BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

pubs.acs.org/biochemistry

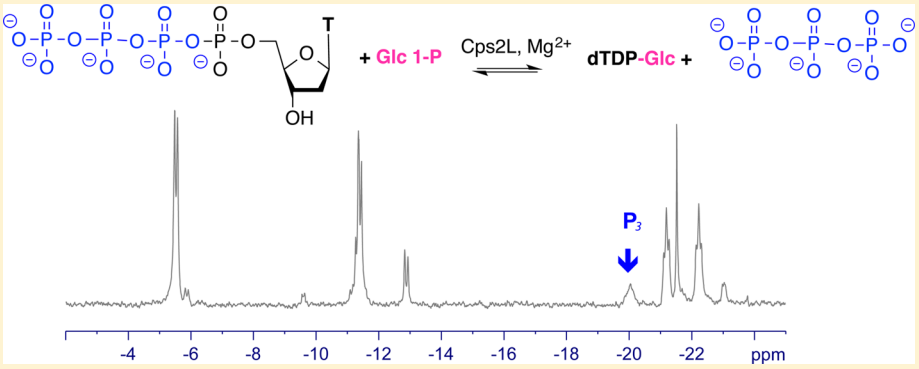
Mechanistic Evaluation of a Nucleoside Tetraphosphate with a Thymidylyltransferase

Stephanie M. Forget,[†] Deborah A. Smithen,[†] Alison Jee,[‡] and David L. Jakeman*[,†,‡]

[†]Department of Chemistry, Dalhousie University, P.O. Box 15000, Halifax, Canada B3H 4R2

[‡]College of Pharmacy, Dalhousie University, P.O. Box 15000, Halifax, Canada B3H 4R2

Supporting Information



ABSTRACT: Pyrimidine polyphosphates were first detected in cells 5 decades ago; however, their biological significance remains only partially resolved. Such nucleoside polyphosphates are believed to be produced nonspecifically by promiscuous enzymes. Herein, synthetically prepared deoxythymidine 5′-tetraphosphate (p_4dT) was evaluated with a thymidylyltransferase, Cps2L. We have identified p_4dT as a substrate for Cps2L and evaluated the reaction pathway by analysis of products using high-performance liquid chromatography, liquid chromatography and tandem mass spectrometry, and ^{31}P nuclear magnetic resonance spectroscopy. Product analysis confirmed production of dTDP-Glc and triphosphate (P_3) and showed no trace of dTTP-Glc and PP_i, which could arise from alternative pathways for the reaction mechanism.

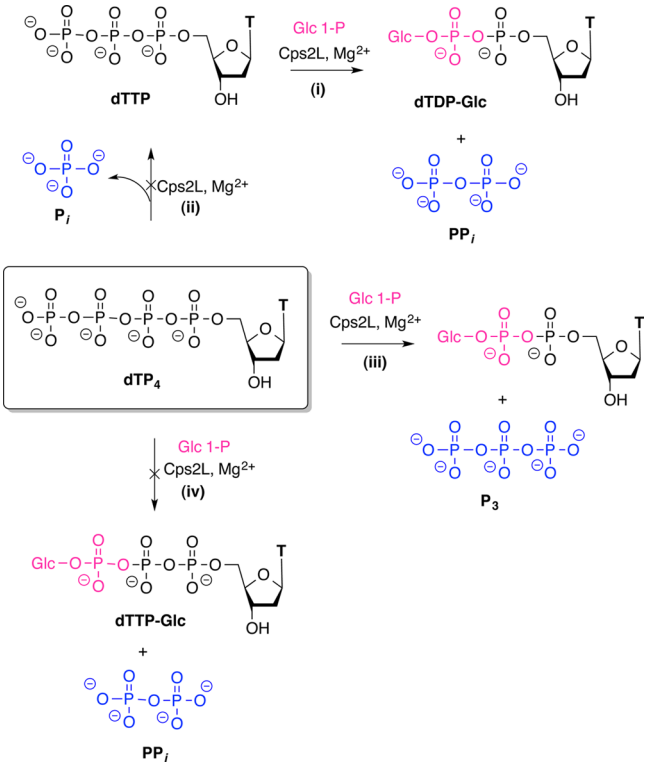
Nature utilizes the phosphate group in fundamental roles in almost all biological processes. The biological roles of naturally occurring mono- and dinucleoside polyphosphates (p_nN and Np_nN, where N is a nucleoside, p is phosphate, and n = 2−7) are not well understood. Although these compounds have been detected within cells at nanomolar concentrations, there remains a debate about the significance of these polyphosphates as they are believed to be the shunt products of promiscuous cellular reactions.[1] The most well-studied are the adenine nucleotides, Ap_nA, which have been linked to various intracellular processes and extracellular signaling pathways, but there have been fewer studies examining the roles of mononucleoside polyphosphates.[1−3] Adenosine 5′-tetraphosphate (p_4A) is present in human plasma and is among the most potent endogenous vasoconstrictors known.[4] Another study identified p_4A as a competitive inhibitor of tryptophanyl-tRNA synthetase, and analysis of a crystal structure suggested that p_4A bound in a manner that mimicked that of the catalytic transition state.[5] p_4A analogues in which select bridging phosphate oxygens have been substituted for methylene linkers were found to be inhibitors of (asymmetrical) dinucleoside tetraphosphatases.[6] Only a few reports have examined the abundance and function of pyrimidine polyphosphates. Pyrimidine dinucleoside polyphosphates have been detected in *Saccharomyces cerevisiae* and *Escherichia coli* and found to accumulate upon a shift in temperature and upon exposure to cadmium.[7]

