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A Kinase-Independent One-Pot Multienzyme Cascade for an Expedient Synthesis of Guanosine 5′-Diphospho-D-mannose

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Abstract: Biomimetic synthesis routes towards the important natural D-mannosyl donor guanosine 5′-diphospho-D-mannose (GDP-Man) rely on kinase-catalyzed nucleotide triphosphate (NTP)-dependent phosphorylations of D-mannose (Man), to give D-mannose 6-phosphate or α-D-mannose 1-phosphate (αMan 1-P) as an intermediate product. A GDP-Man synthesis not requiring the kinase/NTP system would be practical and cost-effective. Here, we have developed a multienzyme cascade towards GDP-Man, characterized in that αMan 1-P was obtained by a diastereoselective phosphatase-catalyzed phosphorylation of Man. α-D-Glucose 1-phosphate (αGlc 1-P), prepared *in situ* through phosphorylase-catalyzed conversion of sucrose in the presence of inorganic phosphate, was used as an expedient phosphoryl donor. The incipient αMan 1-P and guanosine triphosphate (GTP) were converted into GDP-Man by a highly *manno* compared to *gluco* selective nucleotidyltransferase. Pyrophosphatase was additionally required to hydrolyze the pyrophosphate released from the GTP, thus driving the reaction towards GDP-Man. The enzymatic cascade was operated with the αMan 1-P and the GDP-Man formation decoupled from one another (*sequential mode*) or having all steps run concurrently (*simultaneous mode*). Detailed time course analysis revealed that kinetic pull due to the constant removal of the intermediate αMan 1-P in simultaneous-mode reactions was important to promote phosphorylation of Man from αGlc 1-P in high efficiency, avoiding loss of sugar 1-phosphates by hydrolysis. Under optimized conditions for the one-pot transformation involving four enzymes, 100 mM (67 g L^{-1}) GDP-Man was prepared from 140 mM sucrose and phosphate, using 400 mM Man as the phosphoryl acceptor. The product was recovered by anion-exchange and size-exclusion chromatography in ≥95% purity in about 50% yield (100 mg). These results demonstrate for the first time the practical use of a phosphorylase-phosphatase combi-catalyst as an alternative to the canonical kinase for the anomeric phosphorylation of the sugar substrate in nucleoside diphospho-sugar synthesis. Phosphorylation from inorganic phosphate *via* the intermediate αGlc 1-P rather than from NTP, particularly GTP, appears advantageous specifically in cases where the sugar acceptor is a bulk commodity that can be applied in suitable excess to the phosphatase reaction.

Keywords: carbohydrates; guanosine 5′-diphospho-D-mannose (GDP-Man); nucleotide sugars; one-pot multienzyme cascade; phosphorylase-phosphatase combi-catalyst; phosphorylation

Introduction

Guanosine 5′-diphospho-D-mannose (GDP-Man), the nucleotide activated form of D-mannose (Man), has many important biological functions. It is the universal mannosyl donor for mannosyltransferases, a group of glycosyltransferases involved in diverse biosynthetic tasks, including protein glycosylation in eukaryotes and *O*-antigen formation in bacteria.[1] Furthermore, various natural GDP-activated sugars, including GDP-β-L-fucose, GDP-D-rhamnose and GDP-mycosamine, are derived from GDP-Man as the central intermediate.[2] In addition, GDP-Man is required for vitamin C biosynthesis in plants.[3]

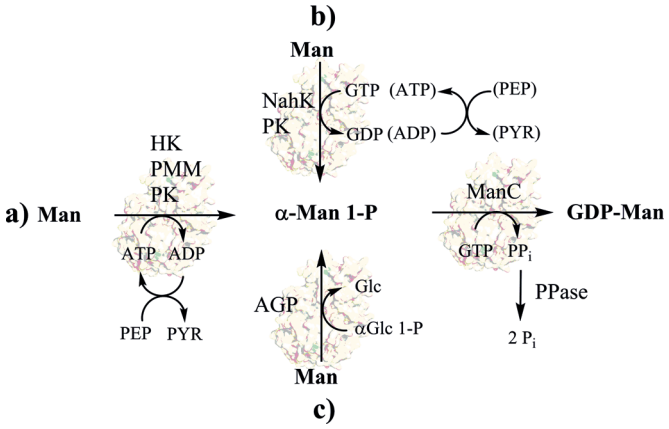
GDP-Man is naturally synthesized *via* two pathways. *De novo* biosynthesis starts with phosphorylation of D-glucose (Glc) by hexokinase (HK, EC 2.7.1.1). D-Glucose 6-phosphate is converted to α-D-

