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Screening and characterization of β-*N*-acetylhexosaminidases for the synthesis of nucleotide-activated disaccharides

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Abstract

Extracellular β-*N*-acetylhexosaminidases from different fungal strains were screened for their ability to synthesize nucleotide-activated oligosaccharides. In combination with GalNAc(β1-*p*NP) as donor the nucleotide sugar UDP-GlcNAc was found to be an effective acceptor substrate for β-*N*-acetylhexosaminidases from *Aspergillus parasiticus*, *Aspergillus flavus*, *Penicillium oxalicum*, *Trichoderma harzianum*, *Aspergillus flavipes*, *Aspergillus tamarii*, and *Aspergillus oryzae*. β-*N*-acetylhexosaminidase from *Trichoderma harzianum* was selected for further studies on the synthesis of the UDP-disaccharide GalNAc(β1–4)GlcNAc(α1-UDP) (UDP-LacdiNAc). The addition of heptakis-(2,6-di-*O*-methyl)-β-cyclodextrin (HM-β-CD) was crucial for the synthesis of this compound by increasing the solubility of the donor substrate GalNAc(β1-*p*NP) in aqueous solutions at room temperature. HM-β-CD also increased the maximum reaction velocity (V_{max}) of the enzyme, which was probably due to the elimination of enzyme inhibitors, e.g., *p*NP and/or GalNAc, by the cyclodextrin derivative. Under optimized conditions GalNAc(β1–4)GlcNAc(α1-UDP) was formed stereo- and regioselectively with an overall yield of 3.5% (17.7 μmol, 15.1 mg). The chemical structure was characterized by ^{1}H and ^{13}C NMR spectroscopy and MALDI-TOF mass spectrometry.

Keywords: Glycosidase; Nucleotide sugars; Transglycosylation; β-*N*-Acetylhexosaminidase; Nucleotide-activated disaccharides; Synthesis

1. Introduction

Nucleotide-activated di- and oligosaccharides are known to be present in higher animals and bacteria and represent a class of glycoconjugates whose biosynthesis and biological function are poorly characterized. Nucleotide-activated oligosaccharides were first isolated from milk of different mammals such as goat [1], man [2–4], pig [5] and sheep [6]. Also hen oviduct was found to be a source of these glycoconjugates [7,8]. Structural analyses have revealed that most of the nucleotide-activated oligosaccharides are based on the nucleotide sugar UDP-*N*-acetyl-α-D-glucosamine (UDP-GlcNAc) and that Gal(β1–4)GlcNAc(α1-UDP) (UDP-LacNAc) is the most prominent compound.

In archaebacteria nucleotide-activated oligosaccharides were identified as precursors in the biosynthesis of different carbohydrate polymers. The biosynthesis of pseudomurein proceeds via GalNAc(β1–3)GlcNAc(α1-UDP) as an intermediate [9]. The nucleotide-activated oligosaccharides GlcA(1–3)GalNAc-UDP and GlcA(1–3)GalNAc(1–4)GalNAc-UDP are involved in the biosynthesis of methanochondroitin [10]. Nucleotide-activated oligosaccharides based on UDP-GlcNAc, UDP-Gal, GDP-ManNAc, and UDP-ManNAc were characterized as precursors in the biosynthesis of the S-layer-glycoproteins [11]. The enzymes synthesizing these compounds have not yet been identified.

In this context access to nucleotide-activated oligosaccharides by chemical and/or enzymatic methods could help to clarify the biosynthesis and elucidate the biological function of these naturally occurring glycoconjugates. It is assumed that Leloir glycosyltransferases are responsible for the synthesis of nucleotide di- and oligosaccharides in vivo by using nucleotide sugars as acceptor substrates. We

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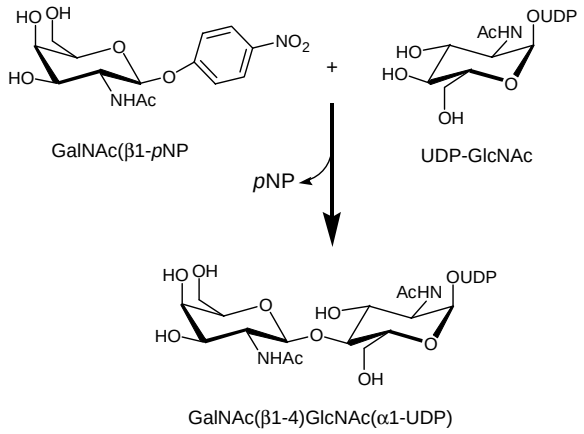

Fig. 1. Synthesis of GalNAc(β1–4)GlcNAc(α1-UDP) with β-*N*-acetylhexosaminidase from *T. harzianum*.

