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Synthesis of Nucleotide Analogues by a Promiscuous Phosphoribosyltransferase

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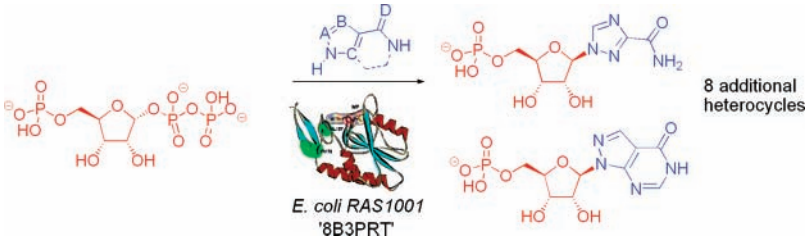
ABSTRACT



An *Escherichia coli* strain overexpressing a mutant variant of a phosphoribosyl transferase was developed as a catalyst for the efficient preparation of a range of purine nucleotide analogues. This system offers an efficient and rapid method for nucleotide analogue synthesis with 100% *β*-selectivity, providing analytically pure product in a single purification step.

Nucleoside analogues are an important class of pharmaceuticals encompassing antiviral, antiparasitic, and anticancer agents.[1−3] In particular, nucleoside analogues remain components of the frontline defenses against some of the most serious viral threats to human health including AIDS (HIV),[4] hepatitis B (HBV),[5] herpes (HSV),[1] and SARS.[6] Most nucleoside analogues are prodrugs for nucleotides, generated by intracellular phosphorylation of the 5′-hydroxyl group to the corresponding mono-, di-, or triphosphates. The pharmacological study and design of new agents are dependent upon the availability of synthetic nucleotides, which enable investigations into structure−activity relationships, kinetics, and other metabolic consequences of these important pharmaceuticals.[7−9]

Nucleotide analogues are generally synthetically prepared via 5′-phosphorylation of their nucleoside congeners, and multiple protective group manipulations are often required to control the regiochemistry of ribose phosphorylation. Nucleoside analogue precursors are themselves the products of multistep pathways, and nucleotide synthetic strategies must address a number of potential challenges including selective activation of the anomeric position for nucleobase addition, control of anomeric stereochemistry (or resolution of diasteromeric products), and ultimate purification of the highly polar water soluble compounds. Intermediates in these pathways are often unstable, particularly the anomerically activated ribosides. As a result, a variety of individualized multistep solutions have been developed for nucleotide analogue syntheses.

Biological systems synthesize purine nucleotides by one of two general strategies: either de novo, via multistep nucleobase elaboration of 5-phospho-D-ribosylamine, or via a base "salvage" strategy. The de novo pathways represent unique linear solutions to the biosynthesis of the various

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natural purines. However, the salvage pathways share the common approach of catalyzing the addition of nucleobases to anomerically activated ribose. Two enzyme classes, purine nucleoside phosphorylase (PNP) and phosphoribosyltransferases (PRTs), catalyze the addition of structurally diverse nucleobases to ribose activated as ribose-1-phophate or 5-phosphoribosyl 1-pyrophosphate, respectively.[10−12] The mechanistic commonality inherent in the salvage solutions to a wide variety of nucleotide biosyntheses has prompted us to investigate the PRT systems for their utility in biocatalytic generation of synthetic nucleotide analogues.

Laboratory-scale biocatalytic generation of nucleotide analogues suffers from potential practical drawbacks including the specificity of the enzymatic systems involved, difficulty in obtaining purified enzymes, and the separation of polar reaction products from polar starting materials and byproducts. To address these concerns, we have developed a biocatalytic system for the synthesis of a range of nucleotide analogues. Herein, we present a one-step biocatalytic process for the synthesis of several nucleotide analogues from commercially available starting materials. *E. coli* whole cell crude extract is used as the biocatalyst, and nucleotides are purified in a single step using anion-exchange chromatography.