mannose 1-phosphate (αMan 1-P) in three steps, catalyzed by glucose 6-phosphate isomerase (EC 5.3.1.9), phosphomannose isomerase (EC 5.3.1.8) and phosphomannomutase (PMM, EC 5.4.2.8) *via* D-fructose 6-phosphate and D-mannose 6-phosphate. Finally, αMan 1-P is transformed to GDP-Man by a GDP-Man pyrophosphorylase (ManC, EC 2.7.7.13). GTP is the second substrate of the reaction and pyrophosphate (PP_i) is a co-product. An alternative route towards GDP-Man is the so-called salvage pathway.[4] It involves the direct conversion of Man into Man 6-P by a nucleotide triphosphate-dependent kinase, like HK. The remaining steps are the same as in the *de novo* biosynthesis.[4]

Promising chemical routes towards GDP-Man comprise either the direct phosphorylation of unprotected Man by phosphoric acid and tetrahydrofuran[5a] or the MacDonald reaction,[5b] in which the peracetylated Man is phosphorylated at the α-anomeric position. After deprotection the αMan 1-P is reacted with guanosine 5′-monophosphomorpholidate in pyrimidine using tetrazole as the catalyst. The main disadvantage of the method is that multiple reactions are performed in separate steps and the overall yield is therefore only moderate (phosphorylation 65%[5c] or 72%,[5a] condensation 76%; overall ~50% based on the Man used). Furthermore, the reaction times are long (1 day for phosphorylation and deprotection,[5c] 2 days for condensation).[5] Conditions for accelerated condensation were reported with other sugar 1-phosphates to form nucleotide sugars,[5i] but to our knowledge not with αMan 1-P.

To overcome these limitations, conversions with cell extracts[6] or biomimetic routes have been considered. Elling and co-workers[7] utilized an enzymatic synthesis according to the salvage pathway, as shown in Scheme 1a.

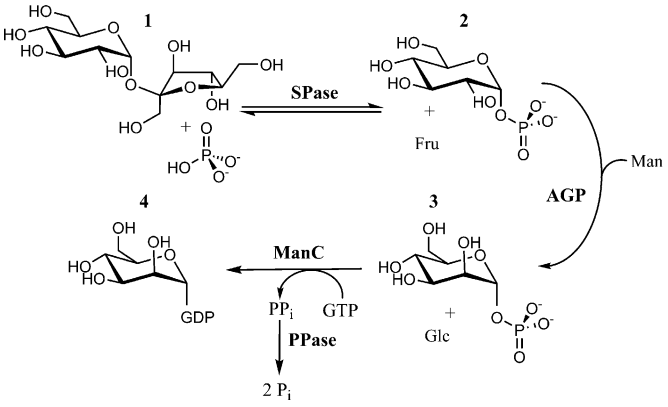
Typically, HK is about 30% as active with Man as compared to Glc.[8] ATP was regenerated using phosphoenol pyruvate (PEP) and pyruvate kinase (PK, EC 2.7.1.40) (Scheme 1a). Conversion of Man 6-P into GDP-Man involved the biosynthetic steps catalyzed by PMM and ManC (Scheme 1a). Pyrophosphatase (PPase, EC 3.6.1.1) was included to shift the equilibrium of the ManC reaction and also to remove end-product inhibition by PP_i. A final product concentration of 4 mM was obtained and the yield was 80% based on the GTP used. The GDP-Man synthesized within 72 h (581 mg) was isolated in 34.3% yield. Using a similar one-pot approach, however, applying instead of purified HK, PMM and ManC whole cells of *E. coli*, each expressing one of the three enzymes recombinantly, Honghong et al.[9] demonstrated formation of GDP-Man (21 mM; 680 mg) from Man (65 mM) and an energy source (D-fructose, 130 mM) within 22 h. The product was not isolated though.



Scheme 1. Routes of GDP-Man synthesis involve αMan 1-P as the central intermediate. Enzymatic methods for αMan 1-P synthesis (a–c) and its conversion into GDP-Man are shown. The abbreviations are: AGP (EC 3.1.3.10), α-D-glucose 1-phosphate phosphatase; PEP, phosphoenol pyruvate; PYR, pyruvate; other abbreviations can be found in the text.

