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Enzyme-mediated synthesis of Molnupiravir: paving the way for the application of biocatalysis in pharmaceutical industry

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Molnupiravir (Lagevrio®) is an orally-administered anti-COVID-19 agent. Due to the urgency to meet the worldwide demand and the growing environmental concern, there is a need for speed in the industrial implementation of novel and efficient bioprocesses for Molnupiravir synthesis. This concept paper aims to review the most relevant milestones that have guided important developments in the enzyme-mediated synthesis of Molnupiravir, including detailed comments on the advantages and drawbacks of the different synthetic routes. Finally, based on a personal perspective, new greener processes for Molnupiravir manufacturing are proposed and discussed.

Introduction

Nucleoside analogs (NAs) have long served as chemotherapeutic agents for the treatment of many different types of cancers and viral infections.[1] Furthermore, emerging viral diseases, from avian flu (H5N1) to SARS (SARS-CoV-1), MERS (MERS-CoV) or COVID19 (SARS-CoV-2), but also other existing viral diseases, such as Hepatitis C, Dengue, or Ebola, require a swift in the development of novel NAs for these and future viral infections.[2]

NAs have been traditionally synthesized using chemical methods, which often require time-consuming multistep processes, including protection-deprotection reaction steps and the presence of undesirable racemic mixtures, which implies a decrease in the final product yield. Additionally, these strategies usually involve the use of expensive and environmentally unfriendly chemical reagents and the presence of hazardous organic solvents.[3] Nowadays, the use of enzyme-mediated processes in the industry is gaining ground over traditional chemical methods, and numerous examples of the practical use of biocatalysts for the synthesis of nucleosides and nucleotides have been extensively described in the literature.[4]

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First-generation routes for Molnupiravir synthesis

In its origin, Molnupiravir (Lagevrio®, MK-4482/EIDD-2801) **(1)** was designed as an anti-influenza drug, however, the global concern about the rapid growth of COVID-19 made that it was repurposed as an oral anti-SARS-CoV-2 agent.[5] Once the potential of Molnupiravir as a therapeutic agent for COVID-19 treatment was confirmed, Merck licensed the drug candidate from Ridgeback Biotherapeutics. Nowadays, Molnupiravir has recently received emergency use authorization for the treatment of mild-to-moderate coronavirus disease (COVID-19) by the U.S. Food and Drug Administration (FDA). Additionally, EU Medicines Agency (EMA) supports Molnupiravir before formal authorization of national authorities. Even though it has been almost a year and a half since Molnupiravir appeared on the global scene, numerous scientific groups from both, academia and the pharma industry, have spent so much effort in the development of novel synthetic routes for Molnupiravir manufacturing.

The first route (route 1a) to Molnupiravir synthesis (published in open literature) started from uridine **(2)** and involved five chemical steps, including the presence of organic solvents, derivatizations, and protections, which led to a low final yield (17% diol deprotection not disclosed) (Scheme 1).[6] By a simple reordering of the reaction steps (route 1b), Steiner et coworkers improved the abovementioned synthetic protocol

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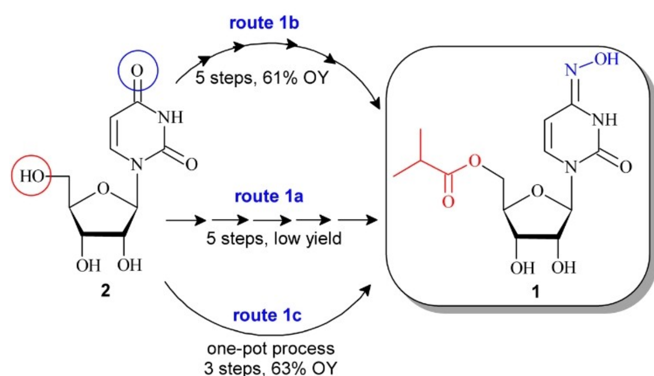


