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Carbohydrate Research 302 (1997) 179–189

CARBOHYDRATE RESEARCH

Primary structure of seven sulfated oligosaccharide-alditols released by reductive β-elimination from oviducal mucins of *Rana temporaria* Characterization of the sequence $HSO_3(3)GlcA(\beta 1-3)Gal$

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Received 17 March 1997; accepted 22 April 1997

Abstract

The mucins isolated from *Rana temporaria* egg jelly coats were found to be composed of Gal, Fuc, GlcNAc, GalNAc and GlcA acid. The primary structure of seven sulfated oligosaccharide-alditols was obtained by 1D/2D NMR analyses ($^1H-^{13}C$). The results show the presence of the sulfated monosaccharides $HSO_3(3)Gal$, $HSO_3(6)GlcNAc$ and $HSO_3(3)GlcA$. The sequence $HSO_3(3)GlcA(\beta 1-3)Gal$, which constitutes the major determinant of the HNK-1 oncofœtal epitope, was characterized. © 1997 Elsevier Science Ltd.

Keywords: Sulfated-oligosaccharides; NMR; Amphibian; Egg jelly coat

1. Introduction

The jelly envelopes deposited around amphibian eggs are composed of mucin-type glycoproteins which constitute the first barrier for fertilizating sperm. They play a role in species specificity of sperm–egg interaction, sperm capacitation, acrosomal reaction and prevention of polyspermy. The jelly coat retains Ca^{2+} ions necessary for successful fertilization and also ensures an embryonic protection [1–8].

Recent works have demonstrated that the carbohydrate chains of these mucins are highly species-specific [8–19]. We previously reported the primary structure of seventeen oligosaccharide-alditols from the oviducal mucin of *Rana temporaria*, which contained sulfate and glucuronic acid. Here we describe new compounds characterized by the presence of O-3 sulfated glucuronic acid.

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PII S0008-6215(97)00129-8

2. Experimental

The jelly coat was lyophilized and the crude material was subjected to alkaline borohydride degradation in 1 M $NaBH_4$, 0.1 M NaOH, pH 12.6 at 37°C for 48 h. The reaction was stopped by DOWEX 50W-X8 (Mesh 25–50, H^+ form) until neutral pH at 4°C. After filtration, the filtrate was adjusted at pH 6.5 and concentrated under vacuum. Boric acid formed was removed by co-distillation (3 times) with anhydrous MeOH.

The material was further fractionated on DOWEX 1-X2 (mesh 200–400, $HCOO^-$ form). After water elution of neutral compounds, acidic oligosaccharides were desorbed with pyridine acetate buffer (pH 7.6), from 50 mM to 1000 mM. Each fraction was applied to a Bio-Gel P2 column (1 × 100 cm) and eluted with water at a flow rate 15 mL h^{-1}. The acidic oligosaccharides were isolated by high performance liquid chromatography (HPLC) on primary-bond silica (Supelco LC-NH2; 4.6 mm × 25 cm, Supelco Inc., Belefonte, USA) using acetonitrile/30 mM potassium phosphate buffer pH 5.2 with a flow rate of 1 mL/min. The oligosaccharide-alditols were detected by spectroscopy UV at 206 nm. Several fractions were obtained at various concentration according to anionic charge of oligosaccharides (Fig. 1). Peaks were collected, desalted on Bio-Gel P2 (1 cm × 80 cm) and lyophilized.

Sugar analysis was carried out by GLC of trifluoroacetylated derivatives of methylglycosides formed by methanolysis in 0.5 M hydrochloric acid in MeOH at 80°C for 24 h according to Zanetta et al. [20].

The 400 MHz ^1H NMR experiments were performed on a Bruker AM-400WB spectrometer with 5 mm ^1H–^{13}C mixed probe head operating in the pulse Fourier-transform mode and controlled by an Aspect 3000 computer. After two exchanges with D_2O (99.95% atoms ^2H, Aldrich) and intermediate lyophilization, the samples were analysed with a spectral width of 3000 Hz for 16 k frequency domain points and time domain data point giving a final digital resolution of 0.365 Hz/point. The chemical shifts are given relative to sodium 4,4′ dimethyl-4-silapentane-1-sulfonate, but were actually measured relative the methyl signal of internal acetone ($\delta = 2.225$ ppm for ^1H and $\delta = 31.55$ ppm for ^{13}C) in D_2O at 300°K. The two dimensional homonuclear correlation spectroscopy (COSY) with simple and double relay transfer, 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 300 ms.

