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Research review paper

Biocatalytic approaches applied to the synthesis of nucleoside prodrugs

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ABSTRACT

Nucleosides are valuable bioactive molecules, which display antiviral and antitumour activities. Diverse types of prodrugs are designed to enhance their therapeutic efficacy, however this strategy faces the troublesome selectivity issues of nucleoside chemistry. In this context, the aim of this review is to give an overview of the opportunities provided by biocatalytic procedures in the preparation of nucleoside prodrugs. The potential of biocatalysis in this research area will be presented through examples covering the different types of nucleoside prodrugs: nucleoside analogues as prodrugs, nucleoside lipophilic prodrugs and nucleoside hydrophilic prodrugs.

Contents

1. Introduction

The aim of this introduction is to show the vast spectrum of possibilities involved in the field of nucleoside prodrugs. There are excellent revisions (Bobeck et al., 2010; De Clercq, 2011; Jordheim et al., 2013; Pertusati et al., 2012; Pradere et al., 2014; Sofia, 2011; Zawilska et al., 2013) on this subject that should be consulted for a much more precise and complete information.

Nucleoside analogues are a family of compounds widely used as antiviral and anticancer drugs that exert their activity through multiple mechanisms of action. In most cases, nucleoside analogues are converted to the corresponding 5′-triphosphate derivatives and incorporated into nucleic acids, causing inhibition of cell proliferation and cell death. Moreover, they can also inhibit different enzymes involved in nucleoside metabolism. In 1969 the first nucleoside based drugs were approved by the Food and Drug Administration (FDA): Cytarabine (1-β-D-arabinofuranosylcytosine, Ara-C) for the treatment of acute myeloid leukaemia and Edoxudine (5-ethyl-2′-deoxyuridine), effective against herpes simplex virus but not further applied. This was the beginning of a long and productive race in the development of new nucleoside derivatives. These researches ended up in several drugs and drug

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candidates and in the present there are more than 25 nucleoside analogues for the treatment of different viral infections and 6 nucleoside analogues approved as cytotoxic agents (Jordheim et al., 2013).

In many cases nucleosides, as many other active compounds, do not show appropriate physicochemical, pharmacokinetic or pharmacodynamic properties and therefore the prodrug approach is advisable. The term prodrug was first introduced in 1958 (Albert, 1958) and can be defined as a compound administered in an inactive pharmacological form and further converted *in vivo* to the parent active drug by metabolic processes. The main purpose of a prodrug is to modify or mask some negative properties of the parent active compound such as chemical or *in vivo* instability, poor water solubility, deficient uptake through cell membranes, toxicity and intolerance to administration (Stella, 2010).

This strategy has been gaining a considerable importance in the development of new medicaments and pharmaceutical market forecasts estimate that about 10% of the available drugs can be classified as prodrugs (Huttunen et al., 2011; Zawilska et al., 2013). Acetanilide is considered the first example of a prodrug used in clinic; it is activated by *in vivo* hydroxylation to paracetamol (*N*-(4-hydroxyphenyl) ethanamide) and it was used as early as in 1867 as antipyretic agent (Bertolini et al., 2006).

There are different classifications of prodrugs, based on polarity, action mechanism or taking into account if the modification is present at the base, sugar or phosphate moieties, but in general prodrugs are divided in two main groups: carrier-linked prodrugs and bioprecursor prodrugs (Zawilska et al., 2013).

In the first group, the active drug is attached to a carrier or promoiety by means of a covalent linker that is biocatalytically or chemically cleaved *in vivo*, releasing both the active parent compound and the carrier. Therefore, the carrier must also fulfil certain requirements such as to be metabolised to non-toxic species, to be nonimmunogenic, easily available and its linkage should be stable enough to reach the target inside the body. The classical approach for prodrug design (Dahan et al., 2014) involves the covalent attachment of either hydrophilic functionalities (*e.g.*, phosphate, sulphate) to increase the water solubility of the parent drug (Stella and Nti-Addae, 2007), or lipophilic moieties (*e.g.*, ester) to increase its passive permeability (Rautio et al., 2008). To the second group of prodrugs, the bioprecursors, belong those compounds that are modified structures of the active drug. They lack of linkers and carrier moieties but need to be chemically or metabolically transformed to the active molecule (Kokil and Rewatkar, 2010).

Since nucleosides and nucleoside analogues are rapidly catabolised to inactive derivatives and display poor bioavailability, the prodrug approach has been extensively explored and this strategy afforded new drug generations. Representative examples of different nucleoside prodrugs are found in Scheme 1.

Regarding lipophilic nucleoside prodrugs, they are expected to show the following advantages: increased metabolic stability, pass through cell membrane by passive diffusion becoming independent of active transporter, better absorption by oral delivery and enhanced circulation times. On the other hand, they may have poorer water solubility and can accumulate in the liver, deriving in higher toxicity and degradation of the drug.

Ara-C is the typical example of a successful application of this kind of strategies. Elacytarabine (Scheme 1) is an Ara-C derivative that carries an elaidic acid moiety attached to the 5′-hydroxyl group, which confers protection against cytidine deaminase action and avoids active transport into cells (Bergman et al., 2004). This prodrug proved to be active in leukaemia, solid tumour cell lines (Bergman et al., 2004) and xenografts (Breistol et al., 1999). Further examples, selected from many reported structures, are the 3′,5′-di-*O*-isobutyryl diester of 2′-α-fluoro-2′-β-*C*-methylcytidine (Mericitabine) (Sofia et al., 2010) and the 2′,3′,5′-tri-*O*-isobutyryl ester of 4′-azidocytidine (Halapiravir) (Brandl et al., 2008).

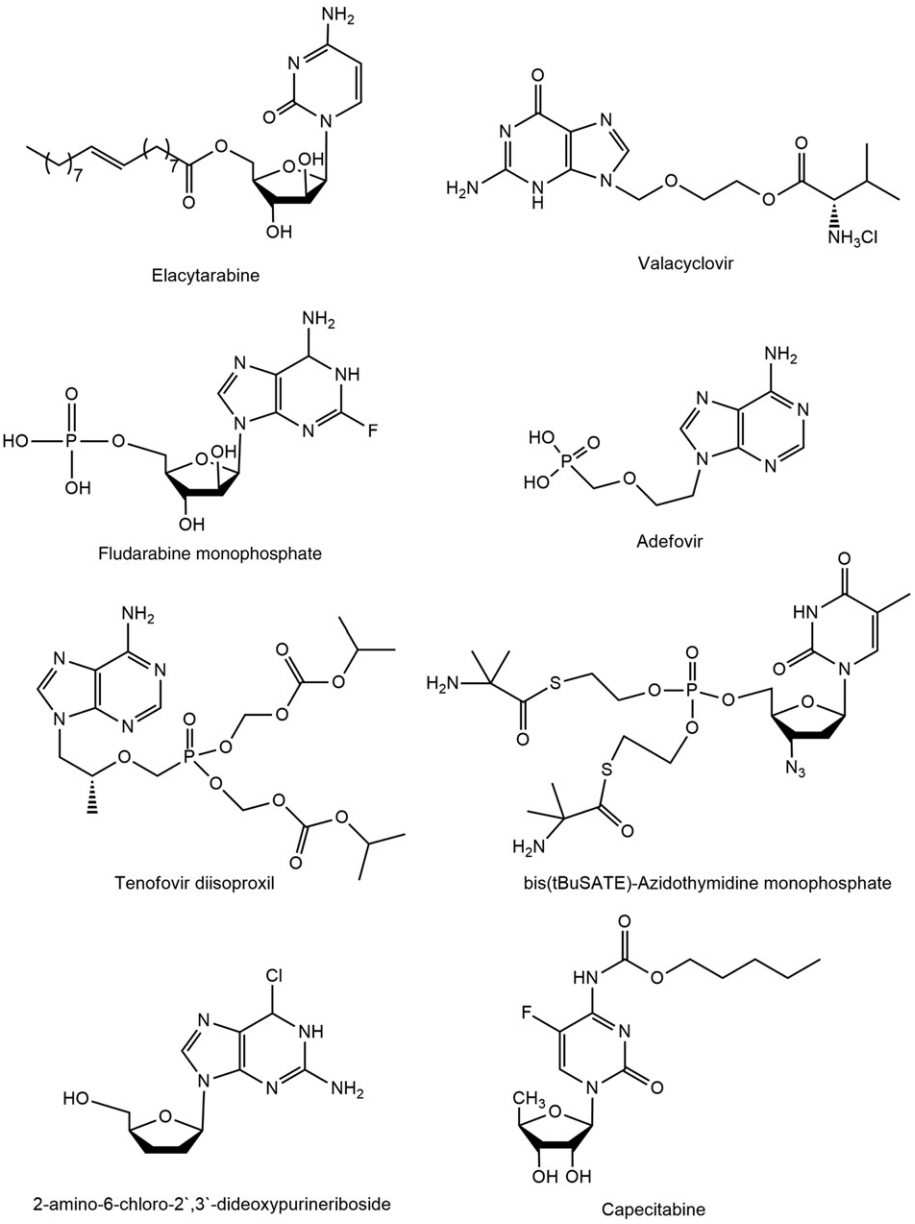
Not only acyl groups but also aminoacyl moieties are used as promoieties in nucleoside prodrugs, mainly either as esters or amides (Vig et al., 2013). Acyclovir is an acyclic nucleoside used as an antiherpetic agent that is phosphorylated to the corresponding monophosphate selectively by virus-specific thymidine kinase. The L-valyl ester of Acyclovir (Valacyclovir, Scheme 1) is probably the first commercial example of amino acid prodrug that utilizes intestinal transporters to increase permeability and therefore, overcomes poor oral bioavailability of the parent compound. After the discovery of Valacyclovir, Valganciclovir, the corresponding prodrug of Ganciclovir, was evaluated and showed an oral bioavailability approximately 10-times higher than that of the parent compound (Anderson et al., 1995; Jung and Dorr, 1999). There are other examples of L-valyl ester prodrugs of nucleoside analogues such as the 5′-*O*-valyl derivative of Didanosine (2′,3′-dideoxyinosine) and the 3′-*O*-valyl ester of the broad spectrum antiviral Valopicitabine (2′-methylcytidine) (Brown, 2009; Pierra et al., 2006).

In order to obtain prodrugs with increased water solubility, a complementary strategy involves the attachment of polar or ionic groups to the active drug. In the nucleoside field the most explored route is the synthesis of nucleoside 5′-monophosphates. To this group of derivatives belongs Fludarabine monophosphate (9-β-D-arabinofuranosyl-2-fluoroadenine-5′-monophosphate, F-araAMP, Scheme 1), a hydrophilic prodrug widely used in the treatment of chronic lymphocytic leukaemia therapies (Robak et al., 2009).

As abovementioned, in most cases nucleosides must be transformed to the corresponding nucleoside 5′-triphosphates in order to become substrate of polymerases and consequently be incorporated into growing nucleic acids. In this metabolic process the rate limiting step is the first phosphorylation that affords the 5′-monophosphates. Their direct delivery would provide a potential way to circumvent this limitation, but unfortunately these compounds are rapidly hydrolysed by phosphatases. To overcome this drawback, the phosphate group has been replaced with the isoelectric and isosteric phosphonate moiety, providing a chemically and enzymatically more stable derivative that can be also further phosphorylated by nucleotide kinases (Pertusati et al., 2012). For example, Adefovir (Scheme 1) is an acyclic nucleoside phosphonate active against hepatitis B virus and HIV, which exerts its activity by inhibiting the enzyme reverse transcriptase (RT).

However, phosphates as well as phosphonates derivatives are ionic at physiological pH and consequently both show very poor cell permeability (Lichtenstein et al., 1960). In order to facilitate cell uptake, their negative charges have been masked generating a new kind of prodrugs (Caron et al., 2010). These compounds are called pronucleotides because upon entering the cell, the masking groups are hydrolysed to release the 5′-nucleotides, which are further phosphorylated to the di- and triphosphate derivatives (Mehellou et al., 2010). This approach is called 'kinase bypass' (Ray and Hostetler, 2011) and it is essential in those cases where the nucleoside analogue does not exert the expected biological property, whilst its triphosphate derivative proves to be active. For example, 3′-*O*-methylthymidine, an analogue of Zidovudine (3′-azido-3′-deoxy thymidine, AZT), is inactive against HIV, whilst its triphosphate is an exceptionally potent inhibitor of HIV-RT, the inactivity of the nucleoside being attributed to poor phosphorylation by host kinases (McGuigan et al., 1993). This strategy was supported by the FDA's approval of the antiviral prodrugs Adefovir dipivoxil and Tenofovir diisoproxil (Scheme 1), both phosphonate pronucleotides acting as reverse transcriptase inhibitors of a crucial virus enzyme in human immunodeficiency virus 1 (HIV-1) and hepatitis B virus infections (Jordheim et al., 2013).

Other phosphate and phosphonate derivatives have been also developed (McGuigan et al., 2013); for example, *S*-acyl-2-thioethyl (SATE) groups are hydrolysed inside cells into the phosphodiester derivative by a nonspecific esterase, which subsequently liberates the nucleoside monophosphate by the action of a phosphodiesterase (Peyrottes et al., 2004). The SATE derivative of AZT, bis(tBuSATE)AZT-MP (Scheme 1),



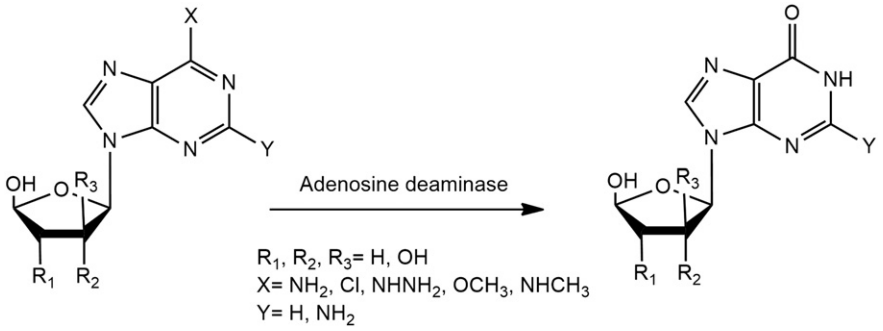
Scheme 1. Representative examples of different types of nucleoside prodrugs.

showed marked antiviral activity in thymidine kinase-deficient CEM cells in which AZT was virtually inactive, reaffirming the hypothesis that it exerts its activity *via* intracellular delivery of AZT 5′-monophosphate (Lefebvre et al., 1995). Phosphoramidates and phosphorodiamidates have been also explored as pronucleotides with improved bioavailability (McGuigan et al., 2013; Mehellou et al., 2009).

Other types of derivatives expand the diversity of nucleoside prodrugs. A particular case is provided by analogues that lack of attached moieties but are considered prodrugs, since they are activated *in vivo* to an active drug. Typical examples are guanine nucleoside prodrugs. Guanosine analogues are frequently used as antiviral and anticancer agents, but they show very poor water solubility. Since some 6-substituted-2-aminopurine nucleoside analogues are transformed to the corresponding guanine derivatives by the action of adenosine deaminase they are potential guanosine prodrugs (Pei et al., 2011). For example, the properties of 2-amino-6-chloro-2′,3′-dideoxypurine riboside (Scheme 1) were evaluated as an adenosine deaminase activated prodrug of 2′-3′-dideoxyguanosine.

In addition, nucleosides and nucleosides derivatives are used as nucleobase prodrugs. An example of a triple prodrug of 5-fluorouracil, an approved antineoplastic drug for the treatment of solid tumours, is Capecitabine (5′-deoxy-5-fluorocytidine carbamate, Scheme 1). This derivative is efficiently absorbed after oral administration (Johnston and Kaye, 2001; Koukourakis et al., 2008) and metabolised by hepatic carboxyesterases, generating 5′-deoxy-5-fluorocytidine, which is further transformed to 5′-deoxy-5-fluorouridine by cytidine deaminase. 5′-Fluorouracil (Miwa et al., 1998) is ultimately formed by the action of thymidine phosphorylase.

