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β-*N*-Acetylhexosaminidase-catalysed synthesis of non-reducing oligosaccharides

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Abstract

A large panel of fungal β-*N*-acetylhexosaminidases was tested for the regioselectivity of the β-GlcNAc transfer onto *galacto*-type acceptors (D-galactose, lactose, 2-acetamido-2-deoxy-D-galactopyranose). A unique, non-reducing disaccharide β-D-Glc*p*NAc-(1 → 1)-β-D-Gal*p* and trisaccharides β-D-Glc*p*NAc-(1 → 4)-β-D-Glc*p*NAc-(1 → 1)-β-D-Gal*p*, β-D-Gal*p*-(1 → 4)-β-D-Glc*p*-(1 → 1)-β-D-Glc*p*NAc and β-D-Gal*p*-(1 → 4)-α-D-Glc*p*-(1 → 1)-β-D-Glc*p*NAc were synthesised under the catalysis of the β-*N*-acetylhexosaminidase from the *Aspergillus flavofurcatis* CCF 3061 with D-galactose and lactose as acceptors. The use of 2-acetamido-2-deoxy-D-galactopyranose as an acceptor with the β-*N*-acetylhexosaminidases from *A. flavofurcatis* CCF 3061, *A. oryzae* CCF 1066 and *A. tamarii* CCF 1665 afforded only β-D-Glc*p*NAc-(1 → 6)-D-Gal*p*NAc.

Keywords: β-*N*-Acetylhexosaminidase; Non-reducing saccharides; Enzymatic transglycosylation; *Aspergillus flavofurcatis*; Regioselectivity of glycosylation

1. Introduction

Fungal β-*N*-acetylhexosaminidases (EC 3.2.1.52, glycoside hydrolase family 20) [1,2] catalyse hydrolysis and also transfer of both β-GlcNAc and β-GalNAc residues to a broad variety of glycosidic and non-glycosidic acceptors, e.g. GlcNAc [3–6], GalNAc [7], oligosaccharides [8], ergot alkaloids [9], thiamine [10], L-serin [11] and many others. Reversed glycosylation using free GlcNAc with the β-*N*-acetylhexosaminidase from *Aspergillus oryzae* was also demonstrated [12].

The ability of glycosidases to form non-reducing sugars is extremely rare. We have previously prepared β-D-Glc*p*NAc-(1 → 1)-β-D-Man*p* with the β-*N*-acetylhexosaminidase from *A. oryzae*, which was probably the first documented case of non-reducing disaccharide synthesis with this glycosidase type [13]. Non-reducing galactooligosaccharide α-D-Gal*p*-(1 → 1)-α-D-Glc*p* was previously prepared by the α-galactosidase from *Candida guilliermondii* H-404 [14]. Another non-reducing disaccharide containing Gal-structures was 6′-sulfo β-D-Gal*p*-(1 → 1)-α-D-Glc*p*, recently synthesised by the β-galactosidase from *Bacillus circulans* [15]. Other two cases of enzymatic glycosylation at the anomeric hydroxyl of the glycosidic acceptor have been described, however, with glycosyltransferases: α-glucosylation of lactose [16,17] and β-galactosylation of xylose [18].

Non-reducing oligosaccharides, such as trehalose, sucrose and its derivatives often occur in the nature [19]. The non-reducing disaccharide everninose (2-*O*-methyl-α-L-lyxopyranosyl 2,6-di-*O*-methyl-β-D-mannopyranoside), isolated from everninomicins [20], belongs to the orthosomycin antibiotics family [21,22] and its chemical synthesis including its α,α-isomer was also accomplished [23].

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New synthetic analogues of trehalosamine exhibit antibacterial and immunoadjuvant properties [24,25]. β-D-Glc*p*NAc-(1 → 1)-α-D-Gal*p*NAc was found to be a structural unit of tunicamicins [26].

The regioselectivity of the glycosyl transfer catalysed by β-*N*-acetylhexosaminidases can be manipulated by changing the structure of the aglycon or the anomeric configuration of the acceptor, e.g. by the so-called "remote anomeric effect". With simple methyl glycosides α/β-Glc*p*-OMe [27], α/β-Gal*p*-OMe [28] or α/β-Glc*p*NAc-OMe [2] as acceptors, the regioselectivity of the β-GlcNAc transfer to C-3, C-4 or C-6 is influenced by the configuration at C-1 of the glycoside acceptor.

In the present paper we have investigated the regioselectivity of glycosylation of reducing *galacto*-type acceptors by β-*N*-acetylhexosaminidases, particularly with respect to the production of rare, non-reducing sugars.

