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TWO-DIMENSIONAL, ^{1}H-N.M.R. STUDY OF PERACETYLATED, REDUCED DERIVATIVES OF THREE OLIGOSACCHARIDES ISOLATED FROM HUMAN MILK

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ABSTRACT

The structures of the peracetylated derivatives of the following alditols obtained from oligosaccharides of human milk have been established by two-dimensional, *J*-resolved and *J*-correlated, ^{1}H-n.m.r. spectroscopy at 360 MHz: β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc-ol, 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-ol, and β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-[β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)]-β-D-Gal*p*-(1→4)-D-Glc-ol.

INTRODUCTION

Recently, we analysed the 360-MHz, ^{1}H-n.m.r. spectra of a series of peracetylated oligosaccharides[1]. Owing to the strong downfield shifts induced by the acetyl substituents, the problems of signal overlap were less severe than is usually the case with the underivatised oligosaccharides. Nonetheless, some groups of signals were still unresolved and could only be analysed by scalar-decoupling difference spectroscopy. A full assignment of all resonances of a medium-sized oligosaccharide required a considerable number of decoupling experiments.

We have now made use of the greatly enhanced resolution of the *J*-resolved, two-dimensional (2D) n.m.r. spectra[2] and of the connectivity information obtainable from the *J*-correlated 2D-spectra[3]. Although the recording of 2D spectra is time-consuming, the net saving in time is substantial, since connectivities between all coupled protons can be derived from a single spectrum. Perhaps a more important advantage of the *J*-correlated spectra is that no irradiation by a second r.f. field is needed for this purpose; hence, coupled nuclei can be related to each other, even if their signals are heavily overlapped.

Our main objective was to elucidate the structure of the peracetylated alditol **3** obtained from the corresponding hexasaccharide of human milk (for formulae,

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TABLE I

CHEMICAL SHIFTS FOR SOLUTIONS OF PERACETYLATED OLIGOSACCHARIDE ALDITOLS IN $CDCl_3$ AT 305 K, AS OBTAINED BY TWO-DIMENSIONAL ^{1}H-N.M.R. SPECTROSCOPY (ACETYL GROUPS ARE NOT INDICATED IN THE FORMULAE)

β-D-*Gal*-(1→3)-*β*-D-*GlcNAc*-(1→3)-*β*-D-*Gal*-(1→4)-D-*Glc*-*ol* (**1**)

H-1	4.48	5.16	4.59	4.27
H-1′				4.30
H-2	5.04	2.86	5.06	5.44
H-3	4.95	4.59	3.81	5.39
H-4	5.34	4.94	5.37	4.12
H-5	3.85	3.62	3.81	5.08
H-6	4.10	4.00	4.03	4.09
H-6′	4.10	4.35	4.17	4.47
H-N		5.70		

α-L-*Fuc*-(1→2)-*β*-D-*Gal*-(1→3)-*β*-D-*GlcNAc*-(1→3)-*β*-D-*Gal*-(1→4)-D-*Glc*-*ol* (**2**)

H-1	5.45	4.35	5.08	4.58	4.29
H-1′					4.36
H-2	4.96	3.83	2.59	5.18	5.43
H-3	5.36	4.93	4.85	3.88	5.41
H-4	5.20	5.24	4.83	5.33	4.11
H-5	4.62	3.83	3.67	3.83	5.07
H-6	1.23	4.09	4.10	4.05	4.11
H-6′		4.09	4.34	4.19	4.48
H-N			6.83		

β-D-*Gal*-(1→3) ——— *β*-D-*GlcNAc*-(1 → 3)

β-D-*Gal*-(1→4)-D-*Glc*-*ol* (**3**)

β-D-*Gal*-(1→4)-*β*-D-*GlcNAc*-(1 ——— 6)

H-1	4.51	4.60	5.09	4.47	4.14
H-1′					4.36
H-2	5.10	3.82	2.91	5.03	5.47
H-3	4.96	5.21	4.56	3.80	5.32
H-4	5.34	3.76	4.91	5.29	4.07
H-5	3.85	3.62	3.62	3.81	5.06
H-6	4.09	4.08	4.04	3.49	4.07
H-6′	4.09	4.12	4.29	3.83	4.49
H-N		6.42	5.77		

see Table I and Figures). On the other hand, we wished to test the general applicability of the two techniques to larger oligosaccharides, since only small ones have been investigated by homonuclear, 2D-n.m.r. spectroscopy hitherto[4-6]. For this reason, we also examined the peracetylated derivatives of the tetra- and penta-

