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# Streamlined Chemo-Enzymatic Synthesis of Molnupiravir via Lipase Catalyst

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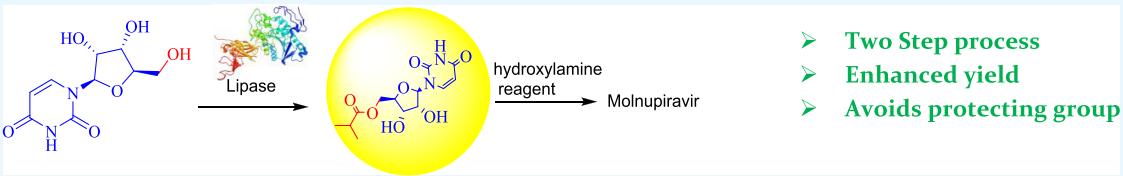


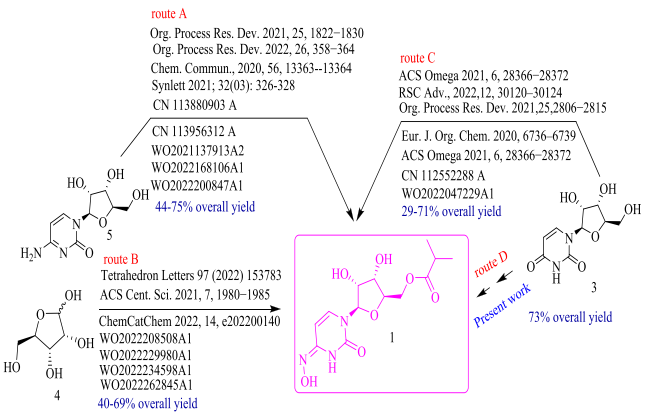

**ABSTRACT:** An enzymatic approach for the synthesis of Molnupiravir has been developed using immobilized lipase as a biocatalyst. This method involves a concise process of the regioselective esterification of uridine with isobutyric anhydride using Lipase (Addzyme-011). This efficient route gets 97% conversion of uridine **3**, with an overall 73% yield of molnupiravir **1** in two steps. The use of inexpensive and easily available lipase makes the synthesis cost-effective and accessible globally, promoting the principles of green chemistry.

## 1. INTRODUCTION

In recent times, active research to develop new antiviral drugs has gained prominence due to emerging viral infections such as MERS, SARS, and COVID-19. To combat these emerging viruses requires the identification and screening of new potential lead molecules to prevent another pandemic in the near future. The immediate development of new drugs and vaccines is critical and time-consuming, due to which repurposing of existing antiviral drugs has gained momentum in drug discovery research. The claudine, *N*4-hydroxycytidine (EIDD-1931), 2′-*C*-methylcytidine, molnupiravir, uprifosbuvir, balapiravir, acalabrutinib, BMS-986094, remdesivir, GS-6620, and ceforanide are being explored for their potential effectiveness against coronavirus and other viruses.[1,2] Molnupiravir is a promising antiviral agent for the current coronavirus and future viral threats. It is an antiviral drug initially developed for influenza treatment and has also shown promising activity against several RNA viruses, including SARS-CoV-2,[3−5] developed by Drive and later acquired by Ridgeback Biotherapeutics comarketed with Merck as MK-4482.[6−8] Numerous scientific groups from academia and the pharmaceutical industry have dedicated significant efforts to develop new synthetic routes for manufacturing Molnupiravir (Scheme1). This has necessitated developing an efficient process to synthesize/produce these drugs.

Regarding the synthesis of Molnupiravir, the next to uridine step consisted of five-step protection of Molnupiravir, the next to uridine step consisted of five-step protection with acetonide, esterification, triazole coupling, hydroxyamine and deprotection (route C),[11] another smoothing multistep reaction process using D-ribose was described here (route B)[10] and

**Scheme 1. Disclosed Routes for Molnupiravir**




in another way the reports used cytidine as a synthon for molnupiravir (route A).[9]

Dey et al., developed a two-step process with novel thionated uridine intermediate using Lawesson's reagent[11a] and Merck and co synthesis of ribose to Isobutyryl uridine engineered ribosyl-1-kinase process involves seven enzymes like, Novozyme 435, 5-*S*-methylthirbose kinase, uridine

