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Exploiting Nucleotidylyltransferases To Prepare Sugar Nucleotides

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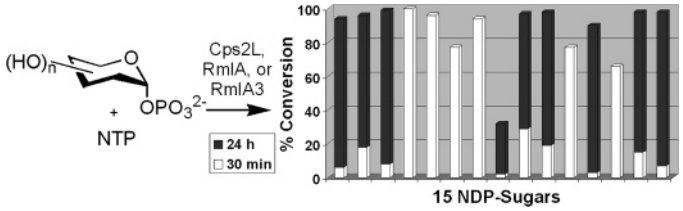
ABSTRACT



Enzymatic approaches to prepare sugar nucleotides are gaining in importance and offer several advantages over chemical synthesis including high yields and stereospecificity. We report the cloning, expression, and purification of two new wild-type thymidylyltransferases and observed catalysis with a wide variety of substrates. Significant product inhibition was not observed with the enzymes studied over a 24 h period, enabling the efficient preparation of 15 sugar nucleotides, clearly demonstrating the synthetic utility of these biocatalysts.

Despite advancements in modern drug discovery, the majority of pharmaceuticals continue to be derived from natural product scaffolds, many of them glycosylated secondary metabolites.[1,2] It is well-established that the carbohydrate portions of these molecules are essential for bioactivity as there exists many cases where aglycons show little or no bioactivity compared to their glycosylated counterparts.[3,4] Although the precise role of the sugar residue varies, carbohydrates have traditionally been implicated in drug pharmacokinetics and have also been shown to impart specific interactions with biological targets.[4,5] Recently, the glycosylation of colchicine, a previously nonglycosylated natural product, has been shown to not only improve bioactivity but also change the mechanism of action.[6]

The in vivo glycosylation of secondary metabolites is mediated by Leloir-type glycosyltransferase enzymes,[7] which rely on sugar nucleotide donors produced by nucleotidylyltransferase enzymes (Scheme 1) that are later modified by sugar processing enzymes. Recent studies have shown that numerous glycosyltransferases are promiscuous with respect to their sugar nucleotide donors, allowing these enzymes to emerge as key glycorandomization tools.[8−11] The first enzyme evolution study of a glycosyltransferase has also been recently reported, demonstrating the increased viability of glycorandomization approaches.[12] In order to effectively make use of Nature's method of glycosylation to expand

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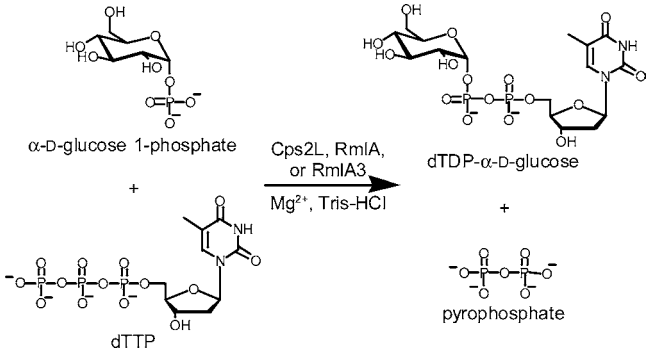
Scheme 1. Primary Metabolic Reaction Catalyzed by Cps2L, RmlA, and RmlA3 α-D-Glucose 1-phosphate Thymidylyltransferases



libraries of carbohydrate-containing drug candidates, access to a wide variety of sugar nucleotides is required. Although advances in chemical approaches towards synthesizing sugar nucleotides have been reported,[13−15] yields are often low and controlling the stereoselectivity of direct coupling approaches has proven problematic.[16−18] The in vitro preparation of sugar nucleotides, through the use of nucleotidylyltransferases, is quickly emerging as an attractive alternative to chemical synthesis,[19−25] although preliminary studies suggested substrate inhibition as a limiting factor in the use of nucleotidylyltransfersaes.[26,27]

The scope and limitations of the nucleotidylyltransferase-catalyzed formation of sugar nucleotides was evaluated with a variety of enzymes, sugar 1-phosphates, and nucleoside triphosphates. We examined the substrate flexibility of three wild-type bacterial thymidylyltransferases: Cps2L (*Streptococcus pneumoniae* R6), RmlA (*Streptococcus mutans* UA159), and RmlA3 (*Aneurinibacillus thermoaerophilus* DSM 10155). Our selection of sugar 1-phosphates was composed of five commercially available α-D-sugar 1-phosphates diverging in structure from α-D-Glc-1-P[28] and one *β*-L-sugar 1-phosphate prepared by chemical synthesis (Figure 1). The α-D-sugar 1-phosphates probe the flexibility of the three enzymes toward changes in configuration at the

