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Carbohydrate Research 305 (1997) 475–481

CARBOHYDRATE RESEARCH

The cofactor Mg^{2+} — a key switch for effective continuous enzymatic production of GDP-mannose using recombinant GDP-mannose pyrophosphorylase

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Received 9 June 1997; accepted 2 October 1997

Abstract

The aim of the presented work is the chemoenzymatic synthesis of GDP-mannose (GDP-Man). Mannose-1-phosphate (Man-1-P) is synthesised by a very convenient chemical method and is activated with GTP and recombinant GDP-mannose pyrophosphorylase (GDP-Man PP) to give GDP-Man. The productivity of the process is improved using reaction engineering techniques. Detailed kinetic studies, modelling of the reaction and simulations of different reaction systems revealed that besides a strong product inhibition the cofactor Mg^{2+} is a key switch for effective enzymatic synthesis. Using a two-stage cascade of enzyme membrane reactors, GDP-Man could be produced continuously with a space-time yield of $28\,g\,L^{-1}\,d^{-1}$ and an enzyme consumption of $0.9\,U\,g^{-1}$, which means a six-fold improvement related to batch synthesis.

Keywords: GDP-mannose; Sugar nucleotides; Sugar phosphates; Chemo-enzymatic synthesis; Reaction engineering; Enzyme membrane reactor

1. Introduction

GDP-mannose (GDP-Man), which was isolated for the first time in 1954 from yeast [1], is one of eight sugar nucleotides involved in the biosynthesis of carbohydrate chains of glycoconjugates in mammalian cells [2,3]. They act as substrates for glycosyltransferases which are used for oligosaccharide synthesis *in vivo* and *in vitro* [4–7]. Many efforts have been made to improve the availability of glycosyltransferases by over-expression of the corresponding genes [8]. But a technological application of oligosaccharide synthesis using glycosyltransferases is still lacking due to the non-availability of large amounts of the sugar nucleotides. Here we report the chemoenzymatic production of GDP-Man, which has been used for

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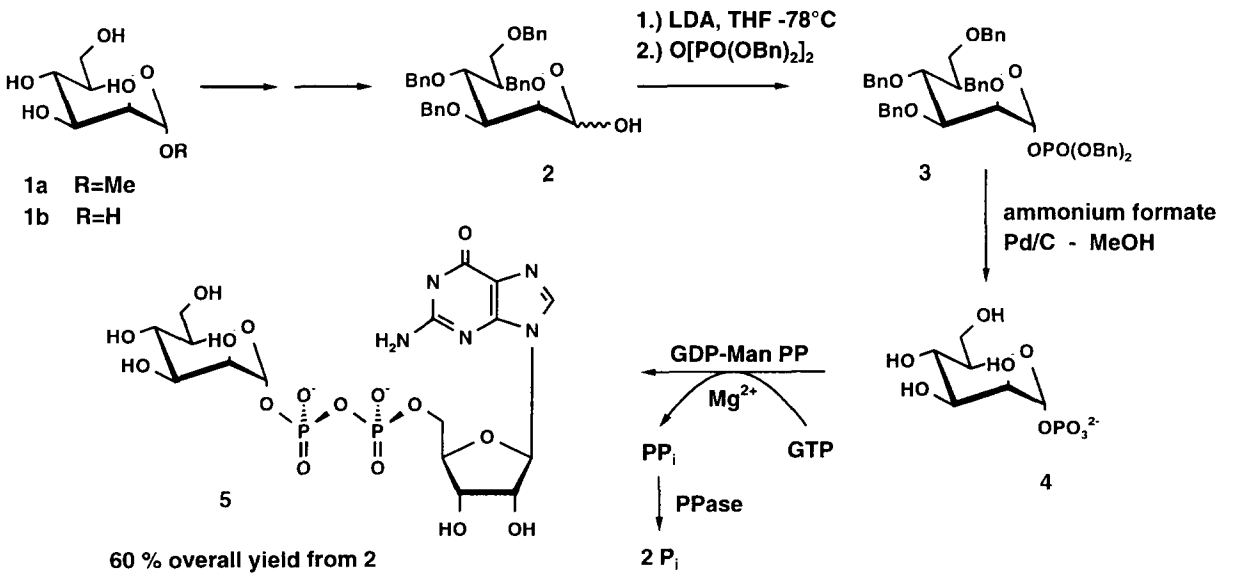
0008-6215/98/$19.00
PII S0008-6215(97)10095-7

