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Comparative study of carbohydrate chains released from the oviducal mucins of the two very closely related amphibian species *Bombina bombina* and *Bombina variegata*

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Abstract

The eggs of amphibians are surrounded by an extracellular matrix, termed jelly coat, which is mainly composed of hydrated mucin-type glycoproteins. These highly glycosylated molecules are synthesized by the oviduct and play an important role in the fertilization process. From a structural and chemical point of view, these oviducal mucins are very different from one species to another and they could be involved in the species-specificity of gamete interactions or could influence the parasite tropism. *Bombina bombina* and *Bombina variegata* are the two most closely related species within the genus, which hybridize readily in nature. Divergence occurred during geographic isolation estimated at 2-7 million years ago. The oviducal mucins of these species have been studied at the carbohydrate level, and the primary structures of 28 compounds have been established by NMR spectroscopy. The carbohydrate chains released from the oviducal mucins of the two species were similar and characterized by the common sequences GlcNAc(β1-3)[Fuc(α1-4)]GlcNAc(β1-6) and GlcNAc(α1-4)Gal(β1-4)Gal(β1-3) attached to GalNAc-ol (core 2). Nevertheless, some differences confirmed the strict species-specificity of amphibian oviducal carbohydrate chains observed previously. On the one hand, the presence of βGal 1,4-linked to βGlcNAc in *B. bombina*, but not in *B. variegata*, can indicate that β4GalT: βGlcNAc and β4GalT: βGal are two distinct glycosyltransferases. On the other hand, deaminoneuraminic acid (Kdn) is present in *B. bombina*, and *N* -glycolylneuraminic acid (NeuGc) in *B. variegata*. Although the enzymes involved in the biosynthesis of Kdn are not as well characterized, it can be suggested that at least one step of the biosynthetic pathway of NeuAc has been disrupted, leading the *B. bombina* oviducal NeuAc-9-synthase to use Man-6-P as a substrate, instead of ManNAc-6-P.

Keywords: Amphibia; *Bombina variegata*; *Bombina bombina*; Sialic acid; *O*-glycan

1. Introduction

Amphibian egg jelly coats, secreted by oviduct cells, are composed of several layers, which are deposited around the oocyte. This extracellular matrix constitutes the first barrier that must be passed through by the fertilizing sperm before reaching the egg plasma membrane. It is generally accepted that the jelly coat plays several important roles in the fertilization process, including sperm-egg binding, prevention of polyspermy (anuran), sperm capacitation, induction of the acrosome reaction, maintenance of divalent cation concentration and recognition of homologous species [1,2].

Previous structural studies have shown that mucins, which are the major constituents of amphibian egg jelly coats, possess species-specific carbohydrate chains [3–14]. This species-specificity of glycosylation supports the observed species-specificity of gamete interaction. Of particular interest are several proposals suggesting the role of sugar chains as sperm receptors [15]. According to the role ascribed to carbohydrates as an evolutionary potential of information, species-specificity could also be involved in a great number

Abbreviations: HPLC, high performance liquid chromatography; COSY, correlation spectroscopy; ROESY, rotating-frame nuclear overhauser enhancement spectroscopy; HMQC, heteronuclear multi-quantum coherence.

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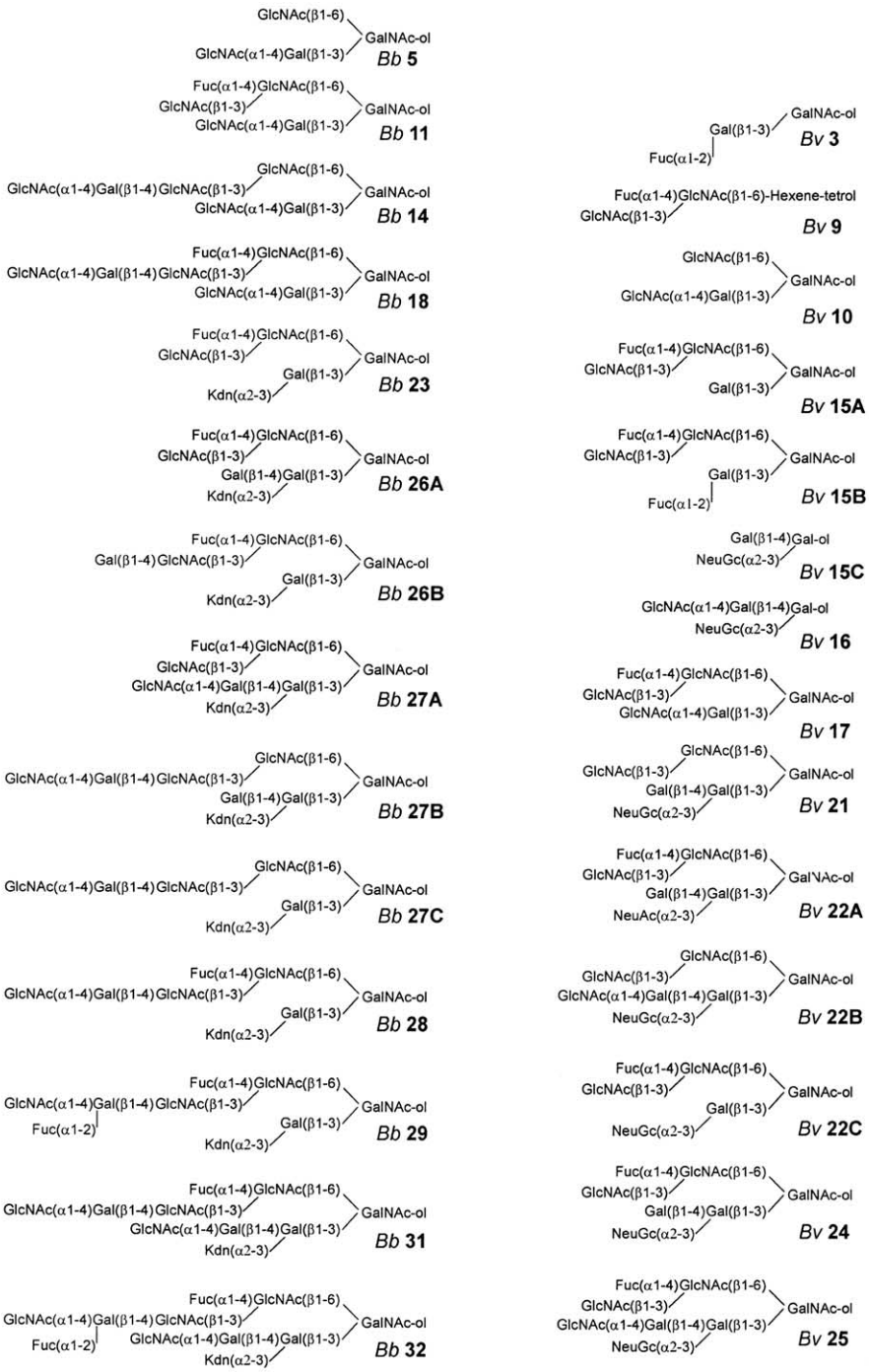

Fig. 1. Summary scheme of the primary structures characterized in *B. bombina* and *B. variegata* oviducal mucins.

of carbohydrate-based specific recognition systems, such as parasitism or microbial pathogenesis. The European fire-bellied toad *Bombina bombina* and yellow-bellied toad *Bombina variegata* are two very closely related species which hybridize readily in nature. Divergence occurred during geographic isolation estimated at 2–7 million years ago [16]. In the present study, we have isolated neutral and acidic oligosaccharides from the oviducal mucins of the two species and have determined their primary structure.

2. Materials and methods

2.1. Sampling of jelly coat mucus

Spawns from *B. bombina* and *B. variegata* were induced by injections of 750 IU of human chorionic gonadotrophin (ORGANON, France).

2.2. Extraction of oligosaccharide-alditols

O-linked oligosaccharides were released from the crude material by reductive β-elimination (0.05 M NaOH, 1 M $NaBH_4$, 37 °C, 48 h). The reaction was stopped by the addition of cation exchange resin (Dowex 50 × 8, 200–400 mesh, H^+ form). The solution was filtered and borate salts were removed by repeated evaporation with methanol. The total material was purified by cation exchange chromatography using a Dowex 50 × 2 column (200–400 mesh, H^+ form) to remove residual peptides. The total fraction of oligosaccharide-alditols was desalted by gel permeation on a Bio-Gel P2 column (80 × 2 cm), and lyophilized. Each oligosaccharide-alditol was isolated on a primary amine-bonded silica column (Supelcosyl™, LC-NH_2; 4.6 mm × 25 cm, Supelco Inc., Bellefonte, USA) by high performance liquid chromatography (HPLC) using gradients of acetonitrile/30 mM potassium phosphate buffer (pH 5.2)/H_2O. Each compound was further purified by gel filtration on a Bio-Gel P2 column (50 × 2 cm).

