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Structural Studies on Phosphorylated Oligosaccharides Derived from Yeast Mannan by $^1H\{^{31}P\}$ Relayed Spin-echo Difference Spectroscopy (RESED)*

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Phosphorylated and nonphosphorylated tri- and tetrasaccharides were isolated from yeast mannan and analyzed by 500-MHz ^1H NMR spectroscopy. Relayed spin-echo difference spectroscopy (RESED) was used to reveal subspectra of the phosphorylated residues. In this way the attachment position of the phospate group could be established. The structures of the phosphorylated compounds turned out to be

6-*O*-phosphate
|
Manα(1-2)Manα(1-2)Man

and

6-*O*-phosphate
|
Manα(1-3)Manα(1-2)Manα(1-2)Man

RESED spectroscopy has proved to be a suitable method in analyzing low amounts of phosphorylated oligosaccharides.

In elucidating the structure of phosphorylated carbohydrates as occurring in lysosomal enzymes (1), yeast proteins (2), or polysaccharides (3) a main problem is the determination of the position of the phosphate group. It is difficult to deduce which monosaccharide is substituted and to deduce which carbon atom of the monosaccharide is involved in the linkage. Recently, we described $^1H\{^{31}P\}$ relayed spin-echo difference spectroscopy (RESED)[1] as a method to reveal a subspectrum of the phosphorylated sugar residue in an oligosaccharide (4). The proposed pulse sequence is a combination of spin-echo difference spectroscopy (SED) (5) and one-dimensional homonuclear Hartmann-Hahn (HOHAHA) spectroscopy (6).

$$\begin{array}{lccccc} ^1H: & 90°(x)\text{-}\tau\text{-d-}180°(y)\text{-d-}\tau\text{-} & & \boxed{\text{MLEV}(y)} & & \text{aq } +/- \\ ^{31}P: & & 90° \quad 90° & \text{on/of} & & \end{array}$$

where AQ is acquisition. By applying a composite ^{31}P 180° pulse together with a proton-refocusing 180° pulse, the proton coupled with ^{31}P will refocus with a phase opposite to that of the remaining protons if the delay τ is equivalent to approximately $1/(2J_{POCH})$. During the MLEV-17 mixing sequence the negative magnetization of this proton is spread out through the scalar coupling network as in the conventional one-dimensional HOHAHA experiment. Subtraction from data of an experiment without the ^{31}P pulses affords a subspectrum of the phosphorylated residue.

In the present study we will demonstrate the usefulness of RESED spectroscopy on low amounts of phosphorylated tri- and tetrasaccharides derived from yeast mannan.

EXPERIMENTAL PROCEDURES

Materials—Bio-Gel P-2 (minus 400-mesh) and Dowex 1-X8 (200–400-mesh) were purchased from Bio-Rad. *Saccharomyces cerevisiae* mannan and *Escherichia coli* alkaline phosphatase were obtained from Sigma. Jack bean α-mannosidase was prepared according to Li and Li (7).

Preparation of Mannooligosaccharides—Acetylation and acetolysis of 2 g of mannan were carried out essentially as described by Kocourek and Ballou (8) in 50 ml of dry pyridine and 50 ml of acetic anhydride at 20 °C for 64 h with shaking and then heated at 100 °C for 4 h. After centrifugation at 10,000 rpm for 10 min the supernatant was evaporated to dryness on a rotary evaporator at 50 °C. Acetolysis of the syrupy residue was carried out in 50 ml of acetic anhydride, 50 ml of acetic acid, and 5 ml of concentrated sulfuric acid at 40 °C for 8 h. The acidic mixture was then neutralized by adding 200 ml of pyridine and evaporated to dryness. To the residue 100 ml of chloroform and 100 ml of water were added. The chloroform layer was recovered and evaporated to dryness. The products were deacetylated in 30 ml of dry methanol and 5 ml of 0.5 M methanolic sodium methoxide. After 30 min at room temperature, 50 ml of ethyl acetate was added to decompose sodium methoxide. Then the solution was neutralized with acetic acid, evaporated, and the residue was taken up in 50 ml of water. The products were then applied to a column (1.2 × 23 cm) of Dowex 1-X8 (200–400-mesh, $HCOO^-$ form). The column was eluted stepwise with 120 ml each of water, 1 M formic acid, 2 M formic acid, and 4 M formic acid, respectively. The obtained fractions were fractionated further on a column (1.8 × 165 cm) of Bio-Gel P-2 (minus 400-mesh) developed with 0.05 M pyridine-acetic acid buffer, pH 5.0. Calibration of the column was carried out with glucose oligomers.

