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Chemical and spectroscopic characterisation of the phosphatidylinositol manno-oligosaccharides from *Mycobacterium bovis* AN5 and WAg201 and *Mycobacterium smegmatis* mc^2 155

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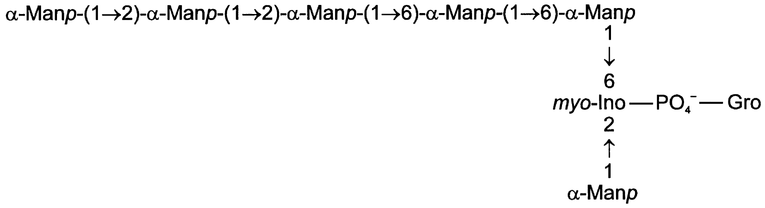
Abstract

Two classes of phosphatidylinositol manno-oligosaccharides (PIMs) were isolated from each of *Mycobacterium bovis* AN5 and WAg201 and *Mycobacterium smegmatis* mc^2 155. The deacylated PIMs (dPIMs), were identified as hexasaccharide (dPIM-6) and disaccharide (dPIM-2) species composed of mannose, *myo*-inositol, glycerol and phosphate residues in the proportions of 6:1:1:1 and 2:1:1:1, respectively. Structural analysis, employing a combination of microanalytical methods, nuclear magnetic resonance spectroscopy, and mass spectrometric techniques established that the sequence of residues within dPIM-6, as shown below, was identical in the three mycobacterial strains investigated.



$$\begin{array}{rcl}
\alpha\text{-Man}p\text{-}(1\rightarrow2)\text{-}\alpha\text{-Man}p\text{-}(1\rightarrow2)\text{-}\alpha\text{-Man}p\text{-}(1\rightarrow6)\text{-}\alpha\text{-Man}p\text{-}(1\rightarrow6)\text{-} & \alpha\text{-Man}p & \\
 & 1 & \\
 & \downarrow & \\
 & 6 & \\
 & myo\text{-Ino} & \text{—}PO_4^-\text{—Gro} \\
 & 2 & \\
 & \uparrow & \\
 & 1 & \\
 & \alpha\text{-Man}p &
\end{array}$$

Enzymic degradation of dPIM-6 with an α-(1→2)-D-mannosidase resulted in the formation of a tetrasaccharide (dPIM-4) whose structure, along with that of dPIM-2, were identified as truncated versions of dPIM-6. The ^{1}H and ^{13}C NMR resonances of both native dPIM species and the dPIM-6 degradation product were fully assigned by homo- and hetero-nuclear chemical shift correlation methods, and the location of the phosphodiester group was determined by a 2D ^{1}H–^{31}P chemical shift correlation experiment. © 1998 Elsevier Science Ltd. All rights reserved

Keywords: Mycobacteria; Structure; Phosphatidylinositol manno-oligosaccharide; NMR spectroscopy

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1. Introduction

Lipoarabinomannan (LAM) is a major complex carbohydrate antigen of the mycobacterial cell wall and is important to bacterial intracellular survival. There is now much evidence that mycobacterial lipoarabinomannan in the *M. tuberculosis*/*M. bovis* complex has profound effects on the host immune system, specifically on macrophage effector functions [1–3], inhibition of MHC antigen presentation [4] and on T-lymphocyte proliferation [5]. An understanding of the chemical structural basis of these immune effects would be valuable in understanding the role of LAMs in mycobacterial infection, and a number of studies have been directed to these ends with partial structures elucidated [6–10]. Three clear size classes of lipoglycan are seen during purification of lipoarabinomannans, namely LAM, 17.4 kDa [11]; LM (lipomannan), and the phosphatidylinositol manno-oligosaccharides (PIMs). It has been reported that LAM and LM are based on the PIM mannose "core" motif [12] and, as such, its structure is of importance in understanding the biosynthesis of LAMs.

In pioneering work, Ballou et al. isolated and characterised a series of glyceryl phosphatidylinositol di-, tri-, tetra-, and penta-manno-oligosaccharides from the chloroform–methanol extracts of *M. bovis* strain BCG [13], *M. tuberculosis* and *M. phlei* [14]. More recently, the deacylated form of PIM from *M. tuberculosis* H37Ra/*M. smegmatis* [15] has been reported [12] to have a structure based on that proposed by Ballou et al., but is shown to contain six mannosyl residues (PIM-6).

In the present work, we report a methodology for the O-deacylation and isolation of the size classes within PIM. An NMR spectroscopy strategy which unambiguously assigns the structure of PIM-6 is described. It involves the acquisition of two-dimensional DQCOSY, ROESY, ^{13}C–^{1}H HMQC, ^{13}C–^{1}H and ^{31}P–^{1}H HMBC and 1D ^{1}H and ^{13}C NMR spectra. We describe structures for the major PIM core oligosaccharides and those produced by specific enzymic degradation.

2. Results and discussion

Isolation and purification.—The lipoglycans produced by *Mycobacterium bovis* strains AN5 and WAg201 and by *Mycobacterium smegmatis* mc^2 155 were separately isolated by using the Triton X-114 phase-separation technique of Severn et al. [16] following mechanical disruption and enzymic digestion with RNase and DNase and ultracentrifugation. These were resolved into various class sizes (LAM, LM and PIM) by size-exclusion chromatography on a Sephacryl S-200 column utilizing a disaggregating buffer containing deoxycholate. The fraction eluting at K_{av} 0.22 was pooled, desalted by ultrafiltration, and lyophilized to give a white amorphous product in 3.7% yield (based on the dry cell weight).

Glycosyl constituent and linkage analysis of the deacylated phosphatidylinositol manno-oligosaccharides.—The PIM fraction from *M. bovis* AN5 was obtained as a heterogeneous mixture of phosphatidylinositols varying in the number of mannose residues and the number and types of fatty acids. Removal of the ester-bound fatty acids by treatment with anhydrous hydrazine permitted the deacylated phosphatidylinositol manno-oligosaccharides (dPIMs) to be resolved into their class sizes by size-exclusion chromatography on a column of Bio-Gel P-2 eluted with pyridinium acetate. Quantitative colourmetric analysis of the eluant revealed the presence of two fractions containing both neutral glycose [17] and phosphate [18]. These corresponded to dPIM-6 (K_{av} 0.40) and dPIM-2 (K_{av} 0.73) and were collected and lyophilized. All subsequent NMR analyses were made on the deacylated, Bio-Gel P-2 purified products.

Quantitative GLC analysis of the reduced and acetylated acid hydrolysates from the deacylated PIMs [19] revealed mannose and *myo*-inositol residues in the approximate molar ratio of 2:1 for dPIM-2 and 6:1 for dPIM-6. Glycerol residues were also observed but at a lower level than expected due to losses during sample preparation.