2.3. Matrix-assisted laser desorption mass spectrometry (MALD-MS)

Molecular weights of 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. The samples were dissolved in water at a concentration of 50–100 pmol/µl and 1 µl of these solutions was mixed with 1 µl of matrix on the target and allowed to crystallize at room temperature. The structures were mass analyzed in the negative mode using 2,5-dihydroxybenzoic acid matrix (10 mg/ml dissolved in water-ethanol, 80:20). External calibration was performed using angiotensin I (1296.7 Da) purchased from Sigma. For each analysis, between 10 and 20 shots were accumulated.

2.4. NMR spectroscopy

^{1}H-NMR experiments were performed on a Bruker ASX 400 WB spectrometer with 5 mm ^{1}H-^{13}C mixed probe head operating in the pulse Fourier-Transform mode and controlled by an Aspect 3000 computer. Chemical shifts were expressed in parts per million downfield from internal sodium 4,4'-dimethyl-4-silapentane-1-sulfonate, but were actually measured by reference to internal acetone (δ = 2.225 ppm in D_2O at 27 °C). The two dimensional homonuclear correlated spectroscopy (COSY) with simple and double relay transfer, the heteronuclear multi-quantum coherence (HMQC) and the rotating-frame nuclear overhauser enhancement spectroscopy (ROESY) were performed using Bruker standard pulse sequence library. For ROESY experiment, the mixing time was set at 300 ms.

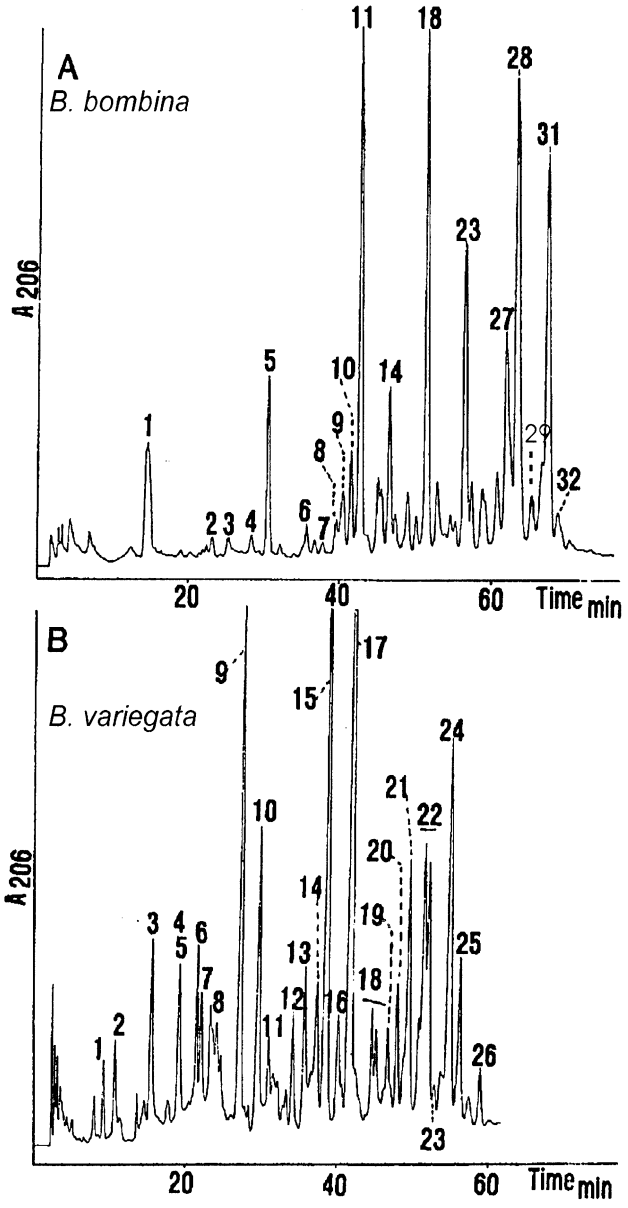

Fig. 2. HPLC profiles of total oligosaccharide fractions obtained from oviducal mucins of (A) *B. bombina* and (B) *B. variegata*.

3. Results

The primary structures of the 28 oligosaccharide-alditols isolated from the mucins of *B. bombina and B. variegata* are reported in Fig. 1 and the corresponding HPLC profiles are depicted in Fig. 2. The chemical shifts of each oligosaccharide-alditol are indicated in Tables 1 and 2. The analyses showed that although compounds *Bb* **5**, *Bb* **11** were identical to *Bv* **10** and *Bv* **17**, respectively, most of these structures are species-specific for *B. bombina* and *B. variegata*. Some short sequences have been previously characterized in other amphibian species. The oligosaccharide-alditols were organized into three main families according to their structural patterns, to facilitate discussion of the NMR data.

Table 1
^{1}H-NMR Chemical shifts of the oligosaccharide-alditols released from *B. bombina* and *B. variegata*

