THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 267, No. 34, Issue of December 5, pp. 24279-24286, 1992
Printed in U.S.A.

Structural Characterization of a Novel Class of Glycophosphosphingolipids from the Protozoan *Leptomonas samueli**

(Received for publication, June 8, 1992)

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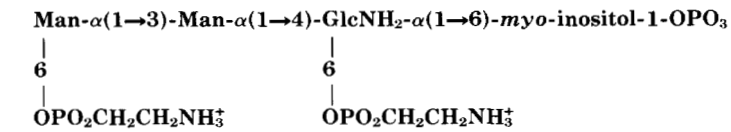
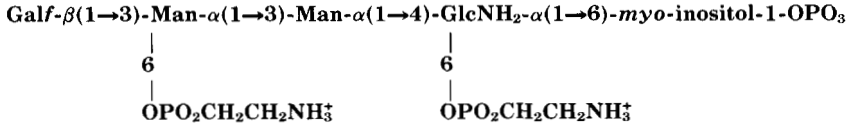
Aqueous phenol extraction of the lower trypanosomatid *Leptomonas samueli* released into the aqueous layer a chloroform/methanol/water-soluble glycophosphosphingolipid fraction. Alkaline degradation and purification by gel filtration chromatography resulted in a tetrasaccharide (phosphatidylinositol (PI)-oligosaccharide A), and a pentasaccharide (PI-oligosaccharide B), each containing 2 mol of 2-aminoethylphosphonate and 1 mol of phosphate. Nuclear magnetic resonance spectroscopy and fast atom bombardment-mass spectrometry suggested that the structure of PI-oligosaccharide A is



and that of PI-oligosaccharide B is as shown.



Both compounds contain an inositol unit linked to ceramide via a phosphodiester bridge. The major aliphatic components of the ceramide portion are stearic acid, lignoceric acid, and C_{20}-phytosphingosine.

These novel glycolipids fall within the glycosylated phosphatidylinositol (GPI) family, since they contain the core structure Manα(1→4)$GlcNH_2$α(1→6)*myo*-inositol-1-PO_4, which is also found in the glycoinositolphospholipids and lipophosphoglycan of *Leishmania* spp., the *L. major* promastigote surface protease, the glycosylphosphatidylinositol anchor of *Trypanosoma brucei* variant surface glycoprotein, and the lipopeptidophosphoglycan of *Trypanosoma cruzi*.

The glycophosphosphingolipids of *Leptomonas* have features in common with the glycolipids of both *Leishmania* and *T. cruzi*, resembling the former by the α(1→3) linkage of mannose to the GPI core, 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*. Thus these data lend some support to the hypothesis that both *T. cruzi* and *Leishmania* evolved from a *Leptomonas*-like ancestor.

Structural studies of phosphoinositol-containing glycolipids have gained new impetus following the discovery that they function as membrane anchors for many eukaryotic cell surface proteins (reviewed by Ferguson and Williams (1988), Low (1989), and Thomas *et al.* (1990)). Well characterized

* This research was supported by grants from Conselho Nacional de Desenvolvimento Cientifico e Technológico (Programa de Formação de Recursos Humanos para Desenvolvimento Technológico and Programa de Apoio as Desenvolvimento Científico e Technológico); Financiadora de Estudos e Projetos, FAPERJ, and Conselho de Ensino de Pós-Graduação-Universidade Federal do Rio de Janeiro. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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examples include rat brain Thy-1 glycoprotein (Homans *et al.*, 1988) and the variant surface glycoprotein of *Trypanosoma brucei* (Ferguson *et al.*, 1988). Other members of the trypanosomatidae family synthesize cell surface glycoinositolglycerolipids which are not covalently linked to protein. These include the heterogeneous lipophosphoglycan (LPG)[1] of *Leishmania* spp., in which the glycoinositolglycerolipid is linked to a phosphorylated polysaccharide (Orlandi and Turco, 1987; Turco *et al.*, 1987; Turco *et al.*, 1989; McConville *et al.*, 1987; McConville *et al.*, 1990b), and a family of low molecular weight glycoinositolphospholipids (GIPLs) (McConville and Bacic, 1989; McConville *et al.*, 1990a) from *Leishmania major* and *Leishmania donovani* which are not linked to either protein or polysaccharide. Epimastigote forms of *T. cruzi* also synthesize a protein and polysaccharide-free phosphoinositol-containing glycolipid, the so-called lipopeptidophosphoglycan (LPPG). Although this material differs from the glycophosphoglycerolipids of *Leishmania* in that it is a glycophosphosphingolipid (Previato *et al.*, 1990; Lederkremer *et al.*, 1990, 1991), the glycan moiety contains the core sequence Manα(1→4)Glc$NH_2\alpha$(1→6)*myo*-inositol-1-PO_4 which is common to all hitherto described members of the glycosylphosphatidylinositol (GPI) lipid family (Ferguson *et al.*, 1991).

