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Hadamard 1D ^{1}H TOCSY and its Application to Oligosaccharides†

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Replacement of a series of *N* consecutive one-site selective excitation NMR experiments by *N* simultaneous *N*-site excitations with phases of component pulses varied according to the $N \times N$ Hadamard matrix offers a sensitivity improvement of the order of $\sqrt{N}$ (for $N = 2, 4, 8, \ldots$) [R. Freeman, *Spin Choreography*, pp. 177–183. Spektrum, Oxford (1996)]. Here this principle was extended to one-dimensional (1D) ^{1}H TOCSY experiments, in the absence and presence of heteronuclear decoupling. The 1D ^{1}H Hadamard TOCSY technique was applied to selected oligosaccharides, representing a class of biomolecules for which multiple 1D TOCSY experiments are the mainstay in ^{1}H NMR spectral assignment and structural characterization. © **1997 John Wiley & Sons, Ltd.**

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INTRODUCTION

Spectral editing by total correlation spectroscopy (TOCSY)[1,2] is a powerful way of unraveling the notoriously congested spectral regions of oligosaccharide ^{1}H NMR spectra, yielding assignable subspectra of the individual glycosyl groups.[3–6] To increase the spectral resolution and minimize the total acquisition time, carrying out a series of one-dimensional (1D) selective TOCSY experiments (one for each glycosyl group) is preferred over a full-blown two-dimensional (2D) TOCSY experiment, provided that the various glycosyl anomeric (H1) resonances are accessible for selective excitation. However, it occurred to us that the sensitivity of the sequential 'one-residue-at-a-time' 1D TOCSY method can be further improved, considering the potential of combining it with Hadamard spectroscopy.

Blechta and Freeman[7] have shown how to retrieve the multiplex advantage of 2D NMR in 1D experiments by Hadamard spectroscopy and thus achieve considerable savings in experimentation time. These 1D experiments use simultaneous multi-site selective excitation, with phase alternation for different excitation sites according to a Hadamard matrix. The basic idea is applicable to all types of selective spectroscopy where the phase of the detected signal can be made to follow the phase of the excitation pulse. This is the case for the 1D TOCSY experiment,[8–10] which is known (e.g. Ref. 11) to produce spectra whose amplitude changes sign with a 180° phase change of the excitation pulse. Such opposite-phase spectra are, in fact, often seen in standard 1D TOCSY experiments as a consequence of opposite phase in the wings (side-lobes) of the selective excitation profile, and are then considered 'artifacts'.

In this paper, we present the combination of 1D TOCSY and Hadamard spectroscopy as applied to oligosaccharide structure determination. Owing to the specific features of the ^{1}H NMR spectra of oligosaccharides, in particular the accessibility of the separate region of anomeric proton resonances to selective excitation, the time savings offered by the Hadamard 1D ^{1}H TOCSY technique are expected to be considerable for these compounds. One should be able to obtain a series of *N* monosaccharide subspectra with increased sensitivity (by a factor of $\sqrt{N}$ over standard 1D TOCSY) in a shorter time.

We shall demonstrate the principle of 1D ^{1}H Hadamard TOCSY experiments on a disaccharide (lactose), and then illustrate the application of the technique for the spectral assignment of a phosphorylated high-mannose undecasaccharide (OL-8P). In particular, the position of the phosphate diester group in the structure is delineated from the mannosyl subspectra obtained by

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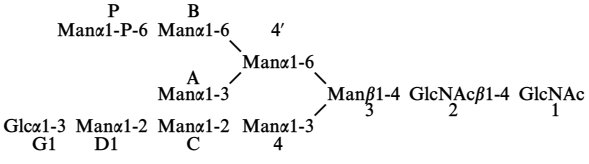
Hadamard 1D ^{1}H TOCSY, with and without the use of ^{31}P decoupling.