2. Experimental

2.1. Materials

p-Nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside and 2-acetamido-2-deoxy-D-galactopyranose were obtained from Senn Co., Switzerland, D-galactose and lactose were purchased from Fluka, Biogel P-2 Gel (200–3000 Da) was from Bio-Rad. All β-*N*-acetylhexosaminidases used in this work originated from the Library of glycosidases of the Laboratory of Biotransformation, Institute of Microbiology in Prague and were prepared by cultivation of the respective fungi as described previously [29–31]. The producing strains are deposited with the Czech Collection of Fungi (CCF) at the Department of Botany, Charles University, Prague.

2.2. Analytical HPLC

Analytical HPLC was carried out on a modular system (Spectra Physics, USA) consisting of an SP 8800 ternary gradient pump, an SP 8880 autosampler and a Spectra Focus scanning UV-Vis detector operating at 200 nm. The analyses were performed on a Lichrospher 100-5 NH_2 column (250 mm × 4 mm) (Watrex, Czech Republic); mobile phase acetonitrile–water 79:21 for β-D-Glc*p*NAc-(1 → 1)-β-D-Gal*p* (**3**), *N*,*N*′-diacetylchitobiose (**4**) and 75:25 for β-D-Glc*p*NAc-(1 → 4)-β-D-Glc*p*NAc-(1 → 1)-β-D-Gal*p* (**5**), *N*,*N*′,*N*″-triacetylchitotriose (**6**), respectively, flow rate of 0.6 ml/min at 24 °C.

2.3. Semi-preparative HPLC

Semi-preparative HPLC was carried out on a modular system (an SP 8810 Ti pump, a Rheodyne injection port with a 100 μl sample loop, a Spectra Focus scanning UV-Vis detector operating at 200 nm; Spectra Physics, USA), a ChromJet 4400 integrator. The analyses were performed on a Lichrospher 100-5 NH_2 column (250 mm × 8 mm) (Watrex, Czech Republic); mobile phase acetonitrile–water 79:21 for **3**, **4** and 75:25 for **5**, **6**, respectively, flow rate of 3.0 ml/min at 24 °C. Authentic standards of **4** and **6** [12] were used for HPLC characterisation (co-chromatography) of respective substances in the mixtures.

2.4. NMR and mass spectroscopy

NMR spectra were recorded on a Varian UNITY *Inova*-400 MHz spectrometer (399.89 MHz for ^{1}H, 100.55 MHz for ^{13}C) in D_2O (99.95% D, Chemtrade, CZ) or $CDCl_3$ at 303 K. The assignments are based on COSY, HMQC, HMBC and 1D-TOCSY experiments. All 2D experiments were done using the manufacturer's software. Chemical shifts are referenced to internal acetone (δ_H 2.030 ppm and δ_C 30.50 ppm) for D_2O as solvent, and in $CDCl_3$ to δ = 7.265 and 77.00 ppm. Digital resolution used (0.0004 ppm for ^{1}H, 0.01 ppm for ^{13}C) justified quoting the proton chemical shifts to three and carbon chemical shifts to two decimal places. Carbon chemical shifts and some proton chemical shifts were determined from 2D spectra. Mass spectra were recorded on the Brucker MALDI-TOF (Bruker Biflex II) mass spectrometer. Optical rotations were measured with Perkin-Elmer 241 polarimeter.

2.5. Transglycosylation reactions on an analytical scale

Screening system: *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**1**, 2.0 mg, 6.0 μmol) and D-galactose (**2**, 8 mg, 44 μmol) or 2-acetamido-2-deoxy-D-galactopyranose (**7**, 6.5 mg, 29 μmol) or lactose (**9**, 10 mg, 29 μmol) as acceptors were dissolved in citrate–phosphate buffer (300 μl, 50 mM, pH 5.0) at 37 °C and the respective β-*N*-acetylhexosaminidase (0.5 U) was added. The reactions were monitored by TLC (mobile phase: propan-2-ol/water/ammonia = 7/2/1, v/v/v) on silica gel plates (Kieselgel 60 F_{254}, Merck). Compounds were visualised by spraying with 5% H_2SO_4 in ethanol.

Due to the complex course of transglycosylation reactions, the reaction mixtures were pre-separated on Bio-Gel P-2 column (48 cm × 1 cm). The samples of disaccharide fractions were submitted to the enzymatic cleavage by the β-*N*-acetylhexosaminidase from *A. flavofurcatis* CCF 3061 (0.5 U) to prove the type of the new glycosidic linkage formed. β-GlcNAc and the remaining monosaccharidic or disaccharidic units resulting from this enzymatic cleavage were analysed by HPLC. The formation of 2-acetamido-2-deoxy-D-glucopyranose was an indirect evidence of a new β-GlcNAc moiety attached to the acceptors. Results of the screening with D-galactose, 2-acetamido-2-deoxy-D-galactopyranose and lactose as acceptors are summarised in Table 1.