The presence of different classes of GPI containing lipids in *Trypanosoma* and *Leishmania* species prompted us to investigate the phosphoinositol-containing glycolipids of the lower trypanosomatids, in the hope that data from these monogenetic organisms would clarify the evolutionary relationships and biological significance of these molecules. In this paper we report the structure of two phosphoinositol oligosaccharides isolated from the glycophosphosphingolipids of *Leptomonas samueli*, a monogenetic kinetoplastid parasite found in the insect *Zelus leucogrammus* Perty, 1834 (Hemiptera: Reduviidae) (Wallace, 1979).

EXPERIMENTAL PROCEDURES

Materials—2-Aminoethylphosphonic acid was obtained from Aldrich Chemical Co. Deuterium oxide (99.9%) was obtained from Goss Scientific (Ingatestone, United Kingdom). Sugars, fatty acids, long chain base standards, glycerol, and thioglycerol were obtained from Sigma (Poole, United Kingdom), and were used without further purification.

Isolation of Glycophosphosphingolipids (GPS)—L. samueli promastigotes were cultured as previously described (Palatnik *et al.*, 1987) and the resulting cells (approximately 5×10^{12}) were extracted with 45% (v/v) aqueous phenol at 80 °C. The aqueous layer was dialyzed, freeze dried, and applied to a column (2 × 100 cm) of Bio-Gel P-60. The excluded material was lyophilized and the GPS were recovered by extraction (twice) with chloroform/methanol/water (10:10:3). The extracts were combined, evaporated to dryness, dissolved in water, and lyophilized. The yield of GPS was about 100 mg.

Isolation of Phosphoinositol Oligosaccharides—The PI-oligosaccharides were isolated from the intact GPS by alkaline hydrolysis (1 M KOH, 48 h at 37 °C) (Smith and Lester, 1974). After neutralization with acetic acid, non-polar material was removed by chloroform extraction, and the aqueous layer was treated with Dowex 50W-X8 (H^+) resin. The mixture was filtered through glass wool and the PI-oligosaccharides were eluted from the resin with water. The eluate was freeze dried, dissolved in water, and fractionated on a column of Bio-Gel P-4.

Carbohydrate Analysis—Neutral and acidic sugars were determined after methanolysis (0.5 M methanolic HCl; 80 °C for 18 h with mannitol as internal standard). Fatty acids were removed by hexane extraction and the methyl glycosides were trimethylsilylated with bis-(trimethylsilyl)trifluoroacetamide/pyridine (1:1, v/v) for 4 h at room temperature (Sweeley *et al.*, 1963). The products were analyzed by gas-liquid chromatography (GC) using a capillary column of OV-101 (25 m × 0.2-mm inner diameter), with helium as the carrier gas at 0.5 kpascal. The column temperature was programmed from 120 to 240 °C at 2 °C min^{-1}.

For analysis of inositol and glucosamine, samples were treated with 3 M methanolic HCl (18 h at 80 °C: arabinitol internal standard). The dried methanolysates were then hydrolyzed with 6 M HCl (18 h; 105 °C), reduced with sodium borohydride, acetylated (acetic anhydride/pyridine (9:1)), and analyzed by GC as above, except that the column temperature was programmed from 120 to 240 °C at 3 °C min^{-1}.

