Carbohydrate Research, 191 (1989) 191–207
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

STRUCTURAL ANALYSIS OF FIVE LACTOSE-CONTAINING OLIGOSACCHARIDES BY IMPROVED, HIGH-RESOLUTION, TWO-DIMENSIONAL ^{1}H-N.M.R. SPECTROSCOPY*

NICOLE PLATZER, DANIEL DAVOUST,

Laboratoire de Chimie Organique Structurale, Université Pierre et Marie Curie, C.N.R.S.–U.A. 455, 4 place Jussieu, F-75230 Paris (France)

MICHEL LHERMITTE,

Unité I.N.S.E.R.M. No 16, 2 place de Verdun, F-59045 Lille (France)

CHANTAL BAUVY, DENISE M. MEYER, AND CHRISTIAN DERAPPE†

Unité I.N.S.E.R.M. No 180, C.N.R.S.-U.A.C. 71, U.E.R. Biomédicale des Saints-Pères, 45 rue des Saints-Pères, F-75270 Paris (France)

(Received December 22nd, 1987; accepted for publication in revised form, August 10th, 1988)

ABSTRACT

The ^{1}H-n.m.r. spectra of five oligosaccharides, lactose, 6′- and 3′-sialyllactose, lacto-*N*-tetraose, lacto-*N*-fucopentaose I, and A-pentasaccharide in deuterium oxide have been obtained, at 500 MHz and 25°. COSY, COSY LR, RECSY, and COSY LR-R determinations, the latter two involving multistep transfer of magnetization revealed the *J* connectivities, including very small interactions which do not result in resolved multiplicity. Complete sets of unambiguous resonance assignments were obtained, including resonances in the poorly resolved region between δ 3.4 and 4.1. In the 2D patterns, each group of signals aligned with the "head group" (generally one of the reporter groups) constitutes a finger-print characteristic of the type of residue and of its position in the carbohydrate chain. Numerous coupling constants were measured from resolution-enhanced 1D spectra. Small long-range glycosidation shifts are most likely representative of slight changes in conformation and/or conformational equilibria. The large, reciprocal, long-range glycosidation shifts shown by the resonances of the two L-fucosyl groups of A-pentasaccharide indicated through-space interactions between these residues.

*This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale (Unités 16 and 180), and from the Centre National de la Recherche Scientifique (Unité Associée 455).
†To whom correspondence should be addressed.

0008-6215/89/$ 03.50

INTRODUCTION

Oligosaccharide chains of glycoproteins and glycolipids are known to play a fundamental role in many biological functions[1]. Many have been isolated from biological sources and their primary structures analyzed by physical or biochemical methods, as lectin affinity, mass spectrometry, or high-resolution nuclear magnetic resonance. This later method is specially useful for studies of oligosaccharides[2,3]. Structural reporter groups give rise to signals outside the envelop of the overlapping resonances, and the chemical shifts of these protons were correlated with specific sequences and branching patterns[4]. More recently, many informations regarding the conformational properties of oligosaccharides led to a better understanding of their biological functions. Indeed, conformational effects are expected to be well reflected in variations of chemical shifts or coupling constants, as well as in nuclear Overhauser enhancements[5]. The assignments of numerous signals within the overlapping envelop is a prerequisite to an extensive exploitation of these parameters. Two-dimensional (2D) correlated spectroscopy allowed assignments of new signals through connectivities with well identified reporter groups for some oligosaccharide alditols[6,7], glycosylamino acids[8], and gangliosides[9].

In the present paper, we describe the application of improved 2D n.m.r. methods derived from correlation spectroscopy through scalar coupling (COSY)[10]. The first method is an extension of relayed correlation spectroscopy (RECSY)[11] involving several steps of magnetization transfer[12,13]. The second one is long-range-correlated spectroscopy (COSY-LR)[14] which emphasizes connectivities through small couplings. The third one combines the advantages of long-range correlated spectroscopy and relayed spectroscopy. Thus, we determined accurately the chemical shifts for most protons of some tri-, tetra-, and penta-saccharides having both anomeric configurations at the reducing end. The few remaining protons were localized in very narrow shift ranges. For these analyses, we selected lactose (**1**),

β-D-Gal*p*-(1→4)-D-Glc
1

α-NeuAc-(2→6)-β-D-Gal*p*-(1→4)-D-Glc
2

α-NeuAc-(2→3)-β-D-Gal*p*-(1→4)-D-Glc
3

β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc
4

α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc
5

α-D-Gal*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc
2 3
↑ ↑
1 1
α-L-Fuc*p* α-L-Fuc*p*
6

6′-sialyllactose (**2**), 3′-sialyllactose (**3**), lacto-*N*-tetraose (**4**), lacto-*N*-fucopentaose I (**5**), and A-pentasaccharide (**6**), which have characteristic structural sequences, usually present in oligosaccharides of glycoproteins or glycolipids. Moreover, some of these compounds exhibit blood-group activity. These oligosaccharides were previously isolated from bovine colostrum[15], human milk[16,17], and human pregnancy urine[18–20]. Some of these results have been reported in a preliminary communication[21].

EXPERIMENTAL

Oligosaccharides. — Compound **1** was purchased from Sigma Chemical Co., compounds **2** and **3** were purified, from commercial neuraminyllactose (Sigma), by descending preparative paper chromatography on Schleicher & Schuell 2043-b paper in 5:5:1:3 (v/v) ethyl acetate–pyridine–acetic acid–water. Compounds **4** and **5** were obtained as described by Grimmonprez[22] and purified by passage through a column of Bio-Gel P-4 (−400 mesh; Bio-Rad) according to Yamashita *et al.*[23]. Compound **6** was isolated, as previously described[24], from human pregnancy urine (blood group A, Lewis a).

