Species specificity of O-linked carbohydrate chains of the oviducal mucins in amphibians: structural analysis of neutral oligosaccharide alditols released by reductive β -elimination from the egg-jelly coats of *Rana clamitans*¹

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The extracellular matrix (the so-called jelly coat) surrounding amphibian eggs mainly comprises highly O-glycosylated proteins. These oviducal mucins have an important role in the fertilization process, and their carbohydrate chains are remarkably species-specific. Alkaline reductive treatment of the jelly-coat material of the frog *Rana clamitans* led to the release of oligosaccharide alditols. The neutral oligosaccharide alditols were fractionated and purified by successive chromatographic techniques. The structures of 27 of them, ranging from three to sixteen monosaccharides, were established by a combination of NMR spectroscopy, methylation analyses and matrix-assisted laser-desorption ionization–time of flight MS. Typically, some of the neutral compounds appeared to possess the core structure:

Gal(β 1–3)[GlcNAc(β 1–6)]Gal(β 1–3)[GlcNAc(β 1–6)]GalNAc-ol (where GalNAc-ol represents *N*-acetylgalactosaminol). Moreover, a novel type of chain termination, characterized by an unusual sequence {Fuc(α 1–2)Gal(α 1–3)Gal(α 1–4)Gal(β 1–3/4)} was observed. Indeed, the most complex representative structure of this series was found to be: Fuc(α 1–2)Gal(α 1–3)Gal(α 1–4)Gal(β 1–3)[Fuc(α 1–2)Gal(α 1–3)Gal(α 1–4)Gal(β 1–4)GlcNAc(β 1–6)]Gal(β 1–3)[Fuc(α 1–2)Gal(α 1–3)Gal(α 1–4)Gal(β 1–4)GlcNAc(β 1–6)]GalNAc-ol.

Key words: glycosylation, NMR spectroscopy, O-glycans, oligosaccharides.

INTRODUCTION

Amphibian eggs are surrounded by a vitellin envelope and several structurally and chemically distinct jelly coats. Jelly-coat layers are synthesized by specific regions of the oviduct, and are sequentially deposited around the oocyte. This jelly coat is the first barrier through which fertilizing sperm must pass before reaching the egg plasma membrane. The jelly coats are made up of several concentric layers of distinct composition and morphology, which can vary widely between species. Mucin-type glycoproteins, highly O-glycosylated, have been shown to be the major components of this jelly coat and to have important roles in fertilization. Many functions have been attributed to the egg jelly components such as sperm binding, sperm capacitation, induction of the acrosome reaction, prevention of species cross-fertilization, block of polyspermy (anurans) and provision of a protective ionic environment for the developing embryo [1–10]. A major glycoprotein from the egg jelly coat of *Bufo japonicus* has been isolated, and was shown to display calcium-binding properties [7]. The isolation and characterization of two highly glycosylated proteins from *Bufo arenarum* egg jelly coat, which contribute towards forming the jelly layers, has been reported [11]. However, the molecular mechanisms underlying such functions are not yet well characterized.

Previous studies have shown a species-specific structural diversity in carbohydrate chains of amphibian egg jelly coats [12–19]. These carbohydrate chains, which represent new pheno-

typic markers of amphibian species, could be involved in species-specific gamete recognition, or in specific host–parasite interaction [20].

A block to cross-fertilization located in the egg jelly of the frog *Rana clamitans* has been observed [21]. If the specificity resides in the jelly, then a study of the exact chemical composition of the molecules would help to elucidate the mechanism of jelly action in fertilization.

In the present study, we have isolated carbohydrate chains from the oviducal mucins of *R. clamitans* and we describe the primary structure of neutral oligosaccharide alditols, which appear to be specific for this species.

EXPERIMENTAL

Sampling of jelly-coat mucus

Eggs from *R. clamitans* were obtained from Amphibians of North America, Charles D. Sullivan Company, Inc. (Nashville, TN, U.S.A.). The jelly-coat material was freeze-dried.

Isolation of oligosaccharide alditols

O-linked oligosaccharides were released from the crude material by alkaline borohydride treatment (1 M NaBH₄, 0.1 M NaOH) at 37 °C for 72 h. The reaction was stopped by the addition of DOWEX 50 × 8 (25–50 mesh; H⁺ form) at 4 °C. The solution

Abbreviations used: HMQC, heteronuclear multiple-quantum coherence; HMBC, heteronuclear multiple-bound coherence; Le^x, Lewis X; ROESY, rotating-frame nuclear Overhauser enhancement spectroscopy; MALDI–TOF, matrix-assisted laser-desorption ionization–time of flight; GalNAc-ol, *N*-acetylgalactosaminol.

¹ We would like to dedicate this paper to the memory of Professor André Verbert.

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was filtered, adjusted to pH 6.5, and then concentrated under vacuum at 25 °C. Borate salts were removed by repeated evaporation with methanol. The resulting sample was applied to a Bio-Gel P-2 (Bio-Rad Laboratories, Richmond, VA, U.S.A.) column and eluted with water. The carbohydrate-containing fractions were revealed with orcinol/sulphuric acid reagent [0.2% (w/v) orcinol in H₂SO₄/water; 1:4, (v/v)] on silica-gel plates (pre-coated silica gel 60; Merck, Gibbstown, NJ, U.S.A.), pooled and concentrated in a rotary evaporator.

Residual peptide material was removed on a column of DOWEX 50 × 2 (200–400 mesh; H⁺ form). Oligosaccharide alditols were applied on a column of DOWEX 1 × 2 (200–400 mesh; HCOO⁻ form). The major fraction, containing neutral oligosaccharide alditols, was obtained after elution with water. Acidic oligosaccharide alditols were also obtained by elution with a discontinuous gradient (50, 100, 200, 500 mM) of pyridine/acetic acid solution, and desalting by gel filtration on a Bio-Gel P-2 column.

Five fractions (termed **FN A** to **FN E**) were obtained from the major neutral sample after gel-filtration chromatography on a Bio-Gel P-6 column. These five fractions were separated on the basis of their behaviour upon TLC on silica-gel plates in the solvent system n-butanol/acetic acid/water (20:10:15, by vol.). Oligosaccharide alditols from the fractions **FN B**, **FN C** and **FN D** were isolated by HPLC on a primary amine-bonded silica column (SupelcosylTM LC-NH₂; 4.6 mm × 250 mm; Supelco Inc., Bellefonte, PA, U.S.A.) using acetonitrile/water gradients with a flow rate of 1 ml/min at room temperature. Oligosaccharide peaks were detected by absorption at 206 nm. After evaporation of acetonitrile under vacuum, the fractions were desalted on a Bio-Gel P-2 column and freeze-dried.

Fractions **C5**, **C8**, **C10**, **C11**, **C15** and **C17** were fractionated further by C₁₈ reverse-phase chromatography on a 5 µm Zorbax ODS column (4.6 mm × 250 mm) run isocratically with water as an eluent at a flow rate of 0.5 ml/min at room temperature.

Analytical procedures

Carbohydrate analyses were performed using GC. Samples were analysed after methanolysis (0.5 M HCl in anhydrous methanol for 24 h at 80 °C) followed by *N*-reacetylation and trimethylsilylation as described by Kamerling et al. [22].

Methylation analyses were performed as described by Ciucanu and Kerek [23]. The partially acetylated methyl glycosides were analysed by GC/MS on a Carlo Erba GC 8000 gas chromatograph coupled with a Finnigan Automass II mass spectrometer, and identified as described by Fournet et al. [24]. MS analyses were performed in electron-impact or chemical-ionization mode using NH₃ as the reactant gas.