Scheme 1. Chemical routes to Molnupiravir synthesis. OY: overall yield.[6–7]

(61 % final yield, fewer isolation/purification steps), including a continuous flow procedure for the final acetonide deprotection that enhanced the selectivity and reproducibility of the process (Scheme 1).[7]

As shown in Scheme 1, initial chemical routes for Molnupiravir synthesis displayed two major constraints: i) acylation of 5′-OH from ribose moiety, and ii) the formation of the oxime on the pyrimidine ring. As a major drawback, some impurities from side reactions were detected in the different reaction steps of classical routes (e.g. uridine, mono and/or diacylated site products, unreacted products, among others). Additionally, according to the published literature,[8] uridine is not an optimal starting material for large scale manufacturing (expensive for use in tonne-scale, limited availability), and this, together with the need for more eco-friendly synthetic processes, made pharma companies try to develop novel and sustainable synthetic alternatives from simple and cheap raw substrates.

More recently, Hu et co-workers have developed a tentative one-pot process for Molnupiravir manufacturing from cytidine[8] (route 1c). As a major advantage, route 1c got the achievement of deprotection and hydroxyamination in one single step leading to a more efficient process (overall yield 63 %, 99.7 % purity). Additionally, other important advantages were shown, such as easier and selective protection of 2′-OH and 3′-OH and amino group of cytidine, or the absence of product degradation usually associated with the deprotection stage.

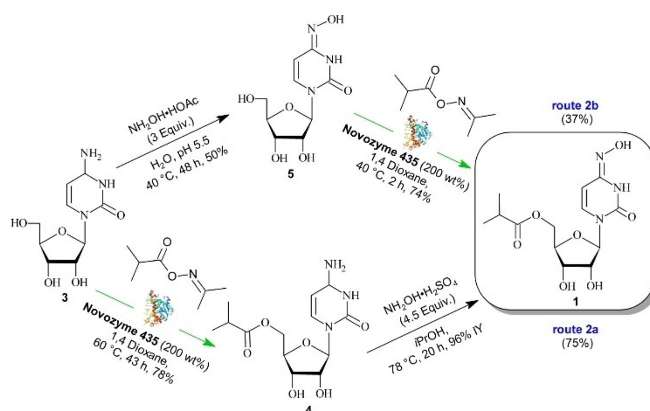
Chemo-enzymatic synthesis of Molnupiravir

As shown above, the presence of by-products from the acylation and the hydroxyamination reactions is one of the most important drawbacks of the chemical routes. In this context, the development of a shorter and protecting group-free route must address this issue. These problems were faced by Vasudevan et co-workers[9] by reducing the previous synthesis to a two-step chemo-enzymatic route starting from cytidine (**3**).

These routes included a lipase-mediated selective acylation followed by a chemical hydroxyamination (route 2a) or *vice versa* (route 2b) (Scheme 2). Both routes (2a and 2b) involve the selective esterification of 5′-OH group of the corresponding nucleoside catalyzed by Novozyme 435 lipase (immobilized *Candida antarctica* Lipase B, EC 3.1.1.3) in the presence of isobutyric oxime ester. Moreover, the direct hydroxyamination of cytidine (**3**) or cytidine isobutyryl ester (**4**) using hydroxylamine was assayed in presence of different solvents. Among all assayed conditions, the use of $NH_2OH{\bullet}H_2SO_4$ in isopropanol led to total mono-hydroxyamination. As a major advantage, a higher overall yield was obtained by route 2a (75 % *vs* 37 %). Unfortunately, longer reaction times and the presence of a final column chromatography step are needed, which increases the final cost and hampers their industrial application. In contrast, shorter reaction times are needed in route 2b. Additionally, it is important to note that *N*-hydroxycytidine (**5**) (route 2b) was obtained by simple crystallization from the reaction mixture (50 %), avoiding the presence of an extra purification step.

