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Structural analysis of three sulfated oligosaccharides isolated from human milk

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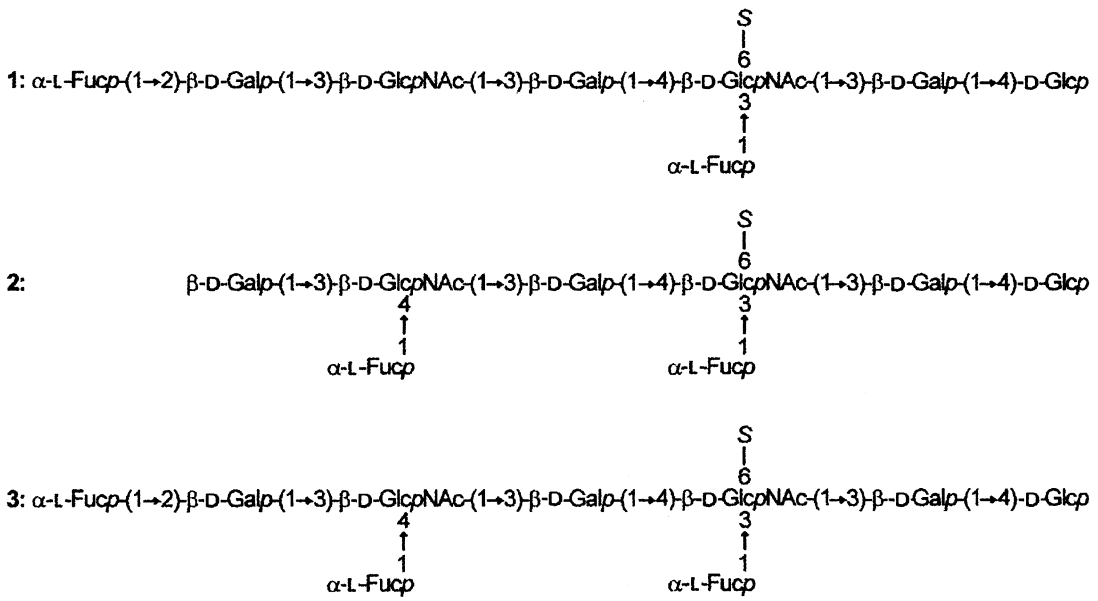
Abstract

The structures of two sulfated octasaccharides and one sulfated nonasaccharide isolated from human milk have been investigated. Using ^{13}C and ^{1}H NMR spectroscopy and ESMS, the following structures **1–3** were established:



Keywords: Sulfated oligosaccharides; Human milk

1. Introduction

Human milk is unique among that of mammals with regard to its high content of complex oligosaccharides. The amount of complex oligosaccharides varies with lactation, decreasing from 25 g L^{-1} (65% of total carbohydrate content in colostrum to 8–12 g L^{-1} in mature milk [1,2]). More than 130 neutral and acidic complex oligosaccharides have been identified

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so far. Even today, the list of oligosaccharides present in milk is not complete. With improving methods of isolation and characterisation, new compounds are continually being isolated.

These oligosaccharides consist of five different monosaccharides: D-glucose, D-galactose, *N*-acetyl-neuraminic acid, L-fucose and 2-acetamido-2-deoxy-D-glucose. Furthermore, they may be sulfated as shown in this article. All milk oligosaccharides so far studied have the lactose structure at their reducing end. The epithelial cells of secretory glands contain a series of glycosyl transferases that form the carbohydrate moiety of mucus with blood group activities [3]. In the mammary gland, these enzymes use lactose as a false acceptor to synthesise complex carbohydrates. Thus, the study of their structure may provide interesting information concerning the specificity of these glycosyltransferases. Here, we describe the isolation and the identification of three minor sulfated oligosaccharides (< 100 $\mu g\ L^{-1}$) from an homologous series, only differentiated by the number and the position of fucosyl residues.

2. Experimental

Isolation of oligosaccharides.—The fractionation of milk oligosaccharides using a combination of gel filtration, anion exchanger and paper chromatography has been previously described [5]. Sulfated oligosaccharides were finally separated by HPLC on primary-bonded silica (Supelcosil LC-NH_2 column, 4.6×250 mm; Supelco Inc., Bellefonte, USA) using MeCN–15 mM potassium phosphate (65:35). The flow rate was 1 mL min^{-1} and the absorbance was measured at 206 nm.

