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Selective Observation of Subspectra of Phosphorylated Residues in Complex Carbohydrates by $^1H\{^{31}P\}$ Relayed Spin-Echo Difference Spectroscopy Based on MLEV-17

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A variety of carbohydrate chains comprising glycoconjugates and polysaccharides bear phosphate and/or sulfate groups (*1–4*). A main problem in the elucidation of the primary structure of these acidic compounds is the determination of the location of the substituents. It is difficult to establish which monosaccharide is substituted and to establish which carbon atom of the monosaccharide is involved in the linkage. The availability of only small amounts of material may be a further complicating factor. In the ^1H NMR spectrum of carbohydrates the signals of the skeleton protons resonating in the crowded bulk region between δ = 3.4 and 3.9 ppm (*5*) often cannot be assigned. Attachment of a phosphate or sulfate group gives rise to shifts of $\Delta\delta$ = 0.25–0.55 ppm (*6*) or $\Delta\delta$ = 0.5–0.7 ppm (*7*), respectively, of the proton attached to the substituted carbon atom. Usually such a shift increment is sufficient to make this proton observable out of the bulk region. The extra signal in the structural reporter group region (*5*) can only be assigned to a sugar residue if the interconnection with a structural reporter group, for example, an anomeric proton, is known. To identify a 6-O sulfated mannose in a N-linked carbohydrate chain Van Kuik *et al.* (*8*) successfully applied the 1D HOHAHA method (*9*) to prove the interconnection between the H-6 and H-6′ signals and the H-1 and H-2 structural reporter groups of this residue. For phosphate, application of the selective π pulse, essential for the 1D HOHAHA method, can be very difficult if the α-proton signal is not shifted out of the bulk region, or if this signal interferes with other signals like mannose H-2 or galactose H-4, often occurring in the region between 3.9 and 4.4 ppm. The use of $^1H\{^{31}P\}$ NMR techniques as decoupling experiments can be helpful to reveal the α proton in this case.

Cohen *et al.* proposed a sequence for selective observation of phosphate ester protons in sugar phosphate mono-esters by $^1H\{^{31}P\}$ spin-echo difference spectroscopy (SED) (*10*):

^1H: $\pi/2(x)$—τ—d—$\pi(y)$—d—τ—AQ+/−

^{31}P: $\pi/2$ $\pi/2$ on/off.

By applying a composite ^{31}P π pulse together with a proton refocusing π pulse, one can refocus the POCH signal with a phase opposite to that of the remaining protons

