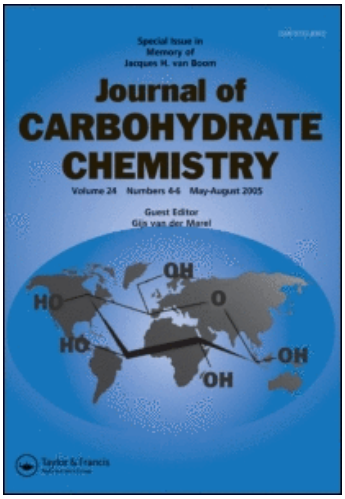

Journal of Carbohydrate Chemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713617200

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To cite this Article Schraml, Jan , De Bruyn, André , Contreras, Roland and Herdewijn, Piet(1997) 'A Versatile NMR Technique for the Identification of Phosphorylation Sites in Oligosaccharides', Journal of Carbohydrate Chemistry, 16: 2, 165 — 170

To link to this Article: DOI: 10.1080/07328309708006518

URL: http://dx.doi.org/10.1080/07328309708006518

J. CARBOHYDRATE CHEMISTRY, 16(2), 165-170 (1997)

A VERSATILE NMR TECHNIQUE FOR THE IDENTIFICATION OF PHOSPHORYLATION SITES IN OLIGOSACCHARIDES

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Received September 10, 1996 - Final Form December 9, 1996

ABSTRACT

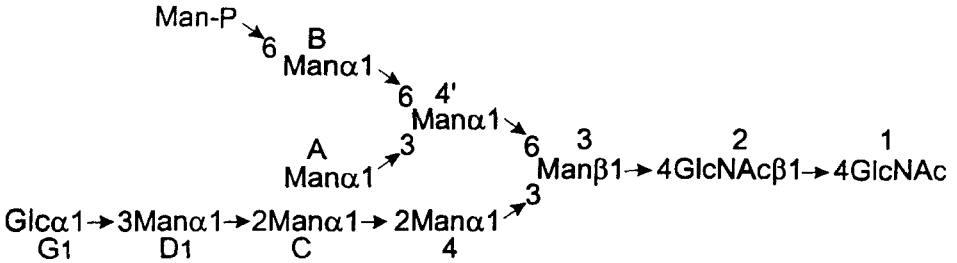
A combination of two well-established NMR methods, namely, phosphorus decoupling and 1D-TOCSY, provides identification of phosphorylation sites in a simple and straightforward manner in small to medium-sized oligosaccharides. The method is demonstrated on Man-P-GlcMan$_7$GlcNAc$_2$, a glycoprotein-derived N-linked type oligosaccharide.

INTRODUCTION

Phosphorylation is encountered in many biologically important events often involving oligo- or polysaccharides.[1] In order to be able to analyse the function of the phosphorylation/dephosporylation process it is important to know which monosaccharide units and which of their oxygen atoms are phosphorylated. The determination of the phosphorylation site is a simple undertaking if it involves anomeric carbon atoms. In such

cases the multiplet of the anomeric proton exhibits heteronuclear coupling (^1H - ^{31}P) which is easily identified by heteronuclear decoupling. Other phosphorylation sites are identified only with considerable difficulty, as the results of 2D ^1H - ^{31}P correlation experiments are difficult to interpret unambiguously due to congestion in the relevant part of the ^1H NMR spectrum.

We have found that the phosphorylation sites may be identified in an easy and reliable way using a combination of $\{^{31}P\}$ decoupling with 1D-TOCSY (or selective TOCSY),[2,3] which is simple to implement on a modern spectrometer. The method is demonstrated here using Man-P-$GlcMan_7GlcNAc_2$ as an example. The compound has the following structure:



RESULTS AND DISCUSSION

The ^1H NMR spectra shown in Fig. 1 clearly illustrate the method. An expansion of the anomeric region of the spectrum is shown on the left while on the right is the relevant part of the spectrum in which signals from all other O-C-H protons are found. The top trace (*a*) is an ordinary ^1H NMR spectrum, the three pairs of traces (*b, c, d*) show 1D TOCSY spectra . In each pair of traces the same anomeric proton line was selectively excited, lower and upper traces within the pair were measured with and without $\{^{31}P\}$ decoupling, respectively.