RESULTS

Materials

Lactose (purchased from Merck, Darmstadt, Germany) was dissolved in 99.96% D_2O (0.75 ml) (Isotec, Miamisburg, OH, USA) to a concentration of 150 mM; the solution was transferred into a 5 mm XA-5-LP NMR tube (Campro Scientific, Veenendaal, The Netherlands).

The phosphorylated high-mannose oligosaccharide (OL-8P) was isolated from cellobiohydrolase I (CBH-I) obtained from the filamentous fungus *Trichoderma reesei* RUTC 30.[12] Briefly, *N*-linked oligosaccharides were released from purified *T. reesei* CBH-I using peptide:*N*-glycosidase F (PNGase F). Oligosaccharide OL-8P was isolated from the PNGase-F digest by Biogel P4 filtration. Preliminary structural characterization of the oligosaccharide involved fluorescent labeling and polyacrylamide gel electrophoretic analysis, sequential exoglycosidase (glucosidase, mannosidase) and alkaline phosphatase digestions, monosaccharide composition analysis and Dionex high-pH anion-exchange chromatographic profiling. The complete structural characterization of OL-8P employed various ^{1}H, ^{13}C and ^{31}P NMR techniques.[12] The resulting structure and letter/number coding of glycosyl residues for oligosaccharide OL-8P are as follows:



About 0.5 mg of pure OL-8P was deuterium exchanged several times in 99.96% D_2O and finally dissolved in 0.75 ml of 99.96% D_2O (Isotec). The solution was transferred into a 5 mm XXA-5-LP NMR tube (Campro Scientific).

NMR methods

NMR experiments were performed on a Varian UNITY-500 instrument (operating at 499.668 MHz for ^{1}H NMR and at 202.276 MHz for ^{31}P NMR), running under VNMR software (version 5.1A). The instrument was equipped with a 5 mm ID PFG (inverse detection with pulsed magnetic field gradients) $^{1}H\{X\}$ probe, tuned for ^{31}P in the 'decoupling' channel.

The 1D TOCSY experiments[13] used the MLEV17 sequence[2] (MLEV = Malcolm Levitt's composite-pulse decoupling cycle) for isotropic mixing of 200–300 ms duration; the mixing time was flanked by two trim pulses of 2 ms each. Selective excitation of anomeric resonances was achieved using 90° E-BURP-1 pulses[14] with bandwidths varying from 10–20 Hz (equivalent to a B_1 field of 15 Hz for 225–450 ms). (E-BURP = excitation by a band-selective, uniform response, pure-phase pulse). The E-BURP-1 pulses were generated by a waveform generator (Varian) using the excitation profiles for shaped pulses calculated by the Pandora's Box program (version 4.1), a part of Varian's VNMR software.[11] Phase alternation of the pulses based on the Hadamard 2 [*H(2)*] or Hadamard 4 [*H(4)*] matrices was also taken care of by the Pandora's Box software. Heteronuclear ^{31}P decoupling was conducted by continuous low-power (B_2 field strength *ca* 50 Hz) on-resonance decoupling throughout the entire pulse sequence. The $\{X\}$ broadband transmitter was set to the frequency of the sole ^{31}P resonance of OL-8P as determined by a ^{31}P NMR measurement. Further details specific to the pertinent experiments are mentioned in the figure captions.

NMR experiments were conducted at 27 °C for lactose and at 33 °C for OL-8P, without sample spinning. Each of the traces in the Hadamard 1D ^{1}H TOCSY experiments of OL-8P was collected in 832 transients (by interleaving the data acquisition using a block size of 64 transients), whereas the traces for lactose were acquired in eight transients each. The acquisition and isotropic mixing times were 4 and 0.3 s, respectively; no additional relaxation delay was inserted before the selective excitation pulse. The FIDs were collected in 25 600 data points each. Data were processed on a Silicon Graphics Indigo2 workstation using the Felix95 software package (BioSym/MSI, San Diego, CA, USA). Typically, line broadening of the spectra was applied using an exponential weighting function (with lb = 0.2 or 0.3); the spectra were zero-filled to 32K points.

