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Note

Fungal β-*N*-acetylhexosaminidases with high β-*N*-acetylgalactosaminidase activity and their use for synthesis of β-GalNAc-containing oligosaccharides

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Abstract

About 60 fungal strains were tested for production of extracellular β-*N*-acetylhexosaminidases. A unique β-*N*-acetylhexosaminidase with the β-GalNAc-ase/β-GlcNAc-ase ratio of 2.3–2.8 was found in the culture filtrates of some strains of *Penicillium oxalicum*. Addition of 20% (w/v) $MgSO_4$ increased the β-GalNAc-ase/β-GlcNAc-ase ratio to the value of 3.35. Cultivation conditions influence this ratio as well. β-*N*-Acetylhexosaminidases from *P. oxalicum* CCF 2430 and *Aspergillus oryzae* CCF 1066 considerably differing in the GalNAc-ase activity were used for the synthesis of the following structures β-D-Gal*p*NAc-(1→4)-D-Glc*p*NAc, β-D-Gal*p*NAc-(1→6)-D-Glc*p*NAc, β-D-Gal*p*NAc-(1→6)-D-Gal*p*NAc, β-D-Gal*p*NAc-(1→4)-α-D-Glc*p*NAcOAll and β-D-Gal*p*NAc-(1→6)-β-D-Gal*p*-(1→4)-α-D-Glc*p*NAcOAll to demonstrate the application of these new enzymes.

Keywords: β-*N*-Acetylhexosaminidases; β-*N*-Acetylgalactosaminidase; β-GalNAc

β-*N*-Acetylhexosaminidases (EC 3.2.1.52) hydrolyse (and also transglycosylate) both β-GlcNAc and β-GalNAc moieties. After numerous experiments aiming to separate and identify both activities,[1] it was concluded that this is a single enzyme having affinity for both substrates. We demonstrate here the possibility of fine tuning the β-GalNAc-ase/β-GlcNAc-ase activity ratio by a rational enzyme source selection together with physiological (cultivation) and environmental modifications. The availability of enzymes with more distinct activities—either β-GalNAc-ase or β-GlcNAc-ase—is very important in their use for synthetic purposes as demonstrated here. A fungal source with the high β-GalNAc-ase activity has now been located and the β-GalNAc-ase/β-GlcNAc-ase ratio improved by the cultivation conditions and by manipulation of the medium. Chitooligomers were found to be optimal inductors of these inducible fungal enzymes.[2,3]

A substantial increase of β-GalNAc-ase activity was observed between the 12th and 13th day of cultivation, when the β-GalNAc-ase/β-GlcNAc-ase ratio reached 2.3–2.8 (Fig. 1). After precipitation of the enzyme by ammonium sulfate (80% saturation) from the cultivation medium[2,3] the ratio decreased to 1.2–1.5. The difference in values may be caused by possible changes in refolding or in the hydration envelope.

Different pH optima for β-GalNAc-ase (pH optimum 4.5) and β-GlcNAc-ase (pH optimum 5.0) were found (Fig. 2). The highest β-GalNAc-ase/β-GlcNAc-ase ratio is at pH value 4.5. These data are important for the design of the reaction conditions when using β-GalNAc-ase for the synthetic purposes.

Fifty-five fungal strains having high β-*N*-acetylhexosaminidase activity were screened for their β-GalNAc-ase/β-GlcNAc-ase ratio (Table 1). Generally, the β-GalNAc-ase/β-GlcNAc-ase ratio in most of β-*N*-

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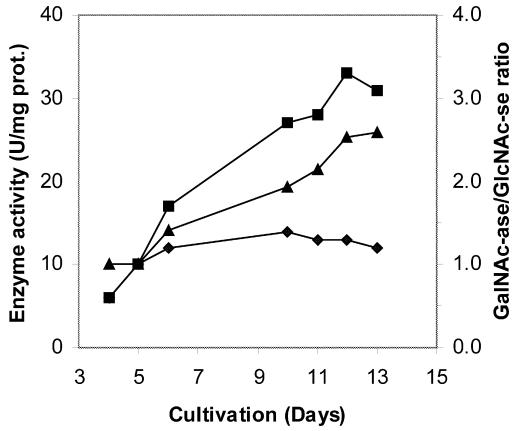


Fig. 1. Induction of extracellular β-*N*-acetylhexosaminidase from *P. oxalicum* CCF 2430 (pH of determination 4.5), (■) β-GalNAc-ase, (◆) β-GlcNAc-ase, (▲) β-GalNAc-ase/β-GlcNAc-ase ratio.