could recently demonstrate that UDP-GlcNAc is indeed an in vitro acceptor substrate for β-1,4-galactosyltransferase 1 (β4Gal-T1) from human and bovine milk synthesizing UDP-LacNAc [12]. Thus, β4Gal-T1 may be responsible for the synthesis of UDP-LacNAc found in both human and bovine milk. In order to extend the synthetic approach to these compounds we explored nucleotide sugars as acceptor substrates of glycosidases [13]. With β-galactosidase from *B. circulans* we synthesized the UDP-disaccharides UDP-LacNAc, Gal(β1–4)Glc(α1–UDP) (UDP-Lactose), and Gal(β1–4)Gal(α1-UDP). The corresponding UDP-trisaccharides Gal(β1–4)Gal(β1–4)GlcNAc(α1-UDP), Gal(β1–4)Gal(β1–4)Glc(α1-UDP), and Gal(β1–4)Gal(β1–4)Gal(α1-UDP) were also formed. The α-galactosidase from *B. adolescentis* yielded Gal(α1–3)Glc(α1-UDP) and Gal(α1–3)Gal(α1-UDP) [14]. In contrast to a previous report by Jourdian and Distler [15] these glycosidases formed the nucleotide di- and trisaccharides with an exclusive stereo- and regioselectivity. In the course of our work we have screened other glycosidases for their ability to use nucleotide sugars as acceptor substrates.

Here we present our results on the screening of β-*N*-acetylhexosaminidases (EC 3.2.1.30) from different microbial sources with the nucleotide sugars UDP-Glc, UDP-GlcNAc, and UDP-Gal as acceptor substrate. The β-*N*-acetylhexosaminidase from *T. harzianum* was selected for the synthesis of GalNAc(β1–4)GlcNAc(α1-UDP) (UDP-LacdiNAc) (Fig. 1).

2. Materials and methods

2.1. Materials

GalNAc(β1-*p*NP), GlcNAc(β1-*p*NP), and nucleotide sugars were obtained from Sigma (Deisenhofen, Germany). All other chemicals were, if not otherwise stated, from Roth (Karlsruhe, Germany). All media components were from Oxoid (Basingstoke Hants, United Kingdom).

2.2. Production and purification of β-*N*-acetylhexosaminidases

The fungal strains used for the production of the extracellular β-*N*-acetylhexosaminidases [16,17] are deposited in the culture collection of fungi (CCF, Department of Botany, Charles University, Prague). The cultures were maintained on the following medium (g/l): agar-agar (20); bacto-peptone (5); malt extract (35). Conical flasks (500 ml) with 100 ml medium were inoculated with the suspension of the spores in 0.1% Tween 80. The flasks were cultivated on a rotary shaker at 28 °C. Medium composition (g/l): mycological peptone (5); KH_2PO_4 (3); $NH_4H_2PO_4$ (5); yeast extract (0.5); chitin hydrolysate (2) [18]. The cultures were harvested after 12 days of cultivation when extracellular β-*N*-acetylhexosaminidase activity reached its maximum. Induction of β-*N*-acetylhexosaminidase [16] with aminosugars from chitin hydrolysate ensured high specific activity practically void of other glycosidases. The enzymes were pre-purified from the culture filtrate by ammonium sulfate fractionation (30–80% saturation cut) that gave preparations of high specific activity directly used for the respective reactions. The specific activity of the enzyme preparations obtained were as follows (U/mg protein): *Aspergillus parasiticus* CCF 3058 (21), *A. flavus* CCF 814 (34), *A. flavipes* CCF 554 (55), *A. tamarii* CCF 1665 (30), *A. oryzae* CCF 147 (49), *Penicillium oxalicum* CCF 2430 (30), and *T. harzianum* CCF 2687 (44).

2.3. Screening of β-*N*-acetylhexosaminidases

The donor substrates GlcNAc(β1-*p*NP) (9 μmol, 3.1 mg) and GalNAc(β1-*p*NP) (6 μmol, 2.1 mg), respectively, were dissolved in 50 mM Na_2HPO_4 buffer (100 μl), pH 6.5, containing 200 mM heptakis-(2,6-di-*O*-methyl)-β-cyclodextrin (HM-β-CD, 20 μmol, 26.6 mg). The acceptor substrates were added to obtain a final concentration of 100 mM: UDP-Glc (10 μmol, 6.1 mg), UDP-GlcNAc (10 μmol, 6.5 mg) and UDP-Gal (10 μmol, 6.1 mg), respectively. The β-*N*-acetylhexosaminidases were tested using the following activities: *A. parasiticus* (0.6 U/ml), *Aspergillus flavus* (0.3 U/ml), *P. oxalicum* (0.3 U/ml), *T. harzianum* (0.2 U/ml), *Aspergillus flavipes* (0.4 U/ml), *Aspergillus tamarii* (0.2 U/ml) and *Aspergillus oryzae* (0.9 U/ml). The reaction mixtures were incubated at 30 °C and the reaction was stopped after 14 h by heating for 5 min at 95 °C. The synthesis yields were determined by HPLC.

2.4. HPLC analysis

HPLC analysis of nucleosides, nucleotides and nucleotide-activated mono-, di- and trisaccharides was conducted as described previously [19]. Samples were fractionated by ion-pair-reversed-phase HPLC on a Hypersil ODS-column (C_{18}, 5 μm, 4.6 mm × 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 pH 5.6, as eluent at a flow rate of 1 ml/min with UV-detection at 260 nm.

