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Chemoenzymatic Synthesis of Uridine 5′-Diphospho-2-acetonyl-2-deoxy-α-D-glucose as C_2-Carbon Isostere of UDP-GlcNAc

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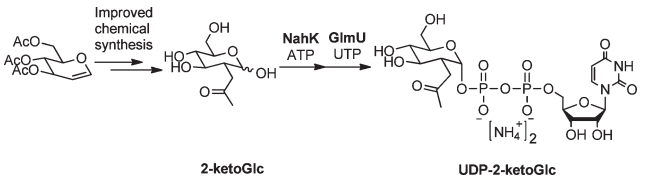

2-ketoGlc, which is the C_2-carbon isostere of GlcNAc, is a novel GlcNAc analogue with a ketone group. The corresponding glycosyltransferase donor substrate, UDP-2-ketoGlc, is necessary for synthesizing 2-ketoGlc-containing molecules and is thus highly important for metabolic polysaccharide remodeling and engineering. We report here the first chemoenzymatic synthesis of UDP-2-ketoGlc using our two-enzyme (NahK and GlmU) system in vitro.

Biomolecules containing sugar moieties are ubiquitous in nature. For example, the sugars *N*-acetylglucosamine (GlcNAc) can be found in living organisms ranging from bacteria to vertebrates where they act as the fundamental components of many important polysaccharides (e.g., bacterial cell walls[1,2] and glycosaminoglycans[3,4]). Chemical modification of GlcNAc residues by an unnatural analogue in such a polysaccharide chain, which requires unnatural sugar nucleotides as critical donor molecules, would thus be a valuable tool to get insight into the mechanism of polysaccharide formation and metabolic pathways.[5,6]

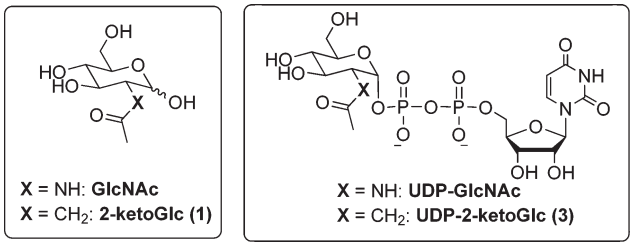

FIGURE 1. Chemical structures of 2-ketoGlc and UDP-2-ketoGlc.

2-Ketosugars, which are C_2-carbon isosteres of the 2-*N*-acetamidosugars, were reported as analogues that possess a ketone group which could be chemoselectively labeled by hydrazide reagents (Figure 1).[7,8] Bertozzi's group demonstrated that a 2-ketoGal (C_2 isostere of GalNAc) has been taken as a substrate for metabolic glycoprotein engineering through the salvage pathway while the GlcNAc counterpart could not be incorporated.[7] The major reason lies in the lack of 1-phosphorylation enzyme for GlcNAc which is critical for the generation of UDP-sugar donor and the competition effect as well. Thus, it would be very promising to have easy access to the UDP-sugar donor of 2-ketoGlc (Figure 1) to realize the incorporation of this GlcNAc analogue.

We have previously developed a methodology for chemoenzymatic synthesis of uncommon GlcNAc/GalNAc-1-phosphate analogues and unnatural UDP-GlcNAc/GalNAc sugar donor analogues library using the enzymes NahK and GlmU.[9−12] NahK, which is an *N*-acetylhexosamine 1-kinase with surprisingly relaxed specificity,[10,11] can convert GlcNAc and its analogues to GlcNAc-1-phosphate and its analogues with high efficiency. GlmU, which is an *N*-acetylglucosamine 1-phosphate uridyltransferase, can transform the sugar-1-phosphates into the corresponding UDP-sugar donors.[12] Here we report the first synthesis of the essential 2-ketoGlc donor molecule UDP-2-ketoGlc (**3**) using a chemoenzymatic route by our two-enzyme system in vitro.

