# Multi-enzyme Cascades for the *In Vitro* Synthesis of Guanosine Diphosphate L-Fucose

Reza Mahour,[a] Pavel A. Marichal-Gallardo,[b] Thomas F. T. Rexer,*[c] and Udo Reichl[d]

Recombinant Leloir glycosyltransferases can be exploited to synthesize a wide range of HMOs using *in vitro* biocatalytic reactions. However, high costs and unavailability of bulk amounts of most nucleotide sugars, such as guanosine diphosphate L-fucose (GDP-Fuc), are major obstacles for the efficient large-scale synthesis. Here, we report two novel multi-enzyme cascades for the synthesis of GDP-Fuc from readily available and low cost precursors. The first cascade was developed to produce GDP-Fuc from guanosine (Guo), fucose (Fuc), polyphosphate ($PolyP_n$) and catalytic amounts of adenine triphosphate (ATP). GDP-Fuc was produced with a final concentration of 7 mM (4.1 g/L) and a reaction yield of 68% from Guo and Fuc within 48 h with a biocatalyst load of 0.34 $g_{enzyme}/g_{product}$. A second cascade, consisting of ten enzymes and eleven reactions was developed to carry out the synthesis from mannose (Man), Guo, $PolyP_n$, L-glutamine (L-Glu) and catalytic amounts of ATP, and nicotinamide adenine dinucleotide phosphate (NADPH). Utilizing this cascade, GDP-Fuc was produced with a final concentration of 7.6 mM (4.5 g/L) and a reaction yield of 72% in a reaction time of 48 h with a biocatalyst load of 0.97 $g_{enzyme}/g_{product}$. Finally, a method for chromatographic purification of GDP-Fuc was established achieving product purities of 90.5%.

## Introduction

Positive effects of functional oligosaccharides on human health have been shown in various studies.[1,2] This concerns, in particular, the diet with human milk oligosaccharides (HMOs) from infancy to adulthood. For instance, it was shown that 2′-fucosyllactose contributes to the cognitive development of infants.[2] Consequently, there are ongoing efforts to include a wide variety of HMOs in infant food formula.[3] Of the more than 200 known HMO structures, around 70% are fucosylated.[4] Fucosylation is catalyzed *in vivo* by fucosyltransferases, members of the Leloir glycosyltransferase class of enzymes. Fucosyltransferases catalyze the transfer of fucose (Fuc) from guanosine diphosphate L-fucose (GDP-Fuc, the activated form of Fuc) to an acceptor oligosaccharide or protein. While most HMOs are produced to date by *in vivo* approaches, i.e., fermentation, the potential of *in vitro* enzymatic synthesis has been demonstrated to yield a wide range of simple and notably complex oligosaccharides using recombinant Leloir glycosyltransferases.[5,6] This synthesis, however, requires high cost sugar nucleotides such as GDP-Fuc (~30 €/mg) which significantly hampers low-cost large-scale manufacturing.[5,7] Therefore, developing scalable synthesis routes for sugar nucleotides is an important step towards the viable enzymatic production of functional oligosaccharides.

*In vivo* GDP-Fuc is either produced *de novo* by the conversion of guanosine diphosphate mannose (GDP-Man) and nicotinamide adenine dinucleotide phosphate (NADPH) as part of the hexosamine pathway or directly from Fuc through the salvage pathway.[8] In one of the first studies on its preparative scale synthesis, 78 mg of GDP-Fuc were produced from 100 mg of GDP-Man and 148 mg of NADPH.[9] By a combination of four different permeabilized microbial cells, Koizumi et al. synthesized GDP-Fuc from mannose (Man) and guanosine monophosphate (GMP) with yields of 17% and 52%, respectively, to a final GDP-Fuc concentration of 18.4 g/L.[10] After the discovery and successful recombinant production of bifunctional L-fucokinase/GDP-L-fucose pyrophosphorylase (FKP), GDP-Fuc was synthesized from Fuc, adenine triphosphate (ATP), and guanosine trisphosphate (GTP).[11–13] Recently, Wang et al. carried out the synthesis from Man, ATP, NADPH, and GTP with a final concentration of 178 mg/L and a yield of 14%.[11,14] In addition to the *in vitro* enzymatic synthesis, the production of GDP-Fuc by the fermentation of genetically engineered bacterial strains has been investigated.[15]

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A key prerequisite for the development of scalable enzymatic synthesis is the availability of inexpensive substrates in bulk amounts. Here, we report the development of two cascades for the synthesis GDP-Fuc from inexpensive precursors. The first cascade consists of five enzymes and seven reactions for the synthesis of GDP-Fuc from Fuc, guanosine (Guo), polyphosphate ($PolyP_n$), and catalytic amounts of ATP. However, due to the relatively high costs of Fuc compared to the other substrates, a second pathway was constructed using mannose (Man) as the sugar source. Therefore, a cascade consisting of ten enzymes and eleven reactions was designed and established to synthesize GDP-Fuc from Man, Guo, $PolyP_n$, L-glutamine (L-Glu), and catalytic amounts of NADPH and ATP. In both cascades, dimethyl sulfoxide (DMSO) was used to solubilize the Guo and avoid hydrogel formation among guanosine-containing molecules. Moreover, an ion exchange chromatography protocol was established to purify GDP-Fuc from the reaction mix to purities comparable to commercial standards. To the best of the authors' knowledge, both cascades presented herein are described for the first time.

## Results

### Solubility of Guo

Guo exhibits a very low solubility (~1.82 mM) in water.[16] Pure dimethyl sulfoxide (DMSO) was found to be able to dissolve Guo up to 1 M at 75 °C and 0.5 M at 25 °C. Consequently, pure DMSO was used to generate Guo stock solutions that were used as substrates for all enzyme reactions resulting in a DMSO concentration of 1 % v/v.

