A biocatalytic platform for the synthesis of 2′-functionalized nucleoside analogues.

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ABSTRACT: Nucleosides functionalized at the 2′-position play a crucial role in therapeutics, serving as both small molecule drugs and modifications in therapeutic oligonucleotides. However, the synthesis of these molecules often presents significant synthetic challenges. In this study, we present an approach to the synthesis of 2′-functionalized nucleosides based on enzymes from the purine nucleoside salvage pathway. Initially active-site variants of DERA aldolase were generated for the highly stereoselective synthesis of D-ribose-5-phosphate analogs with a broad range of functional groups at the 2-position. Thereafter these 2-modified pentose phosphates were converted into 2′-modified purine analogs by construction of one-pot multi-enzyme cascade reactions, leading to the synthesis of guanosine (2′-OH) and adenosine (2′-OH, 2′-Me, 2′-F) analogues. Our findings demonstrate the capability of these biocatalytic cascades to efficiently generate 2′ functionalized nucleosides, starting from simple starting materials.

Introduction

Nucleoside analogues are an important class of pharmaceutical agent showing activity towards a range of biological targets.¹ For therapeutic applications nucleosides are typically modified at a variety of different positions including the ribose sugar, the phosphate backbone or the nucleobase, depending on the desired pharmacological properties.² Particular interest and synthetically challenging is the 2′-modification of the ribose sugar, being utilized in both nucleoside analogue drugs (Figure 1A), and also nucleoside building blocks of therapeutic oligonucleotides (Figure 1B). Therapeutic oligonucleotides are an important new modality that facilitate a precision medicine approach for patients.³⁴ Modifications to the 2′-position of the nucleoside, e.g. 2′-methoxy, 2′-methoxyethoxy (MOE), and 2′-fluoro are all commonly deployed substitutions in therapeutic oligonucleotides (Figure 1B) which impart both resistance to nucleases and improvements in RNA binding affinity.

The preparation of 2′-modified nucleosides often requires multi-step synthesis, with extensive protection/deprotection sequences or generates complex mixtures of regioisomers, making the synthesis of these molecules challenging. We considered a biocatalytic route to access 2′-modified nucleosides since enzymes are often able to carry out complex transformations in an atom efficient manner, under relatively mild conditions, and without the use of protecting groups. Moreover, since different biocatalysts often function under similar temperature and pH in aqueous solution, they can be combined into multi-enzyme cascades allowing for two or more reactions to be carried out in one pot without the need to isolate intermediates. This approach leads to a reduction in waste from intermediate/product isolation and also allows reactions to be coupled together, thereby overcoming potential thermodynamic barriers of individual reactions.⁵ These advantages have resulted in biocatalysis becoming increasingly deployed in the synthesis of active pharmaceutical ingredients (APIs).⁶

Recent reports have shown that enzymes from the nucleoside salvage pathway⁷ (Figure 1C) provide a useful platform for the generation of functionalized nucleosides. In the retrosynthetic direction, this pathway is responsible for the degradation of 2′-deoxynucleosides. Starting with a nucleoside phosphorylase (NP) the nucleobase is removed resulting in the generation of a C-1 phosphate sugar (Figure 1C). In the following step phosphonotomutase (PPM) catalyses transfer of the phosphate group from the 1′ position to the 5′ position, and finally deoxyribose-5-phosphate aldolase (DERA) breaks down the sugar into acetaldehyde and D-glyceraldehyde-3-phosphate via a retroaldol reaction. As all three enzymes catalyze reversible reactions.

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they can be combined in the synthetic direction to generate nucleosides. An excellent example of this approach is the synthesis of the antiviral nucleoside analogue islatravir (Figure 1D). There are additional examples of enzymes on this pathway being used to generate nucleoside analogues such as dideoxyinosine and molnupiravir. Furthermore, there are also examples of other biocatalysts being employed in the synthesis of nucleosides, nucleotide analogues and oligonucleotides.

