (Fig. 1A). PyNP-Y02 was used as biocatalyst for both reactions.

The reaction solutions were directly used for the purification process. Rib-1P and dRib-1P were purified as their barium salts, as previously described [24–26]. Rib-1P and dRib-1P remained in solution during the precipitation steps for base/nucleoside and phosphate with a quite constant concentration (Fig. 1D, E). Almost all Rib-1P and dRib-1P were precipitated with barium. Both TLC and HPLC analysis confirmed the purity of the barium salts of Rib-1P (Rib-1P-Ba) and dRib-1P (dRib-1P-Ba) to be higher than 95%. Only traces of Urd and Thd were detected. The yield of Rib-1P-Ba and dRib-1P-Ba was 13% and 25% (Fig. 1 B), respectively, compared to the amount of substrate added to the enzymatic cleavage reaction.

Compared to the results of Fateev et al [26], yields for barium salts of Rib-1P and dRib-1P were lower. Therefore, the reaction conditions for the enzymatic synthesis of Rib-1P and dRib-1P were modulated. Especially the impact of the phosphate to substrate ratio was investigated further.

Different substrate to phosphate ratios (1:10 to 1:2.5) were tested by keeping the phosphate concentration constant at 500 mM but changing the substrate concentrations between 50 and 200 mM. For both compounds the percentage of conversion was decreasing upon increasing substrate concentration (Fig. 2A, B). By increasing the Urd concentration from 50 to 200 mM, the substrate conversion decreased from 70 to 40% (Fig. 2A), while the yield of Rib-1P-Ba increased from 0% to 25%, (Fig. 2C). An increase of the Thd concentration from 50 mM to 200 mM resulted in decreased conversion percentages from 71 to 43% (Fig. 2B). In contrast, the determined yields of dRib-1P-Ba were 0% at 50 mM and 100 mM substrate concentration and 27% at 200 mM substrate concentration (Fig. 2D). In summary, substrate and phosphate concentrations of 200 mM and 500 mM, respectively, gave the best results to produce barium salts of Rib-1P and dRib-1P.

3.2. Enzymatic synthesis of Ara-1P, $_{2F}$Rib-1P and ^{2F}Ara-1P

Based on the results obtained for Rib-1P and dRib-1P, the production of Ara-1P, $_{2F}$Rib-1P and ^{2F}Ara-1P was examined using substrate and phosphate concentrations of 200 mM and 500 mM. For these syntheses, thermostable PyNP-Y04 was used, as it showed a higher

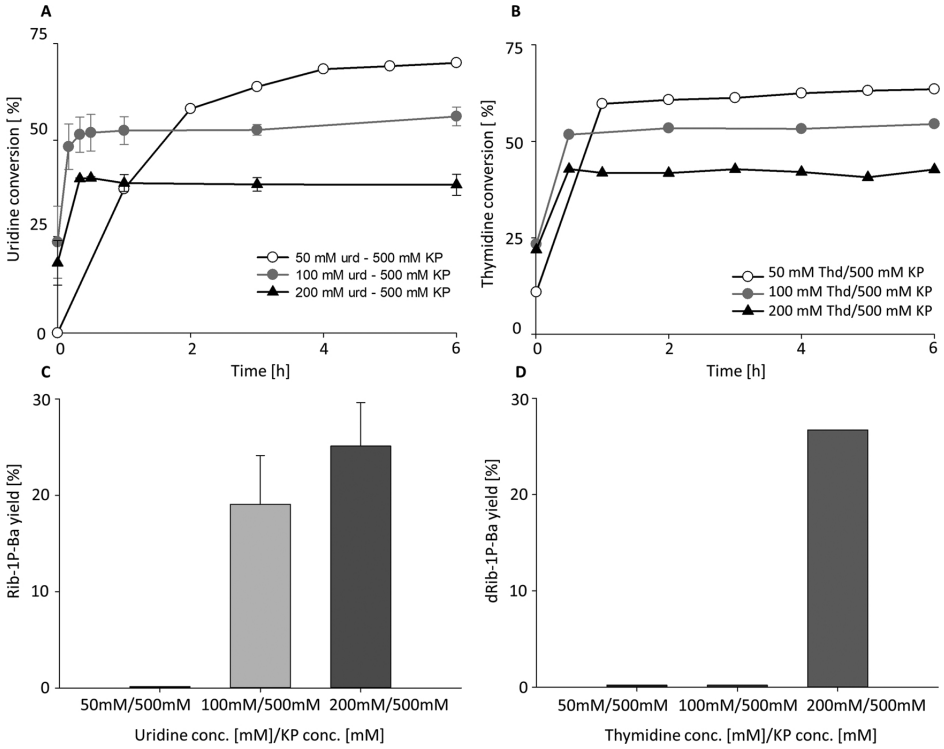


Fig. 2. Optimization of the reaction conditions to produce barium salts of Rib-1P and dRib-1P using thermostable PyNP-Y02. Substrate concentrations of 50 mM, 100 mM and 200 mM were used at a constant phosphate concentration of 500 mM. (A and B) Relative conversion of uridine and thymidine to Rib-1P and Ura(A) and dRib-1P and Thy (B) at varying substrate to phosphate ratios. (C and D) Relative yields of the barium salts of Rib-1P (C) and dRib-1P (D) compared to the starting Urd and Thd concentrations, respectively.

synthesis rate in the phosphorolysis reaction compared to PyNP-Y02 (Suppl. Table 2).

The synthesis of Ara-1P was carried out at 80 °C for 24 h. Conversion of Ara-U was about 37% after 24 h (Fig. 3A). With increasing reaction time, an increasing amount of Ara-1P was observed (Fig. 3C). Extending the reaction time up to 72 h led to an increased conversion of Ara-U to 47%. Equilibrium of the reaction was reached after 48 h (Suppl. Fig. 1). However, with increased incubation time, degradation of Ara-1P to arabinose was observed in TLC experiments. Accordingly, 24 h reactions were used for the production of Ara-1P.

$_{2F}$Rib-1P was synthesized from $_{2F}$Ribo-U at 65 °C for 72 h. After 72 h 34% of $_{2F}$Rib-U was converted to $_{2F}$Rib-1P and Ura (Fig. 3B). Increasing amounts of $_{2F}$Rib-1P were observed with increasing reaction time (Fig. 3C). Pre-synthesis trials were conducted at 80 °C as well, however, two non-specific peaks were detected by HPLC. The formation of these by-products has previously been described in transglycosylation reactions to produce purine nucleosides using $_{2F}$Ribo-U as a sugar donor and they were identified to be Ara-U and Anhydro-U [29]. At high temperature, the chemical formation of the Anhydro-U is observed in correlation with HF group release from the C2′-position of $_{2F}$Ribo-U. Anhydro-U is subsequently hydrolyzed to Ara-U at elevated temperature [29]. These by-products were not observed at a reaction temperature of 65 °C.

Phosphorolysis of ^{2F}Ara-U into ^{2F}Ara-1P and Ura was tested at 65 °C and 80 °C for 72 h using thermostable PyNP-Y04. After 72 h, conversion of ^{2F}Ara-U was higher at 80 °C (27%) than at 65 °C (16%) (Fig. 3B, Suppl. Fig. 1). No by-products or degradation peaks were detected by HPLC. Formation of ^{2F}Ara-1P was followed by TLC (Fig. 3D) and increasing amounts of ^{2F}Ara-1P were confirmed with increasing reaction time.

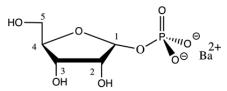
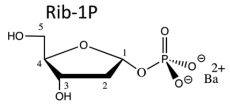
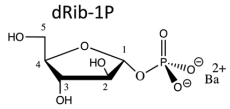
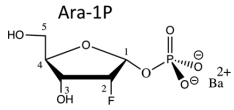
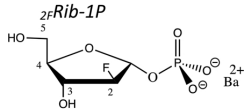
3.3. Purification of Ara-1P, $_{2F}$Rib-1P and ^{2F}Ara-1P as their barium salts

To identify a suitable purification method for the production of modified Pentose-1P-Ba salts, the available purification protocols for Rib-1P and dRib-1P were compared. Relative to the dRib-1P-protocol, the Rib-1P-protocol gave lower final yields for Ara-1P, while for $_{2F}$Rib-1P and ^{2F}Ara-1P the opposite was observed (Fig. 3D). In correlation to the starting substrate concentration, the yield of Ara-1P-Ba was 13% using the dRib-1P-protocol (Fig. 3D). For both, $_{2F}$Rib-1P-Ba and ^{2F}Ara-1P-Ba relative yields were approximately 23% using the Rib-1P-protocol (Fig. 3D). Using the improved protocols with a reaction volume of 25 ml 0.2 g and 0.4 g of Ara-1P-Ba and $_{2F}$Rib-1P-Ba, respectively, were obtained. Using a reaction volume of 50 ml 0.4 g of ^{2F}Ara-1P-Ba were attained. The purity of the Pentose-1Ps was determined by TLC (Suppl. Fig. 2) and HPLC to be > 95%. Traces of base and nucleoside were detected in all preparations.

3.4. Structure confirmation of Rib-1P, dRib-1P, Ara-1P, $_{2F}$Rib-1P and ^{2F}Ara-1P by NMR spectroscopy

The structure of all produced Pentose-1Ps was confirmed by ^{1}H, ^{13}C{^{1}H}, and ^{31}P{^{1}H} NMR spectroscopy (Table 2, Suppl. Fig. 4–8). Additionally, ^{19}F{^{1}H} NMR spectroscopy was applied for structure confirmation of fluoro-modified Pentose-1Ps (Table 2, Suppl. Fig. 7, 8).

Table 2
Structures of Pentose-1Ps were confirmed by NMR spectroscopy.

