Carbohydrate Research, 183 (1988) 19–34
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

SCOPE AND LIMITATIONS OF ROTATING-FRAME NUCLEAR OVERHAUSER ENHANCEMENT SPECTROSCOPY APPLIED TO OLIGOSACCHARIDES

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(Received February 24th, 1988; accepted for publication, April 19th, 1988)

ABSTRACT

Two-dimensional rotating-frame nuclear Overhauser enhancement spectroscopy has been applied to milk oligosaccharides containing the blood-group determinants Le^a, Le^d, or Le^b. The results indicate a large number of dipolar interactions that are consistent with the preferred conformations described for these blood-group determinants. Despite careful experimental conditions, coherent transfer processes are not suppressed completely, allowing only a qualitative evaluation of the nuclear Overhauser data. The ^{1}H- and ^{13}C-n.m.r. spectra are assigned by comparison with literature data and by application of two-dimensional ^{13}C–^{1}H shift-correlation spectroscopy.

INTRODUCTION

With the development of 2D-n.m.r. techniques[1,2] and the use of high-field n.m.r. spectrometers, the complete unravelling of ^{1}H- and ^{13}C-n.m.r. spectra of biomolecules up to the size of small proteins has come within reach. These n.m.r. assignments are important for structural analysis and are a prerequisite to detailed analysis of the conformation of these molecules in solution by n.O.e.-effects[3], which may be obtained in 1D or 2D experiments. However, n.O.e. measurements of oligosaccharides[4,5] and other medium-sized complex molecules are often hampered by an unfavourable value of $\omega_0\tau_c$, the product of the spectrometer angular frequency and the molecular rotational correlation time. As pointed out by Bothner-By *et al.*[6], the observation of transient n.O.e.-effects in the rotating frame overcomes this problem. In the 1D variant, this experiment starts with a 90° pulse, which is immediately followed by a 90°-phase-shifted long spin-locking pulse, which traps the initial equilibrium magnetization in the rotating frame along the spin-lock field. Analogous to the time of irradiation in an n.O.e.-experiment, this spin-locking is retained for a certain period (in the range 50–400 ms), during which time

0008-6215/88/$ 03.50

magnetization may be exchanged through dipolar interactions between protons that are in close proximity. The molecular re-orientation rates are fast compared to the effective Larmor frequency in the rotating frame, resulting in only positive n.O.e.-effects. For the normal n.O.e.-experiment, a 2D variant has been suggested and designated NOESY; analogously, for the spin-lock experiment, a 2D type of experiment has been suggested[6,7] and designated rotating-frame Overhauser enhancement spectroscopy (ROESY).

The unfavourable $\omega_0\tau_c$ condition for n.O.e. experiments with oligosaccharides may be alleviated by a change in temperature[5] or field strength. At the present state of technology, the latter possibility means a decrease in field strength, which will be accompanied by a loss in resolution. Change of temperature may result in enhancement of n.O.e.-efects, but, since the required temperature change can be quite substantial, such a method is not preferred. The rotating-frame n.O.e. experiment may now serve as a better alternative.

The conformations of the Le^a, Le^d, and Le^b blood-group determinants each attached to a synthetic spacer were the subject of an earlier study[8]. A combination of HSEA energy calculations[8,9] and n.m.r. observations indicated a preferred conformation for each of the three determinants. A complete analysis of the ^{1}H-n.m.r. spectra of three fucosylated lacto-*N*-tetraoses containing the above-mentioned structural elements is now presented. These assignments were obtained mainly in combination with the analysis of the respective ^{13}C-n.m.r. spectra and by application of ^{13}C–^{1}H heteronuclear correlation spectroscopy. The milk oligosaccharides are then used as model compounds to evaluate the potency of the ROESY experiment for conformational analysis of oligosaccharides in solution.

EXPERIMENTAL

The fucosylated lacto-*N*-tetraoses (**1**–**3**) were obtained as described[10]. The non-reducing end of each compound consists of a blood-group determinant, *i.e.* **1** Le^a (12 mg), **2** Le^d (28 mg, in combination with 22 mg of a fucosylated lacto-*N*-neotetraose, denoted **2′**, containing the Lewis-X determinant), and **3** Le^b (20 mg).

500-MHz ^{1}H-N.m.r. experiments were performed with a Bruker AM-500 spectrometer (Department of NMR Spectroscopy, University of Utrecht, or SON hf-NMR facility, Department of Biophysical Chemistry, University of Nijmegen) equipped with an Aspect-3000 computer. Prior to analysis, the samples were repeatedly treated with D_2O at pD 7 and at room temperature, with intermediate lyophilization, and finally dissolved in 99.96% D_2O (Aldrich). The experiments were performed at a probe temperature of 27°. Chemical shifts (δ) are expressed in p.p.m. downfield from that for 4,4-dimethyl-4-silapentane-1-sulfonate (DSS), but were measured by reference to internal acetone (δ 2.225 in D_2O at 27°). Resolution enhancement was achieved by Lorentzian-to-Gaussian transformation.

The 2D-^{1}H–^{1}H COSY experiment was performed with the pulse-sequence $90°–t_1–\Delta–90°–\Delta–acq(t_2)$, with the delays Δ inserted to enhance correlation transfer *via* long-range couplings[11]. The value of Δ was set at 0.4 s to optimize their detection for couplings less than 0.3 Hz[12]. The spectrum was obtained in the magnitude mode. A total of 1000 f.i.d.'s was obtained with 8 scans each, and saturation of the residual HDO line was applied during the recycle delay and during Δ. Prior to F.t., non-shifted sine-functions were applied in t_1 and t_2.

Prior to the ROESY experiments, the samples were degassed in the n.m.r. tube by repeated air evacuation and sealed under nitrogen; then acetone-d_6 (99.8% D, Merck) was added to a final concentration of 5% as a deuterium-lock. The ROESY experiments were performed using the sequence 90°–t_1–90°–SL–acq(t_2), wherein SL is a singular spin-lock pulse. An 8-pulse phase-program was applied[7], which included a 180° phase alternation for the spin-lock pulse between consecutive scans and cycling through the x, y, −x, and −y directions of the full pulse sequence. A 90° phase difference between the second 90° pulse and the spin-lock pulse was kept at all times. The application of TPPI[13] allowed for phase-sensitive handling of the data. The spin-lock pulse consisted of a single 200-ms pulse, with a field-strength of 2200 Hz, corresponding to a 90° pulse length of 110 μs. The spectral offset was at δ 4.90 in the ^{1}H-n.m.r. spectrum. A 512 × 4K data matrix was obtained, which was zero-filled to 2K × 4K prior to F.t. For resolution enhancement in t_2, a $\pi/4$ shifted sine-squared function was applied; in t_1 only a cosine-squared function was applied to suppress truncation artifacts. Any attempt to enhance the resolution in ω_1 introduced severe baseline artifacts for the diagonal peaks. A third-order polynomal base-line correction (Bruker software) was included after the first F.t.

