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Synthesis of Nucleotide-Activated Oligosaccharides by β-Galactosidase from *Bacillus circulans*

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The enzymatic access to nucleotide-activated oligosaccharides by a glycosidase-catalyzed transglycosylation reaction was explored. The nucleotide sugars UDP-GlcNAc and UDP-Glc were tested as acceptor substrates for β-galactosidase from *Bacillus circulans* using lactose as donor substrate. The UDP-disaccharides Gal(β1-4)GlcNAc(α1-UDP) (UDP-LacNAc) and Gal(β1-4)Glc(α1-UDP) (UDP-Lac) and the UDP-trisaccharides Gal(β1-4)Gal(β1-4)GlcNAc(α1-UDP and Gal(β1-4)Gal(β1-4)Glc(α1-UDP) were formed stereo- and regioselectively. Their chemical structures were characterized by ^{1}H and ^{13}C NMR spectroscopy and fast atom bombardment mass spectrometry. The synthesis in frozen solution at –5 °C instead of 30 °C gave significantly higher product yields with respect to the acceptor substrates. This was due to a remarkably higher product stability in the small liquid phase of the frozen reaction mixture. Under optimized conditions, at –5 °C and pH 4.5 with 500 mM lactose and 100 mM UDP-GlcNAc, an overall yield of 8.2% (81.8 µmol, 62.8 mg with 100% purity) for Gal(β1-4)GlcNAc(α1-UDP) and 3.6% (36.1 µmol, 35 mg with 96% purity) for Gal(β1-4)Gal(β1-4)GlcNAc(α1-UDP) was obtained. UDP-Glc as acceptor gave an overall yield of 5.0% (41.3 µmol, 32.3 mg with 93% purity) for Gal(β1-4)Glc(α1-UDP) and 1.6% (13.0 µmol, 12.2 mg with 95% purity) for Gal(β1-4)Gal(β1-4)Glc(α1-UDP). The analysis of other nucleotide sugars revealed UDP-Gal, UDP-GalNAc, UDP-Xyl and dTDP-, CDP-, ADP- and GDP-Glc as further acceptor substrates for β-galactosidase from *Bacillus circulans*.

Key words: Glycosidase / Nucleotide sugars / Transglycosylation / UDP-lactose / UDP-*N*-acetyllactosamine / UDP-trisaccharides.

Introduction

Three decades ago nucleotide-activated oligosaccharides were isolated from milk of mammals, including man, and characterized as elongated UDP-*N*-acetyl-α-D-glucosamine (UDP-GlcNAc) derivatives (Jourdian *et al.*, 1961; Kobata, 1962, 1963, 1966; Kobata and Ziro, 1965). Goat colostrum turned out to contain Neu5Ac-Gal(1-6)GlcNAc-UDP and Neu5Ac-Gal(1-4)GlcNAc-UDP (Jourdian *et al.*, 1961). In human milk and colostrum Gal(β1-4)GlcNAc-UDP and Fuc(1–2 or 4)Gal(β1-4)GlcNAc-UDP were found (Kobata, 1963, 1966). Nucleotide-activated disaccharides were also isolated from hen oviduct and identified as Gal(β1-*P*-6)GlcNAc(α1-UDP) and Fuc(1-4)GlcNAc-UDP (Suzuki, 1962; Nakanishi *et al.*, 1967). Although these glycoconjugates are known to be present in higher animals, their biosynthesis and biological function have not yet been elucidated.

In archaebacteria UDP- and GDP-activated oligosaccharides were identified as precursors in the biosynthesis of cell wall components such as pseudomurein (*e. g.* GalNAc(β1-3)GlcNAc-UDP) and the S-layer (*e. g.* saccharide-GlcNAc-UDP, saccharide-Gal-UDP, saccharide-ManNAc-GDP; Hartmann and König, 1989; König *et al.*, 1989, 1994; Hartmann *et al.*, 1993). However, the biosynthetic route is still not known and the enzymes involved have not yet been isolated and characterized.

In this context an access to nucleotide-activated oligosaccharides by chemical and/or enzymatic methods could help to elucidate the biosynthesis and biological function of these naturally occurring glycoconjugates. In contrast to earlier findings (Jourdian and Distler, 1973), we have recently demonstrated that UDP-GlcNAc is also an *in vitro* acceptor substrate for β-1,4-galactosyltransferase (β4GalT1, EC 2.4.1.38) from human and bovine milk, resulting in the formation of Gal(β1-4)GlcNAc(α1-UDP) (UDP-LacNAc; Elling *et al.*, 1999). This result implies that β4GalT1 may be responsible for the biosynthesis of UDP-LacNAc found in human milk.

Jourdian and Distler (1973) reported the enzymatic *in vitro* synthesis of the UDP-disaccharides Gal(β1-3, 4 or 6)GlcNAc-UDP using the transglycosylation activity of β-galactosidase from bovine testes with UDP-GlcNAc as acceptor substrate. Since these products could be further sialylated by incubation with a goat colostrum sialyltransferase and CMP-*N*-acetylneuraminic acid, it was proposed that this might be the pathway for the enzymatic synthesis of UDP-disaccharides and UDP-trisaccharides (Jourdian and Distler, 1973).

In order to investigate improvements in the regioselectivity, we have started to test several glycosidases with

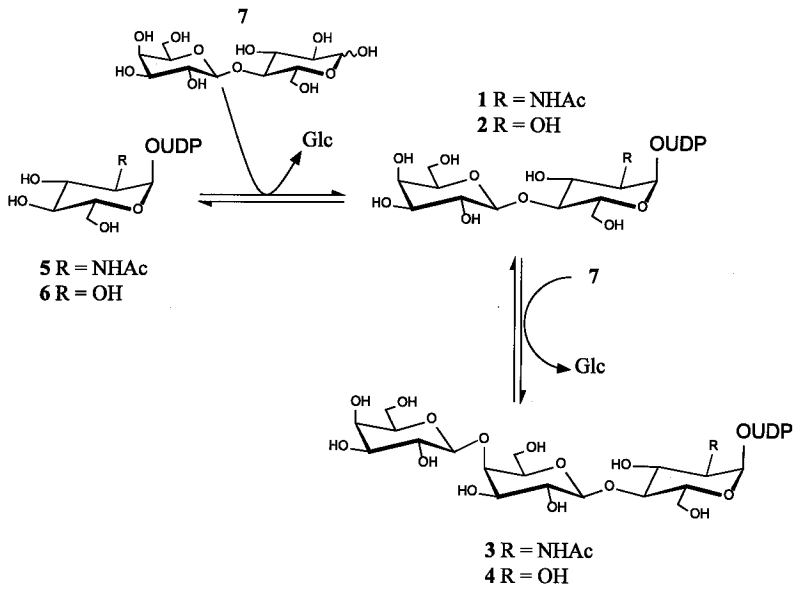


Fig. 1 The Synthesis of UDP-Di- and -Trisaccharides by β-Galactosidase from *Bacillus circulans*. Gal(β1-4)GlcNAc(α1-UDP) [**1**] (UDP-LacNAc) and Gal(β1-4)Gal(β1-4)GlcNAc(α1-UDP) [**3**], and Gal(β1-4)Glc(α1–UDP) [**2**] (UDP-Lac) and Gal(β1-4)Gal(β1-4)Glc(α1-UDP) [**4**] were formed in the transglycosylation reaction of β-galactosidase from *Bacillus circulans* with UDP-GlcNAc [**5**] and UDP-Glc [**6**], respectively, as acceptor substrates and lactose [**7**] as donor substrate.

nucleotide sugars as acceptor substrates in transglycosylation reactions. The present report describes the utilization of UDP-GlcNAc and UDP-Glc as acceptor substrates for β-galactosidase (EC 3.2.1.23) from *Bacillus circulans*, resulting in the selective synthesis of UDP-*N*-acetyllactosamine [Gal(β1-4)GlcNAc(α1-UDP), UDP-LacNAc] and UDP-lactose [Gal(β1-4)Glc(α1-UDP), UDP-Lac] and their corresponding Gal(β1-4)-elongated UDP-trisaccharides (Figure 1).

