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Species-specificity of amphibia carbohydrate chains: the *Bufo viridis* case study

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Abstract

The jelly coat surrounding the eggs of amphibia is composed of oviducal mucins and plays an important role in the fertilization process. From a structural and chemical point of view, these jellies are very different from one species to another. *Bufo viridis* is the 13th amphibia species studied in term of carbohydrate structural analysis. The oligosaccharides have been released from the oviducal mucins by reductive β elimination, purified by various chromatography procedures and analyzed by ^{1}H and ^{13}C 1D–2D NMR spectroscopy. Among the 15 compounds, ten have novel structures, although they possess some well-known structural patterns as blood group epitopes (Le^x, Le^y) or other sequences already observed in other amphibia species. These results reinforce our hypothesis about the strict species-specificity of these carbohydrate chains. It must be noted that such species-specificity does not depend on one particular monosaccharide but it is rather due to a set of particular tri- or tetrasaccharide sequences. Hence, *B. viridis* species could be characterized by the simultaneous presence of a 2,3,6-trisubstituted galactosyl residue, the GlcNAc(β1–3)[Fuc(α1–4)]GlcNAcβ sequence and the Le^x, Le^y or Cad determinants. The anionic charge of the oligosaccharides is carried only by sialic acid α-(2→6)-linked to GalNAc-ol residue as in *Bufo bufo* or in *Bufo arenarum*.

Keywords: NMR; Amphibia; Oviducal mucin; *Bufo viridis*; *O*-Glycan; Blood group

1. Introduction

The amphibia eggs are surrounded by an extracellular matrix named jelly coat which is constituted by concentric layers of material which can vary in number according to the species and in their composition.[1] Jelly coat plays an important role in fertilization steps such as sperm–egg binding, prevention of polyspermy (anurans),[2,3] sperm capacitation, acrosome reaction,[4–6] maintenance of the appropriate divalent cations concentration,[7–9] and recognition of homologous species. The jelly material is mainly composed of glycoproteins of mucin type containing up to 50% of carbohydrates, which display a remarkable species specificity.[10–23] Such an inter-species diversity in glycosylation could certainly be involved in many biological mechanisms, such as the prevention of species cross-fertilization or the symbiotic function towards parasites. So, the biological roles of oligosaccharides seem to be critical and their understanding becomes crucial to further progress. Moreover, the structural analysis of these components can also furnish the basis of molecular taxonomy.

Neutral and acidic O-linked oligosaccharides have been released from egg jelly coats of the anuran *Bufo viridis* by alkali-borohydride treatment. We report here the structures of the main carbohydrate chains which appear to be specific for *B. viridis* through a set of particular tri- or tetrasaccharide sequences.

2. Experimental

Sampling of jelly coat mucus.—Eggs from *B. viridis*, collected in Romania, were obtained from natural spawns. The jelly coat material was lyophilized.

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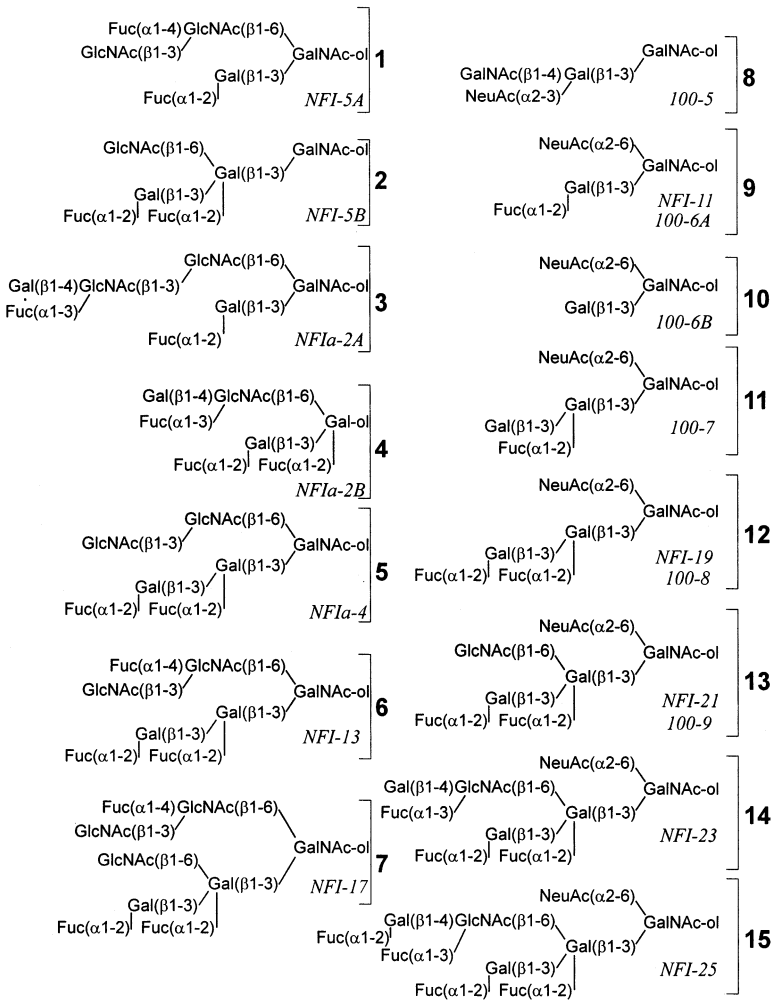
Isolation of oligosaccharide–alditols.—Performed as described in Refs. 10–23. Typically, O-linked oligosaccharides were released from the crude material by reductive β-elimination (0.05 M NaOH, 1 M $NaBH_4$, 37 °C, 48 h). The reaction was quenched by addition of acetone, and borate salts were removed by repeated evaporation with MeOH. The crude material was submitted to a cationic ion-exchange resin chromatography on Dowex 50 × 2 (200–400 mesh, H^+ form) to remove residual peptides. The neutral oligosaccharide–alditols were separated from acidic oligosaccharide–alditols by anionic-exchange chromatography on Dowex 1 × 2 (200–400 mesh, $HCOO^-$ form). After water elution of neutral compounds, acidic oligosaccharides were desorbed with a 100 mM solution of pyridine acetate buffer (pH 6.7). A neutral fraction was subfractionated on Bio-Gel P2 column (90 × 1.5 cm) to give three subfractions *NFI–NFIII*. The neutral and acidic components from the fractions *NFI* and *100 mM*, respectively, were isolated by HPLC on a primary amine-bonded silica column (Supelcosyl LC-NH_2; 4.6 × 250 mm; Supelco Inc, Bellefonte, USA) using MeCN–water or MeCN–water–30 mM potassium phosphate buffer (pH 5.2) as eluent, at a flow rate of 1 mL min^{-1}. The fractions corresponding to peaks 6–10 on the HPLC profile of fraction *NFI* were pooled to give fraction *NFIa*. This latter fraction was submitted to a second HPLC chromatography on the same NH_2-column and eluted with a suitable gradient. All the collected fractions were desalted by gel permeation on a Bio-Gel P2 column (55 × 2 cm), and lyophilized.[24]

Methylation.—The reduced oligosaccharide (200 μg) was methylated according to Ciucanu and Kerek.[25]

Gas chromatography–mass spectrometry (GC–MS).—The partially acetylated methylglycosides were analyzed by GC–MS according to Fournet et al.[26]

NMR spectroscopy.—^{1}H NMR experiments were performed on a Bruker ASX 400 WB spectrometer equipped with 5 mm ^1H–^{13}C mixed probe head operating in the pulse Fourier-Transform mode and controlled by an Aspect 3000 computer. ROESY and ^{13}C NMR experiments were performed on a Bruker DMX



Scheme 1. Oligosaccharide–alditols from the oviducal mucin of *B. viridis*. *NFI* 13, peak 13 of the subfraction I (Bio-gel P2) of the neutral fraction; *NFIa*, pool of *NFI* 6–10; 100-7, peak 7 of the fraction eluted with a 100 mM solution of buffer.