Pentose-1P-Ba salts	NMR spectroscopic data
	**^{1}H NMR** (500 MHz, D_2O): δ = 3.68 (dd, $^{2}J_{H,H}$ = 12.3 Hz, $^{3}J_{H,H}$ = 5.4 Hz, 1H, H-5 A), 3.79 (dd, $^{2}J_{H,H}$ = 12.3 Hz, $^{3}J_{H,H}$ = 3.0 Hz, 1H, H-5B), 4.12 (m_c, 1H, H-3), 4.19 (m_c, 1H, H-2), 4.30 (m_c, 1H, H-4), 5.68 (dd, $^{3}J_{H,H}$ = 4.7 Hz, $^{3}J_{H,H}$ = 4.7 Hz, 1H, H-1) ppm. **^{13}C{^{1}H} NMR** (126 MHz, D_2O): δ = 61.7 (C-5), 69.9 (C-3), 71.8 (d, $^{3}J_{C,P}$ = 5.7 Hz, C-2), 84.9 (C-4), 97.4 (C-1) ppm. **^{31}P{^{1}H} NMR** (203 MHz, D_2O): δ = 2.3 ppm.
Rib-1P 	**^{1}H NMR** (500 MHz, D_2O): δ = 2.08 (d, $^{2}J_{H,H}$ = 14.4 Hz, 1H, H-2 A), 2.37 (ddd, $^{2}J_{H,H}$ = 14.4 Hz, $^{3}J_{H,H}$ = 5.7 Hz, $^{3}J_{H,H}$ = 5.7 Hz, 1H, H-2B), 3.60 (dd, $^{2}J_{H,H}$ = 12.1 Hz, $^{3}J_{H,H}$ = 5.6 Hz, 1H, H-5 A), 3.73 (dd, $^{2}J_{H,H}$ = 12.1 Hz, $^{3}J_{H,H}$ = 3.2 Hz, 1H, H-5B), 4.21–4.24 (m, 2H, H-3, H-4), 5.78 (dd, $^{3}J_{H,H}$ = 5.6 Hz, $^{3}J_{H,H}$ = 5.6 Hz, 1H, H-1) ppm. **^{13}C{^{1}H} NMR** (126 MHz, D_2O): δ = 41.7 (d, $^{3}J_{C,P}$ = 5.4 Hz, C-2), 61.9 (C-5), 71.2 (C-3), 86.1 (C-4), 99.6 (d, $^{2}J_{C,P}$ = 4.4 Hz, C-1) ppm. **^{31}P{^{1}H} NMR** (202 MHz, D_2O): δ = 1.8 ppm.
dRib-1P 	**^{1}H NMR** (500 MHz, D_2O): δ = 3.69 (dd, $^{2}J_{H,H}$ = 12.2 Hz, $^{3}J_{H,H}$ = 6.5 Hz, 1H, H-5 A), 3.84 (dd, $^{2}J_{H,H}$ = 12.2 Hz, $^{3}J_{H,H}$ = 3.3 Hz, 1H, H-5B), 3.92 (dd, $^{3}J_{H,H}$ = 5.8 Hz, $^{3}J_{H,H}$ = 3.2 Hz, 1H, H-3), 4.18 (dd, $^{3}J_{H,H}$ = 3.1 Hz, $^{3}J_{H,H}$ = 1.7 Hz, 1H, H-2), 4.23 (ddd, $^{3}J_{H,H}$ = 6.3 Hz, $^{3}J_{H,H}$ = 6.1 Hz, $^{3}J_{H,H}$ = 3.3 Hz, 1H, H-4), 5.49 (d, $^{3}J_{H,H}$ = 6.6 Hz, 1H, H-1) ppm. **^{13}C{^{1}H} NMR** (126 MHz, D_2O): δ = 61.6 (C-5), 76.5 (C-3), 81.9 (d, $^{3}J_{C,P}$ = 7.0 Hz, C-2), 84.2 (C-4), 102.6 (d, $^{2}J_{C,P}$ = 4.2 Hz, C-1) ppm. **^{31}P{^{1}H} NMR** (203 MHz, D_2O): δ = 1.7 ppm.
Ara-1P 	**^{1}H NMR** (500 MHz, D_2O): δ = 3.70 (d, $^{2}J_{H,H}$ = 11.8 Hz, 1H, H-5 A), 3.88 (d, $^{2}J_{H,H}$ = 11.8 Hz, $^{3}J_{H,H}$ = 3.0 Hz, 1H, H-5B), 4.24 (m_c, 1H, H-4), 4.28 (m_c, 1H, H-3), 4.96 (d, $^{2}J_{H,F}$ = 53.4 Hz, 1H, H-2), 5.77 (m_c, 1H, H-1) ppm. **^{13}C{^{1}H} NMR** (176 MHz, D_2O): δ = 60.9 (C-5), 68.8 (C-3), 82.3 (C-4), 90.0 (d, $^{1}J_{C,F}$ = 187.6 Hz, C-2), 96.1 (C-1) ppm. **^{31}P{^{1}H} NMR** (202 MHz, D_2O): δ = 2.2 ppm. **^{19}F NMR** (659 MHz, D_2O): δ = –121.7 ppm.
$_{2F}$Rib-1P 	**^{1}H NMR** (500 MHz, D_2O): δ = 3.76 (dd, $^{2}J_{H,H}$ = 12.3 Hz, $^{3}J_{H,H}$ = 5.9 Hz, 1H, H-5 A), 3.90 (dd, $^{2}J_{H,H}$ = 12.3 Hz, $^{3}J_{H,H}$ = 3.3 Hz, 1H, H-5B), 4.22 (dd, $^{3}J_{H,F}$ = 24.2 Hz, $^{3}J_{H,H}$ = 5.4 Hz, 1H, H-3), 4.29 (ddd, $^{3}J_{H,H}$ = 5.6 Hz, $^{3}J_{H,H}$ = 5.6 Hz, $^{3}J_{H,H}$ = 3.3 Hz, 1H, H-4), 5.05 (d, $^{2}J_{H,F}$ = 50.2 Hz, 1H, H-2), 5.74 (dd, $^{3}J_{H,F}$ = 9.9 Hz, $^{3}J_{H,H}$ = 7.0 Hz, 1H, H-1) ppm. **^{13}C{^{1}H} NMR** (176 MHz, D_2O): δ = 61.2 (C-5), 74.8 (d, $^{2}J_{C,F}$ = 27.1 Hz, C-3), 84.7 (C-4), 100.3 (d, $^{2}J_{C,P}$ = 3.5 Hz, C-1), 100.8 (dd, $^{1}J_{C,F}$ = 180.4 Hz, $^{3}J_{C,P}$ = 7.6 Hz, C-2) ppm. **^{31}P{^{1}H} NMR** (202 MHz, D_2O): δ = 1.8 ppm. **^{19}F{^{1}H} NMR** (471 MHz, D_2O): δ = –120.9 ppm
^{2F}Ara-1P	

3.5. Stability of Pentose-1Ps at different pH values and temperatures

The stability of Pentose-1Ps was assessed to evaluate their behavior during the purification process or as substrate for enzymatic reactions. All synthesized Pentose-1P-Ba salts were incubated in buffers with different pH values (1–13) and temperatures (room temperature (RT), 40 °C, 60 °C, 80 °C) over a period of 14 days in aqueous solution (Table 3, Suppl. Fig. 3).

All tested Pentose-1P-Ba salts were completely degraded at 80 °C after 14 days. The most unstable compound was dRib-1P-Ba, which is in good accordance with the results of Pontis [36]. The minimum $t_{1/2}$ of 12 h was observed at 80 °C. The maximum $t_{1/2}$ for dRib-1P was 1.2 days at room temperature. ^{2F}Ara-1P-Ba was completely stable with 100% residual amount after 14 days at 60 °C.

Stability of all Pentose-1P-Ba salts was dependent on the pH used. They all showed high stability at pH values between 9–13 with residual amounts ranging between 75% and 100%. At lower pH values, Pentose-1P-Ba salts were less stable. At pH 1, half-lives ranged between 0.5 days (Rib-1P-Ba) to 2 days (^{2F}Ara-1P-Ba). As dRib-1P-Ba was immediately degraded at pH 1, its half-life could not be calculated.

4. Discussion

Here we show the enzymatic synthesis and purification of grams of natural (Rib-1P and dRib-1P) and modified ($_{2F}$Rib-1P, Ara-1P and ^{2F}Ara-1P) Pentose-1Ps using thermostable PyNPs. The synthesis and purification methods earlier described for Rib-1P and dRib-1P were optimized to increase the product yields [26].

Due to the importance of Pentose-1Ps, more and more synthesis routes are being developed. Recently, a special focus on the production of ^{2F}Ara-1P was observed as it is a building block of ^{2F}Ara-nucleosides. The presence of a fluorine atom at the C2′-arabino position of nucleosides or nucleotides is known to exert a wide variety of physicochemical effects [37]. These include a strengthening of the glycosidic bond which has led to a widespread application of C2′-arabino nucleotides in modified oligonucleotides. A number of chemical synthesis routes for ^{2F}Ara-1P [38–40] were recently published. Yamada et al. studied the synthesis of ^{2F}Ara-1P starting from the commercially available 1,3,5-tri-*O*-benzoyl-2-deoxy-2-fluoro-α-D-arabinofuranose (6 steps; three SiO_2 and one ion-exchange column chromatography; 20% combined yield, α/β = 21) and its C1-bromo derivative (35% combined yield, α/β = 1.2) [38,40]. Later, Fateev et al. described the transformation of 1,3,5-tri-*O*-benzoyl-2-deoxy-2-fluoro-α-D-arabinofuranose into ^{2F}Ara-1P without purification of all the intermediate compounds, which gave rise to the desired phosphate at a yield of 45–50% (α/β = ca. 1:1) [39]. The synthesized ^{2F}Ara-1P was used for the synthesis of purine nucleosides employing purine nucleoside phosphorylases as biocatalysts. However, it was shown [39] that chemically synthesized ^{2F}Ara-1P was not a substrate for uridine phosphorylase and thymidine phosphorylase of *E. coli*. Possible explanations are remnants of the chemical synthesis or a mixture of α and β anomers may have blocked the reaction. Therefore, enzymatic synthesis of ^{2F}Ara-1P is a valuable alternative to provide this very important sugar-phosphate on multi-gram scale.

