Synthetic Utility of Yeast Hexokinase. Substrate Specificity, Cofactor Regeneration, and Product Isolation

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Yeast hexokinase (EC 2.7.1.1) catalyzes the phosphorylation of pyranose and furanose analogs of glucose at 0.01−125% of the rate of glucose. The enzyme is highly tolerant of structural changes at C-2 and C-3 of glucopyranose and less tolerant of changes at C-1 and C-4. Preparative phosphorylations were performed on compounds having 0.01−100% of the activity of glucose, using phosphoenolpyruvate and pyruvate kinase to regenerate ATP. The effects of inhibition of hexokinase by phosphoenolpyruvate and acetyl phosphate on cofactor regeneration are discussed.

Introduction

Sugar phosphates are ubiquitous in biology, occurring as monomers in solution, as components of soluble and cell-surface glycoconjugates, and as members of phosphodiester-spaced oligosaccharides. Sugar phosphates play important roles in metabolism,[1,2] enzyme regulation, cell structure,[3] lymphocyte homing,[4] protein translocation,[5] inflammation,[6] and immune response.[7] As components of the lipid A (endotoxin) region of lipopolysaccharides, sugar phosphates contribute to the toxic and immunogenic properties of many Gram-negative bacteria. Sugar phosphates also appear in the antiphagocytic capsules of pathogenic bacteria.[8] In all of these cases, the phosphate group seems to serve as a structural epitope, controlling partitioning among metabolic manifolds, binding to proteins, and biological activity.

Encouraged by the pioneering studies of Sols,[9−11] Cleland,[12,13] and Westwood,[14] and by the more recent work of Wong[15] and Withers,[16] we explored the use of yeast hexokinase (EC 2.7.1.1) for the phosphorylation of unprotected carbohydrates and related compounds, in order to develop efficient methodology for the synthesis of naturally occurring sugar phosphates and their analogs. Although many chemical methods for phosphorylation exist,[17] they all require elaborate manipulation of protecting groups to achieve regioselectivity with carbohydrate substrates. Low yields of phosphorylated product, the need for multistep procedures (e.g., phosphitylation, ligand exchange, oxidation, and deprotection), toxic reagents, and the use of organic solvents are other potential disadvantages of chemical methods. In contrast, hexokinase catalyzes the completely regioselective phosphorylation of unprotected carbohydrates, using metabolic intermediates as reagents and water as the solvent.

Hexokinase has broad substrate specificity and yet is regiospecific for the phosphorylation of the primary hydroxyl group of D-glucose and related structures. Hexokinase accepts as substrates a number of deoxy and

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deoxyfluoro analogs of D-glucose.[12,13,15] It has a slight kinetic preference for α-glucopyranose over β-glucopyranose.[10] Hexokinase catalyzes the phosphorylation of D-mannose as effectively as that of D-glucose, but does not catalyze the phosphorylation of D-galactose.[9] Hexokinase reacts well with furanose forms of sugars, such as D-fructofuranose and D-arabinofuranose, in which the geometry of hydroxyl substituents resembles that of D-glucose.

We have developed a semiquantitative TLC assay that conveniently determines the activity of hexokinase with unnatural substrates for synthetic purposes. On the basis of the results of this assay, preparative gram-scale phosphorylations have been performed on compounds having 0.01−100% of the activity of D-glucose. Both phosphoenolpyruvate and acetyl phosphate were found to inhibit hexokinase, and the effects of this inhibition on cofactor regeneration are discussed.

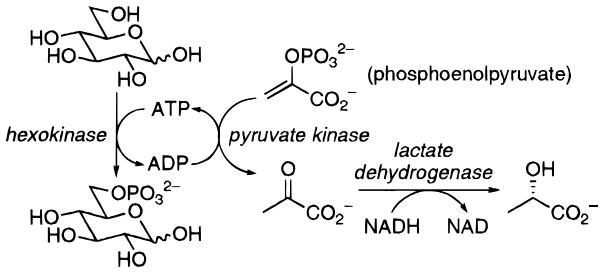
Results and Discussion

Although the two isozymes of yeast hexokinase are similar in their amino acid content, physical characteristics, and substrate specificity and are identical in their mechanism,[11,18] they can still be distinguished by their relative activities with D-fructose and D-mannose.[19] Under fixed assay conditions, isozyme A (or P-I) catalyzes the phosphorylation of D-fructose and D-mannose, respectively, at 2.6 and 0.60 times the rate of phosphorylation of D-glucose. Isozyme B (or P-II), on the other hand, reacts with D-fructose and D-mannose, respectively, at 1.3 and 0.28 times the rate of reaction with D-glucose. On the basis of its relative rates of reaction with D-fructose and D-mannose of 2.3 and 0.69, the enzyme used in this work (from Biozyme) appears to be isozyme A.

