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Structural analysis of the oligosaccharide-alditols released by reductive β-elimination from oviducal mucins of *Rana temporaria*

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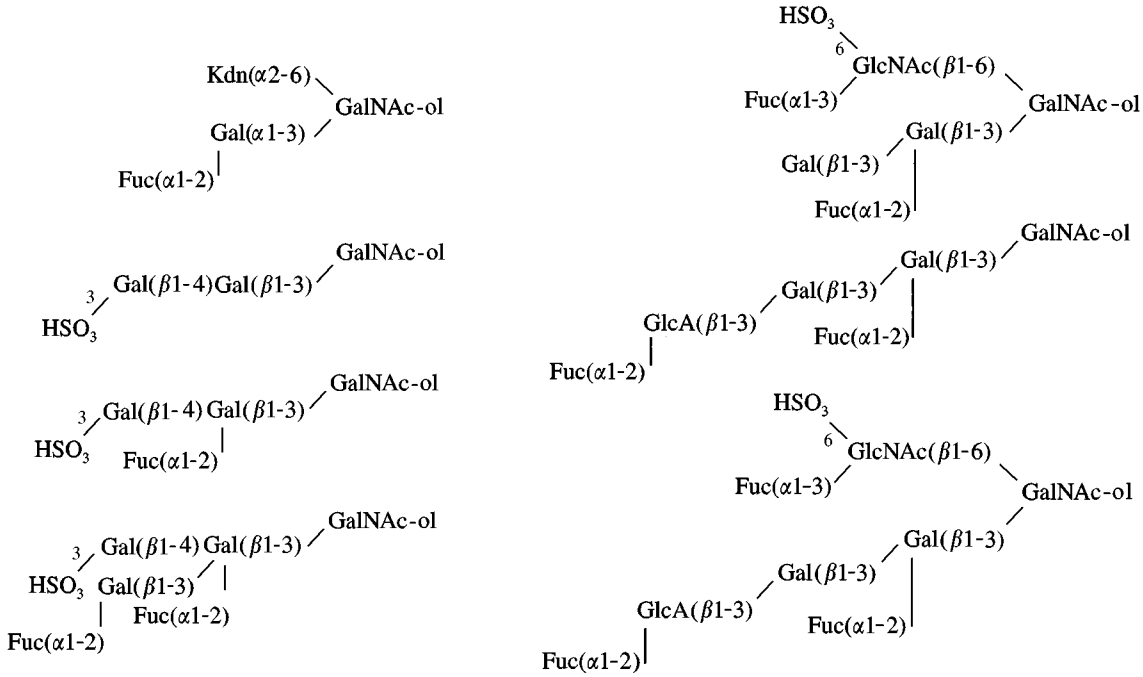
The carbohydrate chains of the mucins which constitute the jelly coat surrounding the eggs of *Rana temporaria* were released by alkaline borohydride treatment. Neutral and acidic oligosaccharide-alditols were purified by ion-exchange chromatography and HPLC. From the structural analysis, based upon ^{1}H and ^{13}C-NMR spectroscopy in combination with MALDI-TOF, the following glycan units are proposed.



Keywords*: ^{1}H-NMR, carbohydrate, mucin, amphibian, *Rana temporaria

***Abbreviations*: MALDI-TOF, matrix assisted laser desorption ionization – time of flight; HPLC, high performance liquid chromatography; COSY, correlation spectroscopy; HSQC, heteronuclear single-quantum coherence spectroscopy; HMQC, heteronuclear multiple-quantum coherence spectroscopy; ROESY, rotating-frame overhauser enhancement spectroscopy; Fuc, fucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; GalNAc-ol, *N*-acetylgalactosaminitol; GlcA, glucuronic acid**

Introduction

The jelly coat surrounding amphibian eggs is composed of mucin-type glycoproteins. These highly glycosylated molecules are synthesized by oviduct cells and play an

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important role in the fertilization process, involving capacitation of the sperm, induction of the acrosome reaction, block of polyspermy (anouran species), and recognition of homologous species [1–4]. The jelly coat is very important for the embryonic protection and retains Ca^{2+} ions necessary for successful fertilization [3, 5]. Our previous studies have shown that the jelly coat glycoproteins present highly species-specific glycosylation patterns and that they contain, for most of the species, 3-deoxy-D-glycero-D-galacto-nonulosonic acid (Kdn) as the only acidic sugar [6–15]. Moreover, some species exhibit, as a major structure, rare human carbohydrate determinants (i.e. Lewis Y in *Pleurodeles waltl* [6]). In the present study, we have isolated neutral and acidic carbohydrate chains from the egg jelly of *Rana temporaria*, and determined the structure of seventeen of them.

Material and methods

The jelly coat was lyophilized and the dry material (2 g) was subjected to alkaline reductive degradation in 0.1 M NaOH containing 1 M $NaBH_4$ (200 ml) at 37 °C for 48 h. The reaction was stopped by DOWEX 50X8 (mesh 25–50, H^+ form) and boric acid was eliminated as its methyl ester in the presence of methanol. Neutral and acidic oligosaccharides were further fractionated on DOWEX 1X2 (mesh 200–400, $HCOO^-$ form). After water elution of neutral compounds, acidic oligosaccharides were desorbed with 50, 100, 200 and 300 mM of pyridine-acetate buffer (pH 6.5) respectively, and fractions were isolated by high-performance liquid chromatography on primary-bond silica (Supelcosyl LC-NH_2; 4.6 mm × 25 cm, Supelco Inc. Bellefonte USA) using acetonitrile/30 mM potassium phosphate, pH 5.2, with a flow rate of 1 ml min^{-1}. Several fractions were obtained at various concentrations of the elution gradient (see HPLC profiles in Fig. 2). The neutral oligosaccharide-alditols were eventually recycled on a 5 μm ODS Zorbax column (25 cm × 0.94 cm I.D.; Du Pont Instruments, Paris, France), with water as eluent. Oligosaccharide-alditols were detected by spectroscopy at 206 nm.

Analytical procedure

Carbohydrate composition was determined according to Zanetta *et al.* [16] and to Clamp *et al.* [17].

^{1}H-NMR spectroscopy was performed on a Bruker ASX 400 WB spectrometer. Chemical shifts are expressed in ppm downfield from internal sodium 4,4′-dimethyl-4-silapentane-1-sulfonate, but were actually measured by reference to internal acetone ($\delta = 2.225$ ppm in D_2O at 25 °C). The two-dimensional homonuclear correlation spectroscopy (COSY), with simple and double relay transfer, the heteronuclear single-quantum coherence (HSQC), the heteronuclear multiple-quantum coherence (HMQC) and rotating-frame nuclear Overhauser enhancement spectroscopy (ROESY) experiments were performed using Bruker standard pulse-sequences. For ROESY experiments, the mixing time was set at 400 ms.

Mass spectroscopy

Mass measurements of matrix O-glycan-alditols were performed by matrix assisted laser desorption and time of flight mass spectrometry on a Vision 2000 (Finnigan Mat, Hemel) instrument in reflection mode (nitrogen laser: 337 nm).

Samples were dissolved in water at a concentration of 50–100 pmol μl^{-1} and 1 μl of these solutions were mixed with 1 μl of matrix onto the target then allowed to crystallize at room temperature.

Monoacidic structures were mass analysed in the positive mode using 2,5-dihydroxybenzoic acid (10 mg ml^{-1} in methanol:water 70:30). The diacidic compound was analysed in the negative mode using 3 aminochinolin matrix (10 mg ml^{-1} in water:ethanol 90:10). Ten to 15 shots were accumulated for each spectrum.

Results

A fractionation of neutral oligosaccharide-alditols on Bio-Gel P-4 resulted in three sub-fractions and only the third one was studied here (**N-III**). Two oligosaccharide-alditols **N-III-1** and **N-III-2** were further purified by preparative reversed phase HPLC on a 5 μm ODS column (Fig. 1). The acidic oligosaccharide-alditols were eluted from the Dowex 1X2 column in seven fractions, with 50, 100, 200, 300, 400, 500 and 600 mM buffer solutions. Figure 2 shows the HPLC profile on a LC-NH_2 column of the four fractions 50 to

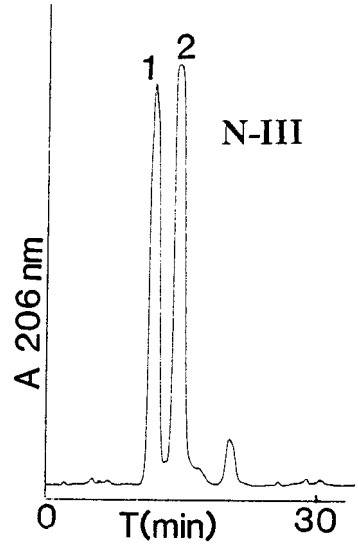


Figure 1. HPLC profile of neutral oligosaccharide-alditols (fraction **N-III**) on a 5 μm ODS column, eluted with water. (1) GalNAc-ol and Gal(β1-3)GalNAc-ol; (2) Gal(α1-3)GalNAc-ol.

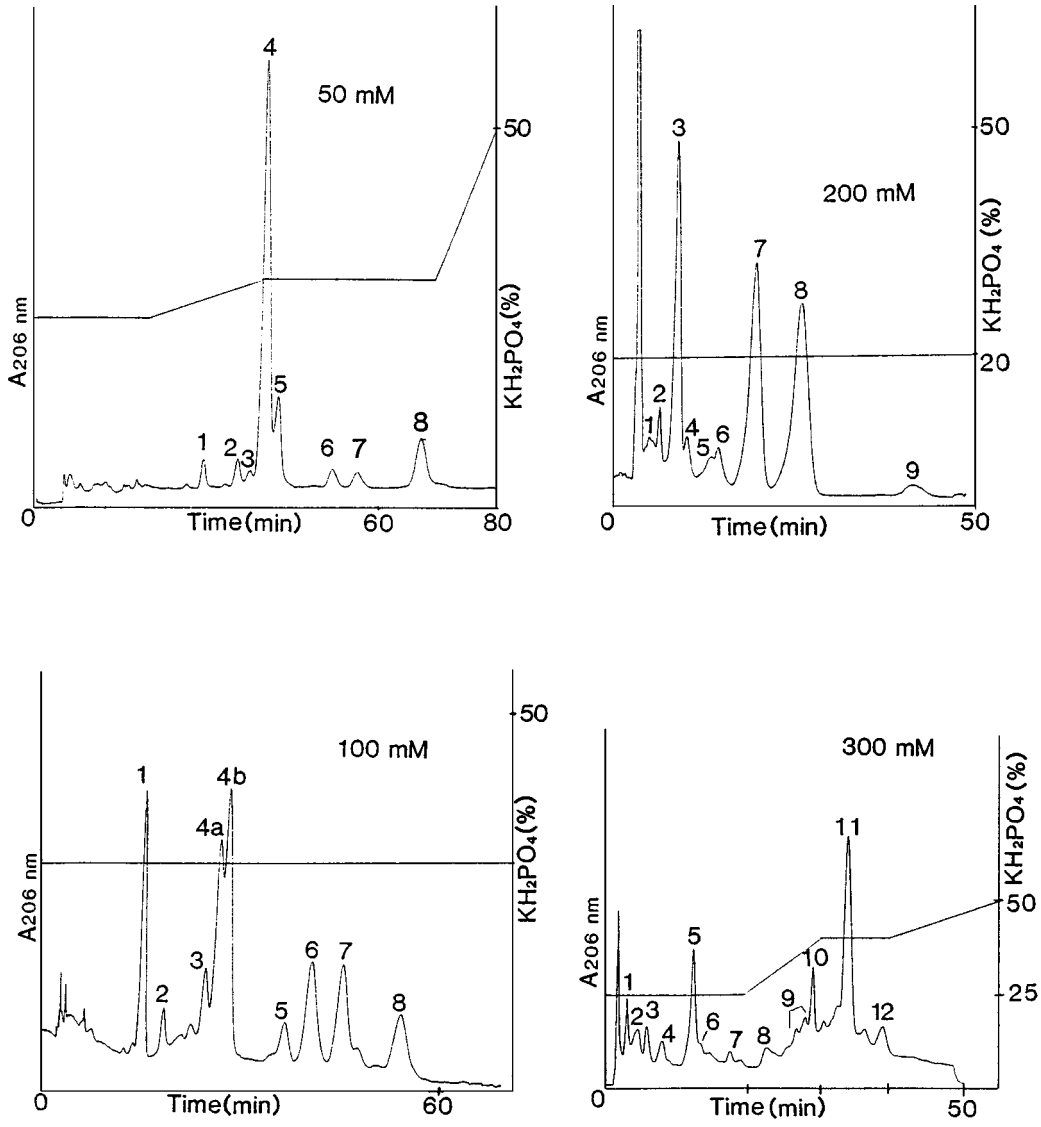


Figure 2. HPLC profiles of oligosaccharide-alditols eluted from the anionic ion exchanger with pyridine acetate buffer. The primary-bonded silica LC-NH_2 (25 × 0.46 cm) was eluted with gradients of acetonitrile-30 mM KH_2PO_4, as depicted on the profiles.