Methylation [19] of the deacylated PIM samples then hydrolysis, reduction, and acetylation produced methylated alditol acetate derivatives that furnished mass spectral data from which the position of the linkages of the component glycosyl residues could be deduced. This procedure gave low estimates for the amount of inositol present in the samples. However, after dephosphorylation of the dPIM samples by treatment with aqueous HF, quantitative yields of the inositol derivative were observed, and this data is presented in Table 1.

By comparison with published data [12], two derivatives were identified from the methylation analysis of the deacylated and dephosphorylated

Table 1
Methylation analysis of dephosphorylated dPIM-6, dPIM-4 and dPIM-2 from *M. bovis* AN5

Constituent sugar and deduced substitution[a]		R_t[b]	Molar Ratio		
			dPIM-6	dPIM-4	dPIM-2
1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol	t-Man	0.81	2.0	2.2	2.0
2,6-di-*O*-acetyl-1,3,4,5-tetra-*O*-methylinositol	2,6-Ino	1.00	0.7	1.0	0.7
1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylmannitol	2-Man	1.26	2.0	Tr[c]	Tr[c]
1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylmannitol	6-Man	1.46	1.9	2.0	Tr[c]

[a] As their partially methylated peracetylated alditol derivatives.
[b] Retention times relative to that of $3,4,5\text{-Me}_3\text{-Ino-1-}d = 1.0$.
[c] Tr, Trace amounts.

PIM-2. 1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol (t-Man) and 2,6-di-*O*-acetyl-1,3,4,5-tetra-*O*-methyl-*myo*-inositol (2,6-Ino) units were present in the approximate molar ratio of 2:1, indicating that PIM-2 contained a branching *myo*-inositol unit substituted with two terminal mannosyl residues.

Similar methylation analysis of the deacylated and dephosphorylated PIM-6 revealed that it was more complex than dPIM-2. Assuming one inositol residue per molecule, it contained four additional mannosyl residues (Table 1), these being two units each of 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylmannitol (2-Man) and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylmannitol (6-Man), indicating that the additional residues in PIM-6 were 2- and 6-linked mannosyl residues in one or more linear oligosaccharides.

Molecular weight analysis.—Electrospray-ionization mass spectrometry (ESIMS) was used to establish the molecular weight of the phosphatidylinositol manno-oligosaccharides. The positive-ion ESIMS of the per-*O*-methylated dPIM-6 gave charged ions at *m*/*z* 1710, 1694 and 1689 Da for the potassium, sodium and ammonium ion adducts, respectively, indicating a molecular mass of 1671 Da. In addition, smaller sets of adduct ions corresponding to molecular species with masses of 1467 and 1263 Da were present, indicating small amounts of per-*O*-methylated dPIM-5 and dPIM-4 within the sample. A further small set of adduct ions, corresponding to a molecular species with a mass of 1452 Da, was also observed. This could result from low levels of fragmentation due to loss of a terminal mannose unit from per-*O*-methylated dPIM-6. Similarly a molecular mass of 855 Da for the per-*O*-methylated dPIM-2 was established from the observed sodium and ammonium ion adducts at *m*/*z* 878 and 873 Da, respectively. This data is in excellent agreement with the calculated masses for the per-*O*-methylated dPIM-6 as a per-*O*-methylated mannohexaosylinositol phosphoglyceride, $C_{71}H_{131}O_{41}P$ (1671 Da) and per-*O*-methylated dPIM-2 as a per-*O*-methylated mannobiosylinositol phosphoglyceride, $C_{36}H_{67}O_{21}P$ (855 Da).

Glycosyl sequence determination.—The sequence of glycosyl residues in dPIM-6 from *M. bovis* AN5 was established using a range of high-resolution NMR techniques. Complete assignment of the ^{1}H and ^{13}C NMR spectra of the oligosaccharides enabled the relative stereochemistry, ring size and anomeric configuration of each of the component glycoses to be determined. The NMR spectra were recorded at 23 °C in D_2O at pD 5.7.

The 1D ^{1}H NMR spectrum of dPIM-6 exhibited six resonances in the low-field region of the spectrum [Fig. 1(A)], and these and the residues from which they come were labelled as **A**–**F** in decreasing order of the chemical shifts (Table 2), while the inositol and glycerol residues and their NMR resonances were labelled as **G** and **H**, respectively.

Six discrete ^{13}C resonances were observed within the 90–110 ppm region of the 1D ^{13}C NMR spectrum of dPIM-6. These anomeric ^{13}C resonances could be correlated with their directly attached protons in a heteronuclear ^{1}H–^{13}C chemical shift correlation (HMQC) experiment (Fig. 3) [20], and their chemical shifts are given in Table 3.

All seven ^{1}H NMR resonances arising from the subspectrum of residue **A** in dPIM-6 were assigned by a two-dimensional homonuclear double quantum filtered chemical shift correlation (DQCOSY) experiment [21,22] [Fig. 2(A)]. The small magnitude of the vicinal couplings for $J_{1,2}$ (1.6 Hz) and $J_{2,3}$ (3.3 Hz) and the large coupling constants for $J_{3,4}$ and $J_{4,5}$ (^3J~10 Hz) measured in the DQCOSY experiment are consistent with this residue being a

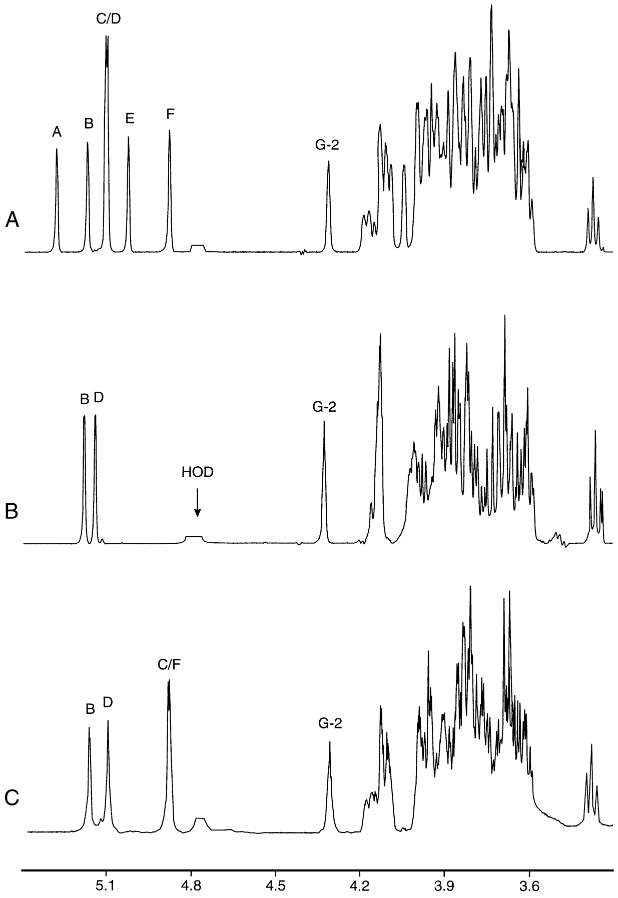


Fig. 1. ^{1}H NMR spectra of the deacylated phosphatidylinositol manno-oligosaccharides derived from *M. bovis* AN5. (A), dPIM-6; (B), dPIM-2; and (C), dPIM-4.

hexopyranose possessing the *manno*-configuration. The value of the heteronuclear one-bond ^{13}C–^{1}H coupling constant ($^1J_{C\text{-}1,H\text{-}1}$) of 173 Hz (Table 3) was characteristic of an α-anomeric configuration; typically coupling constants above 170 Hz are indicative of a α-configuration [23].

