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Review

New developments in nucleoside analogues biosynthesis: A review

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ABSTRACT

The present review deals with current advances in the chemoenzymatic synthesis of biologically important nucleoside analogues (NA), either by the use of microorganisms or enzymes as biocatalysts. The interest in exploiting these biocatalysts is constantly increasing nowadays because of the advantages they have with respect to classic organic chemistry synthesis, such as a fewer number of synthesis steps, an improved chemo-, regio- and stereoselectivity, high catalytic efficiency, and simple subsequent processing. Besides, this technology offers an environmentally friendly alternative in comparison with synthetic chemistry. Thus, the present article gives a brief outline of emerging methodologies for the biosynthesis of NAs commonly used in cancer therapies, such as cladribine, clofarabine, nelarabine, fludarabine, decitabine, cytarabine and floxuridine, and as antivirals: ribavirin, iduviran, vidarabine, acyclovir, lamivudine and emtricitabine from microbial or enzymatic sources, and their potential applications in the biotechnology industry. Also, it points highlights the importance of subsequent modifications of nucleoside analogues by different enzymes used as biocatalysts in order to improve the pharmacological properties of the existing drugs. Moreover, the importance of biocatalyst immobilization for industrial applications is considered.

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1. Introduction

Nucleosides are endogenous compounds that play a key role in different cellular processes such as DNA and RNA synthesis, cell signaling, enzymatic regulation and metabolism. Natural nucleosides are formed by the association of a purine (adenine and guanine) or a pyrimidine (cytosine, thymine and uracil) base with a pentose residue β-D-deoxyribofuranose or β-D-ribofuranose for DNA or RNA, respectively (Scheme 1).

Analogues of these nucleosides are synthetic compounds, chemically modified in some cases, that have been developed to mimic the physiological functions of their natural versions in order to interfere with the cellular metabolism and subsequently be incorporated into the DNA and RNA to inhibit cell division and viral

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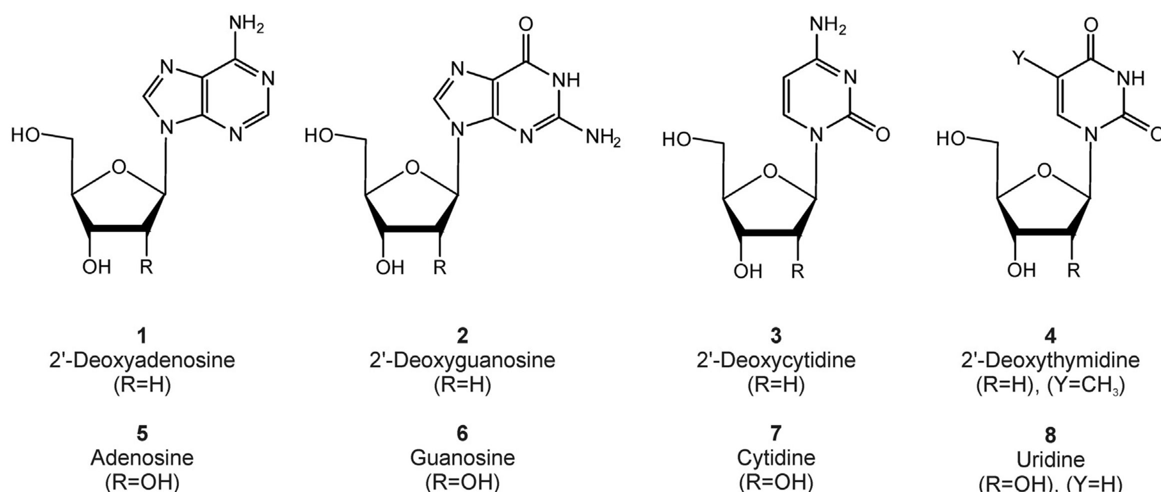
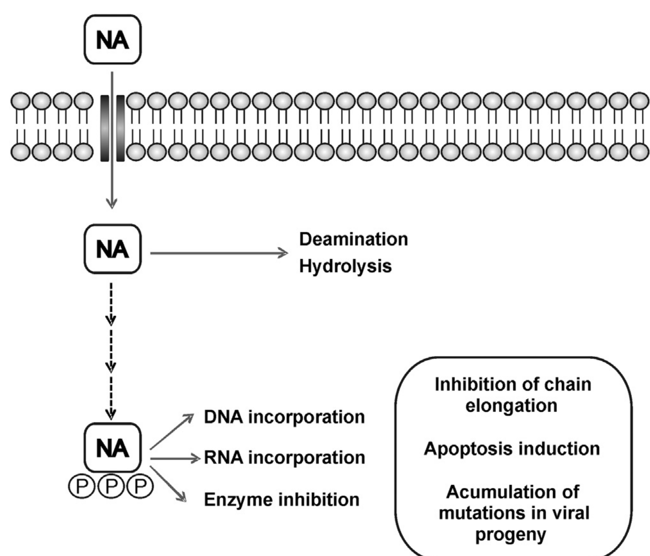


Scheme 1. Natural nucleosides that constitute nucleic acids, R = H: β-D-deoxyribofuranose (DNA); R = OH:β-D-ribofuranose (RNA).



Scheme 2. Mechanism of action of nucleoside analogues. These compounds enter the cell trough a nucleoside transporter mechanism and then they become phosphorylated by nucleoside kinases. The triphosphate metabolite is incorporated in nucleic acids or they inhibit nucleic acid synthesis by inhibiting crucial enzymes. Deaminases and 5′-nucleotidases are catabolic enzymes that may possibly alter the amount of active metabolites.

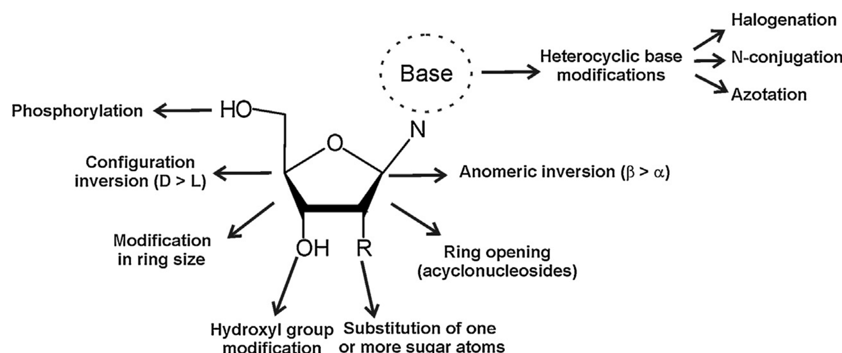
replication [1]. The incorporation of these analogues in DNA can induce suppression of chain elongation, the accumulation of mutations in the viral progeny or induction of apoptosis. This action has potential therapeutic benefits such as inhibition of cancer cell growth and viral replication. In addition to their incorporation into nucleic acids, nucleosides and their analogues can interact and inhibit enzymes such as essential and human viral polymerases, kinases, ribonucleotide reductase, DNA methyltransferases, purine and pyrimidine nucleoside phosphorylases (NPs) and thymidylate synthase (Scheme 2).

Nucleoside analogues (NAs) are mainly synthesized by chemical methods that require the use of organic solvents, multiple reaction steps and the removal of protecting groups, causing unwanted accumulation of racemic mixtures that affect further purification [2]. In this sense, the use of enzymes for the synthesis of these nucleosides has emerged as an alternative due to their high catalytic efficiency, inherent selectivity, and simple subsequent processing. Among the advantages of the use of these biocalatytic or biotechnological methods are very mild reaction conditions, high stereo- and regioselectivity, and the use of an environment-friendly technology [1,3]. The enzymes mostly used for nucleoside synthesis via transglycosylation have been nucleoside phosphorylases (NPs) and *N*-deoxyribosyltransferases (NDTs) obtained either by their isolation or the use whole cell microorganisms in wild type form or genetically engineered to overexpress the enzyme of interest [4–7]. Furthermore, the immobilization of biocatalysts stabilizes, modifies the selectivity and therefore facilitates separation from the reaction medium, which favors the purification of the desired product in a simpler manner [8].

Furthermore, the research and clinical trials required for a new NA to reach its pharmaceutical use involve a very long and costly process. The strategies include the development of lipid-nucleoside conjugates, prodrugs, and liposome preparations [9], being acylation one of the most studied strategies to create biopharmaceuticals with better activity [10–12]. Also, different approaches to create nucleoside derivatives have been studied, such as glycosylation, halogenation and deamination, among others [3]. So, in this review we will discuss the advances in current and alternative methods for the production of nucleoside analogues of clinical interest by biocatalysis.

2. Nucleoside analogues

As aforementioned, these compounds mimic their natural counterparts, which allow them to be incorporated either in DNA or RNA, therefore interfering with cell division or viral replication. Nowadays, NAs represent a major group of antimetabolite cytotoxic drugs in current clinical use [9]. These agents can be used against hepatitis B virus (HBV), hepatitis C virus (HCV), herpes simplex virus (HSV), influenza virus, cytomegalovirus (CMV), and human immunodeficiency virus (HIV), and a broad range of human cancers such as lymphomas, leukemias, pancreatic, bladder, colon, breast and kidney cancer, among others [13,14]. To enter the cell, NAs require specialized nucleoside transporter proteins due to their hydrophilic nature. There is growing evidence that the abundance and tissue distribution of nucleoside transport proteins contributes to cellular specificity and sensitivity to nucleoside analogues [15]. The clinically administered compounds are generally prodrugs that enter the cell and are phosphorylated by deoxynucleoside salvage pathways, and the triphosphate nucleotide is responsible for producing their activity by being incorporated into DNA during replication or repair, acting as chain terminators and stopping viral DNA polymerase (Scheme 2). However, each NA has unique



Scheme 3. Modifications in natural nucleosides structure that make nucleoside analogues.

drug–target interactions that help to explain their differences in activity in various diseases. The most important modifications that can lead from a natural nucleoside to analogues are summarized in Scheme 3.

