UDP-Glucose Analogues as Inhibitors and Mechanistic Probes of UDP-Glucose Dehydrogenase

Robert E. Campbell and Martin E. Tanner*

Department of Chemistry, University of British Columbia, Vancouver, British Columbia V6T 1Z1, Canada

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UDP-glucose dehydrogenase catalyzes the NAD^+-dependent 2-fold oxidation of UDP-glucose to give UDP-glucuronic acid. The putative aldehyde intermediate is not released from the active site and is presumably tightly bound. We have prepared UDP-7-deoxy-α-D-*gluco*-hept-6-ulopyranose, **5**, that contains a methyl ketone at C-6 and cannot be further oxidized by the enzyme. Ketone **5** was found to be a competitive inhibitor of the dehydrogenase from *Streptococcus pyogenes* with a K_I value of 6.7 μM. We have also prepared the secondary alcohols UDP-6*S*-6*C*-methylglucose, **4a**, and UDP-6*R*-6*C*-methylglucose, **4b**. Compound **4a**, but not **4b**, was found to be a slow substrate for the dehydrogenase and was converted into the ketone inhibitor **5**. This is consistent with the notion that the *pro-R* hydride is transferred in the first oxidation step of the normal enzymatic reaction.

Introduction

UDP-glucose dehydrogenase catalyzes the irreversible 2-fold oxidation of UDP-glucose to produce UDP-glucuronic acid (Figure 1).[1] UDP-glucuronic acid serves as an activated donor of glucuronate residues in a variety of biochemical pathways throughout nature. In mammals, glucuronate residues are found as components of glycosaminoglycans (heparin, hyaluronic acid, and condroitin sulfate), which form the "ground substance" or extracellular medium of connective tissues.[2,3] They are also used to solubilize waste materials targeted for excretion in a process known as glucuronidation.[4] In many strains of pathogenic bacteria such as group A streptococci and *Streptococcus pneumoniae* type 3, they are found as components of the polysaccharide capsule that surrounds the organism.[5,6] The capsule is thought to act as a "sugar coating" that masks the normal cell surface features from the immune system of the host. It is well established that virulent strains of encapsulated bacteria become avirulent when capsule formation is disrupted.[7−9] An understanding of the mechanism used by the bacterial UDP-glucose dehydrogenase may allow the design of inhibitors in an effort toward antibiotic design.

The mechanism of the dehydrogenase reaction appears to involve an initial oxidation of UDP-glucose to generate

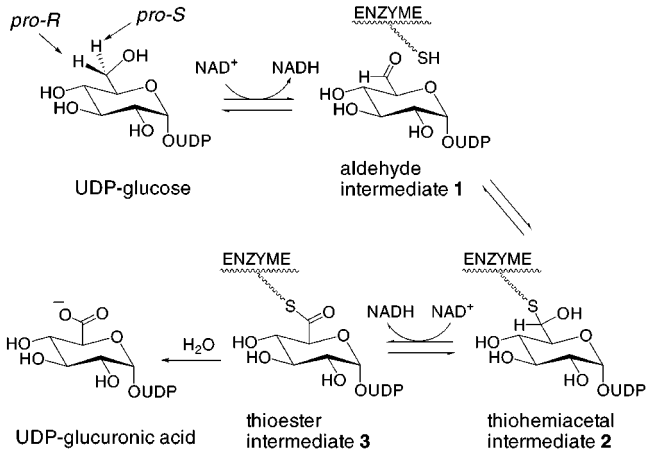

Figure 1. Proposed mechanism for the reaction catalyzed by UDP-glucose dehydrogenase.

the bound aldehyde intermediate **1** (Figure 1).[10−15] This putative intermediate is not released from the active site to any appreciable extent and is inaccessible to external aldehyde trapping reagents present in the solution.[12,14,15] Compound **1** has been independently synthesized and was found to be a kinetically competent substrate for the dehydrogenase reaction (k_{cat} = 1.0 s^{-1}, K_M = 14 μM).[11] This supports the notion that aldehyde **1** is a true intermediate in the reaction and is effectively sequestered or tightly bound in the active site of the enzyme. This binding is likely in the form of a covalent thiohemiacetal linkage with an active site cysteine residue. Mechanistic

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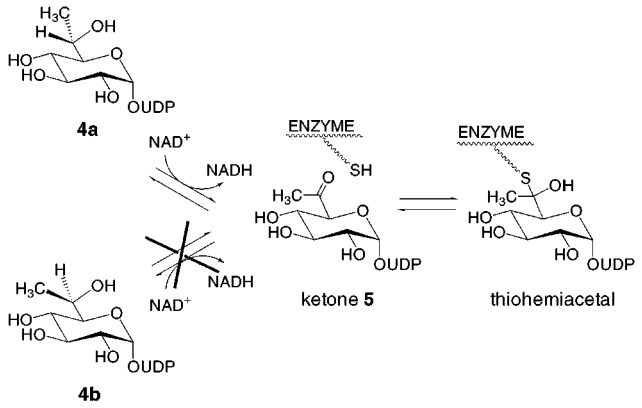


Figure 2. Structures of compounds **4** and **5** and the alternate reaction catalyzed by UDP-glucose dehydrogenase.