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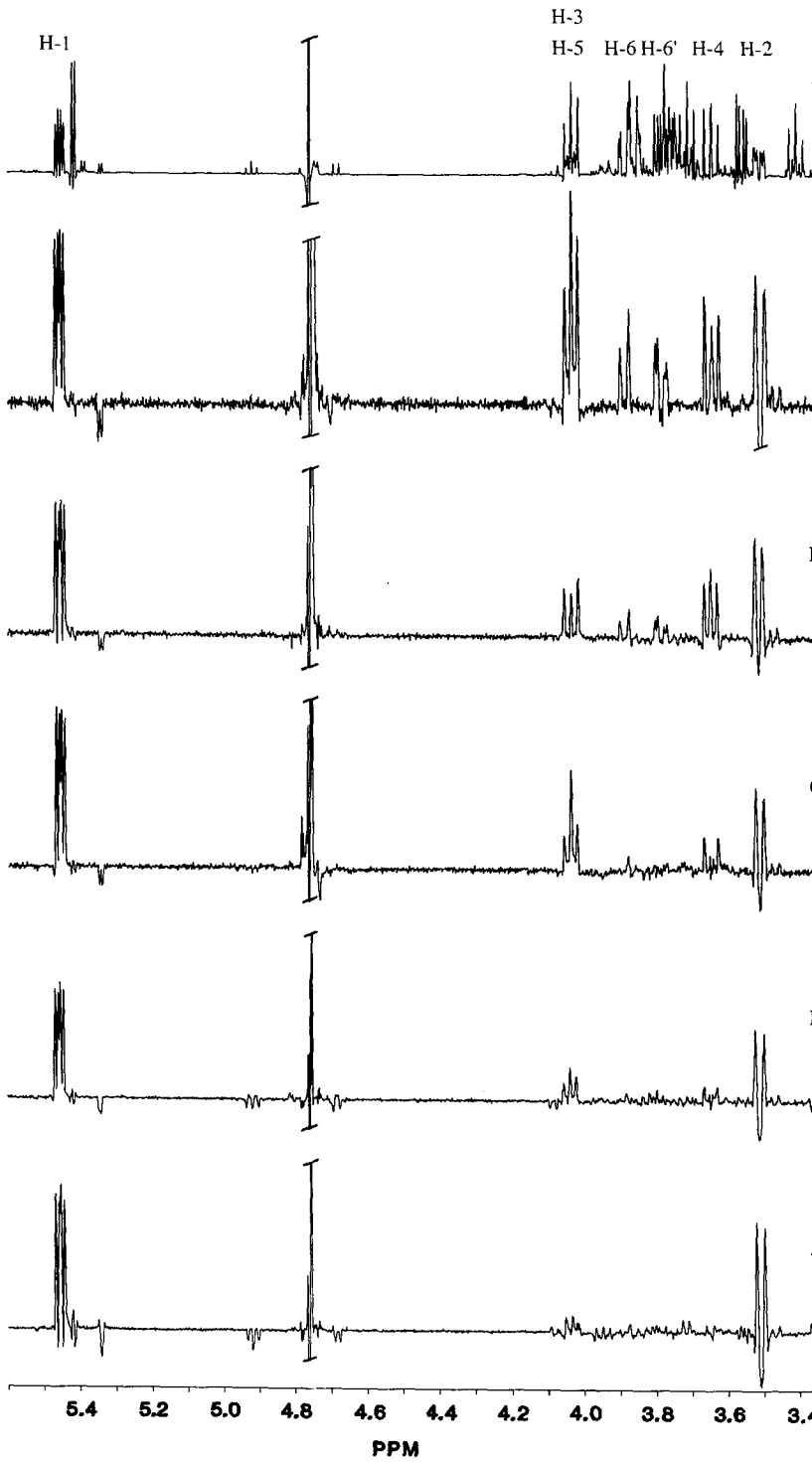
H-1
H-3
H-5
H-6
H-6'
H-4
H-2
F
E
D
C
B
A
5.4
5.2
5.0
4.8
4.6
4.4
4.2
4.0
3.8
3.6
3.4
PPM

if the delay τ is equivalent to $1/2J_{POCH}$. Acquisition of the data after the second delay τ and subtraction from data of an experiment without the ^{31}P pulses yields a spectrum of only POCH signals. If there is only one phosphate group, the POCH signal is selectively inverted by 180°, an effect similar to that observed by application of a selective π pulse in the 1D HOHAHA method. We therefore propose the following sequence:

$$^{1}H: \quad \pi/2(x)—\tau—d—\pi(y)—d—\tau—\overline{\text{MLEV}(y)}\text{AQ}+/-$$

$$^{31}P: \quad \pi/2 \quad \pi/2 \quad \text{on/off.}$$

The MLEV-17 composite spin-lock pulse is an efficient mixing sequence for magnetization transfer between coupled protons (*11*); during this mixing sequence the negative magnetization of the POCH signal is spread out through the scalar coupling network as in the conventional 1D HOHAHA experiment. Subtraction from data of an experiment without the ^{31}P pulses leads to a subspectrum of the phosphorylated residue.

As an example of this relayed spin-echo difference (RESED) method, spectra at various mixing times of maltose 1-phosphate are depicted in Fig. 1 together with the 500 MHz ^{1}H NMR spectrum. At a mixing time of 20 ms (Fig. 1A) only the H-1 and H-2 signals are present. The H-2 atom has a long-range $^{1}H-^{31}P$ coupling as is clear from its multiplet structure (Fig. 1F). At longer mixing times (Figs. 1B–1E) the proton signals subsequently appear. Mixing times of 120 and 160 ms reveal the total subspectrum of the phosphorylated glucose residue.

One of the difficulties of the $^{1}H\{X\}$SED method is the phase modulation of the proton resonances as discussed by Cohen *et al.* (*10*). Applying trim pulses before and after the MLEV sequence destroys unwanted antiphase magnetization of the POCH signal and out-of-phase components of the proton resonances resulting in spectra which can be phased in pure absorption mode. In some cases the trim pulses can lock a magnetization to an opposite phase as for a part of the H-2 multiplet of maltose 1-phosphate in Fig. 1.

