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Sequential ^{1}H and ^{13}C Resonance Assignments for an Octa- and Decasaccharide of the *N*-Acetyllactosamine Type by Multiple-Step Relayed Correlation and Heteronuclear Correlation Nuclear Magnetic Resonance

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400 MHz ^{1}H-NMR and 100 MHz ^{13}C-NMR spectra of a neutral octasaccharide and of a disialyldecasaccharide of the *N*-acetyllactosamine type were studied. The resonance assignments were made by combining multiple-relayed coherence-transfer chemical-shift-correlated spectroscopy (multiple-RELAY-COSY) and $^{1}H/^{13}C$-shift correlated 2D experiments. The complete analysis of the ^{1}H and ^{13}C spectra was performed.

Homonuclear and heteronuclear two-dimensional NMR spectroscopy have proven very useful as a tool for the complete ^{1}H- and ^{13}C-assignment of glycan structures, which is the first stage before studying the oligosaccharide conformation by the use of inter-residue through-space connectivities (nuclear Overhauser effect) experiments. It is generally not possible to obtain all the connectivities between the atoms within the unresolved envelope by conventional COSY experiments. Multi-step-relayed correlation spectroscopy (RECSY) [1] and homonuclear Hartmann-Hahn spectroscopy [2] have recently been reported to correlate directly the resolved anomeric and H-2 protons with remote (H-3, H-4, H-5) protons through a linear network of couplings. The missing ^{1}H-assignments, such as some H-5 and H-6 atoms and multiple overlapping resonances, can be further extracted from heteronuclear COSY experiments, which generally give good dispersions. In the present study, we applied the multiple-step relayed correlation and heteronuclear correlation spectroscopy for the sequential ^{1}H- and ^{13}C-resonance assignments for a neutral octasaccharide and a disialyldecasaccharide of the *N*-acetyllactosamine type. The ^{1}H- and ^{13}C-NMR data from this neutral octasaccharide [3] and related structures [4, 11] have been previously reported, and their preferred solution conformations investigated by means of HSEA calculations and NOE

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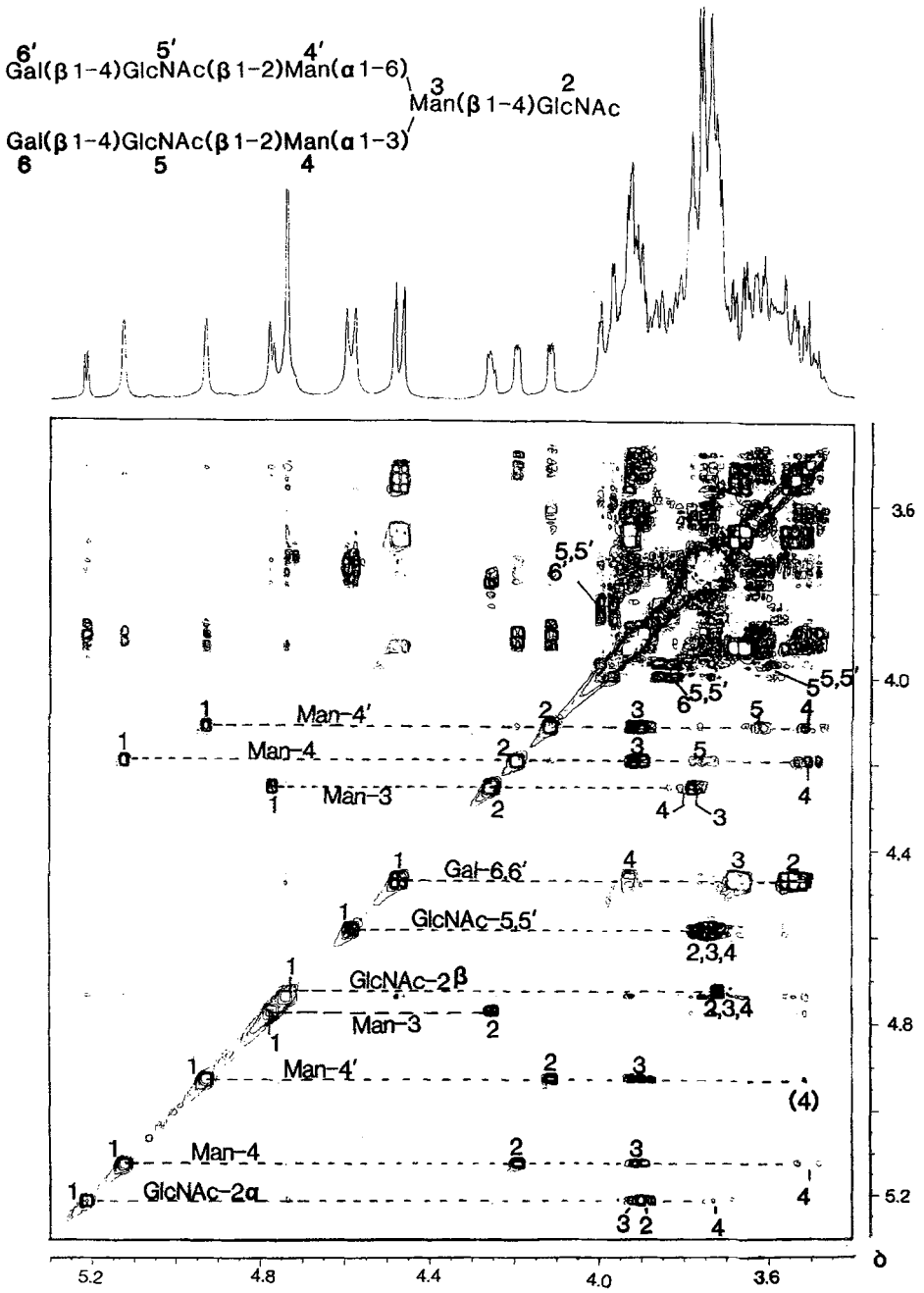

