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A novel phosphatidylinositol manno-oligosaccharide (dPIM-8) from *Gordonia sputi*

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Abstract—The deacylated phosphatidylinositol manno-oligosaccharides (dPIMs) from the glycosyl phosphatidylinositol (GPI) carbohydrate antigen anchor of *Gordonia sputi* were the known 2,6-di-*O*-α-mannopyranosyl-*myo*-inositol glycerophosphate (dPIM-2) and the illustrated novel compound (dPIM-8), which could not be separated from dPIM-7 and dPIM-6, these three compounds being present in the mixture in the molar ratios 1.0:0.65:0.4. dPIM-8 is an analogue of dPIM-2 (and also of dPIM-7 and dPIM-6) in having α-mannopyranose and an α-mannopyranosyl linked heptasaccharide bonded to O-2 and O-6, respectively, of the inositol. The dPIM-8 species has not been found previously.

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

1
↓
6
myo-Ino 1 - PO_4^- - 1 Gro
2
↑
1
α-D-Man*p*

dPIM-8

Keywords: Mycobacteria; *Gordonia sputi*; Phosphatidylinositol manno-oligosaccharide; NMR spectroscopy; MALDI

1. Introduction

Intense research into the architecture of the cell envelope of Mycobacteria and related bacteria has revealed an abundance of immunoregulatory polysaccharides and lipoglycans.[1] In the plasma membrane component there are three carbohydrate-based materials each having the core structural unit 2,6-di-*O*-α-D-mannopyranosyl-1-phosphatidyl-*myo*-inositol (PIM-2).[2] Firstly, this compound appears in extended form with the mannosyl substituent at O-6 elaborated into mannose-based oligosaccharides containing X mannose units, and in this way the PIM-(X + 1) series of products is formed,[3–5] the highest member previously reported being PIM-7.[6] Secondly, lipomannans (LM) occur in which a linear α-(1→6)-linked mannan, substituted with α-(1→2)-linked mannose branches, extends the mannose oligomer emanating from O-6 of the phosphatidyl inositol (PI) unit of PIM-6,[7] and thirdly, even more complex analogues (the LAMs) are found, which contain additional α- and β-linked D-arabinofuranose-based branched oligosaccharides attached to the mannan moieties of LM.[8] According to the bacterial strain involved these compounds may have either inositol phosphate[9] or

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further D-mannose[10] substituents attached to the terminal arabinofuranose residues, and accordingly they are termed AraLAMs or ManLAMs, respectively.

The PIM cores of the mycobacterial lipoglycans are acylated at O-3 and O-2 of the glycerol moiety, the acyl groups often being palmitate and 10-methyloctadecanoate.[11] Additionally, PIM-2 and PIM-6 may carry further fatty acid ester groups at O-3 of the inositol and at O-6 of the mannose residue linked to O-2 of the cyclitol.[12,13] The importance of the lipidic groups in the neighbourhood of the PIM core has been highlighted by the finding that most of the LAM immunoregulatory effects are eliminated on removal of the ester groups by alkaline hydrolysis.[14]

The significance of PIMs as immunomodulators is indicated by the increasing number of reports in the literature on their structures and biological activities. There is now much evidence that mycobacterial PIMs have profound effects in the host immune systems, on the specificities of T-lymphocyte proliferation[12,15] and on adhesion mediation involving lectins on the surface of non-phagocytic cells.[16] Additionally, PIMs, as well as AraLAM, have recently been shown to activate an innate immune response via Toll-like receptor-2 (TLR-2).[17] The antigen-presenting molecule CD1b binds PIM-6 as well as ManLAM from *Mycobacterium leprae* and *Mycobacterium tuberculosis*, and an interaction between this protein and the acyl side chains of PIM-2 has been established.[18]

