Carbohydrate Research, 185 (1989) 1–13
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

COMPLETE ^{1}H- AND ^{13}C-N.M.R. ASSIGNMENTS FOR TWO SULPHATED OLIGOSACCHARIDE ALDITOLS OF HEN OVOMUCIN*

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(Received September 10th, 1987; accepted for publication in revised form, March 8th, 1988)

ABSTRACT

The complete ^{1}H- and ^{13}C-n.m.r. assignments for β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-6-SO_3H-(1→6)-[β-D-Gal*p*-(1→3)]-D-GalNAcol and α-NeuAc*p*-(2→3)-β-D-Gal*p*-(1→3)-[β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-6-SO_3H-(1→6)]-D-GalNAcol were made by a combination of 2-D correlation experiments (Relayed-Cosy; and ^{13}C,^{1}H Correlation-shift n.m.r. spectroscopy), and 1-D n.m.r. spectroscopy. The results illustrate the ability of these methods to locate sulphate and NeuAc groups in anionic mucinous glycoproteins.

INTRODUCTION

Although sulphated mucous glycoproteins represent an important class of biological components, involved particularly in the protection of the tracheo-bronchial tractus[1,2], only few informations are available on the primary structures of oligosaccharides containing sulphate esters[3]. This is essentially due to the low quantities of secreted mucous glycoproteins available and to the difficulties encountered in fractionating the anionic oligosaccharide alditols released by reductive β-elimination. In contrast hen ovomucin represents an important source of sulphated carbohydrates, thus giving the possibility to apply n.m.r. procedures requiring large amounts of material. This was the reason for the choice of this particular O-glycoprotein.

EXPERIMENTAL

The oligosaccharide alditols isolated from hen ovomucin have been previously described by Strecker *et al.*[4]. The 400-MHz ^{1}H-n.m.r. experiments were performed with a Bruker AM-400 spectrometer, equipped with a 5-mm ^{1}H–^{13}C mixt-probe head, operating in the pulsed F.t. mode and controlled by an Aspect 3000 computer (Centre Commun de Mesures, Université de Lille Flandres-Artois).

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*This work was supported by grants from the Université des Sciences et Techniques de Lille Flandres-Artois, by the C.N.R.S., and by the Ministère de l'Éducation Nationale.

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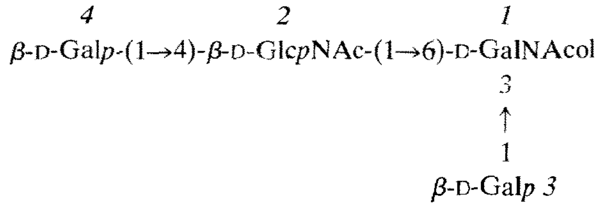
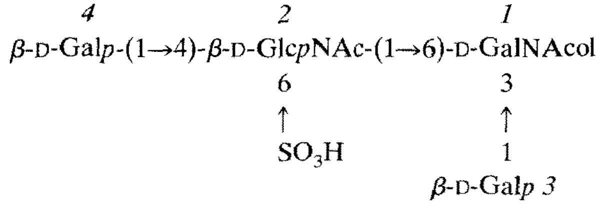
The concentrations of oligosaccharide alditols **1**, **2**, and **3** were respectively 16, 36, and 80 mg/mL. After three exchanges with D_2O (99.96% atoms D, Aldrich) and intermediate lyophilisations, the products were analysed at a spectral width of 3000 Hz for a 16 K frequency-domain points and time-domain data points giving a final digital resolution of 0.365 Hz/point. The 100-MHz ^{13}C-n.m.r. experiments were obtained with the standard Bruker pulse program Powgate with ^{1}H decoupling. The spectral width was 25 000 Hz for 32 K frequency-domain points and time-domain data points giving a final digital resolution of 1.526 Hz/point; a thirty degree pulse (2 μs) and a 0.5-s recycle delay were used. The chemical shifts are given relative to the signal of sodium 4,4-dimethyl-4-silapentane-1-sulphonate, but were actually measured relative to the signal of the methyl group of acetone (δ 2.225 for ^{1}H, and δ 31.40 for ^{13}C).

The 2D-homonuclear Cosy 45 experiments were performed by use of the standard Bruker pulse program Cosy. In these experiments, the spectral width was 1600 Hz, the ^{1}H ninety-degree pulse was 10.6 μs, and 128 W × 2 K data matrix were acquired which were zerofilled prior to F.t. to obtain a 1 × 2 K spectral data matrix; a sine-bell squared fonction was used in both dimensions.



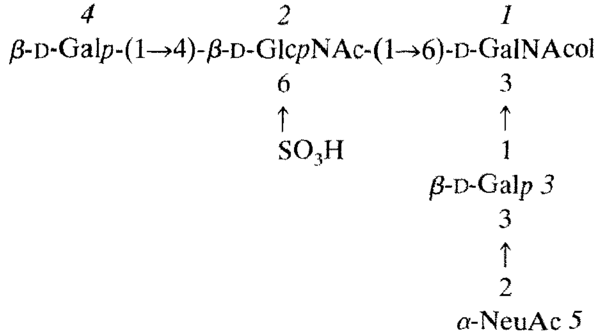
$$\begin{array}{ccc} \mathit{4} & \mathit{2} & \mathit{1}\\ \beta\text{-D-Gal}p\text{-}(1\rightarrow4)\text{-} & \beta\text{-D-Glc}p\text{NAc-}(1\rightarrow6)\text{-} & \text{D-GalNAcol}\\ & & 3\\ & & \uparrow\\ & & 1\\ & & \beta\text{-D-Gal}p\ \mathit{3} \end{array}$$