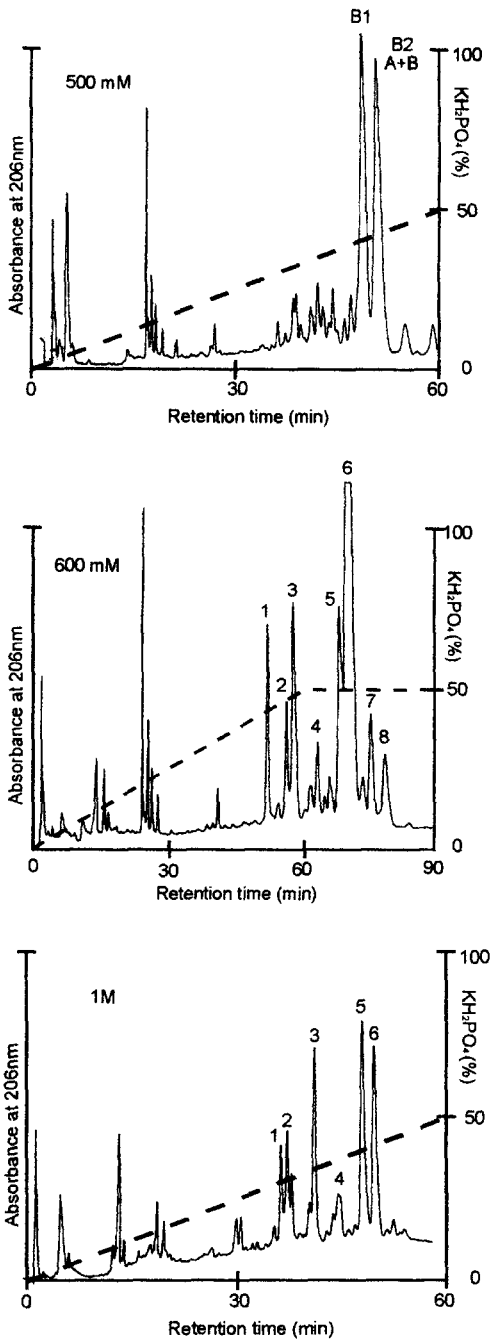


Fig. 1. HPLC profiles of oligosaccharide-alditols released from the egg jelly coat of *Rana temporaria*.

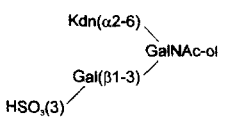
Mass measurements of matrix O-glycan-alditols were performed by matrix assisted laser desorption and time of flight mass spectrometry (MALDI-TOF) on a Vision 2000 (Finnigan Mat, Hemel) instrument in reflection mode (nitrogen laser: 337 nm). Samples were dissolved in water at a concentration of 50–100

pmol μL^{-1} and 1 μL of these solutions were mixed with 1 μL of matrix on to the target then allowed to crystallize at room temperature. The structures were mass analysed in the negative mode using 3-aminochinolin matrix (10 mg mL^{-1} in H_2O:EtOH 90:10). Ten to 15 shots were accumulated for each analysis.

3. Results and discussion

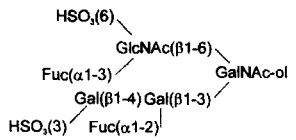
Fig. 1 depicts the HPLC profiles of fractions 500, 600 and 1000 mM desorbed from the anion exchange column. Sixteen major peaks were characterized, but several compounds were actually isolated in two different sub-fractions.

Compound 1 (600 mM-3).—From the methanolysis of compound **1** the molar ratio composition was found to be Kdn, Gal and GalNAc-ol in the molar ratio 1:1:1. The presence of sulfate was deduced from the MALDI-TOF analysis, which exhibited a pseudo molecular ion $[M - H]^-$ at *m/z* 724. The sulfate was discriminated from phosphate on the basis of the absence of coupling between proton and phosphorus. The O-6 substitution of the GalNAc unit with Kdn is clearly defined by the characteristic H-3 ax and H-3 eq signals of the Kdn residue as well as the significant upfield shifted value of the GalNAc-ol H-6b resonance (Fig. 2 and Table 1) [18]. The O-3 sulfation of the Gal unit is deduced from the set of its H-3 and H-4 signals [21]. So, the sequence of compound **1** was determined as follows:



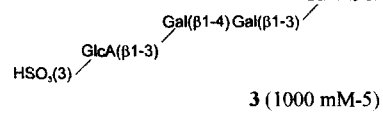
1 (600 mM-3)

Compound 2 (600 mM-1).—The chemical shifts (Fig. 2 and Table 1) of the H-2 and H-5 atom resonances of the GalNAc-ol unit are characteristic of an O-3 and O-6 substituted unit (type 2 core). The structural reporter groups of the Gal residues are superimposable on those observed in the sequence HSO_3(3)Gal(β1–4)[Fuc(α1–2)]Galβ1–3 previously described [18]. The second branch of the compound was characterized by NMR signals already observed in two compounds which possess the common sequence: HSO_3(6)[Fuc(α1–3)]GlcNAc(β1–6) [19]. Therefore, the sequence of compound 600 mM-1 was established as follows:



2 (600 mM-1)

Compound 3 (compound 1000 mM-5).—From the MALDI-TOF analysis, compound **3** contains 2 Gal, 1 GlcA, 1 GalNAc-ol and 1 sulfate residue. The structural reporter groups of GalNAc-ol (H-2, H-4 and H-5) are consistent with an O-3 substituted hexosaminitol unit (Fig. 2 and Table 1). Comparison of the NMR spectrum of **3** with that of compound 200-7 previously identified as HSO_3(3)Gal(β1–4)Gal(β1–3)GalNAc-ol [18] shows similar chemical shifts for the Gal linked to GalNAc-ol, particularly the H-1 and H-4 signals which are respectively observed at $\delta = 4.497$ (H-1, **3**), $\delta = 4.503$ (H-1, 200-7), $\delta = 4.185$ (H-4; **3**) and 4.200 (H-4, 200-7). The attachment of GlcA at O-3 of Gal III is deduced from the chemical shifts of Gal III H-4 signal at $\delta = 4.165$. Indeed, the H-3 and H-4 signals of Gal O-3 substituted with GlcA have been observed at $\delta = 3.82$, 3.78 or 3.82 (H-3) and $\delta = 4.173$, 4.142 or 4.169 (H-4) for compounds having the common sequence Fuc(α1–2)GlcA(β1–3)Gal [18]. Such an observation was also found for compound **4** (see below), for which a complete NMR assignment has been performed by ^1H–^1H correlation spectroscopy. The signal observed at $\delta = 4.327$ ppm was ascribed to GlcA H-3, according to the comparison of the spectra of **4** and **5** and this downfield shifted value can be easily interpreted as resulting from the O-3 sulfation of the Glucuronic acid unit. Consequently, the sequence of **3** was deduced to be following:



3 (1000 mM-5)

Compound 4 (600 mM-6).—From the two-step relayed COSY spectrum depicted in Fig. 3 and Table 1, compound **4** can be defined as an extension of **3** with an additional αFuc, attached at O-2 of Gal II unit. Indeed, the Chemical shifts of the H-2, H-3 and H-4 of this Gal unit perfectly match those of the

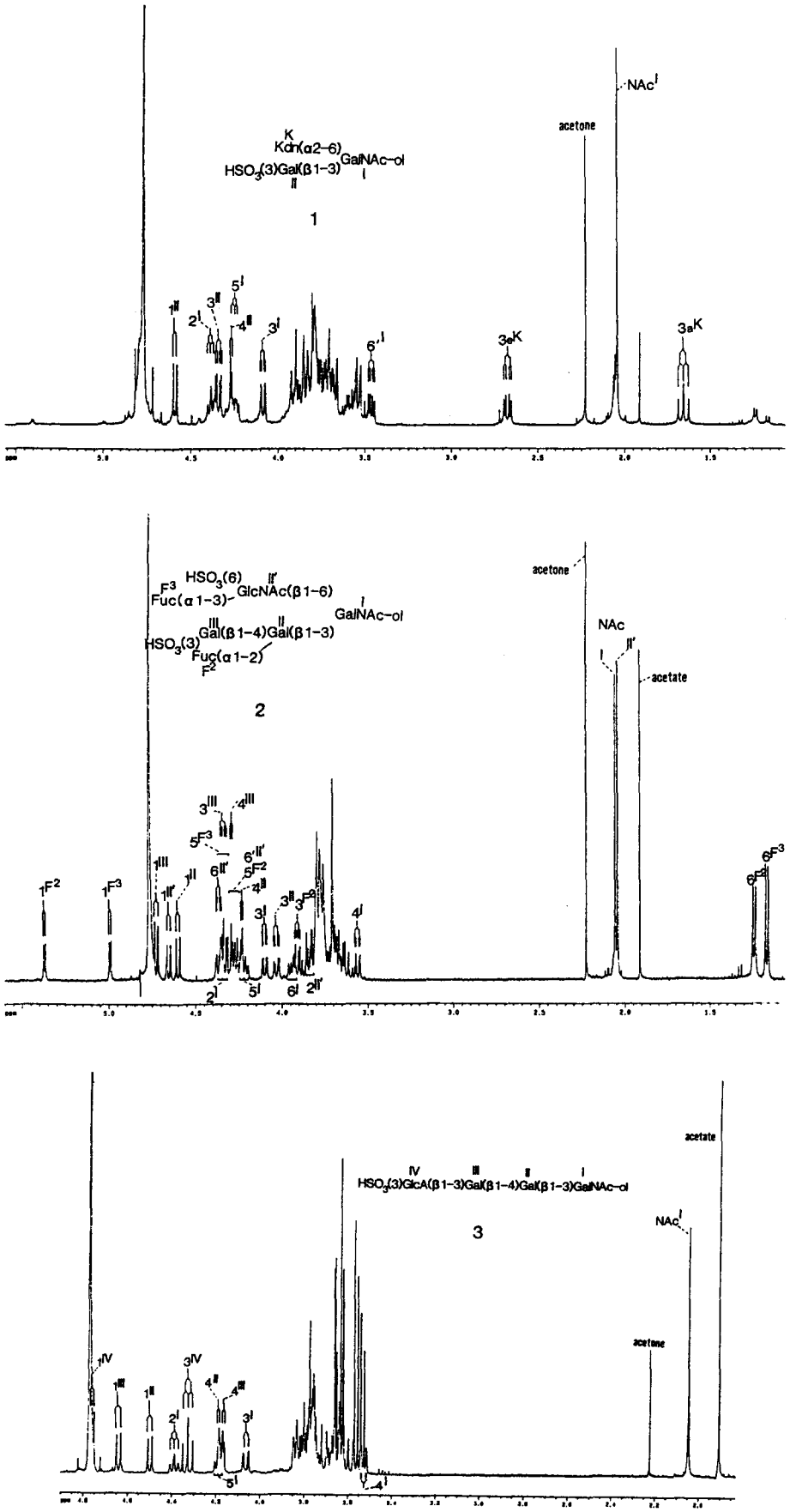
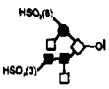
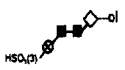
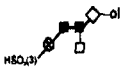
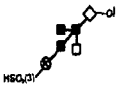


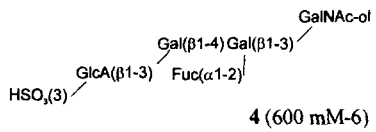
Fig. 2. ^{1}H NMR spectra of compounds **1**, **2**, **3**.

Table 1
^{1}H NMR chemical shifts of the sulfated oligosaccharide-alditols[a]

