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Biocatalytic cascade transformations for the synthesis of *C*-nucleosides and *N*-nucleoside analogs

Martin Pfeiffer[1] and Bernd Nidetzky[1,2]

Nucleosides and their analogs, including those that feature substitution of the canonical *N*-glycosidic by a *C*-glycosidic linkage, provide access to potent antiviral, antibacterial, and antitumor drugs. Furthermore, they are key building blocks of m-RNA vaccines and play a crucial role for vaccine therapeutic effectiveness. As the medicinal applications of nucleosides increase in number and importance, there is a growing need for efficiency-enhanced routes of nucleoside synthesis. Cascade biocatalysis, that is, the application of natural or evolved enzymes promoting complex transformations in multiple steps in one pot and without the need of intermediate purification, emerges as a powerful tool to obtain nucleosides from readily available starting materials. Recent efforts in enzyme discovery and protein engineering expand the toolbox of catalysts active toward nucleosides or nucleotides. In this review, we highlight recent applications, and discuss challenges, of cascade biocatalysis for nucleoside synthesis. We focus on *C*-nucleosides and important analogs of the canonical *N*-nucleosides.

Addresses
[1] Institute of Biotechnology and Biochemical Engineering, Graz University of Technology, NAWI Graz, Petersgasse 12, A-8010 Graz, Austria
[2] Austrian Centre of Industrial Biotechnology (acib), Krenngasse 37, A-8010 Graz, Austria

Corresponding author: Nidetzky, Bernd (bernd.nidetzky@tugraz.at)

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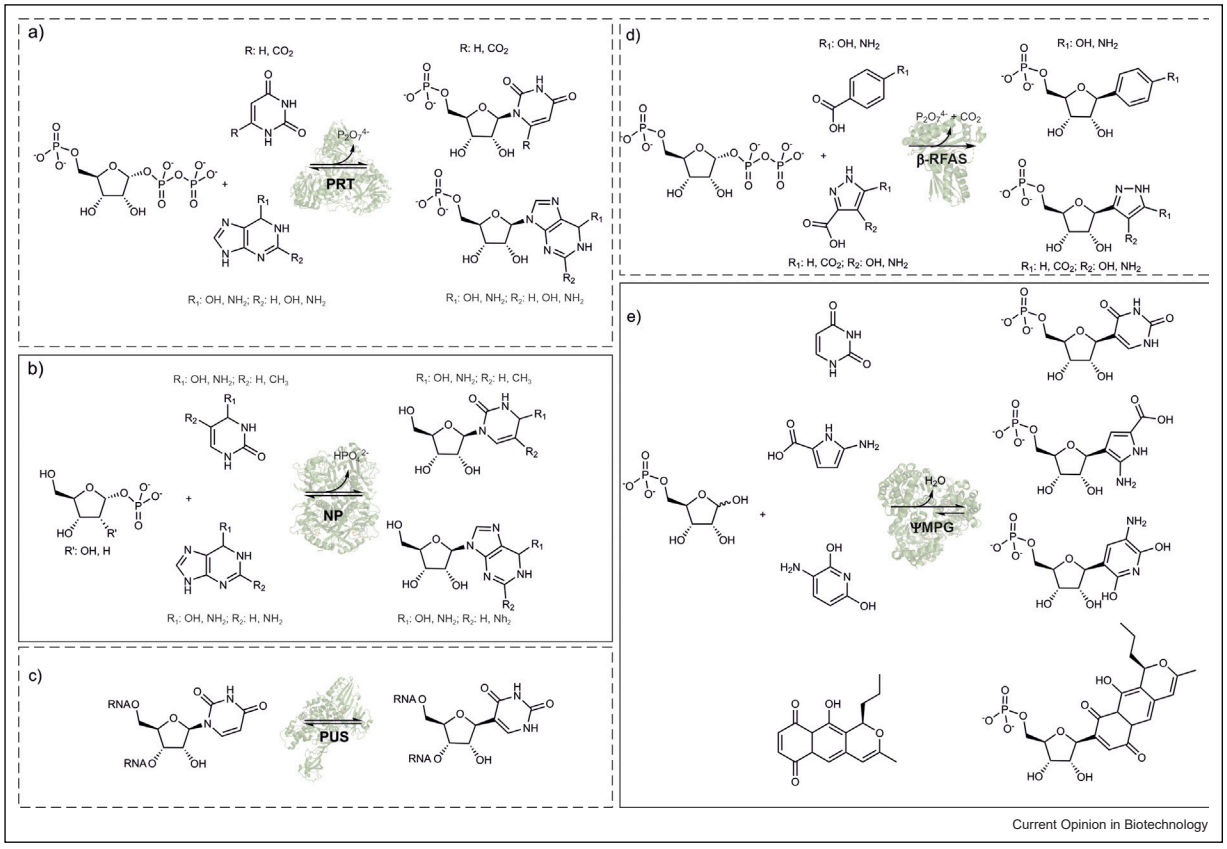
Introduction

