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Hydrolytic and transglycosylation reactions of *N*-acyl modified substrates catalysed by β-*N*-acetylhexosaminidases

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Abstract—The hydrolytic and transglycosylation capabilities of 35 fungal β-*N*-acetylhexosaminidases with *p*-nitrophenyl 2-amino-2-deoxy-β-D-glucopyranoside and its four *N*-acyl derivatives (CH═O, $COCH_2OH$, $COCH_2CH_3$, $COCF_3$) as substrates were tested. The preparation of four novel *p*-nitrophenyl disaccharides from these unnatural substrates catalysed by enzymes from *Aspergillus oryzae*, *Penicillium oxalicum* and *Talaromyces flavus* represents a considerable extension of the synthetic potential of glycosidases.

1. Introduction

The recent dynamic development of glycosciences has brought about the increasing need for new glycostructures. Novel carbohydrate compounds can effectively be synthesized using enzymatic methods.[1] Glycosidases (EC 3.2), although they often exhibit a poor regioselectivity and sometimes low yields, are readily available from different sources. They tolerate environmental stress and thanks to their broad substrate specificity they are able to accept a wide range of donors with different aglycon moieties and acceptors.[2–6]

It has been revealed only recently that besides their natural substrates (glycons), glycosidases are capable of accepting substrates bearing various structural modifications. These modified substrates, often exhibiting important biological properties,[7] find applications in many areas, such as structure-activity relationship studies[8–10] or treatment of glycosidase-induced pathogenic states.[9]

The first papers dealing with the enzymatic recognition of substrates modified at the glycon moiety were published in the early 70s, mostly investigating the substrate specificity of the β-*N*-acetylhexosaminidase from *Aspergillus oryzae* isolated from a digestive amylase preparation Takadiastase®. Substrates bearing 3-*O*-methyl, 6-*O*-methyl, 3,4-di-*O*-methyl and 3,4,6-tri-*O*-methyl groups,[11] as well as a range of *N*-acyl modified substrates[12,13] were studied.

The affinity of various β-*N*-acetylhexosaminidases from mushrooms and marine vertebrates to substrates modified at C-2 amino group was thoroughly studied by Molodtsov and Vafina.[14–16] They isolated and characterised the 'β-*N*-glycylhexosaminidase' from the marine helminth *Chaetopterus variopedatus*[17] and the 'β-*N*-benzoylhexosaminidase' from the scallop *Mizuhopecten yessoensis*.[18] Besides, they detected a high tolerance to *N*-acyl modifications with the β-*N*-acetylhexosaminidase from the fungus *Hohenbuehelia serotina*.[19] A similar study was carried out by Leaback and Walker with the commercial pig epididymal β-*N*-acetylhexosaminidase.[20]

Probably the first use of modified substrates in a transglycosylation reaction was described by Wong and Takayama with *p*-nitrophenyl 6-oxo-β-D-galactopyranoside.[21] C-6 modifications were also studied by MacManus et al.[22] with a set of 10 *p*-nitrophenyl galactopyranosides bearing different functionalities at C-6, such as methyl, alkene, alkyne or fluorine. A β-*N*-acetylhexosaminidase was first used by Hušáková et al. in a reaction with a 6-*O*-acetylated glycosyl donor.[23] To our best knowledge, no case of transglycosylation with *N*-acyl modified substrates catalysed by glycosidases has yet been published.

In the present paper, we report the affinity of a library of 35 fungal β-*N*-acetylhexosaminidases to various *N*-acyl modified substrates from the viewpoint of their hydrolytic and transglycosylation potentials. Four novel *p*-nitrophenyl

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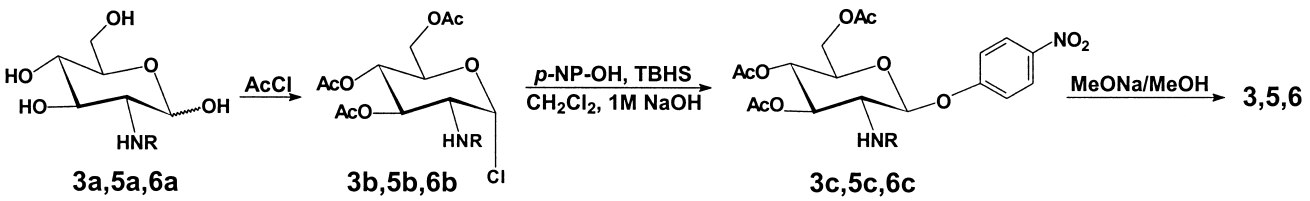


3: R = -CHO
5, **5a**: R = -$COCH_2OH$
5b, **5c**: R = -$COCH_2OAc$
6: R = -$COCH_2CH_3$

Scheme 1.

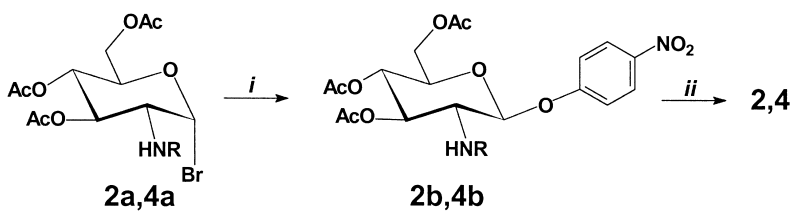
disaccharides were prepared in transglycosylation reactions with these new substrates. The experimental results are discussed regarding molecular models of modified substrates docked in the active centre of the β-*N*-acetylhexosaminidase from *A. oryzae* Culture Collection of Fungi 1066.

2. Results and discussion

Compounds **3**, **5** and **6** were prepared from the corresponding 2-acylamido-2-deoxy-D-glucopyranoses[24–26] using modified method[27] (Scheme 1). The selective formylation with *p*-nitrophenyl formiate[28] as well as those using HCOOH with Ac_2O[29,30] did not lead to the desired product, and so the formylation with a mixed acetic formic anhydride was performed.[24] Substrate **2** was synthesized using modified method[31] (Scheme 2). In the synthesis of substrate **4** (Scheme 2), 1,3,4,6-tetra-*O*-acetyl-2-amino-2-deoxy-β-D-glucopyranose hydrochloride[32] was trifluoroacetylated[33] to give **4a**.

Substrates **2**–**6** were subjected to a screening for hydrolytic cleavage comprising 35 fungal β-*N*-acetylhexosaminidases from the genera of *Acremonium*, *Aspergillus*, *Penicillium* and *Talaromyces* (Table 1).

Compounds **2** and **4** were poor substrates for all β-*N*-acetylhexosaminidases tested. On the other hand, substrates **6**, **3** and **5** (from the best to the worst, respectively) were cleaved by the majority of tested enzymes. The β-*N*-acetylhexosaminidase from *A. oryzae* Culture Collection of Fungi (CCF) 1066 did not substantially hydrolyse any of the modified substrates **2**–**6** at a comparable level to the standard substrate **1** (Table 1). These experimental results were evaluated with regard to the interaction energies calculated from the molecular model of this enzyme with substrates **1**–**6** (Table 2). The decrease of the interaction energy of an enzyme–substrate complex generally reflects better binding. Neither of the respective modified substrates exhibited any substantial steric conflict with the enzyme active centre, as could be seen from minor differences in the steric interaction energies. Surprisingly, the steric interaction energy slightly decreased with the size of the substituent (formyl→acetyl→propionyl, −71, −84 and −114 kJ/mol, respectively). More obvious differences were observed in the electrostatic interaction energies. Here, the standard substrate **1** displayed the strongest electrostatic interaction (−216 kJ/mol), whereas the electrostatic interaction energy of substrate **6** was significantly higher (−83 kJ/mol). Thus, the low cleavage of substrate **6** appears to be a result of a lower binding affinity. With substrates **2**–**5** the situation is different: it may be supposed from the interaction energy values that all these substrates bind well to the active site of the enzyme and that their low



Substrate	R	Reagents (*i*)	Reagents (*ii*)
2	-H	*p*-NP-OAg, dry MeCN, 12 h	MeONa/ MeOH, 3 h
4	-$COCF_3$	*p*-NP-OH, Fétison reagent, 2,6-dimethylpyridine, MeCN, 2 h	sat. NH_4OH: MeOH 1:10, 6 h

Scheme 2.

