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Supporting Information

Diastereoselective Synthesis of Glycosyl Phosphates by Using a Phosphorylase–Phosphatase Combination Catalyst

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SUPPORTING INFORMATION

CONTENT

1 METHODS

1.1 Chemicals and reagents

αGlc 1-*P* (disodium salt hydrate) was from Sigma-Aldrich (Vienna, Austria) with a purity of 97%. Other carbohydrates were obtained in the highest purity (98%) available at commercial suppliers: D-Man (Sigma-Aldrich), L-Fuc (Roth), L-Sor (Roth), sucrose (Roth), D-Xyl (Roth), Xol (Sigma-Aldrich), D-Gal (Roth; Carbosynth), L-Ara (Roth), D-Ara (Sigma-Aldrich), D-GlcNAc (Sigma-Aldrich), D-GalNAc (Sigma-Aldrich), D-GlcUA (Sigma-Aldrich) and D-GlcNH_2 (Sigma-Aldrich). All other chemicals were reagent grade. All oligonucleotides were from Sigma-Aldrich. DNA sequencing was performed at LGC Genomics (Berlin, Germany). Phusion DNA polymerase was from Thermo Fisher Scientific (Waltham, MA, USA). Electrocompetent *E. coli* Origami B (DE3) cells were from Novagen (Merck KgaA, Darmstadt, Germany).

1.2 Enzyme expression and purification

1.2.1 Phosphatase AGP

The AGP from *E. coli* was used. An *N*-terminally *Strep*-tagged enzyme preparation was applied. Enzyme expression, purification and determination of specific activity were performed as described before.[1] One Unit of AGP activity is defined as the amount of enzyme releasing 1 µmol of inorganic phosphate (Pi) from αGlc 1-*P* per minute under the conditions applied.

1.2.2 Phosphatase HAD13

HAD13 from *E. coli* was used. An *N*-terminally *His*-tagged enzyme preparation was applied. Enzyme expression and purification were performed as described before.[1] One Unit of HAD13 activity is defined as the amount of enzyme releasing 1 µmol of Pi from αGlc 1-*P* per minute under the conditions applied.

1.2.3 Phosphatase HAD4

HAD4 from *E. coli* was used. HAD4 was fused to the positively charged binding module Z_{basic2}. Expression and purification of Z_{basic2}_HAD4 were described before.[2] One Unit of HAD4 activity is defined as the amount of enzyme releasing 1 µmol of Pi from αGlc 1-*P* per minute under the conditions applied.

1.2.4 Sucrose phosphorylase (SPase)

The SPase from *Leuconostoc mesenteroides* was used. An *N*-terminally *Strep*-tagged enzyme preparation was applied. *E. coli* BL21-Gold (DE3) cells harboring the pASK-IBA7+[3] expression vector encoding the SPase were cultivated in 1-L baffeled shaken flasks at 37°C and 110 rpm using Lennox-media containing 0.115 mg/mL ampicillin. When OD_{600} had reached 0.8, temperature was decreased to 22°C and gene expression was induced with 200 µg/L anhydrotetracycline for 20 h. Enzyme purification was done as described above for AGP. The specific activity was determined in 50 mM Mes buffer (pH 7.0) at 37°C using 100 mM inorganic phosphate and 100 mM sucrose as substrates. The reaction was started with 0.12 U/mL SPase and consumption of Pi was measured as described under 1.3 Assays. One unit of SPase activity is defined as the amount of enzyme consuming 1 µmol of Pi per minute under the conditions applied.

1.2.5 Phosphatase BT4131

The BT4131 from *Bacteroides thetaiotaomicron* VPI-5482 was used. An *N*-terminally Z_{basic2}-tagged enzyme preparation was applied. Z_{basic2} is a highly positively charged protein module that is useful for purification and immobilization (see ref. 2 and citations therein). The BT4131 gene encoded in a pET-3A-042[4] vector was provided by Prof. Karen N. Allen (Department of Chemistry, Boston, United States). The gene was amplified from the vector by PCR using phusion polymerase and the following pair of primers:

5´-CTCTGTTCCAGGGTCCGACGAAAGCTTTATTTT-3´

5´-GTTAGCAGCCGGATCTCTTAGATAATCCCGAAATG-3´.

BT4131 PCR product was subcloned into the pT7-HAD4[2] expression vector by circular polymerase extension cloning[5,6] using the following pair of primers for vector linearization:

5´-CATTTCGGGATTATCTAAGAGATCCGGCTGCTAAC-3´

5´-AAAATAAAGCTTTCGTCGGACCCTGGAACAGAG-3´.

The resulting vector construct encodes a BT4131 fusion protein where the small module Z_{basic2} is joined by a flexible linker (11 amino acids) to the N-terminus of BT4131. Sequenced expression vector pT7-BT4131 was transformed into *E. coli* BL21-Gold (DE3) and transformants were selected on LB-agar plates containing 0.05 mg/mL kanamycin. Recombinant BT4131 was produced in 1-L baffled shaken flasks at 37°C and 110 rpm using Lennox-media containing 0.05 mg/mL kanamycin. When OD_{600} had reached 0.8, temperature was decreased to 30°C and gene expression was induced with 0.4 mM isopropyl β-D-1-thiogalactopyranoside for 4 h. Enzyme purification was done as described before.[2] The specific activity was determined in 50 mM Mes buffer supplemented with 5 mM $MgCl_2$ (pH 7.0) at 37°C using 20 mM Glc 6-*P* as substrates. The reaction was started with 0.04 mg/mL BT4131 and release of Pi was measured as described under 1.3 assays. One unit of BT4131 activity is defined as the amount of enzyme releasing 1 µmol of Pi from Glc 6-*P* per minute under the conditions applied.

1.3 Assays

Inorganic phosphate was determined colorimetrically at 850 nm.[7] αGlc 1-*P* and αGlc 6-*P* were assayed enzymatically.[8] Fru 6-*P* was determined using mannitol 1-phosphate dehydrogenase.[9] The concentration of sugar phosphate product was determined directly by NMR (see later) or indirectly from the phosphate mass balance for substrate consumed (Δ[αGlc 1-*P*]) and products formed (equation 1).

$$\Delta[\alpha\text{Glc 1-}P] = [\text{sugar phosphate product}] + [\text{Glc 6-}P] + [\text{phosphate}] \quad (1)$$

Unless mentioned, protein concentration was determined using the Roti-Quant assay (Roth) referenced against BSA.[10] The concentration of BT4131and HAD4 was determined spectrophotometrically at 280 nm and a molar extinction coefficient of 14690 M^{-1} cm^{-1} and 25440 M^{-1} cm^{-1} was used, respectively.

1.4 Enzymatic transphosphorylations

1.4.1 HAD4 and HAD13

Reactions were performed at 37 °C in 50 mM Hepes buffer (pH 7.0) containing 15 mM $MgCl_2$. Agitation was at 650 rpm using an Eppendorf (Hamburg, Germany) Thermomixer compact. Twenty mM αGlc 1-*P* and 100 mM D-Man or D-Glc were used. Reactions were started by addition of enzyme (HAD4, HAD13 0.2 U/mL each) and

were incubated for 90 min. Samples were taken in 15 min intervals and were analyzed for phosphate, αGlc 1-*P* and Glc 6-*P*.

1.4.2 AGP

Reactions were performed at 37°C in 50 mM Mes buffer (pH 7.0) in a total volume between 200 µL - 1000 µL. Agitation was at 650 rpm. Twenty mM αGlc 1-*P* and 200 mM acceptor (D-Man, D-Gal, D-GlcNAc, D-$GlcNH_2$, D-$GalNH_2$, D-GlcUA, L-Fuc, D-Xyl, D-Ara, L-Ara, Xol) were used. Reactions were started with addition of 0.2 U/mL AGP and incubated for 75 min. Samples were taken in 15 min intervals, heat-treated (99°C, 5 min) and centrifuged at 20,000 *g* for 5 min. Product analysis was done as described above under 1.3 Assays. NMR analyses (TOCSY, $^{1}H/^{31}P$-HSQC) were done directly from the reaction mixtures (1D proton and phosphorus NMR) or after sugar phosphate product recovery (TOCSY, $^{1}H/^{31}P$-HSQC) as described below in section 1.6.

Full reaction time courses were recorded for each acceptor. Rates of αGlc 1-*P* consumption (r_s) and phosphate release (r_p) were obtained from the time courses. The ratio r_s/r_p is used to characterize phosphoryl transfer, as described in the main text.

Phosphorylated product yields are always based on the limiting αGlc 1-*P* concentration initially present. Unless mentioned, the yield was determined at a αGlc 1-*P* conversion of ≥ 90%.