Units	Chemical shifts (ppm)														
		*Bb***5**	*Bb***11**	*Bb***14**	*Bb***18**	*Bv***22A**	*Bv***22B**	*Bv***22C**	*Bb***23**	*Bv***24**	*Bv***25**	*Bb***26A**	*Bb***26B**	*Bb***28**	*Bb***31**
GalNAc-ol I	H-1	n.d.	3.814	n.d.	3.816	3.818	n.d.	n.d.	n.d.	3.834	n.d.	~3.78	~3.78	3.820	3.823
	H-1'	n.d.	3.751	n.d.	3.751	3.747	n.d.	n.d.	n.d.	3.761	n.d.	~3.78	~3.78	3.749	3.753
	H-2	4.408	4.400	4.401	4.400	4.389	4.389	4.389	4.387	4.389	4.392	4.387	4.387	4.387	4.389
	H-3	4.082	4.076	4.077	4.077	4.06	4.074	4.060	n.d.	4.072	4.086	4.066	4.082	4.068	4.082
	H-4	n.d.	3.565	n.d.	3.565	3.451	3.479	3.427	3.44	3.461	3.501	3.461	3.437	3.440	3.494
	H-5	4.263	4.243	4.247	4.245	4.231	4.231	4.231	4.243	4.231	4.231	4.223	4.223	4.245	4.226
	H-6	n.d.	3.911	n.d.	3.913	3.884	3.884	3.884	n.d.	3.884	n.d.	3.892	3.892	3.899	3.905
	H-6'	n.d.	3.676	n.d.	3.674	3.661	3.661	3.661	n.d.	3.661	n.d.	3.547	3.547	3.649	3.655
	NAc	2.054	2.069	2.065	2.068	2.068	2.068	2.068	2.066	2.069	2.072	2.066	2.066	2.068	2.068
Gal II	H-1	4.520	4.518	4.524	4.52	4.573	4.594	4.538	4.525	4.578	4.598	4.564	4.525	4.526	4.583
	H-2	n.d.	3.600	n.d.	3.600	3.702	3.717	3.607	n.d.	3.717	n.d.	3.70	3.592	3.593	3.712
	H-3	n.d.	3.745	n.d.	3.746	4.23	4.251	4.123	4.093	4.247	n.d.	4.205	4.098	4.097	4.213
	H-4	n.d.	3.973	n.d.	3.975	4.146	4.181	3.940	n.d.	4.150	n.d.	4.142	3.927	3.926	4.173
	H-5	n.d.	3.75	n.d.	~3.80	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	~3.77
GlcNAc III	H-1	4.868	4.875	4.872	4.874	–	–	–	–	–	–	–	–	–	–
	H-2	n.d.	3.903	n.d.	3.906	–	–	–	–	–	–	–	–	–	–
	H-3	n.d.	3.812	n.d.	3.815	–	–	–	–	–	–	–	–	–	–
	H-4	n.d.	3.544	n.d.	3.546	–	–	–	–	–	–	–	–	–	–
	H-5	4.171	4.160	~4.16	4.163	–	–	–	–	–	–	–	–	–	–
	H-6	n.d.	~3.81	n.d.	3.837	–	–	–	–	–	–	–	–	–	–
	H-6'	n.d.	~3.81	n.d.	3.783	–	–	–	–	–	–	–	–	–	–
	NAc	2.104	2.105	2.103	2.107	–	–	–	–	–	–	–	–	–	–
Gal III	H-1	–	–	–	–	4.739	4.863	–	–	4.744	4.870	4.740	–	–	4.864
	H-2	–	–	–	–	3.532	3.572	–	–	3.543	n.d.	3.538	–	–	3.575
	H-3	–	–	–	–	3.676	3.742	–	–	3.685	n.d.	n.d.	–	–	3.751
	H-4	–	–	–	–	3.915	3.988	–	–	3.915	n.d.	n.d.	–	–	3.989
GlcNAc IV	H-1	–	–	–	–	–	4.904	–	–	–	4.904	–	–	–	4.904
	H-2	–	–	–	–	–	3.911	–	–	–	n.d.	–	–	–	3.922
	H-3	–	–	–	–	–	3.814	–	–	–	n.d.	–	–	–	3.821
	H-4	–	–	–	–	–	3.556	–	–	–	n.d.	–	–	–	3.555
	H-5	–	–	–	–	–	~4.2	–	–	–	~4.2	–	–	–	4.180
	H-6	–	–	–	–	–	n.d.	–	–	–	n.d.	–	–	–	~3.78
	H-6'	–	–	–	–	–	n.d.	–	–	–	n.d.	–	–	–	~3.78
	NAc	–	–	–	–	–	2.090	–	–	–	2.094	–	–	–	2.094
GlcNAc II'	H-1	4.547	4.466	4.498	4.475	4.470	4.497	4.470	4.466	4.472	4.469	4.470	4.470	4.476	4.475
	H-2	n.d.	3.867	n.d.	3.870	3.829	3.772	3.829	n.d.	3.84	n.d.	3.838	3.838	3.847	3.855
	H-3	n.d.	4.001	n.d.	4,000	3.982	3.710	3.982	4.064	3.989	n.d.	n.d.	n.d.	n.d.	3.995
	H-4	n.d.	3.705	n.d.	3.719	3.724	3.526	3.724	n.d.	3.722	n.d.	n.d.	n.d.	n.d.	3.731
	H-5	n.d.	3.545	n.d.	3.543	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.535	3.538
	H-6	n.d.	3.987	n.d.	3.986	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.980	n.d.
	H-6'	n.d.	3.845	n.d.	3.839	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.844	n.d.
	NAc	2.068	2.094	2.10	2.095	2.113	2.094	2.106	2.102	2.115	2.111	2.111	2.111	2.104	2.109
GlcNAc III'	H-1	–	4.639	4.614	4.673	4.644	4.584	4.644	4.639	4.644	4.646	4.642	4.655	4.673	4.679
	H-2	–	~3.61	n.d.	3.649	3.61	3.660	3.61	n.d.	~3.61	n.d.	~3.61	3.674	3.652	3.645
	H-3	–	~3.61	n.d.	3.787	3.61	3.575	3.61	n.d.	~3.61	n.d.	~3.61	n.d.	3.786	3.784
	H-4	–	3.203	n.d.	3.454	3.198	3.470	3.198	3.204	3.200	3.201	n.d.	3.217	3.454	3.449
	H-5	–	3.399	n.d.	3.546	3.402	n.d.	3.402	3.401	3.406	3.402	n.d.	3.202	3.554	3.548
	H-6	–	3.975	n.d.	4.094	3.974	n.d.	3.974	n.d.	3.974	n.d.	3.975	n.d.	4.091	4.093
	H-6'	–	3.590	n.d.	3.691	3.592	n.d.	3.592	n.d.	3.592	n.d.	3.587	n.d.	3.693	3.689
	NAc	–	2.009	2.019	2.010	2.006	2.016	2.006	2.003	2.006	2.004	2.005	2.005	2.003	2.003
Gal IV'	H-1	–	–	4.518	4.500	–	–	–	–	–	–	–	4.439	4.498	4.497
	H-2	–	–	n.d.	3.578	–	–	–	–	–	–	–	n.d.	3.581	3.579
	H-3	–	–	n.d.	3.733	–	–	–	–	–	–	–	n.d.	3.734	3.732
	H-4	–	–	n.d.	3.977	–	–	–	–	–	–	–	n.d.	3.980	3.980
	H-5	–	–	n.d.	~3.78	–	–	–	–	–	–	–	n.d.	n.d.	n.d.

Table 1
(continued)

Units	Chemical shifts (ppm)													
		*Bb***5**	*Bb***11**	*Bb***14**	*Bb***18**	*Bv***22A**	*Bv***22B**	*Bv***22C**	*Bb***23**	*Bv***24**	*Bv***25**	*Bb***26A**	*Bb***26B**	*Bb***28**	*Bb***31**
GlcNAc V'	H-1	–	–	4.864	4.863	–	–	–	–	–	–	–	–	4.863	4.864
	H-2	–	–	n.d.	3.913	–	–	–	–	–	–	–	–	3.914	3.912
	H-3	–	–	n.d.	3.778	–	–	–	–	–	–	–	–	n.d.	3.781
	H-4	–	–	n.d.	3.546	–	–	–	–	–	–	–	–	3.545	3.545
	H-5	–	–	~4.18	4.183	–	–	–	–	–	–	–	–	4.183	4.183
	H-6	–	–	n.d.	3.837	–	–	–	–	–	–	–	–	3.838	~3.78
	H-6'	–	–	n.d.	3.783	–	–	–	–	–	–	–	–	3.768	~3.78
	NAc	–	–	2.075	2.070	–	–	–	–	–	–	–	–	2.068	2.070
Fuc F[4]	H-1	–	5.007	–	5.017	5.015	–	5.015	5.012	5.015	5.013	5.015	5.015	5.021	5.020
	H-2	–	3.812	–	3.813	3.797	–	3.797	n.d.	3.808	n.d.	3.808	3.808	3.808	3.811
	H-3	–	3.922	–	3.938	3.912	–	3.912	n.d.	3.919	n.d.	n.d.	n.d.	n.d.	3.933
	H-4	–	3.801	–	3.816	3.806	–	3.806	n.d.	3.800	n.d.	n.d.	n.d.	n.d.	3.811
	H-5	–	4.805	–	4.775	4.809	–	4.809	4.807	4.807	4.812	4.809	~4.79	4.78	4.787
	CH_3	–	1.275	–	1.279	1.274	–	1.274	1.273	1.273	1.274	1.272	1.277	1.276	1.276
K[a]or N[b]	H-3ax	–	–	–	–	1.853	1.853	1.850	1.754	1.870	1.880	1.803	1.754	1.755	1.815
	H-3eq	–	–	–	–	2.741	2.752	2.752	2.720	2.759	2.752	2.680	2.720	2.719	2.681
	H-4	–	–	–	–	3.697	3.794	3.794	n.d.	n.d.	n.d.	n.d.	n.d.	3.608	3.630
	H-5	–	–	–	–	3.856	3.935	3.935	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.534
	H-7	–	–	–	–	n.d.	n.d.	n.d.	n.d.	3.944	n.d.	n.d.	n.d.	n.d.	n.d.
	NAc	–	–	–	–	2.034	–	–	–	–	–	–	–	–	–
	NGc	–	–	–	–	–	4.123	4.123	–	4.123	4.123	–	–	–	–

n.d. = Not determined
[a] = Kdn
[b] = NeuAc or NeuGc.