Figure 1. Contour plot of two-dimensional two-step relayed correlation spectrum (3.4-5.2 ppm) of the octasaccharide. The first arabic number is related to the number of the protons, the superscript corresponds to the sugar residue.

experiments. Our purpose was to assign more accurately the ^{1}H-chemical shifts of the signals included in the bulk of unresolved resonances, showing the use of multiple step relayed correlation NMR spectroscopy.

Experimental

The neutral and acidic oligosaccharides were isolated from the urine of patients suffering from Morquio disease type B [12] and sialidosis [13], respectively.

The 400 MHz ^{1}H-NMR experiments were performed on a Bruker AM-400 WB spectrometer equipped with a 5 mm ^{1}H/^{13}C mixed probe-head, operating in the pulse Fourier transform mode and controlled by an Aspect 3000 computer. After three exchanges with ^2H_2O (99.95% atoms ^{2}H, Aldrich) and intermediate lyophilisations, the products (concentration about 50 mg/0.5 ml $^2H_2$) were analysed with a spectral width of 3000 Hz for 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 using the standard Bruker pulse program POWGATE with ^{1}H broad band composite-pulse decoupling. The spectral width was 23,000 Hz for 32 K frequency domain points and time domain data giving a final digital resolution of 1.387 Hz/ point. A 90° pulse (6 µs) and a 1 sec recycle delay were used. The chemical shifts are given relative to sodium-4,4-dimethyl-4-silapentane-1-sulphonate, but were actually measured to the methyl of internal acetone (δ = 2.225 ppm for ^{1}H and δ = 31.55 ppm for ^{13}C) in ^2H_2O at 300 K.

The 2D homonuclear COSY 45 experiments were performed using the standard Bruker pulse program COSY. In these experiments the spectral width was 1800 Hz. The ^{1}H ninety degrees pulse was 10.6 µs. 256 W x 2 K data matrices were acquired which were zero-filled prior to Fourier transformation to obtain a 1 K x 2 K spectral data matrix, a sine-bell squared function was used in both dimensions.

