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ARTICLE

# Kinetic evaluation of glucose 1-phosphate analogues with a thymidylyltransferase using a continuous coupled enzyme assay

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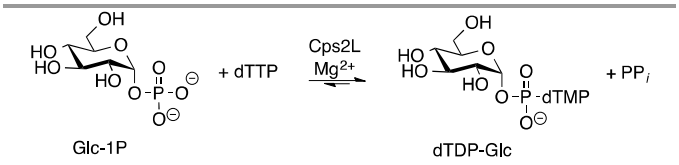
Cps2L, a thymidylytransferase, is the first enzyme in *Streptococcus pneumoniae* L-rhamnose biosynthesis and an antibacterial target. We herein report the evaluation of six sugar phosphate analogues selected to further probe Cps2L substrate tolerance. A modified continuous spectrophotometric assay was employed for facile detection of pyrophosphate ($PP_i$) released from nucleotidylyltransfase-catalysed condensation of sugar 1-phosphates and nucleoside triphosphates to produce sugar nucleotides. Additionally, experiments using waterLOGSY NMR spectroscopy were investigated as a complimentary method to evaluate binding affinity to Cps2L.

## Introduction

*Streptococcus pneumoniae* is a highly infectious, Gram positive bacterium responsible for many invasive pneumococcal diseases, including pneumonia, meningitis and sepsis. A prevalent, pathogenic organism, *S. pneumoniae* has shown widespread clinical resistance to penicillin and chloramphenicol, as well as to synergistic treatments involving β-lactams and aminoglycosides.[1, 2] *S. pneumoniae* cell wall assembly is essential for bacterial survival and virulence; thus, disruption of pathways integral to cell wall biosynthesis is a known mechanism for many clinically used antibiotics and a commonly pursued target for various bacterial enzyme inhibitors. Cps2L (EC 2.7.7.24) is a bacterial thymidylyltransferase (nucleotidylyltransferase) cloned from *S. pneumoniae* that catalyses the first step in the biosynthesis of L-rhamnose (Scheme 1),[3] an essential constituent of the cell wall in many bacterial species.[4] These enzymes are responsible for generating activated sugars in the form of glycosylated nucleoside diphosphates (NDPs), which serve as substrates for glycosyltransferases, and represent attractive antibacterial targets in that they show broad homology across various species.[5, 6] Furthermore, they have been used to prepare sugar nucleotide analogues for enzymatic glycodiversification studies,[7-9] and used to prepare phosphonate[10] and carbacyclic[11] sugar nucleotide analogues that have been put forward as putative glycosyltransferase inhibitors.




**Scheme 1.** The physiological reaction catalysed by Cps2L.

The present study details the enzymatic evaluation glucose 1-phosphate analogues (**1**-**6**, Figure 1) selected to further probe the substrate tolerance of Cps2L. Kinetic evaluation of individual substrates and inhibitors provides the most effective means to probe the Cps2L active site and to identify structure-activity relationships. This follows from the observation that the enzyme is flexible, undergoing significant conformational changes between substrate-bound and product-bound states.[12] We have previously successfully employed a 7-methyl-6-thioguanosine (MESG)-based coupled spectrophotometric kinetic assay,[13] for the quantitation of inorganic phosphate ($P_i$) in solution to measure Cps2L kinetics, however the assay proved challenging un our hands owing to the thermal instability of MESG towards base-catalysed decompositions. Indeed, MESG decomposes at ambient temperature, with a half-life of 4 h at pH 8, which led to difficulties when acquiring kinetic data.[14] A handful of literature methods employ a coupled assay using xanthine oxidase (XO) and purine nucleoside phosphorylase (PNP) to quantify phosphate; these assays have been employed for kinetic evaluation of UDP-*N*-

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acetylglucosamine enolpyruvyl transferase (MurA)[15] and DNA polymerases.[16] For enzymes releasing diphosphate ($PP_i$), such as nucleotidylyltransferases, these reactions are typically coupled to inorganic pyrophosphatase (IPP) to produce two units of phosphate, although alternate methods to detect $PP_i$ have been described.[17]

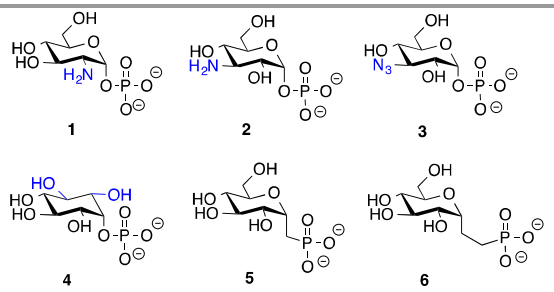


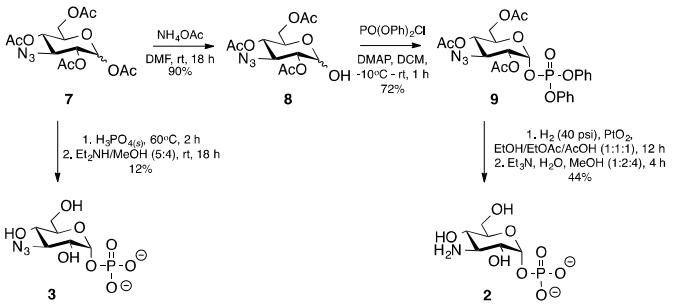
**Figure 1.** Analogues of glucose 1-phosphate evaluated in this study.

Herein we employ a phosphate-detecting assay based on IPP (EC 3.6.1.1), PNP (EC 2.4.2.1), and XO (EC 1.17.3.2) for the kinetic evaluation of Cps2L substrates and inhibitors. Additionally we report the application of waterLOGSY NMR binding studies for the determination of a dissociation constant ($K_d$) for one of the sugar phosphate analogues found to be a Cps2L inhibitor in an effort to explore alternate methods to evaluate binding.

## Results and Discussion

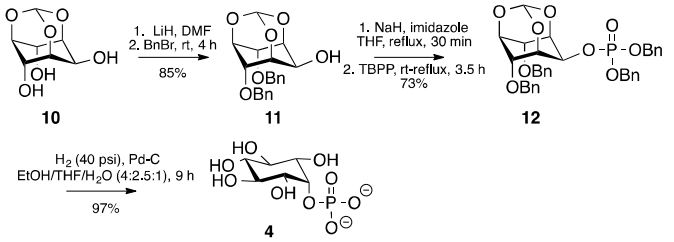
### Synthesis

The synthesis of kanosamine 1-phosphate **2** began with the preparation of 1,2,4,6-tetra-*O*-acetyl-3-azido-3-deoxy-D-glucopyranose **7** following a published procedure.[18] Anomeric deacetylation to give **8** and subsequent phosphorylation using diphenyl chlorophosphate resulted in a mixture of anomers that were separable by silica gel flash chromatography, leading to the isolation of the expected major α-isomer **9**. Hydrogenolysis using Adam's catalyst served to remove the phenyl groups as well as reduce the azide to the amine. The acetyl groups were removed under mild conditions using triethylamine in water and methanol. The final sugar phosphate **2** was purified by anion exchange chromatography, eluting as a zwitterion with an increasing gradient of acetic acid in water. Phosphate 3-azido-3-deoxy-D-glucopyranoside **3** was synthesized beginning with the same intermediate **7**[18] that was phosphorylated using the MacDonald procedure.[19] The resulting crude product was isolated using column chromatography and then deprotected by treatment with diethylamine in methanol at room temperature. Passing through acidic Dowex resin gave the phosphoric acid, which was subsequently converted to the triethylammonium salt of **3** in moderate yield (12% over two steps, Scheme 2).



