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Structural analysis of novel rhamnose-branched oligosaccharides from the glycophosphosphingolipids of *Leptomonas samueli**

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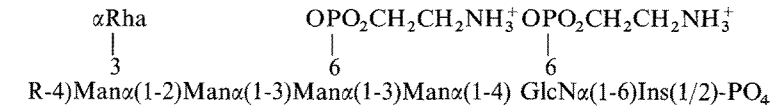
Mild alkaline hydrolysis of the glycophosphosphingolipids of the protozoan *Leptomonas samueli* liberated several phosphoinositol-containing oligosaccharides (PI-oligosaccharides), which were purified by high performance anion exchange chromatography. The oligosaccharides in the resulting four fractions were characterized by methylation analysis, fast atom bombardment mass spectrometry and two-dimensional nuclear magnetic resonance spectroscopy. The oligosaccharides contain the core structure Manα(1-4)GlcNα(1-6)-*myo*-inositol-1-OPO_3, and are substituted with 2 mol of 2-aminoethylphosphonate per mol of oligosaccharide. The nonreducing ends of the oligosaccharides were terminated by rhamnose branched neutral and acidic xylose-containing penta-, hexa-, hepta- and octasaccharides, of which the three most abundant were shown to have the structures:



Where R = Xylβ(1-4)Xylβ(1- (PI-oligosaccharide I)
Xylβ(1-3)Xylβ(1-4)Xylβ(1- (PI-oligosaccharide II)
GlcAα(1-3)Glcα(1-4)Xylβ(1-4)Xylβ(1- (PI-oligosaccharide III)

More tentative structures are also proposed for three minor oligosaccharides.

Keywords: glycophosphosphingolipids; rhamnose-branched oligosaccharide; Trypanosomatidae, *Leptomonas samueli*

Abbreviations: DQCOSY, double quantum filtered correlated spectroscopy; FAB-MS, fast atom bombardment mass spectrometry; GC-MS, gas chromatography-mass spectrometry; GC, gas chromatography; GPI, glycosylphosphatidylinositol; HPAE, high performance anion-exchange chromatography; $[M + H]^+$, protonated molecule; $[M - H]^-$, deprotonated molecule; NOE, nuclear Overhauser enhancement; PAD, pulsed amperometric detection; PI, phospho-inositol; RI, relative intensity; ROESY, rotating frame nuclear Overhauser enhancement spectroscopy; TOCSY, total correlation spectroscopy; AEP, 2-aminoethylphosphonic acid; Glc, glucose; GlcN, glucosamine; GlcNAc, *N*-acetylglucosamine; GlcA, glucuronic acid; Gal, galactose; Man, mannose; Rha, rhamnose; Xyl, xylose; Ins, *myo*-inositol.

Introduction

Parasitic protozoa of the Trypanosomatidae family are the cause of serious diseases of humans and their livestock,

* Dedicated to the Memory of Dr Bernard Fournet, our friend and adviser.

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including leishmaniasis, the various African trypanosomiases and Chagas' disease. All the trypanosomatid parasites of mammals exhibit complex digenetic life-cycles, alternating between an insect vector and their vertebrate host. Other trypanosomatids are monoxenic being restricted to a single invertebrate host, usually an insect or other arthropod, throughout their life cycle [1, 2].

Biochemical comparison between monoxenic and heteroxenic trypanosomatids therefore afford the means of distinguishing between adaptations required for survival in the insect digestive tract and features that have subsequently evolved in response to the new challenges associated with the colonization of vertebrate hosts. Such an approach could potentially identify the molecular determinants of virulence, and might ultimately enable the development of novel chemotherapeutic strategies.

In protozoa, cell surface proteins are frequently attached to membranes via glycosylphosphatidylinositol (GPI) anchors [3]. In the heteroxenic members of the Trypanosomatidae GPI anchors are also sometimes used to attach cell surface carbohydrates to the cell membrane [4]. These GPI anchors are structurally related to the protein linked GPI anchors found in protozoa [3] and in higher eukaryotes [5].

The trypanosomatid parasites of mammals also synthesize cell surface GPIs which do not have covalently attached protein or polysaccharide chains. These structures include the glycoinositolphospholipids of *Leishmania* [6] and the lipopeptidophosphoglycan of *Trypanosoma cruzi* epimastigote forms [7].

Little is known about the mode of anchoring of the cell surface carbohydrates of lower trypanosomatids, though it may be speculated that since GPI anchoring of both proteins and carbohydrates occurs in the heteroxenic trypanosomatids, the same may prove to be the case in the monoxenic species.

This hypothesis is supported by our recent discovery of a novel class of GPI-type glycophosphosphingolipids in the monogenetic trypanosomatid *Leptomonas samueli* [8]. Alkaline hydrolysis of these glycophosphosphingolipids were liberated a mixture of phosphoinositol oligosaccharides and resolved into several fractions by gel filtration chromatography. Two of the fractions (designated A and B) were shown to comprise tetra and pentasaccharide chains containing the core structure Manα(1-3)Manα(1-4-GlcNα(1-6) and were substituted with 2 mol of 2-aminoethylphosphonate (AEP) [8]. A third fraction C, proved to be a mixture of rhamnose branched oligosaccharides. We now report the purification of this fraction by high pH anion exchange chromatography (HPAE), and the characterization of the major phosphoinositol oligosaccharides by nuclear magnetic resonance spectroscopy, methylation analysis and fast atom bombardment mass spectrometry.

Materials and methods

Materials

Deuterium oxide was purchased from Goss Scientific (Ingatestone, UK), and glycerol, dithiothreitol and dithioerythritol were obtained from Sigma Chemical Co. (Poole, UK) and were used without further purification.

Isolation and purification of phosphoinositol (PI) oligosaccharides

Promastigotes of *L. samueli* were cultured as previously described [9]. Glycophosphosphingolipids were extracted and the PI-oligosaccharides liberated from the intact glycophosphosphingolipids by alkaline hydrolysis as previously described [8].

Gel filtration chromatography

The PI-oligosaccharides isolated by alkaline hydrolysis were applied to a column of Bio-Gel P-4 (1 × 100 cm) and eluted with water. Fractions of 1 ml were collected and assayed for carbohydrate by the phenol-sulfuric acid procedure [10].

High pH anion exchange chromatography

The material eluted in the void volume of the Bio-Gel P-4 column was further fractionated by HPAE chromatography using a Carbopac PA-1 preparative column (0.9 × 25 cm, Dionex, Sunnyville, CA) and pulsed amperometric detection (PAD). The mobile phase initially consisted of 80% solution A (0.1 M NaOH) and 20% solution B (0.1 M NaOH and 1 M NaOAc). After injection the proportion of solution B was increased to 100% over a 10 min period by means of a linear gradient. The flow rate was 4 ml min^{-1}. Peaks were collected when an increase in the baseline slope was detected. The four major fractions obtained were neutralized with acetic acid and applied to a 1.5 × 10 cm column of Dowex-50 (8% cross linked; 20–50 mesh) in the H^+ form and eluted with water. The eluates were freeze dried, dissolved in water and further desalted by passage through a column of Bio-Gel P-2 (0.8 × 30 cm).