Despite the proton anomeric resonances arising from residues **C** and **D** being nearly coincident, their respective connectivity pathways from H-1 to H-3 were readily discernible in the DQCOSY spectrum. However, delineation of the spin systems from H-3 to H-6/H-6′ of residues **B**, **C**, **D**, **E** and **F** was complicated by the considerable resonance overlap within the (3.3–4.4 ppm) region of the ^{1}H spectrum. ^{13}C assignments were able to be established independently by observation in the HMBC experiment (spectra not shown) of intraresidue three-bond carbon–proton correlations ($^3J_{C\text{-}H}$) relating H-1 and C-3 and H-1 and C-5 within the pyranose ring system of each of the glycosyl residues (Table 4). Using this information, the H-3 and H-5 resonances in each residue could be identified from the HMQC spectrum (Fig. 3), which aided in the complete assignment of the subspectra arising from residues **B**, **C**, **D**, **E** and **F** in the DQCOSY. From this data all glycoses within dPIM-6 were identified as α-mannopyranosyl residues; the ^{1}H chemical shifts and vicinal proton coupling constants (Table 2) are in agreement with typical published data for such residues [24,25].

A heteronuclear ^{13}C–^{1}H chemical shift correlation experiment (HMQC) permitted unambiguous assignment of all the ^{13}C resonances within dPIM-6 by reference to the determined ^{1}H assignments; the data is presented in Table 3. The ^{13}C chemical shift values enabled the identification of the linkage positions within each of the residues. The relatively deshielded values for the methine carbons C-2**A** (79.3 ppm), C-2**C** (79.7 ppm), C-2**G** (79.4 ppm) and C-6**G** (79.1 ppm) and the hydroxymethyl carbons C-6**D** (66.2 ppm) and C-6**F** (66.9 ppm) in dPIM-6 (Table 2) indicate that these are the sites of glycosylation [26].

The ^{31}P NMR spectrum of dPIM-6 exhibited a single peak (3.4 ppm) arising from the phosphodiester and the 2D ^{1}H–^{31}P HMBC correlation map (Fig. 4) showed strong connectivities between the ^{31}P signal and ^{1}H resonances attributed to H-1 of the inositol residue (H-1**G** at 4.15 ppm) and the H-3s of the glycerol moiety (H-3**H** at 3.93 ppm and H-3′**H** at 3.98 ppm). From the DQCOSY spectrum, five additional proton resonances could be identified with the subspectrum of residue **G**, and based on the discrete chemical shifts of the H-2 (4.33 ppm) and H-5 (3.38 ppm) resonances, this residue was confirmed as the inositol unit [27]. The magnitude of the observed vicinal couplings ($J_{1,2}$ 2.2, $J_{2,3}$ 3.0 Hz and $J_{3,4}$, $J_{4,5}$, $J_{5,6}$, and $J_{6,1}$ ~10 Hz) indicates that H-2 is equatorial, but the other protons are axial, and this residue consequentially must be the *myo*-isomer. The four methylene and one methine proton resonances in the glycerol residue (**H**) were readily identified from the appropriate cross peaks in the DQCOSY spectrum.

The 1D ^{1}H NMR spectrum of dPIM-2 exhibited two discrete anomeric resonances [Fig. 1(B)], and the corresponding anomeric ^{13}C resonances were observed in the 1D ^{13}C NMR spectrum. From the subspectra defined in the DQCOSY experiment, these residues were identified as the mannopyranoses corresponding to **B** and **D** in dPIM-6. The spin systems arising from glycerol and inositol residues were also identified in the DQCOSY

Table 2
^{1}H NMR chemical shifts and coupling constants (Hz) for dPIM-6, dPIM-4 and PIM-2 from *M. bovis* type AN5[a]

Residue	Monosaccharide unit	Oligosaccharide	H-1 ($J_{1,2}$)	H-1′ ($J_{1',2}$ $J_{1,1'}$)	H-2 ($J_{2,3}$)	H-3 ($J_{3,4}$) or ($J_{2,3}$)	H-3′ ($J_{2,3'}$ $J_{3',3'}$)	H-4 ($J_{4,5}$)	H-5 ($J_{5,6}$)	H-6 ($J_{5,6}$ $J_{6,6'}$)	H-6′ ($J_{5,6'}$)
A	→2)-α-Man*p*-(1→	dPIM6	5.30		4.11	3.95		3.69	3.78	3.79	3.84
			(1.6)		(3.3)	(9.3)		(8.9)		(2.1, 12.4)	(6.4)
B	α-Man*p*-(1→	dPIM6	5.19		4.13	3.88		3.69	4.01	3.78	3.84
			(1.6)		(3.6)	(9.4)		(9.4)		(2.3, 11.5)	(6.2)
		dPIM4	5.19		4.13	3.89		3.70	4.03	3.79	3.85
		dPIM2	5.18		4.13	3.88		3.69	4.00	3.78	3.85
C	→2)-α-Man*p*-(1→	dPIM6	5.13		3.99	3.95		3.66	4.00	3.86	3.88
			(1.5)		(3.2)	(9.3)		(10.4)		(2.4, 11.4)	(5.9)
	α-Man*p*-(1→	dPIM4	4.91		3.98	3.85		3.65	4.00	3.78	3.89
D	→6)-α-Man*p*-(1→	dPIM6	5.11		4.15	3.84		3.80	4.20	3.67	4.00
			(1.5)		(3.6)	(10.3)		(10.8)		(2.2, 12.8)	(4.8)
		dPIM4	5.12		4.15	3.83		3.80	4.20	3.68	3.99
	α-Man*p*-(1→	dPIM2	5.14		4.13	3.86		3.75	4.02	3.81	3.84
E	α-Man*p*-(1→	dPIM6	5.04		4.06	3.84		3.63	3.77	3.77	3.89
			(1.7)		(10.6)	(10.1)		(9.3)		(2.3, 12.2)	(5.2)
F	→6)-α-Man*p*-(1→	dPIM6	4.90		4.02	3.86		3.72	3.81	3.74	3.96
			(1.4)		(3.2)	(9.7)		(9.1)		(1.2, 12.7)	(5.6)
		dPIM4	4.91		4.02	3.88		3.72	3.84	3.81	4.00
G	→6)-*myo*-Ino-(1→P	dPIM6	4.15		4.33	3.61		3.66	3.38	3.86	
	2		(2.2)		(3.0)	(10.6)		(11.0)	(9.1)	(9.3)	
	↑	dPIM4	4.15		4.34	3.61		3.66	3.38	3.86	
		dPIM2	4.15		4.33	3.60		3.66	3.37	3.88	
H	Gro-(1→P	dPIM6	3.62	3.70	3.92	3.92	3.98				
			(4.9)	(5.8, 12.0)		(6.7)	(3.2, 9.4)				
		dPIM4	3.62	3.70	3.93	3.93	3.99				
		dPIM2	3.93	3.70	3.93	3.61	3.99				

