Structural analysis of a new series of oligosaccharide-alditols released by reductive β -elimination from oviducal mucins of *Rana utricularia*

Willy MORELLE and Gérard STRECKER¹

Laboratoire de Chimie Biologique et Unité Mixte de Recherche du CNRS 111, Université des Sciences et Technologies de Lille, 59655 Villeneuve d'Ascq Cedex, France

Egg jelly coats from *Rana utricularia* are formed by components secreted along the oviduct. These secretion products overlay the oocytes as they pass along the different oviducal portions. In this study, carbohydrate chains of the jelly coat surrounding the eggs of *R. utricularia* were released by alkali/borohydride treatment. Fractionation of O-linked oligosaccharide-alditols was achieved by a combination of chromatographic techniques comprising anion-exchange chromatography, gel-permeation chromatography and HPLC on a silica column bonded with aminopropyl groups. Structural characterization was performed by one- and two-dimensional ¹H-NMR spectroscopy in combination with matrix-assisted laser-desorption ionization–time of flight MS and methylation analysis. Ten oligosaccharide structures

possessing a core consisting of Gal β (1 $\rightarrow$ 3)GalNAc-ol with or without branching through a GlcNAc residue linked β (1 $\rightarrow$ 6) to the GalNAc residue (core type 2 or core type 1 respectively) are described. The most representative carbohydrate sequences are: GlcNAc(β 1-3)[Fuc(α 1-4)]GlcNAc, GalNAc(α 1-3)[Fuc(α 1-2)]-Gal(β 1-4)GlcNAc(β 1-3)GlcNAc and Gal(β 1-3)GlcNAc(α 1-3)-[Fuc(α 1-2)]Gal(β 1-4)GlcNAc. The carbohydrate chains isolated from *R. utricularia* are quite different from those found in other amphibian species, in which the presence of species-specific material has been characterized. Since the jellies surrounding amphibian eggs are involved in egg–sperm interactions, these structural investigations can provide biochemical support for investigation of the fertilization process.

INTRODUCTION

Amphibian eggs are surrounded by a vitelline envelope opposed to the plasma membrane and a series of structurally and chemically distinct jelly coats; the latter are synthesized by specific regions of the oviduct and are sequentially deposited as the egg is transported toward the cloaca [1,2]. This jelly coat is the first barrier through which fertilizing sperm must pass before reaching the egg plasma membrane. In anurans, the number of jelly coats varies among species from as many as six in *Rana pipiens* [3] to as few as three in *Xenopus laevis* [4]. The jelly coats are made up of several layers of distinct composition and morphology which can vary widely between species [5]. The significance of the variable number of jelly coat layers is not yet understood. Interest in these oviductally derived investments has centred on their obligatory participation in the early events of fertilization [6–8]. This conclusion is based on the observation that, whereas dejellied eggs exposed to sperm exhibit a very low degree of fertilizability, this deficit can be reversed in the presence of solubilized jelly. Factors in the jelly contribute to the ability of the sperm to penetrate the egg extracellular matrix. This was first shown by Kambara [9] and has subsequently been demonstrated in many anurans [10]. The functions that have been suggested to be associated with amphibian jelly coats at fertilization include sperm binding, sperm activation (capacitation and/or induction of the acrosome reaction), prevention of species cross-fertilization, the blocking of polyspermy and provision of a protective environment for the developing embryo [11,12].

Mucin-type glycoproteins have been shown to be the major components of this coat. These highly glycosylated molecules synthesized by oviduct cells are generally considered to play an

important role in fertilization [13–15]. However, the molecular mechanisms underlying such functions are not yet well characterized. A study in *Bufo arenarum* proposed that mannose residues in the envelope vitelline are involved in the recognition of sperm [16]. It has not been clarified, however, at what steps of fertilization these residues function. Further investigation is needed to identify the vitelline envelope components of amphibians that are involved in the recognition and binding of sperm. In *Bufo japonicus*, it has been reported that galactose residues are exposed on the surface of acrosome-reacted sperm [17]. Recently, a major glycoprotein from the egg jelly coat of *B. japonicus* has been isolated and shown to display Ca²⁺-binding properties. The high Ca²⁺-binding capacity of this glycoprotein was abolished by its desialylation. The function of this glycoprotein could be to preserve and modulate Ca²⁺ ions for fertilizing sperm [18]. Previous structural studies have shown that the mucin possesses species-specific glycanic chains [19–23], which probably support the observed species-specificity of gamete interaction. Certainly, the great diversity of known oligosaccharide structures is compatible with generation of species-specificity for gamete interactions. The variety of compositions, sequences, branching patterns, conformations and other features of oligosaccharides associated with glycoproteins provide a staggering number of combinatorial possibilities. Structural analysis of these components should be useful for the study in more detail of the mechanism of jelly action in fertilization.

In a previous study [21], the isolation and characterization of 12 oligosaccharides from the jelly coat of *Rana utricularia* was described. The present paper deals with the determination of the structure of a new series of oligosaccharides isolated from the same source. Their structures were determined by 400 MHz ¹H-

Abbreviations used: MALDI–TOF, matrix-assisted laser desorption ionization–time of flight; GC/MS, gas chromatography/mass spectrometry; GalNAc-ol, N-acetylgalactosaminitol.

¹ To whom correspondence should be addressed.