β-D-Gal-(1→3)-β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-D-Glc-ol

(1)

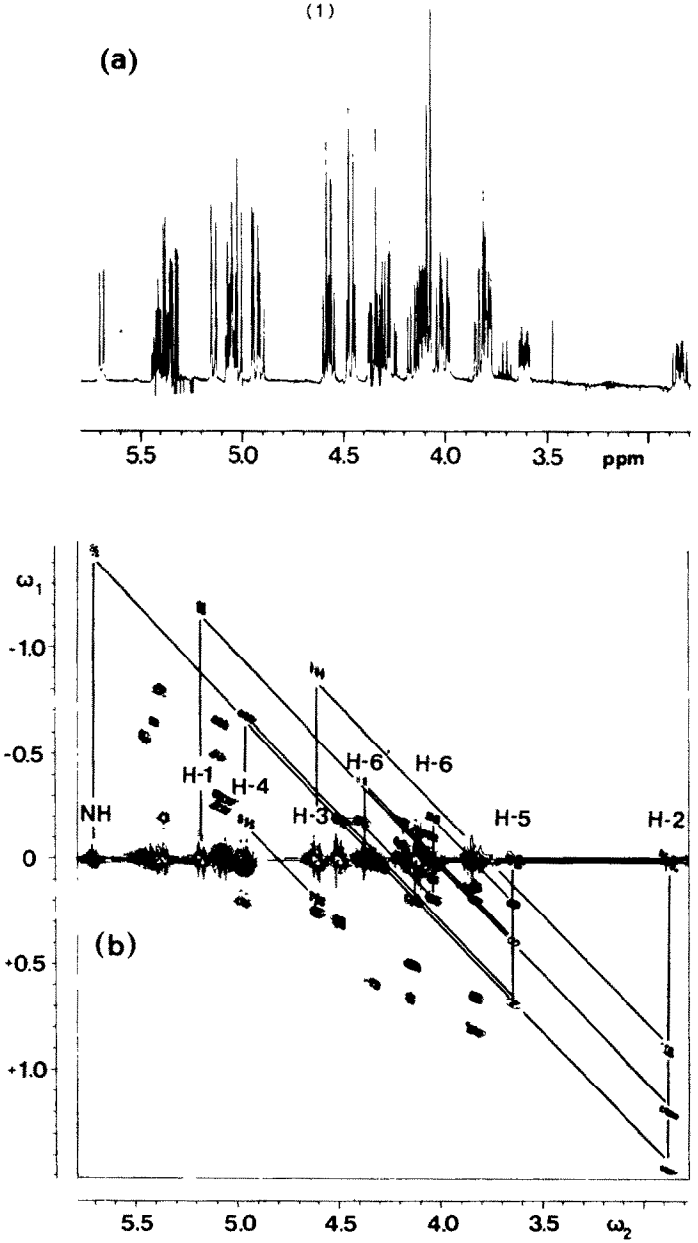

Fig. 1. The sugar ring-proton region of the 360-MHz, ^{1}H-n.m.r. spectra of the peracetylated tetrasaccharide alditol **1** measured in $CDCl_3$ at 305 K. (*a*) The resolution-enhanced 1D spectrum; (*b*) contour plot of the 2D SECSY spectrum; establishment of scalar connectivities is illustrated by lines linking contours exhibited by the GlcNAc residue. Acetyl groups are not indicated in the formula.

saccharide alditols **1** and **2**, whose one-dimensional n.m.r. spectra had already been assigned[1].

Compound **3** represents a rare example of a structure comprising type 1 [β-D-Gal-(1→3)-D-GlcNAc] and type 2 chains [β-D-Gal-(1→4)-D-GlcNAc] in the same molecule[8]. Although closely related structures (containing fucose and sialic acid residues in addition) could be determined by enzymic degradation and methylation analysis[9], the results presented here show that this goal can be achieved more easily by two-dimensional n.m.r. spectroscopy.

