Chemoenzymatic synthesis of nucleoside analogs as potential medicinal agents

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Abstract

Modified nucleosides are important drugs used to treat cancer, viral or bacterial infections. They also serve as precursors for the synthesis of modified oligonucleotides (antisense oligonucleotides (ASOs) or short interfering RNAs (siRNAs)), a novel and effective class of therapeutics. While the chemical synthesis of nucleoside analogs is challenging due to multi-step procedures and low selectivity, enzymatic synthesis offers an environmentally friendly alternative. However, current challenges for the enzymatic synthesis of nucleoside analogs are the availability of suitable enzymes or the high costs of enzymes production.

To address these challenges, this work focuses on the application of thermostable purine and pyrimidine nucleoside phosphorylases for the chemo-enzymatic synthesis of nucleoside analogs. These enzymes catalyze the reversible phosphorolysis of nucleosides into the corresponding nucleobase and pentofuranose-1-phosphate and have already been successfully used for the synthesis of modified nucleosides in small scale. So far, the production of sugar-modified nucleosides has been a major challenge. In this study, it was shown that the synthesis of arabinose- or fluoroarabinose-containing nucleoside analogs by glycosylation starting from modified sugar-1-phosphates is possible with very high yields. This was not possible using transglycosylation reactions. One very evident example is the synthesis of 5-ethynyl-2´-deoxy-2´-fluorouridine arabinoside. It was not obtained by transglycosylation with fluoroarabinofuranosyl uracil as sugar donor whereas almost 50 % of the base was transformed by direct glycosylation with the corresponding 2-deoxy-2-fluoro-arabinofuransoe-1-phosphate.

Halogenated ribo- and deoxynucleosides were produced at mg scale with a reagent grade purity of >95 %. The cytotoxic activity in different blood tumor cell lines (HL-60 and CCRF-CEM) was tested for these nucleoside analogs and it was shown that the enzymatically produced compounds showed similar IC_{50} values as their chemically produced counterparts. Compared to ribonucleosides, deoxyribonucleosides revealed a reduced non-specific cytotoxicity.

In order to transfer the synthesis of modified nucleosides to a larger scale, the expression of thermostable nucleoside phosphorylases was first established by high cell density fed-batch cultivation in a laboratory bioreactor. Almost three times the volumetric yield of the recombinant protein PNP 03 was produced in the fed-batch cultivation in comparison to the shake flask cultures. The biocatalytic process was also optimized; exemplified by the synthesis of halogenated nucleoside analogs using a continuous enzyme membrane reactor. The results of the substrates conversion for nucleoside synthesis were very similar for discontinuous and continuous reactions. The enzymes were stable with natural substrates for several weeks and with modified substrates for up to 7 days. In addition, heat-treated cell lysate achieved a similar result to purified enzymes.

Zusammenfassung

Modifizierte Nukleoside sind wichtige Medikamente zur Behandlung von Krebs, viralen oder bakteriellen Infektionen. Sie dienen auch als Vorläufer für die Synthese von modifizierten Oligonukleotiden (Antisense-Oligonukleotide (ASOs) oder *short-interfering RNAs* (siRNAs)), einer neuen und sehr effektiven Klasse von Therapeutika. Während die chemische Synthese von Nukleosidanaloga aufgrund von Mehrschrittverfahren und geringer Selektivität eine Herausforderung darstellt, bietet die enzymatische Synthese eine umweltfreundliche Alternative. Aktuelle Herausforderungen für die enzymatische Synthese von Nukleosidanaloga sind die Verfügbarkeit geeigneter Enzyme oder hohe Kosten für die Enzymproduktion.

Um diese Herausforderungen zu bewältigen, konzentriert sich diese Arbeit auf die Anwendung von thermostabilen Purin- und Pyrimidinnukleosidphosphorylasen für die chemo-enzymatische Synthese von Nukleosidanaloga. Diese Enzyme katalysieren die reversible Phosphorolyse von Nukleosiden in die entsprechende Nukleobase und Pentofuranose-1-phosphat. Sie wurden bereits erfolgreich für die Synthese von modifizierten Nukleosiden im kleinen Maßstab eingesetzt. Bisher war die Herstellung von zuckermodifizierten Nukleosiden eine große Herausforderung. In dieser Studie wurde gezeigt, dass die Synthese von Arabinose- oder Fluoroarabinose-haltigen Nukleosidanaloga durch Glykosylierung ausgehend von modifizierten Zucker-1-Phosphaten mit sehr hohen Ausbeuten möglich ist. Dies war mit Transglykosylierungsreaktionen nicht möglich. Ein sehr anschauliches Beispiel ist die enzymatische Synthese von 5-Ethinyl-2´-deoxy-2´-fluorouridin arabinosid. Mittels Transglykosylierung mit Fluorarabinofuranosyluracil als Substrat war die Synthese nicht möglich. Hingegen wurden fast 50 % der Base durch direkte Glykosylierung umgesetzt.

Halogenierte Ribo- und Desoxynukleoside wurden im mg-Maßstab mit einer Reinheit von >95 % hergestellt. Die zytotoxische Aktivität dieser Nukleosidanaloga wurde in verschiedenen Bluttumorzelllinien (HL-60 und CCRF-CEM) getestet und es wurde gezeigt, dass die enzymatisch hergestellten Verbindungen ähnliche IC50-Werte aufwiesen wie ihre chemisch hergestellten Pendants. Im Vergleich zu Ribonukleosiden zeigten Desoxyribonukleoside eine reduzierte unspezifische Zytotoxizität.

Um die Synthese von modifizierten Nukleosiden in einen größeren Maßstab zu transferieren, wurde die Expression von thermostabilen Nukleosid-Phosphorylasen zunächst in einen Hochzelldichte-Fed-Batch-Prozess in einem Laborbioreaktor etabliert. In den Kultivierungen des Fed-Batch-Bioreaktors wurde im Vergleich zu Kulturen in Schüttelkolben fast die dreifache volumetrische Ausbeute des rekombinanten Proteins PNP 03 erzeugt. Der biokatalytische Prozess wurde am Beispiel der Synthese von halogenierten Nukleosidanaloga in einem kontinuierlichen Enzymmembranreaktor optimiert. Die Umsatzraten waren für diskontinuierliche und kontinuierliche Reaktionen sehr ähnlich. Die Enzyme waren bei natürlichen Substraten mehrere Wochen und bei modifizierten Substraten bis zu sieben Tage stabil. Mit Hitze-behandeltem Zelllysat wurden ähnliche Ergebnisse erzielt wie mit gereinigtem Enzym.

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List of Publications

I. Heba Yehia*, Sarah Kamel*, Katharina Paulick, Peter Neubauer, Anke Wagner. Substrate spectra of nucleoside phosphorylases and their potential in the production of pharmaceutically active compounds. Curr Pharm Des. 2017; 23(45): 6913-6935.

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II. Sarah Kamel*, Heba Yehia*, Peter Neubauer, Anke Wagner. Enzymatic Synthesis of Nucleoside Analogues by Nucleoside Phosphorylases. In: Fernández-Lucas, J. and Camarasa-Rius, M. (eds) Enzymatic and Chemical Synthesis of Nucleic Acid Derivatives. John Wiley & Sons 1–28.

https://doi.org/10.1002/9783527812103.ch1

III. Heba Yehia*, Sarah Kamel*, Peter Neubauer, Anke Wagner. Glycosylation with α-D-pentofuranose-1-phosphates leads to higher product yields for nucleoside analogs compared to transglycosylation reactions. (In preparation).

IV. Heba Yehia, Hendrik F. T. Klare, Peter Neubauer, Jens Kurreck, Anke Wagner.

Halogenated nucleoside analogs produced in a chemo-enzymatic process are highly active in leukemic cell lines. (In preparation).

*Authors contributed equally to the work

Author's Contribution to the Papers

Paper	Co-author	Contribution
I	Heba Yehia	Writing section 4, preparation of figures and tables
	Sarah Kamel	
	Katharina Paulick	Writing abstract, preparation of figures and tables
	Peter Neubauer	General concept and outlook of the paper
	Anke Wagner	General concept and outlook of the paper, writing section 1-3 and 5
	All co-authors read the paper, revised and corrected the final manuscript	
II	Sarah Kamel	Writing sections 1, 2 and 3, preparation of figures and tables
	Heba Yehia	Writing sections 4, 5 and 6, preparation of figures and tables
	Peter Neubauer	Revising the manuscript
	Anke Wagner	General concept, writing section 7 and proofreading the manuscript
III	Heba Yehia	Experimental work of the transglycosylation reactions, writing the related materials and methods, results and the discussion
	Sarah Kamel	Experimental work of the pentofuranose-1-phosphates synthesis, writing the introduction, related material and methods and results
	Peter Neubauer	Research concept
	Anke Wagner	Supervision and proofreading the written paper
IV	Heba Yehia	Experimental work, writing the paper and preparing the figures
	Hendrik F. T. Klare	NMR measurements and interpretation
	Peter Neubauer	Research concept
	Jens Kurreck	Supervising the cytotoxicity experiments
	Anke Wagner	Research concept, supervision and proofreading the written paper

List of Abbreviations

2CA	2-Chloroadenine
2FA	2-Fluoroadenine
Ade	Adenine
Ado	Adenosine
ALL	Acute lymphocytic (lymphoblastic) leukemia
API	Active pharmaceutical ingredient
*Ap*MTAP	5'-Methythioadenosine phosphorylase from *Aeropyrum pernix*
Ara-1-P	D-Arabinose 1-phosphate
AraA	9-β-D-arabinofuranosyladenine
AraU	1-β-D-arabinofuranosyl uracil
ATP	Adenosine triphosphate
CCRF-CEM	Human peripheral blood acute lymphoblastic leukemia cell line
CFP	6-Chloro-2-fluoropurine
DAD	Diode-array detector
dAdo	2'-deoxyadenosine
DAP	2-Aminoadenine
DCP	2,6-Dichloropurine
DCW	Dry cell weight
dGTP	2'-Deoxyguanosine triphosphate
dGuo	2'-Deoxyguanosine
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
dNTP	2'-Deoxyribonucleoside triphosphate
DOT	Dissolved oxygen tension
dR	2-Deoxyribose
dR-1-P	2-Deoxy-D-ribofuranose 1-phosphate
dTMP	Thymidine monophosphate
dUMP	2'-Deoxyuridine monophosphate
E. coli	*Escherichia coli*
EC	Enzyme commission number
EdU	5-Ethynyl-deoxyuridine
EMR	Enzyme membrane reactor
ENT	Equilibrative nucleoside transporter
EU-FAra	(2'S)-2'-deoxy-2'-fluoro-5-ethynyluridine
EU-FR	2´-Deoxy-2'-fluoro-5-ethynyluridine
EUra	5-Ethynyluracil
FanaU	1-(2'-Deoxy-2'-fluoro-β-D-arabinofuranosyl) uracil
FAra-1-P	2-Deoxy-2-fluoro-α-D-arabinofuranose-1-phosphate
FPLC	Fast protein liquid chromatography
FR-1-P	2-Deoxy-2-fluoro-α-D-ribofuranose-1-phosphate
FU	1-(2'-deoxy-2'-fluoro-β-D-ribofuranosyl) uracil
HBV	Hepatitis B virus

HCV	Hepatitis C virus
HEK293	Human embryonic kidney 293 cells
HIV	Human immunodeficiency virus
HL-60	Human leukemia cell line
HPLC	High-performance liquid chromatography
HRT	Hydraulic residence time
IB	Inclusion bodies
IPTG	Isopropyl β-D-1-thiogalactopyranoside
k_m	Michaelis constant
KP	Potassium phosphate buffer
LB	Lysogeny broth
MRP	Multidrug resistance protein
MSM	Mineral salts medium
MTAP	S-methyl-5'-thioadenosine phosphorylase or 5’-methythioadenosine phosphorylase
MWCO	Molecular weight cut-off
NA	Nucleoside analog
NAD	Nicotinamide adenine dinucleotide
NDT	Nucleoside deoxyribosyltransferase
NMR	Nuclear magnetic resonance
NP	Nucleoside phosphorylase
OAT	Organic anion transporter
OD_{600}	Optical density of a sample measured at a wavelength of 600 nm
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEPT	Peptide transporter
PES	Polyethersulfone
PID	Proportional-integral-derivative controller
PMS	N-methyl dibenzopyrazine methyl sulfate
PNP	Purine nucleoside phosphorylase
PPRT	Pyrimidine phosphoribosyl transferase
PyNP	Pyrimidine nucleoside phosphorylase
q_{CO2}	Specific carbon dioxide production rate
q_{O2}	Specific oxygen uptake rate
R-1-P	D-Ribose 1-phosphate
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
TB	Terrific broth
TCA	1,2,4-triazole-3-carboxamide
TCA cycle	Citric acid cycle or Tricarboxylic acid cycle
Thd	Thymidine
TP	Thymidine phosphorylase
UP	Uridine phosphorylase
Urd	Uridine
V_{max}	Enzyme's maximum rate
XTT	Sodium salt of 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide
α-PF-1-P	α-D-pentofuranose-1-phosphate

1. Introduction

Nucleoside analogs (NAs) (also called: modified nucleosides, nucleoside mimetics) are glycosylamines acting as purine or pyrimidine antagonists. They possess a wide range of applications as therapeutic agents, in diagnostic, food industry or research-relevant fields. Their clinical use started with the approval of 6-mercaptopurine as anticancer agent in 1953 (Burchenal *et al.*, 1953), which is still on the world health organization (WHO) list of essential medicines for the treatment of acute lymphatic leukemia (ALL). Up till now, after more than 60 years of clinical and diagnostic applications, the ongoing research suggests that this class of compounds is still regarded as an interesting one with more potential to be explored.

In structure, NAs show modifications compared to the five canonical nucleosides (Fig. 1). Modifications were introduced to the base or sugar part or to both. Functional group substitutions including halogenation (e.g., cladribine, idoxuridine, gemcitabine), ring opening (e.g., ribavirin, acyclovir), ring expansion (e.g., pentostatin, azepinomycin) or dehydroxylation (e.g., carbovir, abacavir) are examples of the possible variations found in NAs (Fig. 2). As active pharmaceutical ingredients (APIs), NAs are regarded as prodrugs that need to be activated by phosphorylation inside the cells to the triphosphate forms.

The standard for NAs synthesis is still chemical methods which are (i) laborious with many steps that necessitate purification between each step, (ii) environmentally-unfriendly involving many hazardous solvents and chemicals and (iii) not highly efficient due to the production of many byproducts because of the poor regio- and stereoselectivity. In contrast, enzymatic or chemo-enzymatic methods offer a greener, more efficient and selective process. However, a hindrance to a wider application of enzymatic methods is the high cost load of the enzyme. Furthermore, enzymes cannot be used to produce NAs with more complicated modifications due to their inherent substrate specificity.

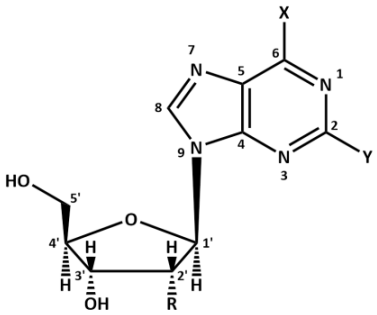
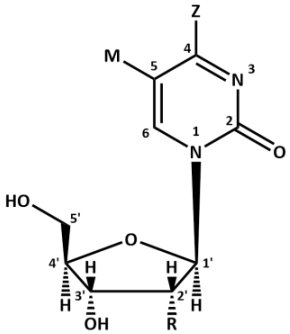
(a) Purine nucleosides	(b) Pyrimidine nucleosides
	
Adenine: X=NH_2, Y=H	Cytosine: Z=NH_2, M=H
Guanine: X=O, Y=NH_2	Thymine: Z=O, M=CH_3
	Uracil: Z=O, M= H
Riboside: R=OH, Deoxyriboside: R=H	

Fig. 1: General structure of nucleosides showing (a) natural purine nucleosides and (b) natural pyrimidine nucleosides.

Different approaches can be envisioned to reduce the cost of the biocatalysts. This can happen by addressing either the enzyme production or how it is used for biocatalysis or both. The former can be

achieved by high cell density cultivations, optimization of the expression vectors and expression conditions and using partially purified proteins, crude cell extracts or whole cells. The latter mainly focuses on reusing the enzymes to make the maximal use of their biocatalytic life span. For this reason, different immobilization techniques were developed as well as devices that permit continuous enzymatic reactions. Additionally, dedicated studies of mathematical modeling of enzyme kinetics enable the *in silico* optimization of the enzymatic reaction conditions.

Recently thermostable enzymes were identified as interesting biocatalysts for the synthesis of NAs. They bear many advantages when compared to mesophilic ones. These include easier purification of the proteins through a heat shock step that disposes most of the proteins of the expression host (typically *E. coli*), performing the reactions at higher temperatures (>45 °C) allowing the better solubility of poorly soluble substrates (many nucleobases) and avoiding the contamination by environmental mesophilic bacteria. Furthermore, there is the possibility of cosolvency with organic solvents in the case of poorly soluble substrates and higher diffusion coefficients that are usually correlated to higher reactions yields (Lasa and Berenguer, 1993; Haki and Rakshit, 2003; Sinisterra *et al.*, 2010).

In the present work, we set up a whole process of producing nucleoside analogs via a chemo-enzymatic approach starting with cheap and commercially-available precursors. The production of thermophilic enzymes was up-scaled from shake flask cultivations to benchtop bioreactor cultivations. The synthesis of halogen-substituted purine NAs using thermophilic NPs was studied in both small scale batch reactions and in enzyme membrane reactors. NA production yields were compared using transglycosylation reactions or direct glycosylation approaches. Finally, the therapeutic potential of halogenated NAs was studied in human leukemia cell lines.

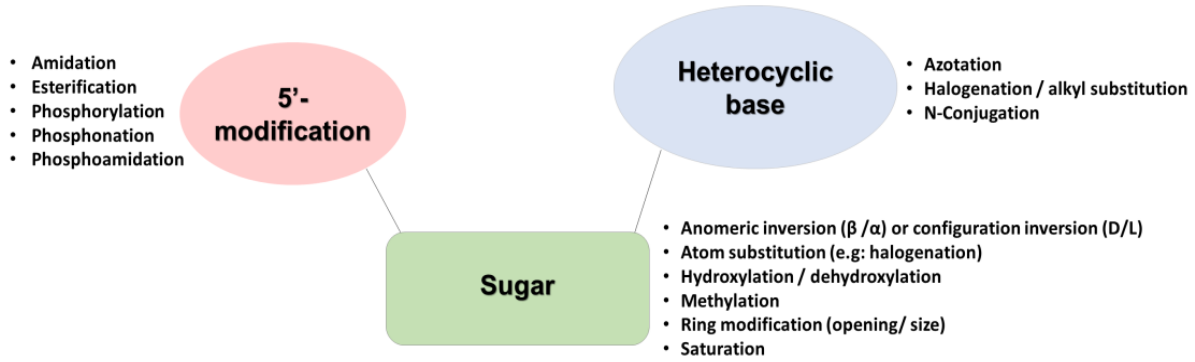


Fig. 2: Possible modifications of nucleosides, adapted from (Wiebe, 2007; Jordheim *et al.*, 2013).

2. Scientific Background

Antimetabolites are compounds with a similar structure to their physiological counterparts, hence, they can affect their production, metabolism or usage and interfere with the involved biological pathways. Therefore, they represent one of the oldest rationally designed therapeutic classes. As drugs, they mainly affect the S phase of the cell cycle by mimicking the role of natural metabolites making them quite eligible for use as antiproliferative agents where the rate of cell division is higher in cancer cells compared to normal cells. This circumstance also ensures that healthy cells are only minimally affected (Avendaño and Menéndez, 2008; Peters, 2014; Jeswani and Paul, 2017). Antimetabolite anticancer drugs are either folate antagonists or nucleoside analogs.

It was the unprecedented drug design approach from Gertrude Elion and George Hitchings that introduced NA into the focus of research. They created antimetabolites that could interfere with nucleic acids production and growth inside the cells and should, as a result, possess anticancer, antiprotozoal, antiviral and antibacterial properties (Fig. 2). They were awarded a Nobel Prize in physiology or medicine in 1988 on account of their achievements in this research area (Elion, 1989).

After more than 60 years of application, ongoing research suggests that the class of NAs is still regarded as an interesting field with promising prospect. Sofosbuvir is one clear example that highlights the potential of this group of therapeutics (Fig. 3). This uridine derivative was developed in 2007 in an endeavor to create compounds that target NS5B RNA-dependent RNA polymerase (RdRp) and was named after its inventor Michael Sofia. In 2013, it was approved by the food and drug administration (FDA), under the trade name Sovaldi®, and later by the European Medicines Agency (EMA) for treating chronic HCV, in combination with ribavirin (another NA; guanosine analog). Almost a year later, another formulation was approved under the name Harvoni® which combines it with ledipasvir (HCV NS5A inhibitor). This actually positioned Sofosbuvir as the fundamental core of HCV treatment regimes. The first year recorded sales of Sovaldi of almost US$ 11 billion, marking the most successful launch in the history of pharmaceuticals (Sofia, 2016).

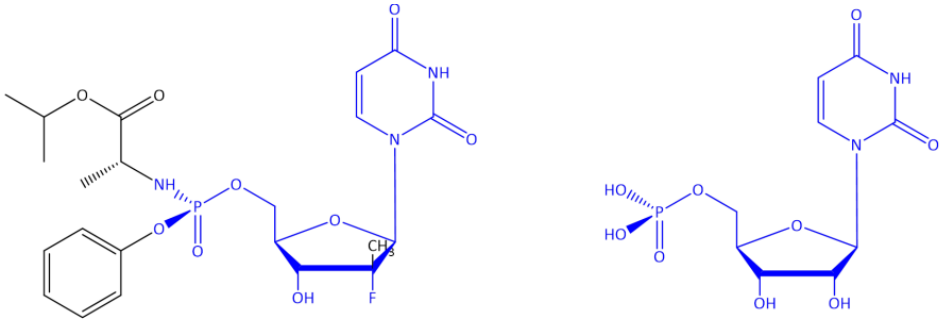


Fig. 3: Structure of Sofosbuvir in comparison to the canonical uridine monophosphate. Common parts are depicted in blue.

Beside the well-known therapeutic value of NAs, there are some other applications that also merit research. The applications are versatile including, but not limited to, universal or inert bases for sequencing or PCR reactions, probes for nucleic acids labeling, and increased stabilization and base stacking by non-specific hybridization (Loakes, 2001; Yang *et al.*, 2018). As they mimic the natural molecules, they help to visualize and study normal cell physiological processes (e.g., proliferation, defense mechanisms, drug resistance). Additionally NA derivatives have an increasing importance as

fluorescent reporters and click chemistry linkers (Loomis and Bell, 1988; Guo *et al.*, 2010; Choi and Berdis, 2016; Pereira *et al.*, 2017).

2.1. Naturally occurring nucleoside analogs

NAs with different modifications (Fig. 4) have been isolated from several marine and terrestrial organisms. Over the years, these substantial discoveries have helped to understand the cellular metabolic pathways on the molecular level and inspired possible modifications for medicinal use.

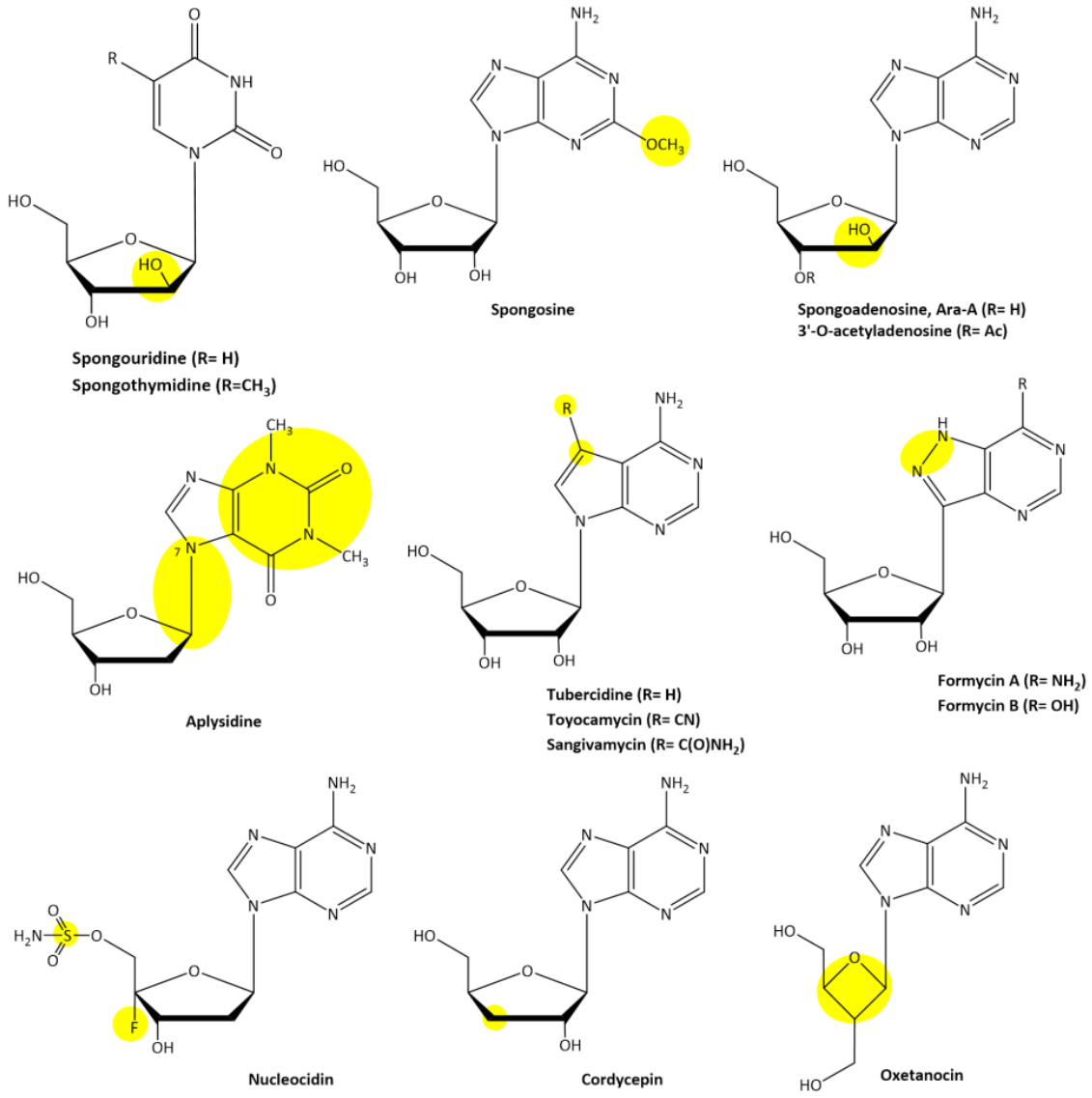


Fig. 4: Examples of naturally occurring non-canonical nucleosides.

Different genera, mainly sponges and actinobacteria, were reported to be the principal producers of such bioactive NAs. For example, the pyrimidine analogs spongothymidine and spongouridine (3-β-D-arabinofuranosyl thymine and uracil) and the adenosine analog spongosine (2-methoxyadenosine) were first obtained from the marine sponge *Cryptotethya crypta* (Bergmann and Feeney, 1950; Bergmann and Burke, 1955; Bergmann and Stempien Jr., 1957). Both compounds were the basis for developing vidarabine and cytarabine which are still in use as antiviral and cytotoxic agents, respectively (Laport *et al.*, 2009). Vidarabine was later discovered, together with its

3'-O-acetyl derivative, in the soft coral *Eunicella cavolini* (yellow gorgonian) some years after it was already approved for therapeutic use (Cimino *et al.*, 1984).

Aplysidine has a unique structure with glycosidic bond attached to N7 and its nucleobase resembling theophylline. It was discovered in the marine sponge *Aplysina* and like theophylline, it can also antagonize adenosine A1 receptors (Kondo *et al.*, 1992; Huang *et al.*, 2014). 7-Deazaadenosine analogs tubercidin, toyocamycin and sangivamycin were isolated from different *Streptomyces* sp. and sponges (Saneyoshi *et al.*, 1965; Uematsu and Suhadolnik, 1970, 1974; Zabriskie and Ireland, 1989). 8-Aza-9-deaza-adenosine and inosine-like formycin A and B were discovered in various *Streptomyces* and *Nocardia* species (Daves and Cheng, 1976) and are still widely utilized as PNP inhibitors. The unique fluorine-containing nucleocidin was extracted from the soil bacterium *Streptomyces calvus* (Thomas *et al.*, 1956; Morton *et al.*, 1969). 3'-Dehydroxyadenosine (cordycepin) was first identified in the ascomycete fungus *Cordyceps militaris* (Cunningham *et al.*, 1950), which is an organism used in the ancient Chinese medicine. A further adenosine analog with an unconventional four-membered sugar scaffold (oxetanocin) was first recorded from the bacterium *Bacillus megaterium* (Shimada *et al.*, 1986). Even a nucleoside antibiotic compound with a more complex structure like tunicamycin was found in *Streptomyces lysosuperificus* (Takatsuki *et al.*, 1971; Ito *et al.*, 1980). Pseudouridimycin is a recently discovered nucleoside analog from actinomycetes extract and has already gained a remarkable attention as the first nucleoside analog that selectively inhibits bacterial RNA polymerase. Therefore, it is considered a promising antibacterial drug candidate that is potent against emergent antibiotic-resisting strains (Chellat and Riedl, 2017; Maffioli *et al.*, 2017).

2.2. Nucleoside analogs as therapeutics

Whether they made it into clinical trials, got approved for use by patients, or just remained a record as a research subject, NAs form a very attractive group for therapeutic value exploration. They can be developed either by a rational design methodology (e.g., immucillins that inhibit the transition state of parasitic nucleoside hydrolase, fludarabine that has better solubility and adenosine deaminase resistance than vidarabine, and clofarabine that combines the advantages of cladribine and fludarabine) or by screening different substitutions. They have been used since the 1960s as the first line of therapy for various pathological conditions, especially as anticancer and antiviral agents on account of their antimetabolite properties (Fig. 5).

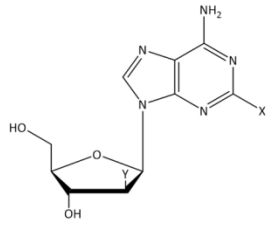
NA drugs are administered either in their basic form or with an additional chemical group that enhances their solubility or bioavailability. This should be distinguished from structural changes to improve their action or protect them from degradation (e.g. halogenation at the C-2 position of purines to prevent deamination). In all cases, NAs are considered prodrugs whose 5'-phosphorylated derivatives express the actual antiproliferative activity.

2.3. Uptake and activation of nucleoside drugs *in vivo*

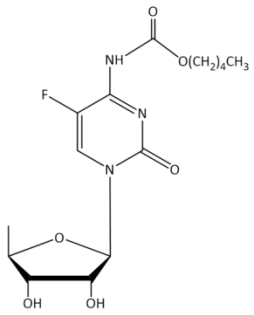
Cellular uptake of NAs, which are generally hydrophilic, occurs through transporters located in the plasma membrane. These are divided into several families that differ in topology, mechanism and substrate specificity:

1) Concentrative Nucleoside Transporter (CNT) has three isoforms and transports zidovudine, ribavirin or stavudine.

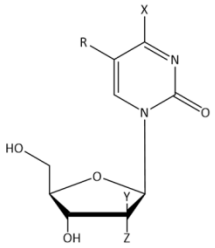
A **Anticancer**



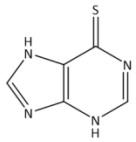
	X	Y	Main use (s)
Cladribine	Cl	H	hairy cell leukemia, B-cell chronic lymphocytic leukemia
Clofarabine	Cl	F	acute lymphoblastic leukemia and acute relapsing myeloid leukemia
Fludarabine	F	OH	chronic lymphocytic leukemia , acute myeloid leukemia



Capecitabine (breast, esophagus, larynx, and gastrointestinal and genitourinary tract cancer)

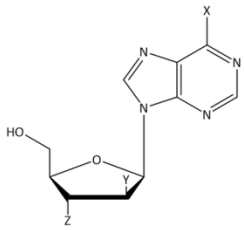


	R	X	Y	Z	Main use (s)
Azacitidine	H	NH_2	H	OH	chronic myelomonocytic leukemia, myelodysplastic syndrome
Cytarabine	H	NH_2	OH	H	acute and chronic myeloid leukemia, acute lymphocytic leukemia and acute promyelocytic leukemia
Floxuridine	OH	F	H	H	colorectal cancer and adenocarcinoma liver metastasis
Gemcitabine	H	NH_2	F	F	non-small cell lung cancer, pancreatic, bladder and breast cancer

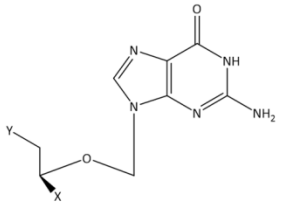


6-mercaptopurine (acute lymphocytic leukemia and immunosuppressive in ulcerative colitis and remitting Crohn's disease)

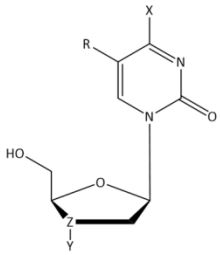
B **Antiviral**



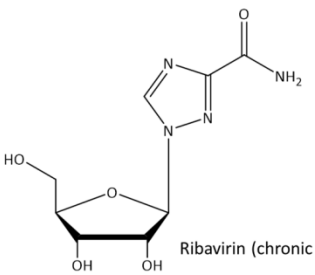
	X	Y	Z	Main use (s)
Didanosine	OH	H	H	HIV
Vidarabine	NH_2	OH	OH	HSV, VZV



	X	Y	Main use (s)
Aciclovir	H	OH	HSV, VZV
Ganciclovir	CH_2OH	OH	CMV
Valaciclovir	H	$OC(O)C(NH_2)CH(CH_3)_2$	CMV, HSV, VZV



	R	X	Y	Z	Main use (s)
Brivudine	CHCHBr	OH	OH	C	HSV, VZV
Edoxudine	CH_2CH_3	OH	OH	C	HSV
Emtricitabine	F	NH_2	H	S	HIV
Idoxuridine	I	OH	OH	C	HSV, VZV
Lamivudine	H	NH_2	H	S	HBV, HIV
Telbivudine	CH_3	OH	OH	C	HBV
Trifluridine	F_3	OH	OH	C	HSV
Zalcitabine	H	NH_2	H	C	HIV
Zidovudine	CH_3	OH	N_3	C	HIV



Ribavirin (chronic HCV)

Fig. 5: Examples of approved nucleoside analogs used as anticancer **(A)** or antiviral drugs **(B)**. Adapted from (Galmarini *et al.*, 2002; Rabuffetti, 2017; Seley-Radtke and Yates, 2018).

2) Equilibrative Nucleoside Transporter (ENT) consists of four members and is responsible for the transport of didanosine, ribavirin or zalcitabine.

3) Organic Anion Transporter (OAT) comprises five isoforms and is responsible for the up-take of ganciclovir, zidovudine, acyclovir, zalcitabine or cidofovir.

4) Organic Cation Transporter (OCT) facilitates the transport of organic cationic substrates, some non-charged molecules and anions, and also the transport of zidovudine or ganciclovir.

5) Peptide Transporter (PEPT) that involves four H^+-dependent transporters that accept anionic, cationic, zwitterionic peptides or valacyclovir.

6) Multidrug Resistance Protein (MRP) ejects nucleotides in an ATP-dependent mechanism. It differs from the other types as its substrate-binding site faces the cytosol. It is responsible for the transport of lamivudine, adefovir or 5-fluorouracil.

Most transporters are not specific to certain NAs. As already indicated, different transporter proteins show different mechanisms of action that move the molecules across the cell membrane. Concentration and equilibration transporters are the most involved in natural nucleosides uptake in cells lacking *de novo* synthesis mechanisms. Their action depends on a substrate gradient over the membrane. Other transporters depend on the availability of two substrates on the different sides of the membrane (e.g. exchange of anions with α-ketoglutarate via OAT) and are denoted as substrate exchange transporters. Concentrative Na^+- or H^+-dependent uptake is another uptake coupled to channels that keep the transmembrane ionic strength. Substrate export by ATP-dependent efflux pumps is an energy-dependent route to extrude substrate against concentration gradient (Pastor-Anglada *et al.*, 2005; Koczor, 2012; Jordheim *et al.*, 2013).

Once inside the cells, NAs first get phosphorylated to the 5'-monophophate derivative by nucleoside kinase (specifically deoxycytidine kinase (dCK), thymidine kinase (TK) and deoxyguanosine kinase (dGK); depending on the location and substrate). This first phosphorylation reaction is considered the rate-determining step for the overall process. It is followed by a second phosphorylation by nucleoside monophosphate kinase and a third one by nucleoside diphosphate kinase. NAs administered as nucleobases (e.g., 6-mercaptopurine, thioguanine) are phosphorylated via phosphoribosyl transferases (e.g., hypoxanthine-guanine phosphoribosyl transferase and adenine phosphoribosyltransferase) (Galmarini *et al.*, 2002; Jordheim *et al.*, 2013).

2.4. Therapeutic applications of nucleoside analogs

NAs with antineoplastic activity result in cell death and/or suppression of cellular propagation and repair by manifesting one or many of several mechanisms of actions against both dividing and quiescent cells. In dividing cells, they are used by polymerases and are integrated into the elongating DNA or RNA strands. The integration causes the (i) termination of the biopolymer growth when the following nucleoside fails to bind, (ii) wrong proteins after translation leading to apoptosis of the concerned cells, or (iii) double helix mismatching that lead to DNA breaks and DNA fragments accumulation as a result of futile mismatch repair cycles. They can also inhibit several nucleoside-acting enzymes which perturbs the normal physiological processes and leads to cytotoxicity (Table 1) (Pizzorno *et al.*, 2003; Tsesmetzis *et al.*, 2018). In resting cells, the activity relies mostly on impairing the repair mechanisms rather than targeting the DNA synthesis machinery. Cladribine and fludarabine, known to be active against non-dividing cells, directly activate caspase-9 and caspase-3 apoptotic pathways in addition to causing single stranded DNA breaks during double helix repair which induces the expression of poly(ADP-ribose) polymerase (PARP) for DNA repair.

PARP production then depletes the energy-providing-molecules NADH and ATP of the involved cells (Galmarini *et al.*, 2002; Sigal *et al.*, 2010).

As previously stated, NAs as antivirals prevent viral replication by inhibition of the viral enzymes (mainly viral polymerases, reverse transcriptase and S-adenosylhomocysteine hydrolase). When NAs act as substrates for polymerases, they integrate into the nascent DNA or RNA and either cause chain termination, or lead to mutations in the following DNA repair process. This can cause cell death when several mutations occur that cross the error catastrophe threshold. Presently, 5 out of 9 infectious human viral diseases are treated with NAs (Campagnola *et al.*, 2015; Savić *et al.*, 2015; Seley-Radtke and Yates, 2018). The most important antiviral groups are 2',3'-dideoxynucleoside analogs and 5-substituted 2'-deoxyuridine derivatives.

Table 1: Effect of different nucleoside analogs on inhibiting pivotal enzymes.

NAs examples	Affected enzyme	Effect
Pentostatin (2'-deoxycoformycin)	Adenosine deaminase (Johnston, 2011)	accumulation of adenosine and deoxyadenosine and imbalance of the nucleoside pools which has toxic effects
fludarabine, cytarabine, vidarabine, nelarabine	DNA polymerases esp. polymerase α (Kolesar *et al.*, 1996; Robak *et al.*, 2005)	retardation or inhibition of DNA synthesis and accumulation of cells in G_1-S phase boundary
fludarabine, cladribine, clofarabine, gemcitabine	Ribonucleotide reductase (Pankiewicz, 2000; Robak *et al.*, 2005)	concentration of deoxynucleotides decreases in the affected cells which increases the likelihood of incorporation of the NA in DNA
5-fluorouracil, floxuridine, capecitabine	Thymidylate synthase (Tsesmetzis *et al.*, 2018)	imbalance of dNTP pools esp. depletion of dTMP and accumulation of dUMP that can be misincorporated into DNA together with NA
decitabine, azacitidine, zebularine	DNA methyltransferases (Ewald *et al.*, 2008)	DNMT binds covalently to DNA containing the NA resulting in: - depleting the global enzyme concentrations and decrease the overall DNA methylation - steric hindrance for further DNA elongation
forodesine, peldesine, 6-mercaptopurine	PNP (Bzowska *et al.*, 2000; Balakrishnan *et al.*, 2006)	increased levels of dGuo and dGTP disturb the concentration of dNTP pools and consequently cause cell death

As parasites are often incapable of *de novo* purine synthesis, inhibiting any of the enzymes of the salvage pathways (e.g., kinases, PPRT, PNP, MTAP, hydrolases) can kill the respective parasitic cell (Lawton, 2005; Vodnala *et al.*, 2016). One drawback, however, is the similarity between the pathways of the host and the eukaryotic parasitic cells which limits the practical use due to high levels of toxicity. Another antiparasitic-acting approach is using compounds that block pathways which only exist in the pathogen without affecting the corresponding enzymes of the host. For example, it was found that PNP from *Plasmodium falciparum* (*Pf*PNP) is involved in both purine and polyamine metabolisms and can recognize the substrate 5'-methylthioinosine that does not exist in human metabolism. Thus, the rationally designed group of immucillins was developed to inhibit the PNPs leading to purine starvation of the parasite and purine-less death (Shi *et al.*, 2004; Chaudhary *et al.*, 2006). However, despite the great impact of parasitic diseases on humans and domestic animals, the present research directed towards antiparasitic drugs discovery and development is not as extensive

as the one for antineoplastic and antiviral purposes. Currently, most of the NAs studied for parasites treatment are originally developed and investigated for cancer research. Furthermore, antineoplastic drugs are being used off-label for veterinary antiparasitic use.

The research on NAs as antibacterial agents has decreased in the past two decades in favor of the expanding anticancer and antiviral screening. Yet, with the immense development of new resistant strains, more focus and new strategies have to be directed towards this worldwide threat (e.g. capuramycin derivatives and pseudouridimycin). In this context, NAs can represent useful bacteriostatic or bactericidal candidates via several mechanisms; such as (i) inhibition of cell wall peptidoglycan synthesis, (ii) inhibition of various enzymes that play a role in nucleoside synthesis or salvage pathways, or (iii) inhibition of enzymes involved in nucleic acid or protein synthesis (Serpi *et al.*, 2016; Maffioli *et al.*, 2017).

Beside the well described acute and chronic fungal infections, newly emerging resistant strains are being reported as the cause for high levels of morbidity and mortality due to nosocomial infections in immunocompromised patients. Consequently, similar to the aforementioned mechanisms for antibacterial therapy, antimicrobial fungal treatments are sought (Serpi *et al.*, 2016). Interestingly, the first reported examples of the class of antifungal chitin synthase inhibitors were polyoxins; a group of pyrimidine analogs (Isono *et al.*, 1965).

Apart from their antimicrobial and cytotoxic activity, some NAs can cross the blood-brain barrier, which can be used to alleviate neuropathological conditions and modulate neuroinflammation. This contributes to their therapeutic effect against some viral infections like HBV, HCV and HIV (Gish, 2006; Savić *et al.*, 2015). Furthermore, nucleosides and nucleotides can be used in enteral immunonutrition to modulate immune system proliferation; promoting the growth of intestinal microflora and the development of lymphocytes, natural killer cells and macrophages (Hu and Yang, 2014). Dietary nucleotides are absorbed as nucleosides in the small intestine and are specially added to infant formulae due to the high rate of growth (Yu, 2002). They are also commonly used as flavor enhancers (Del Arco *et al.*, 2017) yet, they are regarded as the underlying causes for increased uric acid levels and gout. Some purine NAs express T-cell selective immunosuppressive activity and are administered for patients with autoimmune disease (e.g., systemic lupus erythematosus, multiple sclerosis or rheumatoid arthritis) or after organ transplantation (Robak *et al.*, 2006; Jordheim *et al.*, 2013).

2.5. Chemical synthesis of nucleoside analogs

The chemical synthesis is still the state of the art for NAs production in both laboratory and large scale. As nucleosides are polyhydroxylated compounds with multiple active positions, the OH groups have to be first replaced with good leaving (protecting or directing) groups to guarantee the highest β/α product ratios and that the reactions take place at the correct positions. Later, these substituents have to be removed in additional purification steps that add to the laboriousness, time consumption, hazardous solvents and chemical consumption and total cost of the whole production process. Yields depend on the structure of the heterocyclic base, sugar donor, reaction conditions and catalysts. This renders an estimation of the reaction efficiency difficult and a subject of case-by-case judgement. Some harsh reaction conditions are also accountable for the poor yields as either the reactants, intermediates or products do not tolerate them. The acidic degradation of deoxyriboside purines is an example.

Chemical synthesis of NAs follows one of two different approaches (Shelton *et al.*, 2016). In the divergent method, a C-N glycosidic bond already exists and reactions modify a nucleobase or

N-substituted ribosylamine. Hence, the proper β-anomer acquisition is guaranteed. The second, the convergent method, depends on protecting a heterocycle and a sugar scaffold, then connecting them with a glycosidic bond. This is the more commonly employed approach in pyrimidine nucleosides production as it also permits broader structure diversity. Purine nucleosides, on the other hand, are more difficultly acquired due to the formation of several byproducts (N3, N7, N9 sugar addition).

2.6. Enzymatic synthesis of nucleoside analogs

Enzymatic reactions proved themselves as a scientifically-attractive field of study and a convenient, indispensable, efficient and reliable alternative for NAs production over several decades.

In contrast to the conventional chemical methods, enzymes offer a more efficient regio-, stereo- and enantioselective approach. They are a greener and more environmentally-friendly option that avoids using hazardous solvents and chemicals. They are also less laborious and comprise less procedure by avoiding the multiple protection-deprotection steps. This reduces the need for product purification between steps and decreases the number of byproducts. Furthermore, enzymatic reactions often take place at mild conditions of temperature, pH and pressure, which avoids product degradation or the formation of racemic mixtures of multiple isomers. On the other hand, employing enzymatic synthesis has its own drawbacks. The most pronounced of which is the cost load of the enzyme and the great specificity of the enzyme catalysts, which makes it crucial to have a vast library to facilitate different alterations.

Since the cost of enzyme production is the strongest limiting factor that prevents large scale application, economic approaches have been developed to overcome this challenge. They involve using whole cells, immobilized enzymes or continuous enzyme reactor systems. These approaches were shown to enable the reuse of the enzymes or to increase the lastingness thereof.

Many enzyme types and classes were investigated concerning their role in (chemo-)enzymatic nucleoside chemistry. The diverse enzymes with versatile actions allow a broad array of reactions/ modifications to the natural enzymes. In most cases, these reactions were inspired by the physiological pathways of *de novo* nucleoside biosynthesis and salvage. Nucleoside modifying enzymes can be sorted into three categories (Condezo *et al.*, 2006; Li *et al.*, 2010): I. hydrolytic enzymes, II. oxygenases, or III. transferases.

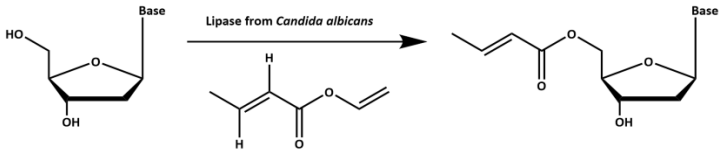
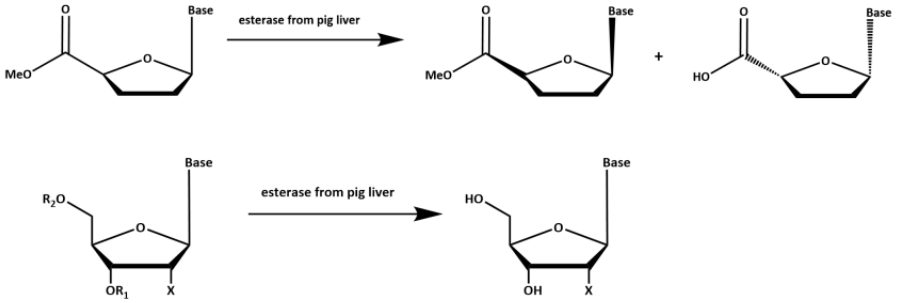
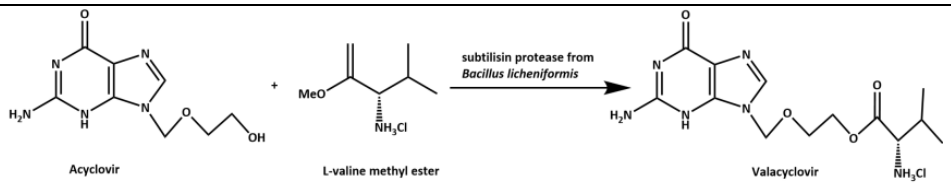
Hydrolytic enzymes can either alter substituents on the heterocyclic base (e.g., adenosine deaminase) or on the sugar moiety (Table 2). As known for hydrolases, they have a wide range of activity and substrates. So, they can catalyze the break of several different chemical bonds in the presence of water and they can also catalyze the reverse chiral stereoselective synthesis. For example, inosine-uridine nucleoside hydrolase, involved in the purine salvage in parasites, catalyzes the N-ribohydrolysis of inosine into hypoxanthine and ribose via the formation of a ribooxocarbenium ion transition state. This founded the basis for the development of some antiparasitic drugs (e.g., p-aminophenyliminoribitol that inhibits the hydrolase enzyme and the transition-state inhibitors immucillins) (Mazumder *et al.*, 2002; Lawton, 2005).

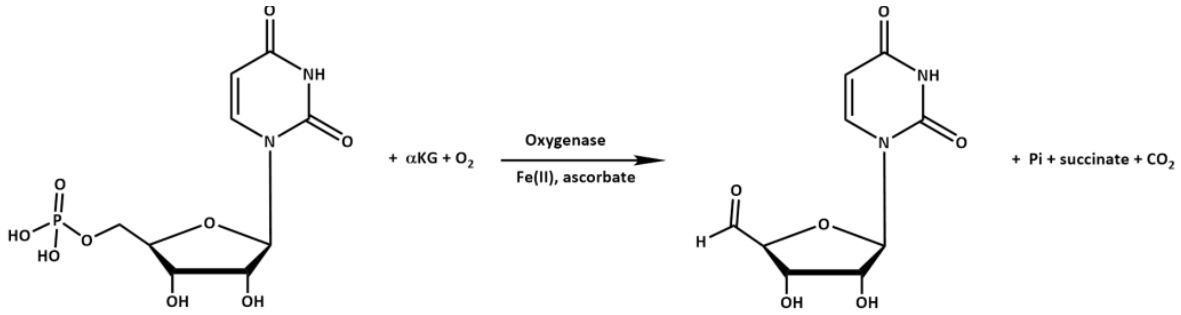
Oxygenases oxidize the hydroxyl groups on the sugar moiety into aldehyde and carboxylic groups (e.g., formation of uridine-5'-aldehyde, a precursor for the antibiotic muramycin that inhibits bacterial cell wall synthesis) (Scheme 1). The reaction is facilitated by non heme, mononuclear Fe(II)/alpha-ketoglutarate dependent oxygenase (Goswami *et al.*, 2017; Huang *et al.*, 2018).

Transferases catalyze the association of a heterobase (sugar acceptor) to the ribosyl moiety liberated from a sugar donor. Physiologically, they have a fundamental role in nucleoside salvaging which

makes them essential for organisms that lack *de novo* synthesis mechanisms. They are divided into two classes: nucleoside deoxyribosyltransferase and nucleoside phosphorylases. Both enzymes exhibit a high degree of regio- and stereoselectivity where the β-stereoisomer of N-9 purine and N-1 pyrimidine glycosylation are predominantly, if not absolutely, formed.

Table 2: Different nucleoside modifications catalyzed by hydrolytic enzymes. Reaction schemes were adapted or from (Li *et al.*, 2010; Lapponi *et al.*, 2016).

Enzyme	Reaction
Adenosine deaminase	 Conversion (inactivation) of vidarabine by adenosine deaminase
Lipase	 Enantioselective acylation/ deacylation of OH groups yielding compounds with improved absorption
Esterase	 Enantioselective hydrolysis to clean up racemic mixtures and deblocking acyl groups
Protease	 Formation of α-branched amino acid derived prodrugs with better chemical stability, aqueous solubility and oral bioavailability
β-galactosidase	5'-O-β-glycosylated prodrugs formation with better solubility and therapeutic efficiency



Scheme 1: Conversion of uridine monophosphate to uridine-5'-aldehyde catalyzed by oxygenase. Adapted from (Goswami *et al.*, 2017).

2.6.1. Nucleoside deoxyribosyltransferases (NDT)

Nucleoside deoxyribosyltransferases (NDT, EC 2.4.2.6) are most commonly found in *Lactobacilli* sp. with less representation in *Lactococci* and some parasites. They catalyze the 2-deoxyribose sugar exchange between two nitrogenous bases (purine/purine, pyrimidine/pyrimidine or purine/pyrimidine). A purine-specific subclass (PDT) was found to exclusively recognize purine bases. NDTs are specific for 2'-deoxyribosyl nucleosides displaying a remarkable tolerance to base structure modifications. However, some studies also showed a catalytic effect on arabinosyl and 2'-fluororibosyl nucleosides (Fernandez-Lucas *et al.*, 2010; Fernández-Lucas *et al.*, 2011).

NDTs are homohexamers with one catalytic site per monomer and two subunits constitute a whole catalytic unit (Armstrong *et al.*, 1996; Anand *et al.*, 2004). It was also found that 3' and 5'-OH groups in the substrates are critical for the catalytic activity by binding to the conserved amino acids Glu-98, Asp-92 and Asn-123 to correctly position the nucleosides within the binding site (Fig. 6) (Kaminski *et al.*, 2008; Fresco-Taboada *et al.*, 2013). The nucleobase binding site differs between NDTs and PDTs. In NDTs, the base binding is achieved via the residues Gln-46, Asp-72, and Tyr-157.

The reaction occurs through a ping-pong double placement mechanism with an intermediate dR-enzyme formation, that later transfers the sugar moiety to the second base. From an industrial perspective, as this enzyme is capable of performing both the cleavage and binding steps (cf. NPs), it is a candidate for immobilization, which is an economical requisite for scalability.