in vitro synthesis of mannose-containing oligosaccharides [9–12], and which is the starting material for the enzymatic synthesis of GDP-fucose using the long biosynthesis pathway [13,14]. GDP-Man or some of its derivatives have been synthesised chemically by the phosphomorpholidate coupling method [15]. Lately, a variant of this method using 1*H*-tetrazole as a catalyst to reduce the reaction time has been described [16]. For enzymatic synthesis a yeast extract containing a GDP-mannose pyrophosphorylase (GDP-Man PP) activity was used [17–20]. All these syntheses with their generally poor yields or long reaction times are difficult to scale up to an effective multigram scale production. Recently, we reported an improved synthesis of GDP-Man (**5**) in a 4-enzyme system using two purified recombinant enzymes (phosphomannomutase, EC 5.4.2.8 and GDP-Man PP, EC 2.7.7.13) starting from D-mannose [21]. Further studies have revealed that a continuous production in an enzyme membrane reactor seems to be the most effective way to produce GDP-Man, though only a few examples of continuous enzymatic production of carbohydrates in enzyme membrane reactors have been reported in the literature [22–30].

The employed strategy shown in Scheme 1 combines the chemical synthesis of mannose-1-phosphate (Man-1-P) (**4**) and an enzymatic step for the activation catalysed by the GDP-Man PP. This strategy makes it possible to improve the productivity of GDP-Man synthesis using reaction engineering techniques considering kinetic requirements in a very simple two-enzyme system. Improvements of methods for Man-1-P synthesis [19,31–34] make this route to be a good alternative for large scale synthesis of GDP-Man when compared to the complete enzymatic pathway starting with D-mannose.

2. Results and discussion

2,3,4,6-Tetra-*O*-benzyl-D-mannopyranose (**2**) is easily available from methyl α-D-mannopyranoside (**1a**) [35] or D-mannose (**1b**) [36]. For phosphorylation we chose a very efficient method using tetrabenzyl pyrophosphate [37] with an excellent yield of 95% for the fully protected Man-1-P (**3**). The cleavage of the protecting groups by catalytic hydrogenation appeared to be difficult as previously mentioned by Pallanca et al. [19]. Many attempts employing standard procedures with hydrogen gas and Pd/C in various solvents or with Pt metal as catalyst have failed, leading either to very low conversion or to decomposition of the product. We found that a very convenient method using ammonium formate as hydrogen donor [38] and Pd/C was successful. The main advantage of this method is the simple workup comprising of filtration of the catalyst, removal of the solvent and drying. This crude product can be used for the enzymatic activation with GTP and GDP-Man PP to give GDP-Man (**5**) in a three-step overall yield of 60% starting from **2**.



Scheme 1. Chemoenzymatic synthesis of GDP-Man. GTP, guanosine-5′-triphosphate; PP_i, pyrophosphate; P_i, phosphate; GDP-Man PP, GDP-mannose pyrophosphorylase (EC 2.7.7.13); PPase, inorganic pyrophosphatase (EC 3.6.1.1).