RESULTS AND DISCUSSION

The often reproduced, "three-dimensional", stacked plots of the *J*-resolved, two-dimensional, ^{1}H-n.m.r. spectra are not very informative; in fact, they are unintelligible for complex compounds. On the other hand, the projections of these spectra on to the ω_2 frequency axis (*i.e.*, the chemical shift axis) produce simple, proton-

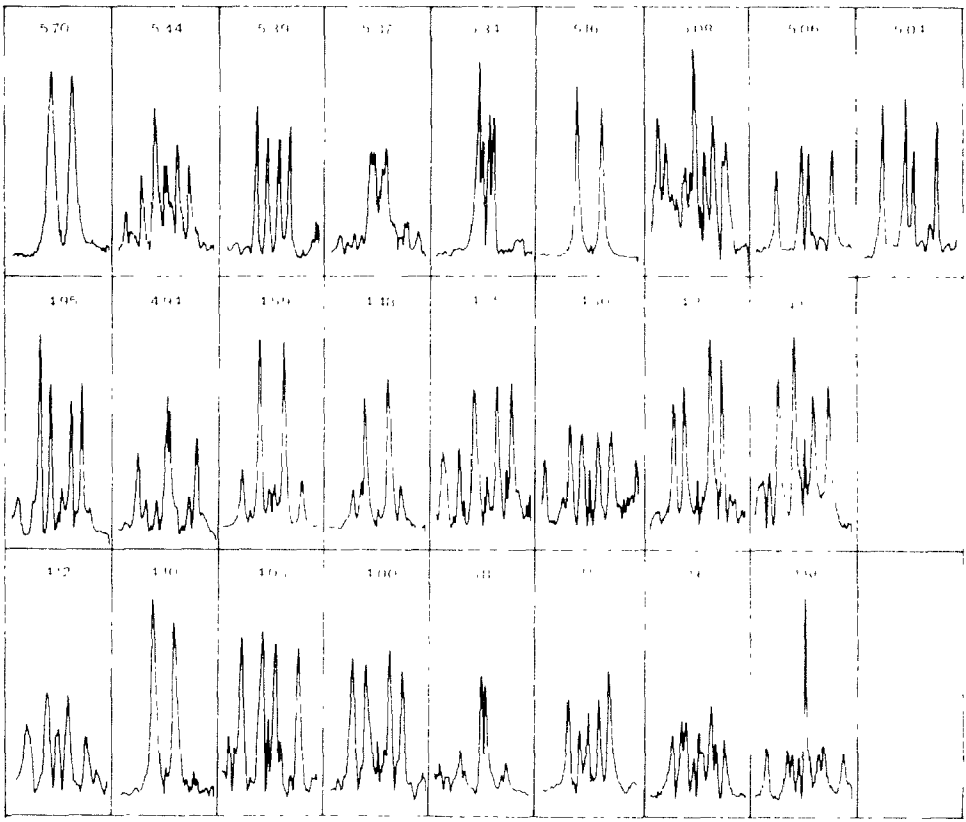

Fig. 2. Cross-sections through the resonances of the sugar ring protons in the 2D J-resolved spectrum of peracetylated **1**.

