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Large-scale preparation of the oligosaccharide phosphate fraction of *Pichia holstii* NRRL Y-2448 phosphomannan for use in the manufacture of PI-88

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Abstract

Mild acid-catalysed hydrolysis of the extracellular phosphomannan of the yeast *Pichia holstii* NRRL Y-2448 produces a high-molecular-weight phosphomannan core, a low-molecular-weight oligosaccharide phosphate fraction, and a neutral oligosaccharide fraction. A method was developed for the large-scale preparation of the oligosaccharide phosphate fraction, consisting predominantly of the pentasaccharide phosphate, 6-*O*-PO_3H_2-α-D-Man-(1→3)-α-D-Man-(1→3)-α-D-Man-(1→3)-α-D-Man-(1→2)-D-Man, for use in the manufacture of the promising new anti-cancer agent, PI-88. Further insights were also gained into the structure of the phosphomannan by HPLC analysis of the time course of the hydrolysis reaction.

Keywords: *Pichia holstii*; Phosphomannan; Oligosaccharide phosphate fraction; PI-88

1. Introduction

The yeast *Pichia (Hansenula) holstii* NRRL Y-2448, when grown aerobically in a nitrogen-limited medium that uses D-glucose as the carbon source and contains an excess of orthophosphate, produces an extracellular phosphomannan that has been the subject of numerous studies.[1–12] The phosphomannan (PS) is composed of a highly branched, high-molecular-weight ($5–39 \times 10^6$) phosphomannan core (PC) whose structure has only recently been elucidated.[11] Oligosaccharide chains composed principally of the repeating pentasaccharide phosphate **1**, are linked α-(1→6) to the terminal phosphate of the PC and make up approximately 90% of the PS. These side chains, which are capped by unphosphorylated residues,[9,11] are susceptible to mild acid hydrolysis of the phosphate diester linkages thus giving ready access to both the PC and **1**. Indeed, the PC and low-molecular-weight oligosaccharide preparations, previously assumed to consist solely of **1**, have been used extensively as tools and probes for studying phosphomannosyl receptors.[13–19]

Recently, some light has been shed on the nature of the phosphorylated side chains.[12] The available data suggests that the majority of these side chains are composed of up to at least ten repeating pentasaccharide phosphate residues. In the remaining side chains, some pentasaccharide phosphate residues are replaced by tetrasaccharide phosphate and to a much lesser extent by hexa-, tri- and disacchar-