NMR spectroscopy in conjunction with matrix-assisted laser-desorption ionization–time of flight (MALDI–TOF) MS and methylation analysis.

MATERIALS AND METHODS

Sampling of jelly coat mucus

Eggs from *R. utricularia* 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 alkali/borohydride treatment in 1 M NaBH₄/0.1 M NaOH at 37 °C for 48 h. The reaction was stopped by the addition of Dowex 50 (X8; 25–50 mesh; H⁺ form) at 4 °C. The solution was filtered, adjusted to pH 6.5 and then concentrated in a rotary evaporator at 25 °C. Borate salts were removed by repeated evaporation with methanol. The resulting sample was applied to a Bio-Gel P-2 (45–90 µm; Bio-Rad Laboratories, Richmond, CA, U.S.A.) column (1 cm × 100 cm) and eluted with water at a flow rate of 22 ml/h. Fractions (7 ml) were collected and analysed for A₂₀₆. The fractions were visualized with orcinol/H₂SO₄ reagent [24] on silica-gel plates (precoated silica gel 60; Merck, Darmstadt, Germany). The carbohydrate-containing fractions were pooled and concentrated in a rotary evaporator at 25 °C. Peptide material was removed on a column (20 cm × 1 cm) of Dowex 50 (X2; 200–400 mesh; H⁺ form). Oligosaccharide-alditols were applied to a column (20 cm × 1 cm) of Dowex 1 (X2; OH[−] form). After a wash with 500 ml 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 in a rotary evaporator at 25 °C and further desalted by gel permeation on a Bio-Gel P-2 column (90 cm × 2.3 cm internal diameter) eluted with deionized water at a flow rate of 14 ml/h. Fractions (4 ml) were collected and analysed for A₂₀₆ and for hexose by orcinol/H₂SO₄ coloration. The carbohydrate-containing fractions were pooled and lyophilized.

Oligosaccharide-alditols were isolated by HPLC on a primary amine-bonded silica column (Supelcosyl LC-NH₂; 4.6 cm × 250 mm; Supelco Inc., Bellefonte, PA, U.S.A.) using acetonitrile/water containing 3 mM potassium phosphate with a flow rate of 1 ml/min at room temperature. Oligosaccharide peaks were detected by measuring A₂₀₆. Acetonitrile was evaporated from all collected fractions by a stream of nitrogen, and the fractions were then concentrated in a rotary evaporator at 25 °C. All the collected fractions were purified by gel permeation on a Sephadex G-10 column (45 cm × 0.5 cm; Pharmacia) equilibrated with deionized water at a flow rate of 6 ml/h, and monitored by measuring A₂₀₆ (LKB 2138 Uvicord S). Fractions (1 ml) were collected and analysed for hexose by orcinol/H₂SO₄ coloration. The carbohydrate-containing fractions were pooled, concentrated in a rotary evaporator at 25 °C and then lyophilized.

Analytical procedures

Carboxyl reduction

Before methylation of compounds (50-II-3, 50-II-4, 50-II-5 and 50-II-6), oligosaccharide-alditols were dissolved in dry DMSO (100 µl). Methyl iodide (100 µl) was added, and the solution was mixed well and allowed to stand for 2 h at room temperature in

order to convert the carboxylic acid group into a methyl ester. After lyophilization, the derivatized oligosaccharide-alditol was dissolved in dry methanol (500 µl) and the methyl ester was reduced using NaB²H₄ at room temperature for 12 h. After the addition of acetic acid to pH 6, the sample was dried under reduced pressure. Boric acid was removed as methyl borate by evaporating repeatedly (5–6 times) with about 5 ml of methanol using a rotary evaporator at 25 °C. The dried material was further purified by gel-filtration chromatography on a column (45 cm × 0.5 cm) of Sephadex G-10 (Pharmacia) equilibrated with deionized water. The column was eluted with deionized water at a flow rate of 6 ml/h, and the eluate monitored by measuring A₂₀₆ (LKB 2138 Uvicord S). Fractions (1 ml) were collected and analysed for hexose by orcinol/H₂SO₄ coloration. The carbohydrate-containing fractions were pooled, concentrated in a rotary evaporator at 25 °C and then lyophilized.

Methylation

The carboxyl-reduced oligosaccharide or the native oligosaccharide (50 µg) was dissolved in 50 µl of a NaOH/DMSO suspension, prepared by stirring anhydrous DMSO with powdered NaOH [25]. After sonication for 30 min, 100 µl of methyl iodide was added and the suspension was sonicated for another 2 h. The methylated product was extracted into chloroform and back-washed with water. After evaporation with a stream of nitrogen, traces of DMSO were removed by lyophilization. The methylated product was subjected to methanolysis (500 µl of 0.5 M HCl in anhydrous methanol and heated at 80 °C for 24 h). After cooling, the solution was dried under a flow of nitrogen, and 50 µl of pyridine and 200 µl of acetic anhydride were added; the sample was kept at room temperature overnight. The reagents were evaporated under nitrogen, and the sample was dissolved in 50 µl of methanol.

Gas chromatography/mass spectrometry (GC/MS)

The partially acetylated methyl glycosides were analysed by GC/MS as follows: capillary column (25 cm × 0.33 mm; Silicone OV101), using helium as carrier gas at a pressure of 50.7 kPa with the following temperature program (120–240 °C at 2 °C/min). GC/MS analyses were carried out on a Nermag R10-10 mass spectrometer (Rueil Malmaison, France). MS analyses were carried out in electron impact (70 eV) or chemical ionization mode using NH₃ as reactant gas.