Assay of Activity of Hexokinase with Unnatural Substrates. Because analogs of glucose can induce a conformational change in hexokinase and thereby promote ATPase activity in the enzyme,[20] it was necessary to design an assay that was general for a wide range of unnatural phosphoryl acceptors and yet one that would not give false positive results for the production of ADP simply through hydrolysis of ATP. We therefore developed a semiquantitative TLC assay that measures the rate of production of "unnatural" sugar phosphates relative to the rate of production of D-glucose 6-phosphate from D-glucose. A positive reaction was evidenced by the appearance and intensification with time of a new TLC spot having the color and R_f value characteristic of a sugar phosphate. Control experiments indicated that the TLC assay could detect the production of sugar phosphate after only 1% conversion.

In order to assess the accuracy of the time-dependent TLC assay, substrates of varying structure and apparent reactivity, based on the TLC assay, were subjected to a continuous spectrophotometric assay for conversion of ATP to ADP (Scheme 1). In this assay, phosphoenolpyruvate and pyruvate kinase react with ADP to regenerate ATP. The pyruvate produced is subsequently reduced by NADH and lactate dehydrogenase.[21] The rates of

Scheme 1



reaction determined spectrophotometrically were generally in agreement, within a factor of 2, with those determined by TLC. Furthermore, the rates determined by TLC were used successfully to predict the amounts of hexokinase required to perform preparative phosphorylations (*vide infra*).

Substrate Specificity of Hexokinase. The effects of substrate structure on the ability of hexokinase to catalyze the phosphorylation of analogs of glucose are summarized in Scheme 2. In general, hexokinase is more tolerant of structural variations the farther they are removed from the site of phosphorylation. Thus, hexokinase is highly tolerant of modifications at C-2 and C-3, somewhat less tolerant of variations at C-1, and relatively intolerant of structural changes at C-4. At C-1, C-2, and C-3, hexokinase tolerates inversion of configuration, deoxygenation, or methylation of the hydroxyl group, with one possible exception being the reported inhibition of hexokinase by 2-*O*-methyl-D-glucose and 2-*O*-methyl-D-mannose.[14] Replacement of the anomeric hydroxyl by fluorine or a thiol group slows reactivity of the substrates even more. At C-2 and C-3, hexokinase seems to accept substrates in which the hydroxyl group has been replaced by fluorine, chlorine, or an amino group or in which the hydroxyl group has been oxidized to a carbonyl group. At C-4, the detrimental kinetic effects of inversion of configuration and replacement of the hydroxyl group by fluorine are more pronounced than at other sites. No other analogs bearing modification at C-4 have been studied with hexokinase. Although branching is tolerated somewhat at C-2 (e.g., **20**), it is not tolerated at C-5. L-Sorbose and D-tagatose, which exist in solution as 98% α-pyranose and 20% β-pyranose and β-furanose forms,[22] respectively (Scheme 3), fail to react with hexokinase. 5-Thio-D-glucose and 5-thio-D-fructose, however, do react with hexokinase at about 1.0−2.5% of the rate of D-glucose.[11,24]

Furanoses that resemble glucopyranose, particularly at sites corresponding to C-4 and C-3 of D-glucose, are good substrates of hexokinase. Although no single secondary hydroxyl group of glucopyranose is essential for substrate activity, it appears that at least two are necessary for productive substrate binding and turnover. To a first approximation, multiple changes in the structure of glucose are multiplicative in their effect on the rate of reaction of a compound with hexokinase.

When the ability of hexokinase to phosphorylate disaccharide derivatives of D-glucose was investigated, mal-

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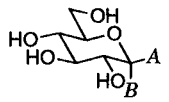
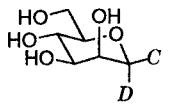
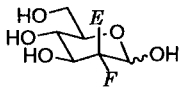
Scheme 2[a]



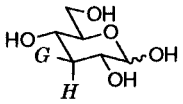
	A	B	v
1α	H	OH	109[b]
1β	OH	H	90[b]
2	H	H	3,[c] 1.4[d]
3	H	F	e
4	SH[f]	H	0.04[d]
5	H	OCH_3	1
6	OCH_3	H	1