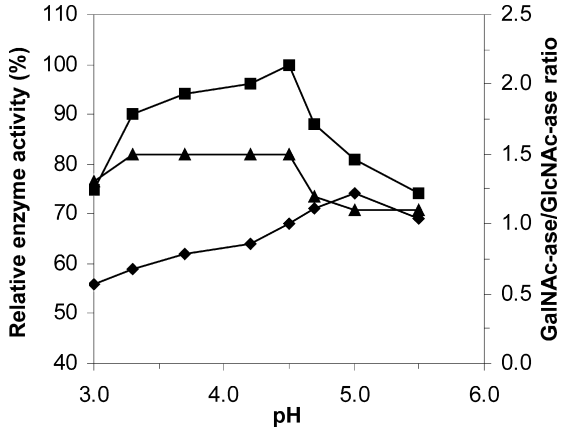


Fig. 2. pH optima of β-*N*-acetylhexosaminidase from *P. oxalicum* CCF 2430, (■) β-GalNAc-ase, (◆) β-GlcNAc-ase, (▲) β-GalNAc-ase/β-GlcNAc-ase ratio.

acetylhexosaminidases is under 0.5. Higher activity of β-GalNAc-ase was found only in very few enzymes tested, the best one being from *Penicillium oxalicum* CCF 2430.

We have observed previously that the addition of some inorganic salts or organic solvents could positively stimulate the β-*N*-acetylhexosaminidase activity.[4,5] Therefore, we also studied the influence of selected inorganic salts, e.g., $FeSO_4$, $MnSO_4$ (Mn^{2+} or Fe^{2+} are often required by glycosidases as cofactors), $(NH_4)_2SO_4$, $MgSO_4$ and LiCl (in higher concentrations influencing water activity and/or enzyme hydration)[5] on β-GlcNAc-ase and β-GalNAc-ase (and their ratio) activity of β-*N*-acetylhexosaminidase from *P. oxalicum* CCF 2430. Addition of $FeSO_4$ (0–10 mM: β-GlcNAc-ase 29% of control, β-GalNAc-ase 35% of control) or $MnSO_4$ (0–1 mM: β-GlcNAc-ase 65% of control, β-GalNAc-ase 72% of control) decreased both activities and their ratio was not notably changed (data not shown). On the contrary, $(NH_4)_2SO_4$, $MgSO_4$ and LiCl suppressed only β-GlcNAc-ase activity. The maximum ratio of 3.35 was observed in the presence of 20% (w/v) of $MgSO_4$ (Fig. 3). Acetonitrile, which was used as cosolvent to solubilize the rather insoluble *p*NP-β-GalNAc,[4] strongly inhibited β-GalNAc-ase activity (10% of acetonitrile (v/v)—more than 60% of the activity was lost). The influence of aminosugars (GlcN·HCl, GlcNAc and GalNAc) on the β-*N*-acetylhexosaminidase from *P. oxalicum* CCF 2430 was studied (Table 2). All tested aminosugars decreased the β-GalNAc-ase/β-GlcNAc-ase ratio. These compounds are known feedback inhibitors of this enzyme. This is an important finding because during enzymatic reactions (synthesis, enzyme determination) these sugars (GlcNAc or GalNAc) are liberated into the reaction medium, thus changing these parameters.