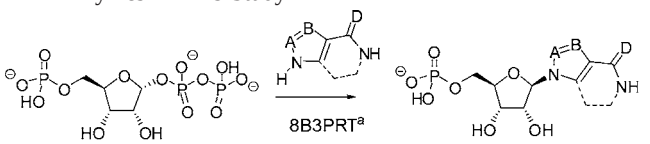
We have recently generated a mutant variant of *E. coli* hypoxanthine phosphoribosyltransferase (HPRT) as a product of a directed evolution study in which an error-prone PCR library of the *hpt* gene in a protein expression host was selected for improved in vivo transformation of triazole carboxamide **13** to ribavirin monophosphate (the nucleotide and peptide sequence is reported in the Supporting Information). To further investigate the synthetic utility of this mutant, designated 8B3PRT (V157A, Y173H), we examined the ability of this enzyme to utilize a structurally diverse range of commercially available purine and purine base analogues (Table 1).

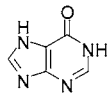
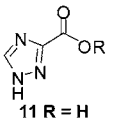
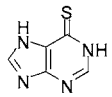
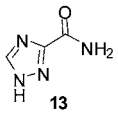
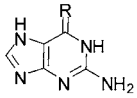
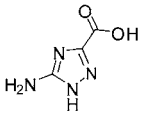
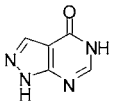
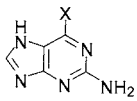
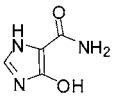
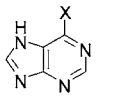
To facilitate the use of 8B3PRT as a practical catalyst, we desired to obviate the need for enzyme isolation and developed our system using a simple cell-free preparation. *E. coli* BL21(DE3) cells harboring plasmid pRAS1001, a pET28a-based plasmid-containing the mutant *hpt* gene, were induced at OD = 0.6 with IPTG and incubated for 3 h. Two milliliter aliquots of cells were pelleted by centrifugation and treated with a commercial detergent (BugBuster, Novagen, Inc.), each providing enough catalyst for 30 mg scale reactions. The cell preparation was added to buffered solutions of phosphoribosyl pyrophosphate (PRPP) and purine base analogues to catalyze the formation of nucleotides in less than 2 h at 37 °C.

Previously reported assays for PRT activity are dependent upon coupled enzyme assays detecting the turnover of natural nucleobases such as hypoxanthine or adenosine. Thus, the assay for nonnatural base analogue turnover required the development of a general in vitro assay for PRPP turnover. For this purpose, a molybdate-based assay for the detection of phosphate was modified to detect for the formation of pyrophosphate by adding an excess of inorganic pyrophosphatase to the enzymatic reactions (Figure 1). In addition to observing efficient turnover for triazole carboxamide **13**, we observed enhanced turnover for a wide variety of purine base analogues. As pyrophosphate turnover is an indirect indication of product formation, percent conversion was additionally determined via ^{1}H NMR, assaying for the disappearance of PRPP and formation of product (Table 1). For this purpose, 10% D_2O was added to reactions, and a W5-WATERGATE water suppression pulse sequence was employed to eliminate the water resonance[13] and accurately measure the ratio of the anomeric protons (H1′). Often, conversion of PRPP was nearly quantitative, and since PRPP is a highly unstable technical grade compound containing contaminating inorganic phosphates and degradation products, we used percent conversion to determine reaction efficiency in lieu of percent yield.

Purine phosphoribosyltransferase mutant 8B3PRT demonstrated enhanced activity and relaxed specificity in processing a wide variety of nucleoside base analogues. In the case of ribavirin monophosphate formation from triazole

Table 1. Base Analogues Assayed for Percent Conversion by PRT Enzymes in This Study



entry	conversion (%)	entry	conversion (%)	entry	conversion (%)
 **1**	87	 **7**	0	 **11 R = H** **12 R = Me**	N.D. N.D.
 **2**	72	 **8**	0	 **13**	68
 **3 R = O** **4 R = S**	N.D. 96	 **9**	N.D.	 **14**	90
 **5 X = Cl** **6 X = Br**	38 66	 **10**	N.D.	 **15 X = Cl** **16 X = Br**	60 34

[a] PRT extracts (6 μL) were added to an aqueous solution of 1 mM PRPP, 30 μL of base analogue (3−25 mM), 12 mM Tris pH 7.3, and 12 mM $MgCl_2$ to a final reaction volume of 600 μL. Percent conversion measured by NMR. N.D. = not determined.