RESULTS

The principles of the TOCSY approach in its 2D, 1D sequential and 1D simultaneous modes are outlined schematically in Fig. 1. When the total duration of the data gathering is of prime consideration, the common 2D TOCSY experiment [Fig. 1(a)] is conveniently replaced by an appropriate set of selective 1D TOCSY experiments [Fig. 1(b)]. However, performing a small number of consecutive soft-pulse experiments (revealing subspectra one glycosyl residue at a time) is not necessarily the most efficient approach to 1D TOCSY in terms of sensitivity. An alternative strategy may be employed that utilizes simultaneous multi-site excitation employing a coding scheme based on Hadamard matrices.[7,15]

Generic *H(2)* matrix	frequency (a)	frequency (b)
Expt (1)	+	+
Expt (2)	+	−

For example, *H(2)* Hadamard excitation employs two separate experiments [Expts (1) and (2)], which are run with two-site selective excitation on frequencies (a) and (b); Expt (2) differs from Expt (1) in that phase inversion of the second component soft pulse [on fre-

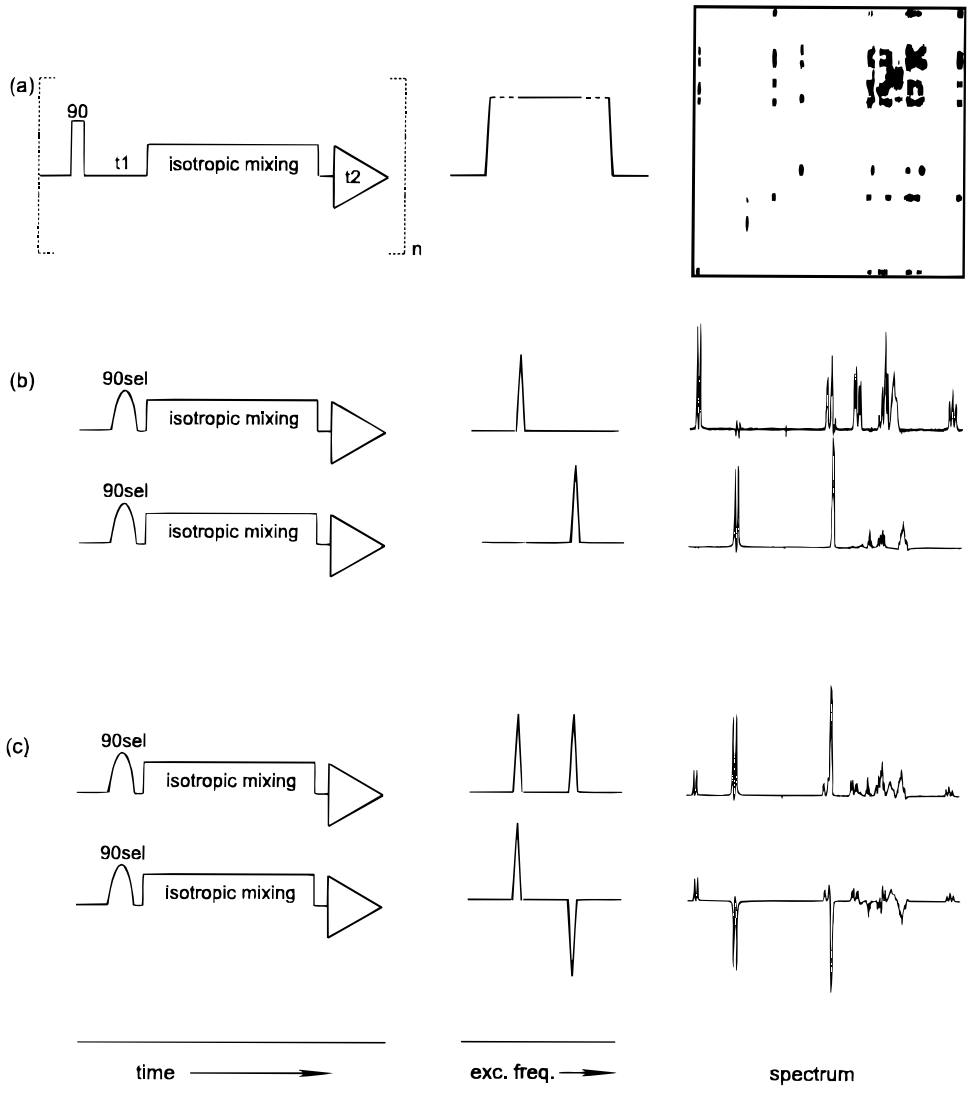


Figure 1. Pulse sequences, frequency-domain excitation profiles and resulting spectra of (a) 2D TOCSY, (b) sequential 1D TOCSY and (c) Hadamard 1D TOCSY experiments

quency (b)] is applied according to the *H(2)* matrix given above. The spectra from these two experiments are combined according to the columns of the matrix. Thus, the sum of spectra (1) and (2) gives the spectrum excited by soft pulse (a), while the difference of spectra (1) and (2) gives the spectrum excited by soft pulse (b) [see Fig. 1(c)]. The sensitivity of the spectra resulting from *H(2)* Hadamard 1D TOCSY is improved by a factor of $\sqrt{2}$ compared with the consecutive approach, i.e. two experiments with one soft pulse at a time.