2-Acetamido-2-deoxy-D-glucopyranose was used as an acceptor for β-*N*-acetylhexosaminidase from *P. oxalicum* CCF 2430 (Scheme 1). The reaction was performed in 20% (w) $MgSO_4$ to increase the β-GalNAc-ase/β-GlcNAc-ase ratio. The reaction gave 2-acetamido-2-deoxy-β-D-galactopyranosyl-(1→4)-β-D-2-acetamido-2-deoxy-D-glucopyranose (**1**, 26.5%) and 2-acetamido-2-deoxy-β-D-galactopyranosyl-(1→6)-2-acetamido-2-deoxy-D-glucopyranose (**2**, 19%). Even though these regioisomers have the same molecular weight, the compounds could be separated simply by gel chromatography on Toyopearl HW40F. This fact could be explained by different exclusion volumes (shape, hydration envelope) of both disaccharides. The results showed that this enzyme represents a β-*N*-acetylhexosaminidase with a very high β-GalNAc-ase/β-GlcNAc-ase ratio. To the best of our knowledge this enzyme has not been used yet for transglycosylation reactions. 2-Acetamido-2-deoxy-D-galactopyranose was used as an acceptor under the same reaction conditions (Scheme 1). In this case only a single isomer 2-acetamido-2-deoxy-β-D-galactopyranosyl-(1→6)-2-acetamido-2-deoxy-D-galactopyranose (**3**) was formed in an excellent yield of 87%.

Acceptors substituted at the reducing end with a group suitable for further modifications, allyl 2-acetamido-2-deoxy-α-D-glucopyranoside (GlcNAcαOAll) and allyl β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-α-D-glucopyranoside (LacNAcαOAll)[6] were used with β-*N*-acetylhexosaminidase from *Aspergillus oryzae* CCF 1066 (Scheme 2) having the β-GalNAc-ase/β-GlcNAc-ase ratio of 0.56. Previously, the transfer of GlcNAc[4,5,7,8] or GalNAc [7,8] residues to different oligosaccharides carrying a β-Glc moiety at the non-reducing end yielding β-(1→4) and/or β-(1→6) linkages was described. The enzymatic transfer of GalNAc onto the β-Gal moiety always afforded β-D-Gal*p*NAc-(1→6)-D-Gal*p*NAc.[9]

Table 1
β-GalNAc-ase/β-GlcNAc-ase ratio of various fungal β-*N*-acetylhexosaminidases

Strain [a]	β-GalNAc-ase/β-GlcNAc-ase ratio
Aspergillus oryzae CCF 147	0.26
A. oryzae CCF 172	0.34
A. oryzae CCF 1066	0.56
A. oryzae CCF 1602	0.26
A. oryzae T IMI 16266ii	0.43
A. flavus CCF 146	0.23
A. flavus CCF 642	0.54
A. flavus CCF 1129	0.66
A. flavus T IMI 124930	0.57
A. parasiticus CCF 141	0.25
A. parasiticus CCF 1298	0.59
A. parasiticus T IMI 15957ix	0.32
A. sojae T IMI 191300	0.51
A. flavofurcatis CCF 107	0.22
A. flavofurcatis T IMI 124938	0.35
A. tamarii CCF 1665	0.30
A. terreus CCF 55	0.50
***A. terreus* CCF 57** [b]	**1.00**
A. terreus T IMI 17294	0.46
***A. terreus* CCF 2539**	**1.01**
A. flavipes CCF 76	0.54
A. flavipes CCIM USA	0.68
***A. flavipes* CCF 2026**	**1.00**
A. flavipes T IMI 171885	0.90
A. niveus CCF 544	0.75
A. niveus T IMI 171878	0.15
A. niger CCIM K1	0.40
A. niger CCIM K2	0.42
A. phoenicis CCF 61	0.49
A. versicolor CCF 2491	0.09
Penicillium spinulosum CCF 2159	0.92
P. melinii CCF 2440	0.12
P. brasilianum CCF 2155	0.75
***P. funiculosum* CCF 1994**	**1.20**
P. funiculosum CCF 2325	0.76
***P. purpurogenum var. rubrisclerotium* CCF 2984**	**1.22**
***P. purpurogenum var. rubrisclerotium* CCF 2985**	**1.15**
P. pittii CCF 2277	0.63
***P. oxalicum* CCF 1667**	**2.00**
***P. oxalicum* CCF 1959**	**1.60**
***P. oxalicum* CCF 2315**	**1.40**
***P. oxalicum* CCF 2430**	**2.30**
***P. oxalicum* CCF 3009**	**1.40**
***P. oxalicum* CCF 2062**	**1.50**
Fusarium oxysporum CCF 377	0.18

Residual enzyme activities: β-Gal <3%, α-Gal, α-Glc and β-Glc <0.6%. CCF Culture Collection of Fungi, Department of Botany, Charles University, Prague; CCIM Culture Collection of the Institute of Microbiology, Prague, Czech Republic; ATCC American Type Culture Collection, Rockville, Maryland, USA; IMI International Mycological Institute, Egham, UK.