2.1. Synthesis methods and industrial biotechnology

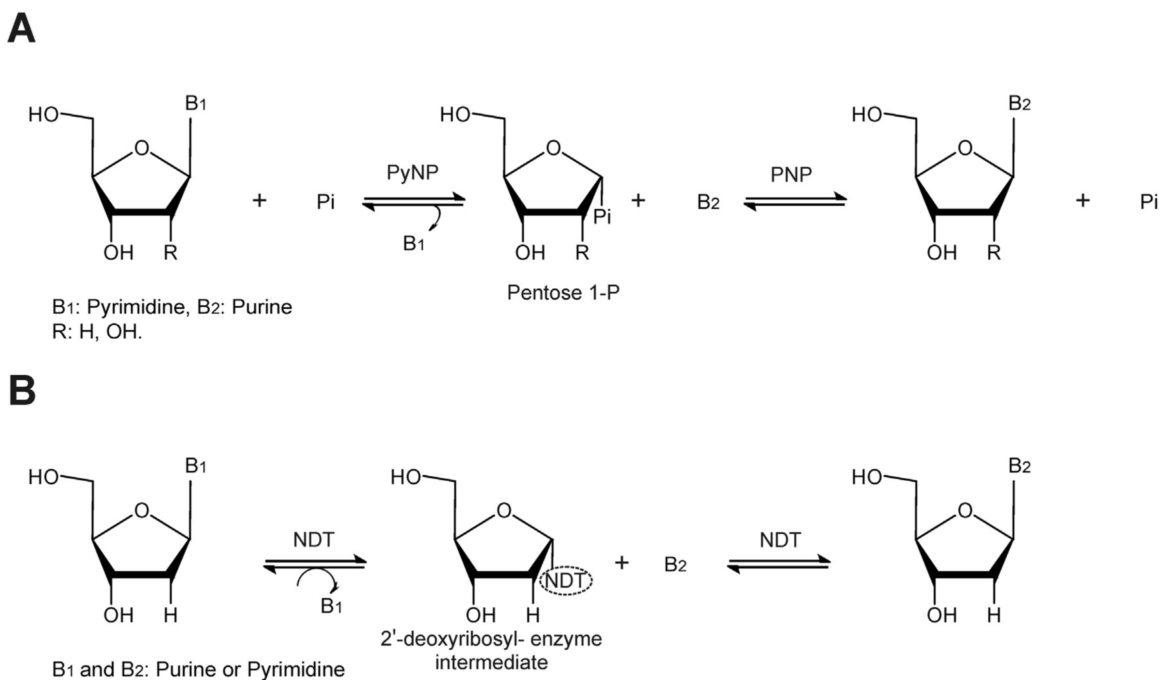
The vast majority of modified nucleosides are synthesized by chemical methods. Despite the progress achieved in the field of synthetic chemistry, due to the complex structure, polyfunctionality and instability of these molecules, a large number of steps are required for their synthesis, involving numerous protection and deprotection stages to control the anomeric configuration [2]. The need for many complex reaction steps for the production of these compounds results in high production costs. Therefore, for nearly four decades new strategies for the synthesis of NA have been sought. These problems can be overcome by successfully using new biocatalytic methodologies [1,3]. The low cost and high productivity of biocatalysis technology allows obtaining modified nucleosides with potential use in cancer and antiviral therapies [1,13,16]. In addition, the potential use of these nucleosides as antibiotics [17–19], radiopharmaceuticals [20–22], or molecular probes [23] has recently been demonstrated. Considering how important nucleoside analogues are in medicine and biotechnology, there is considerable interest in the development of simple and efficient synthesis of these compounds. The use of chemoenzymatic methods undoubtedly improves the price–quality ratio during the production of many medical drugs [24]. Also, the use of enzymes promotes chemo-, regio- and/or stereoselectivity of the reaction raised, promoting the development of the so-called industrial biotechnology to revolutionize drug production systems for pharmaceutical use [25].

However, for the application of these biocatalysts at industrial scale, it is necessary that they can be reused and easily separated from the reaction medium in order to increase process productivity. Previous studies from our group have shown that the use of different biocatalyst immobilization techniques during the bioprocess improves reusability and promotes product recovery [26–28].

Regarding industrial biotechnology, NAs have been synthesized via transglycosylation reactions using two types of enzymes: nucleoside phosphorylases (NPs) and *N*-deoxyribosyltransferases (NDTs), which transfer glycosyl residues from nucleosides to different purine or pyrimidine bases and analogues to acceptor bases. NPs catalyze the reversible phosphorolysis of both ribo- and deoxyribonucleosides by the cleavage of *N*-glycosidic bonds of nucleosides without addition of ATP, to form a free base and its respective activated pentose moiety (pentose-1-P), which is then coupled to the desired modified base either by the same or a different NP to give a NA. They can be classified according to their substrate specificity as purine NPs (PNPs; EC 2.4.2.1) or pyrimidine NPs (PyNPs; EC 2.4.2.2) (Scheme 4A) [8,29]. Alternatively, NDTs (EC 2.4.2.6) catalyze the exchange between the purine or pyrimidine base of 2′-deoxyribonucleosides and free purine or pyrimidine bases. In the reaction, an intermediate of a covalently bound 2-deoxy-α-D-ribofuranosyl moiety is formed, where the glycosidic hydroxyl of 2′-deoxyribose is esterified by a glutamic acid of the active site of NDTs (Scheme 4B) [30]. However, it has been demonstrated that NDTs can accept other related sugar donors as substrates. This property has been exploited to produce nucleoside analogues [31]. These enzymes are also classified into two classes according to their substrate specificity: NDT type I (PDT), specific for purine exchange (Pur ↔ Pur), and NDT type II (NDT), which catalyzes the transfer between purines and/or pyrimidines (Pur ↔ Pur, Pur ↔ Pyr, Pyr ↔ Pyr). Both NPs and NDTs used as biocatalysts come from different sources: *wild type* microorganisms, or using genetic engineering approaches either by the use of recombinant whole cells or recombinant enzymes [32].

Nowadays, the use of multienzymatic systems allows a facile and environmentally friendly technology for the synthesis of medically relevant NAs [33]. Multienzymatic arrangements are composed of either *in vivo* (whole cells) or *in vitro* systems (coupled enzymes). By the combined use of two or more enzymes, much more complex synthetic schemes can be devised. It also allows making irreversible a reversible process, shifting the reaction equilibrium and the partial or total elimination of unwanted products [33–35]. The most common examples of NA synthesis employing multienzymatic approaches have been the use of PNPs in combination with PyNPs or uridine phosphorylases (UPs) or thymidine phosphorylases (TPs). The joint use of both enzymes makes it possible to produce purine nucleosides from pyrimidine ones or vice versa, and represents the most simple multienzymatic process (one-pot, two-step). Besides, another attractive strategy is the use of other types of enzyme in combination with NPs or NDTs such as lipases, deaminases, halogenases, and kinases either as whole cells or enzyme systems [36–39]. More examples have been recently reviewed by Fernandez-Lucas et al., 2015.

Another interesting strategy is to perform enzymatic one-pot multistep reactions, which imitate naturally occurring biosynthetic pathways. In this approach, the biotransformation occurs in one vessel, sequentially or simultaneously, generating less waste formation by avoiding downstream operations [40]. Mikhailopulo et al. obtained NAs from simpler substrates by the concomitant use of several enzymes. The authors described a novel nucleoside synthesis strategy in one-pot transformation of pentoses into nucleosides in the presence of purines or pyrimidines as heterobases. The process is catalyzed by recombinant *E. coli* enzymes: ribokinase (RK) (D-pentose → D-pentose-5-phosphate (D-PF-5P)), phosphopentomutase (PPM) (D-PF-5P → α-D-pentofuranose-1-phosphate (D-PF-1P)), and NPs (D-PF-1P + heterobase → nucleoside) [24]. In another study, the development of an artificial biosynthetic path-



Scheme 4. Enzymatic synthesis of nucleosides and catalytic mechanism of: (A) PNPs and (B) NDTs.

way for the production of 2-deoxyribose-5-phosphate (DR5P) from fructose was achieved by using six recombinant *E. coli* strains expressing thermophilic enzymes [41].

A further strategy for NA production consists in exploiting genetic engineering techniques such as genetic manipulation of gene clusters, pathway engineering and combinatorial biosynthesis, which allows obtaining other compounds of increasing interest such as nucleoside antibiotics [18]. Moreover, the synthesis of the antiretroviral nucleoside analogue didanosine (2′,3′-dideoxyinosine) by bioretrosynthesis, an application of the retrograde evolution hypothesis, for biosynthetic pathway construction with enzymes that undergo structure-guided mutagenesis and directed evolution has been reported [42].

2.2. Nucleoside analogue derivatives

Nucleosides are polyvalent compounds with multiple hydroxyl and amino groups that confer them a highly hydrophilic and polar nature. These features make them suffer from low oral bioavailability. Also, their plasma half-lives are relatively short because of the cleavage of the glycosyl bond by nucleoside phosphorylases or deamination by nucleoside deaminases present in the body. Consequently, large doses and frequent administration are needed to maintain the plasma concentration required for effective treatment. This is why new strategies for NA modification are in continuous search. Chemical modification of parent drugs is one of the most successful strategies to overcome the drawbacks mentioned above. Acylation has proved to be an effective approach, and lipophilic nucleoside prodrugs able to cross the cell membrane and liberate parent agents have been widely investigated [43]. The best known examples are the clinically approved amino acid ester prodrugs of nucleosides valganciclovir, and valacyclovir which have been used as alternatives to the antiviral agents ganciclovir and acyclovir due to their higher therapeutic efficacy [10]. However, modification of only one out of several identical functional groups in a molecule is a difficult task to organic chemists. An example of this problem is the regioselective acylation of polyhydroxyl compounds such as nucleosides [44]. The first reports in this field are from the 1950s and involved nonselective acylations with subsequent tedious separation processes, or time-consuming protection-deprotection steps in selective reactions on ribonucleosides lo and mainly in the 3′-*O*-acylation of deoxyribonucleosides. By employing traditional chemical methods, one-step acylations with high selectivity was only achieved by means of using bulky acylating reagents, which leave secondary hydroxyls free due to steric hindrances [45]. Nowadays, the use of enzymes such as lipases appears as an excellent alternative for the modification of these polyfunctional compounds due to the high regioselectivity, mild reaction conditions, and environmental friendliness of the enzymatic processes [3,46]. Lavandera et al. [47,48] reported that lipase could functionalize its favorable hydroxyl in the acylation of polyhydroxyl compounds, due to the specific structure of its active center. Moreover, solvent engineering strategies were developed to improve the stability and regioselectivity of the enzymes, the use of ionic liquid-containing systems and binary organic solvent mixtures being a successful approach [49–51].