The 50-MHz ^{13}C-n.m.r. experiments were performed at 27° with a Bruker WM-200 spectrometer equipped with a 5-mm broad-band probe-head, operating in the pulsed F.t. mode and controlled by an Aspect 2000 computer (SON hf-NMR-facility, Department of Biophysical Chemistry, Nijmegen University). ^{13}C-N.m.r. chemical shifts (δ) are expressed in p.p.m. relative to that of internal acetone at δ 31.55, with an accuracy of 0.02 p.p.m. The 2D-^{13}C–^{1}H COSY experiment was performed with simultaneous suppression of ^{1}H homonuclear couplings[14,15], using the standard Bruker pulse program XHCORRD, with the phase cycling of the refocussing pulse, as described by Wilde[16]. Refocussing delays, as required in the experiment, were adjusted to an average $^1J_{CH}$ coupling constant of 150 Hz[17]. The ^{1}H and ^{13}C 90° pulse-widths were 8 and 12 μs, respectively. The ^{1}H pulse-widths were determined as described[18]. A 96 × 4K data matrix was acquired which was zero-filled prior to F.t. to obtain a 2K × 8K spectral data matrix. A $\pi/4$ shifted sine-squared function for ^{13}C sub-spectra and a non-shifted sine-bell function for ^{1}H sub-spectra were applied to enhance resolution.

^{13}C-N.m.r. T_1 relaxation values were determined by the inversion-recovery method[19], the data of which were analyzed by a three-parameter fit. The interpretation of these values in terms of rotational mobility was according to Kuhlmann *et al.*[20].

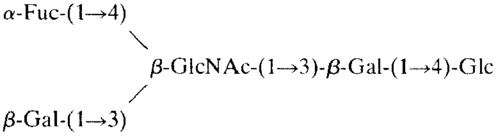
RESULTS AND DISCUSSION

The major parts of the 50-MHz ^{13}C-n.m.r. spectra of **1**, **2** (and **2′**), and **3** are assigned by comparison with data for synthetic compounds containing the same blood-group determinants[8,21] and by comparison with the assignments for lactose[18] (see Table I). For some nearly overlapping ^{13}C-n.m.r. signals, identification was obtained in combination with the ^{1}H-n.m.r. assignments after performing the 2D-^{13}C–^{1}H COSY experiments. This interplay of ^{1}H and ^{13}C signal identification is especially important for the mixture of **2** and **2′**. Since **2** and **2′** are present in unequal amounts, the identification of nearly overlapping signals is considerably facilitated, for the ^{13}C- as well as for the ^{1}H-n.m.r. spectra.

The 500-MHz ^{1}H-n.m.r. spectrum of **3** is displayed in Fig. 1. Starting from ^{13}C-n.m.r. assignments, the 2D-^{13}C–^{1}H COSY experiments for **1**, **2** (and **2′**), and **3** (see Fig. 2, spectrum of **3**) provided a complete list of ^{1}H-n.m.r. shift-positions for all four compounds (see Table II). Several proton signals outside the bulk-region of each ^{1}H-n.m.r. spectrum have been used for 1D spin-decoupling experiments, in order to obtain the chemical shifts of their *J*-coupled neighbouring protons. The results indicate an accuracy of ~0.003 p.p.m. for the ^{1}H chemical shifts in Table II, obtained from the 2D experiments, as was confirmed by the ROESY experiments. The assignments for the protons of the blood-group determinant structures for all compounds are in agreement with those reported for the synthetic compounds con-

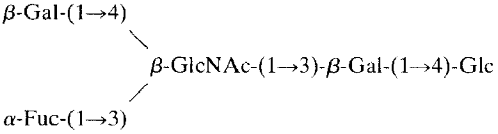
α-Fuc-(1→2)-β-Gal-(1→3)-β-GlcNAc-(1→3)-β-Gal-(1→4)-Glc

1



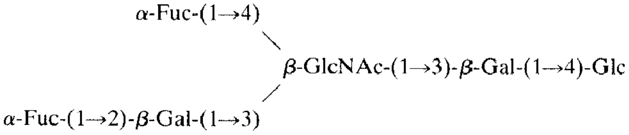
$$\begin{array}{l} \alpha\text{-Fuc-}(1\rightarrow4) \\ \qquad\quad\searrow \\ \qquad\qquad\beta\text{-GlcNAc-}(1\rightarrow3)\text{-}\beta\text{-Gal-}(1\rightarrow4)\text{-Glc} \\ \qquad\quad\nearrow \\ \beta\text{-Gal-}(1\rightarrow3) \end{array}$$

2



$$\begin{array}{l} \beta\text{-Gal-}(1\rightarrow4) \\ \qquad\quad\searrow \\ \qquad\qquad\beta\text{-GlcNAc-}(1\rightarrow3)\text{-}\beta\text{-Gal-}(1\rightarrow4)\text{-Glc} \\ \qquad\quad\nearrow \\ \alpha\text{-Fuc-}(1\rightarrow3) \end{array}$$

2′



$$\begin{array}{l} \qquad\qquad\alpha\text{-Fuc-}(1\rightarrow4) \\ \qquad\qquad\qquad\quad\searrow \\ \qquad\qquad\qquad\qquad\beta\text{-GlcNAc-}(1\rightarrow3)\text{-}\beta\text{-Gal-}(1\rightarrow4)\text{-Glc} \\ \qquad\qquad\qquad\quad\nearrow \\ \alpha\text{-Fuc-}(1\rightarrow2)\text{-}\beta\text{-Gal-}(1\rightarrow3) \end{array}$$

3

TABLE I

^{13}C-N.M.R. CHEMICAL SHIFTS[a] (δ) OF **1–3** TOGETHER WITH THE CHEMICAL SHIFT DIFFERENCES (× 100) BETWEEN **3** AND **1** AND BETWEEN **3** AND **2**

