Structure of three Kdn-containing oligosaccharide-alditols released from oviducal secretions of *Ambystoma tigrinum*: characterization of the carbohydrate sequence Fuc (α1–5) [Fuc (α1–4)] Kdn (α2–3/6)

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Abstract Oligosaccharide-alditols released by reductive β-elimination from the egg jelly coat of *Ambystoma tigrinum* were analyzed by ^{1}H NMR spectroscopy. As observed for five other amphibian species, these carbohydrate chains are highly species-specific, and should support the species-specificity of gamete interaction. The carbohydrate chains of *Ambystoma tigrinum* are characterized by the presence of a new type of sequence: Fuc (α1–5) [(Fuc (α1–4)] Kdn (α2–3/6).

Key words: Amphibian; Egg jelly coat; Oligosaccharide; ^{1}H NMR

1. Introduction

The jelly coat surronding amphibian eggs is composed of mucin-type glycoproteins which constitute the first barrier for fertilisating sperm. Studies have shown that they are implicated to play important role in fertilization, involving capacitation of the sperm, induction of the acrosome reaction, block of polyspermy and recognition of homologous species [1–3]. Recent works have demonstrated that the carbohydrate chains of these mucins are highly species-specific [4–6], that should support the observed species-specificity of gamete interaction. The structural analysis of these components can also furnish the basis of a molecular taxonomy. In the present study, we isolated the carbohydrate chains from egg jelly of *Ambystoma tigrinum*, and determined the structure of three of them. Although our results are preliminary, they support the view that jelly components differ from one species to another and constitute putative ligands for gamete recognition.

2. Materials and methods

2.1. Isolation of oligosaccharide-alditols

Eggs from *Ambystoma tigrinum* were obtained from Amphibians of North America, Charles O'Sullivan Co., Nashville, TN.

The jelly coat was lyophilized and the dry material (900 mg) was submitted to alkaline reductive degradation in 50 mM NaOH containing 1 M $NaBH_4$ (100 ml) at 37°C for 48 h. The reaction was stopped by Dowex 50 X 2 (mesh 25–50; H^+ form) and boric acid was co-distilled in the presence of methanol. Neutral and acidic oligosaccharides were further fractionated on Dowex 1 X 2 (mesh 200–400; $HCOO^-$ form). Acidic oligosaccharides were desorbed with 50 mM pyridine-acetate buffer (pH 6.5) and isolated by HPLC on primary-bonded silica (Supelcosyl LC-NH_2, 4.6 mm × 25 cm; Supelco Inc., Bellefonte, USA) using acetonitrile, 30 mM potassium phosphate, pH 5.2, with a flow rate of 1 ml/min. After an isocratic elution (70:30; 1 h) the concentration of buffer was increased up to 40%, for 1 h.

2.2. Analytical method

2.2.1. Methylation analysis. Before methylation analysis, the oligosaccharide-alditols were hydrolyzed with formic acid (pH 2) at 100°C for 1 h, in order to specifically split the Kdn linkage. The resulting material was reduced with $NaBD_4$, and methylated according to Hakomori [7]. Methyl ether derivatives were liberated with methanol/0.5 M HCl (80°C; 20 h) and O-acetylated at room temperature with a mixture of pyridine/acetic anhydride (1:5; 100 μl). Partially methylated acetylated Kdn-ol derivates were analyzed by GLC-MS.

2.2.2. ^{1}H NMR spectroscopy. ^{1}H NMR spectroscopy was performed on a Bruker ASX400 spectrometer. Chemical shifts are expressed in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) but were actually measured by reference to internal acetone (δ = 2.225 ppm in D_2O at 25°C).

