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Determination of the composition of the oligosaccharide phosphate fraction of *Pichia* (*Hansenula*) *holstii* NRRL Y-2448 phosphomannan by capillary electrophoresis and HPLC

Vito Ferro,[a,*] Caiping Li,[a] Kym Fewings,[a] Maria C. Palermo,[a] Robert J. Linhardt,[b] Toshihiko Toida[c]

[a]*Department of Research & Development, Progen Industries Ltd, PO Box 28, Richlands BC, Qld 4077, Australia*
[b]*Department of Chemistry, Division of Medicinal and Natural Products Chemistry, Department of Chemical and Biochemical Engineering, University of Iowa, Iowa City, IA 52242, USA*
[c]*School of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi, Inage, Chiba 263-8522, Japan*

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Abstract

The promising new anticancer agent, PI-88, is prepared by the sulfonation of the oligosaccharide phosphate fraction of the extracellular phosphomannan produced by the yeast *Pichia* (*Hansenula*) *holstii* NRRL Y-2448. The composition of the oligosaccharide phosphate fraction was determined by capillary electrophoresis (CE) with indirect UV detection using 6 mM potassium sorbate at pH 10.3 as the background electrolyte. Further confirmation of the composition was obtained by HPLC analysis of a sample dephosphorylated by treatment with alkaline phosphatase. The structure of the hexasaccharide component has been determined by isolation and NMR spectroscopic analysis of its dephosphorylated derivative. Additionally, the structure of a second, previously undetected tetrasaccharide component (a hexosamine) has been determined by isolation and NMR spectroscopic analysis of the acetate of its dephosphorylated derivative. It is demonstrated that CE is an ideal method for the quality control of the oligosaccharide phosphate fraction for use in the production of PI-88.

Keywords: *Pichia* (*Hansenula*) *holstii*; Phosphomannan; Oligosaccharide phosphate fraction; Capillary electrophoresis; PI-88

1. Introduction

The novel sulfated oligosaccharide agent known as PI-88 has recently been identified as a promising inhibitor of tumour growth and metastasis[1] and is currently undergoing clinical evaluation in cancer patients. PI-88 is an inhibitor of angiogenesis by virtue of its ability to block heparan sulfate binding of angiogenesis-inducing growth factors. PI-88 also blocks metastasis by inhibiting heparanase,[2] a key enzyme involved in the degradation of the extracellular matrix surrounding tumour cells, thus preventing their spread to other sites via entry into blood vessels and lymphatics. In addition to its anticancer activities, PI-88 shows promise as a potential anticoagulant/antithrombotic agent with a novel mode of action.[3–5] It is a specific ligand for heparin cofactor II, enhancing its ability to inhibit thrombin (factor IIa). However, it does not interact with antithrombin-III and thus shows no anti-Xa or AT-III mediated ant-IIa activity.

PI-88 is prepared[6] by the sulfonation of the oligosaccharide phosphate fraction (OPF) of the extracellular phosphomannan produced by the yeast *Pichia* (*Hansenula*) *holstii* NRRL Y-2448.[7,8] Early structural studies of the OPF found that the principal component is the pentasaccharide phosphate **1**[9] and that smaller amounts of tetrasaccharide[9,10] and hexasaccharide[10] were also present. More recently, NMR and mass spectral studies have demonstrated that it contains

* Corresponding author. Tel.: +61-7-32739100; fax: +61-7-33751168.
E-mail address: vito.ferro@progen.com.au (V. Ferro).

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substantial amounts of tetrasaccharide phosphate **3**, as well as minor amounts of hexa-, tri- and disaccharide phosphates.[7] The presence of the phosphate group in these oligosaccharides prevents their successful separation by size-exclusion chromatography (SEC);[7] thus, the amounts of each component present were not quantified.

An important aspect of the production of PI-88 for clinical trials is the availability of analytical procedures for the characterisation and quality control of the intermediate OPF. We now report on the determination of the composition of the OPF by capillary electrophoresis (CE) with indirect UV detection combined with HPLC analysis of dephosphosphorylated OPF.