Moreover, glycosylated derivatives of many nucleoside drugs [52,53] were reported to significantly mask the toxicity of the parent drugs and/or improve their pharmacokinetic properties. Besides, the cellular uptake of many glycosylated products can be enhanced due to the active absorption mediated by glucose transport systems [54]. Again, the regioselective glycosylation of nucleosides remains a tedious task for organic chemists, owing to the presence of multiple active hydroxyl and amino groups. Nucleoside *O*-glycosylation represents a typical problem in chemical selectivity, considering that the nucleobase (an undesired site of reaction) is usually more nucleophilic than the hydroxyl (the desired site of reaction). This is why the use of enzymes for the reaction catalysis appears so attractive [55]. Other modifications such as halogenation, deamination and deacylation have been extensively studied in previous works [3,38,39,56,57].

2.3. Immobilization

As a general term, immobilization describes many different forms of cell or enzyme confinement to different types of surfaces

or matrixes. As mentioned above, biocatalyst immobilization stabilizes, favors their facile separation from the reaction medium for further reuse and also makes the purification of the final product easier. Different techniques have been employed for whole cell and enzyme immobilization [58].

Microbial whole cells are efficient, ecological, and low-cost catalysts that have been successfully applied in the pharmaceutical, environmental, and food industries, among others. Cell entrapment is the most widely used technique for whole cell immobilization; in essence, the cells are included within a rigid network that prevents cell release but is porous enough to allow the diffusion of substrates and products [58]. There are three types of matrixes used for microorganism stabilization and can be divided into hydrogels (alginate, κ-carrageenan, chitosan), thermogels (agar, agarose, cellulose), and synthetic polymers (polyacrylamide, polyvinyl alcohol, polyurethane) [58–60]. Of these matrixes agarose, alginate and polyacrylamide have been widely used for biocatalyst immobilization for NA synthesis. Additionally, it has been reported that the addition of nanocomposites to immobilization process improves the mechanical properties of biocatalysts obtained [61] facilitating the design of bioreactors for further scale bioprocess [28]. Cell entrapment has some disadvantages such as diffusional problems between substrates and products, and microorganism activity may decrease due to the process of matrix formation. For these reasons, immobilization by entrapment should be optimized for each microorganism.

Enzyme immobilization is one of the leading methods employed in biotechnology and bioengineering. To be used as biocatalysts, enzymes are needed in high amounts, and the process to obtain them is much more expensive. That is why their application requires, in many instances, their recovery and reuse to make an economically feasible process. Moreover, the use of an immobilized enzyme allows simplifying the reactor design and controlling the reaction, considering that the simple act of filtering the enzyme stops the reaction. Enzymes can be stabilized by absorption, covalent conjugation, entrapment, and affinity binding [62].

A large variety of supports can be employed for enzyme immobilization, which can be classified according to their chemical properties [62]. Natural polymers include alginate, chitosan and chitin, collagen, carrageenan, gelatin, cellulose, pectin and sepharose. Inert polymers and inorganic materials are usually used as carrier matrixes. A variety of inorganic solids can be used for the immobilization of enzymes, e.g., alumina, silica, zeolites, and mesoporous silicas [63,64]. Supports, such as agarose beads, zeolites, porous glass, epoxy resins like Sepabeads, offer large areas for enzyme–support interactions. Among the reactive groups, epoxy or glyoxyl groups may be considered very adequate [65]. Examples for the employment of some of these matrixes for immobilizing biocatalysts for NA production are described later in this text.

In the last decades, numerous immobilization methods and supports have been developed to obtain chromatographic stationary phases with immobilized enzymes. These are used in batch-wise experiments or packed into columns and in flow systems such as immobilized enzyme reactors (IMERs). In an IMER the enzyme can be immobilized on a suitable chromatographic support or on the inner surface of silica capillaries, where the catalytic reaction occurs upon substrate injection during its chromatographic elution. In general, IMERs can be coupled with capillary electrophoresis (CE), HPLC and other modes for on-line analysis [66]. A very promising approach has been described in the work by Calleri et al., where they report the development of a novel IMER, consisting of a purine NP from *Aeromonas hydrophila* (AhPNP) immobilized covalently on a fused silica open tubular capillary (OTC) via Schiff base chemistry. This biochromatographic system was used for the evaluation of the substrate specificity on nucleoside libraries. Approximately 60 reactions were run without activity loss, thus confirming the reliability of this IMER as a tool for screening nucleoside analogues. Furthermore, the AhPNP-IMER was highly stable under long-term storage conditions with 100% retained activity after 1 month [67].

The immobilization methods and matrixes used influence the activity of immobilized enzymes. Enzyme properties have to be usually improved before their implementation at industrial scale (where many cycles of high yield processes are desired). Generally, immobilized enzymes to be reused for long times in industrial reactors and, in addition, some other critical enzyme properties have to be improved, such as stability, activity, inhibition of by reaction products, and selectivity towards unnatural substrates [68].

In conclusion, in order to achieve successful biocatalyst immobilization, parameters such as biocatalyst stability, mechanical resistance, operational stability, and reusability should be considered.

3. Clinically relevant nucleoside analogues in chemotherapy and viral treatment

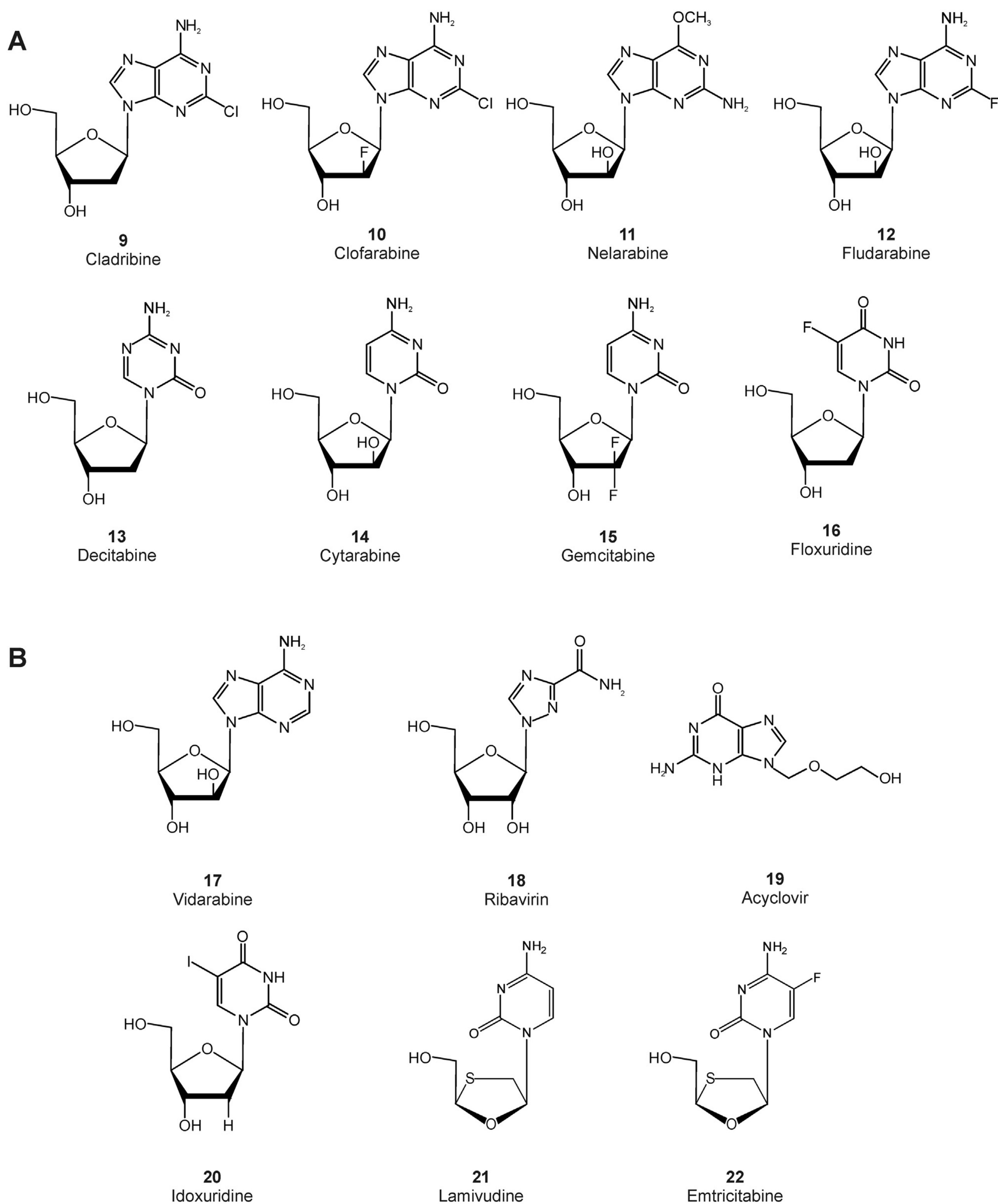
Anticancer and antiviral nucleosides include several analogues of pyrimidine and purine nucleosides. In cancer, the two main purine analogues are cladribine and fludarabine, drugs commonly used in the treatment of low-grade malignant disorders of the blood. Recently, other novel second-generation purine nucleoside analogues, clofarabine and nelarabine, have been synthesized and rapidly introduced into preclinical studies and clinical trials [69]. Regarding pyrimidine analogues, cytarabine was the first NA approved by the FDA in 1969, and it is extensively used in the treatment of acute leukemia; gemcitabine is indicated for the treatment of several solid tumors; and decitabine is used for the treatment of myelodysplastic syndromes (MDS) and is also being studied for other types of cancer. The fluoropyrimidine floxuridine has activity against various types of cancer.