^{1}H-N.m.r. spectroscopy. — Prior to analysis, the oligosaccharides were repeatedly treated with ^2H_2O at p^{2}H 7 at 20°, with intermediate lyophilization. Finally, the samples were dissolved in ^2H_2O (0.4 mL, 99.96 atom % ^{2}H, Sigma). 1D and 2D ^{1}H-n.m.r. spectra were recorded with a Bruker WM 500 spectrometer equipped with a Bruker Aspect-2000 computer. 1D spectra were obtained by use of a 30°-pulse width, 32K data points in time and frequency domains, and a spectral width of 2500 Hz. Resolution enhancement was achieved by Lorentzian to Gaussian transformation[25]. Coupling constants were measured in the 1D spectrum after resolution enhancement. Owing to second order effects, some of these data must be regarded as apparent constants. Chemical shifts (δ) are given relative to the signal of sodium 4,4-dimethyl-4-silapentane-1-sulfonate (indirectly to acetone in ^2H_2O, δ 2.225) with an accuracy of 0.001 p.p.m. for reporter groups. The software used to obtain the COSY, COSY LR, and one-step RECSY diagrams were obtained from Bruker FT NMR 2D programs (version 850 101). For the COSY-LR experiments, a delay of 0.2 s was used. RECSY experiments involving several steps of magnetization transfer were obtained with programs adapted[26] from the Bruker program using the sequences: $90° - t_1 - 90°(\tau - 180 - \tau - 90°)_n - t_2$ ($\tau \sim 30$ ms). The RECSY experiment with two-step transfer, where coherence would be relayed from A → M → X, does not lead to observable connectivities when the coupling constant, J_{AM}, is very small as is the case for $J_{4,5}$ in Gal and GalNAc units. This difficulty was overcome with the following sequence, named COSY LR-R: $90° - t_1 - \tau_m - 90° - \tau_m/2 - 180° - \tau_m/2 - 90°$ acquire ($\tau_m \sim 0.30$ s). The phase cycle was the same as for the one-step RECSY experiment. Experimental conditions for data acquisition in the 2D experiments were as follows: 90° pulse 10.5 μs, time domain in f_2 2K, and 256 spectra were collected with 64 scans. Prior to Fourier

transformation, the time-domain matrix was extended by zero filling in f_1 dimension, and the signals were processed with unshifted sine-bell functions in both dimensions. Data were recorded in magnitude mode. Spectral width were chosen according to the spreadout of the 1D spectrum: for **1** 1200 Hz, **2–4** 2000 Hz, **5** and **6** 2250 Hz resulting in a digital resolution of 1.17 Hz/pt, 1.95 Hz/pt, and 2.19 Hz/pt, respectively. Chemical shifts obtained from 2D diagrams are given with an accuracy of ±0.01 p.p.m.

RESULTS

Lactose (**1**). — Since all the saccharides examined in this work contain the sequence β-D-Gal*p*-(1→4)-D-Glc as common structural element at their reducing end, the complete assignment of the spectra of lactose in both anomeries was first undertaken (Table I). The reporter groups H-1 of α-D-Glc, β-D-Glc, and D-Gal*p* were used as "head groups" for relayed magnetization transfers (anomerization effects upon the H-1 signal of D-Gal*p* are absent).

For the protons of the α-D-Glc group, a series of signals lined up at the frequency of H-1 in one dimension, and at their own frequencies in the other dimension: H-2 (COSY), H-2 and -3 (two transfers), H-2, -3, and -4 (three transfers), and H-2, -3, -4, and -5 (four transfers). The resonances of all the skeleton protons are thus successively detected. The signals of the hydroxymethyl group H-6 and -6′

TABLE I

^{1}H-N.M.R. DATA FOR LACTOSE (**1**)

Protons	*α Anomer*		*β Anomer*	
	δ	J *(Hz)*	*δ*	J *(Hz)*
D-*Glc residue*				
H-1	5.222	$J_{1,2}$ 3.7	4.659	$J_{1,2}$ 7.9
H-2	3.580	$J_{2,3}$ 9.8	3.284	$J_{2,3}$ 9.2
H-3	3.831	$J_{3,4}$ 8.9	3.65[a]	
H-4	3.652	$J_{4,5}$ 9.9		
H-5	3.949		3.609	$J_{5,6a}$ 2.2 $J_{5,6b}$ 5.1
H-6a	3.86_5	$J_{6a,6b}$ −12.3	3.952	$J_{6a,6b}$ −12.3
H-6b	3.85_0		3.801	
β-D-*Gal*p *group*				
H-1	4.447[b]		4.447[b]	$J_{1,2}$ 7.8
H-2	3.548		3.541	$J_{2,3}$ 10.0
H-3	3.660		3.659	$J_{3,4}$ 3.4
H-4	3.923[b]		3.923[b]	
H-5	3.72[b]		3.72[b]	
H-6a,6b	3.75–3.80[b]		3.75–3.80[b]	

[a]H-3 and H-4 could not be differentiated. [b]The resonance frequencies are the same for both anomers.

were identified in the COSY pattern by cross-peaks arising from *J* connectivities with H-5.

For the protons of the β-D-Glc group, two problems were encountered. The resonance frequencies of H-3 and -4 are fortuitously nearly equivalent and scarcely different from the resonance of H-5: the signals correlating successively H-3, -4, and -5 to the "head group" H-1 partially overlap. Furthermore, owing to the mixing of states in the tightly-coupled system, magnetization originating from H-6 and -6′ was precociously relayed to the frequency of H-1 in the fourth transfer. The second reporter group of the β-D-Glc group, H-2, failed to be a useful "head group" for relayed magnetization transfer, probably owing to a short T_2 relaxation time. Of course, the lines within the H-2 multiplet are broader than those of the anomeric signals.