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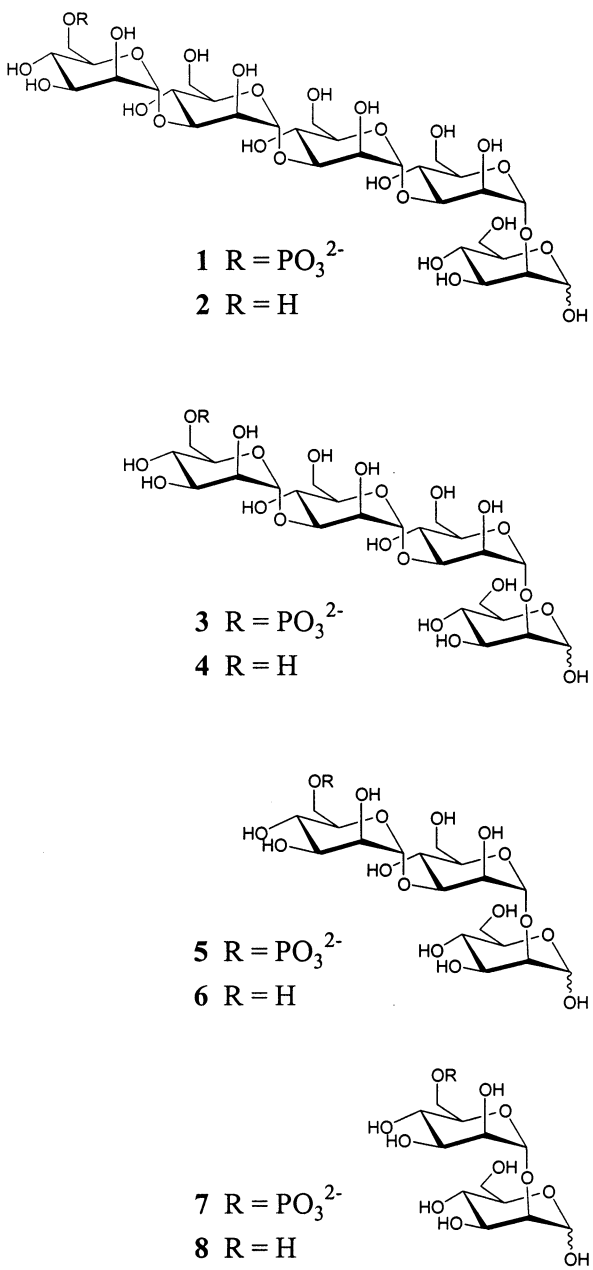
ide phosphates. There is also evidence that the unphosphorylated units capping the side chains are predominantly pentasaccharide and disaccharide residues. In addition, it has been shown that these low-molecular-weight oligosaccharide preparations assumed to consist solely of **1** also contain as much as 20–25% of the tetrasaccharide phosphate **3**, as well as minor amounts of hexa-, tri- and disaccharide phosphates. The presence of the phosphate group in these oligosaccharides prevents their successful separation by size-exclusion chromatography.[12] Such a low-molecular-weight oligosaccharide preparation (free of neutral oligosaccharides) shall be referred to as the oligosaccharide phosphate fraction or OPF (Scheme 1).



Scheme 1.

The product of exhaustive sulfation of the OPF, known as PI-88, has recently been identified as a promising inhibitor of tumour growth and metastasis and is currently in Phase I clinical trials.[20] PI-88 exerts its antimetastatic effects by inhibiting heparanase,[21,22] an endoglucuronidase which cleaves the heparan sulfate side chains of heparan sulfate proteoglycans in the extracellular matrix (ECM) surrounding tumour cells. The degradation of the ECM facilitates the spread of tumour cells by enabling them to both enter into and escape from blood vessels and lymphatics. Heparanase is also implicated in angiogenesis, i.e., the growth of new blood vessels from pre-existing blood vessels surrounding tumours. Heparanase can act directly, by promoting invasion of endothelial cells (vascular sprouting), and indirectly, by releasing heparan sulfate-bound growth factors (e.g., bFGF) and generating heparan sulfate degradation fragments that promote bFGF activity.[21,22] PI-88 is thus also an inhibitor of angiogenesis by virtue of its inhibition of heparanase and by its ability to block heparan sulfate binding of angiogenesis-inducing growth factors.[20] PI-88 also shows promise as a potential anticoagulant/antithrombotic agent with a novel mode of action.[23] It enhances the ability of heparin cofactor II to inhibit thrombin but does not interact with AT-III.

Large quantities of clinical grade PI-88 are required for ongoing pre-clinical studies (including animal toxicology) and human clinical trials, necessitating a reliable supply of the OPF, preferably to good manufacturing practice (GMP) standards. No commercial production of the OPF exists, and to date its preparation has only been described on a laboratory scale. These preparations usually involve the isolation and purification by differential ethanol precipitation of its barium salt and are thus not easily scaleable, and the product obtained is of questionable purity. A

reliable method was thus sought for its preparation in multigram quantities that avoided the use of toxic barium salts, was amenable to scale up to pilot plant scale, and was of sufficient purity for the GMP production of PI-88 for clinical trials.