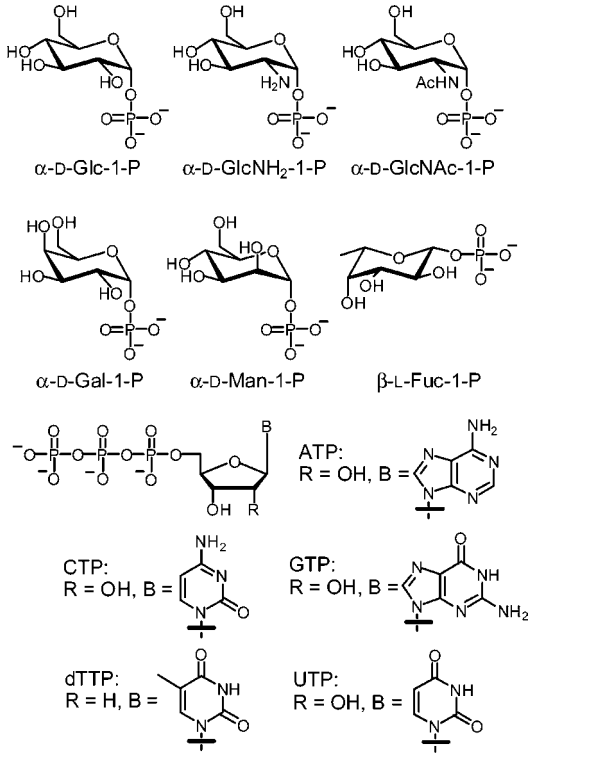


Figure 1. Structures of sugar 1-phosphates and NTPs used in nucleotidylyltransferase substrate flexibility studies.

C2 and C4 stereocenters of the various α-D-sugar 1-phosphates. In addition, synthetic access to *β*-L-Fuc-1-P[29] provided an opportunity to study the flexibility of the nucleotidylyltransferases toward a substrate with a different conformation since *β*-L-Fuc-1-P adopts a $^1C_4$ chair conformation whereas the α-D-sugar 1-phosphates adopt $^4C_1$ chair conformations. It is important to note that *β*-L-sugar nucleotides are key substrates for many family 1 (GT-B) glycosyltransferases[30,31] involved in the biosynthesis of bioactive natural products and thus access to these substrates is of particular significance for glycorandomization studies.

A series of assays including six pyranosyl-1-phosphates and five nucleoside triphosphates (Figure 1) were used to evaluate the substrate flexibility and assess the synthetic utility of the Cps2L, RmlA, and RmlA3 thymidylyltransferases. The enzymatic reactions were conducted using conditions consistent with those described in the literature including millimolar substrate concentrations and the pres-

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(28) Abbreviations: NTP, nucleoside triphosphate; NDP, nucleoside diphosphate; NMP, nucleoside monophosphate; ATP, adenosine triphosphate; CTP, cytidine triphosphate; GTP, guanosine triphosphate; dTTP, deoxythymidine triphosphate; UTP, uridine triphosphate; α-D-Glc-1-P, α-D-glucose 1-phosphate; α-D-GlcNH$_2$-1-P, α-D-glucosamine 1-phosphate; α-D-GlcNAc-1-P, *N*-acetyl-α-D-glucosamine 1-phosphate; α-D-Gal-1-P, α-D-galactose 1-phosphate; α-D-Man-1-P, α-D-mannose 1-phosphate; *β*-L-Fuc-1-P, *β*-;-fucose 1-phosphate.

(29) Synthetic scheme, procedures, and characterization data are available in the Supporting Information.

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ence of inorganic pyrophosphatase.[21,24,25] Aliquots of each reaction were quenched with methanol and centrifuged prior to HPLC and ESI-MS/MS analysis. In the absence of thymidylyltransferase, NTP, sugar 1-phosphate, or $MgCl_2$, no product formation was observed.

