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CHARACTERIZATION OF A NEW OLIGOSACCHARIDE CONTAINING *myo*-INOSITOL FOUND IN PREGNANCY URINE

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ABSTRACT

A new acidic, *myo*-inositol glycoside was isolated from the pregnancy urine of a single donor. Its structure was investigated by methylation analysis, enzyme hydrolysis, mass spectrometry and nuclear-magnetic resonance spectroscopy. The glycoside was identified as *O*-α-*N*-acetylneuraminosyl-(2→3)-*O*-β-D-galactopyranosyl-(1→1L)-*myo*-inositol. Its isolation and characterization are discussed in the light of earlier results.

INTRODUCTION

Several naturally occurring glycoconjugates contain *myo*-inositol covalently bound to other carbohydrates, but only a few have been reported to consist of oligosaccharides. Two such saccharides have been identified from yeast, 1-*O*-α-D-mannopyranosyl-[1] and 6-*O*-α-D-mannopyranosyl-*myo*-inositol[2]; the first was suggested to belong to D-mannosyl group donors, and the second to complex glycolipid catabolites. Another neutral disaccharide, isolated[3] from the juice of the sugar beet, was characterized as 1L-1-*O*-α-D-galactopyranosyl-*myo*-inositol (1-α-galactinol)[3,4], and a trisaccharide from rape-seed meal was identified as *O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-galactopyranosyl-(1→1L)-*myo*-inositol[5]. An isomer of 1-α-galactinol has been obtained from rat mammary-gland; its structure was established as 6-*O*-β-D-galactopyranosyl-*myo*-inositol (6-β-galactinol)[6], and its distribution was investigated in rat tissues[7]. The first *myo*-inositol glycoside found in human material was α-L-fucopyranosyl-*myo*-inositol, the presence of which in normal urine was reported by Lundblad

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and assoc.[8,9]. Three other neutral oligosaccharides containing *myo*-inositol were also isolated, from pregnancy urine, by the same group[10], and were claimed to be pregnancy-dependent, as they disappeared after delivery[11]. So far, only neutral oligosaccharides containing *myo*-inositol have been found in plants, yeast, and mammals. We describe herein the isolation and characterization of a new type of *myo*-inositol glycoside, namely a neuraminyl→galactinol derivative, and its possible dependence on pregnancy is discussed.

EXPERIMENTAL

Material. — Third-term pregnancy urine was collected from a single, non-secretor donor (A blood-group) not subject to any dietary restriction. The urine was kept frozen at −18° until used.

Analytical methods. — The urine (1.5 L) was concentrated by rotary evaporation to 1/15th of the starting volume, centrifuged, and filtered through a column (10 × 110 cm) of Sephadex G-25 (fine). The column was eluted with distilled water containing sodium azide (200 mg/L), to prevent bacterial growth. The elution pattern for neutral hexoses and *N*-acetylneuraminic acid was monitored by the following colorimetric methods: orcinol–sulfuric acid reagent according to the procedure of Vasseur[12] for neutral hexoses, and the diphenyl reagent according to a modified version of Werner and Odin's procedure[13] for sialic acid. The fractions obtained from the gel were desalted by passage through columns (3 × 25 cm) packed with AG 50W-X8 (100–200 mesh, H^+) and AG 3-X4A (100–200 mesh, OH^-) ion-exchange resin at +4°, and submitted to ion-exchange chromatography on a column (2 × 30 cm) of DEAE Sephadex A-25, equilibrated in 0.01M pyridine acetate buffer, pH 6.0. Elution was performed with 0.01M pyridine acetate buffer, pH 5.3, and then by a linear gradient of 0.01 to 0.1M pyridine acetate buffer, pH 5.3. Neutral hexoses and *N*-acetylneuraminic acid were monitored as indicated above. The acidic fraction containing *myo*-inositol underwent preparative paper chromatography (Schleicher and Schüll 2043-b paper) in 5:5:1:3 (v/v) ethyl acetate–pyridine–acetic acid–water (solvent *A*). The papers were stained with the silver dip reagent[14]. After elution with 0.05M pyridine acetate buffer, pH 5.3, all fractions were purified on a column of Bio-Gel P-2 (200–400 mesh). The fractions obtained were monitored by analytical paper chromatography in the following three solvent systems: (*B*) 2:1:2 (v/v, upper phase) ethyl acetate–pyridine–water, (*C*) 6:1:3, (v/v) 1-propanol–ethyl acetate–water, and (*D*) 10:4:3, (v/v) ethyl acetate–pyridine–water.

