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^{1}H and ^{13}C-NMR Analysis of Four Pentasaccharides from Urine of Blood-Group A and B, Le^{b} Adults. Confirmation of the Structure of Two New Oligosaccharides: Fuc(α1-2)[GalNAc(α1-3)]Gal(β1-3)[Fuc(α1-4)]Glc and Fuc(α1-2)[Gal(α1-3)]Gal(β1-3)[Fuc(α1-4)]Glc

JEAN-MICHEL WIERUSZESKI, JEAN-CLAUDE MICHALSKI, JEAN MONTREUIL and GÉRARD STRECKER

Laboratoire de Chimie Biologique et Unité Mixte CNRS n° III, Université des Sciences et Techniques de Lille Flandres-Artois, F-59 655 Villeneuve d'Ascq Cedex, France

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The major pentasaccharides Fuc(α1-2)[GalNAc(α1-3)]Gal(β1-4)[Fuc(α1-3)]Glc and Fuc(α1-2)[Gal(α1-3)]Gal(β1-4)[Fuc(α1-3)]Glc, which are normally present in the urine of blood-group A Le^{b} and B Le^{b} healthy subjects, were each found to be contaminated by a minor component when analysed by ^{1}H-NMR. The determination of these structures, Fuc(α1-2)[GalNAc(α1-3)]Gal(β1-3)[Fuc(α1-4)]Glc and Fuc(α1-2) [Gal(α1-3)]Gal(β1-3)[Fuc(α1-4)]Glc, was based on the results of methylation analysis and $^{1}H/^{13}C$-NMR spectroscopy.

Human urinary oligosaccharides possess the lactose and lacto-*N*-tetraose cores [1, 3], and therefore are very similar to those characterized in human milk [4, 5]. Their main feature is the presence of α(1-3)-linked galactose or *N*-acetylgalactosamine residues, according to the blood-group phenotype of the donors, in contrast to milk oligosaccharides which are devoid of these blood-group determinants. As with milk oligosaccharides, the urinary oligosaccharides result from the transfer of fucose, galactose and *N*-acetylglucosamine residues to free glucose, galactose or lactose [1, 3]. The faeces of blood-group A, breast-fed infants have been found to contain large amounts of blood-group A active oligosaccharides, resulting from the transfer of α-GalNAc residues to the main components of milk [6].

As previously described [1], the major pentasaccharides isolated from human urine possess the following structures: Fuc(α1-2)[GalNAc(α1-3)]Gal(β1-4)[Fuc(α1-3)]Glc (A phenotype) and Fuc(α1-2)[Gal(α1-3)]Gal(β1-4)[Fuc(α1-3)Glc (B phenotype). Despite their apparent

Abbreviations: HPLC, high performance liquid chromatography; GLC, gas liquid chromatography; NMR, nuclear magnetic resonance; COSY, correlation spectroscopy; Gal, D-galactopyranose; GalNAc, 2-acetamido-2-deoxy-D-galactopyranose; Glc, D-glucopyranose; Fuc, L-fucopyranose; LNDFH I, lacto-*N*-difucohexaose I (Le^{b} determinant).

homogeneity in most chromatographic procedures, these oligosaccharides appeared contaminated by a minor component, when analysed by NMR spectroscopy. This observation prompted us to purify the material and to establish its chemical structure.

Materials and Methods

The major urinary pentasaccharides VA and VB were isolated according to [1]. The two isomers contained in the fraction VA were fractionated on an ODS HPLC column (ZORBAX C-18 9.4 mm x 25 cm) using water as eluent. The molar ratio of monosaccharides was determined as described by Zanetta *et al.* [7].

The methylation analyses of the reduced oligosaccharides were performed as described by Paz-Parente *et al.* [8] and the methyl ethers were identified as partially acetylated, methylated methylglycosides by GLC-MS [9]. Fucose residues were removed by mild acid hydrolysis (CF_3COOH, 0.1 N; 100°C; 1 h) in order to compare the results of the methylation analysis with those obtained with the native material.

^{1}H-Nuclear Magnetic Resonance Spectroscopy

For ^{1}H-NMR measurements, the deuterium-exchanged oligosaccharide were dissolved in 0.5 ml of ^2H_2O (99.96% atom ^{2}H, Aldrich). The 400 MHz ^{1}H-NMR experiments were performed with a Bruker AM-400 WB spectrometer, equipped with a 5 mm ^{1}H-/^{13}C mixed probe head, operating in the pulsed Fourier transform mode and controlled by an Aspect 3000 computer (Centre commun de mesures, Université de Lille Flandres Artois). All the spectra were obtained at a probe temperature of 300 K. One dimensional spectra were obtained with 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-NMR experiments were obtained with the standard Bruker pulse programme Powgate with ^{1}H composite pulse decoupling. The spectral width was 22,727 Hz for a 32 K frequency-domain data points and time-domain data points giving a final digital resolution of 1.387 Hz/point; a ninety-degree pulse (6 µs) and a 1 s recycle delay were used. The chemical shifts are given relative to the signal of the methyl group of acetone (δ 2.225 for ^{1}H and δ 31.55 for ^{13}C).

