Chemical characterisation of glycosylinositolphospholipids of Herpetomonas samuelpessoai ☆

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Abstract

The structure of two glycosylinositolphospholipids of the cell surface of the monoxenic protozoan Herpetomonas samuelpessoai have been deduced by methylation analysis, fast-atom bombardment mass spectrometry and two dimensional nuclear magnetic resonance spectroscopy. These glycolipids have features in common with the glycoinositolphospholipids of both Leishmania and Trypanosoma cruzi, resembling the former by the presence of the hybrid type core sequence Man $\alpha 1 \rightarrow 3$(Man $\alpha 1 \rightarrow 6$)Man $\alpha 1 \rightarrow 4$GlcN$\alpha 1 \rightarrow 6$ myo-inositol-1-PO$_4$-lipid, while the 2-aminoethylphosphonate substituent on O–6 of glucosamine and the presence of ceramide in place of glycerol lipids is more reminiscent of T. cruzi. Possible phylogenetic implications of these observations are discussed.

1. Introduction

Glycoinositolphospholipids (GIPLs) that are not linked to either protein or polysaccharide are major cell surface glycolipids in all trypanosomatids investigated to date. These glycolipids differ from those found in plants, yeasts and other fungi, in that their glycan moieties contain the highly conserved core motif Man $\alpha(1 \rightarrow 4)$GlcN, linked to either inositol phosphosphingolipids or to inositol phosphoglycerolipids. Ceramide-linked GIPLs have been found in Trypanosoma cruzi [1] and members of Crithidia, Herpetomonas, Leptomonas and Endotrypanum [2], whereas glycerol linked GIPLs appear to be more

characteristic of *Leishmania* [3] (including the reptilian parasites *Leishmania tarentolae* and *Leishmania adleri*) and *Phytomonas* [2]. In *Leishmania* species pathogenic to humans the conserved glycan core structure is further modified by the addition of mannose or mannose and galactose residues, to form three distinct lineages of GIPLs, designated type 1, type 2 and hybrid, which are expressed to varying extents in different species and developmental stages [4]. In type 1 GIPLs the mannose of the conserved core is substituted by a $(1 \rightarrow 6)$ linked mannose, whereas in type 2 it is substituted by $(1 \rightarrow 3)$ linked mannose. The hybrid type is a branched structure in which 2 mannose substituents are present, linked $(1 \rightarrow 3)$ and $(1 \rightarrow 6)$. In *Leishmania major*, and to some extent in *Leishmania mexicana*, though not in *Leishmania donovani*, the cores of type 2 GIPLs contain a galactofuranosyl residue, and are capped by varying numbers of α and β galactopyranosyl units. The glycan portions of type 1 and type 2 GIPLs are homologous to the GPI protein anchors and the lipophosphoglycan anchor respectively [5]. It seems likely that this classification of GIPLs is not restricted to *Leishmania*, since glycoconjugates from several other parasites appear to conform to the same architecture.

In *T. cruzi* (the causative agent of Chagas' disease in humans), the major cell-surface glycoconjugate of epimastigote forms is a glycolipid, the so called lipopeptidophosphoglycan (LPPG), the glycan core of which is similar to type 1 GIPLs of *Leishmania* [6]. The main chain of this glycan contains a tetramannose structure in which the mannosyl units are linked in a sequence similar to the protein linked anchors of *T. cruzi* [7]. The glycan core of LPPG is further substituted by two nonreducing galactofuranosyl end units and 2-aminoethylphosphonate (AEP) at *O*-6 of glucosamine, and is linked to a ceramide rather than to an alkylacyl glycerol lipid moiety.

We have recently characterised a novel family of GIPLs in the monoxenous trypanosomatid *Leptomonas samueli* [8]. The glycan portions of these compounds are related to type 2 GIPLs of *Leishmania*, but their lipid moieties have a closer affinity to those found in *T. cruzi*. Surprisingly, apart from the presence of two AEP substituents, the oligosaccharide chains of *L. samueli* glycolipids A and B were identical to type two GIPLs of *Leishmania* designated iM2 and GIPL-1 respectively [8]. Although (as in *Leishmania major*) the type 2 core of *L. samueli* oligosaccharide B was capped with a Gal*f* residue, oligosaccharides containing additional galactose residues were not detected. A more complex family of *L. samueli* glycolipids was subsequently characterised, which did not contain galactofuranose, but had a monorhamnosyl side chain 3-*O*-linked to the fourth mannose distal to inositol, and showed also further substitutions by xylose, glucose and glucuronic acid-containing saccharide chains [9].

The biological significance of the structural diversity of glycoinositolphospholipids that share the same core structure is unknown. It is possible that these glycolipids enable the parasite to colonise the insect gut, presumably by mediating attachment to the gut epithelium. Some of the structural variation in the glycolipids of these parasites may be specific adaptations required for survival in the harsh environment of the insect digestive tract, the nature of which will be host dependent and will vary from species to species. This possibility prompted us to investigate the glycoinositolphospholipids of *Herpetomonas samuelpessoai*, a monoxenous trypanosomatid that parasitises the same insect (*Zelus leucogrammus*) as does *L. samueli* [10]. According to our hypothesis we would predict that similar selective pressures in the host habitat would lead to the development of structurally and functionally related GIPLs in both organisms.