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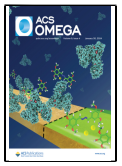





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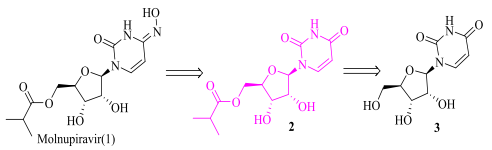
phosphorylase, acetate kinase, pyruvate oxidase, catalase, sucrose phosphorylase, and further required stoichiometric phosphoryl donor adenosine triphosphate and excess sucrose to maintain equilibrium in the reaction, results in the formation of byproduct propionate, enzyme phosphorylases expenses are a significant concern.[10b] Recently Snead and co-workers reported a two-step synthesis of Molnupiravir from cytidine involving direct oxime formation from costly oxime ester and substrate cytidine with 41% overall yield to get the sugar C5−OH using Novozyme 435.[16] The envisioned process offers several advantages such as eliminating the need for a complex and unreliable multistep procedure. Additionally, it avoids the use of a toxic reagent called Lawesson's reagent during intermediate synthesis. This approach provides an alternative strategy to Molnupiravir, as it utilizes uridine instead of the more expensive cytidine. Consequently, our main focus has been on developing an efficient pathway to obtain Molnupiravir (route D) by employing a two-step process that involves the production of a regioselective monoacylated intermediate. Lipase, a highly versatile biocatalyst, has been extensively used for the kinetic resolution of racemic alcohols for the synthesis of enantiopure drugs and drug intermediates.[12] Due to its high chemo-, regio-, and stereoselectivity, which encouraged us toward the synthesis of Molnupiravir, lipase was selected for the biocatalytic regioselective acylation of uridine in this study. The retrosynthetic approach toward the production of Molnupiravir was outlined in (Scheme 2). This strategy involves the

**Scheme 2. Retro-Synthesis of Molnupiravir**




lipase-catalyzed synthesis of **2** as a key step that could be directly converted to **1** in a single step.[10b] The key intermediate compound **2**, in turn, could be easily synthesized selectively by the regioselective acylation of the primary alcohol (Uridine) without any protection for the secondary alcohols using lipase.

## 2. RESULTS AND DISCUSSION

**2.1. Screening of Immobilized Lipases.** The most important step in achieving successful regioselective esterification is the screening of commercially available immobilized lipases. Enzyme immobilization involves improving enzyme stability, increasing enzyme loading per unit volume, facilitating biocatalyst recycling, and simplifying downstream processing. This process heavily depends on the amine functional groups found in various amino acids, which possess diverse characteristics. These amino acids exhibit activity in binding with the appropriate support materials through a range of linkages and interactions. In a similar way, it depends on the support used. Enzymes are immobilized using reversible physical adsorption, cross-linking, entrapment, encapsulation, or covalent bonding.[17,18] Lipases from different sources (SI1.1) were screened for their regioselective esterification reaction of uridine to form uridine monoester in the presence of isobutyric anhydride as the acyl donor. In this case, the intended result is uridine monoester, while the undesirable products are diester and triester.

Among 21 commercial immobilized lipases (SI1.1) screened, Addzyme 011 (TLL) exhibited the best conversion rate of uridine to its uridine monoester ($c$ = 90.89%), followed by Amano (CESL-8) with a conversion rate of $c$ = 88.59%. Although the formation of diester is observed with the selected two enzymes, the percentage of diester formation is minimum compared with other lipase enzymes employed. It is also observed that some enzymes do not carry out any esterification reaction with uridine; this may be related to the binding sites of the enzymes. If the compound does not bind to the catalytic binding sites of the enzymes, then no conversion will be observed. The other enzymes screened and their conversion rates are given in (Table S2).

Thus, to further carryout the synthesis of the final drug intermediate from uridine, Addzyme 011 (TLL) is employed.