2.5. Influence of cyclodextrins on the solubility of GalNAc(β1-pNP) and GlcNAc(β1-pNP)

The influence of cyclodextrin on the solubility of GalNAc(β1-*p*NP) and GlcNAc(β1-*p*NP) was assessed using solutions of 0, 50, 100 and 200 mM cyclodextrin in 50 mM Na_2HPO_4 buffer (pH 6.5). Samples of α-, β-, γ-hydroxypropyl-β- and heptakis-(2,6-di-*O*-methyl)-β-cyclodextrin, respectively, were tested. The cyclodextrin solutions were added to a defined (5 mg) amount of the *p*NP-glycoside substrate at room temperature until they were dissolved completely. The solubility was determined by calculating the concentration of the *p*NP-glycoside substrates in the different volumes of buffer needed for solubilization.

2.6. Activity assay of β-N-acetylhexosaminidases

The activity of the β-*N*-acetylhexosaminidases was determined with GlcNAc(β1-*p*NP) as substrate. The reaction mixture contained 45 mM GlcNAc(β1-*p*NP) (2.25 μmol, 0.77 mg) and 200 mM HM-β-CD (10 μmol, 13.3 mg) in 50 mM Na_2HPO_4 (pH 6.5, 50 μl). The reaction was started by the addition of an aliquot of enzyme solution. The length of the reaction time at 30 °C allowed a conversion rate between 2 and 5%. The reaction was terminated by a pH shift, adding 950 μl glycine/NaOH buffer (250 mM, pH 10.0). The amount of *p*-nitrophenol liberated in 200 μl of the mixture was determined by measuring the absorbance at 405 nm in a microplate reader (MWG Biotech, Ebersberg, Germany). After quantification with a calibration curve ($A_{405\,nm} = 8.99\ [p\text{NP}] + 0.05$) one enzyme unit was defined as the amount of enzyme which hydrolyses 1 μmol of GlcNAc(β1-*p*NP) per minute under the described conditions.

2.7. Kinetic analysis

The kinetic parameters of the β-*N*-acetylhexosaminidase from *T. harzianum* were determined by variation of the donor substrate concentration. GalNAc(β1-*p*NP) was varied between 1 and 50 mM in 50 mM Na_2HPO_4 (pH 6.5, 50 μl) containing 200 mM HM-β-CD (10 μmol, 13.3 mg) and 0.6 U/ml β-*N*-acetylhexosaminidase. The same reaction series was conducted without addition of HM-β-CD varying the concentration of GalNAc(β1-*p*NP) between 1 and 10 mM. After incubation (10 min at 30 °C) the reaction was terminated by a pH shift, adding 950 μl glycine/NaOH buffer (250 mM, pH 10.0). The amount of *p*-nitrophenol formed by the enzyme was determined as described above. One enzyme unit was defined as the amount of enzyme which hydrolyses 1 μmol of GalNAc(β1-*p*NP) per minute under conditions described above.

2.8. Inhibition of β-N-acetylhexosaminidase by GalNAc

The inhibition of β-*N*-acetylhexosaminidase from *T. harzianum* was determined in the absence and presence of HM-β-CD. The enzyme activity was determined with GalNAc(β1-*p*NP) (0.1 μmol, 0.2 mg) dissolved in 50 mM Na_2HPO_4 (pH 6.5, 50 μl). GalNAc was added in variable concentrations between 1 and 1.5 mM before starting the reaction by the addition of 0.6 U/ml enzyme. The same reaction series were conducted with a buffer solution containing 200 mM HM-β-CD (10 μmol, 13.3 mg). After incubation at 30 °C for 10 min the enzyme reactions were stopped and enzyme activities were determined as described above.

2.9. Preparative synthesis of GalNAc(β1–4)GlcNAc(α1-UDP) (3) with β-N-acetylhexosaminidase from T. harzianum

For the synthesis of **3**, 500 μmol (356 mg) of GalNAc(β1-*p*NP) (**1**) (100 mM final concentration), 300 μmol (103 mg) of UDP-GlcNAc (**2**), disodium salt, 60 mM, and 0.64 U/ml β-*N*-acetylhexosaminidase (3.2 U) were dissolved in 5 ml of 50 mM Na_2HPO_4 buffer, pH 6.5 containing 200 mM HM-β-CD. The reaction mixture was incubated at 30 °C and the reaction was stopped after 8 h by heating at 95 °C for 5 min. The pooled samples were analyzed by HPLC and product isolation was conducted as described previously [13].

2.10. Mass spectrometry and NMR spectroscopy

Negative-ion mode MALDI-TOF mass spectrometric analysis and ^1H and ^{13}C NMR experiments were carried out as described previously [13].