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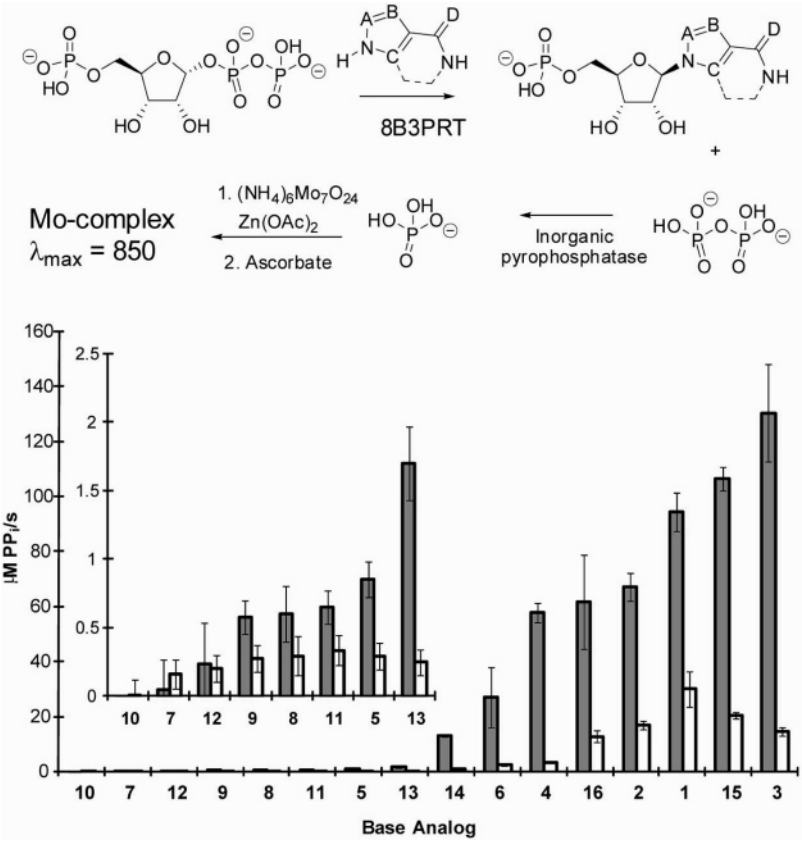

Figure 1. Colorimetric assay for pyrophosphate turnover (above) and measured turnover rates measurement for 8B3PRT (grey) versus wild type HPRT (white). Error bars represent std. dev. of triplicate measurements.

carboxamide **13**, an 8-fold improvement of turnover versus wild type HPRT was observed and for thioguanine **4** the improvement is almost 17-fold (Figure 1). The enhanced activity is likely due to relaxed specificity for base binding given that protein expression of 8B3PRT, as measured by INVISION (InVitrogen Inc.) 6xHis staining of soluble cell extracts, was only 2.8-fold that of wild-type HPRT. The substitution of alanine for valine in V157A, the backbone carbonyl of which is implicated in hydrogen bonding of the N1 proton in hypoxanthine **1**, likely accommodates more favorable substrate interactions. The effect of substitution in the active site can be rationalized by comparison to the structure of *E. coli* HPRT[12] (Figure 2).

