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STRUCTURE OF A NEW HEXASACCHARIDE FROM THE COAGGREGATION POLYSACCHARIDE OF *Streptococcus sanguis* 34

FLOYD C. MCINTIRE*,
Department of Diagnostic and Biological Sciences, School of Dentistry, University of Colorado Health Sciences Center, 4200 East Ninth Avenue, Box C285, Denver, Colorado 80262 (U.S.A.)

C. ALLEN BUSH, SHING-SHING WU,
Department of Chemistry, Illinois Institute of Technology, Chicago, Illinois 60616 (U.S.A.)

SU-CHEN LI, YU-TEH LI,
Department of Biochemistry, Tulane University, School of Medicine, New Orleans, Louisiana 70112 (U.S.A.)

MICHAEL MCNEIL,
Department of Microbiology, Colorado State University, Fort Collins, Colorado 80523 (U.S.A.)

SUSAN S. TJOA, AND PAUL V. FENNESSEY
NIH Clinical Mass Spectrometry Resource, University of Colorado Health Sciences Center, Denver, Colorado 80262 (U.S.A.)

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ABSTRACT

The major constituent of a coaggregation polysaccharide from *Streptococcus sanguis* 34 is a hexasaccharide, isolated as the alditol. The proposed structure is α-D-Gal*p*NAc-(1→3)-β-L-Rha*p*-(1→4)-β-D-Glc*p*-(1→6)-β-D-Gal*f*-(1→6)-β-D-Gal*p*NAc-(1→3)-D-Galol, based upon g.l.c.–m.s. of alditol acetates and partially methylated alditol acetates, f.a.b.–m.s., ^{1}H-n.m.r. spectroscopy, g.l.c.–m.s. of trimethylsilylated (+)- and (−)-2-butyl glycosides, and cleavage by α-*N*-acetylgalactosaminidase. The structural deduction was facilitated by cleavage of the hexasaccharide at the furanoside linkage by 48% hydrogen fluoride, and reduction of the product, to yield α-D-Gal*p*NAc-(1→3)-β-L-Rha*p*-(1→4)-β-D-Glc*p*-(1→6)-D-Galol.

INTRODUCTION

There are many specific, adherence interactions between different bacterial species in dental plaque[1], many of which appear to involve cell-associated lectins on one organism interacting with complementary structures on another[2–6]. The

*To whom correspondence should be addressed.

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coaggregation of *Actinomyces viscosus* T14V (T14V) with *Streptococcus sanguis (mitis)* 34 (Ss 34) depends upon a lectin on T14V interacting with[7-9] a specific carbohydrate on Ss 34, and it is inhibited by β-galactosides and β-*N*-acetylgalactosaminides. The specific carbohydrate has been isolated from cell walls of Ss 34; it is a polysaccharide composed of one major and two very minor oligosaccharides joined by phosphoric diester bridges[6,10].

The complete structure of the major oligosaccharide-alditol (OL-6) is the subject of this article. These studies were facilitated by the availability of a tetrasaccharide-alditol (OL-4) which was produced by the action of 48% hydrogen fluoride on the parent polysaccharide and on OL-6, followed by reduction[10].

RESULTS AND DISCUSSION

The alditol acetates from OL-6 gave, on g.l.c. analysis, 4 peaks, identified by relative-retention indices and by m.s. The peaks were compared with those given by a library of alditol acetates prepared from authentic sugars. Thus, OL-6 was found to contain 1 Rha, 1 Glc, 2 Gal, and 2 GalNAc; OL-4 was shown to contain the same sugars in the ratios of ~1:1:1:1. The determination of absolute configurations[11] gave the L configuration for Rha, and the D configuration for the other sugars.

When OL-6 containing hydrogen on C-1 of the terminal alditol unit was carried through the alditol acetate analysis in which sodium borodeuteride was used for the reduction following hydrolysis, the m.s. clearly indicated that ~50% of the Galol peak contained deuterium. This observation established one Galol at the alditol terminal of OL-6. When OL-4 having deuterium on C-1 of the alditol residue was carried through the alditol acetate analysis with sodium borohydride reduction after hydrolysis, only the Galol contained deuterium. This showed conclusively that Galol was also at the alditol terminal of OL-4. As the difference between OL-6 and OL-4 is one Gal and one GalNAc unit, these observations established the partial sequence of –Gal–GalNAc–Galol for OL-6.