3.1. Compounds with an upper branch represented by the sequence GlcNAc(β1-3)[Fuc(α1-4)]GlcNAc(β1-6)

This first class is represented by compounds *Bb* **11**, **23**, **26A**, **27A** (*Bb* for *B. bombina*) and *Bv* **15A**, **15B**, **17**, **22A**, **22C**, **24**, **25** (*Bv* for *B. variegata*). The mixture of compounds *Bb* **11** and *Bv* **17** was extensively studied by NMR analysis, including COSY, ROESY, HMQC and HMBC experiments. In the first step, confirmatory evidence from ^{1}H-^{1}H COSY, one step- and two step-relayed COSY spectra was obtained for the stereochemistry of each sugar unit, which was determined by consideration of the values of the first four ^3J coupling constants displayed and its *J* connectivities (Fig. 3). *N* -acetylhexosamines (here β-GlcNAc and α-GlcNAc) were distinguished from hexoses (here β-Gal) owing to their characteristic C2 atom resonance observed between 55 and 57 ppm (Table 3). The two step-relayed COSY GlcNAc III' H-1 track (δ 4.639) shows cross-peaks with GlcNAc III' H-2, 3, 4, 5. Such a transfer of magnetization from H-1 to H-5 occurring during a two step-relayed COSY is attributable to a virtual coupling resulting from the superimposition of the H-2 and H-3 atom resonances at δ 3.61. The heteronuclear spectrum (HMQC) of *Bb* **11** contained all the ^{1}H/^{13}C cross-peaks which were assigned by compilation of the ^{1}H chemical shifts previously measured. The relatively low-field position of some ^{13}C resonances, compared to their position in the spectra of the corresponding non-substituted monosaccharides, was caused by glycosylation and revealed the substitution pattern in the polymer. These data showed that GalNAc-ol is 3,6-substituted, Gal II is 4-substituted, GlcNAc II' is 3,4-substituted, whereas Fuc, GlcNAc III and III' are terminal sugar residues. By means of ROESY and HMBC experiments, the sequence of oligosaccharide-alditol *Bb* **11** was achieved. Particularly, the HMBC experiment optimized for long-range $^3J_{C,H}$ couplings established the following intra-residue linkages: Fuc(1→4)GlcNAc II'; GlcNAc III' (1→3)GlcNAc II'; GlcNAc II' (1→6)GalNAc-ol I; GlcNAc III(1→4)Gal II; Gal II(1→3)GalNAc-ol I (Fig. 4). These combined observations, from four different NMR studies, have proven the sugar sequence *GlcNAc(β1-3)[Fuc(α1-4)] GlcNAc(β1-6)[Gal(β1-3)]GalNAc-ol* to be common to the series of eleven compounds quoted above. The lower branches of this series of compounds were identified as Gal(β1-3) (*Bv* **15A**), Fuc(α1-2)Gal(β1-3) (*Bv* **15B**), GlcNAc(α1-4)Gal(β1-3) (*Bb* **11**, *Bv* **17**), Kdn(α2-3) Gal(β1-3) (*Bb* **23**), NeuGc(α2-3)Gal(β1-3) (*Bv* **22C**), Gal(β1-4)[Kdn(α2-3)]Gal(β1-3) (*Bb* **26A**), Gal(β1-4) [NeuAc(α2-3)]Gal(β1-3) (*Bv* **22A**), Gal(β1-4)[NeuGc(α2-3)]Gal(β1-3) (*Bv* **24**), GlcNAc(α1-4)Gal(β1-4)[NeuGc(α2-3)]Gal(β1-3) (*Bv* **25**). As shown in Tables 1 and 2, the Fuc(α1-2)Gal sequence was easily established owing to the well known characteristic chemical shifts of Fuc H-1, H-5, H-6 and Gal H-1 and H-2 [17]. In the same way, sequences Kdn(α2-3)Gal and NeuGc(α2-3)Gal were identified on the basis of the typical H-3 and H-4 atom resonances of the 3-substituted β-Gal unit.

The three oligosaccharide-alditols *Bb* **26A**, *Bv* **22A** and *Bv* **24** just differ due to the presence of Kdn, NeuAc or NeuGc, respectively, α-2,3 linked to the first Gal unit of the dimer Gal(β1-4)Gal (Figs. 5 and 6). These three sialic acids were

Table 2
^{1}H-NMR chemical shifts of the oligosaccharide-alditols released from *B. bombina* and *B. variegata*

Units		Chemical shifts (ppm)												
		*Bv***3**	*Bv***9**	*Bv***10**	*Bv***15A**	*Bv***15B**	*Bv***15C**	*Bv***17**	*Bv***21**	*Bb***27A**	*Bb***27B**	*Bb***27C**	*Bb***29**	*Bb***32**
GalNAc-ol	H-1	n.d.	3.479	n.d.	3.758	3.758	n.d.	3.814	n.d.	3.826	3.826	3.826	~3.78	n.d.
I/Hex-ol	H-1'	n.d.	3.408	n.d.	3.758	3.758	n.d.	3.751	n.d.	3.754	3.754	3.754	~3.78	n.d.
I/Gal-ol	H-2	4.398	4.238	4.407	4.389	4.001	4.298	4.001	4.387	4.387	4.387	4.387	4.387	4.388
I/hexene-tetrol I	H-3	4.09	5.777	4.082	4.057	4.083	4.124	4.076	4.071	4.081	4.069	4.069	4.067	4.093
	H-4	3.521	5.777	n.d.	3.467	3.491	n.d.	n.d.	n.d.	3.503	3.443	3.443	3.44	3.440
	H-5	4.161	4.325	4.262	4.262	4.237	n.d.	4.243	4.229	4.39	4.39	4.39	4.243	4.225
	H-6	n.d.	3.732	n.d.	3.897	3.893	n.d.	n.d.	n.d.	3.920	3.920	3.920	3.867	n.d.
	H-6'	n.d.	3.426	n.d.	3.654	3.677	n.d.	n.d.	n.d.	3.656	3.656	3.656	3.593	n.d.
	NAc	2.046	–	2.068	2.067	2.056	–	2.069	2.065	2.069	2.069	2.069	2.066	2.070
Gal II	H-1	4.582	–	4.547	4.464	4.572	–	4.518	4.576	4.581	4.581	4.526	4.527	4.581
	H-2	n.d.	–	n.d.	3.559	3.679	–	n.d.	n.d.	3.711	3.711	3.595	3.591	n.d.
	H-3	n.d.	–	n.d.	3.676	3.874	–	n.d.	4.246	4.215	4.215	4.098	4.095	n.d.
	H-4	3.928	–	3.972	3.901	3.924	–	n.d.	4.152	4.195	1.195	3.928	3.927	4.176
Gal III	H-1	–	–	–	–	–	4.645	–	4.744	4.862	4.716	–	–	4.864
	H-2	–	–	–	–	–	n.d.	–	n.d.	3.574	3.537	–	–	n.d.
	H-3	–	–	–	–	–	n.d.	–	n.d.	3.749	3.689	–	–	n.d.
	H-4	–	–	–	–	–	n.d.	–	n.d.	3.991	3.920	–	–	n.d.
GlcNAc IV/III	H-1	–	–	4.867	–	–	–	4.873	–	4.902	–	–	–	4.904
	H-2	–	–	n.d.	–	–	–	n.d.	–	3.922	–	–	–	n.d.
	H-3	–	–	n.d.	–	–	–	n.d.	–	3.82	–	–	–	n.d.
	H-4	–	–	n.d.	–	–	–	n.d.	–	3.555	–	–	–	n.d.
	H-5	–	–	4.171	–	–	–	4.160	–	n.d.	–	–	–	n.d.
	H-6	–	–	n.d.	–	–	–	n.d.	–	~3.82	–	–	–	n.d.
	H-6'	–	–	n.d.	–	–	–	n.d.	–	~3.82	–	–	–	n.d.
	NAc	–	–	2.103	–	–	–	2.094	–	2.094	–	–	–	2.093
	H-1	–	4.473	4.520	4.464	4.477	–	4.464	4.495	4.465	4.501	4.501	4.473	4.475
	H-2	–	3.720	n.d.	3.858	3.858	–	n.d.	n.d.	3.853	3.779	3.779	3.844	n.d.
	H-3	–	3.847	n.d.	3.996	4,000	–	n.d.	n.d.	3.995	n.d.	n.d.	n.d.	n.d.
	H-4	–	3.599	3.441	3.720	3.720	–	n.d.	n.d.	3.721	n.d.	n.d.	n.d.	n.d.
	H-5	–	n.d.	n.d.	3.534	3.534	–	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	H-6	–	n.d.	n.d.	3.987	3.987	–	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	H-6'	–	n.d.	n.d.	3.849	3.849	–	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	NAc	–	2.088	2.053	2.099	2.101	–	2.106	2.094	2.107	2.093	2.093	2.102	2.107
GlcNAc III’	H-1	–	4.645	–	4.648	4.648	–	4.639	4.582	4.643	4.64	4.64	4.676	4.687
	H-2	–	3.497	–	3.612	3.612	–	n.d.	n.d.	~3.61	n.d.	n.d.	3.648	n.d.
	H-3	–	3.497	–	3.622	3.622	–	n.d.	n.d.	~3.61	n.d.	n.d.	n.d.	n.d.
	H-4	–	3.123	–	3.207	3.207	–	3.202	n.d.	3.201	n.d.	n.d.	3.439	n.d.
	H-5	–	3.298	–	3.397	3.397	–	3.399	n.d.	3.403	n.d.	n.d.	n.d.	n.d.
	H-6	–	n.d.	–	3.974	3.974	–	n.d.	n.d.	3.976	n.d.	n.d.	n.d.	n.d.
	H-6'	–	n.d.	–	3.593	3.593	–	n.d.	n.d.	3.590	n.d.	n.d.	n.d.	n.d.
	NAc	–	2.005	–	2.006	2.006	–	2.009	2.018	2.002	2.002	2.002	1.997	1.998
Gal IV'	H-1	–	–	–	–	–	–	–	–	–	4.501	4.501	4.527	4.528
	H-2	–	–	–	–	–	–	–	–	–	~3.56	~3.56	3.864	n.d.
	H-3	–	–	–	–	–	–	–	–	–	~3.74	~3.74	n.d.	n.d.
	H-4	–	–	–	–	–	–	–	–	–	3.978	3.978	n.d.	3.988
GlcNAc V'	H-1	–	–	–	–	–	–	–	–	–	4.847	4.847	5.015	5.030
	H-2	–	–	–	–	–	–	–	–	–	3.911	3.911	~3.82	n.d.
	H-3	–	–	–	–	–	–	–	–	–	~3.79	~3.79	n.d.	n.d.
	H-4	–	–	–	–	–	–	–	–	–	~3.54	~3.54	n.d.	n.d.
	H-5	–	–	–	–	–	–	–	–	–	~4.20	~4.20	n.d.	4.187
	H-6	–	–	–	–	–	–	–	–	–	~3.82	~3.82	n.d.	n.d.
	H-6'	–	–	–	–	–	–	–	–	–	~3.82	~3.82	n.d.	n.d.
	NAc	–	–	–	–	–	–	–	–	–	2.069	2.069	2.056	2.053

