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Note

Gradient-enhanced homonuclear 2D NMR techniques applied to oligosaccharides containing manno-hexoses provide improved correlations for protons coupled by small ^3J

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We demonstrate here that gradient-enhanced homonuclear correlation techniques provide much better correlations, particularly between protons coupled by a small $^3J_{HH}$ value.

The reliable structural or conformational analysis of oligosaccharides by proton NMR depends critically on the prior unambiguous assignment of all pyranose protons before any attempt can be made to establish the position of the glycosidic linkage (e.g. via $^3J_{CH}$ [1,2] or inter-residue NOEs). When the oligosaccharides are natural rather than synthetic products, the correct assignment is clearly a prerequisite for meaningful solution NMR studies. Typically, the analysis starts from the anomeric protons and proceeds through the intact spin system by means of various two-dimensional correlation techniques [3] (COSY, TOCSY [4,5] etc.) or one-dimensional equivalents [6], whereby individual anomeric protons are selectively excited by means of shaped, soft excitation pulses [7]. The success of these techniques depends critically on the size of the coupling constants along the spin network since the intensities of correlation peaks are functions of ^3J values. For β-anomeric protons in the *galacto* or *gluco* configurations, the H1-H2 correlation step is trivial because the coupling constant is large (7–8 Hz). For α-anomers the situation is slightly more challenging in view of the smaller $^3J_{H1,H2}$ values (3–4 Hz).

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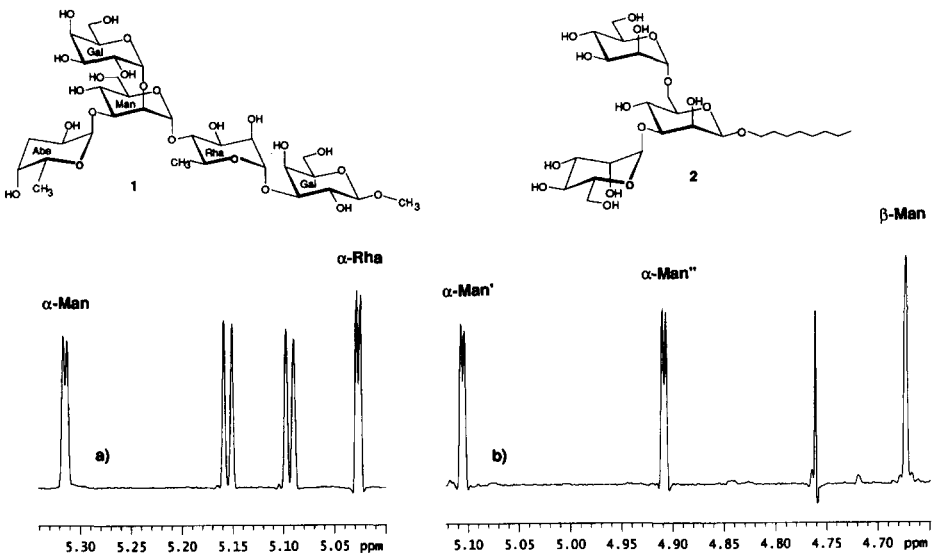

Fig. 1. Partial proton 1D spectra and structures of the oligosaccharides **1** and **2** in aqueous solution. (a) The four α-anomeric protons of **1**. The H1-H2 coupling constants are 1.8 Hz for α-mannose and 1.9 Hz for α-rhamnose. (b) All three anomeric protons of **2**. Chemical shifts and coupling constants of **2** are listed in Table 2.

The difficulties increase significantly, however, for carbohydrates containing mannose and rhamnose moieties in which H2 is equatorial. Values observed in these cases are usually less than 2 Hz, as shown in Fig. 1a for the pentasaccharide α-D-Gal*p*-(1 → 2)-[α-D-Abe*p*]-(1 → 3)-α-D-Man*p*-(1 → 4)-α-L-Rha*p*-(1 → 3)-β-D-Gal*p*-1 → OCH_3 **1** [8]. Even smaller $^3J_{H1,H2}$ values (1.7 Hz) occur in the two α-mannose moieties of the branched trisaccharide, α-D-Man*p*-(1 → 3)-[α-D-Man*p*]-(1 → 6)-β-D-Man*p*-1 → $O(CH_2)_7CH_3$ **2** [9]. However, the β-mannose in trisaccharide **2** (Fig. 1b) provided the worst case; the actual $^3J_{H1,H2}$ value of 0.8 Hz could only be determined under optimal spectral resolution and after extensive resolution enhancement (data not shown).

