ELSEVIER

Carbohydrate Research 309 (1998) 77–87

CARBOHYDRATE RESEARCH

The extracellular polysaccharide of *Pichia (Hansenula) holstii* NRRL Y-2448: the phosphorylated side chains

Lesley A.S. Parolis[a], Haralambos Parolis[a,*], Lennart Kenne[b], Morten Meldal[c], Klaus Bock[c]

[a] *School of Pharmaceutical Sciences, Rhodes University, Grahamstown 6140, South Africa*
[b] *Department of Chemistry, Swedish University of Agricultural Sciences, S-750 07 Uppsala, Sweden*
[c] *Department of Chemistry, Carlsberg Laboratory, Gamle Carlsbergvej 10, 2500 Valby, Denmark*

Received 6 January 1998; accepted 4 April 1998

Abstract

The exopolysaccharide produced by *Pichia (Hansenula) holstii* NRRL Y-2448 is composed of a phosphomannan core to which oligosaccharide diester phosphate side chains are appended. The oligosaccharides of the side chains were released as oligosaccharide phosphates and neutral oligosaccharides by mild hydrolysis with aqueous acetic acid and aqueous hydrogen fluoride, respectively. The liberated oligosaccharide phosphates were studied by NMR spectroscopy and by electrospray and fast atom bombardment mass spectrometry. The structures of the neutral oligosaccharides were determined by 1D and 2D NMR spectroscopic experiments. Further insight into the length of the side chains was obtained from a matrix assisted laser desorption ionisation–time of flight mass spectrometric study of high and low molecular weight fragments obtained from partial acid hydrolysis of the native polysaccharide.

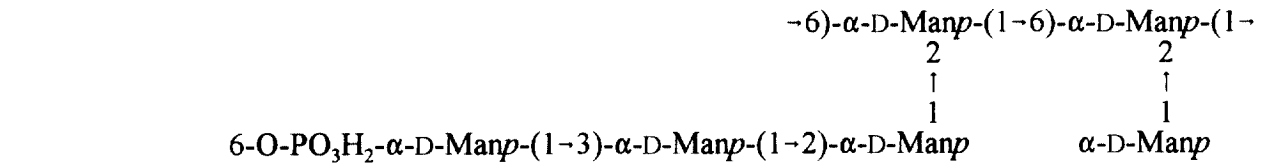
Keywords: *Pichia holstii*; Exopolysaccharide; Phosphomannan; Oligosaccharide side chains; NMR spectroscopy

1. Introduction

The yeast *Pichia (Hansenula) holstii* NRRL Y-2448 produces a viscous, highly branched extracellular phosphomannan, **PS**, when grown in a culture medium containing an excess of orthophosphate [1]. Mild hydrolysis of the **PS** produces a high molecular weight phosphomannan core, **PC** [2–4], and a low molecular weight oligosaccharide phosphate fraction. Both the **PC** and the oligosaccharide phosphate fraction have become valuable tools in studies on mannose-6-phosphate receptors. The structure of the major repeating unit of the **PC** has recently been shown [5] to be the branched hexasaccharide phosphate.

* Corresponding author. Fax: 00 2746 1311205.

0008-6215/98/$19.00
PII S0008-6215(98)00101-3



The low molecular weight fraction, which accounts for approximately 90% of the **PS**, is derived from the phosphorylated side chain oligosaccharides attached to the **PC**. The major oligosaccharide present in this fraction [3,4], and thus the main repeating unit found in the oligosaccharide side chains, is the pentasaccharide phosphate **1**.

a **c** **e** **d** **b**

-6-O-PO_3H_2-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→

1

The length of the oligosaccharide diesterphosphate side chains of the **PS** and the nature of the units terminating these side chains have not been established, although weak binding of the **PS** to mannose-6-phosphate receptors and the presence of a signal for diester phosphate only in the ^{31}P spectrum of the **PS** [5] indicate that units capping the side chains are devoid of monoester phosphate. Furthermore, the structural significance of minor amounts of tetrasaccharide and hexasaccharide phosphate, reported to be present in low molecular weight preparations from partial hydrolysates [6], remains unknown.

The extensive use of low molecular weight oligosaccharide preparations, assumed to consist of oligosaccharide **1**, in research on mannose-6-phosphate receptors [7–13] prompted us to seek answers to these questions. In the present paper we report on further structural studies on the oligosaccharide side chains of the **PS**.

2. Experimental

General methods.—Gel permeation chromatography (GPC) was performed on calibrated columns of Bio-Gel P-2 and P-4 (1.6×94 cm) using 0.1 M NH_4HCO_3 (pH 8.3) as eluent. Compounds were detected by refractive index.

NMR spectroscopy.—Samples were deuterium exchanged by lyophilising solutions in D_2O, and then dissolved in 99.99% D_2O (0.45 mL). Spectra were referenced relative to acetone (δ 2.230 for ^1H and 31.07 for ^{13}C) and to 85% H_3PO_4 (δ 0.0 for ^{31}P). Spectra were recorded at either 27 or 30 °C on either Bruker AM-500, AMX-400 or AMX-600 spectrometers. The 2D NMR pulse programs used were (a) phase sensitive COSY [14] using TPPI with double quantum filter and presaturation during relaxation delay; (b) NOESY [15] using TPPI with presaturation during relaxation delay and a mixing delay of 800 ms; (c) HOHAHA [16] using TPPI with presaturation during relaxation delay and employing the MLEV-17 sequence for mixing, with a mixing delay of 89 ms; (d) HMQC [17] and HMQC-TOCSY [18] using TPPI with presaturation during relaxation delay and GARP decoupling during acquisition; (e) HMBC [19] with no decoupling during acquisition and a mixing delay of 60 ms.

Preparation of oligosaccharide phosphates.—Small-scale hydrolysis of the **PS** (17 mg) in 1% HOAc at 100 °C for 8 h, as described previously [5], followed by GPC of the products on Bio-Gel P-4 gave the elution profile shown in Fig. 1. The yields obtained were **A** 11.7, **B** 3.0, **C** 0.7, and **D** 1.6 mg. The oligosaccharide phosphates previously obtained from a large-scale hydrolysis of the **PS** [5] were also used in this study.

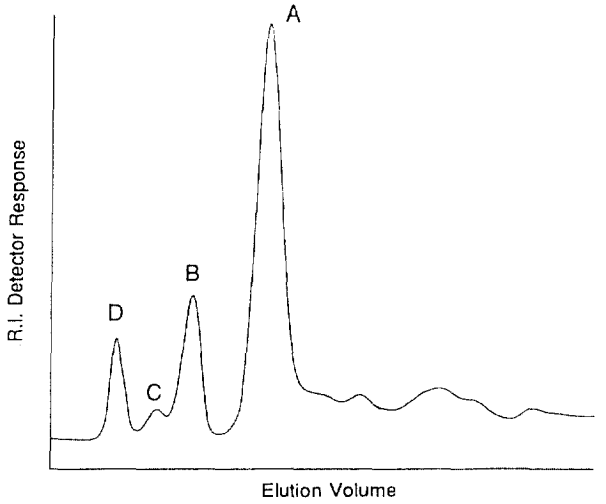


Fig. 1. GPC of the hydrolysate of the **PS** on Bio-Gel P-4.

