Nucleoside Phosphorylases

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Abstract: Nucleoside phosphorylases (NPs) are transferases that catalyse the reversible cleavage of the glycosidic bond of ribo- or deoxyribo nucleosides, in the presence of inorganic phosphate, to generate the base and ribose- or deoxyribose-1-phosphate.

Since pyrimidine as well as purine nucleoside phosphorylases exist, the combination of both enzymes makes possible the generation of purine nucleosides from pyrimidine ones. As a consequence, NPs from different sources, mainly bacterial, have been exploited as tools for the enzymatic synthesis of nucleoside analogues. These molecules are extensively used as antiviral and anticancer agents because of their ability to act as reverse transcriptase inhibitors or chain terminators in RNA or DNA synthesis.

This review covers literature reports from 2000 on, focused mainly on the synthesis of nucleosides by free and immobilised microbial whole cells, along with some examples of modified nucleosides obtained by coupling transglycosylation to other enzymatic reactions.

The biological aspects of NPs are also discussed since they became an interesting target for clinical applications due to their key role in nucleotide metabolism. Finally, brief comments about their structures and catalytic mechanisms are included.

1. INTRODUCTION

a. General Aspects

Nucleoside analogues have been extensively used in cancer and antiviral therapies [1,2] as monomers and, more recently, as building blocks for oligonucleotide therapeutics [3-5].

As antiviral agents, nucleosides inhibit replication of the viral genome whereas anticancer compounds inhibit cellular DNA replication and repair [6]. These molecules exert their action through different mechanism such as inhibition of polymerases activity or acting as chain terminators in RNA or DNA synthesis [7].

For the design of effective, selective and non-toxic drugs, a variety of strategies have been devised. These approaches involve several formal modifications of the naturally occurring nucleosides, in particular alteration of the carbohydrate moiety [8,9].

The first antiviral agents were developed over 40 years ago, however, since 1985, when the anti HIV activity of AZT [10] was discovered, many nucleoside analogues were designed as antiviral drugs against targets such as herpex, hepatitis, HIV and cytomegalovirus [11-14]. Nucleosides like 2',3'-dideoxyinosine (didanosine) [15], 2',3'-dideoxy-cytidine (zalcitabine), 2'-deoxy-3'-thiacytidine (lamivudine), 3'-azido-3'-deoxythymidine (zidovudine), 2',3'-didehydro-3'-deoxythymidine (stavudine) and 6-cyclopropylamino-2',3'-didehydro-2',3'-dideoxyguanosine (abacavir), shown in Fig. (**1**), are used in antiviral treatments [16].

Regarding anticancer nucleoside analogues used in the clinic, representative examples, 2-chloro-2'-deoxyadenosine (cladribine), 2-fluor-9-β-D-arabinofuranosyladenine (fludar-abine), 1-β-D-arabinofuranosylcytosine (cytarabine) and 2',2'-difluorodeoxycytidine (gemcitabine), are also depicted in Fig. (**1**).

Since major problems in these treatments are acquirement of resistance and side effects such as unspecific cytotoxicity [17], new compounds possessing more potent and broad activities are continually under study [18] (Table **1**).

Traditionally, nucleosides are prepared by various chemical methods which often involve difficult, inefficient and time-consuming multistage processes [26,27]. The most common route to synthesise nucleosides include the coupling of the nucleobase (or derivatives thereof) to the carbohydrate moiety. Glycosyl activation and protecting groups on the heterocyclic base and the sugar residue are usually required as well as the control of anomeric configurations, especially when preparing deoxyribosides and arabinosides. Regioeselectivity of C-N glycoside formation is also necessary when other nucleophilic groups are present in the heterocyclic bases [28]. In general, the cost of the production processes affords drugs that are inaccessible for people with small resources or for mass application. The massive availability of antiviral agents may be essential not

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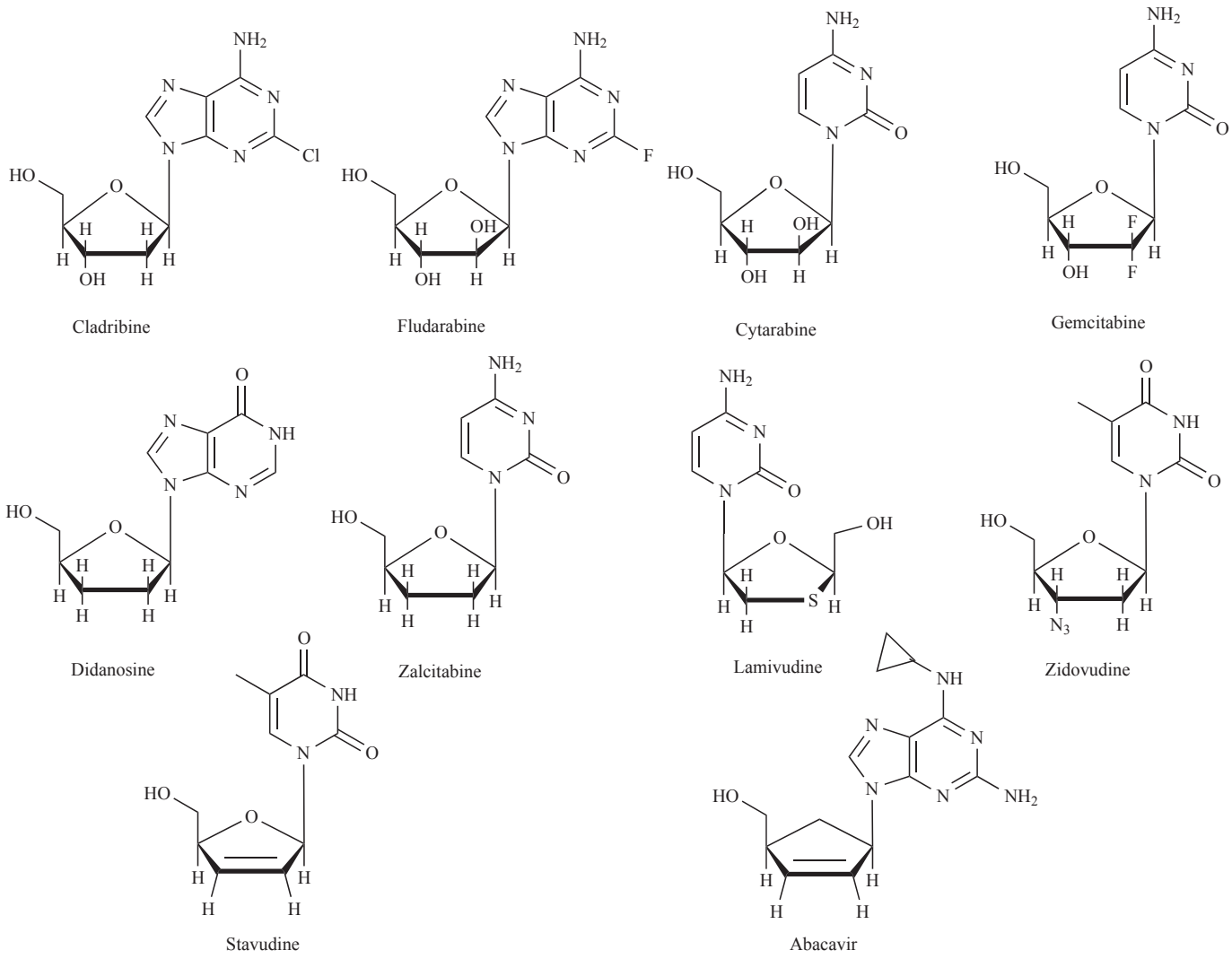


Fig. (1). Antiviral and anticancer nucleoside analogues.

Table 1. New developed bioactive nucleosides

Nucleoside	Bioactivity	Ref.
2,6-Diaminopurine dioxolane (amdoxovir)	Antiviral (HIV-1)	9
Viramidine	Antiviral	15
Nicotinamide riboside	Antitumor	19
3'-C-Triflouromethyl-2',3'-dideoxyadenosine	Antiviral (HBV)	20
3'-C-Ethynyluridine	Antitumor	21
2'-Deoxy-2'-fluorcytidine	Antiviral (HCV)	22
Imidazo(1,2-*c*-) pyrimidine base-modified nucleosides	Anti-cytomegalovirus anti-leukemic	23
Azanucleosides	Bioactivity studies in progress	24
4´-Ethynyl nucleosides	Antiviral (HIV-1)	25

only for epidemics, such as HIV infection, but also for facing the consequences of biological weapons [29].

The enzymatic synthesis of both natural and unnatural nucleosides reported in the last two decades, offer

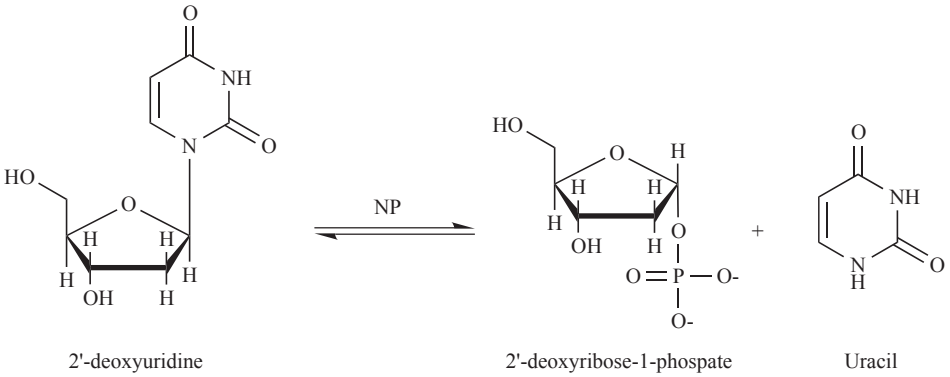


Fig. (2). Phosphorolysis of nucleosides by NPs.

advantages over the chemical methods since highly regio- and stereo controlled reactions occur [30].

b. Glycosyltransfer Reaction

Two classes of enzymes, nucleoside phosphorylases (NPs) and *N*-deoxyribosyltransferases, which transfer glycosyl residues to acceptor bases, have been used in the synthesis of nucleosides [31].

Naturally, NPs are transferases that catalyse the reversible cleavage of the glycosidic bond of ribo- and deoxyribo nucleosides, in the presence of inorganic phosphate, to generate the base and ribose- or deoxyribose-1-phosphate, Fig. (**2**).

NPs are widespread in prokaryotes, yeast and higher organisms and both pyrimidine (PyNP) and purine (PNP) nucleoside phosphorylases exist [32]. *In vivo*, phosphorolysis is highly favoured over synthesis due to coupling with other metabolic pathways. However, the thermodynamic equilibrium for PNP, but not for PyNP, is shifted towards nucleoside synthesis. Therefore, the combination of both enzymes makes possible the generation of purine nucleosides from pyrimidine ones (so called transglycosylation, Fig. (**3**). As a consequence, NPs from different sources, mainly bacterial, have been exploited as tools for the enzymatic synthesis of nucleoside analogues.

In addition, NPs present a biomedical interest due to their key role in nucleoside metabolism mainly in cancer chemotherapies [33].

Reviews dealing with the biocatalysed synthesis of nucleosides using these enzymes or whole cells have been published by Hanrahan and Hutchinson in 1992 [34], Utagawa in 1999 [35] and Prasad *et al.* also in 1999 [36].

This paper covers literature reports from 2000 on, focused mainly on the synthesis of nucleosides by free and immobilised microbial whole cells, together with some examples of modified nucleosides obtained by coupling transglycosylation to other enzymatic reactions.

2. NUCLEOSIDE PHOSPHORYLASES

a. Biological role. A Target for Clinical Application

The phosphorolysis of purine and pyrimidine nucleosides catalysed by PNPs and PyNPs occurs in eukaryotes and prokaryotes metabolism during the so called "salvage pathways". This process makes use of preformed purine and pyrimidine bases as precursors in the production of nucleotides and therefore, provides an alternative to their *de novo* biosynthetic pathway [37].

Although human PNP is widely expressed in human tissues and is present in both the cytosol and the mitochondria, the highest activity is in peripheral red blood

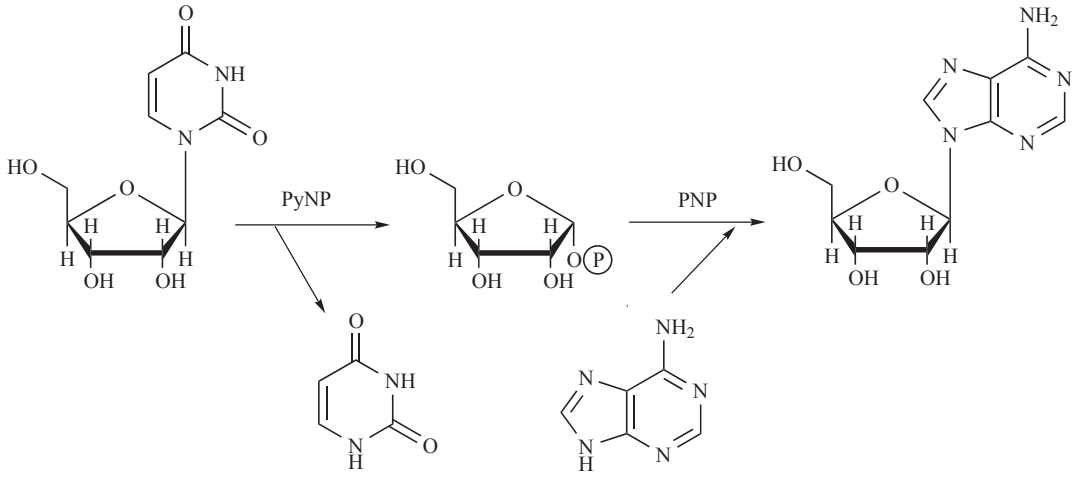


Fig. (3). Synthesis of purine nucleosides from pyrimidine ones.

cells, blood granulocytes and lymphoid tissue [38]. Thymidine phosphorylase (TP) and uridine phosphorylase (UP) are also expressed at high levels in human blood and in tissues such as liver, intestine and brain [39].