2. Results and discussion

Previously described methods for the preparation of the OPF involve mild acid-catalysed hydrolysis of the PS.[8,9] After neutralisation, the phosphorylated products, the OPF and the PC, are converted into their barium salts by the addition of barium acetate. The PC is then precipitated with 0.1 volumes of ethanol and removed by centrifugation or filtration. Addition of a further two volumes of ethanol to the mother liquors then precipitates the OPF. Alternatively, the OPF is isolated by size-exclusion chromatography of the hydrolysate.[10]

The precipitation method has been found to be less than optimal. The PC is incompletely removed during the first precipitation step, resulting in impure OPF which must be further purified by size-exclusion chromatography.[9,17] The product may also be contaminated with incompletely hydrolysed oligosaccharide phosphate chains (predominantly made up of the decasaccharide diphosphate *P*-Man_5-*P*-Man_5) as well as smaller, neutral oligosaccharides.[9,17] The very large volumes of ethanol required are also impractical for scale up. Moreover, the use of toxic barium acetate needs to be avoided. The size-exclusion method is also not well suited for scaling up due to the very large quantities of expensive size-exclusion media that are required, as well as the need to concentrate the large volumes of hydrolysate that are obtained prior to chromatography.

The synthesis began with the PS, which was prepared by fermentation of *P. holstii* NRRL Y-2448.[2,9] The product was obtained by ethanol precipitation as a sticky gum with a high water–ethanol content ($\sim 60\%$) that could be used directly in the hydrolysis step. The ^{1}H NMR (300 MHz) spectrum of an analytical sample of the PS recorded in D_2O at 45 °C was in accord with the literature.[11] This fermentation has been successfully scaled up into a 500-L fermenter to provide up to 20 kg of product per run (wet weight; dry weight ~ 8 kg).

The hydrolysis of the PS was carried out for 6–10 h at 100 °C at a concentration of approximately 40–50 g/L, pH 2.2–2.5 using 1 M HCl as the catalyst and in the presence of KCl. The pH was closely monitored and was found to rise slightly (up to pH 3) over the first hour of reaction as the PS went into solution. Therefore, it was readjusted back to pH 2.2–2.5 by further additions of 1 M HCl. It has been noted[9,11] that under these hydrolysis conditions the side chains attached to the PC are incompletely removed. The ^{1}H NMR spectra of these products usually showed ^{1}H signals at $\delta \sim 5.7$ ($^3J_{H,P} \sim 8$ Hz), indicative of the presence of phosphate diester linkages. However, this was not found to be the case in this instance, as shown by the ^{1}H NMR spectrum of the isolated PC, which displayed no such signals. These incomplete hydrolyses observed previously may have been due to the (unchecked) rise in the pH during the early stages of the reaction.

The hydrolysis was routinely performed on 2.5 kg (wet weight) of PS. The reaction was monitored by HPLC using a size-exclusion column (Tosohaas TSK-Gel G2500PWXL) with refractive index detection. Fig. 1(c) shows a typical chromatogram at the completion of the reaction (6 h). All of the components of the fractions were isolated as described in Section 3 and were identified by comparison of their NMR and/or mass spectral data with those in the literature.[11,12] The fractions were found to contain: the PC (A), *P*-Man_5–*P*-Man_5 (B), OPF (C), a mixture of phosphate and the unphosphorylated oligosaccharides **2**, **4** and **6** (D), and the unphosphorylated disaccharide **8** (E). Fractions F–H correspond to salts (NaCl or KCl) and ethanol.

An analysis of the time course of the reaction (Fig. 1(a–c)) indicates that the initial products formed are the PC, poly-*P*-Man_5 chains, the OPF and the disaccharide **8**. Over the course of the reaction, any remaining phosphorylated side chains attached to the PC are cleaved, the bulk of the poly-*P*-Man_5 chains are hydrolysed to OPF, and some neu-