Results and Discussion

Formation of Nucleotide-Activated Oligosaccharides

UDP-α-D-glucose and UDP-*N*-acetyl-α-D-glucosamine were tested at 30 °C as acceptor substrates with lactose as donor in the transglycosylation reaction of β-galactosidase from *Bacillus circulans*. Thin layer chromatography (TLC) analysis of incubation mixtures revealed in each case two new spots with R_f values of 0.11 and 0.08, very close to the spots of the nucleotide-activated monosaccharides (R_f 0.15), suggesting the formation of nucleotide-activated oligosaccharides. Besides lactose (R_f 0.37), in each case also a spot with a R_f value of 0.24 appeared, most likely representing the transglycosylation product Gal(β1-4)Gal(β1-4)Glc. This trisaccharide has been reported to be formed in the transglycosylation reaction with lactose as donor and acceptor using β-galactosidase from *Bacillus circulans* (Yanahira *et al.*, 1995). The analyses by ion-pair-reversed-phase HPLC on Hypersil ODS confirmed the formation of two nucleotide-activated oligosaccharide products with UDP-GlcNAc (Figure 2A) and with UDP-Glc (Figure 2B) as acceptor substrates, respectively. The HPLC peak for UDP-*N*-acetyllactosamine (UDP-LacNAc) was assigned by comparison with UDP-LacNAc synthesized by β-1,4-galactosyltransferase (Elling *et al.*, 1999). UDP-trisaccharide products were also formed as revealed by structural analysis (see below). Substitution of lactose by *p*-nitrophenyl β-D-galactopyranoside as donor substrate gave similar results (data not shown). Lactose was chosen as acceptor for all subsequent experiments because of its higher stability in aqueous solutions.

Preparative Synthesis of UDP-Activated Di- and Trisaccharides with β-Galactosidase from *Bacillus circulans*

For the preparative synthesis of the UDP-activated di- and trisaccharides using UDP-GlcNAc or UDP-Glc as acceptor substrates and lactose as donor substrate, optimized reaction conditions were used (for a discussion of the conditions, see below).

UDP-*N*-Acetyllactosamine (UDP-LacNAc) and the Corresponding Gal(β1-4)-Elongated UDP-Trisaccharide

The transglycosylation reaction of β-galactosidase in frozen solution at –5 °C gave 31% for UDP-LacNAc (**1**) and 11% for the corresponding Gal(β1-4)-elongated UDP-trisaccharide (**3**) with respect to the acceptor concentration (Figure 1). The overall yield after anion-exchange chromatography and gel filtration was 8.2% (81.8 μmol) for **1**, corresponding to 62.8 mg with a purity of

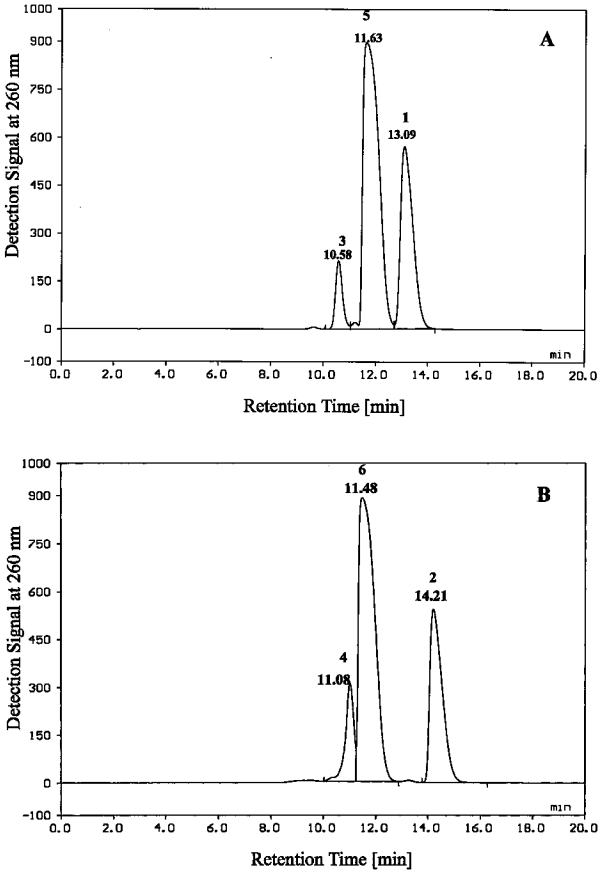


Fig. 2 HPLC-Analysis of Nucleotide-Activated Oligosaccharides Formed by β-Galactosidase from *Bacillus circulans* at 30 °C. Analysis was performed by ion-pair-reversed-phase HPLC [column: Hypersil ODS (250 x 4.6 mm, 5 µm particle size); solvent system: 100 mM potassium acetate, pH 5.6, containing 0.013% (v/v) *n*-octylamine and 5% (v/v) methanol; flow rate: 1 ml/min; UV-detection: 260 nm; Elling and Kula, 1993].
(A) UDP-GlcNAc as acceptor: [**5**], UDP-GlcNAc (11.63 min); [**1**] Gal(β1-4)GlcNAc(α1-UDP) (13.09 min); [**3**] Gal(β1-4)Gal(β1-4)GlcNAc(α1-UDP) (10.58 min).
(B) UDP-Glc as acceptor: [**6**] UDP-Glc (11.48 min); [**2**] Gal(β1-4)Glc(α1-UDP) (14.21 min); [**4**] Gal(β1-4)Gal(β1-4)Glc(α1-UDP) (11.08 min).

100% according to HPLC. The overall yield of isolated **3** was 3.6% (36.1 µmol), corresponding to 35 mg with apurity of 96% according to HPLC.

UDP-Lactose (UDP-Lac) and the Corresponding Gal(β1-4)-Elongated UDP-Trisaccharide

The enzymatic synthesis in frozen solution at –5 °C gave 31% for UDP-Lac (**2**) and 8.4% for the corresponding Gal(β1-4)-elongated UDP-trisaccharide (**4**) with respect to the acceptor concentration (Figure 1). After isolation by anion-exchange chromatography and gel filtration an overall yield of 5.0% (41.3 µmol) of **2** corresponding to 32.3 mg with a purity of 93% according to HPLC was obtained. The overall yield of isolated **4** was 1.6% (13.0 µmol), corresponding to 12.2 mg with a purity of 95% according to HPLC.

Mass Spectrometry

Negative-ion mode FAB-MS analysis was performed on the four purified products to determine their molecular mass. In all cases a typical glycerol spectrum (*m/z* [91 + n^x92 where n = 0,1,2…9) with one additional intense peak in the high-mass region was observed. Pseudo molecular ions were observed at *m/z* 767.9 ([M-H]$^-$, Hex-HexNAc-UDP) for **1** (Figure 3A), *m/z* 727.0 ([M-H]$^-$, Hex_2-UDP) for **2** (Figure 3B), *m/z* 930.8 ([M-H]$^-$, Hex_2-HexNAc-UDP) for **3** (Figure 3C), and *m/z* 889.7 ([M-H]$^-$, Hex_3-UDP) for **4** (Figure 3D). In addition the sodiated pseudo-molecular ions were observed for all four products. The appearance of the free acids as the major products is due to the extensive salt precipitation steps during the product isolation. However, isolation of UDP-LacNAc without salt precipitation as described earlier (Elling *et al.*, 1999) gave the mono-sodiated nucleotide disaccharide as the major product. Fragment ions were observed for **3** at *m/z* 768.7 ([Hex-HexNAc-UDP – H]$^-$), *m/z* 704.6 ([Hex_2-HexNAc-PP – H]$^-$), *m/z* 624.6 ([Hex_2-HexNAc-P – H]$^-$), and *m/z* 403.4 ([UDP – H]$^-$), and for **4** at *m/z* 727.5 ([Hex_2-UDP – H]$^-$), *m/z* 663.5 ([Hex_3-PP – H]$^-$), *m/z* 583.5 ([Hex_3-P – H]$^-$), and *m/z* 403.4 ([UDP – H]$^-$).

NMR Spectroscopy

The interpretation of the ^{1}H and ^{13}C 1D, and 2D (homo and heteronuclear) NMR spectra (Figures 4A, 5A, and 6A) of **1**, Gal(β1-4)GlcNAc(α1-UDP, was carried out as described previously (Elling *et al.*, 1999), and the data are presented in Table 1.

In the case of **2** the replacement of *N*-acetylglucosamine by glucose, with respect to **1**, as deduced from the observed mass, was corroborated in the ^{1}H 1D spectrum (Figure 4B) by the absence of the *N*-acetyl singlet at δ 2.07 as well as the absence of the C-2 resonance at 54 ppm in the ^{13}C 1D spectrum (Figure 5B). By means of 2D TOCSY (20 and 100 ms spinlock time) (not shown), ROESY (Figure 6B), and HMBC (not shown) experiments all resonances observed in the ^{1}H and ^{13}C 1D spectra could be assigned (Table 1). The 2D ROESY and ^{1}H-^{13}C HMBC spectra facilitated the determination of the linkage-types present. The anomeric track of Glc H-1α (A_{H-1}) at δ 5.596 in the 2D TOCSY spectrum (not shown) revealed four cross peaks at δ 3.565 (A_{H-2}), 3.795 (A_{H-3}), 3.687 (A_{H-4}), and 3.980 (A_{H-5}). Both A_{H-6a} and A_{H-6b}, at δ 3.885 and δ 3.906, respectively, were assigned on the A_{H-5} track. The positions of the cross peaks on the anomeric track of Gal H-1β (B_{H-1}) were similar to those observed in **1**. The (β1-4)-linkage between the residues B and A in **2** was confirmed by the observation of the interresidual connectivity between B_{H-1} (δ 4.455) and A_{H-4} (δ 3.687) in the ROESY spectrum (Figure 6B), and corroborated by the two three-bond couplings between B_{H-1} and A_{C-4} (δ 4.455, 78.92) and between B_{C-1} and A_{H-4} (δ 104.1, 3.687) in the HMBC spectrum. These results allowed the identification of **2** as Gal(β1-4)Glc(α1-UDP.