¹H-NMR spectroscopy

¹H-NMR experiments were performed on a Bruker ASX 400 WB spectrometer. Chemical shifts are expressed in p.p.m. downfield from internal sodium 4,4'-dimethyl-4-silapentane-1-sulphonate but were actually measured by reference to internal acetone (δ = 2.225 p.p.m. in ²H₂O at 25 °C). The two-dimensional homonuclear COSY with single- and double-relay transfer was performed using Bruker standard pulse sequences.

MALDI–TOF MS

Molecular mass of the oligosaccharides was measured by MALDI on a Vision 2000 TOF 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 reflectron mode under 6 kV accelerating voltage and positive or negative detection.

Figure 1 HPLC profiles of oligosaccharide-alditols from fraction 50-II (A) and 50-I (B)

Samples were chromatographed on a column (250 mm $\times$ 4.6 mm) of primary amine-bonded silica (Supelcosyl LC-NH2) using gradient elution with acetonitrile/3 mM KH_2PO_4 (·····). Flow rate was 1 ml/min. A_{206} was measured.

The sample was dissolved in water at a concentration of 20 pmol/ μl . A 1 μl volume of the analyte solution was mixed with an equal volume of the matrix solution on to the target then allowed to crystallize at room temperature. We used 2,5-dihydroxybenzoic acid (10 mg/ml dissolved in water/ethanol, 80:20, v/v) for neutral and monoacidic fractions. External calibration was performed using angiotensin I standard purchased from Sigma (molecular mass 1296.7 Da). About 10–15 shots were accumulated for each spectrum.

Methanolysis and trimethylsilylation

Carbohydrate analysis was performed using gas chromatography

with a silicone OV 101 capillary column (25 m $\times$ 0.32 mm internal diameter). Samples were analysed after methanolysis (0.5 M HCl in anhydrous methanol for 24 h at 80 $^{\circ}\text{C}$) followed by N-acetylation and trimethylsilylation by the method of Kamerling et al. [26] modified by Montreuil et al. [27].

RESULTS

Isolation of oligosaccharide-alditols

Oviducal mucins of *R. utricularia* were submitted to alkali/borohydride degradation and the released oligosaccharides were desalted by gel-permeation chromatography. The pool of released

O-linked carbohydrate chains was subjected to Dowex 50 (X2; H⁺ form) chromatography which removed most of the peptides. Both neutral and acidic oligosaccharide-alditols were retained on a column of the strong anionic ion-exchanger Dowex 1 (X2; 200–400 mesh; OH[−] form). They were eluted with 50 mM pyridine/acetate buffer (pH ~ 6). Fractionation by gel permeation on a Bio-Gel P-2 column of fraction 50 mM gave two fractions, 50-I and 50-II. HPLC on primary amine-bonded silica was used to fractionate 50-I into three oligosaccharide-alditol fractions (Figure 1B) and 50-II into six oligosaccharide-alditol fractions (Figure 1A).

Structural determination of glucuronic acid-containing oligosaccharide-alditols (fraction 50-II)

Peaks 1 and 2 isolated from fraction 50-II were identified as Gal(β1-3)GalNAc-ol (50-II-1) and a mixture of GlcNAc(β1-6)[Gal(β1-3)]GalNAc-ol and GalNAc(α1-3)[Fuc(α1-2)]Gal(β1-3)GalNAc-ol (50-II-2). These compounds have been previously described [21]. The primary structures of the oligosaccharide-

Table 1 Molar proportions of monosaccharide methyl ethers in methanolysates of permethylated oligosaccharide-alditols

Methyl ethers	Amount in oligosaccharide-alditol (mol)						
	50-II-3	50-II-4	50-II-5	50-II-6	50-I-1	50-I-2	50-I-3
(1,4,5,6)-Me ₄ -GalN(Me)Ac-ol	1.0	—	—	—	—	—	—
(1,4,5)-Me ₃ -3,6-anhydroGalN(Me)Ac-ol + (1,4,5)-Me ₃ -GalN(Me)Ac-ol*	—	1.0	1.0	1.0	1.0	1.0	1.0
(2,4,6)-Me ₃ -Gal	0.9	0.8	0.9	0.8	—	—	—
6,6'-d ₂ -(2,3,4,6)-Me ₄ -Glc	0.8	0.8	0.9	0.9	—	—	—
(3,4,6)-Me ₃ -GlcN(Me)Ac	—	0.7	0.8	0.7	0.9	—	—
(4,6)-Me ₂ -GlcN(Me)Ac	—	—	0.8	—	0.7	0.7	0.7
(6)-Me-GlcN(Me)Ac	—	—	—	0.8	—	—	—
(2,3,4)-Me ₃ -Fuc	—	—	—	0.9	0.9	1.9	1.8
(4,6)-Me ₂ -Gal	—	—	—	—	0.9	1.8	1.9
(3,4,6)-Me ₃ -GalN(Me)Ac	—	—	—	—	0.8	1.8	0.7
(3,6)-Me ₂ -GlcN(Me)Ac	—	—	—	—	—	0.8	0.8
(2,3,4,6)-Me ₄ -Gal	—	—	—	—	—	—	0.9

* Methanolysis of 3,6-substituted GalNAc-ol leads to the formation of (1,4,5)-Me₃-3,6-anhydroGalN(Me)Ac-ol and (1,4,5)-Me₃-GalN(Me)Ac-ol in the ratio 3:1 [28].