This problem is not limited to H1-H2 coupling constants. Small coupling constants of similar magnitude are encountered frequently between H4 and H5 in aldopyranosyl rings. For example, $^3J_{H4,H5}$ in fucose and galactose is often smaller than 1 Hz and provides a significant challenge for reliable peak assignments past the H4 proton. In our NMR studies of carbohydrates, ranging from mono- to pentasaccharides, we found that gradient-enhanced correlation techniques [10] provided much better correlation information. While this is true for any size of coupling constant, it is especially valuable in cases where small coupling constants are present. Recognizing that β-Man in **2** is a challenging case, we present a comparison of various 2D techniques [3] applied to **1** and **2** dissolved in D_2O.

In solution NMR spectroscopy, gradients are typically used to (1) eliminate or reduce the need for phase-cycling [11], which is essential in conventional (i.e., non-gradient) spectroscopy to remove undesired axial peaks and to select for either N or P type

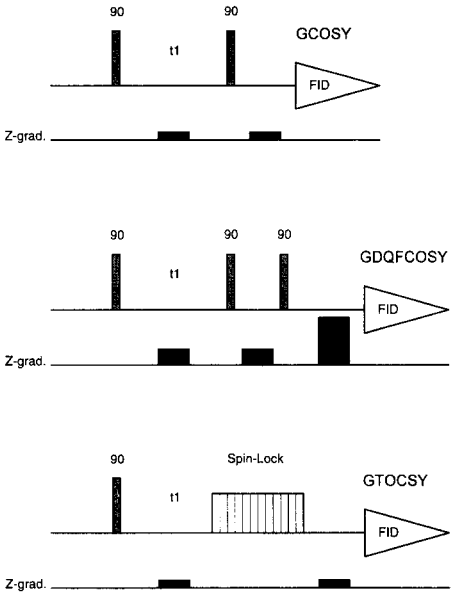

Fig. 2. The pulse sequences of the gradient-enhanced two-dimensional correlation techniques used in this work. Experimental details are reported in the text.

signals; (2) coherence pathway selection as, for example, in the double-quantum filtered COSY experiment [10,12] (GDQF-COSY, Fig. 2) and (3) to eliminate the intense solvent signal in H_2O samples [12,13]. Since each transient results in a clean, artifact-free FID, two-dimensional experiments can be run with a single scan per t1-increment. At 500 MHz, oligosaccharides in D_2O can be measured at a minimum concentration of approximately 5 mM by a single scan gradient-enhanced COSY experiment (GCOSY, Fig. 2). Excellent digital resolution in both dimensions can be achieved within an experimental time of only 17 min. In the case of the GDQF-COSY [10,12], the overall sensitivity is lower (approximately one half) due to coherence pathway elimination. However, its main benefit, the very effective removal of the residual water signal (and other isolated spin systems, e.g., methyl groups not coupled to other protons), far outweighs this disadvantage. The clean elimination of the water signal without baseline distortion is particularly useful when peaks of interest, typically H1 protons of α-pyranoses and H5(α-Fuc) of Lewis-a structures in oligosaccharide mixtures [14], are very close or directly underneath the solvent peak, and the complexity of the spectrum makes it impossible to "move" the water signal by changing the temperature. At a concentration of 5 mM, excellent GDQF-COSY spectra can be obtained with two to three scans per t1-increment, resulting in experimental times that are well below 1 h. We found that, compared to their non-gradient equivalents, GCOSY, GDQF-COSY and GTOCSY (Fig. 2) experiments permitted much better observation of correlations between protons that are coupled by a small coupling constant. Although we chose α-rhamnose and α-mannose in **1** and especially β-mannose in **2** as a "worst case" example to illustrate these findings, it should be emphasized that the same was observed