2.6.2. Nucleoside phosphorylases (NPs)

Nucleoside phosphorylases (NPs) are ubiquitous, extracellular, polymeric enzymes. They have become the most studied enzyme class with respect to nucleoside biosynthesis since the first member was isolated from rat liver (Kalckar, 1945). They facilitate the reversible breakage/formation of a glycosidic bond between nucleobase and sugar moiety in the presence of inorganic phosphate. *In vivo*, the thermodynamic equilibrium directs the reaction towards nucleobase liberation while the opposite occurs *in vitro*. They are classified with respect to their catalytic domains and substrate spectra into two major classes: NP-I that comprises purine nucleoside phosphorylases (PNP, EC 2.4.2.1), 5'-methyl thioadenosine phosphorylase (MTAP, EC 2.4.2.28), uridine phosphorylase (UP, EC 2.4.2.3) and the NP-II family, whose members are pyrimidine nucleoside phosphorylase (PyNP, EC 2.4.2.2) and thymidine phosphorylase (TP, EC 2.4.2.4). The reactions take place through the formation of an α-D-pentofuranose-1-phosphate (α-PF-1-P) intermediate.

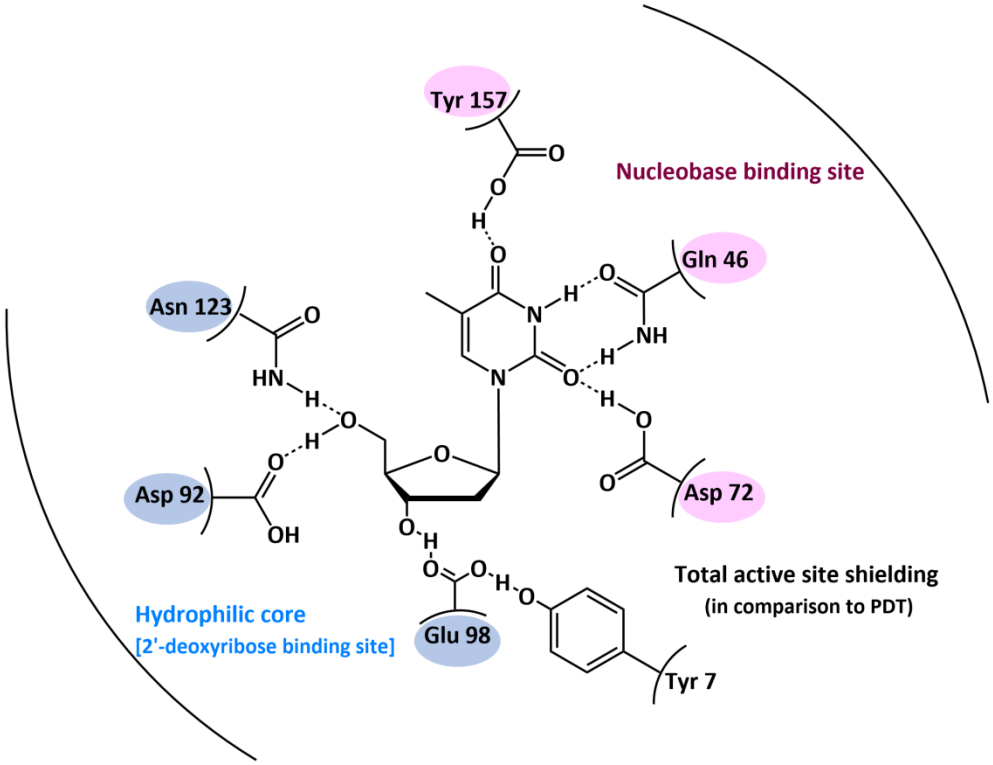


Fig. 6: Active site of NDT from *Lactobacillus leichmannii* with thymidine as substrate. Adapted from Fresco-Taboada and colleagues (Fresco-Taboada *et al.*, 2013).

2.6.2.1. Catalytic mechanism

The catalytic mechanism is quite conserved among the different subclasses. An S_N1, bi-bi, substrate-assisted mechanism is universally proposed (Erion *et al.*, 1997). NPs are specific for β-nucleosides generating α-PF-1-P products. The nucleoside binds in an electron-rich conformation, which leads to a steric or van der Waals strain. Electrons flow from O-4 of the sugar to the nucleobase and a ribooxo-carbenium ion is formed as a transition state that is stabilized by the second substrate (phosphate anions). Interactions of the active site sustain the electron flow to the heterocyclic ring ending in glycosidic bond cleavage (Erion *et al.*, 1997; Caradoc-Davies *et al.*, 2004; Yehia *et al.*, 2017).

2.6.2.2. Active site residues

Detailed information to the structure and active site residues for the different kinds of NPs are summarized in **paper I** and **paper II** of this thesis. Within the NP classes, the amino acid sequences of the catalytic domain display conserved residues and a homologous subunit topology in many cases, despite low homology levels of the overall sequences and quaternary structures. NP-I enzymes exist in several oligomeric states: dimers in case of eukaryotic UPs, trimers as in mammalian PNPs and MTAPs, and hexamers (trimers of dimers) in the cases of prokaryotic PNP, MTAP and UP. NP-I proteins consist of α/β protein folds with superimposing secondary structures among all types. Interestingly, UP has higher structural resemblance to PNPs than PyNPs, that also catalyze pyrimidine nucleoside reactions (Caradoc-Davies *et al.*, 2004). In NP-I members, the phosphate binding site is found in the N-terminus of α-helix. It is characterized by three arginine residues that act towards oxygen atoms of the phosphate anion, main-chain nitrogen atoms and a side chain of OH residues that participate in hydrogen bonds. They have the same pattern of ribose binding to its specific site

that relies on hydrophobic van der Waals interaction. The unique hydrophobic pocket recognized in the sugar binding site of human MTAP, comprising the amino acids His-257 and Val-233, is allegedly responsible for its binding to 5'-deoxy-5'-methylthio group. The nucleobase binding site, however, shows a distinction that accounts for the specificity of UPs despite the common noncovalent π-stacking by the conserved phenyl alanine. The hydrophilic nature of the base pocket forming hydrogen bonds with the purine is replaced with a hydrophobic one in UPs that also contains extra 23 inserted amino acids in the 163-185 region. Therefore, its smaller volume prevents against binding of the double ring structure. A similar remark is noted for trimeric and hexameric PNPs, the latter being more substrate tolerant due to a more open base binding site (Mao *et al.*, 1997; Pugmire and Ealick, 2002; Caradoc-Davies *et al.*, 2004).

NP-II enzymes are homodimers whose subunits consist of α/β/α folds, not related to the ones in NP-I, implying an independent evolution. Like the PyNP from *E. coli* that is known for its narrow substrate spectrum, TP also has a methionine instead of Lys-108 of other PyNPs. This is probably the reason behind their specificity towards 2'-deoxyribosides. On the other side, the NP-II's conserved arginine, lysine and histidine residues provide electrostatic interactions to stabilize the oxocarbenium transition state (Pugmire and Ealick, 2002; X F Gao *et al.*, 2006; Yehia *et al.*, 2017).

2.6.2.3. Substrate spectra of NPs

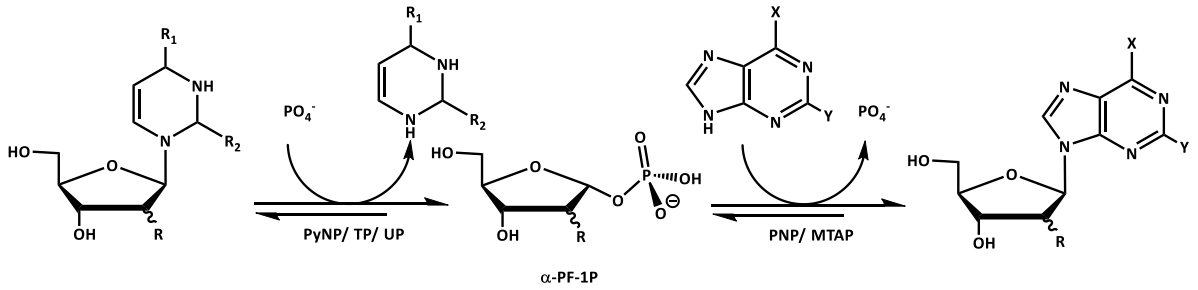
Despite a conserved catalytic mechanism, NPs of different origin show diverse substrate spectra. The native profile for NP-I enzymes is that mammalian trimeric PNPs are specific for oxo-purine nucleosides while bacterial hexameric PNPs recognize both oxo- and aminopurine nucleosides. The other enzymes reflect their names yet, their substrate spectrum can also be variable. Detailed and comprehensive description can be found in **paper I** and **paper II**. General, interesting remarks are:

- MTAPs can usually accept 6-amino and 6-oxopurines. Many of them can also cleave adenosine, 5'-halogenated sugars and longer S-aliphatic chains. Electron donating and withdrawing groups at C2 are tolerated by some. A thermophilic kind, *Ap*MTAP, even accepts C2' modified substrates (Zhou *et al.*, 2013; Yehia *et al.*, 2017).
- PNPs are reported to have a wide substrate spectrum with both base and sugar variations. Yet, the loss of hydroxyl groups at C2' and C3' of the substrates' sugar decreased their reaction rates, in comparison to ribosides, due to the decreased electronegativity on C1'. The unusual N3-C1' and N7-C1' nucleosides have records of being cleaved by different PNPs. Open ring nucleosides (e.g., ribavirin) are also ribosylated by both bacterial and mammalian PNPs (Yehia *et al.*, 2017; Kamel *et al.*, 2018).
- Cytidine is not phosphorolyzed by UP or TP. Interestingly, it was cleaved by the thermozyme MTAP from *Aeropyrum pernix* (Caradoc-Davies *et al.*, 2004; Zhou *et al.*, 2013).
- UPs are, despite their name, not specific for 2'-hydroxyl nucleosides. Electronegative groups at C5 like fluorine render the corresponding nucleosides better substrates compared to the canonical ones because the electron withdrawal aids breaking the glycosidic bond and therefore, the detachment of the base from the enzyme is easier (inductive force). The bigger electronegative substitution with bromine has two coalescing forces, both functioning in a way that the glycosidic bond cleaves easier (both the aforementioned inductive force and steric forces with Ile-220 and Val-221 distorting the riboside binding angle). In case of electron donating groups (e.g. methyl group), these two forces are opposite to each other due to the increased electron density on the base (Caradoc-Davies *et al.*, 2004).

- PyNPs are not as well-studied as PNPs but it is already known that a wide range of C5 and C2' modifications are recognized by many PyNPs (Yehia *et al.*, 2017).
- TP requires the presence of 3'-OH to form hydrogen bonds with NH-group of Thr-123 whereas 2'-OH blocks His-85 from binding to the carbonyl group on C2. Hence, riboside nucleosides are considered as inhibitors (Panova *et al.*, 2007).

2.6.2.4. Synthesis of modified nucleosides by nucleoside phosphorylases

PNP-catalyzed reactions favor the nucleoside synthesis direction more than PyNPs (Fresco-Taboada *et al.*, 2013). This is the basis of transglycosylation reactions where pyrimidine nucleosides act as sugar donors and purine bases as sugar acceptors (Scheme 2).



Scheme 2: Transglycosylation reaction.

This concept involves the combination of sequential two NP-catalyzed reactions in one pot. In a first step, a pyrimidine nucleoside is cleaved by PyNP in the presence of inorganic phosphate to the free base and α-PF-1-P. In the second step, PNP binds the purine base to the activated sugar, giving rise to a purine nucleoside. The phosphate is subsequently liberated again. It is obvious that this could also be carried out using two pyrimidine or two purine bases depending on the reaction's dynamic equilibrium.

Another approach that employs the same reactions is the separation of the two reactions in two pots with a purification step for the α-PF-1-P in between. This is especially helpful when the sugar donor is a poor enzyme substrate (high k_m, low V_{max}) e.g., 2'-deoxy-2'-fluorouridine or 2'-deoxy-2'-fluoroarabinouridine, which are the precursors for the important class of 2'-fluoropurine nucleosides. Further information is given in **paper III**.

2.6.2.5. Production of modified nucleosides in larger scales

Almost all enzymatic syntheses routes described so far were performed in microliter scale. Yet, some few larger scale trials were also documented to produce the antiviral ribavirin. The endogenic synthesis during the cultivation of guanosine-producing *Bacillus* sp. is an example. The ribavirin formamide precursor 1,2,4-triazole-3-carboxamide (TCA) is added during the cultivation where the overexpressed PNP adds the ribofuranosyl scaffold. The highest yield reached 19.1 g L^{-1} in a 7.5 L bioreactor culture (Chen *et al.*, 2009; Ma *et al.*, 2014). Another pilot-scale synthesis scored 97 % TCA conversion into ribavirin over 168 h in a 20 L enzymatic reaction (Sakharov *et al.*, 2015). Another attempt for the synthesis of cytotoxic 2'-deoxyribosides in 40-100 mg scale was done over almost 4 days reaction (Huang *et al.*, 1981, 1983).

The main obstacle against the spreading of alternative large scale synthesis is the cost-effectiveness (Del Arco and Fernández-Lucas, 2018). The time required for sufficient production usually exceeds

the life time of the known enzymes under the given conditions. Pure active enzymes are expensive to obtain and preparing them for re-use is not an easy process. The poor solubility of many NAs precursors acts against good volumetric yields. Harsher conditions of temperature, pH or organic solvents can usually not be applied as the enzymes do not withstand these conditions. Therefore, up-scaling the enzyme expression or increasing the soluble enzyme volumetric yield of the bacterial culture are important tools towards gaining a wider acceptance of the large scale enzymatic synthesis.

2.6.2.6. Application of thermostable enzymes

The increased interest in thermophilic organisms and their inherently stable enzymes that started in the 1970s has contributed to the advancement and new aspects of nucleosides synthesis research. Thermostable enzymes possess rigid structures and express their optimal activity at temperatures around that of their original organisms. However, they retain the same catalytic activity as their mesophilic counterparts. In addition to thermal stability, they also tolerate a wide pH range and chemicals (e.g. organic solvents). With respect to purine nucleosides formation, these characteristics help solving the problem related to nucleobases' poor solubility by using higher temperatures and organic solvents like DMSO, DMF and 2-methyl tetrahydrofuran (Bruins *et al.*, 2001; Almendros *et al.*, 2012). Generally, other advantages involve (i) resisting the contamination by mesophilic enzymes that might lead to proteolysis, (ii) better mass transfer, (iii) less viscous reaction mixture through the use of higher temperatures and consequently lower power is needed for mixing, (iv) higher substrate concentrations and thus higher volumetric yields, and (v) the option to recycle the enzymes due to their higher stability (Bruins *et al.*, 2001; Haki and Rakshit, 2003). These merits compensate the extra costs of energy and heating, and make thermozymes good candidates for commercial and industrial use. As a result, thermophilic enzymes do not require a strictly controlled environment and have a high robustness for up-scaling.

Establishment of a cost-effective industrial process, especially with the current routine protocols of recombinant protein expression in mesophilic host organisms is possible while retaining the properties of the native enzyme (Vieille and Zeikus, 2001). Thereby, they are less expensive to produce than in the original organism, because the protein purification is as simple as adding an affinity tag or a heating step that denatures the mesophilic proteins (Bruins *et al.*, 2001).

So far, thermostable enzymes involved in nucleoside synthesis have been isolated from many bacterial genera and archaea and were successfully immobilized and used in various forms and setups (Sinisterra *et al.*, 2010; Yehia *et al.*, 2017; Del Arco and Fernández-Lucas, 2018). These enzymes belong to different NP classes, however, no thermostable NDTs have been identified so far.

Enzyme recycling is rather significant for rendering the reactions more economical and thus, encouraging their use. Detailed examples are given in **paper I** and **paper II**. In whole cell catalysis, both native and recombinant cells are used. The optimal activity is described to be best when the cells are in the late exponential or the early stationary phase and the nucleoside salvaging is maximized (J A Trelles *et al.*, 2004; Nóbile *et al.*, 2010). Whole cells, per se, can be functionally regarded as immobilized enzyme systems and the cells are retrieved by centrifugation and resuspension. The complexity of whole cells, however, hinders their wide spread application as it increases the chance of byproduct formation. Furthermore, products could be degraded by other enzymes (e.g. deaminases) (J A Trelles *et al.*, 2004; Li *et al.*, 2010).

The application of crude cell lysate may not be particularly beneficial over pure enzyme in terms of recycling but it spares the work and cost of enzyme purification. Crude cell lysates have been widely

used for the synthesis of NAs. It is pronouncedly helpful in case of thermostable enzymes expressed in mesophilic hosts since a heating step is enough to eliminate most of the host proteins (Bruins *et al.*, 2001; Almendros *et al.*, 2012).

Immobilized pure enzymes are in principle the best technique for enzyme recycling. The choice of a proper support material is critical in the beginning but the method affords the advantages of reusability and easier product purification. Good examples were given by different research groups who used magnetically active supports that could be easily removed from the medium (Fernández-Lucas *et al.*, 2013; Zhou *et al.*, 2015). Drawbacks, however, are the decreased volumetric activity due to masking of the active sites, low loading capacity especially if two enzymes are co-immobilized, troublesome multimeric enzyme stability or enzyme leaking over several runs. These reasons necessitate the assessment of the cost-effectiveness by comparing the product amounts vs. the preparation costs.

Enzyme reactors allow the maximum use of the enzyme especially when operated in fed-batch or continuous mode. Unlike the batch reaction method, where the catalytic activity is terminated with the end of the reaction time, the reaction continues as long as the enzyme is still active allowing for making the most of the enzyme's potential. The enzyme is usually attached to an inert support or enclosed in a membrane with a molecular weight cut-off (MWCO) smaller than the enzyme's, which also allows its subsequent retrieval.

3. Aim of the Project

Due to a widespread application of nucleoside analogs in various fields and the still available drawbacks of the chemical synthesis processes, there is a need to optimize available methods for the enzymatic synthesis of NAs. The actual study focuses on the enzymatic synthesis of NAs with thermostable nucleoside phosphorylases. The following research questions were addressed:

1. **Does the two-pot sequential glycosylation reaction afford better final product yields than the one-pot cascade transglycosylation reaction?**

To produce sugar-modified NAs, two approaches were compared: transglycosylation reactions and direct glycosylation reactions. As an example 5-ethynyluracil (EUra) was used as sugar-acceptor, since EUra-containing NAs are of high interest in the field of diagnostics.

2. **Can thermostable nucleoside phosphorylases be efficiently produced in benchtop bioreactors?**

To increase the active protein yield, a suitable fed-batch cultivation method was developed by adjusting critical parameters like glucose feed, concentration of the inducing agent or time of induction. Correspondingly, glucose concentration, culture optical density, dissolved oxygen tension were closely monitored during the cultivation.

3. **Can halogenated NAs be produced in 100 mg scale with high purity?**

Protocols were developed to produce and purify halogenated NAs in batch reactions with high yield and purity. NAs were purified by preparative HPLC and structures were confirmed by NMR.

4. **Can NAs be produced in continuous enzymatic membrane reactors?**

To reduce the enzyme cost, continuous methods to produce NAs were evaluated. A continuous enzyme reactor controlled by a mini computer was established and used for the synthesis of 2'-deoxyadenosine and two NAs. Purified enzymes and heat-treated extracts were applied and compared for their efficiency.

5. **Do NAs produced in an enzymatic process show the same biological activity as NAs produced by a chemical method?**

The purified halogenated NAs were tested for their cytotoxic activity against different human leukemic cell lines using standard assays. IC_{50} values were determined.

4. Materials & Methods

4.1. Bacterial strain

Protein expressions were done using *E. coli* BL21-Gold strain bearing a modified pCTUT7 expression vector (Zhou *et al.*, 2013) carrying NP genes. The cell bank was stored at -80 °C in LB medium containing 25 % glycerol.

Three strains producing three different PyNPs (PyNP 01, 02, 04), two strains producing two PNPs (PNP 01, 02) and one MTAP-producing strain (PNP 03) were used throughout the course of this work. The clones were kindly provided by BioNukleo GmbH.

4.2. Media

Lysogeny broth (LB) and terrific broth (TB) media were prepared according to (Sambrook and Russell, 2001). Bioreactor cultivations were done in mineral salt medium (MSM) (Holme *et al.*, 1970) (g L^{-1}): 14.6 K_2HPO_4, 3.6 NaH_2PO_4.2 H_2O, 2.0 Na_2SO_4, 2.47 $(NH4)_2SO_4$, 0.5 NH_4Cl, 1.0 $(NH_4)_2$-H-citrate, 0.1 thiamine hydrochloride, 2 mM $MgSO_4$, 0.1 mL antifoam PPG2000 and 2 mL trace elements solution. Trace elements solution was composed of (g L^{-1}): 0.5 CaCl.$2H_2O$, 0.18 $ZnSO_4 \cdot 7H_2O$, 0.10 $MnSO_4 \cdot H2O$, 20.1 Na_2-EDTA, 16.7 $FeCl_3 \cdot 6H_2O$, 0.16 $CuSO_4 \cdot 5H_2O$, 0.18 $CoCl_2 \cdot 6H_2O$, 0.085 $Na_2SeO_3 \cdot 5H_2O$, 0.14 $Na_2MoO_4 \cdot 2H_2O$, and 0.725 $Ni(NO_3)_2 \cdot 6H_2O$. For the batch phase 5 g L^{-1} glucose was added while the feeding solution contained 500 or 850 g L^{-1} glucose.

HEK293 cells were cultured in low glucose Dulbecco's modified Eagle's medium (Biowest) containing 10 % fetal bovine serum, 2 mM glutamine, non-essential amino acids, 4.5 mg mL^{-1} glucose and the antibiotics penicillin and streptomycin. HL-60 and CCRF-CEM were cultured in RPMI 1640 w/o L-glutamine supplemented with 10 % fetal bovine serum, 2 mM glutamine and the antibiotics penicillin and streptomycin.

4.3. Protein expression and purification

4.3.1. Cultivation in shake flasks

PyNP 01, 04 and PNP 01, 03 were produced in EnPresso® B medium (Enpresso GmbH, Berlin, Germany) at 30 °C, while PyNP 02 and PNP 02 were produced in TB medium at 37 °C. In both cases, precultures were prepared from glycerol stocks in LB at 37 °C. Antibiotics were added based on the resistance genes. All the cultures were prepared in Ultra Yield™ flasks, covered with AirOtop™ Enhanced seals (Thomson Instrument Company, California, USA) and incubated in incubation shaker (Infors HT, Switzerland) at 200 rpm. The culture volume was 20 % of the flask total filling volume. The IPTG concentration for induction depended on the produced protein (Table 3).

4.3.2. Cultivation in bioreactors

Fed-batch cultures were carried out in a 3.7 L benchtop bioreactor (KLF2000, Bioengineering, Switzerland). *On-line* monitoring involved measuring pH, temperature, dissolved oxygen tension

(DOT), and O_2 and CO_2 in the exhaust gas using the $BlueInOne_{Ferm}$ sensor (BlueSens, Baesweiler, Germany).

A series of cultivations were run for some NPs and the parameters were adjusted in each consequent cultivation to improve the running process in terms of time and volumetric and biomass yield of soluble protein. A general scheme is described here while the variations are illustrated in the results section (Table 3).

Two consecutive precultures were used for every bioreactor cultivation. Preculture 1 was prepared by inoculating 10 mL LB with 1 mL glycerol stock and incubating at 37 °C, 200 rpm for 6 h. Second preculture was prepared in Ultra Yield flask containing 50 mL EnPresso B (A) or 5 % glucose in MSM (B) inoculated with preculture 1 and covered with AirOtop seals. The second preculture was incubated at 30 °C, 200 rpm for 18 h.

The bioreactor cultivations were carried out at 30 °C and comprised three phases: (1) initial batch cultivation in 2 L MSM medium containing 5 g L^{-1} glucose as a sole carbon source, (2) fed-batch phase with exponentially increasing glucose feeding, and (3) protein production phase after induction with IPTG. The pH was tightly controlled at 7.0 ± 0.05 using 25 % (v/v) NH_4OH. The DOT was regulated through the adjustment of stirring rate and air flow rate. Foaming was controlled by adding antifoam (PPG2000) manually in 0.1 mL steps, whenever foam appeared.

After glucose consumption in the batch phase, indicated by zero residual glucose and increased DOT, glucose feeding was started at a rate calculated according to the following equation:

$$F_t\ [g\ L^{-1}] = \frac{\mu_{set} X_0 V_0}{C_f Y_{x/s}}$$ **Equation 1**.

X_0 - cell dry weight (g L^{-1}) at the end of the batch phase (calculated from a correlation between previous OD_{600} and DCW measured values)

V_0 - culture volume (L) at the time of feeding start

μ_{max} - specific growth rate calculated during batch phase (h^{-1})

μ_{set} - 75 % of μ_{max}

C_f - glucose concentration in the feeding solution (g L^{-1})

$Y_{X/S}$ - yield coefficient (g g^{-1}; biomass produced per glucose), calculated from the batch phase

While normally μ_{set} is used in the calculation of the initial feed rate to achieve the targeted specific growth rate within a short time, μ_{max} was used in the bioreactor cultivations (I-III), to have a slower decrease of the specific growth rate, which may better adapt the cells to the induction. In cultivations IV and V, μ_{set}= 75 % μ_{max} was used due to the incidents of glucose accumulation.

The exponential feeding was continued until late growth stage then filter-sterilized IPTG was added in the same concentration as in the shake flask. Only in the case of fed-batch cultivation V (PNP 03), IPTG was added by comparing the OD_{600} in the bioreactor to that in the shake flask at the time of induction. The feeding was then switched to a constant mode with a flow rate = ½ the last flow rate and continued until OD_{600} no less than 100 or until the excessive foaming was no longer suppressed by PPG2000. Only in the fed-batch cultivation IV, the feeding after induction was switched to a linear one with feeding = 0.5 x F_t in an attempt to gain more biomass and hence, more protein while avoiding glucose accumulation.

Extra 2 mM Mg^{2+} (from 1 M stock solution of $MgSO_4$) were added by bolus feed in the fed-batch cultivations IV and V for every increase of OD_{600}= 20.

Samples were taken hourly from the bioreactor for OD_{600} measurement, dry cell weight determination and residual glucose. In fed-batch cultivation V (PNP 03 production), extra samples for

Mg^{2+}, acetate, pyruvate concentrations were analyzed. Samples for protein analysis were collected after induction and stored at -20 °C until analysis (Section 4.3.5).

4.3.3. Determination of cell growth

Cell growth in shake flasks and bioreactors was monitored by measuring the absorbance at 600 nm (OD_{600}) with a UV/Vis spectrophotometer (Ultrospec 3300, Amersham Biosciences, Germany).

The specific growth rate (μ) was calculated during the batch phase between two consecutive OD_{600} measurements.

In bioreactor cultivations, cell growth was also analyzed by determining the dry cell weight (DCW). Therefore, 2 mL were centrifuged at 16,000 g for 5 min at 4 °C (CT 15RE, VWR, Japan). The supernatant was later used for the analysis of extracellular medium components (Section 4.6.2 and 4.6.3) and the cell pellet was washed with 0.9 % $NaCl_{aq}$ (w/v) and dried at 75 °C for 24 h. Measurements were done in duplicate.

Table 3: Overview of thermostable NPs overexpression in fed-batch bioreactor cultivations and shake flasks.

		KLF bioreactor			Shake flask	
Symbol	Protein	Glucose feed ($g\ L^{-1}$)	Preculture*	IPTG (µM)	Medium	IPTG (µM)
I	PyNP 04**	500	A	500	EnPresso B	500
II	PyNP 02	500	A	100	TB	100
III	PNP 02	500	A	100	TB	100
IV	PNP 02	500	B	100	TB	100
V	PNP 03	850	B	100	EnPresso B	20

*A: second preculture was carried out in EnPresso® B, B: second preculture is in 5 % glucose in MSM.

** PyNP 04 has chloramphenicol resistance cassette instead of ampicillin.

4.3.4. Exhaust gas analysis

A mass balance of the gas phase is postulated around the reactor for the calculations according to the following equations:

Oxygen uptake rate $Q_{O2}\,[mol_{O2}\,(L\ h)^{-1}] = \frac{\dot{V}_G^{\alpha}}{V_F \times V_M} \times \left[Y_{O2}^{\alpha} - \frac{1-Y_{O2}^{\alpha}-Y_{CO2}^{\alpha}}{1-Y_{O2}^{\omega}-Y_{CO2}^{\omega}} \times Y_{O2}^{\omega}\right]$ **Equation 2.**

Carbon dioxide production rate $Q_{CO2}\,[mol_{CO2}\,(L\ h)^{-1}] = \frac{\dot{V}_G^{\alpha}}{V_F \times V_M} \times [Y_{CO2}^{\omega} \times \frac{1-Y_{O2}^{\alpha}-Y_{CO2}^{\alpha}}{1-Y_{O2}^{\omega}-Y_{CO2}^{\omega}} - Y_{CO2}^{\alpha}]$ **Equation 3.**

Respiration coefficient RQ $[-] = \frac{Q_{CO2}}{Q_{O2}}$ **Equation 4.**

Specific oxygen uptake rate $q_{O2}\,[mol_{O2}\,(g\ h)^{-1}] = \frac{Q_{O2}}{X}$ **Equation 5.**

Specific carbon dioxide production rate $q_{CO2}\,[mol_{CO2}\,(g\ h)^{-1}] = \frac{Q_{CO2}}{X}$ **Equation 6.**

$\dot{V}_G^{\alpha}$ - gas flow at the inlet ($L\ h^{-1}$)

Y_{O2}^{α}, Y_{CO2}^{α} - oxygen and the carbon dioxide mole fraction in the inlet gas

Y_{O2}^{ω} , Y_{CO2}^{ω} - oxygen and carbon dioxide mole fraction in the exhaust gas

V_F - liquid volume (L)

V_M - molar gas volume of 22.4 L mol^{-1}

4.3.5. Protein analysis and quantification

OD_{600}= 5 cell samples were collected during protein expression. Cells were resuspended and disrupted using lysis buffer with the composition: 50 mM NaH_2PO_4, 300 mM NaCl, 1 mM $MgCl_2$, 1 mg L^{-1} lysozyme (Fluka™), 25 U mL^{-1} benzonase® (Novagen®), 0.1 mM phenylmethylsulfonyl fluoride (PMSF; Roth, Germany).

Protein samples were analyzed on SDS-PAGE as per standard method (Sambrook and Russell, 2001). Protein concentration was measured using a NanoDrop1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, ME, USA).

The unit of PyNP activity expresses the amount that catalyzes the phosphorolysis of 1 µmol uridine per min and for PNP, it is the amount that phosphorolyses 1 µmol adenosine or guanosine per min and the reaction temperature depends on the optimal one for the specific enzyme (Section 4.3.7).

4.3.6. Protein purification

The cell pellet was lysed in 50 mM sodium phosphate buffer, pH 8.0, supplemented with 300 mM NaCl, 1 mM $MgCl_2$, 10 mM imidazole, 1 mg L^{-1} lysozyme, 25 U mL^{-1} benzonase® and 0.1 mM PMSF. Lysis buffer was added at 3 mL g^{-1} of the wet cell weight. Cells were resuspended and left to stand at room temperature for 30 min. Lysed cells were sonicated on ice for 5 min with 30 % power input and with 30 s on/off intervals using an ultrasonicator (UP200S, Hielscher Ultrasonics, Teltow, Germany). Following sonication, the lysate was heated in a water bath at 60 °C (in case of PNP 01, 02 and PyNP 01, 02) or 80 °C (in case of PNP 03 and PyNP 04) for 30 min. Afterwards the solution was centrifuged at 10,000 g, 4 °C (Centrifuge 5810R, Eppendorf, Hamburg, Germany) for 20 min and the supernatant was filtrated through 0.45 µm CME filter (Rotilabo®, Carl Roth, Karlsruhe, Germany).

The clear lysate was purified by Ni-NTA chromatography using gravity column in the case of shake flask protein expressions and Äkta FPLC system (GE Healthcare, Munich, Germany) for fed-batch bioreactor cultivations. Eluate was finally dialyzed overnight against 2 mM phosphate buffer and analyzed by SDS PAGE.

4.3.7. Enzyme activity determination

The activity assay was carried out in 50 mM potassium phosphate (KP) buffer, pH=7 using 1 mM adenosine or guanosine (in the cases of PNP and MTAP) or uridine (in the case of PyNP). To a substrate solution, heated to the enzyme's optimal temperature, enzyme solution was added to the concentration of 0.3 µg mL^{-1} and samples were taken regularly for 30 min. Samples were immediately treated with methanol, centrifuged at 21,500 g for 20 min at 4 °C (CT 15RЄ, VWR, Japan) and analyzed by HPLC (Agilent 1200 series system). The substrate conversion was calculated as:

$$\text{Conversion percentage} = \frac{[\text{Liberated base}]}{[\text{Liberated base} + \text{remaining nucleoside}]} \times 100$$ **Equation 7**.

Only the conversion percentages that showed linear increase of the base release were used for further calculations. 1 U of NP is the amount that phosphorolyzes 1 µmol nucleoside per minute.

4.4. Production of nucleoside analogs

4.4.1. Small scale synthesis of modified nucleosides

4.4.1.1. Transglycosylation reaction

To evaluate and optimize transglycosylation reactions, they were carried out in a volume of 2 mL, with 1 to 5 U of PNP and PyNP. The tested sugar donor nucleosides were uridine (Urd), thymidine (Thd), 1-β-D-arabinofuranosyl uracil (AraU), 1-(2'-deoxy-2'-fluoro-β-D-ribofuranosyl) uracil (FU), 1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl) uracil (FanaU) and the nucleobases were 6-chloro-2-fluoropurine (CFP), 2,6-dichloropurine (DCP), 2-aminoadenine (DAP), 2-fluoroadenine (2FA), 2-chloroadenine (2CA) and everything was dissolved in 2 mM KP. Due to their poor solubility, 2FA and 2CA were dissolved in 20 % DMSO in 2 mM KP. A ratio of 10 mM nucleoside to 1 mM base was applied. The reaction temperature depended on the optimal temperature of the biocatalysts used or the thermolability of the substrates, intermediate or products (Table 4). The assignment of enzymes and nucleosides is extrapolated from the results of the optimum enzymes used to obtain the corresponding sugar-1-phosphate (Kamel *et al.*, 2018).

Table 4: Reaction conditions used for transglycosylation and direct glycosylation experiments.

Sugar donor (Nucleoside/α-PF-1-P)	Temperature (°C)	Enzymes
Urd/ R-1-P	60	PNP 02-PyNP 02
Thd/ dR-1-P	40	PNP 01-PyNP 01
AraU,FanaU/ Ara-1-P, FAra-1-P	80	PNP 03-PyNP 04

To prepare mg amounts of nucleosides, transglycosylation reactions were carried out in 50 mL final volume in 2 mM KP, pH=7. Enzyme amounts of 0.01 mg PyNP mL^{-1} and 0.05 mg PNP mL^{-1} were used. A nucleoside to nucleobase ratio of 2:1 was applied. Due to solubility limitations of 2FA and 2CA, 10 % and 20 % of DMSO, respectively, were added. Uridine was used as a reactant to produce riboside nucleosides and thymidine for deoxyribosides. 25 mM was the concentration of nucleobases CFP and DCP, while the less soluble DAP, 2FA and 2CA were used in 15 mM concentration.

Reactions were analyzed by HPLC and conversion percentage was calculated based on equation 7.

Purification of NAs was done at room temperature by preparative KNAUER HPLC system equipped with a Smartline Detector 2600 and using AZURA P 2.1L pumps with a flow of 21 mL min^{-1} through a Kinetex® 5 µm EVO C18 250 × 21.2 mm RP column.

4.4.1.2. Direct reaction

The reactions were carried out in different ratios of sugar-1-phosphates (in the form of barium salts) and CFP (1:1, 1:2, 1:4, 1:0.1). The ratio of 1:4 was afterwards applied for DCP, DAP, 2FA in 20 mM Tris-HCl; pH 7.4. Biocatalysts combinations and reaction temperatures are set according to table 4. Reactions planned to produce ribosides and deoxyribosides were carried out using 1 U PNP while for modified sugar nucleosides, 5 U of enzyme were used.

4.4.1.3. Synthesis of 5-ethynyluracil (EUrA) nucleoside analogs

Thd, FU and FanaU were used as sugar donors in transglycosylation reactions in 2 mM KP in a final volume of 2 mL. A sugar donor to EUra ratio of 10:1 was applied. PyNP 04 was added in an amount of 0.25 mg mL^{-1}.

Ba salts of dR-1-P, FR-1-P and FAra-1-P were used in a final volume of 2 mL to directly react with EUra in the ratios of 2:1. PyNP 04 was added in the amount of 0.5 mg mL^{-1}.

4.4.2. Production of nucleoside analogs in an enzyme membrane reactor (EMR)

The details of the reactor's design were previously described in (Sitanggang *et al.*, 2014) (Fig. 7). It consists of two pressurized substrate reservoirs and two parallel pressure-withstanding glass reaction containers, containing pH and temperature probes and having a working volume of 90 mL each. The containers' bottom allows the placement of flat ultra-filtration membranes with an active surface area of 12.38×10^{-4} m^2. A proportional-integral-derivative (PID) controller was incorporated to control the flux and thus the hydraulic residence time (HRT). Flux is feed-back regulated in the continuous process by a precision balance. Parameters setting and data storage were managed in the first experiments by the software Laboratory Virtual Instrument Engineering Workbench (LabVIEW) Professional, version 2012. In later experiments, the PID controller regulation was done by a program written in Python through a Raspberry Pi computer.

Different membrane materials (MWCO = 10 kDa) were tested for their ability to influence system pressure or the reaction itself, namely polyethersulfone (PES), permanently hydrophilic polysulphone (UFX-10pHt), regenerated cellulose (RC 70 PP) or composite fluoropolymer membranes (ETNA 10PP).

At 40 °C, 10 mM base (adenine (Ade), CFP or DCP) and 25 mM Thd were dissolved in 2 mM KP buffer (pH=7). PNP 02 and PyNP 02 were added in the ratio of 7:1 with total amounts of 1050 and 150 U, respectively. Stirring speed was set to 250 rpm.

The enzymatic reactions were compared in batch reaction setup and continuous reaction under the same conditions. The reaction was carried out using both pure and cell lysate form of NPs using the natural substrates Thd and Ade. The effect of different residence times (2 to 8 h) was investigated.

To overcome the tubing connections popping out, which secured the system under the LABVIEW script, the maximum pressure allowed was set to 4 bar in the python program. Using the new controlling system, the experiments were established using the natural nucleobase. Afterwards, more experiments were run for modified nucleosides production using either DCP or CFP as nucleobases and Thd as sugar donor in the transglycosylation reaction. Both pure enzymes and cell lysates were tested (PNP 02 and PyNP 02).

Samples were withdrawn via the sampling port where the enzyme is retained behind the membrane (Fig. 7). The samples were analyzed by HPLC (Agilent 1200 series system).

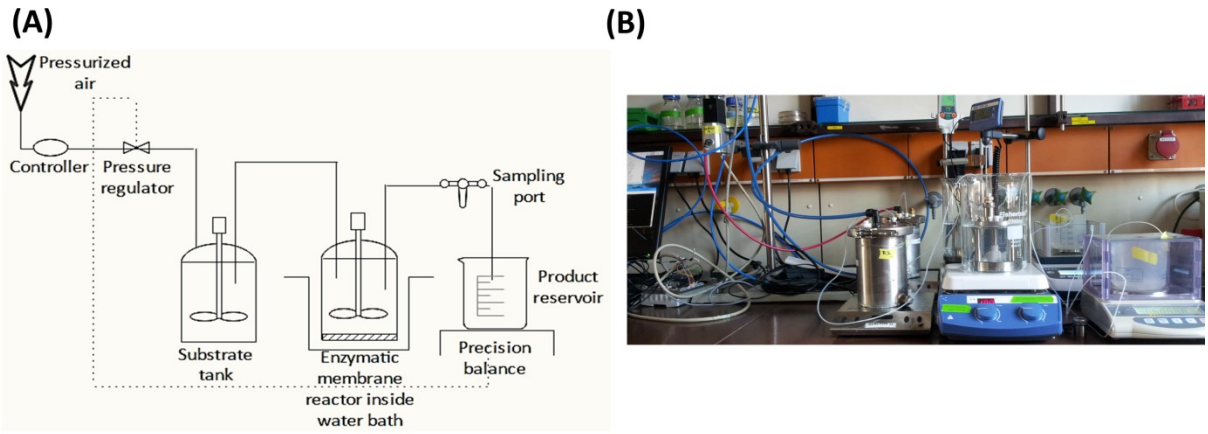


Fig. 7: Enzyme membrane reactor. **(A)** schematic diagram, adapted from (Sitanggang *et al.*, 2014), **(B)** picture while operation showing (controller, substrate tanks, reactor vessels inside water baths, product containers placed over balances).

4.5. Cytotoxicity assay

Cytotoxicity was measured by the XTT colorimetric assay. The measurement took place according to the protocol of the Cell Proliferation Kit II (Roche Diagnostics GmbH). The reagent mixture was prepared by mixing 1 mL of 1 mg L^{-1} of XTT sodium salt (PanReac AppliChem) with 2 µL 12.5 mM of PMS. 50 µL of the reagent solution was added to 100 µL of test samples, incubated at the cell culture CO_2 incubator at 37 °C and the absorbance was measured 4 h later at 450 nm. Cladribine (Carbosynth) was used as a positive control and the sample solvent (phosphate buffered saline PBS) served as the negative control. All experiments were conducted in three independent experiments, in triplicates.

HL-60 was diluted to 1.2×10^5 cells in growth medium and placed in a 96-well flat-bottom plate. Solutions with different concentrations of nucleoside analogs were added and the plate was incubated for 24 h. In total, 1.2×10^5 cells of CCRF-CEM in growth media were placed in a 96-well flat-bottom plate. After the addition of the test NAs, the plate was incubated for 48 h. A total of 1.2×10^5 cells of HEK293 in growth media were placed in a 96-well flat-bottom plate. Test samples were added after 24 h incubation and the plate was further incubated for another 24 h.

4.6. Analytics

4.6.1. High performance liquid chromatography (HPLC) analysis

Enzymatic reactions were followed with an HPLC Agilent 1200 series system equipped with an Agilent DAD detector. The reaction yields were determined by following the nucleobases and nucleosides absorption at 260 nm using a reversed phase C18 column (Kinetex® 5 µm, Evo C18, 100 Å LC column 150 x 4.6 mm, Phenomenex, CA, USA) with the following gradient: from 97 % 20 mM ammonium acetate and 3 % acetonitrile to 60 % 20 mM ammonium acetate and 40 % acetonitrile in 10 min.

4.6.2. Determination of residual glucose concentrations

Residual glucose was measured in cell-free samples, using the supernatant of the samples described in section 4.3.3. Glucose hexokinase FS reagent (DiaSys Diagnostic Systems GmbH, Holzheim, Germany) or enzymatic hexokinase photometric assay using Cedex Bio HT Analyzer as described by the manufacturer were used.

4.6.3. Determination of magnesium, acetate and pyruvate concentrations (performed in Cedex Bio HT Analyzer, Mannheim, Germany).

The measurement of magnesium was performed with Magnesium Bio HT kit (Roche Diagnostics, Germany). It is based on the colorimetric detection with chlorophosphonazo III.

Acetate is detected with an enzymatic photometric assay using Acetate V2 Bio HT (Roche Diagnostics, Germany). The formation of NADH during the enzymatic reaction is measured by an increased absorbance at 340 nm correlating with the concentration of acetate in the sample.

Pyruvate concentration was determined using the Pyruvate Bio HT (Roche Diagnostics, Germany). The photometric assay is based on the lactate dehydrogenase reaction.

The kits were used according to the manufacturer's recommendations.

4.6.4. Validation of nucleoside structure by nuclear magnetic resonance (NMR)

^{1}H, ^{13}C, ^{19}F, and ^{31}P NMR spectra were recorded in DMSO-d_6 or D_2O 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 (^{1}H NMR: δ = 2.50 ppm for DMSO-d_5 and δ = 4.79 ppm for HDO; ^{13}C NMR: δ = 39.52 ppm for DMSO-d_6) (Sinisterra *et al.*, 2010). ^{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 CCl_3F and H_3PO_4 (Harris *et al.*, 2001). Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, m_c = centrosymmetric multiplet, br = broad signal), coupling constants (Hz) and integration.

5. Results

5.1. Expression of thermostable PyNP, PNP and MTAP in benchtop bioreactors

Available nucleoside phosphorylases were successfully expressed in shake flasks. To increase the productivity, the aim was to establish a suitable method to express the enzymes in benchtop bioreactors. Four NPs (2 PyNP, 1 PNPs, 1 MTAP) were overexpressed in *E. coli* BL21-Gold in 3.7 L KLF benchtop scale bioreactor. Despite the same expression vector and hosts used, μ_{max} varied from 0.17 to 0.41 h^{-1}. Lower μ_{max} were recorded for PyNP expressing strains.

In preparatory cultivations, a glucose concentration of 15 g L^{-1} was used in the batch phase. As a consequence, maximum stirring and aeration capacity were reached almost by the end of the batch phase. For the remaining cultivation time, culture had to be discarded or pure oxygen was mixed with air. In cultivations performed at 30 °C, compared to 37 °C, the proportion of soluble protein was higher.

Two different pre-culture schemes were applied. Two consecutive precultures were used. While preculture 1 was prepared in LB, the second preculture was prepared in either EnPresso B (A) or 5 % glucose in MSM (B). No, or at least shorter, lag phases were observed when precultures were prepared in MSM.

Calculation errors in fed-batch cultivations I and II led to a lower OD_{600} and biomass for expression of PyNP 04 and 02 (Table 5). The other measurements, however, were similar to other cultivations.

Among all cultivations, leaky expression of NPs was observed.

The first PNP 02 expression was marked by weak bands on the analysis gel, also depicted in the samples from the flask cultivation. Therefore, the second experiment was carried out with fresh transformants (Apendix I).

Results for the expression of the thermostable PNP 03 (fed-batch cultivation V) are described in more details as they comprise more explicit data from the extracellular analytes (Table 5, Fig. 8).

To minimize the volume changes during feeding and trying to increase the efficiency of the bioreactor, a concentrated glucose feeding solution was attempted in fed-batch cultivation V but 850 g L^{-1} glucose concentration eventually crystallized in the connecting tubes and ended in irregular pumping pattern, independent from the calculations and settings, in contrast to the regular pumping from 500 g L^{-1} glucose feeding solution.

OD_{600} and DCW parameters are both used to evaluate biomass. In general, there was a good linear correlation between the values of OD_{600} and DCW with a high regression fit and the biomass increased in a similar behavior as the OD_{600} (Fig. 8B).

The respiratory quotient (RQ) was approximately 1 over the course of the fed-batch cultivations, i.e. nearly all the oxygen was transformed into carbon dioxide during respiration. The nearly optimal respiration would normally be interrupted at the points were the culture conditions were changed (feed start, induction, changing stirrer speed and air flow) because at these points, the off-gas measurement was unbalanced during abrupt changed gas values. The values for the specific respiratory rates correlated well with the analysis of the unspecific rates. During most of the course of cultivation, the q_{CO2} was nearly equal to the q_{O2}.

Table 5: Overview of different parameters of the bioreactor processes.

Symbol	Protein	μ_{max} (h^{-1})	Final OD_{600}	DCW (g L^{-1})	Batch phase $Y_{x/s}$ (g g^{-1})
I	PyNP 04	0.161	50	18.5	0.66
II	PyNP 02	0.167	85	40	0.39
III	PNP 02	0.432	45	21	0.57
IV	PNP 02	0.415	170	58	0.56
V	PNP 03	0.401	90	42.5	0.38

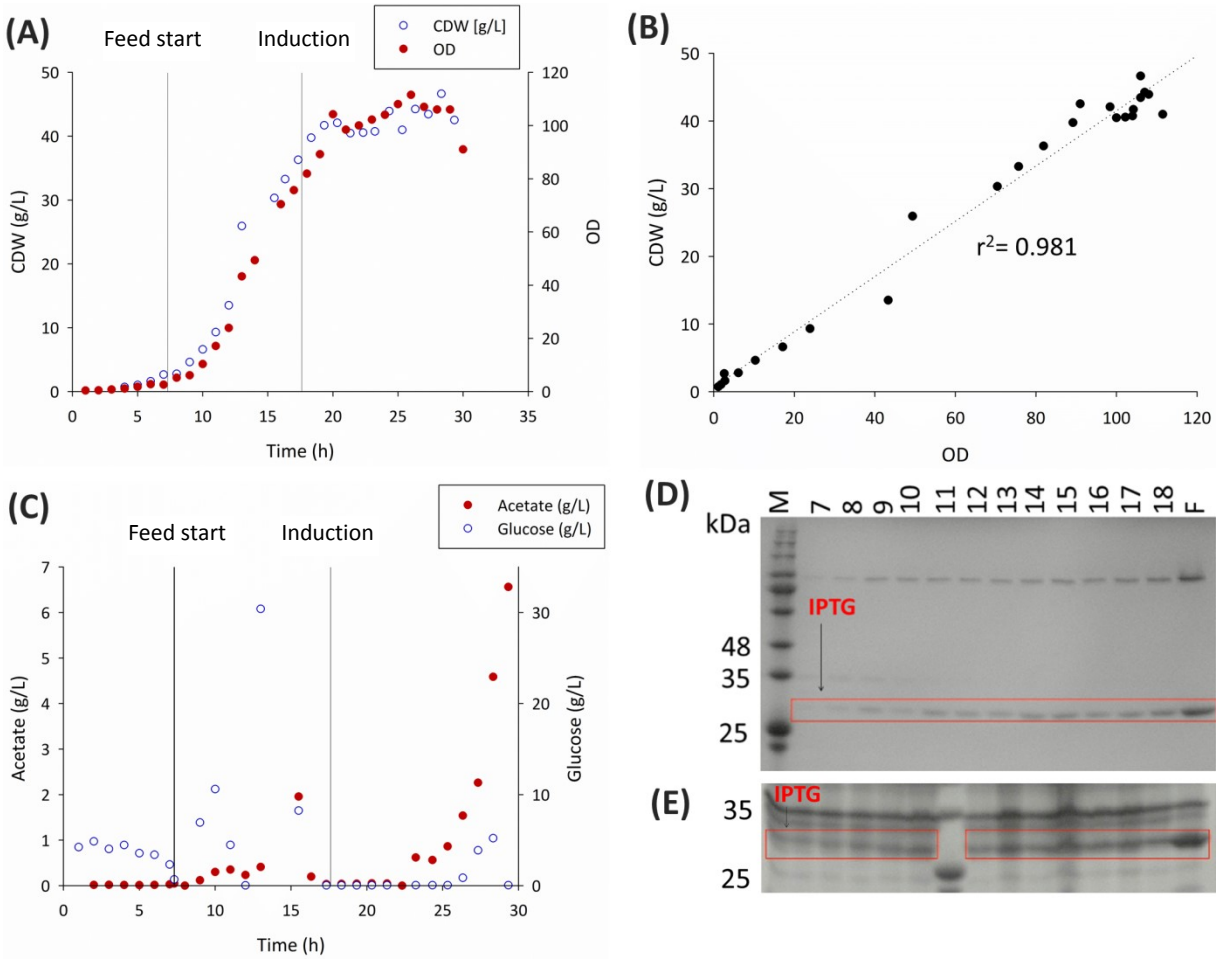


Fig**. 8:** Expression of PNP 03 in a KLF bioreactor. **(A)** Growth of *E. coli* BL21-Gold expressing PNP 03 was followed by measuring OD_{600} and DCW. **(B)** A high correlation between optical density (OD) and dry cell weight (DCW) was observed. **(C)** Acetate concentration relative to residual glucose during the expression of PNP 03 as determined by Cedex BioHT analyzer. **(D,E)** SDS-PAGE analysis of recombinant expression of PNP 03 in *E. coli* BL21-Gold either observed in the soluble protein **(D)** or the insoluble fraction **(E)**. M: marker, F: expression in shake flask, lanes labeling denote time (h).

The Cedex Bio HT Analyzer was used during the expression of PNP 03 (fed-batch cultivation V), which afforded the extra measurements for Mg^{2+}, acetate and pyruvate. Regular extra magnesium boli were added to compensate for the increase of biomass. The highest value of ca. 10.25 mM was obtained at the end of the fed-batch cultivation V. Pyruvate concentrations were also measured, but the determined values were below the detection limit of 8.706 mg L^{-1} over the whole period of cultivation. Acetate concentration increase correlated with glucose overfeeding starting 15 h after inoculation. Afterwards, it declined with substrate limitation during constant feeding. Acetate

concentration increased again around 22 h reflecting that the stationary growth phase was reached (Fig. 8C).

Glucose accumulation in fed-batch cultivation V was a result of the high glucose concentration that crystalized and clogged the tubes therefore, pumping did not proceed according to the settings. The aim of high glucose concentration in the feeding solution was initially to minimize the volume change. However, with this setup, lower concentration and/or continuous heating of the feed reservoir and tubings are prerequisites.

PNP 03 yield obtained in the benchtop bioreactor cultivation was compared to that from shake flask experiments. In shake flask scale, the measured OD_{600} at the time of induction was 7.71 ± 0.44, while before harvesting it was 19.75 ± 3.7. The harvested cells weighed 1.5 ± 0.17 g, from which 7.05 ± 0.2 mg PNP 03 were purified. The activity of the purified protein was determined, according to the enzyme's ability to phosphorolyze adenosine, to be 10.56 U mg^{-1}. The wet cell weight from the bioreactor was 365 g, from which 685 mg PNP 03 were purified. The activity of the purified protein was 8.57 U mg^{-1}.

In addition to the spectrophotometric analysis, the SDS-PAGE gels confirmed the better NPs yield in the shake flask (Fig. 8D and Appendix I) which is compensated in large scale by the better volumetric yield. In both shake flasks and bioreactors, a significant portion of the produced protein is present in the insoluble pellet which is comparable in both scales.