Analytical procedures

Neutral and acidic sugars were determined by GC of their trimethylsilyl ethers following methanolysis at 80 °C [11]. Inositol and glucosamine were determined by GC after methanolysis followed by hydrolysis, reduction and acetylation, as previously described [8]. Total phosphorus was determined by the method of Ames [12] and acid-hydrolysable phosphorus by Bartlett's method [13].

Methylation analysis

The PI-oligosaccharides I and II were methylated by the procedure of Ciukanu and Kerek [14]. The uronic acid containing PI-oligosaccharides III and IV were methylated with methyl iodide in the presence of methysulfinyl anion

[15] and were then reduced with sodium borodeuteride in dimethylsulfoxide. The permethylated oligosaccharides I to IV were methanolysed with 0.5 M HCl in methanol (18 h at 80 °C). The methanolysate was dried in a stream of nitrogen and acetylated with acetic anhydride:pyridine (9:1 by vol) for 24 h at 18 °C, and analysed by GC on an OV-101 capillary column (25 m × 0.2 mm) using helium as carrier gas (0.5 bar). The column oven was temperature programmed from 120–180 °C at 2 °C per min. The acetylated partially methylated methyl glycosides were initially identified by comparison of their retention times with those of authentic standards, and the identifications were confirmed by GC-MS [16].

NMR Spectroscopy

Proton and ^{13}C NMR spectra were obtained at 30 °C using a Varian Unity 500 spectrometer equipped with 5 mm triple resonance and broadband probes. TOCSY [17], double and triple quantum filtered COSY [18, 19] and ROESY [20, 21] spectra were obtained using standard pulse programs and were collected in the phase sensitive mode using the method of States *et al.* [22]. The spin lock field was generated by continuous low power irradiation from the transmitter. For all spectra, the transmitter frequency was located at the same position as the water resonance. A ROESY spectrum of fraction II was obtained at 600 MHz and 30 °C on a Varian Unity 600 spectrometer. Mixing times were 150 ms in the ROESY experiment and 60 ms in the TOCSY spectra, apart from an additional TOCSY spectrum on fraction I with an 80 ms mixing time. Carbon spectra were obtained using the same spectrometer in a 5 mm broad-band probe. The proton spectrum of the mixture was referenced to internal acetone at 2.225 ppm and those of the HPAE-purified fractions via the rhamnose methyl group at 1.268 ppm. Spectral widths of 2080 Hz were used in both domains and typically 16 scans were collected at each of 512 increments in t_1 for correlation spectra. The ^{31}P NMR spectrum of the fraction C mixture was obtained at 81 MHz and 30 °C on a Bruker WM200 spectrometer equipped with a 5 mm dedicated ^{31}P probe, and referenced to external 85% H_3PO_4 at 0 ppm.

A ^{13}C-detected ^1H-^{13}C correlation spectrum [23] of the mixture was obtained at 30 °C and 125 MHz on a Bruker AM500 spectrometer equipped with a 5 mm broad-band probe.

Fast atom bombardment mass spectrometry

Fast atom bombardment mass spectra were recorded using either Kratos MS50 (for the mixture) or Kratos MS80RFA spectrometers (for purified fractions I–IV), both equipped with Ion Tech FAB guns using xenon atoms as the bombarding particles. The liquid matrices were either monothioglycerol for the unseparated mixture or a 1:1 mixture of glycerol and dithiothreitol/dithioerythritol (5:1) for the HPAE purified fractions. Underivatized samples were dissolved in 30% acetic acid to a concentration of about 10 μg μl^{-1} and 1 μl was mixed with an equal volume of matrix on the FAB probe. The spectra of fractions I and III were obtained at resolution of 3000 and those of fractions II and IV a resolution of 1000.

Results

Isolation and analysis of PI-oligosaccharides from L. samueli

In a previous paper [8] we described the purification and characterization of two phosphoinositol oligosaccharides obtained by mild alkaline hydrolysis and Bio-Gel chromatography of a glycophosphosphingolipid from *L. samueli*. Another fraction, eluting in the void volume of the Bio-Gel column was not characterized further at that time. Compositional analysis of this material revealed mannose, xylose, rhamnose, glucose, glucuronic acid, glucosamine, inositol, phosphate and phosphonate. A positive ion FAB mass spectrum was recorded and protonated molecules were observed at *m/z* 1694, 1856, 2032 and 2164, of which *m/z* 1694 was the most abundant. The 500 MHz ^1H NMR spectrum showed not only heterogeneity due to migration of the phosphate group during base cleavage, as noted previously [8] but also a high degree of heterogeneity associated with the oligosaccharide chain which prevented full structural analysis. It was therefore decided to separate this fraction by HPAE-PAD chromatography prior to further characterization.

Fractionation by HPAE-PAD

On preparative Dionex chromatography four major peaks were obtained (Fig. 1) and the collected fractions were designated I, II, III and IV. All four peaks had an unresolved trailing shoulder which may be attributable to heterogeneity arising from the phosphate migration from *O*–1 to *O*–2 of inositol during alkaline cleavage. The ratio of the heights of the major and the trailing peaks was approximately that found in the NMR spectrum for the two α-GlcN H-1 resonances. The shapes of peaks I and III suggested that the corresponding fractions were heterogeneous, probably as a result of structural variation in the oligosaccharide chain. Figure 2 shows the anomeric region of the 500 MHz NMR spectra of the four fractions.

Composition of fractions I, II, III and IV

Compositional analysis showed that all fractions contained mannose, rhamnose, glucosamine, inositol, 2-aminoethylphosphonate and phosphate in the molar ratios 4:1:1:1:2:1. In addition, fraction I contained 2 mol of xylose and a small amount of glucose; fraction II contained 3 mol of xylose; fraction III contained 1 mol glucose, 1 mol glucuronic acid and 2 mol xylose, and fraction IV contained 1 mol glucose, 1 mol glucuronic acid and 3 mol xylose. The methylation analysis of these fractions is summarized in Table 1.

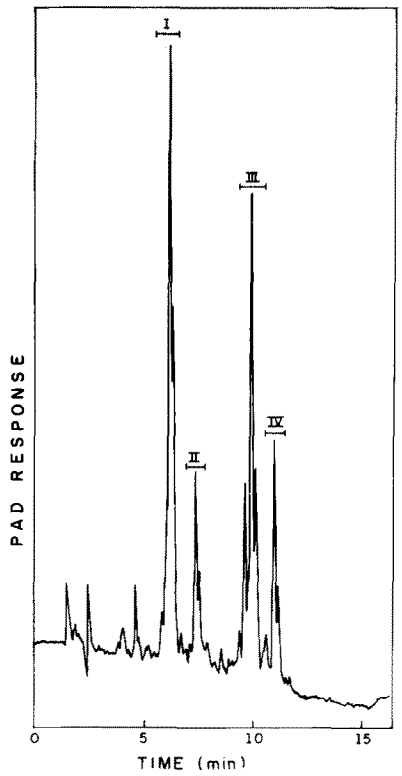

Figure 1. Separation of fractions I–IV by HPAE with PAD.

