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Enzyme-aided construction of medium-sized alditols of complete O-linked saccharides

The constructed hexasaccharide alditol Galβ1–4GlcNAcβ1–6Galβ1–4GlcNAcβ1–6(Galβ1–3)GalNAc-ol resists the action of endo-β-galactosidase from *Bacteroides fragilis*

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Abstract

We have constructed by enzyme-aided in vitro synthesis a hexasaccharide alditol Galβ1–4GlcNAcβ1–6Galβ1–4GlcNAcβ1–6(Galβ1–3)GalNAc-ol and shown that it resists the action of endo-β-galactosidase from *Bacteroides fragilis* under conditions where a related pentasaccharide alditol, GlcNAcβ1–3Galβ1–4GlcNAcβ1–6(Galβ1–3)GalNAc-ol, was completely cleaved. Together with earlier results from this laboratory, our present data imply that endo-β-galactosidase from *B. fragilis*, apparently, can be used to distinguish between GlcNAcβ1–6Gal and GlcNAcβ1–3Gal units within linear backbone sequences of all known types of oligo-(*N*-acetyllactosamino)glycans.

Key words: Endo-β-galactosidase; O-Linked saccharide; GlcNAcβ1–6Gal; In vitro synthesis; NMR; HPAEC

1. Introduction

Endo-β-galactosidase from *Bacteroides fragilis* cleaves linear GlcNAcβ1–3Galβ1–4GlcNAc/Glc sequences at the internal galactosidic linkages [1,2], but fails to hydrolyse branched structures of the type GlcNAcβ1–3(GlcNAcβ1–6)Galβ1–4GlcNAc [2,3], as well as linear GlcNAcβ1–6Galβ1–4GlcNAc/Glc sequences [4].

On the other hand, Hanisch et al. isolated from human skimmed milk mucins a mixture of oligosaccharide alditols with the hexasacharide alditol Galβ1–4GlcNAcβ1–6Galβ1–4GlcNAcβ1–6(Galβ1–3)GalNAc-ol (**9**, see Table 1 for saccharide numbers) as one of the major components [5]. When this mixture was treated with the *B. fragilis* enzyme, a considerable fraction of the alditols was cleaved. Although it was not quite clear from the results whether the hexasaccharide alditol **9** was also cleaved, the notion was generated that linear Rβ1–4GlcNAcβ1–6Galβ1–4GlcNAc sequences bound to the O-linked core 2 structure may be cleavable.

In the present study we describe the enzyme-aided in vitro synthesis and endo-β-galactosidase treatment of the hexasaccharide alditol Galβ1–4-GlcNAcβ1–6Galβ1–4GlcNAcβ1–6(Galβ1–3)GalNAc-ol (**9**). The structure of each saccharide on the synthesis route was confirmed by NMR. The constructed alditol was not cleaved by the endo-β-galactosidase from *B. fragilis*.

2. Materials and methods

2.1. Materials

Galβ1–3GalNAc, galactose (Gal), lactose (Lact), maltotriose (MT), maltotetraose (Mtet), maltopentaose (MP), UDP-GlcNAc and UDP-Gal were purchased from Sigma, St. Louis, MO, USA.

UDP-[6-^{3}H]GlcNAc and UDP-[6-^{3}H]Gal were from Amersham, Buckinghamshire, UK.

2.2. Enzymic methods

The β(1,6)-*N*-acetylglucosaminyltransferase (EC 2.4.1.148) reactions were carried out by incubating the saccharides with hog gastric microsomes as previously described [6].

The galactosylation with bovine milk β(1,4)-galactosyltransferase (EC 2.4.1.90) (Sigma) was carried out essentially as described [7].

The partial reaction with bovine milk β(1,4)-galactosyltransferase was carried out as follows: 750 nmol of substrate was incubated for 6 min at 37°C with 3 μmol of UDP-Gal and 50 mU of the enzyme in 100 μl of 50 mM Tris-HCl, pH 7.5, containing 10 mM $MnCl_2$. The reaction was terminated by adding 200 μl of ethanol.