*Aqueous HF hydrolysis of the **PS**.*—The **PS** (200 mg) was mixed with aq 48% HF at 0 °C and was maintained at this temperature until the **PS** had dissolved whereafter the mixture was placed in a cold room at 5 °C. After 96 h the hydrolysate was poured into a slurry of $CaCO_3$ and solid CO_2 in CH_2Cl_2. The mixture was extracted with H_2O (4×) to remove the carbohydrate material (104 mg). Separation of this material on Bio-Gel P-2 gave the profile shown in Fig. 2. The fractions were collected and rechromatographed on Bio-Gel P-2 to afford **H** (1 mg), **I** (2.3 mg), **J** (6 mg), **K** (59 mg), **L** (10 mg), and **M** (9 mg). Fractions were examined by ^1H and ^{13}C NMR spectroscopy.

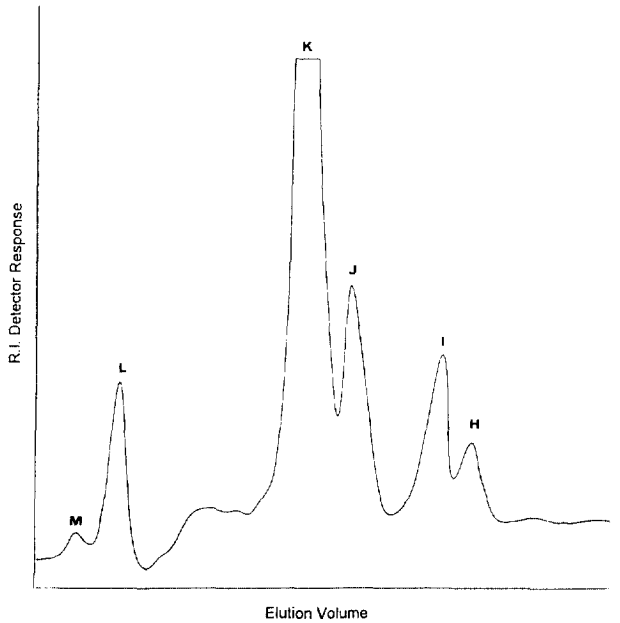


Fig. 2. GPC of the HF hydrolysate of the **PS** on Bio-Gel P-2.

*Preparation of samples of the **PS** for MALDI-TOF-MS analysis.*—The **PS** (30 mg) in aq 1% HOAc was heated at 100 °C for 2 h after which the sample was cooled, the solvent was evaporated off, and the mixture was separated into a low molecular weight fraction **O** (containing oligosaccharides) and a high molecular weight fraction **P** (containing mostly polymeric material). A sample of **P** was retained for MS analysis and the remainder was hydrolysed as before with aq 1% HOAc and again separated by GPC into a high and low molecular weight fraction. The process was repeated a further two times yielding in all four high and four low molecular weight fractions which were analysed by MALDI-TOF-MS.

Mass spectrometry.—MALDI-TOF spectra were obtained on a LINEAR LDI-1700XS spectrometer in matrices of 2,4,6-trihydroxyacetophenone. ESMS spectra were acquired in the negative mode on a VG Quattro triple quadrupole mass spectrometer with aq 50% CH_3CN as the mobile phase at a flow rate of 8 μL/min. Samples were prepared at concentrations of ~20 pmol/μL and 10 μL of the sample were injected. FABMS spectra were recorded on either acetylated or trideuteroacetylated samples with a VG Analytical High Field ZAB-1F mass spectrometer fitted with an M-Scan FAB gun, as previously described [20].

3. Results and discussion

Hydrolysis of the **PS** with aq 1% HOAc at 100 °C as previously described [5], followed by GPC separation of the hydrolysate on Bio-Gel P-4 (Fig. 1), afforded three oligosaccharide phosphate fractions, **A**, **B**, and **C**, in addition to the **PC** fraction, **D**. Fraction **A** was rechromatographed on Bio-Gel P-2 and was then examined in D_2O at 30 °C by NMR spectroscopy (pD 6.05). The ^{31}P NMR spectrum of **A** showed a single resonance at δ 1.49 for a monophosphate ester. The ^1H and ^{13}C NMR spectra (Figs. 3 and 4, respectively) clearly identify the main component of **A** as a pentasaccharide. The ^1H and ^{13}C NMR assignments for the component residues **a**, **b**, **c**, **d**, and **e** (Table 1) of the pentasaccharide were determined from COSY-DQF [14], HOHAHA [16], and HMQC [17] 2D NMR experiments. The sequence of the residues in the pentasaccharide was established from a 2D NOESY [15] experiment as **a**–(1-3)–**c**–(1-3)–**e**–(1-