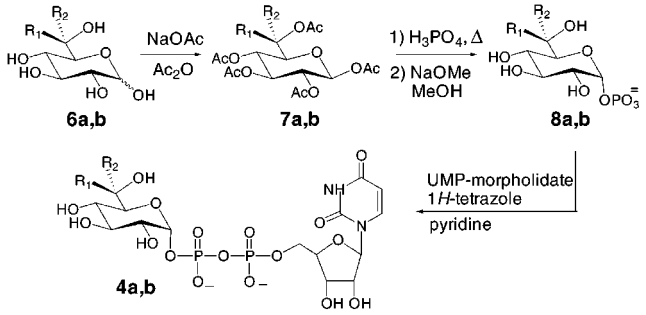
studies indicate that the second oxidation proceeds directly from the thiohemiacetal **2** to give a thioester intermediate, **3**.[10,12,16] A final hydrolysis step generates the product UDP-glucuronic acid and the free enzyme. Support for the involvement of covalent catalysis via cysteine comes from inactivation studies with thiol-directed reagents including UDP-chloroacetol.[12] In the case of the enzyme from *Streptococcus pyogenes*, mutation of a conserved cysteine residue led to a dramatic reduction in catalytic activity.[10] Furthermore, it was shown that the Cys-to-Ser mutant underwent the first two oxidation steps of the reaction to generate the ester equivalent of **3**, but subsequent hydrolysis of the ester was very slow, and the covalent adduct accumulated. This observation strongly supports the proposal that the active site cysteine participates in covalent catalysis in the wild-type reaction.

In this paper we describe studies aimed at generating a mechanism-based reversible inhibitor of the dehydrogenase from group A streptococci. We reasoned that a UDP-glucose analogue such as ketone **5** should mimic the aldehyde intermediate **1** and be tightly bound by the dehydrogenase via a thiohemiacetal linkage (Figure 2). Ketone **5** could be produced in the active site of the enzyme by the oxidation of UDP-6*S*-6*C*-methylglucose, **4a**, and/or UDP-6*R*-6*C*-methylglucose, **4b**. A second oxidation presumably could not take place since it would require the cleavage of a carbon−carbon bond. We have found that ketone **5** is a competitive inhibitor of the enzyme with a K_I value of 6.7 μM. In addition, alcohol **4a** is slowly oxidized by the enzyme to give ketone **5**, whereas alcohol **4b** is not a substrate. This is consistent with the notion that the *pro-R* hydride is transferred in the first oxidation step of the normal enzymatic reaction.

Results

(A) Synthesis of Analogues. UDP-6*S*-6*C*-methylglucose, **4a**, and UDP-6*R*-6*C*-methylglucose, **4b**, were prepared from the corresponding methylglucose diastereomers **6a** and **6b** that had been described previously by Blériot et al. (Scheme 1).[17,18] In their synthesis a nonstereoselective route is reported that allows one to prepare both diastereomers from glucuronolactone in

Scheme 1. Synthesis of 4a and 4b[a]



[a] For series a, R_1 = H and R_2 = CH_3; for series b, R_1 = CH_3 and R_2 = H.

seven steps. The diastereomers may be chromatographically separated when protected as diacetonides. In this work the free sugars **6a** and **6b** were first peracetylated with sodium acetate in acetic anhydride. In each case it was possible to crystallize the β-anomer **7a** or **7b** from the anomeric mixture (α:β = 1:3) that was initially isolated. To confirm the stereochemical assignment made in the previous work,[17,18] the structure of **7b** was determined by X-ray crystallographic analysis and was found to contain an *R*-configuration at C-6 as expected. The peracylated sugars were subjected to a modified MacDonald procedure[19] using neat phosphoric acid at 55 °C and then deprotected using NaOMe/MeOH to give the α-phosphates **8a** and **8b** in 30% yield. These compounds were coupled to UMP-morpholidate in the presence of 1*H*-tetrazole[20] to give UDP-6*S*-6*C*-methylglucose, **4a** (36% yield), and UDP-6*R*-6*C*-methylglucose, **4b** (26% yield).

Ketone **5** was prepared from the known alcohol **9** that is stereoselectively synthesized from glucuronolactone in six steps (Scheme 2). [17,18] Alcohol **9** was oxidized under Swern conditions to give the crude ketone that was directly converted to alkene **10** using the Takai procedure[21] (53% yield from **9**). Alkene **10** was deprotected with TFA to give **11** and then peracetylated using acetic anhydride and sodium acetate to give **12** as a mixture of anomers (α:β = 1:2). A protected version of compound **11** has been previously prepared via an alternate route.[22] It was possible to selectively crystallize the β-anomer of **12**, and an attempt was made to directly introduce the α-phosphate by submitting this anomer to the MacDonald procedure. This proved to be unsuccessful presumably because the acidic conditions promoted the formation of a tertiary carbocation at C-6 and led to the decomposition of the compound. Instead the anomeric mixture of **12** was treated with hydrazine acetate to give the free hemiacetal **13**. This material was dibenzylphosphitylated,[23] oxidized/deprotected by ozonolysis, hydrogenated over Pd/C, and finally deacetylated using 10% triethylamine in methanol/H_2O to give phosphate **14** as

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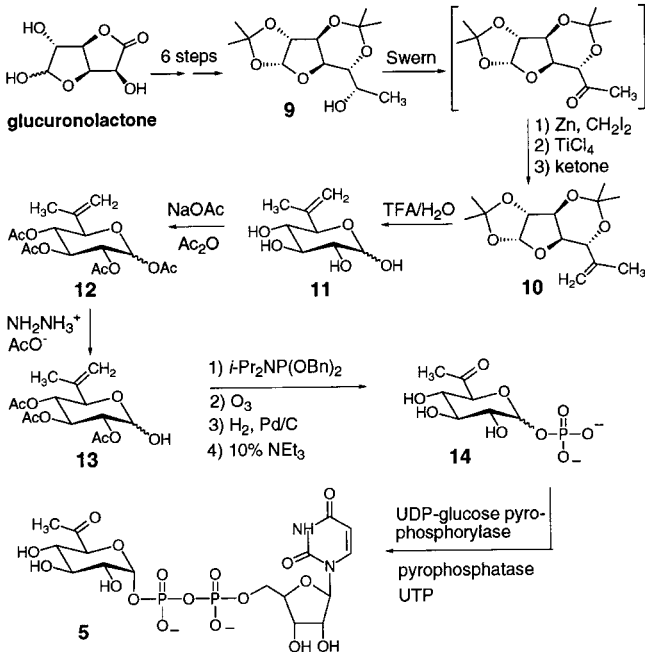
Scheme 2. Synthesis of Ketone 5



a mixture of anomers (α:β = 3:2) in a 40% yield. To introduce the terminal uridine monophosphate group, a mild coupling reaction that operates under nonbasic conditions was desired. In previous work, UDP-glucose pyrophosphorylase was used to prepare aldehyde **1**, indicating that the enzyme accepted glucose 1-phosphate analogues with some structural modification at C-6.[11] It also seemed likely that the enzyme would selectively accept only the α-anomer of **14** and remove the need for a separation of the anomeric mixture. Indeed when **14** was treated with UDP-glucose pyrophosphorylase, UTP, and inorganic pyrophosphatase, the target ketone **5** was cleanly generated as analyzed by HPLC and could be isolated with a 15% yield. It is likely that the pyrophosphorylase coupling reaction could also have been employed in the preparation of **4a** and **4b**; however, this was not attempted.