Table 2 Monosaccharide composition data for oligosaccharide-alditols released from the mucin of the jelly coat

The compositions of 10 oligosaccharide-alditols were deduced from [M + Na]⁺ pseudomolecular ions obtained by positive-ion MALDI-TOF MS or [M − H][−] pseudomolecular ions obtained by negative-ion MALDI-TOF MS. Monosaccharide units were identified on the basis of their vicinal coupling constants by NMR.

Compound	[M + Na] ⁺	[M − H] [−]	Molar ratio					
			GalNAc-ol	Gal	GlcNAc	GalNAc	Fuc	GlcA
50-II-1	408	—	1	1	0	0	0	0
50-II-2a	611	—	1	1	1	0	0	0
50-II-2b	757	—	1	1	0	1	1	0
50-II-3	584	560	1	1	0	0	0	1
50-II-4	787	763	1	1	1	0	0	1
50-II-5	990	966	1	1	2	0	0	1
50-II-6	1136	1112	1	1	2	0	1	1
50-I-1	1163	—	1	1	2	1	1	0
50-I-2	1674	—	1	2	2	2	2	0
50-I-3	1633	—	1	3	2	1	2	0

Table 3 ¹H-NMR chemical shifts of the acidic oligosaccharide-alditols

The monosaccharides are represented by symbolic notation: ◇-ol, GalNAc-ol; ■, βGal; ●, βGlcNAc; □, αFuc; ⊗, GlcA. N.D., not determined. The linkage position is specified by the direction of the connecting bars as follows:

		Chemical shifts (p.p.m.) of:			
Residue	Reporter group	50-II-3	50-II-4	50-II-5	50-II-6
GalNAc-ol (I)	H-1	N.D.	3.790	3.779	3.794
	H-1'	N.D.	3.737	3.730	3.731
	H-2	4.394	4.391	4.392	4.390
	H-3	4.079	4.076	4.074	4.076
	H-4	N.D.	3.457	3.456	3.463
	H-5	4.199	4.277	4.258	4.255
	H-6	N.D.	3.926	3.914	3.904
	H-6'	N.D.	3.683	3.656	3.651
	NAc	2.048	2.068	2.067	2.070
Gal β1-3 (II)	H-1	4.530	4.520	4.520	4.520
	H-2	N.D.	3.719	3.716	3.718
	H-3	N.D.	3.814	3.817	3.818
	H-4	4.178	4.179	4.179	4.177
	H-5	N.D.	N.D.	N.D.	3.39
GlcA β1-3 (III)	H-1	4.672	4.670	4.672	4.673
	H-2	3.422	3.425	3.425	3.425
	H-3	N.D.	3.519	3.521	3.522
	H4	N.D.	3.728	3.729	3.713
GlcNAc β1-6 (II')	H-1	—	4.540	4.494	4.470
	H-2	—	3.703	3.781	3.846
	H-3	—	3.519	3.728	N.D.
	H-4	—	3.431	3.514	N.D.
	H-5	—	3.456	N.D.	N.D.
	H-6	—	3.930	3.923	3.993
	H-6'	—	3.742	3.750	3.85
GlcNAc β1-6 (III')	NAc	—	2.068	2.088	2.106
	H-1	—	—	4.580	4.470
	H-2	—	—	3.672	3.612
	H-3	—	—	3.585	3.612
	H-4	—	—	3.459	3.204
	H-5	—	—	N.D.	3.20
	H-6	—	—	3.923	3.986
Fuc α1-3 (F)	H-6'	—	—	3.750	3.591
	NAc	—	—	2.016	2.106
	H-1	—	—	—	5.012
	H-2	—	—	—	3.806
	H-3	—	—	—	3.919
	H-4	—	—	—	3.80
	H-5	—	—	—	4.103
CH ₃		—	—	—	1.273

alditols (compounds 50-II-3 to 50-II-6) were established by combining the results of methylation analysis (Table 1), ¹H-NMR spectroscopy and MALDI-TOF MS (Table 2). Oligosaccharide-alditols were analysed by MALDI-TOF MS in the positive-ion mode for neutral oligosaccharide-alditols and in the negative-ion mode for acidic oligosaccharide-alditols. The spectra showed characteristic [M + Na]⁺ and [M + K]⁺ pseudomolecular ions in the positive-ion mode and [M − H][−] pseudomolecular ions in the negative-ion mode.

From the MALDI-TOF MS analysis, the presence of a hexuronic unit was observed in compounds 50-II-3, 50-II-4, 50-II-5 and 50-II-6 (Table 2). A comparison of the two-step relayed COSY spectra of compounds 50-II-4 and 50-II-5 showed a distortion in the shape of cross-peaks H-1/H-2/H-3/H-4 which prevented identification of the sugar unit defined by the roman number III in Table 3. Since the other monosaccharide units were easily identified as β -Gal (II) and β -GlcNAc (II', III') on the basis of the set of their vicinal coupling constants and their characteristic H-2 atom resonances, the sugar unit III was indirectly characterized as hexuronic acid. In fact, this sugar was identified as glucuronic acid on the basis of the results obtained after carboxymethylation, reduction with NaB^2H_4 , permethylation, methanolysis, acetylation and GC/MS analysis. This cycle of reaction led to the characterization of 6,6'-d₂-(2,3,4,6)-Me₄-Glc (Figure 2A), allowing the identification of unit III as glucuronic acid in the four oligosaccharide-alditols 50-II-3, 50-II-4, 50-II-5 and 50-II-6. The β -configuration of the glucuronic acid was deduced from the $^3J_{1,2}$ coupling constant (8 Hz).