The 2D-homonuclear COSY 45, COSY with simple, double and triple relay transfers were performed by use of the standard Bruker pulse programme library or the programmes given by B. Perly (C.E.A, Saclay). For all RCT experiments, refocusing delays of 35 ms were chosen and the relaxation delay was 2 s. In all these experiments, the spectral width was 1840 Hz, the ^{1}H ninety-degree pulse was 10.6 µs; 256 W x 2 K data matrices were acquired, which were zero-filled prior to Fourier transform, to obtain a 1 K x 2 K spectral data matrix and a sine-bell squared function was used in both dimensions.

The 2D- ^{13}C/^{1}H COSY experiments were performed with simultaneous suppression of ^{1}H homonuclear couplings by use of the standard Bruker pulse programme XHCORRD. Refocusing delays were adjusted to an average $^1J_{C,H}$ coupling constant of 150 Hz. ^{1}H and ^{13}C

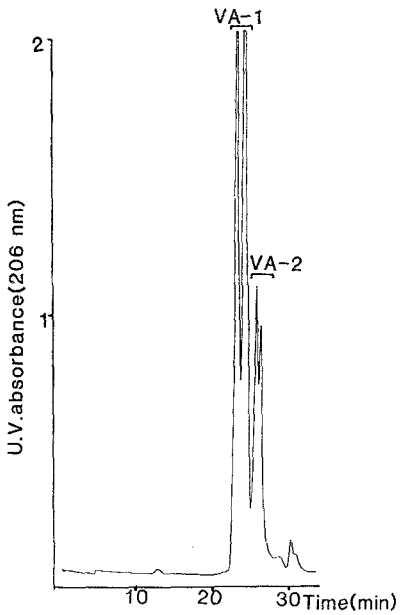


Figure 1. Separation of the oligosaccharides VA-1 and VA-2 by liquid chromatography on a column of ODS-C18. Eluent: water; flow-rate: 0.5 ml/min.

ninety-degree pulse widths were 10.6 and 6 μs. The relaxation delay was 0.8 s. 128 W x 4 K data matrix was acquired which was zero-filled prior to Fourier transform to obtain a 512 W x 4 K spectral data matrix. An exponential function (LB= 1 Hz) for ^{13}C-subspectra and a sine-bell function for ^{1}H-subspectra were applied to enhance the signal/noise ratio.

Immunochemical Determination of Blood Group Activities of Oligosaccharides

The blood group A_1, Lewis a and Lewis b activities were determined with commercial antisera (Diagast Lab., Lille, France) in hemagglutination inhibition assays. The results were given as amounts of oligosaccharides (μg) completely inhibiting hemagglutination of a suspension of 3% (blood group A_1) or 5% (Lewis a and b) erythrocytes. The titers of the antibodies were 32 for anti-A_1 and 16 for anti-Lewis immune sera.

Results

Purification of Isomers VA-1 and VA-2

The fraction VA (400 mg) isolated from 100 l urine was purified by preparative ODS HPLC, yielding two substances (VA-1, 280 mg; and VA-2, 48 mg) (Fig. 1). Each compound furnished α and β anomers and the nature of the products was further verified by recycling the four peaks on the same columns, after total mutarotation.

Table 1. Molar ratios[a] of partially methylated sugars from native and defucosylated oligosaccharides.

	Oligosaccharides					
Methylated sugar	VA	VA-1	VA-2	VA-1 (afuco)	VA-2 (afuco)	VB
2,3,4-Me_3-Fuc	1.6	1.7	1.6	-	-	1.8
3,4,6-Me_3-GalNAc	0.9	0.9	0.9	0.9	0.8	-
2,3,4,6-Me_4-Gal	-	-	-	-	-	1.0
4,6-Me_2-Gal	1.0	1.0	1.0	-	-	1.0
1,2,5,6-Me_4-Glc-ol	0.9	0.8	0.9	-	-	0.9
2,4,6-Me_3-Gal	-	-	-	1.0	1.0	-
1,2,3,5,6-Me_5-Glc-ol	-	-	-	0.7	0.1	-
1,2,3,5,6-Me_5-Glc-ol	-	-	-	-	0.6	-

[a] Molar ratio based on one residue of 4,6-Me_2-Gal (native material) or 2,4,6-Me_3-Gal (defucosylated oligosaccharides).