		 1	 2	 3	 4	 5	 6	 7
GalNAc-ol I	H-1a,1b	n.d.	n.d.	n.d.	3.788	3.806	3.792	3.792
	H-2	4.383	4.348	4.387	4.389	4.388	4.332	4.332
	H-3	4.084	4.098	4.063	4.079	4.080	4.104	4.057
	H-4	n.d.	3.556	3.531	3.528	3.527	3.570	3.534
	H-5	4.244	4.210	4.19	4.150	4.088	4.120	4.120
	H-6a,6b	n.d./3.462	3.940	n.d.	3.676/3.630	3.665/3.636	3.672/3.644	3.672/3.644
	NAc	2.043	2.056	2.049	2.044	2.044	2.047	2.051
Gal II	H-1	4.588	4.698	4.497	4.587	4.591	4.680	4.576
	H-2	n.d.	n.d.	n.d.	3.824	3.816	3.953	3.756
	H-3	4.340	4.030	n.d.	3.972	3.970	4.108	3.967
	H-4	4.269	4.233	4.185	4.193	4.180	4.451	4.138
	H-5	n.d.	n.d.	n.d.	3.760	3.761	n.d.	n.d.
Gal III	H-1	–	4.725	4.639	4.653	4.690	4.700	4.627
	H-2	–	n.d.	n.d.	3.743	3.851	3.781	3.635
	H-3	–	4.336	n.d.	3.810	3.903	3.810	3.857
	H-4	–	4.290	4.165	4.157	3.882	4.187	4.393
Gal V	H-1	–	–	–	–	4.811	4.869	4.954
	H-2	–	–	–	–	3.544	3.558	3.678
	H-3	–	–	–	–	3.693	3.696	3.914
	H-4	–	–	–	–	3.894	3.895	3.862
GlcNAc II′	H-1	–	4.651	–	–	–	–	–
	H-2	–	3.841	–	–	–	–	–
	H-6a	–	4.368	–	–	–	–	–
	H-6b	–	4.280	–	–	–	–	–
	NAc	–	2.041	–	–	–	–	–
GlcA IV	H-1	–	–	4.761	4.761	4.752	4.775	4.719
	H-2	–	–	n.d.	3.589	3.612	3.612	3.636
	H-3	–	–	4.327	4.330	4.329	4.335	4.333
	H-4	–	–	n.d.	3.695	3.765	3.701	3.771
	H-5	–	–	n.d.	3.791	3.772	3.798	3.798
Fuc II′	H-1	–	4.990	–	–	–	–	–
	H-5	–	4.348	–	–	–	–	–
	CH_3	–	1.163	–	–	–	–	–
Fuc II	H-1	–	5.371	–	5.285	5.286	5.397	5.273
	H-2	–	n.d.	–	3.808	3.807	3.780	3.803
	H-3	–	3.912	–	3.923	3.919	3.928	3.913
	H-4	–	n.d.	–	3.831	3.825	3.799	3.831
	H-5	–	4.281	–	4.281	4.277	4.285	4.513
	CH_3	–	1.238	–	1.241	1.241	1.238	1.277
Fuc V	H-1	–	–	–	–	–	–	5.443
	H-2	–	–	–	–	–	–	3.78
	H-3	–	–	–	–	–	–	3.995
	H-4	–	–	–	–	–	–	3.826
	H-5	–	–	–	–	–	–	4.285
	CH_3	–	–	–	–	–	–	1.238
Kdn	H-3a	1.654	–	–	–	–	–	–
	H-3e	2.673	–	–	–	–	–	–

[a] The monosaccharides are represented by this symbolic notation: ◇–ol = GalNAc-ol, △ = αKdn, ■ = βGal, □ = αFuc, ⊗ = βGlcA, and ● = βGlcNAc. The linkage position is specified by the direction of the connecting bars as follows: and n.d. = not determined.

signals observed for compound 200-8 (HSO_3(3)-Gal(β1–4)[Fuc(α1–2)]Gal(β1–3)GalNAc-ol) previously described [18]. As discussed above for **3**, the structural reporter groups of Gal III are typical of an O-3 substitution with GlcA, itself sulfated at O-3 ($\delta_{H\text{-}3} = 4.330$). From these data, the sequence of **4** was established as follows:



4 (600 mM-6)

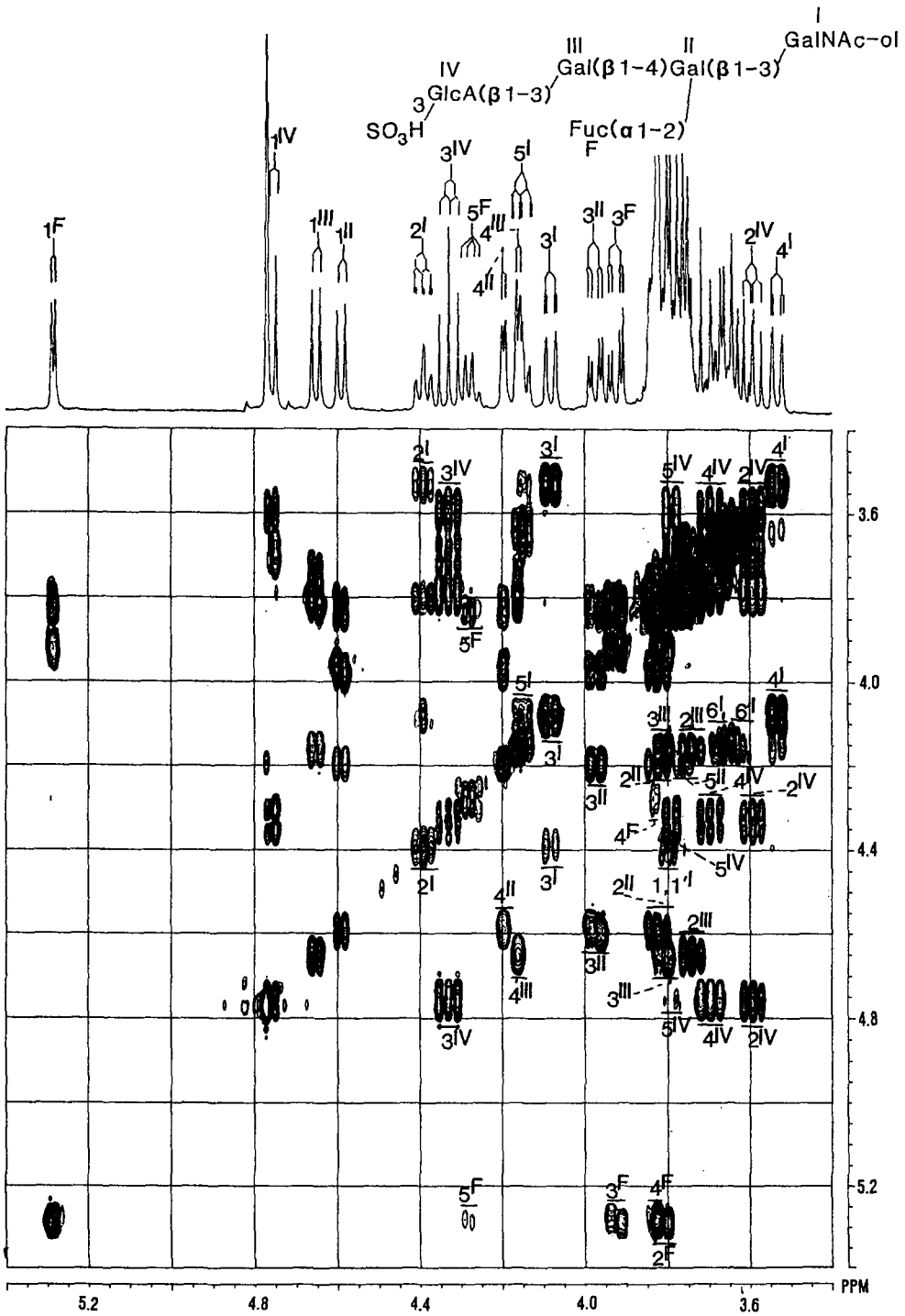
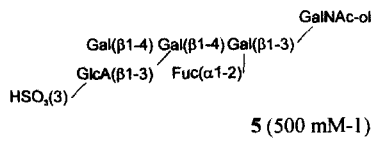