1



$$\begin{array}{ccc} \mathit{4} & \mathit{2} & \mathit{1}\\ \beta\text{-D-Gal}p\text{-}(1\rightarrow4)\text{-} & \beta\text{-D-Glc}p\text{NAc-}(1\rightarrow6)\text{-} & \text{D-GalNAcol}\\ & 6 & 3\\ & \uparrow & \uparrow\\ & SO_3H & 1\\ & & \beta\text{-D-Gal}p\ \mathit{3} \end{array}$$

2



$$\begin{array}{ccc} \mathit{4} & \mathit{2} & \mathit{1}\\ \beta\text{-D-Gal}p\text{-}(1\rightarrow4)\text{-} & \beta\text{-D-Glc}p\text{NAc-}(1\rightarrow6)\text{-} & \text{D-GalNAcol}\\ & 6 & 3\\ & \uparrow & \uparrow\\ & SO_3H & 1\\ & & \beta\text{-D-Gal}p\ \mathit{3}\\ & & 3\\ & & \uparrow\\ & & 2\\ & & \alpha\text{-NeuAc}\ 5 \end{array}$$

3

The 2D-homonuclear Cosy with simple, double, triple, and quadruple relay transfers were performed by use of the standard Bruker pulse program Cosyrct, and the pulse programs Cosydr, Cosytr, and Cosyqr[5]. For example, the Cosyqr experiment (Version 1986) was performed with sequence, $D_1 - 90 - D\phi - 90 - D_2 - 180 - D_2 - 90 - D_3 - 180 - D_3 - 90 - D_4 - 180 - D_4 - 90 - D_5 - 180 - D_5 - 90 -$ FID, where $D_1 = 1$ s, 90 and 180 = 90 and 180° ^{1}H-pulse (90° = 10.6 μs), $D\phi$ = incremental delay (initial = 3 μs), and $D_2 = D_3 = D_4 = D_5 = 30$ ms as a good compromise. In all these experiments, for a spectral width of 1600 Hz, a 256 W × 2 K data matrix was obtained, which was zerofilled to 1 K × 2 K prior to F.t.; a sine-bell squared fonction was used in both dimensions.

The 2D–^{13}C–^{1}H Cosy experiment was performed with simultaneous suppression of ^{1}H-homonuclear couplings[6,7] by use of the standard Bruker pulse program Xhcorrd. In this experiment, the phase cycling of the refocusing pulse described by Wilde and Bolton[8] was used in addition. Refocusing delays were adjusted[9] to an average $^1J_{C,H}$ coupling constant of 150 Hz. ^{1}H and ^{13}C – 90° pulse widths were 10.6 and 6 μs, respectively. A 128 W × 4 K data matrix was acquired which was zerofilled prior to F.t. to obtain a 512 W × 2 K spectral data matrix. An exponential function (LB = 4 Hz) for ^{13}C-subspectra and a sine-bell function for ^{1}H subspectra were applied to enhance the signal/noise ratio.

RESULTS AND DISCUSSION

Figure 1 shows the monodimensional ^{1}H-n.m.r. spectrum of the tetrasaccharide alditol OL-1 (**1**). The signal assignments of the main structural reporter groups have been previously established by van Halbeck *et al.*[10,11], who particularly discriminated the *N*-acetyl methyl protons of GalNAcol from those of GlcNAc. Based on these data and connectivities in the 2D-Cosy and 2D-Relayed Cosy spectra, the assignments could be made, with the exception of H-5,6 Gal^3 and Gal^4 (Table I). The fully decoupled ^{13}C-n.m.r. spectrum of **1**, analysed by comparison with the ^{13}C-Dept and the 2D–^{13}C–^{1}H Cosy spectra, permitted the identification of C-1,6 of GalNAcol and Gal C-6, and C-6 of GlcNAc (Table II). C-5 of Gal^3 and Gal^4 were assigned by comparing the ^{13}C-n.m.r. spectra of OL-1 (**1**) and OL-2 (**2**). In **2** (see below), one of the C-5 signals was shifted upfield, and this decrease was attributed to the attachment of a sulphate group at C-6 of the adjacent GlcNAc residue. C-5 of Gal^3 was considered to be not affected by the addition of the sulphate group, and consequently C-5 of Gal^3 and Gal^4 were found to resonate at δ 76.14 and 76.51, respectively. These assignments are in agreement with the conclusion of Bush *et al.*[12], who based their reasoning on the chemical shift analogies with similar oligosaccharide alditols reported by Shaskhow *et al.*[13]. Except for the new value that we determined for C-6 of GlcNAc at δ 61.28, all the parameters given in Table II are in agreement with those previously reported[12]. In addition, the 2D–^{13}C–^{1}H Cosy spectrum allowed the assignment of H-5,6 of Gal with an accuracy of 0.01 p.p.m.