Neutral hexoses, 6-deoxyhexoses, 2-amino-2-deoxyhexoses, and inositols were qualitatively analyzed by paper chromatography in solvent system (*D*) and in 5:3:2 (v/v) 1-butanol–pyridine–0.1M hydrochloric acid (solvent *E*), after hydrolysis with 2M hydrochloric acid for 2 h at 100°. Inositols and monosaccharides were used as standards.

Monosaccharides and inositols were quantitatively determined by g.l.c. The trimethylsilyl derivatives were prepared according to the method of Chambers and

Clamp[15] and analyzed in a glass column packed with 3% (w/w) of SE-30 on WAW-DMCS Chromosorb (100–200 mesh).

^{1}H-N.m.r. spectra were recorded at 250.13 MHz with a Bruker 54 250 spectrometer operating, in the Fourier-transform mode, at 25°. Chemical shifts refer to an internal standard of sodium 4,4-dimethyl-4-silapentane-1-sulfonate, by indirect reference to acetone in deuterium oxide (δ 2.225). The acquisition parameters were: pulse width, 3 μs (corresponding to a flip angle of ~30°); spectral width, 2500 kHz; and 32 k data points. Shifts and coupling constants were determined with an accuracy of ±0.15 Hz.

Methylation procedure. — The methylation analysis was performed according to the method of Hakomori[16] with potassium *tert*-butoxide as base[17]. The partially methylated alditol acetates[18] were identified by g.l.c.–m.s. with a Hewlett–Packard 5993-B spectrometer operated with 5885 software. The injection port was kept at 270°, and the capillary column (CP-SYL-5 Chrompak, 25 m × 0.25 mm) was connected to the ion source of the mass spectrometer with a platinum restrictor that split the flow at the entry of the ion source. The capillary column was heated from 160 to 260° at a rate of 3°/min. The mass spectra were recorded at an ionization potential of 70 eV. The methylated alditol acetates were identified by a combination of g.l.c. retention times, selected-ion recording, and mass spectra.

The permethylated asialosaccharide was analyzed by g.l.c.–m.s. The capillary column was heated from 200 to 260° at a rate of 2°/min. The sequence analysis of the permethylated, native saccharide was carried out by e.i.m.s. (Rieber 10-10) with a solid-probe inlet. The operating conditions were: ionization current, 200 μA; ionization energy, 70 eV; accelerating voltage, 8.2 kV; and ion-source temperature, 130°.

Enzymic degradations. — *N*-Acetylneuraminic acid was removed by incubation of the oligosaccharide (0.5 μmol) with α-neuraminidase (EC 3.2.1.18, 0.1 unit) from *Vibrio cholerae* (Beringwerke AG) in 0.05M sodium acetate buffer, pH 5.5, 0.15M sodium chloride, and 9mM calcium chloride for 15 h, at 37°. Original and asialo-oligosaccharide (0.1 μmol each), in a final volume of 50 μL, were incubated for 15 h at 37°, with the following β-D-galactosidases (EC 3.2.1.23, 0.4 unit each): Jack bean (Sigma, suspension in 3M ammonium sulfate and 25mM sodium citrate buffer, pH 5.5), *Escherichia coli* (Boehringer, suspension in 3M ammonium sulfate and 0.01M sodium phosphate buffer, pH 6.0), and beef liver (Sigma grade III, in 0.01M sodium phosphate buffer, pH 6.0, and 0.15M sodium chloride). All incubations were performed under a toluene film. Reactions were stopped by heating for 5 min at 100°. After dilution, all samples were desalted on small AG 50W-X8 and AG 3-X4A columns (H^+ and OH^-, respectively), and analyzed by paper chromatography in solvent system *D*. Original and asialosaccharide, D-galactose, and *myo*-inositol were used as standards.