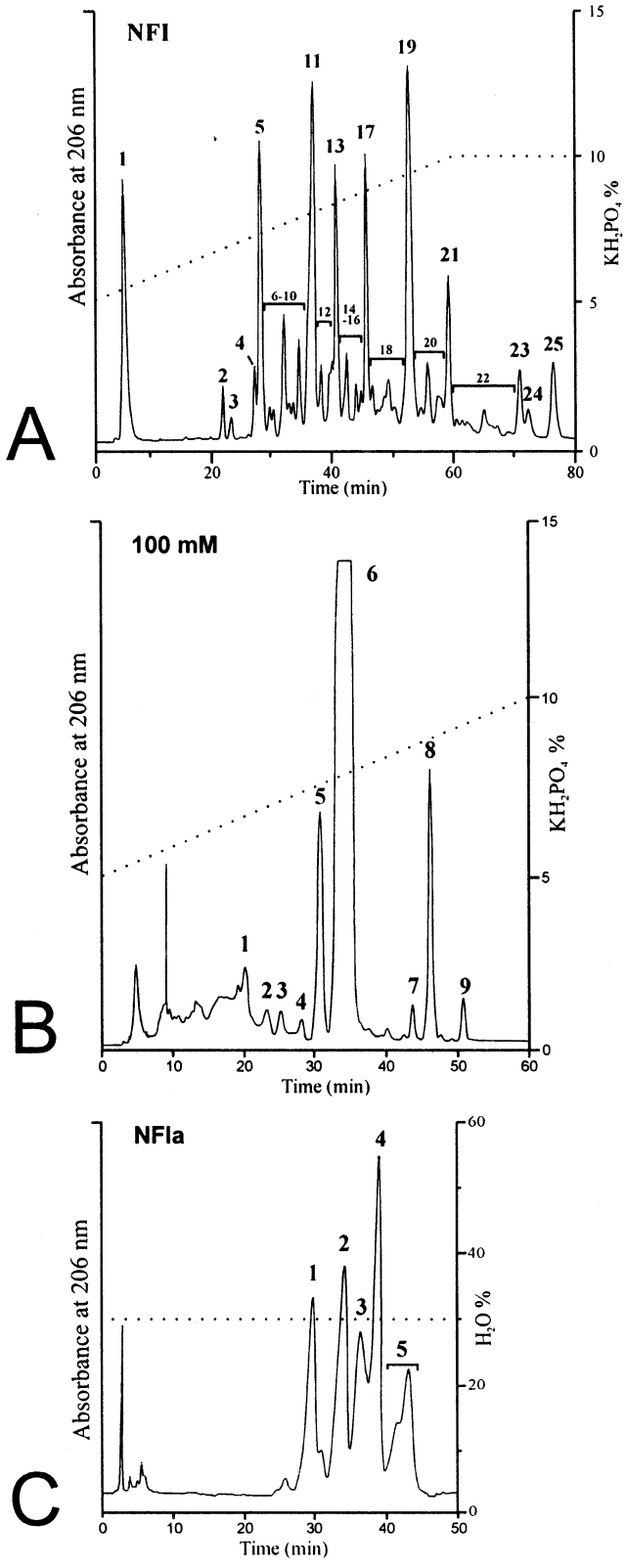


Fig. 1. HPLC profiles of fractions *NFI* (A), *100 mM* (B) and *NFIa* (C). The fraction *NFIa* corresponds to peaks 6–10 from (A).

600 spectrometer. Chemical shifts are expressed in ppm downfield from internal sodium 4,4′-dimethyl-4-silapentane-1-sulfonate but were in fact measured by reference to internal acetone (δ 2.225 ppm in D_2O at 27 °C). The two dimensional homonuclear correlated spectroscopy (COSY) with simple and double relay transfer, the heteronuclear multi quantum coherence (HMQC) and the rotating-frame nuclear Overhauser enhancement spectroscopy (ROESY) were performed using Bruker standard-pulse sequences library.[27,28] For ROESY experiment, the mixing time was set at 300 ms.

3. Results

The major carbohydrate chains released from *B. viridis* egg jelly coats are shown in Scheme 1. Compounds **9** (*NFI-11*/*100-6A*), **12** (*NFI-19*/*100-8*) and **13** (*NFI-21*/*100-9*) were eluted in neutral or acidic 100 mM fractions. Compounds **14** (*NFI-23*) and **15** (*NFI-25*) were also eluted with water, although they also contain a *N*-acetylneuraminic acid unit (Fig. 1). This fact was probably due to an overload of the resin rather than a lactonization of the sialic acid. Among these 15 compounds, five of them (**8**–**12**) have been already characterized in various *Amphibis mucin* (Table 1).[11–13,17–19,29] The other ten oligosaccharides are novel and can be considered as species-specific for *B. viridis*. Compound **15** is the more complex of this homologous series. For this reason, it has been exhaustively studied and its structure has been established unambiguously by NMR, MALDI-TOF analysis and methylation.

Structure of oligosaccharide-alditol **15**.—The ^{1}H NMR spectrum of **15** contains eight signals in the anomeric region, named F^2_{II}, F^2_{III}, F^2_V, F^2, II, III, IV and V. Confirmatory evidence from double relayed COSY spectrum (Fig. 2, Table 2) was obtained for the configurational assignments for the eight sugar residues on the basis of the measurement of vicinal coupling constants: β-galactose (II, III, V), β-*N*-acetylglucosamine (IV) and α-fucose (F^2_{II}, F^2_{III}, F^2_V and F^3). Two other signals at δ 1.671 and 2.719 were assigned to H-3ax and H-3eq atom resonances, respectively, of *N*-acetylneuraminic acid. The MALDI-TOF analysis (Table 4) displays an $[M-H]^-$ pseudomolecular ion at m/z 1786 confirming this monosaccharidic composition (Table 4). The 6-O-sialylated Gal(β1–3)GalNAc-ol core of **15** was assigned on the basis of the following observations: (i)The GalNAc-ol H-2, H-3 and H-5 resonances were observed at δ 4.223, 3.966 and 4.117, respectively; (ii) the GalNAc-ol H-6′ gave rise to a doublet of doublet at δ 3.458, clearly upfield from the bulk of the skeleton proton signals; (iii) the set of chemical shifts of NeuAc H-3ax (δ 1.671) and H-3eq (δ 2.719) were typical for the α-(2→6) linkage to GalNAc-ol.[28] Furthermore, the sets of Gal^{III} H-1 (δ 4.891), H-2 (δ 3.678), H-3 (δ 3.885), Gal^{II} H-3 (δ 4.176), H-4 (δ 3.996) and Fuc F^2_{II} and F^2_{III}

