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4-Deoxy-substrates for β-*N*-acetylhexosaminidases: How to make use of their loose specificity

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β-*N*-Acetylhexosaminidases feature so-called wobbling specificity, which means that they cleave substrates both in *gluco*- and *galacto*- configurations, with the activity ratio depending on the enzyme source. Here we present the new finding that fungal β-*N*-acetylhexosaminidases are able to hydrolyze and transfer 4-deoxy-*N*-acetylhexosaminides with high yields. This clearly demonstrates that the 4-hydroxy moiety at the substrate pyranose ring is not essential for substrate binding to the enzyme active site, which was also confirmed by molecular docking of the tested compounds into the model of the active site of β-*N*-acetylhexosaminidase from *Aspergillus oryzae*. A set of four 4-deoxy-*N*-acetylhexosaminides was synthesized and screened against a panel of β-*N*-acetylhexosaminidases (extracellular and intracellular) from various sources (fungal, human, animal, plant and bacterial) for hydrolysis. The results of this screening are reported here, as well as the structures of three novel 4′-deoxy-disaccharides prepared by transglycosylation reaction with high yields (52% total disaccharide fraction) using β-*N*-acetylhexosaminidase from *Talaromyces flavus*.

Keywords: β-*N*-acetylhexosaminidase/4-deoxy-glycoside/enzymatic synthesis/modified substrate/transglycosylation

Introduction

In recent years, β-*N*-acetylhexosaminidases (EC 3.2.1.52, CAZy GH20) have been intensively studied, especially their structure, function and substrate specificity. Dysfunctions in human hexosaminidases result in serious lysosomal storage disorders (Sandhoff and Tay-Sachs diseases), which draws attention to the structure and catalytic mechanism of these enzymes (Mark and James 2002; Maier et al. 2003; Mark et al. 2003; Lemieux et al. 2006). In the past decade, we have concentrated on the study of substrate specificities and structural aspects of fungal β-*N*-acetylhexosaminidases, with respect to their high synthetic potential for the preparation of bioactive oligosaccharides (Fialová et al. 2004, 2005; Loft et al. 2009). In this paper, we focused on the 4-hydroxyl group of the substrate pyranosyl ring, which can be either axial (GalNAc) or equatorial (GlcNAc). The substrates of both configurations are cleaved by β-*N*-acetylhexosaminidases, with the GlcNAc-ase/GalNAc-ase activity ratio depending on the enzyme source (Weignerová et al. 2003). This phenomenon is called the "wobbling" specificity. In our previous work (Křen et al. 1998), we have shown that, e.g., galactosyltransferase from bovine milk is also able to wobble at the C-4 position and catalyze the transfer of glucose.

4-Deoxy acetamido sugars and their derivatives have been synthesized and studied for various biological activities by several groups. Peracetylated 2-acetamido-2,4-dideoxy-4-fluoro-D-glucosamine (4-F-GlcNAc) was used for the down-regulation of the expression and ligand activity of T-cell receptors (P- and E-selectin), lowering skin inflammatory responses and preventing the dermal dissemination of cutaneous lymphomas (Descheny et al. 2006; Gainers et al. 2007). It was also shown that the treatment of ovarian tumor cells with 4-F-GlcNAc resulted in an inhibition of cellular attachment to galaptin, which plays a vital role in the adhesion of tumor cells (Woynarowska et al. 1994). The structural requirements of mammalian Gal/GalNAc-specific lectins were studied using allyl 3- and 4-deoxy-GalNAc (Ichikawa et al. 1990). Other authors observed the biological role of 4-deoxy hexosaminide derivatives as chain terminators in glycoconjugate (heparan sulfate) or chitin biosynthesis, which makes these compounds potential anti-amyloid and antifungal therapeutic agents (Berkin et al. 2000, 2002, 2005; Kisilevsky et al. 2004; Danac et al. 2007). Recently, Aerts' group presented a novel substrate 4-methylumbelliferyl 4-deoxychitobiose for an accurate and convenient chitotriosidase assay in patients with Gaucher disease (Aguilera et al. 2003; Schoonhoven et al. 2007). Finally, a number of deoxygenated donor/acceptor substrates have been used in structure–function relationship studies of various glycosyltransferases (Srivastava et al. 1990; Brockhausen et al. 1992; Kanie et al. 1993; Du and Hindsgaul 1996; Kajihara et al. 1998).

The first studies of the active site architecture and structure–function relationship in β-*N*-acetylglucosaminidases

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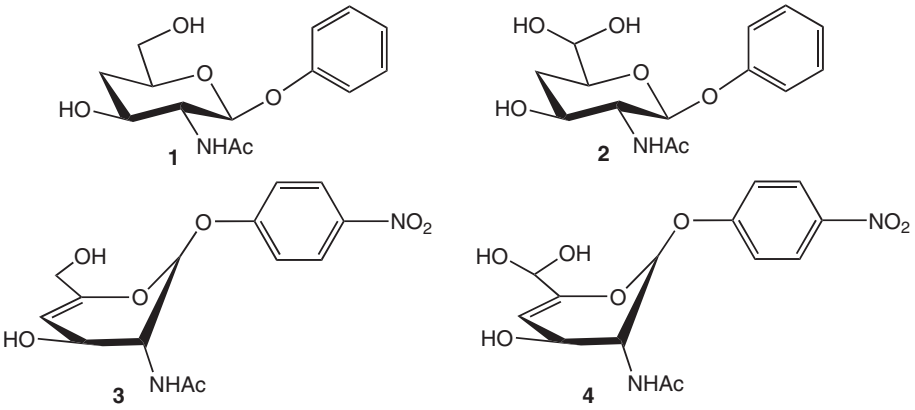

