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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, GDPglucose, 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), and GDP-6-deoxy-Talose (GDP-6deoxyTal) etc.¹ Other GDP-sugars, such as GDP-glucose (GDP-Glc) and GDP-glucosamine (GDP-GlcNH₂), are key intermediates in the biosynthesis of β 1,4-glucans, glucosylglycerate, and legionaminic acid-containing glycoconjugates.⁴

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Supporting Information Available: Experimental details for cloning, over-expression, 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>.

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 requires 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 formation of monosaccharide 1-phosphate from the corresponding monosaccharide and ATP; 2) a pyrophosphorylase-catalyzed formation of sugar-nucleotide and pyrophosphate by-product from nucleotide triphosphate and the monosaccharide 1-phosphate. Taking advantage of promiscuous enzymes involved in these pathways, efficient chemo-enzymatic 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 chemo-enzymatically prepared by combining an *N*-acetylhexosamine 1-kinase (NahK) and an UDP-*N*-acetylglucosamine pyrophosphorylase (GlmU or AGX1) in either a one-pot or a sequential 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 manose 1-phosphate and derivatives were generally used in the formation of GDP-sugars.^{10,11,12} 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 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 towards the formation of GDP-sugars by hydrolyzing the pyrophosphate by-product.

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 pET22b(+) vector. The protein was overexpressed in *E. coli* BL21(DE3), yielding over 80 mg of PFManC per liter 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 by-product 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 Man4N₃ (**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**), as well as glucose (**8**) and 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**), Rha4N₃ (**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-ManNH₂ (**14**, 75%), GDP-ManN₃ (**15**, 81%), GDP-ManF (**17**, 84%), GDP-Glc (**20**, 72%), GDP-2-deoxyGlc (**21**, 76%), and GDP-GlcNH₂ (**22**, 80%) from corresponding mono-saccharides and derivatives (**1–3**, **5**, **8–10**). GDP-Man₄N₃ (**18**), a potential non-radioactive 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 Man₄N₃ (**6**). The system also provided a moderate yield (47%) and a low yield (16%) for the formation of GDP-Tal (**19**) and GDP-GlcN₃ (**23**), respectively, which may be attributed by less optimal PFMnC activity for Tal 1-phosphate and GlcN₃ 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, suggested that substrates with bulkier groups at C2 position are not acceptable for PFMnC.

Concerning the report that PFMnC 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 firstly carried out in Tris-HCl buffer (100 mM, pH 8.0) containing ManNAc or GlcNAc (15 mM), GTP (35 mM), MgCl₂ (10 mM), and NahK_15697 (0.4 mg/mL). After incubation at 37 °C for 24 hr, PFMnC (0.5 mg/mL) and excess of EcPpA were added and the reactions were allowed to proceed at 80 °C for up to 6 hr. Unfortunately, neither of the reactions resulted in detectable GDP-sugars. More experiments are required to further identify the substrate specificity of PFMnC.

In conclusion, we have further investigated the substrate specificity of NahK_15697 and PFMnC 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).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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R¹ = H, OH, NH₂, N₃; R² = H, OH, NH₂, N₃, F; R³ = H, OH, N₃; R⁴ = H, OH

Scheme 1. One-pot three-enzyme synthesis of GDP-sugars

R¹ = H, OH, NH₂, N₃; R² = H, OH, NH₂, N₃, F; R³ = H, OH, N₃; R⁴ = H, OH

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 ManNH₂	14 GDP-ManNH₂	75	84
3 ManN₃	15 GDP-ManN₃	81	92
4 ManNAc	16 GDP-ManNAc	ND ^c
5 ManF	17 GDP-ManF	84	91
6 Man4N₃	18 GDP-Man4N₃	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

substrate	product	yield ^a (%)	scale ^b (mg)
10 GlcNH ₂	22 GDP-GlcNH ₂	80	87
11 GlcN ₃	23 GDP-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.