A summary of the substrate flexibility of these three enzymes, determined after 30 min, is presented in white in Figure 2. The incubation of α-D-Glc-1-P with both dTTP and UTP consistently produced very high levels of conversion (87−100%) with all three enzymes, indicating that UTP was readily accepted as an alternative NTP with α-D-Glc-1-P. Similarly, incubation of all three enzymes with α-D-$GlcNH_2$-1-P with both dTTP and UTP also produced high levels of conversion (77−96%) after 30 min at 37 °C. When the reaction of other α-D-sugar 1-phosphates with dTTP was explored, it was found that α-D-Man-1-P, α-D-Gal-1-P, and α-D-GlcNAc-1-P were also accepted by all three enzymes at reasonable rates after 30 min (>50% in all but one case), demonstrating that changes in configuration at C2 and C4, and even substitution of an acetyl group at C2, were tolerated by all three nucleotidylyltransferases. Interestingly, when both alternative α-D-sugar 1-phosphates and alternative NTPs were incubated with the nucleotidylyltransferases, yields decreased significantly in all cases except with α-D-$GlcNH_2$-1-P and UTP, illustrating that these enzymes readily tolerate one change in either sugar 1-phosphate or NTP.

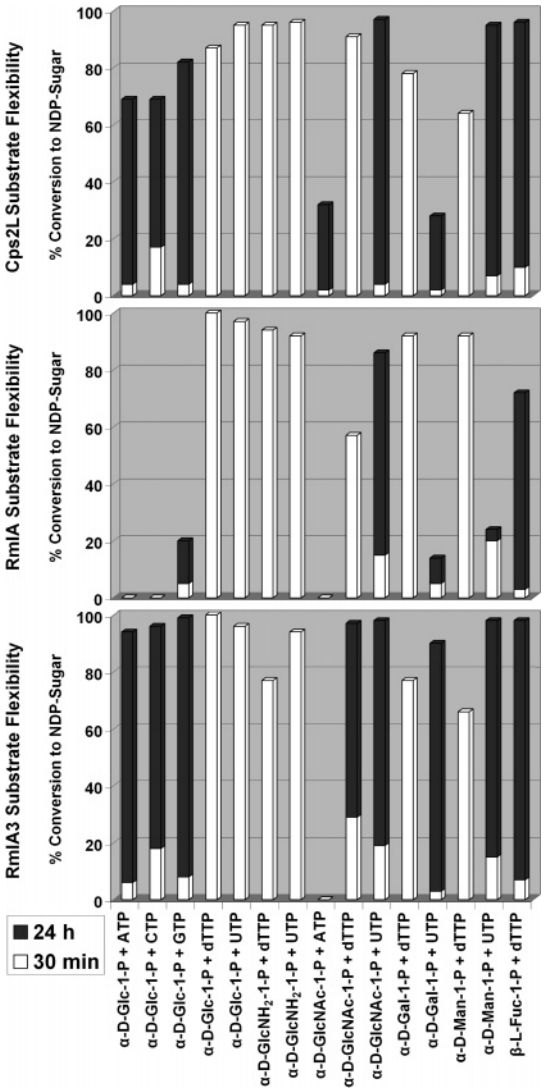

Figure 2. Substrate flexibility of Cps2L, RmlA, and RmlA3 after 30 min and 24 h incubations at 37 °C.

It is significant to note that the synthesized β-L-Fuc-1-P substrate was accepted by all three nucleotidylyltransferases, albeit at conversions ranging from 3 to 10% after 30 min at 37 °C.

The initial incubation of reactions for 30 min at 37 °C provided an excellent survey of relative substrate affinity. In order to more fully assess the synthetic potential of these biocatalysts, reactions resulting in conversions to product of less than 50% after 30 min were incubated for 24 h at 37 °C. A summary of these results, shown in black in Figure 2, illustrates that significant improvements in conversion were obtained for all reactions with all enzymes after incubation for 24 h at 37 °C. All three enzymes were able to synthesize dTDP-β-L-Fuc in >70% conversion after 24 h. In the case of RmlA3, conversions of eight substrates after 30 min ranged from only 3−29%, while conversions after 24 h with the same group of substrates had increased to 90−99%.