Figure 1: A – Examples of 2′-modified nucleoside and nucleotide analogues. B – Examples of common 2′-modifications made to therapeutic oligonucleotides. C - Nucleoside salvage pathway enzymes break down and recycle nucleosides. D – Biocatalytic synthesis of islatravir employing the salvage pathway enzymes. E – This work - proposed synthesis of 2′-functionalized nucleosides using non-natural aldot donors resulting in modifications (R group) being installed at the 2′-position.

Clearly to exploit this pathway for the synthesis of 2′ modified nucleosides, all enzymes in the pathway would need to possess sufficient promiscuity to tolerate a wide range of 2′-modifications. The initial challenge posed by this synthetic cascade is the limited substrate scope of DERA. While PPM and PNP enzymes have been shown to tolerate a small number of 2′-modifications, thus far the approach has been limited to 2′-deoxy nucleosides when starting from DERA, with acetaldehyde as the donor. Engineering DERA would allow the synthesis of 2′-modified D-ribose-5-phosphate analogues starting from D-glyceraldehyde-3-phosphate and an aldehyde donor. The products could then be further modified with the remaining enzymes in the pathway to generate 2′-functionalized nucleosides (Figure 1E).

In general aldolases tend to have a more limited substrate scope for the donor rather than the acceptor, consequently they are often classified based on their donor selectivity with the acetaldehyde-dependent enzyme DERA being a prime example of this. DERA catalyses the reversible aldol condensation of acetaldehyde and D-glyceraldehyde-3-phosphate and is typically only able to accept a small range of donor analogues, such as propanal or glycolaldehyde, often coming at the cost of significantly reduced activity. A recent report demonstrated that screening a diverse panel of DERAs resulted in activity toward six different aldehydes. Recent work has also involved engineering DERA to expand the activity of the wild-type enzyme, resulting in variants able to catalyze Michael addition of nitromethane to α,β-unsaturated ketones.

In this work we demonstrate that mutations in the active site of E. coli DERA result in significantly broadened donor substrate scope allowing for the synthesis of a wide range of 2-modified-5-phosphate sugars. We have used these DERA variants to develop a one-pot synthesis of 2-functionalized α,β-nucleosides using non-natural aldol donors resulting in modifications (R group) being installed at the 2′-position.
pot synthesis of adenosine analogues functionalized at the 2′-position with OH, araOH, Me and F.

**Broadening the Donor Substrate Scope of DERA**

Previous work suggested that the restriction in donor substrate scope of DERA was possibly due to steric constraints in the active site. Moreover, the substrate scope of fructose-6-phosphate aldolase (FSA) was greatly expanded by a “minimalist protein engineering” approach increasing space in the active site. We initially decided to focus on a similar strategy, generating mutations in the active site of DERA with the intention of enlarging the binding pocket for the donor substrate. The previously reported crystal structure of *E. coli* DERA (PDB =1jCL)<sup>16</sup>, which contains the linear aldon product bound to the catalytic lysine residue, was used to identify six active site residues in close proximity to the 2-position of the final product. These residues were mutated to alanine and the resulting variants screened for activity against a broad panel of aldehyde donor substrates (Figure 2).

*Figure 2: A - Assay of DERA variants towards a range of aldehyde donors using DL-G3P as acceptor. Conversion was monitored by HPLC using UV at 220nm after derivatisation with O-benzylhydroxylamine. B - Heatmap showing the average conversions of triplicate repeats for each of the aldolase variants with a range of aldehyde donors generating products (3-14). For FSA the opposite stereochemistry at C2 is generated to the stereochemistry shown.*

Alongside the *Ec*DERA WT and variants we also screened the DERA from *A. chlorophenolicus* (*Ac*DERA<sub>WT</sub>), which was recently shown to have the broadest donor substrate scope of a panel of DERAs<sup>19</sup>, and fructose-6-phosphate aldolase (*Ec*FSA<sub>WT</sub>). The panel of aldehyde donors included halogen (Cl)-substituted, two functional groups found in therapeutic oligonucleotides (OMe and MOE) and two typical protecting groups used in nucleoside chemistry (OBn and OTBDMS). These aldehydes were all screened with DL-glyceraldehyde-3-phosphate (DL-G3P, 1) as the acceptor.