The presence of an additional β-anomeric signal in the

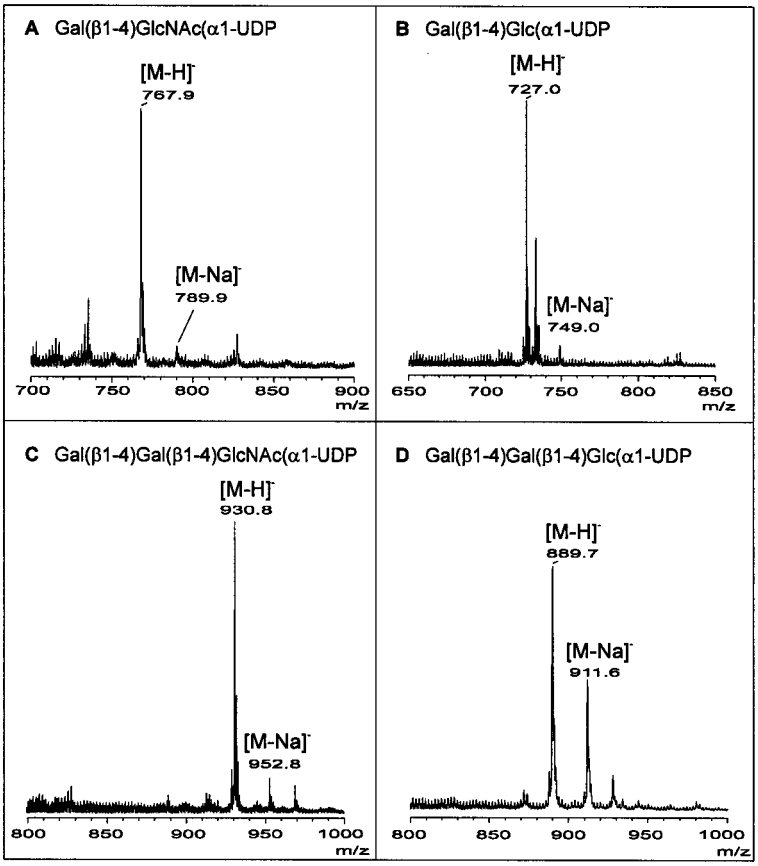


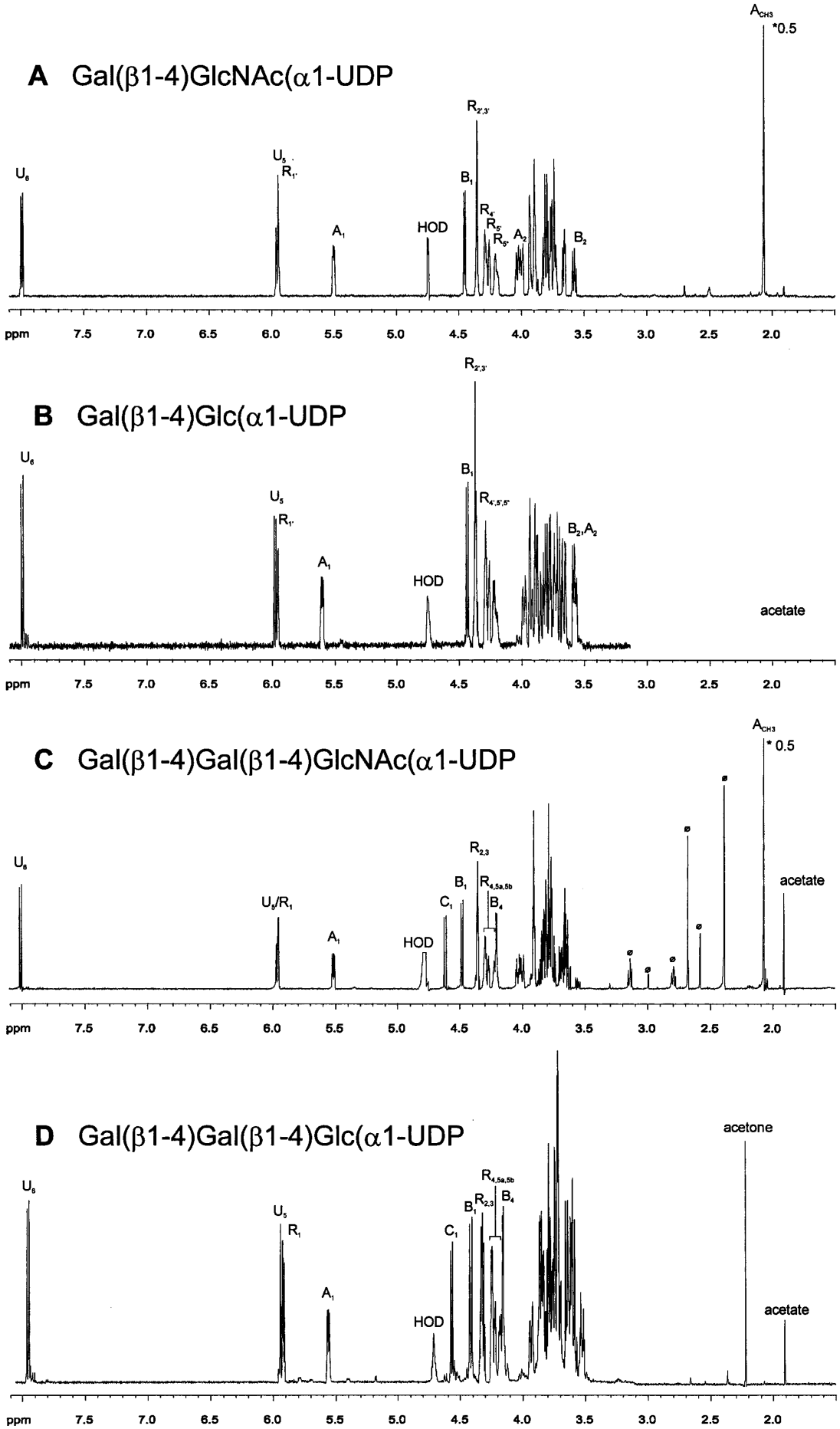
Fig. 3 FAB-MS (Negative-Ion Mode) Spectra of UDP-Di- and Trisaccharides.
Gal(β1-4)GlcNAc(α1-UDP) (**1**, panel A), Gal(β1-4)Glc(α1-UDP) (**2**, panel B), Gal(β1-4)Gal(β1-4)GlcNAc(α1-UDP) (**3**, panel C), and Gal(β1-4)Gal(β1-4)Glc(α1-UDP) (**4**, panel D).

^{1}H 1D spectra (Figures 4C and 4D) of **3** and **4** at δ 4.614 (C_{H-1}) and δ 4.613 (C_{H-1}), respectively, indicated a nucleotide-activated trisaccharide (Table 1). The corresponding anomeric tracks in the TOCSY (100 ms) spectra (not shown) revealed that the additional residue was Gal in both products. The nature of the glycosidic linkages present in **3** and **4** were deduced from the ROESY (250 ms) spectra (Figures 6C and 6D); interresidual cross peaks between C_{H-1} and B_{H-4} (δ 4.614, 4.205 for **3**; δ 4.613, 4.203 for **4**) and between B_{H-1} and A_{H-4} (δ 4.481, 3.791 for **3**; δ 4.457, 3.683 for **4**) reflect (1-4)-linkages only. It should be noted that the pronounced downfield shift of the B_{H-4} resonance when comparing the signals from **3** and **1** (Δδ 0.280) and **4** and **2** (Δδ 0.270) already pointed toward a (β1-4)-linkage between residues C and B in both cases. The same results can be deduced from the ^{13}C NMR data (B_{C-4}: **1**→**3**, δ 69.34–77.30; **2**→**4**, δ 69.17–77.30) (Figures 5C and 5D; Table 1). The combined observations permit the identification of **3** as Gal(β1-4)Gal(β1-4)GlcNAc(α1-UDP) and **4** as Gal(β1-4)Gal(β1-4)Glc(α1-UDP).