5.2. Chemo-enzymatic synthesis of nucleosides: comparison of transglycosylation and direct glycosylation reactions

To produce modified purine nucleosides, transglycosylation reactions were applied. Different sugar donors and CFP were applied to optimize the reaction conditions. Both steps (sugar donor cleavage and second nucleobase glycosylation) were assessed to evaluate the process. Using enzyme concentrations of 1 U PNP and PyNP, the natural nucleosides (Urd, Thd) showed higher percentages of cleavage in comparison to the modified ones (AraU, FanaU) (< 10 %). CFP riboside and deoxyriboside were formed in a percentage of ca. 90 % (Fig. 9). Increasing the enzyme concentrations (5 U PNP and PyNP) for the modified nucleosides (AraU and FanaU) resulted in a corresponding increase in the percentage of nucleoside (sugar donor) cleavage together with a remarkable decline in the corresponding nucleobase concentration. Cleavage of AraU increased from 5 to 17 % while that for FanaU was from ~1 % to 3.5 % (more than 3 folds).

To establish direct glycosylation reactions, different ratios of α-PF-1-P to base were tested. Direct glycosylation gave similar results to the transglycosylation in the cases of riboside and deoxyriboside nucleosides if the molar concentration of α-PF-1-P to base was double or higher (Fig. 10).

The synthesis of sugar-modified CFP-containing NAs was tested in both transglycosylation and glycosylation reactions. In both transglycosylation and glycosylation reactions, no products of interest were observed. The HPLC analysis of reactions involving AraU, FanaU and modified sugars (Ara-1-P and FAra-1-P), showed peaks that do not share spectroscopic features with the aromatic groups detected for the substrates (Fig. 11). This is in contrast to the similar spectroscopic profiles observed for riboside and deoxyriboside nucleosides. Therefore, the newly formed peaks are regarded as products for an unspecific reaction, other than that catalyzed by NPs, since the negative control (substrates without enzyme addition) remained unchanged over 30 h incubation under the same conditions as the reaction.

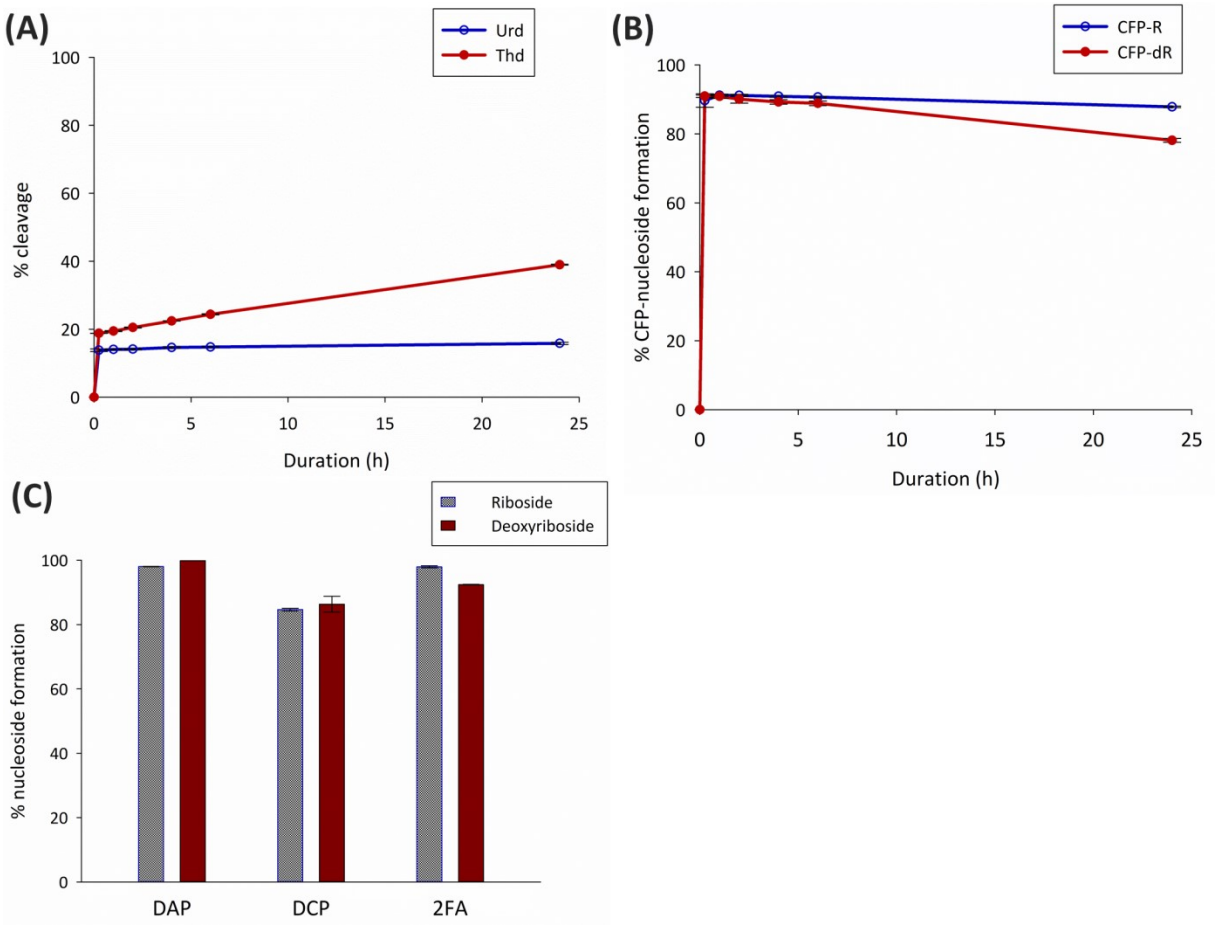


Fig. 9: Transglycosylation reaction to synthesize several purine nucleoside analogs using low concentrations of PNP and PyNP. Sugar donor cleavage **(A)** and the formation of CFP-R and CFP-dR **(B)** were studied. Optimized reaction conditions were applied for the synthesis of DAP, DCP and 2FA containing ribosides and deoxyribosides **(C)**, (n=2).

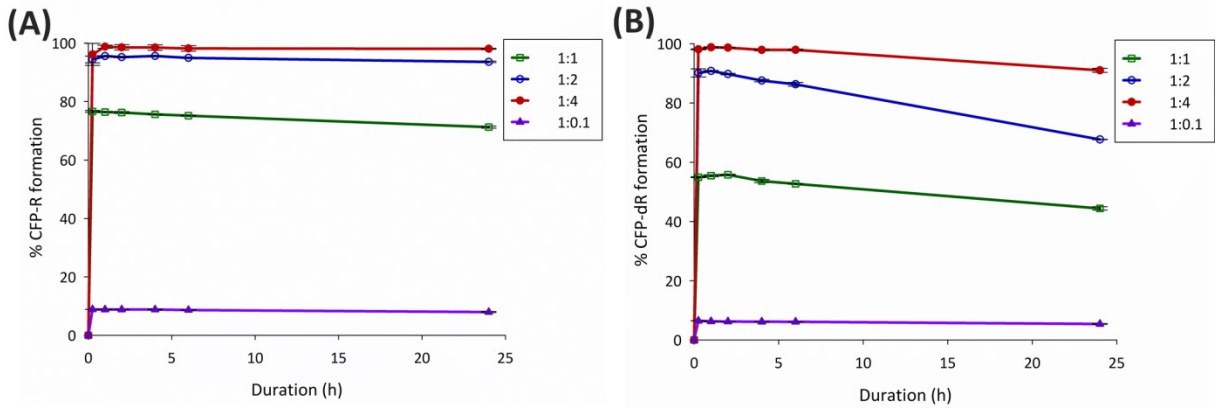


Fig. 10: Direct glycosylation of CFP and **(A)** α-D-ribose-1-phosphate and **(B)** α-D-deoxyribose-1-phosphate in different ratios of base to α-PF-1-P, (n=2).

FU, as a sugar donor reactant, was also investigated and a number of by-products were observed. This has been previously described for FU under high temperature conditions. It was explained by the depletion of a HF molecule and the formation of O^2,2'- anhydro-1-(β-D-arabinofuranosyl)uracil which is later hydrolyzed to AraU (Szeker *et al.*, 2012).

Additionally, the synthesis of EUra-containing nucleosides was studied. EdU production yields in the first three hours did not change whether direct glycosylation or transglycosylation were used (60 % and 55 %, respectively). The difference was quite clear in the cases of the synthesis of fluororiboside

(EUra-FR) and fluoroarabinoside (EUra-FAra) nucleosides. Product yields increased from 18 % and 0 %, respectively, to almost 50 %. It is also noted that the direct reaction with dR-1-P reached the equilibrium state during the first hour, therefore, sampling was stopped after 4 h while in the cases of FR-1-P and FAra-1-P, product formation kept increasing linearly until the last sampling point (24 h) (Fig. 12).

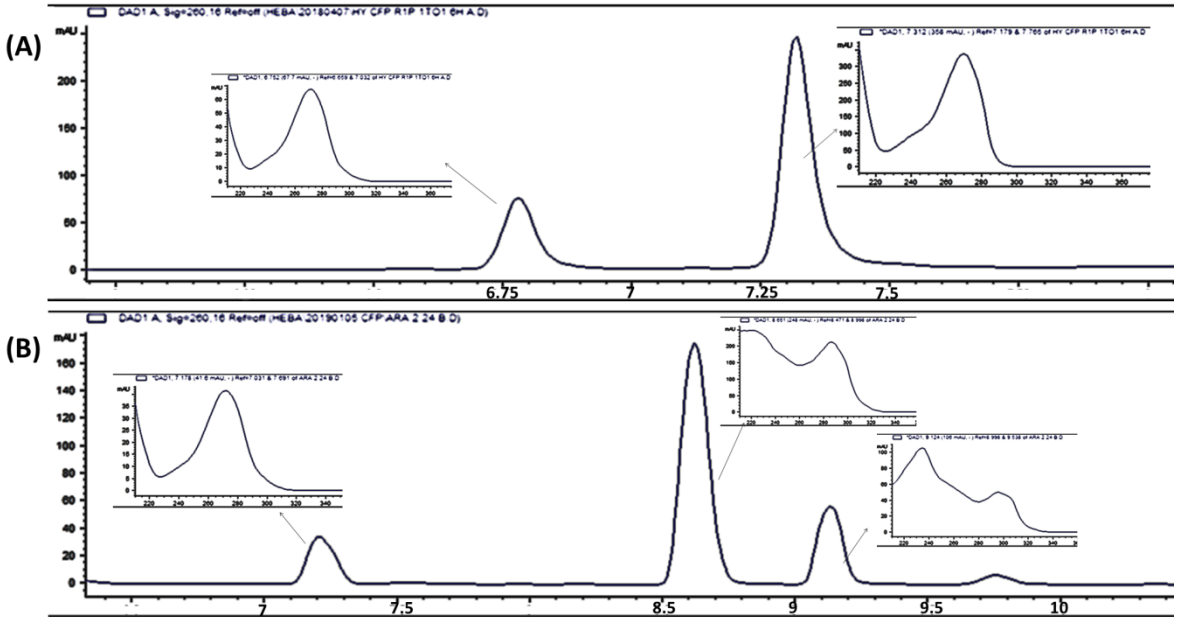


Fig. 11: Chromatograms of **(A)** CFP + R-1-P vs. **(B)** CFP + Ara-1-P reactions illustrating the spectra of the different peaks.

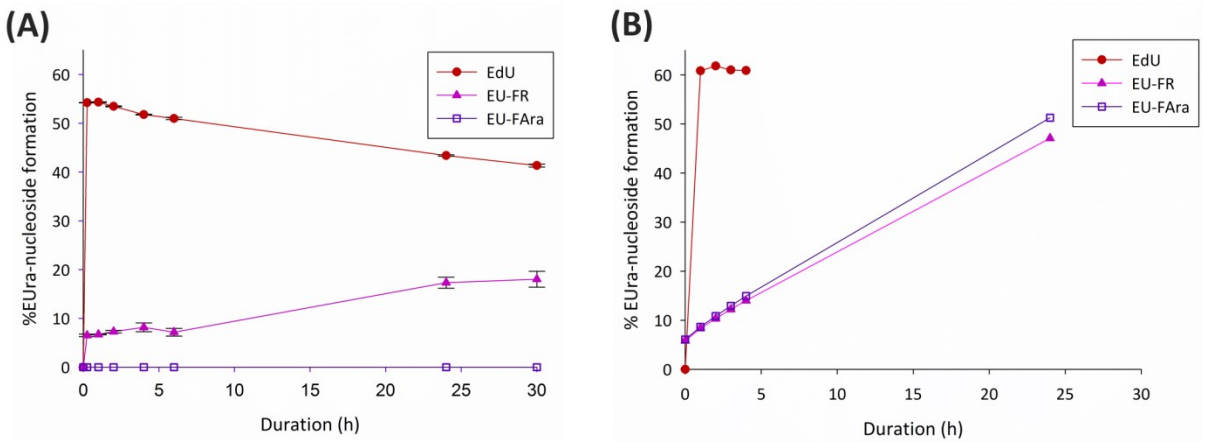


Fig. 12: Synthesis of different EUra nucleosides by **(A)** transglycosylation with Thd, FU and FanaU (n=2) and **(B)** direct glycosylation with dR-1-P, FR-1-P and FAra-1-P.

5.3. Production of natural and modified nucleosides in an enzymatic membrane reactor

In transglycosylation reactions in the EMR, a ratio of sugar donor to nucleobase of 2.5 to 1 was used. As in the small scale experiments, there was an excess of the PNP over PyNP with the ratio of 7:1.

To establish EMRs, the choice of the cut-off membrane might be critical. Hence, different membranes were tested. The tested membrane types were screened for their properties. PES is generally inert and tolerant to a wide pH range and flux rate. Regenerated cellulose can endure different solvents and does not retain proteins. Hydrophilic polysulphone can operate at high temperatures and at a

wide pH range. Fluoropolymer membranes have superior fouling-resistant properties and are easily cleaned for reuse without the need for specific detergents. The different tested membranes had no influence on the enzyme activity or the pressure inside the reactor compartment (Fig. 13). Polyethersulfone (PES) ultrafiltration membrane was selected for further experiments on account of its good enzyme rejection and retaining properties, in addition to its high operational time span and stability (Sitanggang *et al.*, 2014).

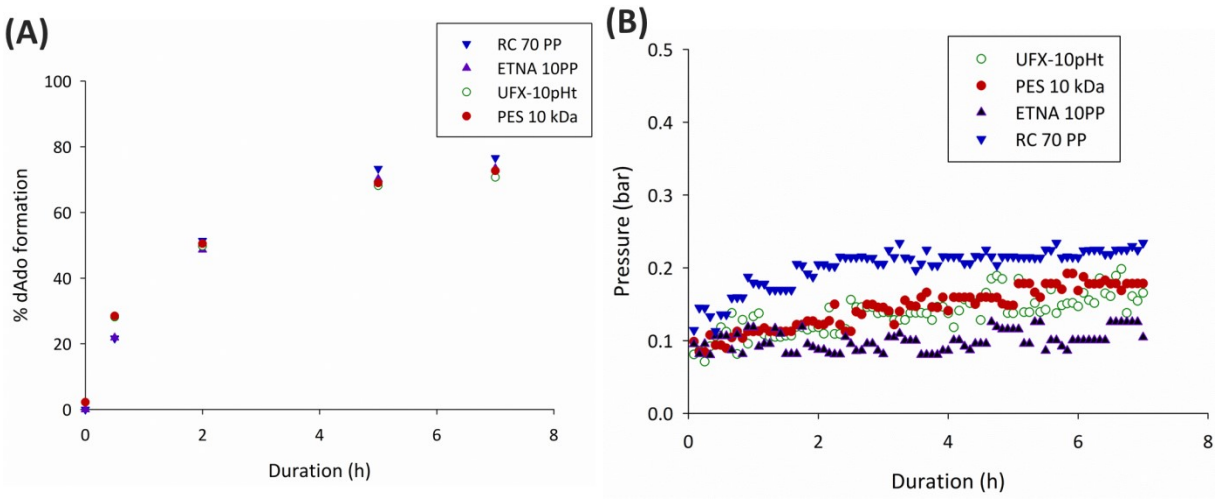


Fig. 13: Effect of different membranes on the enzyme-catalyzed conversion of Ade to dAdo **(A)** and the measured pressure for each (HRT= 7 h) **(B)**.

The formation of dAdo in a transglycosylation reaction was used to establish the EMR. In the first trial experiment, dAdo production was comparable in both batch and continuous mode (ca. 80 % using pure enzymes and ca. 75 % with cell lysate). The time needed to reach the same level increased from 5 h (batch reaction) to 8 h (continuous substrate feeding) with pure enzymes while the cell lysate biocatalysis produced the maximum level at the same duration of both batch and continuous reactions (11 h) (Fig. 14A,B). Thymidine cleavage was the same (ca. 40 %) using pure enzyme or heat-treated *E. coli* lysate. Yet, in batch reaction, the cleavage using pure enzymes kept increasing over the time course which was not observed again in any later experiment.

As the objective of using the continuous production system is to obtain the highest substrate conversion in the shortest possible time, combining batch reaction with continuous substrate feeding was employed. A higher flux/shorter residence time (HRT) than in the starting experiments was used since the reaction equilibrium was already reached. After starting the reactions, they were incubated in batch mode for 6 h to reach dAdo formation of 75 %. Afterwards, substrate feeding was started with a HRT= 4 h. Formation of dAdo was 80 %. Pure enzyme and cell lysate gave almost identical results (Fig. 14C). The same experiment with a higher constant flux (HRT= 2 h) resulted in the reactor instability due to high pressure. The tube junctions burst out leading to the fast depletion of the substrate tanks under the constant flux and the balance back regulation. That is why no HRT below 4 h was used for further experiments.

For the first experiments, the EMRs were controlled by a LabVIEW program and data collection was supported by several National Instruments modules. The LabVIEW program offers a wide range of applications, which however were not needed for the synthesis of NAs in a transglycosylation reaction. Therefore, a cheap and easy-to-handle system was established. Using a python-programmed software, dAdo production was tested. The batch phase was 1-2 h before starting the substrate feeding. Comparing pure enzyme and cell lysate, dAdo formation was 90 % for purified enzyme and only 75 % for the heat-treated *E. coli* extract. In the case of *E. coli* cell lysate,

much reduced activity for both enzymes was noticed on the second day which was overcome by changing the membrane 25 h after starting the reaction. A membrane change was necessary as the membrane was clogged due to the precipitated *E. coli* enzymes from the extract (Fig. 14D). It correlated with strong increase of pressure in the system.

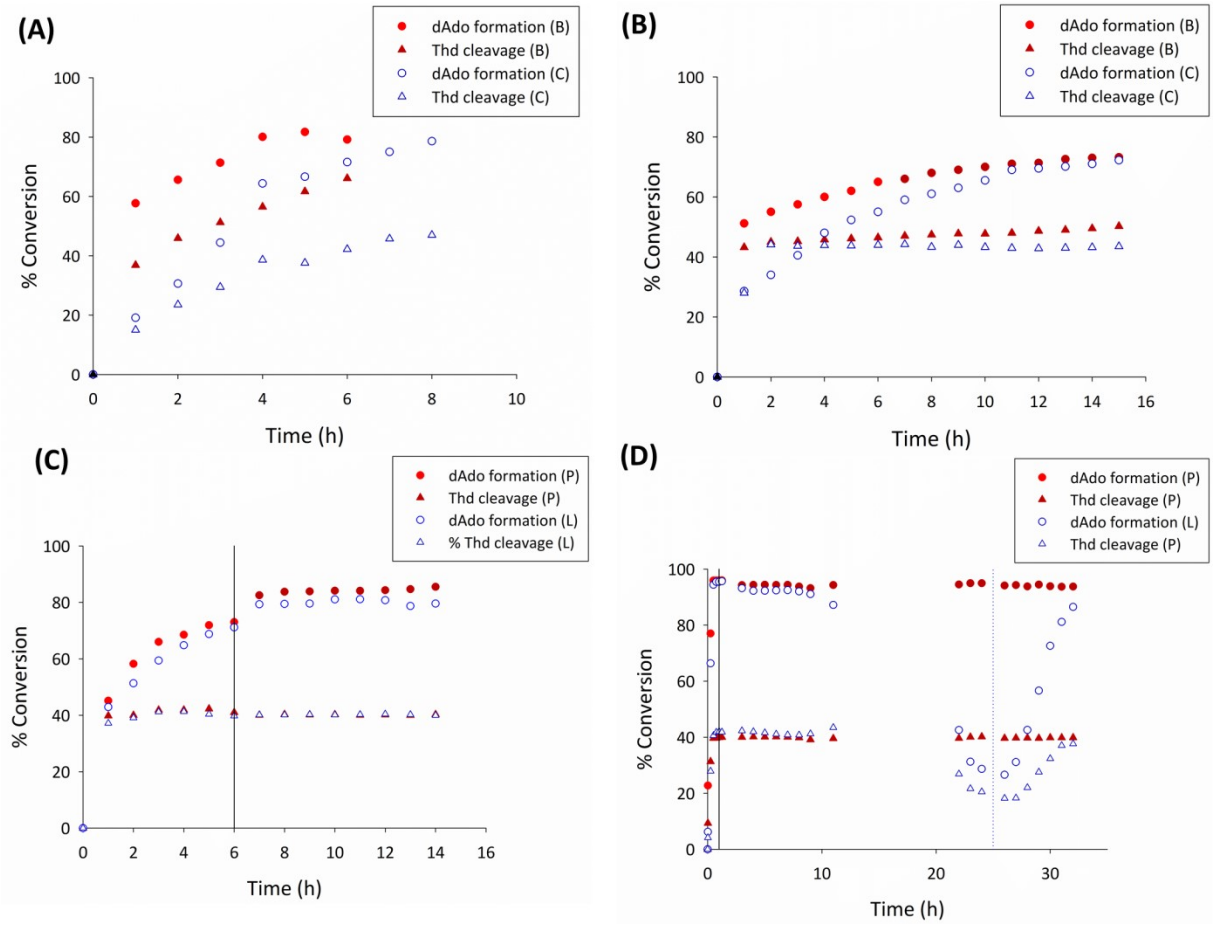


Fig. 14: Production of dAdo from Ade and Thd in EMR at different conditions. Pure PNP 02 and PyNP 02 enzymes **(A)** and heat-treated *E. coli* cell lysates with overexpressed PNP 02 and PyNP 02 **(B)** were used in batch experiment (B) and continuous substrate feeding (C) (HRT= 8 h). **(C)** Continuous substrate feed started after a batch phase of 6 h (HRT= 4 h) for both purified enzymes (P) and cell lysates (L). **(D)** Continuous mode of the EMR was started after 1h (HRT= 4 h). Purified enzymes (P) and cell lysates (L) were applied. The PES membrane was changed for the lysate containing reactor after 25 h (marked by a vertical line).

The synthesis of modified nucleosides (DCP-dR and CFP-dR) was studied using the EMR. In both cases, the maximum conversion was observed after 1 h in the batch phase. Afterwards, substrate feeding started with a HRT of 4 h. DCP-dR formation maintained the maximum level until around 60 h. It was noticed that PyNP 02 activity declined few hours later than PNP 02 activity. CFP-dR formation decreased after almost 20 h while Thd cleavage was steady until 30 h. As the decrease of activity was coupled with turbidity in the reactor vessel, the content was centrifuged and the membrane was changed which resulted in temporary increase in activity around 44 h followed by further decline (Fig. 15).

The final experiment was carried out to evaluate the span of the pure enzymes and the recombinant *E. coli* cell lysate activity against natural substrates (Ade and Thd). The cell lysate was active for up to 4 weeks (~ 40 % Thd cleavage, ~ 85 % dAdo formation) while the pure enzymes exceeded 8 weeks of unaltered activity (40 % Thd cleavage, 92 % dAdo formation).

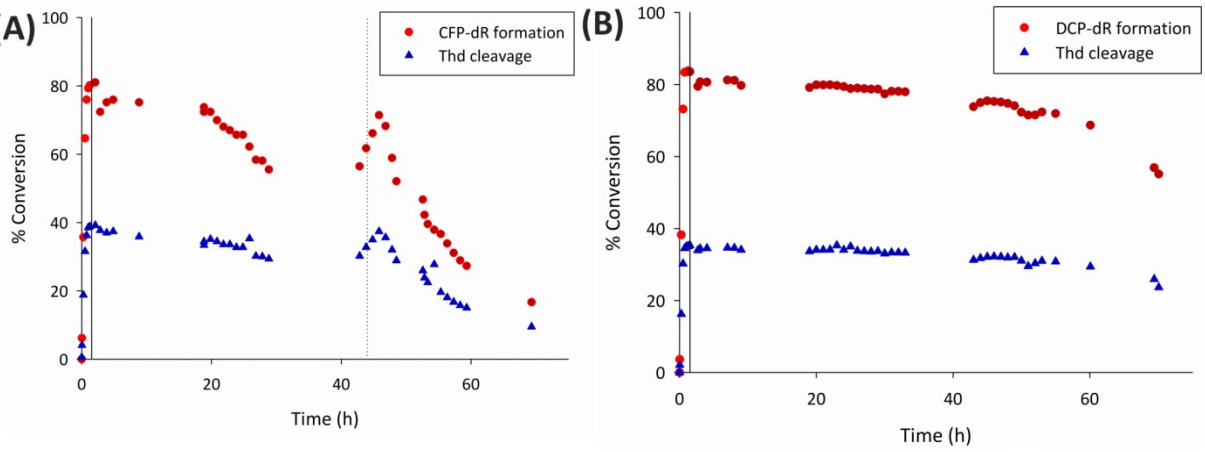


Fig. 15: Synthesis of CFP-dR **(A)** and DCP-dR **(B)** in an EMR using purified PNP 02 and PyNP 02. Substrate feed started after a batch phase of 1 h with HRT of 4 h. Membrane change during CFP-dR synthesis **(A)** occurred at 44 h, marked by a vertical line.

5.4. Production of modified nucleosides in mg scale

Modified nucleosides were produced in batch reactions using the transglycosylation approach. Reactions with thymidine serving as a sugar donor were performed at 40 °C to protect the thermolabile dR-1-P against hydrolysis (Drenichev *et al.*, 2018). High conversion percentages were obtained for all the reactions (~ 60 % or higher) since one of the involved reactants (Urd or Thd) is a natural substrate for NPs (Fig. 16).

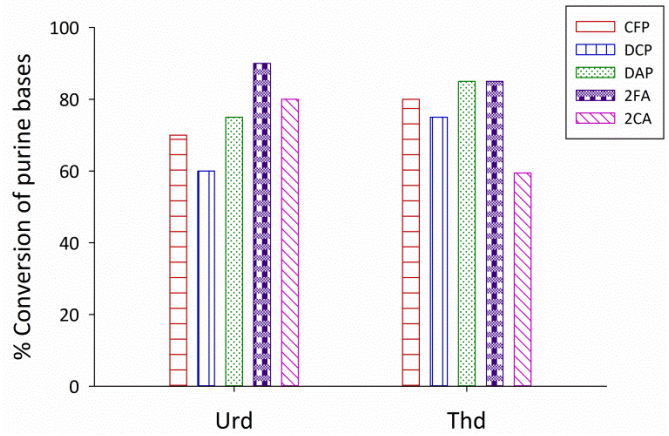


Fig 16: Yields of transglycosylation reactions for different purine bases and Urd or Thd as sugar donors.

The yields and recovery percentages of the produced NAs were calculated relative to the depletion of the purine bases (Table 6). To reach a purity >95 %, the purifications by HPLC for DAP containing nucleosides was repeated twice resulting in the highest loss of product. NMR analysis confirmed the structures for all produced NAs (Appendix II).

Table 6: Production parameters for transglycosylation reactions to synthesize purine analogs.

Compound	Conversion (%)	Product yield		Purity (%)	Recovery (%)
		mg	mmol		
2CA-R	80	129	0.43	99.7	57
2CA-dR	60	91	0.32	99.7	43
2FA-R	90	154	0.54	99.5	72
2FA-dR	85	121	0.45	97	60
CFP-R	70	213	0.7	99	56
CFP-dR	80	248	0.86	99.3	69
DCP-R	60	180	0.56	98.4	45
DCP-dR	75	189	0.62	99.6	50
DAP-R	75	98	0.35	99.3	47
DAP-dR	85	114	0.43	96.1	57

5.5. Cytotoxicity assays

Driven by the well-established therapeutic properties of purine analogs as anticancer drugs, the cytotoxicity of the purified NAs against several hematologic leukemia cell lines was investigated. The acute myeloid leukemia cells line HL-60 was utilized as the initial test cell lines. Human embryonic kidney cells (HEK293) were used as a non-malignant control. Chemically-produced cladribine (purchased from Carbosynth) was used as a positive control. Although cladribine has been extensively studied before, it was included in these tests to check whether the enzymatic synthesis method affected the compounds' activity.

Initial screening experiments were carried out for 10 enzymatically-synthesized NAs against HL-60 and HEK293 cells. Three concentrations were applied to the tested wells (100 µM, 1 µM and 100 nM). The results showed that except for 2-aminoadenine nucleosides, all the higher concentrations of the tested NAs have cytotoxic activity against the leukemia cells under investigation (Fig. 17).

2FA-R, 2FA-dR and 2CA-dR were chosen for further studies. The IC_{50} for these compounds were determined against different hematologic cell lines (HL-60 and CCRF-CEM) with chemically-produced 2CA-dR as a positive control (Table 7). The chemically and enzymatically synthesized 2CA-dR gave comparable results. 2CA-dR and 2FA-dR showed comparable results. While 2CA-dR had a lower IC_{50} values, 2FA-dR showed a higher percentage of killed cells.

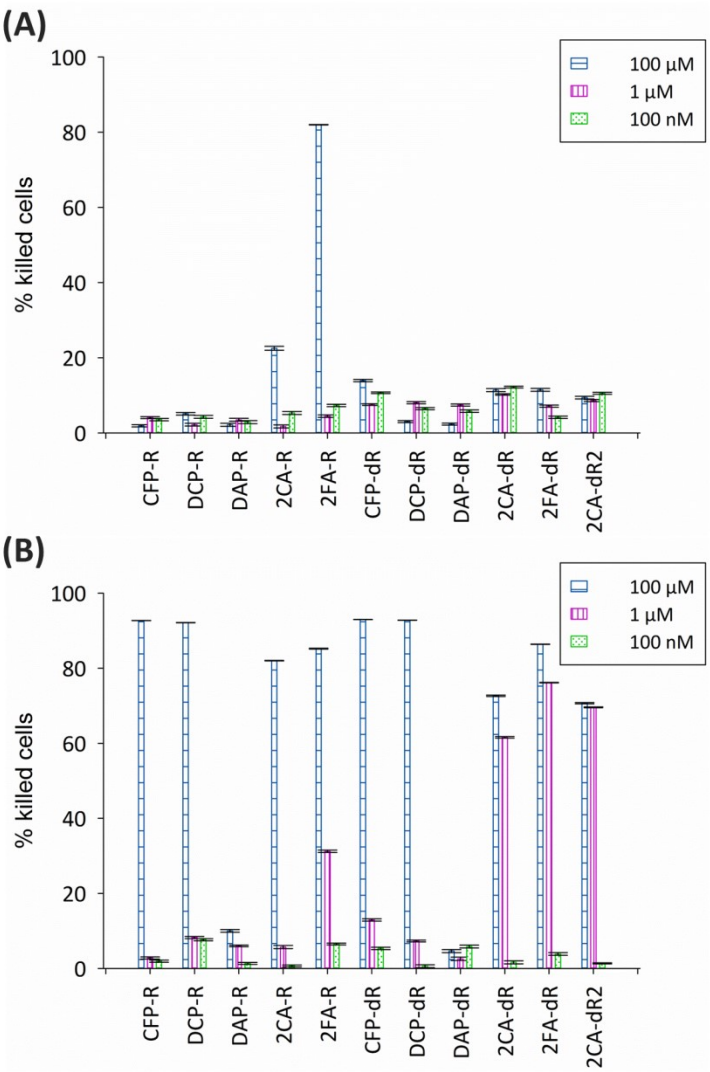


Fig. 17: Cell viability assay (XTT assay) of enzymatically-produced NAs against (A) acute myeloid leukemia (HL-60) cells and (B) normal human embryonic kidney cells (HEK293). The positive control was cladribine produced in a chemical process (2CA-dR2). The results represent the mean ± SD of three independent experiments.

Table 7: Determined IC_{50} (µM) for 2CA-dR, 2FA-R and 2FA-dR. IC_{50} values reflect the concentration that inhibits 50 % of cell growth compared to the negative control (PBS). The IC_{50} values were calculated from viability curves. The results are presented as arithmetic means ± standard error of the mean (S.E.).

Compound	Cell line	
	HL-60	CCRF-CEM
2CA-dR (enzymatically produced)	0.092 ± 0.0382	6.13E-01 ± 0.257
2FA-R	1.26E+00 ± 0.118	1.61E+00 ± 0.415
2FA-dR	0.36634 ± 0.116	7.36E+00 ± 0.693
2CA-dR (chemically-produced: +ve control)	0.18202 ± 0.061	3.88E-01 ± 0.315

6. Discussion

Since, to this day, NPs are still an important subject of research, this study was directed towards the optimization of the enzymatic reaction in small scale and the scale up of the whole process. It was successfully shown that "the golden standard" transglycosylation reaction show high product yields with ribosides or deoxyribosides as sugar donors. With sugar-modified substrates, however, final product yields were low or even no product was detected. Direct glycosylation reactions starting from pentofuranose-1-phosphate were superior in this case and strongly increased product yields were observed in comparison to transglycosylation reactions.

To transfer enzymatic reactions catalyzed by nucleoside phosphorylases into industrial application, both the expression of the biocatalysts and the enzymatic reactions were up-scaled in this study. The up-scaling from 50 mL shake flask cultivations to 3.7 L benchtop bioreactors was shown. Although the protein yields were slightly lower in bioreactor cultivations, better volumetric yields were obtained. Thermostable PyNP, PNP and MTAP were successfully expressed in bioreactor cultivations.

To up-scale the enzymatic production of NAs, both a batch and a continuous approach were evaluated. Both methods led to comparable product yields, however, due to the longevity of the thermostable biocatalysts the productivity was drastically increased in the continuous system. Purified enzymes were stable for more than 2 month producing dAdo in a transglycosylation reaction. Using the batch method, 9 derivatives of cladribine were successfully produced and purified using semi-preparative HPLC. The compounds were validated for their biological activity in different hematologic leukemia cell lines. In general, a higher non-specific toxicity was observed with ribosides in comparison to deoxyribosides.

6.1. Advantages of glycosylation vs. transglycosylation for producing modified nucleosides

To produce NAs using NP biocatalysts, two different routes were investigated in this study: the one-pot, two-steps transglycosylation reaction and the direct glycosylation starting from α-PF-1-P.

The synthesis of different 5-ethynyl uracil (EUra) nucleosides (**Paper III**) was studied in detail. These compounds, mainly deoxyriboside and fluoroarabinoside nucleosides, were already described as sensitive and quick tools for DNA labeling (Salic and Mitchison, 2008; Neef and Luedtke, 2011). The transglycosylation of EUra using Thd afforded 55 % of EdU formation compared to 60 % conversion observed in the direct glycosylation with dR-1-P. Comparable results were observed in transglycosylation reactions with pyrimidines substituted at the 5-position with electronegative groups (e.g. halides) (Serra *et al.*, 2013). The yields for EU-FR and EU-FAra production increased from less than 5 % upon using the respective sugar-modified nucleoside as a sugar donor to almost 50 % when direct glycosylation was adapted. The results for the synthesis of EU-FAra are promising due to its advantageous sensitivity-toxicity balance during the labeling of nucleic acids, especially in the cases of longer analysis times (Neef and Luedtke, 2011; Ligasov and Koberna, 2018).

The products yields normally depend on the substrates, phosphate concentration in the reaction or the nature and affinity of the enzyme (Tono and Cohen, 1962; Utagawa *et al.*, 1985; Taverna-Porro *et al.*, 2008; Fateev *et al.*, 2015). However, yields obtained in this study come in accordance with previous results described for reactions that involve sugar modified scaffolds (Table 8). For example, thermophilic UP showed no activity against 2'-deoxy-2'-fluorouracil and thermophilic PNP had 10

times lower catalytic activity towards AraA in comparison to dAdo and DAP-dR (Sinisterra *et al.*, 2010). Long reaction times, up to 2 months, were needed for 2'-deoxy-2'-fluoronucleosides synthesis by transglycosylation (Tuttle *et al.*, 1993). Conversely, the direct reaction of Ara-1-P or FAra-1-P with different purine bases resulted in product formation of 20 to 70 % (Konstantinova *et al.*, 2011; Fateev *et al.*, 2014). The direct glycosylation can be used for the synthesis of nucleosides with various glycone and aglycone scaffolds (Utagawa, 1999; Fateev *et al.*, 2014) yet, it is regarded especially useful for the synthesis of NAs class harboring a fluorine atom in the 2'-position like clofarabine or gemcitabine (Bonate *et al.*, 2006; Kirk, 2006; Liu *et al.*, 2008).

The results clearly show that the transglycosylation reactions are efficient for the synthesis of riboside and deoxyriboside NAs without the need for the extra steps of α-PF-1-Ps synthesis and purification, while direct glycosylation is advantageous for producing sugar-modified nucleosides. This also suggests that NPs are more tolerant to nucleobase modifications than to sugars' (I A Mikhailopulo and Miroshnikov, 2013).

In both, transglycosylation and nucleoside cleavage reactions, the cleavage of pyrimidine nucleosides (exploited as sugar donors) was in the same order of magnitude as observed in previous studies (Szeker *et al.*, 2012; Kamel *et al.*, 2018). It decreased for nucleosides with C2'-fluorine substitution, which can be explained by the strong electronegativity of the fluorine substituent (in comparison to the native oxygen) resulting in a shorter and hence, stronger glycosidic bond. Additionally, steric properties that include C2' are critical to guarantee the correct conformation of the sugar moiety and hence, the Walden inversion at the tetrahedral chiral C1' atom that mediates the NP-catalyzed SN2 reaction (Stoeckler *et al.*, 1980; I A Mikhailopulo and Miroshnikov, 2011). That is why the direct glycosylation is more convenient for producing sugar-modified nucleosides as it does not have to consider the propriety of all the conditions at once ie: the two steps can be optimized separately.

6.2. Expression of NPs in benchtop bioreactors

Thermostable NPs were expressed in recombinant *E. coli* in high cell density cultivations in benchtop bioreactors. Glucose was used as a sole carbon source and active forms of the proteins were obtained. The cultivations consisted of two steps; pre-induction growth step that include the batch and exponential fed-batch cultivation and post-induction production step.

It is generally postulated that the lag phase can be divided into lag1 and lag2. Lag1 is the acclimatization of cells to the new medium via the formation of efficient tools for carbon utilization. Lag2 starts afterwards when the expression of the genes necessary for amino acids synthesis and ribosomes start and growth in cell size occurs yet, without division. The longer lag phase after EnPresso B starter culture, in comparison to the no/or short lag phase after MSM-glucose preculture, probably reflects the adaptation to the transition from the richer cultivation medium (EnPresso B) that comprises yeast and peptone. Thus, the lack of preformed amino acids in the MSM-glucose medium cause the prolongation of lag1 phase where the cells' machinery focuses on producing the necessary transporters and enzymes for maximal biomass production during the exponential growth phase (Madar *et al.*, 2013; Schultz and Kishony, 2013).

The protein yields obtained in this study were low in comparison to other protein yields reported in literature and even other NPs produced in *E. coli* high cell density cultivations (Choi *et al.*, 2006; Ren *et al.*, 2013) (Table 9). An average of 10 folds higher production levels are noted for several proteins including NPs (mesophilic UP and PNP from *E. coli*) (Zuffi *et al.*, 2004).

Table 8: Some examples of NAs produced in pure form by NPs through either transglycosylation or direct glycosylation with α-PF-1-P.

NA product	Reactants	Catalyst	Duration (h)	Reaction termination	Yield* (%)	Purification method	Purity (%)	Recovery* (%)	Reference
2CA-dR	2CA + dGuo	GA-*E. coli* BMT-1D/1A cells	21	Boiling then centrifugation	65	Silica gel chromatography, crystallization	90	54	(Mikhailopulo *et al.*, 1993)
5'-Deoxy-6-thioguanosine	5'-deoxyadenosine + 6-thioguanine	adenosine deaminase + PNP	190	Boiling	N.D	Silica gel reverse phase chromatography via preparative HPLC	N.D	15	(Chae *et al.*, 1998)
6-Thiopurine-5'-deoxyriboside	5'-dAdo + 6-thiopurine							13.5	
6-Thiopurine-2',5'-dideoxy riboside	6-thiopurine + 2',5'-dideoxythymidine	TP + PNP	12					12	
2',5'-Dideoxy-6-thioguanosine	6-thioguanine + 2',5'-dideoxy-thymidine	TP + PNP	24					18	
2CA-dR	2 CA + dGuo	GA-*E. coli* BMT-4D/1A cells	7	Centrifugation	95	Silica gel chromatography, crystallization	99.6	81	(Barai *et al.*, 2002)
Ribavirin	TCA + Guo	GA-*E. coli* BMT-4D/1A cells	30		90-92	Ion-exchange chromatography, crystallization	99.46	67-70	
Ribavirin	TCA + Guo	Immobilized PNP	36	Decantation (repeated reactions)	92	Ion-exchange chromatography, crystallization	99.9	68	(Konstantinova *et al.*, 2004)
6-Azauridine	6-Azauracil + Ado	Immobilized PNP + UP	4.8		19.3	Silica gel chromatography, crystallization	99	45	
Vidarabine	AraU + Ade		24		45.7		98	47	

*Calculated depending on the nucleobase, N.D: not described

Table 8: Some examples of NAs produced in pure form by NPs through either transglycosylation or direct glycosylation with α-PF-1-P (Continued).

NA product	Reactants	Catalyst	Duration (h)	Reaction termination	Yield* (%)	Purification method	Purity (%)	Recovery* (%)	Reference
DAP-Ara	AraU + DAP	*E. aerogenes* cells	48	Centrifugation	90	Crystallization at 4 °C	N.D	N.D	(Wei *et al.*, 2008)
5-Methyluridine	Guo + Thy	PNP + UP	26	Heat to 90 °C	92 (Guo)	Hot filtration, cold filtration, crystallization	90	84	(Gordon *et al.*, 2011)
Fludarabine	2FA + α/β Ara-1-P	*E. coli* PNP	3	Store at 14 °C for 24 h	95	Product filtration	98	77	(Konstantinova *et al.*, 2011)
Nelarabine	2-amino-6-methoxypurine + α/β Ara-1-P		36	Evaporation till dryness	N.D	silica gel chromatography	N.D	40	
9-β-D-Arabinohypoxanthine	Hypoxanthine + α/β Ara-1-P		N.D				98	81	
Arabinothymidine	Thymine + α/β Ara-1-P						99.4	61	
Clofarabine	2CA + α/β FAra-1-P	*E. coli* PNP	168	Filtration	N.D	silica gel chromatography	99.4	42	(Fateev *et al.*, 2014)
5-Aza-7-deazaguanine-2'- fluoro-arabinoside	5-Aza-7-deazaguanine + α/β FAra-1-P		384				99.3	81	
CFP-R	Urd + CFP	PNP + PyNP (on magnetic beads)	20	Mechanical removal by magnet	75	silica gel column chromatography	98	60	(Zhou *et al.*, 2015)

*Calculated depending on the nucleobase

N.D: not described

The lower protein production can be attributed to the leaky expression that may destabilize the plasmids and increase the cellular metabolic strain. Another possibility is the weak termination sequence downstream of the stop codons which may not be enough to stabilize the mRNA transcript. This is responsible for an increased energy consumption and metabolic burden (Joseph *et al.*, 2015). The considerable inclusion body (IB) formation is another aspect that results from an unbalanced equilibrium between protein aggregation and solubility and is considered a big problem while producing high levels of recombinant heterologous proteins during bioreactor cultivation processes. The underlying reason is the difference between the microenvironment of *E. coli* and that of the native organism like pH, cofactors, redox potential or folding mechanisms. The protein misfolding increases protein aggregation into inclusion bodies (Rosano and Ceccarelli, 2014). The aggregation is favored at higher temperatures due to the strong temperature dependence of hydrophobic interactions that constitute the aggregation (Sorensen and Mortensen, 2005).

In this study, the expression strategy was optimized to increase protein yields for thermophilic NPs. Critical factors are feeding, formation of acetate or IPTG concentration. In all bioreactor cultures, the feeding was decreased after induction as it was shown before that a lower feeding rate leads to higher protein production, despite lower biomass (Norsyahida *et al.*, 2009). This is explained relevant to the metabolic load conferred on the cells after induction, change of host cell physiology, the toxicity of by-products and the general decrease of specific growth rate (Norsyahida *et al.*, 2009; Leone *et al.*, 2015). Furthermore, bioreactor cultivations were carried out at 30 °C to achieve a lower ratio of inclusion bodies formation in comparison to the ones at 37 °C. Finally, the amount of insoluble protein from the bioreactor was quite comparable to shake flask protein production performed in parallel. It was also in good accordance with shake flask cultivations performed previously with thermostable enzymes (Zhou *et al.*, 2013).

Acetate accumulation is known to negatively influence *E. coli* growth and protein production in addition to being considered a carbon sink. In this study, acetate accumulation was only measured during PNP 03 production and it was observed that protein production was still increasing when the cells were harvested, yet the process was stopped due to high accumulation of acetate. Increasing acetate concentrations are explained by an overflow metabolism in the presence of high glucose concentrations. It is also known as the bacterial Crabtree effect (Eiteman and Altman, 2006; Leone *et al.*, 2015). The formation of acetate can be limited using a system that allows for higher k_La values or applying co-aeration with pure oxygen. In the context of acetate formation, BL21 is the host of choice for recombinant protein expression among the different *E. coli* production strains due to a higher level of acetate metabolism and glyoxylate shunt that promotes its use in the TCA cycle. It also lacks two proteases (*lon*, *ompT*) leading to higher protein yields (Marisch *et al.*, 2013). However, glucose limitation is still a must throughout the cultivation as it preserves the growth rate below the maximum specific growth rate. The relevance of glucose limitation was observed for cultivation II (PyNP 02) and III (PNP 02), where lower protein production was detected after glucose accumulation.

In the first experiments, IPTG was added at the same volumetric concentration as in the shake flask. For PNP 03 expression, the IPTG concentration was dependent on the biomass reached before induction. The difference is reflected in the NPs yields (0.311 and 0.15 g L^{-1} for PNP 03 and 02, respectively). IPTG concentration is a critical factor as both too low and too high concentrations are associated with reduced protein production. The latter is due to the toxicity effects resulting from driving metabolic resources to protein production rather than cell maintenance (Yazdani *et al.*, 2004).

Table 9: Examples of some recombinant proteins produced in high cell density *E. coli*.

Protein	Host	Expression vector (promoter/ inducing agent)	Culture conditions and carbon source	Production yields		Reference
				Cells	Protein ($g\ L^{-1}$)	
PvRII (malaria vaccine)	*E. coli* BLR(DE3)pLysS	pET28a(+) (T7 / IPTG)	batch, fed-batch (exponential then linear feed)/ glucose	85 $g\ L^{-1}$ DCW	0.8	(Yazdani *et al.*, 2004)
*Ec*PNP	*E. coli* DH5α	pUC18 (lac / no induction)	batch, fed-batch (constant feed increased on intervals)/ glycerol	Biomass 171±8.2	2.3±0.24	(Zuffi *et al.*, 2004)
*Ec*UP				Biomass 167±10.5	4.8±0.33	
EI-β-gal[1]	Rosetta™ (DE3)	pET (T7 / IPTG)	batch, fed-batch (linear feed)/ glucose	OD_{600} 151	0.234	(Fong and Wood, 2010)
EI-OPH[2]				OD_{600} 129	0.236	
Tyrosinase	*E. coli* JM109	pMFvpt (T5 / IPTG)	fed-batch with exponential feeding/ glycerol	OD_{600}: 102±2	2.89±0.48	(Ren *et al.*, 2013)
Valinomycin	*E. coli* BL21-Gold derivative	pCTUT7 derivative (lac / IPTG)	batch, fed-batch (exponential then constant feed)/ glucose	OD_{600}: 120	0.002	(Li *et al.*, 2015)
rMBP-NAP[3]	*E. coli* TB1	pNEB (lac / IPTG)	batch/ glucose	30 $g\ L^{-1}$ biomass	1.738	(Lu *et al.*, 2015)
antithrom-bosis insulin variant	*E. coli* BL21(DE3)pLysS	pET-3a derivative (lacUV5 / IPTG)	batch, fed-batch (constant feed)/ glycerol	OD_{600}: 66	IB level = 29 %	(Jing *et al.*, 2018)
PNP 03	*E. coli* BL21-Gold	pCTUT7 derivative (lac / IPTG)	batch, fed-batch (exponential then constant feed)/ glucose	OD_{600}: 105	0.311	This study
PNP 02				OD_{600}: 170	0.15	

[1]Elastin-like polypeptide-intein-tagged β-galactosidase

[2]Elastin-like polypeptide-intein-tagged mutant parathion hydrolase

[3]rMBP-NAP: maltose binding protein fused to *Helicobacter pylori* neutrophil activating protein

Because of the denser biomass cultures in the bioreactor, IPTG concentration for induction should be either empirically increased (in comparison to shake flask) or calculated relevant to the biomass as done in fed-batch cultivation V (PNP 03). Alternative approach to increase protein production could be the addition of a second IPTG dose during the production phase or the addition of IPTG to the feeding glucose solution to prevent dilution of the inducing agent in the culture (Norsyahida *et al.*, 2009; Restaino *et al.*, 2013).

The biomass yields in shake flasks and the bioreactor cannot be compared without acknowledging the difference in hydromechanical stress. It is quantified using the parameter local energy dissipation rate (ε_{max}). This is usually one order of magnitude higher in stirred tank reactor than in shake flasks. Whereas bacterial cells are generally regarded as robust in comparison to animal cells or fungi, the cell damage and reduced cell growth due to stress is not a completely negligible factor (Klöckner and Büchs, 2012; Lattermann and Büchs, 2016). This is one of the reasons for the higher protein per biomass yield in shake flasks.

In PNP 02 and 03 overexpression, magnesium, that is detrimental for many enzymes' activities and biosynthetic pathways, was replenished regularly to avoid starvation. Mg^{2+} limitation leads to the impairment of cell membrane integrity, debilitated transport of nitrogen, sulfur and iron, faster rate of 70S ribosome unfolding and degradation, decrease of the cellular protein content and consequent growth cessation (Nierhaus, 2014; Caglar *et al.*, 2017). Its lack was also connected to a decline in carbon source dedication to biomass and an alternative fermentation pathway and acetate build up occurrence (Christensen *et al.*, 2017).

6.3. Producing NAs in continuous enzyme membrane reactor

Biocatalysts are cost driving factors in the enzymatic synthesis routes. To decrease the operational cost, NPs have been subjected to extended or repeated use by whole cell catalysis, immobilization on an inert support or coupling immobilization to a continuous reactor mode. The small lab scale EMR, initially developed for the continuous bioconversion of lactose and fructose using β-galactosidase (Sitanggang *et al.*, 2014), was used for the synthesis of natural and modified nucleosides in this study. Advantages of EMRs are the prolonged use of the enzyme, applying the enzyme in a free unaltered state, avoiding active sites masking compared to immobilization methods and the selective mass transfer across the membrane in case of substrates and products with variable particle sizes (Rios *et al.*, 2004).

Both purified protein and cell lysate supported product formation for extended times (up to 4 weeks) without any additional enzyme addition. This strongly confirms that the NPs enzyme class is a good candidate for larger scale industrial application. The faster decline in activity against CFP in comparison to the natural Ade or DCP nucleobase may conjecture that nucleobases have the same enzyme inhibition-halogen size relationship as the one observed against cultured cell lines; the larger the halogen substitution at C2 of the nucleobase the less the toxicity (Bennett *et al.*, 1966).

There is not much data about using NPs in continuous mode and as mentioned previously, most NPs applications in continuous reactions were carried out using the immobilized form of the enzyme. *B. stearothermophilus* cell lysate immobilized by anion exchange resin was used as a source for both PNP and PyNP to produce 5-methyl uridine (5-MU), the precursor for stavudine and zidovudine antivirals, from thymine and inosine. The reaction ran stable for 17 days without activity decrease or contamination (Hori *et al.*, 1991). Another continuous synthesis trial proceeded by loading the mesophilic PNP from *Aeromonas hydrophila* onto a prepacked silica column and combining that with an analytical or semi-preparative HPLC system via a switching valve allowing a high reaction surface

area, easier handling and automatic fast monitoring. Using this system for one pot-one enzyme transglycosylation reactions, inosine was used to donate ribose moiety to other purine nucleobases. Conversions up to almost 90 % were achieved and plateaued in 10 to 30 min depending on the nucleobase. This agrees to a good extent with the second group of experiments reported in section 5.3. The system showed good storage and longevity capacity as more than 50 reactions could be performed over 10 months (Calleri *et al.*, 2015). The same system was extended furthermore to integrate an additional column possessing mesophilic UP which is connected prior to the PNP. This setup allowed the production of natural nucleosides (Ado and dAdo) in yields of ca. 90 % after 1 h and 20 % of AraA was recorded after 5 h (Cattaneo *et al.*, 2017).

Another NP reactor application, unrelated to NAs production, was developed to establish an easily reusable system for food quality control. More concisely, the freshness of fish was assessed to determine its suitability for consumption (Carsol and Mascini, 1998; Okuma and Watanabe, 2002). Therefore, inosine monophosphate, inosine and hypoxanthine concentrations in fish samples after ATP degradation were monitored. In one study, alkaline phosphatase, NP and xanthine oxidase were immobilized separately on glass beads and packed in columns in series. In another, the enzymes were co-immobilized on chitosan and packed into two columns. In both cases, the systems were fast, reliable and reproducible. 700 samples could be analyzed over a period of 2 months in the first and 1 month in the latter.

6.4. Cytotoxicity of the enzymatically-produced NAs

The activity of the enzymatically-produced NAs against several hematologic leukemia cell lines was tested. The acute myeloid leukemia cells (HL-60) were chosen as the primary test system due to its extensive use in studying myeloid cells differentiation and proliferation (Birnie, 1988; Majda *et al.*, 2011; Shigemi *et al.*, 2013; Saleh *et al.*, 2014; Golshan *et al.*, 2016). Human embryonic kidney cells (HEK293) were used as a non-malignant control for the potential non-specific cytotoxicity (Saleh *et al.*, 2014; Wang *et al.*, 2015; Golshan *et al.*, 2016).