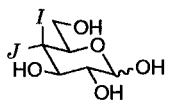
	C	D	v
7	H	H	8[c]
8	H	OCH_3	2



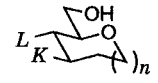
	E	F	v
9	OH	H	65
10	H	H	96,[c] 100[g]
11	H	F	50[d,h]
12	H	Cl	52[h]
13	F	F	53[h]
14	H	OCH_3	i
15	OCH_3	H	i
16	H	NH_2	68[c,g]
17	H	NHAc	0.01
18	NH_2	H	3
19	NHAc	H	< 0.01
20	CH_2OH	OH	1[g]
21	=O		20[g]



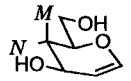
	G	H	v
22	H	OH	100
23	H	H	100
24	F	H	4[h]
25	H	F	4[h]
26	OCH_3	H	53[h]
27	NH_2	H	0.2[d]



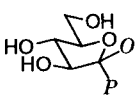
	I	J	v
28	OH	H	~ 0.1[g]
29	H	F	4[h]
30	F	H	0.4[h]



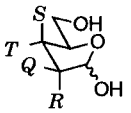
	n	K	L	v
31	1	OH	OH	2
32	1	H	OH	< 0.01
33	1	H	H	< 0.01
34	0	H	OH	< 0.01



	M	N	v
35	H	OH	0.3
36	OH	H	< 0.01



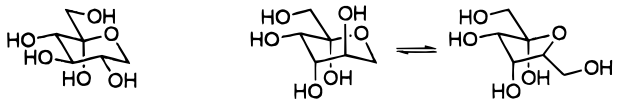
	O	P	v
37	OH[j]	CH_2OH	220
38	OH[f]	CH_3	0.4,[c] 1[k]
39	H	CH_3	2[k]
40	H	CHO	120,[c] 108[l]
41	H	CH_2OH	154,[c] 72,[k] 136[l]
42	CH_2OH	H	2,[c] 56[l]



	Q	R	S	T	v
43	OH	H	H	OH	50, 2[c]
44	H	OH	H	OH	0.02
45	OH	H	OH	H	< 0.01
46	H	OH	OH	H	< 0.01

[a] Initial rates were measured at pH 7.6 and 25 °C, using 50 mM substrate. All rates were normalized to that of an equilibrium mixture (36:64) of α- and β-D-glucopyranose (v = 100). Some rates were calculated assuming 50 mM initial substrate concentration and using values of K_m and V_{max} (measured at pH 7.4−8.0) reported in the literature. No correction was made for possible variations in isozyme composition. [b]Reference 10. [c]Reference 13. [d]Reference 11. [e]Slow substrate, no rate data.[16] [f]Substrate is actually an equilibrium mixture of anomers. [g]Reference 9. [h]Reference 14. [i]Reported inhibitor but not substrate. [j]Substrate is actually a 2:70:5:23 equilibrium mixture of α and β pyranose and furanose forms.[22] [k]Reference 23. [l]Reference 12.

Scheme 3



α-L-sorbopyranose β-D-tagatopyranose β-D-tagatofuranose

tose and cellobiose showed no measurable activity with hexokinase (v < 0.01% of that of D-glucose). Sophorose and laminaribiose, on the other hand, initially appeared to react at 0.01 and 0.03%, respectively, of the rate of D-glucose. Preparative phosphorylations, however, revealed that the only product formed from either sophorose or laminaribiose was D-glucose 6-phosphate. Control experiments demonstrated that the formation of D-glucose 6-phosphate was the result of hexokinase-mediated hydrolysis of disaccharide and subsequent phosphorylation of D-glucose and was not the result of either contamination of hexokinase by β-glucosidase or trace impurities of D-glucose in sophorose or laminaribiose.

Inhibition of Hexokinase by Phosphoenolpyruvate and Acetyl Phosphate. Although the use of phosphoenolpyruvate and pyruvate kinase or acetyl phosphate and acetate kinase to regenerate ATP from ADP *in situ* is well documented,[25−27] we were surprised to find that when D-glucose and 50 mM phosphoenolpyruvate were combined with hexokinase, pyruvate kinase, and catalytic ATP, virtually no reaction occurred. At low concentrations, phosphoenolpyruvate proved to be a reversible linear competitive inhibitor of hexokinase (Figure 1), with K_i = 7.8 mM versus ATP. At higher concentrations, the inhibition of hexokinase by phosphoenolpyruvate was even more pronounced. Although phosphoenolpyruvate is known to inhibit rabbit red blood cell hexokinase (K_i = 14 mM),[28] we have found no other report of the inhibition of hexokinase by phosphoenolpyruvate. However, careful examination of synthetic accounts reporting the pertinent details revealed that only about 1−3% of the expected catalytic activity of hexokinase had been observed in the presence of phosphoenolpyruvate.[15,29,30] When acetyl phosphate was examined, it too inhibited yeast hexokinase competitively with respect to ATP, with K_i = 9.1 mM (Figure 1).