The anomeric kinase NahK (EC 2.7.1.162) is involved in the metabolism of *N*-acetyl-D-glucosamine and *N*-acetyl-D-galactosamine.[10] Because NahK is also active with Man, although less so than on the natural substrates, it was exploited for αMan 1-P synthesis.[11] By using NahK, the step economy for conversion of Man into αMan 1-P was further improved.[12] Li et al.[12a] demonstrated the synthesis of GDP-Man (100 mg) in 94% yield based on the initial Man (15 mM) used, employing a one-pot three-enzyme cascade comprising NahK, ManC and PPase. Since GTP was applied as substrate for both NahK and ManC, the GDP-Man yield on GTP used was only 40%. Note that GTP is by far the most expensive substrate of the overall reaction. Although the more expedient ATP could be used as substrate of NahK in principle, its usage in a one-pot reaction is prohibited by the specificity of ManC which, besides GTP, also utilizes ATP for nucleotidyl transfer to αMan 1-P. Clearly, the resulting ADP-Man is an unwanted side product.[13]

Therefore, a synthetic route able to combine the selectivity of NahK regarding the anomeric phosphorylation of Man with the use of an inexpensive phosphoryl donor would be of high interest for a practical and also cost-effective GDP-Man production. We show here that a phosphatase-catalyzed selective transphosphorylation, in which αMan 1-P is formed from Man and α-glucose 1-phosphate (αGlc 1-P), represents a possible solution (Scheme 1c). GDP-Man is thus prepared in an alternative one-pot reaction involving four enzymes without a kinase (Scheme 2). The phosphorylase-phosphatase cascade required to generate the intermediate αMan 1-P was reported by us in a recent publication.[14] The current study advan-



Scheme 2. Enzymatic cascade for one-pot synthesis of GDP-Man. **1** sucrose, **2** αGlc 1-P, **3** αMan 1-P, **4** GDP-Man; Fru, D-fructose; SPase (EC 2.4.1.7), sucrose phosphorylase (from *Leuconostoc mesenteroides*); AGP (from *Escherichia coli*); ManC (from *E. coli*); PPase (from *E. coli*).

ces the synthetic application of this cascade by demonstrating for the first time its efficient integration with a nucleotidyltransferase/PPase system. We show intensification of GDP-Man synthesis to a final product concentration of 70 mM at an optimized (>99%) utilization of GTP in the overall reaction. A comparative evaluation of the pros and cons of anomeric sugar phosphorylation by phosphorylase-phosphatase and nucleotide triphosphate-dependent kinase systems reveals their complementary use for GDP-Man synthesis. Multistep enzymatic transformations performed in one pot are powerful tools of system biocatalysis for nucleotide-activated sugar synthesis.[7b–d,12b] They are topical in biocatalytic synthesis,[15a–c] including carbohydrates in particular.[12b,15d]

Results and Discussion

The enzymatic cascade applied to the synthesis of GDP-Man is shown in Scheme 2. All enzymes were recombinantly expressed in *E. coli* and used as purified protein preparations. Generally, the enzymes were expressed reasonably well, with purified yields ranging from 10 to 50 mg L^{-1}. The Supporting Information summarizes the results of enzyme production and purification, and it also provides some basic biochemical properties of the enzymes (Figure S1, Table S3). Units (U) of enzyme activity refer to standard assays described in the Supporting Information. Despite being used in a one-pot reaction, the enzymatic cascade can be operated in one of two possible modes. The sequential mode involves synthesis of the intermediate αMan 1-P decoupled from the actual formation of GDP-Man. Alternatively, all individual reactions are made to run simultaneously. Each mode was evaluated in detail for GDP-Man production.

Enzymatic Cascade Operated in a Sequential Mode

This approach was used first because it offered the possibility to analyze separately the two critical transformations of the overall reaction, Man→αMan 1-P and αMan 1-P→GDP-Man. It was determined that all enzymes are active and stable at 37 °C and pH 7.0 (Table S3, Supporting Information), so these conditions were used without further optimization. It was shown before by Wildberger et al.[14] that transphosphorylation from αGlc 1-P, coupled to *in situ* produc-