For the protons of the β-D-Gal*p* group, starting from H-1, the signals of H-2, -3 and -4 were easily assigned. Owing to the low value of the coupling $J_{4,5}$, the magnetization relayed from H-5 to H-1 remained at a low intensity level. The assignment of H-5 was confirmed by use of the COSY-LR method. Returning to the 1D spectrum, analysis of integral intensities showed that the resonances of the protons of the side chain are located in the very narrow range δ 3.75–3.80. As a result of second-order effects among H-5, -6, and -6′, the signals of H-6 and -6′ cannot be distinguished without simulation of subspectra. It is noticeable that the doubling of the H-2 and -3 signals of β-D-Gal*p*, due to anomerization, was clearly observable in the 1D spectrum recorded with resolution enhancement. The results are in agreement with partial data reported earlier for the terminal β-D-Gal group[27] and for the D-Glc residue in the sequence Hex-β-D-Glc[26].

6′-Sialyllactose (**2**). — The combined use of the 2D-n.m.r. methods just described (RECSY and COSY LR) allowed the resonances of all the protons of 6′-sialyllactose to be assigned. The terminal D-Glc group gave for both anomers successive patterns very similar to those observed for lactose. Similarly, magnetization was transferred with good efficiency from H-1 to H-2, -3, and -4 of the D-Gal*p* residue. Cross-peaks correlated the weakly coupled H-5 and -4 in the COSY LR contour plot. The signal of H-5 was shifted to low field with respect to its resonance frequency for lactose. Furthermore, signals corresponding to an isolated network of three spins were recognized in the COSY contour plot, namely, the signal at the low-field extremity of the overlapping envelop (δ 3.975), the signal of H-5, and a third signal in the bulk (δ 3.60). The outer resonances could then be ascribed to H-6 and -6′, one of them being shifted to low field and the other to high field, in comparison to those for lactose. Similar shifts were previously observed upon substitution of D-galactitol at O-6 by a NeuAc group[28]. The doubling of the signal for H-6 due to anomerization was observed. Owing to the first-order character of the three-spin system, H-5, -6, and -6′, accurate values could be determined for the coupling constants $J_{5,6}$ and $J_{6,6'}$.

For the NeuAc group, the signals for H-3*a* and -3*e* were first used as "head groups" in the relay patterns, leading to the successive identification of the signals

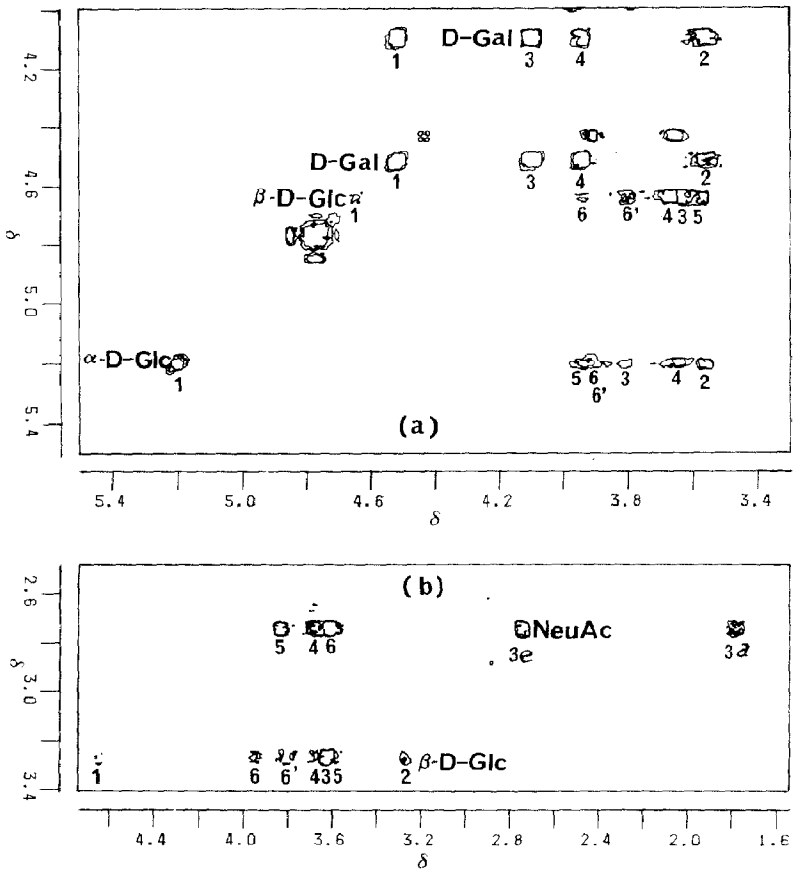

Fig. 1. Partial two-dimensional, shift-correlated spectra (RECSY) for 3'-sialyllactose (**3**). Four steps of magnetization transfer to anomeric protons (a); and to α-NeuAc H-3*e* and β-D-Glc H-2 (b).

for H-4, -5, and -6. The signal of this last proton was fortunately found in the middle of the bulk, free from any overlap interaction, and thus was further used as "head group" for the analysis of the side-chain protons.

