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Inhibition of Guanosine Monophosphate Synthetase by the Substrate Enantiomer L-XMP

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Studies with mirror-image L-enantiomer nucleosides and nucleotides have revealed relaxed enantioselectivities of several cellular kinases and viral polymerases.[1,2] This feature of enzyme–ligand molecular recognition has been exploited in the design of efficacious antiviral L-nucleoside drugs, which have lowered host-cell toxicity.[3–6] For example, lamivudine (2′,3′-dideoxy-3′-thiacytidine, 3TC), an L-nucleoside drug, exploits the relaxed enantioselectivity of human immunodeficiency virus (HIV) reverse transcriptase to inhibit viral replication.[7] Conversely, the enantioselectivities of the majority of nucleotide biosynthesis enzymes have not been characterized. The depletion of cellular nucleotide pools has been shown to result in antiproliferative, antibacterial, and immunosuppressive effects.[8–11]

Guanosine monophosphate synthetase (GMPS), an enzyme involved in de novo nucleotide biosynthesis, catalyzes the amination of xanthosine 5′-monophosphate (XMP) to guanosine 5′-monophosphate (GMP) in the presence of glutamine (the amine source) and ATP.[10,12] GMPS possesses two active sites that are separated by approximately 30 Å, thus suggesting that GMPS undergoes a significant conformational change during catalysis.[13,14] In the amidotransferase active site, a glutamine residue is hydrolyzed to liberate ammonia, which subsequently functions as the nucleophile in the amination of XMP [Eq. (1)]. In the synthetase active site, the 2-carbonyl of XMP is adenylated with ATP [Eq. (2)] to activate the aromatic ring for subsequent aminolysis [Eq. (3)]. Formation of adenyl-XMP is believed to trigger glutamine hydrolysis in the amidotransferase active site.[10,12–16]

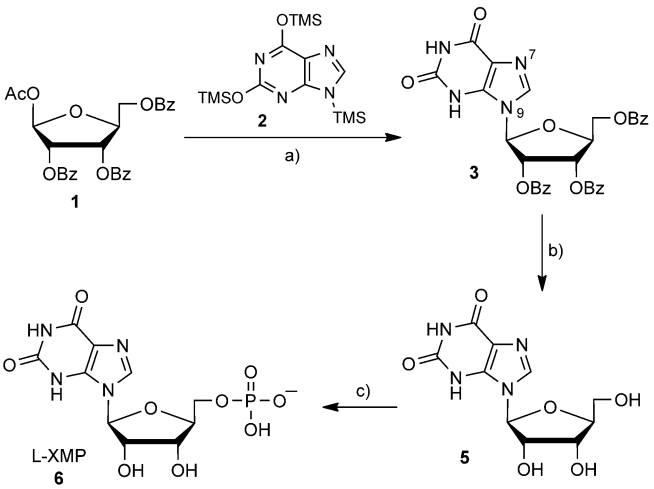
$$\text{glutamine} + H_2O \xrightarrow{\text{GMPS}} \text{glutamate} + NH_3 \quad (1)$$

$$XMP + ATP \rightleftharpoons \text{adenyl} - XMP + PPi \quad (2)$$

$$\text{adenyl} - XMP + NH_3 \xrightarrow{\text{GMPS}} GMP + AMP \quad (3)$$

A crystal structure of *Escherichia coli* GMPS has been solved that reveals a large solvent-accessible synthetase pocket with considerable surface area.[13] Several base-modified D-XMP analogues have been shown to function as substrates for GMPS and be converted to their amine derivatives,[17] and non-hydrolyzable adenyl-XMP analogues have been synthesized.[18] Based on this structural information and our interest in characterizing for the first time the enantioselectivity of GMPS, we hypothesized that L-XMP, the enantiomer of native ligand D-XMP, could target GMPS and modulate enzymatic activity. We hypothesized that L-XMP could incorporate into the synthetase active site and inhibit enzyme function, or less likely, L-XMP could function as a substrate for GMPS and undergo aminolysis to yield L-GMP. In either case, enzymatic synthesis of D-GMP would be affected, either by direct enzyme inhibition or by the activity of a suicide substrate. Given the central importance of GMPS in eukaryote and prokaryote biochemistry, we examined the enantioselectivity of the enzyme.