2. Results and discussion

Recently, a large-scale preparation of the OPF was developed[8] that gave ^{1}H and ^{13}C NMR and mass spectral data in accord with the literature.[7] The OPF obtained from such a preparation[8] was subjected to detailed structural characterisation by chemical and spectroscopic means. Methylation/fragmentation followed by GC–MS analyses of the alditol acetates (data not shown) revealed three major components corresponding to (a) 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl-D-mannitol (from $1\rightarrow2$ Man); (b) 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-mannitol (from $1\rightarrow3$ Man); and (c) 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-mannitol (from nonreducing end Man6P). Ion chromatographic analysis demonstrated that the OPF contained 3.0–7.4% of phosphate, which is consistent with a single phosphate group in each oligosaccharide component. A combination of 1D and 2D (DQF COSY, HMBC, HMQC, NOESY) NMR spectroscopy (data not shown) then confirmed the structure of the major component as the pentasaccharide phosphate **1**.[7] Capillary electrophoresis (CE) was next investigated to determine the proportions of the components of the mixture.

CE has been developed into a powerful and highly efficient technique to analyse a wide variety of molecules, allowing rapid, repetitive, high-resolution analysis with little, if any, sample preparation. The use of CE for carbohydrate analysis has seen a rapid increase in recent years,[11–14] despite the inherent lack of chromophores for conventional UV detection. This apparent drawback has been overcome chiefly by the use of derivatisation strategies that typically introduce a chromophore or fluorophore by reductive amination, thus allowing detection by UV absorbance or fluorescence. Derivatisation reactions, however, may not always be possible or suitable, particularly for quality control purposes because of variable yields. An alternative, commonly used strategy that allows direct sample analysis is that of indirect UV detection, which requires the use of a suitable UV-absorbing background electrolyte (BGE).[15] A number of BGEs have been tested for the separation of low-molecular weight carbohydrates with indirect UV detection, e.g., sorbic acid,[15,16] riboflavin,[17] 5-sulfosalicylic acid,[18] tryptophan[19] and 1-naphthylacetic acid.[20] In some works, poor peak resolutions and poor baseline noise have been reported.

The BGE chosen for the analysis of OPF was 6 mM potassium sorbate since it has been used previously for the analysis of acidic monosaccharides.[21,22] Since the component phosphorylated oligosaccharides are naturally ionised, a relatively low pH of 5–5.8 was used in previous studies.[21,22] However, with OPF, such a low pH gave poor resolution and peak shape, as well as an unacceptably long run time. The previously reported highly alkaline conditions (pH $\sim$12)[15,16] required for neutral saccharides also gave an unstable baseline. The optimised conditions were found to be pH 10.3, capillary temperature of 20 °C, and operating voltage of 18 kV. Although the UV absorbance maximum of sorbic acid is 254 nm, the use of 214 nm as the detection wavelength was preferred as this gave a less noisy baseline. These conditions resulted in a stable baseline, good resolution and good reproducibility, as depicted in Fig. 1.

Fig. 1 surprisingly shows that the OPF is made up of at least seven components. The two major components (Peaks B and C) are presumably due to the penta- and tetrasaccharide phosphates **1** and **3**, respectively, and make up approximately 59 and 28.5%, respectively, of the total carbohydrate content. The amount of **3** is slightly higher than the previous estimate based on ESIMS data.[7] As expected, the electropherogram also indicates that there is a small amount of hexasaccharide phosphate (Peak A, 1%) and disaccharide phosphate **7** (Peak F, 3%). However, the presence of monosaccharide phosphate (Peak G, 3%) and an extra peak in addition to trisaccharide phosphate **5** (Peak D or E, 5.5% total) were not anticipated (the second peak is presumably a previously unidentified tri- or tetrasaccharide phosphate). Peak G was confirmed as mannose 6-phosphate by spiking experiments with pure mannose 6-phosphate standard. Samples originating from phosphomannan batches from several different fermentation runs were similarly analysed. Some samples contained no mannose 6-phosphate, indicating that it is unlikely to be an actual minor component of the phosphorylated side chains of the phosphomannan, but rather a byproduct of the hydrolysis of the larger oligosaccharide phosphates. The exhaustive, acid-catalysed hydrolysis of the phosphomannan has, in fact, been shown to be a good source of mannose 6-phosphate.[23] The amounts of the other minor components did not change appreciably, but some slight variation was seen in the ratio of pentasaccharide

