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Carbohydrate Research 306 (1998) 435–443

CARBOHYDRATE RESEARCH

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

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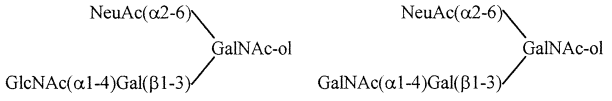
Received 19 August 1997; accepted 3 November 1997

Abstract

The O-linked oligosaccharides of the jelly coat surrounding the eggs of *Rana dalmatina* were released by alkaline borohydride treatment. Low-molecular-mass, monosialyl oligosaccharide-alditols were isolated by anion-exchange chromatography and fractionated by consecutive normal-phase high-performance liquid chromatography on a silica-based alkylamine column. The structures of the oligosaccharide-alditols were determined by 400-MHz ^{1}H-NMR spectroscopy in combination with matrix assisted laser desorption ionization–time of flight analysis. The five structures were identified range in size from trisaccharides to hexasaccharides, possessing a core consisting of Gal(β1–3)GalNAc-ol (core type 1). Novel oligosaccharide-alditols are:



The carbohydrate chains isolated from *Rana dalmatina* are different from those found in other amphibian species, in which the presence of species-specific material has been characterized. Since the role of carbohydrates appears more and more apparent during the fertilization process, the biodiversity of the O-linked oligosaccharides could support such a biological role.

Keywords: Amphibian egg jelly coats; *Rana dalmatina*; NMR; Oligosaccharides

1. Introduction

Amphibian eggs are surrounded by a vitelline envelope and several structurally and chemically distinct jelly coats. The jelly coats are made up of several layers of distinct composition and morphology which are highly variable according to the species or group of amphibians [1]. Chemical analyses have indicated their glycoprotein nature, with 60–70% carbohydrates and 30–40% proteins the relative amount of which is variable according to

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0008-6215/98/$19.00
PII S0008-6215(97)10074-X

species. The low amount of Man, as well as the high proportions of GalNAc, serine and threonine suggested that they were typical mucin-type glycoproteins. These glycoproteins have been shown to play important roles in fertilization [2–4]. The jelly coat is not a simple barrier for a fertilizing sperm but an obligatory participant in the process of sperm–egg fusion, as dejellied eggs cannot be fertilized. Many functions have been attributed to the egg jelly components such as sperm binding, sperm capacitation, sperm activation, induction of the sperm acrosome reaction, prevention of species cross-fertilization, the blocking of polyspermy and provision of a protective environment for the developing embryo [5–10]. In view of the prevalent occurrence of glycoproteins in the amphibian egg coats, the question of how these glycoproteins are involved in any function during fertilization and early development is important but has remained unanswered. Our incomplete understanding of their functions is, in part, due to the difficulty of their purification.

A study of the fertilization-impairing activity of vitelline coat components in *Bufo arenarum* proposed that mannose residues in vitelline coat are involved in the recognition of sperm [11]. In *Bufo japonicus*, the carbohydrate moieties in 36–39 kDa glycoproteins of the vitelline coat, exposed as a result of hydrolysis by the oviducal *pars recta* protease, are involved in binding with fertilizing sperm. GalNAc and/or sialic acid, but not Man, are among the sugar residues functioning in the binding [12]. A major glycoprotein from egg jelly coat of *Bufo japonicus* has been isolated and was shown to display calcium-binding properties. The high Ca^{++} binding capacity of this glycoprotein was abolished by its desialylation. The function of this glycoprotein could be to preserve and modulate Ca^{++} ions for fertilizing sperm [13]. In *Xenopus laevis*, lectins are contained within the cortical granules at fertilization. These lectins bind to oligosaccharide targets on glycoproteins in the egg jelly coat where they participate in the formation of the fertilization envelope which blocks sperm entry [14,15]. Previous structural studies have shown that the mucins possess species-specific glycan chains [16,17], that could support the observed species-specificity of gamete interaction. The structural analysis of these components should be useful to the study of the mechanism of jelly action in fertilization.

In this study, the O-linked sugar chains were released from *Rana dalmatina* egg jelly coats by β-elimination and their oligosaccharide structures were determined by 400-MHz ^{1}H-NMR spectroscopy in conjunction with Matrix-assisted laser desorption ionization-time of flight (MALDI–TOF) mass spectrometry.

