Industrial potential of the enzymatic synthesis of nucleoside analogs: Existing challenges and perspectives

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Nucleoside phosphorylases have progressed from an enzymatic curiosity to a viable synthetic tool. However, despite the recent advances in nucleoside phosphorylase-catalyzed nucleoside synthesis, the widespread application of these enzymes in industrial processes is still lacking. We attribute this gap to three key challenges, which are outlined in this short review. To address these persistent obstacles, we believe that biocatalytic nucleoside synthesis needs to embrace interdisciplinary partnerships with the fields of organic chemistry, process engineering and flow chemistry.

Introduction

Nucleosides and their phosphorylated counterparts, nucleotides, are arguably the most versatile class of biomolecules. Most famously, they represent the backbone of life by encoding information as DNA and RNA. This code manages its own transcription, translation, replication, and proof-reading – an impressive feat for just five nucleosides and their derivatives. In addition, nucleosides play integral roles in cellular energy transfer systems and as cofactors for complex enzymatic transformations. It is therefore unsurprising that medicinal chemists were quick to recognize nucleoside analogues as ideal entry points for drug development campaigns to treat diseases which involve pathways hinging on the participation of nucleosides or nucleotides.[1–4] The most striking examples of such diseases include cancers and viral infections, both of which depend on hijacking the cellular replication machinery for a fast-paced replication of genomic information.[5,6] In many cases, administration of analogues of the natural nucleosides can inhibit key kinases or polymerases to either slow down cancer or viral proliferation, giving the innate immune system time to catch up. Alternatively, such an interference in the replication can introduce excessive mutations, leading to lower viability or even cell death.[7]

Given this pivotal role of nucleosides in medicinal and biological chemistry, it is easy to see why chemical methods to synthesize these compounds have attracted the interest of researchers for several decades[8] and continue to do so in present times.[9] However, nucleosides are less than trivial to make.[10] Their relatively high content of heteroatoms makes many of them poorly soluble in common organic solvents, while their dense arrangement of functional groups (particularly on the ribosyl moiety) generally requires extensive protecting group schemes and demands a high degree of selectivity in key bond-forming steps. As such, truly “efficient” nucleoside synthesis is still considered an unmet challenge in organic chemistry, despite more than seven decades of research in the field.[11]

To circumvent many of the challenges encountered during conventional nucleoside synthesis, increased efforts have recently been made toward the development of biocatalytic methods.[12] As a result of this trend, the greater enzyme family of nucleoside phosphorylases[13] has witnessed a true renaissance.[14,15] Natively, these enzymes perform the phosphorolysis of nucleosides, yielding the corresponding nucleobase and pentose 1-phosphate (Figure 1). Since this transformation is fully reversible,[16] they can also be employed for the synthesis of nucleosides, either from the corresponding sugar phosphate or by transglycosylation from a sacrificial glycosyl donor. Intriguingly, nucleoside phosphorylases exhibit perfect regio- and stereoselectivity over the glycosylation step and permit reactions with unprotected, readily available starting materials. Recent work from our group and others has elucidated the principles of thermodynamic control in these reaction systems[16–19] and demonstrated the application of these enzymes in the synthesis of various pharmaceutically relevant nucleosides, including the anti-HIV drug islatravir[20] and the anti-Covid19 drug molnupiravir,[21,22] among others.[23,24]

Notwithstanding these advances, the widespread application of nucleoside phosphorylases in industrial manufacturing processes is still lacking. This stands in stark contrast to other established classes of enzymes such as lipases and alcohol dehydrogenases which have become a common sight in the pharmaceutical industry and beyond.[25] We attribute this gap to three key challenges: i) the non-trivial downstream processing of enzymatic syntheses involving nucleosides, ii) the difficulties associated with large-scale preparation of these enzymes and their efficient (re-)use, and iii) their somewhat limited substrate scopes. This short review outlines these challenges, briefly discusses recent important advances, and highlights why future efforts will require a closer collaboration between traditionally distant scientific fields.

Downstream processing of enzymatic syntheses

> “*It does, for example, no good to offer an elegant, difficult and expensive process to an industrial manufacturing chemist whose ideal is something to be carried out in a disused bathtub by a one-armed man who cannot read, the product being collected continuously through the drain hole in 100% purity and yield*.” – Sir John Cornforth

Synthetic routes developed in academic laboratories are often at odds with the requirements for an industrial process. This holds particularly true for biocatalytic syntheses, which typically give low substrate and product titers (in the 10 mM range and below), byproduct formation, incomplete conversion of starting materials and/or high salt concentrations.[26] In the case of biocatalytic nucleoside synthesis, product mixtures typically contain residual starting

