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Enzymatic synthesis of nucleotide sugars

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The present review gives a survey on the biosynthetic pathways of nucleotide sugars which are important for the *in vitro* synthesis of mammalian glycoconjugates. With respect to the use of these enzymes in glycotechnology the availability as recombinant enzymes from different sources, the large-scale synthesis of nucleotide sugars and their *in situ* regeneration in combination with glycosyltransferases are summarized and evaluated.

Keywords: **enzymatic synthesis, nucleotide sugars**

Abbreviations: **ADP, Adenosine 5′-diphosphate; CDP, Cytidine 5′-diphosphate; CMP, Cytidine 5′-monophosphate; CMP-Neu5Ac, Cytidine 5′-monophospho-*N*-acetyl-β-D-neuraminic acid; CTP, Cytidine 5′-triphosphate; dTDP, 2′-deoxythymidine 5′-diphosphate; dUDP, 2′-deoxyuridine 5′-diphosphate; EMR, enzyme membrane reactor; Fru, D-fructose; Fru-6-P, D-fructose 6-phosphate; Fuc, L-fucose; Fuc-1-P, β-L-fucose 1-phosphate; Gal, D-galactose; Gal-1-P, α-D-galactose 1-phosphate; GalN, D-galactosamine; GalN-1-P, α-D-galactosamine 1-phosphate; GalNAc, *N*-acetyl-D-galactosamine; GalNAc-1-P, *N*-acetyl-α-D-galactosamine 1-phosphate; GDP, Guanosine 5′-diphosphate; GDP-6-d-4-k-L-Gal, Guanosine 5′-diphospho-6-deoxy-4-keto-β-L-galactose; GDP-6-d-4-k-Man, Guanosine 5′-diphospho-6-deoxy-4-keto-α-D-mannose; GDP-Fuc, Guanosine 5′-diphospho-β-L-fucose; GDP-Fuc PP, Guanosine 5′-diphospho-β-L-fucose pyrophosphorylase; GDP-Man, Guanosine 5′-diphospho-α-D-mannose; GDP-Man DHy, GDP-mannose-4,6-dehydratase (EC 4.2.1.47); GDP-Man PP, GDP-man pyrophosphorylase (EC 2.7.7.13); Glc-1-P, α-D-glucose 1-phosphate; Glc-6-P, D-glucose 6-phosphate; GlcA-1-P, α-D-glucuronic acid 1-phosphate; GlcN, D-glucosamine; GlcN-1-P, α-D-glucosamine 1-phosphate; GlcN-6-P, D-glucosamine 6-phosphate; GlcNAc, *N*-acetyl-D-glucosamine; GlcNAc-1-P, *N*-acetyl-α-D-glucosamine 1-phosphate; GlcNAc-6-P, *N*-acetyl-D-glucosamine 6-phosphate; GlmU, glucosamine uridyltransferase; GTP, Guanosine 5′-triphosphate; LacNAc, N-acetyllactosamine; Le^x, Lewis X antigen; Man, D-mannose; Man-1-P, α-D-mannose 1-phosphate; Man-6-P, D-mannose 6-phosphate; ManNAc, *N*-acetyl-D-mannose; ManNAc-6-P, *N*-acetyl-D-mannose 6-phosphate; NAD$^+$, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide hydride; NADP$^+$, nicotinamide adenine dinucleotide phosphate; NADPH, nicotinamide adenine dinucleotide phosphate hydride; NAS, N-acetoxysuccinimide; Neu5Ac, *N*-acetylneuraminic acid; Neu5Ac-9-P, *N*-acetylneuraminic acid 9-phosphate; NeuAc aldolase, *N*-acetylneuraminic acid aldolase (EC 4.1.3.3); NMPK, nucleoside 5′-monophosphate kinase (EC 2.7.7.4); PEP, phosphoenolpyruvate; PP, pyrophosphorylase; P_i, inorganic phosphate; PP_i, inorganic pyrophosphate; PGM, phosphoglucomutase (EC 2.7.5.1); PK, pyruvate kinase (EC 2.7.1.40); PMI, phosphomannose isomerase (EC 5.3.1.8); PMM, phosphomannomutase (EC 5.4.2.8); Pyr, pyruvate; UDP, uridine 5′-diphosphate; UDP-Gal, uridine 5′-diphospho-α-D-galactose; UDP-GalNAc, uridine 5′-diphospho-*N*-acetyl-α-D-galactosamine; UDP-GalN, uridine 5′-diphospho-α-D-galactosamine; UDP-Glc, uridine 5′-diphospho-α-D-glucose; UDP-Glc DH, UDP-glucose dehydrogenase; UDP-GlcA, uridine 5′-diphospho-α-D-glucuronic acid; UDP-GlcNAc, uridine 5′-diphospho-*N*-acetyl-α-D-glucosamine; UDP-Xyl, uridine 5′-diphospho-α-D-xylose; UMP, uridine 5′-monophosphate**

Introduction

Carbohydrates as part of glycoconjugates play an important role in dynamic physiological processes like cell-cell recognition and tumor development [1]. They are ligands for pathogens and modulate the functions of hormones and antibiotics [2]. The ability of oligosaccharides to mediate inter- and intracellular communication is closely related to their enormous structural variation. In comparison with proteins and nucleic acids saccharides are best suited to carry specific biological information. Not only the sequence and conformation of the monomers of oligosaccharides can be modulated, but also the type of glycosidic linkages and the branching of the sugar chains can be varied. It is this structural complexity, which makes the chemical synthesis of carbohydrates a challenging task [3]. These difficulties can be overcome by the use of the biosynthetic pathways to synthesize oligosaccharides *in vitro*.

In contrast to the formation of proteins and nucleic acids, where all of the monomeres are linked by one enzymatic activity according to a template, there is at least one specific

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glycosyltransferase for each linkage and monosaccharide in oligosaccharide biosynthesis. For some linkages enzyme families have been found in one organism that catalyze the same reaction but differ in kinetic properties, the *in vitro* substrate spectrum and the *in vivo* expression pattern [4–8].

The Leloir-glycosyltransferases catalyze the sequential transfer of monosaccharides from nucleotide sugars to an aglycon or a growing oligosaccharide chain [9,10]. The application of these enzymes allows the straightforward synthesis of carbohydrates and glycoconjugates in high yields and absolute stereo- and regioselectivity.

The first nucleotide sugars were identified 45 years ago and found to act as sugar donors in transfer reactions [11]. In the following years, first syntheses for nucleotide sugars were developed [12–15]. Because the glycosyltransferases were not available, only small amounts of their substrates, preferably containing a radioactive label were needed for analytical studies. In recent years more and more genes encoding glycosyltransferases were cloned and became available for synthetic purposes [16,17]. This development increased the demand for the substrates of these enzymes. Since the 1980s the known syntheses were scaled up and new strategies and technologies were applied to produce large amounts of nucleotide-activated sugars. Table 1 demonstrates this development for the enzymatic synthesis of CMP-*N*-acetylneuraminic acid (CMP-Neu5Ac).

