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The extracellular polysaccharide of *Pichia (Hansenula) holstii* NRRL Y-2448: The structure of the phosphomannan backbone

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Abstract

The phosphomannan core of the exopolysaccharide of *Pichia (Hansenula) holstii* NRRL Y-2448 was isolated after hydrolytic removal of the oligosaccharide phosphate side-chains. The core polysaccharide and its dephosphorylated derivative were subjected to extensive 1D and 2D NMR spectroscopy which yielded information on the linkage sites and on the sequence of the mannosyl residues in the major oligosaccharide repeating unit. The most probable structure for the repeating unit was

-[6-O-PO$_3$H$_2$-α-D-Man-(1→3)-α-D-Man-(1→2)-α-D-Man-(1→2)]-α-D-Man-(1→6)-[α-D-Man-(1→2)]-α-D-Man-(1→6)-

A semiquantitative conformational analysis was performed by Monte Carlo simulations and the result was confirmed by comparison with the experimentally determined NMR data. The distance distribution for the phosphate groups was determined from the modelling and was found to cover the expected range of distances for phosphorylated high-mannose oligosaccharides. © 1996 Elsevier Science Ltd.

Keywords: *Pichia holstii*; Phosphomannan backbone; Repeating oligosaccharide; Conformation; NMR spectroscopy

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1. Introduction

The structure of the extracellular phosphomannan synthesized by the yeast *Pichia (Hansenula) holstii* NRRL Y-2448, when grown in a culture medium containing an excess of orthophosphate, has been the subject of numerous investigations [1–8]. In spite of this, only some of the structural features of the polysaccharide have been unravelled and these have mainly been obtained from mild acid hydrolysis studies [6,8]. Current knowledge indicates that the phosphomannan (**PS**) is composed of a highly branched, high molecular weight phosphomannan core to which are attached chains composed principally of the repeating pentasaccharide phosphate (**1**),

-*P*-6-α-D-Man*p*-(1→3)-α-D-Man*p*-(1→3)-α-D-Man*p*-(1→3)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→

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The phosphomannan core (**PC**) which accounts for only 10% of the total mannose and phosphate content of the **PS** is highly branched and contains (1 → 2), (1 → 3), and (1 → 6) linkages [8]. The precise structure of the **PC**, the length of the pentasaccharide phosphodiester chains, the structure and significance of minor amounts of tetrasaccharide and hexasaccharide phosphates reported to be present in the oligosaccharide fraction of partial hydrolysates [5,6], and the exact nature of the units capping the chains remain largely unresolved. Interest in resolving these questions stems from the widespread use of the pentasaccharide phosphate and the **PC** as tools and probes in research on phosphomannosyl receptors [9–19].

Preparations of both the pentasaccharide phosphate and the **PC** have been used in affinity chromatography to isolate phosphomannosyl receptors [9–12], and pentasaccharide phosphate preparations have been coupled to proteins [13–16] primarily as a means of directing their uptake by cells which express phosphomannosyl receptors. The **PC** has also been used in an ELISA assay to determine the inhibitory potencies of a series of glycosylated peptides to the binding of **PC** to a cation-independent phosphomannosyl receptor [17], and both the **PC** and the pentasaccharide phosphate have been used in binding–inhibition studies involving the lymphocyte homing receptor [18] and serum amyloid P component [19].

In the present communication we report on the structure and molecular modelling of the repeating oligosaccharide unit of the **PC**.

2. Experimental

General methods.—Preparation of alditol acetates and analytical GLC were carried out as previously described [20]. GPC was performed on a dextran-calibrated column of

Sephacryl S500 (1.6 × 96 cm) using 0.1 M aq NH_4HCO_3 (pH 8.3) or 0.1 M aq NaOAc (pH 7.4) as eluent, or on a column of Bio-Gel P-4 (1.6 × 94 cm) or Bio-Gel P-30 (1.6 × 95 cm) using 0.1 M aq NH_4HCO_3 (pH 8.3) as eluent. Compounds were detected by refractive index.

NMR spectroscopy.—Samples were deuterium-exchanged by lyophilizing solutions in D_2O, and then dissolved in 99.99% D_2O (0.45 mL). Spectra were referenced relative to internal acetone (δ 2.23 for ^1H and 31.07 ppm for ^{13}C). Spectra were recorded at 27, 35, and 45 °C on either a Bruker AM 500 or AMX 600 spectrometer. The pD values of the D_2O solutions of the NMR samples were as follows: **PS** 8.00, **PC** 7.2, and **DPC** 8.5. The 2D NMR pulse programs used were as follows: (*a*) Phase-sensitive COSY [21] using TPPI with double quantum filter and presaturation during relaxation delay; (*b*) NOESY [22] using TPPI with presaturation during relaxation delay and mixing time; (*c*) HOHAHA [23] with presaturation during relaxation delay and employing the MLEV-17 sequence for mixing; (*d*) HMQC [24] using TPPI, presaturation during relaxation delay-and GARP decoupling during acquisition; (*e*) HMBC [25] with no decoupling during acquisition. COSY and NOESY experiments on **DPC** were recorded at 500 MHz using spectral widths of 1901 and 2100 Hz, data matrices of 1024 × 4096 and 512 × 2048 with 48 scans per t_1 increment, and relaxation delays of 2 and 1 s, respectively. For the NOESY experiments a mixing delay of 0.1 s was used. All other 2D experiments were recorded at 600 MHz. HOHAHA spectra were obtained using a spectral width of 2403 Hz, a data matrix of 512 × 4096 points with 48 scans per t_1 increment, and a mixing time of 86 ms. HMQC and HMBC spectra were obtained using 512 × 4096 and 256 × 4096 data matrices, respectively, spectral widths of 2403 (^1H) and 16600 Hz (^{13}C) with 64 scans per t_1 increment, and relaxation delays of 1 and 1.5 s, respectively.

Extracellular polysaccharide (PS) of Hansenula (Pichia) holstii.—The polysaccharide was a gift from Dr M.E. Slodki and was used without further purification.

Preparation of the phosphomannan core polysaccharide (PC).—(a) Small-scale preparation. **PS** (17 mg) in aq 1% AcOH (2.5 mL) was heated at 100 °C in a Reacti-Vial for 8 h after which the solution was lyophilized and the mixture was chromatographed on Bio-Gel P-4 using 0.1 M NH_4HCO_3 as eluent. The elution profile is shown in Fig. 3.

