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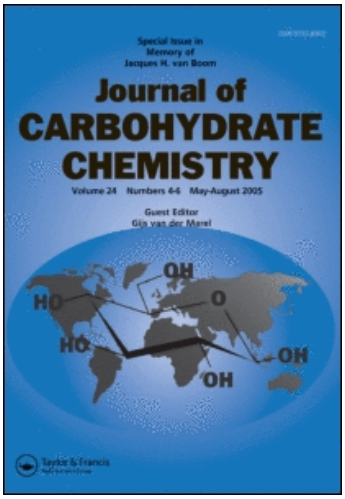

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GALACTOSIDASE-ASSISTED SYNTHESIS EN ROUTE TO TYPE I AND TYPE II STRUCTURES OF CHITOOLIGOMERS [1]

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ABSTRACT

Transgalactosylation of chitobiose and chitotriose led to formation of terminally (β1-3)- and (β1-4)-galactosylated chitooligosaccharides ready for fucosylation to give Lewis[a] and Lewis[x] motifs. Their structures could be assigned employing HPAEC-PAD, methylation, ESI-MS/MS and NMR analyses.

INTRODUCTION

Fucosyltransferases are a group of enzymes which use GDP-β-L-fucose to catalyze the transfer of fucose under inversion of the anomeric configuration to various acceptors and form fucosylated oligosaccharides with (α1-2)-, (α1-3)-, (α1-4)- and (α1-6) linkages.[2] In order to study the acceptor specificity of recombinant human fucosyltransferases further modified oligosaccharide acceptor substrates have to be synthesized. The use of enzymes in oligosaccharide synthesis provides an attractive route, since protection-deprotection sequences are avoided and complete control is exerted over newly generated anomeric centres by the specificity of the particular enzyme used.[3]

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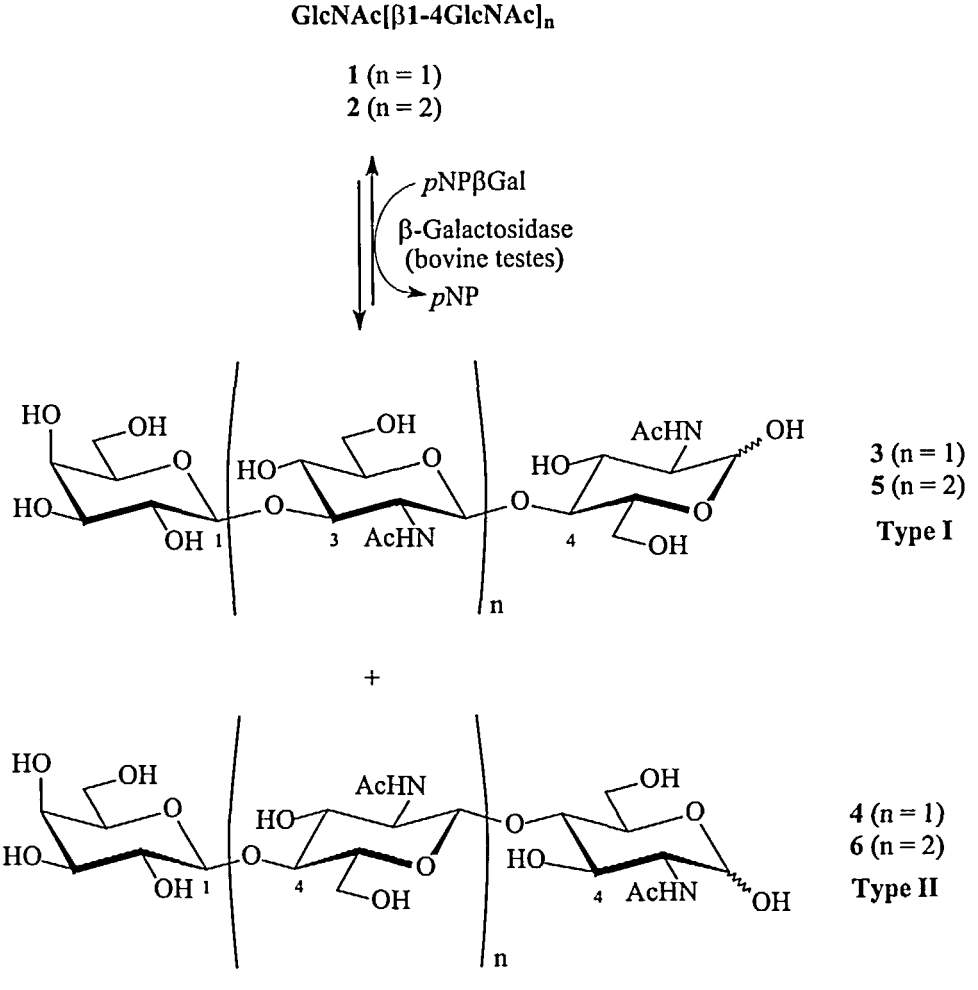
RESULTS AND DISCUSSION