Table 2
(continued)

Units		Chemical shifts (ppm)												
		*Bv***3**	*Bv***9**	*Bv***10**	*Bv***15A**	*Bv***15B**	*Bv***15C**	*Bv***17**	*Bv***21**	*Bb***27A**	*Bb***27B**	*Bb***27C**	*Bb***29**	*Bb***32**
Fuc F^2	H-1	5.256	–	–	–	5.228	–	–	–	–	–	–	5.277	5.277
	H-2	n.d.	–	–	–	3.804	–	–	–	–	–	–	3.791	n.d.
	H-3	n.d.	–	–	–	3.919	–	–	–	–	–	–	n.d.	n.d.
	H-4	n.d.	–	–	–	3.826	–	–	–	–	–	–	n.d.	n.d.
	H-5	4.279	–	–	–	4.275	–	–	–	–	–	–	4.082	n.d.
	CH_3	1.241	–	–	–	1.240	–	–	–	–	–	–	1.216	1.218
Fuc F^4	H-1	–	5.019	–	5.013	5.013	–	5.007	–	5.011	–	–	5.008	5.019
	H-2	–	3.677	–	3.808	3.808	–	n.d.	–	3.811	–	–	3.805	n.d.
	H-3	–	n.d.	–	3.920	3.920	–	n.d.	–	3.919	–	–	n.d.	n.d.
	H-4	–	n.d.	–	3.800	3.800	–	n.d.	–	3.800	–	–	n.d.	n.d.
	H-5	–	4.816	–	4.809	4.809	–	4.805	–	4.811	–	–	~4.77	n.d.
	CH_3	–	1.276	–	1.270	1.270	–	1.275	–	1.273	–	–	1.278	1.278
K[a]or N[b]	H-3ax	–	–	–	–	–	1.785	–	1.869	1.813	1.813	1.783	1.755	1.814
	H-3eq	–	–	–	–	–	2.768	–	2.759	2.680	2.680	2.717	2.719	2.680
	H-4	–	–	–	–	–	n.d.	–	n.d.	3.634	3.634	3.634	3.604	n.d.
	NGc	–	–	–	–	–	4.120	–	4.123	–	–	–	–	–

n.d. = Not determined
[a] = Kdn
[b] = NeuAc or NeuGc.

easily characterized by the presence or the absence of acetyl or glycolyl signals. The H-3, H-4 atom resonances of β-Gal II, as well as the H-3ax, H-3eq signals of NeuAc or NeuGc, are similar to those observed for blood group Cad determinant-containing *O*-glycans [18]. The slight differences are due to the replacement of β-GalNAc by β-Gal. It is worth noting that *Bv* **22A** was not isolated in a pure state (Fig. 6). The presence of NeuAc in *Bv* **22A** was proved by

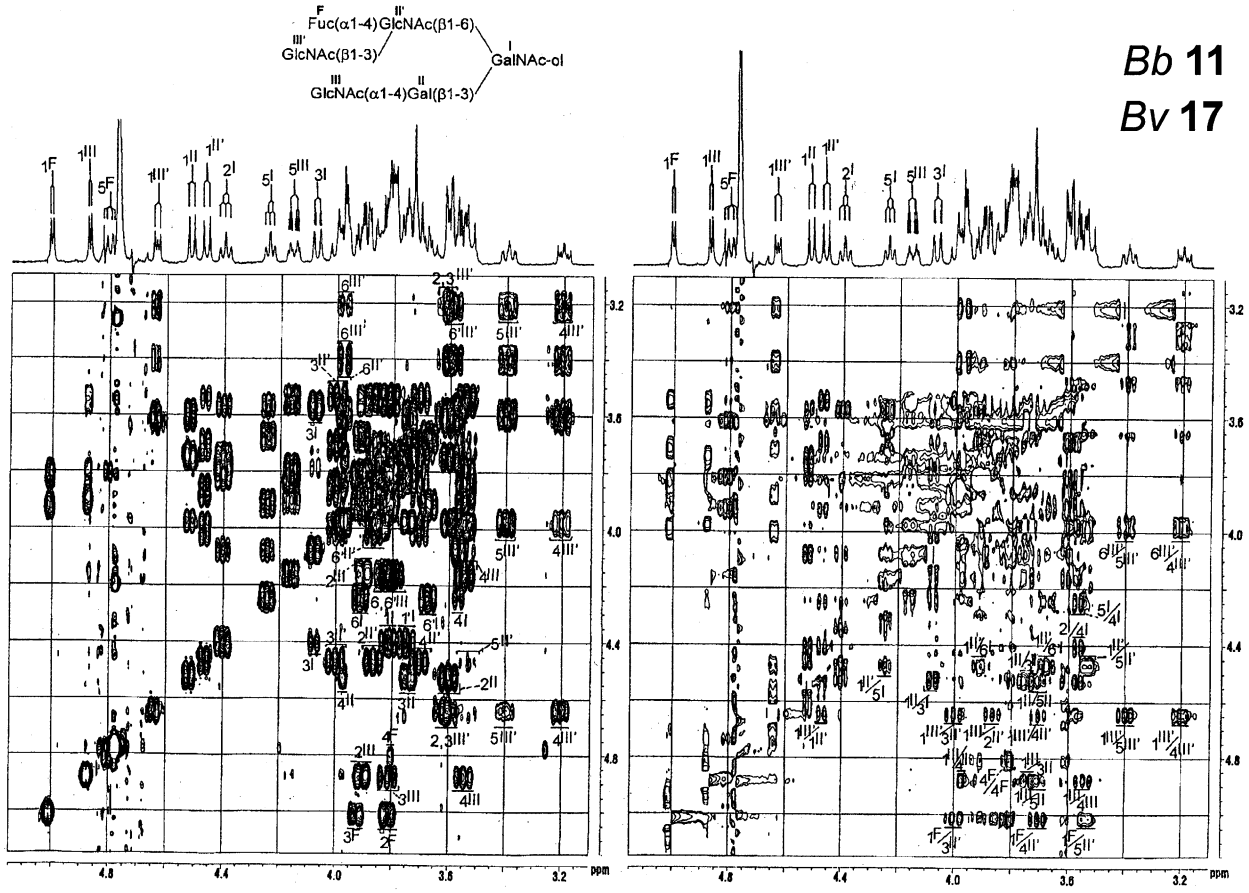

Fig. 3. Double relayed COSY (left) and ROESY (right) spectra of *Bb* **11**, *Bv* **17**.

Table 3
^{13}C-NMR chemical shifts of the oligosaccharide-alditol 11 released from *B. bombina*

Units	Chemical shifts (ppm)		Units	Chemical shifts (ppm)	
GalNAc-ol I	C-1	n.d.	GlcNAc III	C-1	99.37
	C-2	52.65		C-2	55.29
	C-3	78.31		C-3	71.64
	C-4	70.19		C-4	70.91
	C-5	68.79		C-5	68.79
	C-6	72.12		C-6	n.d.
	NAc	23.24		NAc	23.26
Gal II	C-1	105.71	Glc NAc II'	C-1	102.94
	C-2	72.12		C-2	56.82
	C-3	73.30		C-3	77.55
	C-4	77.87		C-4	73.89
	C-5	76.64		C-5	76.73
				C-6	61.21
Fuc F[4]	C-1	99.47		NAc	23.70
	C-2	69.00			
	C-3	70.39	GlcNAc III'	C-1	102.34
	C-4	73.33		C-2	57.28
	C-5	68.02		C-3	74.52
	C-6	16.73		C-4	72.12
				C-5	77.03
				C-6	63.03
				NAc	23.60

n.d. = not determined

measurement of the molecular weight of each component and the observation of characteristic NeuAc H-4 (δ 3.697) and NAc (δ 2.034) atom resonances. Compounds *Bb* **27A** and *Bv* **25** are extensions of *Bb* **26A** and *Bv* **24** with a GlcNAc α1→4 linked to the Gal III unit. The presence of the additional α-GlcNAc unit is evidenced by its H-1 signal observed at δ 4.902 (*Bb* **27A**) or δ 4.904 (*Bv* **25)**, whereas the Gal III H-1 signal is shifted downfield at δ 4.862 or 4.870 (Figs. 7–9). These characteristics were also inferred from the ROESY spectra of *Bb* **18** and *Bb* **31** (see below).