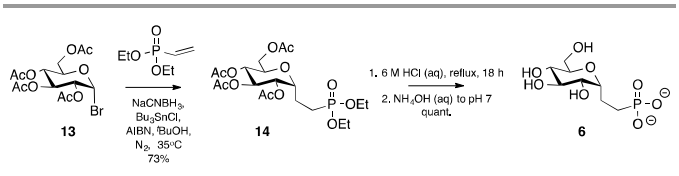
**Scheme 2.** Synthetic scheme for compounds **2** and **3**.

*myo*-Inositol 2-phosphate **4** was prepared using a modified version of Billington's approach[20] using LiH to form the anion of the protected inositol **10** for benzylation at the 4- and 6-positions.[21, 22] The free hydroxyl of **11** was phosphorylated with tetrabenzyl pyrophosphate, and the resulting phosphotriester **12** was deprotected by hydrogenolysis of the benzyl groups and hydrolysis of the orthoester to give the desired *myo*-inositol 2-phosphate **4**, which was isolated as the bistriethylammonium salt (Scheme 3).



**Scheme 3.** Synthetic scheme for compound **4**.

Synthesis of methylene phosphonate **5** was accomplished as previously described.[10] Synthesis of glucose ethylphosphonate **6** was achieved in two steps starting from commercially available acetobromo-α-D-glucose **13** (Scheme 4). Free radical coupling of **13** with diethyl vinyl phosphate,[23] using AIBN as the radical initiator, was performed using reaction conditions recently described by Wagner and co-workers for the synthesis of the analogous galactose 1-*C*-phosphonate.[24] This provided the ethyl-protected phosphonate **14** in a 73% yield. In lieu of a stepwise approach as described in the literature,[24, 25] we examined a one-pot global deprotection under aqueous acidic conditions, which successfully provided fully deprotected sugar **6** in quantitative yield following ion exchange chromatography and purification over Sephadex LH-20 resin.



**Scheme 4.** Synthetic scheme for compound **6**.

### Analytical substrate evaluation with Cps2L using HPLC

Sugar phosphates **1-6** were evaluated as substrates for the thymidylyltransferase Cps2L (Table 1). Previous studies have

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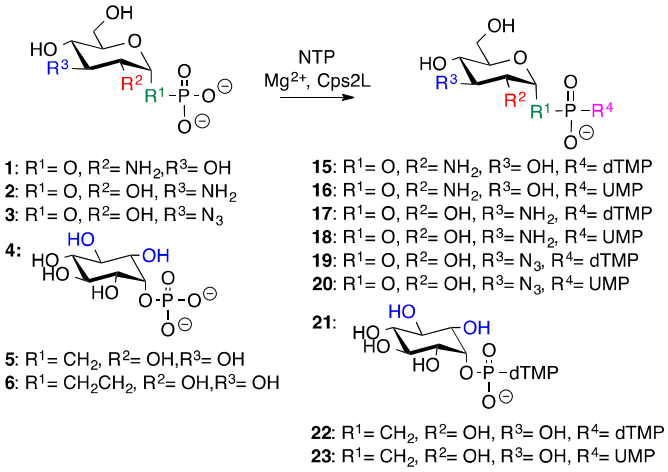
shown that Cps2L has broad substrate tolerance, and can turnover sugar phosphates containing modifications at C-2 and C-3, as well as phosphonate analogues.[3, 10, 26] Reaction progress with recombinant Cps2L was monitored using HPLC, and enzymatic sugar nucleotide production was confirmed using LC-MS[2]. We have previously reported that commercially available glucosamine 1-phosphate **1** was found to efficiently turnover to produce dTDP and UDP sugar nucleotides, **15** and **16**.[3] Both **2** and **3** were identified as Cps2L substrates when coupled with dTTP or UTP; however, UTP conversion required significantly more time and quantitative conversion was not achieved. Studies with RmlA, a Cps2L homologue with 89% overall sequence identity, have shown lower tolerance to sugar phosphates with modifications at C-3 and C-4 compared to C-2 and C-6, and that use of an alternate NTP in conjunction with an alternate sugar 1-phosphate has a cumulative negative influence on enzyme efficiency, a trend that was consistent with our data.[27-31] The relatively sluggish turnover of **3** may be explained by the increased linear length of the azido group, as it has previously been reported that increasing 3-*O* alkoxy chain length in a series of Glc-1P analogues resulted in increasingly sluggish conversion rates.[26]

**Table 1.** Evaluation of glucose 1-phosphate analogues as Cps2L substrates.




| Compound | EU Cps2L | NTP | Product | % conversion | Time (h) |
|---|---|---|---|---|---|
| **1**[a] | 2 | dTTP | **15** | 95 | 0.5 |
| **1**[a] | 2 | UTP | **16**[b] | 95 | 24 |
| **2** | 2 | dTTP | **17**[b] | 100 | 0.5 |
| **2** | 8 | UTP | **18** | 21 | 48 |
| **3** | 8 | dTTP | **19**[b] | 100 | 17 |
| **3** | 8 | UTP | **20** | 22 | 4d |
| **4** | 40 | dTTP | **21** | 18 | 8d |
| **4** | 40 | UTP | - | 0 | >24 |
| **5**[a] | 2 | dTTP | **22** | 95 | 0.5 |
| **5**[a] | 2 | UTP | **23** | 65 | 0.5 |
| **6** | 10 | dTTP | - | 0 | > 24 |

[a]Values obtained previously[3, 10]; [b]These sugar nucleotides were scaled-up and isolated.

*myo*-Inositol 2-phosphate **4** provided a new scaffold to probe Cps2L substrate tolerance; our analysis of the structure of RmlA, which has identical active site residues with Cps2L, in complex with dTDP-Glc suggested that **4** would be accommodated in the active site of the thymidylyltransferase.[32] The resulting inositol-nucleotide, lacking an anomeric centre, would serve as a putative glucosyltransferase inhibitor. Carbaglucose 1-phosphate has been demonstrated to be turned over to produce the UDP-sugar by a bacterial, but not eukaryotic, nucleotidylyltransferase.[33] *myo*-Inositol 2-phosphate **4** was a substrate when coupled with dTTP; however, product formation to produce **21** never surpassed 24% and required high Cps2L concentrations in order to achieve appreciable conversions; product formation was not observed upon incubation with UTP. Signals identified as breakdown products dTDP and dTMP were observable by analysis using HPLC, alongside the peak for the product, sugar nucleotide **21**. Methylene phosphonate **5** has previously been identified as a Cps2L substrate,[10] but phosphonate **6**, which contains an additional methylene linker between the pseudo-anomeric center and the phosphorus, was not a substrate.

### Enzymatic synthesis of sugar nucleotides

We performed scaled-up chemoenzymatic syntheses of three sugar nucleotides that would provide interesting scaffolds for future studies with nucleotidylyltransferases and glycosyltransferases. For instance, the azido group of sugar nucleotide **19** provides a chemical handle for facile derivitisation using click-chemistry.[31, 34] Chemoenzymatic syntheses were performed on a milligram scale in order to isolate the sugar nucleotide products from the reactions of **1** and UTP to yield **16** (26%), of **2** and dTTP to yield **17** (86%), and of **3** and dTTP to yield **19** (33%). The discrepancy in the yields compared to the conversions as observed in the analytical HPLC experiments was attributed either to incomplete conversion to the sugar nucleotide on the larger scale (in the case of **19**) or to the use of different purification procedures. In the purification of **16** and **19**, a final desalting step with a single Sephadex LH-20 column was performed instead of our previous method that was used for the purification of **17**,[10] which utilized a Dowex and then a Sephadex G-10 column,[9] decreasing the overall yield.

