Enzymatic Discrimination of 2-Acetamido-2-deoxy-D-mannopyranose-Containing Disaccharides Using β-*N*-Acetylhexosaminidases

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This paper is dedicated to Prof. David Herbert Gordon Crout on the occasion of his retirement.

Abstract: β-*N*-Acetylhexosaminidase from *Aspergillus oryzae* selectively discriminates mixture of the disaccharides GlcNAcβ(1→4)GlcNAc (**1**) and GlcNAcβ(1→4)ManNAc (**2**). *N,N′*-Diacetylchitobiose (**1**) was selectively hydrolyzed by β-*N*-acetylhexosaminidase, whereas its C-2 epimer (**2**) was completely resistant to the enzyme hydrolysis. Analogous discrimination was observed also with GalNAcβ(1→4)GlcNAc (**3**) and GalNAcβ(1→4)ManNAc (**4**). β-*N*-Acetylhexosaminidases from *A. terreus, A. flavus*, bovine kidney and bovine epididymis displayed the same selectivity, whereas the enzymes from *A. sojae, A. tamarii, Penicillium brasilianum, P. oxalicum, P. funiculosum, P. multicolor, Talaromyces flavus* and jack beans hydrolyzed both types of disaccharides. Molecular modelling of β-*N*-acetylhexosaminidase from *A. oryzae* CCF 1066 and docking experiments with both types of disaccharides revealed that the ManNAc residue causes distortion of disaccharide molecule resulting in a steric conflict with a Trp^{482} that causes diminished stabilization of the oxazolinium transition state by extending the distance of Asp^{345} in the active site. Both ManNAc-containing disaccharides **2** and **4** dock with similar steric energies into the active site but without cleaving and also without notable inhibition. This novel phenomenon enables also the preparative production of both disaccharides **2** and **4** starting from *N, N*'-diacetylchitobiose (**1**) or GalNAcβ(1→4)GlcNAc (**3**) followed by Lobry de Bruyn–Albreda van Ekenstein C-2 epimerization catalyzed by $Ca(OH)_2$. The resultant mixture of the respective epimers **1**, **2** or **3**, **4** that is hardly separable by, e.g., analytical HPLC can be treated with the β-*N*-acetylhexosaminidase from *A. oryzae* and the mixture of monosaccharides and target disaccharide can be easily separated using gel filtration.

Keywords: acetamido sugars; alkali-catalyzed epimerization; *Aspergillus oryzae*; *N,N′*-diacetylchitobiose; molecular modelling

Abbreviations: CCF: Culture Collection of Fungi, Department of Botany, Charles University, Prague; GlcNAc: 2-acetamido-2-deoxy-D-glucopyranose; ManNAc: 2-acetamido-2-deoxy-D-mannopyranose; *p*NP-βGlcNAc: *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside; *p*NP-βGalNAc: *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside; GlcNAcβ(1→4)GlcNAc: 4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-acetamido-2-deoxy-D-glucopyranose; GlcNAcβ(1→4)ManNAc: 4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-acetamido-2-deoxy-D-mannopyranose; GalNAcβ(1→4)GlcNAc: 4-*O*-(2-acetamido-2-deoxy-β-D-galactopyranosyl)-2-acetamido-2-deoxy-D-glucopyranose; GalNAcβ(1→4)ManNAc: 4-*O*-(2-acetamido-2-deoxy-β-D-galactopyranosyl)-2-acetamido-2-deoxy-D-mannopyranose; GlcNAcβ(1→6)GlcNAc: 6-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-acetamido-2-deoxy-D-glucopyranose; GlcNAcβ(1→6)ManNAc: 6-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-acetamido-2-deoxy-D-mannopyranose.
Enzyme: β-*N*-acetylhexosaminidase (EC 3.2.1.52)

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Introduction

2-Acetamido-2-deoxy-D-mannose (ManNAc) is a frequently occurring glycosyl residue in many bacterial capsular polysaccharides and lipopolysaccharides (e.g., from *Haemophillus influenzae* and *Streptococcus pneumoniae*).[1,2] In Gram-positive bacteria, such as *Staphylococcus aureus* H, and *Bacillus subtilis*, the β-ManNAc residue is a component of the "linkage unit" attaching teichoic acids to the peptidoglycan.[2b]

Despite an extensive literature search no disaccharides containing ManNAc at the reducing end have been described – except for GlcNAcβ(1→4)ManNAc (**2**) published in our previous work.[3,4]

