

Purine and pyrimidine salvage pathway in thermophiles: a valuable source of biocatalysts for the industrial production of nucleic acid derivatives

Jon Del Arco & Jesús Fernández-Lucas

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Purine and pyrimidine salvage pathway in thermophiles: a valuable source of biocatalysts for the industrial production of nucleic acid derivatives

Jon Del Arco¹ · Jesús Fernández-Lucas^{1,2} 

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Abstract

Due to their similarity to natural counterparts, nucleic acid derivatives (nucleobases, nucleosides, and nucleotides, among others) are interesting molecules for pharmaceutical, biomedical, or food industries. For this reason, there is increasing worldwide demand for the development of efficient synthetic processes for these compounds. Chemical synthetic methodologies require numerous protection-deprotection steps and often lead to the presence of undesirable by-products or enantiomeric mixtures. These methods also require harsh operating conditions, such as the use of organic solvents and hazard reagents. Conversely, enzymatic production by whole cells or enzymes improves regio-, stereo-, and enantioselectivity and provides an eco-friendly alternative. Because of their essential role in purine and pyrimidine scavenging, enzymes from purine and pyrimidine salvage pathways are valuable candidates for the synthesis of many different nucleic acid components. In recent years, many different enzymes from these routes, such as nucleoside phosphorylases, nucleoside kinases, 2'-deoxyribosyltransferases, phosphoribosyl transferases, or deaminases, have been successfully employed as biocatalysts in the production of nucleobase, nucleoside, or nucleotide analogs. Due to their great activity and stability at extremely high temperatures, the use of enzymes from thermophiles in industrial biocatalysis is gaining momentum. Thermophilic enzymes not only display unique characteristics such as temperature, chemical, and pH stability but also provide many different advantages from an industrial perspective. This mini-review aims to cover the most representative enzymatic approaches for the synthesis of nucleic acid derivatives. In this regard, we provide detailed comments about enzymes involved in crucial steps of purine and pyrimidine salvage pathways in thermophiles, as well as their biological role, biochemical characterization, active site mechanism, and substrate specificity. In addition, the most interesting synthetic examples reported in the literature are also included.

Keywords Extremophiles · Bioprocesses · Salvage pathway · Enzymatic production

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Introduction

Nucleic acids are indispensable molecules for all living organisms, since they are essential for many different biochemical processes involving storage and transfer of genetic information. In this respect, the use of nucleic acid derivatives (analogues of nucleosides, nucleotides, or nucleobases) as active pharmaceutical ingredients (APIs) is extensively reported in the literature (De Clercq 2005; Galmarini et al. 2002; Jordheim et al. 2013; Lapponi et al. 2016; Parker 2009). Additionally, some nucleoside-5'-monophosphates (NMPs) are also commonly used in the food industry. For example, inosine monophosphate (IMP) and guanosine monophosphate (GMP) have been found to elicit an umami taste in humans (Behrens et al. 2011). Moreover, growth and immune system

development in babies are improved by dietary nucleotide supplementation (Hawkes et al. 2006). Moreover, deoxynucleoside-5'-triphosphates (dNTPs) are extensively used as reagents in a wide range of molecular biology applications, such as DNA amplification, real-time PCR, DNA sequencing, DNA microarray experiments, and site-directed mutagenesis, among others. As a result, there is a significant demand for nucleic acid derivatives in food, biomedical, and pharmaceutical industries, and therefore, many different research groups and companies are focused on the development of innovative and efficient synthetic processes.

Traditionally, these molecules have been synthesized by different multi-step chemical methods employing harmful solvents and reagents (Mikhailopulo 2007), leading to poor or moderate yields and low product purity. Nowadays, enzymatic or chemoenzymatic production by whole cells or enzymes offers an environmentally friendly alternative to traditional chemical processes. They also have many advantages, such as one-pot one-step reactions, high stereo- and regioselectivity, and the absence of undesirable by-products (Boryski 2008; Del Arco and Fernández-Lucas 2017; Fernández-Lucas 2015; Fresco-Taboada et al. 2013; Lapponi et al. 2016; Lewkowicz and Iribarren 2006, 2017; Mikhailopulo 2007, Mikhailopulo and Miroshnikov 2010).

Despite the advantages of enzyme-mediated bioconversions, several factors hinder their industrial application: (i) the low stability and short lifespan of the enzyme under the rather harsh operating conditions often needed in some industrial processes, (ii) the high cost of purification of recombinant enzymes, (iii) the poor solubility of some organic substrates in aqueous media, and (iv) difficulties associated to the isolation of the biocatalyst from the reaction medium for reutilization.

To develop more efficient and economical processes, the modern industry increasingly demands new biocatalysts which exhibit activity and stability under aggressive reaction conditions, e.g., extreme pH values, high temperatures, or presence of organic solvents. In this context, enzymes from extremophilic organisms are valuable biocatalysts for the industrial implementation of synthetic bioprocesses (Dumorme et al. 2017; Raddadi et al. 2015; Van Den Burg 2003).

Thermophiles are organisms which can grow at high temperatures, and they can be classified into three different groups: moderate thermophiles (optimal growth from 45 to 70 °C), extreme thermophiles (optimal growth > 70 °C), and hyperthermophiles (optimal growth from 80 to 110 °C) (Zeldes et al. 2015). Enzymes from thermophilic microorganisms, also known as thermozyms, are typically stable and active at high temperatures. Therefore, the use of thermozyms offers the possibility of raising the temperature of the reaction and this helps to prevent microbial contamination, improves substrate solubility, and leads to reduced viscosity in the reaction mixture. As a consequence, substrate diffusion is increased, and higher overall reaction rates are

obtained. Additionally, the production of thermozyms using mesophilic organisms as host strains reduces the difficulty and costs of the purification process. For example, a heat shock treatment can be used to denature most of the mesophilic proteins in the cleared lysates, avoiding tedious multi-step purification processes (Del Arco et al. 2017a, b, c; Haki and Rakshit 2003).

Interestingly, pairwise and multiple sequence alignment, as well as functional and structural comparison between mesophilic and thermophilic enzymes reveal that both, thermophilic and mesophilic enzymes, display similar domain organization, catalytic mechanisms, and active site architectures (Vieille and Zeikus 2001). Taking into an account all aforementioned advantages (easier purification and crystallization, similar activity, and higher thermal stability than mesophilic counterparts, among others), thermostable enzymes have also played a key role as structural models for a better understanding of molecular mechanisms about structure-function relationship in the enzyme families (Vieille and Zeikus 2001).

Purine and pyrimidine metabolism are essential metabolic routes in all living organisms, because nucleobases are key molecules for the synthesis of DNA and RNA, proteins, and other metabolites. Biosynthesis of nucleotides is performed through two different metabolic routes: *de novo* and salvage pathways. Most organisms synthesize purine and pyrimidine nucleotides from simple precursors like glycine, glutamine, or aspartate (*de novo* pathway). Meanwhile, in salvage pathways, the requirement for nucleobases is satisfied by means of different metabolic routes with endogenous and/or exogenous sources of preformed nitrogen bases. Therefore, many different enzymes from purine and pyrimidine salvage pathways can be employed in the synthesis of nucleic acid derivatives. In fact, these natural enzymatic schemes have been reproduced in industry.