### Kinetic evaluation using IPP-PNP-XO coupled spectrophotometric assay

We then evaluated the glucose 1-phosphate analogues **1-6** using a continuous spectrophotometric coupled kinetic assay that was selected as a more robust alternative to MESG-based coupled and to discontinuous HPLC-based assays: Cps2L catalysis was coupled with IPP to produce phosphate, which was in turn used as a substrate for PNP, along with inosine. XO was then used to catalyse the final reaction in the sequence, namely the oxidation

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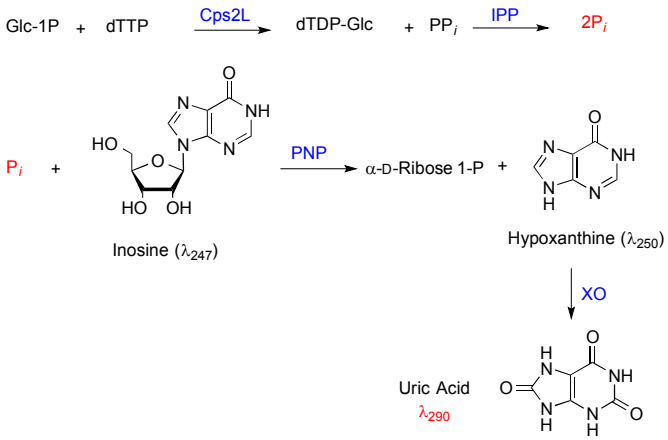
of hypoxanthine to uric acid that produces a change in absorbance at $\lambda_{290}$ such that initial reaction velocities can be monitored spectrophotometrically (Scheme 5). We demonstrated the utility of this assay with nucleotidylyltransferases by determining the kinetic parameters for the Cps2L-catalysed conversion of Glc-1P to dTDP-Glc in the presence of saturating dTTP, from which we established $K_m$, $k_{cat}$, and $k_{cat}/K_m$ values that were comparable to those obtained in previous studies using HPLC and MESG-coupled assays.[10, 35]




**Scheme 5.** Coupled spectrophotometric enzyme assay devised to measure Cps2L activity using PNP and XO.

Sugar phosphates **1**, **2**, and **3**, had $K_m$ values that were an order of magnitude larger than Glc-1P. The observed preference for 2-amino sugar **1** over 3-amino sugar **2** is predominantly a function of the $k_{cat}$ as the apparent binding constants differ only by a factor of 1.5. Comparison of **2** and **3** suggests that substitution of an amino group for an azido group resulted in a 100-fold reduction in $k_{cat}$, but had no effect on the $K_m$ value. This may be rationalized by a nonproductive binding event, where **3** binds to Cps2L with a similar affinity and where reduced $k_{cat}$ is a function of misalignment of the phosphate for efficient catalysis. The $K_m$ value for **1** was unchanged in the presence of dTTP or UTP, and the change in specificity was attributed to the $k_{cat}$ value, which decreased 100-fold with UTP. Kinetic evaluation showed that specificity was affected by both an increase in $K_m$ and a reduction in $k_{cat}$ for C-2 and C-3 modified sugars, which is different from the trend observed in a previous series of compounds, which included galactose 1-phosphate (Gal 1-P), and the phosphonate analogues of Glc-1P and Gal-1P, that were found to have unchanged $K_m$ values in comparison to Glc-1P.[10]

**Table 2.** Kinetic parameters for Cps2L substrates.

| Variable substrate | NTP | $k_{cat}$ ($s^{-1}$) | $K_m$ (μM) | $k_{cat}$ / $K_m$ ($uM^{-1}s^{-1}$) |
|---|---|---|---|---|
| Glc-1P | dTTP | 24 | 83 ± 10 | 0.28 |
| Glc-1P | UTP | 0.016 | 160 ± 7 | 0.0096 |
| **1** | dTTP | 4.5 | 690 ± 29 | 0.0065 |
| **1** | UTP | 0.1 | 700 ± 160 | 0.00015 |
| **2** | dTTP | 0.08 | 1100 ± 100 | 0.000072 |
| **3** | dTTP | 0.0003 | 1100 ± 330 | 0.00000027 |
| **5** | dTTP | 0.55 | 180 ± 17 | 0.031 |
| **5** | UTP | 0.01 | 380 ± 53 | 0.00028 |

A $K_m$ of 180 μ□, a $k_{cat}$ 5.5 $s^{-1}$, and a $k_{cat}/K_m$ of 0.031 □$M^{-1}s^{-1}$ was identified for methylphosphonate **5** when evaluated as a Cps2L substrate. In a previous study, it was determined that **5** had hundred-fold reduced efficiency with Cps2L compared to Glc-1P,[21] whereas the present study found the difference to be ten-fold, suggesting that **5** was a better substrate than previously reported.

*myo*-Inositol 2-phosphate **4** was turned over too slowly to be evaluated as a substrate with the spectrophotometric assay and we wanted to probe its ability to inhibit the physiological reaction based on its poor ability to turnover to product, which required near equimolar concentrations of enzyme and extended reaction times to achieve 20-40% conversion. Inhibition assays with **4** gave the best fit to a mixed inhibition model, from which a $K_i$ value of 8.9 mM was calculated. In light of this result, we attempted to chemoenzymatically prepare the inositol nucleotide product **21** to elaborate our inhibition studies by exploring the possibility of product inhibition. Unfortunately, attempts to isolate **21** were unsuccessful, likely due to poor initial turnover to product; on a milligram scale, a maximum of 11% conversion was observed and purification led to the isolation of 0.86 mg of **21** that was not pure according to analysis by NMR spectroscopy.

**Table 3.** Inhibition constants for the Cps2L physiological reaction.

| Inhibitor | $K_i$ (mM) | Inhibition model |
|---|---|---|
| **4** | 8.9 ± 4.7 | Mixed |
| **6** | 1.2 ± 0.2 | Competitive |

We sought to determine the ability of **6** to act as an inhibitor of the physiological reaction having established that it was not a Cps2L substrate. The kinetic data obtained were fitted to standard inhibition equations by non-linear regression, providing a $K_i$ value of 1 mM for ethylphosphonate **6** (Figure 3, a). The Lineweaver-Burk plot demonstrated that the mode of inhibition was best described using a competitive model with respect to Glc-1P, the natural substrate of Cps2L (Figure 3, b).

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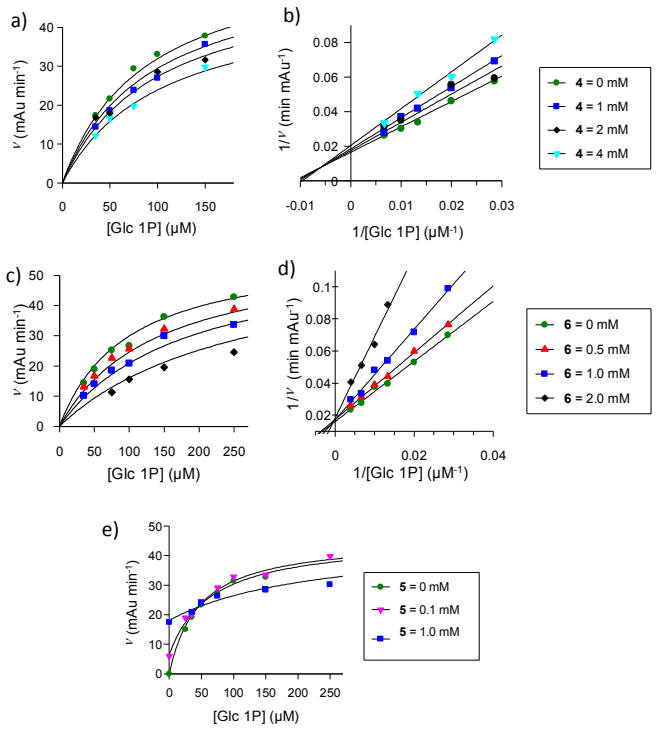


**Figure 2.** Cps2L inhibition by compound **4**: a) Michaelis-Menten and b) Lineweaver-Burk plots; inhibition by compound **6**: c) Michaelis-Menten and d) Lineweaver-Burk plots used to determine $K_i$ and mode of inhibition. e) Competitive substrate model between **5** and Glc-1P.