(B) Enzymatic Studies. Ketone **5** was tested as an inhibitor of the dehydrogenase reaction by following the initial rate of oxidation of UDP-glucose as a function of substrate concentration at six different fixed inhibitor concentrations. The resulting double reciprocal plot intersected on the *Y*-axis, indicating that the inhibition was competitive in nature (Figure 3). A replot of the slopes against [**5**] gave a linear plot showing a K_I value of 6.7 μM (Figure 4, inset). No evidence for slow, tight binding or irreversible inhibition was detected.

UDP-6*S*-6*C*-methylglucose, **4a**, and UDP-6*R*-6*C*-methylglucose, **4b**, were tested as substrates by incubating them with UDP-glucose dehydrogenase and NAD^+ and monitoring the production of NADH using UV spectroscopy at 340 nm. In the case of UDP-6*R*-6*C*-methylglucose, **4b**, no reaction could be detected even at very high enzyme concentrations (1.4 mg/mL). In the case of UDP-6*S*-6*C*-methylglucose, **4a**, however, a slow reaction did occur that reached equilibrium after less than 10% of the substrate was consumed.[24]

To further establish the notion that **4a**, but not **4b**, was oxidized to give **5** in the previous experiment, a sample of **5** was treated with NADH to look for the

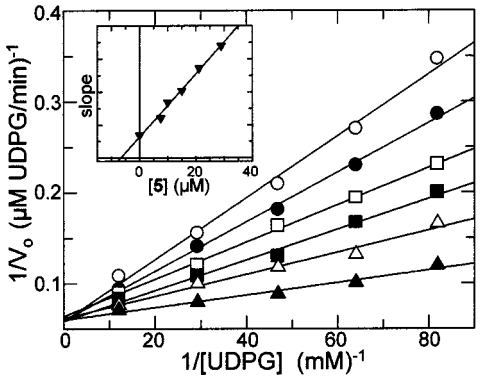


Figure 3. Double reciprocal plot for the inhibition of UDP-glucose dehydrogenase by compound **5**. Inhibitor concentrations are 0 μM (▲), 7.5 μM (△), 10 μM (■), 15 μM (□), 21 μM (●), and 29 μM (○). The inset shows a replot of the slopes as a function of [**5**].

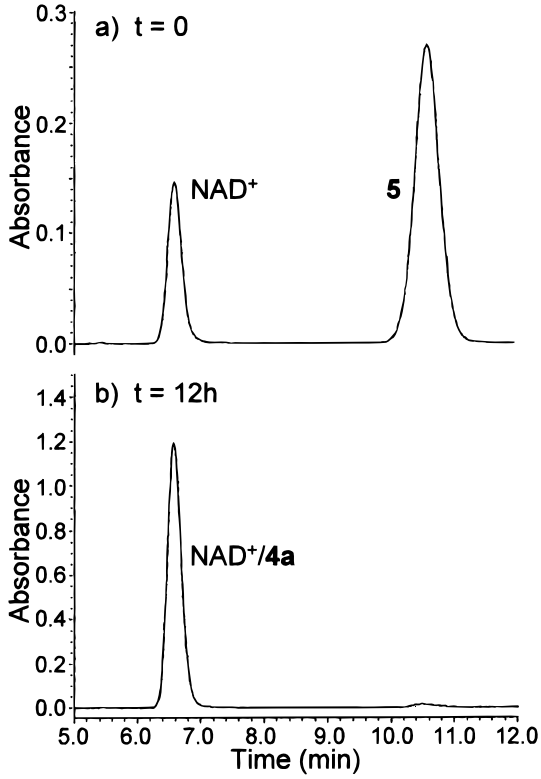


Figure 4. Ion-paired reversed-phase HPLC traces for the reduction of ketone **5** by NADH catalyzed by UDP-glucose dehydrogenase.

formation of **4a** via the reverse reaction. The reaction was followed by ion-paired reversed-phase HPLC[25] as shown in Figure 4. Prior to addition of the enzyme (Figure 4a), peaks corresponding to NAD^+ (present as a minor impurity in NADH) and ketone **5** are observed (NADH itself is not shown in Figure 4). In separate runs it was shown that **4a** coelutes with NAD^+ under all conditions tested and that **4b** coelutes with ketone **5**. After incuba-

(24) Due to the slow rate of the enzymatic reaction and the fact that the equilibrium favored **4a**, it was not possible to obtain accurate steady-state kinetic constants. The equilibrium could be shifted toward the product **5** by the addition of 25 mM NH_2OH, and under these conditions the value of k_{cat}/K_M for the oxidation of **4a** was found to be 10 000 times lower than that for the oxidation of UDP-glucose.

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tion with the dehydrogenase (Figure 4b), the peak eluting at the retention time of **5**/**4b** had disappeared and the peak corresponding to NAD^+/**4a** had increased in intensity. This indicates that ketone **5** had been converted soley to UDP-6*S*-6*C*-methylglucose, **4a**. Appropriate spiking experiments with **4a** and **4b** confirmed this assignment (traces not shown).

