Enzyme-catalyzed synthesis of isosteric phosphono-analogues of sugar nucleotides†

Stephen A. Beaton,[a] Malcolm P. Huestis,[b] Ali Sadeghi-Khomami,[bc] Neil R. Thomas[c] and David L. Jakeman*[ab]

Received (in Cambridge, UK) 13th May 2008, Accepted 20th October 2008
First published as an Advance Article on the web 18th November 2008
DOI: 10.1039/b808078j

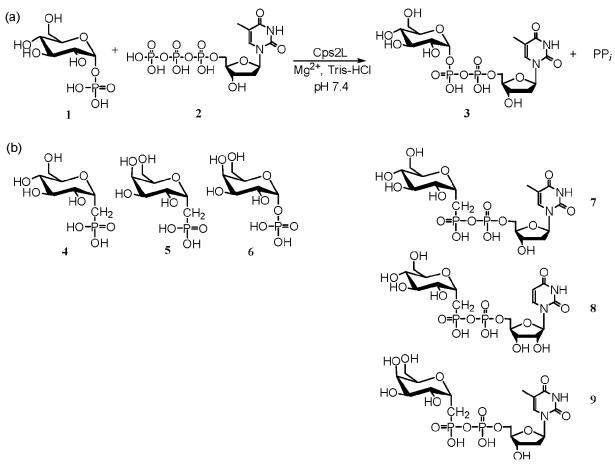
Efficient enzymatic syntheses of isosteric phosphono analogues of sugar nucleotides have been accomplished using a thymidylyltransferase.

Phosphonates are structural mimics of phosphates that have found utility in probing a diverse array of biological processes including the development of clinical antiretroviral and osteoporosis drugs.[1,2] The replacement of a labile phosphate linkage with a hydrolytically more stable phosphonate[3] has facilitated the study of a variety of enzymes catalyzing phosphate bond cleavage[4–8] including enzymes where a transient cleavage of the phosphate occurs during catalysis.[9] Phosphonates have also been developed as inhibitors of ester hydrolysis[10,11] or as reagents for proteomic profiling of serine proteases.[12]

Glycosyltransferases reversibly[13–15] catalyze the transfer of a donor sugar to an acceptor substrate with the concomitant release of a pyrophosphoryl moiety, often in the form of a nucleoside diphosphate,[16] although reversibility for mammalian transferases has yet to be demonstrated. The ability to control glycosylation by developing glycosyltransferase inhibitors will facilitate entry into new classes of therapeutics for a wide variety of diseases.[17] Replacing the anomeric oxygen substituent with a methylene unit in a sugar nucleotide provides an isosteric, non-hydrolyzable, phosphono sugar nucleotide analogue that acts as a mechanism-based inhibitor where glycosyl transfer is compromised. Sugar nucleotide analogues of this type have been shown to be competitive inhibitors of glycosyltransferases[18,19] and surrogate substrates for sugar nucleotide processing enzymes, particularly if the corresponding deoxysugar nucleotide is labile.[20] Many glycosyltransferases are bi-lobal enzymes that undergo significant conformational changes upon catalysis,[21] necessitating access to non-scissile structural analogues of the enzyme substrates to probe enzyme function. Synthetic access to appropriate phosphono sugar nucleotides is limited by the need to perform phosphonate–phosphate coupling steps, reactions that are even more sluggish and low yielding than phosphate–phosphate couplings for the preparation of sugar nucleotides.[20] We envisage the importance of phosphonate analogues of sugar nucleotides to increase as the field of glycobiology develops and additional glycosyltransferase targets are identified.[22,23] We hypothesize that nucleotidylyltransferases will be efficient catalysts for the formation of phosphono analogues of sugar nucleotides. Our study is the first to examine the interactions of phosphono analogues of α-D-glucose-1-phosphate (**4**) and α-D-galactose-1-phosphate (**5**) with nucleoside 5′-triphosphates and a recombinant nucleotidylyltransferase to form isosteric non-hydrolyzable phosphono sugar nucleotides.

The synthesis of **4** was accomplished essentially as described by Nicotra in five facile steps (44%).[24] A similar synthetic route was proposed and executed for the synthesis of **5** in seven steps (22%, ESI†).

Physiologically, Cps2L catalyzes condensation of α-Dglucose-1-phosphate (**1**) and deoxythymidine triphosphate (**2**) to yield dTDP-glucose (**3**, Scheme 1). A series of assays involving **4** and **5** and five nucleoside triphosphates as substrates were used to evaluate the substrate specificity of Cps2L thymidylyltransferase. The enzymatic reactions were carried out using conditions described previously.[25] The enzyme (2 EU) was incubated with phosphonate **4** or **5** (2 mM), $MgCl_2$ (2.2 mM), and dTTP (1 mM) at 37 °C for 24 h.[26] HPLC analysis of quenched aliquots was used to confirm the formation of product based on conversion of NTP. Control reactions were performed, and in the absence of any one reagent, no sugar nucleotide product was formed. Product formation



Scheme 1 (a) Primary physiological metabolic reaction catalyzed by Cps2L; (b) substrates and products examined in this study.