The β(1,3)-*N*-acetylglucosaminyltransferase (EC 2.4.1.149) reactions with human serum were carried out essentially as described [6].

Degradation with β-*N*-acetylhexosaminidase from jack bean

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Abbreviations: Gal, D-galactose; GlcNAc, *N*-acetyl-D-glucosamine; GalNAc, *N*-acetyl-D-galactosamine; NMR, nuclear magnetic resonance spectroscopy; WEFT, water eliminated fourier transformation; DQFCOSY, double quantum filtered correlation spectroscopy; DEPT, distortionless enhancement by polarization transfer; HPAEC, high-pH anion-exchange chromatography; PAD, pulsed amperometric detection.

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(EC 3.2.1.30) (Sigma) was carried out as described [8] with the incubation time of 16 h.

Treatment with endo-β-galactosidase (EC 3.2.1.103) from *Bacteroides fragilis* (Boehringer, Mannheim, Germany) was carried out as follows: the saccharides were incubated for 24 h with 20 mU of the enzyme in 100 μl of 50 mM sodium acetate buffer, pH 5.8, containing 0.2 mg·ml^{-1} bovine serum albumin and 0.5 mg·ml^{-1} NaN_3.

All enzyme reactions, except the partial reactions, were terminated by heating in a boiling water bath for 5 min and the reaction mixtures were desalted by passing through a double bed of Dovex AG 50 W (H^+) and AG 1 (Ac^-) resins.

The reduction of the tetrasaccharide Galβ1–4GlcNAcβ1–6(Galβ1–3)GalNAc was carried out with $NaBH_4$ essentially as described [9]. The completeness of the reaction was controlled by subjecting the borate-free saccharide to ^{1}H NMR.

2.3. Chromatographic methods

Paper chromatography was carried out on Whatman III Chr paper with an upper phase of butanol/acetic acid/water 4:1:5 by vol. Unlabeled markers Gal, Lact, MT, Mtet and MP (Sigma) were stained by alkaline silver nitrate.

Gel permeation chromatography on a column of Bio-Gel P-2 (Bio-Rad. Richmond, CA, USA) (1 × 144 cm) or Bio-Gel P-4 (1 × 145 cm) was carried out with 0.02% aqueous NaN_3. Fractions of 1 ml were collected.

Gel-permeation chromatography on a column of Superdex 75 HR (10 × 300 mm) (Pharmacia, Sweden) was run with water at 1 ml/min using a 2150 HPLC Pump (Pharmacia). The eluent was monitored at 214 nm and the oligosaccharides were quantified against external GlcNAc.

High-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was carried out with a Dionex Series 4500i HPLC system (Dionex, CA, USA) equipped with a CarboPac PA-1 column (4 × 250 mm) preceded by a CarboPac PA-1 guard column, at a flow rate of 1 ml/min at ambient temperature. The column was equilibrated with 40 mM or 60 mM NaOH and the isocratic run was carried out with the same eluent. The eluting saccharides were collected in tubes containing 0.5 ml of 0.4 M acetic acid for immediate neutralization of the eluent. Salts were removed by passing through Dowex AG 50 (H^+) and lyophilization. Due to limited capacity of the column, the samples were chromatographed in several batches containing no more than 150 nmol of saccharides.

2.4. ^{1}H and ^{13}C NMR spectroscopy

Prior to NMR experiments the saccharides were twice dissolved in ^2H_2O and evaporated to dryness. The samples were then dissolved in 600 μl of ^2H_2O (99.996 atom %; Cambridge Isotope Laboratories, Woburn, MA, USA) and the NMR experiments were carried out on a Varian Unity 500 spectrometer at 300 K (for **9** and **10** at 295 K). In recording 1D proton spectra a modified WEFT sequence [10] was used for water suppression. DQFCOSY [11] was used for assignment of overlapping signals.