When the preparation of a nucleoside prodrug is planed, it must be taken into account that nucleosides are polyfunctional molecules carrying amino and hydroxyl groups with similar nucleophilicity and in some cases, labile glycosidic bonds. Therefore, their selective modification requires of protection and deprotection steps and mild conditions. In this regard, biotransformations provide alternative and suitable routes to traditional chemistry approaches, and consequently, several chemoenzymatic strategies have been applied to the synthesis



Scheme 2. Biotransformation performed by adenosine deaminase (ADA).

of the different types of nucleoside prodrugs above discussed. For the particular case of pronucleotides, reports regarding their biocatalysed preparation are not yet available.

Considering the above presented context, the aim of the following sections is to summarize the existing knowledge regarding the syntheses of nucleoside prodrugs addressed by chemo-biocatalysed routes. The focus will be on literature available from 2010 to the present and only subjects that were not formerly reviewed or examples previous to the above indicated date, but considered representative for the discussion, will be included in this work.

2. Nucleoside analogues as prodrugs

According to prodrug definition, nucleoside and nucleoside analogues should be also considered as prodrugs by themselves since in most cases they must be phosphorylated by different kinases to the final triphosphorylated derivatives in order to be incorporated into nucleic acids by viral or human polymerases. However, this criterium will not be used throughout this review, otherwise it should be extended to comprise all the reported biocatalysed synthesis of nucleoside analogues. For this purpose, excellent reviews are available (Fresco-Taboada et al., 2013; Lewkowicz and Iribarren, 2006; Mikhailopulo, 2007; Mikhailopulo and Miroshnikov, 2011).

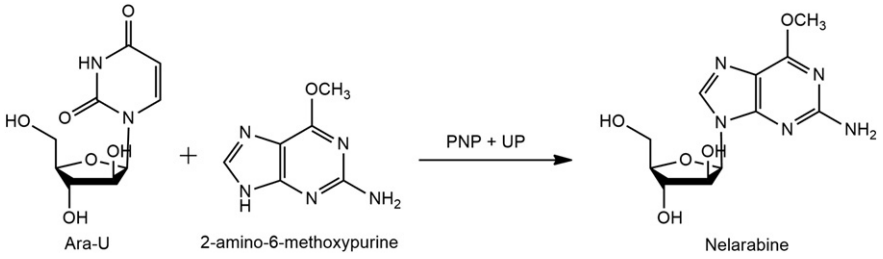
The only exceptions included in this paper are those nucleoside analogues that are *in vivo* activated by routes different to phosphorylation (Maag, 2012; Zhang et al., 2014). For organisative purposes, the chemoenzymatic preparation of these compounds is divided in nucleoside and nucleobase prodrugs.

2.1. As nucleoside prodrugs

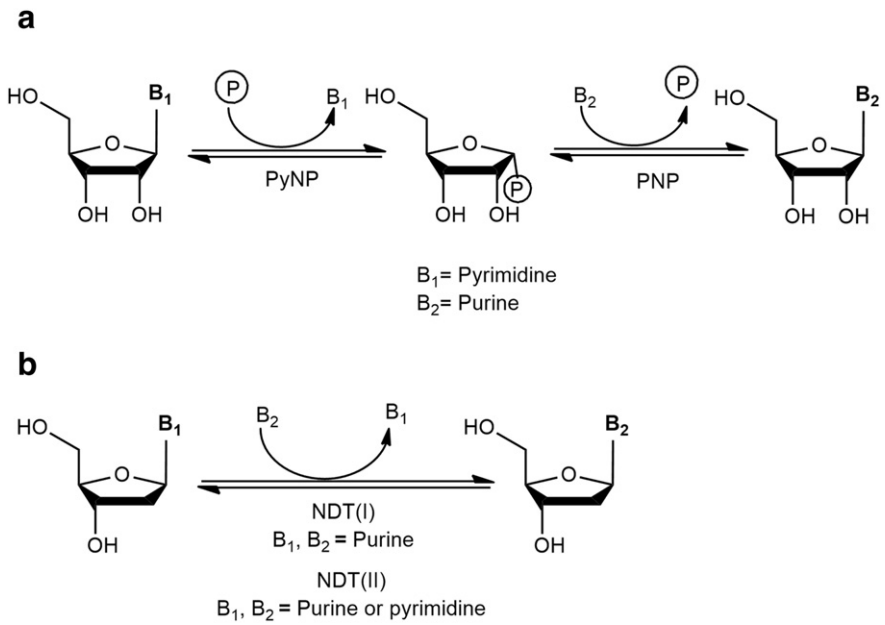
Adenosine deaminase (ADA, EC 3.5.4.4) plays an important role in purine metabolism, catalysing the irreversible hydrolytic deamination of adenosine (Scheme 2, X = NH_2, Y = R_3 = H, R_1 = R_2 = OH) to inosine. It is possible to profit its ubiquity and abundance in human tissues for the activation of nucleoside prodrugs (Pei et al., 2011). 6-Substituted adenosines, like 6-chloro-, 6-hydrazino-, 6-methoxy- and 6-methylaminopurine ribosides (Scheme 2), are ADA substrates exhibiting different degrees of efficacy. In addition, some 2,6-disubstituted purine nucleosides, carrying 2-amino or 2-alkyl groups, but not halides, can be deaminated, whilst nucleosides modified at 2′ or 3′, but not at 5′, are also deaminated by this enzyme (Medici et al., 2008).

Guanosine analogues are frequently used as antiviral and anticancer agents but their poor water solubility requires the development of more soluble prodrugs. One of the most interesting examples is Nelarabine (Scheme 3), which is currently being used for the treatment of acute lymphoblastic leukaemia (Robak and Robak, 2013). This prodrug is rapidly demethoxylated in blood to 9-β-D-arabinofuranosylguanine (Ara-G) by ADA (Kisor, 2006) and it is 8-times more water soluble than the active drug. The chemo-enzymatic synthesis of Nelarabine is shown in Scheme 3 (Herbal et al., 2005; Krenitsky et al., 1992). The key step in this process is the regio- and stereoselective biocatalysed reaction between 2-amino-6-methoxypurine and 1-β-D-arabinofuranosyluracil (Ara-U) using a combination of nucleoside phosphorylases (NPs). Naturally, NPs are transferases that catalyse the reversible cleavage of the glycosidic bond of ribo- and deoxyribonucleosides in the presence of inorganic phosphate, to generate the corresponding base and ribose- or deoxyribose-1-phosphate. Both pyrimidine (PyNP) and purine (PNP, EC 2.4.2.1) nucleoside phosphorylases exist and significant differences in substrate specificity of phosphorylases from mammalian and some lower organisms like protozoan parasites and bacteria are found. Thymidine phosphorylase (TP, EC 2.4.2.4) and uridine phosphorylase (UP, EC 2.4.2.3) are the main members of PyPNs, being thymidine and uridine the corresponding natural substrates. *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. Thus, the combination of both enzymes makes possible the generation of purine nucleosides from pyrimidine ones (the so called transglycosylation, Scheme 4a). This reaction is totally regio- and stereoselective, producing only the corresponding *N*-9/β product (Lewkowicz and Iribarren, 2006).

NPs from different sources have been employed to synthesise 6-mono- and 2,6-di substituted purine nucleosides by transglycosylation. 2,6-Diaminopurine arabinoside (DAPA) (Zuffi et al., 2004) was prepared using UP and PNP cloned and overexpressed in independent *Escherichia coli* strains (74% yield, 2.5 h, 60 °C). The enzymes were co-immobilised on epoxy-activated Sepabeads EC-EP/M resin to make the biocatalyst suitable for industrial scale applications. DAPA, like Nelarabine, is used



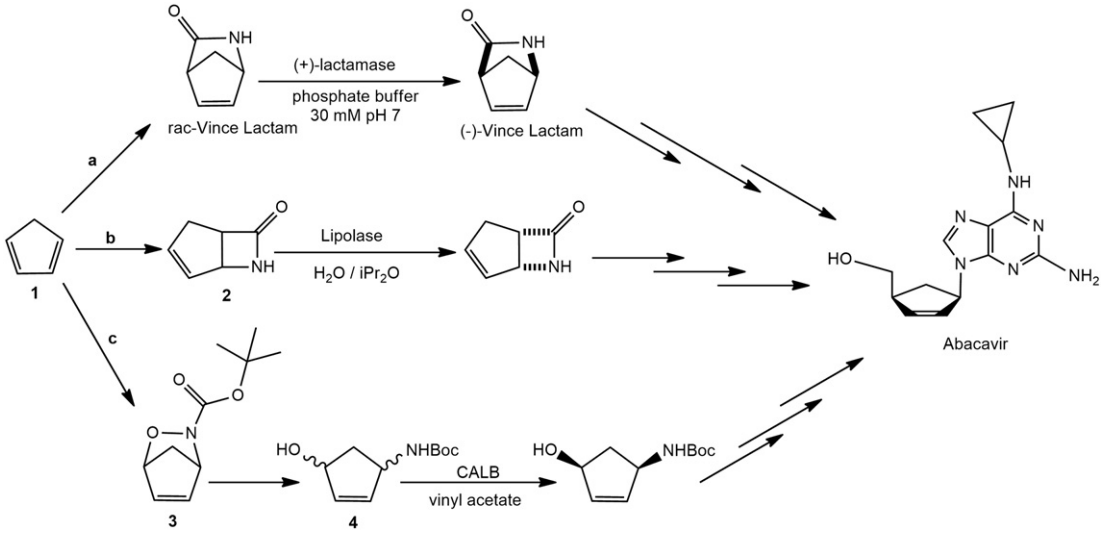
Scheme 3. Chemoenzymatic synthesis of Nelarabine, an Ara-G prodrug, mediated by nucleoside phosphorylases. UP: uridine phosphorylase. PNP: purine nucleoside phosphorylase.



Scheme 4. Biocatalysed nucleoside synthesis. **a**—Transglycosylation catalysed by nucleoside phosphorylases **b**—Nucleobase exchange catalysed by nucleoside-2′-deoxyribosyltransferases. PyNP: pyrimidine nucleoside phosphorylase. PNP: purine nucleoside phosphorylase. NDT I: nucleoside 2′-deoxyribosyltransferase type 1. NDT II: nucleoside 2′-deoxyribosyltransferase type 2.

as Ara-G prodrug. The synthesis of not only DAPA but also 2,6-diaminopurine riboside (DAPR), 2,6-diaminopurine-2′-deoxyriboside (dDAPR) and 2,6-diaminopurine-2′-3′-dideoxyriboside (ddDAPR) was performed by whole cell biocatalysed transglycosylations carried out by our research group (Medici et al., 2006). After cell collection screening, *Aeromonas salmonicida* (95%, 1 h, 60 °C), *Enterobacter aerogenes* (85%, 1 h, 60 °C), *E. coli* BL 21 (66%, 48 h, 45 °C) and *Enterobacter gergoviae* (72%, 48 h, 60 °C) were used to prepare DAPR, dDAPR, ddDAPR and DAPA respectively, using the corresponding uracil nucleosides as substrates. dDAPR was also obtained using thymidine as substrate and *Proteus vulgaris* as biocatalyst (88%, 0.5 h, 45 °C). The use of dDAPR as deoxyguanosine prodrug in L1210 cells (Weckbecker and Cory, 1987) and in ducks (Kitos and Tyrrell, 1995) was reported. ddDAPR is a potent and selective inhibitor of HIV and HBV and moreover, a strong inhibitor of human ADA. Therefore, it is used in combination with other nucleoside drugs, such as Vidarabine (9-β-D-arabinofuranosyladenine, Ara-A), to potentiate their activity in the treatment of herpes simplex and vaccinia virus infections (Balzarini and De Clercq, 1989). DAPR and DAPdR, but not DAPA, were obtained using recombinant whole cells that co-express TP and PNP or UP and PNP. These strains were able to express two genes in two individual plasmids or in tandem in a single plasmid (Ding et al., 2010). Lactose, which is cheap and no toxic, was used to induce protein expression and 70–90% yield was reached after 2-h incubation at 50 °C.

Citrobacter koseri whole cells were immobilised by entrapment techniques in our laboratory, in view to future scale up for industrial applications (Nóbile et al., 2012). The agarose-immobilised cells were used up to 68 times and the storage stability was at least 9 months. By this approach, DAPA (77% yield in 24 h) was prepared. Other microorganisms were reported as sources of biocatalysts useful for the preparation of 6- and 2-amino-6-substituted nucleosides, as potential inosine and guanosine analogues prodrugs: *Aeromonas hydrophila* free



Scheme 5. Chemoenzymatic approaches (**a**, **b**, **c**) to prepare Abacavir from Diels–Alder cycloaddition of cyclopentadiene.

and agarose-entrapped whole cells (Nóbile et al., 2010), as well as cloned and overexpressed free enzymes (Ubiali et al., 2012), thermostable *Geobacillus stearothermophilus* (De Benedetti et al., 2012) and *Aeropyrum pernix* K1 (Zhu et al., 2013).

As alternative to NPs, nucleoside 2′-deoxyribosyltransferases (NDTs, EC 2.4.2.6) have been used in nucleoside synthesis. NDTs catalyse the regio- and stereoselective transference of a 2-deoxy-α-D-ribofuranosyl moiety from purine or pyrimidine 2′-deoxyribonucleosides to free purine or pyrimidine bases (Scheme 4b) and are specific for 2′-deoxyribonucleosides (Anand et al., 2004). Two classes of NDTs exist, depending on their substrate specificity: NDT type I (PDT), specific for purines (Pur ↔ Pur) and NDT type II (NDT), which catalyses the transference between purines and/or pyrimidines (Pur ↔ Pur, Pur ↔ Pyr, Pyr ↔ Pyr) and are widely distributed in *Lactobacilli* species. The use of NDTs instead of NPs as biocatalysts for nucleoside analogues synthesis shows as main advantage the one-pot, one-step, instead one-pot, two-step synthesis by using NPs, since only one enzyme is involved. On the contrary, NPs can be employed in the synthesis of a large range of nucleoside analogues, since they can recognise either ribo or 2′-deoxyribose moieties and many variants at 2′ and 3′ positions (Holguin et al., 1975). Recent studies have shown that some NDTs, such as that from *Lactobacillus reuteri* (LrNDT), can slightly recognise modifications at the deoxyribose moiety. This enzyme was cloned and overexpressed (Fernández-Lucas et al., 2010) and the recombinant free and immobilised LrNDT were employed in the synthesis of several arabinosylnucleosides (Fernández-Lucas et al., 2013). When LrNDT immobilised on magnetic chitosan beads was used, DAPA was obtained in 83% yield at 2 h and the biocatalyst could be recycled for at least 30 consecutive batch reactions, with negligible loss of catalytic activity. The characteristics, applications and potential of NDRT have recently been deeply reviewed (Fresco-Taboada et al., 2013).

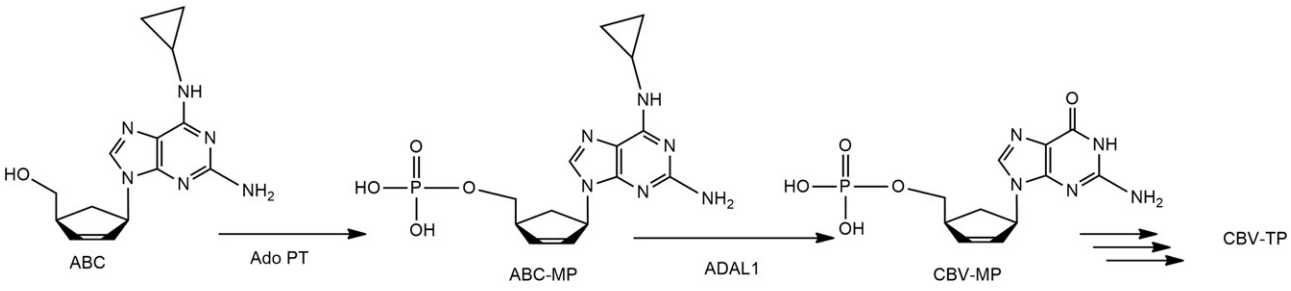
In *de novo* synthesis of nucleoside analogues with modified sugar moieties, the biocatalysed step usually involved is the stereoselective resolution of racemic substrates mediated by hydrolases.