Structural Analysis of the Oligosaccharides VA-1 and VA-2

The two oligosaccharides VA-1 and VA-2 contain two fucose, one *N*-acetylgalactosamine, one galactose and one glucose residue and furnished the same methyl ether derivatives, i.e.: 2,3,4-Me_3-Fuc; 3,4,6-Me_3-GalNAc; 4,6-Me_2-Gal; 1,2,5,6-Me_4-Glc-ol (ratio 2:1:1:1) (Table 1). After removing the fucose residues by mild acid hydrolysis, VA-1 and VA-2 furnished 1,2,3,5,6-Me_5Glc-ol and 1,2,4,5,6-Me_5-Glc-ol respectively, indicating that substance VA-2 possesses the Gal(1-3)Glc sequence, instead of the usual lactose core.

The NMR data from VA-1 and VA-2 are given in Figs. 2-5 and Tables 2 and 3. For VA-1, the H-1 resonances of *N*-acetylgalactosamine and fucose were identified owing to their small $J_{1,2}$ coupling constants (α-anomer) and the examination of the COSY spectrum in which the H-2 signal of the *N*-acetylgalactosamine residue resonates at δ = 4.243 ppm. The location of the *N*-acetylgalactosamine H-2 resonance was further confirmed by ^1H-/^{13}C COSY analysis, in which the correlation was found at δ C-2 *N*-acetylgalactosamine = 50.85 ppm.

The H-1 resonance of the β-Glc residue was recognized by observing its connectivity with the strongly shielded H-2 signal at δ = 3.489 ppm, and the H-1 signal of α-Glc owing to its relative low intensity (β/α =3/2). For the fucose residues, some ambiguities reside in the atypical set of their resonances. The signals at δ = 4.388 ppm and 3.336 ppm correspond to the H-5 resonances of the α-(1-2)-linked fucose residue, doubled by the anomerization

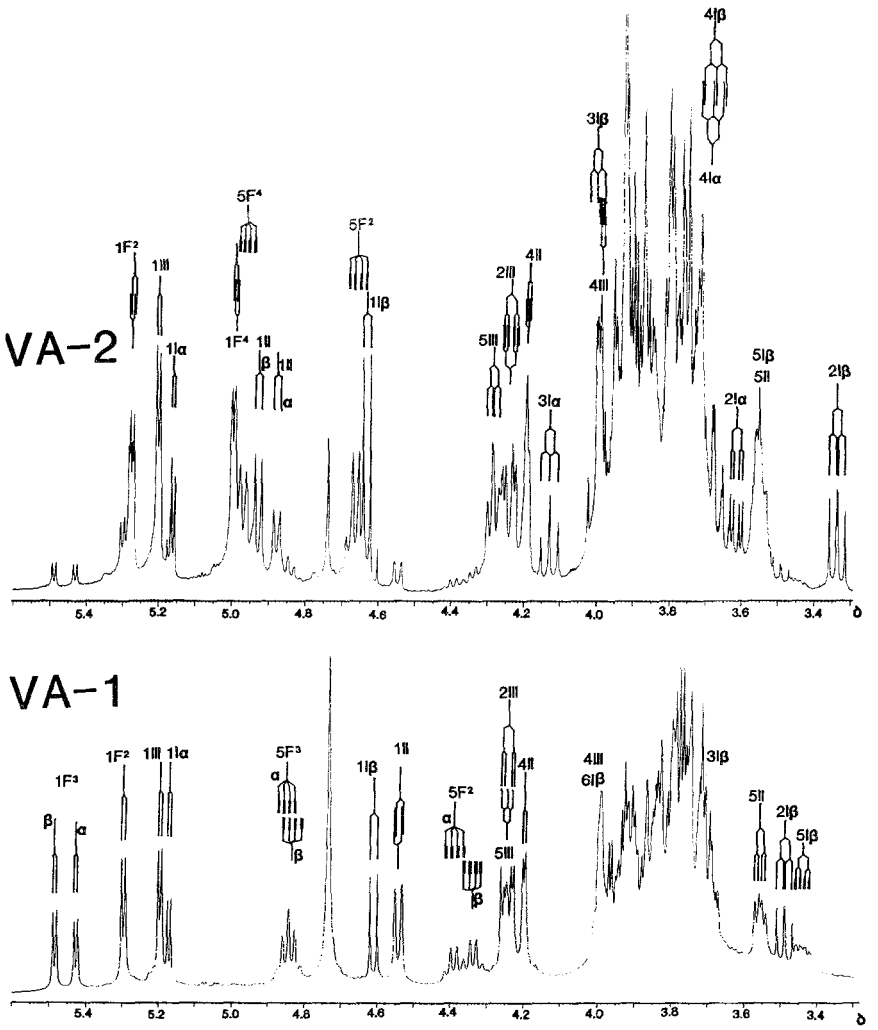


Figure 2. ^{1}H-NMR Spectra at 400 MHz of oligosaccharides VA-1 and VA-2. Arabic numbers: numbering of the proton in the ring. Roman numbers: I for the glucose, II for the galactose, III for the *N*-acetylgalactosamine. F: fucose. VA-2 is contaminated by VA-1.