Atom[b]		δ(**1**)	δ(**2**)	δ(**3**)	δ(**3**) − δ(**1**)	δ(**3**) − δ(**2**)	δ(**2'**)
Glc	1α	93.11	93.08	93.10	(−1)	(2)	93.08
	2α	72.45	72.42	72.45	(0)	(3)	72.42
	3α	72.68	72.67	72.67	(−1)	(0)	72.67
	4α	79.59	79.71	79.60	(1)	(−9)	79.71
	5α	71.45	71.39	71.46	(1)	(7)	71.39
	6α	61.28	61.26	61.27	(−1)	(1)	61.26
Glc	1β	97.04	97.01	97.03	(−1)	(2)	97.01
	2β	75.11	75.08	75.10	(−1)	(2)	75.08
	3β	75.64	75.63	75.63	(−1)	(0)	75.63
	4β	79.51	79.60	79.51	(0)	(−9)	79.60
	5β	76.13	76.15	76.11	(−2)	(−4)	76.15
	6β	61.40	61.38	61.40	(0)	(2)	61.38
Gal^{4}	1	104.25	104.19	104.26	(1)	(7)	104.19
	2	71.51	71.25	71.46	(−5)	(19)	71.25
	3	82.88	83.32	82.85	(−3)	(−47)	83.32
	4	69.89	69.62	69.91	(2)	(29)	69.62
	5	76.13	76.15[c]	76.11	(−2)	(−2)	76.06[c]
	6	62.26	62.24	62.26	(0)	(0)	62.24
GlcNAc	1	104.52	103.84	104.51	(−1)	(−33)	103.76
	2	56.28	57.15	57.05	(77)	(−10)	57.24
	3	78.51	77.20	75.79	(−272)	(−141)	76.41
	4	69.79	73.42	73.10	(331)	(−22)	73.62
	5	76.57	76.51	76.49	(−8)	(−2)	76.15
	6	61.75	60.92	60.80	(−95)	(12)	60.92
	NAc	23.48	23.60	23.50	(2)	(−10)	23.57
	C=O	175.48	175.96	175.41	(−7)	(−55)	175.89
Gal^{3}	1	101.55	104.09	101.92	(37)	(−217)	
	2	77.96	71.79	77.79	(−17)	(600)	
	3	74.81	73.77	74.95	(14)	(118)	
	4	70.44	69.62	70.05	(−39)	(33)	
	5	76.38	76.06	76.06	(−32)	(0)	
	6	62.45	62.91	62.89	(44)	(−2)	
Fuc^{2}	1	100.81		100.85	(4)		
	2	69.36		69.58	(22)		
	3	70.75		70.76	(1)		
	4	73.17		73.29	(12)		
	5	67.79		67.54	(−25)		
	6	16.59		16.63	(4)		
Fuc^{4}	1		99.28	99.07		(−21)	
	2		69.13	69.12		(−1)	
	3		70.42	70.42		(0)	
	4		73.22	73.29		(7)	
	5		68.10	68.31		(21)	
	6		16.69	16.69		(0)	
Gal^{4,3}	1						103.04
	2						72.33
	3						74.36
	4						69.62
	5						76.15
	6						62.75

TABLE I (*continued*)

Atom[b]		$\delta(\mathbf{1})$	$\delta(\mathbf{2})$	$\delta(\mathbf{3})$	$\delta(\mathbf{3}) - \delta(\mathbf{1})$	$\delta(\mathbf{3}) - \delta(\mathbf{2})$	$\delta(\mathbf{2'})$
Fuc^3	1						99.85
	2						69.00
	3						70.49
	4						73.22
	5						67.95
	6						16.63

[a] Expressed relative to the signal for acetone at δ 31.55. [b] A superscript at the name of a sugar residue indicates to which position of the adjacent monosaccharide it is glycosidically linked. [c] Assignments may have to be interchanged.

taining the same determinants[8,21]. Apart from a constant deviation, which is probably due to a differing reference signal, the assignments for **2**′ are in good agreement with previously reported data[21]. A partial assignment for the protons of **1** as presented earlier[7] is in agreement with the present results, except for a reversal of the positions for H-3 of Gal^3 and Gal^4. A drawback of the 2D-^{13}C–^{1}H COSY experiment is the rather poor result for the signals of the hydroxymethyl group. In carbohydrates, the protons of the hydroxymethyl groups usually have resonance positions close to each other, rendering a distorted line-shape in the ^{1}H shift-domain due to higher order effects. Furthermore, in the present 2D-^{13}C–^{1}H COSY experiment, geminal ^{1}H–^{1}H *J*-couplings are not removed[15]. When H-6 and H-6′ of a sugar residue could not be recognized separately, only the average chemical shifts are given for both protons (see Table II); no attempt has been made to identify their exact positions.

The ^{13}C relaxation studies for **3** indicate the average T_1-value for the skeleton carbon atoms to be: Glc, 0.23; Gal^4, 0.20; Gal^3 and GlcNAc, 0.14; Fuc^2 and Fuc^4, 0.15 s. The T_1 value for the CH$_3$ group of GlcNAc, Fuc^2, and Fuc^4 is, taking into account the number of attached protons, larger by a factor of 5 to 9. For the major part of the molecule, τ_c is therefore in the order of 3.5×10^{-10}, indicating $\omega_0\tau_c$ to be 1.1 at 500 MHz. This value predicts very low intensity n.O.e.-effects and, indeed, a NOESY experiment at 500 MHz for **3** did not give any measurable cross-peaks apart from some small-intensity intra-residue n.O.e.-effects.