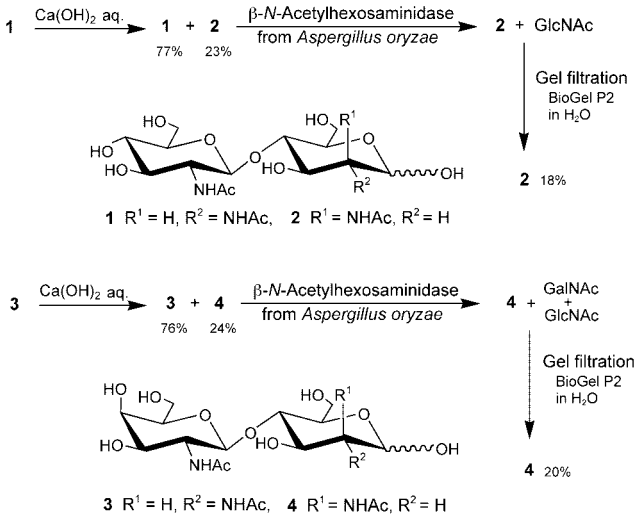
Recently, ManNAc was identified as the strongest monosaccharidic ligand for the natural killer cell activating protein NKR-P1.[3] Nevertheless, the strongest holosaccharidic ligand (besides glycoconjugates and glycodendrimers) is probably the disaccharide GlcNAcβ(1→4)ManNAc (**2**) or GalNAcβ(1→4)ManNAc (**4**).[3]

The interesting biological activities of this saccharide stimulated a high demand for this substance for *in vivo* tests and for the preparation of its derivatives. This compound was prepared for the first time in our laboratory by Lobry de Bruyn–Alberda van Ekenstein [catalyzed by aqueous $Ca(OH)_2$] epimerization of *N, N*'-diacetylchitobiose.[4] The resulting mixture of both 2-epimers (containing only 25% of the desired product **2**) was, however, separable only by multiple preparative HPLC runs. This enabled us to obtain only milligram amount of the compound needed for its spectral characterization and preliminary biological tests.

Numerous attempts for its chemical synthesis based on GlcNAc substitution of the ManNAc derivatives failed. We have also tried the β-*N*-acetylhexosaminidase (EC 3.2.1.52)-catalyzed transfer of a β-GlcNAc residue onto ManNAc that proved to be effective in many previous cases.[5,6] This approach failed as well, despite many enzymes tested. Only the enzymes from *Aspergillus flavofurcatis* CCF 3061 and *A. tamarii* CCF 1665 gave traces of **2** together with the other products as identified by HPLC.

It is generally accepted that glycosidases unable to cleave certain glycosidic linkages are often unable to synthesize the same type of the bond (often regioisomers). Adopting an "inverse approach" led us to the presumption that the enzyme(s) unable to synthesize the respective glycosidic linkage would not be able to hydrolyze it. This proved to be true in our case when the β-*N*-acetylhexosaminidase from *A. oryzae* selectively hydrolyzed *N,N*'-diacetylchitobiose (**1**), whereas the epimer (**2**) remained intact. The resulting mixture of mono- and disaccharides is then easily separable by gel filtration (Scheme 1).

We report here also a possible molecular mechanism by which microbial β-*N*-acetylhexosaminidases can distinguish disaccharides bearing ManNAc at their reducing end. This unique reactivity has been evaluated using mixtures of GlcNAcβ(1→4)ManNAc (**2**) and *N,N*'-diacetylchitobiose (**1**) as well as GalNAcβ(1→4)ManNAc (**4**) and GalNAcβ(1→4)GlcNAc (**3**). This reaction can be employed for a large-scale preparation of the ManNAc-containing disaccharides that are important components of microbial cell walls, and can be utilized as an immunoactive compounds.



Scheme 1. Selective removal of GlcNAcβ(1→4)GlcNAc (**1**) and GalNAcβ(1→4)GlcNAc (**3**) from the epimerization mixtures by β-*N*-acetylhexosaminidase from *Aspergillus oryzae*.

Results and Discussion

Attempted Enzymatic Synthesis of GlcNAcβ(1→4)ManNAc (2)

The reaction mixture after the attempted transglycosylation of ManNAc with β-*N*-acetylhexosaminidase using *p*NP-βGlcNAc as a donor was pre-separated by gel filtration to obtain the disaccharide fraction. This fraction was analyzed by TLC and HPLC for the occurrence of **2**. The authentic sample of this compound prepared previously[4] was used as a standard.

The formation of a minor amount of **2** was observed in two cases only (*Aspergillus flavofurcatis* CCF 3061 and *A. tamarii* CCF 1665) in the HPLC pattern and this was further proved by co-chromatography with the authentic compound. In some cases the formation of the other (unidentified) regioisomers was observed. These data should only demonstrate the difficulty of the enzymatic reaction leading to the desired product.