Chemical syntheses of these molecules have been developed, but the yields are low and organic solvents have to be used [18]. The scale up of chemical syntheses to obtain kg-amounts of nucleotide sugars is therefore not economical. In contrast enzymatic methods including *in situ* regeneration cycles are more attractive. These regeneration cycles not only reduce the cost of sugar nucleotides, but also lessen the problem of product inhibition caused by the resulting nucleoside phosphates [19]. Enzymes for the synthesis and *in situ* regeneration of nucleotide sugars have been described in former reviews [9,20,21]. We have recently summarized the enzymes for the synthesis of primary and secondary nucleotide sugars published until 1996 [22]. The present review summarizes the syntheses of the nine nucleotide sugars, which are the substrates of the Leloir-glycosyltransferases in mammals. The availability of the enzymes and their use as tools in glycobiotechnology are particularly emphasized. The nucleotide sugars referred to are uridine 5′-diphospho-α-D-glucose (UDP-Glc), uridine 5′-diphospho-α-D-glucuronic acid (UDP-GlcA), uridine 5′-diphospho-α-D-galactose (UDP-Gal), uridine 5′-diphospho-*N*-acetyl-α-D-galactosamine (UDP-GalNAc), uridine 5′-diphospho-*N*-acetyl-α-D-glucosamine (UDP-GlcNAc), guanidine 5′-diphospho-β-L-fucose (GDP-Fuc), guanidine 5′-diphospho-α-D-mannose (GDP-Man), cytidine 5′-monophospho-*N*-acetyl-β-D-neuraminic

Table 1. Enzymatic syntheses of CMP-Neu5Ac

Year	*Enzyme source*	*Scale [μmol]*	*Yield [%]*	*Synthesized structure*	*Ref.*
1962	Hog submaxillary glands	65	33	[^{14}C]CMP-Neu5Ac	[11]
1962	*Neisseria meningitidis*	13		[^{14}C]CMP-Neu5Ac	[87]
1966	Swine submaxillary glands	15	70–90	[^{14}C]CMP-Neu5Ac	[88]
1966	Hog submaxillary glands	360	40–50	[^{14}C]CMP-Neu5Ac	[89]
1968	Calf thyroid	400	82	[^{14}C]CMP-Neu5Ac	[90]
1972	Calf brain	36	70	[^{14}C]CMP-Neu5Ac	[91]
1972	Bovine submaxillary glands	160	70–85	[^{14}C]CMP-Neu5Ac	[92]
1979	Frog liver / equine submandibular glands	100	95	[^{14}C]CMP-Neu5Ac	[93]
1985	Calf brain	100	60–70	CMP-Neu5Ac	[94]
1986	Bovine submandibular glands	20	72	CMP-Neu5Ac	[95]
1988	Calf brain	4000	90–95[a] / 62[b] (2,7g)	CMP-Neu5Ac	[86]
1988	Calf brain	500	52	CMP-Neu5Ac	[83]
1990	Calf brain	130 / 65[c]	76 / 15[c]	CMP-Neu5Ac	[96]
1991	*E. coli,* recombinant	3000	97 (2g)	Sialyl-LacNAc	[97]
1991	*E. coli,* recombinant	3100	94 (2.1g)	CMP-Neu5Ac	[98]
1992	*E. coli,* recombinant	2700	90 (1,7g)	CMP-Neu5Ac	[99]
1995	*E. coli*	1240	80 (0,76g)	CMP-Neu5Ac	[100]
1996	*E. coli*	10000	60 (4 g)	CMP-Neu5Ac	[101]

[a] Synthesis yield, not purified product
[b] Overall yield calculated from ManNAc produced in a preliminary synthesis
[c] *In situ* generation of CTP from CMP

acid (CMP-Neu5Ac), and uridine 5′-diphospho-α-D-xylose (UDP-Xyl). Most of the syntheses follow the biosynthetic routes to the nucleotide sugars (Fig. 1). Primary nucleotide sugars are synthesized *in vivo* from the sugar-1-phosphate and a nucleoside triphosphate catalyzed by pyrophosphorylases. Secondary nucleotide sugars are generated by the modification of a primary nucleotide sugar. For some nucleotide sugars additional pathways were elucidated, which represent two step *de novo* syntheses. These salvage pathways allow the cell to activate monosaccharides that come from lysosomal degradation or nutrition. The sugars are phosphorylated in the anomeric position by kinases and activated by pyrophosphorylases (Fig. 1). Salvage pathways were found for GDP-Fuc, UDP-Gal, UDP-GlcA and UDP-GalNAc, which therefore can also be classified as primary nucleotide sugars.

UDP-α-D-glucose (UDP-Glc)

UDP-Glc is synthesized *in vivo* from α-D-glucose-1-phosphate (Glc-1-P) by the action of UDP-Glc pyrophosphorylase (EC 2.7.7.9). There exists another pathway in plants in which sucrose is cleaved by sucrose synthase (EC 2.4.1.13) using nucleoside diphosphates (Fig. 1). UDP is the natural substrate in this reaction yielding UDP-Glc and D-fructose. The substrate spectrum of sucrose synthase comprises dUDP, ADP, dTDP and CDP giving rise to activated glucoses which are partly precursors in bacterial deoxysugar pathways [23]. UDP-Glc was first synthesized from UTP and Glc-1-P by an enzyme preparation from yeast [14]. In the same year this activated sugar was isolated from permeabilized cells of yeast [15]. This fermentative method was further improved [24,25] and is used for the large scale production of UDP-Glc. Some enzymatic syntheses were established too [26]. The described enzymes are very useful for the *in situ* generation of UDP-Glc in the large scale synthesis of UDP-Gal [27] as well as for the *in situ* regeneration of UDP-Gal [28–30] and UDP-GlcA [31–33].

UDP-α-D-galactose (UDP-Gal)

One biosynthetic pathway to UDP-Gal is the epimerisation of UDP-Glc catalyzed by UDP-glucose 4-epimerase (EC 5.1.3.2). Additionally this activated sugar is formed either on the Leloir pathway by uridyl transfer from UDP-Glc to α-D-galactose-1-phosphate (Gal-1-P) catalyzed by galactose-1-phosphate-uridyltransferase (Gal-1-P uridyltransferase) (EC 2.7.7.12) or by the salvage pathway in which Gal-1-P is condensed with UTP by UDP-Gal pyrophosphorylase (EC 2.7.7.8) (Fig. 1). Gal-1-P is formed from galactose by galactokinase (EC 2.7.1.6). The latter pathway was not yet used in preparative synthesis *in vitro,* because of the lacking availability of the UDP-Gal pyrophosphorylase. UDP-Gal has been synthesized more than thirty years ago in a small scale [34,35]. Heidlas et al. [27] used the enzymes of the Leloir pathway to synthesize UDP-Gal in a 4.2 mmol scale yielding 2.5 g (43%) of UDP-Gal. UDP-Glc, the UMP-donor of the uridyltransferase reaction, was regenerated *in situ* by UDP-Glc pyrophosphorylase. We established an alternative method using also Gal-1-P uridyltransferase (Bülter, Elling unpublished results). The side product Glc-1-P was converted to 6-phosphogluconate by phosphoglucomutase (EC 2.7.5.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.27) to shift the equilibrium of the reaction to the product side. The synthesis was performed on a 1.9 mmol scale in a repetitive batch mode [36] in which the partially expensive enzymes were used in 16 subsequent batches without any loss of enzyme activity. The space time yield of the synthesis was 7.1 $g \cdot 1^{-1} \cdot d^{-1}$. The overall yield of the synthesis amounted to 40% and 1.1 gram of pure UDP-Gal was obtained. Whole cells as bioreactors for the synthesis of nucleotide sugars are an attractive alternative. UDP-Gal was synthesized with recombinant *E. coli* cells overexpressing inorganic pyrophosphatase and UDP-Glc pyrophosphorylase. These cells were combined with *Corynebacterium ammoniagenes* that produces UTP from orotic acid. A space time yield of 46 $g \cdot 1^{-1} \cdot d^{-1}$ and 94 g UDP-Gal were obtained [37].