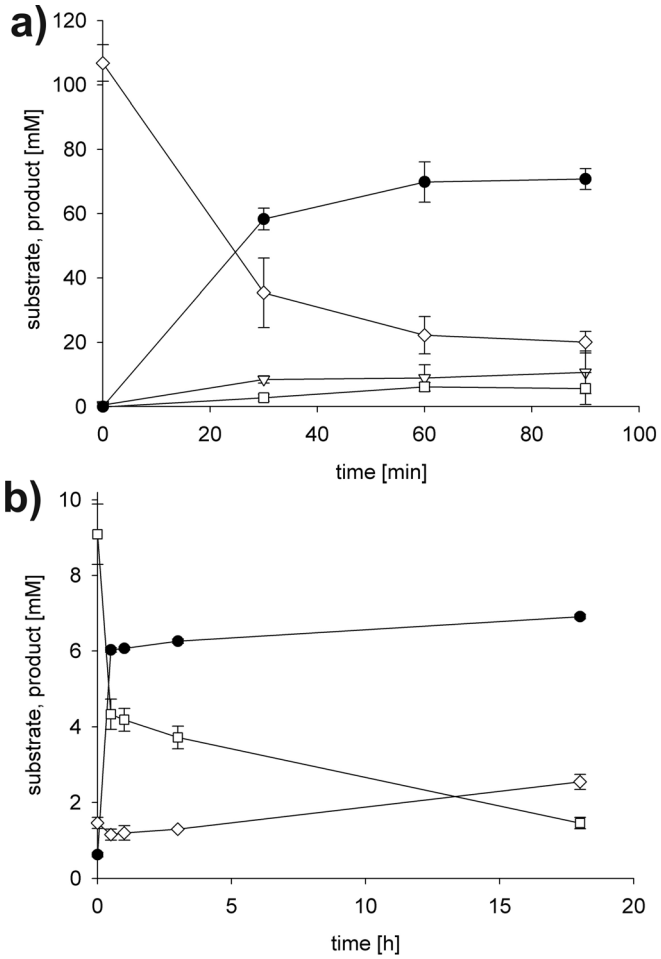


Figure 1. GDP-Man synthesis by the enzymatic cascade operated in a sequential mode. a) Synthesis of αMan 1-P (●) from sucrose (100 mM), phosphate (100 mM; ◇) and Man (400 mM) using SPase (4.5 U mL^{-1}) and AGP (0.3 U mL^{-1}). The intermediate αGlc 1-P (□) is also shown. Besides αMan 1-P, small amounts of Glc 6-P (▽) are formed as side product of the transphosphorylation. b) Conversion of αMan 1-P into GDP-Man, showing also the consumption of GTP (□) and the formation of GDP (◇). Note: the decrease in the αMan 1-P concentration from synthesis a) to conversion into GDP-Man b) is due to dilution by adding ManC (1.7 U mL^{-1}), PPase (3 U mL^{-1}) and GTP (10 mM). (Experiments were performed in triplicates and standard deviations are shown with error bars.).

tion of αGlc 1-P, was best performed when the SPase activity exceeded the AGP activity by about 15-fold. It was furthermore known that use of a Man concentration exceeding that of sucrose and phosphate by about 4-fold was useful to prevent loss of the intermediate αGlc 1-P to the competing enzymatic hydrolysis, also catalyzed by AGP. Figure 1 shows typical time courses of the αMan 1-P and the GDP-Man forming partial reactions of the overall cascade. Synthesis of αMan 1-P proceeded fast and plateaued after about 90 min, giving a final product concentration of 70 mM equivalent to a 70% yield based on the initial phosphate concentration used.

The yield based on the Man used was 18%. αGlc 1-P was present in negligible amounts during the reaction (Figure 1a), indicating that its utilization by AGP was not rate-limiting overall. Glc 6-P was initially (30 min) formed in low amounts but increased gradually to about 15 mM at the end of the reaction. This result is due to the phosphoryl transfer from αGlc 1-P to the Glc released from αGlc 1-P by hydrolysis. Note that therefore the time of stopping the reaction is important. Had we waited longer than 90 min, the relative abundance of Glc 6-P in the product would have increased at the expense of αMan 1-P present (data not shown). A close mass balance for the phosphate in the reaction was obtained.

In the second part of the overall conversion (Figure 1b), GDP-Man was synthesized with a 75% conversion based on the αMan 1-P and GTP present. Most of the product was formed in the first hour of reaction, with only a slight increase in GDP-Man concentration during a prolonged incubation up to 18 h. Importantly, the GTP concentration decreased even after the GDP-Man formation had come to a halt. GTP consumption was partly reflected by an increase in GDP, suggesting it to be caused by hydrolysis of the nucleoside triphosphates, probably catalyzed by AGP. We therefore assayed AGP with GTP as substrate and determined the enzyme to have a low specific activity of 0.10 ± 0.01 U mg^{-1} for GTP conversion. This was considered to be not prohibitive for using the enzyme in simultaneous mode.