Reaction conditions were varied with different acceptors (D-Man, D-Gal, L-Fuc) to determine effects of the acceptor substrate concentration and of the ratio of acceptor/donor substrate on r_s/r_p.

1.5 Sequential and simultaneous combi-catalytic phosphorylation

Reactions were performed at 37°C in 50 mM Mes buffer (pH 7.0) and agitation was at 650 rpm. Sequential phosphorylations involved conversion of sucrose and phosphate (200 mM each) with 8 U/mL SPase in a total volume of 700 µL. Samples taken in 20 min intervals were analyzed for αGlc 1-*P* and phosphate. After 100 min, acceptor substrate (1 M; D-Man) and 0.3 U/mL AGP were added to reach a total volume of 1 mL. Note that addition of the acceptor resulted in about 3-fold increase in total volume, hence dilution of the αGlc 1-*P* formed. Reaction was continued for another 130 min.

Simultaneous phosphorylations were performed in a total volume of 1.2 mL and used sucrose and phosphate at 100 - 150 mM each. Acceptor was present at 1 M (D-Man, D-GlcNAc) or 2 M (D-Gal). Reactions were started by simultaneous addition of 8 U/mL (D-Man, D-GlcNAc, D-Gal) SPase and 0.3 U/mL AGP (D-Man, D-Gal) or 2 U/mL AGP (D-GlcNAc). Samples were taken in 30 min intervals and analysed as described above in assays 1.3.

Preparative scale conversions were performed in a total volume of 10 mL (D-Man) or 1.5 mL (D-GlcNAc, D-Gal). Reactions were stopped by heat-treatment (99°C, 5 min) after 90 min (D-Man, D-Gal) or 300 min (D-GlcNAc). Otherwise reaction conditions were the same as above.

1.6 Recovery of reaction products for product identification

$BaAc_2$ was added to samples in a concentration equal to the initial αGlc 1-*P* concentration used in the reaction. Sugar phosphates and phosphate were subsequently precipitated by dilution (1:1, by volume) with ethanol. After incubation for 1.5 h at 4 °C, the precipitate was collected by centrifugation (4°C, 20,000 *g*, 15 min), washed with ethanol and dried at 40 °C for 2 h. Products were re-dissolved using aqueous Na_2SO_4, and the resulting $BaSO_4$ precipitate was removed.

1.7 Selective hydrolysis of Glc 6-*P* by BT4131

The Reaction was performed in 50 mM Mes supplemented with 5 mM $MgCl_2$ buffer (pH 7.0) at 37°C using Glc 6-*P* and αGlc 1-*P* as substrates. Five U/mL BT4131 was used to start the reaction and Pi, Glc 6-*P* and αGlc 1-*P* concentrations were monitored as described under 1.3 assays.

1.8 Purification of reaction products

Heat-treated reaction mixtures were diluted (10 mL αMan 1-*P*, 1.5 mL αGlcNAc 1-*P*, 0.5 mL αGal 1-*P*) 1:10 (v:v) with 50 mM Mes (5 mM $MgCl_2$, pH 7.0). Hydrolysis of side products (sugar 6-phosphates) was initiated by addition of 1.5 U/mL BT4131. Reactions were incubated at 37°C for 1 h and stopped by heat-treatment (99°C, 5 min). Residual protein was precipitated by centrifugation (4°C, 20,000 *g*, 10 min). Cleared reaction mixture was applied to a FliQ FPLC column (33 x 6.2 mm; 1 mL; Generon, Berkshire, U.K.) packed with SuperQ-650M (Tosh, Tokyo, Japan) anion exchange resin mounted onto an ÄKTA prime plus FPLC system (GE Healthcare, Little Chalfont, U.K.). Columns were beforehand equilibrated with buffer A (10 mM ammonium chloride pH 8.0) and elution of phosphorylated reaction products was performed with 60% buffer B (100 mM ammonium chloride pH 8.0). Purification was monitored by TLC. One µl of each fraction was spotted directly on silica gel plates, developed using 1-propanol/n-butanol/ethanol/H_2O = 2:3:3:2 (v/v/v/v) and stained with thymol sugar stain (5 g/L thymol, 5% H_2SO_4 in ethanol). Fractions containing phosphorylated reaction products were lyophilized (Christ Alpa 1-4; Osterode am Harz, GE). Resulting powder was resolubilized in H_2O (10 mL, αMan 1-*P*, 1.5 mL αGlcNAc 1-*P* and 0.5 mL αGal 1-*P*) and $BaAc_2$ was added to samples in a concentration equal to the initial Pi concentration. Phosphorylated reaction products and Pi were precipitated sequentially; first Pi was precipitated by addition of 20% (v) ethanol. The resulting precipitate was removed by centrifugation (4°C, 20,000 *g*, 15 min) and the cleared supernatant was diluted 1:5 (v:v) with ethanol. After incubation for 1.5 h at 4°C, the precipitate was collected by centrifugation (4°C, 20,000 *g*, 15 min) and washed with ethanol. Residual ethanol and H_2O were removed by lyophilisation. Barium salts of products were resolubilized as described above under 1.6. Purity of isolated compounds was analyzed by ^{1}H NMR, ^{13}C NMR, (see below section 1.9) and phosphate measurements as described in Assays 1.3.

Purification yield, product purity and relative amount of impurities were calculated as follows:

Purification yield % = mass product after purification/mass product in reaction mixture

Product purity % = mass of product/total mass

Relative mass of impurities % = mass of impurity/total mass

1.9 Nuclear magnetic resonance spectroscopy

NMR measurements for product identification were performed in D_2O (99.9% D) at 27 °C on a Bruker DRX 400 AVANCE spectrometer using the Topspin 1.3 software or on a Bruker AV-III 600 spectrometer using the Topspin 3.1 software. Proton and phosphorus spectra were measured at 400.13 MHz, and 161.96 MHz, or at 600.13 MHz and 242.94 MHz, respectively. 1D spectra were recorded with 32,768 data points. Zero filling to 65,536 data points, appropriate exponential multiplication and Fourier transformation led to spectra with ranges of ~5400 Hz (^{1}H), and ~24,000 Hz (^{31}P). ^{31}P spectra were measured with proton decoupling. 2D spectra were recorded with 256 experiments, each with 2048 data points and an appropriate number of scans. Zero filling and Fourier transformation led to spectra with ranges of ~5400 Hz and ~24,000 Hz for proton and phosphorus,

respectively. For combined *in situ* ^{1}H and ^{31}P NMR measurements the reactions are performed directly in a sample tube placed in the Bruker AV-III 600 spectrometer at 25°C. Samples contain 20 mM αGlc 1-*P* and 200 mM of the respective acceptor, 21 U/mL AGP, and 50 mM MES buffer pH 7.0, in D_2O (0.70 mL). Proton and phosphorus spectra are alternatingly taken every 10 min over a time of ca. 10 h. Chemical shifts have been referenced to external acetone (δ_H: 2.225 p.p.m) and 85% aqueous phosphoric acid (δ_P: 0.00 p.p.m.).

NMR measurements of purified products were performed in D_2O (99.9% D) at 27 °C on a Varian (Agilent) INOVA 500-MHz NMR spectrometer (Agilent Technologies, Santa Clara, California, USA) and the VNMRJ 2.2D software was used. ^{1}H NMR spectra (499.98 MHz) were measured on a 5 mm indirect detection PFG-probe, while a 5 mm dual direct detection probe with z-gradients was used for ^{13}C NMR spectra (125.71 MHz). ^{1}H NMR spectra were recorded with pre-saturation of the water signal by a shaped pulse in case of Gal 1-*P*. Standard pre-saturation sequence was used: relaxation delay 2 s; 90° proton pulse; 2.048 s acquisition time; spectral width 8 kHz; number of points 32 k. ^{13}C NMR spectra were recorded with the following pulse sequence: standard ^{13}C pulse sequence with 45° carbon pulse, relaxation delay 2 s, Waltz decoupling during acquisition, 2 s acquisition time. ACD/NMR Processor Academic Edition 12.0 (Advanced Chemistry Development Inc.) was used for evaluation of spectra.