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Table 1. Cleavage of GlcNAcβ(1→4)ManNAc (**2**) by the β-*N*-acetylhexosaminidases.

Source of β-*N*-acetylhexosaminidase	Hydrolytic activity[a]			
	10 min	30 min	60 min	180 min
Aspergillus oryzae CCF 1066	–	–	–	–
A. flavipes CCF 1895	–	+	+	+
A. sojae CCF 3060	+	++	++	+++
A. tamarii CCF 1665	+	++	+++	+++
A. terreus CCF 2539	–	–	–	–
A. flavus CCF 3056	–	–	–	–
Mucor dimorphosporus CCF 2609	–	–	–	–
Penicillium brasilianum CCF 2155	–	–	–	++
P. oxalicum CCF 2430	–	++	++	+++
P. funiculosum CCF 2984	–	+	+	++
P. chrysogenum CCF 1269	–	+	+	++
P. multicolor CCF 2244	+	++	++	+++
Talaromyces flavus CCF 2686	–	+	+	+++
Bovine kidney (Sigma)	–	–	–	–
Bovine epididymis (Sigma)	–	–	–	–
Jack beans (Sigma)	++	+++	+++	+++
Lysozyme (Sigma)	–	–	–	–
Rat plasma	–	–	–	–

[a] + Monosaccharides formation (GlcNAc and ManNAc) from **2** (+ < 10%, ++ > 10%, +++ > 25%); – no hydrolysis.

Selectivity of the Hydrolysis of GlcNAcβ(1→4)ManNAc (2) with Various β-N-Acetylhexosaminidases

A large panel of various β-*N*-acetylhexosaminidases was tested for their ability to hydrolyze the disaccharide **2** (Table 1). Only those enzymes that were found to be completely unable to cleave this disaccharide would be suitable for its discrimination in the presence of *N,N*'-diacetylchitobiose. The selected enzymes, e.g., from *A. oryzae*, *A. terreus,* and *A. flavus,* were incubated with the substrate **2** for a prolonged time (24 hours) without any detectable hydrolysis (TLC, HPLC). All tested β-*N*-acetylhexosaminidases cleaved *N,N*'-diacetylchitobiose.

The animal β-*N*-acetylhexosaminidases were tested for the same reaction mainly to prove the applicability of this immunoactive saccharide *in vivo*. None of the animal β-*N*-acetylhexosaminidases including samples of fresh rat plasma (containing β-*N*-acetylhexosaminidase and lysozyme) hydrolyzed this disaccharide. This proved the potential stability of the substance in body fluids and its possible *in vivo* applicability.

Selectivity of the Hydrolysis of GalNAcβ(1→4)ManNAc (4) by Fungal β-N-Acetylhexosaminidases

Screening for hydrolysis of **4** was performed analogously as in the case of **2** and quite similar results were obtained (data not shown). Therefore, we used for the discrimination of **4** the same enzyme from *A. oryzae* as in the previous case. The disaccharide **4** has not been described so far.

Inhibition of β-*N*-Acetylhexosaminidases by GlcNAcβ(1→4)ManNAc (2)

The compound **2** was tested as a potential inhibitor of fungal β-*N*-acetylhexosaminidases from *A. oryzae* CCF 1066, *P. oxalicum* CCF 2430, *Mucor dimorphosporus* CCF 2609 and *Talaromyces flavus* CCF 2686. Virtually no inhibition was observed (Table 2).

Mechanism of the Discrimination of ManNAc-Containing Disaccharides by β-*N*-Acetylhexosaminidase from *A. oryzae*

The β-*N*-acetylhexosaminidases, similar to most other glycosidases, are rather promiscuous towards an aglycone (or second saccharide). The phenomenon observed by us, e.g., the discrimination according to the reducing saccharide was, therefore, unusual, and we have attempted to explain this effect.

Recently, the β-*N*-acetylhexosaminidase from *A. oryzae* was sequenced and its model based on high homology with the known sequences and the crystal structure[7] was constructed. We carried out a simulation of the docking of the respective disaccharides into this model of the active center of the β-*N*-acetylhexosaminidase from *A. oryzae* and the following data were obtained.