Interestingly, the same authors also designed a similar chemical synthesis of Molnupiravir from **3** in four steps (including acetonide protection-deprotection steps) with a moderately high yield (44 %).[10] Although this novel chemical route improved the first-generation routes,[6–7] the overall yield was lower than that obtained by route 2a. Moreover, it also implies the presence of undesired hydrolyzed impurities due to the protection-deprotection of OH groups from ribose moiety, and the presence of more reagents.

The chemo-enzymatic routes 2a and 2b not only avoid the *N*-hydroxyl acylation of the precursor nucleosides (usually common in chemical methodologies) but also allow control of the hydroxyamination process. However, the required chemical hydroxyamination limits the green potential of this approach. Furthermore, the required long reaction times, the purification of intermediates, and the presence of a final column chromatography step (route 2a) are undesirable operational constraints that need to be overcome for a suitable application at an industrial scale. To this end, several authors have deepened into the tentative scale-up of chemo-enzymatic routes proposed by Vasudevan et co-workers. Hence, the effect of the different variables of the process, such as type of solvent, temperature



Scheme 2. Two-step chemo-enzymatic synthesis of Molnupiravir.[8] IY: isolated yield.

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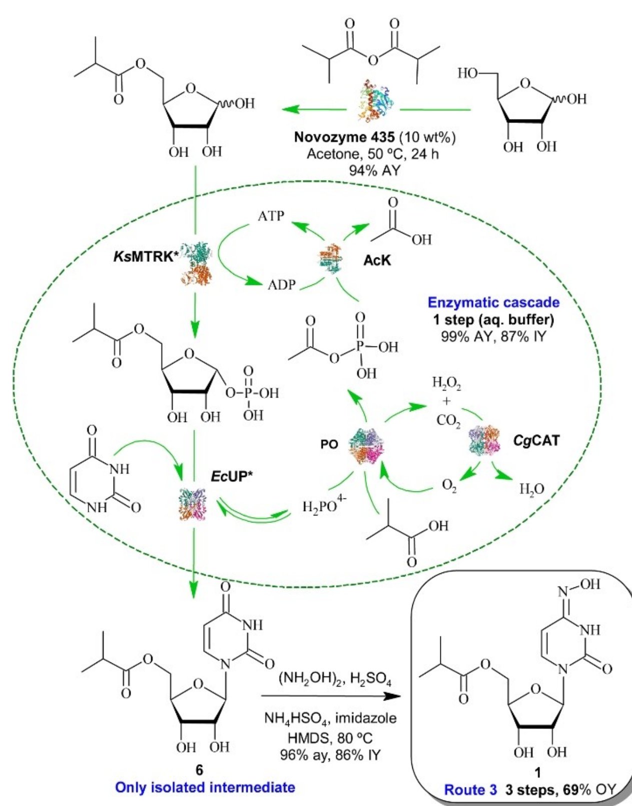
and pH effect, solvent volume, reaction time, catalysts loading, substrates concentration, solubility of substrates and products, and product purification, among other operational features, have been thoroughly studied.[11–12]

On the one hand, Ahlqvist et co-workers developed a supply-centered and chromatography-free chemo-enzymatic synthesis at decagram scale in a 41 % overall yield by following route 2a. Interestingly, they performed an exhaustive solvent screening for the enzymatic acylation to select environmentally preferable alternatives to 1,4-dioxane. Unfortunately, experimental results did not show any practical alternative to the use of 1,4-dioxane. In addition, the study of the effect of solvent volume and enzyme loading on the productivity of the process revealed that an increase in the enzyme amount led to a decrease in substrate conversion.[10] Finally, they deepen into the development of novel conditions for a greener transamination and tested different low-cost recrystallization conditions of intermediates and products from the crude reaction mixture.[11]