Table 1
^{1}H NMR chemical shifts of oligosaccharide-ols **8**–**14**

Residues [a]	Reporter group	Chemical shifts (ppm)						
		8	**9**	**10**	**11**	**12**	**13**	**14**
GalNAc-ol^{I}	H-1,1′	3.774	3.752	3.752	n.d.	n.d.	3.77	3.77
	H-2	4.380	4.377	4.384	4.326	4.237	4.236	4.230
	H-3	4.062	4.077	4.058	4.097	4.055	3.987	3.982
	H-4	3.530	3.538	3.532	3.575	n.d.	3.631	3.632
	H-5	4.145	4.221	4.228	4.193	4.147	4.111	4.122
	H-6	3.635	n.d.	3.862	n.d.	n.d.	3.852	3.86
	H-6′	3.635	3.475	3.491	3.476	3.468	3.468	3.466
	NAc	2.046	2.036	2.047	2.046	2.036	2.061	2.055
Gal^{II}	H-1	4.563	4.580	4.475	4.671	4.695	4.706	4.698
	H-2	3.410	n.d.	3.568	n.d.	n.d.	3.8	3.804
	H-3	4.166	n.d.	n.d.	4.011	4.177	4.177	4.178
	H-4	4.092	n.d.	n.d.	4.219	4.004	4.003	3.999
Gal or GalNAc^{III}	H-1	4.729			4.617	4.890	4.896	4.900
	H-2	3.903			n.d.	n.d.	3.682	3.685
	H-3	3.682			n.d.	n.d.	3.888	3.89
	H-4	3.915			3.922	n.d.	3.907	3.910
	NAc	2.027						
Gal^{V}	H-1							4.456
	H-2							3.501
	H-3							3.657
	H-4							3.901
GlcNAc^{IV}	H-1						4.587	4.616
	H-2						3.709	3.925
	H-3						3.533	3.883
	H-4						3.440	3.870
	H-5						3.470	3.604
	H-6						3.928	4.01
	H-6′						3.747	3.87
	NAc						2.066	2.061
Fuc F_{II}	H-1		5.263		5.398	5.399	5.390	5.396
	H-2		n.d.		n.d.	n.d.	3.760	3.763
	H-3		n.d.		n.d.	n.d.	3.849	3.853
	H-4		n.d.		n.d.	n.d.	3.806	3.806
	H-5		4.266		4.271	4.294	4.301	4.297
	CH_3		1.232		1.231	1.197	1.195	1.096
Fuc F_{III}	H-1					5.323	5.325	5.328
	H-2					n.d.	3.797	3.8
	H-3					n.d.	3.727	3.734
	H-4					n.d.	3.838	3.832
	H-5					4.334	4.331	4.332
	CH_3					1.232	1.231	1.235
Fuc F^3	H-1							5.106
	H-2							3.690
	H-3							3.903
	H-4							3.791
	H-5							4.834
	CH_3							1.178
NeuAc^{N}	H-3 ax	1.931	1.692	1.711	1.692	1.687	1.678	1.684
	H-3 eq	2.686	2.273	2.728	2.725	2.715	2.723	2.727
	H-4	3.599	n.d.	n.d.	n.d.	n.d.	3.671	3.677
	H-5	n.d.	n.d.	n.d.	n.d.	n.d.	3.823	3.83
	H-6	3.828	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	H-7	3.507	n.d.	n.d.	n.d.	3.572	n.d.	n.d.
	H-8	3.88	n.d.	n.d.	n.d.	n.d.	3.87	3.896
	H-9	3.88	n.d.	n.d.	n.d.	n.d.	3.87	3.896
	H-9′	3.604	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	NAc	2.033	2.028	2.033	2.032	2.027	2.034	2.036

n.d., not determined.

[a] The nomenclature of each residue is indicated in Figs. 2–6. F_{II} is the fucose unit attached to the Gal II unit; F^3 is the fucose unit attached in position 3.

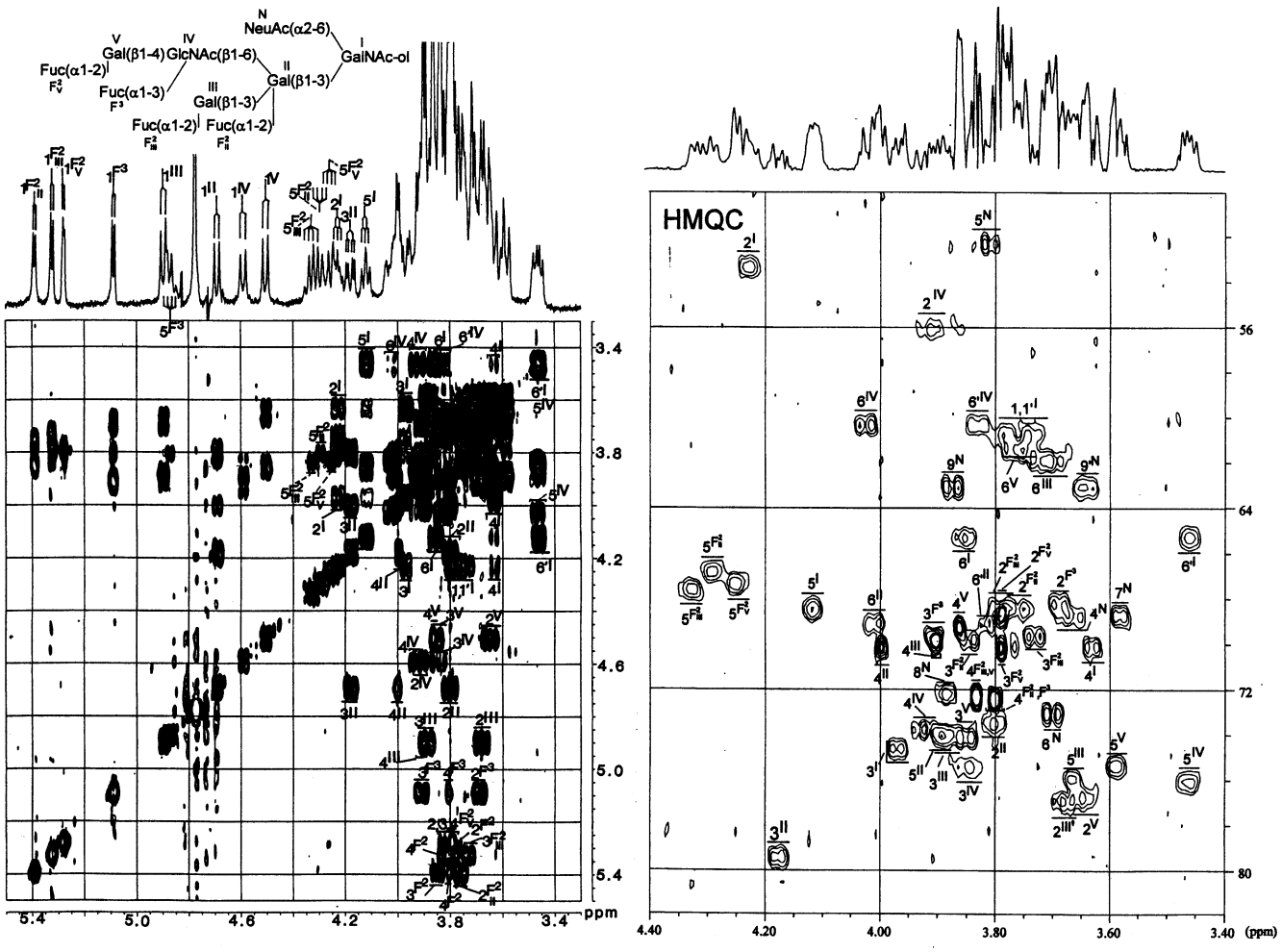


Fig. 2. Double relayed COSY and HMQC spectra of compound **15**.

