



# UDP-*N*-Acetyl- $\alpha$ -D-glucosamine as acceptor substrate of $\beta$ -1,4-galactosyltransferase. Enzymatic synthesis of UDP-*N*-acetylactosamine

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The capacity of UDP-*N*-acetyl- $\alpha$ -D-glucosamine (UDP-GlcNAc) as an *in vitro* acceptor substrate for  $\beta$ -1,4-galactosyltransferase ( $\beta$ 4GalT1, EC 2.4.1.38) from human and bovine milk and for recombinant human  $\beta$ 4GalT1, expressed in *Saccharomyces cerevisiae*, was evaluated. It turned out that each of the enzymes is capable to transfer Gal from UDP- $\alpha$ -D-galactose (UDP-Gal) to UDP-GlcNAc, affording Gal( $\beta$ 1-4)GlcNAc( $\alpha$ 1-UDP (UDP-LacNAc). Using  $\beta$ 4GalT1 from human milk, a preparative enzymatic synthesis of UDP-LacNAc was carried out, and the product was characterized by fast-atom bombardment mass spectrometry and <sup>1</sup>H and <sup>13</sup>C NMR spectroscopy. Studies with all three  $\beta$ 4GalTs in the presence of  $\alpha$ -lactalbumin showed that the UDP-LacNAc synthesis is inhibited and that UDP- $\alpha$ -D-glucose is not an acceptor substrate. This is the first reported synthesis of a nucleotide-activated disaccharide, employing a Leloir glycosyltransferase with a nucleotide-activated monosaccharide as acceptor substrate. Interestingly, in these studies  $\beta$ 4GalT1 accepts an  $\alpha$ -glycosidated GlcNAc derivative. The results imply that  $\beta$ 4GalT1 may be responsible for the biosynthesis of UDP-LacNAc, previously isolated from human milk.

**Keywords:** glycosyltransferase/ $\beta$ -1,4-galactosyltransferase/nucleotide-activated disaccharide/UDP-LacNAc

**Abbreviations:** BSA, bovine serum albumin; FAB-MS, Fast Atom Bombardment Mass Spectrometry; 2D HMBC, two-dimensional Heteronuclear Multiple-Bond Coherence; 2D ROESY, two-dimensional Rotating Frame Nuclear Overhauser Enhancement Spectroscopy; 2D TOCSY, two-dimensional Total Correlation Spectroscopy;  $\beta$ 4GalT,  $\beta$ -1,4-galactosyltransferase;  $\alpha$ -LA,  $\alpha$ -lactalbumin; LacNAc, *N*-acetylactosamine; NMR, Nuclear Magnetic Resonance; UDP-GlcNAc, uridine 5'-diphospho-*N*-acetyl- $\alpha$ -D-glucosamine; UDP-Gal, uridine 5'-diphospho- $\alpha$ -D-galactose; UDP-GalNAc, uridine 5'-diphospho-*N*-acetyl- $\alpha$ -D-galactosamine; UDP-Glc, uridine 5'-diphospho- $\alpha$ -D-glucose; UDP-LacNAc, uridine 5'-diphospho-*N*-acetylactosamine; UDP-Xyl, uridine 5'-diphospho- $\alpha$ -D-xylose.

## Introduction

$\beta$ -1,4-Galactosyltransferase ( $\beta$ 4GalT, EC 2.4.1.38) is the best characterized Leloir-glycosyltransferase with respect to *in vitro* oligosaccharide synthesis. It is commercially available and easily purified from bovine [1] or human milk [2,3]. When bound to the milk protein  $\alpha$ -lactalbumin ( $\alpha$ -LA)  $\beta$ 4GalT forms the lactose synthase complex (EC 2.4.1.22) which has a high affinity for free glucose, thus synthesizing lactose [Gal( $\beta$ 1-4)Glc] in lactating mammary glands [4–6]. A  $\beta$ -1,4-galactosyltransferase gene family has

been reported recently [7] the members of which show different properties. The  $\beta$ -1,4-galactosyltransferase studied in this paper is the  $\beta$ 4GalT1 according to the nomenclature in reference [7].

The broad synthetic potential of  $\beta$ 4GalT1 has been widely exploited for the chemoenzymatic synthesis of oligosaccharides and (neo)glycoconjugates. Thus it was demonstrated that  $\beta$ 4GalT1 transfers Gal from UDP- $\alpha$ -D-galactose (UDP-Gal) to GlcNAc and its  $\beta$ -glycosides, whereas lactose synthase is involved *in vitro* in the galactosylation of Glc and its  $\alpha$ - and  $\beta$ -glycosides [8–14]. In a reaction cycle with three enzymes we have utilized  $\beta$ 4GalT1 from bovine and human milk for the synthesis of *N*-acetylactosamine [Gal( $\beta$ 1-4)GlcNAc; LacNAc] on a gram scale [15], and in combination with recombinant  $\alpha$ 1,3-galactosyltransferase for the

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synthesis of the Galili-epitope Gal( $\alpha$ 1-3)Gal( $\beta$ 1-4)GlcNAc ( $\beta$ 1-O(CH<sub>2</sub>)<sub>8</sub>COOCH<sub>3</sub>) [16].

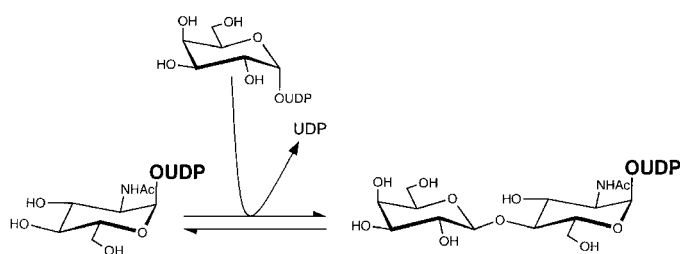
Our current investigations on nucleotide-activated oligosaccharides were strongly inspired by Kobata [17], who in 1963 reported the isolation and identification of a nucleotide-activated disaccharide, Gal( $\beta$ 1-4)GlcNAc( $\alpha$ 1-UDP (UDP-LacNAc), and a nucleotide-activated trisaccharide, Fuc(1-2/4)Gal( $\beta$ 1-4)GlcNAc( $\alpha$ 1-UDP, from human milk and colostrum. In the same period, nucleotide-activated di- and trisaccharides were also identified in goat colostrum [Neu5Ac-Gal(1-6)GlcNAc( $\alpha$ 1-UDP and Neu5Ac-Gal(1-4)GlcNAc( $\alpha$ 1-UDP] [18], and hen oviduct [Gal( $\beta$ 1-*P*-6)GlcNAc( $\alpha$ 1-UDP and Fuc(1-4)GlcNAc( $\alpha$ 1-UDP] [19,20]. The finding of such compounds in hen oviduct demonstrated that nucleotide-activated oligosaccharides are not unique to mammals. The biosynthesis and the physiological function of these nucleotide-activated di- and trisaccharides have not yet been elucidated. In contrast, at the end of the eighties, UDP- and GDP-activated oligosaccharides were found in archaeobacteria and identified as precursors in the biosynthesis of cell wall components such as pseudomurein and the S-layer [21–25].