[a] *Department of Chemistry, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4J3*
[b] *College of Pharmacy, Burbidge Building, Dalhousie University, 5968 College St., Halifax, Nova Scotia, Canada B3H 3J5. E-mail: david.jakeman@dal.ca; Fax: 1 902 494 1396; Tel: 1 902 494 7159*
[c] *School of Chemistry, Centre for Biomolecular Sciences, University of Nottingham, Nottingham, England NG7 2RD*

† Electronic supplementary information (ESI) available: Experimental details, characterization of synthetic products, HPLC, MS/MS data and kinetic plot. See DOI: 10.1039/b808078j

was confirmed by ESI-MS/MS by observing characteristic fragmentation patterns of product ions. Apparent kinetic parameters K_m and k_{cat} for the sugar-1-phosphate analogues were determined by varying phosphonate concentrations and monitoring by discontinuous HPLC using methods described previously.[27]

The incubation of **4** with either dTTP or UTP after 30 min afforded conversions of 95% and 70%, generating products **7** and **8**, respectively, indicating that **4** was readily accepted as an alternative substrate (Fig. 1). Conversely, incubation of Cps2L with **5** and dTTP or UTP produced only **9** (>40%). Significant product was also obtained when assays containing ATP, CTP, or GTP with **4** were conducted although yields remained below 20%, even after extended incubation. These enzymatic conversions are significantly greater yielding, more timely and experimentally more convenient than chemical phosphate–phosphonate coupling reactions using morpholidate-[20] or our attempts using *N*-methylimidazole activation strategies for nucleoside monophosphates.[28] A scaled-up enzymatic reaction containing **4** and dTTP was performed. Initial purification of the product **7** was challenging due either to the presence of salt or to the basic pH that caused the breakdown of the product. A similar instability has been observed by Lowary and co-workers with sugar nucleotides.[29] However, using a weakly acidic ion-pair buffer (tributyl ammonium bicarbonate, pH 6) as we have reported previously for the reversed-phase separation of sugar nucleotides,[30] followed by cation exchange and gel filtration, we were able to purify **7** with minimal loss or degradation of product (Fig. 2).

Apparent kinetic parameters were determined for Cps2L with respect to **4–6** in the forward direction upon coupling with dTTP (Table 1). The K_m values were similar for **4–6**, and are within the same range as previously reported values of K_m for α-D-Glc*p*-1-P and even β-L-Ara*f*-1-P.[27] This indicates that the formation of an activated Michaelis complex is accomplished approximately equally. Similar trends in Michaelis parameters have been shown with a series of deoxyglucose-1-phosphates and a related nucleotidylyltransferase; Pohl observed limited differences between

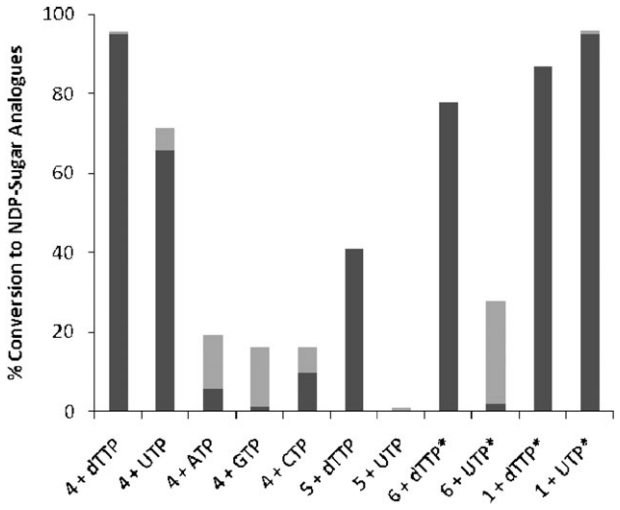


Fig. 1 Substrate conversions by Cps2L after 30 min (•) and 24 h (◦) incubations at 37 °C. (*ref. 27).

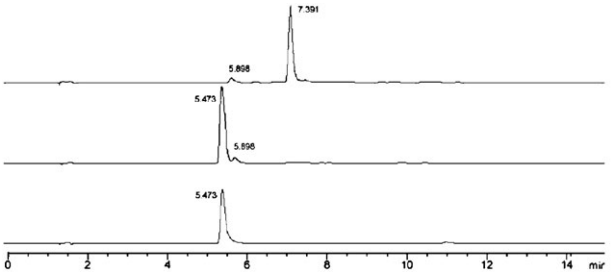


Fig. 2 Reversed-phase ion-pair HPLC traces. HPLC trace for the dTTP control void of enzyme (***top***); the enzyme coupled reaction of **4** and dTTP (***middle***), and the final purified product, **7** (***bottom***).

substrate binding, yet significantly different deoxysugar nucleotide yields.[31] Relative to the physiological substrate (**1**), **4** exhibited a 140-fold decrease in k_{cat}, **5** a 3200-fold decrease and **6** a 8200-fold decrease. Thus the difference between conversions for all these enzymatic reactions lies within the k_{cat} value.