The improved conversions to product observed over a 24 h time period at 37 °C with all three enzymes suggests that Cps2L, RmlA, and RmlA3 are stable over a significant portion of the 24 h time period. It is interesting to note that RmlA3, a thermophilic enzyme, and Cps2L, a mesophilic enzyme, generally demonstrated comparable levels of activity over this period of time at 37 °C. These conversion figures clearly indicate the potential synthetic utility of these three nucleotidylyltransferases, particularly as neither substrate nor product inhibition was observed. Also of particular significance is the ability of all three thymidylyltransferases to synthesize dTDP-β-L-Fuc in high yield (72−98%) after incubation at 37 °C for 24 h. This is potentially surprising when you consider that dTDP-β-L-Rha is a competitive inhibitor of *P. aeruginosa* RmlA with micromolar affinity,[32,33] and the epimerization at C2 and C4 of the hexopyranose ring are the only stereochemical differences between the products. In addition, only minimal degradation (typically <5%) of NTPs and NDP-sugars to NMPs and NDPs was observed over the 24 h incubation period, illustrating the stability of substrates and products at 37 °C. While the substrate flexibility of several other thermophilic nucleotidylyltransferases have been reported[20,34] these results provide incentive to study the substrate flexibility of a psychrophilic enzyme, which would potentially function at even lower temperatures than those reported herein and further minimize the degradation of substrates and products over a given time period.

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In comparison with the substrate flexibility of other characterized nucleotidylyltransferases,[20,24,25,34,35] Cps2L, RmlA, and RmlA3 rank very high in terms of the number of sugar 1-phosphates and NTPs they will accept, and the high conversions to product obtained demonstrate excellent synthetic potential. In particular, E_p (*Salmonella enterica* LT2)[24,25] has demonstrated a broad sugar 1-phosphate flexibility but produced significantly lower conversions with UTP than dTTP in several cases. Thermostable UDPG-PPase (*Pyrococcus furiosus* DSM 3638)[20] again demonstrated broad sugar 1-phosphate flexibility, but produced much lower conversions with dTTP than UTP in all but one case. In addition, UDPG-PPase would not accept ATP, a purine nucleotide, in contrast to Cps2L and RmlA3, which demonstrated catalysis with ATP and α-D-Glc-1-P in excellent yield (69% and 94%, respectively). Interestingly, another thermostable nucleotidylyltransferase, ST0452 (*Sulfolobus tokadaii* strain 7)[34] has demonstrated stringent substrate specificity as no catalysis was observed with α-D-$GlcNH_2$-1-P, α-D-Gal-1-P, or α-D-Man-1-P. In addition, ST0452 would not accept ATP, CTP, or GTP nucleotides with any sugar 1-phosphate. The uridylyltransferase GalU (*Streptococcus pneumoniae*)[35] has also demonstrated stringent specificity with respect to nucleotide base as no catalysis was observed with either ATP or UTP with α-D-Glc-1-P.

Nucleotidylyltransferases involved in secondary metabolism have, in general, been reported to be more substrate stringent than the primary metabolic enzymes described above.[36,37] In the case of BtrD,[36] an aminoglycoside antibiotic thymidylyltransferase, it was found that, out of six sugar 1-phosphates tested, only two α-D-*gluco*-hexopyranosyl-1-phosphates were accepted by the enzyme. In addition, BtrD was also found to be incapable of accepting ATP, CTP, or GTP nucleotide bases with any sugar 1-phosphate. The thymidylyltransferase SgcA1[37] from the enediyne antibiotic C-1027 gene cluster has also demonstrated very stringent specificity accepting only α-D-Glc-1-P of six sugar 1-phosphates and only dTTP and UTP (6% conversion) of the five NTP bases studied.

In summary, Cps2L, RmlA, and RmlA3, three primary metabolic enzymes, have clearly demonstrated their ability to use both dTTP and UTP nucleotides along with six sugar 1-phosphates to efficiently produce both dTDP- and UDP-sugars. In addition, Cps2L and RmlA3 have also demonstrated their utility in the preparation of ADP-, CDP-, and GDP-sugars with conversions to product ranging from 69−99%, illustrating the potential synthetic utility of these nucleotidylyltransferases over others involved in primary and secondary metabolism. In addition, this is the first report to demonstrate the ability of bacterial nucleotidylyltransferases to prepare dTDP-β-L-Fuc with high conversions ranging from 72 to 98% after incubation at 37 °C for 24 h. The lower temperature required for catalysis compared to the heat-stable archaeal UDPG-PPase[20] may offer a significant advantage when utilizing less stable sugar 1-phosphates or NTPs. In conclusion, these three enzymes provide a convenient route to the efficient preparation of a library of fifteen sugar nucleotides with a variety of sugars and nucleotide bases. Kinetic studies on these thymidylyltransferases will provide more insight into their substrate flexibility.

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Supporting Information Available: Full experimental details including Cps2L, RmlA, and RmlA3 cloning, expression, and purification protocols, synthetic procedures, NMR and mass spectrometry characterization data, and HPLC methodology and retention times. This material is available free of charge via the Internet at http://pubs.acs.org.

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