Discussion

The inability to detect any released aldehyde **1** during the course of the normal UDP-glucose dehydrogenase reaction led us to suspect that this compound was tightly bound by the enzyme and that UDP-glucose analogues with carbonyl functionalities at C-6 might serve as potent inhibitors of the enzyme. The observation that ketone **5** is a competitive inhibitor with a K_I value of 6.7 μM, whereas the K_M value for UDP-glucose is 20 μM, indicates that any potential improvement in binding affinity is moderated by the introduction of the methyl group. The methyl group could be causing steric problems in the active site as well as stabilizing the carbonyl form of the compound and thereby lowering the propensity for addition of the active site thiol to generate a thiohemiacetal (relative to an analogous addition to the aldehyde). The latter problem may be addressed by preparing the trifluoromethyl ketone analogue of **5** that should greatly increase the amount of thiohemiacetal formed and possibly lower the value of K_I.

During the course of the normal UDP-glucose dehydrogenase reaction, both the *pro-R* and the *pro-S* hydrogens are sequentially transferred to two molecules of NAD^+ (Figure 1). In the UDP-6*C*-methylglucose diastereomers **4a** and **4b**, these hydrogens have been selectively replaced with methyl groups, and the compounds may be used as a tool to probe the order of the hydride-transfer steps. The observation that **4a**, which retains the "*pro-R*-like" hydrogen, is a substrate (albeit a slowly reacting one) and **4b** is not suggests that the *pro-R* hydride is transferred during the first oxidation of alcohol to aldehyde in the normal *S. pyogenes* dehydrogenase reaction (Figure 2). This observation is in agreement with studies done on the UDP-glucose dehydrogenase from beef liver which report that the *pro-R* hydride is transferred in the first oxidation step.[26]

Experimental Section

General Procedures. Reactions were performed under argon unless otherwise noted. All chemicals were purchased from Sigma, Aldrich, or Lancaster. Preparative thin-layer chromatography (TLC) was performed on glass-backed plates of either 1 or 2 mm thick silica gel 60F254. Amberlite IR-120(plus) resin was used for all counterion exchange (sodium form) and neutralization (hydrogen or pyridinium form) after O-deacetylation or hydrogenation. 7-Deoxy-L-*glycero*-D-*gluco*-heptopyranose, **6a**, 7-deoxy-D-*glycero*-D-*gluco*-heptopyranose, **6b**, and 7-deoxy-1,2:3,5-di-*O*-isopropylidene-L-*glycero*-α-D-*gluco*-heptofuranose, **9**, were prepared as previously described.[17,18] Elemental analyses were performed by the Microanalytical Lab of the University of British Columbia Chemistry Department.

1,2,3,4,6-Pentacetyl-7-deoxy-L-*glycero*-β-D-*gluco*-heptopyranose (7a). To a solution of **6a** (0.277 g, 1.43 mmol) in Ac_2O (12 mL) was added NaOAc (0.79 g, 9.6 mmol), and the solution was refluxed for 1 h, cooled to rt, and once again heated to reflux. The solution was poured into saturated $NaHCO_3$ and extracted with EtOAc, the organic layer washed (brine) and dried (Na_2SO_4), and the solvent removed in vacuo. Flash chromatography (50% Et_2O/50% hexanes) yielded an analytically pure mixture of anomers as a white solid (4β:1α by ^{1}H NMR, 478 mg, 83%). Successive recrystallizations from Et_2O gave the pure β-anomer **7a** (274 mg, 48%): $[\alpha]^{20}_D = -15.2$ ($c = 0.798$, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.63 (d, 1H, $J = 8.3$), 5.20 (dd, 1H, $J = 9.3, 9.3$), 5.13 (dd, 1H, $J = 9.2, 8.3$), 5.11 (dd, 1H, $J = 9.6, 9.4$), 5.03 (dq, 1H, $J = 6.6, 2.0$), 3.56 (dd, 1H, $J = 9.8, 2.0$), 2.10 (s, 3H, Ac), 2.04 (s, 3H, Ac), 2.01 (s, 3H, Ac), 1.98 (s, 3H, Ac), 1.98 (s, 3H, Ac), 1.27 (d, 3H, $J = 6.6$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.4, 170.1, 169.3, 169.2, 169.0, 92.2, 75.7, 73.0, 70.3, 67.5, 65.6, 21.0, 20.8, 20.6, 20.6, 20.5, 15.7; DCI(+) MS (NH_3) *m*/*z* 422 ($M + NH_4^+$, 100%). Anal. Calcd for $C_{17}O_{11}H_{24}$: C, 50.48; H, 5.99. Found: C, 50.69; H, 6.04.

7-Deoxy-L-*glycero*-α-D-*gluco*-heptopyranosyl Phosphate, Sodium Salt (8a). Compound **7a** (100 mg, 0.25 mmol) and 0.5 mL of Et_2O were quickly added to crystalline phosphoric acid (210 mg) that had been dried overnight in vacuo over P_2O_5. The mixture was rapidly stirred and the ether *carefully* removed in vacuo with slight heating. The neat mixture was maintained in vacuo at 50−55 °C with stirring for 2 h. THF (4 mL) was added, the solution was cooled to −10 °C, and NH_4OH was added dropwise (11−12 drops) until the pH was neutral to litmus. Ammonium phosphate precipitate was removed by filtration and washed with THF (20 mL) and the solvent removed under reduced pressure to give a dark syrup. The crude product was dissolved in water and washed with $CHCl_3$. Several drops of pyridine were added, and the water was removed in vacuo followed by dissolution in toluene and solvent removal in vacuo. Preparative TLC (60% $CHCl_3$/35% MeOH/5% H_2O) gave the acetylated free acid of **8a** (47 mg). This compound was immediately dissolved in MeOH (5 mL), and 1 M NaOMe was added to a final concentration of 50 mM. Once TLC analysis indicated the O-deacetylation reaction was complete, cation-exchange resin (hydrogen or pyridinium form) was added until the pH was neutral to litmus. The resin was removed by filtration, washed with MeOH, and solvent was removed in vacuo. Preparative TLC (44% $CHCl_3$/44% MeOH/12% H_2O) followed by passage through cation-exchange resin (sodium form) and lyophilization gave **8a** as a white powder (21 mg, 29%): ^{1}H NMR (400 MHz, D_2O) δ 5.45 (dd, 1H, $J = 6.9, 3.4$), 4.10 (dq, 1H, $J = 6.7, 1.5$), 3.71 (dd, 1H, $J = 9.5, 9.4$), 3.60 (dd, 1H, $J = 10.1, 1.4$), 3.49−3.45 (m, 2H,), 1.22 (d, 1H, $J = 6.6$); ^{13}C NMR (75 MHz, D_2O) δ 94.8 (d, $J = 6.0$), 74.4, 73.3, 72.0 (d, $J = 7.8$), 70.0, 64.7, 19.1; ^{31}P NMR (121.5 MHz, D_2O) δ 0.020 (s); HR-LSI(−) MS (thioglycerol matrix) *m*/*z* calcd for $C_7H_{14}O_9P$ 273.0375, found 273.0368.