About antiviral NAs, these nucleosides have been described for over four decades, and constitute the first-line therapy against specific viral infections. As they can be used for long periods of time in chronic viral infections, NAs with improved bioavailability and reduced side effects have been extensively explored. The purinic analogues vidarabine and acyclovir and the pyrimidinic analogue idoxuridine are effective against herpes simplex virus and varicella zoster virus and have been on the market for a long time. The guanosine analogue ribavirin is effective against both DNA and RNA viruses, being the first- line treatment in combination with PEG-IFN against hepatitis C infection. Also, the discovery of NA effective in HIV therapy marked a breakthrough in AIDS treatment. The cytidine analogues lamivudine and emtricitabine are used in combination with other drugs in HIV therapy.

The nucleoside analogues commonly used in anticancer and antiviral therapies [9,16] that are discussed in this review are represented in Scheme 5.

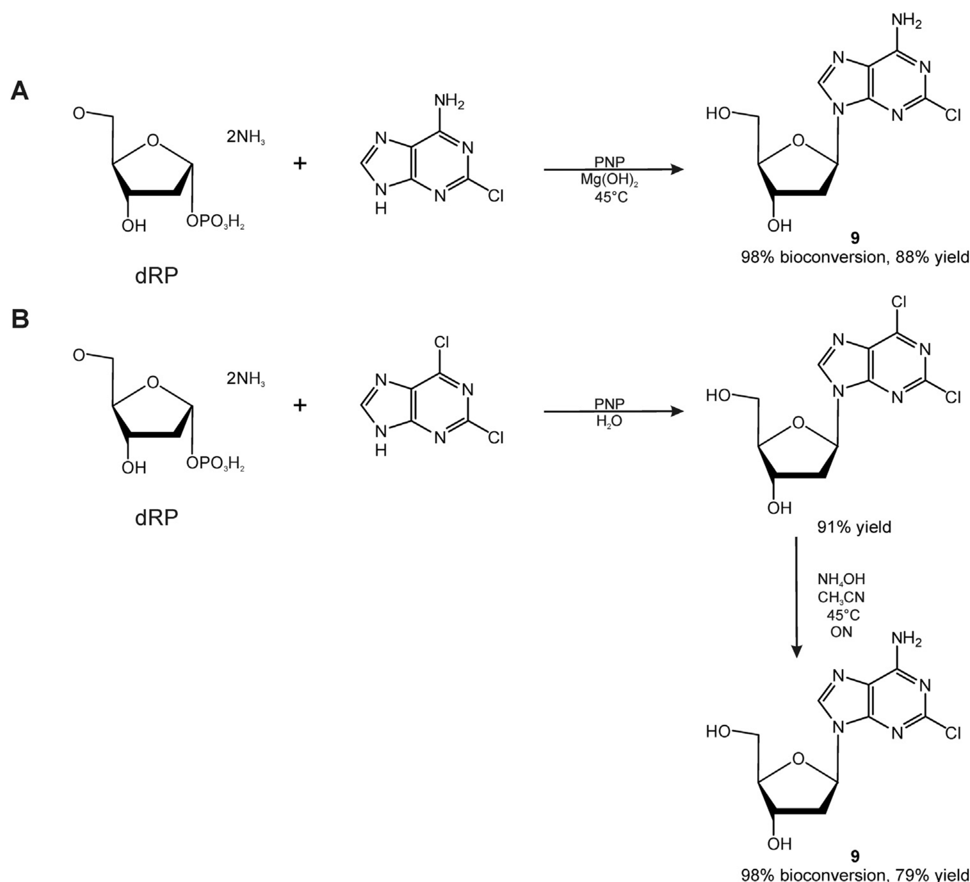
3.1. Purine nucleoside analogues (PNAs)

Cladribine (2-chlorodeoxyadenosine, 2-CdA, Leustatin) is a deoxyadenosine analogue in which the hydrogen atom at the 2-position of the purine ring has been substituted for a chlorine atom (Scheme 5). This substitution makes cladribine resistant to deamination by the enzyme adenosine deaminase (ADA). In cells, it requires phosphorylation by deoxycytidine kinase (DCK) to convert to its cytotoxic form, 2-chlorodeoxyadenosine triphosphate. Thus, in lymphocytes, in which the activity of DCK is high and 50-nucleotidase activity is low, the administration of cladribine results in the accumulation of its nucleotides, inhibiting DNA and RNA synthesis and subsequently enhancing DNA strand break [70]. This NA shows remarkable activity, produces long-lasting com-



Scheme 5. Chemical structure of relevant nucleoside analogues used in: A) cancer therapy and B) viral treatment.

plete remissions, and has been the standard treatment in hairy cell leukemia (HCL), a chronic B-cell lymphoproliferative disorder [71]. Also, it has been shown to be effective in treating chronic lymphocytic leukemia (CLL) [72], indolent non-Hodgkin lymphoma and acute myeloid leukemia [73,74]. It was first one synthesized in the 1960s and since those early reports, different synthesis methods have emerged in the literature. Regarding chemoenzymatic synthesis of 2-CdA, Komatsu and Araki reported an approach using two strategies: (i) via direct enzymatic glycosylation pathway by conversion of chemically synthesized 2-deoxy-alpha-D-ribose 1-phosphate (dRP) to 2-CdA using a PNP from *E. coli* (Scheme 6A); or (ii) a two-step route involving a glycosylation-amination pathway via 9-(2-deoxy-beta-D-ribos-1-yl)-2, 6- dichloropurine (Cl_2Pu-dR). Both methods gave bioconversion values of 98% and achieved good



Scheme 6. Biosynthetic pathways for 2-CdA generation via: A) direct enzymatic glycosylation and B) glycosylation-amination pathway.

yields (88% and 79%, respectively). After the two-step route, cladribine **9** was purified with anion exchange resin [IER (OH)] followed by recrystallization, obtaining the aforementioned yield. This strategy could be useful as an efficient alternative method for the syntheses of various unnatural 2′-deoxynucleosides [75]. Besides, another report states that cladribine was synthesized using intact recombinant *Escherichia coli* overexpressing *Geobacillus stearothermophilus* B-2194 thermostable PNP from various substrates. The advantage of using these cells modified with the thermostable enzyme is that the process can be run at a temperature of 70 °C, which guarantees the maximal concentrations of sparingly soluble substrates. The best ribose donors and base sources were assayed for the reaction, and the best results were obtained with 2-chloroadenine as a modified base and the highest yield was reached when using 2′-deoxypurines as donors of deoxyribose (up to 95% in the case of deoxyguanosine) [76].

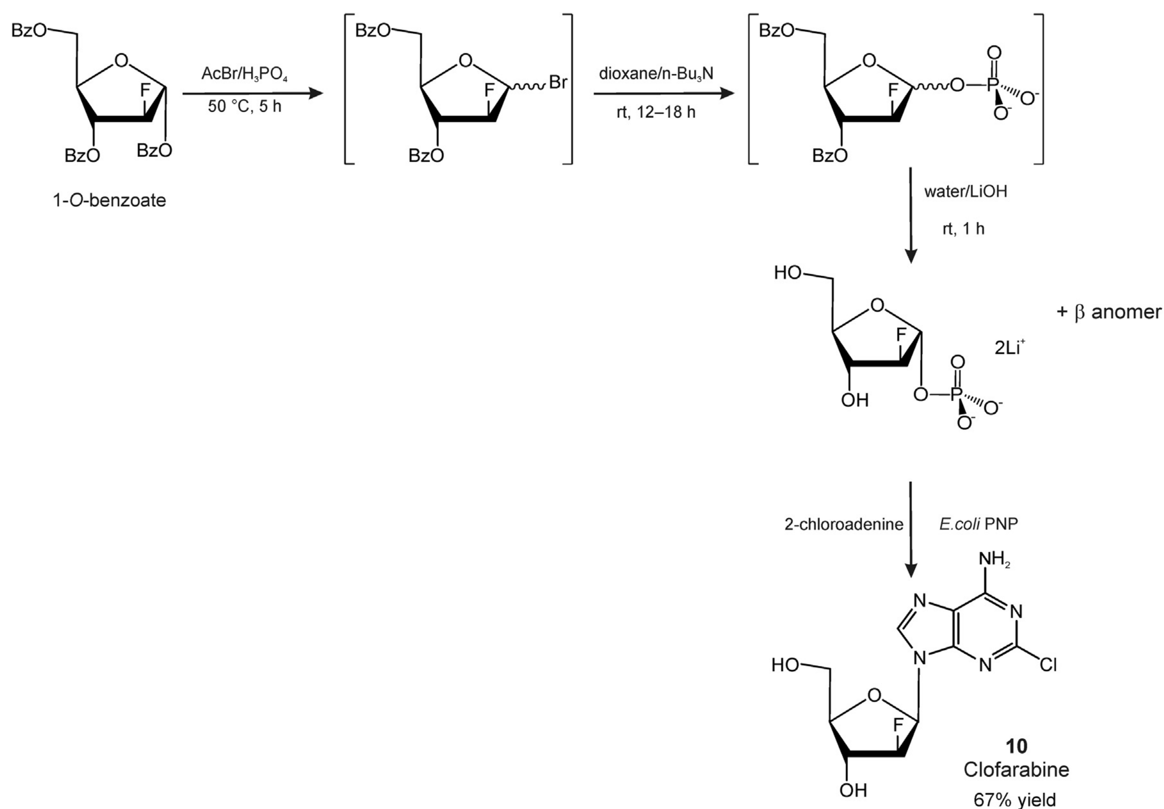
Clofarabine (9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-2-chloroadenine, CAFdA, Clolar, Evoltra) is the most promising PNA in current clinical trials conducted in patients with acute leukemia [77,78]. Among this family of nucleosides, it has recently attracted considerable attention owing to its successful application for the treatment of pediatric acute leukemia [79].