For the purposes of comparison, methylphosphonate **5** was evaluated as an inhibitor of the physiological reaction; at 100 μM **5** no obvious effect on reaction rates was observed, whereas at 1 mM **5** some rate suppression was observed, along with a non-zero rate at 0 □M Glc-1P indicating that **5** was turning over to sugar nucleotide **22** within the timeframe of the kinetic assay (Figure 2, e). Production of **22** was confirmed by HPLC. The obtained rates were fit to a competitive substrate model from which a ten-fold difference in catalytic efficiency between **5** and Glc-1P was calculated, 0.08 □$M^{-1}s^{-1}$ and 0.83 □$M^{-1}s^{-1}$, respectively, which agreed with the kinetic data obtained from the individual substrate assays, confirming that **5** does not inhibit Cps2L.

### Determination of dissociation constants using waterLOGSY NMR binding experiments

As an alternate method to the kinetic coupled assay for the determination of binding, compound **6** was evaluated using waterLOGSY NMR. WaterLOGSY spectra were acquired with **6** in the presence and absence of Cps2L in order to confirm binding to the enzyme, which is determined by a change in phasing for binding signals in the processed spectra; **6** only bound to Cps2L in the presence of dTTP (Figure 3), a result that agrees with the widely accepted ordered Bi-Bi reaction mechanism for Cps2L.[32, 36]

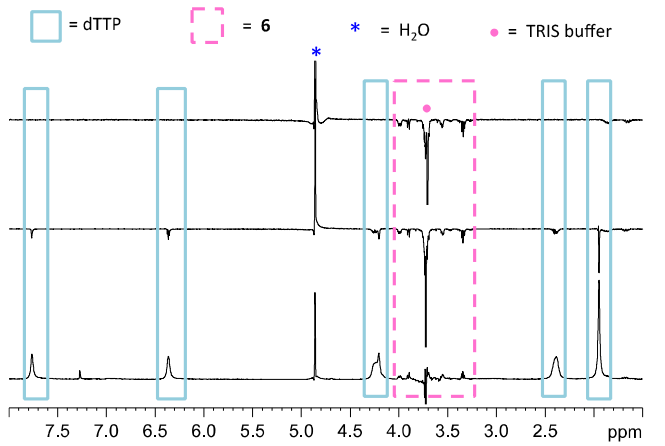


**Figure 3.** WaterLOGSY NMR spectra. Top: **6** + Cps2L, no binding; middle: **6** + dTTP, no binding; bottom: **6** + dTTP + Cps2L, binding.

Having qualitatively established the binding of **6** to Cps2L, we sought to determine the $K_d$ value for the enzyme-ligand complex using a waterLOGSY method described by Dalvit and co-workers.[37] After establishing a lower concentration limit for sugar **6** at which positive signals, indicative of binding, could still be observed, aliquots of a 100 mM aqueous solution of **6** (1 – 8 μL) were titrated into an enzyme and dTTP solution. A waterLOGSY NMR spectrum was recorded after each addition. Control experiments were also carried out without Cps2L in order to generate a correction curve for the contribution of free ligand. Two clearly defined signals were selected for analysis, derived from H-4 (3.35 ppm) and H-6 (3.88 ppm) of ethylphosphonate **6**, and the corrected signal intensities were plotted as a function of the concentration of **6** and fit to a standard dose-response curve by non-linear regression. This provided us with $K_d$ values of 17 (± 4) and 23 (± 8) mM for H-4 and H-6, respectively (Figure 3). This dissociation constant measured from the waterLOGSY experiment was significantly larger than the $K_i$ of 1.2 mM that was determined using the coupled spectrophotometric assay. In our experimental data, the $K_d$ curves are shallow and therefore (likely) overestimate the true dissociation constant. This same observation was made by Fielding *et al.* upon evaluating binding of L-tryptophan to bovine serum albumin (BSA). They attributed overestimated $K_d$ values to effects caused predominantly by spin diffusion, which at high ligand concentration results in the loss of polarization acting against the NOE transfer due to cross relaxation mechanisms, rather than to effects from non-specific binding.[38, 39] Thus, our results corroborate that waterLOGSY signal intensity is not a direct function of ligand binding and, as such, caution must be taken with experimental design and interpretation of results. We also speculate that the charged nature of the phosphonate group may affect NOE transfer mechanisms because of the different nature of the ionic interactions. Nevertheless, both the spectrophotometric kinetic assay and the waterLOGSY NMR binding experiment identified **6** as a weak (mM) binder to Cps2L.

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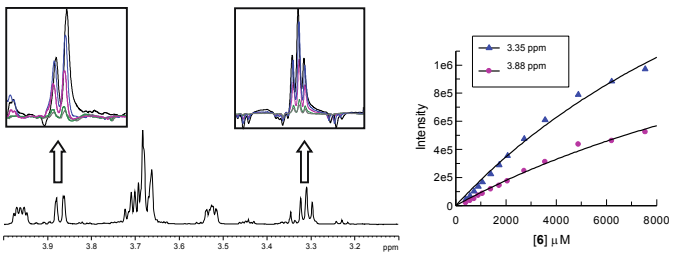


**Figure 4.** Determination of $K_d$ for **6** binding to Cps2L in the presence of dTTP. Left: Change in signal intensity observed in waterLOGSY NMR spectra; Right: Plot of corrected signal intensity *vs.* concentration of **6**: ▲ H-4; ■ H-6.

## Conclusions

Using a spectrophotometric IPP-PNP-XO coupled assay, the substrate tolerance of Cps2L was probed using six Glc-1P analogues: a 2-amino sugar **1**, a 3-amino sugar **2**, a 3-azido sugar **3**, a carbocyclic inositol **4**, and two phosphonates **5** and **6**. The four phosphates were found to be Cps2L substrates, and the rate of conversion followed the trend **1**>**2**>**3**>>**4**. The general trend was that modification at the 2-position was tolerated better than modification at the 3-position. Inositol **4** was a very poor substrate, demonstrating that loss of the ring oxygen or the addition of steric bulk at this position, or a combination of these, was not tolerated within the Cps2L active site. Although isosteric phosphonate **5** had previously been identified as a substrate, we found that non-isosteric compound **6** was not a substrate. NMR binding studies using waterLOGSY experiments were performed with compound **6** which identified it as a weak Cps2L binder, but the method may not be appropriate for quantitation given an apparent overestimation of the $K_d$ value. Inhibition studies using the spectrophotometric assay were performed with **4** and **6**, and both were identified as mM inhibitors.

