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Complete Analysis of the ^{1}H- and ^{13}C-NMR Spectra of Four Blood-group A Active Oligosaccharides

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The fully assigned ^{1}H and ^{13}C-NMR spectra of four group A oligosaccharides by use of multiple-relayed, coherence-transfer chemical-shift-correlated spectroscopy (multiple-RELAY-COSY) and ^{1}H-/^{13}C-correlation spectroscopy are reported. These analyses were performed on the following compounds:

III-A; GalNAcα1-3[Fucα1-2]Gal:
VI-A; GalNAcα1-3[Fucα1-2]Galβ1-3[Fucα1-4]GlcNAcβ1-3Gal:
VII-A-1; GalNAcα1-3[Fucα1-2]Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-1Glycerol:
VII-A-2; GalNAcα1-3[Fucα1-2]Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4Glc.

Human urine is a valuable source for the isolation of blood-group active fucosyl oligosaccharides, the structures of which are related to the Se, Le and A, B, H phenotypes of the subjects. Consequently, these compounds represent good models for immunochemical and physical investigations [1-2].

The structures of blood group active glycolipids [3] and reduced oligosaccharides isolated by alkaline borohydride degradation of mucins [4-6] have been previously established. Moreover, the fully assigned ^{1}H- and ^{13}C-NMR spectra of milk oligosaccharides bearing H, Le^{a}, Le^{b} and Le^{x}(X) determinants have recently been reported [7]. HSEA methods and proton nuclear Overhauser effect (nOe) measurements have led to a well-defined low-energy conformation for the non-reducing terminal blood group active fragments [8-10].

The present paper describes an assignment of the ^{1}H- and ^{13}C-NMR spectra of four blood group A active oligosaccharides in order to extend the database of chemical shifts for carbohydrates of biological interest.

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III-A GalNAcα1-3Gal
2
Fucα1

VI-A Fucα1
4
GalNAcα1-3Galβ1-3GlcNAcβ1-3Gal
2
Fucα1

VII-A-1 Fucα1
4
GalNAcα1-3Galβ1-3GlcNAcβ1-3Galβ1-1Glycerol
2
Fucα1

VII-A-2 Fucα1
4
GalNAcα1-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc
2
Fucα1

Figure 1. Structure of the four blood-group A oligosaccharides.

Materials and Methods

Oligosaccharides presented in Fig. 1 were isolated from urine as described by Strecker *et al.* [11]. Compounds VI-A and VII-A-1, which co-migrate on preparative paper chromatography, were further fractionated on an ODS HPLC column (5 µm ODS Zorbax; 25 cm x 0.94 cm internal diameter; Du Pont Instruments, Paris, France), using water as eluant. Their structures were verified by methylation analysis [12].

400 MHz NMR experiments were performed on a Bruker AM-400 WB spectrometer (Centre Commun de Mesure, Université des Sciences et Techniques de Lille Flandres-Artois), equipped with a 5 mm $^{1}H/^{13}C$ mixed probe-head, operating in the pulse Fourier transform