Different chemoenzymatic approaches for the synthesis of this NA were developed by Fateev et al. In the first one, the chemical synthesis of 2-deoxy-2-fluoro-α-D-arabinofuranose-1-phosphate (2FAra-1P) intermediate was achieved via three-step conversion. Then, condensation of the obtained product with 2-chloroadenine mediated by the recombinant *E. coli* PNP yielded 67% clofarabine **10** (Scheme 7). The second approach is a one-pot enzymatic cascade in which ribokinase (RK) and phosphopentomutase (PPM) turn 2-deoxy-2-fluoro-D-arabinose into the phosphate form 2FAra-1P, followed by its condensation with 2-chloroadenine via *E. coli* PNP, thereby obtaining clofarabine in 48% yield after 24 h [80]

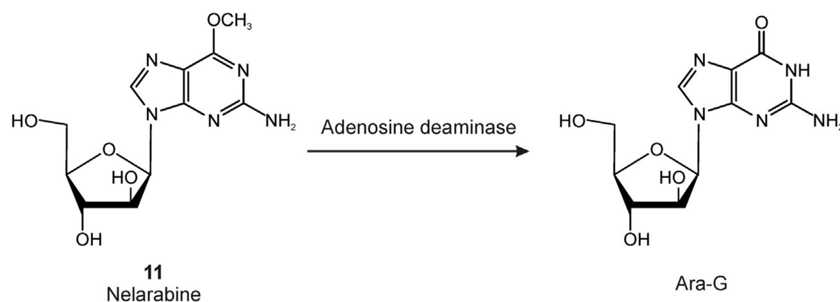
Nelarabine (9-β-D-arabinofuranosylguanine, Ara-G, Arranon, Atriance) is a guanosine analogue in which the hydrogen at the 6-position of the ring is substituted by a metoxy group. Nelarabine is a prodrug of guanine arabinoside (Ara-G) that is 8 times more soluble and is rapidly demetoxilated to Ara-G in blood by adenosine deaminase (Scheme 8). It is a T-cell selective NA and it was rapidly approved by the US FDA in 2005 due to its efficacy against T-cell acute lymphoblastic leukemia (T-ALL) and T-cell lymphoblastic lymphoma (T-LBL)[81].

A chemoenzymatic approach for β-D-arabinofuranosyl purine nucleoside synthesis, among which nelarabine and fludarabine were described using commercially recombinant *E. coli* PNP, was developed. MacDonaldís method was employed for the synthesis of α-D-arabinofuranose 1-phosphate, which then was coupled to the corresponding base using *E. coli* PNP (Scheme 9). The mixture of the isomeric phosphates was tested in the reaction with purine heterocyclic bases catalyzed by recombinant *E. coli* PNP. Interestingly, pyranose phosphate did not interfere with the reaction of furanose phosphate with purine bases. Moreover, this compound was effectively biosynthesized using 2-amino-6-methoxypurine and the mixtures of isomeric phosphates catalyzed by *E. coli* PNP, obtaining 40% yields. Remarkably, nelarabine was obtained at 53% yield when 2-amino-6-methoxypurine and 1-(b-D-arabinofuranosyl)uracil were used as a donor of the arabinofuranose residue and *E. coli* uridine and purine nucleoside phosphorylases as biocatalysts [82].

Fludarabine (9-β-D-arabinosyl-2-fluoroadenine o, Ara-U, Fludara) is currently indicated for use in chronic lymphocytic leukemia (CLL) patients who were refractory to prior treatment



Scheme 7. Chemoenzymatic synthesis of clofarabine.



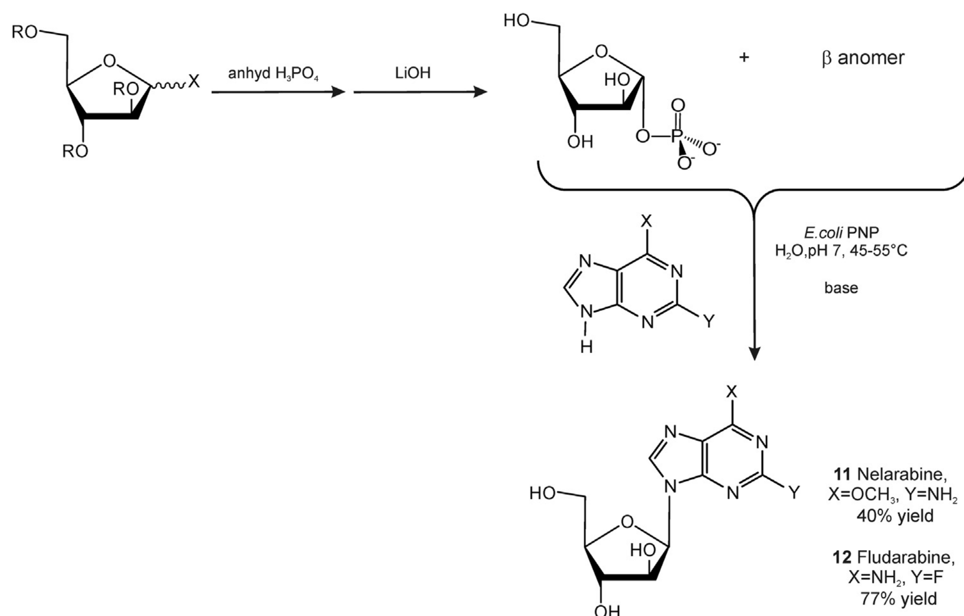
Scheme 8. Deamination of nelarabine to Ara-G.

with alkylating agents [69,77]. Fludarabine **12** is administered to patients as the soluble prodrug fludarabine 5′-monophosphate, which then enters the cells and becomes triphosphorylated by deoxycytidine kinase (dCK) or deoxyguanine kinase (dGK) to exert its inhibitory actions.

As described above, the same chemoenzymatic approach using MacDonaldís method was employed for the synthesis of fludarabine. The mixture of isomeric phosphates was combined with 2-fluoroadenine via *E. coli* PNP at 55 °C for 1 h, obtaining bioconversion values of 98%, and 77% yield after crystallization and recovery (Scheme 9). Interestingly, the rate formation of fludarabine **12** under these conditions was similar to the PNP-catalyzed conversion of α-D-ribofuranose 1-phosphate with 2-fluoroadenine [82]. In a later study, *Citrobacter koseri* cells were used for the production of this compound and other arabinofuranosyl compounds. Cells were immobilized by entrapment in various matrixes, agarose being the one with better results. The best results were obtained with free cells yielding 58% of fludarabine after 14 h at 60 °C [83].

Vidarabine (9-β-D-arabinofuranosyladenine, Ara-A, vira-A) was the first clinically approved antiviral drug, and it is currently only used topically for herpes virus keratitis. Ara-A is metabolized to its 5′-triphosphate (araATP) and in herpes virus-infected cells, treatment with this NA results in selective inhibition of virus replication. The main disadvantages to the clinical use of ara-A include its extremely low water solubility and *in vivo* rapid deamination to 9-β-D arabinofuranosylhypoxanthine (ara-H) by adenosine deaminase. Since ara-H is considerably less potent than ara-A, attempts have been made to develop ara-A derivatives that are deaminase-resistant.

Regarding ara-A biosynthesis, the above-mentioned biocatalyst immobilized *Citrobacter koseri* and was able to produce vidarabine, obtaining 71% yield in 26 h, and was successfully scaled up to 150 mL, obtaining 62.5% yield after 26 h [83]. In a previous report the enzymes Puo-phosphorylase and uridine phosphorylases, obtained by genetic engineering from overproducing *E. coli* BL21(DE3)/pERPUPHO1, were immobilized in aminopropylated macroporous glass and used as biocatalyst for vidarabine production, obtaining 56% yields after 24 h [84]. More recently, the multienzymatic synthesis of this drug, also using arabinosyluracil and adenine, was reported; uridine phosphorylase from *Clostridium*



Scheme 9. Synthesis of nelarabine and fludarabine using MacDonalds method for isomeric phosphates obtention and coupling to the purinic base via *Escherichia coli* PNP.

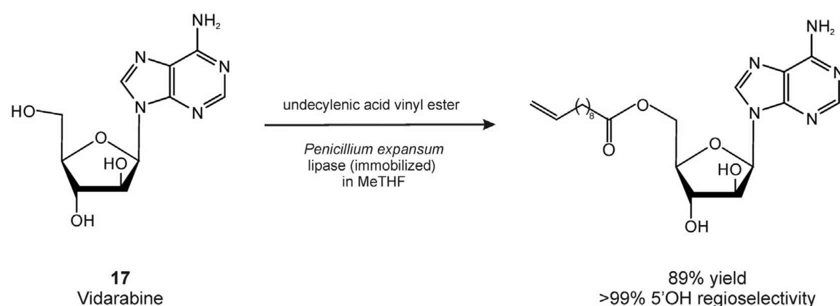
perfringens (CpUP) and a PNP from *Aeromonas hydrophila* AhPNP were used as covalently immobilized biocatalysts. The synthesis of ara-A was scaled up 2 L, and the product was isolated with 53% yield (3.5 g/ L) and 98.7% purity. Using this procedure, a smaller E-factor (mass ratio of waste to the desired product) was achieved in comparison with the classical chemical procedure (E-factor: 423 vs. 1356, respectively) highlighting the "greenness" of this new enzymatic route [85].

Also, the acylation of this NA and other purine analogues, using 2-methyltetrahydrofuran (MeTHF), a biomass-derived compound that represents a promising medium for biocatalysis, was reported. The regioselective acylation of ara-A was performed using immobilized *Penicillium expansum* lipase in MeTHF. Good yields (89%) and excellent 5′OH regioselectivity (>99%) were obtained after 42 h using undecylenic acid vinyl ester as acyl donor (Scheme 10) [86].