2 RESULTS

2.1 Enzyme purification

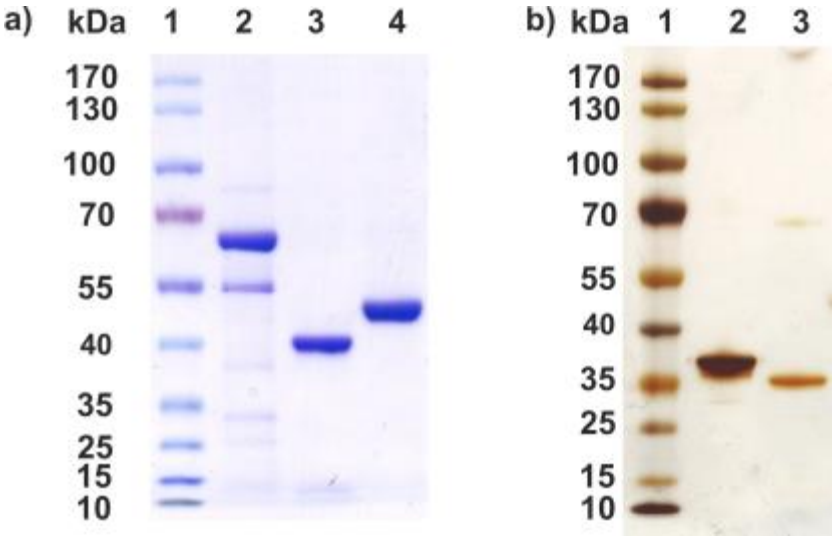

Figure S1. SDS polyacrylamide gel showing the purity of the enzyme preparations used. a) Lane 1: PageRuler Prestained Protein Ladder (Thermo Scientific), Lane 2: *Strep*-tagged SPase (59 kDa), Lane 3: Z_{basic2}_BT4131 (37 kDa). Lane 4: *Strep*-tagged AGP (45 kDa), 3 µg protein was applied to the gel. Protein was detected by coomassie staining b) Lane 1: PageRuler Prestained Protein Ladder (Thermo Scientific), Lane 2: *His*-tagged HAD13 (31 kDa), Lane 3: Z_{basic2}-tagged HAD4 (31 kDa), 1 µg protein was applied to the gel, protein was detected by silver staining

Table S1. Specific activities and yields of used enzyme preparations.

Enzymes	Specific activity U/mg	Yield mg/L
AGP	53 ± 5	1
HAD13	23+3	1
HAD4	19±2	11
SPase	80 ± 5	9
BT4131	5 ± 1	50

2.2 AGP-catalyzed transphosphorylations and product purification

2.2.1 Acceptor substrates

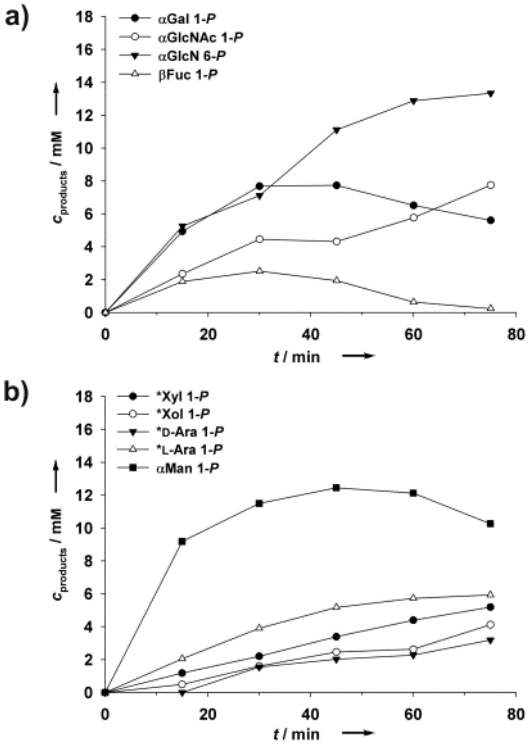

Figure S2. Reaction time courses of AGP (0.2 U/mL) catalyzed phosphorylation of different acceptors (200 mM) using αGlc 1-*P* (20 mM) as donor substrate. Products were characterized by NMR spectroscopies, and the indicated sugar phosphates were identified unambigously (see Figures S3 – S9 and also Figure S12 later). Compounds marked with asterisk are assumed reaction products. Their NMR characterization was not pursued.

2.2.2 Product identification by NMR

Purified phosphorylated products were identified by TOCSY and $^{1}H/^{31}P$-HSQC. The corresponding spectra of αMan 1-*P* (Figure S3), αGlcNAc 1-*P* (Figure S4), αGal 1-*P* (Figure S5), β-L-Fuc 1-*P* (Figure S6) and $\beta GlcNH_2$ 6-*P* (Figure S7), are shown. The corresponding proton and phosphorous NMR chemical shifts are summarized in Table S2 and Table S3. The abundance of starting material (αGlc 1-*P*) and reaction products was analyzed by 1D proton and phosphorus NMR from reaction mixtures (Table S4).

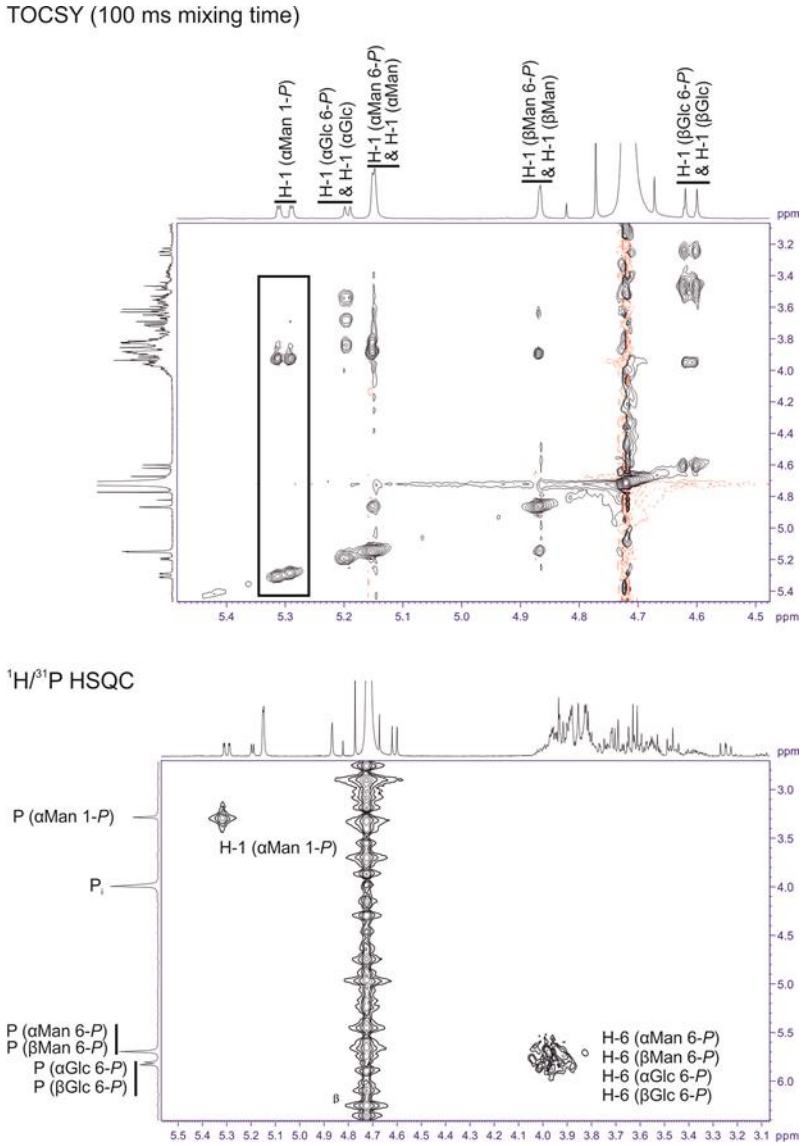

Figure S3. TOCSY and $^{1}H/^{31}P$-HSQC of purified phosphorylated products derived from reactions containing αGlc 1-*P* (20 mM) as donor and D-Man (200 mM) as acceptor substrate. αMan 1-*P* is the main product.