Phosphorolytic cleavage of pyrimidine nucleosides analogues was strongly dependent on the modification in the C2′-position. After 6 h of reaction time, Pentose-1Ps were formed with the following percentages: 43% (dRib-1P), 38% (Rib-1P), 25% (Ara-1P), 8% ($_{2F}$Rib-1P), and 6% (^{2F}Ara-1P). The lower conversion of modified nucleosides compared to the natural nucleoside substrates correlates with the stabilization of the N-glycosidic bond by the presence of a fluoro-atom at the C2′position of the sugar [11]. Crystallographic data have shown decreased length of the N-glycosidic bond of $_{2F}$Ribo-U (1.45 Å) and ^{2F}Ara-U (1.46 Å) as well

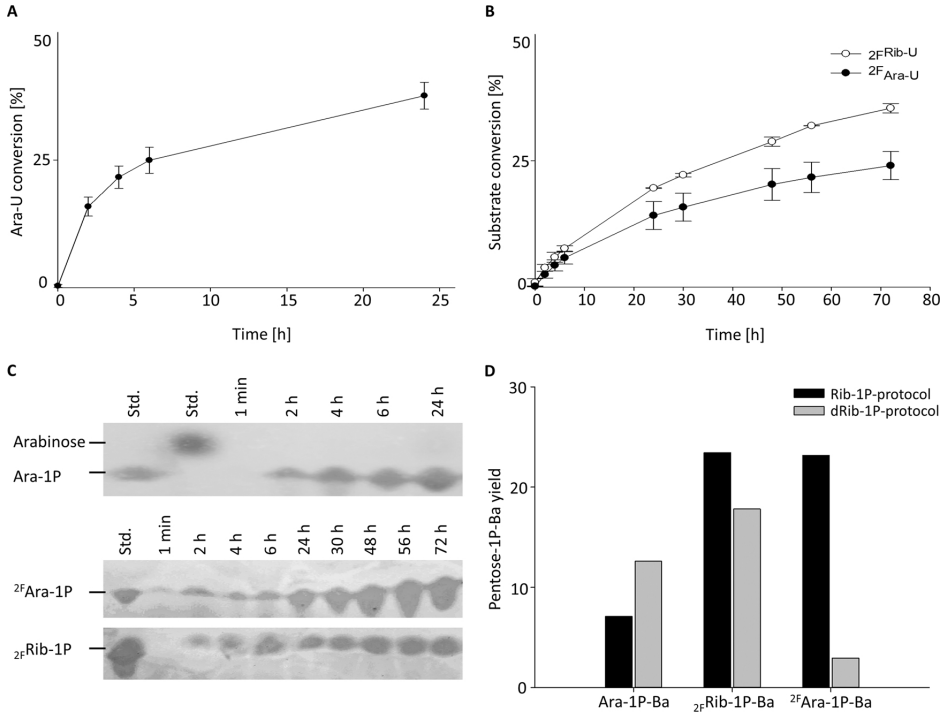


Fig. 3. Chemo-enzymatic synthesis of Ara-1P, $_{2F}$Rib-1P and ^{2F}Ara-1P at substrate and phosphate concentrations of 200 mM and 500 mM, respectively, using the thermostable PyNP-Y04. (A) Relative conversion of Ara-U to Ara-1P and uracil at 80 °C. (B) Relative conversion of $_{2F}$Rib-U and ^{2F}Ara-U at 65 °C and 80 °C, respectively. (C) Formation of Ara-1P, $_{2F}$Rib-1P and ^{2F}Ara-1P during the enzymatic reaction was evaluated colorimetrically by TLC. Standard solutions were used in concentration of 30 mM. Ara-1P was detected by p-anisaldehyde solution. $_{2F}$Rib-1P and ^{2F}Ara-1P were visualized by Hanes reagent. (D) Relative yields of the barium salts of Ara-1P, $_{2F}$Rib-1P and ^{2F}Ara-1P using either the Rib-1P-protocol or dRib-1P-protocol for purification. The relative yield was calculated in relation to the starting concentration of the respective substrate.

Table 3
Stability of Pentose-1P Ba salts at different pH and temperatures after an incubation time of 14 days. To determine $t_{1/2}$ of Pentose-1P-Ba salts at different temperatures 25 mM solutions of the Pentose-1P-Ba salts were prepared in 10 mM KP buffer (pH 7) and incubated at different temperatures (room temperature (RT), 40 °C, 60 °C, 80 °C). To evaluate stability at different pH values, samples were incubated at room temperature in buffers with varying pH values of 1, 3, 5, 7, 9, 11 or 13.

Pentose-1Ps-Ba salt	Residual [%] / $t_{1/2}$ [d]										
	pH							Temp			
	1	3	5	7	9	11	13	RT	40 °C	60 °C	80 °C
Rib-1P-Ba	0 / 0.5	≥25 / 5.5	≥50 / n.r.	≥75 / n.r.	≥50 / n.r.	≥75 / n.r.	100 / n.r.	≥75 / n.r.	100 / n.r.	≥25 / 12.7	0 / 1
$_{2F}$Rib-1P-Ba	0 / 0.8	≥75 / n.r.	≥75 / n.r.	≥75 / n.r.	≥75 / n.r.	≥75 / n.r.	≥75 / n.r.	≥75 / n.r.	≥75 / n.r.	100 / n.r.	0 / 1.5
dRib-1P-Ba	0[a]	0[a]	0[a]	0 / 1.7	100 / n.r.	≥75 / n.r.	≥75 / n.r.	0 / 1.2	0 / 0.5	0 / 0.5	0 / 0.5
Ara-1P-Ba	0 / 0.5	≥25 / 8	≥75 / n.r.	≥75 / n.r.	100 / n.r.	≥75 / n.r.	≥75 / n.r.	≥75 / n.r.	≥75 / n.r.	100 / n.r.	0 / 2.5
^{2F}Ara-1P-Ba	0 / 2	≥75 / n.r.	100 / n.r.	100 / n.r.	100 / n.r.	100 / n.r.	100 / n.r.	100 / n.r.	100 / n.r.	100 / n.r.	0 / 6.5

[a] Total loss occurred right away starting from the first sample; ≥25%: 25%–49%; ≥50%: 50%–74%, ≥75%: 75%–99%; n.r. t1/2 were not reached within 14 days.

as Ara-U (1.47 Å) [43–45] compared to Urd (aver. value 1.49 Å) and Thd (1.48 Å) [46,47]. The strength of the glycosyl N1-C1′ bond is evidently reflected in the rate of phosphorolysis.

Losses during the purification process (up to 80%) were noticed. In previous publications a co-precipitation of phosphate and Rib-1P using barium was described [25]. Increased recovery of Pentose-1Ps was described by an initial precipitation of phosphate as insoluble NH_4MgPO_4 [26]. However, co-precipitation of Pentose-1Ps and phosphate in the presence of NH_4Cl and $MgCl_2$ was previously described due to similar ionization pattern of both compounds at the applied pH [48].

5. Conclusions

Applying thermostable nucleoside phosphorylases, we successfully synthesized gram quantities of modified Pentose-1Ps, $_{2F}$Rib-1P, Ara-1P and ^{2F}Ara-1P, at a purity above 95%. Modified Pentose-1Ps were stable at temperatures up to 60 °C, allowing for downstream processing at elevated temperatures. The availability of the Pentose-1Ps in g-scale and high purity (only α-anomer) will allow for (i) a comparison of substrate activities of nucleoside phosphorylases, (ii) better understanding of nucleoside phosphorylases kinetics (iii) the development of efficient enzymatic methods for the synthesis of biologically valuable

nucleosides, (iv) more detailed metabolic studies and (v) studies of their therapeutic or diagnostic potential.

Conflict of interest

A.W. is the CEO of the biotech startup BioNukleo GmbH. The authors have no other relevant affiliations or financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No conflict of interest is known to the other authors.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.mcat.2018.07.028.

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Supplementary information

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

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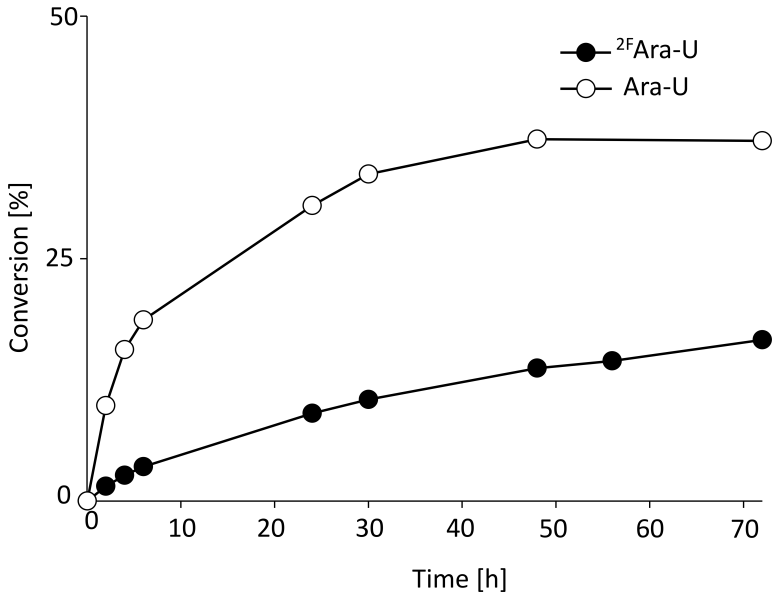
Supplementary table 1. *Buffers used for pentose-1P pH stability testing*

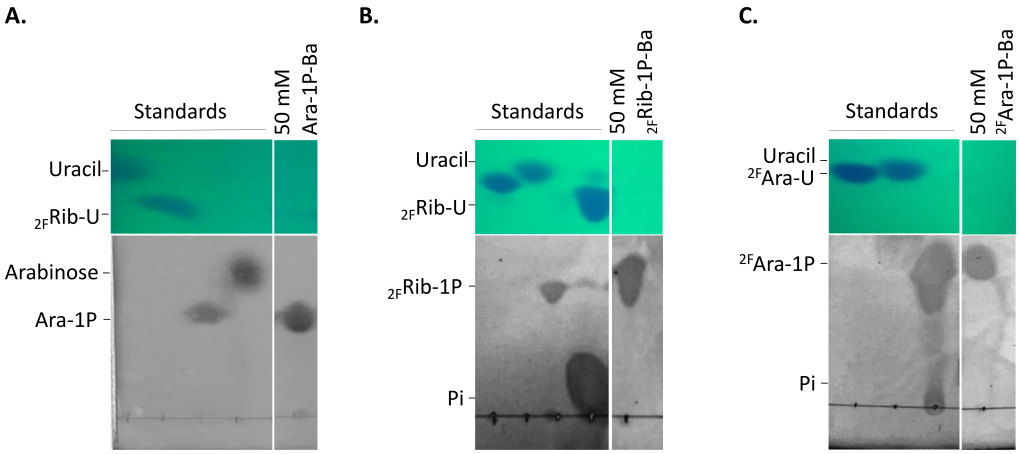
pH	Buffer	pH adjustment
1	HCl/KCl	HCl
3	Sodium acetate	Acetate - Acetic acid
5	Acetate	Acetate - Acetic acid
7	Potassium phosphate	Potassium dihydrogen phosphate
9	Tris	HCl
11	Sodium bicarbonate	NaOH
13	KCl/ NaOH	KCl - NaOH

Supplementary table 2. Activity of both used PyNPs towards the sugar modified nucleosides.