For the ROESY experiments, the pulse scheme is basically the same as for a homonuclear Hartmann–Hahn[22] experiment (HOHAHA), although, in the former experiment, the magnetization transfer is meant to be effected by incoherent dipolar interactions, whereas the latter experiment aims at transfer by coherent interactions through scalar coupling. Consequently, together with ROESY cross-peaks, Hartmann–Hahn type cross-peaks will also be present in the spectrum. The latter cross-peaks pose a serious problem, because ROESY cross-peaks are negative, whereas Hartmann–Hahn type cross-peaks are positive. The interference of the two mechanisms affects the intensity of cross-peaks from scalar-coupled

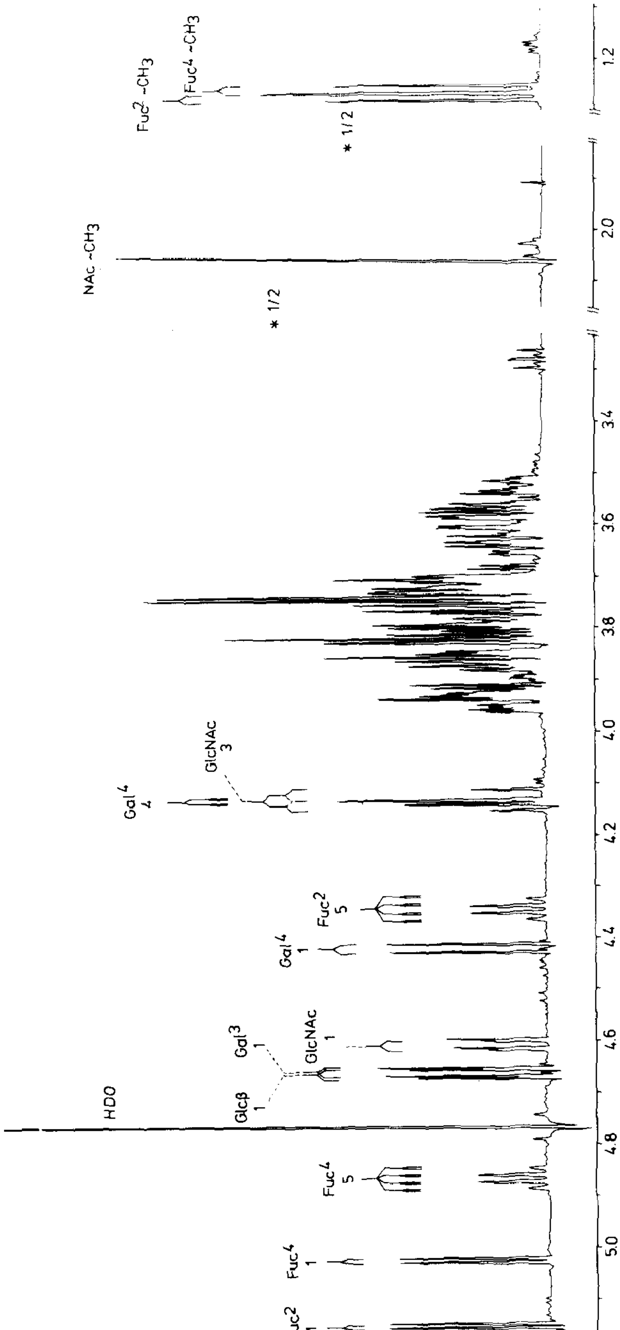


Fig. 1. 500-MHz ^{1}H-n.m.r. spectrum of **3**.

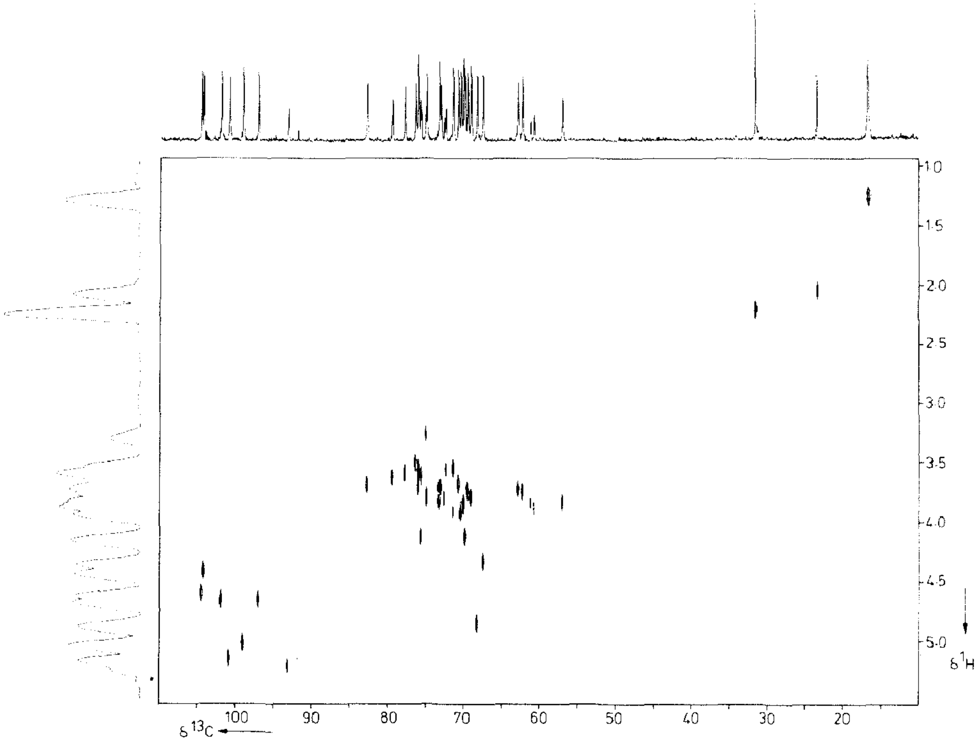


Fig. 2. 2D-^{13}C–^1H COSY spectrum of **3** at a ^1H-n.m.r. frequency of 200 MHz. Included are the projections in the separate dimensions.

TABLE II

^1H-N.M.R. CHEMICAL SHIFTS[a] (δ) FOR COMPOUNDS **1–3**

Carbon atom[b]		*Compound*			
		1	**2**	**3**	**2'**
Glc	1α	5.218	5.219	5.217	5.219
	2α	3.588	3.584	3.573	3.584
	3α	3.819	3.826	3.821	3.826
	4α	3.648	3.650	3.636	3.650
	5α	3.938	3.935	3.935	3.935
	6α	3.855	3.861	3.859	3.861
Glc	1β	4.660	4.661	4.660	4.661
	2β	3.274	3.286	3.263	3.286
	3β	3.633	3.632	3.632	3.632
	4β	3.649	3.651	3.643	3.651
	5β	3.596	3.600	3.57	3.600
	6β				
Gal^4	1	4.419	4.434	4.416	4.434
	2	3.572	3.584	3.567	3.584
	3	3.741	3.719	3.708	3.719
	4	4.137	4.155	4.130	4.155
	5	3.721	3.716	3.708	3.716
	6	3.761	3.769	3.759	3.769