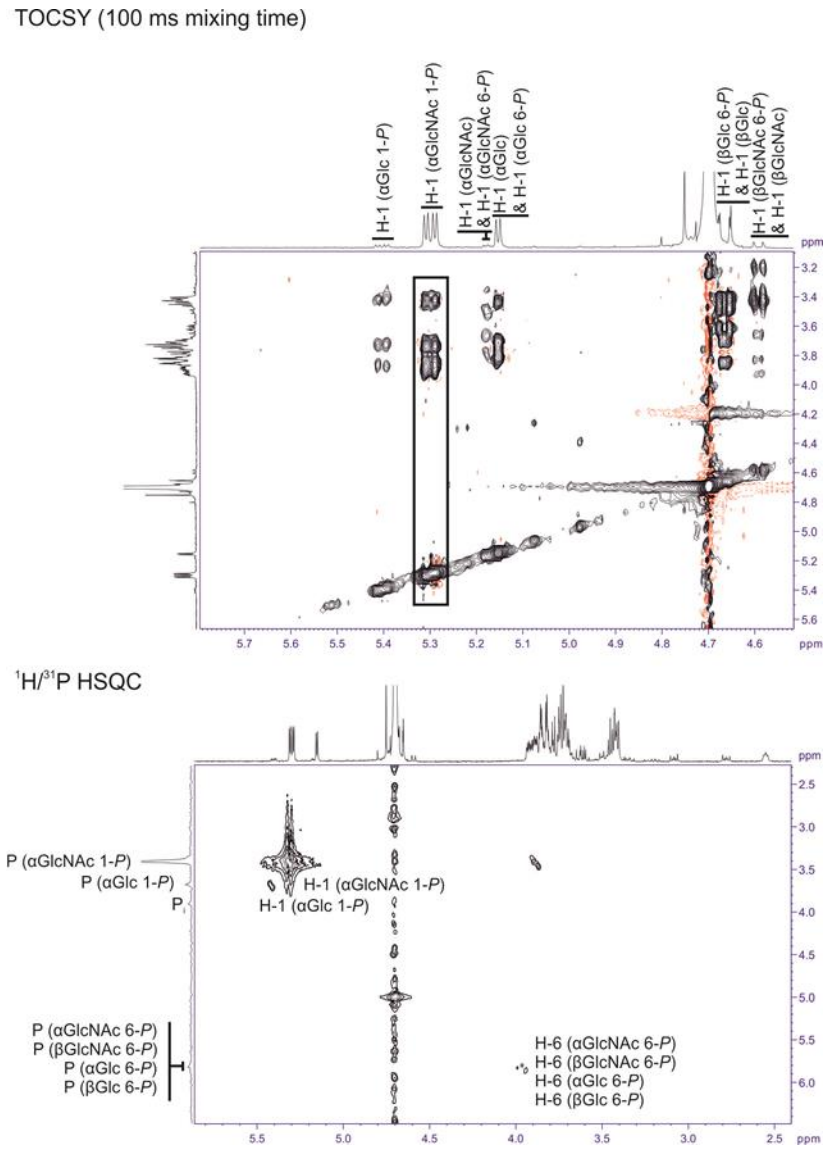

Figure S4. TOCSY and ^{1}H/^{31}P-HSQC of purified phosphorylated products derived from reactions containing αGlc 1-*P* (20 mM) as donor and D-GlcNAc (200 mM) as acceptor substrate. αGlcNAc1-*P* is the main product.

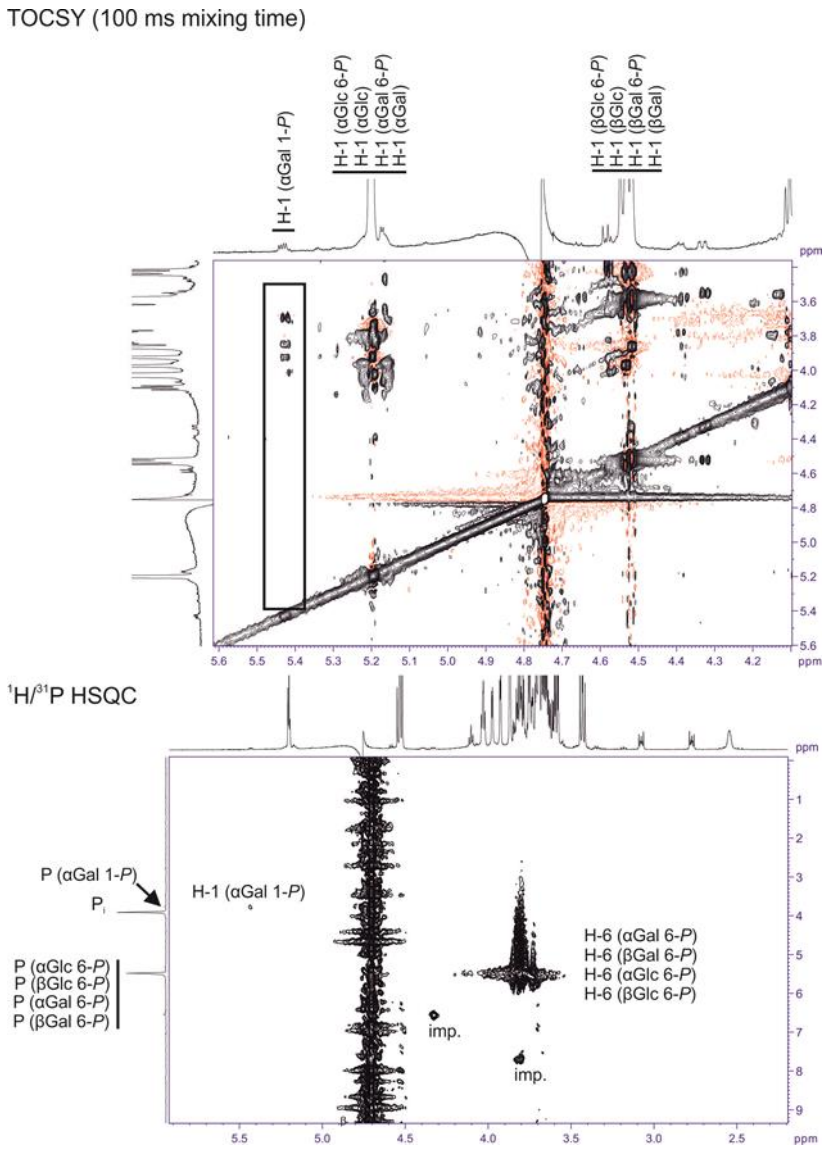

Figure S5. TOCSY and ^{1}H/^{31}P-HSQC of purified phosphorylated products derived from reactions containing αGlc 1-*P* (20 mM) as donor and D-Gal (200 mM) as acceptor substrate. αGal 1-*P* is the main product.

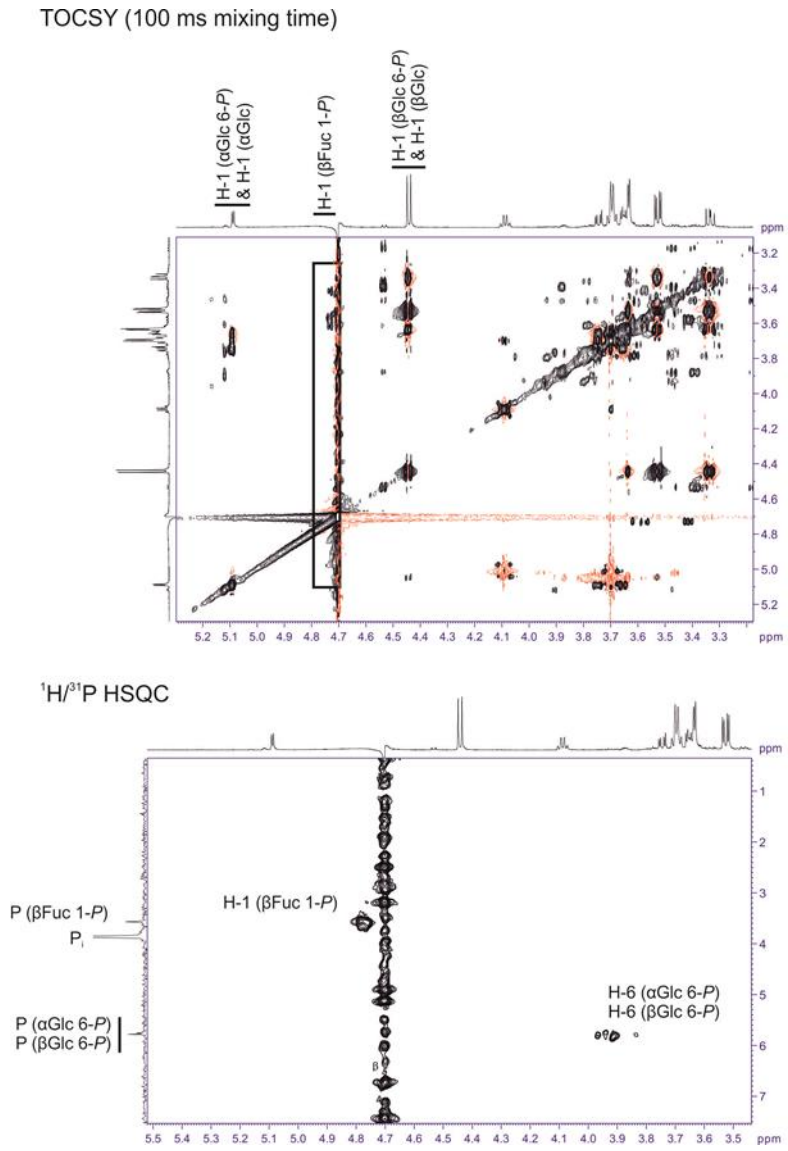

Figure S6. TOCSY and $^{1}H/^{31}P$-HSQC of purified phosphorylated products derived from reactions containing αGlc 1-*P* (20 mM) as donor and L-Fuc (200 mM) as acceptor substrate. β-L-Fuc 1-*P* is the main product.