NMR experiments were performed using a BrukerTM 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 ³H₂O at 25 °C). The two-dimensional homonuclear COSY, with simple- and double-relay transfer, the heteronuclear multiple quantum coherence (HMQC), the heteronuclear multiple bound coherence (HMBC) and the rotating-frame nuclear Overhauser enhancement spectroscopy (ROESY) experiments were performed using Bruker standard-pulse sequences.

Molecular mass of the oligosaccharide alditols was measured by matrix-assisted laser desorption-ionization-time-of-flight (MALDI-TOF) MS on a Vision 2000 TOF instrument (Thermo Finnigan France, Les Ulis, France) in the reflectron mode (nitrogen laser: 337 nm).

Samples were dissolved in water at a concentration of 50–100 pmol/µl. Of the sample solution, 1 µl was mixed with an equal volume of the matrix solution on the target, and then allowed to crystallize at room temperature. Neutral structures were analysed in the positive mode using 2,5-dihydroxybenzoic acid [10 mg/ml in methanol/water; 70:30, (v/v)]. External calibration was performed using an angiotensin I standard purchased from Sigma (molecular mass, 1296.7 Da). Between 10 and 25 shots were accumulated for each spectrum.

In order to complete its analysis, a periodic oxidation/NaBH₄ reduction procedure was applied to compound **B1**. Oxidation with NaIO₄ was performed in 0.01 M sodium acetate buffer, pH 4.5, at 4 °C for 30 min. The reaction was quenched by the addition of ethylene glycol, and the mixture was then adjusted to pH 9 before reduction by NaBH₄ for 3 h at room temperature. The reaction was then stopped by acetic acid addition, and the mixture was purified by repeated evaporation with methanol and desalting on a Bio-Gel P-2 column. The resulting sample was finally analysed by MALDI-TOF spectrometry in the positive mode, as described for neutral structures.

RESULTS

Isolation of neutral oligosaccharide alditols

The jelly-coat material of *R. clamitans* was submitted to alkaline borohydride degradation, and the released oligosaccharides were desalted by gel-filtration chromatography. The pool of released O-linked carbohydrate chains was submitted to a cationic-exchange chromatography procedure to remove residual peptides. Neutral oligosaccharide alditols were then separated from acidic oligosaccharide alditols by anionic-exchange chromatography on DOWEX 1 × 2, HCOO⁻ form. After water elution of neutral compounds, which constitute the major fraction of the isolated oligosaccharide alditols, acidic oligosaccharide alditols were desorbed with a discontinuous gradient of pyridine/acetate solution. These acidic compounds will be the subject of a subsequent study.

The fractionation by gel filtration on a Bio-Gel P-6 column of the neutral pool gave five fractions, namely **FN A** to **FN E**. These fractions differ in terms of their migration behaviour during TLC analysis, indicating different sizes in the oligosaccharides present in each fraction. The fraction **FN A**, which did not migrate, and the fraction **FN E**, which contains only small components, were not analysed. The fractions of medium size, **FN B** to **FN D**, were investigated.

HPLC on a primary-amine-bonded silica column was used to fractionate **FN B** into four oligosaccharide alditol fractions (**B1** to **B4**), **FN C** into 19 oligosaccharide alditol fractions (**C1** to **C19**) and **FN D** into six oligosaccharide alditol fractions (**D1** to **D6**), as shown in Figure 1. All the collected fractions were purified by gel filtration on Bio-Gel P-2 before structural elucidation. HPLC on a C₁₈ reverse-phase Zorbax column was used to sub-fractionate several **FN C** fractions: **C5**, **C8**, **C10**, **C11**, **C15** and **C17**. Elution was performed isocratically with water (Figure 2).

The compounds **C1** and **C5-2** isolated from **FN C** were also present in **FN D**, as peaks **D2** and **D5** respectively. Similarly, the compounds **B1** and **B2** obtained from **FN B** corresponded respectively to peaks **C18** and **C19** of **FN C**. Certain fractions were complex mixtures, and were not characterized.

Structural determination of neutral oligosaccharide alditols

The primary structures of the neutral oligosaccharide alditols obtained from oviducal mucins of *R. clamitans* were established

Figure 1 HPLC profiles of oligosaccharide-alditols from the fractions FN B, FN C and FN D

Samples were chromatographed on an LC-NH₂ column eluted with an acetonitrile/water gradient. The flow rate was 1 ml/min. Absorbance was measured at 206 nm. The first peaks of **FN B**, eluted between 10 and 30 min, were pooled for recycling and ulterior analysis.

Figure 2 HPLC profiles of oligosaccharide alditols from the fractions **C5**, **C8**, **C10**, **C11**, **C15** and **C17**, chromatographed on a Zorbax ODS column eluted isocratically with water

The flow rate was 0.5 ml/min. Absorbance was measured at 206 nm.

by combining the results of ¹H/¹³C-NMR spectroscopies, methylation analysis and MALDI-TOF analysis.

The structures of all the compounds discussed below are shown in Schemes 1 and 2. The oligosaccharide alditols have been gathered in three main series according to their structural patterns. The numbering of sugar units (roman numerals I to IX and II' to V') is exemplified by the structures of compounds presented in NMR spectra (**C12** and **C14**) and in Scheme 2 (**C5-3**, **C5-1**, **C10-1** and **C8-1**).

Neutral oligosaccharide alditols were analysed by MALDI-TOF spectrometry in the positive ion mode. The spectra showed characteristic [M + Na]⁺ pseudomolecular ions (Table 1).

Compounds **D3**, **D5**(= **C5-2**), **C8-2**, **C10-2**, **C10-3**, **C11-4** and **C12**

Inspection of the integrated 600 MHz ¹H NMR spectrum of compound **C12** showed resonances attributable to six α- and three β-linked sugar residues (Scheme 1 and Table 2). These monosaccharides were identified as α-fucose (F), α-galactose (III, IV, IV' and V'), β-galactose (II, III') and β-N-acetylglucosamine (II') as a consequence of TOCSY analysis (Figure 3) and one- and two-step relayed COSY spectra (results

Scheme 1 Structures of neutral oligosaccharide alditols isolated from *R. clamitans* egg jelly coat (first and second series)

not shown), which allowed us to determine the 3J coupling constants that are characteristic of the β -gluco, β -galacto and α -galacto configurations. Moreover, the TOCSY experiment of **C12** permitted the assignment of most of the proton resonances (except the H-5 and H-6,6' belonging to the β -Gal units) and the HMQC experiment allowed the ^{13}C assignments to be made (Figure 3 and Table 2). The H-5 and H-6,6' resonances of the two β -Gal units II and III' were assigned in the HMQC spectrum. The identification of the terminal *N*-acetylgalactosaminitol (GalNAc-ol) unit was on the basis of the observation of its eight ^1H signals. Particularly, the H-3, H-5, H-6 and H-6' resonances are characteristic of the core type 2 structure GlcNAc(β1-6)[Gal(β1-3)]GalNAc-ol [25]. Moreover, the assignment of the most downfield ^{13}C signals indicated their involvement in glycosidic linkages: C-2 for α -Gal IV and V'; C-3 for α -Gal III and IV'; C-4 for β -Gal II and III'; and C-3 and C-6 for GalNAc-ol I. The HMBC experiment optimized for long-range $^3J_{\text{C,H}}$ couplings (Figure 3) established the following inter-residue linkages: α -Fuc(1 → 2)- α -Gal IV,V'; α -Gal IV,V'(1 → 3)- α -Gal III,IV'; α -Gal III,IV'(1 → 4)- β -Gal II,III'; β -Gal III'(1 → 4)- β -GlcNAc II'; β -GlcNAc II'(1 → 6) GalNAc-ol I

and β -Gal II(1 → 3) GalNAc-ol I. On the basis of these data, the structure of compound **C12** was fully established. Nevertheless, the exact assignment of atom resonances belonging to α -Gal units III and IV', on one hand, and units IV and V', on the other hand, was not deduced from this experimentation, but from the comparison of the NMR spectra of compounds **D5**, **C8-2**, **C10-2** and **C11-4** (see below).