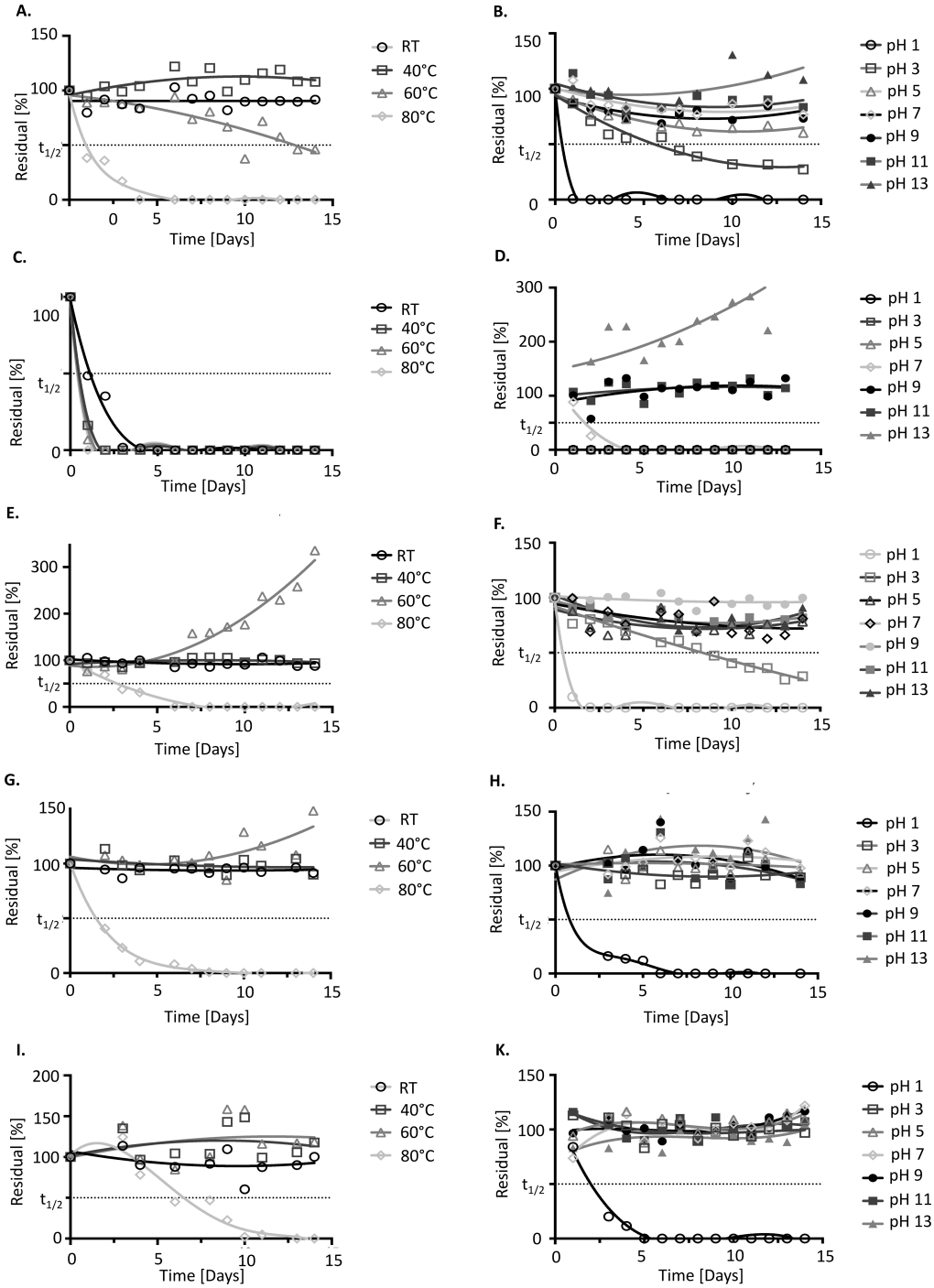
Enzyme	Reaction temp./ Protein conc.	2'-deoxy-2'-fluorouridine ($_{2F}$Ribo-U)	1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl) uracil (^{2F}Ara-U)	1-(β-D-Arabinofuranosyl) uracil (Ara-U)
PyNP-Y04	80°C / 1 mg/mL	57%	52%	81%
PyNP-Y02	50°C/ 0.59 mg/mL	4%	0%	3.3%



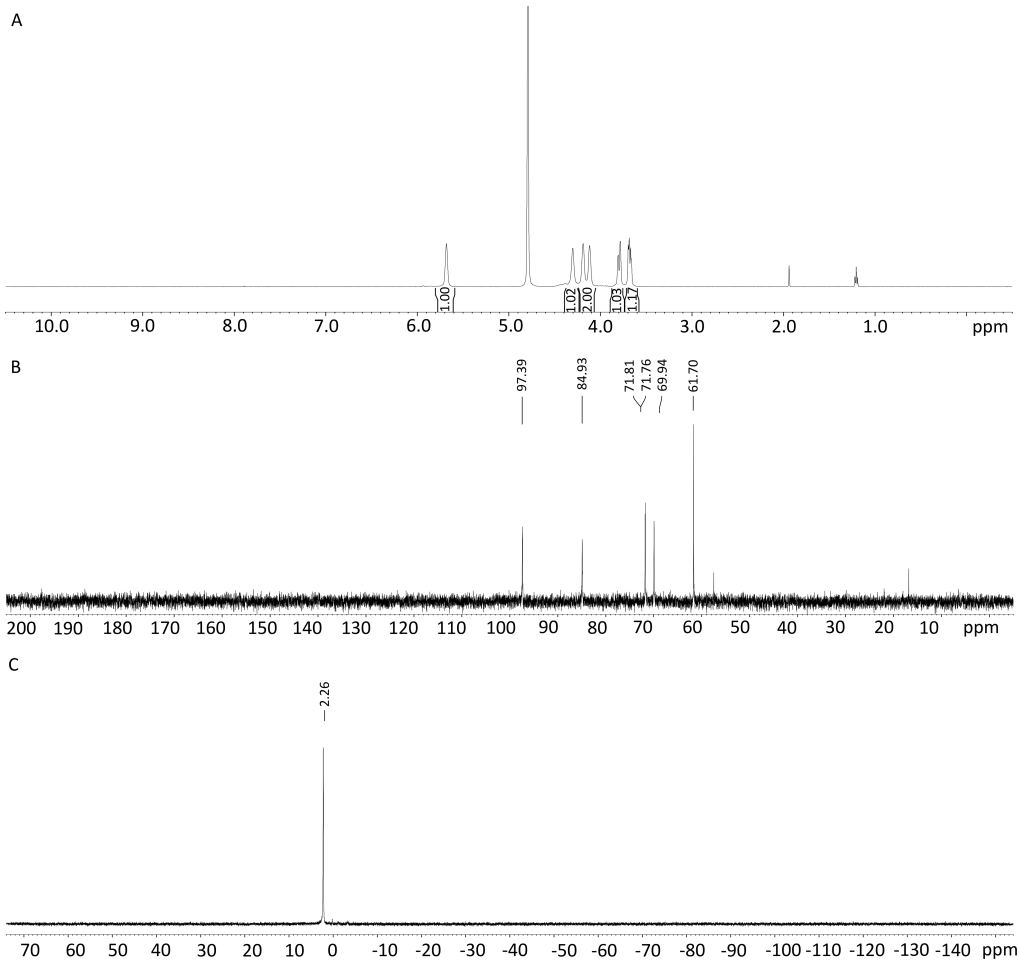
Supplementary figure 1. Relative conversion of ^{2F}Ara-U and Ara-U using thermostable PyNP-Y04 at a reaction temperature of 65°C. Substrate and phosphate concentrations of 200 mM and 500 mM were applied.



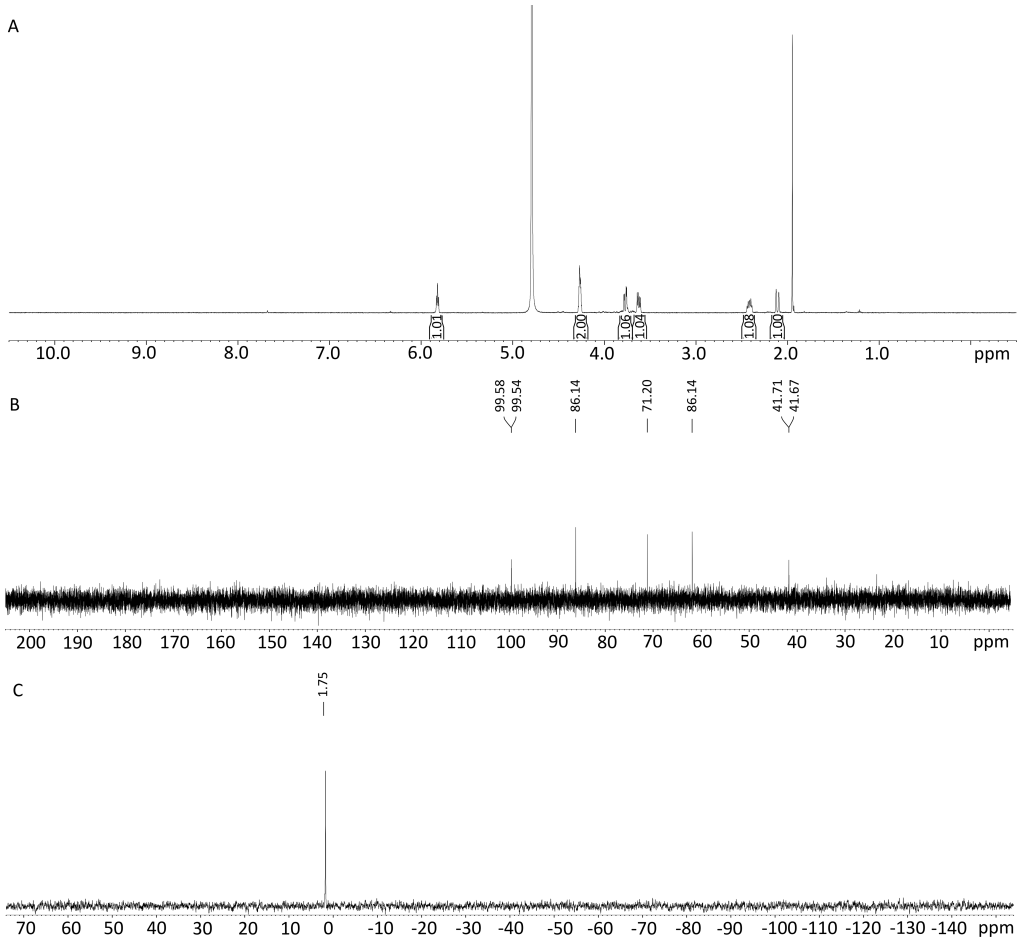
Supplementary figure 2. Purity of barium salts Ara-1P (A), $_{2F}$Rib-1P (B) and ^{2F}Ara-1P (C).