In the present paper we demonstrate that Gal( $\beta$ 1-4)GlcNAc( $\alpha$ 1-UDP (UDP-*N*-acetylactosamine; UDP-LacNAc) can be synthesized by human and bovine  $\beta$ 4GalT1 *in vitro*. This implies that the nucleotide sugar UDP-*N*-acetyl- $\alpha$ -D-glucosamine (UDP-GlcNAc) serves as an acceptor substrate of a mammalian Leloir glycosyltransferase,  $\beta$ 4GalT1, whereby Gal is transferred from UDP-Gal to  $\alpha$ -glycosidated GlcNAc (Fig. 1). The synthesis of UDP-LacNAc by a Leloir glycosyltransferase promises a wide synthetic and biochemical application but also raises questions about the cellular occurrence and physiological function of this compound as will be discussed in this paper.

## Materials and methods

### Materials

Uridine 5'-diphospho- $\alpha$ -D-galactose (UDP-Gal), uridine 5'-diphospho- $\alpha$ -D-glucose (UDP-Glc), uridine 5'-diphospho-*N*-acetyl- $\alpha$ -D-glucosamine (UDP-GlcNAc), uridine 5'-



**Figure 1.** UDP-*N*-acetyl- $\alpha$ -D-glucosamine (UDP-GlcNAc) as acceptor substrate of  $\beta$ -1,4-galactosyltransferase (EC 2.4.1.38) with UDP- $\alpha$ -D-galactose as donor substrate: Enzymatic synthesis of Gal( $\beta$ 1-4)GlcNAc( $\alpha$ 1-UDP (UDP-LacNAc).

diphospho-  $\alpha$ -D-xylose (UDP-Xyl), bovine serum albumin (BSA),  $\alpha$ -lactalbumin ( $\alpha$ -LA), and  $\beta$ -1,4-galactosyltransferase ( $\beta$ 4GalT1) from bovine milk (EC 2.4.1.38) were purchased from Sigma (Deisenhofen, Germany). Uridine 5'-diphospho-*N*-acetyl- $\alpha$ -D-galactosamine (UDP-GalNAc) was synthesized as reported earlier [26]. Human milk and recombinant human  $\beta$ 4GalT1 were obtained as previously described [3,27]. Calf intestinal alkaline phosphatase (EC 3.1.3.1) was from Boehringer Mannheim (Mannheim, Germany). HEPES (*N*-[2-(hydroxyethyl) piperazine]*N'*-(2-ethanesulphonic acid)) was supplied by Roth (Karlsruhe, Germany). D<sub>2</sub>O (99.9 and 99.96 atom % D) was purchased from Isotec, Veendaal, The Netherlands. All other chemicals were from Merck (Darmstadt, Germany).

### Synthesis of UDP-*N*-acetylactosamine with $\beta$ -1,4-galactosyltransferase

In a standard assay, a buffer solution of 200 mM HEPES-NaOH pH 7.0, 1 mM MnCl<sub>2</sub>, 1 mg ml<sup>-1</sup> BSA, 0.01% NaN<sub>3</sub> contained 10 mM UDP-Gal and 10 mM UDP-GlcNAc. The reaction was started by the addition of 0.4 U ml<sup>-1</sup>  $\beta$ 4GalT1. The assay volume was 100  $\mu$ l and the incubation temperature 30 °C. To study the effect of  $\alpha$ -LA, variable amounts of  $\alpha$ -LA were added to the assay as indicated below. Reactions were stopped by heating at 95 °C for 5 min as indicated in the Results and discussion section. Reaction mixtures were diluted with distilled water (1:5) and analyzed by HPLC on a Hypersil ODS column (250  $\times$  4.6 mm, 5  $\mu$ m particle size) (CS Chromatografie Service, Langerwehe, Germany); elutions were performed with 100 mM potassium acetate pH 5.6, containing 0.013% (v/v) *n*-octylamine and 5% (v/v) methanol at a flow rate of 1 ml min<sup>-1</sup>, monitoring the effluent at 260 nm [28]. Yields were determined by the relation between the peak area for uridine 5'-diphospho-*N*-acetylactosamine (UDP-LacNAc) and the sum of the peak areas for UDP-LacNAc and the corresponding substrate (acceptor or donor).

### Preparative Synthesis of UDP-*N*-acetylactosamine with $\beta$ -1,4-galactosyltransferase from human milk

To a solution of UDP-Gal (61 mg Na<sub>2</sub>-salt, 100  $\mu$ mol), UDP-GlcNAc (65.2 mg Na<sub>2</sub>-salt, 100  $\mu$ mol), and BSA (10 mg) in 200 mM HEPES-NaOH, pH 7.0 (10 ml), containing 1 mM MnCl<sub>2</sub>, was added 4 U  $\beta$ 4GalT1 from human milk, and the mixture was incubated for 7 days at 30 °C under sterile conditions. The reaction was stopped by denaturation of the enzyme at 95 °C (5 min), and an aliquot of the solution was diluted (1:5) with distilled water for analysis by HPLC [28]. The yield of the enzymatic synthesis was 14.6% (with reference to the acceptor substrate).

For the isolation of the product, proteins were removed in a 10 ml stirred ultrafiltration cell from Amicon (Witten, Germany) using a YM 10 membrane (cut-off 10 kDa). The protein-free solution was adjusted to pH 6.2, and loaded on

a column ( $1.2 \times 8$  cm) of Sepharose Q FF (Cl<sup>-</sup>-form; Pharmacia, Freiburg, Germany) equilibrated with distilled water. After washing with distilled water (30 ml), UDP-LacNAc was eluted with a linear salt-gradient (75 ml distilled water, 75 ml 1 M LiCl) at a flow rate of  $1 \text{ ml min}^{-1}$ . The fractions containing UDP-LacNAc (UV detection at 260 nm) were pooled and adjusted to pH 7.0. The solution was concentrated at  $35^\circ\text{C}$  *in vacuo* and finally desalted by gel filtration on a column ( $2.6 \times 74.5$  cm) of Bio-Gel P-2 extra fine (Bio-Rad, Deisenhofen, Germany) using distilled water at a flow rate of  $0.25 \text{ ml min}^{-1}$  and at  $4^\circ\text{C}$ . After lyophilisation, the product was analyzed by  $^1\text{H}$  and  $^{13}\text{C}$  nuclear magnetic resonance (NMR) spectroscopy and fast-atom bombardment mass spectrometry (FAB-MS). The overall yield was 5.2% UDP-LacNAc Na<sub>2</sub>-salt (based on the acceptor substrate;  $5.2 \mu\text{mol}$ , 4.2 mg).