TABLE II *(continued)*

Carbon atom[b]		*Compound*			
		1	**2**	**3**	**2′**
GlcNAc	1	4.626	4.704	4.605	4.719
	2	3.824	3.949	3.846	3.958
	3	3.992	4.077	4.130	3.876
	4	3.530	3.755	3.728	3.627
	5	3.495	3.548	3.517	3.548
	6	3.816	3.918	3.861[c]	3.918
	6′			3.924[c]	
	NAc	2.060	2.026	2.064	2.026
Gal^3	1	4.645	4.490	4.659	
	2	3.598	3.482	3.603	
	3	3.832	3.650	3.805	
	4	3.892	3.892	3.862	
	5	3.679	3.63	3.576	
	6	3.770	3.733	3.737	
Fuc^2	1	5.188		5.150	
	2	3.778		3.751	
	3	3.665		3.693	
	4	3.741		3.744	
	5	4.293		4.339	
	6	1.241		1.269	
Fuc^4	1		5.023	5.018	
	2		3.800	3.804	
	3		3.895	3.929	
	4		3.792	3.823	
	5		4.871	4.860	
	6		1.178	1.253	
Gal^{4,3}	1				4.463
	2				3.493
	3				3.956
	4				3.892
	5				n.d.
	6				3.730
Fuc^3	1				5.128
	2				3.691
	3				3.895
	4				3.793
	5				4.832
	6				1.173

[a] Chemical shifts are relative to the signal of DSS (using internal acetone at δ 2.225) in D_2O. [b] A superscript at the name of a sugar-residue indicates to which position of the adjacent monosaccharide it is glycosidically linked. [c] From ROESY experiment.

protons. Therefore, the conditions for the ROESY experiments must be selected to minimize any coherent magnetization transfer processes[7]. Coherent magnetization transfer due to the Hartmann–Hahn mechanism between protons i and j is dependent on the effective field-strength for these protons and thereby on the absolute difference in their respective offsets[7], $|\Delta f_i|$ and $|\Delta f_j|$. For this reason, the offset is positioned at the far low side of the spectrum, in combination with a minimal spin-lock field strength. Composite spin-lock pulse-sequences have been designed to compensate for these undesired offset differences[23] and, thus, the use of a singular spin-lock pulse is obvious. In addition to the Hartmann–Hahn type cross-peaks, COSY type cross-peaks[6] might add to the 2D-n.m.r. spectrum. Because the integral over a COSY type multiplet is zero, these contributions are cancelled by integration over a cross-peak or simply by adding all columns or rows that contribute to that cross-peak.

The ROESY spectrum of **3** with a 200-ms mixing time is depicted in Fig. 3. With the offset at the far low end of the spectrum, the condition $|\Delta f_i| = |\Delta f_j|$ will be met relatively easily only for the skeleton protons since their resonance positions are close to each other (δ 3.5–4.0). No significant positive cross-peaks but a limited

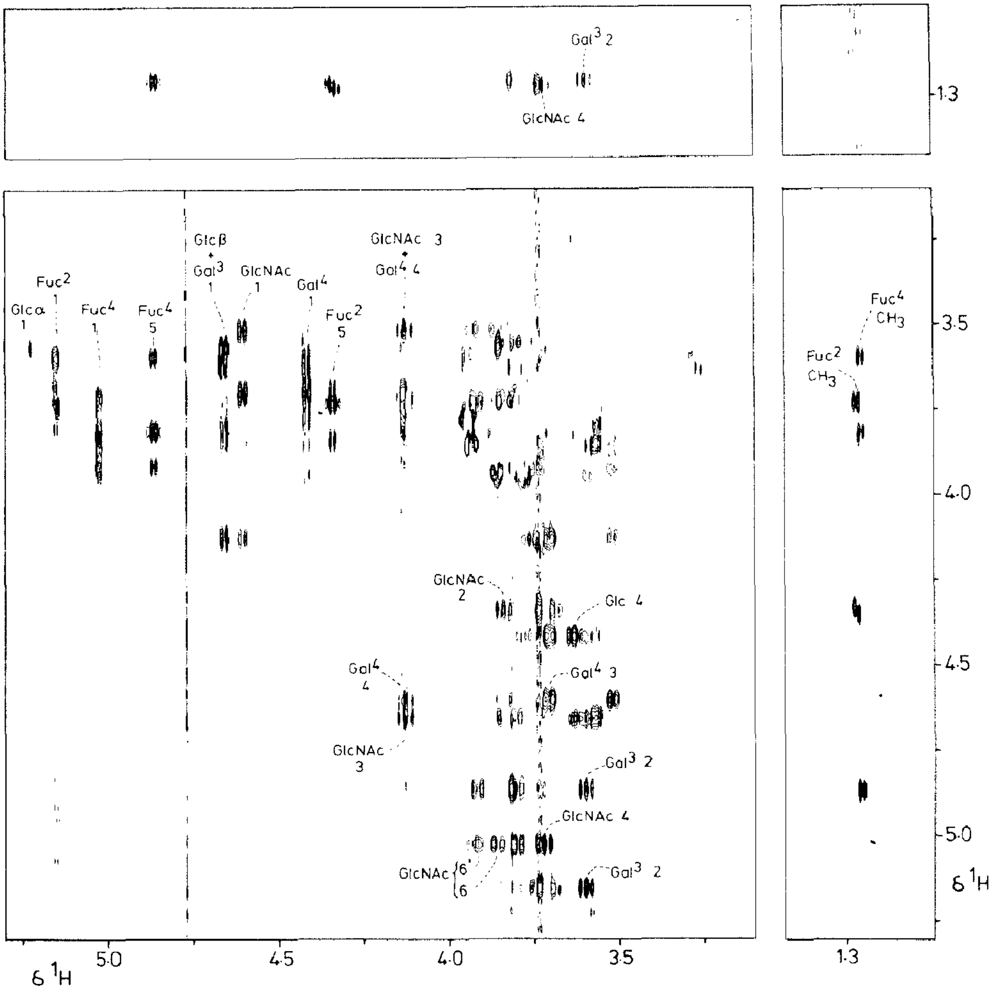


Fig. 3. ROESY spectrum of **3**, with a mixing time of 200 ms. Only the negative peaks are shown for the regions of anomeric and skeleton protons between 5.3 and 3.1 p.p.m. and of the Fuc CH_3 protons.