Figure 2 illustrates the application of *H(2)* Hadamard 1D TOCSY to the disaccharide lactose [Galβ(1–4)Glc]; it should be mentioned that lactose in aqueous solution and ambient temperature is present as a mixture of two anomeric forms Galβ(1–4)Glcα and Galβ(1–4)Glcβ, in the approximate ratio of 1:2. The subspectra of the two β-glycosyl residues were obtained by simultaneous selective excitation of the anomeric resonances of Glcβ and Gal, in the first experiment with the same phase and in the second experiment with opposite phase of the individual soft pulses. The subspectrum shown for Glcβ is the result of summation of Expts (1) and (2), whereas the subspectrum of Gal is the result of the subtraction, Expt (1) – (2).

H(2) matrix for lactose		
	Glcβ H1	Gal H1
Expt (1)	+	+
Expt (2)	+	–

The results of the analogous experiment using four *H(4)* Hadamard 1D ^{1}H TOCSY excitations on OL-8P are shown in Fig. 3. In this case, we chose to excite the anomeric signals of mannosyl residues P, C, D_1 and B. Figure 4 shows the subspectra of these four mannosyl residues, generated by recombining the *H(4)* Hadamard 1D TOCSY traces of Fig. 3. The signal-to-noise ratio of the spectra in Fig. 4 is improved by a factor of 2 over each of the spectra that one would obtain by the corresponding four sequential 1D TOCSY experiments with the equivalent number of scans.

