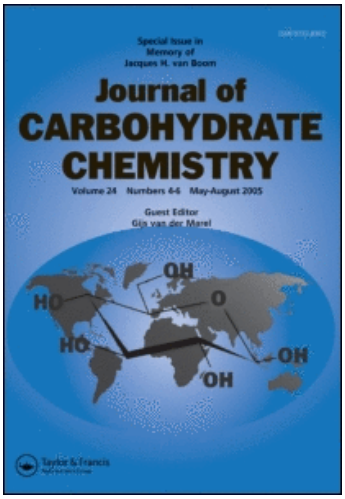

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Identification of Phosphorylation Sites in Polysaccharides by 1-D^{1}H-^{31}P HMQC Experiments

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COMMUNICATION

IDENTIFICATION OF PHOSPHORYLATION SITES IN POLYSACCHARIDES BY 1-D ^{1}H-^{31}P HMQC EXPERIMENTS

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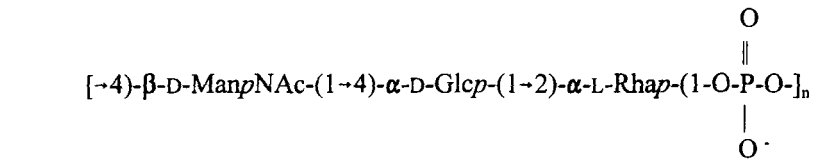
Phosphoric ester groups are found in many bacterial polysaccharides. They may occur as phosphomonoesters, but more often, they appear as phosphodiesters. The reducing end of some polysaccharides, e.g., several capsular polysaccharides from *Escherichia coli* and *Neisseria* species,[1,2] is linked to O-1 of a 2,3-di-O-acylglycerol through a phosphodiester linkage. A few pneumococcal polysaccharides are substituted by choline phosphate,[3] an immunologically significant substituent. The cell-wall polysaccharide from *Bacillus cereus* AHU 1356,[4] the extracellular polysaccharide from *Streptococcus pneumoniae* type 11A[5] and the capsular polysaccharide from *S. pneumoniae* type 23F[6] contain glycerol phosphate diesters. There are also examples of substitution by ribitol phosphate, such as the capsular polysaccharide from *S. pneumoniae* type 11F.[7] The repeating units of teichoic acids are linked by phosphodiester linkages. Examples are the capsular antigens of *Neisseria meningitides* type A[8] and *Haemophilus influenzae* type c.[9] Glycerol phosphate comprises part of the polysaccharide backbone of the capsular polysaccharide from *N. meningitidis* type Z.[10] Ribitol phosphate is part of the backbone of the capsular polysaccharides of *S. pneumoniae* type 34[11] and *H. influenzae* type a.[12] Other alditol phosphates rarely occur in polysaccharides.[13,14]

Phosphorylation sites can be determined by observing splitting of the signals in the ^{13}C spectrum of the polysaccharide due to ^{13}C-^{31}P couplings. However, carbon atoms

adjacent to the site of substitution may also show splitting; this may complicate the interpretation of the results. Phosphorylation sites can also be determined by 2D ^{1}H-^{31}P correlation experiments, e.g., HMQC[15,16] and selective observation of subspectra of phosphorylated residues by $^{1}H\{^{31}P\}$ relayed spin-echo difference spectroscopy (RESED).[17] If there is only one phosphorus resonance involved, as in most cases encountered to date, the recording of a complete, time-consuming 2D HMQC is not necessary. Recently, Schraml, et al.[18] proposed ^{31}P decoupling with 1D-TOCSY to identify phosphorylation positions. There are several limitations to their method: (1) the anomeric protons must be separated so that they can be selectively excited, (2) no severe spectral overlap in the non-anomeric region so that the splitting due to ^{1}H-^{31}P coupling can be easily recognized, and (3) magnetization can be transferred from H-1 to the proton which shows ^{1}H-^{31}P coupling.

Herein, we describe a 1D ^{1}H-^{31}P multiple-quantum coherence experiment for determining the phosphorylation sites in polysaccharides. The analysis the capsular polysaccharide from *Streptococcus pneumoniae* serotype 19F[19] **1** was used to illustrate the utility of the proposed method.



