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Studies on the Substrate Specificity of *Escherichia coli* Galactokinase

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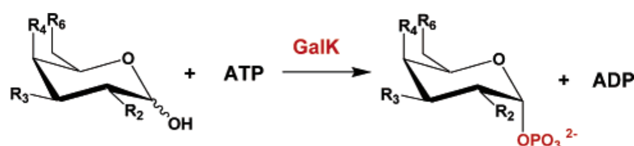
ABSTRACT



In vitro glycorandomization (IVG) technology is dependent upon the ability to rapidly synthesize sugar phosphates. Compared with chemical synthesis, enzymatic (kinase) routes to sugar phosphates would be attractive for this application. This work focuses upon the development of a high-throughput colorimetric galactokinase (GalK) assay and its application toward probing the substrate specificity and kinetic parameters of *Escherichia coli* GalK. The demonstrated dinitrosalicylic assay should also be generally applicable to a variety of sugar-processing enzymes.

Glycosylated bacterial metabolites represent a main class of pharmaceutically important natural products.[1] In many cases, the sugar constituents of these molecules are critical for their mode of action and modification of these carbohydrates can lead to beneficial affects on drug targeting, action, and/or pharmacology. While there are a number of routes for altering glycosylation, one of the most promising for complex metabolites is in vitro glycorandomization (IVG).[2] This method takes advantage of the limitless flexibility of the chemical synthesis of unique sugar precursors with the inherent or engineered substrate promiscuity of enzymes to activate (nucleotidylyltransferase, "E_p") and attach (glycosyltransferases, "GlyT") these precursors to various natural product aglycons (Scheme 1). This method has recently been applied toward the generation of novel nonribosomal peptides and aminocoumarin antibiotics.[2e,3]

Given the key role sugar phosphates play in the glycorandomization process, the rapid synthesis of sugar phosphate libraries would directly contribute to the efficiency of IVG. Many elegant synthetic routes to sugar phosphates already exist, but a tedious process is often unavoidable.[2a−c] The overall yield can be low due to multistep synthetic manipulations, and the purification and/or stability of sugar phosphates are also limiting factors. Thus, an enzymatic (kinase) route would be an attractive alternative, as it is a one-step process and could be coupled, in a single reaction vessel, to IVG.

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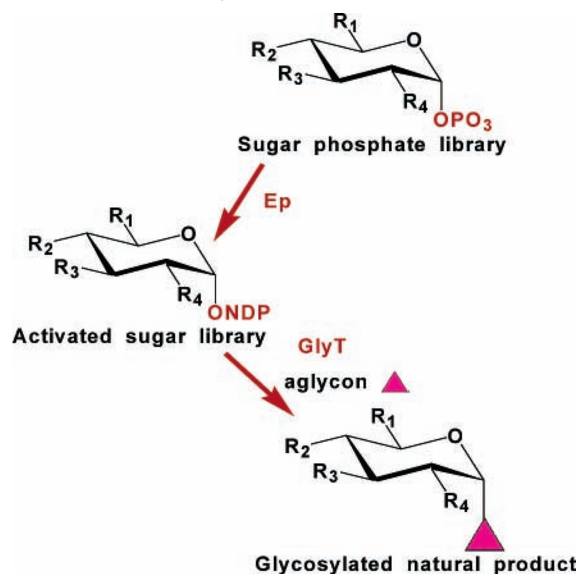
Scheme 1. Schematic Pathway for in Vitro Glycorandomization