Table 1. Screening on the hydrolysis of substrates **2**–**6** with fungal β-*N*-acetylhexosaminidases

Source of enzyme	**2**[a]	**3**[a]	**4**[a]	**5**[a]	**6**[b]
Acremonium persicinum CCF 1850	+	+++	−	+++[c]	***[c]
Aspergillus awamori CCF 763	−	+	+	+	*
A. caelatus CCF 3087	−	+	−	+	*
A. flavipes CCF 869	−	+	−	+	*
A. flavipes CCF 1895	+	+	−	+	nd
A. flavipes CCF 3067	−	+	−	+	nd
A. flavofurcatis CCF 107	−	+	−	+	*
A. flavus CCF 642	−	+	−	+	nd
A. flavus CCF 3056	−	+	−	+	*
A. niger CCIM K2	−	+	nd	+	nd
A. niveus CCF 3057	−	+	−	+	**
A. nomius CCF 3086	−	+	−	+	nd
A. oryzae CCF 147	−	++	−	+	**
A. oryzae CCF 1066	−	++	−	++	*
A. parasiticus CCF 141	−	+	−	+	nd
A. parasiticus CCF 1298	−	+	−	+	**
A. phoenicis CCF 61	−	+	−	++	*
A. sojae CCF 3060	+	+	−	+	*
A. tamarii CCF 1665	−	+	−	−	*
A. terreus CCF 2539	+	+++	−	+++[c]	***
A. terreus CCF 3059	nd	nd	nd	nd	*
A. versicolor CCF 2491	+	+	−	+	nd
Hamigera avellanea CCF 2923	+	+++	−	++	***
Penicillium brasilianum CCF 2155	−	+++	−	+++	***[c]
P. brasilianum CCF 2171	−	+++[c]	−	+++	nd
P. chrysogenum CCF 1269	−	++	−	++	nd
P. funiculosum CCF 1994	nd	nd	−	nd	**
P. multicolor CCF 2244	−	++	−	++	**
P. oxalicum CCF 1959	−	+++	−	++	**
P. oxalicum CCF 2315	−	++	−	++	***[c]
P. oxalicum CCF 2430	−	+++	−	+++	**
P. oxalicum CCF 3009	nd	nd	−	nd	**
P. pittii CCF 2277	−	+++[c]	−	+++	nd
P. spinulosum CCF 2159	−	+++	−	+++	nd
Talaromyces flavus CCF 2686	+	+++[c]	−	+++[c]	**

[a] Hydrolysis of substrates **2**–**5** was spectrophotometrically determined as the liberated *p*-nitrophenol: the ratio of hydrolysis rates of the respective modified substrate and substrate **1** was 24–12% (+++), 11–5% (++), 4–1% (+) or lower than 1% (−).

[b] Hydrolysis of substrate **6** was analysed by TLC: the ratio of hydrolysed substrates **6** and **1** was 100–50% (***), 50–25% (**) or lower than 25% (*). Both substrates were assayed at the starting concentration of 1 mM due to poor water solubility of substrate **6** (sat. solution 1.4 mM) and enzymatic activities were estimated according to TLC.

[c] Three best hydrolysing enzymes for substrates **3**, **5** and **6**.

conversion into the hydrolytic product is caused by the destabilisation of the oxazolinium reaction intermediate in the proposed reaction mechanism,[9] or at another level of the 'dynamic' part of the hydrolytic process.

The three best hydrolysing enzymes for each of substrates **3**, **5** and **6** (Table 1) were selected as potential candidates for catalysing transglycosylation reactions, namely β-*N*-acetylhexosaminidases from *Acremonium persicinum* CCF 1850 (substrates **5** and **6**), *A. terreus* CCF 2539 (**5**), *Penicillium brasilianum* CCF 2155 (**6**), *P. brasilianum* CCF 2171 (**3**), *P. oxalicum* CCF 2315 (**6**), *P. pittii* CCF 2277 (**3**) and *T. flavus* CCF 2686 (**3**, **5**). The β-*N*-acetylhexosaminidase from *A. oryzae* CCF 1066 displayed the lowest hydrolytic potential of all tested enzymes with substrate **6** and, therefore, it was selected for the catalysis of a transglycosylation reaction with substrate **6** as a glycosyl acceptor.

Substrates **3**, **5** and **6** suffered from poor water solubility (saturated solutions at 35 °C: substrate **3**: 3.3 mM, substrate **5**: 10.0 mM, substrate **6**: 1.4 mM). To reach the optimum concentration of substrates for transglycosylation reactions (approx. 50 mM),[1] MeCN was used as a cosolvent in the reaction medium.[1,34,35] It was shown before that MeCN is well tolerated by respective β-*N*-acetylhexosaminidases.[36] The activities of the selected enzymes in MeCN (0–45% v/v) were tested (Table 3).

Table 3. Activity (%) of β-*N*-acetylhexosaminidases in MeCN

β-*N*-Acetylhexosaminidase	5% v/v MeCN[a]	45% v/v MeCN[a]
A. persicinum CCF 1850	84	0
A. terreus CCF 2539	77	33
A. oryzae CCF 1066	107	7
P. brasilianum CCF 2155	125	2
P. brasilianum CCF 2171	81	29
P. oxalicum CCF 2315	93	22
P. pittii CCF 2277	87	43
T. flavus CCF 2686	107	55

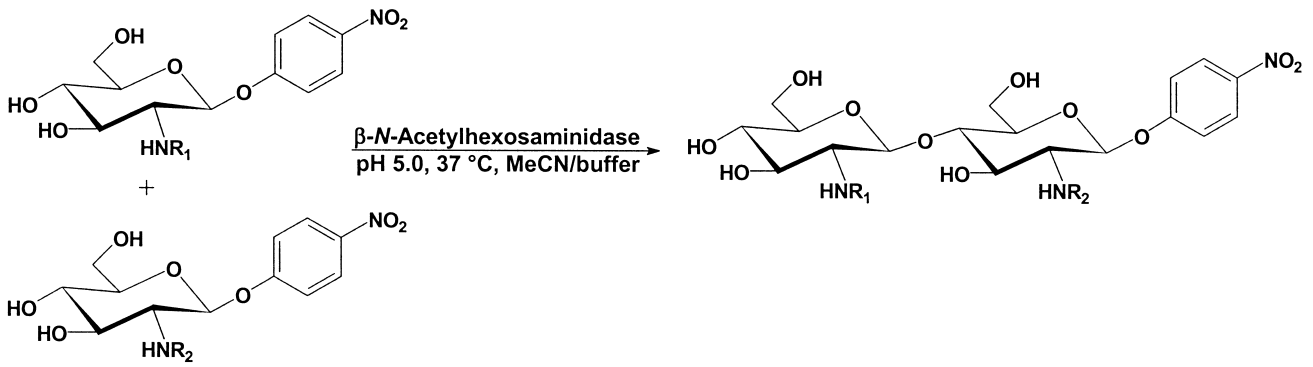
[a] Relative activities correlated to the values obtained in respective reactions without MeCN.

All β-*N*-acetylhexosaminidases were inhibited by 45% v/v MeCN, those from *A. persicinum* CCF 1850 and *P. brasilianum* CCF 2155 were almost totally inactivated. However, at 5% v/v, the cosolvent had a certain activation effect on the β-*N*-acetylhexosaminidases from *A. oryzae* CCF 1066, *P. brasilianum* CCF 2155 and *T. flavus* CCF 2686, the activity of which increased up to 125%.