3. Results and discussion

3.1. GalNAc(β1-pNP) and GlcNAc(β1-pNP) as substrates of β-N-acetylhexosaminidases

Transglycosylation reactions require high concentrations of substrates to obtain sufficient yields of the transglycosylation products [20]. From our studies with β-galactosidase from *B. circulans* we learned that a concentration of at least 20 mM Gal(β1-*p*NP) is necessary to detect the formation of nucleotide disaccharides [13]. However, in the present study the low solubility of the donor substrates GalNAc(β1-*p*NP) (maximum 11 mM) and GlcNAc(β1-*p*NP) (maximum 15 mM) in aqueous solutions at room temperature turned out to be a serious problem in the β-*N*-acetylhexosaminidase-mediated transglycosylation reactions. Cyclodextrins are well known to serve as carrier molecules for unstable or poorly in water-soluble substances [21]. Therefore, cyclodextrins should also improve the solubility of GalNAc(β1-*p*NP) and GlcNAc(β1-*p*NP) by the formation of water-soluble inclusion complexes.

We tested four different cyclodextrins, namely α-, β-, and γ-hydroxypropyl-β-cyclodextrin and heptakis-(2,6-di-*O*-methyl)-β-cyclodextrin (HM-β-CD). The best results were obtained with 200 mM HM-β-CD by which the solubility of both *p*-nitrophenyl glycosides were six-fold increased reaching 90 mM for GlcNAc(β1-*p*NP) and 60 mM for GalNAc(β1-*p*NP) (data not shown).

3.2. Screening of β-*N*-acetylhexosaminidases

The β-*N*-acetylhexosaminidases from *A. parasiticus*, *A. flavus*, *P. oxalicum*, *T. harzianum*, *A. flavipes*, *A. tamarii* and *A. oryzae* were investigated for their ability to form activated disaccharides. Different nucleotide sugars were screened as acceptor substrates in transglycosylation reactions using the donor substrates GlcNAc(β1-*p*NP) and GalNAc(β1-*p*NP), respectively, in the presence of 200 mM HM-β-CD. A pH value of 6.5 was chosen with reference to published data for a β-*N*-acetylhexosaminidase from *A. oryzae* [22]. Table 1 summarizes the results obtained by HPLC analysis of the formed nucleotide-activated disaccharides. UDP-GlcNAc was independently from the enzyme source the best acceptor substrate. However, GlcNAc(β1-*p*NP) as donor substrate was weak and gave maximum yields of only 2%. The best enzyme was that from *T. harzianum* with 22% yield for the combination of GalNAc(β1-*p*NP) and UDP-GlcNAc. The enzyme reached the highest yield after 8 h, whereas yields of only 4 and 5%, respectively, were obtained after 3 h with the enzymes from *A. parasiticus* and *P. oxalicum* (Fig. 2).

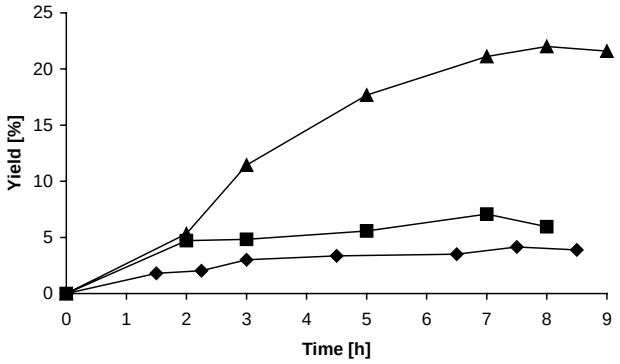

Fig. 2. Formation of nucleotide-activated disaccharides with β-*N*-acetylhexosaminidases from *A. parasiticus* (0.64 U/ml (◆)), *P. oxalicum* (0.29 U/ml (■)) and *T. harzianum* (0.23 U/ml (▲)). Reaction conditions: 100 mM UDP-GlcNAc, 200 mM heptakis-(2,6-di-*O*-methyl)-β-cyclodextrin, 63 mM GalNAc(β1-*p*NP), volume: 100 μl, 30 °C.

3.3. Characterization of β-*N*-acetylhexosaminidase from *T. harzianum*

3.3.1. Michaelis–Menten kinetics

The effect of HM-β-CD on the kinetic properties of β-*N*-acetylhexosaminidase from *T. harzianum* was investigated. Fig. 3 shows the Michaelis–Menten kinetics with GalNAc(β1-*p*NP) as substrate in the presence and absence of HM-β-CD. In the latter case a substrate concentration of up to 10 mM was used due to the limited solubility of GalNAc(β1-*p*NP). However, the K_m values of 3.44 ± 0.49 mM (with HM-β-CD) and 3.21 ± 0.41 mM

Table 1
Screening of β-*N*-acetylhexosaminidases for the formation of nucleotide disaccharides