For DEPT(135) spectra of ^{13}C resonances 45 k points were acquired with a spectral width of 15,000 Hz. The proton–carbon couplings were decoupled during the acquisition period. HMQC spectra [12] were used to assign the ^{13}C resonances of interest.

The ^{1}H and ^{13}C chemical shifts were referenced to internal acetone (2.225 and 31.55 ppm, respectively).

3. Results

3.1. Construction of the hexasaccharide alditol Galβ1–4GlcNAcβ1–6Galβ1–4GlcNAcβ1–6(Galβ1–3)GalNAc-ol

Starting from commercially available disaccharide Galβ1–3GalNAc **1** (see Table 1 for saccharide numbers) the tetrasaccharide alditol Galβ1–4GlcNAcβ1–6(Galβ1–3)GalNAc-ol (**4**) was constructed by successive use of β1,6 GlcNAc transferase of hog gastric mucosal microsomes, bovine milk β1,4 Gal transferase and reduction with $NaBH_4$. The products of all the enzyme reactions were isolated by gel-filtration on a column of Bio-Gel P-2 (data not shown) and characterized by NMR (Table 2). The Galβ1–4GlcNAcβ1–6 branch of **4** was further elongated by human serum β1,3 GlcNAc transferase to GlcNAcβ1–3Galβ1–4GlcNAcβ1–6(Galβ1–3)GalNAc-ol (**5**). The location of the incoming GlcNAc was established by observing that the ^{1}H chemical shifts of Galβ1–3 were unaffected by the substitution, but those of Galβ1–4 showed the displacement of the H-4 signal to the lower field as well as other changes characteristic of the substitution of position 3 by a GlcNAc residue (Table 2) [13,14].

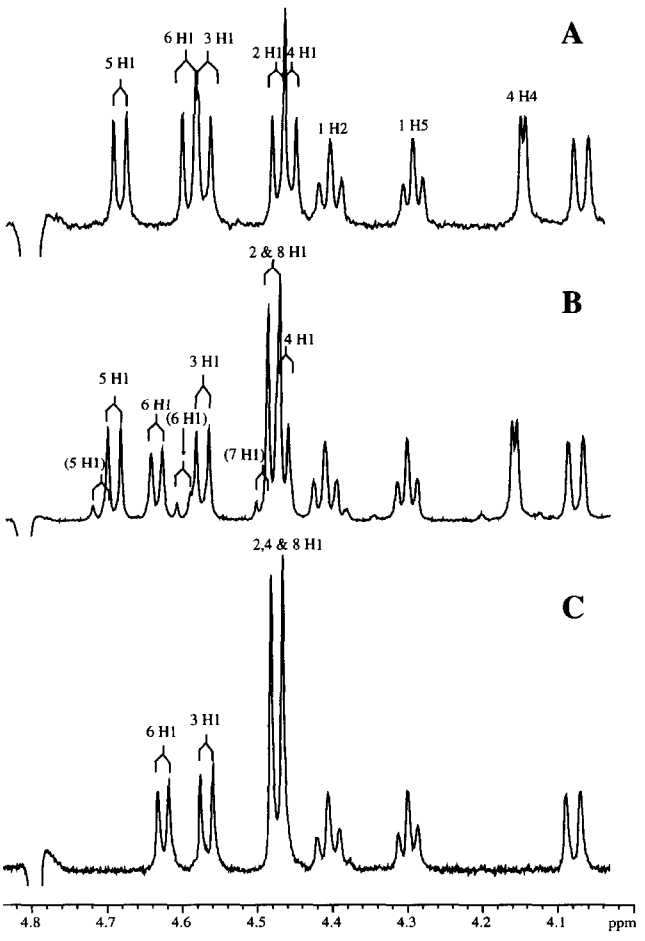
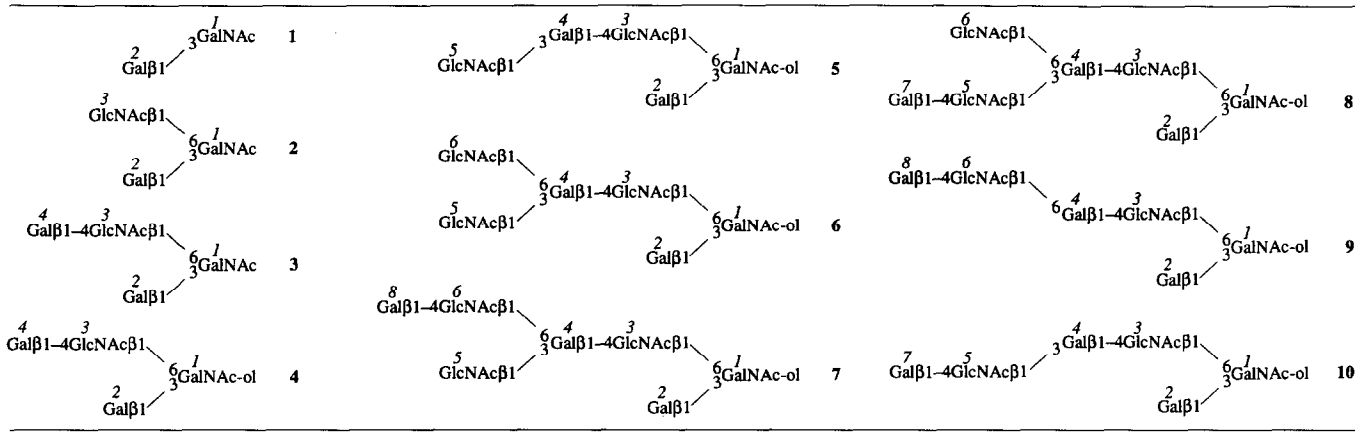