NMR spectroscopy.—NMR experiments were performed on a Bruker ASX 400 WB spectrometer. Chemical shifts are expressed downfield from internal 4,4′-dimethyl-4-silapentane-1-sulfonate, but were actually measured by reference to internal acetone ($\delta = 2.225$ in D_2O at 25 °C). The two-dimensional homonuclear correlation spectroscopy (COSY) and the heteronuclear multiple-quantum coherence (HMQC) were performed using Bruker standard pulse sequences.

Reductive amination.—2-Aminopyridine (500 mg) was added to 100 μL of HOAc, which had been heated at 65 °C and maintained at 80 °C with repeated vortex mixing until 2-aminopyridine completely dissolved, and was prepared freshly before use. This coupling agent (40 μL) was added to 100 nmol of oligosaccharide. The reaction mixture was mixed and heated to 37 °C for 8 h, then cooled to room temperature (rt), and 10 μL of a reducing agent (freshly prepared by mixing 354 mg of borane–dimethylamine complex and 100 μL of HOAc) was added. The reaction mixture was mixed and heated to 37 °C for 24 h, then cooled to rt [5]. To remove the borane–pyridine complex, 75 and 85% MeOH were added in succession to the reaction mixture and evaporated [6]. After addition of 500 μL of deionised water, the pH of the solution was adjusted to 10 with diluted ammonia solution and the excess of reagents was extracted with 500 μL of $CHCl_3$ (ten times). The aq phase was neutralized with dilute acetic acid prior to lyophilisation.

HPLC of derivatised oligosaccharides.—The pyridylamino derivatives were purified by reversed-phase high-performance liquid chromatography (HPLC) on a 5 μm C18 Zorbax column (250×4.6 mm D.I.). The derivatised oligosaccharides were dissolved in 100 μL of deionised water and injected into the column. The column was equilibrated with 0.1% trifluoroacetic acid in water. After injection, isocratic conditions were applied for 10 min with the initial solvent, followed by a gradient to 30% acetonitrile in 0.1% trifluoroacetic acid for 60 min. The flow rate was 0.7 mL min^{-1} and the absorbance was measured at 230 nm. The collected pyridylamino-derivatised oligosaccharides were then lyophilised prior to examination by ESIMS and ESIMS/MS.

Electrospray mass spectrometry.—The electrospray ionization mass experiments were carried out on a Quattro II triple-quadrupole mass spectrometer (Micromass). Samples were dissolved in 49:49:2 water–MeOH–AcOH at a concentration of approximately

50 pmol μL^{-1} and the solution injected into the electrospray ion source by a Harvard syringe pump. Optimisation of the parameters was performed both in the MS and MSMS mode using the (Glc)$\hat{n}$-PA model. Typically, best conditions were defined for a cone voltage of about 60 V, a collision energy range between 30 and 40 eV and an argon collision gas pressure at 4.0 mTorr. Spectra were recorded in MCA (multiple channel acquisition) mode at a scan speed of 10 s and were smoothed once.

3. Results and discussion

Isolation and purification of oligosaccharides.—As previously reported [4], the acidic oligosaccharides were fractionated by anion-exchange chromatography into 22 fractions (Fig. 1). Two minor components (A and B) were stained in fractions 20 mM-4 and 50 mM-1 and subsequently purified by preparative paper chromatography. Subfractions A1 and A2, were finally separated by HPLC.

Fig. 1. Paper chromatography of milk oligosaccharides eluted from an anion exchanger (Dowex 1×2; 200–400 mesh; HCO_2^-) with a discontinuous gradient of pyridine–acetate buffer. Solvent: 5:5:1:3 pyridine–ethyl acetate–acetic acid–water. Migration: 5 days.

Yields were 1.2, 1.4 and 2 mg for A1, A2 and B, respectively, starting from 20 L of pooled human milk.

NMR analysis.—The two-step relayed COSY spectra of the three oligosaccharides are presented in Figs. 2 and 3 and the NMR data are reported in Tables 1–3. On the H-1 tracks of residues II–IV in the 2D spectra, cross-peaks were detected with H-2, 3, 4, allowing sugar units II, IV and VI to be identified as β-Gal ($J_{1,2} = J_{2,3}$ 8 Hz; $J_{3,4}$ 4 Hz; $J_{4,5}$ 1 Hz) and III and V as β-GlcNAc ($J_{1,2} = J_{2,3} = J_{3,4} = J_{4,5}$ 8 Hz; δ C-2 = 56–57 ppm) on the basis of the set of the coupling constants. The terminal reducing unit was characterised as glucose, according to the observation of anomeric protons at δ 5.21 (α anomer) and 4.66 ppm (β anomer).Whereas the α-Glc residue was easily identified on the basis of the presence of triplets for its trans-axial H-3 and H-4 atom resonances, the identical chemical shifts of β-Glc H-3 and H-4 signals prevented such an observation. Nevertheless, the assignment of β-Glc was made according to its characteristic H-2 resonance observed at $\delta = 3.28$ ppm [8–10].