2. Experimental

Sampling of jelly coat mucus.—Eggs from *Rana dalmatina* were obtained from natural spawnings in nature. The jelly coat material was lyophilized.

Isolation of oligosaccharide-alditols.—O-linked oligosaccharides were released from the crude material by alkaline borohydride treatment in 1 M $NaBH_4$, 0.1 M NaOH at 37 °C for 48 h. The reaction was stopped by the addition of Dowex 50×8 (25–50 mesh, H^+ form) at 4 °C. The solution was filtered, adjusted to pH 6.5 and then concentrated under vacuum at 25 °C. Borate salts were removed by repeated evaporation from methanol. The resulting sample was applied to a Bio-Gel P-2 (45–90 μm; Bio-Rad Labs, Richmond, CA, USA) column (1×100 cm) and eluted with water at a flow rate of 22 mL/h. Fractions (7 mL) were collected and analyzed for absorbance at 206 nm. The fractions were revealed with orcinol-sulphuric acid reagent [18] on silica gel plates (pre-coated silica gel 60; Merck; Darmstadt, Germany). The carbohydrate-containing fractions were pooled and concentrated under vacuum at 25 °C. Peptide material was removed on a column (20×1 cm) of Dowex 50×2 (200–400 mesh; H^+ form). Oligosaccharide-alditols were applied on a column (20×1 cm) of Dowex 1×2 ($HCOO^-$ form). After washing with 500 mL of water, oligosaccharide-alditols were eluted with a discontinuous gradient (50, 100 and 200 mM) of pyridine-acetic acid buffer. The fractions (500 mL) were concentrated at 25 °C and further desalted by gel permeation on a Bio-Gel P2 column (90×2.3 cm) eluted with deionized water at a flow rate of 14 mL/h. Fractions (4 mL) were collected and analyzed for absorbance at 206 nm and for hexose by orcinol/sulphuric acid coloration. The carbohydrate-containing fractions were pooled and lyophilized.

Oligosaccharide-alditols were isolated by high performance liquid chromatography (HPLC) on a primary amine-bonded silica column (Supelcosyl LC-NH2, 4.6×250 mm; Supelco Inc., Bellefonte, USA) using acetonitrile/water containing 15 mM

potassium phosphate as the eluent at a flow rate of 1 mL/min at room temperature. Oligosaccharide peaks were detected by absorption at 206 nm. Acetonitrile was evaporated from all collected fractions under a stream of nitrogen and then concentrated under vacuum at 25 °C. All the collected fractions were purified by gel permeation on a Sephadex G-10 column (45×0.5 cm, Pharmacia) equilibrated with deionized water at a flow rate of 6 mL/h, monitored by absorbance at 206 nm (LKB 2138 Uvicord S). Fractions (1 mL) were collected and analyzed for hexose by orcinol/sulphuric acid coloration. The carbohydrate-containing fractions were pooled, concentrated, and then lyophilized.

^{1}H-NMR spectroscopy.—^{1}H-NMR experiments were 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 ($\delta = 2.225$ in D_2O at 25 °C). The two dimensional homonuclear correlated spectroscopy (COSY) with simple and double relay transfer was performed using Bruker standard pulse sequences.

Matrix-assisted laser desorption mass spectrometry (MALD–MS).—Molecular weights of the oligosaccharides were measured by matrix assisted laser desorption ionization (MALDI) on a Vision 2000 time of flight instrument (Finnigan Mat, Bremen, Germany) equipped with a 337 nm UV laser. Samples were mounted on an x–y moveable stage allowing irradiation of selected sample areas. The mass spectra were acquired in the reflectron mode under 6 kV accelerating voltage and negative detection.

The sample was dissolved in water at a concentration of 20 pmoles per microliter. One μL of the analyte solution was mixed with an equal volume of the matrix solution onto the target then allowed to crystallize at room temperature. 2,5-dihydroxy benzoic acid (10 mg/mL dissolved in water–ethanol, 80:20) was used for the monoacidic fractions. External calibration was performed using angiotensin I purchased from Sigma. 10 to 15 scans were accumulated for each spectrum.