H-1 (δ 5.386 and 5.319, respectively) and H-5 (δ 4.289 and 4.325, respectively) proved the existence of the classical Fuc(α1–2)Gal(β1–3)[Fuc(α1–2)]Gal(β1–3) sequence.[12,17,18,20] The ROESY spectrum (Fig. 3) allowed the detection of the H-5 atom resonance for Gal^{II} and Gal^{III} (δ 3.897 and 3.669, respectively) on the basis of the correlations $1^{II}/5^{II}$ and $1^{III}/5^{III}$. This information allowed the correlation of H-5 with H-6 and H-6′ for each Gal residue in the COSY spectrum, and to assign the H-6 and H-6′ chemical shifts. Thus, the C-5 and C-6 of Gal^{II} and Gal^{III} resonances have been assigned on the HMQC spectrum (Gal^{II}, 73.90/69.12; Gal^{III}, 75.99/61.80 ppm). The shielding for C-5 and the deshielding for C-6 of Gal^{III} indicated a C-6 substitution at this unit. In addition, the total set of the structural reporter group of the two Fuc residues, i.e., H-1, H-5 and CH_3 for each residue (Fuc^2_V, δ 5.273/4.249/1.266; Fuc^3, δ 5.083/4.868/1.228), as well as Gal^V H-1 (δ 4.499) and $GlcNAc^{IV}$ NAc group (δ 2.050), were indicative of the presence of the Lewis Y determinant Fuc(α1–2)Gal(β1–4)[Fuc(α1–3)]GlcNAcβ.[29,30] This sequence constituted the C-6 branch of the trisubstituted Gal^{II}.

Structure of oligosaccharide–alditols **13** *and* **14**.— Comparison of the NMR spectra of **13** and **14** with that of **15** clearly showed the successive loss of the unit Fuc^2_V in **14**, and Gal^V, Fuc^2_V, F^3 in **13** (Fig. 4). In accordance with the loss of these sugar units, pseudomolecular ions $[M-H]^-$ at m/z 1640 (**14**) and 1332 (**13**) were observed. The absence of Fuc^2_V in **14** is also reflected by the upfield shifts for the Gal^V H-2 ($\Delta\delta$ −0.144) and H-3 ($\Delta\delta$ −0.190) signals. The NMR parameters of $GlcNAc^{IV}$ in **13** are characteristic of a non-substituted GlcNAc unit. The C-2,3,6 linked Gal^{II} were characterized by methylation analysis, since the COSY spectra (Fig. 4) did not allow to observe the H-5–H-6–H-6′ correlation of Gal^{II}.

Structure of oligosaccharide–alditols **1**, **6** *and* **7**.— These compounds have in common the sequence GlcNAc(β1–3)[Fuc(α1–4)]GlcNAc, already identified in *Rana utricularia*[14] and *Rana ridibunda*.[23] For these three compounds, the characteristic chemical shifts of GalNAc-ol H-6 and H-6′ confirmed the attachment of this sequence at C-6 of the hexosaminitol unit (Figs. 5 and 6, and Table 3). The C-3 branch of compound **1** was

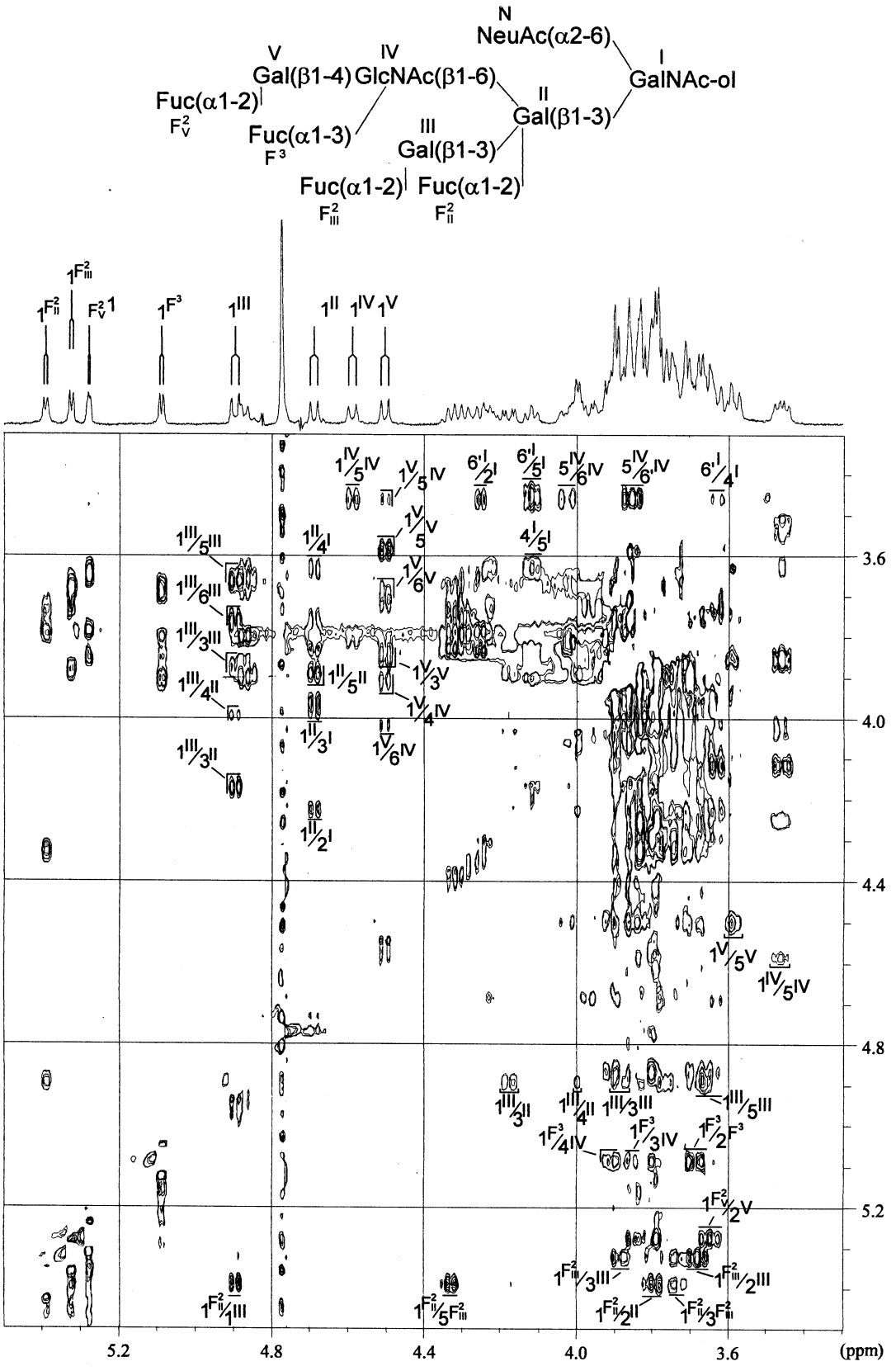


Fig. 3. ROESY spectrum of compound **15**.