Because of the stability of the phosphorylating reagent and low cost of the enzyme, phosphoenolpyruvate and pyruvate kinase were still chosen to be used for cofactor regeneration in preparative scale phosphorylations. By slow continuous addition, phosphoenolpyruvate could be maintained at a low (~5 mM) steady-state concentration,

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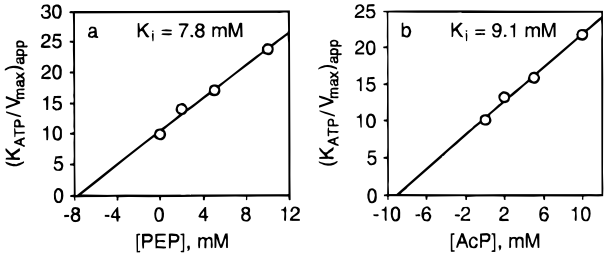


Figure 1. Inhibition of yeast hexokinase by (a) phosphoenolpyruvate and (b) acetyl phosphate. Inhibition is competitive versus ATP.

Table 1. Preparative Phosphorylations Catalyzed by Hexokinase

substrate	product[a]	purified yield, g (%)
D-glucose (**1**)	**47**	1.1 (82)
2-deoxy-D-glucose (**2**)	**48**	1.0 (79)
methyl α-D-glucopyranoside (**5**)	**49**	0.7 (50)
methyl α-D-mannopyranoside (**8**)	**50**	1.0 (73)
D-mannose (**9**)	**51**	1.3 (99)
2-amino-2-deoxy-D-glucose (**16**)	**52**	1.2 (99)
2-acetamido-2-deoxy-D-glucose (**17**)	**53**	0.2 (99)
2-amino-2-deoxy-D-mannose (**18**)	**54**	1.1 (96)
β-D-allose (**22**)	**55**	0.5 (85)
D-glucal (**35**)	**56**	1.1 (83)

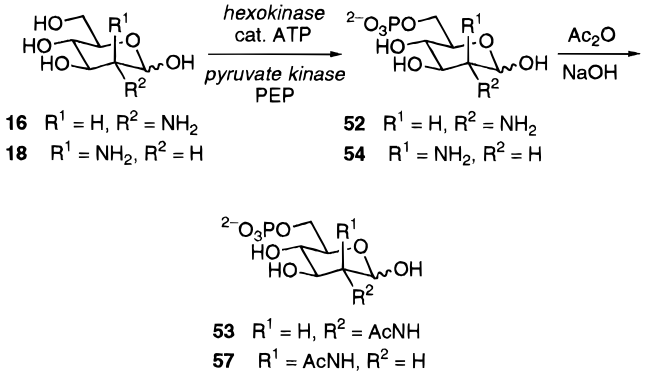
[a] All substrates were phosphorylated on O-6.

and catalytic (0.7 mol %) ATP could be regenerated satisfactorily. No difficulties due to the feedback inhibition of pyruvate kinase by pyruvate[25] were observed. *In situ* regeneration of ATP reduced the overall expense due to cofactor, avoided feedback inhibition of hexokinase by ADP, and simplified the isolation and purification of sugar phosphate products.

Preparative Phosphorylations. Using quantities of hexokinase as determined by the TLC assay, sugars and sugar derivatives representing a variety of structures and having 0.01−100% of the activity of D-glucose were phosphorylated on gram scale. Reactions were typically run overnight, with the addition of phosphoenolpyruvate being rate-limiting. When the introduction of phosphoenolpyruvate was complete, the progress of each reaction could be determined by assay for pyruvate and residual phosphoenolpyruvate. The soluble hexokinase and pyruvate kinase catalysts retained ≥90% of their original activities. Product isolation was readily achieved by anion exchange chromatography using ammonium bicarbonate as eluant.[31] This method proved to be more efficient and convenient than precipitation of products as their barium[32] or lithium[33] salts or anion exchange using formate or borate[34] as eluant. Crude products thus obtained were ≥95% pure, the only significant impurity being ammonium phosphate. Analytical purity was achieved by recrystallizing the sugar phosphates as their bisammonium, bis(cyclohexylammonium), or monosodium salts. Yields of purified products were typically about 85% (Table 1).