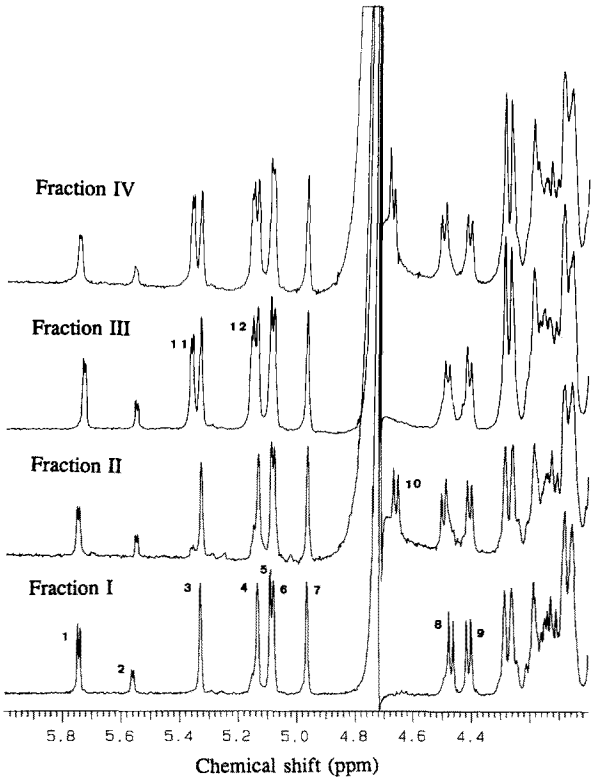

Figure 2. The anomeric region of the 500 MHz ^{1}H NMR spectra of fractions I, II, III and IV obtained at 30 °C. Peak numbering is as follows: 1. α-GlcN(1) H-1; 2. α-GlcN(2) H-1; 3. α-Man(1) H-1; 4. α-Man(3) H-1; 5. α-Man(2) H-1; 6. α-Man(4) H-1; 7. α-Rha H-1; 8. β-Xyl(2) H-1; 9. β-Xyl(1) H-1; 10. β-Xyl (3) H-1; 11. α-GlcA H-1; 12. α-Glc H-1.

Table 1. Methylation analysis of the PI-oligosaccharide fractions from *Leptomonas samueli* glycophosphosphingolipids.

O-Acetylated partially O-methylated methyl glucosides[a]	*PI-oligosaccharide fractions*			
	I	*II*	*III*	*IV*
Methyl 2,3,4-tri-*O*-methylxyloside	0.6	0.7	–	0.6
Methyl 2,3,4-tri-*O*-methylrhamnoside	0.7	0.7	0.8	0.7
Methyl 2,4-di-*O*-methylxyloside	0.1	0.6	–	–
Methyl 2,3-di-*O*-methylxyloside	0.9	0.8	1.7	1.7
Methyl 2,3,4,6-tetra-*O*-methylglucoside	0.1	–	1.0	1.0
Methyl 3,4,6-tri-*O*-methylmannoside	1.0	1.0	1.0	1.0
Methyl 2,4,6-tri-*O*-methylmannoside	0.8	0.7	0.7	0.8
Methyl 2,4,6-tri-*O*-methylglucoside	–	0.2	1.0	–
Methyl 2,6-di-*O*-methylmannoside	1.0	0.9	1.0	1.0
Methyl 4,6-di-*O*-methylglucoside	–	–	–	0.6

[a] *O*-acetylated partially *O*-methylated methylglycosides were identified and quantified by GC-MS.

Characterization of fraction I

Positive mode fast atom bombardment mass spectrometry of fraction I (Fig. 3) revealed an $[M + H]^+$ signal at *m/z* 1694.3 (monoisotopic). In the negative mode a deprotonated molecule was observed at *m/z* 1692.6. This is compatible with a composition of 4 mol hexose, 2 mol pentose, 1 mol hexosamine, 1 mol deoxyhexose, 1 mol inositol, 1 mol phosphate and 2 mol AEP, for which the calculated M_r is 1693.5 (monoisotopic), in good agreement with the compositional data above. A weak signal (relative intensity ≈10% of *m/z* 1694) was observed at *m/z* 1856, which presumably corresponds to the $[M + H]^+$ of an oligosaccharide of identical composition except for the presence of an additional hexose residue.

Fragments observed in the FAB spectrum provided evidence of the sequence of residues and the location of the AEP substituents. The fragmentations observed were predominantly cleavages distal to the glycosidic oxygen, the charge being retained on the inositol-phosphate bearing end of the molecule. These are identified in Fig. 3 as ions of type Y_n in the Domon and Costello system of nomenclature [24].

The Y_n cleavages were especially facile distal to the AEP-substituted residues (producing *m/z* 960 and 529) [8]. The observation of ring cleavage ions (of type $^{1,5}X_n$) 28 U above the Y_n signals provided further support for the assignment. Below *m/z* 1122 Z_n ions (18 U lower than corresponding Y_n) were also present. Both of these subsidiary fragmentations became more intense at lower mass.

The presence of a deoxyhexose branch was inferred from a weak ion *m/z* 1548 (M + H-146) and its location at Man(4) (see Fig. 6) was deduced from the interruption of the regular sequence of glycosidic cleavage ions between *m/z* 1430 and 1122, corresponding to the elimination of the combined residue masses of hexose and deoxyhexose (Fig. 3).