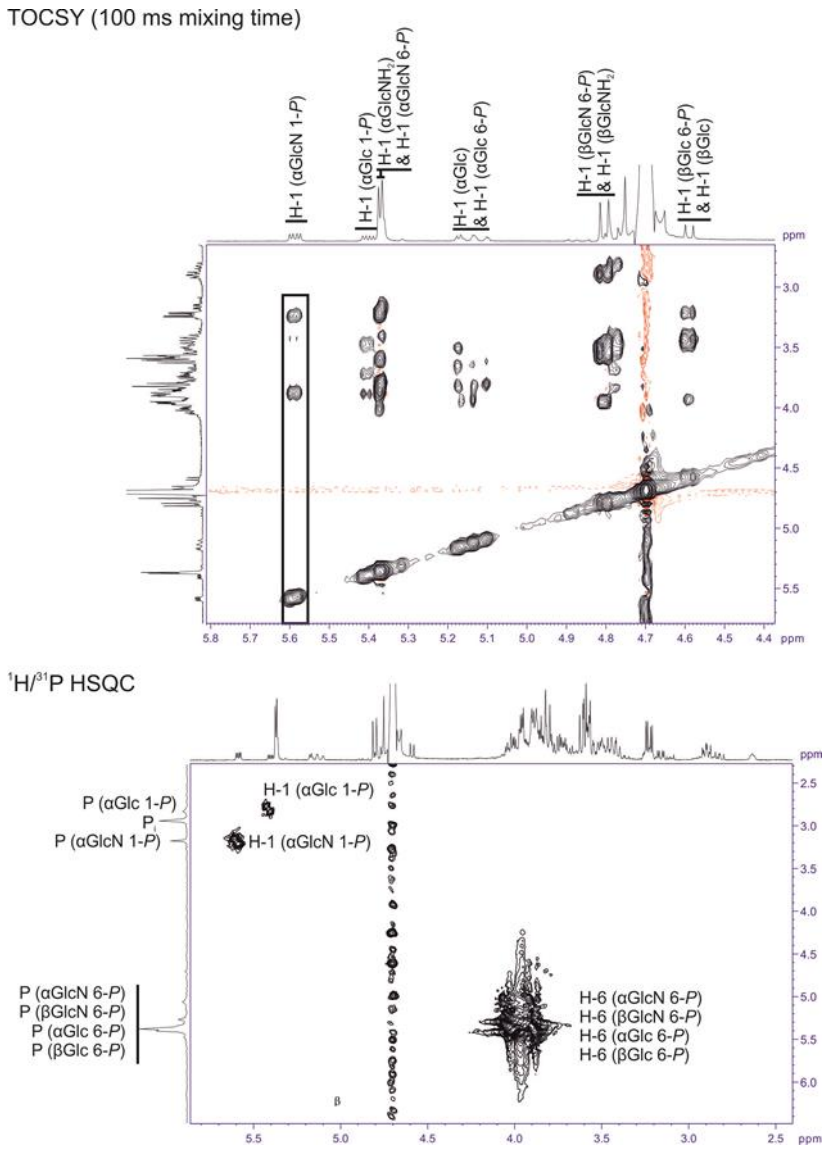

Figure S7. TOCSY and $^{1}H/^{31}P$-HSQC of purified phosphorylated products derived from reactions containing αGlc 1-*P* (20 mM) as donor and D-$GlcNH_2$ (200 mM) as acceptor substrate. $GlcNH_2$ 6-*P* is the main product.

Compositional analysis of product mixtures by NMR

Table S2. Proton and phosphorus NMR chemical shifts of reaction products

	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b	P
αMan 1-*P**	5.30	3.93	3.92	3.59	3.86	3.88	3.70	3.3
$\alpha GlcNH_2$ 1-*P**	5.60	3.24	3.80	3.43	3.80	3.74	3.70	2.8
αGal-1-*P**	5.43	3.69	3.86	3.92	n.d.	3.68	3.63	3.9
β-L-Fuc-1-*P**	4.74	3.40	3.60	3.58	n.d.	1.21		3.55

Table S3. Proton and phosphorus NMR chemical shifts of α-GlcNAc-1-*P*

	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b	CH_3	P
αGlcNAc 1-*P**	5.30	3.83	3.72	3.42	3.90	3.80	3.75	2.04	3.4

*All shifts for αMan 1-*P*[11, 12], $\alpha GlcNH_2$ 1-*P*[13], αGal 1-*P* [12, 14], βFuc-1-*P*[15,16] and αGlcNAc 1-*P*[12, 17] are in good agreement with earlier reported values.

2.2.3 r_s/r_p dependence on the acceptor substrate concentration

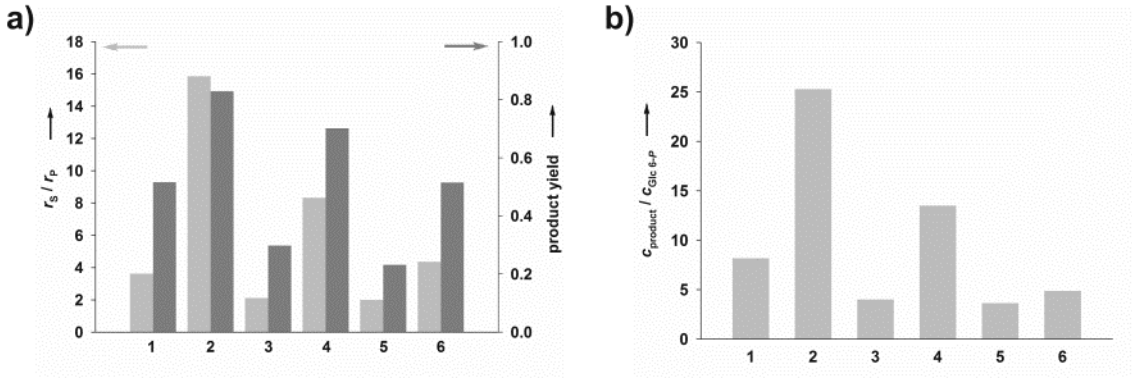

Figure S8. The effect of acceptor substrate concentration on r_s/r_p and on the total yield of phosphorylated product is displayed. (1) 20 mM αGlc 1-*P*, 100 mM D-Man; (2) 20 mM αGlc 1-*P*, 400 mM D-Man; (3) 20 mM αGlc 1-*P*, 100 mM D-Gal; (4) 20 mM αGlc 1-*P*, 400 mM D-Gal; (5) 50 mM αGlc 1-*P*, 200 mM L-Fuc; (6) 50 mM αGlc 1-*P*, 2000 mM L-Fuc. r_s is the rate of αGlc 1-*P* consumption, and r_p is the rate of phosphate release. A ratio r_s/r_p greater than unity indicates phosphoryl transfer to acceptor substrate.