Influence of Temperature and pH on the Reaction Yield

The beneficial influence of a low temperature (15 °C) on the yield of *N*-acetyllactosamine (LacNAc) using *N*-acetylglucosamine as acceptor and lactose as donor was recently reported for β-galactosidase from *Bacillus circulans* (Vetere and Paoletti, 1996). Moreover, with the same acceptor and donor and using β-galactosidase from *E. coli*, an increase in yield was observed from 35% at 25 °C to 53% at −5 °C (frozen aqueous solution) (Hänsler and Jakubke, 1996; Jakubke *et al.*, 1996). Following the latter approach, we compared the transglycosylation reaction of β-galactosidase from *Bacillus circulans* at 30 °C and at −5 °C (frozen aqueous solution),

Fig. 4 500 or 600-MHz 1D ^{1}H NMR Spectra of UDP-Di- and Trisaccharides. ▶
Probes were analyzed at a temperature of 300 K, in D_2O. Gal(β1-4)GlcNAc(α1-UDP) (**1**, panel A), Gal(β1-4)Glc(α1-UDP) (**2**, panel B), Gal(β1-4)Gal(β1-4)GlcNAc(α1-UDP) (**3**, panel C), and Gal(β1-4)Gal(β1-4)Glc(α1-UDP) (**4**, panel D). A = GlcNAc in **1** and **3**, and Glc in **2** and **4**; B = Gal to Glc(NAc); C = Gal to Gal; R = Rib; and U = uracil.


A Gal(β1-4)GlcNAc(α1-UDP
B Gal(β1-4)Glc(α1-UDP
C Gal(β1-4)Gal(β1-4)GlcNAc(α1-UDP
D Gal(β1-4)Gal(β1-4)Glc(α1-UDP
ppm
acetate
acetone
HOD

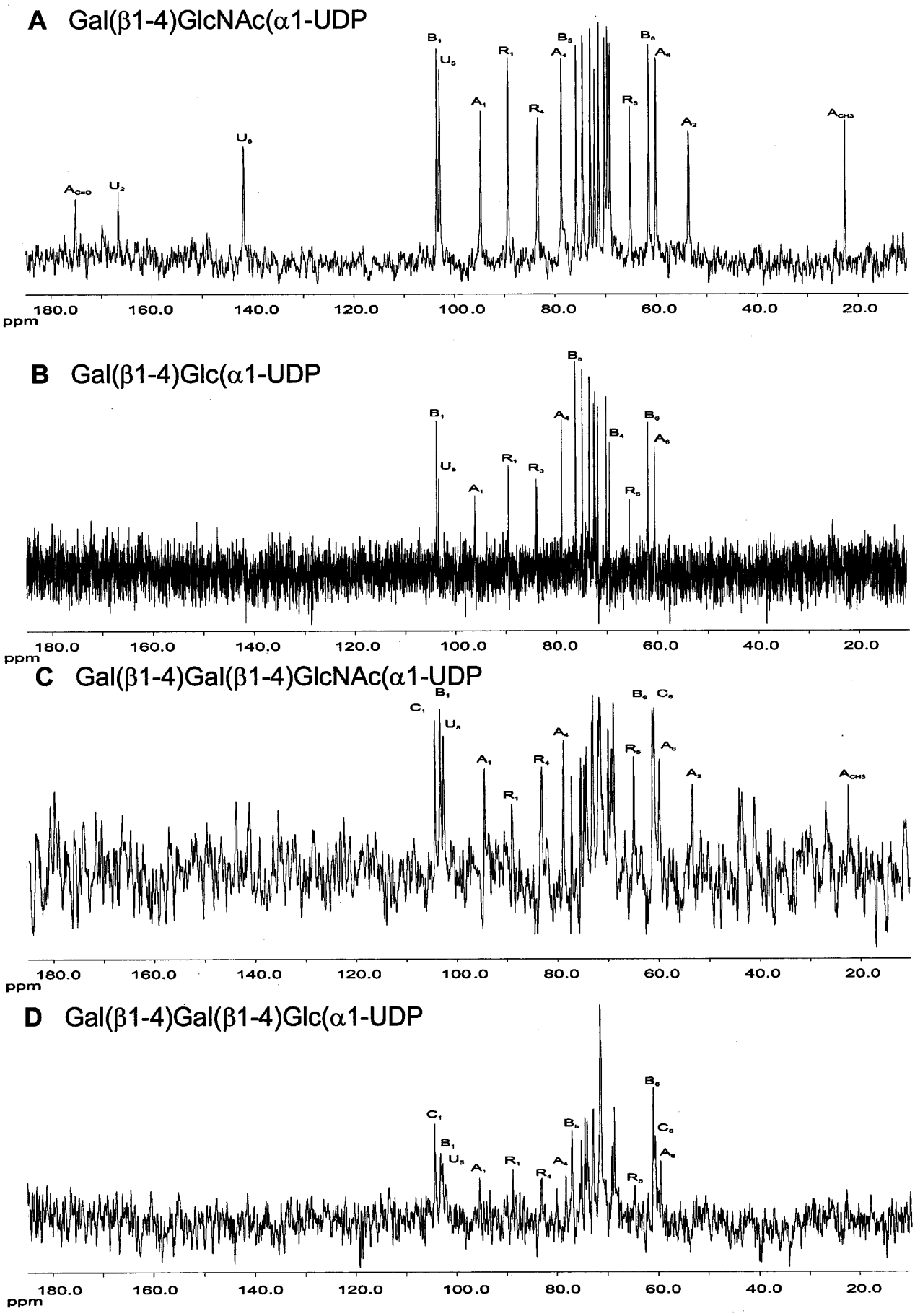

A Gal(β1-4)GlcNAc(α1-UDP
B Gal(β1-4)Glc(α1-UDP
C Gal(β1-4)Gal(β1-4)GlcNAc(α1-UDP
D Gal(β1-4)Gal(β1-4)Glc(α1-UDP
ppm
180.0
160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0

Table 1 ^{1}H and ^{13}C NMR Chemical Shifts of Gal(β1-4)GlcNAc(α1-UDP) (**1**), Gal(β1-4)Glc(α1-UDP) (**2**), Gal(β1-4)Gal(β1-4)GlcNAc(α1-UDP) (**3**), and Gal(β1-4(Gal(β1-4)Glc(α1-UDP) (**4**), prepared with β-Galactosidase (*B. circulans*), Recorded at 300 K.

Atom	^{1}H[b]				^{13}C[c]			
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**
A1	5.510 (3.0,7.2)	5.596 (3.5, 7.1)	5.511 (3.0,7.0)	5.603 (7.2,3.0)	95.04	96.53	94.65	95.64
A2	4.039	3.565	4.034	3.565	53.81	72.45	53.42	71.95
A3	3.813	3.795	3.810	3.778	70.41	72.56	70.01	71.59
A4	3.786	3.687	3.791	3.683	78.98	78.92	78.90	78.48
A5	4.013	3.980	4.006	3.977	72.41	72.14	71.52	71.59
A6a	3.779[d]	3.885[d]	3.773[d]	3.885[d]	60.29	60.26	59.88	58.82
A6b	3.906[d]	3.906[d]	3.901[d]	3.906[d]	–	–	–	–
A CH3	2.071	–	2.072	–	22.77	–	22.36	–
A C=O	–	–	–	–	175.41	–	n.d.	–
B1	4.458 (7.8)	4.455 (7.5)	4.481 (8.0)	4.457 (7.9)	103.80	104.12	103.45	103.30
B2	3.566	3.576	3.651	3.647	71.57	71.88	71.52	71.59
B3	3.648	3.665	3.780	3.775	73.25	73.31	73.22	74.28
B4	3.925	3.933	4.205	4.203	69.34	69.17	77.30	77.30
B5	3.722	3.722	3.931	3.924	76.04	76.33	75.49	75.77
B6a	3.850[d]	3.845[d]	n.d.	n.d.	61.68	61.68	61.28	61.22
B6b	3.791[d]	3.799[d]	n.d.	n.d.	–	–	–	–
C1	–	–	4 – 614 (8.0)	4.613 (7.5)	–	–	104.54	104.49
C2	–	–	3.624	3.625	–	–	71.97	73.04
C3	–	–	3.671	3.665	–	–	73.06	73.22
C4	–	–	3.914	3.905	–	–	68.96	68.88
C5	–	–	n.d.	n.d.	–	–	74.88	75.41
C6a	–	–	n.d.	n.d.	–	–	60.93	60.90
C6b	–	–	n.d.	n.d.	–	–	–	–
R1	5.952 (3.6)	5.958 (3.5)	5.951 (3.5)	5.954 (3.5)	89.58	89.67	89.23	89.05
R2	4.358	4.358	4.354	4.357	74.75	74.85	74.37	74.73
R3	4.358	4.370	4.354	4.377	69.82	69.92	69.36	69.39
R4	4.296	4.299	4.295	4.291	83.65	84.13	83.27	83.31
R5a	4.261	4.281	4.262	4.261	65.39	65.38	64.97	63.70
R5b	4.210	4.266	4.207	4.216	–	–	–	–
U2	–	–	–	–	166.92	166.40	n.d.	n.d.
U4	–	–	–	–	152.10	152.75	n.d.	n.d.
U5	5.962 (8.4)	5.977 (8.4)	5.962 (8.4)	5.977 (8.4)	103.21	103.31	102.83	102.88
U6	7.998 (8.4)	7.988 (8.4)	8.007 (8.4)	7.999 (8.4)	142.02	141.93	n.d.	n.d.