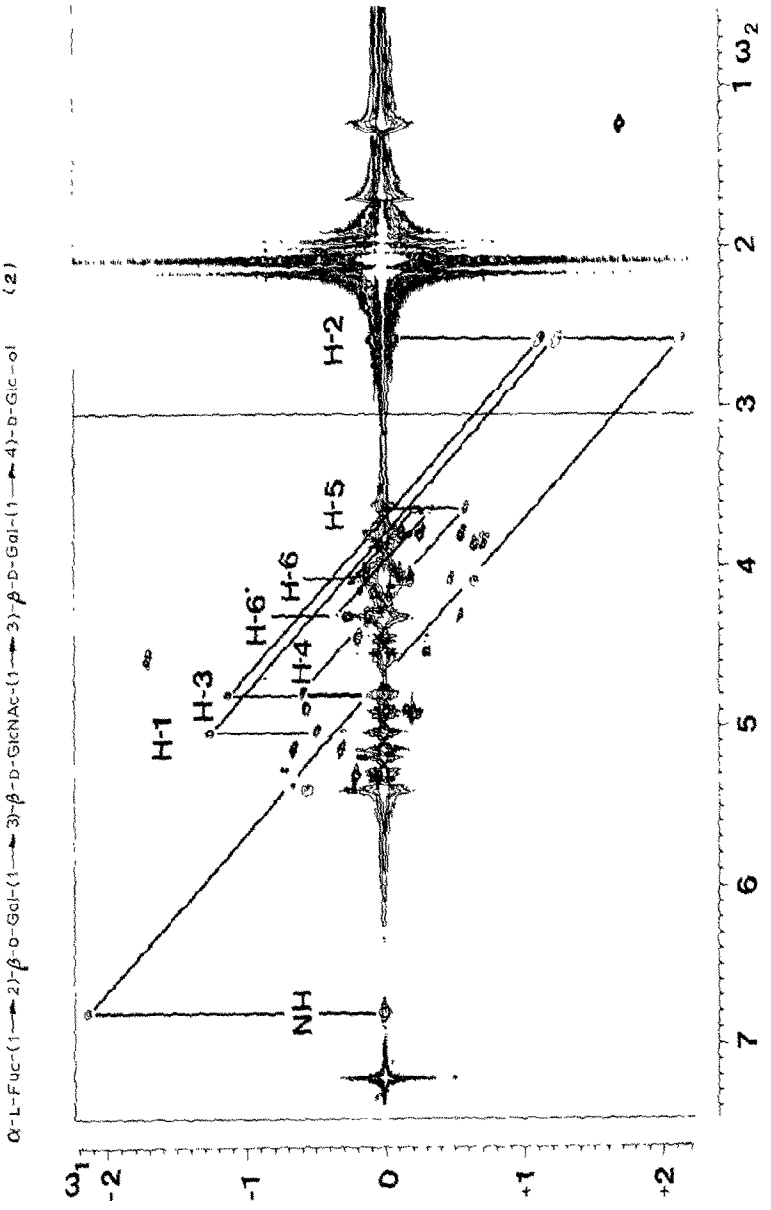

Fig. 3. Contour plot of the 2D SECSY spectrum of the peracetylated pentasaccharide alditol 2. Establishment of scalar-coupling connectivities is illustrated by lines linking contours exhibited by the GlcNAc residue. Acetyl groups are not indicated in the formula.