Structural analogs of the canonical *N*-nucleosides (Figure 1) are important synthetic targets in medicinal chemistry, mostly for inhibitor development but also for application as noncanonical building blocks of synthetic RNA and DNA. Various nucleoside analogs have become widely known as potent inhibitors of RNA or DNA polymerases [1] and of reverse transcriptase [2]. Additionally, they can inhibit enzymes of the nucleoside de novo and salvage pathways, including nucleoside phosphorylases (NPs) [3] and inosine-5′-monophosphate dehydrogenases [4]. The recently developed nucleoside analogs Molnupiravir and Islatravir are promising drug candidates against COVID-19 [5] and HIV infections [1], respectively. Furthermore, nucleoside analogs are integral part of the success story of m-RNA-based therapeutics [6]. Uniform replacement of uridine by the natural *C*-nucleoside pseudouridine (Ψ), or by the synthetic Ψ derivative *N*-1-methyl-pseudouridine (N1mΨ), enhances the translation efficiency and reduces the immunogenicity of therapeutic RNA. This crucial evidence has enabled major breakthroughs in RNA vaccine development. Therefore, Ψ or N1mΨ (Figure 1) are key constituents of the current vaccines used to combat the COVID-19 pandemic [6].

Chemical synthesis of nucleosides is challenging due to the structural complexity and the stereochemical requirements of the different target molecules. Typically, only the β-glycoside stereoisomer is bioactive (Figure 1). The preferred route to *N*-nucleosides involves nucleophilic addition of the nucleobase to an activated pentose intermediate [7,8]. Modification of canonical *N*-nucleosides [9] and de novo synthesis of the nucleoside from achiral starting material [10] constitute alternative approaches. *C*-nucleosides are synthesized by direct coupling of carbohydrates and the nucleobases or alternatively, by stepwise reconstruction of the glycone or aglycone upon the relevant functional moieties [11–19]. Although substantial improvements were made in these synthetic approaches regarding stereoselectivity [12,15] and reaction step economy [10,19], important challenges remain: the requirement of harsh reaction conditions, extensive use of protecting group chemistry, and usage of harmful chemicals. Biocatalysis offers an attractive alternative to pure chemical synthesis, overcoming many of the above-mentioned drawbacks. In the field of *N*-nucleoside synthesis, enzymatic routes have been explored for many decades [20–22]. Over the last years [23–35], however, the detailed characterization of *C*-nucleoside metabolic pathways (e.g. microbial biosynthesis of *C*-nucleoside antibiotics, salvage pathways of *C*-nucleosides) has resulted in the discovery of *C*-nucleoside-active enzymes that expand the toolbox of

Figure 1



Enzymatic reactions for the installment of **(a–b)** a β-*N*-glycosidic and **(c–e)** a β-*C*-glycosidic linkage in nucleoside or nucleotide synthesis. The types of enzyme in solid frame have shown potential for application in biocatalytic synthesis. The other types (dashed frame) have had limited use so far, probably due to the complex requirements of their reactions.

biocatalysis [36••–40]. In this review, we introduce the enzymes of *C*-nucleoside synthesis in the broader context of biocatalytic synthesis of nucleosides. Additionally, we discuss recent advances in biocatalytic cascade transformations for the synthesis of *N*-nucleoside analogs, such as Molnupiravir [40,41••,42] and Islatravir [43••], and the *C*-nucleoside Ψ [36••].

Biocatalytic toolbox for the formation of *N*- and *C*-nucleosides

Enzymatic turnover of the β-*N*-glycosidic linkage is central for the biosynthesis and the recycling of nucleosides and nucleotides. Phosphoribosyl transferases (PRT) such as orotate-PRT are involved in the de novo biosynthesis of pyrimidine nucleosides. By contrast, adenosine-PRT, 6-oxopurine-PRT, and uracil (Ura)-PRT are key enzymes in the purine and pyrimidine salvage pathways (Figure 1a). They catalyze the formation of nucleoside monophosphates from 5-phospho-D-ribosyl-α-1-pyrophosphate (PRPP) and a nucleobase. A S_N2-like (configurationally inverting) nucleophilic displacement of the pyrophosphate by the nucleobase gives the β-*N*-glycosidic product. Although highly abundant in nature, PRTs are not widely applied in biocatalytic synthesis of nucleosides [44–47], perhaps because of the (perceived) difficulty to supply the pyrophosphate-activated ribosyl donor to their reactions. It can be interesting, therefore, to explore synthetic routes to PRPP by in vivo or in vitro cascade biotransformations.