Carbocyclic nucleosides are nucleoside analogues in which the furanosic ring has been replaced by a carbocycle. The substitution of the endocyclic oxygen atom by a CH_2 moiety increases not only the chemical stability of the *N*-glycosidic bond, but also makes these derivatives metabolically resistant to the action of many enzymes, such as NPs. The methods for the synthesis of these compounds involve principally the construction of the cycle through a cycloaddition of several heterodienophiles, to produce bicycle derivatives that are kinetically resolved by enzymatic catalysis (Boutureira et al., 2013). One of the most popular cycloadducts for synthesising carbocyclic nucleosides is 2-azabicyclo[2.2.1]hept-5-en-3-one (γ-lactam, ABH, or Vince lactam, Scheme 5a), which is efficiently synthesised from an aza-Diels–Alder [4 + 2] cycloaddition reaction between cyclopentadiene (**1**) and tosyl cyanide or chlorosulphonyl isocyanate, followed by hydrolysis. ABH has been used for the synthesis of carbocyclic nucleosides for more than 30 years, since Robert Vince recognised the potential of this intermediate to synthesise carbocyclic nucleosides with the required *cis* orientation of both hydroxymethyl and nucleobase groups. A recent review collected the different synthesis and numerous applications of ABH (Singh and Vince, 2012). Enzymatic resolution of ABH has been extensively studied, and there are several enzymes that provide excellent enantioselectivity and facilitate the preparation of enantiopure carbocyclic nucleosides. γ-Lactamase (EC 3.5.2.-) is an amidase, which can be used for the resolution of racemic γ-lactam. *Sulfolobus solfataricus* γ-lactamase showed absolute enantioselectivity towards (+)-γ-lactam. The enzyme was cloned, expressed in *E. coli* and after purification, it was immobilised on microcrystalline cellulose matrix (Avicel) retaining most of its activity (Wang et al., 2014). Zhu et al (2014) reported for the first time a single strain containing two opposite chirally selective γ-lactamases. Both (+) and (−)-γ-lactamase activities were detected in *Bradyrhizobium japonicum* USDA 6 whole cells, a symbiotic nitrogen-fixing bacterium. After expression in *E. coli*, the recombinant enzymes could be easily applied in the preparation of optically pure (+)-ABH or (−)-ABH.

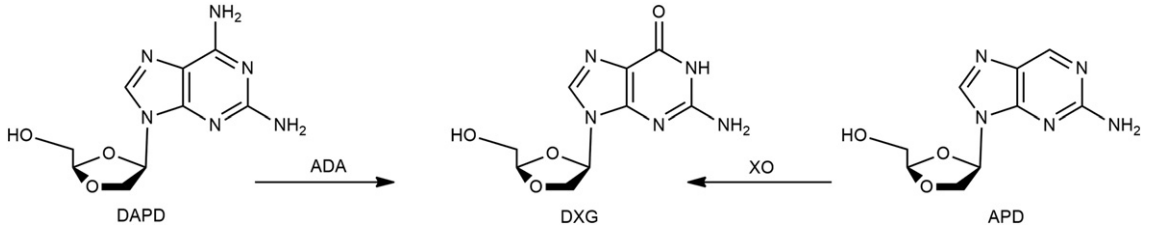
The well-known use of (−)γ-lactam is in the synthesis of Abacavir (ABC, Scheme 5). ABC is a carbocyclic guanosine analogue with inhibitory activity of HIV-RT, acting as a prodrug with improved toxicity profile and higher oral bioavailability. ABC is first phosphorylated by adenosine phosphotransferase (AdoPT) into the cell to ABC monophosphate (Scheme 6), which is then converted to Carbovir monophosphate (CBV-MP), by the enzyme adenosine deaminase-like protein isoform 1 (ADAL1), recently identified to play a physiological role in the salvage routes of 6-substituted purine- or 2-aminopurine nucleoside monophosphate (Murakami et al., 2011). Finally, CBV-MP is anabolised to CBV-TP, a potent inhibitor of HIV-RT that acts both as a competitive inhibitor of dGTP for DNA incorporation and as a DNA chain terminator (Faletto et al., 1997; Stankova et al., 2012).

Boyle et al. (2012) reported the synthesis of ABC from the β-lactame (**2**) obtained by [2 + 2] cycloaddition of chlorosulphonyl isocyanate with cyclopentadiene (Scheme 5b) and subsequent Lipolase (a soluble preparation of *Thermonyces lanuginosus* lipase, produced by submerged fermentation of a genetically modified *Aspergillus oryzae* microorganism) catalysed kinetic resolution. Tardibono et al. (2011) developed a new synthetic route from an acylnitroso-derived hetero Diels–Alder cycloadduct (**3**), which after reduction conducted to the synthesis of racemic aminocyclopentenol (**4**). Kinetic enzymatic resolution of this compound was achieved with commercially available immobilised lipase B from *Candida antarctica* (CALB), previous to the base coupling (Scheme 5c). Using this strategy, compounds having different 4′-carbon side chains analogues of CBV and ABC were prepared and tested for antiviral activity.

(−)-β-D-2,6-Diaminopurine dioxolane (Amdoxovir, DAPD, Scheme 7), a more water soluble prodrug of (−)-β-D-dioxolane guanine (DXG), exhibits good anti HIV-1 activity in humans. These compounds, which are reverse transcriptase inhibitors, belong to a family of nucleoside analogues that possesses a dioxolane ring instead of a classical carbohydrate moiety (Chen et al., 1996). DAPD is susceptible to deamination by ADA, yielding DXG. A related compound, (−)-β-D-2-aminopurine dioxolane (APD), more water soluble than DAPD and with the potential to be metabolised *in vivo* to DXG mediated by



Scheme 6. Abacavir (ABC) *in vivo* transformation to Carbovir triphosphate (CBV-TP). AdoPT: adenosine phosphotransferase. ADAL1: adenosine deaminase like isoform 1.



Scheme 7. Dioxolane guanine (DXG) prodrugs and their metabolic conversion in the active drug. DAPD: Amdoxovir, APD: 2-aminopurine dioxolane, ACPD: 2-amino-6-chloropurine dioxolane, ADA: adenosine deaminase, XO: xanthine oxidase.

xanthine oxidase (XO, EC 1.17.3.2), also exhibits excellent anti-HIV activity (Manouilov et al., 1997).

An efficient and scalable synthesis of the dioxolane nucleoside analogues was achieved using enzymatic resolution (Sznaidman et al, 2005). Starting from commercially available 2,2-dimethoxy- or 2,2-diethoxyethanol (Scheme 8), the synthesis of racemic, ester protected, dioxolane lactone (**1**) was performed in two steps. This compound was subjected to pancreatic porcine lipase (PPL) resolution, obtaining the desired (R)-lactone (**2**) in 98% *ee* (Scheme 8). After its chemical reduction and glycosylation, followed by a selective enzyme-catalysed hydrolysis using CALB, the desired β-anomer was obtained (Zhou et al., 2012). From 2-amino-6-chloropurine dioxolane (ACPD, **3**, X = Cl, Scheme 8) DAPD and APD were prepared by amination or reduction, respectively.

In view of the success of Abacavir, many 6-modifications of Amdoxovir were explored. These new potential prodrugs were achieved from the key intermediate ACPD by treatment with the corresponding amine in methanol (Narayanasamy et al., 2007). All the synthesised prodrugs were more soluble than DXG or DAPD, and the anti-HIV activities were higher.

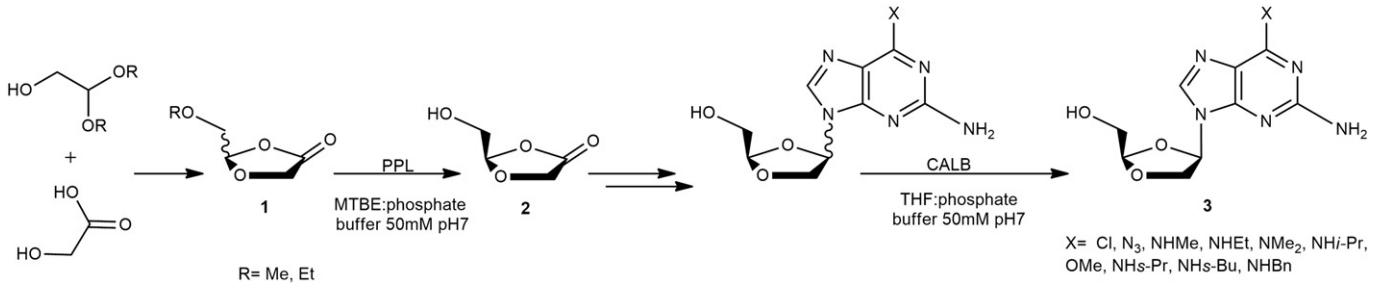
2.2. As base prodrugs

In some cases, nucleosides and nucleoside derivatives are used as nucleobase prodrugs (Zawilska et al., 2013). 6-Methylpurine (MeP) and 2-fluoroadenine (F-Ade) are two potent cytotoxic purine analogues. To exert their activity, the corresponding nucleosides must be phosphorylated by adenine phosphoribosyl transferase (APRT) from the extracellular environment and finally converted to their respective triphosphates by cellular mono- and diphosphate kinases. Incorporation of the corresponding ribonucleoside triphosphate into RNA produces interference in RNA processing, whilst the corresponding 2′-deoxynucleoside monophosphates irreversibly formed inhibit thymidylate synthase and interferes with DNA synthesis, causing inhibition of cell proliferation and cell death (Parker et al., 2011). Since APRT is an enzyme involved in cell purine nucleotide salvage pathway, tumour selective delivery prodrugs were developed.

Human PNP (hPNP) and *E. coli* PNP (ePNP) exhibit different substrate specificities. Whilst hPNP is specific for 6-oxopurines and their nucleosides, ePNP accepts 6-oxo- and 6-aminopurines and their nucleosides, as well as other 6-substituted purine nucleoside analogues (Lewkowicz and Iribarren, 2006). Taking this into account, three nontoxic prodrugs, 6-methylpurine 2′-deoxyriboside (6MePdR), 2-fluoro-2′-deoxyadenosine (F-dAdo) and F-araAMP, were applied to a gene-directed enzyme-prodrug therapy (GDEPT) strategy (Tai et al., 2010). Using the anti-tumour therapy system ePNP/6MePdR, firstly reported by Sorscher et al. (1994), the administered prodrug is converted to a cytotoxic drug only at tumour sites, where ePNP is either expressed or targeted by fusion to a tumour specific antibody or antibody fragment. Similarly, Parker et al. (2003) employed ePNP/F-dAdo and F-araAMP systems. Although 6MePdR and F-dAdo are similar as substrates for ePNP, improved antitumour activity was observed with F-dAdo, since F-Ade is a better substrate of APRT. Additionally, both 6MeP and F-Ade readily diffuse across cell membranes and kill neighbouring tumour cells that do not express ePNP. As alternative, the antitumour effect of mutants hPNP (Afshar et al., 2009) and *Salmonella typhimurium* PNP (Chen et al., 2013) combined with 6MePdR was tested.

6MePdR, F-dAdo and F-araA (Fludarabine) were enzymatically synthesised by nucleoside phosphorylases from different microbial sources. Liang et al. (2010) cloned and overexpressed *E. coli* BL21 TP and PNP in *E. coli* DH5α and the recombinant strain was used to produce 6MePdR, using thymidine as 2-deoxyribose donor, reaching 95% yield in one hour. The production of arabinonucleosides from Ara-U and the corresponding bases by microbial transglycosylation reaction was reported by Nóbile et al. (2012). *C. koseri* (CECT 856) was selected to carry out the biotransformation and immobilised by entrapment techniques. The agarose-immobilised whole cells could be used up to 68 times and no significant loss of activity was detected after 9 months. Employing this methodology, F-araA was obtained in 58% yield after 14 h.

A similar approach to the one above described is the use of *E. coli* cytosine deaminase (eCD, EC 3.5.4.1) as a cancer gene therapy strategy (Lehouritis et al., 2013). CD is found in many bacteria and fungi but not in mammalian cells. It can not only catalyse the deamination of cytosine to uracil but also convert the antifungal agent 5-fluorocytosine (5-FC) to the antitumour drug 5-fluorouracil (5-FU). This compound is one of the approved antineoplastic drugs for the treatment of solid tumours, such as breast, colorectal, and gastric cancers. Similar to 6MeP and F-Ade, when 5-FU is transformed to 5-fluorouridine triphosphate or to 5-fluoro-2-deoxyuridine-5-monophosphate, both RNA and protein syntheses are inhibited (Pałasz and Cież, in press). Because of its



Scheme 8. Chemoenzymatic synthesis of dioxolane nucleosides. PPL: porcine pancreatic lipase. CALB: *Candida antarctica* lipase B. DAPD: (−)-β-D-2,6-diaminopurine dioxolane.

short plasma half-life and poor tumour selectivity, several oral 5-FU prodrugs, such as Tegafur (1-(2-tetrahydrofuryl)-5-fluorouracil), Carmofur (1-hexylcarbamoyl-5-fluorouracil), Doxifluridine (5′-deoxy-5-fluorouridine, DFUR) and Capecitabine (5′-deoxy-5-fluorocytidine carbamate), were developed to overcome these problems (Jhansi Rani et al., 2012; Mazzaferro et al., 2013). DFUR, which was previously developed for the treatment of breast, colorectal and other cancers, shows a tumour-selective activity as a result of its selective conversion to 5-FU by human TP, an enzyme with higher activity in tumour than in normal tissues (Shimma et al., 2000). In a first chemo-enzymatic approach to prepare DFUR (Scheme 9), Bavaro et al. (2009) employed 2′,3′,5′-tri-*O*-acetyluridine (**1**, Scheme 9) as starting material and two biocatalysed steps were involved. In the initial one, the substrate was regioselectively hydrolysed by *Pseudomonas fluorescens* lipase and after four chemical steps, 5′-deoxyuridine (**2**) was obtained. Further *one-enzyme*, one-pot transglycosylation catalysed by immobilised UP from *Bacillus subtilis*, afforded DFUR in about 40% conversion. Alternatively, TP from *E. coli*, immobilised on PEI (polyethylenimine) activated Sepabeads® and stabilised with 20% oxidised dextran, was used in view of preparative applications (Serra et al., 2013).

3. Nucleoside lipophilic prodrugs

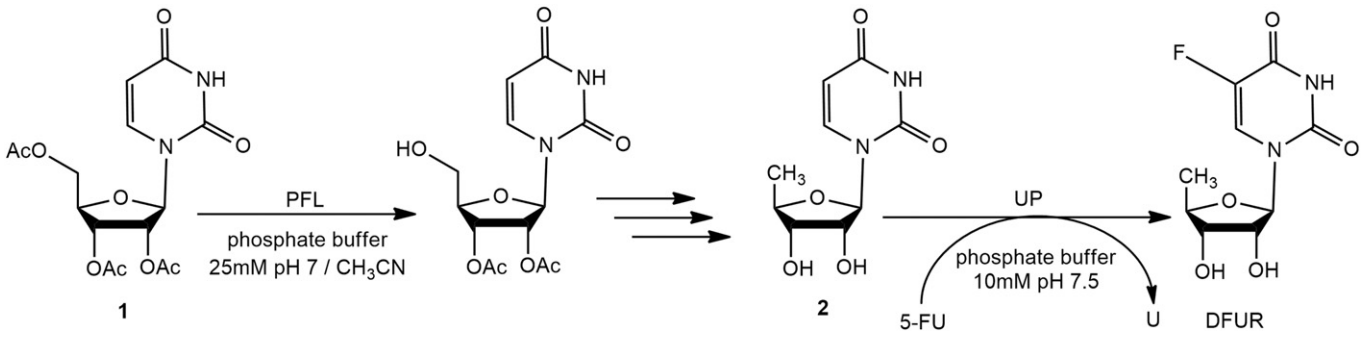
The potential of hydrolytic enzymes in the field of nucleosides has been previously reviewed by Ferrero and Gotor (2000a,b) and by Zong and coworkers (Li et al, 2010a). In particular, lipases (EC 3.1.1.3) are powerful tools for the preparation of lipophilic nucleosides derivatives, due to the ability of these enzymes to catalyse highly regioselective acylations. Other useful applications of hydrolases in the field of nucleosides, such as separation of β-D/L-nucleosides mixtures (Martínez Montero et al., 2010) and selective hydroxyl or amino group protection with synthetic purposes (Palacio et al., 2013) are out of the scope of the present review and consequently, they will not be covered.