effect. These values are in agreement with those found with a series of oligosaccharides bearing the blood group A determinant (unpublished results). Therefore, the examination of the successive COSY, Relayed COSY and Double Relayed COSY spectra (Fig. 4) allows correlation of the H-1/H-5 atoms of the α-(1-2)-linked fucose residue, and consequently, to recognize the α-(1-3)-linked fucose resonances. The H-1 signal from Fucα(1-3)- is strongly deshielded and doubled by the anomerization effect of the reducing glucose residue.

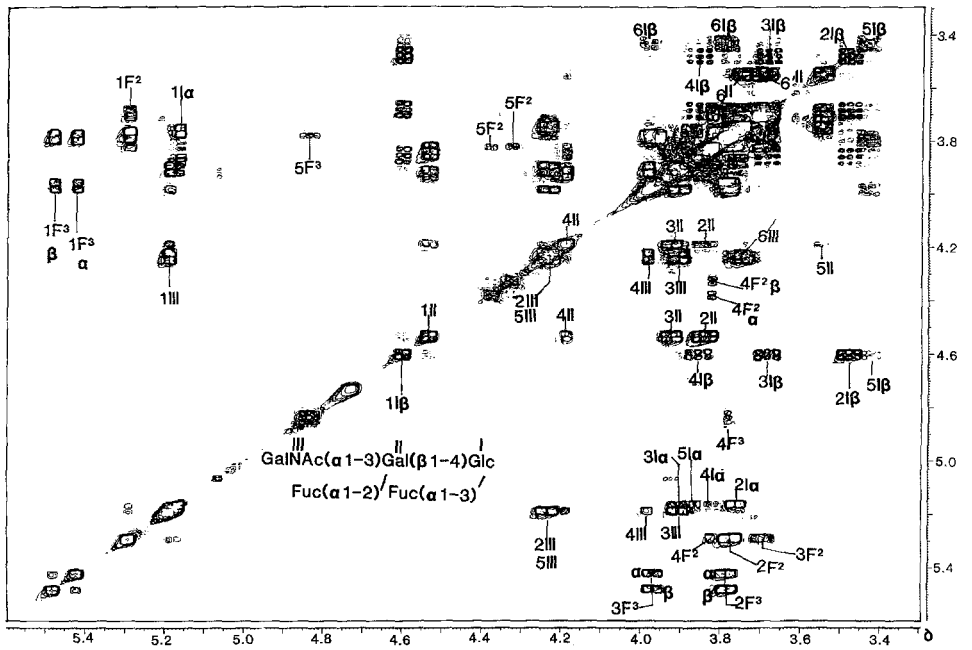


Figure 3. 400 MHz Triple-step-Relayed COSY spectrum of oligosaccharide VA-1.

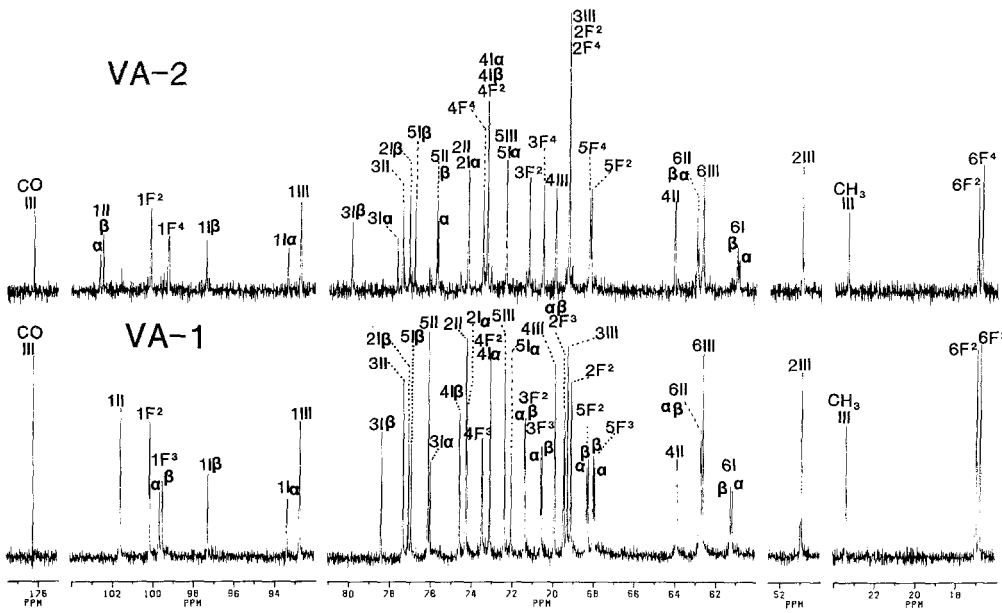


Figure 4. ^{13}C-NMR spectra at 100 MHz of oligosaccharides VA-1 and VA-2. For abbreviations, see Fig. 2.