Enzymatic Digestions—Digestions with jack bean α-mannosidase and *E. coli* alkaline phosphatase were performed as described previously (9).

Analytical Methods—Hexose was determined by the orcinol-sulfuric acid method of Hewitt (10). Phosphate was determined by the method of Chan *et al.* (11) as modified by Ames and Dubin (12). Mannose 6-phosphate was estimated enzymatically according to Gawehn (13) and Asikin and Koeppe (14) as described previously (9).

500-MHz ^1H NMR Spectroscopy—Oligosaccharides were repeatedly exchanged in ^2H_2O (99.96% ^2H, Aldrich) with intermediate lyophilization. All spectra were recorded on a Bruker AM-500 spectrometer (Department of NMR Spectroscopy, Utrecht University, The Netherlands) operating at 500 MHz at a probe temperature of

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[1] The abbreviations used are: RESED, $^1H\{^{31}P\}$ relayed spin-echo difference spectroscopy; HOHAHA, homonuclear Hartmann-Hahn; MLEV, Malcom Levitt.

27 °C. Resolution enhancement of the one-dimensional proton spectra was achieved by Lorentzian-to-Gaussian transformation. The RESED spectra were recorded on a 5-mm inverse broadband probe head with the pulse sequence: $90°(x)(^1H)$-τ-$90°(^{31}P)$-$180°(y)(^1H)$-$90°(^{31}P)$-τ-MLEV(y)-aq (where aq is acquisition). (4). Subtraction from data of an experiment with a similar pulse sequence without the ^{31}P pulses revealed the subspectrum of the phosphorylated sugar residue. The ^{31}P 90° pulse length (125.759 MHz) was 12 μs; the ^1H 90° pulse, supplied through the decoupler channel, was 27 μs. The delay τ was 34 ms, and the MLEV-17 mixing sequence (15) had a duration of 120 ms (for P-1) or 200 ms (for P-2).

RESULTS AND DISCUSSION

Preparation of Mannooligosaccharides—By controlled acetolysis of yeast mannan the 1-6 linkages of the backbone were cleaved, yielding a mixture of mannose oligosaccharides containing 1-2 and 1-3 linkages with or without phosphate residues (16). The deacetylated acetolysis products (330 mg obtained from 2 g of mannan) were fractionated on a Dowex 1-X8 column. The neutral oligosaccharides were recovered in the water eluate (D-1) and those containing phosphate in the 1 M formic acid eluate (D-2) with the recoveries of 290 and 53 mg of mannose equivalents, respectively. The molar ratio of mannose to phosphate was 3.9:1 for D-2. By gel filtration on Bio-Gel P-2 fraction D-1 was resolved into two major peaks, N-1 and N-2, eluting at the positions of tetra- and trisaccharides, respectively (data not shown). Fraction D-2 was resolved into two major peaks, P-1 and P-2, eluting at the positions close to those of hexa- and tetrasaccharides, respectively (Fig. 1). All fractions were further purified by rechromatography under the same conditions. The yields of P-1 and P-2 were 25.7 and 12.9 mg of mannose equivalents, respectively.

Chemical analysis indicated that the molar ratio of mannose to phosphate was 4.2:1 for P-1 and 3.1:1 for P-2. Enzymatic estimation indicated that P-1 contains 0.9 mol and P-2 contains 0.6 mol of mannose 6-phosphate/mol of oligosaccharide. After digestion of these oligosaccharides (0.5 mg of mannose equivalents) with 4 units of alkaline phosphatase for 40 h, P-1 and P-2 eluted on Bio-Gel P-2 each as a single peak at the positions of tetra- and trisaccharides, respectively (data not shown). These results suggested that P-1 is a mannotetraose and P-2 is a mannotriose, both containing 1 mol of phosphate.