### Selection of enzymes

A literature search using the BRENDA enzyme database was carried out to identify and select enzymes for the construction of the pathway.[17] The criteria employed for selection were overlapping pH and temperature activity ranges. The enzymes selected are detailed in Table 1. Moreover, the catalyzed reactions and activity parameters are shown as reported in the literature.

### Synthesis of GDP-Fuc from Fuc and Guo

Pathway design: The constructed pathway for the synthesis of GDP-Fuc is illustrated in Figure 1. The cascade contains five enzymes and seven reactions (see Table 1 regarding the enzymes used in this work). There are three ATP-dependent kinase reactions. Consequently, an excess amount of ATP is needed to facilitate the full conversion of Fuc, Guo, and GMP. As ATP is relatively expensive compared to Guo, a regeneration cycle of ATP from ADP was established by exploiting the promiscuity of the polyphosphate kinase (PPK3) towards diphosphate nucleotides and by using $PolyP_n$ as the phosphate

**Table 1.** Enzymes used in this study and their reported activity range.[a]

| Enzyme | EC | Reaction | pH | *T* [°C] | Co-fact. | Ref. |
|---|---|---|---|---|---|---|
| | | Cascade 1: Enzymes for the synthesis of GDP-Fuc from Fuc and Guo | | | | |
| FKP | 2.7.1.52<br>2.7.7.30 | $Fuc + ATP \rightleftarrows Fuc1P + ADP$<br>$GTP + Fuc1P \rightleftarrows GDP - Fuc + PPi$ | 7.5 | 25–37 | $Mg^{2+}$ | [11, 12, 18] |
| PPK3 | 2.7.4.1 | $GDP + PolyP_n \rightleftarrows GTP + PolyP_{n-1}$<br>$ADP + PolyP_n \rightleftarrows ATP + PolyP_{n-1}$ | 5–11 | 30–40 | $Mg^{2+}$ | [19, 20] |
| GSK | 2.7.4.73 | $Guo + ATP \rightleftarrows GMP + ADP$ | 7.2 | 32 | $Mg^{2+}$ | [21] |
| GMPK | 2.7.4.8 | $GMP + ATP \rightleftarrows GDP + ADP$ | 7.4 | 30 | $Mg^{2+}$ | [22] |
| PPA | 3.6.1.1 | $PPi \rightleftarrows 2\ Pi$ | 7–8 | 25–35 | $Mg^{2+}$ | [20] |
| | | Cascade 2: Additional enzymes for synthesis of GDP-Fuc from Man and Guo | | | | |
| WCAG | 1.1.1.271 | $GDP4dehydro6deoxyMan + NADPH \rightleftarrows GDP - Fuc + NADP^+$ | 7.5 | 37 | $Mg^{2+}$ | [14] |
| GLDH | 1.4.1.2 | $LGlut + NADP^+ \rightleftarrows NADPH + NH_3 + AKG$ | 8 | 37 | | [23] |
| GLK | 2.7.1.2 | $Man + ATP \rightleftarrows Man6P + ADP$ | 7–8 | 25–35 | $Mg^{2+}$ | [24] |
| MANC | 2.7.7.13 | $GTP + Man1P \rightleftarrows GDPMan + PPi$ | 7–8 | 25–35 | $Mg^{2+}$ | [24] |
| GMD | 4.2.1.47 | $GDPMan \rightleftarrows GDP4dehydro6deoxyMan + H_2O$ | 7.5 | 37 | $Mg^{2+}$ | [14] |
| MANB | 5.4.2.8 | $Man6P \rightleftarrows Man1P$ | 7–8 | 25–35 | $Mg^{2+}$ | [24] |

[a] Abbreviations are as follows: GSK, guanosine-inosine kinase; GMPK, guanylate kinase; PPK3, polyphosphate kinase; MANC, mannose-1-phosphate guanylyltransferase; MANB, phosphomannomutase; GLK, glucokinase; PPA, inorganic diphosphatase; FKP, bifunctional fucokinase/L-fucose-1-P-guanylyltransferase; GMD, GDP-mannose 4,6-dehydratase; WCAG, GDP-L-fucose synthase; GLDH, glutamate dehydrogenase.

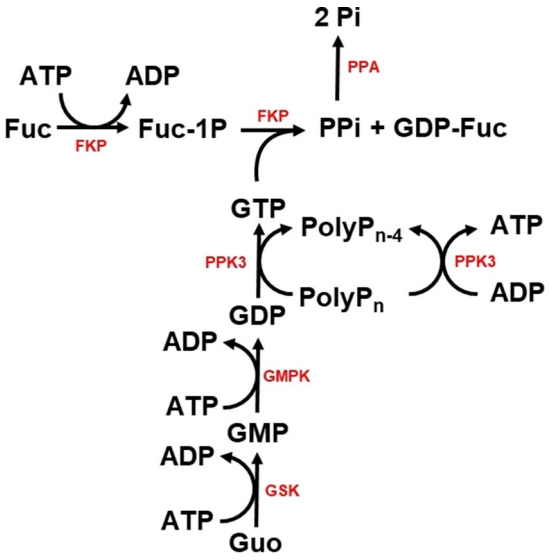


**Figure 1.** Cascade of five enzymes and seven reactions for the synthesis of GDP-Fuc from Fuc, Guo, PolyP$_n$, and a catalytic amount of ATP. The names of the enzymes are in red. Abbreviations are as follows: PPi, diphosphate; Pi, phosphate. Promiscuity of PPK3 allows regeneration of ATP and GTP by only one enzyme.

source. In addition, inorganic diphosphatase (PPA) was used to hydrolyze diphosphate and drive the reaction towards GDP-Fuc.