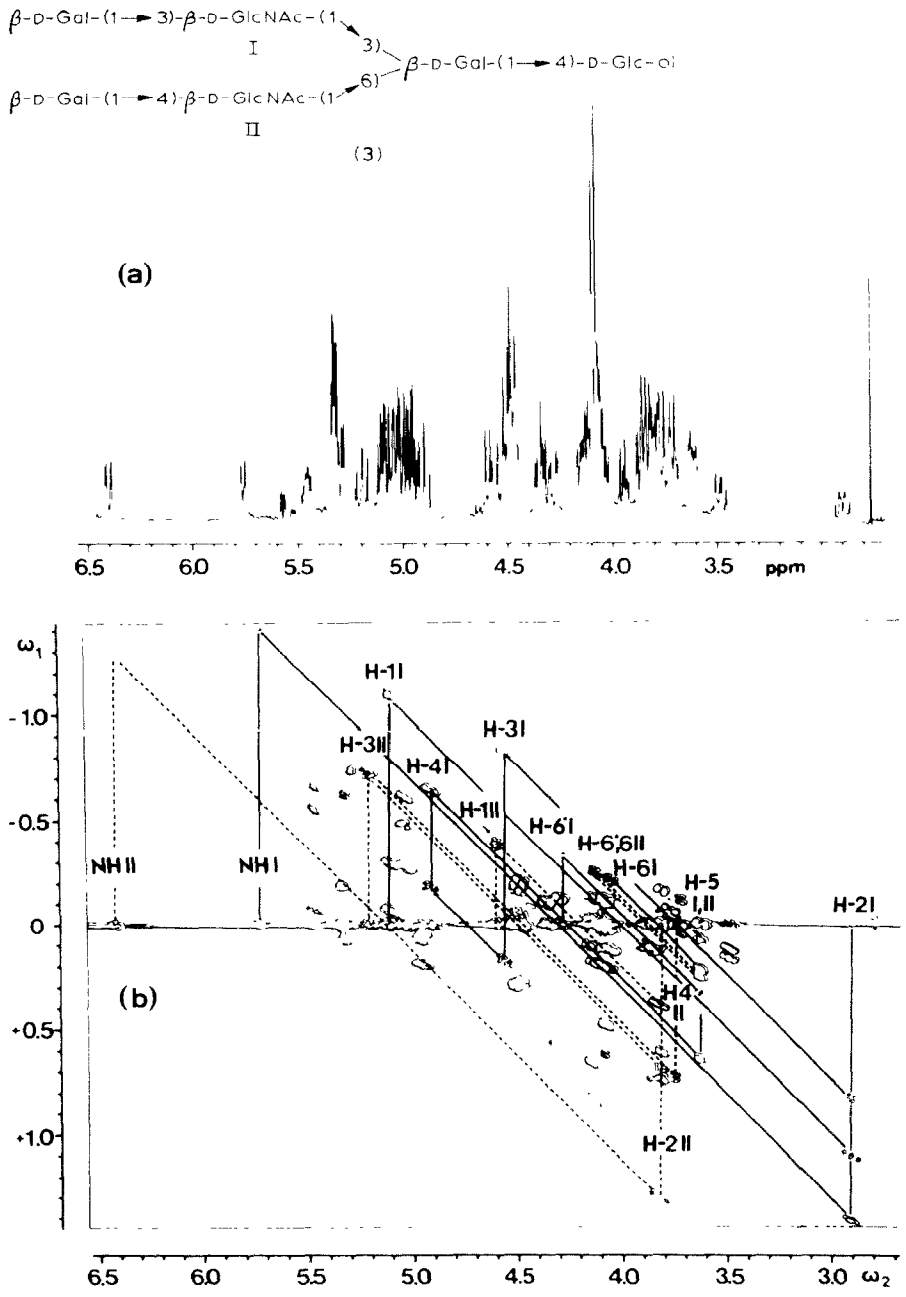

Fig. 4. The sugar ring-proton region of the 360-MHz, ^{1}H-n.m.r. spectra of the peracetylated hexasaccharide alditol 3. (*a*) The resolution-enhanced 1D spectrum; (*b*) contour plot of the 2D SECSY spectrum; establishment of scalar-coupling connectivities is illustrated by lines linking contours exhibited by the GlcNAc residues I (——) and II (– – –). Acetyl groups are not indicated in the formula.

proton-decoupled spectra consisting of singlet resonances, and the cross sections through these singlets parallel to the ω_1 axis yield the multiplets of individual resonances. Whereas such a spreading of the interproton scalar-coupling information in the second dimension may suffice to assign spectra of simple molecules, additional information on connectivities between coupled protons is required for unequivocal structure determination of a complex compound. This is particularly true if the molecules under question contain repeating units of similar type, such as saccharide residues representing formally identical 7-spin systems.

The multiplets of the sugar-ring protons of **1** are shown in Fig. 2. Most of them are first-order, or nearly so. Even though they were not extensively overlapped in the one-dimensional spectrum (Fig. 1a), the increase in resolution in the two-dimensional spectrum is evident. Scalar-coupling connectivities between these multiplets have been established with the aid of the SECSY variant[3] of 2D, *J*-correlated spectroscopy. The contour plot (Fig. 1b) shows the SECSY spectrum of **1** viewed from the top of the signals. The contours corresponding to resonances of the particular protons are displayed parallel to the ω_2 axis in the centre of the plot. If two nuclei, A and B, are coupled to each other, they exhibit off-line contours (correlation contours) at the chemical shifts ω_2^A and ω_2^B. The distances of these contours from the central line are $-$ and $+\omega_1 = 0.5\ (\omega_2^A - \omega_2^B)$ for the low-field resonance A and the high-field resonance B, respectively. This means that these contours can be correlated by linking by lines of 135° slope. If one of these nuclei is further coupled to others, additional contours occur at its chemical shift, and this simple correlation procedure can be continued until the entire coupled spin-system of the given sugar residue is traced. When $\omega_2^A - \omega_2^B$ is small, or in other crowded situations, contour overlap prevents this easy type of analysis. In such cases, the connectivity information can be obtained from cross sections[7] through the basic peaks parallel to the ω_1 axis, as mentioned above for *J*-resolved spectra. Contour plots of the SECSY spectra of compounds **2** and **3** are shown in Figs. 3 and 4b, respectively, and the chemical shifts of the assigned resonances for **1**–**3** are given in Table I. For **1** and **2**, the numerical values can be compared with those obtained from one-dimensional spectra[1]. The deviations are rather small; out of the total of 64 chemical shifts compared, 33 are identical, 29 deviate by ± 0.01 p.p.m., and 2 differ by ± 0.02 p.p.m. Hence, notwithstanding the lower digital resolution of the 2D spectra, the accuracy of the chemical shifts derived therefrom is satisfactory for practical purposes of signal assignments. Therefore, the primary structure of **1** and **2** can be derived from their 2D spectra in a manner exactly as described[1] for conventional spectra.