On the other hand, Paymode et co-workers focused their work on the development of practical reactions based on route 2b, and also assayed the effect of different purification conditions. As a result, a highly crystalline hydrate of **5** was obtained, which implies an easy purification of this important intermediate (an added value for the industrial implementation). Finally, a tentative scale-up at 10–100 g scale, including several eco-friendly solvents, like water and 2-methyl tetrahydrofuran, highlighted the industrial viability of this approach.[12]

In this context, the development of novel eco-friendly synthetic routes for Molnupiravir manufacturing, which meet the demands for large-scale implementation, is nowadays a hot spot for pharma industries and research groups. As an example of this green tendency, Merck has developed a multi-enzymatic cascade for the synthesis of uridine isobutyryl ester **(6)** (precursor of Molnupiravir) starting from cost-effective and easily available raw materials.[13–14] Considering previously reported constraints, the authors envisioned that an ideal synthesis should start with the acylation of 5-OH group of ribose, but it also should circumvent the challenges of the late-stage acylation step. This synthetic approach involves a well-known lipase-mediated acylation of the primary alcohol, followed by nucleobase coupling. To reach this last goal, the authors proposed an enzymatic cascade (Scheme 3, route 3) which implies: i) the phosphorylation of the 1-OH of the 5-isobutyrylated ribose via *Klebsiella spp.* 5-methylthioribose kinase (*Ks*MTRK, EC 2.7.1.100) to generate the 1-ribosylated intermediate, and ii) the uracil coupling through a transglycosylation catalyzed by an *E. coli* uridine phosphorylase (*Ec*UP, EC 2.4.2.3) (Scheme 3).

Interestingly, the initial activity of both enzymes was significantly improved by directed evolution campaigns, leading to engineered variants with an 80–100 fold increased catalytic efficiency. More interestingly, to overcome the problem of the costly molecules used for the phosphorylation, a phosphate recycling cascade to regenerate ATP and for removing the inorganic phosphate was coupled to the main two-step reaction (Scheme 3). Additionally, the authors also included the use of



Scheme 3. Three-step chemo-enzymatic synthesis of Molnupiravir synthesis. *Ks*MTRK*: evolved *Klebsiella spp.* 5-methylthioribose kinase. *Ec*UP*: evolved *E. coli* uridine phosphorylase. *Cg*CAT: *Corynebacterium glutamicum* catalase (Roche #11650645103). AcK: Acetate kinase (ACK-101, Codexis). PO: pyruvate oxidases from *Aerococcus urinaeequi* (PO3, Codexis), *Streptococcus sanguinis* (PO5, Codexis), or *Weisella confusa* (PO6, Codexis). AY: Assay yield.

hexamethyldisilazane (HMDS) as a solvent for the chemical preparation of the oxime. As a result, a highly efficient and sustainable route for Molnupiravir synthesis (route 3, overall yield 69 %), which starts from simple materials and also avoids tedious and unproductive chromatography purifications, was developed. Even though this methodology offered a more sustainable alternative for Molnupiravir synthesis, it is worth mentioning that, again, the last step for Molnupiravir manufacturing requires the chemical hydroxyamination of **6**, which decreases the greener potential of this catalytic approach. Furthermore, the necessary use of up to six enzymes considerably increases the final production costs and, therefore, the implementation at an industrial scale. Moreover, all the required enzymes are provided by Codexis, and are not currently commercially available to other manufacturers.

Enzymatic synthesis of Molnupiravir

Despite the previous chemical or chemo-enzymatic procedures have been implemented by different pharma companies, the worldwide environmental concern forces industries to develop novel and more eco-friendly processes. As shown below, the main six lab-scale synthetic methodologies for Molnupiravir

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manufacturing are compared in terms of green chemistry, by using the semi-quantitative Eco-Scale approach to greenness evaluation of the different routes of synthesis. Eco-Scale, assumes that an "ideal" system, has a score of 100, which is 100% safe.[15] The higher the value of EcoScale, the more sustainable the process will be.

