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Carbohydrate Research 305 (1998) 517–523

CARBOHYDRATE RESEARCH

β-Glucosylation of chitooligomers by galactosyltransferase

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Received 2 June 1997; accepted 6 August 1997

Abstract

Galactosyltransferase from bovine milk was found to be able to utilise UDP-Glc to transfer Glc onto GlcNAc and chitooligomers $[\text{-}\beta\text{-GlcNAc-}(1 \rightarrow 4)\text{-}]_n$, $n = 2\text{–}4$. β-Glucosylated products were used in binding studies with NKR-P1A protein cloned from rat natural killer cells. © 1998 Elsevier Science Ltd.

Keywords: β1–4 Galactosyltransferase; β-Glucopyranosyl; Chitooligomer; NKR-P1A protein; UDP-Glucose

1. Introduction

Receptors related to C-type animal lectins seem to play various functions in natural killer (NK) cells [1]. We have recently shown that rat NKR-P1 antigen is a carbohydrate-binding protein with remarkably high affinity for both calcium and certain monosaccharides (such as GlcNAc) [2]. In our current experiments aimed at the understanding of the biological relevance of this unusual reactivity, chitooligomers and their various derivatives were investigated as useful ligands for the mapping of the carbohydrate-binding site of NKR-P1. Chitooligomers $[\text{-}\beta\text{-GlcNAc-}(1 \rightarrow 4)\text{-}]_n$, $n = 2\text{–}9$ bind to this protein with high affinity [3]. To investigate the role of the NHAc group on the terminal glycosyl unit, chitooligomers having the terminal NHAc group replaced by hydroxyl, i.e., containing a terminal $\beta\text{-}(1 \rightarrow 4)$-Glc*p* unit, were prepared, using the selective $\beta\text{-}(1 \rightarrow 4)$-Glc*p* transfer catalysed by $\beta\text{-}(1 \rightarrow 4)$-galactosyltransferase from bovine milk (GalT) recently used in the preparation of glucosylated alkaloids [4]. Glycosyltransferases are often considered as being somewhat stringent towards the distal one to two saccharidic residues and also very specific for the nucleoside of the glycosyl donor. There are, however, more and more examples demonstrating that glycosyltranferases can work with both unnatural donors and/or acceptors while maintaining strict regio- and stereoselectivity and high yields. The substrate specificity of $\beta\text{-}(1 \rightarrow 4)$-galactosyltransferase from bovine milk (GalT) has been extensively studied and often reviewed [5]. The possibility of Glc transfer by galactosyltransferase was demonstrated by Andree and Berliner [6], and by

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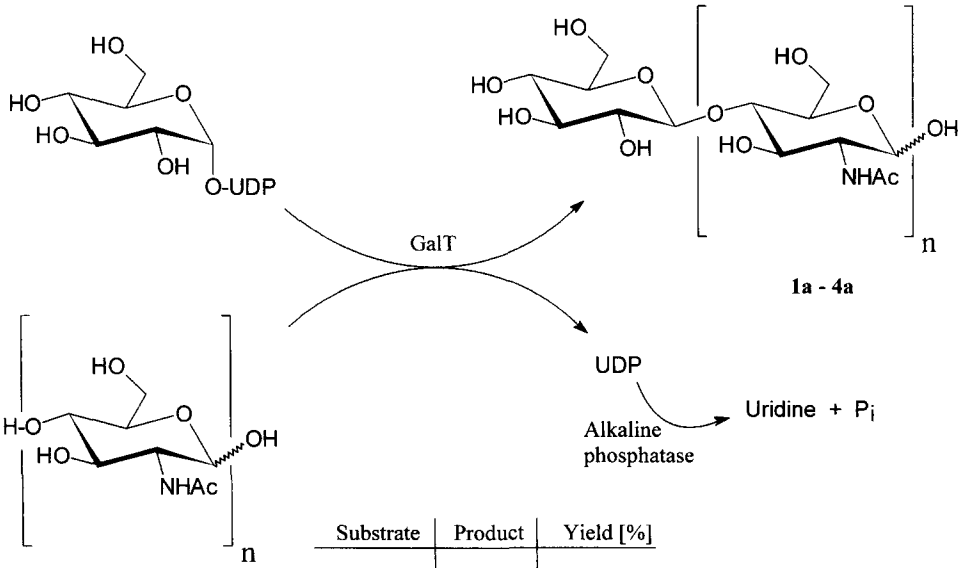
PII S0008-6215(97)00248-6