2. Materials and methods

2.1. Isolation of glycoinositolphospholipids

H. samuelpessoai promastigotes were cultured as described by Palatnik et al. [11], and harvested cells (2×10^{11}) were extracted with 45% aqueous phenol at 80°C. After dialysis and lyophilisation, the aqueous phase was redissolved in distilled water and applied to a column of Bio-Gel P-100, and the excluded fraction collected and freeze dried. GIPLs were recovered by extraction with chloroform/methanol/water (10:10:3), as previously described [8].

2.2. Isolation of phosphoinositol oligosaccharides

PI-oligosaccharides were isolated from the intact GIPLs by hydrolysis with 1 M KOH (72 h at 37°C) [12]. After neutralisation with acetic acid, non-polar material was removed by chloroform extraction and the aqueous layer was treated with Dowex 50W-X8 (H$^+$) resin, and the PI-oligosaccharides were eluted from the resin with water, desalted on a column of TSK HW 40, using as eluent 0.5% acetic acid at a flow rate of 1 ml min^{-1}. The material eluting in the void volume was concentrated, passed through a C$_{18}$ Sep-Pak cartridge (Millipore), and recovered by lyophilisation.

2.3. High pH anion exchange chromatography

The PI-oligosaccharides were fractionated by HPAE chromatography on a Dionex model BioLC carbohydrate analyser equipped with a pulsed amperometric detector (pulse potential E1 = 0.05 V) using a Carbopac PA-1 column (0.9 × 250 cm; Dionex, Sunnyville, CA). The flow rate was 4 ml min^{-1}, and the initial solvent comprised 87% 100 mM NaOH (solvent A) and 13% 1 M sodium acetate in 100 mM NaOH (solvent B), maintained isocratically for 30 min. The proportion of solvent B was then increased to 21% over a 10 min period, maintained at that proportion for a further 20 min, before being increased to 50% by means of a 10 min linear gradient. Fractions were collected, immediately neutralised with acetic acid, concentrated and desalted by passage through a column of Sephadex G-10 (Pharmacia).

2.4. Analytical procedures

Neutral and acidic sugars were determined after methanolysis (0.5 M methanolic HCl, 24 h at 80°C) by gas chromatography (GC) of their trimethylsilyl ethers as previously described [8]. The absolute configurations of the neutral monosaccharides were determined by GC of their trimethylsilylated 2-butyl glycosides [13]. Inositol and glucosamine were quantitated by GC after methanolysis (3 M methanolic HCl, 18 h at 80°C), borohydride reduction and acetylation. Fatty acids and long chain bases were identified by GC and GC-MS after methanolysis as described by Gaver and Sweeley [14]. Total neutral sugars were quantitated by the phenol/sulphuric acid procedure [15]. Total and acid hydrolysable phosphorus was determined by the methods of Ames [16] and Bartlett [17] respectively. Long chain bases in the methanolysates of GIPLs were quantitated by the procedure of Lauter and Trams [18] using C$_{18}$ phytosphingosine as standard.

2.5. Methylation analysis

Prior to methylation, the PI-oligosaccharides were converted to their triethylamine salts by passage through a column of Dowex 50W-X2 resin in the triethylamine form, so as to increase their solubility in dimethysulphoxide. After freeze drying the PI-oligosaccharides were methylated with lithium methylsulphinylmethanide anion according to the procedure of Parente et al. [19]. The methylated oligosaccharides were hydrolysed with 4 M trifluoroacetic acid (4 h, 100°C), reduced, acetylated and the partially methylated alditol acetates were analysed by GC-MS as previously described [20]. O-methylated, O-acetylated methylglycosides were prepared by methanolysis of the permethylated oligosaccharides (0.5 M HCl, 24 h, 80°C), then acetylated, and characterised by GC-MS [21].

2.6. Nuclear magnetic resonance spectroscopy

Proton NMR spectra were obtained as previously described [9] at 500 MHz using a Varian Unity 500 spectrometer. Proton spectra were referenced to internal acetate anion at 1.908 ppm. The TOCSY and ROESY spectra were obtained with mixing times of 80 ms and 150 ms respectively. The one-dimensional ^{31}P NMR spectrum was obtained at 202 MHz on the same spectrometer using a 5 mm broad-band probe with broadband proton decoupling.

2.7. Fast atom bombardment mass spectrometry

Liquid secondary ionisation mass spectra (LSIMS) were obtained on a Kratos concept II HH spectrometer (Kratos Analytical, Manchester, UK) equipped with a Kratos LSIMS source and a caesium ion gun operated at 13.5 kV bombarding energy. Thioglycerol or glycerol were used as liquid matrices, and the

instrument was operated at 8 kV accelerating voltage at low (1000) resolution. Native materials were analysed in the negative ion mode and permethylated samples in the positive. The magnet was scanned at 30 s per decade of mass and several scans were accumulated and averaged using the DS90 data system. The positive ion collision-induced dissociation spectrum of permethylated PI-oligosaccharide A was obtained on a Finnigan TSQ 70 triple stage quadrupole instrument. Protonated molecules generated by fast atom bombardment ionisation underwent low energy collisions with the helium target gas in the second (radio frequency only) quadrupole, and the daughter ions were analysed by scanning the third quadrupole.

