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Efficient Enzymatic Synthesis of Guanosine 5′-Diphosphate-Sugars and Derivatives

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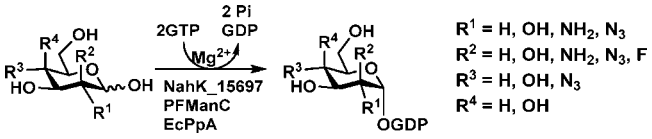
ABSTRACT



An *N*-acetylhexosamine 1-kinase from *Bifidobacterium infantis* (NahK_15697), a guanosine 5′-diphosphate (GDP)-mannose pyrophosphorylase from *Pyrococcus furiosus* (PFManC), and an *Escherichia coli* inorganic pyrophosphatase (EcPpA) were used efficiently for a one-pot three-enzyme synthesis of GDP-mannose, GDP-glucose, their derivatives, and GDP-talose. This study represents the first facile and efficient enzymatic synthesis of GDP-sugars and derivatives starting from monosaccharides and derivatives.

Glycosyltransferases are key enzymes responsible for the assembly of carbohydrates. Most of these enzymes require activated sugar-nucleotides as donor substrates. Thus, development of facile protocols for efficient synthesis of such molecules are of great significance and has been an active field of research.[1] Among guanosine 5′-diphosphate (GDP)-activated sugars, GDP-mannose (GDP-Man) is essential for the biosynthesis of mannosyl donor dolichol phosphate β-D-mannose (Dol-P-Man) involved in the synthesis of eukaryotic *N*-glycans, glycosylphospho-inositol (GPI) anchors, and O-mannosylated glycoproteins,[2] as well as bacterial cell-surface polysaccharides.[3] GDP-Man is also a fundamental metabolic intermediate for the biosynthesis of many other natural GDP-sugars, including GDP-mannuronic acid (GDP-ManA), GDP-L-fucose (GDP-Fuc), GDP-6-deoxy-Talose (GDP-6deoxyTal), etc.[1] Other GDP-sugars, such as GDP-glucose (GDP-Glc) and GDP-glucosamine (GDP-GlcNH$_2$), are key intermediates in the biosynthesis of β1,4-glucans, glucosylglycerate, and legionaminic acid containing glycoconjugates.[4]

Chemical synthesis of sugar-nucleotides generally suffers from tedious protection/deprotection steps, low total yields, and long reaction times.[5] On the other hand, enzymatic approaches following *de novo* biosynthetic pathways require multiple enzymes and laborious separation processes. Recently, salvage biosynthetic pathways of several sugar-nucleotides were discovered, which usually involve two enzyme-catalyzed steps: (1) a kinase-catalyzed

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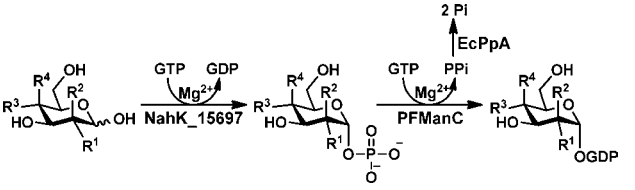
formation of monosaccharide 1-phosphate from the corresponding monosaccharide and ATP; (2) a pyrophosphorylase-catalyzed formation of sugar-nucleotide and pyrophosphate byproduct from nucleotide triphosphate and the monosaccharide 1-phosphate. Taking advantage of promiscuous enzymes involved in these pathways, efficient chemoenzymatic approaches were developed for preparative-scale synthesis of sugar-nucleotides and their non-natural derivatives. For example, a bifunctional L-fucose 1-kinase/GDP-Fuc pyrophosphorylase (FKP) from *Bacteroides fragilis* was applied successfully for the synthesis of GDP-Fuc and derivatives.[6] In addition, monosaccharide 1-kinases and a promiscuous UDP-sugar pyrophosphorylase (BLUSP) were used efficiently for one-pot enzymatic synthesis of UDP-hexose and derivatives from simple hexose and derivatives.[7] Furthermore, a panel of UDP-HexNAc and derivatives were chemoenzymatically prepared by combining an *N*-acetylhexosamine 1-kinase (NahK) and an UDP-*N*-acetylglucosomine pyrophosphorylase (GlmU or AGX1) in either a one-pot or a sequenctial manner.[8,9]