Palcic and Hindsgaul [7] who, however, were not able to prove spectrally the nature and position of the sugar transferred. Recently, this transfer was fully confirmed (MS, ^{1}H NMR and ^{13}C NMR) [4]. The concomitant transfer of Gal and Glc in the presence of a UDP-Gal-generating system (UDP-Glc and UDP-Glc 4′-epimerase) was also observed. We present here the novel enzymatic preparations of a series of terminally β-glucosylated chitooligomers ($n = 1$–4).

2. Results and discussion

In an analytical experiment GlcNAc (**1**), chitobiose (**2**), chitotriose (**3**) and chitotetraose (**4**) (Scheme 1) were subjected to glucosyl transfer under conditions analogous to those described previously [4] using UDP-Glc as a glycosyl donor. Liberation of UDP was detected by ion exchange TLC [9]. After 20 h (37 °C), the first spots corresponding to new products were detected by TLC (silica gel) and also UDP was formed. When alkaline phosphatase was added to remove the feedback inhibition of UDP by its conversion into uridine, and more donor (UDP-Glc) and GalT were added, the reaction proceeded further to be completed within 4–6 days (Scheme 1). The reaction was faster with higher chitooligomers; the K_M values of GalT for chitooligomers (for the Gal transfer) being 3.6, 2.5 and 1.2 mM for GlcNAc, $(GlcNAc)_2$ and $(GlcNAc)_3$, respectively [8]. V_{max} for **2**, **3** (Gal transfer by GalT) drops only to the value 80–90% of that of **1**. Andree and Berliner [6] estimated the Glc transfer rate to GlcNAc was 0.3% of the Gal transfer rate, but our results compared to Ref. [8] suggest that this rate can be about 10× higher. Also previously published results [4] support the fact that Glc transfer is at least one order higher than the reported value of 0.3%.

The semipreparative reactions were performed on a 0.02–0.05 mmol scale. For efficient and rapid Glc-1 → 4-transfer, the high concentration of UDP-Glc (0.2 M) is essential as K_M of GalT for this substrate is obviously much higher than that for UDP-Gal. Such large quantities acidic substrate (two equivalents) exceed the buffering capacity of cacodylate buffer (50 mM, pH 7.5) used. To maintain the pH optimum for the enzymes, it is necessary to neutralise the acidity by the addition of 1 M NaOH (approx. 50 μL per 1 mL of the mixture). The pH should be carefully checked during the reaction and especially after each addition of the new substrates. The concentration of Mn^{2+} should be kept under 5



Substrate	Product	Yield [%]
n = 1 (**1**)	**1a**	27
n = 2 (**2**)	**2a**	30
n = 3 (**3**)	**3a**	25
n = 4 (**4**)	**4a**	22

Scheme 1.

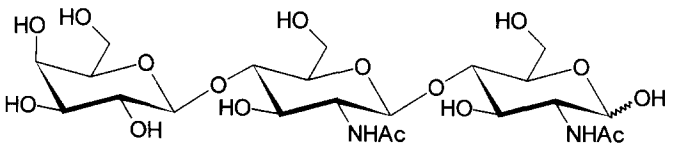
mM. Although a higher concentration (optimum 13 mM) promotes the enzyme activity, it simultaneously accelerates the decomposition of the UDP-Glc.