In pioneering work, Ballou et al. isolated and characterised a series of deacylated glycosyl phosphatidylinositol di-, tri-, tetra- and penta-mannosides (dPIM-2 to dPIM-5) from the chloroform–methanol extracts of *M. bovis* strain BCG,[4] *M. tuberculosis* and *M. phlei*.[19] In dPIM-2 the two mannosyl units are glycosidically attached at positions 2 and 6 of the *myo*-inositol ring,[5] and the more highly glycosylated forms each retain the mannosyl substituent at O-2, but have elongated mannose-based chains at O-6.[19] More recently, the deacylated form of a PIM from *M. tuberculosis* H37Ra/*M. smegmatis* has been determined by chemical methods based on methylation analysis[3] to have the pentasaccharide α-D-Man*p*-(1→2)-[α-D-Man*p*-(1→2)]$_2$-[α-D-Man*p*-(1→6)]$_2$ linked to O-6 of the *myo*-inositol ring, as well as a mannosyl substituent at O-2 and the usual glycerophosphate at O-1. That is, it is a dPIM-6 compound with structure akin to those proposed for the dPIM set of compounds analysed by Ballou et al.[4,5,19] By use of an NMR spectroscopic strategy we have identified the same dPIM-6 in *M. bovis* AN5 and *M. smegmatis* mc^2 glycosyl phosphatidylinositols.[20] Comparative analyses of PIMs isolated from *M. bovis* BCG, *M. tuberculosis* H37Rv and *M. smegmatis* 607 strains have shown that within the general structure there is a great deal of heterogeneity, including variation in the acylation states of PIM-2[12] and PIM-6.[13]

Gordonia sputi is a Gram-positive coryneform bacterium,[21,22] which has been identified as an opportunistic pathogen causing systemic infections in humans. As a member of the actinomycetes, a taxon including the extensively studied facultative intracellular pathogen *M. tuberculosis*, the Gordonia have characteristic cell envelope architecture, dominated by lipids and lipoglycans. The latter compounds, apparently structurally related to LAM, have been identified in representative organisms in other genera within the actinomycete, including *Corynebacterium matruchotii*,[23] *Dietzia maris*,[24] *Gordonia rubropertincta*,[25] *Amycolatopsis sulphurea*,[26] *Rhodococcus rhodnii*,[27] *Rhodococcus equi*[28] and *Rhodococcus ruber*.[29]

To define further the distribution and diversity of phosphatidylinositol manno-oligosaccharides within the non-mycobacterial Actinomycetes lineage we have investigated the PIMs obtainable from *G. sputi* ATCC 33609, and now report on the use of high resolution NMR spectroscopy to determine the structure of a novel component, dPIM-8.

2. Results and discussion

The mixture of PIMs was extracted from disrupted cells and deacylated (to become 'dPIMs') by treatment with anhydrous hydrazine. The components were purified by gel permeation chromatography (GPC) to give two major fractions, of which the lower molecular weight fraction was identified as dPIM-2 by the finding that its ^{1}H and ^{13}C NMR spectra were identical to those previously published.[20] The larger fraction, containing higher molecular weight material, showed eight anomeric signals of unequal intensity (and a trace of a ninth) in the ^{1}H-NMR spectrum (Fig. 1). Analysis of this fraction (referred to as 'dPIM-8m') revealed the presence of mannose and *myo*-inositol in the ratio of 3.3:1, which is appreciably smaller than expected on the basis of the results reported below—perhaps because the mannose decomposed partially under the acidic conditions used for the hydrolysis. The absolute configuration of the mannose was shown to be D by GLC analysis of the derived acetylated (+)-2-butyl pyranosides.[30]