Fig. 3. COSY spectrum of compound **4**.

Compound **5** *(500 mM-1).*—The 2D NMR spectrum (Fig. 4 and Table 1) of **5** shows the presence of 3 Gal (II, III, V), 1 GlcA (IV), 1 GalNAc-ol (I) and 1 Fuc (F) units. The O-3 sulfation of GlcA was verified because of the downfield shifted value of its H-3 signal ($\delta = 4.329$). By comparison of the spectrum with that of **4**, a deshielding effect can be observed which affects the Gal III H-4 signal ($\Delta\delta = +0.275$ ppm), and shows the possible O-4 substitution with the additional Gal V unit. As indicated by the set of the H-2, H-3 and H-4 atom resonances the Gal V unit occurs in a terminal position. The NOE contacts which are observed on the ROESY spectrum are: Fuc H-1 → Gal II H-2; Fuc H-1 → Gal II H-3; Gal II H-1 → GalNAc-ol H-2; Gal II H-1 → GalNAc-ol H-3; Gal III H-1 → Gal II H-4 (strong); GlcA IV H-1 → Gal III H-3 and Gal V H-1 → Gal III H-4. On the basis of these data, the following sequence was deduced:



5 (500 mM-1)

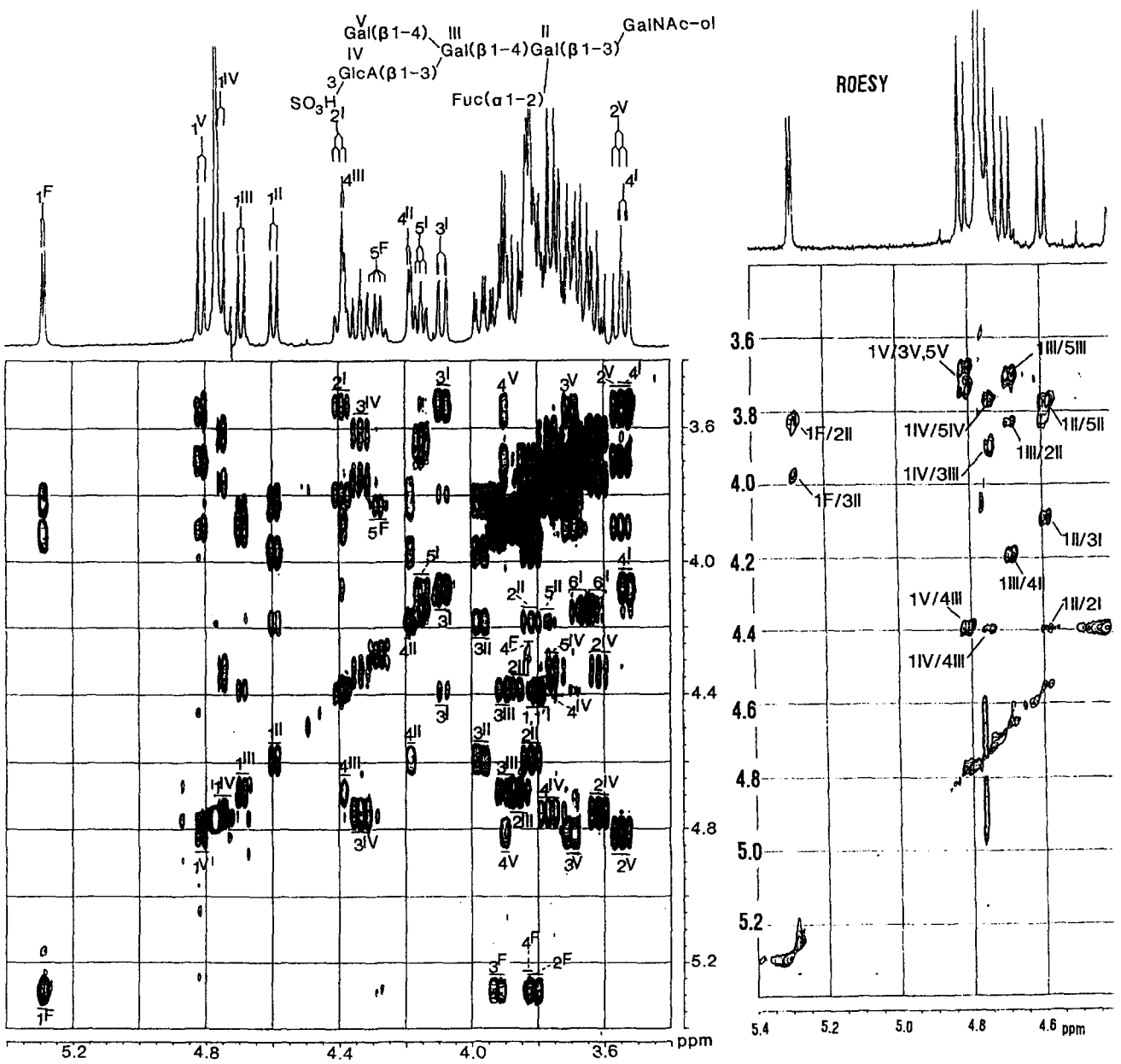
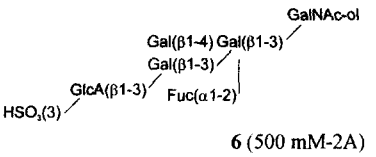