In this study, we have examined the reaction of Cps2L (EC 2.7.7.24), a thymidylyltransferase cloned from *Streptococcus pneumoniae*, with a monopyrimidine polyphosphate, deoxythymidine 5′-tetraphosphate [p_4dT (Scheme 1)]. Physiologically, Cps2L catalyzes the coupling of α-D-glucose 1-phosphate (Glc 1-P) and deoxythymidine 5′-triphosphate (dTTP) to produce deoxythymidine 5′-diphospho-α-D-glucose (dTDP-Glc). The promiscuity of Cps2L[8−10] and other nucleotidylyltransferases[11−16] with respect to the sugar donor and nucleotide has previously been documented. Many aspects of the Cps2L mechanism, and more generally of nucleotidylyltransferase mechanisms, have previously been dissected.[17] The enzymatic reaction is widely accepted to proceed via an ordered Bi-Bi reaction mechanism, in which binding of dTTP precedes binding of the sugar phosphate.[8,18,19] The Michaelis−Menten kinetics of the physiological reaction and for various sugar phosphate analogues have been described previously.[20−22] This

Received: November 20, 2014
Revised: January 30, 2015
Published: February 3, 2015

DOI: 10.1021/bi501438p
Biochemistry 2015, 54, 1703−1707

Scheme 1. Catalysis by Cps2L[a]



[a](i) Physiological reaction and (ii–iv) plausible reaction pathways and products for the reaction between Glc 1-P and p_4dT catalyzed by Cps2L.

class of enzyme possesses an allosteric site with a purported role in regulation upon binding of deoxythymidine 5′-diphospho-L-rhamnose, a downstream product.[18] p_4dT contains an additional phosphate group relative to the physiological substrate, dTTP, which may provide new insights into the pathway of phosphate transfer in nucleotidylyltransferase reactions, in particular determining whether the active site can accommodate additional ionic charge and increased steric bulk. Herein, we discuss the possible pathways by which p_4dT may act as a Cps2L substrate and propose a reaction pathway based upon analysis using HPLC, LC–MS^2, and NMR spectroscopy.