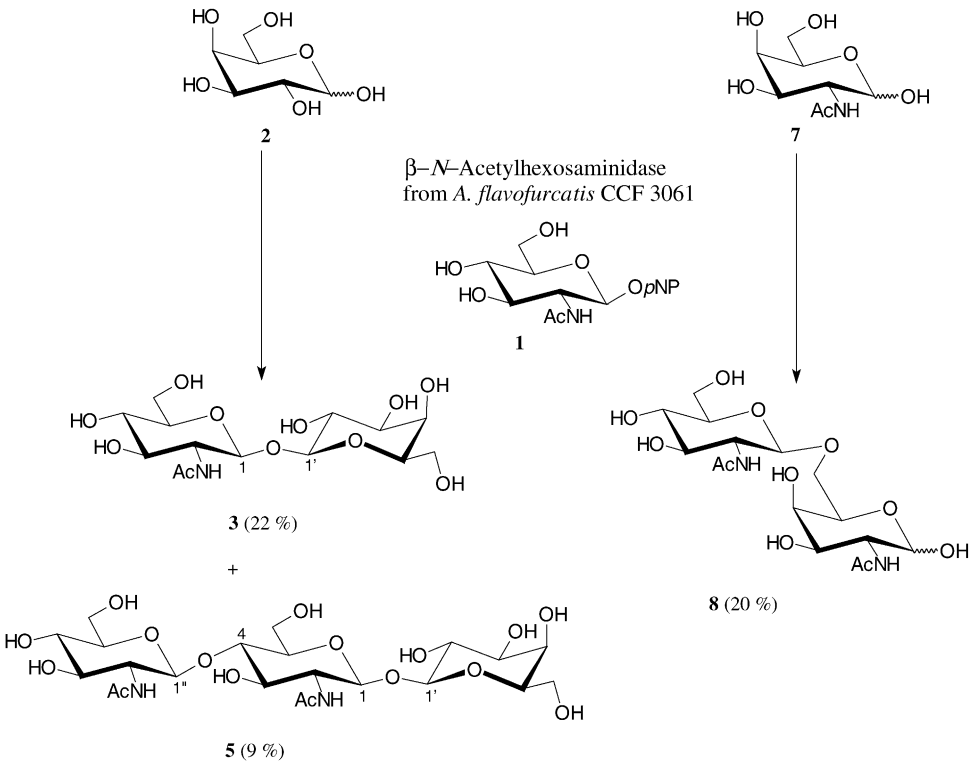
Table 1
Screening of D-galactose (Gal), 2-acetamido-2-deoxy-D-galactopyranose (GalNAc) and lactose (Lac) transglycosylation with β-*N*-acetylhexosaminidases (product formation)

Source of β-*N*-acetylhexosaminidase	Gal[a]	GalNAc[a]	Lac[a]
Acremonium persicinum CCF 1850	+	+	+
Aspergillus awamori CCF 763	++	++	+
A. caelatus CCF 3087	+	+	–
A. flavipes CCF 76	–	–	–
A. flavipes CCF 544	–	–	–
A. flavipes CCF 869	–	–	–
A. flavipes CCF 1895	++	+	+
A. flavipes CCF 2026	+	+	–
A. flavipes CCF 3067	+	+	+
A. flavofurcatis CCF 107	+	++	+
***A. flavofurcatis* CCF 3061**	**+++**	**+++**	**++**
A. flavus CCF 146	–	–	–
A. flavus CCF 814	–	–	–
A. flavus CCF 1129	++	++	+
A. flavus CCF 3056	+	+	–
A. fumigatus CCF 1059	–	–	–
A. niveus CCF 3057	–	–	–
A. nomius CCF 3086	+	++	+
A. oryzae CCF 147	–	–	–
A. oryzae CCF 1063	+++	++	+
***A. oryzae* CCF 1066**	**++**	**+++**	**++**
A. oryzae CCF 3062	+	+	+
A. parasiticus CCF 1298	++	++	+
A. parasiticus CCF 3058	+	+	–
A. phoenicus CCF 61	–	–	–
A. sojae CCF 3060	+	+	–
***A. tamarii* CCF 1665**	**++**	**+++**	**+**
A. tamarii CCF 3085	++	++	+
A. terreus CCF 3059	–	–	–
Penicillium brasilianum CCF 2155	+	+	+
P. funiculosum CCF 1994	+	++	+
P. funiculosum CCF 2325	–	–	–
P. funiculosum CCF 2984	+	+	+
P. funiculosum CCF 2985	+	+	+
P. chrysogenum CCF 1269	–	–	–
P. multicolor CCF 2244	–	–	–
P. oxalicum CCF 1959	+	+	+
P. oxalicum CCF 2315	+	+++	–
P. oxalicum CCF 2430	++	++	+
P. oxalicum CCF 3009	+	+	–
Talaromyces avellaneus CCF 2923	–	–	–
T. flavus CCF 2324	+	++	+
T. flavus CCF 2686	+	+	–
Trichoderma harzianum CCF 2687	+++	+	–

[a] Transfer of β-GlcNAc from the donor *p*NP-β-GlcNAc, 0.05 M citrate–phosphate buffer (pH 5.0), ratio donor:acceptor (1:8, donor concentration 0.028 M), 37 °C, 5.0 h (+++ >20% yield, ++ >10% yield, + <10% yield; – no transglycosylation activity observed).