The two-step relayed COSY spectrum (results not shown) of **D5**(= **C5-2**) revealed the presence of the trisaccharidic sequence Fuc(α1-2)Gal(α1-3)Gal(α1-4). The structural-reporter groups relative to the GalNAc-ol unit (H-3, H-5, H-6, H-6') are characteristic of the core type 2 structure, GlcNAc(β1-6)[Gal(β1-3)]GalNAc-ol [25]. Moreover, the chemical shifts of the H-3 and H-4 atoms of GlcNAc II' are characteristic of a terminal β -GlcNAc unit. These observations indicate that the trisaccharide Fuc(α1-2)Gal(α1-3)Gal(α1-4) is attached to the β -Gal II unit.

The second compound **C8-2** also showed resonances attributable to the trisaccharidic sequence Fuc(α1-2)-Gal(α1-3)Gal(α1-4), and to a core type 2 structure. The presence of a second α -1,2-linked fucose unit can lead to two possible

Scheme 2 Structures of neutral oligosaccharide alditols isolated from *R. clamitans* egg jelly coat (third series)

structures, the first one with a sequence of Fuc(α1-2)Gal β-1,3-linked to GalNAc-ol, and the second one with the sequence Fuc(α1-2)Gal(β1-4)GlcNAc β-1,6-linked to GalNAc-ol. These

sequences have been largely reported in the literature, and are characterized by their Fuc and Gal II or III' H-1 atom resonances. Here, the Gal II and Fuc F_{II} H-1 resonances perfectly match those observed for the sequence Fuc(α1-2)Gal(β1-3)GalNAc-ol [25]. These observations confirmed that the Fuc(α1-2)-Gal(α1-3)Gal(α1-4)Gal(β1-4) sequence is attached to the GlcNAc II' unit.

The examination of the COSY spectra of **D5** and **C8-2** provided some details to allow us to locate the Fuc-Gal-Gal sequence to the upper or the lower branch. Particularly, the H-5 and H-6,6' atom resonances of Gal IV or V' were observed at δ = 4.227 p.p.m. and 3.76 p.p.m. for **D5**, and δ = 4.213 p.p.m. and 3.744 p.p.m. for **C8-2**. Since H-4/H-5 cross peaks are generally observable in the case of the α-galacto configuration, it became possible to follow these correlations to the anomeric proton via H-3 and H-2.

The COSY spectrum of compound **C10-2** (results not shown) indicated the absence of terminal fucose and the presence of two sequences Gal(α1-3)Gal(α1-4). Since the structural reporter-group signals of the GalNAc-ol unit confirm the nature of the core type 2, the structure of **C10-2** is evident. Despite the low resolution of the signals, the H-1 and H-2 of Gal IV and V' are clearly discernible. Moreover, the H-5 resonance of Gal IV and Gal V' can be easily assigned by comparison with the spectra of **D5** and **C8-2**. The correlations H-5/H-4/H-3 were also observed, but interrupted between H-3 and H-2.

The anomeric protons of Gal IV and V' were finally assigned as a consequence of the inspection of the relayed COSY spectrum of **C11-4** (results not shown). The integration of the anomeric region revealed the presence of one α-fucose and four α-galactose units. Consequently, one α-Gal unit occurs in the terminal position, which is confirmed by the upfield value of the H-1 atom of one of the two α-1,3-linked galactose units. The location of the fucose unit was deduced from the chemical shift values of the H-5 and H-6 resonances of the Gal IV and V'. The Gal IV H-5 and H-6,6' signals perfectly match those observed for Gal IV in compound **D5**, whereas the H-5 and H-6,6' resonances of Gal V' possess new chemical shifts, which result from the loss of the fucose unit. Since the structure of **C11-4** is now established, a comparison of the COSY spectra of **C11-4** and **C10-2** allows us to determine the chemical shifts of the Gal IV and V' H-2 and H-1 signals. From this comparison, it can be concluded that the signals relative to the α-1,3-linked galactose of the 1,3 branch (lower branch) are systematically downfield-shifted with respect to the 1,6 branch (upper branch). This observation led to the complete assignment of the TOCSY spectrum of **C12**.

The structures of compounds **D3** (1 α-Fuc, 2 α-Gal, 1 β-Gal and 1 GalNAc-ol) and **C10-3** (mixture of two isomers: 1 α-Fuc, 3 α-Gal, 2 β-Gal, 1 β-GlcNAc and 1 GalNAc-ol) were established by comparison of their COSY spectra (results not shown) with those of the five compounds studied above.

Compounds **C14**, **C15-6**, **C16**, **C17-3**, **B1** (= **C18**) and **B2** (= **C19**)

This second series of compounds is characterized by the presence of the backbone Gal(β1-3)Gal(β1-3) which has been previously characterized in the carbohydrate chains released from the mucins of *Bufo bufo* [26] and *R. arvalis* [27] (Scheme 1 and Table 3).

The structure of the major compound (**C14**) of the series was established as a result of the analysis of the two-step relayed COSY and the ROESY spectra (Figure 4). The ¹H-NMR spectrum shows the presence of ten anomeric protons (ratio 2:2:1:1:1:1:1:1:1:1). As reported above for compound **C12**, the presence of 2 α-Fuc, 4 α-Gal, 3 β-Gal, 1 β-GlcNAc and 1 GalNAc-ol units was deduced from the measurement of ³J coupling

Table 1 Monosaccharide composition data for neutral oligosaccharide-alditols released from the oviducal mucin of *R. clamitans*

The compositions of oligosaccharide alditols were deduced from $[M+Na]^+$ pseudomolecular ions obtained by positive-ion MALDI-TOF MS. Monosaccharide units were identified on the basis of their vicinal coupling constants by NMR and of GC carbohydrate analysis after methanolysis and trimethylsilylation. The experimental $[M+Na]^+$ values reported are in agreement with the deduced values calculated from the average masses of native oligosaccharide residues and useful average masses for O, H and Na elements.

Compound	$[M+Na]^+$ Measured value	$[M+Na]^+$ Calculated value	Molar ratio				
			GalNAc-ol	Gal	GlcNAc	GalNAc	Fuc
D3	879.2	878.7	1	3	0	0	1
D5 = C5-2	1082.7	1081.9	1	3	1	0	1
C8-2	1390.4	1390.2	1	4	1	0	2
C10-2	1422.0	1422.2	1	6	1	0	0
C10-3 A,B	1406.2	1406.2	1	5	1	0	1
C11-4	1568.5	1568.4	1	6	1	0	1
C12	1714.6	1714.5	1	6	1	0	2
C14	1876.8	1876.6	1	7	1	0	2
C15-6	2023.8	2022.8	1	7	1	0	3
C16	2080.3	2079.8	1	7	2	0	2
C17-3	2242.7	2242.0	1	8	2	0	2
B1 = C18	2566.4	2566.3	1	10	2	0	2
B2 = C19	2712.6	2712.4	1	10	2	0	3
D1	611.7	611.5	1	1	1	0	0
C1 = D2	757.4	757.6	1	1	1	0	1
C5-3	1123.6	1123.0	1	2	2	0	1
C6	1269.2	1269.1	1	2	2	0	2
C10-4	1431.5	1431.3	1	3	2	0	2
C2	919.5	919.8	1	2	1	0	1
C5-1	1082.6	1081.9	1	3	1	0	1
C3	960.5	960.8	1	1	1	1	1
C7	1123.1	1123.0	1	2	1	1	1
C10-1	1326.4	1326.2	1	2	1	2	1
C5-4	1123.6	1123.0	1	2	1	1	1
C8-1	1326.4	1326.2	1	2	2	1	1