300 mM, which were further analysed. The numbering of the various acidic fractions takes into account the concentration of the elution buffer (50, 100, 200 and 300), together with the appearance in HPLC elution profile. Some of the fractions were in too low concentration or too complex to allow an extensive study. The order of the description of the oligosaccharide-alditols in the text was set afterward in such a way as to write them in a logical sequence.

The structures of oligosaccharide-alditols were established on the basis of NMR. Mass spectroscopy (MALDI-TOF) was used for confirming the presence of an anionic group (phosphate or sulfate, $\Delta MW = 80$). The discrimination between these two groups was achieved by NMR, by analysing a putative ^{31}P-^{1}H coupling constant, the absence of which allowing to identify the anionic unit as sulfate. Monosaccharide units were identified on the basis of their vicinal coupling constants, and the linkage by HMQC spectroscopy. In some cases, the sequence was determined using the ROESY pulse sequence NMR.

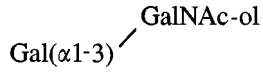
The fraction **N-III-1** contains a mixture of the two well known compounds GalNAc-ol and Gal(β1-3)GalNAc-ol (Table 1) in a ratio 3:2.

The acidic fraction **100-4a** also contains a compound of low molecular weight, namely Kdn(α2-6)GalNAc-ol, which was identified on the basis of its NMR spectrum (Table 1). Indeed, the chemical shifts relative to the GalNAc-ol unit are identical to those observed for reference compound NeuAc(α2-6)GalNAc-ol [18]. The α-2,6-linked Kdn shows specific H-3ax and H-3eq signals at $\delta = 1.660$ and 2.671 ppm [6].

Table 1. ^{1}H-NMR chemical shifts of the oligosaccharide-alditols (N-III-1a), (N-III-1b), (N-III-2), (100-4a), (50-4), (50-5a) and (50-5b) containing core type 1 and 8. The monosaccharides are represented by this symbolic notation: ◇-ol, GalNAc-ol; ■, *β*Gal; ■, *α*Gal; ▲, Kdn; □, Fuc. ND, not determined. The linkage position is specified by the direction of the connecting bars as follows: 

Residue	*Reporter group*	*Chemical shifts (ppm) in*						
								
		N–III–1a	N–III–1b	N–III–2	100–4a	50–4	50–5a	50–5b
GalNAc-ol **I**	H-1,1′	ND	3.801/3.735	3.795/3.709	ND	3.794/3.703	3.76/3.72	3.826/3.760
	H-2	4.251	4.390	4.373	4.243	4.365	4.377	4.421
	H-3	3.849	4.064	3.935	3.841	3.925	4.052	3.884
	H-4	3.390	3.510	3.714	3.403	3.713	3.522	3.669
	H-5	3.928	4.192	4.054	4.013	4.104	4.232	3.992
	H-6,6′	ND	3.680/3.629	3.756/3.652	ND	3.827/3.503	3.845/3.468	3.85/3.474
	NAc	2.055	2.050	2.045	2.052	2.038	2.045	
Gal *β*1-3 **II**	H-1,1′	–	4.477	–	–	–	4.471	–
	H-2	–	3.564	–	–	–	3.567	–
	H-3	–	3.677	–	–	–	3.668	–
	H-4	–	3.903	–	–	–	3.899	–
	H-5	–	3.729	–	–	–	3.72	–
	H-6,6′	–	3.78	–	–	–	3.77	–
Gal *α*1-3 **II**	H-1,1′	–		5.170	–	5.150	–	5.142
	H-2	–		3.868	–	3.86	–	3.976
	H-3	–		3.866	–	3.86	–	∼ 4.11
	H-4	–		4.028	–	4.025	–	4.039
	H-5	–		4.054	–	4.051	–	4.100
	H-6,6′	–		3.756	–	3.75	–	∼ 3.76
Fuc *α*1-2 **F**	H-1,1′	–		–	–	–	–	5.293
	H-2	–		–	–	–	–	3.790
	H-3	–		–	–	–	–	3.888
	H-4	–		–	–	–	–	3.850
	H-5	–		–	–	–	–	4.061
	CH_3	–		–	–	–	–	1.241
Kdn *α*2-6 **K**	H-3ax	–		–	1.660	1.661	1.653	1.670
	H-3eq	–		–	2.671	2.670	2.686	2.690
	H-4	–		–	–	–	3.58	3.58
	H-5	–		–	–	–	3.52	3.52
	H-6	–		–	–	–	3.66	3.66
	H-7	–		–	–	–	3.87	3.87
	H-8	–		–	–	–	3.82	3.82
	H-9,9′	–		–	–	–	3.90/3.70	3.90/3.70

The compound **N-III-2** (Fig. 3, Tables 1 and 4) is composed of GalNAc-ol and Gal in the ratio 1:1. The anomeric configuration of the α-Gal unit is evident, from the shape of its H-1 resonance ($J_{1,2} \cong 3$ Hz), despite its distortion due to a virtual long-range coupling. The ^{13}C-NMR data reported in Table 4 clearly show the C-3 substitution of the GalNAc-ol unit, and, therefore, lead to propose the following structure:



N-III-2

The compound **50-4 (= 100-7),** (Fig. 4 and Table 1), possesses Kdn and Gal respectively α-2,6- and α-1,3-linked to GalNAc-ol. This conclusion results from the observation of typical Kdn H-3ax, H-3eq, GalNAc H-3, H-6 and H-6′

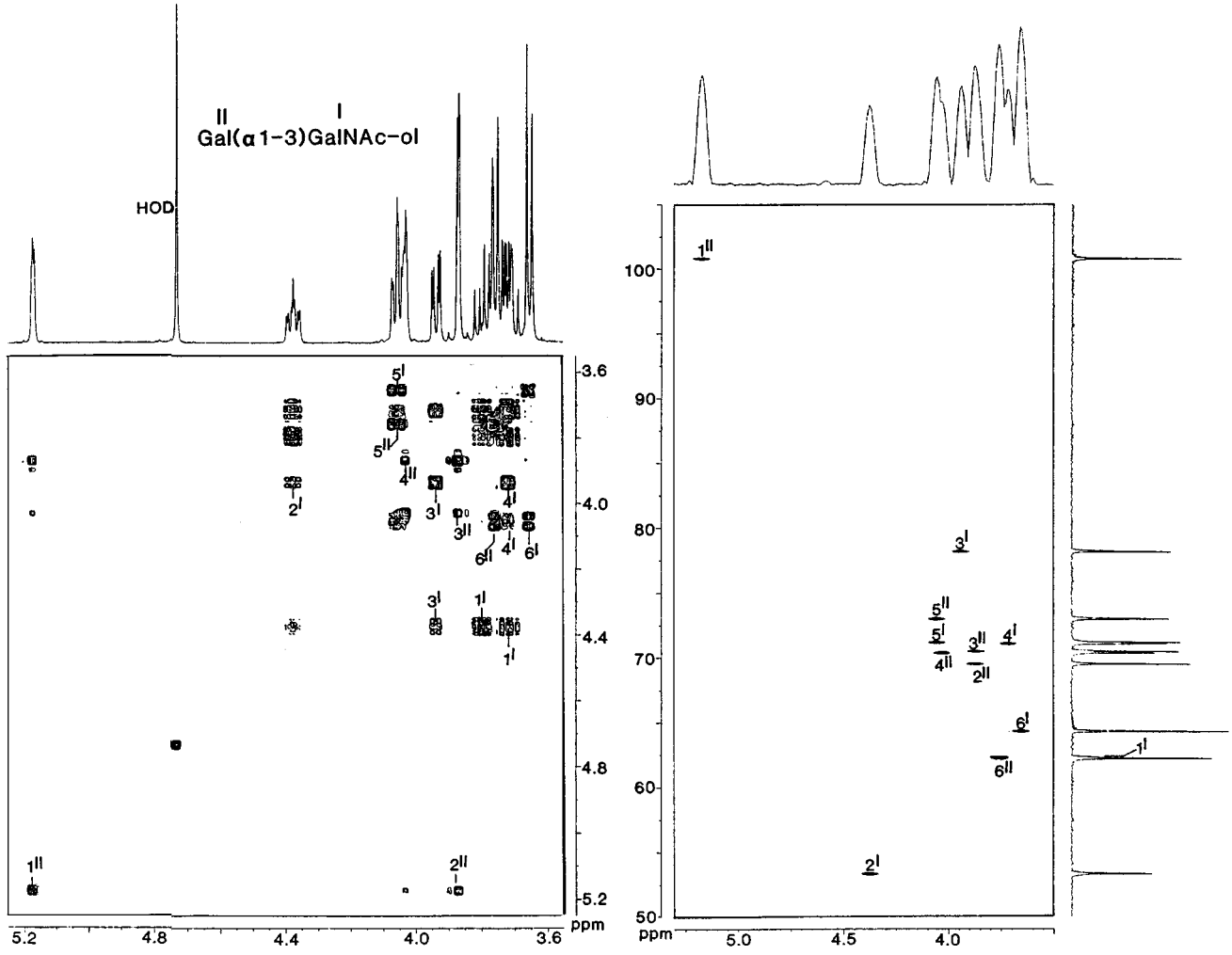
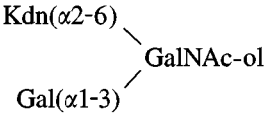


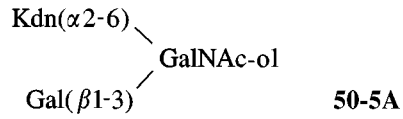
Figure 3. Homonuclear and heteronuclear COSY spectra of compound **N-III-2**.

and Gal H-1 ($J_{1,2} \sim 3$ Hz), H-5 atom resonances which are characteristic for each structural element of the following compound:

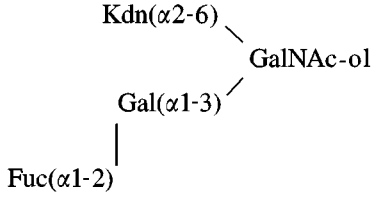


50-4

The NMR spectrum of fraction **50-5** confirms that it contains two different oligosaccharide-alditols, according to the presence of two GalNAc-ol H-2 and H-5 signals (Fig. 5; Tables 1 and 4). Both structures contain Kdn O-6 linked to GalNAc-ol, according to the H-6 and H-6′ atom resonances of the hexitol. The α-Gal residue II-B was identified on the basis of the $J_{1,2}$ coupling constant (= 3 Hz), and the set of the vicinal coupling constants observed for $J_{2,3}$, $J_{3,4}$ and $J_{4,5}$ coupling constants (respectively 8, 3 and 1 Hz). The β-Gal residue II-A ($J_{1,2} = 8$ Hz; $J_{4,5} = 1$ Hz) occurs in the terminal non-reducing position, whereas the α-Gal residue II-B is O-2 substituted, as shown by its C-2 resonance deshielded at 79.15 ppm. Since this deshielding is correlated with the occurrence of an additional Fuc unit, the sequences of the two compounds in the fraction can be established as follows:



50-5A



50-5B

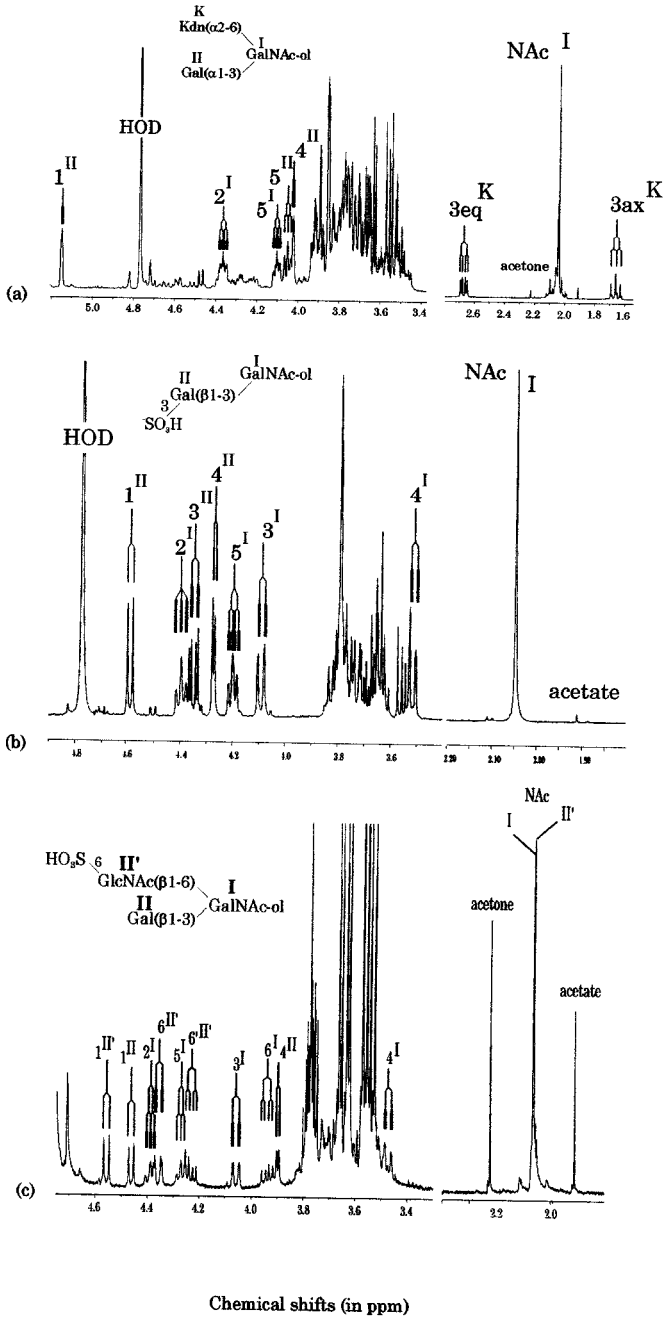
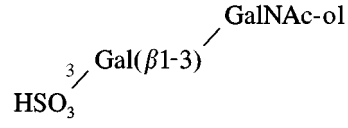


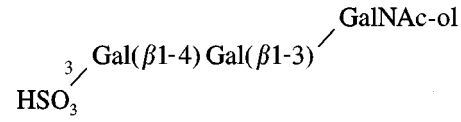
Figure 4. ^{1}H-NMR spectra of compounds **50-4** (a), **200-3** (b) and **300-5** (c).

The ^{1}H NMR spectrum of compound **200-3** shows the presence of resonances relative to Gal H-1, H-3 and H-4, which are characteristic of an O-3 sulfated Gal unit [19], and to GalNAc-ol H-2 and H-5, themselves significant of an O-3 substituted GalNAc unit (Fig. 4 and Table 2). These observations point us to propose the following structure:



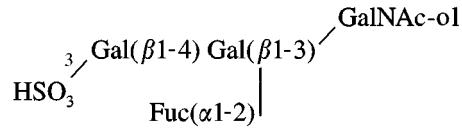
200-3

MALDI analysis of compound **200-7** shows a [M-H + 2K]$^+$ ion at *m/z* 704 (Fig. 6) which may correspond to a sulfated trisaccharide-alditol with GalNAc-ol, Gal and SO_3H in the molar proportions of 1:2:1. The downfield-shift resonances of Gal III H-3 and H-4 (Fig. 7; Tables 2 and 5) are attributable to the C-3 substitution by a sulfate residue [19]. The Gal II unit is itself substituted on the 4 position, as indicated by the downfield-shift resonance of H-4 (δ = 4.200 ppm) and C4 (δ = 79.35 ppm). The ROESY spectrum indicated nOe contacts between Gal III H-1/Gal II H-4 and Gal II H-1/GalNAc-ol I H-3, an observation that led us to propose the following sequence:



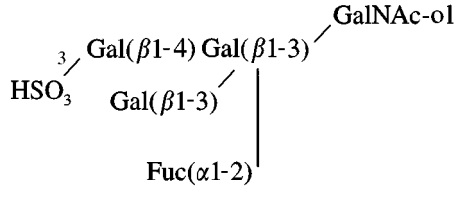
200-7

MALDI analysis of fraction **200-8** shows a [M-H + 2K]$^+$ ion at *m/z* 850 (Fig. 6), which indicates the compound to be an extension of **200-7** with a Fuc unit (ΔMW = 146). The set of Gal III H-3 and H-4 resonances is identical to that of compound **200-7** and is characteristic of an O-3 sulfatation (Fig. 8 and Table 2). On the HMQC spectrum, the di-substitution of Gal II at position 2 and 4 can be deduced from the C-2 and C-4 atom resonance observed at δ = 80.25 and 78.39 ppm, respectively (Table 5). In addition, nOe effects between Fuc H-1 and Gal II H-1, H-2, H-3 signals, Gal III H-1 and Gal II H-3, H-4 signals (Fig. 9) confirm the α-1,2-linkage of Fucose to the Gal II unit:



200-8

Structure **100-4b**: From the MALDI analysis which shows a [M-H + 2K]$^+$ ion at *m/z* 1012 (Fig. 6), the compound **100-4b** can be considered as an extension of **200-8** with in addition a hexose unit, identified as galactose on the basis of the 2D COSY spectrum (Fig. 10 and Table 2). The C-2, C-3 and C-4 atom resonances of Gal II are shifted downfield (Table 5), which shows C-3 at the point of substitution by the Gal III′ unit. The nOe contacts (Fuc H-1 → Gal II H-1, H-2, H-3; Gal III′ H-1 → Gal IIH-3, H-4; Gal III H-1 → Gal II H-3, H-4; Gal II H-1 → GalNAc-ol H-2, H-3) depicted in Fig. 9 are in good agreement with the following sequence:



100-4B

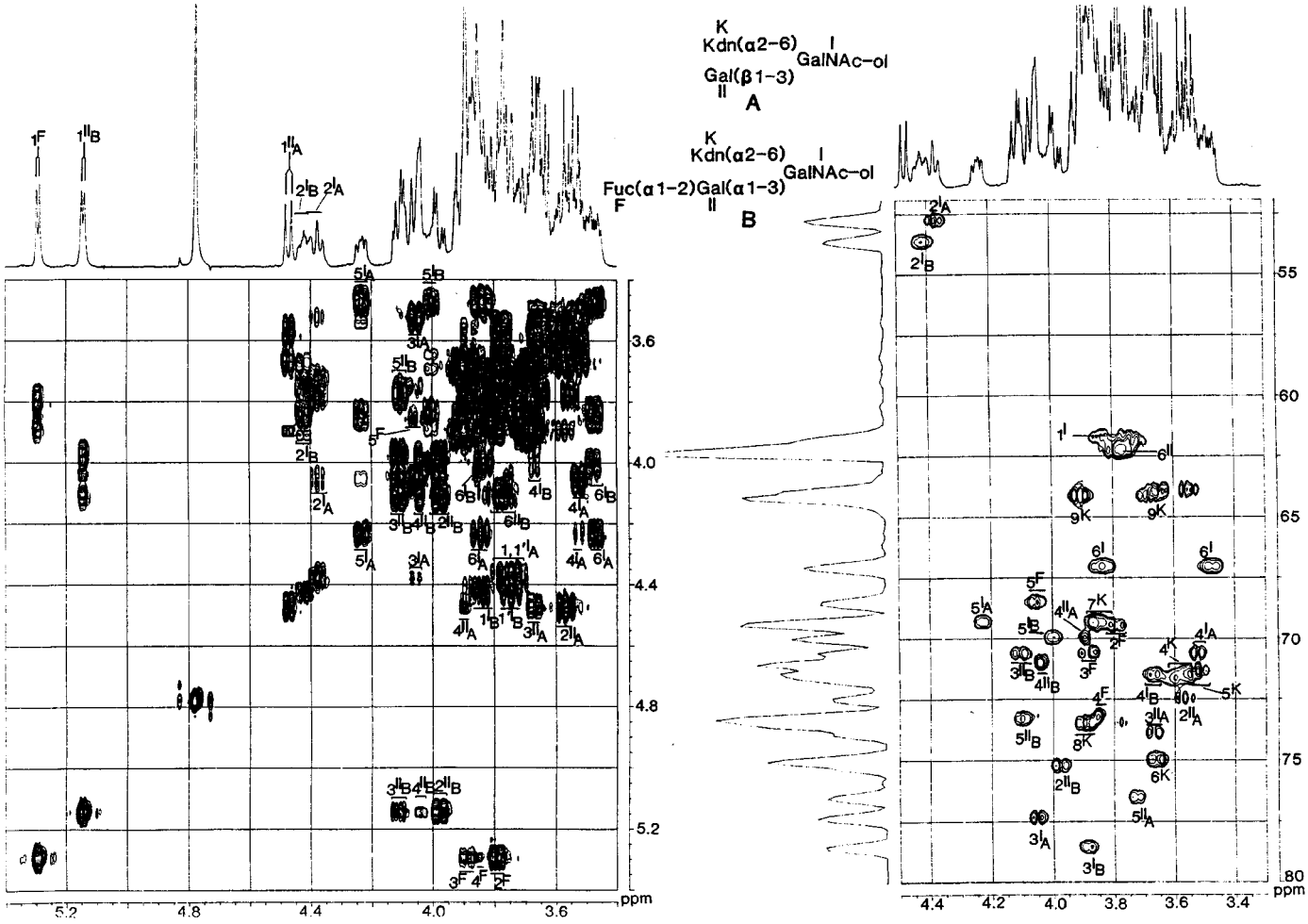
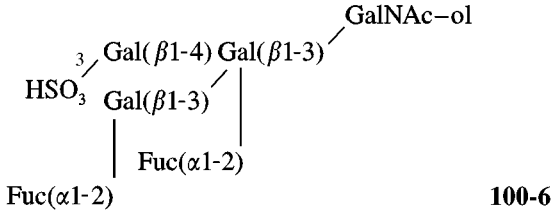


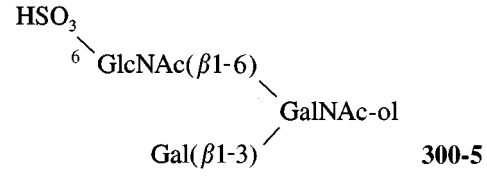
Figure 5. Homonuclear and heteronuclear COSY spectra of fraction **50-5**.

The MALDI spectrum of compound **100-6** (Fig. 6) shows a $[M\text{-}H + 2K]^+$ pseudo-molecular ion at m/z 1158 corresponding to an extension of **100-4b** with in addition a Fuc unit (ΔMW: 146). A comparison of the HMQC spectra of both compounds **100-4b** and **100-6** (Figs 10 and 11; Tables 2 and 5) indicates that Gal III′ C-2 is deshielded from 72.44 to 75.19 ppm, leading to the linkage of the additional Fuc unit at position 2 of Gal III′:



100-6

The NMR spectrum of fraction **300-5** (Fig. 5) is superimposable with that of compound II [20] previously described as:

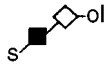
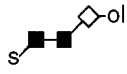
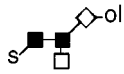
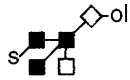
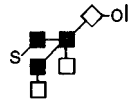
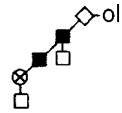
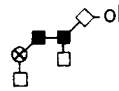


300-5

The presence of a sulfate group at O-6 of GlcNAc is responsible for a significant downfield shift for GlcNAc H-6 and H-6′ atom resonances (Table 3) [21].

Compound **200-9**, with $[M\text{-}H + 2K]^+$ at *m/z* 890 (Fig. 6), is an extension of **300-5** with α-1,2-linked fucose, as was clearly demonstrated by the set of structural reporter-group signals for Gal(β1-3) (δH-1 = 4.577) and Fuc(α1-2) (δH-1 = 5.245, δH-5 = 4.305, δCH_3 = 1.242) [22]. As shown above for compound **300-5**, the presence of a sulfate group at O-6 of GlcNAc is attested by the typical downfield shift of H-6 and H-6′ atom resonances (Fig. 12 and Table 3).