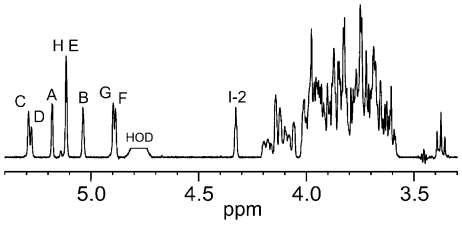


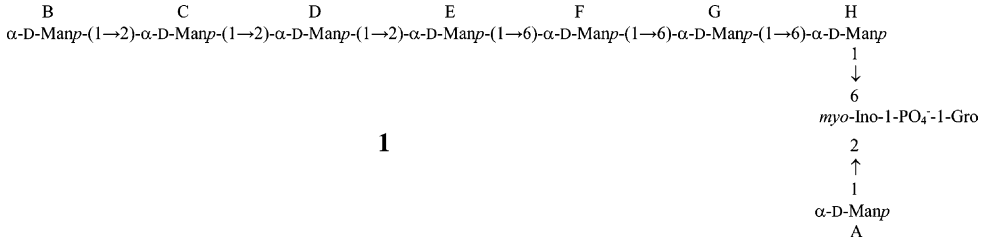
Figure 1. ^{1}H NMR spectrum of the deacylated phosphatidylinositol manno-oligosaccharides dPIM-8m from *Gordonia sputi*. The signals of the anomeric protons of mannose units A–H of dPIM-8 (**1**) and H-2 of the inositol are identified.

The negative mode MALDI mass spectrum of dPIM-8m revealed three $(M-H)^-$ ions (*m/z* 1629, 1467 and 1305) with the relative intensities 1:0.6:0.5. While this suggested that the sample contained dPIM-8, dPIM-7 and dPIM-6 [calculated $(M-H)^-$ values = 1629, 1467 and 1305] in these ratios, the possibility that the second and third components were derived by fragmentation of dPIM-8 during mass spectrometry could not be ignored. To address this possibility the sample was methylated and a negative mode MALDI spectrum corroborated the conclusion that it comprised a mixture of dPIM-8, dPIM-7 and dPIM-6 [found *m/z* 2064, 1860 and 1656; calculated $(M-H)^-$ values = 2064, 1860, 1656], and indicated ratios of 1.0:0.7:0.3. From the molecular masses it is concluded that each component underwent methylation at each hydroxyl group except that of the phosphate diester function.

The complex nature of the dPIM-8m mixture resulted in severe overlaps in the NMR spectra of the three components and enforced long data collection times and the use of linear prediction of experimental data to enhance the resolution in the second dimension. By this means, and by use of a combination of homo- and heteronuclear two-dimensional experiments, all the resonances in the ^1H and ^{13}C NMR spectra of the three components of the mixture were assigned.

For dPIM-6 there was almost complete identity between its NMR spectral features and those of the mannose residues was readily determined by comparison of their ^{13}C chemical shifts with those of mannose,[33] a downfield shift of 4–7 ppm being observed for the carbon atoms bearing a glycosyl substituent. Determination of the inter-residue sequence of the sugar residues was carried out by use of ^1H, ^1H NOESY and ^1H, ^{13}C HMBC spectra. The connectivities are presented in Table 2 and indicate that the glycosidic linkages are as shown in structure **1**.

Integration of the signals of the anomeric atoms in the decoupled 2D ^1H, ^{13}C HMQC spectrum (Fig. 2) afforded ratios for the three component dPIM-8m as follows: A, 1.0:B, 1.1:C + D, 1.3:E, 0.7:F + G, 1.9:H, 1.0. Calculations of these ratios for a mixture of dPIM-8 (1.0), dPIM-7 (0.65) and dPIM-6 (0.4) give the ratios A, 1:B, 1:C + D, 1.3:E, 1:F + G, 2:H, 1, which, apart from an unaccounted for discrepancy in the figures for mannose unit E, are consistent with the conclusions drawn about the composition of the dPIM-8m mixture. When the complementary set of ratios were measured from Figure 1 the following normalised ratios were obtained: A, 1.0:B, 1.0:C + D, 1.4:E + H, 1.9:F + G, 2.0, whereas the expected figures are 1:1:1.3:2:2. This confirms the ratios of the components of the mixture dPIM-8m and suggests that the intensity measurement for unit E derived from the HMQC spectrum (Fig. 2) (0.7 instead of 1.0) is anomalously small.



1

nose moieties A, B and E–H of compound **1** and, similarly for dPIM-7, there was almost complete identity between its resonances and those of units A, B and D–H. It is concluded therefore that the smallest component of the mixture (dPIM-6) has the structure of dPIM-8 less the mannobiose at the exposed end of the oligosaccharide chain, while dPIM-7 is the major component devoid of a single mannose at that position.