3. Results

The acidic oligosaccharides released by reductive β-elimination were fractionated into four major components on an LC–NH_2 column (Fig. 1). The primary structures of the oligosaccharide-alditols obtained from oviductal mucins of *Rana dalmatina*

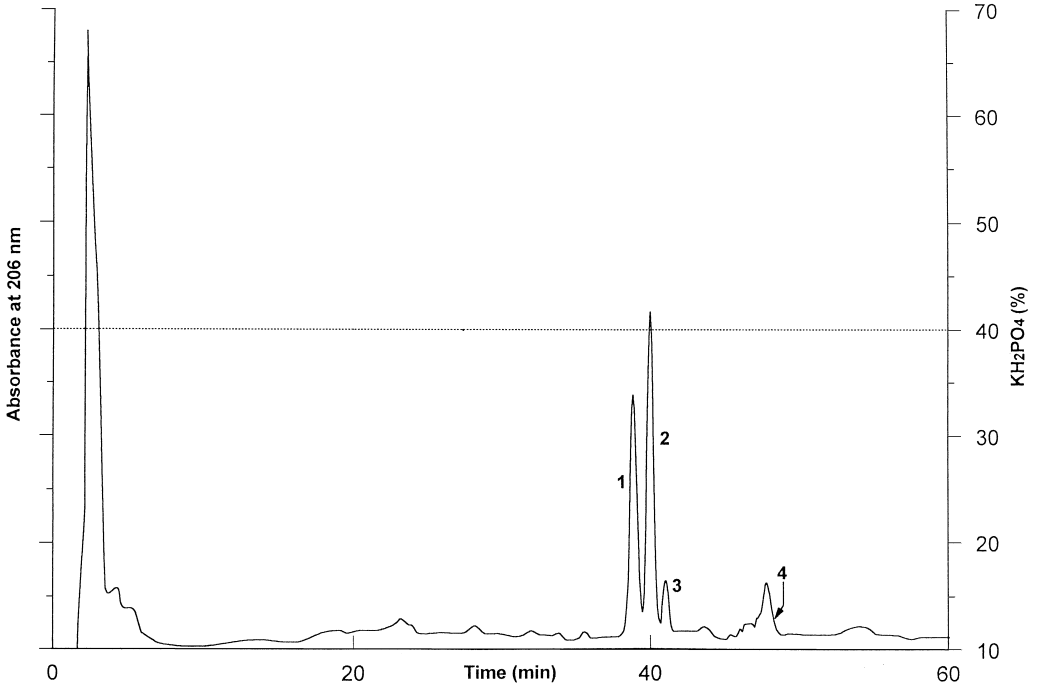
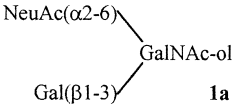
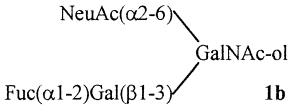


Fig. 1. Separation of acidic oligosaccharide-alditols released from the egg jelly coat of *Rana dalmatina* by HPLC. Samples were chromatographed on a column (4.6×250 mm) of primary amine-bonded silica (Supelcosyl LC–NH_2; Supelco Inc., Bellefonte, USA) using gradient elution with acetonitrile/water containing 15 mM potassium phosphate, as depicted on the profile. Flow rate was 1 ml/min. Absorbance was measured at 206 nm.

were established by combining the results of matrix assisted laser desorption ionization–time of flight (MALDI–TOF) analysis (Table 1) and ^{1}H-NMR spectroscopy (Table 2). Acidic oligosaccharide-alditols were analysed by MALDI–TOF spectrometry in the negative ion mode. The spectra showed characteristic $[M–H]^-$ pseudomolecular ions (Table 1). Fraction **1** contained a mixture of two compounds composed of NeuAc, Gal, GalNAc-ol (1:1:1) and NeuAc, Gal, Fuc and GalNAc-ol (1:1:1:1), respectively (Table 1). Their relevant ^{1}H-NMR parameters perfectly matched those observed for NeuAc(α2–6)[Gal(β1–3)]GalNAc-ol and NeuAc(α2–6)[Fuc(α1–2)Gal(β1–3)]GalNAc-ol [19].