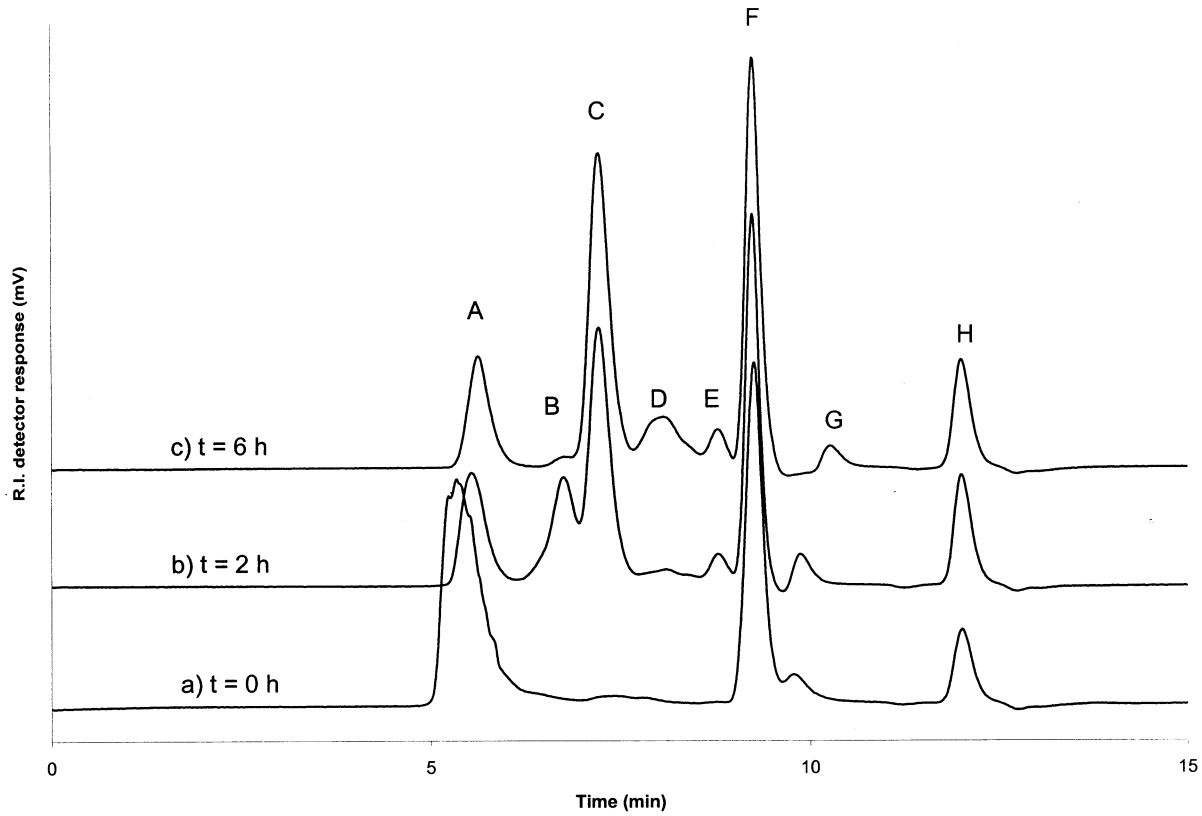


Fig. 1. HPLC analysis of the phosphomannan hydrolysate at time = 0, 2 and 6 h.

tral oligosaccharides (**2**, **4** and **6**) and phosphate are produced. Interestingly, the amount of disaccharide **8** formed increases only slightly over time. Attempts to convert the remaining traces of fraction B into the OPF by prolonging the reaction time did not yield more OPF, but rather more unphosphorylated products and phosphate (fraction D). This indicated that the phosphate group of the OPF was being cleaved. The optimal reaction time was therefore determined to be 6–10 h. The early appearance of **8** in the reaction mixture, in a relatively unchanging amount over time, confirms that in the PS the disaccharide **8** is present mostly as an unphosphorylated capping unit on the side chains and undergoes rapid hydrolysis. The lack of pentasaccharide **2** in the initial stages of the reaction also seems to indicate that this pentasaccharide may not be a major unphosphorylated capping unit as has been suggested.[12] Pentasaccharide **2** probably arises to a large extent from dephosphorylation of **1**. Similarly, the oligosaccharides **4** and **6** arise in a similar manner from their respective phosphates **3** and **5**, which are incorporated in smaller amounts into the side chains.