1

The 1D ^{31}P NMR spectrum showed a single resonance. The complete ^{1}H assignment will be published elsewhere. Figure 1 shows the 1D ^{1}H NMR spectra of the polysaccharide: (a) 1D ^{1}H spectrum, (b and c) two 1D TOCSY[20,21] spectra that correspond to the residues involved in phosphorylation, and (d) the 1D ^{1}H-^{31}P HMQC spectrum. The two peaks observed in the 1D ^{1}H-^{31}P HMQC spectrum (d) were identified as the H-1 of Rha and the H-4 of ManNAc. Thus, the phosphorylation sites were determined as the oxygen atoms on C-1 of Rha and C-4 of ManNAc. The experiment showed that these two glycosyl residues were linked through a phosphodiester bond since they are connected to the same phosphorus atom.

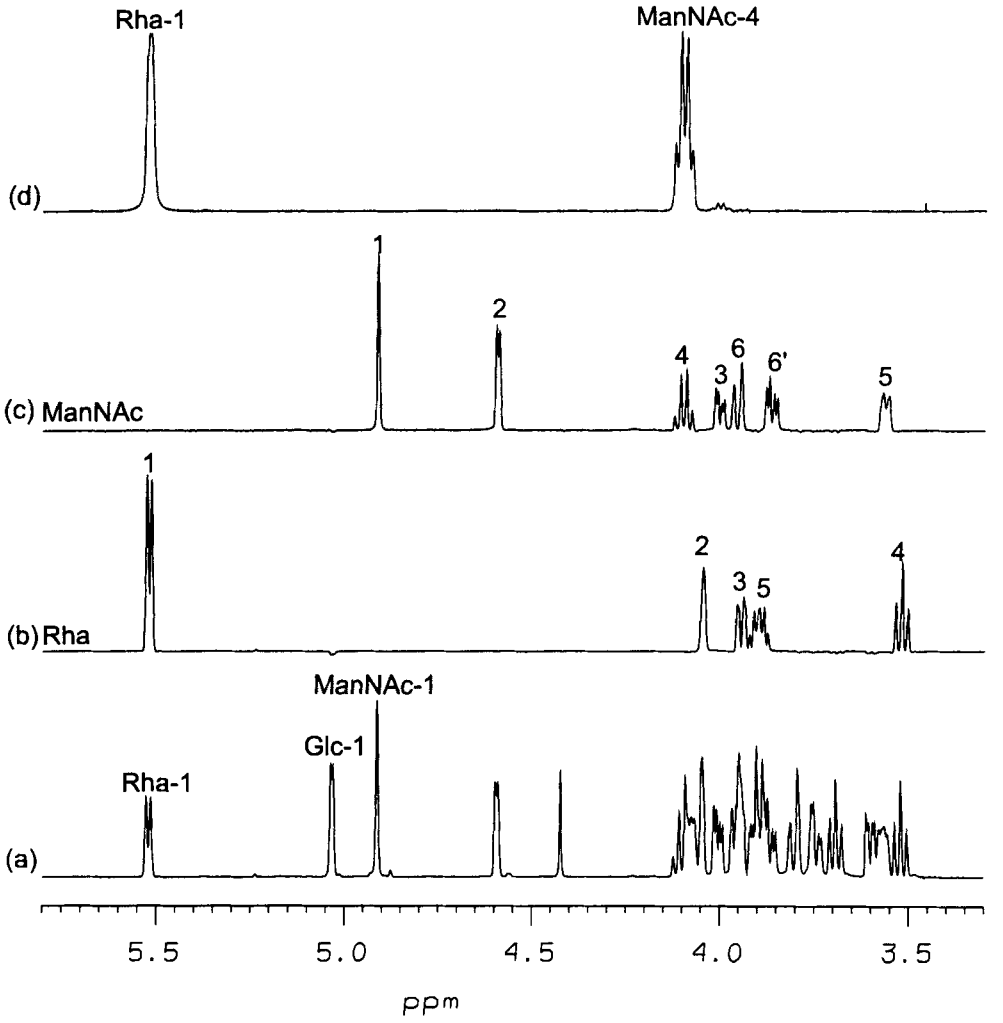


Figure 1. ^{1}H NMR spectra of *S. pneumoniae* type 19F capsular polysaccharide recorded at 600 MHz and 60 °C. (a) 1D ^{1}H spectrum. (b) and (c) 1D TOCSY spectra of Rha and ManNAc, respectively, recorded with a mixing time of 230 ms. The spectrum in (b) resulted from the simultaneous and selective excitation of the anomeric and the CH_3 protons. The spectrum (c) resulted from selective excitation of the C-2 proton. (d) 1D {^{1}H, ^{31}P} HMQC spectrum.

The proposed method is easy to execute on modern NMR spectrometers capable of performing ^{1}H-^{31}P correlation experiments. The 2 D ^{1}H-^{31}P HMQC pulse sequence is provided by the manufacturer. On Varian spectrometers a 2D ^{1}H-^{31}P HMQC experiment is converted to a non-selective 1D ^{1}H-^{31}P HMQC experiment by setting "ni" and "phase" to 1. The delay used to allow ^{1}H-^{31}P couplings to evolve was optimized for $^{3}J(^{31}P\text{-O-C-}^{1}H)$ couplings (1-20 Hz) to minimize peak intensity due to $^{4}J(^{31}P\text{-O-C-}^{1}H)$ couplings (< 1 Hz). In our experiment the optimum delay was set to 50 ms for a ^{1}H-^{31}P coupling of 10 Hz; no peaks due to ^{4}J couplings were observed. The interpretation of 1D ^{1}H-^{31}P HMQC spectra is straightforward providing that the ^{1}H assignments are available.