Enzymatic Cascade Operated in a Simultaneous Mode

Conditions for the simultaneous-mode reaction were largely based on the evidence from Figure 1. The SPase/AGP activity ratio of 15 was maintained, but the volumetric AGP activity was decreased to 0.03 U mL^{-1} for it to become rate-limiting for the overall conversion. To ensure fast utilization of the *in situ* formed αMan 1-P, ManC activity was used in 40-fold excess over AGP activity. GTP was present in a limiting concentration (12 mM). Sucrose and phosphate were applied in 1.7-fold molar excess over GTP. Results of the one-pot reaction are summarized in Figure 2a. GTP was rapidly consumed and converted quantitatively into GDP-Man. We note the actual GDP-Man production rate (0.13 mM min^{-1}) to have been higher (4-fold) than expected from the limiting AGP activity present. The effect is explained by enhanced turnover of AGP under conditions in which an external acceptor (here: Man) replaces water during dephosphorylation of the enzyme in the catalytic cycle. Figure 2a also shows that the product was stable over 20 h of incubation. The commercial preparation of GTP contains GDP (~12%). No additional GDP was formed in the reaction (Figure 2a). The

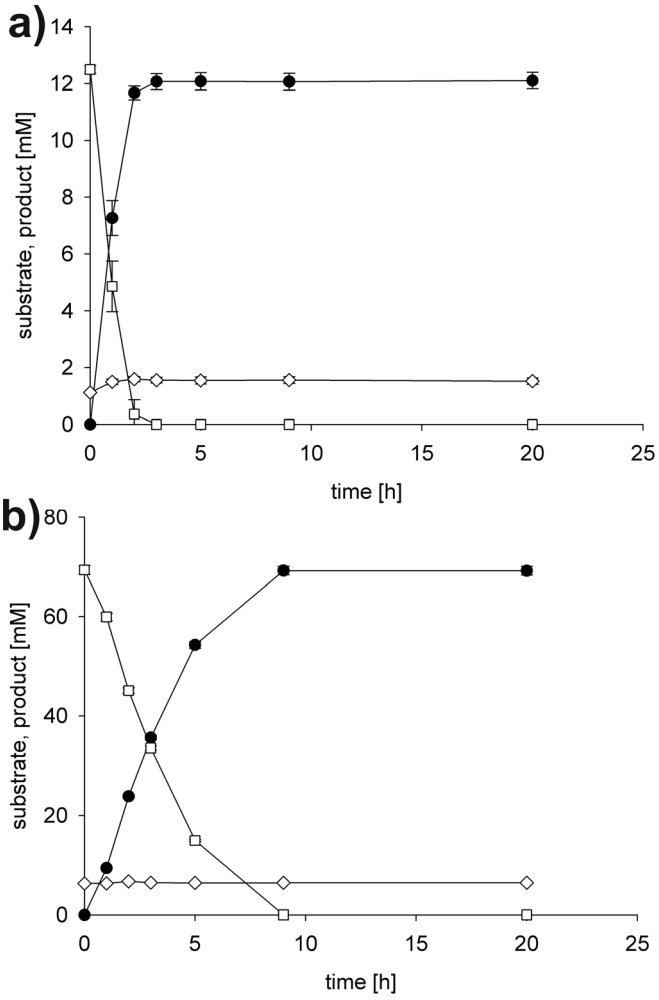


Figure 2. GDP-Man synthesis by the enzymatic cascade operated in a simultaneous mode using GTP as the limiting substrate concentration, 12 mM in a) and 70 mM in b). Reaction mixtures contained AGP (0.03 U mL^{-1}), SPase (0.45 U mL^{-1}), ManC (1.25 U mL^{-1}) and PPase (6 U mL^{-1}). The concentrations of sucrose and phosphate were 1.7 times that of the GTP used. Man was present at 400 mM. Symbols: (●) GDP-Man, (□) GTP, (◇) GDP. (Experiments were performed in triplicates and standard deviations are shown with error bars.).

small GTP hydrolase activity of AGP did not, therefore, impair the synthesis.