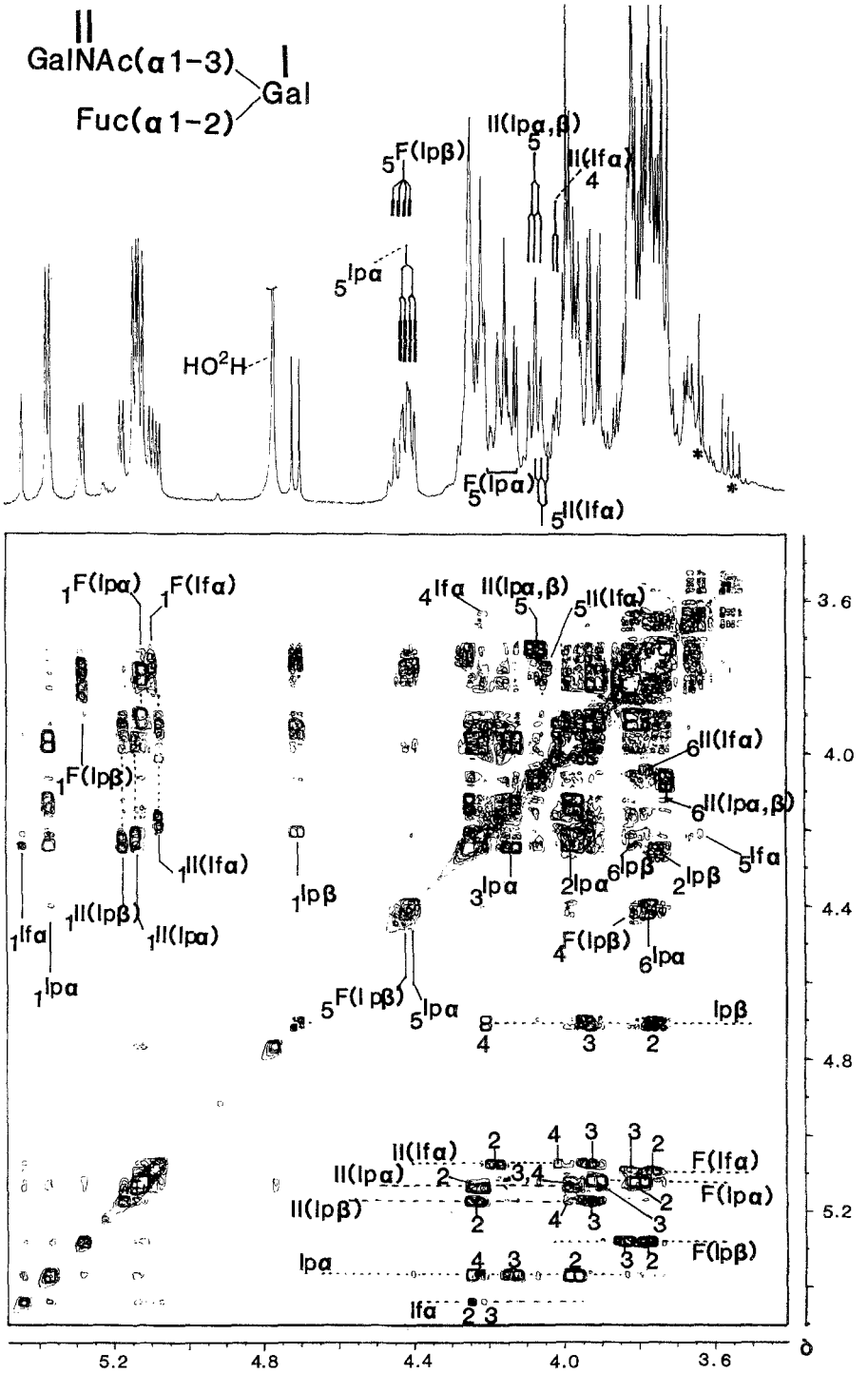

Figure 2. 400 MHz Partial ^{1}H-NMR double-relayed COSY spectrum of the trisaccharide III-A.
Key: $1^{II(Ipa)}$ indicates the chemical shift of H-1 of sugar II attached to a-D-galactopyranose (Ipa), etc. F = fucose.

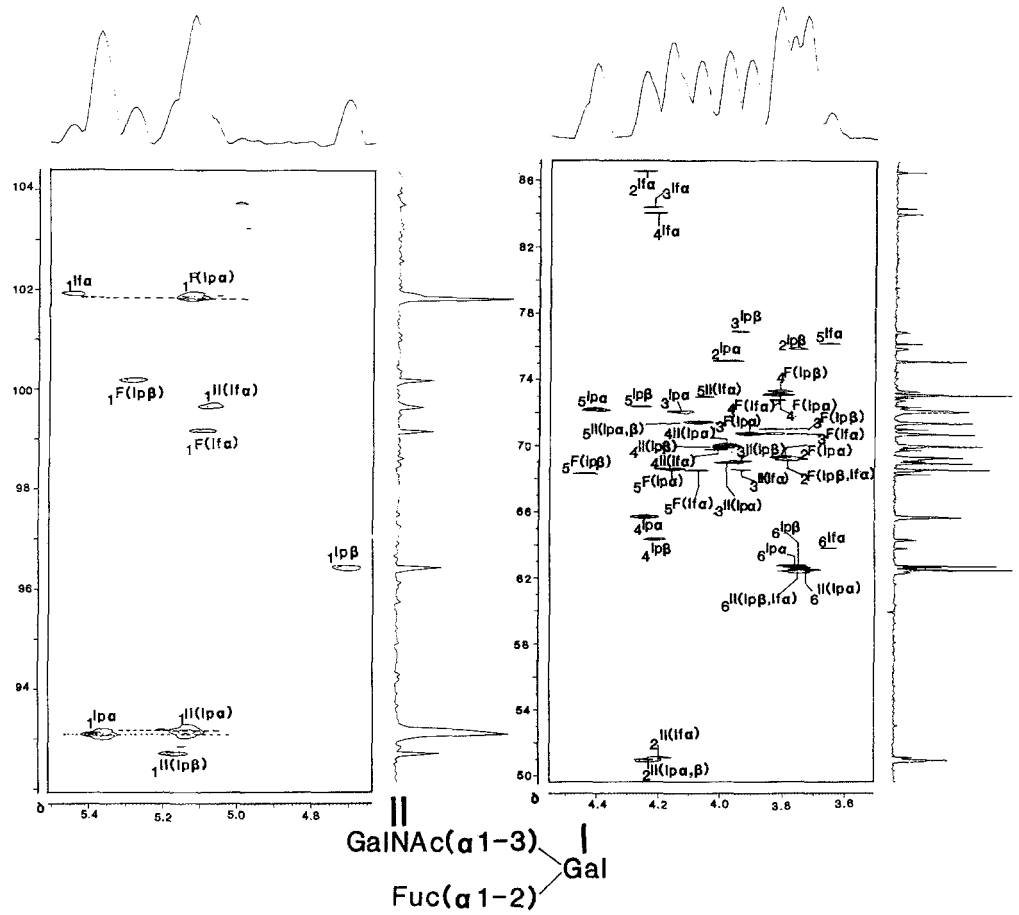

Figure 3. 2D-Heteronuclear-correlated NMR spectrum of trisaccharide III-A. On the left, 4.65-5.50 ppm for proton and 92-104 ppm for carbon (anomeric protons); on the right, 3.50-4.50 ppm for proton and 50-86 ppm for carbon.

mode and controlled by an Aspect 3000 computer. Each oligosaccharide (12-45 mg) was dissolved in 0.45 ml of ^2H_2O after three exchanges with ^2H_2O (99.96% atoms ^2H, Aldrich, Milwaukee, MI, USA) and intermediate lyophilisations. The pulse programmes used for homonuclear and heteronuclear correlation spectroscopy have been previously described [13].