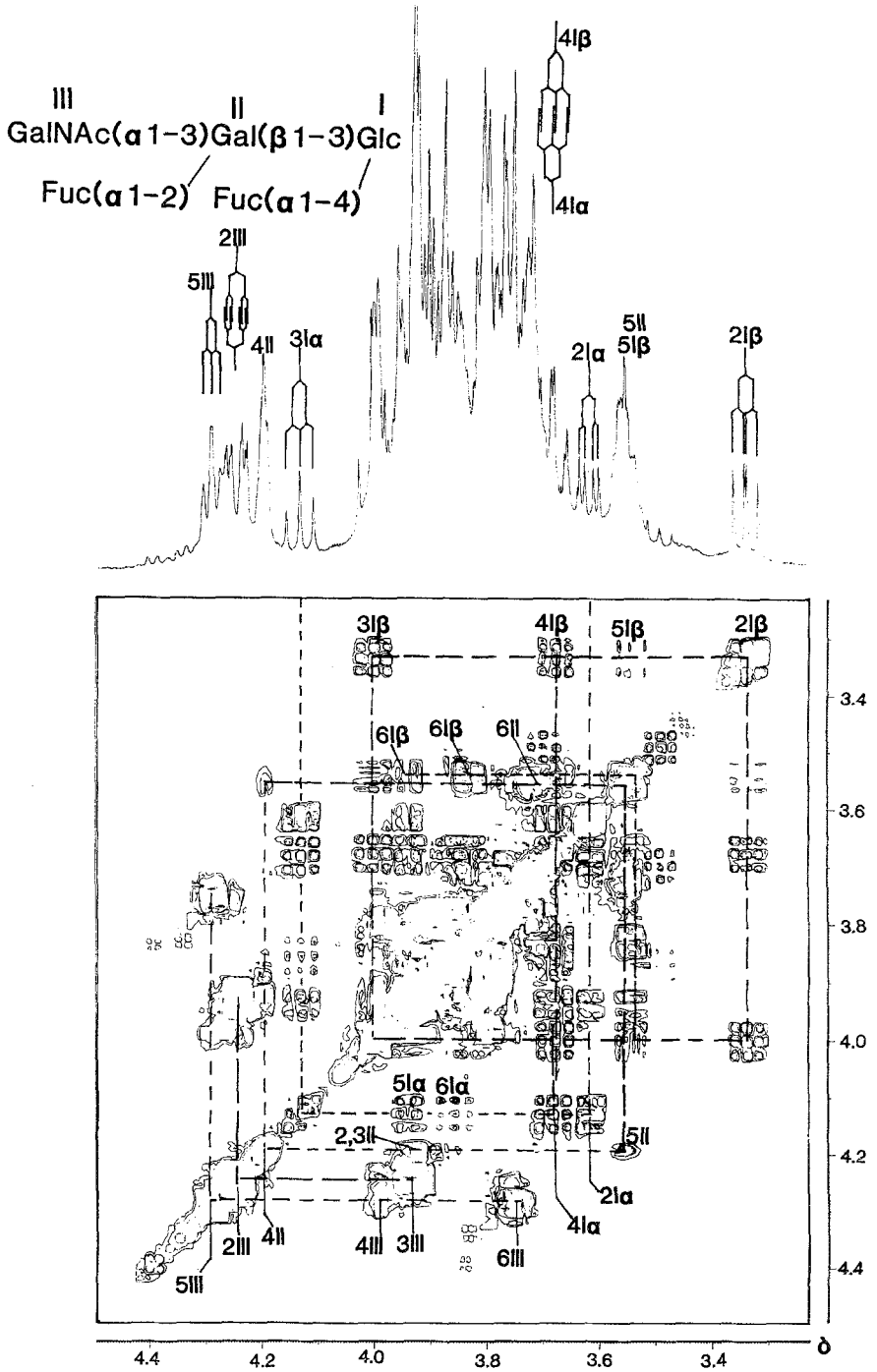


Figure 5. 400 MHz Two-step-Relayed COSY spectrum of oligosaccharide VA-2.

Table 2. ^{1}H-NMR chemical shifts of urinary oligosaccharides.