It is known that phosphonate analogues have a wider bond angle and shorter bond lengths between the C–O–P atoms and the C–C–P atoms, respectively.[3] By titration, we determined that the pK_a2 of phosphonate (**4**) was half a unit higher than the physiological substrate (**1**). These geometric and ionization changes presumably account for the decrease in turnover efficiency for **4** *versus* **1**, but this modification has less effect on the levels of conversion than the change in stereochemistry at C4 (**1** *versus* **6**). This suggests that the stereochemistry at C4 is of greater importance in terms of the overall catalytic efficiency than the ionization state of the phosphoryl centre; thus the configuration of the sugar affects the reactivity more than the electron density on the phosphoryl centre. This is corroborated by the fact that an even lower k_{cat} was observed for a furanosyl sugar phosphate, and highlights the importance of sugar stereochemistry.[27]

Two contrasting observations result from the comparison between the phosphono sugar-1-phosphate analogues relative to the parent sugar-1-phosphates. First, the phosphonate **4** has a lower turnover efficiency in comparison to **1**, and second, the phosphonate **5** has a higher turnover efficiency, relative to **6**. It is interesting that a stereochemical change at C4, distant from the reactive site, can partially be countered by modification of the phosphate moiety (**5**). Potentially, the change in stereochemistry at C4 may perturb the trajectory of the phosphate, and replacing the anomeric oxygen with a methylene unit may partially realign the nucleophile trajectory as a result of the differences in geometry of the phosphonate. Increases in pK_a2 values were also observed for a series of 3-*O*-alkyl glucose-1-phosphate Cps2L substrates, indicating that the enzyme is capable of accepting substrates with modified pK_a2 values.[32] Our results clearly demonstrate that the anomeric oxygen substituent is not critical for nucleotidylyltransferase activity and further elaboration at this site will potentially result in structurally and functionally important sugar nucleotide analogues.

A study on the conversion of carbaglucose-1-phosphate using a catalytically analogous nucleotidylyltransferase revealed that the carbocyclic analogue of the physiological substrate displayed a 725-fold decrease in k_{cat}/K_m with respect

Table 1 Apparent kinetic parameters for Cps2L substrates with dTTP

Parameter	**1**[27]	**4**	**5**	**6**	β-L-Ara*f*-1-P[27]
V_{max} (μM min^{-1})	2.818	0.4321	0.493	0.1022	0.2801
K_m (μM)	139	124	176	107	90
k_{cat} (min^{-1})	27.6	0.198	0.00849	0.00335	0.00218
k_{cat}/K_m (μM^{-1} min^{-1})	0.199	0.00166	0.0000483	0.0000313	0.0000242

to the physiological substrate.[33] In contrast, the phosphonate analogue **4**, exhibited a 120-fold decrease in k_{cat}/K_m. The presence of the methylene functionality as a non-scissile isosteric analogue of the glycosidic linkage in these sugar nucleotide analogues (**7–9**), and the facile access to these compounds by enzymatic coupling, will enable their use as substrates for subsequent sugar nucleotide processing enzymes to generate specific isosteric glycosyltransferase inhibitors.

In conclusion, nucleotidylyltransferases have been shown to solve the issues of low yields and sluggish reaction times for the formation of phosphono analogues of sugar nucleotides. Cps2L is the first nucleotidylyltransferase to couple dTTP and UTP nucleotides with a phosphonate analogue of glucose-1-phosphate to produce phosphono analogues of dTDP- and UDP-sugars. It is also capable of producing a phosphono analogue of dTDP-Gal. The enzymatic production of these products indicate that increases in the second ionization constant (pK_a2) does not significantly hinder the catalytic activity. In the case of the galacto-configured substrates, the phosphonate is a better substrate than the phosphate. Furthermore, the ready conversion of the phosphono analogue of glucose-1-phosphate to phosphono sugar nucleotide analogues indicates the potential of efficient enzymatic transformations to generate novel glycosyltransferase probes.

We thank the Canadian Institutes of Health Research, the Natural Sciences and Engineering Research Council, The Royal Society (UK) and the Mizutani Glycoscience Foundation of Japan for funding. We thank Jessica Pearson for purification of the enzyme.

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26 Abbreviations: dTTP, deoxythymidine triphosphate; UTP, uridine triphosphate; ATP, adenosine triphosphate; CTP, cytidine triphosphate; GTP, guanosine triphosphate; dTDP, deoxythymidine diphosphate; α-D-Glc-1-P, α-D-glucose-1-phosphate; α-D-Gal-1-P, α-D-galactose-1-phosphate; β-L-Ara*f*-1-P, β-L-arabinofuranose-1-phosphate; EU, Enzyme Unit.
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