To test the synthetic ability of β-*N*-acetylhexosaminidases with modified glycosyl donors and to optimise reaction conditions, reactions with substrates **3**, **5** and **6** were first tested on an analytical scale and monitored by TLC and HPLC. For each substrate, three best hydrolysing enzymes (Table 1) were tested and classified regarding the following criteria: (i) the most effective product formation; (ii) the lowest impairment of the enzymatic activity by MeCN. As a result, the following transglycosylation reaction schemes were proposed: (i) substrates **3** or **5** with the β-*N*-acetylhexosaminidase from *T. flavus* CCF 2686 in 45 and 5% v/v MeCN, respectively; (ii) substrate **6** with the

Table 2. Interaction energies of substrates with the β-*N*-acetylhexosaminidase from *A. oryzae* CCF 1066

Substrate	Total interaction energy (kJ/mol)	Steric contribution (kJ/mol)	Electrostatic contribution (kJ/mol)
1	−299	−84	−216
2	−251	−87	−164
3	−227	−71	−156
4	−226	−123	−103
5	−274	−111	−163
6	−194	−114	−83



Product	R	Source of enzyme	MeCN [%]	Yield [%]
7	$R_1 = R_2 =$ -CHO	*T. flavus* CCF 2686	45	16
8	$R_1 = R_2 =$ -$COCH_2OH$	*T. flavus* CCF 2686	5	78
9	$R_1 = R_2 =$ -$COCH_2CH_3$	*P. oxalicum* CCF 2315	45	24
10	R_1 = -$COCH_2CH_3$, R_2 = Ac	*A. oryzae* CCF 1066	45	1.8

Scheme 3.

β-*N*-acetylhexosaminidase from *P. oxalicum* CCF 2315 in 45% v/v MeCN; (iii) substrates **1** and **6** with the β-*N*-acetylhexosaminidase from *A. oryzae* CCF 1066 in 45% v/v MeCN.

The above reactions were performed on a semi-preparative scale (Scheme 3) under conditions optimised by HPLC analyses. Products **7**, **8** and **9** were obtained in reasonable yields (78% for product **8**) and with a high selectivity (exclusive formation of β(**1**→**4**) bonds). The formation of product **10** was unexpected. Due to the molar ratio of substrates **1**: **6**=1:2 and the low hydrolysis rate of substrate **6** by the β-*N*-acetylhexosaminidase from *A. oryzae* CCF 1066 we assumed the formation of *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranosyl-(**1**→**4**)-2-deoxy-2-propionamido-β-D-glucopyranoside as a major product. However, the enzyme preferred to transfer the modified glycosyl moiety to *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside and only products **9** and **10** were isolated from the reaction mixture. This demonstrates the high affinity of the β-*N*-acetylhexosaminidase from *A. oryzae* CCF 1066 to the modified substrate **6**.

3. Conclusion

The acetamido group is a crucial structural feature of substrates for β-*N*-acetylhexosaminidases, which are quite sensitive to its modification. This study clearly demonstrates for the first time that besides cleavage, fungal β-*N*-acetylhexosaminidases are able to catalyse synthetic transglycosylation reactions with *N*-acyl modified substrates. They tolerate certain sterical changes at C-2 (shorter or longer acyls, a hydroxyl instead of a hydrogen). Nevertheless, they do not accept highly electronegative acyls, for example, trifluoroacetyl, nor the free amino group. Transglycosylation products were obtained selectively and in good yields. Moreover, the β-*N*-acetylhexosaminidase from *A. oryzae* CCF 1066 exhibited a higher affinity to the modified substrate **6** than to the standard substrate **1**. This concept widely extends the synthetic potential of β-*N*-acetylhexosaminidases.

4. Experimental

4.1. Materials

Citrate/phosphate buffer (Mc Ilvaine) pH 5.0 was prepared by mixing 0.1 M citric acid (24.3 mL) and 0.2 M Na_2HPO_4 (25.7 mL), by diluting with water to 100 mL and adjusting pH to 5.0. The fungal strains producing β-*N*-acetylhexosaminidases (EC 3.2.1.52) originated from the CCF, Department of Botany, Charles University, Prague, or from the Culture Collection of the Institute of Microbiology (CCIM), Prague. The strains were cultivated as described previously.[37,38] Flasks (500 mL) with medium (100 mL) were inoculated with the suspension of spores in 0.1% Tween 80 and cultivated on a rotary shaker at 28 °C. Three media were used. Mineral medium (for *A. oryzae* CCF 1066), [g/l]: yeast extract (0.5), KH_2PO_4 (3), $NH_4H_2PO_4$ (5), $(NH_4)_2SO_4$ (2), GlcNAc (5), NaCl (15), pH 6.0. Peptone medium (for *Aspergillus awamori* CCF 763, *A. niger* CCIM K2, *A. phoenicis* CCF 61 and *P. chrysogenum* CCF 1269), [g/l]: yeast extract (0.5), mycological peptone (5), KH_2PO_4 (3), $NH_4H_2PO_4$ (5), casein acid hydrolysate (7.5), pH 6.0. In inductor supplemented medium (for all other strains in Table 1), the casamino acids were replaced by crude chitin hydrolysate (2 g/l). After sterilisation each flask was supplemented with 0.5 mL 10% $MgSO_4 \cdot 7H_2O$. Enzymes were isolated by fractional $(NH_4)_2SO_4$ precipitation (20–80% sat.) and the precipitates were directly used for respective reactions.

4.2. Methods

4.2.1. Analytical methods. TLC was performed on pre-coated Merck silica gel DC-Alufolien Kieselgel 60 F_{254}

plates. The spots were visualised by charring with 5% H_2SO_4 in EtOH. Optical rotation was measured on a Perkin–Elmer 241 polarimeter at 589 nm. CD spectra were measured on Jobin–Yvon/Spex CD 6 spectrophotometer in the spectral region of 184–400 nm in H_2O (compounds **7**, **8**) or MeOH (compounds **9**, **10**). ^{1}H- and ^{13}C NMR spectra were recorded on a Varian INOVA-400 spectrometer (399.89 MHz and 100.55 MHz, respectively) at 30 °C in the indicated solvents. Chemical shifts are expressed in the δ-scale and were measured with digital resolution justifying the reported values to three or two decimal places, respectively. HMQC and HMBC readouts are accurate to one decimal place only. Residual solvent signals were used for referencing ($CDCl_3$: δ_H 7.265, δ_C 77.00; CD_3OD: δ_H 3.330, δ_C 49.30), internal acetone (δ_H 2.030, δ_C 30.50) was used for D_2O solutions. The reported assignment is based on gCOSY, HMQC, HMBC, and 1D-TOCSY experiments. Positive-ion electrospray (ESI) mass spectra were recorded on an LCQ^{DECA} ion trap mass spectrometer (Finnigan, San Jose, USA) equipped with an ESI ion source. Spray voltage was set at 5.0 kV, tube lens voltage was −10 V. Samples dissolved in 40% MeCN were continuously infused into the ion source via a linear syringe pump at a flow rate of 3 μL/min. Full scan spectra were acquired over the *m*/*z* range of 150–1000 Da. A 0.1% solution of Ultramark 1621 (PCR, Inc., Gainesville, FL) in MeCN was used to calibrate the *m*/*z* scale of the instrument. Analytical HPLC was carried out on a Spectra Physics modular analytical system (San Jose, USA) comprised of an SP 8800 ternary gradient pump, an SP 8880 autosampler and a Spectra Focus scanning UV/VIS detector. Preparative HPLC was performed on a Spectra Physics modular preparative system (San Jose, USA) comprised of an SP 8810 Ti pump, a Rheodyne injection port with a 100 μL sample load, a Spectra 100 variable wavelength UV/VIS detector and a ChromJet SP 4400 integrator. A Lichrospher 100-5 NH_2 column (Watrex, CR, 250×4 mm—analytical and 250×8 mm—preparatory) with a mobile phase MeCN–H_2O 79:21 was used at ambient temperature. Compounds were detected at 200 nm.

4.2.2. Molecular modelling. The primary sequence of the β-*N*-acetylhexosaminidase from *A. oryzae* CCF 1066 was aligned with the known X-ray structures of the β-*N*-acetylhexosaminidases from *Serratia marcescens* and *Streptomyces plicatus*, extracted from the Brookhaven Protein Database (PDB entry: 1QBA and 1HP4, respectively). The sequence data are available in DDBJ/EMBL/GeneBank databases (http://www.ncbi.nlm.nih.gov/) under the accession AY091636. 3D models were generated by Modeller6 package.[40] For model refinement and minimisation, the SYBYL package with the TRIPOS force field (TRIPOS Associates Inc.) were used. The complete modelling including the alignment and energy minimisation was done exactly as described previously.[39] The docking of ligands was performed as described earlier.[39] The positioning of the ligand in the arbitrary site was done with the DOCK module included in SYBYL/MAXIMIN2 that calculates energies of interaction based on steric contributions from the TRIPOS force field and on electrostatic contributions from any atomic charges present in the ligand. Exact positioning of the ligand was done by a two step procedure, that is, energy minimisation followed by a molecular dynamics, in exactly the same manner for all ligands described.[39] The non-binding interaction energy between the model and the ligands within the optimised complex was calculated using the TRIPOS force field. This estimation of a real interaction energy neglects solvation and desolvation effects.