3'-Sialyllactose (**3**). — Fig. 1 shows the result of the three-step relayed coherence transfers to various reporter groups for H-1 of the α-D-Glc, β-D-Glc, and β-D-Gal*p*; H-2 of β-D-Glc; H-3 of β-D-Gal*p*; and H-3 of α-NeuAc units. It was possible to obtain assignments for all the protons of the D-Glc residue of both anomers, for the skeleton protons of the α-NeuAc group, and for the ring protons of the β-D-Gal*p* residue but H-5. In fact, the resonance of this last proton, very slightly coupled to H-4, failed to give correlation cross-peaks at the frequency of H-3 in the RECSY experiment (n = 1), as well as at the frequency of H-1 with the three-step sequence (RECSY, n = 3). It was anticipated that a sequence involving long-range COSY associated with one further step of magnetization transfer (COSY LR-R) would result in significant transfers of magnetization from H-5 to

H-3. Fig. 2 compares the cross-sections obtained at the frequency of H-3 in a RECSY experiment (n = 1) and in the COSY LR-R experiment. In the first case, $J_{1,2}$ and $J_{2,3}$ values (~8–10 Hz) led to good transfer efficiency, whereas the $J_{3,4}$ values (~3 Hz) led to poor transfer efficiency. In the second case, the transfer of magnetization was optimized for $J_{3,4}$, and sufficient magnetization was transferred through the very small coupling interaction $J_{4,5}$ (unresolved in the 1D spectrum) to allow the identification of H-5.

In the 1D spectrum, the integrated intensities showed that the signals of the unassigned protons (the side-chain protons of the β-D-Gal*p* and α-NeuAc units) are confined to narrow regions, in the ranges δ 3.57–3.60 (one proton), 3.69–3.78

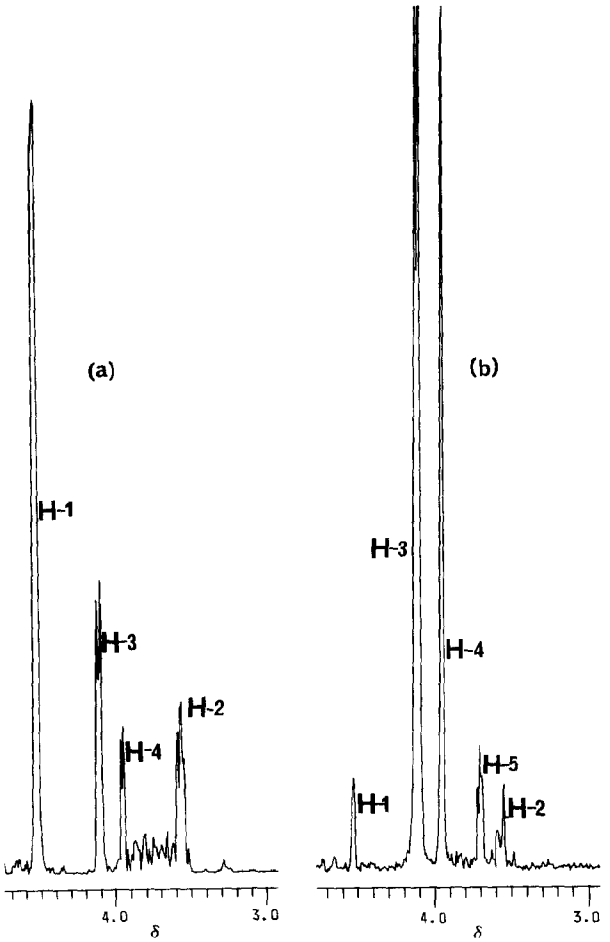

Fig. 2. Two-dimensional, shift-correlated spectra for 3′-sialyllactose (**3**). Cross section at the frequency of H-3: (a) Classical two-steps of magnetization transfer (RECSY), and (b) two steps of magnetization transfer with introduction of fixed delays to enhance signals arising from small *J* connectivities (COSY LR-R).

TABLE II

^{1}H-N.M.R. DATA[a] FOR 6′- (**2**) AND 3′-SIALYLLACTOSE (**3**)

Protons	*6′-Sialyllactose*			*3′-Sialyllactose*		
	α Anomer	*α/β*[b]	*β Anomer*	*α Anomer*	*α/β*[b]	*β Anomer*
D-*Glc residue*						
H-1	5.222		4.666	5.219		4.661
$J_{1,2}$	3.8		8.1	3.8		7.8
H-2	3.600		3.307	3.58		3.281
$J_{2,3}$	8.7		9.0			9.2
H-3	3.84		3.65	3.84		3.63
H-4	3.62		~3.60	3.67		3.65
H-5	3.94		~3.62	3.96		3.60
H-6a	3.88		3.953	3.87 ±0.02[c]		3.96
H-6b	3.86		3.79			3.80
β-D-*Galp residue*						
H-1		4.425			4.530	
$J_{1,2}$		8.0			7.8	
H-2	3.537		3.528		3.57	
$J_{2,3}$		10.0			9.9	
H-3		3.66		4.117		4.113
$J_{3,4}$		4.1			3.1	
H-4		3.932			3.956	
H-5		3.81			3.71	
$J_{5,6a}$		1.7				
H-6a	3.977		3.973		3.72–3.78[c]	
H-6b		3.60				
$J_{6a,6b}$		−10.3				
α-*NeuAc group*						
H-3*a*		1.743			1.800	
$J_{3a,4}$		11.8			12.3	
$J_{3a,3e}$		12.5			12.3	
H-3*e*		2.710			2.757	
$J_{3e,4}$		4.9			4.5	
H-4		3.64			3.68	
$J_{4,5}$		9				
H-5		3.86			3.84	
$J_{5,6}$		10.5				
H-6		3.720			3.62	
$J_{6,7}$		1.8				
H-7		3.563			3.58	
H-8		3.88			3.88 ±0.02	
H-9a		3.64			3.69	
H-9b		3.85			3.88 ±0.02	
N-Ac		2.028			2.030	

[a] δ Values; J values given in Hz. [b] Data given in this column correspond to protons which do not undergo the anomeric effect. [c] Chemical shifts of H-5, -6a, and -6b could not be more accurately specified.