The 2D homonuclear COSY with simple and double relay transfers was performed using the standard Bruker pulse program COSYRCT and the pulse program COSYDR (Bruno Perly, Cea Saclay, personal communication). For example, the COSYDR experiment was performed using the sequence: D_1-90-D_ϕ-90-D_2-180-D_2-90-D_3-180-D_3-90-FID; where D_1 = 2 sec; 90, 180 = 90°, 180° 1H pulse (90° = 10.6 µsec); D_ϕ = incremental delay (initial = 3 µsec); $D_2 = D_3$ = 35 ms. In all experiments for a spectral width of 1800 Hz, 256 W x 2 K data matrices were obtained, which were zero-filled to 1 K x 2 K prior to Fourier-transformation, a sine-bell squared function was used in both dimensions.

The 2D heteronuclear-correlated experiments were performed with simultaneous ^{1}H broad band decoupling using the standard Bruker pulse program XHCORRD. Refocusing delays were adjusted to an average JC-H coupling constant of 142 Hz. Spectral windows of 10,000 Hz, with 4096 data points, for ^{13}C, and 900 Hz, with 128 data points, for ^{1}H were employed. ^{1}H and ^{13}C- 90° pulse width was 10.6 and 6 µsec respectively. A 128 W x 4 K data matrix was acquired which was zero-filled prior to Fourier-transformation to obtain a 512 W x 4 K spectral data matrix. The F1 domain was multiplied by a sine-bell function and the F2 domain by a line-broadening function (LB = 1 Hz) prior to processing.