Three different multienzyme systems for the *in situ* regeneration of UDP-Gal have been developed for the synthesis of *N*-acetyllactosamine (LacNAc). Two of these contain four enzymes and start with the conversion of UDP, which is released from UDP-Gal by β1,4galactosyltransferase 1, to UDP-Glc by pyruvate kinase (PK) (EC 2.7.7.40) and UDP-Glc pyrophosphorylase. The equilibrium of the latter is shifted by the cleavage of pyrophosphate to phosphate catalyzed by inorganic pyrophosphatase (EC 3.6.1.1). The energy source for these cycles is phosphoenolpyruvate (PEP), which is relatively expensive. The generation of UDP-Gal is accomplished by UDP-Glc 4-epimerase [28], which can be replaced by Gal-1-P uridyltransferase showing a better equilibrium constant for the formation of UDP-Gal [29].

We have developed a regeneration cycle for UDP-Gal which includes only two enzymes [30]. Sucrose synthase converts UDP and sucrose to UDP-Glc and fructose. UDP-Glc is epimerized to UDP-Gal by UDP-Glc 4-epimerase. The addition of PEP is not necessary in this cycle and phosphate, being a potent enzyme inhibitor is not a side product. Sucrose represents the energy source for this cycle with a free energy of hydrolysis of 29.5 $kJ \cdot mol^{-1}$ (ATP: −30.5; PEP: −61.9) The reaction cycle of the LacNAc synthesis was further combined with a recombinant α1,3galactosyltransferase (Fig. 2). With this extended synthesis we produced the xenotransplantation antigen Gal(α1–3)Gal(β1–4)GlcNAc(β1-O-$(CH_2)_8COOCH_3$ in 82% yield [38]. Recently, α1,3-galactosylated epitopes were synthesized in a similar approach using recombinant α1,3galactosyltransferase [39].

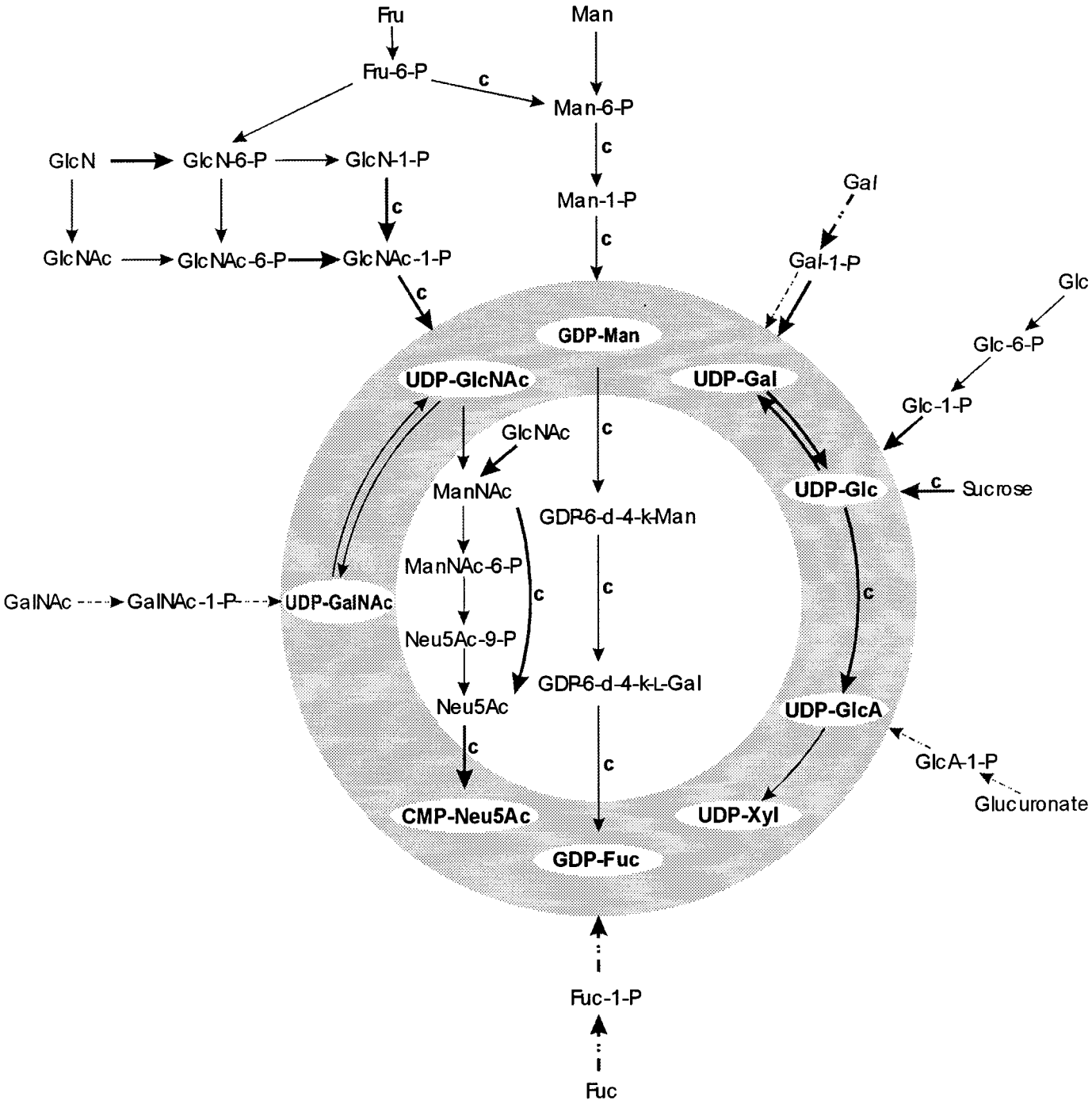

Figure 1. Biosynthetic pathways to the nine nucleotide sugars, that are the most important in mammalian glycoconjugate biosynthesis. Pathways from mammalians, microorganisms and plants are combined in this figure. **C:** The gene encoding the enzyme for this reaction has been cloned; ⇢ : The reaction is part of a salvage pathway; →: The enzymatic reaction was used in large scale preparative synthesis; GDP-6-d-4-k-Man: GDP-6-deoxy-4-keto-α-D-mannose; GDP-6-d-4-k-L-Gal: GDP-6-deoxy-4-keto-β-L-galactose

