¹H- and ¹³C-n.m.r. spectroscopy of 2-oxo-3-deoxy-D-glycero-D-galactononulosonic acid-containing oligosaccharide-alditols bearing Lewis X, Lewis Y and A-Lewis Y determinants isolated from the jelly coat of *Pleurodeles waltl* eggs

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Three acidic oligosaccharide-alditols carrying Lewis X, Lewis Y and A-Lewis Y determinants were isolated from the jelly coat of *Pleurodeles waltl* eggs. These compounds possess the following structures. Gal β 1-4(Fuc α 1-3)GlcNAc β 1-3[2-oxo-3-deoxy-D-glycero-D-galactononulosonic acid (KDN) α 2-6]GalNAc-ol; Fuc α 1-2Gal β 1-4(Fuc α 1-3)GlcNAc β 1-3(KDN α 2-6)GalNAc-ol and Fuc α 1-2(GalNAc α 1-3)Gal β 1-4(Fuc α 1-3)GlcNAc β 1-3(KDN α 2-6)GalNAc-ol. The complete ¹H-n.m.r.-spectrum assignment for the three compounds and the ¹³C-n.m.r. analysis of the A-Lewis Y determinant-containing heptasaccharide are reported.

INTRODUCTION

Previous reports on bird [1] and fish [2,3] mucins have shown that these materials contain easily accessible sources of carbohydrates. In addition, the diversity of their chemical structures allows the prediction of probable diversity in their functions. The jelly coat of amphibian eggs represents an abundant source of sialylated mucins which have not yet been extensively investigated. Recently, we characterized 2-oxo('keto')-3-deoxy-D-glycero-D-galactononulosonic acid (KDN) in the jelly coat of *Pleurodeles waltl* [4]. KDN is a deaminated sialic acid which was first isolated from rainbow trout eggs [2,3] and later obtained from the capsular polysaccharide of *Klebsiella ozaenae* serotype K4 [5]. In a preliminary paper, we described the structure of the carbohydrate units released by reductive β -elimination, followed by the examination of the chemical shift of their structural reporter groups obtained by ¹H n.m.r. spectroscopy [4]. These oligosaccharide chains have been shown to carry Lewis X, Lewis Y and A-Lewis Y determinants and this biological material constitutes an easily available source of these tissue antigens. In the present paper, we report the complete ¹H-n.m.r.-spectrum assignment for the three major KDN-containing oligosaccharide-alditols and the ¹³C-n.m.r. analysis of the heptasaccharide with the A-Lewis Y determinant.

MATERIALS AND METHODS

Eggs from *P. waltl* were obtained from natural spawnings in the aquarium. The jelly coat material was lyophilized and dry material (160 mg) was subjected to alkaline reductive degradation in 50 mM-NaOH containing 1.0 M-NaBH₄ (10 ml) at 37 °C for 24 h. The reaction was stopped by addition of Dowex 50 $\times$ 8 (25–50 mesh; H⁺ form) at 4 °C. The solution was filtered, adjusted to pH 5.5 with 0.1 M-NaOH and then concentrated. Boric acid was distilled as a methyl ester in the presence of methanol. The material was then desalted on a Bio-Gel P-2 column (2 cm $\times$ 50 cm).

Analytical procedures

Oligosaccharide-alditols were isolated by h.p.l.c. on primary-amine-bonded silica (Supelcosyl LC-NH₂ 4.6 mm $\times$ 25 mm; Supelco Inc., Bellefonte, CA, U.S.A.) using a solution of acetonitrile/30 mM-KH₂PO₄ buffer, pH 5.2 (65:35, v/v), at a flow rate of 1 ml/min. Oligosaccharides were detected by u.v. spectroscopy at 206 nm.

Carbohydrate composition was determined according to the method of Zanetta *et al.* [6].