The reaction mixture was fractionated by gel filtration (Biogel P2). The observation of $[M + Na]^+$ and $[M + K]^+$ ions in electrospray ionisation mass spectra (ESIMS) of compounds **1a**, **2a**, **2b**, **3a**, **3b** and **4a** at *m/z* of 162 mass units higher than in parent compounds indicated the attachment of one hexose unit. Its nature was inferred from characteristic vicinal proton–proton coupling constants found by COSY and 2D *J*-resolved experiments. According to $J_{1,2}$ values, all anomeric centres have β-configuration except the reducing end unit which is a mixture of α/β anomers. Comparison of ^{13}C NMR spectra of new compounds with those of chitobiose, chitotriose [9], and model compound **2b**, **3b** suggested that the introduced sugar unit was attached to C-4.

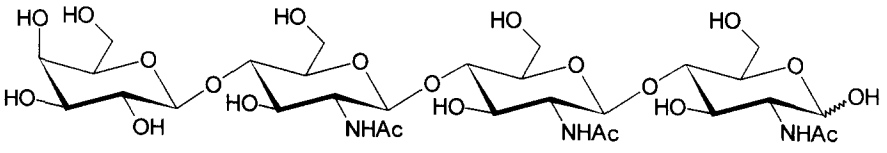
We have prepared also two derivatives of chitobiose and chitotriose bearing the β-Gal*p*-(1 → 4) unit, **2b** and **3b** (Scheme 2) by transgalactosylation of starting material **2** and **3** with β-galactosidase from *Bacillus circulans* using *p*-nitrophenyl β-D-galactopyranoside as a donor. This enzyme is known to maintain rather strict regioselectivity transferring the β-Gal*p* unit onto C-4 of the glycosides with Glc*p* configuration [10,11]. This reaction has been used for the enzymatic preparation of *N*-acetyllactosamine from *N*-acetyllactosamine from GlcNAc [10]. These compounds were also used for the inhibition assays to test the influence of the βGal*p* configuration in the terminal position of the respective chitooligomers.

Compound **1a** (*N*-acetylcellobiosamine) was prepared previously by nine-step synthesis from 2-acetamido-3-*O*-acetyl-1,6-anhydro-2-deoxy-β-D-glucopyranose by Koenigs–Knorr reaction with acetobromoglucose [16]. Compound **2b** was prepared previously by galactosylation of **2** using GalT from bovine milk and it was used for ^{13}C NMR spectral studies [17].

The inhibition assays (Fig. 1) revealed that compounds **1a**, **2a** and **3a** were comparable to their analogues **2**, **3**, **4** as inhibitors of the binding of rat natural killer cell lectin, NKR-P1A, to its high affinity ligand, $GlcNAc_{23}BSA$ [2]. The concentrations causing 50% inhibition in this assay (IC_{50}) were nearly identical for **1a** and **2** and for **2a** and **3** (approx. 10^{-7} M and 4×10^{-8} M, respectively). The IC_{50} for compound **4** was only slightly lower than for the Glc analogue **3a** (2×10^{-9} and 7×10^{-9} M, respectively). Therefore, we can conclude that the terminal NHAc group is not essential for the binding to this lectin, however, its substitution with an OH group somewhat lowers the affinity of the compound for the receptor. In contrast to this type of tolerance by the receptor, the terminal substitution by β-(1 → 4)-linked Gal results in sharp decrease in the inhibitory potency with IC_{50} in the range of 10^{-5} M for both **2b** and **3b** (see Fig. 1). As recently pointed out by Drickamer [12], the anomeric configuration of C-4 hydroxyl is critical to distinguish the group of evolutionarily older lectins from the more recently developed species [12]. In this respect, NKR-P1A seems to be somewhat unusual in its ability to distin-



2b



3b

Scheme 2.