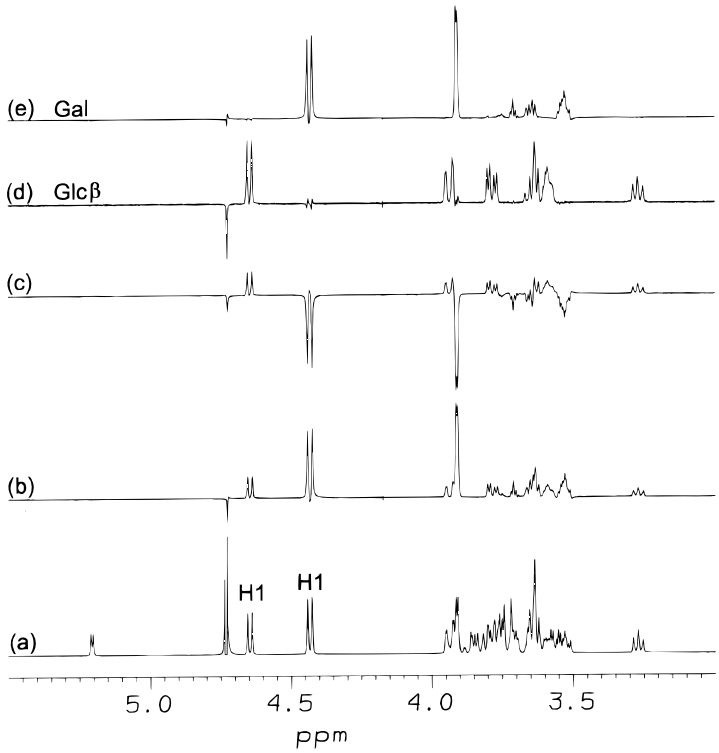


Figure 2. *H(2)* Hadamard 1D TOCSY of lactose, Galβ(1–4)Glc. Lactose, in D_2O at 27 °C, is present as a 1 : 2 mixture of the α- and β-anomers of the disaccharide. (a) ^1H reference spectrum of lactose; (b) 1D TOCSY spectrum obtained by simultaneous excitation of Glcβ H1 and Gal H1 with selective 90° pulses of the same phase (++); (c) 1D TOCSY spectrum obtained by simultaneous excitation of Glcβ H1 and Gal H1 with selective 90° pulses of opposite phase (+−); (d) Individual 1D TOCSY spectrum of Glcβ obtained by co-adding traces (b) and (c); (e) Individual 1D TOCSY spectrum of Gal obtained by subtracting traces (b) and (c).

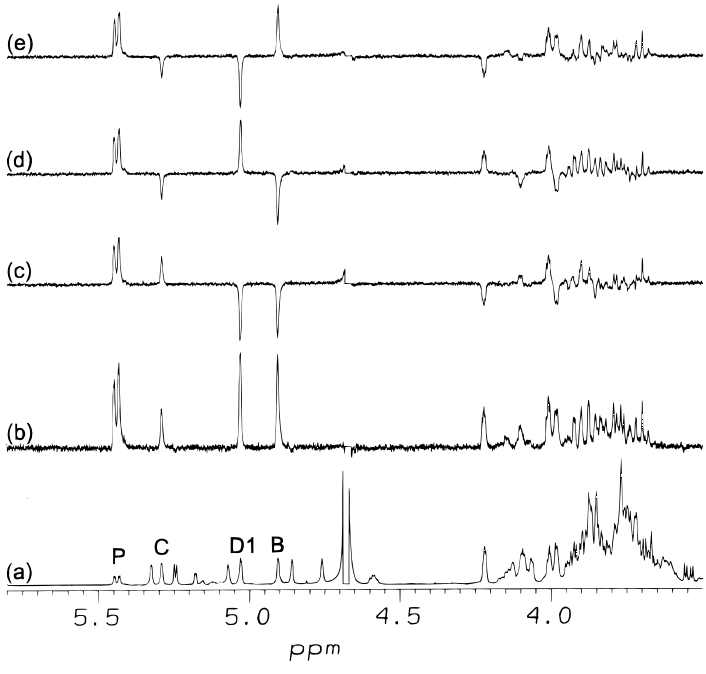


Figure 3. *H(4)* Hadamard 1D TOCSY of oligosaccharide OL-8P in D_2O at 33 °C. (a) ^1H reference spectrum of OL-8P; (b) 1D TOCSY spectrum obtained by simultaneous excitation of Man-P, Man-C, Man-D_1 and Man-B H1 with selective 90° pulses of the same phase (++++); (c) 1D TOCSY spectrum obtained by simultaneous excitation of Man-P, Man-C, Man-D_1 and Man-B H1 with selective 90° pulses of alternating phases (++−−); (d) 1D TOCSY spectrum obtained by simultaneous excitation of Man-P, Man-C, Man-D_1 and Man-B H1 with selective 90° pulses of alternating phases (+−+−); (e) 1D TOCSY spectrum obtained by simultaneous excitation of Man-P, Man-C, Man-D_1 and Man-B H1 with selective 90° pulses of alternating phases (+−−+).