The synthesis of the type I [Gal(β1-3)GlcNAc] (**3, 5**) and type II [Gal(β1-4)GlcNAc] (**4, 6**) structures of chitooligomers are based on transgalactosylation of **1** and **2** using β-galactosidase from bovine testes (E.C. 3.2.1.23) along the previously reported procedure (Scheme 1).[4]

The bovine testes enzyme is commercially available but too expensive to be used in large scale chemistry. A simplified purification procedure [5] was followed according to the initial steps described by Distler and Jourdian.[6] Glucose, *N*-acetyl galactosamine and



Scheme 1. Transgalactosylation of chitobiose and chitotriose.

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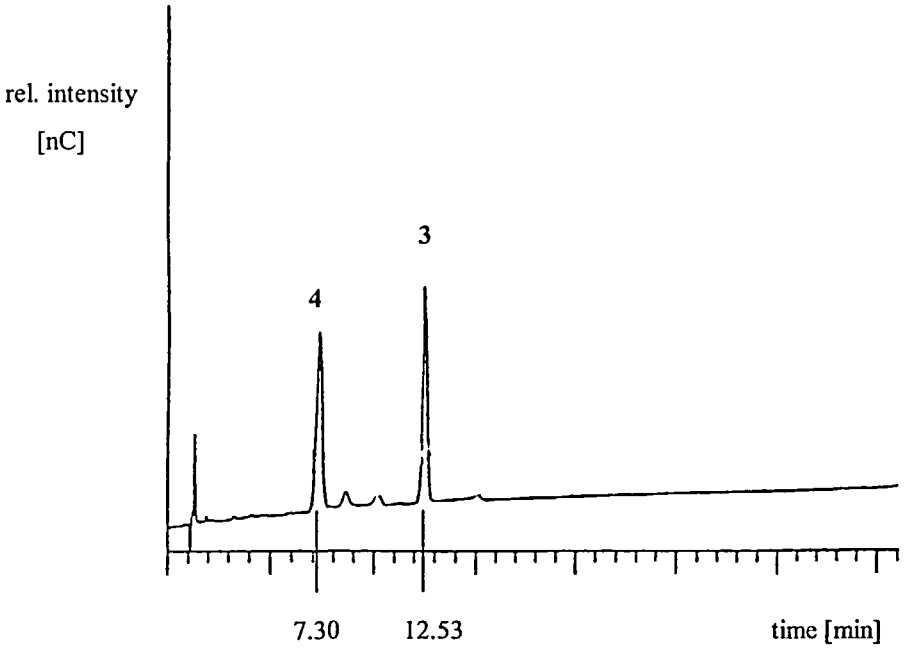

Figure 1. HPAEC-PAD analysis of regiospecifically galactosylated chitobioses Gal(β1-3)GlcNAc(β1-4)GlcNAc (**3**) and Gal(β1-4)GlcNAc(β1-4)GlcNAc (**4**).