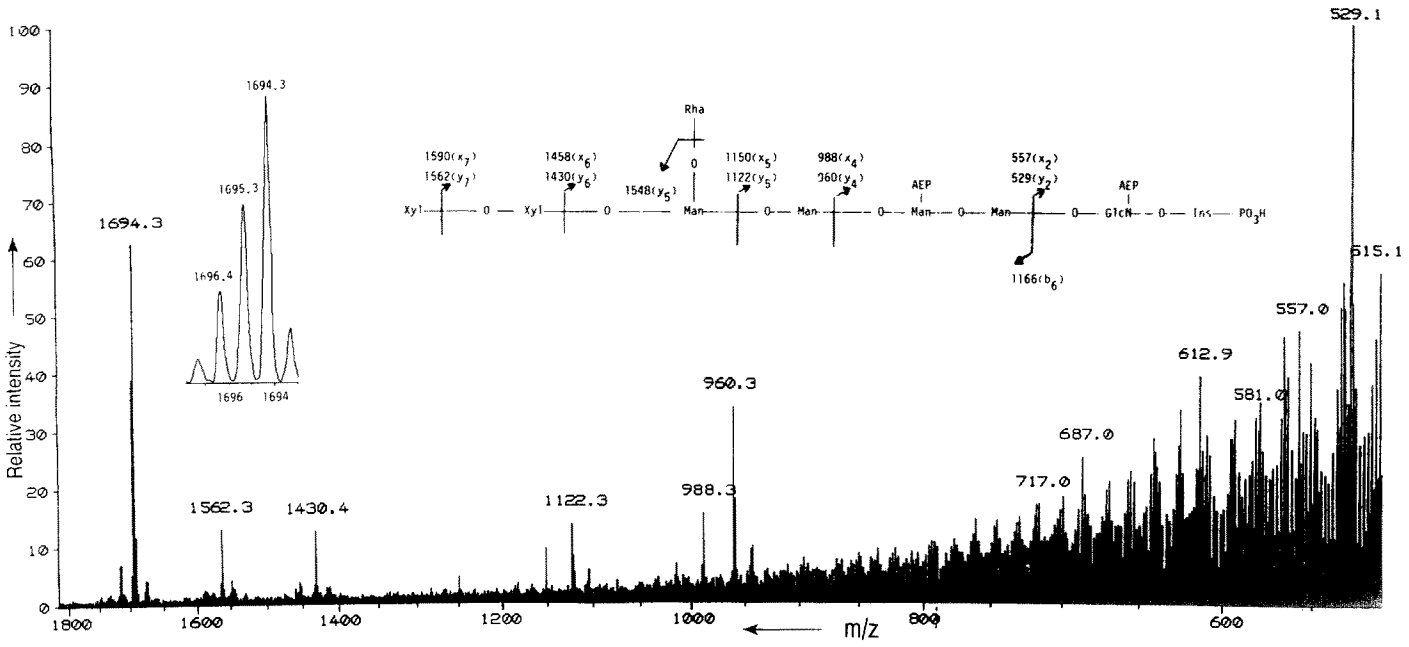

Figure 3. Fast Atom Bombardment Mass Spectrum of Fraction I. The inset shows an expansion of the molecular ion region.

Almost complete assignments of the proton NMR spectra were achieved from DQCOSY and TOCSY spectra of fraction III (Fig. 4), although some tentative assignments, given in square brackets, were made by comparison with data obtained from the mixture (Table 2). Sugar configuration was established by analysis of inter-proton coupling constants (obtained from the 2D spectra). Xylose was distinguished from glucose by the presence of the lowfield H-5e. Anomeric configurations were established from $^3J_{1,2}$ and chemical shift data.

Comparison between literature values and the proton chemical shifts for the α-rhamnose residue and a β-xylose residue with an anomeric proton resonance at 4.476 ppm (β-Xyl(2)) (Table 2), showed that these residues were terminal [25–30]. In a ROESY spectrum (using a 150 ms spin lock generated by continuous low power irradiation), the β-Xyl(2) H-1 showed intra-residue NOEs to H-2, H-3 and H-5a and an inter-residue NOE to β-Xyl(1) H-4, establishing the Xyl(2)β(1-4)Xyl(1)β(1- substructure. The ^{1}H chemical shift of β-Xyl(2) at 4.476 ppm was close to that found in the nonreducing residue of -4)Xylβ (reported as 4.457 ppm for β-xylobiose in [29]), in agreement with the presence of a β(1-4)-linkage.

In the ROESY spectrum of fraction III (Fig. 5) additional NOEs were observed between β-Xyl(2) H-1 and β-Xyl(1) H-5e. Mass spectrometric evidence showed that β-Xyl(1) is linked to the branch point mannose, identified as -3,4)-Man*p*-(1- from methylation analysis data (Table 1). No inter- or intra-residue NOE were observed from β-Xyl(1) H-1 in the ROESY spectrum of fraction I, but an NOE to α-Man(4) H-4 at 3.931 ppm and another to a resonance at 3.827 ppm, tentatively assigned as α-Man(4) H-6 were observed in the ROESY spectrum of fraction III.

The α-rhamnose H-1 showed an inter-residue NOE to α-Man(4) H-2 and an intra-residue NOE to H-2. Unfortunately, α-Man(4) H-3 is almost coincident with α-Rha H-2, obscuring any inter-residue NOE. Inter-residue NOEs to an equatorial β-hydrogen are frequently observed [31].

Analysis of the region of the ROESY spectrum of fraction I containing the α-Man linkage data was complicated by the overlap of resonances. A somewhat better spectrum was obtained for fraction III. Any α-Man(4) H-1 ROESY crosspeak to its own H-2 was obliterated by a strong α-Man(4) H-1/H-2 TOCSY artefact, but an inter-residue NOE to α-Man(3) H-2 was observed. TOCSY artefacts can be readily distinguished from ROESY crosspeaks by their different phases: the ROESY peaks are 180° out of phase with the diagonal whilst TOCSY peaks are in phase. In the ^{1}H-^{13}C correlation spectrum of the mixture, α-Man(3) H-2 was found to resonate at a low field, consistent with a Man(4)α(1-2)Man(3)α(1- linkage. The α-Man(3) H-1 showed an intra-residue NOE to H-2 and a NOE to the α-Man(2) H-3, which also appeared (with opposite phase) in the α-Man(2) H-1 track due to strong TOCSY transfer through α-Man(2) H-2. Any NOE transfer between α-Man(3) H-1 and α-Man(2) H-2 was hidden by the strong α-Man(4) H-1/H-2 TOCSY artefact, but a Man(3)α(1-3)Man(2)α(1- linkage) is proposed. α-Man(2) forms part of the core structure described previously [8] so the spectral analysis is not repeated here.

A minor component, present at about the 10% level was also observed and contributed additional anomeric resonances at 5.154 and 4.498 ppm (Table 2). The TOCSY