Enzyme source	Yield (%)[a]			
	Donors	Acceptors		
		UDP-Glc	UDP-GlcNAc	UDP-Gal
A. parasiticus CCF 3058	GlcNAc(β1-*p*NP)	–[b]	2.0	–
	GalNAc(β1-*p*NP)	–	4.0	–
A. flavus CCF 814	GlcNAc(β1-*p*NP)	–	n.d.[c]	n.d.
	GalNAc(β1-*p*NP)	–	n.d.	n.d.
P. oxalicum CCF 2430	GlcNAc(β1-*p*NP)	–	0.7	–
	GalNAc(β1-*p*NP)	–	5.0	–
T. harzianum CCF 2687	GlcNAc(β1-*p*NP)	–	1.9	–
	GalNAc(β1-*p*NP)	0.4	22.0	1.0
A. flavipes CCF 554	GlcNAc(β1-*p*NP)	–	0.9	–
	GalNAc(β1-*p*NP)	1.4	1.9	–
A. tamarii CCF 1665	GlcNAc(β1-*p*NP)	–	2.0	–
	GalNAc(β1-*p*NP)	–	1.9	–
A. oryzae CCF 147	GlcNAc(β1-*p*NP)	–	–	–
	GalNAc(β1-*p*NP)	–	–	–

Screening conditions are described in Section 2.
[a] With reference to the acceptor substrate concentration.
[b] No product formation.
[c] Not determined.

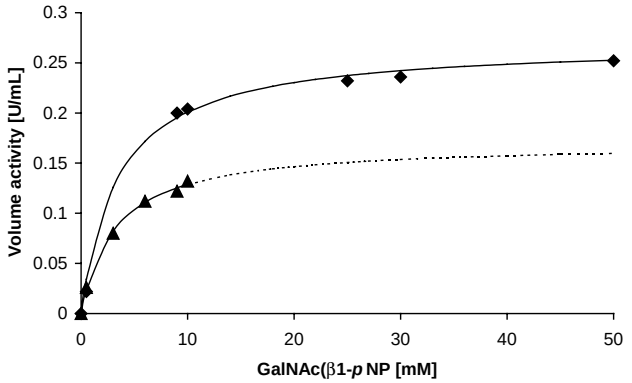

Fig. 3. Michaelis–Menten kinetics of β-*N*-acetylhexosaminidase from *T. harzianum* with GalNAc(β1-*p*NP) as substrate in the presence of 200 mM (◆) and without (▲) heptakis-(2,6-di-*O*-methyl)-β-cyclodextrin. Kinetic data: (◆) $K_m = 3.44 \pm 0.49$ mM; $V_{max} = 0.27 \pm 0.007$ U/ml; $R^2 = 0.999$; (▲) $K_m = 3.21 \pm 0.41$ mM; $V_{max} = 0.17 \pm 0.007$ U/ml; $R^2 = 0.999$.

(without HM-β-CD) do not differ significantly. We concluded that HM-β-CD does not affect the affinity of the substrate for the enzyme, however, the comparison of the V_{max} values revealed differences. The enzyme had a higher V_{max} in the presence of HM-β-CD (0.27 U/ml compared to 0.17 U/ml). We hypothesized that the higher activity of the enzyme in the presence of HM-β-CD may be due to the elimination of inhibitory by-products, e.g., *p*NP and/or GalNAc, which are formed by hydrolysis of the donor substrate during the transglycosylation reaction.

3.3.2. Inhibition with GalNAc

In order to test our hypothesis, the inhibitory effect of GalNAc in the absence and presence of HM-β-CD was investigated. Fig. 4 shows that GalNAc is a strong competitive inhibitor ($K_I = 0.21 \pm 0.04$ mM) of the β-*N*-acetylhexosaminidase from *T. harzianum*. Similar results were reported for GlcNAc as inhibitor of the

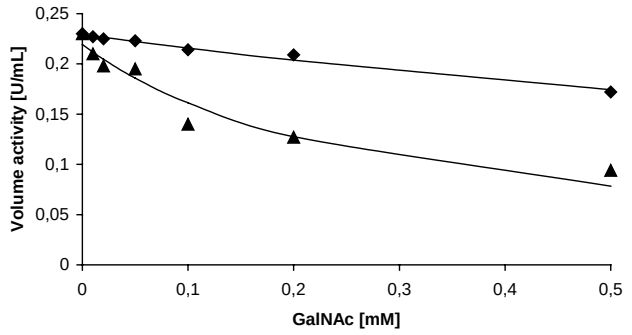

Fig. 4. Inhibition kinetics of β-*N*-acetylhexosaminidase from *T. harzianum* with GalNAc(β1-*p*NP) as substrate and GalNAc as inhibitor in the presence of 200 mM (◆) and without (▲) heptakis-(2,6-di-*O*-methyl)-β-cyclodextrin. Kinetic data: (◆) $K_m = 3.44 \pm 0.49$ mM; $K_I = 1.19 \pm 0.08$ mM; $V_{max} = 0.31 \pm 0.002$ U/ml; $R^2 = 0.999$; (▲) $K_m = 3.44 \pm 0.49$ mM; $K_I = 0.21 \pm 0.04$ mM; $V_{max} = 0.29 \pm 0.01$ U/ml; $R^2 = 0.995$.