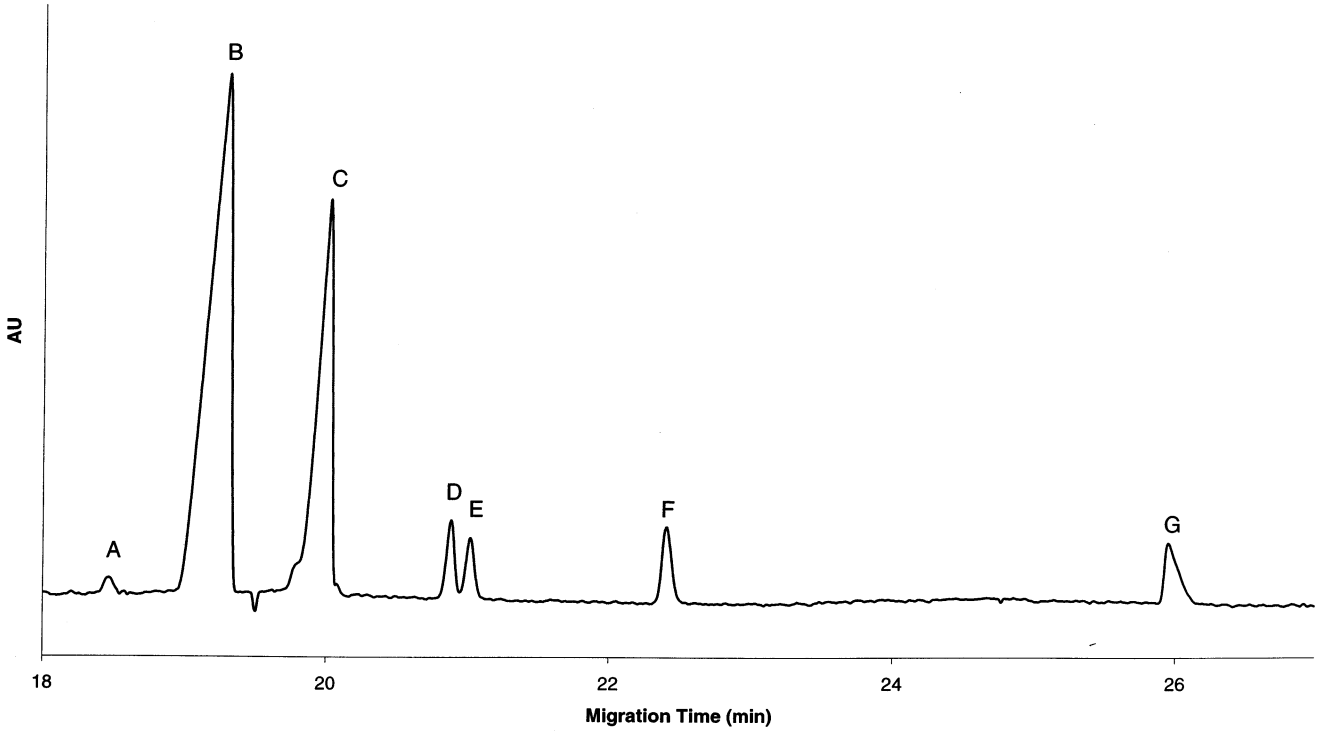


Fig. 1. Capillary electropherogram of the oligosaccharide phosphate fraction.

phosphate **1** (Peak B) to tetrasaccharide phosphate **3** (Peak C) ranging from ~2:1 to 3:2. The amount of Peak D relative to Peak E was also variable. Only one trisaccharide (**6**) and one tetrasaccharide (**4**) have previously been isolated from phosphomannan hydrolysates, either from the neutral oligosaccharides released[8] or from specific dephosphorylation of the OPF by treatment with aqueous HF.[7]

Other than the commercially available mannose 6-phosphate, pure samples of the oligosaccharide phosphates were not available (because they are not amenable to chromatographic separation). Other means were thus sought to confirm the identity of the peaks. Direct HPLC analysis of the OPF was not successful, giving a single peak with either an SEC column or a carbohydrate (cation-exchange) column. However, the corresponding neutral oligosaccharides are amenable to HPLC analysis and can be isolated by preparative SEC on Bio-Gel P-2.[7,8]

A sample of the OPF was thus dephosphorylated by treatment with alkaline phosphatase. The resulting neutral oligosaccharides were then analysed by HPLC using a Rezex RNO-Oligosaccharide column (Fig. 2). The various peaks were readily identified by comparison with the pure oligosaccharides **2**, **4**, **6** and **8**, previously isolated as byproducts of the hydrolysis.[8] The results mirrored those seen in the CE analysis, with the exception that only one peak was clearly discernible in the trisaccharide region (for **6**).