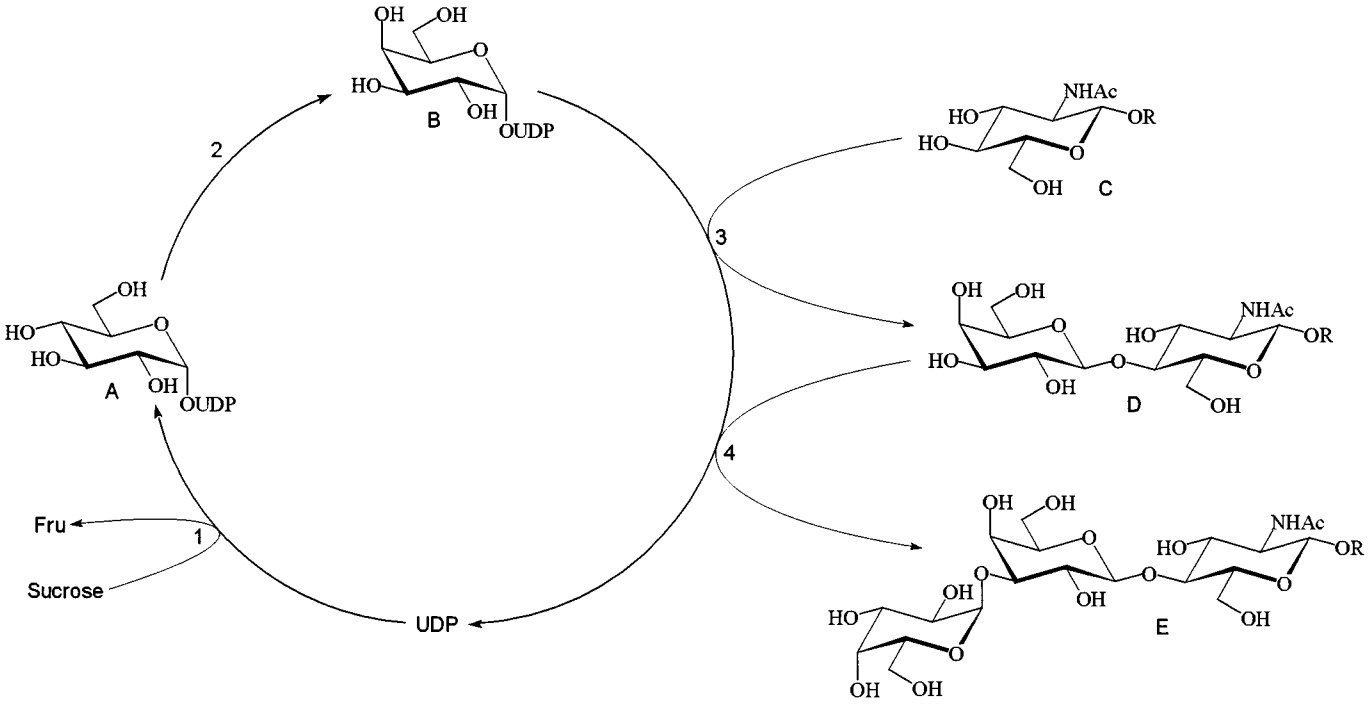

Figure 2. Enzymatic synthesis of the xenotransplantation antigen. Gal(α1–3)Gal(β1–4)GlcNAc(β1-O-$(CH_2)_8COOCH_3$ **(E)** [38] with *in situ* UDP-Gal regeneration [30]. **1:** Sucrose synthase, **2:** UDP-Glc 4-epimerase; **3:** β1–4galactosyltransferase, **4:** α1–3galactosyltransferase; **A:** UDP-Gal, **B:** UDP-Glc, **C:** GlcNAc(β1-O-$(CH_2)_8COOCH_3$; **D:** Gal(β1–4)GlcNAc(β1-O-$(CH_2)_8COOCH_3$, **R:** -$(CH_2)_8COOCH_3$

UDP-*N*-acetyl-α-D-glucosamine (UDP-GlcNAc)

The biosynthesis of UDP-GlcNAc starts generally from fructose-6-phosphate (Fru-6-P), which is converted to D-glucosamine-6-phosphate (GlcN-6-P) by glutamine:fructose-6-phosphate amidotransferase/glucosaminesynthase (EC 2.6.1.16) (Fig. 1). In eucaryotes GlcN-6-P is acetylated to *N*-acetyl-D-glucosamine-6-phosphate (GlcNAc-6-P) by glucosamine-6-phosphate-*N*-acetyltransferase (EC 2.3.1.4). GlcNAc-6-P is isomerized to *N*-acetyl-α-D-glucosamine-1-phosphate (GlcNAc-1-P) by phosphoacetylglucosamine mutase (EC 5.4.2.3) and UDP-GlcNAc pyrophosphorylase (EC 2.7.7.23) converts GlcNAc-1-P and UTP to UDP-GlcNAc. In eubacteria GlcN-6-P is first isomerized into α-D-glucosamine-1-phosphate (GlcN-1-P) by phosphoglucosamine mutase, then acetylation by glucosamine-1-phosphate-acetyltransferase produces GlcNAc-1-P and finally UDP-GlcNAc is synthesized from GlcNAc-1-P and UTP by UDP-GlcNAc pyrophosphorylase. The latter pathway was elucidated three decades ago [40–42] and has been confirmed recently by the purification of the *glmU* gene product glucosamine uridyltransferase (GlmU) from *E. coli* [43]. This bifunctional enzyme catalyzes the last two steps of the biosynthesis of UDP-GlcNAc converting GlcN-1-P to UDP-GlcNAc [44]. Furthermore phosphoglucosamine mutase activity was detected in a crude extract of *E. coli* [44].

GlcNAc from lysosomal degradation of oligosaccharides is directly phosphorylated to GlcNAc-6-P by GlcNAc kinase (EC 2.7.1.59), which was recently purified to homogeneity from rat liver [45] and is widely distributed in mammalian tissues. This enzyme activity was also detected in bacteria [46]. GlcNAc kinase from *E. coli* was purified 130 fold and characterized [46]. Moreover glucosamine-6-phosphate-*N*-acetyltransferase, which also produces GlcNAc-6-P, was detected in bacteria. The presence of these enzymes indicates that a *N*-acetylglucosamine phosphomutase activity should also be present in bacteria for the anabolic utilisation of GlcNAc-6-P. However this mutase was not yet found in bacteria. In summary the pathway outlined above for the biosynthesis of UDP-GlcNAc in bacteria is probably the most important but not the only one.

Since the enzymes of the biosynthetic pathways are neither commercially available nor easy to prepare, syntheses following these routes depend on the activities present in crude cell extracts. Extracts from yeast were used to synthesize UDP-GlcNAc [47] and [^{14}C]-UDP-GlcNAc [48] from GlcNAc-6-P. More recently this synthesis was scaled up for the production of 1.7 mmol (1.1 g) UDP-GlcNAc in a 17% yield [49]. GlcNAc-6-P was synthesized chemically from GlcN-6-P obtained from D-glucosamine (GlcN) using commercially available hexokinase [48,49]. UDP-GlcNAc was synthesized in a 0.6 mmol scale from GlcNAc-1-P in a 60% yield using an enzyme extract from calf liver [50].

An alternative approach made use of side activities of commercially available enzymes [51,52]. The synthesis started with the phosphorylation of GlcN by yeast hexokinase. GlcN-6-P was isomerized to GlcN-1-P using rabbit muscle phosphoglucomutase (PGM) (EC 2.7.5.1). UDP-Glc pyrophosphorylase from yeast converted this product to UDP-GlcN, which was finally acetylated chemically. In this way 1.2 µmol (3.5 millicurie) of [^{32}P]UDP-GlcNAc [51] were produced with 70% yield. A serious draw back of this synthetic approach is the inhibition of UDP-Glc pyrophosphorylase by UTP and GlcN-6-P [52]. Furthermore the relative activities of the enzymes towards the non physiological substrate are low (1% for PGM, 3% for UDP-Glc pyrophosphorylase) the high costs of UDP-Glc pyrophosphorylase not withstanding [49]. By means of a hollow fiber reactor it was possible to reuse the enzymes involved in the synthesis of UDP-GlcN in several batches [52]. Apart from this economical effect the use of a two step method and the incorporation of a UTP regeneration system maintained low concentrations of the inhibiting reactants in the presence of UDP-Glc pyrophosphorylase. In this way 100 µmol (60 mg) UDP-GlcN were synthesized with 16% yield.