RESULTS AND DISCUSSION

Ten portions (1.5 L each) of pooled urine from one pregnant donor were concentrated, filtered, and fractionated by gel chromatography on Sephadex G-25, neutral hexoses and *N*-acetylneuraminic acid being monitored (see Fig. 1) The eluate was pooled and divided into Fractions I to VII, which were analyzed for their carbohydrate content (data not shown). The ten Fractions IV (total 950 mg) contained *myo*-inositol (1:0.1 D-glucose residue by g.l.c.). They were desalted and fractionated on a DEAE-Sephadex A-25 column, as indicated in the Experimental section. The material eluted by 0.045-0.052M pyridine acetate buffer, pH 5.3, which contained *N*-acetylneuraminic acid and *myo*-inositol, was submitted to preparative paper chromatography in solvent *A*. Fractions having a mobility lower than that of α-*N*-acetylneuraminyl-(2→3)-lactose were eluted and purified by gel filtration on a Bio-gel P-2 column. One of these fractions (**1**) was homogeneous in all four solvents: R_{Lactose} 0.41 (*A*), 0.40 (*B*), 0.80 (*C*), and 0.17 (*D*).

Sugar analysis of **1** indicated D-galactose, *myo*-inositol, and *N*-acetylneuraminic acid in the molar proportion of 1.0:1.0:1.0. They accounted for more than 98% of the dry weight. The inositol residue was characterized as *myo*-inositol on the basis

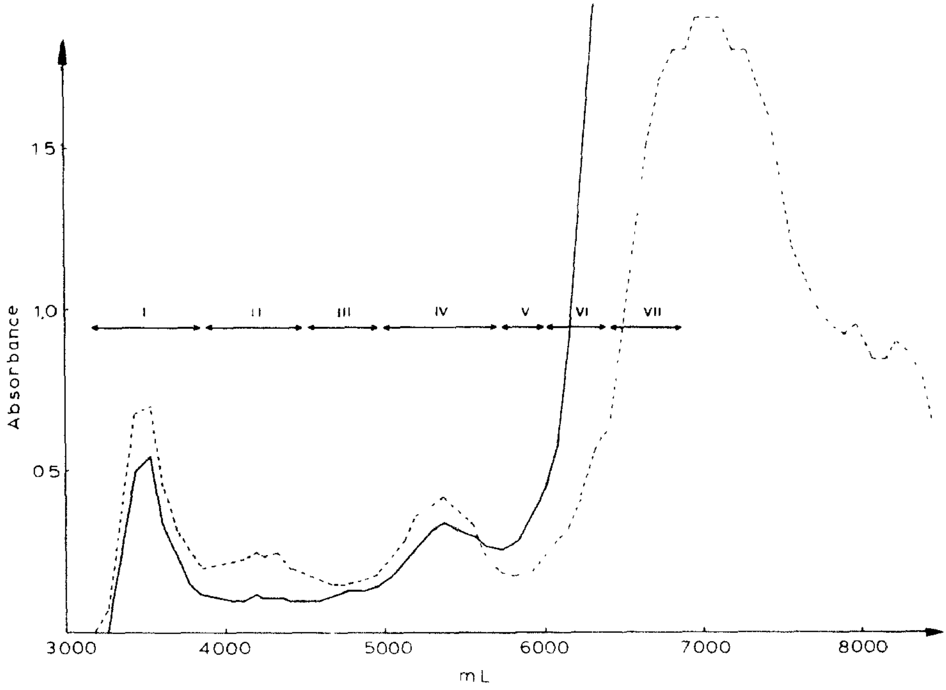


Fig. 1. Gel chromatography on a Sephadex G-25 (fine) column of a filtrate of urine (1.5 L) from a nonsecretor, pregnant woman. The column was eluted with water containing sodium azide (200 mg/L) Fractions (40 mL) were collected, and assayed for neutral hexoses[12] and sialic acid[13] The material was pooled and concentrated to give Fractions I to VII as indicated.

of identical mobility on paper chromatography in solvents *D* and *E*, and identical retention time on g.l.c. (Me_3Si derivatives), after acid hydrolysis.