In summary, we report a simple and reliable way to identify phosphorylation positions in polysaccharides. Although the method was demonstrated for a polysaccharide, it can be applied to any saccharide substituted with a phosphate ester. The method takes the advantage of high sensitivity resulted from ^{1}H detection and the 100% natural abundance of ^{31}P. A 1D ^{1}H-^{31}P HMQC spectrum usually takes a couple of minutes to record. If more than one ^{31}P resonance is involved, a selective 1 D ^{1}H-^{31}P HMQC experiment can be used by replacing the hard (short) ^{31}P pulse by a soft (long) one.

Experimental

The *S. pneumoniae* serotype 19F (SmithKline Beecham Biologicals, Belgium) capsular polysaccharide (~20 mg) was exchanged twice in 99.96% D_2O with intermediate lyophilization. The sample was dissolved in 0.7 mL of 99.96% D_2O and transferred to a 5-mm NMR tube (Wilmad 535-pp). The NMR spectra were recorded on a Varian UnityPlus 600 spectrometer operating at 599.88 MHz for ^{1}H and 242.814 for ^{31}P. The spectrometer was equipped with a 5-mm inverse (^{1}H, X) probe with z-gradients. All experiments were performed at 60 °C to reduce the viscosity of the solution to narrow the signals in the spectra. The proton spectral width was 3000 Hz and 16384 complex data points were collected. Chemical shifts were referenced to 4,4 dimethyl-4-silapentane-1-sulfonate (DSS). The 1D TOCSY experiments used the MLEV17[21] sequence for isotropic mixing; the effective spinlock field strength was 7.9 KHz. Selective excitation was achieved using 90° E-BURP-1 pulse[22] generated by a waveform generator using the excitation profiles calculated by the Pandora's Box program (Varian). The delay used to allow for the ^{1}H-^{31}P couplings to evolve in the 1D ^{1}H-^{31}P HMQC experiment was 50 ms, which was the optimum for the $^{3}J(^{31}P\text{-O-C-}^{1}H)$ a coupling of 10 Hz.

Data were processed on a Silicon Graphics Indy workstation using Felix 95.0 software (BIOSYM/Molecular Simulations, San Diego). A Lorentzian-to-Gaussian weighting function (lb=-0.5, gb=0.05) was applied.

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