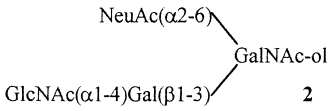


1a



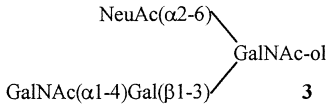
1b

The MALDI–TOF spectrum of compound **2** showed a $[M–H]^-$ pseudo-molecular ion at m/z 878, which indicated the following molar proportions: HexNAc, Hex, NeuAc, HexNAc-ol (1:1:1:1). The HexNAc and Hex units were identified as α-GlcNAc and β-Gal on the basis of the set of the vicinal coupling constants $J_{1,2}$ to $J_{4,5}$ (S, L, L, L and L, L, S, S, respectively; S < 4Hz, L < 6Hz), as shown in Fig. 2. The set of chemical shifts of NeuAc H-3ax at $\delta = 1.671$ ppm and H-3eq at $\delta = 2.729$ is typical for the (α2 → 6) linkage to GalNAc-ol, the H-6 and H-6′ resonances of which ($\delta = 3.850$ and 3.515, respectively) reflect this substitution (Fig. 2 and Table 2). The chemical shifts of Gal H-1, H-4 and GlcNAc H-1, H-2, H-3, H-4 were identical with those observed for the neutral oligosaccharide-alditol GlcNAc(α1–4)Gal(β1–3)-GalNAc-ol [20], proving the attachment of α-GlcNAc at O-4 of β-Gal. On the basis of these observations, the structure of compound **2** was deduced to be the following:



2

Compound **3** was also composed of HexNAc, Hex, NeuAc and GalNAc-ol in the ratio 1:1:1:1. From the NMR analysis (Fig. 3 and Table 2), the HexNAc unit is clearly identified as α-GalNAc on the basis of the set of its vicinal coupling constants ($J_{1,2}$∼4Hz; $J_{2,3}$∼8Hz; $J_{3,4}$∼3Hz; $J_{4,5}$∼1Hz). The introduction of GalNAc in an (α1–4) linkage to Gal causes the downshift of the Gal H-4 at $\delta = 4.976$. The observed shift effect for GalNAc-ol H-6 and H-6′ reflects the attachment of NeuAc at the 6 position. Therefore, the structure of **3** was established as follows:



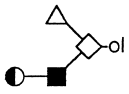
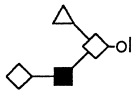
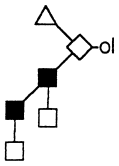
3

The MALDI analysis of compound **4** showed a $[M–H]^-$ pseudo molecular ion at 1129, which indicated the following molar proportions: Fuc, Gal, NeuAc, GalNAc-ol (2:2:1:1). From the NMR spectrum (Fig. 4), the core structure NeuAc(α2–6)[Gal(β1–3)]GalNAc-ol was established by NeuAc H-3ax and H-3eq signals at $\delta = 1.681$ and 2.725, respectively, in conjunction with GalNAc-ol H-6 and H-6′ at $\delta = 3.84$ and 3.463, respectively. The sequence Fuc(α1–2)Gal(β1–3)[Fuc(α1–2)]Galβ was deduced from the set of Gal II H-3 and H-4 resonances observed at $\delta = 4.182$ and 4.009, respectively. For the isomeric sequence Fuc(α1–2)Gal(β1–4)[Fuc(α1–2)]Galβ, these values were found at $\delta = 4.010$ and 4.270, respectively (unpublished). The assignment of the Fuc H-1 resonances

Table 1
Monosaccharide composition data for oligosaccharide-alditols released from the mucin of the jelly coat. The compositions of five oligosaccharide-alditols were deduced from $[M–H]^-$ pseudomolecular ions obtained by negative ion MALDI–TOF spectrometry. Monosaccharide units were identified on the basis of their vicinal coupling constants by NMR

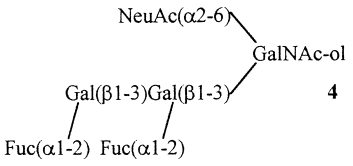
Compounds	$[M–H]^-$	Molar ratio					
		GalNAc-ol	Gal	GlcNAc	GalNAc	Fuc	NeuAc
1a	675	1	1	0	0	0	1
1b	821	1	1	0	0	1	1
2	878	1	1	1	0	0	1
3	878	1	1	0	1	0	1
4	1129	1	2	0	0	2	1

Table 2
^{1}H-NMR chemical shifts of the oligosaccharide-alditols

Residue	Reporter	Chemical shifts (ppm)		
				