Fig. 1. Anomeric regions of ^{1}HNMR spectra of (A) saccharide **6**. (B) Products of mono-β1,4-galactosylation of saccharide **6** (saccharides **7** and **8**). Peak labels refering to the minor product of the reaction (saccharide **8**) are shown in parenthesis. (C) Saccharide **9**. See Table 1 for saccharide structures and denotation system.

The pentasaccharide alditol **5** was then incubated with UDP-GlcNAc and β1,6 GlcNAc transferase of hog gastric microsomes. The hexasaccharide alditol GlcNAcβ1–6(GlcNAcβ1–3)Galβ1–4GlcNAcβ1–6(Galβ1–3)-GalNAc-ol (**6**) formed was separated from the unreacted pentasaccharide alditol by gel-filtration on a column of

Table 1
Structures of the saccharides and denotation of monosaccharide residues



Bio-Gel P-4 (not shown). The newly formed linkage was confirmed to be GlcNAcβ1–6 by proton NMR (Table 2 and Fig. 1A) and by comparing the ^{13}C DEPT(135) NMR spectra of **5** and **6** (Fig. 2). In DEPT(135) spectrum the signals of C-6 of every monosaccharide unit and C-1 of GalNAc-ol appear as negative peaks. In the spectrum of **5** five overlapping negative signals are seen around 62 ppm. These are the carbons carrying two protons and an unsubstituted hydroxyl group (C-6 of residues 2–5 and C-1 of residue 1; see Table 1 for denotation system). Substitution of C-6 causes displacement of the signal to a lower field, near 69–72 ppm [15]. Only one negative signal (C-6 of residue 1, 72.0 ppm) is found on this area in the spectrum of **5**, but in the spectrum of **6** a new negative signal (C-6 of 4) has appeared at 70.1 ppm. Whitfield et al. have reported a similar chemical shift for the C-6 of galactose substituted by β1,6-GlcNAc [16].