The absolute configurations of the neutral monosaccharides were determined by GC of their trimethylsilylated (−)-2-butylglycosides (Gerwig *et al.*, 1978).

Fatty Acid Analysis—Samples were transesterified with 0.5 M methanolic HCl for 18 h at 80 °C. The fatty acid methyl esters were extracted into hexane and analyzed by GC (before and after trimethylsilylation) using an OV-101 capillary column, with temperature programming from 180 to 310 °C at 5 °C min^{-1}.

Long Chain Sphingosine Bases—Long chain sphingosine bases were released from GPS by methanolysis (1 M methanol HCl made 10 M with respect to water) for 18 h at 80 °C (Carter and Gaver, 1967). The pH was adjusted to 11 with aqueous NaOH, and the methanolysate was extracted three times with 2 volumes of diethyl ether. The combined extracts were dried with sodium sulfate, evaporated under nitrogen, dissolved in methanol and *N*-acetylated with acetic anhydride (Gaver and Sweeley, 1966). The *N*-acetyl derivatives were *O*-trimethylsilylated by treatment with bis-(trimethylsilyl)trifluoroacetamide/pyridine (1:1, v/v) for 4 h at room temperature and analyzed by capillary GC and by GC-MS.

Gas Chromatography Mass Spectrometry (GC-MS)—Capillary GC-MS was performed with a Riber-Mag R10-10 instrument (Rueil Malmainson, France) using a DB-1 capillary column (30 m × 0.2 mm, inner diameter). The GC oven temperature was programmed either from 180 to 310 °C (for fatty acid methyl esters and trimethylsilylated *N*-acetylated sphingosine bases), or from 100 to 240 °C (for trimethylsilylated methylglycosides) at 5 °C min^{-1} in both cases. Both electron impact and chemical ionization spectra (using ammonia as reagent gas) were recorded.

Other Analytical Methods—Total neutral sugars were analyzed by the phenol/sulfuric acid procedure (Dubois *et al.*, 1956). Total phosphorus was determined by the method of Ames (1966) and acid hydrolyzable phosphorus by the method of Bartlett (1959). The procedure of Lauter and Trams (1962) was used for the quantitative analysis of the long chain bases in methanolysates of GPS. C_{18}-phytosphingosine was used as standard.

Nuclear Magnetic Resonance Spectroscopy (NMR)—Samples were exchanged by repeated lyophilization from deuterium oxide before final dissolution in 0.5 ml of D_2O. All spectra were obtained at 30 °C in 5-mm tubes and probes. ^{31}P spectra were obtained on a Bruker WM200 spectrometer and were referenced to external 85% H_3PO_4 at 0 p.p.m. One-dimensional ^{13}C spectra were obtained on a Bruker WP200 spectrometer and were referenced to external dioxane at 67.4 p.p.m. One- and two-dimensional proton NMR spectra were obtained on Varian Unity 600 and Bruker AM500 spectrometers. Proton chemical shifts were referenced to internal 3-(trimethylsilyl)propionic 2,2,3,3-d_4 acid at 0 p.p.m.. All correlation spectra were obtained in the magnitude mode. Standard microprograms provided with the current software were used for all pulse sequences except for the 600 MHz ROESY experiment.

^1H correlation spectra were run at 500 or 600 MHz, using the COSY-45 pulse sequence (Aue *et. al.*, 1976). For the spectrum of the PI-oligosaccharide A, 512 experiments, each with 4 transients of 2048 data points, were performed with spectral windows of 2304 Hz in both domains. The relaxation delay between pulse trains was 1.5 s and the experiment was tuned for a 5-Hz coupling constant. The data were zero-filled in f_1 to give digital resolution of 2.25 Hz/point in f_1 and f_2 and the spectrum was processed using unshifted sine bell window functions in both domains. Similar spectral parameters were used for the triple relayed COSY of PI-oligosaccharide A, except that 32 transients were collected in each experiment. The experiment was tuned for coupling constants of 6 and 8 Hz.