The tested NAs, except for 2-aminoadenine nucleosides, were active against the leukemia cells under investigation at the higher concentration (100 μM). This is in good accordance with the previous postulation that halogenation is an important means to boost anticancer properties (Faderl *et al.*, 2005; Liu *et al.*, 2005; Rivero, Britos, *et al.*, 2012) since the lack thereof will make the compound susceptible to deamination by adenosine deaminase (ADA) (Pui *et al.*, 2005; Ma *et al.*, 2011). However, the high dose required for the activity of 6-chloro-2-fluoropurine and 2,6-dichloropurine derivatives and 2-chloroadenosine (*cf*. 2'-deoxy-2-chloroadenosine; cladribine) is an important hindrance for their qualification as potential clinical agents due to the expected toxicity.

A non-selective toxicity was observed for 2-fluoroadenosine and 2-chloroadenosine in this study. As per previous reports, 2-fluoroadenosine and its products from PNP activity (2-fluoroadenine) and kinase activity (2-fluoroadenosine triphosphate) exhibit non-selective toxicity (Skipper *et al.*, 1959; Bennett *et al.*, 1966; Avramis and Plunkett, 1983; Bonate *et al.*, 2006; de Giuseppe *et al.*, 2012) and have been partially accounted responsible for fludarabine (9-β-D-arabinofuranosyl-2-fluoroadenine monophosphate) nephrotoxicity (Avramis and Plunkett, 1983).

The less non-selective toxicity of 2-chloroadenosine complies with an old hypothesis that the toxicity decreases with larger halogen substitution at the C2-position of the purine ring (Bennett *et al.*, 1966). The deoxyriboside counterparts of 2-fluoroadenosine and 2-chloroadenosine showed a strongly decreased non-specific toxicity. Percentage of killed HEK293 cells was 11.4 % for both 2CA-dR and 2FA-dR at 100 μM in comparison to 22.5 and 82 % for 2CA-R and 2FA-R, respectively, at the same

concentration. The higher toxicity of ribosides can be connected to one of the following reasons: (i) local dNTPs and NTP pools show differences in concentrations inside cells. NTPs accumulate to higher concentrations at any given time (Potenski and Klein, 2014; Kong *et al.*, 2018) so, they are more available for activity, (ii) RNA polymerase is more prone to proofreading errors (10^{-5} error per base replicated) in comparison to DNA polymerase (10^{-9} to 10^{-10}) (Garwal, 1996; Sydow and Cramer, 2009).

Of the main issues of cell toxicity studies was to prove that impurities from the enzymatic process do not interfere with biological activity. Therefore, commercially available cladribine produced in a chemical process was compared to cladribine produced by NPs in this study. Comparable IC_{50} values of 0.18202 ± 0.061 and 0.092 ± 0.0382 µM, respectively, were calculated against HL-60. This is in good accordance with formerly published data described for the same cell lines (Table 10).

Table 10: Different recorded IC_{50} values (µM) for cladribine (2CA-dR) against the cell lines, also used in this study.

HL-60	CCRF-CEM
0.092 ± 0.0382 (this study)	6.13E-01 ± 0.257 (this study)
>0.05 (Stachnik *et al.*, 2005)	0.09 ± 0.05 (Lotfi *et al.*, 2001)
0.0041 (Shigemi *et al.*, 2013)	0.015 (Huang *et al.*, 1981)
0.041 (Månsson *et al.*, 1999)	0.003 (Carson *et al.*, 1980; Montgomery *et al.*, 1992)
0.0700 ± 0.0060 (Nishi *et al.*, 2013)	

7. Conclusions and Outlook

7.1. Conclusions

In the presented study, a production chain for NAs was successfully established. The expression of NPs was up-scaled from shake flask scale to benchtop bioreactor. The synthesis of halogenated NAs was up-scaled in batch and continuous reactions. In continuous reactions, the applied amount of enzyme was drastically reduced. Furthermore, the activity of NAs produced in an enzymatic process was successfully shown.

Four different NPs were produced in benchtop bioreactors using mineral salt medium with glucose as a single carbon source. The soluble protein amounts were comparable to those from the shake flasks with the advantage of a reduced ecological footprint. Almost three times the protein volumetric yield of the PNP 03 was obtained in the bioreactor (140 mg L^{-1} in shake flask vs. 311 mg L^{-1} in KLF) over approximately the same period of time (ca. 30 h cultivation). 685 mg his-tagged N03 was obtained recording a biomass productivity of 6.78 mg g_{DCW}^{-1}.

Whereas the transglycosylation method was efficient for producing riboside and deoxyriboside nucleosides (>60 % yields based on nucleobase amount in tested cases), the reaction between nucleobases and the corresponding α-PF-1-P was more superior for sugar modified nucleosides (arabinosides and fluoroarabinosides). The synthesis of EUra-containing NAs, studied by either one pot-two enzyme transglycosylation or cascade two pot direct glycosylation, is a clear example. This directly demonstrates that the NPs are more tolerant to nucleobase modifications than those of the sugar moiety and allows the conclusion that only NAs with natural sugar scaffolds could be efficiently produced using another nucleoside as a sugar donor.

NP catalyzed reactions were suitable for up-scaling from µL scale to 100 mL scale or continuous reactions in EMRs showing that the enzymes behave similarly with volumetric expansion. In the presence of the organic solvent DMSO in a concentration up to 20 % v/v, no inhibitory effect on the enzyme activity was observed.

The application of thermostable NPs in continuous EMRs has a high potential for NAs production. NPs were active for more than 8 weeks with natural substrates and up to 7 days with modified substrates. Conversion yields were comparable to the results from batch reactions. Heat-treated cell lysates showed the same nucleoside analog formation results as the pure enzymes in the continuous reaction mode. This is another very prominent finding that supports the suitability of thermostable NPs-catalyzed chemo-enzymatic nucleoside analogs synthesis for commercial use overcoming the need for the extra and cost-intensive purification steps.

In comparison to the chemically produced nucleoside analog cladribine, the enzymatically produced one exhibited similar IC_{50} values in different hematologic cancer cell lines. The results were also in the same order of magnitude as shown in previous reports. Thereby, it can be concluded that the chemo-enzymatic synthesis is a safe and efficient way to produce biologically active NAs in addition to its many advantages over the chemical synthesis.

7.2. Outlook

This study opens room for several interesting improvements. Up-scaling of the expression of NPs was successfully shown, however, some challenges remain. On the one hand, the active units/ mg is decreased in bioreactors compared to shake flask although higher cell density and protein amounts were obtained in the KLF. On the other hand, a significant proportion of inclusion bodies was observed in both shake flask and KLF cultivations. This could be experimented by one of several strategies; cold-shock expression, co-expression of chaperons or investigating the effect of different promotors.

The probability of using DMSO encourages testing how much the enzymes could endure to make the reactions more cost-effective as higher substrate concentrations can be used. Performing reactions that are more concentrated allows making the maximum use of the enzymes and realizing higher amounts of products with poorly soluble substrates (ex: 2FA, 2CA, EU). Pulse feeding of substrates could also be performed after the equilibrium state is reached.

Utilizing NPs in EMR has been depicted to rely on many variables like flux rate or pressure in addition to the other factors controlling the enzymatic reaction (substrate concentration, enzyme concentration, enzyme half-life, turnover number). Studying the effects of these variables would certainly help understand the nature of the reaction. It would be very advantageous if the reaction in the EMR could be mathematically modelled to allow for a better optimization.

Incorporating a further step of kinase-catalyzed reactions in one pot with the transglycosylation reaction also paves the way to a very interesting investigation. Using this approach, the overall reaction can be balanced towards the target product.

8. References

Almendros, M., Berenguer, J. and Sinisterra, J. V. (2012). *Thermus thermophilus* nucleoside phosphorylases active in the synthesis of nucleoside analogues. *Applied and Environmental Microbiology*. 78(9):3128–3135.

Anand, R., Kaminski, P. A. and Ealick, S. E. (2004). Structures of purine 2'-deoxyribosyltransferase, substrate complexes, and the ribosylated enzyme intermediate at 2.0 Å resolution. *Biochemistry*. 43(9):2384–2393.

Del Arco, J., Cejudo-sanches, J., Esteban, I., Clemente-suárez, V. J., Hormigo, D., Perona, A. and Fernández-lucas, J. (2017). Enzymatic production of dietary nucleotides from low-soluble purine bases by an efficient , thermostable and alkali-tolerant biocatalyst. *Food Chemistry*. 237:605–611.

Del Arco, J. and Fernández-Lucas, J. (2018). Purine and pyrimidine salvage pathway in thermophiles: a valuable source of biocatalysts for the industrial production of nucleic acid derivatives. *Applied Microbiology and Biotechnology*. 102:7805–7820.

Armstrong, S. R., Cook, W. J., Short, S. A. and Ealick, S. E. (1996). Crystal structures of nucleoside 2-deoxyribosyltransferase in native and ligand-bound forms reveal architecture of the active site. *Structure*. 4(1):97–107.

Avendaño, C. and Menéndez, J. C. (2008). Antimetabolites. in Avendaño, C. and Menéndez, J. C. (eds) *Medicinal Chemistry of Anticancer Drugs*. Elsevier 9–52.

Avramis, V. I. and Plunkett, W. (1983). 2-Fluoro-ATP: A toxic metabolite of 9-β-D-arabinosyl-2-fluoroadenine. *Biochemical and Biophysical Research Communications*. 35–43.

Balakrishnan, K., Nimmanapalli, R., Ravandi, F., Keating, M. J. and Gandhi, V. (2006). Forodesine, an inhibitor of purine nucleoside phosphorylase, induces apoptosis in chronic lymphocytic leukemia cells. *Blood*. 108(7):2392–2398.

Barai, V. N., Zinchenko, A. I., Eroshevskaya, L. A., Kalinichenko, E. N., Kulak, T. I. and Mikhailopulo, I. A. (2002). A universal biocatalyst for the preparation of base- and sugar-modified nucleosides via an enzymatic transglycosylation. *Helvetica Chimica Acta*. 85(7):1901–1908.

Bennett, L. L., Vail, M. H., Chumley, S. and Montgomery, J. A. (1966). Activity of adenosine analogs against a cell culture, line resistant to 2-fluoroadenine. *Biochemical Pharmacology*. 15(11):1719–1728.

Bergmann, W. and Burke, D. C. (1955). Contributions to the study of marine products. XXXIX. The nucleosides of sponges. III. Spongothymidine and Spongouridine. *Journal of Organic Chemistry*. 20(11):1501–1507.

Bergmann, W. and Feeney, R. J. (1950). The isolation of a new thymine pentoside from sponges. *Journal of the American Chemical Society*. 72(6):2809–2810.

Bergmann, W. and Stempien Jr., M. F. (1957). Contributions to the study of marine products . XLIII . The nucleosides of sponges . V . The synthesis of spongosine. *Journal of Organic Chemistry*. 22(12):1575–1577.

Birnie, G. D. (1988). The HL60 cell line: a model system for studying human myeloid cell differentiation. *The British Journal of Cancer. Supplement*. 9:41–45.

Bonate, P. L., Arthaud, L., Cantrell Jr., W. R., Stephenson, K., Secrist 3rd, J. A. and Weitman, S. (2006). Discovery and development of clofarabine: a nucleoside analogue for treating cancer. *Nature Reviews Drug Discovery*. 5(10):855–863.

Bruins, M., Janssen, A. and Boom, R. (2001). Thermozymes and their applications. *Applied Biochemistry and Biotechnology*. Humana Press Inc. 90(2):155–186.

Burchenal, J. H., Murphy, M. L., Ellison, R. R., Sykes, M. P., Tan, T. C., Leone, L. A., Karnof-Sky, D. A., Craver, L. F., Dargeon, H. W. and Rhoads, C. P. (1953). Clinical evaluation of a new antimetabolite, 6-mercaptopurine, in the treatment of leukemia and allied diseases. *Blood*. 8(11):965–999.

Bzowska, A., Kulikowska, E. and Shugar, D. (2000). Purine nucleoside phosphorylases: properties,

functions, and clinical aspects. *Pharmacology & Therapeutics*. 88(3):349–425.

Caglar, M. U. et al. (2017). The *E . coli* molecular phenotype under different growth conditions. *Scientific Reports*. 7:45303.

Calleri, E., Cattaneo, G., Rabuffetti, M., Serra, I., Bavaro, T., Massolini, G., Speranza, G. and Ubiali, D. (2015). Flow-synthesis of nucleosides catalyzed by an immobilized purine nucleoside phosphorylase from *Aeromonas hydrophila*: Integrated systems of reaction control and product purification. *Advanced Synthesis & Catalysis*. 357(11):2520–2528.

Campagnola, G., Mcdonald, S., Beaucourt, S., Vignuzzi, M. and Peersen, B. (2015). Structure-function relationships underlying the replication fidelity of viral RNA-dependent RNA polymerases. 89(1):275–286.

Caradoc-Davies, T. T., Cutfield, S. M., Lamont, I. L. and Cutfield, J. F. (2004). Crystal structures of *Escherichia coli* uridine phosphorylase in two native and three complexed forms reveal basis of substrate specificity, induced conformational changes and influence of potassium. *Journal of Molecular Biology*. 337(2):337–354.

Carsol, M. A. and Mascini, M. (1998). Development of a system with enzyme reactors for the determination of fish freshness. *Talanta*. 47(2):335–342.

Carson, D. A., Wasson, D. B., Kaye, J., Ullman, B., Martin Jr., D. W., Robins, R. K. and Montgomery, J. A. (1980). Deoxycytidine kinase-mediated toxicity of deoxyadenosine analogs toward malignant human lymphoblasts in vitro and toward murine L1210 leukemia in vivo. *Proceedings of the National Academy of Sciences USA*. 77(11):6865–6869.

Cattaneo, G., Rabuffetti, M., Speranza, G., Kupfer, T., Peters, B., Massolini, G., Ubiali, D. and Calleri, E. (2017). Synthesis of adenine nucleosides by transglycosylation using two sequential nucleoside phosphorylase-based bioreactors with on-line reaction monitoring by using HPLC. *ChemCatChem*. 9(24):4614–4620.

Chae, W. G., Chan, T. C. K. and Chang, C. J. (1998). Facile synthesis of 5'-deoxy- and 2',5'-dideoxy-6-thiopurine nucleosides by nucleoside phosphorylases. *Tetrahedron*. 54(30):8661–8670.

Chaudhary, K., Ting, L. M., Kim, K. and Roos, D. S. (2006). *Toxoplasma gondii* purine nucleoside phosphorylase biochemical characterization, inhibitor profiles, and comparison with the *Plasmodium falciparum* ortholog. *Journal of Biological Chemistry*. 281(35):25652–25658.

Chellat, M. F. and Riedl, R. (2017). Pseudouridimycin : The first nucleoside analogue that selectively inhibits bacterial RNA polymerase. *Angewandte Chemie International Edition in English*. 16(56):13184–13186.

Chen, N., Xing, C. G., Xie, X. X. and Xu, Q. Y. (2009). Optimization of technical conditions of producing ribavirin by *Bacillus subtilis. Annals of Microbiology*. 59(3):525–530.

Choi, J. and Berdis, A. J. (2016). Visualizing nucleic acid metabolism using non-natural nucleosides and nucleotide analogs. *BBA - Proteins and Proteomics*. 1864(1):165–176.

Choi, J. H., Keum, K. C. and Lee, S. Y. (2006). Production of recombinant proteins by high cell density culture of *Escherichia coli. Chemical Engineering Science*. 61(3):876–885.

Christensen, D. G., Orr, J. S., Rao, C. V and Wolfe, J. (2017). Increasing growth yield and decreasing acetylation in *Escherichia coli* by optimizing the carbon-to-magnesium ratio in peptide-based media. *Applied and Environmental Microbiology*. 83(6):e03034-16.

Cimino, G., De Rosa, S. and De Stefano, S. (1984). Antiviral agents from a gorgonian, *Eunicella cavolini*. *Experientia*. 40(4):339–340.

Condezo, L. A., Fernández-Lucas, J., García-Burgos, C. A., Alcántara, A. R. and Sinisterra, J. V (2006). Enzymatic synthesis of modified nucleosides. in Patel, R. N. (ed.) *Biocatalysts in the Pharmaceutical and Biotechnology Industries*. CRC Press 403–426.

Cunningham, K. G., Manson, W., Spring, F. S. and Hutchinson, S. A. (1950). Cordycepin, a metabolic product isolated from cultures of *Cordyceps militaris* (Linn.) Link. *Nature*. 949.

Daves, G. D. and Cheng, C. C. (1976). The chemistry and biochemistry of C-nucleosides. in Ellis, G. P.

and West, G. B. B. T.-P. in M. C. (eds) *Progress in Medicinal Chemistry*. Elsevier 303–349.

Drenichev, M., Alexeev, C., Kurochkin, N. and Mikhailov, S. (2018). Use of nucleoside phosphorylases for the preparation of purine and pyrimidine 2′-deoxynucleosides. *Advanced Synthesis & Catalysis*. 360(2):305–312.

Eiteman, M. A. and Altman, E. (2006). Overcoming acetate in *Escherichia coli* recombinant protein fermentations. *Trends in Biotechnology*. 24(11):530–536.

Elion, G. B. (1989). The Purine Path to Chemotherapy. *Science*. 244:41–47.

Erion, M. D., Stoeckler, J. D., Guida, W. C., Walter, R. L. and Ealick, S. E. (1997). Purine nucleoside phosphorylase. 2. Catalytic mechanism. *Biochemistry*. 36(39):11735–11748.

Ewald, B., Sampath, D. and Plunkett, W. (2008). Nucleoside analogs: molecular mechanisms signaling cell death. *Oncogene*. 27(50):6522–6537.

Faderl, S., Gandhi, V., Keating, M. J., Jeha, S., Plunkett, W. and Kantarjian, H. M. (2005). The role of clofarabine in hematologic and solid malignancies—Development of a next-generation nucleoside analog. *Cancer*. 103(10):1985–1995.

Fateev, I., Antonov, K., Konstantinova, I., Muravyova, T., Seela, F., Esipov, R., Miroshnikov, A. and Mikhailopulo, I. (2014). The chemoenzymatic synthesis of clofarabine and related 2′-deoxyfluoroarabinosyl nucleosides: the electronic and stereochemical factors determining substrate recognition by *E. coli* nucleoside phosphorylases. *Beilstein journal of organic chemistry*. 10:1657–1669.

Fateev, I. V, Kharitonova, M. I., Antonov, K. V, Konstantinova, I. D., Stepanenko, V. N., Esipov, R. S., Seela, F., Temburnikar, K. W., Seley-Radtke, K. L., Stepchenko, V. A., Sokolov, Y. A., Miroshnikov, A. I. and Mikhailopulo, I. A. (2015). Recognition of artificial nucleobases by *E. coli* purine nucleoside phosphorylase versus its Ser90Ala mutant in the synthesis of base-modified nucleosides. *Chemistry*. 21(38):13401–13419.

Fernandez-Lucas, J., Acebal, C., Sinisterra, J. V, Arroyo, M. and de la Mata, I. (2010). *Lactobacillus reuteri* 2′-deoxyribosyltransferase, a novel biocatalyst for tailoring of nucleosides. *Applied and Environmental Microbiology*. 76(5):1462–1470.

Fernández-Lucas, J., Fresco-Taboada, A., Acebal, C., de la Mata, I. and Arroyo, M. (2011). Enzymatic synthesis of nucleoside analogues using immobilized 2′-deoxyribosyltransferase from *Lactobacillus reuteri*. *Applied Microbiology and Biotechnology*. 91(2):317–327.

Fernández-Lucas, J., Harris, R., Mata-Casar, I., Heras, A., Mata, I. and Arroyo, M. (2013). Magnetic chitosan beads for covalent immobilization of nucleoside 2′-deoxyribosyltransferase: application in nucleoside analogues synthesis. *Journal of Industrial Microbiology & Biotechnology*. 40(9):955–966.

Fong, B. A. and Wood, D. W. (2010). Expression and purification of ELP-intein-tagged target proteins in high cell density E . coli fermentation. *Microbial Cell Factories*. 9(77).

Fresco-Taboada, A., de la Mata, I., Arroyo, M. and Fernandez-Lucas, J. (2013). New insights on nucleoside 2′-deoxyribosyltransferases: a versatile biocatalyst for one-pot one-step synthesis of nucleoside analogs. *Applied Microbiology and Biotechnology*. 97(9):3773–3785.

Galmarini, C. M., Mackey, J. R. and Dumontet, C. (2002). Nucleoside analogues and nucleobases in cancer treatment. *The Lancet Oncology*. 3(7):415–424.

Gao, X. F., Huang, X. R. and Sun, C. C. (2006). Role of each residue in catalysis in the active site of pyrimidine nucleoside phosphorylase from *Bacillus subtilis*: A hybrid QM/MM study. *Journal of Structural Biology*. 154(1):20–26.

Garwal, K. A. N. A. (1996). Fidelity of RNA polymerase II transcription controlled by elongation factor TFIIS. *Proceedings of the National Academy of Sciences USA*. 93:13677–13682.

Gish, R. G. (2006). Treating HCV with ribavirin analogues and ribavirin-like molecules. *Journal of Antimicrobial Chemotherapy*. 57(1):8–13.

de Giuseppe, P. O., Martins, N. H., Meza, A. N., dos Santos, C. R., Pereira, H. D. M. and Murakami, M. T. (2012). Insights into phosphate cooperativity and influence of substrate modifications on binding and catalysis of hexameric purine nucleoside phosphorylases. *PLoS ONE*. 7(9):1–11.

Golshan, A., Hassanzadeh, S., Mojdekanloo, M. and Tayarani-Najaran, Z. (2016). Effects of *Biebersteinia multifida* hydro-ethanol extract on proliferation and apoptosis of human prostate cancer and human embryonic kidney cells. *Avicenna Journal of Phytomedicine*. 6(6):671–677.

Gordon, G. E. R., Bode, M. L., Visser, D. F., Lepuru, M. J., Zeevaart, J. G., Ragubeer, N., Ratsaka, M., Walwyn, D. R. and Brady, D. (2011). An integrated chemo-enzymatic route for preparation of β-thymidine, a key intermediate in the preparation of antiretrovirals. *Organic Process Research & Development*. American Chemical Society 15(1):258–265.

Goswami, A., Liu, X., Cai, W., Wyche, T. P., Bugni, T. S., Meurillon, M., Peyrottes, S., Perigaud, C., Nonaka, K., Rohr, J. and Van Lanen, S. G. (2017). Evidence that oxidative dephosphorylation by the nonheme Fe(II), α-ketoglutarate:UMP oxygenase occurs by stereospecific hydroxylation. *FEBS Letters*. 591(3):468–478.

Guo, J., Yu, L., Turro, N. J. and Ju, J. (2010). An integrated system for DNA sequencing by synthesis using novel nucleotide analogues. *Accounts of Chemical Research*. 43(4):551–563.

Haki, G. D. and Rakshit, S. K. (2003). Developments in industrially important thermostable enzymes: a review. *Bioresource Technology*. 89(1):17–34.

Harris, R. K., Becker, E. D., De Menezes, S. M. C., Goodfellow, R. and Granger, P. (2001). NMR nomenclature. Nuclear spin properties and conventions for chemical shifts (IUPAC Recommendations 2001). *Pure and Applied Chemistry*. 73(11):1795–1818.

Holme, T., Arvidson, S., Lindholm, B. and Pavlu, B. (1970). Enzymes: laboratory-scale production. *Process Biochemistry*. 5:62–66.

Hori, N., Watanabe, M., Sunagawa, K., Uehara, K. and Mikami, Y. (1991). Production of 5-methyluridine by immobilized thermostable purine nucleoside phosphorylase and pyrimidine nucleoside phosphorylase from *Bacillus stearothermophilus* JTS 859. *Journal of Biotechnology*. 17(2):121–131.

Hu, G. and Yang, F. (2014). Biological activities of nucleosides and their analogues in dietary foods. *Chemical Rapid Communications*. 2(2):22–28.

Huang, M.-C., Hatfield, K., Roetker, A. W., Montgomery, J. A. and Blakley, R. L. (1981). Analogs of 2'-deoxyadenosine: Facile enzymatic preparation and growth inhibitory effects on human cell lines. *Biochemical Pharmacology*. 30(19):2663–2671.

Huang, M. C., Montgomery, J. A., Thorpe, M. C., Stewart, E. L., Secrist, J. A. and Blakley, R. L. (1983). Formation of 3-(2'-deoxyribofuranosyl) and 9-(2'-deoxyribofuranosyl) nucleosides of 8-substituted purines by nucleoside deoxyribosyltransferase. *Archives of Biochemistry and Biophysics*. 222(1):133–144.

Huang, R., Chen, Y., Zeng, Z., Gao, C., Su, X. and Peng, Y. (2014). Marine Nucleosides: Structure, Bioactivity, Synthesis and Biosynthesis. *Marine Drugs*. 12(12):5817–5838.

Huang, Y., Liu, X., Cui, Z., Wiegmann, D., Niro, G., Ducho, C., Song, Y., Yang, Z. and Van Lanen, S. G. (2018). Pyridoxal-5'-phosphate as an oxygenase cofactor: Discovery of a carboxamide-forming, α-amino acid monooxygenase-decarboxylase. *Proceedings of the National Academy of Sciences*. 115(5):974–979.

Isono, K., Nagatsu, J., Kobinata, K., Sasaki, K. and Suzuki, S. (1965). Studies on polyoxins, antifungal antibiotics. Part I. Isolation and characterization of Polyoxins A and B. *Agricultural and Biological Chemistry*. 29(9):848–854.

Ito, T., Takatsuki, A., Kawamura, K., Sato, K. and Tamura, G. (1980). Isolation and structures of components of Tunicamycin. *Agricultural and Biological Chemistry*. 44(3):695–698.

Jeswani, G. and Paul, S. D. (2017). Recent advances in the delivery of chemotherapeutic agents. in Alexandru Mihai Grumezescu (ed.) *Nano- and Microscale Drug Delivery Systems*. Elsevier 281–298.

Jing, J., Chen, Y., Sheng, L. and Wu, M. (2018). Optimized production of insulin variant, a recombinant platelet aggregation inhibitor, by high cell-density fermentation of recombinant *Escherichia coli. Protein Expression and Purification*. 152:7–12.

Johnston, J. B. (2011). Mechanism of action of pentostatin and cladribine in hairy cell leukemia.

Leukemia & Lymphoma Lymphoma. 52(S2):43–45.

Jordheim, L. P., Durantel, D., Zoulim, F. and Dumontet, C. (2013). Advances in the development of nucleoside and nucleotide analogues for cancer and viral diseases. *Nature Reviews Drug Discovery*. 12(6):447–464.

Joseph, B., Pichaimuthu, S., Srimeenakshi, S., Murthy, M., Selvakumar, K. and Manjunath, S. (2015). An overview of the parameters for recombinant protein expression in *Escherichia coli*. *Journal of cell science and therapy*. 6(5):221.

Kalckar, H. M. (1945). Enzymatic synthesis of a nucleoside. *Journal of Biological Chemistry*. 158(3):723–724.

Kamel, S., Weiss, M., Klare, H. F. T., Mikhailopulo, I. A., Neubauer, P. and Wagner, A. (2018). Chemo-enzymatic production of α-D-pentofuranose-1-phosphates using thermostable pyrimidine nucleoside phosphorylases. *Molecular catalysis*. 458:52–59.

Kamel, S., Yehia, H., Neubauer, P. and Wagner, A. (2018). Enzymatic synthesis of nucleoside analogues by nucleoside phosphorylases. in Fernández-Lucas, J. and Camarasa-Rius, M. (eds) *Enzymatic and Chemical Synthesis of Nucleic Acid Derivatives*. John Wiley & Sons 1–28.

Kaminski, P. A., Dacher, P., Dugué, L. and Pochet, S. (2008). *In vivo* reshaping the catalytic site of nucleoside 2'-deoxyribosyltransferase for dideoxy- and didehydronucleosides via a single amino acid substitution. *Journal of Biological Chemistry*. 283(29):20053–20059.

Kirk, K. L. (2006). Fluorine in medicinal chemistry: Recent therapeutic applications of fluorinated small molecules. *Journal of Fluorine Chemistry*. 127(8):1013–1029.

Klöckner, W. and Büchs, J. (2012). Advances in shaking technologies. *Trends in Biotechnology*. 30(6):307–314.

Koczor, C. (2012). The role of transporters in the toxicity of nucleoside and nucleotide analogs. *Expert Opinion on Drug Metabolism & Toxicology*. 8(6):665–676.

Kolesar, J. M., Morris, A. K. and Kuhn, J. G. (1996). Review : Purine nucleoside analogues: Fludarabine, pentostatin, and cladribine: Part 1: Fludarabine. *Journal of Oncology Pharmacy Practice*. 2(3):160–181.

Kondo, K., Shigemori, H., Ishibashi, M. and Kobayashi, J. (1992). Aplysidine, a new nucleoside from the Okinawan marine sponge *Aplysina* Sp. *Tetrahedron*. 48(35):7145–7148.

Kong, Z., Jia, S., Chabes, A. L., Appelblad, P., Lundmark, R., Moritz, T. and Chabes, A. (2018). Simultaneous determination of ribonucleoside and deoxyribonucleoside triphosphates in biological samples by hydrophilic interaction liquid chromatography coupled with tandem mass spectrometry. *Nucleic Acids Research*. 46(11):e66.

Konstantinova, I. D., Leont'eva, N. A., Galegov, G. A., Ryzhova, O. I., Chuvikovskii, D. V, Antonov, K. V, Esipov, R. S., Taran, S. A., Verevkina, K. N., Feofanov, S. A. and Miroshnikov, A. I. (2004). Ribavirin: Biotechnological synthesis and effect on the reproduction of Vaccinia virus. *Russian Journal of Bioorganic Chemistry*. 30(6):553–560.

Konstantinova, I. D., Antonov, K. V, Fateev, I. V, Miroshnikov, A. I., Stepchenko, V. A., Baranovsky, A. V and Mikhailopulo, I. A. (2011). A chemo-enzymatic synthesis of β-D-arabinofuranosyl purine nucleosides. *Synthesis*. (10):1555–1560.

Laport, M. S., Santos, O. C. and Muricy, G. (2009). Marine sponges: potential sources of new antimicrobial drugs. *Current Pharmaceutical Biotechnology*. 10:86–105.

Lapponi, M. J., Rivero, C. W., Zinni, M. A., Britos, C. N. and Trelles, J. A. (2016). New developments in nucleoside analogues biosynthesis: A review. *Journal of Molecular Catalysis B: Enzymatic*. 218–233.

Lasa, I. and Berenguer, J. (1993). Thermophilic enzymes and their biotechnological potential. *Microbiologia*. 9:77–89.

Lattermann, C. and Büchs, J. (2016). Design and operation of microbioreactor systems for screening and process development. in Mandenius, C. (ed.) *Bioreactors: Design, Operation and Novel Applications*. Wiley-VCH (Wiley Online Books) 35–76.

Lawton, P. (2005). Purine analogues as antiparasitic agents. *Expert Opinion on Therapeutic Patents*.

15(8):987–994.

Leif, R. C., Stein, J. H. and Zucker, R. M. (2004). A short history of the initial application of anti-5-BrdU to the detection and measurement of S phase. *Cytometry*. 58A(1):45–52.

Leone, S., Sannino, F., Tutino, M. L., Parrilli, E. and Picone, D. (2015). Acetate: Friend or foe? Efficient production of a sweet protein in *Escherichia coli* BL21 using acetate as a carbon source. *Microbial Cell Factories*. 14:106.

Li, J., Jaitzig, J., Lu, P., Süssmuth, R. D. and Neubauer, P. (2015). Scale-up bioprocess development for production of the antibiotic valinomycin in *Escherichia coli* based on consistent fed-batch cultivations. *Microbial Cell Factories*. 14(83).

Li, N., Smith, T. J. and Zong, M.-H. (2010). Biocatalytic transformation of nucleoside derivatives. *Biotechnology Advances*. 28(3):348–366.

Ligasov, A. and Koberna, K. (2018). DNA replication : from radioisotopes to click chemistry. *Molecules*. 23(11):3007.

Liu, M.-C., Luo, M.-Z., Mozdziesz, D. E. and Sartorelli, A. C. (2005). Synthesis and biological evaluation of 2- and 7-substituted 9-deazaadenosine analogues. *Nucleosides, Nucleotides and Nucleic Acids*. 24(1):45–62.

Liu, P., Sharon, A. and Chu, C. K. (2008). Fluorinated nucleosides: Synthesis and biological implication. *Journal of Fluorine Chemistry*. 129(9):743–766.

Loakes, D. (2001). The applications of universal DNA base analogues. *Nucleic Acids Research*. 29(12):2437–2447.

Loomis, C. R. and Bell, R. M. (1988). Sangivamycin, a nucleoside analogue, is a potent inhibitor of protein kinase C. *The Journal of Biological Chemistry*. 263(4):1682–1692.

Lotfi, K., Månsson, E., Chandra, J., Wang, Y., Xu, D., Knaust, E., Spasokoukotskaja, T., Liliemark, E., Eriksson, S. and Albertioni, F. (2001). Pharmacological basis for cladribine resistance in a human acute T lymphoblastic leukaemia cell line selected for resistance to etoposide. *British Journal of Haematology*. 113:339–346.

Lu, J., Song, Q., Ji, Z., Liu, X., Wang, T. and Kang, Q. (2015). Fermentation optimization of maltose-binding protein fused to neutrophil-activating protein from *Escherichia coli* TB1. *Electronic Journal of Biotechnology*. 18(4):281–285.

Ma, J., Wang, S., Zhao, M., Deng, X.-S., Lee, C.-K., Yu, X.-D. and Liu, B. (2011). Therapeutic potential of cladribine in combination with STAT3 inhibitor against multiple myeloma. *BMC Cancer*. 11(1):1–11.

Ma, Y., Yang, S., Liu, L., Xu, Q., Zhang, C., Chen, N. and Xie, X. (2014). Fermentative production of ribavirin by overexpressing purine nucleoside phosphorylase in *Bacillus*. *Journal of Chemical and Pharmaceutical Research*. 6(5):1377–1384.

Madar, D., Dekel, E., Bren, A., Zimmer, A., Porat, Z. and Alon, U. (2013). Promoter activity dynamics in the lag phase of *Escherichia coli*. *BMC Systems Biology*. 7:136.

Maffioli, S. I. et al. (2017). Antibacterial nucleoside-analog Inhibitor of bacterial RNA polymerase. *Cell*. 169(7):1240–1248.

Majda, K., Lubecka, K., Kaufman-Szymczyk, A. and Fabianowska-Majewska, K. (2011). Clofarabine (2-chloro-2'-fluoro-2'-deoxyarabinosyladenine)-biochemical aspects of anticancer activity. *Acta Poloniae Pharmaceutica- Drug Research*. 68(4):459–466.

Månsson, E., Spasokoukotskaja, T., Sällström, J., Eriksson, S. and Albertioni, F. (1999). Molecular and biochemical mechanisms of fludarabine and cladribine resistance in a human promyelocytic cell line. *Cancer Research*. 59(23):5956–5963.

Mao, C., Cook, W. J., Zhou, M., Koszalka, G. W., Krenitsky, T. A. and Ealick, S. E. (1997). The crystal structure of *Escherichia coli* purine nucleoside phosphorylase: a comparison with the human enzyme reveals a conserved topology. *Structure*. 5(10):1373–1383.

Marisch, K., Bayer, K., Scharl, T., Mairhofer, J., Krempl, P. M., Hummel, K., Razzazi-Fazeli, E. and Striedner, G. (2013). A comparative analysis of industrial *Escherichia coli* K-12 and B Strains in high-glucose batch cultivations on process-, transcriptome- and proteome level. *PLoS ONE*. 8(8):e70516.

Mazumder, D., Kahn, K. and Bruice, T. C. (2002). Computer simulations of trypanosomal nucleoside hydrolase: Determination of the protonation state of the bound transition-state analogue. *Journal of the American Chemical Society*. 124(30):8825–8833.

Mikhailopulo, I. A., Zinchenko, A. I., Kazimierczuk, Z., Barai, V. N., Bokut, S. B. and Kalinichenko, E. N. (1993). Synthesis of 2-chloro-2'-deoxyadenosine by microbiological transglycosylation. *Nucleosides and Nucleotides*. 12(3–4):417–422.

Mikhailopulo, I. A. and Miroshnikov, A. I. (2011). Biologically important nucloesides: modern trends in biotechnology and application. *Mendeleev Communications*. 21(2):57–68.

Mikhailopulo, I. A. and Miroshnikov, A. I. (2013). Some recent findings in the biotechnology of biologically important nucleosides. *Biotechnologia Acta*. 6(4):63–77.

Montgomery, J. A. (1987). The Biological Bases for the Design of Anticancer Agents. in Cory, J. G. and Szentivanyi, A. (eds) *Cancer Biology and Therapeutics*. Springer, Boston, MA 23–42.

Montgomery, J. A., Shortnacy-Fowler, A. T., Clayton, S. D., Riordan, J. M. and Secrist 3rd, J. A. (1992). Synthesis and biologic activity of 2'-fluoro-2-halo derivatives of 9-beta-D-arabinofuranosyladenine. *Journal of Medicinal Chemistry*. 35(2):397–401.

Morton, G. O., Lancaster, J. E., Van Lear, G. E., Fulmor, W. and Meyer, W. E. (1969). The structure of nucleocidin. 3. (A new structure). *Journal of the American Chemical Society*. 91(6):1535–1537.

Neef, A. B. and Luedtke, N. W. (2011). Dynamic metabolic labeling of DNA in vivo with arabinosyl nucleosides. *Proceedings of the National Academy of Sciences*. 108(51):20404–20409.

Nierhaus, K. (2014). Mg2+,K+, and the Ribosome. *Journal of Bacteriology*. 196(22):3817–3819.

Nishi, R., Yamauchi, T., Negoro, E., Takemura, H. and Ueda, T. (2013). Combination of guanine arabinoside and Bcl-2 inhibitor YC137 overcomes the cytarabine resistance in HL-60 leukemia cell line. *Cancer Science*. 104(4):502–507.

Nóbile, M., Terreni, M., Lewkowicz, E. and Iribarren, A. M. (2010). *Aeromonas hydrophila* strains as biocatalysts for transglycosylation. *Biocatalysis and Biotransformation*. 28(5–6):395–402.

Norsyahida, A., Rahmah, N. and Ahmad, R. M. (2009). Effects of feeding and induction strategy on the production of BmR1 antigen in recombinant *E. coli. Letters in Applied Microbiology*. 49(5):544–550.

Okuma, H. and Watanabe, E. (2002). Flow system for fish freshness determination based on double multi-enzyme reactor electrodes. *Biosensors & bioelectronics*. 17(5):367–372.

Pankiewicz, K. W. (2000). Fluorinated nucleosides. *Carbohydrate Research*. 327(1–2):87–105.

Panova, N. G., Alexeev, C. S., Kuzmichov, A. S., Shcheveleva, E. V, Gavryushov, S. A., Polyakov, K. M., Kritzyn, A. M., Mikhailov, S. N., Esipov, R. S. and Miroshnikov, A. I. (2007). Substrate specificity of *Escherichia coli* thymidine phosphorylase. *Biochemistry (Mosc)*. 72(1):21–28.

Pastor-Anglada, M., Cano-Soldado, P., Molina-Arcas, M., Lostao, M. P., Larráyoz, I., Martínez-Picado, J. and Casado, F. J. (2005). Cell entry and export of nucleoside analogues. *Virus Research*. 107(2):151–164.

Pereira, P. D. et al. (2017). Quantification of cell cycle kinetics by EdU (5-ethynyl-2'-deoxyuridine)-coupled-fluorescence-intensity analysis. *Oncotarget*. 8(25):40514–40532.

Peters, G. J. (2014). Novel developments in the use of antimetabolites. *Nucleosides, Nucleotides and Nucleic Acids*. 33(4–6).

Pizzorno, G., Diasio, R. and Cheng, Y. (2003). Purine analogs. in Kufe, D. W. et al. (eds) *Holland-Frei Cancer Medicine*. 6th edn. Hamilton (ON): BC Decker.

Potenski, C. J. and Klein, H. L. (2014). How the misincorporation of ribonucleotides into genomic DNA can be both harmful and helpful to cells. *Nucleic Acids Research*. 42(16):10226–10234.

Pugmire, M. J. and Ealick, S. E. (2002). Structural analyses reveal two distinct families of nucleoside phosphorylases. *Biochemical Journal*. 361:1–25.

Pui, C., Jeha, S. and Kirkpatrick, P. (2005). Clofarabine. *Nature Reviews Drug Discovery*. 4:369–370.

Rabuffetti, M. (2017). *Purine nucleoside phosphorylases as biocatalysts and pharmacological targets*. Università degli Studi di Milano. Doctoral thesis. doi: 10.13130/rabuffetti-marco_phd2017-03-28

Ren, Q., Henes, B., Fairhead, M. and Thöny-Meyer, L. (2013). High level production of tyrosinase in recombinant Escherichia coli. *BMC Biotechnology*. 13:18.

Restaino, O. F., Bhaskar, U., Paul, P., Lin, L., De Rosa, M., Dordick, J. S. and Linhardt, R. J. (2013). High cell density cultivation of a recombinant *E. coli* strain expressing a key enzyme in bioengineered heparin production. *Applied Microbiology and Biotechnology*. 97(9):3893–3900.

Rios, G. M., Belleville, M. P., Paolucci, D. and Sanchez, J. (2004). Progress in enzymatic membrane reactors – a review. *Journal of Membrane Scince*. 242:189–196.

Rivero, C. W., Britos, C. N., Lozano, M. E., Sinisterra, J. V and Trelles, J. A. (2012). Green biosynthesis of floxuridine by immobilized microorganisms. *FEMS Microbiology Letters*. 331(1):31–36.

Robak, P. and Robak, T. (2013). Older and new purine nucleoside analogs for patients with acute leukemias. *Cancer Treatment Reviews*. 39(8):851–861.

Robak, T., Korycka, A., Kasznicki, M., Wrzesien-Kus, A. and Smolewski, P. (2005). Purine nucleoside analogues for the treatment of hematological malignancies: pharmacology and clinical applications. *Current Cancer Drug Targets*. 5(6):421–444.

Robak, T., Lech-Maranda, E., Korycka, A. and Robak, E. (2006). Purine nucleoside analogs as immunosuppressive and antineoplastic agents: mechanism of action and clinical activity. *Current Medicinal Chemistry*. 13(26):3165–3189.

Rosano, G. L. and Ceccarelli, E. A. (2014). Recombinant protein expression in *Escherichia coli*: Advances and challenges. *Frontiers in Microbiology*. 5:Article 172.

Sakharov, V., Baykov, S., Konstantinova, I., Esipov, R. and Dorogov, M. (2015). An efficient chemoenzymatic process for preparation of ribavirin. *International Journal of Chemical Engineering*. 2015:Article ID 734851.

Saleh, A. M., Aljada, A., Rizvi, S. A. A., Nasr, A., Alaskar, A. S. and Williams, J. D. (2014). *In vitro* cytotoxicity of *Artemisia vulgaris* L. essential oil is mediated by a mitochondria-dependent apoptosis in HL-60 leukemic cell line. *BMC Complementary and Alternative Medicine*. 14:226.

Salic, A. and Mitchison, T. J. (2008). A chemical method for fast and sensitive detection of DNA synthesis *in vivo*. *Proceedings of the National Academy of Sciences of the United States of America*. 105(7):2415–2420.

Sambrook, J. and Russell, D. W. (2001). *Molecular cloning : a laboratory manual*. 3rd edn. New York: Cold Spring Harbor Laboratory Press.

Saneyoshi, M., Tokuzen, R. and Fukuoka, F. (1965). Antitumor activities and structural relationship of tubercidine toyocamycin and their derivatives. *Gann Japanese Journal of Cancer Research*. 56:219–222.

Savić, D., Stanković, T., Lavrnja, I., Podolski-Renić, A., Banković, J., Peković, S., Stojiljković, M., Rakić, L., Ruždijić, S. and Pešić, M. (2015). Purine nucleoside analogs in the therapy of cancer and neuroinflammation. *Molecular inhibitors in targeted therapy*. 1:3–14.

Schultz, D. and Kishony, R. (2013). Optimization and control in bacterial lag phase. *BMC Biology*. 11:120.

Seley-Radtke, K. L. and Yates, M. K. (2018). The evolution of nucleoside analogue antivirals: A review for chemists and non-chemists. Part 1: Early structural modifications to the nucleoside scaffold. *Antiviral Research*. 154:66–86.

Serpi, M., Ferrari, V. and Pertusati, F. (2016). Nucleoside derived antibiotics to fight microbial drug resistance: New utilities for an established class of drugs? *Journal of Medicinal Chemistry*. 59(23):10343−10382.

Serra, I., Bavaro, T., Cecchini, D. A., Daly, S., Albertini, A. M., Terreni, M. and Ubiali, D. (2013). A comparison between immobilized pyrimidine nucleoside phosphorylase from *Bacillus subtilis* and thymidine phosphorylase from *Escherichia coli* in the synthesis of 5-substituted pyrimidine 2′-deoxyribonucleosides. *Journal of Molecular Catalysis B: Enzymatic*. 95:16–22.

Shelton, J., Lu, X., Hollenbaugh, J. A., Cho, J. H., Amblard, F. and Schinazi, R. F. (2016). Metabolism, biochemical actions, and chemical synthesis of anticancer nucleosides, nucleotides, and base analogs.

Chemical Reviews. 116(23):14379–14455.

Shi, W. X., Ting, L. M., Kicska, G. A., Lewandowicz, A., Tyler, P. C., Evans, G. B., Furneaux, R. H., Kim, K., Almo, S. C. and Schramm, V. L. (2004). *Plasmodium falciparum* purine nucleoside phosphorylase - Crystal structures, immucillin inhibitors, and dual catalytic function. *Journal of Biological Chemistry*. 279(18):18103–18106.

Shigemi, H., Yamauchi, T., Tanaka, Y. and Ueda, T. (2013). Novel leukemic cell lines resistant to clofarabine by mechanisms of decreased active metabolite and increased antiapoptosis. *Cancer Science*. 104(6):732–739.

Shimada, N., Hasegawa, S., Harada, T., Tomisawa, T., Fujii, A. and Takita, T. (1986). Oxetanocin, a novel nucleoside from bacteria. *Journal of Antibiotics*. 39(11):1623–1625.

Sigal, D. S., Miller, H. J., Schram, E. D. and Saven, A. (2010). Beyond hairy cell: the activity of cladribine in other hematologic malignancies. *Blood*. 116(16):2884–2896.

Sinisterra, J., Alcántara, A., Almendros, M., Hernáiz, M. J., Sánchez-Montero, J. and Trelles, J. (2010). Enzyme-catalyzed synthesis of nonnatural or modified nucleosides. in Flickinger, M. C. (ed.) *Encyclopedia of Industrial Biotechnology: Bioprocess, Bioseparation, and Cell Technology*. New York: John Wiley & Sons.

Sitanggang, A. B., Drews, A. and Kraume, M. (2014). Continuous synthesis of lactulose in an enzymatic membrane reactor reduces lactulose secondary hydrolysis. *Bioresource Technology*. 167:108–115.

Skipper, H. E., Montgomery, J. A., Thomson, J. R. and Schabel, F. M. (1959). Structure-activity relationships and cross-resistance observed on evaluation of a series of purine analogs against experimental neoplasms. *Cancer Research*. 19(4):425–437.

Sofia, M. J. (2016). Sofosbuvir: The Discovery of a curative therapy for the treatment of hepatitis C virus. in Fischer, J. and Childers, W. E. (eds) *Successful Drug Discovery*. 163–188.

Sorensen, H. P. and Mortensen, K. K. (2005). Advanced genetic strategies for recombinant protein expression in *Escherichia coli*. *Journal of Biotechnology*. 115(2):113–128.

Stachnik, K., Grieb, P. and Skierski, J. S. (2005). Cytotoxic effects of cladribine and tezacitabine toward HL-60. *Acta Biochimica Polonica*. 52(2):561–565.

Stoeckler, J. D., Cambor, C. and Parks, R. E. (1980). Human erythrocytic purine nucleoside phosphorylase: reaction with sugar-modified nucleoside substrates. *Biochemistry*. 19(1):102–107.

Sydow, J. F. and Cramer, P. (2009). RNA polymerase fidelity and transcriptional proofreading. *Current Opinion in Structural Biology*. 19:732–739.

Szeker, K., Zhou, X., Schwab, T., Casanueva, A., Cowan, D., Mikhailopulo, I. A. and Neubauer, P. (2012). Comparative investigations on thermostable pyrimidine nucleoside phosphorylases from *Geobacillus thermoglucosidasius* and *Thermus thermophilus*. *Journal of Molecular Catalysis B: Enzymatic*. 84:27–34.

Takatsuki, A., Arima, K. and Tamura, G. (1971). Tunicamycin, a new antibiotic. I. Isolation and characterization of tunicamycin. *Journal of Antibiotics*. 24(4):215–223.

Taverna-Porro, M., Bouvier, L. A., Pereira, C. A., Montserrat, J. M. and Iribarren, A. M. (2008). Chemoenzymatic preparation of nucleosides from furanoses. *Tetrahedron Letters*. 49(16):2642–2645.

Thomas, S., Singleton, V. L., Lowery, J. A., Sharpe, R. W., Pruess, L. M., Porter, J., Mowat, J. and Bohonos, N. (1956). Nucleocidin, a new antibiotic with activity against trypanosomes. *Antibiotics Annual*. 716–721.

Tono, H. and Cohen, S. S. (1962). The activity of nucleoside phosphorylase on 1-β-D-arabinosyluracil within *Escherichia coli*. *Journal of Biological Chemistry*. 237(4):1271–1282.

Trelles, J. A., Fernández-Lucas, J., Condezo, L. A. and Sinisterra, J. V (2004). Nucleoside synthesis by immobilised bacterial whole cells. *Journal of Molecular Catalysis B: Enzymatic*. 30(5):219–227.

Tsesmetzis, N., Paulin, C. B. J., Rudd, S. G. and Herold, N. (2018). Nucleobase and nucleoside analogues: Resistance and re-sensitisation at the level of pharmacokinetics, pharmacodynamics and metabolism. *Cancers*. 10(7):240.

Tuttle, J. V, Tisdale, M. and Krenitsky, T. A. (1993). Purine 2'-deoxy-2'-fluororibosides as antiinfluenza virus agents. *Journal of Medicinal Chemistry*. 36(1):119–125.

Uematsu, T. and Suhadolnik, R. J. (1970). Nucleoside antibiotics. VI. Biosynthesis of the pyrrolopyrimidine nucleoside antibiotic toyocamycin by *Streptomyces rimosus*. *Biochemistry*. 9(5):1260–1266.

Uematsu, T. and Suhadolnik, R. J. (1974). *In vivo* and enzymatic conversion of toyocamycin to sangivamycin by *Streptomyces rimosus*. *Archives of Biochemistry and Biophysics*. 162(2):614–619.

Utagawa, T., Morisawa, H., Yamanaka, S., Yamazaki, A., Yoshinaga, F. and Hirose, Y. (1985). Mechanism of purine arabinoside synthesis by bacterial transarabinosylation reaction. *Agricultural and Biological Chemistry*. 49(8):2425–2430.

Utagawa, T. (1999). Enzymatic preparation of nucleoside antibiotics. *Journal of Molecular Catalysis B: Enzymatic*. 6(3):215–222.

Vieille, C. and Zeikus, G. J. (2001). Hyperthermophilic enzymes: sources, uses, and molecular mechanisms for thermostability. *Microbiology and molecular biology reviews*. 65(1):1–43.

Vodnala, M., Ranjbarian, F., Pavlova, A., de Koning, H. P. and Hofer, A. (2016). *Trypanosoma brucei* methylthioadenosine phosphorylase protects the parasite from the antitrypanosomal effect of deoxyadenosine: implications for the pharmacology of adenosine antimetabolites. *Journal of Biological Chemistry*. 291(22):11717–11726.

Wang, C., Zhang, Z., Wang, Y. and He, X. (2015). Cytotoxic indole alkaloids against human leukemia cell lines from the toxic plant *Peganum harmala*. *Toxins*. 7(11):4507–4518.

Wei, X., Ding, Q., Ou, L., Zhang, L. and Wang, C. (2008). Two-step enzymatic synthesis of guanine arabinoside. *Chinese Journal of Chemical Engineering*. 16(6):934–937.

Wiebe, L. I. (2007). Applications of nucleoside-based molecular probes for the in vivo assessment of tumour biochemistry using positron emission tomography (PET). *Brazilian Archives of Biology ans Technology*. 50(3):445–459.

Yang, Z., Kim, H., Le, J. T., Mclendon, C., Bradley, K. M., Kim, M., Hutter, D., Hoshika, S., Yaren, O. and Benner, A. (2018). Nucleoside analogs to manage sequence divergence in nucleic acid amplification and SNP detection. *Nucleic Acids Research*. 46(12):5902–5910.

Yazdani, S. S., Shakri, A. R. and Chitnis, C. E. (2004). A high cell density fermentation strategy to produce recombinant malarial antigen in *E. coli*. *Biotechnology Letters*. 26(24):1891–1895.

Yehia, H., Kamel, S., Paulick, K., Wagner, A. and Neubauer, P. (2017). Substrate spectra of nucleoside phosphorylases and their potential in the production of pharmaceutically active compounds. *Current pharmaceutical design*. 23(45):6913–6935.

Yu, V. (2002). Scientific rationale and benefits of nucleotide supplementation of infant formula. *Journal of Paediatrics and Child Health*. 38(6):543–549.

Zabriskie, T. M. and Ireland, C. M. (1989). The isolation and structure of modified bioactive nucleosides from *Jaspis johnstoni*. *Journal of Natural Products*. 52(6):1353–1356.

Zhou, X., Szeker, K., Janocha, B., Böhme, T., Albrecht, D., Mikhailopulo, I. A. and Neubauer, P. (2013). Recombinant purine nucleoside phosphorylases from thermophiles: preparation, properties and activity towards purine and pyrimidine nucleosides. *FEBS Journal*. 280(6):1475–1490.