As an alternative to the PRT-dependent salvage pathway, many organisms possess an additional route, based on NP, for the recycling of nucleoside components. As shown in Figure 1b, NP catalyzes the reversible phosphorolysis of a nucleoside to its corresponding purine or pyrimidine base and α-D-ribose 1-phosphate (Rib1P). The Rib1P substrate for the reverse NP reaction in synthesis is readily accessible from a sacrificial nucleoside used as ribosyl donor in a concurrent NP phosphorolysis reaction [48,49]. Alternatively, D-ribose (Rib) can be phosphorylated at the C5 by ribokinase (RK) using adenosine 5′-triphosphate (ATP). Phosphopentomutase-catalyzed isomerization gives Rib1P. The Rib phosphorylation–isomerization

[36••,43••,50••] has been instrumental in the development of biocatalytic applications of NPs and the enzymes are now a preferred choice for nucleoside synthesis. Furthermore, NPs appear to exhibit inherently relaxed substrate specificity [51]. This together with the significant success of NP-directed evolution [43••,50••] toward expanded substrate scope (e.g. modified pentoses or nucleosides), has promoted NP applications in nucleoside analog synthesis.

In contrast to NPs that are well established for *N*-nucleoside synthesis, enzymes for *C*-nucleoside synthesis are less explored. Although naturally occurring *C*-nucleosides are known for many decades, the relevant pathways of their metabolic turnover (i.e. biosynthesis, degradation) have only recently been elucidated [23–35]. The evidence reveals three distinct types of enzymes responsible for the formation of the defining β-*C*-glycosidic bond. Each type belongs to a unique enzyme family unrelated to the others in sequence and structure. The mechanisms used appear to be also different [25•,26,31•,32,36••,51–55].

First, pseudouridine synthases (PUS) are found in all domains of life and are responsible for the post-transcriptional modification of RNA. They catalyze the site-specific isomerization of RNA-bound uridine into its *C*-nucleoside isomer Ψ (Figure 1c). Unfortunately, the strict requirement for RNA as a substrate, and the lack of activity with free nucleosides or nucleotides hinders the use of PUS in biocatalysis [56–58].

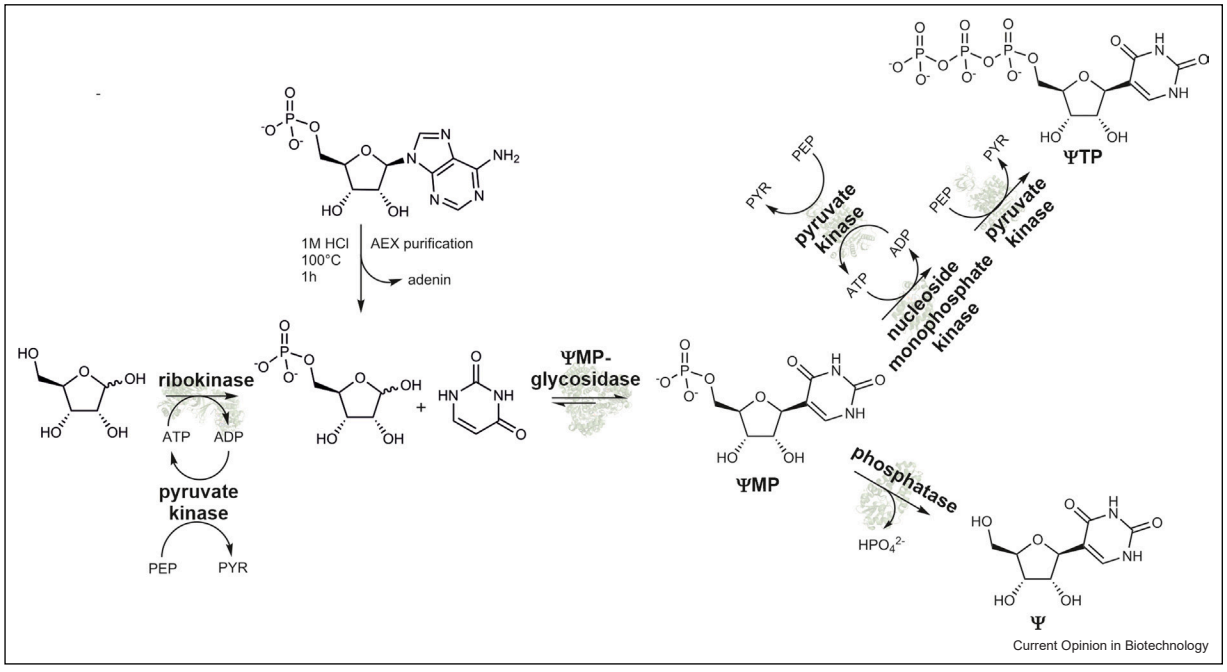
Second, the 4-(D-ribofuranosyl)aminobenzene synthases (β-RFAS) are biosynthetic enzymes found in the methanopterin [54,55] and pyrazole-based *C*-nucleoside antibiotic pathways (e.g. formycin A, pyrazomycin) [23,29•,31•]. β-RFAS catalyze β-*C*-ribosidic bond formation between PRPP and various acceptor substrates featuring a carboxylic group, depending on the enzyme. Examples are aromatic acceptors (e.g. para-aminobenzoic acid) or pyrazole-derived carboxylic acids (Figure 1d). Pyrophosphate release coupled to decarboxylation involves substantial driving force to make these reactions effectively irreversible. However, as already mentioned above in connection to PRTs, not-well-established access to the PRPP substrate presents a limitation. The strict requirement for carboxylic acid group in the acceptor additionally restricts the substrate scope. Applicability of β-RFAS to biocatalytic synthesis remains to be demonstrated.