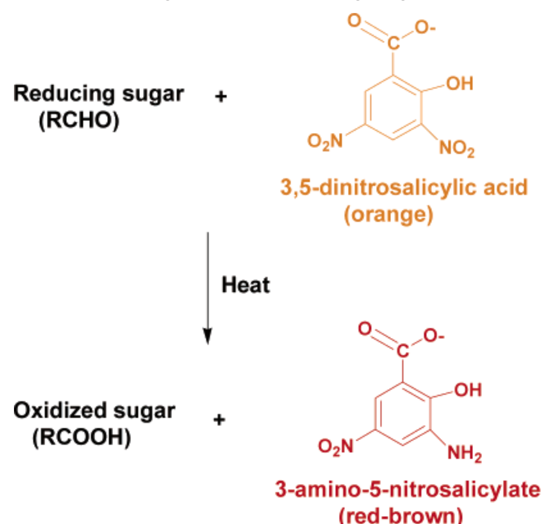


One such kinase, galactokinse (GalK), catalyzes the formation of α-D-galactose-1-phosphate (Gal-1-P) from galactose and ATP. Previous studies have focused upon purification and characterization of GalK from various sources.[4] Limited substrate specificity studies reveal that galactose, 2-deoxygalactose, galactosamine, ATP, 2′-dATP, and 3′-dATP are possible substrates for certain members of this class.

In an effort to expand upon these studies, herein we describe a novel high-throughput assay based on dinitrosalicylic (DNS) acid,[5] and the application of this assay toward the determination of the previously undetermined substrate specificity and kinetic parameters of GalK from *Escherichia coli*. This work reveals that native *E. coli* GalK has a limited substrate scope but clearly provides a foundation from which to launch the pursuit of directed evolution experiments designed to enhance the promiscuity of GalK.

The *galK* gene from *E. coli* K-12 was subcloned into pET-15b vector and overexpressed in BL21 (DE3).[6] The purified

Scheme 2. Schematic Reaction of Dinitrosalicylic (DNS) Acid Reagent and a Reducing Sugar



N-His_6−GalK fusion was used for directly for all studies presented.[7] Our colorimetric assay for GalK is based upon an oxidation−reduction reaction between DNS acid (oxidant) and a reducing sugar (Scheme 2). The reduced product 3-amino-5-nitrosalicylate has a red-brown color, which can be quantified by the absorbance at 575 nm ($\epsilon_{575} = 758$ M^{-1} cm^{-1}).

To test the utility of this assay for GalK, galactose (8 mM) and ATP (10 mM) were incubated with GalK at 30 °C.[8] At different time points, the free sugar/sugar−phosphate ratio was assessed by both TLC[9] and the DNS assay.[10] As a control, assays without GalK gave no change in absorbance at 575 nm, while assays without galactose gave no absorbance at 575 nm. Moreover, DNS assays containing solely the GalK product galactose-1-phosphate lacked absorbance at 575 nm, suggesting that product sugar phosphates should not affect the DNS assay. As confirmation, the product of

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(6) GalK gene was cloned from *E. coli* K-12 and inserted into pET-15b downstream of His-6 coding sequence after digestion with *Nde*I and *BamH*I. pGalK/pET15b-BL21 (DE3) was grown on LB_{Amp100} plate overnight at 37 °C. A single colony was transferred into 100 mL of LB_{Amp100} medium and grown at 37 °C overnight, which was used to inoculate 3 L of superbroth and incubated at 37 °C with shaking. At $OD_{600} = 0.6$, 1 M IPTG was added (1 mM final concentration) and the culture was incubated overnight. The cells were harvested by centrifugation and washed twice with sodium phosphate buffer.

(7) GalK−His_6 was purified via a cobalt affinity column at 4 °C. The crude cell extract, in sodium phosphate buffer, was sonicated 5 × 45 s on ice. The solution was passed through a cobalt resin column (50 mM $CoCl_2$). The bound recombinant proteins were eluted by elution buffer (200 mM imidazole in sodium phosphate buffer). The active fractions were concentrated and further resolved via gel filtration chromatography. The fractions containing homogeneous GalK were collected and concentrated, and the final concentration of protein was determined by Bio-Rad protein assay.