(b) Scaled-up preparation. **PS** (1 g) in aq 1% AcOH (150 mL) was heated under reflux for 10 h, after which the solution was cooled, the pH was adjusted to 8 with NH_4HCO_3, and the solution was lyophilized. The lyophilized material in H_2O (8 mL) was dialyzed (mol wt cut-off 3500) against distilled H_2O (5 × 100 mL) to afford a combined diffusate (873 mg) and a retentate (24 mg). The diffusate was chromatographed on Bio-Gel P-4 using 0.1 M aq NH_4HCO_3 as eluent. The fraction eluted in the void volume (53 mg) was further separated on columns of Bio-Gel P-30 and Sephacryl S500, using the same eluent, to afford a major fraction (**PC**, 40 mg).

Dephosphorylation of ***PC***.—**PC** (15 mg) in buffer (supplied with enzyme, 2 mL), pH 9.8, was treated with alkaline phosphatase (30 units, 30 μL, Sigma) and the solution was maintained at 37 °C for 5 h, after which the mixture was lyophilized. The treatment with enzyme was repeated twice for 72 and 48 h after which the lyophilized residue was purified on Sephacryl S500 using 0.1 M aq NH_4HCO_3 as eluent. The yield of **DPC** (dephosphorylated **PC**) was 11 mg.

3. Results and discussion

The elution profile of the **PS** on Sephacryl S500 (dextran-calibrated) using either 0.1 M aq NH_4HCO_3 (pH 8.3) or 0.1 M aq NaOAc (pH 7.4) as eluent showed it to be polydisperse, displaying several maxima in the molecular weight range $5-20 \times 10^6$. Molecular weight estimates of the **PS** by light scattering and sedimentation diffusion experiments have indicated values of $33-39 \times 10^6$ and 15×10^6, respectively [8]. The ^1H NMR spectra of material obtained from the different molecular weight regions were identical. The ^{31}P NMR spectrum of the **PS** showed a single resonance for phosphodiester at δ −1.26 supporting earlier reports [5,6] of the absence of phosphomonoester groups in the **PS**. Hydrolysis of the **PS** with 4 M aq CF_3CO_2H followed by GLC analysis of the derived alditol acetates showed only Man to be present. The ^1H NMR spectrum of the polysaccharide recorded in D_2O at 45 °C (Fig. 1) showed signals for H-1 of α-linked mannopyranosyl residues at δ 5.669 ($^3J_{H,P}$ 7.7 Hz), 5.136 (2 H), 5.120, and 5.069 while the DEPT spectrum (Fig. 2) showed major C-1 signals at 103.36, 103.25, 102.88, 102.84, and 95.57 ($^2J_{C,P}$ 5.2 Hz) ppm. In addition five major signals were observed for C-5 resonances at 74.71, 74.41, 74.29, 74.22, and 73.12 ($^3J_{C,P}$ 7.2 Hz) ppm, four signals for unsubstituted C-6, and a broad signal for a substituted C-6 at 66.00 ppm. Resonances for linkage carbons were also observed at 79.92, 79.86, 79.24, and 78.97 ($^3J_{C,P}$ 1.9 Hz) ppm. These data are consistent with previous reports [6] that the **PS** consists predominantly of chains to which the pentasaccharide phosphate **1** repeating units are joined via α-glycosyl phosphate linkages. The ^1H and ^{13}C NMR spectra of the **PS** are dominated by the signals of the poly(pentamannosyl phosphate) chains and give no indication of the presence of the **PC** to which these are attached. Previously described methods [5] for preparing the **PC** were found to be unsatisfactory.

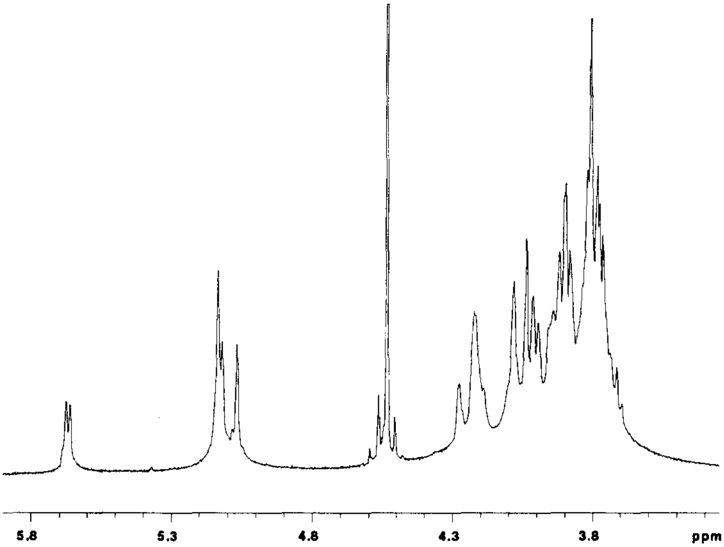

Fig. 1. ^1H NMR spectrum of the **PS** of *Pichia holstii* Y-2448.

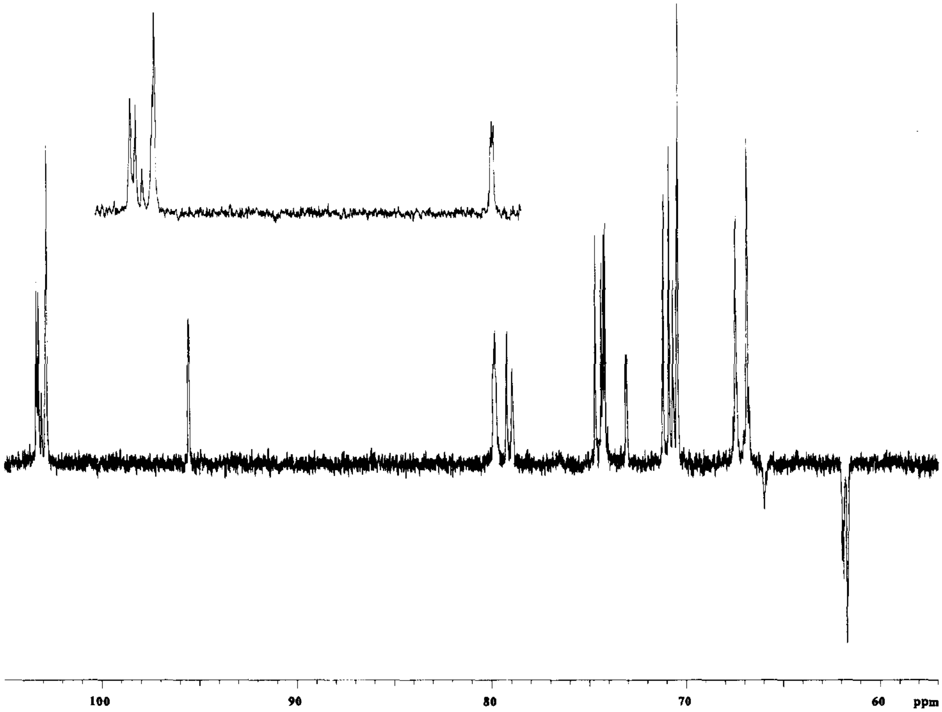

Fig. 2. DEPT spectrum of the **PS** of *Pichia holstii* Y-2448.