Preceding this work, a synthesis of L-XMP (**6**), the enantiomer of natural ligand D-XMP, had not been reported. Our synthesis of L-XMP (**6**) started from L-arabinose, which was elaborated to 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-L-ribofuranoside (**1**) by reported methods (Scheme 1).[19] A Vorbrüggen coupling with



Scheme 1. Synthesis of L-XMP. *Reagents and conditions:* a) TMSOTf, CH_2Cl_2, reflux, 70% (for **3**), 21% (for N^7-isomer **4**, not shown); b) NH_3, MeOH, 55 °C (sealed tube), 93%; c) $POCl_3$, $PO(OMe)_3$, proton sponge; aq TEAB, 19%.

trimethylsilyl-protected xanthine **2** gave a separable mixture of protected L-xanthosine isomers **3** (N^9 isomer) and **4** (N^7 isomer, not shown).[20,21] Deprotection of the benzoyl protecting groups of **3** by using ammonia afforded L-xanthosine (**5**). Selective phosphorylation of the 5′-OH of **5** utilizing phosphorous oxychloride gave L-XMP (**6**).[22]

E. coli GMPS was overexpressed and purified (Figure S1) and an HPLC-based assay was developed to quantitate enzymatic reaction products. GMPS was incubated with test substrates and ammonium acetate (ammonia source), and the reaction was terminated at various time points by addition of ethylenediaminetetraacetic acid (EDTA). GMPS protein was then removed by a molecular weight spin column (30 kDa), and enzymatic reaction products were analyzed by reversed-phase

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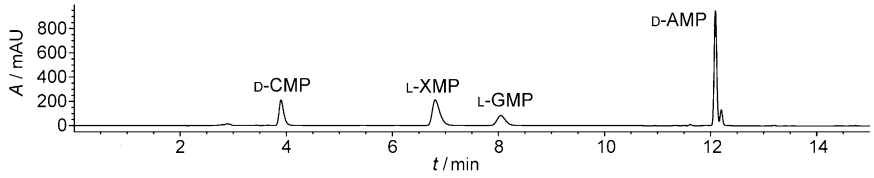

Figure 1. HPLC analysis of L-XMP conversion to L-GMP by GMPS.

HPLC. Surprisingly, we found that incubation of L-XMP and GMPS yielded a new peak of identical retention time as D-GMP (Figure 1). Characterization of this new peak (MS analysis, Figure S3) confirmed that L-XMP was converted to L-GMP by GMPS, demonstrating that turnover of the opposite enantiomer substrate was possible.

Biochemical characterization of the kinetics of L-XMP conversion to L-GMP by GMPS, as well as D-XMP conversion to D-GMP, was measured by fitting the individual GMP/cytosine 5′-monophosphate (CMP, an external standard) ratios from each sample into the slope-intercept equation from the calibration plot. Initial velocity measurements of GMP production as a function of time were measured at a variety of substrate (XMP) concentrations, and at fixed saturating concentrations of the non-varied substrates ATP and ammonium acetate (Figure 2A). Fitting these data to the Michaelis–Menten equation and analysis by nonlinear regression allowed measurement of kinetic parameters (Table 1).

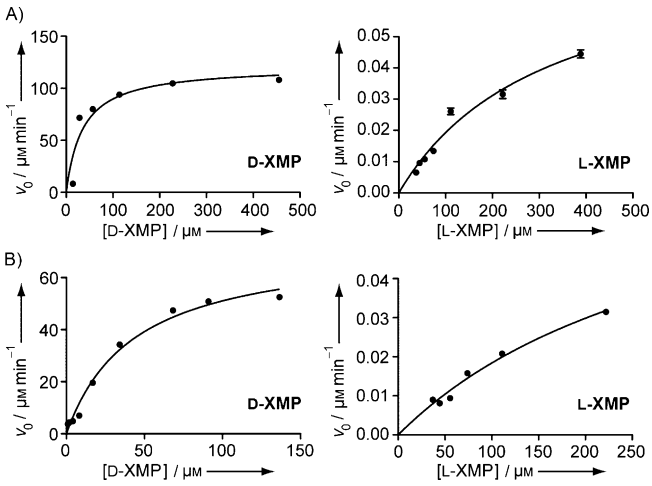

Figure 2. Initial velocities versus substrate concentration plots for D-XMP and L-XMP as ligands for GMPS as measured by A) HPLC analysis and B) UV/Vis analysis.