3. Results

3.1. Chemical composition of GIPLs from H. samuelpessoai cells

Sugar analysis of the GIPLs obtained from of *H. samuelpessoai* promastigotes revealed the presence of rhamnose (Rha), xylose (Xyl), mannose (Man), glucose (Glc), glucosamine (GlcN), glucuronic acid (GlcA) and inositol (Ins) in the molar ratio 1.0:1.6:3.7:0.6:0.9:0.2:1.0. Mild alkaline hydrolysis resulted in a mixture of PI-oligosaccharides and a chloroform-soluble lipid moiety. The sugar composition of the water-soluble PI-oligosaccharides was similar to that of the intact GIPL.

3.2. Characterisation of the lipid moiety

Methanolysis of the chloroform-soluble material produced a mixture of fatty acid methyl esters and long chain bases, which were characterised by GC and GC-MS as summarised in Table 1. The major fatty acid was eicosapentanoic acid (approx. 42%). Other quantitatively significant components were 2-hydroxyoctadecanoic acid (approx. 19%) and a branched eicosahexadecanoic acid (approx. 16%). The remaining components were saturated fatty acids of 18–27 carbon atoms. The long-chain bases were identified by electron ionisation and chemical ionisation mass spectrometry, and comprised a C_{22} phytosphingosine, and two isomeric C_{21} phytosphin-

Table 1
Long chain base and fatty acid compositions of the glycoinositolphospholipids from *H. samuelpessoai*

	%
Long chain base	
C_{21}-phytosphingosine	55.2
C_{21}-phytosphingosine br [a]	21.7
C_{22}-phytosphingosine	23.1
Fatty acid	
18:0	3.7
19:0	1.7
18:0 (2-hydroxy)	19.0
24:0	2.0
25.0	41.9
26:0	4.5
26:0 br [b]	15.5
27:0	9.3
27:0 br [b]	2.7

[a] Branched phytosphingosine.
[b] Branched fatty acid.

gosines. The C_{21} phytosphingosines have been identified in several other lower trypanosomatids, including *Crithidia luciliae*, *L. samueli* and *Endotrypanum schaudinni* [2], but in lower concentrations. No alkylglycerols were detected.

3.3. HPAE chromatography of the liberated phosphoinositol-oligosaccharides

Separation by HPAE chromatography resulted in four main carbohydrate fractions which were designated A, B, C and D which represented respectively 63%, 20%, 12% and 5% of the total. Fraction B was a mixture of at least three components. Fractions A, C and D by contrast appeared to represent relatively homogenous PI-oligosaccharides as judged by FAB MS and NMR data. The monosaccharide compositions of the Dionex fractions determined by sugar analysis are summarised in Table 2. In this paper we report the structure determination of the PI-oligosaccharides A and C.

3.4. Characterisation of phosphoinositol-oligosaccharide A

On negative ion FAB MS of the native PI-oligosaccharide A a deprotonated molecule (M-H$^-$) was observed at m/z 1615 (nominal), consistent with a

Table 2

Neutral sugar analysis (molar ratio) of the Dionex purified phosphoinositol-oligosaccharides of glycoinositolphospholipids from *H. samuelpessoai*

Sugar	PI-oligosaccharides			
	A	B	C	D
Rhamnose	1.0	1.1	1.0	1.0
Xylose	1.1	1.0	1.3	1.3
Mannnose	4.0	3.4	4.3	3.7
Glucose	1.2	0.8	0.0	0.2

[a] Determined by GC as trimethylsilyl derivatives of methylglucosides.

composition of five residues of hexose, one each of pentose, deoxyhexose, non-acetylated glucosamine and inositol phosphate together with a single aminoethylphosphonate (AEP) substituent (calculated $M-H^- = 1615.4685$ (mono-isotopic)). Abundant