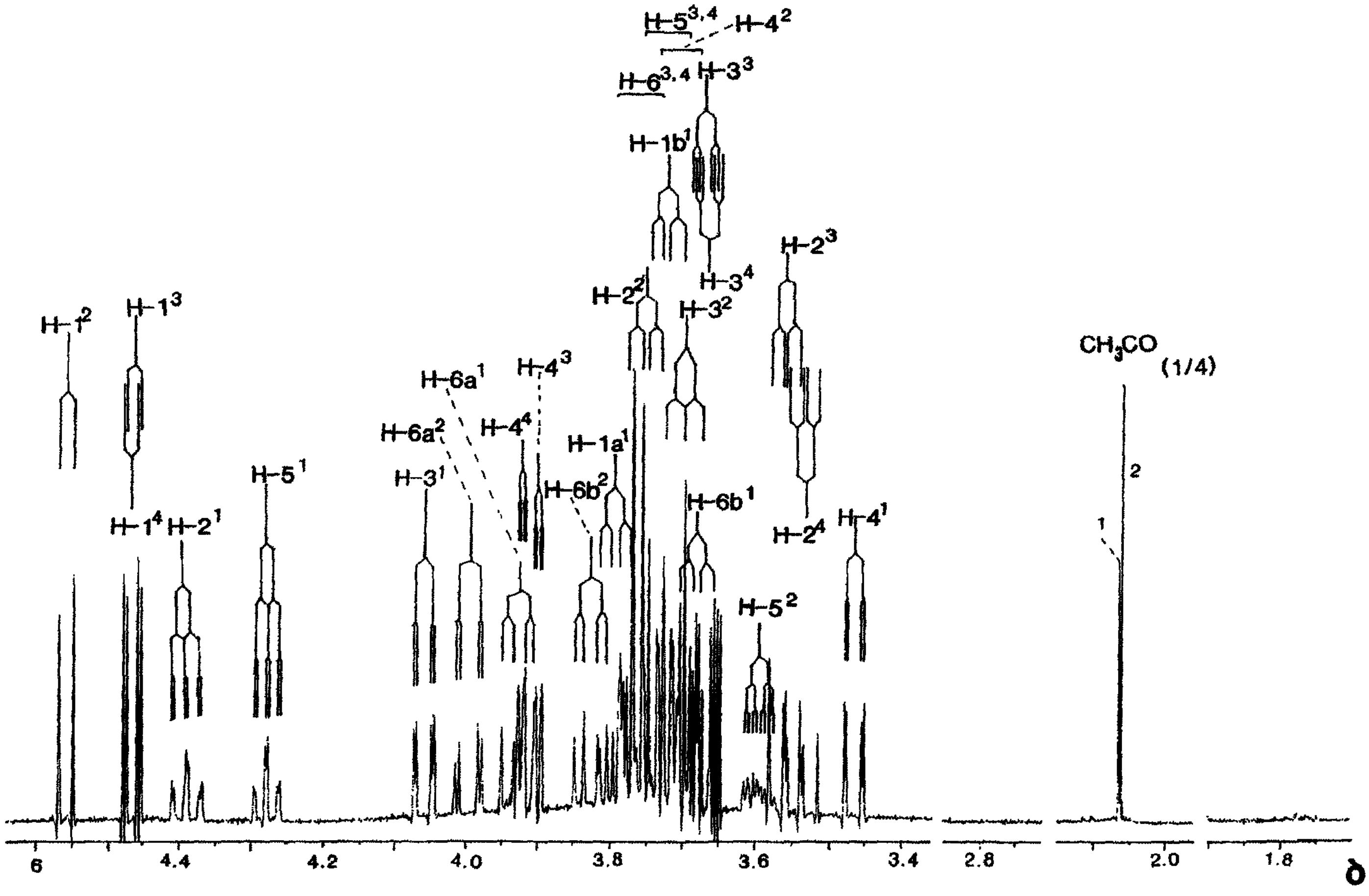


Fig. 1. 400-MHz ^{1}H-n.m.r. spectrum for oligosaccharide alditol **1**.