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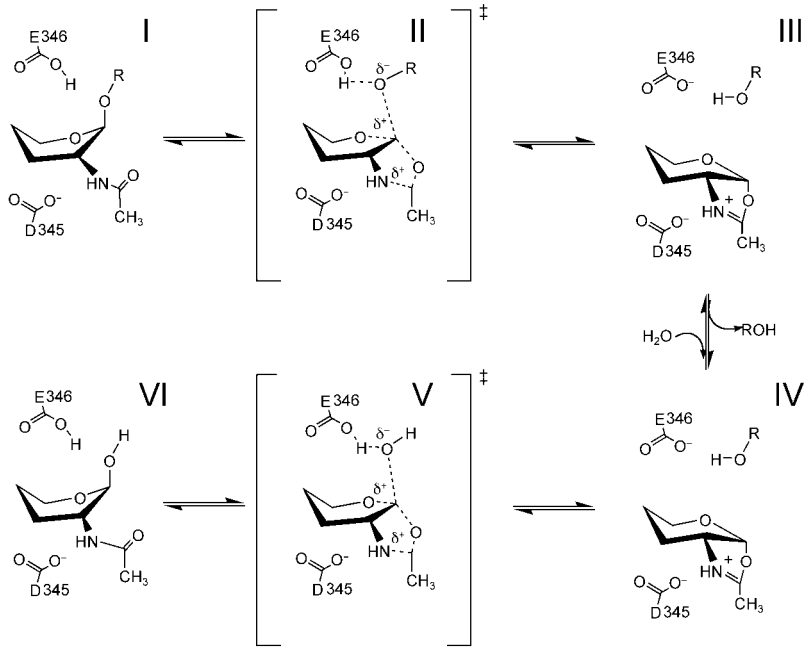
Table 2. Inhibition of β-*N*-acetylhexosaminidases by GlcNAcβ(1→4)ManNAc (**2**).

Substrate	Residual activity [%]			
	*p*NP-GlcNAc		GlcNAcβ(1→4)GlcNAc (**1**)	
Inhibitor concentration[a] β-*N*-Acetylhexosaminidase	0.04 mM	0.4 mM	0.04 mM	0.4 mM
Aspergillus oryzae CCF 1066	90	98	86	102
Penicillium oxalicum CCF 2430	97	82	102	109
Mucor dimorphosporus CCF 2609	95	85	95	71
Talaromyces flavus CCF 2686	96	99	79	78

[a] The concentration of both substrates was 0.4 mM.



Scheme 2. Proposed catalytic mechanism of β-*N*-acetylhexosaminidase from *A. oryzae*.[8]

N,N′-Diacetylchitobiose (**1**) (non-reducing GlcNAc moiety) participates in the hydrogen bonding between Asp^{345}, Tyr^{445} and the acetamido group, which may eventually stabilize the oxazolinium transition state (Scheme 2). The non-reducing end of the disaccharide is locked into the active site owing to the hydrogen bonds of Arg^{193}, Trp^{482} and $Glu^{519}$with the C-4 and C-5 OH groups (Figure 1). The calculated steric interaction energy was $-101.6\ kJ \cdot mol^{-1}$ (Table 3).

On the other hand, the molecule of **2** is slightly distorted and, therefore, there is a steric conflict with Trp^{482}. The reorientation of the ligand due to this steric conflict during the molecular mechanics simulation leads to a 0.3 Å higher distance between Asp^{345} and the acetamido group that obviously results in a diminished stabilization of the oxazolinium transition state. The other interactions stabilizing the non-reducing carbohydrate moiety seem to remain intact. Due to this steric conflict the steric interaction energy is considerably higher ($-53.1\ kJ \cdot mol^{-1}$) (Table 3) than that in the case of *N, N*'-diacetylchitobiose. This may also explain the facts that the enzyme does not attack the substrate (**2**) but it shows virtually no inhibition (Table 2).

The situation of the analogous β(1→6) linked disaccharides is completely different. We were unable to observe the discrimination of **5** and **6** despite extensive screening of our β-*N*-acetylhexosaminidase library (40 enzymes tested, data not shown). Only minor differences in the hydrolysis rate were observed. In the case of the β-*N*-acetylhexosaminidase from *A. oryzae* the hydrolysis of **6** is even somehow faster than that of **5** as observed by HPLC.