Preliminary NMR analysis of the reaction product showed that GDP-Man was formed in high purity. In particular, no GDP-glucose was found within limits of detection. Product specificity of the enzymatic cascade can be explained by the high αMan 1-P compared to αGlc 1-P substrate preference of ManC used.[16] Based on catalytic efficiency (k_{cat}/K_m), we determined ManC to be 20 times more specific for utilization of αMan 1-P (Table S4, Supporting Information). In addition, the excellent specificity of AGP for utilization of αGlc 1-P ($k_{cat}/K_m = 1360\ mM^{-1}\,s^{-1}$)[17] ensures that the αGlc 1-P concentration is kept low (≤ 0.01 mM) during the reaction.

These results reveal clear advantages of running the enzymatic cascade in a simultaneous mode. The requirement of meticulous reaction control during the synthesis of αMan 1-P is eliminated. Formation of side products of the phosphorylase-phosphatase reaction is suppressed, presumably because αGlc 1-P does not accumulate. The yield on the GTP used is higher. The reaction set-up is simpler. However, utilization of Man was a problem that needed special attention. In Figure 2a, the GDP-Man yield on the Man added was only 2.5%. The product titers of 12 mM (8 $g\,L^{-1}$) were also relatively low. Clear targets for enhanced performance of the enzymatic cascade were thus identified and reactions were therefore performed at elevated substrate concentrations of up to 100 mM GTP.

Biocatalytic Process Intensification

Using the base conditions of Figure 2a regarding the enzyme activities used, conversions were done at 30, 50, 70, 80 and 100 mM GTP. Sucrose and phosphate were employed in 1.7-fold excess over GTP in each case. Figure 2b shows a typical time course from these experiments.

GTP was completely utilized and GDP-Man was produced from it in >99% yield. The reaction proceeded at an almost constant rate up to 75% conversion, only to slow down slightly afterwards to reach the full conversion (Figure 2b). Typical volumetric productivities at ≥90% GTP conversion were about 9 $mM\,h^{-1}$. A final GDP-Man concentration of up to ~100 mM was obtained. This exceeds by a factor of about 7 the highest product concentration so far reported from the kinase-dependent synthesis.[12a]

The GDP-Man yield on the Man used in the reaction was 40% or lower, depending on the initial GTP concentration. While this still leaves room for improvement, the yield is clearly acceptable for preparative synthesis of GDP-Man. The reagent price of GTP exceeds that of Man by three orders of magnitude. A synthesis that maximizes the GDP-Man yield on the GTP used therefore strongly improves the cost effectiveness of the process. Minimizing the GTP demand at the expense of the amount of Man used appears to be a useful strategy. *In situ* regeneration of the GTP used in substoichiometric amounts in kinase-dependent cascade transformations provides an alternative option for decreasing the GTP input to the reaction.[7,18] However, reaction complexity must be kept in mind and the sacrificial substrates applied to nucleotide triphosphate regeneration (e.g., PEP; Scheme 1a)[7,11a,18] are also relatively expensive when compared to sucrose and P_i.

Preparative Synthesis of GDP-Man

Using 70 mM GTP under the conditions of Figure 2b, GDP-Man was synthesized in a total volume of 4 mL. The product solution (70 mM; 50 $g\,L^{-1}$; 180 mg) was purified by a combination of anion-exchange (AEX) and size exclusion chromatography (SEC) (Figure 3). GDP-Man (90 mg; sodium salt) was obtained as