Digestion of **1** with α-*N*-acetylneuraminidase completely converted **1** into a neutral disaccharide, as shown by paper chromatography in solvent *D*. The total release of the *N*-acetylneuraminosyl residue indicated an α configuration. Treatment of the disaccharide with various β-D-galactosidases quantitatively released D-galactose and *myo*-inositol, as shown by paper chromatography in solvent *D*, whereas this treatment was ineffective for **1**. This result suggested for **1** a carbohydrate sequence α-NeuAc-(2→?)-β-D-Gal-(1→?)-*myo*-inositol.

Methylation analysis of **1** according to Hakomori[16] gave 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol and a penta-*O*-methyl-*myo*-inositol, identified by g.l.c.–m.s. No deuterium-labelled alditol was detected, indicating the absence of a reducing terminal residue, and consequently the presence of *myo*-inositol in terminal position. The permethylated asialo derivative (**4**) was analyzed by g.l.c.–m.s. and its mass spectrum is given in Fig. 2. Ions at *m*/*z* 233 and 201 (A-series), and 293 (**5**, J_1 fragment) were derived from the inositol moiety. Only weak signals at *m*/*z* 219, 187, and 155 were observed for the hexose moiety, indicating that cleavage of the glycoside linkage in the A-series did not correspond to a major fragmentation of the hexosyl→*myo*-inositol structure. Similar findings have been reported for 6-β- and 1-α-galactinol[7], and also for 1L-*O*-L-fucopyranosyl-*myo*-inositol[3]. Other fragments of the spectra observed at *m*/*z* 45, 75, 88, and 101 were characteristic of the permethyl ethers of reduced mono- and di-saccharides[19,20]. The permethylated derivative of **1** was analyzed by m.s. (Fig. 3) with a direct-inlet system. No molecular ion was observed, but a fragment having *m*/*z* 770 (M − 59) was present. The detection of strong ions at *m*/*z* 376 and 344 confirmed the presence of a terminal *N*-acetylneuraminic acid residue. Fragments at *m*/*z* 596, 580 (weak), and also 520 indicated the presence of a sialylhexose sequence. The ions at *m*/*z* 233 and 201 were derived from the inositol moiety. The weak J_1 fragment at *m*/*z* 293 cannot have been produced by the original trisaccharide, because the α-sialyl residue is (2→3)-linked[19], but might correspond to a partially desialylated **1**.

The ^{1}H-n.m.r. spectrum of **1** was compared to those of α-*N*-acetylneuraminyl-(2→3)-lactose (**2**), lactose, and α-D-galactopyranosyl-(1→1L)-*myo*-inositol (**3**) (see Table I). For the *myo*-inositol residue of **1**, signals were identified for H-2eq, H-3ax, and H-5ax. The chemical shifts ($\Delta\delta$ 0.02) were very close to those observed

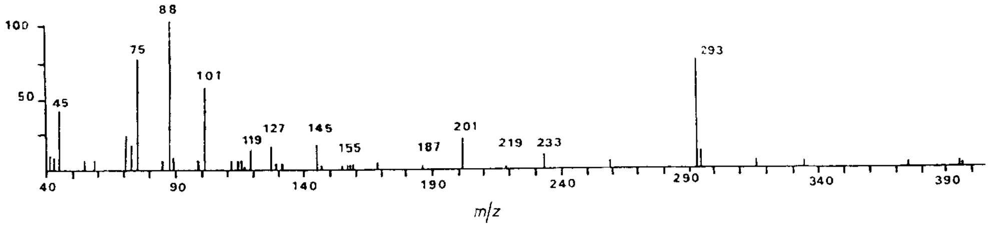
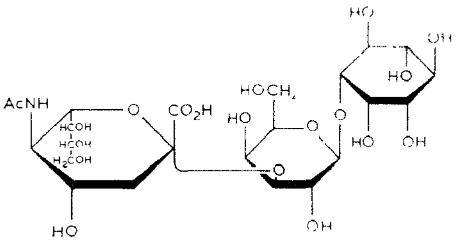


Fig. 2. Mass spectrum of permethylated asialosaccharide 4.