Plotting in absolute-value mode can improve the appearance of the spectrum, but this can result in subtraction artifacts leading to patterns similar to real signals after absolute-value calculation. In Fig. 2 for the RESED method applied to glucose 1-phosphate, a mixing time of 40 ms reveals the H-2, H-3, and part of the H-4 signals. It is obvious that the applied mixing time cannot afford magnetization transfer from H-1 to H-6; the H-6 signal in Fig. 2A is a subtraction artifact. After absolute-value calculation the appearance in Fig. 2B of the H-2 and H-4 signals has improved, but it is uncertain whether the H-6 signal is an artifact or not. A line broadening of 3

FIG. 1. The 500 MHz ^{1}H NMR spectra of maltose 1-phosphate (2 mg in 450 μl D_2O). The experiments were carried out on a Bruker AM-500 equipped with a 5 mm inverse broadband probehead. The ^{31}P $\pi/2$ pulse length (125.759 MHz) was 12 μs, the ^{1}H $\pi/2$ pulse, supplied through the decoupler channel, was 27 μs. (A)–(E) The $^{1}H\{^{31}P\}$RESED spectra (64 scans each) with mixing times of 20, 40, 80, 120, and 160 ms, respectively (d = 12 μs). The delay τ was 34 ms, half its theoretical value calculated from the $^{1}H-^{31}P$ coupling constant of 7.4 Hz. (F) The ^{1}H NMR spectrum, 40 scans. Resolution enhancement of the spectra was achieved by Lorentzian-to-Gaussian transformation. The assignment of the phosphorylated residue is indicated.