### Mass spectrometry

Negative-ion mode Fast Atom Bombardment Mass Spectrometric (FAB-MS) analysis of the reaction product was performed on a JEOL JMS-SX/SX 102A four sector instrument operated at an acceleration voltage of 6 kV. The JEOL MS-FAB 10D FAB gun was operated at an emission current of 10 mA, producing a beam of 4 keV Xe atoms.  $1 \mu\text{l}$  sample ( $10 \mu\text{g}$  in  $0.1 \text{ ml}$  5% acetic acid) was mixed with  $1 \mu\text{l}$  thioglycerol matrix, and linear mass scans over 1000 dalton were recorded. Recorded data were processed using JEOL complement software (Bijvoet Center, Department of Mass Spectrometry).

### NMR spectroscopy

Prior to analysis the reaction products were repeatedly exchanged in D<sub>2</sub>O (99.9 atom % D) with intermediate lyophilisation and finally dissolved in  $450 \mu\text{l}$  D<sub>2</sub>O (99.96 atom % D). Proton-decoupled 75.469-MHz  $^{13}\text{C}$  NMR spectra were recorded on a Bruker AC-300 spectrometer at a probe temperature of 300 K. Chemical shifts ( $\delta$ , ppm) are referenced to external acetone ( $\delta$  31.08). Resolution enhanced  $^1\text{H}$  1D and 2D NMR spectra were recorded on Bruker AMX-500 or Bruker AMX-600 instruments (Department of NMR spectroscopy, Utrecht University) at a probe temperature of 300 K. Chemical shifts ( $\delta$ ) were expressed in ppm relative to internal acetate ( $\delta$  1.908, acetone  $\delta$  2.225). HOD signal suppression was achieved by applying a WEFT pulse sequence in 1D  $^1\text{H}$  experiments and by pre-saturation for 1 s in 2D experiments. 2D TOCSY (total correlation spectroscopy) spectra were recorded by using MLEV-17 mixing sequences with effective spin-lock times between 20 and 100 ms. 2D ROESY (rotating frame nuclear Overhauser enhancement spectroscopy) spectra were recorded with a mixing time of 250 ms [29]. The spin-lock field strength corresponded to a  $90^\circ$  pulse of about  $115 \mu\text{s}$ . A proton detected  $^{13}\text{C}$ - $^1\text{H}$  2D HMBC (heteronuclear multiple-bond coherence) experiment was performed at a  $^1\text{H}$

frequency of 600.140 MHz (150.916 MHz for  $^{13}\text{C}$ ) using a pulse sequence as described by Summers *et al.* [30]. The delay time for the detection of long-range  $^{13}\text{C}$ - $^1\text{H}$  couplings was set to 60 ms.  $^1\text{H}$  1D and 2D spectra were processed on Silicon Graphics IRIS work stations (Indigo 2 and O2) using Bruker UXNMR software (Bijvoet Center, Department of NMR Spectroscopy).  $^{13}\text{C}$  1D spectra were elaborated on Silicon Graphics IRIS work stations (Indigo 2 and O2) using TRITON software (Bijvoet Center, Department of NMR Spectroscopy).

### Effect of different parameters on UDP-LacNAc synthesis

First, the effect of the  $\text{MnCl}_2$  concentration (1–20 mM) and the amount of  $\alpha$ -LA on UDP-LacNAc synthesis with  $\beta$ 4GalT1 from human or bovine milk was investigated using the assay as described above.

Secondly, the concentration of the acceptor substrate UDP-GlcNAc was varied at a constant concentration of 10 mM UDP-Gal. The assay was performed as described above with an incubation time of 4.5 days.

Thirdly, the synthesis of UDP-LacNAc with  $\beta$ 4GalT1 was performed using the assay as described above with 10 mM UDP-Gal and 40 mM UDP-GlcNAc in the presence of  $2 \text{ U ml}^{-1}$  calf intestinal alkaline phosphatase at  $30^\circ\text{C}$  [31].

### Substrate specificity of $\beta$ -1,4-galactosyltransferase

In a standard assay, a 10 mM donor nucleotide sugar/10 mM acceptor nucleotide sugar buffer solution (200 mM HEPES-NaOH, pH 7.0, 1 mM  $\text{MnCl}_2$ ,  $1 \text{ mg ml}^{-1}$  BSA, 0.01%  $\text{NaN}_3$ ;  $100 \mu\text{l}$ ) containing 0, 1 or  $8 \text{ mg ml}^{-1}$   $\alpha$ -LA, was incubated with  $0.4 \text{ U ml}^{-1}$  recombinant human  $\beta$ 4GalT1, bovine  $\beta$ 4GalT1, and human milk  $\beta$ 4GalT1, respectively. After an incubation period of 6 days, the reaction was stopped by denaturation of the enzymes at  $95^\circ\text{C}$  (5 min) and the mixture was analyzed by HPLC [28]. The following donor/acceptor combinations of nucleotide sugars were tested: UDP-Gal/UDP-Glc, UDP-Gal/UDP-Xyl, UDP-Gal/UDP-GalNAc, UDP-Gal/UDP-Gal, UDP-Glc/UDP-GlcNAc, UDP-Glc/UDP-Glc, UDP-Xyl/UDP-Xyl, UDP-GalNAc/UDP-GlcNAc, and UDP-GalNAc/UDP-Glc. Control experiments contained only the donor substrate and were treated in the same way as described above.