TABLE III

^{1}H-N.M.R. DATA[a] FOR LACTO-*N*-TETRAOSE (**4**) AND LACTO-*N*-FUCOPENTAOSE I (**5**)

Protons	*Lacto-N-tetraoseN-*			*Lacto-*N*-fucopentaose I*		
	α Anomer	*α/β*[b]	*β Anomer*	*α Anomer*	*α/β*[b]	*β Anomer*
D-*Glc residue*						
H-1	5.218		4.654	5.218		4.660
$J_{1,2}$	3.7		8.0	3.7		7.9
H-2	3.57		3.277	3.57		3.277
$J_{2,3}$			9.2			
H-3	3.82		3.63	3.82		3.62
H-4	3.63		3.63 ±0.02	3.64		3.65
H-5	3.94			3.94		3.60
$J_{5,6a}$						2.1
H-6a	3.87 ±0.02		3.948	3.87 ±0.02		3.944
H-6b			3.82			3.81
$J_{6a,6b}$						−12.2
β-D-*Galp residue*						
H-1		4.420			4.419	
$J_{1,2}$		7.9			7.9	
H-2		3.59			3.56	
H-3		3.73			3.71	
$J_{3,4}$		3.2			3.5	
H-4		4.152			4.136	
H-5, H-6a, H-6b		3.70–3.80			3.70–3.80	
β-D-*GlcpNAc residue*						
H-1[c]		4.727			4.621	
H-2		3.89			3.81	
$J_{2,3}$					10.6	
H-3		3.81			3.986	
$J_{3,4}$					8.3	
H-4		3.57			3.529	
$J_{4,5}$		10			10.0	
H-5		3.48			3.49	
H-6a		3.90			3.89	
$J_{5,6a}$		2.4			2.2	
H-6b		3.78			3.78	
$J_{5,6b}$		5.0			4.5	
N-Ac		2.026			2.055	
β-D-*Galp group (terminal)*						
H-1		4.420			4.644	
$J_{1,2}$		7.9			7.6	
H-2		3.523			3.58	
$J_{2,3}$		10.0				
H-3		3.64			3.83	
$J_{3,4}$					3.7	
H-4		3.91			3.881	
H-5		3.70			3.70–3.80	
H-6a,6b		3.70–3.80				
α-L-*Fuc group (terminal)*						
H-1					5.186	
$J_{1,2}$					4.1	
H-2					3.75	
H-3					3.64	
H-4					3.72	
H-5					4.289	
$J_{5,6}$					6.7	
H-6					1.233	

[a]*δ* Values; *J* values in Hertz. [b]Data given in this column correspond to protons which do not undergo the anomeric effect. [c]Multiplet due to virtual coupling[36].

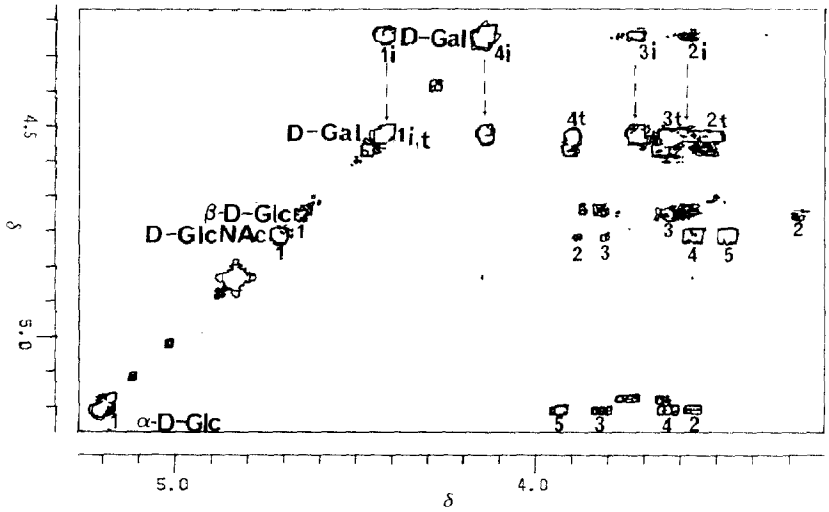

Fig. 3. Partial two-dimensional, shift-correlated spectrum (RECSY) for lacto-*N*-tetraose (**4**) with four steps of magnetization transfer. The signals for the β-D-Gal*p* units are differenciated by subscripts "i" (intrachain) and "t" (terminal).

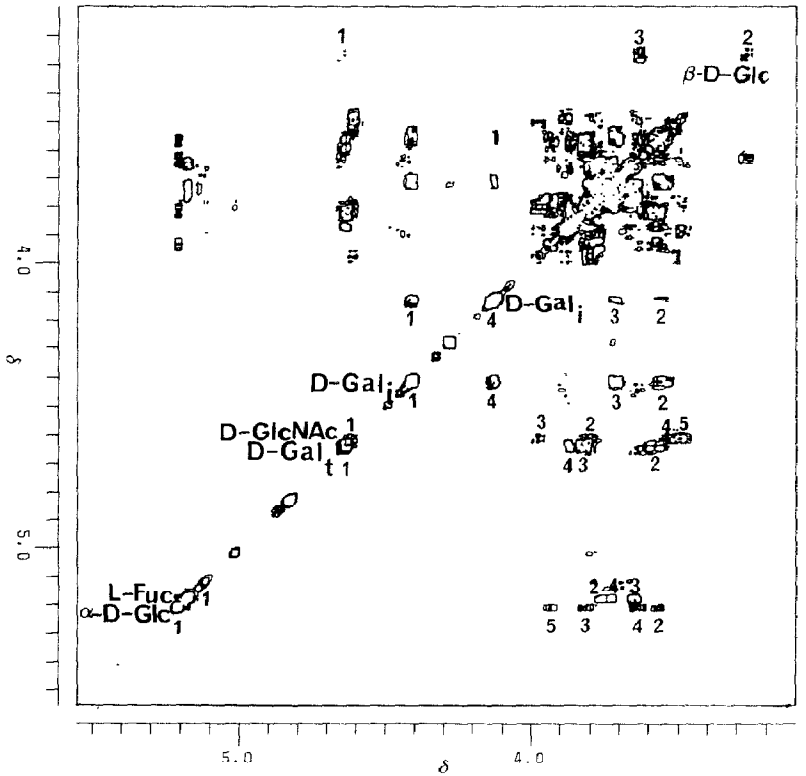

Fig. 4. Partial two-dimensional, shift-correlated spectrum (RECSY) for lacto-*N*-fucopentaose I (**5**) with four steps of magnetization transfer. The signals for the β-D-Gal*p* units are differenciated by subscripts "i" (intrachain) and "t" (terminal).