The ^{1}H NMR spectra of these products usually showed ^{1}H signals at δ ~ 5.7 ($^3J_{H,P}$ ~ 8 Hz) indicative of the presence of diester phosphate linkages. Such signals could have arisen either from incomplete removal of oligosaccharide phosphate side-chains or contamination of the **PC** with high molecular weight chains composed of repeating pentamannosyl phosphate residues. A protocol was therefore developed for the complete removal of the side chains and involved hydrolysis of the **PS** with aq 1% AcOH for 8 h at 100 °C, followed by GPC separation of the hydrolysate on Bio-Gel P-4. The elution profile is shown in Fig. 3. The oligosaccharide (B + C + D) to **PC** (A) ratio was ~ 10:1. When the procedure was scaled-up to 1 g it was necessary to increase the time of the hydrolysis to 10 h and to purify further the **PC** fraction by sequential elution from columns of Bio-Gel P-30 and Sephacryl S500. The ^{1}H NMR spectrum of **PC** at 45 °C (Fig. 4) showed signals for H-1 resonances at δ 5.281 (broad), 5.157 (minor), 5.129, 5.115, 5.092, 5.070 (minor), 5.058, and 5.050. The ^{31}P NMR spectrum of the **PC** gave a single resonance at δ 2.02 indicative of the presence of phosphomonoester only. The DEPT spectrum (Fig. 5) showed C-1 signals at 103.34, 102.95 (2C), 101.37 (broad), and 99.17 (2 C, broad) ppm, and signals for unsubstituted C-6 at 61.96 and for substituted C-6 at 65.19 and 66.56 ppm. The latter and a signal at 73.20 ppm showed coupling of 3.8 and 6.9 Hz, respectively. These data locate the phosphate group at C-6 of a mannosyl residue in the **PC** and identify the signal at 73.20 ($^3J_{C,P}$ 6.9 Hz) as that for C-5 of the residue bearing the 6-phosphate group. The COSY spectrum of the **PC** was

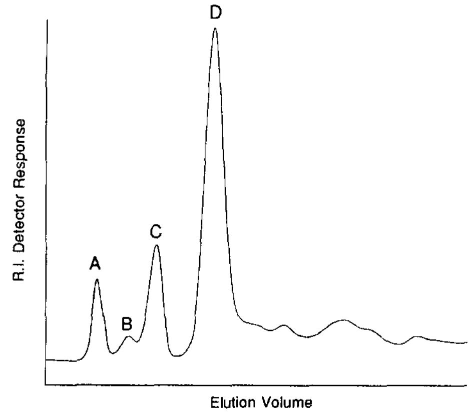

Fig. 3. GPC elution profile of an acetic acid hydrolysate of the **PS** on Bio-Gel P-4 (see text for conditions); A, core polysaccharide (**PC**); D, pentasaccharide fraction; C, decasaccharide fraction; B, higher oligosaccharide fraction.

complex showing no fewer than eleven H-1/H-2 cross-peaks. It was evident from the spectra that the **PC** was not homogeneous in structure possibly due to the presence of phosphate in non-stoichiometric amounts. In spite of the complexity of the 2D NMR spectra of the **PC** it was possible to assign the resonances of several spin systems including, importantly, those of the phosphorylated residue. In order to facilitate the analysis it was decided to study first the dephosphorylated **PC** before continuing with the NMR examination of the **PC**. The **PC** was therefore dephosphorylated by repeated treatment with alkaline phosphatase.

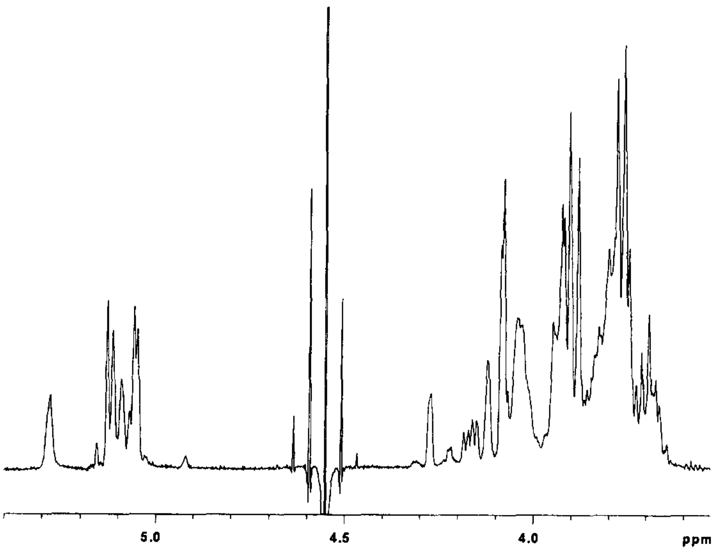

Fig. 4. ^{1}H NMR spectrum of the **PC**.