Purine salvage pathway

Purine salvage is an essential mechanism for the recovery of purine nucleobases in organisms (Fig. 1). Because *de novo* pathways for purine synthesis are absent in the majority of parasites, enzymes involved in purine salvage have become attractive therapeutic targets for the design of inhibitors against parasitic diseases (Del Arco and Fernández-Lucas 2017; El Kouni 2003). Consequently, scientific reports on the functional and structural characterization of these enzymes have provided a lot of valuable information about their substrate specificity and reaction mechanism(s). Because of this, many different enzymes from these metabolic pathways, such as purine nucleoside phosphorylases (PNPs), nucleoside kinases (NKs), purine phosphoribosyltransferases (purine PRTs), deaminases (DA), and nucleoside 2'-deoxyribosyltransferases (NDTs), have been

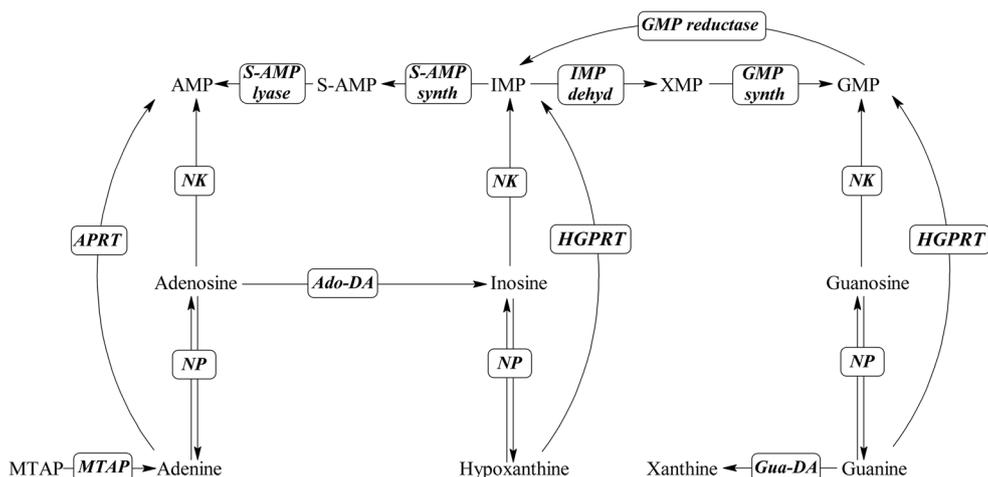


Fig. 1 Purine salvage pathway in *Haloferax volcanii* (Stuer-Lauridsen and Nygaard 1998). NK, nucleoside kinase; MTAP, S-methyl-5'-thioadenosine phosphorylase; Gua-DA, guanine deaminase; Ado-DA, adenosine deaminase; Gua-DA, guanine deaminase; APRT, adenine phosphoribosyltransferase; HGPRT, hypoxanthine-guanine

phosphoribosyltransferase; S-AMP lyase, succinyladenylate lyase; S-AMP synth, succinyladenylate synthetase; GMP reductase, guanosine-5'-monophosphate reductase; IMP dehyd, inosine-5'-monophosphate dehydrogenase; GMP synth, guanosine-5'-monophosphate synthetase

successfully employed as biocatalysts for the synthesis of nucleic acid components. As a consequence, the search for these types of enzymes in thermophilic microorganisms is now the main goal of many different research projects.

Purine nucleoside phosphorylases (PNPs)

PNP (EC 2.4.2.1) belongs to the family of nucleoside phosphorylases (NPs) (Bzowska et al. 2000; Lewkowicz and Iribarren 2006; Pugmire and Ealick 2002). PNP catalyzes the reversible phosphorolysis of the C–N glycosidic bond of nucleosides and 2'-deoxynucleosides to generate ribose- or deoxyribose-1-phosphate along with the corresponding purine nucleobase (Pugmire and Ealick 2002) (Fig. 2). It is a key enzyme in the purine salvage pathway, being responsible for the recovery of purine bases from endo- and/or exogenous sources.

According to their oligomeric state, two different forms of PNPs have been identified: low-molecular-mass PNPs with trimeric quaternary structure and high-molecular-mass PNPs, which are hexamers. Trimeric PNPs are strictly specific for 6-oxopurine nucleosides (i.e., inosine, guanosine, and derivatives) and bases (i.e., hypoxanthine, guanine, and derivatives), whereas hexameric PNPs can only recognize 6-oxo and 6-aminopurine derivatives. Trimeric PNPs usually originate from mammals and higher organisms, while hexameric PNPs are more common in bacteria and protozoan (Lewkowicz and Iribarren 2006; Yehia et al. 2017). For this reason, bacterial and protozoan PNPs have been studied as therapeutic targets for the treatment of parasitic diseases such as malaria, leishmaniasis, Chagas disease, or sleeping sickness (Berg et al. 2010; El Kouni 2003).

PNPs display a high promiscuity in substrate recognition and can act on many different nucleobase and nucleoside analogs. However, regarding ribose moiety recognition, PNPs are highly specific for ribonucleosides and display low activity values when 2'-deoxynucleosides or 2', 3', or 5' modified nucleosides (Fig. S1) are employed as glycosyl donors (Lewkowicz and Iribarren 2006; Yehia et al. 2017). Consequently, different protein engineering strategies, such as site-directed mutagenesis and directed evolution, have been tried out in attempts to improve the recognition of ribose moiety variants, e.g., 3'-deoxynucleosides, 5'-modified nucleosides, 2',3'-dideoxynucleosides, 2'-arabinosyl nucleosides, or

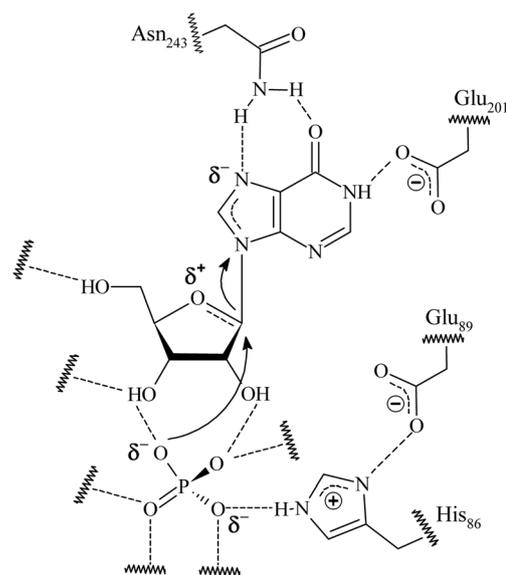


Fig. 2 Catalytic mechanism of human PNP (Pugmire and Ealick 2002)

halogenated 2'-deoxynucleosides (Fig. S1) (Birmingham et al. 2014; Lewkowicz and Iribarren 2006; Yehia et al. 2017).

Due to the wide range of substrates they accept and the possibility of using them in combination with other enzymes such as kinases (NK) or pyrimidine nucleoside phosphorylases (PyNP), PNPs have been extensively employed in the synthesis of modified nucleosides and nucleotides (Lewkowicz and Iribarren 2006; Yehia et al. 2017; Fernández-Lucas 2015). Considering the advantages of the thermophilic enzymes, in recent decades several research groups have focused on the search for NPs from thermophilic strains.

Okuyama et al. (1996) purified and characterized a trimeric PNP (which they named PNP I) and a PyNP from *Bacillus stearothermophilus* TH 6-2. Likewise, they applied isolated NPs to the enzymatic phosphorolysis of inosine and guanosine (PNP I) or thymidine and uridine (PyNP). In 1997, the same authors cloned, expressed, and purified the recombinant PNP I and PNP II from *B. stearothermophilus* (Hamamoto et al. 1997a, b). This second PNP displays a substrate specificity different from that of PNP I, acting more efficiently towards adenosine than on other nucleosides. The presence of two different PNP genes encoded in *B. stearothermophilus* genome suggests that the expression levels of both enzymes could be regulated by intracellular nucleoside concentrations.

A well-known strategy to synthesize nucleosides is the transglycosylation reaction carried out by two NPs. Thereby, the combined use of PyNP and PNP in one-pot, two-step reactions to produce purine nucleosides from pyrimidine nucleosides, or vice versa, is frequently used as a synthetic strategy (Fig. 3).