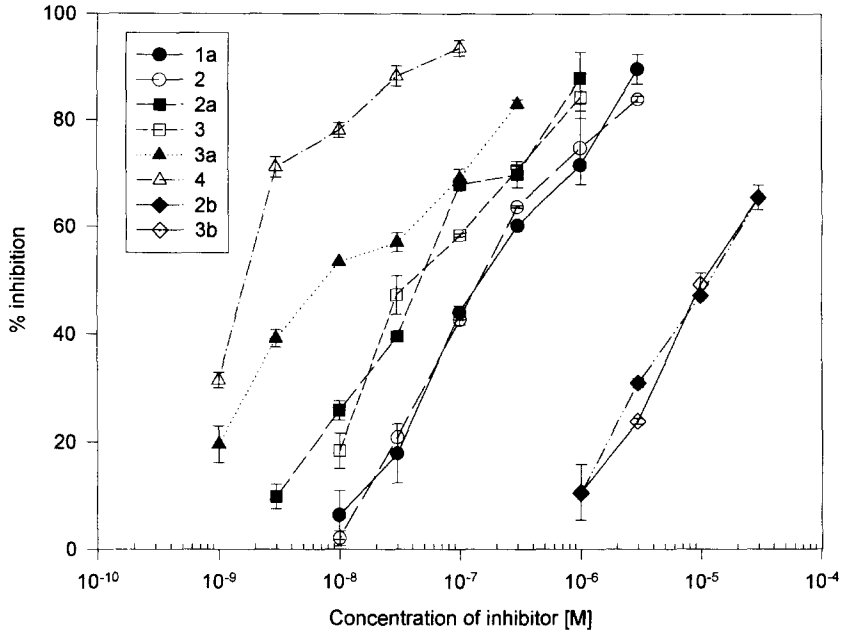


Fig. 1. Potencies of the oligosaccharides studied as the inhibitors of binding of rat natural killer cell lectin, NKR-P1A, to microplate wells coated with the high affinity ligand, $GlcNAc_{23}BSA$. In order to check the performance of the inhibition assay, GlcNAc and Gal were also tested, and provided the inhibition at concentrations described previously (Ref. [2], approx. 3×10^{-7} and 1×10^{-4} M, respectively). The individual oligosaccharides are labeled using the designation adopted in this study.

guish only neutral carbohydrates according to the C-4 configuration (such as Gal vs. Glc in the example here), but not their N-acetylated analogues (both GlcNAc and GalNAc are good ligands for this lectin [2]).

3. Experimental

Chitooligomers **2–4** were prepared by chitin hydrolysis. Chitin obtained from crab shell (Sigma, pract. grade) was fragmented to superfine particles by treatment with a hammer mill. Hydrolysis and neutralisation were performed according to Rupley [13]. The mixture was treated by ultrafiltration (1 kDa membrane) to reach 25% (w/w) of chitooligomers in the mixture. Most of the salt resulting from neutralisation with NaOH was removed by electrodialysis. The mixture was then fractionated by gel filtration on the BioGel P4 (BioRad, USA) [14].

β-(1 → 4)-Galactosyltranferase from bovine milk (EC 2.4.1.22) and UDP-Glc were from Sigma, alkaline phosphatase from calf intestine (EC 3.1.3.1) was from Boehringer, solvents and other chemicals were of p.a. purity.

NMR spectra were measured on a Varian VXR-400 spectrometer (399.95 MHz for ^{1}H, 100.58 MHz for ^{13}C) in deuterium oxide at 25 °C. The sodium salt of 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid was used as an internal reference for both nuclei. Chemical shifts are given in the δ-scale; digital resolution was 0.0002 and 0.006 ppm, respectively. J values are given in Hz. COSY, delay-COSY, and HOM2DJ experiments were performed using the manufacturer's software. Carbon multiplicities were determined by APT (Attached Proton Test).

Positive-ion electrospray ionisation mass spectra (ESIMS) were recorded on a double-sector instrument Finnigan MAT 95 (Finnigan MAT, Bremen, FRG) of BE geometry (magnetic sector preceding the electrostatic one) equipped with the Finnigan ESI source. Samples dissolved in a mixture of acetonitrile–water–methanol = 3:2:5 were continuously fed through a stainless capillary held at 3.0 kV into the electrospray ion source via a linear syringe pump at a rate of 30 μL/min. A mixture of polypropylene glycols Mr = 425 was used to calibrate the m/z scale of mass spectrometer.