Uridine 5′-(7-deoxy-L-*glycero*-α-D-*gluco*-heptopyranosyl Diphosphate), Sodium Salt (4a). Compound **8a** (22 mg, 0.074 mmol) was converted to its pyridinium counterion form and maintained in vacuo overnight. Trioctylamine (33 μL, 0.076 mmol) and pyridine (2−5 mL) were added, and the solvent was removed in vacuo. The process of dissolution in pyridine, solvent removal, and equalization with argon was repeated twice. UMP-morpholidate (84 mg, 0.12 mmol) and 1*H*-tetrazole (17 mg, 0.24 mmol) were added to the flask, dissolved, and concentrated to dryness twice from pyridine, and the final volume of pyridine (0.5 mL) was added. After 2 days at rt the solvent was removed in vacuo and the residue dissolved in water and washed twice (Et_2O). The crude product was applied to a column (1.5 cm × 18 cm) of DE-52 anion-exchange resin and eluted with a linear gradient from 0 to 300 mM LiCl with detection at 254 nm. The relevant fraction (30−40 mL) was applied to a column (110 × 2.5 cm) of Biogel P-2 (200−400 mesh) and eluted with distilled water. Fractions containing product were pooled, concentrated in vacuo to 2−3 mL, and applied to the Biogel P-2 column. Counterion exchange to sodium and lyophilization yielded **4a** as a white powder (17 mg, 36%): ^{1}H NMR (400 MHz, D_2O) δ 7.91 (d, 1H, $J = 8.1$), 5.96−5.95 (m, 2H), 5.58 (dd, 1H, $J = 7.1, 3.4$), 4.34−4.16 (m, 5H), 4.11 (q, 1H, $J = 6.6$), 3.71 (dd, 1H, $J = 9.4, 9.2$), 3.63−3.59 (m, 1H), 3.53−3.46 (m, 2H), 1.25 (d, 3H, $J = 6.6$);

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^{13}C NMR (75 MHz, D_2O) δ 167.7, 153.0, 141.7, 103.0, 95.9 (d, J = 6.7), 88.7, 83.4 (d, J = 8.9), 74.9, 74.0 73.4, 71.9 (d, J = 8.8), 69.9, 69.8, 65.1 (d, J = 5.4), 64.6, 19.1; ^{31}P NMR (121.5 MHz, D_2O) δ −10.8 (d, 1P, J = 20.8), −12.6 (d, 1P, J = 20.7); HR-LSI(−) MS (thioglycerol matrix) m/z calcd for $C_{16}H_{24}N_2O_{17}P_2Na$ 601.0448, found 601.0466.

1,2,3,4,6-Pentacetyl-7-deoxy-D-*glycero*-*β*-D-*gluco*-heptopyranose (7b). Compound **6b** (0.412 g, 2.12 mmol) was subjected to complete acetylation as described for the preparation of **7a**. Flash chromatography (50% Et_2O/50% hexanes) gave **7b** and its α-anomer (3β:1α by ^{1}H NMR, 688 mg, 80%). Successive recrystallizations (Et_2O/hexanes) gave **7b** (429 mg, 50%): $[\alpha]^{20}_D$ = +23.4 (c = 0.798, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.68 (d, 1H, J = 8.2), 5.20 (dd, 1H, J = 9.3, 9.3), 5.05 (dd, 1H, J = 9.4, 8.2), 5.00 (dd, 1H, J = 10.0, 9.2), 4.90 (dq, 1H, J = 6.7, 2.3), 3.76 (dd, 1H, J = 10.1, 2.4), 2.09 (s, 3H, Ac), 2.03 (s, 3H, Ac), 2.03 (s, 3H, Ac), 2.00 (s, 3H, Ac), 1.98 (s, 3H, Ac), 1.23 (d, 3H, J = 6.7); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.2, 170.0, 169.6, 169.3, 168.9, 91.7, 75.4, 72.9, 70.2, 68.7, 68.5, 21.1, 20.8, 20.6, 20.5, 20.5, 13.5; DCI(+) MS (NH_3) m/z 422 (M + NH_4^+, 100%). Anal. Calcd for $C_{17}O_{11}H_{24}$: C, 50.48; H, 5.99. Found: C, 50.56; H, 6.05.