constants. The chemical shifts of GalNAc-ol H-6,6', both observed at $\delta \approx 3.67$ p.p.m., are characteristic of core type 1: Gal(β 1-3)GalNAc-ol [25]. A two-dimensional ROESY experiment was used to sequence the oligosaccharide alditol (Figure 4). Starting from the terminal fucose unit, strong inter-residue ROESY cross-peaks were observed between Fuc H-1 and Gal V, IX H-2 (and H-3), Gal V, IX H-1 and Gal IV, VIII H-3 (and H-4), Gal IV H-1 and Gal III H-4, Gal VIII H-1 and Gal VII H-4, Gal III H-1 and Gal II H-3, Gal II H-1 and GalNAc-ol I H-3. Since the expected ROESY contact between GlcNAc H-1 and Gal II H-6 was not observable, the attachment of GlcNAc VI to C-6 of Gal II was verified by methylation analysis, which clearly indicated the presence of 2,4-dimethylgalactose. The other methylation results are in agreement with the conclusions drawn from the ROESY experiment. From these analyses, and particularly from the ROESY spectrum, which indicated the sequence Gal IV $\rightarrow$ Gal III $\rightarrow$ Gal II $\rightarrow$ GalNAc-ol I and Gal VIII $\rightarrow$ Gal VII, it was possible to clearly assign the Gal IV and Gal VIII H-1 resonances.

Compound **C16** is characterized by the presence of an additional β -GlcNAc unit (unit II'), the H-3 and H-4 resonances of which are characteristic of a terminal unit. Moreover, the H-6 and H-6' resonances of GalNAc-ol (at $\delta = 3.934$ and 3.692 p.p.m.) clearly show the presence of the core type 2.

The COSY spectrum of compound **C17-3** compared with **C16** indicates the presence of an additional Gal III' unit, occurring in the terminal position ($\delta_{H-3} = 3.667$ p.p.m.; $\delta_{H-4} = 3.919$ p.p.m.). The GlcNAc II' H-4 resonance is downfield-shifted in the bulk, and is not observable. Indeed, the C-4 substitution of GlcNAc with β -Gal is characterized by the occurrence of GlcNAc H-2, H-3 and H-4 signals at $\delta \approx 3.75$ p.p.m., as found for those observed

for GlcNAc VI of **C16**, whereas a C-3 substitution should cause the downfield shift of the H-3 resonance.

Compound **B2** (= **C19**) contains three α -Fuc, six α -Gal, four β -Gal, two β -GlcNAc and one GalNAc-ol unit. Both Gal IV' and VIII H-1 resonances are upfield-shifted with respect to the H-1 resonance of the α -Gal of the 1,3 branch. The chemical shifts of the β -anomeric protons (β -Gal and β -GlcNAc) are identical or similar to those observed in **C17-3**. From these observations, the structures of compounds **C16**, **C17-3** and **B2** were clearly established.

From the MALDI-TOF analysis (Table 1), **C15-6** contains three Fuc, seven Gal, one GlcNAc and one GalNAc-ol. Its COSY spectrum (not shown), compared with **C14**, indicated the presence of one additional anomeric proton at $\delta = 5.41$ p.p.m. and the downfield shift of Gal II H-2, H-3 and H-4 at $\delta = 3.804$ p.p.m., 4.018 p.p.m. and 4.169 p.p.m. respectively. These NMR parameters have been shown to be characteristic of the sequence Gal(β 1-3)[Fuc(α 1-2)]Gal(β 1-3) [28]. Moreover, the GalNAc-ol I H-6 and H-6' resonances at $\delta \approx 3.94$ p.p.m. and ≈ 3.68 p.p.m. respectively are typical of the core type 2. The structure of **C15-6** was confirmed by methylation analysis, which showed, in particular, the di-substitution (at C-3 and C-6) of GalNAc-ol and the presence of 4,6-dimethylgalactose.

From the MALDI-TOF analysis (Table 1), compound **B1** (= **C18**) contains two Fuc, ten Gal, two GlcNAc and one GalNAc-ol. The comparison of its NMR spectrum with that of compound **B2** indicates that these two structures only differ in the number of fucose residues. To establish the position of the two remaining fucose units, this compound **B1** was submitted to a periodic oxidation in order to separate the upper 1,6 branch from the lower 1,3 branch of the core type 2. The sugar residue susceptible

Table 2 ^1H -NMR chemical shifts of oligosaccharide-alditols D3, D5, C8-2, C10-2, C10-3 A and B, C11-4, and $^1\text{H}/^{13}\text{C}$ -NMR chemical shifts of compound C12

nd: not determined; *, data from the HMQC experiment.