4.3. Synthesis of substrates

4.3.1. General procedure. Compound **3a**[24] (**5a**[25] or **6a**;[26] 22.0 mmol) was dissolved in acetyl chloride (15 mL) and refluxed under argon. After 12 h, the reaction mixture was diluted with CH_2Cl_2 (80 mL) and extracted with ice cold water (100 mL) and ice cold sat. $NaHCO_3$ (2 × 100 mL), all within 10–15 min. The organic phase was dried with Na_2SO_4 and evaporated in vacuo. The crude compound **3b** (**5b** or **6b**), tetrabutyl ammonium hydrogen sulphate (22.0 mmol) and *p*-nitrophenol (44.0 mmol), were dissolved in a mixture of CH_2Cl_2 (80 mL) and 1 M NaOH (80 mL) and stirred vigorously for 4 h. The reaction was monitored by TLC (AcOEt–hexane 4:1). The mixture was extracted with CH_2Cl_2 (400 mL), the organic phase was separated, washed with water (2×400 mL), dried with Na_2SO_4 and evaporated in vacuo, yielding the crude acetate **3c** (**5c** or **6c**), which was further purified.

4.3.2. *p*-Nitrophenyl 2-deoxy-2-formamido-β-D-glucopyranoside (3). Following Section 4.3.1, compound **3c** (2.45 g, 5.39 mmol; 25%) was prepared from the starting material **3a** (4.56 g, 22.0 mmol) and purified by column chromatography on Merck silica gel 60 (40–63 μm) (AcOEt–hexane 3:1). The deacetylation according to Zemplén[41] yielded the title compound **3** (1.69 g, 5.15 mmol; 96%). White crystals; $[\alpha]_D^{23}=-27.7$ (*c* 0.141, MeOH) (Lit.[12] $[\alpha]_D^{18}=-24.2$ (DMF)); according to NMR it is a mixture of 73% *trans*- and 27% *cis*-amide. ^{1}H NMR (CD_3OD) *trans*-amide: δ 3.468 (dd, 1H, *J*=9.8, 8.7 Hz, H-4), 3.564 (ddd, 1H, *J*=9.8, 5.7, 2.3 Hz, H-5), 3.661 (dd, 1H, *J*=10.3, 8.7 Hz, H-3), 3.572 (dd, 1H, *J*=12.1, 5.7 Hz, H-6u), 3.954 (dd, 1H, *J*=12.1, 2.3 Hz, H-6d), 4.023 (ddd, 1H, *J*=10.3, 8.4, 1.1 Hz, H-2), 5.290 (d, 1H, *J*=8.4 Hz, H-1), 7.206 and 8.225 (4H, AA′BB′, Σ*J*=9.3 Hz, *p*-NP), 8.213 (d, 1H, *J*=1.1 Hz, CH=O); *cis*-amide: δ 3.472 (dd, 1H, *J*=10.4, 8.5 Hz, H-4), 3.473 (dd, 1H, *J*=10.3, 7.9 Hz, H-2), 3.551 (ddd, 1H, *J*=10.4, 5.5, 2.2 Hz, H-5), 3.552 (dd, 1H, *J*=10.3, 8.5 Hz, H-3), 3.757 (dd, 1H, *J*=12.1, 5.5 Hz, H-6u), 3.953 (dd, 1H, *J*=12.1, 2.2 Hz, H-6d), 5.182 (d, 1H, *J*=7.9 Hz, H-1), 7.237 and 8.242 (4H, AA′BB′, Σ*J*=9.2 Hz, *p*-NP), 8.138 (s, 1H, CH=O); ^{13}C NMR (CD_3OD, HMQC readouts) *trans*-amide: δ 55.6 (C-2), 61.9 (C-6), 71.1 (C-4), 74.7 (C-3), 77.9 (C-5), 99.2 (C-1), 117.0 (2×C-*ortho*), 125.9 (2×C-*meta*), 163.7 (CH=O); *cis*-amide: δ 59.6 (C-2), 62.9 (C-6), 71.1 (C-4), 74.7 (C-3), 77.9 (C-5), 99.1 (C-1), 117.0 (2×C-*ortho*), 126.0 (2×C-*meta*), 167.2 (CH=O); ESI-MS: *m*/*z* calcd for $C_{13}H_{16}N_2O_8Na$ $[M+Na]^+$ 351.1, found 351.3.

4.3.3. *p*-Nitrophenyl 2-deoxy-2-glycoloylamido-β-D-glucopyranoside (5). Following Section 4.3.1, compound **5c** (3.20 g, 6.07 mmol; 28%) was prepared from the starting material **5a** (6.14 g, 22.0 mmol) and purified by column chromatography on silica gel (AcOEt–PE 2:1). White powder; ^{1}H NMR ($CDCl_3$): δ 2.070 (6H, 2×Ac), 2.086 (3H, Ac), 2.155 (3H, Ac), 3.937 (ddd, 1H, *J*=9.8, 5.6, 2.6 Hz, H-5), 4.192 (dd, 1H, *J*=12.3, 2.6 Hz, H-6u), 4.270 (ddd, 1H,

J=10.7, 8.6, 8.1 Hz, H-2), 4.290 (dd, 1H, J=12.3, 5.6 Hz, H-6d), 4.432 and 4.611 (2H, AB, J=15.5 Hz, CH_2O-), 5.168 (dd, 1H, J=9.8, 9.3 Hz, H-4), 5.370 (d, 1H, J=8.1 Hz, H-1), 5.397 (dd, 1H, J=10.7, 9.3 Hz, H-3), 6.275 (d, 1H, J=8.6 Hz, NH), 7.076 and 8.194 (4H, AA′BB′, ΣJ=9.3 Hz, *p*-NP); ^{13}C NMR ($CDCl_3$, HMQC and HMBC readouts): δ 20.5 (4×Ac), 54.2 (C-2), 61.9 (C-6), 62.7 (CH_2O-), 68.4 (C-4), 71.2 (C-3), 72.6 (C-5), 98.3 (C-1), 116.7 (2×C-*ortho*), 125.7 (2×C-*meta*), 143.2 (C-*para*), 161.3 (C-*ipso*), 167.6 (2-CO), 169.2 (4-CO), 169.5 (CH_2OCO), 170.3 (6-CO), 171.1 (3-CO); ESI-MS: *m/z* calcd for $C_{20}H_{24}N_2O_{12}Na$ $[M+Na]^+$ 549.1, found 549.2. The deacetylation according to Zemplén[41] and crystallisation from hot water yielded the title compound **5** (1.59 g, 4.43 mmol; 73%). White crystals; $[\alpha]_D^{23}=-28.9$ (*c* 0.135, H_2O).

4.3.4. *p*-Nitrophenyl 2-deoxy-2-propionamido-β-D-glucopyranoside (6). Following Section 4.3.1, compound **6c** (3.6 g, 7.5 mmol; 34%) was prepared from the starting material **6a** (5.18 g, 22.0 mmol) and purified by crystallisation from hot EtOH. The deacetylation according to Zemplén[41] and crystallisation from hot water yielded the title compound **6** (1.67 g, 4.7 mmol; 62%). White crystals; $[\alpha]_D^{23}=-17.3$ (*c* 0.196, MeOH) (Lit.[12] $[\alpha]_D^{18}=-19.7$ (DMF)); ^{1}H NMR (CD_3OD): δ 1.141 (t, 3H, J=7.7 Hz, Et), 2.227 (dq, 1H, J=16.7, 7.7 Hz), 2.278 (dq, 1H, J=16.7, 7.7 Hz), 3.453 (dd, 1H, J=9.8, 8.6 Hz, H-4), 3.537 (ddd, 1H, J=9.8, 5.7, 2.3 Hz, H-5), 3.626 (dd, 1H, J=10.4, 8.6 Hz, H-3), 3.744 (dd, 1H, J=12.1, 5.7 Hz, H-6u), 3.952 (dd, 1H, J=12.1, 2.3 Hz, H-6d), 3.982 (dd, 1H, J=10.4, 8.5 Hz, H-2), 5.229 (d, 1H, J=8.5 Hz, H-1), 7.195 and 8.226 (4H, AA′BB′, ΣJ=9.3 Hz, *p*-NP); ^{13}C NMR (CD_3OD): δ 11.4 ($\mathbf{C}H_3CH_2$), 31.5 ($CH_3\mathbf{C}H_2$), 58.2 (C-2), 63.4 (C-6), 72.5 (C-4), 76.0 (C-3), 79.2 (C-5), 100.8 (C-1), 118.0 (2×C-*ortho*), 127.1 (2×C-*meta*), 144.9 (C-*para*), 164.5 (C-*ipso*), 178.3 (C=O); ESI-MS: *m/z* calcd for $C_{15}H_{20}N_2O_8Na$ $[M+Na]^+$ 379.1, found 379.2.