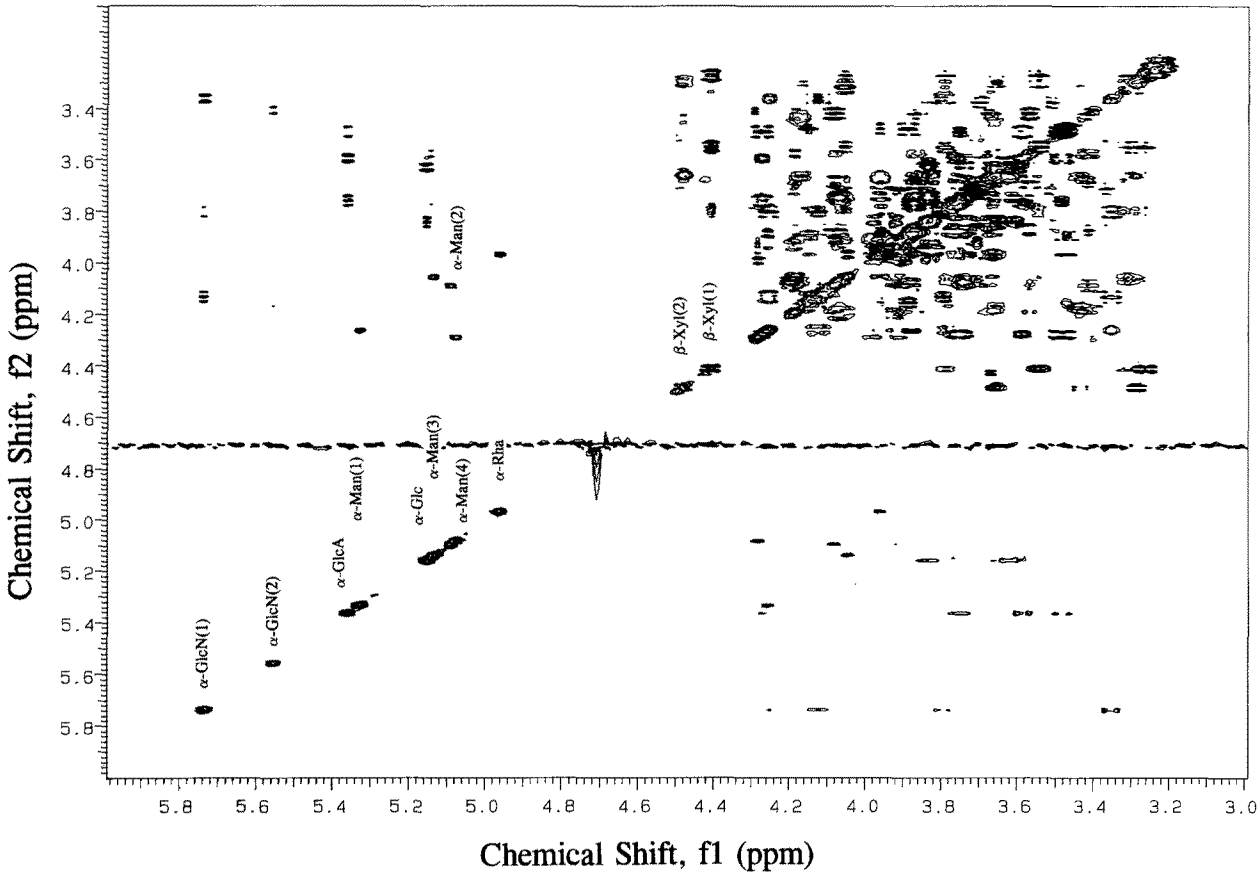

Figure 4. Partial 500 MHz TOCSY spectrum of fraction III obtained at 30 °C and with a 60 ms mixing time. The spin systems are labelled. Only positive contours have been plotted.

spectrum enabled partial assignments of these spin systems, which were consistent with a homologous containing an additional α-Glc residue, designated here as fraction Ia. An analysis of the chemical shifts of the β-Xyl(2) residue in fraction Ia suggested a Glcα(1-4)Xyl(2)β(1- linkage). The signal at *m/z* 1856 in the positive mode FAB spectrum of fraction I is also consistent with the presence of this species.

Characterization of fraction II

Protonated or deprotonated molecules were obtained on fast atom bombardment mass spectrometry at *m/z* 1826.6 ($[M + H]^+$) in the positive mode and 1824 ($[M - H]^-$) in the negative mode respectively. These imply a composition of 4 mol hexose, 3 mol pentose, 1 mol deoxyhexose, 1 mol hexosamine, 1 mol inositol, 1 mol phosphate and 2 mol AEP (calculated M_r = 1825.5), which is in agreement with the analytical results, and suggests that this oligosaccharide differs from oligosaccharide I by the presence of an extra pentose residue. The signal-to-noise in this spectrum was poorer than in the spectrum of fraction I, but signals at *m/z* 1722 and 1694 ($^{1,5}X_8$ and Y_8) were consistent with the terminal location of the additional pentose. Apart from the Y_5 ion at *m/z* 1680, attributable to the loss of the deoxyhexose branch, the fragmentation appeared very similar to that of fraction I.

Consistent with the mass spectrometric data from this fraction, NMR analysis revealed the presence of an additional β-xylose residue with an anomeric resonance at 4.663 ppm (Table 2). The chemical shifts for this residue were compatible with a terminal location, whereas the chemical shifts of the β-Xyl(2) residue were considerably perturbed compared with fraction I. The 600 MHz ROESY spectrum showed crosspeaks between β-Xyl(3) H-1 and a resonance at 3.64 ppm, and between β-Xyl(2) H-1 and β-Xyl(1) H-4 and H-5e, consistent with a Xyl(3)β(1-3)Xyl(2)β(1-4)Xyl(1)β-(1- substructure at the non-reducing terminus. Minor resonances assignable to the GlcAα(1-3)Glcα(1- substructure found in fraction III, suggested some cross contamination.

Characterization of fraction III

Positive ion fast atom bombardment mass spectrometry of fraction III produced a protonated molecule at *m/z* 2032.4, consistent with the composition of 5 mol hexose, 2 mol pentose, 1 mol hexuronic acid, 1 mol deoxyhexose, 1 mol hexosamine, 1 mol inositol, 1 mol phosphate and 2 mol AEP, for which the calculated M_r is 2031.6 (monoisotopic). Weaker $[M + H]^+$ ions were present at *m/z* 2164 and 2326. The major species at *m/z* 2032 corresponds to the addition of a hexose and hexuronic acid residue to oligosaccharide I.

Table 2. Proton NMR assignments of the *Leptomonas samueli* PI-oligosaccharide fractions[a].