Notable are the single-step syntheses of methyl α-mannopyranoside 6-phosphate (**50**) and mannose 6-phosphate (**51**), which have been shown to have potent antiinflammatory activity in the central nervous system.[6] The best chemical syntheses of **50** and **51** require three or four steps to form product in only 40−46% overall yield.[35] Also notable is the preparative phosphorylation of the extremely unreactive substrate, 2-acetamido-2-deoxy-D-glucose (**17**, v = 0.01% of that of D-glucose). Although **17** has previously been reported as an inhibitor of hexokinase,[20,36] it is infact a slow substrate, and 2-acetamido-2-deoxy-D-glucose 6-phosphate (**53**) was isolated in 99% yield. A more practical route to **53**, however, is phosphorylation of 2-amino-2-deoxy-D-glucose (**16**) to give **52**, followed by Schotten−Baumen acylation of **52** to give **53** (Scheme 4). This method has also been used to convert 2-amino-2-deoxy-D-mannose (**18**) to 2-amino-2-deoxy-D-mannose 6-phosphate (**54**) and 2-acetamido-2-deoxy-D-mannose 6-phosphate (**57**). Compounds **52**−**54** and **57** are biosynthetic precursors to the nucleotide-linked carbohydrates required as glycosyl donors for the enzymatic synthesis of hexosamine and *N*-acetylhexosamine-containing mammalian cell-surface oligosaccharides.[37] Compound **57** is also a biosynthetic precursor to *N*-acetylneuraminic acid,[2] a carbohydrate shown to be particularly prevalent on the surface of tumor cells[38] and known to have antiallergic activity.[39]

Scheme 4



In conclusion, yeast hexokinase is extremely stable, even when used in soluble form, and is useful for the preparative phosphorylation of a wide range of pyranoid and furanoid analogs of glucose. Products are most conveniently isolated by anion exchange chromatography, using ammonium bicarbonate as the eluant. Although we report for the first time the inhibition of yeast hexokinase by ATP-regenerating reagents, phosphoenolpyruvate and acetyl phosphate, this inhibition is easily overcome by introducing the regenerating reagent in a rate-limiting, continuous fashion.

Experimental Section

Materials and Methods. NAD (crystalline, 99%) and ATP (disodium salt) were purchased from Sigma. Hexokinase (yeast, HK-2B), pyruvate kinase (rabbit muscle, PK3), and lactate dehydrogenase (rabbit muscle, LDHR2) were purchased

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from Biozyme Laboratories and were used without additional purification. Compounds **31**,[40] **32**,[41] and **34**[42] were made according to previously reported procedures or slight modifications thereof.[43,44] ^{1}H, ^{13}C, and ^{31}P NMR spectra were acquired at nominal resonance frequencies of 250 and 101 MHz, respectively, using D_2O as the solvent. FAB-MS data were obtained using Cs^+ (20 eV) as the ionizing beam and glycerol/NaI as the matrix. Elemental analyses were performed by Robertson Microlit Laboratories, Inc. TLC was performed on 6.7 cm × 2 cm normal-phase TLC plates (250 μm sorbent thickness) from either Analtech (Silica Gel HL) or Whatman (K6F silica gel 60 Å). Silica gel chromatography was performed on Merck grade 9385, 230−400 mesh, 60-Å silica gel.

TLC Assay for Hexokinase Activity. In a final volume of 1.00 mL of 0.10 M sodium phosphate buffer, pH 7.6, at rt, were combined 50 mM glucose or unnatural substrate, 5.0 mM ATP, 5.0 mM $MgCl_2$, and 1.0 or 10 U of hexokinase. Hexokinase (20 or 200 μL of a 50 U mL^{-1} solution) was added last to initiate the reaction. Control reactions containing all of the components but hexokinase in the same final volume were also run. Reactions were monitored periodically by TLC (9.5 mM tetrabutylammonium hydroxide in 80% aqueous acetonitrile, anisaldehyde stain) for the presence of sugar phosphate. Under these conditions, R_f values of glucose, ATP, and glucose 6-phosphate were 0.53, 0.16, and 0.30, respectively.