Considering Eco-Scale calculation (Table 1), it can be concluded that alternative enzyme-mediated approaches to Molnupiravir (2a and 2b) and the chemical route 1c have the highest Eco-Scale values among the other reported methods, with good to excellent yields and operational simplicity. However, all displayed scores are below average (threshold = 50), which is the minimum value acceptable from the point of view of the Eco-Scale algorithm. In table 1 can be observed that the main penalties lie in the excessive use of solvents, their toxicity, corrosive and dangerous nature. In fact, the use of organic solvents accounts for about 80% of all currently reported synthetic routes.

Recently, Burke et co-workers (Manchester University) have paved the way for a total greener synthesis of Molnupiravir by replacing the chemical hydroxyamination with a simple enzyme-mediated transformation of **3** to **5**, catalyzed by an evolved *E. coli* cytidine deaminase (*Ec*CD*, EC 3.5.4.5) (Scheme 4).[16] This work started from the hypothesis that *Ec*CD could use hydroxylamine as the nucleophile for the deamination reaction instead of water (Scheme 4, route 4). Unfortunately, as expected, **5** was further hydrolyzed and converted to **2** by *Ec*CD. To overcome this undesired side-effect the authors developed an engineered cytidine aminotransferase (*Ec*CD*) through the evolutionary adaption of the hydrolytic wild-type *Ec*CD. After three rounds of evolution, they identified a novel variant (*Ec*CD_{T123G}) which considerably improved the *N*-hydroxycytidine **(5)**:uridine **(2)** ratio up to 48 fold (8:1). To assess the potential of *Ec*CD* for scalable manufacturing, the synthesis of **5** was successfully performed at high substrate concentrations ([substrates] = 100 g/l, 90% conversion, 24 h). Even though this brilliant two-step enzymatic strategy offers a promising sustainable alternative for *N*-hydroxycytidine synthesis, the presence of **2** (by-product) in the reaction medium needs to be addressed for a suitable scale-up.

However, it is interesting to point out that Manchester University and Sterling Pharmaceuticals are working on the industrial development of this two-step enzymatic synthesis, including the optimization of biocatalysts recycling. As a major advantage, the use of the engineered catalyst *Ec*CD* is not restricted by intellectual property, and is also available for purchase at a relatively moderate price.

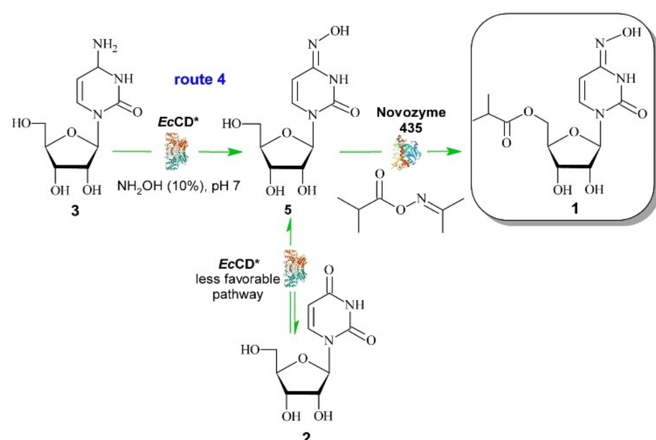
Another possible scenario for the enzymatic synthesis of Molnupiravir can be the use of a transglycosylation reaction to get **5** from a nucleoside donor and *N*-hydroxycytosine. This hypothetical two-step enzymatic synthesis would start from non-expensive natural nucleosides, like cytidine, and could be performed by different enzymes, such as nucleoside hydrolases (NHs, EC numbers 3.2.2.1-3, 3.2.2.7-8, 3.2.2.16),[17] nucleoside phosphorylases (NPs, EC numbers 2.4.2.1-4),[18] nucleoside ribosyltransferases (NRTs, EC number 2.4.2.5),[19] or evolved nucleoside 2′-deoxyribosyltransferases (NDTs, EC number 2.4.2.6) (Scheme 5, route 5).[20] These enzymes display a high transglycosilation capability (especially NPs and NRTs) and high promiscuity in the nucleobase recognition (NHs, NRTs and NDTs), which offer the possibility to use a broad range of purine and pyrimidine nucleosides as starting substrates.