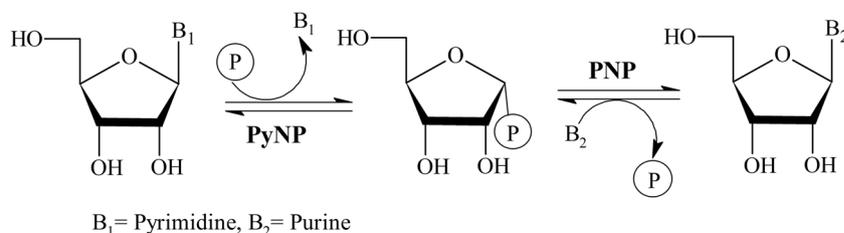
Hori et al. (1991) developed a one-pot, two-step process for the enzymatic production of 5-methyluridine from inosine and thymine by employing co-immobilized PNP and PyNP from *B. stearothermophilus* JTS 859 in a sequential reaction. A similar approach was used by Taran et al. (2009) by combining partially purified preparations of PNP II and PyNP from *Geobacillus stearothermophilus* B-2194 (basonym *B. stearothermophilus*) covalently attached to aminopropylsilochrom AP-CPG-170. Immobilized derivatives exhibited high enzymatic activity and stability at 70 °C and were reused up to 20 times in the synthesis of 2'-deoxyadenosine. Finally, as a proof of concept, immobilized biocatalysts were used in the enzymatic synthesis of several APIs, such as 2-chloro-2'-deoxyadenosine (cladribine, antitumoral FDA drug), 2-fluoro-2'-

deoxyadenosine (prodrug used in suicide gene therapy in cancer treatment), and arabinosyl adenine (vidarabine, ara-A, an FDA-approved drug for the treatment of herpes simplex and varicella zoster viruses, and a potential precursor of fludarabine and clofarabine, FDA-approved drugs for cancer treatment).

Trelles et al. (2005) carried out a microbial screening to find transglycosylation activities in a microorganism library of more than 30 microorganisms. As a result, *B. stearothermophilus* ATCC 12980 was chosen as the best biocatalyst for the enzymatic production of different 6-substituted purine nucleosides, such as 6-chloropurine, 6-methoxypurine, 6-mercaptopurine, and 6-iodopurine ribonucleosides. Furthermore, *B. stearothermophilus* ATCC 12980 whole cells were immobilized in different matrices, such as agar, agarose, and polyacrylamide and efficiently employed in the synthesis of different 6-substituted purine ribo and 2'-deoxyribonucleosides from uridine or thymidine at different reaction times (1–24 h) with a conversion percentage ranging from 29% (6-iodopurine riboside) to 96% (6-aminopurine riboside) (Trelles et al. 2005). More recently, the synthesis of anti-HCV compounds has been carried out using *G. stearothermophilus* (basonym *B. stearothermophilus*) whole cells (Rivero et al. 2012). In this work, the authors tested the ability of *G. stearothermophilus* CECT 43 as a biocatalyst for the synthesis of different 6-halogenated purine ribo and 2'-deoxyribonucleosides. The authors also tested different reaction parameters, such as molar ratio, temperature, and reaction time. Moreover, to evaluate the potential of this microorganism as an industrial biocatalyst, *G. stearothermophilus* CECT 43 whole cells were immobilized by entrapment in 2–5% (w/v) agarose and 15–25% (w/v) polyacrylamide. In view of the results obtained, agarose at 4% was selected as the best option for the immobilization process. The immobilized derivative was shown to be active and stable for 6 months (when stored at 4 °C) and reusable during 70 cycles, leading to a production of 379 mg/L of 6-chloropurine-2'-deoxyribonucleoside.

In 2004, Tahirov et al. (2004) solved the three-dimensional structure of PNP from *Thermus thermophilus* HB8 (*Tt*PNP) and shed light on its unusual substrate specificity. Although the *Tt*PNP structure seems to be similar to those of hexameric PNPs (e.g., PNPs from *Escherichia coli*) and should also bind 6-oxo and 6-aminopurines, functional characterization revealed a strict specificity for guanosine (similar to trimeric PNPs), and no activity was observed on adenosine (Fig. 4). The reason of this unexpected specificity for guanosine could

Fig. 3 Transglycosylation reaction catalyzed by the PyNP-PNP multi-enzymatic system



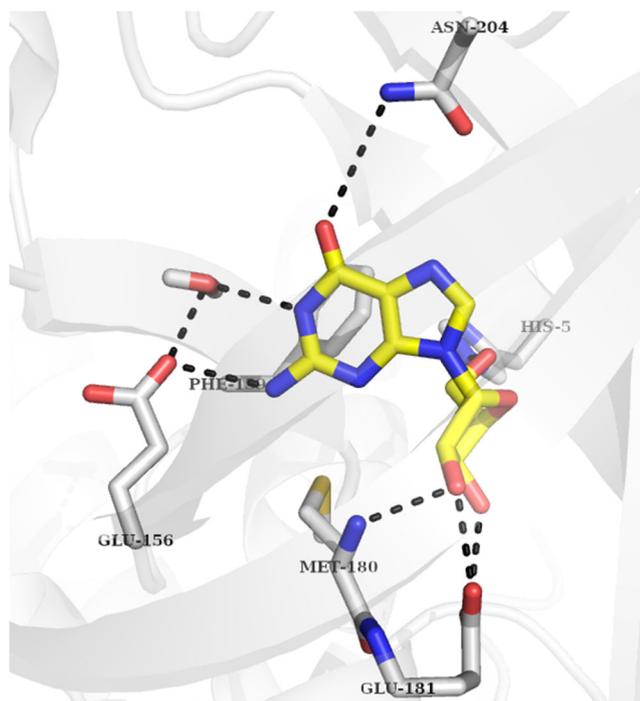


Fig. 4 Overall structure of *Tt*PNP (cartoon) in complex with guanosine (sticks) (PDB access code 1ODJ). The probable hydrogen bonds are shown in dotted lines

be different interaction between Asn204 and Glu156 residues and corresponding nucleobase. When *Tt*PNP binds adenosine, the nucleobase is mainly recognized by Asn204 residue and reaction evolves in unfavorable way, whereas when *Tt*PNP binds guanosine, Glu156 is the main residue involved in nucleobase recognition and the reaction proceeds towards nucleoside hydrolysis.

Interestingly, recent studies described the presence of a second trimeric PNP in *T. thermophilus* HB8 (Tomoike et al. 2013). However, other studies suggest that it could be a monomer in solution (Almendros et al. 2012). This novel PNP displays strict activity on adenosine, showing that *T. thermophilus* PNPs have an atypical reversal substrate specificity when compared to that of their corresponding trimeric and hexameric homologs (Bzowska et al. 2000; Lewkowicz and Iribarren 2006; Pugmire and Ealick 2002).

In a very interesting study, Almendros et al. (2009) tested several strains of *T. thermophilus* in their search for thermostable biocatalysts for the synthesis of ribo and 2'-deoxyribonucleosides. As a result, *T. thermophilus* HB27, PRQ-16, PRQ-25, B, Fiji3A1, and VG7 were selected as the most relevant candidates. Additionally, the reaction mixtures did not show the presence of any by-products arising from the action of adenosine deaminases, a common problem in mesophiles.

As a follow-up of this previous work, Almendros et al. (2012) performed the cloning, expression, production, and purification of recombinant PNP I and PNP II from *T.*

thermophilus HB27. The biochemical characterization revealed that both enzymes are active at extremely high temperatures (optimum activity at 95 °C) and display optimal activity between pH 4–6. Analytical ultracentrifugation and substrate specificity studies showed *Tt*PNP I and *Tt*PNP II as unusual PNPs. On the one hand, *Tt*PNP I was shown to be a hexameric high-molecular-mass PNP with an atypical strict specificity over 6-oxopurines, as expected from previous reports (Tahirov et al. 2004). On the other hand, *Tt*PNP II was shown to be a monomeric low-molecular-mass PNP with a remarkable preference for 6-amino and 2,6-diaminopurines. Since no monomeric low-molecular-mass PNP has been reported to date, *Tt*PNP II represents the first milestone in this respect (Almendros et al. 2012). Moreover, both enzymes displayed a remarkable stability in the presence of organic solvents, such as DMFA, DMSO, or 2-MeTHF, which are usually employed to increase the solubility of some purine bases and nucleosides.