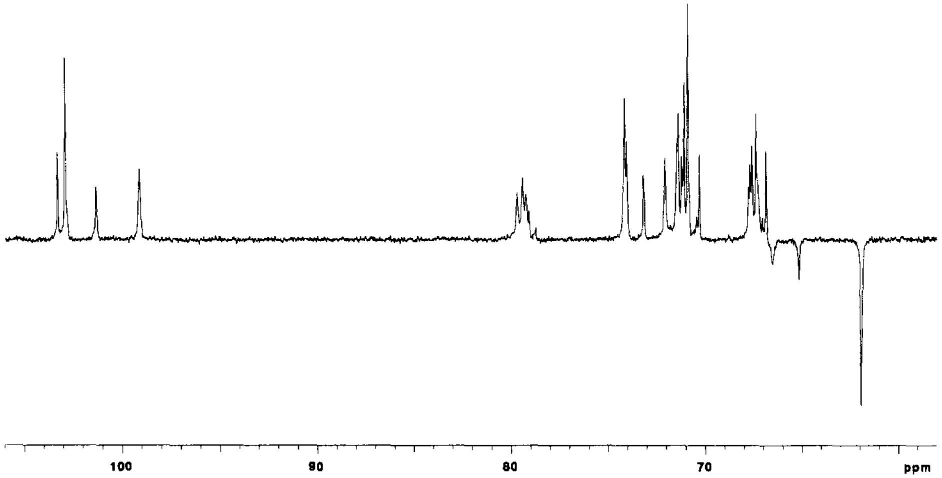

Fig. 5. DEPT spectrum of the **PC**.

The NMR spectra of the dephosphorylated core polysaccharide (**DPC**) were simpler than those of the **PC**, confirming the non-stoichiometry of phosphate in the **PC**. The ^{1}H NMR spectrum of the **DPC** at 45 °C (Fig. 6) showed major signals for H-1 resonances, at δ 5.290 (**A**), 5.157 (**B**, $^{3}J_{1,2}$ 1.5 Hz), 5.117 (**C**), 5.088 (**D**), and 5.059 (**E** and **F**, 2 H), and a minor signal at 5.071 (**G**). The ^{13}C NMR spectrum of the **DPC** (Fig. 7) showed, inter alia, C-1 signals at 103.17 (2 C), 103.07, 101.60, and 99.39 (2 C) ppm, signals for substituted carbons at 79.69 (2 C), 79.36, and 79.01, and a signal for two substituted C-6

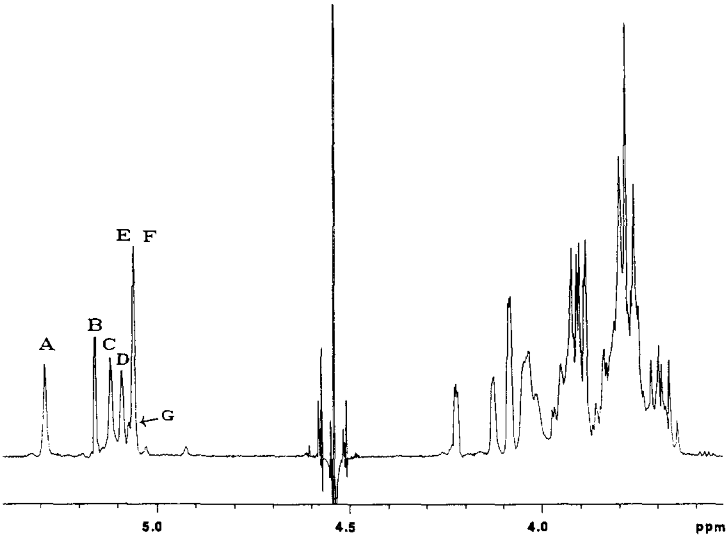

Fig. 6. ^{1}H NMR spectrum of the **DPC**.

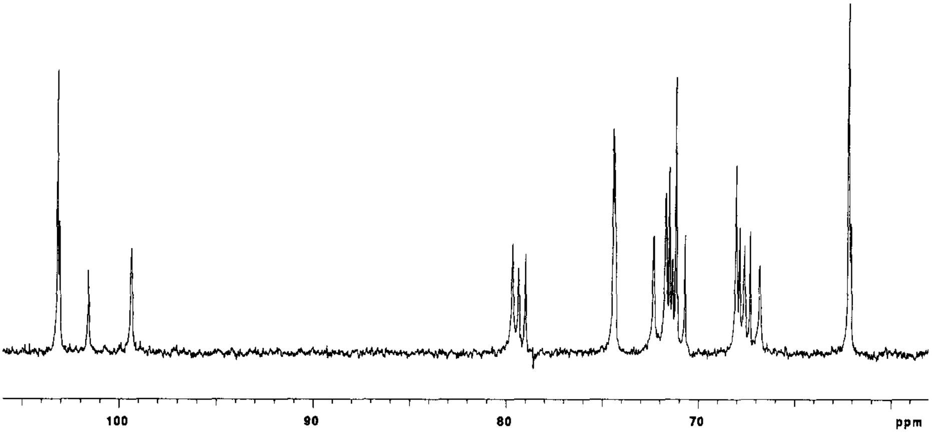

Fig. 7. ^{13}C NMR spectrum of the **DPC**.

atoms (DEPT) at 66.82 ppm. The $^{1}J_{C,H}$ coupling constants for the anomeric carbon atoms ranged from 171.7 to 173.6 Hz indicating that all the mannosyl residues were α-linked. These data suggest that the **DPC** consists predominantly of a hexasaccharide repeating unit.

The linkage positions and the sequence of the mannosyl residues in the repeating unit of the **DPC** were established from 2D NMR experiments. The ^{1}H chemical shifts for each mannosyl residue were established from ^{1}H–^{1}H correlation experiments (COSY [21] and HOHAHA [23]) while the ^{13}C chemical shifts were established from ^{1}H–^{13}C correlation experiments (HMQC [24] and HMBC [25]). It was necessary to repeat the COSY, HOHAHA, and NOESY [22] experiments at several temperatures in order to distinguish between overlapping resonances. The ^{1}H and ^{13}C data listed in Table 1 were obtained from experiments recorded at 35 °C. The residues of the repeating unit have been labelled **A**–**F** in decreasing order of chemical shift of the H-1 resonances. The minor H-1 resonance at δ 5.065 has been designated **G**.

The chemical shifts of residue **A** and particularly of residues **C** and **D** were not apparent from the weak cross-peaks observed in the ^{1}H–^{1}H correlation spectra recorded at 27 °C and it was necessary to establish their chemical shifts from spectra recorded at 35 and 45 °C. On the other hand, the shifts of **B**, **E**, and **F** were easily traced from the strong cross-peaks which were evident in the spectra recorded at all three temperatures. This behaviour suggests that the polysaccharide has segmental motion and that residues **B**, **E**, and **F** are present in more flexible parts of the molecule (e.g., side chains) while **C** and **D**, and possibly **A**, are present in a more rigid part of the molecule (e.g., backbone).