Two (for oligosaccharides A1 and A2) or three α-L-fucose units (for oligosaccharide B) were identified on the basis of the presence of methyl resonances at $\delta = 1.15, 1.23$ and 1.17 ppm, respectively, and the set of coupling constants $J_{1,2}$ to $J_{4,5}$ that are characteristic of the α-galacto configuration. The H-1, H-5 and H-6 atom resonances of terminal α-L-fucose units at different positions allow the direct recognition of the linkages [7]. For example, the presence of α-(1→2)-, α-(1→3) and α-(1→4)-linked fucose units in oligosaccharide B (Fig. 3) can be directly deduced from the chemical shifts of these structural-reporter-group values. Moreover, the fucosylation at C-4 or C-3 of the *N*-acetyl glucosamine unit in the sequence β-D-Gal-(1→3/4)-D-GlcNAc gives rise to downfield shifts for the α-L-fucose H-5 atom resonance, at δ 4.82 (1→3-linkage) and 4.80 ppm (1→4-linkage) [7]. The Lewis^b determinant is characterized by the presence of α-(1→2)- and α-(1→4)-linked fucose units attached to β-D-Gal and β-D-GlcNAc. In such a case, the

Table 1
^{1}H and ^{13}C NMR data for oligosaccharide A1 [a]

Sugar		δ (ppm) of														
		H-1	H-2	H-3	H-4	H-5	H-6	H-6′	$COCH_3$	C-1	C-2	C-3	C-4	C-5	C-6	$COCH_3$
→4)-α-D-Glc*p*	Iα	5.219	3.576	3.83	3.65	3.94	3.86	3.86		92.90	72.4	72.7	79.6	70.9	n.d.	–
→4)-β-D-Glc *p*	Iβ	4.661	3.276	3.64	3.64	3.64	n.d.	n.d.		96.96	74.74	75.6	79.6	75.9	61.3	–
→3)-β-D-Gal*p*-(1→	II	4.434	3.587	3.707	4.176	3.72	3.74	3.74		103.94	71.3	83.1	69.4	76.2	62.2–63	–
→3,4,6)-β-D-GlcNAc-(1→	III	4.752	3.969	3.88	3.99	3.80	4.31	4.29	2.019	102.9	57.3	75.73	74.0	73.3	67.5	23.4
α-L-Fuc-(1→	F^3	5.108	3.678	3.88	3.77	4.823	1.146	–		100.00	68.8	70.2	73.2	67.9	16.5	–
→3)-β-D-Gal*p*-(1→	IV	4.490	3.468	3.718	4.091	n.d.	n.d.	n.d.		102.67	71.8	83.1	69.8	76.2	62.2-63	–
→3)-β-D-GlcNAc-(1→	V	4.627	3.797	3.99	3.54	3.48	3.88	3.78	2.049	104.32	56.2	78.5	69.8	76.3	61.2	23.3
→2)-β-D-Gal*p*-(1→	VI	4.648	3.85	3.83	3.89	n.d.	n.d.	n.d.		101.27	77.8	74.2	70.1	76.2	62.2-63	–
α-L-Fuc-(1→	F^2	5.185	3.765	3.65	3.72	4.300	1.229	–		100.61	69.07	70.7	73.0	67.7	16.5	–

[a] n.d., not determined. Downfield ^{13}C signals indicating the C-substitutions are underlined.