The core structure of compound 50-II-3 was identified as type 1 (C-3 monosubstituted GalNAc-ol residue) from the set of ^1H -NMR chemical shifts of the GalNAc-ol H-2 and H-5 atoms [29]. The methylation analysis (Figure 2B) furnished *inter alia* a (2,4,6)-Me₃-Gal derivative. An acetyl group in the C-3 position prevented the formation of fragment B₁ (m/z 204). Fragment B₁ is rapidly converted into fragment B₂ (m/z 159) for the neutral monosaccharides [30]. Consequently, the sequence of 50-II-3 was deduced to be: GlcA(β 1-3)Gal(β 1-3)GalNAc-ol.

The chemical shifts of the H-2, H-5 and H-6,6' atoms of GalNAc-ol for compounds 50-II-4, 50-II-5 and 50-II-6 involve the structure of the GlcNAc(β 1-6)[Gal(β 1-3)]GalNAc-ol core type. From the MALDI-TOF MS analysis (Table 2) and the COSY spectra, compounds 50-II-4, 50-II-5 and 50-II-6 can be considered to be extensions of compound 50-II-3 with one GlcNAc (50-II-4), two GlcNAc (50-II-5) and two GlcNAc plus one Fuc (50-II-6) units.

For compound 50-II-5, the sequence GlcNAc(β 1-3)-GlcNAc(β 1-6)GalNAc-ol is easily deduced from the methylation analysis (Table 1) and a comparison of NMR data with those for the neutral oligosaccharide-alditol GlcNAc(β 1-3)-GlcNAc(β 1-6)[Gal(β 1-3)]GalNAc-ol previously described [21].

Compound 50-II-6 can be considered to be an extension of compound 50-II-5 with an additional Fuc residue. The methylation analysis (Table 1) led to the identification of (6)-Me-GlcN(Me)Ac, which is in favour of the attachment of Fuc at the O-4 position of GlcNAc. This conclusion is also based on the conservation of sequence observed between compounds 50-I-1 and 50-II-5. From these data, the structures of these three oligosaccharide-alditols were deduced to be the following:

Figure 2 Mass spectra of 6,6'-d₂-(2,3,4,6)-Me₄-Glc (A) and (2,4,6)-Me₃-Gal (B)

CD₂OMe, C²H₂OMe.

Structural determination of neutral oligosaccharide-alditols from fraction 50-I

Fraction 50-I, obtained by gel-permeation chromatography of the material eluted from the anionic column (Dowex 1 X2) with 50 mM pyridine/acetate buffer, contains a series of neutral penta- to nona-saccharide-alditols. These neutral compounds were strongly retained on the anionic exchanger which was used in the OH⁻ form. The material eluted between 15 and 22 min on the LC-NH₂ column (Figure 1B) corresponds to the oligosaccharide-alditols described in a previous paper [21].

The MALDI-TOF MS analysis of compound 50-I-1 shows a $[M + \text{Na}]^+$ at m/z 1163 (Table 2), which indicates the presence of Hex, HexNAc, deHex, HexNAc-ol in the proportions 1:3:1:1. Methanolysis of the compound confirmed this analysis and confirmed the nature of the carbohydrate units (Fuc, GlcNAc, GalNAc, Gal, GalNAc-ol in the proportions 1:2:1:1:1). From the ^1H -NMR spectra, the presence of the blood-group determinant GalNAc(α 1-3)[Fuc(α 1-2)]Gal β 1-3 is easily verified by the corresponding structural reporter group observed at δ = 5.381 p.p.m. (H-1 Fuc) and δ = 5.189 p.p.m. (H-1 GalNAc)

(Table 4). The chemical shifts of the anomeric protons observed at $\delta = 4.480$ p.p.m. and $\delta = 4.584$ p.p.m. (Table 4) match those of the sequence GlcNAc(β 1-3)GlcNAc(β 1-6) present in compound 50-II-5. Moreover, the methylation analysis clearly shows the points of substitution (Table 1). From this analysis, the structure of compound 50-I-1 was deduced to be the following:

With the pseudomolecular ion $[M + Na]^+$ at m/z 1674 (Table 2), compound 50-I-2 can be considered to be an oligosaccharide-alditol composed of Hex, HexNAc, Fuc and GalNAc-ol in the molar proportions 2:4:2:1. Examination of the NMR spectrum (Figure 3 and Table 4) shows the presence of two α -GlcNAc(III, V') and two β -GlcNAc(II', III') units. The chemical shifts of the anomeric protons of the Gal (II, IV'), Fuc (FII, FIV') and GalNAc(III, V') units point to the occurrence of two blood-group A determinants [31]. The linkages were established by methylation analysis (Table 1), and the linkage configurations were determined by measuring the coupling constants. The presence of the core GlcNAc(β 1-6)[Gal(β 1-3)]GalNAc-ol is revealed by the typical chemical shifts for GalNAc-ol H-2 and H-5 atoms, as well as by the splitting of H-6,6' atoms into two resonances observed at $\delta = 3.90$ p.p.m. and 3.68 p.p.m. Since the chemical shift of the β -1,3-linked galactose unit II (at $\delta = 4.695$ p.p.m.) is characteristic of the blood-group epitope attached to GalNAc-ol, the additional GlcNAc III' unit is necessarily included in the dimer GlcNAc(β 1-3)GlcNAc(β 1-6), which constitutes the main feature of the series of oligosaccharides released from the mucin of *R. utricularia* [21]. This 1,3 linkage is also confirmed by the methylation analysis. For compound 50-I-2, these results provide the structure:

The MALDI-TOF spectrum of compound 50-I-3 shows a $[M + Na]^+$ pseudomolecular ion at m/z 1633 (Table 2) corresponding to an oligosaccharide-alditol composed of three Hex, two deHex, three HexNAc and one HexNAc-ol units. The H-1 signals observed at $\delta = 5.380$ p.p.m., 5.188 p.p.m. and 4.695 p.p.m. match those of α -Fuc, α -GlcNAc and β -Gal engaged in the sequence GalNAc(α 1-3)[Fuc(α 1-2)]Gal(β 1-3)GalNAc-ol (Figure 4) [21,22,31]. The quality of the two-step relayed COSY spectrum was too poor to be able to observe some atoms of the sugar ring. Nevertheless, it was possible to characterize the sugar unit IV' as α -GlcNAc, the H-2 and H-3 atoms of which were both observed at 3.86 p.p.m. This chemical shift is in agreement with an O-3 substitution. The GlcNAc II' H-1 resonance, observed at $\delta = 4.618$ p.p.m., is characteristic of a 4-substituted GlcNAc unit (chain type II), by comparison with the chain type I for which the anomeric proton is always shifted downfield. At least, the values of the H-4 resonances of the two Gal II and III' units, observed at $\delta = 4.22$ and 4.28 p.p.m. respectively, are characteristic of a 3,4-substitution. By combining these NMR data with the results of the methanolysis analysis (GlcNAc,

Table 4 ^1H -NMR chemical shifts of the neutral oligosaccharide-alditols

The monosaccharides are represented by symbolic notation: $\diamond$ -ol, GalNAc-ol; $\blacksquare$, β Gal; $\bullet$, β GlcNAc; $\square$, α Fuc; $\diamond$, α GlcNAc; $\bullet$, α GlcNAc; N.D., not determined. The linkage position is specified by the direction of the connecting bars as follows:

Residue	Reporter group	Chemical shifts (p.p.m.) of:		
		50-I-1	50-I-2	50-I-3
GalNAc-ol (I)	H-1; 1'	N.D.	3.77	N.D.
	H-2	N.D.	N.D.	4.29
	H-3	N.D.	4.077	4.07
	H-4	N.D.	3.573	N.D.
	H-5	N.D.	4.21	4.21
	H-6	N.D.	3.90	N.D.
	H-6'	N.D.	3.68	N.D.
	Nac	2.039	2.046	2.046
Gal β 1-3 (II)	H-1	4.67	4.695	4.695
	H-2	N.D.	3.891	N.D.
	H-3	N.D.	4.013	4.01
	H-4	N.D.	4.218	4.203
GalNAc α 1-3 (III)	H-1	4.189	5.188	5.188
	H-2	N.D.	4.250	4.248
	H-3	N.D.	3.93	3.93
	H-4	N.D.	4.019	4.02
	H-5	N.D.	4.157	4.16
	H-6, 6'	N.D.	3.77	N.D.
	Nac	2.042	2.049	2.048
GlcNAc β 1-6 (II')	H-1	4.480	4.525	4.618
	H-2	N.D.	3.793	N.D.
	Nac	2.062	2.096	2.073
GlcNAc β 1-6 (III')	H-1	4.584	4.623	—
	H-2	N.D.	3.685	—
	H-3	N.D.	3.76	—
	Nac	2.039	2.038	—
GlcNAc β 1-4 (III'; IV')	H-1	—	4.595	4.613
	H-2	—	3.895	N.D.
	H-3	—	3.98	N.D.
	H-4	—	4.218	4.273
GalNAc α 1-3 (V')	H-1	—	5.176	—
	H-2	—	4.238	—
	H-3	—	3.891	—
	H-4	—	3.996	—
	H-5	—	4.209	—
	H-6, 6'	—	3.76	—
	Nac	—	2.019	—
GlcNAc α 1-3 (IV')	H-1	—	—	5.242
	H-2	—	—	3.86
	H-3	—	—	3.86
	Nac	—	—	2.019
Gal β 1-3 (V')	H-1	—	—	4.524
Fuc α 1-2 F(II)	H-1	5.381	5.381	5.380
	H-2	N.D.	3.799	N.D.
	H-3	N.D.	3.799	N.D.
	H-5	4.31	4.317	4.32
	CH ₃	1.348	1.232	1.232
Fuc α 1-2 F(III'; IV')	H-1	—	5.347	5.325
	H-2	—	3.808	N.D.
	H-3	—	3.781	N.D.
	H-5	—	4.101	4.20
	CH ₃	—	1.252	1.240