(8) Sugar solution (0.5 mL, 8 mM), ATP (10 mM), and $MgCl_2$ (5 mM) in 50 mM sodium phosphate buffer (pH = 7.5) was incubated at 30 °C for 5 min, and then GalK (final concentration is 5.6 μM) was added to the reaction system. At various time points, aliquots were analyzed via chromatography (ref 9) and the DNS assay (ref 10).

(9) Reaction was monitored by TLC using 10 mM tetrabutylammonium hydroxide in 80% aqueous acetonitrile (anisaldehyde stain). R_f values of galactose, ATP, and galactose-1-phosphate were 0.51, 0.12, and 0.28, respectively. LC-MS analysis was also consistent with the formation of Gal-1-P.

(10) During the reaction process, 50 μL of reaction solution was taken out periodically and transferred to 100 μL of DNS reagent solution (45mM) to assess the reaction progress. Each DNS reaction solution was placed in a bath of boiling water for 15 min and subsequently cooled to room temperature followed by the addition of 15 μL of a 40% potassium sodium tartrate solution to stabilize the color. The absorbance at 575 nm was recorded, and a plot of change in absorbance at 575 nm as a function of time was obtained.

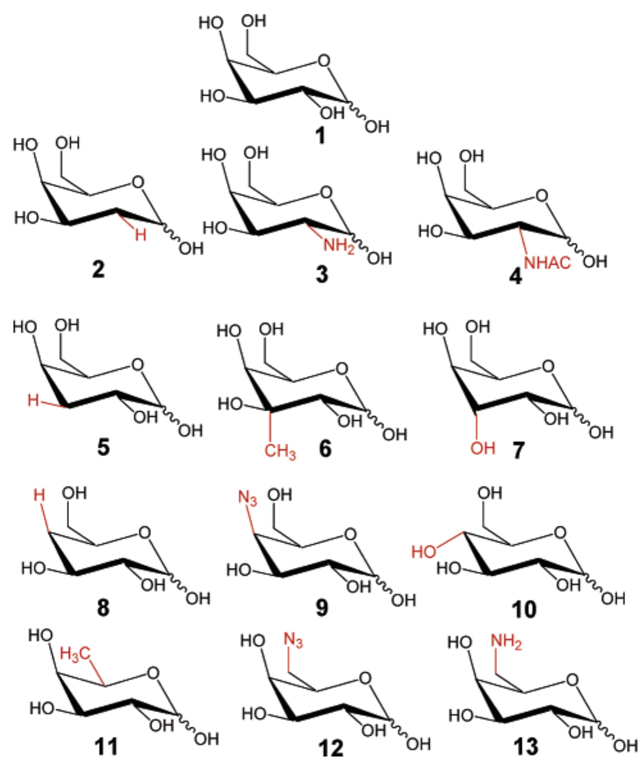


Figure 1. Potential GalK substrates tested in this study.

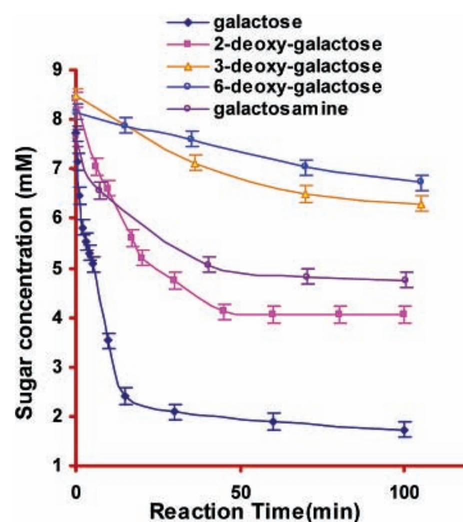


Figure 2. Progress curve of the decrease in free sugar substrate concentration as a function of reaction time.