After neutralisation of the hydrolysate and adjustment of the pH to 9–9.5 with 1 M NaOH, the OPF and unphosphorylated oligosaccharides were separated from the PC by ultrafiltration through a 10,000 nominal molecular weight cut off (NMWCO) membrane. The OPF, unphosphorylated oligosaccharides and salts permeated through the membrane whilst the high-molecular-weight PC was retained. The separation was achieved in diafiltration mode (against eight diavolumes of purified water) with a tangential or crossflow system. Such a system is readily scaled up by increasing the available membrane area and the flow rate. Membranes of NMWCOs ranging from 3,000 to 100,000 were also successfully trialed, however, the 10,000 NMWCO membrane was the most efficient.

Purification of the permeate was achieved by ion-exchange chromatography on DEAE-Spherilose resin equilibrated with 0.01 M NH_4HCO_3. The OPF and traces of fraction B

bound to the resin whilst the unphosphorylated products were washed through. The OPF was then eluted off with 0.25 M NH_4HCO_3. The pooled fractions containing the OPF were concentrated and desalted by reverse osmosis prior to lyophilisation. The reverse osmosis step was critical in rapidly reducing the large volumes obtained (50–60 L per hydrolysis) to manageable levels for lyophilisation and is a key step in any further scale up. Moreover, the reverse osmosis was also able to remove the bulk of the inorganic salts in the fractions, resulting in a very pure final product containing only a few per cent of inorganic salts. The OPF was obtained as a hygroscopic powder that was approximately 93% pure by HPLC (single carbohydrate peak) and could be used without further purification for the production of PI-88. Analytical samples could be obtained by further desalting on columns of Bio-Gel P-2, followed by lyophilisation. This procedure has successfully been used to provide 500–600 g of OPF per hydrolysis on a routine basis, and there is potential for further scale up to pilot plant scale because all the process steps are directly scaleable.

The ^1H and ^{13}C NMR spectra of the OPF were in accord with the published spectra[12] and displayed the expected major resonances for **1** as well as a number of minor resonances due to the presence of the homologous oligosaccharide phosphates **3**, **5** and **7**. The ^{31}P NMR spectrum gave a single peak at δ 3.44, indicative of phosphate monoester.[24] In addition, the mass spectrum, obtained in the negative-ion mode by the electrospray ionisation technique (ESIMS), gave peaks for the expected major $[M-H]^-$ ions at m/z 907.3 and 745.3, respectively, for **1** and **3**, as well as peaks at m/z 583.3 and 421.3 for **5** and **7**, respectively.[12] Interestingly, the reported peak for hexasaccharide phosphate was not clearly discernible, presumably due to the very small amount present. Its presence was detected, however, in the mass spectrum of fraction B (m/z 979.3, $[M-2H]^{2-}$, P_2-Man_{11}).[12]

In conclusion, we have developed a procedure for preparing the oligosaccharide phosphate fraction of the extracellular phosphomannan of *P. holstii* in multigram quantities to GMP standard, which is amenable to scale up to pilot plant scale for use in the manufacture of PI-88 for clinical trials. We have also shed further light on the structure of the phosphomannan.