Zhou, X., Mikhailopulo, I., Cruz Bournazou, M. N. and Neubauer, P. (2015). Immobilization of thermostable nucleoside phosphorylases on MagReSyn® epoxide microspheres and their application for the synthesis of 2,6-dihalogenated purine nucleosides. *Journal of Molecular Catalysis B: Enzymatic*. 115:119–127.

Zhou, X., Szeker, K., Jiao, L.-Y., Oestreich, M., Mikhailopulo, I. A. and Neubauer, P. (2015). Synthesis of 2,6-dihalogenated purine nucleosides by thermostable nucleoside phosphorylases. *Advanced Synthesis & Catalysis*. 357(6):1237–1244.

Zuffi, G., Ghisotti, D., Oliva, I., Capra, E., Frascotti, G., Tonon, G. and Orsini, G. (2004). Immobilized biocatalysts for the production of nucleosides and nucleoside analogues by enzymatic transglycosylation reactions. *Biocatalysis and Biotransformation*. 22(1):25–33.

9. Theses

1. It is possible to up-scale the expression of recombinant nucleoside phosphorylases from shake flask scale to benchtop bioreactor scale.
2. Three times the protein volumetric yield of the PNP N03 was obtained in the bioreactor in comparison to the shake flask over approximately the same duration.
3. Two different approaches for nucleoside analogs synthesis can be employed. The one pot-two enzyme transglycosylation is suitable for the synthesis of riboside and deoxyriboside nucleosides while the direct glycosylation is better used for sugar modified nucleosides (arabinosides and fluoroarabinosides).
4. Different 5-ethynyluracil containing nucleosides that are used to detect cell proliferation and nucleic acids synthesis were successfully produced enzymatically with nucleoside phosphorylases. Substrate conversion was ca. 50 % on producing the fluoroarabinoside counterpart by direct glycosylation with 2-deoxy-2-fluoro-α-D-arabinofuranose-1-phosphate that was otherwise, not formed by transglycosylation.
5. Using thermostable nucleoside phosphorylases in continuous reactors offers many advantages as the amount of needed enzyme is drastically reduced. nucleoside phosphorylases can be used for more than 2 months with natural substrates and up to 7 days with modified ones.
6. Cell lysates of the recombinant *E. coli* can be used instead of purified enzymes with the same efficiency. Hence, thermostable nucleoside phosphorylases are more cost-effective biocatalysts for potential industrial applications.
7. Reactions performed in µl scale can be up-scaled to 50 mL without a decrease in product formation. The organic solvent dimethyl sulfoxide DMSO in a concentration up to 20 % v/v has no inhibitory effect on the enzyme's activity.
8. A purification protocol applying semi-preparative HPLC was successfully developed during this work. Nucleoside analogs with purity >95 % (reagent grade) were obtained.
9. The enzymatically produced NAs give similar results to chemically synthesized ones in biological tests against leukemia cell lines.
10. Nucleoside phosphorylases from thermophilic organisms are promising candidates for large scale, economical use. Producing and utilizing them in larger scale is possible with the advantage of a lower biocapacity and ecosystem burden.

10. Publications

Paper I

Yehia, H., Kamel, S., Paulick, K., Wagner, A. and Neubauer, P. (2017). Substrate spectra of nucleoside phosphorylases and their potential in the production of pharmaceutically active compounds. *Current pharmaceutical design*. 23(45):6913–6935.

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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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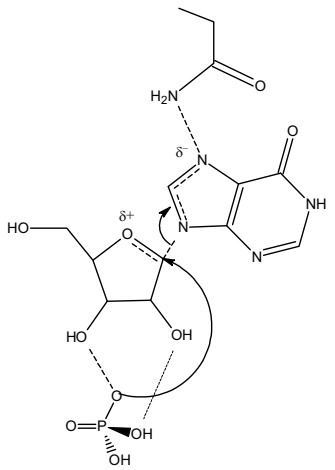


Scheme 1. Proposed catalytic mechanism of human PNP. Figure adapted from Pugmire and Ealick [1].

(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.

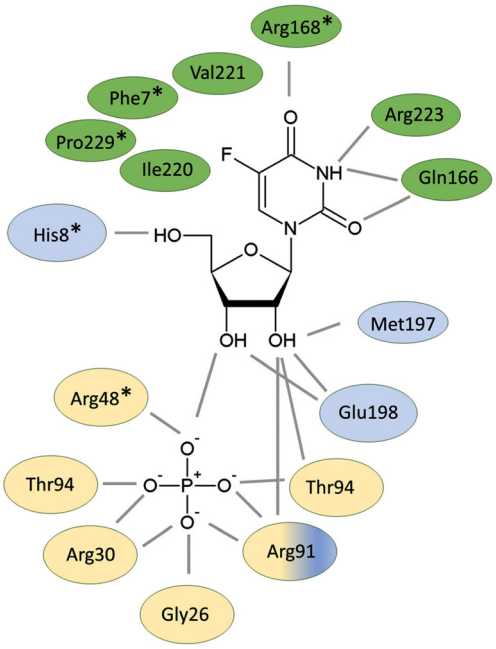


Fig. (1). Schematic drawing of the active site of *E. coli* uridine phosphorylase. Residues binding the base, the sugar-moiety and phosphate are shown in dark grey, light grey and white, respectively. Residues from the adjacent subunit are marked with *.

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 inter-monomeric 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].

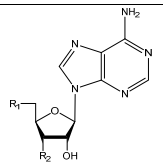
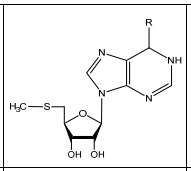
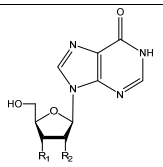
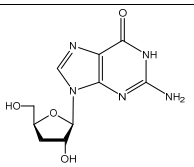
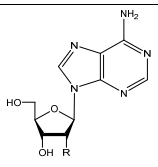
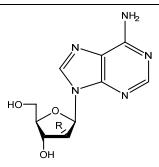
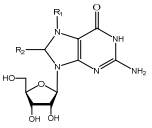
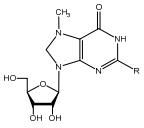
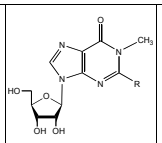
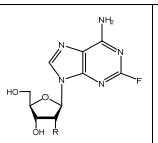
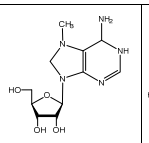
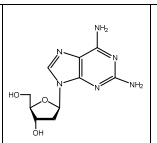
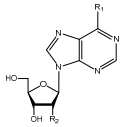
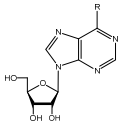
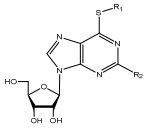
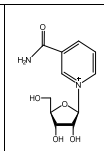
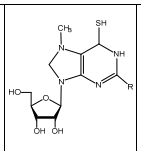
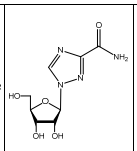
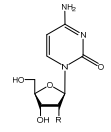
MTAPs from different sources exhibit a range of substrate tolerance that does not always comply with the empirical strict specificity. For example, 6-oxopurine guanosine and inosine are accepted by some organisms. *Pyrococcus furiosus* can use both nucleosides. A slightly lower substrate affinity was 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 4: R_1= CH_3, R_2= CH_3 2: R_1= $CH_3(CH_2)_2$, R_2= H 5: R_1= $CH(CH_3)_2$, R_2= H 3: R_1= $CH_3(CH_2)_3$, 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....

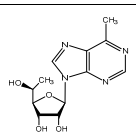
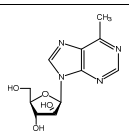
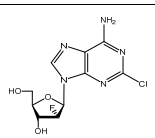
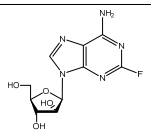
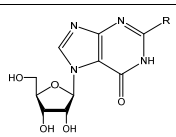
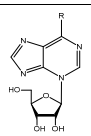
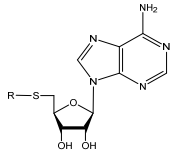
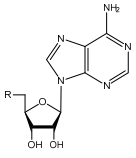
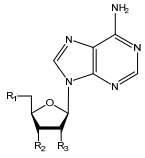
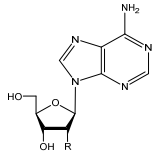
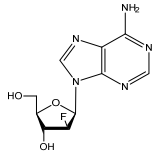
Base-modified nucleosides							
							B. acetylicum
					[52]		*B. subtilis*
			[3]	15 [53]			*B. taurus*
						16,17 [37]	*D. geothermalis**
1, 2 [54,55] 3-6 [55]	7-10 [55]	13,14 [31]	[3,56]	15 [3]			*E. coli*
1,2 [54]							*F. tularensis*
						16,17 [37]	*G. thermoglucosidasius**
		11[57], 12 [58]		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....

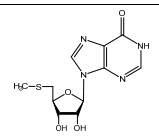
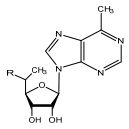
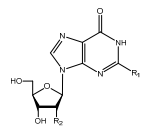
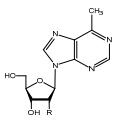
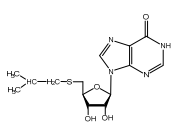
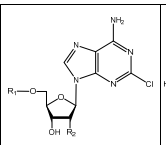
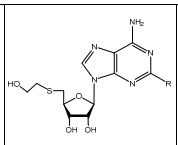
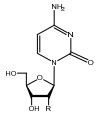
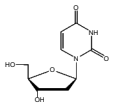
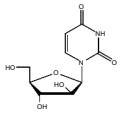
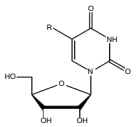
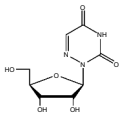
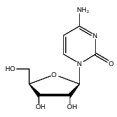
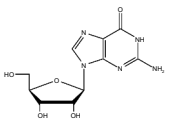
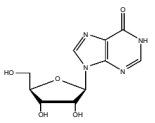
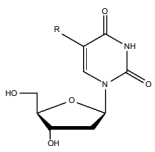
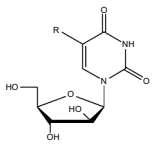
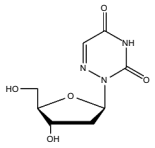
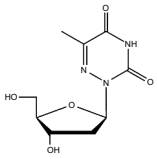
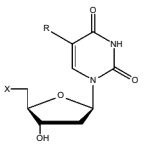
	7 [54,77]					*S. solfataricus**
5 [78]	7[79]	13 [79,80] 14, 15, 16 [80]	17,18 [79]			*T. brucei*
Base-modified nucleosides						
						
[68]						*C. acidophila**
[69]						*H. sapiens*
Sugar- and base-modified nucleosides						
							
1: R= OH (allo) 2: R= OH (tallo)	3: R_1= H, R_2= OH 4: R_1= NH_2, 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].

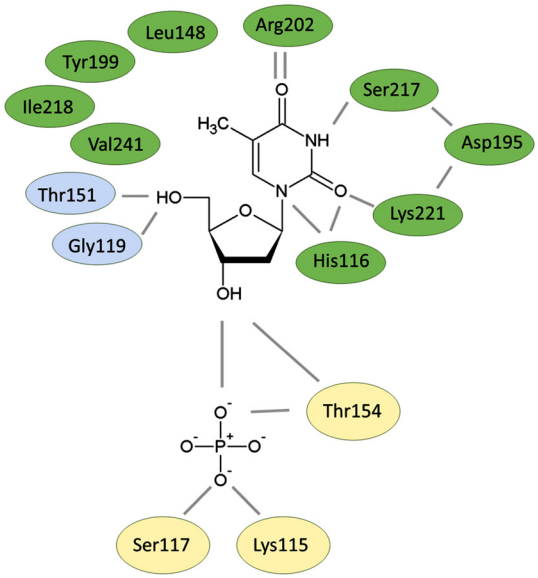


Fig. (2). Schematic drawing of the active site of human thymidine phosphorylase. Residues binding the base, the sugar-moiety and phosphate are shown in dark grey, light grey and white, respectively.

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*.

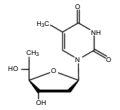
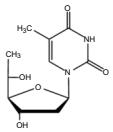
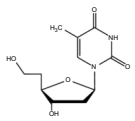
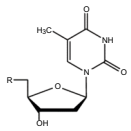
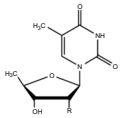
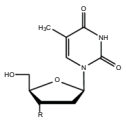
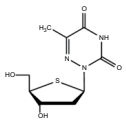
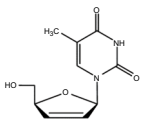
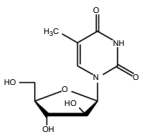
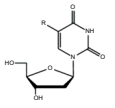
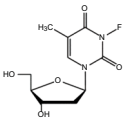
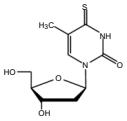
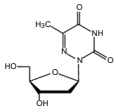
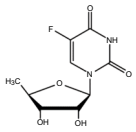
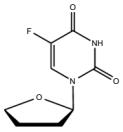
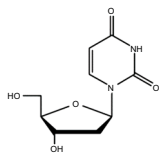
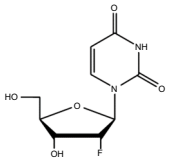
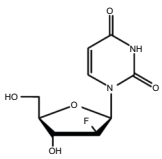
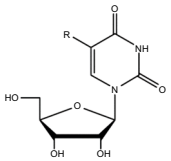
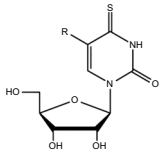
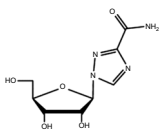
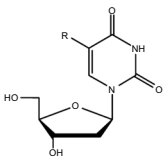
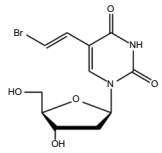
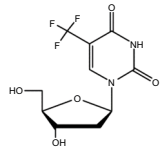
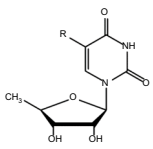
sugar-modified nucleosides: 5′-moiety					
					
			1: R = NH2 2: R = SH 3: R = Cl 4: R = F	5: R = H 6: R = OH	
			4 [139,140]		*H. sapiens*
[130]	[130]	[130]	1-3 [130,131]	5, 6 [130,131,138]	*E. coli*
Sugar-modified nucleosides: 3′-moiety, ring-modifications					
					
7: R = NH_2 8: R= SH					
7, 8 [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.

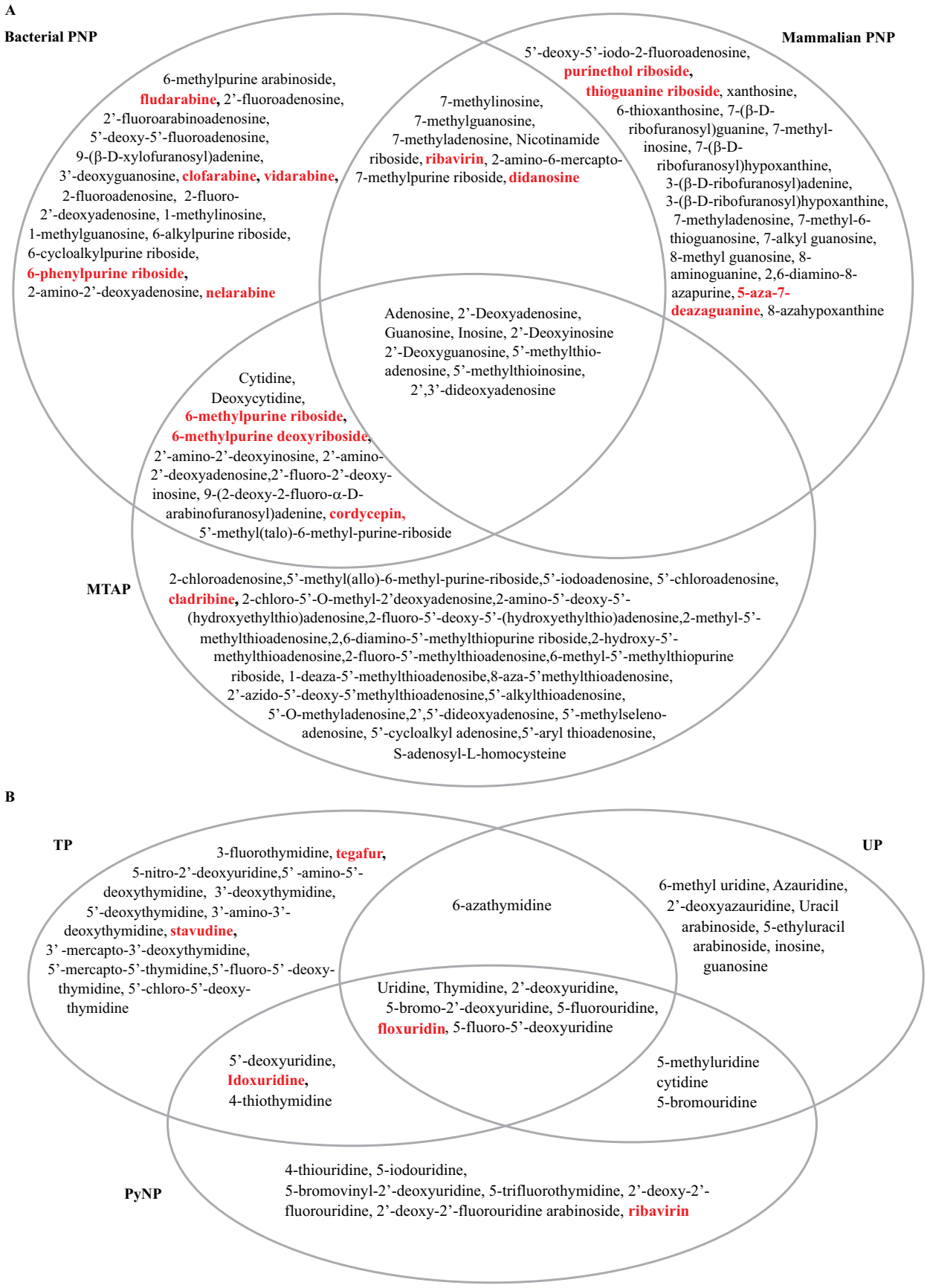


Fig. (3). Comparison of substrates phosphorolyzed by members of NP subfamilies. A) hexameric PNP, trimeric PNP and MTAP, B) UP, TP and PyNP. Drugs or compounds with therapeutic activity are written in bold and are underlined.

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.

REFERENCES

[1] Pugmire MJ, Ealick SE. Structural analyses reveal two distinct families of nucleoside phosphorylases. Biochem J 2002; 25: 1-25.

[2] Kalckar HM. The enzymatic synthesis of purine ribosides. J Biol Chem 1947; 167: 477-86.

[3] Bzowska A, Kulikowska E, Shugar D. Purine nucleoside phosphorylases: Properties, functions, and clinical aspects. Pharmacol Ther 2000; 88: 349-425.

[4] Vita A, Huang CY, Magni G. Uridine phosphorylase from *Escherichia coli* B: Kinetic studies on the mechanism of catalysis. Arch Biochem Biophys 1933; 226: 687-92.

[5] Jensen KF, Nygaard P. Purine nucleoside phosphorylase from *Escherichia coli* and *Salmonella typhimurium*. Purification and some properties. Eur J Biochem 1975; 51: 253-65.

[6] Tozzi MG, Camici M, Mascia L, Sgarrella F, Ipata PL. Pentose phosphates in nucleoside interconversion and catabolism. FEBS J 2006; 273: 1089-1101.

[7] Sgarrella F, Poddie FP, Meloni MA, Sciola L, Pippia P, Tozzi MG. Channelling of deoxyribose moiety of exogenous DNA into carbohydrate metabolism: role of deoxyriboaldolase. Comp Biochem Physiol B Biochem Mol Biol 1997; 117: 253-57.

[8] Hammer-Jespersen K, Munch-Petersen A. Multiple regulation of nucleoside catabolizing enzymes: Regulation of the *deo* operon by the *cytR* and *deoR* gene products. MGG Mol Gen Genet 1975; 137: 327-35.

[9] Tozzi MG, Sgarrella F, Ipata PL. Induction and repression of enzymes involved in exogenous purine compound utilization in *Bacillus cereus*. Biochim Biophys Acta - Gen Subj 1981; 678: 460-66.

[10] Hammond DJ, Gutteridge WE. Purine and pyrimidine metabolism in the trypanosomatidae. Mol Biochem Parasitol 1984; 13: 243-61.

[11] Silva RG, Nunes JES, Canduri F, *et al*. Purine nucleoside phosphorylase: a potential target for the development of drugs to treat T-cell-and apicomplexan parasite-mediated diseases. Curr Drug Targets 2007; 8: 413-22.

[12] Furukawa T, Yoshimura A, Sumizawa T, *et al*. Angiogenic factor. Nature 1992; 356: 668-668.

[13] Akiyama S, Furukawa T, Sumizawa T, *et al*. The role of thymidine phosphorylase, an angiogenic enzyme, in tumor progression. Cancer Sci 2004; 95: 851-57.

[14] Dandanell G, Szczepanowski RH, Kierdaszuk B, Shugar D, Bochtler M. *Escherichia coli* Purine Nucleoside Phosphorylase II, the Product of the *xapA* Gene. J Mol Biol 2005; 348: 113-25.

[15] Roosild TP, Castronovo S, Fabbiani M, Pizzorno G. Implications of the structure of human uridine phosphorylase 1 on the development of novel inhibitors for improving the therapeutic window of fluoropyrimidine chemotherapy. BMC Struct Biol 2009; 9: 14.

[16] Larson ET, Mudeppa DG, Gillespie JR, *et al*. The crystal structure and activity of a putative trypanosomal nucleoside phosphorylase reveal it to be a homodimeric uridine phosphorylase. J Mol Biol 2010; 396: 1244-59.

[17] Ling F, Inoue Y, Kimura A. Purification and characterization of a novel nucleoside phosphorylase from a *Klebsiella sp.* and its use in the enzymatic production of adenine arabinoside. Appl Environ Microbiol 1990; 56: 3830-34.

[18] Mikhailopulo IA, Miroshnikov AI. Biologically important nucleosides : modern trends in biotechnology and application. Mendeleev Commun 2011; 21: 57-68.

[19] Guan R, Ho M-C, Almo SC, Schramm VL. Methylthioinosine phosphorylase from *Pseudomonas aeruginosa*. Structure and annotation of a novel enzyme in quorum sensing. Biochemistry 2011; 50: 1247-54.

[20] Buckoreelall K, Wilson L, Parker WB. Identification and characterization of two adenosine phosphorylase activities in *Mycobacterium smegmatis*. J Bacteriol 2011; 193: 5668-74.

[21] Hatanaka M, Del Giudice R, Cedric L. Adenine formation from adenosine by mycoplasmas: adenosine phosphorylase activity. Proc Natl Acad Sci 1975; 72: 1401-5.

[22] Miech RP, Senft AW, Senft DG. Pathways of nucleotide metabolism in *Schistosoma mansoni* - VI adenosine phosphorylase. Biochem Pharmacol 1975; 24: 407-11.

[23] Savarese TM, el Kouni MH. Isolation and substrate specificity of an adenine nucleoside phosphorylase from adult *Schistosoma mansoni*. Mol Biochem Parasitol 2014; 194: 44-47.

[24] Heyworth PG, Gutteridge WE, Ginger CD. Purine metabolism in *Trichomonas vaginalis*. FEBS Lett 1982; 141: 106-10.

[25] Yamada EW. The phosphorolysis of nucleosides by rabbit bone marrow. J Biol Chem 1961; 236: 3043-46.

[26] Caradoc-Davies TT, Cutfield SM, Lamont IL, Cutfield JF. Crystal structures of *Escherichia coli* uridine phosphorylase in two native and three complexed forms reveal basis of substrate specificity, induced conformational changes and influence of potassium. J Mol Biol 2004; 337: 337-54.

[27] Dontsova M V, Gabdoulkhakov AG, Molchan OK, *et al*. Structural biology and crystallization communications preliminary investigation of the three-dimensional structure of *Salmonella typhimurium* uridine phosphorylase in the crystalline state. Acta Cryst 2005; 61: 337-40.

[28] Lashkov AA, Gabdoulkhakov AG, Shtil AA, Mikhailov AM. Crystallization and preliminary X-ray diffraction analysis of *Salmonella typhimurium* uridine phosphorylase complexed with 5-fluorouracil. Acta Crystallogr Sect F Struct Biol Cryst Commun 2009; 65: 601-3.

[29] Lashkov AA, Zhukhlistova NE, Gabdoulkhakov AH, *et al*. The X-ray structure of *Salmonella typhimurium* uridine nucleoside phosphorylase complexed with 2,2′-anhydrouridine, phosphate and potassium ions at 1.86 Å resolution. Acta Crystallogr Sect D Biol Crystallogr 2010; 66: 51-60.

[30] Canduri F, Silva RG, dos Santos DM, *et al*. Structure of human PNP complexed with ligands. Acta Crystallogr Sect D Biol Crystallogr 2005; 61: 856-62.

[31] Bennett EM, Li C, Allan PW, Parker WB, Ealick SE. Structural basis for substrate specificity of *Escherichia coli* purine nucleoside phosphorylase. J Biol Chem 2003; 278: 47110-18.

[32] Trembacz H, Jezewska MM. Specific adenosine phosphorylase from hepatopancreas of gastropod *Helix pomatia*. Comp Biochem Physiol B 1993; 104: 481-87.

[33] Sgarrella F, Frassetto L, Allegrini S, *et al*. Characterization of the adenine nucleoside specific phosphorylase of *Bacillus cereus*. Biochim Biophys Acta 2007; 1770: 1498-1505.

[34] Mcelwain MC, Williams M V, Pollack ' AJD. *Acholeplasma laidlawii* B-PG9 adenine-specific purine nucleoside phosphorylase that accepts ribose-1-phosphate, deoxyribose-1-phosphate, and xylose-1-phosphate. J Bacteriol 1988; 170: 564-67.

[35] Shirae H, Yokozeki K. Purifications and properties of orotidine-phosphorolyzing enzyme and purine nucleoside phosphorylase from *Erwinia carotovora* AJ 2992. Agric Biol Chem 1991; 55: 1849-57.

[36] Shi W, Ting LM, Kicska GA, *et al*. *Plasmodium falciparum* purine nucleoside phosphorylase: crystal structures, immucillin inhibitors, and dual catalytic function. J Biol Chem 2004; 279: 18103-6.

[37] Zhou X, Szeker K, Janocha B, *et al*. Recombinant purine nucleoside phosphorylases from thermophiles: Preparation, properties and activity towards purine and pyrimidine nucleosides. FEBS J 2013; 280: 1475-90.

[38] Morisawa H, Yamanaka S, Yamazaki A, Yoshinaga F, Hirose Y. Properties of Nucleoside Phosphorylase from *Enterobacter aerogenes*. Agric Biol Chem 1985; 49: 3239-46.

[39] Nannemann DP, Kaufmann KW, Meiler J, Bachmann BO. Design and directed evolution of a dideoxy purine nucleoside phosphorylase. Protein Eng Des Sel 2010; 23: 607-16.

[40] Chaudhary K, Ting LM, Kim K, Roos DS. *Toxoplasma gondii* purine nucleoside phosphorylase biochemical characterization, inhibitor profiles, and comparison with the Plasmodium falciparum ortholog. J Biol Chem 2006; 281: 25652-58.

[41] Ting LM, Gissot M, Coppi A, Sinnis P, Kim K. Attenuated *Plasmodium yoelii* lacking purine nucleoside phosphorylase confer protective immunity. Nat Med 2008; 14: 954-58.

[42] Cobb SL, Deng H, Hamilton JT, McGlinchey RP, O'Hagan D. Identification of 5-fluoro-5-deoxy-D-ribose-1-phosphate as an intermediate in fluorometabolite biosynthesis in *Streptomyces cattleya*. Chem Commun 2004: 592-93.

[43] Bagarolo ML, Porcelli M, Martino E, Feller G, Cacciapuoti G. Multiple disulfide bridges modulate conformational stability and flexibility in hyperthermophilic archaeal purine nucleoside phosphorylase. Biochim Biophys Acta 2015; 1854: 1458-65.

[44] Almendros M, Berenguer J, Sinisterra J-V. *Thermus thermophilus* nucleoside phosphorylases active in the synthesis of nucleoside analogues. Appl Environ Microbiol 2012; 78: 3128-35.

[45] Bzowska A, Kulikowska E, Darzynkiewicz E, Shugar D. Purine nucleoside phosphorylase. Structure-activity relationships for substrate and inhibitor properties of N-1-, N-7-, and C-8-substituted analogues; differentiation of mammalian and bacterial enzymes with N-1-methylinosine and guanosine. J Biol Chem 1988; 263: 9212-17.

[46] Kulikowska E, Bzowska A, Wierzchowski J, Shugar D. Properties of two unusual, and fluorescent, substrates of purine-nucleoside phosphorylase: 7-methylguanosine and 7-methylinosine. Biochim Biophys Acta - Protein Struct Mol Enzymol 1986; 874: 355-63.

[47] Lee J, Filosa S, Bonvin J, Guyon S, Aponte RA, Turnbull JL. Expression, purification, and characterization of recombinant purine nucleoside phosphorylase from *Escherichia coli*. Protein Expr Purif 2001; 22: 180-88.

[48] Bzowska A, Kulikowska E, Shugar D. Formycins A and B and some analogues: selective inhibitors of bacterial (*Escherichia coli*) purine nucleoside phosphorylase. Biochim Biophys Acta - Protein Struct Mol Enzymol 1992; 1120: 239-47.

[49] Todorova NA, Schwarz FP. Effect of the phosphate substrate on drug-inhibitor binding to human purine nucleoside phosphorylase. Arch Biochem Biophys 2008; 480: 122-31.

[50] Ducati RG, Santos DS, Basso LA. Substrate specificity and kinetic mechanism of purine nucleoside phosphorylase from *Mycobacterium tuberculosis*. Arch Biochem Biophys 2009; 486: 155-64.

[51] Zang Y, Wang WH, Wu SW, Ealick SE, Wang CC. Identification of a subversive substrate of *Trichomonas vaginalis* purine nucleoside phosphorylase and the crystal structure of the enzyme-substrate complex. J Biol Chem 2005; 280: 22318-25.

[52] Xie XX, Xia JG, He KF, Lu LN, Xu QY, Chen N. Low-molecular-mass purine nucleoside phosphorylase: characterization and application in enzymatic synthesis of nucleoside. antiviral drugs. Biotechnol Lett 2011; 33: 1107-12.

[53] Cheng J, Farutin V, Wu Z, *et al*. Purine nucleoside phosphorylase-catalyzed, phosphate-independent hydrolysis of 2-amino-6-mercapto-7-methylpurine ribonucleoside. Bioorg Chem 1999; 27: 307-25.

[54] Hassan AE, Abou-Elkhair RA, Parker WB, Allan PW, Secrist 3rd JA. 6-Methylpurine derived sugar modified nucleosides: Synthesis and evaluation of their substrate activity with purine nucleoside phosphorylases. Bioorg Chem 2016; 65: 9-16.

[55] Hassan AE, Abou-Elkhair RA, Riordan JM, *et al*. Synthesis and evaluation of the substrate activity of C-6 substituted purine ribosides with *E. coli* purine nucleoside phosphorylase: palladium mediated cross-coupling of organozinc halides with 6-chloropurine nucleosides. Eur J Med Chem 2012; 47: 167-74.

[56] Wielgus-Kutrowska B, Kulikowska E, Wierzchowski J, Bzowska A, Shugar D. Nicotinamide riboside, an unusual, non-typical, substrate of purified purine-nucleoside phosphorylases. Eur J Biochem 1997; 243: 408-14.

[57] Parks RE, Agarwal RP. Purine nucleoside phosphorylase. Enzym 1972; 7: 483-514.

[58] Lewis AS, Lowy BA. Human erythrocyte purine nucleoside phosphorylase: molecular weight and physical properties. A Theorell-Chance catalytic mechanism. J Biol Chem 1979; 254: 9927-32.

[59] Rejman D, Panova N, Klener P, Maswabi B, Pohl R, Rosenberg I. N-phosphonocarbonylpyrrolidine derivatives of guanine: a new

class of bi-substrate inhibitors of human purine nucleoside phosphorylase. J Med Chem 2012; 55: 1612-21.

[60] Silva RG, Carvalho LP, Oliveira JS, *et al*. Cloning, overexpression, and purification of functional human purine nucleoside phosphorylase. Protein Expr Purif 2003; 27: 158-64.

[61] Silva RG, Pereira JH, Canduri F, de Azevedo Jr WF, Basso LA, Santos DS. Kinetics and crystal structure of human purine nucleoside phosphorylase in complex with 7-methyl-6-thio-guanosine. Arch Biochem Biophys 2005; 442: 49-58.

[62] Furihata T, Kishida S, Sugiura H, Kamiichi A, Iikura M, Chiba K. Functional analysis of purine nucleoside phosphorylase as a key enzyme in ribavirin metabolism. Drug Metab Pharmacokinet 2014; 29: 211-14.

[63] May RA, Hoffee P. Purine nucleoside phosphorylases purified from rat liver and Novikoff hepatoma cells. Arch Biochem Biophys 1979; 193: 398-406.

[64] Silva RG, Santos DS, Basso LA, *et al*. Purine nucleoside phosphorylase activity in rat cerebrospinal fluid. Neurochem Res 2004; 29: 1831-35.

[65] Aberg AM, Ronquist G, Haney M, Waldenstrom A. Effects of some modulators on purine nucleoside phosphorylase activity in myocardial tissue. Scand J Clin Lab Invest 2010; 70: 8-14.

[66] Fateev IV, Antonov K V, Konstantinova ID, *et al*. The chemoenzymatic synthesis of clofarabine and related 2'-deoxyfluoroarabinosyl nucleosides: the electronic and stereochemical factors determining substrate recognition by E. coli nucleoside phosphorylases. Beilstein J Org Chem 2014; 10: 1657-69.

[67] Bzowska A, Kulikowska E, Poopeiko NE, Shugar D. Kinetics of phosphorolysis of 3-(β-d-ribofuranosyl)adenine and 3-(β-d-ribofuranosyl)hypoxanthine, non-conventional substrates of purine-nucleoside phosphorylase. Eur J Biochem 1996; 239: 229-34.

[68] Carteni'-Farina M, Oliva A, Romeo G, *et al*. 5'-Methylthioadenosine phosphorylase from *Caldariella acidophila*. Purification and properties. Eur J Biochem 1979; 101: 317-24.

[69] Zappia V, Oliva A, Cacciapuoti G, Galletti P, Mignucci G, Carteni-Farina M. Substrate specificity of 5'-methylthioadenosine phosphorylase from human prostate. Biochem J 1978; 175: 1043-50.

[70] Toorchen D, Miller RL. Purification and characterization of 5'-deoxy-5'-methylthioadenosine (MTA) phosphorylase from human liver. Biochem Pharmacol 1991; 41: 2023-30.

[71] Fabianowska-Majewska K, Duley J, Fairbanks L, Simmonds A, Wasiak T. Substrate specificity of methylthioadenosine phosphorylase from human liver. Acta Biochim Pol 1994; 41: 391-95.

[72] White MW, Vandenbark AA, Barney CL, Ferro AJ. Structural analogs of 5'-methylthioadenosine as substrates and inhibitors of 5'-methylthioadenosine phosphorylase and as inhibitors of human lymphocyte transformation. Biochem Pharmacol 1982; 31: 503-7.

[73] Koszalka GW, Krenitsky TA. 5′-Methylthioadenosine (MTA) phosphorylase from promastigotes of *Leishmania donovani*. In: Nyhan WL, Thompson LF, Watts RWE, Eds. Purine Pyrimidine Metab Man V Part B Basic Sci Asp.; Boston, MA: Springer US 1986; pp. 559-63.

[74] Savarese TM, Crabtree GW, Parks Jr. RE. 5'-Methylthioadenosine phosphorylase-I. Substrate activity of 5'-deoxyadenosine with the enzyme from sarcoma 180 cells. Biochem Pharmacol 1981; 30: 189-99.

[75] Cacciapuoti G, Bertoldo C, Brio A, Zappia V, Porcelli M. Purification and characterization of 5'-methylthioadenosine phosphorylase from the hyperthermophilic archaeon *Pyrococcus furiosus*: substrate specificity and primary structure analysis. Extremophiles 2003; 7: 159-68.

[76] Ferro AJ, Wrobel NC, Nicolette JA. 5-Methylthioribose 1-phosphate: a product of partially purified, rat liver 5'-methylthioadenosine phosphorylase activity. Biochim Biophys Acta 1979; 570: 65-73.

[77] Cacciapuoti G, Porcelli M, Bertoldo C, De Rosa M, Zappia V. Purification and characterization of extremely thermophilic and thermostable 5'-methylthioadenosine phosphorylase from the archaeon *Sulfolobus solfataricus*. Purine nucleoside phosphorylase activity and evidence for intersubunit disulfide bonds. J Biol Chem 1994; 269: 24762-69.

[78] Bacchi CJ, Goldberg B, Rattendi D, Gorrell TE, Spiess AJ, Sufrin JR. Metabolic effects of a methylthioadenosine phosphorylase substrate analog on African trypanosomes. Biochem Pharmacol 1999; 57: 89-96.

[79] Vodnala M, Ranjbarian F, Pavlova A, de Koning HP, Hofer A. *Trypanosoma brucei* methylthioadenosine phosphorylase protects the parasite from the antitrypanosomal effect of deoxyadenosine: Implications for the pharmacology of adenosine antimetabolites. J Biol Chem 2016; 291: 11717-26.

[80] Ghoda LY, Savarese TM, Northup CH, *et al*. Substrate specificities of 5'-deoxy-5'-methylthioadenosine phosphorylase from *Trypanosoma brucei* brucei and mammalian cells. Mol Biochem Parasitol 1988; 27: 109-18.

[81] Leer J. Uridine phosphorylase from *Escherichia coli*. Physical and chemical characteristics. Eur J Biochem 1976; 75: 217-24.

[82] Avraham Y, Grossowicz N, Yashphe J. Purification and characterization of uridine and thymidine phosphorylase from *Lactobacillus casei*. Biochim Biophys Acta 1990; 1040: 287-93.

[83] Molchan OK, Dmitrieva NA, Romanova DV, Lopes LE, Debabov VG MA. Isolation and initial characterization of the uridine phosphorylase from *Salmonella typhimurium*. Biochem 1998; 63: 195-99.

[84] Mordkovich NN, Safonova TN, Manuvera VA, *et al*. Physicochemical characterization of uridine phosphorylase from *Shewanella oneidensis* MR-1. Dokl Biochem Biophys 2013; 451: 187-89.

[85] Lee CS, Jimnez BM, Sullivan WJO. Purification and characterization of uridine (thymidine) phosphorylase from *Giardia lamblia*. Mol Biochem Parasitol 1988: 271-77.

[86] Liu M, Cao D, Russell R, Handschumacher RE, Pizzorno G. Expression, characterization, and detection of human uridine phosphorylase and identification of variant uridine phosphorolytic activity in selected human tumors. Cancer Res 1998; 58: 5418-24.

[87] Pontis H, Degerstedt G, Reichard P. Uridine and deoxyuridine phosphorylases from ehrlich ascites tumor. Biochim Biophys Acta 1961; 51: 138-47.

[88] Bose R YE. Uridine phosphorylase activity of isolated plasma membranes of rat liver. Can J Biochem 1977; 55: 528-33.

[89] el Kouni MH, Naguib FNM, Niedzwicki JG, Iltzsch MH, Cha S. Uridine phosphorylase from *Schistosoma mansoni*. J Biol Chem 1988; 263: 6081-86.

[90] Zhu S, Song D, Gong C, *et al*. Biosynthesis of nucleoside analogues via thermostable nucleoside phosphorylase. Appl Microbiol Biotechnol 2013; 97: 6769-78.

[91] Temmink OH, de Bruin M, Turksma AW, Cricca S, Laan AC, Peters GJ. Activity and substrate specificity of pyrimidine phosphorylases and their role in fluoropyrimidine sensitivity in colon cancer cell lines. Int J Biochem Cell Biol 2007; 39: 565-75.

[92] Yan R. Uridine phosphorylase in breast cancer: a new prognostic factor? Front Biosci 2006; 11: 2759.

[93] el Kouni MH, Naguib FNM, Naguib FNM. Differences in Activities and Substrate Specificity of Human and Murine Pyrimidine Nucleoside Phosphorylases: Implications for Chemotherapy with 5-Fluoropyrimidines. Cancer Res 1993; 53: 3687-93.

[94] Yano S, Kazuno H, Suzuki N, *et al*. Synthesis and evaluation of 6-methylene-bridged uracil derivatives. Part 1: Discovery of novel orally active inhibitors of human thymidine phosphorylase. Bioorganic Med Chem 2004; 12: 3431-41.

[95] Cui H, Ruda GF, Carrero-Lérida J, Ruiz-Pérez LM, Gilbert IH, González-Pacanowska D. Exploring new inhibitors of *Plasmodium falciparum* purine nucleoside phosphorylase. Eur J Med Chem 2010; 45: 5140-49.

[96] Mitterbauer R, Karl T, Adam G. *Saccharomyces cerevisiae* URH1 (encoding uridine-cytidine N-ribohydrolase): Functional complementation by a nucleoside hydrolase from a protozoan parasite and by a mammalian uridine phosphorylase. Appl Environ Microbiol 2002; 68: 1336-43.

[97] Johansson M. Identification of a novel human uridine phosphorylase. Biochem Biophys Res Commun 2003; 307: 41-46.

[98] Watanabe S-I, Hino AH, Wada K, Eliason JF, Uchida T. Purification, cloning, and expression of murine uridine phosphorylase. 1995; 270: 12191-96.

[99] Veres Z, Neszmelyi A, Szabolcs A, Denes G. Inhibition of uridine phosphorylase by pyrimidine nucleoside analogs and consideration of substrate binding to the enzyme based on solution conformation as seen by NMR spectroscopy. Eur J Biochem 1988; 181: 173-81.

[100] Bzowska A, Ananiev AV, Ramzaeva N, *et al*. Purine nucleoside phosphorylase: Inhibition by purine N(7)- and N(9)-acyclo-nucleosides; and substrate properties of 7-β-D-ribofurano-sylguanine and 7-β-D-ribofuranosylhypoxanthine. Biochem Pharmacol 1994; 48: 937-47.

[101] Wielgus-Kutrowska B, Bzowska A. Kinetic properties of *Cellulomonas sp.* purine nucleoside phosphorylase with typical and non-typical substrates: implications for the reaction mechanism. Nucleosides Nucleotides Nucleic Acids 2005; 24: 471-76.

[102] Bromley JR, Warnes BJ, Newell CA, *et al*. A purine nucleoside phosphorylase in *Solanum tuberosum L.* (potato) with specificity for cytokinins contributes to the duration of tuber endodormancy. Biochem J 2014; 458: 225-37.

[103] Stoeckler JD, Cambor C, Parks RE. Human erythrocytic purine nucleoside phosphorylase: reaction with sugar-modified nucleoside substrates. Biochemistry 1980; 19: 102-7.

[104] Utagawa T, Morisawa H, Yamanaka S, Yamazaki A, Yoshinaga F, Hirose Y. Properties of nucleoside phosphorylase from *Enterobacter aerogenes*. Agric Biol Chem 1985; 49: 3239-46.

[105] Shirae H, Yokozeki K. Purification and properties of purine nucleoside phosphorylase from *Brevibacterium acetylicum* ATCC 954. Agric Biol Chem 1991; 55: 493-99.

[106] Eustaquio AS, McGlinchey RP, Liu Y, *et al*. Biosynthesis of the salinosporamide A polyketide synthase substrate chloroethylmalonyl-coenzyme A from S-adenosyl-L-methionine. Proc Natl Acad Sci U S A 2009; 106: 12295-300.

[107] Barsacchi D, Cappiello M, Tozzi MG, *et al*. Purine nucleoside phosphorylase from bovine lens: purification and properties. Biochim Biophys Acta 1992; 1160: 163-70.

[108] Moyer TP, Fischer AG. Purification and characterization of a purine-nucleoside phosphorylase from bovine thyroid. Arch Biochem Biophys 1976; 174: 622-29.

[109] Pegg AE, Williams-Ashman HG. Phosphate-stimulated breakdown of 5'-methylthioadenosine by rat ventral prostate. Biochem J 1969; 115: 241-47.

[110] Araki T, Ikeda I, Matoishi K, *et al*. Method for producing cytosine nucleoside compounds., 2005.

[111] Stepchenko VA, Seela F, Esipov RS, Miroshnikov AI, Sokolov YA, Mikhailopulo IA. Enzymatic synthesis of 2′-deoxy-β-d-ribonucleosides of 8-azapurines and 8-Aza-7-deazapurines. Synlett 2012; 23: 1541-45.

[112] Appleby TC, Mathews II, Porcelli M, Cacciapuoti G, Ealick SE. Three-dimensional Structure of a Hyperthermophilic 5′-Deoxy-5′-methylthioadenosine Phosphorylase from Sulfolobus solfataricus. J Biol Chem 2001; 276: 39232-42.

[113] Kung PP, Zehnder LR, Meng JJ, *et al*. Design, synthesis, and biological evaluation of novel human 5'-deoxy-5'-methylthioadenosine phosphorylase (MTAP) substrates. Bioorg Med Chem Lett 2005; 15: 2829-33.

[114] Firestone RS, Cameron SA, Tyler PC, Ducati RG, Spitz AZ, Schramm VL. Continuous fluorescence assays for reactions involving adenine. Anal Chem 2016; 88: 11860-67.

[115] Scocca JJ. Purification and substrate specificity of pyrimidine nucleoside phosphorylase from *Haemophilus influenzae*. J Biol Chem 1971; 246: 6606-10.

[116] Walter MR, Cook WJ, Cole LB, *et al*. Three-dimensional structure of thymidine phosphorylase from *Escherichia coli* at 2,8 A resolution. J Biol Chem 1990; 265: 14016-22.

[117] Saunders PP, Wilson BA, Saunders GF. Purification and comparative properties of a pyrimidine nucleoside phosphorylase from *Bacillus stearothermophilus*. J Biol Chem 1969; 244: 3691-97.

[118] Gao XF, Huang XR, Sun CC. Role of each residue in catalysis in the active site of pyrimidine nucleoside phosphorylase from *Bacillus subtilis*: A hybrid QM/MM study. J Struct Biol 2006; 154: 20-26.

[119] Szeker K, Zhou X, Schwab T, *et al*. Comparative investigations on thermostable pyrimidine nucleoside phosphorylases from *Geobacillus thermoglucosidasius* and *Thermus thermophilus*. J Mol Catal B Enzym 2012; 84: 27-34.

[120] Voorde J Vande, Gago F, Vrancken K, Liekens S, Balzarini J. Characterization of pyrimidine nucleoside phosphorylase of *Mycoplasma hyorhinis*: implications for the clinical efficacy of nucleoside analogues. Biochem J 2012; 445: 113-23.

[121] Bronckaers A, Aguado L, Negri A, *et al*. Identification of aspartic acid-203 in human thymidine phosphorylase as an important residue for both catalysis and non-competitive inhibition by the small molecule "crystallization chaperone" 5'-O-tritylinosine (KIN59). Biochem Pharmacol 2009; 78: 231-40.

[122] Omari K El, Bronckaers A, Liekens S, Erez-Erez M-J, Balzarini J, Stammers DK. Structural basis for non-competitive product inhibition in human thymidine phosphorylase: implications for drug design. Biochem J 2006; 399: 199-204.

[123] Liekens S, Balzarini J, Hernández AI, *et al*. Thymidine Phosphorylase is noncompetitively inhibited by 5′- O -trityl-inosine (KIN59) and related compounds. Nucleosides, Nucleotides and Nucleic Acids 2006; 25: 975-80.

[124] Mitsiki E, Papageorgiou AC, Iyer S, *et al*. Structures of native human thymidine phosphorylase and in complex with 5-iodouracil. Biochem Biophys Res Commun 2009; 386: 666-70.

[125] Norman RA, Barry ST, Bate M, *et al*. Crystal structure of human thymidine phosphorylase in complex with a small molecule inhibitor. Structure 2004; 12: 75-84.

[126] Pugmire MJ, Ealick SE. The crystal structure of pyrimidine nucleoside phosphorylase in a closed conformation. Structure 1998; 6: 1467-79.

[127] Balaev V V., Lashkov AA, Gabdulkhakov AG, *et al*. Structural investigation of the thymidine phosphorylase from *Salmonella typhimurium* in the unliganded state and its complexes with thymidine and uridine. Acta Crystallogr Sect F Struct Biol Commun 2016; 72: 224-33.

[128] Kono A, Hara Y, Sugata S, Matsuhima Y, Ueda T. Substrate specificity of a thymidine phosphorylase in human liver tumor. Chem. Pharm. Bull. 1984; 32: 1919-21.

[129] Schinazi RF, Peck A, Sommadossi J-P. Substrate specificity of *Escherichia coli* thymidine phosphorylase for pyrimidine nucleosides with anti-human immunodeficiency virus activity. Biochem Pharmacol 1992; 44: 199-204.

[130] Panova NG, Alexeev CS, Kuzmichov AS, *et al*. Substrate specificity of *Escherichia coli* thymidine phosphorylase. Biochem 2007; 72: 21-28.

[131] Hatano A, Harano A, Takigawa Y, *et al*. Kinetic parameters and recognition of thymidine analogues with varying functional groups by thymidine phosphorylase. Bioorg Med Chem 2008; 16: 3866-70.

[132] Panova NG, Alexeev CS, Polyakov KM, Gavryushov SA, Kritzyn AM, Mikhailov SN. Substrate specificity of thymidine phosphorylase of *E. coli*: role of hydroxyl groups. Nucleosides, Nucleotides and Nucleic Acids 2008; 27: 1211-14.

[133] Reigan P, Edwards PN, Gbaj A, *et al*. Aminoimidazolyl-methyluracil analogues as potent inhibitors of thymidine phosphorylase and their bioreductive nitroimidazolyl prodrugs. J Med Chem 2005; 48: 392-402.

[134] Kubilus J, Lee LD, Baden HP. Purification of thymidine phosphorylase from human amniochorion. Biochim Biophys Acta 1978; 527: 221-28.

[135] Hoffee PA, Blank J. Thymidine phosphoryalse from *Salmonella typhimurium*. Methods Enzym 1978; 51: 437-42.

[136] Blank JG, Hoffee PA. Purification and properties of thymidine phosphorylase from *Salmonella typhimurium*. Arch Biochem Biophys 1975; 168: 259-65.

[137] Desgranges C, Razaka G, Rabaud M, Bricaud H. Catabolism of thymidine in human blood platelets: purification and properties of thymidine phosphorylase. Biochim Biophys Acta 1981; 654: 211-18.

[138] Serra I, Bavaro T, Cecchini DA, *et al*. A comparison between immobilized pyrimidine nucleoside phosphorylase from *Bacillus subtilis* and thymidine phosphorylase from *Escherichia coli* in the synthesis of 5-substituted pyrimidine 2 -deoxyribonucleosides. J Mol Catal B Enzym 2013; 95: 16-22.

[139] Grierson JR, Brockenbrough JS, Rasey JS, *et al*. Evaluation of 5'-deoxy-5'-[F-18]fluorothymidine as a tracer of intracellular thymidine phosphorylase activity. Nucl Med Biol 2007; 34: 471-78.

[140] Bijnsdorp I V., de Bruin M, Laan AC, Fukushima M, Peters GJ. The role of platelet-derived endothelial cell growth factor/thymidine phosphorylase in tumor behavior. Nucleosides, Nucleotides and Nucleic Acids 2008; 27: 681-91.

[141] Yang K, Yoshimura G, Mori I, Sakurai T KK. Thymidine phosphorylase and breast carcinoma. Anticancer Res 2002; 22: 2355-60.

[142] Bronckaers A, Gago F, Balzarini J, Liekens S. The dual role of thymidine phosphorylase in cancer development and chemotherapy. Med Res Rev 2009; 29: 903-53.

[143] Panova N, Kosiova I, Petrova M, *et al*. Nucleoside phosphonic acids in thymidine phosphorylase inhibition: structure - activity relationship. Nucleic Acids Symp Ser 2008; 52: 665-66.

[144] Gbaj A, Edwards PN, Reigan P, Freeman S, Jaffar M, Douglas KT. Thymidine phosphorylase from *Escherichia coli*: Tight-binding inhibitors as enzyme active-site titrants. J Enzyme Inhib Med Chem 2006; 21: 69-73.

[145] Panova NG, Shcheveleva EV, Alexeev CS, *et al*. Use of 4-thiouridine and 4-thiothymidine in studies on pyrimidine nucleoside phosphorylases. Mol Biol 2004; 38: 770-76.

[146] Ogiuchi Y, Maruoka Y, Ando T, Kobayashi M, Ogiuchi H. Thymidylate synthase, thymidine phosphorylase and orotate phosphoribosyl transferase levels as predictive factors of chemotherapy in oral squamous cell carcinoma. Acta Histochem Cytochem 2008; 41: 39-46.

[147] Focher F, Spadari S. Thymidine phosphorylase : A two-face Janus in anticancer chemotherapy. Curr Cancer Drug Targets 2001; 1: 141-53.

[148] Sugata S, Kono A, Hara Y, Karube Y MY. Partial purification of a thymidine phosphorylase from human gastric cancer. Chem Pharm Bull 1986; 34: 369-75.

[149] Hori N, Watanabe M, Yamazaki Y, Mikami Y. Purification and characterization of thermostable purine nucleoside phosphorylase of *Bacillus stearothermophilus* JTS 859. Agric Biol Chem 1989; 53: 3219-24.

[150] Timothy H. Trana, S. Christoffersenb, Paula W. Allanc, William B. Parkerc, Jure Piskurb I, Serrad, M. Terrenid and SEE. The crystal structure of *Streptococcus pyogenes* uridine phosphorylase reveals a distinct subfamily of nucleoside phosphorylases. Biochemistry 2011; 50: 6549-58.

[151] Zhou M, Pugmire MJ, Vuong BQ, Ealick SE. Cloning, expression and crystallization of pyrimidine nucleoside phosphorylase from *Bacillus stearothermophilus*. Acta Crystallogr D Biol Crystallogr 1999; 55: 287-90.

[152] Hamamoto T, Noguchi T, Midorikawa Y. Purification and characterization of purine nucleoside phosphorylase and pyrimidine nucleoside phosphorylase from *Bacillus stearothermophilus* TH 6-2. Biosci Biotechnol Biochem 1996; 60: 1179-80.