the GalK reaction coelutes on TLC or HPLC with Gal-1-P and was also confirmed via LC-MS. Use of the DNS assay allowed a progress curve of the change in sugar concentration as a function of time to be obtained by converting the absorbance to the corresponding sugar concentration by using standard curves (Figure 2).[11] The initial velocity was determined by the slope value of the linear phase in the progress curve, and the kinetic parameters for galactose and ATP were determined (V_{max} = 1.65 μmol min^{-1} mg^{-1}, and K_m for galactose and ATP were calculated to be 2.1 and 2.5 mM, respectively).[12] The results are comparable with previously published kinetic parameters for GalK from different species (0.5−1.5 mM for K_m of galactose and 0.15−5 mM for K_m of ATP).[4]

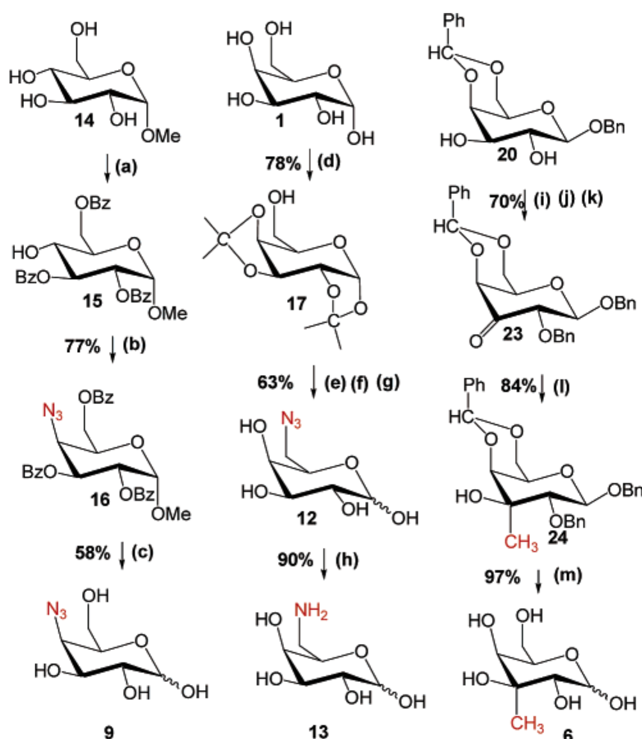
To test the utility of this assay toward other putative substrates, a sugar library, including galactose (**1**) and other monosaccharide analogues, was tested (Figure 1). Six of the alternative free sugars examined (3-deoxy-D-galactose, **5**; 3-*C*-methyl-D-galactose, **6**; 4-deoxy-D-galactose, **8**; 4-azido-4-deoxy-D-galactose, **9**; 6-azido-6-deoxy-D-galactose, **12**; and 6-amino-6-deoxy-D-galactose, **13**) were synthesized, while the other six (2-deoxy-D-galactose, **2**; 2-amino-deoxy-D-galactose, **3**; 2-*N*-acetamido-2-deoxy-D-galactose, **4**; D-gulose, **7**; D-glucose, **10**; 6-deoxy-D-galactose, **11**) were commercially available. Of the synthetically derived analogues, **5** and **8** were prepared as previously described,[2b,13j−l] while the routes to **6**, **9**, **12**, and **13** are illustrated in Scheme 3.[13] The DNS assay was then used to monitor the reaction progress.[8,10] As a control, assays without GalK and assays without sugars were also analyzed for each substrate. We found the DNS assay to be very sensitive to the change in sugar concentration, amenable to a multiwell plate format, and, as a first approximation, easily discernible by the naked eye (Figure 3). This assay is also advantageous as it directly

(11) Standard curve was prepared by making a series of standards (sugar: 0.5, 1, 2, 2.5, 3, 3.5, 4, 5, 6, 7, and 8 mM respectively) and ATP (10 mM) in sodium phosphate buffer (final volume 50 μL and pH = 7.5) and submitting them to the DNS assay (ref 10).