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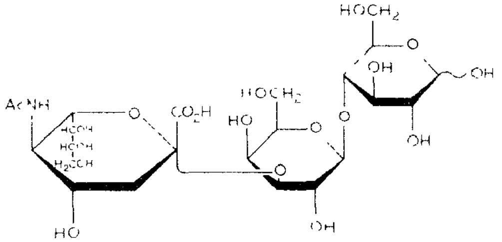

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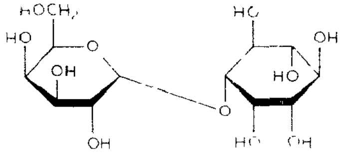

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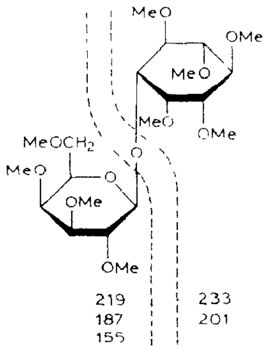

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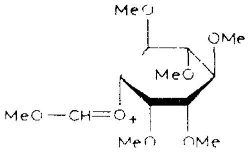

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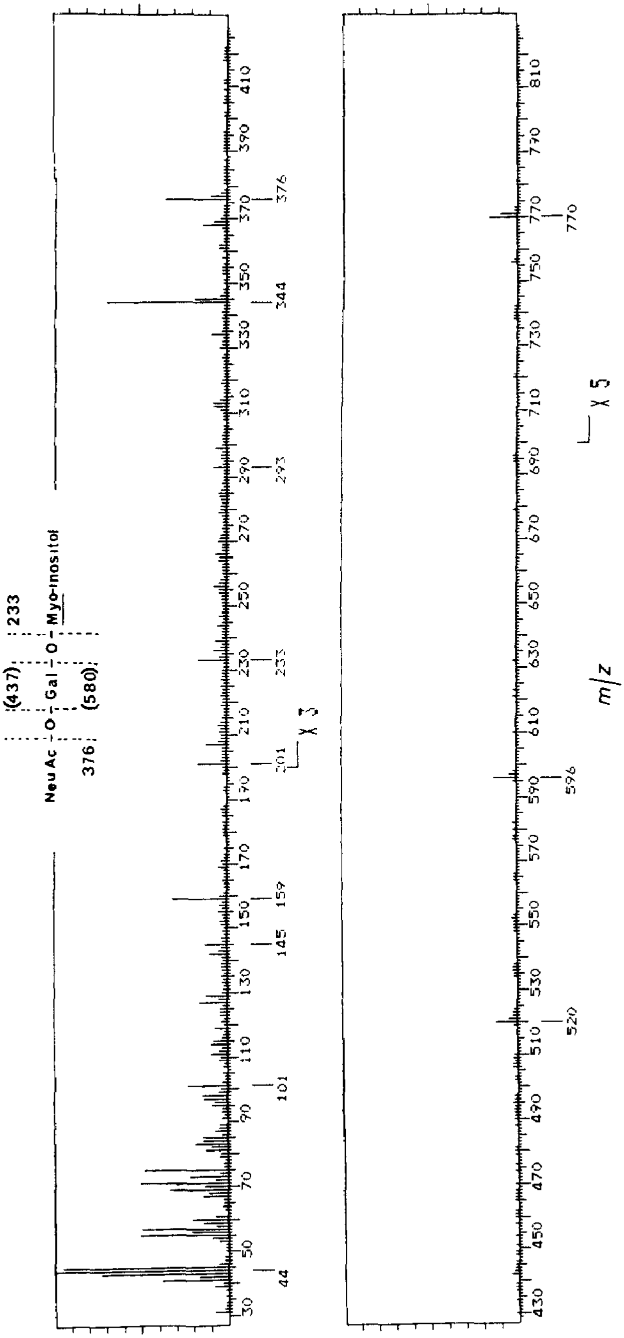
for the same protons of **3**, and the coupling constants of **1** were also very similar to those of **3**. These results suggest a (1→1L) linkage between the *myo*-inositol and D-galactose residues of **1**. For the D-galactose residue of **1**, the chemical shifts and coupling constants were very different from those observed[20] for the same residue of **3**, and correspond to those for a β-D-galactopyranosyl residue[21]. The H-1′ signal for lactose is at δ 4.50 (*J* 7.6 Hz). A relatively large shift-increment for H-3′ (δ 4.115