Table 1. ^{1}H-NMR Chemical shifts for trisaccharide III-A.

	H-1	H-2	H-3	H-4	H-5	H-6	NAc
GalNAc(α1-3)	5.137	4.225	3.976	3.978	4.066	3.727	2.044
Fuc(α1-2)	5.120	3.796	3.912	3.811	4.155	1.225	-
Gal*p*, α	5.369	3.972	4.134	4.244	4.405	3.768	-
GalNAc(α1-3)	5.172	4.236	3.935	3.990	4.066	3.746	2.049
Fuc(α1-2)	5.279	3.777	3.842	3.802	4.432	1.205	-
Gal*p*, β	4.707	3.754	3.940	4.206	4.256	3.754	-
GalNAc(α1-3)	5.074	4.181	3.932	4.015	4.052	3.746	2.044
Fuc(α1-2)	5.092	3.763	3.828	3.811	4.070	1.215	-

Results

Oligosaccharide III-A

The 2D COSYDR spectrum of III-A is shown in Fig. 2. The pattern of anomeric proton signals is rather complex, since nine resonances are observable. Six of them can be explained by the occurrence of α- and β-anomers of the reducing galactopyranosidic residue and the anomerisation effect reported for fucose and *N*-acetylgalactosamine residues. The three other anomeric protons are related to a reducing galactofuranosidic residue. Since the H-1 resonances of the α-anomers of fucose, galactose and *N*-acetylgalactosamine residues possess very similar $^3J_{1,2}$ coupling constants, their assignment was made by comparing the homonuclear and heteronuclear COSY spectra (Figs. 2 and 3). Indeed, the H-2 signal of *N*-acetylgalactosamine is known to resonate at a relatively low field, while its C-2 resonance must appear in the range of 50-55 ppm. This is the case for three signals connected at δ = 4.181/51.12, 4.225/50.93 and 4.236/50.87 ppm, respectively, and which correspond to the H-2/C-2 cross-peaks of *N*-acetylgalactosamine. The anomeric protons of the α and β-Gal*p* residues were recognized owing to the connectivities extended until the H-4 atom (at δ = 4.244 and 4.206 ppm) on the COSYDR spectrum, while the structural reporter groups from the fucose residue were assigned *via* the connectivity between the H-5 and the H-4 atoms. Consequently, the three major doublets observed at δ = 5.369, 5.137 and 5.120 ppm, which occur in the ratio 1:1:1, were assigned to the H-1 resonances of galactose, *N*-acetylgalactosamine and fucose of the major anomer (α) of compound III-A. The second and the third anomers (β-Gal*p* and α-Gal*f*, respectively) are in the ratio 3:2, according to the intensity of the anomeric proton at δ = 4.707 and 5.439 ppm, and the assignment of fucose and *N*-acetylgalactosamine attached to these two galactose residues should be deduced from their respective intensity. The ratio of the three isomers, αGal*p*:βGal*p*:αGal*f* was approximately 5:3:2. Traces of a fourth isomer with a reducing β-Gal*f* residue could be detected owing to the presence of four distinct cross-peaks, fucose H-5/H-6 (not shown). This fourth isomer did not exceed 5%, and no traces of other connectivities were detected on the COSY spectra.

Table 2. ^{13}C-NMR Chemical shifts for trisaccharide III-A.

	C-1	C-2	C-3	C-4	C-5	C-6	CO	CH_3
GalNAc(α1-3)	93.15	50.93	68.95	69.99	71.41	62.50	176.00	23.31
Fuc(α1-2)	101.84	69.28	70.72	73.10	68.57	16.60	-	-
Gal*p*, α	97.07	75.13	72.04	65.69	72.13	62.75	-	-
GalNAc(α1-3)	92.68	50.87	69.05	69.88	71.41	62.34	175.76	23.20
Fuc(α1-2)	100.15	69.15	70.99	73.30	68.29	16.51	-	-
Gal*p*, β	96.42	75.88	76.88	64.32	72.35	62.65	-	-
GalNAc(α1-3)	99.65	51.12	68.53	69.73	72.92	62.41	176.06	23.31
Fuc(α1-2)	99.13	69.17	70.72	72.77	68.49	16.60	-	-
Gal*f*, α	101.88	86.52	84.35	84.00	76.19	68.80	-	-

The COSYDR spectrum enabled establishment of the assignment of most of the ^{1}H-resonances, and, particularly, gave easily the connectivities between the H-5 and H-6 atoms of galactose and *N*-acetylgalactosamine residues. The missing resonances, mainly related to the Gal*f*-containing isomer, were extracted from the ^{1}H-/^{13}C-COSY spectrum (Fig. 3). Previous identification of the H-6 resonances of galactose and *N*-acetylgalactosamine residues on the COSY spectra, enabled verification that the C-6 atom of *N*-acetylgalactosamine resonates at a higher field than the C-6 of galactose, by contrasting the results obtained for the three other oligosaccharides (see below). The combination of the 2D homonuclear and heteronuclear COSY experiments provides a complete list of ^{1}H and ^{13}C-NMR shift positions for the trisaccharide III-A (Tables 1 and 2).