To synthesize free 2-ketoGlc (**1**) as a NahK substrate, the key intermediate *β-gluco*-type 2-iodo compound **6** was previously prepared as a minor isomer from the glucal by reaction with NIS or I_2; however, the yield for this desired isomer was normally less than 10%.[7,8,13] We designed an improved route as shown in Scheme 1. *tert*-Butyldiphenylsilyl groups of **5**[14] were converted to acetyl by TBAF followed by peracetylation. Keck radical reaction of **6** with

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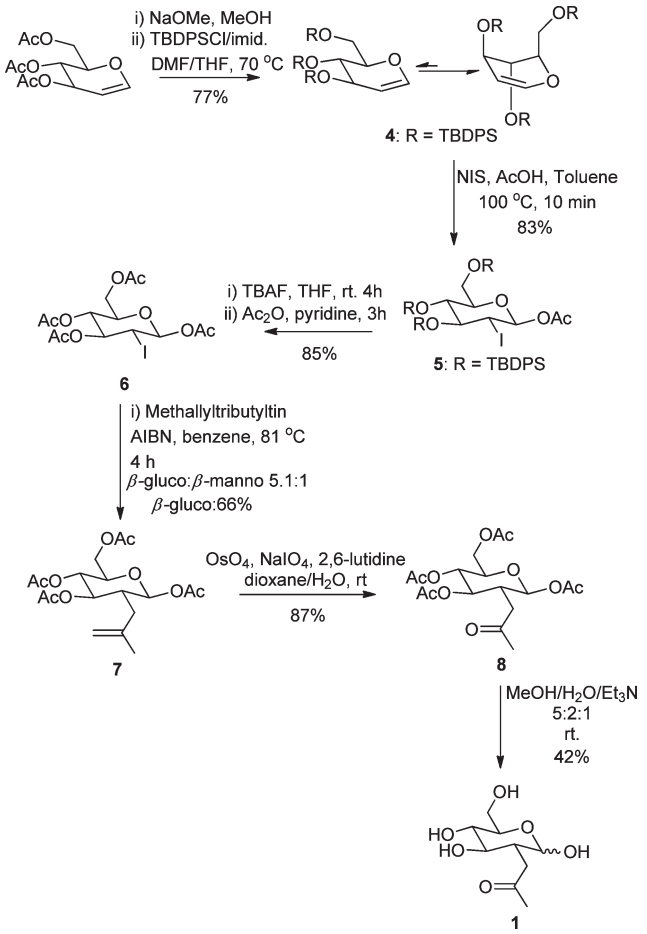
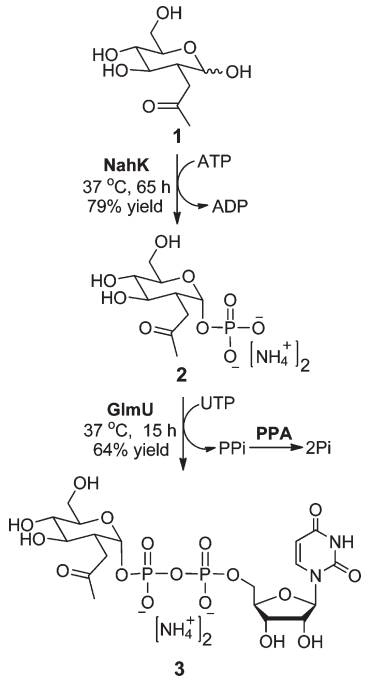
SCHEME 1. Improved Synthesis of 2-ketoGlc



methallyltributyltin afforded compound **7** in 66% yield with the *β-manno* type as a side product. Longer refluxing time (overnight as reported in ref 7) may result in the removal of anomeric acetyl group. Oxidative cleavage of olefin **7** by OsO_4-NaIO_4 in the presence of weak base[15] furnished peracetylated 2-ketoGlc **8** in good yield which was further deprotected under mild conditions to provide the desired 2-ketoGlc **1**. It is worth noting that although moderate yield was observed for the deacetylation reaction, other methods (MeONa/MeOH or K_2CO_3/MeOH) did not give our desired free sugar (< 10%). The NMR data of compound **8** were in disagreement with those reported by Bertozzi and co-workers.[7] However, our HRMS and COSY experiments confirmed that our structural assignment was correct (see the Experimental Section and Supporting Information). In their paper, H-3 was missing, the coupling constant for H-4 was not consistent with the *gluco*-type configuration, and the chemical shifts of H-6 were not correct.