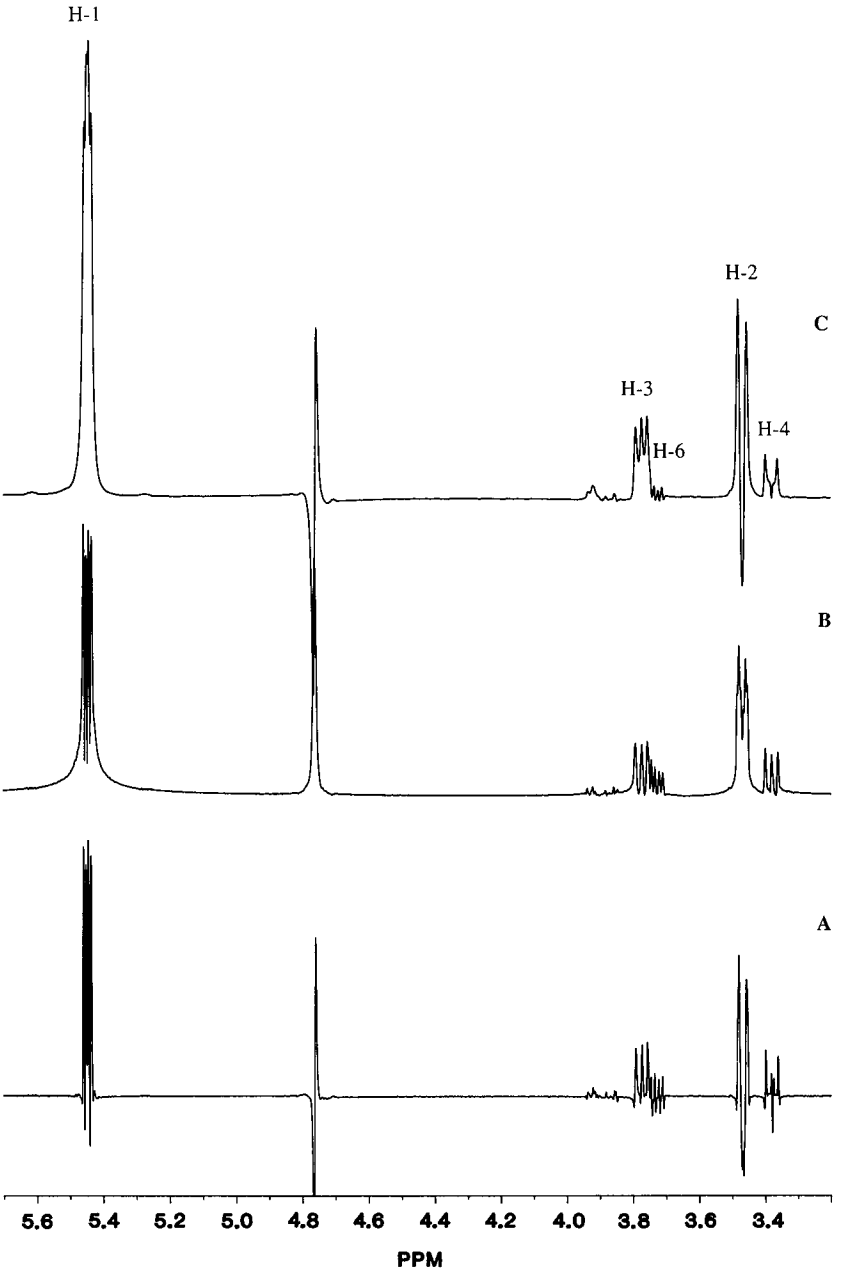

FIG. 2. The 500 MHz $^{1}H\{^{31}P\}$ RESED spectra (40 scans) of glucose 1-phosphate (2 mg in 450 μl D_2O). The mixing time is 40 ms, the delay τ is 34 ms. (A) Pure absorption-mode resolution-enhanced spectrum. (B) Magnitude spectrum of (A). (C) Pure absorption-mode spectrum after a line broadening of 3 Hz.

Hz (Fig. 2C) partially prevents subtraction artifacts, but it cancels opposite phase magnetizations of the H-4 quartet.

The value of the interval τ should equal $1/2J_{POCH}$ for maximal inversion of the POCH signal (*10*). The spectra shown in Figs. 1 and 2 are recorded with $\tau = 34$ ms, which is half the theoretical value calculated from the $^{31}P-^{1}H$ coupling constant of 7.4 Hz. We found the value of τ not to be very critical for this experiment, which is important, since the coupling constant will often be unknown. Keeping this delay short (about half its theoretical value) is most convenient since phase modulation of the proton resonances is kept as small as possible.

In the RESED method magnetization transfer between coupled protons will be very efficient due to the applied MLEV mixing sequence (*11*); therefore we consider this method to be more sensitive than INEPT-like sequences (*12*). We applied the RESED method successfully on low amounts of 6-O-phosphorylated oligosaccharides derived from yeast mannan; these results will be published elsewhere.

REFERENCES

1. E. G. BERGER, E. BUDDECKE, J. P. KAMERLING, A. KOBATA, J. C. PAULSON, AND J. F. G. VLIEGENTHART, *Experientia (Basel)* **38,** 1129 (1982).
2. W. TANNER AND L. LEHLE, *Biochim. Biophys. Acta* **906,** 81 (1987).
3. L. KENNE AND B. LINDBERG, *in* "The Polysaccharides" (G. O. Aspinale, Ed.), Vol. 2, p. 287, Academic Press, New York, 1983.
4. I. A. NIEDUSZYNSKI, *in* "Polysaccharides" (E. D. T. Atkins, Ed.), Chap. 4, VCH Publishers, Deerfield Beach, 1985.
5. J. F. G. VLIEGENTHART, L. DORLAND, AND H. VAN HALBEEK, *Adv. Carbohydr. Chem. Biochem.* **41,** 209 (1983).
6. J. BREG, P. ROMIJN, H. VAN HALBEEK, J. F. G. VLIEGENTHART, R. A. VISSER, AND C. A. G. HAASNOOT, *Carbohydr. Res.* **174,** 23 (1988).
7. M. J. HARRIS AND J. R. TURVEY, *Carbohydr. Res.* **15,** 57 (1970).
8. J. A. VAN KUIK, J. BREG, C. E. M. KOLSTEEG, J. P. KAMERLING, AND J. F. G. VLIEGENTHART, *FEBS Lett.* **221,** 150 (1987).
9. D. G. DAVIS AND A. BAX, *J. Am. Chem. Soc.* **107,** 7197 (1985).
10. J. S. COHEN, C. CHEN, AND A. BAX, *J. Magn. Reson.* **59,** 181 (1984).
11. A. BAX AND D. G. DAVIS, *J. Magn. Reson.* **65,** 355 (1985).
12. L. D. FIELD AND B. A. MESSERLE, *J. Magn. Reson.* **66,** 483 (1986).