Ara-C (**1**, Scheme 10) is a potent agent employed in the treatment of acute myelogenous leukaemia and non-Hodgkin's lymphoma. However, it is deaminated by plasma deaminases and its high hydrophilicity renders it ineffective in solid tumours. These stability and bioavailability limitations may be circumvented through prodrugs based on regioselective Ara-C acylation, such as 5′-*O*-elaidyl-Ara-C and 5′-*O*-L-valyl-Ara-C (Chhikara and Parang, 2010), whose chemical synthesis faces the troublesome discrimination of similar reactive amino and hydroxyl groups.

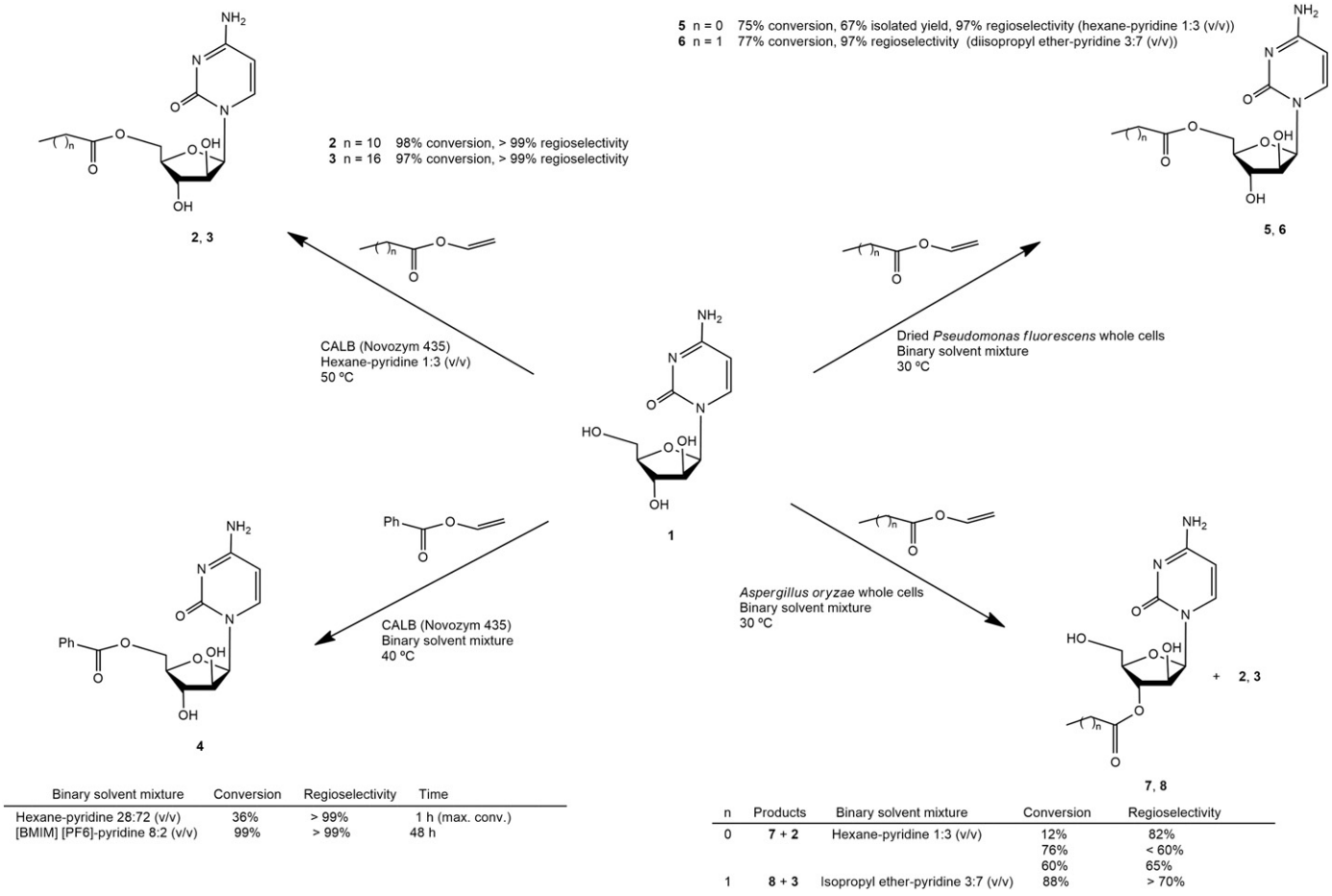
Continuing with their previous work on Ara-C enzymatic acylation, Zong, Zhao and coworkers obtained Ara-C derivatives bearing fatty acyl moieties at 5′ (**2** and **3**, Scheme 10; Li et al., 2010b,c). In both works a screening of different commercial hydrolases was carried out, exhibiting CALB the best results in terms of conversion and regioselectivity (>99% of 5′-regioisomer). To assay the effect of the reaction medium on the biotransformation, a set of binary solvents of different polarity was tested and diisopropyl ether (IPE)-pyridine 1:3 (v/v) and hexane-pyridine 1:3 (v/v) allowed the highest conversion and regioselectivity. Since the latter mixture gave also the highest initial reaction rate, it was chosen for further experiments to adjust experimental parameters such as molar excess of acylating agent (vinyl laurate or vinyl stearate, Scheme 10), temperature and initial water content (a_w). This conducted to the obtention of 5′-*O*-lauroyl- (**2**) and 5′-*O*-stearoyl-Ara-C (**3**) at very high substrate conversions (98% and 97%, respectively). The same biocatalyst was employed to obtain 5′-*O*-benzoyl-Ara-C (**4**; Li et al., 2010d) by applying a similar methodology; in this case, a binary reaction medium contaning a ionic liquid (1-butyl-3-methylimidazolium hexafluorophosphate, [BMIM] [PF_6]) led to a much higher substrate conversion than hexane-pyridine 1:3, but at a much lower rate (Scheme 10).

In the search of a less expensive biocatalyst for regioselective acylation, Zhao, Yu and coworkers (Li et al., 2012a,b) employed lyophilized *P. fluorescens* whole-cells (other names of this microorganism: *Pseudomonas cepacia*; *Burkholderia cepacia*) as a source of hydrolytic enzymes. The authors studied the effect of culture conditions (carbon and nitrogen sources and their concentration, surfactant, culture time) on both the growth and the catalytic behaviour of the bacterial cells. The whole-cell biocatalyst prepared under the optimal culture conditions gave 5′-*O*-acetyl- (**5**) and 5′-*O*-propanoyl-Ara-C (**6**) at good substrate conversions and with high regioselectivities (Scheme 10); however, higher conversions, yields and reaction rates had been previously reached by this research group using CALB (Li et al., 2006a,b). In contrast to the regiopreference for nucleosides 3′-hydroxyl exhibited by extracellular lipases from *Pseudomonas* sp. (Ferrero and Gotor, 2000a; Li et al, 2010a), the whole-cell biocatalyst showed regioselectivity towards 5′-hydroxyl. This fact was explained by the authors considering that cell-bound- and intracellular lipases may exhibit a regiopreference different to that of the extracellular lipase. Interestingly, IPE-pyridine 3:7 (v/v) was chosen as the reaction medium to obtain 5′-*O*-propanoyl-Ara-C (**6**) using *P. fluorescens* whole cells because the hexane-pyridine 1:3 system gave lower conversions, unlike CALB-catalysed propanoylation, in which both mentioned solvent mixtures afforded similar conversions. The authors also tested the stability of the whole cells in IPE-pyridine 3:7 and found that the biocatalyst retained around 30% of its initial activity after four batches (12 h each batch; Li et al., 2012b).

When the source of whole cells was the filamentous fungus *A. oryzae* (Li et al., 2012c; Yang et al., 2014), 3′-*O*-acylated Ara-C derivatives were the main products (**7** and **8**, Scheme 10). Supply of Spans (sorbitan fatty acid ester surfactants) to the cell culture as inductors of lipase production conducted to biotransformations with higher regioselectivity than by using Tweens (polysorbate surfactants), but the opposite effect was observed in relation to substrate conversion (Scheme 10, Li et al., 2012c). These results indicate that more than one type of micellium-bound hydrolase may be produced by the microorganism and the use of Spans may favour the production of a lipase with higher regiopreference towards 3′-hydroxyl group. In the synthesis of the propanoylated Ara-C derivative **8** (Yang et al., 2014), a screening of several experimental variables (reaction medium, excess of vinyl propanoate, temperature, shaking speed) was carried out to find the best performance of the *A. oryzae* whole cell biocatalyst (88% Ara-C conversion, >70% regioselectivity, Scheme 10);



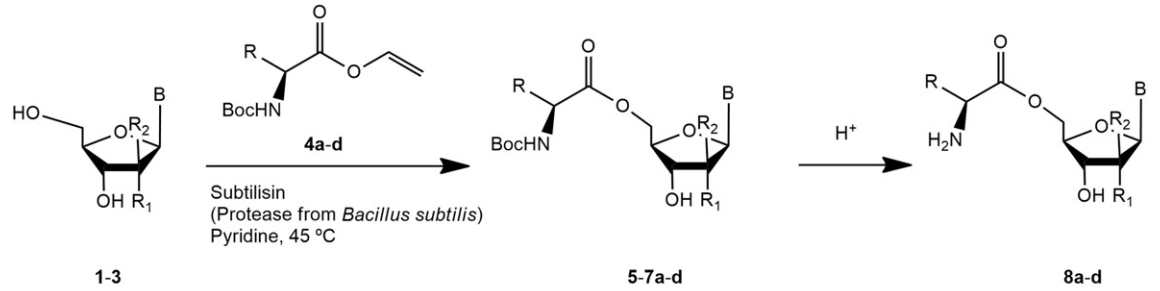
Scheme 9. Chemoenzymatic synthesis of Doxifluridine. PFL: *Pseudomonas fluorescens* lipase. UP: uridine phosphorylase from *Bacillus subtilis*.



Binary solvent mixture	Conversion	Regioselectivity	Time
Hexane-pyridine 28:72 (v/v)	36%	> 99%	1 h (max. conv.)
[BMIM] [PF6]-pyridine 8:2 (v/v)	99%	> 99%	48 h

n	Products	Binary solvent mixture	Conversion	Regioselectivity
0	**7** + **2**	Hexane-pyridine 1:3 (v/v)	12%	82%
			76%	< 60%
			60%	65%
1	**8** + **3**	Isopropyl ether-pyridine 3:7 (v/v)	88%	> 70%

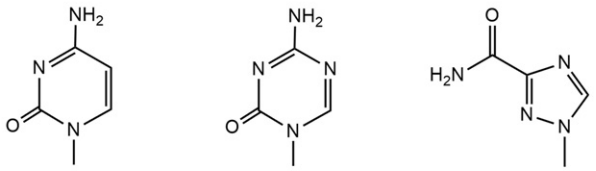
Scheme 10. Biocatalytic preparation of Cytarabine lipophilic derivatives using isolated enzymes and whole cells.



1,**5** R_1 = OH, R_2 = H, B = C
2,**6**,**8** R_1 = H, R_2 = OH, B = 5-AzC
3,**7** R_1 = H, R_2 = OH, B = Riv

a R = Ph
b R = Me
c R = iPr
d R = iBu

5a-**d** 74-84% isolated yield
6a 83% conversion, 76% isolated yield
6b-**d** 53-71% isolated yield
7a-**d** 79-82% conversion



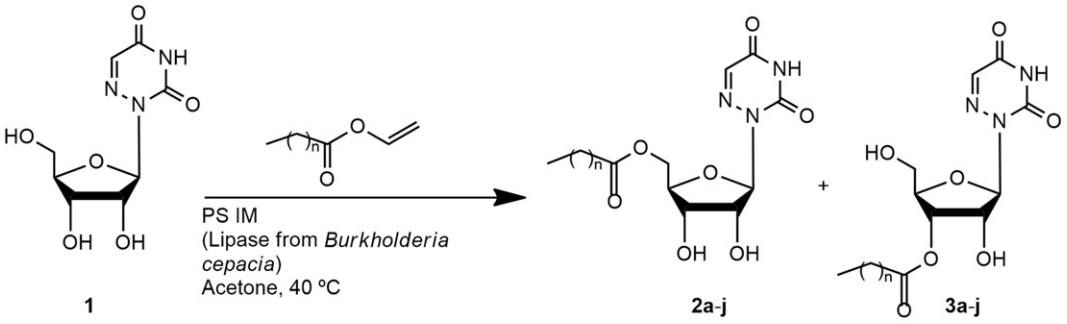
Scheme 11. Protease-catalysed conjugation of bioactive nucleosides with α-aminoacids.

this involved the use of a very high molar vinyl propionate/Ara-C ratio (90).

Conjugation of bioactive nucleosides with aminoacids can provide derivatives with higher bioavailability, as it has been reported for Ara-C and other nucleosides, such as Gemcitabine, Levovirin and Valopicitabine (Chhikara and Parang, 2010; Li et al., 2008; Mackman and Cihlar, 2004). Wu, Lin and coworkers (Xu et al., 2014) have studied a chemoenzymatic approach to obtain L-aminoacid ester prodrugs of 5-Azacytidine (**2**, Scheme 11). A set of commercial hydrolases was screened in pyridine at 50 °C employing vinyl *N*-Boc-L-phenylalanyl as the acyl donor and the best result was attained with subtilisin, the alkaline protease of *B. subtilis*, which catalysed 5′-*O*-aminoacylation with high regioselectivity and substrate conversion (83%). The solvent affected the conversion and regioselectivity of the reaction and it was observed a loss of regioselectivity in DMSO and DMF. Boc group of 5-Azacytidine derivatives **6a–d** was chemically deprotected to afford the corresponding 5′-aminoacylated prodrugs **8a–d**. Subtilisin catalysis was also applied to obtain 5-*O*-(*N*-Boc-L-aminoacyl) esters from Ara-C (**5a–d**) and from biological active Ribavirin (**7a–d**).

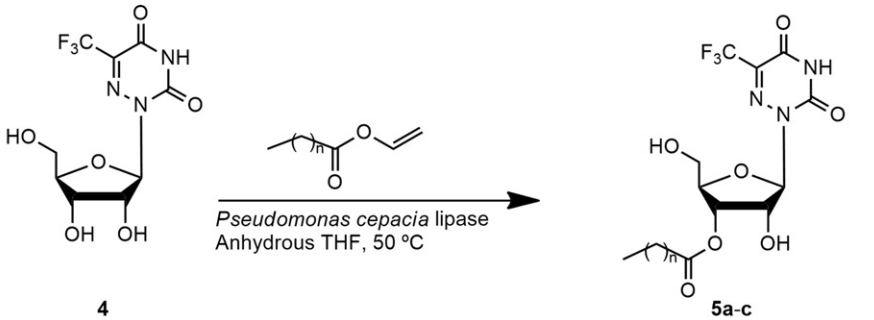
6-Azauridine (6-AU, **1**, Scheme 12) possesses antiviral activity against a wide range of viruses and it is also employed as antineoplastic drug (Crance et al., 2003). Searching for 6-AU potential prodrugs, and complementing their previous works on 5-*O*-acyl-6-AU derivatives obtained through CALB- (Wang and Zong, 2009b) and *Thermomyces lanoginosus* lipase (TLL) catalysis (Wang et al., 2009a), these researchers assayed *B. cepacia* lipase (PS IM Amano, immobilised) (Wang et al., 2012a,b) and studied a set of aliphatic vinyl esters (C2–C18) as acyl donors. A reversal of the previously commented PSL regiopreference for 3′-hydroxyl group was observed when short chain acylating agents (C2–C4) were employed, whilst longer acyl chains (C6–C18) gave predominantly the corresponding 3′-*O*-acyl derivative; the best regioselectivity was found with the stearoyl (C18) moiety (Scheme 12). Employing medium- and long chain acylating agents (C6–C14), *P. cepacia* lipase also catalysed highly regioselective 3′-*O*-acylation of Trifluridine (**4**) (Wang et al., 2011).