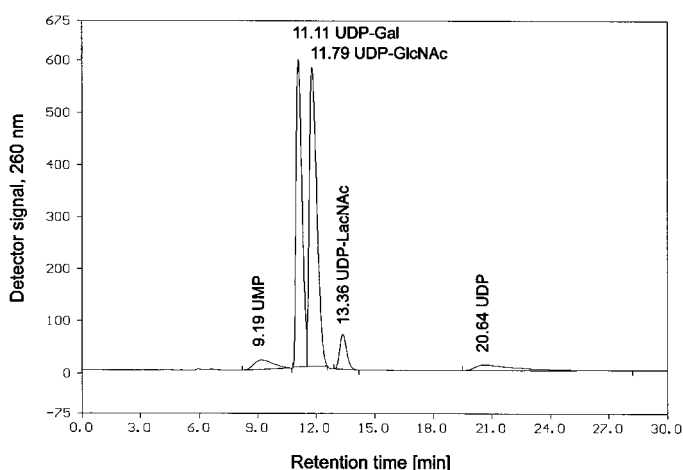
## Results and discussion

### UDP-N-Acetyl- $\alpha$ -D-glucosamine as acceptor substrate of $\beta$ -1,4-galactosyltransferase

$\beta$ -1,4-Galactosyltransferase ( $\beta$ 4GalT1) accepts a wide spectrum of derivatives of the natural donor UDP-Gal and the natural acceptor GlcNAc [12]. In all reported cases the enzyme catalyzes the transfer of galactose or its derivative to the HO-4 position of a  $\beta$ -linked GlcNAc or its derivative.

It was also stated that  $\beta$ 4GalT1 is not the suitable biocatalyst for preparative synthesis when *N*-acetyl- $\alpha$ -D-glucosaminides are used as acceptors [32,33].

In our initial experiments  $\beta$ 4GalT1 from bovine and human milk were tested for their ability to accept UDP-*N*-acetyl- $\alpha$ -D-glucosamine (UDP-GlcNAc) as acceptor substrate (Fig. 1). After 7 days of incubation at 30 °C, HPLC profiling revealed in each case the appearance of a product eluting after UDP-Gal and UDP-GlcNAc; the yields were 15% and 14.6% (based on acceptor), respectively. The retention time of the product peak corresponded to that of Gal( $\beta$ 1-4)GlcNAc( $\alpha$ 1-UDP (UDP-LacNAc) synthesized via a transgalactosylation reaction from lactose and UDP-GlcNAc in the presence of  $\beta$ -galactosidase from *Bacillus circulans* [Zervosen A, Nieder V, Gutiérrez Gallego R, Kamerling JP, Vliegthart JFG, Elling L, unpublished results]. To exclude the possibility of contamination of the milk  $\beta$ 4GalT1 preparations used in this work with other enzymes, the experiment was repeated with purified recombinant human  $\beta$ 4GalT1 [27]. After an incubation time of 6 days the same HPLC profile was obtained with a product in a yield of 9.7% (based on the acceptor substrate) (Fig. 2). In order to characterize the product, a preparative enzymatic synthesis was performed with  $\beta$ 4GalT1 from human milk. After 7 days of incubation at 30 °C, a product was formed in a yield of 14.6% (HPLC analysis; based on the acceptor substrate). Isolation of the product led to an overall product yield of 5.2% (5.2  $\mu$ mol, 4.2 mg).



**Figure 2.** Synthesis of UDP-LacNAc with recombinant human  $\beta$ 4GalT1 as biocatalyst, as analyzed by HPLC (column: Hypersil ODS column (250  $\times$  4.6 mm, 5  $\mu$ m particle size); solvent system: 100 mM potassium acetate pH 5.6, containing 0.013% (v/v) *n*-octylamine and 5% (v/v) methanol; flow rate: 1 ml min<sup>-1</sup>; UV-detection: 260 nm) [28]. A buffer solution of 200 mM HEPES-NaOH pH 7.0, 1 mM MnCl<sub>2</sub>, and 0.01% NaN<sub>3</sub> contained 10 mM UDP-Gal and 10 mM UDP-GlcNAc. The incubation was carried out in the presence of 0.4 U ml<sup>-1</sup>  $\beta$ 4GalT1 and 1 mg ml<sup>-1</sup> BSA for 6 days at 30 °C.

## Structural studies of UDP-*N*-acetylglucosamine

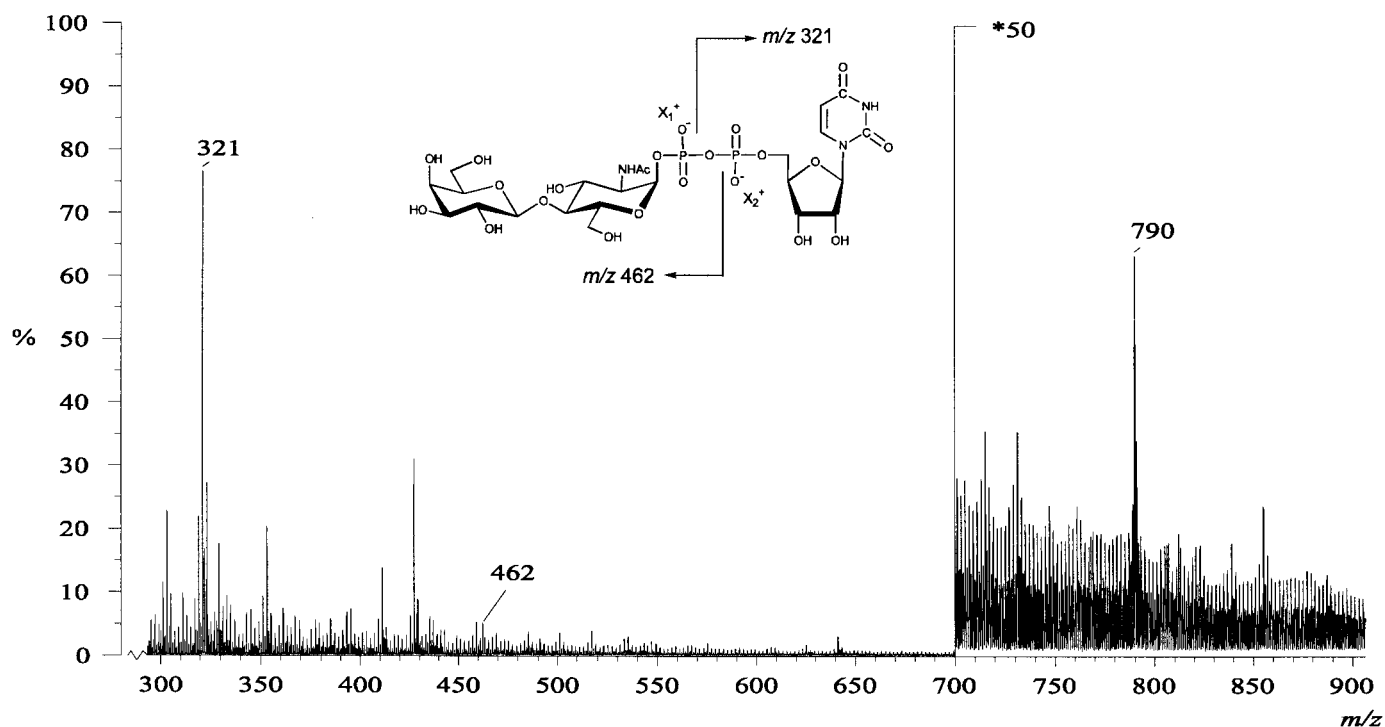
The negative-ion mode FAB-MS spectrum of the nucleotide-activated saccharide showed one intense peak in the high-mass region at  $m/z$  790.0 corresponding to the [M-Na]<sup>-</sup> pseudo-molecular ion of Hex-HexNAc-UDP ( $X_{1,2}$  = Na, Fig. 3). Two fragment ions were found at  $m/z$  462.0 and 321.0 belonging to Hex-HexNAc-*P* and UMP, respectively.