Fraction I	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6/H5'*	*H-6'*
Ins-1-P	4.087	4.195	3.576	3.688	3.434	3.876	
Ins-2-P	–	–	–	–	–	3.846	
α-GlcN(1)	5.755	3.355	4.132	3.806	4.262	4.126	[4.126]
α-GlcN(2)	5.567	3.38	4.153	3.779	–	–	–
α-Man(1)	5.336	4.265	3.886	[3.968	3.952]	–	–
α-Man(2)	5.094	4.091	3.983	[3.703	4.191	4.070	3.936]
α-Man(3)	5.139	4.059	3.966	3.741	–	–	–
α-Man(4)	5.083	4.296	3.998	3.929	–	[3.827]	–
α-Rha	4.968	3.971	3.889	3.478	4.157	1.268	
β-Xyl(1)	4.415	3.272	3.552	3.800	4.059	3.317	
β-Xyl(2)	4.475	3.255	3.430	3.627	3.966	3.307	
Minor components							
β-Xyl	4.498	3.316	3.691	3.506	–	–	–
α-Glc	5.154	3.549	3.680	3.592	–	–	–
Fraction II	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6/H5'*	*H-6'*
Ins-1-P	4.087	4.195	3.576	3.688	3.426	3.876	
Ins-2-P	–	–	–	–	–	3.846	
α-GlcN(1)	5.752	3.358	4.126	3.811	–	–	–
α-GlcN(2)	5.554	–	–	–	–	–	–
α-Man(1)	5.334	4.273	3.880	3.968	–	–	–
α-Man(2)	5.094	4.096	3.901	[3.703]	–	–	–
α-Man(3)	5.138	4.066	[3.961]	–	–	–	–
α-Man(4)	5.083	4.297	3.991	3.932	–	[3.827]	–
α-Rha	4.968	3.976	3.891	3.478	4.151	1.268	
β-Xyl(1)	4.410	3.268	3.546	3.792	4.056	–	
β-Xyl(2)	4.498	3.465	3.632	3.685	4.001	[3.301]	
α-Man(3)	4.663	3.323	3.459	3.632	3.971	3.310	
Minor components							
β-Xyl?	4.472	3.654	–	–	–	–	
β-Xyl?	4.444	3.654	–	–	–	–	
Fraction III	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6/H5'*	*H-6'*
Ins-1-P	4.197	4.084	3.568	3.687	3.426	3.868	
Ins-2-P	–	–	–	–	–	3.846	
α-GlcN(1)	5.733	3.354	4.127	3.796	4.256	4.116	–
α-GlcN(2)	5.553	3.390	4.162	–	–	–	–
α-Man(1)	5.328	4.257	3.890	3.970	–	–	–
α-Man(2)	4.999	4.084	3.908	3.703	–	–	–
α-Man(3)	5.141	4.066	3.961	[3.741]	–	–	–
α-Man(4)	5.078	4.284	3.986	[3.932]	–	[3.827]	–
α-Rha	4.962	3.983	3.889	3.473	4.148	1.268	–
β-Xyl(1)	4.406	3.265	3.545	3.792	4.056	3.318	–
β-Xyl(2)	4.475	3.288	3.656	3.661*	4.169	3.427	
α-Glc	5.153	3.606	3.836	3.569	–	–	–
α-GlcA	5.348	3.588	3.753	3.490	4.271		
Minor components							
β-Xyl?	4.496	3.380	3.520	3.715	4.200	–	
β-Xyl?	4.431	3.338	3.690	3.690	–	–	
α-Glc?	5.149	3.631	3.777	–	–	–	–

(*continued*)

Table 2. (*continued*)

Fraction IV	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6/H5'*	*H-6'*
Ins-1-P	–	–	–	–	–	–	–
Ins-2-P	–	–	–	–	–	–	
α-GlcN(1)	5.751	–	–	–	–	–	
α-GlcN(2)	5.559	–	–	–	–	–	
α-Man(1)	5.334	–	–	–	–	–	
α-Man(2)	5.093	–	–	–	–	–	
α-Man(3)	5.139	–	–	–	–	–	
α-Man(4)	5.085	–	–	–	–	–	
α-Rha	4.969	–	–	–	–	1.268	
β-Xyl(1)	4.412	–	–	–	–	–	
β-Xyl(2)	4.498	–	–	–	–	–	–
β-Xyl(3)	4.676	–	–	–	–	–	–
α-Glc	5.161	–	–	–	–	–	–
α-GlcA	5.363	–	–	–	–	–	–

[a] Residues are numbered from the Ins outwards, i.e. towards the nonreducing terminal α-GlcN(1) and α-GlcN(2) refer to components phosphorylated at Ins *O*-1 or *O*-2 respectively.
[ ] Assigned tentative.
* Assignment on the basis of a ROESY crosspeak with the β-Xyl(2) H-5e: an intense peak was observed in the β-Xyl(2) H-1 trace of the TOCSY spectrum corresponding to [H-3 + H-4].

