Characterisation of a bacterial galactokinase with high activity and broad substrate tolerance for chemoenzymatic synthesis of 6-aminogalactose-1-phosphate and analogues

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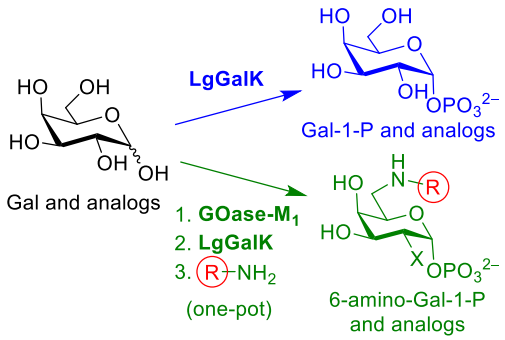
Abstract

Glycosyl phosphates are important intermediates in many metabolic pathways and are substrates for diverse carbohydrate active enzymes. There is a need to develop libraries of structurally similar analogues that can be used as selective chemical probes in glycomics. Here we explore chemoenzymatic cascades for the fast generation of glycosyl phosphate libraries without protecting group strategies. The key enzyme is a new bacterial galactokinase (LgGalK) cloned from *Leminorella grimontii* which was produced in *E. coli* and shown to catalyse 1-phosphorylation of galactose. LgGalK displayed a broad substrate tolerance, being able to catalyse the 1-phosphorylation of a number of galactose analogues, including 3-deoxy-3-fluorogalactose and 4-deoxy-4-fluorogalactose, which are first reported substrates for wild-type galactokinase. LgGalK and galactose oxidase variant M_1 were combined in a one-pot two-step system to synthesise 6-oxogalactose-1-phosphate and 6-oxo-2-fluorogalactose-1-phosphate, which were subsequently utilised to produce a panel of 30 substituted 6-aminogalactose-1-phosphate derivatives by chemical reductive amination in a one-pot three-step chemoenzymatic process.

Graphical abstract



Keywords

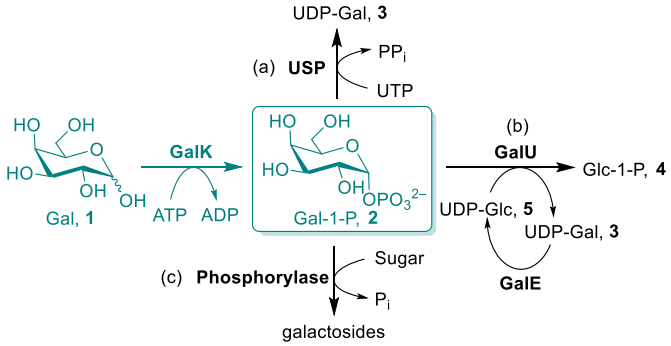
sugars ● biocatalysis ● kinases ● oxidoreductases ● carbohydrates

Introduction

Glycosyl phosphates are critically important in carbohydrate metabolism, and participate in primary metabolism (e.g., gluconeogenesis and glycolysis),[1] as well as in secondary metabolism (e.g., glycosylated natural product synthesis).[2] Furthermore, they can be involved in the regulation of metabolic processes.[3] In glycan biosynthesis, glycosyl-1-phosphates are key precursors to complex glycans, acting as natural glycosylation agents.

Galactose-1-phosphate (Gal-1-P, **2**) is a key intermediate in the metabolism of galactose (Gal, **1**). It can be used as an activated sugar donor to synthesise uridine diphosphate galactose (UDP-Gal, **3**) either by UDP-sugar pyrophosphorylase (USP) (Scheme 1a) in the salvage pathway,[4-6] or by galactose-1-phosphate uridyltransferase (GalU) and UDP-glucose-4-epimerase (GalE) in the Leloir pathway (Scheme 1b).[7] The resulting product **3** is a sugar donor for diverse galactosyltransferases. Phosphate **2** can also be used by phosphorylases to produce galactosides (Scheme 1c) *in vitro*. For example, BiGalHexNAcP is able to utilise **2** and its analogues to generate β-1,3-linked galactosides and their derivatives containing T-antigen and galacto-N-biose.[8] Therefore, stereoselective glycosidic phosphorylation can be considered as the key activation step for glycosylation.