		2	**3**	**4**
GalNAc-ol **I**	H-1	3.806	3.807	n.d.
	H-1′	3.753	3.75	n.d.
	H-2	4.394	4.394	4.24
	H-3	4.074	4.077	4.06
	H-4	3.563	3.564	3.576
	H-5	4.248	4.244	4.15
	H-6	3.850	3.846	3.84
	H-6′	3.515	3.522	3.463
	NAc	2.054	2.054	2.042
Gal (β1–3) **II**	H-1	4.521	4.521	4.699
	H-2	3.618	3.620	3.817
	H-3	3.743	3.738	4.182
	H-4	3.966	3.976	4.009
	H-5	n.d.	n.d.	n.d.
Gal (β1–3) **III**	H-1	—	—	4.893
	H-2	—	—	3.686
	H-3	—	—	3.891
	H-4	—	—	3.919
	H-5	—	—	n.d.
GalNAc (α1–4) **III**	H-1	—	4.901	—
	H-2	—	4.170	—
	H-3	—	4.007	—
	H-4	—	4.048	—
	H-5	—	4.394	—
	H-6,6′	—	3.74	—
	NAc	—	2.121	—
GlcNAc (α1–4) **III**	H-1	4.867	—	—
	H-2	3.891	—	—
	H-3	3.825	—	—
	H-4	3.539	—	—
	H-5	4.190	—	—
	H-6	3.775	—	—
	H-6′	3.775	—	—
	NAc	2.125	—	—
Fuc (α1–2) **F(II)**	H-1	—	—	5.326
	H-2	—	—	3.796
	H-3	—	—	3.73
	H-4	—	—	n.d.
	H-5	—	—	4.33
	CH_3	—	—	1.235
Fuc (α1–2) **F(III)**	H-1	—	—	5.403
	H-2	—	—	3.76
	H-3	—	—	3.76
	H-4	—	—	n.d.
	H-5	—	—	4.30
	CH_3	—	—	1.201
NeuAc (α2–6)	H-3 ax	1.671	1.684	1.681
	H-3 eq	2.729	2.728	2.721
	H-4	3.682	3.682	3.67
	H-5	3.821	3.819	3.80
	H-6	3.69	3.69	n.d.
	H-7	n.d.	3.587	n.d.
	H-8	3.88	3.87	n.d.
	H-9	3.88	3.87	n.d.
	H-9′	3.62	3.642	n.d.
	NAc	2.034	2.034	2.033

The monosaccharides are represented by this symbolic notation: ◇-ol = GalNAc-ol; ■ = βGal; ◐ = αGlcNAc; □ = αFuc; △ = αNeuAc.
n.d. = not determined.
The linkage position is specified by the direction of the connecting bars as follows: 

was deduced from a previous observation concerning the sequence Fuc*(α1–2)Gal(β1–3)-[Fuc(α1–2)Gal-ol in which the anomeric proton of the terminal Fuc* unit resonates at $\delta = 5.48$ [21]. On the basis of these observations, the structure of **4** was deduced to be the following



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4. Discussion

The carbohydrate chains from *Rana dalmatina* jelly coat mucus glycoprotein eggs were released in the form of reduced oligosaccharides by alkaliborohydride treatment. Fractionation of the mixture of O-linked oligosaccharide-alditols was achieved by a combination of chromatographic techniques comprising gel-permeation chromatography on Bio-Gel P-2, anion exchange chromatography on Dowex 1×2 and normal-phase