The f.a.b.-m.s. indicated clearly that OL-6 is a hexasaccharide-alditol and also revealed the positions of Rha and both GalNAc units in the sequence. The f.a.b.-m.s. of the OL-6 sample gave two major ions in the high-mass region, at *m/z* 1059 and 1081. These ions are consistent with the molecular ion + (H^+) and + (Na^+), respectively, of a hexasaccharide-alditol containing 2 GalNAc, 1 Rha, 1 Glc, and 2 Gal units. The nominal molecular weight of a compound having this composition is 1058. More-extensive sequence-information was obtained from f.a.b.-m.s. analysis, shown in Fig. 1, of the peracetylated derivative of OL-6, which showed an $(M + H)^+$ of 1815 and two series of ions, *i.e.*, *m/z* 330, 560, 848, and 1423, and 375, 662, and 950. These represent sequential fragmentation from the nonreducing and reducing ends, respectively, and can be depicted as follows.

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      330   560   848          1423
GalNAc—Rha—Hex—Hex—GalNAc—hexitol
                  950  662      375
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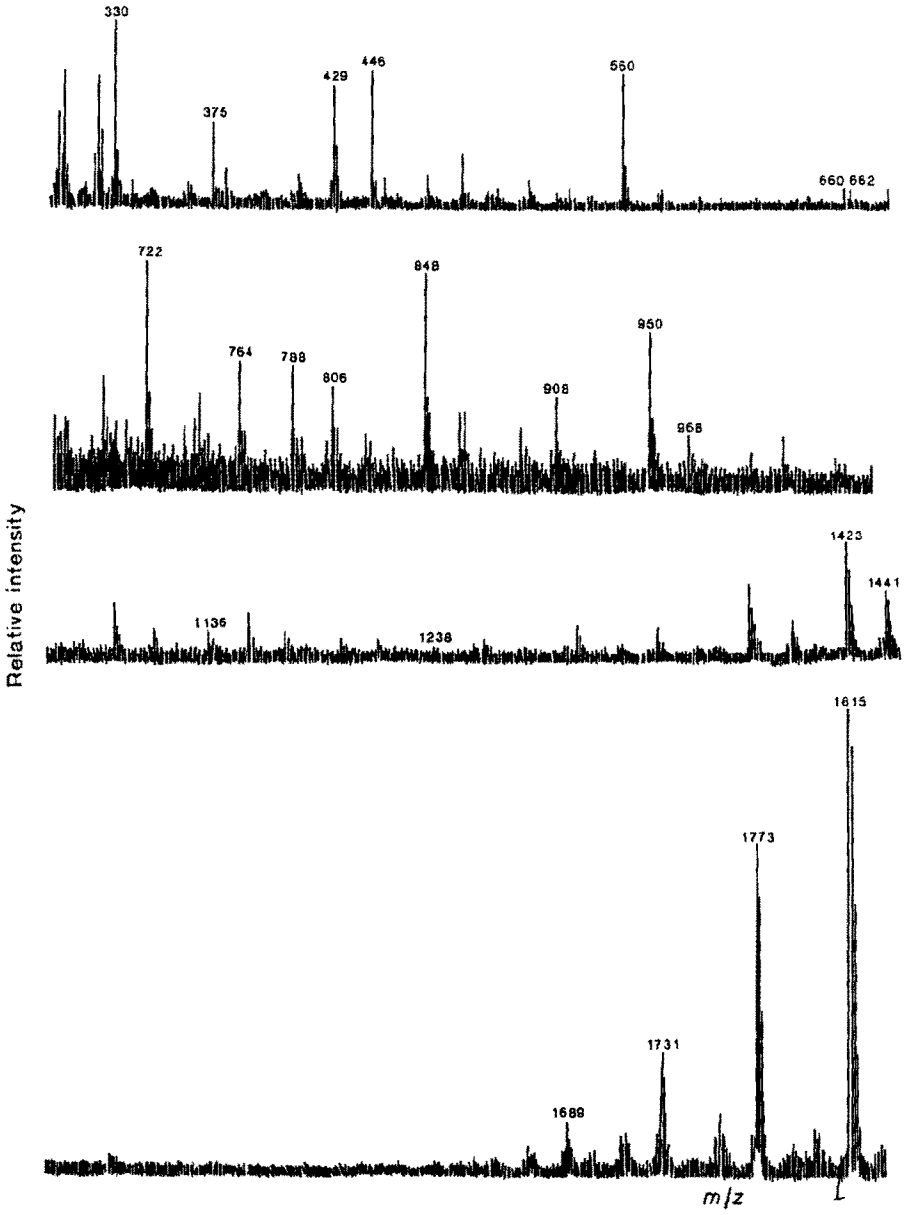