		VA-1	VA-2	VB-1	VB-2
Gal	H-1	4.544(α)	4.877(α)	4.563(α)	4.875(α)
(β1-4 or		4.541(β)	4.928(β)	4.561(β)	4.943(β)
1-3)	H-2	3.847	3.912(α)	3.858	3.912(α)
			3.903(β)		3.902(β)
	H-3	3.936	3.91	3.970	3.961(α)
					3.963(β)
	H-4	4.196	4.194(α)	4.259	4.259
			4.190(β)		
	H-5	3.555	3.55	3.596	3.596
	H-6	3.72	3.73	3.74	3.74
	H-6'	3.75	3.75	3.76	3.76
GalNAc	H-1	5.196	5.201	5.244	5.44
or	H-2	4.243	4.243(α)	3.880	3.880
Gal			4.239(β)		
(α1-3)	H-3	3.914	3.934	3.95	3.95
	H-4	3.990	3.998	3.95	3.95
	H-5	4.244	4.312	4.218	4.218
	H-6	3.73	3.73	3.72	3.72
	H-6'	3.77	3.77	3.75	3.75
	NAc	2.039	2.040	—	—
Glc α	H-1	5.170	5.159	5.171	5.157
	H-2	3.770	3.613	3.768	3.611
	H-3	3.890	4.125	3.898	4.122
	H-4	3.826	3.679	3.83	3.674
	H-5	3.905	3.935	3.92	3.930
	H-6,6'	3.87	3.860	3.87	3.952
Glc β	H-1	4.610	4.632	4.611	4.630
	H-2	3.489	3.335	3.487	3.335
	H-3	3.694	3.997	3.688	3.997
	H-4	3.867	3.674	3.87	3.68
	H-5	3.438	3.540	3.440	3.539
	H-6	3.805	3.826	3.992	3.94
	H-6'	3.995	3.936	3.683	3.83
Fuc[2]	H-1	5.296	5.278(α)	5.282	5.263(α)
			5.273(β)		5.258(β)
	H-2	3.784	3.777	3.790	3.790
	H-3	3.703	3.876	3.696	3.87
	H-4	3.833(α)	3.840	3.821	n.d.
		3.828(β)			
	H-5	4.388(α)	4.662	4.385(α)	4.665
		4.336(β)		4.331(β)	
	CH_3	1.292	1.291	1.285(α)	1.285(α)
				1.283(β)	1.272(β)
Fuc[3]	H-1	5.427(α)		5.417(α)	
		5.484(β)		5.474(β)	
	H-2	3.793		3.795	
	H-3	3.977		3.970	
	H-4	3.788		3.782	
	H-5	4.851(α)		4.872(α)	
		4.835(β)		4.854(β)	

Table 2 (continued)

		VA-1	VA-2	VB-1	VB-2
	CH_3	1.279(α) 1.276(β)		1.255(α) 1.251(β)	
Fuc[4]	H-1		4.998(α) 4.994(β)		4.988
	H-2		3.791		3.791
	H-3		3.907		3.907
	H-4		3.90		3.91
	H-5		4.969		4.970
	CH_3		1.295		1.267

The H-5 signals of *N*-acetylgalactosamine, galactose and β-Glc resonate away from the bulk of the proton resonances, which allows easy observation of the H-3, H-4, H-5 and H-6 connectivities. When reported on the ^1H-/^{13}C COSY spectrum, these values permit assignation all of the carbon atoms (Table 3 and Fig. 3).

For oligosaccharide VA-2, our attention was first drawn to the characteristic set of the H-2 and H-3 resonances of the glucose residue. The H-2 signal of α-Glc is shifted upfield away from the bulk at δ =3.613 ppm, while the H-3 resonance is downfield shifted at δ =4.125 ppm (Fig. 5). The H-2 and H-3 atoms of β-Glc are similarly shifted when compared with VA-1. The assignment of the fucose residues was made according to the fact that the H-5 atom of the α(1-2)-linked fucose residue resonates at a higher field than those of Fucα(1-4)-, and, consequently, the examination of the COSY spectra furnished most of the connectivities (result not shown).

Concerning the galactose and *N*-acetylgalactosamine residues, the occurrence of the H-5 resonances away from the bulk of the resonances, as in VA-1, largely facilitated the assignment of the connected protons.

The main characteristics of the ^{13}C-NMR spectrum of VA-2, in comparison with those of VA-1, are the downfield shift of the C-3 atom of both α-Glc and β-Glc residues (due to the attachment of galactose at C-3 of glucose), and the upfield shift of the C-4 atom of β-Glc. The anomerization effect observed in VA-1 for the signals of the α(1-3)-linked fucose is reported, in VA-2, to the C-1 and C-5 resonances of the galactose residue.

Structural Analysis of VB-1 and VB-2

The two isomers VB-1 and VB-2 were not efficiently purified on the ODS HPLC column, and, therefore, the mixture (12 mg) was directly analysed by NMR spectroscopy (Fig. 6 and Tables 2 and 3).