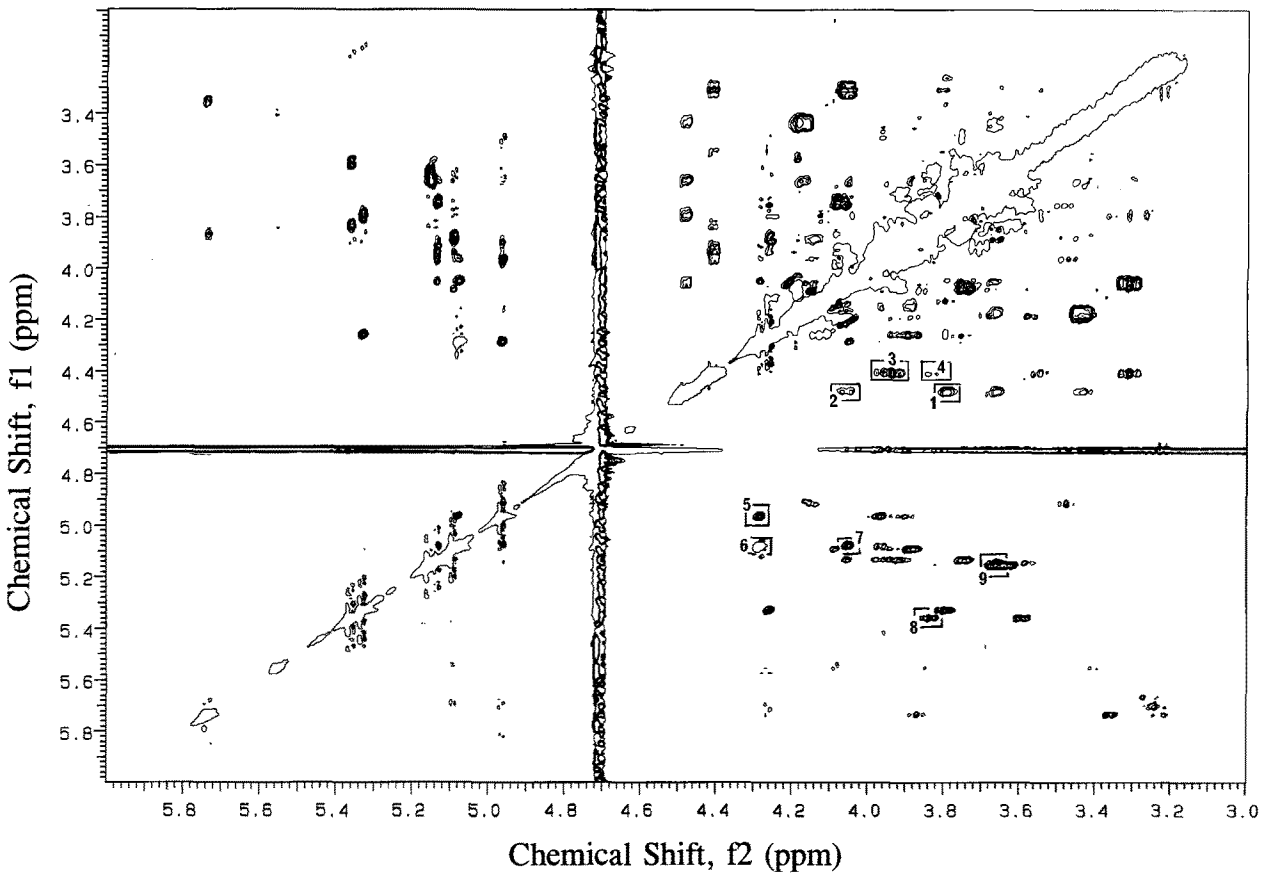

Figure 5. Partial 500 MHz ROESY spectrum of fraction III obtained at 30 °C and with a 2.5 kHz, 150 ms spin lock generated by continuous low power irradiation. NOE peaks are plotted positive and only one contour is plotted for the diagonal and TOCSY artefacts. A number of peaks comments on in the text are boxed and numbered: 1. the NOE between β-Xyl(2) H-1 and β-Xyl(1) H-4; 2. the NOE between β-Xyl(2) H-1 and β-Xyl(1) H-4; 3. the NOE between β-Xyl(1) H-1 and α-Man(4) H-4; 4. the NOE tentatively identified as between β-Xyl(1) H-1 and α-Man(4) H-6; 5. the NOE between α-Rha H-1 and α-Man(4) H-2; 6. the TOCSY artefact between α-Man(4) H-1 and H-2; 7. the NOE between α-Man(4) H-1 and α-Man(3) H-2; 8. the NOE between α-GlcA H-1 and α-Glc H-3; 9. the NOE between α-Glc H-1 and β-Xyl(2) H-4.

Fragment ions at *m/z* 1856 (Y_9), 1722 (X_8), 1694 (Y_8) and 1562 (Y_7) suggest that the nonreducing terminal sequence is hexuronic acid-hexose-pentose. A weak ion at *m/z* 1886 (Y'_5) was consistent with a deoxyhexose branch. Most of the other fragment ions (*m/z* 1430, 1122, 960, and 529) were similar to those in fraction I.

Weak signals present at *m/z* 1407, 1284 and 1250 could not be assigned. These may be related to the peak in the Dionex chromatogram with slightly shorter retention time which was collected along with the main peak. An attempt to obtain a 'pure' spectrum by collisional activation of *m/z* 2032 and analysis of the daughters by means of a scan at constant B/E was unsuccessful.

Compared with fraction I, the NMR spectrum of fraction III contained spin systems for an α-GlcA residue (with an anomeric proton resonance at 5.348 ppm) and an α-Glc residue (anomeric proton at 5.153 ppm) (Table 2), in accord with the mass spectrometric and analytical data. Chemical shift comparisons with model systems [30], were consistent with a terminal location for the α-GlcA residue, which was also supported by methylation analysis (Table 1). The chemical shifts of the β-Xyl(2) residue were strongly perturbed compared with fraction I. Analysis of the ROESY spectrum of fraction III (Fig. 5) showed an inter-residue NOE from α-GlcA H-1 to a resonance at 3.839 ppm assigned as α-Glc H-3. The α-Glc H-1 showed an inter-residue NOE to a resonance at 3.662 ppm, consistent with either H-3 or H-4 of β-Xyl(2). This linkage was assigned as (1-4) on the basis of the lack of 2,4-dimethylxylose in the methylation analysis of this fraction (Table 1). Structurally important NOEs are listed in Table 3. Signals attributable to two minor components were detected in the FAB-MS and the NMR spectra of this fraction. These components had $[M + H]^+$ at *m/z* 2164 (RI ≈ 5% of *m/z* 2032) and *m/z* 2326 (RI ≈ 10% of *m/z* 2032) corresponding to an additional pentose or an additional (pentose + hexose) unit compared to the major component. In the 2D NMR spectra an additional α-Glc and two additional β-Xyl spin systems were observed. These are tentatively assigned to components containing an extra Glcα(1-4)Xylβ(1- disaccharide or Xylβ(1- monosaccharide linked to *O*-4 of the α-Glc residue of oligosaccharide III, by analogy with data obtained from the glucuronoxylan of *L. samueli* [9].

Characterization of fraction IV

Because only a limited amount of sample was available, the positive ion FAB spectrum of fraction IV was obtained at low resolution, so as to maximize sensitivity. Thus the $[M + H]^+$ signal observed at *m/z* 2165.0 represents an average, not a monoisotopic value, and was consistent with a composition of 5 mol hexose, 3 mol pentose, 1 mol hexoronic acid, 1 mol hexosamine, 1 mol deoxyhexose, 1 mol inositolphosphate and 2 mol AEP. Although the signal to noise ratio was poor, the presence of fragment ions at *m/z* 1932 and 1694 was compatible with a structure in which the terminal hexose of oligosaccharide Ia was substituted with hexuronic acid and pentose, which could also explain the presence of 4,6-dimethyglucoside in the methylation analysis.

Discussion

Several phosphoinositol oligosaccharides were liberated from the glycophosphosphingolipids of the monoxenic

Table 3. Inter- and intra-residue NOEs involving anomeric resonances from ROESY spectra of PI-oligosaccharide III.

From	*Inter-residue*	*Intra-residue*
α-GlcN(1) H-1 to	3.868 (Ins(1) H-6)	3.358 (α-GlcN(1) H-2)
α-GlcN(2) H-1 to	3.846 (Ins(2) H-6)	3.406 (α-GlcN(2) H-2)
α-GlcA H-1 to	3.839 (α-Glc H-3)	3.592 (α-GlcA H-2)
α-Man(1) H-1 to	3.796 (α-GlcN H-4)	4.258 (α-Man(1) H-2)
		3.882 (α-Man(1) H-3 via TOCSY)
α-Glc H-1 to	3.662 (β-Xyl(2) H-4)	3.626 (α-Glc H-2)
α-Man(3) H-1 to	3.916 (α-Man(2) H-3)	4.064 (α-Man(3) H-2)
		3.966 (α-Man(3) H-3 via TOCSY)
		3.741 (α-Man(3) H-4 via TOCSY)
α-Man(2) H-1 to	3.884 (α-Man(1) H-3)	4.086 (α-Man(2) H-2)
α-Man(4) H-1 to	4.052 (α-Man(3) H-2)	4.280 (and TOCSY α-Man(4) H-2)
		3.976 (α-Man(4) H-3 via TOCSY)
α-Rha H-1 to	4.280 (α-Man(4) H-2)	3.897 (α-Rha H-3 via TOCSY)
	3.959 (α-Man(4) H-3 and α-Rha H-2)	
β-Xyl(1) H-1 to	3.934 (α-Man(4) H-4)	3.307 (β-Xyl(1) H-5a)
(4.402)	3.827 (α-Man(4) H-6??)	3.543 (β-Xyl(1) H-3)
β-Xyl(2) H-1 to	4.056 (β-Xyl(1) H-5e)	3.658 (β-Xyl(2) H-3)
(4.476)	3.790 (β-Xyl(1) H-4)	3.430 (β-Xyl(2) H-5a)