Residues	Reporter group	D3	D5(= C5-2)	C8-2	C10-2	C10-3A	C10-3B	C11-4	C12	(^{13}C)
GalNAc-ol I	H-1	nd	≈ 3.82	3.783	3.807	≈ 3.81	≈ 3.81	≈ 3.80	3.802	(61.89)
	H-1'				3.745					
	H-2	≈ 4.39	4.390	4.405	4.387	≈ 4.39	≈ 4.39	4.389	4.387	(52.86)
	H-3	nd	4.070	4.084	4.069	4.057	4.069	4.069	4.068	(79.08)
	H-4	nd	3.552	3.497	3.549	3.462	3.543	3.556	3.552	(70.66)
	H-5	nd	4.193	4.260	4.198	4.202	4.202	4.185	4.196	(68.92)
Gal II	H-6,6'	nd	3.936	3.924	3.927	3.93	3.93	3.935	3.936	(72.42)
			3.687	3.702	3.687	3.69	3.69	3.688	3.684	
	NAc	2.051	2.060	2.056	2.057	2.061	2.061	2.059	2.057	(23.46)
	H-1	4.543	4.534	4.569	4.534	4.530	4.530	4.530	4.531	(104.64)
	H-2	nd	3.578	3.675	3.578	3.575	3.575	3.588	3.574	(72.04)
	H-3	nd	3.770	3.873	3.771	nd	nd	3.773	3.775	(73.60)
Gal III	H-4	nd	4.075	3.922	4.073	nd	nd	4.077	4.074	(77.86)
	H-5	nd	nd	nd	nd	nd	nd	nd	3.793*	(76.78)
	H-6,6'	nd	nd	nd	nd	nd	nd	nd	3.905*	(61.65)
	H-1	4.985	4.988	—	4.991	4.944	4.993	4.984	4.99	(101.35)
	H-2	nd	≈ 4.03	—	4.000	3.831	4.019	4.013	4.003	(68.30)
	H-3	nd	≈ 4.03	—	4.050	nd	nd	4.065	4.045	(75.30)
Gal IV	H-4	nd	≈ 4.03	—	4.286	nd	nd	4.223	4.221	(66.97)
	H-5	≈ 4.39	4.385	—	4.402	≈ 4.36	≈ 4.36	nd	4.393	(72.04)
	H-6,6'	nd	≈ 3.72	—	≈ 3.72	≈ 3.73	≈ 3.73	≈ 3.73	≈ 3.73	(61.87)
	H-1	5.202	5.205	—	5.186	—	5.205	5.206	5.206	(96.27)
	H-2	nd	3.962	—	3.888	—	3.966	3.967	3.959	(75.92)
	H-3	nd	4.166	—	3.966	—	≈ 4.16	4.167	4.166	(70.46)
GlcNAc II'	H-4	nd	4.047	—	4.026	—	nd	4.048	4.047	(70.87)
	H-5	nd	4.227	—	4.194	—	nd	4.230	4.234	(72.42)
	H-6,6'	nd	3.76	—	≈ 3.74	—	nd	3.755	3.76	(62.66)
	H-1	—	4.540	4.571	4.554	4.561	4.561	≈ 4.55	4.550	(102.90)
	H-2	—	3.719	3.755	3.753	≈ 3.76	≈ 3.76	3.76	3.757	(56.44)
	H-3	—	3.536	3.675	nd	nd	nd	3.677	3.696*	(74.00)
Gal III'	H-4	—	3.433	3.696	nd	nd	nd	nd	3.701*	(80.23)
	H-5	—	nd	3.586	3.567	nd	nd	3.585	3.592*	(76.25)
	H-6	—	3.933	4.017	nd	nd	nd	nd	nd	(61.46)
	H-6'	—	3.715	3.855	3.850	—	—	—	—	
	NAc	—	2.060	2.056	2.057	2.061	2.061	2.059	2.057	(23.46)
	H-1	—	—	4.522	4.522	4.528	4.528	4.522	4.523	(104.71)
Gal IV'	H-2	—	—	3.551	3.557	≈ 3.55	≈ 3.55	3.547	3.549	(72.04)
	H-3	—	—	3.755	3.748	nd	nd	3.751	3.761	(73.60)
	H-4	—	—	4.067	4.073	nd	nd	4.073	4.066	(78.27)
	H-5	—	—	nd	nd	nd	nd	nd	3.793*	(76.78)
	H-6,6'	—	—	nd	nd	nd	nd	nd	3.905*	(61.65)
	H-1	—	—	4.990	4.991	4.985	4.944	4.991	4.99	(101.35)
Gal V'	H-2	—	—	≈ 4.01	3.987	4.019	3.831	3.984	4.003	(68.30)
	H-3	—	—	≈ 4.01	4.019	nd	nd	4.025	4.036	(75.41)
	H-4	—	—	4.221	4.285	nd	nd	4.284	4.221	(66.97)
	H-5	—	—	4.373	4.370	≈ 4.39	≈ 4.39	nd	4.364	(72.04)
	H-6,6'	—	—	3.714	≈ 3.72	≈ 3.73	≈ 3.73	≈ 3.73	≈ 3.73	(61.87)
	H-1	—	—	5.198	5.176	5.205	—	5.179	5.198	(96.27)
Fuc IV or II	H-2	—	—	3.955	3.863	3.966	—	3.865	3.959	(75.92)
	H-3	—	—	4.157	3.940	≈ 4.16	—	3.94	4.157	(70.46)
	H-4	—	—	4.025	4.004	nd	—	nd	4.027	(70.87)
	H-5	—	—	4.213	4.164	nd	—	4.166	4.215	(72.42)
	H-6,6'	—	—	3.744	≈ 3.73	nd	—	3.742	3.75	(62.66)
	H-1	5.226	5.226	5.222	—	—	5.226	5.226	5.223	(101.66)
Fuc V'	H-2	nd	3.800	3.799	—	—	3.800	3.800	3.798	(69.67)
	H-3	nd	3.893	3.927	—	—	nd	3.895	3.888	(70.84)
	H-4	nd	3.818	3.823	—	—	nd	nd	3.826	(73.02)
	H-5	nd	4.104	4.277	—	—	4.102	4.105	4.104	(68.49)
	CH ₃	1.238	1.240	1.244	—	—	1.239	1.241	1.236	(16.76)
	H-1	—	—	5.222	—	5.226	—	—	5.223	(101.66)
Fuc V'	H-2	—	—	3.799	—	3.800	—	—	3.798	(69.67)
	H-3	—	—	3.879	—	nd	—	—	3.888	(70.84)
	H-4	—	—	3.823	—	nd	—	—	3.826	(73.02)
	H-5	—	—	4.099	—	4.102	—	—	4.104	(68.49)
	CH ₃	—	—	1.246	—	1.239	—	—	1.236	(16.76)

Figure 3 Homonuclear (TOCSY) and heteronuclear COSY spectra (HMQC and HMBC) of compound C12

For further details, see the Experimental section.

Table 3 ¹H-NMR chemical shifts of oligosaccharide alditols C14, C15-6, C16, C17-3, B1 and B2

nd, not determined; assignments denoted by asterisks may need to be interchanged.

Residues	Reporter group	C14	C15-6	C16	C17-3	B1 (= C18)	B2 (= C19)
GalNAc-ol I	H-1	≈ 3.80	≈ 3.79	3.787	3.726	nd	≈ 3.75
	H-1'	≈ 3.76					
	H-2	4.384	4.387	4.388	4.381	4.388	4.386
	H-3	4.043	4.065	4.026	4.018	nd	4.023
	H-4	3.528	3.552	3.489	3.481	3.492	3.492
	H-5	4.153	4.201	4.239	4.230	nd	4.234
	H-6,	≈ 3.67	≈ 3.94	3.934	3.927	nd	nd
	H-6'		≈ 3.68	3.692	3.710		3.701
Gal II	NAc	2.060	2.060	2.049	2.044	2.050	2.050
	H-1	4.538	4.584	4.528	4.519	4.524	4.523
	H-2	3.734	3.804	3.732	3.729	nd	3.732
	H-3	3.857	4.018	3.847	3.844	nd	3.850
Gal III	H-4	4.125	4.169	4.124	4.117	nd	4.120
	H-1	4.703	4.694	4.700	4.691	4.700	4.683
	H-2	3.620	3.627	3.623	3.618	nd	3.623
	H-3	3.759	3.740	3.760	3.755	nd	3.760
Gal IV	H-4	4.082	4.071	4.080	4.076	nd	4.082
	H-1	5.018	5.014	5.019	5.013	5.019	5.019
	H-2	4.005	≈ 4.02	4.010	≈ 4.01	nd	4.006
	H-3	4.053	4.032	4.069	≈ 4.02	nd	4.055
Gal V	H-4	4.227	4.219	4.239	4.222	nd	4.230
	H-5	4.387	4.385	4.388	4.393	4.388	nd
	H-6,6'	3.715	≈ 3.72	3.716	3.710	nd	≈ 3.72
	H-1	5.199	5.207	5.199	5.192	5.199	5.199
Gal VI	H-2	3.956	3.969	3.957	3.951	nd	3.957
	H-3	4.158	4.167	4.158	4.156	nd	4.159
	H-4	4.028	4.039	4.028	4.025	nd	4.029
	H-5	4.377	4.221	4.214	4.210	nd	4.213
GlcNAc VII	H-6,6'	≈ 3.73	≈ 3.75	3.753	3.748	nd	≈ 3.75
	H-1	4.612	—	4.597	4.593	4.597	4.598
	H-2	3.749	—	≈ 3.74	3.736	nd	3.741
	H-3	3.933	—	≈ 3.74	≈ 3.74	nd	nd
Gal VIII	H-4	3.607	—	≈ 3.74	nd	nd	nd
	NAc	2.060	—	2.062	2.060*	2.060	2.060
	H-1	4.524	—	4.52	4.519	4.524	4.523
	H-2	3.549	—	3.55	3.545	nd	3.551
Gal IX	H-3	3.752	—	3.756	3.749	nd	3.755
	H-4	4.064	—	4.067	4.062	nd	4.068
	H-1	4.992	—	4.991	4.986	4.992	4.991
	H-2	3.995	—	≈ 4.01	3.991	nd	3.994
Gal X	H-3	4.028	—	≈ 4.01	≈ 4.02	nd	4.030
	H-4	4.219	—	4.208	nd	nd	4.220
	H-5	4.371	—	4.371	4.380	nd	4.379
	H-6,6'	3.715	—	nd	nd	nd	≈ 3.72
Fuc V and/or V'	H-1	5.199	—	5.199	5.192	5.199	5.199
	H-2	3.956	—	3.957	3.951	nd	3.957
	H-3	4.158	—	4.158	4.156	nd	4.159
	H-4	4.028	—	4.028	4.022	nd	4.029
Fuc IX	H-5	4.377	—	4.214	4.210	nd	4.213
	H-6,6'	≈ 3.73	—	3.753	3.748	nd	≈ 3.75
	H-1	5.226	5.224	5.226	5.220	5.224	5.226
	H-2	3.800	3.797	3.799	3.795	nd	3.799
Fuc II	H-3	3.892	3.885	3.893	3.887	nd	3.892
	H-4	3.827	3.823	3.826	3.825	nd	3.826
	H-5	4.099	4.091	4.100	4.095	nd	4.100
	CH ₃	1.241	1.24	1.238	1.234	1.240	1.240
Fuc IX	H-1	5.226	—	5.226	5.220	5.224	5.226
	H-2	3.800	—	3.799	3.795	nd	3.799
	H-3	3.892	—	3.893	3.887	nd	3.892
	H-4	3.827	—	3.826	3.825	nd	3.826
Fuc II	H-5	4.099	—	4.100	4.095	nd	4.100
	CH ₃	1.241	—	1.238	1.234	1.240	1.240
	H-1	—	5.410	—	—	—	—
	H-2	—	3.837	—	—	—	—
Fuc II	H-3	—	3.758	—	—	—	—
	H-4	—	nd	—	—	—	—
	H-5	—	4.218	—	—	—	—
	CH ₃	—	1.24	—	—	—	—