A set of biological active purine nucleosides (**1a–d**, Scheme 13a), such as antitumoural 2-chloro- (**1b**) and 8-chloroadenosine (**1d**), has been acylated by means of highly regioselective CALB catalysis, affording the corresponding 5′-*O*-undecylenyl (10-undecenoyl) derivatives **2a–d** (Gao et al., 2011). Owing to the presence of the double bond in the acyl moiety, nucleosides esters **2a–d** are potential monomers to obtain polymeric prodrugs allowing controlled release (Li et al., 2010b). Expanding the repertoire of biocatalysts for nucleoside regioselective acylation, the lipase from *Penicillum expansum* (PEL) was assayed for the undecylenylation of a set of biological active purine ribo- and arabinonucleosides (Scheme 13b, Gao et al., 2012). Commercial crude

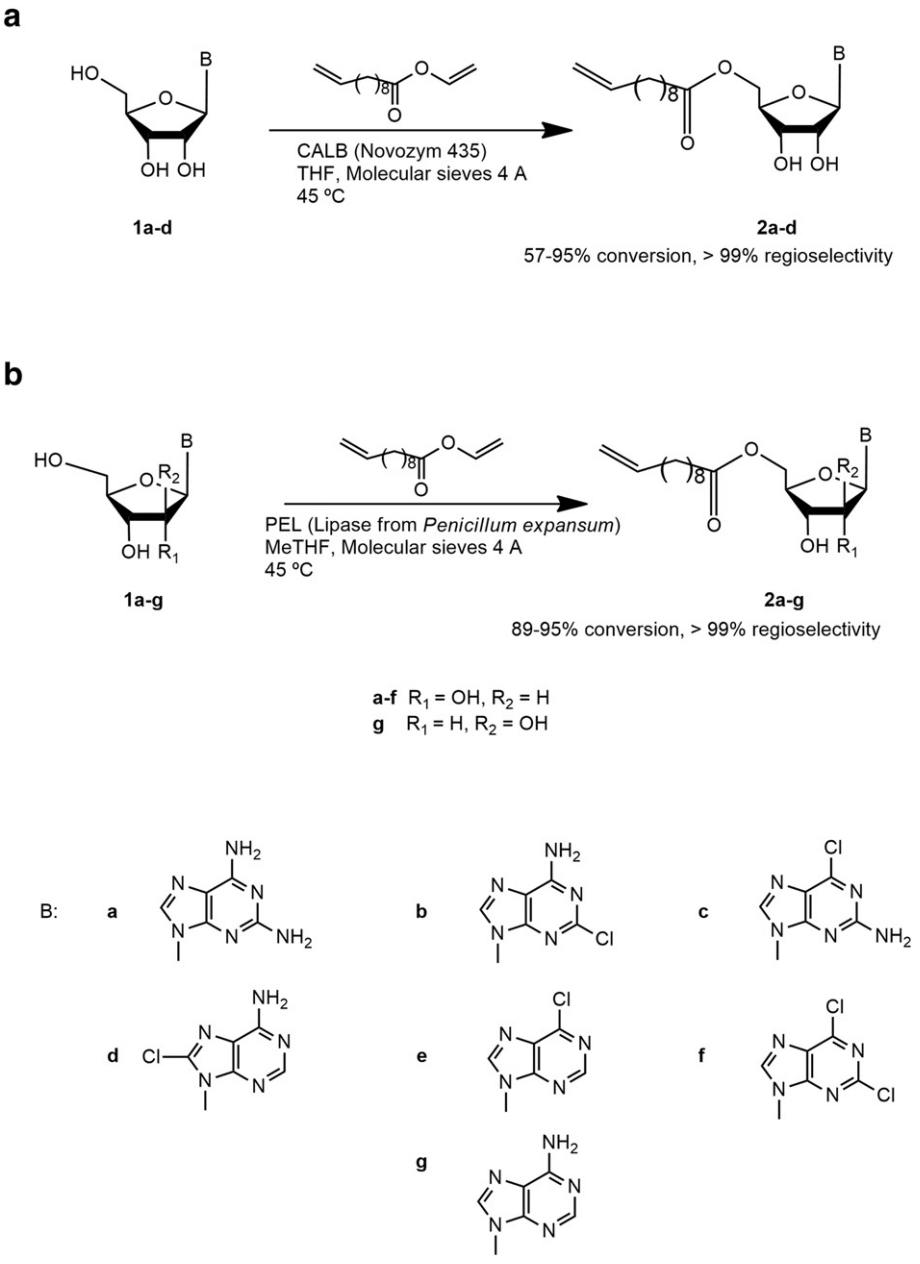


	n	5′-ester (**2**) (%)	3′-ester (**3**) (%)
a	0	70	19
b	1	76	15
c	2	64	19
d	4	38	43
e	6	13	63
f	8	13	66
g	10	11	74
h	12	10	75
i	14	7	77
j	16	4	80

98- >99% **1** conversion



Scheme 12. Preparation of 6-azauridine and trifluridine lipophilic derivatives catalysed by *Pseudomonas cepacia* lipase.



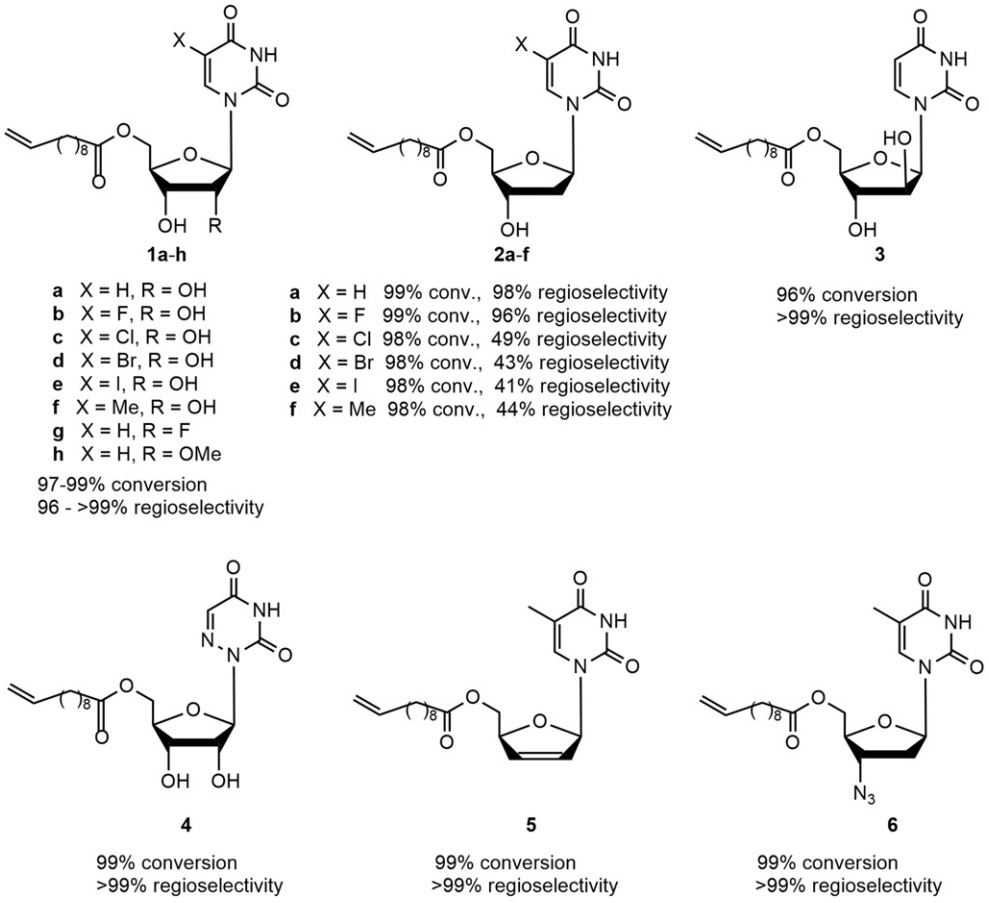
Scheme 13. Undecenylation of bioactive purine nucleosides catalysed by: **a**—*Candida antarctica* lipase B; **b**—*Penicillum expansum* lipase.

PEL was immobilised on a macroporous adsorbent acrylic resin and the resulting biocatalyst screened for the undecylenylation of 8-chloroadenosine (**1d**) in several organic solvents. Best substrate conversion, reaction rate and thermal stability of the biocatalyst were reached in biomass-derived 2-methyltetrahydrofuran, thus, this solvent was employed as the reaction medium to optimize experimental parameters of the biotransformation (enzyme amount, molar excess of acylating agent, nucleoside amount, temperature) and to extend the undecylenylation to other purine nucleosides (**1e**–**g**). In all examples depicted in Scheme 13b, PEL exhibited high regioselectivity towards 5′-hydroxyl group.

PEL-catalysed undecylenylation in 2-methyltetrahydrofuran was also applied to biological active pyrimidine nucleosides, conducting to products **1**–**6** (Scheme 14, Gao et al., 2013); for some 2′-deoxynucleosides (**2c**–**f**), it was observed that substitution at 5 in the base affected the regioselectivity of the enzyme. A qualitatively similar effect of the substituents on the regioselectivity was also found in CALB-catalysed acylation of a set of pyrimidine 2′-deoxynucleosides (Scheme 15, Wang et al., 2013), and higher regioselectivities towards 5′-acylation were attained both in the hexanoylation and myristoylation than in the decanoylation. In the case of 2′-*O*-methyluridine (**1**, Scheme 15), the corresponding esters **2a**–**c** were formed with exclusive regioselectivity at 5′ regardless of the acylating agent chain length (Wang et al., 2013). 2′-Halo substituted pyrimidine nucleosides, such as **3**, were almost quantitatively acylated with very high regioselectivity. Again, results of similar profile were found by assaying TLL (Bi et al., 2014), although with this biocatalyst the highest 5′-regioselectivity was reached in the myristoylation (Scheme 15). By the other hand, whilst CALB afforded the highest regioselectivity in acetone, TLL gave the highest in THF.

Since predictability is rather limited, results previously presented in this section illustrate the need of screening experimental parameters in order to get the most satisfactory results.

In addition to acylations involved in the previous examples, lipases also catalyse deacylations, providing a regioselectivity complementary to that achieved by direct acylation of nucleosides. Deacylation can be



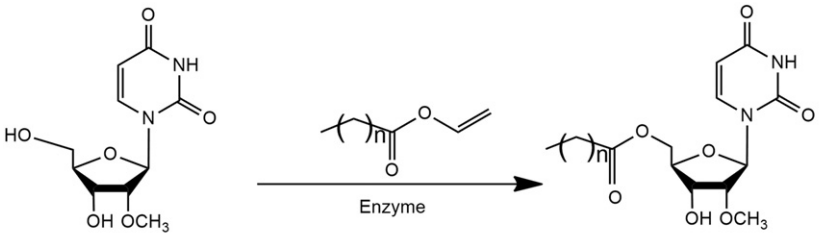
Scheme 14. *Penicillum expansum* lipase-catalysed undecylenylated of pyrimidine nucleosides.

carried out by hydrolysis or alcoholysis and in our laboratory we have studied CALB-catalysed deacylation of triacetylated arabinonucleosides **1** and **2** (Scheme 16). It was found that the reaction medium modulates the regioselectivity of deacetylation: whilst hydrolysis in buffer phosphate affords 2′,3′-di-*O*-acetylated products (**3** and **4**), alcoholysis conducts to 2′-mono-*O*-acetylated compounds (**5** and **6**). In particular, **4** has been reported as a useful topical prodrug of Ara-A, suitable for the treatment of herpes virus infections (Sabaini et al., 2010). Interestingly, this effect of the reaction medium on deacylation regioselectivity contrasts with CALB-catalysed deacylation of ribonucleosides (Baldessari and Iglesias, 2012): 2′,3′-di-*O*-acylated ribonucleosides are formed in alcohol, whilst hydrolysis provides full deacylated nucleosides. These results suggest a crucial role of furanose stereochemistry in enzyme substrate recognition.

Continuing with their previous work to gain insight into the molecular basis of CALB regioselectivity in nucleosides acylation (Lavandera et al., 2005), Ferrero, Gotor and coworkers (Martínez Montero et al., 2012) studied the levulinylation of a set of nucleosides catalysed by this enzyme. In order to rationalise the observed 5′-*O*-regioselectivity, these researchers performed a molecular modelling study and a NMR-based conformational analysis of nucleosides, concluding that the base moiety (pyrimidine or purine) does not exert a significant role on the selectivity and the rate of the acylation. Based on the molecular modelling, it was demonstrated that the North (*N*, 2′-*exo*) conformation of nucleosides is probably the key catalytic intermediate responsible for the 5′-*O*-regioselectivity. In relation with CALB behaviour in ribonucleosides alcoholysis, a molecular modelling carried out by us on 2′,3′,5′-tri-*O*-acetyluridine suggests that the high regioselectivity towards 5-*O*-deacetylation without further deacetylation could be explained on the basis of an inverse orientation of the substrate in CALB active site (Gudiño et al., 2012).

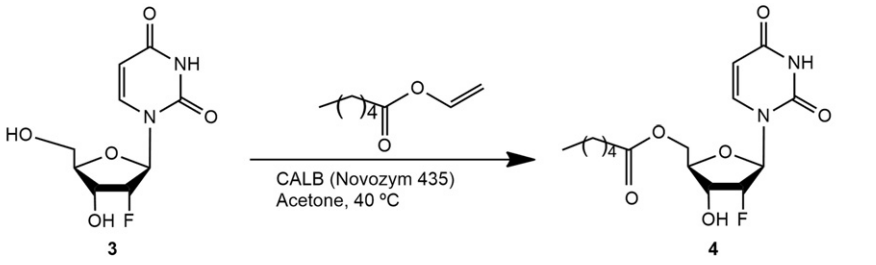
In regard to acyclonucleosides, Acyclovir (ACV), owing to its limited bioavailability (20%) shows moderate antiviral efficacy after oral administration. Hence, it is necessary and feasible to design a prodrug for improving oral absorption (Zhang et al., 2014). To carry out the biocatalysed synthesis of Valacyclovir, McClean et al. (2011) screened 62 immobilised and free commercial enzyme preparations and 19 of them were able to catalyse the hydrolysis of Valacyclovir to Acyclovir. Among them, subtilisin A (subtilisin protease from *Bacillus licheniformis*, also known as subtilisin Carlsberg) and ChiroCLEC™BL (a cross-linked form of the same enzyme), displayed the highest activity. However, all attempts to esterify L-valine with Acyclovir failed probably because of the poor solubility of L-valine and Acyclovir in both organic and aqueous solvents. Testing L-valine methylester as substrate, enantiopure Valacyclovir was finally produced in the presence of ChiroCLEC™BL in neat methyl L-valinate or with tert-butanol as cosolvent (Scheme 17).

