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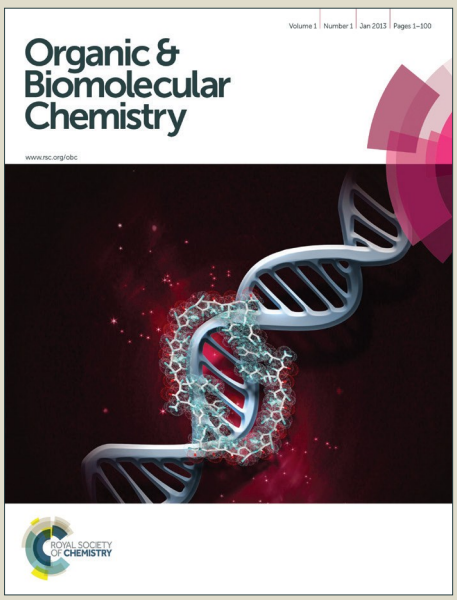

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ARTICLE

Polyphosphate-Containing Bisubstrate Analogues as Inhibitors of a Bacterial Cell Wall Thymidylyltransferase

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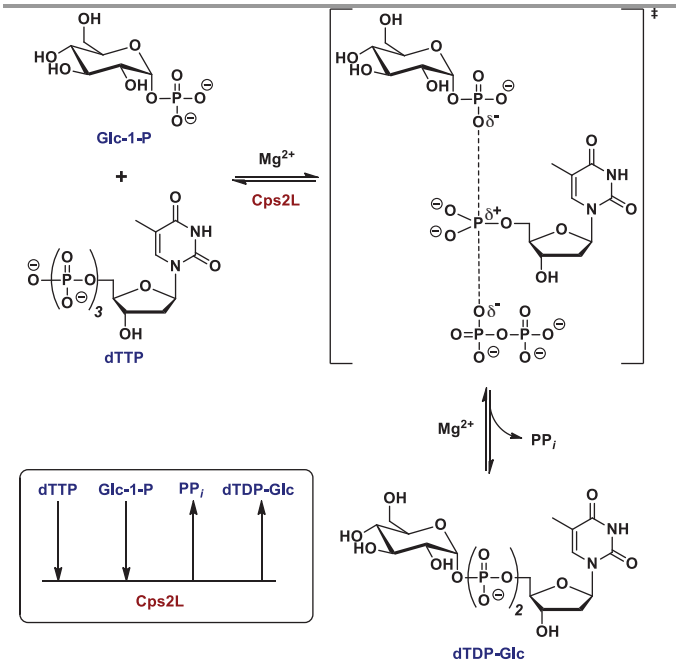
A series of polyphosphate containing sugar nucleotide analogues were synthesized and evaluated as bisubstrate inhibitors of α-D-glucose 1-phosphate thymidylyltransferase Cps2L, the first enzyme in *Streptococcus pneumoniae* L-rhamnose biosynthesis, and a novel antibacterial target. WaterLOGSY NMR spectroscopy demonstrated binding of bisubstrate analogues to Cps2L and a spectrophotometric coupled assay was used to determine apparent K_i values.

Introduction

Streptococcus pneumoniae is a highly infectious, Gram-positive bacterium that is considered to be the most important pathogen in many pneumococcal infections including community-acquired pneumonia, bacteremia and bacterial meningitis. [1] Management of such infections used to be relatively straightforward through treatment with penicillin and other microbial agents; however, in 1967 the first case of penicillin non-susceptibility in *S. pneumoniae* was described and by the 1990's, pneumococcal isolates were found to exhibit high levels of resistance to penicillin and other β-lactam antibiotics. [2-4] The rapid development and spread of this resistance in many countries has been of great concern in recent years and so the development of novel antibiotics that bypass known mechanisms of resistance is of the utmost importance.