Synthesis of GDP-Fuc from Fuc and Guo: All reactions in the cascade were performed at pH 7.5. The time course of reaction intermediates is shown in Figure 2. A steep decline of Guo and a production of GMP, GDP, GTP, and GDP-Fuc was observed. GDP-Fuc was produced to a final concentration of 7 mM (4.1 g/L) equivalent to a yield of 68% after 48 h from Guo and Fuc, respectively. The biocatalyst load was 0.34 $g_{enzyme}/g_{product}$. The formation of GDP-Fuc was confirmed by MALDI-TOF (see supplementary file (SI); Figure SI 5). As ATP was 12-fold less abundant than the stoichiometric amount required, the results also confirm the efficient *in situ* regeneration of ATP from ADP and PolyP$_n$.

### Synthesis of GDP-Fuc from Man and Guo

Synthesis of GDP-Fuc from GDP-Man: To avoid using comparatively high cost Fuc as substrate, a second cascade was established to synthesize GDP-Fuc from Man. Man is around 40-fold cheaper than Fuc. First, a two-enzyme cascade containing GDP-mannose-4,6-dehydratase (GMD) and GDP-L-Fucose synthase (WCAG) was established to study the conversion of GDP-Man to GDP-Fuc (see Figure 3). It is known that GDP-Fuc has an inhibitory effect on GMD, resulting in low conversion yields.[25,26]

With the goal of improving the conversion yield, the role of pH was investigated in a broader range (pH 7.0, 7.5, 8.0, 8.5, and 9.0; Figure 4). It was observed that virtually full conversion of GDP-Man to GDP-Fuc was achieved at pH $\geq$ 8.0. In contrast, at pH 7.0 and 7.5, even a longer incubation time did not result in higher conversion yields (data not shown).

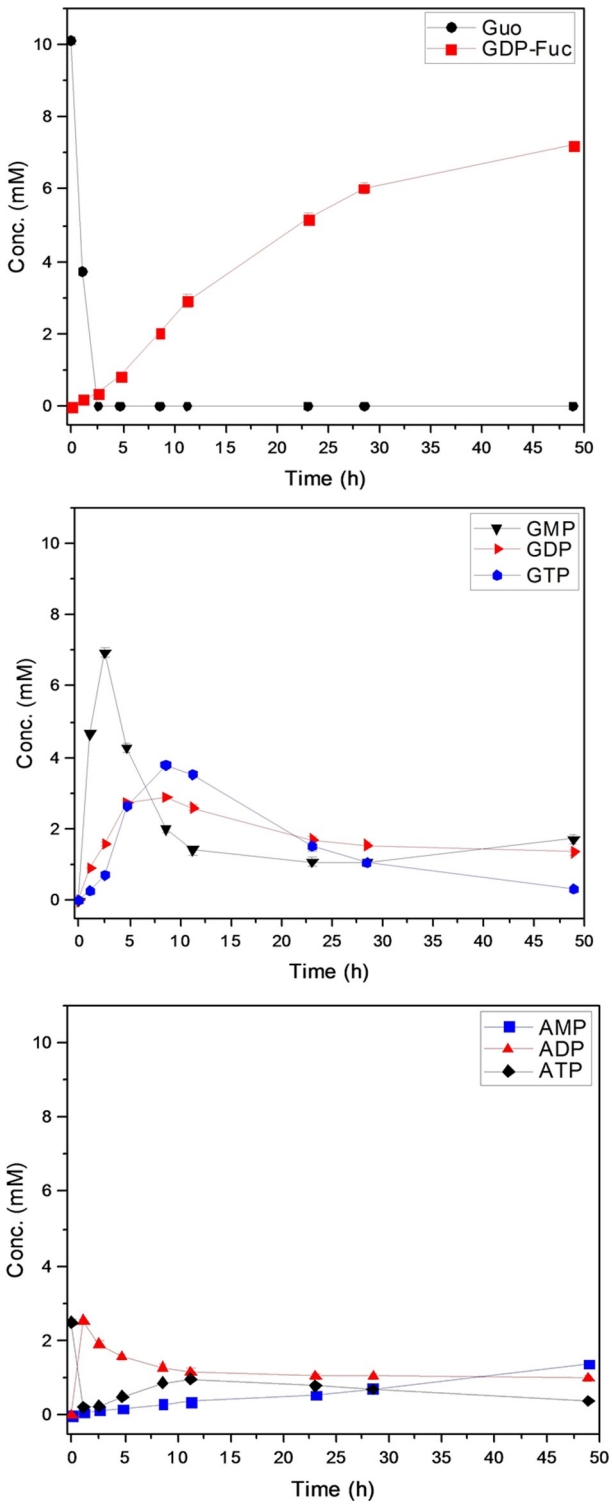


**Figure 2.** Time course of reactions using cascade 1 (see Figure 1) to synthesize GDP-Fuc. The reaction mixture for synthesis of GDP-Fuc consisted of 200 mM Tris-HCl (pH 7.5), 10 mM Fuc, 10 mM Guo (in DMSO), 2.5 mM ATP, 7.5 mM PolyP$_n$, 45 mM $MgCl_2$, and the following enzymes: GSK (0.22 μg/μL), GMPK (0.78 μg/μL), PPK3 (0.05 μg/μL), FKP (0.31 μg/μL), and PPA (0.03 μg/μL) in a final volume of 200 μL and 37 °C. The final DMSO content of the reaction matrix was 1% v/v. (a) Shows the consumption of Guo and production of GDP-Fuc, (b) shows the reaction time courses of GMP, GDP, and GTP, (c) shows the reaction time courses of ATP, ADP, as well as AMP. The production of AMP is mainly due to chemical conversion of ADP to ATP during the reaction. Experiments were performed in triplicates and error bars represent the standard error. The values of standard errors are listed in Table SI 2.