[a] More strains (5–10) from each species were tested, however only the most representative examples are given in this table.

[b] Bold entries—β-GalNAc-ase/β-GlcNAc-ase ratio >1.0.

All the above experiments also corroborated the general view that β-*N*-acetylhexosaminidases do not accept axial 4-OH group in the galacto- (Gal, GalNAc) configuration—presumably because of steric hindering of this group.

1. Experimental

1.1. General methods

Reactions were monitored by thin layer chromatography (TLC) analysis on silica gel plates (Kieselgel 60 F_{254}, Merck). Compounds were visualised by spraying with 20% H_2SO_4 in EtOH, followed by charring at 150 °C. Column chromatography was performed on silica gel 60 M (0.040–0.063 mm, Merck), Toyopearl HW40F (100 Da–10 kDa, Tosoh Corp.) or Biogel P-2 (200–3000 Da, Bio-Rad). NMR spectra were measured on Brucker AMX 400 and 500 spectrometers, data are given in ppm, coupling constants (*J*) in Hz. Mass spectra were recorded on the Brucker MALDI-TOF mass spectrometer.

p-Nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside, *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside and 2-acetamido-2-deoxy-D-galactopyranose were obtained from Senn Co., Switzerland, β-*N*-acetylhexosaminidases were prepared as described previously.[2,3] Briefly, respective microorganisms were grown in shaked liquid cultures in the media with the chitin hydrolysates as inducers, typically at 28 °C for 10 days.[2] The enzymes were isolated from the culture supernatant and purified by fractional precipitation with $(NH_4)_2SO_4$ (30–80% saturation cut).

1.2. Enzyme assay

Glycosidase activities were assayed in 50 mM citrate–phosphate buffer pH 4.5 as described previously.[10]

1.3. 2-Acetamido-2-deoxy-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucopyranose (1) and 2-acetamido-2-deoxy-β-D-galactopyranosyl-(1→6)-2-acetamido-2-deoxy-D-glucopyranoside (2)

2-Acetamido-2-deoxy-D-glucopyranose (97 mg, 0.438 mmol) and *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside (30 mg, 0.088 mmol) were dissolved in 50 mM citrate–phosphate buffer pH 4.5 (0.5 mL) and 20% (w/v) of $MgSO_4$ was added. The reaction mixture was incubated with β-*N*-acetylhexosaminidase from *P. oxalicum* CCF 2430 (8.5 U) at 37 °C. After 3.5 h the reaction was stopped by heating at 100 °C for 10 min, extracted with ethyl ether (2 × 5 mL) and lyophilised. The residue was loaded to a Toyopearl HW40F column (2.6 × 80 cm, flow rate 25 mL/h) and

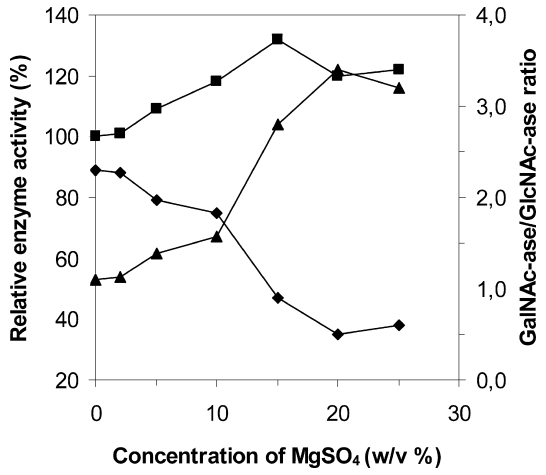


Fig. 3. Influence of $MgSO_4$ on β-GalNAc-ase/β-GlcNAc-ase ratio activity, (■) β-GalNAc-ase (= 100%), (◆) β-GlcNAc-ase, (▲) β-GalNAc-ase/β-GlcNAc-ase ratio.