The chemical synthesis of glycosyl phosphates is possible, but it requires several protection and deprotection steps. In addition, regio- and stereoselectivity can be difficult to control[9-11] and overall yields are generally low. On the other hand, enzymatic synthesis requires only one step from the free sugar, and is generally highly regio- and stereoselective. Enzymatic phosphorylation can also be utilised in one-pot enzymatic and chemoenzymatic cascades, which have become of great interest recently. [12,13]



Scheme 1. The role of galactokinase in sugar synthesis as a gateway enzyme for the production of Gal-1-P (**2**), which is a precursor of multiple biosynthetic pathways: (a) salvage pathway biosynthesis, (b) Leloir pathway biosynthesis; (c) phosphorylase catalysed galactoside synthesis.

Galactokinase (EC 2.7.1.6, GalK), which belongs to the GHMP kinase family (galactokinase, homoserine kinase, mevalonate kinase and phosphomevalonate kinase),[14] catalyses the ATP-dependent phosphorylation at the anomeric carbon of **1** to produce **2** (Scheme 1).[15] A variety of galactokinases from diverse organisms including both eukaryotes[16-20] and prokaryotes[21-24] have been identified and characterised, exhibiting different biochemical properties and substrate specificities. Several bacterial galactokinases, including EcGalK,[21] SpGalK,[22] BiGalK,[23] and MtGalK,[24] showed robust catalytic activity and demonstrated their use in the biocatalytic synthesis of nucleotide sugars,[25] as well as biologically significant glycans and their analogues,[26,27] but the substrate range has been limited to analogues that are structurally closely related to the parent sugar, often differing by only a single substitution.

Herein, a novel bacterial galactokinase from *Leminorella grimontii* (LgGalK) was overproduced and biochemically characterised. Its substrate specificity was investigated, which revealed a broad tolerance, making it suitable for the synthesis of several analogues of **2**. We also demonstrate its use in the synthesis of a panel of substituted 6-aminogalactose-1-phosphate in one-pot chemoenzymatic cascades, in combination with a galactose oxidase (GOase) variant and a chemical reductive amination step. We showed that in a simple three-step procedure, a diverse library of complex sugar phosphate analogues can be generated, providing a useful chemical probe toolkit for glycomics studies.

Results and Discussion

Cloning, expression and purification of LgGalK

The galactokinase gene (1167 bp) was amplified from the genomic DNA of *Leminorella grimontii*. A phylogenetic analysis (Figure 1) revealed that the closest characterised homologue of LgGalK is EcGalK from *E. coli* (68% identity), followed by MtGalK from *Meiothermus taiwanensis* (42%), while BiGalK from *Bifidobacterium infantis* was found to possess the lowest identity (32%). According to the amino acid sequence alignment (Supporting Information, Figure S1), all three typically conserved glycine-rich functional motifs of GHMP superfamily members were found in LgGalK, consistent with GalK belonging to this class.

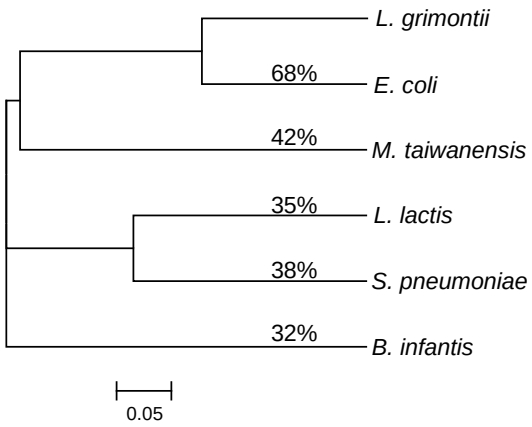


Figure 1. Phylogenetic tree of characterised galactokinases.

The open reading frame coding for LgGalK was cloned into a pET-30a vector with N-terminal His-tag. The recombinant protein was produced in *E. coli* BL21(DE3) cells (induced with 1 mM IPTG at 18°C for 20 hours) and purified by nickel affinity chromatography. SDS-PAGE analysis (Figure 2) indicates that the purified LgGalK has an apparent molecular weight of around 40 kDa, matching with the theoretical molecular mass of 42 kDa.