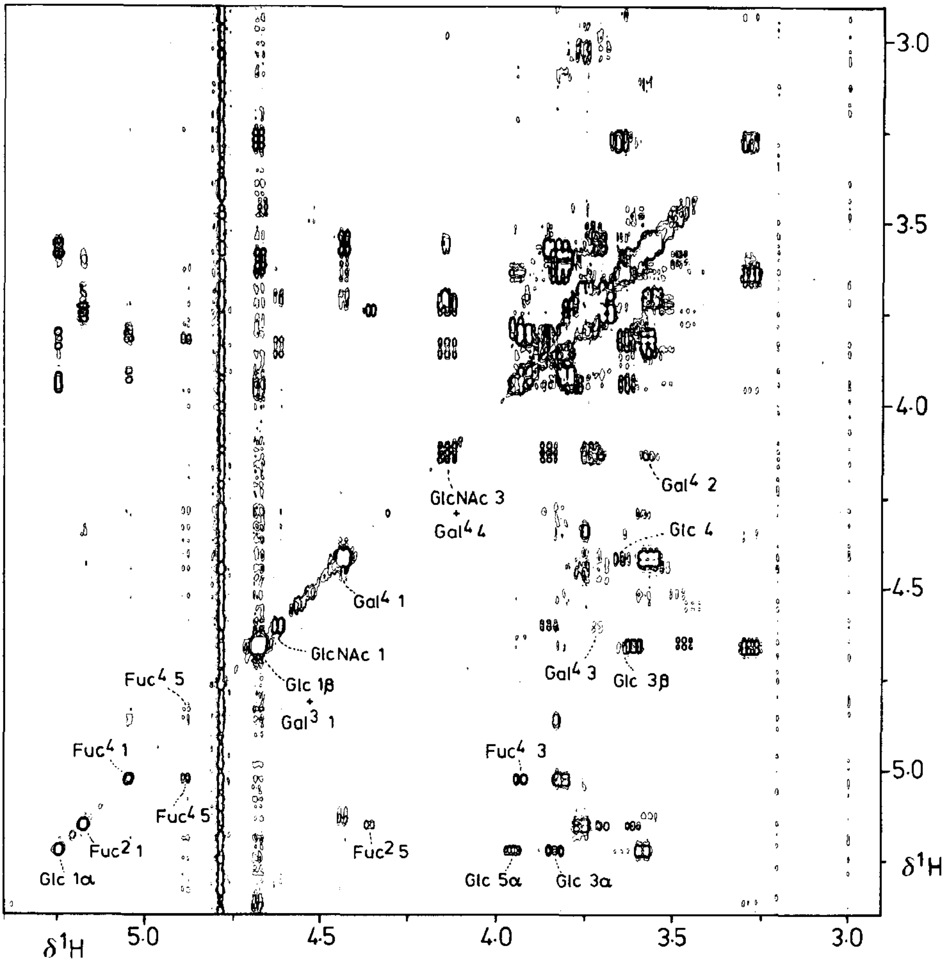


Fig. 4. 2D-^{1}H–^{1}H COSY spectrum of **3**, with the experiment optimized for detection of cross-correlations through long-range J-couplings by insertion of two delays of 0.4 s (see Experimental).

number of *negative* cross-peaks are observed in this region, indicating that here the Hartmann–Hahn condition is still met relatively well and many, mostly intraresidue, n.O.e.-effects are annulled by Hartmann–Hahn-effects with an opposite sign. An unexpected *positive* cross-peak with an intensity in the order of the ROESY effects is present between Fuc^4 H-1 and H-5 (not shown). This cross-peak is due to a small long-range coupling between these protons across the ring-oxygen atom and the position of the spectral offset relative to the chemical shifts of these protons being very favourable for Hartmann–Hahn transfer. This long-range coupling for Fuc^4 is proven by the ^{1}H–^{1}H COSY experiment on **3**, designed to enhance cross-peaks from small couplings (see Fig. 4). This spectrum not only displays a coupling between H-1 and H-5 for Fuc^4, but also shows several other ^4J and ^5J couplings. By consequence, *via* ^4J and ^5J coupling between nearly overlapping signals, the Hartmann–Hahn magnetization transfer process may be a more frequently occurring complicating factor for the application of the ROESY experiment than a first inspection of chemical shifts and ^3J coupling constants would suggest. The occurrence of such couplings for oligosaccharides has been demonstrated[12].

From the foregoing, it is concluded that the experimental conditions are sufficient to suppress the Hartmann–Hahn transfer mechanism, to an extent that n.O.e.-effects are dominant in most cases. In the expanded regions of the ROESY spectrum of **3** (see Fig. 3), a large number of cross-peaks are identified from their position along the ω_1-axis (see Table III). Analogous to the NOESY experiment, many cross-peaks are present as a consequence of intra-residue n.O.e.-effects (not indicated in Fig. 3; for clarity, only the positions of the diagonal peaks and peaks that stem from interglycosidic n.O.e.-effects are indicated). In Fig. 3, the anomeric protons of the α-residues show effects only on their H-2 and H-3, the former effect being the larger, whereas for all β-residues large effects are observed from H-1 on their H-3 and H-5 and a small effect on their H-2. For the Fuc residues, any interactions of H-1 with H-4 cannot be excluded since H-2 and H-4 coincide. These intra-residue effects are qualitatively in agreement with the proton–proton distances and do not point to significant contributions of HOHAHA relay artifacts[24,25]. Apart from these intra-residue n.O.e.-effects, several interglycosidic effects are observed (see Fig. 3). These are from H-1 of each residue to protons on the other side of the glycosidic linkage, *i.e.*, from Gal^4 H-1 to Glc H-4, from GlcNAc H-1 to Gal^4 H-3 and H-4, from Gal^3 H-1 to GlcNAc H-3, from Fuc^4 H-1 to GlcNAc H-4, H-6, and H-6′, and from Fuc^2 H-1 to Gal^3 H-2 and H-3. In all these cases, the intensity of the n.O.e.-effect on the proton of the substituted carbon atom is the largest, in accordance with the linkage positions. This renders

TABLE III

CROSS-PEAKS IDENTIFIED IN THE ROESY SPECTRUM OF **3** (SEE FIGS. 3 AND 5)