TLC was carried out using Silica Gel 60 GF_{254} (Merck) with the solvent system n-propanol–water–conc. ammonia (7:2:1), twice developed. The spots were visualised by UV (nucleosides) and by charring with 5% H_2SO_4 in EtOH. Depletion of UDP-Glc was followed by ion-exchange TLC on PEI cellulose

(Merck) that was developed consecutively with 0.3 M lithium chloride (1 min), 1 M lithium chloride (10 min) and then 1.6 M lithium chloride (47 min) without drying in between [15]. The spots were observed under UV light.

NKR-P1 protein preparation and the inhibition assay (tens of micrograms of the studied compounds were necessary) were performed as described previously [2].

2-Acetamido-4-O-(β-D-glucopyranosyl)-2-deoxy-D-glucopyranose (**1a**). GlcNAc (**1**) (10 mg, 0.045 mmol), UDP-Glc (48 mg, 0.048 mmol) were dissolved in sodium cacodylate buffer (50 mM, pH 7.5, 400 μL) containing manganese(II) chloride (5 mM). The pH of the mixture was checked by a pH metre and adjusted to the value of 7.5 with 1 M NaOH if necessary. Galactosyltransferase (GalT) (0.4 U in 18 μL storage buffer) was added and the mixture was incubated at 37 °C for 30 h. Then another UDP-Glc (12 mg, 0.02 mmol), GalT (0.12 U) and alkaline phosphatase (20 U) were supplemented. The pH was checked after all additions. Another addition, GalT (0.12 U), UDP-Glc (12 mg), was done after another 24 h. The reaction was terminated by boiling (5 min) after total 96 h and fractionated by gel filtration on the column packed with BioGel P2 (2.6 × 80 cm) eluted with water at 24 °C. The fractions were lyophilised to give **1a** (4.3 mg, 27%) as white amorphous powder: $[\alpha]_D^{23} = +9$, *c* 0.1, water, (+12.9, *c* 1, water, Ref. [16]); ^{1}H NMR (D_2O): δ 2.053 (3 H, s, Ac, β), 3.330 (1 H, dd, *J* 9.3, 7.9, H-2′β), 3.425 (1 H, *J* 9.7, 9.0, H-4′β), 3.526 (1 H, dd, *J* 9.3, 9.0, H-3′β), 4.547 (1 H, d, *J* 7.9, H-1′β), 4.725 (1 H, d, *J* 8.2, H-1β), 2.051 (3 H, s, Ac, α), 3.318 (1 H, dd, *J* 9.3, 8.0, H-2′α), 3.417 (1 H, dd, *J* 9.7, 9.0, H-4′α), 3.502 (1 H, dd, *J* 9.3, 9.0, H-3′α), 4.542 (1 H, d, *J* 8.0, H-1′α), 5.214 (1 H, d, *J* 2.8, H-1α). ESIMS *m/z* 405 (calculated for $C_{14}H_{24}NO_{11}Na$, 405) $[M + Na]^+$, 421, (calculated for $C_{14}H_{24}NO_{11}K$, 421) $[M + K]^+$.