2.2.4 Time courses of αMan 1-*P* formation at varied donor/acceptor substrate ratio

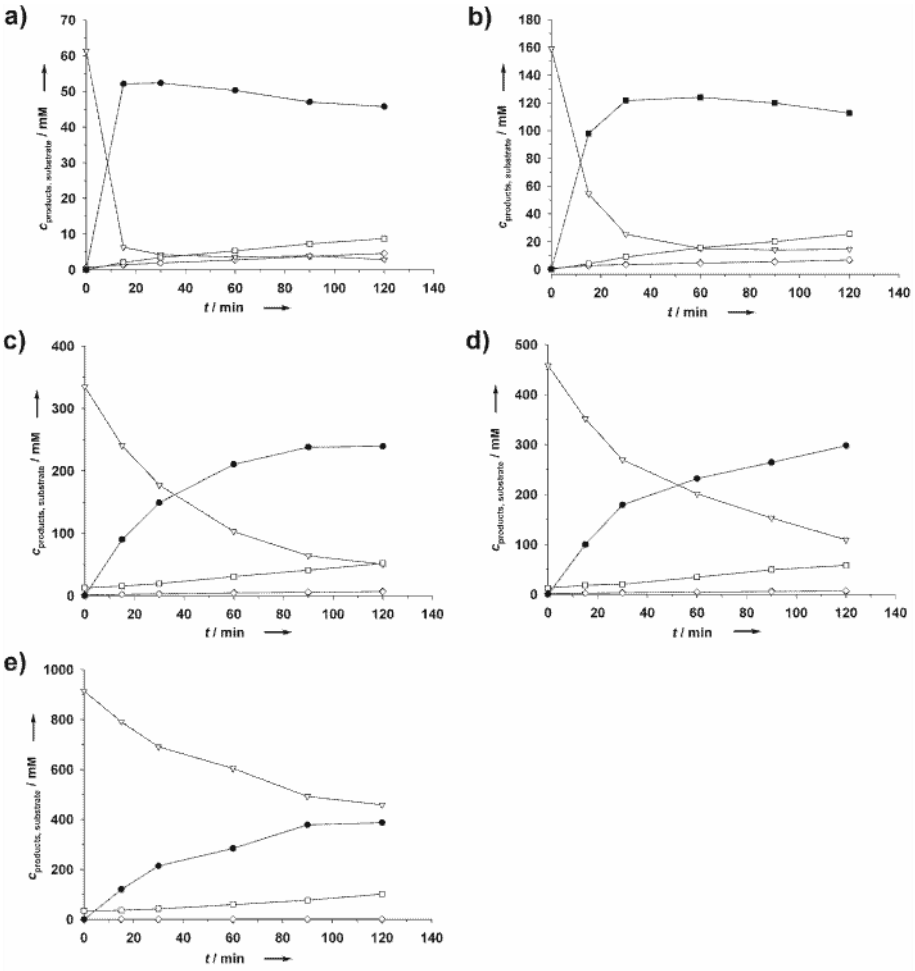

Figure S9. Reaction time courses for αMan 1-*P* (●) production are shown. The acceptor (D-Man) concentration was kept constant (1000 mM), the concentrations of αGlc 1-*P* (∇) and AGP were varied. Panel (a) 61 mM αGlc 1-*P* and 0.8 U/mL AGP; (b) 160 mM αGlc 1-*P* and 1.6 U/mL AGP; (c) 350 mM αGlc 1-*P* and 3.2 U/mL AGP; (d) 470 mM αGlc 1-*P* and 4.0 U/mL AGP; (e) 950 mM αGlc 1-*P* and 8 U/mL AGP. Phosphate (□) and Glc 6-*P* (◊) are also indicated.

2.2.5 In-situ ^{31}P NMR measurements

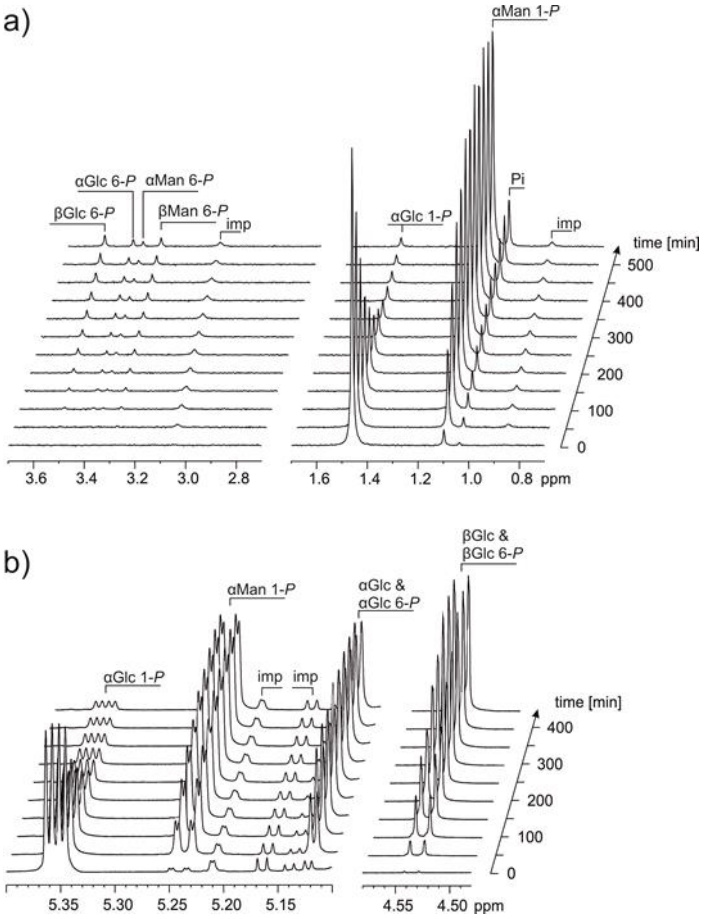

Figure S10. *In situ* ^{31}P and ^{1}H NMR measurements of AGP catalyzed phosphoryl transfer from αGlc 1-*P* to the acceptor D-Man. Panel a) shows a stack plot of *in situ* ^{31}P NMR spectra. In panel b) the region of anomeric aldohexose proton signals in concomitantly recorded *in situ* ^{1}H NMR spectra are presented. It is shown that αGlc 1-*P* is consumed and αMan 1-*P* is preferentially formed in the reaction. Free phosphate, Man 6-*P* and Glc 6-*P* are byproducts that are initially accumulated in very small amounts. After complete consumption of the αGlc 1-*P*, the αMan 1-*P* concentration decreases to give larger amounts of the byproducts. Unknown, probably aldohexose impurities were present in trace amount, and they were also phosphorylated (imp). Formation of free glucose (b) indicates the preferred formation of the αGlc, while the βGlc is subsequently generated by mutarotation.

2.2.6 Dependence of Glc 6-*P* yield on r_s/r_p

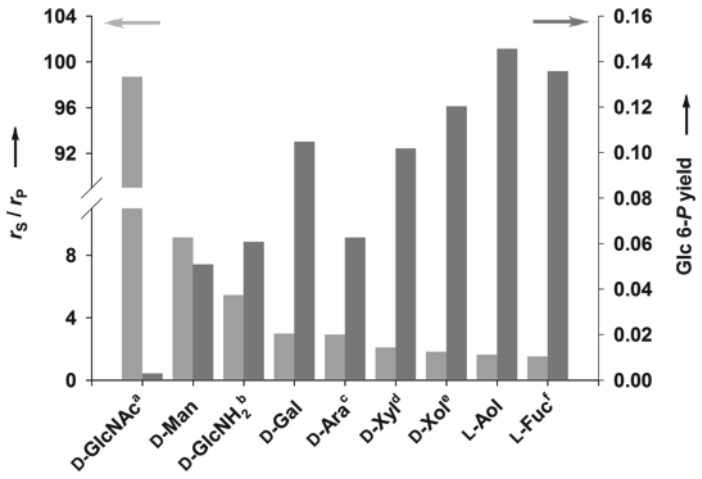

Figure S11. Transphosphorylation ratio (r_s/r_p) and Glc 6-*P* yield in reactions with different acceptor substrates. Conditions were the same as in Figure S2. Light gray bars show r_s/r_p; dark gray bars show the Glc 6-*P* yield which was calculated at a αGlc 1-*P* conversion of ≥ 90%, except [a]38%, [b]81%, [c]69%, [d]86%, [e]89% and [f]75%. r_s is the rate of αGlc 1-*P* consumption, and r_p is the rate of phosphate release. A ratio r_s/r_p greater than unity indicates phosphoryl transfer to acceptor substrate.

2.2.7 Sequential and simultaneous combi-catalytic sugar phosphorylation

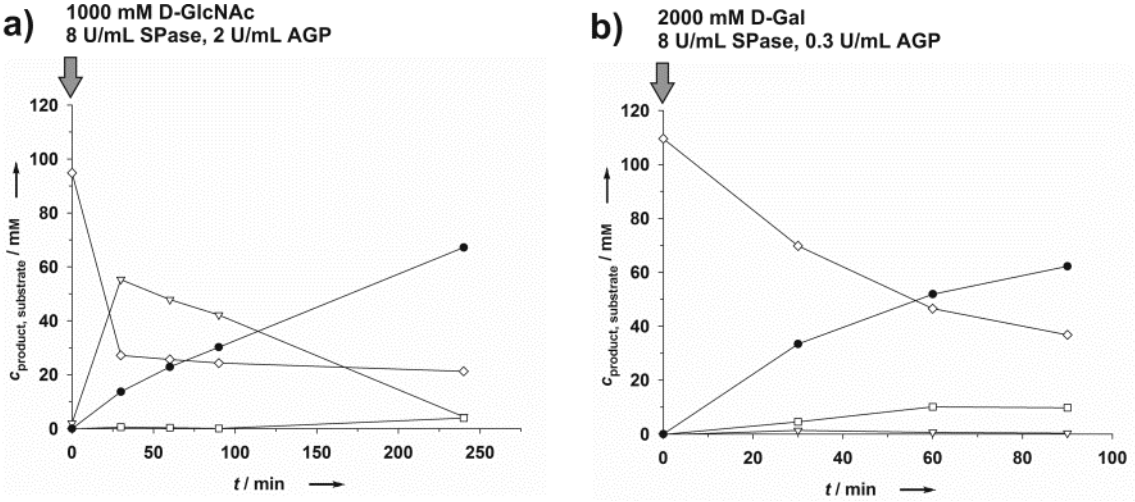

Figure S12. Simultaneous-mode combi-catalytic phosphorylation of D-GlcNAc and D-Gal. The symbols indicate αGlcNAc 1-*P* or αGal 1-*P* (●), αGlc 1-*P* (▽), phosphate (◊) and Glc 6-*P* (□).