This tentative shortcut for the production of Molnupiravir intermediates (**3** or **4**) was addressed by Merck as an alternative

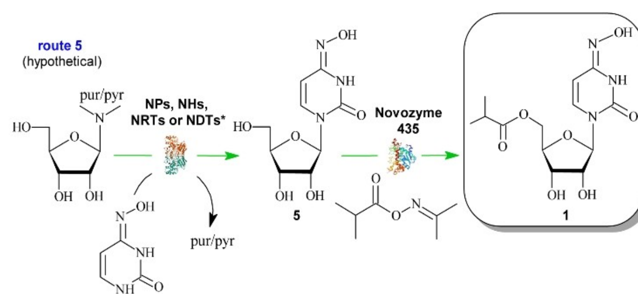
Table 1. Eco-scale calculation for the different synthetic routes (r1-3) for Molnupiravir manufacturing. [a]

Eco-Scale parameter	1a	1b	1c	2a	2b	3
1. yield	−41.5	−19.5	18.5	−29.5	−20	−15.5
2. price of reaction components	0	−5	0	−5	−5	−40
3. safety (hazard warning symbols)	−75	−85	40	−30	−25	−40
4. technical setup	−1	−1	0	0	0	0
5. temperature/time	−5	−3	1	−4	−4	−3
6. workup and purification	−6	−9	11	−4	−4	−9
Eco-Scale score	0	0	29.5	27.5	42	0

[a] Raw data from each scheme are collected from the literature. http://ecoscale.cheminfo.org/calculator.



Scheme 4. Two-step synthesis of Molnupiravir involving *Ec*CD* and Novozyme 435. *Ec*CD*: engineered *E. coli* cytidine aminotransferase.



Scheme 5. Two-step synthesis of Molnupiravir involving a transglycosylation (NPs, NHs, NRTs, NDTs*) and lipase-mediated acylation (Novozyme 435). NPs: nucleoside phosphorylases. NHs: nucleoside hydrolases. NRTs: nucleoside ribosyltransferases. NDTs*: evolved nucleoside 2′-deoxyribosyltransferases.

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to route 3. However, the enzymatic deamination of **5** from *E. coli* native enzymes and the inefficient isolation of **5** from water led them to discard this option.

Regarding the first operational constraint, this undesired drawback can be overcome by employing pure enzymes instead partially purified enzymes. Even though the use of partially purified enzymes is a common industry practice (to decrease production costs), initial raw substrates (nucleosides and nucleobases) can be modified by residual *E. coli* enzymes. In this context, the use of pure enzymes is mandatory. A feasible alternative to get a low-cost downstream protein purification is the use of hyperthermophilic enzymes (that usually display high thermal stability), which allow an easy purification through a heat shock treatment that denatures most of the host proteins of the mesophilic host (*E. coli*).[19,21] Another operational alternative could be the use of magnetic matrixes for the one-step purification-immobilization of his-tagged proteins by immobilized metal affinity chromatography (IMAC) onto magnetic spheres. This strategy would allow not only an easy purification of recombinant proteins from crude extracts, but also an efficient methodology to get a reusable catalyst.[22] Additionally, the second major disadvantage (inefficient isolation of **5**) has been recently addressed by Paymode et co-workers (see above). In this regard, the authors considerably improved the feasibility of the purification process, leading to an easily purified highly crystalline hydrate of **5**.[12]

Similar to the enzymatic hydroxyamination proposed by Burke et co-workers, this methodology would involve the presence of by-products (released nucleobase) as a major drawback. However, this undesired constraint can be used to favor the transglycosylation reaction in the desired way. To this effect, several synthetic strategies have been previously described, such as the irreversible phoshorolysis of 7-methylguanosine iodide to 7-methylguanine[23] or the use of guanosine as nucleoside donor at neutral pH values.[24] In both cases, due to the nature of released nucleobase (7-methylguanine can not be recognized by NPs; guanine precipitates as a result of low water solubility), the equilibrium is shifted away from the reverse reaction. Additionally, both nucleobases are poorly water-soluble and easily separate the reaction, remaining **5** as single species in solution.