**Figure 3.** Cascade of two enzymes for the synthesis of GDP-Fuc from GDP-Man.

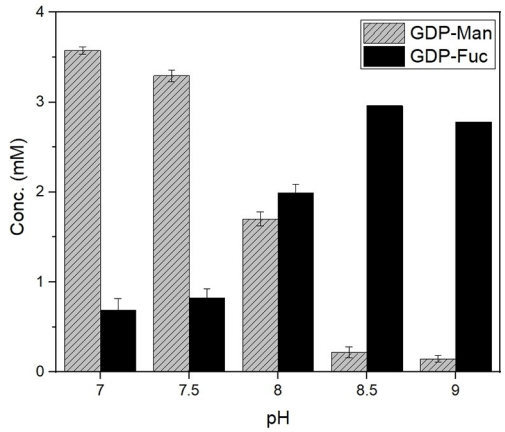


**Figure 4.** Conversion of GDP-Man to GDP-Fuc after 5 h (end of the reactions) with a two-enzyme cascade. The experiments were carried out at 37 °C. Concentrations were as follows: 150 mM Tris-HCl, 10 mM $MgCl_2$, 3–4 mM GDP-Man, 4 mM NADPH, WCAG (0.45 μg/μL), and GMD (1.03 μg/μL) in a the final volume of 33 μL. Experiments were carried out in triplicates and error bars represent the standard error.

Pathway design for synthesis of GDP-Fuc from Man and Guo: The second cascade was developed to synthesize GDP-Fuc from Man, Guo, $PolyP_n$, L-Glu, and catalytic amounts of NADPH and ATP (see Figure 5). L-Glu was used as a substrate for the *in situ* regeneration of the expensive NADPH. Beforehand, the synthesis of GDP-Man from Man, ATP, and Guo using the first part of the cascade was verified (see SI - Figure SI 2).

Synthesis of GDP-Fuc from Man and Guo: Since a pH over 8.0 was necessary to achieve high yields of GDP-Fuc from GDP-Man, a pH of 8.5 was selected to carry out the cascade reactions. The time course of the reaction intermediates is shown in Figure 6. The reaction starts with immediate conversion of Guo to GMP, and further into GDP and GTP. The

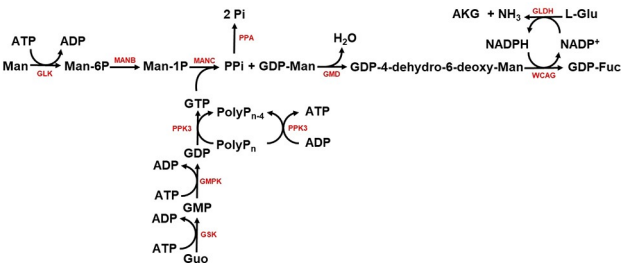


**Figure 5.** Cascade 2 for the synthesis of GDP-Fuc from Man, Guo, $PolyP_n$, L-Glu, and catalytic amounts of ATP and NADPH. Man6-P, mannose 6-phosphate; Man-1P, mannose 1-phosphate; $H_2O$, water; GDP-4-dehydro-6-deoxy-Man, guanosine diphosphate-4-keto-6-deoxy -mannose; $NH_3$, ammonia; AKG, α-ketoglutaric acid.