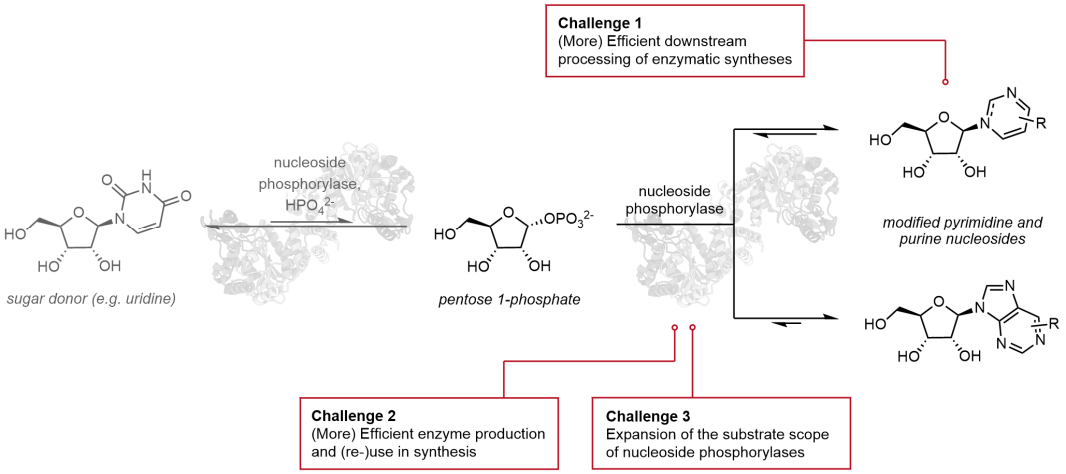

Figure 1. Current challenges in nucleoside phosphorylase-catalyzed nucleoside synthesis.

material, excessive water as solvent and buffer salts, in addition to the desired product. Historically, this has necessitated extensive chromatographic purification to obtain the product in sufficient purity, which went hand-in-hand with significant waste accumulation in the form of solvent. From a sustainability or efficiency perspective, this sub-optimal downstream processing of enzymatic reaction mixtures has thus far prevented nucleoside phosphorylase-catalyzed transformations from setting themselves apart from their chemical counterparts.[10]

To date, there have been relatively few studies which include the development of a downstream processing workflow in a biocatalytic nucleoside synthesis scheme. Indeed, most studies do not go beyond showing that the desired product is present in the reaction mixture and relegate the isolation of the product to potential future scale-up studies. Notable exceptions to this trend are the syntheses of islatravir[20] and molnupiravir[21] reported by Merck. While islatravir was prepared through a slurry-to-slurry process going from a suspension of the insoluble nucleobase to a suspension of the insoluble nucleoside (which only required filtration), the more soluble key intermediate of molnupiravir was accessed by an elegant extraction and recrystallization process. In both processes, a clever exploitation of solubility properties enabled a chromatography-free isolation of the product in high purity. While similar strategies based on the precipitation of purine nucleosides have been employed in early examples by Zuffi *et al.*[27] and Ubiali *et al.*,[28] comparable approaches have thus far been lacking for pyrimidine nucleosides. To fill this gap, we believe that a closer partnership between biochemists and chemical engineers will be necessary to weigh the requirements of the biocatalytic reaction against those of the overall process, including an efficient downstream processing strategy.

New approaches for efficient enzyme preparation

> "The more we recycle, the less we need to mine."
> – Wilfred Visser

Despite the impressive biocatalytic synthesis routes that have been developed in recent decades (and that have also found application in industry), there are still prejudices against enzymatic catalysts. This is often due to observed or perceived instabilities (to temperature, pH, cosolvents or shearing forces), the relatively high price of enzymes, or their limited reusability compared to organo-catalysts or transition metals. While the use of thermostable nucleoside phosphorylases[29,30] resolves many of the stability issues classically associated with enzymes, such as resistance to high temperatures[31] or cosolvent contents,[32] immobilization approaches typically grant further stability and enable a reuse, ultimately resulting in a drastic reduction of overall cost. In the field of nucleoside biocatalysis, enzymes have primarily been immobilized by binding to an ion exchange resin[33] or by covalent binding to a solid support.[34,35] Indeed, the use of such classically immobilized nucleoside phosphorylases in flow reactors has been demonstrated in several proof-of-concept studies for the synthesis of pyrimidine and purine nucleosides.[36–38] However, due to persistent drawbacks (primarily costly resins as well as large losses of enzyme activity by immobilization), several alternative immobilization techniques have been developed in recent years. For instance, the covalent but reversible immobilization of a purine nucleoside phosphorylase on agarose microbeads activated with thiol groups allowed a recycling of the resin after the enzyme was inactivated.[39] In a different approach, cross-linking based self-immobilization[40] of *Escherichia coli* uridine phosphorylase even eliminated the need for an external support.[41]

Building on the early advances in enzyme immobilization, the past decade has seen the emergence of *in situ* immobilization as a viable alternative to classical approaches. *In situ* immobilization typically provides a much smoother and simpler purification process and retains higher activities compared to classical methods. Among the different approaches (see e.g. the review by Rehm and colleagues),[42] inclusion body-based approaches have recently attracted the most attention. Although inclusion bodies are relatively heterogenous in their composition and typically occur as undesired side products during protein expression, they can preserve enzymatic activity and act as biological solid supports. The formation of such catalytically active inclusion bodies is typically induced by the addition of specific aggregation-supporting tags to the target protein, although naturally occurring catalytically active inclusion bodies may also exist.[43] Recently, several suitable tags have been identified[44] which induce the formation of such

inclusion bodies and their application to a variety of different proteins has been shown.[45] Notably, a very recent study from the Krauss group[46] demonstrated the robust use of catalytically active inclusion bodies in flow chemistry which is an important step towards their wide-spread application. Although such engineered inclusion bodies have not been used in the field of nucleoside synthesis so far, we believe that they offer a valuable alternative to the use of purified or classically immobilized enzymes. However, their successful implementation in synthetic processes will require a union of biochemistry and flow technology.