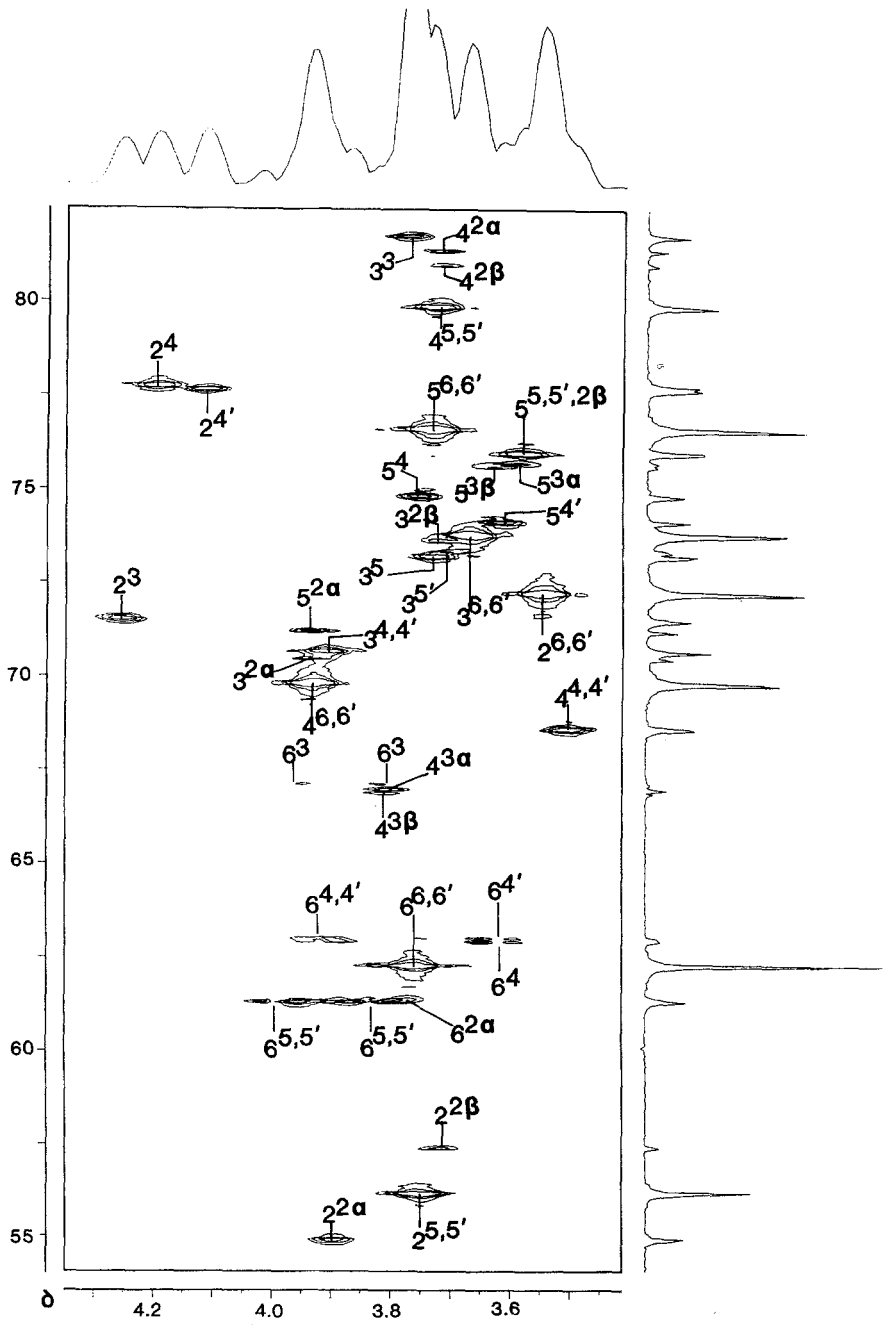

Figure 2. Contour plot of the heteronuclear COSY spectrum (3.4-4.4/50-85 ppm) of the octasaccharide.