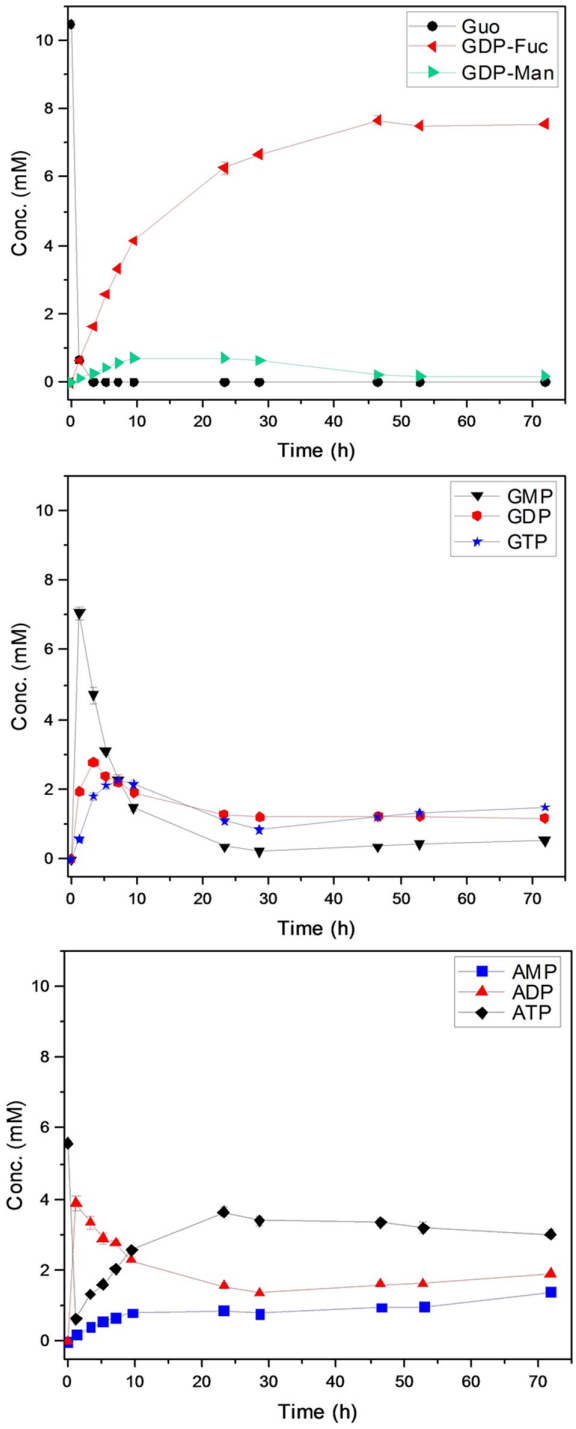


**Figure 6.** Time course of reaction products and intermediates for the synthesis of GDP-Fuc from Man, Guo, $PolyP_n$, L-Glu, and catalytic amounts of ATP and NADPH. The experiments were carried out at 37 °C and 200 mM Tris-HCl (pH 8.5), 75 mM $MgCl_2$, 10.5 mM Man, 10.5 mM Guo, 50 mM L-Glu, 1 mM NADPH, 5.5 mM ATP, 13.5 mM $PolyP_n$, GSK (0.11 μg/μL), GMPK (0.49 μg/μL), PPK3 (0.02 μg/μL), GLK (0.33 μg/μL), MANB/C (0.17 μg/μL), WCAG (0.07 μg/μL), GMD (0.17 μg/μL), PPA (0.03 PPA), and 10 units of GLDH (2.99 μg/μL) in a final volume of 200 μL. The final DMSO content of the reaction matrix was 1 % v/v. (a) shows the consumption of Guo, the production of GDP-Man and its consumption for synthesis of GDP-Fuc, (b) shows the production of GMP followed by its consumption for production of GDP and GTP, (c) shows the concentration of ATP, ADP as well as AMP; The production of AMP is mainly due to chemical conversion of ADP to ATP. Experiments were carried out in triplicates and error bars represent the standard error. The values of standard errors are listed in Table SI 3.

higher concentration of GDP-Fuc with regard to GDP-Man during the reaction time demonstrates the high catalytic activity of GMD and WCAG. GDP-Fuc was produced with a concentration of 7.6 mM (4.5 g/L) and a reaction yield of 72% after 48 h with a biocatalyst load of 0.97 $g_{enzyme}/g_{product}$. ATP and NADPH were used 5.7-fold and 10.5-fold less than stoichiometric amounts, respectively. The production of GDP-Fuc was verified by MALD-TOF mass spectrometry (see SI - Figure SI 6). Furthermore, to understand the effect of pH on the overall performance of the cascade, pH values of 7.0, 7.5, 8.0, 8.5, and 9.0 were screened and it was found out that pH 8.5 and 9.0 resulted in the highest conversion yield (see SI - Figure SI 4).

### Chromatographic purification of the GDP-Fuc cascade product

As a first screening step, purified commercially available GDP-Fuc was injected into a strong quaternary amine anion exchanger column (HiTrap Q HP) to assess its capture and subsequent elution with a gradient.

An injection of 5 μL of GDP-Fuc (7.9 mM) using 50 mM Tris pH 7.4 resulted in successful capture of GDP-Fuc. It was possible to recover the product by using a gradient elution at around 6% of desorption buffer (50 mM Tris, 1.0 M NaCl, pH 7.4) with a conductivity of 7.6 mS/cm at the peak apex (Figure 7A, top panel).

Injection of a GDP-Fuc cascade product (50 μL) using the same setup showed impurities in the flow-through and GDP-Fuc was collected as a first and fully resolved peak in the gradient at the same retention time (Figure 7A, bottom panel) as the previously injected purified standard. The GDP-Fuc was further confirmed by injecting the cascade product spiked with the purified commercial standard (not shown).

Due to the early elution of GDP-Fuc in the gradient at a conductivity close to that of the equilibration buffer, it was decided to increase the pH from 7.4 to 8.0 in order to achieve a higher product retention. Another set of experiments with the purified commercial standard and a 10-fold increase of loaded cascade product (0.5 mL) was performed at pH 8.0 (Figure 7B) with a modified gradient (step 1:15% desorption buffer over 25 CV; step 2:100% desorption buffer over 5 CV). As observed, a conductivity exceeding 11 mS/cm was needed to desorb GDP-Fuc loaded at pH 8.0 compared to around 7.6 mS/cm at pH 7.4. Despite the expected change of the desorption profile due to the higher loading, GDP-Fuc was fully resolved in the gradient.

The purity of GDP-Fuc in the loaded cascade product was estimated to be around 25% based on the chromatographic UV signal at 260 nm (Figure 7B, bottom panel). The purified GDP-Fuc was collected and re-injected using the same method. In stark contrast to the cascade product, the purity of GDP-Fuc after ion exchange chromatography was estimated to be 90.5% (Figure 7C) and comparable to that of the commercial standard (91.8%).

## Discussion

The objective of this study was to develop two multi-enzyme cascades to synthesize GDP-Fuc from inexpensive and readily available precursors. Guo is used in the food industry as raw