## Experimental

### General methods and instrumentation: Organic synthesis

All chemicals and reagents were purchased from commercial sources and were used as received, unless otherwise noted. Syntheses that required anhydrous conditions were performed under an inert atmosphere of dried high purity nitrogen. Dry THF and DCM were obtained using a MB-Solvent Purification System (Department of Chemistry, University of Saskatchewan). Ethyl acetate, hexanes and dichloromethane were obtained crude and purified *via* distillation, under air at 1 atm pressure, before use. (Department of Chemistry, Dalhousie University). HPLC grade methanol was employed where stated. Glassware was dried overnight in an oven set at 120 °C and assembled under a stream of inert gas. Analytical thin layer chromatography was performed using silica gel 60 F254 precoated glass plates (Merck); compound spots were visualized by ultraviolet light at 254 and/or 365 nm and/or by charring after treatment with chromosulphuric acid solution or Vanillin stain. Flash chromatography was performed with Merck silica gel 60 (230–400 mesh). Lyophilisation of samples was carried out using an Edward Freeze-Dryer. NMR spectra were recorded on a Bruker 500 MHz spectrometers. All $^{1}H$, $^{13}C$ and $^{31}P$ chemical shifts are reported in ppm using tetramethylsilane (0.00 ppm) or the solvent signal [$CDCl_3$ ($^{1}H$ 7.26 ppm; $^{13}C$ 77.16 ppm); $D_2O$ ($^{1}H$ 4.79 ppm)] as the internal reference or MeOD ($^{13}C$ 49.50 ppm in $D_2O$) or 85% aq. $H_3PO_4$ ($^{31}P$ 0.00 ppm) as an external reference. Splitting patterns are indicated as follows: br, broad; s, singlet; d, doublet; t, triplet; at, apparent triplet; q, quartet; m, multiplet. All coupling constants (*J*) are reported in Hertz (Hz). All waterLOGSY NMR spectra were recorded on a 700 MHz spectrometer equipped with a 1.7 or 5 mm cryoprobe at the Biomolecular Magnetic Resonance Facility, National Council of Canada, Halifax. Mass spectra were recorded by Mr. Xiao Feng using ion trap (ESI TOF) instruments or recorded on an API Qstar XL pulsar hybrid LC/MS/MS (System Applied Biosystem/MDS Sciex) and VG 70SE (VG Analytical Inc. Ltd) at the Saskatchewan Structural Sciences Centre.

### 2,4,6-tri-*O*-Acetyl-3-azido-3-deoxy-α-D-glucopyranoside diphenylphosphate (9)

DMAP (0.097 g, 0.78 mmol) was added to a solution of **8** (0.220 g, 0.664 mmol) in $CH_2Cl_2$ (5.0 mL) and stirred at rt for 15 min. The reaction mixture was cooled to 10 °C prior to the dropwise addition of diphenyl chlorophosphate (0.165 mL, 0.797 mmol). After stirring for 1 h, the reaction mixture was diluted with $CH_2Cl_2$ (10 mL) and washed successively with ice cold water (2 × 15 mL), ice cold 0.5 M HCl (2 × 15 mL), and saturated aqueous $NaHCO_3$ (2 × 15 mL). The organic fraction was dried ($Na_2SO_4$), concentrated *in vacuo*, and purified by flash chromatography (40–50% EtOAc in petroleum ether) to give **9** as colorless oil (0.198 g, 72%) along with its β–isomer (0.016 g, 5%). α-anomer: $^{1}H$ NMR ($CDCl_3$): δ 7.39 (m, 4H), 7.27 (m, 6H), 6.08 (dd, *J* 6.5, 3.0 Hz, 1H), 5.03 (t, *J* 10.5 Hz, 1H), 4.18 (dd, *J* 12.5, 4.0 Hz, 1H), 4.04-4.07 (m, 1H), 3.98 (t, *J* 10.5 Hz, 1H), 3.93 (dd, *J* 12.5, 2.0 Hz, 2H), 2.15 (s, 3H), 2.03 (s, 3H), 1.97 (s, 3H) ppm; $^{31}P\{^{1}H\}$ ($CDCl_3$): δ -13.82 ppm. HRFABMS: found $[M-H]^-$ 484.3751, $C_{19}H_{23}N_3O_{10}P$ requires $[M-H]^-$ 484.3739. β-anomer: $^{1}H$ NMR ($CDCl_3$): δ 7.24-7.39 (m, 10H), 6.40 (d, *J* 4.0 Hz, 1H), 5.06 (t, *J* 10.0 Hz, 1H), 4.57-4.62 (m, 1H), 4.25 (dd, *J* 12.5, 4.0 Hz, 1H), 3.98-4.07(m, 3H), 2.15 (s, 3H), 2.09 (s, 6H) ppm; $^{31}P\{^{1}H\}$ ($CDCl_3$): δ -12.85 ppm; HRFABMS: found $[M-H]^-$ 484.3748, $C_{19}H_{23}N_3O_{10}P$ requires $[M-H]^-$ 484.3739.

### 3-Amino-3-deoxy-α-D-glucopyranosyl phosphate (2)

To a solution of α-**9** (0.300 g, 0.619 mmol) in 10 mL of EtOH:EtOAc:AcOH (1:1:1), $PtO_2$ (0.14 g, 0.62 mmol) was added and the reaction mixture was degassed and charged with $H_2$ (40-60 psi) and stirred for 12 h. The mixture was filtered through a bed of celite, which was washed with MeOH, and the solvent was evaporated. The resulting residue was dissolved in a 4:2:1 mixture of MeOH:$H_2O$:triethylamine (10 mL) and stirred at rt for 4 h. The reaction mixture was neutralized with Dowex 50W-X8 ($H^+$ form), filtered, and the pH brought to 9 with a dilute solution of $NH_4OH$. The product was purified by

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anion exchange chromatography using AG1-X8 resin eluted with a gradient of acetic acid (0 - 200 mM) as the zwitterion. Fractions containing product **2** were pooled and lyophilized to produce **2** as a white solid (0.070 g, 44%). $^1$H NMR ($D_2O$): δ 5.43 (dd, *J* 7.0, 3.5 Hz, 1H), 3.68-3.83 (m, 4H), 3.62 (t, *J* 10.0 Hz, 1H), 3.39 (t, *J* 10.5 Hz, 1H) ppm; $^{31}$P{$^1$H} ($D_2O$): δ 1.37 ppm. HRFABMS: found [M-H]$^-$ 258.1444, $C_6H_{13}NO_8P$ requires [M-H]$^-$ 258.1432.

**Triethylammonium 3-azido-3-deoxy-α-D-glucopyranosyl phosphate (3)**

A mixture of crystalline phosphoric acid (0.50 g, 5.1 mmol, dried over $P_2O_5$ under vacuum overnight) and **7** (0.24 g, 0.64 mmol) was heated at 60 °C for 2 h under vacuum. The reaction mixture was cooled to rt and THF (12 mL) was added. The resulting suspension was cooled to 0 °C and ammonium hydroxide (1.2 mL, 14.8 N) was added, resulting in precipitate that was removed by filtration. The residue was washed with THF, and the filtrate evaporated to dryness under reduced pressure to obtain crude protected phosphate, from which column chromatography (silica gel 70-150 mesh, eluent; $CHCl_3$:MeOH:$H_2O$, 10:10:1) yielded 2,4,6-tri-*O*-acetyl-3-azido-3-deoxy-α/β-D-glucopyranosyl phosphate (0.040 g, α:β, *ca*. 1:1). The obtained protected phosphate (0.040 g) was suspended in methanol (20 mL) and cooled to 0 °C. Diethylamine (2.5 mL) was added and the resulting reaction mixture was stirred at rt for 48 h. The solvents were removed under reduced pressure and the resulting residue was dissolved in distilled water (5 mL) and passed through a bed of Dowex 50W-X8 ($H^+$ form). An excess of triethylamine was added to the eluate, which was then evaporated under reduced pressure to yield triethylammonium salt of **3** (0.030 g, 12%) as a colorless solid. Spectra matched the previously reported data. [40] $^1$H NMR ($D_2O$, 500 MHz): δ 5.45-5.38 (m, 1H), 3.93-3.87 (m, 1H), 3.81 (d, *J* 12.3 Hz, 1H), 3.78-3.67 (m, 2H), 3.50 (dd, *J* 10.1, 1.7 Hz, 1H), 3.42 (t, *J* 9.8 Hz, 1H), 3.15 (q, *J* 7.3 Hz, 6H), 1.22 (t, *J* 7.3 Hz, 9H) ppm; $^{13}$C NMR ($D_2O$, 125 MHz): δ 93.8 (d, *J* 6.0 Hz), 72.1, 70.5 (d, *J* 8.2 Hz), 68.1, 65.7, 60.1 ($CH_2$), 46.6 ($CH_2$), 8.2 ppm; $^{31}$P{$^1$H} NMR ($D_2O$, 202.5 Hz): δ 1.29 ppm. HRMS (ESI$^-$): found [M-H]$^-$ 284.0277, $C_6H_{11}N_3O_8P$ requires [M-H]$^-$ 284.0278.