Table 2
^{1}H and ^{13}C NMR data for oligosaccharide A2 [a]

Sugar		δ (ppm) of														
		H-1	H-2	H-3	H-4	H-5	H-6	H-6′	$COCH_3$	C-1	C-2	C-3	C-4	C-5	C-6	$COCH_3$
→4)-α-D-Glc*p*	Iα	5.217	3.574	3.83	3.64	3.94	3.86	3.86	–	93.03	72.4	72.7	79.6	70.92	n.d.	–
→4)-β-D-Glc *p*	Iβ	4.662	3.279	3.64	3.64	3.64	n.d.	n.d.	–	97.00	74.94	75.6	79.6	76.1	61.5	–
→3)-β-D-Gal*p*-(1→	II	4.435	3.580	3.704	4.176	3.72	3.74	3.74	–	α104.07 β104.21	71.30	83.3	69.52	76.20	62.3-63	–
→3,4,6)-β-D-GlcNAc-(1→	III	4.752	3.967	3.89	3.987	3.80	4.37	4.13	2.020	103.53	57.03	75.75	73.99	73.3	67.43	23.4
α-L-Fuc-(1→	F^3	5.114	3.675	3.90	3.775	4.805	1.152	-	–	99.90	68.78	70.10	73.25	67.98	16.7	-
→3)-β-D-Gal*p*-(1→	IV	4.505	3.500	3.719	4.109	3.62	3.74	3.74	–	102.81	71.7	83.3	69.54	75.9	62.3–63	–
→3,4)-β-D-GlcNAc-(1→	V	4.690	3.961	4.073	3.760	3.54	3.93	n.d.	2.031	103.82	57.10	77.35	73.3	76.6	61.05	23.7
α-L-Fuc-(1→	F^4	5.033	3.790	3.89	3.802	4.883	1.171	–	–	99.09	69.03	70.10	73.25	67.98	16.8	–
β-D-Gal*p*-(1→	VI	4.515	3.481	3.635	3.88	3.62	3.74	3.74	–	103.66	71.7	76.7	69.88	75.9	62.3–63	–

[a] n.d., not determined. Downfield ^{13}C signals indicating the C-substitutions are underlined.

Table 3
^{1}H and ^{13}C NMR data for oligosaccharide B [a]

Sugar		δ (ppm) of												
		H-1	H-2	H-3	H-4	H-5	H-6	H-6′	$COCH_3$	C-1	C-2	C-3	C-4	C-5	C-6	$COCH_3$
→4)-α-D-Glc*p*	Iα	5.218	3.576	3.83	3.64	3.94	3.86	3.86		92.61	72.4	72.7	79.6	n.d.	n.d.	–
→4)-β-D-Glc *p*	Iβ	4.661	3.280	3.64	3.64	3.64	n.d.	n.d.		96.43	74.77	75.6	79.6	n.d.	61.5	–
→3)-β-D-Gal*p*-(1→	II	4.434	3.587	3.70	4.174	3.72	3.74	3.74		103.62	71.30	82.9	69.20	76.2	62.3–63	–
→3,4,6)-β-D-GlcNAc-(1→	III	4.749	3.966	3.88	3.986	3.79	4.35	4.31	2.019	103.43	56.9	75.9	73.9	73.3	67.3	23.5
α-L-Fuc-(1→	F^3	5.108	3.681	3.88	3.77	4.819	1.152	–		99.67	68.8	70.1	73.3	67.8	16.7	–
→3)-β-D-Gal*p*-(1→	IV	4.490	3.464	3.70	4.086	3.62	3.74	3.74		102.26	71.74	82.9	69.44	75.6	62.3–63	–
→3,4)-β-D-GlcNAc-(1→	V	4.607	3.829	4.134	n.d.	3.51	3.88	n.d.	2.054	103.95	57.0	75.61	73.3	75.9	60.6	23.6
α-L-Fuc-(1→	F^4	5.033	3.800	3.92	3.83	4.871	1.256	–		98.57	69.1	70.1	73.3	67.8	16.7	–
→2)-β-D-Gal*p*-(1→	VI	4.661	3.602	3.81	3.85	3.59	3.73	3.073		101.42	77.7	74.9	70.0	76.0	62.3–63	–
α-L-Fuc-(1→	F^2	5.149	3.747	3.68	3.72	4.343	1.273	–		100.45	69.5	70.7	73.3	67.3	16.7	–

[a] n.d., not determined. Downfield ^{13}C signals indicating the C-substitutions are underlined.

H-1 and H-6 atom resonances of α-L-fucose units possess significant chemical shifts, compared with those of α-L-fucose present in H or Le[a] determinants (Tables 1–3).