Table 2
^{1}H and ^{13}C NMR chemical shifts of oligosaccharide-ol **15**

Residues	Reporter groups	Chemical shifts	
GalNAc-ol^{I}	H-1,1′/C-1	3.77/60.73	
	H-2/C-2	4.223/53.24	
	H-3/C-3	3.966/**74.66** [a]	
	H-4/C-4	3.629/70.12	
	H-5/C-5	4.117/68.45	
	H-6/C-6	3.852/**65.25**	
	H-6′	3.458	
	NAc	2.050/22.89	
Gal^{II}	H-1/C-1	4.684/100.43	(7.8 Hz) [b]
	H-2/C-2	3.799/**73.48**	
	H-3/C-3	4.176/**79.42**	
	H-4/C-4	3.996/70.20	
	H-5/C-5	3.897/**73.90**	
	H-6/C-6	4.006/**69.12**	
	H-6′	3.784	
Gal^{III}	H-1/C-1	4.891/101.24	(7.8 Hz)
	H-2/C-2	3.678/**76.96**	
	H-3/C-3	3.885/74.13	
	H-4/C-4	3.902/69.76	
	H-5/C-5	3.669/75.99	
	H-6/C-6	3.72/61.80	
	H-6′	3.72	
GlcNAc^{IV}	H-1/C-1	4.584/101.33	(8.0 Hz)
	H-2/C-2	3.910/56.03	
	H-3/C-3	3.830/**75.42**	
	H-4/C-4	3.910/**73.79**	
	H-5/C-5	3.463/76.11	
	H-6/C-6	4.027/60.25	
	H-6′	3.83	
	NAc	2.050/22.89	
Gal^{V}	H-1/C-1	4.499/100.73	(7.8 Hz)
	H-2/C-2	3.645/**76.77**	
	H-3/C-3	3.847/74.13	
	H-4/C-4	3.861/69.25	
	H-5/C-5	3.593/75.38	
	H-6/C-6	3.763/61.80	
	H-6′	3.763	
NeuAc^{N}	H-3ax/C-3	1.671/41.03	
	H-3eq	2.719	
	H-4/C-4	3.689/68.73	
	H-5/C-5	3.817/52.21	
	H-6/C-6	n.d./73.05	
	H-7/ C-7	n.d./68.72	
	H-8/C-8	3.873/72.21	
	H-9,9′/C-9	3.873, n.d./63.03	
	NAc	2.030/22.49	
Fuc F^{2}_{II}	H-1/C-1	5.386/98.07	(3.7 Hz)
	H-2/C-2	3.756/68.38	
	H-3/C-3	3.845/69.82	
	H-4/C-4	3.796/72.29	
	H-5/C-5	4.289/66.82	
	H-6/C-6	1.190/15.98	
Fuc F^{2}_{III}	H-1/C-1	5.319/99.93	(3.9 Hz)
	H-2/C-2	3.793/68.30	
	H-3/C-3	3.725/69.66	
	H-4/C-4	3.833/72.42	
	H-5/C-5	4.325/67.58	
	H-6/C-6	1.228/15.98	
Fuc F^{3}_{IV}	H-1/C-1	5.083/99.10	(3.9 Hz)
	H-2/C-2	3.685/68.21	
	H-3/C-3	3.905/69.65	
	H-4/C-4	3.799/72.42	
	H-5/C-5	4.868/67.01	
	H-6/C-6	1.228/15.98	
Fuc F^{2}_{V}	H-1/C-1	5.273/99.86	(2.2 Hz)
	H-2/C-2	3.81/68.6	
	H-3/C-3	3.81/70.08	
	H-4/C-4	3.81/72.29	
	H-5/C-5	4.249/67.34	
	H-6/C-6	1.266/15.98	

[a] Linkage carbons are indicated in bold.
[b] $J_{1,2}$, in Hz, are indicated in brackets.

clearly identified as the short sequence Fuc(α1–2)Gal, due to the significant chemical shift of H-2, H-3 and H-4 resonances observed for the Gal^{II} unit (Table 3). The sequence Fuc(α1–2)Gal(β1–3)[Fuc(α1–2)]Gal(β1–3) was easily assigned in compounds **6** and **7**, following previous observations,[12,17,18,20] and the presence of an additional GlcNAc^{IV} unit β-(1→6)-linked to Gal^{II} of **7** was showed by a methylation analysis which released a 4-OMe-Gal methyl ether derivative.

Structure of oligosaccharide–alditols **1**, **2**, **3**, **4** *and* **5**.—Compound **2** has been observed in a mixture with oligosaccharide–alditols **1** (Fig. 5). According to the relative intensity of the signals visualized on the NMR spectrum (Fig. 5 and Table 3), the main significant chemical shifts which evidence the structure proposed for compound **2** were assigned. Typically, the chemical shifts of GalNAc-ol H-6 and H-6′ both observed at δ 3.67, confirm the presence of the type 1 core Gal(β1–3)GalNAc. The second oligosaccharide-alditol (**1**) contains the sequences GlcNAc(β1–3)[Fuc(α1–4)]GlcNAc (upper branch) and Fuc(α1–2)Gal (lower branch). By recycling the peaks 6–10 of the neutral fraction *NFI* (see Section 2), five peaks were isolated. MALDI-TOF and NMR analyses of the second peak, namely *NFIa*-2, showed a mixture of the two compounds, **3** and **4**, containing, respectively, two Fuc, two Gal, two GlcNAc, one GalNAc-ol and three Fuc, two Gal, one

Table 3
^{1}H NMR chemical shifts of the oligosaccharide-ols **1–7**

Residues [a]	Reporter group	Chemical shifts (ppm)						
		1	**2**	**3**	**4**	**5**	**6**	**7**
GalNAc-ol or Gal-ol^{I}	H-1,1′	3.76	n.d.	3.783	3.783	3.778	3.77	3.76
	H-2	4.381	n.d.	4.401	4.000	4.233	4.228	4.223
	H-3	4.062	n.d.	4.081	4.134	4.040	4.034	3.970
	H-4	3.470	n.d.	3.489	3.911	3.613	3.601	3.60
	H-5	4.218	n.d.	4.237	4.311	4.178	4.17	4.131
	H-6	3.878	3.667	3.911	3.949	3.903	3.882	3.878
	H-6′	3.65	3.667	3.673	3.723	3.692	3.684	3.694
	NAc	2.039	2.046	2.052		2.044	2.041	2.056
Gal^{II}	H-1	4.555	4.677	4.571	4.664	4.684	4.679	4.682
	H-2	3.659	3.772	3.674	3.743	3.815	4.811	3.789
	H-3	3.855	4.156	3.872	3.883	4.180	4.174	4.164
	H-4	3.903	3.989	3.921	3.858	4.018	4.014	4.004
Gal^{III} or $^{IV'}$	H-1		4.884	4.448		4.899	4.895	4.898
	H-2		3.662	3.496		3.694	3.688	3.681
	H-3		3.866	3.650		3.896	3.890	3.884
	H-4		3.888	3.896		3.908	3.910	3.910
GlcNAc$^{II'}$	H-1	4.460		4.603	4.497	4.513	4.486	4.488
	H-2	3.838		3.942	3.778	3.795	3.859	3.857
	H-3	3.982		3.872	3.779	3.745	3.997	4.001
	H-4	3.700		n.d.	n.d.	3.518	3.717	3.723
	H-5	3.519		n.d.	n.d.	3.464	3.535	3.540
	H-6	n.d.		n.d.	n.d.	3.927	3.979	n.d.
	H-6′	n.d.		n.d.	n.d.	3.747	3.846	n.d.
	NAc	2.081		2.083	2.083	2.072	2.087	2.081
GlcNAc or Gal$^{III'}$	H-1	4.634		4.664	4.457	4.582	4.640	4.642
	H-2	3.58		n.d.	3.540	3.671	3.61	3.6
	H-3	3.58		n.d.	3.668	3.585	3.61	3.6
	H-4	3.187		n.d.	3.923	3.459	3.203	3.08
	H-5	3.377		n.d.	n.d.	3.464	3.390	3.397
	H-6	3.954		n.d.	n.d.	3.927	3.969	3.594
	H-6′	3.58		n.d.	n.d.	3.747	3.61	3.6
	NAc	1.989		2.001		2.019	2.005	2.006
GlcNAc^{IV}	H-1		4.568					4.574
	H-2		3.701					3.708
	H-3		3.514					3.529
	H-4		3.420					3.436
	NAc		2.039					2.056
Fuc F_{II}	H-1	5.212	5.374	5.226	5.478	5.417	5.412	5.395
	H-2	3.785	3.743	3.804	3.799	3.765	3.76	3.758
	H-3	3.898	3.831	3.917	3.80	3.866	3.86	3.846
	H-4	3.800	3.782	3.826	3.80	3.803	3.801	3.801
	H-5	4.254	4.278	4.273	4.290	4.270	4.269	4.268
	CH_3	1.226	1.170	1.244	1.212	1.200	1.198	1.188
Fuc F_I or F_{III}	H-1		5.313		5.033	5.334	5.329	5.329
	H-2		3.776		3.805	3.800	3.794	3.792
	H-3		3.709		3.80	3.740	3.736	3.729
	H-4		3.789		3.80	3.811	3.818	3.82
	H-5		4.307		4.050	4.334	4.333	4.323
	CH_3		1.209		1.228	1.236	1.232	1.227
Fuc F^3 or F^4	H-1	4.995		5.114	5.102		5.007	5.008
	H-2	3.792		3.690	3.690		3.804	3.804
	H-3	3.900		3.905	3.895		3.913	3.915
	H-4	3.782		3.787	3.787		3.796	3.800
	H-5	4.788		4.826	4.826		4.789	4.786
	CH_3	1.257		1.172	1.172		1.269	1.271

n.d., not determined.