GDP-Man PP was obtained by fermentation of *Escherichia coli* BL21(DE3)pLysS strain on a 20 L scale. A specific activity of 350 U L^{-1} was attained. The purification of the catalyst to achieve homogeneity has been described [21]. However, for synthetic purposes a two step purification including ultrafiltration (UF-membrane, cut off 10 kDa) and anion-exchange chromatography (Q-Sepharose) is sufficient to remove hydrolysing activities fully. The enzyme can be stored as a suspension in $(NH_4)_2SO_4$ at 4 °C for months without any significant loss of activity. Before use, the $(NH_4)_2SO_4$ should be washed out by ultrafiltration due to its strong inhibitory effect on the enzyme ($K_i = 0.85\,mM \pm 0.08\,mM$).

For a detailed kinetic study of GDP-Man PP as basis for modelling and simulation of the system an HPLC assay was used in this study. The Nucleotidyltransferase Substrate Screening Assay (NUSSA) [39] we used for screening of pyrophosphorylases gave slightly different results for the determination of the kinetic parameters of GDP-Man PP before [21]. This might be due to unknown interactions of substrates, cofactors and enzymes in this multienzyme assay. The reinvestigation leads to the kinetic parameters shown in Table 1. Eq. 1 shows the kinetic model that was used for calculation of the kinetic parameters and simulation of different reaction systems. All reactions were carried out in the presence of inorganic pyrophosphatase (EC 3.6.1.1), so that the reverse reaction has not to be considered. A very strong product inhibition by GDP-Man as well as pyrophosphate (PP_i) is observed. But the central coherence is given by the cofactor Mg^{2+}. The activity of the enzyme does not depend directly on the concentration of Mg^{2+} as usually observed for cofactor dependent enzymes but on the ratio of the concentration of Mg^{2+} and the substrate GTP. A maximum in enzyme activity appears at a ratio of about 1. Initial reaction velocities with varying Mg^{2+} concentrations at three different GTP concentrations were determined. Fig. 1 shows the activity as function of the Mg^{2+}/GTP ratio due to complex formation. The complex of Mg^{2+} and GTP is very strong, so all Mg^{2+}, which is not bound to the enzyme, should be complexated by GTP if enough GTP is present. A surplus of Mg^{2+} may lead to a non-constructive binding to a further Mg^{2+}-binding site of the enzyme, and in the consequence its activity is lowered. To consolidate this hypothesis the following experiment was made: During the progress of a batch synthesis the GTP concentration will decrease and the ratio of Mg^{2+}/GTP will increase. The addition of an exact amount of a strong chelating agent such as EDTA should decrease the free Mg^{2+} concentration and shift the Mg^{2+}/GTP ratio to its optimal value. Therefore the reaction velocity should increase after addition of EDTA. Fig. 2 shows the experimental results, which are in accordance with the above hypothesis. Nevertheless, it should be emphasised that all considerations concerning the mechanism of the enzyme reaction are strictly hypothetical.

$$v = v_{max} \times \frac{\left(\frac{c_{Mg}}{c_{GTP}}\right)^2}{K_M^{Mg} + \left(\frac{c_{Mg}}{c_{GTP}}\right)^2 + \frac{\left(\frac{c_{Mg}}{c_{GTP}}\right)^3}{K_I^{Mg}}} \times \frac{c_{Man\text{-}1\text{-}P}}{K_M^{Man\text{-}1\text{-}P} + c_{Man\text{-}1\text{-}P}} \times \frac{c_{GTP}}{K_M^{GTP} + c_{GTP} + \frac{K_M^{GTP} c_{GDP\text{-}Man}}{K_I^{GDP\text{-}Man}} + \frac{K_M^{GTP} c_{PP}}{K_I^{PP}}}$$

Equation 1. Kinetic model of the GDP-Man PP reaction used for simulations. The kinetic parameters are given in Table 1. The term containing the Mg^{2+}/GTP ratio is accorded to a theoretic study of Yano et al. [45] for a substrate surplus inhibition during microbial growth. The model is valid for Mg^{2+}/GTP ratios between 0–2.5. For larger ratios a constant activity of 10 U/mL was found.