A = GlcNAc in **1** and **3**, and Glc in **2** and **4**; B = Gal to Glc(NAc); C = Gal to Gal; R = Rib, U = uracil. Coupling constants (Hz) are given between brackets.

[a] In the case of ^{1}H NMR spectroscopy, A1 means H-1 of residue A. In the case of ^{13}C NMR spectroscopy, A1 means C-1 of residue A; etc.

[b] In ppm relative to the signal of internal acetate (δ 1,908, acetone δ 2.225).

[c] In ppm relative to the signal of external acetone (δ 31.08).

[d] The assignment of H6a and H6b may have to be interchanged within one residue.

respectively, with UDP-GlcNAc as acceptor. With UDP-GlcNAc as acceptor a maximum yield (with reference to the acceptor) of 22% after 90 min for the UDP-disaccharide and 5.5% after 150 min for the UDP-trisaccharide was obtained at 30 °C (Figure 7A). It is obvious that the formation of the UDP-trisaccharide follows that of the UDP-disaccharide. At the maximum yield of UDP-disaccharide formation the ratio between the UDP-di- and trisaccharide was 81:19. The transglycosylation reaction with UDP-GlcNAc turned out to be much slower at – 5 °C, however, higher product yields were obtained (Figure 7B). In addition, UDP-Glc was tested as acceptor substrate at – 5 °C. After 42 h, UDP-LacNAc and UDP-Lac were formed in yields of 31% and 29%, respectively (Figure 7B). The maximum yields for the UDP-trisaccharides Gal(β1-4)Gal(β1-4)GlcNAc(α1-UDP) and Gal(β1-4)Gal-(β1-4)Glc(α1-UDP) were 10% after 88 h and 16% after 78

◀ **Fig. 5** 75-MHz 1D ^{13}C NMR Spectra of UDP-Di- and Trisaccharides.
Probes were analyzed at a temperature of 300 K, in D_2O. Gal(β1-4)GlcNAc(α1-UDP) (**1**, panel A), Gal(β1-4)Glc(α1-UDP) (**2**, panel B), Gal(β1-4)Gal(β1-4)GlcNAc(α1-UDP) (**3**, panel C), and Gal(β1-4)Gal(β1-4)Glc(α1-UDP) (**4**, panel D). A = GlcNAc in **1** and **3**, and Glc in **2** and **4**; B = Gal to Glc(NAc); C = Gal to Gal; R = Rib; and U = uracil.

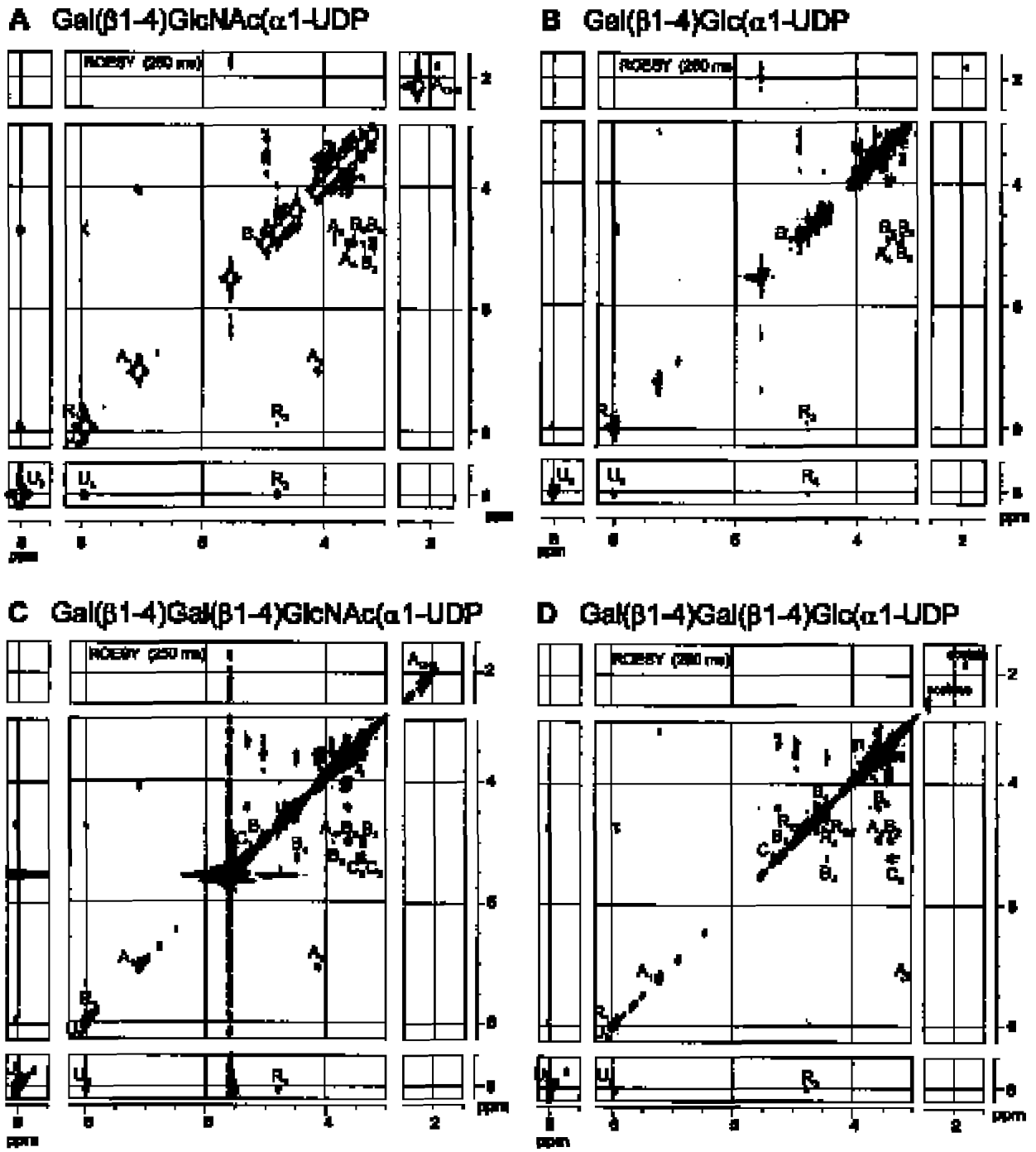


Fig. 6 500 or 600-MHz 2D ROESY NMR Spectra of UDP-Di- and Trisaccharides.
Probes were analyzed at a spin lock time of 250 ms, probe temperatures of 300 K, in D_2O. Gal(β1-4)GlcNAc(α1-UDP) (**1**, panel A), Gal(β1-4)Glc(α1-UDP) (**2**, panel B), Gal(β1-4)Gal(β1-4)GlcNAc(α1-UDP) (**3**, panel C), and Gal(β1-4)Gal(β1-4)Glc(α1-UDP) (**4**, panel D). A = GlcNAc in **1** and **3**, and Glc in **2** and **4**; B = Gal to Glc(NAc); C = Gal to Gal; R = Rib; and U = uracil.

h, respectively (Figure 7B). At the maximum yield of UDP-disaccharide formation the ratio between the UDP-di- and trisaccharides was 82:18 with UDP-GlcNAc (very similar to that observed at 30°C) and 72:28 with UDP-Glc as acceptor. These results indicate that UDP-Lac is preferentially galactosylated over UDP-LacNAc.

As stated earlier, factors that cause an increase in yield of hydrolase-catalyzed reactions are a reduced water concentration in the frozen state and a high concentration of solutes in the small liquid phase of a solid frozen reaction mixture (Hänsler and Jakubke, 1996; Jakubke *et al.*, 1996). The low temperature causes an increased product stability, due to a decreased competitive product hydrolysis, as it is observed for the synthesis of the UDP-disaccharides at −5 °C up to an incubation period of 60 h (Figure 7B).