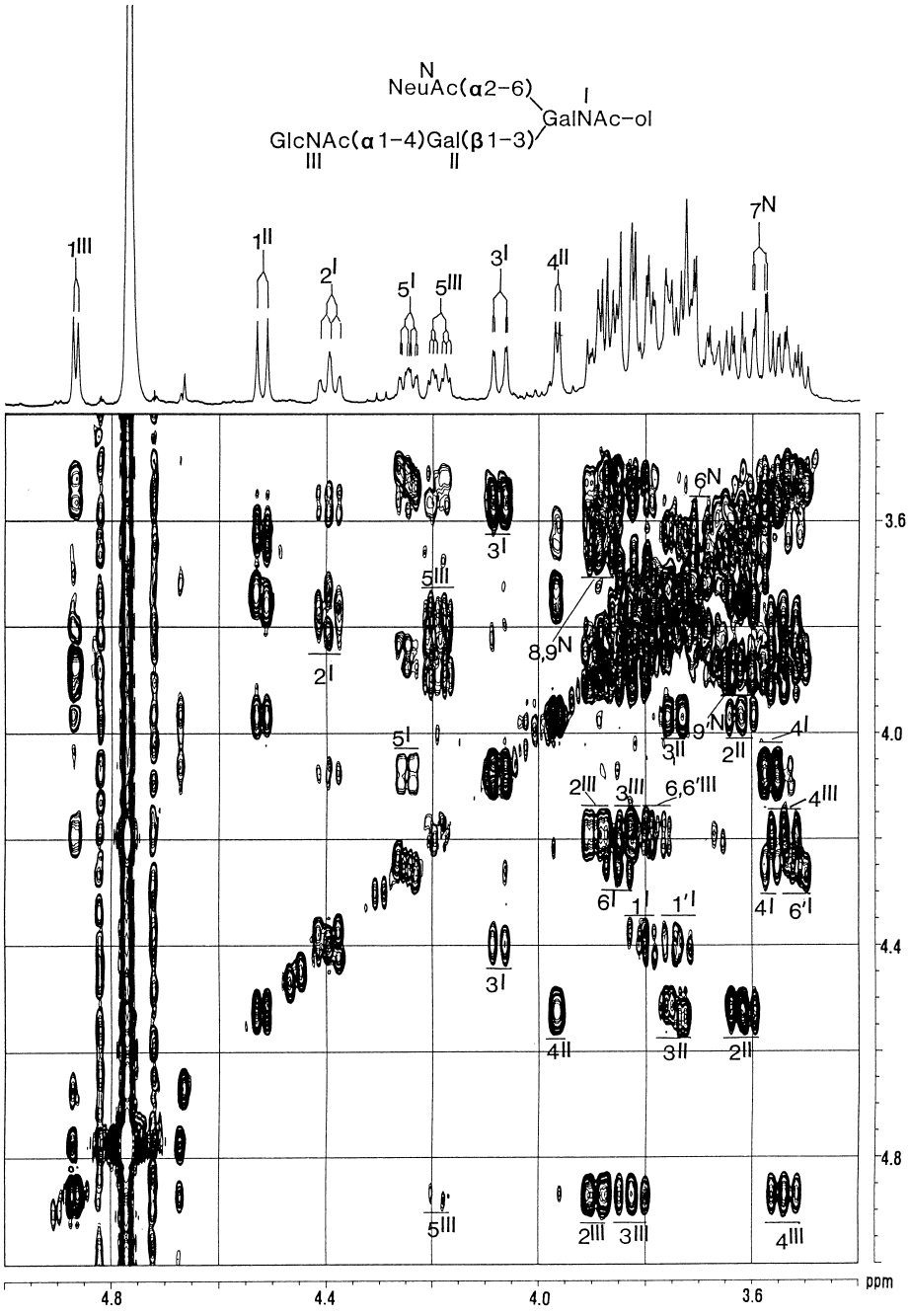

Fig. 2. Double-relayed COSY spectrum of oligosaccharide-alditol **2**.

high-performance liquid chromatography on a silica-based alkylamine column. Five oligosaccharide-alditols were characterized and some of them exhibit new types of structures. These O-glycans form a series of sialyl oligosaccharides. The core structure is exclusively Gal(β1–3)GalNAc-ol. Sialic acid is always (α2–6)-linked to GalNAc-ol. The species-specific structural diversity, which has previously been observed in carbohydrate chains of amphibian egg jellies, was partially verified for *Rana dalmatina*. Whereas compound **4** is present in the mucin of *Xenopus laevis* [22], compounds **2** and **3** are novel, characteristic of *Rana dalmatina*. Moreover, the carbohydrate chains found in this species strongly differ from those which are characteristic of *Rana temporaria* [16], although a close phylogenetic relationships between these two species is known. A compilation of dozens of carbohydrate chains which are characteristic of eight other amphibian species does not allow to deduce a taxonomic or phylogenetic relationship between these species. Therefore, the species-specificity of