The isolation and determination of the structure of the (neutral) hexasaccharide, which had not been done previously because of its low abundance, were next pursued. The neutral oligosaccharide fraction from a large-scale hydrolysis of the phosphomannan,[8] represented by the unbound effluent from the ion-exchange chromatography step, was collected, concentrated by reverse osmosis and freeze dried. Analysis of this sample by HPLC revealed a similar distribution of oligosaccharides as seen in the alkaline phosphatase experiment, with the exception of the amount of disaccharide **8**. The increased amount of **8** is due to it being the major non-phosphorylated capping unit of the phosphomannan side chains.[7,8] The sample was then subjected to repeated SEC on a column of Bio-Gel P-2, resulting in the isolation of 13 mg of material, corresponding to the hexasaccharide (Peak A, Fig. 2) that was of sufficient purity (~80% by HPLC) for NMR spectroscopic analysis. This material was confirmed as being predominantly hexasaccharide by ESIMS (positive-ion mode, m/z 1012.9 $[M + Na]^+$). Its structure was then firmly established as the hexasaccharide **10**, in which an extra D-mannose unit is (1→3)-linked at the nonreducing end, by a combination of 1D (^1H and ^{13}C) and 2D (gCOSY, gHSQC) NMR spectroscopy and comparison with the literature[7,24] (NMR data are presented in Table 1). The corresponding hexasaccharide phosphate is thus presumably **9**, with the phosphate group at the terminal 6-OH of the nonreducing end.

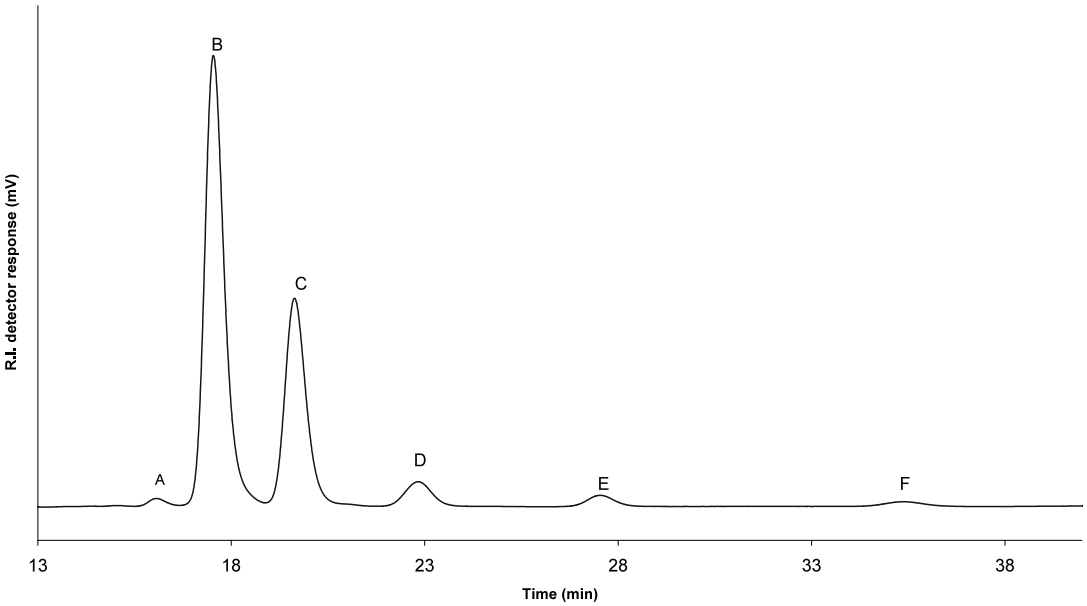


Fig. 2. HPLC chromatogram of the neutral oligosaccharides obtained upon dephosphorylation of the oligosaccharide phosphate fraction by alkaline phosphatase.