*2.6. Synthesis of 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1 → 1)-β-D-galactopyranoside (β-D-Glc*p*NAc-(1 → 1)-β-D-Gal*p*) (**3**) and 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1 → 4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1 → 1)-β-D-galactopyranoside (β-D-Glc*p*NAc-(1 → 4)-β-D-Glc*p*NAc-(1 → 1)-β-D-Gal*p*) (**5**)*

D-Galactose (**2**, 201 mg, 1.120 mmol) as an acceptor and *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**1**, 48 mg, 0.140 mmol) as a donor were dissolved in citrate–phosphate buffer (50 mM, pH 5.0, 5.0 ml). The reaction mixture was incubated with the β-*N*-acetylhexosaminidase from *A. flavofurcatis* CCF 3061 (6 U) at 37 °C. After 4.5 h the reaction was stopped by heating (100 °C, 10 min). The liberated *p*-nitrophenol was extracted with diethyl ether (2 × 10 ml). The reaction mixture was lyophilised and separated on a Biogel P-2 column (120 cm × 2.5 cm, flow rate 20.5 ml/h, water). The fraction containing disaccharides (58 mg) was further separated by semi-preparative HPLC yielding **3** (12 mg, 0.031 mmol, 22%) and **4** (14 mg, 0.033 mmol, 23.5%) with retention



Scheme 1.

times 32.63 and 21.72 min, respectively. The fraction containing trisaccharides **5** and **6** (20 mg) was also separated by semi-preparative HPLC affording compounds **5** (7.5 mg, 0.012 mmol, 9%) and **6** (10.6 mg, 0.018 mmol, 13%) with retention times 31.72 and 22.44 min, respectively (Scheme 1).

Compound **3**: ^{1}H NMR (D_2O): 1.829 (3H, s, 2-Ac), 3.26 (2H, m, H-4, H-5), 3.300 (1H, dd, J = 8.0, 10.0 Hz, H-2′), 3.353 (1H, dd, J = 8.9, 10.3 Hz, H-3), 3.430 (1H, dd, J = 3.5, 10.0 Hz, H-3′), 3.464 (1H, ddd, J = 1.0, 4.8, 7.2 Hz, H-5′), 3.53 (1H, m, H-6a), 3.541 (1H, dd, J = 8.6, 10.3 Hz, H-2), 3.56 (2H, m, H-6′), 3.697 (1H, dd, J = 1.0, 3.5 Hz, H-4′), 3.709 (1H, dd, J = 1.7, 12.3 Hz, H-6b), 4.468 (1H, d, J = 8.0 Hz, H-1′), 4.673 (1H, d, J = 8.6 Hz, H-1). ^{13}C NMR (D_2O): 22.52 (2-Ac), 55.53(C-2), 60.90 (C-6), 61.32 (C-6′), 68.85 (C-4′), 70.07 (C-4), 70.47 (C-2′), 72.85 (C-3′), 74.09 (C-3), 75.52 (C-5′), 76.24 (C-5), 98.20 (C-1), 100.01 (C-1′), 175.24 (2-CO); MALDI-TOF MS: C_{14} $H_{25}NO_{11}$ (383.14): m/z 383.20 $[M + H]^+$; $[\alpha]_D^{20} = -6.98$ (c = 0.63, H_2O). Vicinal coupling constants showed that both sugars moieties responsible for two contiguous spin systems found by COSY have β-*galacto* and β-*gluco* configurations. A crosspeak between C-1 and H-1′ observed in HMBC indicated a (1 → 1) glycosidic bond.