4′-O-(β-D-Glucopyranosyl)-di-N-acetylchitobiose (**2a**). di-*N*-Acetylchitobiose (**2**) (8.5 mg, 0.02 mmol), UDP-Glc (24 mg, 0.04 mmol) were dissolved in sodium cacodylate buffer (50 mM, pH 7.5, 200 μL) containing manganese(II) chloride (5 mM). The pH of the mixture was checked by the pH metre and adjusted to 7.5. GalT (0.2 U) was added and the mixture was incubated at 37 °C. After 2 days, another UDP-Glc (6 mg, 0.01 mmol), GalT (0.06 U) and alkaline phosphatase (20 U) were supplemented. The reaction was terminated by boiling (5 min) after total 72 h and fractionated by gel filtration as above. The fractions were lyophilised to give **2a** (3.3 mg, 30%) as white amorphous powder: $[\alpha]_D^{23} = -28.0$, *c* 0.3, water; ^{1}H NMR (D_2O): 2.053 (3 H, s, Ac), 2.079 (3 H, s, Ac), 3.322 (1 H, dd, *J* 9.2, 7.9, H-2″), 3.419 (1 H, dd, *J* 9.4, 9.2, H-4″), 3.522 (1 H, dd, *J* 9.2, 9.2, H-3″), 4.540 (1 H, d, *J* 7.9, H-1″), 4.617 (1 H, d, *J* 8.2, H-1′α), 4.626 (1 H, d, *J* 8.2, H-1′β), 4.712 (1 H, d, *J* 7.9, H-1β), 5.205 (1 H, d, *J* 3.0, H-1α); ^{13}C NMR (D_2O): 25.02 (q, 2 C), 56.42 (d, C-2β), 58.16 d, 59.00 (d, C-2α), 62.55 t, 62.79 t, 62.92 t, 63.49 t, 72.12 d, 72.19 d, 72.31 d, 72.68 d, 74.92 d, 76.02 d, 77.66 d, 77.69 d, 78.40 d, 81.26 d, 81.45 d, 82.24 d, 93.36 (d, C-1β), 97.70 (d, C-1α), 104.19 d, 105.43 d, 177.40 s, 177.53 s, 177.81 s. ESIMS *m/z* 609 (calculated for $C_{22}H_{38}N_2O_{16}Na$, 609) $[M + Na]^+$, 625 (calculated for $C_{22}H_{38}N_2O_{16}K$, 625) $[M + K]^+$.

4″-O-(β-D-Glucopyranosyl)-tri-N-acetylchitotriose (**3a**). Tri-*N*-acetylchitotriose (**3**) (12.5 mg, 0.02 mmol), UDP-Glc (24 mg, 0.04 mmol) were dissolved in sodium cacodylate buffer (50 mM, pH 7.5, 200 μL) containing manganese(II) chloride (5 mM) and the pH of the mixture was adjusted to 7.5. GalT (0.2 U) was added and the mixture was incubated at 37 °C. After 2 days, another UDP-Glc (6 mg, 0.01 mmol), GalT (0.06 U) and alkaline phosphatase (20 U) were supplemented. The reaction was terminated by boiling (5 min) after total 72 h and fractionated by gel filtration as above. The fractions were lyophilised to give **3a** (4.7 mg, 30%) as white amorphous powder: $[\alpha]_D^{23} = -43$, *c* 0.35, water; ^{1}H NMR (D_2O): 2.049 (3 H, s, Ac), 2.073 (6 H, s, 2 × Ac), 3.318 (1 H, dd, *J* 9.2, 7.9, H-2″), 3.415 (1 H, dd, *J* 9.8, 8.0, H-4‴), 3.519 (1 H, dd, *J* 9.2, 9.0, H-3‴), 4.536 (1 H, d, *J* 7.9, H-1‴), 4.591 (1 H, d, *J* 8.3), 4.600 (1 H, d, *J* 8.3), 4.613 (1 H, d, *J* 8.2), 5.198 (1 H, d, *J* 2.8, H-1α); ^{13}C NMR (D_2O): 24.87 q, 24.99 (q, 2 C), 56.39 (d, C-2β), 57.79 d, 58.01 d, 58.86 (d, C-2α), 62.63 t, 62.69 t, 62.75 t, 63.63 t, 71.99 d, 72.19 d, 74.74 d, 74.86 d, 75.90 d, 76.61 d, 77.26 d, 77.36 d, 77.54 d, 77.59 d, 78.11 d, 78.76 d, 81.25 d, 81.73 d, 81.78 d, 81.93 d, 82.37 d, 93.18 (d, C-1β), 97.53 (d, C-1α), 104.03 d, 104.10 d, 105.30 d, 177.20 s, 177.32 s, 177.42 s. ESIMS *m/z* 812 (calculated for $C_{30}H_{51}N_3O_{21}Na$, 812) $[M + Na]^+$, 828 (calculated for $C_{30}H_{51}N_3O_{21}K$, 828) $[M + K]^+$.