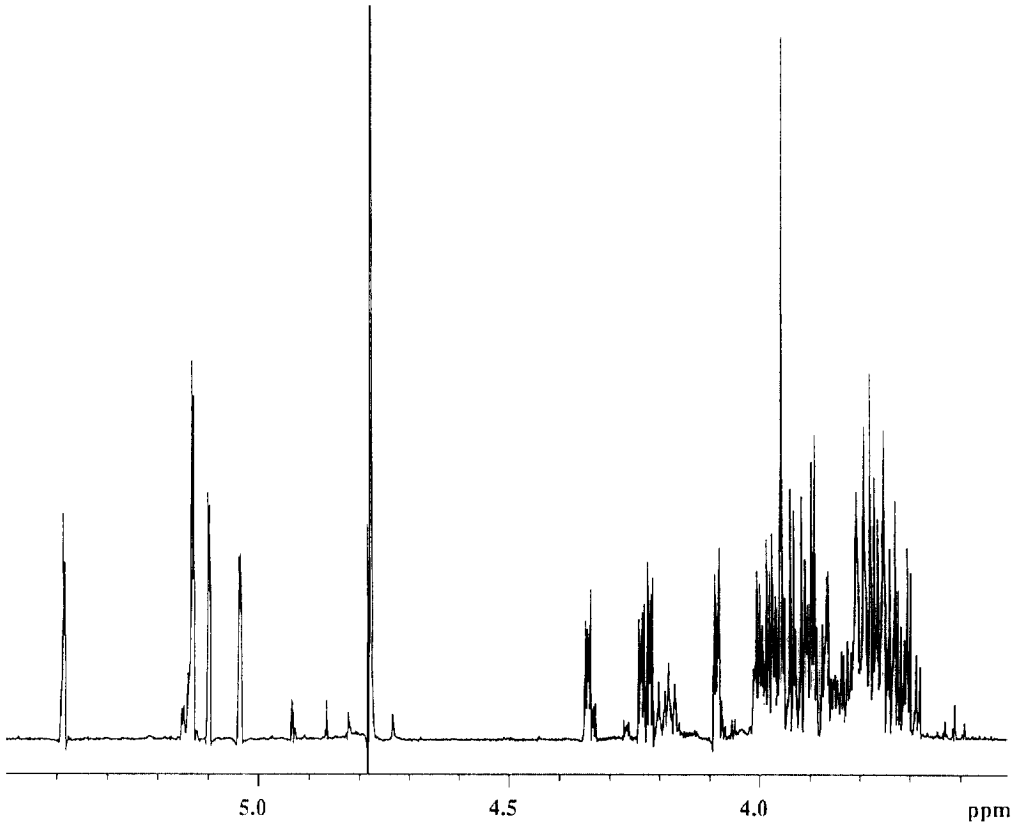


Fig. 3. ^{1}H NMR spectrum of fraction **A**.

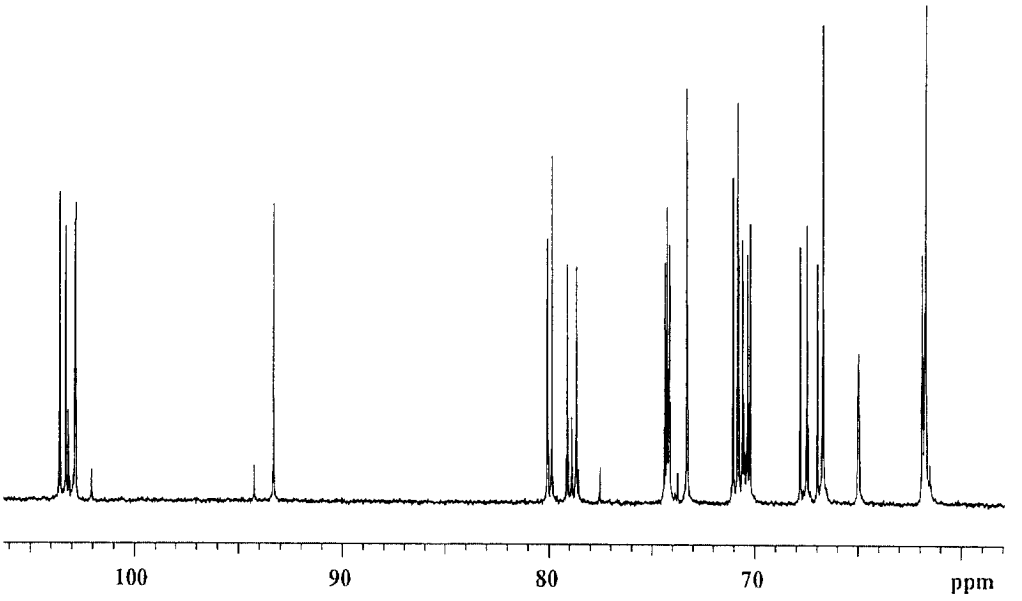


Fig. 4. ^{13}C NMR spectrum of fraction **A**.

3)-**d**-(1-2)-**b**. The location of the monophosphate ester group was established at C-6 of the non-reducing terminal residue **a**. This assignment follows from the downfield location of the signal assigned to C-6 of **a**, the upfield location of C-5 of **a**, the two- and three-bond ^{13}C–^{31}P coupling constants for C-6 and C-5, respectively, and the deshielding of the H-6a and H-6b resonances of **a**. The above data establish the main component of **A** as the pentasaccharide phosphate **1**.

Table 1
^1H and ^{13}C chemical shift data[a] for *P. holstii* pentasaccharide phosphate **A** (major anomeric component)

Residue		H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6a,b/C-6
→2)-α-Man~OH	**b**	5.372 (1.7)[b] 93.3	3.946 **79.9**	3.931 70.7	3.691 67.8	3.793 73.2	3.890, 3.762 61.8
→2)-β-Man~OH	**b**	4.905 94.3	4.070 **78.6**	3.751 73.2	3.598 67.4	3.389 77.5	3.901, 3.711 61.7
α-Man-6-PO_4	**a**	5.082 (1.7) 103.6	4.069 (3.4) 70.8	3.890 (10) 71.1	3.684 67.5	3.942 73.3	4.168, 3.966 65.0
→3)-α-Man	**c**	5.115 (1.8) 103.3	4.329 (3.3) 70.2	3.979 (10) **80.1**	3.714 66.7	3.834 74.2	3.903, 3.740 61.9
→3)-α-Man	**e**	5.114 (1.8) 102.8	4.203 (3.2) 70.6	3.984 (9) **79.1**	3.781 66.7	3.769 74.1	3.861, 3.746 61.8
→3)-α-Man	**d**	5.021 (1.8) 102.8	4.221 (3.3) 70.3	3.948 (9) **78.7**	3.739 67.0	3.792 74.4	3.890, 3.762 61.8

[a] Chemical shifts in ppm relative to acetone at δ 2.230 for ^1H and 31.07 for ^{13}C, at 27 °C.
[b] Coupling constants in Hz.
Linkage carbons are in bold.

Table 2
Negative ion ESMS of fractions from GPC of a partial hydrolysate of **PS**

Fraction	*m/z*	*m/z*	*m/z*	*m/z*	*m/z*
A	1069.2 (*P*-Man_6)	907.2 (*P*-Man_5)	745.2 (*P*-Man_4)	583.4 (*P*-Man_3)	421.2 (*P*-Man_2)
B	979.4 (P_2-Man_{11})	898.4 (P_2-Man_{10})	817.6 (P_2-Man_9)	736.4 (P_2-Man_8)	655.5 (P_2-Man_7)
C_1	949.5 (P_3-Man_{16})	895.9 (P_3-Man_{15}) 893.5[a] (P_4-Man_{20})	841.8 (P_3-Man_{14}) 853.7 (P_4-Man_{19})	787.5 (P_3-Man_{13}) 813.5 (P_4-Man_{18})	733.2 (P_3-Man_{12}) 772.4 (P_4-Man_{17})
C_2		893.8 (P_4-Man_{20}) 892.6[a] (P_5-Man_{25}) 892.0[a] (P_6-Man_{30}) 891.6[a] (P_7-Man_{35})	853.7 (P_4-Man_{19}) 860.9 (P_5-Man_{24}) 865.9 (P_6-Man_{29}) 869.2 (P_7-Man_{34})	812.9 (P_4-Man_{18}) 828.6 (P_5-Man_{23}) 838.9 (P_6-Man_{28}) 846.2 (P_7-Man_{33})	796.2 (P_5-Man_{22}) ~811 (P_6-Man_{27})

[a] Anticipated ions not unambiguously identified.

