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Enzyme-Catalyzed Synthesis of Furanosyl Nucleotides

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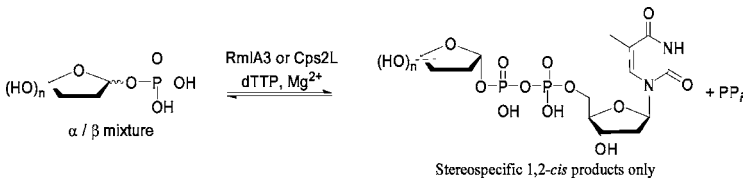
ABSTRACT



A bacterial α-D-glucopyranosyl-1-phosphate thymidylyltransferase was found to couple four hexofuranosyl-1-phosphates, as well as a pentofuranosyl-1-phosphate, with deoxythymidine 5′-triphosphate, providing access to furanosyl nucleotides. The enzymatic reaction mixtures were analyzed by electrospray ionization mass spectrometry and NMR spectroscopy to determine the anomeric stereochemistry of furanosyl nucleotide products. This is the first demonstration of a nucleotidylyltransferase discriminating between diastereomeric mixtures of sugar-1-phosphates to produce stereopure, biologically relevant furanosyl nucleotides.

Glycoconjugates are important constituents of cellular matrices and mediate interactions of the cell with external stimuli.[1] In higher organisms, sugar functionalities are exclusively found in a pyranose ring form (excluding nucleic acid components), while in lower organisms including protozoae,[2] fungi,[3] bacteria,[4] and archaebacteria,[5] there exist a significant proportion of furanoside-containing glycoconjugates. Greater insight into the biosynthesis of furanoconjugates and polyhexofuranosides offers the potential of designing selective medicinal entities to treat infections from organisms producing these glycoconjugates, as well as enzymatic tools to probe these biosynthetic catalysts.[6]

The galactofuranosyltransferases responsible for the biosynthesis of polyhexofuranosides require galactofuranosyl nucleotides;[7] however, the chemical synthesis of these sugar nucleotides requires the coupling of activated nucleoside 5′-monophosphates with furanosyl-1-phosphates, which are often fickle.[8] Enzymatic approaches toward the synthesis of hexopyranosyl nucleotides have demonstrated significant utility,[9] as the nucleotidylyltransferases responsible for the physiological production of α-D-glucose deoxythymidine 5′-diphosphate from α-D-glucopyranosyl-1-phosphate and deoxythymidine 5′-triphosphate possess inherently broad substrate specificity and are amenable to mutagenesis, further altering substrate specificity.[10,11] Access to dTDP-furanoses may also

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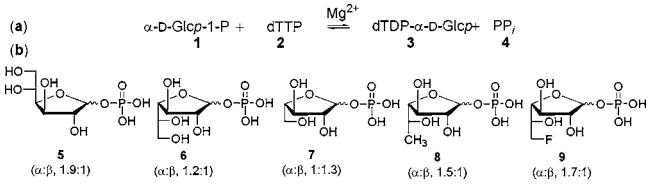
enable substrate specificity studies on natural product glycosyltransferases involved in the biosynthesis of furanoside-containing natural products such as the gilvocarcins.[12] Herein, we report the ability of a wild-type recombinant thymidylyltransferase to catalyze the formation of stereopure furanosyl nucleotides when provided with anomeric mixtures of furanosyl-1-phosphates. The potential of nucleotidylyltransferases to select one sugar-1-phosphate diastereomer over another is an area of carbohydrate-active enzymology that remains largely unexplored.

Five furanosyl-1-phosphates[13] (Scheme 1b) were evaluated as potential substrates for the bacterial thymidylyltransferase

Scheme 1. (a) Physiological Reaction Catalyzed by α-D-Glucopyranosyl-1-phosphate Thymidylyltransferase Cps2L, and (b) Furanosyl-1-phosphates Used in This Study[13]



Cps2L (*Streptococcus pneumoniae*).[14,15] The enzyme (10 EU) was incubated with furanosyl-1-phosphate (2 mM), $MgCl_2$ (2.2 mM), and dTTP[16] (1 mM) at 22 °C for 24 h. The extent of product formation was determined by HPLC analysis of quenched aliquots of the enzymatic reaction mixtures.[14,17] Control reactions with any one reagent absent from the reaction mixtures failed to produce any sugar nucleotide product. Inorganic pyrophosphatase (0.5 EU) was included in each reaction to hydrolyze pyrophosphate, limiting any potential reversibility. A summary of these enzymatic studies is presented in Figure 1 and Table 1. Product formation was confirmed by HILIC-ESI-MS/MS by observing the characteristic fragmentation patterns of molecular ions.[18]