Based on the model of Valacyclovir, Valganciclovir as well as other nucleoside analogue prodrugs have been designed. Lobucavir is a cyclobutyl guanine nucleoside analogue developed by Bristol Myers Squibb some 20 years ago for the treatment of herpes virus and hepatitis B (Ireland et al., 1997). A prodrug of Lobucavir, in which one of the two hydroxyls is coupled to valine, was reported by Patel (2008). The aminoacylation of 3′-hydroxyl group (Scheme 18) could be achieved by enzymatic transesterification of Lobucavir using ChiroCLEC™BL and ZL-valine p-nitrophenyl ester as acyl donor. The rate and yield of the coupling reaction were very dependent on the choice of solvent. The best yield (61%) was obtained using acetone-DMF 70:30 as solvent. Alternatively, high yield (84%) and selectivity were observed when

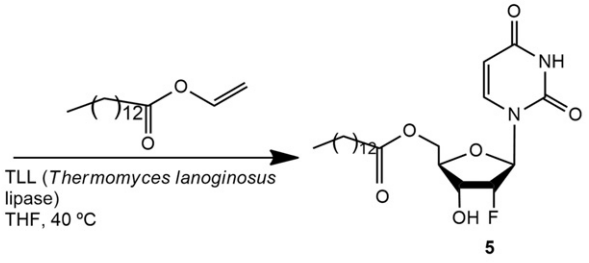


Enzyme and reaction conditions

CALB (Novozym 435), anhydrous acetone, 40 ºC	> 99% conversion, > 99% regioselectivity
TLL (*Thermomyces lanoginosus* lipase), anhydrous THF, 40 ºC	> 99% conversion, > 99% regioselectivity

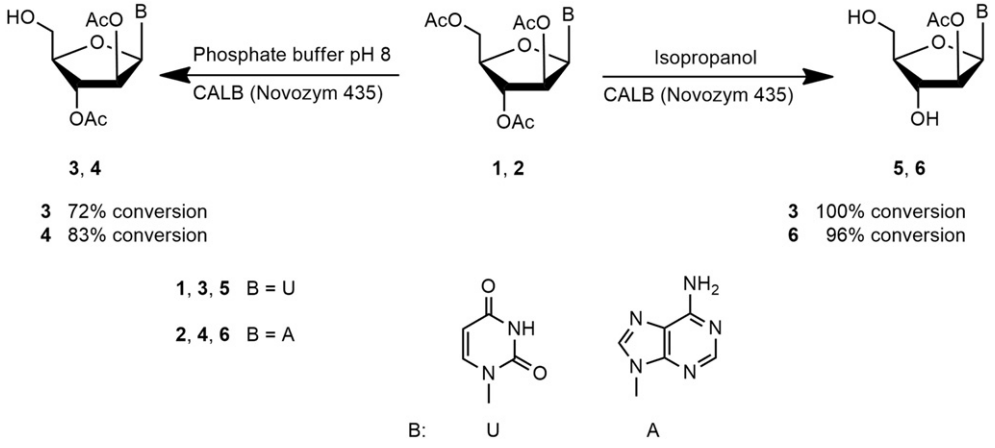


99% conversion, 99% regioselectivity

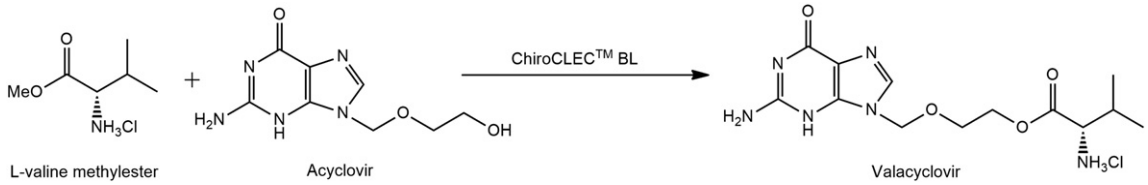


99% conversion, 99% regioselectivity

Scheme 15. Lipase-catalysed acylation of pyrimidine 2′-deoxynucleosides.



Scheme 16. *Candida antarctica* lipase B-catalysed deacetylation of arabinonucleosides.



Scheme 17. Valacyclovir enzymatic synthesis from Acyclovir. ChiroCLEC™BL: cross-linked form of *Bacillus licheniformis*.

ChiroCLEC™PC (cross-linked crystals of *P. cepacia* lipase) or Amano lipase PS30 (also *P. cepacia* lipase) immobilised on Accurel polypropylene and ZL-valine trifluoroethyl ester were used.

4. Nucleoside hydrophilic prodrugs

4.1. Monophosphates

Phosphate prodrugs provide diverse advantages in the formulation and development of poorly water-soluble compounds. Phosphate group is chemically stable, its synthesis has been broadly reported, and its presence increases the solubility of the parent drug in several orders of magnitude (Heimbach et al., 2007). Furthermore, phosphate ester prodrugs are readily cleaved by endogenous phosphatases releasing rapidly the pharmacologically active component.

The most emblematic example of nucleoside phosphate prodrug is F-araAMP (Scheme 1), that is a fluorinated nucleoside 5′-monophosphate analogue of the antiviral agent Ara-A. The presence of 2-fluorine in the purine moiety and a phosphate group in the arabinose moiety results in an increased aqueous solubility and enhanced resistance to adenosine deaminases compared to Ara-A.

As previously mentioned, F-araAMP is indicated for the treatment of B-cell chronic lymphocytic leukaemia, in particular of patients who have not responded to or whose disease has progressed during treatment with at least one standard alkylating agent containing regimen (Ricci et al., 2009) and in non-Hodgkin's lymphoma (Anderson and Perry, 2007). Compared to F-araA, its phosphate prodrug is markedly more soluble in water and in aqueous buffers (Heimbach et al., 2007). After administration, F-araAMP is rapidly dephosphorylated by serum phosphatases and converted into the parent drug, which is then transported into cells by nucleoside transport systems. Intracellularly, it is rephosphorylated by deoxycytidine kinase to produce F-araAMP and later on accumulated as its biologically active 5′-triphosphate derivative (Ricci et al., 2009).

Several biocatalytic strategies were followed in the synthesis of unnatural nucleotides employing enzymes with phosphorylating activity such as kinases, phosphotransferases, phosphoribosyltransferases and acid phosphatases.

5′-Monophosphorylation catalysed by cellular viral kinases is the rate-limiting step in the pharmacological activation of anticancer and antiviral drugs. Given their key function, kinases have been subject to many functional and structural studies in the last years, and the information collected represents a powerful toolbox for the development of enzymatic processes for the preparation of non-natural nucleosides 5′-monophosphates.

Humans as other mammals have four different deoxyribonucleoside kinases (dNKs). There are two cytosolic dNKs, thymidine kinase 1 (TK1, EC 2.7.1.21) and deoxycytidine kinase (dCK, EC 2.7.1.74), and two mitochondrial dNKs, deoxyguanosine kinase (dGK, EC 2.7.1.113) and thymidine kinase 2 (TK2, EC 2.7.1.21) with distinct but overlapping substrate specificities (Table 1A).

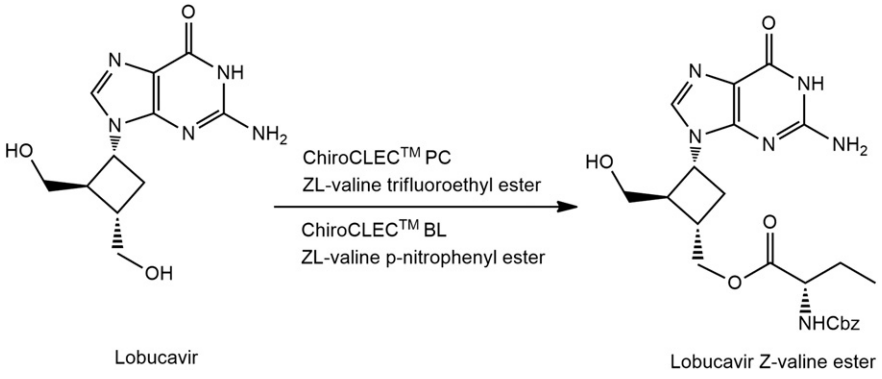
TK1 has a restricted substrate preference phosphorylating only thymidine and deoxyuridine. 5-Halogenated deoxyuridines as 5-fluoro, 5-chloro, 5-bromo and 5-iodo are as good substrates for TK1 as thymidine (Prichard et al., 2007) and also AZT is efficiently phosphorylated (Sharif et al., 2012).

TK2 has the same substrate preference as TK1 but is also active on dC. Singularly, TK2 phosphorylates Brivudine, dC and L-enantiomer of deoxynucleosides such as L-thymidine and L-dC, with comparable specificity constants (Deville-Bonne et al., 2010).

Mitochondrial dGK is only active on purine deoxyribonucleosides: dG and dI, and less efficiently on dA and dC. Anticancer purine analogues such as Cladribine (2-chloro-2′-deoxyadenosine), Ara-G and Clofarabine (2-chloro-2′-fluoro-2′-deoxyadenosine) are phosphorylated as efficiently as dG (Sjöberg et al., 1998). dGK exhibits a relaxed enantioselectivity, accepting LdG, retaining the 20% of the activity, compared to dG. Remarkably, LdA is better substrate than dA with efficiency higher than that of dG (Gaubert et al, 1999).

Cytosolic dCK has broad substrate specificity and phosphorylates deoxynucleosides (dA, dG and dC) and cytidine, using both ATP and UTP as phosphate donors, being dC the preferred substrate. Cytidine analogues such as Ara-C, Gemcitabine, Zalcitabine (2′,3′-dideoxycytidine, ddC) and unnatural L-nucleosides (*e.g.*, β-L-2′,3′-dideoxy-3′-thiacytidine, Lamividune) as well as diverse purine analogues, like Cladribine, F-araA and Clofarabine are also activated by dCK.

There are three ribonucleoside kinases (rNKs) in human cells: cytosolic adenosine kinase and two uridine-cytidine kinases (nuclear



Scheme 18. Lobucavir Z-valine ester prodrug. ChiroCLEC™PC: cross-linked crystals of *Pseudomonas cepacia* lipase. ChiroCLEC™BL cross-linked crystals of subtilisin Carlsberg.

Table 1
A) Natural substrates of the human deoxyribonucleoside kinases (dNKs) and ribonucleoside kinases (rNKs). B) Summary of nucleoside analogues used in antiviral and anticancer therapies and enzymes carrying out their phosphorylation.

A

Enzyme	Natural substrates
dNK	
TK1	Thymidine, deoxyuridine
TK2	Thymidine, deoxyuridine, deoxycytidine
dCK	Deoxycytidine, deoxyadenosine, deoxyguanosine
dGK	Deoxyguanosine, deoxyadenosine, deoxyinosine
rNK	
ADK	Adenosine, deoxyadenosine
UCK1	Uridine, cytidine
UCK2	Uridine, cytidine

B

Drug	Enzyme
Anticancer	
Cladribine	dCK, dGK, Dm-dNK
Clofarabine	dCK, dGK
Cytarabine	dCK, Dm-dNK
Ara-G	dGK
Fludarabine	dCK
Gemcitabine	dCK, Dm-dNK
Clofarabine	dCK, dGK
Antiviral	
Didanosine	cN-II
Zalcitabine	dCK
Lamivudine	dCK
AZT	TK1
Brivudine	TK2, Dm-dNK
Stavudine	TK1
Acyclovir	Viral TK, 5′-NT
Gancyclovir	Viral kinases
Ribavirin	AK, 5′-NT

UCK1 and cytosolic UCK2). Adenosine kinase (ADK, EC 2.7.1.20) is a widely distributed enzyme that catalyses the phosphorylation of adenosine and in low extent, dA, using ATP as phosphate donor. This enzyme is responsible for the phosphorylation of Ribavirin (Wu et al., 2005), the immunosuppressive drug Mizoribine (Yokota, 2002) and the anticancer *C*-nucleoside, Tiazofurin (Fridland et al., 1986). ADK is also active on 6-modified purine ribosides, such as F-Ado, 8-azaadenosine, 6-methylpurine riboside and Aristeromycin (Long et al., 2008).

In addition to their natural substrates, UCK1 and UCK2 kinases have been shown to phosphorylate a range of uridine and cytidine analogues but do not phosphorylate deoxyribonucleosides and purine ribonucleosides (Suzuki et al., 2004). These kinases are most likely to contribute to the initial phosphorylation of 2′-methylcytidine nucleosides together with dCK (Golitsina et al., 2010).

Cytosolic 5′-nucleotidase II is a mammalian nucleotidase that specifically catalyses the dephosphorylation of 6-hydroxypurine nucleoside 5′-monophosphates (IMP, dIMP, GMP, dGMP), regulating the cellular pool of IMP and GMP. This enzyme exhibits also phosphotransferase activity, phosphorylating antiviral and antitumour nucleoside analogues such as Didanosine (2′,3′-dideoxyinosine), Carbovir (Johnson and Fridland, 1989), Acyclovir (Keller et al., 1985) and Ribavirin (Wu et al., 2005).

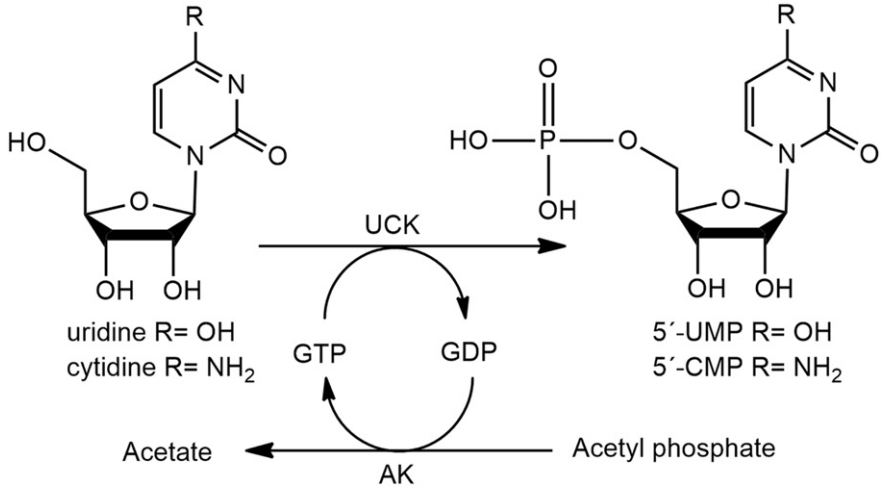
A summary of the activity of deoxy- and ribonucleoside kinases on pharmacologically active nucleosides is shown in Table 1B.

On the other hand, *Drosophila melanogaster* has only one kinase (Dm-dNK, EC: 2.7.1.145) that phosphorylates all 2′-deoxynucleosides with exceptionally high turnover rates, displaying higher affinity for pyrimidine nucleosides than for purine ones. In addition, Dm-dNK is active on non-natural nucleosides, confirming its potential in the biocatalysed synthesis of modified nucleotides, as well as suicide gene in gene therapy (Ma et al., 2011). Deoxypyrimidine nucleoside analogues such as Ara-C, Ara-T, Brivudin and 5-fluoro-2′-deoxyuridine are phosphorylated by Dm-dNK with 58, 53, 54 and 92% relative activities compared to thymidine, respectively (Munch-Petersen et al., 2000). Albeit converted less efficiently, Gemcitabine is phosphorylated with a turnover number of 6.50 s^{-1}, being its efficiency about 4-fold higher than dCK (Knecht et al., 2009). Deoxyadenosine and Cladribine are also good substrates of the enzyme whilst its activity on dG drops (Munch-Petersen et al., 2000).

Dm-dNK was recently applied as biocatalyst to the preparative synthesis of dA-, Ara-A- and F-araAMP. Different enzyme preparations (free, adsorbed on epoxy Sepabeads-PEI, and Sepabeads-PEI cross-linked with aldehyde dextran) were evaluated. After optimization, the cross-linked Dm-dNK preparation proved to be the best biocatalyst affording 98% yield of Ara-AMP in 12 h and 91% of F-araAMP in 4 h (Serra et al., 2014).

Recently, Qian et al. (2014) reported a new method for the production of 5′-UMP and 5′-CMP from the corresponding nucleosides employing uridine/cytidine kinase from *E. coli* K-12 and acetate kinase (AK) from *Lactobacillus bulgaris* ATCC 11842 to regenerate GTP (Scheme 19). Employing recombinantly expressed and isolated enzymes, 97% conversion was achieved in 3 h for 5′-UMP and 13 h in case of 5′-CMP.