Before ¹H-n.m.r. analysis, the oligosaccharide-alditol fractions were repeatedly exchanged with ²H₂O at p²H 7, and lyophilized. ¹H-N.m.r. spectroscopy was performed on a Bruker AM-400 WB spectrometer. The indicated probe temperature was 27 °C. Chemical shifts (δ) are expressed in p.p.m. down-field from the internal standard, sodium-4,4-dimethyl-4-silapentane-1-sulphonate (DSS), but were actually measured by reference to the internal standard acetone (δ = 2.225 in ²H₂O at 27 °C).

The two-dimensional homonuclear and heteronuclear correlated spectroscopy (COSY) experiments were performed using the standard Bruker pulse library [7].

RESULTS AND DISCUSSION

Characteristics of the crude jelly-coat mucin and the effect of alkaline borohydride treatment

As shown in Table 1, the jelly-coat mucin contains about 50 % carbohydrate material, including 9 % KDN. Oligosaccharides released from the mucin by alkaline borohydride treatment were desalted on a Bio-Gel P-2 column and fractionated on primary-amine-bonded silica [4]. Samples of each component (3–6 mg) were isolated starting from 160 mg of dry mucin. Fraction II-3 was a mixture of two compounds, the structure of which was not investigated (Fig. 1).

N.m.r. analysis of oligosaccharide-alditols

The double-relayed COSY spectra of compounds II-1, II-2 and II-4 and the ¹H/¹³C- heteronuclear COSY spectrum of II-4

Table 1. Carbohydrate composition of the jelly coat of *Pleurodeles waltl* eggs, and of oligosaccharide-alditols released by reductive elimination

Molar ratios were determined on the basis of 1 Gal residue.

Carbohydrate	Crude jelly-coat mucin		Oligosaccharide-alditols (molar ratios)		
	Composition (%)	Molar ratio	II-1	II-2	II-4
Gal	6.6	1.0	1.0	1.0	1.0
GlcNAc	8.4	1.0	0.9	0.9	0.9
GalNAc	20.2	2.5	—	—	0.9
GalNAc-ol	—	—	0.9	0.9	1.0
Fuc	7.0	1.14	1.1	2.1	2.0
KDN	9.4	1.0	0.9	0.9	1.1
Total	51.6

Fig. 1. Fractionation of oligosaccharide-alditols on a Supelcosyl LC-NH₂ column **Fig. 2.** Double-relayed COSY spectrum of compound II-1

The arabic numeral indicates the position of the ¹H in the ring of the monosaccharide given with a roman numeration. F indicates a fucose residue with the linking locant indicated as a superscript.

are depicted in Figs. 2–5. For oligosaccharide-alditols II-1 and II-2, the primary structure has been established by comparing their ¹H-n.m.r. spectra with those of reference compounds bearing Lewis X and Lewis Y determinants [8,9].

Fig. 3. Double-relayed COSY spectrum of compound II-2

See legend to Fig. 2.

Fig. 4. Double-relayed COSY spectrum of compound II-4

See legend to Fig. 2.

The ^1H -n.m.r. spectrum of the compound II-1, together with the results of the sugar analysis, indicates that the component is a pentasaccharide-alditol containing Gal, GlcNAc, KDN and GalNAc-ol.

The chemical-shift values (Table 2) for H-6 and H-6' (δ 3.814 and 3.474) and H-5 (δ 4.161) of GalNAc-ol are indicative of the core structure GlcNAc β 1-3[sialic acid(Sia) α 2-6]GalNAc-ol [8,9]. The structural-reporter-group resonances of Fuc, namely H-1, H-5 and H-6, are identical to those assigned for the Gal β 1-4(Fuc α 1-3)GlcNAc(β 1-) structural element that was previously described [8]. The double-relayed COSY spectrum of II-1 furnished most of the ^1H assignments, except for the H-7 to H-9 resonances of KDN, which are included between 3.8 and 3.9 p.p.m. (as confirmed on the ^1H - and ^{13}C -COSY spectrum of II-4).