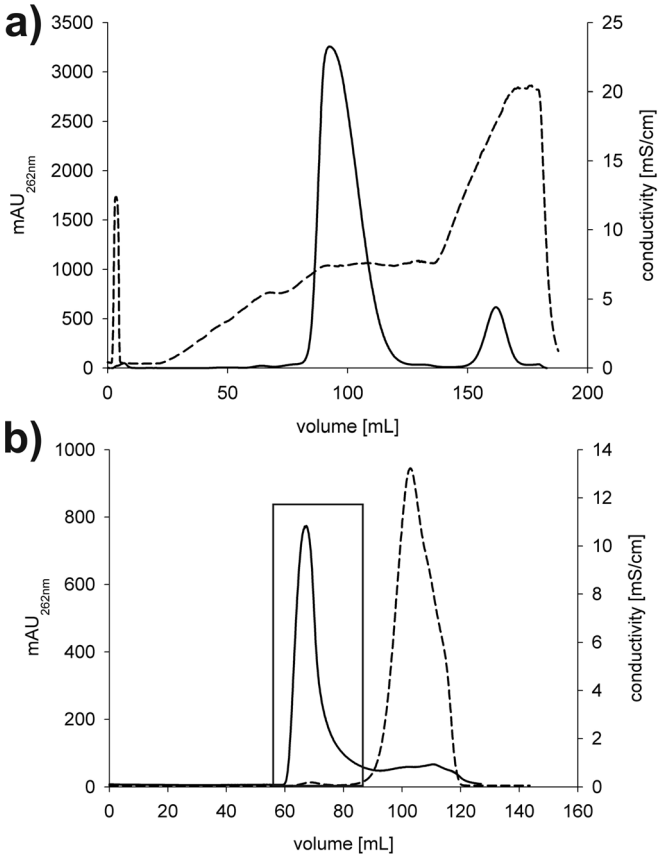


Figure 3. Purification of GDP-Man by AEX a) and SEC b) is shown. For details of the protocols used, see the Experimental Section. The solid line shows the absorbance trace, the dashed line is the conductivity trace due NaOAc in a) and NaOAc in b). The boxed region in panel b) shows elution of pure GDP-Man.

a white powder (Figure S2, Supporting Information) after lyophilization. The purity was at least 95% (HPLC, capillary electrophoresis, NMR), and the overall yield was 50% (90 mg). Losses of product occur mainly during the size-exclusion step. ^{1}H and ^{13}C NMR analysis of the product confirmed its identity (Figures S3 and S4, Supporting Information).

Conclusions

We show in this study of GDP-Man synthesis that diastereoselective transphosphorylation from αGlc 1-P to Man presents an interesting and cost-effective alternative to direct nucleotide triphosphate-dependent phosphorylation of Man to generate the central αMan 1-P intermediate of the overall biocatalytic cascade reaction. We further show that the four-enzyme system comprising SPase, AGP, ManC and PPase is operated most effectively by running all enzymatic steps simultaneously in a one-pot conversion, giving GDP-Man in the highest so far reported product titers (100 mM). The yield of GDP-Man on the GTP used was maximized to its theoretical limit (>99%). The utilization of Man, by contrast, was not as efficient (≤40% yield), thus suggesting complementary synthetic uses for an enzymatic cascade involving the anomeric kinase or the combination of SPase and AGP. The kinase cascade, possibly expanded by an enzyme module for nucleotide triphosphate regeneration,[7,11a,18] is clearly the system of choice when effective utilization of the sugar acceptor is required. Using inexpensive bulk sugars like Man, however, the phosphorylase-phosphatase cascade offers the interesting advantage that the input of GTP can be minimized. Since AGP presents a broad range of aldohexose substrates phosphorylated at the anomeric position,[14] the phosphorylase-phosphatase cascade described here might be applicable, with a case-specific adaptation of the nucleotidyltransferase, to the synthesis of nucleotide-activated sugars other than GDP-Man. Emerging high throughput-amenable techniques of reaction analysis are expected to facilitate the development and optimization of the multi-enzyme catalyzed transformations.[19]

Experimental Section

Enzymatic Cascade Reaction in Sequential and Simultaneous Mode of Operation

Reactions were performed at 37 °C on a Thermomixer Comfort (Eppendorf, Hamburg, Germany) in 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 7.0) and agitation at 650 rpm. Sequential reactions involved conversion of sucrose and P_i (100 mM each) by SPase (4.5 U mL^{-1}) and phosphoryl transfer from αGlc 1-P to Man (400 mM) by AGP (0.3 U mL^{-1}) in a total volume of 400 μL. Samples (taken every 30 min, 30 μL) were quenched by heat treatment at 99 °C for 5 min and analyzed for Glc 6-P, αGlc 1-P and P_i (see the Supporting Information for assays used). After 90 min the reaction was stopped by heat inactivation (99 °C, 5 min) and 10 mM GTP, 5 mM $MgCl_2$, PPase (3 U mL^{-1}), ManC (1.7 U mL^{-1}) and 100 mM MES buffer (pH 7.0) supplemented with 10 mM $MgCl_2$ were added to reach a final volume of 2 mL and 10 mM of αMan 1-P. Samples taken (0, 1, 3, 6 and 18 h; 10 μL each) were analyzed by HPLC and CE.