Fig. 1. β-*N*-Acetylhexosaminidase substrates **1**–**4**.

using deoxygenated substrates were performed in the 1970s. Mega et al. (1973) determined the contribution of the binding energies of the substrate hydroxyl moieties into the active site of β-*N*-acetylglucosaminidase from *Aspergillus oryzae* by determining the enzyme kinetic parameters for these phenyl deoxy-hexosaminides (incl. compound **1** presented here) and calculating the respective binding energy differences. Later, a similar experiment with β-*N*-acetylglucosaminidase from pig epididymis was described by Kolesnikov et al. (1976), using *p*-nitrophenyl deoxy-hexosaminides as substrates. The results of these projects suggested that the pyranosyl 4-hydroxy group is not crucial to the activity of this enzyme.

The aim of this work is to study the influence of the 4-hydroxyl group (and its absence) in the reaction catalyzed by β-*N*-acetylhexosaminidases from diverse sources and to develop a method for the enzymatic synthesis of complex 4′-deoxy-disaccharides. Here we report on the synthesis of 4-deoxy-glycopyranosides **1** – **4** (Figure 1) and on their use as substrates for a panel of one prokaryotic and 27 eukaryotic β-*N*-acetylhexosaminidases. The results of the activity assays were compared with computational modeling experiments. Furthermore, a high-yielding transglycosylation reaction is reported, using 4-deoxy-glycoside **1** as a glycosyl donor catalyzed by a fungal β-*N*-acetylhexosaminidase.

Results

Synthesis of 4-deoxy-glycopyranosides 1–4

Starting compounds phenyl and *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranosides (**7**, **8**) were prepared from chloride **6** (Horton 1966). Following the procedure described by Berkin et al. (2002), providing straightforward access to all four desired compounds **1**–**4** (Figure 1), we prepared phenyl and *p*-nitrophenyl 2-acetamido-3,4-di-*O*-acetyl-2-deoxy-β-D-glucopyranosides **11a** and **11b** (instead of the originally used methyl glycosides), which were oxidized by SO_3-pyridine in DMSO/TEA to yield α,β-unsaturated aldehydes **12a** and **12b** (Figure 2). Reduction of the carbonyl group in **12a** and **12b** by $NaBH_4$ in MeOH yielded α,β-unsaturated derivatives **3** and **13**, respectively. Catalytic hydrogenation of the double bond of **13** on Pd/C yielded 4-deoxy-glycoside **1**; hydrogenation of unsaturated aldehyde **14** yielded 4-deoxy derivative **2**. Compound **4** was obtained by deacetylation of **12b**.

Screening for hydrolysis of 4-deoxy-glycosides by β-N-acetylhexosaminidases

Four 2-acetamido-2,4-dideoxy-β-D-hexopyranosides **1**–**4** were tested as substrates with a large series of β-*N*-acetylhexosaminidases (24 fungal extracellular enzymes produced in the Laboratory of Biotransformation and four commercial enzymes from non-fungal sources, including a human one). The 4,5-unsaturated glycosides **3** and **4** were not cleaved by any of the enzymes tested. 4-Deoxy-hexosaminides **1** and **2** proved to be good β-*N*-acetylhexosaminidase substrates (Table I). Compound **1** in particular appeared to be a very good substrate for fungal β-*N*-acetylhexosaminidases (up to 85% hydrolysis rate relative to phenyl 2-acetamido-2-deoxy-β-D-glucopyranoside [Ph-GlcNAc]), especially for the enzymes from the *Penicillium* and *Talaromyces* genera (Table I). These results were supported by an in silico experiment, in which glycosides **1**–**4** were docked into the active site of a model of fungal β-*N*-acetylhexosaminidase (*A. oryzae*). Generally, fungal enzymes were able to catalyze the hydrolysis of 4-deoxy-glycosides more efficiently than those of plant, bacterial or mammalian origin.

Transglycosylation reactions using 4-deoxy-N-acetylhexosaminide 1 as a glycosyl donor

The conditions of the transglycosylation reaction using compound **1** as a glycosyl donor, GlcNAc (**5**) as an acceptor, and β-*N*-acetylhexosaminidase from *Talaromyces flavus* CCF 2686 were first optimized at analytical scale by varying the concentrations and ratios of the reaction components. The reactions were monitored by thin-layer chromatography (TLC), and the following reaction conditions were identified as the most efficient: 75 mM donor **1**, 300 mM acceptor **5**, incubation for 5—6 h at 35°C. The preparative transglycosylation reaction was performed using the optimum conditions described above, yielding a mixture of three 4-deoxy-disaccharides (Figure 3) with an outstanding yield of 52% of total disaccharide fraction. The reaction mixture was separated by size exclusion chromatography, the disaccharide fraction eluted from the column as a single peak. For the separation of individual products, a new