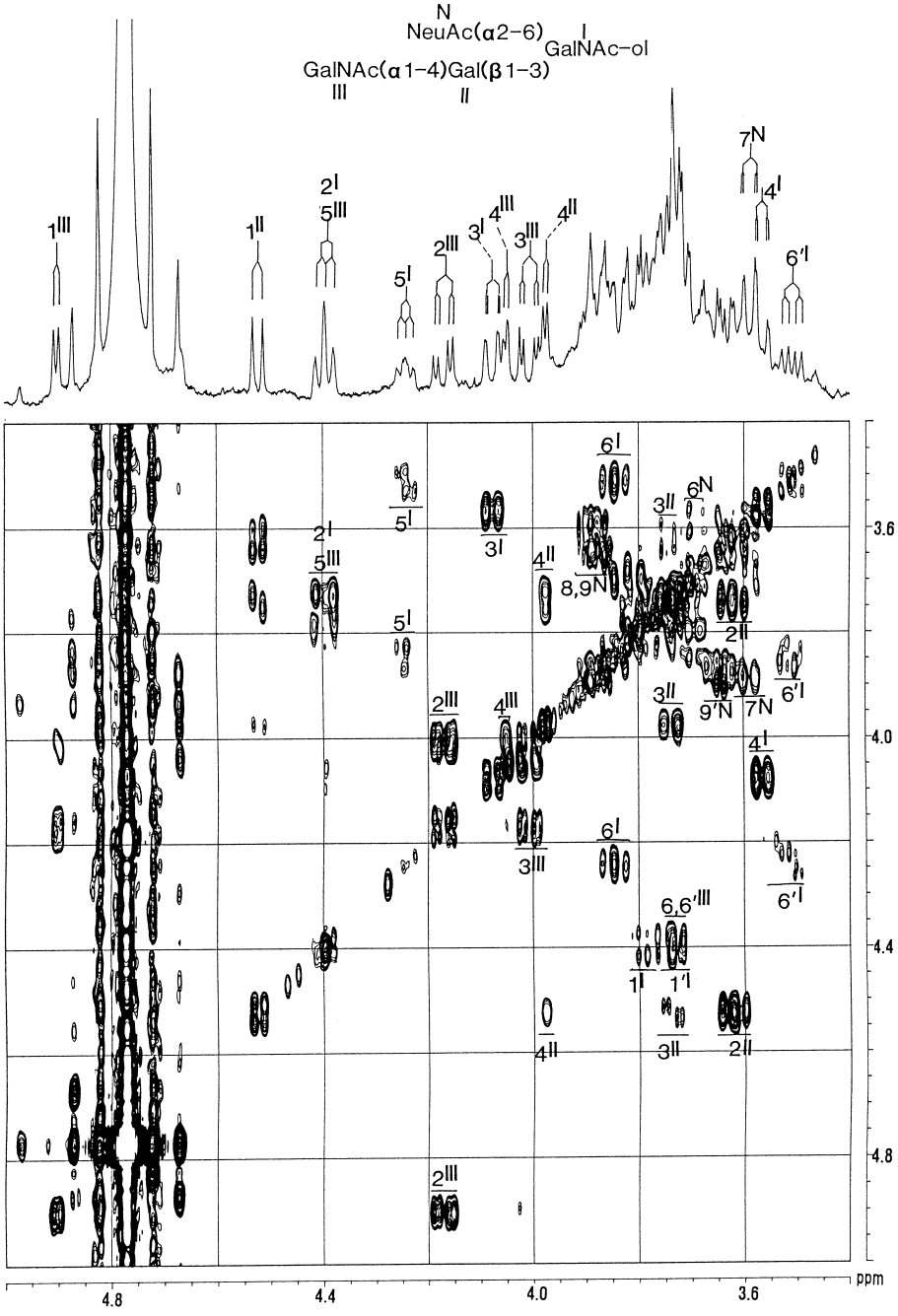

Fig. 3. COSY spectrum of oligosaccharide-alditol **3**.