Table 1
Values of kinetic parameters of the GDP-Man PP reaction determined using the model of eq 1. The value of v_{max} was determined to be 200 U $mL^{-1} \pm 165$ U mL^{-1}

	K_m	K_i
Man-1-P	$15\,\mu M \pm 3\,\mu M$	—
GTP	$40\,\mu M \pm 9\,\mu M$	—
GDP-Man	—	$9\,\mu M \pm 2\,\mu M$
PPi	—	$16\,\mu M \pm 4\,\mu M$
Mg^{2+}	2 ± 2	0.1 ± 0.1

But regardless of the real mechanistic reasons the kinetic properties of the reaction system lead to important conclusions concerning the type of the reactor for an effective enzymatic production of GDP-Man. It is shown above that a constant high reaction velocity can only be reached at a constant ratio of Mg^{2+} and GTP. Therefore a synthesis in a batch reactor is not satisfactory for production because the Mg^{2+}/GTP ratio increases with conversion. Feeding of chelating agents is very difficult,

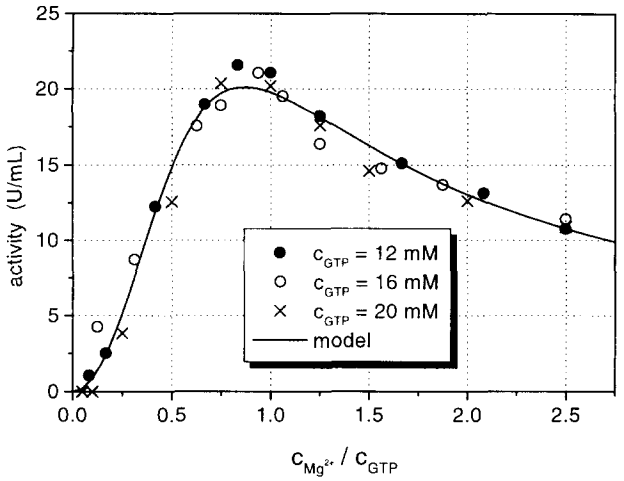


Fig. 1. Influence of the ratio Mg^{2+}/GTP on GDP-Man PP activity. The measurements were carried out by a HPLC standard assay procedure with the given GTP concentrations.

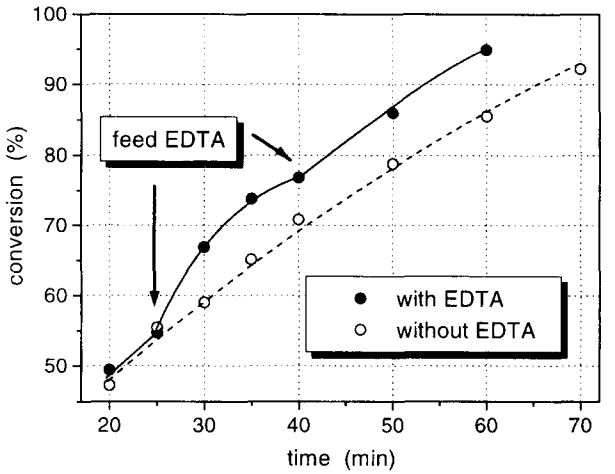


Fig. 2. Batch synthesis of GDP-Man. $c_{Man\text{-}1\text{-}P}$ = 5 mM, c_{GTP} = 10 mM, c_{Mg} = 10 mM, GDP-Man PP: 1 U/mL, PPase: 2 U/mL. Addition of EDTA: equiv. to 2 mM at 25 and 40 min.