Figure 3 COSY spectrum of oligosaccharide-alditol 50-I-2

GalNAc; 2:1) and the methylation analysis (2,3-substituted Gal, 4-substituted GlcNAc, 3-substituted GlcNAc, terminal Gal), the structure of compound 50-I-3 was deduced to be the following:

DISCUSSION

In this study, the carbohydrate chains from *R. utricularia* oviducal mucins were released in the form of reduced oligosaccharides by

alkali/borohydride treatment. Fractionation of the mixture of O-linked oligosaccharide-alditols was achieved by a combination of chromatographic techniques including gel-permeation chromatography on Bio-Gel P-2, anion-exchange chromatography on Dowex 1 (X2) and straight-phase HPLC on a silica-based alkylamine column. The structures of ten O-linked carbohydrate chains were determined using ^1H -NMR spectroscopy, MALDI-TOF MS and methylation analysis.

Oviducal mucins from amphibians present a large spectrum of glycans which can be considered species- or subspecies-specific [19–23]. The oligosaccharide-alditols released from *R. utricularia* mucin are no exception to this rule, as can be observed from the list presented in Figure 5. The structural features of these oligosaccharides are as follows. They have type-1, -2 or -8 cores, i.e. $\text{Gal}(\beta 1-3)\text{GalNAc-ol}$, $\text{GlcNAc}(\beta 1-6)[\text{Gal}(\beta 1-3)]\text{GalNAc-ol}$

Figure 5 Oligosaccharide-alditols released from *R. utricularia* egg jelly coat

* See reference [27]. Compounds with terminal Gal-ol result from a peeling reaction.

bound by two Kdn residues, the most prevalent sugar residue in Kdn-rich glycoproteins. Toads such as *B. bufo* [22,23] are characterized by the presence of NeuAc and NeuGc. Kdn is the specific sialic acid of several amphibian mucins, such as *Pleurodeles waltlii* [39] and *Ambystoma mexicanum* [19]. To investigate further whether egg jelly plays a role in fertilization through its capacity to retain bivalent cations, it would be of interest to determine whether the glucuronic acid-containing oligosaccharide-alditols of *R. utricularia* bind Ca^{2+} with high affinity.

Carbohydrates, which make up more than 50 % of amphibian egg jelly coats, are often thought of as potential recognition markers, although no definitive evidence has yet been found for this in amphibians. Such a function is well documented for the zona pellucida of mammalian species. A number of recent studies have shown that carbohydrate moieties present within the zona pellucida and the corresponding carbohydrate-binding proteins

on the sperm surface [40] regulate the sperm–zona pellucida interaction [41]. Specific carbohydrate moieties mediate sperm–zona pellucida interaction in mice [42,43], rats [44] and hamsters [45]. Recently, Alves et al. [46] have shown that sulphated polysaccharides from the egg jelly of sea urchins are species-specific inducers of the acrosomal reaction. Much of the experimental evidence now available points to a major function for carbohydrates in sperm–egg interaction.

In conclusion, the structures of the carbohydrate chains that characterize the mucin of *R. utricularia* are original and species-specific. The pattern of glycan structures found for each amphibian species may represent the basis of species-specific recognition phenomena. These structural analyses also reveal the existence of new glycosylation pathways and consequently new glycosyltransferase activities to explain them. For instance, the $\text{Gal}(\beta 1-3)\text{GlcNAc}(\alpha 1-3)$ sequence (compound 50-I-2) requires a new β -1,3-galactosyltransferase acting on $\text{GlcNAc}(\alpha 1-3)$.

Scheme 1 Biosynthetic pathway for compound 50-II-6

This research was supported by the Centre National de la Recherche Scientifique (UMR 111, Relations Structure-Fonction des Constituants Membranaires, Directeur Professor André Verbert) and by the Ministère de l'Enseignement Supérieur et de la Recherche.

