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Note

Facile enzymatic synthesis of sugar 1-phosphates as substrates for phosphorylases using anomeric kinases

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ABSTRACT

Three sugar 1-phosphates that are donor substrates for phosphorylases were produced at the gram scale from phosphoenolpyruvic acid and the corresponding sugars by the combined action of pyruvate kinase and the corresponding anomeric kinases in good yields. These sugar 1-phosphates were purified through two electrodialysis steps. α-D-Galactose 1-phosphate was finally isolated as crystals of dipotassium salts. α-D-Mannose 1-phosphate and 2-acetamido-2-deoxy-α-D-glucose 1-phosphate were isolated as crystals of bis(cyclohexylammonium) salts.

Phosphorylases are a class of enzymes that phosphorolyze glycosyl linkages to generate sugar 1-phosphates.[1–4] Because their reactions are reversible and highly regiospecific, they are expected to be practical catalysts for producing particular oligosaccharides. Because sugar 1-phosphates are donor substrates for reverse phosphorolysis, their availability is important for the utilization of phosphorylases.

According to the CAZy classification based on their amino acid sequences, phosphorylases belong to one of the following families: glycosyltransferase (GT) 4 and 35 and glycoside hydrolase (GH) 13, 65, 94, 112, and 130.[5] The majority of phosphorylases phosphorolyze α- or β-D-glucosides to generate α- or β-D-glucose 1-phosphate (αGlc1*P* or βGlc1*P*) with anomeric retention or inversion.[1–4] Preparative methods of αGlc1*P* or βGlc1*P* as pure crystalline forms such as those in salts have been well documented. These compounds have been generated by phosphorylases from commercially produced sugar resources. αGlc1*P* has been reported to be produced from starch by glycogen phosphorylase as a dipotassium salt.[6] It has also been produced from sucrose by sucrose phosphorylase.[7] βGlc1*P* has been produced as a bis(cyclohexylammonium) salt from trehalose by inverting trehalose phosphorylase.[8]

Several phosphorylases act on glycosides other than glucosides. The GH112 family comprises phosphorylases that act on β-D-galactosides to generate α-D-galactose 1-phosphate (αGal1*P*) (**1**), including 1,3-β-galactosyl-*N*-acetylhexosamine phosphorylase (EC 2.4.1.211)[9,10] and 1,4-β-D-galactosyl-L-rhamnose phosphorylase (EC 2.4.1.247).[11] The GH130 family comprises phosphorylases that act on β-D-mannosides to generate α-D-mannose 1-phosphate (αMan1*P*) (**2**), including 1,4-β-mannosyl-glucose phosphorylase (EC 2.4.1.281),[12] β-1,4-mannooligosaccharide phosphorylase (EC 2.4.1.319),[13] and 1,4-β-mannosyl-*N*-acetylglucosamine phosphorylase (EC 2.4.1.320).[14,15] The GH94 family mainly comprises phosphorylases that act on β-D-glucosides, although it also includes *N*,*N*′-diacetylchitobiose phosphorylase (EC 2.4.1.280)[16] to generate 2-acetamido-2-deoxy-α-D-glucose 1-phosphate (αGlcNAc1*P*) (**3**).

Although sugar 1-phosphates can be chemically synthesized,[17,18] enzymatic methods are preferred for their practical synthesis on a large scale. However, it is difficult to produce sugar 1-phosphates using phosphorylases except for αGlc1*P* and βGlc1*P* because there are no abundantly available resources to be phosphorolyzed by known enzymes. Although an attempt to change cellobiose phosphorylase into lactose phosphorylase to obtain αGal1*P* from lactose has been reported,[19] the catalytic efficiency of the mutant enzyme was too low to be used as a practical catalyst.

Abbreviations: αGal1*P*, α-D-galactose 1-phosphate; αGlc1*P*, α-D-glucose 1-phosphate; αGlcNAc1*P*, 2-acetamido-2-deoxy-α-D-glucose 1-phosphate; αMan1*P*, α-D-mannose 1-phosphate; βGlc1*P*, β-D-glucose 1-phosphate; GH, glycoside hydrolase; GlcNAc, 2-acetamido-2-deoxy-α-D-glucose; GT, glycosyltransferase; PEP, phosphoenolpyruvic acid; TLC, thin layer chromatography.

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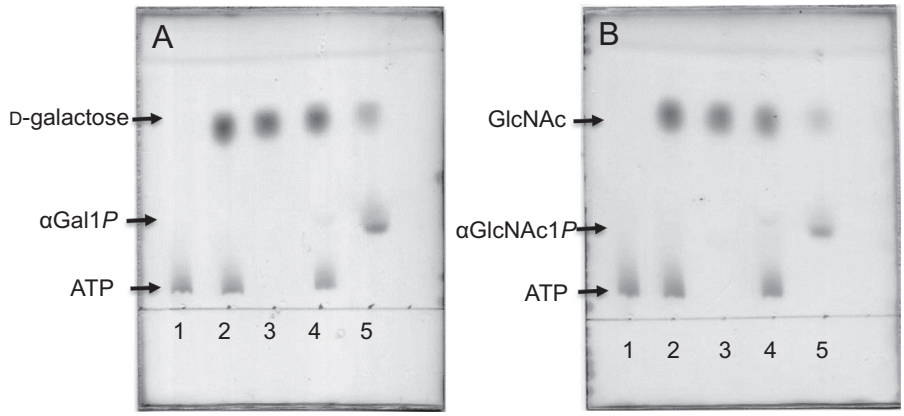
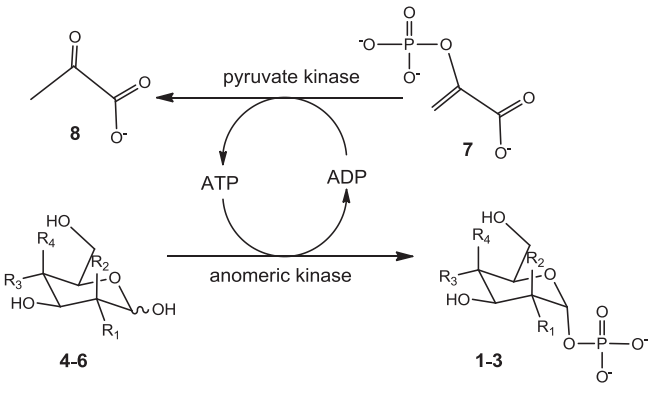