Nevertheless, such a simple synthetic route has not yet been developed for the synthesis of GDP-Man and other GDP-sugars, mainly due to the lack of suitable monosaccharide 1-kinases. As a result, chemically prepared or commercially available mannose 1-phosphate and derivatives were generally used in the formation of GDP-sugars.[10−12] We recently found that a NahK from *Bifidobacterium infantis* ATCC15697 (NahK_15697) could phosphorylate a number of monosaccharides including mannose and derivatives.[13] Taking advantage of this and the promiscuity of NahK_15697 and a GDP-Man pyrophosphorylase from *Pyrococcus furiosus* DSM3638 (PFManC),[12] we present here an efficient one-pot three-enzyme system for quick preparative-scale synthesis of GDP-sugars and their derivatives.

As shown in Scheme 1, three enzymes were used in one pot to synthesize GDP-Man, GDP-Glc, their derivatives, and GDP-Tal. The first enzyme was NahK_15697, which catalyzed the formation of monosaccharide 1-phosphates. The second enzyme was PFManC, which catalyzed the reversible formation of GDP-sugars and pyrophosphate from monosaccharide 1-phosphates and guanosine 5′-triphosphate (GTP). The last enzyme was an inorganic pyrophosphatase cloned from *Escherichia coli* (EcPpA).[14] It drove the reaction toward the formation of GDP-sugars by hydrolyzing the pyrophosphate byproduct.

Scheme 1. One-Pot Three-Enzyme Synthesis of GDP-Sugars



Genetic analysis showed that the DNA sequence of the archaeal enzyme PFManC contains numerous rare codons. To increase the heterologous protein expression level in *E. coli*, the DNA sequence of PFManC was codon optimized. The synthetic gene obtained by custom synthesis was cloned into the pET22b(+) vector. The protein was overexpressed in *E. coli* BL21(DE3), yielding over 80 mg of PFManC per liter of cell culture after purification.[15]

Besides GTP, it was reported that PFManC could also utilize ATP to form ADP-sugars.[12] In order to avoid unexpected byproduct formation in the one-pot system, GTP, instead of ATP, was used as the phosphate donor for NahK_15697 (Scheme 1). To our delight, GTP was a suitable substrate for NahK_15697. As shown in Table S1 and Figure S2, except for Man4N_3 (**6**) which had a relatively low yield of 36%, NahK_15697 was able to use GTP as a phosphate donor for high-yield (> 53%) phosphorylation of all other monosaccharides and derivatives tested including mannose (**1**) and its derivatives (**2**−**5**), talose (**7**), and glucose (**8**) as well as its C2-derivatives (**9**−**12**). The results confirmed previously reported broad substrate specificity of NahK toward both monosaccharides and phosphate donors.[8,13,16] We also tested a number of C6 modified substrates, including Rha (**25**), Rha4N_3 (**26**), PerNAc (**27**), 6-deoxyTal (**28**), and ManA (**29**), but none was a suitable substrate (Table S1 and Figure S2) for NahK_15697 when either ATP or GTP was used as the phosphate donor. The results imply that the C6 hydroxyl group may play essential roles in substrate recognition by NahK_15697.

The synthesis of GDP-sugars was carried out using the one-pot three-enzyme system shown in Scheme 1.[17] As listed in Table 1,[18] the system was quite efficient in synthesizing GDP-Man (**13**, 94%), GDP-ManNH_2 (**14**, 75%),

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(15) See Supporting Information for details about cloning, overexpression, and purification of PFManC.

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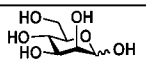
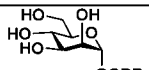
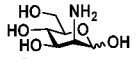
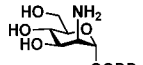
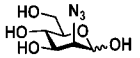
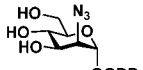
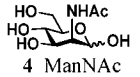
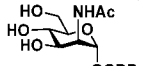
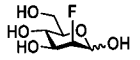
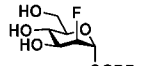
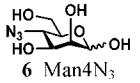
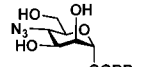
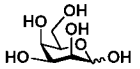
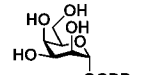
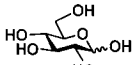
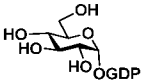
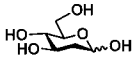
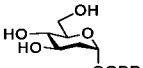
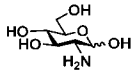
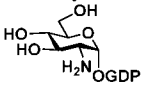
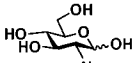
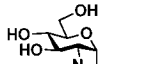
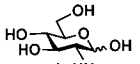
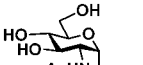
(17) See Supporting Information for reaction details.