Expanding the substrate spectrum of nucleoside phosphorylases

"*We're seeing a move toward making things that either chemistry cannot make or can't make efficiently but biology does*." – Dr. Frances Arnold

In principle, wild-type nucleoside phosphorylases are relatively promiscuous, enabling the preparation of nucleoside analogs that are otherwise challenging to access with chemical methods.[47] Indeed, the literature harbors a range of examples, including the pharmaceutically relevant nucleosides cladribine, ribavirine,[48,49] vidarabine[37] and nelarabine.[39] This inherent promiscuity may explain why comparably few studies exist which describe the engineering of nucleoside phosphorylases to broaden their substrate spectrum. In fact, structure-function studies on the human nucleoside phosphorylases (predominantly showing decreases in activity) currently account for the majority of literature examples.[50–54] However, the limits of this natural promiscuity have thus far prevented these enzymes from becoming the go-to option for nucleoside synthesis. As such, accessing nucleoside targets outside the natural substrate spectrum currently requires either an extensive engineering campaign (as pursued by Merck in their synthesis of islatravir)[20] or the serendipitous discovery of active homologs by screening (as exemplified by our work on the diversification of 4'-methylated nucleosides).[18] Since the three-dimensional structure of nucleoside phosphorylases as well as all active site residues are highly conserved,[13] it is typically very challenging to rationalize which amino acid exchanges could yield improved activities. As a result, engineering efforts of nucleoside phosphorylases currently rely on random mutations introduced by error-prone PCR or extensive screening of single-site mutation libraries. For instance, Nannemann *et al.*'s[55] engineering of the human purine nucleoside phosphorylase for the synthesis of dideoxy nucleosides provided a more than ten-fold improved variant over four rounds of semi-rational engineering and evolution. In the impressive examples mentioned above, Merck achieved an 80-fold improvement of activity of *E. coli* uridine phosphorylase for the production of 5-isobutyryl uridine en route to molnupiravir[21] and a approx. 1000-fold improvement of activity of *E. coli* purine nucleoside phosphorylase for the synthesis of islatravir.[20] In both studies, extensive screening of single-site mutation libraries and their combination provided highly active enzyme variants over four to five rounds of evolution. While it is unclear if similar engineering campaigns could expand the promiscuity of nucleoside phosphorylases to provide true "generalists", we expect that recent advances in sequencing technology[56] and the advent of reliable computational methods for enzyme design[57] will accelerate the expansion of the substrate scope of these enzymes.

Concluding remarks

"*If you want to go fast, go alone, if you want to go far, go together*." – African proverb

As outlined above, we believe that biocatalytic nucleoside synthesis stands to profit immensely by embracing interdisciplinary science. This holds particularly true for the combination of biocatalysis and organic chemistry. While either discipline by itself has, thus far, fallen short of achieving "efficient" nucleoside synthesis, we are convinced that a union of the two fields will be well equipped to overcome the existing challenges. Impressive demonstrations of such efforts have recently made headlines in various subfields of chemistry,[58] and we expect nucleoside synthesis to follow these developments soon. Indeed, the recent work of Benkovics *et al.*[59] provides an inspirational example to this end. In their synthesis of the cyclic dinucleotide MK-1454, the combination of organocatalytic fluorination and thiophosphorylation with enzymatic triphosphorylation and cyclization provided the tools for a highly selective synthesis of an enormously challenging target on the process scale.

In our opinion, the key value of biocatalysis for nucleoside/nucleotide preparation lies in the preparation of moderately modified core structures, which can be subsequently elaborated by chemical means. For instance, the biocatalytic synthesis of nucleosides bearing reactive handles (e.g. halogen or alkyne substituents) can serve as a springboard for the rapid introduction of further molecular complexity, e.g. by crosscoupling.[60,61] Such approaches could also benefit from other upcoming disciplines such as artificial intelligence and big data management as this facilitates rational reaction optimization in both biocatalysis and synthetic chemistry.[62] Thus, we believe that the field of nucleoside biocatalysis currently stands at a turning point where the rapid progress in adjacent fields will act as a catalyst for innovation.

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Conflict of Interest

A.K. is CEO of the biotech company BioNukleo GmbH. S.W. is a scientist at BioNukleo GmbH and P.N. is a member of the advisory board.

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