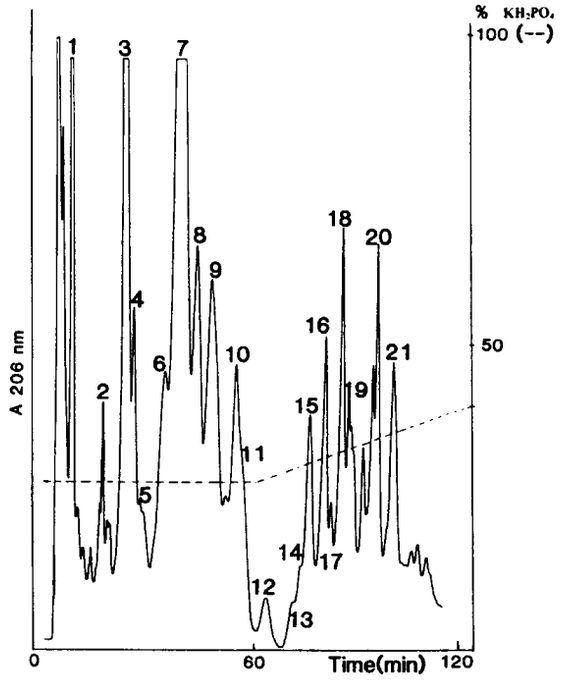

Fig. 1. HPLC separation of oligosaccharide-alditols released from the egg jelly coat of *Ambystoma tigrinum*.

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Abbreviations: Kdn, 3-deoxy-D-glycero-D-galacto-nonulosonic acid; GLC-MS, gas liquid chromatography coupled with mass spectroscopy; HPLC, high performance liquid chromatography.

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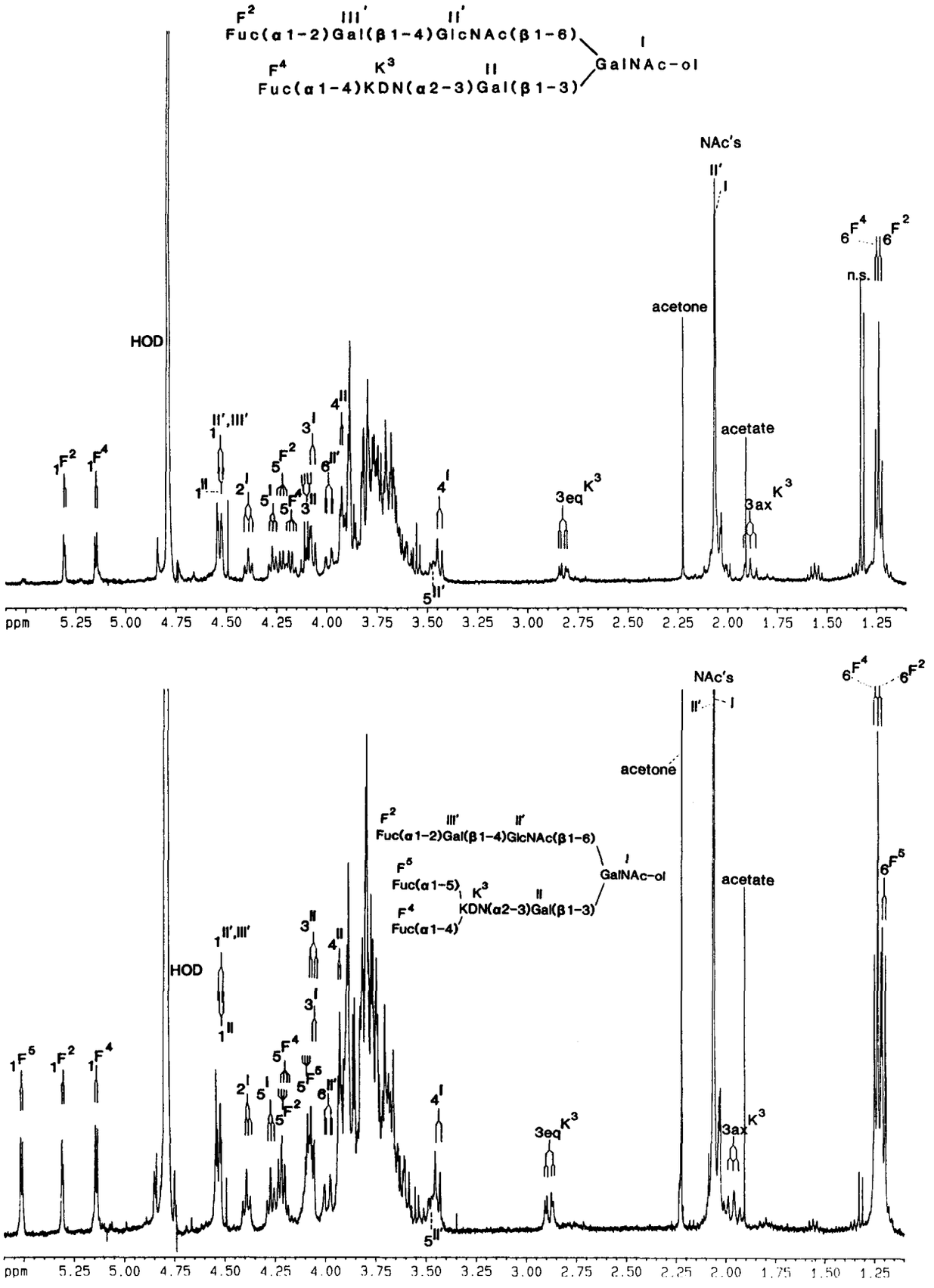
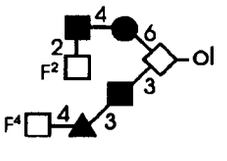
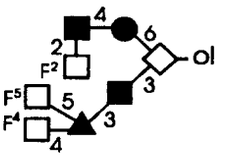
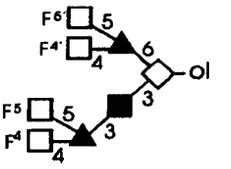