The ^1H and ^{13}C chemical shifts of the main component of dPIM-8m (i.e., dPIM-8 itself) are recorded in Table 1, together with the $^3J_{H,H}$ and $^1J_{C,H}$ values for the anomeric H and C atoms for which eight signals were observed in the decoupled 2D ^1H, ^{13}C HMQC spectrum as illustrated in Figure 2. From the $J_{H\text{-}1,H\text{-}2}$[31] and $J_{C\text{-}1,H\text{-}1}$[32] values it can be concluded that all are α-linked, and the linkage pattern of the individual mannose moieties A, B and E–H of compound **1** and, similarly for dPIM-7, there was almost complete identity between its resonances and those of units A, B and D–H. It is concluded therefore that the smallest component of the mixture (dPIM-6) has the structure of dPIM-8 less the mannobiose at the exposed end of the oligosaccharide chain, while dPIM-7 is the major component devoid of a single mannose at that position.

The presence of a phosphodiester linkage in dPIM-8m was corroborated by the occurrence in the ^{31}P NMR spectrum of a singlet at δ 0.03 and by correlations in the ^1H,^{31}P HSQC spectrum between the phosphorus signal and H-1 of the inositol residue and H-1,1′ of the glycerol residue. As with other GPI-anchor compounds, therefore, the glyceryl phosphate is 1→1 phosphate diester linked to the inositol. The complete structure of the main component of dPIM-8m is therefore as shown in structure **1**. This conclusion was further tested using the product of dephosphorylation of the dPIM-8m fraction, which was made by treatment with cold, aqueous hydrogen fluoride.[34] The fully de-esterified material, after purification on a column of DEAE Sepharose, showed no ^{31}P NMR signals but it retained the eight anomeric proton resonances in the ^1H spectrum. Only the resonance of

Table 1. ^{1}H and ^{13}C NMR chemical shifts for dPIM-8 from *Gordonia sputi*

Sugar residue	$^{1}H/^{13}C$					
	1	2	3	4	5	6
A α-D-Man*p*-(1→	5.18 (1.9) 102.1 [172.9]	4.12 71.0	3.87 71.2	3.69 67.4	4.00 73.6	3.77, 3.84 61.7
B α-D-Man*p*-(1→	5.04 (1.8) 103.0 [172.1]	4.06 70.8	3.83 71.1	3.63 67.6	3.75 74.0	3.73, 3.89 61.9
C →2)-α-D-Man*p*-(1→	5.29 (1.6) 101.4 [174.0]	4.09 79.3	3.94 70.8	3.67 67.8	3.75 74.0	n.r. 61.9
D →2)-α-D-Man*p*-(1→	5.28 (1.7) 101.4 [173.0]	4.08 79.5	3.94 70.8	3.66 67.9	3.75 74.0	n.r. 61.9
E →2)-α-D-Man*p*-(1→	5.11 (n.r.) 98.9 [173.4]	3.98 79.6	3.95 71.0	3.69 67.7	3.69 73.5	3.76, 3.88 61.7
F →6)-α-D-Man*p*-(1→	4.89 (1.7) 100.3 [172.4]	3.98 70.8	3.83 71.6	3.72 67.4	3.82 72.0	3.73, 3.95 66.8
G →6)-α-D-Man*p*-(1→	4.90 (1.8) 100.4 [172.4]	4.01 70.8	3.86 71.5	3.72 67.4	3.82 71.5	3.76, 3.96 66.3
H →6)-α-D-Man*p*-(1→	5.12 (n.r.) 102.4 [173.3]	4.14 70.8	3.83 71.5	3.80 67.2	4.18 71.6	3.67, 3.98 66.1
→2,6)-*myo*-Ino-(1→P	4.14 77.4	4.33 79.2	3.60 70.6	3.65 73.5	3.37 73.7	3.85 79.0
Gro-(1→P	3.92, 3.97 67.2	3.90 71.6	3.61, 3.69 62.9			

$^{3}J_{H1,H2}$ and $^{1}J_{C,H}$ values in hertz are given in parentheses and square brackets, respectively. n.r. indicates not resolved.