β-*N*-acetylhexosaminidase from *A. oryzae* ($K_I = 1.6$ mM) [23]. In contrast, the presence of 200 mM HM-β-CD reduced the inhibition by GalNAc as revealed by the six-fold higher K_I value of 1.19 ± 0.08 mM (Fig. 4). We concluded that HM-β-CD is able to complex GalNAc and to eliminate it as a competitive inhibitor which favors higher yields in the formation of nucleotide-activated oligosaccharides in transglycosylation reactions.

3.4. Preparative synthesis of GalNAc(β1–4)GlcNAc(α1-UDP) with β-N-acetylhexosaminidase from T. harzianum

The transglycosylation reaction of β-*N*-acetylhexosaminidase from *T. harzianum* yielded 22.1% of GalNAc (β1–4)GlcNAc(α1-UDP) with respect to the acceptor concentration. The overall yield after product isolation by anion exchange chromatography and gel filtration was 3.5% (17.7 μmol) corresponding to 15.1 mg of the product with a purity of 91% according to HPLC.

3.5. Mass spectrometry

Negative-ion mode MALDI-TOF mass spectrometric analysis of the purified product gave only one peak in the mass-region of interest at *m*/*z* 809.16 (data not shown), which corresponds to the deprotonated pseudo-molecular ion of $HexNAc_2$-UDP ($[M–H]^-$). The appearance of the free acid, rather than the sodium-adduct was observed before and is due to the extensive salt precipitation steps during product isolation [13].

3.6. NMR spectroscopy

The 1D ^{1}H NMR spectrum of GalNAc(β1–4)GlcNAc(α1-UDP) (Fig. 5) showed four signals downfield of the HOD signal ($\delta = 4.751$). Two of these signals, at $\delta = 7.993$ (U6) and $\delta = 5.963$ (U5), were attributed to the uracil ring protons based on literature [24,25]. The anomeric signal at $\delta = 5.960$ ($^3J_{1,2} = 3.6$ Hz) was assigned to the ribose residue R (β configuration, furanose ring form), whereas the remaining anomeric signal at $\delta = 5.499$ ($^3J_{1,2} = 3.0$ Hz, $^3J_{1,P} = 7.0$ Hz) was assigned to the GlcNAc residue A (α configuration, pyranose ring form) linked to the phosphate group. Two chemical shifts, upfield of the HOD resonance could also be assigned directly; the singlet at $\delta = 2.064$, representing six protons, originating from the *N*-acetyl protons of GlcNAc residue A and GalNAc residue B, and the anomeric doublet at $\delta = 4.562$ ($^3J_{1,2} = 8.1$ Hz), which reflects the H1 of GalNAc residue B (β configuration, pyranose ring form). The ^{13}C NMR spectrum (not shown) revealed only three distinct signals in the anomeric region at $\delta = 103.47$ (B1), 103.42 (U5) and 89.49 (R1). The anomeric signal corresponding to residue A could not be determined. Furthermore three signals were observed in the downfield region at $\delta = 167.20$ (U2), 152.57 (U4) and