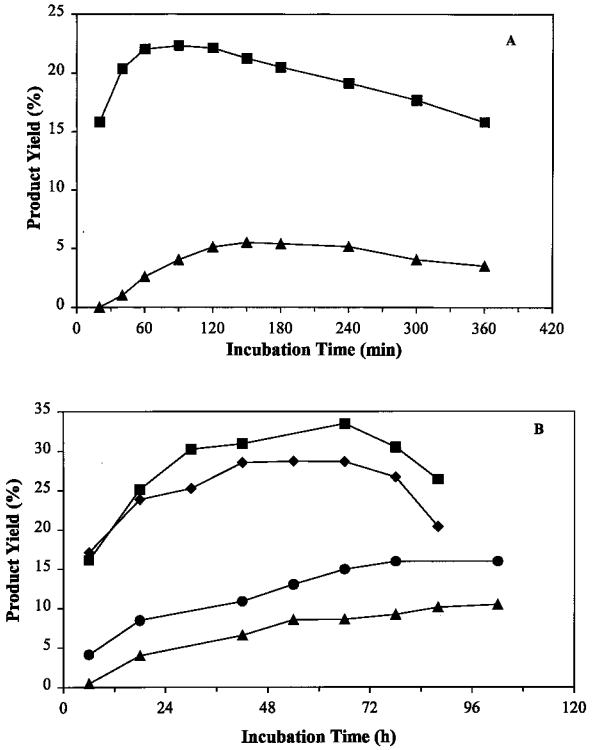


Fig. 7 Comparison of UDP-Oligosaccharide Synthesis with β-Galactosidase from *Bacillus circulans* at 30°C and at – 5 °C.
(A) Formation of Gal(β1-4)GlcNAc(α1-UDP) (■) and Gal(β1-4)Gal(β1-4)GlcNAc(α1-UDP) (▲) at 30°C. Lactose (500 mM) and UDP-GlcNAc (100 mM) were incubated with 10 U/ml of the enzyme.
(B) Formation of Gal(β1-4)GlcNAc(α1-UDP) (■), Gal(β1-4)Gal(β1-4)GlcNAc(α1-UDP) (▲), Gal(β1-4)Glc(α1-UDP) (◆) and Gal(β1-4)Gal(β1-4)Glc(α1-UDP) (●) at – 5 °C. Lactose (500 mM), UDP-GlcNAc (100 mM), and UDP-Glc (100 mM) were incubated with 10 U/ml of the enzyme.

The concentration of substrates in the small liquid phase of a frozen solution is dependent on the volume ratio of the liquid phase and the solid ice phase. In a solid frozen solution the liquid/ice volume ratio is low. If a solution with a low concentration of reactants and an enzyme is frozen to a solid state, the reactants and the enzyme are concentrated in the remaining small liquid phase resulting in an acceleration of the enzymatic reaction (Hänsler and Jakubke, 1996). However, in the transglycosylation reaction performed at – 5 °C the relative high solute concentrations (500 mM lactose and 100 mM UDP-GlcNAc) gave no solid state of the frozen reaction mixture. Thus, the slurry reaction mixture gave a relative high liquid/ice volume ratio. In order to reduce the liquid/ice volume ratio, the experiment with UDP-Glc as acceptor was repeated at –20 °C with a lower donor (200 mM lactose) and a higher (100 U/ml) enzyme concentration (data not shown). A higher reaction rate was expected by a smaller liquid phase with higher concentrations of reactants and enzyme. However, the rate of UDP-Lac and UDP-trisaccharide formation was much slower than that at – 5 °C reaching 26% and 16% yield, respectively, after 16 days. The relatively low reaction rate may be caused by a reduced enzyme activity which is caused by a restricted flexibility and mobility of the enzyme in a frozen reaction mixture as already pointed out for the tetrameric β-galactosidase from *E. coli* with a molecular mass of 465 kDa (Hänsler and Jakubke, 1996). For comparison, the molecular mass for β-galactosidase from *Bacillus circulans* has been reported to be 247 kDa (Usui *et al.*, 1996).

The transglycosylation reaction using UDP-GlcNAc as acceptor substrate was further investigated at – 5 °C at pH 7.0 and 4.5, respectively (data not shown). The yields were similar for both pH values, however, the reaction rate for the product formation was higher at the higher pH value. At pH 7.0, 28% of UDP-LacNAc was obtained after 1 day and 9.1% of the corresponding UDP-trisaccharide after 2 days. At pH 4.5 maximum yields of 29% and 9.5% for UDP-LacNAc and the corresponding UDP-trisaccharide, respectively, were obtained after 2 days. Since at pH 7.0 a higher rate of product hydrolysis was observed, pH 4.5 was chosen for further optimization at –5 °C.

Variation of Donor and Acceptor Concentration

The influence of different concentrations of lactose and UDP-GlcNAc was investigated at 30 °C and – 5 °C. The ratios of UDP-di- and trisaccharides were calculated at the maximum UDP-disaccharide formation, and are summarized in Tables 2 and 3.

Both in frozen solution at – 5 °C and at 30 °C the yields (with respect to the acceptor concentration) of UDP-LacNAc (Table 2) and Gal(β1-4)Gal(β1-4)GlcNAc(α1-UDP) (data not shown) were significantly higher when 500 mM donor substrate was used. The reaction rate for UDP-disaccharide formation was, however, the highest with 100 mM lactose at both temperatures. This may be due to a substrate inhibition at high lactose concentrations. One effect of an increased yield of the UDP-disaccharide is a

Table 2 Effects of the Variation of the Donor Concentration on the Yields of UDP-Di and Trisaccharides at –5 °C and 30 °C.

Temperature (°C)	[Lactose] (mM)	Time (h)	Yield (%) UDP-disaccharide	Yield (%) UDP-trisaccharide	Ratio di:tri
30 °C	500	1.5	22.3	5.1	4.4:1
30 °C	100	0.33	10.0	0.9	11.1:1
30 °C	50	0.5	9.4	–[a]	–
– 5 °C	500	42	33.2	7.5	4.4:1
– 5 °C	100	18	27.7	4.4	6.3:1
– 5 °C	50	18	22.8	2.9	7.9:1

The ratio of formed UDP-di- and trisaccharides was calculated at the maximum yield of UDP-LacNAc. Lactose (50 to 500 mM) and UDP-GlcNAc (100 mM) were incubated with 10 U/ml enzyme in 20 mM acetate buffer pH 4.5 at –5 °C and 20 mM NaH_2PO_4/NaOH pH 7.0 at 30 °C, respectively.
[a] No UDP-trisaccharide formation was detected by HPLC at 50 mM lactose.

Table 3 Effects of the Variation of the Acceptor Concentration on the Yields of UDP-Di and Trisaccharides at –5 °C and 30 °C.

Temperature (°C)	[UDP-GlcNAc] (mM)	Time (h)	Yield (%) UDP-disaccharide	Yield (%) UDP-trisaccharide	Ratio di:tri
30 °C	100	1.5	24.5 [24.5][a]	6.0 [6.0][a]	4.1:1
30 °C	50	1.17	22.0 [11.0]	5.4 [2.7]	4.1:1
30 °C	25	1.0	22.2 [5.6]	5.0 [1.25]	4.4:1
–5 °C	100	42	33.9 [33.9]	5.6 [5.6]	6.1:1
–5 °C	50	42	32.1 [16.0]	7.5 [3.75]	4.3:1
–5 °C	25	66	29.6 [7.4]	11.5 [2.9]	2.6:1

The ratio of formed UDP-di- and trisaccharides was calculated at the maximum yield of UDP-LacNAc. Lactose (500 mM) and UDP-GlcNAc (25 to 100 mM) were incubated with 10 U/ml enzyme in 20 mM acetate buffer pH 4.5 at –5 °C and 20 mM NaH_2PO_4/NaOH pH 7.0 at 30 °C, respectively.
[a] Product concentration.

concomitant increase of the UDP-trisaccharide concentration, which results in a decrease of the ratio between UDP-di- and trisaccharides (Table 2). Interestingly, at 30 °C no formation of the UDP-trisaccharide could be observed with 50 mM lactose, indicating that the product is immediately hydrolyzed and a certain threshold substrate concentration of the UDP-disaccharide must be reached. For comparison, with 50 mM lactose at – 5 °C higher product yields are obtained for the UDP-disaccharide resulting in the formation of UDP-trisaccharide. In summary, the yield of 40.7% for the formation of UDP-di- plus trisaccharides at – 5 °C is significantly higher than the yield for the synthesis at 30 °C (27.4%). No further increase of the summarized yields was observed, which indicates that equlibrium has been reached under these conditions.