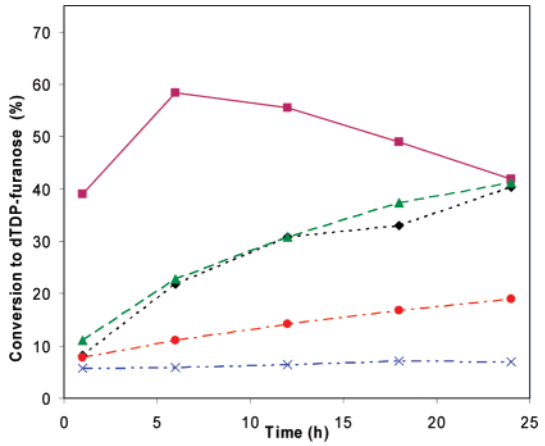


Figure 1. Cps2L-catalyzed conversion of furanosyl-1-phosphates to dTDP-furanoses: (■) L-Ara*f*-1-P; (▲) D-Fuc*f*-1-P; (◆) D-Gal*f*-1-P; (●) D-6F-Gal*f*-1-P; (×) D-Glc*f*-1-P. The decrease of dTDP-L-Ara*f* after 6 h demonstrates the instability of dTDP-furanoses over the duration of the assay.

The Cps2L enzyme coupled the furanosyl-1-phosphates and dTTP to form dTDP-furanoses with conversions lower than that of the physiological reaction with α-D-glucopyranosyl-1-phosphate and many alternative hexopyranosyl-1-phosphates,[14] likely due to either the lack of optimal interactions within the Cps2L binding site for furanosyl-1-phosphates or a rate-limiting step during the chemistry of the reaction (*vide infra*). We observed similar levels of conversion with the homologous RmlA3 enzyme from *Aneurinibacillus thermoaerophilus*[19] (75% identity), which is consistent with the comparable activities of these two enzymes with hexopyranosyl-1-phosphate substrates,[14,15] and also the identical active-site residues, based on the crystal structure of the homologous *Pseudomonas aeruginosa* RmlA.[20] Greater conversions were observed with D-galactofuranosyl-1-phosphate and L-arabinofuranosyl-1-phosphate, while D-glucofuranosyl-1-phosphate was converted the least efficiently, demonstrating that configuration at C4 of the furanosyl-1-phosphate is important for catalysis. Only trace furanosyl nucleotide product peaks were observed when using the wild-type thymidylyltransferases with uridine 5′-triphosphate, in contrast to studies with hexopyranosyl-1-phosphates,[14] where a significant conversion to hexopyranosyl nucleotide was observed upon coupling with UTP. In contrast, the α-D-galactopyranosyl-1-phosphate uridyltransferase responsible for the production of α-D-galactose uridine 5′-diphosphate was able to convert α-D-galactofuranosyl-1-phosphate into the corresponding furanosyl nucleotide in 79% yield.[21]

^{1}H NMR spectra of reaction mixtures with L-Ara*f*-1-P as substrate (Figure S1) clearly demonstrated the conversion of only the *β*-anomer (1,2-*cis*-phosphate) to the sugar

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(16) Abbreviations: HILIC-ESI-MS/MS, hydrophilic interaction liquid chromatography-electrospray ionization tandem mass spectrometry; NTP, nucleoside 5′-triphosphate; NDP, nucleoside 5′-diphosphate; NMP, nucleoside 5′-monophosphate; dTTP, deoxythymidine 5′-triphosphate; UTP, uridine 5′-triphosphate; α-D-Glc*p*-1-P, α-D-glucopyranose-1-phosphate; dTDP-α-D-glc*p*, deoxythymidine α-D-glucopyranose; α/*β*-D-Glc*f*-1-P, α/*β*-D-glucofuranose-1-phosphate; α/*β*-D-Gal*f*-1-P, α/*β*-D-galactofuranose-1-phosphate; α/*β*-L-Ara*f*-1-P, α/*β*-L-arabinofuranose-1-phosphate; α/*β*-D-Fuc*f*-1-P, α/*β*-D-6-deoxygalactofuranose-1-phosphate; α/*β*-D-6Ffuc*f*-1-P, α/*β*-D-6-deoxy-6-fluorogalactofuranose-1-phosphate; dTDP-α-D-glc*f*, deoxythymidine α-D-glucofuranose; dTDP-α-D-gal*f*, deoxythymidine α-D-galactofuranose; dTDP-α-L-ara*f*, deoxythymidine α-L-arabinofuranose 1 EU = the amount of enzyme needed to catalyze 1 mol of substrate to product per minute.
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Table 1. Cps2L-Catalyzed Formation of dTDP-Furanoses[a]