Fig. 1. Fast-atom-bombardment mass spectrum of peracetylated OL-6.

The combination of these two series of ions provided the sequence for OL-6, but they do not distinguish between hexose isomers (Glc *vs.* Gal). The f.a.b.-m.s. of underivatized OL-4 showed that this compound contains the four sugars from the nonreducing end of OL-6; it had $(M + H)^+$ and $(M + Na)^+$ ions at *m/z* 694 and 716, respectively.

When the sequence information from f.a.b.-m.s. and alditol acetate analyses was brought together, there was only one possible position for Glc. Thus, the complete sequence was established as GalNAc–Rha–Glc–Gal–GalNAc–Galol.

The g.l.c.-m.s. analysis of the partially methylated alditol acetates of OL-6 and OL-4 not only established points of linkage and most of the ring structures, but also confirmed the positions of both the Gal and the Glc in the sequence. This analysis gave six different derivatives from OL-6 and four from OL-4, shown in Table I. Whenever possible, the relative g.l.c. retention-times and the m.s. of these derivatives were compared with those of the same derivatives prepared from authentic saccharides.

With regard to sequence, the identification of 1,5-di-*O*-acetyl-2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-galactitol confirmed that the nonreducing terminal was GalNAc. The presence of a mono-*O*-acetyl-penta-*O*-methylgalactitol in both OL-6 and OL-4 confirmed that Gal was at the reducing terminal of both oligosaccharides.

To establish the position of linkage to Galol in OL-6, it was necessary to use OL-6 having deuterium at Galol C-1, and authentic reference compounds with deuterium. Without deuterium, 3- and 4-*O*-acetylpenta-*O*-methylgalactitol have identical m.s. and retention-times. With deuterium at C-1, the m.s. of the mono-*O*-acetylpenta-*O*-methylgalactitol from OL-6 was identified with the 3-*O*-acetyl reference compound; it was very different from the 4-*O*-acetyl reference compound.

In the partially methylated alditol acetates from OL-6 (see Table I), the positions of the *O*-acetyl groups indicated the following: both GalNAc units and

TABLE I

RETENTION TIMES OF PARTIALLY METHYLATED ALDITOL ACETATE DERIVATIVES

Alditol derivatives[a]	*Relative retention index*[b]		
	Standards	*OL-6*	*OL-4*
1,2,4,5,6-Gal	—	16.66	
1,2,3,4,5-Gal			16.92
2,4-Rha	17.29	17.31	17.30
2,3,6-Glc	18.45	18.47	18.51
2,3,5-Gal	18.86	18.91	
3,4,6-Gal2NAcMe	20.79	20.78	20.75
3,4-Gal2NAcMe		22.08	

[a]The numbers indicate the positions of the *O*-methyl groups. All other hydroxyl groups of the alditol are acetylated; *e.g.*, 1,2.4,5,6-Gal = 3-*O*-acetyl-1,2,4,5,6-penta-*O*-methylgalactitol. [b]Methylene units.

Rha are pyranosides; the linkage is at O-3 of Rha and O-6 of nonterminal GalNAc; the nonterminal Gal is a furanoside with linkage at O-6. Although a suitable Gal*f* reference compound was not available for direct comparison, the identity of our derivative was confirmed by comparison of its rather unusual mass spectrum with that published[12] for 1,4,6-tri-*O*-acetyl-2,3,5-tri-*O*-methylgalactitol. The Glc could be either a pyranoside with linkage at O-4, or a furanoside with linkage at O-5. The conclusions on ring structure were confirmed, and the ambiguity with regard to Glc was resolved, by the ^{1}H-n.m.r. analysis.