The 1D <sup>1</sup>H NMR spectrum (Fig. 4) showed four signals downfield of the HOD signal ( $\delta$  4.754). Two of these signals, at  $\delta$  8.002 and  $\delta$  5.969 (<sup>3</sup>*J* 8.1 Hz), were attributed to the uracil-ring protons based on literature [34,35]. The anomeric signal at  $\delta$  5.969 (<sup>3</sup>*J*<sub>1,2</sub> 2.6 Hz) was assigned to the ribose residue R ( $\beta$  configuration, furanose ring form), whereas the remaining anomeric signal at  $\delta$  5.511 (<sup>3</sup>*J*<sub>1,2</sub> 3.2 Hz, <sup>3</sup>*J*<sub>1,P</sub> 7.2 Hz) was assigned to the GlcNAc residue A ( $\alpha$  configuration, pyranose ring form) linked to the phosphate group. Two chemical shifts, upfield of the HOD resonance could also be assigned; the singlet at  $\delta$  2.072 originating from the *N*-acetyl protons of GlcNAc A and the anomeric doublet at  $\delta$  4.465 (<sup>3</sup>*J*<sub>1,2</sub> 7.5 Hz) which reflects the Gal residue B ( $\beta$  configuration, pyranose ring form).

The <sup>13</sup>C NMR revealed four signals in the anomeric region at  $\delta$  103.70 (B<sub>C-1</sub>), 103.20 (U<sub>C-5</sub>), 95.00 (A<sub>C-1</sub>), and 89.80 (R<sub>C-1</sub>) (Figure 4). Furthermore, four signals were observed in the downfield region at  $\delta$  175.55 (A<sub>C=O</sub>), 166.70 (U<sub>C-2</sub>), 152.30 (U<sub>C-4</sub>), and 142.10 (U<sub>C-6</sub>). Typical GlcNAc resonances were found at  $\delta$  22.72 (A<sub>NAc-CH<sub>3</sub></sub>) and  $\delta$  53.89 (A<sub>C-2</sub>). The signals at  $\delta$  60.30, 61.62 and 65.13 reflected the presence of three hydroxymethyl groups, the latter of which is substituted.

By means of 2D TOCSY, ROESY and HMBC experiments all <sup>13</sup>C and almost all <sup>1</sup>H resonances in the 1D spectra could be assigned (Table 1). In the TOCSY spectra (not shown) the three identified anomeric <sup>1</sup>H signals were used to assign the complete spin systems; a mixing time of 100 ms allowed the identification of all resonances corresponding to a single residue, whereas a mixing time of 20 ms made the sequential assignment possible.

To establish the linkage type between residues B and A, 2D ROESY and HMBC experiments were performed. In the ROESY spectrum (not shown) the anomeric track of residue B revealed several cross-peaks at  $\delta$  3.998, 3.79, 3.737, 3.659 and 3.573. The cross-peak at  $\delta$  3.573 was attributed to TOCSY transfer to H-2 of the same residue. The cross-peaks at  $\delta$  3.737 and 3.659 were identified as intraresidual connectivities to H-5 and H-3, respectively. The remaining cross-peaks at  $\delta$  3.998 and 3.79 were assigned as interresidual contacts to H-5 and H-4/3 of residue A, respectively. In the <sup>13</sup>C-<sup>1</sup>H HMBC spectrum (Fig. 4) the visualization of the interresidual three-bond connectivities over the glycosidic bond yielded the unambiguous determination of the B( $\beta$ 1-4)A sequence via two long-range couplings between A<sub>C-4</sub> and B<sub>H-1</sub> ( $\delta$  79.12 and 4.465) and between B<sub>C-1</sub> and A<sub>H-4</sub> ( $\delta$  103.70 and 3.767).



**Figure 3.** FAB-MS (negative-ion mode) of UDP-LacNAc, Gal( $\beta 1-4$ )GlcNAc( $\alpha 1$ -UDP).

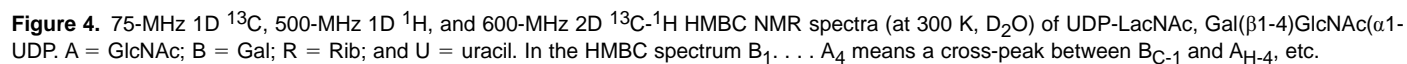
#### Evaluation of different conditions for UDP-*N*-acetylactosamine synthesis

In order to gain further insight into the enzymatic synthesis of UDP-LacNAc from UDP-GlcNAc and UDP-Gal with  $\beta 4\text{GalT1}$  as a biocatalyst, the influence of different parameters was investigated. In earlier reports an optimal synthesis of Gal( $\beta 1-4$ )GlcNAc with  $\beta 4\text{GalT1}$  from human and bovine milk was achieved by enzyme stabilization with BSA and a concentration of  $\text{Mn}^{2+}$  between 20 and 50 mM [36,37]. In another study, an inhibitory effect of  $\text{Mn}^{2+}$  concentrations  $>4$  mM at pH 8.0 on the activity of bovine milk  $\beta 4\text{GalT1}$  was found with 10 mM GlcNAc and 0.25 mM UDP-Gal [38].

In the present study, applying incubation periods of 7 days, a  $\text{Mn}^{2+}$  concentration of 1 mM at pH 7.0 was found to be optimal for UDP-LacNAc synthesis using the human or bovine  $\beta 4\text{GalT1}$ . It was noted for the bovine enzyme that  $\text{Mn}^{2+}$ , at concentrations between 10 and 20 mM, and a pH between 7.5 and 7.9 catalyzes efficiently the decomposition of UDP-Gal (within 4 h) to UMP and Gal 1,2-cyclic phosphate, leading to a decrease in the product formation. These results are in accordance with studies on the stability of UDP-Gal [39]. However, UDP-GlcNAc was found to be stable for at least 9 h under the same conditions [39]. Based on these data, we suggest that a  $\text{Mn}^{2+}$  concentration of 1 mM reflects more the physiological conditions and may explain the biosynthesis of UDP-LacNAc, previously described to occur in human milk [17].