EXPERIMENTAL PROCEDURES

General Methods. All reagents and anhydrous solvents were purchased from commercial suppliers and used as received unless otherwise indicated. Recombinant Cps2L was prepared as previously described.[23] NMR spectra were acquired on a Bruker 500 MHz NMR spectrometer at the Nuclear Magnetic Resonance Research Resources center located at Dalhousie University. ^1H NMR spectra were calibrated to D_2O (δ 4.79) as an internal reference; ^{31}P NMR spectra were calibrated to external phosphoric acid (δ 0). Splitting patterns are indicated as follows: br, broad; s, singlet; d, doublet; t, triplet; at, apparent triplet; q, quartet; m, multiplet. A Hach H160 portable pH meter equipped with a PH47-SS probe was used to measure the pH of solutions in NMR tubes. High-resolution mass spectra were recorded using ion trap (ESI TOF) instruments or recorded on an API Qstar XL pulsar hybrid LC/MS/MS instrument (System Applied Biosystem/MDS Sciex). Low-resolution mass spectra were obtained using an Applied Biosystems hybrid triple-quadrupole linear ion trap (Qtrap 2000) mass spectrometer equipped with an electrospray ionization (ESI) source. The capillary voltage was set to −4500 kV with a declustering potential of −60 V, and the curtain gas was set to 10 (arbitrary units). The mass spectrometer was coupled to an Agilent 1100 HPLC instrument fitted with a Phenomenex Kinetex 2.6 μ (150 mm × 2.10 mm) that was run using an isocratic method with a 70/30 CH_3CN/2 mM aqueous ammonium acetate (pH 5.5) mixture at a flow rate of 120 μL min^{-1}. Enzymatic reactions were analyzed using reversed-phase HPLC performed on a Hewlett-Packard Series 1050 instrument using an Agilent Zorbax 5 μm Rx-C18 column (150 cm × 4.6 mm). Compounds bearing a nucleobase chromophore were monitored at an absorbance of 254 nm using a previously described method.[23]

Deoxythymidine Tetraphosphate Tributylammonium Bicarbonate Salt (p_4dT). Activated 4 Å molecular sieves were added to separate solutions of deoxythymidine 5′-monophosphate (dTMP, NBu_4 salt, 330 mg, 0.50 mmol) in anhydrous DMF (4 mL) and trimetaphosphate (NBu_4 salt, 726 mg, 0.75 mmol) in anhydrous DMF (6 mL), while being gently stirred under nitrogen for 2 h. *N*-Methylimidazole (0.2 mL, 2.5 mmol) and 1-phenylsulfonyl-2,3-dimethylimidazolium triflate (234 mg, 0.6 mmol) were then added to the trimetaphosphate solution, while it was being stirred at room temperature under nitrogen for 25 min. The resulting mixture was then added dropwise, over 5 min, to the dTMP solution at 0 °C, while it was being stirred under nitrogen for 4 h and warmed to room temperature, before being cooled to 0 °C and quenched with a 0.05 M aqueous triethylammonium acetate solution (10 mL). The reaction mixture was then poured into water (10 mL), washed with chloroform (3 × 20 mL), and concentrated to give the crude product, which was purified using reversed-phase flash chromatography (Biotage purification system) over C18 silica, eluting with 25 to 65% MeOH/buffer (10 mM aqueous tributylammonium bicarbonate) over 25 column volumes at a flow rate of 4 mL/min. The major product-containing fractions (eluting at ∼50% MeOH/buffer) were then combined and concentrated to a volume of ∼5 mL and lyophilized to give the product (p_4dT·5HNBu$_3$, 159 mg, 21% yield) as a white solid. Spectral data matched literature data:[24] ^1H NMR (D_2O, 500 MHz) δ 7.83 (s, 1H), 6.40 (t, 1H, J = 7.0 Hz), 4.74–4.73 (m, 1H), 4.32–4.22 (m, 3H), 3.19–3.16 (m, 30H, salt), 2.46–2.35 (m, 2H), 1.98 (s, 3H), 1.74–1.68 (m, 30H, salt), 1.46–1.38 (m, 30H, salt), 0.98 (t, 45H, J = 7.3 Hz, salt); ^{13}C NMR (D_2O, 175 MHz) δ 167.9, 153.1, 138.7, 113.1, 86.9 (d, J = 9.3 Hz), 86.2, 72.4, 66.9 (d, J = 5.6 Hz), 54.0 (CH_2, salt), 39.9, 26.5 (CH_2, salt), 20.6 (CH_2, salt), 14.1 (CH_3, salt), 13.0; $^{31}P\{^1H\}$ NMR (D_2O, 202.4 MHz) δ 11.0 (d, 1P, J = 18 Hz), 11.9 (d, 1P, J = 18 Hz), 23.2–23.6 (m, 2P); LRMS 560.9 $(M - H)^-$; HRMS m/z 560.9477 found, m/z 560.9483 calcd for $C_{10}H_{17}N_2P_4O_{17}$.