**2.2. Effect of Organic Solvents.** The solubility of a compound plays a crucial role in achieving a high yield and conversion rates. It directly influences the conversion rate, thus, affecting the overall yield. To enhance the solubility of the substrate, both polar and nonpolar organic solvents were employed (Table S1). To investigate the impact of organic solvents on uridine conversion, a range of solvents with varying log $P$ values were evaluated. Uridine, being a biological product, exhibits high solubility in polar solvents, such as water and methanol. However, the use of methanol or water was deemed unsuitable due to issues such as hydrolysis, loss of enzyme activity, problems in workup, and purification challenges. In a study by Zieniuk et al., they underscored the significance of substrate solubility in organic solvents for achieving high conversion rates. Their research demonstrated a direct correlation between lipase activity and the presence of organic solvents. This highlights the critical role of solvents in mediating bond interactions between substrates and active sites, subsequently influencing the enzyme activity. Solvents with varying log $P$ values can either hinder or alter the conformation of enzyme active sites, leading to reduced or no activity, or even denaturation and degradation.[13] Through our screening process, it was observed that uridine showed minimal solubility in chloroform and ethyl acetate and was slightly soluble in dichloromethane, acetonitrile, and 1,4-dioxane, although it was sparingly soluble in THF, DMF, and DMSO. Solvents with partial solubility showed the higher formation of diester compounds, while solvents in which uridine did not dissolve resulted in no conversion. The desired product, uridine monoester, was obtained most effectively with THF (96% conversion), which also exhibited minimal formation of the uridine diester. Some organic solvents hindered the conversion of uridine to its mono- or diester forms. Additional data on the conversion rates achieved with other organic solvents are depicted in Table S3. Finally, satisfactory yields were observed when using THF for both the reaction and isolation processes.

**2.3. Equivalence Studies for Acyl Donors.** The equivalence study for isobutyric anhydride was carried out with (4, 6, 8, and 10 equiv) at constant uridine and THF to find out at which equivalence of the lipase enzymes gives the best conversion. It was found that with the increase in the equivalence of the acyl donor, the conversion rate also increases with a reduction in the uridine concentration. The maximum conversion of uridinetouridine monoester was observed at 10 equiv (95.6% conversion) of the isobutyric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anhydride. The formation of the Uridine diester is completely absent in this case (Figure S1).[12a−d]

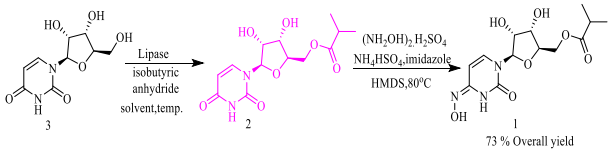
**2.4. Effect of Reaction Time.** The optimization of reaction time was carried out using Addzyme 011 (TLL) and uridine catalyzed transesterification reaction in THF was carried out for 6 days (24, 48, 72, 96, 120, 144 h). The samples were collected in each mentioned time interval and immediately analyzed with HPLC-PDA. The maximum conversion was achieved after 96 h with a conversion rate of 97.16% for uridine monoester, and thereafter, a decline in the conversion rate was observed. The advancement in time also shows that there is a formation of uridine diester but the uridine diester formation was minimum at 96 h with full consumption of uridine. Thus, 96 h was concluded as the optimum time to perform the other studies (Figure S2).[12a−d]

**2.5. Effect of Temperature.** The temperature has a great impact on the catalytic activity of lipase. To study the impact of temperature on the conversion rate of substrate **3** to **2**, Uridine and enzyme at different temperatures (20, 30, 40, and 50 °C) were taken. The conversion efficiency of the reaction was estimated with HPLC-PDA analysis and observed to be optimum after 96 h. It was observed that the conversion rate of uridine monoester increased with an increase in temperature and maximum conversion was obtained at 40 °C with a 94.72% conversion rate (Figure S3). The effect of temperature on the enzyme activity of immobilized enzymes was carried out by Zoltan et al. noted that enzymes are typically sourced from mesophilic microorganisms, which thrive at specific optimal temperatures. It was previously assumed that enzymes would perform best at temperatures close to the microbes' growth conditions. However, their experiments revealed a different outcome: enzymes indeed exhibit peak conversion rates at their optimal temperature, but as the temperature increases gradually, the reaction rate begins to decline. This suggests that temperature may influence the conformational structure of enzymes.[14] In-depth reviews by Chattopadhyay and Mazumdar show that with the rise in temperature, the enzymes start to lose their properties due to a break in the hydrogen bond, which eventually leads to the expansion in the active sites leading to lose binding with the substrate and eventually lose the enzyme activity.[15]