In 2007, the production and characterization of a PNP from the hyperthermophilic archaeon *Pyrococcus furiosus* (*Pf*PNP) was reported as the first archaeal PNP (Cacciapuoti et al. 2007). *Pf*PNP is a hexamer, active and stable at extremely high temperatures (optimum temperature at 120 °C, and *T_m* ranging from 110 to 120 °C), with a very interesting resilience to the presence of SDS. Despite the fact that *Pf*PNP is a homo-hexamer, this enzyme displays similar substrate specificity to that of trimeric mammalian PNPs which are specific for guanosine and inosine. Furthermore, the results of site-directed mutagenesis on Cys254 and Cys256 strongly suggested that this Cys pair forms an intra-subunit disulfide bond which plays an important role in the extremely high thermostability of *Pf*PNP.

By the same token, the production of thermostable PNPs from *Deinococcus geothermalis* (*Dg*PNP) and *G. thermoglucosidasius* (*Gt*PNP) was reported by Zhou et al. (2013). In this exhaustive study, the authors analyzed the thermostability, substrate specificity and kinetic properties of both enzymes. Thermal studies revealed that *Dg*PNP was active and stable at 55 °C for 8 h, whereas *Gt*PNP was stable and active at 70 °C during the same time period. Similar to high-molecular-mass PNPs, *Dg*PNP and *Gt*PNP recognized both 6-oxo and 6-aminopurine nucleosides. Additionally, both enzymes were tested against cytidine, 2'-deoxycytidine and 2'-amino and 2'-fluoro modified nucleosides (Fig. S1). According to previous experimental data, several fluoropurine nucleosides were produced by a multi-enzymatic PyNP-PNP system (Zhou et al. 2013). In this regard, the transglycosylation reaction catalyzed by *Tt*PNP and *Dg*PNP (*Tt*PyNP/*Dg*PNP) led to the formation of 2'-fluoro-2'-deoxyadenosine (24% conversion at 55 °C and 24 h). Moreover, the tandem reaction catalyzed by *Tt*PyNP/*Gt*PNP led to the synthesis of 2'-fluoroarabinosyl adenine from 2'-fluoroarabinosyl uracil and adenine (14% conversion at 80 °C and 24 h).

5'-Methylthioadenosine phosphorylases (MTAPs)

MTAPs (EC 2.4.2.28) catalyze the reversible phosphorolysis of 5'-deoxy-5'-methylthioadenosine (MTA) in the presence of inorganic phosphate (Fig. 5). Despite the fact that MTAP is an essential enzyme in the metabolism of polyamines, it is also important for the salvage of adenine, and many authors consider MTAP to be part of the purine salvage pathway (Fig. 1). Because of their similarity to PNPs, some authors suggest that MTAPs may belong to the class of low- and high-molecular-mass PNPs. Nevertheless, there is no consensus on this issue.

In 1994, the presence of a MTAP in the thermoacidophilic archaeon *Sulfolobus solfataricus* (*SsMTAP*) was reported for the first time (Cacciapuoti et al. 1994). *SsMTAP* displayed an optimal activity at 120 °C, with negligible loss of activity when incubated at 100 °C for 2 h, and a half-life of 5 and 15 min after incubation at 130 or 140 °C. In addition, the enzyme showed stability and activity in the presence of some denaturing agents (9 M urea, 4 M guanidine hydrochloride, or 0.075% SDS), organic solvents (negligible loss of activity in presence of 50% v/v methanol, 50% v/v ethanol, or 50% v/v dimethylformamide), and detergents (1% v/v Triton X-100) when stored at room temperature for 24 h. In addition, *SsMTAP* was shown not only to be capable of acting on MTA but also to be active towards inosine, guanosine, and adenosine, and its efficiency was higher than that of MTA. *SsMTAP* was crystallized as a hexamer with three disulfide bonds and this fact can account for its extreme thermal stability (Appleby et al. 2001; Cacciapuoti et al. 1999).

More recently, Cacciapuoti et al. (2003) reported the production, purification, and characterization of an MTAP from the hyperthermophilic archaea *Pyrococcus furiosus* (*PfMTAP*). As expected, the enzyme is active and stable at extremely high temperatures (maximum activity at 125 °C, 98% of retained activity after 5 h at 100 °C, and half-life of 43 min at 130 °C). Furthermore, like *SsMTAP*, *PfMTAP* is a hexamer with a molecular mass of 180 kDa and is active using 5'-methylthioadenosine, adenosine, inosine, and guanosine. However, the kinetic analysis reveals that *PfMTAP* shows a greater preference for 6-aminopurine nucleosides. *PfMTAP* is

also resistant to protein denaturant agents, such as detergents, organic solvents, and salts, when stored at high temperature (Cacciapuoti et al. 2003).

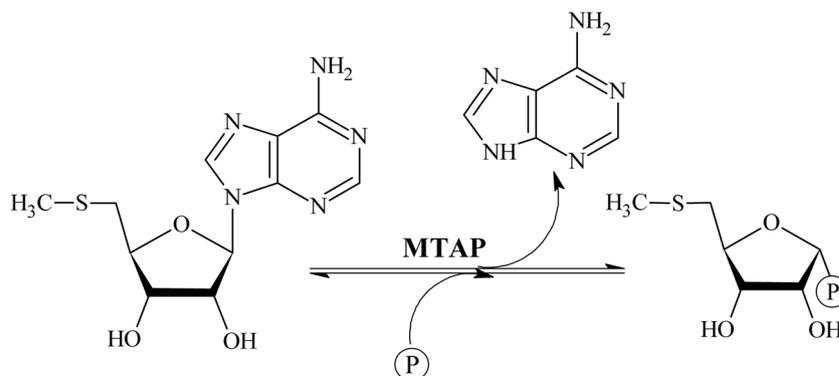
Interestingly, Cacciapuoti et al. (2005) discovered the presence of a second MTAP encoded in the genome of *S. solfataricus* (*SsMTAP* II), which is strictly specific for MTA. This finding suggests that *SsMTAP* II may not play a very important role in purine scavenging in *S. solfataricus*. Furthermore, these authors studied the substitution of several Cys residues by site-directed mutagenesis and demonstrated that they are essential for the enzyme stability.

Despite the great functional and structural knowledge of thermophilic MTAPs, there are not many examples of their use as biocatalysts in the literature. One interesting approach was developed by Zhou et al. (2013). They cloned, produced, purified, and also characterized the MTAP from hyperthermophile *Aeropyrum pernix* (*ApMTAP*). *ApMTAP* displayed high activity at extremely high temperatures (> 99 °C) and was stable for more than 69 h at 90 °C. To evaluate its substrate specificity, kinetic analysis of phosphorolysis of inosine and adenosine was performed in 50 mM phosphate, pH 7.0, at 80 °C. Results derived from these experiments showed adenosine to be the best substrate (lower K_M and k_{cat} values than inosine). Finally, the authors performed the synthesis of 2'-deoxy-2'-fluoroadenosine by employing a sequential transglycosylation reaction catalyzed by *TtPyNP* and *ApMTAP* at 80 °C and 24 h (yield 24%, 80 °C, and 24 h).