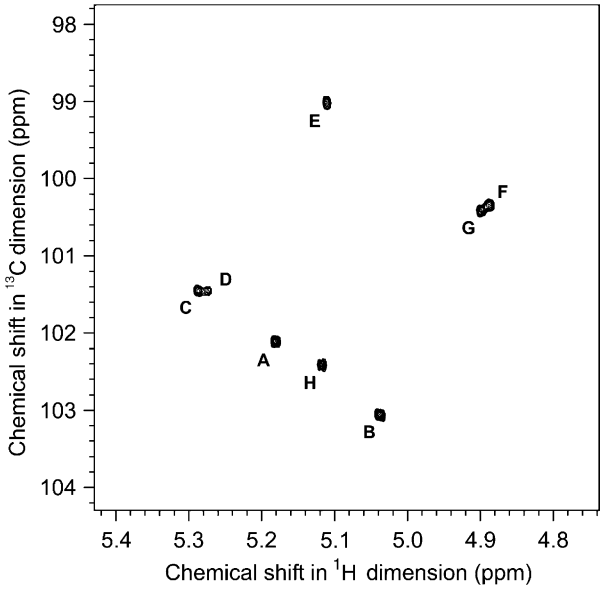


Figure 2. The anomeric atom resonances of the decoupled ^{1}H, ^{13}C HMQC spectrum of dPIM-8m (**1**) isolated from *Gordonia sputi*. The labels A–H identify the signals of the individual mannose moieties.

the A mannose unit had undergone significant change in chemical shift by the dephosphorylation [0.05 ppm upfield, i.e., from δ 5.18 to 5.13 (Table 1)] and consequently overlaps the resonances of the E and H protons. The other anomeric protons changed position of resonance by no more that 0.01 ppm. Integration of the sets of resonances of the fully de-esterified material gave the following ratios: A + E + H, 3.0:B, 1.0:C + D, 1.4:F + G, 2.3, whereas those ratios calculated for the dephosphorylated dPIM-8m (assuming the previously determined ratios of the components: 1:0.65:0.4) are 3:1:1.3:2.

In the MALDI mass spectrum (positive mode) of the fully de-esterified material three molecular ions with *m*/*z* 1499, 1337.5 and 1175.5 were observed, corresponding to the $(M+Na)^{+}$ ions for the completely dephosphorylated dPIM-8, dPIM-7 and dPIM-6, respectively. Methylation analysis of this material (methylation followed by hydrolysis, reduction and acetylation) revealed the presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-mannitol, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-mannitol and 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl-D-mannitol as well as 2,6-di-*O*-acetyl-1,3,4,5-tetra-*O*-methyl-*myo*-inositol in the

Table 2. Interglycosidic correlations from the anomeric atoms observed in the NOESY and HMBC spectra of dPIM-8 from *Gordonia sputi*

Sugar residue	δ_H or δ_C			
	Anomeric atom	NOE to	HMBC to	Connectivity
A α-D-Man*p*-(1→	5.18	4.33		A, H1 to Ino, H2
			79.2	A, H1 to Ino, C2
	102.1		4.33	A, C1 to Ino, H2
B α-D-Man*p*-(1→	5.04	4.09		B, H1 to C, H2
			79.3	B, H1 to C, C2
	103.0		4.09	B, C1 to C, H2
C →2)-α-D-Man*p*-(1→	5.29	4.08		C, H1 to D, H2
			79.5	C, H1 to D, C2
	101.4		4.08	C, C1 to D, H2
D →2)-α-D-Man*p*-(1→	5.28	3.98		D, H1 to E, H2
			79.6	D, H1 to E, C2
	101.4		3.98	D, C1 to E, H2
E →2)-α-D-Man*p*-(1→	5.11	3.73, 3.95		E, H1 to F, H6
			66.8	E, H1 to F, C6
	98.9		3.73, 3.95	E, C1 to F, H6
F →6)-α-D-Man*p*-(1→	4.89	3.76, 3.96		F, H1 to G, H6
			66.3	F, H1 to G, C6
	100.3		3.76, 3.96	F, C1 to G, H6
G →6)-α-D-Man*p*-(1→	4.90	3.67		G, H1 to H, H6
			66.1	G, H1 to H, C6
	100.4		3.67, 3.98	G, C1 to H, H6
H →6)-α-D-Man*p*-(1→	5.12	3.85		H, H1 to Ino, H6
			79.0	H, H1 to Ino, C6
	102.4		3.85	H, C1 to Ino, H6