During the course of the repeated SEC, it became clear by HPLC analysis of the fractions that there was indeed an extra component present in the mixture eluting close to the trisaccharide ($t_R = 22.3$ min vs. $t_R = 22.9$ min for trisaccharide **6**, data not shown). When analysing the bulk sample, this peak was buried under the trisaccharide peak (Peak D) and thus not visible. It eluted towards the end of the tetrasaccharide fraction (Peak C, $t_R = 19.7$ min) and the beginning of the trisaccharide fraction (Peak D). Unfortunately, all attempts to isolate it directly failed and only mixed fractions were obtained. One such fraction which contained only **4** (95%) and the unidentified compound (5%) was examined by ESIMS (positive-ion mode). This showed a molecular-ion peak corresponding to tetrasaccharide (m/z 688.9 $[M + Na]^+$), but no peaks of the expected intensity were discernible for a trisaccharide.

A second fraction which contained the unidentified compound (6.5%), **4** (85%) and **2** (8.5%) was then peracetylated under standard conditions, and the minor component corresponding to the peracetate of the un-

Table 1
^{13}C and ^{1}H NMR chemical shift data for hexasaccharide **10** (major anomeric component)

Residue	C-1/H-1	C-2/H-2	C-3/H-3	C-4/H-4	C-5/H-5	C-6/H-6a,b
I	93.3	79.8 [b]	70.8	67.8	73.2	61.8
	5.38 (1.6) [a]	3.95	3.96	3.68	3.79	[c]
II	102.8	70.3	78.7 [b]	66.9	74.0	61.8
	5.03 (1.9)	4.23	3.95	3.73	[d]	[c]
III [e]	103.0	70.4	78.9 [b]	66.9	74.3	61.8
	5.12	4.24	4.01 (3.0, 9.4)	3.76	[d]	[c]
IV [e]	103.0	70.4	78.9 [b]	66.9	74.3	61.8
	5.12	4.24	4.02 (2.8, 9.4)	3.76	[d]	[c]
V	102.8	70.4	78.8 [b]	66.9	74.2	61.8
	5.13	4.24	3.97	3.78	[d]	[c]
VI	103.1	70.8	71.1	67.6	74.1	61.9
	5.14 (1.6)	4.07 (1.8, 3.4)	3.88	3.64 (9.8, 9.8)	3.79	[c]

[a] Selected coupling constants in Hz.
[b] Linkage carbons.
[c] Chemical shifts for H-6's: δ 3.72–3.78 and δ 3.84–3.92.
[d] Chemical shifts for H-5's: δ 3.78–3.85.
[e] Values may have to be interchanged.

Table 2
^{13}C and ^{1}H NMR chemical shift data for compound **11**

Residue	C-1/H-1	C-2/H-2	C-3/H-3	C-4/H-4	C-5/H-5	C-6/H-6a,b
I	76.2	80.4 [a]	72.8	65.3	74.2	62.28
	5.35	3.99 (2.6) [b]	5.03 (10.2)	5.19 (10.0)	3.71 (3.4, 3.8)	4.08, 4.08
II	99.2	70.8	74.0 [a]	67.4	71.0	62.35
	4.84 (2.3)	5.17 (3.4)	4.20 (9.8)	5.18 (10.3)	4.12 (6.7, 2.4)	4.00, 4.36 (12.3)
III	99.2	70.8	75.3 [a]	66.6	69.5 [c]	61.8
	5.04 (1.9)	4.99 (3.7)	3.96 (9.9)	5.34 (9.9)	3.86 (3.6, 2.9)	4.03, 4.17 (13.3)
IV	99.0	69.7	68.4	65.7	69.6 [c]	62.0
	4.92 (1.9)	5.01 (3.3)	5.16 (10.0)	5.25 (10.0)	3.90 (4.7, 2.4)	4.02, 4.25 (12.3)

Other ^{13}C NMR data: δ 20.6–21.0 (13 C, OCO*C*H$_3$), 23.4 (NHCO*C*H$_3$), 169.4–170.6 (14 C, C=O). Other ^{1}H NMR data: δ 1.95–2.18 (13s, COC*H*$_3$), 2.02 (s, NHCOC*H*$_3$), 7.23 (d, $J_{1,NH}$ 9.7 Hz, NH).

[a] Linkage carbons.

[b] Selected coupling constants in Hz.