The branched hexasaccharide alditol **6** was then subjected to a partial reaction with bovine milk β(1,4)-galactosyltransferase. The non-, mono- and digalactosylated saccharides formed in the reaction were separated by HPAEC in an isocratic run with 60 mM NaOH (Fig. 3A, peaks 1, 2 and 3, respectively). The two monogalactosylated isomers **7** and **8**, however, were unseparable, but were distinguished in the proton NMR spectrum (Fig. 1B). After exhaustive β-*N*-acetylhexosaminidase treatment a mixture of isomeric hexasaccharide alditols **9** and **10** was obtained, that could be separated by HPAEC in an isocratic run with 40 mM NaOH (Fig. 3B). The GlcNAc released in the reaction (peak 3) was then removed by passing the pool of peaks 2 and 3 through the

Table 2
^1H chemical shifts of structural reporter groups of saccharides **1**–**10** at 300 K

Reporter group	Residue	Saccharides									
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**[a]	**10**[a]
H-1	1	5.216 (α)	5.197 (α)	5.196 (α)							
		4.693 (β)	4.664 (β)	4.664 (β)	n.d.[b]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	2	4.493/4.437	4.489/4.431	4.488/4.431	4.466	4.464	4.466	4.465	4.465	4.466	4.464
	3	–	4.570/4.564	4.593/4.587	4.561	4.558	4.563	4.559	4.559	4.562	4.555
	4	–	–	4.472	4.471	4.458	4.450	4.453	4.453	4.466	4.457
	5	–	–	–	–	4.684	4.679	4.677	4.695	–	4.702
	6	–	–	–	–	–	4.585	4.621	4.584	4.620	–
	7	–	–	–	–	–	–	–	4.480	–	4.480
	8	–	–	–	–	–	–	4.465	–	4.466	–
H-2	1	4.277 (α)	4.286 (α)	4.287 (α)							
		4.989 (β)	3.972 (β)	3.974 (β)	4.392	4.393	4.393	4.396	4.396	4.396	4.396
H-4	1	4.248 (α)	4.214 (α)	4.215 (α)							
		4.181 (β)	4.148 (β)	4.147 (β)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	2	3.912	3.914/3.907	3.912/3.907	3.902	3.901	3.903	n.d.	n.d.	3.904	n.d.
	4	–	–	3.929	3.927	4.150	4.138	4.144	4.144	n.d.	4.155
H-5	1	4.140 (α)	4.011 (α)	4.233 (α)	4.281	4.280	4.284	4.287	4.287	4.288	4.282

For saccharide structure and denotation see Table 1.
[a] Chemical shifts measured at 295 K; [b] n.d., not determined.

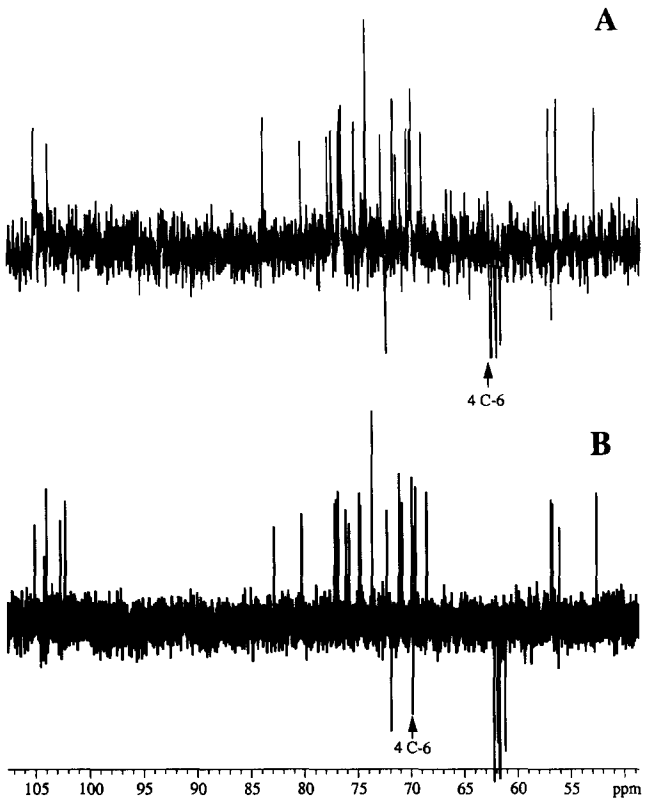


Fig. 2. (A) DEPT(135) spectrum of pentasaccharide alditol **5**. (B) DEPT(135) spectrum of hexasaccharide alditol **6**. The C-6 signal of galactose 4 has shifted to a lower field due to substitution of the carbon by a GlcNAc residue.