4.3.5. *p*-Nitrophenyl 2-amino-2-deoxy-β-D-glucopyranoside (2). 3,4,6-Tri-*O*-acetyl-2-amino-2-deoxy-α-D-glucopyranosyl bromide hydrobromide (**2a**)[42] (8.98 g, 20.0 mmol) was dissolved in dry MeCN (150 mL), silver *p*-nitrophenolate (5 g, 20.3 mmol) was added and the mixture was stirred at ambient temperature overnight. The reaction was monitored by TLC (AcOEt–hexane 4:1). The insoluble materials were removed by filtration through Celite and the filtrate was evaporated in vacuo yielding the crude acetate **2b**, which was purified by column chromatography on silica gel (AcOEt–hexane 4:1). Acetate **2b** (3.2 g, 6.9 mmol; 35%) was deprotected according to Zemplén[41] and crystallised from hot MeOH affording the title compound **2** (991 mg, 3.3 mmol; 48%). Yellowish crystals; $[\alpha]_D^{23}=-91.1$ (*c* 0.214, H_2O) (Lit.[42] $[\alpha]_D^{20}=-42$ (*c* 0.4, MeOH) for hydrochloride); ^{1}H NMR (D_2O): δ 2.737 (dd, 1H, J=9.9, 8.2 Hz, H-2), 3.278 (m, 1H, H-3), 3.296 (m, 1H, H-4), 3.471 (ddd, 1H, J=9.6, 5.7, 2.3 Hz, H-5), 3.556 (dd, 1H, J=12.4, 5.7 Hz, H-6u), 3.730 (dd, 1H, J=12.4, 2.3 Hz, H-6d), 4.960 (d, 1H, J=8.2 Hz, H-1), 7.026 and 8.039 (4H, AA′BB′, ΣJ=9.4 Hz, *p*-NP); ^{13}C NMR (D_2O): δ 56.49 (C-2), 60.72 (C-6), 69.67 (C-4), 75.72 (C-3), 76.54 (C-5), 100.52 (C-1), 116.61 (2×C-*ortho*), 126.25 (2×C-*meta*), 142.72 (C-*para*), 161.92 (C-*ipso*); ESI-MS: *m/z* calcd for $C_{12}H_{16}N_2O_7Na$ $[M+Na]^+$ 323.1, found 323.2.

4.3.6. *p*-Nitrophenyl 2-deoxy-2-trifluoroacetamido-β-D-glucopyranoside (4). Crude syrupy 1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-trifluoroacetamido-∝-D-glucopyranosyl bromide (**4a**)[33] (318 mg, 0.685 mmol) was dried with 4A molecular sieve (250 mg) in vacuo for 2 h. Dry *p*-nitrophenol (191.6 mg, 1.4 mmol) was mixed with molecular sieve A4 (290 mg), the mixture was suspended in dry MeCN (4 mL) under argon and stirred for 10 min. 2,6-Dimethylpyridine (160 μL) was added dropwise and after 10 min of stirring, Fétison reagent (50% Ag_2CO_3/celite; 221 mg, 0.368 mmol) was added. After 10 more minutes the solution of **4a** in MeCN (3×2 mL) was added via canula. The reaction was monitored by TLC (AcOEt–hexane 1:1). After 2 h the reaction mixture was diluted with CH_2Cl_2 (50 mL), extracted with water (50 mL), 0.02 M H_2SO_4 (50 mL) and with water (3×50 mL) again. The organic phase was dried with Na_2SO_4, concentrated in vacuo and recrystallised twice from hot EtOH yielding *p*-nitrophenyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-trifluoroacetamido-β-D-glucopyranoside (**4b**) (142 mg, 0.272 mmol; 40%). White crystals; ^{1}H NMR ($CDCl_3$): δ 2.075 (s, 3H, 4-Ac), 2.086 (s, 3H, 3-Ac), 2.097 (s, 3H, 6-Ac), 3.975 (ddd, 1H, J=9.8, 5.6, 2.6 Hz, H-5), 4.177 (dd, 1H, J=12.4, 2.6 Hz, H-6u), 4.304 (dd, 1H, J=12.4, 5.6 Hz, H-6d), 4.313 (ddd, 1H, J=10.6, 8.8, 8.1 Hz, H-2), 5.182 (dd, 1H, J=9.8, 9.2 Hz, H-4), 5.336 (d, 1H, J=8.1 Hz, H-1), 5.405 (dd, 1H, J=10.6, 9.2 Hz, H-3), 6.706 (d, 1H, J=8.8 Hz, 2-NH), 7.076 and 8.204 (4H, AA′BB′, ΣJ=9.3, *p*-NP); ^{13}C NMR ($CDCl_3$): δ 20.37 (4-Ac), 20.50 (3-Ac), 20.64 (6-Ac), 54.63 (C-2), 61.87 (C-6), 68.14 (C-4), 70.95 (C-3), 72.44 (C-5), 97.58 (C-1), 114.42 (CF_3, $J_{C,F}$=287.9 Hz), 116.63 (2×C-*ortho*), 125.78 (2×C-*meta*), 143.29 (C-*para*), 157.48 (2-C=O, $J_{C,F}$=37.9), 161.09 (C-*ipso*), 169.30 (4-C=O), 170.51 (6-C=O), 170.99 (3-C=O); ESI-MS: *m/z* calcd for $C_{20}H_{21}F_3N_2O_{11}Na$ $[M+Na]^+$ 545.1, found 545.2. Compound **4b** (142 mg, 0.272 mmol) was mixed with sat. NH_4OH–MeOH 1:10 (5 mL) and left for 6 h at ambient temperature (TLC MeOH–CH_2Cl_2 1:2).[43] The evaporation in vacuo afforded **4** (107 mg, 0.270 mmol; 99%). Yellowish crystals; $[\alpha]_D^{23}=-9.2$ (*c* 0.229, H_2O) (Lit.[13] $[\alpha]_D^{25}=-10.8$ (*c* 0.157, DMF)).

4.4. Solubility of the *N*-acyl modified substrates 3, 5 and 6

The solubility of substrates **3**, **5** and **6** in MeCN/buffer mixture was measured by a gradual addition of MeCN to a suspension of substrates (1–2 mg) in citrate/phosphate buffer (pH 5.0) at 37 °C, so that the final substrate concentration would be about 50 mM and the MeCN concentration would not exceed 50% v/v, which is generally the highest possible concentration tolerated by most fungal β-*N*-acetylhexosaminidases. Substrates **3** (50 mM) and **6** (46 mM) were soluble in 45% v/v MeCN, whereas for substrate **5** (50 mM), 5% v/v MeCN was sufficient.

4.5. Enzymatic methods

4.5.1. Enzyme activity assay.[2] The reaction mixture containing substrate **1** (2 mM, starting concentration) and

β-*N*-acetylhexosaminidase (0.02–0.03 U) in buffer was incubated with shaking at 35 °C for 10 min. Liberated *p*-nitrophenol was determined spectrophotometrically (420 nm) under alkaline conditions (0.1 M Na_2CO_3). 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. The activity towards substrates **2**–**5** was determined analogously, with the amount of enzyme being 0.07–0.15 U. In the case of substrate **6**, reaction mixtures containing 1 mM substrate **6** and β-*N*-acetylhexosaminidase (0.07 U) in buffer at 35 °C were analysed by TLC after 10 min (AcOEt–MeOH–sat. NH_4OH 7:3:1). In the enzymatic screening, the enzymes were classified according to the ratio of hydrolysis rates of the respective modified substrate and substrate **1** assayed under the same conditions.