Purine phosphoribosyltransferases (purine PRTs)

Purine bases originating from the hydrolysis of nucleosides by PNPs, MTAP, NHs, or NDTs (el Kouni 2003; Fresco-Taboada et al. 2013; Schramm and Grubmeyer 2004) are used by purine PRTs to synthesize the corresponding NMPs. These enzymes catalyze the reversible transfer of the 5-phosphoribosyl group from 5-phospho- α -D-ribose-1-pyrophosphate (PRPP) to N9 in 6-amino or 6-oxopurines such as adenine, guanine, hypoxanthine, and xanthine. A divalent metal ion, usually Mg^{2+} , is necessary for the transfer reaction. PRTs are essential for purine scavenging because they are involved in the

Fig. 5 Phosphorolysis of MTA catalyzed by MTAP



formation and cleavage of the C–N glycosidic bonds in adenosine-5'-monophosphate (AMP), IMP, GMP, and xanthosine-5'-monophosphate (XMP). All the abovementioned purine PRTs display a conserved PRPP-binding motif (formed by a 13 residue “fingerprint” region), so they belong to the class of type I PRTs (Del Arco and Fernández-Lucas 2017). Nonetheless, as the enzymatic activity towards adenine differs from that towards hypoxanthine, guanine, or xanthine, they are also classified in terms of their substrate specificity. Thus, 6-aminopurine PRTs (APRTs) are specific for 6-aminopurines (adenine, 2-fluoroadenine, or 2-chloroadenine, among others) (Fig. S1) (Del Arco and Fernández-Lucas 2017; Del Arco et al. 2017b; Esipov et al. 2016; Iglesias et al. 2015), while 6-oxopurine PRTs (HPRT, GPRT, XPRT, HGPRT, and HGXPRT) can use different 6-oxopurine bases (hypoxanthine, guanine, xanthine, and other 6-oxopurine analogs) as substrates (Del Arco and Fernández-Lucas 2017; Scism et al. 2007, Scism and Bachmann 2010).

As mentioned above, adenine is the preferred substrate of APRTs. However, this enzyme can recognize several 6-aminopurine analogs (2,6-diaminopurine, 6-amino-2-hydroxypurine, 6-methylpurine, zeatin, isopentenyladenine, benzyladenine, and 8-azaadenine) as substrates (Fig. S1). In spite of the mentioned substrate promiscuity, some examples of the use of APRTs for the production of NMP analogs from 6-aminopurine derivatives have been published (Del Arco and Fernández-Lucas 2017; Esipov et al. 2016).

Hansen et al. (2014) tested the biocatalytic potential of an APRT from the thermoacidophilic archaeon *S. solfataricus*, SsAPRT. This enzyme displays the highest activity at pH 7.5–8.5; however, a distinct peak of activity at pH 4.5 was also noted. In a similar way, Del Arco et al. (2017b) produced and biochemically characterized APRT from *T. thermophilus* HB8, T_{HB8} APRT. T_{HB8} APRT was shown to be active and stable in a pH range of 5–6, and in a temperature range from 60 to 90 °C (optimal activity pH 6 and 70–80 °C). More recently, the substrate specificity of an APRT from *T. thermophilus* HB27 (T_{HB27} APRT) has been tested (Esipov et al. 2016). T_{HB27} APRT displays activity towards different 6-aminopurine derivatives, such as 2,6-diaminopurine, 2-fluoroadenine, 2-chloroadenine, 2-methoxyadenine, or N1-methyladenine (Fig. S1). Interestingly, these authors also include a multi-enzymatic system composed of ribokinase gene from *Thermus* sp. 2.9 and phosphoribosylpyrophosphate synthetase (PRPP-synthetase) from *T. thermophilus* HB27 as the PRPP source (Fig. 6). The production of 2-fluoroadenosine-5'-monophosphate ($\approx 37\%$) and 2-chloroadenosine-5'-monophosphate ($\approx 30\%$) from D-ribose and the corresponding nucleobase was carried out by this thermophilic cascade system (Esipov et al. 2016).

In 6-oxopurine PRTs, the oligomerization state seems to play a key role not only in catalysis but also in both stabilization of the active conformation and overall protein stability.

Thermostable 6-oxopurine PRTs are described in the literature as dimers and tetramers (Del Arco et al. 2017c, Del Arco and Fernández-Lucas 2017; Hansen et al. 2014; Sinha and Smith 2001), as illustrated by dimeric HGXPRT from *S. solfataricus* and tetrameric HGXPRT from *T. thermophilus*.

Purine PRTs are active over a broad variety of 6-oxopurine bases; thus, some examples of the use of thermophilic 6-oxopurine PRTs as biocatalysts for 5'-NMPs production have been reported. For example, HGPRTs from *Thermoanaerobacter tengcongensis*, TtHGPRT (Chen et al. 2003) and *S. solfataricus*, SsHGPRT (Hansen et al. 2014), HGXPRT from *Pyrococcus horikoshi*, PhHGXPRT (de Souza Dantas et al. 2008), and XPRT and HGXPRT from *T. thermophilus* HB8, TtXPRT (Del Arco et al. 2017b, c) and TtHGXPRT (Del Arco et al. 2017c, Del Arco et al. 2017c). It is interesting to note the unusually high activity and stability showed by TtHGXPRT under alkaline conditions (pH 8–11). This allows it to be used for the synthesis of several dietary nucleoside-5'-monophosphates, such as IMP and GMP, from high concentrations of poorly water-soluble purine bases. More recently, TtHGXPRT was covalently attached to glutaraldehyde-activated MagReSyn®Amine magnetic microspheres (Del Arco et al. 2018a) (Fig. 7). Two different approaches allowed the preparation of highly active biocatalysts: (i) enzyme immobilization by the N-terminus at pH 8.5 yielding M T_{t} HGXPRT1-3 derivatives and (ii) a multi-point covalent immobilization strategy through solvent-exposed lysine residues at pH 10 (M T_{t} HGXPRT4-5 derivatives). As observed, the thermal stability and reusability of magnetic derivative M T_{t} HGXPRT3 was higher than that of its magnetic counterpart M T_{t} HGXPRT4-5. Accordingly, M T_{t} HGXPRT3 was used in the synthesis of ribonucleoside-5'-monophosphates with an application as food additives.

More interestingly, Acosta et al. (2018) developed a multi-enzymatic system for the one-pot production of nucleic acid derivatives of interest in the pharmaceutical and food industries. Firstly, the 2'-deoxyribosyltransferase from *Lactobacillus delbrueckii*, LdNDT, performed the transglycosylation reaction using 2'-deoxyinosine or 2'-deoxyguanosine as the 2'-deoxyribose donor and different purine analogs as acceptors, to get different modified nucleosides (Fig. 8). In a second step, TtHGXPRT converted the released hypoxanthine or guanine to IMP or GMP. As a result, the combined use of LdNDT and TtHGXPRT shifted the equilibrium of the transglycosylation reaction towards product formation and transformed the resulting by-products into high added-value compounds (Fig. 8).

Purine deaminases (DAs)

Deaminases catalyze the deamination process in molecules by substituting the amino group for a keto group DAs from the purine salvage pathway in extremophiles have not been

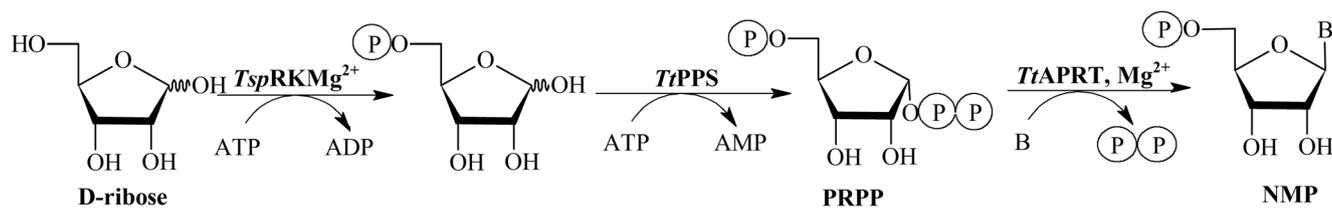


Fig. 6 Synthesis of substituted adenosine-5'-monophosphates catalyzed by the *TspRK/TtPPS/TtAPRT* multi-enzymatic cascade system (Esipov et al. 2016)

studied extensively, with most research having been carried out with those obtained from methanogens. For example, Worrell and Nagle (1990) performed an extensive characterization of the purine salvage route in *Methanobacterium thermoautotrophicum*. After studying the purine base and nucleoside recycling reactions, adenine (EC 3.5.4.2) and adenosine DA (EC 3.5.4.4) activities were detected. However, no activity was reported for guanine DA (EC 3.5.4.3). Adenosine deaminase was highly active in *M. thermoautotrophicum*, but interconversion of adenine to hypoxanthine (adenine deaminase) was slightly detected. Likewise, the detection of phosphorylation products from adenosine, guanosine, and inosine demonstrated the presence of nucleoside kinases. Moreover, since adenosine deaminase activity was also much higher than adenosine kinase activity, adenosine may be metabolized through inosine to IMP.