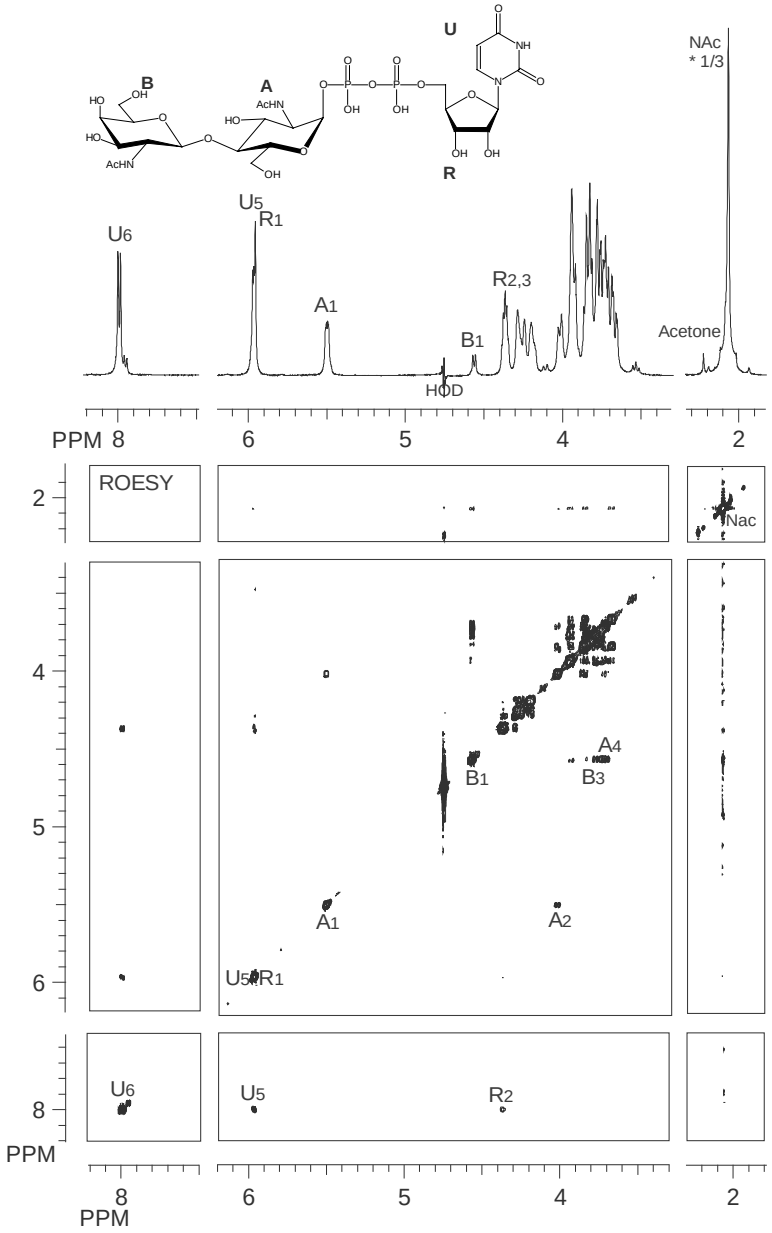

Fig. 5. The 500 MHz 1D ^{1}H and 2D ROESY NMR spectra of GalNAc(β1–4)GlcNAc(α1-UDP). The sample was analyzed at a temperature of 300 K in D_2O. A: GlcNAc; B: GalNAc; R: Rib; U: uracil.

142.51 (U6). Typical GlcNAc and GalNAc resonances were found at $\delta = 22.77$ (ACH_3 and BCH_3), $\delta = 53.74$ (A2) and 53.29 (B2).

By means of 2D TOCSY, ROESY and HMBC NMR experiments all ^{1}H resonances and almost all ^{13}C resonances in the corresponding 1D spectra could be assigned (Table 2). In the TOCSY spectrum (not shown) the three identified anomeric ^{1}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 10 ms made the sequential assignment possible.

To establish the linkage type between residues B and A, 2D ROESY and HMBC NMR experiments were performed. In the ROESY spectrum (Fig. 5) the anomeric track of residue B ($\delta = 4.562$) revealed a cross peak at $\delta = 3.709$, which could be assigned as interresidual contact to H4 of residue A. In the ^{13}C–^{1}H HMBC spectrum (not shown) the visualization of the interresidual three-bond connectivities over the glycosidic bond yielded unambiguous determination of the B(β1–4)A sequence via two long range couplings between B_{H1} and A_{C4} ($\delta =$ 4.562, 79.54) and between B_{C1} and A_{H4} ($\delta =$ 103.47, 3.709).

Table 2
^{1}H and ^{13}C NMR chemical shifts of GalNAc(β1–4)GlcNAc(α1-UDP) recorded in D_2O at 300 K

Atom[a]	^{1}H NMR[b]	^{13}C NMR[b]
A1	5.499	n.d.
A2	4.019	53.74
A3	3.849	70.41
A4	3.709	79.54
A5	3.930	72.12
A6a	3.838[c]	60.41
A6b	3.669[c]	
ACH_3	2.064	22.77
B1	4.562	103.47
B2	3.927	53.29
B3	3.771	71.44
B4	3.947	–
B5	3.489	76.03
B6a	3.833[c]	61.50
B6b	3.762[c]	
BCH_3	2.064	22.77
R1	5.960	89.49
R2	4.366	n.d.
R3	4.366	69.86
R4	4.288	83.81
R5a	4.241	65.38
R5b	4.201	
U2	–	167.20
U4	–	152.57
U5	5.963	103.42
U6	7.993	142.51

A: GlcNAc; B: GalNAc to GlcNAc; R: ribose; U: uracil. n.d.: not determined.

[a] In the case of ^{1}H NMR spectroscopy, A1 means H1 of residue A. In the case of ^{13}C NMR spectroscopy, A1 means C1 of residue A, etc.

[b] In ppm relative to the signal of external acetone ($\delta = 2.225$ for ^{1}H and $\delta = 31.08$ for ^{13}C).

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

4. Conclusions

We have established the access to nucleotide disaccharides by β-*N*-acetylhexosaminidase-catalyzed transglycosylation reactions. The reported compound will facilitate further investigations on the biochemical role of these glycoconjugates. Our future work will concentrate on the use of these novel glycoconjugates as building block substrates of Leloir glycosyltransferases as well as investigations on their biochemical function as inhibitors of glycosyltransferases and nucleotide sugar transporters.