(12) To determine the K_m for galactose, the sugar concentration was varied over a range of 1−6 mM, and ATP was kept at a constant saturating concentration of 10 mM. A series of reactions were set up, and the DNS assay was monitored reaction as mentioned above. The initial velocity was determined by the slope of the linear phase in the progress curve. K_m of ATP was determined where ATP was a variable substrate (0.5−10 mM) and galactose concentration was fixed at a saturating concentration of 10 mM. Kinetics data were analyzed by Enzyme Kinetics Module, Version 1.1. The saturation plots and Lineweaver−Burke plots are shown in Supporting Information.

(13) (a) **3-Methyl-α-D-galactose (6).** ^{1}H NMR (CD$_3$OD): δ 4.56−4.48 and 4.14−4.08 (m, 1H), 3.84−3.34 (m, 5H), 1.40−1.14 (m, 3H). ^{13}C NMR (CD$_3$OD): δ 96.2, 74.9, 74.4, 74.0, 73.7, 73.7, 17.8. ESI-MS: calcd for $C_7H_{14}O_6Na$, 217.1; found, *m/z* 217.1 [M + Na]$^+$. (b) **4-Azido-4-deoxy-α-D-galactose (9).** ^{1}H NMR (D$_2$O): δ 4.57 (d, J = 7.2 Hz, 1H), 3.49 (dd, J = 7.2, 9.8 Hz, 1H), 3.93 (dd, J = 9.8, 3.9 Hz, 1H), 4.00 (d, J = 3.9 Hz, 1H), 3.74 (m, 1H), 3.76 (m, 2H). ^{13}C NMR (D$_2$O): δ 96.738, 72.092, 73.459, 62.935, 73.765, 61.239. MS: calcd for $C_6H_{11}N_3O_5$, 205.1; found, *m/z* 203.9 [M − H]$^-$. (c) **6-Azido-6-deoxy-α-D-galactose (12).** ^{1}H NMR (D$_2$O): δ 5.28 (d, J = 3.7 Hz, 1H), 3.81 (dd, J = 3.7, 10.3 Hz, 1H), 3.87 (dd, J = 10.3, 3.2 Hz, 1H), 3.97 (dd, J = 3.2, 0.8 Hz, 1H), 4.21 (m, 1H), 3.52 (m, 2H). ^{13}C NMR (D$_2$O): δ 92.609, 68.421, 69.266, 69.879, 69.166, 51.149. MS: calcd for $C_6H_{11}N_3O_5$, 205.1; found, *m/z* 206 [M + H]$^+$. (d) **6-Amino-6-deoxy-α-D-galactose (13).** ^{1}H NMR (D$_2$O): δ 5.30 (d, J = 3.5 Hz, 1H), 3.81 (dd, J = 3.5, 10.3 Hz, 1H), 3.87 (dd, J = 10.3, 3.2 Hz, 1H), 3.99 (d, J = 3.2 Hz, 1H), 4.27 (m, 1H), 3.35 (m, 1H), 3.26 (m, 1H). ^{13}C NMR (D$_2$O): δ 92.580, 68.274, 69.131, 70.064, 66.445, 40.579. MS: calcd for $C_6H_{13}NO_5$, 179.1; found, *m/z* 180.0 [M + H]$^+$. (e) May, J. A., Jr.; Sartorelli, A. C. *J. Med. Chem.* **1979**, *22*, 2(8), 971. (f) Cappi, M. W.; Moree, W. J.; Qiao, L.; Marron, T. G.; Weitz-Schmidt, G.; Wong, C.-H. *Bioorg. Med. Chem.* **1997**, *5* (2), 283. (g) Ziegler, T.; Eckhardt, E.; Strayle, J.; Herzog, H. *Carbohydr. Res.* **1994**, *253*, 167. (h) Zhang, Z.; Magnusson, G. *J. Org. Chem.* **1996**, *61*, 2383. (i) Lichtenthaler, F. W.; Oberthur, M.; Peters, S. *Eur. J. Org. Chem.* **2001**, *20*, 3849. (j) Andreana, P. R.; Sanders, T.; Janczuk, A.; Warrick, J.; Wang, P. G. *Tetrahedron Lett.* **2002**, *43*, 6525. (k) Thomas, S. S.; Plenkiewicz, J.; Ison, E. R.; Bols, M.; Zou, W.; Szarek, W. A.; Kisilevsky, R. *Biochim. Biophys. Acta* **1995**, *1272* (1), 37. (l) Kucar, S.; Zamocky, J.; Bauer, S. *Collect. Czech. Chem. Commun.* **1975**, *40* (2), 457.