eluted with water to give **1** (9.9 mg, 0.023 mmol, 26.5%) and **2** (7.2 mg, 0.017 mmol, 19%). NMR and MS data were identical with data published previously (**1** and **2**).[7]

1.4. 2-Acetamido-2-deoxy-β-D-galactopyranosyl-(1 → 6)-2-acetamido-2-deoxy-D-galactopyranose (3)

2-Acetamido-2-deoxy-D-galactopyranose (97 mg, 0.438 mmol) and *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside (50 mg, 0.146 mmol) were dissolved in 50 mM citrate–phosphate buffer pH 4.5 (0.5 mL), and 20% (w/v) of $MgSO_4$ was added. The reaction mixture was incubated with β-*N*-acetylhexosaminidase from *P. oxalicum* CCF 2430 (5.6 U) at 37 °C. After 3.5 h the reaction was stopped by heating at 100 °C for 10 min, followed by the work up as above, yielding **3** (54 mg, 0.127 mmol, 87%). NMR and MS data were identical with the data published previously (**3**).[11]

1.5. Allyl 2-acetamido-2-deoxy-β-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-α-D-glucopyranoside (4)

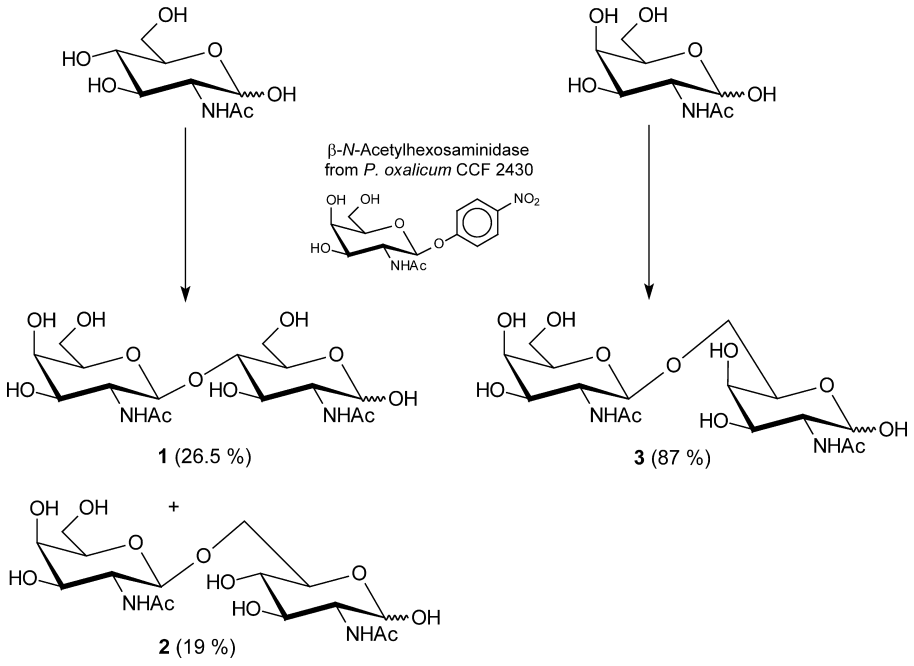
Allyl 2-acetamido-2-deoxy-α-D-glucopyranoside (75 mg, 0.287 mmol) and *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside (25 mg, 0.073 mmol) were dissolved in 50 mM citrate–phosphate buffer pH 5.0 (2.5 mL). The reaction mixture was incubated with β-*N*-acetylhexosaminidase from *A. oryzae* CCF 1066 (2 U) at 37 °C. After 4 h the reaction was stopped by heating at 100 °C for 10 min, extracted with ethyl ether (2 × 5 mL) and lyophilised. The residue was loaded to a Biogel P2 column (2.6 × 120 cm, flow rate 25 mL/h) and eluted with water to give **4** (26.5 mg, 0.057 mmol, 78%). Compound **4** was identified and characterised after peracetylation (Ac_2O–Py, 2 h, 60 °C; silica gel chromatography, 10/0.2 $CHCl_3$–MeOH).