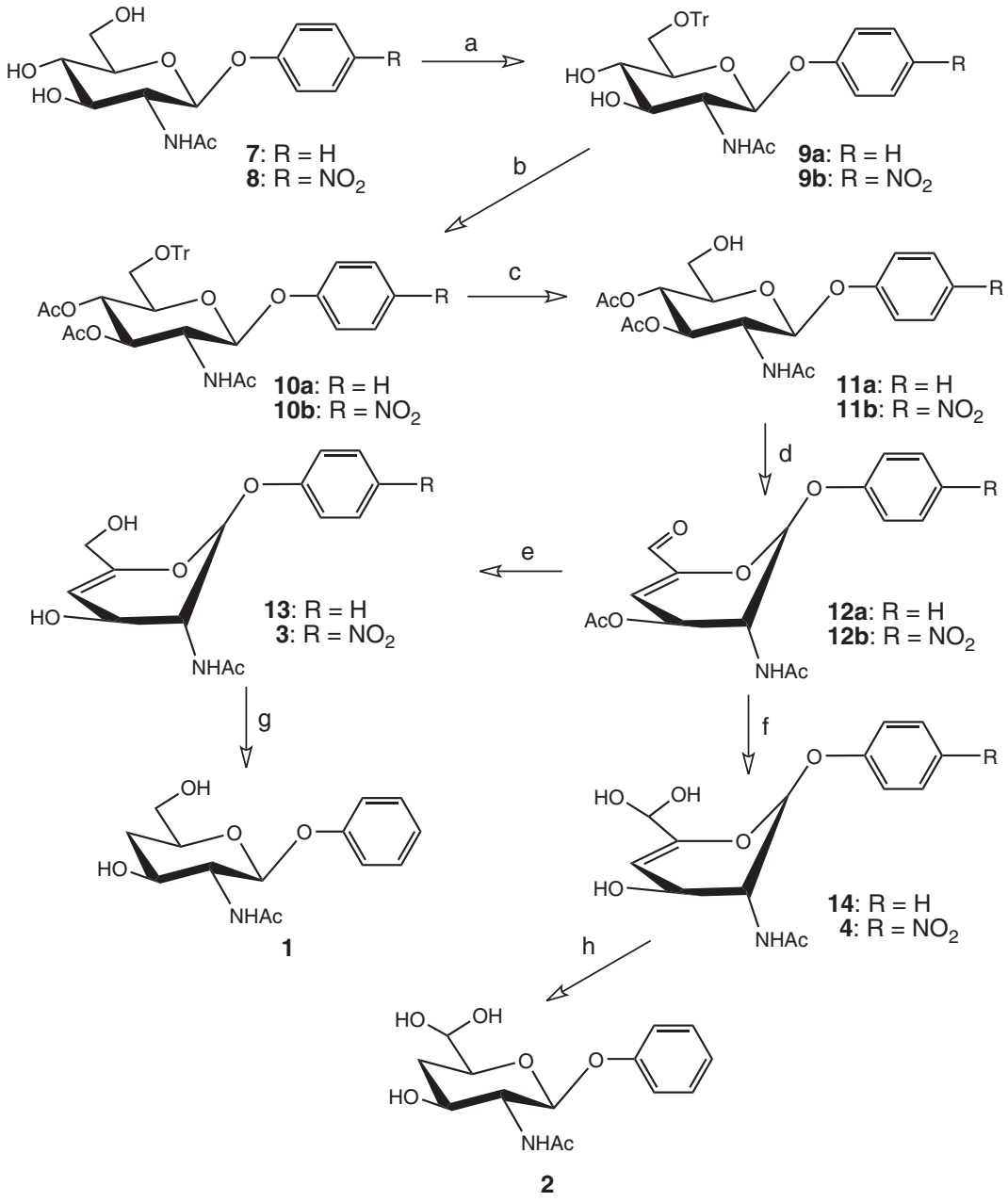

Fig. 2. Synthesis of compounds **1**–**4**. Reagents and conditions: (a) TrCl, Py, r.t., 2 d, 85%; (b) Ac_2O, Py, r.t., 12 h, 95%; (c) $HCOOH/Et_2O$ (1:1), 1.5 h, r.t., 90%; (d) SO_3·Py, $DMSO/Et_3N$, 1.5 h, r.t., 93%; (e) $NaBH_4$, MeOH, 20 min, r.t., 64%; (f) K_2CO_3, $MeOH/H_2O$ (10:1), 1 h, r.t., 90%.; (g) H_2-Pd/C, MeOH, 3 d, r.t., 60%; (h) H_2-Pd/C, MeOH, 4 h, r.t., 90%.

semipreparative ion-exchange high-performance liquid chromatography (HPLC) method was developed, capable of separating even the 1-4 and 1-6 regioisomers (Figure 4, Supplementary material). The disaccharides were eluted in a mobile phase containing 9 mM sulfuric acid, which required neutralization. Final purification and desalting was accomplished on a microcrystalline cellulose column (Avicel® PH-101). Finally, after lyophilization, three novel 4′-deoxy-disaccharides 4-deoxy-β-GlcNAc-(1→4)-GlcNAc (**15**) 7%, 4-deoxy-β-GlcNAc-(1→6)-GlcNAc (**16**) 6% and 4-deoxy-β-GlcNAc-(1→6)-4-deoxy-β-GlcNAc-*O*-Ph (**17**) 14% were obtained in sufficient purity for mass spectrometry (MS) and nuclear magnetic resonance (NMR) characterization.

In an analogous preparative transglycosylation reaction, containing only substrate **1** both as glycosyl donor and acceptor (200 mM) and catalyzed by β-*N*-acetylhexosaminidase from *T. flavus* CCF 2686, the phenyl disaccharide **17** was obtained in a similar yield (14%). The reaction was stopped after 2 h since the longer incubation time leads to the undesired cleavage of the product. The reaction mixture was separated on a Biogel P2 column, and the product **17** was eluted as a pure compound, lyophilized and characterized.