Ribavirin (1-β-ribofuranosyl-1,2,4-triazole-3-carboxamide, Virazole) is a guanosine (ribonucleic) analogue mainly used as an antiviral agent in combination with PEG interferon α in the treatment of hepatitis C [87,88]. This compound has inhibitory activity against a broad spectrum of RNA viruses and is usually included among the different drugs able to affect viral nucleic acid synthesis. Recently, ribavirin activity for the treatment of different types of cancer, including leukemia and lymphoma [89], and also breast cancer [90], has been demonstrated, making this compound relevant not only as an antiviral, but also as an antitumor agent. It is not surprising then why this nucleoside analogue has been so widely studied. Ribavirin has been synthetized using *Enterobacter aerogenes* free PNP [12] and free cells [29], *Brevibacterium acetylicum* free cells [91] and free PNP [92] and *Erwinia carotovora* free cells [93]. More recently, different approaches using immobilized PNP and cells have been studied, Konstantinova et al. described an efficient method for ribavirin production using guanosine and 1, 2, 4-triazole-3-carboxamide (TCA) as donor base and immobilized *E. coli* PNP, obtaining 36.8 mM of ribavirin with a molar yield of 92% after 36 h [84]. In a later study, *E. coli* immobilized cells with the same substrates yielded 72 mM ribavirin after 4 h of reaction time at pH 7.0, 60 °C, the same conditions as those of the previously mentioned assays [6]. Our group reported the biosynthesis of this compound using *Escherichia coli* ATCC 12407 as biocatalyst. This microorganism was stabilized by immobilization in agarose, and a packed-bed reactor was developed for bioprocess scale-up. In this work, when the reactions were carried out at 30 °C, a significant increase of over 40% was achieved in comparison with higher temperatures. Noteworthy, this was the first time that ribavirin biotransformation was performed at 30 °C, favored by the use of the soluble substrate uridine, also allowing easier bioprocess scale-up. By using a packed bed reactor, 95 mg of ribavirin was achieved [94]. Furthermore, our group reported the synthesis of this compound using an extremophilic microorganism as biocatalyst. *Geobacillus kaustophilus* ATCC 8005 entrapped in an agarose matrix supplemented with the nanocomposite bentonite. This immobilized biocatalyst was stable for more than 580 h without activity loss, significantly improving operational stability and mechanical properties over those of the conventional agarose matrix. Additionally, a packed-bed bioreactor for bioprocess scale-up was designed, which was able to produce 370 mg L^{-1} of ribavirin [95].

In a series of preclinical evaluations, the alanine ester of ribavirin showed improved bioavailability and reduced side effects. So, an enzymatic process was developed for the regioselective acylation of ribavirin with the oxime ester of L-carbobenzyloxy(Cbz)-alanine using Novozym 435 (*C. antarctica* lipase B or Chirazyme L-2). After 24 h at 60 °C, the acylated product was isolated with 85% yield [96]. In the same manner, Kryger et al. proposed the synthesis of a macromolecular prodrug of ribavirin. The synthesis of ribavirin acrylate was carried out by a chemoenzymatic approach using acetone oxime acrylate, ribavirin and Novozym 435 lipase. Optimized reaction and purification procedures afforded overall product recovery yields over 85%. The effect of this ribavirin MP was tested in erythrocytes, hepatocytes and macrophages by an internalization assay, which showed reduced uptake by erythrocytes without altering drug interaction with the other relevant hepatic cell lines, which suggests an effective elimination of the main side effect of ribavirin [97].

Acyclovir (9-(2-Hydroxyethoxymethyl) guanine, acycloguanosine, ACV) synthesis and antiviral activity was reported in 1977. It is currently indicated for the treatment of HSV and VZV infections. However, acyclovir has a number of drawbacks such as poor solubility in water and low oral adsorption. For these reasons, different galactose-functionalized polymer–acyclovir conjugates with different linkers were developed. Polymerizable acyclovir derivatives were synthesized by enzymatic transesterification of acyclovir with divinyl dicarboxylates using CAL-B or amano



Scheme 10. Regioselective acylation of vidarabine catalyzed by *Penicillium expansum* lipase.

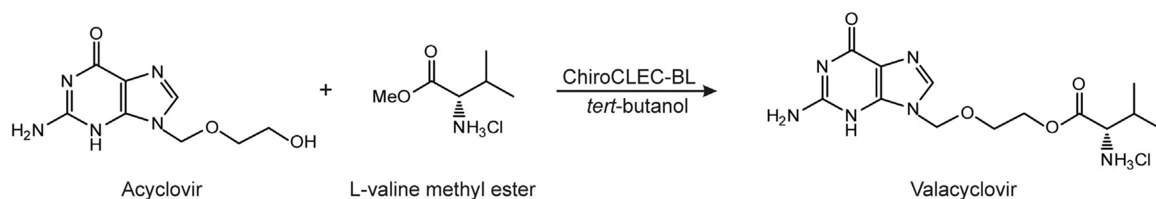
protease from *Aspergillus melleus* (PS) as catalyst in anhydrous acetone or DMSO at 50 °C and 250 rpm. Then, vinyl acyclovir esters or amides with vinyl D-galactose esters were copolymerized by radical polymerization using 2,2′azoisobutyronitrile (AIBN) as initiator in DMSO, and five corresponding galactose-functionalized polymer–acyclovir conjugates with different linkers were obtained. In vitro release experiments showed that the release rate of acyclovir could be efficiently controlled by controlling the type of acyclovir-linked bond (an ester bond or an amide bond) and the relative linker length between acyclovir and the main polymer chain. Confocal laser scanning microscopy images revealed that resultant galactose-functionalized polymer–acyclovir conjugates had evident targeting to hepG2 cells, and the cellular internalization of acyclovir could be enhanced by changing the linker length [98].

In a different approach McClean et al. assayed the hydrolysis of the L-valine ester prodrug of acyclovir (valacyclovir) to acyclovir using 62 immobilized and free commercial enzyme preparations. From this screening 19 of them proved to be competent catalysts for the hydrolysis of valacyclovir to acyclovir. The subsequent studies focused on subtilisin A (subtilisin protease from *Bacillus licheniformis*, also known as Subtilisin Carlsberg) and ChiroCLEC-BL (a cross-linked form of the same enzyme), which displayed the highest activity. However, all attempts to esterify L-valine with acyclovir failed to yield the desired product with the enzymes tested. By testing L-valine methyl ester instead, enantiopure valacyclovir was finally produced in the presence of ChiroCLEC-BL with the best rates observed in neat methyl L-valinate or with *tert*-butanol cosolvent (Scheme 11).

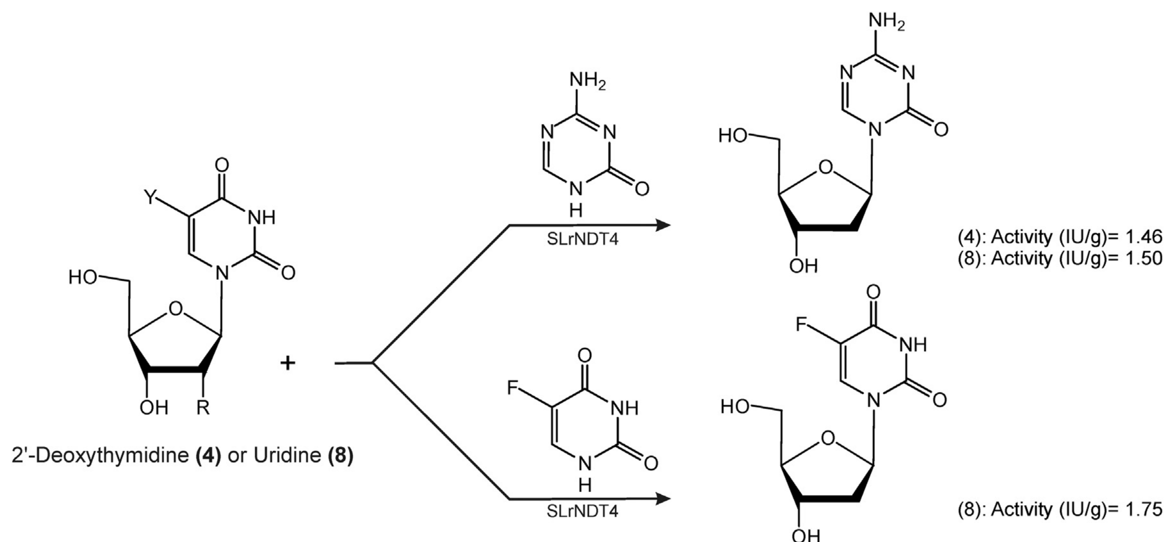
3.2. Pyrimidinic nucleoside analogues

Decitabine (5-aza-2′-deoxycytidine, 5-aza-dCyd, Dacogen) is a methyltransferase inhibitor that it is used for the treatment of myelodisplactic syndromes (MDS) and chronic myelomonocytic leukemia (CMML). Low doses of this compound also showed promising results in the treatment of chronic myeloid leukemia (CML) and hemoglobinopathies, whereas its efficacy in solid tumors is very limited [99]. Nowadays, it is the most widely used inhibitor of DNA methylation, frequently altered in cancerous cells [100]. Fernández-Lucas et al. described the enzymatic synthesis of various natural and therapeutic nucleosides effective against cancer and viral diseases, among which decitabine was produced using the recombinant *Lactobacillus reuteri* 2′-deoxyribosyltransferase attached covalently to Sepabeads EC-EP303 (SLrNDT4) as biocatalyst (Scheme 12) [5]. Regarding the enzymatic activity of the biocatalyst, 1 IU was defined as the amount of enzyme producing 1 μmol/min of 2′-deoxyadenosine using 2.6–22.0 mg of immobilized biocatalyst (containing 11.25 μg of immobilized LrNDT) in a 1.5- solution containing 5 mM 2′-deoxyuridine and 5 mM adenine in 50 mM MES buffer, pH 6.5. This biocatalyst was efficiently recycled for several batch reactions, pointing out its importance for a potential industrial application.