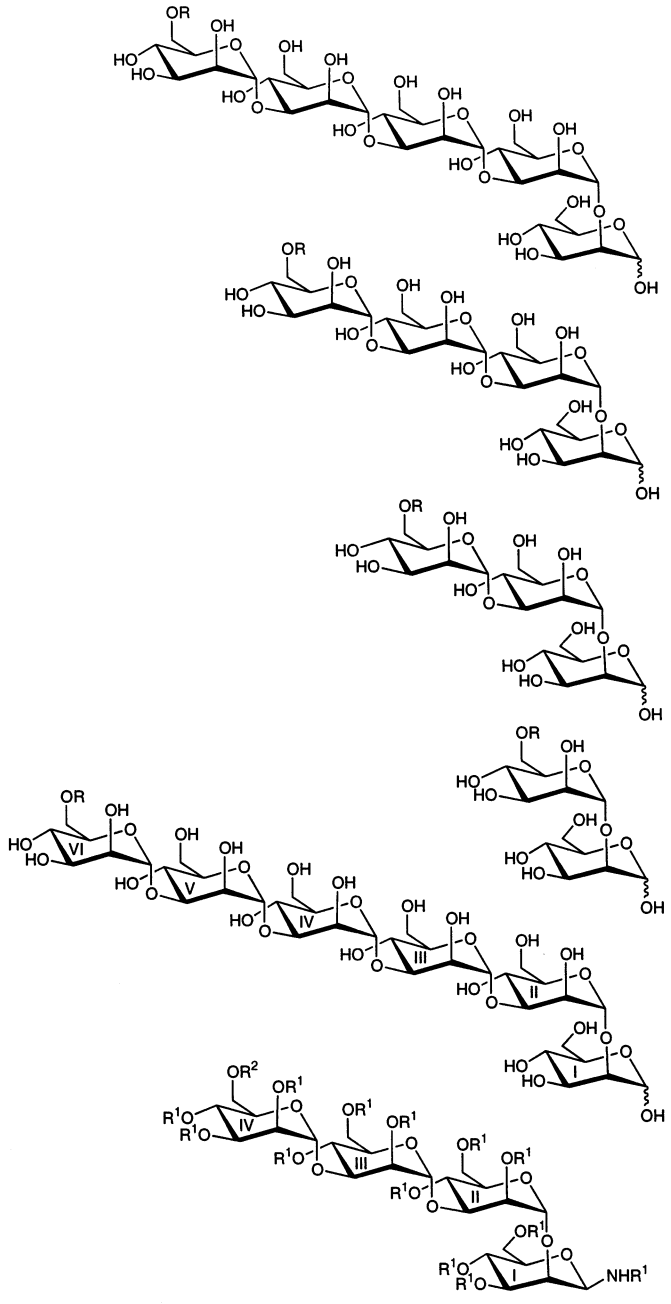
[c] Values may have to be interchanged.

known compound was isolated by repeated reversed-phase HPLC. The product, which was ~84% pure by HPLC, was identified as the tetramannosylamine peracetate **11** by ESIMS (m/z 1276.3 $[M + Na]^+$) and by a combination of 1D (^{1}H and ^{13}C) and 2D (gCOSY, gHSQC) NMR spectroscopy (NMR data are presented in Table 2). In the ^{1}H NMR spectrum of **11**, the presence of the amide group is confirmed by the doublet at δ 7.23 ($J_{1,NH}$ 9.7 Hz) that is coupled to the reducing end anomeric proton at δ 5.35 (d, $J_{1,2}$ 0, $J_{1,NH}$ 9.7 Hz). Further confirmation is provided by the ^{13}C NMR spectrum that shows that the corresponding anomeric carbon atom is shifted upfield to δ 76.2, and that there is a single *N*-acetyl signal at δ 23.4. The unidentified compound is thus the tetramannosylamine **12**, which suggests that one of either Peak D or Peak E in the electropherogram of the OPF (Fig. 1) corresponds to the tetramannosylamine phosphate **13**, whilst the other corresponds to trisaccharide phosphate **5**. There is no evidence to indicate the presence of other glycosylamines in the OPF, and its origin and possible role in the intact phosphomannan polysaccharide is a matter for speculation. Its presence may have implications for the biosynthesis and release of the phosphomannan from the yeast cells.

In conclusion, the composition of the OPF from *P.* (*Hansenula*) *holstii* NRRL Y-2448 phosphomannan has been determined by CE and HPLC, and the hexasaccharide component has been isolated and characterised by NMR spectroscopy. In addition, the presence of a tetramannosylamine component has been detected, and its structure has been characterised by NMR spectroscopy of its peracetate derivative. In so doing, further light has been shed on the structure of the phosphomannan, and a CE method has been developed that is suitable for quality control of the OPF for use in the manufacture of PI-88. It has been demonstrated that CE with indirect UV detection is an ideal method for the analysis of closely related phosphorylated oligosaccharides as it allows rapid separations with high resolution and minimal sample preparation.