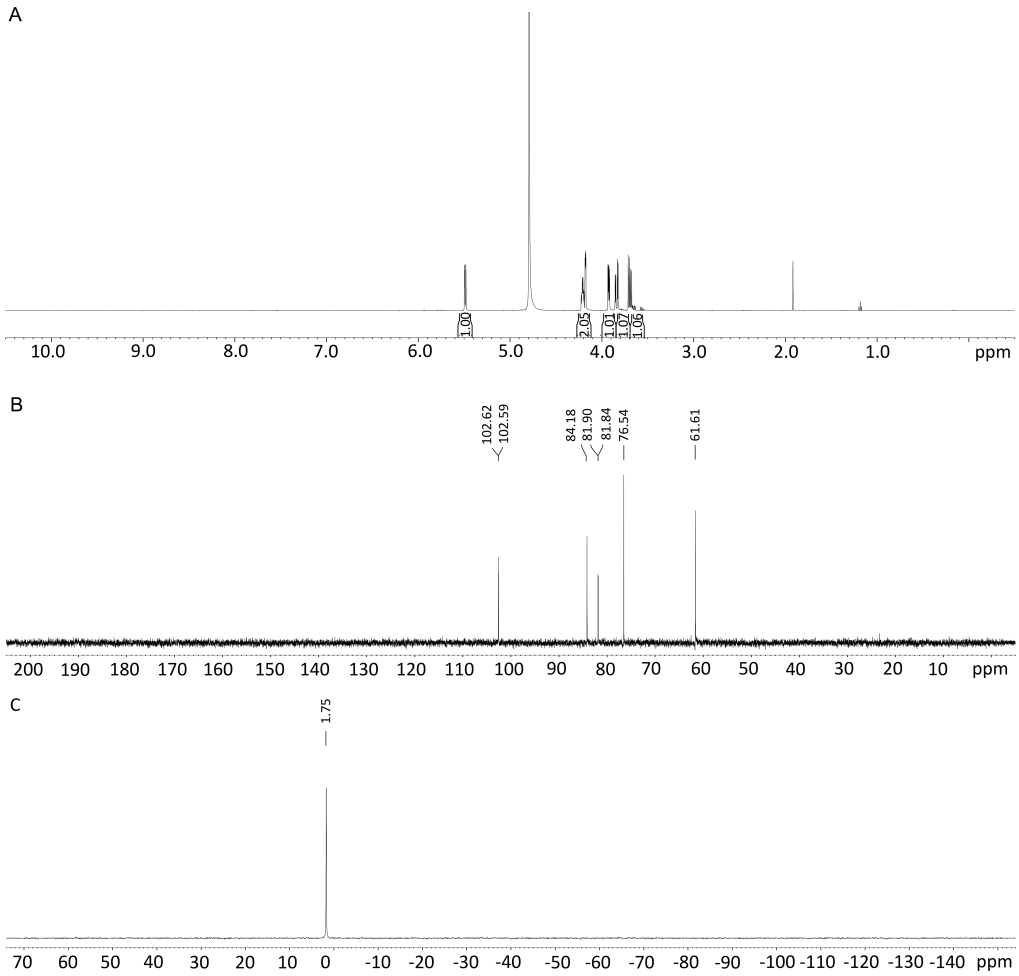
Supplementary figure 3. Temperature (A, C, E, G, I) and pH (B, D, F, H, K) stability of Rib-1P (A, B), dRib-1P (C, D), Ara-1P (E, F), $_{2F}$Rib-1P (G, H) and ^{2F}Ara-1P (I, K). Data points were fitted through a non-linear regression model and $t_{1/2}$ life was estimated and plotted on the curves.



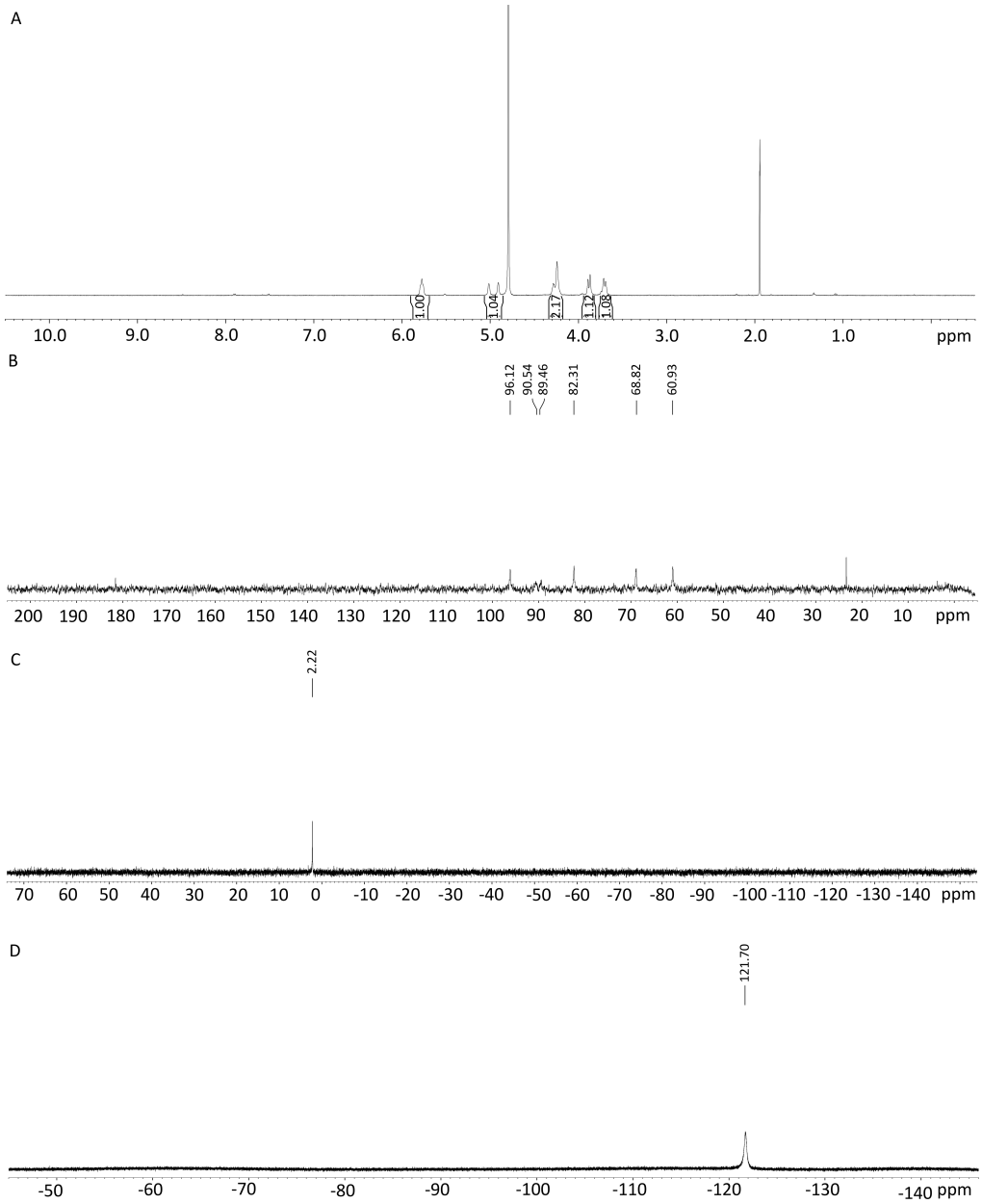
Supplementary figure 4. NMR spectra of Rib-1P. (A) ^{1}H NMR (500 MHz, D_2O). (B) $^{13}C\{^{1}H\}$ NMR (126 MHz, D_2O). (C) $^{31}P\{^{1}H\}$ NMR (203 MHz, D_2O).



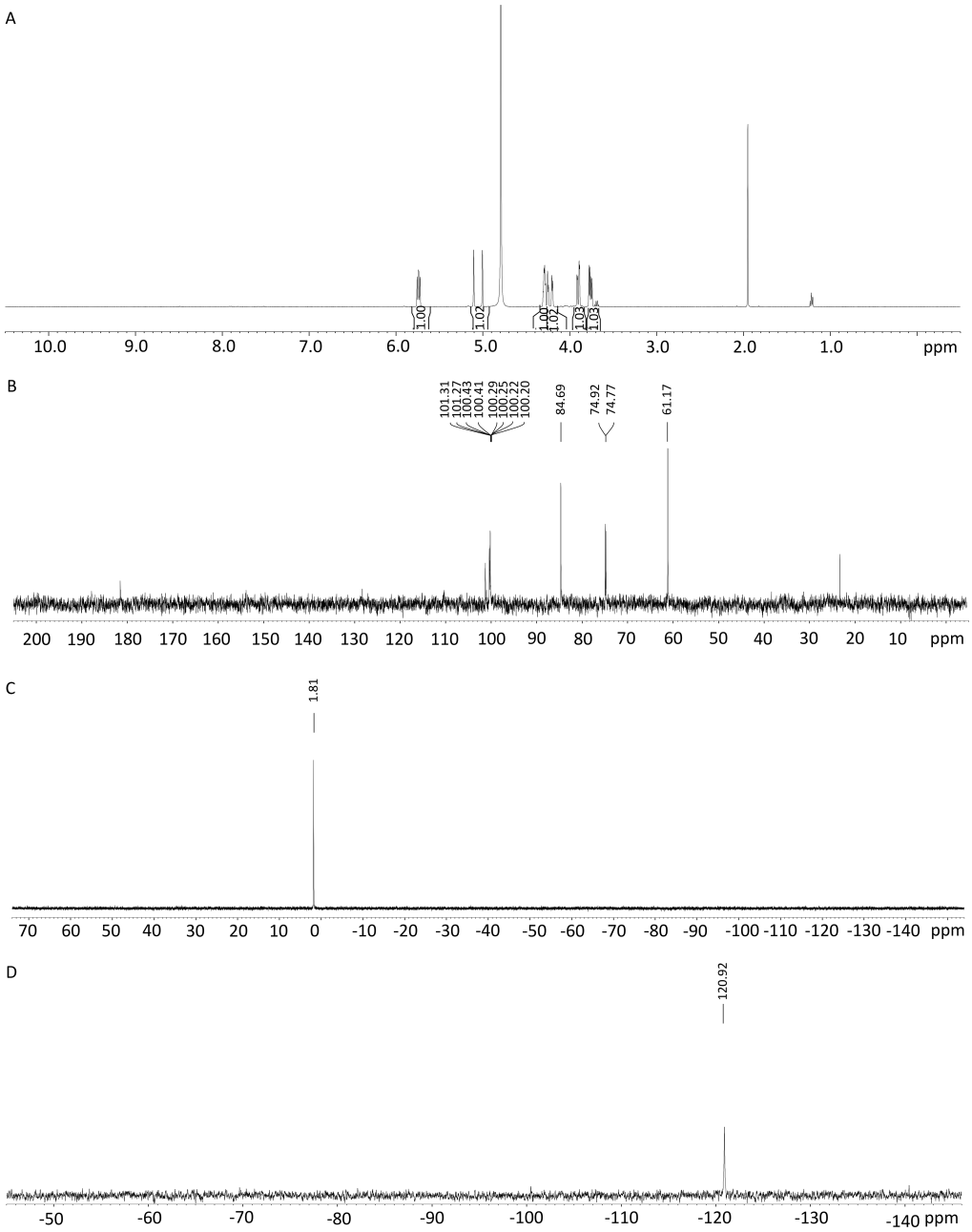
Supplementary figure 5. NMR spectra of dRib-1P. (A) ^{1}H NMR (500 MHz, D_2O). (B) $^{13}C\{^{1}H\}$ NMR (126 MHz, D_2O). (C) $^{31}P\{^{1}H\}$ NMR (202 MHz, D_2O).