3. Experimental

General.—NMR spectra were recorded using a Varian Unity-400, XL-300 or -200 spectrometer for solutions in D_2O and are referenced to internal acetone (δ 2.225 for ^1H and δ 31.0 for ^{13}C) or to external H_3PO_4 (δ 0.00 for ^{31}P). Mass spectra were obtained by electrospray-ionization (ESIMS) on a Fisons VG Quattro II mass spectrometer. Analytical HPLC was performed on a Waters Alliance 2690 separations module using a Tosohaas TSK-Gel G2500PWXL 6 μm (300 × 7.8 mm) size-exclusion column with a flow rate of 1.0 mL/min and 0.05 M NaCl as the mobile phase. Detection was with a Waters 2410 refractive index detector. Reverse osmosis was performed on a Millipore Proscale system (operating temperature 15–22 °C, feed pressure 1200 kPa, inlet flow rate 8 L/min) equipped with two Helicon RO4 cartridges with Nanomax 50 membrane (membrane area 0.37 m^2/cartridge). Thin-layer chromatography (TLC) was performed on E. Merck Kieselgel 60 F_{254} aluminium-backed sheets with specified eluents. Compounds were detected by charring with 10% aq H_2SO_4. All reagents used were analytical grade and were used without further purification. All water used was purified in house to USP purified water standard. DEAE-Spherilose and Bio-Gel P-2 were from Isco and BioRad, respectively.

Phosphomannan from P. holstii *NRRL Y-2448 (PS).*—The extracellular polysaccharide from *P. holstii* NRRL Y-2448 was prepared by fermentation according to the literature procedure[2,9] and was isolated by EtOH precipitation as a gum which was used without further purification. A typical yield from a 400-L fermentation was 20 kg. The moisture–EtOH content was determined to be approximately 60% by loss on drying. An analytical sample was prepared by dissolving lyophilised

PS (244 mg) in deionised water (100 mL) and dialysing against two changes of deionised water (4 L) followed by lyophilisation to give a white amorphous solid (218 mg). ^{1}H NMR (300 MHz, 45 °C): δ 5.68 (bd, 1 H, $^3J_{H,P} \sim 7.5$ Hz), 5.12 (bs, 3 H), 5.06 (bs, 1 H).

Hydrolysis of the phosphomannan.—A solution of KCl (600 g) in water (60 L), acidified to pH 2.4 with 1 M HCl, was prepared in a 75 L Chemap stainless steel reaction vessel. The PS (2.49 kg wet weight) was added in portions to the solution via the addition portal, and the mixture was heated at 100 °C for 7 h with vigorous stirring. The pH was monitored hourly and was readjusted to pH 2.3 after 1 h by further addition of 1 M HCl. The progress of the reaction was monitored by HPLC. At the completion of the reaction, the hydrolysate was cooled to rt and then the pH was adjusted to pH 9.5 by the addition of 1 M NaOH.

Diafiltration of the hydrolysate.—The hydrolysate was diluted to 70 L and transferred to a 150-L stainless steel tank. It was then diafiltered against eight diavolumes of purified water using an ultrafiltration system fitted with two Sartorius Hydrosart 10K (NMWCO 10,000) filter cassettes (membrane area 0.6 m^2/cassette) configured in series. Operating parameters were: inlet pressure 200 kPa, outlet pressure 150 kPa, retentate cross flow rate 15.6 L/min, permeate flux rate 66–87 L/h/m^2 (16–22 °C). The progress of the separation was monitored by HPLC. The permeate (630 L, conductivity = 1.1 mS/cm) was split into six batches for processing by ion-exchange chromatography.