Table 3 (contd.)

Residues	Reporter group	C14	C15-6	C16	C17-3	B1 (= C18)	B2 (= C19)
GlcNAc II'	H-1	—	4.533	4.539	4.538	4.555	4.555
	H-2	—	3.775	3.708	3.75	nd	3.750
	H-3	—	3.653	3.532	nd	nd	≈ 3.68
	H-4	—	3.448	3.432	nd	nd	nd
	H-5	—	nd	3.459	nd	nd	nd
	H-6	—	nd	3.939	nd	nd	nd
	H-6'	—	—	3.759	—	—	—
	NAc	—	2.060	2.064	2.055*	2.066	2.066
Gal III'	H-1	—	4.529	—	4.465	4.524	4.523
	H-2	—	3.574	—	3.536	nd	3.551
	H-3	—	3.765	—	3.667	nd	3.755
	H-4	—	4.071	—	3.919	nd	4.068
Gal IV'	H-1	—	4.983	—	—	4.992	4.991
	H-2	—	≈ 4.02	—	—	nd	3.994
	H-3	—	4.016	—	—	nd	4.030
	H-4	—	4.219	—	—	nd	4.220
	H-5	—	4.362	—	—	4.370	4.379
	H-6,6'	—	≈ 3.72	—	—	nd	≈ 3.72
Gal V'	H-1	—	5.197	—	—	5.179	5.199
	H-2	—	3.955	—	—	nd	3.957
	H-3	—	4.146	—	—	nd	4.159
	H-4	—	4.023	—	—	nd	4.029
	H-5	—	4.221	—	—	nd	4.213
	H-6,6'	—	≈ 3.75	—	—	nd	≈ 3.75

to periodate oxidation in the moderate conditions used is the polyol (GalNAc-ol in this case), resulting in an opening of its C4–C5 bond. This selective periodic oxidation was followed by NaBH₄ reduction, and analysis by MALDI–TOF spectrometry in the positive-ion mode. Treatment of compound **B1** with the NaIO₄/NaBH₄ procedure resulted in four products characterized by their [M+Na]⁺ pseudomolecular ions (Figure 5), which correlate with the predicted masses of the expected structures. The corresponding structures, derived from the upper branch (products a and b) or from the lower part (products c and d) of compound **B1** are the following: product a, (at *m/z* 775.2) = Gal-Gal-Gal-GlcNAc-ethanediol; product b (at *m/z* 921.3) = Fuc-Gal-Gal-Gal-GlcNAc-ethanediol; and product c (at *m/z* 1670.7) is characterized by the loss of one of the two fucose units of product d, which, at *m/z* 1816.8, is Fuc-Gal-Gal-Gal[Fuc-Gal-Gal-Gal-GlcNAc]Gal-TheNAc-ol (where TheNAc-ol represents 2-acetamido-2-deoxy-L-threitol).

These results indicate that **B1** is a mixture of the compounds **B1-A** and **B1-B**, the structures of which are shown in Scheme 1. Moreover, the comparison between chemical shifts of Gal V' residues of both compounds **B1** and **B2** showed a shielding of $\Delta\delta = -0.02$ p.p.m. of the H-1 chemical shift in the case of compound **B1**. This is in favour of the unfucosylated form of the upper branch of compound **B1**. Therefore the compound **B1-A** seems to be the major compound of the mixture, as is also indicated by the MS results (major peaks a and d), whereas compound **B1-B** may possess two possible structures.

Compounds D1, C1(= D2), C5-3, C6, C10-4, C2, C5-1, C3, C7, C10-1, C5-4 and C8-1

The third series of oligosaccharide alditols (Scheme 2, Tables 4 and 5) is characterized by the presence of the two following inner-cores: GlcNAc(β 1-6)[Gal(β 1-3)]GalNAc-ol and Gal(β 1-3)[GlcNAc(β 1-6)]Gal(β 1-3)GalNAc-ol. They are

extended with different sequences or sugar units, such as the sequence Fuc(α 1-3)GlcNAc(β 1-6), the Lewis X determinant Gal(β 1-4)[Fuc(α 1-3)]GlcNAc(β 1-6), the GalNAc unit β 1,3-linked to the Gal II, III or III'.

The monosubstitution (at C-3) or the di-substitution (at C-3 and C-6) of GalNAc-ol was deduced from the characteristic chemical shifts of the GalNAc-ol H-6,6' protons. The sequence Gal(β 1-3)[GlcNAc(β 1-6)]Gal(β 1-3)GalNAc-ol, which was established by methylation analysis, is characterized by the typical downfield-shifted resonances of Gal II H-2 ($\delta \approx 3.71$ p.p.m.), H-3 ($\delta \approx 3.82$ p.p.m.) and H-4 ($\delta \approx 4.16$ p.p.m.) (Table 4). The presence of the GlcNAc IV unit affects the H-5 and H-6,6' resonances of Gal II. The attachment of α -Fuc to C-3 of GlcNAc was verified by methylation analysis of compound **C1**, which showed the presence of 4,6-dimethyl-GlcNAc. A comparison between the **C5-3** and **C1** spectra indicated that the C-3 fucosylation of GlcNAc II' is reflected by the downfield shift of the GlcNAc H-2 resonance, from $\delta = 3.696$ p.p.m. to $\delta = 3.843$ p.p.m., and the H-3 resonance, from $\delta = 3.526$ p.p.m. to $\delta = 3.628$ p.p.m.. The C-3 fucosylation of GlcNAc II' (**C1**) or of GlcNAc IV (**C5-3** and **C10-4**) gives rise to characteristic resonances of the Fuc H-1, observed at $\delta \approx 4.99$ and 4.97 p.p.m. respectively. Such a property is evident in the case of compound **C6**, which possesses these two fucose units (Table 4).

The Lewis X determinant is present in compounds **C5-1** and **C10-4**. In the case of **C5-1**, the attachment of GlcNAc IV to C-6 of Gal II was deduced from the characteristic downfield shift of the Gal II H-6,6' resonances. Moreover, the core structure Gal(β 1-3)GalNAc-ol was deduced from the GalNAc-ol H-6,6' signals at $\delta \approx 3.65$ p.p.m. The structural reporter-group signals of the fucose unit present in the Lewis X sequences match with the data from the literature [25]. The comparison of the NMR spectra of **C6** and **C10-4** clearly indicates that both contain the sequence Fuc(α 1-3)GlcNAc(β 1-6)[Gal(β 1-3)]Gal(β 1-3)-GalNAc-ol. Consequently, the Lewis X determinant, in compound **C10-4**, is attached to C-6 of GalNAc-ol.