Oligosaccharide VI-A

Methylation of the reduced oligosaccharide VI-A gave a mixture of 3,4,6-Me_3-GalN(Me)Ac, 2,3,4-Me_3-Fuc, 4,6-Me_2-Gal, 6-Me-GlcN-(Me)Ac and 1,2,4,5,6-Me_5-Gal-ol (ratio 1:1.6 :1:1:1), which is in accordance with the structure shown in Fig. 1.

The NMR analysis confirmed the occurrence of α(1-2)- and α(1-4)-linked fucose residues, and most of the H-1 resonances were unambiguously recognized by successive examination of the COSY 45, COSYRCT and COSYDR spectra (Fig. 4). Only the H-5 and H-6 were extracted from the heteronuclear COSY spectrum, and the complete assignment of the ^{1}H and ^{13}C spectra is given in Tables 3 and 4.

Oligosaccharide VII-A-1

Methylation analysis of the reduced oligosaccharide VII-A-1 furnished a mixture of 3,4,6-Me_3-GalN(Me)Ac, 2,3,4-Me_3-Fuc, 4,6-Me_2-Gal, 6-Me-GlcN(Me)Ac and 2,4,6-Me_3-Gal (ratio 1:1.8:1:1:1), without the formation of methylated hexitol deriving from a reducing

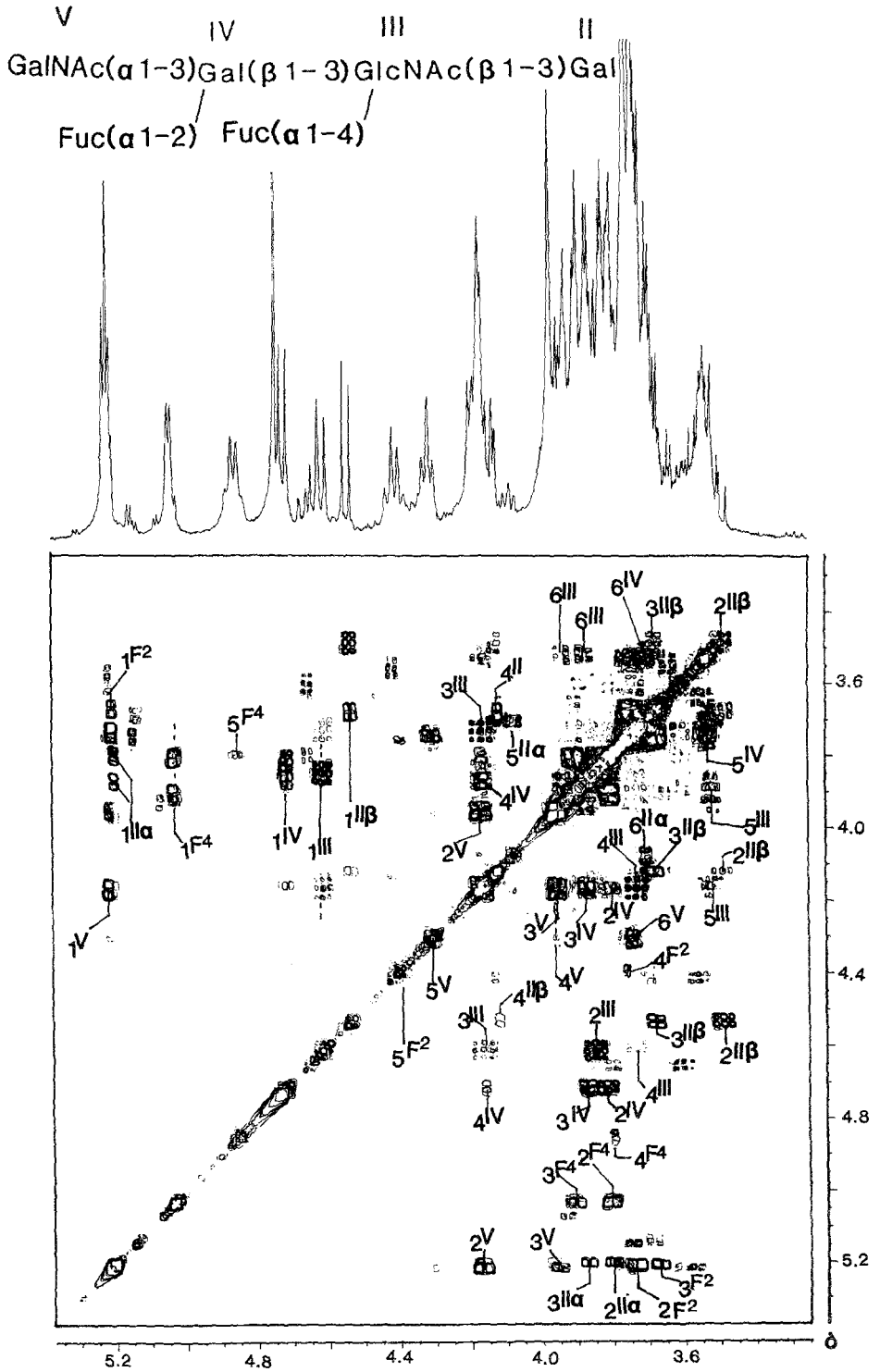

Figure 4. 400 MHz Partial ^{1}H-NMR double-relayed COSY spectrum of hexasaccharide VI-A.