[a] Observed first-order chemical shifts and coupling constants (Hz), measured at 23 °C in D_2O (pD 5.7).

Table 3
^{13}C NMR chemical shifts and $^1J_{C,H}$ values (Hz) for dPIM-6, dPIM-4 and PIM-2 from *M. bovis* type AN5[a]

Residue	Monosaccharide unit	Oligosaccharide	C-1 ($J_{C\text{-}1,H\text{-}1}$)	C-2	C-3	C-4	C-5	C-6
A	→2)-α-Man*p*-(1→	dPIM6	101.6 (173)	79.3	71.0	67.9	74.1	61.7
B	α-Man*p*-(1→	dPIM6	102.2 (173)	71.1	71.3	67.4	73.6	61.9
		dPIM4	102.0	71.0	71.3	67.6	73.6	61.7
		dPIM2	102.3	70.9	71.3	67.6	73.7	61.9
C	→2)-α-Man*p*-(1→	dPIM6	99.1 (173)	79.7	71.0	67.6	73.7	62.0
	α-Man*p*-(1→	dPIM4	100.3	70.8	71.5	67.5	73.6	61.8
D	→6)-α-Man*p*-(1→	dPIM6	102.5 (173)	70.9	71.6	67.3	71.7	66.2
		dPIM4	102.3	70.9	71.6	67.5	71.8	66.2
	α-Man*p*-(1→	dPIM2	102.4	71.1	71.3	67.4	73.6	61.5
E	α-Man*p*-(1→	dPIM6	103.1 (173)	70.9	71.2	67.7	74.2	61.8
F	→6)-α-Man*p*-(1→	dPIM6	100.5 (173)	70.8	71.4	67.9	71.5	66.9
		dPIM4	100.2	70.8	71.4	67.6	71.6	66.5
G	→6)-*myo*-Ino-(1→P	dPIM6	77.6	79.4	70.7	73.7	73.8	79.1
	2	dPIM4	77.3	79.3	70.7	73.6	73.7	79.1
	↑	dPIM2	77.7	79.4	70.8	73.7	73.9	79.0
H	Gro-(1→P	dPIM6	63.1	71.7	67.2			
		dPIM4	63.1	71.7	67.3			
		dPIM2	63.1	71.8	67.3			

[a] Assignments were determined by ^{13}C–^1H chemical shift correlation (HMQC) unless otherwise indicated, measured at 23 °C in D_2O (pD 5.7).

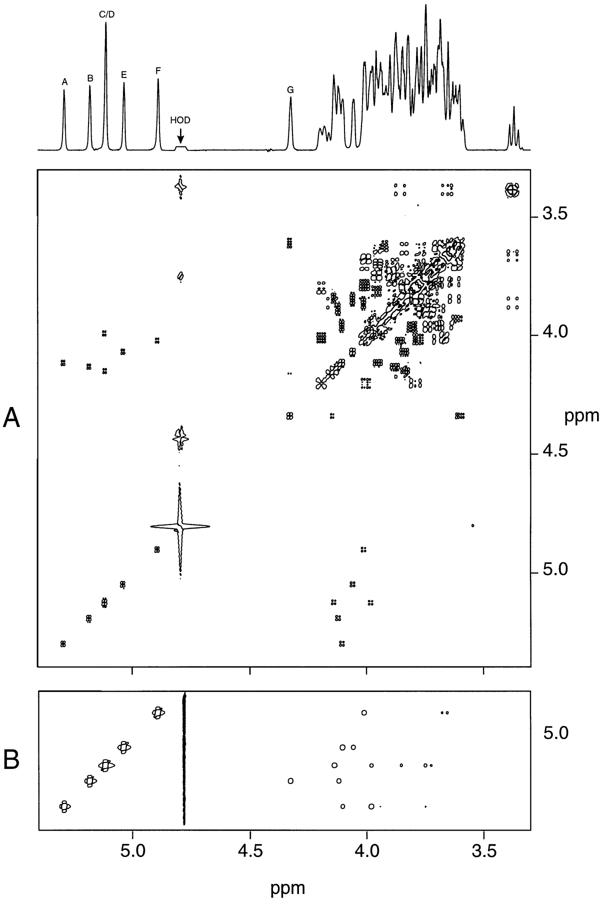


Fig. 2. Two-dimensional NMR spectra of deacylated PIM-6 derived from *M. bovis* AN5. (A), double quantum phase-sensitive COSY contour map and (B), partial NOESY contour map, showing correlations for the anomeric protons.

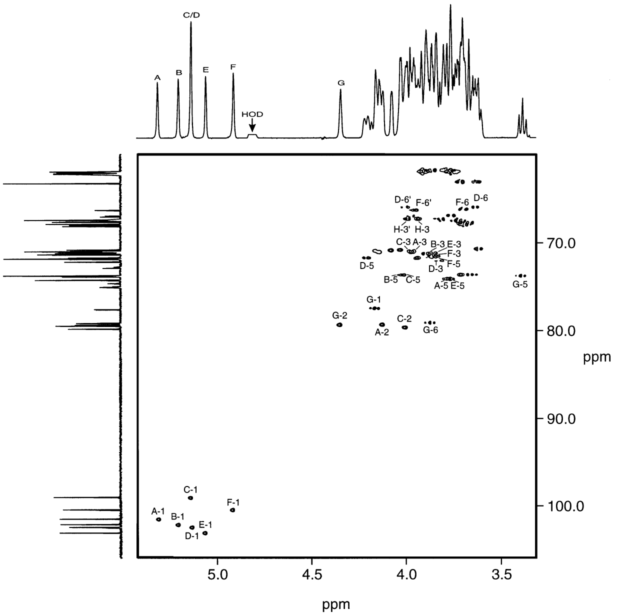


Fig. 3. HMQC spectra of deacylated PIM-6 derived from *M. bovis* AN5 showing the ^{1}H–^{13}C correlations.