HPLC Assays and Specific Activity. HPLC instrumentation and methods were as described previously.[10] The specific activity was measured from a reaction mixture containing p_4dT or dTTP (1 mM), Glc 1-P (2 mM), $MgCl_2$ (1.1 mM), Cps2L (0.95 μM), and IPP (0.5 EU). The volume was increased to 50 μL using Tris-HCl (20 mM stock solution at pH 7.5). The final sample pH was 7.5. Reactions were quenched by the addition of MeOH (10 μL) to withdrawn reaction mixture aliquots (10 μL) at 1, 2, and 5 min. Specific activity was defined in terms of enzyme units (EU) as the amount of product produced (millimoles) per unit time (1 min) per unit volume (1 mL) of

DOI: 10.1021/bi501438p
Biochemistry 2015, 54, 1703–1707

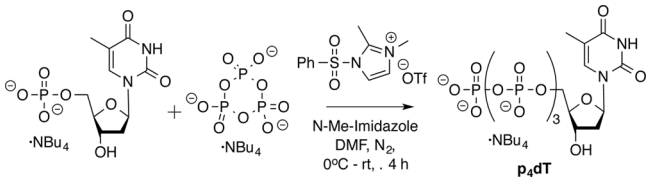
enzyme solution. The percent conversion was determined by integration of the HPLC trace as follows: % conversion = (area under the product peak)/(area under the product peak + area under the p_4dT peak).

^{31}P NMR Experiments. A reaction mixture contained p_4dT (5 mM), Glc 1-P (5 mM), $MgCl_2$ (100 μM), D_2O (10% volume), and Cps2L (95 μM). The volume was increased to 500 μL using Tris-HCl (20 mM stock solution at pH 7.5). The final sample pH was 7.0. For reaction progress determination using HPLC analysis at λ_{254}, 10 μL of the reaction mixture was quenched with 10 μL of MeOH and then centrifuged at 14000 rpm for 5 min. After incubation at 37 °C for 5 h, conversion of 25% to dTDP-Glc was achieved. The production of dTDP-Glc was confirmed by LC–MS^2 analysis. The reaction mixture was transferred to a Shigemi NMR tube. ^{31}P{^{1}H} NMR spectra were recorded for 256 scans at pH 7, 8.7, 9.2, and 9.6, and 3200 scans at pH 10; the pH of the solution was adjusted by titration with aqueous NaOH (0.2 M). Finally, the reaction mixture was spiked with P_3 (2 mM), and an additional NMR spectrum was recorded.

RESULTS AND DISCUSSION

The synthesis of p_4dT was accomplished by coupling dTMP with an activated phenylsulfonylimidazolium salt of trimetaphosphate, following a procedure described by Mohamady et al. (Scheme 2).[25] Reversed-phase (C18) column chromatography facilitated isolation of p_4dT in a 21% yield.

Scheme 2. Synthesis of p_4dT



Three pathways delineating possible outcomes for the activity of p_4dT as a Cps2L substrate are illustrated in Scheme 1. In the first proposed pathway (ii, Scheme 1), p_4dT is hydrolyzed, potentially enzymatically, releasing monophosphate (P_i) and dTTP, which then reacts with Glc 1-P to produce dTDP-Glc in the same manner as the physiological reaction (i, Scheme 1). A second pathway (iii, Scheme 1) would proceed via the reaction of p_4dT and Glc 1-P to produce dTDP-Glc, releasing triphosphate (P_3); this requires Glc 1-P to attack the α-phosphorus of p_4dT, resulting in departure of P_3, a process that closely resembles the accepted reaction mechanism.[18] Finally, the third scenario (iv, Scheme 1) would produce an alternate sugar nucleotide, dTTP-Glc, which would produce deoxythymidine 5′-triphospho-α-D-glucose (dTTP-Glc) from the attack of Glc 1-P at the β-phosphorus of p_4dT, releasing diphosphate (PP_i), a product released in the physiological reaction.