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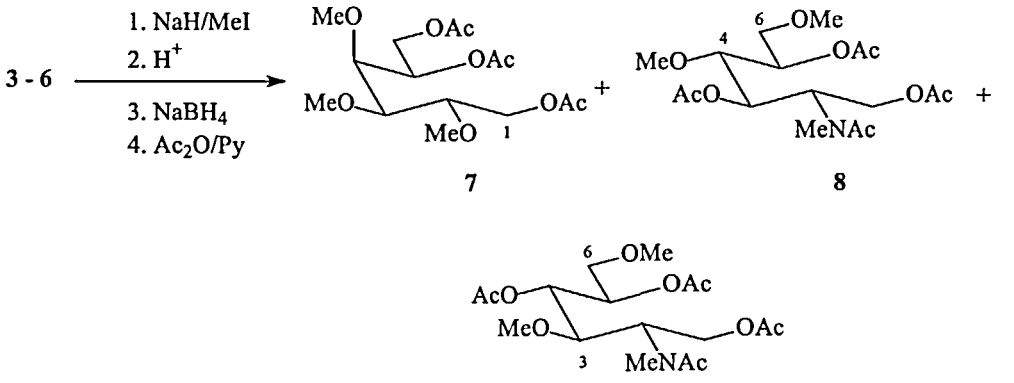
N-acetyl glucosamine[7] are described to be excellent acceptors whereas mannose, L-arabinose and L-fucose were only slightly active.[6] Further, it is known that β-galactosidase from bovine testes hydrolyses 1-3-, 1-4-, and 1-6-linkages of *N*-acetyllactosamine derivatives.[6]

In the enzymatic preparation of 2-acetamido-2-deoxy-3-*O*-(β-D-galactopyranosyl)-D-galactose the equilibrium was shifted to result in both 1-3- and 1-6 linkages,[5] whereas only the formation of a 1-3 linkage was observed in synthesizing T-antigen derivatives.[4] However, a formation of 1-4 linkages was never reported under these conditions. To our surprise the enzymatic galactosylation of chitobiose **1** and chitotriose **2** gave evidence for formation of terminally 1-3- as well as 1-4-galactosylated derivatives. A 1:1 ratio of **3** and **4** was detected with chitobiose and a 1:1.4 ratio of **5** and **6** with chitotriose.

The assignment of the structures is based on HPAEC-PAD analysis (Fig. 1 exemplifies the plot for the chitobiose-derived products **3** and **4**) and confirmed by NMR analysis.



Ratio of methylated peracetylated alditols

Galactosylated chitooligomers	7	8	9
Gal(β1-3)GlcNAc(β1-4)GlcNAc (3)	1	1	1
Gal(β1-4)GlcNAc(β1-4)GlcNAc (4)	1	-	2
Gal(β1-3)GlcNAc[(β1-4)GlcNAc]$_2$ (5)	1	1	2
Gal(β1-4)GlcNAc[(β1-4)GlcNAc]$_2$ (6)	1	-	3

Scheme 2 / Table 1. Methylation analysis.

At present only a plausible explanation may be given. Apparently due to its equatorial OH-4 the terminal GlcNAc residue is recognized by the enzyme like the equatorial OH-3 in GalNAc. However, further experiments are needed to prove the polar and spatial requirements of the known acceptor substrates for this enzyme.

The transgalactosylation reactions of **1** and **2** were performed on a semi-preparative scale, and the separation of the regiospecifically galactosylated chitooligomers **3**+**4** and **5**+**6** turned out to be a challenging problem which could be solved only by employing HPAEC-PAD chromatography. Structural analysis was performed after separation employing methylation analysis as well as ESI-MS/MS of the permethylated alditols obtained by reduction of the galactosylated chitooligomers. The β