500-MHz ^1H NMR Spectroscopy—For NMR spectroscopy of each fraction approximately 100 nM were available. The ^1H NMR spectrum of the nonphosphorylated trisaccharide (N-2) together with the ^1H NMR and RESED spectra of the phosphorylated trisaccharide (P-2) are presented in Fig. 2. The ^1H NMR data of the structural reporter groups are listed in Table 1. Comparison with compound 65 in Ref. 17 reveals N-2 (Fig. 2*A*) to be the 1-6-linked branch of an oligomannoside type of carbohydrate chain. The chemical shift of the H-1 signal at 5.044 ppm and that of the H-2 signal at 4.065 ppm are typical for a terminal α(1-2) linked Man $\underline{D}_1$ residue. The values for the H-1 and H-2 signals at 5.300 and 4.109 ppm, respectively, are indicative for an α(1-2)-linked Man $\underline{C}$ residue, which is α(1-2)-substituted (17). Taking into account the intensity of the remaining H-1 signal at 5.371 ppm it is clear that the reducing Man $\underline{4}$ residue is almost completely in its α

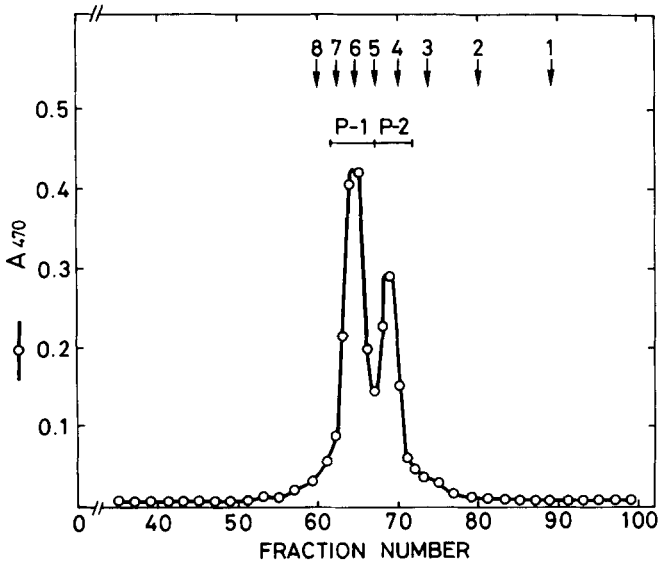

FIG. 1. **Gel permeation chromatography of the acidic oligosaccharides.** The acidic oligosaccharides (D-2, 51 mg) were applied to a Bio-Gel P-2 column (1.8 x 165 cm) which was then developed with 0.05 M pyridine-acetic acid buffer, pH 5.0. Fractions of 3.1 ml were collected and aliquots thereof were used for monitoring neutral sugar. The *arrows* denote the elution positions of glucose oligomers with *numbers* indicating the number of glucose units.

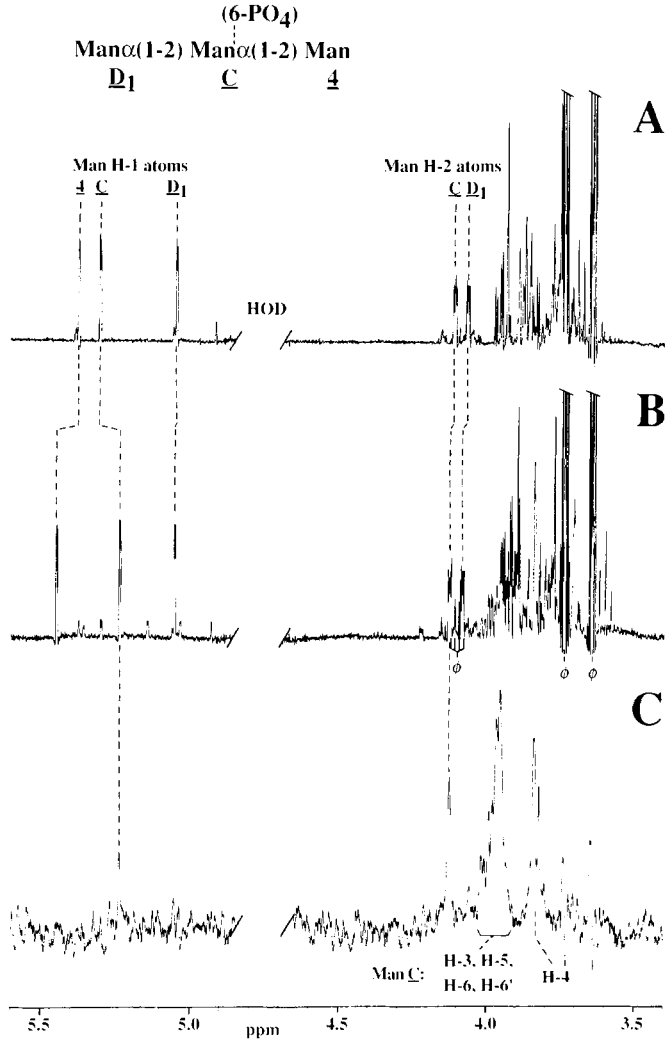

FIG. 2. **500-MHz ^1H NMR spectra of trisaccharides derived from yeast mannan.** *A*, resolution-enhanced one-dimensional spectrum of N-2 (972 scans). *B*, resolution-enhanced one-dimensional spectrum of P-2 (656 scans). *C*, RESED spectrum of P-2; in total 9000 scans were recorded, and the mixing time was 200 ms. The *numbers* and *letters* in the spectra refer to the corresponding residues in the structures. ϕ denotes impurity.