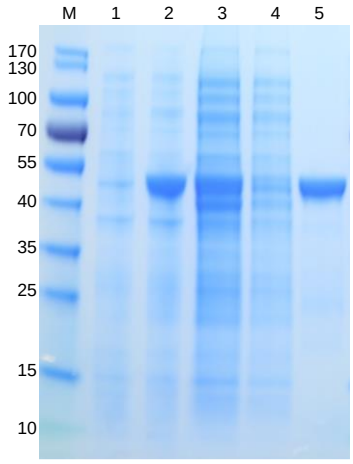


Figure 2. SDS-PAGE analysis of LgGalK. M: molecular weight marker; 1: before induction; 2: after induction; 3: supernatant; 4: flow-through; 5: purified LgGalK.

Biochemical characterisation of LgGalK

In order to characterise the properties of LgGalK, its activity against **1** was measured by the DNS assay[19] under a range of different conditions. The optimal temperature was found to be 50°C, with high activity between 40 and 55°C (Figure 3A). Enzymatic activity gradually increased from pH 6.0 to 9.0, but sharply decreased at pH 10.0 (Figure 3B). Similar to other characterised galactokinases, LgGalK requires Mg^{2+} as a cofactor, and the effect of several other divalent metal ions was also tested (Figure 3C). Cu^{2+} and Zn^{2+} decreased kinase activity considerably, while Ni^{2+}

and Fe^{2+} could substitute Mg^{2+} as cofactor with almost no reduction of activity. Furthermore, the optimal Mg^{2+} concentration was determined to be around 1 mM, with very modest decrease in activity as the Mg^{2+} concentration increased (Figure 3D). The apparent kinetic constants of LgGalK towards **1** were determined using the DNS assay by varying the concentration of **1** (1, 2, 4, 6, 8 mM) with saturation concentration of ATP (20 mM). The initial velocity was obtained by measuring the slope value of the linear phase in the progress curve. The Michaelis-Menten plot was generated to obtain the kinetic parameters for **1**: K_M = 1.3±0.4 mM, k_{cat} = 23.7±1.8 s^{-1}, k_{cat}/K_M = 18.1 $mM^{-1}{\cdot}s^{-1}$.

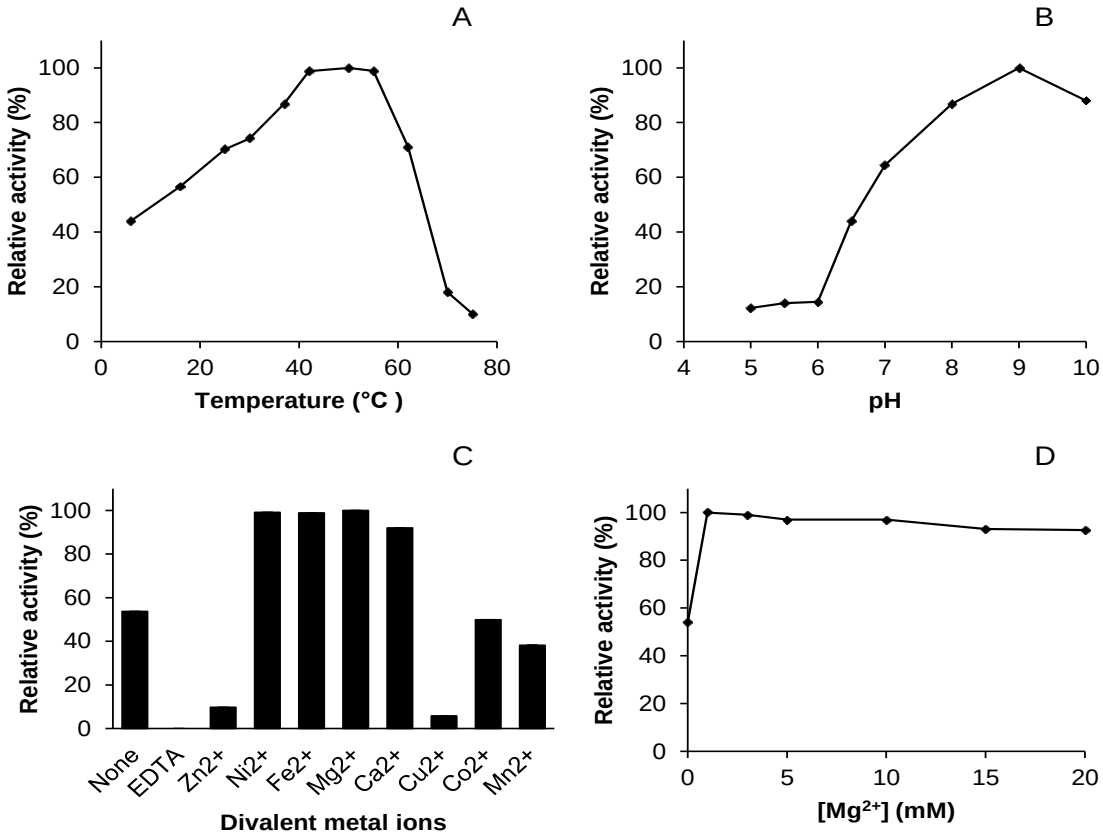
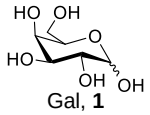
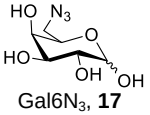
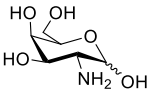
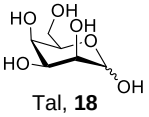
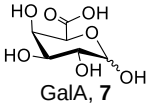
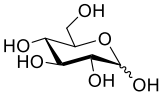
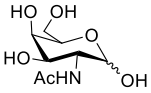
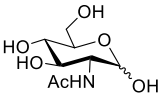
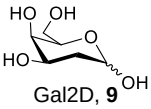
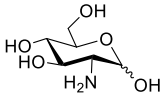
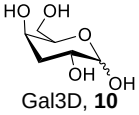
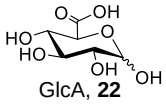
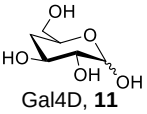
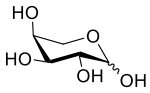
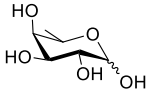
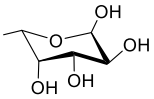
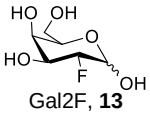
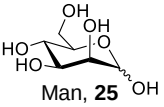
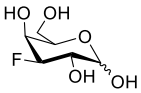
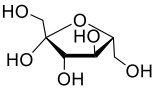
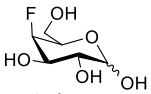
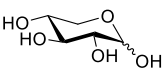
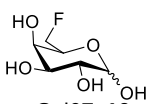