spectrum of dPIM-2. With the exception of H-5, H-6 and H-6′ of residue **D**, the chemical shifts observed for all protons in dPIM-2 were identical (within 0.05 ppm) with the corresponding resonances in dPIM-6 (Table 2). The upfield shifts of H-5**D** and H-6′**D** (0.18 and 0.16 ppm, respectively) and downfield shift of H-6**D** (0.14 ppm) can be attributed to glycosyl substitution at O-6**D** in dPIM-6. Comparison of the ^{13}C NMR data from dPIM-2 and dPIM-6 (Table 3) showed no significant shifts for the carbon resonances in residues **B**, **G** and **H** (i.e., less than 0.4 ppm). However, consistent with residue **D** being glycosidically substituted at O-6 in dPIM-6 and terminal in dPIM-2, an upfield shift for C-6**D** (4.7 ppm) and downfield shift of 1.9 ppm for C-5**D** was observed in dPIM-2 [26].

The sequences of the residues within dPIM-2 and dPIM-6 were established by measuring inter-residue ^{1}H–^{1}H NOEs [28,29] and long-range ^{1}H–^{13}C couplings and the data is given in Table 4. NOEs were measured using two-dimensional ROESY experiments [Fig. 2(B)] [30] and were employed qualitatively to establish short ($\leq$3Å) through-space connectivities between the anomeric proton of each mannose residue with the aglyconic protons of the adjacent glycosidically linked residue. The long-range ^{1}H–^{13}C couplings across the glycosidic linkages, obtained by a proton-detected HMBC experiment [31] allowed the sequence of residues to be determined independently.

Thus, the anomeric proton resonance of the α-Man*p* residue **A** (5.30 ppm) was connected by a transglycosidic NOE cross peak to H-2**C** (3.99 ppm) [Fig. 2(B)], suggesting that residue **A** is linked to the O-2 position of residue **C**. The occurrence of cross-peaks relating H-1**E**/H-2**A**, H-1**C**/H-6**F** and H-1**F**/H-6**D** established the partial linear sequence **E**-(1→2)-**A**-(1→2)-**C**-(1→6)-**F**-(1→6)-**D**. Furthermore, the H-1 proton resonances of residues **B** and **D** showed strong NOEs to protons within the inositol unit, at H-2**G** (4.33 ppm) and H-6**G** (3.86 ppm), respectively, indicating the sequence **D**-(1→6)-**G**-(1←2)-**B** in which the inositol residue (**G**) is a branch point within dPIM-6.

In addition to the inter-residue NOEs, all anomeric proton resonances exhibited single intra-residue NOEs to their adjacent H-2. The absence of any corresponding 1,3-*syn*-axial correlations was consistent with the assigned α-conformation.

The NOE connectivities and the indicated linkage positions in dPIM-6 are in agreement with the results from long range ^{1}H–^{13}C coupling experiments

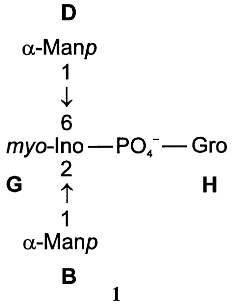
Table 4
Long-range ^1H–^{13}C and ^1H–^1H-NOE intra- and interresidue correlations obtained for dPIM-6 from *M. bovis* type AN5[a]

Residue	Monosaccharide unit	Observed atom	NOE contacts		Long-range ^1H–^{13}C correlations	
			Intraresidual	Interresidual	Intraresidual	Interresidual
A	→2)-α-Man*p*-(1→	H-1	4.11, **A**2	3.99, **C**2	71.0, **A**3; 74.1, **A**5	79.7, **C**2
		C-1				3.99, **C**2
B	α-Man*p*-(1→	H-1	4.13, **B**2	4.33, **G**2	71.3, **B**3; 73.6, **B**5	79.4, **G**2
		C-1				4.33, **G**2
C	→2)-α-Man*p*-(1→	H-1	3.99, **C**2	3.74, **F**6; 3.96, **F**6′	71.0, **C**3; 73.7, **C**5; 79.7, **C**2	66.9, **F**6
		C-1				
D	→6)-α-Man*p*-(1→	H-1	4.15, **D**2	3.87, **G**6	71.5, **D**5; 71.6, **D**3	79.1, **G**6
		C-1				
E	α-Man*p*-(1→	H-1	4.06, **E**2	4.11, **A**2	71.2, **E**3; 74.2, **E**5	79.3, **A**2
		C-1				4.11, **A**2
F	→6)-α-Man*p*-(1→	H-1	4.02, **F**2	3.67, **D**6	70.8, **F**2; 71.4, **F**3; 71.5, **F**5	66.2, **D**6
		C-1				3.67, **D**6; 4.00, **D**6′
G	→6)-*myo*-Ino-(1→P 2 ↑	H-1			79.4, **G**2;	
		C-1			4.33, **G**2; 3.86, **G**6	
H	Gro-(1→P	H-3			63.1, **H**1;	
		H-3′			63.1, **H**1	

[a] Measured at 23 °C in D_2O (pD 5.7).

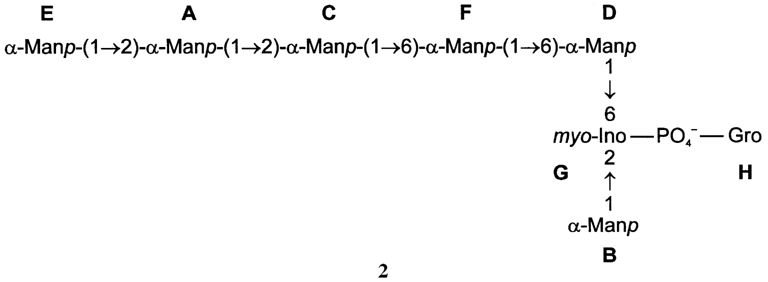
(HMBC). dPIM-2 exhibited the NOEs for H-1**B**/H-2**G**, H-1**D**/H-6**G**, analogous to those seen for dPIM-6 (Table 4). This indicates dPIM-2 to be a truncated form of dPIM-6 possessing only two mannopyranosyl residues linked at the 2- and 6-positions of the *myo*-inositol unit.