Since NPs play a key role in nucleotide metabolism, they are required for normal cellular function. Therefore, it has been suggested that inhibitors (usually modified nucleosides) of specific NP may be used as chemotherapeutics [40]. Patients lacking PNP have severe T-cell immune deficiency while B-cells function is unaffected. PNP deficiency results in very low uric acid concentrations and high amount of deoxyguanosine. T-cells accumulate dGTP by the action of 2'-deoxycytidine kinase, which allosterically inhibits ribonucleoside reductase resulting in DNA synthesis turn off [41]. Then, the inhibition of human PNP is used to selectively kill T-cells in leukaemia and lymphomas, to suppress the T-cell response after organ transplantation or to treat T-cell mediated autoimmune diseases [42].

Inhibition of human PNP is also important in antiviral therapies based on nucleoside analogues since avoids degradation of the active compounds. Therefore, NPs inhibitors are often supplied together with the active drug [43].

Due to the significant differences in substrate specificity of phosphorylases from mammalian and other lower organisms like protozoan parasites and bacteria, they are also potential targets in the treatment of diseases such as malaria [44].

Bacterial PNPs are also of biomedical interest because of their potential in anticancer treatments based on gene therapy strategies. For example, tumour cells transfected with the *E. coli* PNP gene are able to cleave non-toxic nucleoside prodrugs to highly cytotoxic bases [45].

The interest in TPs increased when it was discovered that it is identical to PD-ECGF, the platelet-derived endothelial cell growth factor [46], one of the angiogenesis tumour factors [47]. Thus, inhibition of TP is postulated to slow solid tumour growth and metastasis [48].

Finally, inhibition of human liver UP raises blood uridine levels and produces a protective effect against the toxicity of chemotherapeutic agents, like 5-fluoruracil, without reducing its antitumour activity [49].

b. Quaternary Structure and Active Sites

Pugmire and Ealick [37] defined, based on structural studies, two distinct families of NPs: NP-I and NP-II.

NP-I family includes enzymes that share a common single domain subunit, with either a trimeric or a hexameric quaternary structure and accept both purine and pyrimidine substrates.

PNP is the most studied member of this family and an exhaustive review was published by Browska *et al.* [32]. PNP has been isolated and characterised from a wide variety of mammalian (bovine brain [50], rabbit liver [51], calf spleen [52], human erythrocytes [53]), bacterial (*E. coli* [54], *Erwinia* [55]*, Bacillus cereus* [56]) and recently, yeast [57] and protozoan parasite [58] species.

Two main forms of PNPs have been identified:

1. Low molecular mass (LMM) trimeric PNPs specific for 6-oxopurines and their nucleosides (guanosine and inosine as natural substrates). Enzymes of this type can be found in many mammalian tissues and in some microorganisms like *Bacillus stearothermophilus* [59], *Mycobacterium tuberculoses* [60], *Cellulomonas sp.* [61] and *Saccharomices cereviseae* [57].
2. High molecular mass (HMM) hexameric PNPs showing broader specificity. They accept both 6-oxo and 6-amino purines and their nucleosides and are found in microorganisms, mainly bacteria and also protozoan parasites [44]. In general, adenosine is a much better substrate than inosine and guanosine.

In some bacterial species such as *Bacillus subtilis* and *Bacillus stearothermophillus*, both trimeric and hexameric forms have been found [62].

Human and *E. coli* PNPs are, respectively, the most representative enzymes of LMM and HMM forms, and all PNPs sequences reported from various sources showed homologies with any of these two enzymes [63].

The active site of LMM PNP [64] is buried in the protein structure and can be divided in three parts: the phosphate, the pentose and the base binding sites. Phosphate binding site is positively charged. Pentose binding site is rather hydrophobic, being OH2' and OH3' hydrogen bonded to Met219 and Tyr88, respectively, while OH5' has contact with His257. Other interactions involving pentose ring are hydrophobic, Fig. (**4a**). In the base binding site, O6 is hydrogen bonded to NH of Asn243 and to Glu201 via a water molecule, being Glu201 also linked to NH1.

The active site of HMM PNPs also comprises 3 binding sites [65], Fig. (**4b**). The most buried part is the phosphate binding site and is more positively charged than the one of hPNP. The pentose is in close proximity to the phosphate and only its OH5' makes no direct contact with it. OH5', OH3' and O4' are linked to the protein by unique hydrogen bonds while OH2' makes three direct contacts with it. Hydrophobic Van der Walls interactions between the pentose and the enzyme were also observed.

The nucleoside is bound in the high syn glycosidic bond conformation and the sugar generally adopts a C4' endo sugar pucker. Since the orientation of C1' respect to C4'O4'C2' plan is important, arabinosides are poorer substrate than ribosides and xylosides are not accepted [66].

The base binding site is largely exposed to solvent and a network of water molecules is observed. Non specific Π-Π interactions and the absence of Asn243 and Glu201 seems to account for the broader specificity of these enzymes [66]. Asp204 can make contacts with O6 of 6-oxo purines, $NH_2$6 of 6-amino analogues or N7 of analogues without donor or acceptor substituents.

These structural studies agree with experimental data that shows that hexameric PNPs are more selective for sugar moieties while trimeric ones discriminates better base estructures.

UP is another member of the NP-I family. The amino acid sequence is highly conserved among several bacterial, human and mouse enzymes. UP is a hexamer (but may be considered as a trimer of dimers coupled by K^+) [67], is specific for pyrimidine nucleosides and presents higher affinity for ribosides over 2'-deoxyribosides and for uracil over other pyrimidine bases [68].

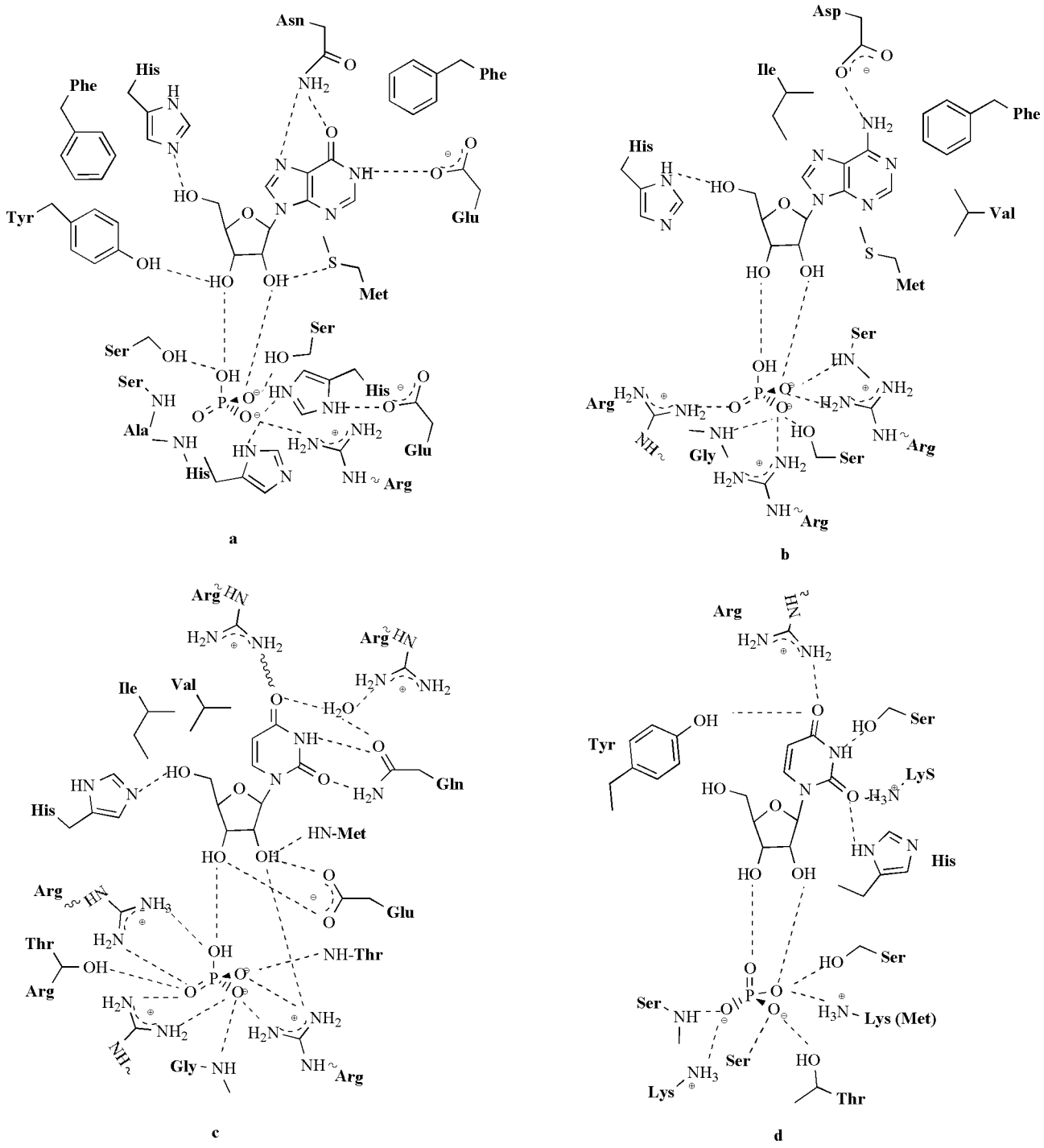


Fig. (4). Representative active site of NPs. a) LMM PNP, b) HMM PNP, c) UP, d) PyNP.

Both ribose and phosphate binding pockets of UP are similar to those of *E. coli* PNP [69], and the higher affinity of UP for ribosides over 2'-deoxyribosides may be explained by the three hydrogen bonds formed by the OH2', Fig. (**4c**).

Base binding pocket is smaller and therefore, purines are excluded and bulky groups, such as the methyl group of thymine, produce distortions and reduction of the catalytic efficiency. The key residue in the base binding site is Gln166 which forms two hydrogen bonds with uracil, one between the amide N and O2 and the other between the carbonyl O and N3, Fig. (**4c**). This enzyme as well as any other known NPs, does not cleave cytidine. The amino group at the 4 position, which is expected to carry a δ^+ charge on the amino protons, is repulsed by the positively charged side-chain of Arg168. In addition, N3 is deprotonated at physiological pH and therefore would be unable to form a hydrogen bond with Gln166, which reduces the binding affinity [70].

Recently [71], the ability of human deoxycytidine kinase (dCK) for degrading 2'-deoxynucleosides (including dC) to 2'-deoxyribose-1-P and the corresponding free base was

reported. Unlike NPs, dCK lucks of deoxyribose transferase activity and therefore, provides an irreversible reaction.

NP-II family is composed by enzymes that display a dimeric quaternary structure and are specific for pyrimidine analogues. Members from lower organisms accept both thymidine and uridine while those from higher species, including humans, are selective for thymidine.

TP is the most widespread enzyme of this family. It has been shown that the selectivity for the ribose moiety, as well as for 5-substituents of the pyrimidine ring varies among species. TPs from rat liver [72], mouse liver [73], horse liver [74], *E. coli* [75] and *Lactobacillus* [76] showed selectivity for the 2'-deoxyribose moiety, while the presence of 5-groups in the pyrimidine ring was less important.

Several organisms such as *Bacillus stearothermophilus* [77] and *Haemophilus influenzae* [78] are reported to contain a single **PyNP** that does not discriminate at the 2'-position of the ribose.

PyNPs and TPs show significant sequence similarity and detailed structural information was provided by the crystal structure of *E. coli* TP [79] and *Bacillus stearothermophilus* PyNP [80]. Each subunit of the TP dimer consists in a large mixed α-helical and β-sheet domain (α/β domain) separated by a large cleft from a smaller α-helical domain (α domain). The active site of each subunit consists of a pyrimidine binding site in the α domain and a phosphate binding site across the cleft in the α/β domain. The distance between the phosphate and pyrimidine binding site is too large and the catalysis does not occur unless the α and α/β domains move together from an open (empty) to a closed (substrate bound) conformation.

The interaction of the substrates with residues in the active site of *Bacillus stearothermophilus* PyNP was reported by Pugmire and Ealick [80] and is shown in Fig. (**4d**). Phosphate forms hydrogen-bonds with Lys81, Ser83, Lys108, Ser110 and Thr120. In the pyridine binding site the O2, O4 and N3 of the pyrimidine base are engaged in direct interactions with Arg108, Ser183, Tyr165 and Lys187.

The only difference when comparing the active site residues in the crystal structure of *E. coli* TP and *B. stearothermophillus* PyNP is the presence of Lys108 in the last one. The side chain of Lys108 forms a hydrogen bond with O1 of the phosphate, which in turn forms a hydrogen bond with the OH2' of the ribose moiety. In *E. coli* TP as well as in human TP, Lys is changed by Met creating a different hydrogen bonding pattern with the phosphate oxygen that binds to the OH2', which may contribute to different 2' position specificity.

Another residue in the active site that is conserved in all members of NP-II family, for which thymidine is substrate, is His (82 in *B. stearothermophilus*, 85 in *E. coli* and 116 in human) that plays an important role as reported recently [81]. It is suggested that a proton from His imidazole ring interacts with the O2 of thymidine, which makes the pyrimidine base a better leaving group. This proton comes from the deprotonation of phosphate affording the dianion that acts as nucleophile. Unlike UP, PyNP and TP show no selectivity for 5-substituents of the pyrimidine ring. The environment near this position consists of a hydrophobic pocket with enough room and flexibility to accommodate different groups.