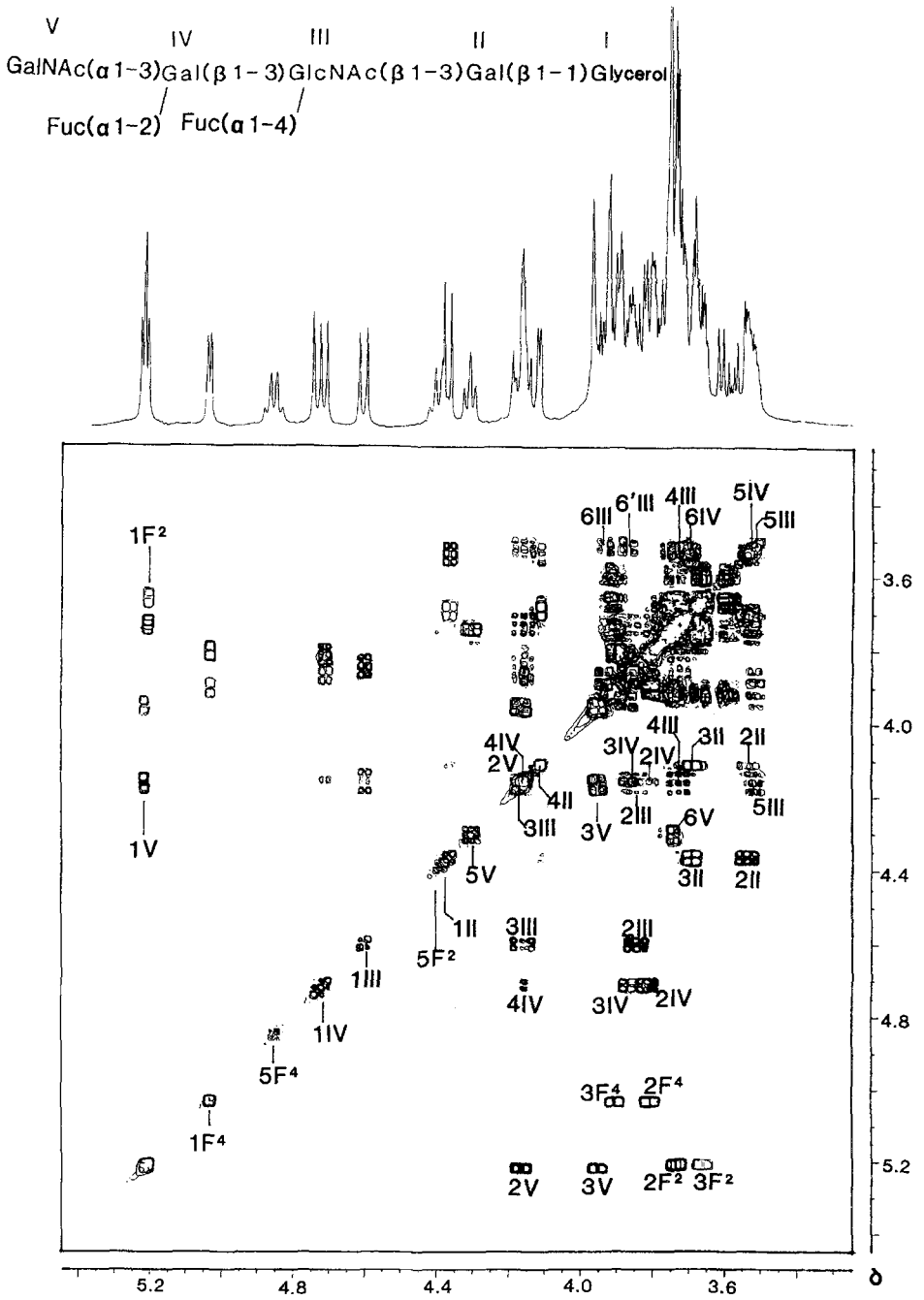

Figure 5. 400 MHz Partial ^{1}H-NMR COSYRCT spectrum of heptasaccharide VII-A-1.

Table 3. ^{1}H-NMR Chemical shifts for oligosaccharides VI-A, VII-A-1 and VII-A-2[a]