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1-3-linkage of compounds **3** and **5** led to formation of 4,6-di-*O*-methyl-2-acetamidoglucitol derivatives indicating a GlcNAc residue monosubstituted at O-3. In contrast, compounds **4** and **6** gave derivatives characteristic for an O-4 substituted GlcNAc residue indicating a β1-4-linkage (Table 1). These results could be confirmed by ESI-MS/MS of the corresponding permethylated alditols. The molecular ions of their isomers related to **3** and **5** were identical and found at *m/z* 779. The same method applied to the isomers obtained from **4** and **6** gave molecular ions at *m/z* 1024. A distinction between 3- and 4-*O*- galactosylated compounds could be performed by CID. The type I compounds **3** and **5** led to the well known preferred elimination[8] of the 3-linked substituent at the GlcNAc to give an intense fragment at *m/z* 543. A signal at *m/z* 259, characteristic for a hexose (Hex) residue confirmed this elimination (Figures 2a and 2b). In the 4-linked isomers of type II (**4** and **6**) these signals were not observed.

There is increasing interest among biochemists to explore functions and substrate structures for glycosyltransferases and this warranted the study of modified acceptor oligosaccharides in vitro. Therefore, these type I and type II derivatives of chitooligomers (**3** to **6**) were tested as acceptor substrates for recombinant human fucosyltransferases III and VI, and after fucosylation novel chitooligomers with terminal Lewis^a and Lewis^x motifs could be obtained.[9]

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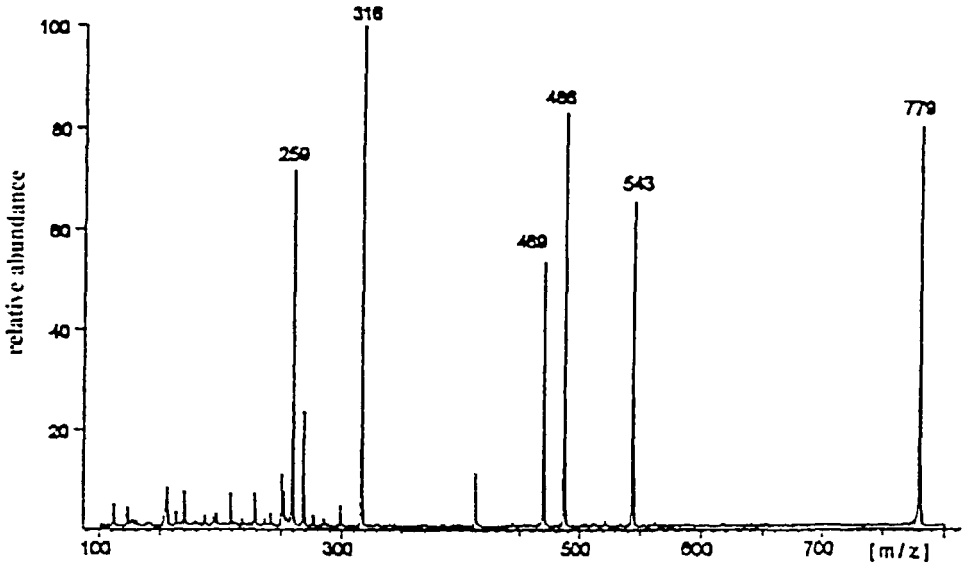
EXPERIMENTAL

Enzyme assay for β-galactosidase. The rate of hydrolysis of the substrate *p*-nitrophenyl β-D-galactopyranoside was determined by measuring the absorbance of the liberated nitrophenol in alkaline solutions described by Kuby and Lardy.[10] Incubation mixtures contained the following components in total volumes of 0.1 mL: sodium phosphate-citrate buffer, pH 4.3, as prepared by McIlvaine, 25 μL; *p*-nitrophenyl β-Gal, 0.5 μmol, and 1 to 4 units of enzyme. Control tubes contained the same components but lacked either substrate or enzyme. Incubations were conducted for 30 min at 37 °C and were terminated by the addition of 1 mL of 0.25 M glycine buffer, pH 10. Absorbance was measured in cells with a 1 cm light path at 400 nm. One unit (U) is defined as the hydrolysis of 1 μmol of *p*NPβGal/min under the above conditions. Protein concentrations were determined by the method of Bradford.[11]