Human TP shows a similar folding to those reported for other members of the NP-II family [82] and the overall structure shows more similarity to *B. stearothermophilus* PyNP than to *E. coli* TP. One difference is the presence of Val241 instead of Fen207 in the hydrophobic pocket what may explain the more discriminating characteristic of this enzyme.

c. Catalytic Mechanisms

Several mechanisms have been proposed to explain the catalytic activity of PNPs [32].

A sequential mechanism (all substrates bind to the enzyme before any product is released) has been proposed for all PNPs, but it is less clear if the reaction proceeds via ordered or random binding. In the case of *E. coli* PNP, phosphorolysis occurs via a random mechanism while in the synthetic direction pentose-1-phosphate binds obligatory prior to the purine base, which is in agreement with the active site structure.

Kinetic studies of trimeric PNP indicate that bond cleavage precedes bond making and therefore a SN1 mechanism was proposed [83]. The transition state, Fig. (**5**), has the purine ring rigidly fixed into the living group pocket and C1' has oxocarbenium character. Although, a transition state involving an oxocarbenium ion is generally accepted, different electronic states of the purine base have been suggested.

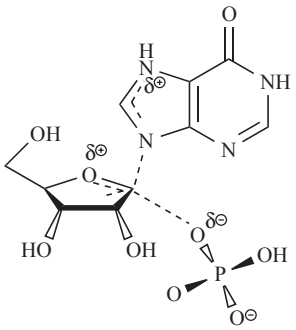


Fig. (5). Transition state PNP-catalysed phosphorolysis.

Protonation of N7 by a chain of proton transfer process from bulk water via immobilised water molecules and the Asn243 was hypothesized. This mechanism, analogous to the non enzymatic acid-catalysed hydrolysis, produces electron withdrawal from the scissile N9-C1' bond and therefore favours its cleavage.

A negatively charged purine base in the transition state has also been proposed but different distributions of the negative charge in the purine ring have been postulated [61, 84, 85].

An alternative mechanism was suggested by Ealick [37], in which the β nucleoside binds in a high-energy + anticlinal torsion angle of the glycosidic bond, with the ribose moiety in the uncommon C4' endo sugar pucker. This high energy conformation produces steric strain, which encourages glycosidic cleavage. The glycosidic bond is further

weakened by electron flow from O4' of the ribose to the purine ring. The result is an oxocarbenium ion that is stabilised by the negative charges of the phosphate. This phosphate ion binds on the α-side of the ribose ring, where it is positioned to participate in a SN1 nucleophilic attack at the C1' position. The flow of electrons from the glycosidic bond to the purine ring is probably stabilised by a hydrogen bond between N7 and active-site-residue Asn.

More recently, Canduri *et al.* [86] proposed a new catalytic mechanism for hPNP based on high resolution crystallographic data, which connects previously reported discrepancies. In this model the phosphate dianion, formed by interaction with His86, attacks the C1' of the pentose and weakens the glycosidic bond, causing a shift of electrons into the purine ring. The negative charge in this mechanism, unlike those described above, is localized at the purine O6 forming an anion intermediate. This O6 makes a strong electrostatic interaction with Asn243 which also pulls electrons from the glycosidic bond. In this model, Glu201 also participates and its role is to prevent reconstitution of the keto tautomer via hydrogen bond interaction with NH1.

For hexameric PNP [54], structural studies showed that N7 is probably at hydrogen bond distance from Asp204. The phosphate group stabilises the conformation change that locates Arg217 in contact with Asp204 inducing proton transfer, which allows formation of a salt bridge between the side chains. Base protonation leads to charge flow, producing the ribooxocarbenium ion character of the ribose. The negatively charged phosphate group stabilises the transition state and the reaction is completed when the phosphate ion captures the oxycarbocation.

These catalytic mechanisms proposed for PNP activity are likely representative for all members of the NP-I family. The conservation of key residues and interactions with substrates in the phosphate and ribose binding pocket suggest that ribooxocarbenium ion formation during UP mediated catalysis may be similar to that proposed for *E. coli* PNP. It is also possible that protonation of uracil occurs, to make the base a better living group [70] through a water molecule that forms a hydrogen bond with O4 of uracil and Arg223 side-chain. Similarly, modified uracil bases containing strong electronegative substitutients at the 5 position, increase reaction rate of UP because the electron deficient bases are better living groups [86].

For NP-II family, kinetic studies using *E. coli* TP and rabbit muscle TP have shown that the enzyme follows a sequential mechanism whereby phosphate is the first substrate to bind and 2-deoxyribose-1-phosphate is the last product to leave [87]. This mechanism is in accordance with the active close conformation and the stabilization provided by phosphate and 2-deoxyribose-1-phosphate.

Structural studies on *B. stearothermophilus* PyNP have led Pugmire and Ealick [37,80] to propose a catalytic SN1 mechanism for this family, similar to the one proposed for NP-I members, in which the pyrimidine nucleoside is bound in a high energy conformation causing the weakening of the glycosidic bond. This bond is further weakened by flow of electrons from O4' of the ribose moiety to the pyrimidine ring stabilised by Arg168 and Lys187, while the phosphate ion is positioned to attack the C1' of the ribose to yield 2-deoxyribose-1-phosphate and the free pyrimidine base. These authors suggest that His82 in *B. stearothermophilus* PyNP could be protonating N1 of the cleaved pyrimidine base. However, when Mendieta *et al.* [81] studied the role of His 85 in *E. coli* TP reported that protonation on N1 was not observed, and suggested an alternative mechanism. The negative charge on the O2 of pyrimidine base increases during the reaction and doubly protonated His85 contributes to its stabilization in the three complexes, enzyme-substrate, enzyme-transition state and enzyme-products. In the first step, the O2 base interacts with a proton from the protonated histidine. The weakening of the glycosidic bond generates an intermediate oxycarbocation in the sugar, which in a fast second step reacts with the phosphate dianion affording 2-deoxyribose-1-phosphate and the enol form of the base, which would then spontaneously tautomerise to the more stable keto form, Fig. (**6**).

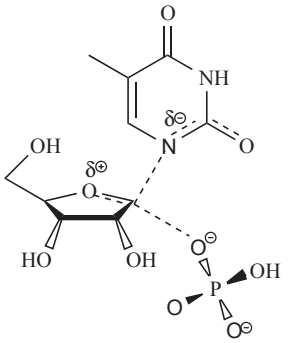


Fig. (6). Transition state TP-catalysed phosphorolysis

Using kinetic isotope effects and computational chemistry, Schramm [88] has defined the transition state structures for several members of the NP-families and has designed transition state analogues that are among the most powerful enzyme inhibitors.

Birck and Schramn [89] reported that human TP, unlike other NPs, proceeds through an SN2-like transition state with bond orders of 0.50 to the thymine living group and 0.33 to the attacking oxygen nucleophile. The observed values of kinetic isotope effects can only be explained assuming a symmetric transition state were the leaving group is replaced in synchrony with the attacking phosphate nucleophile. The pKa of the 2-carbonyl oxygen of thymidine (-2.98) may account for the above mentioned bond orders, since at physiological pH it is difficult to protonate this position to activate the leaving group and therefore, a higher degree of nucleophile participation is required.

d. Screening and Immobilisation

The first reports on nucleoside synthesis catalysed by NPs have been carried out employing only isolated enzymes but later on, the use of whole cells increased due to the benefits derived, such as avoidance of isolation and purification of the enzymatic system and improved activity, stability and cost [90].

In particular, microorganisms supply a large diversity of biocatalysts with differential substrate specificities. In order to make use of this enzymatic diversity for the synthesis of bioactive compounds, a screening process capable of

Table 2. Screening of microorganisms for nucleoside production

Microorganisms	Adenosine		Deoxyadenosine	
	%	time(h)	%	time(h)
Achromobacter cycloclastes	79	24	82	4
Aeromonas salmonicida	78	1	66	1
Arthrobacter oxydans	-	-	-	-
Bacillus cereus	-	-	ND	ND
Bacillus stearothermophilus	77	24	91	4
Brevibacterium helvolum	-	-	-	-
Citrobacter freundii	81	1	60	1
Citrobacter amalonaticus	89	1	ND	ND
Enterobacter cloacae	58	4	77	4
Enterobacter gergoviae	92	1	60	4
Erwinia amylovora	78	1	71	4
Erwinia carotovora	50	4	ND	ND
Escherichia coli BL21	94	1	73	1
Klebsiella planticola	84	4	64	1
Klebsiella sp.	76	1	67	4
Lactobacillus acydophilus	78	24	97	4
Lactobacillus animalis	-	-	75	4
Norcardia	-	-	ND	ND
Proteus rettgeri	86	1	49	1
Proteus vulgaris	83	1	25	1
Pseudomona putida	-	-	68	1
Pseudomona stuzeri	-	-	99	1
Serratia macescens	71	4	-	-
Serratia rubidaea	79	4	-	-
Streptomyces badius	-	-	65	4
Streptomyces sp.	-	-	-	-
Xanthomonas traslucens	23	4	ND	ND

identifying the strains with the proper transglycosylation activity has been developed [91].

The development of the screening assay was based on three basic steps, as proposed by Ogawa and Shimizu [92]: determination of the desired enzymatic activity; choice of the group of microorganisms to be screened and design of a proper, convenient and sensitive assay that allows to screen as many microorganisms as possible.

The selected activity was the pyrimidine-purine transglycosylation reaction since, as mentioned previously, the equilibrium of the phosphorolysis catalysed by pyrimidine phosphorylases is shifted towards the products to a higher extent than that catalysed by purine phosphorylases [32]. As a consequence, the pentafuranosyl transfer from pyrimidine nucleosides to purine bases is preferred.

Regarding the microorganisms, at least two species per available genera were selected in order to test a proper diversity.

The screening procedure was designed using the synthesis of adenosine as the model system, the reaction volume was minimised to 50 µl and samples were taken by duplicate. This biotransformation can be performed within a wide range of temperatures (30 - 65ºC) but a strict control of this experimental variable is necessary when interference of other deleterious enzymatic activities is present. For instance, the action of some hydrolases, like deaminases, can be minimised by conducting the reaction at 60ºC, since most of NPs maintain over 70% of their activity [93]. One of the exceptions is TP whose activity decreases when the temperature overcomes 50ºC. Therefore, reactions involving this enzyme were carried out at 45ºC. This problem can be avoided using 2'-deoxyuridine as an alternative source of the deoxyribosyl moiety.

After incubation at different times, samples were centrifuged and the supernatants were analysed by semiquantitative TLC. Samples affording product yields over 10 % were further quantified by HPLC. To identify the products, either comparison with available reference samples or HPLC-MS was performed.

The screening was tested for the synthesis of the natural nucleosides adenosine and 2'-deoxyadenosine from uridine and 2'-deoxyuridine respectively using conditions previously optimised for the synthesis of adenosine with *E. coli* BL21 [94]. Results are shown in Table **2**.

E. coli BL21, *Enterobacter gergoviae* and *Citrobacter amalonaticus* were selected as the best biocatalysts for adenosine synthesis while *E. coli* BL21 and *Klebsiela planticola* were selected for deoxyadenosine.

After selection of the microorganisms, further optimisation of the reaction conditions can be carried out using, for example, factorial design. Growth conditions can be also analysed in order to get optimal reaction conditions and reproducible assays. The determination of the number of viable cells/weight of biocatalyst ratio showed that, in the case of *E. coli* BL21, cells harvested in middle/late exponential phase gave higher transglycosylation activity and therefore, the reaction time could be reduced to the half [95].

Microorganisms selected by screening can be used directly as biocatalysts or as sources of free enzymes, which can be obtained by cloning, expression and purification as reported by Ubiali *et al.* [96].

In cases were industrial application has been hampered by the low specific activity associated with the wild type microorganisms, genetically modified strains that co-express NPs at very high level were developed [97]. This strategy enables the optimization of nucleosides preparation in terms of productivity and cost as well as the direct implementation of the process at preparative scale.

Recently [98], Oliva *et al.* used pentapeptide scanning mutagenesis to cause random insertion of a 5 aminoacid cassette in the UP from *E. coli*. This mutagenesis approach appears to be useful for the rapid preparation of mutants that present altered enzymatic activities.

Although, free bacterial cells or extracted and purified NPs are appropriate for carrying out transglycosylation reactions at laboratory scale [99], for industrial applications the use of immobilised biocatalysts, that provide increased productivity and easy of handling, is preferred. Properly designed immobilisation on solid supports enables the reuse of the catalyst and may increase the stability of enzymes and cells under a wide range of experimental conditions [100].

Few examples describing the immobilisation of isolated NPs are reported. One of the first ones deals with the immobilisation of UP and PNP from *Bacillus stearothermophilus* on an anionic exchange resin applied to the synthesis of 5-methyluridine [101]. Another example was provided by Zuffi *et al.* [102] co-immobilising phosphorylases from *E. coli* on epoxy activated solid supports by covalent linkages.

The stabilisation of the quaternary structure of multimeric enzymes, which requires proper assembly of all the subunits to preserve activity, was achieved by Guisan and co-workers [103]. These authors used different immobilisation strategies to stabilise the multimeric UP and PNP from *B. subtilis*. The simplest one was ionic adsorption employing supports such as modified agarose and epoxy resins. However, the use of supports bearing ionic groups directly bound to the surface may establish a weak interaction with the enzyme. To overcome this drawback that can restrict experimental conditions, this group has also assessed covalent multi-subunits attachments using two different supports: Eupergit C and Sepabeads (epoxy hydrophobic resins), and agarose beads activated with glyoxyl groups (glyoxyl-agarose), a hydrophilic matrix. The mechanism of immobilisation on both supports is different. On epoxy resins, the immobilisation occurs at first by hydrophobic interaction and, afterwards, by covalent-bond formation between the nucleophile group of the enzyme and the epoxy functions of the support [104,105]. Immobilisation on glyoxyl-agarose beads occurs at alkaline pH, via the area richest in lysines on the protein surface [106]. Unlike PNP immobilisation, poor activity was obtained by UP immobilisation because of distortions of the protein structure. The solution was achieved following a strategy previously reported for others enzymes [107] using supports coated with polycationic polymers, e.g., polyethylenimine, allowing the enzyme to be fully covered by the polymer [108]. Further cross-linking with aldehyde dextran, which reacts both with the free amino groups of the enzyme and with the polyamine, permits the complete stabilization of the multimeric enzyme and prevents its desorption under any reaction conditions. This UP derivative and the previously mentioned, PNP on glyoxyl-agarose, showed high activity and were more stable than the corresponding free enzymes at pH 10 and 45ºC [103]. These biocatalysts were successfully used to improve the preparation of 2'-deoxynucleosides.