The *Ec*DERA<sub>L20A</sub> and F76A variants showed significant improvement over both *Ec*DERA<sub>WT</sub> and FSA<sub>WT</sub>. For both variants, conversions ranging from 34-91% were obtained towards all substrates screened with the exception of OTBDMS which showed no activity towards any of the enzyme variants. For the majority of substrates *Ec*DERA<sub>F76A</sub> gave higher conversion than *Ec*DERA<sub>L20A</sub>. The double alanine mutation at these two positions *Ec*DERA<sub>L20A/F76A</sub> showed increased conversion with some of the larger aldehyde substrates (MOE, OBn, heptanal) at the cost of decreased conversion with a selection of other functional groups (OH, Cl, OMe).

**Computational Rationalization of Substrate Scope**

To rationalize the increase in activity of the three active variants, models were generated *in silico* and compared to
that of the E. coli wild type DERA. The binding pockets volumes for all four enzymes were analyzed using fpocket\textsuperscript{23} and compared. As predicted, mutation of the selected residues to alanine resulted in an increase in the volume of the active site for all variants. The WT enzyme has a binding pocket volume of 317 Å\textsuperscript{3}, this increases to 416 Å\textsuperscript{3} for L20A, 447 Å\textsuperscript{3} for F76A and 529 Å\textsuperscript{3} for L20A/F76A. These increases in binding pocket size seem to correlate with the trend of activity observed, with only smaller donor molecules like glycolaldehyde and propanal being active with the WT, whereas the variants with significantly increased binding pocket volumes are able to accommodate larger substrates. Particularly bulky substrates such as benzoxy-lacetaldehyde or methoxy(ethoxy)acetaldehyde also show better activity with the larger binding pockets of F76A and L20A/F76A.

To determine whether the expected products are able to fit inside the active site and occupy a reasonable binding mode an example product functionalized with 2-MOE was representatively covalently docked into the three variants using autodock\textsuperscript{29} and compared to WT ligand in the crystal structure (Figure 3). For all three variants the 2-MOE functionalized products were able to occupy a binding mode similar to that of the wild type product (2-deoxy) and in all cases the larger functional group added at the 2-position was able to occupy the newly generated binding pockets. This further supports the hypothesis that an increase in the volume of the active site is responsible for the increase in substrate scope.

Figure 3: The binding pockets for DERA and the three variants. Each of the variants are shown with the 2-MOE substrate covalently docked into the active site. Catalytic residues are shown in yellow, residues targeted for mutation are shown in orange, binding pocket sizes calculated by pocket

are shown for each enzyme. A – Wild Type E. coli crystal structure from 1jCL. B – L20A variant. C – F76A variant. D–L20A/F76A variant.

Synthesis of 2-Modified D-Ribose-5-Phosphates and L-Lyxose-5-Phosphates

Glyceraldehyde-3-phosphate is both expensive and relatively unstable, degrading to methyl glyoxal and free phosphate at neutral pH\textsuperscript{44}. One way to offset these problems is to generate D- or L-glyceraldehyde-3-phosphate from D- or L-glyceraldehyde respectively in situ using a kinase, an approach that has been employed previously when using FSA aldolase \textsuperscript{25,26}. L-G3P (1a) can be generated from L-glyceraldehyde (15) using glycerokinase from Cellumonas sp\textsuperscript{27} (scheme 1A) and D-G3P (1b) can be generated from D-glyceraldehyde (16) using dihydroxyacetone kinase from Citrobacter freundii\textsuperscript{28} (Scheme 1B). These kinase enzymes were therefore combined into two step cascades with the active EcDERA variants.