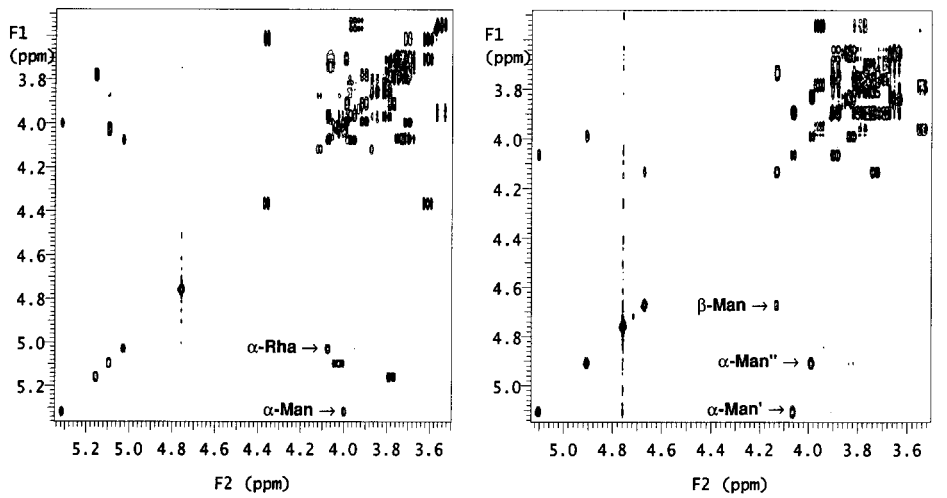

Fig. 3. The GCOSY spectra of **1** (20 mM, *left*) and **2** (10 mM, *right*) in aqueous solution showing the H1/H2 interactions. The water signal was neither presaturated nor treated in any other way.

in cases of small $^3J_{H4,H5}$ values, allowing the connection of the H5/H6a/H6b portion to the rest of the pyranose molecule.

The GCOSY spectra of **1** and **2** are shown in Fig. 3. In both cases a strong correlation peak can be observed between H1 and H2 of all moieties, including the 0.8 Hz β-Man residue of **2**. In Fig. 4, a comparison between GCOSY, GDQF-COSY and the conventional COSY experiment, optimized for small J (or long-range) interactions (COSYLR [15]), is shown for the trisaccharide **2**. The solvent signal was not treated in any way other than by coherence selection in the GDQF-COSY experiment (neither presaturation nor any post-acquisition water-elimination routine). All 2D spectra and traces through the β-Man anomeric proton are recorded and depicted in absolute value mode with identical vertical display parameters. Even in the single-scan GDQF-COSY experiment, a very clean H1/H2 correlation can be observed although all peak intensities, diagonal and cross-peaks, are reduced significantly by the coherence selection. The solvent peak and its t1-ridge are completely eliminated by this process. This removal of a large portion of the magnetization reaching the receiver would allow for a much higher receiver gain setting, and consequently even better signal-to-noise ratios. However, to facilitate comparisons between the different techniques, this was not done (i.e., acquisition and processing parameters affecting the peak intensities are identical in all three experiments). The signal-to-noise ratios obtained with the different techniques for the H1/H2 cross-peaks are summarized in Table 1. Even at a four-fold shorter experimental time, the GCOSY produced cross-peak intensities that are more than double those observed in COSYLR for the two α-pyranoses and almost double for the β-pyranose. In fact, all cross-peak intensities are even higher in the GDQF-COSY than in the COSYLR if the experiments are compared with an equal number of transients. While the long-range COSY does reveal the desired interactions, it is an inferior alternative that greatly reduces the overall sensitivity of the experiment. Not surprisingly, the COSY