Cytarabine (1-β-D-arabinofuranosylcytosine, Ara-C, Cytosar-U) is used for the treatment of acute leukemias and lymphomas, especially acute nonlymphocytic leukemia. Again, its triphosphorylated metabolite (ara-CTP) exerts its cytotoxic activity. The main drawback of this NA is that it is susceptible to inactivation by cytidine deaminases present in the body, causing very short plasma half-life and then, the need for continuous intravenous infusion to maintain plasma levels and to provide maximum therapeutic efficacy [101]. To avoid these problems, different Ara-C prodrugs have been prepared. Enzymatic acylation is a great approach for the preparation of these nucleosides with increased activity. Li et al. performed an enzymatic screening with different commercial hydrolases for Ara-C acylation with vinyl stearate (VS) in different binary organic solvents. Among all the enzymes tested, *Candida antarctica* lipase B immobilized on a macroporous acrylic support (Novozym 435) showed the highest 5′-OH regioselectivity (>99.9%) and conversion (90.8%). Interestingly, they found that the initial rate and the substrate conversion were correlated with the polarity of the cosolvents. Therefore, the use of the binary solvents hexane–pyridine significantly enhanced the operational stability of the immobilized lipase [102]. The same group continued to investigate this approach but using whole cells as biocatalysts, considering the benefits they offer with respect to enzymes, such as elimination of enzyme purification and immobilization, which account for a large part of the final cost and also provide a natural environment for enzymes, protecting them from a rapid deactivation in non-aqueous solvents. The use of lyophilized *Pseudomonas fluorescens* as whole-cell biocatalyst for ara-C acylation using the irreversible acyl donor vinyl propionate (VP) was reported. After continuing assaying various binary solvents, as a general rule they determined that the catalytic activity of the cells increased with the polarity of the organic solvents used. As a result, good yields (77.1%) and 5′-OH regioselectivity (97.3%) were obtained using isopropyl ether:pyridine (30:70) [51]. In a later work, the fungus *Aspergillus oryzae* was used as whole cell catalyst for the acylation of ara-C with vinyl acetate in hexane–pyridine solvents. Different carbon sources and growth phases were evaluated and found to influence bioconversion. The activity of cell-bound lipase drastically increased in the early stage of cell growth and declined in the late stage, independently of the culture media used. These fungus cells showed more specificity towards the 3′-OH group, which was potentiated by the addition of the sorbitan fatty acid esters Span 80 (81% 3′-OH regioselectivity). Contrastingly, the highest lipase activity but lower 3′-regioselectivity was obtained using Tween 85. The cells can recognize both the 3′- and 5′-OH groups of the nucleoside substrate, giving two monoester regioisomers with different molar ratios [103]. The authors continued to optimize the reaction conditions using *Aspergillus oryzae* whole cell systems. In addition, the desired 3′-*O*-propional derivative of ara-C was synthesized with 88.3% yield and regioselectivity >70% [104]. These results showed



Scheme 11. Valacyclovir synthesis from Acyclovir transesterification using ChiroCLEC-BL.



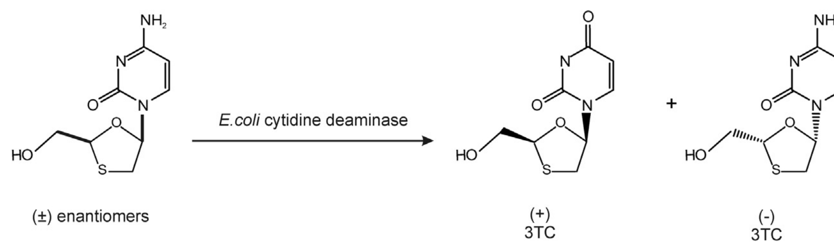
Scheme 12. Synthesis of different 2′-deoxynucleosides by immobilized biocatalyst SLrNDT4.

that the use of whole cells represents a promising tool for the green and economic synthesis of nucleoside esters in the organic solvent mixture, considering the high cost of enzyme preparation and immobilization.

Gemcitabine (2′,2′-difluoro 2′-deoxycytidine, dFdC, Gemzar) is an analogue of cytosine arabinoside (Ara-C) with potent antitumor activity. This NA is indicated as a single agent in the treatment of patients with metastatic pancreatic cancer and in combination chemotherapy in non-small cell lung cancer [3], bladder cancer and breast cancer. It has also been successfully used in the treatment of other tumors such as ovarian cancer, mesothelioma and head and neck cancers. It enters the membrane trough a nucleoside transport mechanism and is triphosphorylated (dFdCTP). This triphosphorylated metabolite competes with deoxycytidine triphosphate (dCTP) as an inhibitor of DNA polymerase. The dFdCDP is a potent inhibitor of ribonucleoside reductase, resulting in depletion of deoxyribonucleotide pools necessary for DNA synthesis, thereby potentiating the effects of dFdCTP. The dFdCTP is incorporated into DNA and after the incorporation of one more nucleotide leads to DNA strand termination. This extra nucleotide may be important in hiding the dFdCTP from DNA repair enzymes, as the incorporation of dFdCTP into DNA appears to be resistant to the normal mechanisms of DNA repair. Gemcitabine can be effectively inactivated mainly by the action of deoxycytidine deaminase [105].

The synthesis of polymer-drug conjugates was attempted by Zhou et al. First, different commercial lipases, organic solvents and substrate molar ratios were tested for gemcitabine acylation with divinyl dicarboxylates of different chain length. Then, four polymerizable vinyl gemcitabine esters were obtained by highly selective transesterification in acetone using CAL-B as catalyst. Subsequently, radical homopolymerization of the obtained 5′-*O*-vinyladipyl-gemcitabine monomers and their copolymerization with different saccharides such as galactose, glucose and lactose was performed. Three saccharide-functionalized polymer–gemcitabine conjugates with 5.7–15.3 wt% gemcitabine content were obtained, among which the conjugates with galactose or lactose as pendants had potential hepatoma-targeting function [106].

Lamivudine ((2′R-cis)-2′,3′-dideoxy-3′-thiacytidine, 3TC, Epivir, Zeffix) is a cytidine analogue that functions as a reverse transcriptase inhibitor (NRTIs). It inhibits both HIV types (1 and 2) of reverse transcriptase (RT) and also HBV RT. It is intracellular phosphorylated to 3TC 5′-triphosphate and, after removal of the diphosphate group, 3TC 5′-monophosphate, is incorporated at the 3′-end of the viral DNA chain, acting as chain terminator. It is currently indicated for the treatment of HBV and HIV infections. In HIV treatment, it is administrated in combination with other anti-HIV agents such as zidovudine and abacavir [16] [107]. When it was under development, it was discovered that the unnatural (−)-enantiomer had the same antiviral activity as but less associated toxicity than the natural one, therefore it was selected for further use. This is why the synthesis of this NA was directed towards the production of this isomer. The enzymatic resolution of a racemic mixture achieved by the use of cytidine deaminase from *E. coli* that was cloned, overexpressed, isolated and immobilized onto Eupergit-C, was reported. The (+) isomer was selectively deaminated from the mixture, and after a two-column process, optically pure 3TC was obtained with 76% crystalline yield (Scheme 13) [108]. More recently, the asymmetrical synthesis of 3TC was developed using a three-step surfactant-treated subtilisin Carlsberg-catalyzed dynamic kinetic resolution protocol. Taking into account that it had been previously demonstrated that cal B catalyzes the cyclization of sulfanylacetate and acetaldehyde forming the intermediates 1.2-oxanthiolan-5-one derivatives, they investigated its activity in the synthesis of 3TC. As they obtained the opposite isomer of 3TC, they screened different lipases and



Scheme 13. Production of lamivudine (3TC) using an immobilized *Escherichia coli* cytidine deaminase.

found that some of them have the same stereochemical preference. Then, the protease subtilisin Carlsberg (STS), known to have the opposite selectivity, was assayed. When the compounds 1,4-dithiane-2,5-diol and a benzoyl protected aldehyde were mixed in the presence of triethylamine (TEA) as the base and phenyl acetate as the acyl donor in the presence of STS, the desired intermediate was obtained with 45% enantiomer purity. Then, after Vorbrüggen coupling and deprotection, lamivudine was obtained with 40% overall yield. After optimization of different reaction parameters such as solvent, temperature and molar yield of the substrates, 89% yield and 82% enantiomerical purity were obtained [109].

Emtricitabine (2′,3′-dideoxy-5′-fluoro-3′-thiacytidine, (−)-FTC, Emtriva) is the last NRTI that was approved by the FDA for the treatment of HIV infection in adults and children (2003). It is very similar to lamivudine (3TC) in its structure and in its mechanism of action. It is also effective for the treatment of HBV infections but has not yet been approved. It is used in combination with tenofovir, or in combination with emtricitabine, tenofovir and efavirenz.

Osborne et al. reported the preparation of the (−)-FTC isomer using an immobilized esterase from a microbial source. Racemic FTC butyrate ester (±) was treated with a cholesterol esterase immobilized on Accurel PP, resulting in the cleavage of the required isomer to the corresponding alcohol (−)-1, which when isolated as the hydrochloride, gave 31% yield (and 98% enantiomer selectivity determined by chiral HPLC) (Scheme 14). The immobilized biocatalyst was recycled 14 times, and the process was successfully scaled up allowing the resolving of 200 g/L of racemic FTC butyrate using 1-pentanol/potassium dihydrogen phosphate buffer as solvents to give (−)-FTC HCl (98% enantiomer selectivity, 2.17 kg, 31% molar yield based on racemic FTC butyrate) [110].

Floxuridine (5-Fluoro-2′-deoxuridine, FdUrD, FUDF) is currently used in the treatment of colorectal, pancreatic, breast, head and neck cancer [13,111]. Fluorinated pyrimidines and their nucleosides constitute a very important class of antitumor agents.