The disruption of cell wall biosynthesis is a known mechanism of action for many clinically used antibiotics and a commonly pursued target given that cell wall assembly is essential for bacterial survival and virulence. Cps2L is a bacterial thymidylyltransferase (nucleotidylyltransferase) cloned from *S. pneumoniae* that catalyses the first committed step in L-rhamnose biosynthesis in many pathogenic bacteria, [5, 6] a necessary sugar constituent of the bacterial cell wall. This process involves the condensation of α-D-glucose 1-phosphate (Glc-1-P) and deoxythymidine triphosphate (dTTP) to yield dTDP-glucose and pyrophosphate (PP_i), Scheme 1. [5, 7] Such nucleotidylyltransferases represent attractive antimicrobial targets in that they display broad substrate specificity and homology across various bacterial species. [8, 9]

The physiological reaction catalysed by Cps2L (Scheme 1) is known to follow an ordered Bi-Bi mechanism characteristic of nucleotidylyltransferases and, following step-wise addition of the substrates, proceeds through a highly negatively charged trigonal bipyramidal transition state. [8, 10-12]

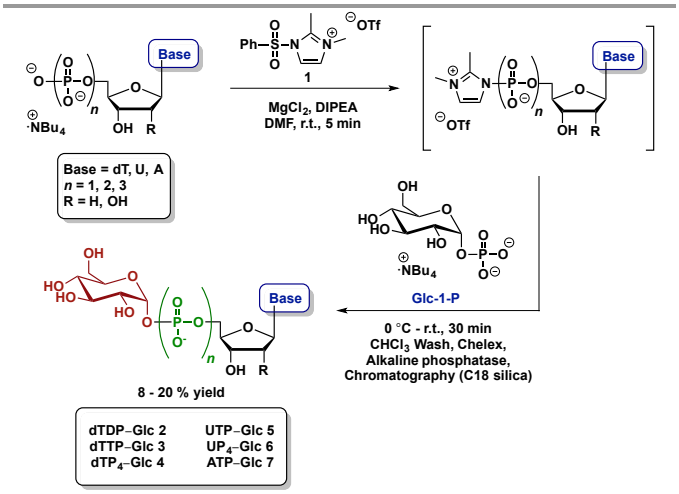


Scheme 1. Physiological reaction catalyzed by Cps2L and ordered Bi-Bi mechanism (inset).

Cognizant that enzymes function to preferentially stabilize the transition state that lies between substrates and reaction products, it would be expected that a species that closely resembles the transition state, but could not be turned-over, would act as a potent non-covalent inhibitor. [13] Species comprising of structural components of both substrates would also be considered bisubstrate analogues. [14-20] Studies carried out concerning the transition states of phosphoryl transfer

enzymes have demonstrated the dominant role of charge balance with regard to transition state stabilization. [21-24] Phosphotransfer enzymes will prioritize the retention of charge at the expense of the preferred native geometry within the active site. [22] With these paradigms in mind, we set out to synthesize inhibitors of Cps2L that possess charge complimentary to that of the binding site, with the hypothesis being that compounds possessing the greatest amount of negative charge would act as the best inhibitors. Furthermore, structurally related compounds including diuridine tetraphosphate (Up_4U) have been approved for clinical use. [25]

We herein report the synthesis of a series of sugar nucleotide analogues that vary in choice of nucleobase and length of polyphosphate linker with a view to mimicking the highly anionic environment within the active site. All compounds were evaluated for their ability to bind Cps2L using water-ligand observed via gradient spectroscopy (WaterLOGSY) NMR and were subsequently examined as inhibitors of Cps2L using a coupled spectrophotometric enzyme assay.



Scheme 2. Synthesis of sugar nucleotides **2-7** using sulfonylimidazolium salt (**1**) as the coupling reagent.