7-Deoxy-D-*glycero*-α-D-*gluco*-heptopyranosyl phosphate, Sodium Salt (8b). Compound **7b** (100 mg, 0.25 mmol) was treated according to the modified MacDonald procedure described for the preparation of **8a**. Preparative TLC gave the acetylated free acid of **8b** (73 mg), which was immediately subjected to O-deacetylation and purification as described. This procedure gave **8b** as a white powder (23 mg, 31%): ^{1}H NMR (400 MHz, D_2O) δ 5.43 (dd, 1H, J = 7.1, 3.7), 4.09 (dq, 1H, J = 6.7, 2.4), 3.90 (dd, 1H, J = 10.1, 2.3), 3.71 (dd, 1H, J = 9.4, 9.4), 3.48−3.45 (m, 1H), 3.32 (dd, 1H, J = 10.0, 9.3), 1.16 (d, 3H, J = 6.8); ^{13}C NMR (75 MHz, D_2O) δ 94.4 (d, J = 5.3), 74.2, 73.6, 72.1 (d, J = 6.9), 71.0, 66.7, 15.5; ^{31}P NMR (121.5 MHz, D_2O) δ 1.18 (s); HR-LSI(−) MS (thioglycerol matrix) m/z calcd for $C_7H_{14}O_9P$ 273.0375, found 273.0373.

Uridine 5′-(7-deoxy-D-*glycero*-α-D-*gluco*-heptopyranosyl Diphosphate), Sodium Salt (4b). Compound **8b** (16 mg, 0.053 mmol) was subject to coupling with UMP-morpholidate and purification as described for the preparation of **4a**. This procedure yielded **4b** as a white powder (8.6 mg, 26%): ^{1}H NMR (400 MHz, D_2O) δ 7.93 (d, 1H, J = 8.1), 5.99−5.98 (m, 2H), 5.60 (dd, 1H, J = 7.3, 3.4), 4.37−4.17 (m, 5H), 4.13 (dq, 1H, J = 6.7, 2.6), 3.93 (dd, 1H, J = 10.2, 2.4), 3.75 (dd, 1H, J = 9.6, 9.3), 3.52 (ddd, 1H, J = 9.8, 3.3, 3.1), 3.39 (dd, 1H, J = 10.1, 9.2), 1.21 (d, 3H, J = 6.6); ^{13}C NMR (75 MHz, D_2O) δ 166.6, 152.1, 141.8, 102.9, 95.8 (d, J = 6.5), 88.6, 83.5 (d, J = 8.9), 74.9, 74.0, 73.3, 71.8 (d, J = 8.2), 70.8, 69.9, 66.8, 65.2 (d, J = 5.0), 15.6; ^{31}P NMR (121.5 MHz, D_2O) δ −10.8 (d, 1P, J = 20.3), −12.5 (d, 1P, J = 20.8); HR-LSI(−) MS (thioglycerol matrix) m/z calcd for $C_{16}H_{24}N_2O_{17}P_2Na$ 601.0448, found 601.0461.

6,7-Dideoxy-1,2:3,5-di-*O*-isopropylidene-6-*C*-methyl-α-D-*gluco*-hept-6-enofuranose (10). To a stirring solution of oxalyl chloride (0.182 mL, 4.0 mmol) in CH_2Cl_2 (20 mL) was added DMSO (0.31 mL, 4.0 mmol) in CH_2Cl_2 (5 mL). This solution was stirred for 2 min at −60 °C before alcohol **9** (0.50 g, 1.8 mmol) in CH_2Cl_2 (10 mL) was added via dropping funnel and stirred for 15 min at −60 °C. Triethylamine (1.27 mL, 9.1 mmol) was added via syringe, and the solution was stirred for 5 min at −60 °C and allowed to warm to rt. Water (50 mL) was added, the aqueous solution was extracted (CH_2Cl_2), the organic layer was washed (brine) and dried (Na_2SO_4), and solvent was removed in vacuo. This procedure gave the ketone as a pale yellow oil (0.45 g, 92%). ^{1}H NMR indicated that this material was >90% pure, but due to instability to silica gel, further purification and characterization were not pursued.

To a rapidly stirring slurry of Zn dust (2.6 g, 40 mmol) in THF (40 mL) was added CH_2I_2 (1.8 mL, 22 mmol). After 30 min, the gray slurry was cooled to 0 °C, and 1.0 M $TiCl_4$ in CH_2Cl_2 (2.9 mL, 2.9 mmol) was added via syringe. This solution was stirred for 30 min at rt before the ketone (610 mg, 2.2 mmol) in THF (7 mL) was added dropwise via syringe. After 1 h, the reaction was diluted with Et_2O and 5% HCl/H_2O. The solution was extracted (Et_2O), the organic layer was washed (saturated $NaHCO_3$, brine) and dried (Na_2SO_4), and solvent was removed in vacuo. The residue was purified by flash chromatography (hexanes and then 90% hexanes/10% EtOAc) to yield **10** as a clear oil (344 mg, 57%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.97 (d, 1H, J = 3.7), 5.05, (s, 1H), 4.88 (d, 1H, J = 1.0), 4.53 (d, 1H, J = 3.7), 4.31 (dd, 1H, J = 7.2, 3.7), 4.17 (d, 1H, J = 3.8), 3.89 (d, 1H, J = 7.2), 1.76 (s, 3H), 1.44 (s, 3H, Me_2C), 1.33 (s, 3H, Me_2C), 1.32 (s, 3H, Me_2C), 1.28 (s, 3H, Me_2C); ^{13}C NMR (75 MHz, $CDCl_3$) δ 142.5, 111.9, 111.6, 106.3, 100.8, 83.7, 82.0, 75.0, 74.3, 27.1, 26.5, 24.0, 23.7, 18.6; DCI(+) MS (NH_3) m/z 271 (M + H^+, 33%). Anal. Calcd for $C_{14}O_5H_{22}$: C, 62.20; H, 8.20. Found: C, 62.15; H, 8.28.