fragment ions at m/z 527 and 555 ($^{1,5}X_2$ and Y_2 in the Domon and Costello system of nomenclature) [22], suggested a terminal glucosamine-inositol phosphate structure with an AEP substituent located on the glucosamine residue [6,8,9]. Other signals were too similar in intensity to the matrix derived chemical background to be confidently assigned as fragment ions. On permethylation, a protonated molecule was observed in the positive ion FAB spectrum at m/z 1931. The expected molecular mass of the protonated permethylated PI-oligosaccharide is 2094, which suggests that the AEP substituent is lost under the conditions of the methylation [23]. This was confirmed by a second experiment, in which the PI-oligosaccharide was N-acetylated prior to methylation, which resulted in an intense protonated molecule at the expected m/z of 2094. Facile cleavage was observed distal to the glucosamine, which
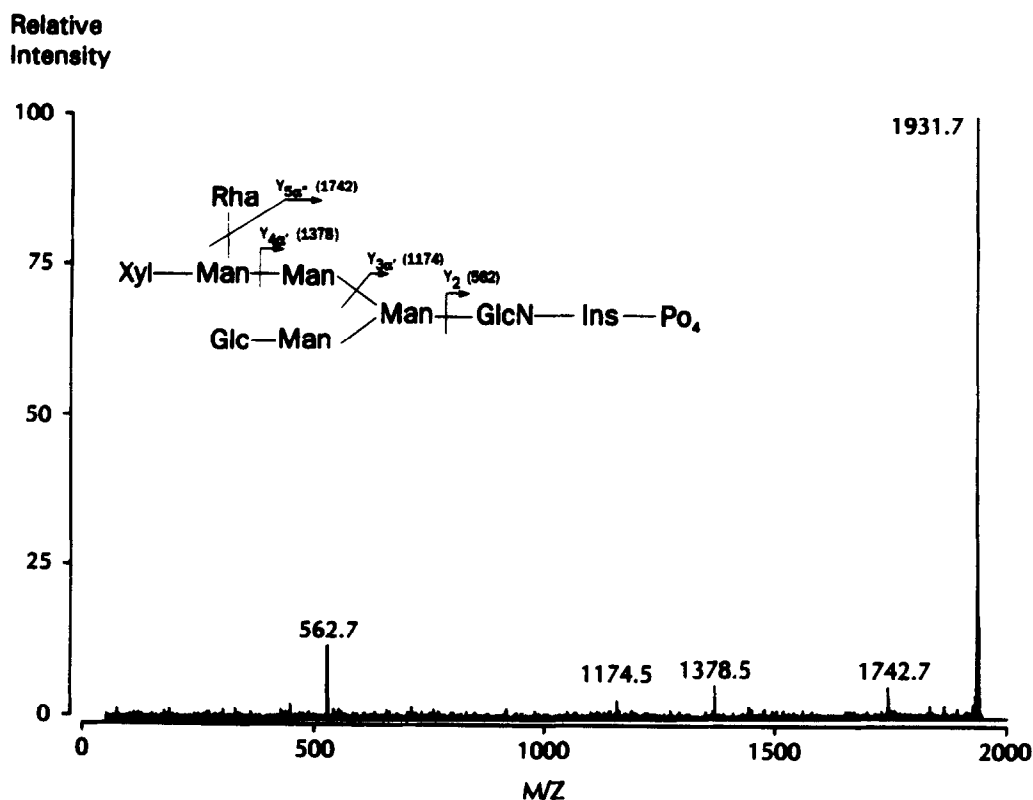

Fig. 1. Fragment ions observed in the daughter ion spectrum of m/z 1931 from permethylated PI-oligosaccharide A. For clarity the fractional parts of m/z values have been omitted and the masses rounded down to their nominal values.

produced the fragments m/z 562 and 590 (Y_2 and Z_2); other fragments were difficult to assign unambiguously because of the high background produced by the ionisation of the liquid matrix. Collisional activation of the protonated molecule at m/z 1931 using a triple quadrupole instrument provided some evidence of the sequence of residues. Four major reducing terminal-containing fragments were observed in the daughter ion spectrum (for clarity the measured values have been rounded to monoisotopic nominal values) (Fig. 1); the ion at m/z 1378 was consistent with the loss of the combined residue masses of hexose, pentose and deoxyhexose by a Y type process (ion $Y_{4\alpha'}$), which together with m/z 1742 (loss of a terminal deoxyhexose by a Y type mechanism, $Y_{5\alpha''}$), suggested the presence of a hexose substituted with both deoxyhexose and pentose at the reducing terminal. A further cleavage producing m/z 1174 ($Y_{3\alpha'}$) shows that this structure is attached to another hexose residue. The mass increment between m/z 562 (already assigned as the Y_2 cleavage at hexosamine) and m/z 1174 provides tentative evidence of a dihexosyl branch on the remaining hexose. Because this experiment was performed at low collision energy, it is unsurprising that ring cleavage fragments were not observed.

A portion of the permethylated PI-oligosaccharide A was hydrolysed, reduced and acetylated and the partially methylated alditol acetates were characterised by GC-MS. This data is summarised in Table 3. Three terminal residues were identified: rhamnose, xylose and glucose. The proportion of 3,4,6-tri-O-methyl-1,2,5-tri-O-acetyl mannitol suggested that two internal mannose residues were present, both monosubstituted in position O-2. Two further mannose

derivatives were detected, disubstituted respectively in positions 3,4 and 3,6. The methylation analysis is thus consistent with the pattern of branching deduced from the FAB MS data.

3.5. NMR Spectroscopy of phosphoinositol-oligosaccharide A

Two signals were observed in the one-dimensional 202-MHz ^{31}P spectrum of PI-oligosaccharide A, one at high field assigned as a phosphomonoester and one at low field attributed to the single AEP residue. The one dimensional proton NMR spectrum of this PI-oligosaccharide confirmed the presence of an AEP residue (N-CH$_2$ at δ 3.23; $^3J_{PCCH} = 13$ Hz and P-CH$_2$ at δ 2.03; $^2J_{PCH} = 22$ Hz), and also contained a resonance characteristic of the methyl group of a 6-deoxyhexose (δ 1.266). In the anomeric region, resonances were observed for a glucosamine (at low field), five mannoresidues, one residue with α-glucopyranose or galactopyranose configuration and one residue with β-glucopyranose or galactopyranose configuration. Assignments were obtained by the application of DQFCOSY [24,25] and TOCSY methods [26], and are summarised in Table 4. The β-glucopyranose residue was shown to be xylopyranose by the observation of a low field H-5e and the α-glucopyranose residue to be glucopyranose. The configuration of the sugars was established from the patterns of inter-proton coupling constants. The chemical shifts of the β-xylopyranose, α-rhamnopyranose and α-glucopyranose are very similar to those of the xylose and rhamnose found in the PI-oligosaccharide A of *Leptomonas samueli* [9] and with those of methyl glycoside model systems [27–