In the same way, the structural determination of compound II-2 was made by comparison of its spectrum with that of the oligosaccharide Fuc α 1-2Gal β 1-4(Fuc α 1-3)GlcNAc β 1-3(NeuAc α 2-6)GalNAc-ol [9].

Indeed, the ^1H resonances of the Lewis sequence, which is common to KDN- and NeuAc-containing oligosaccharides, are identical. The double-relayed COSY spectrum of II-2 allowed the assignment of the ^1H resonances, except for the GlcNAc

residue for which a strong virtual coupling between the H-1 and H-3 atoms abolishes the appearance of cross-peaks on the two-dimensional spectrum.

For compound II-4, the quantity of material (6 mg) was sufficient for recording a heteronuclear COSY spectrum (Fig. 5), which allowed the assignment of all the proton and carbon resonances. The additional GalNAc residue was identified on the basis of the C-2 resonance observed at 50.92 p.p.m., and the characteristic low $J_{4,5}$ coupling constant. As for II-2, the strong virtual coupling of GlcNAc H-1 with its H-3 prevented the observation of corresponding cross-peaks on the homonuclear and heteronuclear COSY spectra. The C-1 and C-2 resonances were visible on the one-dimensional ^{13}C n.m.r. and the C-1/H-1 and C-2/H-2 cross-peaks were hardly detected by decreasing the height of the contour plot against the limit of the background. Nevertheless, the C-3/H-3 cross-peak remained definitively undetectable.

Analysis of the ^{13}C -n.m.r. spectrum of II-4 confirms the attachment of GalNAc to the C-3 atom of Gal III, owing to the observation of the Gal III C-3 resonance which is down-field shifted at 77.27 p.p.m. The up-field shifted value (63.87 p.p.m.) observed for the Gal III C-4 resonance is also characteristic for the GalNAc α 1-3Gal sequence [10]. The comparison of the ^{13}C -

n.m.r. spectrum of (α 2-6)-linked KDN with (α 2-6)-linked NeuAc residue [1] essentially shows a down-field effect of +18.06 p.p.m. for C-5, +2.24 p.p.m. for C-4 and +1.0 p.p.m. for C-6, while the other carbon resonances are not affected ($\Delta\delta < 0.4$ p.p.m.) by deamination (Table 3).

The present study furnished the complete ^1H -n.m.r. assignment of KDN-containing oligosaccharides bearing Lewis X, Lewis Y and A-Lewis Y antigen determinants. 'KDNology' [11] is a new

interesting chapter of glycobiology. Indeed, oligo(KDN) glycan units have been recognized to be involved in the mechanisms of egg-sperm interaction [12]. KDN, which occurs at the non-reducing terminus in the O-linked oligosaccharides of salmon egg mucins, has been postulated also to protect eggs from bacterial invasion and from degradation by exosialidases [2]. It would be too premature to conclude from our results that a similar role exists for KDN in *Pleurodeles*. Moreover, in

Table 2. ^1H -n.m.r. chemical shifts for compounds II-1, II-2 and II-4

Abbreviation: n.d., not determined.