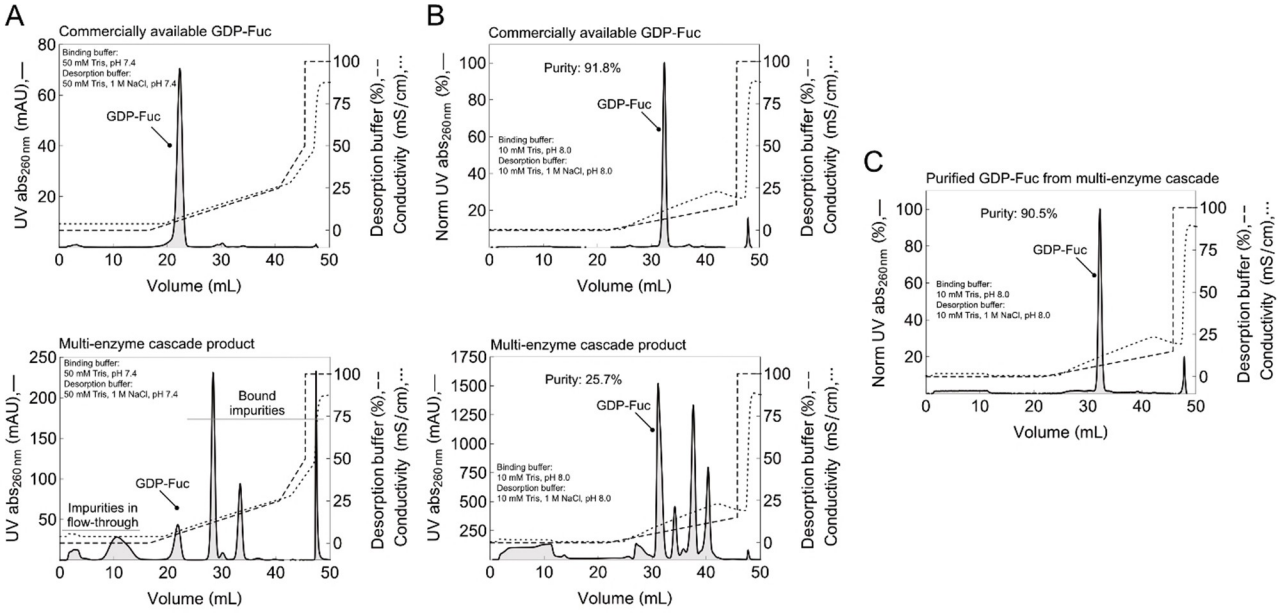


**Figure 7.** Chromatographic purification of GDP-Fuc produced in a multi-enzyme cascade by bead-based anion exchange chromatography. A 1 mL column packed with a strong quaternary amine anion exchanger and a bead size of 34 μm was used. Panel A depicts preliminary scouting experiments with a purified commercial GDP-Fuc standard and the cascade product at pH 7.4. Panel B shows an optimized gradient at pH 8.0 and a 10-fold load increase. GDP-Fuc was identified as the second peak in the gradient from the cascade product. Panel C shows the purified GDP-Fuc cascade product with a purity grade (90.5%) similar to that of the commercial standard (91.8%).

material to produce GMP as a flavor enhancer and is available in bulk amounts.[27] However, using Guo in aqueous solutions is limited by its hydrophobic nature, which results in low solubility. Guo has unique self-assembly properties due to multiple hydrogen bonding interactions resulting in hydrogel formation.[28] DMSO was found to be able to solubilize Guo and avoid hydrogel formation in aqueous solutions. This is due to interactions between the alcohol, amide, amine, and ether as functional groups of Guo are unfavourable in DMSO.[29] The good solubility of the Guo-containing compounds is illustrated by closed mass balances at each of the reaction measurement time points in our experiments (see Figure 2 and Figure 6). DMSO is especially suitable as a solvent due to its low toxicity, i.e., it is widely used in the field of biotechnology as an extractant and solvent as well as in medicine as a therapeutic to reduce detumescence and as a carrier substance for drug delivery.[30]

For the production of GDP-Fuc, at first a cascade was established successfully to start the synthesis from Fuc (~4 €/g), Guo (~0.1 €/g), $PolyP_n$ (0.05 €/g), and catalytic amounts of ATP (~0.4 €/g). As Fuc is relatively expensive compared to the other substrates, a second cascade was developed to produce GDP-Fuc from Man (~0.1 €/g). However, the benefit of lower substrate costs comes at the costs of using ten enzymes instead of five plus an additional co-factor regeneration loop.

To avoid the stoichiometric usage of high-cost co-substrates ATP and NADPH, *in situ* regeneration cycles were implemented. ATP and NADPH are widely used co-substrates for the enzymatic *in vitro* synthesis of chemicals. Thus, multiple examples for their *in situ* regeneration have been developed.[31] In both cascades established in this study, ATP recycling from $PolyP_n$ was implemented building on our previous work.[20,32,33] In our second cascade, a NADPH recycling loop using L-Glu as substrate was implemented that allowed using NADPH 10.5-fold less than the stochiometric amount. L-Glu is used in the food industry as a flavor enhancer and thus, readily available at low costs (~0.07 €/g).[34]

The costs for the production of purified protein using *E. coli* were previously estimated to be around 1.1 €/g.[35] Accordingly, here the cost contribution of enzymes is around 1.5 € per liter of reaction for cascade 1 and 4.7 € for cascade 2. Consequently, the cost of enzymes can be estimated to be around 0.03 € and 1.1 € per gram of GDP-Fuc, respectively. Thus, compared to the costs of substrates used, the cost contribution of the enzymes to the total synthesis costs is minor (see Table SI 4 and 5).

From the experimental data, no single rate-limiting steps were identified. However, the turnover of Guo to GMP is completed after a maximum of 3 h in both cascades and thus, the concentration of GSK can be reduced to some extent without severely affecting the productivity in future reactions.

Previous work on the enzymatic one-pot production of GDP-Fuc from GDP-Man showed low conversion yields due to inhibition of GMD by GDP-Fuc.[10,14] It has been suggested that GDP-Fuc is a competitive inhibitor of GMD by binding to the active site.[25,26,36] Both enzymes, GMD and WCAG, belong to the short-chain dehydrogenases/reductases family and during the conversion of GDP-Man to GDP-Fuc, both liberation and consumption of hydrogen ions are part of the reaction mechanism.[26,37] Once hydrogen ions are involved in a reaction, dissociation constants of ligands and enzymes are a function of the pH value.[38] Therefore, a pH screening was carried out to evaluate its effect on the performance of the two-enzyme cascade (GMD and WCAG) to synthesize GDP-fuc from GDP-Man and NADPH. Interestingly, it was found that at alkaline conditions in the range pH 8.0–9.0, an almost quantitative conversion of GDP-Man to GDP-Fuc can be obtained. This is possibly due to a low binding of GDP-Fuc to GMD at alkaline pH values.