The third type is represented by a family of enzymes forming β-*C*-glycosides from ribose 5′-phosphate (Rib5P). A pyrimidine nucleobase [32,33], a pyridine heterocycle, a 2-amino-1H-pyrrole-5-carboxylic acid [29•], or polyaromatic polyketide [26,37] can serve as the second substrate, depending on the enzyme (Figure 1e). The enzymes are found in diverse biological contexts, including the catabolic pathway of Ψ or the biosynthesis of *C*-nucleoside antibiotics, such as showdomycin and oxazinomycin [26,34,59]. The physiological degradation of Ψ is promoted by the Ψ-5′-monophosphate (ΨMP) glycosidase YeiN. Interestingly, inspite of its biological role and representing a net hydrolysis of the Ψ β-*C*-riboside, the isolated YeiN reaction involves an equilibrium far on the side of the ΨMP substrate, not the Rib5b and Ura products, as one might anticipate. This has important bearings on the use of YeiN in synthesis as discussed later. Mechanistically, YeiN catalyzes β-*C*-glycosidic bond formation via a covalent intermediate that features the open-chain ribose phosphate linked to an active-site lysine of the enzyme [32]. The imino-intermediate can undergo C–C bond formation with Ura in a Mannich-like reaction. The C–C bond formation with polyaromatic polyketides may involve a different reaction mechanism, via a Michael addition that does not strictly require the covalent intermediate [26]. As the Rib5P substrate is obtainable readily from Rib by phosphorylation with RK, YeiN and related enzymes of the Ψ-5′-monophosphate glycosidase protein family (ΨMPG, InterPro entry: IPR007342) [34] are promising for use in biocatalysis. Indeed, the first examples of enzymatic production of a *C*-nucleoside applied this class of enzyme [36••,37].

Biocatalytic cascade reactions for synthesis of Ψ-5′-monophosphate and pseudouridine

First-time example of a biocatalytic cascade reaction for *C*-nucleoside synthesis is presented by the multistep one-pot transformation of Rib and Ura into Ψ [36••]. Both Rib and Ura are readily accessible starting materials. As shown in Figure 2, the Rib is converted to Rib5P using RK under recycling of ATP by the pyruvate (PYR) kinase reaction. The YeiN reaction gives ΨMP. The substrate scope of YeiN enables a limited set of modifications on the sugar phosphate and nucleobase substrate. The equilibrium position of the C–C coupling favors the β-*C*-nucleoside formation [36••]. The 5′-phosphate group of the product is removed by an unspecific phosphatase, to obtain Ψ from ΨMP. Preparation of the nucleoside triphosphate (e.g. pseudouridine 5′-triphosphate (ΨTP)) is of additional interest. ΨTP is used as substrate of RNA polymerases during in vitro transcription reactions for the production of therapeutic RNAs. The 5′-monophosphate is iteratively phosphorylated in a one-pot reaction that combines nucleoside monophosphate kinase (ΨMP → pseudouridine 5′-diphosphate (ΨDP)) and PYR kinase (ΨDP → ΨTP). Phosphoenolpyruvate is used as phosphate donor and ATP is present in catalytic amounts (6 mol%). Interestingly, a specific nucleoside diphosphate kinase was not necessary, given the ability of the PYR kinase to phosphorylate ΨDP from ATP. The cascade reactions typically afford the products (ΨMP, Ψ, and ΨTP) in

Figure 2



Biocatalytic cascade routes to ΨMP, Ψ, and ΨTP. Phosphoenolpyruvate = PEP, AEX= anion exchange chromatography.