Supplementary figure 6. NMR spectra of Ara-1P. (A) ^{1}H NMR (500 MHz, D_2O). (B) $^{13}C\{^{1}H\}$ NMR (126 MHz, D_2O). (C) $^{31}P\{^{1}H\}$ NMR (203 MHz, D_2O).



Supplementary figure 7. NMR spectra of $_{2F}$Rib-1P**.** (A) ^{1}H NMR (500 MHz, D_2O). (B) $^{13}C\{^{1}H\}$ NMR (176 MHz, D_2O). (C) $^{31}P\{^{1}H\}$ NMR (202 MHz, D_2O). (D) ^{19}F NMR (659 MHz, D_2O).



Supplementary figure 8. NMR spectra of ^{2F}Ara-1P. (A) ^{1}H NMR (500 MHz, D_2O). (B) $^{13}C\{^{1}H\}$ NMR (176 MHz, D_2O). (C) $^{31}P\{^{1}H\}$ NMR (202 MHz, D_2O). (D) ^{19}F NMR (471 MHz, D_2O).

Paper IV

Glycosylation with α-D-pentofuranose-1-phosphates leads to higher product yields for derivatives of 5-ethynyl-2′-deoxyuridine compared to transglycosylation reactions

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Abstract:

5-Ethynyluracil containing nucleosides like 5-ethynyl-2′-deoxyuridine are widely used for metabolic labeling of DNA. The standard production route is still based on chemical methods. In a first attempt, we used thermostable nucleoside phosphorylases to produce 5-ethynyl-2′-deoxyuridine and its sugar-modified derivatives in a one-pot transglycosylation reaction. For 5-ethynyl-2′-deoxyuridine and 2′-fluoro-5-ethynyluridine 41% and 18% of ethynyluracil was converted into the corresponding products, respectively. However, no product was obtained for (2′S)-2′-deoxy-2′-fluoro-5-ethynyluridine. Therefore, an alternative approach was used to produce 5-ethynyl-2′-deoxyuridine derivatives using natural and modified α-D-pentofuranose-1-phosphates and 5-ethynyluracil as substrates for thermostable nucleoside phosphorylases. Using this synthesis route, the efficient production of 5-ethynyluracil containing nucleoside analogues was successful. Percentages of conversion were 60%, 47% and 51% for 5-ethynyl-2′-deoxyuridine, 2′-fluoro-5-ethynyluridine and (2′S)-2′-deoxy-2′-fluoro-5-ethynyluridine, respectively. Also, for 5-ethynyl-2′-deoxyuridine and 2′-fluoro-5-ethynyluridine significantly higher product yields were obtained compared to transglycosylation reactions.

1 Introduction

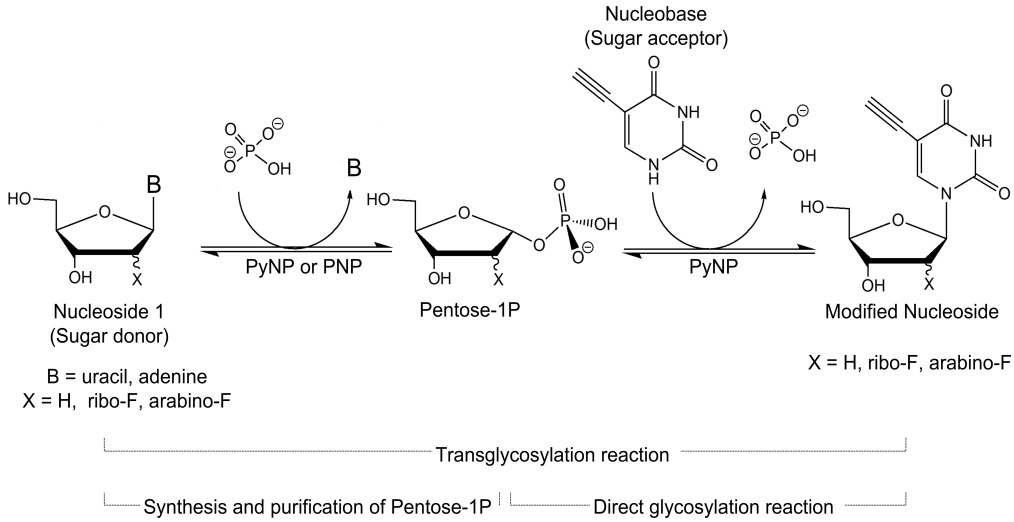
Nucleoside analogues (NAs) are important molecules used in the treatment of several viral and bacterial infections as well as cancer [1–4]. Additionally, they have been used in several biotechnological applications like DNA and RNA labelling [5,6]. 5-Bromo-2’-deoxyuridine (BrdU) is one of the commonly used nucleoside analogues for molecular DNA labelling [7–9]. After incorporation into cellular DNA, it can be detected by immunostaining with antibodies. Due to several drawbacks of BrdU, like poor tissue penetration or high cytotoxicity, alternatives were developed.

The introduction of the bioorthogonal chemical reporting concept revolutionized the study of biological macromolecules in their native environment [10]. The concept is based on the application of small chemical groups (like azide or alkyne) that are efficiently up-taken into cells. Furthermore, these molecules are efficiently incorporated into functional biomolecules without affecting any cellular or biochemical reactions [10]. Modified molecules can be detected by the coupling with a reporter molecule by copper(I)-catalyzed azide-alkyne cycloaddition (CuAAZ), also known as copper-catalyzed click reaction [11,12]. This method was initially modified from the Huisgen 1,3-dipolar cycloaddition [11]. It is, however, more advantageous as it does not require high temperatures or prolonged reaction times. Due to its high stereoselectivity only 1,4-disubstituted isomers are formed. In contrast, the Huisgen reaction leads to a mixture of both 1,4- and 1,5-disubstituted isomers.

5-ethynyl modified nucleoside analogues, particularly 5-ethynyl-2'-deoxyuridine (EdU) and (2′S)-2′-deoxy-2′-fluoro-5-ethynyluridine (^{2F}Ara-EdU), are widely used for DNA labeling [10,13–15]. The introduction of EdU was considered a big advancement in the field of metabolic labelling by applying click chemistry. Since then, EdU is being used in cell proliferation and differentiation studies, cell cycle analysis or to study repair mechanisms [13]. In 2011, ^{2F}Ara-EdU was reported to be less cytotoxic while maintaining DNA incorporation properties and efficient detection [10]. Therefore, it is one of the most suitable compounds for longer-term imaging.

As most nucleoside analogues, EdU and ^{2F}Ara-EdU are so far only synthesized chemically through multistep and laborious procedures [16,17]. Enzymatic synthesis routes have not yet been described for derivatives of EdU although they are widely applied to produce nucleoside analogues. One-pot two-steps transglycosylation reactions were extensively studied as an efficient method for the synthesis of nucleosides and their analogues [4,18–20]. The reaction includes the exchange of two nucleobases over a pentose-sugar moiety using nucleoside phosphorylases (NPs). Despite the advantages of the transglycosylation reaction, it showed low product yields especially for nucleosides with modifications on the sugar moiety [4,18]. As a substitution, the direct enzymatic glycosylation of nucleobases with α-D-pentofuranose-1-phosphates (Pentose-1Ps) using NPs was previously reported (**Scheme 1**) [18,19,21]. The availability of modified Pentose-1Ps was for a long time the bottleneck of this approach. The recently described method for the efficient and environmentally-friendly production of natural and modified Pentose-1Ps, however, creates new opportunities for future applications [22].

Here we present the enzymatic synthesis of EdU, 2′-fluoro-5-ethynyluridine ($_{2F}$Rib-EdU) and ^{2F}Ara-EdU using thermostable NPs. While transglycosylation for the synthesis of EdU works in principle, the synthesis of $_{2F}$Rib-EdU and ^{2F}Ara-EdU, respectively, were not successful. Therefore, 2-deoxy-α-D-ribofuranose-1-phosphate (dRib-1P), 2-deoxy-2-fluoro-α-D-ribofuranose-1-phosphate ($_{2F}$Rib-1P) and 2-deoxy-2-fluoro-α-D-arabinofuranose-1-phosphate (^{2F}Ara-1P) were enzymatically synthesized and used for the direct enzymatic glycosylation with 5-ethynyluracil with yields of the corresponding nucleoside analogues between 47 and 60%.



Scheme 1. Schematic representation of the investigated enzymatic approaches for the synthesis of 5-ethynyl-nucleoside derivatives: transglycosylation reaction and direct glycosylation. Substrates for the direct glycosylation were Pentose-1Ps that were produced in a chemo-enzymatic process beforehand

2 Methods

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, United States) reversed phase C18 column (150 × 4.6 mm). Thermostable nucleoside phosphorylases PyNP-Y02 (E-PyNP-0002), PyNP-Y04 (E-PyNP-0004) and PNP-N02 (E-PNP-0002) were obtained from BioNukleo (Berlin, Germany) and used as recommended by the manufacturer.

Synthesis of modified nucleosides by transglycosylation

EdU, ^{2F}Ara-EdU and $_{2F}$Rib-EdU were synthesized in a one-pot transglycosylation reaction using pyrimidine nucleoside phosphorylase (PyNP) and purine nucleoside phosphorylase (PNP) as biocatalyst and 5-ethynyluracil as sugar acceptor. Sugar donors were thymidine (Thd) or 2'-deoxyadenosine (dAdo) for the synthesis of EdU, and 1-(2'-deoxy-2'-fluoro-β-D-

arabinofuranosyl)uracil (^{2F}Ara-U) and 2'-deoxy-2'-fluorouridine ($_{2F}$Rib-U) for the synthesis of ^{2F}Ara-EdU and $_{2F}$Rib-EdU, respectively.

Using Thd, $_{2F}$Rib-U, ^{2F}Ara-U as sugar donors, 2 ml reaction mixtures of 10 mM sugar donor, 1 mM sugar acceptor and 5U of PyNP-Y04 in 2mM potassium phosphate (KP) buffer (pH 7) were prepared. The reaction mixture was incubated at 40°C for 30 h. With dAdo as a sugar donor, a final reaction volume of 2 mL was used. A reaction mixture of 10 mM sugar donor, 1 mM sugar acceptor, 5U of PyNP-Y04 and 5U PNP-N02 in 2mM KP buffer (pH 7) was prepared. The reaction was incubated at 40°C for 30 h.