approximate molar ratios 2.2:3.6:1.5:1.0. For pure, fully de-esterified PIM-8 these ratios would be 2:3:3:1, and for the proposed mixture of de-acylated PIM-8, -7 and -6 in the ratios 1:0.65:0.4 (the average of the two sets already given) the figures would be 2:3:2.3:1. These data are taken to confirm the correctness of the proposed composition of the mixture dPIM-8m.

The structures of the oligosaccharide components of the PIMs isolated from *G. sputi*, and described here, differ somewhat from the usual pattern of oligomers of the Acetomycetes' PIMs. Although the core structure (PIM-2) appears to be invariable among the Acetomycetes, the presence of the α-(1→6) linked mannotetraose unit emanating from O-6 of the phosphatidyl inositol (PI) unit in the structures now described is novel. Furthermore, the α-(1→2) linked mannose, mannobiose and mannotriose units completing the structures of PIM-6, -7 and -8, respectively, suggest that they are added sequentially in the biosynthesis of these species.

3. Experimental

3.1. Gas chromatographic and mass spectrometric methods

GLC analyses were performed using a Hewlett–Packard 5890 Series II chromatograph equipped with a flame ionisation detector. The compounds were separated on a Supelco 24018 fused silica column (0.25 mm × 15 m) using dry hydrogen as carrier gas and a temperature programme of 180 °C (1 min) rising to 220 °C at a rate of 3 °C min^{-1}. GLC–MS analyses were performed using a Phenomenex fused silica column (0.25 mm × 30 m; S/N 024257) with helium as carrier gas and a Hewlett–Packard model 5970 mass spectrometer. A temperature programme of 170 °C for 3 min rising to 220 °C at a rate of 3 °C min^{-1} was used. MALDI mass spectra were acquired using an Applied Biosystems Voyager-DE Pro MALDI-TOF mass spectrometer in the delayed extraction and reflector modes. Positive and negative ion spectra were collected with an acceleration voltage of 20,000 V, a grid voltage of 76% and zero voltage on the guide wire. The extraction delay time was set to 100 ns. 2,5-Dihydroxybenzoic acid in H_2O was used as the matrix.

3.2. NMR methods

NMR spectra of the deacylated dPIM-8m sample were recorded at 25 °C at 11.4–12.4 mM in D_2O in a Shigemi Advanced Microtube matched with D_2O (BMS-005TV) on a Varian Unity 500 Hz spectrometer equipped with a 5 mm Inverse probe. ^1H and ^{13}C chemical shifts are reported in parts per million with respect to external sodium 4,4-dimethyl-4-silapentanesulfonate (δ_H 0.00) and

external dioxane (δ_C 67.40), respectively. For assignment of signals, both two-dimensional homo- and hetero-nuclear NMR experiments were employed, all utilising standard pulse sequences. Experiments included ^{1}H, ^{1}H DQFCOSY, ^{1}H, ^{1}H TOCSY with mixing times ranging from 10 to 140 ms, ^{13}C-decoupled and ^{13}C-coupled inverse ^{1}H-detected ^{1}H, ^{13}C HMQC experiments as well as ^{1}H, ^{13}C HMQC–TOCSY experiments with mixing times up to 140 ms. For sequence information, a NOESY experiment with a mixing time of 200 ms and a ^{1}H, ^{13}C HMBC experiment with a 60 ms delay, were used. All FIDs were generally subjected to zero filling and multiplication with Gaussian weighting factor (F2 dimension) and a shifted sine-bell function (F1 dimension) prior to Fourier transformation. Linear prediction (2–4 times) was also applied on the data points in the second dimension to enhance the resolution in the spectra. ^{31}P NMR spectra were recorded at 121.5 MHz on a Bruker Avance spectrometer, with phosphoric acid (2% H_3PO_4 in D_2O, δ_P 0.00) as external reference. A gradient enhanced inverse ^{1}H-detected ^{1}H, ^{31}P HSQC experiment was used to obtain the ^{1}H, ^{31}P correlations.