Ion-exchange chromatography of the permeate (fraction C).—A column of DEAE-Spherilose (30 L) was equilibrated with 0.01 M NH_4HCO_3 at a flow rate of 1.5 L/min. The permeate ($\sim$100 L per run, six runs) was loaded onto the column, and the neutral fraction was washed from the column with 0.01 M NH_4HCO_3 until the conductivity of the effluent was within 0.2 mS/cm of the baseline ($\sim$100 L). The product was then eluted off using 0.25 M NH_4HCO_3. One-litre fractions were collected and monitored by HPLC. The appropriate fractions from each chromatography run were combined and concentrated to 20 L by reverse osmosis. Reverse osmosis was continued in diafiltration mode against purified water until the conductivity of the permeate was $\leq$0.2 mS/cm, and the solution was then concentrated to a final volume of 6 L. Lyophilisation then gave the OPF as a white, hygroscopic powder (566 g). The product was of sufficient purity ($\sim$93% by HPLC) for PI-88 synthesis. An analytical sample was prepared by desalting on a column of Bio-Gel P-2 (5 × 100 cm) using 0.1 M NH_4HCO_3 as eluent, followed by lyophilisation. ESIMS: m/z 907.3 ($[M-H]^-$, **1**), 745.3 ($[M-H]^-$, **3**), 583.3 ($[M-H]^-$, **5**), 421.3 ($[M-H]^-$, **7**). ^{31}P NMR (162 MHz): δ 3.44 (s). ^{1}H and ^{13}C NMR data were in accord with the published spectra.[12] ^{1}H NMR (300 MHz): δ 5.38 (s, 0.9 H, α anomer of reducing Man residue), 5.13 (s, 2 H), 5.09 (s, 1 H), 5.04 (s, 1 H), 4.93 (s, 0.1 H, β anomer of reducing Man residue).

Isolation of oligosaccharides from fractions D and E.—A sample of the unbound effluent from the ion-exchange chromatography step (2 L) was concentrated to dryness under vacuum. The residue was purified by size-exclusion chromatography on a column of Bio-Gel P-2 (5 × 100 cm) using 0.1 M NH_4HCO_3 as eluent. The fractions were monitored by TLC (3:2:1 EtOAc–MeOH–water). The following pure oligosaccharides were isolated as white amorphous solids after lyophilisation: **2** (R_f = 0.19, 240 mg), **4** (R_f = 0.25, 303 mg), **6** (R_f = 0.33, 229 mg) and **8** (R_f = 0.42, 310 mg). HPLC analysis showed that fraction E was made up of **8** (R_t = 9.2 min) whilst fraction D contained **6** (R_t = 8.4 min), **4** (R_t = 8.1 min) and **2** (R_t = 7.9 min). The ^{1}H and ^{13}C NMR spectra of the oligosaccharides were in accord with the published spectra.[12]

High-molecular-weight core polysaccharide (fraction A, PC).—A sample of the retentate from the ultrafiltration step (50 mL) was diafiltered against six diavolumes (300 mL) of water using an Amicon miniplate-3 bioconcentrator (NMWCO 3,000). HPLC analysis indicated that the retentate contained only a single peak (R_t = 5.6 min). The retentate was filtered and lyophilised to give the PC as a white, amorphous solid (137 mg). ^{1}H NMR (300 MHz): δ 5.26 (bs, 1 H), 5.08 (bs, 3 H), 5.01 (bs, 2 H).

Isolation of fraction B.—A sample of the permeate from the ultrafiltration step was concentrated under vacuum and was then purified by size-exclusion chromatography on a column of Bio-Gel P-2 (10 × 100 cm) using 0.1 M NH_4HCO_3 as eluent. The fractions were monitored by TLC (7:3 EtOH–1 M ammonium acetate), $R_f = 0.1$ (vs 0.3 for OPF) and HPLC ($R_t = 6.7$ min). Fractions containing pure product (single peak by HPLC) were pooled and lyophilised to give fraction B (predominantly P-Man_5–P-Man_5) as a white, amorphous powder. ^{31}P NMR (162 MHz) δ −1.34 (s), 4.60 (s). ^{1}H NMR (300 MHz) δ 5.66 (d, $^3J_{H,P}$ 7.8 Hz), 5.37 (s, 1 H), 5.10–4.86 (8 H). ESIMS: m/z 979.3 ($[M-2H]^{2-}$, P_2-Man_{11}), 898.3 ($[M-2H]^{2-}$, P_2-Man_{10}), 817.4 ($[M-2H]^{2-}$, P_2-Man_9), 736.4 ($[M-2H]^{2-}$, P_2-Man_8).