To confirm the activity of p_4dT as a Cps2L substrate, a reaction with Glc 1-P was monitored by HPLC.[23] While the reaction did not reach completion, a new signal with a retention time (t_R) of 5.6 min emerged in the HPLC trace recorded at 1 h, and an accumulation of this product was observed over 5 h, whereupon integration of the traces indicated 25% conversion (Figure 1). The t_R of the observed product matched that of a dTDP-Glc standard (Figure SI1 of the Supporting Information), and furthermore, LC–MS^2 analysis of the reaction

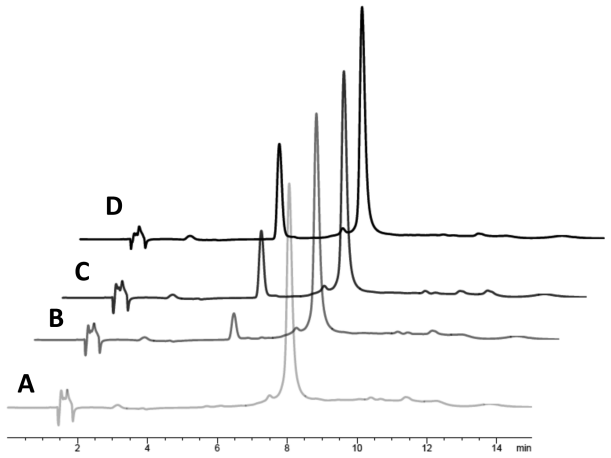


Figure 1. Thymidylyltransferase-catalyzed production of dTDP-Glc (t_R = 5.7 min) from p_4dT (t_R = 8.0 min) and Glc 1-P: (A) 1 min, dTDP-Glc (0%); (B) 1 h, dTDP-Glc (8%); (C) 3 h, dTDP-Glc (19%); and (D) 5 h, dTDP-Glc (25%). The sample comprised p_4dT (5 mM), Glc 1-P (5 mM), $MgCl_2$ (100 μM), D_2O (10% volume), and Cps2L (95 μM) in buffered H_2O.

mixture confirmed the product mass and characteristic fragmentation expected for dTDP-Glc (Figure SI2 of the Supporting Information); these data ruled out pathway iv, as no evidence of dTTP-Glc was observed. We measured the specific activities of dTTP and p_4dT under the same conditions; a specific activity of 0.2 EU was found for dTTP and 0.02 EU for p_4dT. Thus, the rate of turnover to product was reduced 10-fold for p_4dT in comparison to that of dTTP.

To distinguish whether pathway ii or iii resulted in the production of dTDP-Glc, ^{31}P NMR spectra documenting the reaction progress were recorded to analyze the reaction products. The detection of P_i and PP_i in the reaction mixture would indicate that hydrolysis of p_4dT to dTTP was occurring (pathway ii). Alternatively, the detection of P_3 would confirm that p_4dT was acting as a substrate (pathway iii). All samples used for NMR analysis were prepared with 100 μM Mg^{2+} (an essential cofactor), which resulted in an approximately 100-fold reduction in rate compared to that of a reaction mixture containing the standard 1 mM Mg^{2+}, with a measured specific activity of 0.0003 EU. The 10-fold reduction in magnesium concentration overcame broadening of the ^{31}P signals caused by the presence of Mg^{2+}. However, signal overlap (Figure 2D) rendered the ambiguous distinction of PP_i or P_3 from p_4dT signals (Figure 2D) when the reaction mixture was analyzed directly at pH 7.0.

To address the issue of signal overlap in ^{31}P NMR spectra, solutions were titrated from pH 7 to 10 using aqueous NaOH (0.2 M). Spectra showing the changes in chemical shift as a function of pH are shown in the Figure SI3 of the Supporting Information. In a standard sample (Figure 2A), it is apparent that PP_i and P_3 shifts are well-resolved at pH 10. Adjustment of the reaction sample (Figures 1D and 2D) to pH 10 deconvoluted the signals between δ −20 and −25, allowing the identification of the β-phosphorus P_3 signal at δ −20. Spiking the solution with P_3 resulted in an increase in intensity of the β-phosphorus signal at δ −20 and an increase in the intensity of the α-phosphorus signal at δ −5. A similar analysis of the physiological reaction mixture, whereupon the same NMR experiments were run to observe the products from the reaction of dTTP with Glc 1-P, clearly showed the production