2-ketoGlc (**1**) was taken as a good substrate for both enzymes as shown in Scheme 2: 1-phosphorylation was carried out for a longer time (65 h), and additional NahK

SCHEME 2. Enzymatic Production of UDP-2-ketoGlc Using NahK and GlmU Systems



was added during the reaction course to fulfill the greatest conversion (79%).[16] The product 2-ketoGlc-1-P (**2**) was purified by silica gel chromatography and Bio-Gel P-2 column. Pyrophosphorylation of **2** was catalyzed by GlmU in the presence of UTP. Yeast inorganic pyrophosphatase (PPA) was added to drive the reaction forward by degrading the byproduct pyrophosphate (PPi). The resulting UDP-2-ketoGlc was purified by DEAE-cellulose anion exchange resin and Bio-Gel P-2 column for desalting purpose. The good yield of both reactions indicates that the amide -NH- of GlcNAc may not be critical for the recognition of GlcNAc by NahK and GlcNAc-1-phosphate by GlmU in the UDP-GlcNAc biosynthesis. By making these changes to NahK reaction (longer reaction time and additional enzyme), we were able to broaden the scope of substrates of our system which can be utilized in the synthesis of more UDP-Glc derivatives with C-2 modifications (e.g., UDP-2-azido-Glc was also synthesized successfully in our laboratory).

In conclusion, we have successfully demonstrated a chemoenzymatic synthesis of UDP-2-ketoGlc based on our two-enzyme UDP-sugar synthesis system and an improved chemical route to 2-ketoGlc. The structure assignment of compound **8** was corrected. To the best of our knowledge, this is the first chemoenzymatic synthesis of UDP-2-ketoGlc, and most notably, we were able to broaden the substrate specificity of our enzymes by manipulating the reaction conditions to further expand our UDP-sugar donor library.

Experimental Section

2-Deoxy-2-iodo-1,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside (6). To a solution of compound **5** (2 g, 1.91 mmol) in THF (50 mL) was added 1 M TBAF solution in THF (9 mL, 9 mmol). The reaction mixture was stirred at rt for 4 h. The solvent was removed under reduced pressure to afford the triol as a dark brown syrup which was redissolved in anhydrous pyridine

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(30 mL) and treated with acetic anhydride (4 mL) and DMAP (20 mg, cat.). The mixture was stirred at rt for 3 h. Then the solvent was removed under reduced pressure, and the residue was purified by chromatography (hexanes/EtOAc 5:1−4:1) to afford compound **6** (744 mg, 85%) as a clear syrup. NMR data were consistent with ref 13.

2-Deoxy-2-(methallyl)-1,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranoside (7). Compound **6** (820 mg, 1.789 mmol) was dissolved in 20 mL of anhydrous benzene, and methallyltributyltin (4.32 g, 12.53 mmol) was added under Ar. The reaction mixture was degassed by bubbling Ar through the solution for 30 min. AIBN (59 mg, 0.358 mmol) was added, and the reaction mixture was heated at 81 °C for 4 h. The solvent was removed under reduced pressure, and the residue was partitioned between CH_3CN (100 mL) and pentane (200 mL). The CH_3CN layer was separated and concentrated under reduced pressure. The residue was purified by chromatography (hexanes/EtOAc 5:1) to afford compound **7** (459 mg, 66%) as a clear syrup, while *β-manno* (93 mg, 13%) type was isolated as a byproduct. NMR data were consistent with ref 7.