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References

[1] Anderson, R. F.; Cadmus, M. C.; Benedict, R. G.; Slodki, M. E. *Arch. Biochem. Biophys.* **1960**, *89*, 289–292.
[2] Jeanes, A.; Pittsley, J. E.; Watson, P. R.; Dimler, R. J. *Arch. Biochem. Biophys.* **1961**, *92*, 343–350.
[3] Slodki, M. E.; Wickerham, L. J.; Cadmus, M. C. *J. Bacteriol.* **1961**, *82*, 269–274.
[4] Jeanes, A.; Watson, P. R. *Can. J. Chem.* **1962**, *40*, 1318–1325.
[5] Jeanes, A.; Pittsley, J. E.; Watson, P. R.; Sloneker, J. H. *Can. J. Chem.* **1962**, *40*, 2256–2260.
[6] Slodki, M. E. *Biochim. Biophys. Acta* **1962**, *57*, 525–533.
[7] Dintzis, F. R.; Babcock, G. E.; Tobin, R. *Solution Properties of Natural Polymers*; Chemical Society: London, 1968; pp. 195–206 special publication 23.
[8] Slodki, M. E.; Ward, R. M.; Boundy, J. A. *Biochim. Biophys. Acta* **1973**, *304*, 449–456.
[9] Bretthauer, R. K.; Kaczorowski, G. J.; Weise, M. J. *Biochemistry* **1973**, *12*, 1251–1256.
[10] Slodki, M. E. In *Industrial Polysaccharides: Genetic Engineering: Structure/Property Relations and Applications*; Yalpani, M., Ed.; Elsevier: Amsterdam, 1987; pp. 109–119.
[11] Parolis, L. A. S.; Duus, J.Ø.; Parolis, H.; Meldal, M.; Bock, K. *Carbohydr. Res.* **1996**, *293*, 101–117.
[12] Parolis, L. A. S.; Parolis, H.; Kenne, L.; Meldal, M.; Bock, K. *Carbohydr. Res.* **1998**, *309*, 77–87.
[13] Sahagian, G. C.; Distler, J. J.; Jourdian, G. W. *Methods Enzymol.* **1982**, *83*, 392–396.
[14] Steiner, A. W.; Rome, L. H. *Arch. Biochem. Biophys.* **1982**, *214*, 681–687.
[15] Brown, W. J.; Farquhar, M. G. *Cell* **1984**, *36*, 295–307.
[16] Baba, T.; Watanabe, K.; Arai, Y. *Carbohydr. Res.* **1988**, *177*, 153–161.
[17] Murray, G. J.; Neville, Jr., D. M. *J. Biol. Chem.* **1980**, *255*, 11942–11948.
[18] Baba, T.; Watanabe, K.; Yonezawa, N.; Hiroto, M.; Arai, Y. *Carbohydr. Res.* **1988**, *177*, 163–172.
[19] Yoshida, T.; Lee, Y. C. *Carbohydr. Res.* **1994**, *251*, 175–186.
[20] Parish, C. R.; Freeman, C.; Brown, K. J.; Francis, D. J.; Cowden, W. B. *Cancer Res.* **1999**, *59*, 3433–3441.
[21] Hulett, M. D.; Freeman, C.; Hamdorf, B. J.; Baker, R. T.; Harris, M. J.; Parish, C. R. *Nat. Med.* **1999**, *5*, 803–809.
[22] Vlodavsky, I.; Friedmann, Y.; Elkin, M.; Aingorn, H.; Atzmon, R.; Ishai-Michaeli, R.; Bitan, M.; Pappo, O.; Peretz, T.; Michal, I.; Spector, L.; Pecker, I. *Nat. Med.* **1999**, *5*, 793–802.
[23] Podger; D. M. *AATD-Advances in Anticoagulant, Antithrombotic and Thrombolytic Drugs*; IBC 11th Annual International Symposium; Waltham MA, October 2000.
[24] Gorenstein, D. G.; Shah, D. O. In *Phosphorus-31 NMR Principles and Applications*; Gorenstein, D. G., Ed.; Academic: Orlando, 1984; pp. 549–591.