DOI: 10.1021/bi501438p
Biochemistry 2015, 54, 1703–1707

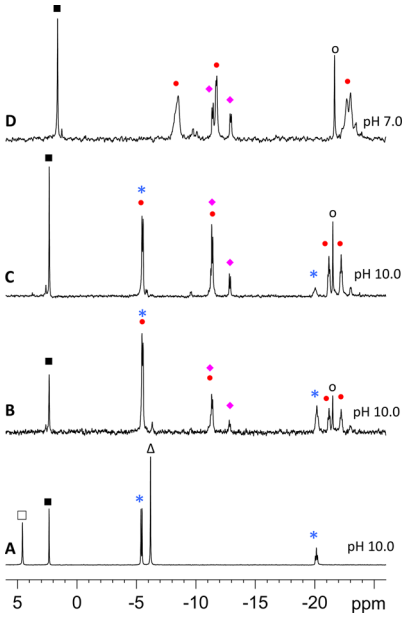


Figure 2. Thymidylyltransferase-catalyzed production of triphosphate (P_3) from p_4dT. $^{31}P\{^1H\}$ NMR (202 MHz, 10% D_2O in H_2O) spectra. Spectra A–C were acquired with 256 scans, while spectrum D was acquired with 3200 scans. (A) Reference sample containing ~5 mM P_3, PP_i, P_i, and Glc 1-P at pH 10 with 95 μM Cps2L. (B) Same as panel C with the addition of 2 mM P_3. (C) Reaction progress at 5 h recorded after adjustment to pH 10. (D) Same sample that was used for Figure 1D. Reaction progress recorded at 5 h at pH 7: (□) P_i, (■) Glc 1-P, (∗) P_3, (△) PP_i, (red circle) p_4dT, (red diamond) dTDP-Glc, and (○) trimetaphosphate (internal reference). The signal at δ −6.2 in spectrum B is a small amount of PP_i that is present in the commercial P_3.

of PP_i at δ −6 (Figure SI4 of the Supporting Information). An additional HPLC experiment demonstrating the stability of p_4dT in the presence and absence of Cps2L over a time period of 24 h demonstrated a maximal breakdown to dTTP of 6%, an observation that offers further evidence against pathway ii (Figure SI5 of the Supporting Information). The reverse reaction of a uridylyltransferase to produce nucleoside polyphosphates has been described previously;[6] uridine 5′-tetraphosphate and uridine 5′-pentaphosphate were enzymatically synthesized by the reaction of uridine 5′-diphophonoglucose with P_3 or tetraphosphate using a uridylyltransferase from *S. cerevisiae*.

CONCLUSIONS

To the best of our knowledge, this study represents the first pathway analysis of a thymidylyltransferase with a nucleoside tetraphosphate substrate. It demonstrates that p_4dT serves as a Cps2L substrate, with a 10-fold decrease in activity compared to that of the physiological reaction, and strongly supports a pathway in which a nucleophilic phosphate attacks the α-phosphate of p_4dT releasing P_3. This demonstrates that Cps2L can accommodate an additional phosphate group in its active site while maintaining catalytic proficiency. This discovery may have implications for the design of nucleotidylyltransferase inhibitors and other enzymes capable of recognizing nucleoside polyphosphates.

ASSOCIATED CONTENT

Supporting Information

HPLC and NMR spectra for p_4dT. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: david.jakeman@dal.ca.

Author Contributions

S.M.F. and D.A.S. contributed equally to this work.

Funding

This work was supported in part by the Natural Sciences and Engineering Research Council of Canada and the Canadian Institutes of Health Research.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We are grateful to an anonymous referee for their diligent and insightful comments.