The chemical shifts of H-1 to H-3 of residues **C**, **D** and **G**, H-1 to H-4 of residues **A** and **F**, and H-1 to H-5 of residues **E** and **B** were obtained from the COSY spectrum while the shifts for H-1 to H-6a,6b of **A**, **B**, **E**, and **F**, H-1 to H-5 of **C** and **D**, and H-1 to H-5 of **G** were readily assigned from the cross-peaks observed in the H-2 trace of HOHAHA experiments. These assignments were supported by the intraresidue NOEs observed between H-1 and H-2, and H-2 and H-3 for all the residues. The ^{13}C

Table 1
^{1}H and ^{13}C chemical shift data for **DPC** at 35 °C

Atom	→ 2-Man (**A**)	Man (**B**)	→ 2,6-Man (**C**)	→ 2,6-Man (**D**)	Man (**E**)	→ 3-Man (**F**)	(**G**)
H-1	5.297	5.153	5.121	5.097	5.056	5.053	5.065
C-1	101.40	103.02	99.12	99.12	103.02	102.91	
H-2	4.126	4.078	4.048	4.030	4.084	4.226	4.075
C-2	**79.23**	70.94	**79.52**	**79.52**	70.94	70.49	
H-3	3.923	3.893	3.936	3.930	3.817	3.961	3.853
C-3	71.11	71.25	71.49	71.25	71.42	**78.78**	
H-4	3.734	3.655	3.836	3.834	3.686	3.769	3.650
C-4	67.78	67.78	67.32	67.32	67.61	67.10	
H-5	3.752	3.789	3.797	3.787	3.767	3.801	3.792
C-5	74.12	74.12	72.09	72.09	74.12	74.12	
H-6a	3.770	3.769	4.027	4.027	3.895	3.897	3.893
H-6b	3.902	3.904	3.685	3.698	3.768	3.767	
C-6	61.85	61.98	**66.52**	**66.52**	61.98	61.98	

resonances for residues **A**–**F** were assigned by comparing the ^{1}H assignments above with the ^{1}H–^{13}C correlation data obtained from an HMQC [24] experiment. The chemical shifts for C-6/H-6a,6b of residues **C** and **D** could not be established unambiguously. Connectivities were not observed between H-5 of these residues and their respective H-6a and H-6b resonances because of severe cross-peak overlap in both the COSY and HOHAHA spectra. However, the two sets of unassigned chemical shifts at 66.52/4.027,3.685 and 66.52/4.027,3.698 could be assigned to C-6/H-6a,6b of **C** and **D**, respectively, as will be explained later.

The linkage sites of the residues in the repeating unit of the **DPC** were identified by comparing the ^{13}C chemical shift data with literature values for methyl α-mannosides [26]. The significant deshielding of the resonances for C-2 of residue **A**, C-3 of residue **F**, and C-2 and C-6 of residues **C** and **D** identify these as the linkage sites. The sequence of the residues in the repeating unit was established from NOESY and HMBC experiments. Part of the NOESY contour map is shown in Fig. 8. All the residues showed characteristic intramolecular NOEs between H-1 and H-2 and between H-2 and H-3. Intense interresidue NOEs between the anomeric protons and the protons across the glycosidic linkages were observed for all the residues. H-1 of **A** showed an intense interresidue NOE to H-2 of **D** and weaker NOEs to H-1 of both **D** and **F**, H-1 of **B** showed an intense interresidue NOE to H-3 of **F**, H-1 of **C** showed an interresidue NOE to H-6b of **D** as well as an NOE to H-1 of **E**, H-1 of **D** showed an interresidue NOE to H-6b of **C** and an NOE to H-1 of **A**, H-1 of **E** showed an intense interresidue NOE to H-2 of **C**, and H-1 of **F** showed an interresidue NOE to H-2 of **A**.

The ^{1}H and ^{13}C NMR data (Table 1) and the observed NOEs (Figs. 8 and 9) indicate that the primary structure of the **DPC** is comprised of the hexasaccharide repeating unit shown in Fig. 9. The three-bond interresidue heteronuclear correlations (Table 2)

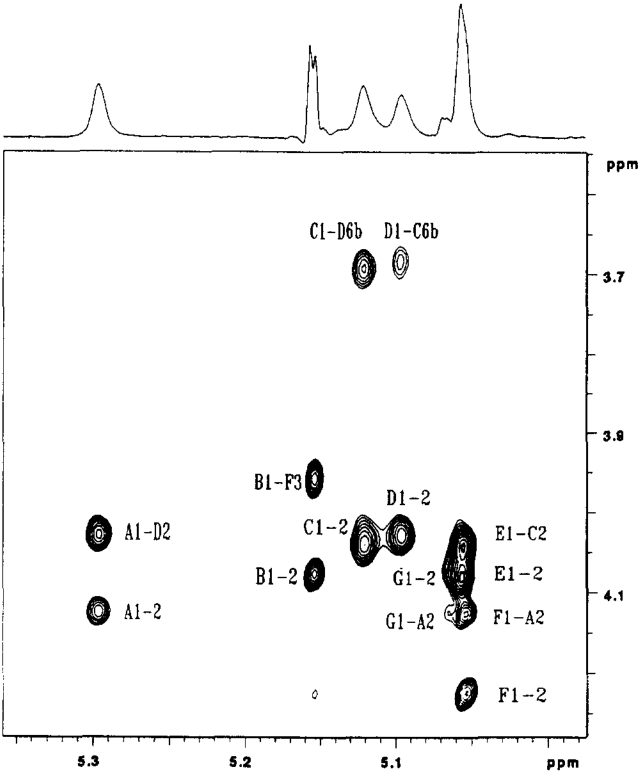

Fig. 8. Partial NOESY contour map of the **DPC**. A1-2 connotes an intraresidue NOE between H-1 and H-2 of **A**; A1-D2 connotes an interresidue NOE between H-1 of **A** and H-2 of **D**, etc.

established from an HMBC experiment on the **DPC** are consistent with the above structure for the hexasaccharide repeating unit.