Fig. 4. COSY and ROESY spectra of compound **5**.

Compounds 6 and 7 (500 mM-2).—The structures of two compounds present in this fraction were found to be the following:



6 (500 mM-2A)

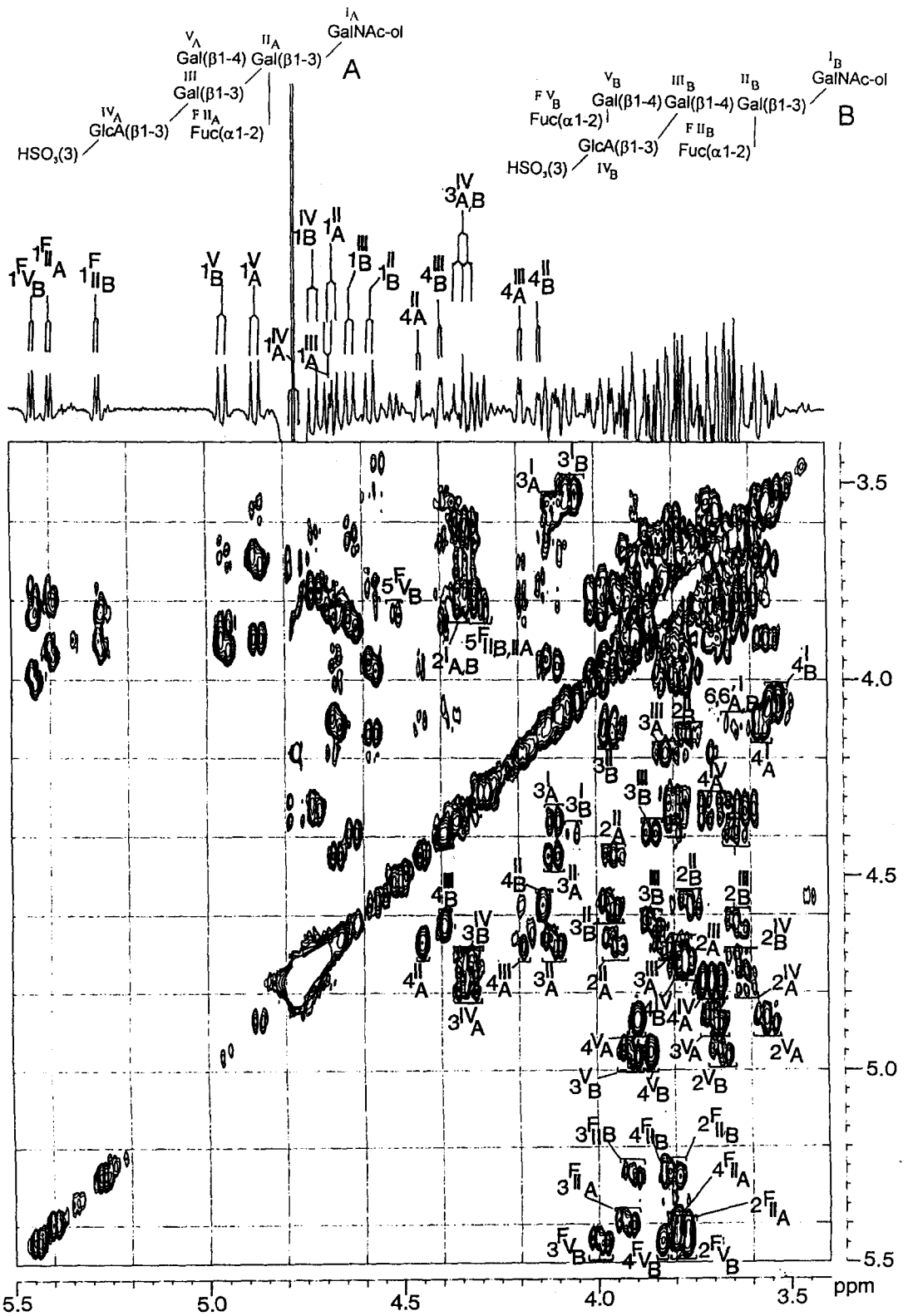
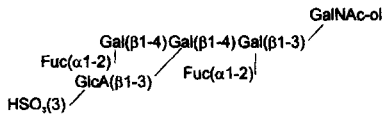


Fig. 5. COSY spectrum of compounds **6** and **7**.