1.6. Allyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-galactopyranosyl-(1 → 4)-2-acetamido-3,6-di-*O*-acetyl-2-deoxy-α-D-glucopyranoside (4a)

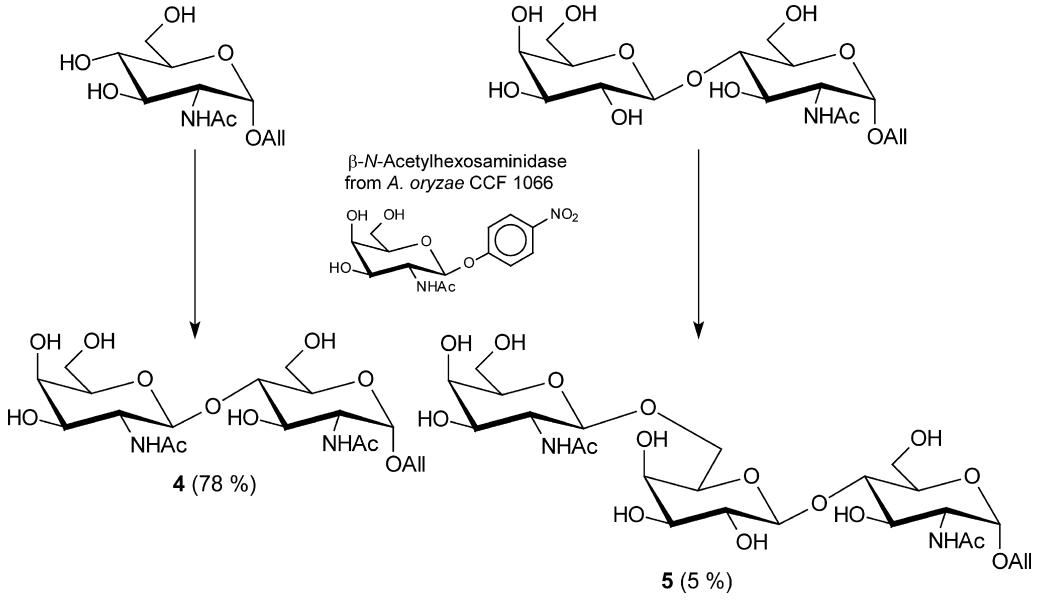
$[\alpha]_D^{20} = +27.0$ ($c = 0.6$, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$): $\delta = 5.85–5.74$ (m, 1 H, CH=), 5.68 (d, 1 H, N′H), 5.63 (d, 1 H, NH), 5.24 (dd, 1 H, 4′-H), 5.17 (dd, 1 H, 3-H, $J_{3,4} = 9.5$), 5.11 (dd, 1 H, 3′-H, $J_{2',3'} = 11.1$, $J_{3',4'} = 3.5$), 4.75 (d, 1 H, 1-H, $J_{1,2} = 3.6$), 4.58 (d, 1 H, 1′-H, $J_{1',2'} = 8.4$), 4.42–4.33 (m, 2 H, CH_2=), 4.30 (dd, 1 H, 6a-H, $J_{5,6a} = 2.0$), 4.22 (dd, 1 H, 6b-H, $J_{5,6b} = 4.2$, $J_{6a,6b} = 11.0$), 4.16 (ddd, 1 H, 2-H, $J_{2,3} = 8.9$), 4.06–3.95 (m, 2 H, 6′a,b), 3.95–3.91 (m, 2 H, CH_2), 3.89–3.85 (m,

Table 2
Influence of aminosugars (GlcN·HCl, GlcNAc and GalNAc) on β-*N*-acetylhexosaminidase activities

	β-GalNAc-ase (%)	β-GlcNAc-ase (%)	β-GalNAc-ase/β-GlcNAc-ase ratio (%)
GlcN·HCl (mmol/L)			
0 (control)	100	66	1.5
5	78	63	1.2
10	71	53	1.3
15	46	44	1.0
GlcNAc (mmol/L)			
5	57	45	1.3
10	60	45	1.3
15	47	40	1.2
GalNAc (mmol/L)			
10	81	61	1.3
15	77	75	1.0
50	75	68	1.1
100	42	58	0.7



Scheme 1.



Scheme 2.