because this would lead to a very complicated continuous feeding profile. An alternative, which is much easier to handle, is the use of a continuous stirred tank reactor (CSTR). In a steady state this reactor operates with a constant feed and discharge, so that all concentrations remain constant. This allows to adjust the optimal Mg^{2+} concentration in the feed leading to a constant Mg^{2+}/GTP ratio corresponding to the maximum in Fig. 1. To overcome its strong product inhibition PP_i is hydrolysed in situ by inorganic pyrophosphatase to inorganic phosphate (P_i) which is not inhibitory. The product inhibition by GDP-Man could be diminished by use of an excess GTP, because GDP-Man was identified as a competitive inhibitor. Furthermore the production was carried out in a two step CSTR cascade approximating the behaviour of a plug flow reactor, which is more efficient for reactions showing product inhibition. Simulation experiments show that the space-time yield (STY) for the continuous production of GDP-Man can be increased by 2-3 fold in a two step CSTR cascade compared with a single CSTR (data not shown). For enzymatic reactions, enzyme membrane reactors (EMR) which behave as CSTR are well established [40–42]. The enzyme as a macromolecule is held back by an ultrafiltration membrane, which can be passed by low-molecular-mass substrates and products. This leads to decoupling of the residence time of reactants and enzyme and consequently to a high utilisation of the enzyme and a simplified purification procedure. Fig. 3 shows the production unit containing two linearly connected EMR. GDP-Man was continuously produced on a gram-scale for a period of more than 50 h with a STY of 28 g L^{-1} d^{-1} (Fig. 4). The behaviour of this reactor and batch reactors as well (data not shown) can be described with good accuracy by the model. To attain a conversion of 60% in EMR 1 and 95% in EMR 2, the ratios Mg^{2+}/GTP were set up to 1.1 and 1.4, respectively. Ratios lower than 1 have to be strictly avoided to prevent the system from wash out. In contrast to unstable reaction conditions at ratios lower than 1, higher ratios cause a self stabilisation of the system. The cascade is a compromise between a loss of activity in one EMR due to a not optimal Mg^{2+}/GTP ratio and the overcoming of the strong product inhibition. But the large improvement of this technique is demonstrated by a comparison of enzyme consumptions[1] of the different reactors. The enzyme consumption in a two-step EMR cascade (0.9 U $g_{product}^{-1}$) is only 15% of that in a batch reactor (5.8 U $g_{product}^{-1}$) and 45% of that in a single EMR (2.0 U $g_{product}^{-1}$). These results clearly highlight the importance of the Mg^{2+}-switch together with an appropriate understanding of reaction engineering.

3. Experimental

General methods.—^1H, ^{13}C and ^{31}P NMR spectra were recorded with a Bruker AM-400 spectrometer (University of Bonn) at the frequencies indicated, employing standard pulse angles and

[1] Enzyme consumption = k_{des}·[E]/STY.

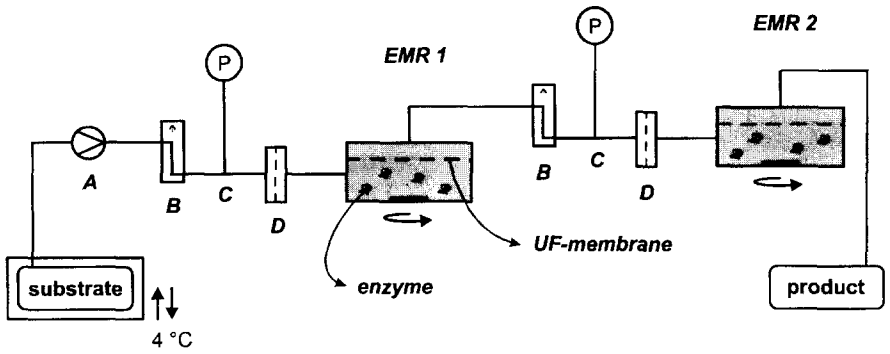


Fig. 3. Two-stage EMR cascade production unit for continuous production of GDP-Man. A: high precision piston pump; B: bubble trap; C: pressure gauge; D: sterile filter; EMR: enzyme membrane reactor.