Results and Discussion

Synthesis of a series of sugar nucleotide analogues (**2-7**), varying in choice of nucleobase and length of polyphosphate linker, were identified as targets. Specifically, thymidine (dT) polyphosphates **2-4** contain the physiological nucleobase. To evaluate the role of the nucleobase versus the role of the polyphosphate linker in determining enzyme affinity, uridine (U) derivatives **5** and **6**, and adenosine (A) derivative **7** were also synthesized since Cps2L has previously been shown to turnover both uridine and adenosine nucleoside triphosphates.[5] We chose the coupling method described by Mohamady *et al.*, [26] involving use of a sulfonylimidazolium salt (**1**) to facilitate reaction between glucose 1-phosphate and the desired nucleotide, Scheme 2, due to the impressive isolated yields. Treatment of a nucleotide with coupling salt **1** is proposed to produce a highly reactive imidazolium salt intermediate that reacts immediately with sugar 1-phosphates to generate the corresponding sugar nucleotides. Following the reaction, purification of the crude products was achieved in a step-wise fashion: a chloroform wash was employed to remove organic-soluble compounds. Chelex treatment was used to remove metal cations. Alkaline phosphatase (ALP) affected enzyme-assisted breakdown of any remaining nucleotide reagent, and finally, reversed-phase column chromatography over C18 silica was employed to remove aforementioned breakdown products and isolate the compounds of interest. Whilst conversion to the desired sugar-nucleotides appeared to be moderate in each case (according to analysis of the crude products using ^{1}H and ^{31}P NMR spectroscopy, difficulties relating to the co-elution of structurally-related impurities during purification using reversed-phase column chromatography were encountered. Nevertheless, the isolated yields (8-20%) were comparable to other recent approaches for phosphate-phosphonate coupling. [27]

WaterLOGSY NMR [28] was used to qualitatively confirm that sugar nucleotide analogues **2-7** bound Cps2L. WaterLOGSY NMR measures n*O*e transfer from irradiated water to ligands and to non-binding small molecules; protein-bound water molecules possess an opposite n*O*e relative to unbound water molecules free in solution, thus enabling a distinction between ligands and non binders. The result is a net opposite phasing in processed spectra for ligands versus nonbinding small molecules. NMR experiments were carried out between each of the chemically synthesized compounds (**2-7**) and Cps2L in the presence of the enzyme cofactor Mg^{2+}. In addition, benzoic acid was added to each experiment as a non-binding control, as it does not interact with Cps2L it was used to identify the phase of non-binding molecules during processing (Fig. 1). Analysis of the resulting spectra (Fig. 1) revealed that all sugar-nucleotide analogues (**2-7**) demonstrated binding to Cps2L as a result of signals phasing opposite to the non-binding control, benzoic acid. dTDP-Glc (**2**), the natural product of the physiological reaction catalyzed by Cps2L, showed the clearest binding interaction of both sugar and nucleotide moieties as evidenced by strong, well defined peaks.

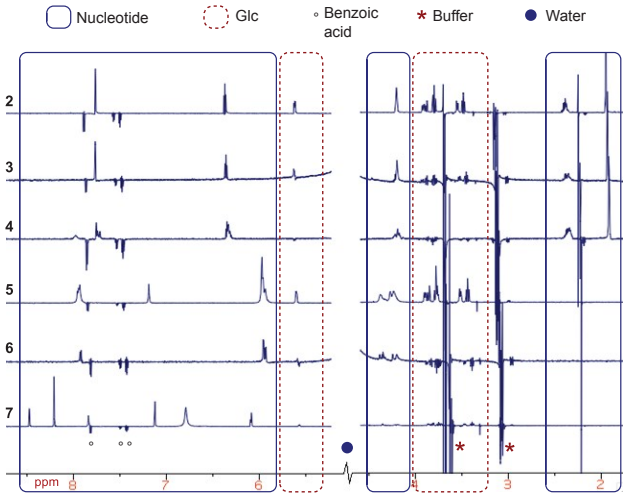


Fig. 1. WaterLOGSY NMR Spectra confirming binding of compounds **2**-**7** to Cps2L. The water-derived peak at 4.79 (●) was removed for clarity. Positively-phased peaks indicate binding, negatively-phased peaks indicate non-binding.

Increasing the length of the polyphosphate linker from two to three units in compound **3** and then four units in compound **4** resulted in the sugar-derived peaks becoming more dispersive, potentially indicative of a weaker binding interaction, whilst the nucleotide-derived peaks continued to demonstrate a strong binding interaction. This can be rationalized by consideration of the ordered Bi-Bi-mechanism of action reported for Cps2L, whereby the nucleotide portion of the substrate is proposed to bind initially. The sugar moiety would then be tethered to the enzyme substrate complex via the polyphosphate linker, whereby a longer linker would likely result in less magnetization being transferred to the sugar portion of the molecule, thus producing a weaker n*O*e effect. A similar trend was observed with the uridine-based compounds (**5** and **6**), with a longer polyphosphate linker resulting in more dispersive glucose-derived peaks in compound **6**. ATP-Glc (**7**) was observed to bind Cps2L with similar intensity compared to tetraphosphates **4** and **6**: however, given that ATP is a substantially less reactive substrate than dTTP and UTP, [5] orientation of **7** is likely non-optimal.