Mass spectrum of permethylated trisaccharide **1**, analyzed by a direct-inlet system.

TABLE I

^{1}H-N.M.R DATA[a] OF COMPOUNDS **1** AND **3**, AND *myo*-INOSITOL

Atoms	*Compound* **1**	**3**	myo-*Inositol*
H-1	3.600	3.630 (2.6; 10.0)	3.520[b] (2.8, 9.8)
H-2	4.255 (2.6)	4.277 (2.6)	4.051 (2.8)
H-3	3.520 (2.6, 10.0)	3.520 (2.6; 10.0)	
H-4		3.670	3.615[c] (9.0)
H-5	3.307 (9.2)	3.327 (9.2)	3.266 (9.2)
H-6		3.760	
H-1′	4.590	5.140 (3.8)	
H-2′		3.860 (3.8; 10.2)	
H-3′	4.115	3.960 (3.2; 10.2)	
H-4′		4.015	
H-5′		4.195	
H-6′		3.730 (6.0)	
H-3″ax	1.797		
H-3″eq	2.765		
C*H*$_3$CO	2.029		

[a] Chemical shifts (δ) at 250.13 MHz relative to internal reference standard of sodium 4,4-dimethyl-4-silapentane-1-sulfonate (*J* values in Hz in parentheses), for solutions in D$_2$O. [b] May also be attributed to H-3. [c] May also be attributed to H-6.

instead of 3.96) indicated substitution at O-3 of the D-galactose residue; the corresponding signal was observed at δ 4.121 for **1** (data not shown). For the *N*-acetylneuraminosyl acid group, the δ values for H-3ax and -3eq suggested an α anomer. The chemical shift for the *N*-acetyl group protons was in agreement with previous results[21]. Thus, structure **1** was established. It is identical to that of α-neuraminyl-(2→3)-lactose, in which a *myo*-inositol substitutes the D-glucose residue. This, therefore, suggests that **1** also originates from the action of sequential glycosyltransferases on *myo*-inositol.

The linkage of the β-D-galactopyranosyl to the *myo*-inositol residue, as determined by ^{1}H-n.m.r. disagrees with the results of Naccarato *et al.*[6,7] for 6-β-galactinol of animal origin. Nevertheless, this linkage is supported by our previous findings[22], which demonstrated the presence, in the urine of various donors, of two and sometimes three disaccharides having an *O*-hexosyl-*myo*-inositol structure. In addition, incubation of lactose and *myo*-inositol with rat-mammary-gland β-D-galactosidase or *Escherichia coli* β-D-galactosidase gives 5- and 6-β-galactinol[23], and also an unknown isomer[24], the mass spectrum of which was identical with that of 6-β-galactinol. This *in vitro* synthesis may occur *in vivo* as well, and any disaccharide that is an isomer of 6-β-galactinol may be an acceptor for an α-(2→3)-sialyltransferase. In the case of 1-β-galactinol, the trisaccharide obtained would be identical to **1**

Although **1** described herein may exist in normal urine, it is present only in minute amounts, as no one has reported its excretion by normal individuals. Its presence and characterization in pregnancy urine is probably related to the generally enhanced excretion of neutral[11,25] and acidic[26–28] oligosaccharides during pregnancy and subsequent lactation, as reported for rats and humans.

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