6,7-Dideoxy-6-*C*-methyl-D-*gluco*-hept-6-enopyranose (11). Compound **10** (330 mg, 1.22 mmol) was dissolved in 50% TFA/H_2O (30 mL) and stirred at room temperature for 3 h, and then solvent was removed in vacuo. The residue was redissolved and solvent removed (H_2O and then repeated with toluene) before purification by flash chromatography (EtOAc, then 95% EtOAc/5% MeOH, and then 90%EtOAc/10% MeOH). This procedure gave **11** as a solid white foam (225 mg, 97%): ^{1}H NMR (400 MHz, D_2O) δ 5.20 (d, 0.35H, J = 3.6), 5.12−5.10 (m, 2H), 4.65 (d, 0.65H, J = 7.8), 4.18 (d, 0.35H, J = 10.0), 3.83−3.78 (m, 0.65H), 3.69 (dd, 0.35H, J = 9.3, 9.2), 3.56 (dd, 0.35H, J = 9.5, 3.7), 3.49−3.43 (m, 1.65H), 3.25 (ddd, 0.65H, J = 7.5, 7.5, 2.4), 1.74 (s, 1.9H), 1.74 (s, 1.1H); ^{13}C NMR (75 MHz, D_2O) δ 141.1, 140.9, 117.9, 117.7, 96.2, 92.5, 80.2, 75.8, 75.6, 74.5, 72.9, 71.8, 71.0, 70.8, 16.7; DCI(+) MS (NH_3) m/z 208 (M + NH_4^+, 39%). Anal. Calcd for $C_8O_5H_{14}$: C, 50.52; H, 7.42. Found: C, 50.55; H, 7.48.

1,2,3,4-Tetracetyl-6,7-dideoxy-6-*C*-methyl-D-*gluco*-hept-6-enopyranose (12). The free sugar **11** (270 mg, 1.42 mmol) was subjected to complete acetylation as described above for the preparation of **7a**. Flash chromatography (70% hexanes/30% EtOAc) gave **12** as a white solid (1α:2β by ^{1}H NMR, 390 mg, 77%). Recrystallization (Et_2O/hexanes) gave the pure β-anomer of **12** (153 mg, 31%). The β-anomer prepared in this manner was used for all characterizations and the attempted MacDonald reaction; however, it was later determined that the anomeric mix was sufficient for subsequent steps: ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.70 (d, 1H, J = 8.3), 5.23 (dd, 1H, J = 9.5, 9.5), 5.07 (dd, 1H, J = 9.5, 8.3), 5.03 (dd, 1H, J = 9.7, 9.6), 4.94 (s, 2H), 3.95 (d, 1H, J = 9.8), 2.05 (s, 3H, Ac), 1.99 (s, 3H, Ac), 1.97 (s, 3H, Ac), 1.93 (s, 3H, Ac), 1.70 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.0, 169.3, 169.3, 168.9, 139.2, 117.0, 91.5, 78.8, 72.6, 70.4, 68.9, 20.8, 20.5, 20.5, 20.5, 16.6; DCI(+) MS (NH_3) m/z 376 (M + NH_4^+, 34%). Anal. Calcd for $C_{16}O_9H_{22}$: C, 53.63; H, 6.19. Found: C, 53.71; H, 6.07.

2,3,4-Tetracetyl-6,7-dideoxy-6-*C*-methyl-*β*-D-*gluco*-hept-6-enopyranose (13). A solution of the peracetylated sugar **12** (60 mg, 0.17 mmol) and hydrazine acetate (18.6 mg, 0.20 mmol) in dimethyl formamide (0.6 mL) was heated at 50 °C for 1 min. After 20 min at rt, the solution was diluted (EtOAc), washed (brine), and dried (Na_2SO_4), and solvent removed in vacuo. Flash chromatography (60% hexanes/40% EtOAc) gave **13** (45 mg, 85%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.54 (dd, 0.7H, J = 9.9, 9.8), 5.42 (d, 0.7H, J = 3.6), 5.24 (dd, 0.3H, J = 9.6, 9.6), 5.03−4.94 (m, 3H), 4.87 (dd, 0.7H, J = 7.4, 3.6), 4.84 (dd, 0.3H, J = 9.7, 8.0), 4.73 (d, 0.3H, J = 10.1), 4.42 (d, 0.7H, J = 10.1), 3.88 (d, 0.3H, J = 9.9), 3.30 (broad s, 1H), 2.06 (s, 3H, Ac), 1.99 (s, 3H, Ac), 1.94 (s, 3H, Ac), 1.94 (s, 3H, Ac), 1.74 (s, 0.9H), 1.72 (s, 2.1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.9, 170.3, 170.2, 169.6, 161.8, 140.3, 139.8, 116.7, 116.6, 95.3, 90.1, 78.4, 73.4, 73.2, 72.1, 71.3, 69.6, 69.6, 69.4, 20.7, 20.7, 20.6, 16.7; DCI(+) MS (NH_3) m/z 334 (M + NH_4^+, 38%). Anal. Calcd for $C_{14}O_8H_{20}$: C, 53.16; H, 6.37. Found: C, 53.32; H, 6.47.

7-Deoxy-D-*gluco*-hept-6-ulopyranosyl Phosphate, Sodium Salt (14). To a stirring solution of **13** (82 mg, 0.26 mmol) in CH_2Cl_2 (5 mL) were added 1*H*-tetrazole (73 mg, 1.0 mmol) and dibenzyl *N*,*N*-diisopropylphosphoramidite (0.22 mL, 0.65 mmol). After 2 h at rt, the reaction mixture was diluted (Et_2O), washed (ice-cold brine), and dried (Na_2SO_4), and solvent was removed in vacuo. The residue was immediately dissolved in CH_2Cl_2 (20 mL) and cooled to −78 °C, and ozone was bubbled through the solution until it turned blue. Argon was bubbled through the solution until the color disappeared, dimethyl