Diagonal peak	*N.O.e. effect*
Glc 1α	Glc 2α, Glc 3α
Glc 1β	Glc 2β, Glc 3α, Glc 5β
Gal^4 1	Gal^4 2, Gal^4 3, Gal^4 5, Glc 4α, Glc 4β
Gal^4 4	Gal^4 3, Gal^4 5, GlcNAc 1
GlcNAc 1	GlcNAc 2, GlcNAc 3, GlcNAc 5, Gal^4 3, Gal^4 4
GlcNAc 3	GlcNAc 1, GlcNAc 5, Gal^3 1
GlcNAc CH$_3$	Gal^3 1, Fuc^2 2, Fuc^2 3
Gal^3 1	Gal^3 2, Gal^3 3, Gal^3 4, Gal^3 5, GlcNAc 3
Fuc^2 1	Fuc^2 2, Fuc^2 3, Gal^3 2, Gal^3 3
Fuc^2 5	Fuc^2 3, Fuc^2 4, Fuc^2 CH$_3$, GlcNAc 2
Fuc^2 CH$_3$	Fuc^2 4, Fuc^2 5, GlcNAc 4
Fuc^4 1	Fuc^4 2, Fuc^4 3, GlcNAc 4, GlcNAc 6, GlcNAc 6′
Fuc^4 5	Fuc^4 3, Fuc^4 4, Fuc^4 CH$_3$, Gal^3 2
Fuc^4 CH$_3$	Fuc^4 4, Fuc^4 5, Gal^3 2

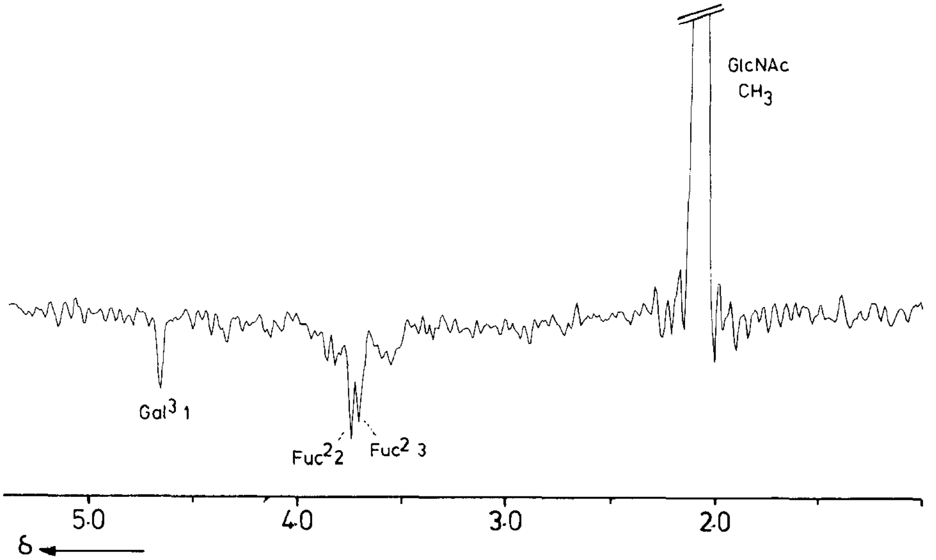


Fig. 5. Added cross-section in ω_1 of the 500-MHz ROESY spectrum of **3** at the GlcNAc CH_3 chemical shift.

the ROESY experiment equally suitable for structure determination as the NOESY experiment[26].

In addition to these "obvious" n.O.e.-effects, dipolar connectivities are observed that are more remote and are indicative of the overall conformation of **3**. These effects are from Fuc^4 CH_3 and H-5 to Gal^3 H-2, from Fuc^2 CH_3 to GlcNAc H-4, from Fuc^2 H-5 to GlcNAc H-2, and from GlcNAc CH_3 to Fuc^2 H-2 and H-3 and to Gal^3 H-1. The n.O.e.-effects from GlcNAc CH_3 are small, but observable in both ω_1 (Fig. 5) and ω_2 dimensions. Fuc^2 H-2 and H-3 are strongly coupled, and inspection of a molecular model of **3** suggests that the n.O.e.-effect from GlcNAc CH_3 is probably only on H-3. To account for the n.O.e.-effects involving the NAc group, its orientation must be such that the N–H proton is nearly *trans* with respect to GlcNAc H-2.

The conformation of a synthetic oligosaccharide comprising the Lewis^b determinant has been estimated before by a combination of HSEA energy calculations and n.m.r. measurements, including n.O.e.-effects[8]. To support the calculated conformation, only the n.O.e.-effects of Gal^4 H-1 to GlcNAc H-6 and H-6′ (not resolved) and of Fuc^4 H-5 to Gal^3 H-2 were described. In the present analysis, these interactions are confirmed by the ROESY experiment, but several other interglycosidic n.O.e.-effects are observed in addition. However, these effects are in general accord with the conformation that was predicted by the HSEA calculations[8], *i.e.*, the Fuc^2 residue is folded backwards across the GlcNAc residue. The HSEA energy-calculations demonstrated the interaction of the NAc group with Fuc^2 to be important, but, since the orientation of this acetamido group was