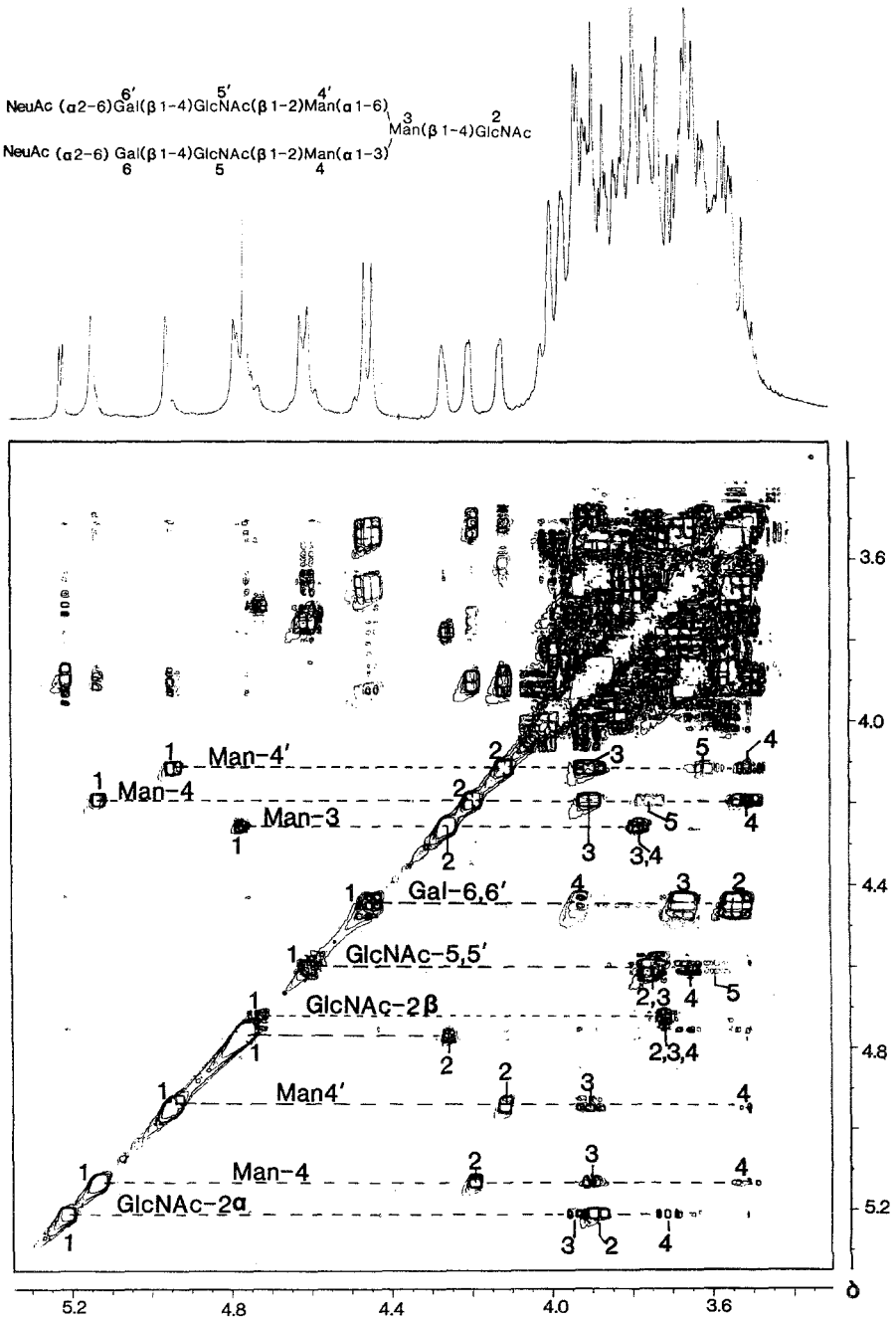

Figure 3. Contour plot of the two-dimensional two-step relayed correlation spectrum (3.4-5.2 ppm) of the decasaccharide.