From these above observations, the three oligosaccharides were clearly defined as oligomers composed of one lactose and two Gal-GlcNAc units, respectively β-D-Gal-(1→3)-D-GlcNAc in non-reducing terminal position (for H, Lewis[a] and Lewis[b] determinants) and β-D-Gal-(1→4)-D-GlcNAc (for Lewis[x] determinant). Since the downfield chemical shifts of both Gal^{II} and Gal^{IV} H-4 resonances indicate a C-3 substitution [8,9], the sugar chains are evidently linear.

Consequently, oligosaccharide B should possess the sequence of the VI^2Fuc, V^4Fuc, III^3Fuc-*p*-lacto-*N*-hexaose previously described [8]. This inference is contradicted by the downfield shift of Gal^{IV} H-1, observed at $\delta = 4.490$ ppm instead of 4.424 ppm [8]. Moreover, the H-6 and H-6′ resonances observed at $\delta = 4.35$ and 4.31 ppm are significative of 6-*O*-sulfonation of β-GlcNAc, which is responsible for the downfield shift of the anomeric proton of the neighbouring Gal unit [9]. Similar observations were made for oligosaccharides A_1 and A_2, in which a sulfate group occurs at position 6 of $GlcNAc^{III}$. It can be also notified that the occurrence of a phosphate can be secluded, since $^1H/^{31}P$ coupling is not observable.

Data obtained from 2D $^{13}C/^1H$ spectra reveal the nature of linkage sites, according to the ^{13}C signals which are downfield shifted and are underlined in Tables 1–3. For example, the observation of $GlcNAc^{III}$ C-3, C-4 and C-6 atom resonances at 75.9, 73.9 and 67.3 ppm (Table 3) clearly assigns these carbons as the sites of substitution.

ESIMS analysis.—In order to confirm the location of the sulfate group, the 2-aminopyridine labelled oligosaccharides were analysed by ESIMS and ESIMS/MS, in the negative mode (Fig. 4). The pseudo molecular ions $[M–H]^-$ observed at m/z 1521 or 1667 clearly indicated the following molar compositions: 2 Fuc, 3 Gal, 2 GlcNAc, 1 H_2SO_4, 1 Glc-ol, 1 2-AP, for compounds A1,