3.2. Compounds with an upper branch represented by the sequence GlcNAc(β1-3)[Fuc(α1-4)]GlcNAc(β1-6) extended with Gal(β1-4) and GlcNAc(α1-4)Gal(β1-4) units

This series of oligosaccharide-alditols is represented by compounds *Bb* **26B** (additional β-Gal unit) and *Bb* **18**, **28**, **29**, **31**, **32** (additional GlcNAc(α1-4)Gal unit). It is worth noting that oligosaccharide-alditols isolated from *B. variegata* are devoid of these extended upper branches.

Compounds *Bb* **18** and *Bb* **31** were extensively analyzed by multiple step-relayed COSY and ROESY experiment in order to define the characteristics of their structural reporter groups. The entire β-Gal IV' and α-GlcNAc V' spin system was unambiguously assigned from the 2D COSY spectra (Figs. 7 and 8). From the comparison of NMR spectra of *Bb* **11** and **18**, the relatively downfield position of GlcNAc III' H-4 (δ 3.45 vs. δ 3.20 ppm) and H-6 (δ 4.09 vs. δ 3.97 ppm) is consistent with a C4 substitution. Similarly, the Gal IV' unit, which exhibited the same NMR data as the Gal II unit of

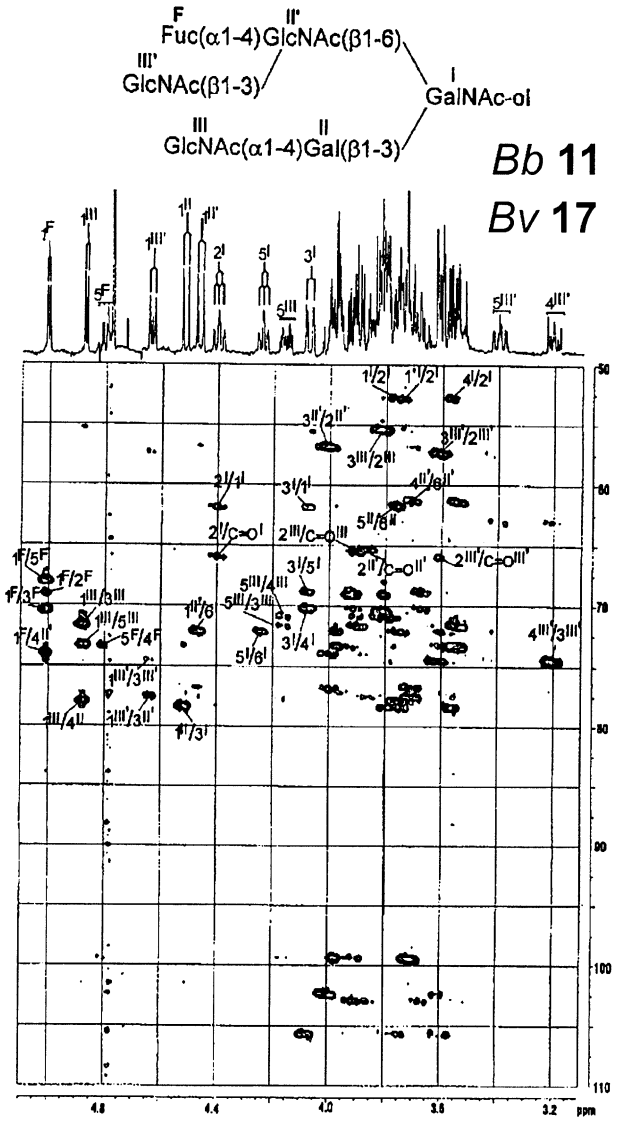

Fig. 4. HMQC of *Bb* **11**, *Bv* **17**.

the lower branch, appeared as C4 substituted. These 1→4 linkages were confirmed by the ROE contacts observed between Gal IV' H-1 and GlcNAc III' H-4, on the one hand, and between GlcNAc V' H-1 and Gal IV' H-4, on the other hand. The upper branch described in compound *Bb* **18** was also observed in *Bb* **28** and **31**. The lower branches of *Bb* **18**, **28** and **31** are similar to those of *Bb* **11**, **23** and **27A**, respectively. Compounds *Bb* **29** and **32** are α1→2 fucosylated extensions of *Bb* **28** and **31**, respectively. In Fig. 9, the ^{1}H-NMR spectra of *Bb* **29** and **32** are depicted. The attachment of the α-Fuc residue via an α1→2 linkage to β-Gal IV' was established from the chemical shifts of Fuc H-1 at δ 5.277, H-5 at δ 4.082 and CH_3 at δ 1.216. As compared to *Bb* **28** and **31**, downshifts for β-Gal IV' H-1 (Δδ + 0.029) and H-2 (Δδ + 0.284) were observed on the COSY spectrum of *Bb* **29**, whereas the 1D NMR spectrum of *Bb* **32** showed similar data for Fuc H-1, CH_3 and Gal IV' H-1.

Compound *Bb* **26B** has been observed in a mixture with other oligosaccharide-alditols. Comparing *Bb* **26B** with **29**

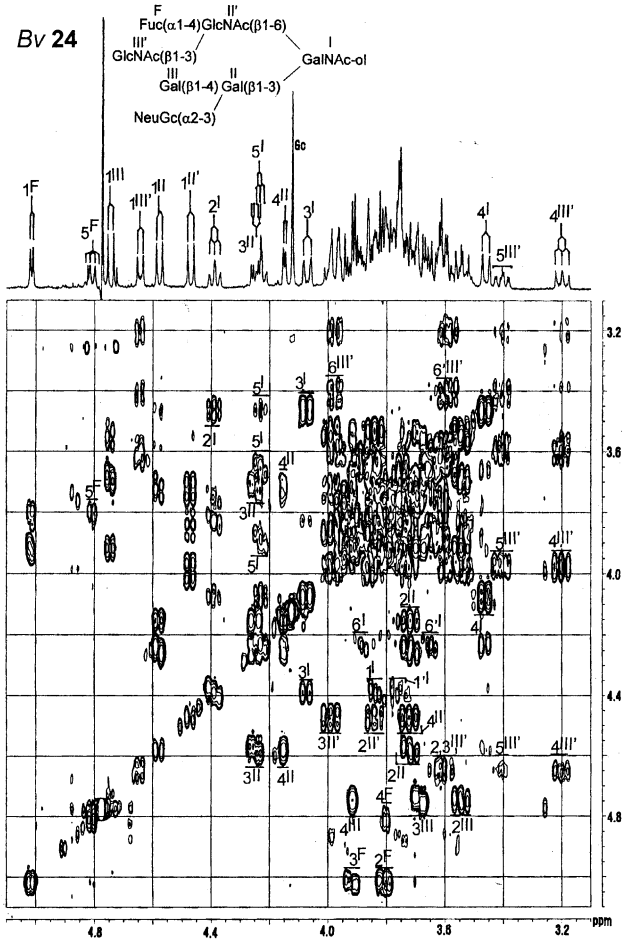

Fig. 5. Double relayed COSY spectrum of *Bv* **24**.

and **32**, the presence of terminal Gal IV' is indicated by the Gal H-1 signal at δ 4.439 ppm, whereas the structural reporter group signals relative to the lower branch perfectly match those of *Bb* **23**, **28** and **29**.

3.3. Compounds with an upper branch devoid of an α1→4 linked Fuc unit (oligosaccharide-alditols Bb 14, 27B, 27C and Bv 21, 22B)

The upper branch of compound *Bv* **21** contains the sequence GlcNAc(β1-3)GlcNAc(β1-6), previously described in other amphibians, such as *Rana utricularia* [11] and *Bufo viridis* [4] as confirmed by the set of GlcNAc II' H-1 (δ 4.495), NAc (δ 2.094) and GlcNAc III' H-1 (δ 4.582), NAc (δ 2.018). The COSY spectrum of the fraction *Bv* **22** (Fig. 6) is more informative, since it allows to discriminate, on the same spectrum, all the H-1 to H-4 atom resonances of the GlcNAc II' and III' units present in the sequence GlcNAc(β1-3)GlcNAc (*Bv* **22B**).