3. Experimental

General.—Mass spectra were obtained by electrospray-ionisation (ESIMS) on a Fisons VG Quattro II mass spectrometer. Analytical HPLC was performed on a Waters Alliance 2690 Separations Module using a Rezex RNO-Oligosaccharide 12-μm (200 × 10 mm) column (mobile phase: water, flow rate: 0.3 mL/min, column temperature: 80 °C). Detection was with a Waters 2410 refractive index detector. Reversed-phase HPLC was performed using a Waters Nova-Pak C_{18} 4-μm (3.9 × 150 mm) column with detection by UV absorbance at 230 nm (Waters 2487 UV detector). 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. 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 a Helicon RO4 cartridge with Nanomax 50 membrane (membrane area 0.37 m^2). All reagents were analytical grade and were used without further purification. All water was purified in house to USP purified water standard. DEAE-Spherilose, Bio-Gel P-2, alkaline phosphatase and mannose 6-phosphate were from ISCO, BioRad, ICN and Sigma, respectively. The oligosaccharide phosphate fraction (OPF) from *P.* (*Hansenula*) *holstii*



1 R = PO_3^{2-}
2 R = H

3 R = PO_3^{2-}
4 R = H

5 R = PO_3^{2-}
6 R = H

7 R = PO_3^{2-}
8 R = H

9 R = PO_3^{2-}
10 R = H

11 $R^1 = R^2 = Ac$
12 $R^1 = R^2 = H$
13 $R^1 = H$ $R^2 = PO_3^{2-}$

NRRL Y-2448 phosphomannan was prepared as previously described.[8]

Capillary electrophoresis.—Capillary electrophoresis was performed on a Beckman P/ACE 5000 System equipped with a P/ACE UV Absorbance Detector. Beckman eCAP™ fused silica capillaries (50 μm i.d., 375 μm o.d., length 107 cm, 100 cm to detector) were used in these analyses. The background electrolyte (BGE) was 6 mM potassium sorbate, pH 10.3 (adjusted with KOH), capillary temperature was 20 °C and operating voltage 18 kV. Samples were prepared by dissolution in water at a concentration of 1 mg/mL and were injected by pressure injection for 10 s. Detection was by indirect UV absorbance at 214 nm. The capillary was rinsed between each run for 10 min with BGE solution. BGE solutions were replaced after approximately 12 analyses.

NMR spectroscopy.—One-dimensional (1D) ^1H and ^{13}C NMR and two-dimensional (2D) gCOSY and gHSQC experiments on compounds **10** and **11** were performed on a Varian Unity 400 spectrometer with standard Varian software at 25 °C. Spectra were referenced relative to internal acetone (δ 2.225 for ^1H and δ 31.0 for ^{13}C) for solutions in D_2O (**10**), or to the solvent peaks (δ 7.23 for ^1H and δ 77.0 for ^{13}C) for solutions in $CDCl_3$ (**11**). 1D ^1H and ^{13}C NMR and 2D DQF

COSY, NOESY, HMQC and HMBC experiments on the OPF were performed on a JEOL 400 MHz spectrometer with standard JEOL software at 30 °C.

Methanolysis of oligosaccharides.—Oligosaccharide samples were thoroughly dried in vacuo over P_2O_5 and dissolved in 0.5 mL of dry methanolic 1 M HCl using screw-capped tubes with Teflon-lined septa in the screw cap. Nitrogen gas was bubbled through the solution for 15 s, and then the tubes were capped. After methanolysis for 24 h at 80 °C, the acidic solution was neutralised by the addition of 0.15 mL of pyridine, and amino sugars were N-reacetylated by the addition of 0.1 mL of Ac_2O. The sample solution was evaporated with nitrogen gas flow at 35 °C. The residue was dried for 16 h in vacuo over P_2O_5. Finally, the sample was trimethylsilylated with 30 μL of 2:1 *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA)–pyridine at 80 °C for 1 h. Gas chromatography with flame-ionisation detection was carried out on a Hitachi G-3000 gas chromatograph equipped with DB-1 fused silica capillary column (375 μm i.d. × 25 m). GC–MS analyses were performed on a Hewlett–Packard gas chromatograph model 5890 series II with a DB-1 fused silica capillary column (375 μm i.d. × 25 m), followed by detection with a mass-selective detector model 5971 equipped with a Chemistation data system. All analyses were performed in the electron-impact ionisation mode, and an ionising voltage of 70 eV was used. The temperatures of the injection port and the detector were set at 280 °C. The column temperature was programmed from 120 to 270 °C at the rate of 20 °C/min and then held isothermally at 270 °C.