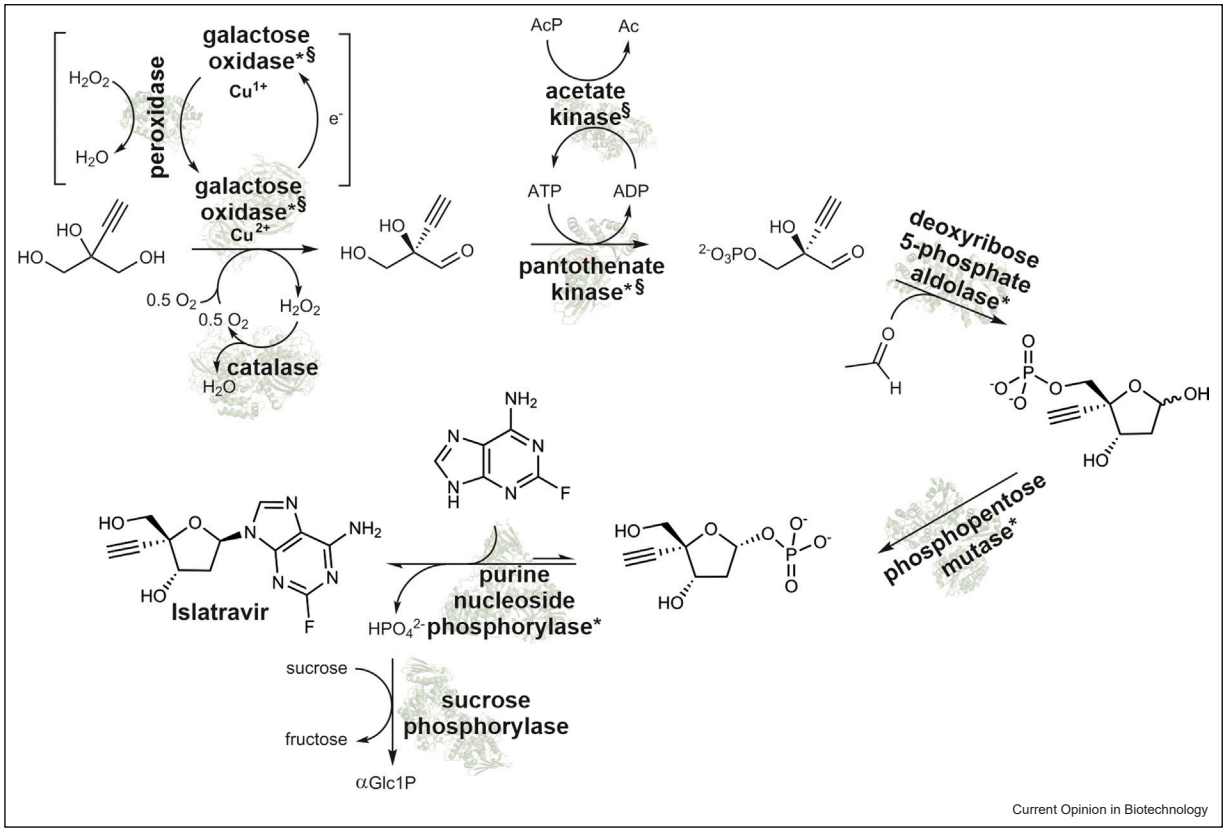
excellent yields of between 80% and 90%, based on the limiting nucleoside used (5–15 mM) and with Rib(5P) present in a 2.5-fold molar excess. The products were recovered in high purity (> 85%, 10–60 mg) from small-scale reactions (15 mL). The isolated ΨTP could be utilized for RNA polymerase-catalyzed synthesis of RNA featuring uniform substitution of uridine by Ψ. Various analogs of Ψ were synthesized based on tolerance of YeiN for structural variation in the substrates used: the 5′-monophosphates of 2-deoxy-Rib, 2-deoxy-2-fluoro-Rib, D-xylose, and D-arabinose are accepted as Rib5P analogs. 3-Methyluracil, 6-aminouracil, 4-thiouracil, and 2-thiouracil are used as analogs of Ura.