A similar one-pot reaction strategy to prepare 2′-deoxynucleoside-5′-monophosphates was followed by Zou et al. (2013). In this study, *Lactobacillus delbrueckii* NDT-II, *B. subtilis* dCK and *E. coli* K-12 AK were

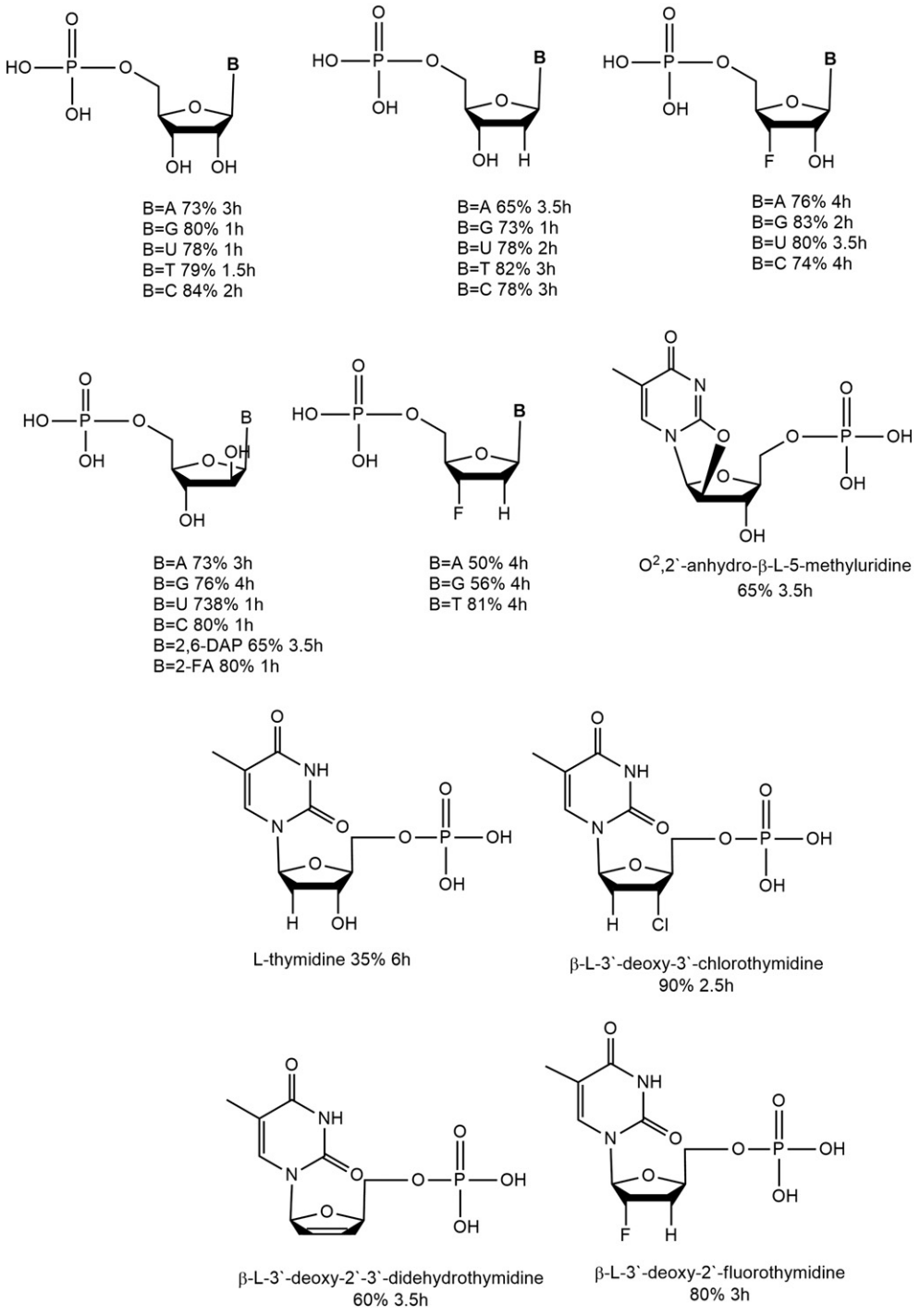


Scheme 19. Enzymatic cascade applied to the biosynthesis of 5′-UMP and 5′-CMP. UCK: uridine kinase; AK: acetate kinase.

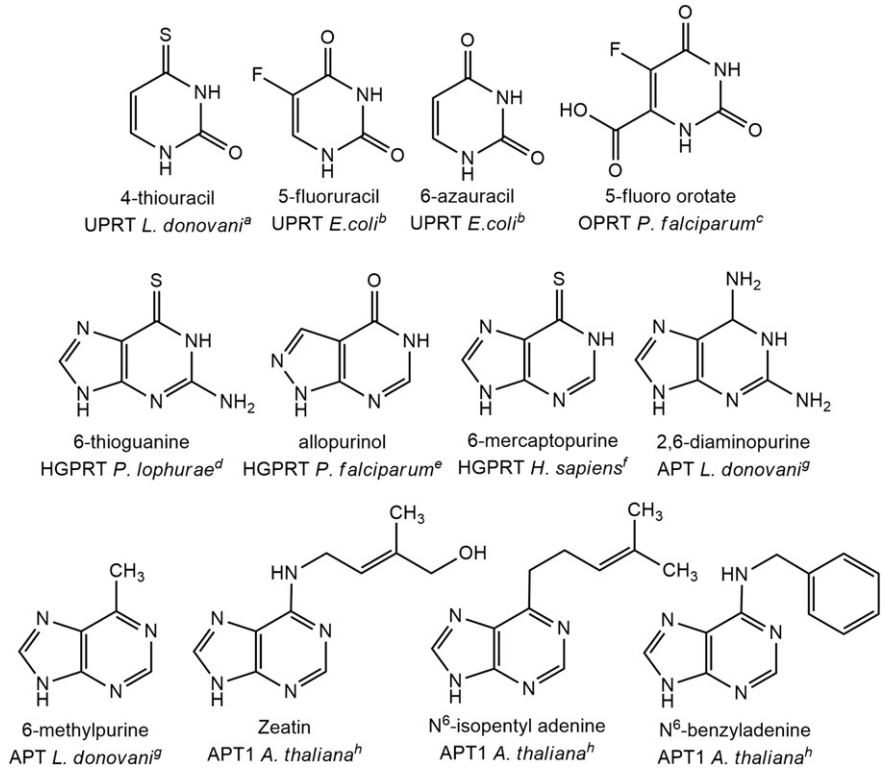
individually expressed in *E. coli* BL21, and the three cell-free extracts were combined to synthesise 5′-dAMP, 2,6-diaminopurine-2′-deoxynucleoside-5′-monophosphate (5′-dDAMP) and 5′-dCMP, from thymidine as deoxyribose donor, GTP as phosphate donor, acetyl phosphate to regenerate GTP and the corresponding nucleobase. Under optimal conditions 9.01 mM of 5′-dAMP in 7 h, 8.68 mM 5′-dDAMP in 10 h and 6.23 mM 5′-dCMP in 2 h were obtained.

Alternatively, the phosphorylation of modified nucleosides was catalysed by nucleoside phosphotransferases (EC 2.7.1.77, NPT) present in crude enzyme preparations from carrot, wheat germ, *Serratia marcescens* and *Pantoea agglomerans* 47/3 (previously *Erwinia herbicola* 47/3). These enzymes hold a wide substrate specificity concerning phosphate acceptors and donors, utilizing diverse phosphate monoesters (NMPs) or p-nitrophenylphosphate (p-NPP).

Several studies dated in the 1970s and 1980s reported the formation of phosphate monoesters of 2′- and 3′-*O*-methylcytidine, 6-methyluridine, Formycine, 2′-deoxy-5-fluorouridine, Acyclovir and 5-aminoimidazolribofuranoside in good yields employing NPT (crude extracts or purified enzyme). However, the formation of 2′-*O*- and 3′-*O*-monophosphates and the presence of contaminating activities (5′-nucleotidases, phosphorylases, deaminases, *etc.*) in the crude extracts undermined current use of these enzymes in synthetic applications.



Scheme 20. Nucleoside and nucleoside analogues 5′-monophosphate synthesis by *P. agglomerans* 47/3 whole cells. A: adenine, G: guanine, U: uracil, T: thymine, C: cytosine.

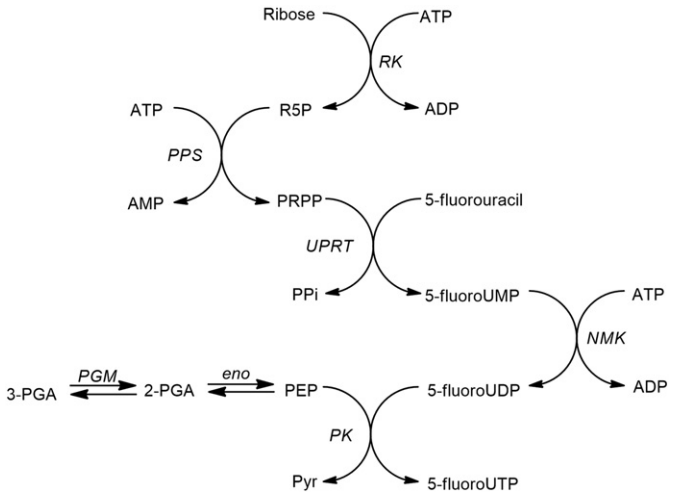


Scheme 21. Substrate acceptance of pyrimidine and purine analogues by phosphoribosyltransferases. References: a: Soysa et al. (2013); b: Rasmussen et al. (1986); c: Krungkrai et al. (2004); d: Schimandle et al. (1987); e: Keough et al. (1999); f: Krenitsky et al. (1969); g: Turttle and Krenitsky (1980); h: Allen et al. (2002).

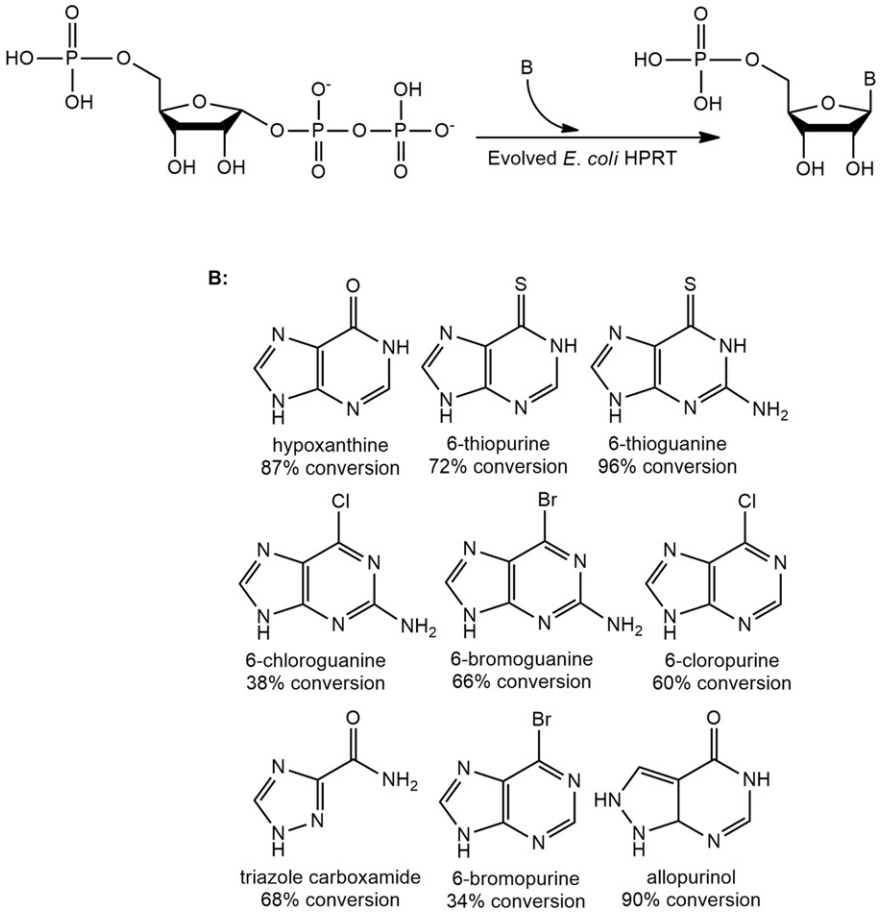
In particular, NPT activity present in *P. agglomerans* 47/3 whole cells or cell extracts were employed in the preparation of 5′-monophosphoesters of 3′-fluoro-3′-deoxyribonucleosides and 3′-fluoro-2′,3′-dideoxyribonucleosides (Scheme 20; Zinchenko et al., 1990), arabinonucleosides (Scheme 20; Barai et al., 2004) and β-L-nucleosides with p-PNP as phosphate donor (Scheme 20; Birischevskaya et al., 2007) in good yields and mild conditions. 2′,3′-Dideoxyuridine and ddC were also selectively 5′-monophosphorylated by *P. agglomerans* 47/3 whole cells, although lower yields were achieved (Zhernosek et al., 2006). Unfortunately, the lack of protein sequences in Protein Data Bank makes difficult their further characterization and utilization in a recombinant form. Moreover, their pH range of activity, cellular localization and phosphohydrolase activity, suggest they may have been wrongly categorised, belonging to a broader group of enzymes with phosphatase/phosphotransferase activity such as acid phosphatases (Class B) family.

In nucleoside salvage pathways, nucleoside phosphorylases (NPs) and phosphoribosyltransferases (PRTs) share a common approach catalysing the addition of nucleobases to activated riboses. In particular, PRTs catalyse the condensation of structurally diverse nucleobases to 5′-phosphoribosyl-1-pyrophosphate, producing selectively β-nucleotides and pyrophosphate. For the salvage of purine bases, organisms generally contain one PRT specific for adenine (EC 2.4.2.7) and one or more 6-oxo PRTs responsible for the salvage of hypoxanthine, xanthine and guanine. For pyrimidine bases, two PRTs are found: uracil phosphoribosyltransferase (EC 2.4.2.9) and orotate phosphoribosyltransferase (EC 2.4.10). Substrate scope of 6-oxopurine PRTs varies with the organism studied and is divided according to nucleobase specificity. In mammals, *Leishmania* and other kinetoplastids, a single PRT is present and accepts hypoxanthine and guanine (HGPRT, EC 2.4.2.8). *E. coli* possesses two 6-oxopurine PRTs, one for hypoxanthine (HPRT) and another for guanine and xanthine (GXPRT). Characteristically, protozoa parasites PRTs, such as *Plasmodium falciparum*, *Toxoplasma gondii* and *Tritichomonas foetus* have one single 6-oxopurine PRT that accepts hypoxanthine, guanine and xanthine (HGXPRT) (Ullman et al., 2010). Highly specific xanthine PRTs are found in Gram positive bacteria, like *B. subtilis*, *Leishmania* and other trypanosomatids (XPRT, EC 2.4.2.22) (Arent et al., 2006; Ullman et al., 2010) whilst specific guanine phosphoribosyltransferase (GPRT) is so far only in *Giardia lamblia* (Munagala et al., 2000).