(18) All NMR and MS data are available in the Supporting Information.

Table 1. Synthesis of GDP-Sugars Using the One-Pot Three-Enzyme System Shown in Scheme 1

substrate	product	yield [a] (%)	scale [b] (mg)
 **1** Man	 **13** GDP-Man	94	102
 **2** $ManNH_2$	 **14** $GDP\text{-}ManNH_2$	75	84
 **3** $ManN_3$	 **15** $GDP\text{-}ManN_3$	81	92
 **4** ManNAc	 **16** GDP-ManNAc	ND [c]	
 **5** ManF	 **17** GDP-ManF	84	91
 **6** $Man4N_3$	 **18** $GDP\text{-}Man4N_3$	33	37
 **7** Talose	 **19** GDP-Tal	47	51
 **8** Glc	 **20** GDP-Glc	72	78
 **9** 2-deoxyGlc (2-deoxyMan)	 **21** GDP-2 deoxyGlc	76	80
 **10** $GlcNH_2$	 **22** $GDP\text{-}GlcNH_2$	80	87
 **11** $GlcN_3$	 **23** $GDP\text{-}GlcN_3$	16	18
 **12** GlcNAc	 **24** GDP-GlcNAc	ND	

[a] Isolated yields from P-2 column. [b] The mass of isolated product. [c] ND, not detected.

$GDP\text{-}ManN_3$ (**15**, 81%), GDP-ManF (**17**, 84%), GDP-Glc (**20**, 72%), GDP-2-deoxyGlc (**21**, 76%), and $GDP\text{-}GlcNH_2$ (**22**, 80%) from corresponding monosaccharides and derivatives (**1**–**3**, **5**, **8**–**10**). $GDP\text{-}Man4N_3$ (**18**), a potential nonradioactive probe for investigating the activity of mannosyltransferases,[11] was synthesized with a moderate yield of 33%, most likely due to the less optimal activity of NahK_15697 for $Man4N_3$ (**6**). The system also provided a moderate yield (47%) and a low yield (16%) for the formation of GDP-Tal (**19**) and $GDP\text{-}GlcN_3$ (**23**), respectively, which may be attributed by less optimal PFManC activity for Tal 1-phosphate and $GlcN_3$ 1-phosphate. On the other hand, the synthesis of GDP-ManNAc (**16**) and GDP-GlcNAc (**24**) using the one-pot three-enzyme system was not successful, suggesting that substrates with bulkier groups at the C2 position are not acceptable for PFManC.

Concerning the report that PFManC exhibited optimal activity at 80 °C and was able to synthesize GDP-GlcNAc from GlcNAc 1-phosphate,[12] a one-pot two-step strategy was also tested for the preparation of GDP-ManNAc and GDP-GlcNAc. In general, reactions were first carried out in Tris-HCl buffer (100 mM, pH 8.0) containing ManNAc or GlcNAc (15 mM), GTP (35 mM), $MgCl_2$ (10 mM), and NahK_15697 (0.4 mg/mL). After incubation at 37 °C for 24 h, PFManC (0.5 mg/mL) and an excess of EcPpA were added and the reactions were allowed to proceed at 80 °C for up to 6 h. Unfortunately, neither of the reactions resulted in detectable GDP-sugars. More experiments are required to further identify the substrate specificity of PFManC.

In conclusion, we have further investigated the substrate specificity of NahK_15697 and PFManC using chemically or enzymatically prepared compounds and have developed an efficient one-pot three-enzyme system to quickly obtain GDP-Man, GDP-Glc, their non-natural derivatives, and GDP-Tal from simple monosaccharides and derivatives in preparative scale. These structurally defined GDP-sugars and derivatives are excellent compounds for investigating the substrate specificity of glycosyltransferases (e.g., mannosyltransferases).

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Supporting Information Available. Experimental details for cloning, overexpression, and purification of NahK_15697, PFManC, EcPpA, chemical synthesis of monosaccharide derivatives, and enzymatic synthesis of GDP-sugars and derivatives, as well as NMR and HRMS data and spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.