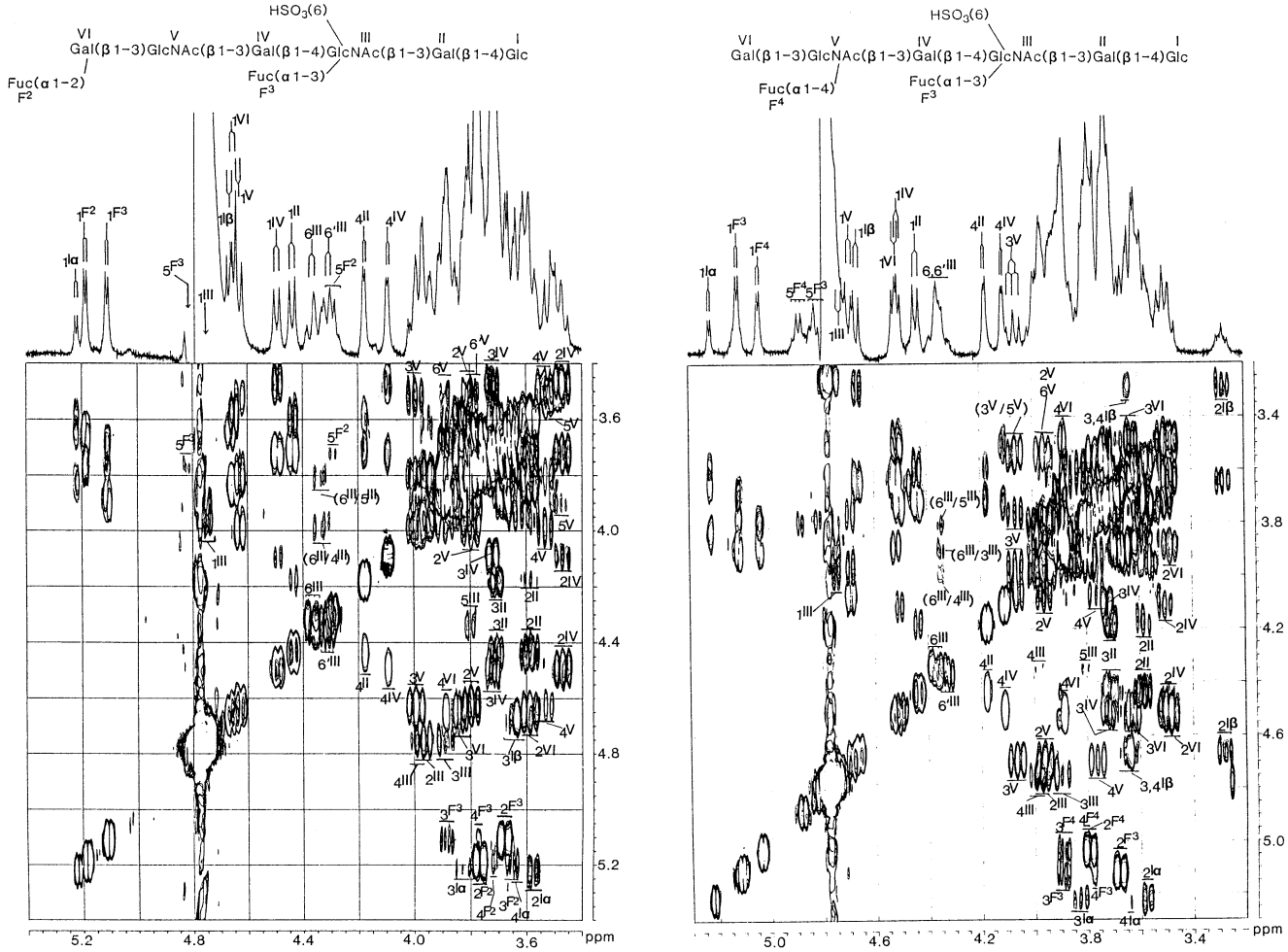

Fig. 2. Double-relayed COSY spectra of compounds A1 (left) and A2 (right).

A2, and 3 Fuc, 3 Gal, 2 GlcNAc, 1 H_2SO_4, 1 Glc-ol, 1 2-AP, for compound B.

Moreover, the ESIMS/MS showed series of B and Y ions characterised by the presence of a sulfate group. ESIMS/MS was performed in negative mode in order to specifically collect sulfate-containing secondary fragments. For compound B for example, the series of Y ions (1521, 1359, 1010, 848) is interrupted at m/z 848, allowing us to presume that sulfate group may be attached to inner GlcNAc, Gal or Glc-ol units. In the same way, the series of B ions (1409, 1247) is interrupted at m/z 1247. So, since all the secondary Y or B fragments observed systematically contain the inner residue of GlcNAc, it confirms the attachment of the sulfate group to this GlcNAc residue of compound B. The same observations are done on compounds A1 and A2. The series of Y ions from compounds A1 and A2 are both interrupted at m/z 848, whereas the B series interrupted at m/z 848, whereas the B series are both interrupted at m/z 1101, confirming the attachment of the sulfate group to the inner residue of GlcNAc.

In conclusion, the evidence obtained by two different methods demonstrated that the