Immobilised cell technologies have been widely developed since the 1980s, using both wholly artificial and naturally occurring methods [109]. In the first, cells are artificially entrapped in or attached to various supports where they keep or not a viable state, depending on the degree of harmfulness of the immobilization procedure. Polysaccharide gel matrixes, more particularly Ca-alginate hydrogels [110] are by far the most frequently used materials for harmless cell entrapment. In this method, the cells are enclosed in a polymeric matrix which is porous enough to allow the diffusion of substrates and products. Cell attachment to an organic or inorganic matrix may be obtained by covalently binding cells to the support using cross-linking agents such as glutaraldehyde or carbodiimide, being these immobilization procedures generally incompatible with cell viability.

The spontaneous adsorption of microbial cells to different types of carriers provides natural immobilised systems in which cells are attached to their support by weak, generally non-specific contacts such as electrostatic interactions. In suitable environmental conditions, this initial adsoption step may be followed by colonization of the support, leading to the formation of a biofilm in which microorganisms are entrapped within a matrix of extracellular polymers they themselves secreted.

Regarding the application to nucleoside synthesis of immobilised whole cells, few articles have been reported, such as the use of immobilised *E. coli* [111-114] and *Xanthomonas campestris* [115]. More recently, it was explored the activity and stability of *Enterobacter gergoviae* [116], *E. coli* BL21 [117] and *Citrobacter amalonaticus* [118] immobilised by entrapment techniques using different supports like naturally occurring polysaccharides extracted from seaweed like agar, agarose and alginate [119]. The last one has the inherent disadvantage, for the phosphate depending transglycosylation, of its instability in phosphate media. Besides, the use of a synthetic matrix polyacrylamide [120] has also been reported. Immobilization variables (cell load, shaking speed and polymer concentration), storage and mechanical stability, reusability and productivity were studied using the synthesis of adenosine as reaction model.

Almost all of the immobilised biocatalysts afforded similar yields (near 90%) to those obtained using the

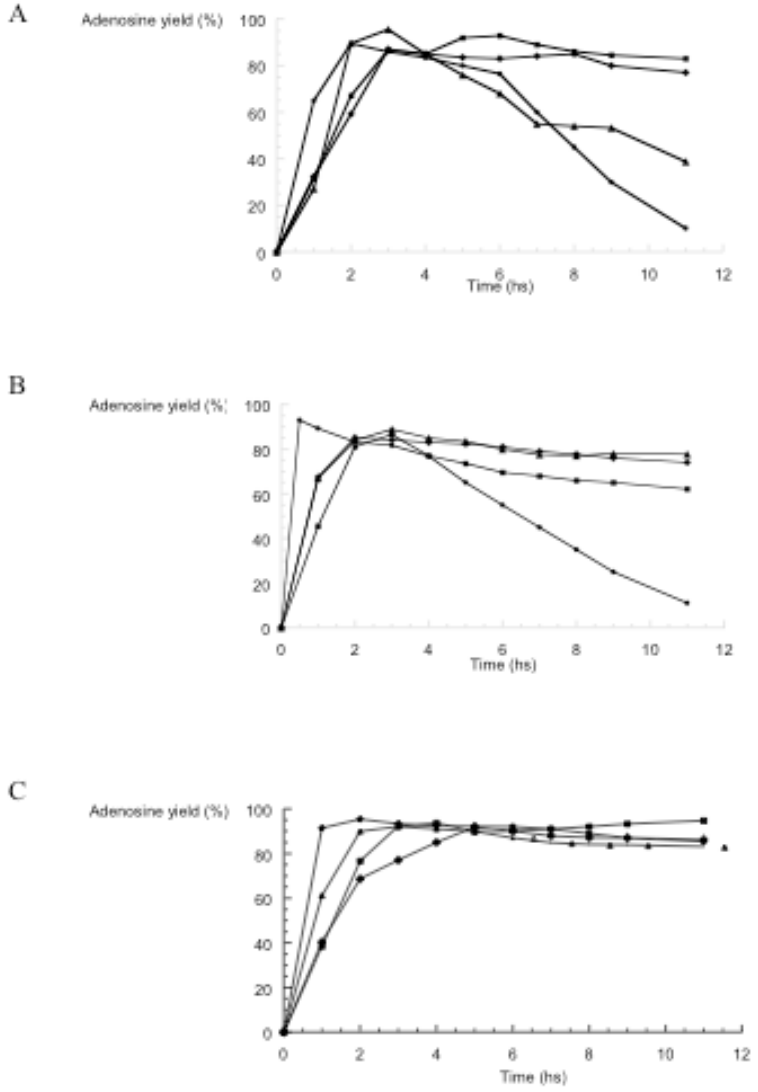


Fig. (7). Time course of adenosine synthesis biocatalysed by free and immobilised A) *Enterobacter gergoviae*, B) *Escherichia coli* BL21, C) *Citrobacter amalonaticus* (_) free cells, (■) 2 % agarose support, (_) 2 % agar support, (_) 8 % polyacrylamide support.

corresponding free cells but longer reaction times were required, Fig. (**7**). This slower kinetic may be attributed to intraparticullar diffusion restriction.

Longer reaction times to achieve maximum yields were also observed when cell load was low and kinetic conditions prevail. In contrast, at higher cell loading, the reaction rate decreases probably due to diffusion restriction produced by cell multilayers.

In general, the activity of each immobilised micro-organism was similar for all the matrixes tested, which suggests the absence of dramatic diffusion restriction of reagent and products. However, each biocatalyst presented different reuse behaviour depending on the support, probably due to differences in the cell wall and /or membrane composition, Fig. (**8**) [121].

In the case of free cells, after few cycles the yield is dramatically reduced probably because the number of viable cells decreased. In contrast, the immobilisation enhanced the stability of the biocatalysts. *E. coli* immobilised on agarose showed no loss of activity during the first 26 cycles and could be used up to 30 times before adenosine yield dropped to 50 % of the initial value. Agar-immobilised *Enterobacter gergoviae* allowed to carry out 35 cycles subsequently, while *Citrobacter amalonaticus* immobilised on polyacrylamide could be reused for 61 cycles.

Finally, a comparison of the storage stability between the most suitable catalysts obtained for each microorganism was also analysed, Fig. (**9**). *E. coli* on agarose and *E. gergoviae* on agar kept their activity over 6 months while *C. amalonaticus* on polyacrylamide was the less stable, probably due to toxicity caused by monomers. These results suggest that the described biocatalysts fulfil the requirements for industrial applications; it means: high activity, proper longevity, absence of by-products and short reaction time.

3. SYNTHETIC APPLICATIONS

a. Coupled Enzymatic Systems

The need for developing new processes for the production of modified nucleosides in high yields has derived in an increasing number of research works dealing

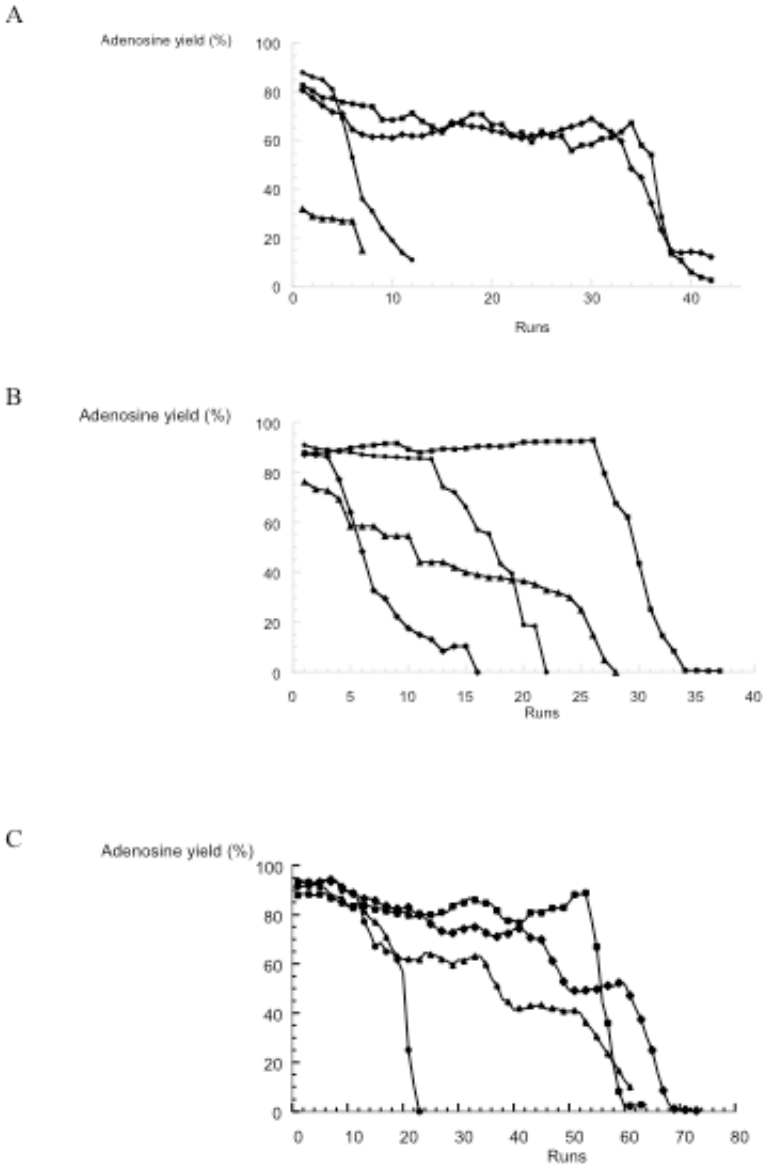


Fig. (8). Storage Stability reuse A) *Enterobacter gergoviae,* B) *Escherichia coli* BL21, C) *Citrobacter amalonaticus* (_) free cells, (■) 2 % agarose support, (_) 2 % agar support, (_) 8 % polyacrylamide support.

with enzymatic reactions that can be coupled to transglycosylation. These additional activities can shift the reaction equilibrium, modify the pyrimidine nucleoside reagent or transform the purine nucleoside product. The following examples show some representative coupled-systems.

Uracil Thymine Dehydrogenase / Dihydrouracil Dehydrogenase

Using these enzymes, the pyrimidine base formed after transglycosylation is converted into a compound incapable of acting as substrate of PNs. As a result, the reaction equilibrium is shifted towards the formation of a desired product [122].

By the action of uracil thymine dehydrogenase, uracil is irreversibly converted into barbituric acid. If barbiturase is present in the reaction system, barbituric acid is further decomposed into urea and malonic acid. Uracil may also be converted by dihydrouracil dehydrogenase into dihydrouracil and then into N-carbamoyl-β-alanine by the action of dihydropyrimidinase. These two strategies were applied to the synthesis of 2'-deoxyguanosine.

Xanthine Oxidase (XO)

The synthesis of pyrimidine nucleosides carried out by biocatalysed transglycosylations usually affords low yields due to the involved reactions equilibrium. To overcome this difficulty, hypoxanthine nucleosides can be used as starting materials since the released base can be converted into uric acid by xanthine oxidase. The reaction proceeds unidirectionally to form pyrimidine nucleosides since uric acid is not accepted by NPs, Fig. (**10**).

XO-mediated prodrug activation has been also used to increase the bioavailability, solubility and selectivity of a

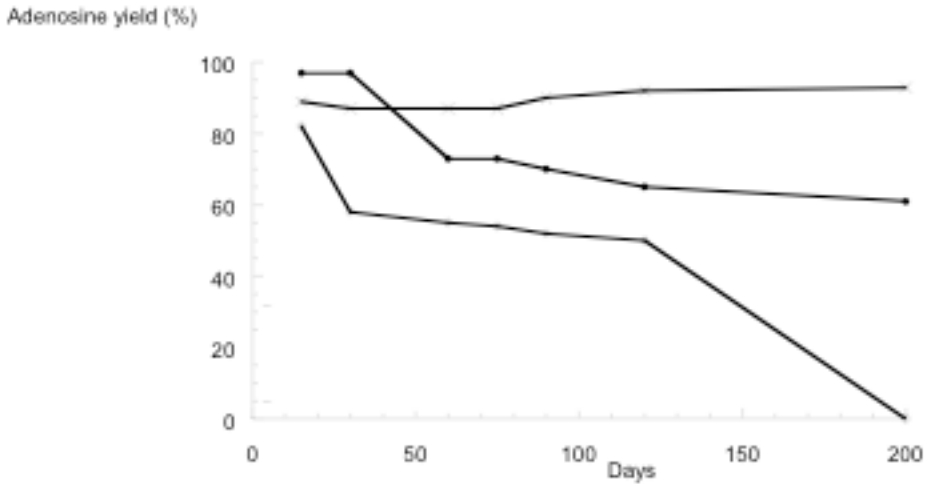


Fig. (9). Biocatalyst (_)Enterobacter gergoviae immobilised on agar (○) *Citrobacter amalonaticus* immobilised on polyacrylamide (x) *Escherichia coli* BL21 immobilised on agar.