Scheme 1

For both cascades, enzyme loadings were optimized (supplementary 8.2.1) and the cascades were then screened towards a selection of the substrates from the initial panel (Figure 4). In general a similar pattern of activity for the WT and the two variants was seen compared to the initial screen. However, for both cascades, when starting from enantiomerically pure glyceraldehyde only a single product peak was observed in the HPLC traces (compared to two peaks for DL-G3P) which suggest the formation of a single product diastereomer at the 2-position as desired (supplementary 11.15.1).

Interestingly when comparing the two reactions, the cascade starting from L-glyceraldehyde performed better than with D-glyceraldehyde. One possible explanation for this difference is due to the presence of native E. coli enzymes co-expressed with the EcDERA variants. For example, D-G3P is a substrate for triose phosphate isomerase (TIM), a highly active enzyme that is rate-limited only by diffusion

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of G3P into the active site. Even small quantities of this enzyme present from E. coli remaining after purification are likely to be sufficient to isomerize a significant proportion of D-G3P into dihydroxyacetone phosphate (DHAP) thus lowering conversions (Scheme 3). Despite this side reaction, the D-ribose-5-phosphate analogues were all generated with conversions ranging from 22-66% for all the substrates screened.

![Figure 4: A Synthesis of l-lyxose-5-phosphate analogues via two step biocatalytic cascades using CsGK and EcDERA variants. B Synthesis of d-ribose-5-phosphate analogues via two step biocatalytic cascades using CfDHAK and EcDERA variants. Heatmaps show average conversions for triplicate repeats.](https://doi.org/10.26434/chemrxiv-2023-26t6z)

**Synthesis of 2-Fluoro Pentose-5-Phosphates**

One modification of particular interest for therapeutic oligonucleotides is 2-fluoro, which is used in several currently approved therapeutic oligonucleotides (lumasiran, inclisiran, etc.). Synthesis of the 2-F modified R5P requires fluoroacetaldehyde as the donor substrate. In view of the difficulty of handling aldehyde we decided instead to generate it in situ using an oxidase.

Initial work showed that fluoroacetaldehyde could be generated from fluoroethanol using methanol oxidase from *Pichia pastoris* - PpAO *WT* (supplementary 9.1). This system was then combined with both kinase enzymes, CfDHAK or CsGK, and screened with the EcDERA *WT* alongside the two best performing variants (Scheme 2).

**Scheme 2**

Combining the oxidase, kinase and aldolase enabled the synthesis of fluorinated products 19a and 19b (Scheme 2). In contrast to the previous aldehyde substrates, with fluoroacetaldehyde the WT enzyme gave higher conversion than both the F76A and L20A/F76A variants presumably due to the small size of the fluorine substituent. The HPLC traces for this cascade (supplementary 9.2.2, 9.3.2) contained multiple peaks, suggesting the presence of a
Inhibition of TIM Activity by Addition of phosphoenolpyruvate

As highlighted above, the isomerization of D-G3P 1a to DHAP 20 catalyzed by TIM presented a problem for the the cascade to generate D-ribose-5-phosphate analogues. (Scheme 3). Phosphoenolpyruvate (PEP) has been previously shown to inhibit TIM\(^a\) and can also be used as a phosphate donor substrate for pyruvate kinase in order to implement an ATP recycling system. We reasoned that addition of phosphoenolpyruvate to the cascade could serve to both recycle ATP for the kinase step as well as increase overall conversion to product by inhibiting the formation of DHAP via TIM.

Scheme 3: Isomerization of D-G3P (1a) into DHAP (19) via TIM reduces conversion.

Reactions were screened using the F76A variant, both with and without the PEP/PK recycling system present (Figure 5). A 2-fold excess of PEP was added to ensure efficient inhibition of TIM throughout the reaction while still enabling recycling of ATP. Addition of the PEP/PK recycling system resulted in an increase in conversion for all substrates screened, more closely matching conversions seen for the l cascade. In addition to the increase in conversion to product, the amount of DHAP side product also decreased (supplementary 10.4).