Fig. 2. ^{1}H NMR spectra of oligosaccharide-alditols 16 and 18.

Table 1
^{1}H NMR chemical shifts for oligosaccharide-alditols

		 16	 18	 21
Residue	Reported group	Chemical shifts		
Fuc^2	H-1	5.308	5.308	-
	H-5	4.225	4.225	-
	H-6	1.230	1.228	-
Fuc^4	H-1	5.150	5.140	5.137
	H-5	4.180	4.210	4.211
	H-6	1.247	1.246	1.242
$Fuc^{4'}$	H-1	-	-	5.127
	H-5	-	-	4.175
	H-6	-	-	1.230
$Fuc^{5, 5'}$	H-1	-	5.512	5.508
	H-5	-	4.080	4.080
	H-6	-	1.206	1.205
Gal^3	H-1	4.528	4.529	4.539
	H-2	3.601	3.605	3.603
	H-3	4.091	4.082	1.082
	H-4	3.929	3.932	3.93
Gal^4	H-1	4.535	4.535	-
$GlcNAc^6$	H-1	4.535	4.535	-
	H-5	3.468	3.465	-
	H-6	3.986	3.989	-
	NAc	2.066	2.067	-
Kdn^3	H-3ax	1.885	1.961	1.964
	H-3eq	2.821	2.885	2.878
Kdn^6	H-3ax	-	-	1.826
	H-3eq	-	-	2.833
GalNAc-ol	H-2	4.390	4.392	4.381
	H-3	4.068	4.067	4.056
	H-4	3.438	3.444	3.522
	H-5	4.271	4.272	4.236
	H-6	-	-	3.436
	NAc	2.061	2.063	2.037

