A General Enzymatic Method for the Synthesis of Natural and "Unnatural" UDP- and TDP-Nucleotide Sugars

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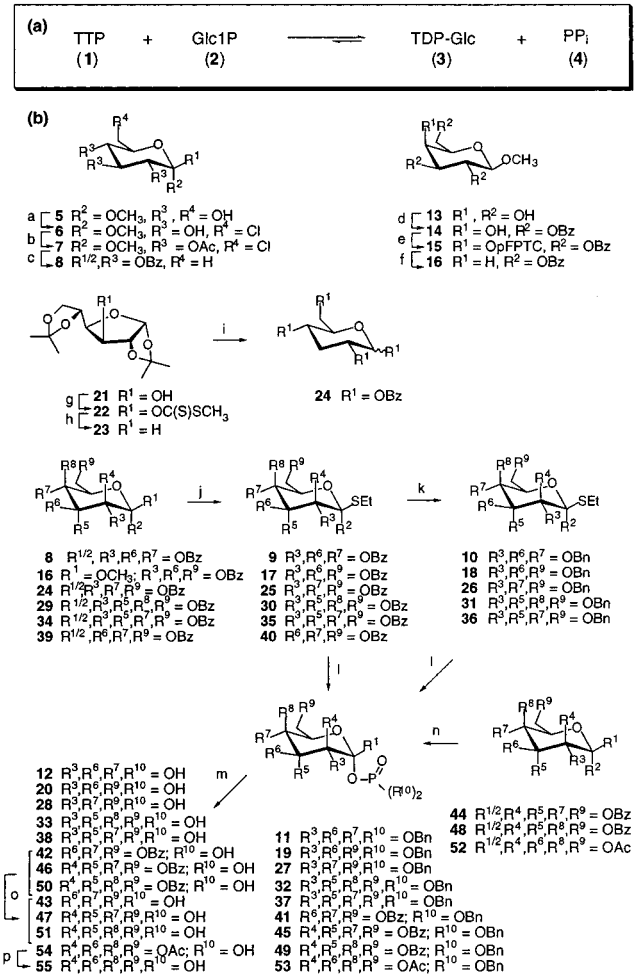
Received April 26, 2000

Many bioactive metabolites possess unusual carbohydrates required for molecular recognition.[1] The glycosyltransferases which incorporate these essential ligands are known to rely almost exclusively upon UDP- and TDP-nucleotide sugars and some have demonstrated promiscuity toward the sugar donor.[2] These discoveries have led to the exploitation of the carbohydrate biosynthetic machinery to manipulate metabolite glycosylation,[3] revitalizing interest in methods to expand the repertoire of available UDP- and TDP-sugar nucleotides.[4] We now report that a substrate specificity reevaluation of *Salmonella enterica* LT2 α-D-glucopyranosyl phosphate thymidylyltransferase (E_p) reveals this enzyme can convert a wide array of α-D-hexopyranosyl phosphates to their corresponding UDP- *and* TDP-nucleotide sugars. Thus, we present a general chemoenzymatic method to rapidly generate these reagents, the significance of which is in providing a substrate set for developing in vitro glycosylation systems.[5]

The selected enzyme for this study is a member of the prevalent nucleotidylyltransferase family responsible for the reversible conversion of α-D-glucopyranosyl phosphate (Scheme 1a, **2**) and NTP (e.g. **1**) to the corresponding NDP-sugar nucleotide (**3**) and pyrophosphate (**4**). Of the many nucleotidylyltransferases studied, the **3**-forming thymidylyltransferases have received the least attention.[6] The best characterized thymidylyltransferase (E_p) is from *Salmonella* in which substrate specificity studies were limited to only a few available hexopyranosyl phosphates.[6a] To extend these studies, we overexpressed the *rml*A-encoded E_p in *E. coli* to provide the desired E_p as >5% of the total soluble protein.[7] The corresponding E_p was purified to near homogeniety with a specific activity of 110 U mg^{-1}, a 2-fold improvement over the previously reported values.[6a,8]

Scheme 1. (a) The Reaction Catalyzed by E_p and (b) the Syntheses of α-D-Hexopyranosyl Phosphates[a]



[a] (a) Ph_3P, CCl_4; (b) Ac_2O, pyr; (c) (i) $LiAlH_4$, (ii) AcOH/HCl, (iii) BzCl, pyr; (d) BzCl, pyr; (e) pFPTC-Cl, DMAP; (f) (*n*-Bu)$_3$SnH; (g) (i) NaH, imidazole; (ii) CS_2; (iii) CH_3I; (h) AIBN, (*n*-Bu)$_3$SnH; (i) (i) CF_3CO_2H, (ii) BzCl, pyr; (j) EtS-TMS, ZnI_2; (k) (i) NaOMe; (ii) NaH, BnBr; (l) $(BnO)_2P(O)OH$, CF_3SO_3H, NIS; (m) H_2, Pd/C; (n) (i) HBr; (ii) $(BnO)_2P(O)OH$, silver triflate, 2,4,6-collidine; (o) NaOH; (p) AcOH/HCl. In each case, cation exchange provided the Na^+ salt.