**4,6-Di-*O*-benzyl-*myo*-inositol-1,3,5-orthoformate (11)**

To a solution of *myo*-inositol 1,3,5-orthoformate **10** (0.60 g, 3.2 mmol) in DMF (30 mL), lithium hydride (0.100 g, 12.6 mmol) was added followed by benzyl bromide (0.82 mL, 6.9 mmol) and stirred at rt for 4 h. Water was added to reaction mixture and stirred for 30 min. Solvents were evaporated under reduced pressure to give a crude residue (1.5 g). The residue was taken in ethyl acetate and washed with water followed by brine. The organic layer was dried over anhydrous sodium sulfate and filtered. Solvent was removed from filtrate under reduced pressure. The crude product was purified by flash column chromatography (EtOAc/hexanes, 3:7) to afford dibenzyl ether **11** (1.0 g, 85%) as a white solid. Spectra matched the previously reported data.[22] $^1$H NMR ($CDCl_3$, 500 MHz): δ 7.4-7.2 (m, 10H), 5.44 (d, *J* Hz, 1H), 4.64 (d, *J* 11.5 Hz, 2H), 4.56 (d, *J* 11.5 Hz, 2H), 4.44 (br s, 1H), 4.35 (t, *J* 3.6 Hz, 2H), 4.23-4.18 (m, 3H), 3.0 (d, *J* 11.7 Hz, 1H) ppm.

**4,6-Di-*O*-benzyl-2-*O*-dibenzylphosphate-*myo*-inositol 1,3,5-orthoformate (12)**

To a solution of dibenzyl ether **11** (0.18 g, 0.50 mmol) in THF (5.0 mL) containing a trace amount of imidazole, sodium hydride (0.030 gm, 0.75 mmol, 60% in mineral oil) was added and refluxed for 30 min. The reaction mixture was cooled to rt and a solution of tetrabenzyl pyrophosphate (0.35 gm, 0.65 mmol) in THF (7.0 mL) was added and refluxed for 3.5 h. The reaction mixture was cooled to rt and filtered, and the residue was washed with ethyl acetate and filtrate was collected. The filtrate was concentrated under reduced pressure and the crude residue was purified by flash column chromatography (EtOAc/hexanes, 3:7) to obtain dibenzyl phosphate **12** (0.23 g, 73%) as a white solid. Spectra matched the previously reported data. [20] $^1$H NMR ($CDCl_3$, 500 MHz): δ 7.4-7.2 (m, 20H), 5.51 (s, 1H), 5.14-5.05 (m, 4H), 4.97 (dd, *J* 6.9, 0.8 Hz, 1H), 4.60-4.52 (m, 4H), 4.44-4.41 (m, 1H), 4.40-4.37 (m, 2H), 4.33 (t, *J* 3.4 Hz, 2H) ppm.

**Bis(triethylammonium) *myo*-inositol 2-phosphate (4)**

To a solution of protected inositol phosphate **12** (0.108 g, 0.171 mmol) in EtOH:THF:$H_2O$ (4:2.5:1, 15 mL), Pd-C (0.040 g, 10 wt %) was added, and the mixture was hydrogenated in a Parr apparatus ($H_2$ pressure 40-50 psi) for 9 h. The reaction mixture was passed through a short column of celite and washed with ethanol followed by water. The filtrate was evaporated to dryness under reduced pressure to obtain crude 2-phosphate-*myo*-inositol-1,3,5-orthoformate (0.05 g). This was taken in TFA:$H_2O$ (4:1, 5 mL) and stirred at rt for 3 h. The solvents were removed under reduced pressure, and the residue dissolved in water (6 mL) and passed through a column of Dowex 50W-X8 resin ($H^+$ form). An excess of triethylamine was added to the eluate, which was evaporated under reduced pressure to obtain the bistriethylammonium salt of **4** as a white solid (0.077 g, 97%). $^1$H NMR ($D_2O$, 500 MHz): δ 4.53-4.49 (m, 1H), 3.66 (t, *J* 9.65 Hz, 2H), 3.50 (d, *J* 10.1 Hz, 2H), 3.24 (t, *J* 9.30 Hz, 1H), 3.16 (q, *J* 7.20 Hz, 12H), 1.24 (t, *J* 7.20 Hz, 18H) ppm; $^{13}$C NMR ($D_2O$, 125 MHz): δ 76.7 (d, *J* 5.9 Hz), 74.4, 72.7, 71.1 (d, *J* 2.9 Hz), 46.6 ($CH_2$), 8.2 ppm; $^{31}$P{$^1$H} ($D_2O$, 202 MHz): δ 2.2 ppm; HRMS (ESI$^-$): found [M-H]$^-$ 259.0206, $C_6H_{12}O_9P$ requires [M-H]$^-$ 259.0213.

**Diethyl 2-(2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl) ethylphosphonate (14)**

Tributyltin chloride (59 µL, 0.219 mmol, 0.3 equiv.), sodium cyanoborohydride (69 mg, 1.09 mmol, 1.5 equiv.), diethylvinyl phosphonate (1.12 mL, 7.3 mmol, 10 equiv.) and AIBN (108 mg, 0.657 mmol, 0.9 equiv.) were added successively to a solution of acetobromo-α-D-glucose **13** (300 mg, 0.73 mmol, 1 equiv.) in anhydrous *tert*-butanol (8 mL), with stirring under nitrogen. The reaction mixture was then warmed to 35 °C, with stirring under nitrogen for 3 days before concentrating and separating between dichloromethane (50 mL) and water (50

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mL). The aqueous phase was extracted with dichloromethane (2 x 50 mL) and the combined organic extracts were washed with brine (100 mL), dried over anhydrous sodium sulfate and concentrated to give the crude product, which was purified using column chromatography over silica ($R_f$ ~ 0.15 in 80% ethyl acetate/hexanes), eluting with 50-100% ethyl acetate/hexanes then 2% methanol/ethyl acetate, to give **13** as a clear colourless oil with spectra that matched reported data (264 mg, 73% yield). [23] $^1$H NMR ($CDCl_3$, 500 MHz) δ 5.28 (t, 1H, *J* 9.0 Hz), 5.07 (dd, 1H, *J* 9.5, 5.5 Hz), 4.95 (t, 1H, *J* 9.0 Hz), 4.20 (dd, 1H, *J* 12.5, 5.5 Hz), 4.14-4.06 (m, 6H), 3.79-3.76 (m, 1H), 2.07 (s, 3H), 2.04 (s, 3H), 2.02 (s, 3H), 2.00 (s, 3H), 1.91-1.76 (m, 3H), 1.70-1.61 (m, 1H), 1.32 (td, 6H, *J* 7.0, 4.0 Hz) ppm; $^{31}$P{$^1$H} NMR ($CDCl_3$, 202.4 MHz) δ 31.1 ppm; $^{13}$C NMR ($CDCl_3$, 125 MHz) δ 170.6, 170.1, 169.7, 169.6, 72.6 (d, *J* 17 Hz), 70.3, 70.2, 68.9 (d, *J* 17 Hz, 2 x C), 62.4, 61.9, 61.8, 21.9, 20.79 (2 x C), 20.77, 20.7, 19.1 (d, *J* 4 Hz), 16.6 (d, *J* 6 Hz, 2 × C) ppm; LRMS: 519.1 $(M+Na)^+$.