TABLE I

^{1}H-N.M.R. CHEMICAL SHIFTS AND COUPLING CONSTANTS FOR OLIGOSACCHARIDE ALDITOLS FROM HEN OVOMUCIN

	Chemical shifts (δ)												
	Gal^3	*Gal*^{4}	*GlcNAc*	*GalNAcol*	*Gal*^{3}	*Gal*^{4}	*GlcNAc*	*GalNAcol*	*Gal*^{3}	*Gal*^{4}	*GlcNAc*	*NeuAc*	*GalNAcol*
	1				**2**				**3**				
H-1	4.464	4.469	4.560	3.791	4.462	4.538	4.583	3.793	4.527	4.537	4.581		3.817
H-1′				3.728				3.74[a]					3.747
H-2	3.560	3.538	3.751	4.390	3.571	3.555	3.783	4.385	3.609	3.528	3.756		4.384
H-3	3.674	3.669	3.698	4.060	3.667	3.677	3.693	4.058	4.111	3.675	3.692		4.066
H-3*a*												1.801	
H-3*e*												2.774	
H-4	3.901	3.925	3.700	3.466	3.898	3.927	3.73[a]	3.471	3.925	3.925	3.73	3.70[a]	3.437
H-5	3.72[a]	3.72[a]	3.594	4.280	3.72[a]	3.72[a]	3.78[a]	4.271	3.69[a]	3.73[a]	3.79[a]	3.862	4.252
H-6a	3.77	3.77	3.998	3.933	3.78	3.78	4.419	3.943	3.75	3.75	4.412	3.73	3.937
H-6b	3.75	3.75	3.828	3.680	3.75	3.75	4.349	3.674	3.75	3.75	4.373		3.665
H-7												3.61	
H-8												3.89	
H-9a												3.62	
H-9b												3.57	
CH_3CO			2.064	2.066			2.065	2.065			2.064	2.032	2.066
	Coupling constants (Hz)												
$J_{1,1'}$				−11.2				−11.2					−11.3
$J_{1,2}$	7.6	7.8	8.2	7.0	7.7	7.8	8.2	6.9	7.7	7.8	8.2		6.9
$J_{1',2}$				8.0				8.0					7.9
$J_{2,3}$	9.6	9.6	10.5	1.4	9.8	9.7	10.5	1.6	9.7	9.7	10.5		1.5
$J_{3a,3e}$												−12.4	
$J_{3a,4}$												12.1	
$J_{3e,4}$												4.8	
$J_{3,4}$	3.4	3.4	[b]	9.6	3.4	3.4	[b]	9.6	3.4	3.4	[b]		9.7
$J_{4,5}$	1	1	7.4	1.3	1	1	[b]	1.2	1	1	[b]	9.9	1.3
$J_{5,6a}$	[b]	[b]	2.4	4.9	[b]	[b]	2.3	4.9	[b]	[b]	2.4	10.0	4.9
$J_{5,6b}$	[b]	[b]	5.1	7.4	[b]	[b]	[b]	7.5	[b]	[b]	[b]		7.4
$J_{6a,6b}$			−12.0	−10.4			−10.4	−10.5			−10.4		−10.5

[a] Error: ±0.01 p.p.m. [b] Not determined.

TABLE II

THE CHEMICAL SHIFT (δ) FOR THE CARBON ATOMS OF THE OLIGOSACCHARIDE ALDITOLS FROM HEN OVOMUCIN

Compound	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*C-7*	*C-8*	*C-9*	*CO*	*CH_3CO*
1											
β-D-Gal*p*-(1→3)	105.04	72.27	73.67	69.92	76.14	62.19					
β-D-Gal*p*-(1→4)	104.03	72.13	73.67	69.71	76.51	62.19					
β-D-Glc*p*NAc-(1→6)	102.74	56.14	73.67	79.66	75.90	61.28				175.73	23.53
D-GalNAcol	61.70	52.60	77.17	71.85	69.59	68.54				175.61	23.12
2											
β-D-Gal*p*-(1→3)	104.98	72.24	73.65	69.60	76.11	62.17					
β-D-Gal*p*-(1→4)	103.66	72.14	73.65	69.77	72.00	62.17					
β-D-Glc*p*NAc-6-SO_3H-(1→6)	102.80	56.14	73.74	76.49	78.63	67.46				175.69	23.53
D-GalNAcol	61.70	52.59	77.08	69.98	68.62	73.56				175.58	23.12
3											
α-NeuAc-(2→3)	174.81	100.83	40.94	69.37	52.83	74.08	69.24	72.94	63.73	176.11	23.21
β-D-Gal*p*-(1→3)	104.77	70.53	76.81	68.43	75.97	62.16					
β-D-Gal*p*-(1→4)	103.62	72.12	73.50	69.75	71.41	62.16					
β-D-Glc*p*NAc-6-SO_3H-(1→6)	102.77	56.07	73.71	76.46	78.50	67.40				175.69	23.58
D-GalNAcol	61.69	52.57	77.05	69.69	68.25	73.62				175.53	23.13