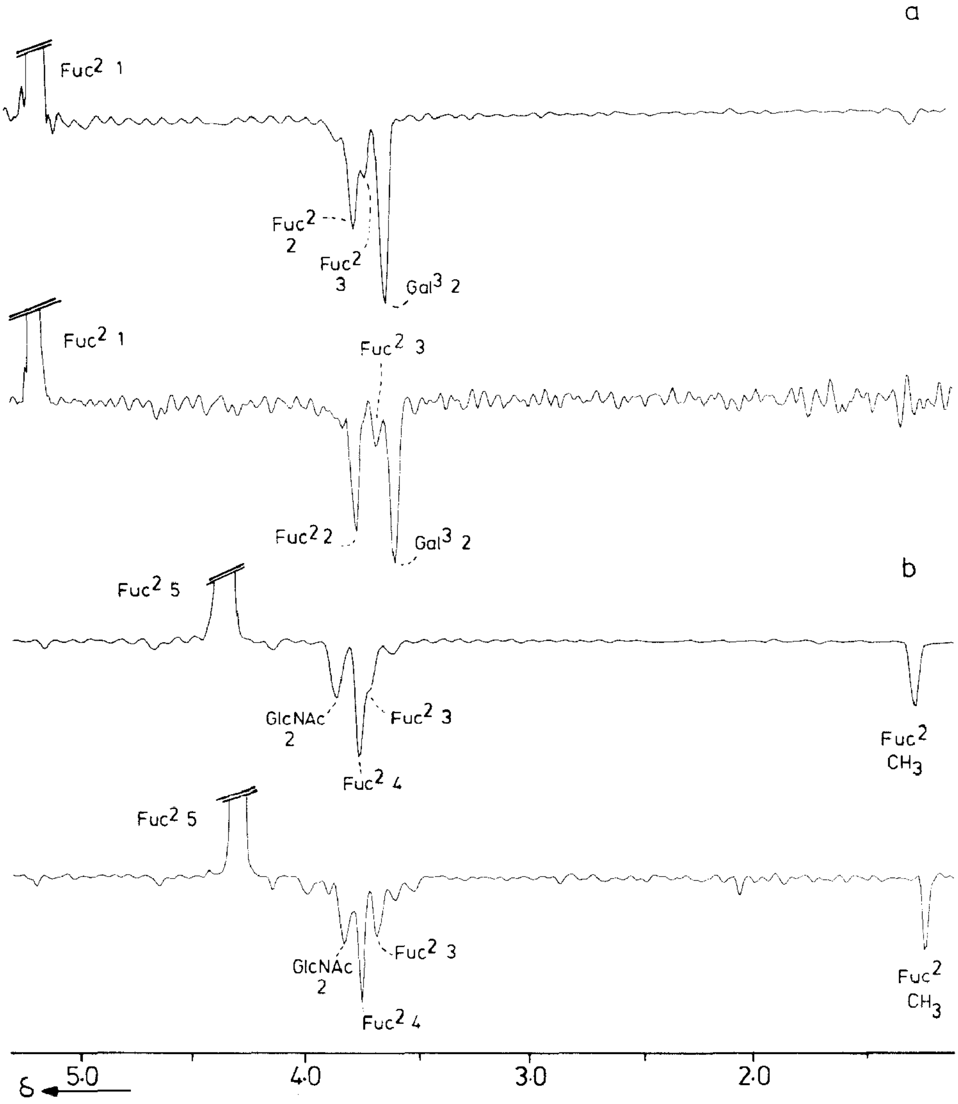


Fig. 6. Added ω_I-cross-sections from the 500-MHz ROESY spectra of compounds **3** (upper) and **1** (lower) for Fuc^2 H-1 (a) and Fuc^2 H-5 (b).

chosen arbitrarily, no significance was attached to the interaction. However, the present results confirm the involvement of the NAc group in the conformation. The HSEA calculations predicted a distance of ~4Å between the two Fuc CH_3 groups, but the ROESY experiment does not indicate any interaction between the two Fuc residues.

A ROESY spectrum of **1**, obtained under identical conditions as for **3**, shows features that are very similar to those for **3**, including the interglycosidic n.O.e.-effects related to the linkage positions. In addition, the n.O.e.-effects of Fuc^2 H-5 on GlcNAc H-4 and of Fuc CH_3 on GlcNAc H-2 are observed for **1**, with similar intensities as for **3**. Owing to an increased noise level for the spectrum, no cross-peaks are observed for the NAc signal. Cross-sections in ω_1 of the ROESY spectra of **1** and **3** for Fuc^2 H-1 and H-5 are shown in Fig. 6. The very similar intensities of the interglycosidic n.O.e.-effects for these Fuc^2 protons are strong evidence that the orientation of the α-Fuc-(1→2)-β-Gal-(1→3) structural unit in **1** is retained in **3**, despite the attachment of α-Fuc-(1→4) to GlcNAc.

From Table I, it can be seen that, on completion of **3** by the introduction of Fuc^4 into **1**, the ^{13}C-n.m.r. shift-effects (see Table I) are confined to signals of GlcNAc, Gal^3, and Fuc^4, without changing the Fuc^2 chemical shift positions. On the other hand, when completing **3** by the introduction of Fuc^2 into the monofucosylated compound **2**, ^{13}C-n.m.r. shift-effects are observed for Gal^4 C-2, C-3, and C-4, as well as for Glc C-4α and C-4β. These effects can be understood by taking into account the backfolding of the Fuc^2 residue along GlcNAc. The mechanism of the long-range effects on Glc are uncertain, but are probably connected with a slight reorientation of the β-GlcNAc-(1→4)-Gal linkage by steric interaction with Fuc^2, possibly *via* the NAc group. This interaction is retained in the presence of Fuc^4, as is evidenced further by the identical ^{13}C- and ^1H shifts in **1** and **3** for the NAc group.

Concluding remarks. — The extensive ^3J, ^4J, and ^5J coupling systems of carbohydrates, with concomitant possibilities for strong coupling between skeleton protons, render the HOHAHA mechanism, and relay artifacts derived therefrom[24,25], a drawback of the ROESY experiment for quantitative conformation analysis. The dependence of intra-residue n.O.e.-intensities on experimental conditions prevents their use for distance calibration for inter-residue n.O.e.-interactions. Experimental conditions should be devised that circumvent the HOHAHA mechanism. Recently[27], a 2D-rotating-frame experiment has been described, wherein the mixing time consists of a repetitive pulse-sequence with the period ($\beta - \tau$), wherein β is ~30° pulse and $\tau \sim 10 \times \beta$, determining the duty cycle. This composite spin-lock pulse results in a substantial reduction of HOHAHA cross-peaks in contrast to the ROESY peaks. As an additional complication for quantitative analysis of the ROESY experiment described here, with the spectral offset at the far low side of the spectrum, the degree of spin-locking and the rotating-frame relaxation rate may vary considerably across the spectrum[28,29].

The results presented in this paper indicate that, qualitatively, the ROESY experiment is suitable as a substitute for the NOESY experiment for analysis of neutral oligosaccharides in case the latter experiment does not give usable results, *i.e.*, in the molecular mass range up to 1500. For charged oligosaccharides or glycopeptides, an increased "drag" from the water-contact may produce an apparently larger molecular mass[30] and negative n.O.e.-effects may be observable for smaller molecules.

ACKNOWLEDGMENT

This research was supported by the Netherlands Foundation for Chemical Research (SON/ZWO).

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