Table 2. ^{1}H-NMR chemical shifts of the oligosaccharide-alditols (200-3), (200-7), (200-8), (100-4b), (100-6), (50-7A) and (50-7B) containing core type 1. The monosaccharides are represented by this symbolic notation: (◇-ol, GalNAc-ol; ⊗, GlcA; ■, *β*Gal; S-■, *β*Gal-3-S; □, Fuc. ND, not determined. The linkage position is specified by the direction of the connecting bars as follows: 

Residue	*Reporter group*	*Chemical shifts (ppm) in*						
								
		200-3	200-7	200-8	100-4b	100-6	50-7A	50-7B
GalNAcol **I**	H-1,1′	ND	~ 3.74	~ 3.8	3.799	3.811	~ 3.79	~ 3.80
	H-2	4.396	4.389	4.387	4.359	4.320	4.344	4.397
	H-3	4.091	4.063	4.079	4.106	4.052	4.113	4.086
	H-4	3.516	3.525	3.528	3.570	3.557	3.571	3.534
	H-5	4.201	4.176	4.143	4.118	4.101	4.136	4.136
	H-6	ND	3.63	3.64	~ 3.65	3.634	3.64–3.67	3.64–3.67
	NAc	2.049	2.052	2.049	2.051	2.053	2.051	2.045
Gal *β*1-3 **II**	H-1,1′	–	4.503	4.598	4.673	4.653	4.663	4.593
	H-2	–	3.639	3.817	3.952	3.650	3.789	3.837
	H-3	–	3.795	3.988	4.114	4.086	4.017	3.995
	H-4	–	4.200	4.208	4.455	4.468	4.222	4.198
	H-5	–	3.761	3.774	3.790	~ 3.7	3.77	3.77
	H-6,6′	–	~ 3.7	~ 3.8	~ 3.8	~ 3.8	ND	ND
Gal *β*1-3/4 **III**	H-1,1′	–	–	–	4.864	4.967	4.636	4.600
	H-2	–	–	–	3.563	3.677	3.695	3.687
	H-3	–	–	–	3.689	3.901	3.817	3.778
	H-4	–	–	–	3.895	3.848	4.173	4.142
	H-5	–	–	–	3.662	3.637	3.68	3.68
	H-6,6′	–	–	–	~ 3.83	~ 3.8	ND	ND
Gal-3-S *β*1-4 **III**	H-1,1′	4.592	4.700	4.710	4.730	4.730	–	–
	H-2	ND	3.739	3.725	3.816	3.800	–	–
	H-3	4.348	4.342	4.236	4.334	4.339	–	–
	H-4	4.274	4.276	4.276	4.297	4.303	–	–
	H-5	ND	3.715	3.719	3.741	~ 3.7	–	–
	H-6,6′	ND	~ 3.65	~ 3.8	~ 3.8	~ 3.8	–	–
GlcA *β*1-3 **IV**	H-1,1′	–	–	–	–	–	4.790	4.755
	H-2	–	–	–	–	–	3.560	3.552
	H-3	–	–	–	–	–	3.730	3.726
	H-4	–	–	–	–	–	3.530	3.528
Fuc *α*1-2 **F**^{2,3}	H-1,1′	–	–	5.286	5.406	5.350	5.345	5.273
	H-2	–	–	3.807	3.792	3.768	3.788	3.816
	H-3	–	–	3.926	3.925	3.889	3.91	3.925
	H-4	–	–	3.832	3.803	3.782	3.78	3.831
	H-5	–	–	4.282	4.289	~ 4.30	4.277	4.285
	CH_3	–	–	1.242	1.237	1.270	1.235	1.244
Fuc *α*1-2 **F**^{2,3,3}	H-1,1′	–	–	–	–	5.456	5.336	5.336
	H-2	–	–	–	–	3.769	3.788	3.788
	H-3	–	–	–	–	3.917	3.907	3.907
	H-4	–	–	–	–	3.975	3.831	3.831
	H-5	–	–	–	–	4.514	4.545	4.561
	CH_3	–	–	–	–	1.225	1.221	1.226

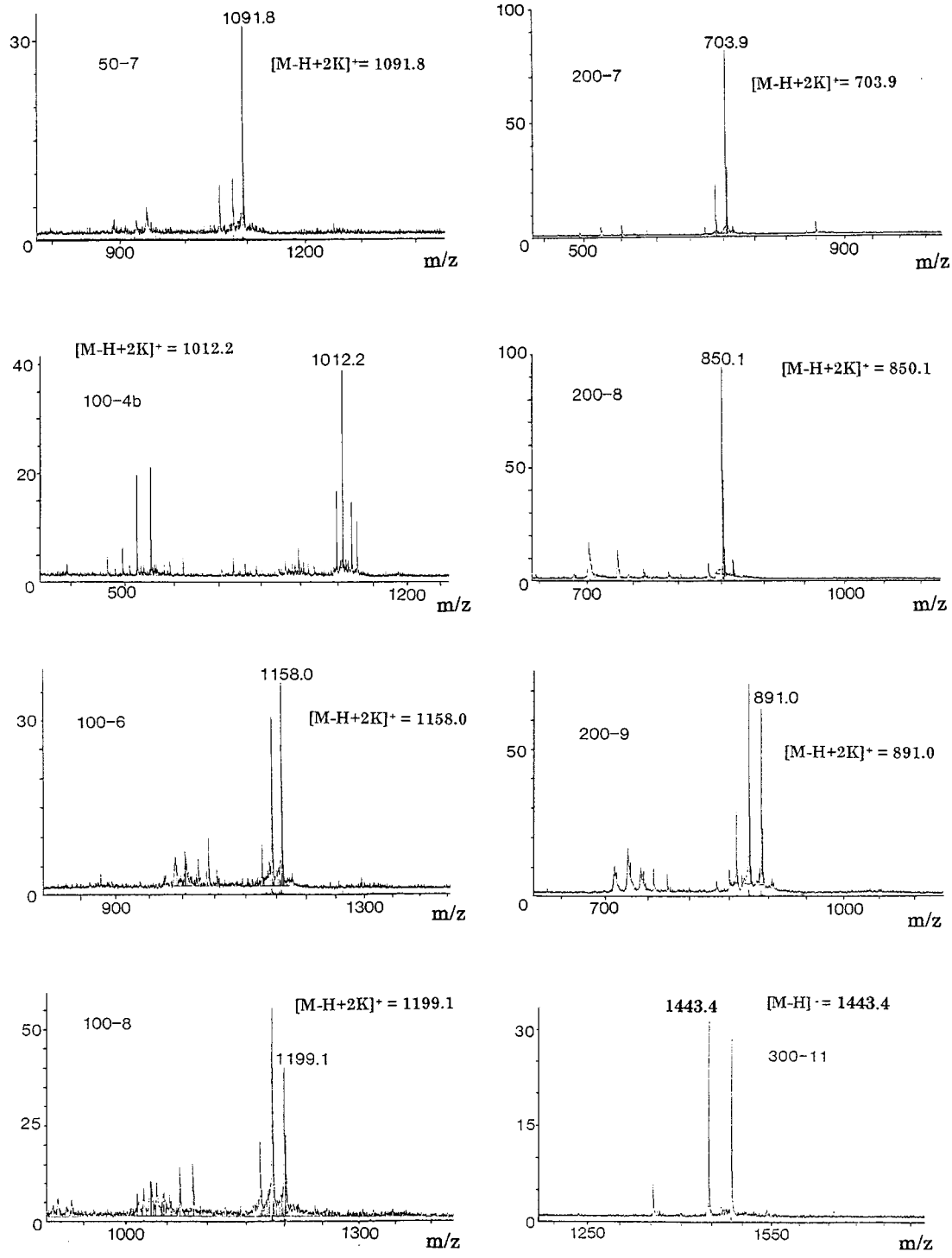


Figure 6. MALDI analyses of acidic oligosaccharide-alditols. Analyses were performed in the positive mode (pseudo-molecular ion $[M\text{-}H + 2K]^+$, except for compound 300-11.

The MALDI analysis of compound **100-8** shows a $[M\text{-}H + 2K]^+$ at *m/z* 1199 (Fig. 6), which is in agreement with the following molar proportions: GlcNAc, Gal, Fuc, GalNAc and sulfate (1:2:2:1:1). The homo and heteronuclear NMR spectra (Fig. 13; Tables 3 and 6) confirm the presence of a sulfate group at O-6 of GlcNAc, owing to its characteristic H-6 and C-6 atom resonances. The downfield shift of the GlcNAc C-3 resonance is attributable to the presence of the α-1,3-linked fucose unit. The second branch of the glycan contains two galactose units. The former (Gal II) is

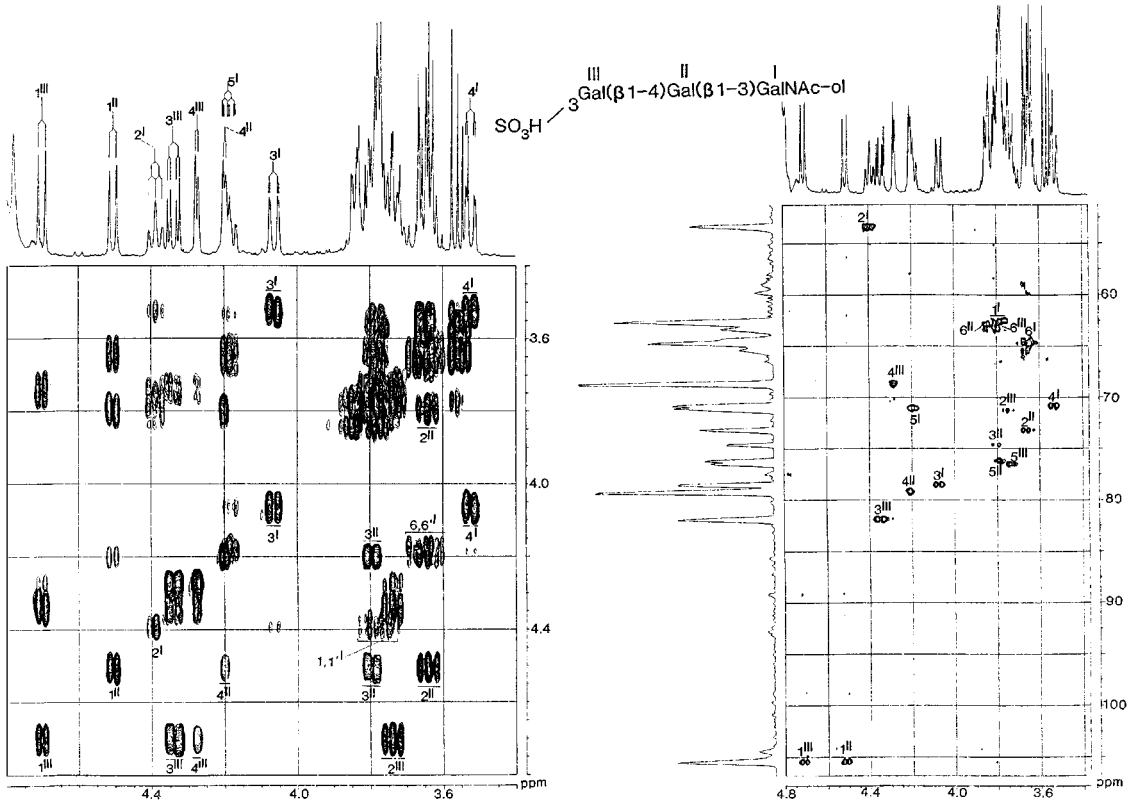


Figure 7. Homonuclear and heteronuclear COSY spectra of compound **200-7**.