REFERENCES

- Schmell, E. D., Gulyas, B. J. and Hedrick, J. L. (1983) in *Mechanism and Control of Animal Fertilization*, (Hartman, J. F., ed.), pp. 365–413, Academic Press, New York
- Wischmütz, E. W. (1966) *Adv. Morphogen.* **5**, 131–179
- Shivers, C. A. and James, J. M. (1970) *Anat. Rec.* **166**, 541–556
- Steinke, J. H. and Benson, D. G. (1970) *J. Morphol.* **130**, 57–63
- Salthe, S. (1963) *J. Morphol.* **113**, 161–171
- Wolf, D. P. and Hedrick, J. L. (1971) *Dev. Biol.* **25**, 360–376
- Raisman, J. S. and Cabada, M. O. (1977) *Dev. Growth Differ.* **19**, 227–232
- Hedrick, J. L. and Nishihara, T. (1991) *J. Electron. Microsc. Tech.* **17**, 319–335
- Kambara, S. (1953) *Annotationes Zool. Jpn.* **26**, 78–84
- Metz, C. B. (1967) in *Fertilization*, vol. 1 (Metz, C. B. and Monroy, A., eds.), pp. 163–236, Academic Press, New York
- Katagiri, C. (1987) *Zool. Sci.* **4**, 1–14
- Wasserman, P. M., Florman, H. M. and Greve, J. M. (1985) in *Biology of Fertilization*, vol. 2 (Metz, C. B. and Monroy, A., eds.), pp. 341–360, Academic Press, New York
- Gwatkin, R. B. L. (1976) in *The Cell Surface in Animal Embryogenesis and Development* (Poste, G. and Nicolson, G. L., eds.), pp. 1–54, Elsevier/North-Holland Biomedical Press, Amsterdam
- Lopo, A. C. (1983) in *Mechanism and Control of Animal Fertilization* (Hartman, J. F., ed.), pp. 269–324, Academic Press, New York
- Wasserman, P. M. (1988) *Annu. Rev. Biochem.* **57**, 415–442
- Valz-Gianinet, J. N., del Pino, E. J. and Cabada, M. O. (1991) *Dev. Biol.* **146**, 416–422
- Takamune, K. (1987) *Gamete Res.* **18**, 215–223
- Shimoda, Y., Kitajima, K., Inoue, S. and Inoue, Y. (1994) *Eur. J. Biochem.* **223**, 223–231
- Strecker, G., Wieruszkeski, J.-M., Michalski, J.-C., Alonso, C., Leroy, Y., Boilly, B. and Montreuil, J. (1992) *Eur. J. Biochem.* **207**, 995–1002
- Maes, E., Florea, D., Delplace, F., Lemoine, J., Plancke, Y. and Strecker, G. (1997) *Glycoconj. J.* **14**, 127–146
- Morelle, W. and Strecker, G. (1997) *Biochem. J.* **321**, 879–887
- Morelle, W. and Strecker, G. (1997) *Glycobiology* **7**, 777–790
- Morelle, W. and Strecker, G. (1997) *Glycobiology* **7**, 1129–1151
- Humbel, R. and Collaert, M. (1975) *Clin. Chim. Acta* **60**, 143–145
- Ciucanu, I. and Kerek, F. (1984) *Carbohydr. Res.* **131**, 209–217
- Kamerling, J. P., Gerwig, G. J., Vliegthart, J. F. G. and Clamp, J. R. (1975) *Biochem. J.* **151**, 491–495
- Montreuil, J., Bouquelet, S., Debray, H., Fournet, B., Spik, G. and Strecker, G. (1986) in *Carbohydrate Analysis, A Practical Approach* (Chaplin, M. F. and Kennedy, J. F., eds.), p. 143, IRL Press, Oxford
- Wieruszkeski, J.-M., Michalski, J.-C., Montreuil, J., Strecker, G., Peter-Katalinic, J., Egge, H., Van Halbeek, H., Mutseers, J. H. G. M. and Vliegthart, J. F. G. (1987) *J. Biol. Chem.* **262**, 6650–6657
- Kamerling, J. P. and Vliegthart, J. F. G. (1992) in *Biological Magnetic Resonance*, vol. 10 (Berliner, L. J. and Reben, J., eds.), pp. 1–287, Plenum Press, New York and London
- Fournet, B., Strecker, G., Leroy, Y. and Montreuil, J. (1981) *Anal. Biochem.* **116**, 489–502
- Dua, V. K., Rao, B. N. N., Wu, S. S., Dube, V. E. and Bush, C. A. (1986) *J. Biol. Chem.* **261**, 1599–1608
- Finne, J. and Krusius, T. (1976) *FEBS Lett.* **66**, 94–97
- Lhermitte, M., Rahmoune, H., Lamblin, G., Roussel, P., Strang, A. M. and Van Halbeek, H. (1991) *Glycobiology* **1**, 277–293
- Brockhausen, I. (1995) in *Glycoproteins* (Montreuil, J., Schachter, H. and Vliegthart, J. F. G., eds.), pp. 201–259, Elsevier Science, Amsterdam
- Florea, D., Maes, E. and Strecker, G. (1997) *Carbohydr. Res.* **302**, 179–189
- Ishihara, K., Hosono, J., Kanatani, H. and Katagiri, C. (1984) *Dev. Biol.* **105**, 435–442
- Inoue, S., Kanamori, A., Kitajima, K. and Inoue, Y. (1988) *Biochem. Biophys. Res. Commun.* **153**, 172–176
- Shimoda, Y., Kitajima, K., Inoue, S. and Inoue, Y. (1994) *Eur. J. Biochem.* **223**, 223–231
- Strecker, G., Wieruszkeski, J.-M., Michalski, J.-C., Alonso, C., Boilly, B. and Montreuil, J. (1992) *FEBS Lett.* **298**, 39–43
- Sinowatz, F., Gabius, H. J. and Amselgruber, W. (1988) *Histochemistry* **88**, 395–399
- Boldt, J., Howe, A. M., Parkerson, J. B., Gunter, L. E. and Kuehn, E. (1989) *Biol. Reprod.* **40**, 887–896
- Shur, B. D. and Hall, N. G. (1982) *J. Cell Biol.* **95**, 674–679
- Lambert, H. (1984) *J. Reprod. Fertil.* **70**, 281–284
- Shalgi, R., Mitityahu, A. and Nebel, L. (1986) *Biol. Reprod.* **34**, 446–452
- Ahuja, K. K. (1985) *Am. J. Anat.* **174**, 207–233
- Alves, A.-P., Mulloy, B., Diniz, J. A. and Mourao, P. A. S. (1997) *J. Biol. Chem.* **272**, 6965–6971