Superdex column. By NMR analysis (Fig. 1C and Table 2) the peaks in Fig. 3B were identified to as Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAcβ1–6(Galβ1–3)GalNAc-ol (**10**) (peak 1, 29 nmol), Galβ1–4GlcNAcβ1–6Galβ1–4GlcNAcβ1–6(Galβ1–3)GalNAc-ol (**9**) (peak 2, 151 nmol) and GlcNAc (peak 3).

3.2. *Treatment of Galβ1–4GlcNAcβ1–6Galβ1–4 [6-^{3}H]GlcNAcβ1–6(Galβ1–3)GalNAc-ol (9) with* Bacteroides fragilis *endo*-β-*galactosidase*

Using the conditions of Hanisch et al. [5], 90 nmol of the hexasaccharide alditol **9** was incubated for 24 h with 20 mU of endo-β-galactosidase from *B. fragilis*. The digest was subsequently analysed by paper chromatography (Fig. 4A), where only the unchanged substrate was detected, indicating that hexasaccharide alditol **9** is completely resistant to the action of the enzyme. Under the conditions used the related pentasaccharide alditol GlcNAcβ1–3[6-^{3}H]Galβ1–4GlcNAcβ1–6(Galβ1–3)GalNAc-ol (**5**) was completely cleaved (Fig. 4B).

4. Discussion

The present data show that hexasaccharide alditol Galβ1–4GlcNAcβ1–6Galβ1–4GlcNAcβ1–6(Galβ1–3)-GalNAc-ol (**9**) is not cleaved by endo-β-galactosidase from *B. fragilis*. This supports our previous data on other oligo-(*N*-acetyllactosamino)glycans [4,17]. We conclude that endo-β-galactosidase from *B. fragilis* is unable to cleave the galactosidic linkages in GlcNAcβ1–6Galβ1–4GlcNAc sequences. It is noteworthy that 6-sulfation of the galactose residue also inhibits the cleavage [1].

In constructing the alditol **9** the elongation of the tetrasaccharide alditol **4** was carried out by human serum β1,3 GlcNAc transferase. Our data show that this enzyme adds the GlcNAc specifically to the 1–6-linked arm of Galβ1–4GlcNAcβ1–6(Galβ1–3)GalNAc-ol. In contrast, Brockhausen et al. have found in hog gastric mucosal microsomes an elongation enzyme which links GlcNAc to Galβ1–3 of the core 1 (or core 2) galactose [18,19].

In constructing the linear GlcNAcβ1–6Gal sequence of alditol **9**, we made use of the branch specificity of bovine milk β(1,4)-galactosyltransferase. Previous work from this and other laboratories have shown that GlcNAcβ1–6(GlcNAcβ1–3)Gal-R, where R is H, GlcNAc, Glc or Glc-OMe, react preferentially at the 1–6-linked arm [8,20,21]. The same was true in the present experiments with GlcNAcβ1–3(GlcNAcβ1–6)Galβ1–4GlcNAcβ 1–6(Galβ1–3)GalNAc-ol (**6**).

Although HPAE chromatography has been used for separation of reduced neutral saccharides only in a few cases [22], the method proved to be very efficient in separating alditols of different size as well as the isomeric hexasaccharide alditols **9** and **10**.