**Bis(ammonium) 2-(α-D-glucopyranosyl) ethylphosphonate (6)**

Fully protected compound **14** (185 mg, 0.373 mmol) was dissolved in 6 M aq. HCl (12 mL) and heated to reflux temperature, with stirring for 24 h. After this time the solution was concentrated *in vacuo* then water (10 mL) was added and concentrated again. The crude product was then dissolved in water (1 mL) and passed through an amberlite-IR-120 ($H^+$) column and the acidic fractions were combined and neutralized with concentrated (35%) aq. Ammonium hydroxide solution. The resulting salt was purified over Sephadex LH-20 resin, eluting with water, to give **6** (115 mg, 100% yield) as a white solid with spectral data that matched literature data. [25] $^1$H NMR ($D_2O$, 500 MHz) δ 4.03-3.98 (m, 1H), 3.88 (dd, 1H, *J* 12.5, 1.5 Hz,), 3.76-3.66 (m, 3H), 3.54-3.51 (m, 1H), 3.35 (t, 1H, *J* 9.3 Hz), 1.94-1.83 (m, 2H), 1.80-1.71 (m, 1H), 1.58-1.49 (m, 1H) ppm; $^{13}$C NMR ($D_2O$, 125 MHz) δ 76.8 (d, *J* 16.3 Hz), 73.8, 73.0, 71.9, 71.1, 61.8, 24.3 (d, *J* 133.6 Hz), 19.2 (d, *J* 3.3 Hz) ppm; $^{31}$P{$^1$H} NMR ($D_2O$, 202.4 MHz) δ 25.6 ppm; LRMS: 271.1 $(M-H)^+$; HRMS: 271.0580 Found, 271.0588; calculated for $C_8H_{16}PO_8$.

**General methods and instrumentation: Enzymatic analysis**

Cps2L[3] and PNP[35] were overexpressed, isolated, and quantified as previously described. Microbial xanthine oxidase (XO) and recombinant inorganic pyrophosphatase (IPP) expressed in *Escherichia coli* were obtained from Sigma-Aldrich. IPP stock solutions (0.1 EU/μL) were prepared in Milllipore water; thawed aliquots were kept in a fridge and were used for up to 1 month after thawing. XO stock solutions (120 U/mL) were prepared in Tris·HCl (pH 7.5, 25 mM) and stored at −30 °C; aliquots were used immediately after thawing. Kinetic reactions were performed in 96-well plates and were monitored by a SPECTRAmax Plus[384] Microplate Reader spectrophotometer with SoftMax Pro version 4.8. Non-linear regression analysis was performed using using GraFit 5.0.4., Erathacus Software.

**Analytical HPLC assays**

HPLC analysis of enzymatic reactions was performed as previously described,[1] with a Hewlett Packard Series 1050 instrument using an Agilent Zorbax 5 μM Rx-C18 column (150 × 4.6 mm) and monitoring at an absorbance wavelength of 254 nm. A linear gradient from 90/10 A/B to 40/60 A/B over 8.0 min followed by a plateau at 40/60 A/B over 2.0 min at 1.0 $mL/min^{-1}$ was used, where A is an aqueous buffer containing 12 mM $Bu_4NBr$, 10 mM $KH_2PO_4$ and 5% HPLC grade $CH_3CN$ and B is HPLC grade $CH_3CN$.

**Chemoenzymatic synthesis and purification of sugar nucleotides**

**Disodium uridine 5'-(2-amino-2-deoxy-α-d-glucopyranosyl bisphosphate) (16)**

A reaction containing **1** (2.5 mg, 9.6 μmol), UTP (10.1 mg, 18.4 μmol), $MgCl_2$ (2.2 mM), IPP (5 EU), in TRIS·HCl (final volume of 550 μL, 25 mM, pH 7.4) was initiated by the addition of Cps2L (725 EU). Complete conversion to product was observed by HPLC at 20 h. The quenching and purification procedure was the same as described for **17** but with a different gradient for the reversed phase chromatography. The following gradient was used: 100/0 A/B over 2 CV, linear increase to 80/20 A/B over 18 CV, and finally 0/100 A/B for 2 CV. Desalting was accomplished on an Sephadex LH-20 column. Sugar nucleotide **16** (1.5 mg) was obtained as the disodium salt (2.5 μmol, 26 %). $^1$H and $^{31}$P NMR matched previously reported data.[41]

**Disodium eoxythymidine 5'-(3-amino-3-deoxy-α-D-glucopyranosyl bisphosphate) (17)**

A reaction containing **2** (3.3 mg, 12.6 μmol), dTTP (6.1 mg, 12.6 μmol), $MgCl_2$ (2.2 mM final concentration), and 18 EU IPP was initiated by the addition of Cps2L (150 EU) in Tris·HCl buffer (50 mM, pH 7.5, 1 mL reaction volume). The enzymatic reaction was performed at 37 °C and reaction progress was monitored by HPLC. No product breakdown was observed over the incubation period. Additional Cps2L (25 EU after 18 h, 25 EU after 32 h) was added to the enzymatic reaction mixture. Additional IPP (10 EU after 32 h) was added at 50% conversion. The enzymatic reaction was stopped once 100% conversion was reached (72 h). Alkaline phosphatase (2 EU) was subsequently added to the mixture, and allowed to incubate for 1.5 h at 37 °C. After the set time, the protein was precipitated with an equivalent volume of methanol (1.5 mL) and the precipitate was washed with two 1 mL portions of methanol. The supernatants were combined, concentrated and re-dissolved in aqueous tributylammonium bicarbonate buffer (10 mM, ~2 mL) for purification via C18 ion-pair reversed-phase chromatography by a previously described method. [10] All UV fractions containing sugar nucleotides, as judged by HPLC, were combined and concentrated to ~2 mL in volume and passed through a cation-exchange column (Dowex® 50W-X8 cation exchange resin 100-200 mesh, $Na^+$ form, 18 mm × 18 cm) in order to generate the sodium salt of the desired sugar nucleotide. Further desalting of the product mixture was

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performed via gel filtration by using a Sephadex G10 column (1.5 cm × 100 cm) with water as the eluent to afford **17** with an isolated yield of 6.1 mg (0.011 mmol, 86.1%). $^1$H NMR ($D_2O$, 500 MHz): δ 8.34 (s, 1H, NH-3), 7.63 (s, 1H, H-6), 6.24 (t, 1H , *J* 7 Hz, H-1), 5.54 (dd, 1H, *J* 7.5, 3 Hz, H-1''), 4.51 (dt, 1H, *J* 6, 3 Hz, H-3'), 4.08-4.11 (m, 3H, H-4', H-5'a and H-5'b), 3.81-3.85 (m, 1H, H-2"), 3.76 (dd, 1H, *J* 12.2, 2.5 Hz, H-6"b), 3.70 (dd, 1H, *J* 12.2, 4 Hz, H-6"a), 3.64 (ddd, 1H, *J* 10.1, 4.5, 3 Hz, 5"-H), 3.53 (t, 1H, *J* 10.4 Hz, H-3"), 3.38 (t, 1H, *J* 9.4 Hz, H-4"), 2.21-2.33 (m, 2H, H-2'a and H-2'b), 1.82 (br s, 3H, $CH_3$) ppm; $^{13}$C (125 MHz, $D_2O$): δ 167.7 (C-4), 152.9 (C-2), 138.3 (C-6), 113.9 (C-5), 85.3 (d, *J* 8.8 Hz, C-4'), 85.0 (C-1'), 73.2 (C-3"), 72.8 (d, *J* 12.4 Hz, C-2"), 72.0 (d, *J* 4.5 Hz, C-1"), 71.1 (C-3'), 68.8 (C-5"), 65.7 (C-4"), 65.5 (d, *J* 5.4 Hz, C-5'), 59.5 (C-6"), 38.9 (C-2'), 12.7 ($CH_3$) ppm; $^{31}$P{$^1$H} (202.5 MHz, $D_2O$): δ -13.1 (d, 1P, $J_{P\beta,P\alpha}$ 21 Hz, P-α), -11.3 (d, 1P, $J_{P\alpha,P\beta}$ 21 Hz, P-β) ppm; HRMS (ESI$^-$): found [M-H]$^-$ 562.0821, $C_{16}H_{26}N_3O_{15}P_2$ requires [M-H]$^-$ 562.0845.