Table I. Hydrolysis of phenyl 4-deoxy-*N*-acetylhexosaminides **1**, **2** by β-*N*-acetylhexosaminidases[a]

Enzyme source	Ph-glycoside 1[b]	Ph-glycoside 2[b]
Acremonium persicinum CCF 1850	++	+
Aspergillus awamori CCF 763	+++	+
Aspergillus flavofurcatis CCF 3061	+	−
Aspergillus flavus CCF 642	++	−
Aspergillus niger CCIM K2	++	−
Aspergillus niveus CCF 3057	+	−
Aspergillus nomius CCF 3086	++	−
Aspergillus oryzae CCF 147	++	−
A. oryzae CCF 1066	++	−
Aspergillus parasiticus CCF 1298	++	−
Aspergillus tamarii CCF 3085	++	−
Aspergillus versicolor CCF 2491	+	−
Fusarium oxysporum CCF 377	+++	+
Penicillium brasilianum CCF 2171	++	+
Penicillium chrysogenum CCF 1269	+++	+
Penicillium oxalicum CCF 1959	++++	+
P. oxalicum CCF 2315	++++	+
P. oxalicum CCF 2430	+++	+
Penicillium pittii CCF 2277	++++	+
Talaromyces flavus CCF 2573	++++	++
T. flavus CCF 2686	++++	++
Talaromyces ohiensis CCF 2229	++++	++
Talaromyces striatus CCF 2232	+++	+
Trichoderma harzianum CCF 2687	+++	−
Canavalia ensiformis (Jack Bean)	++	+
Streptococcus pneumoniae	+	−
Bovine kidney	+	−
Human placenta (HEX A)	+	−

[a]*p*-Nitrophenyl glycosides **3** and **4** were not cleaved by any of the enzymes.
[b](−) <3%, (+) 4–9%, (++) 10–24%, (+++) 25–50%, (++++) >50% hydrolysis rate related to hydrolysis of standard substrate phenyl *N*-acetyl-β-D-glucosaminide **7**.

Docking and simulations of molecular dynamics

Binding energies and overall changes in the active site of β-*N*-acetylhexosaminidase from *A. oryzae* were analyzed during the simulation (10 ns) of molecular dynamics (MD). Table II presents binding parameters, binding energies and active-site interactions calculated after 10 ns of MD. Notable changes are observed in the interactions with the C-4 and C-3 of the substrate pyranose ring. In contrast to *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (*p*NP-GlcNAc) and similar to *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside (*p*NP-GalNAc), the lack of C-4 hydroxyl in 4-deoxy-substrates causes the absence of hydrogen bonding with active sites Arg 193 and Glu 418. This causes an altered position of the substrate pyranose ring in the active site.

Hydrogen bonding involving the hydroxyl at position C-3 is altered even more profoundly. For substrates **2** and **4**, no hydrogen bonding with Arg 193 is observed as the amino acid is significantly shifted in the direction of the substrate diol group. In comparison, *p*NP-GalNAc also lacks this interaction, but there the C-3 hydroxyl is stabilized by an extra hydrogen bond with Arg 308. Supposedly, these changes in the enzyme active site influence the charge distribution during the formation of the oxazolinium reaction intermediate.

Substrate **1** exhibits the highest binding energy of all 4-deoxy compounds tested—its value lies between that of *p*NP-GalNAc and *p*NP-GlcNAc. Correspondingly, the position of **1** at the active site after 10 ns of MD was similar to *p*NP-GlcNAc. As a result, substrate **1** was the most promising candidate for hydrolysis by β-*N*-acetylhexosaminidase from *A. oryzae*. Substrates **2**–**4** show considerably lower binding energies, approaching the range of non-specific binding (*cf.* **2**, **3**). This is caused by diminished hydrogen bonding and by the loss of stacking with Trp 517 and Trp 482 as a result of an improper spacial orientation of the substrates. Consequently, these substrates are not hydrolyzed by β-*N*-acetylhexosaminidases, though they apparently bind to the active site as indicated by their relatively high binding energies.

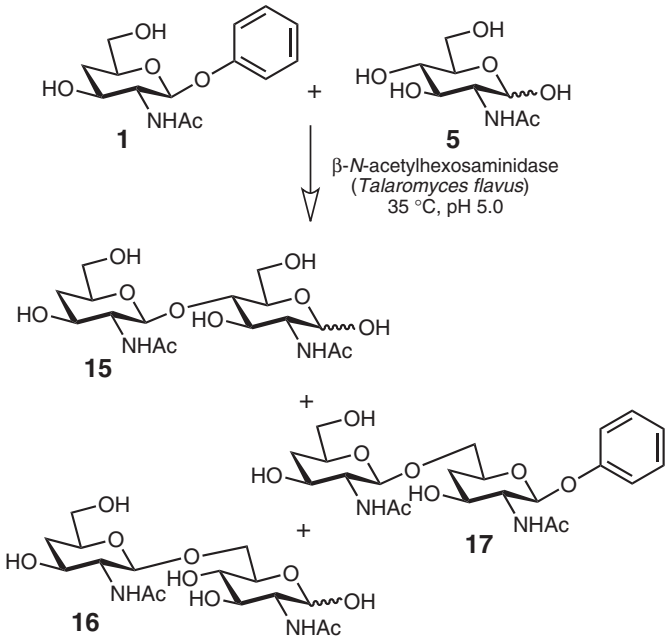

Fig. 3. Transglycosylation reaction catalyzed by β-*N*-acetylhexosaminidase from *T. flavus* using substrate **1** as glycosyl donor and GlcNAc (**5**) as acceptor.