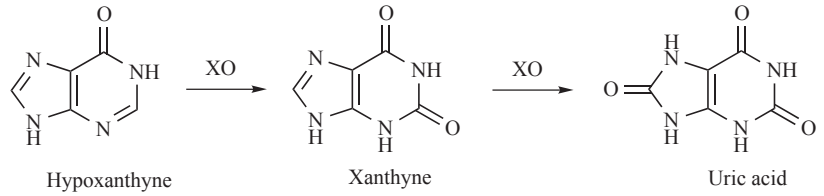


Fig. (10). Enzymatic activity of XO.

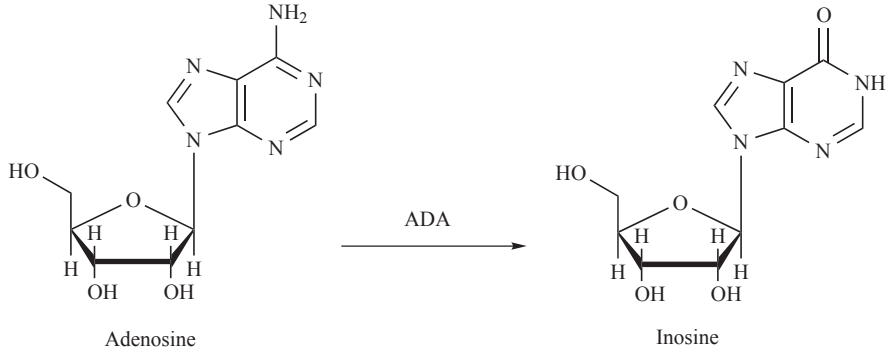


Fig. (11). Enzymatic activity of ADA.

number of antivirals. For example, to enhance brain delivery of anti HIV-nucleoside hypoxanthine 2'-fluor-dideoxy-arabinoside, purine 2'-fluor-dideoxyarabinoside was orally administrated [123].

On the other hand, a series of potential tumour-selective XO-activated prodrugs of the known TP inhibitor 6-amino-5-bromouracil, lacking the carbonyl at C2 and/or C4, were evaluated [124].

Adenosine Deaminase (ADA)

ADA catalyses the irreversible hydrolytic deamination of adenosine and other 6-substituted purine nucleosides to their hypoxanthine derivatives, Fig. (**11**).

This strategy has been extensively used to prepare suitable nucleoside reagents as well as to modified nucleoside products [125,126]. Frequently, ADA is also used in chemoenzymatic synthesis of nucleosides as a key step for the removal of groups at the 6 position [127].

ADA is an ubiquous enzyme due to its important role in purine metabolism. In order to profit this naturally occurring enzymatic activity, ADA-mediated prodrugs have been designed. One of the most interesting examples is the use of 2,6-diaminopurine nucleosides as precursors of guanosine anticancer analogues [128].

Unlike the advantages provided by ADA, its presence can be deleterious in antiviral therapies based on nucleosides. Structural studies helped to design adenosine analogues with differential behaviour to ADA action [129]. Similarly, ADA activity can also be undesired when microbial catalysed transglycosylation is performed, but this

biotransformation can be successfully carried out at 60ºC since ADA activity drops at temperatures higher than 50ºC.

Phosphopentomutase (PPM)

Unlike transglycosylation, were two NPs must be used, nucleoside synthesis can be performed using only one NP and α-pentose-1-phosphate as substrate. Since this kind of compounds is unstable, they can be generated *in situ* from pentoses-5-phosphate by the action of PPM [130], Fig. (**12**).

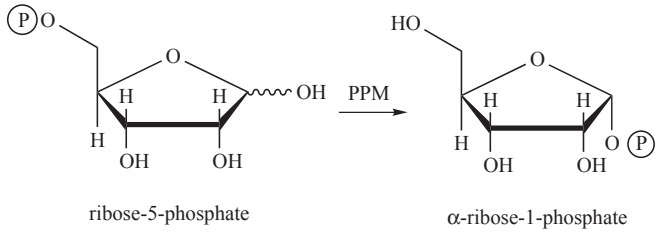


Fig. (12). Enzymatic activity of PPM.

This enzyme catalyses the phosphate group migration from position 5 to 1 affording the α-anomer necessary for the subsequent reaction with NP. This strategy was successfully applied to the one pot synthesis of thymidine [131].

2-Deoxyribose-5-Phosphate Aldolase (DERA)

Aldolases have attracted the interest of organic chemists because of their ability to catalyse the formation of C-C bonds by an aldol addition reaction between an aldehyde and a ketone, with a high degree of stereochemical control.

DERA catalyses the aldolic condensation between acetaldehyde and D-glyceraldehyde-3-phosphate and determines the S configuration of the newly formed stereogenic centre [132] Fig. (**13**). The 2'-deoxyribose-5-phosphate produced was used to prepare 2'-deoxyri-bonucleosides with PPM and the corresponding NP.

Since naturally DERA catalyse the degradation of deoxynucleosides, the synthetic direction can be favoured by addition of acetaldehyde and therefore, an acetaldehyde resistant enzyme should be employed. A DERA with this characteristic from *K. pneumonia* was cloned and expressed in *E. coli* by Horinouchi *et al*. [133]. Other benefit of this reaction is that D-glyceraldehyde-3-phosphate can be generated from cheap sugars, such as glucose. This intermediate is produced stereoselectively by triosephosphate isomerase from prochiral dihydroxyacetone phosphate formed in the glycolysis process.

b. Nucleoside Synthesis

Since biocatalysis showed several advantages over chemical synthesis for the industrial nucleoside production, numerous related research papers and patent applications were written during the last years.

Historically, enzymatic transglycosylation [134] preceded microbial one [35,135]. Due to the broader substrate specificity and greater thermal stability of NPs from prokaryotic microorganisms, they were preferred respect to those from mammal sources [136]. The use of immobilised biocatalysts was explored later on, especially for industrial applications [137].

More recently, the construction of genetically modified bacteria that express high levels of NPs recombinant forms, were advantageously applied to both the production of isolated enzymes and the direct use of the whole cells as biocatalysts [97,138].

Regarding to the need of nucleosides, it is not only restricted to their use as antiviral or anticancer therapies. It is expected that in the near future, the need for 2'-deoxyribonuleosides will grow due to the increasing demand in new medical and biotechnological fields. 2'-Deoxyribo-nucleosides are building blocks of promising antisense drugs for cancer therapy, are synthetic intermediate for antiviral agents, and are also precursors of the indispensable 2'-deoxyribonucleotides used for PCR applications. For example, the current 2'-deoxy-ribonucleotides sources include hydrolysed herring and salmon sperm DNA, which are not suitable for sudden high demands.

Yokoseki and Tsuji [126] have selected intact cells of *Enterobacter aerogenes* AJ 11125 as the best strain for the synthesis of 2'-deoxyadenosine and 2'-deoxyguanosine from 2'-deoxyuridine achieving 75% and 2.9% yield respectively in 4 hours. The later low productivity was attributed to the poor solubility of guanine.

More efficient synthesis of 2',3'-dideoxy-adenosine, -inosine and -guanosine was carried out by Shiragami *et al.* [139] using *E. coli* ATCC 10798.

PNP and UP from *B. subtilis* immobilised using a multipoint covalent strategy [96,140] resulted in a biocatalyst stable under extreme conditions of pH necessary for the synthesis of 2'-deoxyguanosine and 2'-deoxyinosine from 2'-deoxyuridine. Thus, at 45ºC and pH 10, the first compound was prepared in 4 hours and 87% yield and the latter one was obtained with 92% yield in 24 h.

Recently, the covalent co-immobilisation of PNP and UP on a solid matrix functionalised with epoxy groups afforded a biocatalyst with high specific activity, thermal stability and solvent concentration compatibility that could be reused for numerous reaction cycles [141]. In this way, 2'-deoxyguanosine, 2'-deoxyadenosine, 2-fluoradenine- and 2-aminoadenine arabinosides were prepared in yields near 70 %.

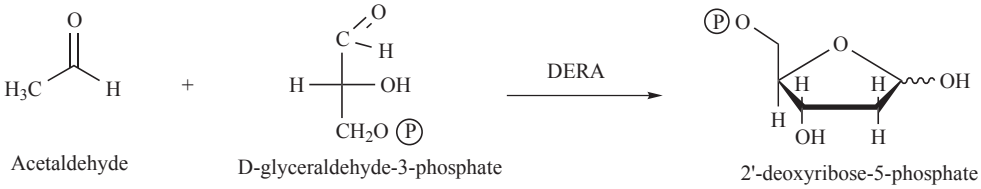
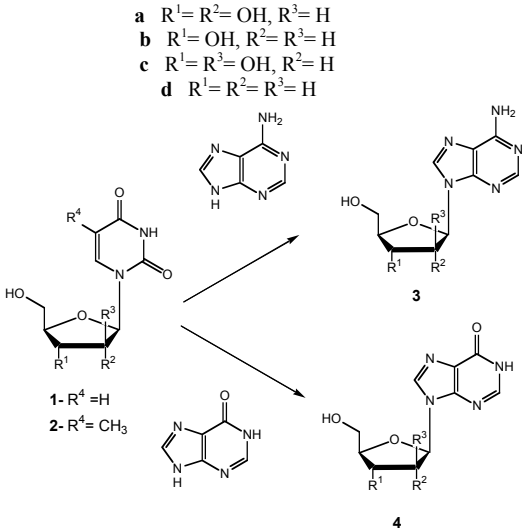


Fig. (13). Enzymatic activity of DERA.

Table 3. Purine nucleoside synthesis using free and immobilised *E. coli* BL21 as biocatalysts



Entry	Matrix	Nucleoside	Product	Time(hs)	Temp.(ºC)	Yield (%)
1	none	1a	3a	3	45	67
2	agarose	1a	3a	6	45	65
3	none	1a	3a	0.5	60	94
4	agarose	1a	3a	2	60	89
5	none	1a	4a	1	60	50
6	agarose	1a	4a	2	60	17
7	none	1b	3b	3	45	85
8	agarose	1b	3b	3	45	77
9	none	1b	3b	2	60	72
10	agarose	1b	3b	3	60	76
11	none	1b	4b	2	60	57
12	agarose	1b	4b	9	60	44
13	none	2b	3b	2	45	94
14	agarose	2b	3b	2	45	85
15	none	2b	4b	2	45	70
16	agarose	2b	4b	3	45	57
17	none	1c	3c	24	60	85
18	agarose	1c	3c	24	60	75
19	none	1c	4c	24	60	32
20	agarose	1c	4c	48	60	40
21	none	1d	3d	21	60	10
22	agarose	1d	3d	21	60	7

Enterobacter aerogenes [35] and *E. coli* BL21 [95] whole cells have also been employed to synthesise arabinonucleosides. The last mentioned strain was further studied, optimising reaction conditions using both free and immobilised cells (Table **3**).

The tested biocatalyst afforded high yields of **3a**, **3b** and **3c** but poor activity was observed when **1d** was used as starting material. With **1a** as the sugar donor, the yield of **3a** was lower at 45ºC than at 60ºC. Moreover, the presence of inosine and hypoxanthine in the experiment carried out at 45ºC revealed the action of ADA. Both **1b** and **2b** were used as substrate for the synthesis of **3b**; starting from **1b** similar yields of **3b** at both 45 and 60 ºC were obtained, suggesting that is a good substrate for both TP and UP. Starting from **2b,** the natural substrate for TP, the reaction is more efficient at 45°C. It was also observed that 2'-deoxynucleoside formation is faster than ADA activity.

Since UP is involved in the biotransformation of arabinonucleosides, the temperature of choice was 60ºC [95]. In all cases, the adenosine analogues were obtained in higher yields than those of hypoxanthine ones, which is in agreement with the previously reported base selectivity of bacterial PNP [136].

Other immobilised bacteria such as *Citrobacter amalonaticus* on polyacrylamide [111] and *Enterobacter gergoviae* on agar [114] were also tested for the synthesis of purine nucleosides (Table **4**).

6-Substituted purine ribosides are of interest as pharmaceutical targets in cancer chemotherapy and are also useful intermediates in the synthetic routes of several biological active nucleosides [142]. Chemical synthesis of these compounds has been carried out using the corresponding inosine analogues as substrates. However, the removal of sugar protecting groups is carried out under

Table 4. Synthesis of different adenine nucleosides

Microorganims	Matrix	Pyrimidine Nucleoside	Time (h)	Adenine nucleoside yield (%)
E. gergioviae	Agar	**1a**	3	87
E. gergioviae	Agar	**1b**	3	68
E. gergioviae	Agar	**1d**	24	0
E. gergioviae	Agar	**2b**	6	78
E. gergioviae	Agar	**1c**	24	0
C. amalonaticus	Polyacrylamide	**1a**	3	91
C. amalonaticus	Polyacrylamide	**1b**	1	23
C. amalonaticus	Polyacrylamide	**1d**	24	10
C. amalonaticus	Polyacrylamide	**2b**	1	17
C. amalonaticus	Polyacrylamide	**1c**	24	55

conditions that affect the 6-substituted purine ring. Hence, Roncaglia *et al.* [143] developed a novel enzymatic procedure for the deprotection of base labile chlorinated purine ribosides.

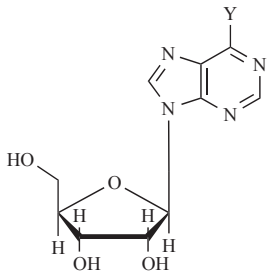
An alternative solution to this problem can be also provided by the use of transglycosylation reactions. The screening methodology described in section **2**.*d* was applied to the preparation of 6-chloro, 6-methoxy, 6-iodo and 6-mercaptopurine ribosides [91]. Several strains afforded positive reactions, but only *Bacillus stearothermophilus* provided suitable yields for all the nucleoside targets (Table **5**). The wide range of acceptance of purine bases showed by *Bacillus stearothermophilus* are in agreement with the two forms of PNP present in this microorganism.

immobilised NPs and whole cells [145,146]. For example, recombinant PNP from *E. coli* BL21 (DE3)/pERPUPH01 immobilised on aminopropylated silochrome could be reused for 15 cycles and was successfully applied to the synthesis of this modified nucleoside [147].