Chemo-enzymatic synthesis and purification of Pentose-1Ps as barium salts

Pentose-1Ps were synthesized and purified as their barium salts as previously described [22]. Thd, ^{2F}Ara-U and $_{2F}$Rib-U were used for the synthesis of dRib-1P, ^{2F}Ara-1P and $_{2F}$Rib-1P, respectively. To a mixture of 200 mM nucleoside in 500 mM KP buffer (pH 7), PyNP-Y02 or PyNP-Y04 were added in concentrations of 0.1 – 1 mg/ml. Reaction temperatures between 40°C and 80°C were used. All Pentose-1Ps were purified as their barium salts and used for direct glycosylation reactions.

Synthesis of 5-ethynyluracil-containing nucleoside by direct glycosylation

EdU, ^{2F}Ara-EdU and $_{2F}$Rib-EdU were synthesized in a direct glycosylation reaction using dRib-1P, ^{2F}Ara-1P or $_{2F}$Rib-1P, respectively, and 5-ethynyluracil as a sugar acceptor in a ratio of 2:1 (sugar: sugar acceptor). EdU was synthesized in a final volume of 2 mL with 8 mM 5-ethynyluracil, 16 mM dRib-1P-Ba salt and 0.1 or 0.5 mg/mL PyNP-Y04 at 40°C for 5h. ^{2F}Ara-EdU and $_{2F}$Rib-EdU were enzymatically-synthesized at 50°C in a total reaction volume of 2 mL with 6 mM 5-ethynyluracil and 12 mM ^{2F}Ara-1P-Ba salt or $_{2F}$Rib-1P-Ba salt at 50°C for 24h and PyNP-Y04 concentration of 0.5 mg/mL.

High performance liquid chromatography (HPLC)

To monitor the enzymatic synthesis of Pentose-1Ps, HPLC analyses were performed. Conversion percentages were calculated as previously reported [22].

For the synthesis of 5-ethynyuracil-containing nucleoside analogues, HPLC analysis was performed with the following gradient: from 97% 20 mM ammonium acetate and 3% acetonitrile to 72% 20 mM ammonium acetate and 28% acetonitrile in 11 min. The reaction yields were determined by quantifying the nucleosides and nucleobases at 260 nm (**Formula 1**). Retention times under these conditions were as follows: Uracil (2.9 min), Thd (5.6 min),

Thymine (Thy) (4.3 min), 5-ethynyluracil (4.4 min), dAdo (6.2 min), adenine (4.6 min), $_{2F}$Rib-U (5.06 min), ^{2F}Ara-U (5.38 min), EdU (6.01 min), $_{2F}$Rib-EdU (4.11 min), ^{2F}Ara-EdU (4.12 min).

Formula 1.

$$\text{Reaction yield} = \frac{\text{Conc. of the formed nucleoside [mM]}}{\text{Conc. of the residual nucleobase [mM]} + \text{Conc. of the formed nucleoside [mM]}} \times 100$$

Thin layer chromatography (TLC)

Pentose-1Ps were detected colorimetrically by TLC as previously described [22]. 2 µl of the standard mixtures and the purified Pentose-1P were loaded on silica plates (Merck). Mobile phase of n-propanol, ammonia and H_2O (11: 2: 7) was used for dRib-1P reactions. A ratio of the same solvents of 11: 2: 5 was used for $_{2F}$Rib-1P and ^{2F}Ara-1P. Fluoro-modified Pentose-1Ps were detected colorimetrically using Hanes reagent, whereas dRib-1P was detected using *p*-anisaldehyde-sulphoric acid solution.

3 Results

Synthesis of EdU in a transglycosylation reaction

Initially, a one-pot transglycosylation reaction setup was used to enzymatically produce EdU, with either Thd or dAdo as sugar donors and 5-ethynyluracil as sugar acceptor (**scheme 1**). Based on preliminary results, transglycosylation reactions were performed in 2 mM phosphate buffer using a 10:1 ratio of the sugar donor to the sugar acceptor. Thermostable NPs were applied as biocatalyst.

Two strategies were examined to produce EdU. In the first approach only one enzyme was applied. PyNP-04 was used for both the cleavage of the sugar donor thymidine and the subsequent formation of EdU from the dRib-1P intermediate and 5-ethynyluracil. In the second approach, dAdo was used as sugar donor. It was cleaved by PNP-N02 to form the dRib-1P intermediate. The latter was then used by PyNP-Y04 to produce EdU from 5-ethynyluracil.

In the one enzyme approach, the cleavage percentage for Thd was 30% (**Fig. 1A**). Using dAdo as sugar donor a percentage of cleavage of 26% was observed (**Fig. 1A)**. Product yields for EdU were 41% and 16% with Thd and dAdo as sugar donors, respectively (**Fig. 1B**). As higher product yields were obtained with the one-enzyme strategy, it was used for reactions with sugar-modified substrates

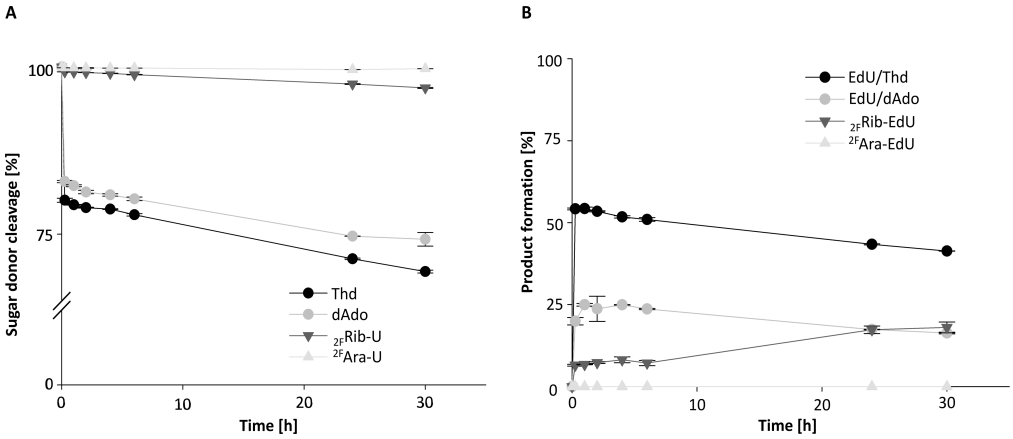


Figure 1 . Enzymatic synthesis of 5-ethynyl-deoxyuridine (●, ●), 2′-fluoro-5-ethynyluridine (▼) and (2′S)-2′-deoxy-2′-fluoro-5-ethynyluridine (▲) in one-pot transglycosylation reactions using thermostable nucleoside phosphorylase. 5-Ethynyl-deoxyuridine was produced either with Thd (●) or dAdo (●) as sugar donors. For the synthesis of 2′-fluoro-5-ethynyluridine and (2′S)-2′-deoxy-2′-fluoro-5-ethynyluridine, $_{2F}$Rib-U or ^{2F}Ara-U were used as sugar donors, respectively. The one-pot transglycosylation reaction was performed at 40°C and the reaction was monitored over a period of 30 h. (**A**) The cleavage of the sugar donors Thd, dAdo, $_{2F}$Rib-U or ^{2F}Ara-U. Pyrimidine nucleosides were converted by PyNP-Y04 and dAdo by PNP-N02. (**B**) The formation of the of 5-ethynyl-containing nucleoside analogues by PyNP-Y04. Thd: thymidine; dAdo: 2'deoxyadenosine; $_{2F}$Rib-U: 2'-deoxy-2'-fluorouridine; ^{2F}Ara-U: 1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)uracil.

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Transglycosylation reaction for the synthesis of 2'-fluoro-5-ethynyluracil nucleoside derivatives

$_{2F}$Rib-EdU and ^{2F}Ara-EdU were synthesized using PyNP-Y04 and the sugar donors $_{2F}$Rib-U and ^{2F}Ara-U, respectively. A percentage of cleavage to their corresponding Pentose-1P and uracil after 30 h was 3% and 0.5%, respectively, (**Fig. 1A**). The cleavage reaction progressed very slowly over the monitored reaction time of 30 h and equilibrium was not reached. The formation of the products $_{2F}$Rib-EdU and ^{2F}Ara-EdU was correlating with sugar donor cleavage. Product yields after 30 h were 18% and 0% for $_{2F}$Rib-EdU and ^{2F}Ara-EdU, respectively (**Fig. 1B**). The formation of 6.5% $_{2F}$Rib-EdU was already detected 15 min after starting the reaction. Afterwards, product formation slowly increased to 18% till the end of the reaction. An equilibrium was not reached within 30 h.

Chemo-enzymatic synthesis and purification of dRib-1P, $_{2F}$Rib-1P, and ^{2F}Ara-1P

As product yields for $_{2F}$Rib-EdU and ^{2F}Ara-EdU were low, the direct glycosylation approach starting from purified Pentose-1Ps, was evaluated. Therefore, dRib-1P, $_{2F}$Rib-1P, and ^{2F}Ara-1P were enzymatically synthesized and purified as their corresponding barium salts as previously described [22]. PyNP-Y02 was used as a biocatalyst for the synthesis of dRib-1P using Thd as a substrate. Due its higher tolerance to sugar-modifications, PyNP-Y04 was chosen to produce $_{2F}$Rib-1P and ^{2F}Ara-1P. The percentages of enzymatic cleavage of Thd, $_{2F}$Rib-U and ^{2F}Ara-U were 43%, 35% and 22%, respectively (**Fig. 2A**). A reaction time of 6 h was chosen for Thd due to the instability of dRib-1P [23]. A reaction time of 72 h was used for $_{2F}$Rib-U and ^{2F}Ara-U. For Thd cleavage, the reaction proceeded very fast and equilibrium was reached after 1 h. The formation of both $_{2F}$Rib-1P and ^{2F}Ara-1P was steady over the reaction duration. Equilibrium was not reached within 72h.

Pentose-1Ps were purified as barium salts after phosphate depletion by manganese ammonia precipitation. Based on the starting substrate concentrations, final yields for Pentose-1P-Ba salts were 22% for dRib-1P-Ba and 23% for both $_{2F}$Rib-1P-Ba and ^{2F}Ara-1P-Ba after purification (**Fig. 2A**). Purity of the Pentose-1Ps was determined by TLC and HPLC to be higher than 95% (**Fig. 2B and 2C**). TLC showed clear bands for each of the tested Pentose-1Ps with no detectable traces of the nucleoside or the nucleobase. Residues of phosphate, however, were detected in $_{2F}$Rib-1P-Ba.