4.5.2. Analytical transglycosylation reactions. The reaction mixture contained 11–13 mg of substrate **3**, **5** (starting concentration 50 mM) or **6** (starting concentration 46 mM) in MeCN/buffer (45% v/v for substrates **3**, **6**; 5% v/v for substrate **5**). The reaction was started by the addition of β-*N*-acetylhexosaminidase (0.8–8.2 U) and was incubated at 37 °C with shaking for 24 h. Aliquots were analysed by TLC (AcOEt–MeOH–sat. NH_4OH 7:3:1) and by HPLC (reactions with substrates **3** and **5**).

4.5.3. *p*-Nitrophenyl 2-deoxy-2-formamido-β-D-glucopyranosyl-(1→4)-2-deoxy-2-formamido-β-D-glucopyranoside (7). Substrate **3** (82 mg, 0.250 mmol) was dissolved in 45% v/v MeCN/buffer (5 mL) and after the addition of the β-*N*-acetylhexosaminidase from *T. flavus* CCF 2686 (13.6 U) the mixture was shaken at 37 °C. After 3 h the reaction was stopped by diluting with MeOH and boiling for 5 min. The reaction mixture was evaporated and chromatographed on a silica gel column (AcOEt–MeOH–sat. NH_4OH 7:3:1.5) yielding the title compound **7** (10.5 mg, 0.020 mmol; 16%). White crystals; $[\Theta]$=+35.0[†] (194 nm), −4.01 (220 nm),+1.25 (280 nm), −0.662 (340 nm). According to NMR the ratio of *trans*-/*cis*-amide isomers was 74:26, however, four species containing all possible combinations were distinguished, albeit not completely; ^{1}H NMR (D_2O) *trans*-/*trans*-amide: δ 3.271 (dd, 1H, *J*=10.2, 10.1 Hz, H-3′), 3.306 (ddd, 1H, *J*=8.3, 5.3, 2.2 Hz, H-5′), 3.415 (dd, 1H, *J*=10.2, 8.3 Hz, H-4′), 3.512 (dd, 1H, *J*=12.2, 4.5 Hz, H-6u), 3.545 (dd, 1H, *J*=12.2, 5.3 Hz, H-6′u), 3.559 (ddd, 1H, *J*=8.4, 4.5, 3.2 Hz, H-5), 3.606 (dd, 1H, *J*=10.5, 10.2 Hz, H-3), 3.626 (ddd, 1H, *J*=10.1, 8.4, 0.7 Hz, H-2′), 3.679 (dd, 1H, *J*=10.5, 8.4 Hz, H-4), 3.691 (dd, 1H, *J*=12.2, 3.2 Hz, H-6d), 3.724 (dd, 1H, *J*=12.2, 2.2 Hz, H-6′d), 3.915 (ddd, 1H, *J*=10.2, 8.4, 0.6 Hz, H-2), 4.473 (d, 1H, *J*=8.4 Hz, H-1′), 5.114 (d, 1H, *J*=8.4 Hz, H-1), 6.965 and 8.022 (4H, AA′BB′, Σ*J*=9.3 Hz, *p*-NP), 8.004 (d, 1H, *J*=0.6 Hz, 2-CH═O), 8.056 (d, 1H, *J*=0.7 Hz, 2′-CH═O); *cis*-/*cis*-amide: δ 3.120 (dd, 1H, *J*=10.2, 8.0 Hz, H-2′), 3.267 (dd, 1H, *J*=9.7, 8.6 Hz, H-4′), 3.306 (ddd, 1H, *J*=9.7, 5.3, 2.2 Hz, H-5′), 3.391 (dd, 1H, *J*=10.2, 8.6 Hz, H-3′), 3.444 (dd, 1H, *J*=10.0, 8.2 Hz, H-2), 3.547 (dd, 1H, *J*=12.2, 5.3 Hz, H-6′u), 3.725 (dd, 1H, *J*=12.2, 2.2 Hz, H-6′;d), 4.438 (d, 1H, *J*=8.3 Hz, H-1′), 5.126 (d, 1H, *J*=8.5 Hz, H-1), 7.000 and 8.033 (4H, AA′BB′, Σ*J*=9.3 Hz, *p*-NP), 7.946 (s, 2H, 2×CH═O); *cis*-/*trans*-amide and *trans*-/*cis*-amide: δ 3.920 (dd, 1H, *J*=10.4, 8.3 Hz, H-2), 4.479 (d, 1H, *J*=8.3 Hz, H-1′), 5.151 (d, 1H, *J*=8.5 Hz, H-1), 8.027 (4H, AA′BB′, Σ*J*=8.6 Hz, *p*-NP), 7.818 (s, 2H, 2×CH═O); ^{13}C NMR (D_2O) *trans*-/*trans*-amide: δ 53.64 (C-2), 54.54 (C-2′), 60.04 (C-6), 60.72 (C-6′), 69.88 (C-3′), 71.98 (C-5), 73.38 (C-4′), 75.03 (C-3), 76.09 (C-5′), 78.79 (C-4), 98.30 (C-1), 101.20 (C-1′), 116.71 (2×C-*ortho*), 126.22 (2×C-*meta*), 142.89 (C-*para*), 161.74 (C-*ipso*), 165.02 (CH═O), 165.08 (CH═O); *cis*-/*cis*-amide: δ 58.32 (C-2), 59.24 (C-2′), 59.67 (C-6), 60.68 (C-6′), 69.74 (C-3′), 71.83 (C-3), 73.11 (C-4′), 75.03 (C-5), 75.09 (C-5′), 78.46 (C-4), 97.85 (C-1), 100.65 (C-1′), 116.71 (2×C-*ortho*), 126.22 (2×C-*meta*), 142.92 (C-*para*), 161.48 (C-*ipso*), 168.43 (CH═O), 168.52 (CH═O); long-range coupling between aldehyde protons and the respective H-2 protons was observed in the *trans*-series. CH═O also showed coupling to C-2 and H-2 to the aldehydic carbonyl (HMBC); ESI-MS: *m*/*z* calcd for $C_{20}H_{27}N_3O_{13}Na$ $[M+Na]^+$ 540.1, found 540.3.

4.5.4. *p*-Nitrophenyl 2-deoxy-2-glycoloylamido-β-D-glucopyranosyl-(1→4)-2-deoxy-2-glycoloylamido-β-D-glucopyranoside (8). Substrate **5** (41 mg, 0.114 mmol) was dissolved in 5% v/v MeCN/buffer (2.3 mL), the β-*N*-acetylhexosaminidase from *T. flavus* CCF 2686 (3.16 U) was added and the mixture was shaken at 37 °C. After 100 min the reaction was stopped by diluting with MeOH and boiling for 5 min. The reaction mixture was evaporated and chromatographed on a silica gel column (AcOEt–MeOH–sat. NH_4OH 7:3:1.5) yielding the title compound **8** (25.6 mg, 0.044 mmol; 78%). Colourless syrup; $[\Theta]$=+36.2 (194 nm), −4.52 (220 nm), +1.11 (280 nm), −0.534 (340 nm); ^{1}H NMR (D_2O): δ 3.276 (dd, 1H, *J*=9.9, 8.4 Hz, H-4′), 3.317 (m, 1H, H-5′), 3.478 (dd, 1H, *J*=12.3, 2.6 Hz, H-6u), 3.479 (dd, 1H, *J*=10.4, 8.4 Hz, H-3′), 3.548 (dd, 1H, *J*=12.3, 5.6 Hz, H-6′u), 3.553 (m, 1H, H-5), 3.567 (dd, 1H, *J*=9.4, 8.3 Hz, H-4), 3.627 (dd, 1H, *J*=10.4, 8.4 Hz, H-2′), 3.682 (dd, 1H, *J*=12.3, 10.9 Hz, H-6d), 3.728 (dd, 1H, *J*=12.3, 2.1 Hz, H-6′d), 3.734 (dd, 1H, *J*=10.5, 8.3 Hz, H-3), 3.867 and 3.893 (2H, AB, *J*=16.6 Hz, CH_2O-), 3.920 (dd, 1H, *J*=10.5, 8.4 Hz, H-2), 3.936 and 3.961(2H, AB, *J*=16.6 Hz, CH_2O–), 4.506 (d, 1H, *J*=8.4 Hz, H-1′), 5.170 (d, 1H, *J*=8.4 Hz, H-1), 6.959 and 8.025 (4H, AA′BB′, Σ*J*=9.3 Hz, *p*-NP); ^{13}C NMR (D_2O): δ 54.71 (C-2), 55.63 (C-2′), 60.20 (C-6), 60.89 (C-6′), 61.28 (2×CH_2O–), 70.08 (C-4′), 72.19 (C-3), 73.58 (C-3′), 75.20 (C-5), 76.23 (C-5′), 79.21 (C-4), 98.51 (C-1), 101.42 (C-1′), 116.85 (2×C-*ortho*), 126.36 (2×C-*meta*), 143.02 (C-*para*), 161.87 (C-*ipso*), 175.86 (C═O), 176.03 (C═O); ESI-MS: *m*/*z*$[M+Na]^+$ 600.2, found 600.3.