Figure 3. Biochemical characterisation of LgGalK: (A) temperature-activity profile, (B) pH-activity profile, (C) effect of divalent metal ions, (D) effect of Mg^{2+} concentration.

Substrate specificity study of LgGalK

The formation of **2** from **1** was monitored by TLC, and further confirmed by HRMS. Furthermore, ^{13}C-labelled galactose ($^{13}C_6$-**1**) was used as substrate and ^{13}C NMR analysis confirmed the formation of $^{13}C_6$-**2** (Supporting Information) The conversion was measured by the DNS assay, giving a value of 72% (Table 1) under the optimised conditions identified above (except for the concentration of Mg^{2+} which was increased to 5 mM).

Table 1. Substrate specificity of LgGalK towards different monosaccharides.

Substrate	MS[a]	TLC[a]	Conv. (%)	Substrate	MS[a]	TLC[a]	Conv. (%)
 Gal, **1**	+	+	72[b]	 Gal6N$_3$, **17**	–	–	NA
 GalN, **6**	+	+	68[b]	 Tal, **18**	+	+	41[b]
 GalA, **7**	–	–	NA	 Glc, **19**	–	–	NA
 GalNAc, **8**	–	–	NA	 GlcNAc, **20**	–	–	NA
 Gal2D, **9**	+	+	<5[b]	 GlcN, **21**	–	–	NA
 Gal3D, **10**	+	+	19[b]	 GlcA, **22**	–	–	NA
 Gal4D, **11**	+	+	<5[b]	 Ara, **23**	–	–	NA
 Gal6D, **12**	+	+	57[b]	 Fuc, **24**	–	–	NA
 Gal2F, **13**	+	+	80[c]	 Man, **25**	–	–	NA
 Gal3F, **14**	+	+	27[c]	 Fru, **26**	–	–	NA
 Gal4F, **15**	+	+	21[c]	 Xyl, **27**	–	–	NA
 Gal6F, **16**	+	+	28[c]				

[a] + : evidence of product formation; – : no evidence of product formation; NA: no activity;
[b] conversion determined by the DNS assay; [c] conversion determined by ^{19}F NMR.