Besides the deaminases from methanogens, Bauer and Carlberg (1973) looked for enzymes with deaminase activity in crude extracts of *Halobacterium cutirubrum*. Guanine, guanosine, adenine, adenosine, and 2'-deoxyadenosine were tested as substrates, and deaminase activity was exclusively detected with the last two substrates. Particularly, an apparent preference for deaminating 2'-deoxyadenosine instead of adenosine was observed. Later on, further studies were carried out to

determine the substrate specificity for adenosine analogs. The enzyme was able to deaminate 8-azaadenosine, whereas no activity was detected for 6-chloropurineribofuranoside and 8-aminoadenosine (Bauer and Carlberg 1973). These results agree with those reported for both *Halobacterium volcanii* and *Halobacterium halobium* (Stuer-Lauridsen and Nygaard 1998) (Fig. 1). In this case, unlike in *M. thermoautotrophicum*, the presence of an enzyme for guanine deamination was also found. The authors suggested that in both halobacteria, adenine is converted into adenosine by adenosine phosphorylase, which later undergoes deamination to inosine by adenosine DA. Finally, the guanine derived from guanosine catabolism is transformed into xanthine by guanine deaminase (Stuer-Lauridsen and Nygaard 1998).

Hermann et al. (2007) predicted the deaminase activity of an unknown protein (Tm0936) from *Thermotoga maritima* using theoretical chemistry methods. According to the relative docking scores, this enzyme could be active on different adenosine derivatives (adenosine, S-adenosyl-L-homocysteine, or 5-methylthioadenosine). In a recent study, Hitchcock et al. (2013) identified several Tm0936 homologs and predicted possible binding substrates also by molecular docking. As a result, adenosine, 5-methylthioadenosine, S-adenosyl-L-homocysteine, and 5'-deoxyadenosine were proposed as

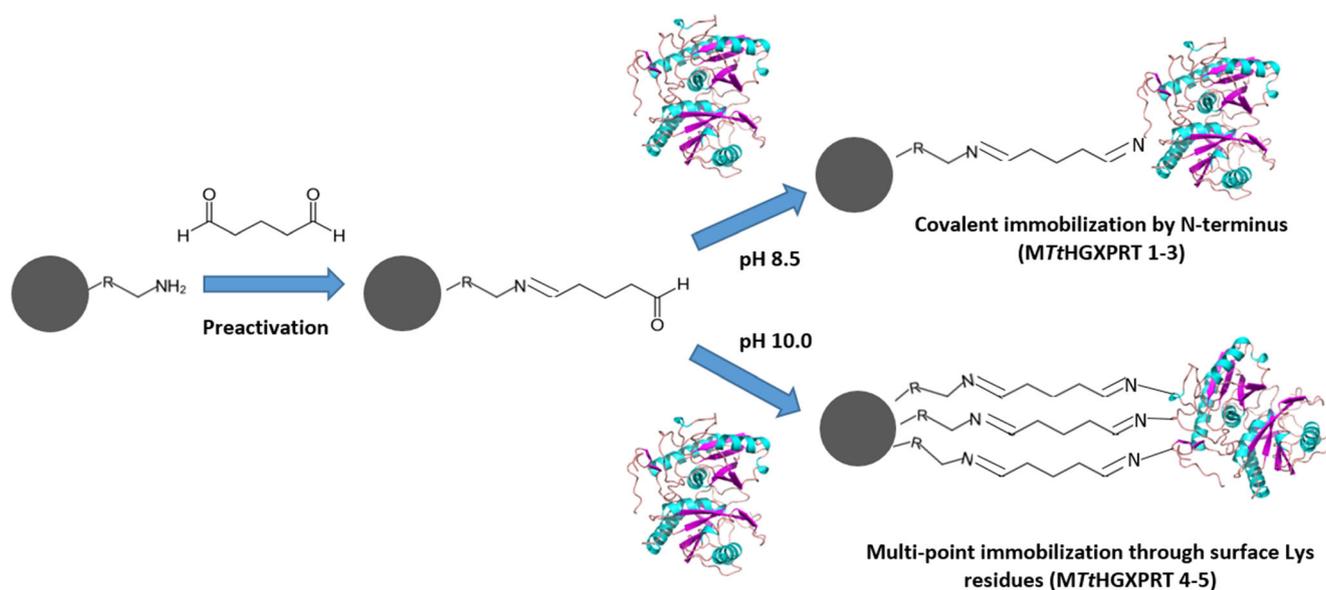


Fig. 7 Schematic representation of immobilization of recombinant *TtHGXPRT* onto glutaraldehyde-activated MagReSyn@amino microspheres

bind uridine. Thus, in view of these results, this enzyme should be renamed as *Ti*CK instead of *Ti*UCK.

Recent studies (Tanaka et al. 2016; Tomoike et al. 2011) described the presence of an unusual amino acid (Tyr93) in the active site of *Ti*CK, while in other UCKs histidine (His) is commonly found at this position. In view of this finding, the authors suggested that the strict specificity for cytidine can be attributed to this single residue. When Tyr93 was replaced by histidine (Y93H) or glutamine (Y93Q), *Ti*CK recovered the phosphorolytic activity towards uridine. Conversely, when Tyr93 was replaced by a smaller residue, such as Phe or Leu, uridine could not be accepted by *Ti*CK either (Tanaka et al. 2016; Tomoike et al. 2011) (Fig. 10).

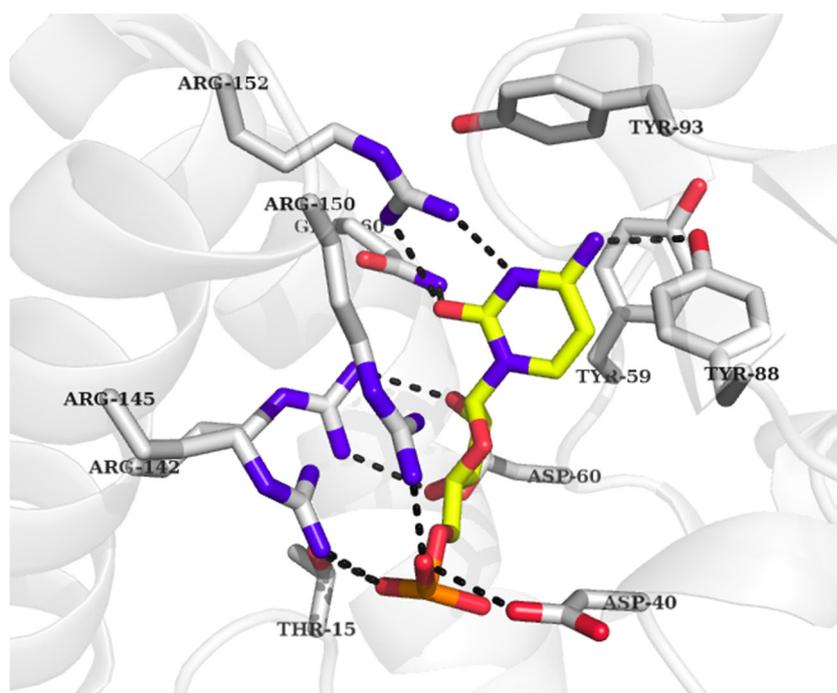
Thymidylate kinase (TMPK, EC 2.7.4.9) can be considered as a bridging enzyme between de novo and salvage pathways. In the de novo pathway, it catalyzes the ATP-Mg²⁺-dependent phosphorylation of deoxythymidine monophosphate (dTMP) to deoxythymidine diphosphate (dTDP). Meanwhile, in the salvage pathway, TMPK is responsible for the conversion of thymidine to dTMP (Arnér and Eriksson 1995). TMPK is active towards a broad range of substrate analogs, and the resulting diphosphate analog is incorporated into the newly synthesized DNA strand, which leads to chain termination (Galmarini et al. 2002; Jordheim et al. 2013). Because of this, TMPK serves as an attractive therapeutic target for the development of antibacterial, antiparasitic, and anticancer drugs. Therefore, it is not surprising that many different crystal structures of TMPKs from hyperthermophiles have been reported, such as those from *Sulfolobus tokodaii* (Biswas et al. 2017) and *T. thermophilus* (Chaudhary et al. 2013).