All of the NMR spectra of **A** showed several minor resonances in addition to those attributed to **1** indicating additional minor components in **A**. In order to establish the nature of the minor oligosaccharide components, **A** was investigated by electrospray mass spectrometry (ESMS). Table 2 lists the $[M-H]^-$ ions observed in the negative ion spectrum of **A**. In addition to the anticipated major $[M-H]^-$ ion at *m/z* 907.2 for the pentasaccharide phosphate **1**, peaks for the $[M-H]^-$ ions for a tetrasaccharide phosphate (*m/z* 745.2), a trisaccharide phosphate (*m/z* 583.4), a disaccharide phosphate (*m/z* 421.2), and a hexasaccharide phosphate (*m/z* 1069.2) were observed. The ratio of the intensities of the ions at *m/z* 745.2 and 907.2 suggested that the tetrasaccharide phosphate constituted 20–25% of **A**. The other three oligosaccharide phosphates appeared to be present in very small amounts. The proportion of the tetrasaccharide phosphate was greater than was anticipated from the earlier report mentioned above [6]. We were therefore concerned that, although ES is a soft ionisation technique that would not be expected to cause glycosidic cleavage, the tetrasaccharide phosphate and the previously unreported tri- and disaccharide phosphates may have resulted from such cleavage of the pentasaccharide phosphate. The ESMS experiments were therefore repeated at lower ionisation energies and the ratio of the ions at *m/z* 745.2 and 907.2 was monitored. As there was no significant variation in the ratio of these ions in all of the experiments performed it was concluded that the tetrasaccharide phosphate was a genuine component of **A** and had not resulted from the loss of hexose from the pentasaccharide phosphate. The most significant change in the ESMS spectra was the dramatic increase in the $[M-2H]^{2-}$ ion at *m/z* 453.4 as the ionisation energy was decreased.

Indeed it was the base peak in the experiment with the lowest ionisation energy. Further proof that the tetra- and lower oligosaccharide phosphates were genuine components of **A** was obtained from FABMS experiments on trideuteroacetylated **A** [20]. The major molecular ions in the positive ion spectrum occurred at *m/z* 1691 and 1394. These represent the $[M+NH_4]^+$ ions for the pentasaccharide and tetrasaccharide phosphate, respectively, in which the phosphate groups are monodeuteroacetylated. The respective $[M+Na]^+$ ions occurred at *m/z* 1696 and 1399, respectively. Smaller $[M+NH_4]^+$ and $[M+Na]^-$ ions occurred at *m/z* 1097 and 1102, respectively, confirming the presence of trisaccharide phosphate in **A**. No molecular ions were observed for a disaccharide phosphate in the positive ion FAB spectra. Very small $[M+Na]^+$ ions were observed at *m/z* 1993, 2290, 2587, 2884, and 3181 indicating the presence of hexa-, hepta- octa-, nona-, and decasaccharide phosphates in fraction **A**. The negative ion FAB spectra showed major pairs of $[M-H]^-$ ions in which the phosphate groups were monodeuteroacetylated and nondeuteroacetylated at *m/z* 1673 and 1628, and 1376 and 1331 for a pentasaccharide phosphate and a tetrasaccharide phosphate, respectively. In addition similar pairs of $[M-H]^-$ ions, but of very low intensity, were observed for di-, tri-, hexa-, hepta-, octa-, nona- and decasaccharide phosphates. Both the positive and negative ion FAB spectra suggested a ratio of 1:4 for the tetrasaccharide phosphate and pentasaccharide phosphate in **A**.

The oligosaccharide fraction **B** was rechromatographed on Bio-Gel P-4 and was then examined by NMR spectroscopy in D_2O at 30 °C (pD 5.68). The proton decoupled ^{31}P spectrum of **B** showed signals at δ 1.52 and −1.29 for a mono- and diester phosphate, respectively. The ^1H NMR spectrum (Fig. 5) showed H-1 resonances at δ 5.682 (1 H, $J_{1,2}$ 1.3, $J_{H\text{-}P}$ 7.9 Hz), 5.385 (0.9 H, $J_{1,2}$ 1.5 Hz, α-anomer of reducing Man residue), 5.132 (2 H), 5.126 (1 H), 5.121 (1 H), 5.109 (1 H, $J_{1,2}$ 1.8 Hz), 5.103 (1 H, $J_{1,2}$ 1.8 Hz), 5.064 (1 H, $J_{1,2}$ 1.8 Hz), 5.037 (1 H, $J_{1,2}$ 1.8 Hz), and 4.930 (0.1 H, β-anomer of reducing Man residue). These results together with the NMR data for the pentasaccharide phosphate (*P*-Man_5) identify **B** as the decasaccharide diphosphate,