	H-1	H-2	H-3	H-4	H-5	H-6	NAc
VI-A							
GalNAc(α1-3)	5.220	4.172	3.960	3.970	4.308	3.737	2.024
Fuc(α1-2)	5.210	3.744	3.674	3.754	4.400	1.294	-
Gal(β1-3)	4.715	3.823	3.876	4.160	*3.537*	*3.754*	-
Fuc(α1-4)	5.036	3.810	3.910	4.806	4.853	1.294	-
GlcNAc(β1-3)	4.625(α)	3.852	4.172	3.732	3.502	3.928	2.063(α)
	4.607(β)					3.860	2.066(β)
Gal α	5.203	3.806	3.876	*4.180*	4.080	3.719	-
Gal β	4.537	3.495	3.684	4.124	*3.685*	*3.745*	-
VII-A-1							
GalNAc(α1-3)	5.218	4.164	3.958	3.971	4.308	4.737	2.023
Fuc(α1-2)	5.208	3.744	3.674	3.754	4.395	1.292	-
Gal(β1-3)	4.714	3.822	3.877	4.160	*3.537*	*3.754*	-
Fuc(α1-4)	5.034	3.811	3.910	3.800	4.853	1.292	-
GlcNAc(β1-3)	4.604	3.852	4.164	3.702	3.536	3.928	2.058
						3.860	-
Gal(β1-1)	4.370	3.548	3.701	4.117	*3.676*	3.760	-
Glycerol	*3.908*	*3.928*	*3.693*				
	3.78		*3.589*	-	-	-	-
lacto-*N*-difucohexaose I [6]							
Fuc(α1-2)	5.150	3.751	3.693	3.744	4.339	1.269	-
Gal(β1-3)	4.659	3.603	3.805	3.862	3.576	3.737	-
Fuc(α1-4)	5.018	3.804	3.929	3.823	4.860	1.253	-
GlcNAc(β1-3)	4.605	3.846	4.130	3.728	3.517	3.861	2.064
						3.924	
Gal(β1-4)	4.416	3.567	3.708	4.130	3.708	3.759	-
Glc α	5.217	3.573	3.821	3.636	3.935	3.859	-
Glc β	4.660	3.263	3.632	3.643	3.57	n.d.	-
VII-A-2							
GalNAc(α1-3)	5.218	4.172	3.960	3.970	4.308	3.740	2.023
Fuc(α1-2)	5.208	3.742	3.668	3.762	4.390	1.292	-
	(+0.058)	(-0.009)	(-0.025)	(+0.018)	(+0.051)	(+0.023)	
Gal(β1-3)	4.712	3.820	3.879	4.166	3.538	3.755	-
	(+0.053)	(+0.217)	(+0.094)	(+0.304)	(-0.038)		
Fuc(α1-4)	5.034	3.813	3.911	3.806	4.853	1.292	-
	(+0.016)	(+0.009)	(-0.018)	(-0.017)	(-0.007)	(+0.039)	
GlcNAc(β1-3)	4.604(α)	3.852	4.170	3.740	3.536	3.880	2.062
			(+0.040)		3.955		
	4.600(β)						
Gal(β1-4)	4.417	3.558	3.706	4.136	3.56	3.72	-
Glc α	5.218	3.576	3.827	3.63	3.94	3.86	-
Glc β	4.658	3.278	3.636	3.64	3.61	3.955	-

[a] Values in italics were extracted from the ^{1}H, ^{13}C COSY spectra.

Table 4. ^{13}C-NMR Chemical shifts for oligosaccharides VI-A, VII-A-1 and VII-A-2.

	C-1	C-2	C-3	C-4	C-5	C-6	CO	CH_3
VI-A								
GalNAc(α1-3)	92.25	51.20	68.86	69.87	72.19	62.90	175.99	23.33
Fuc(α1-2)	100.61	69.21	71.00	73.36	67.74	16.70	-	-
Gal(β1-3)	101.66	75.53	77.17	63.55	75.74	62.70	-	-
Fuc(α1-4)	99.15	69.12	70.50	73.36	68.39	16.81	-	-
GlcNAc(α1-3)	104.48	57.06	75.74	73.36	76.49	60.79	175.62	23.63
Gal α	93.78	68.86	80.02	70.65	70.24	62.37	-	-
Gal β	97.98	72.39	83.16	70.01	75.94	62.18	-	-
VII-A-1								
GalNAc(α1-3)	92.24	51.19	68.87	69.87	72.18	62.90	175.99	23.33
Fuc(α1-2)	100.61	69.21	71.00	73.35	67.73	16.70	-	-
Gal(α1-3)	101.66	75.53	77.17	63.55	75.73	62.70	-	-
Fuc(α1-4)	99.14	69.11	70.50	73.35	68.39	16.81	-	-
GlcNAc(β1-3)	104.57	57.04	75.67	73.35	76.49	60.80	175.63	23.60
Gal(β1-1)	104.57	71.36	82.99	69.93	75.94	62.21	-	-
Glycerol	71.75	72.09	63.59	-	-	-	-	-
Difuco-lacto-*N*-hexaose [7]								
Fuc(α1-2)	100.85	69.58	70.76	73.29	67.54	16.63	-	-
Gal(α1-3)	101.92	77.79	74.95	70.05	76.06	62.89	-	-
Fuc(α1-4)	99.07	69.12	70.42	73.29	68.31	16.69	-	-
GlcNAc(β1-3)	104.51	57.05	75.79	73.10	76.49	60.80	175.41	23.50
Gal(β1-4)	104.26	71.46	82.85	69.91	76.11	62.26	-	-
Glc α	93.10	72.45	72.67	79.60	71.46	61.27	-	-
Glc β	97.03	75.10	75.63	79.51	76.11	61.40	-	-
VII-A-2								
GalNAc(α1-3)	92.27	51.22	68.88	69.89	72.21	62.91	176.01	23.35
Fuc(α1-2)	100.64 (-0.21)	69.23 (-0.36)	71.04 (+0.28)	73.36 (+0.07)	67.75 (+0.21)	16.72 (+0.09)	-	-
Gal(α1-3)	101.68 (-0.24)	75.57 (-2.22)	77.20 (+3.25)	63.57 (-6.48)	75.76 (-0.30)	62.72 (-0.17)	-	-
Fuc(α1-4)	99.16 (+0.09)	69.13	70.51 (+0.09)	73.36 (+0.07)	68.42 (+0.11)	16.83 (+0.14)	-	-
GlcNAc(β1-3)	104.57	57.02	75.66 (-0.13)	73.36 (+0.26)	76.51	60.81	175.58	23.62
Gal(β1-4)	104.28	71.48	82.89	69.93	76.12	62.27	-	-
Glc α	93.12	72.47	72.69	79.61	71.48	61.29	-	-
Glc β	97.05	75.13	75.66	79.53	76.12	61.41	-	-