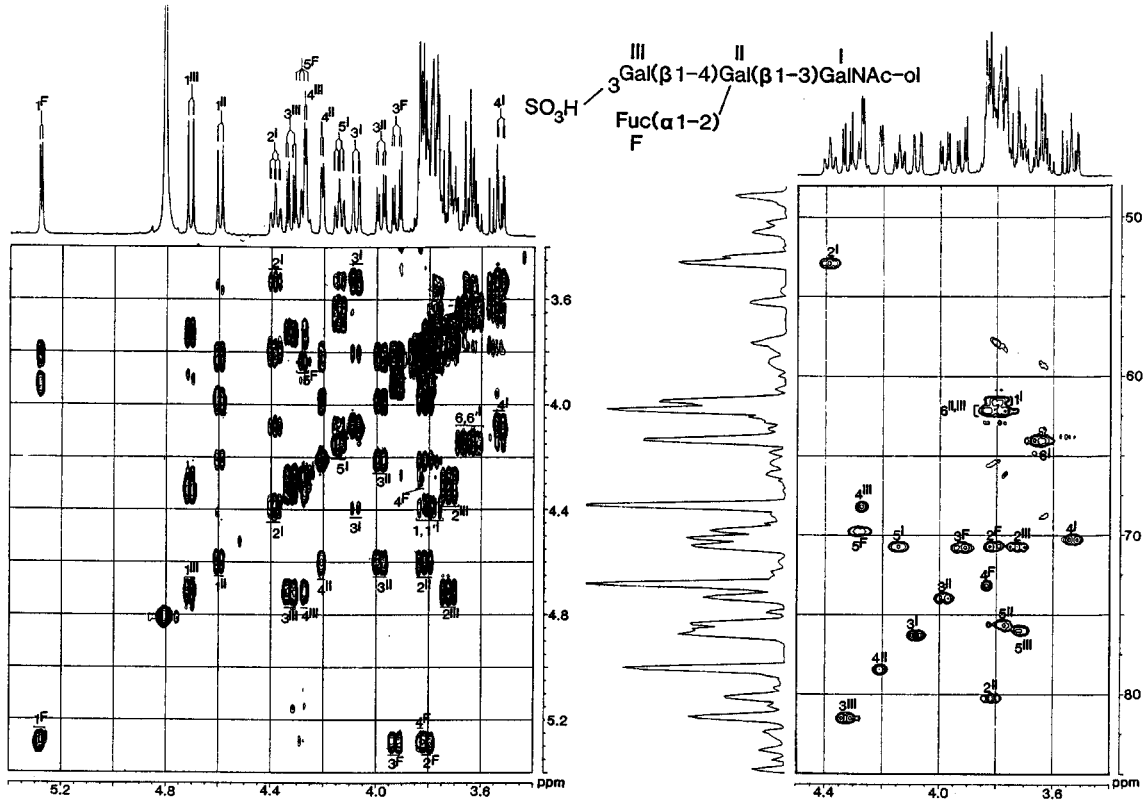


Figure 8. Homonuclear and heteronuclear COSY spectra of compound **200-8**.

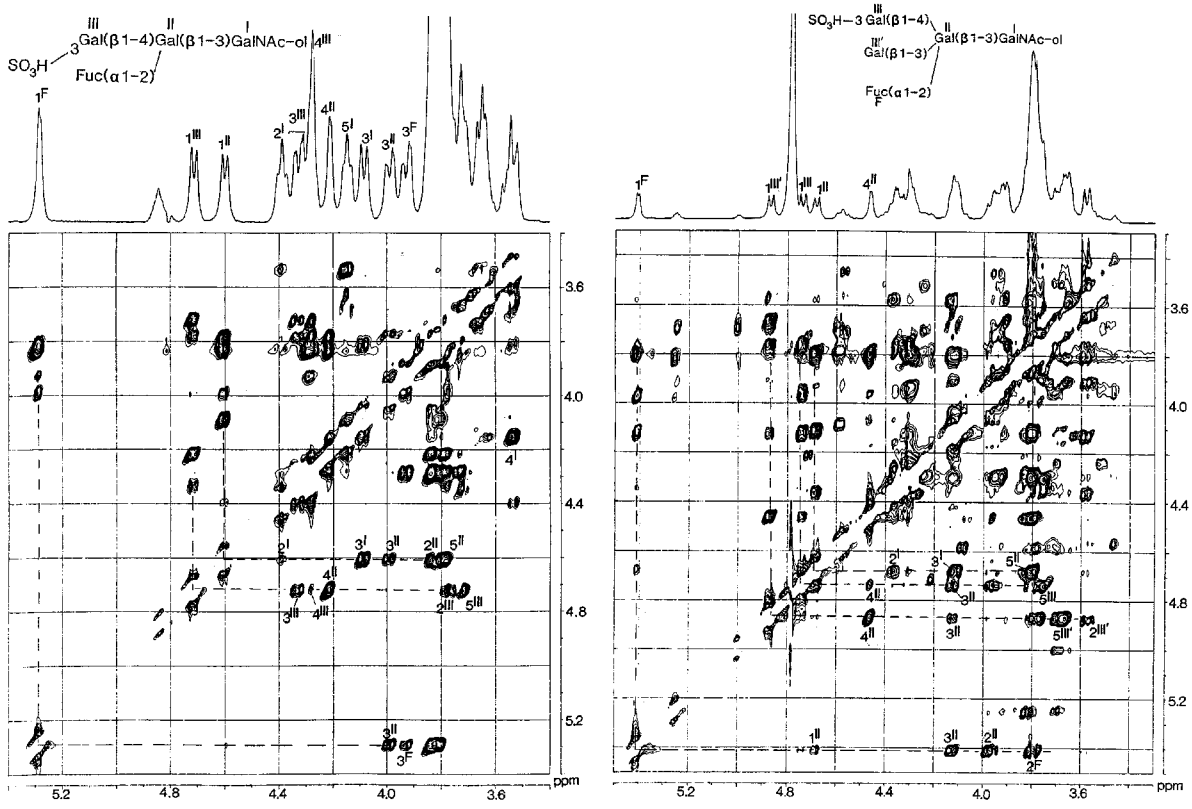


Figure 9. ROESY spectra of compounds **200-8** and **100-4b**.

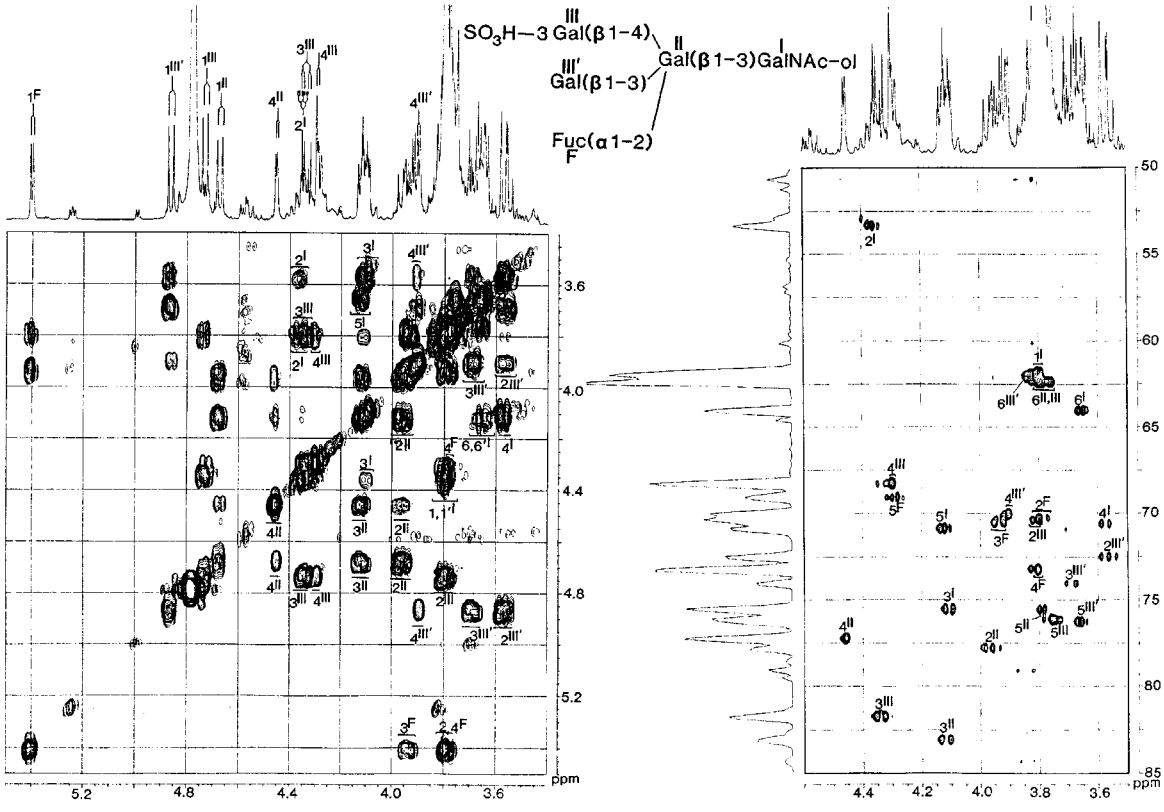


Figure 10. Homonuclear and heteronuclear COSY spectra of compound **100-4b**.

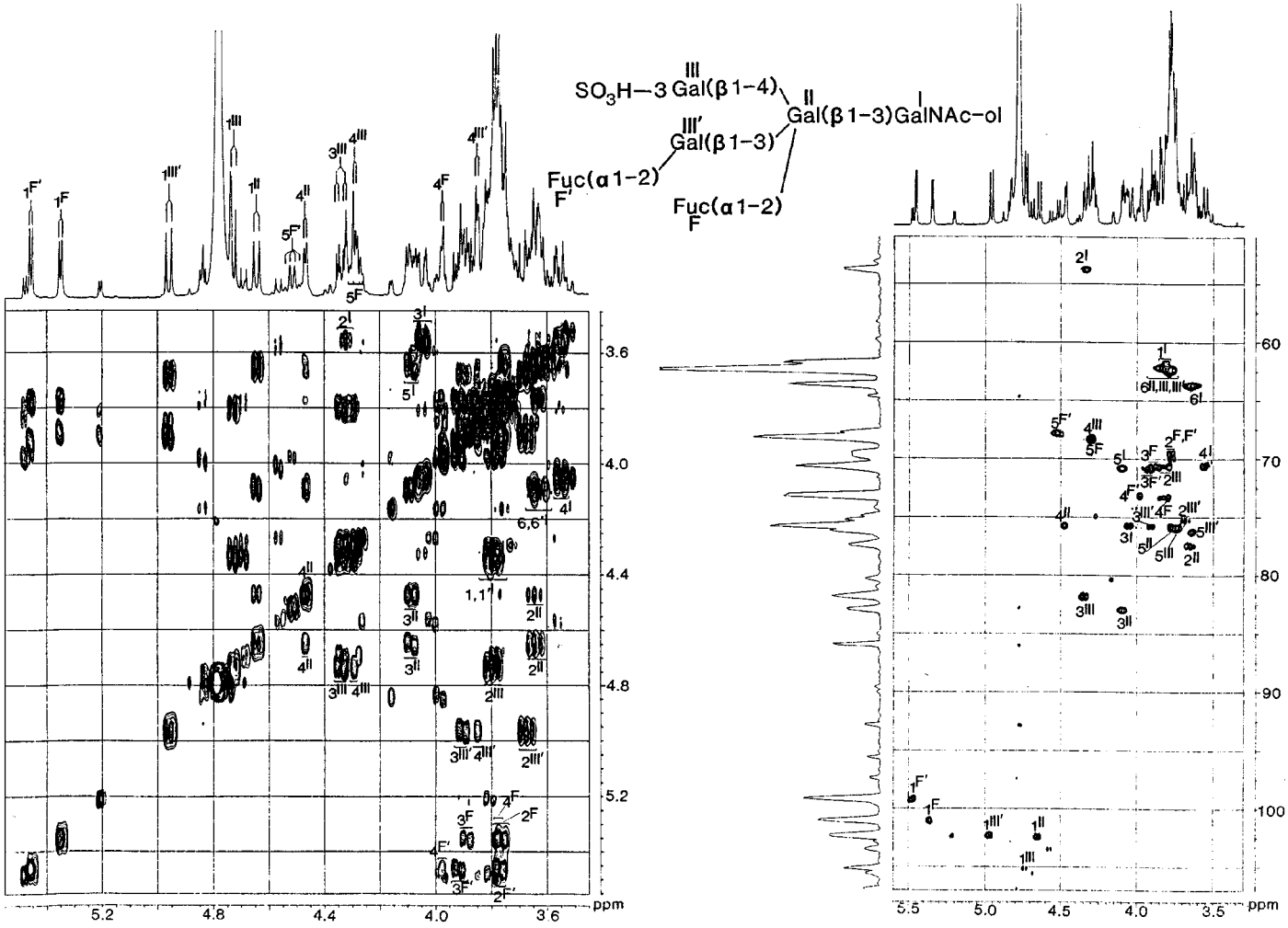
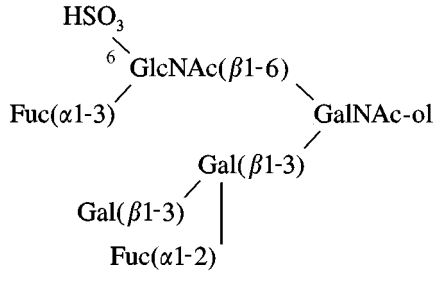