7 (500 mM-2B)

The MALDI-TOF analysis confirms the presence of two compounds, having pseudomolecular ions $[M-H]^-$ at *m/z* 1148 (3 Gal, 1 Fuc, 1 HexA, 1 GalNAc-ol, 1 sulfate) and 1294 (3 Gal, 2 Fuc, 1 HexA, 1 GalNAc-ol, 1 sulfate). The monosaccharides were identified on the basis of the set of their vicinal coupling constants, as depicted on the relayed COSY spectrum (Fig. 5). The H-2, H-3 and H-4 atom resonances of the 11 monosaccharides and the 2 GalNAc-ol residues characterized on the 2D spectrum were fully assigned (Table 1), and the values for most of the ^{13}C resonances were recorded from the HMQC spectrum (Fig. 6 and Table 2). The relative low-field position of some ^{13}C resonances, compared with their position in the spectra of the corresponding non-substituted monosaccharides, were caused by glycosylation and revealed the substitution pattern in

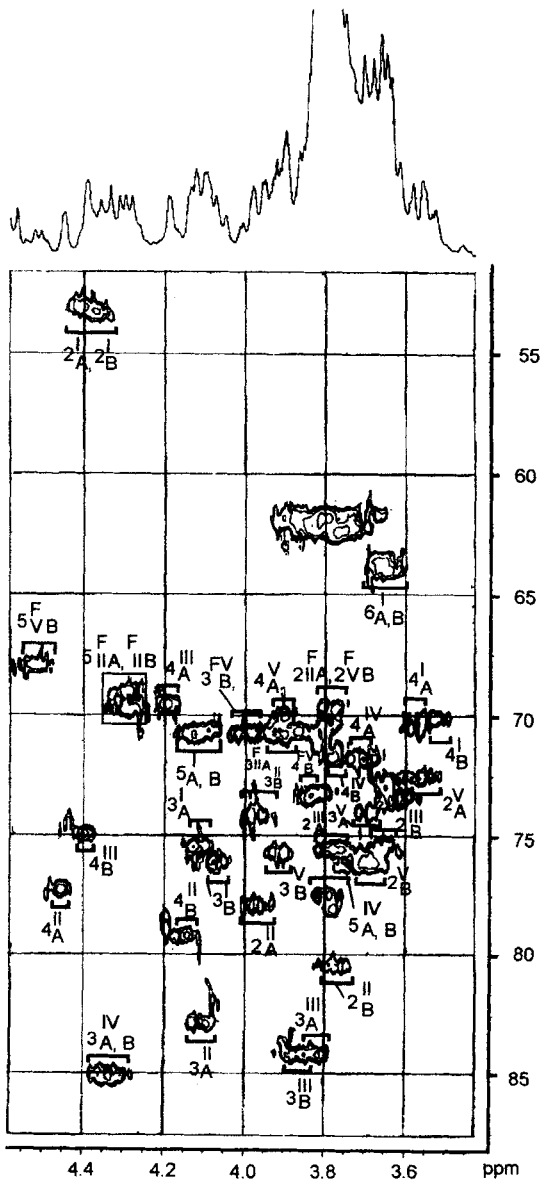
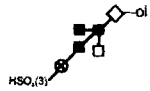


Fig. 6. HMQC spectrum of compounds **6** and **7**.

Table 2
^{13}C NMR chemical shifts of oligosaccharide-alditols **6** and **7** (symbols as in Table 1)

Residue		 **6**	 **7**
GalNAc-ol I	C-1	~61.5	~61.5
	C-2	53.07	53.25
	C-3	75.36	76.02
	C-4	70.49	70.05
	C-5	70.73	70.73
	C-6	~63.7	~63.7
	NAc	23.65	23.65
Gal II	C-1	102.65	103.38
	C-2	~71	80.43
	C-3	82.77	74.12
	C-4	77.23	79.23
	C-5	n.d.	n.d.
	C-6	~61.5	~61.5
Gal III	C-1	105.49	105.72
	C-2	~71	75.40
	C-3	84.07	84.07
	C-4	69.50	74.85
Gal V	C-1	104.31	102.19
	C-2	72.60	76.11
	C-3	73.91	75.74
	C-4	~70	~70.6
	C-5	~73	~73
	C-6	~62	~62
GlcA IV	C-1	104.64	105.31
	C-2	~73	~73
	C-3	85.05	85.05
	C-4	71.75	71.75
	C-5	77.50	77.50
Fuc II	C-1	101.45	102.36
	C-2	69.64	69.64
	C-3	~70	70.65
	C-4	n.d.	~69
	C-5	69.04	69.74
	C-6	16.79	16.98
Fuc V	C-1	–	99.56
	C-2	–	69.64
	C-3	–	70.65
	C-4	–	73.19
	C-5	–	67.85
	C-6	–	16.79

the polymer [22]. These data show the following substitutions: 3-GalNAc-ol; 2, 3, 4-Gal II_A; 3-Gal III_A; 3-GlcA IV_A; 2, 4-Gal II_B; 3, 4-Gal III_B; 3-GlcA IV_B, 2-Gal V_B and Gal V_A, Fuc II_A, Fuc II_B and Fuc V_B as terminal sugar units. From the ROESY spectrum depicted in Fig. 7 the following connectivities were easily observed: H-1 V_A → H-4 II_A; H-1 II_A → H-3 I_A; H-1 Fuc II_A → H-1, H-2, H-3 II_A and H-1