Table 3
O-methylalditol acetates formed on methylation analysis of *H. samuelpessoai* PI-oligosaccharides A and C [a]

O-methyl derivative	PI-oligosaccharides	
	A	C
2,3,4-tri-O-methyl-1,5-di-O-acetyl xylitol	0.7	0.3
2,3,4-tri-O-methyl-1,5-di-O-acetyl rhamnitol	0.9	0.3
2,3,4,6-tetra-O-methyl-1,5-di-O-acetyl glucitol	0.7	-
3,4,6-tri-O-methyl-1,2,5-tri-O-acetyl mannitol	2.0	1.8
2,6-di-O-methyl-1,3,4,6-tetra-O-acetyl mannitol	1.0	1.0
2,4-di-O-methyl-1,3,5,6-tetra-O-acetyl mannitol	0.7	0.2

[a] The partially methylated alditol acetates were identified and quantified by GC-MS.

Table 4

Proton NMR assignment for the *H. samuelpessoai* phosphoinositol-oligosaccharide A [a]

	H-1	H-2	H-3	H-4	H-5	H-6	H-6'
Ins	4.077	4.199	3.574	3.689	3.411	3.861	
GlcN	5.700	3.325	4.070	3.796	4.254	4.129	n.d.
Glc	5.129	3.543	3.738	3.387	[3.841]	n.d.	n.d.
Man(1)	5.437	4.094	4.003	[3.771]	n.d.	n.d.	n.d.
Man(2)	5.286	4.216	3.904	[3.90]	n.d.	n.d.	n.d.
Man(3)	5.134	4.051	3.980	[3.840]	n.d.	n.d.	n.d.
Man(4)	5.080	4.290	4.000	[3.930]	n.d.	[3.837]	n.d.
Rha	4.968	3.963	3.899	3.478	4.148	1.266	
Xyl	4.392	3.240	3.426	3.645	3.240	3.923	

[a] Data obtained at 30°C; [] Assignment tentative

29], indicating nonreducing terminal locations, in agreement with the methylation analysis results (Table 3).

The sequence and linkages of the sugar residues were established from a ROESY experiment [26] obtained at 500 MHz and with a spin-locked mixing time of 150 ms. These data are summarised in Table 5. The Rha H-1 showed inter-residue n.O.e.s to Man(4) H-2 and H-3 and although the inter-residue n.O.e.s from the β-Xyl H-1 could not be unambiguously assigned because of spectral crowding, the data are consistent with the presence of a nonreducing trisaccharide branch Rha(α1 → 3)[Xyl(β1 →

Table 5

Summary of intra- and inter-residue n.O.e. data determining sugar sequence and linkage for the *H. samuelpessoai* phosphoinositol-oligosaccharide A

From	Inter-residue	Intra-residue
GlcN H-1	H-2 @ 3.325	Ins H-6 @ 3.861
		Ins H-1 @ 4.063
Man(2) H-1	{H-2 @ 4.216}	GlcN H-4 @ 3.796
	{H-3 @ 3.909}	
Man(1) H-1	{H-2 @ 4.093}	Glc H-1 @ 5.130
	{H-3 @ 4.003}	Man(2) H-2 @ 4.214
	{H-4 @ 3.770}	Man(2) H-3 @ 3.904
Glc H-1	H-2 @ 3.547	Man(1) H-1 @ 5.347
		Man(1) H-2 @ 4.094
Man(3) H-1	H-2 @ 4.050	4.000; 3.749 - not assigned
Man(4) H-1	{H-2 @ 4.290}	Man(3) H-2 @ 4.050
Rha H-1	H-2 @ 3.964	Man(4) H-2 @ 4.290
		Man(4) H-3 @ 3.972
Xyl H-1	H-2 + H-5a @ 3.251	[Man(4) H-4 @ 3.932]
	H-3 @ 3.251	[Man(4) H-6 @ 3.837]
		Rha H-2 @ 3.964

4)]Man(4) similar to that found in *Leptomonas samueli* PI-oligosaccharide [9]. An n.O.e was observed between Man(4) H-1 and Man(3) H-2, consistent with the presence of a Man(4)(α1 → 2)Man(3) linkage. Inter-residue n.O.e.s. from the α-Man(3) H-1 could not be assigned and this linkage is discussed further below. An inter-residue n.O.e. was observed between the α-Glc H-1 and Man(1) H-1 and H-2, consistent with the presence of a nonreducing terminal Glc(α1-2)Man(1) disaccharide, and established, in conjunction with the methylation analysis, α-Man(2) as the -3,6)-Man branchpoint. Man(1) shows a weak crosspeak both to the Man(2) H-2 and to the expected Man(1) H-1/Man(2) H-3 crosspeak, indicating that the Glc(α1-2)-Man(1) disaccharide is attached to the Man(2) O-3 and implying that the Xyl/Rha terminated branch is attached through the Man(2) O-6, although the n.O.e.s from the Man(3) H-1 were not assigned. Man(2) H-1 shows an n.O.e. to a 10 Hz triplet assigned as GlcN H-4 and GlcN H-1 shows an inter-residue n.O.e. to Ins H-6, thus completing the structure. The low field position of the GlcN H-5 (4.254) resonance is consistent with the presence of an AEP substituent on GlcN O-6 as deduced from the FAB MS data.