Compound	Chemical shift (p.p.m.)									
	H-1	H-2	H-3	H-4	H-5	H-6	H-7	H-8	H-9	CH ₃
II-1										
KDN α 2-6	—	—	1.652(ax) 2.676(eq)	3.603	3.524	3.660	n.d.	n.d.	n.d.	—
Gal β 1-4	4.444	3.494	3.660	3.902	3.62	3.77 3.72	—	—	—	—
Fuc α 1-3	5.132	3.700	3.902	3.792	4.818	1.176	—	—	—	—
GlcNAc β 1-3	4.645	3.981	3.897	4.950	3.603	4.020 3.858	—	—	—	2.066
GalNAc-ol	3.612 3.586	4.244	3.978	3.634	4.161	3.814 3.474	—	—	—	2.023
II-2										
KDN α 2-6	—	—	1.655(ax) 2.675(eq)	3.603	3.524	3.669	n.d.	n.d.	n.d.	—
Fuc α 1-2	5.276	3.805	3.774	3.836	4.256	1.273	—	—	—	—
Gal β 1-4	4.491	3.651	3.858	3.871	3.61	3.77 3.72	—	—	—	—
Fuc α 1-3	5.113	3.699	3.915	3.810	4.871	1.236	—	—	—	—
GlcNAc β 1-3	4.631	3.96	n.d.	n.d.	3.467	4.034 3.853	—	—	—	2.065
GalNAc-ol	3.630 3.568	4.247	3.971	3.599	4.184	3.814 3.474	—	—	—	2.024
II-4										
KDN α 2-6	—	—	1.654 2.674	3.599	3.520	3.664	3.858	3.893	3.92 3.83	—
GalNAc α 1-3	5.198	4.242	3.924	3.998	4.227	3.757	—	—	—	2.035
Fuc α 1-2	5.297	3.783	3.713	3.849	4.336	1.303	—	—	—	—
Gal β 1-4	4.552	3.871	3.941	4.200	3.577	3.75 3.70	—	—	—	—
Fuc α 1-3	5.128	3.699	3.915	3.788	4.871	1.274	—	—	—	—
GlcNAc β 1-3	4.634	3.96	n.d.	3.937	3.467	4.053 3.858	—	—	—	2.066
GalNAc-ol	3.625 3.555	4.246	3.970	3.599	4.180	3.810 3.474	—	—	—	2.024

Table 3. ^{13}C -n.m.r. chemical shifts for compound II-4

	Chemical shift (p.p.m.)										
	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	CO	CH ₃
KDN α 2-6	175.07 (174.87)*	101.76 (101.69)	41.05 (41.50)	71.54 (69.30)	71.27 (53.21)	74.88 (73.86)	69.25 (69.54)	73.39 (73.00)	64.08 (63.67)	—	—
GalNAc α 1-3	92.73	50.92	70.48	69.86	72.32	62.56	—	—	—	175.72	23.31
Fuc α 1-2	100.18	69.10	71.34	73.08	68.26	16.92	—	—	—	—	—
Gal β 1-4	101.53	74.16	77.27	63.87	76.05	62.69	—	—	—	—	—
Fuc α 1-3	99.83	69.10	69.25	73.39	68.19	16.74	—	—	—	—	—
GlcNAc β 1-3	103.06	57.83	n.d.	75.19	76.43	61.46	—	—	—	176.23	23.70
GalNAc-ol	61.41	52.77	77.84	70.81	69.39	66.77	—	—	—	175.07	23.41

* Values for NeuAc(α 2-6) [1].

Fig. 5. Heteronuclear ^1H - ^{13}C -COSY spectrum of compound II-4

Pleurodeles, KDN is the only Sia-derivative that is present, suggesting that it may have a similar function to that of Sia. In that case, one has to characterize the specific enzyme able to

hydrolyse the KDN linkages, since KDN has been shown to be resistant to sialidase action [13].

This research was supported in part by Firma Merck, Preclinical Research and Pharmaceutical Branch, Darmstadt (Director: Dr. K. Irmscher) and by IGODA, Barcelona (Director: Dr. J. Piulats), by the Centre National de la Recherche Scientifique (Unité Mixte no. 111: Relations structure-fonction des constituants membranaires; Director: Professor A. Verbert), by the University des Sciences et Technologies de Lille, and by the Ministère de l'Éducation Nationale. We are grateful to the Conseil Régional du Nord Pas-de-Calais, the Centre National de la Recherche Scientifique, the Ministère de la Recherche et de l'Enseignement Supérieur, the Ministère de l'Éducation Nationale and the Association pour la Recherche sur le Cancer for their contributions to the acquisition of the 400 MHz n.m.r. spectrometer. We are indebted to Miss Catherine Alonso (CNRS technician) for her skillful technical assistance.

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Received 23 December 1991/23 April 1992; accepted 28 April 1992