The COSY-45 spectrum of PI-oligosaccharide B was obtained at 600 MHz, with spectral widths of 3610 Hz in both domains; 256

[1] The abbreviations used are: LPG, lipophosphoglycan; LPPG, lipopeptidophosphoglycan; GIPL, glycoinositolphospholipid; GPS, glycophosphosphingolipid; GPI, glycosylphosphatidylinositol; PI, phosphatidylinositol; 2-AEP, 2-aminoethylphosphonate; Gal*f*, galactofuranose; $GlcNH_2$, glucosamine; GC, gas chromatography; FAB, fast atom bombardment; MS, mass spectrometry; NOE, nuclear Overhauser effect.

spectra averaging 16 transients were collected. A 3-s relaxation delay was allowed between pulse-acquisition sequences, the data zero-filled in f_1 to 1024 points and processed with sine bell window functions in both domains.

A triple-relayed COSY spectrum of PI-oligosaccharide B was obtained under similar conditions and utilized a 30-ms delay between relay steps, tuning the experiment for coupling constants of 8 Hz. The triple quantum filtered COSY spectrum of PI-oligosaccharide B was obtained using spectral windows of 3339.2 Hz in both domains and 600 increments of 6 transients per free induction decay were collected using the States method to obtain a phase-sensitive spectrum. The delay between pulse sequences was 1.5 s.

$^1H^{13}C$ correlation spectra were obtained at 125 MHz with the Bruker AM500 spectrometer in the carbon-detected mode using the pulse sequence of Bax and Morris (1981). For PI-oligosaccharide A the spectral windows were 12,500 Hz in f_1 and 4,700 Hz in f_2. The data were zero-filled in f_1 to give final digital resolutions of 6.1 and 8 Hz/point in f_2 and f_1, respectively. A relaxation delay of 1.2 s was allowed before each pulse-acquisition sequence, and the experiment was tuned for $^1J_{C\text{-}H}$ of 150 Hz. The same conditions were used for the C-H correlation spectrum of PI-oligosaccharide B, except that the spectral width in the proton domain was reduced to 2,380 Hz and the number of measurements in f_1 halved to 256.

The ROESY spectrum of PI-oligosaccharide B was obtained at 600 MHz. The experiment was modified to generate the spin-lock field by continuous low-power irradiation. 400 increments (of 9600 data points) were acquired over a 4800 Hz sweep width in both domains, 16 transients being collected at each increment. A phase-sensitive spectrum was generated with the States-Haberkorn method. The relaxation delay between pulse sequences was 2 s and the residual water peak was eliminated by presaturation. The spectrum was zero-filled to 1024 points in f_1 before transformation.

Fast Atom Bombardment-Mass Spectrometry—FAB mass spectra were acquired using a Kratos MS80 RFA mass spectrometer, fitted with an Ion Tech saddle field gun using xenon atoms as the bombarding particles. The sample (approximately 10 μg dissolved in 1 μl of 30% acetic acid) was mixed with a similar volume of matrix on the stainless steel target. The matrices used were either glycerol or a 1:1 mixture of glycerol and thioglycerol. The instrument was operated at 4 kV accelerating voltage, and the magnet was scanned at 30 s/decade from a start mass of 3600. Between 10 and 20 scans were acquired and averaged using the DS90 raw data software. The mass axis was calibrated with cesium iodide clusters, the reference data being acquired immediately after the sample spectrum. Collision spectra were recorded in the positive ion mode only. Collision-induced dissociation was achieved by admitting helium to the first field-free region gas cell so as to attenuate the ion beam to 30% of its initial value. The decomposition products were analyzed by means of computer generated B/E-linked scans. In these experiments the magnet was scanned at 10 s/decade from a start mass of 2000, and the spectra were obtained by averaging 50 raw data scans.