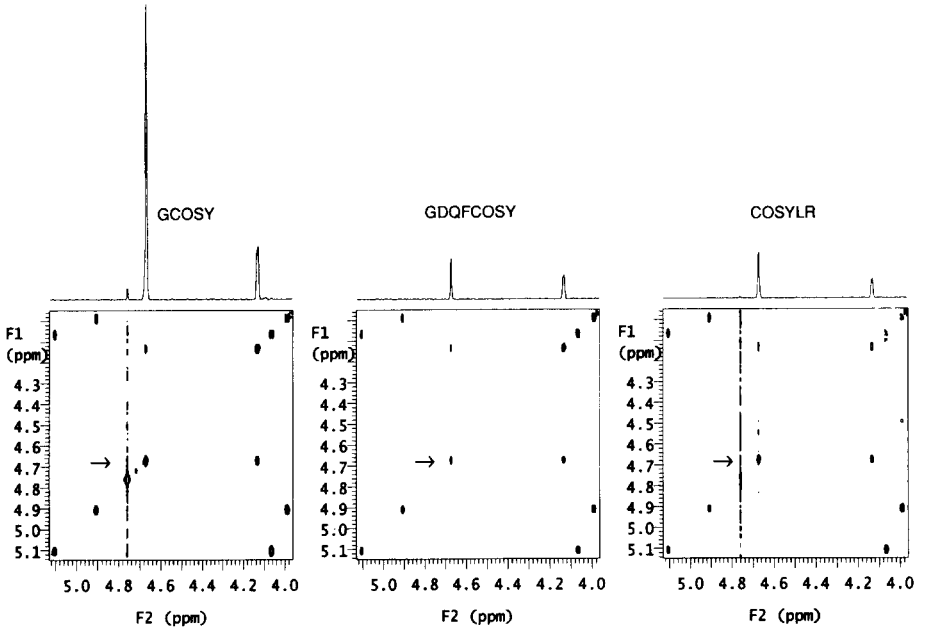

Fig. 4. A comparison between the gradient-enhanced techniques GCOSY GDQF-COSY and the conventional long-range COSY (COSYLR) experiment of the trisaccharide **2** with cross sections (indicated by arrows) through the H1 resonance of β-Man. The gradient techniques are the result of one scan per t1-increment (17 min total experimental time), whereas in the conventional spectrum 4 scans per t1-increment (68 min) were recorded. The vertical scales in all three panels and cross sections are identical. The only means of eliminating the solvent resonance is by double-quantum filtering in the middle panel.

without delays for small J enhancement is even worse, especially in case of the 0.8 Hz β-mannose correlation (Table 1).

The relative intensities of the α-pyranose cross-peaks are, within experimental error, the same in all three techniques, which is not surprising given their very similar coupling

Table 1
A comparison of the signal-to-noise ratios obtained for the H1/H2 crosspeaks in α-D-Man*p*-(1 → 3)-[α-D-Man*p*]-(1 → 6)-β-D-Man*p*-1 → $O(CH_2)_7CH_3$ (**2**) with several two-dimensional COSY techniques

	GCOSY		GDQF-COSY		COSYLR	COSY
Transients per t1 increment [a]	1	4	1	4	4	4
Experimental time (min)	17	68	17	68	68	68
α-D-Man′ ($J_{1,2}$ = 1.68 Hz) [b]	311:1	640:1	118:1	266:1	149:1	132:1
α-D-Man″ ($J_{1,2}$ = 1.75 Hz)	310:1	650:1	118:1	252:1	136:1	138:1
β-D-Man ($J_{1,2}$ = 0.8 Hz)	118:1	226:1	45:1	91:1	67:1	37:1

[a] Values obtained with 4 transients per t1 increment show, within experimental error, the expected two-fold increase compared to the experiments acquired with 1 transient only.
[b] The individual sugar rings are designated as follows: α-D-Man(1 → 3) single-primed; α-D-Man(1 → 6), double-primed.

Table 2
^{1}H chemical shifts [a] and coupling constants [b] of the trisaccharide α-D-Man*p*-(1 → 3)-[α-D-Man*p*]-(1 → 6)-β-D-Man*p*-1 → O(CH$_2$)$_7$CH$_3$ (**2**) [c]

	Chemical shifts						
	H1	**H2**	**H3**	**H4**	**H5**	**H6a** [d]	**H6b** [d]
α-D-Man′ [e]	5.11	4.07	3.90	3.66	3.80	3.89	3.75
α-D-Man″	4.91	3.99	3.83	3.66	3.69	3.89	3.76
β-D-Man-OR [f]	4.67	4.13	3.73	3.80	3.55	3.96	3.79
	Coupling constants						
	J_{12}	J_{23}	J_{34}	J_{45}	J_{56a}	J_{56b}	J_{6a6b}
α-D-Man′	1.7	3.4	9.7	9.7	2.1	6.1	−12.0
α-D-Man″	1.7	3.5	9.2	9.5	2.1	5.8	−12.0
β-D-Man-OR	0.8	3.2	9.7	9.7	5.1	1.9	−11.2

[a] Chemical shifts in ppm relative to 0.1% acetone at 2.225 ppm measured in a separate sample under exactly the same experimental conditions.
[b] Coupling constants in Hz, measured in the 1D spectrum where possible, otherwise in the appropriate z-filtered 1D-TOCSY spectra. The coupling constants were found to be within ±0.1 Hz where a comparison between 1D and 1D-TOCSY was possible.
[c] All data were recorded on a Varian Unity spectrometer operating at 500 MHz. The sample concentration was 10 mM in D_2O, temperature 30.0 ± 0.1°C.
[d] Geminal protons are not assigned stereospecifically. The larger chemical shift was arbitrarily assigned to H6a.
[e] See footnote b Table 1.
[f] The chemical shifts of *n*-octyl are: 3.85/3.65 (H1/H1′), 1.60 (H2), 1.3 (H3-H7), 0.88 ppm (CH_3).