DEPT experiments for correlation of ^{13}C data if necessary. Optical densities were measured with a UV 160 (Shimadzu, Duisburg, Germany) spectrophotometer. All chemical reactions were monitored by TLC on Silica Gel 60 F_{254} aluminium sheets (Merck, Darmstadt, Germany) with visualisation by an anisaldehyde–H_2SO_4 reagent [43]. GDP-Man synthesis and initial reaction velocity measurements were monitored by HPLC using a method according to a previously reported procedure [44]: A reversed phase Hypersil ODS-5 μm column (CS, Langerwehe, Germany) with a precolumn of the same material was used at a temperature of 40 °C with a MeOH gradient in 100 mM KH_2PO_4 (Merck, Darmstadt, Germany), 8 mM tetrabutylammonium hydrogensulfate (Serva, Heidelberg, Germany), pH 5.3. Tetrabenzyl pyrophosphate was obtained from Fluka (Neu Ulm, Germany) and IPTG from Roth (Karlsruhe, Germany). GTP

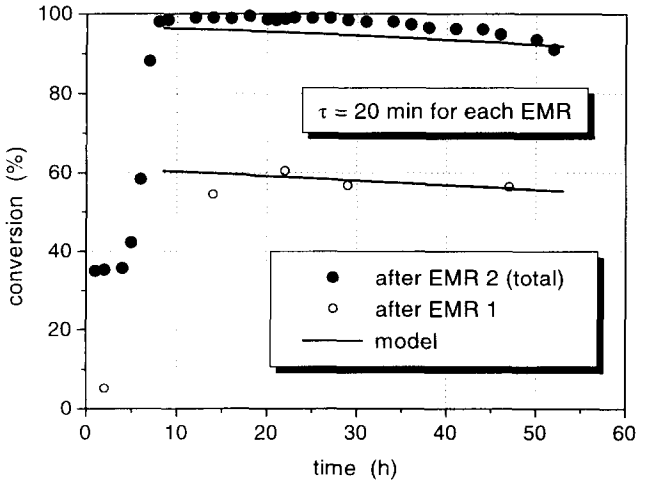


Fig. 4. Results of the continuous production of GDP-Man in a two-stage EMR cascade. $c_{Man\text{-}1\text{-}P} = 1.2$ mM, $c_{GTP} = 2.4$ mM, $c_{Mg} = 1.8$ mM, GDP-Man PP: 0.12 U/mL, PPase: 1 U/mL. Enzyme deactivation: $k_{des} = 0.0083\ h^{-1}$. The line shows the calculated conversion using the model (eq 1) and the parameters of Table 1.

(dilithium salt) and inorganic pyrophosphatase (yeast, EC 3.6.1.1) were purchased from Boehringer Mannheim (Germany). 2,3,4,6-Tetra-*O*-benzyl-D-mannopyranose was from Sigma. All other chemicals were from Sigma as well or Aldrich. Kinetic parameters were identified using a simplex algorithm provided by Scientist® 2.0 (MicroMath®).

Production of GDP-Man PP.—The fermentation of *E. coli* BL21(DE3)pLysS expressing the enzyme was carried out in a 30 L Chemap® Bioreactor at 37 °C; pH was adjusted to 7.3 (NaOH); 470 rpm; oxygen supply of 17 L min^{-1} (after 6 h: 30 L min^{-1}). The fermenter was inoculated with 1 L inoculum grown in five 1 L shaking flasks on 200 mL of LB-medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 5 g L^{-1} NaCl) also containing the antibiotics ampicilline and chloramphenicol. After induction with 0.4 mM IPTG at 8 h and growth for further 2.5 h, the cells were separated by a KA2 cell separator (Westfalia Separator AG, Oelde, Germany). A total of 218 g of wet cells were obtained, and these were suspended in a 20% suspension with Tris-HCl buffer (pH 8.0) and disintegrated in a ball mill (Netzsch, Selb, Germany) with glass beads ($d = 0.3$ mm). After centrifugation the crude extract (protein: 13.5 mg mL^{-1}; activity: 6.1 U mL^{-1}; specific activity: 0.46 U mg^{-1}) can be stored at −20 °C or purified as described [21] by ultrafiltration (membrane: YM 10 Amicon, Witten, Germany) and anion-exchange chromatography (Q-Sepharose FF; linear gradient of KCl, 100–500 mM).