Similarly, nicotinamide riboside (NAR), an intermediate of the biosynthetic pathway to obtain NAD (a cofactor of numerous enzymes) and NAR analogues that have been described as antitumor agents, can also be prepared utilizing NPs [148]. Their chemical syntheses afforded α/β anomeric mixtures while enzymatic procedures were stereoselective.

Deazapurine nucleosides have received attention as pharmaceutical agents because of their stability toward some enzymes involved in nucleosides inactivation e.g. ADA and

Table 5. 6-Substituted purine nucleoside production catalysed by *Bacillus stearothermophilus*



Y	Yield (%)	Time (h)
OCH_3	65	4
SH	54	24
I	39	24
Cl	72	48
NH_2	96	24
OH	69	4

6-Modified purine nucleosides were also obtained using thymidine as ribose donor affording similar yields in short reaction time. Furthermore, *Bacillus stearothermophilus* was immobilised by entrapment techniques using agar, agarose and polyacrylamide as supports, showing that when the matrix employed was agar or agarose, the biocatalyst activity was maintained.

Recent studies have suggested that the major action mechanism of Ribavirin, riboside of 1,2,4-triazole-3-carboxamide [144], is through lethal mutagenesis of the viral RNA genome because its triphosphate is incorporated by RNA polymerase instead of ATP or GTP. This result is in line with experimental data that show that 1,2,4-triazole-3-carboxamide is also accepted by PNP, since the structure of the carboxamide residue mimics C6 and N1 of the purine ring. Ribavirin is industrially prepared by both free and

PNP. Some 9-deazapurines have therapeutic value as inhibitors of human PNP whereas some 7-deazapurines showed inhibitory effects on *E. coli* PNP [32].

Benzimidazole (1,3-deazapurine) derivatives are particularly attractive because they are involved in different biological activities such as inhibition of phosphodiesterase IV [149] and proton pumps [150] and also display antiarrythmic, antihelmintic and antiviral properties [151]. Different modifications on this heterocycle have been reported with the aim of decreasing the cytotoxicity and increasing bioavailability. For example, polyhalogenated benzimidazole nucleosides and modified benzimidazole L-nucleosides have been used for treating herpes and citomegalovirus infection [152]. It has already been shown that benzimidazole derivatives have good affinity for PNP but on the contrary, the corresponding ribosides are poor

substrates for the phosphorolysis due to the higher stability of their glycosidic bond compared to those of purine nucleosides [153].

E. coli BMT-4D/1A [154] has been previously used to produce benzimidazole riboside and 2'-deoxyriboside from guanosine and deoxyguanosine in 70 and 90% yield, respectively. However, Bentancor *et al*. [155] carried out the transglycosylation reaction using available and more soluble nucleosides, uridine and thymidine as reagents. Several microorganisms produced the target nucleosides in suitable yield (75-95%) and *Aeromonas* was further used to study the optimisation of experimental conditions. The optimal temperature found was 60ºC, as for most of the purine ribosides reported in the literature [35]. Both, yield and reaction rate, do not significantly depend on the nucleoside:base (N:B) proportion; therefore, to avoid reagent excess, the selected condition was 1:1. Regarding the synthesis of benzimidazole 2'-deoxyriboside, *Serratia rubidae* produced 85% yield in 5 h.

In the chemical synthesis of guanine nucleosides that are used as antivirals, the coupling of the base to the carbohydrate moiety has been problematic because mixtures of the N7 and N9 regioisomers are formed [156].

In order to obtain nucleoside by enzymatic transglycosylation, the solubility of the substrate must preferably be higher or equal to the enzyme K_M. As the solubility of guanine in water is very low, this method cannot be employed in practice and alternative strategies have been developed.

Mikami *et al*. [157] had prepared glyoxal-guanosine and -2'-deoxyguanosine as intermediates in high yields. The corresponding base, glyoxal-guanine, soluble in water, was prepared and used as substrate of NPs from *B. stearothermophilus* JTS 859. Further alkali treatment enabled to obtain de desired guanosine compounds.

Other strategy involves the use of less polar 2-amino-6-substituted purines like 2,6-diaminopurine and 2-amino-6-chloropurine, which are cheap and commercially available in large quantities and can be transformed into guanine by chemical or enzymatic procedures after nucleoside preparation. Conformational locked guanine nucleosides [5] and guanine arabinoside [158] are recent examples of chemical preparation starting from the corresponding 2-amino-6-chloropurine derivative while several guanosine analogues were prepared by biocatalysed transglycosylation using pyrimidine nucleosides and 2,6-diaminopurine followed by ADA deamination.

Since 2,6-diaminopurine nucleosides can also be deaminated *in vivo* by animal and human ADA [159,160] to produce guanosine analogues, they are also used as prodrugs.

Kawashima *et al*. [161] used *E. aerogenes* AJ 11125 to obtain 2,6-diaminopurine-2'-deoxyriboside followed by treatment with isolated ADA to prepare 2'-deoxyguanosine. Medici *et al*. [162] reported the preparation of 2,6-diaminopurine-2'-deoxyriboside by *Proteus vulgaris* in 88% yield in 30 min. at 45º from thymidine, while using *E. cloacae* as biocatalyst, 80% yield was obtained from 2'-deoxyuridine either at 60 or 45ºC.

2,6-Diaminopurine-2',3'-dideoxyriboside is a potent and selective inhibitor of HIV and human ADA [163]. Therefore, it is also used in combination with 9-β-D-arabinofuranosyladenine [164] in therapies against AIDS, herpes simplex and vaccinia virus. Utagawa [35] reported that microbial synthesis can be performed by *E. coli* whole cells at 50ºC. Medici *et al*. [162] obtained 2,6-diaminopurine-2',3'-dideoxyriboside with bacteria belonging to *Escherichia* and *Aeromonas* genera at 45 ºC. For example, *E. coli* BL21 afforded 65% yield in 48 h.

2,6-Diaminopurine arabinoside like 2-amino-6-methoxypurine arabinoside [160] are potential prodrugs. Since uracil arabinoside is accepted only by UP [165], bacteria containing this enzyme such as *Citrobacter, Enterobacter* and *Aeromonas* genera, afforded high yields of 2,6-diaminopurine arabinoside. *Enterobacter gergoviae* for example, gave this compound in 72% yield after 48 h [162].

To obtain 3'-deoxyguanosine, Barai *et al*. [166] used 3'-deoxycytidine as donor of the sugar moiety, 2,6-diaminopurine as purine base and whole cells of *E. coli* BM-11 and BMT-4D/1A as biocatalysts, Fig. (**14**). The combined action of cytidine deaminase (CDA), UP, PNP and ADA afforded 68% yield of the guanosine analogue.

The natural precursor for the synthesis of several pyrimidine nucleosides such as zidovudine and stavudine [14, 167] is thymidine. As above discussed, deoxynucleosides are relatively expensive and difficult to obtain. Holi *et al*. [106] used *E. coli* cells and 2'-deoxyuridine to synthesise thymidine in 67% yield but this approach has the disadvantage of using an expensive substrate. Nair and Pal [168] reported the use of a thermostable bacteria, *B. stearothermophilus,* which unlike *E. coli*, can be used at near 55ºC where the solubility of deoxynucleosides is much higher and the reaction kinetic faster. The substrate employed was 2'-deoxyinosine and XO was added to shift the equilibrium towards the products. Thymidine was

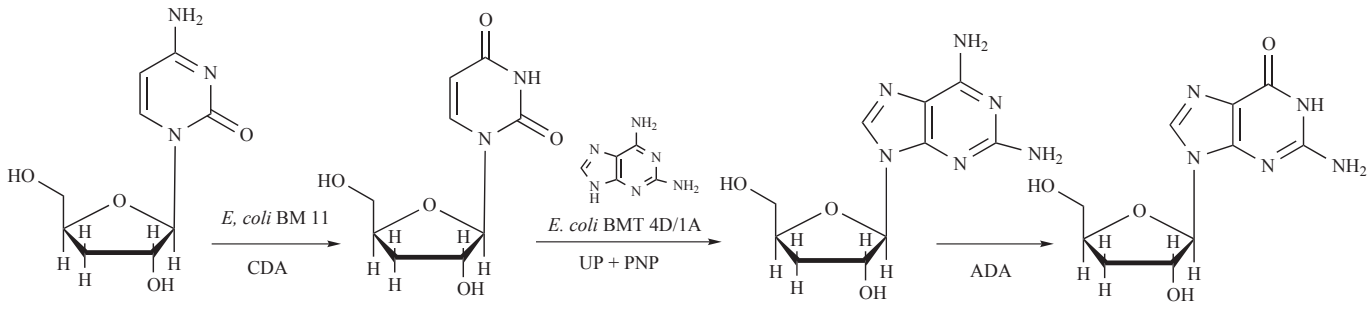


Fig. (14). Synthesis of 3'-deoxyguanosine from 3'-deoxycytidine.

obtained in 70 % yield and other pyrimidine deoxynucleosides such as 5-fluoro-2'-deoxyuridine, 5-azauridine and uridine arabinoside could be prepared according to this procedure.

As cytosine is not accepted by TP as substrate, 2-deoxycytidine was obtained by chemical synthesis from 2'-deoxyuridine. Araki *et al*. [169] used a recombinant bacteria with reduced CDA activity containing modified NP capable of synthesising cytosine nucleosides. Ishibashi *et al*. [170] improved this procedure adding zinc salt to the reaction mixture to improve cytosine nucleoside yields.

Another approach to avoid expensive 2'-deoxynucleosides as substrates, is the use of 2'-deoxyribose-1-phosphate. To shift the equilibrium towards glycosylation, Araki *et al*. [171] proposed to add a metal cation capable of forming a sparingly water-soluble salt with phosphoric acid to precipitate this by-product and remove it of the reaction system. The metals tested were calcium, barium, lithium, aluminium or magnesium. Using this strategy, thymidine [172] and 2',3'-dideoxy-3'-fluorguanosine [173] were prepared from the corresponding sugar-1-phosphate and the base. A similar approach was applied by Ouwerkerk [131] to prepare thymidine and 2'-deoxyuridine. The addition of $MnCl_2$ improved the overall yield from 14 to 60%. Nagahara *et al*. [174] proposed to improve the reaction conversion by using ion exchange resins that act as a phosphate trapping agent.

To produce pentose-1-phosphate, pentose-5-phosphate and microbial cells containing heat stable phosphopentomutase and substantially no phosphatase activities [175] have been used. In a fully biocatalysed procedure, pentose-5-phosphate was produced from acetaldehyde and D-glyceraldehyde-3-phosphate using DERA and triosephosphate isomerase activities of *Klebsiella pneumoniae* B-4-4 and then transformed to 2'-deoxyribonucleosides through coupling the enzymatic reactions of PPM and NPs [133,176].

Finally, NPs were recently [177] used to solve racemic mixtures of chemically prepared 2-deoxy-2-fluorarabinoside-1-phosphate by the preparation of purine 2'-deoxy-2'-fluor-β-D-arabinonucleosides with high both stereoselectivity and yield.