3.3. Bacterial cell culture and dPIM isolation

G. sputi ATCC 33609 was obtained from Environmental and Scientific Research, Porirua, New Zealand. The organism was grown on blood agar plates for 72 h at 37 °C and the cells were collected by scraping the agar. They were killed by heating in aqueous suspension to 100 °C for 3 h before being harvested by filtration through filter paper (Whatman #54). After lyophilisation 2.08 g of whole dry cells was recovered.

The lipoglycans were isolated from disrupted cells using the Triton X-114 phase separation technique previously described.[35] Thus, the cells were washed with tris 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 extracts were combined and incubated at 37 °C to induce phase separation, and the 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 after ultracentrifugation. The lipoglycan species were resolved into their class sizes by gel permeation chromatography on a Sephacryl S-200 column using a disaggregating buffer containing deoxycholate as eluant. The fractions containing the phosphatidylinositol manno-oligosaccharides (PIMs) were eluted at K_{av} 0.22 and were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.[36,37] They were combined and desalted by ultrafiltration (Amicon Centriplus YM-3) before lyophilisation. The PIMs were recovered as an amorphous white solid (73 mg, 3.5% based on the dry cell weight).

For the *O*-deacylation of the PIMs, a sample (66.5 mg) was dried under vacuum overnight and suspended in anhydrous hydrazine (1 mL). After being stirred at room temperature for 30 min, the mixture was cooled, and cold acetone (~5 mL, −70 °C) was slowly added to destroy excess hydrazine and precipitate the product. The deacylated products (dPIMs) were recovered by low-speed centrifugation (4 g, 30 min) and the pellet was washed three times with acetone, dissolved in water and lyophilised.

Purification of the dPIMs (20.8 mg) was performed by gel-permeation chromatography (GPC) on a Bio-Gel P2 column eluted with pyridinium acetate buffer (0.07 M, pH 5.4). This yielded an inseparable mixture of dPIM-8, dPIM-7 and dPIM-6 (5.8 mg, 28%) in the ratios 1:0.6:0.5 (mass spectrometric determination). A further fraction contained dPIM-2 (1.5 mg, 7%), which gave ^{1}H and ^{13}C NMR spectral data identical to those previously reported.[20]

3.4. Chemical analysis of dPIM-8m

The *O*-deacylated phosphatidyl inositol mannan mixture dPIM-8m (1 mg) was hydrolysed by treatment with HCl (4 M) at 120 °C for 15 min. Subsequent reduction of the derived sugars with sodium borohydride, and acetylation, generated mannitol and inositol hexaacetates, which were identified by GLC using the authentic compounds for comparison. The absolute configuration of the mannose was established as D by GLC analysis of the acetylated (+)-2-butyl and (+,−)-2-butyl pyranosides derived directly from the mixed hydrolysis products, essentially as described by Leontein et al.[30]

To cleave the phosphate ester a sample of dPIM-8m (3.4 mg) was transferred to a small Eppendorf tube, dissolved in HF (100 μL, 40% aqueous) and left for 48 h at 6 °C.[31] The solution was neutralised by addition to saturated aqueous $Ca(OH)_2$ (100 mL) and the mixture was evaporated almost to dryness. The supernatant, recovered after centrifugation (2000 rpm, 5 min), was lyophilised and the dephosphorylated PIMs were further purified on a DEAE Sepharose column eluted with water to give 1.3 mg (42%) of product.

Methylation analysis was performed on the mixture of deacylated, de-phosphorylated PIMs (1.3 mg) using iodomethane and sodium methylsulfinylmethanide in dimethyl sulfoxide according to the method of Hakomori.[38] The methylated products were recovered by partitioning between chloroform and water and were hydrolysed, reduced and acetylated to give hexa-methylated/acetylated mannitols and 2,6-di-*O*-acetyl-1,3,4,5-tetra-*O*-methyl-*myo*-inositol, which were identified by GLC–MS.

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