[153] Vande Voorde J, Quintiliani M, McGuigan C, Liekens S, Balzarini J. Inhibition of pyrimidine and purine nucleoside phosphorylases by a 3,5-dichlorobenzoyl-substituted 2-deoxy-d-ribose-1-phosphate derivative. Biochem Pharmacol 2012; 83: 1358-63.

[154] Voorde J Vande, Liekens S, Gago F, Balzarini J. The pyrimidine nucleoside phosphorylase of *Mycoplasma hyorhinis* and how it may affect nucleoside-based therapy. Nucleosides, Nucleotides and Nucleic Acids 2014; 33: 394-402.

[155] Zaks A, Dodds DR. Application of biocatalysis and biotransformations to the synthesis of pharmaceuticals. Drug Discov Today 1997; 2: 513-31.

[156] el Kouni MH, el Kouni MM, Naguib FNM. Differences in activities and substrate specificity of human and murine pyrimidine nucleoside phosphorylases: Implications for chemotherapy with 5-fluoropyrimidines. Cancer Res 1993; 53.

[157] Hori N, Watanabe M, Yamazaki Y, Mikami Y. Purification and characterization of thermostable pyrimidine nucleoside phosphorylase from *Bacillus stearothermophillus* JTS 859. Agric Biol Chem 1990; 54: 763-68.

[158] Mikhailopulo IA. Biotechnology of nucleic acid constituents - State of the art and perspectives. Curr Org Chem 2007; 11: 317-35.

[159] Mikhailopulo IA, Miroshnikov AI. New trends in nucleoside biotechnology. Acta Naturae 2010; 2: 36-59.

[160] Lewkowicz ES, Martínez N, Rogert MC, Porro S, Iribarren AM. An improved microbial synthesis of purine nucleosides. Biotechnol Lett 2000; 22: 1277-80.

[161] Zuffi G, Ghisotti D, Oliva I, *et al*. Immobilized biocatalysts for the production of nucleosides and nucleoside analogues by enzymatic transglycosylation reactions. Biocatal Biotransformation 2004; 22: 25-33.

[162] Hori N, Watanabe M, Sunagawa K, Uehara K, Mikami Y. Production of 5-methyluridine by immobilized thermostable purine nucleoside phosphorylase and pyrimidine nucleoside phosphorylase from *Bacillus stearothermophilus* JTS 859. J Biotechnol 1991; 17: 121-31.

[163] Zhou X, Mikhailopulo IA, Cruz Bournazou MN, Neubauer P. Immobilization of thermostable nucleoside phosphorylases on MagReSyn® epoxide microspheres and their application for the synthesis of 2,6-dihalogenated purine nucleosides. J Mol Catal B Enzym 2015; 115: 119-27.

[164] Trelles JA, Bentancor L, Schoijet A, *et al*. Immobilized *Escherichia coli* BL21 as a catalyst for the synthesis of adenine and hypoxanthine nucleosides. Chem Biodivers 2004; 1: 280-88.

[165] Krenitsky TA, Rideout JL, Koszalka GW, *et al*. Pyrazolo[3,4-d]pyrimidine ribonucleosides as anticoccidials. 1. Synthesis and activity of some nucleosides of purines and 4-(alkylthio)pyrazolo[3,4-d]pyrimidines. J Med Chem 1982; 25: 32-35.

[166] Krenitsky TA, Koszalka GW, Tuttle J V. Purine nucleoside synthesis: an efficient method employing nucleoside phosphorylases. Biochemistry 1981; 20: 3615-21.

[167] Condezo LA, Fernandez-Lucas J, Garcia-Burgos CA, Alcantara AR SJ. Enzymatic synthesis of modified nucleosides. CRC Press 2006.

[168] Vieille C, Zeikus GJ. Hyperthermophilic enzymes: sources, uses, and molecular mechanisms for thermostability. Microbiol Mol Biol Rev 2001; 65: 1-43.

[169] Mikhailopulo A, Miroshnikov AI. Some recent findings in the biotechnology of biologically important nucleosides. Biotechnol Acta 2013; 6: 63-77.

[170] Parker WB, Allan PW, Ealick SE, *et al*. Design and evaluation of 5'-modified nucleoside analogs as prodrugs for an *E. coli* purine nucleoside phosphorylase mutant. Nucleosides Nucleotides Nucleic Acids 2005; 24: 387-92.

[171] Isaksen GV, Hopmann KH, ??qvist J, Brandsdal BO. Computer simulations reveal substrate specificity of glycosidic bond cleavage in native and mutant human purine nucleoside phosphorylase. Biochemistry 2016; 55: 2153-62.

[172] Maynes JT, Yam W, Jenuth JP, *et al*. Design of an adenosine phosphorylase by active-site modification of murine purine nucleoside phosphorylase. Enzyme kinetics and molecular dynamics simulation of Asn-243 and Lys-244 substitutions of purine nucleoside phosphorylase. Biochem J 1999; 344 Pt 2: 585-92.

[173] Nóbile M, Médici R, Terreni M, Lewkowicz ES, Iribarren AM. Use of *Citrobacter koseri* whole cells for the production of arabinonucleosides: A larger scale approach. Process Biochem 2012; 47: 2182-88.

[174] Gordon GER, Visser DF, Brady D, Raseroka N, Bode ML. Defining a process operating window for the synthesis of 5-methyluridine by transglycosylation of guanosine and thymine. J Biotechnol 2011; 151: 108-13.

[175] Tuttle J V, Tisdale M, Krenitsky TA. Purine 2'-deoxy-2'-fluororibosides as antiinfluenza virus agents. J Med Chem 1993; 36: 119-25.

[176] Isaksen GV, Hopmann KH, Åqvist J, Brandsdal BO. Computer simulations reveal substrate specificity of glycosidic bond cleavage in native and mutant human purine nucleoside phosphorylase. Biochemistry 2016; 55: 2153-62.

[177] Stachelska-Wierzchowska A, Wierzchowski J, Bzowska A, Wielgus-Kutrowska B. Site-selective ribosylation of fluorescent nucleobase analogs using purine-nucleoside phosphorylase as a catalyst: effects of point mutations. Molecules 2015; 21: 44.

[178] Mikleušević G, Štefanić Z, Narczyk M, Wielgus-Kutrowska B, Bzowska A, Luić M. Validation of the catalytic mechanism of *Escherichia coli* purine nucleoside phosphorylase by structural and kinetic studies. Biochimie 2011; 93: 1610-22.

[179] Mahmoud Ghanem, Lei Li, Corin Wing A, Schramm* VL. Altered thermodynamics from remote mutations altering human toward bovine purine nucleoside phosphorylase. Biochemistry 2008; 47: 2559-64.

[180] Núnez S, Wing C, Antoniou D, Schramm VL, Schwartz SD. Insight into catalytically relevant correlated motions in human purine nucleoside phosphorylase. J Phys Chem A 2006; 110: 463-72.

[181] Murkin AS, Birck MR, Rinaldo-Matthis A, Shi W, Taylor EA, Schramm VL. Neighboring group participation in the transition state of human purine nucleoside phosphorylase. Biochemistry 2007; 46: 5038-5049.

[182] Edwards PN. A kinetic, modeling and mechanistic re-analysis of thymidine phosphorylase and some related enzymes. J Enzyme Inhib Med Chem 2006; 21: 483-518.

[183] Fernández-Irigoyen J, Santamaría M, Sánchez-Quiles V, *et al*. Redox regulation of methylthioadenosine phosphorylase in liver cells: molecular mechanism and functional implications. Biochem J 2008; 411: 457-65.

[184] Torini JR, Brandão-Neto J, DeMarco R, Pereira HD. Crystal structure of *Schistosoma mansoni* adenosine phosphorylase/5'-methylthioadenosine phosphorylase and its importance on adenosine salvage pathway. PLoS Negl Trop Dis 2016; 10: e0005178.

[185] Oliva I, Zuffi G, Barile D, *et al*. Characterization of *Escherichia coli* uridine phosphorylase by single-site mutagenesis. J Biochem 2004; 135: 495-99.

[186] Oliva I, Zuffi G, Orsini G, Tonon G, De Gioia L, Ghisotti D. Mutagenesis of *Escherichia coli* uridine phosphorylase by random pentapeptide insertions. Enzyme Microb Technol 2004; 35: 309-14.

[187] Veĭko VP, Siprashvili ZZ, Ratmanova KI, Gul'ko LB. Study of the role of histidine residues in the function of uridine phosphorylase from *Escherichia coli* K-12 by protein engineering. Bioorg Khim 1995; 21: 834-37.

[188] D. V. Chebotaev, L. B. Gul'ko and VPV. Protein engineering of uridine phosphorylase from *Escherichia coli* K-12. II. A Comparative Study of the Properties of Hybrid and Mutant Forms of Uridine Phosphorylases. Russ J Bioorganic Chem Transl from Bioorganicheskaya Khimiya Orig Russ Text 2001; 27: 160-66.

[189] Miyadera K, Sumizawa T, Haraguchi M, *et al*. Role of thymidine phosphorylase activity in the angiogenic effect of platelet- derived endothelial cell growth factor/thymidine phosphorylase. Cancer Res 1995; 55: 1687-16.

[190] Moghaddam A, Zhang H-T, Fant T-PD, *et al*. Thymidine phosphorylase is angiogenic and promotes tumor growth. Biochemistry 1995; 92: 998-1002.

[191] Yue H, Tanaka K, Furukawa T, Karnik SS, Li W. Thymidine phosphorylase inhibits vascular smooth muscle cell proliferation via upregulation of STAT3. Biochim Biophys Acta 2012; 1823: 1316-23.

[192] Appleby TC, Erion MD, Ealick SE. The structure of human 5′-deoxy-5′-methylthioadenosine phosphorylase at 1.7 Å resolution provides insights into substrate binding and catalysis. Structure 1999; 7: 629-41.

Paper II

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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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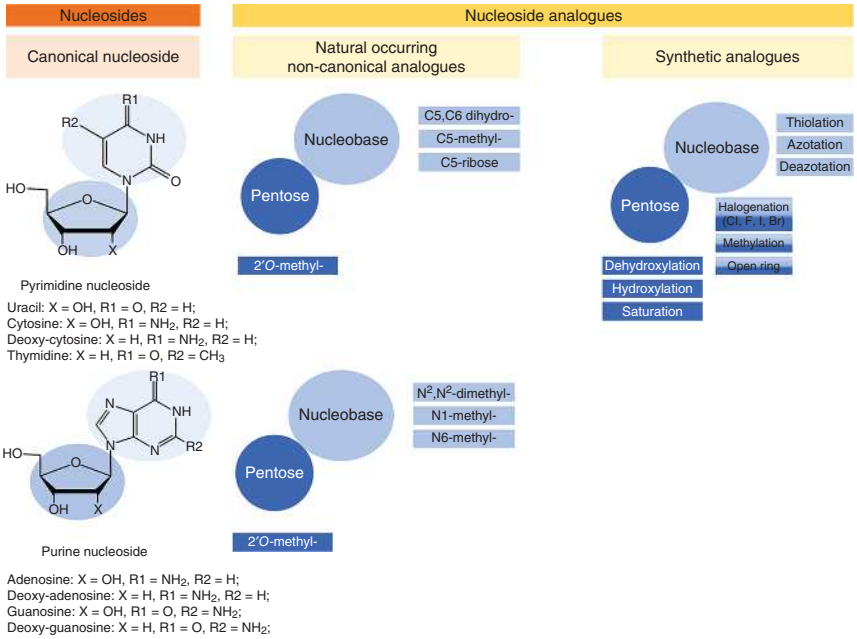

Figure 1.1 Classification of nucleosides and nucleoside analogues. Canonical (unmodified) nucleosides are the building blocks of DNA and RNA. Non-canonical (naturally modified on pentose moiety, base moiety or both) are mainly occurring in RNA. Synthetic nucleosides are used in the treatment of viral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

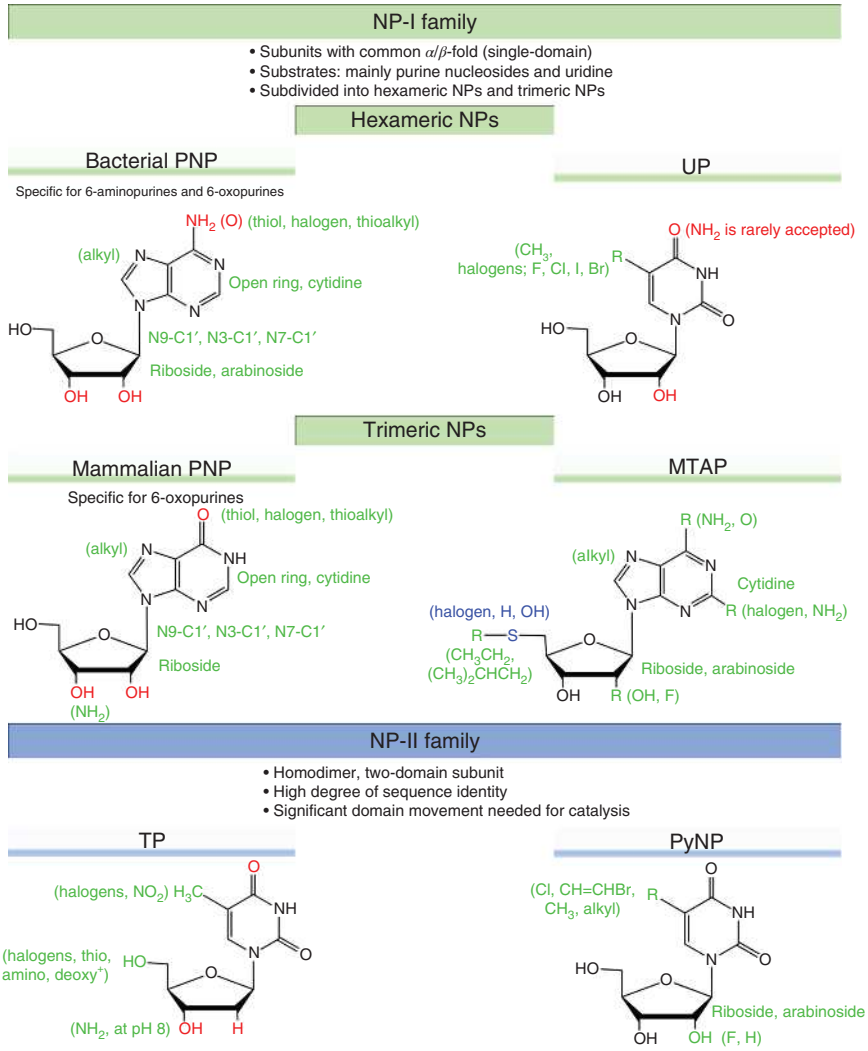

Figure 1.2 Classification of nucleoside phosphorylases (NPs) and their substrate affinities. NPs are classified into two main families: NP-I and NP-II. NP-I family is further subdivided into hexameric and Trimeric NPs. NP-II family is subdivided into two main classes: TP and PyNP. Residues labeled in 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

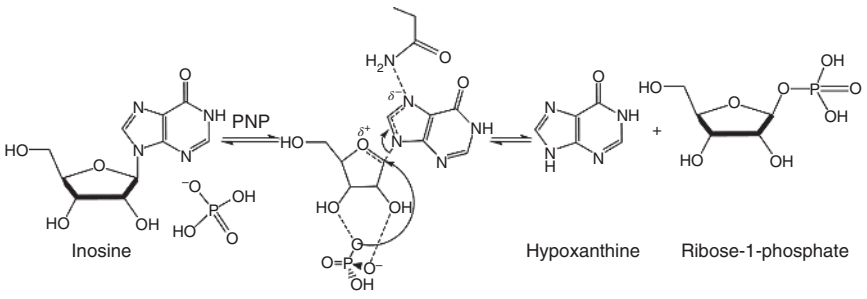

Figure 1.3 Catalytic mechanism described for PNP.

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,

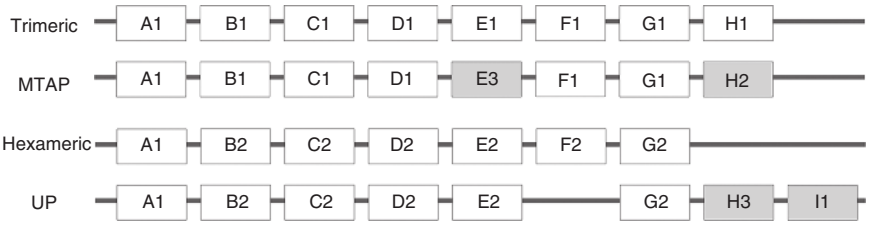

Figure 1.4 Structural motifs described for members of the NP-I family. Highlighted in grey are domains that are unique for either MTAP or UP. Trimeric- trimeric PNP, Hexameric-hexameric PNP.

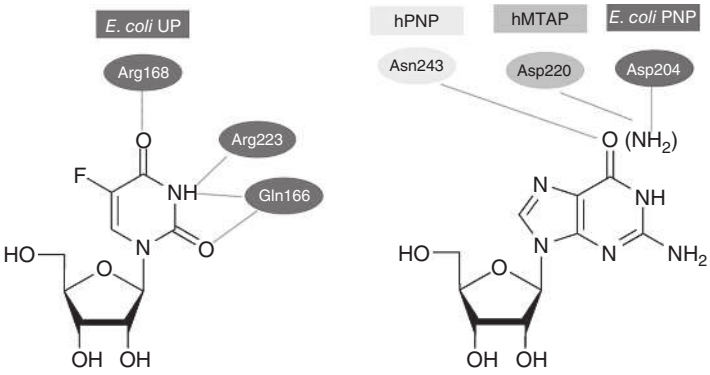

Figure 1.5 Schematic presentation of amino acid residues that define substrate specificity of different nucleoside phosphorylases.

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.

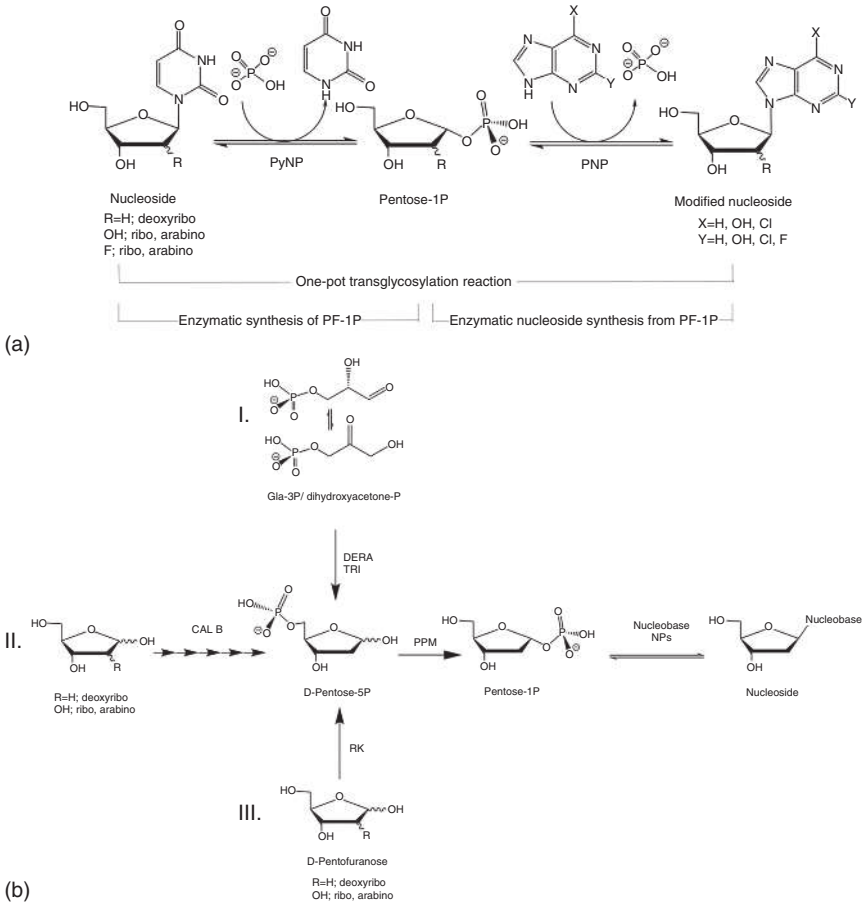

Figure 1.6 Approaches for the synthesis of nucleosides and nucleoside analogues. (a) One-pot transglycosylation reaction. (b(I)) Enzymatic synthesis starting from glyceraldehyde-3-phosphate. (b(II)) Chemo-enzymatic synthesis starting from deoxyribose, ribose, or arabinose. (b(III)) Enzymatic synthesis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

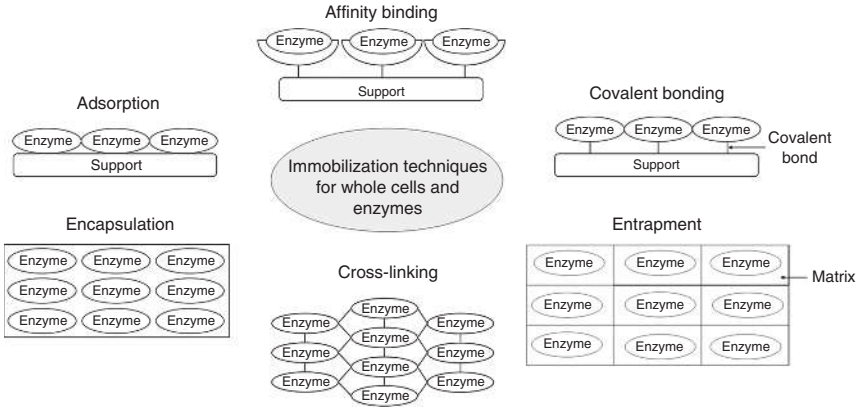

Figure 1.7 Immobilization techniques applied for whole cells and purified enzymes. Source: Adapted from Sirisha et al. 2016 [111] and Zhao et al. 2015 [115].

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]			Floxuridine [57, 126, 127]		
Cordycepin [34, 108]					
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.

References

1 Savić, D., Stanković, T., Lavrnja, I. et al. (2015). Purine nucleoside analogs in the therapy of cancer and neuroinflammation. *Mol. Inhib. Target Ther.* 1 (1): 3–14.

2 Jordheim, L.P., Durantel, D., Zoulim, F., and Dumontet, C. (2013). Advances in the development of nucleoside and nucleotide analogues for cancer and viral diseases. *Nat. Rev. Drug Discovery* 12 (6): 447–464.

3 Agris, P.F. (2015). The importance of being modified: an unrealized code to RNA structure and function. *RNA* 21 (4): 552–554.

4 Rozenski, J., Crain, P.F., and McCloskey, J.A. (1999). The RNA modification database: 1999 update. *Nucleic Acids Res.* 27 (1): 196–197.

5 Charette, M. and Gray, M.W. (2000). Pseudouridine in RNA: what, where, how, and why. *IUBMB Life (International Union Biochem Mol Biol Life)* 49 (5): 341–351.

6 Galmarini, C.M., Mackey, J.R., and Dumontet, C. (2002). Nucleoside analogues and nucleobases in cancer treatment. *Lancet Oncol.* 3 (7): 415–424.

7 De Clercq, E. (2016). Approved antiviral drugs over the past 50 years. *Clin. Microbiol. Rev.* 29 (3): 695–747.

8 Vande Voorde, J., Balzarini, J., and Liekens, S. (2014). An emerging understanding of the Janus face of the human microbiome: enhancement versus impairment of cancer therapy. *J. Antimicrob. Chemother.* 69 (10): 2878–2880.

9 Mikhailopulo, I.A. and Miroshnikov, A.I. (2013). Some recent findings in the biotechnology of biologically important nucleosides. *Biochem. Biotechnol. Mod. Med.* 328–353.

10 Levene, P.A. and Medigrecenau, F. (1911). On nucleases. *J. Biol. Chem.* 9 (5): 389–402.

11 Levene, P.A. and Medigrecenau, F. (1911). On nucleases. *J. Biol. Chem.* 9 (1): 65–83.

12 Jones, W. (1911). Concerning nucleases. *J. Biol. Chem.* 9 (2): 129–138.

13 Jones, W. (1911). On the physiological agents which are concerned in the nuclein fermentation, with special reference to four independent desamidases. *J. Biol. Chem.* 9 (2): 160–180.

14 Levene, P.A., Yamagawa, M., and Weber, I. (1924). On nucleosidases. I. General properties. *J. Biol. Chem.* 60 (3): 693–706.

15 Levene, P.A. and Weber, I. (1924). On nucleosidases. II. Purification of the enzyme. *J. Biol. Chem.* 60 (3): 707–715.

16 Levene, P.A. and Weber, I. (1924). On nucleosidases. III. The degree of specificity of nucleosidase and the distribution of it in various organs and in various species. *J. Biol. Chem.* 60 (3): 717–720.

17 Kalckar, H.M. (1947). The enzymatic synthesis of purine riboside. *J. Biol. Chem.* 167 (2): 477–486.

18 Manson, L.A. and Lampen, J.O. (1951). The metabolism of deoxyribose nucleosides in *Escherichia coli*. *J. Biol. Chem.* 193 (2): 539–547.

19 Yehia, H., Kamel, S., Paulick, K. et al. (2017). Substrate Spectra of Nucleoside Phosphorylases and their Potential in the Production of Pharmaceutically Active Compounds. *Curr. Pharm. Des.* 23 (45): 6913–6935.

20 Pugmire, M.J. and Ealick, S.E. (2002). Structural analyses reveal two distinct families of nucleoside phosphorylases. *Biochem. J* 361: 1–25.

21 Hori, N., Watanabe, M., Yamazaki, Y., and M. (1989). Purification and characterization of second thermostable purine nucleoside phosphorylase in *Bacillus stearothermophilus* JTS 859. *Agric. Biol. Chem.* 53 (12): 3219–3224.

22 Seeger, C., Poulsen, C., and Dandanell, G. (1995). Identification and characterization of genes (xapA, xapB, and xapR) involved in xanthosine catabolism in *Escherichia coli*. *J. Bacteriol.* 177 (19): 5506–5516.

23 Senesi, S., Falcone, G., Mura, U. et al. (1976). A specific adenosine phosphorylase, distinct from purine nucleoside phosphorylase. *FEBS Lett.* 64 (2): 1715–1722.

24 Bzowska, A., Kulikowska, E., and Shugar, D. (2000). Purine nucleoside phosphorylases: properties, functions, and clinical aspects. *Pharmacol. Ther.* 88 (3): 349–425.

25 Bzowska, A., Ananiev, A.V., Ramzaeva, N. et al. (1994). Purine nucleoside phosphorylase: inhibition by purine N(7)- and N(9)-acyclonucleosides; and substrate properties of 7-β-D-ribofuranosylguanine and 7-β-D-ribofuranosylhypoxanthine. *Biochem. Pharmacol.* 48 (5): 937–947.

26 Bzowska, A., Kulikowska, E., Poopeiko, N.E., and Shugar, D. (1996). Kinetics of phosphorolysis of 3-(β-d-ribofuranosyl)adenine and 3-(β-d-ribofuranosyl)hypoxanthine, non-conventional substrates of purine-nucleoside phosphorylase. *Eur. J. Biochem.* 239 (1): 229–234.

27 Wielgus-Kutrowska, B., Kulikowska, E., Wierzchowski, J. et al. (1997). Nicotinamide riboside, an unusual, non-typical, substrate of purified purine-nucleoside phosphorylases. *Eur. J. Biochem.* 243 (1–2): 408–414.

28 Furihata, T., Kishida, S., Sugiura, H. et al. (2014). Functional analysis of purine nucleoside phosphorylase as a key enzyme in ribavirin metabolism. *Drug Metab. Pharmacokinet.* 29 (2): 211–214.

29 Xie, X.X., Xia, J.G., He, K.F. et al. (2011). Low-molecular-mass purine nucleoside phosphorylase: characterization and application in enzymatic synthesis of nucleoside antiviral drugs. *Biotechnol. Lett* 33 (6): 1107–1112.

30 Zhou, X., Szeker, K., Janocha, B. et al. (2013). Recombinant purine nucleoside phosphorylases from thermophiles: preparation, properties and activity towards purine and pyrimidine nucleosides. *FEBS J* 280 (6): 1475–1490.

31 Almendros, M., Berenguer, J., and Sinisterra, J.-V. (2012). *Thermus thermophilus* nucleoside phosphorylases active in the synthesis of nucleoside analogues. *Appl. Environ. Microbiol.* 78 (9): 3128–3135.

32 Hassan, A.E., Abou-Elkhair, R.A., Riordan, J.M. et al. (2012). Synthesis and evaluation of the substrate activity of C-6 substituted purine ribosides with *E. coli* purine nucleoside phosphorylase: palladium mediated cross-coupling of organozinc halides with 6-chloropurine nucleosides. *Eur. J. Med. Chem.* 47 (1): 167–174.

33 Stoeckler, J.D., Cambor, C., and Parks, R.E. (1980). Human erythrocytic purine nucleoside phosphorylase: reaction with sugar-modified nucleoside substrates. *Biochemistry* 19 (1): 102–107.

34 Shirae, H. and Yokozeki, K. (1991). Purifications and properties of orotidine-phosphorolyzing enzyme and purine nucleoside phosphorylase from *Erwinia carotovora* AJ 2992. *Agric. Biol. Chem.* 55 (7): 1849–1857.

35 Nannemann, D.P., Kaufmann, K.W., Meiler, J., and Bachmann, B.O. (2010). Design and directed evolution of a dideoxy purine nucleoside phosphorylase. *Protein Eng. Des. Sel.* 23 (8): 607–616.

36 Cacciapuoti, G., Bertoldo, C., Brio, A. et al. (2003). Purification and characterization of 5′-methylthioadenosine phosphorylase from the hyperthermophilic archaeon *Pyrococcus furiosus*: substrate specificity and primary structure analysis. *Extremophiles* 7 (2): 159–168.

37 Cacciapuoti, G., Porcelli, M., Bertoldo, C. et al. (1994). Purification and characterization of extremely thermophilic and thermostable 5′-methylthioadenosine phosphorylase from the archaeon *Sulfolobus solfataricus*. Purine nucleoside phosphorylase activity and evidence for intersubunit disulfide bonds. *J. Biol. Chem.* 269 (40): 24762–24769.

38 Appleby, T.C., Mathews, I.I., Porcelli, M. et al. (2001). Three-dimensional structure of a hyperthermophilic 5′-deoxy-5′-methylthioadenosine phosphorylase from *Sulfolobus solfataricus*. *J. Biol. Chem.* 276 (42): 39232–39242.

39 Hassan, A.E., Abou-Elkhair, R.A., Parker, W.B. et al. (2016). 6-Methylpurine derived sugar modified nucleosides: synthesis and evaluation of their substrate activity with purine nucleoside phosphorylases. *Bioorg. Chem.* 65: 9–16.

40 Toorchen, D. and Miller, R.L. (1991). Purification and characterization of 5′-deoxy-5′-methylthioadenosine (MTA) phosphorylase from human liver. *Biochem. Pharmacol.* 41 (12): 2023–2030.

41 Fabianowska-Majewska, K., Duley, J., Fairbanks, L. et al. (1994). Substrate specificity of methylthioadenosine phosphorylase from human liver. *Acta Biochim. Pol.* 41 (4): 391–395.

42 Ghoda, L.Y., Savarese, T.M., Northup, C.H. et al. (1988). Substrate specificities of 5′-deoxy-5′-methylthioadenosine phosphorylase from Trypanosoma brucei brucei and mammalian cells. *Mol. Biochem. Parasitol.* 27 (2–3): 109–118.

43 Bacchi, C.J., Goldberg, B., Rattendi, D. et al. (1999). Metabolic effects of a methylthioadenosine phosphorylase substrate analog on *African trypanosomes*. *Biochem. Pharmacol.* 57 (1): 89–96.

44 Zhou, M., Pugmire, M.J., Vuong, B.Q., and Ealick, S.E. (1999). Cloning, expression and crystallization of pyrimidine nucleoside phosphorylase from *Bacillus stearothermophilus*. *Acta Crystallogr. D Biol. Crystallogr.* 55 (Pt 1: 287–290.

45 Saunders, P.P. and Wilson, B.A. (1969). Purification and comparative properties of a pyrimidine nucleoside phosphorylase from *Bacillus stearothermophilus*. *J. Biol. Chem.* 244 (13): 3691–3697.

46 Kono, A., Hara, Y., Sugata, S. et al. (1984). Substrate specificity of a thymidine phosphorylase in human liver tumor. *Chem. Pharm. Bull.* 32 (5): 1919–1921.

47 Schinazi, R.F., Peck, A., and Sommadossi, J.-P. (1992). Substrate specificity of *Escherichia coli* thymidine phosphorylase for pyrimidine nucleosides with anti-human immunodeficiency virus activity. *Biochem. Pharmacol.* 44 (2): 199–204.

48 Panova, N.G., Alexeev, C.S., Kuzmichov, A.S. et al. (2007). Substrate specificity of *Escherichia coli* thymidine phosphorylase. *Biochemistry (Mosc)* 72 (1): 21–28.

49 Hatano, A., Harano, A., Takigawa, Y. et al. (2008). Kinetic parameters and recognition of thymidine analogues with varying functional groups by thymidine phosphorylase. *Bioorg. Med. Chem.* 16: 3866–3870.

50 Panova, N., Kosiova, I., Petrova, M. et al. (2008). Nucleoside phosphonic acids in thymidine phosphorylase inhibition: structure–activity relationship. *Nucleic Acids Symp. Ser.* 52 (1): 665–666.

51 Avraham, Y., Grossowicz, N., and Yashphe, J. (1990). Purification and characterization of uridine and thymidine phosphorylase from Lactobacillus casei. *Biochim. Biophys. Acta* 1040: 287–293.

52 Gao, X.F., Huang, X.R., and Sun, C.C. (2006). Role of each residue in catalysis in the active site of pyrimidine nucleoside phosphorylase from *Bacillus subtilis*: a hybrid QM/MM study. *J. Struct. Biol.* 154 (1): 20–26.

53 Leer, J.C., Hammer-Jespersen, K., and Schwartz, M. (1977). Uridine phosphorylase from *Escherichia coli*. Physical and chemical characterization. *Eur. J. Biochem.* 75: 217–224.

54 Szeker, K., Zhou, X., Schwab, T. et al. (2012). Comparative investigations on thermostable pyrimidine nucleoside phosphorylases from *Geobacillus thermoglucosidasius* and *Thermus thermophilus*. *J. Mol. Catal. B: Enzym.* 84: 27–34.

55 Voorde, J.V., Gago, F., Vrancken, K. et al. (2012). Characterization of pyrimidine nucleoside phosphorylase of *Mycoplasma hyorhinis*: implications for the clinical efficacy of nucleoside analogues. *Biochem. J* 445 (1): 113–123.

56 Scocca, J.J. (1971). Purification and substrate from specificity of pyrimidine nucleoside phosphorylase from *Haemophilus influenzae*. *J. Biol. Chem.* 246 (21): 6606–6610.

57 Serra, I., Bavaro, T., Cecchini, D.A. et al. (2013). A comparison between immobilized pyrimidine nucleoside phosphorylase from *Bacillus subtilis* and thymidine phosphorylase from *Escherichia coli* in the synthesis of 5-substituted pyrimidine 2-deoxyribonucleosides. *J. Mol. Catal. B: Enzym.* 95: 16–22.

58 Hamamoto, T., Noguchi, T., and Midorikawa, Y. (1996). Purification and characterization of purine nucleoside phosphorylase and pyrimidine nucleoside phosphorylase from *Bacillus stearothermophilus* TH 6-2. *Biosci. Biotechnol., Biochem.* 60 (7): 1179–1180.

59 Dandanell, G., Szczepanowski, R.H., Kierdaszuk, B. et al. (2005). *Escherichia coli* purine nucleoside phosphorylase II, the product of the xapA gene. *J. Mol. Biol.* 348 (1): 113–125.

60 Erion, M.D., Stoeckler, J.D., Guida, W.C. et al. (1997). Purine nucleoside phosphorylase. 2. Catalytic mechanism. *Biochemistry* 36 (39): 11735–11748.

61 Mao, C., Cook, W.J., Zhou, M. et al. (1998). Calf spleen purine nucleoside phosphorylase complexed with substrates and substrate analogues. *Biochemistry* 37 (20): 7135–7146.

62 Larson, E.T., Mudeppa, D.G., Gillespie, J.R. et al. (2010). The crystal structure and activity of a putative trypanosomal nucleoside phosphorylase reveal it to be a homodimeric uridine phosphorylase. *J. Mol. Biol.* 396: 1244–1259.

63 Balaev, V.V., Lashkov, A.A., Gabdulkhakov, A.G. et al. (2016). Structural investigation of the thymidine phosphorylase from *Salmonella typhimurium* in the unliganded state and its complexes with thymidine and uridine. *Acta Crystallogr. F Struct. Biol. Commun.* 72 (3): 224–233.

64 Friedkin, M. and Roberts, D. (1954). The enzymatic synthesis of nucleosides. I. Thymidine phosphorylase in mammalian tissue. *J. Biol. Chem.* 207 (1): 245–256.

65 Friedkin, M. and Roberts, D. (1954). The enzymatic synthesis of nucleosides. II. Thymidine and related pyrimidine nucleosides. *J. Biol. Chem.* 207 (1): 257–266.

66 Mikhailopulo, I.a. and Miroshnikov, A.I. (2011). Biologically important nucleosides: modern trends in biotechnology and application. *Mendeleev Commun.* 21 (2): 57–68.

67 Mikhailopulo, I.A. (2007). Biotechnology of nucleic acid constituents-state of the art and perspectives. *Curr. Org. Chem.* 11 (4): 317–335.

68 Mikhailopulo, I.A. and Miroshnikov, A.I. (2010). New trends in nucleoside biotechnology. *Acta Naturae* 2 (2): 36–59.

69 MacDonald, D. (1986). Separation of the anomers of glycosyl phosphates. *Carbohydr. Res.* 6: 376–381.

70 MacDonald, D. (1962). A new route to glycosyl phosphates. *J. Org. Chem.* 27 (3): 1107–1109.

71 MacDonald, D.L. (1966). Preparation of glycosyl phosphates: beta-D-glucopyranosyl phosphate. *Carbohydr. Res.* 3: 117–120.

72 Yamada, K., Matsumoto, N., and Hayakawa, H. (2009). Stereoselective synthesis of 2-deoxy-2-fluoroarabinofuranosyl-α-1-phosphate and its application to the synthesis of 2′-deoxy-2′-fluoroarabinofuranosyl purine nucleosides by a chemo-enzymatic method. *Nucleosides Nucleotides Nucleic Acids* 28 (11–12): 1117–1130.

73 Timmons, S.C. and Jakeman, D.L. (2008). Stereospecific synthesis of sugar-1-phosphates and their conversion to sugar nucleotides. *Carbohydr. Res.* 343 (5): 865–874.

74 Mikhailopulo, I.A. and Cramer, F. (1982). Conformation of N(9)-(β-D-arabinofuranosyl) adenine 5′-monophosphate (ara-AMP) in anhydrous dimethylsulphoxide monitored by 13C NMR. *FEBS Lett.* 144 (1): 109–111.

75 Ouwerkerk, N., Steenweg, M., De Ruijter, M. et al. (2002). One-pot two-step enzymatic coupling of pyrimidine bases to 2-deoxy-D-ribose-5-phosphate. A new strategy in the synthesis of stable isotope labeled deoxynucleosides. *J. Org. Chem.* 67 (5): 1480–1489.

76 Ouwerkerk, N., van Boom, J.H., Lugtenburg, J., and Raap, J. (2000). Chemo-enzymatic synthesis of thymidine 13C-labelled in the 2′-deoxyribose moiety. *Eur. J. Org. Chem.* 861–866.

77 Taverna-Porro, M., Bouvier, L.A., Pereira, C.A. et al. (2008). Chemoenzymatic preparation of nucleosides from furanoses. *Tetrahedron Lett.* 49 (16): 2642–2645.

78 Miroshnikov, A.I., Esipov, R.S., Muravyova, T.I. et al. (2010). A new strategy for the synthesis of nucleosides: one-pot enzymatic transformation of D-pentoses into nucleosides. *Open Conf. Proc. J.* 1 (1): 98–102.

79 Kamel, S., Weiss, M., Klare, H.F.T. et al. (2018). Chemo-enzymatic production of α-D-pentofuranose-1-phosphates using thermostable pyrimidine nucleoside phosphorylases. *Mol. Catal.* 458: 52–59.

80 Kalckar, H.M. (1945). Enzymatic synthesis of a nucleoside. *J. Biol. Chem.* 158 (3): 723–724.

81 Nobile, M., Terreni, M., Lewkowicz, E., and Irabarren, A.M.I. (2010). Aeromonas hydrophila strains as biocatalysts for transglycosylation. *Biocatal. Biotransform.* 28 (5–6): 395–402.

82 Bestetti, G., Cali', S., Ghisotti, D., et al. (2000). Recombinant bacterial strains for the production of natural nucleosides and modified analogues thereof. WO2000039307 A2, filed 23 December 1999 and issued 6 July 2000.

83 Rogert, M.C., Trelles, J.A., Porro, S. et al. (2002). Microbial synthesis of antiviral nucleosides using *Escherichia coli* BL21 as biocatalyst. *Biocatal. Biotransform.* 20 (5): 347–351.

84 Utagawa, T. (1999). Enzymatic preparation of nucleoside antibiotics. *J. Mol. Catal. B: Enzym.* 6 (3): 215–222.

85 Trelles, J.A., Fernández, M., Lewkowicz, E.S. et al. (2003). Purine nucleoside synthesis from uridine using immobilised *Enterobacter gergoviae* CECT 875 whole cells. *Tetrahedron Lett.* 44 (12): 2605–2609.

86 Luo, W., Liu, Y., Zhu, X. et al. (2011). Cloning and characterization of purine nucleoside phosphorylase in *Escherichia coli* and subsequent ribavirin biosynthesis using immobilized recombinant cells. *Enzyme Microb. Technol.* 48 (6–7): 438–444.

87 Carballeira, J.D., Fernandez-Lucas, J., Quezada, M.A. et al. (2009). Biotransformations. In: *Encyclopedia of Microbiology*, 212–251. Elsevier.

88 Pal, S. and Nair, V. (1997). Enzymatic synthesis of thymidine using bacterial whole cells and isolated purine nucleoside phosphorylase. *Biocatal. Biotransform.* 15 (2): 147–158.

89 Wei, X., Ding, Q., Zhang, L. et al. (2008). Induction of nucleoside phosphorylase in *Enterobacter aerogenes* and enzymatic synthesis of adenine arabinoside. *J. Zhejiang Univ. Sci. B* 9 (7): 520–526.

90 Lee, S.H. and Sartorelli, A.C. (1981). Conversion of 6-thioguanine to the nucleoside level by purine nucleoside phosphorylase of sarcoma 180 and sarcoma 180/TG ascites cells. *Cancer Res* 41 (3): 1086–1090.

91 Singh, P.P., Joshi, S., Russell, P.J. et al. (2011). Purine nucleoside phosphorylase mediated molecular chemotherapy and conventional chemotherapy: a tangible union against chemoresistant cancer. *BMC Cancer* 11 (1): 368.

92 Bentancor, L., Trelles, J.A., Nóbile, M. et al. (2004). Benzimidazole as deazapurine analogue for microbial transglycosylation. *J. Mol. Catal. B: Enzym.* 29 (1): 3–7.

93 Quintiliani, M., Balzarini, J., and McGuigan, C. (2013). Design, synthesis, and biological evaluation of C1-phosphonamidate analogues of 2-deoxy-D-ribose-1-phosphate. *Tetrahedron* 69 (43): 9111–9119.

94 Rubira, M.-J., Jimeno, M.-L., Balzarini, J. et al. (1998). Synthesis and NMR conformational studies of stable analogues of 2-deoxy-alfa-D-ribose-1-phosphate. *Tetrahedron* 54: 8223–8240.

95 Kamel, S., Weiss, M., Klare, H.F.T. et al. (2018). Chemo-enzymatic production of α-D-pentofuranose-1-phosphates using thermostable pyrimidine nucleoside phosphorylases. *Mol. Catal.* 458: 52–59.

96 Barai, V.N., Zinchenko, A.I., Eroshevskaya, L.A. et al. (2002). A universal biocatalyst for the preparation of base- and sugar-modified nucleosides via an enzymatic transglycosylation. *Helv. Chim. Acta* 85 (7): 1901–1908.

97 Zhou, X., Szeker, K., Jiao, L.-Y. et al. (2015). Synthesis of 2,6-dihalogenated purine nucleosides by thermostable nucleoside phosphorylases. *Adv. Synth. Catal.* 357 (6): 1237–1244.

98 Hanrahan, J.R. and Hutchinson, D.W. (1992). The enzymatic synthesis of antiviral agents. *J. Biotechnol.* 23 (2): 193–210.

99 Tono, H. and Cohen, S.S. (1962). The activity of nucleoside phosphorylase on 1-β-D-arabinosyluracil within *Escherichia coli*. *J. Biol. Chem.* 237 (4): 1271–1282.

100 Esipov, R.S., Gurevich, A.I., Chuvikovsky, D.V. et al. (2002). Overexpression of *Escherichia coli* genes encoding nucleoside phosphorylases in the pET/Bl21 (DE3) system yields active recombinant enzymes. *Protein Expr. Purif.* 24 (1): 56–60.

101 Hori, N., Watanabe, M., Sunagawa, K. et al. (1991). Production of 5-methyluridine by immobilized thermostable purine nucleoside phosphorylase and pyrimidine nucleoside phosphorylase from *Bacillus stearothermophilus* JTS 859. *J. Biotechnol.* 17 (2): 121–131.

102 Lewkowicz, E.S., Martínez, N., Rogert, M.C. et al. (2000). An improved microbial synthesis of purine nucleosides. *Biotechnol. Lett* 22: 1277–1280.

103 Trelles, J.A., Bentancor, L., Schoijet, A. et al. (2004). Immobilized *Escherichia coli* BL21 as a catalyst for the synthesis of adenine and hypoxanthine nucleosides. *Chem. Biodivers.* 1 (2): 280–288.

104 Krenitsky, T.A., Rideout, J.L., Koszalka, G.W. et al. (1982). Pyrazolo[3,4-d]pyrimidine ribonucleosides as anticoccidials. 1. Synthesis and activity of some nucleosides of purines and 4-(alkylthio)pyrazolo[3,4-d]pyrimidines. *J. Med. Chem.* 25 (1): 32–35.

105 Krenitsky, T.A., Koszalka, G.W., and Tuttle, J.V. (1981). Purine nucleoside synthesis: an efficient method employing nucleoside phosphorylases. *Biochemistry* 20 (12): 3615–3621.

106 Condezo, L.A., Fernandez-Lucas, J., Garcia-Burgos, C.A. et al. (2006). Enzymatic synthesis of modified nucleosides. In: *Biocatalysis in the Pharmaceutical and Biotechnology Industries* (ed. R. Patel), 401–424. CRC Press.

107 Vieille, C. and Zeikus, G.J. (2001). Hyperthermophilic enzymes: sources, uses, and molecular mechanisms for thermostability. *Microbiol. Mol. Biol. Rev.* 65 (1): 1–43.

108 Utagawa, T., Morisawa, H., Yamanaka, S. et al. (1985). Properties of nucleoside phosphorylase from *Enterobacter aerogenes*. *Agric. Biol. Chem.* 49 (11): 3239–3246.

109 Zuffi, G., Ghisotti, D., Oliva, I. et al. (2004). Immobilized biocatalysts for the production of nucleosides and nucleoside analogues by enzymatic transglycosylation reactions. *Biocatal. Biotransform.* 22 (1): 25–33.

110 Zhou, X., Mikhailopulo, I.A., Cruz Bournazou, M.N., and Neubauer, P. (2015). Immobilization of thermostable nucleoside phosphorylases on MagReSyn epoxide microspheres and their application for the synthesis of 2,6-dihalogenated purine nucleosides. *J. Mol. Catal. B: Enzym.* 115: 119–127.

111 Sirisha, V.L., Jain, A., and Jain, A. (2016). Enzyme immobilization: an overview on methods, support material, and applications of immobilized enzymes. *Adv. Food Nutr. Res.* 79: 179–211.

112 Brady, D. and Jordaan, J. (2009). Advances in enzyme immobilisation. *Biotechnol. Lett* 31 (11): 1639–1650.

113 Bickerstaff, G.F. (1997). Immobilization of enzymes and cells: some practical considerations. In: *Immobilization of Enzymes and Cell* (ed. G.F. Bickerstaff), 1–11. Totowa, NJ: Humana Press.

114 Mazid, M.A. (1993). Biocatalysis and immobilized enzyme/cell bioreactors. *Nat. Biotechnol.* 11 (6): 690–695.

115 Zhao, X., Qi, F., Yuan, C. et al. (2015). Lipase-catalyzed process for biodiesel production: enzyme immobilization, process simulation and optimization. *Renewable Sustainable Energy Rev.* 44 (Supplement C): 182–197.

116 Trelles, J.A., Fernández-Lucas, J., Condezo, L.A., and Sinisterra, J.V. (2004). Nucleoside synthesis by immobilised bacterial whole cells. *J. Mol. Catal. B: Enzym.* 30 (5–6): 219–227.

117 Rocchietti, S., Ubiali, D., Terreni, M. et al. (2004). Immobilization and stabilization of recombinant multimeric uridine and purine nucleoside phosphorylases from *Bacillus subtilis*. *Biomacromolecules* 5 (6): 2195–2200.

118 Fernández-Lafuente, R., Hernández-Justiz, O., Mateo, C. et al. (2001). Biotransformations catalyzed by multimeric enzymes: stabilization of tetrameric ampicillin acylase permits the optimization of ampicillin synthesis under dissociation conditions. *Biomacromolecules* 2 (1): 95–104.

119 Serra, I., Serra, C.D., Rocchietti, S. et al. (2011). Stabilization of thymidine phosphorylase from *Escherichia coli* by immobilization and post immobilization techniques. *Enzyme Microb. Technol.* 49 (1): 52–58.

120 Serra, I., Daly, S., Alcantara, A.R. et al. (2015). Redesigning the synthesis of vidarabine via a multienzymatic reaction catalyzed by immobilized nucleoside phosphorylases. *RSC Adv.* 5 (30): 23569–23577.

121 Calleri, E., Ubiali, D., Serra, I. et al. (2014). Immobilized purine nucleoside phosphorylase from *Aeromonas hydrophila* as an on-line enzyme reactor for biocatalytic applications. *J. Chromatogr. B* 968: 79–86.

122 Calleri, E., Cattaneo, G., Rabuffetti, M. et al. (2015). Flow-Synthesis of nucleosides catalyzed by an immobilized purine nucleoside phosphorylase from *Aeromonas hydrophila*: integrated systems of reaction control and product purification. *Adv. Synth. Catal.* 357 (11): 2520–2528.

123 Chen, N., Xing, C.G., Xie, X.X., and Xu, Q.Y. (2009). Optimization of technical conditions of producing ribavirin by *Bacillus subtilis*. *Ann. Microbiol.* 59 (3): 525–530.

124 Ma, Y., Yang, S., Liu, L. et al. (2014). Fermentative production of ribavirin by overexpressing purine nucleoside phosphorylase in Bacillus. *J. Chem. Pharm. Res.* 6 (5): 1377–1384.

125 Sakharov, V., Baykov, S., Konstantinova, I. et al. (2015). An efficient chemoenzymatic process for preparation of ribavirin. *Int. J. Chem. Eng.* 2015: 5, 734851.

126 el Kouni, M.H., el Kouni, M.M., and Naguib, F.N.M. (1993). Differences in activities and substrate specificity of human and murine pyrimidine nucleoside phosphorylases: implications for chemotherapy with 5-fluoropyrimidines. *Cancer Res.* 53 (16): 3687–3693.

127 Desgranges, C., Razaka, G., Rabaud, M., and Bricaud, H. (1981). Catabolism of thymidine in human blood platelets purification and properties of thymidine phosphorylase. *Biochem. Biophys. Acta* 654: 211–218.

128 Fateev, I.V., Antonov, K.V., Konstantinova, I.D. et al. (2014). The chemoenzymatic synthesis of clofarabine and related 2′-deoxyfluoroarabinosyl nucleosides: the electronic and stereochemical factors determining substrate recognition by *E. coli* nucleoside phosphorylases. *Beilstein J. Org. Chem.* 10 (1): 1657–1669.

129 Blank, J.G. and Hoffee, P.A. (1975). Purification and properties of thymidine phosphorylase from *Salmonella typhimurium*. *Arch. Biochem. Biophys.* 265: 259–265.

130 Hoffee, P.A. and Blank, J. (1978). Thymidine phosphoryalse from *Salmonella typhimurium*. *Methods Enzymol.* 437–442.

131 Averett, D.R., Koszalka, G.W., Fyfe, J.A. et al. (1991). 6-Methoxypurine arabinoside as a selective and potent inhibitor of varicella-zoster virus. *Antimicrob. Agents Chemother.* 35 (5): 851–857.

132 Focher, F. and Spadari, S. (2001). Thymidine phosphorylase: a two-face Janus in anticancer chemotherapy. *Curr. Cancer Drug Targets* 141–153.

133 Panova, N.G., Alexeev, C.S., Polyakov, K.M. et al. (2008). Substrate specificity of thymidine phosphorylase of *E. coli*: role of hydroxyl groups. *Nucleosides Nucleotides Nucleic Acids* 27 (12): 1211–1214.

134 Vande Voorde, J., Liekens, S., and Balzarini, J. (2013). *Mycoplasma hyorhinis*–encoded purine nucleoside phosphorylase: kinetic properties and its effect on the cytostatic potential of purine-based anticancer drugs. *Mol. Pharmacol.* 84 (6): 865–875.

135 Fateev, I.V., Kharitonova, M.I., Antonov, K.V. et al. (2015). Recognition of artificial nucleobases by *E. coli* purine nucleoside phosphorylase versus its Ser90Ala mutant in the synthesis of base-modified nucleosides. *Chem. Eur. J.* 21 (38): 13401–13419.

136 Mikhailopulo, A. and Miroshnikov, A.I. (2013). Some recent findings in the biotechnology of biologically important nucleosides. *Biotechnol. Acta* 6 (4): 63–77.