The performance of the two developed cascades is compared to previously published enzymatic cascades in Table 2. Pfeiffer et al. achieved a high concentration of 50 g/L GDP-Man, however, GTP was used as substrate.[39] The high costs of GTP (~21 €/g) can significantly hamper large-scale application. In stark contrast, the utilization of Guo (~0.1 €/g) or even GMP (~0.1 €/g), as shown here, substantially reduces substrate costs around 200-fold.

Koizumi et al. obtained the highest reported GDP-Fuc concentration (18.4 g/L) using whole cell catalysis at a 15 L scale by using Man and GMP (see Table 2).[10] However, drawbacks are low synthesis yields (17% and 52% regarding Man and GMP, respectively) and very high biocatalyst loads (215 g/L) in the form of four different permeabilized microbial cells. Moreover, to avoid low conversions of GDP-Man to GDP-Fuc, the reactions were carried out in two separate reaction vessels.

It was possible to purify the product of our GDP-Fuc cascades by ion exchange chromatography. This method is one of the most widely used purification techniques in industry thanks to its robustness, scalability, and costs.[40] Here, it was possible to fully resolve the GDP-Fuc product using a gradient elution and its purity was increased from 25% to 90.5%, matching that of a commercial standard (91.8%). These preliminary results demonstrate that GDP-Fuc produced in a multi-enzyme cascade can be efficiently purified in an inexpensive manner with readily available industrial materials using a simple method.

To further reduce costs prior to scaling up the GDP-Fuc synthesis, an optimization of the amounts of enzymes, substrates and co-substrates used should be carried out through a more comprehensive screening of reaction conditions. To keep experimental work to a minimum, a design of experiments approach seems especially wellsuited for this purpose.

## Conclusion

Fuc is an important building block for many types of oligosaccharides such as HMOs. The Fuc donor for enzymatic assembly of fucosylated oligosaccharides is the activated nucleotide sugar GDP-Fuc. Until now, high costs of GDP-Fuc have hampered the enzymatic *in vitro* synthesis of fucosylated oligosaccharides beyond the milligram scale. In this work, two multi-enzyme cascades were established to synthesize GDP-Fuc from low-cost and readily available precursors. In both cascades, Guo was used as a substrate by employing DMSO as a co-

**Table 2.** Comparison of reported processes for the *in vitro* biocatalytic production of GDP-Man and GDP-Fuc.

| Stoichiometric compounds | Catalytic Compound | Phosphate source | Conc. [g/L] | Reaction time [h] | Yield [%] | Biocat. Load [$g_{enz}/g_{prodt}$] | Mode of operation | Reaction condition | Scale [mL] | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | GDP-Man | | | | | | |
| 1.2 mM Man-1P; 2.4 mM GTP | | | 0.4 | 0.34 | 60 | | Continuous | Tris-HCl, pH 8, 1.8 mM $MgCl_2$; 2 mM DTT; 25 °C | 3 | [41] |
| 15 mM Man; 35 mM GTP | | | 14.1 | 24 | 94[a] | 0.04 | Batch | 100 mM Tris-HCl, pH 8; 10 mM $MgCl_2$; 37 °C | 12 | [42] |
| 119 mM sucrose; 119 mM Pi; 400 mM Man; 70 mM GTP | | | 50 | 20 | 100 | 0.01 | Batch | 100 mM MES, pH 7; 10 mM $MgCl_2$; 37 °C | 4 | [39] |
| 0.8 mM GDP; 6 mM Man; 0.8 mM ADP | | 4 mM $PolyP_n$ | 0.3 | 4 | 71 | 3.1 | Batch | 50 mM Tris-HCl, pH 7.5; 10 mM $MgCl_2$; 30 °C | 1 | [32] |
| 12.8 mM Guo; 10 mM Man | 5.8 mM ATP | 13.5 mM $PolyP_n$ | 4 | 45 | 52 | 0.35 | Batch | 200 mM Tris-HCl, pH 8.5; 75 mM $MgCl_2$; 37 °C | 0.2 | This work |
| | | | | GDP-Fuc | | | | | | |
| 27.5 mM GDP-Man; 33 mM NADPH | | | 12.9 | 2 | 80 | 0.22 | 2-step batch | 50 mM Tris-HCl, pH 7.5; 10 mM $MgCl_2$; 37 °C | 6 | [9] |
| 333 mM fructose; 166 mM Man; 56 mM GMP | | | 18.4 | 22 | 51 | 11.6 | 2-step batch | 25 g/L $KH_2PO_4$, pH 7.2; 5 g/L $MgSO_4.7H_2O$; 32 °C | 30 | [10] |
| 91 mM Fuc; 54 mM ATP; 53 mM GTP | | | | 48 | | | Batch | 50 mM Tris-HCl, pH 7.5; 50 mM $MgCl_2$; 25 °C | 10 | [43] |
| 10.5 mM Man; 10.5 mM Guo; 50 mM L-Glu | 5.5 mM ATP; 1 mM NADPH | 13.5 mM $PolyP_n$ | 4.5 | 48 | 72 | 0.97 | Batch | 200 mM Tris-HCl, pH 8.5; 75 mM $MgCl_2$; 37 °C | 0.2 | This work |
| 10 mM Fuc; 10 mM Guo | 2.5 mM ATP | 4.5 mM $PolyP_n$ | 4.1 | 48 | 70 | 0.34 | Batch | 200 mM Tris-HCl, pH 7.5; 45 mM $MgCl_2$; 37 °C | 0.2 | This work |

[a] Yield after gel filtration; DTT, dithiothreitol; MES, 2-(N-morpholino)ethanesulfonic acid.