2.2.8 Selective hydrolysis of Glc 6-*P* by BT4131

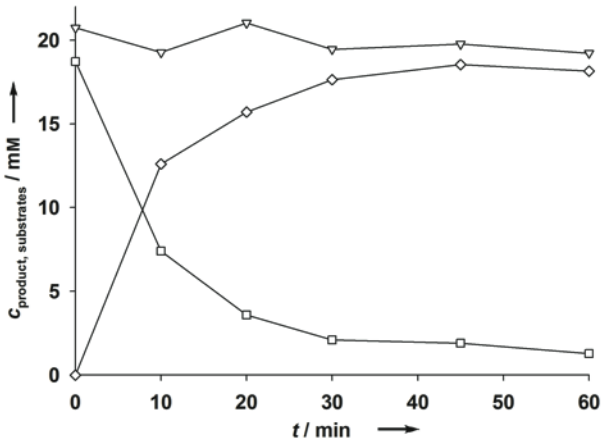

Figure S13. Time course of selective hydrolysis of Glc 6-*P* in an equimolar mixture of αGlc 1-*P* and Glc 6-*P* by (1.5 U/mL) BT4131 is shown. Symbols indicate αGlc 1-*P* (▽), phosphate (◊) and Glc 6-*P* (□).

2.2.9 NMR Spectra of purified reaction products

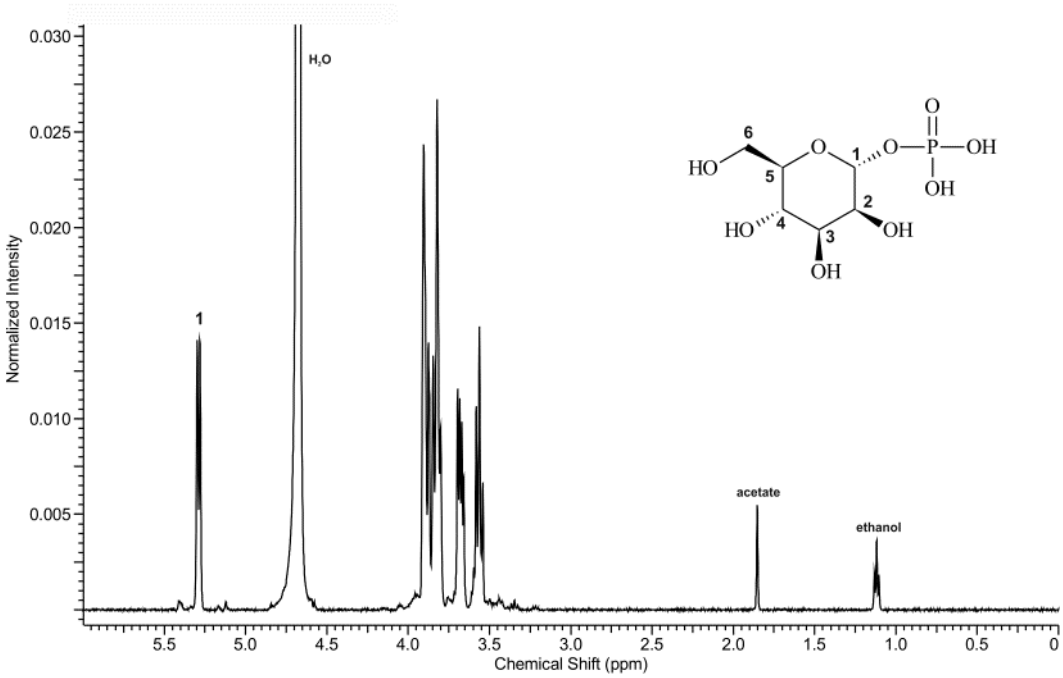

Figure S14. Man 1-*P*; ^{1}H NMR (500 MHz, D_2O, 27°C).

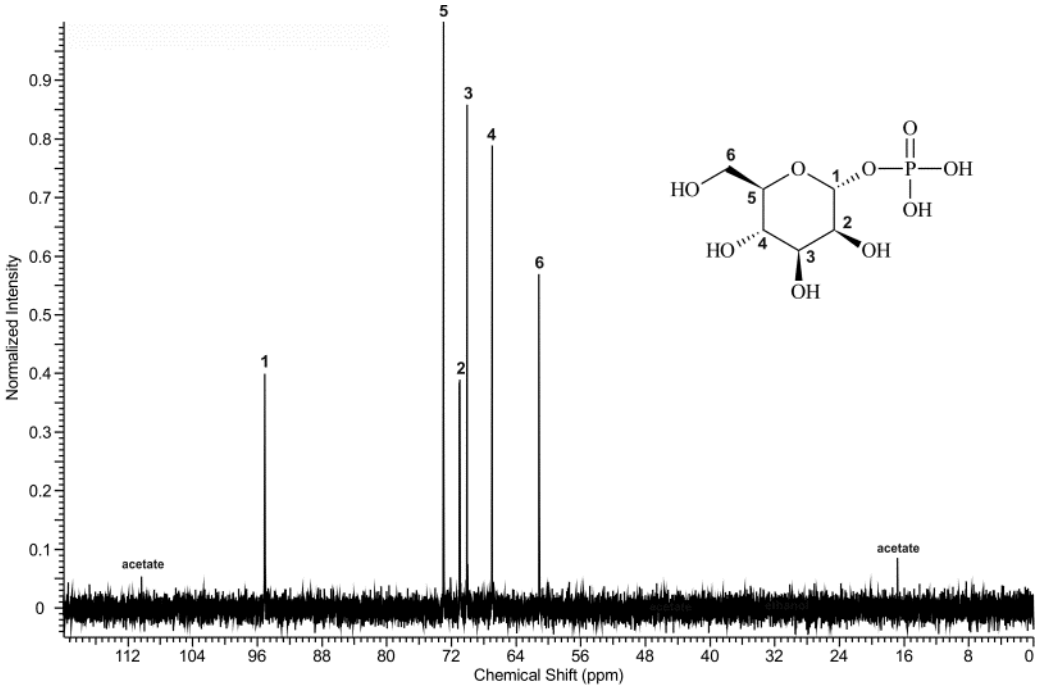

Figure S15. Man 1-*P*; ^{13}C NMR (126 MHz, D_2O, 27°C).

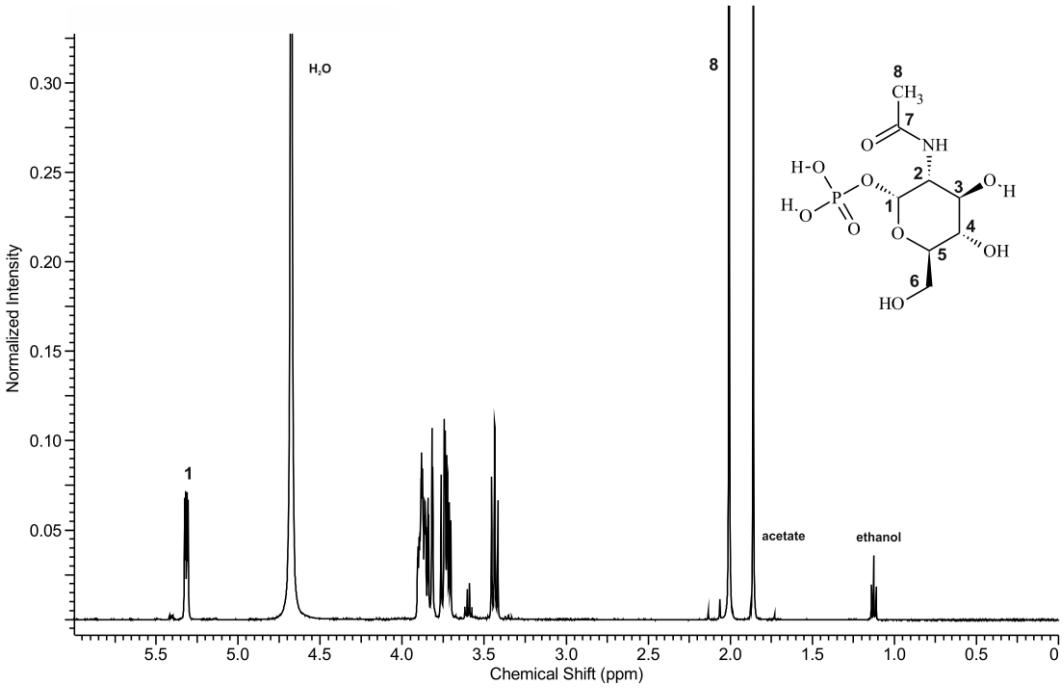

Figure S16. GlcNAc 1-*P*; ^{1}H NMR (500 MHz, D_2O, 27°C).