Adenosine 5′-monophosphate was considered as an alternative source of Rib5P upon acid hydrolysis [38–40]. A disadvantage is that the Rib5P thus released requires isolation, to remove potential inhibitors (e.g. purine base, adenine) and to reduce the high-salt load, before it can be used in the enzymatic reaction. The general concept of cascade reaction in Figure 2 seems to be applicable to other members of the ΨMPG superfamily. Various natural product *C*-ribosides may thus become accessible through biocatalytic synthesis [29•,30,35,37].

Biocatalytic cascade reaction for synthesis of Islatravir

Islatravir (MK-8591) is an *N*-nucleoside analog (Figure 3). It is Merck's investigational nucleoside reverse transcriptase translocation inhibitor under evaluation for the treatment and prevention of HIV-1. A synthetic route solely based on biocatalytic steps was described. It starts from 2-ethynylglycerol and uses 9 enzymes in total (Figure 3) [43••]. A desymmetrizing oxidation of 2-ethynylglycerol is catalyzed by an engineered galactose oxidase. The enzyme was evolved in 12 rounds of directed evolution that selected for enhanced activity and *S* stereoselectivity. Indeed, the reaction by the oxidase proceeded with up to 99% enantiomeric excess and a yield of 68% (200 mM, 22.8 g/L). The galactose oxidase (from *Fusarium graminearum*) was used as an immobilized enzyme preparation. The His_6-tagged enzyme was immobilized onto Nuvia IMAC resin (Bio-Rad) charged with nickel. Use of the galactose oxidase required the soluble addition of peroxidase and catalase. Peroxidase is used to maintain the galactose oxidase in its active Cu^{2+} oxidation state. Catalase destroys H_2O_2. The O_2 substrate of the galactose oxidase is supplied by bubbling air. Following the initial oxidation, a coimmobilized preparation of pantothenate kinase (from *Escherichia coli*) and acetate kinase (AcK) is added to phosphorylate the aldehyde intermediate. The pantothenate kinase was evolved in three steps for activity toward (*R*)-2-ethynylglyceraldehyde. The two His_6-tagged kinases were immobilized on nickel-charged Nuvia IMAC resin (Bio-Rad). Oxidation and phosphorylation needed to be performed sequentially due to lack of selectivity (single phosphorylation of only one primary hydroxy group) on the part pantothenate kinase. ATP used at 1.5 mol% is regenerated from acetyl phosphate

Figure 3



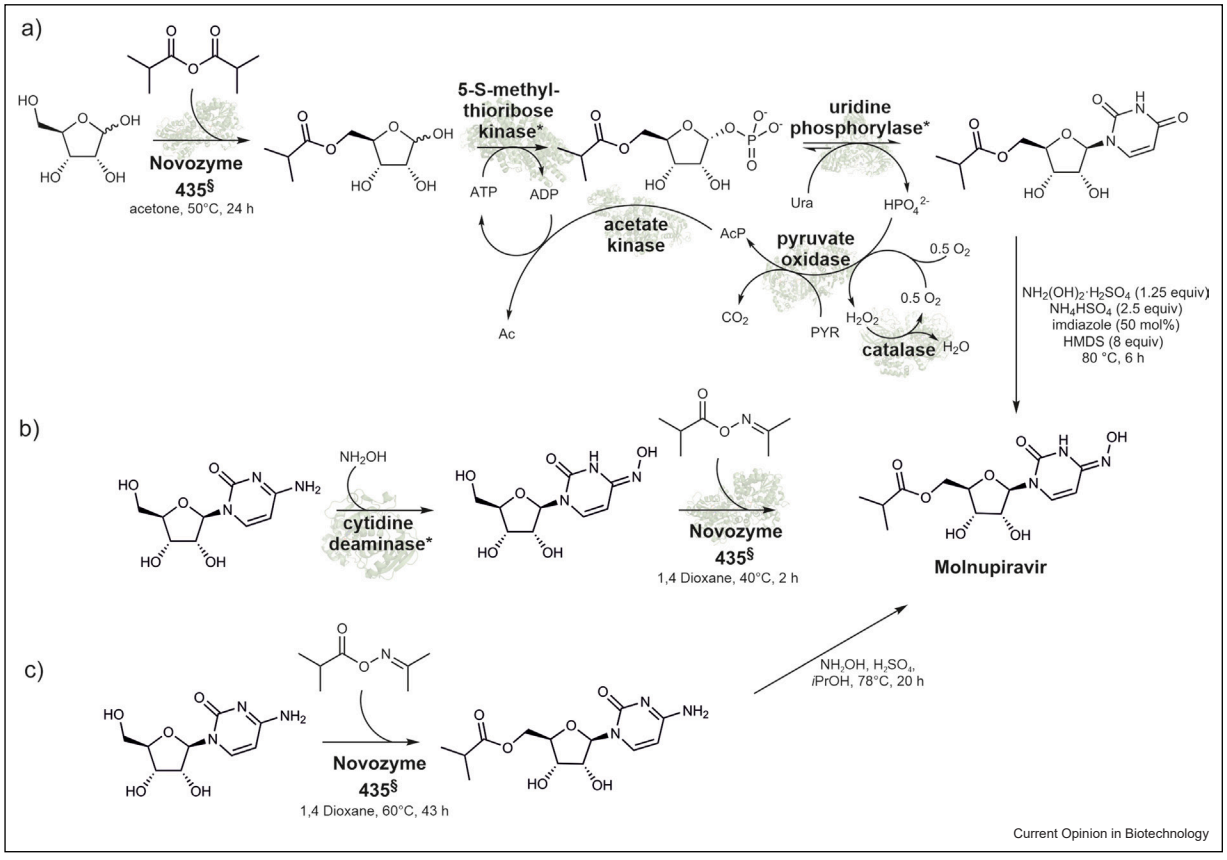
Biocatalytic cascade route toward Islatravir. α-D-glucose 1-phosphate = αGlc1P, acetate = Ac. Evolved enzymes are highlighted with * and immobilized enzymes with §.