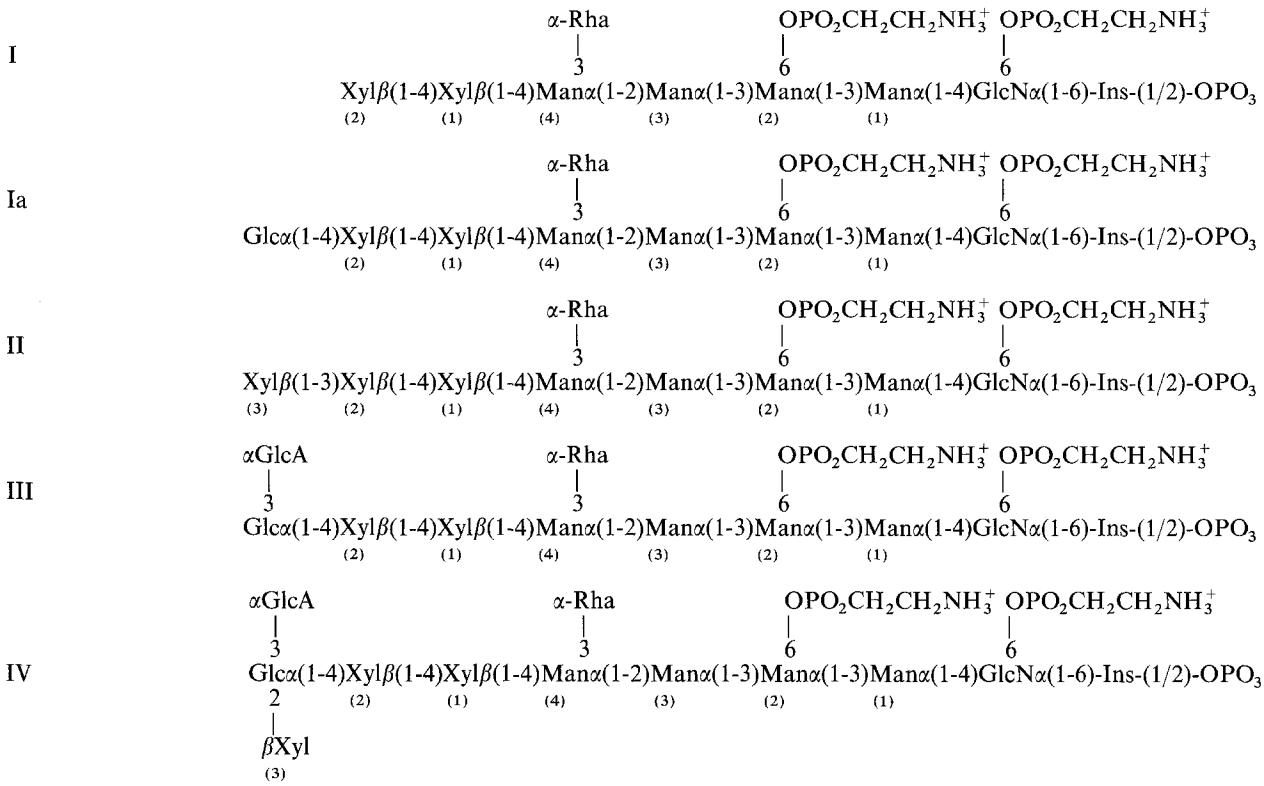

Figure 6. Structures of the PI-oligosaccharides I, Ia, II, III and IV.

trypanosomatid parasite, *Leptomonas samueli* by mild alkaline hydrolysis and were separated by HPAE chromatography. The structures of the purified oligosaccharides were determined by methylation analysis, NMR spectroscopy and fast atom bombardment mass spectrometry. The use of this combination of techniques enabled an almost complete assignment of the NMR spectra of the major oligosaccharides (Table 2). All were rhamnose-branched oligosaccharides (Fig. 6) containing the core sequence of Manα(1-4)GlcNα(1-6)Ins-1-OPO_3 which, in the intact glycophosphosphingolipid, is covalently linked to a ceramic structure. The lipopeptidophosphoglycan of *T. cruzi* is also ceramide linked, but the long chain base is spinganine [7] rather than the phytosphingosine found in *L. samueli* glycophosphosphingolipids [8].

Two mol of AEP were present per mol of oligosaccharide. One of the AEP substituents is attached to *O*–6 of the glucosamine residue and the other is linked to *O*–6 of the second mannose distal to inositol. Substitution with AEP is also observed in lipopeptidophosphoglycan though only a single substituent is present, linked to *O*–6 of glucosamine [7]. AEP linked to *O*–6 of mannose and *N*-acetylglucosamine has also recently been demonstrated in the glycan chains of apolipophorin III from *Locusta migratoria* [32].

All previously characterized GPI structures from trypanosomatids have carbohydrate chains containing predominantly mannose or mannose plus galactose (either Gal*f* or Gal*p*) residues. In contrast *L. samueli* synthesizes glycolipids bearing a monorhamnosyl sidechain which is 3-*O*-linked to the fourth mannosyl residue distal to inositol (Fig. 6). This mannose residue is further substituted by varying nonreducing termini to build a series of either neutral chains (containing xylose; Xylβ(1-3)Xyl or Glcα(1-4)Xyl units) or acidic chains (containing GlcAα(1-3)Glcα(1-4)Xyl or GlcAα(1-3)[Xylβ(1-2)]Glcα(1-4)Xyl units).

The function of these molecules from *L. samueli* is unknown. It is possible that the presence of a negatively charged glycocalyx affords the organism some protection against the hydrolytic environment of the insect gut in the same way that lipophosphoglycan has been shown to increase the survival time of *L. major* in the stomach of the sandfly *Phlebotomus papatasi* [33]. There is also a growing body of evidence that GPI-linked oligosaccharides mediate the process of attachment of trypanosomatid parasites to the gut epithelium of their insect hosts [34–36].

The phylogenetic significance of the structural diversity of the GPI-related molecules of trypanosomatids is likewise unknown. If it is accepted that these molecules are implicated in the process of attachment to and colonization of the insect gut then it is plausible that some of this structural variation is a consequence of the necessity to bind to lectin-like receptors on the surface of the gut epithelium, the nature of which will vary between hosts of different species. In this context it is possibly significant that *Herpetomonas samuelpessoai*, a protozoan that shares the

same insect host (*Zelus leucogramus*) as *L. samueli* [37] and synthesizes both a xylose-rich polysaccharide [38]. The similarities between the glycoconjugates of *L. samueli* and *H. samuelpessoai* may thus be the result of convergent evolution in which organisms of differing phylogeny synthesize similar structures in response to common selective pressures. Studies of other genera and species of trypanosomatids that inhabit the same invertebrate host are needed to clarify the relationship between glycoconjugate structure and host-parasite interaction.

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