ABBREVIATIONS

Bu, *n*-butyl; Cps2L, thymidylyltransferase from *St. pneumoniae*; DMF, dimethylformamide; dTDP-Glc, deoxythymidine 5′-diphospho-α-D-glucose; dTMP, deoxythymidine 5′-monophosphate; dTTP, deoxythymidine 5′-triphosphate; dTTP-Glc, deoxythymidine 5′-triphospho-α-D-glucose; ESI, electrospray ionization; EU, enzyme units; Glc 1-P, α-D-glucose 1-phosphate; HPLC, high-performance liquid chromatography; IPP, inorganic pyrophosphatase; LC, liquid chromatography; MS^2, tandem mass spectrometry; NMR, nuclear magnetic resonance; P_3, triphosphate; p_4A, adenosine 5′-tetraphosphate; p_4dT, deoxythymidine 5′-tetraphosphate; P_i, monophosphate; PP_i, diphosphate; t_R, retention time; TOF, time-of-flight; Tris, tris(hydroxymethyl)amino methane

REFERENCES

(1) Fraga, H., and Fontes, R. (2011) Enzymatic synthesis of mono and dinucleoside polyphosphates. *Biochim. Biophys. Acta 1810*, 1195–1204.

(2) McLennan, A. G. (2000) Dinucleoside polyphosphates: Friend or foe? *Pharmacol. Ther. 87*, 73–89.

(3) McLennan, A. G., Barnes, L. D., Blackburn, G. M., Brenner, C., Guranowski, A., Miller, A. D., Rovira, J. M., Rotllan, P., Soria, B., Tanner, J. A., and Sillero, A. (2001) Recent progress in the study of the intracellular functions of diadenosine polyphosphates. *Drug Dev. Res. 52*, 249–259.

(4) Toelle, M., Jankowski, V., Schuchardt, M., Wiedon, A., Huang, T., Hub, F., Kowalska, J., Jemielity, J., Guranowski, A., Loddenkemper, C., Zidek, W., Jankowski, J., and van der Giet, M. (2008) Adenosine 5′-Tetraphosphate Is a Highly Potent Purinergic Endothelium-Derived Vasoconstrictor. *Circ. Res. 103*, 1100–1108.

(5) Retailleau, P., Weinreb, V., Hu, M., and Carter, C. W., Jr. (2007) Crystal Structure of Tryptophanyl-tRNA Synthetase Complexed with Adenosine 5′-Tetraphosphate: Evidence for Distributed Use of Catalytic Binding Energy in Amino Acid Activation by Class I Aminoacyl-tRNA Synthetases. *J. Mol. Biol. 369*, 108–128.

(6) Guranowski, A., Starzynska, E., Pietrowska-Borek, M., Jemielity, J., Kowalska, J., Darzynkiewicz, E., Thompson, M., and Blackburn, G. (2006) Methylene analogues of adenosine 5′-tetraphosphate: Their chemical synthesis and recognition by human and plant mononucleoside tetraphosphatases and dinucleoside tetraphosphatases. *FEBS J. 273*, 829–838.