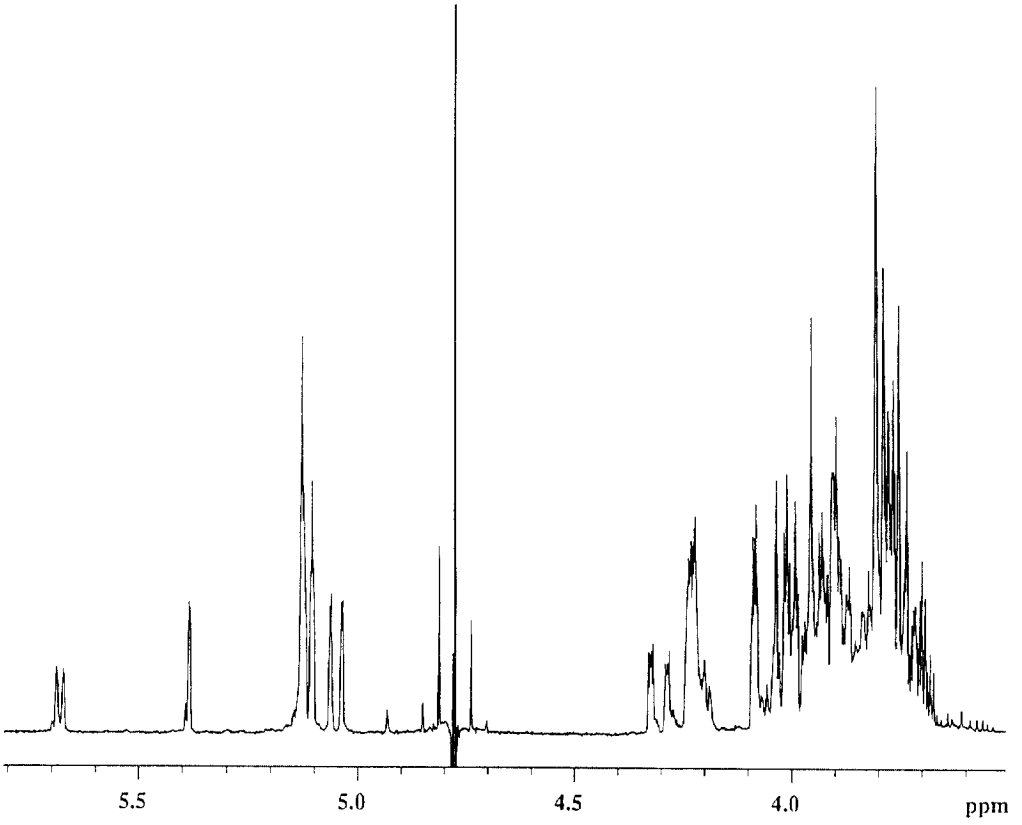


Fig. 5. ^1H NMR spectrum of fraction **B**.

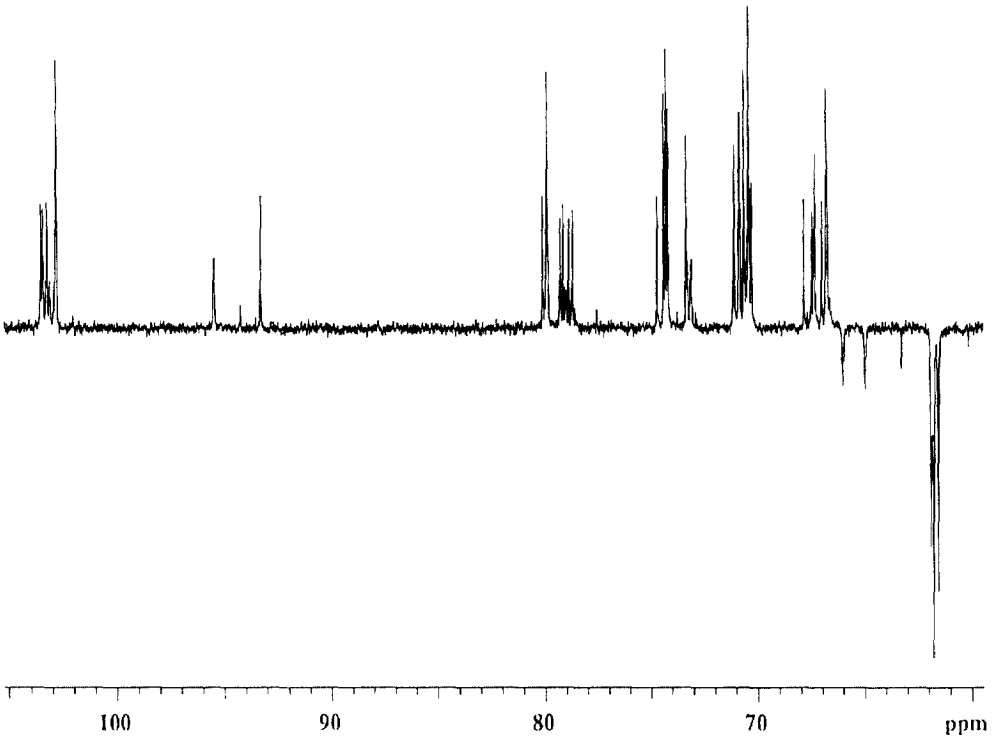


Fig. 6. ^{13}C DEPT spectrum of fraction **B**.

P-Man_5–*P*-Man_5, in which the reducing terminal Man of one *P*-Man_5 unit is linked through an α-glycosidic bond to the 6-phosphate group of another *P*-Man_5 unit. The ^{13}C DEPT spectrum of **B** (Fig. 6), which shows, inter alia, signals at 95.5 ppm ($^2J_{C\text{-}P}$ 5.3 Hz) for C-1 of the Man residue involved in the glycosyl phosphate linkage, 66.0 ($^2J_{C\text{-}P}$ 4.3 Hz) and 65.0 ppm ($^2J_{C\text{-}P}$ 4.3 Hz) for two phosphorylated C-6, 73.1 ($^3J_{C\text{-}P}$ 7.2 Hz) and 73.3 ppm ($^3J_{C\text{-}P}$ 7.6 Hz) for two C-5, 79.0 ppm ($^3J_{C\text{-}P}$ 8.1 Hz) for C-2 of the 2-substituted Man residue involved in the glycosylphosphate linkage, is consistent with the suggested decasaccharide diphosphate.

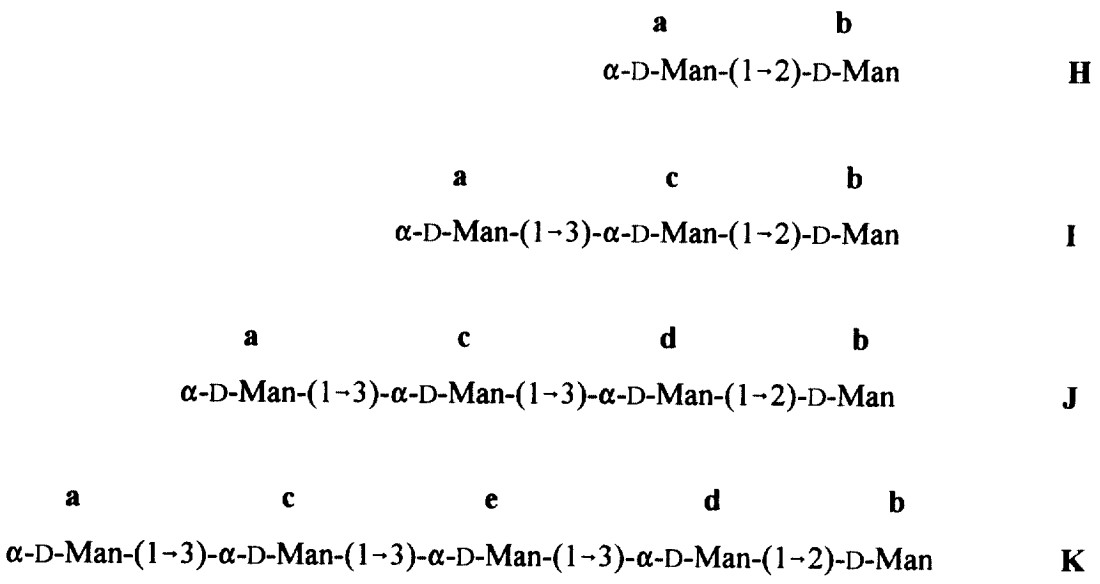
Examination of fraction **B** by ESMS showed the peaks listed in Table 2. The major peaks at *m/z* 898.4 and 817.6 represent the $[M-2H]^{2-}$ ions of the *P*-Man_5–*P*-Man_5 and *P*-Man_4–*P*-Man_5 (or *P*-Man_5–*P*-Man_4) oligosaccharides, respectively. These ions occurred in the approximate ratio of 1.5:1 which is consistent with the approximate 4:1 ratio of pentasaccharide phosphate to tetrasaccharide phosphate found in fraction **A**. The much smaller peaks at *m/z* 979.4, 736.4, and 655.5 represent oligosaccharides with the composition shown in parentheses in Table 2. The negative ion FAB spectrum of acetylated **B** confirmed the ESMS results showing prominent $[M-H]^-$ ions at *m/z* 3099.8 and 2811.5 for P_2-Man_{10} and P_2-Man_9, respectively, and very small peaks for P_2-Man_7 (*m/z* 2233.9), P_2-Man_8 (*m/z* 2523.5), and P_2-Man_{11} (*m/z* 3387.5). In addition minute $[M-H]^-$ ions were observed for P_2-Man_n where $n=12$–15. All the $[M-H]^-$ ions above were accompanied by ions which were 42 amu higher and which corresponded to monoacetylation of the monoester phosphate groups.