The 300-MHz, ^{1}H-n.m.r. spectrum of OL-6 showed, in the 4.5–5.1-p.p.m. region, five isolated resonances which are characteristic of anomeric proton (H-1) resonances. None of the signals of the "reducing" terminal Galol were readily identified. For each anomeric-proton resonance, the corresponding H-2 signal and many of the other methine proton resonances in the crowded region of 3.6–4.1 p.p.m. could be assigned by difference decoupling (see Table II). In the (upfield) methyl region, the resonances of the two acetamido methyl groups of GalNAc were identified, as well as the methyl group of rhamnose which is coupled to rhamnose H-5 at 3.428 p.p.m. The acetamido protons at δ 8.095 and 8.345 were observed in a separate experiment using H_2O–D_2O as the solvent.

TABLE II

^{1}H-N.M.R. CHEMICAL SHIFTS AND COUPLING CONSTANTS OF OL-6 AND OL-4 OLIGOSACCHARIDE-ALDITOLS IN D_2O AT 70°

Residue	*Chemical shifts ($J_{i,i+1}$) of*[a]							
	H-1	*H-2*	*H-3*	*H-4*	*H-5*	*H_{6a},H_{6b}*	*N–CH_3CO*	*Amide H*[b]
	OL-6 Hexasaccharide							
GalNAc	5.095	4.228	3.991	—	4.194	3.754	2.058	8.095
	(4.0)	(11.0)	(11.0)		(6.5)			(9.0)
Rha	4.832	4.206	3.657	3.46	3.428	1.342		
	(0.5)	(3.0)	(8.8)		(6.0)			
Glc	4.499	3.344	3.651	3.64	3.547	3.813, 3.945		
	(8.0)	(8.8)	(8.8)		(5.8, 3.5)	(−12.5)		
Gal*f*	5.055	4.118	—	—	—	—		
	(1.5)	(3.6)						
GalNAc	4.558	3.893	—	—	—	—	2.071	8.345
	(8.1)	(10.0)						(9.0)
	OL-4 Tetrasaccharide							
GalNAc	5.094	4.226	3.986	4.01	4.174	3.752	2.058	
	(3.7)	(11.0)	(11.0)		(6.6)			
Rha	4.829	4.201	3.653	3.45	3.429	1.340		
	(0.5)	(3.5)	(9.5)		(6.0)			
Glc	4.499	3.331	3.647	3.63	3.546	3.807, 3.941		
	(8.1)	(9.5)	(9.5)		(5.8, 3.5)	(−12.5)		

[a]Observed chemical shifts (p.p.m.) are reported relative to 4,4-dimethyl-4-silapentane-1-sulfonate (DSS); coupling constants in Hz. [b]Measured at 24°.

The most-downfield anomeric doublet, at 5.095 p.p.m., was assigned to a GalNAc residue by decoupling of its corresponding H-2 resonance to the acetamido proton at 8.095 p.p.m. This residue was further identified as a nonreducing-terminal α-GalNAc-pyranoside by comparison of the chemical shifts and coupling constants with those of the same residue in blood group-A-active oligosaccharides[13]. This assignment was confirmed by ^{1}H-n.m.r. data on the tetrasaccharide OL-4 described later, and given in Table II.

The large coupling constants of two doublets, at 4.499 and 4.558 p.p.m., are characteristic of β-pyranosides. The chemical shift of the doublet at 4.558 p.p.m. was observed to be temperature-dependent and it was conclusively identified as that of β-GalNAc-pyranoside by decoupling of the H-2 resonance to the acetamido-proton signal at 8.345 p.p.m. The chemical shifts of H-1 and H-2 are typical of β-GalNAc, as reported by Welti *et al.*[14]. The second doublet was assigned as that of β-Glc*p* on the basis of chemical shifts of H-3 and H-4 and the $J_{1,2}$, $J_{2,3}$, and $J_{3,4}$ values, which are very similar to those of 4-substituted D-glucose in lactose[15] and of GlcNAc in type-2 chains[16]. Thus, this analysis established that the Glc was a 4-linked pyranosyl residue, rather than a 5-linked furanosyl residue.