Outlook and future perspectives

Despite the major acceleration of biocatalyst development in recent years paved the way for the totally green synthesis of Molnupiravir, some operational issues need to be addressed for an efficient scale-up.

Firstly, an efficient and cost-effective enzyme-mediated alternative to chemical hydroxyamination needs to be developed. Although the enzymatic approach developed by Burke et co-workers (Scheme 4, route 4) or the proposed transglycosylation (Scheme 5, route 5) could be efficient and sustainable alternatives, we can not forget that chemical hydroxyamination is a low-cost and very efficient synthetic procedure, considerably less environmentally-harmful than other chemical procedures. A tentative cost-effective alternative for Molnupiravir manufacturing could be the development of an enzymatic cascade based on routes 4 or 5. The envisioned cascades would allow a one-pot one-step greener synthesis, but also a more efficient and cost-effective process (reversible reactions can be driven to completion, absence of intermediates and therefore a simplification in product purifications, the demand of time, costs, and chemicals for product recovery may be reduced, among other advantages).

However, given that the *in situ* crystallization of *N*-hydroxycytidine allows easy product isolation and downstream, the scenario of this hypothetical multi-enzymatic cascade needs to address two major operational challenges: i) an organic solvent is needed to dissolve the acyl donors used in the enzymatic acylation of *N*-hydroxycytidine; ii) the lipase-mediated acylation needs to be selective for *N*-hydroxycytidine.

Regarding the first operational issue (organic solvent tolerance), since high percentages of organic solvents (80–95% v/v) are mandatory for the acylation step, the different enzymes involved in the cascade must be active and resistant against denaturation caused by organic cosolvents. These conditions do not appear as a major obstacle for lipases,[9,11–14] but can constitute a major challenge for the other biocatalysts. Moreover, the possibility of 5'-OH acylation of both, cytidine (or other starting nucleosides) and *N*-hydroxycytidine, and/or the over-acylation of *N*-hydroxycytidine seems likely (second challenge). Therefore, enzyme discovery and engineering of tailor-made enzymes are needed for this envisioned cascade, by finding, developing, or enhancing different enzyme features (activity, substrate selectivity, or biocatalyst stability) under required operational conditions. The natural diversity represents a wealthy source of extremophilic biocatalysts, which can be mined using metagenomics.[13,19,25] Alternatively, when a starting candidate enzyme is available, protein engineering,[13,16,20] or enzyme immobilization[26] is usually required for tailoring the activity or stability of the abovementioned enzymes. Based on previous findings of the required enzymes, the first goal seems achievable.

Additionally, if the second major challenge (specific acylation) can not be addressed, the sequential cascade may be promoted at the late stage of the first reaction (transglycosylation or hydroxyamination), by adding the acyl donor to the reaction mixtures only when high percentages of *N*-hydroxycytidine are observed.

To summarize, notwithstanding great findings in the search of an "ideal" totally enzymatic synthesis of Molnupiravir, several operational constraints still need to be improved before any large-scale implementation. By harnessing advances in modern biocatalysis, the scientific community has powerful tools for developing novel enzymatic processes with the potential to accelerate production, and therefore the global distribution, of Molnupiravir. Additionally, it is worth mentioning that future biocatalytic approaches should also consider novel strategies based on simple raw materials of unlimited availability that will minimize the time needed for manufacturing and supplying Molnupiravir.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: Molnupiravir · (chemo)enzymatic processes · eco-friendly · industrial scale-up

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