1 H, 2′-H), 3.85–3.82 (m, 1 H, 5-H), 3.82–3.75 (m, 1 H, 5′-H), 3.64 (dd, 1 H, 4-H), 2.10–1.85 (m, 21 H, 5 CH_3CO, 2 CH_3CONH); ^{13}C NMR (100.62 MHz, $CDCl_3$): $\delta = 169.5$ (C=O), 132.4 (CH=), 116.9 (CH_2=), 101.7 (1′-C), 96.7 (1-C), 77.7 (4-C), 72.1 (3-C), 70.9 (5′-C), 70.1 (3′-C), 68.5 (5-C, CH_2), 66.5 (4′-C), 61.8 (6-C), 52.5 (2-C, 2′-C), 25.1–20.0 (CH_3); MALDI-TOF MS: $C_{29}H_{42}N_2O_{16}$ (674.253): m/z 675.15 $[M+H]^+$.

1.7. Allyl 2-acetamido-2-deoxy-β-D-galactopyranosyl-(1→6)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-α-D-glucopyranoside (5)

Allyl β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-α-D-glucopyranoside (35 mg, 0.083 mmol) and *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside (10 mg, 0.029 mmol) were dissolved in citrate–phosphate buffer 50 mM, pH 5.0 (1 mL). The mixture

was incubated with β-*N*-acetylhexosaminidase from *A. oryzae* CCF 1066 (2 U) at 37 °C. After 90 min the reaction was stopped by heating at 100 °C for 10 min, and the work up like in the preceeding section afforded **5** (3.2 mg, 0.005 mmol, 17%). Compound **5** was identified and characterised after peracetylation (Ac_2O–Py, 2 h, 60 °C; silica gel chromatography, 10/0.2 $CHCl_3$–MeOH).

1.8. Allyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-galactopyranosyl-(1→6)-2,3,4-tri-*O*-acetyl-β-D-galactopyranosyl-(1→4)-2-acetamido-3,6-di-*O*-acetyl-2-deoxy-α-D-glucopyranoside (5a)

$[\alpha]_D^{20} = +35.7$ ($c = 0.1$, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 5.84$–5.75 (m, 1 H, CH=), 5.69 (d, 1 H, N″H), 5.62 (d, 1 H, NH), 5.3–5.17 (m, 4 H, 4′-H, 4″-H, CH_2=), 5.15 (dd, 1 H, 3-H, $J_{3,4} = 9.3$), 4.92 (dd, 1 H, 3″-H, $J_{2'',3''} = 10.4$), 4.85 (dd, 1 H, 2′-H, $J_{2',3'} = 10.5$), 4.81 (dd, 1 H, 3′-H), 4.74 (d, 1 H, 1-H, $J_{1,2} = 3.5$), 4.42 (d, 1 H, 1″-H, $J_{1'',2''} = 7.8$), 4.39 (d, 1 H, 1′-H, $J_{1',2'} = 7.8$), 4.36–4.30 (m, 2 H, 6a,b-H), 4.14 (ddd, 1 H, 2-H, $J_{2,3} = 10.4$), 4.10–3.96 (m, 4H, 6′a,b-H, 6″a,b-H), 3.94–3.88 (m, 3 H, 2″-H, CH_2), 3.82–3.78 (m, 1 H, 5-H), 3.77–3.73 (m, 1 H, 5″-H), 3.65 (dd, 1 H, 4-H), 3.57 (m, 1 H, 5′-H), 2.10–1.86 (m, 30 H, 8 CH_3CO, 2 CH_3CONH); ^{13}C NMR (100.62 MHz, $CDCl_3$): $\delta =$ 170.2 (C=O), 132.8 (CH=), 117.6 (CH_2=), 101.2 (1′-C), 100.7 (1″-C), 96.6 (1-C), 77.2 (4-C), 74.1 (3′-C), 72.5 (3-C), 72.1 (5′-C), 71.5 (5″-C), 71.2 (2′-C), 70.9 (3″-C), 70.5 (5-C), 69.5 (2″-C), 69.1 (6″-C), 68.7 (4″-C), 68.2 (CH_2), 67.9 (6′-C), 66.9 (4′-C), 61.7 (6-C), 52.5 (2-C), 24.5–20.0 (CH_3); MALDI-TOF MS: $C_{41}H_{58}N_2O_{24}$ (962.338): m/z 986.04 $[M + Na]^+$.

Acknowledgements

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