Compound **5**: ^{1}H NMR (D_2O): 1.811 (s, 3H, 2-Ac), 1.840 (s, 3H, 2″-Ac), 3.236 (1H, dd, J = 8.4, 9.7 Hz, H-4″), 3.26 (1H, m, H-5″), 3.284 (1H, dd, J = 7.9, 10.1 Hz, H-2′), 3.301 (1H, ddd, J = 2.2, 4.6, 9.6 Hz, H-5), 3.343 (1H, dd, J = 8.4, 10.3 Hz, H-3″), 3.408 (1H, dd, J = 8.1, 9.6 Hz, H-4), 3.415 (1H, dd, J = 3.5, 10.1 Hz, H-3′), 3.44 (1H, m, H-6b), 3.450 (1H, dt, J = 0.9, 7.1 Hz, H-5′), 3.492 (1H, dd, J = 8.1, 10.3 Hz, H-3), 3.517 (1H, dd, J = 8.4, 10.3 Hz, H-2″), 3.52 (1H, m, H-6″a), 3.54 (2H, m, H-6′), 3.563 (1H, dd, J = 8.2, 10.3 Hz, H-2), 3.622 (1H, dd, J = 2.2, 12.2 Hz, H-6a), 3.652 (1H, dd, J = 0.9, 3.5 Hz, H-4′), 3.70 (1H, m, H-6″b), 4.361 (1H, d, J = 8.4 Hz, H-1″), 4.440 (1H, d, J = 7.9 Hz, H-1′), 4.658 (1H, d, J = 8.2 Hz, H-1). ^{13}C NMR (D_2O) HMQC and HMBC readouts: 24.7 (2″-Ac), 24.8 (2-Ac), 54.9 (C-2), 55.6 (C-2″), 60.3 (C-6), 60.8 (C-6″), 61.2 (C-6′), 68.8 (C-4′), 70.1 (C-4″), 70.6 (C-2′), 72.8 (C-3), 72.9 (C-3′), 73.8 (C-3″), 74.8 (C-5), 75.5 (C-5′), 76.2 (C-5″), 79.6 (C-4), 98.1 (C-1), 100.0 (C-1′), 101.7 (C-1″), 174.8 (2″-CO), 175.2 (2-CO); MALDI-TOF MS: $C_{22}H_{38}N_2O_{16}$ (586.22): m/z 586.28 $[M + H]^+$; $[\alpha]_D^{20} = -20.75$ (c = 0.4, H_2O). ^{1}H NMR spectrum consists of three spin systems (COSY). As judged from the extracted vicinal couplings, one of them has β-*galacto* and two β-*gluco* configuration. Heteronuclear couplings (HMBC) of H-1′ to C-1 and H-1″ to C-4 determine the glycosidic bonds thus confirming the structure **5**.

*2.7. Synthesis of 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1 → 6)-2-acetamido-2-deoxy-D-galactopyranose (β-D-Glc*p*NAc-(1 → 6)-D-Gal*p*NAc) (**8**)*

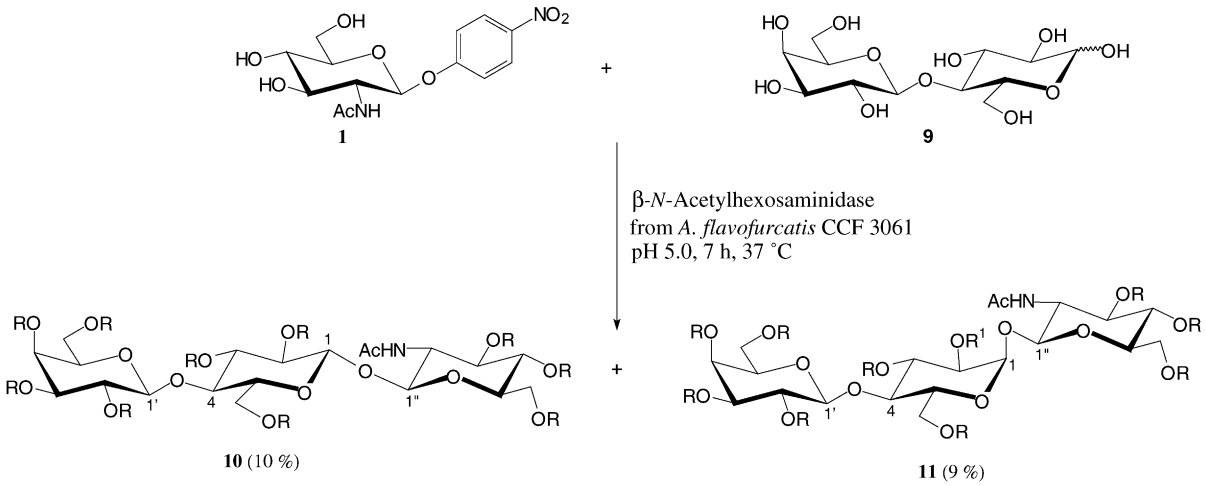
2-Acetamido-2-deoxy-D-galactopyranose (**7**, 270 mg, 1.224 mmol) and glycosyl donor **1** (52.3 mg, 0.153 mmol) were dissolved in citrate–phosphate buffer (50 mM, pH

5.0, 4.0 ml). The reaction mixture was incubated with the β-*N*-acetylhexosaminidase from *A. flavofurcatis* CCF 3061 (5.5 U) at 37 °C. The reaction was terminated after 5 h by heating (100 °C, 10 min), extracted with diethyl ether (2× 5 ml) and lyophilised, loaded on a Biogel P-2 column (120 cm × 2.5 cm, flow rate 19.5 ml/h) and eluted with water to give **8** (12.6 mg, 0.030 mmol, 20%) (Scheme 1). Yields of **8** using the enzymes from *A. tamarii* CCF 1665 and *A. oryzae* CCF 1066 were 17.5% and 14%, respectively. NMR and MS data were identical with the data published previously (**8**) [7].