4‴-O-(β-D-Glucopyranosyl)-tetra-N-acetylchitotetraose (**4a**). Tetra-*N*-acetylchitotetraose (**4**) (10 mg, 0.012 mmol), UDP-Glc (15 mg, 0.024 mmol) were dissolved in sodium cacodylate buffer (50 mM, pH 7.5, 200 μL) containing manganese(II) chloride (5 mM) and the pH of the mixture was adjusted to 7.5. GalT (0.12 U) was added and the mixture was incu-

bated at 37 °C. After 1 day, another UDP-Glc (5 mg, 0.008 mmol), GalT (0.12 U) were added. Following day, UDP-Glc (5 mg, 0.008 mmol), GalT (0.12 U) and alkaline phosphatase (2 U) were supplemented. The reaction was terminated by boiling (5 min) after total 72 h and fractionated by gel filtration on the column (2.6 × 80 cm) packed with BioGel P4. The fractions were lyophilised to give **4a** (3.7 mg, 22%) as white amorphous powder: ^{1}H NMR (D_2O): 2.040 (3 H, Ac), 2.062 (6 H, 2 × Ac), 2.064 (3 H, Ac), 3.310 (1 H, dd, *J* 9.1, 7.9, H-2''''), 3.407 (1 H, dd, *J* 9.5, 9.3, H-4''''), 3.511 (1 H, dd, *J* 9.3, 9.1, H-3''''), 3.986 (1 H, dd, *J* 13.0, 2.7), 4.527 (1 H, d, *J* 7.9, H-1''''), 4.582 (1 H, d, *J* 8.3), 4.587 (1 H, d, *J* 8.2), 4.603 (1 H, d, *J* 8.6), 4.698 (1 H, d, *J* 8.2, H-1β), 5.190 (1 H, d, *J* 2.5, H-1α). ESIMS *m/z* 1015 (calculated for $C_{38}H_{64}N_4O_{26}Na$, 1015) $[M + Na]^+$, (calculated for $C_{38}H_{64}N_4O_{26}K$, 1031) 1031 $[M + K]^+$.

4′-O-(β-D-Galactopyranosyl)-di-N-acetylchitobiose (**2b**). di-*N*-Acetylchitobiose (**2**) (80 mg, 0.19 mmol) and *p*-nitrophenyl β-D-galactopyranoside (200 mg, 0.66 mmol) were dissolved in McIlvain buffer (mix 13.22 mL of 0.05 M sodium monohydrogen phosphate and 6.78 mL of 0.025 M citric acid to give pH 6.2) (1 mL, pH 6.2) and β-galactosidase from *B. circulans* (24 U, Biolacta FN5, Daiwa Kasei, Japan) was added. The mixture was incubated 3 h at 37 °C. The reaction was then stopped by boiling and the product was fractionated by gel filtration on BioGel P2 to give **2b** (11 mg, 10%) as a white amorphous solid: $[\alpha]_D^{23} = -21$, *c* 0.56, water; ^{1}H NMR (D_2O): 2.050 (3 H, s, Ac), 2.076 (3 H, s, Ac), 3.549 (1 H, dd, *J* 10.0, 7.8, H-2″), 3.676 (1 H, dd, *J* 10.0, 3.3, H-3″), 3.935 (1 H, dd, *J* 3.3, 1.0, H-4″), 4.489 (1 H, d, *J* 7.8, H-1″), 4.617 (1 H, d, *J* 8.0, H-1′α), 4.626 (1 H, d, *J* 8.2, H-1′β), 4.709 (1 H, d, *J* 7.9, H-1β), 5.203 (1 H, d, *J* 2.8, H-1α); ^{13}C NMR (D_2O): 24.71 (q, Ac β), 24.97 (q, Ac), 25.01 (q, Ac α), 58.49 (d, C-2β), 57.99 (d, C-2′), 58.96 (d, C-2α), 61.74 (t, C-6′), 62.84 (t, C-6α), 62.92 (t, C-6β), 63.88 (t, C-6″), 71.39 (d, C-4″), 72.09 d, 72.81 d, 73.79 d, 74.95 d, 75.33 d, 77.41 d, 77.63 d, 78.20 d, 80.99 (d, C-4′), 82.12 (d, C-4α), 82.57 (d, C-4β), 93.31 (d, C-1β), 97.71 (d, C-1α), 104.19 (d, C-1′), 105.73 (d, C-1″), 177.29 (s, C=O β), 177.39 (s, C=O′), 177.56 (s, C=O α). ESIMS *m/z* 609 (calculated for $C_{22}H_{38}N_2O_{16}Na$, 609) $[M + Na]^+$, 625 (calculated for $C_{22}H_{38}N_2O_{16}K$, 625) $[M + K]^+$.