The ^1H- and ^{13}C-NMR spectra of VB-1 and VB-2 were found to be very similar to those observed for VA-1 and VA-2, with the exceptions concerning the H-2 and C-2 resonances of the α(1-3)-linked galactose residue (Tables 2 and 3).

Table 3. ^{13}C-NMR Chemical shifts of urinary oligosaccharides.

		VA-1	VA-2	VB-1	VB-2
Gal[II]	C-1	101.50	102.58(α)	101.52	102.61(α)
(β1-4 or			102.44(β)		102.48(β)
β1-3)	C-2	74.18	74.16	74.14	74.14
	C-3	77.25	77.36	77.61	77.75
	C-4	63.63	64.02	64.51(α)	64.67(α)
				64.48(β)	64.62(β)
	C-5	76.03	75.64(α)	75.85	75.85
			75.70(β)		
	C-6	62.69(α)	62.89(α)	62.70(α)	62.86(α)
		62.65(β)	62.94(β)	62.66(β)	62.92(β)
GalNAc	C-1	92.68	92.75	94.23	94.23
or	C-2	50.85	50.93	69.33	69.33
Gal	C-3	69.20	69.23	70.74	70.74
(α1-3)	C-4	69.82	69.88	70.57	70.57
	C-5	72.29	72.30	72.40	72.40
	C-6	62.56	62.64	62.51	62.57
	CO	176.13	176.16	-	-
	CH_3	23.32	23.33	-	-
Glcα	C-1	93.32	93.35	93.33	93.33
	C-2	74.15	74.16	74.15	74.02
	C-3	75.93	77.61	76.03	77.61
	C-4	73.01	73.23	73.02	73.21
	C-5	71.97	72.30	71.97	72.32
	C-6	61.11	60.87	61.10	61.24
Glcβ	C-1	97.20	97.35	97.22	97.35
	C-2	76.98	77.03	77.00	77.00
	C-3	78.32	79.84	78.42	79.85
	C-4	74.51	73.23	74.50	73.21
	C-5	76.85	76.77	76.87	76.76
	C-6	61.20	60.96	61.19	60.93
Fuc[2]	C-1	100.06	100.11	100.16	100.16
	C-2	69.03	69.23	69.05	69.24
	C-3	71.28	71.18	71.35	71.25
	C-4	73.01	73.23	73.02	73.21
	C-5	68.25(α)	68.14	68.20(α)	68.14(α)
		68.18(β)		68.14(β)	68.07(β)
	C-6	16.89	16.99	16.86	16.91
Fuc[3]	C-1	99.57(α)		99.66(α)	
		99.44(β)		99.54(β)	
	C-2	69.38(α)		69.38(α)	
		69.34(β)		69.33(β)	
	C-3	70.51(α)		70.51(α)	
		70.44(β)		70.44(β)	
	C-4	73.39(α)		73.43(α)	
		73.37(β)		73.41(β)	
	C-5	67.85(α)		67.82(α)	
		67.92(β)		67.89(β)	
	C-6	16.72		16.70	
Fuc[4]	C-1		99.24		99.22(α)
					99.27(β)
	C-2		69.23		69.26
	C-3		70.48		70.51
	C-4		73.43		73.43
	C-5		68.22		68.20
	C-6		16.78		16.73