*2.8. Synthesis of β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 1)-2-acetamido-2-deoxy-β-D-glucopyranoside (β-D-Gal*p*-(1 → 4)-β-D-Glc*p*-(1 → 1)-β-D-Glc*p*NAc) (**10**) and β-D-galactopyranosyl-(1 → 4)-α-D-glucopyranosyl-(1 → 1)-2-acetamido-2-deoxy-β-D-glucopyranoside (β-D-Gal*p*-(1 → 4)-α-D-Glc*p*-(1 → 1)-β-D-Glc*p*NAc) (**11**)*

β-*N*-Acetylhexosaminidase from *A. flavofurcatis* CCF 3061 (11 U) was added to the solution of lactose **9** (194 mg, 0.567 mmol) and glycosyl donor **1** (24.3 mg, 0.071 mmol) in citrate–phosphate buffer (50 mM, pH 5.0, 3.0 ml) and the mixture was incubated at 37 °C. After 7 h the reaction was stopped by heating, *p*-nitrophenol was extracted with diethyl ether, the mixture was lyophilised and separated on a Biogel P-2 column (120 cm × 2.5 cm, flow rate 15.0 ml/h, water). The trisaccharide fraction (15.5 mg) was acetylated with pyridine/Ac_2O (2 h, 60 °C). The anomeric hydroxyls of reducing sugars (**6a** and other unidentified regioisomers) were selectively 1-deacetylated with piperidine in THF (5%, 10 ml) at 0 °C for 16 h [18]. This selective deprotection of reducing sugars lowered their R_f on the silica gel chromatography ($CHCl_3$/MeOH = 10/0.6), which enabled to separate them from the non-reducing ones (Scheme 2) [13].

Compound **10** was characterised as peracetate **10a** (7.0 mg, 7.2 μmol, 10%). Compound **10a**: ^{1}H NMR ($CDCl_3$): 1.925, 1.972, 2.019, 2.021, 2.036, 2.049, 2.052, 2.067, 2.091, 2.147, 2.155 (all s, each 3H, Ac), 3.627 (1H, ddd, J = 2.2, 5.1, 9.7 Hz, H-5), 3.700 (1H, ddd, J = 8.1, 8.6, 10.4 Hz, H-2″), 3.717 (1H, ddd, J = 2.2, 4.9, 9.9, H-5″), 3.794 (1H, dd, J = 9.0, 9.7 Hz, H-4), 3.877 (1H, ddd, J = 1.1, 6.5, 7.1 Hz, H-5′), 4.081 (1H, dd, J = 7.1, 11.2 Hz, H-6′a), 4.115 (1H, dd, J = 2.2, 12.2 Hz, H-6″a), 4.134 (1H, dd, J = 6.5, 11.2 Hz, H-6′b), 4.142 (1H, dd, J = 5.1, 12.1 Hz, H-6a), 4.509 (1H, dd, J = 2.2, 12.1 Hz, H-6b), 4.258 (1H, dd, J = 4.9, 12.2 Hz, H-6″b), 4.501 (1H, d, J = 7.8 Hz, H-1′), 4.758 (1H, d, J = 7.8 Hz, H-1), 4.873 (1H, dd, J = 7.8, 9.3 Hz, H-2), 4.970 (1H, dd, J = 3.4, 10.4 Hz, H-3′), 4.975 (1H, d, J = 8.1 Hz, H-1″), 5.039 (1H, dd, J = 9.2, 9.9 Hz, H-4″), 5.115 (1H, dd, J = 7.8, 10.4 Hz, H-2′), 5.214 (1H, dd, J = 9.0, 9.3 Hz, H-3), 5.351 (1H, dd, J = 1.1, 3.4 Hz, H-4′), 5.392 (1H, dd, J = 9.2, 10.4 Hz, H-3″), 5.534 (1H, d, J = 8.6 Hz, NH-2″). ^{13}C NMR ($CDCl_3$) HMQC readouts: 54.6 (C-2″), 60.7 (C-6′), 61.5 (C-6), 61.9 (C-6″), 66.6 (C-4′), 68.4 (C-4″), 69.1 (C-2′), 70.7 (C-5′), 70.9 (C-3′), 71.0 (C-2), 71.9 (C-3″, C-5″), 72.4 (C-3), 73.0 (C-5), 75.7 (C-4), 97.6 (C-1″), 97.9 (C-1), 100.9 (C-1′); MALDI-TOF MS: $C_{40}H_{55}NO_{26}$ (965.30): *m/z* 965.60 $[M+H]^+$; $[\alpha]_D^{20} = -0.75$ (c = 0.4, $CHCl_3$). Three isolated spin systems were picked up by COSY and 1D-TOCSY experiments. According to the respective vicinal coupling constants they corresponded to two β-*gluc*o, β-*galacto* and 2-acetamido-2-deoxy-β-*gluco* units. Up-field resonating H-4 indicated a β-Gal-(1 → 4)-β-Glc glycosidic bond.