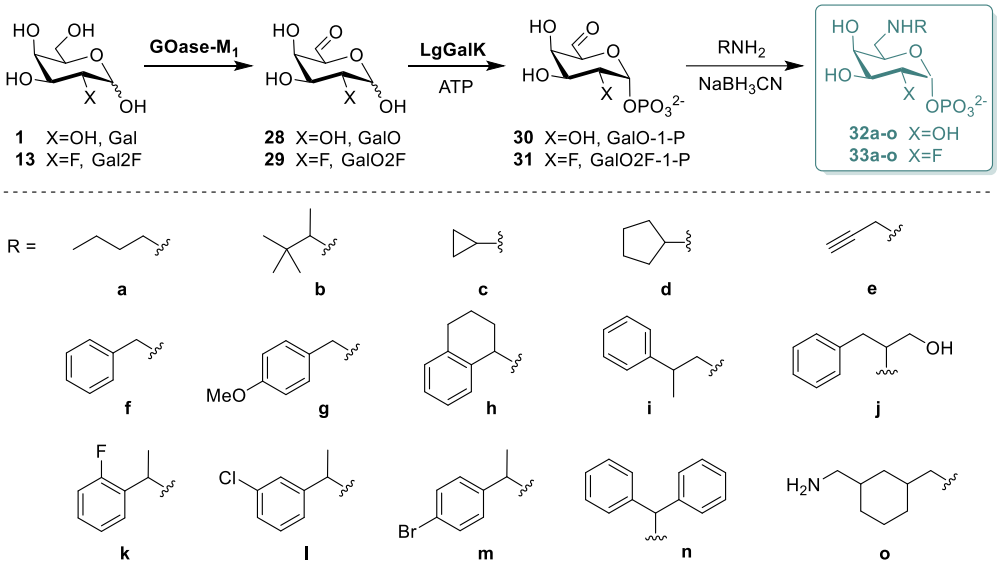
In order to study the substrate specificity, 22 different monosaccharides (**6-27**), including deoxygalactoses **9-12** and deoxyfluorogalactoses **13-16**, were tested as substrates of LgGalK (Table 1). The formation of products in the LgGalK reactions was monitored by TLC and HRMS (Supplementary Information). LgGalK was found to have some activity against 10 substrates to generate the corresponding sugar-1-phosphates: galactosamine **6**, 2-deoxygalactose **9**, 3-deoxygalactose **10**, 4-deoxygalactose **11**, 6-deoxygalactose **12**, 2-deoxy-2-fluorogalactose **13**, 3-deoxy-3-fluorogalactose **14**, 4-deoxy-4-fluorogalactose **15**, 6-deoxy-6-fluorogalactose **16** and talose **18**. Conversions for fluorinated substrates were determined by ^{19}F NMR (Supplementary Information) and with the DNS assay. As shown in Table 1, Gal2F **13** was the best substrate giving 80% conversion, followed by GalN **6** (68%), however only weak or even no activity was identified for Gal2D **9** and GalNAc **8**, respectively. This is consistent with previous observations that galactose derivatives substituted at the C-2 position by small polar groups are substrates for GalKs. Furthermore, 57% and 28% conversions were measured for Gal6D **12** and Gal6F **16**, while no product was found for GalA **7**, demonstrating that LgGalK has a similar tolerance towards C-6 modifications compared to C-2. Interestingly, Gal3F **14** and Gal4F **15** also serve as substrates for LgGalK (albeit with low activity), which has not been reported previously for wild-type galactokinases.

Chemoenzymatic synthesis of 6-aminogalactose-1-phosphate analogues

Galactose oxidase (GOase, EC 1.1.3.9), belonging to the class of copper-dependent alcohol oxidases, is able to oxidise specifically the C-6 hydroxyl group of galactose to generate an aldehyde group, which can be used as a reactive functional handle for further chemistry. In particular, variant M_1 (GOase-M_1)[28] was selected as it was engineered to retain high activity against galactose and improved expression in *E. coli*, making it the most suitable candidate for biocatalysis. To demonstrate the potential of LgGalK as a highly active and promiscuous enzyme for biocatalytic cascade applications, we explored its combination with GOase-M_1, to access a broader range of modified derivatives of **2**.