In additional experiments, the effect of higher acceptor concentrations on the yield of UDP-LacNAc was investigated. In Figure 5 it is shown that the yield of UDP-LacNAc can be further increased by increasing the concentration of UDP-GlcNAc. At 30 mM UDP-GlcNAc and 10 mM UDP-Gal a yield of 19.3% (based on UDP-Gal) after 4.5 days was obtained. Figure 5 also suggests that higher yields can be obtained at even higher UDP-GlcNAc concentrations. In comparison to the former conditions with 10 mM for each of the substrates the yield could be increased more than two-fold when incubated for 4.5 days.

The addition of calf intestinal alkaline phosphatase as used in other glycosyltransferase-mediated syntheses [31] has a beneficial effect on the yield of UDP-LacNAc. In Figure 6 it is shown that the yield increases up to 80% (based on UDP-Gal) within 19 days. However, at such long incubation periods decomposition of UDP-Gal has to be considered. Therefore, the yields based on the donor substrate were corrected by comparison with the yields based on the acceptor substrate. A yield of 17.4% based on acceptor substrate after 19 days corresponds to a corrected yield of 69.6% based on donor substrate indicating that 69.6% of UDP-Gal are converted to UDP-LacNAc and 10.4% of UDP-Gal are decomposed within 19 days (Fig. 6). It is obvious that decomposition of UDP-Gal is not a severe problem within 4.5 days of incubation. The yield does not increase very much after 4.5 days when compared to Figure 5 in the absence of alkaline phosphatase; this may



**Table 1.**  $^1\text{H}$  and  $^{13}\text{C}$  NMR chemical shifts of Gal( $\beta 1-4$ )GlcNAc ( $\alpha 1$ -UDP, prepared with  $\beta 1,4$ -galactosyltransferase from human milk, recorded at 300 K in  $\text{D}_2\text{O}$ . Coupling constants (Hz) are given between brackets.

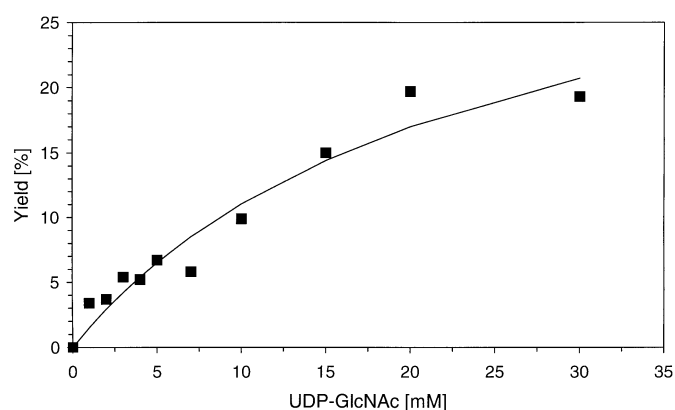
Residue	H or C	$^1\text{H}^a$	$^{13}\text{C}^b$
GlcNAc	A <sub>1</sub>	5.511 (3.2, 7.2)	95.00
	A <sub>2</sub>	4.032	53.89
	A <sub>3</sub>	3.806	70.29
	A <sub>4</sub>	3.767	79.12
	A <sub>5</sub>	3.998	72.37
	A <sub>6(a)</sub>	n.d. <sup>c</sup>	60.30
	A <sub>6b</sub>	3.885 <sup>c</sup>	—
	A <sub>CH3</sub>	2.072	22.72
	A <sub>C=O</sub>	—	175.55
	B <sub>1</sub>	4.465 (7.5)	103.70
Gal	B <sub>2</sub>	3.573	71.66
	B <sub>3</sub>	3.659	73.11
	B <sub>4</sub>	3.933	68.97
	B <sub>5</sub>	3.737	75.98
	B <sub>6(a)</sub>	3.872 <sup>c</sup>	61.62
	B <sub>6b</sub>	3.792 <sup>c</sup>	—
	R <sub>1</sub>	5.969 (2.6)	89.80
Rib	R <sub>2</sub>	4.364	74.76
	R <sub>3</sub>	4.364	69.69
	R <sub>4</sub>	4.289	83.93
	R <sub>5(a)</sub>	4.265	65.13
	R <sub>5b</sub>	4.212	—
Uracil	U <sub>2</sub>	—	166.70
	U <sub>4</sub>	—	152.30
	U <sub>5</sub>	5.969 (8.1)	103.20
	U <sub>6</sub>	8.002 (8.1)	142.10

<sup>a</sup>In ppm relative to the signal of internal acetate ( $\delta$  1.908).<sup>b</sup>In ppm relative to the signal of external acetone ( $\delta$  31.08).<sup>c</sup>The assignment of H-6a and H-6b may have to be interchanged within one residue; n.d. = not determined.

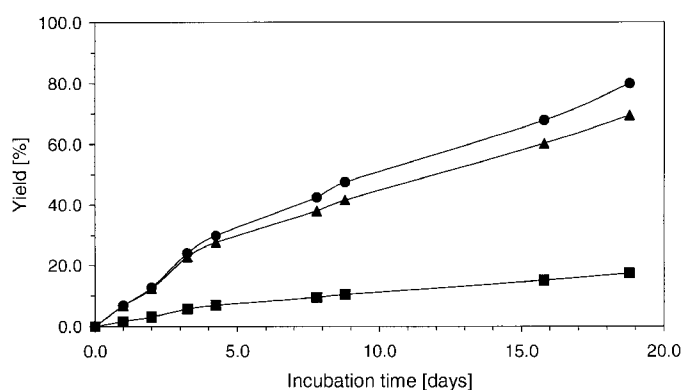
be due to differences in commercial preparations of the used bovine enzyme. However, alkaline phosphatase should be added in order to obtain a maximum yield.