The small values of both $J_{1,2}$ and $J_{2,3}$ of the two remaining anomeric-proton resonances, at 4.832 and 5.055 p.p.m., suggested that both are potential candidates for assignment as H-1 of rhamnose, which has an equatorial proton at C-2. The n.O.e. data were useful in resolving the assignment to H-1 of rhamnose, as the f.a.b.-m.s. data indicated that this residue was attached to an acetamidohexose. Irradiation of the anomeric-proton resonance of the nonreducing-terminal α-GalNAc gave, in addition to the intra-ring n.O.e. at GalNAc H-2, enhancement of the resonances of both Rha H-2 and H-3, which are related by decoupling to the 4.832-p.p.m. resonance, proving that the latter resonance should be assigned to a rhamnose residue attached to the nonreducing-terminal GalNAc. For most glycosidic linkages, the largest n.O.e. on irradiation of the anomeric-proton resonance appears at the proton linked to the aglycon carbon atom (Rha H-3 in this case), but it may appear at the proton attached to the adjacent carbon atom, especially if that proton is equatorial, as is[13] Rha H-2. This effect has been observed[17] in the n.m.r. spectra of blood-group B glycolipids in Me_2SO and of blood-group A oligosaccharide[13] in D_2O, for which n.O.e. is observed between both H-3 and H-4 of the β-Gal residue and H-1 of the α residue linked at O-3. The anomeric configuration of the rhamnose can be established by n.O.e. observed on irradiation of the resonance of Rha H-1. Enhancement was seen, not only at the resonance of Rha H-2, but also at those of H-3 and H-5. The latter two are close to H-1 only if it is axial, as it is in the β-pyranoside. The chemical shifts and coupling constants of H-1 and H-2 of the β-Rha, which has the β-*manno* configuration, are similar to those of the β-Man in the asparagine *N*-linked glycopeptides[18].

The anomeric doublet at 5.055 p.p.m., which is left to be assigned to the remaining galactose residue, cannot be that of a galactopyranoside, as $J_{2,3}$ is small. Therefore, this resonance was assigned to a galactofuranoside, consistent with the

evidence from m.s. of the partially methylated hydrolysis products, which indicated that the hexasaccharide contains a furanosyl residue. The anomeric configuration of this residue could be assigned by the small, vicinal coupling-constant between H-1 and H-2, which implies that these protons are *trans*, corresponding to the configuration of the β-galactofuranoside[19–21].

Comparison of the n.m.r. spectrum of OL-4 with that of the parent hexasaccharide-alditol (see Table II) showed three anomeric-proton signals. The Galol signals are buried in the other methine-proton signals in both cases. The chemical shifts of the resonances assigned are very similar to those of the three nonreducing-terminal residues, α-GalNAc, β-Rha, and β-Glc.

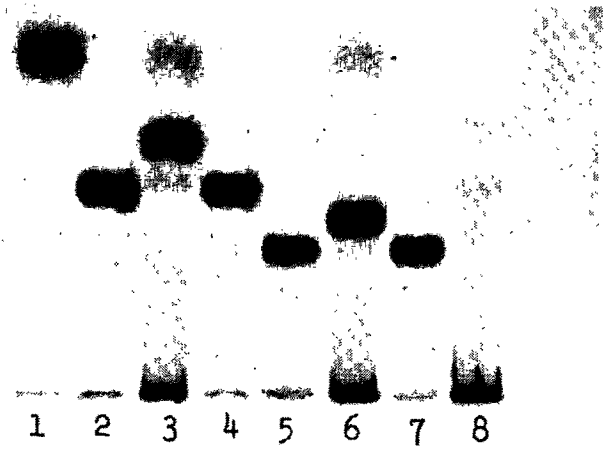

Fig. 2. Cleavage of OL-6 and OL-4 by α-*N*-acetylgalactosaminidase and not by β-*N*-acetylhexosaminidase, demonstrated by thin-layer chromatography. Key: 1, GalNAc; 2, OL-4; 3, OL-4 + α-*N*-acetylgalactosaminidase; 4, OL-4 + β-*N*-acetylhexosaminidase; 5, OL-6; 6, OL-6 + α-*N*-acetylgalactosaminidase; 7, OL-6 + β-*N*-acetylhexosaminidase; and 8, both enzymes.