The variation of the UDP-GlcNAc concentration at – 5 °C and 30 °C gave only minor changes for the yield of UDP-LacNAc (Table 3). Whereas the ratio of UDP-di- and trisaccharide was not changed at 30 °C, an interesting result was achieved for the formation of Gal(β1-4)Gal(β1-4)GlcNAc(α1-UDP) at –5 °C. The yield of the UDP-trisaccharide increased when the UDP-GlcNAc concentration was lowered, as indicated by the decrease of the product ratio (Table 3). It is obvious that this result reflects an effect of the freezing of the reaction mixture. A possible reason may be, that the solubility of UDP-GlcNAc, UDP-di- and trisaccharide, and the buffer salts in the small liquid phase of the frozen reaction mixture is limited. At a low concentration of UDP-GlcNAc (25 mM) the solubilizing capacity of the small liquid phase does not limit the product formation, allowing the synthesis of 7.4 mM UDP-LacNAc and 2.9 mM UDP-trisaccharide. The primary effect of doubling the UDP-GlcNAc concentrations to 50 and 100 mM is a doubling of the UDP-LacNAc concentration to 16 mM and 33.9 mM (Table 3). However, the formation of the UDP-trisaccharide does not follow this tendency suggesting that at these concentrations the small liquid phase of the frozen reaction mixture limits further solubilization of the products.

Jourdian and Distler (1973) reported the formation of UDP-disaccharides with 74% yield (with respect to the donor substrate) after 6 h using 33 U/ml β-galactosidase from bovine testes, 2 mM *p*-nitrophenyl β-D-galactopyranoside as donor substrate and 550 mM UDP-GlcNAc as acceptor substrate at 37 °C and pH 7.1. Although a relatively high yield was obtained, the transglycosylation proceeds with a very low reaction rate at 37 °C and at a very high concentration of enzyme and UDP-GlcNAc. Our current investigations with β-galactosidase from bovine testes could not confirm the data reported by Jourdian and Distler (1973). We found 14.9% of UDP-disaccharide after 16 h, using 100 mM UDP-GlcNAc, 500 mM lactose and 0.33 U/ml β-galactosidase from bovine testes (V. Nieder, M. Kutzer, R. Gutiérrez Gallego, J.P. Kamerling, J.F.G. Vliegenthart, L. Elling, unpublished data).

In conclusion, the enzymatic synthesis of Gal(β1-4)Glc(NAc)(α1-UDP and Gal(β1-4)Gal(β1-4)Glc(NAc)(α1-UDP) with β-galactosidase from *Bacillus circulans* at – 5 °C results in high overall product yields and a remarkably high product stability.

Donor Substrate Spectrum of β-Galactosidase from *Bacillus circulans*

Additional nucleotide sugars were tested as acceptor substrates at 30 °C. Reaction mixtures were analyzed by HPLC for the formation of nucleotide di- and trisaccharides. Table 4 shows that UDP-Gal, UDP-GalNAc, UDP-Xyl and nucleotide-activated glucoses are further accep-

Table 4 Nucleotide Sugars as Acceptor Substrates of β-Galactosidase from *Bacillus circulans.*

Nucleotide sugars as acceptor substrates	Yield (%) (Retention time in min)	
	Nucleotide disaccharide	Nucleotide trisaccharide
UDP-GlcNAc	29.4 (16.2)	5.7 (20.4)
UDP-Glc	23.3 (14.7)	4.9 (18.2)
UDP-Gal	15.6 (15.4)	1.1 (20.2)
UDP-GalNAc	12.8 (16.1)	0.9 (20.4)
UDP-GlcA	n.d.[a]	n.d.[a]
UDP-Xyl	5.7 (12.2)	n.d.[a]
dTDP-Glc	14.8 (13.9)	1.0 (17.5)
CDP-Glc	10.8 (21.5)	n.d.[a]
ADP-Glc	14.9 (20.8)	0.7 (27.1)
GDP-Glc	7.2 (18.8)	n.d.[a]
GDP-Man	n.d.[a]	n.d.[a]
CMP-Neu5Ac	n.d.[a]	n.d.[a]

100 mM nucleotide sugars were mixed with 500 mM lactose, 10 U/ml enzyme in 20 mM phosphate buffer pH 7.0 and incubated for 2 h at 30 °C. After heat incubation at 95 °C for 5 min the reaction mixtures were analyzed by HPLC using a silica gel column (Nucleosil 100-5, ET 250/40, 5 µm, 250 x 4 mm) and isocratic elution with acetonitrile/0.1 M NH_4C1 75:25 at room temperature at a flow rate of 1 ml/min (UV-detection at 260 nm, sample volume 7 µl).
[a] Product formation was not detected.

tor substrates of β-galactosidase from *Bacillus circulans*. With UDP-Xyl only the UDP-disaccharide is formed. The presence of other bases than uracil in nucleotide-activated glucoses affects the product formation resulting in lower yields for the nucleotide-activated disaccharides and only minor or no formation of the nucleotide-activated trisaccharides. Isolation and characterization of all products is in progress in order to investigate them as donor substrates or inhibitors of glycosyltransferases.

Concluding Remarks

The enzymatic access to UDP-di- and trisaccharides by transglycosylation reactions of glycosidases will facilitate investigations on the biological role of nucleotide-activated oligosaccharides. The major goals of our future work are to utilize nucleotide-activated di- and oligosaccharides as *in vitro* building block substrates of Leloir glycosyltransferases and to investigate their biochemical function as inhibitors of glycosyltransferases and nucleotide sugar transporters. As nucleotide-activated building blocks they also promise novel applications of prokaryotic and eukaryotic glycosyltransferases. Work is in progress to examine different *N*-acetylglucosaminyltransferases and sucrose synthase for their ability to transfer *en bloc* LacNAc or lactose from UDP-LacNAc or UDP-Lac, respectively, onto specified acceptor structures. In addition, the cellular appearance and the physiological function of UDP-activated oligosaccharides as well as inhibition studies of GlcNAcTs and nucleotide sugar transporters can be addressed.

Materials and Methods

Materials

β-Galactosidase from *Bacillus circulans* was purchased from Daiwa Kasei KK (Biolacta N5™, Osaka, Japan). Lactose and all nucleotide sugars used in this study were from Sigma (Deisenhofen, Germany). Acetic acid, potassium acetate and sodium acetate were from Roth (Karlsruhe, Germany). All other chemicals were from Merck (Darmstadt, Germany).

Analytical Methods

TLC analysis of reaction mixtures was performed on silica gel 60_{F254} plates (Merck) using acetonitrile/0.1 M NH_4Cl (75:25) as eluent. Saccharides and nucleotide sugars were visualized by spraying with a solution containing 20 mg naphthoresorcinol, 40 mg diphenylamine, and 400 µl H_2SO_4 in 10 ml ethanol, and subsequently color development for 5–10 min at 100 °C. Nucleotide sugars could also be localized under UV-light at 254nm.

Reversed-phase HPLC of nucleosides, nucleotides and nucleotide-activated mono-, di- and trisaccharides was conducted as described previously (Elling and Kula, 1993). Samples were fractionated by ion-pair-reversed-phase HPLC on a Hypersil ODS-column (C_{18}, 5 µm, 4.6 mm x 250 mm; Macherey & Nagel, Düren, Germany) using 0.1 M potassium acetate, containing 5%(v/v) methanol and 0.013% (v/v) *n*-octylamine, adjusted with acetic acid to pH5.6, as eluent at a flow rate of 1 ml/min and UV-detection at 260 nm. Additionally, HPLC of nucleotide-activated mono-, di- and trisaccharides was carried out at room temperature on a silica gel column (Nucleosil 100-5, ET 250/40, 5 µm, 250 x 4 mm; Macherey & Nagel) using acetonitrile/0.1 M NH_4Cl (75:25) as eluent at a flow rate of 1 ml/min and UV-detection at 260 nm.

Synthesis of Nucleotide-Activated Oligosaccharides Using β-Galactosidase

Transglycosylation Reaction at 30 °C and –5 °C For the transglycosylation reaction at 30 °C, 500 mM lactose and 100 mM UDP-GlcNAc (di-sodium salt) or UDP-Glc (di-sodium salt) in 20 mM KH_2PO_4/KOH (100 µl), pH 7.0, were incubated with 10 U/ml β-galactosidase. For the transglycosylation reaction at – 5 °C, the same reactions were carried out in 20 mM sodium acetate/acetic acid (100 µl), pH 4.5. The pH of the buffers was adjusted at 25°C. Prior to incubation at – 5 °C the solution was divided into aliquots of 10 µl and immediately frozen in liquid N_2. The enzyme reactions were terminated at the indicated incubation periods (see Results and Discussion) by heating for 5min at 95°C. The samples were analyzed by TLC and/or HPLC (Hypersil ODS) after addition of 90 µl of distilled water.