Figure 11. Homonuclear and heteronuclear COSY spectra of compound **100-6**.

substituted at both C-2 (δC-2 = 77.44) and C-3 (δC-3 = 83.37), whereas the latter occurs in a terminal non-reducing position, since none of its ^{13}C resonance is deshielded. On the basis of these observations, the structure of **100-8** was deduced to be the following:



100-8

With the pseudomolecular ion $[M\text{-}H + 2K]^+$ at *m/z* 1092 (Fig. 6), compound **50-7** can be considered as a hexasaccharide-alditol composed of Gal, Fuc, GalNAc-ol and hexuronic acid in the molar ratio of 2:2:1:1. The examination of the NMR spectra (Figs 14, 15 and Tables 2 and 5) provides new information, and, particularly, shows that two isomers occur in the fraction. Indeed, two C-2 atom resonances, relative to GalNAc-ol, are observed at δ = 53.57 and 52.88 ppm, respectively. The hexuronic acid was identified as glucuronic acid on the basis of the set of its vicinal coupling constant ($J_{1,2} = J_{2,3} = J_{3,4} = J_{4,5}$ = 8 Hz). Actually, two glucuronic acid units can be characterized with δH-1 = 4.790, δC-1 = 103.67 for the first one, and δH-1 = 4.755, δC-1 = 103.76, for the second one. The HMQC spectrum (Fig. 15) allows us to distinguish two Gal II units, substituted at C-2 and C-3, for Gal II-A, and C-2 and C-4, for Gal II-B, respectively. In both isomers, the Gal III and GlcA IV possess the same substitution, at C-3 for the former, and C-2 for the latter. These conclusions were drawn from the observation of the chemical shifts of the corresponding ^{13}C resonances depicted in Table 5. Consequently, the two isomers can be considered as the extension of the sequences: Gal(β1-3)[Fuc(α1-2)]Gal(β1-3)GalNAc-ol (A)

Table 3. ^{1}H-NMR chemical shifts of the oligosaccharide-alditols (300-5), (200-9), (100-8) and (300-11) containing core type 2. The monosaccharides are represented by this symbolic notation: ◇-ol, GalNAc-ol; ■, *β*Gal; ⊗, GlcA; S-●, GlcNAc-6-S, □, Fuc. ND, not determined. The linkage position is specified by the direction of the connecting bars as follows: 

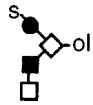
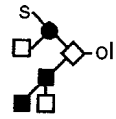
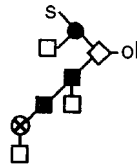
Residue	*Reporter group*	*Chemical shifts (ppm) in*			
					
		300-5	200-9	100-8	300-11
GalNAc-ol **I**	H-1,1′	ND	ND	3.79	3.78
	H-2	4.389	4.393	4.335	4.342
	H-3	4.060	4.079	4.085	4.095
	H-4	3.473	3.512	3.57	3.560
	H-5	4.271	ND	4.198	4.204
	H-6,6′	3.939	3.73	3.93/3.70	3.933/3.689
	NAc	2.067	2.053	2.050	2.053
Gal *β*1-3 **II**	H-1,1′	4.463	4.577	4.660	4.658
	H-2	ND	3.684	3.786	3.781
	H-3	ND	3.876	4.008	4.012
	H-4	3.898	3.916	4.219	4.211
	H-5	ND	ND	∼ 3.76	3.75
	H-6,6′	ND	ND	∼ 3.75	3.74
Gal *β*1-3 **III**	H-1	–	–	4.615	4.630
	H-2	–	–	3.613	3.694
	H-3	–	–	3.65	3.820
	H-4	–	–	3.920	4.169
	H-5	–	–	∼ 3.68	3.69
	H-6,6′	–	–	∼ 3.75	3.74
GlcNAc-6-S *β*1-6**II**′	H-1,1′	4.559	4.575	4.592	4.594
	H-2	ND	3.728	3.876	3.856
	H-3	ND	3.56	3.650	3.665
	H-4	ND	3.587	3.621	3.611
	H-5	ND	ND	3.685	3.685
	H-6,6′	4.360/4.233	4.360/4.233	4.261/4.246	4.368/4.256
	NAc	2.065	2.059	2.037	2.041
GlcA *β*1-3 **IV**	H-1,1′	–	–	–	4.784
	H-2	–	–	–	3.554
	H-3	–	–	–	3.732
	H-4	–	–	–	3.71
	H-5	–	–	–	3.70
Fuc *α*1-2 **F**^{2,3}	H-1,1′	–	5.245	5.380	5.335
	H-2	–	3.804	3.80	3.787
	H-3	–	3.893	3.89	3.886
	H-4	–	3.824	3.800	3.802
	H-5	–	4.305	ND	4.268
	CH_3	–	1.242	1.229	1.234
Fuc *α*1-2 **F**^{2,3,3}	H-1,1′	–	–	–	5.335
	H-2	–	–	–	3.787
	H-3	–	–	–	3.913
	H-4	–	–	–	3.836
	H-5	–	–	–	4.545
	CH_3	–	–	–	1.222
Fuc *α*1-3 **F**^{3,6}	H-1,1′	–	–	4.986	4.990
	H-2	–	–	3.687	3.687
	H-3	–	–	3.837	3.837
	H-4	–	–	3.800	3.800
	H-5	–	–	ND	4.327
	CH_3	–	–	1.158	1.161

Table 4. ^{13}C-NMR chemical shifts of the oligosaccharide-alditols (N-III-1a), (50-5a), and (50-5b) containing core type 1 and 8. The monosaccharides are represented by this symbolic notation: ◇-ol, GalNAc-ol; ■, *β*Gal; ■, *α*Gal; ▲, Kdn; □, Fuc. ND, not determined. The linkage position is specified by the direction of the connecting bars as follows: 

Residue	*Reporter group*	*Chemical shifts (ppm) in*		
				
		N-III-2	50-5a	50-5b
GalNAc-ol **I**	C-1	62.34	61.66	61.60
	C-2	53.31	52.68	53.60
	C-3	78.19	77.26	78.50
	C-4	71.11	70.47	71.40
	C-5	71.19	69.23	69.86
	C-6	64.31	66.96	66.96
	CH_3	23.35	23.27	23.42
Gal *β*1-3 **II**	C-1	–	105.25	–
	C-2	–	72.43	–
	C-3	–	73.74	–
	C-4	–	69.88	–
	C-5	–	76.44	–
	C-6	–	62.18	–
Gal *α*1-3 **II**	C-1	100.78	–	100.61
	C-2	69.53	–	79.15
	C-3	70.50	–	70.55
	C-4	70.39	–	70.97
	C-5	72.99	–	73.24
	C-6	62.23	–	62.18
Fuc *α*1-2 **F**	C-1	–	–	101.40
	C-2	–	–	69.37
	C-3	–	–	70.57
	C-4	–	–	73.18
	C-5	–	–	68.42
	C-6	–	–	16.80
Kdn *α*2-6 **K**	C-1	–	ND	ND
	C-2	–	ND	ND
	C-3	–	41.12	41.12
	C-4	–	71.52	71.42
	C-5	–	71.29	71.29
	C-6	–	74.94	74.94
	C-7	–	69.27	69.27
	C-8	–	73.43	73.48
	C-9	–	64.06	64.06

and Gal(*β*1-4)[Fuc(α1-2)]Gal(*β*1-3)GalNAc-ol (B) with the same terminal disaccharide Fuc(α1-2)GlcA(*β*1-3). The exact assignment of NMR parameters relative to GlcA IV (A) and IV (B) is based on the presence of the sequence A in compound **300-11** (see below). On the basis of these observations, the structures of the oligosaccharide-alditols present in the fraction **50-7** were deduced to be the following:

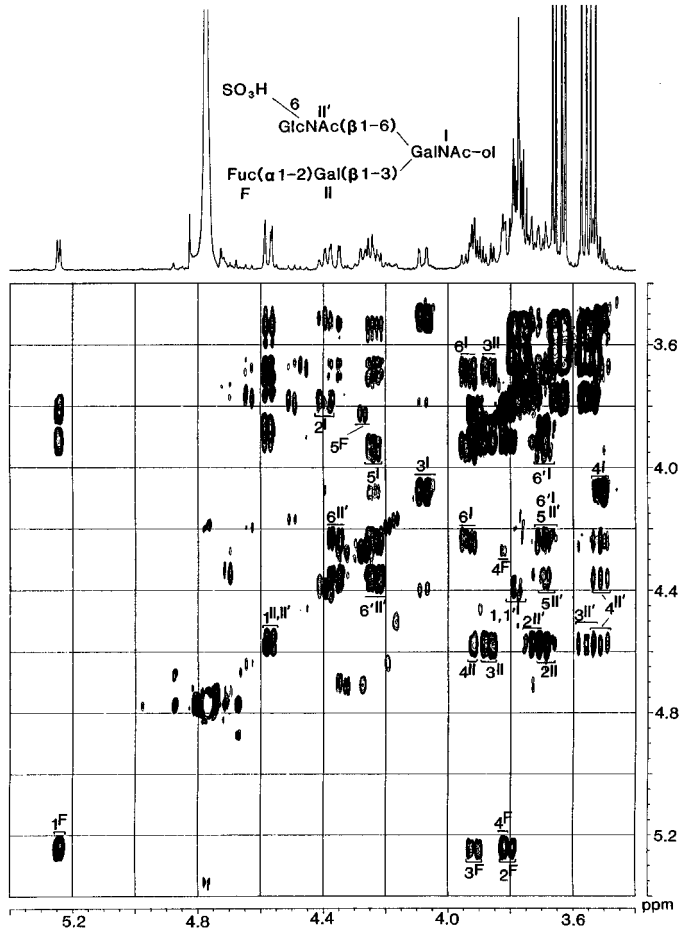
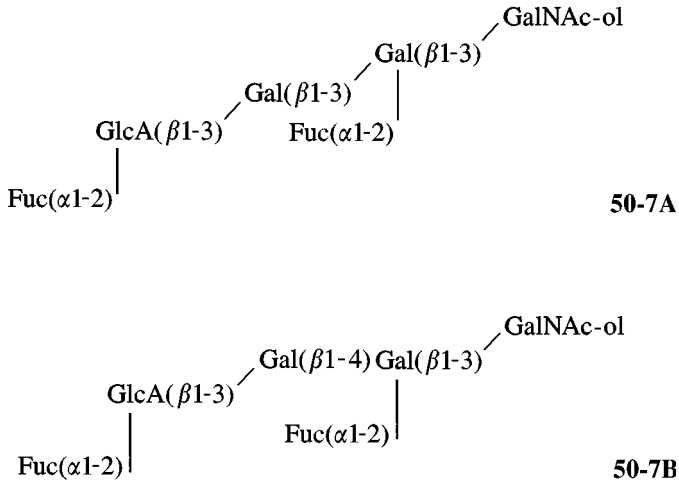


Figure 12. COSY spectrum of compound **200-9**.