The ^{1}H NMR chemical shifts (Table 2) of the struc-

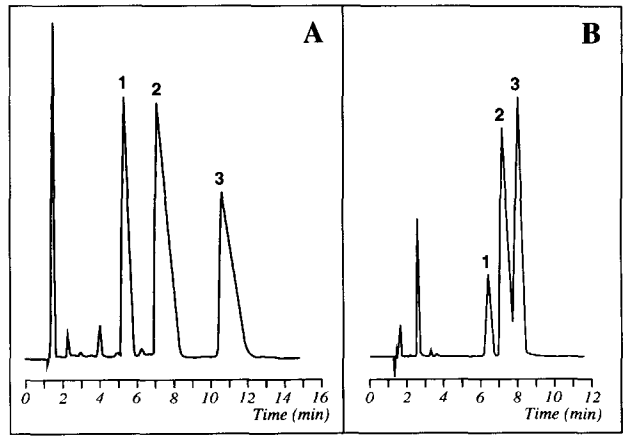


Fig. 3. (A) Separation by HPAEC of saccharides resulting from partial galactosylation of saccharide **6** by bovine milk β1,4-galactosyltransferase: peak 1 is the non-galactosylated saccharide **6**, peak 2 is the monogalactosylated product (mixture of saccharides **7** and **8**), and peak 3 is the digalactosylated product. (B) Separation by HPAEC of saccharides **9** and **10** (peaks 1 and 2, respectively) resulting from β-*N*-acetylhexosaminidase digestion of peak 2 in A. Peak 3 is GlcNAc liberated in the reaction.

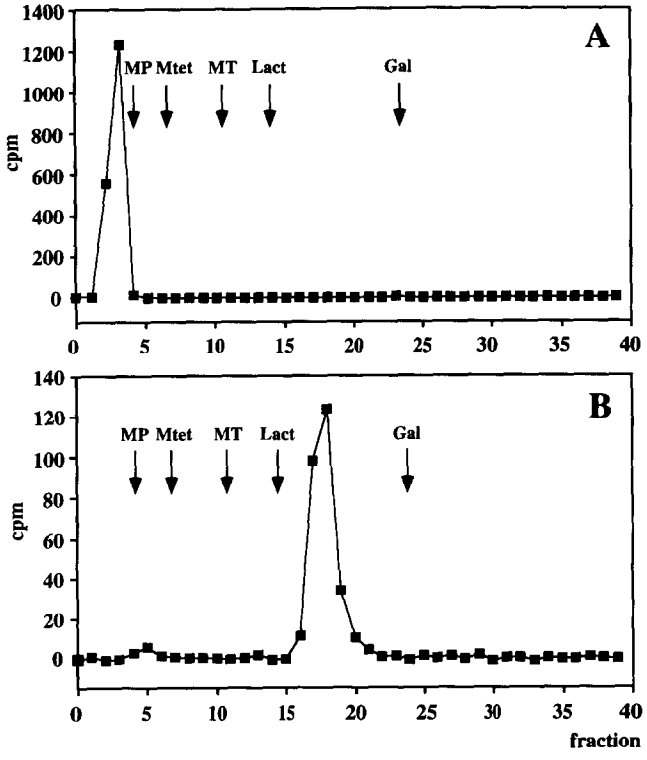


Fig. 4. Paper chromatographic analysis of *B. fragilis* endo-β-galactosidase digests of (A) Galβ1–4GlcNAcβ1–6Galβ1–4GlcNAcβ1–6(Galβ1–3)GalNAc-ol (**9**) and (B) GlcNAcβ1–3Galβ1–4GlcNAcβ1–6(Galβ1–3)GalNAc-ol (**5**)

tural reporter groups of the constructed tetrasaccharide alditol **4** were similar to those determined by a number of groups for the same structure [23–26]. The chemical shifts of larger alditols **5–10** were compared to NMR data of related alditols [24,27,28] and to those of oligo-(*N*-acetyllactosamino)glycans related to the distal end of the molecules studied [14,29]. The chemical shifts of the Galβ1–4GlcNAcβ1–6Gal sequence of the hexasaccharide alditol **9** were also compared to the spectrum of Galβ1–4GlcNAcβ1–6Galβ1–4GlcNAc (Maaheimo et al., unpublished data). In all cases the data obtained was in good agreement with the reference values.

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