Symbols : ◇-ol = GalNAc-ol; ■ = Gal; ● = GlcNAc; □ = Fuc; ▲ = Kdn

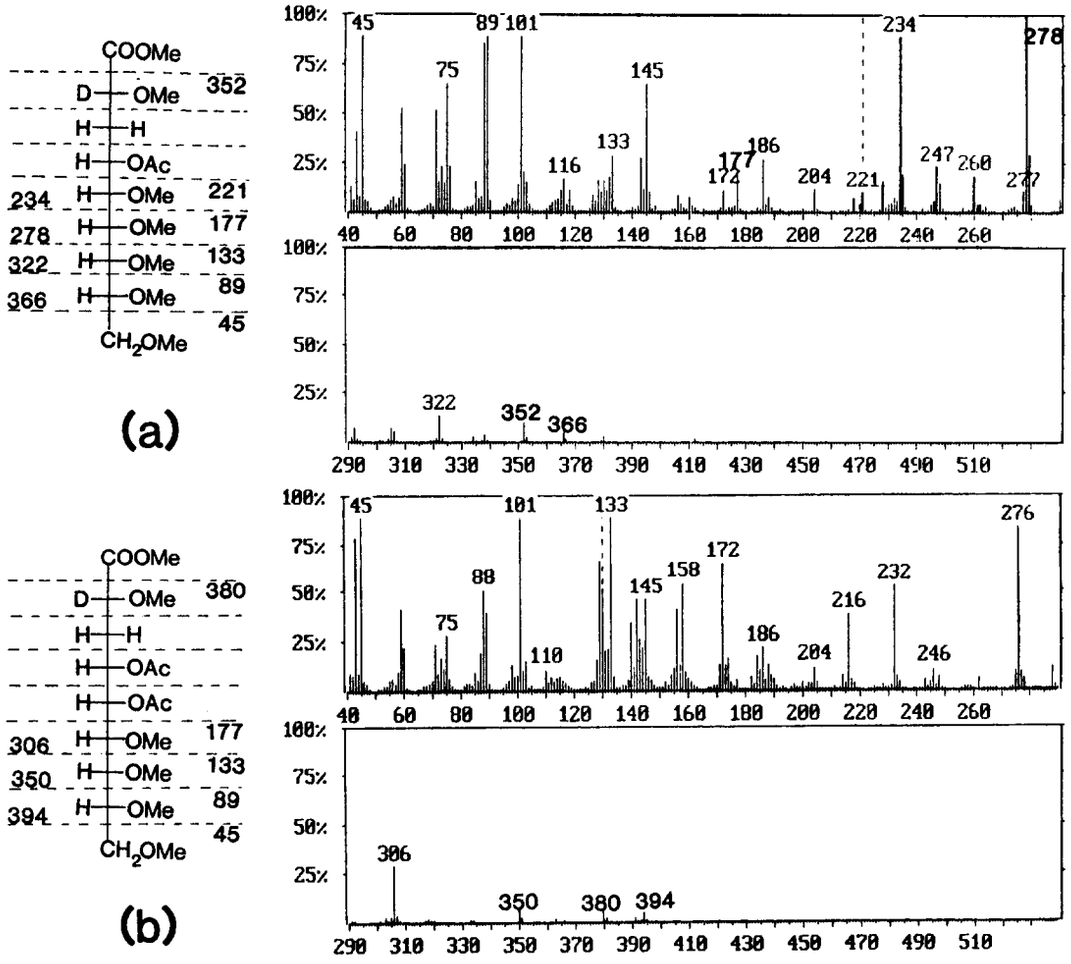


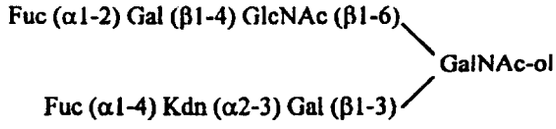
Fig. 3. Mass spectrometry of hexa (a) and pentamethyl (b) derivates of Kdn-ol.

3. Results

The HPLC separation of acidic oligosaccharide-alditols provided 21 fractions (Fig. 1). Some of them were mixtures of several compounds, as further verified by NMR analysis. Nevertheless, three oligosaccharide-alditols (peaks 16, 18 and 21) retained our attention with respect to their unusual and quite new structures.

3.1. Oligosaccharide-alditol 16 (Fig. 2 and Table 1)

The occurence of the GlcNAc (β1–6) [Gal (β1–3)] GalNAc-ol core structure is reflected by the typical GalNAc-ol H-2 and H-5 structural reporter groups signals at δ = 4.390 and 4.271 ppm, respectively [8]. The Fuc (α1–2) Gal (β1–4) GlcNAc sequence is indicated by GlcNAc^6 H-1 at δ = 4.535 ppm, Gal^4 H-1 at δ = 4.535 and Fuc^2 H-1 at δ = 5.308 ppm [9]. The attachment of a sialic acid residue (actually Kdn) at C-3 of Gal^3 induces an upfield shift for Gal^3 H-3, observed at δ = 4.091 ppm. The presence of Kdn was verified according to [4]. The second Fuc residue is attached at C-4 of Kdn, as verified by methylation analysis, which furnishes the 4-mono-*O*-acetyl-2,5,6,7,8,9-hexa-*O*-methyl Kdn-ol (Fig. 3). This observation allows the chemical shifts of the Fuc^4 H-1, H-5 and H-6 structural reporter groups signals to be precisely determined at δ = 5.150, 4.180 and 1.230 ppm, respectively. On the basis of these observation, the sequence of the oligosaccharide-alditol can be established as shown in Scheme 1.