The foregoing NMR spectroscopic evidence, together with data from methylation analysis and ESIMS, clearly established the structures of the deacylated phosphatidylinositol di- and hexa-mannosyl oligosaccharides derived from *Mycobacterium bovis* AN5 as **1** and **2**, respectively:



$$\begin{array}{ccc} & \textbf{D} & \\ & \alpha\text{-Man}p & \\ & 1 & \\ & \downarrow & \\ & 6 & \\ & myo\text{-Ino}—PO_4^-—Gro & \\ & 2 & \\ \textbf{G} & \uparrow & \textbf{H} \\ & 1 & \\ & \alpha\text{-Man}p & \\ & \textbf{B} & \end{array}$$

1



$$\begin{array}{l} \qquad\textbf{E}\qquad\qquad\textbf{A}\qquad\qquad\textbf{C}\qquad\qquad\textbf{F}\qquad\qquad\textbf{D} \\ \alpha\text{-Man}p\text{-}(1\rightarrow2)\text{-}\alpha\text{-Man}p\text{-}(1\rightarrow2)\text{-}\alpha\text{-Man}p\text{-}(1\rightarrow6)\text{-}\alpha\text{-Man}p\text{-}(1\rightarrow6)\text{-}\alpha\text{-Man}p \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad 1 \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad \downarrow \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad 6 \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad myo\text{-Ino}—PO_4^-—Gro \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad 2 \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad \textbf{G}\quad \uparrow \qquad\qquad \textbf{H} \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad 1 \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad \alpha\text{-Man}p \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad \textbf{B} \end{array}$$

2

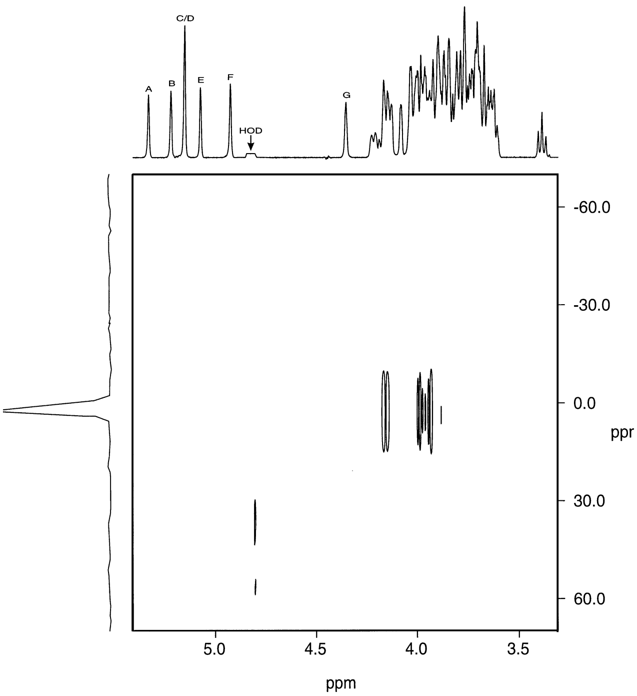


Fig. 4. ^{1}H–^{31}P HMBC spectra of deacylated PIM-6 derived from *M. bovis* AN5 showing the observed long range heteronuclear shift correlations.

The ^{1}H and ^{13}C NMR spectra of dPIM-2 and dPIM-6 isolated from *M. bovis* WAg201 and *M. smegmatis* mc^2 155 were identical to those obtained from *M. bovis* AN5 and indicated the dPIM structures for all three species to be identical.

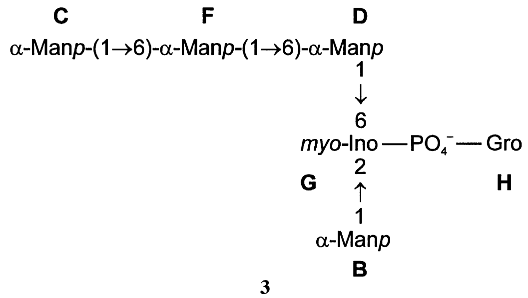
Selective degradation of dPIM-6.—The foregoing structural conclusions were supported by analysis of the degradation product obtained upon treatment of dPIM-6 with a specific α-(1→2)-D-mannosidase [32]. On size-exclusion chromatography (Bio-Gel P-2) this enzymatically-produced, deacylated phosphatidylinositol manno-oligosaccharide (K_{av} 0.46), was retained less than dPIM-2 (K_{av} 0.73) but more than dPIM-6 (K_{av} 0.40). Quantitative analysis of its acid hydrolysis products as alditol acetate derivatives showed it to be composed of mannose and *myo*-inositol residues in the observed molar ratio of 4.6:1. Glycerol residues were also observed but at a lower level than expected due to losses during sample preparation. Results obtained from the methylation analysis of the dephosphorylated, deacylated, phosphatidylinositol manno-oligosaccharide revealed the presence of 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-mannitol (6-Man), 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-mannitol (t-Man), and 2,6-di-*O*-acetyl-1,3,4,5-tetra-*O*-methyl-*myo*-inositol (2,6-Ino) units in the approximate molar ratio of 2:2:1, suggesting the oligosaccharide to be predominantly a tetramannosyl inositol glycerophosphate (dPIM-4) based on the dPIM-2 structure but with two additional 6-linked mannosyl residues on one of the mannosyl residues attached to the inositol moiety.

Electrospray-ionization mass spectrometry established the molecular weight of per-*O*-methylated dPIM-4 as 1263 Da. The positive-ion electrospray mass spectrum gave charged ions at m/z 1286 and 1281 Da arising from the sodium and ammonium ion adducts, respectively. This is in agreement with the calculated mass for $C_{53}H_{99}O_{32}P$ (1263 Da).

The ^{1}H [Fig. 1(C)] and ^{13}C NMR spectra of dPIM-4 were fully assigned, and the NMR data is presented in Tables 2 and 3, respectively. The 1D ^{1}H NMR spectrum of dPIM-4 exhibited only four anomeric resonances, and from the subspectra defined in the DQCOSY experiment, these were identified as the residues corresponding to **B**, **D**, **C** and **F** in dPIM-6. The spin systems arising from the glycerol and inositol residues were also identified in the DQCOSY spectrum of dPIM-4. A comparison of the ^{1}H NMR data (Table 2) from dPIM-4 and dPIM-6 showed significant upfield shifts for the H-1 and H-3 resonances of residue **C** in dPIM-4 (0.22 and 0.10 ppm, respectively) (Table 2), whereas no significant shift was observed for H-2**C**. The shielding of H-1**C** and H-3**C** can be attributed to a β-effect occurring upon glycosidic substitution at O-2**C**. Similarly, an observed upfield shift for C-2**C** (8.9 ppm) and downfield shifts of 1.2 and 0.5 ppm for C-1**C** and C-3**C**, respectively, in dPIM-4 were indicative of residue **C** being glycosidically substituted at O-2 in dPIM-6 and terminal in dPIM-4.