The oligosaccharide material in fraction **C** (Fig. 1) was collected in two parts designated $\mathbf{C_2}$, which contained the material collected from the leading edge of the peak to the middle of the peak, and $\mathbf{C_1}$, which contained the material collected from the middle to the trailing edge of the peak. ^1H NMR examination of $\mathbf{C_1}$ and $\mathbf{C_2}$ indicated that they consisted of higher multiples of the pentasaccharide phosphate. The spectrum of $\mathbf{C_1}$ showed a signal at δ 5.683 ($^3J_{H\text{-}P}$ 7.3 Hz) for H-1 of the Man involved in the glycosyl phosphate linkage, and at δ 5.387 (α) and 4.934 (β) for H-1 of the reducing Man. The integration of the signals was consistent with a pentadecasaccharide (P_3-Man_{15}). Examination of fraction $\mathbf{C_1}$ by ESMS identified the ions listed in Table 2. The major peaks at *m/z* 895.9 and 841.8 corresponded to the $[M-3H]^{3-}$ ions for the oligosaccharides P_3-Man_{15} and P_3-Man_{14},

respectively, and were present in an approximate ratio of 1.9:1. These corresponded to the oligosaccharides *P*-Man_5–*P*-Man_5–*P*-Man_5 and *P*-Man_4–*P*-Man_5–*P*-Man_5 (and/or the other two variations). The other $[M-3H]^{3-}$ ions (Table 2) found in $\mathbf{C_1}$ were present in much smaller amounts, as were the $[M-4H]^{4-}$ ions. It was not possible to identify an $[M-4H]^{4-}$ ion for the oligosaccharide P_4-Man_{20} (*m/z* 893.5) as such a peak would have been swamped by the major ion at *m/z* 895.9. The negative ion FAB spectrum of acetylated $\mathbf{C_1}$ confirmed the ESMS results showing major $[M-H]^-$ ions at *m/z* 4621.5 and 4333.1 for P_3-Man_{15} and P_3-Man_{14}, respectively. The spectrum also showed a small peak at *m/z* 6143 confirming the suspected presence of P_4-Man_{20} in $\mathbf{C_1}$.

The ^{1}H NMR spectrum of $\mathbf{C_2}$ showed signals as for $\mathbf{C_1}$ at δ 5.683 ($^3J_{H\text{-}P}$ 7.3 Hz) for H-1 of the Man involved in the glycosyl phosphate linkage, and at δ 5.387 (α) and 4.934 (β) for H-1 of the reducing Man. The ions observed in the ESMS spectrum of $\mathbf{C_2}$ are listed in Table 2 together with the composition of the oligosaccharides from which they arise. The major peak at *m/z* 893.9 is consistent with the $[M-4H]^{4-}$ ion for $(P\text{-}Man_5)_4$. The peak was approximately 6 amu in width at the base and covered the range 892.4–895.4 amu at half height. Thus the anticipated ions for $[M-5H]^{5-}$ at *m/z* 892.6 for P_5-Man_{25}, for $[M-6H]^{6-}$ at *m/z* 892.0 for P_6-Man_{30}, and for $[M-7H]^{7-}$ at *m/z* 891.6 for P_7-Man_{35}, could not be distinguished. Their presence was suggested by the appearance of prominent ions for P_5-Man_{23}, P_5-Man_{24}, P_6-Man_{28}, P_6-Man_{29}, P_7-Man_{33}, and P_7-Man_{34}. The negative ion FAB spectrum of acetylated $\mathbf{C_2}$ identified P_4-Man_{20} (*m/z* 6143) and P_4-Man_{19} (*m/z* 5855) as the most prominent oligosaccharides in the mixture and also proved conclusively the presence of P_5-Man_{25}. Oligosaccharides of higher molecular masses could not be detected by FABMS.

The MS data obtained on fractions **A**–**C** indicate quite clearly that the separation of the components of the partial hydrolysate by GPC was strongly influenced by the number of phosphate ester groups present in the oligosaccharides. It was thus not possible to separate the mixture of oligosaccharide phosphates present in fraction **A** by GPC for further structural studies due to the retarding influence of the phosphate group on the separation. The FABMS results revealed that all the oligosaccharides with a monoester phosphate group were eluted together irrespective of their molecular weights. In order, therefore, to separate and determine the structures of the tetra and other oligosaccharides detected by the MS studies, the **PS** was treated with aqueous 48% HF at 0 °C and the neutral oligosaccharides produced were separated by GPC on Bio-Gel P-2 (Fig. 2). The ^{1}H NMR spectra of the isolated oligosaccharides, designated **H**, **I**, **J**, and **K**, are shown in Fig. 7 while the NMR data are collected in Table 3. The structures of the oligosaccharides were established by 2D NMR spectroscopy (phase sensitive COSY, HOHAHA, HMQC, and NOESY and/or HMBC) as



The residues **a**–**e** are labelled in the same manner as for the pentasaccharide phosphate **1**.