Recently, the bifunctional glucosamine uridyltransferase (GlmU) from *E. coli* was used for the synthesis of [^{14}C]UDP-GlcNAc in a 18 µmol (1 millicurie) scale [53]. In this synthesis radioactively labeled acetic acid was transferred to Coenzyme A by Coenzyme A synthetase. The cofactor was used for acetylation of GlcN-1-P by GlmU with subsequent activation to [^{14}C]UDP-GlcNAc. The yield of the synthesis before purification was 90%. A second approach combines the pyrophosphorylase activity of GlmU with a marginal side activity of GalNAc-1-P kinase from pig kidney towards GlcNAc (0.18% of the activity towards GalNAc) [54,55] to synthesize the 5-Azido derivative of UDP-GlcNAc and the radiolabeled activated sugar [56].

The *in situ* regeneration system for UDP-GlcNAc includes the enymes pyruvate kinase and UDP-GlcNAc pyrophosphorylase that synthesize UDP-GlcNAc from UDP produced by a GlcNAc transferase. Early attempts to utilize this regeneration cycle failed due to the presence of phosphateses in the UDP-GlcNAc pyrophosphorylase containing cell extracts [57]. The cloning of *glmU* from *E. coli* solved this problem. Overexpressed GlmU was used in the regeneration cycle for UDP-GlcNAc as part of the enzymatic synthesis of hyaluronic acid [32]. Recently the genes encoding UDP-GlcNAc pyrophosphorylase from *Saccharomyces cerevisia, Candida albicans* and from human were cloned and expressed in *E. coli* JM109 [58] but were not yet used in synthesis or regeneration.

UDP-*N*-acetyl-α-D-galactosamine (UDP-GalNAc)

The nucleotide sugar UDP-GalNAc is synthesized *in vivo* by epimerisation of UDP-GlcNAc (Fig. 1). The UDP-GlcNAc 4-epimerase (EC 2.7.7.23) activity responsible for this reaction was found in a partially purified preparation of UDP-Glc 4-epimerase (EC 5.1.3.2) from rat liver [59] and in a highly purified UDP-Glc 4-epimerase from porcine submaxillary glands [60]. The latter preparation showed equal activity with UDP-Glc and UDP-GlcNAc as substrate. Furthermore a chinese hamster ovary cell mutant was isolated, that had simultaneously lost UDP-glucose and UDP-GlcNAc 4-epimerase activity [61]. In summary these results indicate that only one enzyme is responsible for the epimerisation of UDP-Glc and UDP-GlcNAc in mammals.

In yeasts and bacteria UDP-Glc 4-epimerase (EC 5.1.3.2) does not accept UDP-GlcNAc as substrate [60]. In these organisms the epimerisation of UDP-GlcNAc is catalyzed by an independent UDP-GlcNAc 4-epimerase (EC 5.1.3.7) [62]. The UDP-GlcNAc 4-epimerase was only used for a small scale synthesis of UDP-GalNAc, because the unfavourable equilibrium constant (K_{eq}: 0.3) and the difficult separation of UDP-GalNAc and UDP-GlcNAc limited the yield as well as the scale of the reaction [63].

In a crude extract of rat liver perfused with ^{14}C-α-D-galactosamine-1-phosphate (^{14}C-GalN-1-P) the intermediates and the product of a salvage pathway to UDP-GalNAc were detected [64]. The enzymes postulated for this pathway were not characterized until recently a GalNAc-1-P kinase was isolated from pig kidney [54,55]. Furthermore a UDP-GlcNAc pyrophosphorylase was partially purified from pig kidney, that catalyzes the formation of either UDP-GlcNAc or UDP-GalNAc from UTP and GlcNAc-1-P or GalNAc-1-P respectively [65]. The K_m-value of this UDP-GlcNAc pyrophosphorylase for GalNAc-1-P as substrate is rather high (1.1 mM) suggesting that it is a side activity.

GalNAc-1-P kinase from pig kidney has been used for the two step synthesis of the photoaffinity analog [^{32}P]5-azido-UDP-GalNAc and for the preparation of radiolabeled UDP-GalNAc [56]. GalNAc-1-P kinase was used to phosphorylate radioactively labeled GalNAc to GalNAc-1-P. The purified product of this reaction and photolabeled UTP were converted to labeled UDP-GalNAc by the GlmU protein from *E. coli.* GlmU has also a low pyrophosphorylase activity with GalNAc-1-P as substrate (K_m: 3.7 mM, k_{cat}: 0.1 s^{-1}) [66]. As far as we know, a large scale synthesis of UDP-GalNAc with enzymes from the salvage pathway has not been carried out.

Preparative syntheses of UDP-GalNAc were achieved by a chemoenzymatic approach including the enzymatic synthesis of UDP-α-D-galactosamine (UDP-GalN) and a subsequent chemical acetylation of this product. The enzyme galactose-1-P uridyltransferase (EC 2.7.7.12) from the Leloir-pathway of UDP-Gal was used in these approaches to convert UDP-Glc and α-D-galactosamine-1-phosphate (GalN-1-P) to Glc-1-P and UDP-GalN. The general problem of the enzymatic reaction is the unfavourable equilibrium constant with the unnatural substrate (K_{eq}: 0.26) [67].

The reported syntheses therefore included an excess of substrates and / or further enzymatic steps for the conversion of the side product Glc-1-P to shift the equilibrium. The combination with phosphoglucomutase (EC 2.7.5.1) was used to convert Glc-1-P to Glc-6-P in the presence of excess concentrations of UDP-Glc [68]. In a 60 μmol scale 65% synthesis yield was obtained. A quantitative acetylation of the purified UDP-galactosamine was achieved with acetic anhydride [68]. In the second approach Glc-1-P was used for the regeneration of UDP-Glc by UDP-Glc pyrophosphorylase [27]. UDP-galactosamine was acetylated without prior purification using *N*-acetoxysuccinimide. The synthesis yielded 2.7 mmol (1.75 g) UDP-GalNAc (34%).

We developed a chemoenzymatic synthesis in which UDP-Glc was generated *in situ* from the readily available substrates sucrose and UMP by nucleoside monophosphate kinase (NMPK) (EC 2.7.7.4) and sucrose synthase (Fig. 3) [67]. The equilibrium of the uridyltransferase reaction was forced to the product side by phosphoglucomutase and glucose-6-phosphate dehydrogenase (EC 1.1.1.49). UTP and the cofactor NAD^+ were regenerated by PK and lactate dehydrogenase (EC 1.1.1.27), respectively. The enzymatic synthesis was performed in a repetitive batch mode [36]. Thus the partially expensive enzymes could be used for seven batches. UDP-GalN was acetylated with *N*-acetoxysuccinimide. The yield of the synthesis in a 130 μmol scale was 34%. As far as we know an *in situ* regeneration cycle for UDP-GalNAc has not been applied in combination with GalNAc transferases.

UDP-α-D-glucuronic acid (UDP-GlcA)

UDP-GlcA is synthesized *in vivo* from UDP-Glc by UDP-glucose dehydrogenase (UDP-Glc DH) (EC 1.1.1.22). In plants a salvage pathway exists as part of the inositol oxidation pathway from Glc-6-P to UDP-GlcA via *myo*-inositol and glucuronate-1-P [69]. It permits the plant to by-pass UDP-Glc in the synthesis of UDP-GlcA. Extracts from plants were not used for preparative synthesis. All published syntheses include UDP-Glc DH from mammalian liver. With one exception these have been restricted to small scale, because of the competitive inhibition of the enzyme by the product UDP-GlcA (K_i: 6 μM) [70,71]. Simon et al. produced UDP-GlcA in a 0.1 mmol scale achieving a yield of 68% [72]. [β^{32}P]5-azido-UDP-glucuronic acid was synthesized from the corresponding UDP-Glc derivative in a 0.1 μmol scale [73]. The only large scale synthesis was run in a 1.5 mmol scale yielding 890 mg (87%) UDP-GlcA [74]. Despite the reported product inhibition large amounts of enzyme (100 U) and the employment of 4 equivalents of pyruvate were sufficient to achieve a complete conversion.