Figure 4 Double relayed COSY spectrum (top panel), and extension (5.26 to 4.48 p.p.m. in F2 and 4.27 to 3.53 p.p.m. in F1) of the ROESY spectrum (bottom panel) of compound C14

The presence of β -GalNAc in compounds C3, C7, C10-1, C5-4 and C8-1 is deduced from the set of its vicinal coupling constants measured on the COSY spectra (results not shown). The H-4 resonance, found at $\delta = 3.93$ p.p.m., is clearly observed in the COSY spectrum of C10-1. The chemical shifts of the H-1

Figure 5 MALDI-TOF analysis of compound B1 after mild periodic oxidation and reduction

Analysis was performed in the positive mode (pseudomolecular ions; $[M + Na]^+$)

to H-4 resonances of β -GalNAc are typical of a terminal non-reducing unit, whereas the downfield shifts observed for the H-3 and H-4 resonances of Gal II (C3 and C10-1), Gal III (C5-4 and C8-1) and Gal III' (C7 and C10-1) can be attributed to a C-3 or a C-4 substitution of β -Gal (Table 5). The 1,3 linkage was proved conclusively by methylation analysis, which clearly showed the presence of 2,4,6-trimethylgalactose.

DISCUSSION

In the present study, the carbohydrate chains from *R. clamitans* oviducal mucins were released by reductive β -elimination. Neutral oligosaccharide alditols, which constitute the main fraction of the isolated oligosaccharide alditols, were separated from acidic oligosaccharide alditols and fractionated by a sequence of chromatographic techniques. The structures of 27 of them, ranging in size from a trisaccharide to a hexadecasaccharide, were determined using NMR spectroscopy, MALDI-TOF MS and methylation analyses.

The remarkable species-specific structural diversity, which has been observed in carbohydrate chains of other amphibian-egg jellies, was verified for *R. clamitans*, as shown in Schemes 1 and 2. Indeed, the structures presented are novel and characteristic of *R. clamitans*, even if some parts of their sequences have been observed already in other amphibians.

The structural features of these oligosaccharide alditols can be summarized as follows. They possess type-1 or -2 cores, i.e. Gal(β 1-3)GalNAc-ol and GlcNAc(β 1-6)[Gal(β 1-3)]GalNAc-ol respectively. In these O-glycans, the Gal(β 1-3)GalNAc-ol core can be extended at the Gal residue with a Gal(β 1-3) residue. The sequence Gal(β 1-3)Gal(β 1-3)GalNAc-ol is also present in the oviducal mucins of other amphibian species: *B. bufo* [15,26], *R. temporaria* [14], *R. arvalis* [27], *R. ridibunda* [29] and *Xenopus laevis* [13]. As reported for some of these species previously [26,27], a GlcNAc unit β -1,6 linked to Gal II may be observed in the case of *R. clamitans*.

Several carbohydrate chains of *R. clamitans* (first and second series; see Scheme 1) contain the blood-group P₁ determinant Gal(α 1-4)Gal(β 1-4)GlcNAc β -. This sequence has been found previously in *Ambystoma mexicanum* [30] in a fucosylated form {-Fuc(α 1-2)[Gal(α 1-4)]Gal(β 1-4)GlcNAc-}, which could be considered as the hybrid P₁/H blood group determinant. However, in *R. clamitans*, the P₁ determinant is extended at the α -Gal residue with a Gal(α 1-3) residue or with a Fuc(α 1-2)Gal(α 1-3)

Table 4 ^1H -NMR chemical shifts of oligosaccharide-alditols **D1**, **C1**, **C5-3**, **C6**, **C10-4**, **C2** and **C5-1**

nd, not determined.

Residues	Reporter group	D1	C1 (= D2)	C5-3	C6	C10-4	C2	C5-1
GalNAc-ol I	H-1	nd	3.78	3.784	3.788	3.783	nd	3.574
	H-1'		3.744	3.725	3.726	3.722		nd
	H-2	4.395	4.393	4.385	4.386	4.384	4.384	4.382
	H-3	4.060	4.062	4.024	4.022	4.022	4.044	4.043
	H-4	3.468	3.456	3.491	3.481	3.474	3.528	3.523
	H-5	4.279	4.277	4.226	4.227	4.220	4.154	4.155
	H-6,6'	3.931	3.931	3.934	3.936	3.930	nd	3.65
		nd	3.679	3.694	3.691	3.686		
	NAc	2.066	2.050	2.058	2.043	2.046	2.046	2.044
	H-1	4.464	4.463	4.532	4.530	4.529	4.612	4.534
Gal II	H-2	3.542	3.559	3.722	3.712	3.713	nd	3.715
	H-3	nd	nd	3.825	3.824	3.824	nd	3.829
	H-4	3.900	3.900	4.166	4.168	4.166	4.168	4.165
	H-5	nd	nd	3.879	3.883	nd	nd	nd
	H-6	nd	nd	3.975	3.958	nd	nd	3.956
	H-6'			3.879	3.883			3.858
Gal III	H-1	—	—	4.610	4.610	4.607	4.603	4.609
	H-2	—	—	3.606	3.608	3.606	nd	3.603
	H-3	—	—	nd	3.671	3.657	nd	3.688
	H-4	—	—	nd	3.927	3.924	3.925	3.924
Gal V	H-1	—	—	—	—	—	—	4.448
	H-2	—	—	—	—	—	—	3.494
	H-3	—	—	—	—	—	—	3.651
	H-4	—	—	—	—	—	—	3.896
GlcNAc IV	H-1	—	—	4.586	4.587	4.587	4.542	4.609
	H-2	—	—	3.824	3.824	3.823	nd	3.909
	H-3	—	—	3.658	3.668	3.661	nd	3.909
	H-4	—	—	3.530	3.526	2.522	nd	≈ 3.58
	H-5	—	—	nd	nd	nd	nd	≈ 3.58
	H-6	—	—	3.952	nd	nd	nd	4.006
	H-6'			3.764				3.858
	NAc	—	—	2.033	2.033	2.031	2.042	2.044
Fuc IV	H-1	—	—	4.974	4.975	4.973	4.980	5.098
	H-2	—	—	3.690	3.690	3.690	nd	3.684
	H-3	—	—	nd	3.827	3.824	nd	3.898
	H-4	—	—	nd	3.795	3.796	nd	3.789
	H-5	—	—	4.321	4.324	4.320	4.324	4.830
	CH ₃	—	—	1.156	1.158	1.155	1.157	1.171
Fuc II'	H-1	—	4.987	—	4.993	5.112	—	—
	H-2	—	3.691	—	3.690	3.684	—	—
	H-3	—	3.994	—	3.827	3.902	—	—
	H-4	—	nd	—	3.795	3.790	—	—
	H-5	—	4.326	—	4.324	4.881	—	—
	CH ₃	—	1.158	—	1.158	1.174	—	—
GlcNAc II'	H-1	4.539	4.546	4.536	4.547	4.560	—	—
	H-2	nd	3.843	3.696	3.837	3.920	—	—
	H-3	nd	3.628	3.526	3.635	3.822	—	—
	H-4	3.452	3.516	3.431	3.514	≈ 3.88	—	—
	H-5	nd	3.482	3.470	3.489	3.596	—	—
	H-6	nd	3.942	3.924	3.927	4.011	—	—
	H-6'		3.749	3.734	3.657	3.856		
	NAc	2.066	2.066	2.063	2.064	2.064	—	—
Gal III'	H-1	—	—	—	—	4.448	—	—
	H-2	—	—	—	—	3.493	—	—
	H-3	—	—	—	—	3.653	—	—
	H-4	—	—	—	—	3.898	—	—

sequence. The occurrence of a peripheral additional sugar or sequence linked to a blood-group determinant has been already observed for several amphibian species, i.e. *B. bufo* [15,26] and *X. laevis* [31].