^{1}H and ^{13}C NMR spectra of fraction **L** (Fig. 2) indicated it to be primarily a mixture of the decasaccharide diester phosphate α-D-Man-(1→3)-α-D-Man-(1→3)-α-D-Man-(1→3)-α-D-Man-(1→2)-α-D-Man-1-*O*-PO_2H-*O*-6-α-D-Man-(1→3)-α-D-Man-(1→3)-α-D-Man-(1→3)-α-D-Man-(1→2)-D-Man and the corresponding nonasaccharide diester phosphate. The ^{1}H NMR spectrum of **L** showed a signal at δ 5.677 (J_{H-P} 7.7 Hz) for H-1 of an α-mannosyl diester phosphate linkage, a H-1 signal (0.9 H) for a reducing α-mannopyranosyl residue at δ 5.382 and a H-1 signal (0.1 H) for a β-mannopyranosyl residue at δ 4.930. The ^{13}C NMR spectrum of **L** showed a C-1 signal at 95.5 ppm ($^2J_{C-P}$ 5.7 Hz), a signal for C-6 at 66.1 ppm ($^2J_{C-P}$ 3.8 Hz), and a signal for C-5 at 73.1 ppm ($^3J_{C-P}$ 8.1 Hz). The ^{1}H NMR spectrum of fraction **M** (Fig. 7) suggested that this contained the **PC** [5] with residual diester phosphate side chains.

In order to probe further the composition and length of the phosphorylated side chains attached to the **PC**, the **PS** was hydrolysed with aqueous 1% CH_3CO_2H at 100 °C for 2 h and the mixture obtained was separated on Bio-Gel P-4 into an oligosaccharide fraction (**O**), and a polysaccharide fraction (**P**), most of which eluted in the void volume. The two fractions were then examined by MALDI-TOF-MS. The higher molecular weight fraction **P** showed clusters of ions for *P*-Man_{4-6}, P_2-Man_{8-11}, P_3-Man_{13-16}, P_4-Man_{18-21}, P_5-Man_{23-26}, P_6-Man_{28-31}, P_7-Man_{33-36}, and probably for P_8-Man_{38-41}, P_9-Man_{43-46}, and P_{10}-Man_{48-51}. While the molecular masses of the latter two clusters of ions could not be determined accurately, ions with m/z ~8000 and 8800 were definitely present. The lower molecular weight fraction **O** showed prominent ions for *P*-Man_4 and *P*-Man_5 and ions of much lower intensity for *P*-Man_{2-3} and *P*-Man_{6-10}. Prominent ions were also observed for P_2-Man_9 and P_2-

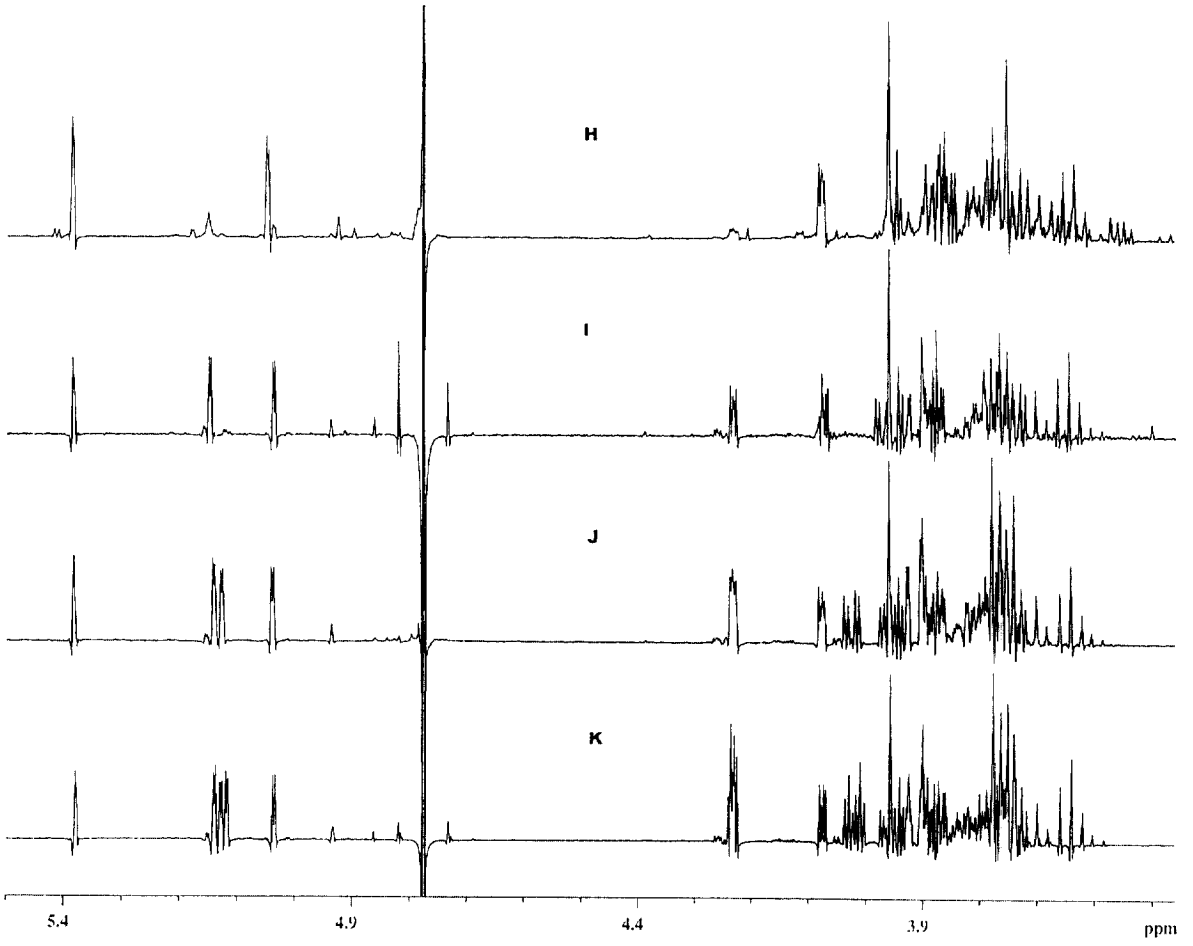


Fig. 7. ^{1}H NMR spectra of the oligosaccharides **H**–**K** obtained from HF hydrolysis of the **PS**.