TABLE I

^1H chemical shifts of structural reporter groups of the constituent monosaccharides of oligosaccharides derived from yeast mannan

Chemical shifts are given in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate but were actually measured indirectly to acetone in ^2H_2O (δ = 2.225 ppm).

Residue	Reporter group	Chemical shift in			
		N-2	P-2	N-1	P-1
Man 4	H-1α	5.371	5.447	5.372	5.447
Man C	H-1	5.300	5.235	5.298	5.240
	H-2	4.109	4.126	4.112	4.126
Man D_1	H-1	5.044	5.051	5.032	5.040
	H-2	4.065	4.083	4.221	4.240
Man E	H-1			5.142	5.164
	H-2			4.068	4.073

anomeric form. These assignments are in accordance with earlier work on mannooligosaccharides (18). Therefore, the structure of N-2 is as follows.

Manα(1-2)Manα(1-2)Man
D_1 C 4

It is not possible to decide which residue of P-2 (Fig. 2*B*) is phosphorylated on the basis of chemical shifts of the H-1 and H-2 signals only, since all structural reporter groups are shifted. Although attachment of a phosphate group gives rise to shifts of $\Delta\delta$ = 0.25–0.55 ppm of the proton attached to the substituted carbon atom (19), no signal has shifted out of the bulk region. This excludes the application of one-dimensional HOHAHA spectroscopy as was successfully employed by Van Kuik *et al.* (20) to prove the interconnection between the H-6 and H-6′ signals and the H-1 and H-2 structural reporter groups of a 6-*O*-sulfated mannose residue. The effect of a sulfate group, $\Delta\delta$ = 0.5–0.7 ppm (21), is enough to shift a skeleton proton out of the bulk region between 3.4 and 4.0 ppm. In the case of P-2, application of the selective 180° pulse, essential for the one-dimensional HOHAHA method, is impossible. However, overlap with other signals will not disturb the inversion of the proton coupled to ^{31}P with the SED sequence. Therefore, a subspectrum of the phosphorylated residue can be revealed by RESED spectroscopy. From comparison of the RESED spectrum in Fig. 2*C* with the one-dimensional spectrum in Fig. 2*B* it is evident that the Man C residue is phosphorylated. The mixing time of 200 ms allowed magnetization transfer from the proton coupled to ^{31}P to the H-1 and H-2 structural reporter groups of this residue. With short mixing times the H-6 and H-6′ signals are found at 3.95 ppm indicative of a 6-*O*-phosphorylated residue. Therefore the structure of P-2 is as follows.

6-*O*-phosphate
|
Manα(1-2)Manα(1-2)Man
D_1 C 4

The ^1H NMR spectrum of the nonphosphorylated tetrasaccharide (N-1) together with the ^1H NMR and RESED spectra of the phosphorylated tetrasaccharide (P-1) is presented in Fig. 3. The ^1H NMR data of the structural reporter groups are listed in Table I. The chemical shifts at δ = 5.142 and δ = 4.068 ppm of N-1 (Fig. 3*A*) are indicative of the H-1 and H-2, respectively, of a terminal α(1-3)-linked mannose residue (17). The shift increment of the Man D_1 H-2 ($\Delta\delta$ = 0.156 ppm) compared to the terminal Man D_1 in N-2 is typical for an α(1-3) substitution (17, 18). The structure of N-1 is an extension of N-2 with an α(1-3)-linked Man E residue.