Table 1. Kinetic parameters of GMPS enzymatic activity in the presence of enantiomeric substrates D-XMP and L-XMP.[a]

Substrate	k_{cat} [s^{-1}]	K_m [μM]	k_{cat}/K_m [$\mu M^{-1} s^{-1}$]
D-XMP[b]	$(4.8\pm0.3)\times10^{-2}$	35.3 ± 8.5	1.4×10^{-3}
D-XMP[c]	$(4.3\pm0.7)\times10^{-2}$	24.9 ± 6.6	1.7×10^{-3}
L-XMP[b]	$(3.2\pm0.3)\times10^{-6}$	316.7 ± 55.6	1.0×10^{-8}
L-XMP[c]	$(3.1\pm0.7)\times10^{-6}$	329.9 ± 104.9	9.4×10^{-9}

[a] Performed in triplicate. Values shown are the mean ± SD. [b] HPLC analysis. [c] UV/Vis analysis.

Analysis of D-XMP revealed an apparent K_m value of 35.3 μM, which was similar to previously reported K_m values for *E. coli* GMPS (29 μM and 166 μM).[23,24] The turnover number (k_{cat}) was found to be 4.8×10^{-2} s^{-1}, which was comparable to a previous report of 9.4×10^{-2} s^{-1}.[24] Analysis of L-XMP revealed an apparent K_m value of 316.7 μM, which is approximately ten times higher than that of the natural enantiomer. Surprisingly, the k_{cat} value was measured at 3.2×10^{-6} s^{-1}, which is a 15000-fold difference in turnover number compared with D-XMP. The specific activity (k_{cat}/K_m) of L-XMP decreased 140000-fold from the natural enantiomer (1.4×10^{-3} $\mu M^{-1} s^{-1}$ for D-XMP versus 1.0×10^{-8} $\mu M^{-1} s^{-1}$ for L-XMP). These results suggest that L-XMP might also inhibit GMPS.

To confirm the values derived from the HPLC assay, a known continuous UV spectrophotometric assay was also employed.[23,25] This assay monitors a reduction in 290 nm absorbance resulting from conversion of XMP ($\varepsilon_{290}=4800$ $M^{-1} cm^{-1}$) to GMP ($\varepsilon_{290}=3300$ $M^{-1} cm^{-1}$). UV-based kinetic data was calculated analogously to the HPLC-derived data (Figure 2B). Analysis of both D-XMP and L-XMP revealed nearly identical results to the HPLC assay (for L-XMP: $k_{cat}/K_m=9.4\times10^{-9}$ $\mu M^{-1} s^{-1}$ (UV) versus 1.0×10^{-8} $\mu M^{-1} s^{-1}$ (HPLC); Table 1).

Although L-XMP conversion to L-GMP by GMPS was demonstrated, the weak affinity of L-XMP for GMPS, coupled with its slow turnover number, suggested possible enzyme inhibition by this ligand. To probe for GMPS inhibition, we performed enzymatic activity experiments with our xanthosine-based molecules to demonstrate reduction of GMPS-mediated amination of D-XMP. Addition of a fixed concentration of inhibitor to varying D-XMP concentrations, followed by UV–visible analysis and fitting to the competitive inhibition equation (or uncompetitive for decoyinine towards XMP),[10] yielded the K_i data shown in Table 2. Evaluation of the known GMPS uncompetitive inhibitor decoyinine revealed a K_i value of 54.1 μM, which was similar to a previous report (26 μM).[26] Mizoribine (bredinin trade name), a known immunosuppresive drug and competitive GMPS inhibitor, was found to be more potent in our hands ($K_i=1.8$ μM); this activity is similar to reports of the same compound against GMPS isolated from rat Walker sarcoma cells ($K_i=10$ μM).[11] L-XMP (**6**) inhibition results were quite interesting, revealing that L-XMP is almost seven times more potent

Table 2. GMPS inhibition by known inhibitors and xanthosine analogues.[a]

Inhibitor	K_i [μM]	Inhibitor	K_i [μM]
decoyinine	54.1 ± 14.5	mizoribine	1.8 ± 0.7
D-xanthosine	>1500	L-xanthosine	>500
L-XMP	7.5 ± 1.8		

[a] Performed in triplicate. Values shown are the mean ± SD.