Scheme 2.

Compound **11a** behaved during C-1 deprotection with piperidine/THF in a rather surprising way as the C-2 acetate on the α-glucopyranosyl moiety was selectively removed to afford decaacetate **11b** (as determined later with MS and NMR). However, β-isomer **10a** remained during this treatment stable. Attempts to peracetylate (py/Ac_2O) **11b** furnished only mixtures of acetates. Acetylation of **11b** (or peracetylation of **11**) does not seem to be feasible probably due to steric hindrances of this position (steric conflict of two axial bonds at αC-1 and C-2). Peracetylation of β-isomer **10** proceeds smoothly and the compound is stable. Therefore, compound **11** was characterised as its decaacetate **11b** (6.5 mg, 6.7 μmol, 9%) (Scheme 2).

Compound **11b**: ^{1}H NMR ($CDCl_3$): 1.958, 1.970, 2.024, 2.032, 2.049, 2.071, 2.090, 2.102, 2.126, 2.153 (all s, 3H each, Ac), 3.575 (1H, ddd, J = 8.0, 8.0, 10.7 Hz, H-2″), 3.679 (1H, dd, J = 8.4, 9.2 Hz, H-4), 3.758 (1H, dd, J = 3.8, 8.7 Hz, H-2), 3.729 (1H, m, H-5″), 3.899 (1H, ddd, J = 1.1, 6.3, 6.8 Hz, H-5′), 4.113 (1H, dd, J = 6.8, 11.3 Hz, H-6′a), 4.115 (1H, dd, J = 7.1, 14.1 Hz, H-6a), 4.200 (2H, m, H-6″), 4.207 (1H, dd, J = 6.3, 11.3 Hz, H-6′b), 4.511 (1H, d, J = 7.9 Hz, H-1′), 4.418 (1H, dd, J = 4.2, 14.1 Hz, H-6b), 4.965 (1H, dd, J = 3.4, 10.5 Hz, H-3′), 5.106 (1H, dd, J = 7.9, 10.5 Hz, H-2′), 5.053 (1H, dd, J = 9.3, 10.0 Hz, H-4″), 5.093 (1H, d, J = 8.0 Hz, H-1″), 5.314 (1H, d, J = 3.8 Hz, H-1), 5.378 (1H, dd, J = 1.1, 3.4 Hz, H-4′), 5.406 (1H, dd, J = 8.4, 8.7 Hz, H-3), 5.481 (1H, dd, J = 9.3, 10.7 Hz, H-3″), 5.780 (1H, d, J = 7.4 Hz, NH-2″). ^{13}C NMR ($CDCl_3$) HMQC readouts: 55.8 (C-2″), 60.9 (C-6′), 61.6 (C-6″), 62.2 (C-6), 66.7 (C-4′), 68.1 (C-5), 68.5 (C-4″), 69.2 (C-2′), 70.5 (C-3), 70.9 (C-3′, C-5′), 71.2 (C-3″), 71.9 (C-5″), 76.4 (C-4), 76.8 (C-2), 91.4 (C-1), 99.8 (C-1″), 100.9 (C-1′); MALDI-TOF MS: $C_{38}H_{53}NO_{25}$ (923.29): m/z 923.60 $[M + H]^+$; $[\alpha]_D^{20} = -1.5$ (c = 0.2, $CHCl_3$). The analysis of vicinal couplings observed in three spin systems present in ^{1}H NMR spectrum (COSY, 1D-TOCSY) determined the presence of α-*gluco*, β-*galacto* and 2-acetamido-2-deoxy-β-*gluco* moieties in the molecule. The molecular weight and 10 singlets observed in the acetyl region of ^{1}H NMR mean that the compound is decaacetate. Chemical shift of H-2 (3.758 ppm) indicates that C-2 position is not acetylated in this compound (compared to the respective shift in **10a** being 4.873 ppm).

3. Results and discussion

The transglycosylation activity of 43 extracellular fungal β-*N*-acetylhexosaminidases was screened for their ability to transfer the β-GlcNAc moiety onto *galacto*-type acceptors, e.g. D-galactose (**2**), 2-acetamido-2-deoxy-D-galactopyranose (**7**) and lactose (**9**) (Table 1). The β-*N*-acetylhexosaminidases from *Aspergillus flavofurcatis* CCF 3061, *A. tamarii* CCF 1665 and *A. oryzae* CCF 1066 gave the best yields of products and thus they were selected for semi-preparative procedures.