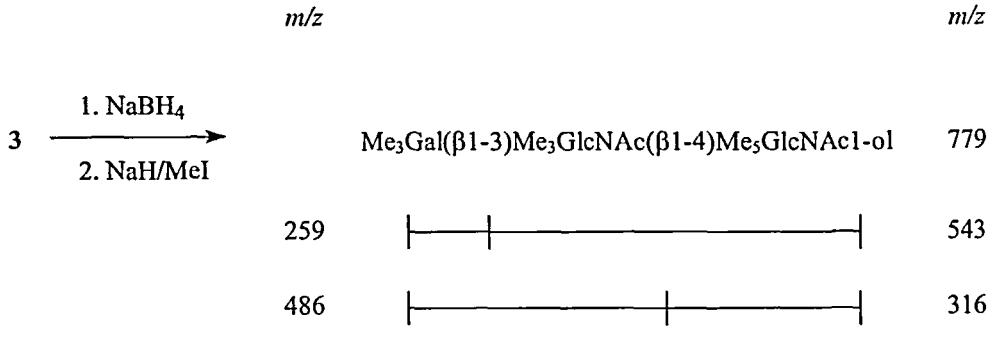


Figure 2a. ESI-MS/MS analysis: CID spectrum of permethylated alditol obtained from **3**. *m/z* of Na-adduct cation given; fragmentation pattern indicated.

Purification procedure of β-galactosidase. All manipulations were performed at 0 - 4 °C unless otherwise stated. Bovine testes (210 g) were obtained from a slaughterhouse and stored at –20 °C until use. The testes were thawed and homogenised with a blender. 0.1 M Acetic acid (210 mL) was added, and the pH was adjusted to 4.0 by dropwise addition of 2 M HCl. The homogenate was stirred and centrifuged for 20 min at 10,000 g. Ammonium sulfate was added to the crude extract to 40% saturation,

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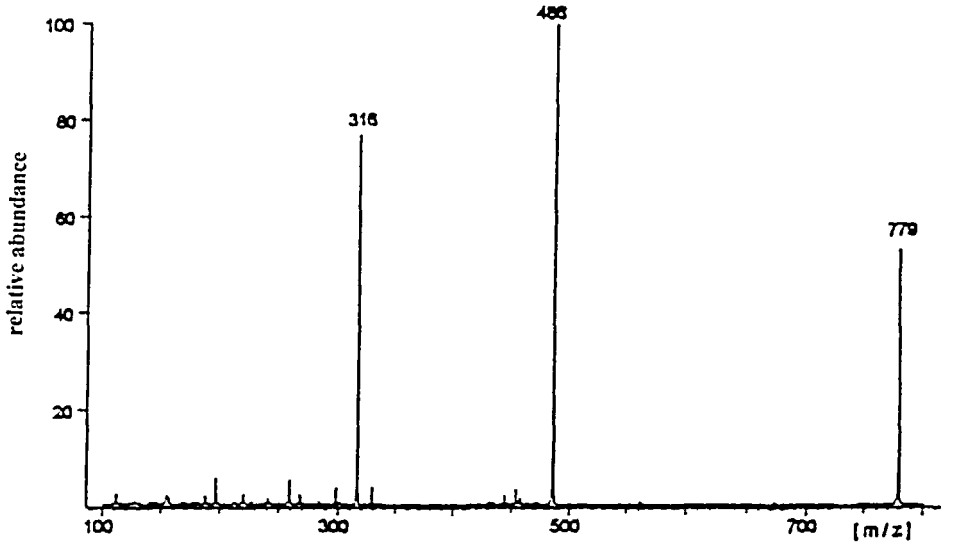

m/z *m/z*

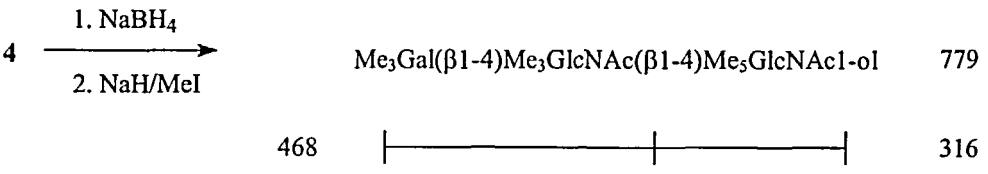

Figure 2b. ESI-MS/MS analysis: CID spectrum of permethylated alditol obtained from **4**. *m/z* of Na-adduct cation given; fragmentation pattern indicated.