Reaction mixtures for conversions in simultaneous mode contained 100 mM MES, 10 mM $MgCl_2$, GTP (12, 30, 50, 70, 80 and 100 mM), sucrose and P_i in a 1.7-fold excess over GTP and 400 mM Man. Reactions were started by simultaneously adding SPase (0.45 U mL^{-1}), AGP (0.03 U mL^{-1}), ManC (1.25 U mL^{-1}) and PPase (6 U mL^{-1}). Samples taken (0, 1, 2, 3, 5, 9 and 20 h; 10 μL each) were quenched by acetonitrile addition (1:10, v/v) and analyzed by HPLC and CE.

Preparative Synthesis of GDP-Man

This was performed in a total volume of 4 mL applying the same conditions as used in simultaneous reactions. The GTP concentration was 70 mM. The reaction was run for 20 h and stopped by heat inactivation (95 °C for 5 min). Precipitated protein was removed by centrifugation (4 °C, 20,000 *g*, 15 min). To isolate GDP-Man from the reaction, AEX was used. Per run 1 mL of the cleared reaction mixture was applied to a FliQ FPLC column (3×1 mL; 33×6.2 mm; Generon, Maidenhead, U.K.) packed with SuperQ-650 M (Tosh, Tokyo, Japan) anion-exchange resin. The column was mounted onto an ÄKTA prime plus FPLC system (GE Healthcare, Little Chalfont, U.K.) and operated with a flow rate of 0.5 mL min^{-1}. The column was pre-equilibrated with buffer A [20 mM sodium acetate (NaOAc), pH 4.6]. Elution was done by applying a continuous gradient from 0% to 30% of buffer B (1 M NaOAc, pH 4.6) in a volume of 30 mL. After complete elution of GDP-Man at 30% of buffer B, a further step to 100% of buffer B was used to elute the bound GDP. Elution was monitored by absorbance at 262 nm. Fractions containing GDP-Man were pooled and concentrated to a final volume of 2 mL under reduced pressure using a rotavapor (Heidolph Laborota 4001, Schwabach, Germany) at 40 °C. To remove excess NaOAc the pooled and concentrated fractions were split into 4×500 μL portions and applied to SEC using a XR 16/100 column (16×1000 mm; GE Healthcare, Little Chalfont, U.K.) packed with Sephadex G10 material (exclusion limit <700 Da) equilibrated in H_2O and mounted to an ÄKTA prime plus FPLC system (GE Healthcare, Little Chalfont, U.K.). Compound elution was performed using H_2O. It was monitored by absorbance at 262 nm and by measuring the conductivity. Fractions containing salt free GDP-Man were pooled and stored at 4 °C. To maximize the yield fractions that contained GDP-Man and NaOAc were pooled, concentrated and reapplied to SEC. Pooled fractions containing pure GDP-Man were lyophilized and washed with EtOH. Excess EtOH was removed after centrifugation at 4 °C and 20,000 *g* for 10 min. The remaining precipitate was lyophilized, resulting in a dry white powder. The isolated yield

thus achieved was 50% (90 mg GDP-Man) with a purity of 95%.

High Pressure Liquid Chromatography (HPLC)

Samples were analyzed on an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, USA) equipped with a 50×4.6 mm Kinetex C18 reversed Phase HPLC column (Phenomenex, Torrance, USA) and a UV detector (λ = 252 nm). The analytical method comprised an injection volume of 1–5 µL, an isocratic flow of 87.5% 20 mM potassium phosphate buffer supplemented with 40 mM tetrabutylammonium bromide, at pH 5.9 and 12.5% acetonitrile at a flow rate of 2 mL min^{-1} for 4 min. GTP [retention time (RT): 3.36 min], GDP (RT: 1.01 min), GDP sugars (RT: 0.62 min), GMP (RT: 0.45 min) were analyzed with this method and referenced against authentic standards.

Nuclear Magnetic Resonance (NMR) Spectroscopy

Ten mg of purified GDP-Man were dissolved in 500 µL of D_2O and analyzed using a Varian (Agilent) INOVA 500-MHz NMR spectrometer (Agilent Technologies, Santa Clara, USA). The VNMRJ 2.2D software was used for measurements. ^{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). 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. HSQC spectra were measured with 128 scans per increment and adiabatic carbon 180° pulses. ACD/NMR Processor Academic Edition 12.0 (Advanced Chemistry Development Inc., Toronto, Canada) was used for evaluation of spectra.

For more experimental details, see the Supporting Information.

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