Table 2
Effect of the donor/acceptor molar ratios on the yields of transglycosylation reactions

Molar ratio[a,b]	Transglycosylation products (yield)[c]		
	Acceptors		
Donor/acceptor	Gal	GalNAc	Lac
1:5–6	**3** (8%)	**8** (11%)	**10** (4%)
1:7	**3** (18%), **5** (9%)	**8** (16%)	**10** (6%), **11** (3%)
1:8	**3** (22%), **5** (9%)	**8** (20%)	**10** (10%), **11** (9%)

3, β-D-Glc*p*NAc-(1 → 1)-β-D-Gal*p*; **5**, β-D-Glc*p*NAc-(1 → 4)-β-D-Glc*p*NAc-(1→1)-β-D-Gal*p*; **8**, β-D-Glc*p*NAc-(1 → 6)-D-Gal*p*NAc; **10**, β-D-Gal*p*-(1 → 4)-β-D-Glc*p*-(1→1)-β-D-Glc*p*NAc; **11**, β-D-Gal*p*-(1 → 4)-α-D-Glc*p*-(1 → 1)-β-D-Glc*p*NAc.

[a] *p*NP-β-GlcNAc—used as an donor in all cases—final concentration 0.028 M.

[b] Molar ratio 1:2–4—no transglycosylation products were formed.

[c] Yields (%) of transglycosylation reactions obtained with the β-*N*-acetylhexosaminidase from *A. flavofurcatis* CCF 3061.

The yields of transglycosylation reactions catalysed by the respective β-*N*-acetylhexosaminidases were improved by optimising the molar ratios of the donor (**1**) to the acceptors (**2**, **7**, **9**). When the molar ratios of donor to acceptors were 1:2–4 (acceptor concentration 0.056–0.112 M) no transglycosylation products were formed. Increasing the molar ratios of reactants (1:6–8) and acceptor concentration (0.17–0.22 M) improved the yields of **3**, **5**, **8**, **10** and **11** and the formation of side products (**4**, **6**) was considerably suppressed (Table 2).

The reaction with D-galactose as acceptor catalysed by the β-*N*-acetylhexosaminidase from *A. flavofurcatis* CCF 3061 afforded two main products β-D-Glc*p*NAc-(1 → 1)-β-D-Gal*p* (**3**) (22%) and β-D-Glc*p*NAc-(1 → 4)-β-D-Glc*p*NAc(1 → 1)-β-D-Gal*p* (**5**) (9%) (Scheme 1). Moreover, two side products, i.e. *N*,*N*′-diacetylchitobiose (**4**) and *N*,*N*′,*N*″-triacetylchitotriose (**6**), were obtained as determined by HPLC co-chromatography with the authentic standards. The analogous reactions catalysed by the β-*N*-acetylhexosaminidases from *A. oryzae* CCF 1066 and *A. tamarii* CCF 1665 afforded non-reducing disaccharide **3** in 17% and 12% yields or trisaccharide **5** in 7% and 6% yields, respectively. With 2-acetamido-2-deoxy-D-galactopyranose as an acceptor, all three enzymes gave the single product, e.g. β-D-Glc*p*NAc-(1 → 6)-D-Gal*p*NAc (**8**). Traces of **4** could simply be separated by gel chromatography on a Biogel P-2. With lactose as an acceptor, entirely new non-reducing lacto-oligosaccharides β-D-Gal*p*-(1 → 4)-β-D-Glc*p*-(1 → 1)-β-D-Glc*p*NAc (**10**) and β-D-Gal*p*-(1 → 4)-α-D-Glc*p*-(1 → 1)-β-D-Glc*p*NAc (**11**) were formed together with side product **6**. Glycosylation of lactose by the β-*N*-acetylhexosaminidases from *A. flavofurcatis*, *A. tamarii* and *A. oryzae* was directed exclusively to its C-1 position. Besides **6**, other unidentified regioisomers were observed (HPLC) in a trace amount.

The regioselectivity of the β-GlcNAc transfer to *galacto*-type acceptors by β-*N*-acetylhexosaminidases was strongly

influenced by the change of substituent at the C-2 position. The use of 2-acetamido-2-deoxy-D-galactopyranose (**7**) as an acceptor afforded exclusively the reducing disaccharide **8**. The replacement of 2-acetamido-2-deoxy-D-galactopyranose by D-galactose or lactose led to the formation of the non-reducing oligosaccharides **3**, **5**, **10** and **11**.

4. Conclusions

This work demonstrates on the large panel of fungal β-*N*-acetylhexosaminidases that *galacto*-type carbohydrates are rather poor acceptors for transglycosylation reaction with these enzymes. An interesting result of this study is the capacity of these enzymes to synthesise unique non-reducing oligosaccharides. We assume that analogous non-reducing sugars may be plausibly "fished-out" from other glycosidase-generated reaction mixtures using appropriate technique, e.g. peracetylation/selective C-1 deacetylation.

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