(three protons), and 3.86–3.89 (two protons). Assuming a similar behavior for the three protons, H-5, -6, and -6′, of the β-D-Gal*p* group in 3′-sialyllactose (**3**) and lactose (**1**), on one hand, of the side-chain protons of the NeuAc group in both 3′- (**3**) and 6′-sialyllactose (**2**) on the other hand, the following tentative assignments were made: the resonances of the two methylene protons of the β-D-Gal*p* residue in the range δ 3.69–3.78 (thus, they contribute with H-5 to a second-order, three-spin system); the resonances of the α-NeuAc side-chain protons H-7 (δ ~3.58), H-8, and one of the methylene proton H-9a (δ 3.88), and the last proton H-9b in the range δ 3.69–3.78. To gain confirmatory evidence of these assignments, a decoupling experiment, performed with the irradiation frequency set at δ 3.88, resulted in the collapse of the high-field signal, δ 3.58, into a doublet with the small coupling constant characteristic of the H-6–H-7 interaction in the NeuAc group[29,30], and spectral modifications occurred at δ ~3.69 while the range δ 3.71–3.78 was left unperturbed. The 1D spectrum recorded with resolution enhancement showed the doubling of the H-3 signal of the β-D-Gal*p* residue owing to long-range anomeric effect.

The results for the two sialyl compounds **2** and **3** are presented in Table II. They are in agreement with the partial data previously reported[31–34] and with those recently obtained for **3** by Lerner and Bax[35] with high-sensitivity, heteronuclear methods (^{1}H–^{13}C).

Lacto-N-*tetraose* (**4**) *and lacto*-N-*fucopentaose I* (**5**). — The analysis of the 2D patterns, involving up to three-step relayed-coherence transfers, obtained from **4** and from its fucosyl derivative **5** allowed the assignments of all the resonances of the constituent sugar residues, apart from H-5, -6 and -6′ of the β-D-Gal*p* groups (see Table III and Figs. 3 and 4). For the two compounds both anomers of the D-Glc residue gave patterns similar to those observed for lactose (**1**). The resonances for all the ring protons of the β-D-Glc*p*NAc residue were easily assigned, starting from the signal of the reporter group H-1 as "head group". Furthermore, since the signal for H-5 was found, for both **4** and **5**, free from any overlapping interactions at the high-field limit of the region of the skeleton protons, correlations were directly obtained in the COSY contour plot for H-6 and -6′.

Some difficulties arose for the β-D-Gal*p* residue which is present twice in the structure of these oligosaccharides. In **4**, the two H-1 protons usually used as "head groups" resonate at the same frequency. Fortunately, substitution at C-3 of the intrachain residue caused, at the adjacent position, a low-field shift which singled out H-4 as a new "head group". The magnetization transfers observed, at the frequency of this proton, in the multistep RECSY experiments allowed the assignment of the resonances of the protons of the intrachain residue. Thus, the cross-peaks appearing at the common frequency of the two anomer protons could be arranged into two independent series, one corresponding to the already-assigned protons of the intrachain β-D-Gal*p* residue, the other to the proton of the terminal β-D-Gal*p* group. For compound **4**, the COSY LR contour plot showed a distinct new correlation between H-4 and H-5 of the terminal β-D-Gal*p* group. For the other β-D-Gal*p*

residue, the correlation was not distinguished, probably owing to the overlap of the H-3 and H-5 resonances. Finally, inspection of the integrated intensities in the 1D spectra of **4** and **5** showed that H-5, -6, and -6′ of the two β-D-Gal*p* residues resonate in the narrow chemical-shift range δ 3.70–3.80.

As regards the L-fucopyranosyl groups, three reporter groups are expected[27], *i.e.*, H-1, H-5, and the methyl group. Nevertheless, H-5 fails to be significantly coupled to H-4. The signals of H-2, -3, and -4 were assigned starting from that of H-1 as "head group". Generally, the results of the present study are in agreement with the partial data previously reported[28,32,33]. Nevertheless, we observed some differences with the data obtained by Rao *et al.*[37]. For compound **4**, H-3 of the β-D-Glc*p*NAc residue was found more shielded by 0.05 p.p.m. For compound **5**, discrepancies of a similar magnitude occurred for the chemical shifts of H-2, -3, and -4 of the α-L-Fuc*p* group. Furthermore, the assignments given for several protons of the β-D-Gal*p* and β-D-Glc*p*NAc residues of **5** were revised. The resonance assignments were reversed for the H-3 protons of the two β-D-Gal*p* residues. In the β-D-Glc*p*NAc residue, the high-field signal exhibited characteristic couplings to three different protons, and consequently was assigned to H-5, which is thus slightly more shielded than H-4. The resonance observed at δ 3.944 had to be assigned to H-6 of the β-D-Glc*p* residue and not to H-6 of the β-D-Glc*p*NAc residue. The assignments are in general agreement with the conformation proposed for the type 1 determinant[37,38]. Upon L-fucosylation at O-2 of the terminal β-D-Gal*p* group, insignificant changes were observed for the fragment β-D-Gal*p*-(1→4)-D-Glc. A similar deshielding occur for H-1 and -3 of the β-D-Gal*p* residue fucosylated at O-2. For the β-D-Glc*p*NAc residue, the opposite changes that occur for H-1 and -3 situated on both sides of the acetamido group account well for moderate changes in the equilibrium orientation of the plane of the amide group.