GOase-M_1 and LgGalK were combined in a one-pot system to synthesise 6-oxogalactose-1-phosphate (GalO-1-P, **28**) from **1**. When the two enzymes were added to the reaction mixture simultaneously, only the formation of Gal-1-P **2** was observed (by HRMS, data not shown), suggesting that the sugar phosphate is not a substrate for GOase-M_1. However, carrying out the same reaction in a one-pot two-step fashion, by adding LgGalK only after completion of the oxidation by GOase-M_1 (Scheme 3), formation of the desired product **30** was detected by HRMS and further confirmed by ^{13}C NMR starting from $^{13}C_6$-**1** (Supporting Information). Encouraged by this result, we also tested the other substrates for LgGalK identified above (**6**, **10**, **12-16** and **18**), in a one-pot two-step system. Only **13** was efficiently converted to 6-oxo-

2-deoxy-2-fluorogalactose-1-phosphate (GalO2F-1-P, **31**) as monitored by HRMS (Supporting Information).



Scheme 2. Chemoenzymatic synthesis of 6-aminogalactose-1-phosphate analogues **32a-o** and **33a-o**. With diamine **o**, reductive amination proceeded only at one amino group.

The reactivity of the aldehyde group of **30-31** can be exploited to perform further chemical derivatisations, most prominently reductive aminations, as previously demonstrated.[29-31] Therefore, we extended our procedure to a one-pot three-step protocol by including a chemical reductive amination with $NaBH_3CN$ and a panel of 15 different amines (Scheme 3), in the same aqueous medium after the biotransformation. The formation of all 30 different 6-amino substituted products **32a-o** and **33a-o** were confirmed by HRMS (Table S1, Supplementary Information), giving access to a wide array of functionally diverse modified monosaccharides to be tested against diverse carbohydrate-binding proteins and enzymes.

Conclusion

A new bacterial galactokinase (LgGalK) with high catalytic efficiency and broad substrate specificity was overproduced and characterised. The effects of temperature, pH and divalent metal ions on its activity were investigated, leading to the identification of the optimal biochemical conditions: 50°C, pH 9.0, 1 mM Mg^{2+}. Kinetic studies of LgGalK towards Gal afforded the kinetic constants for the reaction (in particular K_M = 1.3±0.4 mM and k_{cat} = 23.7±1.8 s^{-1}). LgGalK was shown to catalyse the anomeric carbon phosphorylation of a broad variety of monosaccharides (**6**, **9-16** and **18**) to yield the corresponding sugar-1-phosphates.

Additionally, LgGalK could phosphorylate 6-oxo derivatives **28** and **29**, produced by GOase-M_1. Combination with chemical reductive amination in a one-pot three-step chemoenzymatic system afforded a panel of 30 different 6-aminogalactose-1-phosphate analogues (**32a-o**, **33a-o**).

Experimental section

General

Leminorella grimontii strain DSMZ 5078 (lyophilized culture pellet) was purchased from DSMZ (Germany). Gene primers were synthesised by Genescript (Nanjing, China). Primestar HS DNA polymerase is from Takara (Dalian, China). Restriction enzymes (*Eco*RI, *Xho*I) are from Thermo Fisher Scientific (Shanghai, China). *E. coli* BL21(DE3) cells, *E. coli* Top10 cells and the pET30a vector are from Novagen. Galactose oxidase variant M_1 was prepared as described previously.[31]
Deoxygalactoses **10-11** and deoxyfluorogalactoses **13-16** were supplied by Carbosynth (United Kingdom). Other chemical reagents and solvents were purchased from Sigma-Aldrich and used without further purification, unless stated otherwise.

Analytical methods

^{13}C and ^{19}F NMR spectra were recorded in H_2O/D_2O using a Bruker AV400 (400, 101 and 376 MHz respectively) spectrometers and AV500 (500, 126 and 470 MHz respectively). ^{13}C chemical shifts (δ) are quoted in ppm relative to residual solvent peaks as appropriate. ^{19}F spectra were externally referenced to $CFCl_3$. The coupling constants (*J*) were recorded in Hertz (Hz). HRMS data were obtained from a Bruker APEX III FT-ICR-MS. Samples were run in HPLC grade MeOH or MeCN.