Enzyme assay.—The determination of the initial reaction velocity was carried out in a total volume of 200 μL Tris-HCl buffer (pH 8) containing 1 mM Man-1-P, 1 mM GTP and 1 mM $MgCl_2$. The reaction was started by addition of the enzyme solution and was allowed to continue at 25 °C for 5 min. The solution was then heated for 5 min in a boiling waterbath to stop the reaction by enzyme

denaturation. After centrifugation of the precipitated protein the concentration of the product GDP-Man is determined by HPLC.

Preparation of dibenzyl 2,3,4,6-tetra-O-benzyl-α-D-mannopyranosyl phosphate (**3**).—To a solution of compound **2** (4.9 g, 9.1 mmol) in absolute THF (150 mL) was added 2 M LDA in 1:1:1 hexane-THF-ethylbenzene (6 mL) at −78 °C. The solution was stirred at that temperature for 1 h and tetrabenzylpyrophosphate (6.8 g, 12.7 mmol) in absolute THF was added slowly at −78 °C. After 3 h at −78 °C the solution was slowly brought to room temperature, and TLC (36:1 $CHCl_3$–MeOH; R_f 0.80) indicated complete conversion. A precipitate was filtered off and the solvents were removed under reduced pressure. The residue was purified by flash chromatography [Silica Gel 60, 230–400 mesh (Merck, Darmstadt, Germany), 36:1 $CHCl_3$–MeOH] and **3** was obtained as a colourless oil (6.9 g, 95%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.38–7.13 (m, 30 H, Ar-H), 5.78 (dd, $^3J_{1,P}$ 6.1, $J_{1,2}$ 2.0 Hz, 1 H, H-1), 5.02 (d, J_{gem} 8.5 Hz, 2 H, POBn-CH_2), 4.96 (d, J_{gem} 8.5 Hz, 2 H, POBn-CH_2), 4.86 (d, J_{gem} 10.6 Hz, 1 H, Bn-CH_2), 4.66 (s, 2 H, Bn-CH_2-6), 4.60 (d, J_{gem} 11.6 Hz, 1 H, Bn-CH_2), 4.54–4.42 (m, 4 H, Bn-CH_2), 4.04 (dd, $J_{4,3(5)}$ 10.6 Hz, 1 H, H-4), 3.89 (ddd, $J_{5,6a}$ 4.9, $J_{5,6b}$ 1.7 Hz, 1 H, H-5), 3.81 (dd, $J_{3,2}$ 3.7 Hz, 1 H, H-3), 3.72–3.68 (m, 2 H, H-2,6a), 3.56 (dd, $J_{6b,6a}$ 12.5 Hz, 1 H, H-6b); ^{13}C NMR (100 MHz, $CDCl_3$): δ 138.4–135.6 (Ar-C), 128.6–127.6 (Ar-CH), 96.3 (d, $^2J_{1,P}$ 5.8 Hz, C-1), 78.9 (C-5), 75.2 (Bn-CH_2), 74.4 (d, $^3J_{2,P}$ 8.7 Hz, C-2), 74.1 (C-4), 73.9 (C-3), 73.4, 72.7, and 72.2 (Bn-CH_2), 69.5 (d, $^2J_{C,P}$ 5.4 Hz, PO-Bn-CH_2), 69.4 (d, $^2J_{C,P}$ 5.4 Hz, PO-Bn-CH_2), 68.6 (C-6); ^{31}P{^{1}H} NMR (121.5 MHz, $CDCl_3$): δ −3.12.