Addition of the PEP/PK recycling system enables not only the use of catalytic amounts of ATP but also significantly reduces the effects of TIM on the cascade, enabling the generation of D-R5P analogues in higher conversions.

Semi-Preparative Scale Synthesis of D-Ribose-5-Phosphate and L-Lyxose-5-Phosphate Analogaues

Following the development of successful analytical scale reactions a number of key substrates were then carried through on a semi-preparative scale using EcDERA\(^{19a}\) with a substrate loading of 20 mM glyceraldehyde.

For the synthesis of L-lyxose-5-phosphates 11a-13a (Scheme 4A) the ATP recycling system was omitted since the PEP based inhibition of TIM was not needed for L-G3P. Reactions were instead carried out at 20 mM substrate loading with stoichiometric amounts of ATP. The initial substrate chosen to analyse was the 2-OMe, due to its diagnostic singlet peak in \(^1\)H NMR. Conditions for the scale-up reaction of L-lyxose-5-phosphates were optimized via design of experiment (DOE) (supplementary 11.2.1). The most significant factors were found to be both aldolase and donor substrate concentrations.

For the synthesis of D-ribose-5-phosphates 11b,12b (Scheme 4B) reactions were again carried out at 20 mM substrate loading with the PEP/PK recycling system. The reaction conditions were again optimized by DOE, (supplementary 11.2.2) For all reactions of both cascades products were purified by anion exchange chromatography.
For the synthesis of L-lyxose-5-phosphate analogues, 2-OMe and MOE products 11a and 12a were generated in good conversions (62%, 61% respectively) and isolated in reasonable yields (28%, 39% respectively). For the benzylated product 13a, conversion of 53% was obtained alongside an isolated yield of 25%.

For the D-cascade, 2-OMe and 2-MOE products 11b and 12b were obtained in high analytical yields (74%, 62% respectively) and good isolated yields (46%, 47%). Interestingly for 11b, the scale up analytical yield was higher than any of the previous optimization reactions carried out in 200 µL volumes. When compared to the L-lyxose-5-phosphate products, D-ribose-5-phosphate analogues contained a small amount (<10%) of pyruvate present in the purified sample, which is a by-product of the pyruvate kinase recycling system.

Preparative scale syntheses of the 2-F products were also carried out (Scheme 4C/D). For these reactions several alterations had to be made to the reaction conditions. To overcome issues with oxygen limitation posed by the oxidase, the concentration of substrate was decreased to 10 mM and the reaction was carried out as a series of smaller volume biotransformations (10 x 500 µL). For 2-F-L-lyxose-5-phosphate 19a a conversion of 62% was obtained whereas the 2-F-D-ribose-5-phosphate 19b was generated with a conversion of 46%.

Mass spectrometry (ESI-) analysis confirmed the presence of desired [M-H] ions in both products. While HPLC analysis seemed to show a mixture of diastereomers in a ratio of 80:20 for 2-F-L-lyxose-5-phosphate and 50:50 for 2-F-D-ribose-5-phosphate, the 1H NMR spectra showed a more complex mixture of products. The fluorine NMR analysis showed the expected four diastereomers (supplementary 11.3.4, 11.4.4), alongside multiple unknown fluorine peaks. These extra peaks correspond to diastereomers at the 3’ position or small amounts of fluorinated side products that were not eliminated during purification.

The stereochemistry of the isolated products was determined by comparison to previous work and by the use of 1H-1H NOESY NMR (supplementary 11.5.2). All products were assigned the 2R, 3R stereochemistry, in agreement with previous work. 19

**Biocatalytic Synthesis of 2’-Functionalised Adenosine and Guanosine Analogues**

Finally, to demonstrate the application of this cascade to generate nucleosides functionalized at the 2’-position the aldolase step was combined with phosphopentomutase (EcPPMWT) and purine nucleoside phosphorylase (EcPNPWT) to synthesize guanosine and adenosine analogues in a one pot reaction (Scheme 5).