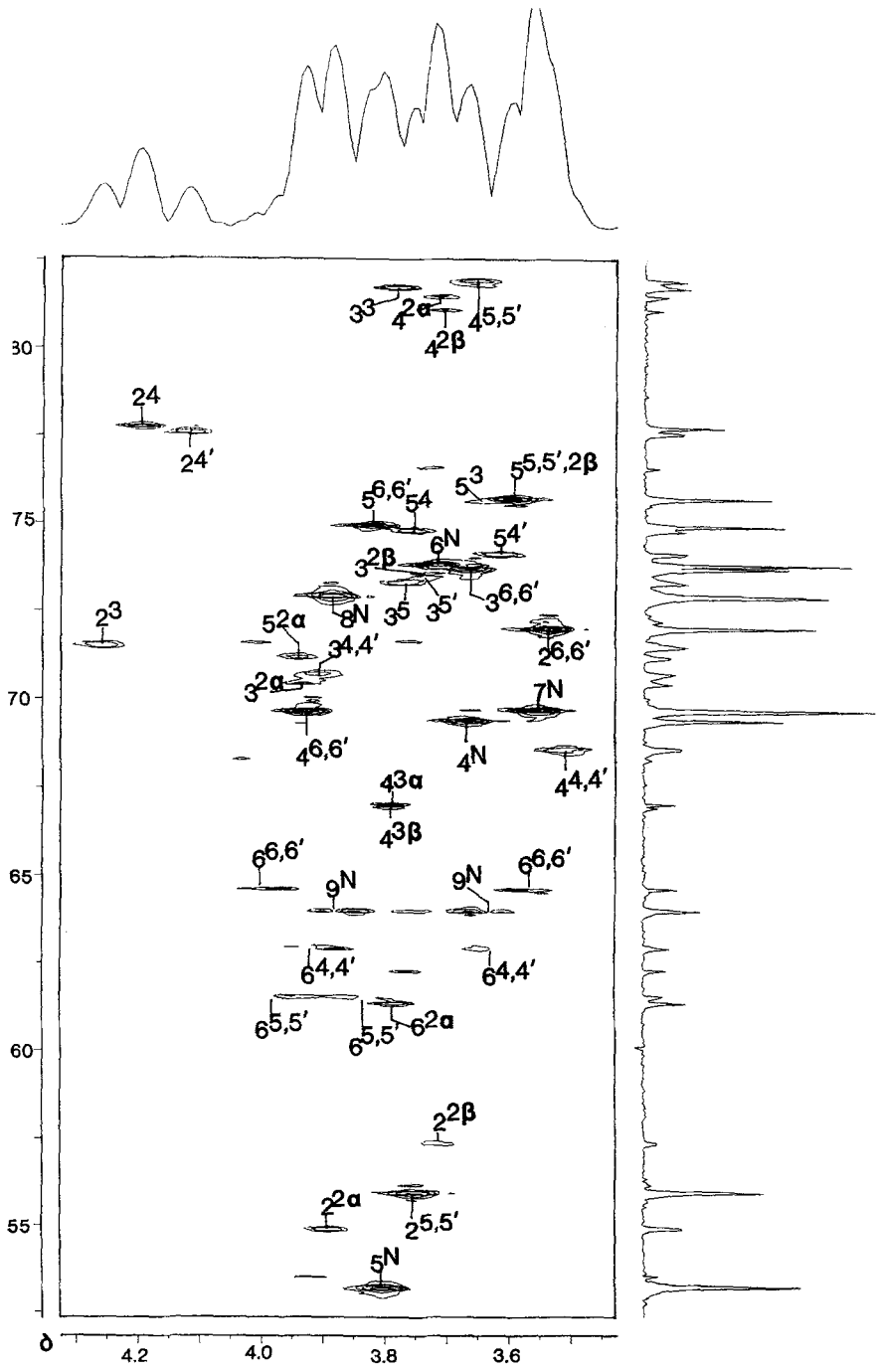

Figure 4. Contour plot of the heteronuclear COSY spectrum (3.2-4.4/50-85 ppm) of the decasaccharide.

Results

Spectrum of the Octasaccharide

The H-1 and H-2 resonances for galactose and *N*-acetylglucosamine residues are directly observed on the conventional 2D COSY 45 spectrum of the octasaccharide, while the connectivities can be extended to the H-3 atoms for the mannose residues due to the presence of their H-2 resonances away from the unresolved region of the spectrum. The cross-peaks between galactose H-2, H-3 and H-4 can also be easily recognized. In the extended part of the spectrum (not shown), the connectivities between the H-3, H-4 and H-5 atoms of the Man-**4** and **4′** residues can hardly be analyzed. These connectivities were easily deduced from the analysis of the RCT and the two-step RELAYED-COSY spectra (Fig. 1), which furnished the chemical shifts of the H-4 and H-5 atoms with a good accuracy. This two-step RELAYED-COSY spectrum also points to the overlap of the H-2, H-3 and H-4 signals of the three *N*-acetylglucosamine residues and, consequently, was not further exploitable. The H-6 resonances of Man-**4** and **4′** appeared on the three-step RELAYED-COSY spectrum (not shown), but the overlap of their cross-sections with the H-3 and H-5 signals made difficult any unambiguous assignment. We have also directly assigned the H-5 and H-6 resonances of Man-**3** on the COSY 45 spectrum showing their typical ABC system appearing in an accessible region of the contour plot. The other resonances were extracted from the heteronuclear correlated NMR spectrum (Fig. 2). Their ^{1}H-chemical shifts was suggested by the two-step RELAYED-COSY spectrum but measured with a relatively good accuracy (± 0.01 ppm) on the 2D ^{1}H/^{13}C-spectrum. The C-6 resonances were established by a DEPT experiment, which allowed *inter alia*, determination of the ^{13}C-chemical shift of the C-6 atom of the Man-**3** residue at $\delta = 67.13$ ppm (α-anomer). For reasons of symmetry, a problem was to assign unambiguously the resonances relevant to one or the another antenna. It was only possible for the C-2, C-4 and C-5 atoms of the Man-**4** and **4′** residues, for which a broadening signal was observed for the relevant signal reliably assigned to the (1-6)-antenna, due to its doubling by the anomerization effect [14]. Consequently, for each ambiguity affecting the choice between resonances related to the **4,4′, 5,5′** and **6,6′** residues, an enlarged and lowest-intensity signal was assigned to the sugar of the (1-6)-antenna.

Spectrum of Disialyldecasaccharide

The ^{1}H and ^{13}C assignments for the disialyldecasaccharide were made according to the same procedure (Fig. 3 and 4). The two-step RELAYED-COSY spectrum contains three cross-peaks for the GlcNAc-**5,5′** residues, which should be normally assigned to the H-2, H-3 and H-4 atoms, respectively. That is contradicted by the analysis of the ^{1}H-^{13}C correlated NMR spectrum, in which the H-2 and H-3 atoms possess identical chemical shifts. Therefore, the third signal observed at δ 3.60 ppm is well related to the H-5 resonance, which results from the choice of the delays D_2 and D_3 (35 ms) allowing an additional transfer of magnetization.