This situation might be well explained also by the docking experiments into the active site model. We could observe that the position of the non-reducing GlcNAc of both disaccharides in the active site is virtually identical and the respective hydrogen bonds

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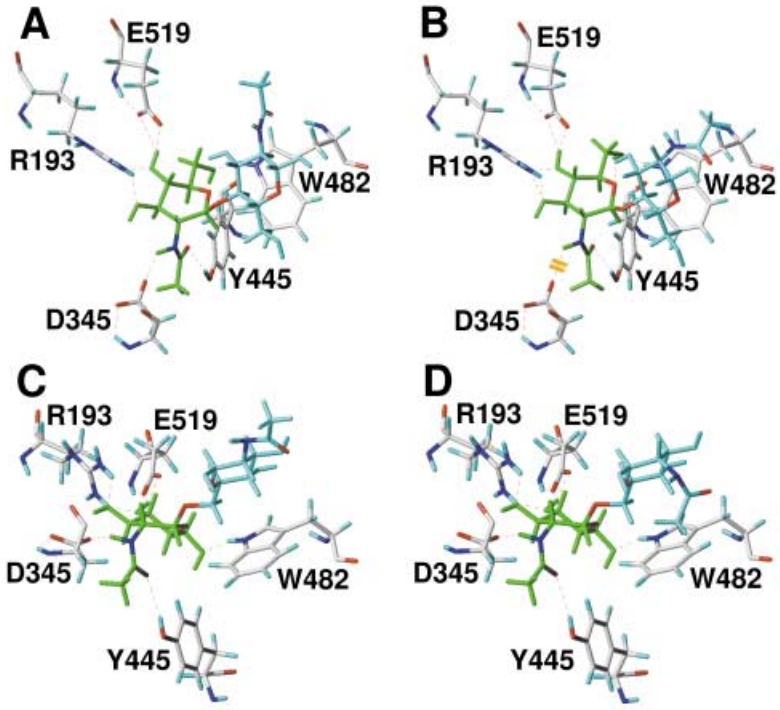
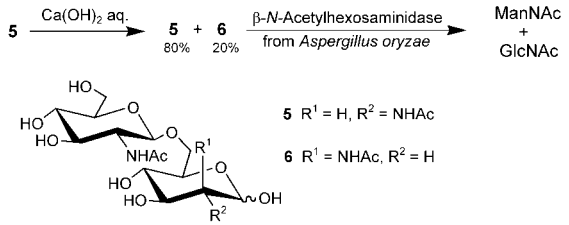


Figure 1. A simulation of the docking of the respective disaccharides into the model of the active center of β-*N*-acetylhexosaminidase from *A. oryzae*. (**A**) GlcNAcβ(1→4)GlcNAc (**1**), (**B**) GlcNAcβ(1→4)ManNAc (**2**), (**C**) GlcNAcβ(1→6)GlcNAc (**5**), (**D**) GlcNAcβ(1→6)ManNAc (**6**).



Scheme 3. Action of β-*N*-acetylhexosaminidase from *A. oryzae* upon β(1→6) linked hexosamine disaccharides.

Table 3. Interaction energies of GlcNAcβ(1→4)GlcNAc (**1**), GlcNAcβ(1→4)ManNAc (**2**), GlcNAcβ(1→6)GlcNAc (**5**) and GlcNAcβ(1→6)ManNAc (**6**) with β-*N*-acetylhexosaminidase from *Aspergillus oryzae* (molecular modelling).

	Interaction energy [kJ · mol^{-1}]		
Substrate	Total	Steric	Electrostatic
1	– 268.8	– 101.6	– 166.8
2	– 246.2	– 53.1	– 193.1
5	– 165.1	– 43.1	– 122.1
6	– 227.8	– 103.7	– 126.2

remain conserved. There is, however, a different situation at the reducing carbohydrate moiety, where, in the case of **6**, a new hydrophobic interaction of CH_3 and of NHAc group with Trp^{482} can be observed. This is reflected also by substantial lowering of the steric interaction energy for **6** (– 103.7 kJ · mol^{-1}) compared to **5** (– 43.1 kJ · mol^{-1}).

The discrimination of ManNAc-containing disaccharides by the β-*N*-acetylhexosaminidases is a rather unexpected and unusual phenomenon. It can be effectively utilized for the production of rare ManNAc-containing saccharides.

Conclusions

We have shown that the β-*N*-acetylhexosaminidase from *Aspergillus oryzae* and some other β-*N*-acetylhexosaminidases from a filamentous fungi selectively discriminate between disaccharides containing ManNAc and GlcNAc at the reducing end. Disaccharides containing the ManNAc moiety (at reducing end) are resistant to the cleavage. This unusual effect was explained by docking experiments of the respective disaccharide into the active site model of the β-*N*-acetylhexosaminidase from *A. oryzae*. This method opens a possibility for the preparative-scale synthesis of these disaccharides that are interesting for their immunoactive properties.

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Experimental Section

β-*N*-Acetylhexosaminidase

All β-*N*-acetylhexosaminidases (EC 3.2.1.52) used in this work, besides the commercial ones, originated from the library of fungal glycosidases of the Laboratory of Biotransformation in Prague and were prepared by cultivation of the respective fungi as described previously.[9] The producing strains are deposited with the Czech Collection of Fungi (CCF) at the Department of Botany of the Charles University, Prague.