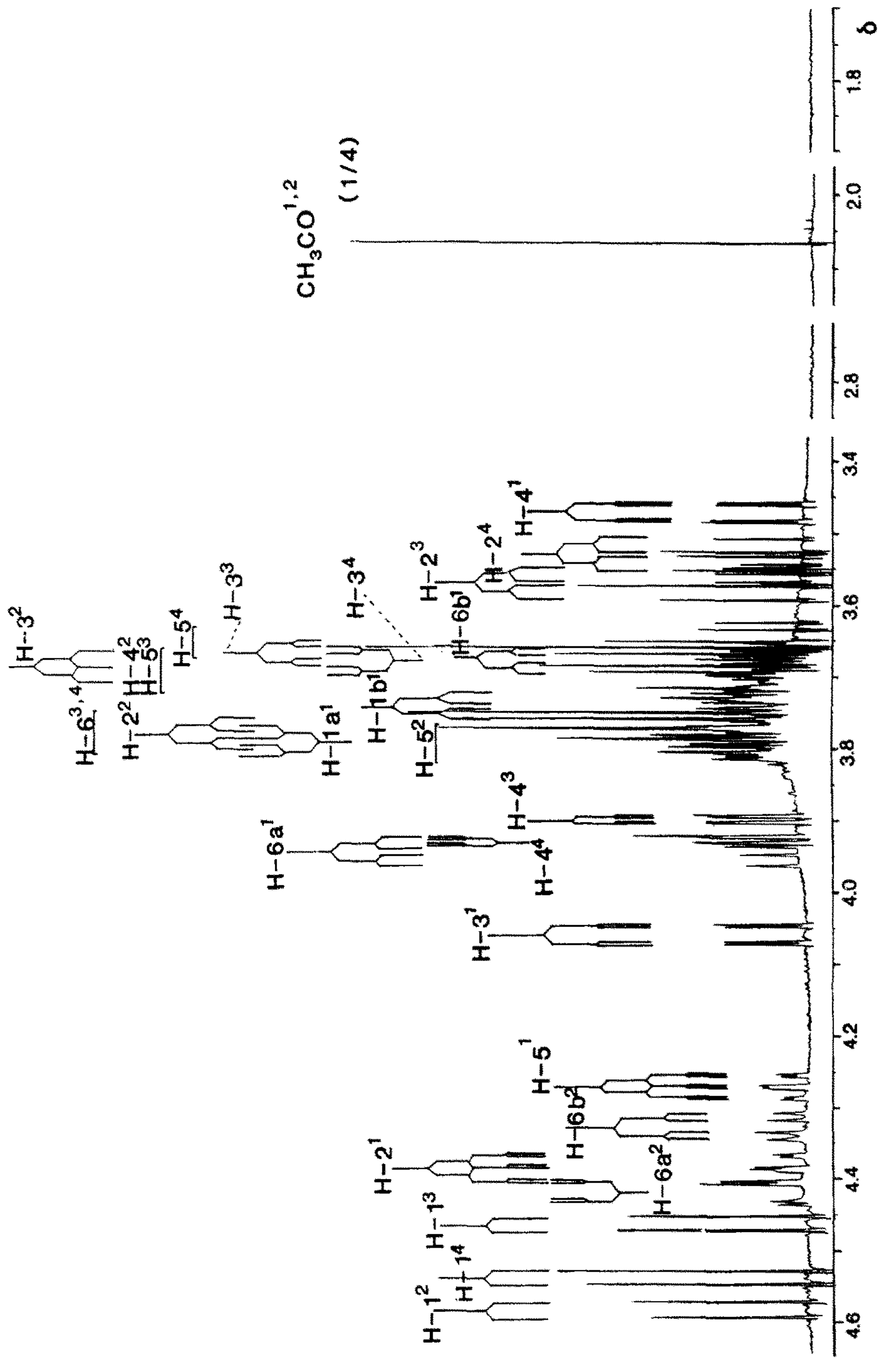


Fig. 2. 400-MHz ^{1}H-n.m.r. spectrum for oligosaccharide alditol **2**.

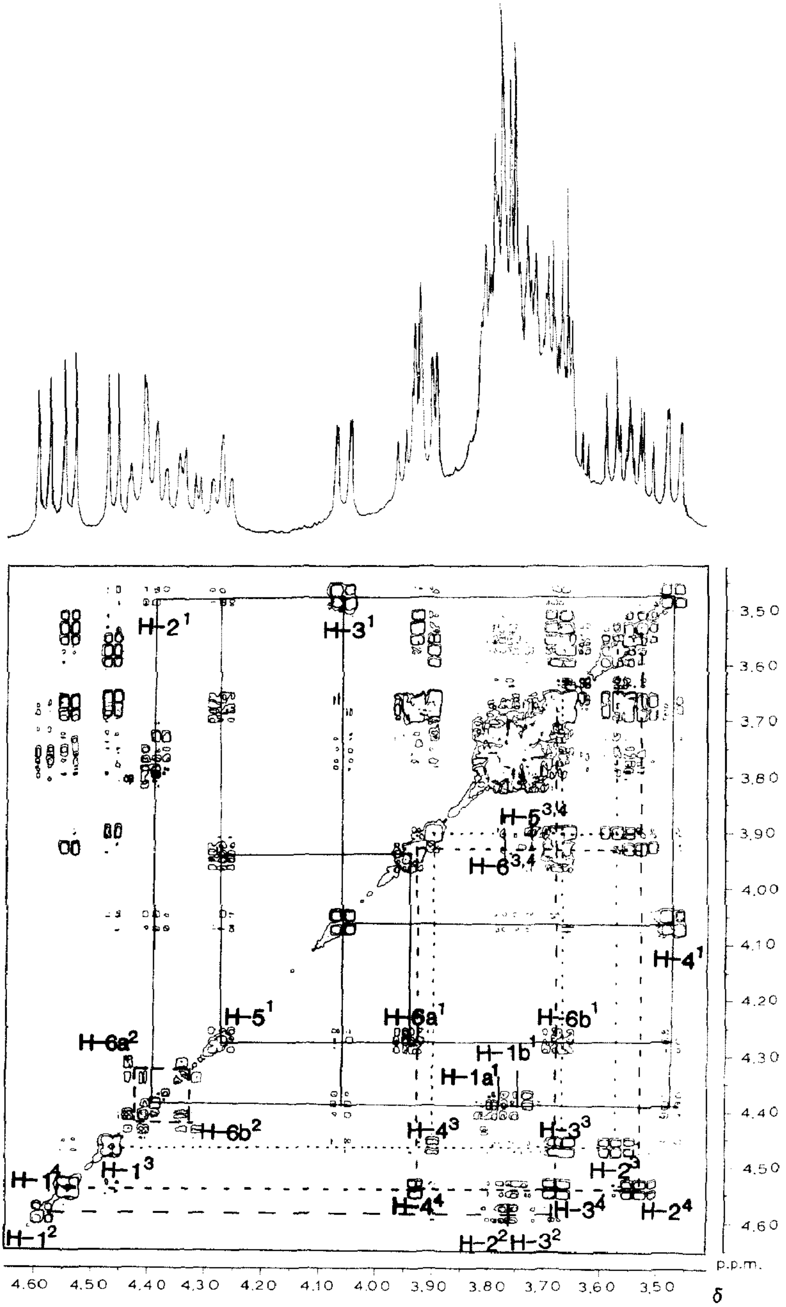


Fig. 3. 2D-Homonuclear Cosy with double-relay transfer (Cosydr) spectrum for oligosaccharide alditol **2**.