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solvent to avoid hydrogel formation in the reaction matrix. At first, a cascade of five enzymes and seven reactions was developed to synthesize GDP-Fuc from Guo, Fuc, $PolyP_n$ and catalytic amounts of ATP. GDP-Fuc was produced with a final concentration of 7 mM (4.1 g/L) and a reaction yield of 68% from Guo and Fuc in a batch process of 48 h and a biocatalyst load of 0.34 $g_{enzyme}/g_{product}$. To further reduce the costs of substrates, a second cascade that uses Man instead of Fuc as the sugar precursor and consists of ten enzymes and eleven reactions was developed. Here, GDP-Fuc was produced with a final concentration of 7.6 mM (4.5 g/L) and a reaction yield of 72% from Guo, Man, $PolyP_n$, L-Glu, and catalytic amounts of ATP and NADPH after 48 h with a biocatalyst load of 0.97 $g_{enzyme}/g_{product}$. Finally, a robust and scalable method for GDP-Fuc purification by ion exchange chromatography was established achieving purities equivalent to the quality of commercial standards. In conclusion, the developed enzyme cascades and the purification method enable the efficient synthesis and purification of GDP-Fuc significantly below current production costs.

## Experimental Section

**Material and methods**. Chemicals and standard methods for analytics are described in the supplementary file (SI). All prices are the list prices as of October 2020 from Carbosynth Ltd (Compton, United Kingdom) with the exception of $PolyP_n$ for which the list price was taken from Merck (Darmstadt, Germany). Enzymes were expressed in *E. coli* and purified using ion metal affinity chromatography as detailed in SI.

**Multi-enzyme experiments**. All enzymatic reactions were performed in 1.5 mL Eppendorf safe-lock tubes (Eppendorf, Germany) at 37 °C and 550 rpm in Eppendorf Thermomixer comfort incubators (Eppendorf, Germany). For reaction time course measurements, aliquots were taken and quenched at 90 °C in MiliQ water for 3 min. All initial concentrations given below are calculated from weighted samples and might slightly deviate from measured initial concentrations.

First cascade starting from Fuc and Guo: The reaction mixture for synthesis of GDP-Fuc contained 200 mM Tris-HCl (pH 7.5), 10 mM Fuc, 10 mM Guo (in DMSO), 2.5 mM ATP, 7.5 mM $PolyP_n$, 45 mM $MgCl_2$, and the following enzymes: GSK (0.22 μg/μL), GMPK (0.78 μg/μL), PPK3 (0.05 μg/μL), FKP (0.31 μg/μL), and PPA (0.03 μg/μL) in a final volume of 200 μL. The final DMSO content of the reaction matrix was 1% v/v.

Second cascade starting from Man and Guo-Production of GDP-Fuc from GDP-Man: The reaction mixtures contained 150 mM Tris-HCl (various pH values), 10 mM $MgCl_2$, 3–4 mM GDP-Man, 4 mM NADPH, WCAG (0.45 μg/μL), and GMD (1.03 μg/μL) in a final volume of 33 μL. To evaluate the role of the pH value on the conversion of GDP-Man to GDP-Fuc, the following pH values were tested: 7.0, 7.5, 8.0, 8.5, 9.0.

Second cascade starting from Man and Guo-Production of GDP-Fuc from Man and Guo with *in situ* regeneration of NADPH: The cascade reactions contained 200 mM Tris-HCl (pH 8.5), 75 mM $MgCl_2$, 10.5 mM Man, 10.5 mM Guo, 50 mM L-Glu, 1 mM NADPH, 5.5 mM ATP, 13.5 mM $PolyP_n$, GSK (0.11 μg/μL), GMPK (0.49 μg/μL), PPK3 (0.02 μg/μL), GLK (0.33 μg/μL), MANB/C (0.17 μg/μL), WCAG (0.07 μg/μL), GMD (0.17 μg/μL), PPA (0.03 μg/μL), and 10 units of GLDH (2.99 μg/μL) in a final volume of 200 μL. The final DMSO content of the reaction matrix was 1% v/v.

**Chromatographic purification of GDP-Fuc with a bead-based anion exchanger**. For the purification of the GDP-Fuc product from the enzymatic cascades, an ÄKTA Pure 25 liquid chromatography system was used at room temperature and controlled by the software UNICORN v6.3 (Cytiva; Uppsala, Sweden). The UV absorbance was monitored at 260 nm. The column used was a pre-packed 1 mL strong anion exchanger (HiTrap Q HP; 29-0513-25; Cytiva; Uppsala, Sweden) with a bead size of 34 μm. The column was operated at 1–4 mL/min (155–624 cm/h). GDP-Fuc purification was performed in bind-elute mode. In short, (A) Equilibration: after a washing step of 10 column volumes (CV) with water, the column was equilibrated with equilibration buffer (10 mM Tris, pH 8.0) for 10 CV at 3 mL/min. (B) Sample injection: the 0.2 μm clarified cascade product was diluted at least 10-fold in equilibration buffer and fed to the column at 2 mL/min. After sample injection, a wash step (2 mL/min) followed with equilibration buffer for 10 CV or until baseline UV absorbance was achieved. (C) Elution: the bound compounds were eluted at 1 mL/min using a combination of different steps and gradients, most notably a first step of 15% desorption buffer (10 mM Tris, 1.0 M NaCl, pH 8.0) for 25 CV followed by a second step to 100% desorption buffer for 5 CV. The column was finally stripped with 2 M NaCl at 3 mL/min for 10 CV and re-equilibrated for subsequent runs.

## Notes

RM and TFTR are the inventors of a pending patent on the described topic.

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## *Conflict of Interest*

The authors declare no conflict of interest.

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