Since there is no difference between the two spectra of traces *b*, which were excited by selective irradiation of H-1 (anomeric proton) of Man-4', one can conclude that this monosaccharide unit is not phosphorylated. A similar picture was obtained when any other anomeric proton resonance was excited except for the two cases shown on traces *c*

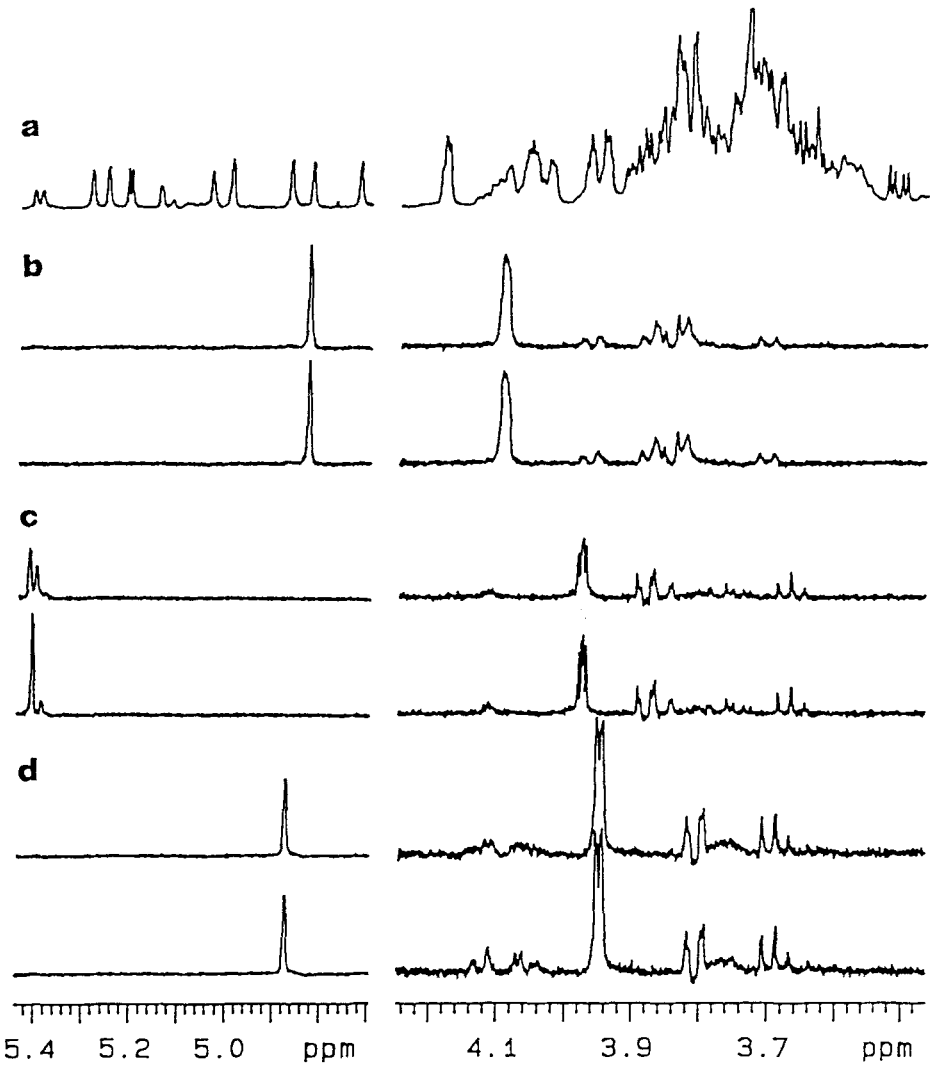


Figure 1. Partial 500 MHz ^{1}H NMR spectra. Trace (*a*) standard ^{1}H NMR spectrum, traces (*b*), (*c*), and (*d*) selective (1D) TOCSY spectra measured with coherent low-power $\{^{31}P\}$ decoupling (lower spectrum) and without $\{^{31}P\}$ decoupling (upper spectrum).

and *d*. The difference between the anomeric parts of the two spectra belonging to trace *c* identifies the H-1 proton of mannose, denoted in the formula as Man-P at one end of the phosphate link. The other end of the link is identified by the difference in the O-C-H regions belonging to the traces *d*, which were excited by a selective pulse aimed at H-1

proton of Man-B. From a series of routine 1D TOCSY experiments carried out with varying mixing times, the signal around δ = 4.1 (which changes with the {31P} decoupling) is identified as H-6 of this unit. As expected, both protons (A and B) of H-6 are affected by the decoupling. Hence, the site of phosphorylation is identified as the oxygen atom on C-6 carbon of Man-B.