sulfide (1 mL) was added, and the solution was stored overnight at −20 °C. Solvent was removed in vacuo, the residue was redissolved in 50% MeOH/50% EtOAc (20 mL), and 10% Pd/C (40 mg) was added. The solution was repeatedly degassed in vacuo, with H_2 being used to equalize the pressure each time. After the solution was stirred for 1 h under H_2, a small amount of cation-exchange resin (pyridinium form) was added, and the solution was filtered and concentrated in vacuo. The residue was dissolved in 1:1 MeOH/H_2O containing 10% NEt_3, and the reaction was stirred at rt for 3 h. Two successive rounds of preparative TLC (45% MeOH/45% $CHCl_3$/10% H_2O), exchange to a sodium counterion, and lyophilization gave **14** as a white powder (3α:2β by ^{1}H NMR, 33.1 mg, 40%). Material prepared in this manner contained a persistent impurity (10 mol % by ^{1}H NMR) displaying spectral properties consistent with *i*-$Pr_2NPO_3Na_2$: ^{1}H NMR (400 MHz, D_2O) δ 5.48 (dd, 0.60H, J = 6.7, 3.5), 4.92 (dd, 0.40H, J = 8.0, 7.3), 4.41 (d, 0.60H, J = 10.2), 4.06 (d, 0.40H, J = 9.7), 3.78 (dd, 0.60H, J = 9.5, 9.3), 3.58−3.44 (m, 2H), 3.52 (d, 1.3H, J = 10.6, $(Me_2CH)_2NPO_3Na_2$), 3.35 (dd, 0.40H, J = 8.7, 8.2), 2.31 (s, 3H); ^{13}C NMR (75 MHz, D_2O) δ 211.0, 210.1, 97.7 (d, J = 3.7), 94.8 (d, J = 4.4), 79.9, 76.4, 75.8, 74.1 (d, J = 5.4), 73.2, 71.8 (d, J = 5.8), 71.6, 71.4, 52.8 (d, J = 4.1, $(Me_2CH)_2NPO_3Na_2$), 28.3, 28.1; ^{31}P NMR (121.5 MHz, D_2O) δ 3.07 (s, 0.4P), 2.56 (s, 0.1P), 0.26 (s, 0.6P); HR-LSI(−) MS (thioglycerol matrix) *m*/*z* calcd for $C_7H_{12}O_9P$ 271.0219, found 271.0221.

Uridine 5′-(7-deoxy-α-D-*gluco*-hept-6-ulopyranosyl Diphosphate), Sodium Salt (5). Compound **14** (18.5 mg, 0.059 mmol) and uridine-5′-triphosphate (32.6 mg, 0.059 mmol) were dissolved in 14.5 mL of 100 mM Hepes (pH 7.5) containing 5.0 mM $MgSO_4$ and 1.0 mM dithiothreitiol. The reaction was initiated by addition of UDP-glucose pyrophosphorylase (30 units) and inorganic pyrophosphatase (30 units). After 24 h, a second portion of each enzyme was added (20 units). Ion-paired reversed-phase HPLC indicated the reaction had gone to completion after 48 h, at which time the reaction mixture was diluted 2-fold with water and purified by anion-exchange and size-exclusion chromatography as described above for the preparation of **4a**. Sodium exchange and lyophilization afforded **5** as a white powder (5.4 mg, 15%): ^{1}H NMR (400 MHz, D_2O) δ 7.91 (d, 1H, J = 8.1), 5.98−5.93 (m, 2H), 5.62 (dd, 1H, J = 7.3, 3.4), 4.46 (d, 1H, J = 10.2), 4.35−4.12 (m, 5H), 3.80 (dd, 1H, J = 9.6, 9.3), 3.57 (ddd, 1H, J = 9.8, 3.3, 3.2), 3.48 (dd, 1H, J = 10.2, 9.2), 2.31 (s, 3H, H/D exchange in D_2O gives m, <1H); ^{31}P NMR (121.5 MHz, D_2O) δ −10.9 (d, 1P, J = 20.2), −12.7 (d, 1P, J = 20.1); HR-LSI(−) MS (thioglycerol matrix) *m*/*z* calcd for $C_{16}H_{22}N_2O_{17}P_2Na$ 599.0291, found 599.0268.

Enzymatic Studies. UDPGDH (1.5 unit/mg) was prepared as previously reported.[12] All enzyme kinetic experiments were performed at 30 °C in 50 mM triethanolamine/HCl (pH 8.7), with 2 mM dithiothreitol. Initial rates were determined by following the reduction of NAD^+ at 340 nM with a Varian Cary 3E UV−vis spectrophotometer followed by least-squares analysis with Cary 3 software version 3.0. For the K_I determination of **5**, the enzymatic reaction was initiated by addition of UDPGDH (final concentration 0.011 mg/mL) to assay buffer (1.0 mL) containing 500 μM NAD^+ and varying concentrations of both **5** and UDPG. The standard error of the reported K_I value is less than 10%. To test **4a** and **4b** as substrates, UDPGDH (final concentration 1.4 mg/mL) was added to assay buffer (0.60 mL) containing either **4a** or **4b** and 10.0 mM NAD^+. Both **4a** and **4b** were also tested as substrates with 25 mM NH_2OH in the assay buffer. To measure the kinetic constants for turnover of **4a**, UDPGDH (final concentration 0.48 mg/mL) was added to assay buffer (0.60 mL) containing 25 mM NH_2OH, 10 mM NAD^+, and varying concentrations of **4a**. Figure 3 was constructed using the program Grafit.

Ion-Paired Reversed-Phase HPLC. HPLC experiments were performed with a Radial-pak C-18 column with detection at 260 nM. The column was eluted by washing for 10 min with 100 mM K_2PO_4 (pH 6.9) and 5.0 mM tetrabutylammonium hydrogen sulfate followed by a linear gradient of 0−50% acetonitrile in the same buffer. To determine the product of the enzymatic reduction of **5**, UDPGDH (final concentration 1.1 mg/mL) was incubated at 30 °C with 10.0 mM NADH and 1.0 mM **5** in a total volume of 0.380 mL of assay buffer. A control sample without UDPGDH was also prepared. Aliquots were removed at timed intervals and analyzed by ion-paired reversed-phase HPLC as described. Peaks were identified by spiking aliquots with **5**, **4a**, **4b**, or NAD^+.

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Supporting Information Available: ^{1}H NMR spectra for compounds **4**, **5**, **8**, and **14** and X-ray crystal structure data for compound **7b**. This material is available free of charge via the Internet at http://pubs.acs.org.

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