In situ regeneration cycles in combination with glucuronyltransferases have been reported. Wherease a crude liver homogenate was used for the synthesis of β-glucuronides [31], a well defined enzyme combination was established for the synthesis of hyaluronic acid [32,33]. In the latter approach both UDP-GlcA and UDP-GlcNAc were regenerated using the enzymes UDP-Glc DH from *E. coli* K5 and GlmU from *E. coli* K12 overexpressed in *E. coli* [33]. The K_m-values of the recombinant UDP-Glc DH for UDP-Glc and NAD^+ were 15 μM and 199 μM, respectively.

UDP-α-D-xylose (UDP-Xyl)

The biosynthesis of UDP-Xyl consists of the decarboxylation of UDP-GlcA catalyzed by UDP-GlcA carboxylyase (EC 4.1.1.35). The enzyme from wheat germ was purified and characterized [75,76]. Unlike the enzyme from *Crypto-*

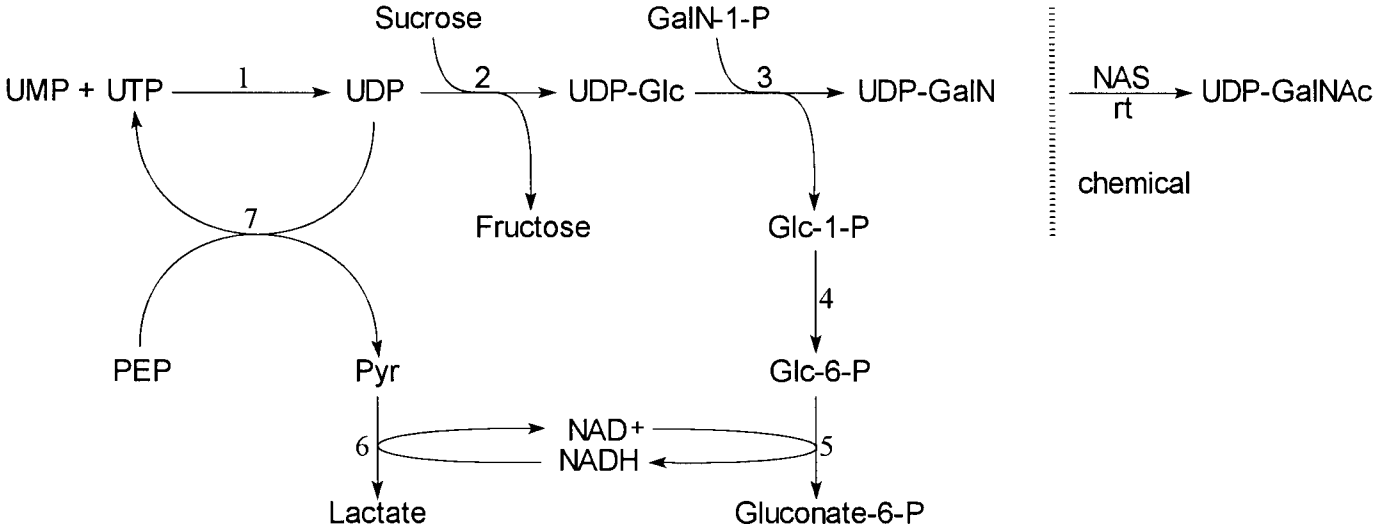

Figure 3. Chemoenzymatic synthesis of UDP-GalNAc [67]. **1:** Nucleosidemonophosphate kinase; **2:** Sucrose synthase; **3:** Galactose-1-phosphate uridyltransferase; **4:** Phosphoglucomutase; **5:** Glucose-6-phosphate dehydrogenase; **6:** Lactate dehydrogenase; **7:** Pyruvate kinase; **NAS:** *N*-acetoxysuccinimide

coccus laurentii, which has an absolute requirement for catalytic amounts of NAD^+ and is inhibited by NADH, no activation or inhibition was detected with the wheat germ enzyme [75]. The K_m value for UDP-GlcA was 0.3 mM and the carboxylyase was inhibited by the product UDP-Xyl (20% at a concentration of 0.2 mM UDP-Xyl) [75]. Recently, the UDP-glucuronic acid carboxylyase was purified 1200 fold from wheat [77]. This preparation was used for the synthesis of 5-azido-[^{32}P]UDP-Xyl in a 20 nmol scale with a 50% yield. To the best of our knowledge no preparative enzymatic synthesis of UDP-Xyl has been published so far.

CMP-*N*-acetyl-β-D-neuraminic acid (CMP-Neu5Ac)

The metabolic route leading to the synthesis of CMP-Neu5Ac starts from Fru-6-P and follows the pathway to UDP-GlcNAc (Fig. 1). UDP is released from UDP-GlcNAc and the aminosugar is simultaneously isomerized to *N*-acetyl-D-mannosamine (ManNAc) by UDP-*N*-acetylglucosamine-2-epimerase (EC 5.1.3.14). ManNAc is then phosphorylated to ManNAc-6-P by ManNAc-kinase (EC 2.7.1.60). The latter two steps are catalyzed by a bifunctional enzyme, which has been recently purified [45] and sequenced [78]. ManNAc can enter the pathway alternatively by epimerisation from GlcNAc catalyzed by GlcNAc-2-epimerase (EC 5.1.3.8). ManNAc-6-P is further converted to *N*-acetyl-D-neuraminic acid 9-phosphate (Neu5Ac-9-P) by Neu5Ac-9-P synthase and Neu5Ac-9-P is dephosphorylated to Neu5Ac by Neu5Ac-9-P phosphatase. All enzymes involved in the biosynthesis of Neu5Ac are localized in the cytoplasm. In contrary, the activation of Neu5Ac with CTP to the nucleotide sugar catalyzed by CMP-Neu5Ac-synthetase occurs in the nucleus by an enzyme recently cloned [79]. In a wide range of bacteria Neu5Ac is synthesized from ManNAc by *N*-acetylneuraminic acid aldolase (NeuAc aldolase) (EC 4.1.3.3) [80]. The gene from *E. coli* K12 C600 was cloned and expressed in *E. coli* M-13 [81]. NeuAc aldolase was used in several enzymatic syntheses of Neu5Ac starting from ManNAc [82–86]. ManNAc was prepared by chemical epimerisation of GlcNAc. Two gram scale syntheses of Neu5Ac were published. Simon *et al.* synthesized Neu5Ac in a 6 mmol scale from ManNAc in a synthesis yield of 90% without purification [87]. Neu5Ac aldolase and *N*-acetylglucosamine-2-epimerase were used in a continuous production of Neu5Ac from GlcNAc in a 140 mmol scale [82]. 15 g of Neu5Ac were produced and purified in a 35% yield. This synthesis was scaled up to yield 1.3 kg of Neu5Ac [83].