[a] The nomenclature of each residue is indicated in Figs. 2–6. In the case of peeling, GalNAc I is degraded and Gal II is reduced in Gal-ol I.

Table 4
Monosaccharide composition for oligosaccharide-ols released from the mucin of the egg jelly coat

Compounds	$[M+Na]^+$	$[M-H]^-$	Molar ratio GalNAc-ol	Gal-ol	GlcNAc	Gal	Fuc	NeuAc
3	1268		1	0	2	2	2	0
4	1170		0	1	1	2	3	0
5	1268		1	0	2	2	2	0
7	1617		1	0	3	2	3	0
8		878	1	0	1	1	0	1
9		821	1	0	0	1	1	1
10		675	1	0	0	1	0	1
12		1129	1	0	0	2	2	1
13		1332	1	0	1	2	2	1
14		1640	1	0	1	3	3	1
15		1786	1	0	1	3	4	1

The composition of oligosaccharide–alditols were deduced from $[M+Na]^+$ pseudomolecular ions and $[M-H]^-$ pseudomolecular ions obtained, respectively, by positive-ion and negative-ion MALDI-TOF spectrometry. Monosaccharide units were identified on the basis of their vicinal coupling constants by NMR.

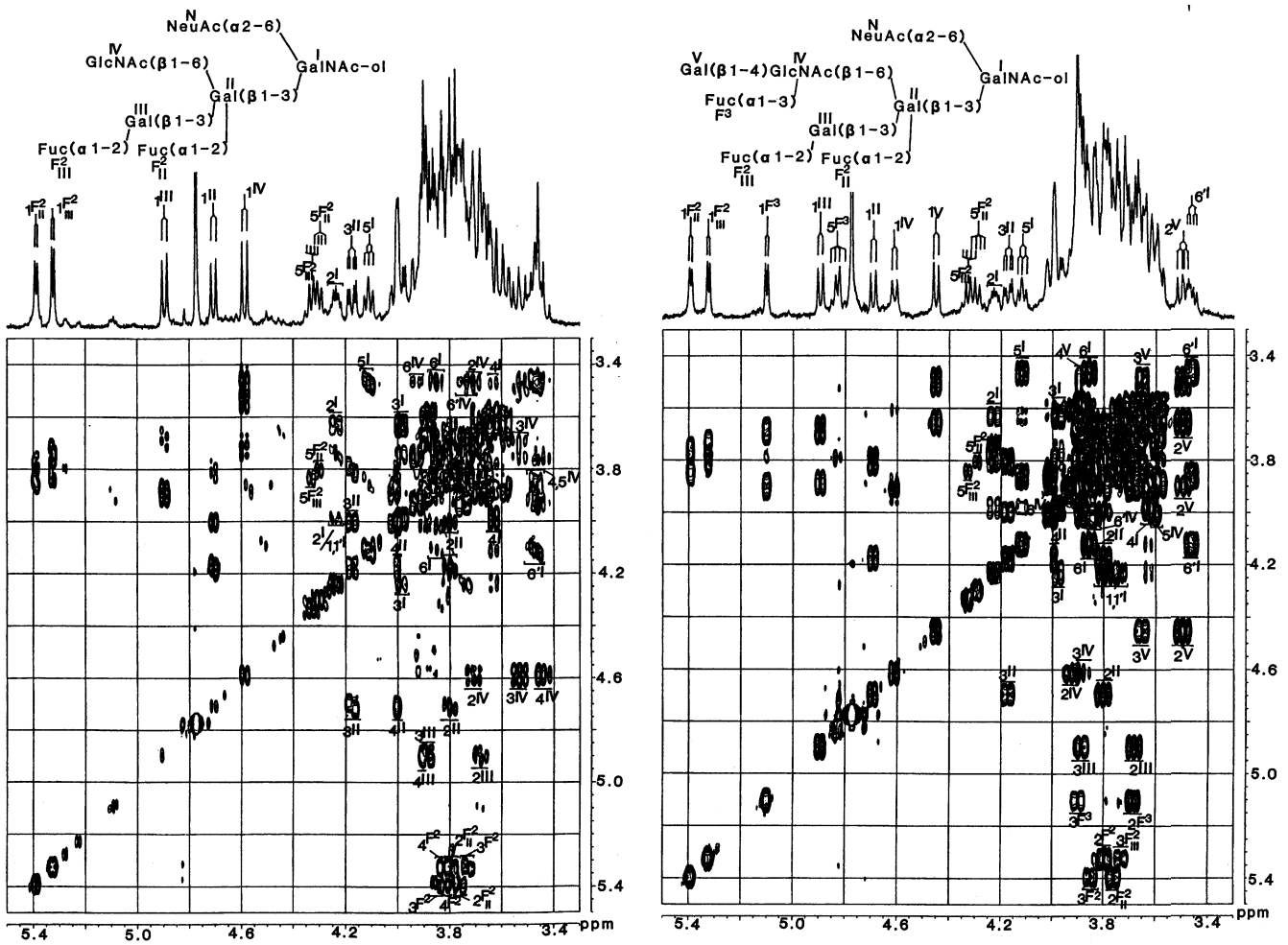


Fig. 4. Relayed COSY spectra of compound **13** (left) and **14** (right).