**Butylammonium deoxythymidine 5'-(3-azido-3-deoxy-α-D-glucopyranosyl bisphosphate) (19)**

A reaction containing **5** (6.0 mg, 21.2 μmol), dTTP (18.8 mg, 32.1 μmol), $MgCl_2$ (2.2 mM), and 5 EU IPP in Tris·HCl buffer (50 mM, pH 7.5, 1.5 mL final volume) was initiated by the addition of Cps2L (250 EU). Additional Cps2L (100 EU at 18 h, 100 U at 24 h, and 100 U at 32 h) and additional IPP (5 EU at 18, and 5 EU at 24 h) were added at the indicated times. The reaction was observed to plateau at 70% conversion after 24 h, with no further product formation after this time although additional Cps2L had been added to the reaction mixture. At 48 h, with no further reaction progress, alkaline phosphatase (8 EU) was added and the reaction incubated at rt for 20 h. The purification procedure was the same as described for **12**, with a few modifications. A SiliaSep 12 g C-18 column was used with an altered gradient for the reverse phase chromatography; the gradient was 20/80 A/B over 2 CV, linear increase to 80/20 A/B over 20 CV, and finally 0/100 A/B for 3 CV, where A is 10 mM aqueous tributylammonium bicarbonate buffer (pH 6.5) and B is HPLC-grade methanol. The combined fractions containing sugar nucleotide, as judged by HPLC analysis, were concentrated to a volume of ~ 2 mL and passed through a Sephadex LH-20 gel filtration column (3.5 × 22 cm) with water as the eluent for desalting. Sugar nucleotide **19** was isolated with 0.5 equivalents of tributylammonium salt (4.6 mg, 7.3 μmol, 33 %). $^1$H NMR ($D_2O$, 500 MHz): δ 7.65 (br d, 1H, *J* 1.1 Hz, H-6), 6.24 (t, 1H, *J* 7.0 Hz, H-1'), 5.48 (dd, 1H, *J* 7.2, *J* 3.2 Hz, H-1"), 4.52 (m, 1H, H-3'), 4.04-4.10 (m, 3H, H-5'a, H-5'b, H-4'), 3.81 (m, 1H, H-5"), 3.73 (dd, 1H, *J* 12.5, 2.2 Hz, H-6"a), 3.61-3.68 (m, 2H, H-3" and H-6"b), 3.45 (dt, 1H, *J* 10.3, 3.1 Hz, H-2"), 3.35 (t, 1H, *J* 9.9 Hz, H-4"), 2.21-2.31 (m, 2H, H-2'a and H-2'b),1.82 (br d, 3H, *J* 1.0 Hz, $CH_3$) ppm; $^{13}$C ($D_2O$ 125, MHz): δ 166.5 (C-4), 151.6 (C-2), 137.2 (C-6), 111.7 (C-5), 94.8 (d, *J* 6.8 Hz, C-1"), 85.3 (d, *J* 8.9 Hz, C-5'), 84.9 (C-1"), 72. 5 (C-5"), 70.9 (C-3'), 70.5 (d, *J* 8.9 Hz, C-2"), 67.8 (C-4"), 65.7 (C-3"), 65.4 (d, *J* 6.4 Hz, C-4'), 59.9 (C-6"), 38.6 (C-2'), 11.6 ($CH_3$) ppm; $^{31}$P{$^1$H} ($D_2O$, 202.5 MHz): δ -13.0 (d, 1P, $J_{P\beta,P\alpha}$ 21 Hz, P-β), -11.3 (d, 1P $J_{P\alpha,P\beta}$ 21 Hz, P-α) ppm; HRMS (ESI$^-$): found [M-H]$^-$ 588.0731, $C_{16}H_{24}N_5O_{15}P_2$ requires [M-H]$^-$ 588.0750.

**IPP-PNP-XO coupled kinetic assays**

Stock solutions containing Tris·HCl (pH 7.5, 25 mM), Glc-1P or compounds **1-6** (25-750 μM), dTTP (1 mM), $MgCl_2$ (5.7 mM), inosine (1 mM), IPP (1.7 EU/mL), PNP (1 μM), and XO (1.5 EU/mL), were allowed to pre-incubate at rt for five min, in order to consume contaminating $P_i$ present in the solutions. The concentrations of substrate varied within the indicated range to be maintained at a level in which the change in absorbance could be detected by UV spec (typically ≥ 25 μM), and keeping the concentrations lower than that of the co-susbtrate (<1 mM). Higher concentrations of dTTP made accurate quantification more challenging due to the presence of background phosphate. An appropriate concentration of Cps2L was then added to initiate the reaction. In all cases, initial reaction velocity was monitored spectrophotometrically over 10 min at a wavelength of 290 nm. Typically, rates were linear for these initial 10 min. Observed initial kinetic rates were halved to account for the 2 equivalents of $P_i$ derived from each $PP_i$ unit produced in the Cps2L reaction. Rates were converted from absorbance units (mAu) to concentration units (μM) using a phosphate standard curve. The standard curve was generated using identical conditions to those described for kinetic assays, except with variable $P_i$ (10-100 μM $NaH_2PO_4·H_2O$) instead of sugar phosphate. $\lambda_{290}$ values were taken once the reaction had reached completion (after approximately 7 min) and plotted against phosphate concentration producing data that was fit by linear regression, providing a slope that was used as the conversion factor between mAu and μM units. Inhibition assays were run using the same techniques as described above, using variable Glc-1P (25-250 mM), except with an appropriate concentration of inhibitor included in the stock solution.

***K*$_d$ determination using waterLOGSY NMR spectroscopy**

Aliquots (1-8 μL) of a 100 mM ethylphosphonate **6** solution were titrated into a solution containing dTTP (2 mM), $MgCl_2$ (1.3 mM), *d*TRIS·HCl (pH 7.5, 20 mM), $D_2O$ (10% *vol*), Cps2L (60 μM), and ethylphosphonate **6** (initial concentration of 400 μM). After each addition, a waterLOGSY NMR spectrum was recorded (128 scans). WaterLOGSY NMR spectra were also acquired without Cps2L to generate a correction curve for the contribution of free ligand to the observed signal intensities; these samples contained dTTP (2 mM), $MgCl_2$ (1.3 mM), *d*TRIS·HCl (pH 7.5, 20 mM), $D_2O$ (10% *vol*), and ethylphosphonate **6** (400, 600, or 1600 μM). Corrected signal intensities were plotted as function of ethylphosphonate concentration and fit to a standard dose-response curve[4] by non-linear regression to determine $K_d$ values. Values of 17 (±4) and 23 (±8) mM were obtained from the signal intensities corresponding to H-4 and H-6, respectively.

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