Preparation of dicyclohexylammonium α-D-mannopyranosyl phosphate (**4**).—To a suspension of compound **3** (2.55 g, 3.18 mmol) and 10% Pd-C (3.5 g) in anhydrous MeOH (100 mL) [Caution: exreme fire hazard!] was added under Ar ammonium formate (5.76 g, 91 mmol, 4.8 equiv each benzyl group). The suspension was stirred over night, when TLC (6:4 CH_3CN–0.1 M aq NH_4Cl) indicated complete conversion. Cyclohexylamine (540 μL, 6.4 mmol) was added and the catalyst was filtered off. The filter cake was washed with MeOH (100 mL) and then with water (50 mL). The filtrates were collected separately. MeOH of the first filtrate was carefully removed under reduced pressure at room temperature, and the residue was dried under vacuum. The aqueous filtrate was lyophilised. The combined white solids can be used for enzymatic activation with GDP-Man PP without further purification. A NMR analysis of the residue resulting from the MeOH filtrate was consistent with published data [34]. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.43 (dd, $^3J_{1,P}$ 6.3, $J_{1,2}$ 1.8 Hz, 1 H, H-1), 4.00 (m, 1 H, H-2), 3.95 (dd, $J_{3,4}$ 9.5, $J_{3,2}$ 3.2 Hz, 1 H, H-3), 3.92–3.84 (m, 2 H, H-5,6b), 3.79 (dd, $J_{6a,6b}$ 11.6, $J_{6a,5}$ 5.3 Hz, 1 H, H-6a), 3.70 (dd, $J_{4,3(5)}$ 9.5 Hz, 1 H, H-4), 3.2 (m, 2 H, cyclohexyl H-1), 2.1–1.1 (m, 20 H, cyclohexyl H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 98.3 (d, $^2J_{1,P}$ 5.4 Hz, C-1), 76.1 (C-5), 73.3 (d, $^3J_{2,P}$ 8.4 Hz, C-2), 72.5 (C-3), 69.2 (C-4), 63.5 (C-6), 53.0 (cyclohexyl C-1), 33.0, 26.9, and 26.4 (cyclohexyl C).

Reaction conditions for preparations of GDP-Man (**5**).—Reactions in a batch or continuously operated reactor were carried out in Tris-HCl buffer at pH 8.0, 2 mM DTT and 1 mg mL^{-1} BSA. All solutions were passed through a sterile filter (0.2 μm, Sartorius, Göttingen, Germany). The temperature was maintained at 25 °C in all cases. The concentration of substrates, enzyme and cofactor is given below the corresponding figures. The course of all reactions was followed by HPLC. GDP-Man could be synthesised in an overall yield of 60% related to **2** and was isolated and identified as described before [21].

Continuous production of GDP-Man (**5**) *in a two-stage EMR cascade.*—The enzyme membrane reactors (volume 3 mL, Plexiglas) fitted with ultrafiltration membranes (YM 10, Amicon, Witten, Germany) preceded by sterile filters (0.2 μm, Sartorius, Göttingen, Germany) were sterilised with 0.1% peracetic acid. Subsequently bovine serum albumin (3 mg) was fed through the bubble traps into each EMR. The substrate solution was pumped into the system using a high precision piston pump (P 500, Pharmacia, Germany). The flow was set to 9 mL h^{-1} corresponding to a residence time of $\tau = 20$ min for each reactor. The outlet was collected in a fraction collector (RediFrac, Pharmacia LKB, Germany). Samples were taken with syringes through the bubble trap behind the first reactor to determine the conversion of the first EMR, and at the outlet.

Acknowledgements

The authors wish to thank Dipl.-Ing. Holger Paschold and Ina Dahmen for technical assistance

during the fermentation, and Heike Offermann and Ursula Mackfeld for their skilful help with the chemical synthesis and the continuous production experiments.

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