TLC

Thin layer chromatography was carried out using silica Gel 60 GF_{254} (Merck) with the solvent system 2-propanol/water/28% ammonia (7/2/1, v/v), twice developed. The spots were visualized by charring with 5% H_2SO_4 in ethanol.

HPLC

The analysis were carried out using an HPLC system consisting of a solvent-delivery system 600 (Waters), a photo-diode array detector 996 (Waters) and a Polymer IEX H^+-form column (8 μm, 250 × 8 mm, Waters). As mobile phase 9 mM H_2SO_4 was employed at a flow rate 0.5 mL · min^{-1} and 35 °C. Compounds were detected at 210 nm. Retention times were 9.56 min for *N,N*'-diacetylchitobiose (**1**), 10.04 min for GlcNAcβ(1→4)ManNAc (**2**), 10.59 min for GalNAcβ(1→4)GlcNAc (**3**), 11.21 min for GalNAcβ(1→4)ManNAc (**4**), 10.49 min for GlcNAcβ(1→6)GlcNAc (**5**) and 9.58 min for GlcNAcβ(1→6)ManNAc (**6**).

NMR

NMR spectra were recorded on a Varian UNITY *Inova*-400 MHz spectrometer (399.90 MHz for ^1H, 100.55 MHz for ^{13}C) in D_2O (99.95% D, Chemtrade) 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. Acetone signal [δ_H and δ_C (30.50)] was used as a reference. Carbon chemical shifts and some proton chemical shifts were determined from 2D spectra with lower accuracy.

Optical Rotation

Optical rotation was measured on a Perkin-Elmer 241 polarimeter.

4-*O*-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-2-acetamido-2-deoxy-D-mannopyranose (2)

N,N'-Diacetylchitobiose (**1**, 500 mg, 1.18 mmol) obtained by acidic cleavage of chitin[10] was dissolved in a saturated aqueous $Ca(OH)_2$ solution (25 mL) and left overnight at a laboratory temperature.[4] Dowex 50WX2 in H^+ form (Supelco, USA) was used for the neutralization and the removal of Ca^{2+} ions. The mixture was evaporated and dissolved in citrate-phosphate buffer (50 mM, pH 5.3, 25 mL) and the β-*N*-acetylhexosaminidase from *A. oryzae* (20 U) was added and the mixture was incubated at 37 °C (typically 2 hours) until all *N,N*'-diacetylchitobiose had disappeared (TLC). The enzyme was then deactivated by 5 min boiling and the mixture was evaporated to 1 – 2 mL. The sample was loaded onto Bio Gel P2 (BioRad, USA) column (2.6 × 80 cm, flow rate 12 mL · h^{-1}, dead volume 190 mL) eluted with H_2O. The isolated yield of **2** was 18% and its structure was confirmed by NMR, MS and optical rotation as described previously.[4]

4-*O*-(2-Acetamido-2-deoxy-β-D-galactopyranosyl)-2-acetamido-2-deoxy-D-glucopyranose (3)

The compound **3** was prepared using a significantly modified procedure[11] for a transglycosylation reaction catalyzed by β-*N*-acetylhexosaminidase from *A. oryzae* CCF 1066. *p*-Nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside (12.5 mg, 73 mmol) and 2-acetamido-2-deoxy-β-D-glucopyranoside (31.5 mg, 287 mmol) were dissolved in citrate-phosphate buffer (50 mM, pH 5.0, 1.25 mL) and the enzyme (2 U) was added. The mixture was incubated at 37 °C for 1 h and the reaction was stopped by 5 min boiling. The reaction mixture was extracted with diethyl ether (3 × 250 μL) to remove the bulk of the liberated *p*-nitrophenol and after partial evaporation loaded onto Toyopearl (HW-40F, TOSOH Corp., Japan) column (2.6 × 80 cm, flow rate 12 mL · h^{-1}, dead volume 220 mL) eluted with H_2O. The isolated yield of **3** was 84.5% (13.1 mg) referred to *p*NP-βGalNAc.

4-*O*-(2-Acetamido-2-deoxy-β-D-galactopyranosyl)-2-acetamido-2-deoxy-D-mannopyranose (4)

The compound **3** (51 mg, 0.12 mmol) was epimerized under the same conditions as above. The enzymatic separation of **3** and **4** was done analogously as in the case of **1** and **2**. The isolated yield of **4** was 21.6% (11 mg). The structure was determined by NMR. $[\alpha]_{589}^{20}$: + 3.9° (*c* 0.73 in water).