Another characteristic feature of the *R. clamitans* mucin consists of the presence of the peripheral trisaccharide Fuc(α 1-2)Gal(α 1-3)Gal(α 1-4), directly attached in the first series of compounds (**D3** to **C12**) to the β -Gal II unit of the type-1 or

-2 cores, and/or to the β -Gal III' unit of the type-2 core. The second series of compounds (**C14** to **B2**), characterized by the presence of the backbone Gal(β 1-3)Gal(β 1-3), also possesses this peripheral trisaccharide, which can be attached to the β -Gal III and the β -Gal VII units of the type-1 or -2 cores, and/or to the β -Gal III' unit of the type-2 core. This new structural characteristic is well represented in the compound **B2**, which comprises 16 monosaccharidic units, and particularly this tri-

Table 5 ^1H -NMR chemical shifts of oligosaccharide-alditols **C3**, **C7**, **C10-1**, **C5-4** and **C8-1**

nd, not determined; the asterisks denote that assignments may have to be interchanged.

Residues	Reporter group	C3	C7	C10-1	C5-4	C8-1
GalNAc-ol I	H-1	3.753	≈ 3.75	3.755	3.787	3.768
	H-1'	nd			3.726	3.720
	H-2	4.399	4.394	4.398	4.383	4.387
	H-3	4.046	4.059	4.045	4.043	4.021
	H-4	3.441	3.448	3.433	3.527	3.487
	H-5	4.265	4.269	4.250	4.150	4.226
	H-6	3.925	3.925	3.917	3.650	3.932
	H-6'	3.670	3.673	3.669		3.690
	NAc	2.045	2.054	2.049	2.045	2.055
Gal II	H-1	4.449	4.461	4.447	4.731	4.532
	H-2	3.591	3.559	3.589	3.714	3.706
	H-3	3.729	3.673	3.732	3.815	3.807
	H-4	4.131	3.898	4.132	4.164	4.165
	H-5	nd	nd	nd	3.871	3.872
	H-6	nd	nd	nd	3.965	3.973
	H-6'				3.892	3.889
Gal III	H-1	—	—	—	4.540	4.590
	H-2	—	—	—	3.641	3.641
	H-3	—	—	—	3.741	3.739
	H-4	—	—	—	4.143	4.148
GalNAc III or IV	H-1	4.594	—	4.589	4.628	4.623
	H-2	3.945	—	3.944	3.936	3.933
	H-3	3.750	—	3.749	3.749	3.752
	H-4	3.940	—	3.930	3.933	nd
	Nac	2.041	—	2.039	2.039*	2.028*
GlcNAc V	H-1	—	—	—	4.599	4.580
	H-2	—	—	—	3.834	3.818
	H-3	—	—	—	3.681	3.655
	H-4	—	—	—	3.525	3.523
	H-5	—	—	—	3.488	3.449
	H-6	—	—	—	3.957	3.947
	H-6'	—	—	—	3.754	3.738
	NAc	—	—	—	2.035*	2.032*
		—	—	—	4.978	4.968
Fuc V	H-1	—	—	—	3.689	3.685
	H-2	—	—	—	3.825	3.824
	H-3	—	—	—	3.796	3.793
	H-4	—	—	—	4.323	4.317
	H-5	—	—	—	1.155	1.152
	CH ₃	—	—	—		
Fuc II'	H-1	4.987	5.097	5.097	—	—
	H-2	3.690	3.963	3.681	—	—
	H-3	3.826	3.888	3.886	—	—
	H-4	3.800	3.776	3.775	—	—
	H-5	4.324	4.819	4.818	—	—
	CH ₃	1.157	1.154	1.153	—	—
GlcNAc II'	H-1	4.540	4.559	4.553	—	4.532
	H-2	3.837	≈ 3.92	3.915	—	3.706
	H-3	3.619	3.832	3.822	—	3.525
	H-4	3.515	≈ 3.92	nd	—	3.430
	H-5	3.489	3.594	nd	—	3.500
	H-6	3.937	4.003	nd	—	3.936
	H-6'	3.748	3.843			3.751
	NAc	2.066	2.068	2.067	—	2.060
Gal III'	H-1	—	4.432	4.429	—	—
	H-2	—	3.516	3.509	—	—
	H-3	—	3.720	3.720	—	—
	H-4	—	4.103	4.103	—	—
	H-5	—	nd	nd	—	—
	H-6,6'	—	nd	nd	—	—
GalNAc IV'	H-1	—	4.612	4.612	—	—
	H-2	—	3.930	3.932	—	—
	H-3	—	3.741	3.737	—	—
	H-4	—	3.934	3.930	—	—
	NAc	—	2.032	2.030	—	—

saccharidic sequence repeated in a tri-antennary structure. However, different intermediates of the biosynthesis of the complete glycan were also isolated. Thus a novel type of chain termination, constituted by the sequence Fuc(α 1–2)Gal(α 1–3)-Gal(α 1–4)Gal(β 1–3/4), is shown here.

The structural features of the third series of compounds (Scheme 2) are the sequence Fuc(α 1–3)GlcNAc(β 1–6) and the Lewis X (Le^x) determinant Gal(β 1–4)[Fuc(α 1–3)]GlcNAc(β 1–6). O-glycans containing Le^x determinants have been already found in the oviducal mucins of some other amphibian species, such as *Pleurodeles waltl* [32] or *A. tigrinum* [33] and, more recently, these two sequences were observed in *X. laevis* [31]. However, in the case of *R. clamitans*, the unfucosylated Gal(β 1–4)GlcNAc(β 1–6) sequence is not observed. Therefore it appears that the synthesis of Le^x by *R. clamitans* may be achieved in a different manner to that observed in the human system [34], with a fucosylation of the GlcNAc unit before its galactosylation. Biosynthetic pathways that are distinct from those of the human system have been described previously for the biosynthesis of Lewis determinants by *Helicobacter pylori* [35].

Another characteristic of this same series of compounds is the presence of a GalNAc unit β 1,3-linked to the β -Gal II, III or III' residues. Such a linkage has been rarely observed until now in amphibian species. Moreover, it is noteworthy that this GalNAc unit can be attached to the Le^x determinant (compounds C7 and C10-1).

Since the present study was performed on the neutral pool of oligosaccharide alditols, the next study on the acidic compounds will contribute to the characterization of this species. However, these results already confirm our previous conclusions concerning the species-specificity of the O-linked glycans released from the amphibian-egg jelly coats. Among all the characterized neutral compounds released from the mucin of *R. clamitans*, most of them have novel structures, even if they include some known structural patterns, such as Le^x or P_1 determinants. These glycans can be considered as phenotypic markers of the species. Such a species specificity has been observed for all amphibian species studied up to now [12–19, 26–29]. These structural analyses also suggest the existence of new glycosylation pathways and, consequently, new glycosyltransferase activities.

From a general point of view, it is noteworthy that the presence of galabiose (Gal α 1–4Gal) in animal N- or O-glycans was rarely observed, while this sequence is normally found in terminal and internal positions of mammalian glycolipids bearing the P_1 or P_k determinants, as described in [36]. Moreover, the glycolipids containing galabiose are known to be targets on host cells for infection by some pathogenic microbes, whereas galabiose-containing glycoproteins were found only when the mammals were infected by tapeworm *Echinococcus granulosus* [36]. Therefore the diversity in glycan structures may reflect the diversity of glycosyltransferases involved in their biosynthesis. From a biological point of view, the species specificity and diversity of glycans could be related to the host–parasite interaction that led to the selection of glycosyltransferase activities during evolution [20].

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