Initial studies showed that the five enzyme cascade (Scheme 5A) was able to generate the ribonucleoside adenosine 25 in good conversions (Table 1). Furthermore as C/DHAKWT is enantiospecific, D-glyceraldehyde can also be substituted with 2 equivalents of DL-glyceraldehyde (Scheme 5E). While the 2′-substrate scope of wild-type PPM and PNP is likely to be restricted we found that in addition to 2′-OH both 2′-Me 27 and 2′-F 28 substitutions were accepted, by both enzymes, albeit with lower conversions (Table 1). Alongside this, a 10x excess of the D-glyceraldehyde starting material was required to give reasonable conversions. This is presumably due to the lower activity of the PPM and PNP steps, alongside the instability of the phosphorylated intermediates, particularly the corresponding γ phosphate. The higher activity of the cascade towards the wild-type substrate 2′-OH suggests that, in principle, this excess would not be required for 2′-Me and 2′-F analogues if more active variants of PPM and PNP were obtained.

For the 2′-Me adenosine 27 changing the aldolase from EcDERAWT to EcDERA32FSA gave an increase in conversion from 17% to 38%. DOE was used to determine the effects of enzyme loading on conversion for the three main enzymes (DERA, PPM, PNP) (supplementary 13). This revealed that the concentration of PNP was by far the most limiting of the three enzymes. By increasing the concentration of PNP in these reactions an increase in conversions for both 2′-Me (38% to 65%) and 2′-F (6% to 20%) was observed (Table 1). Addition of a recently published PNP variant which was engineered to give a small increase in activity to 2′-F adenosine33 resulted in a further increase in conversion to 28% (Table 1). The lower conversions seen for the fluorinated analogues are in part due to a large proportion of this product being converted to 2′-F-inosine (presumably by contaminant adenosine deaminase) this issue is compounded by the relatively large amounts of protein required and highlights the need for further engineering, particularly of the PNP enzyme. In addition to 2′-OH adenosine 2′-OH guanine was synthesized using the optimal conditions from the adenosine reaction (Table 1). While some product was generated, the conversion for the synthesis of guanosine is notably lower than for adenosine, this is likely due in part to its poorer solubility under the reaction conditions.

As the stereochemistry of the 2′-position is set by the aldolase used, changing to a different aldolase allows for alteration of this stereochemistry. Thus replacement of DERA by EcFSAWT resulted in the synthesis of the nucleoside analogue vidarabine (2′-ara-OH adenosine, 29) (Table 1). Vidarabine was generated in a diastereomeric ratio of 90:10, vidarabine:adenosine.
Finally, to characterize the 2'-OH, 2'-araOH, 2'-Me and 2'-F adenosine analogues by 1H NMR 5 ml scale reactions were carried out and the products were purified by semi-preparative HPLC. For adenosine the 1H NMR spectra obtained was identical to the commercial standard. For the 2'-OH and 2'-Me analogues, the 1H NMR spectra confirmed the presence of a single diastereomer at the 2' position. For vidarabine, the 1H NMR spectrum was identical to the commercial standard. For 2'-F adenosine 28, despite the aldolase step generating an epimeric mixture of 2'-F diastereomers, the 2'-F adenosine product was synthesized in a diastereomeric ratio of 98:2 favouring the desired “down” stereochemistry of the fluorine. These isomers were unable to be separated by semi-prep HPLC and were isolated together. This observation suggests that while the aldolase exhibits poor stereoselectivity, one or both of the final two enzymes in the cascade are stereoselective for the desired fluorine diastereomer. Alongside the desired 2'-F adenosine we were also able to isolate the 2'-F inosine side product (supplementary 14,5), thereby demonstrating the potential to generate 2'-functionalized inosine analogues by addition of a deaminase enzyme to the cascade.