constants. This indicates that gradients do not "magically" change the dependence of cross-peaks on the size of the coupling constant involved. It seems much more likely that the very clean, artifact-free FIDs lead to a far superior signal-to-noise ratio of the entire spectrum (diagonal peaks as well as cross-peaks produced by large *and* small coupling constants). The improvement is dramatic: between 1.8 and 2.3 fold in comparing 1-transient GCOSY and COSYLR, or between 3.4 and 4.8 fold when the GCOSY is recorded with four transients. This advantage should not be underestimated, especially in the context of increasing the dimensionality of experiments and the inherent need for very few transients per increment to avoid excessively long experiments. Furthermore, the excellent performance of gradient-enhanced correlation techniques has great potential in observing homonuclear long-range effects (^4J, ^5J) within sugar rings [21].

In cases like the trisaccharide **2**, with all three pyranose residues of the same type (mannose), a complete assignment of the proton resonances is not possible by homonuclear correlation experiments providing information about directly coupled protons only, and published data are therefore incomplete [9]. However, by extending the gradient-enhanced approach to the well-known total correlation spectroscopy (TOCSY [16,17]), complete and unambiguous assignments are very easy to achieve (Table 2). TOCSY also benefits from gradient enhancement, although to a lesser extent regarding time savings, since the minimum required phase cycle in the conventional experiment is very short [10]. However, improved sensitivity of correlation peaks is observed consistently. A

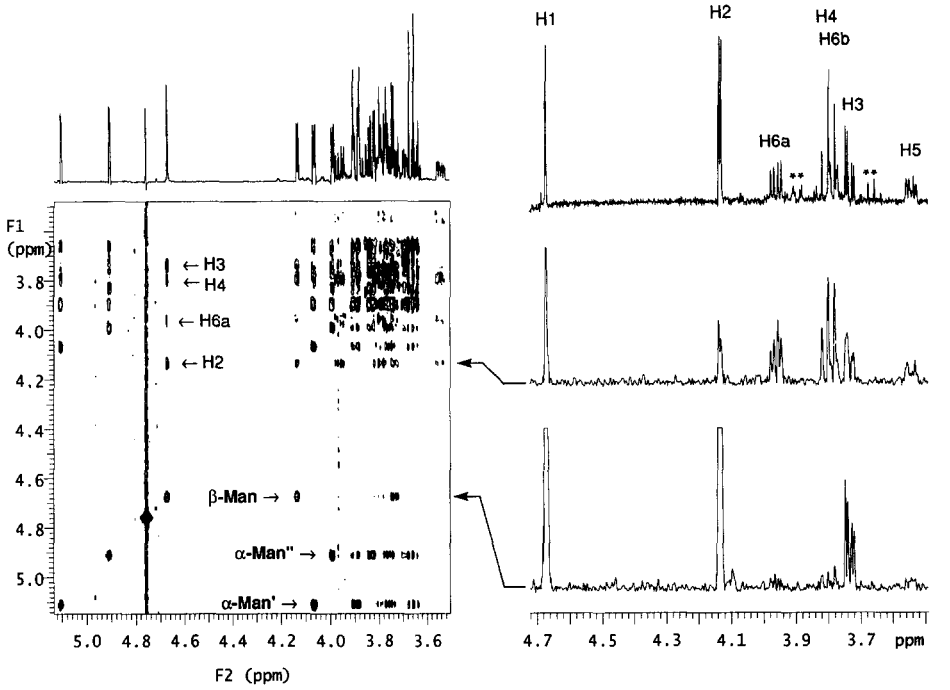

Fig. 5. The GTOCSY spectrum of **2** together with traces taken through the resonances of H1 (β-Man) and H2 (β-Man) as indicated. The 1D spectrum is depicted above the 2D plot and the z-filtered 1D-TOCSY spectrum obtained by selectively inverting H2 (β-Man) is shown as well. Spurious signals due to incomplete cancellation are indicated with stars.

meaningful quantitative comparison is more difficult than for GCOSY given the large number of correlations, but the peaks obtained with a single transient GTOCSY match approximately those obtained with 4 transients in a non-gradient TOCSY experiment.