2-Deoxy-2-(acetonyl)-1,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranoside (8). To solution of compound **7** (127 mg, 0.329 mmol) in dioxane/H_2O (v/v 3:1, 6 mL) were added successively 2,6-lutidine (76 μL, 0.658 mmol), OsO_4 (2.5% in 2-methyl-2-propanol, 83 μL, 0.0066 mmol), and $NaIO_4$ (281 mg, 1.315 mmol). The reaction mixture was stirred at rt overnight. The mixture was partitioned between CH_2Cl_2 (30 mL) and H_2O (15 mL), and the aqueous phase was extracted with CH_2Cl_2 (10 mL). The combined organic layer was dried over Na_2SO_4, filtered, and concentrated under reduced pressure, and the residue was purified by chromatography (hexanes/EtOAc 2:1) to afford compound **8** (111 mg, 87%) as a white solid: ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.70 (d, J = 9.2 Hz, 1H, H-1), 5.10 (dd, J = 10.8, 9.2 Hz, 1H, H-3), 5.03 (app t, J = 9.7 Hz, 1H, H-4), 4.31 (dd, J = 12.4, 4.5 Hz, 1H, H-6a), 4.08 (dd, J = 12.4, 2.1 Hz, 1H, H-6b), 3.78−3.81 (m, 1H, H-5), 2.52−2.58 (m, 1H, H-2), 2.42 (m, 2H, CH_2), 2.12 (s, 3H), 2.09 (s, 3H), 2.08 (s, 3H), 2.01 (s, 3H), 2.01 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 205.2, 170.8, 170.6, 169.8, 169.0, 93.7, 73.5, 72.8, 68.9, 62.0, 41.4, 40.9, 30.0, 20.9, 20.86, 20.8, 20.7; HRMS (ESI) calcd for $C_{17}H_{24}O_{10}Na$ $(M + Na)^+$ 411.1262, found 411.1249 m/z.

2-Deoxy-2-(acetonyl)-*β*-D-glucopyranose (1). Compound **8** (63.5 mg, 0.164 mmol) was dissolved in a mixture of MeOH/H_2O/Et_3N (v/v/v 5:2:1, 5 mL) and stirred at rt for 18 h. The solvent was removed under reduced pressure, and the residue was purified by chromatography (CH_2Cl_2/MeOH 15:1) to afford 2-ketoGlc **1** as a colorless syrup (α and β mixture ~1:1 as indicated by ^{1}H NMR, 15 mg, 42%): ^{1}H NMR (500 MHz, D_2O, α and β mixture ~1:1) δ 5.24 (d, J = 3.3 Hz, 1H, H-1α), 4.69 (d, J = 8.7 Hz, 1H, H-1β), 3.91 (dd, J = 12.3, 2.1 Hz, 1H), 3.74−3.87 (m, 5H), 3.59 (dd, J = 10.9, 9.1 Hz, 1H), 3.39−3.45 (m, 3H), 2.88 (dd, J = 17.4, 5.5 Hz, 1H), 2.76 (dd, J = 16.2, 5.5 Hz, 1H), 2.65−2.71 (m, 2H), 2.28 (s, 3H), 2.276 (s, 3H), 2.24−2.30 (m, 1H), 2.06−2.12 (m, 1H); ^{13}C NMR (125 MHz, D_2O, α and β mixture ~1:1) δ 215.7, 215.3, 97.3 (C-1α), 93.3 (C-1β), 76.7, 75.4, 72.6, 72.5, 71.6, 71.4, 61.7, 61.6, 46.6, 43.0, 42.5, 42.4, 30.6 (2 CH_3); HRMS (ESI) calcd for $C_9H_{16}O_6Na$ $(M + Na)^+$ 243.0839, found 243.0829 m/z.