Scheme 1.

3.2. Oligosaccharide-alditol 18 (Fig. 2 and Table 1)

The sequence Fuc (α1–2) Gal (β1–4) GlcNAc (β1–6) [Kdn (α2–3) Gal (β1–3)] GalNAc-ol, is evident from the set of H-1 signals already assigned for compound 16. The presence of an additional Fuc residue is verified by its H-1, H-5 and H-6

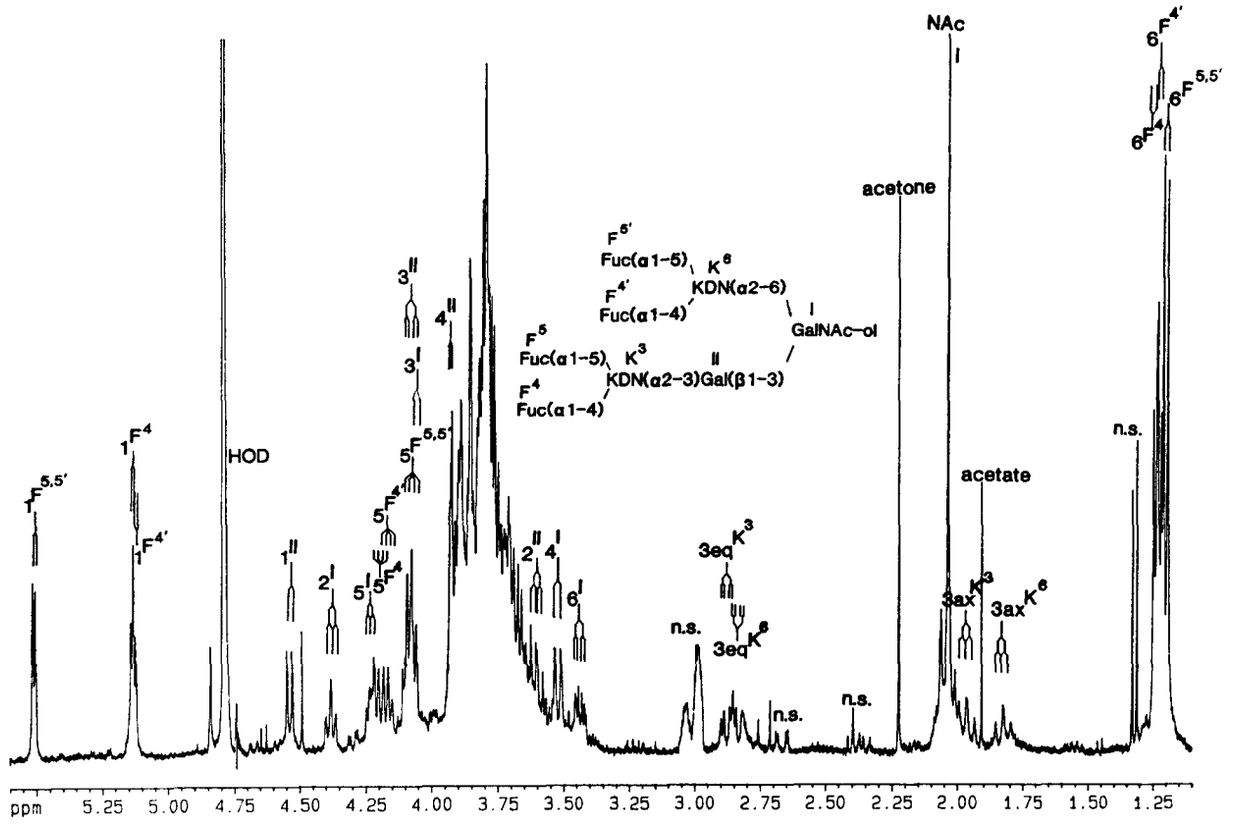
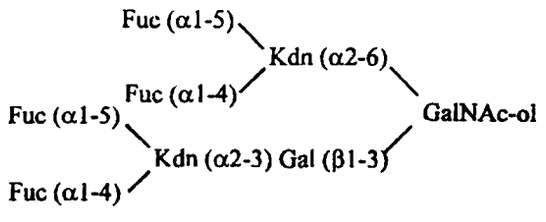


Fig. 4. ^{1}H NMR spectrum of oligosaccharide-alditol 21.

signals at δ = 5.512, 4.080 and 1.206 ppm (Fig. 2). The methylation analysis gives 4,5-di-*O*-acetyl-2,6,7,8,9-penta-*O*-methyl Kdn-ol (Fig. 3), that shows the Kdn unit to be C-4 and C-5 substituted with two Fucose residues. Consequently, this compound can be conceived as an extension of compound 16 with an α1–5-linked Fuc residue attached to Kdn.