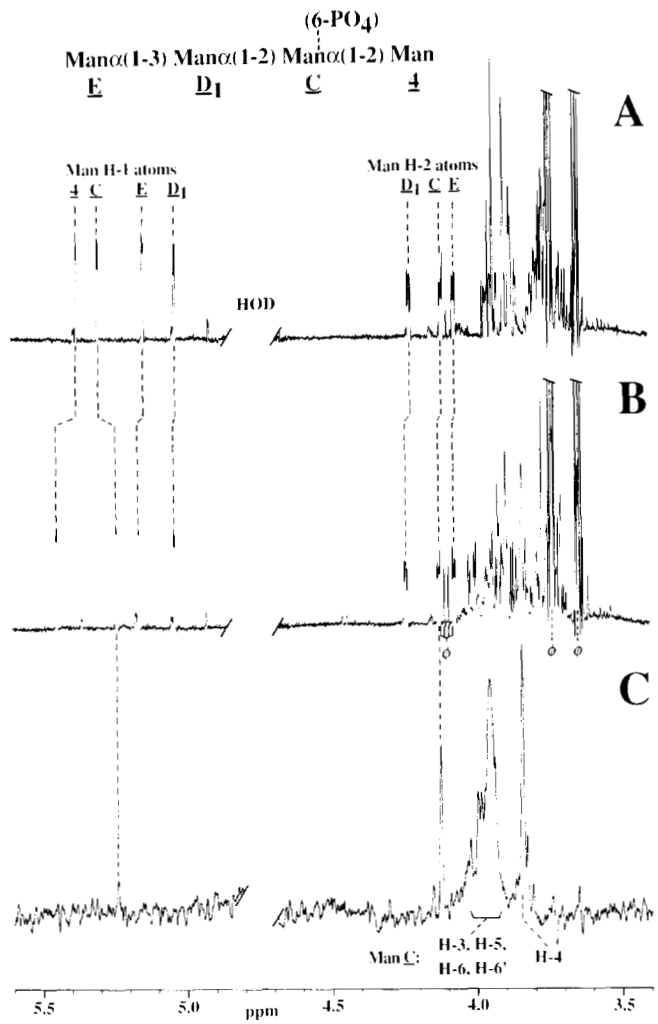

FIG. 3. **500-MHz ^1H NMR spectra of tetrasaccharides derived from yeast mannan.** *A*, resolution-enhanced one-dimensional spectrum of N-1 (896 scans). *B*, resolution-enhanced one-dimensional spectrum of P-1 (608 scans). *C*, RESED spectrum of P-1; in total 18,000 scans were recorded, and the mixing time was 120 ms. The *numbers* and *letters* in the spectra refer to the corresponding residues in the structures. ϕ denotes impurity.

Manα(1-3)Manα(1-2)Manα(1-2)Man
E D_1 C 4

As in P-2 all structural reporter group signals of P-1 are shifted (Fig. 3*B*) when compared to those of N-1 (Fig. 3*A*). Assignment of the position of the phosphate group on the basis of the chemical shifts of structural reporter groups only is impossible here too. The RESED spectrum in Fig. 3*C* clearly is identified as a subspectrum of the Man *C* residue. Analogous to P-2 the Man *C* residue is 6-*O*-phosphorylated resulting in the following structure for P-1.

6-*O*-phosphate
|
Manα(1-3)Manα(1-2)Manα(1-2)Man
E D_1 C 4

It is remarkable that in P-1 and P-2 the H-1 of the 6-*O*-phosphorylated Man C residue is shifted upfield ($\Delta\delta$ = 0.06 ppm), whereas the other structural reporter groups are shifted downfield compared to N-1 and N-2, respectively. The downfield shift of the α anomeric signal of Man 4 ($\Delta\delta$ = 0.08 ppm) can probably be explained by an influence of the phosphate group on the conformation of this residue. This is supported

by the observation that in the spectra of the nonphosphorylated compounds (Figs. 2*A* and 3*A*) no coupling is present on the Man 4 αH-1 signal, whereas in the spectra of the phosphorylated compounds (Figs. 2*B* and 3*B*) a coupling of 1.7 Hz on this signal occurs.

In the RESED pulse sequence the value of the intervals between the 180° pulses and the 90° pulse or MLEV sequence should equal $1/(2J_{POCH})$ for maximal inversion of the proton coupled with ^{31}P. However, the value of this interval was found not to be very critical for this experiment (4). In the present study the ^{31}P-^{1}H coupling constant is not estimated; the interval was set to the relatively short value of 34 ms. The ^{31}P-^{1}H coupling constant probably is smaller than the coupling constant calculated from the delay of 34 ms (J = 14.7 Hz). A longer interval calculated from smaller couplings would give rise to loss of sensitivity due to T2 relaxation processes; therefore, it is better to keep this delay short.

The present work shows the applicability of RESED spectroscopy to identify a phosphorylated residue in an oligosaccharide. It is possible to reveal a subspectrum of this residue in low amounts of material. The MLEV-17 mixing sequence (15), even at a mixing time of 120 ms for P-1, allows transfer of magnetization through small couplings as occurring in the Man C residue.

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