Spectrophotometric Assay for Hexokinase Activity. To 1.465 mL of 0.10 M sodium phosphate buffer, pH 7.6, containing 14.4 U of pyruvate kinase, 2.15 U of lactate dehydrogenase, 50 mM substrate, 2.36 mM ATP, 3.30 mM $MgCl_2$, 13.2 mM KCl, and 0.393 mM NADH, at 25 °C, was added 50 μL of a 0.75 or 7.5 U mL^{-1} solution of hexokinase. The absorbance at 334 nm was recorded continuously for about 10 min, and the activity of hexokinase was calculated using $\epsilon_{334} = 6.18$ L mmol^{-1} cm^{-1} as the molar absorptivity of NADH.

General Procedure for Preparative Phosphorylations. In a 125-mL Erlenmeyer flask equipped with a stir bar were combined substrate (4.3 mmol), 17 mg (0.031 mmol) of ATP, 51 mg (0.25 mmol) of $MgCl_2 \cdot 6H_2O$, 37 mg (0.50 mmol) of KCl, 23 mg (0.15 mmol) of dithiothreitol, 6.5 mg (0.10 mmol) of NaN_3, and 20 mL of deoxygenated, deionized water. The pH of the solution was adjusted to 7.6 with aqueous potassium hydroxide, and the solution was sparged with nitrogen. Hexokinase (45−60 U, determined with the actual compound being phosphorylated) and pyruvate kinase (45−60 U) were added. To this solution (final volume 25 mL), under an inert atmosphere, was added by syringe pump 25 mL of a deoxygenated solution of 4.7 mmol of phosphoenolpyruvate,[45] pH 7.6. Upon completion of the addition of phosphoenolpyruvate, the reaction was assayed for phosphoenolpyruvate and pyruvate.

After completion of the reaction, the solution was adjusted to pH 9.0 using aqueous potassium hydroxide, and barium acetate (1.8 g, 6.85 mmol) dissolved in 4 mL of water was added. The mixture was stirred for 20 min and then filtered to remove barium phosphate. Residual barium was removed by adding 30−35 mmolar equiv of Dowex-50W (H^+), stirring, and filtering. The solution was adjusted to pH 9.0 with aqueous potassium hydroxide and brought to the brink of precipitation by the addition of methanol. The solution was applied to a 150-mL column of Dowex-1×8−100 (HCO_3^-), which was then rinsed with 200−300 mL of 50% aqueous methanol, eluted with 3 L of 65 mM ammonium bicarbonate in 50% aqueous ethanol to remove carboxylate anions, and then eluted with 3 L of 200 mM aqueous ammonium bicarbonate. The latter eluant was concentrated by rotary evaporation at 40 °C to yield sugar phosphate as its diammonium salt. Aminosugar phosphates were recovered as their monoammonium salts.

For storage or FAB-MS analysis, sugar phosphates were converted to their monosodium or bis(cyclohexylammonium) salts. Monosodium salts were formed by stirring sugar phosphates in water with excess Dowex-50W (H^+), filtering, raising the pH of the solution to 4.2 with aqueous sodium hydroxide, and removing the solvent by rotary evaporation. Bis(cyclohexylammonium) salts were formed by dissolving sugar phosphates in water with an excess of Dowex-50W (H^+), stirring, filtering, adding 2 equiv of cyclohexylamine, and removing the solvent by rotary evaporation. The bis(cyclohexylammonium) salts were recrystallized from water−2-propanol.

D-Glucose 6-Phosphate (47). Phosphorylation of **1** according to the general procedure gave 1.04 g (80%) of **47** diammonium salt: ^{1}H NMR δ 5.28 (d, J = 3.8 Hz, 0.3H, H-1α), 4.70 (d, J = 8.0 Hz, 0.7H, H-1β), 4.08−3.92 (m, 2H, H-6,6′), 3.81−3.51 (m, 3.3H), 3.33 (t, J = 9.6 Hz, 0.7H), in agreement with authentic material purchased from Sigma.

2-Deoxy-D-glucose 6-Phosphate (48). Phosphorylation of **2** according to the general procedure yielded 0.95 g (79%) of **48** bis(cyclohexylammonium) salt that was a 1:1 mixture of α and β anomers: ^{1}H NMR δ 5.37 (d, J = 3.2 Hz, 0.5H), 4.94 (dd, J = 1.8, 9.8 Hz, 0.5H), 4.11−3.85 (m, 3H), 3.73 (m, 0.5H), 3.55 and 3.47 (2 t, J = 9.7 Hz, 0.5H each), 3.44 (m, 0.5H), 3.15 (m, 2H), 2.27 (ddd, J = 1.9, 5.0, 12 Hz, H-2eqβ), 2.14 (dd, J = 5.1, 13 Hz, H-2eqα), 1.98 (m, 4H), 1.80 (m, 4H), 1.74 (dt, J = 3.2, 13 Hz, 0.5H, H-2axα), 1.54 (dt, J = 10, 12 Hz, 0.5H, H-2axβ), 1.34 (m, 12H), in agreement with authentic material (sodium salt) purchased from Sigma; ^{31}P NMR δ 4.31.