Discussion

For synthesizing 4-deoxy-β-*N*-acetylhexosaminides, we followed the procedure by Berkin et al. (2002) based on oxidation-β-elimination. This method provided an easy access to compounds **1**–**4** (Figure 2) with similar yields to the reported methyl glycosides (Berkin et al. 2002). Due to the prolonged reaction time, over-reduction of **14** directly led to alcohol **1**. Glycosides **2**, **3** and **4** have been prepared in this way for the first time. This reaction pathway impedes the presence of *p*-nitrophenyl leaving group in substrates **1** and **2**. Instead, these compounds were prepared as phenyl glycosides, which, fortunately enough, have a similar reaction kinetics to the standardly used *p*-nitrophenyl glycosides.

Phenyl glycoside **1** was a very good substrate for fungal β-*N*-acetylhexosaminidases, (Table I), whereas aldehyde **2** was hydrolyzed much more slowly, and unsaturated compounds **3** and **4** were not cleaved at all. β-*N*-Acetylhexosaminidase from *T. flavus*, used in the transglycosylation reaction with **1**, exhibits extremely broad substrate specificity, as shown in previous studies (Fialová et al. 2004, 2005; Loft et al. 2009). The higher tolerance of β-*N*-acetylhexosaminidases of *Penicillium* sp. to the configuration of C-4 hydroxyl is presumably related to

Table II. Binding parameters of compounds docked into the model of β-*N*-acetylhexos-aminidase from *A. oryzae* during molecular dynamics experiments

Substrate	Estimated binding energy (kcal/mol)	Number of hydrogen bonds	Distance from catalytic Glu (nm)	π–π of aglycon with Trp 482	π–π stacking of pyranosyl ring with Trp 517
*p*NP-GlcNAc	−8.06	8	0.41	+	+
*p*NP-GalNAc	−6.69	6	0.54	+	+
1	−7.31	5	0.40	+	−
2	−5.74	6	0.61	−	−
3	−4.16	6	0.42	−	−
4	−6.98	4	0.60	−	−

their exceptionally high GalNAc-ase/GlcNAc-ase activity ratio (>1) (Weignerová et al. 2003). The ability of bacterial, plant and mammalian β-*N*-acetylhexosaminidases to cleave substrates **1** and **2** was much lower than that observed in the fungal enzymes. Presumably, fungi produce extracellular glycosidases to hydrolyze polymeric substrates in order to access low-molecular-weight sugars from the environment, and a high flexibility in the substrate specificity of these enzymes is of advantage. In contrast, (intracellular) enzymes of other origins play a more specific role in cell metabolism, e.g., glycoprotein processing, and therefore their specificity needs to be more focused.

Molecular modeling using the model of β-*N*-acetylhexosaminidase from *A. oryzae* (Ettrich et al. 2007) correlates well with "wet" experiments, suggesting that substrate **1** is the only substrate expected to be hydrolyzed, and showing the distortion of the active site caused by the docking of compounds **3** and **4**, which are not able to reach the conformation necessary to form the oxazoline intermediate (Figures 5 and 6, Supplementary material). Moreover, molecular dynamics simulations revealed that a successful cleavage of substrates by hexosamininidase from *A. oryzae* requires a fixed position of the C-3 hydroxyl of the substrate pyranose ring in the active site. This is mainly ensured by Arg 193 and Asp 345, and this condition is not met in substrates **2** and **4**.

Glycosylation of GlcNAc (**5**) with 4-deoxy-glycoside **1** as a glycosyl donor catalyzed by β-*N*-acetylhexosaminidase from *T. flavus* yielded three novel 4′-deoxy-disaccharides (**15**, 7%; **16**, 6%; **17**, 14%). The isolated yield of the disaccharide fraction was exceptionally high in this reaction, namely 52%. Respective disaccharides were formed in ca 1:1:1 ratio, as determined by the TLC and HPLC. The transglycosylation mode was strongly preferred over simple cleavage since the formation of the hydrolytic product, 2-acetamido-2,4-dideoxy-*xylo*-pyranose, was negligible. The relatively poor reaction regioselectivity may be improved by a selective protection of acceptor hydroxyls (Weignerová et al. 1999). As anticipated, using compound **1** both as a glycosyl donor and acceptor, the transglycosylation reaction catalyzed by β-*N*-acetylhexosaminidase from *T. flavus* yielded the disaccharide **17** (14%). An analogous transfer of a 4-deoxy-glycoside using *N*-acetylglucosaminyltransferase I was described by Srivastava et al. (1990); however, this reaction was only performed at an analytical scale, with the yield of 7% (220 μg) estimated from ^{1}H NMR signal integration. In contrast, our method using β-*N*-acetylhexosaminidase is robust, and the donor for the glycosidase reaction is considerably cheaper than the modified nucleoside required for the transferase-catalyzed reaction.

Materials and methods

All NMR and MS data are in the Supplementary material available online.

*Synthesis of 4-deoxy-hexosaminides **1–4***

*Phenyl 2-acetamido-2-deoxy-β-*D*-glucopyranoside (7)* was prepared from **5** according to published procedure (Roy and Tropper 1990).

p-*Nitrophenyl 2-acetamido-2-deoxy-β-*D*-glucopyranoside (**8**)* was prepared following the method by Begbie (1969).

p-*Nitrophenyl 2-acetamido-2-deoxy-6-*O*-triphenylmethyl-β-*D*-glucopyranoside (**9b**)* was prepared according to published procedure (Ekborg et al. 1982).

p-*Nitrophenyl 2-acetamido-3,4-di-*O*-acetyl-2-deoxy-β-*D*-glucopyranoside (**11b**)* was prepared according to Matta and Barlow (1975).