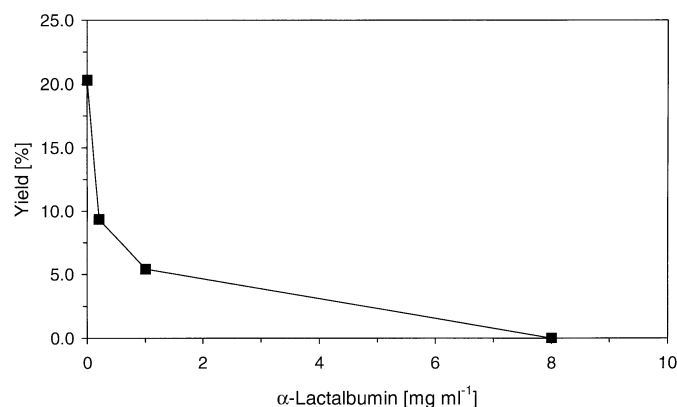
### Effect of $\alpha$ -lactalbumin and substrate specificity

As reported previously, the modification of the  $\beta 4\text{GalT1}$  activity by  $\alpha$ -lactalbumin ( $\alpha$ -LA) depends on the type and the concentration of the acceptor.  $\alpha$ -LA acts as an inhibitor when the GlcNAc concentration is varied near its  $K_m$  value of 5.8 mM [6,38,40,41] and as an activator when the GlcNAc concentration is well below the  $K_m$  value ( $<2\text{mM}$ ) [37,40,41]. The effect of  $\alpha$ -LA on the UDP-LacNAc formation when using bovine  $\beta 4\text{GalT1}$  is shown in Figure 7. The yield of UDP-LacNAc decreases up to an  $\alpha$ -LA concentration of 1 mg ml<sup>-1</sup> (70  $\mu\text{M}$ ), and the synthesis is completely inhibited at 8 mg ml<sup>-1</sup> (560  $\mu\text{M}$ ). In fact, our results can be compared to the classical studies by Brew *et al.* [40] which indicated 85% inhibition of the LacNAc synthesis at 2 mg

**Figure 5.** Effect of increasing UDP-GlcNAc concentrations on the yield of UDP-LacNAc. The yield is based on the initial UDP-Gal concentration. A buffer solution of 200 mM HEPES-NaOH pH 7.0, 1 mM  $\text{MnCl}_2$ , and 0.01%  $\text{NaN}_3$  contained different concentrations of UDP-GlcNAc and 10 mM UDP-Gal. Incubations were carried out in the presence of 0.4 U ml<sup>-1</sup>  $\beta 4\text{GalT1}$  from bovine milk and 1 mg ml<sup>-1</sup> BSA for 4.5 days at 30 °C.

ml<sup>-1</sup> (140  $\mu\text{M}$ )  $\alpha$ -LA. On the other hand, Berliner *et al.* [42] demonstrated a 100% activation of bovine  $\beta 4\text{GalT1}$  in the presence of 0.4 mg ml<sup>-1</sup> (30  $\mu\text{M}$ )  $\alpha$ -LA and 2 mM GlcNAc. In this study it was also stated that the enzymatic conversion of glucosamine acceptors carrying longer *N*-acyl chains (e.g., *N*-octanoyl-) is inhibited by  $\alpha$ -LA concluding that  $\alpha$ -LA binds to a hydrophobic region of  $\beta 4\text{GalT1}$  and that both binding sites are juxtaposed. The effect of  $\alpha$ -LA on the conversion of UDP-GlcNAc may also be similarly interpreted. Although being an  $\alpha$ -glycosidated acceptor,

**Figure 6.** Effect of alkaline phosphatase (2 U ml<sup>-1</sup>) on the yield of UDP-LacNAc. A buffer solution of 200 mM HEPES-NaOH pH 7.0, 1 mM  $\text{MnCl}_2$ , and 0.01%  $\text{NaN}_3$  contained 40 mM UDP-GlcNAc and 10 mM UDP-Gal. Incubations were carried out in the presence of 0.4 U ml<sup>-1</sup>  $\beta 4\text{GalT1}$  from bovine milk and 1 mg ml<sup>-1</sup> BSA for several days at 30 °C. ●, Yield based on donor substrate; ■, yield based on acceptor substrate; ▲, corrected yield indicating the conversion of UDP-Gal into the product UDP-LacNAc. The difference between the yield based on donor substrate and the corrected yield is due to partial decomposition of UDP-Gal.



**Figure 7.** Effect of  $\alpha$ -lactalbumin on the formation of UDP-LacNAc by  $\beta$ 4GalT1 from bovine milk. A buffer solution of 200 mM HEPES-NaOH pH 7.0, 1 mM MnCl<sub>2</sub>, and 0.01% NaN<sub>3</sub> contained 10 mM UDP-Gal and 10 mM UDP-GlcNAc. Incubations were carried out at 30 °C in the presence of 0.4 U ml<sup>-1</sup>  $\beta$ 4GalT1, 1 mg ml<sup>-1</sup> BSA and varying concentrations of  $\alpha$ -lactalbumin.

the nucleotide moiety of the activated sugar may occupy a part of the  $\alpha$ -LA binding site, thereby contributing to the binding of the acceptor substrate. Elucidation of the exact mechanism must await three-dimensional structural analysis of  $\beta$ 4GalT1.

In order to investigate further the substrate specificity of  $\beta$ 4GalT1 other nucleotide sugars in different donor/acceptor combinations in the absence and presence of  $\alpha$ -lactalbumin were tested. The results are summarized in Table 2 for recombinant human  $\beta$ 4GalT1. The effect of  $\alpha$ -LA on the formation of UDP-LacNAc (Fig. 7) supports the well-known role of  $\alpha$ -LA as a modulator of  $\beta$ 4GalT1 decreasing the enzyme's affinity for *N*-acetyl-D-glucosamine [6]. However, although it has been shown previously [6] that  $\alpha$ -LA increases the affinity of  $\beta$ 4GalT1 for glucose, UDP-Glc was not found to be an acceptor substrate for the enzyme in the presence of  $\alpha$ -LA (UDP-lactose could not be detected by HPLC). This result was not surprising because  $\beta$ 4GalT1 accepts  $\alpha$ - and  $\beta$ -glucosides in the presence of  $\alpha$ -LA provided that the aglycon is small [8,32,42]. In earlier studies it was demonstrated that Glc $\alpha$ 1-*P* is not an acceptor in the presence of  $\alpha$ -LA [42,43] implying that a negative charge hinders the recognition by the enzyme. However, in the donor/acceptor combination UDP-Gal/UDP-Glc UDP is formed in the presence and absence of  $\alpha$ -LA indicating that UDP-Gal is enzymatically hydrolyzed. A control experiment with UDP-Gal as acceptor (20 mM UDP-Gal in the reaction) also exhibited hydrolysis. In addition, Table 2 clearly indicates that no other combination of nucleotide sugars with or without  $\alpha$ -LA gives rise to the formation of a nucleotide-activated oligosaccharide. In some combinations, UDP is produced by enzymatic hydrolysis. With UDP-Xyl and UDP-GalNAc as acceptors UDP is only formed in the presence of  $\alpha$ -LA. In earlier studies, xylose

**Table 2.** Substrate specificity of recombinant human  $\beta$ 4GalT1 with different donor/acceptor combinations of nucleotide monosaccharides. In a standard assay, a 10 mM donor nucleotide sugar/10 mM acceptor nucleotide sugar buffer solution (200 mM HEPES-NaOH, pH 7.0, 1 mM MnCl<sub>2</sub>, 1 mg ml<sup>-1</sup> BSA, 0.01% NaN<sub>3</sub>; 100  $\mu$ l) containing 0, 1 or 8 mg ml<sup>-1</sup>  $\alpha$ -LA, was incubated with 0.4 U ml<sup>-1</sup> recombinant human  $\beta$ 4GalT1. After an incubation period of 6 days, the reaction was stopped by denaturation of the enzymes at 95 °C (5 min) and the mixture was analyzed by HPLC [28]. Control experiments contained only the donor nucleotide sugar.