The structure of compound **3** can be deduced almost entirely from its spectra; only a few details require comparative spectral data on related substances. Identification of the spin systems by analysing 2D *J*-resolved and SECSY spectra in the manner described above has shown the presence of a 4-glycosylated glucitol and of two types of both β-D-galactose and 2-acetamido-2-deoxy-β-D-glucose residues.

The glucitol unit was easily recognised by the occurrence of signals for two methylene groups, and the signal for the methine proton at the glycosylation site was

identified by its high-field position, as compared with other methine protons of this spin system that were deshielded by their geminal acetyl groups. For symmetry reasons, sites 3 and 4 are indistinguishable as far as coupling patterns are concerned, but they are differentiated by the chemical shifts of the neighbouring protons. Hence, connectivities of the high-field methine resonance at δ 4.07 with those at δ 5.32, 5.47, 4.36, and 4.14 strongly suggested O-4 as the glycosylation site, since very similar δ values have been obtained[1] for H-3, H-2, H-1, and H-1′, respectively, in a series of seven 4-glycosylated glucitols[1].

Of the two types of galactose residue, one is represented by two chemical-shift-equivalent units, as follows from integration of the one-dimensional spectrum. The lack of any signal shifted to high field shows that both these units are terminal, *i.e.*, that the structure is a biantennary one. This conclusion is corroborated by the high-field shifts of the H-3, H-6, and H-6′ resonances of the third galactose residue, indicating glycosylation at both O-3 and O-6.

2-Acetamido-2-deoxyglucose and galactose resonances have been discriminated by the connectivities of the former with the NH doublet. The glycosylation site is at O-3 in one of the two 2-acetamido-2-deoxyglucose residues and at O-4 in the other, as evidenced by the high-field shifts of the signals of H-3 and H-4, respectively; these shifts can be estimated by comparison with the data[1] for the terminal residue of the peracetylated trisaccharide alditol, β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-D-Glc-ol. Anomeric proton resonances can be used to determine whether the given GlcNAc residue is linked to O-3 or O-6 of the disubstituted galactose residue: the linkage to O-6 results[11-14] in an upfield shift of the H-1 signal by ~0.3 p.p.m., as compared with the alternative (1→3) linkage. This difference for the two GlcNAc residues of **3** amounts to 0.49 p.p.m. Thus, type 1 and type 2 chains can be located unequivocally in the "upper" [(1→3)-linked] and "lower" [(1→6)-linked] branch, respectively, of **3**. Although the sequence and linkage analysis of **3** has also been made by mass spectrometry[10], the correct location of the two types of chains was beyond the scope of that technique.

Thus, the combination of two-dimensional, *J*-resolved and *J*-correlated, ^{1}H-n.m.r. spectroscopy enables the primary structures of relatively complex oligosaccharides to be determined completely.

EXPERIMENTAL

The preparation of the peracetylated, reduced oligosaccharides **1**–**3** has been described elsewhere[10]. For n.m.r. spectroscopy, the samples were dried *in vacuo* and then dissolved in $CDCl_3$ containing a trace of Me_4Si.

The 360-MHz, ^{1}H-n.m.r. spectra were recorded at 305 K with a Bruker HX-360 spectrometer. For the conventional, one-dimensional spectra (recorded with quadrature detection), a 16k memory capacity was used. Digital resolution was 0.35 Hz. For resolution enhancement, the free-induction decays were multiplied by the Lorentzian–Gaussian transformation function[15] included in the Bruker software package.

The software used for 2D spectra was the Bruker November 1980 version based on the programmes developed by Nagayama *et al.*[2]. The data size of the time-domain, 2D, *J*-resolved spectrum was a 128 × 8192 matrix. To improve the spectral resolution, these data were multiplied in both the t_1 and t_2 directions with the Gaussian window function. Fourier transformation was performed with zero filling in both directions. The 512 × 2048 data matrix of the time-domain, 2D, *J*-correlated (SECSY[3]) spectra was processed in a similar way. The latter were analysed in the form of contour plots and cross-sections through individual resonances parallel to the ω_1-axis[7]. Individual multiplets were obtained in cross-sections through resonances of the 2D, *J*-resolved spectra.

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