RESULTS

Isolation and Chemical Composition of Glycophosphosphingolipids of L. samueli—Hot phenol-water extraction of *L. samueli* cells released into the aqueous layer a glycolipid fraction that was soluble in chloroform/methanol/water (10:10:3, v/v) but not in chloroform or in chloroform/methanol (2:1, v/v). Analysis showed that this fraction contained: glucuronic acid (not quantified), mannose, xylose, glucosamine, inositol, rhamnose, galactose, glucose, phosphorus; long chain base and fatty acids in the molar proportions listed in Tables I and II. The levels of phosphorus measured by the procedure of Ames (1966) were 3-fold higher than detected by Bartlett's method, suggesting the presence of acid stable C-P linkages (Horiguchi, 1984). The equimolar proportions of acid labile phosphorus (1 mol), inositol (1 mol), and long chain base (1 mol) indicated that the preparation contained exclusively glycophosphosphingolipids (GPS). This was confirmed by treating it with 1 M KOH (48 h at 37 °C), conditions that hydrolyze inositol phosphoceramide to inositol monophosphate and ceramide. GC analysis of the hydrolysate following partition between chloroform and water showed that long chain bases and fatty acids were present only in the chloroform phase.

TABLE I

Chemical compositions of GPS from L. samueli cells

Component	Inositol
	mol/mol
Rhamnose[a]	0.8
Xylose[a]	3.5
Mannose[a]	5.2
Galactose[a]	0.8
Glucose[a]	0.8
Glucosamine[b]	0.9
Inositol[b]	1.0
Glucuronic acid[a]	—[c]
Phosphorus[d]	
Ames' method	3.0
Bartlett's method	1.0
Long chain bases[d]	0.9

[a] Determined by GC as trimethylsilyl derivatives of methylglycosides.
[b] Determined by GC as alditol acetate derivatives.
[c] Present but not quantified.
[d] Determined by the colorimetric methods described in experimental procedures.

TABLE II

Fatty acid and sphingosine base compositions of GPS from L. samueli

	%
Fatty acid composition	
14:0	2.8
16:0	6.6
18:0	21.2
19:0 br[a]	7.7
19:0	5.8
22:0	3.3
23:0	7.4
24:0	26.0
25:0	8.5
26:0	10.7
Sphingosine base composition	
C_{20}-phytosphingosine	72
C_{21}-phytosphingosine	28

[a] Branched fatty acid.

Identification of the Fatty Acids—The GPS of *L. samueli* was methanolyzed and the resulting fatty acid methyl esters were analyzed by GC and GC-MS. Nine of the 10 peaks were characterized as unbranched saturated fatty acids ranging in carbon number from 14 to 26 (Table II). Stearic acid (18:0) and lignoceric acid (24:0) amounted to 50% of the total. The remaining peak was identified by GC-MS as the methyl ester of a branched 19-carbon acid.

Long Chain Base Composition—Tetramethylsilyl derivatives of the *N*-acetylated long chain bases obtained from GPS were analyzed by GC and GC-MS. The major component was identified as a C_{20}-phytosphingosine derivative. The electron impact mass spectrum of this compound was similar to that of *N*-acetyltrimethylsilyl C_{18}-phytosphingosine, except that the fragments $(M\text{-}174)^+$ and $(M\text{-}276)^+$ were shifted up in mass by 28 units compared to the corresponding ions from the C_{18}-phytosphingosine (Thorpe and Sweeley, 1967). Similarly, 2 minor peaks in the chromatogram were characterized by their electron impact mass spectra as C_{21}-phytosphingosines. Chemical ionization mass spectrometry using ammonia as reagent gas resulted in protonated molecules at the expected masses (m/z 604 and m/z 618 for C_{20} and C_{21}-phytosphingosine derivatives, respectively) thus confirming the identifications.