(AcP) used in 1.5-fold molar excess of substrate. The intermediate 2-ethynylglyceraldehyde 3-phosphate is obtained in 97% yield. After filtering off the solid enzymes, the subsequent reaction steps are carried out simultaneously. An engineered deoxyribose 5-phosphate aldolase (from *Shewanella halifaxensis*, 2 rounds of directed evolution for acetaldehyde tolerance and expression, 25-fold enhancement) catalyzes a diastereoselective aldol condensation with acetaldehyde to yield (3*S*,4*R*)-4-ethynyl-deoxyribose 1-phosphate. 1,5-Isomerization is catalyzed by an engineered phosphopentose mutase (from *Escherichia coli*) that had undergone 2 rounds of directed evolution for activity enhancement (68-fold). The final step involves *N*-β-ribosylation of 2-fluoro-adenine by a purine NP (from *Escherichia coli*). The enzyme was enhanced in specific activity for the particular reaction by 344-fold through 4 rounds of directed evolution. To pull the phosphorylase reaction to high conversion toward Islatravir, strategy of in situ product removal was developed. The phosphate released is used by sucrose phosphorylase and 'trapped' as α-D-glucose 1-phosphate. The nucleoside product crystallizes from the reaction mixture and is filtered off (> 95% purity). The overall cascade reaction was performed at 0.5-g scale. Islatravir is obtained as a single stereoisomer in 51% yield from 2-ethynylglycerol.

Biocatalytic cascade reaction for synthesis of Molnupiravir

The *N*-nucleoside analog Molnupiravir (Figure 4) is an orally administered antiviral drug active against a broad spectrum of viruses. It increases the mutation rate in viral RNA and thus impairs viral replication in the host [5]. A number of synthetic routes to Molnupiravir have been reported [42]. Here we focus on the major strategies of biocatalysis applied (Figure 4). One route proceeds from Rib via a uridine isobutyric acid ester intermediate. Chemical hydroxyamination gives the product [50••]. The other routes start from cytidine and involve either enzymatic [41••] or chemical [60] amino hydroxylation as the key step. The Rib route (Figure 4a) is performed in three steps. First, Rib acylation is catalyzed by Novozyme 435 in 96% yield. Second, an enriched preparation of the esterified Rib (327 mM) is converted to esterified uridine in a 5-enzyme cascade. The main reactions, phosphorylation and nucleoside transfer, are catalyzed by two engineered enzymes, a 5-*S*-methylthioribose kinase (from *Klebsiella spp.*) evolved

Figure 4



Synthetic routes toward Molnupiravir. Acetate = Ac. Evolved enzymes are highlighted with * and immobilized enzymes with §.