The idea that salvage pathways have a common mechanistic solution for the synthesis of nucleosides 5′-monophosphates has driven the study of PRTs as biocatalysts in the synthesis of modified nucleotides, though not many enzymatic syntheses using these enzymes have been reported lately. Scheme 21 compiles the activity of diverse PRTs on modified nucleobases. PRTs have been employed in *in vitro*

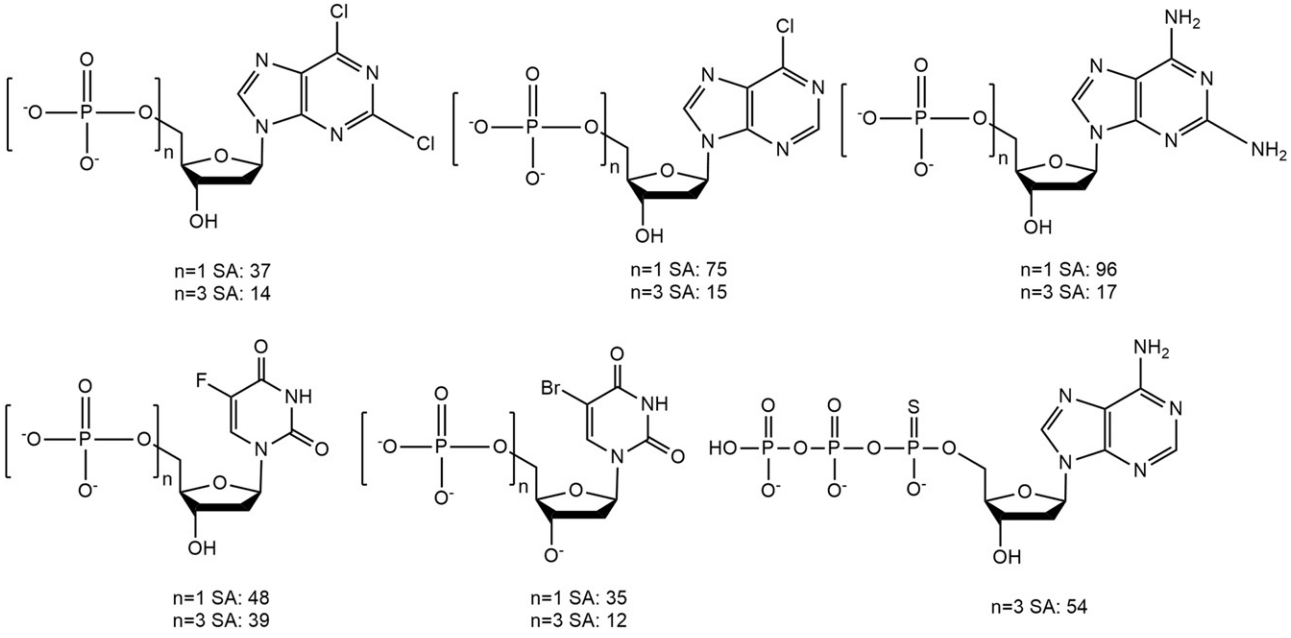


Scheme 22. Enzymatic synthesis of 5-fluorouridine-5′-triphosphate using a mixture of enzymes comprising ribokinase (RK, EC 2.7.1.15), D-ribosyl-α-1-pyrophosphate synthetase (PPS, EC 2.7.6.1), uracil PRT (UPRT, EC 2.4.2.9), nucleoside monophosphate kinase (NMK, EC 2.7.4.4), adenylate kinase (AK, EC 2.7.4.3), pyruvate kinase (PK, EC 2.7.1.40), 3-phosphoglycerate mutase (PGM, EC 5.4.2.1) and enolase (eno, EC 4.2.1.11).



Scheme 23. Synthesis of nucleoside analogues 5′-monophosphates by evolved *E. coli* HPRT (hypoxanthine phosphoribosyl transferase). B: assayed bases.

enzymatic strategies for the preparation of modified nucleotide triphosphates, using enzymes from pentose phosphate and nucleoside salvage pathways, and their incorporation in RNA for spectroscopic (NMR, fluorescence) studies investigated (Alvarado et al., 2014; Zeiner et al., 2008). 5-Fluorouridine 5′-triphosphate was obtained in 88% isolated yield from D-ribose, 5-fluorouracil, ATP and 3-phosphoglycerate on a cascade reaction depicted in Scheme 22. Using a similar approach, 8-azaguanosine 5′-triphosphate (60% isolated yield), 2-fluoroadenosine 5′-triphosphate (90% isolated yield) and deuterated 3′,4′,5′,5′-labelled NTPs (78–90% isolated yield) were prepared (Scott et al., 2004).

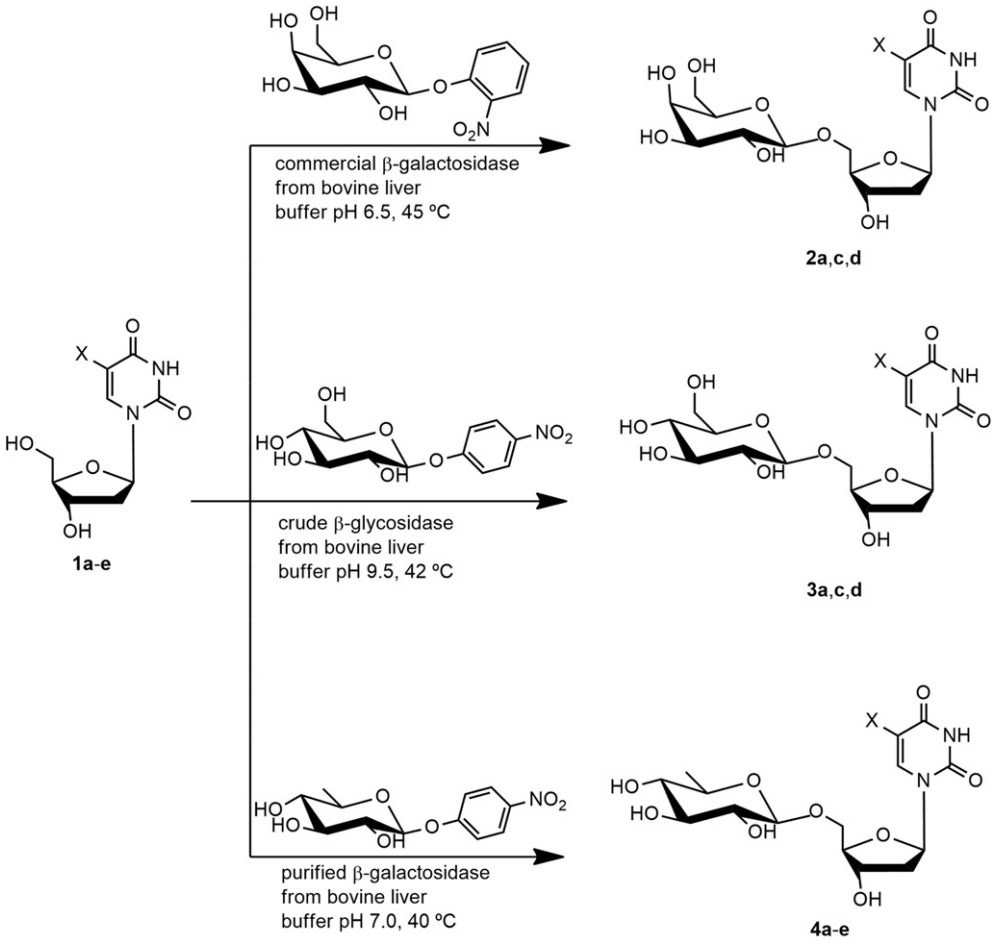


Scheme 24. Specific activities (SA) of new enzyme variants expressed in nmol min^{-1} mg^{-1} of protein.

Protein engineering techniques were applied to alter the substrate specificity of phosphoribosyltranferases. Scism et al. (2007) generated a variant of HPRT from *E. coli* (V157A, Y173H) with improved activity on triazole carboxamide to produce Rivabirin monophosphate. In addition, the variant HPRT showed to accept structurally diverse purine analogues in good conversion (Scheme 23). Using the same evolved *E. coli* HPRT variant, Scism and Bachmann (2010) reported the use of a five enzymes covalently linked enzyme aggregate (CLEA), consisting in a purine nucleotide pathway comprising ribokinase, phosphoribosyl pyrophosphate synthetase and the HPRT variant, adenylate kinase and pyruvate kinase system to regenerate ATP. Allopurinol, 6-thiopurine, 6-chloropurine and purine 5′-monophosphates were obtained in 98, 100, 100 and 48% conversion, in 2 h.

A new chimeric enzyme with phosphodeoxyribosyltransferase activity was built through a structure-based design by Kaminski and Labesse (2013) using two distantly related enzymes: nucleoside deoxyribosyltransferase (EC 2.4.2.6) from *Lactobacilli* and deoxyribonucleoside 5′-nucleotide nucleosidase (EC 3.2.2.-) from rat. The concept was based on several structural similarities shared by both enzymes, such as the adoption of a Rossmann fold and a common catalytic triad. Mechanistically, the hydrolysis of substrates in both enzymes proceeds through the formation of an α-deoxyribose-5-phosphate-enzyme covalent intermediate *via* a conserved glutamate residue. The new variants are able to transfer α-deoxyribose 5-phosphate, α-deoxyribose 5-triphosphate or α-deoxyribose 5-O-(1-thiotriphosphate) between cytosine or adenine, and modified nucleobases like 5-fluorouracil, 5-bromouracil, 2,6-diaminopurine, 6-chloropurine, and 2,6-dichloropurine (Kaminski and Labesse, 2013) with varying degree of efficacy (Scheme 24).

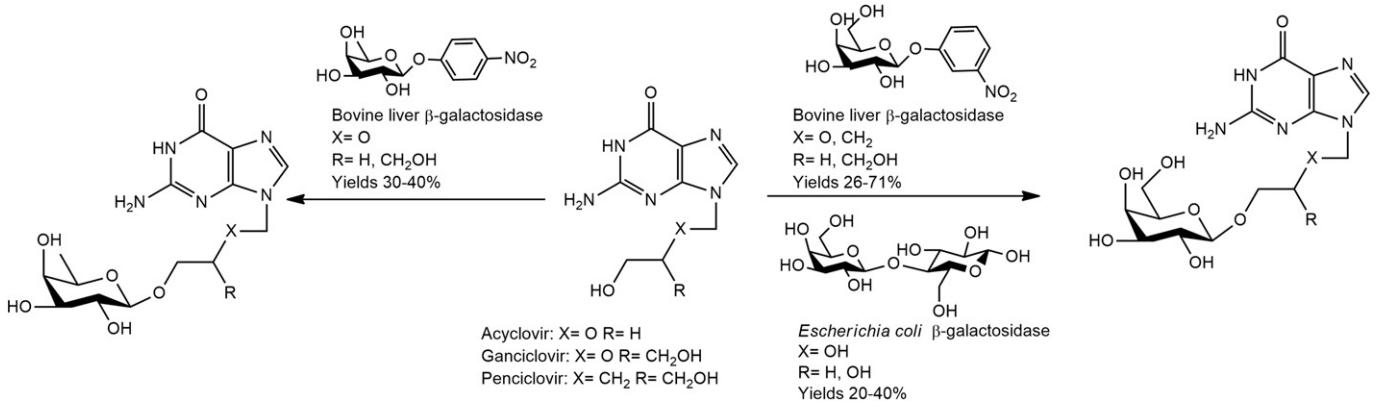
Finally, nonspecific acid phosphatases (NSAPs, E.C. 3.1.3.2) are a group of enzymes that are able to hydrolyse a broad range of organic phosphoesters at acid or neutral pH. These enzymes are widely distributed among enteric bacteria and, along with their inherent activity, some acid phosphatases also exhibit phosphotransferase activity. The reaction proceeds in a reversible manner, whilst extending the reaction times the dephosphorylation reaction occurs.



a X = F
b X = Cl
c X = Br
d X = I
e X = (*E*)-BrCH=CH$_2$

2a	75% conv.,	53% isolated yield,	> 99% regioselectivity
2c	45% conv.,	38% isolated yield,	96% regioselectivity
2d	60% conv.,	46% isolated yield,	92% regioselectivity
3a	72% conv.,	34% isolated yield,	> 99% regioselectivity
3c	49% conv.,	10% isolated yield,	> 99% regioselectivity
3d	22% conv.,	8% isolated yield,	> 99% regioselectivity
4a	51% conv.,		97% regioselectivity
4b	49% conv.,		95% regioselectivity
4c	44% conv.,		93% regioselectivity
4d	60% conv.,		92% regioselectivity
4e	59% conv.,		66% regioselectivity

Scheme 25. Enzymatic glycosidation of pyrimidine nucleosides.



Scheme 26. Enzymatic glycosidation of pyrimidine acyclic nucleosides.

The use of NSAPs in the regioselective synthesis of nucleoside 5′-monophosphate, in particular inosine 5′-monophosphate (5′-IMP), was first reported by Asano et al. (1999) using whole cells of *Morganella morganii* and inorganic pyrophosphate (PPi) as phosphate donor. Similar results were reported by Wever's group using an acid phosphatase from *Shigella flexneri* (PhoN-Sf) (Tanaka et al., 2003; van Herk et al., 2005). In 2012 Liu et al. (2012) investigated the synthesis of 5′-IMP employing a recombinant NSAP from *Escherichia blattae*. Employing the purified enzyme, 5′-IMP was produced in 40 min and 45% yield.

We have recently reported the phosphotransferase activity of NSAPs from wild type and recombinant *Raoultella planticola* and *E. aerogenes* strains (Medici et al., 2014). Natural and modified nucleosides were assessed as substrates using disodium hydrogen pyrophosphate as phosphate donor. Generally, *E. aerogenes* cells were more effective in the phosphorylation of purine nucleosides, whereas *R. planticola* cells showed higher activity on 2′-deoxynucleosides and arabinosides. NSAPs from *E. aerogenes* and *R. planticola* were subsequently heterologously expressed in acid phosphatase deficient *E. coli* BL21 cells and employed as whole cells. Using the recombinant strains, the reaction times were significantly reduced. In some cases, higher yields were also obtained as competitive side reactions present in the cells were circumvented. In particular, 2.1 mM of F-araAMP were obtained in 50 min.

Envisioning larger scale applications and using 5′-IMP as test case, Babich et al. (2012) developed a continuous process employing an immobilised enzyme on a packed-bed reactor. Pho-Sf was covalently immobilised on polymethacrylate porous beads functionalysed with epoxy linkers. On average, operating at a flow rate of 0.02–0.03 ml min^{-1}, 0.5 g of pure 5′-IMP was recovered after 5 days.

4.2. Glycosyl derivatives

Glycosylation of nucleosides may improve their pharmacokinetic properties and reduce their toxicity. Glycosidases (EC 3.2.1) allow a simple synthetic approach to obtain nucleoside glycosylated derivatives, although it presents the drawbacks of competitive glycosyl donor hydrolysis and the need of nucleoside excess, in order to shift equilibrium towards the target derivatives. The use of glycosidases in the field of nucleosides has already been reviewed (Li et al., 2010a) and in the period covered in this section, this strategy has been applied to biological active pyrimidine nucleosides (Scheme 25). In most cases, a screening of experimental parameters (enzyme amount, pH, temperature, nucleoside/glycosyl donor molar ratio) was conducted to improve the yields of the desired products. Most of assays were carried out by using commercial β-galactosidase from bovine liver (Ye et al., 2012; Zeng et al., 2010), which was also purified prior to be assayed (Yan et al., 2012); moreover, crude β-glycosidase from bovine liver prepared in the laboratory was also tested as biocatalyst (Ye et al., 2011). In this way, galactosylated (**2**), glucosylated (**3**) and fucosylated (**4**) pyrimidine nucleosides were obtained. Conversions and yields of glycosylated nucleosides **2**–**4** shown in Scheme 25 were moderate but in most cases very high or excellent regioselectivity could be obtained.

Water-soluble glycosidic prodrugs may also overcome solubility and oral bioavailability problems of acyclic nucleosides. Bovine liver β-galactosidase was also used to prepare galactosylated and fucosylated derivatives of Acyclovir, Ganciclovir and Penciclovir (Scheme 26) using *o*-nitrophenyl-β-galactopyranoside (*o*NPGal) and *p*-nitrophenyl-β-fucopyranoside (*p*NPF) as glycosyl donors (Yan et al., 2012, 2014). The enzyme showed an apparent dependence on the nucleoside structure, being Penciclovir, which contains a methylene group instead of an oxygen atom at 2′, the best accepted substrate, affording 71% yield in 5 h. Even though nitrophenyl glycosides are often used as sugar donors, the utilization of disaccharides is advantageous because of their low costs, good solubility and high stability. Blazek et al. (2012) reported the galactosylation of a set of acyclic nucleosides using β-galactosidase from *E. coli* as biocatalyst and lactose as sugar donor. From the 18 acyclic nucleosides studied, higher yields were achieved when the substrate contained a sterically non-hindered hydroxyl group. Longer aliphatic chains increased the yield of the glycosyl nucleosides and in the case of analogues occurring as diols, only the primary hydroxyl group was involved in the galactosylation.

5. Conclusion

This review testifies current interest in nucleoside prodrugs and efforts to find methodologies improving their preparation. In this sense, the examples covered in this work show how biocatalytic procedures may be applied to obtain a wide variety of nucleoside prodrugs, providing alternative routes to traditional chemistry methodologies to overcome selectivity and stability issues derived from nucleoside chemistry.

Despite the important contributions that biocatalysis has so far done to the field of nucleoside prodrugs, the application of the new advances achieved in biocatalysis, such as protein engineering, methodologies for the search of novel activities and deeper knowledge about different enzyme activities involved in nucleoside metabolism, envisages a growth of biocatalysed syntheses of tailored nucleoside prodrugs.

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