**2.6. Effect of Enzyme Concentration.** Different amounts (10, 20, 40, 80, and 120 mg/mL) of immobilized TLL with a fixed substrate concentration in THF were used to investigate the effect of enzyme concentration on the conversion rate. It was found that, with the increase in the enzyme amount, the conversion rate increased and reached a peak with a conversion efficiency of uridinetouridine monoester observed to be 96.06% at 40 mg/mL and then gradually started forming diester with 80,120 mg/mL lipase (Figure S4).[12a−d]

**2.7. Molnupiravir Synthesis.** The synthesis of molnupiravir was carried out using intermediate 2 which was synthesized at well-optimized conditions with substrate uridine using lipase Addzyme 011 (TLL) and isobutyric anhydride in THF at 40 °C for 96 h, showing excellent regioselectivity at primary alcohol (Scheme 3). Next, the conditions for conversion of 2 to 1 hydroxylamine reagent were used to convert the uracil's amidic carbonyl to an oxime, using cost-effective hexamethyldisilazane (HMDS) as the solvent and mild dehydrating agent. The addition of imidazole catalyzed the reaction by facilitating the formation of TMS-imidazole, a silylation catalyst, enhancing conversion rates.[19−21] Merck and Co reported an enzymatic process using ribose with 69% overall yield.[10b] Hu et al., one-pot chemical synthesis from cytidine involves protection and deprotection steps,[9c] which achieved a 63% overall yield, and Ahlqvist et al. reported a two-step chemo-enzymatic process using Novozyme 435 for acylating using a costly oxime ester and substrate cytidine with 41% overall yield,[16] the main limitation of these methods lies in their expensive substrates and relatively poor yields. The current research, outlined in this paper, aims to overcome these challenges by focusing on the utilization of low-cost uridine and reduction in the number of steps with good overall yields for the synthesis of Molnupiravir.

**Scheme 3. Synthesis of Molnupiravir**




## 3. CONCLUSIONS

The optimization of a cost-effective synthetic route for the synthesis of Molnupiravir through the biocatalysis process was achieved after evaluating the effect of reaction parameters like lipase, solvent, enzyme concentration (lipase), temperatures, number of equivalents of isobutyric anhydride (acyl donor), etc. It was observed that TLL lipase in THF at 40 °C for 96 h, 10 equiv isobutyric anhydride showed excellent regioselectivity at primary alcohol. The desired mono acylated 2 intermediate was isolated in 89% yield and with an overall yield of 73% of 1. In conclusion, we have successfully demonstrated the regioselective acylation of the Molnupiravir intermediate. The use of immobilized enzymes makes this method simple, convenient, and eco-friendly, and it is feasible to execute the current plan on an industrial scale as well.

## 4. EXPERIMENTAL SECTION

**4.1. Analysis.** We conducted enzymatic reactions using a "LABTOP shaking incubator" set at a speed of 200 rpm. For obtaining $^{1}H$ and $^{13}C$ NMR spectra, we utilized instruments with frequencies of 400 and 500 MHz. The chemical shifts (denoted as $\delta$) were measured in parts per million (ppm) by using TMS (tetramethylsilane) as the internal standard (TMS = 0) and CDCl3 (deuterated chloroform) as a reference with a chemical shift of 7.26 ppm. Thin-layer chromatography (TLC) for all reactions was performed on plates prepared by Merck, and we used SRL silica gel with a mesh size of 100−200 for column chromatography. HPLC method was optimized to evaluate the selectively identifying uridine and its related product from the reaction mixture. The enantiomeric excesses (ee) were determined by a waters HPLC system equipped with a 2424 PDA detector using a Chiral IC-3 column (4.6 mm × 250 mm, 3 $\mu$m, Daicel Chemical Industries, Japan) at 260 nm. The HPLC chiral separation was achieved on Chiral Pack IC-3 (4.6 × 250, 3 $\mu$m) with the isocratic mode of separation. The mobile phase consists of hexane and IPA (75:25, v/v) at a flow rate of 1.2 mL/min with a detection wavelength of 260 nm with a run time of 40 min.