The GTOCSY spectrum of **2** is shown in Fig. 5, recorded with a mixing time of 250 ms and a spin-lock field strength of ca. 5 kHz. For both of the α-anomeric protons of **2**, correlations across the entire ring system to H6a and H6b were readily observed. Even correlations between H1 of β-Man and its H2, H3 and H4 resonances can easily be detected in a single-transient GTOCSY experiment (experimental time 19 min). The remaining resonance assignments can be found without any difficulties by looking at the correlations starting from H2 of β-Man at 4.13 ppm. Independent corroboration of the correctness of the assignments was obtained for all three sugar rings by recording selective 1D TOCSY [18,19] spectra starting at H2 of β-Man (Fig. 5) or H1 of the two α-Man residues, respectively. By using 16 calculated z-filter values [20], coupling constants were recorded with high accuracy, i.e. within ± 0.1 Hz of the values in the 1D spectrum where comparisons were possible.

In summary, the use of gradient-enhanced 2D correlation techniques not only offers the well-known advantage of very short experimental times but, equally important, also markedly improves the intensity of correlation peaks between sugar protons, which is particularly useful when small coupling constants are present.

1. Experimental

Spectra were recorded on a Varian Unity 500 spectrometer with a commercial Varian 5 mm z-gradient inverse-detection triple-resonance probe. For data acquisition and processing VNMR software version 4.3A was used. The sample concentrations were 20 mM (**1**) and 10 mM (**2**) in D_2O. All spectra were recorded under temperature controlled conditions at 30.0 ± 0.1°C.

1D-TOCSY spectra were recorded by selectively inverting the H2 resonance of β-Man (or H1 of each of the two α-mannose rings) by a Gaussian pulse of 189 ms duration and by subtracting a spectrum with the same pulse off-resonance (difference method). The mixing time was 200 ms at a field strength of 5 kHz. Sixteen z-filter values were calculated in each case according to Subramanian and Bax [20] based on the chemical shifts of H2, H3, H4, H5 and H6b and 16 scans were accumulated per z-filter value (8 on, 8 off resonance). A 2 ms trim pulse preceded the spin-lock period. The resulting FIDs were coadded before processing with a resolution-enhancing Gaussian function of width 1/5 and shifted by 1/8 of the acquisition time.

All 2D spectra were recorded non-spinning in absolute-value mode with a sweep width of 2500 Hz in both dimensions and 4K data points in F2 (no zero-filling, except for GTOCSY with zero-filling to 8K) and 512 (zero-filled to 1K) data points in F1, resulting in digital resolutions of 1.2 (GTOCSY: 0.6) and 4.9 Hz/pt, respectively. All depicted gradient-enhanced experiments used one single transient per t1-increment (experimental time 17 min; GTOCSY, 19 min), whereas the conventional spectra (COSYLR, COSY, TOCSY) were recorded with 4 transients per t1-increment (68 min). To provide a meaningful comparison in Table 1, the gradient spectra were also recorded with four transients to provide real experimental signal-to-noise values rather than calculated ones. All gradients were rectangular in shape, applied in the *z*-direction and of the following strength and duration: GCOSY: 3 G/cm for 2 ms, GDQF-COSY: 8, 8 and 24 G/cm for 2 ms each, GTOCSY 4 G/cm for 2 ms. Gradient rise and fall times were 10 μs in all cases. All pulses were 90° and of duration 7.5 μs. The long-range COSY experiment was recorded with a 80 ms delay on each side of the second 90° pulse. The GTOCSY experiment was recorded with a 250 ms mixing time at a field strength of 5 kHz. This spin-lock field is sufficient to cover the limited frequency range of the carbohydrate proton resonances, and excellent spin-locking conditions were obtained without noticeable heating of the sample (chemical shifts did not change more than 0.01 ppm compared to the 1D spectrum).

All 2D data sets were processed identically. The absolute value mode requires the use of filters that keep the line widths narrow, thereby enhancing the resolution and not the signal-to-noise ratio. Therefore, the FIDs were multiplied by unshifted squared sine-bell functions of width t2/2 and t1/2, respectively. Signal-to-noise ratios were calculated with a VNMR-internal calculation routine. The same 100 Hz range (from 4.3 to 4.5 ppm) was chosen in each case as the area providing pure noise only. Care was taken to ensure that in all cases the cross-section with the highest intensity of the desired cross-peak was chosen. The maximum was found in exactly the same cross-section in all experiments indicating that gradients of various strength and occurrence in the pulse sequences do not introduce even small changes in chemical shifts.

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