A-Pentasaccharide (**6**). — The 1D spectrum of **6** is complicated owing to the presence of an α-L-fucopyranosyl group linked to O-3 of the end reducing D-glucose residue. The anomeric effect resulted in the doubling of several signals from this group. Nevertheless, nearly all resonances were ascribed (see Table IV), and the contour plot corresponding to four magnetization transfers is shown in Fig. 5.

Glycosylation at O-3 of the reducing D-Glc residue causes changes in chemical shifts, which resulted in widespread resonances. Starting from the signal of H-1 as "head group", assignments were obtained for all the ring protons. Interestingly, the signal of H-5 became well resolved from the bulk and could be used as a new "head group" to which H-6 and -6′ are related by cross-peaks in the COSY contour plot. For the α anomer of the D-Glc residue, the signals of H-2, -3, and -4 were assigned in the same way, but no distinguishable, relayed signal was obtained for H-5, possibly owing to overlap with either H-4 or -3.

For the β-D-Gal*p* and α-D-Gal*p*NAc residues, as previously outlined, magnetization transfers occur with good efficiency from H-1 to H-4 only, leading for each group to the assignment of H-2, -3, and -4. For the β-D-Gal*p* residue, once more a

TABLE IV

^{1}H-N.M.R. DATA[a] FOR A-PENTASACCHARIDE (**6**)

Protons	*α Anomer*	*α/β*[b]	*β Anomer*
D-*Glc residue*			
H-1	5.186		4.612
$J_{1,2}$	3.8		8.1
H-2	3.77		3.488
H-3	3.90		3.68
H-4	3.84		3.87
H-5	3.90–3.95[c]		3.446
H-6a			3.999
H-6b			3.82
α-L-*Fuc*p-*(1→3) group*			
H-1	5.433		5.491
$J_{1,2}$	4.0		3.8
H-2		3.79	
H-3		3.98	
H-4		3.83	
$J_{3,4}$		3.5	
H-5	4.391		4.337
$J_{5,6}$	6.8		6.8
H-6		1.293	
β-D-*Gal*p *residue*			
H-1		4.546	
$J_{1,2}$		7.6	
H-2		3.85	
H-3		3.93	
$J_{3,4}$		3.1	
H-4		4.201	
H-5		3.557	
H-6a		3.75	
H-6b		3.72	
α-D-*GalpNAc group*			
H-1		5.193	
$J_{1,2}$		3.5	
H-2		4.245	
$J_{2,3}$		11	
H-3		3.91	
$J_{3,4}$		3.5	
H-4		3.99	
H-5		4.253	
H-6a,6b		3.76–3.80	
N-Ac		2.039	
α-L-*Fuc*p-*(1→2) group*			
H-1		5.297	
$J_{1,2}$		4.0	
H-2		3.78	
H-3		3.69	
H-4		3.82	
H-5		4.871	
$J_{5,6}$		6.8	
H-6		1.280	

[a] δ Values; J values given in Hz. [b] Data given in this column correspond to protons which do not undergo the anomeric effect. [c] Chemical shifts of H-5, -6a, and -6b could not be more accurately specified.

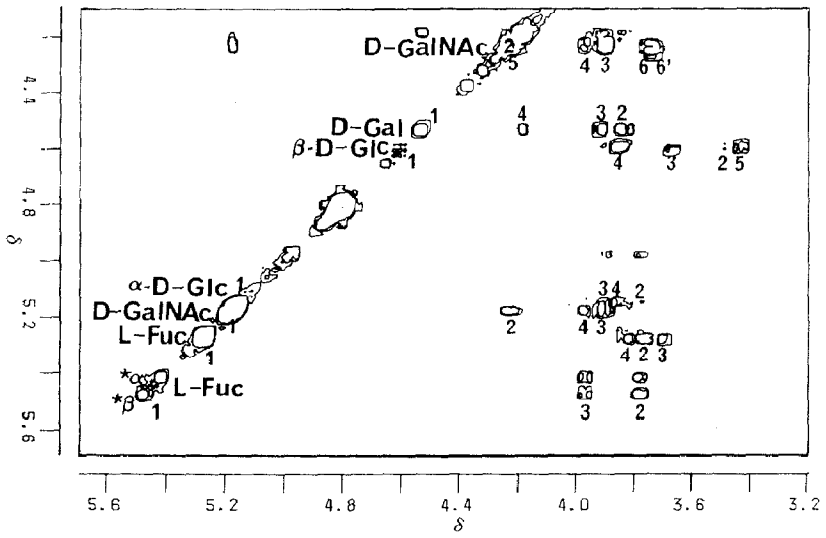

Fig. 5. Partial two-dimensional, shift-correlated spectrum (RECSY) for the A-pentasaccharide (**6**) with four steps of magnetization transfer from the anomeric protons, from GalNAc H-5, and from Gal H-4. For the α-L-Fuc*p*-(1→2) group, *α and *β are used to differenciate signals originating from the two anomers.

second reporter group, H-4, was available to confirm the assignments. Furthermore, two groups of isolated three-spin systems, characteristic for H-5, -6, and -6′ of the β-D-Gal*p* and α-D-Gal*p*NAc residues, were detected by their *J* connectivities in the COSY contour plot. Unambiguous assignment to each residue relied on two observations: (a) new cross-peaks emerged at the frequencies of the D-GalNAc H-4 and H-5 in the COSY LR contour plot, and (b) the resonance of H-4 Gal was slightly narrowed when the signal at δ 3.557 was irradiated.