4″-O-(β-D-Galactopyranosyl)-tri-N-acetylchitotriose (**3b**). tri-*N*-Acetylchitotriose (**3**) (80 mg, 0.13 mmol) and *p*-nitrophenyl β-D-galactopyranoside (200 mg, 0.66 mmol) were dissolved in McIlvain buffer (1 mL, 0.05 M, pH 6.2) and β-galactosidase from *B. circulans* (24 U, Biolacta FN5, Daiwa Kasei, Japan) was added. The mixture was incubated 3 h at 37 °C. The reaction was then stopped by boiling and the product was fractionated by gel filtration on BioGel P2 to give **3b** (27 mg, 26.5%) as a white amorphous solid: $[\alpha]_D^{23} = -114$, *c* 0.1, water; ^{1}H NMR (D_2O): 2.056 (3 H, s, Ac), 2.080 (6 H, s, 2 × Ac), 3.555 (1 H, dd, *J* 9.9, 7.8, H-2‴), 3.942 (1 H, dd, *J* 3.3, 1.1, H-4‴), 4.484 (1 H, d, *J* 7.8, H-1‴), 4.597 (1 H, d, *J* 8.2), 4.606 (1 H, d, *J* 7.9), 4.621 (1 H, d, *J* 7.8), 4.624 (1 H, d, *J* 7.8), 4.711 (1 H, d, *J* 8.3, H-1β), 5.205 (1 H, d, *J* 2.6, H-1α); ^{13}C NMR (D_2O): 24.72 q, 24.95 q, 25.01 q, 56.49 d, 57.89 d, 57.97 d, 58.97 d, 62.74 t, 62.84 t, 62.32 (t, C-6‴), 71.39 (d, C-4‴), 72.08 d, 72.84 d, 73.79 d, 74.94 d, 75.33 d, 77.35 d, 77.44 d, 77.65 d, 78.21 d, 80.99 d, 81.82 d, 81.66 d, 81.99 d, 82.45 d, 93.49 (d, C-1β), 97.67 (d, C-1α), 101.00 d, 104.1 d, 104.12 d, 104.17 d, 105.73 (d, C-1‴), 177.29 s, 177.92 s, 177.55 s. ESIMS *m/z* 812 (calculated for $C_{30}H_{51}N_3O_{21}Na$, 812) $[M + Na]^+$, 828 (calculated for $C_{30}H_{51}N_3O_{21}K$, 828) $[M + K]^+$.

Acknowledgements

This study was supported in part by the Volkswagen Stiftung (Project No. I/71 188), the Deutscher Akademischer Austauschdienst, and the Grant Agency of the Czech Republic (Grant No. 203/96/1267 and 303/96/0639).

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