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References

[1] Jourdian GW, Shimizu F, Roseman S. Isolation of nucleotide-oligosaccharides containing sialic acid. Fed Proc 1961;20:161.
[2] Kobata A. Isolation and identification of two novel uridine nucleotide oligosaccharide conjugates from human milk. Biochem Biophys Res Commun 1962;7:346–50.
[3] Kobata A. The acid-soluble nucleotides of milk. II. Isolation and identification of two novel uridine nucleotide oligosaccharide conjugates from human milk. J Biochem 1963;53:167–75.
[4] Kobata A. The acid-soluble nucleotides of milk. IV. The chemical structure of UDP-X_3. J Biochem 1966;59:63–6.
[5] Kobata A, Ziro S. The acid-soluble nucleotides of milk. III. Occurrence of UDP-*N*-acetyllactosamine and UDP-xylose in pig's milk and colostrum. Biochim Biophys Acta 1965;107:405–13.
[6] Denamur RE, Gaye PJ-B. Isolement et identification de 4 UDP-trisaccharide dans la glande mammaire et le colostrum de brebis. Eur J Biochem 1971;19:23–35.
[7] Nakanishi Y, Shimizu S, Takahashi N, Sugiyama M, Suzuki S. Structure and distribution of a disaccharide-carrying nucleotide and related nucleotides in hen oviduct. J Biol Chem 1967;242:967–76.
[8] Suzuki S. A novel uridine nucleotide containing *N*-acetylglucosamine and galactose. J Biol Chem 1962;237:1393.
[9] Hartmann E, König H. Comparison of the biosynthesis of the methanobacterial pseudomurein and the eubacterial murein. Naturwissenschaften 1990;77:472–5.
[10] Hartmann E, König H. Nucleotide-activated oligosaccharides are intermediates of the cell wall polysaccharide of *Methanosarcina barkeri*. Biol Chem Hoppe Seyler 1991;372:971–4.
[11] Hartmann E, Messner P, Allmeier G, König H. Proposed pathway for biosynthesis of the S-layer glycoprotein of *Bacillus alvei*. J Bacteriol 1993;175:4515–9.
[12] Elling L, Zervosen A, Gutiérrez Gallego R, Nieder V, Malissard M, Berger EG, et al. UDP-*N*-acetyl-α-D-glucosamine as acceptor substrate of β-1,4-galactosyltransferase. Enzymatic synthesis of UDP-*N*-acetyllactosamine. Glycoconjugate J 1999;16:327–36.
[13] Zervosen A, Nieder V, Gutiérrez Gallego R, Kamerling JP, Vliegenthart JFG, Elling L. Synthesis of nucleotide-activated oligosaccharides by β-galactosidase from *Bacillus circulans*. Biol Chem 2001;382:299–311.
[14] Nieder V, Marx SP, Gutiérrez Gallego R, Kamerling JP, Vliegenthart JFG, Elling L. Synthesis of nucleotide-activated disaccharides with β-galactosidase from *Bacillus circulans* and α-galactosidase from *Bifidobacterium adolescentis*. J Mol Catal B Enzymatic 2003;21:157–66.
[15] Jourdian GW, Distler JJ. Formation in vitro of uridine-5′-(oligosaccharide)-1-pyrophosphates. J Biol Chem 1973;248:6781–7.
[16] Hunková Z, Kren V, Ščigelová M, Weignerová L, Scheel O, Thiem J. Induction of β-*N*-acetylhexosaminidase in *Aspergillus oryzae*. Biotechnol Lett 1996;18:725–30.
[17] Hunková Z, Kubátová A, Weignerová L, Kren V. Induction of extracellular glycosidases in filamentous fungi and their potential use in chemotaxonomy. Czech Mycol 1999;51:71–87 (in English).
[18] Rupley JA. The hydrolysis of chitin by concentrated hydrochloric acid, and the preparation of low molecular weight substrates for lysozyme. Biochim Biophys Acta 1964;83:245–55.
[19] Elling L, Kula MR. Purification of sucrose synthase from rice and its protein-chemical characterization. J Biotechnol 1993;29:277–86.
[20] Nilsson KGI. Synthesis with glycosidases. In: Khan SH, O'Neill RA, editors. Modern methods in carbohydrate synthesis, vol. 1. Frontiers

in Natural Product Research Series. Amsterdam: Harwood Academic Publishers, 1996. p. 518–47.
[21] Aree T, Saenger W, Leibnitz P, Hoier H. Crystal structure of heptakis(2,6-di-*O*-methyl)-β-cyclodextrin dihydrate: a water molecule in an apolar cavity. Carbohydr Res 1999;315:199–205.
[22] Singh S, Scigelova M, Vic G, Crout DHG. Glycosidase-catalyzed oligosaccharide synthesis of di-, tri-, and tetra-saccharides using the *N*-acetylhexosaminidase from *Aspergillus oryzae* and the β-galactosidase from *Bacillus circulans*. J Chem Soc, Perkin Trans 1996;1:1921–6.
[23] Rajnochová E, Dvorakova J, Hunkova Z, Kren V. Reverse hydrolysis catalysed by β-*N*-acetylhexosaminidase from *Aspergillus oryzae*. Biotechnol Lett 1997;19:869–72.
[24] Köplin R, Brisson JR, Whitfield C. UDP-galactofuranose precursor required for formation of the lipopolysaccharide O antigen of *Klebsiella pneumoniae* serotype O1 is synthesized by the product of the $rfbD_{KPO1}$ gene. J Biol Chem 1997;272:4121–8.
[25] Yoon JH, Ajisaka K. The synthesis of galactopyranosyl derivatives with β-galactosidases of different origins. Carbohydr Res 1996;292: 153–63.