3.6. Characterisation of phosphoinositol-oligosaccharide C

Negative ion FAB MS of PI-oligosaccharide C showed a deprotonated molecule at m/z 1629, suggesting a similar composition to PI-oligosaccharide A, except that the mass difference of 14 between the two implies the replacement of either a hexose by a hexuronic acid or the pentose by a second deoxyhexose residue. The prominent Y_2 fragment at m/z 527 was again observed, indicating the presence of the AEP-glucosamine-inositol phosphate sequence. FAB MS of the permethylated oligosaccharide yielded a protonated molecule at m/z 1945, (average value: calculated monoisotopic value = 1944.919) in agreement with the data on the native sample, assuming loss of the AEP substituent during methylation. Most of the fragment ions observed in this spectrum were assignable to the X and Y series. Ions at m/z 1798 and 1770 were attributed to the loss of a pentose residue from the nonreducing terminal by X and Y cleavages, which therefore suggested that the m/z

difference of 14 from PI-oligosaccharide A is attributable to the presence of a hexuronic acid residue. This is confirmed by signals at m/z 1712 and 1740, $^{1,5}X_{4\beta}$ and $Y_{4\beta}$) corresponding to the loss of a non reducing hexuronic acid residue. The remaining fragments in the spectrum show that the structure is similar to that of PI-oligosaccharide A, except that the dihexosyl branch on the hexose distal to the glucosamine is replaced by a hexuronosylhexosyl unit. This fragmentation data is summarised in Fig. 2.

The presence of the uronic acid residue is consistent with the increased retention of this PI-oligosaccharide on Dionex chromatography, compared to PI-oligosaccharide A.

The data from methylation analysis are qualitatively similar to that obtained from PI-oligosaccharide A, (Table 3) apart from the absence of the derivative corresponding to the terminal glucose. However the yields from the terminal xylose and rhamnose residues were poor as were those from the 3,6 disubstituted mannose residue. The poor yield of the 3,6-linked mannose could be taken as evidence that the uronic acid is attached to this residue; however this is difficult to reconcile with the LSIMS and NMR data, which suggest a terminal location.

3.7. NMR spectroscopy of phosphoinositol-oligosaccharide C

The proton NMR spectrum (Table 6) of the PI-oligosaccharide C was closely similar to that of PI-oligosaccharide A, except that the two-dimensional spectra indicated an α-GlcA in place of the α-Glc, consistent with the late elution of this fraction
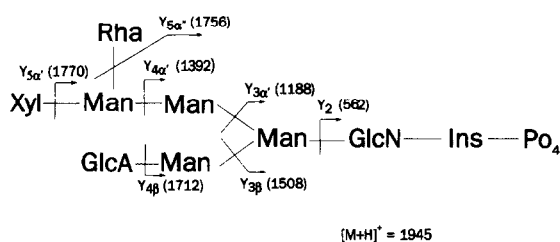

[M+H]$^+$ = 1945

Fig. 2. Fragment ions observed in the LSIMS spectrum of permethylated PI-oligosaccharide C. Most of the Y type ions were accompanied by $^{1,5}X$ ions at m/z $Y_n + 28$. The following were omitted from the figure for reasons of clarity: m/z 1798 ($^{1,5}X_{5\alpha'}$); m/z 1784 ($^{1,5}X_{5\alpha''}$); m/z 1420 ($^{1,5}X_{4\alpha'}$); m/z 1216 ($^{1,5}X_{3\alpha'}$); m/z 1740 ($^{1,5}X_{4\beta}$); m/z 1536 ($^{1,5}X_{3\beta}$) and m/z 590($^{1,5}X_2$).

Table 6
Proton NMR assignments for the *H. samuelpessoai* phosphoinositol-oligosaccharide C [a]

	H-1	H-2	H-3	H-4	H-5	H-6	H-6'
Ins	4.080	4.189	3.572	n.d.	n.d.	n.d.	
GlcN	5.700	3.338	4.075	3.758	[4.257]	[4.138]	n.d.
GlcA	5.129	3.592	3.748	3.503	4.114		
Man(1)	5.373	4.085	n.d.	n.d.	n.d.	n.d.	n.d.
Man(2)	5.202	4.246	[3.957]	n.d.	n.d.	n.d.	n.d.
Man(3)	5.124	4.047	n.d.	n.d.	n.d.	n.d.	n.d.
Man(4)	5.080	4.290	3.992	n.d.	n.d.	n.d.	n.d.
Rha	4.968	3.963	3.899	3.478	4.148	1.266	
Xyl	4.392	3.240	3.426	3.640	3.240	3.920	

[a] Data obtained at 500 MHz and at 30°C; [] Assignment tentative.

from the anion exchange column and the FAB MS data. The proton chemical shifts of the α-GlcA residue were similar to those of a monosaccharide model system [30], indicating a terminal location. The linkages and sequence were deduced by analogy with PI-oligosaccharide A, because of the extreme similarity of their NMR spectra and from the FAB MS and methylation data.