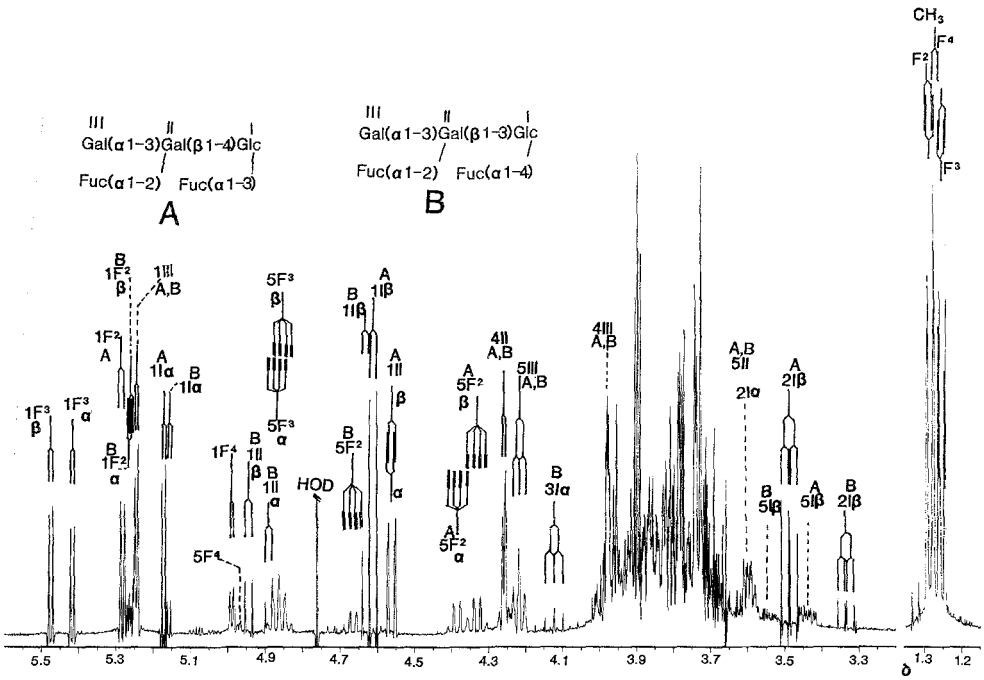


Figure 6. ^{1}H-NMR Spectrum at 400 MHz of the mixture of VB-1 (A) and VB-2 (B) (ratio 3:1).

Specific Inhibition of Erythrocyte Agglutination by Oligosaccharides

Direct hemagglutination assays with oligosaccharides (Table 4) showed that the oligosaccharides VA-1 and VA-2 are poor inhibitors of anti A_1, Lewis a and b antibodies, in comparison of the A active oligosaccharide III A and Le^b active oligosaccharide LNDFH I. These differences in inhibitory activities may be explain in terms of specific conformation of the epitopes, respectively, masked by α-GalNAc, for Lewis b or α(1-4)- or α(1-3)-linked fucose, for A determinants. The structures of VA-1 and VA-2 should be rather related to A Le^y and A Le^b determinants even though glucose replaces the *N*-acetylglucosamine. Specific monoclonal antibodies directed against authentic A Le^y and A Le^b epitopes have been previously defined by Clausen *et al.* [10].

Discussion and Conclusion

Our results point to the occurrence of a new core, Gal(β1-3)Glc, to which blood-group determinants can be transferred (see Fig. 7). Although the analogy between urine and milk oligosaccharides is evident, this core has never been observed for the latter. Milk oligosac-

Table 4. Hemagglutination inhibition assays.

Oligosaccharides		Erythrocytes/Antibodies			
		A_1/ Anti-A_1	A_1/ Anti-A_1	O Le^a/ Anti-Le^a	O Le^b/ Anti-Le^b
GalNAcα1-3Gal 2 Fucα1	III-A	60[a]	30	>2500	>2500
Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc 4 Fucα1	LNDFH	>2500	>2500	60	6
GalNAcα1-3Galβ1-4Glc 2 3 Fucα1 Fucα1	VA-1	500	500	500	500
GalNAcα1-3Galβ1-3Glc 2 4 Fucα1 Fucα1	VA-2	500	1000	500	125

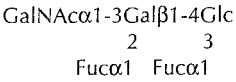
[a] μg of oligosaccharide giving a total inhibition of hemagglutination.

charides result from the activity of glycosyltransferases of the mammary gland toward lactose. Urinary oligosaccharides have a similar origin, since they are excreted in high yields during galactosemia caused by galactose diet [1, 3]. Nevertheless, they are devoid, in this case, of terminal reducing glucose, which indicates that galactose is the acceptor. Epithelial tissues of the intestine may catalyse such sugar transfers on free galactose or lactose. The occurrence of the Gal(β1-3)Glc sequence among the urinary material may result from a transgalactosylase activity of a lactase or a β-galactosidase as has been described for bacteria [11]. From a methodological point of view, our results, and particularly, the complete assignment of the ^{1}H- and ^{13}C-NMR spectra, were largely helped by the use of the Multiple Relayed COSY experiments, which furnished most of the ^{1}H-NMR data.

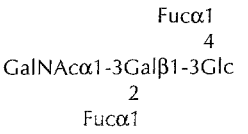
Note Added in Proof

After submitting this paper for publication, a report entitled "Structural analysis of five lactose-containing oligosaccharides by improved, high-resolution, two-dimensional ^{1}H-NMR spectroscopy", by Platzer N, Davoust D, Lhermitte M, Bauvy C, Meyer D and Derappe C (1989) Carbohydr. Res 191:191-207, described the ^{1}H-NMR parameters of oligosaccharide VA-1. The reported values are in good agreement with ours, except for the H-5 and H-6 resonances of α(1-2)- and α(1-3)-linked fucose residues. Nevertheless, our values are confirmed by the connectivities observed between the H-5 and H-4 resonances of α(1-3)-linked fucose, clearly observed on the COSY spectrum depicted in Fig. 3.

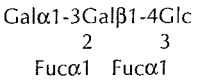
VA-1



VA-2



VB-1



VB-2

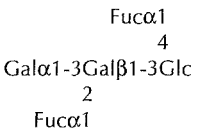


Figure 7. Structures of the urinary oligosaccharides.

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