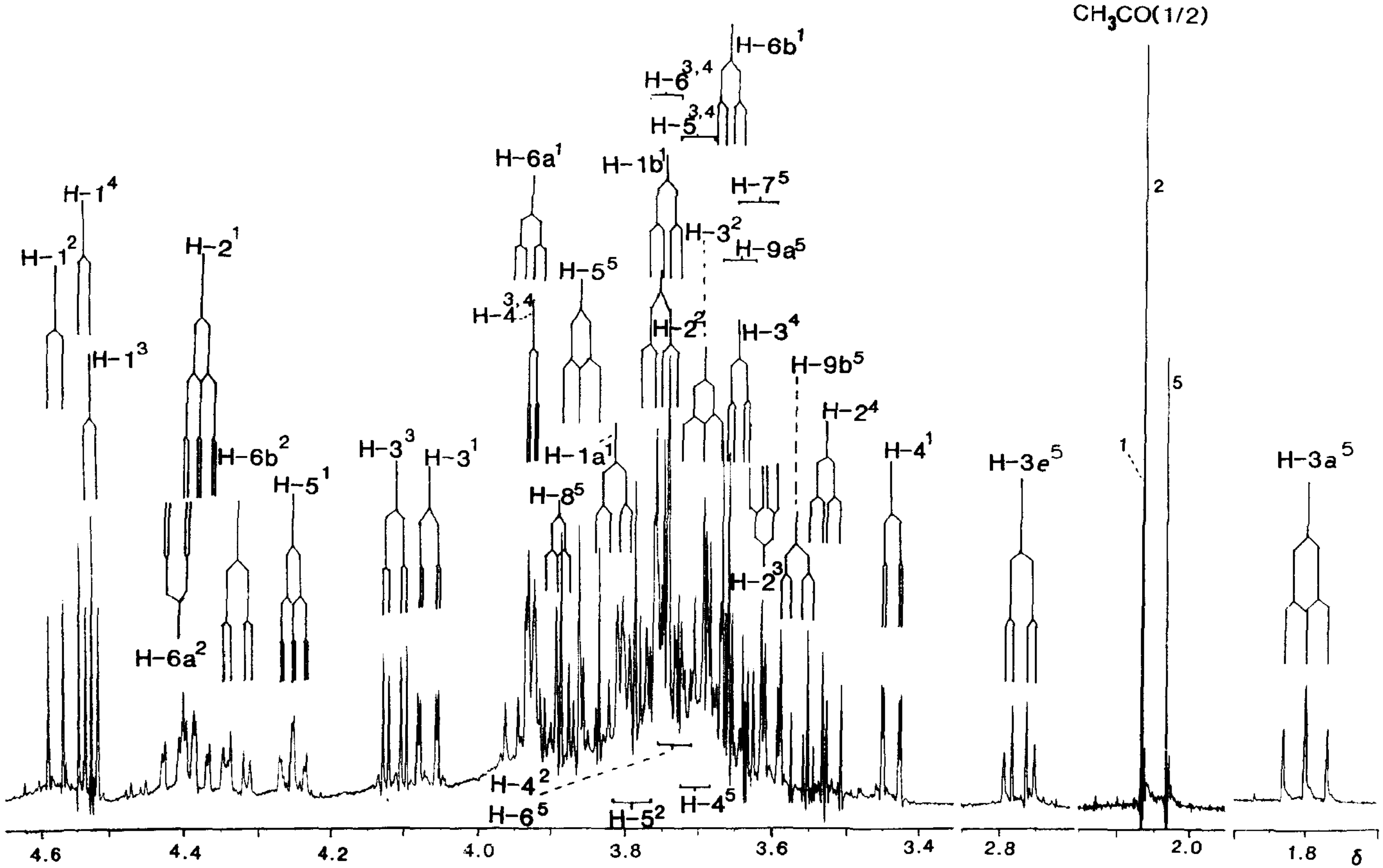


Fig. 4. 400-MHz ^{1}H-n.m.r. spectrum for oligosaccharide alditol **3**.