For the decasaccharide, the conventional 2D COSY 45 was not analyzable in the range 3.5-4 ppm, due to the introduction of the six additional cross-peaks related to the *N*-acetylneuraminic acid residues. Therefore, the RCT, two-step RELAYED-COSY and heteronuclear COSY experiments were essentially used, which furnished all the parameters, except for the Man-**3** H-6 atom (Tables 1 and 2).

Table 1. ^{1}H Chemical shifts for the two oligosaccharides (internal standard: acetone, $\delta = 2.225$).

Octasaccharide	H-1	H-2	H-3	H-4	H-5	H-6	H-7	H-8	H-9	NAc
GlcNAc-2 α	5.210	3.887	3.928	3.717	3.935	3.78	-	-	-	2.061
GlcNAc-2 β	4.723	3.717	3.73	3.709	3.58	n.d.[a]	-	-	-	2.058
Man-3	4.775(α) 4.764(β)	4.257(α) 4.246(β)	3.775	3.803(α) 3.818(β)	3.576(α) 3.646(β)	3.787(6) 3.982(6′)	-	-	-	-
Man-4	5.122	4.191	3.902	3.508	3.750	3.623(6)	-	-	-	-
Man-4′	4.924(α) 4.929(β)	4.112	3.910(α) 3.883(β)	3.496	3.621	3.623(6) 3.920(6′)	-	-	-	-
GlcNAc-5	4.582	3.750	3.732	3.724	3.576	3.834(6) 3.998(6′)	-	-	-	2.052
GlcNAc-5′	4.582	3.750	3.709	3.724	3.576	3.834(6)	-	-	-	2.049(α) 2.046(β)
Gal-6	4.466	3.535	3.664	3.926	3.732	3.74(6) 3.78(6′)	-	-	-	-
Gal-6′	4.470(α) 4.472(β)	3.535	3.664	3.926	3.732	3.74(6) 3.78(6′)	-	-	-	-

Decasaccharide	H-1	H-2	H-3	H-4	H-5	H-6	H-7	H-8	H-9	NAc
GlcNAc-2 α	5.208	3.881	3.928	3.711	3.935	3.78	-	-	-	2..060
GlcNAc-2 β	4.722	3.711	3.732	3.709	3.59	n.d.	-	-	-	2.057
Man-3	4.778(α) 4.768(β)	4.259(α) 4.249(β)	3.784	3.787	3.60	n.d.	-	-	-	-
Man-4	5.133	4.192	3.902	3.508	3.754	3.615(6) 3.920(6´)	-	-	-	-
Man-4´	4.946	4.114	3.914(α) 3.887(β)	3.496	3.609	3.615(6) 3.920(6´)	-	-	-	-
GlcNAc-5	4.602	3.750	3.648	3.654	3.592	3.826(6) 3.990(6´)	-	-	-	2.066
GlcNAc-5´	4.602	3.750	3.648	3.654	3.592	3.826(6) 3.990(6´)	-	-	-	2.062(α) 2.057(β)
Gal-6	4.440	3.531	3.666	3.922	3.818	3.560(6) 4.006(6´)	-	-	-	-
Gal-6´	4.440(α) 4.442(β)	3.531	3.666	3.922	3.818	3.560(6) 4.006(6´)	-	-	-	-
NeuAc	-	-	1.721(3a) 2.665(3e)	3.660	3.804	3.717	3.560	3.888	3.631(9) 3.881(9´)	2.029

[a] n.d., not determined.

Table 2. ^{13}C Chemical shifts for the two oligosaccharides (internal standard: acetone, $\delta = 31.55$).

Octasaccharide	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C8	C-9	C=O	CH_3
GlcNAc-2 α	91.77	54.88	70.47	81.33	71.21	61.32	-	-	-	175.68	23.29
GlcNAc-2 β	96.16	57.41	73.63	80.97	75.64	61.44	-	-	-	175.95	23.59
Man-3	101.64(α)	71.50(α)	81.71	67.00(α)	75.74(α)	67.13(α)	-	-	-	-	-
	101.67(β)	71.46(β)		66.91(β)	75.69(β)	66.91(β)	-	-	-	-	-
Man-4	100.78	77.76	70.69	68.60	74.81	62.91	-	-	-	-	-
Man-4´	98.34	77.69	70.69	68.63	74.17	62.97	-	-	-	-	-
GlcNAc-5	100.73	56.15	73.21	79.89	75.97	61.32	-	-	-	175.86	23.65
GlcNAc-5´	100.73	56.15	73.33(α)	79.91	76.01	61.32	-	-	-	175.88	23.72
			73.36(β)								
Gal-6	104.73	72.01	73.72	69.81	76.59	62.25	-	-	-	-	-
Gal-6´	104.76	72.01	73.72	69.81	76.59	62.25	-	-	-	-	-