50-7A

50-7B

The pseudo molecular ion $[M-H]^-$ observed at *m/z* 1443 (Fig. 6) indicated that compound **300-11** is an octasaccharide-alditol composed of Gal, Fuc, hexuronic acid, HexNAc,

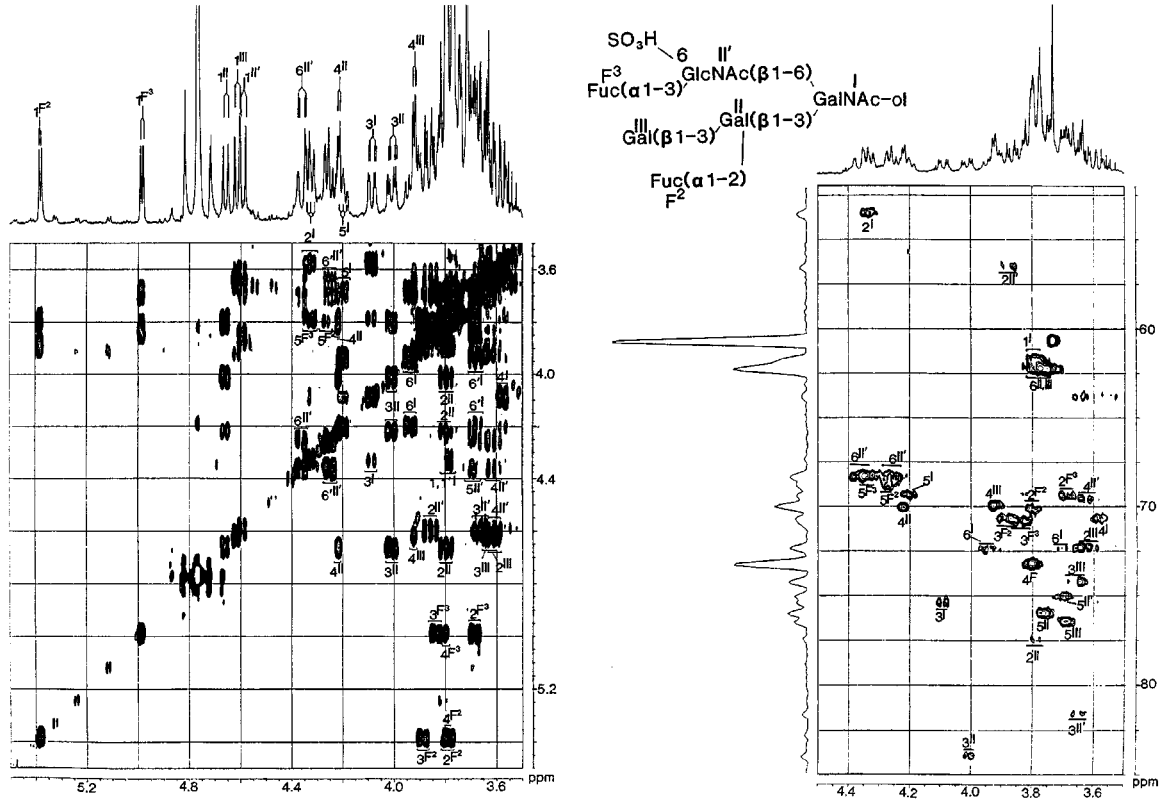


Figure 13. Homonuclear and heteronuclear COSY spectra of compound **100-8.**

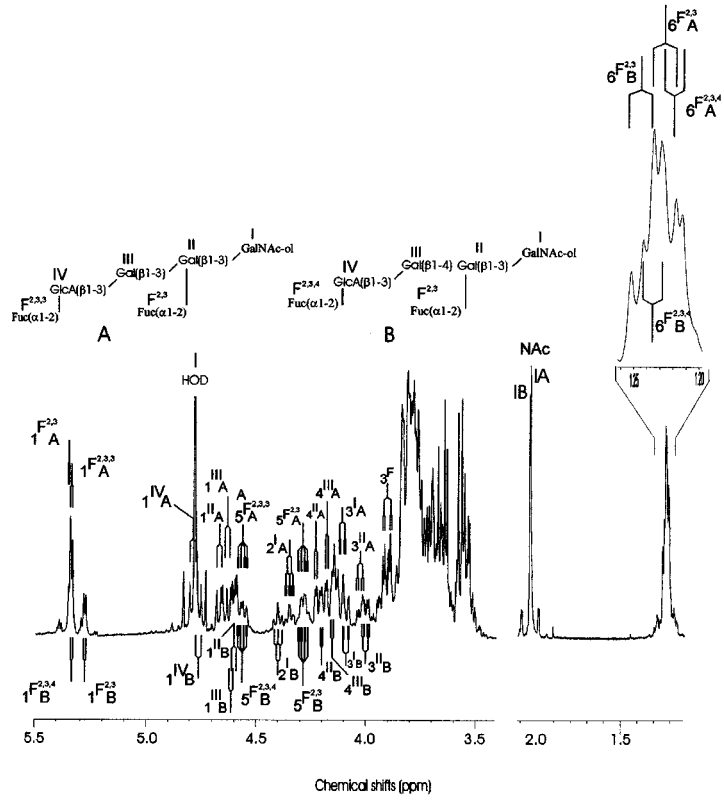
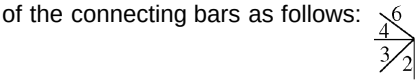
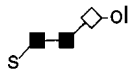
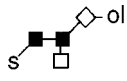
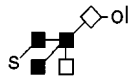
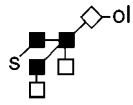
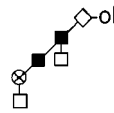
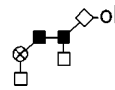


Figure 14. 1H-NMR spectrum of fraction **50-7**.

Table 5. ^{13}C-NMR chemical shifts of the oligosaccharide-alditols (200-7), (200-8), (100-4b), (100-6), (50-7A) and (50-7B) containing core type 1. The monosaccharides are represented by this symbolic notation: ◇-ol, GalNAc-ol; ⊗, GlcA; ■, βGal; S-■, βGal-3-S; □, Fuc. ND = not determined. The linkage position is specified by the direction of the connecting bars as follows: 

Residue	*Reporter group*	*Chemical shifts (ppm) in*					
							
		200-7	200-8	100-4b	100-6	50-7A	50-7B
GalNAc-ol **I**	C-1	~ 62	61.56	61.85	61.71	61.9	61.9
	C-2	53.35	52.84	53.28	53.70	53.57	52.88
	C-3	78.64	76.24	75.39	75.60	75.22	76.41
	C-4	70.83	70.12	70.51	70.51	70.44	70.20
	C-5	71.21	70.60	70.81	70.75	70.80	70.80
	C-6	64.8	64.03	63.98	63.64	64.0	64.0
	CH_3	23.57	23.57	23.63	23.57	23.56	23.56
Gal β1-3 **II**	C-1	105.84	103.48	102.64	102.36	102.50	103.78
	C-2	73.26	80.25	77.71	77.44	77.89	80.30
	C-3	74.5	73.91	83.07	82.97	83.67	73.86
	C-4	79.35	78.39	77.15	75.63	70.08	78.01
	C-5	76.27	75.57	75.51	75.84	76.2	76.2
	C-6	~ 62	62.08	62.30	~ 62	62.2	62.2
Gal β1-3/4 **III**	C-1	–	–	104.33	102.26	105.64	105.95
	C-2	–	–	72.44	75.19	71.85	72.03
	C-3	–	–	73.92	75.76	82.76	82.63
	C-4	–	–	69.93	70.5	69.96	70.08
	C-5	–	–	76.24	76.21	75.8	75.8
	C-6	–	–	61.94	~ 62	62.2	62.2
Gal-3-S β1-4 **III**	C-1	105.84	105.34	105.41	105.14	–	–
	C-2	71.30	70.60	73.17	70.56	–	–
	C-3	82.13	81.49	81.67	81.81	–	–
	C-4	68.76	68.11	68.18	68.14	–	–
	C-5	76.66	75.93	76.10	75.84	–	–
	C-6	~ 62	62.08	62.30	~ 62	–	–
GlcA β1-3 **IV**	C-1	–	–	–	–	103.67	103.76
	C-2	–	–	–	–	79.61	78.93
	C-3	–	–	–	–	77.5	75.5
	C-4	–	–	–	–	73.26	73.26
	C-5	–	–	–	–	77.5	77.5
	C-6	–	–	–	–	ND	ND
Fuc α1-2 $\mathbf{F}^{2,3}$	C-1	–	–	101.27	100.97	101.42	102.47
	C-2	–	–	70.24	69.58	69.68	69.68
	C-3	–	–	70.39	70.64	70.79	70.79
	C-4	–	–	73.17	73.18	73.40	73.28
	C-5	–	–	69.00	68.14	68.92	69.87
	C-6	–	–	16.77	17.09	16.8	16.8
Fuc α1-2 $\mathbf{F}^{2,3,3}$ or $\mathbf{F}^{2,3,4}$	C-1	–	–	–	99.09	100.29	100.29
	C-2	–	–	–	69.58	69.68	70.79
	C-3	–	–	–	70.81	70.79	70.79
	C-4	–	–	–	73.04	73.28	73.28
	C-5	–	–	–	67.72	68.26	68.26
	C-6	–	–	–	16.96	16.8	16.8

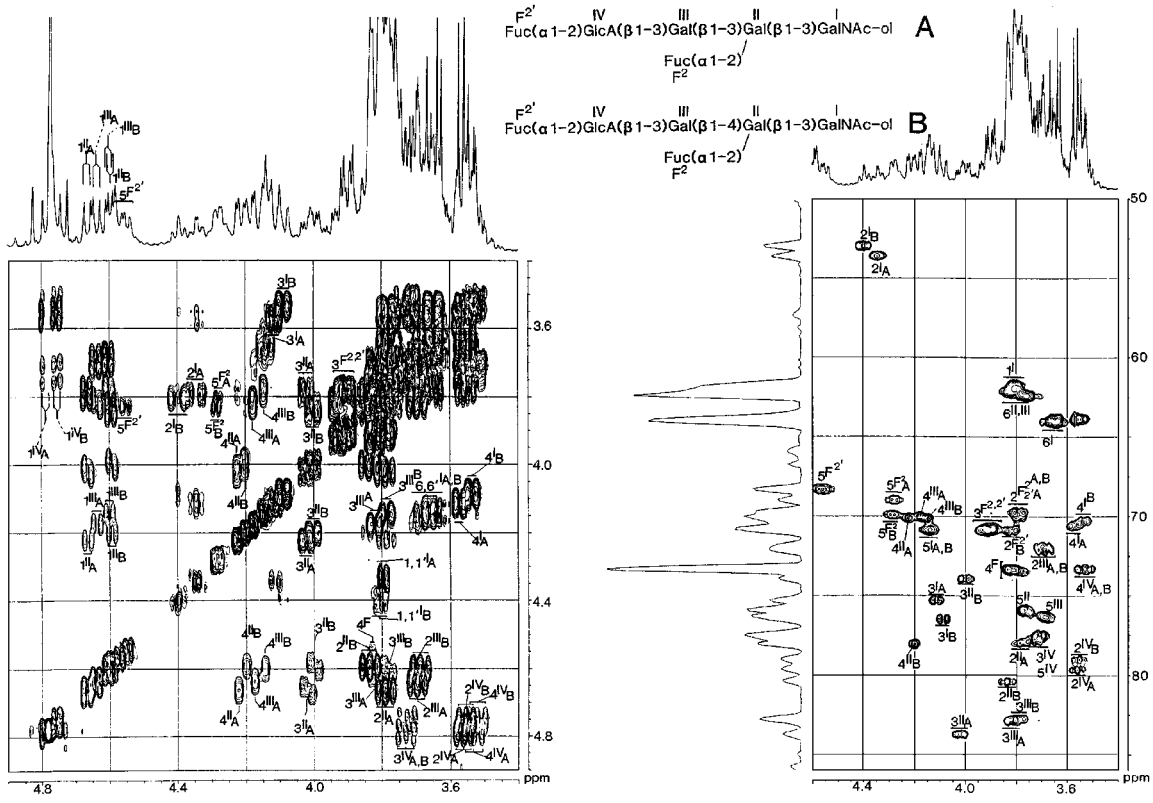


Figure 15. Homonuclear and heteronuclear COSY spectra of fraction **50-7**.