monosaccharide. The ^{1}H-NMR spectrum of VII-A-1 (Fig. 5) does not exhibit the doubling of the anomeric proton characteristic for native, reducing oligosaccharides. In addition, NMR parameters (Tables 3 and 4) are quite similar to those observed for VI-A, except for the H-1, H-2 and H-3 signals related to Gal^{II}. So the presence of an aglycon of low molecular weight was inferred, since it does not affect the chromatographic mobility of the compound. The

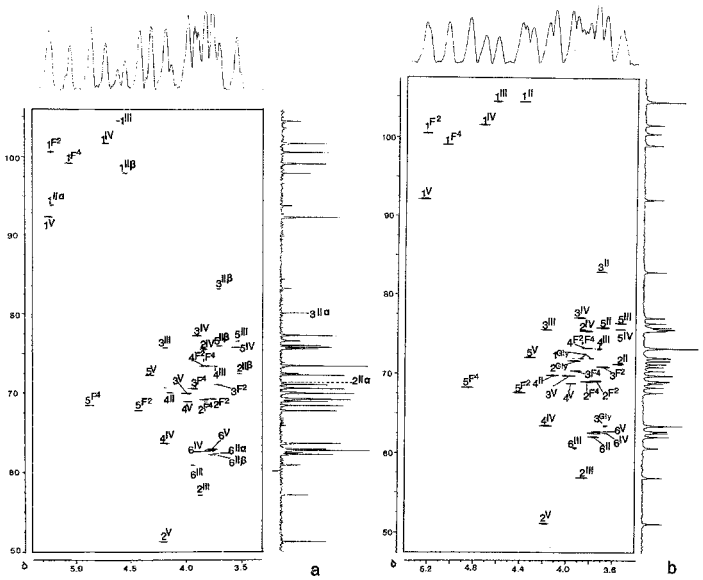

Figure 6. 2D-Heteronuclear-correlated NMR spectrum of heptasaccharide VII-A-1 (a) and VII-A-2 (b). 3.40-5.40 ppm for proton and 60-87 or 60-77 ppm for carbon. F = fucose; G = glycerol.

nature of the aglycon was established by examining the DEPT spectrum (not shown) and the heteronuclear COSY spectrum (Fig. 6). The DEPT experiment clearly indicates the presence of two additional primary carbons, at δ = 63.59 and 71.75 ppm, respectively. The heteronuclear COSY spectrum shows the presence of a third additional ^{13}C resonance, at δ = 72.09 ppm, connected with a proton at δ = 3.928 ppm. Consequently, the aglycon was identified as a residue of glycerol, and the large shift increment of one of the two primary carbons confirms the Gal^{II} residue to be linked at the C-1 position of this polyol. This carbon was arbitrarily numbered C-1, since the isomer of this unsymmetrical glycerol molecule is hard to delineate.

Oligosaccharide VII-A-2

For the oligosaccharide VII-A-2, the combination of the 2D techniques also easily furnished the complete assignment of the ^{1}H- and ^{13}C-NMR spectra (Fig. 7 and Tables 3 and 4).

Since this oligosaccharide is an extension of lacto-*N*-difucohexaose with an additional α(1-3)-linked *N*-acetylgalactosamine residue, the NMR parameter of this blood group Le^{b} active hexasaccharide [7] is also reported in Table 2 in order to estimate the shift increments related to the presence of the α-*N*-acetylgalactosamine residue.