4.5.5. *p*-Nitrophenyl 2-deoxy-2-propionamido-β-D-glucopyranosyl-(1→4)-2-deoxy-2-propionamido-β-D-glucopyranoside (9). Substrate 6 (60.8 mg, 0.171 mmol) was dissolved in 45% v/v MeCN/buffer (3.7 mL) and the β-*N*-acetylhexosaminidase from *P. oxalicum* CCF 2315 (51 U) was added. The mixture was shaken at 37 °C. After 16 h the substrate was practically consumed and the reaction was stopped by diluting with MeOH (10 mL) and boiling for 5 min. The solids were filtered off; the reaction mixture was evaporated to dryness, dissolved in water and extracted into a solid phase on Amberlite XAD-4 resin (BDH Chemicals,

[†] Molar elipticity $[\Theta]$ is reported in units of 10^3 deg cm^2/dmol.

Ltd). After washing with water, *p*-nitrophenyl glycosides were eluted with MeOH, the eluent was evaporated and separated on Sephadex LH-20 (25–100 μm, Pharmacia) with a mobile phase of MeOH–H_2O 4:1 and a flow rate of 22 mL/h, which afforded the title compound **9** (11.8 mg, 0.021 mmol; 24%). White crystals; $[\Theta]$=+56.6 (194 nm), −5.95 (220 nm),+1.83 (280 nm), −0.669 (340 nm); ^{1}H NMR (CD_3OD): δ 1.136 (t, 3H, *J*=7.6 Hz, 2-Et), 1.189 (t, 3H, *J*=7.6 Hz, 2′-Et), 2.232 (dq, 1H, *J*=15.2, 7.6 Hz, 2-CH_2-u), 2.246 (dq, 1H, *J*=15.2, 7.6 Hz, 2-CH_2-d), 2.305 (dq, 1H, *J*=15.2, 7.6 Hz, 2′-CH_2-u), 2.330 (dq, 1H, *J*=15.2, 7.6 Hz, 2′-CH_2-d), 3.335 (dd, 1H, *J*=9.8, 8.3 Hz, H-4′), 3.390 (ddd, 1H, *J*=9.8, 6.4, 2.3 Hz, H-5′), 3.479 (dd, 1H, *J*=10.3, 8.3 Hz, H-3′), 3.601 (ddd, 1H, *J*=9.7, 4.6, 2.0 Hz, H-5), 3.674 (dd, 1H, *J*=11.9, 4.6 Hz, H-6′u), 3.674 (dd, 1H, *J*=9.7, 8.2 Hz, H-4), 3.695 (dd, 1H, *J*=12.2, 4.6 Hz, H-6u), 3.777 (dd, 1H, *J*=10.3, 8.5 Hz, H-2′), 3.786 (dd, 1H, *J*=10.4, 8.2 Hz, H-3), 3.874 (dd, 1H, *J*=12.2, 2.0 Hz, H-6d), 3.943 (dd, 1H, *J*=11.9, 2.2 Hz, H-6′d), 4.059 (dd, 1H, *J*=10.4, 8.4 Hz, H-2), 4.556 (d, 1H, *J*=8.5 Hz, H-1′), 5.220 (d, 1H, *J*=8.4 Hz, H-1), 7.181 and 8.221 (4H, AA′BB′, Σ*J*=9.3 Hz, *p*-NP); ^{13}C NMR (CD_3OD): δ 10.2 (Et), 10.5 (Et′), 30.5 (CH2′), 30.6 (CH2), 56.4 (C-2), 57.3 (C-2′), 61.8 (C-6), 62.7 (C-6′), 72.3 (C-4′), 74.0 (C-3), 76.0 (C-3′), 77.1 (C-5), 78.2 (C-5′), 81.0 (C-4), 100.2 (C-1), 103.3 (C-1′), 118.0 (2×C-ortho), 126.9 (2×C-meta), 144.3 (C-para), 163.7 (C-ipso), 177.2 (2′-C=O), 177.8 (2-C=O); ESI-MS: *m/z* calcd for $C_{24}H_{35}N_3O_{13}Na$ $[M+Na]^+$ 596.2, found 596.5.

4.5.6. *p*-Nitrophenyl 2-deoxy-2-propionamido-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (10). Substrates **1** (40.4 mg, 0.118 mmol) and **6** (80.6 mg, 0.226 mmol) were dissolved in 45% v/v MeCN/buffer (4.9 mL) and the β-*N*-acetylhexosaminidase from *A. oryzae* CCF 1066 (48 U) was added. The mixture was shaken at 37 °C. The enzyme was added twice more (24 U after 7 h and 12 U after 13 h). After 15.5 h substrate **1** was consumed and the reaction was stopped by diluting with MeOH (10 mL) and boiling for 5 min. The solids were filtered off and the reaction mixture was evaporated to dryness, dissolved in water and loaded onto Amberlite XAD-4 resin. After washing with water, *p*-nitrophenyl glycosides were eluted with MeOH, the eluent was evaporated and separated on Sephadex LH-20 (mobile phase MeOH–H_2O 4:1, flow rate 24 mL/h) affording a mixture of **9** and **10**, which was further separated by preparative HPLC. Besides product **9** (2.9 mg, 0.005 mmol; yield 4.3% referred to substrate **1**), the title compound **10** (1.2 mg, 0.002 mmol; yield 1.8%) was obtained. White crystals; $[\Theta]$=+91.8 (194 nm), −9.25 (220 nm),+2.75 (280 nm), −1.23 (340 nm); ^{1}H NMR (CD_3OD): 1.188 (t, 3H, *J*=7.6 Hz, Et), 1.986 (s, 3H, Ac), 2.293 (dq, 1H, *J*=15.1, 7.6 Hz, half of CH_2), 2.338 (dq, 1H, *J*=15.1, 7.6 Hz, half of CH_2), 3.335 (dd, 1H, *J*=9.6, 8.3 Hz, H-4′), 3.393 (ddd, 1H, *J*=9.6, 6.2, 2.2 Hz, H-5′), 3.479 (dd, 1H, *J*=10.3, 8.3 Hz, H-3′), 3.600 (ddd, 1H, *J*=9.7, 4.6, 2.0 Hz, H-5), 3.674 (dd, 1H, *J*=11.9, 6.2 Hz, H-6′u), 3.676 (dd, 1H, *J*=9.7, 8.2 Hz, H-4), 3.691 (dd, 1H, *J*=12.2, 4.6 Hz, H-6u), 3.778 (dd, 1H, *J*=10.3, 8.5 Hz, H-2′), 3.780 (dd, 1H, *J*=10.3, 8.2 Hz, H-3), 3.869 (dd, 1H, *J*=12.2, 2.0 Hz, H-6d), 3.945 (dd, 1H, *J*=11.9, 2.2 Hz, H-6′d), 4.047 (dd, 1H, *J*=10.3, 8.5 Hz, H-2) 4.556 (d, 1H, *J*=8.5 Hz, H-1′), 5.224 (d, 1H, *J*=8.5 Hz, H-1). 7.186 and 8.223 (4H, AA′BB′, Σ*J*=9.3 Hz, *p*-NP); ^{13}C NMR (CD_3OD, HMQC and HMBC readouts): δ 10.4 ($\mathbf{C}H_3CH_2$), 23.0 (Ac), 30.6 ($CH_3\mathbf{C}H_2$), 56.7 (C-2), 57.5 (C-2′), 61.8 (C-6), 62.8 (C-6′), 72.4 (C-4′), 74.2 (C-3), 76.1 (C-3′), 77.1 (C-5), 78.5 (C-5′), 81.1 (C-4), 100.2 (C-1), 103.4 (C-1′), 118.0 (2×C-*ortho*), 126.9 (2×C-*meta*), 144.7 (C-*para*), 164.2 (C-*ipso*), 174.2 ($CH_3\mathbf{C}O$), 177.8 ($CH_3CH_2\mathbf{C}O$); ESI-MS: *m/z* calcd for $C_{23}H_{33}N_3O_{13}Na$ $[M+Na]^+$ 582.2, found 582.4.