CMP-Neu5Ac was synthesized from Neu5Ac comprising only one reaction step (Table 1). CMP-Neu5Ac-synthetase was partially purified from mammalian and microbial sources. The gene encoding this enzyme from *E. coli* was cloned and expressed in *E. coli* with a 36 fold increase in activity [103,104]. More recently the synthetase encoding gene was isolated from *E. coli* O / K1 and overexpressed in *E. coli* W3110 [99]. The expression was 833 fold higher than that published previously [104] and the specific activity in crude cell extracts was 0.25 U · mg^{-1}. In another approach the gene for CMP-Neu5Ac synthetase from *E. coli* K235 was overexpressed 1000 fold in *E. coli* Sure [100]. The specific enzyme activity was 0.05 U · mg^{-1} in crude cell extracts. In some of the synthetic approaches the rather expensive substrate CTP was synthesized enzymatically from CMP using nucleoside monophosphate kinase [84,105] or adenylate kinase [87,97]. Catalytic amounts of ATP or CTP were regenerated *in situ* by pyruvate kinase. One attempt to combine the enzymatic reactions to a one step synthesis from CMP to CMP-Neu5Ac resulted in a low yield due to phosphatase activity in the preparation of CMP-Neu5Ac-synthetase from calf brain [97]. This combined synthesis should be possible using the recombinant enzyme, which was only used for gram scale syntheses of CMP-Neu5Ac from Neu5Ac and CTP so far [99,100]. Another large scale synthesis used CMP-Neu5Ac synthetase from non recombinant *E. coli* K-235 / CS1 [102]. The specific activity in crude cell extracts was 0.05 U · mg^{-1} [101], which is equal to the activity of the recombinant strain published by Shen et al. [100]. The *in situ* regeneration of CMP-NeuAc was achieved in a synthesis of sialyl-*N*-acetyllactosamine (Sialyl-LacNAc) [98]. CMP, the side product of the sialyltransferase-reaction in this synthesis was converted to CDP by NMPK. CTP and ATP were regenerated by PK and recombinant CMP-NeuAc-synthetase produced CMP-NeuAc. The byproduct inorganic pyrophosphate (PP_i) of the synthetase reaction was cleaved to P_i by inorganic pyrophosphatase. This regeneration cycle was also used in the synthesis of α2-3-sialyllactose at the 150 mmol scale [106]. The overall yield of the synthesis was 68% and 77 g of the product were isolated. A fusion protein which had both CMP-Neu5Ac synthetase and α-2,3-sialyltransferase activity was used in this synthesis. The fusion protein was constructed using the genes cloned from *Neisseria meningitidis*. The construct was expressed in *E. coli* BMH71-18 and the specific activity of the CMP-Neu5Ac synthetase in crude cell extract was 0.32 U · mg^{-1}.

GDP-α-D-mannose (GDP-Man)

D-mannose-6-phosphate (Man-6-P) is an intermediate in the biosynthesis of GDP-Man. It is derived either from mannose catalyzed by hexokinase or from Fru-6-P catalyzed by phosphomannose isomerase (EC 5.3.1.8) (PMI). Phosphomannomutase (EC 5.4.2.8) (PMM) isomerizes Man-6-P to α-D-mannose-1-phosphate (Man-1-P), that is activated to GDP-Man by GDP-mannose pyrophosphorylase (EC 2.7.7.13) (GDP-Man PP). Most of the syntheses developed so far made use of crude enzyme preparations. Satisfactory yields of GDP-Man could only be obtained by suppression of the reactions leading to glycolysis via Fru-6-

P. A PMI-deficient mutant of *Salmonella typhimurium* was the enzyme source for the synthesis of [^{14}C]GDP-Man from [^{14}C]Man on a 0.5 μmol scale yielding 60% of the labeled activated sugar [107]. A similar synthesis was performed on a 12 μmol scale yielding 50%. The enzyme source was cells of *Arthrobacter viscosus* that had been frozen to inactivate PMI [108]. In another strategy [^{14}C]GDP-Man was synthesized from [^{14}C]Glc and [^{14}C]Fru catalyzed by an extract from yeast [109]. The monosaccharides entered the pathway to GDP-Man by hexokinase-catalyzed phosphorylation at the C-6 hydroxyl group. The competing glycolytic activities were minimized by the procedure of extract preparation. With 200 μmol (50 mC_i) of the labeled monosaccharides the yield of activated sugar was 20–30%. The same method for the preparation of yeast extract was used to prepare [^{14}C]GDP-Man from [^{14}C]Man [110]. PMI, a zinc-requiring enzyme was inactivated by addition of EDTA to the reaction mixture. Syntheses on a 4.3 nmol (1.4 μC_i) scale yielded 60% of the labeled nucleotide sugar before purification. GDP-Man was synthesized from Man-1-P [72] using a crude GDP-Man PP preparation from yeast [13]. With 100 μmol of Man-1-P 36 mg of GDP-Man (54%) were produced. A similar synthesis on a 20 μmol scale yielded 37% of the activated sugar [111]. GDP-Man PP was purified to homogeneity from pig liver [112]. The activity of the enzyme towards GTP and Glc-1-P (100%) was even higher than towards GTP and Man-1-P (79%). The enzyme was used to synthesize 8-azido-[^{32}P]GDP mannose on an analytical scale (0.5 μmol) (1 mC_i). Recently the genes encoding GDP-Man PP and PMM from *Salmonella enterica* were cloned and overexpressed in *E. coli* BL21(DE3) [113]. The specific activities were 0.1 U · mg for PMM and 0.3–0.6 U · mg^{-1} for GDP-Man PP. Using the recombinant enzymes a well defined enzyme system could be applied to the synthesis of GDP-Man from Man (Fig. 4). It comprised hexokinase for the phosphorylation of Man to Man-6-P. The phosphoryldonor ATP was regenerated from ADP by PK and the equilibrium of GDP-Man PP was shifted to the product side by inorganic pyrophosphatase. The synthesis was performed in a repetitive batch mode, that allowed the repetitive use of enzymes in three subsequent batch syntheses. In this way 1.2 mmol Man were converted to 580 mg GDP Man (80%) with an enzyme consumption of 138 U · g^{-1} product. The overall yield of purified nucleotide sugar was 23% (200 mg). A typical feature of recombinant GDP-Man PP is the influence of the Mg^{2+} / GTP ratio [114]. The enzyme showed the highest activity at a Mg^{2+} / GTP ratio of 1 and a significant decrease of activity at Mg^{2+} / GTP ratios higher than 1 (50% residual activity at a ratio of 2.6). As a consequence GDP-Man PP activity decreased during synthesis in a batch mode because of the consumption of GTP. In addition the kinetic characterisation of GDP-Man PP revealed a strong product inhibition by GDP-Man (K_i = 9 μM) which also lowered the enzyme activity during batch synthesis and increased enzyme consumption. A continuous synthesis in a two stage cascade of enzyme membrane reactors (EMR) solved both problems [114]. The EMR allowed the adjustment of a constant Mg^{2+} / GTP ratio. The combination of two reactors in a cascade was chosen to overcome product inhibition. The space-time yield of the synthesis was 28 g · L^{-1} · d^{-1} and the enzyme consumption in the two step EMR cascade (0.9 U · $g_{product}^{-1}$) was sixfold lower than in an optimized batch reactor. Starting from 95 mmol Man-1-P 300 mg (57%) of crude GDP-Man were produced. The regeneration of GDP-Man in combination with α1-2-mannosyltransferase has been reported [115]. Man-1-P was activated to GDP-Man by GDP-Man PP, which was converted by the α1-2-mannosyltransferase to the mannosylsaccharide and GDP. The latter was converted to GTP by PK and PEP. The side product PP_i was hydrolyzed by inorganic pyrophosphatase to shift the equilibrium and to avoid the inhibition of GDP-Man PP (K_i = 16 μM) [114].