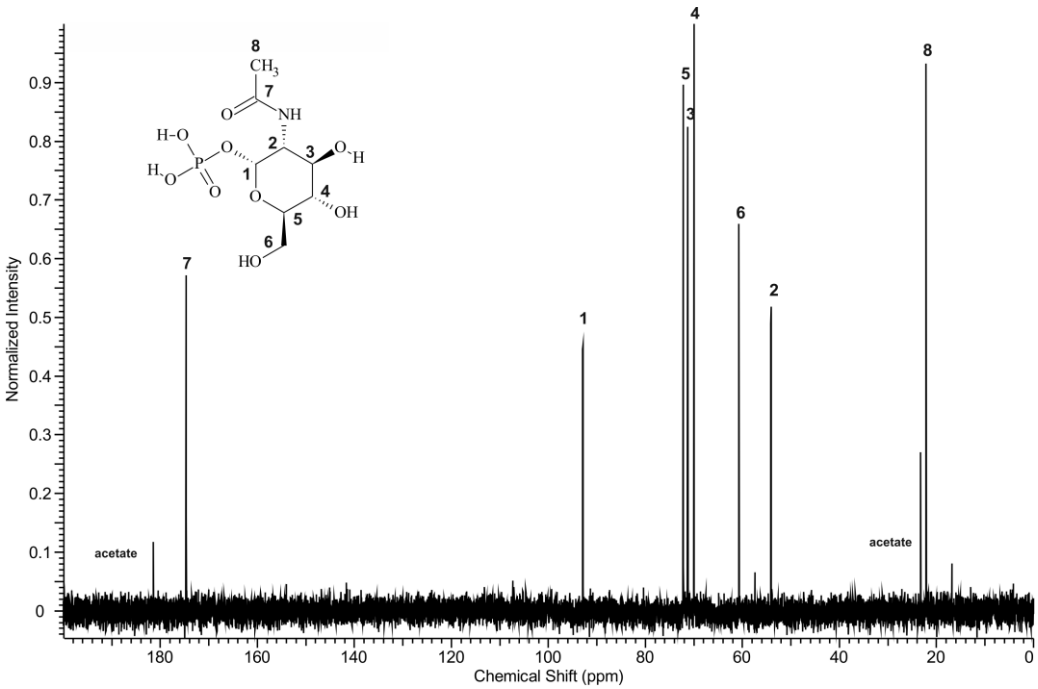

Figure S17. GlcNAc 1-*P*; ^{13}C NMR (126 MHz, D_2O, 27°C).

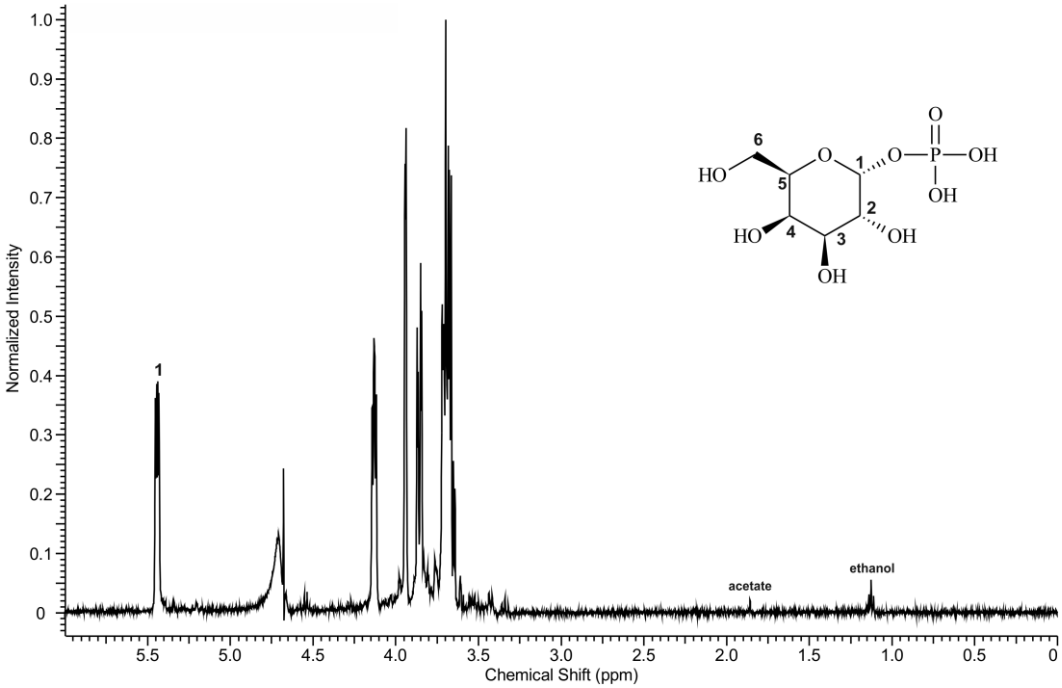

Figure S18. Gal 1-*P*; ^{1}H NMR (500 MHz, D_2O, 27°C)

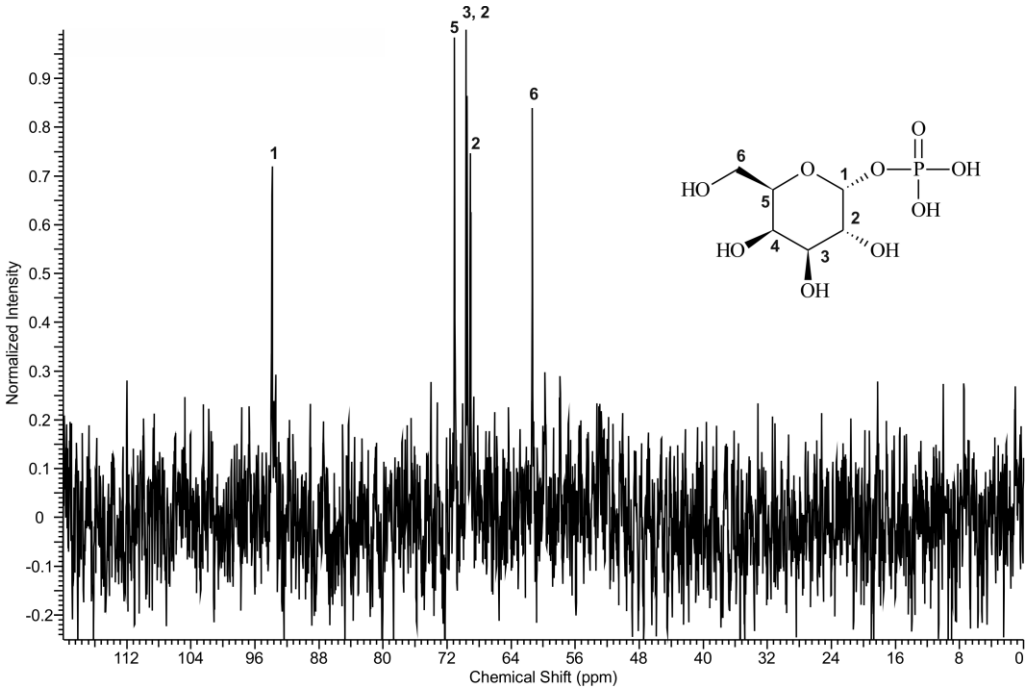

Figure S19. Gal 1-*P*; ^{13}C NMR (126 MHz, D_2O, 27°C).

Table S4. Carbon NMR chemical shifts of reaction products

	C-1	C-2	C-3	C-4	C-5	C-6a	$COCH_3$	CH_3
*αMan 1-*P*	95.10	70.98	70.07	66.68	72.99	61.15		
*αGlcNAc 1-*P*	92.90	54.11	71.32	70.02	72.23	60.71	174.71	22.17
*αGal 1-*P*	93.86	69.07	69.66	96.49	71.09	61.36		

*Chemical shifts are in good agreement with earlier reported values.[12]

Table S5. Purified reaction products

product	**scale**	**purity**[a]	**purification yield**[b]	**impurities**[c]			
				αGlc 1-*P*	acetate	ethanol	Pi
αMan 1-*P*	130 mg	95%	40%	3%	< 1%	< 1%	1%
αGlcNAc 1-*P*	40 mg	87%	70%	2%	10%	1%	0.3%
αGal 1-*P**	6 mg	85%	32%	3%	<1%	<1%	12%

[a] product purity % = mass of product / total mass

[b] purification yield % = mass product after purification / mass product in reaction mixture

[c] relative mass of impurities % = mass of impurity / total mass

* Smaller scale product purification that was not optimized for yield.

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