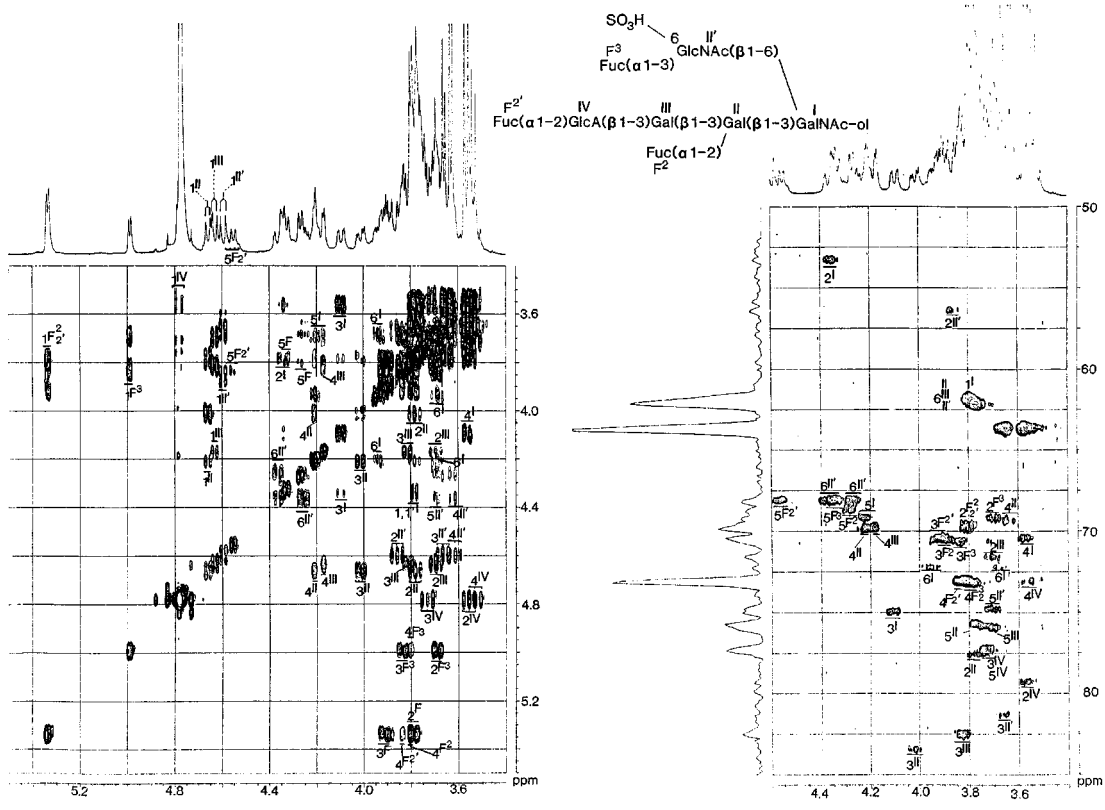
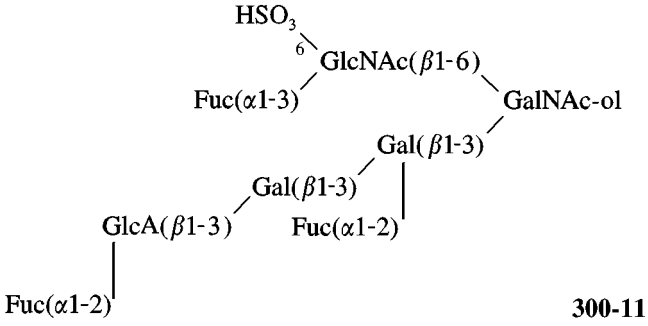


Figure 16. Homonuclear and heteronuclear COSY spectra of compound **300-11**.

GalNAc-ol and SO_3H in the molar ratio : 2:3:1:1:1:1. The NMR spectra (Fig. 16; Tables 3 and 6) clearly confirm the presence of GlcNAc and GlcA according to the set of their vicinal coupling constants. Moreover, the chemical shifts of GalNAc-ol H-6 and H-6′ are characteristic of a type 2 core structure GlcNAc(β1-6)[Gal(β1-3)]GalNAc-ol. A comparison of the spectrum of **300-11** with those of **100-8** and **50-7** indicates the presence of the sequences Fuc(α1-3)GlcNAc-6-S(β1-6) and Fuc(α1-2)GlcA(β1-3)Gal(β1-3)[Fuc(α1-2)] Gal(β1-3). This conclusion results from the observation of some significant resonances such as GlcNAc C-3 and C-6, GlcA C-2, Gal III C-3 and Gal II C-2 and C-3, which match those of the two reference compounds. On the basis of these observations, the structure of compound **300-11** was established as follows:



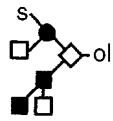
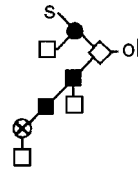
300-11

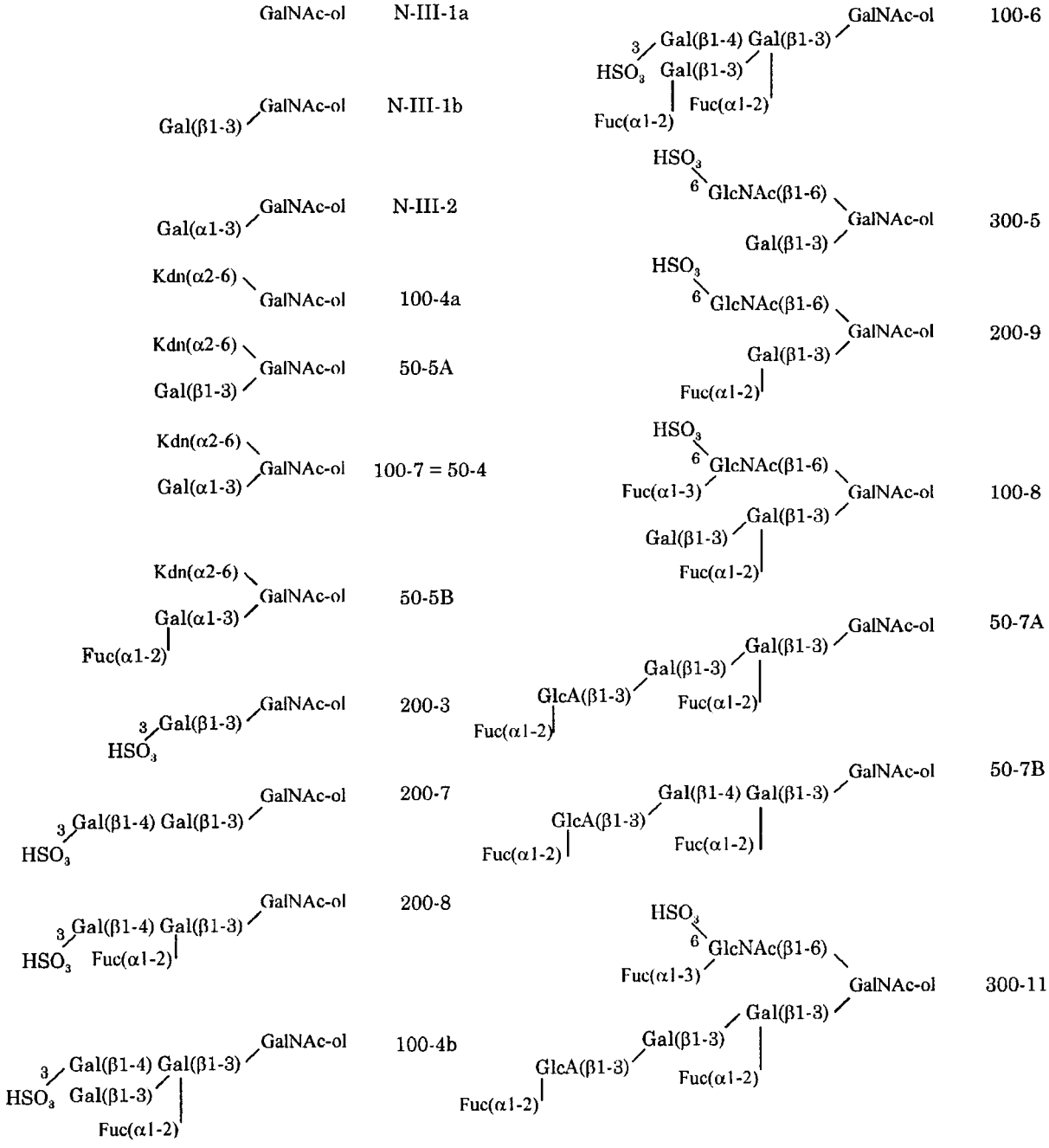
Discussion

The aim of this study was to extend the hypothesis that glycanic chains of amphibian oviducal mucins are highly species-specific. The results obtained for *Rana temporaria* confirm this point, since almost all oligosaccharide-alditols released by reductive β-elimination are novel (Scheme 1). By comparison with the six amphibian species previously examined [5–15], the main characteristics of *Rana temporaria* mucin are the following: 1) The anionic charge of the mucin is carried by Kdn, but also by sulfate and glucuronic acid; 2) the core structure Gal(α1-3)GalNAc, previously found in human bronchial mucus [23] and *Ambystoma tigrinum* mucin [14], is described here for the third time; 3) as generally occurs in carbohydrate chains released from a single species, the different structures derive one from the other according to a specific sequence of biosynthesis. For instance, compounds **200-8**, **100-4b** and **100-6** are related to **200-7** by successive addition of Fuc, Gal and Fuc again, respectively; 4) new fucosyltransferase (FucT) activities can be defined on the basis of new sequences: an α-1,2 FucT acting on terminal GlcA unit (compound **50-7A,B** and **300-11**), or an α-1,3 FucT able to act directly on terminal GlcNAc (or sulfated GlcNAc), as testified by the presence of compounds **100-8**, **200-9** and **300-11**.

By comparison with other amphibian species, at least six different fucosyltransferase activities, such as Kdn: (α1-4)

Table 6. ^{13}C-NMR chemical shifts of the oligosaccharide-alditols (100-8) and (300-11) containing core type 2. The monosaccharides are represented by this symbolic notation: ◇-ol, GalNAc-ol; ■, βGal; ⊗, GlcA; S-●, GlcNAc-6-S; □, Fuc. ND = not determined. The linkage position is specified by the direction of the connecting bars as follows: 

Residue	*Reporter group*	*Chemical shifts (ppm) in*	
			
		100-8	300-11
GalNAc-ol **I**	C-1	62.0	62.1
	C-2	53.42	53.28
	C-3	75.42	74.99
	C-4	70.63	70.46
	C-5	69.35	69.21
	C-6	72.26	72.27
	CH_3	23.6	23.5
Gal β1-3 **II**	C-1	102.37	102.33
	C-2	77.44	77.72
	C-3	83.37	83.57
	C-4	70.09	69.95
	C-5	75.93	75.76
	C-6	62.2	62.2
Gal β1-3 **III**	C-1	105.59	105.44
	C-2	72.31	71.57
	C-3	74.14	82.59
	C-4	69.90	69.95
	C-5	76.44	75.97
	C-6	62.2	62.2
GlcNAc-6-S β1-6 **II′**	C-1	102.68	102.49
	C-2	56.49	56.42
	C-3	81.54	81.38
	C-4	69.53	69.40
	C-5	74.96	74.83
	C-6	68.23	68.18
	CH_3	23.6	23.5
GlcA β1-3 **IV**	C-1	–	103.45
	C-2	–	79.36
	C-3	–	77.14
	C-4	–	73.12
	C-5	–	77.14
	C-6	–	ND
Fuc α1-2 **F**^{2,3}	C-1	101.08	101.13
	C-2	70.00	69.74
	C-3	70.61	70.45
	C-4	73.14	73.2
	C-5	68.8	68.73
	C-6	16.80	16.8
Fuc α1-2 **F**^{2,3,3}	C-1	–	100.08
	C-2	–	69.74
	C-3	–	70.70
	C-4	–	73.2
	C-5	–	68.16
	C-6	–	16.8
Fuc α1-3 **F**^{3,6}	C-1	101.31	101.15
	C-2	69.34	69.23
	C-3	70.76	70.70
	C-4	73.14	73.2
	C-5	68.20	68.14
	C-6	16.50	16.50



Scheme 1

FucT [7], Fuc(α1-4) Kdn: (α1-2) Fuc T [13], Fuc(α1-4)Kdn: (α1-3) Fuc T [7,13], Kdn: (α1-5) Fuc T [14], Gal(α1-3)Gal: (α1-2) Fuc T [10] and Gal (α1-3)[Fuc(α1-2)]Gal : (α1-2) Fuc T [10], can be identified. According to these observations, amphibian tissues could become an excellent model for studying the relation between the structure and the specificity of this class of enzyme.

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