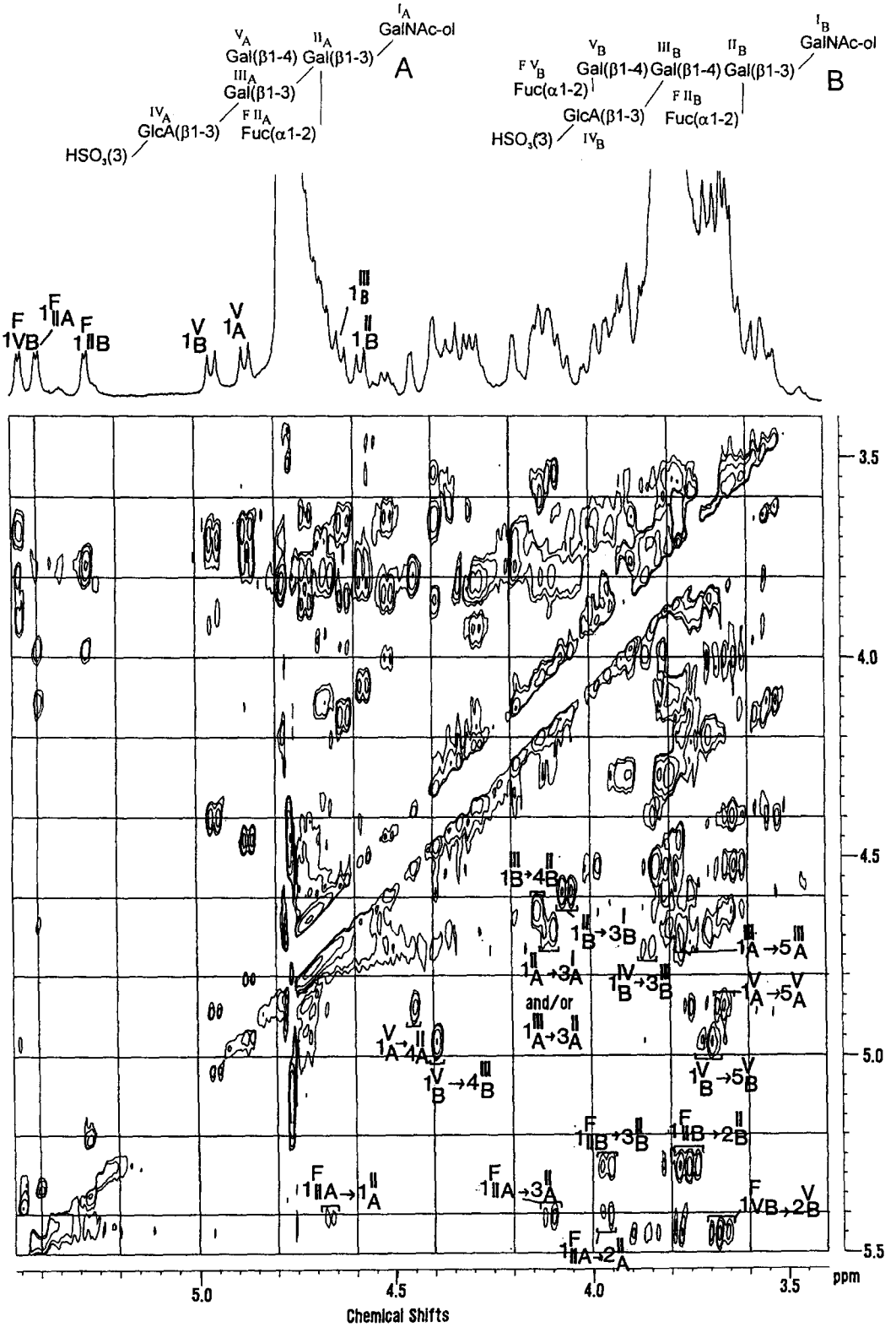


Fig. 7. ROESY spectrum of compounds **6** and **7**.

Fuc $V_B \rightarrow$ H-2 V_B; H-1 $V_B \rightarrow$ H-4 III_B; H-1 $III_B \rightarrow$ H-4 II_B; H-1 $II_B \rightarrow$ H-3 I_B; H-1 $IV_B \rightarrow$ H-3 III_B; H-1 Fuc $II_B \rightarrow$ H-2 II_B, H-3 II_B.

From the data obtained on the basis of these three different NMR experiments, the sequences proposed above were fully established.

In conclusion, this study has confirmed the structural species-specificity of carbohydrate chains which represent more than 75% of the oviducal mucins of Amphibians. [8–18]. In some cases, these mucins are available sources of 'human' antigens, such as Le^X and Le^Y (*Pleurodeles waltl*) or blood group substances [10]. This polymorphism should be of great interest for recognizing phenotypic variants of some species [14,19]. The mucin of *Rana temporaria* is characterized by the presence of sulfate and glucuronic acid which are responsible of the negative charge of the macromolecule. The sulfated oligosaccharide HSO_3(3)GlcA(β1–3)Gal is a part of the HNK-1 epitope, first described as an antigen of human natural killer cells [23–26].

Acknowledgements

This research was supported by the Centre National de la Recherche Scientifique (Unité Mixte No. 111, Relations structure-fonction des constituants membranaires, Director Professor André Verbert) and by the Ministère de l'enseignement Supérieur et de la Recherche. Doina Florea is a fellow of the European Community (Tempus programma PEC JEP-4398/92–95; Coordination: Dr. Rita Barot). We thank Dr. Jérôme Lemoine (UMR111), for recording the MALDI-TOF spectra.

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