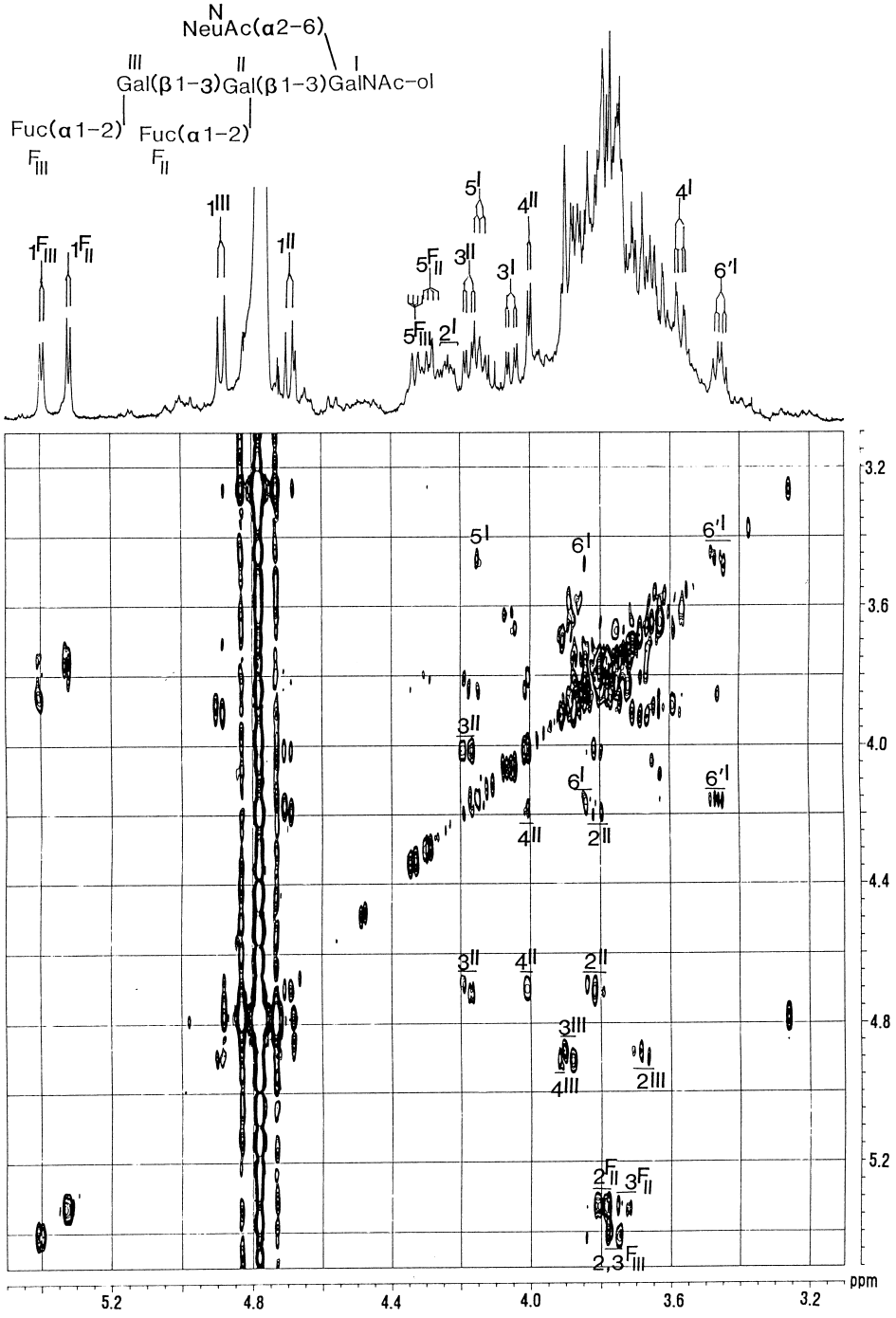

Fig. 4. Double-relayed COSY spectrum of oligosaccharide-alditol **4**.

the carbohydrate chains may be related to the activity levels and specificities of transferases in the glycosylation process of oviducal mucins. It has been found that the molecular basis of the human histo-blood group AB system is explained by the finding that the α-GalNAc and α-Gal transferases differ only by four amino acids and the genes by a few base pairs [23]. Therefore, the expression of the structural biodiversity which characterizes the carbohydrate chains of amphibian oviducal mucins could be relevant to similar substitutions.

Acknowledgements

This research was supported by the Centre National de la Recherche Scientifique (UMR 111, Relations Structure–Fonction des constituants

membranaires, Director Professor André Verbert) and by the Ministère de l'Enseignement Supérieur et de la Recherche.

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