and after stirring for 1 h, the precipitate was collected by centrifugation for 20 min at 10,000 g and dissolved in acetate buffer, pH 4.3. The solution was incubated at 50 °C for 15 min before centrifugation for 10 min at 20,000 g. The supernatant solution was dialysed against acetate buffer, pH 4.3 overnight and lyophilised. The specific activity was 30 mU/mg, and the total activity was 31 U.

High-pH anion-exchange chromatography analysis (HPAEC-PAD). A Dionex BioLC System (Dionex, Sunnyvale, CA, USA) equipped with a CarboPac 1 column (4 × 250 mm) was used in combination with pulsed amperometric detector (detector potentials E-1, E-2, E-3 were + 0.05, + 0.60 and -0.60 V; t-1 = 480 ms, t-2 = 120 ms, t-3 = 60 ms; output range = 300 nA). The oligosaccharide mixtures were injected

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onto the column after desalting on a Biogel P4 column. Elution was performed applying a linear gradient from 0-20 % 0.1 M NaOH over 40 min and to 100 % 0.1 M NaOH containing 0.6 M sodium acetate within 10 min; the flow rate was 1 mL/min.

Methylation analysis. For methylation analysis, the compounds were permethylated,[12] purified, hydrolysed, reduced and peracetylated according to literature.[13]

ESI-MS/MS analysis. The reduced and permethylated samples were dissolved in acetonitrile saturated with NaCl and injected at a flow rate of 1 μL/min into the electrospray chamber (Finnigan MAT TSQ 700 triple quadrupole mass spectrometer equipped with a Finnigan electrospray ion source). For CID experiments, parent ions were selectively transmitted by the first mass analyzer and directed into the collision cell.

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Transgalactosylation. The acceptor substrates (**1** or **2**) (1 equiv, 150 mM) and *p*-nitrophenyl β-D-galactopyranoside (1.5 equiv, 225 mM) were dissolved in 50 mM sodium phosphate-citrate buffer (pH 4.3). The reaction mixture was incubated with β-galactosidase from bovine testes (2 U/ mmol donor) at 37 °C for 48 h. The reaction was terminated by heating to 90 °C for 5 min. The desired product was isolated from a Biogel P2 column with water.

3′-*O*-(β-D-Galactopyranosyl)-di-*N*-acetylchitobiose (3) and 4′-*O*-(β-D-Galactopyranosyl)-di-*N*-acetylchitobiose (4). Di-*N*-acetylchitobiose (**1**, 50 mg, 0.118 mmol) was incubated following the general transgalactosylation method to give a 1:1 mixture of **3** and **4** (16 mg, 0.027 mmol); di-*N*-acetylchitobiose (**1**, 9 mg, 0.021 mmol) was reisolated; based on the reacted disaccharide the yield was 28 %; ^{1}H NMR (400 MHz, D_2O) δ 5.16 (d, 0.54 H, $J_{1\alpha,2}$ = 2.5 Hz, H-1α), 4.67 (d, 0.46 H, $J_{1\beta,2}$ = 8.1 Hz, H-1β), 4.53 (d, 1 H, $J_{1',2'}$ = 7.6 Hz, H-1′), 4.44 (d, 0.5 H, $J_{1'',2''}$ = 8.1 Hz, H-1′′), 4.41 (d, 0.5 H, H-1′′), 2.04 (s, 1.5 H, Ac′), 2.03 (s, 1.5 H, Ac′), 2.01 (s, 3 H, Ac); ^{13}C NMR (125.77 MHz, D_2O): 104.1 and 103.5 (C-1′′), 101.9 and 101.7 (C-1′), 95.2 (C-1β), 90.8 (C-1α); $C_{22}H_{38}N_2O_{16}$ (586.22), FAB *m/z* 587. Separation of approximately 10 μg of the product **3** and **4** for structural analysis was performed applying HPAEC-PAD analysis. Reduction and permethylation of **3** and **4** gave the corresponding alditols with $C_{34}H_{64}N_2O_{16}$ (756.43); ESI-MS/MS showed *m/z* 779 [M(red/per)+Na]$^+$.