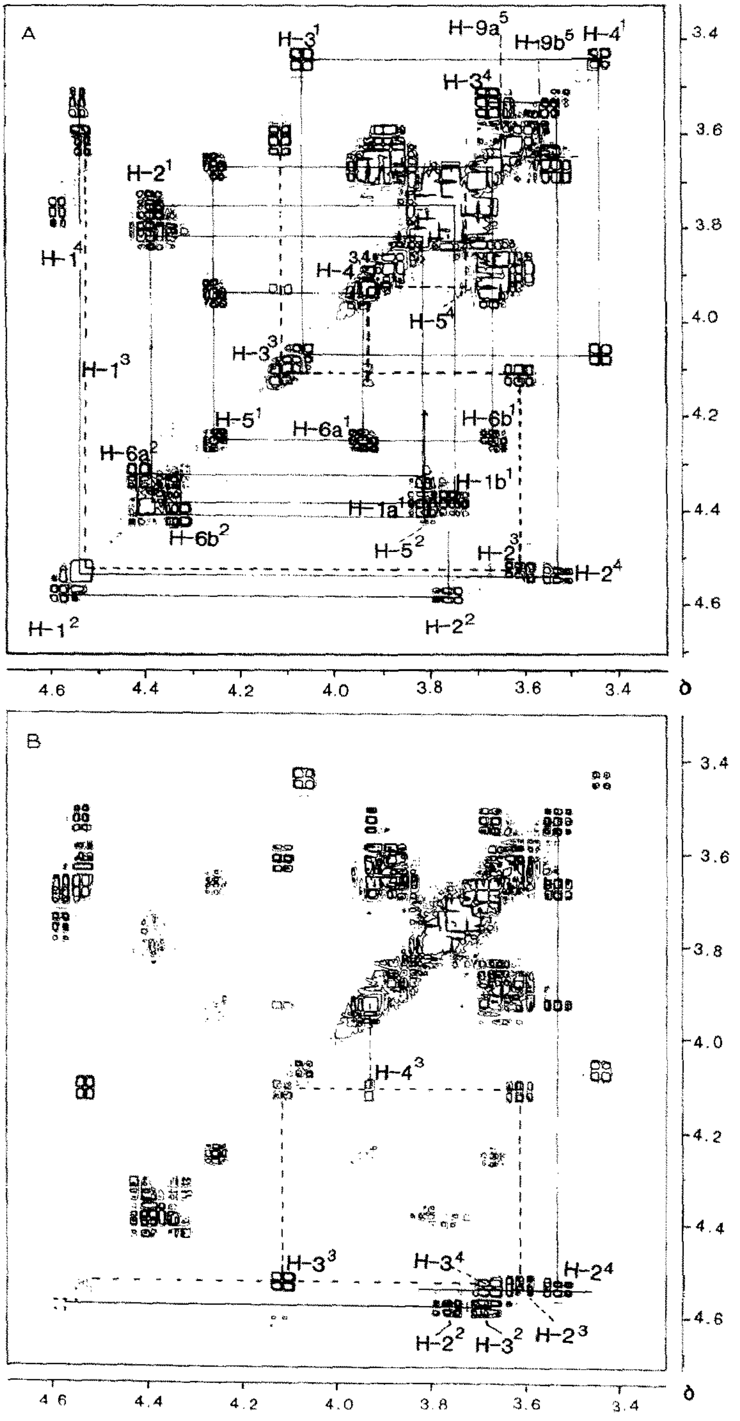


Fig. 5. 2D-Homonuclear Cosy (A) and Cosysr (B) spectrum for oligosaccharide alditol **3**.