3.3. Oligosaccharide-alditol 21 (Fig. 4 and Table 1)

The substitution pattern of GalNAc-ol is clear from the GalNAc-ol H-2, H-5 and H-6′ resonances at δ = 4.381, 4.236 and 3.436 ppm, respectively, which are significant for the core structure Kdn (α2–6) [Gal (β1–3)] GalNAc-ol. The α-2,3 and α-2,6-linked Kdn can be recognized on the basis of their H-3 ax and H-3 eq signals (Table 1). The four fucose units, namely Fuc^4, Fuc$^{4'}$, Fuc^5 and Fuc$^{5'}$, exhibit similar structural reporter groups signals to those observed for compound 18. The methylation analysis also confirms the Kdn unit to be C-4 and C-5 substituted. These results lead to the sequence of oligosaccharide-alditol compound 21 as shown in Scheme 2.



Scheme 2.

4. Discussion

Previous work has shown that the carbohydrate chains of the mucins from amphibian oviducal secretions are highly species-specific. The present findings confirm these observations and establish the structure of three representative glycan chains specific for *Ambystoma tigrinum*.

As observed for other species, the presence of Kdn among amphibians is a general feature. Exceptions are represented by *Bufo japonicus* [6] and *Bufo bufo* (personal results), the mucins of which contain exclusively NeuAc. Another characteristic is the presence of Kdn substituted with Fuc, which implies the existence of amphibian-specific fucosyltransferases. The activities of these new enzymes can be deduced from the diversity of structures depicted in Fig. 5.

If it is confirmed that the egg–sperm interaction is species-specific and is mediated by carbohydrate-type lectin interactions, our structural investigations can provide biochemical support for exploring the fertilization process.

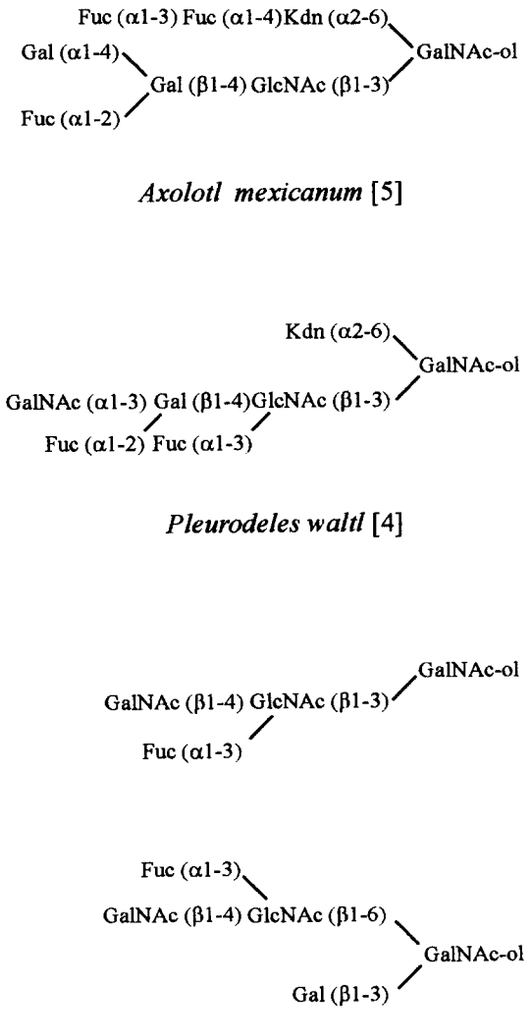


Fig. 5. Structure of oligosaccharides which are characteristic of the egg jelly coat of *Pleurodeles waltl* [4], *Axolotl mexicanum* [5] and *Ambystoma maculatum* [10].

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