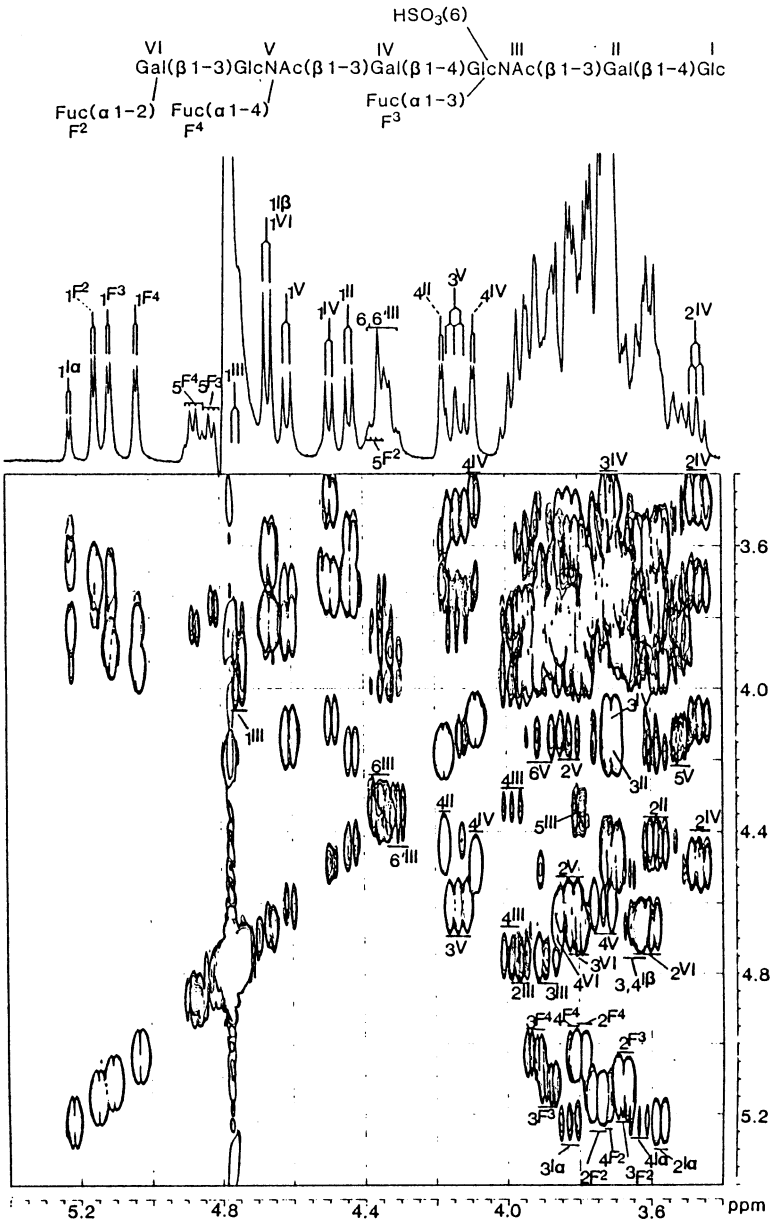

Fig. 3. Double-relayed COSY spectrum of compounds B.

three sulfated oligosaccharides isolated from human milk have structures as shown in Scheme 1. These three oligosaccharides represent new examples of the presence of the core *p*-lacto-*N*-hexaose, previously characterised as di- or trifucosyl derivatives [8,10].