4 α-anomer: ^1H NMR: δ = 1.810, 1.837(2 × 3H, s, CH_3CO-2, CH_3CO-2′), 3.49 (1H, m, H-5′), 3.497 (1H, dd, *J* = 4.5, 12.1 Hz, H-6a), 3.552 (1H, dd, *J* = 9.1, 9.9 Hz, H-4), 3.54 (1H, m, H-3′), 3.56 (2H, m, H-6′), 3.577 (1H, dd, *J* = 2.1, 12.1 Hz, H-6b), 3.663 (1H, ddd, *J* = 2.1, 4.5, 9.9, H-5), 3.693 (1H, dd, *J* = 8.5, 10.8 Hz, H-2′), 3.709 (1H, m, H-4′), 3.927 (1H, dd, *J* = 4.8, 9.1 Hz, H-3), 4.096 (1H, dd, *J* = 1.8, 4.8 Hz, H-2), 4.291 (1H, d, *J* = 8.5 Hz, H-1′), 4.893 (1H, d, *J* = 1.8 Hz, H-1); ^{13}C NMR: δ = 22.1, 22.4 (2 × q, CH_3CO-2, CH_3CO-2′), 52.8 (C-2, C-2′), 61.2 (C-6′), 62.3 (C-6), 67.8 (C-3), 67.9 (C-4′), 70.6 (C-5), 70.7 (C-3′), 75.5 (C-5′), 77.0 (C-4), 93.0 (C-1), 101.9 (C-1′).

4 β-anomer: ^1H NMR: δ = 1.834, 1.851 (2 × 3H, s, CH_3CO-2, CH_3CO-2′), 3.251 (1H, ddd, *J* = 2.1, 4.7, 9.9 Hz, H-5), 3.451 (1H, dd, *J* = 9.4, 9.9 Hz, H-4), 3.470 (1H, dd, *J* = 4.7, 12.0 Hz, H-6a), 3.49 (1H, m, H-5′), 3.52 (1H, m, H-3′), 3.56 (2H, m, H-6′), 3.622 (1H, dd, *J* = 2.1, 12.0 Hz, H-6b), 3.684 (1H, dd, *J* = 8.3, 10.8 Hz, H-2′), 3.709 (1H, m, H-4′), 3.725 (1H, dd, *J* = 4.6, 9.4 Hz, H-3), 4.255 (1H, dd, *J* = 1.7, 4.6 Hz, H-2), 4.268 (1H, d, *J* = 8.3 Hz, H-1′), 4.786 (1H, d, *J* = 1.7 Hz, H-1); ^{13}C NMR: δ = 22.3, 22.4 (CH_3CO-2, CH_3CO-2′), 52.9 (C-2′), 53.5 (C-2), 61.2

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(C-6′), 62.2 (C-6), 67.9 (C-4′),70.9 (C-3′), 71.0 (C-3), 75.3 (C-5), 75.5 (C-5‘), 76.6 (C-4), 93.2 (C-1), 102.0 (C-1′).

Preparation of GlcNAcβ(1→6)GlcNAc (5) and GlcNAcβ(1→6)ManNAc (6)

The compound **5** was prepared as described previously.[12]

The mixture of **5** and **6** was prepared by $Ca(OH)_2$-catalyzed epimerization of **5** (53.6 mg, 0.13 mmol) under the same conditions as described above which gave a 20% yield (13 mg) of **6** under the thermodynamic equilibrium.

Cleavage of GlcNAcβ(1→4)ManNAc (2) by the β-*N*-Acetylhexosaminidases

Compound **2** (1 mg, 2.3 μmol) was dissolved in citrate-phosphate buffer (50 mM, pH 5.0, 100 μL) and 0.5 U of the respective β-*N*-acetylhexosaminidase was added. For use of rat plasma substrate **2** (1 mg, 2.3 μmol) was dissolved in fresh rat plasma (0.1 mL). The reactions were incubated at 37 °C and monitored by TLC and HPLC. For use of lysozyme (Sigma, from chicken egg white, EC 3.2.1.17): the substrate **2** (1 mg) was dissolved in citrate-phosphate buffer (50 mM, pH 6.2, 200 μL) at 37 °C and 5 U (and after 20 hours another 40 U) of lysozyme were added (Table 1). The reactions were monitored by TLC and HPLC. The β-*N*-acetylhexosaminidase activity was assayed using *p*NP-βGlcNAc.[13] One unit of β-*N*-acetylhexosaminidase activity was defined as the amount releasing 1 μmol of *p*-nitrophenol per minute at pH 5.0 and 37 °C.