Table 3
^{1}H and ^{13}C chemical shift data[a] for *P. holstii* derived oligosaccharides **H–K** (major anomeric component)

Residue		H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6a,b/C-6
Disaccharide **H**							
α-Man	**a**	5.047	4.076	3.856	3.645	3.754	
		103.0	70.8	71.1	67.8	74.0	61.8[b]
→2)-α-Man~OH	**b**	5.380	3.958	3.951	3.670	3.780	
		93.3	**79.9**	70.8	67.6	73.2	61.7[b]
Trisaccharide **I**							
α-Man	**a**	5.148	4.072	3.890	3.648	3.79	
		103.0	70.8	71.1	67.8	74.1	61.7[c]
→2)-α-Man~OH	**b**	5.379	3.963	3.951	3.702	3.79	
		93.3	**79.9**[f]	70.8	67.7	73.3	61.7[c]
→3)-α-Man	**c**	5.037	4.229	3.968	3.758	3.79	
		102.9	70.4	**78.7**	67.0	74.2	61.9
Tetrasaccharide **J**							
α-Man	**a**	5.141	4.076	3.890	3.645	3.795	
		103.1	70.8	71.1	67.7	74.2	61.9
→2)-α-Man~OH	**b**	5.378	3.960	3.949	3.703	3.81	
		93.3	**79.9**	70.8	67.8	73.3	61.8[d]
→3)-α-Man	**c**	5.127	4.230	4.021	3.763	3.82	
		102.9	70.5	**78.9**	67.0[e]	74.3	61.7[d]
→3)-α-Man	**d**	5.039	4.231	3.958	3.755	3.78	
		102.8	70.4	**78.8**	67.0[e]	74.1	61.7[d]
Pentasaccharide **K**							
α-Man	**a**	5.143	4.077	3.889	3.644	3.799	3.913, 3.751
		103.2	70.8	71.1	67.7	74.3	61.9
→2)-α-Man~OH	**b**	5.380	3.954	3.953	3.703	3.809	3.852
		93.4	**79.9**	70.8	67.8	73.3	61.7
→3)-α-Man	**c**	5.129	4.234	4.020	3.762		
		102.9	70.5	**78.9**	67.0	74.4	61.7
→3)-α-Man	**d**	5.039	4.233	3.964	3.753		3.716
		102.9	70.4	**78.8**	67.0	74.2	61.7
→3)-α-Man	**e**	5.121	4.237	4.026	3.760	3.780	
		103.1	70.5	**79.0**	67.0	74.4	61.7

[a] Chemical shifts in ppm relative to acetone at δ 2.230 for ^{1}H and 31.07 for ^{13}C, at 30 °C.
[b,c,d,e] Values may have to be interchanged.
[f] Linkage carbons are in bold.

Man_{10} and ions of much lower intensity for P_2-Man_8 and P_2-Man_{11}.

4. Conclusion

The accumulated MS data suggest that the majority of the chains attached to the core phosphomannan **PC** are composed of up to at least ten repeating pentasaccharide phosphate residues. In other chains pentasaccharide phosphate residues are replaced by a tetrasaccharide phosphate and to a much lesser extent by hexa-, tri-, and disaccharide phosphates. The penta- to tetrasaccharide phosphate ratio observed in fractions **A** and **B** (Fig. 1) suggests that some chains may contain more than one tetrasaccharide phosphate residue. The MS data unfortunately did not furnish definitive information on the nature of the residues capping the chains. Such residues are not expected to contain terminal 6-*O*-phosphate groups since the **PS** shows very weak binding to mannose-6-phosphate receptors. An indication of the nature of the units terminating the side chains was forthcoming from a comparison of the elution volumes of the neutral oligosaccharides **H–K** with the minor component eluted after fraction **A** (Fig. 1). This comparison indicated the presence of at least **H** and **K** in the aqueous acetic acid hydrolysate of the **PS**. As it is unlikely that the aqueous acetic acid caused hydrolysis of linkages other than glycosyl phosphate, the presence of **H** and **K** in the hydrolysate of the **PS** strongly suggests that these are chain capping units. The presence of oligosaccharide residues other than repeating pentasaccharide monophosphate in the side chains indicates that, under the stress conditions of growth in excess orthophosphate, the biosynthetic control of the assembly of the side chain is not stringent.

Acknowledgements

The authors thank the Foundation for Research Development (Pretoria) for a sabbatical grant and for continued financial support to H.P. We are grateful to Professors A. Dell and H. Morris and Mr S. Haslam (Imperial College, London) for the preparation of derivatives for FABMS and for the acquisition of the FAB mass spectra. The FABMS work was supported by the Wellcome Trust and the BBSRC.

References

[1] A. Jeannes, J.E. Pittsley, P.R. Watson, and R.J. Dimler, *Arch. Biochem. Biophys.*, 92 (1961) 433–450.
[2] M.E. Slodki, *Biochim. Biophys. Acta*, 57 (1962) 525–533.
[3] R.K. Bretthauer, G.J. Kaczorowski, and M.J. Wiese, *Biochemistry*, 12 (1973) 1251–1256.
[4] M.E. Slodki, in M. Yalpani (Ed.), *Industrial Polysaccharides: Genetic Engineering, Structure/Property Relations and Applications*, Elsevier, Amsterdam, 1987, pp 109–119.
[5] L.A.S. Parolis, J.Ø. Duus, H. Parolis, M. Meldal, and K. Bock, *Carbohydr. Res.*, 293 (1996) 101–117.
[6] M.E. Slodki, R.M. Ward, and J.A. Boundy, *Biochim. Biophys. Acta*, 304 (1973) 449–456.
[7] R.J. Youle, G.J. Murray, and D.M. Neville, Jr., *Proc. Natl. Acad. Sci. USA*, 76 (1979) 5559–5562.
[8] G.J. Murray and D.M. Neville, Jr., *J. Biol. Chem.*, 255 (1980) 11942–11948.
[9] T. Yoshida and Y.C. Lee, *Carbohydr. Res.*, 251 (1994) 175–186.
[10] A.M. Leichtner and M. Krieger, *J. Cell Sci.*, 68 (1984) 183–194.
[11] S.A. Weston and C.R. Parish, *J. Immunol.*, 146 (1991) 4180–4186.
[12] R.W. Loveless, G. Floyd-O'Sullivan, J.G. Raynes, C.-T. Yuen, and T. Feizi, *EMBO J.*, 11 (1992) 813–819.
[13] S.A. Weston and C.R. Parish, *Eur. J. Immunol.*, 22 (1992) 1975–1981.
[14] U. Piantini, O.W. Sørensen, and R.R. Ernst, *J. Am. Chem. Soc.*, 104 (1982) 6800–6801.
[15] R. Baumann, G. Wider, R.R. Ernst, and K. Wüthrich, *J. Magn. Reson.*, 44 (1981) 402–406.
[16] A. Bax and D.G. Davis, *J. Magn. Reson.*, 65 (1985) 355–360.
[17] A. Bax, R.H. Griffey, and B.L. Hawkins, *J. Magn. Reson.*, 55 (1983) 301–315.
[18] L. Lerner and A. Bax, *J. Magn. Reson.*, 69 (1986) 365–380.
[19] A. Bax and M.F. Summers, *J. Am. Chem. Soc.*, 108 (1986) 2093–2094.
[20] A. Dell and P.R. Tiller, *Biochem. Biophys. Res. Commun.*, 135 (1986) 1126–1134.