The sequence GlcNAc(α1-4)Gal(β1-4)GlcNAc(β1-3) GlcNAc(β1-6) was established as the upper branch of the two minor compounds *Bb* **27B** and **27C**. Indeed, the fraction *Bv* **27** is mainly composed of the oligosaccharide-alditol **27A**, characterized by relevant ^{1}H-NMR parameters of the

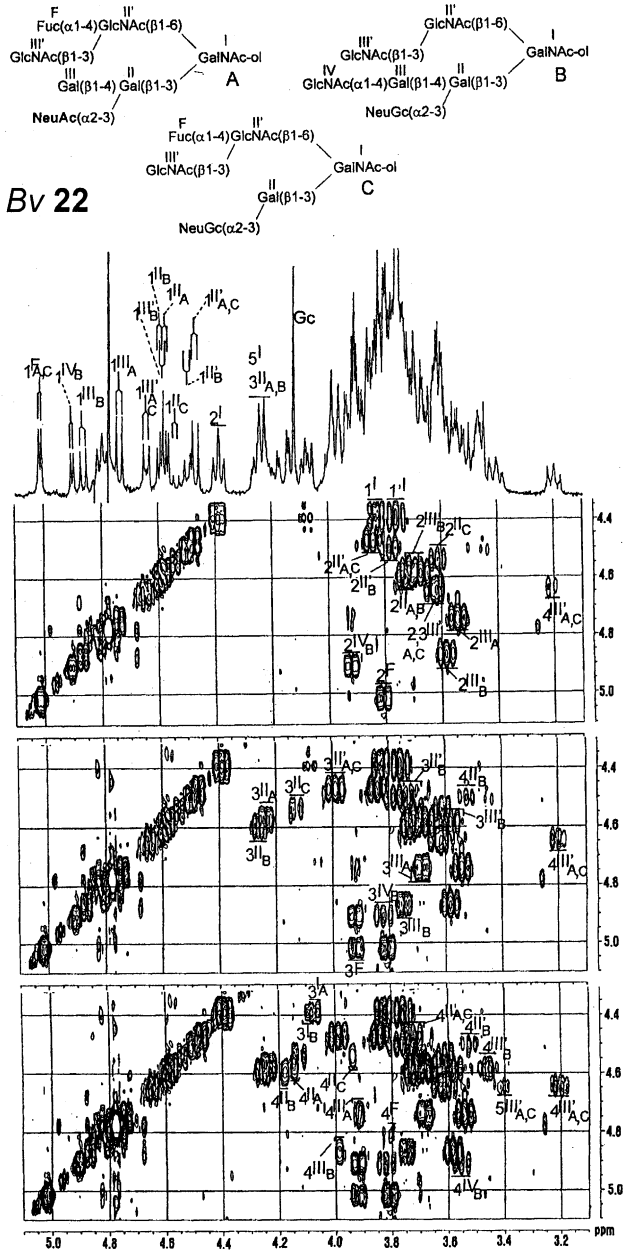

Fig. 6. Part of non-relayed, relayed and double relayed COSY spectra of fraction *Bv* **22**.

upper branch assigned to *Bv* **11**, **23** or *Bv* **15A**, **17**, **25** and the lower branch assigned to *Bb* **31** and **32**. Moreover, the pseudo-molecular ions $[M-H]^-$ observed at *m* / *z* 1405.5 and 1567.5 showed the presence of two minor compounds composed of 3 HexNAc, 2 Gal, 1 Kdn, 1 HexNAc-ol (*Bv* **27C**) and 3 HexNAc, 3 Gal, 1 Kdn, 1 HexNAc-ol (*Bv* **27B**). The ^{1}H-NMR spectrum of the fraction *Bv* **27** contains information relative to the sequences Kdn(α2-3)Gal (Gal H-3 at δ 4.09 and Gal H-4 at δ 3.93) and GlcNAc(α1-4)Gal(β1-4)GlcNAc(β1-3) (α-GlcNAc V' H-1 at δ 4.847, β-Gal IV' H-1 at δ 4.501 and β-GlcNAc III' at δ 4.64). The sequence Gal(β1-4)[NeuAc(α2-3)]Gal(β1-3), ascribed as the lower branch of compound **27B**, was deduced from the observation

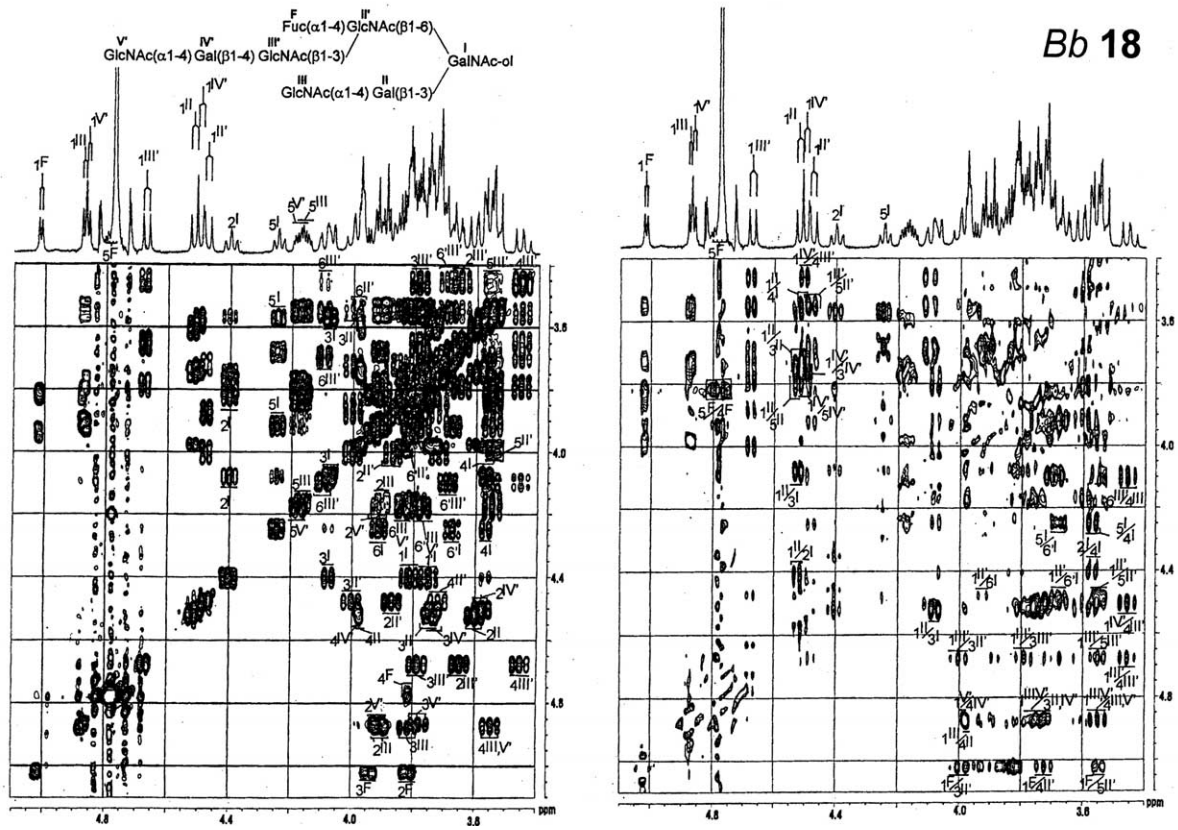

Fig. 7. Double relayed COSY (left) and ROESY (right) spectra of *Bb* **18**.