DOI: 10.1021/bi501438p
Biochemistry 2015, 54, 1703–1707

(7) Coste, H., Brevet, A., Plateau, P., and Blanquet, S. (1987) Non-adenylylated bis(5′-nucleosidyl) tetraphosphates occur in *Saccharomyces cerevisiae* and in *Escherichia coli* and accumulate upon temperature shift or exposure to cadmium. *J. Biol. Chem. 262*, 12096−12103.
(8) Forget, S. M., Bhattasali, D., Hart, V. C., Cameron, T. S., Syvitski, R. T., and Jakeman, D. L. (2012) Synthesis and enzymatic evaluation of ketose phosphonates: The interplay between mutarotation, monofluorination and acidity. *Chem. Sci. 3*, 1866−1878.
(9) Timmons, S. C., Hui, J. P., Pearson, J. L., Peltier, P., Daniellou, R., Nugier-Chauvin, C., Soo, E. C., Syvitski, R. T., Ferrieres, V., and Jakeman, D. L. (2008) Enzyme-catalyzed synthesis of furanosyl nucleotides. *Org. Lett. 10*, 161−163.
(10) Loranger, M. W., Forget, S. M., McCormick, N. E., Syvitski, R. T., and Jakeman, D. L. (2013) Synthesis and evaluation of L-rhamnose-1C-phosphonates as nucleotidylyltransferase inhibitors. *J. Org. Chem. 78*, 9822−9833.
(11) Barton, W. A., Biggins, J. B., Jiang, J., Thorson, J. S., and Nikolov, D. B. (2002) Expanding pyrimidine diphosphosugar libraries via structure-based nucleotidylyltransferase engineering. *Proc. Natl. Acad. Sci. U.S.A. 99*, 13397−13402.
(12) Jiang, J. Q., Biggins, J. B., and Thorson, J. S. (2000) A general enzymatic method for the synthesis of natural and "unnatural" UDP- and TDP-nucleotide sugars. *J. Am. Chem. Soc. 122*, 6803−6804.
(13) Bae, J., Kim, K. H., Kim, D., Choi, Y., Kim, J. S., Koh, S., Hong, S. I., and Lee, D. S. (2005) A practical enzymatic synthesis of UDP sugars and NDP glucoses. *ChemBioChem 6*, 1963−1966.
(14) Zhang, Z., Tsujimura, M., Akutsu, J., Sasaki, M., Tajima, H., and Kawarabayasi, Y. (2005) Identification of an extremely thermostable enzyme with dual sugar-1-phosphate nucleotidylyltransferase activities from an acidothermophilic archaeon, *Sulfolobus tokodaii* strain 7. *J. Biol. Chem. 280*, 9698−9705.
(15) Moretti, R., Chang, A., Peltier-Pain, P., Bingman, C. A., Phillips, G. N., Jr., and Thorson, J. S. (2011) Expanding the Nucleotide and Sugar 1-Phosphate Promiscuity of Nucleotidyltransferase RmlA via Directed Evolution. *J. Biol. Chem. 286*, 13235−13243.
(16) Mizanur, R. M., Zea, C. J., and Pohl, N. L. (2004) Unusually broad substrate tolerance of a heat-stable archaeal sugar nucleotidyltransferase for the synthesis of sugar nucleotides. *J. Am. Chem. Soc. 126*, 15993−15998.
(17) Singh, S., Phillips, G. N., Jr., and Thorson, J. S. (2012) The structural biology of enzymes involved in natural product glycosylation. *Nat. Prod. Rep. 29*, 1201−1237.
(18) Blankenfeldt, W., Asuncion, M., Lam, J. S., and Naismith, J. H. (2000) The structural basis of the catalytic mechanism and regulation of glucose-1-phosphate thymidylyltransferase (RmlA). *EMBO J. 19*, 6652−6663.
(19) Melo, A., and Glaser, L. (1965) The nucleotide specificity and feedback control of thymidine diphosphate D-glucose pyrophosphorylase. *J. Biol. Chem. 240*, 398−405.
(20) Forget, S. M., Jee, A., Smithen, D. A., Jagdhane, R., Anjum, S., Beaton, S. A., Palmer, D. R., Syvitski, R. T., and Jakeman, D. L. (2015) Kinetic evaluation of glucose 1-phosphate analogues with a thymidylyltransferase using a continuous coupled enzyme assay. *Org. Biomol. Chem. 13*, 866−875.
(21) Ko, K. S., Zea, C. J., and Pohl, N. L. (2004) Surprising bacterial nucleotidyltransferase selectivity in the conversion of carbaglucose-1-phosphate. *J. Am. Chem. Soc. 126*, 13188−13189.
(22) Ko, K. S., Zea, C. J., and Pohl, N. L. (2005) Strategies for the chemoenzymatic synthesis of deoxysugar nucleotides: Substrate binding versus catalysis. *J. Org. Chem. 70*, 1919−1921.
(23) Timmons, S. C., Mosher, R. H., Knowles, S. A., and Jakeman, D. L. (2007) Exploiting nucleotidylyltransferases to prepare sugar nucleotides. *Org. Lett. 9*, 857−860.
(24) Kore, A. R., Senthilvelan, A., Srinivasan, B., and Shanmugasundaram, M. (2013) Facile protection-free one-pot chemical synthesis of nucleoside-5′-tetraphosphates. *Nucleosides, Nucleotides Nucleic Acids 32*, 411−420.
(25) Mohamady, S., Desoky, A., and Taylor, S. D. (2012) Sulfonyl imidazolium salts as reagents for the rapid and efficient synthesis of nucleoside polyphosphates and their conjugates. *Org. Lett. 14*, 402−405.

DOI: 10.1021/bi501438p
Biochemistry 2015, 54, 1703−1707