The proposed method is simple to implement. In fact, 1D-TOCSY pulse sequences are now a standard part of manufacturer provided software, and the inclusion of $\{^{31}P\}$ decoupling is rather trivial, providing the hardware (especially the probe) is capable of performing such ^{1}H - $\{^{31}P\}$ experiments.

Several conditions have to be considered for optimizing the experiment (in addition to usual requirements when setting up the common selective TOCSY experiment). The selective excitation can be accomplished by any appropriate method, either by the use of shaped pulses or by a DANTE pulse sequence.[4] It is, however, important to select long enough mixing times. The mixing must assure that the magnetization propagates throughout the whole coupling network in each and every monosaccharide unit. In our experience mixing times of 200 to 300 ms are adequate.

Phosphorus decoupling should use as little power as possible. Otherwise, additional heating can contribute to the heating already produced by spin-lock field and is likely to severely worsen the spectral resolution and cause changes in the chemical shifts. Continuous low-power on-resonance decoupling meets the minimum power requirements and, for the usual range of $^{3}J(^{31}P\text{-}O\text{-}C\text{-}^{1}H)$ coupling constants (1 - 20 Hz) should yield fully decoupled spectra (the weak line seen in the lower trace *c* is due to an impurity and not to incomplete decoupling). High power broad-band decoupling (as usual in e.g., HMQC[5]) even during acquisition time only, does not allow long enough acquisition to achieve the needed resolution. If several phosphate links are present, shaped decoupling pulses can be created to suit the particular case, or, even better, the phosphorus lines can be irradiated one after the other and thus both phosphorus atom and phosphorylation sites can be determined for each of the phosphate links present.

As the results described above indicate, the interpretation of the spectra is simple and straightforward, providing the assignment of the selectively excited lines (usually anomeric lines) is known from other sources.

EXPERIMENTAL

The NMR spectra were measured on a Varian UNITY-500 spectrometer operating at 499.693 MHz for ^{1}H NMR and at 202.276 MHz for ^{31}P NMR. ^{1}H NMR spectra were referenced to the methyl line of free acetate as described by Van Halbeek (δ = 1.91).[6] Standard Varian software version vnmr 5.1 was used throughout. All experiments were performed at 33 °C without sample spinning in a 5 mm "inverse detection" probe (with 7.4 μs being a typical value for 90° pulse for ^{1}H NMR). Shaped pulses were generated by a waveform generator (Varian) using the shaped pulses calculated by the Pandora box program. The sample, Man-P-GlcMan$_7$GlcNAc$_2$ (0.5 mg), was twice deuterium exchanged in 99.98% D_2O before being placed into a 5 mm Wilmad tube (0.7 mL of D_2O solution). In 1D TOCSY measurements the selected anomeric proton line was excited by a full Gaussian 90° pulse of 182 ms duration. Trim pulses of 2 ms flanked the MLEV-17 spin-lock[3] of mixing time 300 ms, the spectral width was 3100 Hz, and acquisition time 2 s; no weighting used, 5000 transients accumulated). The {^{31}P} decoupling was performed in a continuous manner throughout the whole pulse sequence, the broad-band transmitter was set to the ^{31}P freqency as determined by a ^{31}P NMR measurement. The decoupling power was kept low, corresponding to magnetic field ($\gamma B_2/h$) of approximately 50 Hz.

ACKNOWLEDGEMENTS

We thank Prof. Herman Van Halbeek for his advice during the preparation of this manuscript. Research was supported by the Belgian IWT-COT, the FGWO and the National Lottery. Partial financial support from Granting Agency of the Czech Republic (grant no. 203/96/0567) is gratefully acknowledged.

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