Pyrimidine nucleoside phosphorylases (PyNPs)

PyNPs are involved in the recovery of pyrimidine bases in prokaryotic and eukaryotic organisms. In the same way than their purine counterparts, PNPs and MTAPs, PyNPs catalyze the cleavage of the glycosidic C–N bond in pyrimidine nucleosides through a reversible phosphorolytic mechanism, where the P_i attacks the C-1' position of the ribose moiety. Depending on their substrate specificity, PyNPs can be sorted into (i) uridine phosphorylases (UPs, EC 2.4.2.3), which display a remarkable preference for uracil nucleosides as substrates and higher affinity for uridine than 2-deoxyuridine); (ii) thymidine phosphorylases (TPs, EC 2.4.2.4), which show a preference for thymidine as well as 5-substituted pyrimidine nucleosides as substrates; and (iii) non-specific PyNPs (EC 2.4.2.2) (Lewkowicz and Iribarren 2006; Yehia et al. 2017). UPs and TPs usually exist as hexamers and dimers, respectively. Both PyNPs are found in higher organisms and some bacteria such as *E. coli*. In contrast, PyNPs from lower organisms are more promiscuous and can recognize both uracil and thymine (deoxy)nucleosides. These general-purpose PyNPs are usually homodimers.

Likewise, PyNP from thermophilic *B. stearothermophilus* (*Bs*PyNP) is formed by two identical subunits, exhibiting a molecular weight of 46,271 Da (Okuyama et al. 1996). The phosphorolytic reaction is carried out with both uridine and thymidine, indicating that *Bs*PyNP acts as a general-purpose PyNP. In this regard, *Bs*PyNP can use both uridine and thymidine, as well as their derivatives, as pentose-1-phosphate donors for the synthesis of nucleoside analogs. It was later reported that *Bs*PyNP shares 41.5 and 38.1% sequence

Fig. 10 Tridimensional representation of *Ti*CK (cartoon) complexed with cytidine-5'-monophosphate (CMP) (sticks) (PDB access code 3ASZ). Dot lines represent hydrogen bonds



identity with *E. coli* and human TP, respectively. In contrast, no significant similarities were noted with *E. coli* uridine phosphorylase (Okuyama et al. 1996; Tomoki et al. 1996).

In 2012, Almendros et al. (2012) characterized a homodimeric PyNP from *T. thermophilus* (*TtPyNP*), which was active in a broad temperature range, even though it did not show great thermostability. The activity decreased by half after 2 h of incubation at 80 °C, probably due to the lability of the interaction among both monomers. Additionally, the enzymatic activity on nucleobase and nucleoside analogs was tested, revealing the capability of *TtPyNP* to accept thymine, uracil, and 2,4-dioxypyrimidines as substrates. The enzymatic activity towards arabinosyl nucleosides was found to be significantly lower than for 2'-deoxyribonucleosides (e.g., 55% of conversion was reached when 2'-deoxyuridine is used as donor, whereas 3% was obtained from arabinosyl uracil). In addition, *TtPyNP* was not active against 3' and 5' substituted nucleosides, such as 3-azido-5-phosphoribose, probably due to inadequate fitting of the pentose moiety into the active site. Finally, *TtPyNP* did not recognize pyrimidine ring substitutions at position 6, while the reaction proceeded for some 5-substituted derivatives (e.g., 5-iodouracil) (Fig. S1). Therefore, this lack of stringency on position 5 can account for its capacity to accept both uracil and thymine as substrates. As *TtPyNP* is active on 5-iodouracil, 2,6-diaminopurine, and arabinosides, it could be applied to the synthesis of nucleoside analogs used for the treatment of human diseases (Almendros 2012).

In 2012, Szeker et al. (2012) performed the cloning, production, and purification of PyNPs phosphorylases from *T. thermophilus* (*TtPyNP*) and *G. thermoglucosidasius* (*GtPyNP*). Although both enzymes displayed high thermal stability, *TtPyNP* is clearly more thermostable than *GtPyNP*, exhibiting a half-life of more than 24 h when stored at 80 °C. In addition, kinetic analyses on both enzymes revealed similar K_M and k_{cat} values for uridine phosphorylase. In contrast, *TtPyNP* showed a k_{cat} value eight times higher than that of *GtPyNP* when thymidine was used as the substrate. In addition, to evaluate the potential of both enzymes as industrial biocatalysts, their ability to catalyze the phosphorytic cleavage of several 2'-fluoro-2'-deoxynucleoside analogs was tested. In light of the results obtained, *TtPyNP* could be used in combination with a PNP for the synthesis of valuable APIs, such as 2'-fluoro-2'-deoxyguanosine.

More recently, Zhou et al. (2015a) used a multi-enzymatic approach for synthesizing several 2,6-dihalogenated nucleoside analogs, such as 2'-deoxynucleosides 2-chloro-2'-deoxyadenosine (cladribine), 2-fluoro-2'-deoxyadenosine, 2,6-dichloro-2'-deoxyribose and 2,6-diaminopurine-2'-deoxyribose, or the arabinosyl nucleosides ara-2,6-diaminopurine and ara-2,6-dichloropurine, among others. The co-immobilization of *TtPyNP* and PNP from *G. thermoglucosidasius* (*GtPNP*) on MagReSyn® epoxide-

activated spheres also led to an easy recovery of the biocatalysts. Finally, as a proof of concept, the enzymatic synthesis of 2,6-dichloro-2'-deoxyribose was successfully scaled up in a reaction volume of 50 mL with an overall 60% yield.

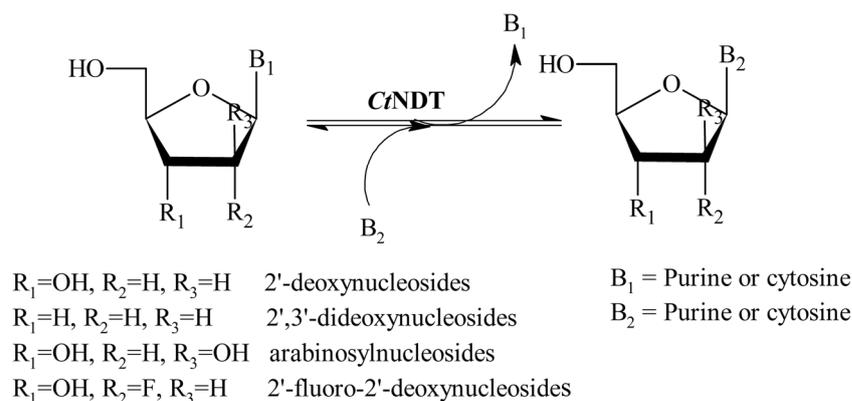
In 2015, the production of 2,6-dihalogenated purine nucleosides was performed through the same multi-enzymatic cascade system *TtPyNP/GtPNP* (Zhou et al. 2015b). For that purpose, the authors co-immobilized *TtPyNP* and *GtPNP* onto epoxy-activated magnetic microspheres and optimized certain immobilization and operational parameters (e.g., pH and temperature, enzyme loading, binding time, reaction time, thermostability, and substrate ratio). Finally, they applied *TtPyNP/GtPNP* to the enzymatic production of some 2,6-dihalogenated purine nucleosides with high conversion percentages (78.5–85.5%) and productivities (1.5–2.0 g/L/h).