As above-mentioned, the biocatalyst *Lactobacillus reuteri* NDT attached to Sepabeads EC-EP303 (SLrNDT4) was able to synthesize this NA [5]. Recently, our group reported several advances in the biosynthesis of this compound, using immobilized *Lactobacillus animalis* as biocatalyst. In the first approach, the best results were obtained using alginate hydrogels, specifically calcium alginate 4% (w/v). This biocatalyst showed an operational stability of 44 h in batch process and 144 h at a prepilot scale, obtaining 0.8 mg/L of floxuridine [112]. In the latter case, the natural matrix previously developed was improved by using strontium as cross-linking agent, obtaining a novel biocatalytic system consisting of *L. animalis* immobilized in Sr-alginate. This biocatalyst showed bioconversion parameters higher than 80% and was also able to obtain a related nucleoside (5-bromouracil-2′-deoxyriboside) in nonconventional media at shorter reaction times. The developed Sr-alginate matrix was an efficient alternative to stabilize microorganisms and could be used to produce a broad spectrum of nucleoside analogues from substrates with low solubility in water [113]. Also, as previously mentioned, the use of bionanocomposites employing natural polysaccharides such as alginate and nanoclays such as bentonite are a promising alternative to developing stabilized biocatalysts. Different mechanical parameters such as swelling ratio, compressive strength and fracture frequency were optimized, favoring scale-up. The developed and improved immobilized biocatalyst was efficiently used for bioprocess scale-up obtaining floxuridine, showing a productivity of 596 mg per gram of biocatalyst. Moreover, storage stability and reusability of the biocatalysts were improved by more than 90% compared with control conditions. In addition, immobilized lactic acid bacteria [28]. Lastly, an immobilized biocatalyst with 2′-*N*-deoxyribosyltransferase activity (NDT) from *L.animalis* was developed from cell free extracts, resulting in a derivative with an activity of 2.6 U/g for the enzymatic synthesis of floxuridine. The obtained activity was better than previously reported using immobilized enzymes. Furthermore, this biocatalyst was satisfactorily used to obtain other halogenated pyrimidine and purine 2′-deoxynucleosides [114]

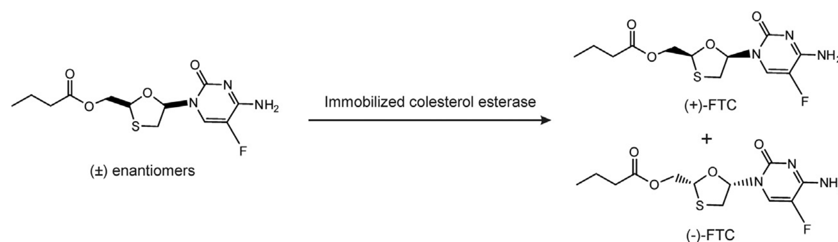
In a different approach, in an attempt to improve the pharmacological effects of the drug and avoid undesired adverse effects [115] Li et al. reported the use of various lipases in different ionic liquid-containing systems for the enzymatic acylation of this nucleoside and its analogues. The selected *Pseudomonas cepacia* lipase (PSC-L) mediated floxuridine benzoylation, obtaining excellent conversion (>99%) and great 3′-regioselectivity (92%) in anhydrous THF with the addition of 1-butyl-2,3-dimethylimidazolium hexafluorophosphate ([C_4MIm]PF_6) [116]. The enzyme performances were significantly enhanced in ionic liquid-containing systems compared to pure organic solvents (Scheme 15). In a later report they examined the substrate recognition and activity of the poorly studied *Thermomyces lanuginosus* lipase (TLL) in different solvents. TLL was effective in the laurotalion of different nucleosides, obtaining 71% 5′OH- regioselectivity, after 3 h at 40 °C, in THF. The catalytic activity and regioselectivity of TLL in the acylation of nucleosides depended strongly on the substrate structure [117].

Additionally, the regioselective galactosylation of floxuridine (FUdR) catalyzed by a commercial β-galactosidase from bovine liver with a high yield (75%) and an excellent 5′OH-regioselectivity (> 99%) was reported [118].

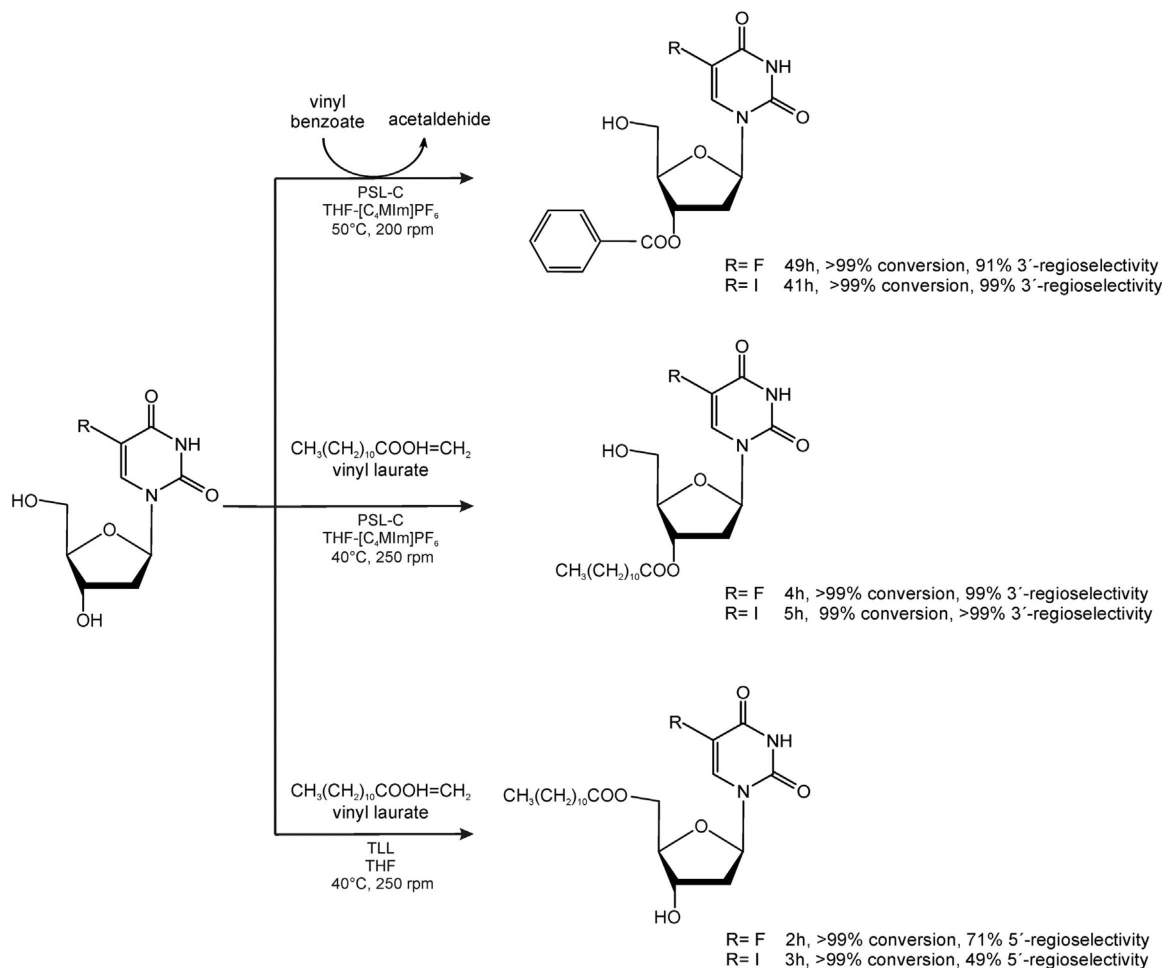
Idoxuridine (5-Iododeoxyuridine, IdUrd, IDU, iduviran) was the first clinically effective antiviral nucleoside analogue synthesized in 1959. It is used in the topical treatment of herpetic keratitis and ocular herpes virus infection (HSV-1, HSV-2) and it is also active against varicella zoster virus (VZV). In the previously mentioned study [5], this compound was obtained using the developed immobilized biocatalyst *Lactobacillus reuteri* NDRT SLrNDT4.

The acylation of this NA and others was assayed using the above mentioned lipases. PSC-L mediated the vinyl laurate acylation of this compound by obtaining complete bioconversion after 6 h and 3′OH-regioselectivity (99%) (Scheme 15) [119]. Also, TLL was effective in the laurolation of different nucleosides, but idoxuridine showed only 49%3′OH- regioselectivity, after 3 h at 40 °C in THF. [117].

Besides esterification, a different approach using the glycosylation of this and other pyrimidinic NAs has been studied. A simple and regioselective method for 5′-OH regioselective galactosylation



Scheme 14. Racemic resolution of (±)-FTC butyrate using an immobilized cholesterol esterase.



Scheme 15. Regioselective acylation of floxuridine and idoxouridine using different lipases.

using a commercial β-galactosidase from bovine liver and also a less expensive biocatalyst, the crude β-glycosidase extract from the same source, was reported. As a result, a 5′-*O*-galactosylated derivative of idoxuridine was obtained after 20 h at 45 °C in phosphate saline buffer (100 mM, pH 6.5) with 60% yield and 92% 5′-regioselectivity. For the crude extract, 46% yield and 87% 5′OH regioselectivity were obtained after 23 h at the same reaction conditions [120].

4. Concluding remarks and future prospects

The use of biocatalysis remains an interesting strategy for NA synthesis. Much progress has been made since the early techniques for the preparation of these compounds, and their modification allows obtaining versatile products with enhanced activity. Chemoenzymatic approaches to the synthesis of nucleosides have proved to be an important instrument to produce a large variety of structurally diverse nucleosides. A biocatalyst allows the replacement of multistep chemical reactions, and great progress in nucleoside analogue synthesis has been achieved by combining chemical methods and biochemical transformations. This combination represents a very promising technology and also shows high efficiency for biotechnological process development. Enzyme application in multistep reactions employing recombinant whole cells expressing multiple enzymes or sequential bio/chemocatalyzed transformations provides efficient pathways for the synthesis of complex compounds in a one-pot process. Enzymes continue to be mostly used in industrial applications and nowadays with the development of modern biotechnology; these proteins will become available more inexpensively, permitting the industrial preparation of nucleoside derivatives via biotransformations. Furthermore, biocatalysts are able to perform highly chemo-,

stereo-, and regioselective synthesis of nucleosides, providing an ecofriendly technology in comparison with classic organic chemistry. In a near future, immobilized enzymes are expected to play an important role as more valuable, recoverable and reusable industrial biocatalysts for biotechnological, medical and pharmaceutical applications, gradually replacing current chemical manufacture of nucleoside analogues.

Acknowledgements

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