substrates	conversion/% (24 h)	HPLC t_R/min	product ion[b] $[M - H]^-$ m/z	major fragment[b] m/z	product
5 + **2**	7	5.76	563	321 $[TMP - H]^-$	dTDP-α-D-Glc*f*
6 + **2**	40	5.83	563	321 $[TMP - H]^-$	dTDP-α-D-Gal*f*
7 + **2**	58 (6 h) 66[c]	5.91	533	321 $[TMP - H]^-$	dTDP-β-L-Ara*f*
8 + **2**	41	6.01	547	321 $[TMP - H]^-$	dTDP-α-D-Fuc*f*
9 + **2**	19	6.02	565	321 $[TMP - H]^-$	dTDP-α-D-6F-Gal*f*
1 + 2	87[d]	5.66[d]	563	321 $[TMP - H]^-$	**3**
1 + UDP	95[d]	5.32[d]	565	323 $[UMP - H]^-$	UDP-α-D-Glc*p*
α-D-Man*p*-1-P + UDP	95[d]	5.43[d]	565	403 $[UDP - H]^-$	UDP-α-D-Man*p*

[a] Percentage conversion = $(A_P/(A_P + A_T + A_D)) \times 100$, where A_P = dTDP-furanose product peak area, A_T = dTTP peak area, and A_D = degradation product peak area. [b] Electrospray ionization enhanced product ion spectra presented in Supporting Information. [c] Accounting for α/β ratio. [d] Data from refs 14 and 23.

nucleotide. Significant decomposition of the non-productive anomer was not observed after reaction at ambient temperature for 21 h. This is the first report of a nucleotidylyltransferase preferentially selecting between a mixture of anomeric phosphates. Further evidence for the preferential recognition of one furanosyl-1-phosphate was obtained by analysis of tandem mass spectra, based on the observation by Wolucka and co-workers[22] that a hexopyranosyl nucleotide with a 1,2-*cis* configuration fragments primarily into a nucleoside 5′-monophosphate, whereas a 1,2-*trans*-configured hexopyranosyl nucleotide fragments into a nucleoside 5′-diphosphate.

Significantly more intense nucleoside 5′-monophosphate fragments were observed in the tandem mass spectrum of all quenched reaction aliquots, indicating that the furanosyl nucleotide products formed were likely 1,2-*cis*-linked sugar nucleotides. Upon fragmenting UDP-α-D-mannose, UDP-α-D-glucose, and dTDP-α-D-glucose standards, we observed UDP, UMP, and dTMP anions, respectively. This provides evidence for the formation of a productive enzyme complex with only 1,2-*cis*-configured furanosyl-1-phosphate diastereomers. The observation that both enzymes were able to convert only one of the phosphates into furanosyl nucleotides is of significance since the stereoselective production of hexofuranosyl-1-phosphates is confounded by both anomeric and steric effects.[24]

To determine if the decrease in the conversions observed with the furanosyl phosphates resulted from reduced binding affinity or rate limitations to the chemistry, kinetic parameters were determined for Cps2L with respect to α-D-Glc-1-P and β-L-Ara*f*-1-P in the forward direction upon coupling with dTTP. Using discontinuous HPLC monitoring to ascertain initial reaction rates, we determined that for α-D-Glc-1-P the K_m was 139 μM, consistent with reported K_m values from homologous enzymes,[25,26] and the k_{cat}/K_m was 0.199 min^{-1} μM^{-1}. For β-L-Ara*f*-1-P, the K_m was 89 μM and the k_{cat}/K_m was 0.0000245 min^{-1} μM^{-1}. To the extent that K_m represents substrate binding, these data imply that Cps2L binds both pyranosyl and furanosyl-1-phosphates with similar affinity, but that the bond forming and breaking steps are severely retarded with the latter series of nonphysiological substrates.

In conclusion, the catalysis observed by the Cps2L thymidylyltransferase clearly demonstrates a broader sugar-1-phosphate substrate specificity than previously established for this class of enzyme. It is also the first demonstration of a thymidylyltransferase able to discriminate between a mixture of anomeric sugar-1-phosphate diastereomers and convert one of them into a physiologically relevant furanosyl nucleotide. This ability to utilize anomeric mixtures of sugar-1-phosphates to stereospecifically furnish sugar nucleotides is important in light of the recent observation that a glycosyltransferase was able to utilize both α and β sugar nucleotide diastereomers.[27] These results provide new opportunities for the enzymatic preparation of furanosyl nucleotides to facilitate an understanding of the biochemical pathways involved in production of furanoconjugates and furanosylated natural products, particularly in view of the recent directed evolution successes with glycosyltransferases involved in natural product biosynthesis.[28]

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Supporting Information Available: Experimental details for the enzyme assays, including Michaelis−Menten traces, NMR, and HILIC-ESI-MS/MS characterization data are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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