Inhibition of the β-*N*-Acetylhexosaminidases by GlcNAcβ(1→4)ManNAc (2)

The disaccharide **2** was tested as a potential inhibitor of fungal β-*N*-acetylhexosaminidases from *A. oryzae* CCF 1066, *P. oxalicum* CCF 2430, *Mucor dimorphosporus* CCF 2609 and *Talaromyces flavus* CCF 2686 (phylogenetically distant species) (Table 2).

The mixtures of either *p*NP-βGlcNAc or GlcNAcβ(1→4)GlcNAc (**1**) (final concentration 0.4 mM) and the disaccharide **2** (final concentration 0.04 or 0.4 mM) in citrate-phosphate buffer (50 mM, pH 5.0, 50 μL) were supplemented with 0.5 U of the respective β-*N*-actylhexosaminidase. The mixtures were incubated at 37 °C for 5 minutes. Controls were identical without **2**. The activity was determined according to the released *p*-nitrophenol (detection at 420 nm) [13] or by HPLC measuring the released GlcNAc.

Transglycosylation Activities of Fungal β-*N*-Acetylhexosaminidases towards ManNAc

The reaction components *p*NP-βGlcNAc (2.6 mg, 7.6 μmol) and ManNAc (7.4 mg, 33.7 μmol) were dissolved in citrate-phosphate buffer (50 mM, pH 5.0, 130 μL) and 0.5 U of respective enzyme (*Aspergillus flavofurcatis* CCF 3061, *A. persicinum* CCF 1850, *A. tamarii* CCF 1665, *A. oryzae* CCF 1063, *A. oryzae* CCF 1066, *A. sojae* CCF 3060, *A. flavus* CCF 3056, *Penicillium oxalicum* CCF 2315, *P. parasiticus* CCF 1298, *P. funiculosum* CCF 1994, *P. funiculosum* CCF 1995, *P. funiculosum* CCF 2984, *Talaromyces flavus* CCF 2686) was added. The reaction mixture was incubated at 37 °C for 8 hours and the reaction was monitored by HPLC.

Hydrolysis of the Mixture of GlcNAcβ(1→6)ManNAc (6) and GlcNAcβ(1→6)GlcNAc (5) by β-*N*-Acetylhexosaminidases

The reaction mixture of **5** and **6** (2.4 mg) was dissolved in citrate-phosphate buffer (50 mM, pH 5.0, 0.2 mL) and 1 U of the respective β-*N*-acetylhexosaminidase was added. The reaction was monitored by HPLC. All enzymes tested (as in the Table 1) hydrolyzed both disaccharides at a similar rate and no discrimination was observed.

Molecular Modelling

The complete primary sequence of the β-*N*-acetylhexosaminidase from *A. oryzae* that was cloned by us[14] was aligned with the known X-ray structure of the β-*N*-acetylhexosaminidase from *Seratia marcescens* and *Streptomyces plicatus*, extracted from the Brookhaven Protein Database (PDB entry: 1QBA and 1HP4, respectively, http://www.rcsb.org/pdb/). These proteins show a high degree of primary structure similarity: 44%. 3D Models constituted by all non-hydrogen atoms were generated by Modeller6 package.[15] For model refinement and minimization the SYBYL package with the TRIPOS force field (TRIPOS Associates Inc.) was used. Finally, the tertiary structure models were checked with Pro Check[16]. With the complete modelling including the alignment and energy minimization, we used exactly the parameters and methods published previously.[7]

For the docking experiments our model structure was fitted onto the crystal structure of 1QBB, a bacterial chitobiase complexed with *N,N*'-diacetylchitobiose (**1**). Compound **1** was placed in an arbitrary position according to the ligand coordinates in the bacterial chitobiase complex. 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 electrostatic contributions from any atomic charges present in the ligand. Exact positioning of the ligand was done by a two-step procedure, energy minimization followed by a molecular dynamics. The ligand-protein system was minimized by 1000 interactions with the Powell minimizer and the TRIPOS force field including electrostatic interactions based on Hückel[18] partial charge distributions using a dielectric constant with a distance dependent function $\varepsilon = 4r$ and non-bonded interaction cut-off of 8 Å.[17,18] A molecular dynamics simulation at 290 K followed the minimization with the NTV ensemble over 15 ps. The resulting structure was then minimized with the same parameters as above to the convergence of the energy gradient less than 0.04 $kJ \cdot mol^{-1}$. For the other ligands described in this paper the procedure was exactly the same. The non-bonded interaction energy between the model and the ligands within optimized complex was calculated using the TRIPOS force field. This estimation of real interaction energy neglects solvation and desolvation effects.

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