*Phenyl 2-acetamido-2-deoxy-6-*O*-triphenylmethyl-β-*D*-glucopyranoside (**9a**)*. Ph_3CCl (0.488 g, 1.750 mmol) was added to a solution of phenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**7**) (0.4 g, 1.347 mmol) in anhydrous pyridine (8 mL). The mixture was stirred at room temperature for 48 h. The resulting mixture was concentrated under reduced pressure, the residue co-evaporated with toluene and the crude product purified by flash chromatography (CH_2Cl_2/MeOH 10:1) yielding **9a** (0.615 g, 85%) as a white solid.

*Phenyl 2-acetamido-3,4-di-*O*-acetyl-2-deoxy-*β-D*-glucopyranoside (**11a**)*. Compound **9a** (0.590 g, 1.095 mmol) was dissolved in CH_2Cl_2 (20 mL), treated with Ac_2O (1 mL) and pyridine (2 mL) and the reaction mixture stirred overnight at room temperature. The mixture was washed sequentially with an ice-cooled saturated solution of $NaHCO_3$ (2 × 25 mL) and H_2O, dried (Na_2SO_4) and concentrated under reduced pressure to yield crude **10a** (0.670 g, 98%) as a white solid, which was used in the next reaction step without further purification. A solution of 1:1 HCOOH–Et_2O (50 mL) was added to compound **10a** (0.670 g, 1.075 mmol) and the mixture stirred for 1.5 h at room temperature. The mixture was concentrated under reduced pressure and the residue co-evaporated with toluene to yield a white solid. Flash chromatography (CH_2Cl_2/MeOH 10:1) on silica gel yielded **11a** (0.390 g, 95%) as a white, amorphous solid.

Phenyl 2-acetamido-3-O-*acetyl-2,4-dideoxy*-α-L-threo-*hex*-4-*enodialdo*-1,5-*pyranoside (**12a**)*. Et_3N (5 mL) at 0°C was added to a solution of **11a** (0.870 g, 2.283 mmol) in dimethyl sulfoxide (DMSO) (11 mL). Then, SO_3-pyridine (2.9 g, 18.221 mmol) in DMSO (22 mL) was added dropwise under stirring over 1.5 h. The mixture was diluted with CH_2Cl_2 (150 mL) and washed sequentially with ice-cooled aqueous tartaric acid (sat), aq $NaHCO_3$ (sat) and H_2O, dried (Na_2SO_4) and concentrated under reduced pressure to yield a residue. The residue was subjected to flash chromatography on silica gel (CH_2Cl_2/MeOH 10:1) to yield **12a** (0.680 g, 93%) as a white amorphous solid.

p-*Nitrophenyl 2-acetamido-3*-O-*acetyl-2,4-dideoxy*-α-L-threo-*hex*-4-*enodialdo*-1,5-*pyranoside (**12b**)*. Compound **12b** (0.180 g, 88%) was prepared via the same procedure as **12a**, starting from **8b** (0.240 g, 0.563 mmol).

Phenyl 2-acetamido-2,4-dideoxy-α-L-threo-*hex*-4-*enopyranoside (**13**)*. $NaBH_4$ (0.151 g, 3.992 mmol) was added to a solution of **12a** (0.680 g, 2.125 mmol) in MeOH (20 mL). The mixture was stirred for 20 min at room temperature and concentrated under reduced pressure; the residue was co-evaporated with MeOH several times. The resulting residue was subjected to flash chromatography on silica gel (CH_2Cl_2/MeOH 10:1) to yield **13** (0.380 g, 64%) as a white amorphous solid.

p-*Nitrophenyl 2-acetamido-2,4-dideoxy*-α-L-threo-*hex*-4-*enopyranoside (**3**)*. Compound **3** (0.090 g, 96%) was prepared via the same procedure as **13**, starting from **12b** (0.105 g, 0.288 mmol).

Phenyl 2-acetamido-2,4-dideoxy-β-D-xylo-*hexopyranoside (**1**)*. A mixture of **13** (0.360 g, 1.290 mmol) and 10% Pd–C (0.400 g) in MeOH (30 mL) was subjected to a hydrogen-balloon pressure for 3 days. The mixture was filtered through Celite 521 (Aldrich), the residue washed with MeOH and the combined filtrate and washings concentrated under reduced pressure to give a residue which was purified by flash chromatography on silica gel (CH_2Cl_2/MeOH 10:1) to yield **1** (0.220 g, 60.6%) as a white amorphous solid.

Phenyl 2-acetamido-2,4-dideoxy-α-L-threo-*hex-4-enodialdo-1,5-pyranoside (**14**)*. K_2CO_3 (0.052 g, 0.376 mmol) was added to a solution of **12a** (0.120 g, 0.376 mmol) in MeOH/H_2O (20 mL, 9:1, *v*/*v*) and the mixture stirred for 1 h at room temperature. Amberlite IR-120 was added to the mixture to neutral pH, the ion exchanger was then filtered off and the filtrate evaporated to dryness by co-evaporation with toluene, yielding pure **14** (0.108 g, 98%).

p-*Nitrophenyl 2-acetamido-2,4-dideoxy*-α-L-threo-*hex-4-enodialdo-1,5-pyranoside (**4**)*. Compound **4** (0.110 g, 98%) was prepared via the same procedure as **14**, starting from **12b** (0.120 g, 0.329 mmol).