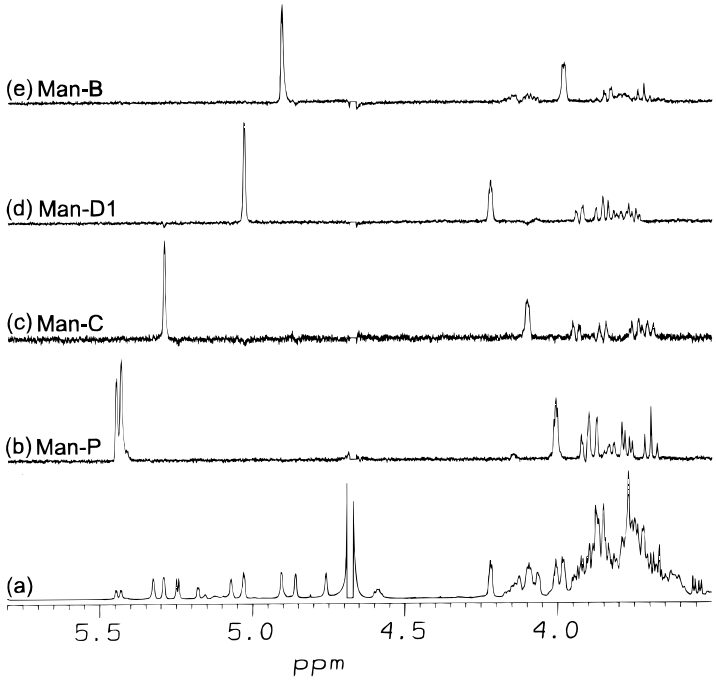


Figure 4. *H(4)* Hadamard 1D TOCSY of oligosaccharide OL-8P in D_2O at 33 °C. (a) ^1H reference spectrum of OL-8P; (b)–(e) individual 1D TOCSY spectra of Man-P, Man-C, Man-D_1 and Man-B, respectively, obtained by combination of traces (b)–(e) in Fig. 3 according to the Hadamard *H(4)* matrix.

H(4) matrix for OL-8P				
	Man-P H1	Man-C H1	Man-D_1 H1	Man-B H1
Expt (1)	+	+	+	+
Expt (2)	+	+	−	−
Expt (3)	+	−	+	−
Expt (4)	+	−	−	+

The subspectrum of Man-P is the result of Expt (1) + (2) + (3) + (4); the subspectrum of Man-C is the result of Expt (1) + (2) − (3) − (4); the subspectrum of Man-D_1 is the result of Expt (1) − (2) + (3) − (4); and the subspectrum of Man-B is the result of Expt (1) − (2) − (3) + (4). It is worth mentioning that the acquisition time per Hadamard trace was approx-