**4.2. Reagents.** We obtained various chemicals for our research from different suppliers. Uridine, HMDS, imidazole, hydroxylamine sulfate, and isobutyric anhydride were procured from TCI Chemicals. Addzyme011, Addzyme 017, and Addzyme 022 were acquired from Advance Enzyme Pvt. Ltd.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Other enzymes like L-4447, *Rhizopus niveus*, *Porcine pancreas*, *Mucor miehei*, *Rhizopus oryzae*, *Aspergillus niger*, *Pseudomonas Fluorescence*, *Pseudomonas Cepacia* was sourced from SIGMA, while CES L 1-8 was obtained from Amano Chem. Ltd. We purchased chemicals and solvents necessary for our synthesis and extraction processes from commercial sources such as SIGMA (located in St. Louis, Missouri, USA), TCI Chemicals, and SRL Pvt. Ltd. For our HPLC analysis, we utilized high-performance liquid chromatography (HPLC)-grade solvents that were provided by Finar Limited.

**4.3. Experimental Procedure for the Synthesis of 2.** The process of selectively introducing an acyl group into a specific region was conducted under well-defined conditions. Initially, uridine (10 g, 40.98 mmol) was dissolved in THF (200 mL), followed by the addition of isobutyric anhydride (64 mL, 409 mmol). To this mixture, TLL lipase (3 g) was added, and the reaction mixture was maintained at 40 °C within a shaking incubator for a duration of 96 h. This led to the complete consumption of the substrate, which was tracked using thin-layer chromatography. The reaction mixture was then subjected to filtration, and the solvent was removed by using a rotary vacuum evaporator. The resultant product, which consisted of the desired monoacylated compound, was separated from minor impurities, including the diacylated counterpart, through column chromatography employing 100−200 mesh. This purification procedure yielded a white solid weighing 11.45 g, achieving an 89% yield.

**4.4. Experimental Procedure for the Synthesis of 1.** To the RB flask containing with inert atmosphere, HMDS (45.0 mL, 216 mmol) and imidazole (0.918 g, 13.5 mmol) were charged. The reaction temperature was raised to 80 °C, and. After the complete dissolution of the reaction mixture, ammonium hydrogen sulfate (7.76 g, 68 mmol) was added, and the temperature was adjusted to 75 °C. The mixture was stirred for 30 min, and then, hydroxylamine sulfate (5.53 g, 50.0 mmol) was added. Next, uridine (8.5 g, 27 mmol) was added, and the reaction mixture was stirred at 75−85 °C for 6 h; then, the mixture was transferred to a separatory funnel using 25 mL of water and heptane (2 × 5 mL) to complete the transfer. After the organic phase was washed with (2 × 10 mL) water, formic acid (4 mL, 105 mmol) was added and stirred at 50 °C for 1 h. Water (25 mL) was added with stirring, and the mixture was transferred to a separatory funnel using water (10 mL) and heptane (10 mL). The combined aqueous extracts were basified with ammonium hydroxide (0.8 mL, 48.6 mmol). EtOAc (40 mL) and ammonium sulfate (26.93 g, 203.85 mmol) were added, and the mixture was heated to 50 °C to give two homogeneous phases. The phases were separated at 50 °C, and the aqueous phase was extracted with EtOAc (2 × 45 mL). The resulting layer was refluxed for 15 min and gradually cooled to 60 °C for 30 min, and MTBE (50 mL) was added. The mixture was then cooled to 0 °C for 2 h and filtered, and the filter cake was washed with MTBE (35.0 mL) and dried to get 1 with 7.29g in 82% isolated yield.

**4.5. Statistical Analysis.** The values of analysis were calculated by taking the average of three experiments and were represented as avg ± SD. Statistical analysis was also performed with GraphPad Prism for analysis of variance (ANOVA) with adhoc Dunnet's test. A significant difference was established with respect to the control taken in the experiment with at least $p < 0.05$.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c06872.

Detailed experimental procedures; analytical data; and copies of the $^{1}H$ and $^{13}C$ NMR spectra (PDF)

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### Notes

The authors declare no competing financial interest.

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