*Phenyl 2-acetamido-2,4-dideoxy-β-D-xylo-1,5-dialdo-hexopyranoside (**2**)*. Pd/C (0.150 g, 10% Pd *w*/*w*) was added to a solution of **14** (0.150 g, 0.511 mmol) in MeOH (20 mL) and the resulting mixture stirred under hydrogen for 4 h. The mixture was filtered through Celite 521 (Aldrich), the residue washed with MeOH, and the combined filtrate and washings were concentrated under reduced pressure to obtain a residue, which was purified by flash chromatography on silica gel (CH_2Cl_2/MeOH 10:1) to yield **2** (0.136 g, 90%) as a white amorphous solid.

Hydrolysis of 4-deoxy-hexosaminides by β-N-acetylhexosaminidases

General: pH 5.0 citrate/phosphate buffer (McIlvaine) was prepared by mixing 0.1 M citric acid (24.3 mL) and 0.2 M Na_2HPO_4 (25.7 mL), diluting with water to 100 mL and adjusting the pH to 5.0. The fungal strains producing β-*N*-acetylhexosaminidases (EC 3.2.1.52) originated from the Culture Collection of Fungi (CCF), Department of Botany, Charles University in Prague or from the Culture Collection of the Institute of Microbiology (CCIM), Prague. The strains were cultivated in liquid media as described previously (Fialová et al. 2004). Enzymes were obtained by $(NH_4)_2SO_4$ precipitation (80% sat) of the cultivation media, and the precipitates were used directly for screening. β-*N*-Acetylhexosaminidases from non-fungal sources (*Canavalia ensiformis*, *Streptococcus pneumoniae*, bovine kidney and human placenta (HexA)) were purchased from Sigma. HPLC was carried out on a modular system consisting of DeltaChrom SDS 020 and 030 pumps (Watrex, CZ), a Spectra 100 Variable UV/VIS CE detector (Thermo Separation Products, USA), Basic Marathon Plus autosampler (Watrex, CZ). The software Clarity AS (Chromservis, CZ) was used for evaluation.

Enzyme activity assay—phenyl glycosides. The reaction mixtures (total volume 50 µL) containing Ph-GlcNAc **7** as a standard substrate or 4-deoxy-glycosides **1**, **2** (starting concentration 2 mM) and β-*N*-acetylhexosaminidase (1.6–2.5 mU for **7**, 6.4–10 mU for **1** and **2**) in pH 5.0 McIlvaine buffer were incubated in a Thermomixer (Eppendorf, DE) with shaking at 35°C for 10 min. The reaction was stopped by heating to 100°C for 3 min and centrifuged (13,000 rpm, 10 min) to remove the denatured proteins. The supernatant (40 µL) was mixed with HPLC mobile phase (40 µL; MeCN:NaOAc buffer, 50 mM, pH 5.0 = 35:65), and the amount of liberated phenol was determined by HPLC. The HPLC analysis was performed on a C18 (Phenomenex, USA) column, 250 × 4.6 mm, at ambient temperature (mobile phase MeCN:NaOAc buffer, 50 mM pH 5.0 = 35:65, flow rate 0.8 mL/min, injection volume 20 µL), and phenol was detected at 275 nm (R_t (phenol) = 5.8 min, R_t (**7**, **1** and **2**) = 2.7 min). In the enzymatic screening, 28 β-*N*-acetylhexosaminidases were tested (24 fungal, four from other sources), the enzymes classified according to the ratio of hydrolysis rates of the modified substrates **1** and **2**) and substrate **7** assayed under the same conditions and extrapolated to the same amount of enzyme. One unit of enzymatic activity was defined as the amount of enzyme that releases 1 µmol of phenol per minute under the above conditions. Each reaction was prepared in duplicate, and each was analyzed twice.

Enzyme activity assay—p-nitrophenyl glycosides. The reaction mixtures (total volume 55 µL) containing *p*NP-GlcNAc (**8**) as a standard substrate (2 mM, starting concentration) and β-*N*-acetylhexosaminidase (0.6–0.8 mU) in McIlvaine buffer pH 5.0 were incubated in 96-well microtitration plates with shaking at 35°C. After 10 min, the reaction was stopped by adding 1 M Na_2CO_3 (150 µL). Liberated *p*-nitrophenol was detected spectrophotometrically at 420 nm (Sunrise Absorbance Reader, Tecan, A). Their activity towards modified substrate **3** or **4** was determined analogously, with the amount of enzyme being 12–16 mU. One unit of enzymatic activity was defined as the amount of enzyme that releases 1 µmol of *p*-nitrophenol per minute under the above conditions. In the enzymatic screening,

28 β-*N*-acetylhexosaminidases were tested (24 fungal, four from other sources). They were classified according to the ratio of the hydrolysis rates of modified substrate **3** or **4** and substrate **8** assayed under the same conditions and extrapolated to the same amount of enzyme. Each reaction was performed in triplicate.

Analytical transglycosylation reaction

The reaction mixtures (total volume 250 μL) contained 1.7–5.1 mg of donor **1** (25–75 mM) and 6.9–16.5 mg of acceptor **5** (125–300 mM) in McIlvaine buffer pH 5.0. The reaction was started by the addition of β-*N*-acetylhexosaminidase from *T. flavus* CCF 2686 (0.2–0.7 U) and was incubated at 35°C with shaking for 6 h. The reaction progress was monitored by TLC (propane-2-ol: water: ammonia = 7:2:1) on aluminum sheets precoated with Silica Gel 60 (F_{254} Merck, G), and the plates were visualized by UV light (254 nm) and charring with a mixture of 5% sulfuric acid in ethanol.