Gene cloning

Genomic DNA was isolated from lyophilized *Leminorella grimontii* culture pellets based on the method described by Mahuku.[32] The DNA primer sequences used to amplify the gene coding region of the putative galactokinase (DNA GeneBank accession number: JMPN1000011.1) were designed according to genomic sequence as follows: forward primer 5'-GGAATTCATGAACGCGCTCGTTGCC-3' (*Eco*RI restriction site underlined), reverse primer 5'-CCGCTCGAGTTAACATAGGCTGGCTCCTGCT-3' (*Xho*I restriction site underlined). DNA amplification was performed utilising Primestar HS DNA polymerase (Takara, Dalian, China) according to the manufacturer's instructions. Briefly, 28 PCR cycles consisting of denaturation at 95°C for 10 sec, annealing at 55°C for 30 sec, and elongation at 72°C for 2 min were performed to amplify the gene. Restriction endonucleases *Eco*RI and *Xho*I (Thermo Fisher Scientific, Shanghai, China) were used to digest PCR products simultaneously, which subsequently were ligated into a pre-digested pET-30a expression vector (Novagen). The resulting construct (pET-30a-LgGalK) was transformed into *E. coli* TOP10 competent cells and transformants were selected on LB medium supplemented with 50 μg mL^{-1} kanamycin. Colonies harboring the expected plasmid construct were screened by DNA sequencing

(Genscript, Nanjing) and used for further experiments. Plasmid extraction, restriction endonuclease digestion, DNA purification, ligation, and transformation procedures were executed according to the manufacturer's instructions.

Protein production and purification

The pET-30a-LgGalK vector was transformed into *E. coli* BL21(DE3) for protein expression. *E.coli* transformants containing the vector were grown in LB medium at 37°C until the optical density at 600 nm (OD_{600}) of the culture reached 0.5-1.0. Protein expression was induced by addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG, final concentration 1 mM), followed by incubation at 18°C for 20 h with shaking at 250 rpm. The cells were harvested by centrifugation at 6000 rpm at 4°C for 20 min, then resuspended in lysis buffer (100 mM NaCl, 50 mM Tris, 1% v/v Triton X-100, pH 8.0). A protease inhibitor cocktail (1% v/v) was subsequently added to the cell suspension. The cells were disrupted by sonication (20 sec on, 20 sec off, 20 cycles). The supernatant of cell lysate was obtained by centrifugation at 15000 rpm at 4°C for 20 min. LgGalK was purified by nickel affinity chromatography on an ÄKTA purification system equipped with a HisTrap™ FF 5ml Ni^{2+}-NTA affinity column (GE Healthcare). The supernatant was loaded on column pre-equilibrated with 5 column volumes of binding buffer (50 mM Tris, 500 mM NaCl, 10mM imidazole, pH 8.0). Subsequently, the column was washed with 20 column volumes of binding buffer and 10 column volumes of washing buffer (50 mM Tris, 500 mM NaCl, 50 mM imidazole, pH 8.0). The target protein was eluted with elution buffer (50 mM Tris, 500 mM NaCl, 500 mM imidazole, pH 8.0). The fractions containing purified protein were collected and concentrated by centrifugation at 4000 rpm at 4°C for 1h, then dialyzed against dialysis buffer (20 mM Tris, pH 8.0) overnight at 4°C. The purified enzyme was either snap-frozen using liquid nitrogen or dissolved in 18% glycerol before stored at –80°C for further experiments. Protein concentration was measured by the Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific) and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to evaluate protein purity. Initial activity tests of purified LgGalK was performed using the method described by Duan *et al.*[33] (Supporting Information).

Biochemical characterisation of LgGalK

Influence of temperature. The enzymatic reaction was performed for 30 min in a total volume of 50 µL, containing **1** (8 mM), ATP (10 mM), $MgCl_2$ (5 mM), Tris buffer (50 mM, pH 8.0), LgGalK (13.6 µg). The mixtures were quenched by adding 100 µL DNS reagent (1 g 3,5-dinitrosalicylic acid and 30 g $KNaC_4H_4O_6{\cdot}4H_2O$ dissolved in 100 mL 0.4 M NaOH), and then heated at 95-100°C for 5 min. Subsequently, they were cooled on ice and further transferred into 96-well plates. Absorbance at 575 nm was measured and the conversion rate was calculated as described previously.[21] All assays were executed in triplicates.

Influence of pH. The enzymatic reaction was performed at 50°C for 30 min in a total volume of 50µL, containing **1** (8 mM), ATP (10 mM), $MgCl_2$ (5 mM), LgGalK (13.6 µg) in different buffers (50 mM, pH varying between 5.0 and 10.0). Quenching and assay were performed as described above. All reactions were performed in triplicates.