Selection of Donor Substrate To select the most suitable donor substrate, 20 mM *p*-nitrophenyl β-D-galactopyranoside or 20 mM lactose were incubated with 0.3 U β-galactosidase at 30 °C in 20 mM KH_2PO_4/KOH pH 7.0 (100 µl), containing 100 mM UDP-Glc as acceptor substrate. Aliquots of 5 µl were taken directly after mixing and after 5 and 15 min of incubation. After the addition of 95 µl distilled water the reaction was stopped by heating for 5 min at 95°C. The samples were analyzed by HPLC (Hypersil ODS).

Influence of pH and Temperature on the Reaction Yield in Frozen Solutions The effect of pH on the synthesis at –5° C was investigated with UDP-GlcNAc as acceptor substrate. The buffers used were 20 mM NaH_2PO_4/NaOH, pH 7.0, and 20 mM sodium acetate/acetic acid, pH 4.5; in both cases the pH was adjusted at 25 °C.

To test the effect of carrying out the reaction at –20 °C, β-galactosidase (10 U) was added to 100 mM glycyl-glycine buffer (100 µl), pH 4.5, containing 200 mM lactose and 100 mM UDP-Glc. Aliquots of 20 µl were incubated at –20 °C after freezing in liquid N_2. The enzyme reactions were stopped after 7, 9, 12, 14, and 16 days of incubation by heating for 5 min at 95°C, and the mixtures were analyzed by HPLC (Hypersil ODS) after the addition of 80 µl distilled water, respectively.

Variation of Donor and Acceptor Concentration The concentration of the donor substrate lactose was varied between 50 and 500 mM with 100 mM UDP-GlcNAc as acceptor and 10 U/ml β-galactosidase. A buffer of 20 mM NaH_2PO_4/NaOH, pH 7.0, was used for incubations at 30 °C. Samples were also incubated at – 5 °C in 20 mM sodium acetate/acetic acid, pH 4.5, after freezing in liquid N_2. At the indicated incubation periods (see Results and Discussion) samples were heated for 5 min at 95°C, and analyzed by HPLC (Hypersil ODS). The acceptor substrate concentrations were varied between 25 and 100 mM UDP-GlcNAc in the presence of 500 mM lactose and 10 U/ml β-galactosidase. The enzymatic syntheses were performed at 30°C and – 5 °C as described above with a final volume of 100 µl, respectively.

Preparative Synthesis of UDP-Activated Di- and Trisaccharides with β-Galactosidase

UDP-GlcNAc as Acceptor Substrate To a solution of lactose (5 mmol, 1710 mg) in 20 mM sodium acetate/acetic acid (10 ml), pH 4.5, were added UDP-GlcNAc (1 mmol, 697.5 mg disodium-

salt) and β-galactosidase (100 U). The solution was immediately divided into 20 aliquots, each vial containing 500 µl, then frozen in liquid N_2 and incubated for 2 days at –5° C. The pH value of the buffer was adjusted at 25 °C prior to freezing. The enzyme reaction was stopped by heating all vials for 5 min at 95 °C. To quantify the different products, the reaction mixtures were pooled and analyzed by HPLC (Hypersil ODS). Then, denatured protein was removed by ultrafiltration with Microcon 10 (Amicon, Witten, Germany), and the nucleotide-activated sugars were separated from the neutral sugars and buffer salts by anion-exchange chromatography on a Sepharose Q FF column (Cl— form, 2.6 x 32 cm; Pharmacia, Freiburg, Germany). To this end the pool (10 ml) was divided into 4 aliquots of 2.5 ml, and each aliquot was loaded onto the column, equilibrated with distilled water. After washing with distilled water, nucleotide-activated mono-, di- and trisaccharides were eluted with 0.4 M NaCl, pH 2.0, at a flow rate of 4 ml/min. The pooled fractions (UV detection at 254 nm) were adjusted to pH 7.0 with 0.1 M NaOH and subsequently concentrated *in vacuo* at 35°C to a volume of 4 ml. After addition of an equal volume of ethanol, the precipitated NaCl was removed by centrifugation. The ethanol in the supernatant was evaporated *in vacuo* at 35°C, and the resulting solution was again mixed with ethanol and worked-up. Finally, the mixture of nucleotide-activated mono-, di- and trisaccharides was separated by gel filtration on a Bio-Gel P-2 extra fine column (2.6 x 74.5 cm; Bio-Rad, München, Germany) at room temperature with distilled water as eluent at a flow rate of 0.25 ml/min and UV detection at 254 nm. The pooled fractions containing the UDP-di- and trisaccharide, respectively, were lyophilized, and the residues, dissolved in 1.5 ml 0.1 M NaCl, were again purified on Bio-Gel P-2. The overall yield of isolated UDP-LacNAc was 8.2% (81.8 µmol) corresponding to 62.8 mg with a purity of 100% according to HPLC (Hypersil ODS). The overall yield of isolated Gal(β1-4)Gal(β1-4)GlcNAc(α1-UDP was 3.6% (36.1 µmol) corresponding to 35 mg with apurity of 96% according to HPLC (Hypersil ODS).

UDP-Glc as Acceptor Substrate To a solution of lactose (3.8 mmol, 1318 mg) in 20 mM sodium acetate/acetic acid (7.7 ml), pH 4.5, UDP-Glc (0.82 mmol, 500 mg di-sodium salt) and β-galactosidase (77 U) were added. The synthesis, HPLC analysis and the product isolation protocols were as described above. The overall yield of isolated UDP-Lacwas 5.0% (41.3 µmol) corresponding to 32.3 mg with a purity of 93% according to HPLC (Hypersil ODS). The overall yield of isolated Gal(β1-4)Gal(β1-4)Glc(α1-UDP) was 1.6% (13.0 µmol) corresponding to 12.2 mg with a purity of 95% according to HPLC (Hypersil ODS).

Acceptor Substrate Spectrum of β-Galactosidase Different nucleotide sugars (100 mM of UDP-Glc, UDP-GlcNAc, UDP-Gal, UDP-GalNAc, UDP-GlcA, UDP-Xyl, dTDP-Glc, CDP-Glc, ADP-Glc, GDP-Glc, GDP-Man, CMP-Neu5Ac) were tested as acceptor substrates by incubation with 500 mM lactose and 10 U/ml β-galactosidase in 20 mM NaH_2PO_4/NaOH, pH 7.0, at 30 °C. Reactions were stopped after 2 h of incubation by heating for 5 min at 95°C, and the mixtures were analyzed by HPLC (Nucleosil 100-5).

Mass Spectrometry

Negative-ion mode fast atom bombardment mass spectrometric (FAB-MS) analysis of the reaction products was performed on a JEOL JMS-SX/SX 102A four-sector instrument operating 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. One µl samples (10 µg in 0.1 ml 5% acetic acid) were mixed with 1.0 µl glycerol 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_2O (99.9 atom % D, Isotec) with intermediate lyophilization and finally dissolved in 450 µl D_2O (99.96 atom % D, Isotec). Proton-decoupled 75 469 MHz ^{13}C NMR spectra were recorded on a Bruker AC-300 spectrometer at a probe temperature of 300 K. Chemical shifts (δ, ppm) are referenced to external acetone (δ 31.08). Resolution enhanced ^1H 1D and 2D NMR spectra were recorded on Bruker AMX-500 or AMX-600 instruments (Department of NMR Spectroscopy, Utrecht University) at a probe temperature of 300 K. Chemical shifts (δ) are expressed in ppm relative to internal acetate (δ 1.908, acetone δ 2.225). HOD signal suppression was achieved by applying a WEFT pulse sequence (Hård *et al.*, 1992) in 1D ^1H experiments and by pre-saturation for 1 s in 2D experiments. 2D TOCSY spectra were recorded by using MLEV-17 mixing sequences with effective spin-lock times between 20 and 100 ms. 2D ROESY spectra were recorded with a mixing time of 250 ms. The spin-lock field strength corresponded to a 90° pulse of about 120 µs. Proton detected ^{13}C-^1H 2D HMBC experiments were performed at a ^1H frequency of 500 139 or 600 140 MHz (125 769 or 150 916 MHz for ^{13}C, respectively) using a pulse sequence as described (Summers *et al.*, 1986). The delay time for the detection of long-range ^{13}C-^1H couplings was set to 60 ms. ^1H 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}C 1D spectra were elaborated on Silicon Graphics IRIS work stations (Indigo 2 and O2) using TRITON software (Bijvoet Center, Department of NMR Spectroscopy).

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