137 Shirae, H. and Yokozeki, K. (1991). Purification and properties of purine nucleoside phosphorylase from *Brevibacterium acetylicum* ATCC 954. *Agric. Biol. Chem.* 55 (2): 493–499.

Paper III

Heba Yehia, Sarah Kamel, Peter Neubauer, Anke Wagner. Glycosylation with α-D-pentofuranose-1-phosphates leads to higher product yields for nucleoside analogs compared to transglycosylation reactions. (In preparation).

III

Glycosylation with α-D-pentofuranose-1-phosphates leads to higher product yields for nucleoside analogs 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. No product was obtained for (2′S)-2′-deoxy-2′-fluoro-5-ethynyluridine. Therefore, an alternative approach was used to produce derivative of 5-ethynyl-2′-deoxyuridine 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 successfully shown. 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. Product yields increased by a factor of 1.5 and 2.5 for 5-ethynyl-2′-deoxyuridine and 2′-fluoro-5-ethynyluridine compared to transglycosylation reactions.

Keywords:

EdU, ^{2F}Ara-EdU, $_{2F}$Rib-EdU, thermostable nucleoside phosphorylase, transglycosylation, direct glycosylation.

List of abbreviations:

NA: nucleoside analogue; BrdU: 5-bromo-2′-deoxyuridine; CuAAZ: copper(I)-catalyzed azide-alkyne cycloaddition; EdU: 5-ethynyl-2′-deoxyuridine; ^{2F}Ara-EdU: (2′S)-2′-deoxy-2′-fluoro-5-ethynyluridine; $_{2F}$Rib-EdU: 2′-deoxy-2′-fluoro-5-ethynyluridine; NP: Nucleoside phosphorylase; Pentose-1P(s): α-D-pentofuranose-1-phosphate(s); dRib-1P: 2-deoxy-α-D-ribofuranose-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; Thd: thymidine; dAo: 2′-deoxyadenosine; ^{2F}Ara-U: 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)uracil; $_{2F}$Rib-U: 2′-deoxy-2′-fluorouridine; PyNP: pyrimidine nucleoside phosphorylase; PNP: purine nucleoside phosphorylase; KP: potassium phosphate buffer; Pi: inorganic phosphate; Pentose-1P-Ba: barium salt of α-D-pentofuranose-1-phosphates; FISH: Fluorescence in situ hybridization; CldU: 5-chloro-2'-deoxyuridine; IdU: 5-iodo-2′-deoxyuridine.

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 biomolecule 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 stereoselective only 1,4-disubstituted isomer 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), were previously reported to be 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 procedure [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 reaction were extensively studied as an efficient method for the synthesis of nucleosides and their analogues [4,18–20]. It is 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, 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 was efficient for the synthesis of EdU, only few or no product was observed for $_{2F}$Rib-EdU and ^{2F}Ara-EdU. 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. Direct glycosylation showed improved yields for the three nucleoside analogues of interest with product yields of 47 to 60%.

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 nucleoside in a transglycosylation reaction

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 – 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 and $_{2F}$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.

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

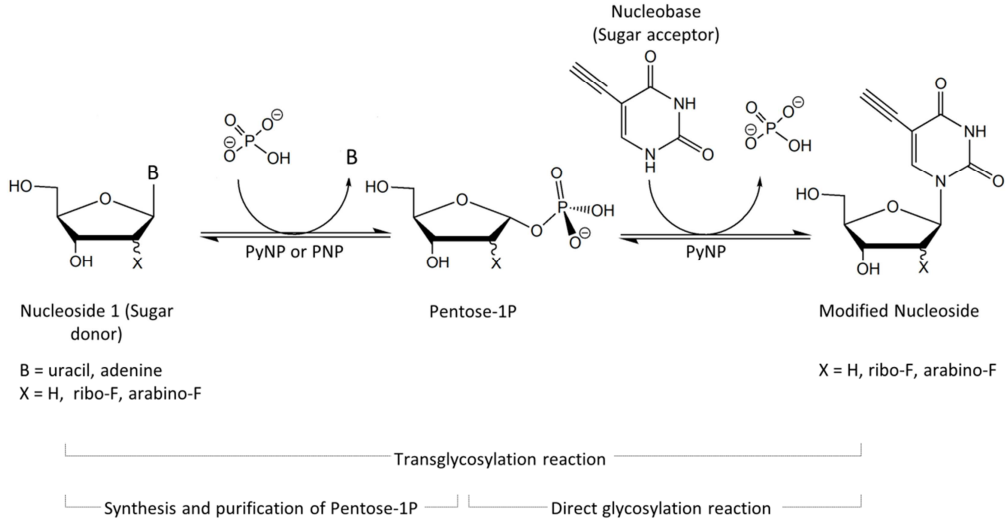
Thin layer chromatography (TLC)

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 the dRib-1P was detected using *p*-anisaldehyde-sulphoric acid solution.

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.



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-1P that were produced in a chemo-enzymatic process beforehand.

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 is 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.

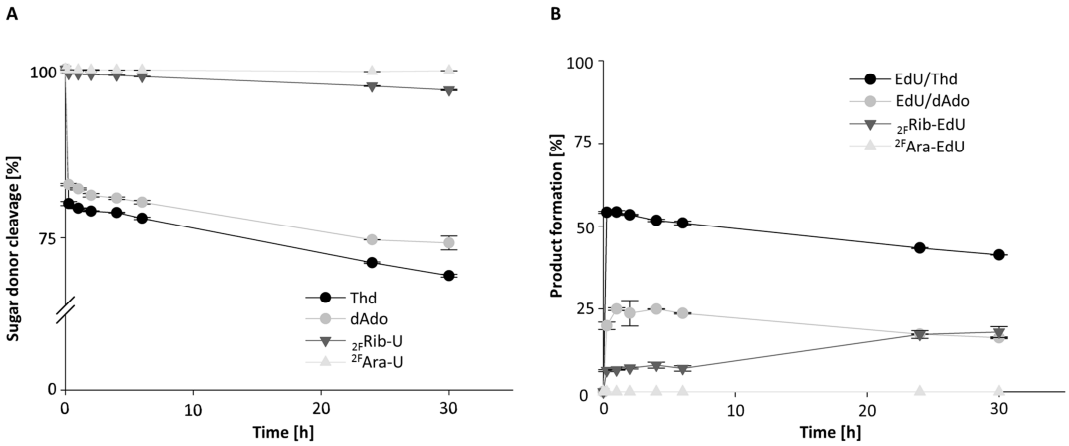


Figure 1. Enzymatic synthesis of 5-ethynyl-deoxyuridine (●, ●), 2′-fluoro-5-ethynyluridine (▼) and (2′S)-2′-deoxy-2′-fluoro-5-ethynyluridine (▲) in one-pot transglycosylation reactions using thermostable nucleoside phosphorylase. 5-Ethynyl-deoxyuridine was produced either with Thd (●) or dAdo (●) as 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.

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 magnesia 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.

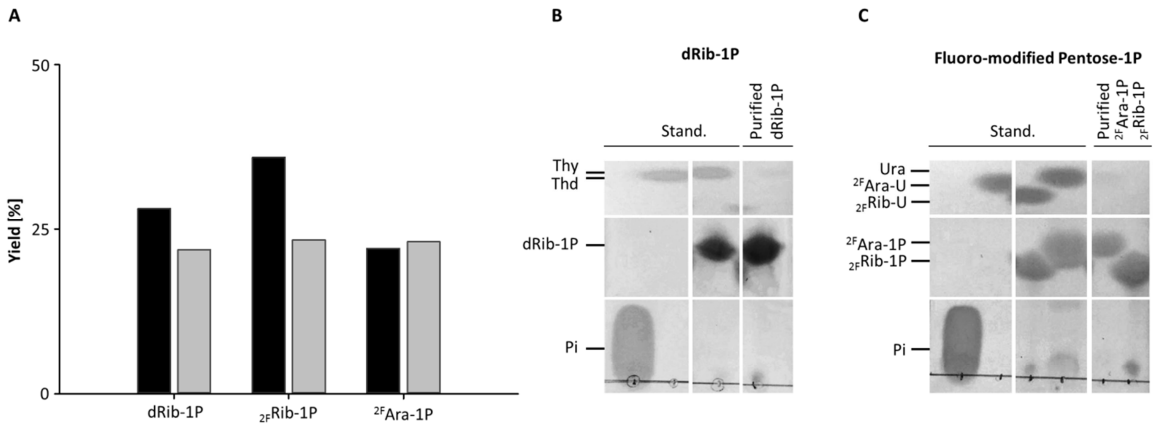


Figure 2. Enzymatic synthesis of natural and modified Pentose-1P. (A) Product yield of Pentose-1Ps after the enzymatic reaction (black bars) and the purified Pentose-1P as Ba salts (grey bars). (B) Assessment of the purity of the synthesized dRib-1P-Ba salts by thin layer chromatography (TLC). (C) Assessment 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 3h. Varying enzyme concentrations did not significantly influence the final product yields.

As higher product yields were obtained with the 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. 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 folds for $_{2F}$Rib-EdU and EdU respectively.

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].

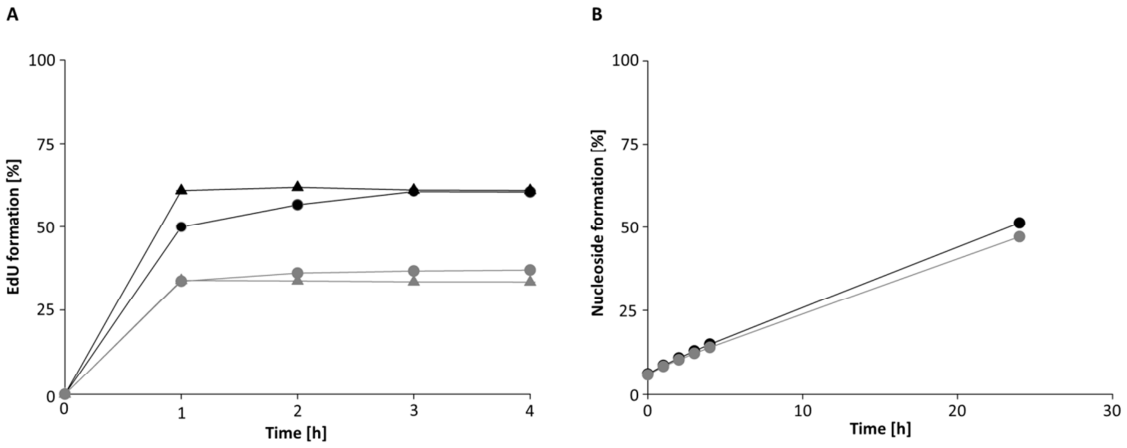


Figure 3. Enzymatic synthesis of 5-ethynyl-containing nucleoside analogues by direct glycosylation reactions using thermostable nucleoside phosphorylase PyNP-Y04. (A) EdU synthesis [%] was performed with 8mM 5-ethynyluracil as a sugar acceptor. dRib-1P concentrations of 8 mM (●,▲) and 16 mM (●,▲) and 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.

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].

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 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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References

[1] L.P. Jordheim, D. Durantel, F. Zoulim, C. Dumontet, Advances in the development of nucleoside and nucleotide analogues for cancer and viral diseases., Nat Rev Drug Discov. 12 (2013) 447–464.

[2] K.L. Seley-Radtke, M.K. Yates, The evolution of nucleoside analogue antivirals: A review for chemists and non-chemists. Part 1: Early structural modifications to the nucleoside scaffold, Antiviral Res. 154 (2018) 66–86.

[3] E. De Clercq, Approved Antiviral Drugs over the Past 50 Years, Clin Microbiol Rev. 29 (2016) 695–747.

[4] I.A. Mikhailopulo, A.I. Miroshnikov, New trends in nucleoside biotechnology, Acta Naturae. 2

(2010) 36–59.

[5] A. Gbaj, E. V. Bichenkova, L. Walsh, H.E. Savage, A.R. Sardarian, L.L. Etchells, A. Gulati, S. Hawisa, K.T. Douglas, New concepts of fluorescent probes for specific detection of DNA sequences: Bis-modified oligonucleotides in excimer and exciplex detection, Libyan J Med. 4 (2009) 152–159.

[6] S. Kumar, A. Sood, J. Wegener, P.J. Finn, S. Nampalli, J.R. Nelson, A. Sekher, P. Mitsis, J. Macklin, C.W. Fuller, Terminal phosphate labeled nucleotides: Synthesis, applications, and linker effect on incorporation by DNA polymerases, Nucleosides, Nucleotides and Nucleic Acids. 24 (2005) 401–408.

[7] K. M, T. Y, N. S, Application of bromodeoxyuridine (BrdU) and anti-BrdU monoclonal antibody for the analysis of tumor cell kinetics by flow cytometry, Nippon Rinsho Japanese J Clin Med. 50 (1992) 2333–2337.

[8] R.C. Leif, J.H. Stein, R.M. Zucker, A short history of the initial application of anti-5-BrdU to the detection and measurement of S phase, Cytometry. 58A (2004) 45–52.

[9] P. Taupin, BrdU immunohistochemistry for studying adult neurogenesis: Paradigms, pitfalls, limitations, and validation, Brain Res Rev. 53 (2007) 198–214.

[10] A.B. Neef, N.W. Luedtke, Dynamic metabolic labeling of DNA in vivo with arabinosyl nucleosides, 2011.

[11] L. Liang, D. Astruc, The copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) “click” reaction and its applications. An overview, Coord Chem Rev. 255 (2011) 2933–2945.

[12] R. Mehta, P. Singhal, H. Singh, D. Damle, A.K. Sharma, Insight into thermophiles and their wide-spectrum applications, 3 Biotech. 6 (2016) 1–9.

[13] A. Salic, T.J. Mitchison, A chemical method for fast and sensitive detection of DNA synthesis in vivo., Proc Natl Acad Sci U S A. 105 (2008) 2415–2420.

[14] U. Rieder, N.W. Luedtke, Alkene-tetrazine ligation for imaging cellular DNA, Angew Chemie - Int Ed. 53 (2014) 9168–9172.

[15] A.B. Neef, N.W. Luedtke, An azide-modified nucleoside for metabolic labeling of DNA, ChemBioChem. 15 (2014) 789–793.

[16] Y.F. Shealy, C.A. O’Dell, G. Arnett, W.M. Shannon, Synthesis and Antiviral Activity of the Carbocyclic Analogues of 5-Ethyl-2’-deoxyuridine and of 5-Ethynyl-2‘-deoxyuridine, J Med Chem. 29 (1986) 79–84.

[17] D. Qu, G. Wang, Z. Wang, L. Zhou, W. Chi, S. Cong, X. Ren, P. Liang, B. Zhang, 5-Ethynyl-2’-deoxycytidine as a new agent for DNA labeling: Detection of proliferating cells, Anal Biochem. 417 (2011) 112–121.

[18] I.A. Mikhailopulo, A.I. Miroshnikov, Some recent findings in the biotechnology of biologically important nucleosides, Biotechnol Acta. 6 (2013) 328–353.

[19] S. Kamel, H. Yehia, P. Neubauer, A. Wagner, Enzymatic synthesis of nucleoside analogues by nucleoside phosphorylases, in: J. Fernández-Lucas, M.-J. Camarasa (Eds.), Enzym Chem Synth Nucleic Acid Deriv, Wiley-VCH, Weinheim, 2018: pp. 1–28.

[20] M.J. Lapponi, C.W. Rivero, M.A. Zinni, C.N. Britos, J.A. Trelles, New developments in nucleoside analogues biosynthesis: A review, J Mol Catal B Enzym. 133 (2016) 218–233.

[21] A.I. Miroshnikov, R.S. Esipov, T.I. Muravyova, I.D. Konstantinova, I. V. Fateev, I.A. Mikhailopulo, A New strategy for the synthesis of nucleosides: one-pot enzymatic transformation of D-pentoses into nucleosides, Open Conf Proc J. 1 (2010) 98–102.

[22] S. Kamel, M. Weiss, H.F.T. Klare, I.A. Mikhailopulo, P. Neubauer, A. Wagner, Chemo-enzymatic production of α-D-pentofuranose-1-phosphates using thermostable pyrimidine nucleoside phosphorylases, Mol Catal. 458 (2018) 52–59.

[23] M. Drenichev, C. Alexeev, N. Kurochkin, S. Mikhailov, Use of Nucleoside Phosphorylases for the Preparation of Purine and Pyrimidine 2'-Deoxynucleosides.pdf, Adv Synth Catal. 360 (2018) 305–312.

[24] H. Tono, S.S. Cohen, The Activity of Nucleoside Phosphorylase on 1-β-d-Arabinosyluracil within Escherichia coli, J Biol Chem. 237 (1962) 1271–1282.

[25] M. Taverna-Porro, L.A. Bouvier, C.A. Pereira, J.M. Montserrat, A.M. Iribarren, Chemoenzymatic preparation of nucleosides from furanoses, Tetrahedron Lett. 49 (2008) 2642–2645.

[26] T. Utagawa, H. Morisawa, S. Yamanaka, A. Yamazaki, F. Yoshinaga, Y. Hir'osh, Mechanism of purine arabinoside synthesis by bacterial transarabinosylation reaction, Agric Biol Chem. 49 (1985) 2425–2430.

[27] I. V. Fateev, M.I. Kharitonova, K. V. Antonov, I.D. Konstantinova, V.N. Stepanenko, R.S. Esipov, F. Seela, K.W. Temburnikar, K.L. Seley-Radtke, V.A. Stepchenko, Y.A. Sokolov, A.I. Miroshnikov, I.A. Mikhailopulo, Recognition of artificial nucleobases by E. coli purine nucleoside phosphorylase versus its Ser90Ala mutant in the synthesis of base-modified nucleosides, Chem - A Eur J. 21 (2015) 13401–13419.

[28] I. Serra, T. Bavaro, D.A. Cecchini, S. Daly, A.M. Albertini, M. Terreni, D. Ubiali, A comparison between immobilized pyrimidine nucleoside phosphorylase from Bacillus subtilis and thymidine phosphorylase from Escherichia coli in the synthesis of 5-substituted pyrimidine 2 - deoxyribonucleosides, "Journal Mol Catal B, Enzym. 95 (2013) 16–22.

[29] X.F. Gao, X.R. Huang, C.C. Sun, Role of each residue in catalysis in the active site of pyrimidine nucleoside phosphorylase from Bacillus subtilis: A hybrid QM/MM study, J Struct Biol. 154 (2006) 20–26.

[30] H. Yehia, S. Kamel, K. Paulick, P. Neubauer, A. Wagner, Substrate Spectra of Nucleoside Phosphorylases and their Potential in the Production of Pharmaceutically Active Compounds, Curr Pharm Des. 23 (2017) 6913–6935.

[31] I.A. Mikhailopulo, A.I. Miroshnikov, Biologically important nucleosides: Modern trends in biotechnology and application, Mendeleev Commun. 21 (2011) 57–68.

[32] J. V Tuttle, M. Tisdale, T.A. Krenitsky, Purine 2'-deoxy-2'-fluororibosides as antiinfluenza virus agents, J Med Chem. 36 (1993) 119–125.

[33] K. Yamada, N. Matsumoto, H. Hayakawa, Stereoselective synthesis of 2-Deoxy-2-fluoroarabinofuranosyl-α-1-phosphate and its application to the synthesis of 2'-deoxy-2'-fluoroarabinofuranosyl purine nucleosides by a chemo-enzymatic method, Nucleosides, Nucleotides and Nucleic Acids. 28 (2009) 1117–1130.

[34] J.D. Stoeckler, C. Cambor, R.E. Parks, Human erythrocytic purine nucleoside phosphorylase: reaction with sugar-modified nucleoside substrates, Biochemistry. 19 (1980) 102–107.

[35] C.S. Alexeev, I. V. Kulikova, S. Gavryushov, V.I. Tararov, S.N. Mikhailov, Quantitative Prediction of Yield in Transglycosylation Reaction Catalyzed by Nucleoside Phosphorylases, Adv Synth Catal. 360 (2018) 3090–3096.

Paper IV

Heba Yehia, Hendrik F. T. Klare, Peter Neubauer, Jens Kurreck, Anke Wagner. Halogenated nucleoside analogs produced in a chemo-enzymatic process are highly active in leukemic cell lines. (In preparation).

IV

Halogenated nucleoside analogs produced in a chemo-enzymatic process are highly active in leukemic cell lines

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Abstract

Nucleoside analogs are an important class of drugs to treat viral infections or cancer. Chemical synthesis is the ´Golden Standard´ for the production of nucleoside analogs but due to its disadvantages, enzymatic production routes have been developed. In this study, the cytotoxic potential of cladribine produced either by chemical or enzymatic methods were compared using leukemic cell lines HL-60 and CCRF-CEM. HEK-293 cells were used as negative control. IC_{50} values were comparable for both chemically and enzymatically produced cladribine. Values of 0.182±0.061 µM and 0.087±0.037 µM, respectively, were calculated applying HL-60 cells. Using CCRF-CEM cells, IC_{50} values of 0.388±0.315 µM and 0.613±0.257 were determined, respectively. As enzymatically-produced cladribine showed efficient inhibition of the tested leukemic cell lines, halogenated and aminated derivatives of cladribine were produced using the same chemo-enzymatic process. Among the tested compounds, diaminopurine riboside and deoxyriboside did not affect the growth of either HL-60 or HEK-293. Five out of nine compounds showed efficient inhibition of HL-60 cells only at the highest tested concentration of 100 µM. While seven of them did not lead to toxicity in the control cell line HEK-293. Non-specific toxicity was only observed for 2-fluoroadenine and 2-chloroadenine ribosides. Deoxyribosides, in comparison, did not show non-specific toxicity. 2FA deoxyriboside was the only compound with a comparable inhibition pattern to cladribine despite that the percentage of killed cells was increased.

Keywords

Nucleoside analogs, anticancer, nucleoside phosphorylases, thermophilic, antimetabolite, enzymatic synthesis, transglycosylation.

List of abbreviations

DNA: deoxyribonucleic acid; RNA: ribonucleic acid; NP: nucleoside phosphorylase; PyNP: pyrimidine nucleoside phosphorylase, PNP: purine nucleoside phosphorylase; Urd: uridine; Thd: thymidine; DMSO: dimethyl sulfoxide; NA: nucleoside analog; NTP: ribonucleotide; dNTP: 2'-deoxynucleotide; 2CA: 2-chlroadenine, 2FA: 2-fluoroadenine; DAP: 2-aminoadenine; DCP:2,6-dichloropurone; CFP: 6-chloro,2-fluoropurine, R:riboside; dR: deoxyriboside; 2CA-dR_{enz}: enzymatically-produced cladribine; 2CA-dR_{chem}: chemically-produced cladribine.

1. Introduction

Nucleosides are ubiquitous molecules that show multiple functions. They are building block of nucleic acids (DNA and RNA), energy storage entities, regulators for the central and peripheral nervous system or modulators of lymphocytes proliferation and immune function. They are produced by *de novo* biosynthesis or salvaged from catabolic and excretion pathways. Natural nucleosides are classified either according to the type of nitrogenous base into purines and pyrimidines nucleosides or based on the sugar part into ribosides and deoxyribosides.

Nucleoside analogs constitute a valuable class of therapeutics, similar to their physiological counterparts, but show modifications at the base or sugar moiety. Exploiting their antimetabolite properties, purine analogs have been employed for more than 50 years for several indications like cancer, viral infections, protozoal infections, hyperuricemia, gout and kidney stones or inflammation [1–4]. The latest FDA-approved nucleoside analog sofosbuvir shows the importance of nucleoside analogs. It is used to treat hepatitis C viral infection and is superior to previous treatment schemes. Since its introduction into the market in 2013, research is still ongoing to employ it into new treatment regimens and cocktails.

In vivo nucleoside analogs are activated to 5'-phosphorylated nucleotides. Different mechanisms of action are observed for nucleoside analogs in both proliferating and quiescent cells. On the one hand, they inhibit cellular propagation or viral replication by integrating in the growing strand of DNA or RNA blocking the binding of more nucleotides. On the other hand, they integrate into the elongating nucleic acid strand and lead to mismatching of the double helices and hence, mutations occur. Furthermore, it is observed that they inhibit important enzymes that control nucleic acids synthesis such as DNA and RNA polymerases, kinases, ribonucleotide reductase, DNA methyltransferases, reverse transcriptases. They can also interfere with other metabolic processes like cell signaling, enzyme regulation and metabolism [4,5]. The clinically-used nucleosides analogs usually combine more than one of these mechanisms. Cladribine or clofarabine, for example, in their active form terminate DNA replication by incorporation into growing nucleic acid strands and additionally block different enzymes like ribonucleotide reductase, thymidylate synthase or DNA polymerases [5–7].

The standard production route for nucleoside analogs in pharmaceutical industry is still the chemical synthesis. It usually involves many protection-deprotection steps, and the use of hazardous chemicals and solvents. Usually many byproducts and low yields are observed [8,9]. Due to the drawbacks of the chemical synthesis, alternative methods were developed. Enzymatic synthesis routes are environmentally-friendly, highly selective and efficient. The most commonly applied enzyme-catalyzed reaction is the one-pot transglycosylation with purine and pyrimidine nucleoside phosphorylases [9,10]. Here, the sugar moiety is exchanged between two nucleobases in the presence of inorganic phosphate. The phosphate ions attack the glycosidic bond of the starting nucleoside and a phosphorylated sugar intermediate and a free base are formed. The sugar moiety is subsequently transferred to the second nucleobase and the nucleoside of interest is formed.

So far, mainly NPs of mesophilic organisms have been applied for the synthesis of nucleoside analogs. However, enzymes from thermophilic bacteria or archaea are of increasing interest as they are active over a wide temperature and pH range which enhances solubility of substrates. Furthermore, a better mass transfer and less viscous reactions are achieved. Also due to a simple purification procedure thermophilic enzymes are a cost-effective biocatalyst for industrial process [9,11].

NP-catalyzed reactions have been investigated since the 1940s, however, the biological properties of enzymatically produced nucleoside analogs were only rarely described [12–14]. The impact of the

production and purification process on the biological activity cannot be excluded, yet not accounted for.

In present study we present a comparison of the biological activity of cladribine produced in a chemical or enzymatic process. As similar IC_{50} values were observed, halogenated and aminated derivatives of cladribine (2CA-dR) were produced in an enzymatic process. Compounds were purified by HPLC to purities >95% and structures were confirmed by NMR. Biological activity assays in a hematologic tumor cell lines showed similar inhibition pattern for 2FA-dR and 2CA-dR. Hence, IC_{50} values and maximum percentage of killed cells was studied in more detail. High non-specific toxicity for 2FA and 2CA ribosides was noted while replacing ribose moiety by a deoxyribose moiety led to reduced non-specific cytotoxicity.

2. Experimental:

2.1. General

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), and VWR (Darmstadt, Germany). Water was purified and deionized by a purification system from Merck Millipore to reach a resistivity of 18.2 MΩ.cm at 25°C (Schwalbach, Germany). Thermostable nucleoside phosphorylases (PyNP-Y01, PyNP-Y02, PNP-N01 and PNP-N02) were obtained from BioNukleo and used as recommended by the manufacturer. Cell lines HEK-239, HL-60 and CCRF-CEM were purchased from German Collection of Microorganisms and Cell Cultures GmbH.

2.2 Enzymatic production of halogenated purine nucleosides

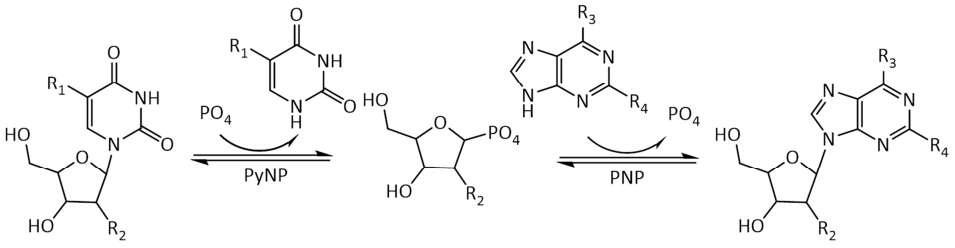
Enzymatic synthesis of purine nucleoside analogs was performed as reported [10,15]. Uridine (Urd) or thymidine (Thd) were used as sugar donors for transglycosylation reaction (**Scheme 1**) while bases were: 2-chloroadenine (2CA), 2-fluoroadenine (2FA), 2-aminoadenine (DAP), 2,6-dichloropurine (DCP), 6-chloro-2-fluoropurine (CFP). Sugar donor concentration was 30-50 mM and base concentration was 15-25 mM (**Table 1, Table 2**). As catalysts, thermostable PyNPs and PNPs were applied: PyNP-Y01 and PNP-N01 were used for Thd-containing reactions to produce deoxyribosides at 40°C while PyNP-Y02 and PNP-N02 were used in case of Urd-containing reactions to produce ribosides at 60°C. Nucleoside phosphorylases were heterologously expressed in *E. coli* and originate from thermophilic bacteria with temperature optima of 50°C (PNP-N01, PyNP-Y01) and 60°C (PNP-N02, PyNP-Y02). Enzymes were purified by affinity chromatography. Enzyme concentrations between 0.01 and 0.05 mg/mL were used for PyNP and PNP, respectively. Enzymatic reactions were performed in 2 mM potassium phosphate (KP) buffer at pH 7 in a reaction volume of 50 ml. Regular samples were taken to monitor enzymatic reactions by high performance liquid chromatography (HPLC).

Table 1: Conditions used for the halogenated purine nucleosides synthesis using uridine as sugar donor.

	2CA	**2FA**	**CFP**	**DCP**	**DAP**
Uridine [mM]	30 mM	30 mM	50 mM	50 mM	30 mM
Base [mM]	15 mM	15 mM	25 mM	25 mM	15 mM
Enzymes/ final enzyme conc. [mg/ml]	PyNP-Y02, PNP-N02/0.01, 0.05				

Table 2: Conditions used for halogenated purine nucleosides synthesis using thymidine as sugar donor.

	2CA	2FA	CFP	DCP	DAP
Thymidine [mM]	30 mM	30 mM	50 mM	50 mM	30 mM
Base [mM]	15 mM	15 mM	25 mM	25 mM	15 mM
Enzymes/ final enzyme conc. [mg/ml]	PyNP-Y01, PNP-N01/ 0.01, 0.05				



	R_1	R_2		R_3	R_4
Uridine	H	OH	2-chloroadenine	NH_2	Cl
Thymidine	CH_3	H	2-fluoroadenine	NH_2	F
			6-chloro-2-fluoroadenine	F	Cl
			2,6-dichloroadenine	Cl	Cl
			2-aminoadenine	NH_2	NH_2

Scheme 1: Transglycosylation reaction

2.3 Cell culture

HEK293 cells were cultured in low glucose Dulbecco's modified Eagle's medium (Biowest) containing 10% fetal bovine serum, 2 mM glutamine, non-essential amino acids, 4.5 mg/ml glucose and the antibiotics penicillin and streptomycin. HL-60 and CCRF-CEM were cultured in RPMI 1640 w/o L-Glutamine supplemented with 10% fetal bovine serum, 2 mM glutamine and the antibiotics penicillin and streptomycin. Cells were grown at 37°C in a humidified atmosphere with 5% CO_2.

2.4 XTT cell proliferation assay

Cytotoxicity was measured by the XTT assay at 450 nm as suggested by the manufacturer (Roche Diagnostics GmbH). Phosphate buffered saline was used as negative control. For HL-60, CCRF-CEM and HEK 293 cells 1.2×10^5 cells in growth media were transferred to each well of a 96-well flat-bottom plate. Subsequently, modified nucleosides were added in varying concentrations. XTT assay was performed after 24 h for HL-60 and HEK 293 cells and after 48 h for CCRF-CEM cells.

At least three replications for each nucleoside concentration/buffer were performed. All experiments were conducted in three independent experiments.

2.5 Analytical high performance liquid chromatography (HPLC)

Enzymatic reactions were followed with HPLC Agilent 1200 series system equipped with an Agilent DAD detector. The reaction yields were determined by following the nucleobases and nucleosides absorption at 260 nm using a reversed phase C18 column (Kinetex® 5 µm, Evo C18, 100 Å LC column 150 x 4.6 mm, Phenomenex, CA, USA) with the following gradient: from 97% 20 mM ammonium acetate and 3% acetonitrile to 60% 20 mM ammonium acetate and 40% acetonitrile in 10 min [16]. Nucleoside depletion and base formation were quantified at 260 nm using pure compound

standards. The retention times were as follows: Urd, 3.2 min; Ura, 2.4 min; Thd, 4.7 min; Thy, 4 min; 2CA-R, 6.2 min; 2CA-dR, 6.5 min; 2CA, 5.69 min; 2FA-R, 5.6 min; 2FA-dR, 5.9 min; 2FA, 5.01 min; CFP-R, 7.5 min; CFP-dR, 8.2 min; CFP, 7 min; DCP-R, 8.2 min; DCP-dR, 8.8 min; DCP, 7.6 min; DAP-R, 4.8 min; DAP-dR, 5.1 min; DAP, 3.9 min. The % of conversion of nucleosides was calculated per **Formula 1** [17].

Formula 1: $$\text{Conversion } \% = \frac{\text{Conc.of the product [mM]}}{\text{Conc.of the product [mM]+ Conc.of the substrate [mM]}} \times 100$$

2.6 Purification of nucleoside analogs

Nucleoside analogs were purified at room temperature using a KNAUER HPLC system equipped with a Smartline Detector 2600 and an AZURA P 2.1L pump. A flow rate of 21 mL/min and a Kinetex® 5 µm EVO C18 250*21.2 mm RP column were used. Deionized water and acetonitrile were used as eluents while the gradient was modified from the analytical method.

2.7 NMR analysis

^{1}H, ^{13}C, ^{19}F, and ^{31}P NMR spectra were recorded in DMSO-d_6 or 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 (^{1}H NMR: δ = 2.50 ppm for DMSO-d_5 and δ = 4.79 ppm for HDO; ^{13}C NMR: δ = 39.52 ppm for DMSO-d_6) [18]. ^{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 CCl_3F and H_3PO_4 [19]. Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, m_c = centrosymmetric multiplet, br = broad signal), coupling constants (Hz) and integration.

3. Results and discussion

3.1. Enzymatic synthesis of cladribine

Cladribine was produced in a transglycosylation reaction as previously described [10]. Thermophilic nucleoside phosphorylases PNP-N01 and PyNP-Y01 were used at a reaction temperature of 40°C. In a reaction volume of 50 mL 60% of 2-chloroadenine were converted to cladribine. Conversion of the sugar donor thymidine was 40%. After HPLC purification and lyophilization, 90 mg of cladribine were produced (**Table 3**). Purity was determined by HPLC to be >99% and structure was confirmed by NMR (**Supplementary table 1**).

3.2. Biological activity of cladribine produced in a chemo-enzymatic process

As byproducts of the enzymatic synthesis could interfere with its biological activity, cladribine produced in a chemo-enzymatic process (2CA-dR_{enz}) was tested in leukemic cell lines. Initially, the acute myeloid leukemia cell line (HL-60) was chosen due to its well-known use in studying myeloid cells differentiation and proliferation [20–22]. Human embryonic kidney cells (HEK-293) were used as a non-malignant control for the potential selective cytotoxicity [21–23]. Commercially available cladribine produced in a chemical process (2CA-dR_{chem}) was used as a positive control.

Comparable IC_{50} values of 0.18202 ± 0.061 and 0.087 ± 0.037 µM were determined for 2CA-dR_{chem} and 2CA-dR_{enz} in HL-60 cells. Additionally, the dose-response behavior for the 2 compounds is the same. For example: the ratio of killed cells at the highest drug concentration (500 µM) was 78.82 and 71.43%, respectively (**Supplementary Fig. 1a**). To validate the results, the biological activity of 2CA-dR was also determined in T-cell leukemia cell line CCRF-CEM. Comparable results for cladribine produced in a chemical or enzymatic process was confirmed (**Table 4**). IC_{50} was calculated to be 0.613

± 0.257 and 0.388 ± 0.315 µM for 2CA-dR_{enz} and 2CA-dR_{chem}, respectively. Likewise, the dose-response curves are very similar (**Suppl. Fig. 1b**). Values obtained for HL-60 and CCRF-CEM were in accordance with previously published data (**Table 4**). The treated HEK cells behaved almost identical for both compounds (**Suppl. Fig. 2b**).

These results were the basis for testing more enzymatically-produced nucleoside analogs being derivatives of cladribine.

Table 3: Efficiency of the enzymatic synthesis of purine nucleoside analogs.

Compound	Conversion (%)	Product yield		Purity (%)	Recovery (%)
		mg	mmol		
2CA-R	80	129	0.43	99.7	57
2CA-dR	60	91	0.32	99.7	43
2FA-R	90	154	0.54	99.5	72
2FA-dR	85	121	0.45	97	60
CFP-R	70	213	0.7	99	56
CFP-dR	80	248	0.86	99.3	69
DCP-R	60	180	0.56	98.4	45
DCP-dR	75	189	0.62	99.6	50
DAP-R	75	98	0.35	99.3	47
DAP-dR	85	114	0.43	96.1	57

* All reactions were carried out in 50 mL volume

* % conversion was calculated based on amount of reacted base

Table 4: Comparison of IC_{50} values determined for cladribine (2CA-dR)

IC_{50} value [µM] for HL-60	**IC_{50} value [µM] for CCRF-CEM**
0.087 ± 0.037 (2CA-dR_{enz}; this study)	0.613 ± 0.257 (2CA-dR_{enz}; this study)
0.18202 ± 0.061 (2CA-dR_{chem}; this study)	0.388±0.315 (2CA-dR_{chem}; this study)
>0.05 [24]	0.09 ± 0.05 [25]
0.041 [26]	0.015 [12]
0.0700 ± 0.0060 [27]	0.003 [28]

3.3. Chemo-enzymatic production of derivatives of cladribine

Results for 2CA-dR_{enz} were very promising. Therefore, derivatives of cladribine were produced in an enzymatic process to study the effect of the modifications on the biological activity. Modifications were either in position 2 or 6 of the purine base (**Scheme 1**). Both ribosides and deoxyribosides were tested.

Cladribine derivatives were produced using thermostable nucleoside phosphorylases in transglycosylation reactions with either uridine or thymidine as sugar donor. 70% to 90% of the purine base substrates were converted to the purine nucleoside analogs of interest. From reaction volumes of 50 mL 90 to 250 mg of purified product were obtained. Losses from the purification process were calculated to be below 30%. Purity of the products were determined to be >95% by HPLC (**Table 3**). Structure were confirmed by HPLC (**Supplementary table 1**).

3.4. Screening for biological activity of cladribine derivatives in leukemic cell lines

Nine enzymatically-produced cladribine derivatives were initially screened using HL-60 while HEK-293 cells served as a toxicity control. Concentrations of 100 µM, 1 µM and 100 nM were applied. Except

for 2-aminoadenine nucleosides, cytotoxic activity against HL-60 cells was noted at the concentration of 100 µM (**Suppl. Fig. 2a**). At 1 µM, only 2FA nucleosides and 2CA-dR had an effect. Percentages of killed cells were 31.23% (2FA-R), 76.16% (2FA-dR), 61.57% (2CA-dR_{enz}) and 69.61% (2CA-dR_{chem}). Beside activity in HL-60 cells, 2FA-R showed remarkable non-selective toxicity on HEK-293 cells at a concentration of 100 µM with a percentage of killed cells >80% (**Suppl. Fig. 2b**). At a concentration of 1 µM, unspecific cytotoxicity was not observed anymore (**Suppl. Fig. 2b,d**). The non-specific toxicity is not observed in the deoxyriboside counterpart with 11.4% killed HEK-293 cells at a concentration of 100 µM. A less pronounced, yet similar, observation was found in 2CA nucleosides where 2CA-R showed double the percentage of killed cells as 2CA-dR at 100 µM concentration (**Suppl. Fig. 2b**).

3.4. Biological activity of 2FA derivatives in comparison to cladribine

As 2FA-dR showed promising results and was studied in more detail in comparison to cladribine. In HL-60 cells, an IC_{50} value of 0.335 ± 0.082 µM was determined for 2FA-dR while in CCRF-CEM, the IC_{50} value was 7.36 ± 0.693 µM. Compared to cladribine, the IC_{50} value against HL-60 cells decreased by a factor of 4. The percentage of killed cells, however, was slightly higher using 2FA-dR. Values of 86.4 and 72.7% were calculated for 2FA-dR and 2CA-dR, respectively (**Suppl. Fig. 2a,c**).

Discussion

Using transglycosylation reactions, nine derivatives of cladribine were successfully produced and purified using semi-preparative HPLC. Final purities were above 95% (reagent grade). The compounds were validated for their biological activity in different hematologic leukemia cell lines. Cladribine and 2FA-dR showed comparable results and IC_{50} values in a low µM range. While for cladribine a slightly lower IC_{50} value was calculated, 2FA-dR showed an increased percentage of killed cells. In general, a higher non-specific was observed for with ribosides in comparison to deoxyribosides.

Derivatives of cladribine have been widely studied as substrates for NPs because of their potential medicinal applications [9,15,17]. Double halogenated purine bases were only studied as substrates for NPs from extreme environments. Both DCP and CFP were converted to the respective riboside and deoxyriboside nucleosides by thermostable PNP from *Geobacillus thermoglucosidasius*. Product yields were in the range of 50-70% [10]. In accordance with our results, CFP nucleobase and deoxyriboside nucleosides showed higher product yields than DCP and ribosides, respectively [10]. 2,6-Diaminopurine, the precursor for the enzymatic synthesis of guanine-derivatives by adenosine deaminase, is one of the best reported NP substrates. In this study, 75% and 85% molar conversion of DAP-R and DAP-dR was observed, which is in good accordance with literature. 73% and 85% of DAP conversion to DAP-dR was obtained with *Enterobacter aerogenes* [29] and *Geobacillus stearothermophilus* whole cells [30,31]. Enzymatic synthesis of cladribine was also much studied so far. Based on the applied reaction conditions, 55% to 90% of cladribine formation was reported with different *E. coli* strains [32,33] and mesophilic PNP from *Aeromonas hydrophila* and *Geobacillus* sp. [34]. In this study, a conversion percentage of 60% was observed which was probably due to the low solubility of the purine base (2CA).

To validate if impurities from the enzymatic process interfere with biological activity, commercially available cladribine produced in a chemical process was compared to cladribine produced by NPs. Comparable IC_{50} values were calculated for cell lines HL-60 and CCRF-CEM. For CCRF-CEM, the IC_{50} value was higher than the literature data, but in good accordance with formerly published data for HL-60 cells (**Table 4**).

The activity of several cladribine derivatives was screened against several hematologic leukemia cell lines. Except for DAP nucleosides, NAs were active against the leukemia cells under investigation at the higher concentration (100 µM). This conforms with the observation that halogenation is an

important prerequisite for anticancer activity [35,36] whereas amino groups make the compound susceptible to deamination by adenosine deaminase [37,38]. IC_{50} for DAP-dR in L1210 leukemic cells was reported to be 140 µM after 72 h [39] and 300 µM in 48 h [40]. In contrast, IC_{50} values were determined to be in the low nM range for 2CA-dR and 2FA-dR in this study and literature, respectively (**Table 4**). The same goes for 2FA-dR whose IC_{50} values fall in the micromolar scale [12,28].

Non-selective toxicity was observed for 2FA-R and 2CA-R in this study. 2FA-R and its metabolites; 2-fluoroadenine (product of PNP activity) and 2-fluoroadenosine triphosphate (product of kinase activity) are already known to have non-selective toxicity [5,41,42]. These compounds have been accounted responsible for fludarabine (9-β-D- arabinofuranosyl-2-fluoroadenine monophosphate) nephrotoxicity [41]. The less non-selective toxicity of 2-chloroadenosine complies with an old hypothesis that the toxicity decreases with larger halogen substitution at the C2-position of the purine ring [43]. 2-Haloadenosine ribosides and arabinosides were shown to lose their activity with an increase in halogen size (F > Cl > Br) [43,44].

The deoxyriboside counterpart of 2-fluoroadenosine and 2-chloroadenosine showed a strongly decreased non-specific toxicity. The higher toxicity of ribosides can be connected to local differences in concentration in dNTPs and NTP pools inside cells. NTPs accumulate to higher concentrations [45,46] so, they are more available for activity. Furthermore, RNA polymerase is more prone to proofreading errors (10^{-5} error per base replicated) in comparison to DNA polymerase (10^{-9} to 10^{-10}) [47,48].

4. Conclusions

Enzymatic synthesis of nucleoside analogs does not influence the compounds' cytotoxic activity. The outlook of such study as per the synthesis involves: (i) increasing the volumetric yield of the synthesis for example: by cosolvency of the buffer with DMSO which did not show any detriment of the enzymes' activity or using the respective pentofuranose-1-phosphate which is freely soluble, (ii) using different reaction setups or forms of the enzymes that spare on the enzyme's use which is the major laborious and cost-intensive step in production for example: immobilization, crude extract, whole cells, continuous enzyme reactors. With respect to activity, solid tumor and virostatic tests are to be included.

Conflict of Interest

A.W. is 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.

References

1. Robak, P., and Robak, T. (2013) Older and new purine nucleoside analogs for patients with acute leukemias. *Cancer Treat. Rev.*, **39** (8), 851–861.
2. Quan, D.J., and Peters, M.G. (2004) Antiviral therapy : nucleotide and nucleoside analogs. *Clin. Liver Dis.*, **8** (2), 371–385.
3. Kumar, V., Harjai, K., and Chhibber, S. (2013) Potential anti-inflammatory role of 2-chloroadenosine treatment during acute lung inflammation in BALB/c mice suffering from Klebsiella pneumoniae B5055-induced acute lung infection. *Crit. Care*, **17** (Suppl 4), P68–P68.
4. Jordheim, L.P., Durantel, D., Zoulim, F., and Dumontet, C. (2013) Advances in the development of

nucleoside and nucleotide analogues for cancer and viral diseases. *Nat. Rev. Drug Discov.*, **12** (6), 447–464.

5. Bonate, P.L., Arthaud, L., Cantrell Jr., W.R., Stephenson, K., Secrist 3rd, J.A., and Weitman, S. (2006) Discovery and development of clofarabine: a nucleoside analogue for treating cancer. *Nat. Rev. Drug Discov.*, **5** (10), 855–863.
6. Bellezza, I., Tucci, A., and Minelli, A. (2008) 2-Chloroadenosine and human prostate cancer cells. *Anticancer Agents Med Chem*, **8** (7), 783–789.
7. Galmarini, C.M., Mackey, J.R., and Dumontet, C. (2002) Nucleoside analogues and nucleobases in cancer treatment. *Lancet Oncol.*, **3** (7), 415–424.
8. Trelles, J.A., Bentancor, L., Schoijet, A., Porro, S., Lewkowicz, E.S., Sinisterra, J. V., and Iribarren, A.M. (2004) Immobilized *Escherichia coli* BL21 as a catalyst for the synthesis of adenine and hypoxanthine nucleosides. *Chem. Biodivers.*, **1** (2), 280–288.
9. Yehia, H., Kamel, S., Paulick, K., Wagner, A., and Neubauer, P. (2017) Substrate spectra of nucleoside phosphorylases and their potential in the production of pharmaceutically active compounds. *Curr. Pharm. Des.*, **23** (45), 6913–6935.
10. Zhou, X., Szeker, K., Jiao, L.-Y., Oestreich, M., Mikhailopulo, I.A., and Neubauer, P. (2015) Synthesis of 2,6-Dihalogenated Purine Nucleosides by Thermostable Nucleoside Phosphorylases. *Adv. Synth. Catal.*, **357** (6), 1237–1244.
11. Del Arco, J., and Fernández-Lucas, J. (2018) Purine and pyrimidine salvage pathway in thermophiles: a valuable source of biocatalysts for the industrial production of nucleic acid derivatives. *Appl. Microbiol. Biotechnol.*, **102**, 7805–7820.
12. Huang, M.-C., Hatfield, K., Roetker, A.W., Montgomery, J.A., and Blakley, R.L. (1981) Analogs of 2'-deoxyadenosine: Facile enzymatic preparation and growth inhibitory effects on human cell lines. *Biochem. Pharmacol.*, **30** (19), 2663–2671.
13. Huang, M.C., Montgomery, J.A., Thorpe, M.C., Stewart, E.L., Secrist, J.A., and Blakley, R.L. (1983) Formation of 3-(2'-Deoxyribofuranosyl) and 9-(2'-Deoxyribofuranosyl) Nucleosides of 8-Substituted Purines by Nucleoside Deoxyribosyltransferase. *Arch. Biochem. Biophys.*, **222** (1), 133–144.
14. Chae, W.G., Chan, T.C.K., and Chang, C.J. (1998) Facile synthesis of 5'-deoxy- and 2',5'-dideoxy-6-thiopurine nucleosides by nucleoside phosphorylases. *Tetrahedron*, **54** (30), 8661–8670.
15. Mikhailopulo, I.A., and Miroshnikov, A.I. (2013) Some recent findings in the biotechnology of biologically important nucleosides. *Biotechnol. Acta*, **6** (4), 63–77.
16. Szeker, K., Zhou, X., Schwab, T., Casanueva, A., Cowan, D., Mikhailopulo, I.A., and Neubauer, P. (2012) Comparative investigations on thermostable pyrimidine nucleoside phosphorylases from Geobacillus thermoglucosidasius and Thermus thermophilus. *J. Mol. Catal. B Enzym.*, **84**, 27–34.
17. Zhou, X., Szeker, K., Janocha, B., Böhme, T., Albrecht, D., Mikhailopulo, I.A., and Neubauer, P. (2013) Recombinant purine nucleoside phosphorylases from thermophiles: preparation, properties and activity towards purine and pyrimidine nucleosides. *FEBS J.*, **280** (6), 1475–1490.
18. Sinisterra, J., Alcántara, A., Almendros, M., Hernáiz, M.J., Sánchez-Montero, J., and Trelles, J. (2010) Enzyme-catalyzed synthesis of nonnatural or modified nucleosides, in *Encyclopedia of Industrial Biotechnology: Bioprocess, Bioseparation, and Cell Technology* (eds.Flickinger, M.C.), John Wiley & Sons, New York.
19. Harris, R.K., Becker, E.D., De Menezes, S.M.C., Goodfellow, R., and Granger, P. (2001) NMR nomenclature. Nuclear spin properties and conventions for chemical shifts (IUPAC

Recommendations 2001). *Pure Appl. Chem.*, **73** (11), 1795–1818.

20. Shigemi, H., Yamauchi, T., Tanaka, Y., and Ueda, T. (2013) Novel leukemic cell lines resistant to clofarabine by mechanisms of decreased active metabolite and increased antiapoptosis. *Cancer Sci.*, **104** (6), 732–739.
21. Saleh, A.M., Aljada, A., Rizvi, S.A.A., Nasr, A., Alaskar, A.S., and Williams, J.D. (2014) In vitro cytotoxicity of Artemisia vulgaris L. essential oil is mediated by a mitochondria-dependent apoptosis in HL-60 leukemic cell line. *BMC Complement. Altern. Med.*, **14**, 226.
22. Golshan, A., Hassanzadeh, S., Mojdekanloo, M., and Tayarani-Najaran, Z. (2016) Effects of Biebersteinia multifida hydro-ethanol extract on proliferation and apoptosis of human prostate cancer and human embryonic kidney cells. *Avicenna J. Phytomedicine*, **6** (6), 671–677.
23. Wang, C., Zhang, Z., Wang, Y., and He, X. (2015) Cytotoxic Indole Alkaloids against Human Leukemia Cell Lines from the Toxic Plant Peganum harmala. *Toxins (Basel).*, **7** (11), 4507–4518.
24. Stachnik, K., Grieb, P., and Skierski, J.S. (2005) Cytotoxic effects of cladribine and tezacitabine toward HL-60. *Acta Biochim. Pol.*, **52** (2), 561–565.
25. Lotfi, K., Månsson, E., Chandra, J., Wang, Y., Xu, D., Knaust, E., Spasokoukotskaja, T., Liliemark, E., Eriksson, S., and Albertioni, F. (2001) Pharmacological basis for cladribine resistance in a human acute T lymphoblastic leukaemia cell line selected for resistance to etoposide. *Br. J. Haematol.*, **113**, 339–346.
26. Månsson, E., Spasokoukotskaja, T., Sällström, J., Eriksson, S., and Albertioni, F. (1999) Molecular and Biochemical Mechanisms of Fludarabine and Cladribine Resistance in a Human Promyelocytic Cell Line. *Cancer Res.*, **59** (23), 5956–5963.
27. Nishi, R., Yamauchi, T., Negoro, E., Takemura, H., and Ueda, T. (2013) Combination of guanine arabinoside and Bcl-2 inhibitor YC137 overcomes the cytarabine resistance in HL-60 leukemia cell line. *Cancer Sci.*, **104** (4), 502–507.
28. Montgomery, J.A., Shortnacy-Fowler, A.T., Clayton, S.D., Riordan, J.M., and Secrist 3rd, J.A. (1992) Synthesis and biologic activity of 2'-fluoro-2-halo derivatives of 9-beta-D-arabinofuranosyladenine. *J. Med. Chem.*, **35** (2), 397–401.
29. Yokozeki, K., and Tsuji, T. (2000) A novel enzymatic method for the production of purine-2'-deoxyribonucleosides. *J. Mol. Catal. B Enzym.*, **10** (1–3), 207–213.
30. De Benedetti, E.C., Rivero, C.W., Britos, C.N., Lozano, M.E., and Trelles, J.A. (2012) Biotransformation of 2,6-diaminopurine nucleosides by immobilized Geobacillus stearothermophilus. *Biotechnol. Prog.*, **28** (5), 1251–1256.
31. Médici, R., Lewkowicz, E.S., and Iribarren, A.M. (2006) Microbial synthesis of 2,6-diaminopurine nucleosides. *J. Mol. Catal. B Enzym.*, **39** (1–4), 40–44.
32. Barai, V.N., Zinchenko, A.I., Eroshevskaya, L.A., Kalinichenko, E.N., Kulak, T.I., and Mikhailopulo, I.A. (2002) A Universal Biocatalyst for the Preparation of Base- and Sugar-Modified Nucleosides via an Enzymatic Transglycosylation. *Helv. Chim. Acta*, **85** (7), 1901–1908.
33. Lapponi, M.J., Rivero, C.W., Zinni, M.A., Britos, C.N., and Trelles, J.A. (2016) New developments in nucleoside analogues biosynthesis: A review. *J. Mol. Catal. B Enzym.*, **133**, 218–233.
34. Taran, S., Verevkina, K., Esikova, T., Feofanov, S., and Miroshnikov, A. (2008) Synthesis of 2-chloro-2'-deoxyadenosine by microbiological transglycosylation using a recombinant Escherichia coli strain. *Appl. Biochem. Microbiol.*, **44** (2), 162–166.
35. Rivero, C.W., De Benedetti, E.C., Sambeth, J.E., Lozano, M.E., and Trelles, J.A. (2012) Biosynthesis of anti-HCV compounds using thermophilic microorganisms. *Bioorg. Med. Chem. Lett.*, **22** (19),

6059–6062.