Isolation and Analysis of PI Oligosaccharides from GPS of L. samueli—Gel filtration chromatography of the PI-oligosac-

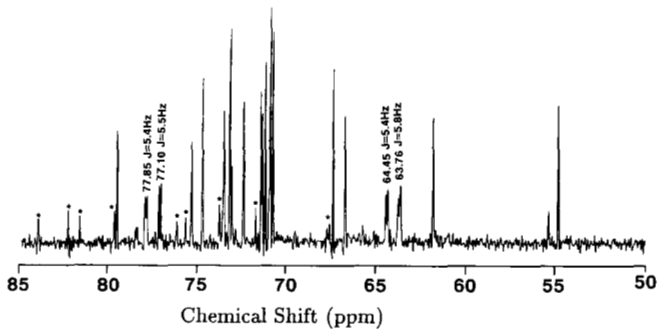

FIG. 1. **Partial 50 MHz ^{13}C NMR spectra of PI-oligosaccharide A obtained at 30 °C.** Peaks showing $^{31}P^{13}C$ couplings are indicated. Peaks marked with an *asterisk* arise from cross-contamination by PI-oligosaccharide B.

charides obtained by alkaline treatment of GPS with 1 M KOH produced several carbohydrate containing fractions, of which the most significant were designated PI-oligosaccharides A, B, and C. FAB-MS and NMR data suggested slight cross-contamination of PI-oligosaccharide A with PI-oligosaccharide B, and of PI-oligosaccharide B with fraction C; which accounts for some of the minor resonances in the NMR spectra. Fraction C proved to be a mixture of several components, the purification and characterization of which will form the subject of a separate communication.[2]

Analytical data demonstrated that all three fractions contained glucosamine, inositol, and phosphorus in the molar ratio of 1:1:3. Only 1 mol of phosphorus was detected by Bartlett's method, indicating that the oligosaccharides each contained 2 mol of phosphonate. Apart from inositol and glucosamine the main monosaccharides detected were mannose (fraction A), mannose and galactose (fraction B), and mannose, xylose, rhamnose, glucose, and glucuronic acid (fraction C).

The one-dimensional ^{31}P spectra of the PI-oligosaccharides A and B showed four resonances, with two low field signals of similar intensity at about 25 p.p.m. and two resonances at about 3 p.p.m. of unequal intensity. These were assigned to the two 2-aminoethylphosphonic acid substituents and to the phosphate group on the inositol ring. The heterogeneity of the inositol phosphorylation was reflected in corresponding heterogeneity of the $GlcNH_2$ and *myo*-inositol spin systems, and may be due to phosphate migration from C-1 to C-2 of the inositol ring during alkaline cleavage.

Characterization of PI-oligosaccharide A—FAB-MS of PI-oligosaccharide A in the positive and negative ion mode produced protonated and deprotonated molecular ions at *m/z* 960.5 and 958.2, respectively. This is compatible with a composition of 2 hexose, 1 hexosamine, and 1 inositol phosphate residues, together with two 2-aminoethylphosphonate substituents (calculated M_r = 959.2). Weak signals at *m/z* 1122 (in the positive mode) and *m/z* 1120 (in the negative mode) provided evidence of cross-contamination with PI-oligosaccharide B.

The 50 MHz one-dimensional ^{13}C NMR spectrum of PI-oligosaccharide A (Fig. 1) showed a total of 26 intense resonances, with three in the anomeric region, one (54.88 p.p.m) typical of C-N in amino sugars (Bock and Pedersen, 1983), and two at high field (25.07 and 36.16 p.p.m.). The highest field resonance showed a 133 Hz coupling constant ascribable to $^1J_{C\text{-}P}$ in the 2-aminoethylphosphonic acid substituents,

TABLE III

Assignments of the proton and carbon NMR spectra of PI oligosaccharide A

$OPO_2CH_2CH_2NH_3^+$ (6) and $OPO_2CH_2CH_2NH_3^+$ (6)

α-Man-(1→3)-α-Man(1→4)-α-$GlcNH_2$-(1→6)-Ino-1-OPO_3

	(2)	(1)	major	minor	major	minor
H-1	5.108	5.358	5.599	5.494	4.201	3.785
H-2	4.085	4.235	3.404	3.391	4.205	4.540
H-3	3.900	3.891	4.157	4.162	3.544	3.580
H-4	3.706	3.739	3.829	3.825	3.689	3.711
H-5	3.914	3.754	4.303	n.d	3.427	n.d
H-6	4.187	3.960	4.15	n.d	3.912	n.d
H-6'	4.065	3.770	4.13	n.d		
P-CH_2	2.03		2.03	($^2J_{PCH}$= 22Hz)		
N-CH_2	3.23		3.23	($^3J_{PCCH}$= 13Hz)		