REFERENCES

[1] Balfour, H. H. *Antiviral Drugs*; Alastair, J. J.; Wood, M. D., Ed.; **1999;** Vol. *340*, pp. 1255-1268.
[2] Perigaud, C.; Gosselin, G.; Imbach, J. L. *Nucleosides Nucleotides*, **1992**, *11*, 903.
[3] Kagiyama, S.; Kagiyama, T.; Phillips, M. I. *Curr. Opin. Mol. Ther.*, **2001**, *2*, 258.
[4] Iribarren, A. M.; Sproat, B. S.; Neuner, P.; Sulston, I.; Ryder, U.; Lamond, A. I. *Proc. Natl. Acad. Sci. USA*, **1990**, *87*, 7747.
[5] Rosenbohm, C.; Pederson, D. S.; Frieden, M.; Jensen, F. R.; Arent, S.; Larsen, S.; Koch, T. *Bioorg. Med. Chem.*, **2004**, *12*, 2385.
[6] Van Rompay, A. R.; Johansson, M.; Karlsson, A. *Pharmacol. Ther.*, **2003**, *100*, 119.
[7] Holy, A. *Cas Lek Cesk*, **2001**, *140*, 583.
[8] Hori, N.; Uehara, K.; Mikami, Y. *Biosci. Biotech. Biochem.*, **1992**, *56*, 580.
[9] Gallo, M.; Monteagudo, E.; Cicero, D. O.; Torres, H. N.; Iribarren, A. M. *Tetrahedron*, **2001**, *57*, 5707.
[10] Herdewijn, P.; De Clercq, E. Design of Anti-AIDS drugs, 1990, Vol 14, Ed. Elsevier, pp. 141- 158.
[11] Zoulim F. *AntiviralRes.*, **2004**, *64*, 1.
[12] Brady, R. C.; Bernstein, D. I. *Antiviral Res.*, **2004**, *61*, 73.
[13] Tanm Y He, S. L.; Huang, Y.; Gale, M. *Curr. Op in. Pharmacol.*, **2004**, *4*, 465.
[14] Tan, X. L.; Chu, C. K.; Boudinot, F. D. *Adv. Drug Deliv. Rev.*, **1999**, *39*, 117.
[15] Yokozeki, K.; Shirae, H.; Kobayashi, K.; Shiragami, H.; Irie, Y. (1990), U. S. Patent Nº 4, 970, 148.
[16] De Clercq, E. *Mini Rev. Med. Chem.*, **2002**, *2*, 163.
[17] Balint, G. A. *Pharmacol. Ther., **2001**, 89*, 17.
[18] De Clercq, E. *Biochim. Biophys. Acta*, **2002**, *1587*, 258.
[19] Franchetti, P.; Pasqualini, M.; Petrelli, R.; Ricciutelli, M.; Vita, P.; Cappellacci, L. *Bioorg. Med. Chem. Lett.*, **2004**, *14*, 4655.
[20] Jeanot, F.; Gosselin, G.; Standring, D.; Bryant, M.; Sommadossi, J. P.; Loi, A. G.; Colla, P. L.; Mathe, C. *Bioorg. Med. Chem.*, **2002**, *10*, 3153.
[21] Hrdlicka, P. J.; Andersen, N. K.; Jepsen, J. S.; Hansen, F. G.; Hansen, F. G.; Haselmann, K. F.; Nielsen, C.; Wengel, J. *Bioorg. Med. Chem.*, **2005**, *13*, 2597.
[22] Shi, J.; Du, J.; Ma, T.; Pankiewicz, K. W.; Patterson, S. E.; Tharnish, P. M.; Mc Brayer, T. R.; Stuyver, L. J.; Otto, M. J.; Chu, C. K.; Schinazi, R. F.; Watanabe, K. A. *Bioorg. Med. Chem.*, **2005**, *13*, 1641.
[23] Kifli, N.; De Clercq, E.; Balzarini, J.; Simons, C. *Bioorg. Med. Chem.*, **2004**, *12*, 4245.
[24] Qiu, X.L.; Qing, F.L. *Bioorg. Med. Chem.*, **2005**, *13*, 277.
[25] Summerer, D.; Marx, A. *Bioorg. Med. Chem. Lett.*, **2005**, *15*, 869.
[26] Ichikawa, E.; Kato, K. *Curr. Med. Chem*., **2001**, *8*, 385.
[27] Townsend, L.B.; Tipson, R.S. Eds. Nucleic Acid Chemistry, Part 3 Section 2, Wiley New York **1986**.
[28] Woltermann, C. J.; Lapin, Y. A.; Kunnen, K. B.; Tueting, D. R.; Sanchez, I. H. *Tetrahedron*, **2004**, *60*, 3445.
[29] Kern, E. R. *Antiviral Res.*, **2003**, *57*, 35.
[30] Garcia-Junceda, E.; Garcia-Garcia, J. F.; Bastida A.; Fernandez-Mayoralas, A. *Biorg. Med. Chem.*, **2004**, *12*, 1817.
[31] Wong, C. H.; Whitesides, G. M. Enzymes in Synthetic Organic Chemistry, Tetrahedron Organic Chemistry Series JE Baldwin, PD Magnus Eds Pergamon UK **1994** Vol. *12*, 290-295.
[32] Browska, A.; Kulikowska, E.; Shugar, D. *Pharmacology & Therapeutics*, **2000**, *88*, 349.
[33] Priego, E.M.; Mendieta, J.; Gago, F.; Balzarini, J.; De Clercq, E.; Camarasa, M. J.; Perez-Perez, M. J. *Nucleosides Nucleotides*, **2003**, *22*, 951.
[34] Hanrahan, J. R.; Hutchinson, D. W. *J. Biotech.*, **1992**, *23*, 193.
[35] Utagawa, T. *J. Mol. Catal. B: Enzym.,* **1999**, *6*, 215.
[36] Prasad, A. K.; Trikha, S.; Parmar, V. S. *Bioorg. Chem.,* **1999**, *27*, 135.
[37] Pugmire, M. J.; Ealick, S. E. *Biochem. J*., **2002**, *361*, 1.
[38] Roberts, E. L. L.; Newton, R. P.; Axford, A. T., *Clin. Chim. Acta*, **2004**, *344*, 109.
[39] Klein, R. S.; Lenzi, M.; Lim, T. H.; Hotchkiss, K. A.; Wilson, P.; Schwartz, E. L. *Biochem. Pharmacol.*, **2001**, *62*, 1257.
[40] Andricopulo, A. D.; Yunes, R. A. *Chem. Pharm. Bull.*, **2001**, *49*, 10.
[41] Montgomery, J. A. *Med. Res. Rev.*, **1993**, *13*, 209.
[42] Stoeckler, J. D. In: Development in cancer chemotherapy; R. J. Glazer Ed., Boca Raton, CRC Press **1984**, 35-60.
[43] Balzarini, J.; Robins, M. J.; Zou, R. M.; Herdewijn, P.; De Clercq, E. *Biochem. Biophys. Res. Commun*, **1987**, *145*, 277.
[44] Munagala, N. R.; Wang, C. C. *Mol. Biochem. Parasitol.*, **2003**, *127*, 143.
[45] Parker, W. B.; Allan, P. W.; Hassan, A. E. A.; Secrist III, J. A.; Sorscher, E. J.; Waud, W. R. *Cancer Gene Therapy*, **2003**, *10*, 23.
[46] Reigan, P.; Edwards, P. N.; Gbaj, A.; Cole, C.; Barry, S. T.; Page, K. M.; Ashton, S. E.; Luke, R. W. A.; Douglas, K. T.; Stratford, I. J.; Jaffar, M.; Bryce, R. A.; Freeman, S. J. *J. Med. Chem.*, **2005**, *48*, 392.
[47] Sengrupta, S.; Sellers, L. A.; Matheson, H. B.; Fan, T. P. *Br. J. Pharmacol*, **2003**, *139*, 219.
[48] McNally, V. A.; Gbaj, A.; Douglas, K. T.; Stratford, I. J.; Jaffar, M.; Freeman, S.; Bryce, R. A. *Bioorg. Med. Chem. Lett.*, **2003**, *13*, 3705.
[49] Cao, D.; Pizzorno, G. *Drugs Today (Barc.)*, **2004**, *40*, 431.
[50] Lewis, A. S.; Glaut, M. D. *Biochem*, **1976**, *15*, 445.
[51] Lewis, A. S.; Glaut, M. D. *J. Biol. Chem*., **1976**, *251*, 407.
[52] Koellner, G.; Stroh, A.; Raszewski, G.; Holy, A.; Bzowska, A. *Nucleosides Nucleotides*, **2003**, *22*, 1699.

[53] Filgueira de Azevedo, W.; Canduri, F.; Marangoni dos Santos, D.; Guimarães Silva, R.; Simões de Oliveira, J.; Sório de Carvalho, L. P.; Basso, L. A.; Mendes, M. A.; Palma, M. S.; Santos, D. S. *Biochem. Biophys. Res. Commun.*, **2003**, *308*, 545.

[54] Koellner, G.; Bzowska, A.; Wielgus-Kutrowska, B.; Luié, M.; Steiner, T. *J. Mol. Biol.*, **2002**, *315*, 351.

[55] Shereo, H.; Yokoseki, K., *Agric. Biol. Chem*., **1991**, *55*, 1849.

[56] Gelpen, R. W.; Sadof, H. L. *J. Biol. Chem.*, **1971**, *246*, 1475.

[57] Lecoq, K.; Belloc, I; Desgranges, C.; Konrad, M.; Daignan-Fornier, B. *J. Bacteriol.*, **2001**, 4910.

[58] Munagala, N.; Wang, C. C. *Biochem.*, **2002**, *41*, 10382.

[59] Hamamoto, T.; Okuyama, K.; Noguchi, T.; Midorikawa, Y. *Biosci Biotechnol Biochem*, **1997**, *61*, 272.

[60] Nolasco, D. O.; Canduri, F.; Pereira, J. H.; Cortinóz, J. R.; Palma, M. S.; Oliveira, J. S.; Basso, L. A.; F de Azevedo Jr, W.; Santos, D. S. *Biochem. Biophys. Res. Commun.*, **2004**, *324*, 789.

[61] Wielgus-Kutrowska, B.; Bzowska, Agnieszka; Tebbe, J.; Koellner, G.; Shugar, D. *Biochim. Biophys. Acta,* **2002**, *1597*, 320.

[62] Jensen, K. F. *Biochim. Biophys. Acta*., **1978**, *525*, 346.

[63] Jensen, K. F. *Eur. J. Biochem*., **1976**, *67*, 377.

[64] Ealick, S. E.; Rule, S. A.; Carter, D. C.; Greenhough, T. J.; Babu, Y. S.; Cook, W. J.; Habash, J.;Helliwell, J. R.; Stoeckler, J. D.; Parks Jr, R. E. *J. Biol. Chem*., **1990**, *265*, 1812.

[65] Koellner, G.; Lui_, M.; Shugar, D.; Saenger, W.;Bzowska, A. *J. Mol. Biol*., **1998**, *280*, 153.

[66] Bennett, E. M.; Li, C.; Allan, P. W.; Parker, W. B.; Ealick, S. E. *J. Biol. Chem.*, **2003**, *278*, 47110.

[67] Harding, M. J. *Acta Crystallogr Sect D*, **2002**, *58*, 872-874.

[68] Leer, J. C.; Hammer-Jespersen, K.; Schwartz, M. *Eur. J. Biochem*., **1977**, *75*, 21.

[69] Oliva, I.; Zuffi, G.; Barile, D.; Orsini, G.; Tonon, G.; De Gioia, L.; Ghisotti, D. J. *Biochem*, **2004**, *135*, 495.

[70] Caradoc-Davies, T. T.; Cutfield, S. M.; Lamont, I. L.; Cutfield, J. F. J. *Mol. Biol.*, **2004**, *337*, 337.

[71] Usova, E.; Maltseva, T.; Földesi, A.; Chattopadhayaya, J.; Eriksson, S. J. *Mol. Biol.*, **2004**, *244*, 1347.

[72] Yamada, E.W. *J. Biol. Chem.*, **1968**, *243*, 1649.

[73] Krenitsky, T. A.; Barclay, M.; Jacquez, J.A. *J. Biol. Chem.*, **1964**, *239*, 805.

[74] Friedkim, M.; Roberts, D. *J. Biol. Chem*., **1954**, *207*, 245.

[75] Razzell, W. E.; Khorana, H. G. *Biochim. Biophys. Acta*, **1958**, *28*, 562.

[76] Avraham, Y.; Grossowicz, N.; Yashphe, J. *Biochim. Biophys. Acta*, **1990**, *1040*, 287.

[77] Hamamoto, T.; Noguchi, T.; Midorikawa, Y. *Biosci. Biotechnol. Biochem*., **1996**, *60*, 1179.

[78] Scocca, J. J. *J. Biol. Chem.*, **1971**, *246*, 6606.

[79] Walter, M. R.; Cook, W. J.; Cole, L. B.; Short, S. A.; Koczalk, G. W.; Klenistsky, T. A.; Ealick, S. E. *J. Biol. Chem.*, **1990**, *265*, 14016.

[80] Pugmire, M. J.; Ealick, S. E. *Structure*, **1998**, *6*, 1467.

[81] Mendieta, J.; Martín-Santamaría, S.; Priego, E. M.; Balzarini, J.; Camarasa, M. J.; Pérez-Pérez, M. J.; Gago, F. *Biochem.*, **2004**, *43*, 405.

[82] Norman, R. A.; Barry, S. T.; Bate, M.; Breed, J.; Colls, J. G.; Emill, R. J.; Luke, R. W. A.; Minshull, C. A.; McAlister, M.S. B.; McCall, El. J.; McMiken, H. H. J.; Paterson, D. S.; Timms, D.; Tucker, J. A.; Pauptit, R. A. *Structure*, **2004**, *12*, 75.

[83] Federov, A.; Shi, W.; Kieska, G.; Federov, E.; Tyler, P. C.; Furneaus, R. H.; Hanson, J. C.; Gainsford, G. J.; Larese, J. Z.; Scramm, V. L.; Almo, S. C. *Biochem*., **2001**, *40*, 853.

[84] Porter, D. J. T. *J. Biol. Chem*., **1992**, *267*, 7342.

[85] Erion, M. D.; Stoeckler, J. D.; Guida, W. C.; Walter, R. L.; Ealick, S. E. *Biochem.*, **1997**, *36*, 11735.

[86] Canduri F.; Fadel, V.; Basso, L. A.; Palma, M. S.; Santos, D. I.; Filgueira de Azevedo Jr., W. *Biochem. Biophys. Res. Commun.*, **2005**, *327*, 646.

[87] Krenitsky, T. A.; Tuttle, J. V. *Biochim. Biophys. Acta*, **1982**, *703*, 247.

[88] Schramm, V. L. *Arch. Biochem. Biophys.*, **2005**, *433*, 13.

[89] Birck, M. R.; Schramm, V. L. *J. Am. Chem. Soc.*, **2004**, *126*, 2447.

[90] Demain, A. L. *Trends Biotechnol.*, **200**0, *18*, 26.

[91] Trelles, J. A.; Valino, A. L.; Runza, V.; Lewkowicz, E. S.; Iribarren, A. M. *Biotechnol. Lett.,* **2005,** *27*, 759.

[92] Ogawa, J.; Shimizu, S. *Trends Biotechnol.*, **1999**, *17*, 13.

[93] Ciuffreda, P.; Loseto, A.; Santaniello, E. *Tetrahedron,* **2002***, 58*, 5767.

[94] Lewkowicz, E.; Martínez, N.; Rogert, M. C.; Porro, S.; Iribarren, A. M. *Biotechnol. Lett.*, **2002,** *22*, 1277.

[95] Rogert, M. C.; Trelles, J.; Porro, S.; Lewkowicz, E.; Iribarren, A. M. *Biocat. Biotrans*, **2002**, *20*, 347.

[96] Ubiali, D.; Rocchietti, S.; Scaramozzino, F.; Terreni, M.; Albertini, A. M.; Fernández–Lafuente, R.; Guisán, J. M.; Pregnolato, M. *Adv. Synth. Catal.*, **2004**, *346*, 1361.

[97] Spoldi, E.; Ghisotti, D.; Cali, S.; Grisa, M.; Orsini, G.; Tonon, G.; Zuffi, G. *Nucleosides Nucleotides*, **2001**, *20*, 977.

[98] Oliva, I.; Zuffi, G.; Orsini, G.; Tonon, G.; De Gioia, T.; Ghisotti, D. *Enzyme Microbiol. Technol.*, **2004**, *35*, 309.