Sugar-nucleotide analogues **2**-**7** were evaluated as inhibitors of Cps2L at various concentrations (0-200 μM), against varying concentrations of dTTP, using a 7-methyl-6-thioguanosine (MESG)-based coupled spectrophotometric inhibition assay. [29, 30] The kinetic data obtained were fit to standard inhibition equations by non-linear regression, using GraFit analysis software, providing a K_i value for each of the compounds (**2**-**7**) evaluated, Table 1. A Lineweaver-Burk plot was also obtained in each case in order to demonstrate the mode of inhibition best described for each compound (ESI).

Table 1. Inhibition of nucleotidylyltransferase Cps2L by sugar nucleotide analogues **2**-**7**.

Entry	Analogue	*n* (PO_3^-)	K_i (μM)	Kinetic model
1	**2** (dT)	**2**	111[a]	C
2	**3** (dT)	**3**	144	C
3	**4** (dT)	**4**	85[a]	C
4	**5** (U)	**3**	710	NC
5	**6** (U)	**4**	470	NC
6	**7** (A)	**3**	778	NC

[a]Average K_i from two inhibition assays; C: Competitive; Non-Competitive.

Results from this series of Cps2L inhibition assays revealed all compounds evaluated (**2**-**7**) to be micromolar inhibitors of the physiological reaction (Table 1) at constant Glc-1-P concentration of 1 mM with an apparent K_m for dTTP of 50 μM (ESI). Within the series, deoxythymidine-based analogues (**2**-**4**, entries 1-3) demonstrated the highest levels of inhibition (K_i 85 μM, entry 3) and were all found to be competitive-type inhibitors with respect to the dTTP substrate. The uridine and adenine derivatives were significantly less potent inhibitors with non-competitive inhibition. Increasing the number of linear phosphate groups from three to four within the compounds also had a positive effect on the inhibitory activity of these compounds, with tetraphosphate analogues providing the most potent K_i values for both the deoxythymidine and uridine-based compounds (compare entries 2 and 3; and 4 and 5). However, dTDP-Glc (**2**) demonstrated greater inhibition than analogue **3**. The increase in inhibition observed for the tetraphosphates over the triphosphate analogues supports the original hypothesis that either by increasing the number of phosphate groups, we would more closely mimic the transition state of the normal physiological reaction or provide a bisubstrate-like species that provides binding interactions from both substrates thereby inhibiting Cps2L. The nucleotide-based inhibitors **2**-**7** were found to be 5-fold more potent than hexose-phosphonate based-inhibitors, [30, 31] providing kinetic evidence, in addition to the WaterLOGSY binding data, that Cps2L prioritizes binding of the nucleoside base over the hexose.

In conclusion, a series of six sugar nucleotide analogues were obtained via chemical synthesis and evaluated as inhibitors of thymidylyltransferase Cps2L. WaterLOGSY NMR was used to confirm that all compounds bind Cps2L, with the nucleotide portion of each molecule indicating a strong binding interaction with the enzyme in the resulting spectra. The most potent inhibitors possessed a deoxythymidine base and/or a tetraphosphate linker, and are the more effective Cps2L inhibitors reported to date. Enhancement of the nucleobase binding interaction via derivatization, or substitution of the polyphosphate linker with non-scissile isosteres may produce more potent inhibitors. Of significance is that the straightforward 1D WaterLOGSY NMR experiment provided qualitative insight into the relative importance of the nucleoside versus hexose binding that was corroborated through a detailed kinetic study.

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Notes and references

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Electronic Supplementary Information (ESI) available: HPLC traces and retention times, Synthetic methods and NMR spectra of synthetic compounds and kinetic plots. See DOI: 10.1039/b000000x

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