*Preparation of 4-deoxy-disaccharides: 2-acetamido-2,4-dideoxy-β-D-xylo-hexopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucopyranose (**15**), 2-acetamido-2,4-dideoxy-β-D-xylo-hexopyranosyl-(1→6)-2-acetamido-2-deoxy-D-glucopyranose (**16**) and phenyl 2-acetamido-2,4-dideoxy-β-D-xylo-hexopyranosyl-(1→6)-2-acetamido-2,4-dideoxy-β-D-xylo-hexopyranoside (**17**)*

Phenyl glycoside **1** (41 mg, 0.15 mmol, 75 mM) and acceptor **5** (132 mg, 0.6 mmol, 300 mM) were dissolved in McIlvaine buffer pH 5.0 (1952 μL). β-*N*-Acetylhexosaminidase from *T. flavus* CCF 2686 (6 U) was added, and the mixture was shaken at 35°C. After 5.5 h, the reaction was stopped by heating to 100°C for 3 min. The reaction mixture was centrifuged (13,000 rpm, 10 min) and loaded onto a Biogel P2 column (Bio-Rad, USA) (120 cm × 2.5 cm, water, flow rate 7 mL/h). Fractions containing a mixture of disaccharides were collected, lyophilized and further purified by cation exchange semipreparative HPLC. The mixture of disaccharides was separated on a Polymer IEX H^+ column (Watrex, CZ), 250 × 8 mm, at ambient temperature (mobile phase 9 mM sulfuric acid, injection 13 × 70 μL, UV detection 210 nm). The flow rate was 0.5 mL/min during the first 20 min of the run, then it was increased to 1.2 mL/min to elute the last compound (R_t (**15**) = 12.9 min, R_t (**16**) = 14.6 min, R_t (**17**) = 60.9 min). The fractions containing the separated disaccharides were collected, immediately neutralized (100 mM sodium hydroxide), dried under vacuum and desalted on a microcrystalline cellulose (Avicel PH-101, Fluka) column (25 cm × 1 cm, mobile phase MeCN: water = 7:3); this step was repeated twice to remove the salt completely. The presence of salt (Na_2SO_4) was verified by the formation of a precipitate with $BaCl_2$. After lyophilization, disaccharides **15**, **16** and **17** were obtained as white solids in the following yields: **15**, 7% (3.7 mg, 0.009 mmol); **16**, 6% (3.4 mg, 0.008 mmol); and **17**, 14% (4.3 mg, 0.009 mmol); relative to substrate **1**.

*Preparation of phenyl 2-acetamido-2,4-dideoxy-β-D-xylo-hexopyranosyl-(1→6)-2-acetamido-2,4-dideoxy-β-D-xylo-hexopyranoside (**17**)*

Substrate **1** (43 mg, 0.16 mmol, 200 mM) was dissolved in McIlvaine buffer pH 5.0 (764 μL), and after the addition of β-*N*-acetylhexosaminidase from *T. flavus* CCF 2686 (1 U), the mixture was shaken at 35°C. After 2 h, the reaction was stopped by heating to 100°C for 3 min. The reaction mixture was centrifuged (13,000 rpm, 10 min) and loaded onto a Biogel P2 column (Bio-Rad, USA) (120 cm × 2.5 cm, water, flow rate 10 mL/h). The disaccharide **17** was freeze-dried and obtained as a white solid (5 mg, 14%).

Docking and simulations of molecular dynamics

Substrates were built in YASARA, force field parameters were derived using the AutoSMILES approach (Krieger et al. 2004). In the first step, YASARA calculated semi-empirical AM1 Mulliken point charges that were corrected by the assignment of AM1BCC atom types and improved AM1BCC charges by fragments of molecules with known RESP charges, to more closely resemble the actual RESP charges. The corresponding bond, angle and torsion potential parameters were taken from the General AMBER force field. Docking of substrates was done manually by overlaying with chitobiose docked in the active site of β-*N*-acetylhexosaminidase from *A. oryzae* (the model was reported previously) (Ettrich et al. 2007). Substrate–enzyme complexes were neutralized and minimized in water (TIP3P) with YASARA. MD simulation was started in YASARA with the following parameters: NPT ensemble (287 K, 0.997 g/mL), at pH 7, intramolecular forces were calculated every 1.5 fs, intermolecular—every 3 fs, force field Yamber2, Particle Mesh Ewald algorithm was used for long-range interaction and cutoff 0.78 nm for short-range, at the periodic boundary conditions. Estimated binding energies of the final enzyme–substrate complexes were calculated with AutoDock4 (Morris et al. 1996).

Supplementary Data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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Abbreviations

CCF, Culture Collection of Fungi; CCIM, Culture Collection of the Institute of Microbiology; DMSO, dimethyl sulfoxide; GalNAc, *N*-acetyl-D-galactosamine; GlcNAc, *N*-acetyl-D-glucosamine; 4-F-GlcNAc, 2-acetamido-2,4-dideoxy-4-fluoro-D-glucosamine; MD, molecular dynamics; MS, mass spectrometry; NMR, nuclear magnetic resonance; Ph-GlcNAc, phenyl 2-acetamido-2-deoxy-β-D-glucopyranoside; Ph-GalNAc, phenyl 2-acetamido-2-deoxy-β-D-galactopyranoside; *p*NP-

GlcNAc, *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside; *p*NP-GalNAc, *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside; TLC, thin-layer chromatography.

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