The ROESY spectrum of dPIM-4 exhibited NOEs for H-1**B**/H-2**G**, H-1**D**/H-6**G**, H-1**C**/H-6**F**, H-1**F**/H-6**D** analogous to those seen for dPIM-6. This indicates dPIM-4 to be a truncated form of dPIM-6 possessing a linear trisaccharide of α(1→6)-linked mannopyranosyl residues in addition to a terminal α-mannopyranosyl residue linked at the 6-and 2-position of the *myo*-inositol unit, respectively.

The complete structure of dPIM-4 is, therefore, that shown in **3** over.



3

3. Conclusions

The accumulated evidence presented herein permits the structure of the deacylated phosphatidylinositol manno-oligosaccharide dPIM-6 isolated from *Mycobacterium bovis* AN5 and WAg201 and *Mycobacterium smegmatis* mc^2 155 to be assigned the structure **2** shown above. This is consistent with the structure of the phosphatidylinositol pentamannosides, obtained from *M. tuberculosis* and *M. phlei* [14] and *M. bovis* strain BCG [13] by Ballou et al., as being a lower homologue of dPIM-6 lacking one of the 2-linked mannosyl residues. Furthermore, the structure is in total agreement with that reported for dPIM-6 from *M. tuberculosis* H37Ra/*M. smegmatis* found by Chatterjee et al. [12].

4. Experimental

General methods.—Commercial reagents and solvents were analytical grade. Aqueous solutions were concentrated under reduced pressure at bath temperatures below 40 °C.

The gel filtration properties of the eluted materials were expressed in terms of their distribution coefficients, $K_{\rm av} = (V_{\rm e} - V_0)/(V_{\rm t} - V_0)$, where V_0 is the void volume of the system, $V_{\rm e}$ is the elution volume of the specific material, and $V_{\rm t}$ is the total volume of the system.

Bacterial cell culture.—*M. bovis* AN5 was obtained from Central Veterinary Laboratories, Weybridge, UK and is used in the production of Tuberculin by the Central Animal Health Laboratory, Wallaceville, New Zealand. WAg201 is a wild type strain of *M. bovis* isolated in New Zealand. *M. smegmatis* mc^2 155 was obtained from Dr. W.R. Jacobs (Albert Einstein College of Medicine of Yeshiva University, New York City). All strains were grown as pellicles on modified Reid's synthetic medium [33], and the cells were killed by heating to 100 °C for 3 h before being harvested on coarse Whatman filter paper.

Isolation procedure.—Lipoglycans were recovered from disrupted cells of *M. bovis* AN5 and WAg201 and *M. smegmatis* mc^2 155 using the Triton X-114 phase-separation technique previously described [16]. Briefly, *M. bovis* cells were washed with Tris AN5 buffered saline, disrupted by passage through a French press, and then treated with RNase and DNase. The cell debris was extracted twice with aqueous Triton X-114 solution at 4 °C. The extract was then incubated at 37 °C to induce phase separation, and lipoglycan was recovered from the lower Triton X-114 rich phase by precipitation with ethanol and centrifugation. It was further purified by Proteinase K treatment and recovered as a precipitated lipoglycan gel in 5.7% yield (based on dry cell weight) after ultracentrifugation. The lipoglycan species were resolved into their various class sizes by size-exclusion chromatography on a Sephacryl S-200 column using as eluant a disaggregating buffer containing deoxycholate. The fractions containing the phosphatidylinositol manno-oligosaccharide (PIM) eluted at $K_{\rm av}$ 0.22 and were identified by SDS-PAGE. These were pooled and desalted by ultrafiltration before lyophilization. PIM was recovered as an amorphous white solid in a 3.7% yield (based on dry cell weight).

O-Deacylation and dephosphorylation of PIM.—For O-deacylation, PIM (20 mg) was suspended in anhydrous hydrazine (1 mL) and stirred at room temperature for 30 min, after which the reaction mixture was cooled, and cold acetone (3 vol., −70 °C) was slowly added to destroy excess hydrazine and precipitate the product. The deacylated PIM was then recovered by low-speed centrifugation (4×*g*, 30 min), and the pellet was washed three times with acetone, dissolved in water, and lyophilized.

The deacylated PIM was fractionated on a Bio-Gel P-2 column (Bio-Rad, 2.6×140 cm) eluted with pyridinium acetate (0.05 M, pH 4.5) at 1 mL/min, and the collected fractions (1.0 mL) were assayed colourimetrically for neutral glycoses [17] and phosphate [18], and two fractions containing both were separately pooled and lyophilized. This yielded 7.9 mg of purified dPIM-6 (a 39.5% yield based on PIM) and 4.2 mg of dPIM-2 (21% yield).

Aqueous hydrogen fluoride (48%, 3 drops) was added to samples of each deacylated PIM (~1 mg) in a polyethylene Eppendorf tube, and the mixture was stored at 4 °C for 48 h. After this time, the mixture was concentrated under a stream of argon then dried overnight in vacuo at room temperature over potassium hydroxide. A little distilled water was added to the samples which were then lyophilized.

Constituent glycosyl analysis.—Quantitative determination of constituent glycoses involved analysis of their derived alditol acetates by capillary gas–liquid chromatography (GLC) as previously described [16]. Thus, samples were subjected to reductive hydrolysis with aqueous trifluoroacetic acid (TFA, 3 M) containing 4-methylmorpholineborane (MMB) [19], and the resulting alditols and inositol were acetylated with acetic anhydride in the presence of TFA at 50 °C for 10 min. The solution was evaporated to dryness, taken up in dichloromethane (2.5 mL) and washed with water. The organic layer was evaporated, and the resulting peracetylated alditols and inositol were analyzed by GLC using a Hewlett–Packard 5890 Series II chromatograph with a Supelco 2330 column (15 m×0.25 mm) at 220 °C employing a flame-ionisation detector (FID). The carrier gas was dry hydrogen (total flow 100 mL min^{-1}, split flow 20 mL min^{-1}). Identification of components was by comparison of retention times with authentic standards.

Glycosyl linkage analysis.—Samples of deacylated and deacylated, dephosphorylated oligosaccharides (~1 mg) were dissolved in dry dimethylsulfoxide (2 M, 200 μL), purged with argon, and potassium (methylsulfinyl)methanide (200 μL) was added. After stirring the mixture for 45 min at room temperature, iodomethane (150 μL) was added, and the mixture stirred for a further 45 min. The methylated products were recovered by partitioning the reaction mixture between water (4×2 mL) and chloroform (2 mL), followed by concentration of the organic phase.