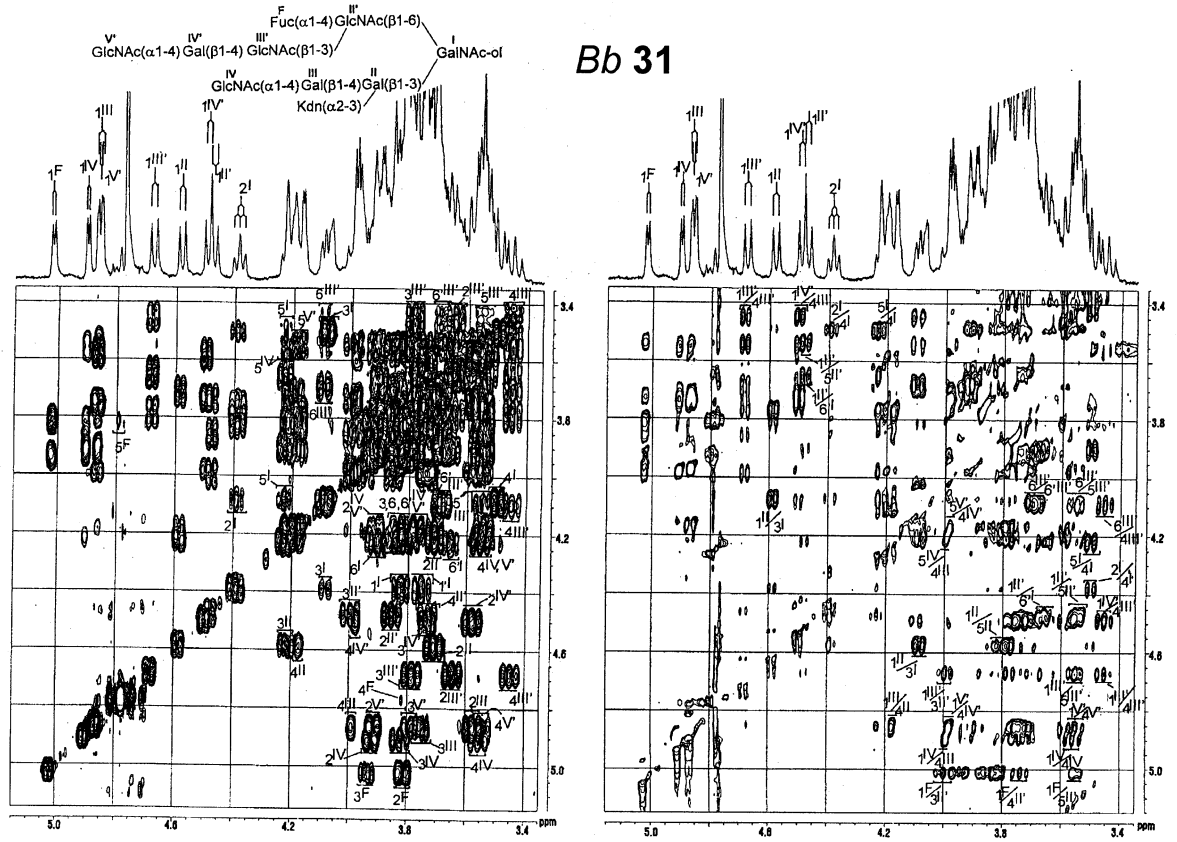

Fig. 8. Double relayed COSY (left) and ROESY (right) spectra of *Bb* **31**.

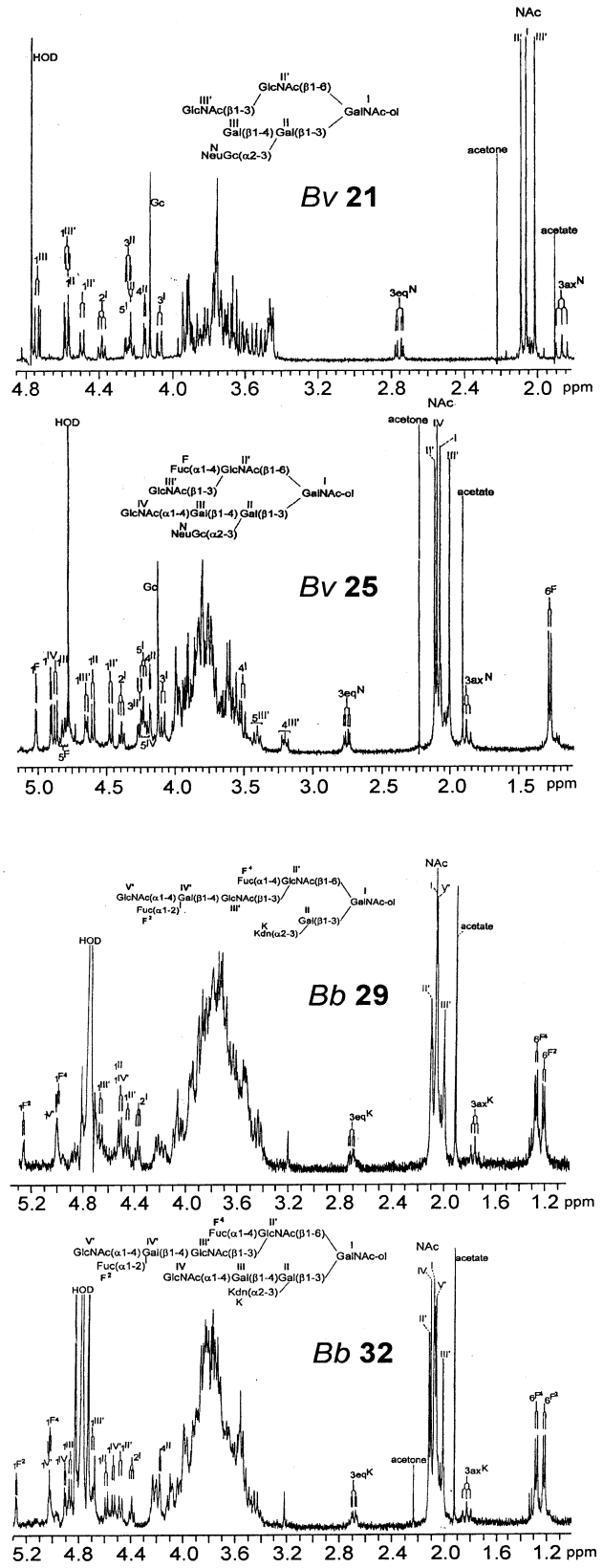

Fig. 9. ^{1}H-NMR spectra of *Bb* **29**, *Bb* **31**, *Bv* **21** and *Bv* **25**.

of the peak at *m* / *z* 1567.5, but its structural reporter groups (Gal II_B H-3, H-4) are actually superimposed with the Gal II_A H-3, H-4 resonances. As for the sequence GlcNAc(β1-3)GlcNAc(β1-6), the only significant reporter groups observable on the two step-relayed COSY spectrum are the H-1 and H-2 atom resonances of GlcNAc II' unit, at δ 4.50 and 3.78, respectively, as it was previously well observed for compound *Bv* **22B**.

3.4. Miscellaneous structures

This series of compounds is composed of glycans previously described, such as *Bb* **5**, *Bv* **3**, *Bv* **10** [17] or resulting from peeling reaction, such as *Bv* **9**, **15C** and **16**.

4. Discussion

This study was initiated in order to confirm the species-specificity of the O-linked glycans released from the mucins of lower vertebrates, and particularly Amphibia. The two closely related species, *Bombina bombina* and *B. variegata*, possess very similar glycan structures. The GlcNAcβ1-3(Fucα1-4)GlcNAcβ1-6 sequence, previously identified as a minor structure in *Rana utricularia* [11] and *Rana ridibunda* [12], is the major element of *Bombina*. The GlcNAc(α1-4)Gal(β1,4)Gal(β1,3) sequence is also characteristic of the two species. The most surprising observation was the presence of α2,3-linked Kdn in *B. bombina* mucins, a sugar which was until now considered as a marker of Urodeles (*Pleurodeles waltl* [13], *Ambystom maculatum* [6], *Ambystoma mexicanum* [14], *Ambystoma tigrinum* [7]). Moreover, the mucins of the second species, *B. variegata*, are devoid of Kdn, and replaced with either NeuGc (major sialic acid) or NeuAc (minor). The biosynthetic pathway of Kdn is very similar to that of NeuAc, though Kdn-9-P synthase and NeuAc-9-P synthase are generally considered to be two different enzymes [19]. Nevertheless, it has been recently shown that the human NeuAc-9-P synthase uses ManNAc-6-P and Man-6-P as substrates to generate the phosphorylated forms of NeuAc and Kdn [20], while the mouse enzyme cannot catalyze the synthesis of Kdn or Kdn-9-P from Man or Man-6-P and PEP [21]. Here we show that a change of sialic acid phenotype accompanies the speciation which led to the formation of the two species *B. bombina* and *B. variegata*. Although the enzymes involved in the biosynthesis of Kdn are not as well characterized, it can be suggested that at least one step of the biosynthetic pathway of NeuAc has been disrupted, leading to the *Bombina bombina* oviducal NeuAc-9-P synthase utilizing Man-6-P as a substrate. Further biochemical and/or genetic investigations will attempt to compare the respective biosynthetic pathways of Kdn and NeuGc in these two *Bombina* sub-species. Another difference between these two species is the absence of Gal β1,4-linked to the dimer GlcNAc(β1-3)GlcNAc in *B. variegata*, and consequently, the absence of sequence GlcNAc(α1-4)[Fuc(α1-2)]Gal(β1-4)GlcNAc(β1-3)[Fuc(α1-4)]GlcNAc(β1-6) in this species.

As expected, the glycan chains extracted from these two oviducal mucins are novel, and can be considered as pheno-

typic markers of the two species. Such species-specificity was observed in all amphibian species studied up to now, and the diversity in glycan structures reflects the evolution of the biosynthetic machinery in the toads.

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