The α-L-Fuc*p* group (1→2)-linked to the β-D-Gal*p* residue is remote from the reducing end of the oligosaccharide, and consequently each of the three reporter groups gave rise to a single signal. Within this group, magnetization was transferred with sufficient efficiency from H-1 to H-4 in the RECSY patterns. The signal of H-5 was assigned in the 1D spectrum on the basis of its integrated intensity and was correlated to the methyl group signal in the COSY experiment.

For the α-L-Fuc*p* group (1→3)-linked to the reducing D-Glc residue, H-1 and H-5 gave two well-differentiated resonances owing to strong long-range anomeric effects. They were ascribed to the relevant anomer on the basis of their relative intensity. Inspection of the integrated intensities of the H-1 D-Glc signal showed that the β anomer was present in slightly greater amount than the α anomer. The change in orientation at the anomeric D-Glc center did not influence the other protons. The resonance of the methyl group partially overlaps the signal from the other α-L-Fuc*p* group. The signals of H-2 and -3 were detected in the appropriate 2D patterns by their connectivities to H-1. In contrast, it was not possible to obtain

a significant magnetization transfer from H-4 to H-1, probably owing to the weakness of the coupling interaction between H-4 and H-3. Nevertheless, in a COSY LR experiment, connectivities were observed between H-4 and H-5 for both anomers. Inspection of the integrated intensities in the 1D spectrum showed that the unassigned signals of H-5, -6a, and -6b of the α-D-Glc residue lie in the range δ 3.90–3.95.

The present results were compared with those of Lemieux *et al.*[5] for the trisaccharide glycoside, α-L-Fuc*p*-(1→2)-[α-D-Gal*p*NAc-(1→3)]-β-D-Gal*p*OR. (These data were referenced to the signal of acetone at δ 2.480 and a correction of −0.255 p.p.m. is needed.) The chemical shifts measured for the protons of the α-D-Gal*p*NAc and β-D-Gal residues closely match those previously reported. Concerning the α-L-Fuc*p* (1→2)-linked group, the values are very similar for H-1, -2, and the protons of the methyl group, whereas discrepancies occurred for the resonances of H-3, -4, and -5. In particular, the last-named proton undergoes a strong deshielding effect (~±0.4 p.p.m.). Data for the α-L-Fuc*p* (1→3)-linked group were compared with those given in the library of chemical shift data[28] for the sequence α-L-Fuc*p*-(1→3)-[β-D-Hex-(1→4)]-D-Hexol. Discrepancies occurred not only for H-1 and -5, chemical shifts of which were found to be strongly dependent on the anomeric form of the reducing end sugar, but also for all the other protons, the largest shift increment (+0.3 p.p.m.) appearing for H-3. As previously outlined by Hindsgaul and Lemieux[39], such deshielding is expected for the protons that are within the Van der Waals radius of oxygen atoms of other sugar units. As a whole, these comparisons led to the conclusion that the relatively fixed conformation of the blood-group A determinant, α-L-Fuc*p*-(1→2)-[α-D-Gal*p*NAc-(1→3)]-β-D-Gal*p*, is well maintained in the composite pentasaccharide with, as shown by Lemieux *et al.*[5] and Bush *et al.*[40], the β face of the α-L-fucopyranosyl ring directed towards the α-D-Gal*p*NAc group, while interactions occur between the two α-L-fucopyranosyl rings affecting mainly the environment of their α face.

DISCUSSION

The 2D n.m.r. techniques used in this study offer a general and complete solution to the problem of resonances hidden in the clustered domain δ 3.5–4.1. They eliminate the need to have recourse to various solvents, such as dimethyl sulfoxide, which spread out resonances, but are susceptible to modify the three-dimensional structure. The oligosaccharides may be easily studied without derivatization or reduction of the end reducing-sugar residue. Thus, these results provide extended data sets for the development of a chemical-shift library[28]. It is noticeable that the results rely only on the firmly established assignments of a few well-known reporter groups. Internal cross-checking of the results is often possible. The determination of chemical shifts through heteronuclear correlations, which may be considered as a more direct procedure, rely more heavily on numerous chemical-shift comparisons between chemically related compounds. The importance of extended

assignments will lie in their use whenever protons, the signals of which are buried in the bulk, play an essential part to define the relative orientation of the individual oligosaccharides. In particular, they allow the detection of long-range glycosidation shifts where a new residue is juxtaposed to an area remote from the glycosidic linkage, and they are a basic prerequisite for extensive nuclear Overhauser studies.

In the 2D patterns, each group of signals correlating protons within the same network of spins constitutes a fingerprint characteristic of the type of monomer and of its position in the carbohydrate chain. It should be noted that the gross multiplicity of each resonance is usually recognizable in the area of contour density. The usefulness of such a fingerprint method will be for the recognition of particular sequences in extended oligosaccharides when overlapping of signals occurs, even in the region of the anomer signals. The combination of several methods of two-dimensional, correlated spectroscopy will allow the examination of heterogeneous mixtures. In particular, it should be noted that small amounts of contaminants may be easily detected and characterized.

The versatility of the methods described in this paper is well demonstrated by the analysis of five oligosaccharides containing up to five residues. They share an end reducing D-Glc residue and, thus, each spectrum was the superimposition of two spectra originating from the two anomers. Short-range and long-range anomeric effects and glycosidation effects were detected, and conformation implications were analyzed.

ACKNOWLEDGMENTS

The authors thank Doctor B. Perly for generously making available the programs used in multistep RECSY experiments prior to publication.

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