Influence of divalent metal ions. The enzymatic reaction was performed at 50°C for 30 min in a total volume of 50 µL, containing **1** (8 mM), ATP (10 mM), LgGalK (13.6 µg) in Tris buffer (50 mM, pH 9.0). Different metal ions (Zn^{2+}, Ni^{2+}, Fe^{2+}, Ca^{2+},

Cu^{2+}, Co^{2+}, Mn^{2+}) or EDTA (5 mM) or different concentrations of Mg^{2+} ions (1, 3, 5, 10, 15, 20 mM) were added to the buffer. Quenching and assay were performed as described above. All reactions were performed in triplicates.

Kinetic study. The enzymatic activity assay was carried out at 50°C in a total volume of 50 µL, containing **1** (1, 2, 4, 6, 8 mM), ATP (20 mM), $MgCl_2$ (5mM) and LgGalK (0.17 µg) in Tris buffer (50 mM, pH 9.0). The initial velocity was calculated by determining the slope of linear phase in the progress curve over 5 min (1 min intervals). All assays were performed in triplicates. The data were fitted to the Michaelis-Menten equation and apparent kinetic parameters were calculated by GraphPad Prism 7.03.

Substrate specificity of LgGalK

The typical assay for substrate specificity was carried out in a total volume of 50 µL of Tris buffer (50 mM, pH 9.0) containing different monosaccharides **1**, **6-27**(8 mM), ATP (10 mM), $MgCl_2$ (5 mM) and LgGalK (13.6 µg), at 50°C for 2 h. The reactions were quenched by heating at 95-100°C for 5 min followed by centrifugation at 12000 rpm for 5 min. The supernatant was analysed by silica gel thin-layer chromatography (TLC) developed with *n*-BuOH/AcOH/H_2O 2:1:1 and stained with *p*-anisaldehyde (6 mL *p*-anisaldehyde, 10 mL conc. H_2SO_4 and 2 mL AcOH dissolved in 180 mL EtOH). Kinase activity of LgGalK against substrates 1, 3, 6-13, 15, 27 was also further confirmed by HRMS. The supernatant was diluted 10-fold in HPLC grade water, a sample (2 µL) was flow-injected into MeCN/H_2O 1:1 (+0.1% formic acid) for analysis on an Agilent 1200 series LC system coupled to an Agilent 6520 QTOF mass spectrometer (ESI negative mode). The conversions of the fluorinated substrates (**13-16**) were measured by ^{19}F NMR, by diluting the supernatant (50 µL) with MeOH/H_2O 1:1 (450 µL) with addition of a sealed glass capillary containing D_2O for the lock. The conversions of non-fluorinated substrates (**1**, **6-12**, **17-27**) were measured by the DNS assay.

Chemoenzymatic synthesis of 6-aminogalactose-1-phosphate analogues

One-pot two-enzyme synthesis of 6-oxogalactose-1-phosphate (30) and 2-fluoro analogue (31). The reaction mixture containing sodium phosphate buffer (35.5 µL, 100 mM, pH 7.4), substrate **1** or **13** (4 µL, 100 mM), horseradish peroxidase (1.5 µL, 1 mg mL^{-1}), catalase (1.0 µL, 1 mg mL^{-1}) and GOase-M_1 (1.0 µL, 9.4 mg mL^{-1}) was incubated at 25°C, 300 rpm for 2 h. Then, ATP (2.5 µL, 200 mM), $MgCl_2$ (2.5 µL, 100 mM) and LgGalK (2 µL, 6.8 mg mL^{-1}) were added to reach the total volume of 50 µL and incubated at 50°C for 2 h. The reaction was stopped by removing the enzymes through a vivaspin column (10 kDa molecular weight cut-off), centrifuged at 12000 rpm for 5 min. Products **30** and **31** were analysed by ^{13}C NMR or^{19}F NMR and HRMS (ESI negative mode).

One-pot three-step chemoenzymatic synthesis of 6-aminogalactose-1-phosphate analogues (32a-o and 33a-o). The enzymatic reaction mixture and process were the same as described above for the preparation of **30** and **31**. After the removal of proteins, 15 different amines (2.0 µL or 2.0 mg) were added into each reaction mixture. AcOH/H_2O (1:1) was added to adjust the pH around 7.0, followed by a solution of $NaBH_3CN$ (5.0 µL, 1 M). The mixture was incubated at 25°C for 20 h. Products **32a-o** and **33a-o** were analysed by HRMS (ESI negative mode).

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