4. Discussion

Cloning of *H. samuelpessoai*, a trypanosomatid isolated from the predatory hemipteran *Zelus leucogrammus* resulted in cultures consisting primarily of promastigotes and opisthomastigotes, forms which are characteristic of the genus *Herpetomonas* [31]. The surface proteins and enzyme activities expressed by *H. samuelpessoai* are similar to those of *Herpetomonas* species isolated from other hosts [32]. Mice immunised with live cultures of *H. samuelpessoai* are protected against challenge with infective forms of *T. cruzi* [33], which suggests that the two organisms share common antigens. This immunological relationship to *T. cruzi* extends to other *Herpetomonas* species as demonstrated by the observation of a high proportion of positive results (98.7%) when *Herpetomonas muscarum muscarum* replaces *T. cruzi* as the whole cell antigen in immunofluorescence tests [34]. The nature of these common epitopes is however unknown.

In the present study, two PI-oligosaccharides isolated from the glycophosphosphingolipids of *H. samuelpessoai* were characterised. Their structures are depicted in Fig. 3. They can be regarded as being

PI-oligosaccharide	Structure

A

α-Rha(1→3)⟍
⟍α-Man(1→2)α-Man(1→6)⟍   OPO₃CH₂CH₂NH₃*
β-Xyl(1→4)⟋   (4)   (3)   ⟍ |
                                      ⟍   6
                                      α-Man(1→4)α-GlcN(1→6)-Ins-1-OPO₃⁻
                  α-Glc(1→2)α-Man(1→3)⟋   (2)
                       (1)

C

α-Rha(1→3)⟍
⟍α-Man(1→2)α-Man(1→6)⟍   OPO₃CH₂CH₂NH₃*
β-Xyl(1→4)⟋   (4)   (3)   ⟍ |
                                      ⟍   6
                                      α-Man(1→4)α-GlcN(1→6)-Ins-1-OPO₃⁻
                 α-GlcA(1→2)α-Man(1→3)⟋   (2)
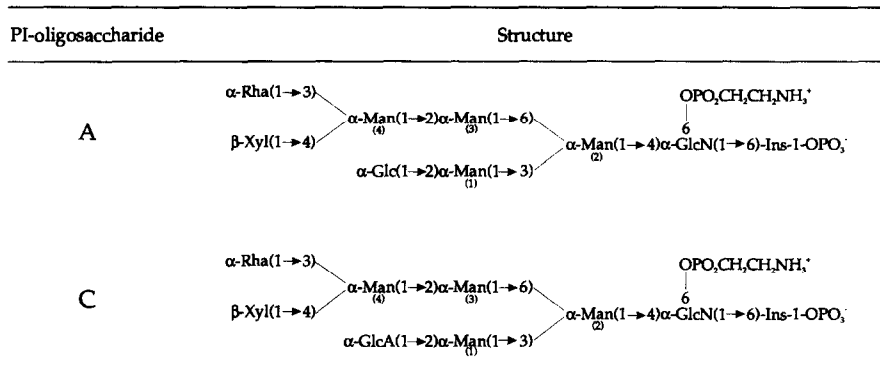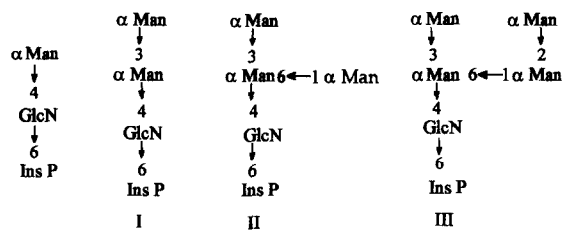   (1)

Fig. 3. Structures of phosphoinositol-oligosaccharides A and C isolated from glycoinositolphospholipids of *H. samuelpessoai*.

formally related to the iM4 hybrid type of GIPL, found in *Leishmania* [4], differing by the addition of either an α-D-Glc (in PI-oligosaccharide A) or an α-D-GlcA (in PI-Oligosaccharide C), to the α(1 → 3)-mannosyl branch. In both PI-oligosaccharides the terminal α(1 → 2) linked mannopyranose of the α(1 → 6)-mannose branch is doubly substituted by α-L-rhamnose and β-D-xylose.
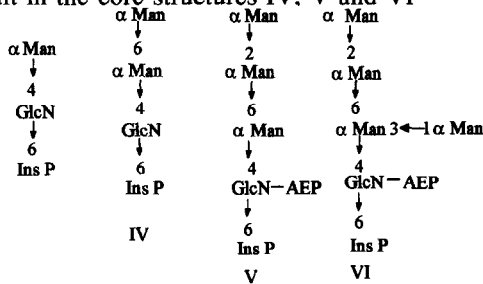
This is the first description of the occurrence of hybrid type oligosaccharides in a monoxenous trypanosomatid; previous reports having been in the dixenous species *Leishmania mexicana, L. donovani* [4] and *L. adleri* [35]. There are however many differences between *H. samuelpessoai* and *Leishmania* species. The insect host of *Herpetomonas* is a hemipteran [10], whereas the vectors of *Leishmania* are sandflies. The lipid moiety of the *Herpetomonas* GIPLs is a ceramide while those of *Leishmania* are alkylglycerols [3]; finally *Herpetomonas* GIPLs are substituted by AEP, which is absent from *Leishmania* species. These features, which differentiate *Herpetomonas* from *Leishmania* are simultaneously suggestive of a relationship to *T. cruzi*, an organism also containing glycophosphosphingolipid and AEP, transmitted by hemiptera, and, as indicated above, having considerable immunological relationship to *Herpetomonas*. How is the apparently contradictory presence of hybrid type GIPLs to be explained in the face of these phenotypic similarities? We propose that the core structure Manα(1 → 4)GlcN(1 → 6)Ins-P was present in the common ancestors of