Substrates demonstrating significant activity with 8B3PRT possess an electronegative atom in the 6-position on the purine base. This is consistent with previous studies[12] proposing that lysine residue K135 facilitates substrate binding through hydrogen bonding with the C6 carbonyl of the natural substrate hypoxanthine **1** in addition to the 6-substituted purine analogues. Likewise, this is consistent with the absence of substantial activity for bases such as adenine **7** and benzimidazole **8**. Aspartate residue D107 has been postulated as the essential general base for deprotonation of N7 of the respective hypoxanthine tautomer.[12] This is illustrated by favorable turnover observed for substrates possessing a hydrogen bond donor at positions N7 in addition to N9 in the azole ring. Both imidazole and triazole heterocylces are accepted as substrates, which both provide a proton at N7. However, this does not appear to be a strict requirement for biocatalysis, given the observed turnover of the pyrazole system of allopurinol **14**. Substitution in ring positions C2 and N3 are optional in substrates, as evident in

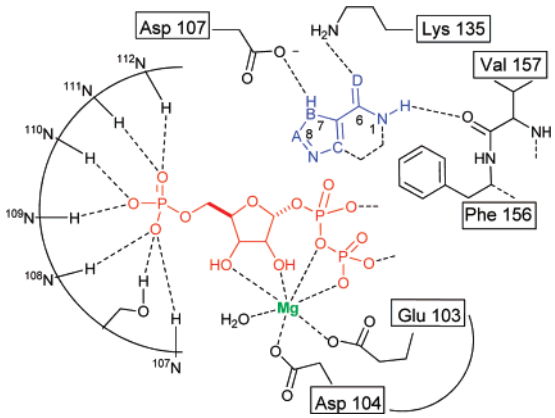

Figure 2. Proposed 8B3PRT model for binding purine analogues based on the X-ray crystal structure of *E. coli* HPRT (PDB entry 1G9S). Enzyme 8B3PRT is a V157A, Y173H double mutant.

catalysis of the triazole carboxamide base **13**. Last, it may be noted that, as with hypoxanthine **1**, all of the substrates were capable of providing a degree of π-stacking interactions with phenylalanine F156.[12]

As nucleotides are not easily resolved under standard reversed-phase HPLC conditions, the nucleotide analogues synthesized in this study were purified by a rapid anion exchange method. The entire reaction mixtures (1 mL) were applied to a quaternary amine column (Mono Q, GE Biosciences), and eluted with an increasing concentration gradient of triethyl ammonium carbonate or triethyl ammonium formate. These volatile buffers can be largely removed in vaccuo subsequent to pH neutralization. Purified nucleotide monophosphates were confirmed by ^{1}H NMR, ^{13}C NMR, and mass spectrometry (see the Supporting Information). The enzymatically catalyzed reactions were completely β-selective, as evidenced by the formation of a single anomeric product by NMR and comparison of coupling constants and chemical shifts of biocatalytically generated nucleotides to known nucleotides.[14,15]

Of note, several of the nucleobase analogues were converted to activated nucleotides with significant clinical relevance including ribavirin monophosphate, allopurinol monophosphate, and mercaptopurine monophosphate.[16−18] Some of these compounds have been previously synthesized from the nucleosides by chemical 5′-phosphorylation, entailing multiple protective group addition and removal steps. Furthermore, the nucleosides themselves are synthesized by multiple steps. For instance the most concise synthesis of ribavirin we could identify was via 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose[19] in three steps in 32% overall yield subsequent to 5′-phosphorylation.[20] Similarly, for chloropurine monphophate,[21,22] thioguanine monophosphate,[23,22] and mercaptopurine monophosphate,[24,22] yields of 54%, 31%, and 64% have been reported. However, the 8B3PRT biocatalytic method described herein forms the anomeric linkage and phosphorylation in a single step from commercially available starting materials in comparatively high yields (Table 1). Since the nucleosides used for phosphorylation reactions entail lengthy multistep syntheses,[19,25] biocatalytic generation of nucleotides using 8B3PRT represents a substantial improvement in efficiency. Additionally, the total syntheses of 6-bromopurine monophosphate and allopurinol monophosphate have not yet been reported.

To the best of our knowledge, this study represents the first application of a promiscuous HPRT for the synthetic preparation of nucleotides. The 8B3PRT system catalyzes the β-selective synthesis of purine nucleotide analogues which can be purified rapidly in a single step. While whole cell catalyzed reactions can potentially be complicated by cell transport and/or toxicity of substrates or products to the cell, this method obviates those problems by using a crude cell-free preparation.

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Supporting Information Available: Methods and spectroscopic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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