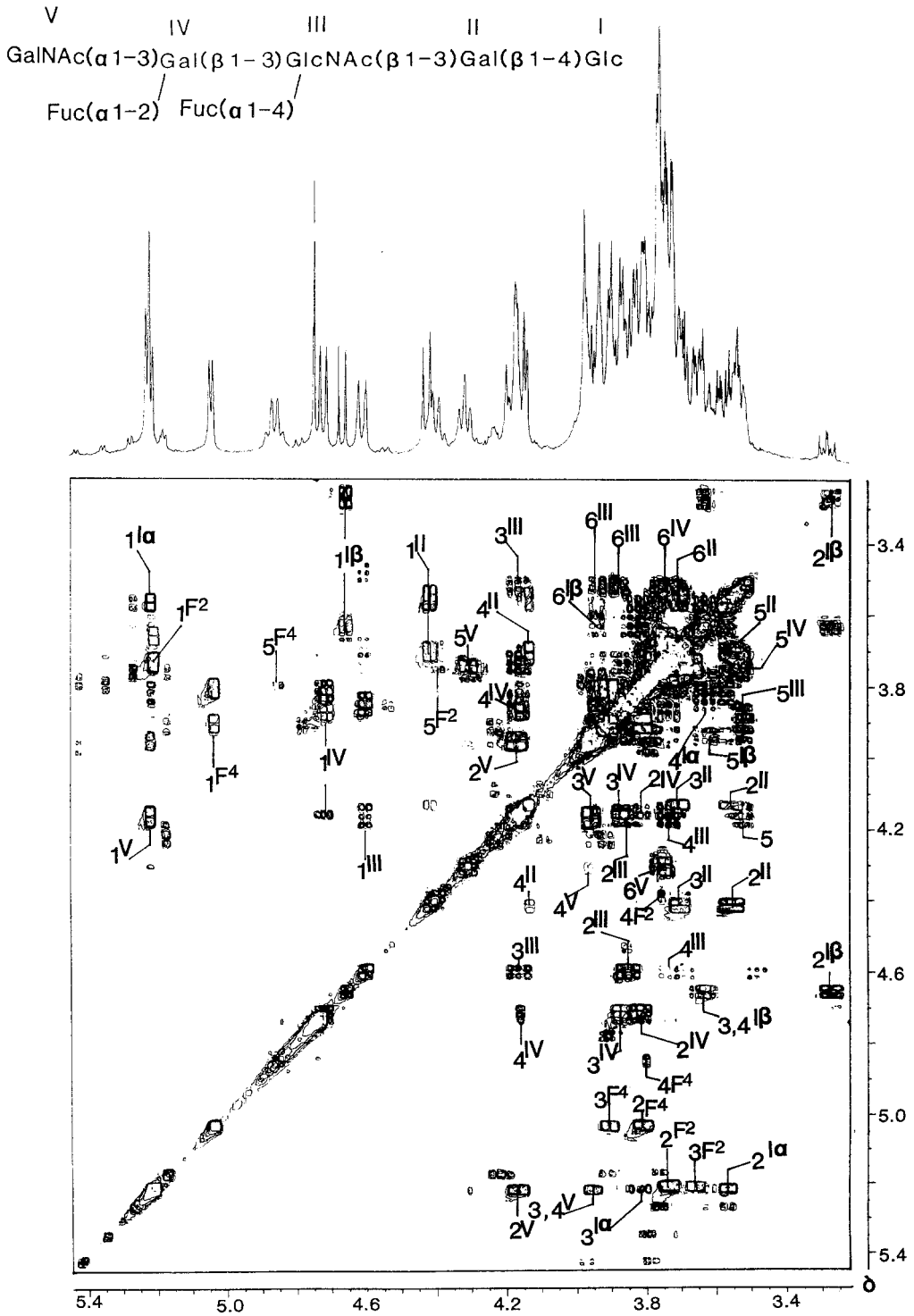

Figure 7. 400 MHz Partial ^{1}H-NMR double-relayed COSY spectrum of oligosaccharide VII-A-2.

Discussion and Conclusion

Numerous two-dimensional NMR techniques presently allow the complete determination of proton and carbon resonance assignments in oligosaccharides [14-16]. These detailed analyses are a prerequisite for further conformational studies using nuclear Overhauser effects. Two-dimensional $^{13}C/^{1}H$-chemical shift correlation spectroscopy is the most efficient procedure for assigning all of the resonances, but has severe experimental limitations due to the relative insensitivity of conventional ^{13}C-NMR spectroscopy methods. Among the new pulse sequences recently described, we have chosen to apply 2D-multistep relayed correlation spectroscopy and the classical ^{1}H-/^{13}C-correlation spectroscopy, because homonuclear Hartman-Hahn spectroscopy or indirect detection methods require specific equipment not yet available in our laboratory. The major limitations of the multiple-relayed COSY are the decay of magnetization during the evolution period, the difficulty to choose a convenient mixing time valid for all types of monosaccharide units and the inevitable overlap of cross-peaks from protons having identical chemical shifts.

In the case of the oligosaccharides described above, the only limitation was due to the low coupling constant $J_{4,5}$ of the galactose and *N*-acetylgalactosamine residues, which prevented the transfer of magnetization over the H-4 atom. For these reasons, the three and four relay-COSY experiments did not furnish additional assignments, except for the *N*-acetylglucosamine and glucose units, for which a mixing time of 30 ms allowed the transfer of magnetization up to the sixth atom of hydrogen. But the bad delineation of the cross-peaks led us to prefer the ^{1}H-/^{13}C-COSY experiments for assigning the H-5 and H-6 atoms by examination of the cross-sections in the F1 dimension (accuracy of 0.004 ppm, with 3 Hz/point).

With regard to the reference product, lacto-*N*-difucohexaose I (Le^b determinant), the main glycosidation shift in VI-A, VII-A-1 and VII-A-2 is observed for C-4 of Gal^{IV} (-6.4 ppm), instead of the downfield shift expected for C-3 (+3.2 ppm). It can be related to the bigger nOe effect affecting galactose H-4 rather than galactose H-3, after saturation of galactose H-1, which is attributed to a shorter distance between *N*-acetylgalactosamine H-1 and galactose H-4 than galactose H-3 [8, 10]. A second important glycosidation shift is observed for C-1 for Fuc^4 (+2.1 ppm), which probably results from steric hindrances between Fuc^2, Fuc^4 and *N*-acetylgalactosamine. Consequently, these model compounds are of great interest for further conformational studies involving HSEA methods and measurement of nOe effects.

The structure of VII-A-2 is new, but this type of substance is not unique, since urinary glycerol-containing oligosaccharides have been previously described [17]. This type of material is probably the result of transglycosylation to free glycerol in the intestine rather than the product of catabolism of a new class of glyceroglycolipids. Indeed, such a phenomenon has been reported for infants fed on breast milk [18] or during induced Galactosuria [19-21].

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