Scheme 3. Preparation of 3-*C*-Methyl-D-galactose, 4-Azido-4-deoxy-D-galactose, and 6-Azido- and 6-Amino-D-galactose[a]



[a] Reaction conditions: (a) BzCl, pyridine; (b) Tf_2O, then NaN_3, DMF; (c) MeONa, then 2 N H_2SO_4; (d) anhydrous $CuSO_4$, acetone, concentrated H_2SO_4; (e) anhydrous pyridine, TsCl; (f) NaN_3, DMF; (g) 80% CF_3COOH; (h) 10% Pd/C, H_2; (i) $SnO(Bu)_2$, PMBCl; (j) BnBr, NaH, then CAN; (k) Dess−Martin oxidant; (l) CH_3MgBr; (m) 10% Pd/C, H_2.

follows substrate disappearance and thereby eliminates the chance of false positive results, which have been observed in other sugar kinase-coupled assays.[14]

The substrate specificity studies revealed that GalK is highly active with D-galactose (Figure 2), as expected, and,

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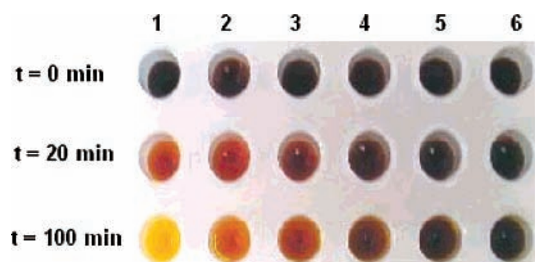

Figure 3. Reaction progress monitored by DNS assay. Lane 1: **1**. Lane 2: **2**. Lane 3: **3**. Lane 4: **5**. Lane 5: **11**. Lane 6: **10**.

consistent with galactokinases from other species,[4] moderately active (a <10-fold decrease in k_{cat}) with substrates substituted at C-2 (**2**, $k_{cat} = 30\ min^{-1}$, $K_m = 3.6$ mM; **3**, $k_{cat} = 11.7\ min^{-1}$, $K_m = 2.9$ mM). However, the extent of tolerance of C-2 alteration (e.g., increasing C-2 steric bulk, **4**) is limited. GalK is also weakly active (a <20-fold decrease in k_{cat}) with substrates lacking hydrogen-bonding potential at either C-3 or C-6 (**5**, $k_{cat} = 5.1\ min^{-1}$, $K_m = 6.4$ mM; **11**, $k_{cat} = 2.9\ min^{-1}$, $K_m = 4.9$ mM), extending the known substrates for this class of enzyme.

It is interesting to note that the lack of tolerance for C-4 changes in the substrate, yet given the typically high concentrations of cellular glucose, this stringent C-4 control clearly supports the need for the alternative (and typically highly regulated) metabolic entry of glucose via the dual action of hexokinase and phosphoglucomutase. Moreover, the demonstrated DNS assay can be used for the rapid screening of various sugar processing enzymes and presents a convenient screen for future directed evolution work of GalK as a new component of IVG.

Supporting Information Available: Saturation plots, Lineweaver−Burk plots, and synthetic details for **5**, **6**, **9**, **12**, **13**, and **16**−**24**. This material is available free of charge via the Internet at http://pubs.acs.org.

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