Most of the α-D-hexopyranosyl phosphates examined were synthesized from free sugars while **2**, **56**, and **57** were commercially available. For most synthetically derived glycosyl phosphates (Scheme 1b), a general phosphorylation strategy from the appropriately protected precursor relied upon (i) anomeric activation via the ethyl-thio-β-D-pyranoside (**9**, **17**, **25**, **30**, **35**, and **40**[9]), (ii) deprotection/reprotection (**10**, **18**, **26**, **31**, and **36**), (iii) phosphorylation (**11**, **19**, **27**, **32**, **37**, and **41**), and (iv) complete deprotection (**12**, **20**, **28**, **33**, **38**, and **43**). The overall yield of this four-step phosphorylation strategy ranged from 19 to 28% including the final ion exchange. Alternatively, phosphorylation (**45**, **49**, and **53**) via the glycosyl halide followed by

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(2) Gal, D-galactose; Glc, D-glucose; Man, D-mannose; NTP, nucleotide triphosphate; pFPTC, pentafluorophenoxythiocarbonyl; TDP, thymidine diphosphate; TMP, thymidine monophosphate; TTP, thymidine triphosphate; UDP, uridine diphosphate.

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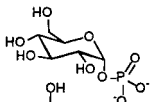
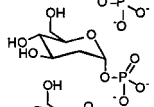
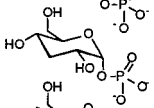
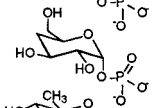
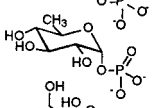
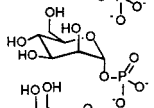
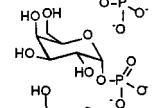
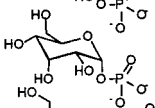
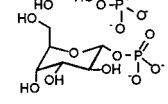
(6) (a) Lindquist, L.; Kaiser, R.; Reeves, P. R.; Lindberg, A. A. *Eur. J. Biochem.* **1993**, *211*, 763−770. (b) Gallo, M. A.; Ward J.; Hutchinson, C. R. *Microbiol.* **1996**, *142*, 269−275.

(7) The gene *rml*A was previously known as *rfb*A (Reeves et al. *Trends Microbiol.* **1996**, *4*, 495−502).

(8) An $(NH_4)_2SO_4$ precipitate of *E. coli*-*prfb*A-C crude extracts was dialyzed against buffer B (20 mM Tris·HCl, 1 mM EDTA, pH 7.5). The dialysate was resolved by anion exchange (DE52, 3 × 15 cm, 50 mL buffer B wash followed by a linear gradient of 0−500 mM NaCl, 1.0 mL min^{-1}) and the E_p fractions combined, concentrated, and further resolved by FPLC gel filtration (S-200, 2 × 70 cm, 50 mM Tris·HCl, 200 mM NaCl, pH 7.5). The purified E_p was stored in aliquots (−80 °C) until used.

10.1021/ja001444y CCC: $19.00
Published on Web 06/30/2000

Table 1. E_p-Catalyzed Conversion of Substrates

Substrate	TTP Conv. (%)[a]	Reten. (min)[b]	UTP Conv. (%)[a]	Reten. (min)[b]
2[c] 	99.3 ± 0.1	4.1[d]	99.5 ± 0.7	3.7[d]
43 	25.5 ± 0.4[e]	4.2	22.3 ± 0.4[e]	3.7
28 	96.2 ± 0.9[f]	4.3	6.5 ± 0.3[f]	3.7
20 	98.3 ± 1.6	4.4	99.3 ± 0.4	3.9
12 	98.2 ± 1.7	4.3	99.1 ± 0.8	3.9
56[c] 	99.5 ± 0.1[g]	4.1	17.9 ± 1.7[g]	3.7
57[c] 	56.8 ± 0.4[g]	4.2	32.7 ± 2.7[g]	3.7[d]
38 	14.8 ± 0.1	4.0	-[h]	-[h]
47 	5.4 ± 0.4	4.0	-[h]	-[h]
31, 51 and 55	-[h]	-[h]	-[h]	-[h]

[a] Percent conversion = $[A_P/(A_P + A_T)] \times 100$, where A_P = the NDP-sugar product peak integration and A_T represents the NTP peak integration. HRMS for all observed products reported in the Supporting Information. [b] Standard retention times: TDP, 4.5 min; TTP, 7.2 min; UDP, 4.0 min; UTP, 6.1 min. [c] Commercially available. [d] Coelutes with commercially available standard. [e] Product hydrolysis observed (**43**, 7.6% TDP and 10.2% UDP). [f] Adjusted for the 2:1 α/β-**28**. [g] In contrast to previously published studies (ref 6a). [h] No products observed.