in 3 rounds for α-stereoselective anomeric phosphorylation of the esterified Rib (> 100-fold improvement) and a uridine phosphorylase evolved in 5 rounds to tolerate the isobutyric acid side chain at the position O5 of Rib1P (80-fold enhancement of specific activity). The ATP (0.5 mol%) was recycled from AcP using AcK. The phosphate released in the phosphorylase reaction was recycled through the decarboxylating pyruvate oxidase reaction that converts pyruvate (1.25-fold excess) and phosphate (10 mol%) in the presence of O_2 into AcP, CO_2, and H_2O_2. The generated H_2O_2 is removed by catalase providing in situ generation of O_2. Additionally, O_2 is supplied by sparging air at 0.5 slpm. Besides providing the AcP for ATP regeneration, the pyruvate oxidase reaction provides thermodynamic pull for the β-*N*-glycosylation to complete. The multistep one-pot reaction gives the intermediate product (5′-isobutyril-uridine) at > 280 mM concentration. The product is extracted into 2-methyl-tetrahydrofuran (THF) extraction and subsequently crystallized (≥ 99.5% purity, ~87% yield). Finally, the amidic carbonyl of uridine is converted to the corresponding oxime by chemical hydroxyamination in hexamethyldisilazane solvent in a yield of up to 96%. Molnupiravir is thus obtained from Rib in an overall yield of 69% (Figure 4a) [50].

The cytidine routes (Figure 4b,c) developed by Burke et al. [41••] and Vasudevan et al. [60] circumvent the synthetic installment of the β-*N*-glycosidic bond due to judicious choice of substrate. In the first route, an engineered cytidine deaminase (3 rounds of directed evolution directed toward activity and selectivity) catalyzes the formation of *N*-4-hydroxy-cytidine in the presence of hydroxylamine. The key feature of the evolved enzyme is that it prefers the addition of hydroxylamine over deamination to give uridine. At a substrate concentration of 500 mM or lower, the product ratio of *N*-4-hydroxy-cytidine and uridine was about 8:1. When the cytidine concentration was increased to 750 mM and the temperature lowered to 4 °C, the *N*-1-hydroxy-cytidine crystallized spontaneously in situ. This enabled the intermediate product to be recovered directly from the mixture in 85% yield and 98% purity at 5-g scale. Further scale-up to 150 g was performed using enzyme as lyophilized cell extract instead of purified preparation. The yield was 71%. Although not immediately shown by

the authors, the literature demonstrates 5′-acylation of *N*-4-hydroxy-cytidine with isobutyric acid [60]. Catalytic acylation from the isobutyric acid methylester using Novozyme 435 proceeds in 74% yield [60]. Thus, an overall yield for the biocatalytic cascade reaction in two steps is estimated as 52%. Significant potential for a streamlined production of Molnupiravir is suggested. In an alternative route, Vasudevan et al. [60] (Figure 4c) combine the biocatalytic acylation of cytidine using Novozyme 435 with chemical hydroxamination of the cytidine ester. The two-step synthesis has an overall yield of 75%. Interestingly, inversion of the reaction steps, so that the hydroxyamination of cytidine is performed before the esterification, gives a yield of only 35%. This effect is due to reduced efficiency of the hydroxyamination. The synthetic routes of Burke et al. [41••] and Vasudevan et al. [60] both offer substantial improvement over the first reported synthesis route that involved 5 chemical steps with an overall yield of just 17% [42].

Conclusions and outlook

Multienzyme one-pot cascade transformations are powerful tools for the synthesis of nucleosides as active pharmaceutical ingredients. Protein engineering can adapt the enzymes to the requirements of the cascade reaction in terms of specificity and selectivity [61,62], typically within just a few months [49••]. A general synthetic strategy toward analogs of the canonical nucleosides, bearing structural modifications on the β-ribosyl or nucleobase part of the molecule, involves a sugar 1-phosphate intermediate [43••,50••]. The intermediate is obtained directly by α-selective anomeric phosphorylation or through a two-step process of 5-phosphorylation and 1,5-isomerization. Engineering approaches targeting the regeneration of the phosphorylation agent ATP in combination with in situ product removal for equilibrium shift help to make the cascade reactions highly efficient overall. Product concentrations of around 200 g/L can be obtained in excellent yields. Installment of the required modifications directly on a natural nucleoside represents an interesting alternative concept of biocatalysis for nucleoside analog synthesis [41••]. Discovery of enzymes for C–C coupling promotes *C*-nucleoside synthesis through cascades involving Rib5P as intermediate [36••]. Biocatalytic cascade transformations emerge as sustainable alternatives to purely chemical routes of nucleoside synthesis.

Editorial disclosure statement

Given his role as Guest Editor, Bernd Nidetzky had no involvement in the peer review of the article and has no access to information regarding its peer-review. Full responsibility for the editorial process of this article was delegated to Byung-Gee Kim.

Credit authorship contribution statement

M.P., B.N.: design of study, writing of the article.

Data Availability

No data were used for the research described in the article.

Conflict of interest statement

Nothing declared.

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Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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