Methylated dPIM samples were hydrolysed with aqueous trifluoroacetic acid (2 M, 200 μL) for 1 h at 120 °C. After cooling, the solutions were evaporated to dryness then two aliquots of toluene (500 μL) were added and evaporated. The samples were reduced with sodium borohydride (200 μL, 10 mg mL^{-1} in 1 M ammonium hydroxide) for 1 h at 50 °C. After cooling, acetone (250 μL) was added to quench any remaining reductant, then the samples were evaporated to dryness. Since acid-catalysed acetylation avoids the need to remove borate from the samples, the reaction was performed using a mixture of acetic acid (40 μL), ethyl acetate (200 μL), acetic anhydride (600 μL) and perchloric acid (60%, 23 μL) for 15 min at room temperature. Excess acetic anhydride was decomposed by the addition of water (2 mL) and 1-methylimidazole (40 μL). The partially methylated alditol acetate derivatives produced were recovered by partitioning the reaction mixture between water (3×4 mL) and dichloromethane (1 mL), followed by concentration of the organic phase. Derivatised samples were analysed by GLC using the conditions described above. Identification of components was by comparison of retention times with authentic standards and for selected samples by GLC–EIMS using a Hewlett–Packard MSD 5970 on a Hewlett–Packard Ultra-2 column (20 m×0.2 mm) at 50 °C (3 min), 35 °C/min to 180 °C (2 min), 50 °C/min to 220 °C (15 min), and electron ionisation at 70 eV. The identity of 2,6-di-*O*-acetyl-1,3,4,5-tetra-*O*-methylinositol was confirmed by GLC–EIMS; characteristic ions at *m*/*z* 200, 191 and 75 Da were consistent with those observed by Chatterjee et al. [12].

Enzymic degradation of dPIM-6.—An α-(1→2)-D-mannosidase preparation that had been isolated from Taka-Diastase® as described by Yamamoto et al. [32] was supplied by Dr. R. Trimble. dPIM-6 (36 mg) in acetate buffer (5 mL, 0.01M, pH 5.2) containing α-(1→2)-D-mannosidase (0.44 mg) was heated at 30 °C, and after 16 h the reaction mixture was concentrated, applied to a Bio-Gel P-2 column and eluted with pyridinium acetate (0.05 M, pH 4.5) at 1 mL/min. The collected fractions (1.0 mL) were assayed colourimetrically for neutral glycoses [17] and phosphate [18], and the fractions of the only peak containing both, which eluted at K_{av} 0.46, were pooled and lyophilized to give dPIM-4 as an white amorphous solid (29 mg). A later eluting peak, containing only neutral glycose, was confirmed as mannose by ^{1}H NMR spectroscopy.

SDS polyacrylamide gel electrophoresis.—Lipoglycan mixtures, column eluants and purified LAM, LM and PIM were analyzed in a discontinuous SDS polyacrylamide gel system [34] employing a tricine buffer [35]. The gels were 0.75 mm thick and contained 4 and 12% polyacrylamide in the stacking and separating gels, respectively. Samples (approximately 100 μg) were diluted to a volume of 50 μL with distilled water and mixed 4:1 with sample buffer (62.5 mM Tris-HCl pH 6.8, 20% glycerol, 5% SDS) and aliquots containing LAM (10 μL), LM (10 μL), PIM (5 μL) and the dye, bromophenol blue (5 μL, 0.05% in 4:1 water:sample buffer) were loaded on to the gel. Electrophoresis was performed at 100 mV until the dye front was approximately 1 cm from the bottom of the gel, and the lipoglycan bands were visualized by silver staining [36].

Nuclear magnetic resonance spectroscopy.— Nuclear magnetic resonance spectra were obtained on a Varian Unity 500 spectrometer, and data was processed using Felix software (version 95, Biosym). Solutions of the saccharide samples in deuterium oxide (0.7 mL) were prepared at a concentration of 10–40 mg/mL and adjusted to pD 5.7 subsequent to lyophilization from D_2O. All spectra were measured on the deacylated PIM samples in 5-mm tubes at 23 °C.

^1H NMR spectra were recorded at 500 MHz using a spectral width of 2.3 kHz, an acquisition time of 3.2 s, a 16 K data block and a 90° pulse. Broadband proton-decoupled ^{13}C NMR spectra were recorded at 125 MHz using a spectral width of 25 kHz, a 32 K data block, and a 90° pulse employing WALTZ decoupling [37].

Chemical shifts are expressed relative to the methyl resonance of internal acetone (δH, 2.225 ppm; C, 31.07 ppm). Proton-coupled ^{13}C NMR spectra were determined by gated coupling [38].

Complete assignment of spectra was accomplished using two-dimensional homo- and heteronuclear chemical shift correlation techniques. Double quantum filtered [22] homonuclear two-dimensional chemical shift correlated spectroscopy (COSY) [21] was performed in the phase-sensitive mode. Data was acquired over a frequency range of 2.3 kHz by using data sets of 512×2048 points and 32 scans were acquired for each value of t_1.

Two-dimensional ROESY [30] experiments were performed in a phase-sensitive mode over a spectral width of 3.0 kHz, using a data set of 512×2048 points, 32 scans and a mixing time of 450 ms for each experiment.

Heteronuclear two-dimensional ^{13}C and ^1H chemical shift-correlated spectra were measured in the ^1H detected mode via multiple quantum coherence (HMQC) [39] using data sets of 512×1024 points and spectral widths of 12.7 and 2.3 kHz for ^{13}C and ^1H domains, respectively. Ninety-six scans were acquired for each t_1 value and ^{13}C decoupling during ^1H acquisition was achieved using the GARP-1 composite pulse [40]. Heteronuclear multiple bond correlated (HMBC) experiments were carried out using the pulse sequence described by Bax and Summer [31] and employed a delay of 60 ms for the evolution of long range couplings.

^{31}P NMR spectra were measured at 202 MHz by employing spectral widths of 10 kHz and a 90° pulse and aqueous phosphoric acid (85%) was used as the external standard (δP 0.0 ppm). ^1H–^{31}P correlations (HMBC) were made in the ^1H detected mode by using a data matrix of 16×1024 points, sweep widths of 10 kHz for ^{31}P and 13 kHz for ^1H, and a mixing time of 60 ms.

Electrospray-ionization mass spectrometry.— Samples for electrospray mass spectrometry were analyzed on a VG Platform II electrospray mass spectrometer with an electrospray ion source. Per-*O*-methylated deacylated phosphatidylinositol manno-oligosaccharide samples were first dissolved in chloroform and then run using methanol as diluent. Injection volumes were 10 μL, and the flow rate was set at 20 μL/min. Samples were analysed in the positive-ion mode using a cone voltage of 20 V and the electrospray tip voltage was typically 3.5 kV. The mass spectrometer was scanned from *m*/*z* 1000 to 2000 with a scan time of 10 s. Data was collected using a multichannel analysis mode.

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