As mentioned above, the resonances at δ 3.685 and 3.698 (Table 1) were assigned to

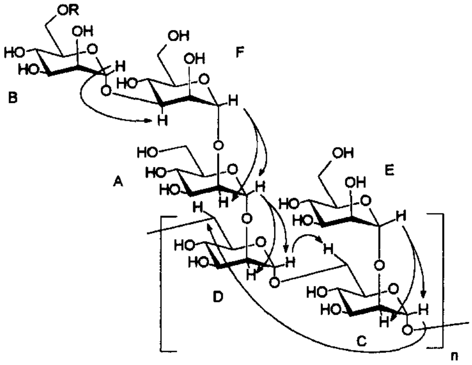

Fig. 9. Hexasaccharide repeating unit of the **PC** (R = PO_3^{2-}) and **DPC** (R = H) showing the interresidue NOEs observed for the latter.

Table 2
Three-bond interresidue heteronuclear correlations for **DPC**

Residue	Anomeric H	Anomeric C	Correlation to C	Correlation to H
A	5.297		79.52 (C-2, **D**)	
		101.40		4.030 (H-2, **D**)
B	5.153		78.78 (C-3, **F**)	
		103.02		3.961 (H-3, **F**)
C	5.121		66.52 (C-6, **C** or **D**)	
D	5.097		66.52 (C-6, **C** or **D**)	
E	5.056		79.52 (C-2, **C**)	
F	5.053		79.23 (C-2, **A**)	

units **C** and **D**, respectively. These assignments were based on the assumption that the **DPC** was composed of a repeating unit oligosaccharide. Reversing these assignments would imply that the **DPC** is composed of two different block structures of approximately equal size. One block would consist of repeating (1 → 6)-linked **D** units to which side chains of **B**–**F**–**A** were linked, and the other block would consist of (1 → 6)-linked **C** units to which were attached side chains of **E**. The repeating unit structure proposed above is regarded as the more likely of the two.

The ^{1}H chemical shift data for the residues in the **DPC** greatly facilitated the analysis of the COSY, HOHAHA, and NOESY spectra of the **PC**. The ^{1}H chemical shifts for the residues in the non-phosphorylated repeating units present in the **PC** resembled closely those of the equivalent residues in the **DPC**. Assignment of the chemical shifts for the resonances of the residues present in the hexasaccharide 6-phosphate repeating units in the **PC** could now be made with certainty and are collected in Table 3. The residues have been designated **Ap**, **Bp**, etc., to facilitate comparison with those in the **DPC**. The downfield location of the H-5 and H-6a,6b resonances of **Bp** in the **PC** when compared with the location of these resonances for **B** in the **DPC** clearly identifies C-6 of **Bp** as the site of the phosphate ester group. The structure of the repeating unit of the **PC** is shown in Fig. 9.

The extent of phosphorylation of the hexasaccharide repeating unit was readily determined from the ratio of the H-2 resonances of residues **Fp** (δ 4.283) and **F** (δ

Table 3
^{1}H Chemical shift data for **PC** at 27 °C

Atom	→ 2-Man (**Ap**)	Man (**Bp**)	→ 2,6-Man (**Cp**)	→ 2,6-Man (**Dp**)	Man (**Ep**)	→ 3-Man (**Fp**)	Man (**Gp**)
H-1	5.295	5.124	5.125	5.108	5.055	5.042	5.064
H-2	4.125	4.085	4.053	4.084	4.089	4.283	4.078
H-3	3.915	3.914			3.819	3.938	3.857
H-4	3.747	3.742			3.682	3.741	
H-5		3.942			3.764	3.830	
H-6a		4.174			3.902	3.890	
H-6b		4.059			3.759	3.771	

4.226) in the **PC** (Fig. 4). The value determined (0.85 phosphate per hexasaccharide unit, or 1 phosphate/7.0 Man residues) was lower than previously reported values (1 phosphate/6.2 Man residues) [8]. This is not surprising as core samples prepared by previously described methods were shown to be contaminated with pentasaccharide phosphate side-chains.

The minor (fractional) ^{1}H signals **G** and **Gp** observed in the spectra of the **DPC** (Fig. 6) and the **PC** (Fig. 4), respectively, indicate that some variation exists in the repeating units of both polysaccharides. Although it is not possible to indicate the precise nature of this variation, the ^{1}H chemical shift values for **G** are very similar to those of residues **B** and **E** (Table 1) and clearly identify **G** as a terminal unit. Moreover the NOESY spectrum of the **DPC** shows interresidue NOEs between H-1 of **G** and H-1 and H-2 of **A**. These data suggest that the sequence **G**(1 → 2)**A**(1 → 2)**D** must be present in the **DPC**. Such a sequence, however, would not be consistent with the 0.85:0.15 integral ratio observed for H-2 of **Fp** and **F** in the ^{1}H spectrum of the **PC** (Fig. 4). One could speculate that if **G** were linked to O-2 of residue **E** then the chemical shifts of H-1, H-2, and H-3 of **E** would be expected to be deshielded and then to resemble and overlap with those of **A**. It is therefore possible that the sequence **G**(1 → 2)**E**(1 → 2)**C** could be present in some of the oligosaccharide repeating units.

In order to understand the properties of the phosporylated core polysaccharide a molecular modelling study was performed. The broad lines and very similar chemical shifts in the spectra of the **DPC** complicate a conformational analysis based on NOE or spin–spin coupling data. The comparison of the modelling study and the experimental data can therefore only be of a qualitative nature.

The chemical shifts of the core structure can give some information about preferred conformations. The overall conformation of the **PC** or the **DPC** is predominantly determined by the (1 → 6) linkage, and especially the rotation about the C-5–C-6 bond. The conformation of the hydroxymethyl group can normally be examined using the proton–proton coupling constants, but for a large structure with very broad lines, due to the slow tumbling of the polysaccharide, this is not possible. The shape of the signals corresponding to the two H-6 protons of residues **C** and **D** indicate that they have similar coupling constants, and thus predominately populate the gg conformation [27]. Another indication for a large population of the gg conformation is the downfield chemical shift of the H-4 signal for residues **C** and **D** at ~ 3.83 ppm [27,28]. The unsubstituted Man residues, such as **B** or **E**, have H-4 resonating at ~ 3.67 ppm, and measurement of coupling constants for this type of residue has shown [27,29] that the main populated conformers are gg and gt in about equal amount. The downfield location of H-4 most likely arises from the proximity of O-6 to H-4 in the gg conformation and is in agreement with observations for Man-(1 → 6)-Man disaccharides [29]. The gg conformation is populated to a large extent, but the gt conformation is still likely to be populated to some extent.