[99] Hutchinson, D.W. *Trends Biotechnol*, **1990**, *8*, 348.

[100] Gupta, M.N. *Biotechnol. Appl. Biochem.*, **1991**, *14*, 1.

[101] Hori, N.; Watanabe, M.; Sunagawa, K.: Uehara, K.; Mikami, Y. *J. Biotech.*, **1991**, *17*, 121.

[102] Zuffi, G.; Ghisotti, D.; Oliva, I.; Capra, E.; Frascotti, G.; Tonon, G.; Orsini, G. *Biocat. Biotrans.*, **2004**, *22*, 25.

[103] Rocchietti, S.; Ubiali, D.; Terreni, M.; Albertini, A. M.; Fernández-Lafuente, R.; Guisán J.M.; Pregnolato, M. *Biomacromolecules,* **2004**, *5*, 2195.

[104] Guisán, J.M. *Enzyme Microb. Technol.*, **1988**, *10*, 375.

[105] Katchalsky-Katzir, E.; Kraemer, D. M. *J. Mol. Catal. B:Enzym. Catal. B: Enzymol*, **2000**, *10*, 157.

[106] Guisán, J.M.; Alvaro, G.; Fernández-Lafuente, R.; Rosell, C.M.; García, J.L.; Tagliani, A. *Biotechnol. Bioeng.*, **1993**, *42*, 455.

[107] Mateo, C.; Abian, O.; Fernández-Lafuente, R.; Guisán, J. M. *Biotechnol. Bioeng*, **2000**, *68*, 98.

[108] Torres, R.; Mateo, C.; Fuentes, M.; Palomo, J. M.; Ortiz, C.; Fernández-Lafuente, R.; Guisán, J. M.; Tam, A.; Daminati, M. *Biotechnol Prog*., **2002**, *18*, 1221.

[109] Junter, G. A.; Jouenne, T. *Biotechnol. Adv.*, **2004**, *22*, 633.

[110] Gerbsch, N.; Buchholz, R. *FEMS Microbiol Rev.*, **1995**, *16*, 259.

[111] Gaevaia, L. V.; Zhukov, V. N. *Prikl Biokhim Mikrobiol.* **1987**, *23*, 309.

[112] Hori, N.; Watanabe, M.; Sunagawa, K.; Uehara, K.; Mikami, Y. *J. Biotechnol.* **1991**. *17*, 121.

[113] Holy, A.; Votruba, I. *Nucleic Acids Symp. Ser.* **1987**, *18*, 69.

[114] Ewing, D. F.; Holy, A.; Votruba, I.; Humble, R. W.; Mackenzie, G.; Hewedi, F.; Shaw, G. *Carbohydr. Res.* **1991**, *21*, 109

[115] Iaskovich, G. A.; Iakovleva, E. P. *Prikl Biokhim Mikrobiol.* **1999**, *35*, 146.

[116] Trelles, J. A.; Fernández, M.; Lewkowicz, E. S.; Iribarren, A. M.; Sinisterra, J. V. *Tetrahedron Lett.*, **2003**, *44*, 2605.

[117] Trelles, J. A.; Bentancor, L.; Schoijet, A.; Porro, S.; Lewkowicz, E. S.; Sinisterra, J. V.; Iribarren, A. M. *Chem. Biodiv.*, **2004**, *1*, 280.

[118] Trelles, J. A.; Lewkowicz, E. S.; Sinisterra, J. V.; Iribarren, A. M. *Int. J. Biotechnol.*, **2004**, *6*, 376.

[119] Hoffman, A.S. *Adv. Drug Deliv. Rev*, **2002**, *43*, 3.

[120] Hemachander, C.; Bose, N.; Puvanakrishnan, R. *Process Biochem.*, **2001**, *36*, 629.

[121] Trelles, J. A.; Fernández-Lucas, J.; Condezo, L. A.; Sinisterra, J. V. *J. Mol. Cat. B: Enzymat., **2004**, 30*, 219.

[122] Mikami, Y.; Matsumoto, S.; Yoshinaka, S.; Sunaga, Y.; Hasegawa, A., USP 6017736

[123] Shanmuganathan, K.; Koudriakova, T.; Nampalli, S.; Du, J.; Gallo, J. M. *J. Med. Chem.,* **1994**, *37*, 821.

[124] Reigan, P.; Gbaj, A.; Chinje, E.; Stratford, I. J.; Douglas, K. T.; Freeman, S. *Bioorg. Med. Chem Lett.*, **2004**, *14*, 5247.

[125] Kewn, S.; Wang, L. H.; Hoggard, P. G.; Rousseau, F.; Hart, R.; MacNeela, J. P.; Khoo, S. H.; Back, D. J. *Antimicrob. Ag. Chemother.*, **2003**, 255.

[126] Yokozeki, K.; Tsuji, T. *J. Mol. Catal. B:Enzym. Cat. B: enzymat.*, **2000**, *10*, 207.

[127] Grupta, M.; Nair, V. *Tetrahedron Lett.*, **2005**, *46*, 1165.

[128] Weckbecker, G.; Cory, J. G. *Cancer Res.,* **1987**, *47*, 2218.

[129] Heranadez, S.; Ford, H.; Marquez, V. E. *Bioorg. Med. Chem.,* **2002**, *10*, 2723.

[130] Barbas III, C. F.; Wong, C. H. *Bioorg. Chem.,* **1991**, *19*, 261.

[131] Ouwerkerk, N.; Steenweg, M.; Ruijter, M.; Brower, J.; van Boom, J. H.; Lugtenburg, J.; Raap, J. *J. Org. Chem.*, **2002**, *67*, 1480.

[132] Wong, C. H.; García-Junceda, E.; Chen, L.; Blanco, O.; Gijsen, H. J. M.; Steensma, D. H. *J. Am. Chem. Soc.,* **1995**, *117*, 3333.

[133] Horinouchi, N.; Ogawa, J.; Sakai, T.; Kawano, T.; Matsumoto, S.; Sasaki, M.; Mikami, Y.; Shimizu, S. *Appl. Environm. Microbiol.*, **2003**, 3791.
[134] Hutchinson, D. W. *Trends Biotechnol.*, **1990**, *8*, 348.
[135] Cotticelli, G.; Magri, P.; Grisa, M.; Orsini, G.; Tonon, G.; Zuffi, G. *Nucleosides Nucleotides*, **1990**, *18*, 1135.
[136] Mao, C.; Cook, W. J.; Zhou, M.; Koszalka, G.W.; Krenitsky, T. A.; Ealick, S.E. S*tructure*, **1997**, *5*, 1373.
[137] Barai, V. N.; Zinchenko, A. I.; Eroshevskaya, L. A.; Kalinichenko, E. N.; Kulak, T. I.; Mikhailopulo, I. A. *Helv. Chim. Acta,* **2002**, *85*, 1901.
[138] Esipov, R. S.; Gurevich, A. I.; Chuvinovsky, D.V.; Chupova, L. A.; Muravyova, T. I.; Niroshnikov, A.I. *Protein Expression and Purification*, **2002**, *24*, 56.
[139] Shiragami, H.; Irie, Y.; Yasuda, N. *USP Application*, **2001**, 6306647, 43.
[140] Pregnolato, M.; Terreni, M.; Albertini, A.; Guisan, J. M.; Lafuente, R. F.; Frigerio, M. WO patent 030086619.
[141] Tonon, G.; Capra, E.; Orsini, G.; Zuffi, G. USP Application **2004** 01424308.
[142] Wang, J. F.; Zhang, L. R.; Yang, Z. J.; Zhang, L. H. *Bioorg. Med. Chem.*, **2004**, *12*, 1425.
[143] Roncaglia, D. I.; Schmidt, A. M.; Iglesias, L. E.; Iribarren, A. M. *Biotechnol. Lett.,* **2001***, 23*, 1439.
[144] Vo, N. V.; Young, K. C.; Lai, M. M. C. *Biochem.,* **2003**, *42*, 10462.
[145] Iaskovich, G. A.; Iakovleva, E. P. *Prikl Biokhim. Mikrobiol.,* **1999**, *35*, 146.
[146] Konstantinova, I. D.; Leoneva, 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.; Miroshniko, A. I. *Bioorg. Khim.*, **2004**, *30*, 613.
[147] Konstantinova, I. D.; Esipov, R. S.; Muraveva, T. I.; Taran, S. A.; Verevkina, K. N.; Gurevich, A.; Feofanov, S. A.; Miroshniko, A. I. *RU Patent,* **2004**, 2230118.
[148] Freídlos, F.; Jarman, M.; Davies, L. C.; Boland, M. P.; Hnox, R. *J. Biochem. Pharmacol.*, **1992**, *44*, 25.
[149] Cheng, J. B.; Cooper, K.; Duplantier, A. J.; Eggler, J. F.; Kraus, K. G.; Marshall, S. C.; Marfat, A.; Masamune, H.; Shirley, J. T.; Tickner, J. E.; Umland, J. P. *Bioorg. Med. Chem. Lett.*, **1995**, *5*, 1965.
[150] Kugishima, H.; Horie, T.; Imafuku, K. *J. Heterocyclic Chem.*, **1994**, *31*, 1557.
[151] Salluja, S.; Zou, R.; Drach, J.C.; Townsend, L.B.- *J. Med. Chem.*, **1996**, *39*, 881.
[152] [1] Zou, R.; Kawashima, E.; Freeman, G. A.; Koszalka, G. W.; Drach, J. C.; Townsend, L. B. *Nucleosides Nucleotides*, **2000**, *19*, 125.
[153] Doskocil, J.; Holy, A. *Coll. Czech. Chem. Comm.*, **1977**, *42*, 370.
[154] Mickhailopulo, I. L.; Kazimierczuk, Z.; Zinchenko, A. I.; Barai, V. N.; Romanova, V.; Eroshevskaya, L. A. *Nucleosides Nucleotides,* **1995**, *14*, 477.
[155] Bentancor, L.; Trelles, J. A.; Nobile, M.; Lewkowicz, E. S.; Iribarren, A. M. *J. Mol. Catal. B:Enzym.,* **2004**, *29*, 3.
[156] Koshkin, A. A.; Fensholdt, J.; Pfundheller, H. M.; Lomholt, C. *J. Org. Chem.,* **2001**, *66*, 8504.
[157] Mikami, Y.; Matsumoto, S.; Hayashi, Y.; Sato, T. *USP Application,* **2005**, 0037470.
[158] Yu, X. J.; Li, G.; Qi, X. X.; Deng, Y. Q. *Bioorg. Med. Chem. Lett.*, **2005**, *15*, 683.
[159] Mahmoudian, M.; Eaddy, J.; Dawson, M. *Biotechnol. Appl. Biochem.*, **1999**, *29*, 229.
[160] Kitos, T. E.; Tyrrell, D. L. J. *Biochem. Pharmacol*., **1995**, *49*, 1291.
[161] Kawashima, E.; Teui, Y. S.; Ishido, Y.; Yokoseki, K. *Nucleic Acids Symp. Ser.*, **2000**, *44*, 25.
[162] Medici, R.; Lewkowicz, E.; Iribarren, A. M. *J. Mol. Catal. B: Enzym*, **2006**, *39*, 40.
[163] Pauwel, R.; Baba, M.; Balzarini, J.; Herdewijin, P.; Desmyter, J.; Robins, M.; Zou, R. M.; Madej, D.; De Clercq, E. *Biochem. Pharmacol.*, **1988**, *37*, 1317.
[164] Balzarini, J.; De Clercq, E. *Biochem. Biophys. Res. Commun.*, **1989**, *159*, 61.
[165] Krenitsky, T. A.; Koszalka, G. W.; Tuttle, J. V.; Rideout, J. L.; Elion, G. B. *Carbohydrate Research*, **1981**, *97*, 139.
[166] Barai, V. N.; Zinchenko, A. I.; Eroshevskaya, L. A.; Zhernosek, E.V.; Balzarini, J.; De Clercq, E.; Mikhailopulo, I. A. *Nucleosides, Nucleotides & Nucleic Acids*, **2003**, *22*, 5.
[167] Choo, H.; Chong, Y.; Chu, C. K. *Bioorg. Med. Chem. Lett.,* **2003**, *13*, 1993.
[168] Nair, V.; Pal, S. *USP Application*, **2001**, 6316228.
[169] Araki, T.; Ikeda, I.; Matoishi, K.; Abe, R.; Olkawa, T.; Matsuba, Y.; Ishibashi, H.; Nagahara, K.; Fukuiri, Y. *USP Application*, **2003**, 0307405.
[170] Ishibashi, D.; Nagahara, K.; Fukuiri, Y.; Matsuba, Y. *JP Patent Application*, **2004**, 034086.
[171] Araki, T.; Ikeda, I.; Takahashi, K.; Ito, K.; Asano, T.; Nikumaru, S.; Nakamura, T.; Ishibashi, H.; Nagahara, K.; Fukuiri, Y. *USP Application,* **2004**, 0219646.
[172] Komatsu, H.; Awano, H. *J. Org. Chem.,* **2002**, *67*, 5419.
[173] Komatsu, H.; Araki, T. *Tetrahedron Lett.*, **2003**, *44*, 2899.
[174] Nagahara, K.; Ishibashi, D.; Fukuiri, Y.; Matsuba, Y. *J. Patent Application*, **2004**, 041073
[175] Mikami, Y.; Sasaki, Y.; Sunaga, Y.; Sato, T. *J.P. Application*, **2002**, 095494.
[176] Ogawa, J.; Saito, K.; Sakai, T.; Horinouchi, N.; Kawano, T.; Matsumoto, S.; Sasaki, M.; Mikami, Y.; Shimizu, S. *Biosci. Biotechnol. Biochem.*, **2003**, *67*, 933.
[177] Yamada, K.; Matsumoto, N.; Hayakawa, H. *W.O. Patent*, **2005,** 040181.