4.5.7. NMR characterisation of transglycosylation products. All prepared disaccharides showed the expected pseudomolecular ions in the MS spectra. According to $J_{1,2}$ values (^{1}H NMR), the anomeric configuration was β in all cases. H-1 and H-1′ protons were differentiated on the basis of the coupling of the former to C-*ipso* of *p*-NP. The (**1→4**)-linkage was inferred from heteronuclear couplins of H-1′ to C-4 or H-4 to C-1′ observed in HMBC. The downfield shift of C-4 with respect to the parent compound provided a supporting argument. The attachment of a substituent to C-2 is apparent from the coupling of H-2 and side chain protons to the same carbonyl.

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References and notes

1. Křen, V.; Thiem, J. *J. Chem. Soc. Rev.* **1997**, *26*, 463–473.
2. Mega, T.; Ikenaka, T.; Matsushima, Y. *J. Biochem.* **1970**, *68*, 109–117.
3. Monsan, P.; Paul, F. *FEMS Microbiol. Rev.* **1995**, *16*, 187–192.
4. Bucke, C. *J. Chem. Technol. Biotechnol.* **1996**, *67*, 217–220.
5. Ščigelová, M. Ph.D. Thesis, Charles University Prague, 1997.
6. Ščigelová, M.; Crout, D. H. G. *Enzyme Microb. Technol.* **1999**, *25*, 3–14.
7. Krist, P.; Herkommerová-Rajnochová, E.; Rauvolfová, J.; Semeňuk, T.; Vavrušková, P.; Pavlíček, J.; Bezouška, K.; Petruš, L.; Křen, V. *Biochem. Biophys. Res. Commun.* **2001**, *287*, 11–20.
8. Williams, S. J.; Withers, S. G. *Carbohydr. Res.* **2000**, *327*, 27–46.
9. Horsch, M.; Mayer, C.; Sennhauser, U.; Rast, D. M. *Pharmacol. Ther.* **1997**, *76*, 187–218.
10. Muzzarelli, R. A. A. Analytical biochemistry and clinical significance of *N*-acetyl-β-D-glucosaminidase and related enzymes. In *Chitin and Chitinases*; Jollès, P., Muzzarelli, R. A. A., Eds.; Birkhäuser Verlag: Basel, 1999; pp 235–247.
11. Mega, T.; Ikenaka, T.; Matsushima, Y. *J. Biochem.* **1972**, *71*, 107–114.

12. Yamamoto, K. *J. Biochem.* **1973**, *73*, 631–635.
13. Yamamoto, K. *J. Biochem.* **1973**, *73*, 749–753.
14. Molodtsov, N. V.; Vafina, M. G. *Int. J. Biochem.* **1974**, *5*, 235–237.
15. Molodtsov, N. V.; Vafina, M. G. *Int. J. Biochem.* **1974**, *5*, 239–240.
16. Molodtsov, N. V.; Vafina, M. G. *Comp. Biochem. Physiol., B* **1974**, *48*, 257–260.
17. Molodtsov, N. V.; Vafina, M. G. *Biochim. Biophys. Acta* **1974**, *364*, 296–303.
18. Molodtsov, N. V.; Vafina, M. G. *Comp. Biochem. Physiol., B* **1975**, *51*, 313–315.
19. Molodtsov, N. V.; Vafina, M. G. *Carbohydr. Res.* **1976**, *47*, 188–194.
20. Leaback, D. H.; Walker, P. G. *Biochem. J.* **1967**, *104*, 70.
21. Takayama, S.; Wong, C.-H. *Curr. Org. Chem.* **1997**, *1*, 109–126.
22. MacManus, D. A.; Grabowska, U.; Biggadike, K.; Bird, M. I.; Vulfson, E. N.; Gallagher, T. *J. Chem. Soc., Perkin Trans. 1* **1999**, 295–305.
23. Hušáková, L.; Riva, S.; Casali, M.; Nicotra, S.; Kuzma, M.; Huňková, Z.; Křen, V. *Carbohydr. Res.* **2001**, *331*, 143–148.
24. Khanna, I. K.; Weier, R. M.; Lentz, K. T.; Swenton, L.; Lankin, D. C. *J. Org. Chem.* **1995**, *60*, 960–965.
25. Kuboki, A.; Okazaki, H.; Sugai, T.; Ohta, H. *Tetrahedron* **1997**, *53*, 2387–2400.
26. Inouye, Y.; Onodera, K.; Kitaoka, S.; Hirano, S. *J. Am. Chem. Soc.* **1956**, *78*, 4722–4724.
27. Singh, S.; Packwood, J.; Samuel, C. J.; Critchley, P.; Crout, D. H. G. *Carbohydr. Res.* **1995**, *279*, 293–305.
28. Yamamoto, K. *Bull. Chem. Soc. Jpn.* **1972**, *45*, 1253–1254.
29. Sheehan, J. C.; Yang, D.-D. H. *J. Am. Chem. Soc.* **1958**, *80*, 1154–1158.
30. Greig, C. G.; Leaback, D. H.; Walker, P. *J. Chem. Soc.* **1961**, *1*, 879–883.
31. Yasukochi, T.; Inaba, C.; Fukase, K.; Kusumoto, S. *Tetrahedron Lett.* **1999**, *40*, 6585–6589.
32. Bergmann, M.; Zervas, L. *Chem. Ber.* **1931**, *64*, 975–980.
33. Wolfrom, M. L.; Bhat, H. B. *J. Org. Chem.* **1967**, *32*, 1821–1823.
34. Sauerbrei, B.; Thiem, J. *Tetrahedron Lett.* **1992**, *33*, 201–204.
35. Scheckermann, C.; Wagner, F.; Fischer, L. *Enzyme Microb. Technol.* **1997**, *20*, 629–634.
36. Kubisch, J.; Weignerová, L.; Kötter, S.; Lindhorst, T. K.; Sedmera, P.; Křen, V. *J. Carbohydr. Chem.* **1999**, *18*, 975–984.
37. Huňková, Z.; Křen, V.; Ščigelová, M.; Weignerová, L.; Scheel, O.; Theim, J. *Biotechnol. Lett.* **1996**, *18*, 725–730.
38. Huňková, Z.; Kubátová, A.; Weignerová, L.; Křen, V. *Czech. Mycol.* **1999**, *51*, 71–88.
39. Hušáková, L.; Herkommerová-Rajnochová, E.; Semeňuk, T.; Kuzma, M.; Rauvolfová, J.; Přikrylová, V.; Ettrich, R.; Plíhal, O.; Bezouška, K.; Křen, V. *Adv. Synth. Catal.* **2003**, *345*, 735–742.
40. Sali, A.; Blundell, T. L. *J. Mol. Biol.* **1993**, *234*, 779–815.
41. Zemplén, G.; Pacsu, E. *Chem. Ber.* **1929**, *62*, 1613–1614.
42. May, E. L.; Mosettig, E. *J. Org. Chem.* **1950**, *15*, 890–895.
43. Burke, S. D.; Danheiser, R. L. Oxidizing and Reducing Agents. Handbook of Reagents for Organic Synthesis; Wiley: Chichester, 1999; P 361.