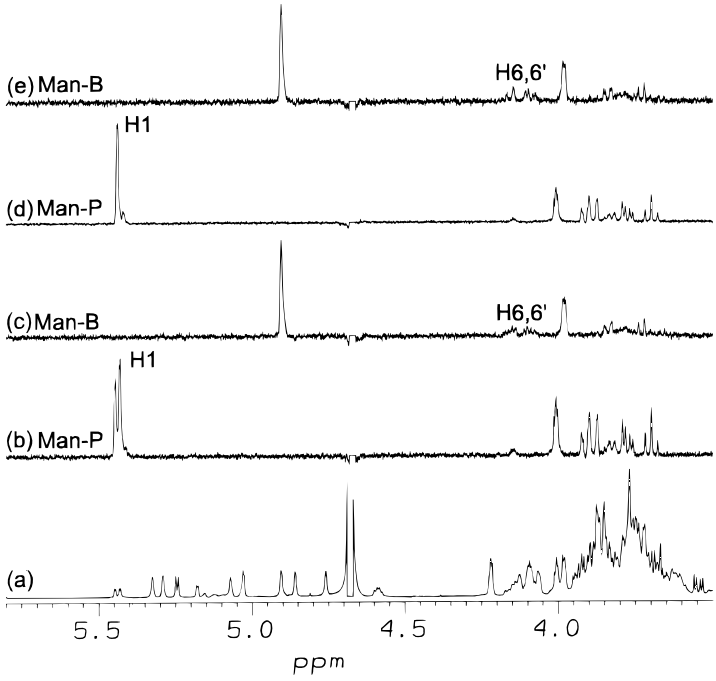


Figure 5. *H(2)* Hadamard 1D TOCSY of oligosaccharide OL-8P in D_2O at 33 °C, without and with ^{31}P decoupling. (a) ^1H reference spectrum of OL-8P; (b) and (c) Individual 1D TOCSY spectra of Man-P and Man-B, respectively, obtained by adding and subtracting traces obtained by simultaneous excitation of Man-P and Man-B H1 with selective 90° pulses of the same and opposite phase, respectively; (d) and (e) individual ^{31}P-decoupled 1D TOCSY spectra of Man-P and Man-B, respectively, obtained by adding and subtracting traces obtained by simultaneous excitation of Man-P and Man-B H1 with selective 90° pulses of the same and opposite phase, respectively, under ^{31}P decoupling conditions.

imately 1 h, whereas a 2D TOCSY data set (832 transients per experiment; 256 experiments of 2K data points per FID) would require 47.5 h of data accumulation.

In order to locate the phosphate group in the structure of OL-8P, an *H(2)* Hadamard 1D TOCSY experiment was performed on the H1 signals of Man-P and Man-B, in the absence and presence of ^{31}P decoupling. Figure 5 contains the subspectra of Man-P and Man-B; the subspectra of Man-P are the results of summation, whereas the subspectra of Man-B are the result of subtraction of the appropriate Hadamard spectra. Figure 5 illustrates that the signals of anomeric and, more importantly, non-anomeric protons split by ^{31}P,^{1}H scalar coupling can be readily identified by comparison of 1D TOCSY subspectra in the absence and presence of ^{31}P decoupling. In the subspectra of the OL-8P residues, Man-P H1 (doublet of doublets at 5.44 ppm, with $^{3}J_{H1,H2} = 1.7$ Hz and $^{3}J_{HP} = 8.1$ Hz) and Man-B H6 and H6′ are the only signals that sharpen significantly upon ^{31}P decoupling. These H6 protons, at 4.16 and 4.10 ppm, respectively, exhibit a coupling pathway to the H1 proton of Man-B (at 4.91 ppm) [Figs 4(e) and 5(c)]; the collapse of the multiplets of both these H6 protons due to ^{31}P decoupling is shown in Fig. 5(e). These observations identify the location of the phosphate group in OL-8P between Man-P C1 and Man-B C6.

CONCLUSIONS

This paper presents a procedure for obtaining 1D TOCSY-type correlation information on oligosaccharides by simultaneous excitation of several anomeric sites with selective radiofrequency pulses that are phase-alternated individually according to a Hadamard matrix. The 1D TOCSY correlation spectra for individual glycosyl residues are obtained by recombining these scans with the corresponding permutation of signs.

Hadamard 1D ^{1}H TOCSY retrieves the sensitivity advantage that is lost on going from 2D TOCSY to the consecutive 1D TOCSY version. If the full Hadamard matrix is employed (N experiments with simultaneous excitation of N sites), the sensitivity is improved by $\sqrt{N}$ compared with that of N consecutive scans using a single soft pulse each. More importantly, N pieces of correlation information are obtained in a time much shorter than that required by the corresponding 2D TOCSY experiment. Our results show that the theoretically predicted improvements in signal-to-noise ratio are indeed achieved. The Hadamard 1D ^{1}H TOCSY experiment is readily implemented on modern NMR spectrometers.

Thus, Hadamard 1D ^{1}H TOCSY, with multi-site simultaneous excitation, brings together the advantages of 1D and 2D NMR, improving upon consecutive 1D TOCSY in terms of sensitivity and upon 2D TOCSY in data acquisition time (and digital resolution). Although by no means limited to applications for oligosaccharides, the experiment should prove particularly useful for carbohydrates.

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