complete deprotection gave the glycosyl phosphates **47**, **51**, and **55** in an overall yield ranging from 37 to 47%. Our 6-deoxy precursor (**8**) was synthesized by $LiAlH_4$ reduction and subsequent benzoylation of the previously described halide **7**.[10] For the 4-deoxy progenitor, deoxygenation at C-4 was accomplished by selective benzoylation of methyl β-D-galactopyranoside (**13**) to provide the desired tribenzoylated **14** (54%) as well as the tetrabenzoylated derivative (19%) as previously described.[11] Subsequent C-4 activation (**15**) and $(n\text{-Bu})_3SnH$ reductive 4-deoxygenation were accomplished as previously described to give the desired 4-deoxy precursor **16**.[12] The 3-deoxy predecessor **24** was synthesized from 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**21**) by reduction of the previously reported furanose **22**,[13] while the 2-deoxy precursor **39** derived from a commercial source.

To evaluate the synthetic utility of the purified thymidylyltransferase, E_p, α-D-hexopyranosyl phosphate, Mg^{2+}, and NTP were incubated at 37 °C for 30 min and the extent of product formation determined by HPLC. The results of these assays are illustrated in Table 1. Confirmation of product formation was based upon HPLC coelution with commercially available standards and/or HPLC isolation and high-resolution mass spectroscopy of the product.[14] As controls, no product formation was observed in the absence of E_p, glycosyl phosphate, Mg^{2+}, or NTP.[15,16]

The fundamental goal of this work is to assess the utility of E_p as a catalyst/reagent to simplify the synthesis of useful nucleotide sugars. Table 1 clearly illustrates E_p can accomplish this task in that of the twelve glycosyl phosphates tested (which include all possible α-D-hexoses and monodeoxy α-D-glucoses), eight with TTP and six with UTP provide appreciable product under the conditions described.[17] An examination of accepted α-D-hexopyranosyl phosphates with TTP suggests E_p prefers pyranosyl phosphates which are predicted to exist predominately as $^4C_1$ conformers (**3**, **12**, **20**, **28**, **43**, **56**, and **57**), while those predicted to not adopt the $^4C_1$ conformation show little or no activity (**31**, **38**, **47**, **51**, and **55**).[18] Regarding specific interactions required for conversion, analysis of the corresponding deoxy series (**12**, **20**, **28**, and **43**) implicates only a single critical hydroxyl (C-2), the removal of which impairs the yield by >70%. A similar trend is observed in the UTP series with two obvious exceptions, glycosides **28** and **56**. Cumulatively, these results may suggest that, while the C-2 hydroxyl is universally critical for turnover, alterations at C-3 in the context of UTP result in adverse cooperativity.

In conclusion, the presented work clearly demonstrates the pliable nature of E_p and its potential for the synthesis of desired nucleotide sugars. Thus, these studies will broadly impact efforts to understand and exploit the biosynthesis of glycosylated bioactive natural products.[19] Moreover, this work suggests E_p specificity may be governed by conformation rather than specific hydrogen bonds. Efforts are in progress to expand the scope of this methodology.

Acknowledgment. We gratefully acknowledge Professor Hung-wen Liu (Department of Chemistry, University of MN) for *E. coli*-p*rfb*A-C and Dr. George Sukenick (NMR Core Facility, SKI) for mass spectral analyses. J.S.T. is an Alfred P. Sloan Research Fellow and Rita Allen Foundation Scholar. This work was supported in part by grants from the National Institutes of Health (GM58196 and CA84374), the Mizutani Foundation for Glycoscience, and a Cancer Center Support Grant (CA-08748).

Supporting Information Available: Experimentals, E_p purification, and characterization data for **5**−**55** and nucleotide sugar products (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA001444Y

(9) The α/β-**40** mixture (1:1.5) was chromatographically resolved.

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(14) For select compounds, product peaks (ref 15) were lyopholized and submitted directly for HRMS (FAB) analysis.

(15) A reaction containing 5 mM NTP, 10 mM sugar phosphate, and 5.5 mM $MgCl_2$ in a total volume of 50 μL 50 mM potassium phosphate buffer, pH 7.5 at 37 °C, was initiated by the addition of 3.52 U E_p (1 U = the amount of protein needed to produce 1 μmol of TDP-D-glucose min^{-1}). The reaction was incubated with slow agitation for 30 min at 37 °C, quenched with MeOH (50 μL), and centrifuged (5 min, 14000 × *g*) and the supernatant was stored at −20 °C until analysis by HPLC.

(16) Samples (20 μL) were resolved on a Sphereclone 5u SAX column (250 × 4.6 mm) fitted with a guard column (30 × 4.6 mm) using a linear gradient (20−60 mM potassium phosphate buffer, pH 5.0, 1.5 mL min^{-1}, A_{275} nm).

(17) These yields might be further optimized by using pyrophosphatase to drive the equilibrium of the reaction (ref 4b).

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