GDP-β-L-fucose (GDP-Fuc)

GDP-fucose is synthesized *in vivo* via two different pathways. The *de novo* synthesis is evolutionarily conserved and was identified in bacteria [116–118], mammals [119,120], seaworm [121] and plants [122]. It starts from GDP-Man which is converted to GDP-Fuc in three steps. GDP-Man is converted to GDP-6-deoxy-4-keto-α-D-mannose by GDP-mannose-4,6-dehydratase (GDP-Man DHy) (EC 4.2.1.47). The second reaction is an isomerisation to GDP-6-deoxy-4-keto-β-L-galactose, which is finally reduced to GDP-Fuc.

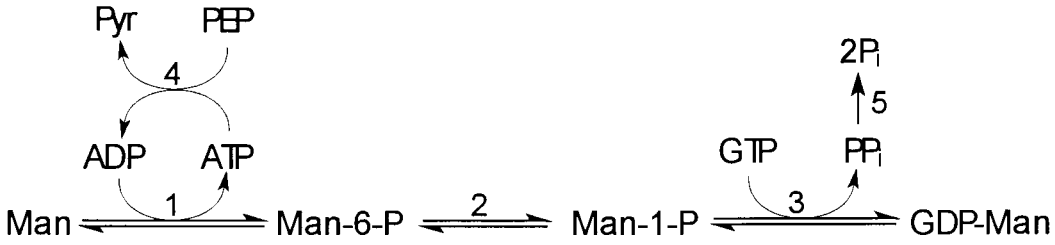

Figure 4. Enzymatic synthesis of GDP-Man [113]. **1:** Hexokinase; **2:** Phosphomannomutase; **3:** GDP-mannose pyrophosphorylase; **4:** Pyruvate kinase; **5:** Inorganic pyrophosphatase

The last two steps of the pathway are catalyzed by the bifunctional enzyme GDP-6-deoxy-4-keto-mannose 3,5-epimerase 4-reductase (fucose synthetase). In *E. coli* the enzymes of this pathway are encoded in the colanic acid cluster, which is responsible for the synthesis of the fucose containing exopolysaccharide colanic acid. GDP-Man DHy has been purified from porcine thyroid [123] and *Klebsiella pneumoniae* [124]. The gene encoding GDP-Man DHy from *Arabidopsis thaliana* was expressed in *E. coli* XL1 Blue MRF' [125]. Recently, also the gene from *E. coli* strain INVF1 was expressed in *E. coli* [126] and in another approach the gene from *E. coli* K12 was expressed in *E. coli* strain GI934 [127]. Also the human gene was cloned and expressed in *E. coli* BL21(DE3) [127]. In another approach the human gene was cloned and expressed in Lec13 cells [128]. The GDP-Man DHy from human, *E. coli* and *K. pneumoniae* were shown to utilize $NADP^+$ and not NAD^+ as a cofactor. One potential regulatory mechanism of the pathway is the inhibition of GDP-Man DHy by the final product GDP-Fuc [129]. Fucose synthetase was first detected in porcine liver [130]. More recently the human gene encoding FX protein was cloned and the protein was identified as fucose synthetase [131]. The gene from *E. coli* K12 was expressed in the *E. coli* K12 strain SØ874 [132] and the fucose synthetase was crystallized [133]. Fucose synthetase from *E. coli* and human can utilize NADH or NADPH, but in both cases NADPH is used more efficiently [132]. The recombinant enzymes GDP-Man DHy and fucose synthase were not yet used for preparative synthesis.

The salvage pathway to GDP-Fuc was first proposed after studies in rats showing that radiolabeled L-fucose could be incorporated into glycoproteins [134,135]. These results suggested a salvage pathway from L-fucose via β-L-fucose-1-phosphate (Fuc-1-P) to GDP-Fuc. The enzymes L-fucose kinase (EC 2.7.1.52) and GDP-fucose pyrophosphorylase (GDP-Fuc PP) (EC 2.7.7.31), involved in this pathway, were partially purified from pig liver [136,137]. The L-fucose kinase preparation was used to synthesize Fuc-1-P from L-fucose on a 1 mmol scale [136]. The reaction yielded only 7.5% of Fuc-1-P in 75% purity. From the conversion of 14 μmol Fuc-1-P catalyzed by the GDP-Fuc PP preparation 3 mg (36%) GDP-Fuc could be isolated [137]. These syntheses were optimized by improving the isolation of the enzymes to yield 40% Fuc-1-P and 60% of GDP-Fuc [138]. GDP-Fuc was synthesized directly from L-fucose with an extract from hog submaxillary glands in a 0.1 mmol scale [139]. The synthesis yielded 81% GDP-Fuc. Using an extract from porcine submaxillary glands Fuc-1-P was synthesized on a 3 mmol scale [140]. The addition of an ATP-regeneration system consisting of PEP and PK reduced the concentration of the L-fucose kinase inhibitor ADP to catalytic amounts and therefore doubled the yield of the synthesis to 80%. In the same study GDP-Fuc was synthesized on a 50 μmol scale yielding only 23%, because of instability of the product during purification. L-fucose kinase was purified 23000 fold from porcine thyroid [141]. Recently L-fucose kinase was purified to homogeneity from pig kidney [142], but the enzyme was not yet used for synthesis. The enzymes of the long pathway to GDP-Fuc have been used for the synthesis of GDP-Fuc from GDP-Man in a 100 μmol scale achieving a yield of 10–30% [143]. When performed on an analytical scale the same synthesis yielded 70–90% of GDP-Fuc. Presumably the product inhibition of GDP-Man DHy limited the yield of the large scale approach. The enzyme source was a crude extract from *Aerobacter aerogenes.* In a similar synthesis on a 70 μmol scale a crude extract of *Agrobacterium radiobacter* was used [144]. The yield of the synthesis was not published. On an analytical scale the recombinant enzymes GDP-Man DHy and fucose synthetase from either *E. coli* or human were shown to convert GDP-Man to GDP-Fuc [127]. The recombinant enzymes are also of interest for the regeneration of GDP-Fuc in oligosaccharide synthesis. Until now only crude enzyme preparations were used in regeneration cycles developed for the synthesis of sialyl Lewis^x (sialyl Le^x) [145]. The regeneration cycle, which was based on the salvage pathway combined the PK / PEP regeneration of GTP with GDP-Fuc PP partially purified from porcine thyroids. This regeneration was only suitable for analytical scale synthesis of sialyl Le^x. For the synthesis of 5–10 mg of sialyl Le^x another regeneration cycle was used which included the enzymes of the *de novo* pathway to GDP-Fuc. GDP-Man PP from dried yeast cells and a crude extract from *Klebsiella pneumonia* containing GDP-Fuc generating enzymes were combined with the PK / PEP regeneration of GTP and alcohol dehydrogenase from *Thermoanaerobium brockii* for the regeneration of $NADP^+$ [145].

Conclusions

In the present review we have shown that many enzymes for the synthesis of nucleotide sugars are described in biosynthetic pathways. Some enzymes are available as highly expressed recombinant proteins, however, only few of them are characterized as suitable biocatalysts for the development of industrial processes. In this respect a choice must be made among the enzymes from different organisms. We think that the enzymes from microbial sources are best suited for nucleotide sugar synthesis in glycotechnology. In future, this may also hold for microbial glycosyltransferases synthesizing oligosaccharide structures with an industrial impact. However, as far as these are not available glycotechnology should rely on recombinant mammalian glycosyltransferases produced in suitable hosts.

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