modern trypanosomatids, and early in evolution became substituted by α-Manp linked either (1 → 3) or (1 → 6), giving rise to two divergent families of structures, from which were derived the GIPLs of *Leishmania* species and *Endotrypanum* [36] on the one hand, and those of *T. cruzi* and *H. samuelpessoai* on the other. Structures I, II and III depict a possible series of glycosylations which could have resulted in the characteristic core structures of *Leishmania*.

```
              α Man         α Man              α Man    α Man
               ↑             ↑                  ↑        ↑
  α Man        3             3                  3        2
   ↑          α Man      α Man6←1 α Man      α Man 6←1 α Man
   4           ↑             ↑                  ↑
  GlcN         4             4                  4
   ↑          GlcN          GlcN               GlcN
   6           ↑             ↑                  ↑
  Ins P        6             6                  6
              Ins P         Ins P              Ins P

   I            II            III
```

Structure I is found in the promastigotes of *L. major, L. mexicana*, and *Endotrypanum* and may be further substituted by units of β-Galf, α-Galp, β-Galp and β-Arap [4,36]. Structure II occurs in *L. donovani* and *L. mexicana*, with and without substitution of glucosamine by ethanolamine phosphate [4]. Structure III occurs in *L. mexicana, L. donovani* and *L. adleri*, sometimes with an ethanolamine phosphate substituent [4,35].

In the *T. cruzi/H. samuelpessoai* structural family glycosylations of the common precursor could result in the core structures IV, V and VI

```
                    α Man        α Man       α Man
                      ↓            ↓           ↓
        α Man         6            2           2
          ↓         α Man        α Man       α Man
          4           ↓            ↓           ↓
        GlcN          4            6           6
          ↓         GlcN         α Man      α Man 3←—1 α Man
          6           ↓            ↓           ↓
        Ins P         6            4           4
                    Ins P       GlcN—AEP    GlcN—AEP
                                  ↓           ↓
          IV                      6           6
                                Ins P       Ins P

                                  V          VI
```

Core structure IV, in which the common precursor is substituted by an $\alpha(1 \rightarrow 6)$ linked Man$p$ unit is the probable intermediate for the synthesis of structure V, which in *T. cruzi* is substituted at glucosamine by AEP [6]. Further substitutions of V by Gal$f$ $(1 \rightarrow 3)$ and Man$p(1 \rightarrow 2)$ give rise to oligosaccharides present in *T. cruzi* LPPG. Additional $\alpha(1 \rightarrow 3)$ mannosylation of V results in structure VI, which is the core sequence of *H. samuelpessoai* GIPLs. Further substitution by α-Rha, β-Xyl and α-GlcA or α-Glc units results in the PI-oligosaccharides A and C described in this paper.

Leptomonas samueli (a trypanosomatid species that parasitises the same insect as *H. samuelpessoai*) is intermediate between *Leishmania* and *T. cruzi / Herpetomonas* with respect to these structural and phylogenetic relationships.The GIPLs of *Leptomonas* [8,9] have the *Leishmania* type core structure I. However, further substitutions of this core involves the addition of complex chains of $\alpha(1 \rightarrow 3)$ linked Man, $\beta(1 \rightarrow 4)$linked Xyl and terminal units of Rha, Glc and GlcA. The presence of Xyl, GlcA and Rha units in both *Herpetomonas* and *Leptomonas* (even though linked to different core structures) may represent a convergent adaptive response to the similar selective pressures experienced by two species of different phylogeny sharing the same insect host. Although the core structure of the *Leptomonas samueli* glycolipids are of the type found in *Leishmania*, the organism is clearly related to the *T. cruzi/Herpetomonas* group because its GIPLs are AEP-containing and ceramide-linked.

Although glycosylphosphatidylinositol lipid structures constitute a small proportion of the vast array of phenotypic traits which vary between trypanosomatid species and genera, their cell surface location and high levels of expression is suggestive of a possible involvement in the interaction of host and parasite. In consequence we would predict that the structures of these molecules would be peculiarly sensitive to the effects of the varying internal environments encountered in hosts of different species. Comparative investigation of these molecules in a range of monoxenous and dixenous trypanosomatids of varying host specificities is thus likely to afford considerable insight into the structural evolution of these molecules, and their role in mediating the adaptation of parasites to their hosts.

Acknowledgements

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