Acknowledgements

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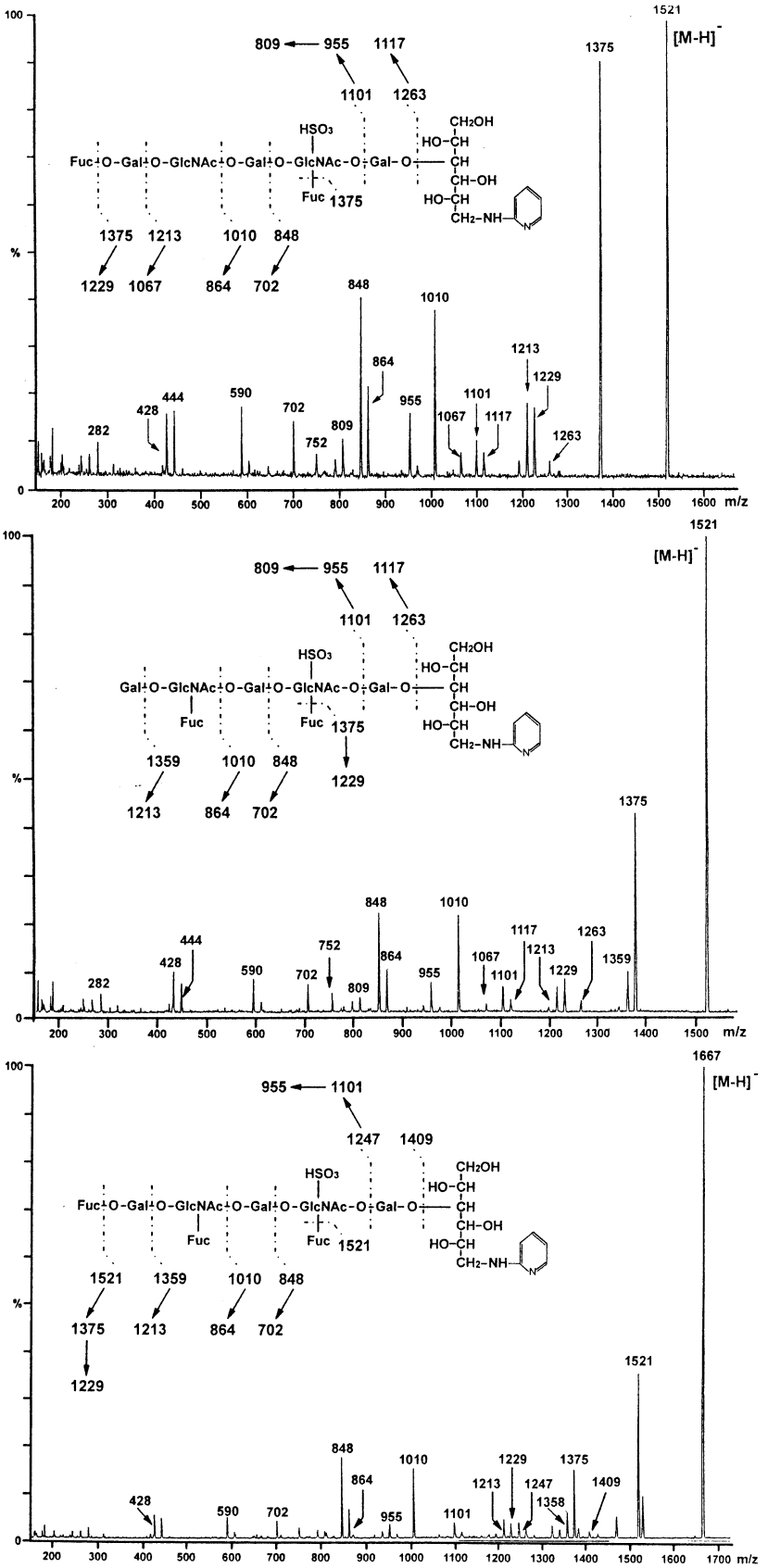
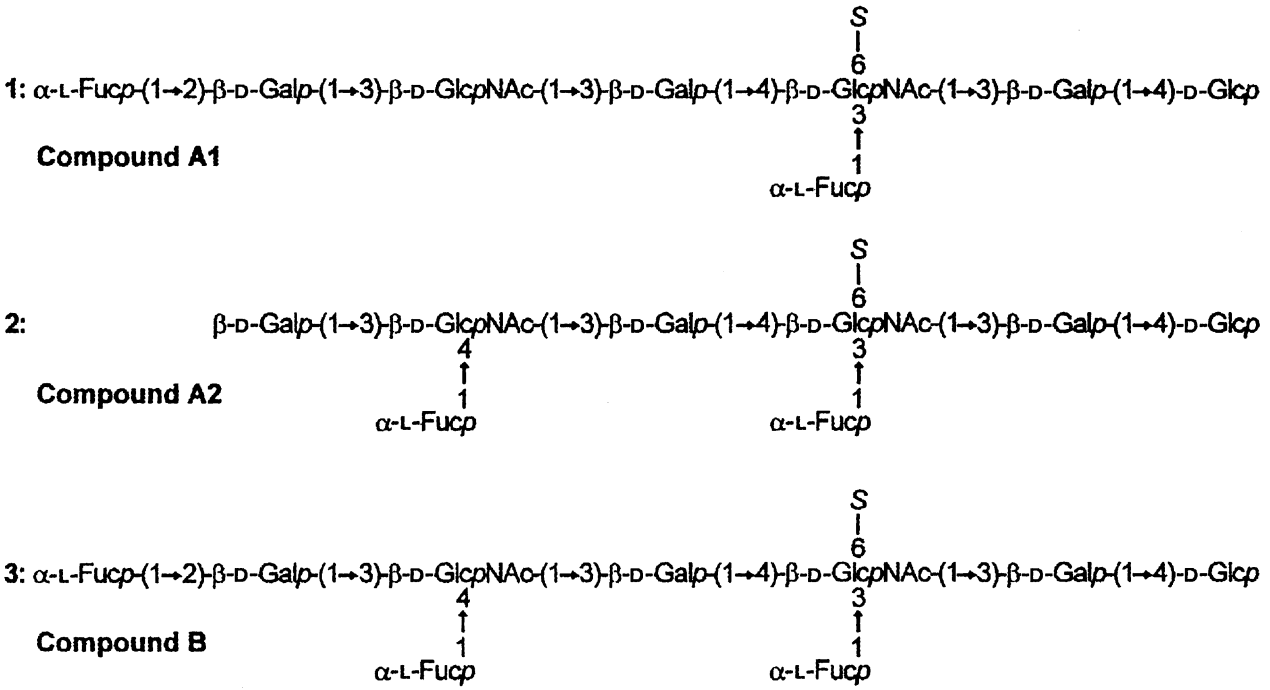


Fig. 4. ESIMS/MS spectra of compounds A1, A2 and B after derivatization with 2-aminopyridine.



Scheme 1. Structure of sulfated oligosaccharides **1–3** isolated from human milk.

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