Conclusions

By targeting the active site of EcDERAT, simple mutations (F76A, L20A) have been shown to significantly increase donor substrate scope and allow for the synthesis of a broad range of d-pentose-5-phosphate analogues functionalized at the 2-position. Addition of a kinase to generate glyceraldehyde-3-phosphate in situ resulted in the syn-

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Table 1. Biocatalytic synthesis of 2'-functionalized nucleoside via one pot cascades

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<tr>
<th>Entry</th>
<th>2'-Modification</th>
<th>Nucleobase</th>
<th>Sugar Equivalents</th>
<th>Aldolase loading (variant)</th>
<th>PPM Loading (mg/ml)</th>
<th>PNP Loading (mg/ml)</th>
<th>Conversion (%)</th>
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a: sugar equivalents as determined by the equivalents of D-Glyceraldehyde relative to nucleobase
b: reaction carried out with 2 mM DL-Glyceraldehyde as opposed to 1 mM D-Glyceraldehyde, conversion reported with respect to limiting nucleobase.
c: reaction repeated with best performing PNP variant from recently published paper<sup>31</sup>
thesis of 2-functionalised D-ribose-5-phosphate and L-lyxo-sose-5-phosphate analogues via a two-step cascade. Furthermore, addition of an alcohol oxidase led to the formation of fluoroacetaldehyde \textit{in situ} and subsequent generation of 2-F analogues via a three step one pot reaction. To characterize the various pentose-5-phosphate products semi-preparative biotransformations were carried out. This resulted in the synthesis of 2-OMe, MOE, OBn and F analogues in good conversions and isolated yields. With the exception of fluorine, all products were generated as single diastereomers at 2- highlighting the excellent stereo-selectivity of DERA.

To demonstrate the utility of the purine nucleoside salvage pathway enzymes for synthesizing nucleosides functionalized at the 2′-position the EcDERA\textsubscript{F76A} variant was combined with EcPPM\textsubscript{WT} and EcPNP\textsubscript{WT}. This allowed the synthesis of the ribonucleosides adenosine and guanine as well as 2′-Me and 2′-F adenosine analogues. These nucleo-side analogues can be generated in a one pot reaction starting from relatively simple starting materials. By changing the aldolase used from EcDERA\textsubscript{F76A} to EcFSA\textsubscript{WT}; stereoch- emical control can be imparted at the 2′-position allowing for the synthesis of the nucleoside analogue vidarabine.

While PPM and PNP have been shown to tolerate a small range of 2′-functionalization such as 2′-NH\textsubscript{2}, araOH and 2′-F, cascades utilizing DERA have thus far been limited to the synthesis of 2′-deoxy nucleosides. This work shows that a much broader range of 2′-functionalization is possible via engineering of the aldolase. We consider this to be the first step towards the repurposing of this pathway towards generating a much broader range of nucleoside analogues. Future work engineering PPM and PNP for increased substrate scope at the 2′-position will hopefully increase the range of nucleosides able to be generated via this approach.

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**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>AO</td>
<td>Alcohol Oxidase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>DERA</td>
<td>2-deoxy-d-ribose-5-phosphate aldolase</td>
</tr>
<tr>
<td>DHAK</td>
<td>Dihydroxyacetone kinase</td>
</tr>
<tr>
<td>DOE</td>
<td>Design of Experiment</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray Ionisation</td>
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<tr>
<td>FSA</td>
<td>Fructose-6-Phosphate aldolase</td>
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<tr>
<td>G3P</td>
<td>Glyceraldehyde-3-phosphate</td>
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<tr>
<td>GK</td>
<td>Glycerokinase</td>
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</tbody>
</table>

**REFERENCES**


**HPLC** = High Performance Liquid Chromatography

**MOE** = Methoxy(ethoxy)

**NMR** = Nuclear Magnetic Resonance

**PPM** = Phosphopentomutase

**PNP** = Purine Nucleoside Phosphorylase

**R5P** = Ribose-5-Phosphate

**TIM** = Triosephosphate Isomerase

**WT** = Wild Type