Methyl α-D-Glucopyranoside 6-Phosphate (49). Phosphorylation of **5** according to the general procedure yielded 0.65 g of **49** monosodium salt as a white solid: mp 138−140 °C dec; ^{1}H NMR δ 4.81 (d, J = 3.6 Hz, 1H, H-1), 4.09 (m, 2H, H-6,6′), 3.76 (m, 1H, H-5), 3.65 (t, J = 9 Hz, 1H), 3.59 (dd, J = 3.5, 9.4 Hz, 1H, H-2), 3.52 (t, J = 9.4 Hz, 1H), 3.43 (s, 3H, OCH_3); ^{31}P NMR δ 3.94; IR (KBr) 3464, 1637, 1471, 1404, 1366, 1263, 1203, 1049, 931 cm^{-1}; FAB-MS m/z 319 (M + Na$^+$), 297 (M + H$^+$), 275 (free acid + H$^+$). Anal. Calcd for $C_7H_{14}O_9NaP \cdot H_2O$: C, 26.76; H, 5.13; P, 9.86. Found: C, 26.94; H, 5.34; P, 10.00.

Methyl α-D-Mannopyranoside 6-Phosphate (50). Phosphorylation of **8** according to the general procedure yielded 0.97 g (73%) of **50** monosodium salt as a white solid: mp 145−147 °C; ^{1}H NMR δ 4.77 (br s, 1H, H-1), 4.05 (m, 2H, H-6,6′), 3.94 (dd, J = 1.6, 3.0 Hz, 1H, H-2), 3.86−3.80 (m, 2H, H-4, 3), 3.65 (m, 1H, H-5), 3.42 (s, 3H, OCH_3); ^{31}P NMR δ 4.58; IR (KBr) 3526, 2917, 1448, 1389, 1353, 1323, 1197, 1022, 970, 930 cm^{-1}; HRMS calcd for $C_7H_{15}O_9NaP$ (M + H$^+$) 297.0348, found 297.0351.

D-Mannose 6-Phosphate (51). Phosphorylation of **9** according to the general procedure yielded 1.34 g (99%) of **51** monosodium salt as a white solid: ^{1}H NMR δ 5.19 (d, J = 1.58 Hz, 0.6H, H-1α), 4.92 (d, J = 1.02 Hz, 0.4H, H-1β), 4.02−3.99 (m, 2H, H-6,6′), 3.94 (m, 1H, H-2), 3.86 (m, 2H), 3.72 (m, 1H), in agreement with the literature;[35] ^{31}P NMR δ 4.90; HRMS calcd for $C_6H_{13}NaO_9P$ (M + H$^+$) 283.0205, found 283.0195.

2-Amino-2-deoxy-D-glucose 6-Phosphate (52). Phosphorylation of **16** according to the general procedure yielded 1.22 g (100%) of **52**[30,46−48] monoammonium salt: ^{1}H NMR δ 5.46 (d, J = 3.6 Hz, 0.7H, H-1α), 4.46 (d, J = 8.5 Hz, 0.3H, H-1β), 4.17−4.04 (m, 2H, H-6,6′), 4.01 (m, 0.7H, H-5α), 3.92 (dd, J = 9.3, 10.5 Hz, 0.7H, H-3α), 3.71 (dd, J = 8.5, 10.5, 0.3H, H-3β), 3.66−3.57 (m, 1.3H, H-4α,4β,5β), 3.34 (dd, J = 3.6, 10.5 Hz, 0.7H, H-2α), 3.01 (dd, J = 8.5, 10.5 Hz, 0.3H, H-2β), in agreement with authentic material (sodium salt) purchased from Sigma; ^{31}P NMR δ 4.49; HRMS calcd for $C_6H_{15}NO_8P$ (M + H$^+$) 260.0529, found 260.0535.

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(43) Compound **31** was prepared by deacetylation of 3,4,6-tri-*O*-acetyl-D-glucal, followed by catalytic hydrogenation.

(44) Deoxygenation (with double-bond migration) of 3,4,6-tri-*O*-acetyl-D-glucal with $LiClO_4$−Et_3SiH (Wustrow, D. J.; Smith, W. J., III; Wise, L. D. *Tetrahedron Lett.* **1994**, *35*, 61−64) was followed by catalytic hydrogenation and deacetylation to give **32**.