The size of any relevant polysaccharide fragment does not allow for a full simulation Molecular Dynamics study including water. It is, however, possible to perform Monte Carlo simulations with a simple force field (HSEA) [30] or MD studies in vacuo (eg., using Discover).

A Monte Carlo simulation of a 16-repeat of the **PC** was therefore performed. This fragment contains 96 monosaccharide residues and 16 phosphates (in total 1793 atoms). The HSEA force-field calculations were performed without inclusion of charges and were thereby based only on the simple HSEA force-field. A phosphate residue was constructed with a symmetric coordinated phosphate and P–O distances of 1.52 Å. Monte Carlo [31] simulations were carried out at elevated temperatures up to 1200 K and 200,000 MC steps in order to sample as large a part of the conformational space as possible. Generally, an MC simulation starting in an extended low-energy structure would approach a more compact structure quite quickly. This can be attributed to the fact that, even if the extended structure has the lowest enthalpy, the entropy of such a structure will force the molecule to adopt a more compact fold (Fig. 10). It is also worthy of note that the probability is very low that all (1 → 6) linkages would be in an 'extended' conformation at the same time. During the MC run, the hydroxymethyl group of the **C** and **D** units visited all three staggered conformations, but with a preference for the gg conformation. The comparison of the ω angle in different residues in the backbone showed quite large variations indicating that the number of MC steps should be increased in order to get the most realistic average properties for each linkage. This, however, is not necessary as the averaging can also be performed using the repeating nature of the polysaccharide and by performing averaging over several identical linkages. Doing this for two separate MC simulations of 200,000 steps each, it could be seen that such averaging gave comparable results for the ω angles for the **C** and **D** residues, with gg:gt:tg of 50:29:21 for **C** and 61:26:13 for **D**. The small difference between the residues of the backbone is feasible, because small differences are observed between the chemical shifts of H-6b and H-5 of the two residues (Table 1).

The observed interresidue NOEs confirm that the conformational space for the individual glycosidic linkages in the polysaccharide is similar to those observed in the disaccharide fragments. This is clearly observed for the (1 → 2) glycosidic linkages, showing strong NOE from H-1 across the linkage to H-2 and also a weak NOE between the two anomeric protons. For the (1 → 3) linkage a strong NOE is observed from H-1 across the linkage to H-3.

The observed ^{31}P spin–spin couplings to C-5 and C-6 of 3.8 and 6.9 Hz, respectively, are in reasonable agreement with the values of 4.6 and 6.4 Hz measured for methyl α-D-mannopyranoside 6-phosphate by Bernlind et al. [32]. They concluded, based on carbon and proton coupling constants, that the phosphate group occupies all three staggered conformations (g –, g +, t 0.2:0.2:0.6) and that the hydroxymethyl group has conformer populations of 0.7:0.3:0 for gg:gt:tg in the monosaccharide. Similar behaviour would be expected here. The average values from MC are 0.09:0.19:0.7 for the phosphate linkage and 54:28:18 for the hydroxymethyl group. The general overestimation of the tg conformation for hydroxymethyl groups is due to the high temperature used in the MC calculations, which affects the distribution of the population in high and low energy areas. The agreement is still reasonable enough for the data to be used in a qualitative description of the conformational space.

Mannose 6-phosphate receptors which are known to bind to the **PC** are bivalent [33], therefore it is of interest to estimate the phosphate–phosphate distances in the core structures. During the MC simulation the distances between all the phosphate groups were monitored and the results are presented in Fig. 11. In Fig. 11 (bottom) it is seen