Because the polysaccharide from which OL-6 was obtained was shown to have an affinity for a specific lectin which had a strong preference for β-GalNAc over α-GalNAc[10], it was, of interest to obtain additional evidence as to the anomeric nature of the terminal GalNAc. This was achieved by use of α-*N*-acetylgalactosaminidase (EC 3.2.1.49) and β-*N*-acetylhexosaminidase (EC 3.2.1.52). The data in Fig. 2 show conclusively that the nonreducing terminal of both OL-6 and OL-4 is α-GalNAc.

The foregoing analyses are all in agreement with the decision that the complete structures of OL-6 and OL-4 are α-D-Gal*p*NAc-(1→3)-β-L-Rha*p*-(1→4)-β-D-Glc*p*(1→6)-β-D-Gal*f*-(1→6)-β-D-Gal*p*NAc-(1→3)-D-Galol, and α-D-Gal*p*NAc-(1→3)-β-L-Rha*p*-(1→4)-β-D-Glc*p*-(1→6)-D-Galol, respectively.

The structure of OL-6 is a new one, never reported previously.

EXPERIMENTAL

Materials. — Both OL-4 and OL-6 were available in two forms: (*a*) with hydrogen and (*b*) with deuterium on C-1 of the alditol residue[10]. α-*N*-Acetylgalactosaminidase was isolated from limpet by Uda *et al.*[22], and β-*N*-acetylhexosaminidase from jack bean by Li and Li[23]. (*R*)-(−)- and (*S*)-(+)-2-butanol were purchased from Aldrich Chemical Co. (Milwaukee, WI).

Analysis for component sugars. — Alditol acetates were prepared by hydrolyzing the oligosaccharide (~1 mg) followed by reduction[24] and acetylation[25]. The residue containing the alditol acetates was dissolved in freshly distilled ethyl acetate (1 mL) and analyzed by g.l.c. and g.l.c.-m.s.

Methylation. — The procedure of Waeghe *et al.*[25] was used for permethylation; the permethylated oligosaccharides (~1 mg) were purified by the method of Narui *et al.*[26], and were hydrolyzed in 2M trifluoroacetic acid[25] (TFA; 300 μL) for 1 h at 120°, cooled to room temperature, and the TFA evaporated under nitrogen at room temperature. In order to remove the final traces of water, methanol (1 mL) was added to the residue and evaporated to dryness. The partially methylated sugars were reduced, and the alditols acetylated. The products were dissolved in ethyl acetate for analysis by g.l.c. and g.l.c.-m.s.

Terminal-alditol analysis. — Because of the volatility of the mono-*O*-acetyl-penta-*O*-methylalditols and their often unsatisfactory recovery through the regular analysis, the following procedure[27] was used to identify only the terminal alditol residue. The oligosaccharide-alditol (~1 mg) was permethylated as already described. The dried residue was hydrolyzed with 2M TFA (300 μL) for 1 h at 120°, and the TFA was evaporated under nitrogen. The dry hydrolyzate was then acetylated and the product analyzed by g.l.c.-m.s.

Gas–liquid chromatography. — All of the samples were analyzed by using a capillary gas chromatograph (Carlo Erba, or Pye) fitted with a DB1 column (30 m × 0.25 mm o.d.) (J & W Scientific). The injector of each instrument was modified to fit a van den Berg dry injector[28], and the heated zone was held at 250°. Samples (1–2 μL) were placed on the injector tip, and the solvent was evaporated before the tip was lowered into the thermal zone. The detector, or transfer line for m.s., was also held at 250°. The oven was held for 4 min at an initial isothermal temperature of 140°, and then programmed to 250° at 2°/min. The relative retention-index (methylene units) was determined for each component by the addition of hydrocarbons to each sample and interpolation of each unknown peak between those of two hydrocarbons.