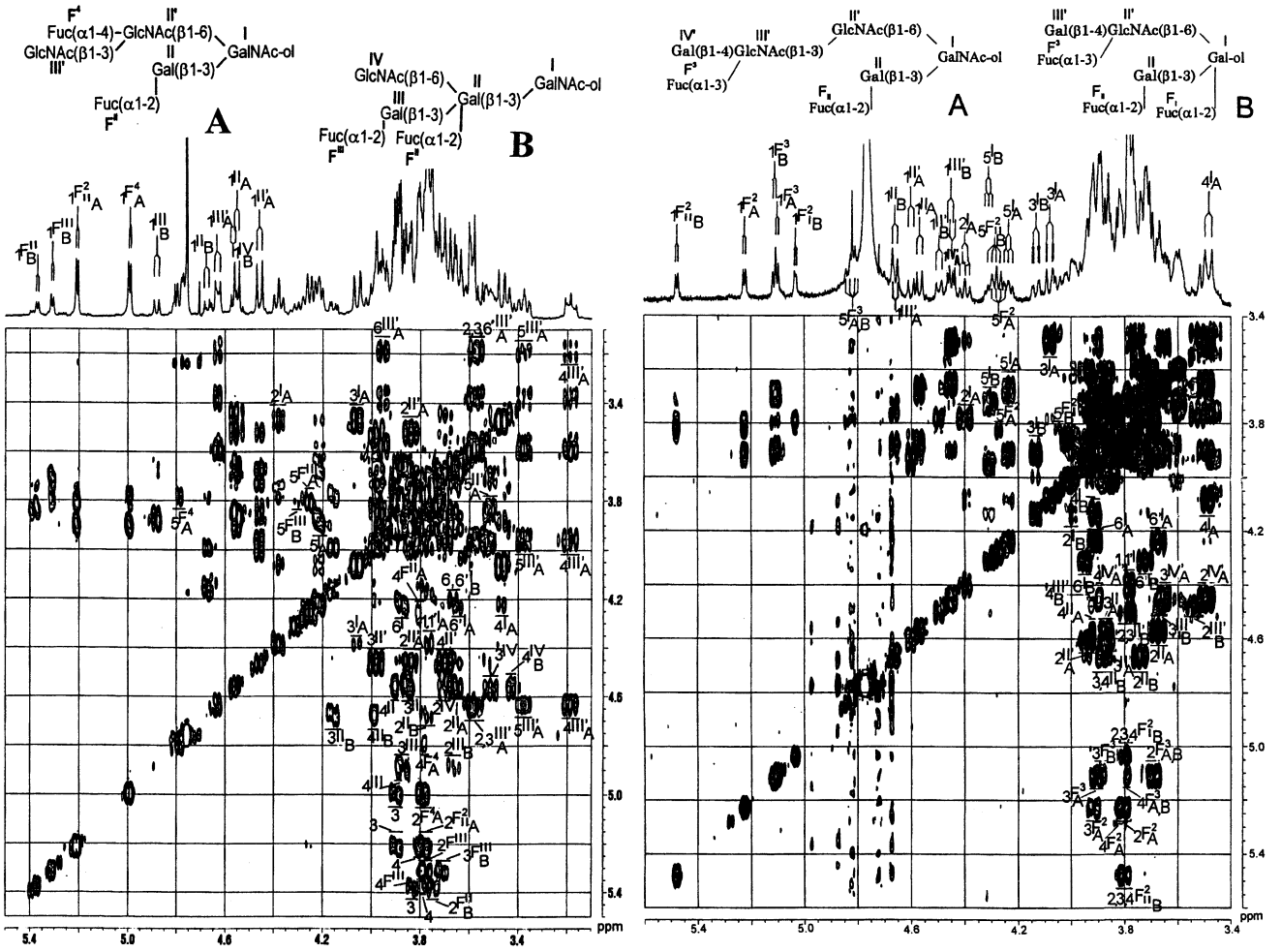


Fig. 5. Double relayed COSY spectra of compounds **1** (A) and **2** (B) (left) and **3** (A) and **4** (B) (right).

GlcNAc and one Gal-ol residues (Fig. 5 and Table 3). The structures of **3** and **4** were established on the basis of the following observations: (i) Assignment of the sequences Fuc(α1–2)Gal(β1–3)GalNAc-ol and Fuc(α1–2)Gal(β1–3)[Fuc(α1–2)]Gal-ol, on the basis of the compilation of previous data;[12,17,18,20] (ii) identification of two different Fuc units, the NMR parameters of which are characteristic of the Lewis X determinant; (iii) characterization of 2,3,6-OAc-1,4,5-OMe-Gal-ol, 3-OAc-4,6-OMe-GlcN(Me)Ac and 3,4-OAc-6-OMe-GlcN(Me)Ac among the methyl ethers obtained by methylation. Consequently, combination of methylation, MALDI-TOF analysis and NMR confirmed the structure of compounds **3** and **4**. The NMR spectrum of **5** (Table 3) revealed the four anomeric protons which are characteristic of the sequence Fuc(α1–2)Gal(β1–3)[Fuc(α1–2)]Gal(β1–3)GalNAc-ol (see above) and two other signals, at δ 4.513 and 4.582, previously assigned for the sequence GlcNAc(β1–3)GlcNAc(β1–6)GalNAc-ol.[13,14] From these observations, the structure of **5** has been established.

4. Discussion

This study was initiated to confirm the species-specificity of the O-linked carbohydrate chains released from the mucins of lower vertebrates. Fifteen oligosaccharides were isolated from the oviducal mucin of *B. viridis*, from which 11 are novel, although they are composed of arrangements of tri-, tetra- and pentasaccharidic sequences previously reported from other amphibian mucins. The sequence Fuc(α1–2)Gal(β1–3)[Fuc(α1–2)]GlcNAc(β1–6)Gal(β1–3) has been reported from *Bufo bufo*[13] and *Rana arvalis*[20] mucins, but the specificity of *B. viridis O*-glycans resides in an association of this sequence with the Le^x and the Le^y epitopes. The Le^x and Le^y epitopes themselves have been observed in *Pleurodeles waltl*[10] and *Ambystoma tigrinum*,[22] attached to O-3 of the core GlcNAc(β1–3)GalNAc-ol (*P. waltl*) or O-6 of GalNAc-ol (*A. tigrinum*). The sequence GlcNAc(β1–3)[(Fuc(α1–4))]-GlcNAc(β1–6) is well represented in *B. viridis*. This sequence, which has also been reported from *R. utricu-*

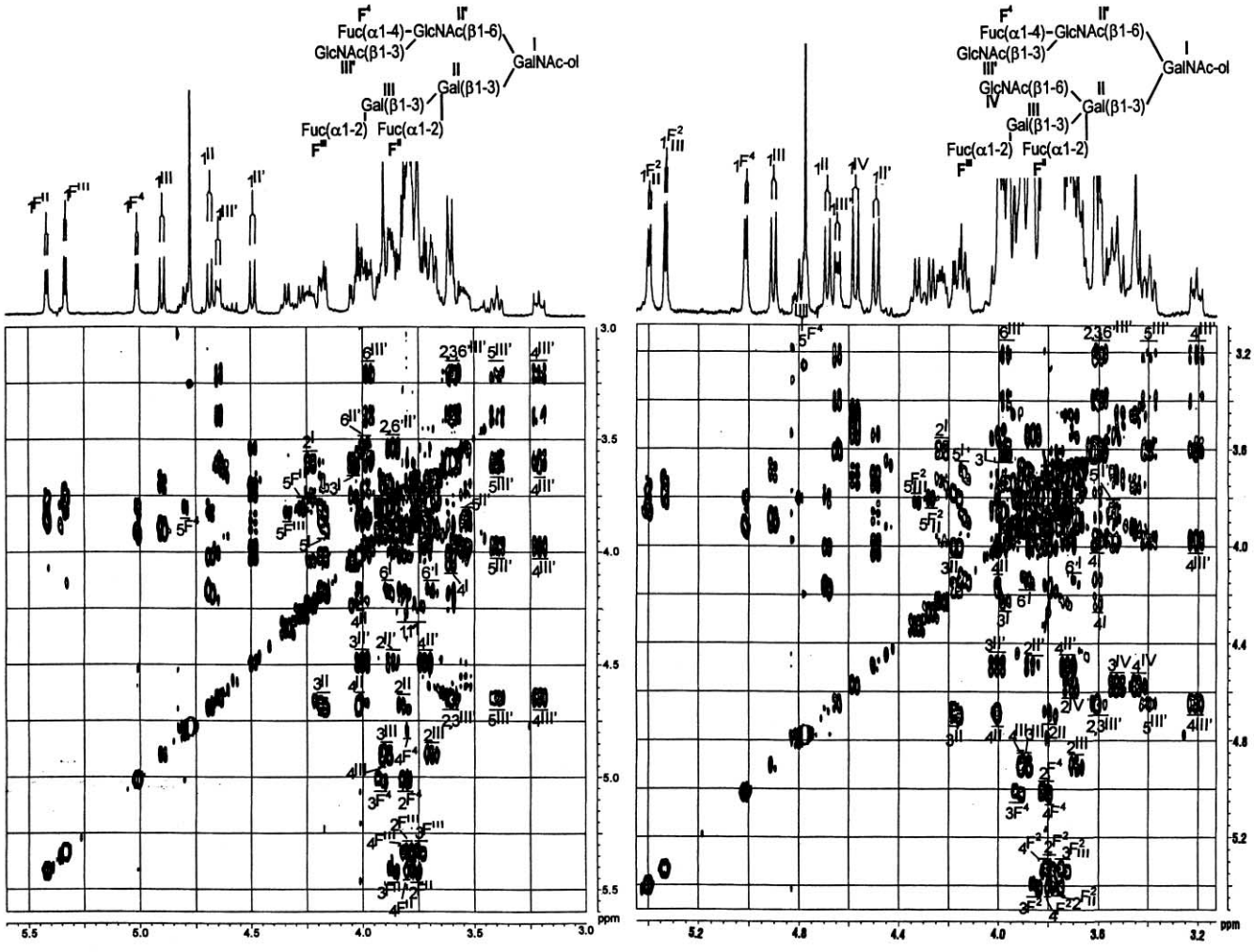


Fig. 6. Double relayed COSY spectra of compounds **6** (left) and **7** (right).

laria,[14] exhibits characteristic NMR features, such as a strong coupling between the H-1, H-2 and H-3 resonances of the β-(1→3)-linked unit to GlcNAc, and a significantly H-4 signal upfield from the bulk resonance.