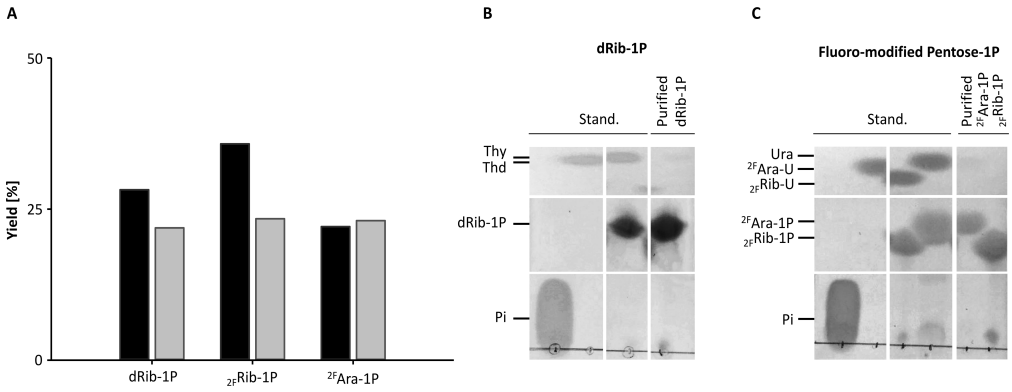

Figure 2. Enzymatic synthesis of natural and modified Pentose-1P. (**A)** Product yield of Pentose-1Ps after the enzymatic reaction (black bars) and the purified Pentose-1P as Ba salts (grey bars). (**B)** Assessment of the purity of the synthesized dRib-1P-Ba salts by thin layer chromatography (TLC). **(C)** Assessment of the purity of the synthesized fluoro-modified Pentose-1P-Ba salts ($_{2F}$Rib-1P-Ba and ^{2F}Ara-1P-Ba) by TLC. Thd: thymidine; Thy: thymine; dRib-1P: 2-deoxy-α-D-ribofuranose-1-phosphate; Pi: inorganic phosphate; $_{2F}$Rib-U: 2'-deoxy-2'-fluorouridine; ^{2F}Ara-U: 1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)uracil; $_{2F}$Rib-1P: 2-deoxy-2-fluoro-α-D-ribofuranose-1-phosphate; ^{2F}Ara-1P: 2-deoxy-2-fluoro-α-D-arabinofuranose-1-phosphate.

Synthesis of 5-ethynyluracil-containing modified nucleosides in a direct glycosylation reaction

To produce EdU, $_{2F}$Rib-EdU and ^{2F}Ara-EdU in a direct glycosylation reaction, 5-ethynyluracil and Pentose-1Ps were used as substrate. The percentage of EdU formation was 60% and 37% using ratios for dRib-1P-Ba and base of 2:1 and 1:1, respectively (**Fig. 3A**). For both reaction conditions, equilibrium was reached after 1 to 3 h. Varying enzyme concentrations did not significantly influence the final product yields.

As higher product yields were obtained with a ratio of 2:1 for Pentose-1P to base, the same conditions were applied for the synthesis of $_{2F}$Rib-EdU and ^{2F}Ara-EdU. However, no product formation was observed with enzyme concentrations of 0.1 mg/mL. Therefore, final enzyme concentrations were increased to 0.5 mg/ml. Yields for $_{2F}$Rib-EdU and ^{2F}Ara-EdU were 47% (2.8 mM) and 51% (3 mM) after 24h, respectively (**Fig. 3B**). A reaction equilibrium was not reached over the reaction duration. Compared to transglycosylation reactions product yields increased 2.5 and 0.9 fold for $_{2F}$Rib-EdU and EdU respectively.

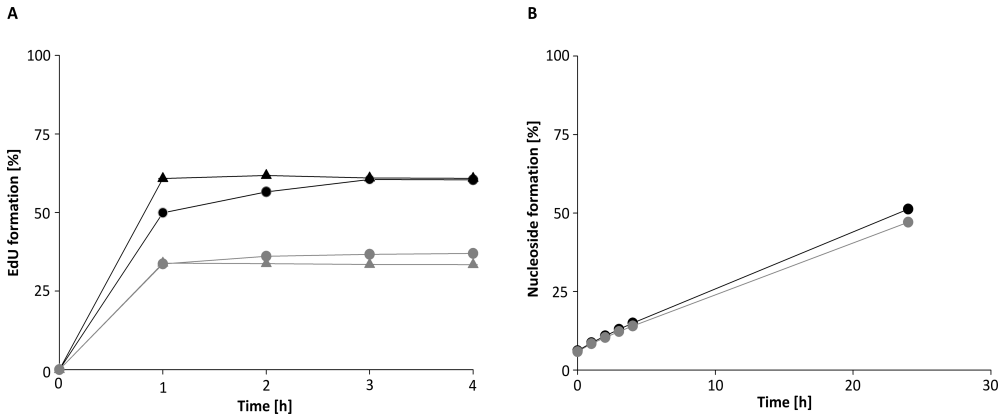

Figure 3. Enzymatic synthesis of 5-ethynyl-containing nucleoside analogues by direct glycosylation reactions using thermostable nucleoside phosphorylase PyNP-Y04. (**A**) EdU synthesis [%] was performed with 8mM 5-ethynyluracil as a sugar acceptor. dRib-1P concentrations of 8 mM (●,▲) and 16 mM (●,▲) and the enzyme concentration of 0.1 mg/ml (●, ●) and 0.5 mg/ml (▲,▲) were applied. Reaction was performed at 40°C for 4h. (**B**) Synthesis of $_{2F}$Rib-EdU (●) and ^{2F}Ara-EdU (●) using a ratio of 2:1 Pentose-1P to 5-ethynyluracil (sugar acceptor) and enzyme concentration of 0.5mg/ml. Reaction was performed at 50°C for 24h. The Concentration of the formed nucleoside and the depleted nucleobase was evaluated by HPLC. EdU: 5-ethynyl-deoxyuridine; dRib-1P: 2-deoxy-α-D-ribofuranose-1-phosphate; $_{2F}$Rib-EdU: 2′-fluoro-5-ethynyluridine; ^{2F}Ara-EdU: (2′S)-2′-deoxy-2′-fluoro-5-ethynyluridine.

4 Discussion

In this study, the efficient enzymatic synthesis of 5-ethynyluracil-containing nucleoside analogues using thermostable NPs was shown. In a glycosylation reaction using purified Pentose-1P analogues and 5-ethynyluracil as substrates, product yields between 47% to 60% were reached for EdU, $_{2F}$Rib-EdU and ^{2F}Ara-EdU. The results are in accordance with previous reports where direct enzymatic glycosylation of different nucleobases using PNPs or PyNPs was successfully demonstrated [24–27].

During the past decade, the use of nucleoside analogues with 5-ethynyluracil as base moiety has been of increasing interest as a powerful tool to track DNA replication and cell proliferation [13]. EdU is a highly sensitive, quick and efficient labelling tool with several prominent features. The small size of the linker azide group allows good tissue penetrability hence, denaturation treatment is not required and therefore the DNA structure is preserved. Despite the advantages of EdU, it is a toxic antimetabolite that might cause DNA instability, cell-cycle arrest or necrosis [10]. ^{2F}Ara-EdU, a Less toxic derivative, was described as suitable alternative [10]. However, a widespread use is hampered due to the complex and inefficient chemical synthesis route. Therefore, more efficient synthesis routes are of big interest.

Transglycosylation reaction is widely described for the synthesis of nucleosides and their analogues. It is generally applied when natural nucleosides or arabinosides are used as sugar donors [4,18,19]. In this study, EdU was produced with moderate yield in a transglycosylation reaction with PyNP-Y04 as biocatalyst. Hence, the base 5-ethynyluracil was well accepted by the applied enzyme. The electron withdrawing ethynyl group (C≡CH) at the 5-position is comparable to halogen, alkene and haloalkenyl groups that were tolerated well by other PyNPs [28]. Furthermore it is less bulky than some of these substituents, which enhances the acceptance by the enzymes [28–30].

In the case of fluororiboside and fluoroarabinoside synthesis, transglycosylation was described to show only low product yields even if extremely extended reaction times and excessive amounts of enzyme were used [31]. When 2'-deoxy-2'-fluorouridine was used as a sugar donor to produce different modified 2'deoxy-2'fluoro-purine nucleosides with potential antiviral and cytotoxic activity, reaction times reached almost two months in some cases and the amount of enzyme was almost 6000 U of TP and double that of PNP to achieve acceptable yields [32]. In this study, only low or no product formation was observed for $_{2F}$Rib-EdU and ^{2F}Ara-EdU synthesis using the transglycosylation approach. Low yields can be mainly correlated to the low cleavage percentage of the sugar donor nucleosides. While the cleavage percentages for ^{2F}Ara-U and $_{2F}$Rib-U were only 0.5% and 3%, respectively, it was around 30%

for the natural nucleoside thymidine. This might be attributed to the high electronegativity of the fluorine substitution on 2´-position of the sugar moiety. It leads to a shorter and hence, stronger glycosidic bond [33]. Consequently, a different conformation of the sugar moiety is formed which affects the Walden inversion at the tetrahedral C1' atom and the S_N2 reaction catalyzed by NPs [31,34].

Direct glycosylation in contrast, was as a viable approach to drastically increase product yields. This approach offers many advantages as it allows for an independent optimization of reaction conditions for each step (cleavage and glycosylation reactions). One critical factor of transglycosylation reactions is the phosphate concentration. High phosphate ions concentration shifts the equilibrium towards the nucleoside cleavage and synthesis of the Pentose-1P. Therefore, its presence favors the cleavage reaction but hinders the glycosylation reaction starting from the Pentose-1P intermediate [35]. If the sugar donor is a good substrate for the first enzyme, this critical balance is overcome and the equilibrium is shifted towards the synthesis of the second nucleoside. However, in cases where K_m values are high, the reaction fails to proceed towards nucleoside 2 as the pentose-1P production via phosphorolysis becomes the rate determining step [33].

5 Conclusions

It is well known that fluorine atom in the 2'-position has a favorable effect on the stability and the bioavailability of the corresponding compounds. However, the availability of these compounds is limited by the ease of their production. So far, both chemical and enzymatic methods have shown several drawbacks and led to low product yields. The application of glycosylation reactions is a promising strategy to produce base and sugar modified nucleosides with high product yields. It is not only limited to EdU derivatives but could be transferred to the production of therapeutic nucleoside analogues with modification at the sugar moiety.

Conflict of Interest

A.W. is CEO and P.N. a shareholder of the biotech startup BioNukleo GmbH. The authors have no other relevant affiliations, financial interest or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No conflict of interest is known to the other authors.

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