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2-Acetamido-2-deoxy-D-glucose 6-Phosphate (53). To a solution of 0.35 mmol of **52** in 7.00 mL of 1 N aqueous NaOH, at 0 °C, was added alternating portions of 0.14 mmol of acetic anhydride and 0.14 mL of 1 N NaOH. The solution was shaken for 5 min after each addition of acetic anhydride and was chilled back to 0 °C after each addition of NaOH. A pH ≥ 12 was maintained. When TLC (butanol−acetic acid−water 3:1:1, ninhydrin stain) indicated complete consumption of starting material, the solution was acidified with Dowex-50W (H^+), filtered, and adjusted to pH 9.0 with aqueous NaOH. Purification by anion exchange chromatography gave **53**[30,47] diammonium salt (85.0 mg, 73%): ^{1}H NMR δ 5.21 (d, J = 3.4 Hz, 0.6H, H-1α), 4.71 (d, J = 8.8 Hz, 0.4H, H-1β), 4.03−3.90 (m, 1H, 3.6H), 3.82−3.55 (m, 2.4H), 2.05 (s, 3H, CH_3); ^{31}P NMR δ 2.88; HRMS calcd for $C_8H_{16}NO_9P$ ($M + H^+$) 302.0643, found 302.0641.

Enzymatic phosphorylation of **17** (0.43 mmol) using 1.0 U of hexokinase gave, after 147 h, 0.16 g (100%) of **53** as a white solid.

2-Amino-2-deoxy-D-mannose 6-Phosphate (54). Phosphorylation of **18** according to the general procedure yielded 1.14 g (96%) of **54**:[48] ^{31}P NMR δ 4.01.

β-D-Allose 6-Phosphate (55). Phosphorylation of **22** according to the general procedure gave 0.516 g (85%) of **55**[49] monosodium salt: mp 125−126 °C; ^{1}H NMR (400 MHz) δ 4.82 (d, J = 8.3 Hz, 1H, H-1), 4.12 (t, J = 2.8 Hz, 1H), 4.04 (m, 1H), 3.93 (m, 1H), 3.82 (dd, J = 3.7, 10.4 Hz, 1H), 3.65 (dd, J = 2.7, 10.0 Hz, 1H), 3.37 (dd, J = 3.0, 8.3 Hz, 1H); ^{31}P NMR δ 3.55; HRMS calcd for $C_6H_{12}Na_2O_9P$ (monosodium salt + Na^+) 305.0014, found 305.0025.

D-**Glucal 6-Phosphate (56).** According to the general procedure, **35** was phosphorylated with 11 U of hexokinase for 72 h to yield 1.09 g (83%) of **56** bis(cyclohexylammonium) salt: mp 150 °C dec; ^{1}H NMR δ 6.42 (dd, J = 1.6, 6.1 Hz, 1H, H-1), 4.81 (dd, J = 2, 6.2 Hz, 1H, H-2), 4.28 (dt, J = 1.9, 7.3 Hz, 1H, H-3), 4.15 (ddd, J = 3.4, 8.2, 12.3 Hz, 1H, H-6), 4.00 (ddd, J = 1.7, 6.1, 12.3 Hz, 1H, H-6′), 4.90 (br d, J = 11 Hz, 1H, H-5), 3.82 (dd, J = 7.2, 10.0 Hz, 1H, H-4); ^{13}C NMR δ 147.12 (C-1), 106.80 (C-2), 81.62 (d, J_{CP} = 6.0 Hz, C-6), 71.87, 71.68, 65.59 (d, J_{CP} = 4.6 Hz, C-5), 53.54 (CN), 33.62, 27.49, 26.97; ^{31}P NMR δ 5.77; IR (KBr) 3440, 2941, 1648, 1560, 1457, 1228, 1137, 1059, 968, 939 cm^{-1}. Anal. Calcd for $C_{18}H_{37}N_2O_7P\cdot H_2O$: C, 48.86; H, 8.88; N, 6.33; P, 7.00. Found: C, 49.05; H, 8.84; N, 6.12; P, 6.88.

2-Acetamido-2-deoxy-D-mannose 6-Phosphate (57). Acetylation of **54** according to the procedure described for the chemical synthesis of **53**, until ninhydrin assay[50] detected no free amine remaining, gave 98.8 mg (84%) of **57**:[2] ^{31}P NMR (162 MHz) δ 3.94.

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