In conclusion, this study has confirmed the structural species-specificity of the carbohydrate chain. As in the *P. waltl* and *A. tigrinum*, *B. viridis* oviducal mucins are available sources of Le^x and Le^y antigens. On the basis of the comparative studies performed on amphibia, it appears that Kdn is generally found in Urodelans, whereas the anionic charges in anurans can be carried by NeuAc, NeuGc (*N*-glycolylneuraminic acid), GlcA and sulfate groups. Moreover, many glycanic patterns have been characterized in particular species. For example, The HSO_3(3)GlcA(β1–3)Galβ sequence, which is a part of the HNK-1 epitope and the Fuc(α1–2)GlcA(β1–3)Galβ sequence have been identified in *Rana temporaria*[15,16] and not in other amphibia. The Fuc(α1–4)[Fuc(α1–5)]Kdn sequence is characteristic of the *A. tigrinum* species.[22] Recent studies, including the present case of *B. viridis*, confirm the species-specificity of amphibia carbohydrates, it must emphasized that such a specificity can be due to specific arrangements of oligosaccharidic sequences. For example, *B. viridis* and *R. arvalis*[20] both possess the Fuc(α1–2)Gal(β1–3)[Fuc(α1–2)][GlcNAc(β1–6)]Gal(β1–3) sequence but the carbohydrate chains of *R. arvalis* are devoid of Le^x and Le^y epitopes, and the anionic charges are carried by sulfate groups, instead of sialic acid. Such a polymorphism testifies the presence of specific inherited enzymatic equipment, with different specificities, and carbohydrates could be considered as markers for the species. The symbiotic function of carbohydrates has been often evoked as playing an important role during host-parasite interactions.[31] In this regard, it has been recently shown that *Xenopus laevis* and *Xenopus fraseri* each carry separate species-specific protopolystoma which do no occur in other *Xenopus* species.[32]

With these results in hand, we will now attempt to find a possible correlation between the species-specific glycotypes of amphibia and these host-specificities.

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References

1. Salthe, S. N. *J. Morphol.* **1963**, *113*, 161–171.
2. Elinson, R. P. *J. Embryol. Exp. Morph.* **1974**, *32*, 325–335.
3. Oterino, J.; Toranzo, G. S.; Zelarayan, L.; Bühler, M. I. *Zygote* **1997**, *5*, 267–271.
4. Jego, P.; Joly, J.; Boisseau, C. *Reprod. Nutr. Develop.* **1980**, *20*, 557–567.
5. Hoshi, M.; Nishigaki, T.; Ushiyama, A.; Okinaga, T.; Chiba, K.; Matsumoto, M. *Int. J. Dev. Biol.* **1994**, *38*, 167–174.
6. Jaiswal, B. S.; Cohen-Dayag, A.; Tur-Kaspa, I.; Eisenbach, M. *FEBS Lett.* **1998**, *427*, 309–313.
7. Shimoda, Y.; Kitajima, K.; Inoue, S.; Inoue, Y. *Eur. J. Biochem.* **1994**, *223*, 223–231.
8. Fraser, L. R. *Human Reprod.* **1998**, *13*, 9–19.
9. Sardet, C.; Roegiers, F.; Dumollard, R.; Rouviere, C.; McDougall, A. *Biophys. Chem.* **1998**, *72*, 131–140.
10. Strecker, G.; Wieruszeski, J. M.; Michalski, J. C.; Alonso, C.; Boilly, B.; Montreuil, J. *FEBS Lett.* **1992**, *298*, 39–43.
11. Strecker, G.; Wieruszeski, J. M.; Fontaine, M. D.; Plancke, Y. *Glycobiology* **1994**, *4*, 605–609.
12. Strecker, G.; Wieruszeski, J. M.; Plancke, Y.; Boilly, B. *Glycobiology* **1995**, *5*, 137–146.
13. Morelle, W.; Strecker, G. *Glycobiology* **1997**, *7*, 1129–1151.
14. Morelle, W.; Strecker, G. *Biochem. J.* **1997**, *321*, 879–887.
15. Maes, E.; Florea, D.; Delplace, F.; Lemoine, J.; Plancke, Y.; Strecker, G. *Glycoconjugate J.* **1997**, *14*, 127–146.
16. Florea, D.; Maes, E.; Strecker, G. *Carbohydr. Res.* **1997**, *302*, 179–189.
17. Morelle, W.; Cabada, M. O.; Strecker, G. *Eur. J. Biochem.* **1998**, *252*, 253–260.
18. Morelle, W.; Guyétant, R.; Strecker, G. *Carbohydr. Res.* **1998**, *306*, 435–443.
19. Maes, E.; Florea, D.; Coppin, A.; Strecker, G. *Eur. J. Biochem.* **1999**, *264*, 301–313.
20. Coppin, A.; Maes, E.; Flahaut, C.; Coddeville, B.; Strecker, G. *Eur. J. Biochem.* **1999**, *266*, 370–382.
21. Coppin, A.; Maes, E.; Morelle, W.; Strecker, G. *Eur. J. Biochem.* **1999**, *266*, 94–104.
22. Maes, E.; Plancke, Y.; Delplace, F.; Strecker, G. *Eur. J. Biochem.* **1995**, *230*, 146–156.
23. Mourad, R.; Morelle, W.; Neveu, A.; Strecker, G. *Eur. J. Biochem.* **2001**, *268*, 1990–2003.
24. Montreuil, J.; Bouquelet, S.; Debray, H.; Fournet, B.; Lemoine, J.; Michalski, J. C.; Spik, G.; Strecker, G. In *Carbohydrate Analysis, A Practical Approach*; Chaplin, M. F.; Kennedy, J. F., Eds. Glycoproteins; IRL: Oxford, 1986; p. 143.
25. Ciucanu, I.; Kerek, F. *Carbohydr. Res.* **1984**, *131*, 209–217.
26. Fournet, B.; Strecker, G.; Leroy, Y.; Montreuil, J. *Anal. Biochem.* **1981**, *116*, 489–502.
27. Wagner, G. *J. Magn. Reson.* **1983**, *55*, 151–156.
28. Bax, A. D.; Davis, D. G. *J. Magn. Reson.* **1985**, *63*, 207–213.
29. Kamerling, J. P.; Vliegenthart, J. F. G. *Biol. Magn. Reson.* **1992**, *10*, 1–194.
30. van Halbeek, H.; Gerwig, G. J.; Vliegenthart, J. F. G.; Tsuda, R.; Hara, M.; Akiyama, K.; Schmid, K. *Biochem. Biophys. Res. Commun.* **1985**, *131*, 507–514.
31. Gagneux, P.; Varki, A. *Glycobiology* **1999**, *98*, 747–755.
32. Tinsley, R. C.; Jackson, J. A. *Int. J. Parasitol.* **1998**, *28*, 1573–1582.