36. Liu, M.-C., Luo, M.-Z., Mozdziesz, D.E., and Sartorelli, A.C. (2005) Synthesis and Biological Evaluation of 2- and 7-Substituted 9-Deazaadenosine Analogues. *Nucleosides, Nucleotides and Nucleic Acids*, **24** (1), 45–62.
37. Ma, J., Wang, S., Zhao, M., Deng, X.-S., Lee, C.-K., Yu, X.-D., and Liu, B. (2011) Therapeutic potential of cladribine in combination with STAT3 inhibitor against multiple myeloma. *BMC Cancer*, **11** (1), 1–11.
38. Pui, C., Jeha, S., and Kirkpatrick, P. (2005) Clofarabine. *Nat. Rev. Drug Discov.*, **4**, 369–370.
39. Weckbecker, G., and Cory, J.G. (1987) 2, 6-Diaminopurinedeoxyriboside as a Prodrug of Deoxyguanosine in L1210 Cells. *Cancer Res.*, **47** (9), 2218–2223.
40. Weckbecker, G., and Cory, J.G. (1989) Metabolic activation of 2,6-diaminopurine and 2,6-diaminopurine-2'-deoxyriboside to antitumor agents. *Adv. Enzyme Regul.*, **28**, 125–144.
41. Avramis, V.I., and Plunkett, W. (1983) 2-Fluoro-ATP: A toxic metabolite of 9-β-D-arabinosyl-2-fluoroadenine. *Biochem. Biophys. Res. Commun.*, **113** (1), 35–43.
42. de Giuseppe, P.O., Martins, N.H., Meza, A.N., dos Santos, C.R., Pereira, H.D.M., and Murakami, M.T. (2012) Insights into Phosphate Cooperativity and Influence of Substrate Modifications on Binding and Catalysis of Hexameric Purine Nucleoside Phosphorylases. *PLoS One*, **7** (9), 1–11.
43. Bennett, L.L., Vail, M.H., Chumley, S., and Montgomery, J.A. (1966) Activity of adenosine analogs against a cell culture, line resistant to 2-fluoroadenine. *Biochem. Pharmacol.*, **15** (11), 1719–1728.
44. Montgomery, J.A. (1987) The Biological Bases for the Design of Anticancer Agents, in *Cancer Biology and Therapeutics* (eds.Cory, J.G., and Szentivanyi, A.), Springer, Boston, MA, pp. 23–42.
45. Kong, Z., Jia, S., Chabes, A.L., Appelblad, P., Lundmark, R., Moritz, T., and Chabes, A. (2018) Simultaneous determination of ribonucleoside and deoxyribonucleoside triphosphates in biological samples by hydrophilic interaction liquid chromatography coupled with tandem mass spectrometry. *Nucleic Acids Res.*, **46** (11), e66.
46. Potenski, C.J., and Klein, H.L. (2014) How the misincorporation of ribonucleotides into genomic DNA can be both harmful and helpful to cells. *Nucleic Acids Res.*, **42** (16), 10226–10234.
47. Sydow, J.F., and Cramer, P. (2009) RNA polymerase fidelity and transcriptional proofreading. *Curr. Opin. Struct. Biol.*, **19**, 732–739.
48. Garwal, K.A.N.A. (1996) Fidelity of RNA polymerase II transcription controlled by elongation factor TFIIS. *Proc. Natl. Acad. Sci. USA*, **93**, 13677–13682.

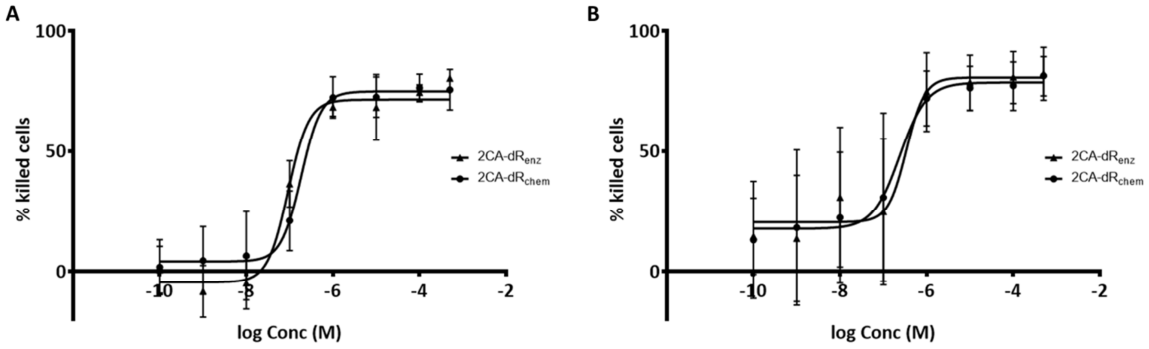
Supplementary material

Supplementary table 1: Structures of halogenated purine nucleosides were confirmed by NMR spectroscopy.

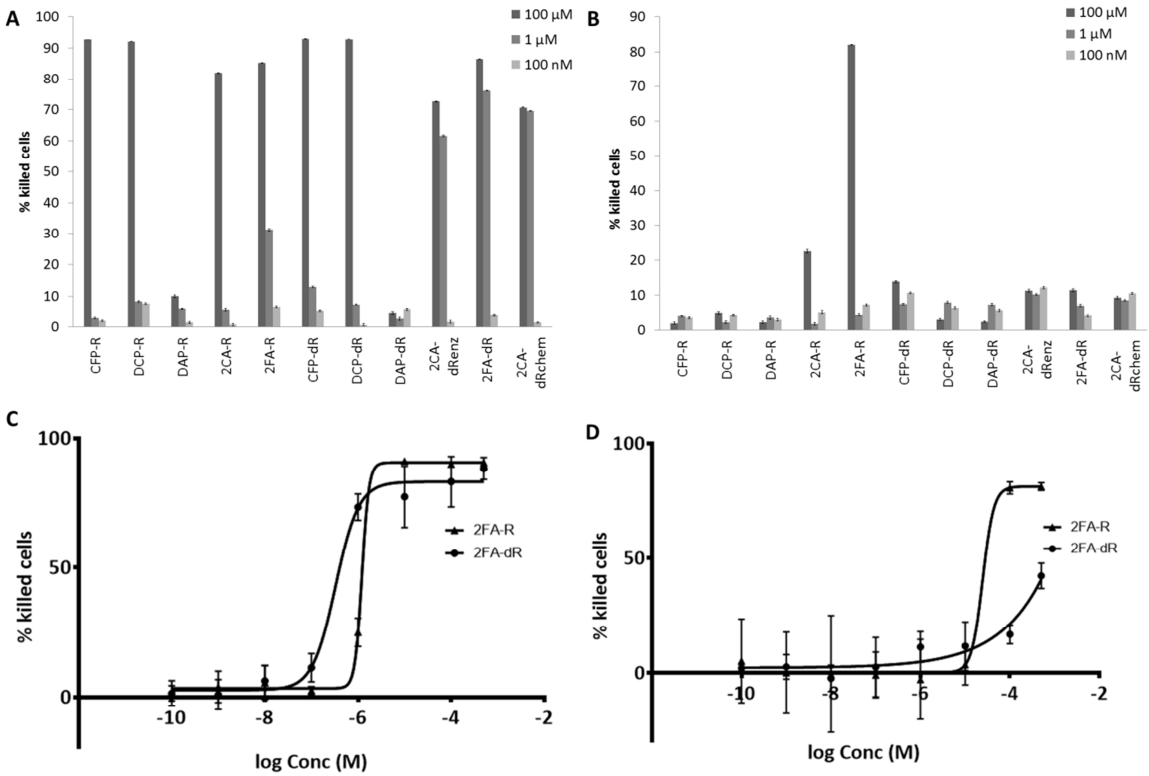
Compound	NMR spectroscopic data
2CA-R	**^{1}H NMR** (500 MHz, DMSO-d_6): δ = 3.55 (ddd, ^{2}J = 12.0 Hz, ^{3}J = 6.0 Hz, ^{3}J = 4.0 Hz, 1H, H-5'A), 3.66 (ddd, ^{2}J = 12.0 Hz, ^{3}J = 4.4 Hz, ^{3}J = 4.4 Hz, 1H, H-5'B), 3.94 (ddd, ^{3}J = 3.8 Hz, ^{3}J = 3.8 Hz, ^{3}J = 3.5 Hz, 1H, H-4'), 4.13 (m_c, 1H, H-3'), 4.52 (m_c, 1H, H-2'), 5.05 (dd, ^{3}J = 5.6 Hz, ^{3}J = 5.6 Hz, 1H, 5'-OH), 5.19 (br s, 1H, 3'-OH), 5.46 (br s, 1H, 2'-OH), 5.81 (d, ^{3}J = 6.0 Hz, 1H, H-1'), 7.84 (br s, 2H, 6-NH_2), 8.38 (s, 1H, H-8) ppm. **^{13}C{^{1}H} NMR** (126 MHz, DMSO-d_6): δ = 61.4 (C-5'), 70.4 (C-3'), 73.6 (C-2'), 85.7 (C-4'), 87.4 (C-1'), 118.2 (C-5), 140.0 (C-8), 150.3 (C-4), 153.0 (C-2), 156.8 (C-6) ppm.
2CA-dR	**^{1}H NMR** (500 MHz, DMSO-d_6): δ = 2.28 (ddd, ^{2}J = 13.3 Hz, ^{3}J = 6.2 Hz, ^{3}J = 3.3 Hz, 1H, H-2'A), 2.64 (ddd, ^{2}J = 13.3 Hz, ^{3}J = 7.5 Hz, ^{3}J = 5.8 Hz, 1H, H-2'B), 3.51 (ddd, ^{2}J = 11.7 Hz, ^{3}J = 5.6 Hz, ^{3}J = 4.7 Hz, 1H, H-5'A), 3.60 (ddd, ^{2}J = 11.7 Hz, ^{3}J = 4.9 Hz, ^{3}J = 4.9 Hz, 1H, H-5'B), 3.86 (ddd, ^{2}J = 4.5 Hz, ^{3}J = 4.5 Hz, ^{3}J = 2.9 Hz, 1H, H-4'), 4.38 (m_c, 1H, H-3'), 4.95 (dd, ^{3}J = 5.6 Hz, ^{3}J = 5.6 Hz, 1H, 5'-OH), 5.30 (d, ^{3}J = 4.0 Hz, 1H, 3'-OH), 6.26 (dd, ^{3}J = 7.3 Hz, ^{3}J = 6.3 Hz, 1H, H-1'), 7.80 (br s, 2H, 6-NH_2), 8.35 (s, 1H, H-8) ppm. **^{13}C{^{1}H} NMR** (126 MHz, DMSO-d_6): δ = 39.2 (C-2'), 61.6 (C-5'), 70.7 (C-3'), 83.5 (C-1'), 87.9 (C-4'), 118.1 (C-5), 139.8 (C-8), 150.0 (C-4), 152.9 (C-2), 156.8 (C-6) ppm.
2FA-R	**^{1}H NMR** (500 MHz, DMSO-d_6): δ = 3.54 (ddd, ^{2}J = 12.0 Hz, ^{3}J = 5.9 Hz, ^{3}J = 4.2 Hz, 1H, H-5'A), 3.66 (ddd, ^{2}J = 12.0 Hz, ^{3}J = 4.5 Hz, ^{3}J = 4.5 Hz, 1H, H-5'B), 3.93 (ddd, ^{3}J = 3.8 Hz, ^{3}J = 3.7 Hz, ^{3}J = 3.7 Hz, 1H, H-4'), 4.12 (ddd, ^{3}J = 4.3 Hz, ^{3}J = 4.3 Hz, ^{3}J = 3.9 Hz, 1H, H-3'), 4.51 (ddd, ^{3}J = 5.5 Hz, ^{3}J = 5.5 Hz, ^{3}J = 5.3 Hz, 1H, H-2'), 5.05 (dd, ^{3}J = 5.6 Hz, ^{3}J = 5.6 Hz, 1H, 5'-OH), 5.19 (d, ^{3}J = 4.7 Hz, 1H, 3'-OH), 5.46 (d, ^{3}J = 6.0 Hz, 1H, 2'-OH), 5.78 (d, ^{3}J = 5.9 Hz, 1H, H-1'), 7.86 (br s, 2H, 6-NH_2), 8.34 (s, 1H, H-8) ppm. **^{13}C{^{1}H} NMR** (126 MHz, DMSO-d_6): δ = 61.4 (C-5'), 70.3 (C-3'), 73.5 (C-2'), 85.6 (C-4'), 87.4 (C-1'), 117.5 (d, $^{4}J_{C,F}$ = 3.6 Hz, C-5), 139.9 (C-8), 150.6 (d, $^{3}J_{C,F}$ = 20.3 Hz, C-4), 157.6 (d, $^{3}J_{C,F}$ = 20.9 Hz, C-6), 158.5 (d, $^{1}J_{C,F}$ = 202.8 Hz, C-2) ppm. **^{19}F{^{1}H} NMR** (471 MHz, DMSO-d_6): δ = –52.1 ppm.
2FA-dR	**^{1}H NMR** (500 MHz, DMSO-d_6): δ = 2.26 (ddd, ^{2}J = 13.3 Hz, ^{3}J = 6.2 Hz, ^{3}J = 3.3 Hz, 1H, H-2'A), 2.65 (ddd, ^{2}J = 13.3 Hz, ^{3}J = 7.4 Hz, ^{3}J = 6.0 Hz, 1H, H-2'B), 3.50 (ddd, ^{2}J = 11.7 Hz, ^{3}J = 5.5 Hz, ^{3}J = 5.5 Hz, 1H, H-5'A), 3.59 (ddd, ^{2}J = 11.7 Hz, ^{3}J = 5.0 Hz, ^{3}J = 5.0 Hz, 1H, H-5'B), 3.85 (m_c, 1H, H-4'), 4.38 (m_c, 1H, H-3'), 4.95 (dd, ^{3}J = 5.6 Hz, ^{3}J = 5.6 Hz, 1H, 5'-OH), 5.31 (d, ^{3}J = 4.2 Hz, 1H, 3'-OH), 6.23 (dd, ^{3}J = 6.8 Hz, ^{3}J = 6.8 Hz, 1H, H-1'), 7.83 (br s, 2H, 6-NH_2), 8.31 (s, 1H, H-8) ppm. **^{13}C{^{1}H} NMR** (126 MHz, DMSO-d_6): δ = 39.2 (C-2'), 61.6 (C-5'), 70.7 (C-3'), 83.5 (C-1'), 87.9 (C-4'), 117.5 (d, $^{4}J_{C,F}$ = 3.6 Hz, C-5), 139.7 (C-8), 150.3 (d, $^{3}J_{C,F}$ = 20.0 Hz, C-4), 157.6 (d, $^{3}J_{C,F}$ = 21.2 Hz, C-6), 158.5 (d, $^{1}J_{C,F}$ = 201.5 Hz, C-2) ppm. **^{19}F{^{1}H} NMR** (471 MHz, DMSO-d_6): δ = –52.1 ppm.

CFP-R	**^{1}H NMR** (500 MHz, DMSO-d_6): δ = 3.59 (ddd, ^{2}J = 12.0 Hz, ^{3}J = 5.4 Hz, ^{3}J = 4.0 Hz, 1H, H-5'A), 3.70 (ddd, ^{2}J = 12.0 Hz, ^{3}J = 5.1 Hz, ^{3}J = 4.2 Hz, 1H, H-5'B), 3.99 (ddd, ^{3}J = 4.1 Hz, ^{3}J = 4.0 Hz, ^{3}J = 4.0 Hz, 1H, H-4'), 4.18 (ddd, ^{3}J = 4.9 Hz, ^{3}J = 4.9 Hz, ^{3}J = 4.9 Hz, 1H, H-3'), 4.52 (ddd, ^{3}J = 5.3 Hz, ^{3}J = 5.1 Hz, ^{3}J = 5.1 Hz, 1H, H-2'), 5.07 (dd, ^{3}J = 5.4 Hz, ^{3}J = 5.4 Hz, 1H, 5'-OH), 5.25 (d, ^{3}J = 5.4 Hz, 1H, 3'-OH), 5.59 (d, ^{3}J = 5.7 Hz, 1H, 2'-OH), 5.94 (d, ^{3}J = 4.9 Hz, 1H, H-1'), 8.95 (s, 1H, H-8) ppm. **^{13}C{^{1}H} NMR** (126 MHz, DMSO-d_6): δ = 60.8 (C-5'), 69.8 (C-3'), 74.0 (C-2'), 85.6 (C-4'), 88.3 (C-1'), 130.5 (d, $^{4}J_{C,F}$ = 4.9 Hz, C-5), 146.4 (C-8), 150.6 (d, $^{3}J_{C,F}$ = 18.5 Hz, C-6), 153.5 (d, $^{3}J_{C,F}$ = 17.4 Hz, C-4), 156.1 (d, $^{1}J_{C,F}$ = 214.1 Hz, C-2) ppm. **^{19}F{^{1}H} NMR** (471 MHz, DMSO-d_6): δ = –51.7 ppm.
CFP-dR	**^{1}H NMR** (500 MHz, DMSO-d_6): δ = 2.37 (ddd, ^{2}J = 13.5 Hz, ^{3}J = 6.4 Hz, ^{3}J = 4.2 Hz, 1H, H-2'A), 2.71 (ddd, ^{2}J = 13.5 Hz, ^{3}J = 6.3 Hz, ^{3}J = 6.3 Hz, 1H, H-2'B), 3.53 (dd, ^{2}J = 11.8 Hz, ^{3}J = 4.5 Hz, 1H, H-5'A), 3.61 (dd, ^{2}J = 11.8 Hz, ^{3}J = 4.5 Hz, 1H, H-5'B), 3.89 (ddd, ^{3}J = 4.3 Hz, ^{3}J = 4.3 Hz, ^{3}J = 3.7 Hz, 1H, H-4'), 4.43 (ddd, ^{3}J = 5.7 Hz, ^{3}J = 3.9 Hz, ^{3}J = 3.9 Hz, 1H, H-3'), 4.94 (br s, 1H, 5'-OH), 5.37 (br s, 1H, 3'-OH), 6.36 (dd, ^{3}J = 6.4 Hz, ^{3}J = 6.4 Hz, 1H, H-1'), 8.89 (s, 1H, H-8) ppm. **^{13}C{^{1}H} NMR** (126 MHz, DMSO-d_6): δ = 39.3 (C-2'), 61.1 (C-5'), 70.1 (C-3'), 84.3 (C-1'), 88.1 (C-4'), 130.5 (d, $^{4}J_{C,F}$ = 5.0 Hz, C-5), 146.5 (d, $^{5}J_{C,F}$ = 2.9 Hz, C-8), 150.6 (d, $^{3}J_{C,F}$ = 18.5 Hz, C-6), 153.5 (d, $^{3}J_{C,F}$ = 17.4 Hz, C-4), 156.1 (d, $^{1}J_{C,F}$ = 214.2 Hz, C-2) ppm. **^{19}F{^{1}H} NMR** (471 MHz, DMSO-d_6): δ = –51.7 ppm.
DCP-R	**^{1}H NMR** (500 MHz, DMSO-d_6): δ = 3.59 (ddd, ^{2}J = 12.0 Hz, ^{3}J = 5.4 Hz, ^{3}J = 4.0 Hz, 1H, H-5'A), 3.71 (ddd, ^{2}J = 12.0 Hz, ^{3}J = 4.6 Hz, ^{3}J = 4.6 Hz, 1H, H-5'B), 3.99 (ddd, ^{3}J = 4.1 Hz, ^{3}J = 3.9 Hz, ^{3}J = 3.9 Hz, 1H, H-4'), 4.18 (ddd, ^{3}J = 4.9 Hz, ^{3}J = 4.9 Hz, ^{3}J = 4.9 Hz, 1H, H-3'), 4.52 (ddd, ^{3}J = 5.3 Hz, ^{3}J = 5.1 Hz, ^{3}J = 5.1 Hz, 1H, H-2'), 5.08 (dd, ^{3}J = 5.4 Hz, ^{3}J = 5.4 Hz, 1H, 5'-OH), 5.25 (d, ^{3}J = 5.4 Hz, 1H, 3'-OH), 5.59 (d, ^{3}J = 5.7 Hz, 1H, 2'-OH), 5.97 (d, ^{3}J = 4.9 Hz, 1H, H-1'), 8.98 (s, 1H, H-8) ppm. **^{13}C{^{1}H} NMR** (126 MHz, DMSO-d_6): δ = 60.7 (C-5'), 69.8 (C-3'), 74.0 (C-2'), 85.7 (C-4'), 88.3 (C-1'), 131.0 (C-5), 146.4 (C-8), 149.8 (C-6), 151.1 (C-2), 153.1 (C-4) ppm.
DCP-dR	**^{1}H NMR** (500 MHz, DMSO-d_6): δ = 2.38 (ddd, ^{2}J = 13.5 Hz, ^{3}J = 6.4 Hz, ^{3}J = 4.2 Hz, 1H, H-2'A), 2.71 (ddd, ^{2}J = 13.5 Hz, ^{3}J = 6.2 Hz, ^{3}J = 6.2 Hz, 1H, H-2'B), 3.54 (ddd, ^{2}J = 11.8 Hz, ^{3}J = 5.0 Hz, ^{3}J = 5.0 Hz, 1H, H-5'A), 3.62 (ddd, ^{2}J = 11.8 Hz, ^{3}J = 5.0 Hz, ^{3}J = 5.0 Hz, 1H, H-5'B), 3.89 (ddd, ^{3}J = 4.3 Hz, ^{3}J = 4.3 Hz, ^{3}J = 3.8 Hz, 1H, H-4'), 4.43 (m_c, 1H, H-3'), 4.93 (dd, ^{3}J = 5.5 Hz, ^{3}J = 5.5 Hz, 1H, 5'-OH), 5.36 (d, ^{3}J = 4.4 Hz, 1H, 3'-OH), 6.39 (dd, ^{3}J = 6.4 Hz, ^{3}J = 6.4 Hz, 1H, H-1'), 8.92 (s, 1H, H-8) ppm. **^{13}C{^{1}H} NMR** (126 MHz, DMSO-d_6): δ = 39.4 (C-2'), 61.1 (C-5'), 70.1 (C-3'), 84.3 (C-1'), 88.2 (C-4'), 131.0 (C-5), 146.5 (C-8), 149.7 (C-6), 151.0 (C-2), 152.8 (C-4) ppm.
DAP-R	**^{1}H NMR** (500 MHz, DMSO-d_6): δ = 3.53 (ddd, ^{2}J = 12.1 Hz, ^{3}J = 5.8 Hz, ^{3}J = 3.7 Hz, 1H, H-5'A), 3.64 (ddd, ^{2}J = 12.1 Hz, ^{3}J = 3.1 Hz, ^{3}J = 3.1 Hz, 1H, H-5'B), 3.90 (ddd, ^{3}J = 3.5 Hz, ^{3}J = 3.2 Hz, ^{3}J = 3.2 Hz, 1H, H-4'), 4.09 (dd, ^{3}J = 4.7 Hz, ^{3}J = 3.1 Hz, 1H, H-3'), 4.51 (dd, ^{3}J = 5.5 Hz, ^{3}J = 5.5 Hz,1H, H-2'), 5.09 (br s, 1H, 3'-OH), 5.35 (br s, 1H, 2'-OH), 5.43 (br s, 1H, 5'-OH), 5.71 (d, ^{3}J = 6.2 Hz, 1H, H-1'), 5.71 (br s, 2H, 2-NH_2), 6.76 (br s, 2H, 6-NH_2), 7.91 (s, 1H, H-8) ppm. **^{13}C{^{1}H} NMR** (126 MHz, DMSO-d_6): δ = 61.7 (C-5'), 70.7 (C-3'), 73.2 (C-2'), 85.5 (C-4'), 87.0 (C-1'), 113.6 (C-5), 136.2 (C-8), 151.4 (C-4), 156.2 (C-2), 160.0 (C-6) ppm.

DAP-dR	**^{1}H NMR** (500 MHz, DMSO-d_6): δ = 2.19 (ddd, ^{2}J = 13.1 Hz, ^{3}J = 6.0 Hz, ^{3}J = 2.8 Hz, 1H, H-2'A), 2.58 (ddd, ^{2}J = 13.1 Hz, ^{3}J = 8.0 Hz, ^{3}J = 5.7 Hz, 1H, H-2'B), 3.51 (dd, ^{2}J = 11.8 Hz, ^{3}J = 4.2 Hz, 1H, H-5'A), 3.58 (dd, ^{2}J = 11.8 Hz, ^{3}J = 4.5 Hz, 1H, H-5'B), 3.84 (ddd, ^{3}J = 4.2 Hz, ^{3}J = 4.2 Hz, ^{3}J = 2.5 Hz, 1H, H-4'), 4.35 (m_c, 1H, H-3'), 5.27 (d, ^{3}J = 3.4 Hz, 1H, 3'-OH), 6.16 (dd, ^{3}J = 8.0 Hz, ^{3}J = 6.0 Hz, 1H, H-1'), 6.18 (br s, 2H, 2-NH_2), 7.25 (br s, 2H, 6-NH_2), 8.00 (s, 1H, H-8) ppm. 5'-OH not observed. **^{13}C{^{1}H} NMR** (126 MHz, DMSO-d_6): δ = 39.4 (C-2'), 61.8 (C-5'), 70.9 (C-3'), 83.1 (C-1'), 87.7 (C-4'), 112.9 (C-5), 136.6 (C-8), 151.0 (C-4), 154.7 (C-2), 158.1 (C-6) ppm.



Suppl. Fig 1. XTT assay results showing the effect of chemically and enzymatically produced cladribine (2CA-dR$_{chem}$ and 2CA-dR$_{enz}$) on (A) HL-60 leukemic cells; IC$_{50}$ (2CA-dR$_{chem}$) = 0.182 ± 0.061 µM, IC$_{50}$ (2CA-dR$_{enz}$) = 0.087 ± 0.037 µM and (B) CCRF-CEM cells; IC$_{50}$ (2CA-dR$_{chem}$) = 0.388±0.315 µM, IC$_{50}$ (2CA-dR$_{enz}$) = 0.613±0.257 µM. The results shown represent the mean ± SD of three independent trials.



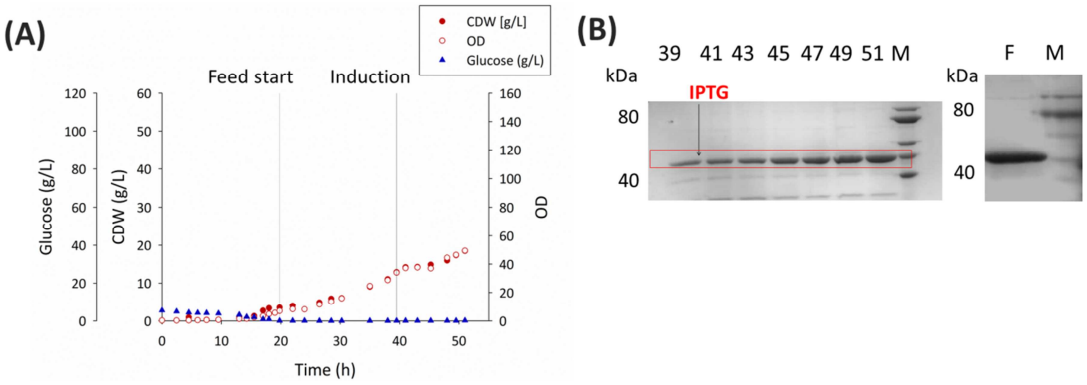
Suppl. Fig. 2. Cytotoxicity assay of several nucleoside analogues against (A) acute myeloid leukemia (HL60) cells, (B) normal human embryonic kidney cells (HEK-293), dose-response of effective drugs versus (C) HL-60, (d) HEK-293. The viability was assessed by XTT assay and the positive control in the first tests is chemically-produced cladribine (2CA-dR$_{chem}$). The results shown represent the mean ± SD of three independent trials.

Appendix I: Overview for the different NPs overexpression results

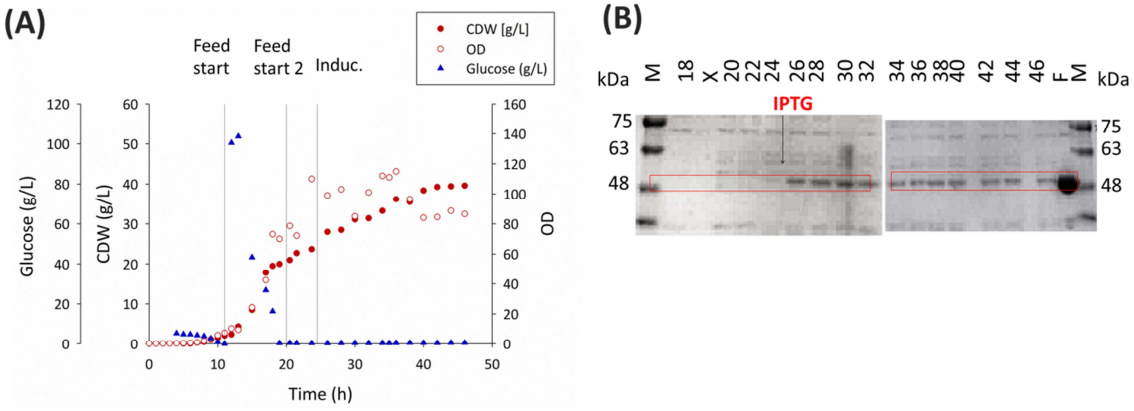
Results listed here are to be viewed together with the relative data *vide supra* (Section 5.1) (Table 5).

Figures show the Overexpression of recombinant NPs in *E. coli* BL21-Gold; **(A)** OD, DCW and extracellular glucose concentration in KLF cultivations, **(B)** SDS-PAGE analyses of protein. M: marker, F: expression in shake flask, lanes labeling denote time (h).

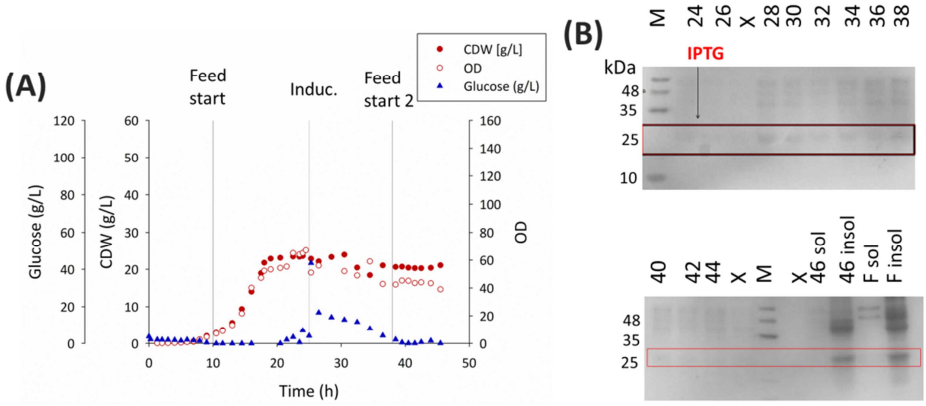
➢ Fed-batch cultivation I: PyNP 04 (48.4 kDa)



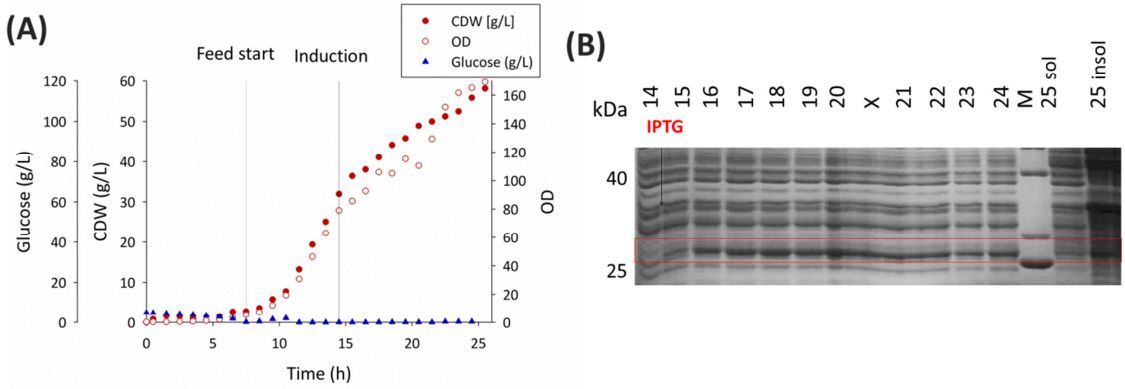
➢ Fed-batch cultivation II: PyNP 02 (46 kDa)



➢ Fed-batch cultivation III: PNP 02 (26 kDa)



➢ Fed-batch cultivation IV: PNP 02 (26 kDa)



Appendix II: Structure elucidation results

2-chloroadenosine: 2-chloroadenine (67.8 mg, 0.4 mmol) was dissolved in DMSO and uridine was added (195.3 mg, 0.8 mmol) in 2 mM potassium phosphate buffer pH=7. Enzymes were added and the reaction took place for 3 h at 60 °C. The total yield was 80 % which was dried in vacuo: **^{1}H NMR** (500 MHz, DMSO-d_6): δ = 3.55 (ddd, ^{2}J = 12.0 Hz, ^{3}J = 6.0 Hz, ^{3}J = 4.0 Hz, 1H, H-5'A), 3.66 (ddd, ^{2}J = 12.0 Hz, ^{3}J = 4.4 Hz, ^{3}J = 4.4 Hz, 1H, H-5'B), 3.94 (ddd, ^{3}J = 3.8 Hz, ^{3}J = 3.8 Hz, ^{3}J = 3.5 Hz, 1H, H-4'), 4.13 (m_c, 1H, H-3'), 4.52 (m_c, 1H, H-2'), 5.05 (dd, ^{3}J = 5.6 Hz, ^{3}J = 5.6 Hz, 1H, 5'-OH), 5.19 (br s, 1H, 3'-OH), 5.46 (br s, 1H, 2'-OH), 5.81 (d, ^{3}J = 6.0 Hz, 1H, H-1'), 7.84 (br s, 2H, 6-NH_2), 8.38 (s, 1H, H-8) ppm. **^{13}C{^{1}H} NMR** (126 MHz, DMSO-d_6): δ = 61.4 (C-5'), 70.4 (C-3'), 73.6 (C-2'), 85.7 (C-4'), 87.4 (C-1'), 118.2 (C-5), 140.0 (C-8), 150.3 (C-4), 153.0 (C-2), 156.8 (C-6) ppm.

2-chloro-2'-deoxyadenosine: 2-chloroadenine (67.8 mg, 0.4 mmol) was dissolved in DMSO and thymidine was added (193.7 mg, 0.8 mmol) in 2 mM potassium phosphate buffer pH=7. Enzymes were added and the reaction took place for 5 h at 40 °C. The total yield was 60 % which was dried in vacuo: **^{1}H NMR** (500 MHz, DMSO-d_6): δ = 2.28 (ddd, ^{2}J = 13.3 Hz, ^{3}J = 6.2 Hz, ^{3}J = 3.3 Hz, 1H, H-2'A), 2.64 (ddd, ^{2}J = 13.3 Hz, ^{3}J = 7.5 Hz, ^{3}J = 5.8 Hz, 1H, H-2'B), 3.51 (ddd, ^{2}J = 11.7 Hz, ^{3}J = 5.6 Hz, ^{3}J = 4.7 Hz, 1H, H-5'A), 3.60 (ddd, ^{2}J = 11.7 Hz, ^{3}J = 4.9 Hz, ^{3}J = 4.9 Hz, 1H, H-5'B), 3.86 (ddd, ^{2}J = 4.5 Hz, ^{3}J = 4.5 Hz, ^{3}J = 2.9 Hz, 1H, H-4'), 4.38 (m_c, 1H, H-3'), 4.95 (dd, ^{3}J = 5.6 Hz, ^{3}J = 5.6 Hz, 1H, 5'-OH), 5.30 (d, ^{3}J = 4.0 Hz, 1H, 3'-OH), 6.26 (dd, ^{3}J = 7.3 Hz, ^{3}J = 6.3 Hz, 1H, H-1'), 7.80 (br s, 2H, 6-NH_2), 8.35 (s, 1H, H-8) ppm. **^{13}C{^{1}H} NMR** (126 MHz, DMSO-d_6): δ = 39.2 (C-2'), 61.6 (C-5'), 70.7 (C-3'), 83.5 (C-1'), 87.9 (C-4'), 118.1 (C-5), 139.8 (C-8), 150.0 (C-4), 152.9 (C-2), 156.8 (C-6) ppm.

2-fluoroadenosine: 2-fluoroadenine (114.8 mg, 0.7 mmol) was dissolved in DMSO and uridine was added (366.3 mg, 1.5 mmol) in 2 mM potassium phosphate buffer pH=7. Enzymes were added and the reaction took place for 5 h at 60 °C. The total yield was 90 % which was dried in vacuo: **^{1}H NMR** (500 MHz, DMSO-d_6): δ = 3.54 (ddd, ^{2}J = 12.0 Hz, ^{3}J = 5.9 Hz, ^{3}J = 4.2 Hz, 1H, H-5'A), 3.66 (ddd, ^{2}J = 12.0 Hz, ^{3}J = 4.5 Hz, ^{3}J = 4.5 Hz, 1H, H-5'B), 3.93 (ddd, ^{3}J = 3.8 Hz, ^{3}J = 3.7 Hz, ^{3}J = 3.7 Hz, 1H, H-4'), 4.12 (ddd, ^{3}J = 4.3 Hz, ^{3}J = 4.3 Hz, ^{3}J = 3.9 Hz, 1H, H-3'), 4.51 (ddd, ^{3}J = 5.5 Hz, ^{3}J = 5.5 Hz, ^{3}J = 5.3 Hz, 1H, H-2'), 5.05 (dd, ^{3}J = 5.6 Hz, ^{3}J = 5.6 Hz, 1H, 5'-OH), 5.19 (d, ^{3}J = 4.7 Hz, 1H, 3'-OH), 5.46 (d, ^{3}J = 6.0 Hz, 1H, 2'-OH), 5.78 (d, ^{3}J = 5.9 Hz, 1H, H-1'), 7.86 (br s, 2H, 6-NH_2), 8.34 (s, 1H, H-8) ppm. **^{13}C{^{1}H} NMR** (126 MHz, DMSO-d_6): δ = 61.4 (C-5'), 70.3 (C-3'), 73.5 (C-2'), 85.6 (C-4'), 87.4 (C-1'), 117.5 (d, $^{4}J_{C,F}$ = 3.6 Hz, C-5), 139.9 (C-8), 150.6 (d, $^{3}J_{C,F}$ = 20.3 Hz, C-4), 157.6 (d, $^{3}J_{C,F}$ = 20.9 Hz, C-6), 158.5 (d, $^{1}J_{C,F}$ = 202.8 Hz, C-2) ppm. **^{19}F{^{1}H} NMR** (471 MHz, DMSO-d_6): δ = –52.1 ppm.

2-fluoro-2'-deoxyadenosine: 2-fluoroadenine (114.8 mg, 0.7 mmol) was dissolved in DMSO and thymidine was added (338 mg, 1.4 mmol) in 2 mM potassium phosphate buffer pH=7. Enzymes were added and the reaction took place for 5 h at 40 °C. The total yield was 85 % which was dried in vacuo: **^{1}H NMR** (500 MHz, DMSO-d_6): δ = 2.26 (ddd, ^{2}J = 13.3 Hz, ^{3}J = 6.2 Hz, ^{3}J = 3.3 Hz, 1H, H-2'A), 2.65 (ddd, ^{2}J = 13.3 Hz, ^{3}J = 7.4 Hz, ^{3}J = 6.0 Hz, 1H, H-2'B), 3.50 (ddd, ^{2}J = 11.7 Hz, ^{3}J = 5.5 Hz, ^{3}J = 5.5 Hz, 1H, H-5'A), 3.59 (ddd, ^{2}J = 11.7 Hz, ^{3}J = 5.0 Hz, ^{3}J = 5.0 Hz, 1H, H-5'B), 3.85 (m_c, 1H, H-4'), 4.38 (m_c, 1H, H-3'), 4.95 (dd, ^{3}J = 5.6 Hz, ^{3}J = 5.6 Hz, 1H, 5'-OH), 5.31 (d, ^{3}J = 4.2 Hz, 1H, 3'-OH), 6.23 (dd, ^{3}J = 6.8 Hz, ^{3}J = 6.8 Hz, 1H, H-1'), 7.83 (br s, 2H, 6-NH_2), 8.31 (s, 1H, H-8) ppm. **^{13}C{^{1}H} NMR** (126 MHz, DMSO-d_6): δ = 39.2 (C-2'), 61.6 (C-5'), 70.7 (C-3'), 83.5 (C-1'), 87.9 (C-4'), 117.5 (d, $^{4}J_{C,F}$ = 3.6 Hz, C-5), 139.7 (C-8), 150.3 (d, $^{3}J_{C,F}$ = 20.0 Hz, C-4), 157.6 (d, $^{3}J_{C,F}$ = 21.2 Hz, C-6), 158.5 (d, $^{1}J_{C,F}$ = 201.5 Hz, C-2) ppm. **^{19}F{^{1}H} NMR** (471 MHz, DMSO-d_6): δ = –52.1 ppm.

6-chloro-2-fluoropurine riboside: 6-chloro-2-fluoropurine (216 mg, 1.3 mmol) was mixed with uridine (610 mg, 2.5 mmol) in 2 mM potassium phosphate buffer pH=7. Enzymes were added and the

reaction took place for 8 h at 60 °C. The total yield was 70 % which was dried in vacuo: **^{1}H NMR** (500 MHz, DMSO-d_6): δ = 3.59 (ddd, ^{2}J = 12.0 Hz, ^{3}J = 5.4 Hz, ^{3}J = 4.0 Hz, 1H, H-5'A), 3.70 (ddd, ^{2}J = 12.0 Hz, ^{3}J = 5.1 Hz, ^{3}J = 4.2 Hz, 1H, H-5'B), 3.99 (ddd, ^{3}J = 4.1 Hz, ^{3}J = 4.0 Hz, ^{3}J = 4.0 Hz, 1H, H-4'), 4.18 (ddd, ^{3}J = 4.9 Hz, ^{3}J = 4.9 Hz, ^{3}J = 4.9 Hz, 1H, H-3'), 4.52 (ddd, ^{3}J = 5.3 Hz, ^{3}J = 5.1 Hz, ^{3}J = 5.1 Hz, 1H, H-2'), 5.07 (dd, ^{3}J = 5.4 Hz, ^{3}J = 5.4 Hz, 1H, 5'-OH), 5.25 (d, ^{3}J = 5.4 Hz, 1H, 3'-OH), 5.59 (d, ^{3}J = 5.7 Hz, 1H, 2'-OH), 5.94 (d, ^{3}J = 4.9 Hz, 1H, H-1'), 8.95 (s, 1H, H-8) ppm. **^{13}C{^{1}H} NMR** (126 MHz, DMSO-d_6): δ = 60.8 (C-5'), 69.8 (C-3'), 74.0 (C-2'), 85.6 (C-4'), 88.3 (C-1'), 130.5 (d, $^{4}J_{C,F}$ = 4.9 Hz, C-5), 146.4 (C-8), 150.6 (d, $^{3}J_{C,F}$ = 18.5 Hz, C-6), 153.5 (d, $^{3}J_{C,F}$ = 17.4 Hz, C-4), 156.1 (d, $^{1}J_{C,F}$ = 214.1 Hz, C-2) ppm. **^{19}F{^{1}H} NMR** (471 MHz, DMSO-d_6): δ = –51.7 ppm.

6-chloro-2-fluoropurine-2'-deoxyriboside: 6-chloro-2-fluoropurine (216 mg, 1.3 mmol) was mixed with thymidine (606 mg, 2.5 mmol) in 2 mM potassium phosphate buffer pH=7. Enzymes were added and the reaction took place for 3 h at 40 °C. The total yield was 80 % which was dried in vacuo: **^{1}H NMR** (500 MHz, DMSO-d_6): δ = 2.37 (ddd, ^{2}J = 13.5 Hz, ^{3}J = 6.4 Hz, ^{3}J = 4.2 Hz, 1H, H-2'A), 2.71 (ddd, ^{2}J = 13.5 Hz, ^{3}J = 6.3 Hz, ^{3}J = 6.3 Hz, 1H, H-2'B), 3.53 (dd, ^{2}J = 11.8 Hz, ^{3}J = 4.5 Hz, 1H, H-5'A), 3.61 (dd, ^{2}J = 11.8 Hz, ^{3}J = 4.5 Hz, 1H, H-5'B), 3.89 (ddd, ^{3}J = 4.3 Hz, ^{3}J = 4.3 Hz, ^{3}J = 3.7 Hz, 1H, H-4'), 4.43 (ddd, ^{3}J = 5.7 Hz, ^{3}J = 3.9 Hz, ^{3}J = 3.9 Hz, 1H, H-3'), 4.94 (br s, 1H, 5'-OH), 5.37 (br s, 1H, 3'-OH), 6.36 (dd, ^{3}J = 6.4 Hz, ^{3}J = 6.4 Hz, 1H, H-1'), 8.89 (s, 1H, H-8) ppm. **^{13}C{^{1}H} NMR** (126 MHz, DMSO-d_6): δ = 39.3 (C-2'), 61.1 (C-5'), 70.1 (C-3'), 84.3 (C-1'), 88.1 (C-4'), 130.5 (d, $^{4}J_{C,F}$ = 5.0 Hz, C-5), 146.5 (d, $^{5}J_{C,F}$ = 2.9 Hz, C-8), 150.6 (d, $^{3}J_{C,F}$ = 18.5 Hz, C-6), 153.5 (d, $^{3}J_{C,F}$ = 17.4 Hz, C-4), 156.1 (d, $^{1}J_{C,F}$ = 214.2 Hz, C-2) ppm. **^{19}F{^{1}H} NMR** (471 MHz, DMSO-d_6): δ = –51.7 ppm.

2,6-dichloropurine riboside: 2,6-dichoropurine (236 mg, 1.2 mmol) was mixed with uridine (610 mg, 2.5 mmol) in 2 mM potassium phosphate buffer pH=7. Enzymes were added and the reaction took place for 24 h at 60 °C. The total yield was 60 % which was dried in vacuo: **^{1}H NMR** (500 MHz, DMSO-d_6): δ = 3.59 (ddd, ^{2}J = 12.0 Hz, ^{3}J = 5.4 Hz, ^{3}J = 4.0 Hz, 1H, H-5'A), 3.71 (ddd, ^{2}J = 12.0 Hz, ^{3}J = 4.6 Hz, ^{3}J = 4.6 Hz, 1H, H-5'B), 3.99 (ddd, ^{3}J = 4.1 Hz, ^{3}J = 3.9 Hz, ^{3}J = 3.9 Hz, 1H, H-4'), 4.18 (ddd, ^{3}J = 4.9 Hz, ^{3}J = 4.9 Hz, ^{3}J = 4.9 Hz, 1H, H-3'), 4.52 (ddd, ^{3}J = 5.3 Hz, ^{3}J = 5.1 Hz, ^{3}J = 5.1 Hz, 1H, H-2'), 5.08 (dd, ^{3}J = 5.4 Hz, ^{3}J = 5.4 Hz, 1H, 5'-OH), 5.25 (d, ^{3}J = 5.4 Hz, 1H, 3'-OH), 5.59 (d, ^{3}J = 5.7 Hz, 1H, 2'-OH), 5.97 (d, ^{3}J = 4.9 Hz, 1H, H-1'), 8.98 (s, 1H, H-8) ppm. **^{13}C{^{1}H} NMR** (126 MHz, DMSO-d_6): δ = 60.7 (C-5'), 69.8 (C-3'), 74.0 (C-2'), 85.7 (C-4'), 88.3 (C-1'), 131.0 (C-5), 146.4 (C-8), 149.8 (C-6), 151.1 (C-2), 153.1 (C-4) ppm.

2,6-dichloropurine-2'-deoxyriboside: 2,6-dichloropurine (236 mg, 1.3 mmol) was mixed with thymidine (606 mg, 2.5 mmol) in 2 mM potassium phosphate buffer pH=7. Enzymes were added and the reaction took place for 3 h at 40 °C. The total yield was 75 % which was dried in vacuo: **^{1}H NMR** (500 MHz, DMSO-d_6): δ = 2.38 (ddd, ^{2}J = 13.5 Hz, ^{3}J = 6.4 Hz, ^{3}J = 4.2 Hz, 1H, H-2'A), 2.71 (ddd, ^{2}J = 13.5 Hz, ^{3}J = 6.2 Hz, ^{3}J = 6.2 Hz, 1H, H-2'B), 3.54 (ddd, ^{2}J = 11.8 Hz, ^{3}J = 5.0 Hz, ^{3}J = 5.0 Hz, 1H, H-5'A), 3.62 (ddd, ^{2}J = 11.8 Hz, ^{3}J = 5.0 Hz, ^{3}J = 5.0 Hz, 1H, H-5'B), 3.89 (ddd, ^{3}J = 4.3 Hz, ^{3}J = 4.3 Hz, ^{3}J = 3.8 Hz, 1H, H-4'), 4.43 (m_c, 1H, H-3'), 4.93 (dd, ^{3}J = 5.5 Hz, ^{3}J = 5.5 Hz, 1H, 5'-OH), 5.36 (d, ^{3}J = 4.4 Hz, 1H, 3'-OH), 6.39 (dd, ^{3}J = 6.4 Hz, ^{3}J = 6.4 Hz, 1H, H-1'), 8.92 (s, 1H, H-8) ppm. **^{13}C{^{1}H} NMR** (126 MHz, DMSO-d_6): δ = 39.4 (C-2'), 61.1 (C-5'), 70.1 (C-3'), 84.3 (C-1'), 88.2 (C-4'), 131.0 (C-5), 146.5 (C-8), 149.7 (C-6), 151.0 (C-2), 152.8 (C-4) ppm.

2-aminoadenosine: 2-aminoadenine (113 mg, 0.8 mmol) was mixed with uridine (366 mg, 1.5 mmol) in 2 mM potassium phosphate buffer pH=7. Enzymes were added and the reaction took place for 3 h at 60 °C. The total yield was 75 % which was dried in vacuo: **^{1}H NMR** (500 MHz, DMSO-d_6): δ = 3.53 (ddd, ^{2}J = 12.1 Hz, ^{3}J = 5.8 Hz, ^{3}J = 3.7 Hz, 1H, H-5'A), 3.64 (ddd, ^{2}J = 12.1 Hz, ^{3}J = 3.1 Hz, ^{3}J = 3.1 Hz, 1H, H-5'B), 3.90 (ddd, ^{3}J = 3.5 Hz, ^{3}J = 3.2 Hz, ^{3}J = 3.2 Hz, 1H, H-4'), 4.09 (dd, ^{3}J = 4.7 Hz, ^{3}J = 3.1 Hz, 1H, H-3'), 4.51 (dd, ^{3}J = 5.5 Hz, ^{3}J = 5.5 Hz,1H, H-2'), 5.09 (br s, 1H, 3'-OH), 5.35 (br s, 1H, 2'-OH), 5.43 (br s, 1H, 5'-OH), 5.71 (d, ^{3}J = 6.2 Hz, 1H, H-1'), 5.71 (br s, 2H, 2-NH_2), 6.76 (br s, 2H, 6-NH_2), 7.91 (s, 1H, H-8)

ppm. **^{13}C{^{1}H} NMR** (126 MHz, DMSO-d_6): δ = 61.7 (C-5'), 70.7 (C-3'), 73.2 (C-2'), 85.5 (C-4'), 87.0 (C-1'), 113.6 (C-5), 136.2 (C-8), 151.4 (C-4), 156.2 (C-2), 160.0 (C-6) ppm.

2-amino-2'-deoxyadenosine: 2-aminoadenine (113 mg, 0.8 mmol) was mixed with thymidine (363 mg, 1.5 mmol) in 2 mM potassium phosphate buffer pH=7. Enzymes were added and the reaction took place for 5 h at 40 °C. The total yield was 85 % which was dried in vacuo: **^{1}H NMR** (500 MHz, DMSO-d_6): δ = 2.19 (ddd, ^{2}J = 13.1 Hz, ^{3}J = 6.0 Hz, ^{3}J = 2.8 Hz, 1H, H-2'A), 2.58 (ddd, ^{2}J = 13.1 Hz, ^{3}J = 8.0 Hz, ^{3}J = 5.7 Hz, 1H, H-2'B), 3.51 (dd, ^{2}J = 11.8 Hz, ^{3}J = 4.2 Hz, 1H, H-5'A), 3.58 (dd, ^{2}J = 11.8 Hz, ^{3}J = 4.5 Hz, 1H, H-5'B), 3.84 (ddd, ^{3}J = 4.2 Hz, ^{3}J = 4.2 Hz, ^{3}J = 2.5 Hz, 1H, H-4'), 4.35 (m_c, 1H, H-3'), 5.27 (d, ^{3}J = 3.4 Hz, 1H, 3'-OH), 6.16 (dd, ^{3}J = 8.0 Hz, ^{3}J = 6.0 Hz, 1H, H-1'), 6.18 (br s, 2H, 2-NH_2), 7.25 (br s, 2H, 6-NH_2), 8.00 (s, 1H, H-8) ppm. 5'-OH not observed. **^{13}C{^{1}H} NMR** (126 MHz, DMSO-d_6): δ = 39.4 (C-2'), 61.8 (C-5'), 70.9 (C-3'), 83.1 (C-1'), 87.7 (C-4'), 112.9 (C-5), 136.6 (C-8), 151.0 (C-4), 154.7 (C-2), 158.1 (C-6) ppm.