Methylation analysis–acetylation and GC–MS analysis.—The sample was permethylated by the Hakomori method,[25] and permethylation was carried out repeatedly to obtain permethylated oligosaccharide. The product was subjected to acetolysis in 80% AcOH containing 1 M HCl at 80 °C for 24 h, and the resultant partially O-methylated monosaccharides were reduced by 5% $NaBH_4$ in 10 mM NaOH. After removal of borate by evaporation as methylborate, partially O-methylated alditols were acetylated.[26] The partially methylated alditol acetates were analysed by GC–MS under the same conditions as described above.

Ion-chromatography.—A sample of the OPF was hydrolysed with 2.5 M trifluoroacetic acid at 100 °C for 6 h under nitrogen. After centrifugation at 1800*g* for 10 min, the sample was dried at 50 °C under a stream of nitrogen and dissolved in water. Inorganic phosphate was analysed by ion-chromatography using a TSK gel IC-anion-PW (4.6 mm i.d. × 50 mm) suppressor column and a Dowex 50W-X (H +) (5.0 mm i.d. × 200 mm) column. The temperature of the separation was controlled at 30 °C, and the mobile phase was 1.42 mM $NaHCO_3$ and 1.5 mM Na_2CO_3 at a flow rate of 1.0 mL/min. Detection was by suppressed conductivity using a Tosoh model CM-8 (Tosoh Co., Tokyo).

Dephosphorylation of the OPF with alkaline phosphatase.—A sample of the OPF (100 mg) was dissolved in buffer (1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 10 mM diethanolamine, 1 mL), pH 9.5, and treated with alkaline phosphatase (520 units, 0.4 mg) at 26 °C for 24 h, by which time TLC (7:3 EtOH–1 M ammonium acetate) indicated complete consumption of the starting material. The mixture was then passed through a column of DEAE-Spherilose (1.3 × 3.8 cm) equilibrated with 0.01 M NH_4HCO_3. The fractions containing product were then combined, desalted by passage through a column of Sephadex G-25M (Pharmacia PD-10) and analysed by HPLC.

Isolation of hexasaccharide **10**.—A sample of the unbound effluent from the ion-exchange chromatography step from a large-scale hydrolysis of phosphomannan[8] (50 L) was concentrated by reverse osmosis and then lyophilised. The residue was subjected to SEC 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 HPLC. The hexasaccharide-enriched fractions were combined, concentrated and subjected to repeated SEC on columns of Bio-Gel P-2 (firstly 5 × 100 cm and then 2.5 × 50 cm) using water as eluent. The hexasaccharide **10** was isolated as a white, amorphous solid (13 mg) after lyophilisation. The purity was ~80% by HPLC ($t_R = 16.2$ min). ESIMS: m/z 1012.9 $[M + Na]^+$. ^1H and ^{13}C NMR data are presented in Table 1. Also isolated was a fraction containing compounds **2** (8.5%, $t_R = 17.6$ min), **4** (85%, $t_R = 19.7$ min) and **12** (6.5%, $t_R = 22.3$ min).

Isolation of peracetate **11**.—The above fraction containing compounds **2** (8.5%), **4** (85%) and **12** (6.5%) was lyophilised and then acetylated by treatment with excess Ac_2O–pyridine (25 °C, 24 h). Workup (CH_2Cl_2) gave a residue (846 mg) that was subjected to repeated reversed-phase HPLC employing a linear gradient of 30–50% MeCN–water over 35 min (flow rate: 2 mL/min). This resulted in the isolation of the peracetate **11** as a white, amorphous solid (11 mg, $t_R = 11.6$ min). The purity was ~84.5% by reversed-phase HPLC. ESIMS: m/z 1276.3 $[M + Na]^+$. ^1H and ^{13}C NMR data are presented in Table 2.

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