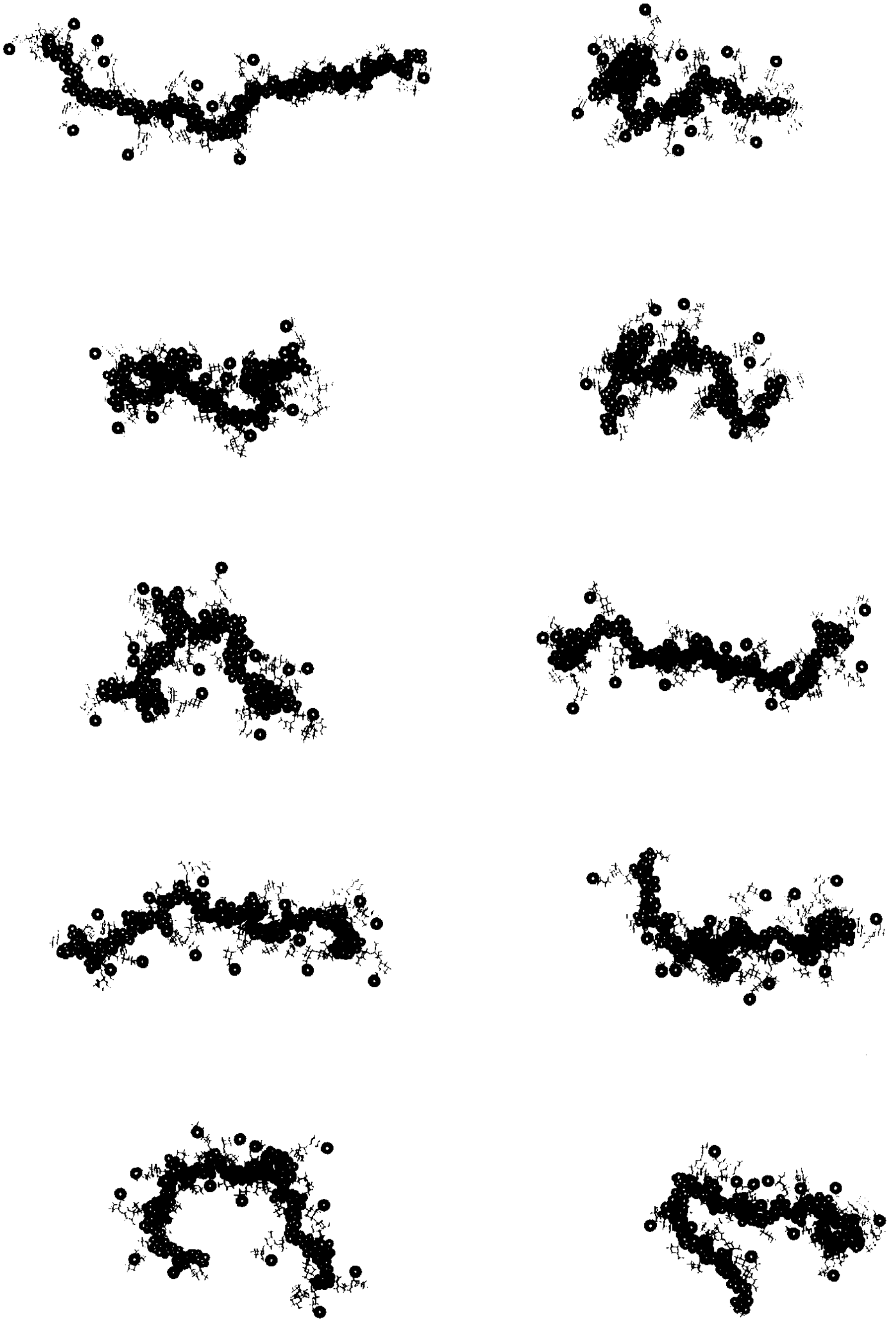

Fig. 10. Conformations generated by selecting randomly from a Monte Carlo simulation, followed by energy minimization. The backbone of (1 → 6)-linked residues and the phosphorus atoms are shown in CPK.

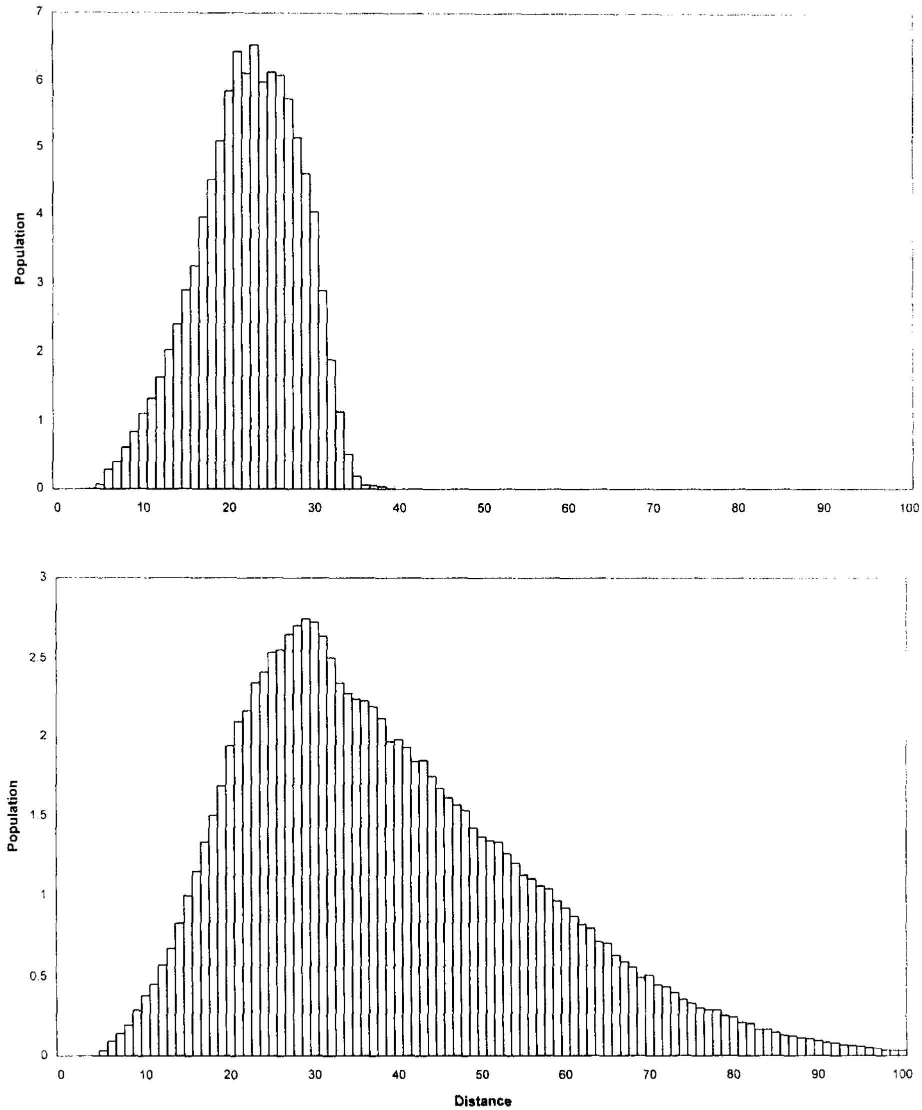

Fig. 11. Population of distances between phosphate groups (P to P) for adjacent phosphate groups (top) and between all groups (bottom) in the core structure based on Monte Carlo simulation of a 16-repeat oligosaccharide (see text).

that essentially all distances between 6 and 100 Å are covered by this 16-repeat sequence, as any pair of phosphates could potentially bind to a receptor, and not only two consecutive ones. In Fig. 11 (top) the more restricted distance distribution between two consecutive phosphates on branches is presented.

Investigation of the **PC** conformation, using Molecular Dynamics in the Discover program (Biosym, San Diego) and the CVFF force field (100–200 ps), indicates similar overall behaviour. The fold seems more compact, even using slightly elevated temperatures (450 K) and a dielectric constant of 80. This could be due to the difference in temperature or an effect of the force field. Proper treatment would require simulation explicitly including water molecules, but this is not currently possible for a large polysaccharide fragment.

4. Conclusion

The phosphomannan core of *Pichia holstii* was prepared in pure form by mild hydrolysis and the structure of the major repeating oligosaccharide has been determined to be the highly branched phosphorylated mannose hexasaccharide presented in Fig. 9. The pentamannosyl phosphate side-chains of the **PS** are attached to the core through phosphate diester linkages involving the terminal α-(1 → 3)-linked mannose 6-phosphate on the mannose trisaccharide branch. The presence of the three α-(1 → 2)-linked mannose residues in the repeating unit of the **PC** (Fig. 9) explains the strong binding of the core to concanavalin A [5]. The fact that the **PS** does not bind concanavalin A indicates that the pentamannosyl phosphate side-chains of the **PS** shield the core from such interaction. Monte Carlo simulation and MD-calculations on a fragment of the **PC** showed the inter phosphate distances to be in the range expected between the two binding subunits for the calcium-independent mannose 6-phosphate receptor, thus accounting for the relatively high affinity of the receptor towards the phosphomannan core.

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