than decoyinine inhibition against *E. coli* GMPS ($K_i = 7.5$ μM). Both D-xanthosine and L-xanthosine nucleosides were also tested and neither molecule inhibited GMPS; this suggests that 5′-monophosphorylation is required for inhibition. Mizoribine does not require phosphorylation for GMPS inhibition. Our results suggest that L-XMP can inhibit GMPS enzymatic activity with potency similar to or slightly better than other known inhibitors.[11,26–28]

To understand the molecular interactions of D-XMP and L-XMP within the GMPS active site, energy minimized three-dimensional conformations of the biochemical reaction intermediates (adenyl-D-XMP and adenyl-L-XMP; Figure S4 B) were docked into the crystal structure of *E. coli* GMPS (Surflex-dock in the SYBYL software suite; PDB ID: 1GPM[13]). The molecule of AMP observed in the X-ray crystal structure was extracted and re-docked into GMPS with a calculated similarity of 0.908 (1.0 is the theoretical maximum), showing reliability in docking accuracy (Figure S4 A). Several stabilizing molecular interactions were observed between adenyl-D-XMP and GMPS, such as hydrogen bonds between Lys856 and the xanthine nucleobase; Arg875, Arg765, and Glu768 to ribose alcohols; and Asn761 to the phosphate of D-XMP (Figure 3 A). The considerable size of the GMPS pocket readily accommodated the docking of adenyl-L-XMP; however, the L-ribose sugar occupied a substantially different position within the synthetase domain (Figure 3 B). No longer present were many of the key molecular interactions between the nucleobase and ribose alcohols as evident by a decreased consensus score, which is an estimate of the overall ligand binding affinity (CScore = 7.68 for D-XMP versus 6.16 for L-XMP). One compensating molecular interaction was observed for adenyl-L-XMP, which was a hydrogen bond between Asn761 to a ribose alcohol. The conformation of the L-ribose sugar in adenyl-L-XMP also forces C^2 of the nucleobase to be positioned approximately 2.0 Å away from the region in space occupied by the natural adenyl-D-XMP ligand. This perturbation to nucleobase conformation may deter aminolysis of the adenylated unnatural monophosphate, thereby slowing enzyme turnover. Additionally, the loss of key hydrogen-bonding interactions may also contribute to the loss in enzyme efficiency. Nonetheless, our observation of the synthesis of L-GMP from L-XMP implies that the large size of the synthetase pocket must allow some movement of adenyl-L-XMP to obtain the correct conformation for amination.

In conclusion, the synthesis of L-GMP from L-XMP provides new insight into the substrate promiscuity of GMPS. GMPS was also inhibited by L-XMP at low micromolar levels; this is comparable to the behavior of other known inhibitors. These results provide new insight into GMPS–ligand interactions that will be useful for future inhibitor designs.

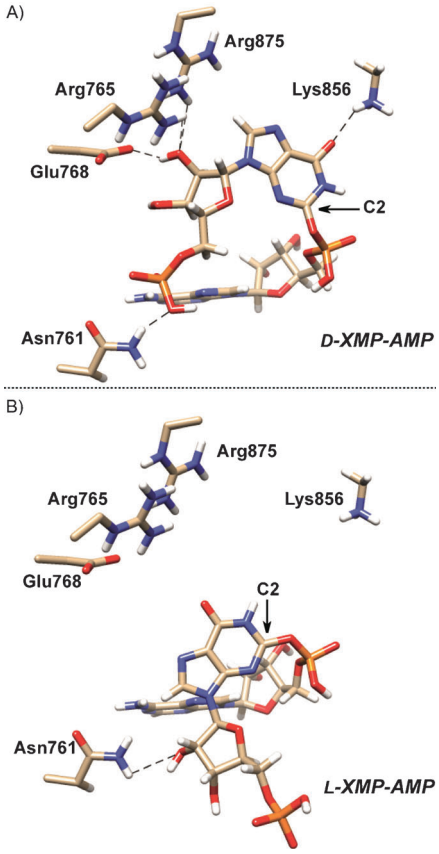

Figure 3. Docking of A) adenyl-D-XMP and B) adenyl-L-XMP intermediates into the *E. coli* GMPS synthetase binding pocket. Key hydrogen-bonding interactions between the adenylated ligands and GMPS are marked with dashed lines. The 2-position of the xanthine nucleobase is marked (2.0 Å conformational shift).

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