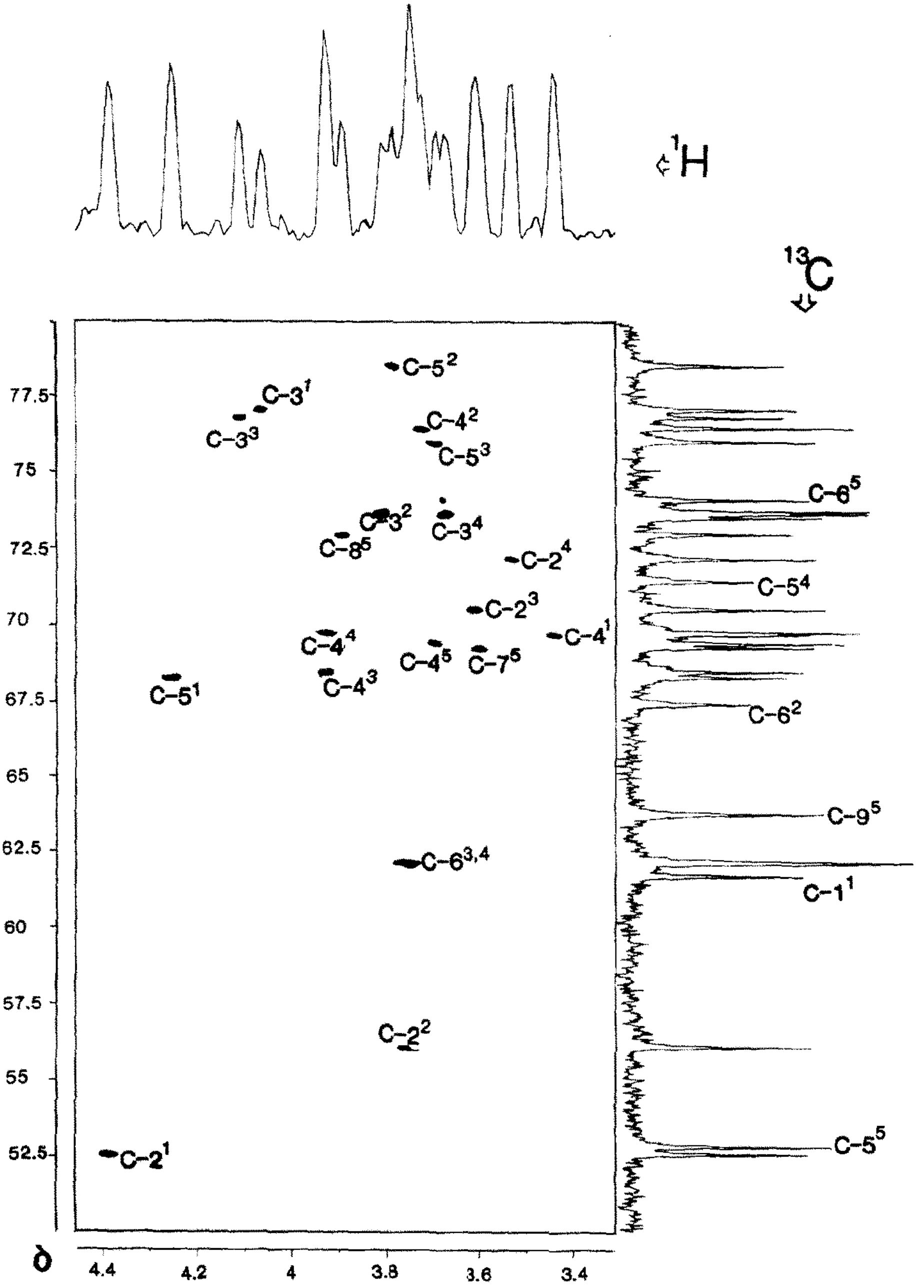


Fig. 6. Heteronuclear 2-D chemical-shift correlation spectrum for oligosaccharide alditol **3**.

The ^{1}H-n.m.r. spectrum of the sulphated oligosaccharide alditol OL-2 (**2**) is given in Fig. 2. In comparison with that of **1**, H-1 of Gal^4 was shifted downfield from δ 4.469 to 4.538. The most significant increment was observed for H-6a,6b of GlcNAc, which were shifted downfield by 0.421 and 0.521 p.p.m., respectively. The coupling constant $J_{6a,6b}$ decreased from 12 to 10.4 Hz, which suggested a steric hindrance due to the electronegative sulphate group. For **2**, the 2D-Double-relayed Cosy spectrum furnished most of the ^{1}H-signal assignments (Fig. 3). In addition to the ^{13}C-n.m.r. parameters, the 2D–^{13}C–^{1}H-Cosy spectrum allowed the definition of the Gal H-5,6 resonances, which did not appear in the 2D-Cosy spectra. Strongly marked features observed in the ^{13}C-chemical shifts were the downfield shifts of C-6 (+ 6.18) and C-5 (+ 2.73) of GlcNAc, and C-6 (+ 5.02) of GalNAcol, while upfield shifts were observed for C-4 (− 3.27) of GlcNAc, C-4 (− 1.87) of GalNAcol, and C-5 (− 4.51) of Gal^4.

For the oligosaccharide alditol OL-3 (**3**) (Fig. 4), the NeuAc residue present in α-(2→3) linkage to Gal^3 was characterized by the set of its H-3 chemical shifts ($\delta_{H\text{-}3a}$ 1.801; $\delta_{H\text{-}3e}$ 2.774) in combination with the downfield shift of H-3 of Gal^3, which underwent at δ 4.111. The comparison of the 2D-Cosy and 2D–Relayed-Cosy spectra of **3** (Fig. 5), which clearly established the connectivities between H-1,2,3,4 of Gal^3 and Gal^4, allowed the easy assignments of these signals. As for **1** and **2**, H-5,6 of Gal were recognized in the 2D–^{13}C–^{1}H-Cosy spectrum (Fig. 6). The signals of the carbon atoms of NeuAc were also easily recognized by comparing the ^{13}C-n.m.r. spectra of **2** and **3**. The chemical shifts of the NeuAc carbon atoms are very similar to those of free NeuAc or sialyllactose[14]. The ^{1}H signals of NeuAc were assigned by examination of the 2D–^{13}C–^{1}H-Cosy spectrum with an accuracy of 0.01 p.p.m. The only difficulty was to assign the *N*-acetylmethyl protons of GalNAcol and GlcNAc. They could only be discriminated on the basis of the increased relaxation time of GalNAcol and GlcNAc already observed for **1**, and which was assumed to also occur for **3**. This assignments of the GalNAcol and GlcNAc *N*-acetylmethyl signals at δ 2.066 and 2.064, respectively, are in accordance with the analysis of the 2D–^{13}C–^{1}H-Cosy spectrum, which furnished corresponding ^{13}C-chemical shifts at δ 23.58 and 23.13; these values were also found for **1** and **2**. By comparing with the spectrum of **2**, the additional signals observed at δ 23.21 and 176.11 were assigned to the CH_3 and CO carbons, respectively, of the NeuAc *N*-acetylmethyl group.

These n.m.r. data illustrate the possibility of determining the exact location of sulphate and NeuAc groups in anionic mucinous glycoproteins. Similar observations concerning the polysulphated proteoglycans have been recently reported[15–18]. As an easily available material, hen ovomucin represents a suitable model for the establishment of the n.m.r. parameters of the class of sulphated oligosaccharides.

ACKNOWLEDGMENTS

The authors thank Mrs. Catherine Alonso (CNRS technician) for skilfull technical assistance. They are grateful to the Conseil Régional de la Région Nord-

Pas-de-Calais, the Centre National de la Recherche Scientifique, the Ministère de l'Éducation Nationale, and the Ministère de la Recherche et de l'Enseignement Supérieur for financing the n.m.r. spectrometer.

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