2-Deoxy-2-(acetonyl)-α-D-glucosyldiammonium Phosphate (2). The reaction mixture (1.249 mL) contained 40 mM GlcNAc or its analogues, 50 mM ATP, 10 mM $MgCl_2$, and 1.5 mg/mL NahK in 100 mM Tris−HCl buffer (pH 9.0). After incubation at 37 °C for 48 h, additional NahK was added to a final concentration of 2.25 mg/mL. The mixture was incubated at 37 °C for another 17 h. Upon completion, the reaction mixture was briefly boiled for 2 min and then centrifuged to remove protein. The supernatant was concentrated under reduced pressure, and the residue was purified by normal-phase silica gel column chromatography using gradient CH_2Cl_2/5 mM NH_4HCO_3 in methanol as eluent (CH_2Cl_2/5 mM NH_4HCO_3 in methanol 1:1−0:1). The fractions containing the products were collected and concentrated under reduced pressure. The residue was further purified by Bio-Gel P-2 column. The fractions containing products were combined and lyophilized to provide compound **2** as a white foam (13.2 mg, 79%, diammonium salt): ^{1}H NMR (500 MHz, D_2O) δ 5.47 (dd, J = 7.7, 3.0 Hz, 1H), 3.89−3.95 (m, 2H), 3.81 (dd, J = 12.4, 5.2 Hz, 1H), 3.67 (dd, J = 11.0, 9.1 Hz, 1H), 3.45 (app t, J = 9.6 Hz, 1H); 2.88 (dd, J = 18.0, 5.5 Hz, 1H), 2.78 (dd, J = 18.0, 7.2 Hz, 1H), 2.30 (s, 3H), 2.27−2.34 (m, 1H); ^{13}C NMR (125 MHz, D_2O) δ 214.5 (d), 94.8, 72.7, 71.7, 70.7, 60.9, 42.3 (d), 41.4, 29.9 (d); ^{31}P NMR (202 MHz, D_2O): δ 2.39; HRMS (ESI) calcd for $C_9H_{16}O_9P$ $(M - H)^-$ 299.0537, found 299.0545 m/z.

Uridine 5′-Diphospho-2-acetonyl-2-deoxy-α-D-glucopyranose Diammonium Salt (3). The reaction was carried out in a cocktail (3.952 mL) containing 10 mM 2-ketoGlc-1-P (**2**), 15 mM UTP, 10 mM $MgCl_2$, 100 mM Tris−HCl (pH 7.5), 1 mg/mL GlmU, and 1 U/mL inorganic pyrophosphatase. After incubation at 37 °C for 15 h, the mixture was heated in boiling water for 2 min followed by centrifugation to remove protein. The supernatant was directly loaded on DEAE-cellulose anion-exchange resin using gradient ammonium bicarbonate as eluent (0 to 250 mM). The fractions containing the products were collected and freeze-dried, and the residue was further purified by Bio-Gel P-2 column. The fractions containing products were combined and freeze-dried to provide compound **3** as a white foam (16.3 mg, 64%): ^{1}H NMR (400 MHz, D_2O) δ 8.01 (d, J = 8.1 Hz, 1H), 6.00−6.02 (m, 2H), 5.58 (dd, J = 7.2, 2.9 Hz. 1H), 4.39−4.43 (m, 2H), 4.19−4.32 (m, 3H), 3.87−3.95 (m, 2H), 3.82 (dd, J = 12.4, 4.4 Hz, 1H), 3.67 (dd, J = 10.6, 9.1 Hz, 1H), 3.49 (app t, J = 9.7 Hz, 1H), 2.76−2.92 (m, 2H), 2.29 (s, 3H), 2.26−2.36 (m, 1H); ^{13}C NMR (100 MHz, D_2O) δ 214.1, 166.2, 151.8, 141.7, 102.7, 96.0 (d), 88.4, 83.3 (d), 73.8, 73.1, 71.5, 70.4, 69.6, 64.9 (d), 60.5, 42.1 (d), 41.2, 29.9; ^{31}P NMR (162 MHz, D_2O) δ = −11.5 (d, J = 19.9 Hz), −12.9 (d, J = 19.9 Hz); HRMS (ESI) calcd for $C_{18}H_{27}N_2O_{17}P_2$ $(M - H)^-$ 605.0790, found 605.0814 m/z.

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra for compounds **1**−**3** and **6**−**8** and ^{31}P NMR spectra for compounds **2** and **3**. This material is available free of charge via the Internet at http://pubs.acs.org.