Donor substrate	Acceptor substrate	Formation <sup>a</sup> of NAO <sup>b</sup> /UDP		
		Addition of $\alpha$ -lactalbumin (mg ml <sup>-1</sup> )		
		None	1	8
UDP-Gal	UDP-Glc	-/+	-/+	-/+
UDP-Gal	UDP-Xyl	-/-	-/+	-/+
UDP-Gal	UDP-GalNAc	-/-	-/+	-/+
UDP-GalNAc	UDP-Glc	-/-	-/-	-/-
UDP-GalNAc	UDP-GlcNAc	-/-	-/-	-/-
UDP-Glc	UDP-GlcNAc	-/-	-/-	-/-
UDP-Glc	UDP-Glc	-/-	-/-	-/-
UDP-Xyl	UDP-Xyl	-/-	-/-	-/-
UDP-Gal	UDP-Gal	-/-	-/+	-/+

<sup>a</sup>- no formation, + formation, of NAO and UDP, respectively.

<sup>b</sup>NAO: nucleotide activated oligosaccharide.

was reported to be a good acceptor in the presence of  $\alpha$ -LA [36,42] and has been recently identified as the first acceptor of bovine milk  $\beta$ 4GalT1 giving rise to two disaccharide products (Gal( $\beta$ 1-4)Xyl and Gal( $\beta$ 1- $\beta$ 1)Xyl) [14]. UDP-GalNAc was reported to serve as a good donor substrate of  $\beta$ 4GalT1 from bovine milk in the presence of a high  $\alpha$ -LA concentration (8 mg ml<sup>-1</sup>) and GlcNAc as acceptor yielding GalNAc( $\beta$ 1-4)GlcNAc [44]. In our experiments the donor/acceptor combinations UDP-GalNAc/UDP-GlcNAc and UDP-GalNAc/UDP-Glc gave neither a nucleotide disaccharide nor UDP, respectively, in the presence or absence of  $\alpha$ -LA (Table 2). UDP-Glc was also identified to be a donor substrate of bovine  $\beta$ 4GalT1 with GlcNAc as acceptor [45,46]. A transfer reaction to UDP-GlcNAc or hydrolysis of UDP-Glc could not be detected in our experiments. Similar results were also obtained for  $\beta$ 4GalT1 from human and bovine milk (data not shown).

The findings of UDP-GlcNAc as an acceptor substrate may also be interpreted with respect to previous findings concerning the donor specificity of  $\beta$ 4GalT1. It has been shown that in addition to UDP-Gal also other naturally occurring UDP-sugars can serve *in vitro* as donor sub-



strates of  $\beta 4\text{GalT}$  [47]. These were interpreted as side activities which can be exploited for synthesis. UDP-Glc and UDP-GalNAc were utilized as donor substrates of bovine  $\beta 4\text{GalT1}$  [46,48] for the synthesis of Glc( $\beta 1-4$ )GlcNAc( $\beta 1$ -OR and, in the presence of  $\alpha$ -LA, for the synthesis of GalNAc( $\beta 1-4$ )GlcNAc [44].

More experimental evidence concerning the ability to synthesize UDP-LacNAc may come from a new human  $\beta 4\text{GalT}$  family [7]. The new members,  $\beta 4\text{GalT2}$ ,  $\beta 4\text{GalT3}$  [7],  $\beta 4\text{GalT4}$  [49],  $\beta 4\text{GalT5}$  [50,51], and  $\beta 4\text{GalT6}$  [52] show a different response of activity towards the modulation by  $\alpha$ -LA.  $\beta 4\text{GalT3}$  and 5 are insensitive to  $\alpha$ -LA and transfer little if any Gal from UDP-Gal to Glc. They do not show any inhibition of LacNAc synthesis, however,  $\beta 4\text{GalT2}$  reacts like the bovine  $\beta 4\text{GalT}$ . On the other hand,  $\beta 4\text{GalT4}$  activity is increased by  $\alpha$ -LA for Glc and GlcNAc as acceptor substrates [49] and is required together with  $\beta$ -1,3-*N*-acetylglucosaminyltransferase for the efficient biosynthesis of poly-*N*-acetyllactosamine in core 2 branched O-glycans [53].  $\beta 4\text{GalT6}$  is involved in the biosynthesis of lactosylceramide [52]. Whether one of these new  $\beta 4\text{GalT}$  family members has a higher affinity and activity towards UDP-GlcNAc as an acceptor substrate remains to be investigated. In this context it may also be elucidated whether UDP-LacNAc plays a donor substrate role in the biosynthesis of poly-LacNAc glycans in glycoproteins and glycolipids.

### General remarks

The observation that UDP-GlcNAc is *in vitro* an acceptor substrate of  $\beta 4\text{GalT1}$  from human milk is of biochemical importance and gives a plausible explanation for the biosynthesis of UDP-LacNAc identified in human milk [17]. The biochemical and physiological role of UDP-LacNAc is still unclear, but can now be addressed since we have established the preparative access to this compound by using  $\beta 4\text{GalT1}$  (present paper) as well as by using  $\beta$ -galactosidase from *Bacillus circulans* in a transgalactosylation reaction (Zervosen A, Nieder V, Gutiérrez Gallego R, Kamerling JP, Vliegthart JFG, Elling L, unpublished results). The enzymatic synthesis of UDP-LacNAc as a nucleotide-activated building bloc may promise novel applications of prokaryotic and eukaryotic *N*-acetylglucosaminyltransferases (GlcNAcTs) in oligosaccharide synthesis, e.g., synthesis of poly-*N*-acetyllactosamine (poly-LacNAc) or branched N- and O-glycan structures. Work is in progress in our laboratory to check different *N*-acetylglucosaminyltransferases for their ability to transfer LacNAc *en bloc* from UDP-LacNAc onto specified acceptor structures. In addition, the cellular appearance and the physiological function of UDP-LacNAc as well as inhibition studies of GlcNAcTs and nucleotide sugar transporters can be addressed.

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