3′′-*O*-β-Galactopyranosyl)-tri-*N*-acetylchitotriose (5) and 4′′-*O*-(β-D-Galactopyranosyl)-tri-*N*-acetylchitotriose (6). Tri-*N*-acetylchitotriose (**2**, 20 mg,

0.032 mmol) was incubated following the general transgalactosylation method to give a 1:1.4 mixture of **5** and **6** (5 mg, 6.33 μmol); tri-*N*-acetylchitotriose (**2**, 4 mg, 6.37 μmol) was reisolated; based on the reacted trisaccharide the yield was 25 %; ^{1}H NMR (400 MHz, D_2O) δ 5.10 (d, 0.6 H, $J_{1\alpha,2}$ = 2.0 Hz, H-1α), 4.61 (d, 0.4 H, $J_{1\beta,2}$ = 8.1 Hz, H-1β), 4.56-4.49 (m, 2 H, H-1′, H-1′′), 4.38 (d, 0.5 H, $J_{1'''',2''''}$ = 8.1 Hz, H-1′′′), 4.36 (d, 0.5 H, $J_{1'''',2''''}$ = 7.6 Hz, H-1′′′), 1.98-1.96 (m, 9 H, Ac); ^{13}C NMR (125.77 MHz, D_2O): 103.9 and 103.1 (C-1′′′), 101.7 (C-1′, C-1′′), 95.3 (C-1β), 90.9 (C-1α); $C_{30}H_{51}N_3O_{21}$ (789.74), FAB *m/z* 790. Separation of approximately 10 μg of the products **5** and **6** for structural analysis was performed applying HPAEC-PAD analysis. Reduction and permethylation of **5** and **6** gave the corresponding alditols with $C_{45}H_{83}N_3O_{21}$ (1001.55); ESI-MS/MS gave *m/z* 1024 $[M(red/per)+Na]^+$.

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REFERENCES

1. Reported in part at the First EuroConference on Carbohydrates in Drug Research (ECCDR1) Stintino, Sardinia, Italy 9/99.
2. C. Breton, R. Oriol and A. Imberty, *Glycobiology*, **8**, 87 (1998).
3. J. Thiem, *FEMS Microbiol. Rev.*, **16**, 193 (1995).
4. U. Gambert and J. Thiem, *Carbohydr. Res.*, **299**, 85 (1997).
5. L. Hedbys, E. Johansson, K. Mosbach and P.-O. Larsson, *Carbohydr. Res.*, **186**, 217 (1989).
6. J. J. Distler and G. W. Jourdian, *J. Biol. Chem.*, **248**, 6772 (1973).
7. A. Alessandrini, E. Schmidt and F. Zilliken and P.György, *J. Biol. Chem.*, **220**, 71 (1956).
8. H. Egge and J. Katalinic, *J. Mass Spectrom. Rev.*, 331 (1987).
9. M. Nimtz, E. Grabenhorst, U. Gambert, J. Costa, V. Wray, M. Morr, J. Thiem and H. S. Conradt, *Glycoconjugate J.*, **15**, 873 (1998).
10. S. A. Kuby and H. A. Lardy, *J. Am. Chem. Soc.*, **75**, 890 (1973).
11. M. Bradford, *Anal. Biochem.*, **72**, 248 (1976).
12. S. Hakamori, *J. Biochem.*, **55**, 205 (1964).
13. M. Nimtz, G. Noll, E.-P. Pâques and H.S. Conradt, *FEBS Lett.*, **271**, 14 (1990).