Figure 1. TLC analysis of the reactions of anomeric kinases with and without the ATP-regeneration system. (A) The reaction of galactokinase with D-galactose; (B) the reaction of *N*-acetylhexosamine 1-kinase with GlcNAc. Lane 1, standard ATP; lanes 2 and 4, without the ATP-regenerating system (50 mM ATP), *t* = 0 and 20 h, respectively; lanes 3 and 5, with ATP-regeneration system (1 mM ATP), *t* = 0 and 20 h, respectively.

Anomeric kinases are enzymes that phosphorylate the anomeric position of sugars with the consumption of ATP. The enzyme galactokinase (EC 2.7.1.6) produces αGal1*P* from D-galactose (**4**).[20] αMan1*P* and αGlcNAc*P* can be produced by *N*-acetylhexosamine 1-kinase (EC 2.7.1.162) from D-mannose (**5**) and 2-acetamido-2-deoxy-D-glucose (GlcNAc) (**6**), respectively.[21] However, it is often noted that kinases are inhibited by high concentrations of ATP, their phosphate donor substrate.[22,23] The substrate inhibition effect restricts the concentration of the substrate to low values, thus resulting in low production efficiency. To avoid this problem, we propose to perform the kinase reaction in the presence of an ATP-regenerating system.[24] Pyruvate kinase (EC 2.7.1.40) with phosphoenolpyruvic acid (PEP) (**7**) is one of the most commonly used ATP-regenerating systems.[24] This enzyme transfers the phosphate group of PEP to ADP to generate pyruvic acid (**8**) and ATP. We confirmed that the reactions of both galactokinase and *N*-acetylhexosamine 1-kinase with 1 mM ATP in the presence of the ATP-regeneration system were much faster than those with 50 mM ATP (Fig. 1). Here we document the facile synthesis of αGal1*P*, αGlcNAc1*P*, and αMan1*P* using kinases and the ATP-regenerating system, followed by the isolation of these compounds by electrodialysis[25] and crystallization.

The enzymatic reactions are described in Scheme 1. Each sugar 1-phosphate was generated with good yields (>90%) from PEP by the combination of pyruvate kinase and each corresponding anomeric kinase. The resultant reaction mixtures contained sugar 1-phosphates, pyruvic acid, ATP/ADP, and unreacted monosaccharide. The first electrodialysis using a 100 Da molecular mass cutoff membrane effectively removed pyruvic acid from the mixture. After the second electrodialysis with a 300 Da molecular mass cutoff membrane, sugar 1-phosphates were recovered in the dialysate solution. The electrodialysis is scale-up ready by increasing the surface area of the membranes and replaces the chromatographic step often reported for the purification of sugar 1-phosphates. After the purification, the countercations were replaced using a cation exchange resin, and the sugar 1-phosphates were crystallized as dipotassium salts or bis(cyclohexylammonium) salts in water/ethanol or water/acetone as pure sugar 1-phosphates.



	R_1	R_2	R_3	R_4
1,4	OH	H	H	OH
2,5	H	OH	OH	H
3,6	NHAc	H	OH	H

Scheme 1.

With the protocol mentioned above, αGal1*P*, αGlcNAc1*P*, and αMan1*P*, which are donor substrates for phosphorylases, are easily prepared at the gram scale. The facile production of these compounds will advance the research on phosphorylases by supplying their substrates.

1. Experimental

1.1. Enzymes and chemicals

Pyruvate kinase from rabbit muscle was purchased from Oriental Yeast Co., Ltd (Tokyo, Japan). Galactokinase[26] and *N*-acetylhexosamine 1-kinase[21] from *Bifidobacterium longum* subsp. *longum* JCM1217 were prepared as described previously. PEP monosodium salt and ATP disodium salt were purchased from Wako Pure Chemicals Industry (Osaka, Japan). Other chemicals used were of reagent grade.

1.2. The reactions of anomeric kinases with and without the ATP-regeneration system

The enzymatic reactions were carried out in reaction mixtures (100 μL) at 30 °C. The composition of each reaction mixture without the ATP-regeneration system was 50 mM ATP (pH adjusted to 7.0 with NaOH), 5 mM $MgCl_2$, 50 mM monosaccharide (D-galactose and GlcNAc), and anomeric kinase (9.4 μg/mL galactokinase and 100 μg/mL *N*-acetylhexosamine 1-kinase, respectively). That with the ATP-regeneration system was 1 mM ATP, 50 mM PEP (pH adjusted to 7.0 with NaOH), 5 mM $MgCl_2$, 50 mM monosaccharide (D-galactose and GlcNAc), anomeric kinases (9.4 μg/mL galactokinase and 100 μg/mL *N*-acetylhexosamine 1-kinase, respectively), and 0.42 U/mL pyruvate kinase. After the reactions for 20 h, aliquots (1 μL) of the reaction mixtures were spotted on a thin layer