Mass spectrometry (g.l.c.–m.s.). — Samples were introduced into the mass spectrometer *via* a capillary g.l.c. column as already described. Mass spectra were obtained on a Vacuum Generator (VG) MM-16, low-resolution mass spectrometer. The instrument was operated in the electron-impact mode with an ionization potential of 70 eV. The source was held at 200° and a mass range of 700 to 20 was scanned in a repetitive manner. Data were acquired on a VG 2000 data system.

Fast-atom-bombardment mass spectrometry. — The f.a.b.-mass spectra were obtained on a Vacuum Generator (VG) 7070 E HF fitted with an Ion Tech (FAB11NF) saddle-field source. The source was run at 8 kV, using xenon gas to form the atom beam. The mass spectrometer was scanned from *m/z* 2000 to *m/z* 80 in a repetitive mode. The f.a.b. target was prepared by using a 1:1 glycerol–2-thioglycerol matrix. The hexasaccharide (~2 μg) was dissolved in methanol (~25 μL), and the solution added to the matrix on the target in 1- or 2-μL aliquots. Spectra were acquired by using a VG 2000 data system. The peracetyl derivatives were formed by adding acetic anhydride (25 μL) to the oligosaccharide (~0.5 mg) and heating for 4 h at 60°; the mixture was blown dry, and the residue dissolved in methanol for analysis. The f.a.b.-mass spectra of the peracetyl derivatives were obtained on a VG ZAB-HF instrument, using techniques described previously[29].

^{1}H-N.m.r. spectroscopy. — Samples (~2 mg) were dissolved in D_2O and lyophilized. This procedure was repeated three times, in order to ensure exchange of hydroxyl protons. The samples were then dissolved in 0.35 mL of high-purity D_2O (Merck Sharp and Dohme) in a 5-mm tube, and spectra were recorded at 300 MHz with a Nicolet Spectrometer equipped with a 1280 computer and a pulse programmer. Spin-difference decoupling-spectra (s.d.d.s) were obtained by subtracting control from irradiated spectra and the difference curves were compared with simulated spectra[17]. Nuclear Overhauser effect (n.O.e.) difference spectroscopy on OL-6 was done at 70° to obtain[17] large, positive n.O.e. value. The n.O.e. experiment used a 3-s presaturation of the irradiated resonance, followed by acquisition with the irradiation turned off. The observed chemical shifts are reported relative to internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS), using acetone as an internal standard (δ = 2.225 *vs.* DSS).

Signals of exchangeable amide protons were determined in 9:1 H_2O–D_2O with[30] 0.01M TFA. Spectra were observed with selective irradiation, using the Redfield 2–1–4–1–2 pulse sequence[31].

Determination of configuration of sugars. — Trimethylsilylated (*R*)-(−)- and (*S*)-(+)-2-butyl glycosides were prepared by the method of Gerwig *et al.*[11] with the following modifications: (*i*) silver carbonate was not used to neutralize the HCl; instead, the butanol–HCl was evaporated to dryness under nitrogen; (*ii*) for the preparation of trimethylsilyl ethers, the dry 2-butyl glycosides were dissolved in pyridine (250 μL), and hexamethyldisilizane (50 μL) and then chlorotrimethylsilane (50 μL) were added. The mixture was sealed, and kept for 15–20 h at room temperature; sediment was then removed by centrifugation, and the clear supernatant liquor was used directly for g.l.c.–m.s. In this case, the g.l.c. conditions were a 4-min hold at 140°, followed by a temperature program from 140 to 180° at 1°/min; all other conditions of g.l.c.-m.s. were as already described.

Enzymic release of GalNAc from nonreducing terminal. — The oligosaccharide (16 μg) was incubated with 0.01 unit of α-*N*-acetylgalactosaminidase or 0.8 unit of β-*N*-acetylhexosaminidase in a final volume of 30 μL of 25mM sodium citrate buffer, pH 4.0. After incubating for 16 h at 37°, the incubation mixture (15 μL) was

applied to a silica gel-60 plate (Merck) and developed with 1:1:1 1-butanol–acetic acid–water. Mono- and oligosaccharides on the thin-layer plate were revealed by spraying with diphenylamine–aniline reagent[32].

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