In another sequential synthetic scheme, the enzymatic synthesis of 2,6-diaminopurine nucleoside and 5-methyluridine was performed using two thermostable NPs from *Aeropyrum pernix* K1 (*ApUP* and *ApPNP*). Both enzymes (i) were shown to be active and stable at extremely high temperatures (maximum activity at 90–100 °C), (ii) displayed negligible loss of activity after being stored for 1 h at 90–100 °C and in a broad pH 4–9 range (activity > 80%), and (iii) retained an activity > 60% when stored at pH 6–10 (Zhu et al. 2013).

Nucleoside 2'-deoxyribosyltransferases (NDTs)

Nucleoside 2'-nucleoside deoxyribosyltransferases (EC 2.4.2.6), also called *N*-deoxyribosyltransferases or trans-*N*-deoxyribosylases, belong to the glycosyltransferases family, as do NPs. NDTs catalyze the transglycosylation reaction of the 2'-deoxyribose moiety between purine and/or pyrimidine bases (Fresco-Taboada et al. 2013; Kaminski 2002) (Figs. 8 and 11). NDTs are key enzymes in the nucleobases salvage pathway, being responsible for the recovery of both purine and pyrimidine bases. In this regard, several protozoan and bacterial NDTs have been reported (Bosch et al. 2006; Crespo et al. 2017; Del Arco et al. 2018b; Fernández-Lucas et al. 2010; Fresco-Taboada et al. 2013, 2018; Kaminski 2002; Lawrence et al. 2009; Méndez et al. 2018; Miyamoto et al. 2007; Steenkamp and Halbich 1992). NDTs can be classified into two classes: type I NDTs (PDTs) specific for purines (Pur↔Pur), such as adenine, guanine, or hypoxanthine, and type II NDTs (NDTs), which catalyze the transfer of 2'-deoxyribose among purines and/or pyrimidines (Pur↔Pur, Pur↔Pyr, Pyr↔Pyr) (Fresco-Taboada et al. 2013; Kaminski 2002). These enzymes are strictly specific for 2'-deoxynucleosides, regioselective (N-1 glycosylation in pyrimidine and N-9 in purine), as well as stereoselective (β -anomers are exclusively formed). However, recent studies have shown that NDTs are able to ribosylate in different positions of purine

Fig. 11 Enzymatic synthesis of nucleoside analogs by *Ct*NDT



and pyrimidine rings (Fernández-Lucas et al. 2010; Fresco-Taboada et al. 2013).

Despite the potential of NDTs as industrial biocatalysts, only one thermophilic NDT has been described to date: NDT from *Chroococcidiopsis thermalis* PCC 7203, *Ct*NDT. *Ct*NDT is a homotetramer active on purine bases and 2'-deoxynucleosides, but it also acts on cytosine and 2'-deoxycytidine (Del Arco et al. 2018b). All of these unique features prevent the strict classification of *Ct*NDT into either a type I or a type II NDT and further suggest that oligomeric state and structure should complement and expand this functional classification in the future. Moreover, *Ct*NDT displays high activity values (80–100%) in the presence of 20% of several water-miscible co-solvents, such as ethyl acetate, acetone, acetonitrile, chloroform, DMF, DMSO, methanol, ethanol, 2-propanol, ethylene glycol, glycerol, and propylene glycol. *Ct*NDT also displayed activity and stability at a wide range of acid-neutral pH values (from 3 to 7) and high temperatures (from 50 to 100 °C). Finally, as a proof of concept, the enzymatic synthesis of several therapeutic nucleosides, such as arabinosyl adenine (vidarabine), arabinosyl cytosine (cytarabine), 2',3'-dideoxyinosine (didanosine), and 2'-fluoro-2'-deoxyguanosine, was carried out by *Ct*NDT (Fig. 11) (Fig. S1).

Uracil phosphoribosyltransferases (UPRTs)

UPRTs catalyze the reversible transfer of the 5-phosphoribosyl group from PRPP to the N1 of uracil, in the presence of Mg^{2+} (Fig. 12). Since uridine-5'-monophosphate, UMP, is a common precursor of all pyrimidine nucleotides, UPRT plays a key role in the pyrimidine salvage pathways (Del Arco and Fernández-Lucas 2017, Del Arco et al. 2017a). This enzyme displays a marked preference for uracil and its derivatives, recognizing neither thymine nor cytosine as substrates (Del Arco and Fernández-Lucas 2017; Del Arco et al. 2017a). These enzymes display great potential as biocatalysts; however, the high cost and low stability of PRPP hinders their industrial application.

Several thermophilic UPRTs have been described, such as dimeric UPRT from *Bacillus caldolyticus*, tetrameric UPRTs from *S. solfataricus*, *Aquifex aeolicus*, and *T. thermophilus* (Del Arco et al. 2017a). Among them, we wish to highlight the thermal activity and stability of UPRTs from *B. caldolyticus* (relative activity $\approx 60\%$ at 70 °C for 10 min), *Sulfolobus shibatae* (negligible loss of activity at 80 °C for 1 h), and *T. thermophilus* (negligible loss of activity at 70 °C for 4 h and a half-life over 70 min at 80 °C) (Del Arco et al. 2017a).

Del Arco et al. (2017a) characterized the UPRT from *T. thermophilus*, *Tt*UPRT. As expected, the enzyme showed great activity and stability across broad ranges of temperature (from 50 to 80 °C), pH (from 5.5 to 9), and ionic strength (from 0 to 500 mM sodium chloride). Interestingly, substrate specificity studies revealed that *Tt*UPRT is active towards thymine, 5-halogenated uracil derivatives, 5-hydroxymethyluracil, and 6-methyluracil (Fig. S1). This report described for the first time the application of an UPRT to the synthesis of therapeutic nucleotide analogs, such as 5-bromouridine-5'-monophosphate, 5-chlorouridine-5'-monophosphate, 5-iodouridine-5'-monophosphate, 5-hydroxy-methyluridine-5'-monophosphate, and 6-methyluridine-5'-monophosphate.

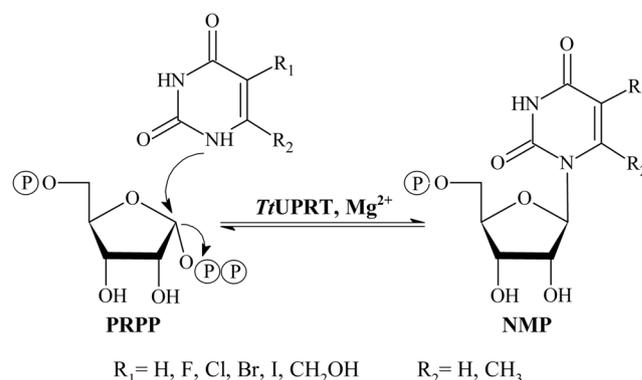


Fig. 12 Enzymatic production of pyrimidine nucleoside-5'-monophosphate analogs by *Tt*UPRT

Due to its ability to act on 5-substituted pyrimidines (a position commonly substituted in nucleotide analogs used for the treatment of human diseases), *Tt*UPRT could be applied to cancer treatment through the so-called suicide gene therapy, based on the introduction into cancer cells of the genes encoding cytosine deaminase (CD) and UPRT.

Conclusion

Purine and pyrimidine salvage enzymes are very interesting biocatalysts for the synthesis of nucleoside and nucleotide analogs. They can complement or replace other less efficient and non-environmentally friendly synthetic strategies. Enzymes from thermophiles are active and stable under extreme temperatures, broad pH ranges, and the presence of organic solvents. Because of this, the search for thermozymes is a primary focus of interest in many biotechnological firms and academic research groups. We hope that this mini-review will help other scientists to appreciate the potential of thermophilic purine and pyrimidine salvage enzymes as biocatalysts for the industrial production of nucleic acid derivatives.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals by any of the authors.

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