Decasaccharide	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	C=O	CH_3
GlcNAc-2 α	91.76	54.88	70.44	81.44	71.17	61.36	-	-	-	175.68	23.30
GlcNAc-2 β	96.16	57.37	73.58	81.10	75.64	61.46	-	-	-	175.95	23.61
Man-3	101.64(α)	71.50(α)	81.71	67.01(α)	75.72	67.23(α)	-	-	-	-	-
	101.67(β)	71.46(β)		66.95(β)		67.09(β)					
Man-4	100.79	77.79	70.72	68.58	74.80	62.89	-	-	-	-	-
Man-4´	98.26	77.64	70.68	68.61	74.14	62.96	-	-	-	-	-
GlcNAc-5	100.58	55.95	73.30	81.83	75.72	61.54	-	-	-	175.89	23.72
GlcNAc-5´	100.64	55.95	73.39	81.88	75.72	61.54	-	-	-	175.89	23.72
Gal-6	104.73	72.01	73.72	69.66	74.93	64.60	-	-	-	-	-
Gal-6´	104.76	72.01	73.72	69.66	74.93	64.60	-	-	-	-	-
NeuAc	174.45	101.34	41.25	69.38	53.17	73.82	69.74	72.91	64.00	176.12	23.12

Discussion

The use of multiple RELAYED COSY experiments provides the assignments of protons H-1 and H-4 (and H-5 for mannose), but is itself limited by resonance overlappings. The homonuclear Hartmann-Hahn spectra should present similar disadvantages. The use of concerted homonuclear and heteronuclear correlation spectroscopy is another alternative when sufficient amounts of material are available. Nevertheless, the relative insensitivity of these ^{13}C conventional heteronuclear chemical shift correlation experiments will be overcome by the inverse detection procedure [15].

Our data are very similar to those published by Paulsen *et al.* [3] for the ^{1}H-chemical shifts from the neutral octasaccharide for which we just assigned some reliable additional parameters to GlcNAc-**2** and Man-**4,4´** residues. The only observed discrepancy concerns the ^{13}C-chemical shifts of C-4 Gal-**6,6´** and C-3 Man-**4,4´** atoms, that we respectively assigned at δ 69.81 and 70.69 ppm, instead of the opposite values. These new assignments can be clearly deduced from the comparison of homonuclear and heteronuclear COSY spectra, since the H-4 atom of Gal-**6,6´** resonates at a lower field than that of H-3 of Man-**4,4´**.

The NMR data from the sialyldecasaccharide show that the α(2-6)-sialylation induces a non-equivalence of the H-6 signals of the galactose residues ($\Delta\delta$ = -0.20 and +1.24 ppm) and increments of the chemical shifts of Gal H-5 ($\Delta\delta$ = +0.18 ppm), GlcNAc H-4 ($\Delta\delta$ = -0.07), Man-**4** H-1 ($\Delta\delta$ = +0.011 ppm) and Man-**4´** H-1 ($\Delta\delta$ = +0.017 ppm). Despite their low values, these two latter chemical shift effects are of great interest for locating the *N*-acetylneuraminic acid residue in monosialyl di-antennary glycans [10].

The large chemical shift effects observed for C-4 of *N*-acetylglucosamine ($\Delta\delta$ = +1.95 ppm), C-5 of galactose ($\Delta\delta$ = -2.35 ppm) are identical to those for the trisaccharide NeuAcα(2-6)Galβ(1-4)GlcNAc [7]. Particularly, the large downfield shift of the C-4 resonance of *N*-acetylglucosamine has been discussed in terms of spatial conformation in which the *N*-acetylneuraminic acid residue [7, 14, 16] is "folded" over the inner sugar residues.

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