Carbon assignments

			major	minor	
C-1	103.49	101.83	95.90	97.17	77.10*
C-2	70.81	70.66	54.88	55.42	72.36
C-3	71.11	79.47	71.28		71.37
C-4	67.44	66.78	75.31		73.13
C-5	73.08	74.69	70.66		73.47
C-6	64.45*	61.87	63.76*		77.85*
P-CH_2	25.07		25.07	($^1J_{PC}$ = 133Hz)	
N-CH_2	36.16		36.16	($^2J_{PCC}$ not resolved)	

*, shows coupling to phosphorus (about 5 Hz).

which was verified by comparison with the spectrum of authentic 2-aminoethylphosphonic acid. Four resonances (at 77.85, 77.10, 64.45, and 63.76 p.p.m.) showed small (about 5 Hz) couplings attributable to $^2J_{P\text{-}O\text{-}C}$ or $^3J_{P\text{-}O\text{-}C\text{-}C}$ (O'Connor *et al.*, 1979). A DEPT-135 spectrum showed that the two high field resonances and the resonances at 61.87, 64.45, and 63.76 p.p.m. arose from methylene groups, and indicated that the 2-aminoethylphosphonate groups were located on O-6s of 2 of the sugar residues and that no (1→6)-linked sugars were present. The FAB-MS data discussed below suggest that the AEP substituents are located on the terminal mannose and the glucosamine, so the resonance at 61.87 p.p.m. was assigned as C-6 of the central Man residue. The carbon assignments are summarized in Table III, and were obtained by means of a C-H correlation experiment using the pulse sequence of Bax and Morris (1981).

The one-dimensional ^1H NMR spectrum (Fig. 2) showed four low field resonances assigned to anomeric protons; a major and minor resonance ($^3J_{1,2}$ = 3.5 Hz approximately) due to the $\alpha GlcNH_2$ and two resonances ($^3J_{1,2}$ about 1.4 Hz) assigned to mannose residues. No *N*-acetylmethyl resonance was visible. Two multiplets (each 4H) centered at 3.23 and 2.03 p.p.m. were visible. The spectrum was assigned from COSY-45 and triple-relayed COSY experiments at 500 MHz (Fig. 3), and these assignments are listed in Table IV. Some of the expected cross-peaks were not observed in the triple-relayed COSY spectrum due to the range of coupling constants present. The $\alpha GlcNH_2$ H-4/H-5 cross-peak was weak but was visible at a low contour level. The tightly overlapping Man(1) spin system complicated assignment and was finally resolved

[2] J. O. Previato, L Mendonça-Previato, C. Jones, R. Wait, and B. Fournet, unpublished data.

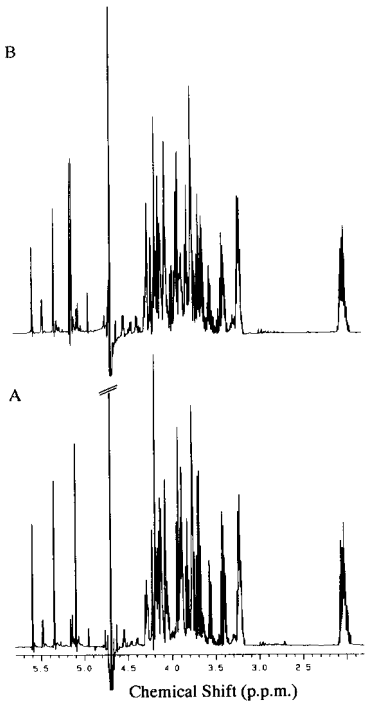

FIG. 2. **Partial 600 MHz ^{1}H NMR spectra of (*A*) PI-oligosaccharide A and (*B*) PI-oligosaccharide B, obtained at 30 °C.** The resonance from residual water has been truncated.

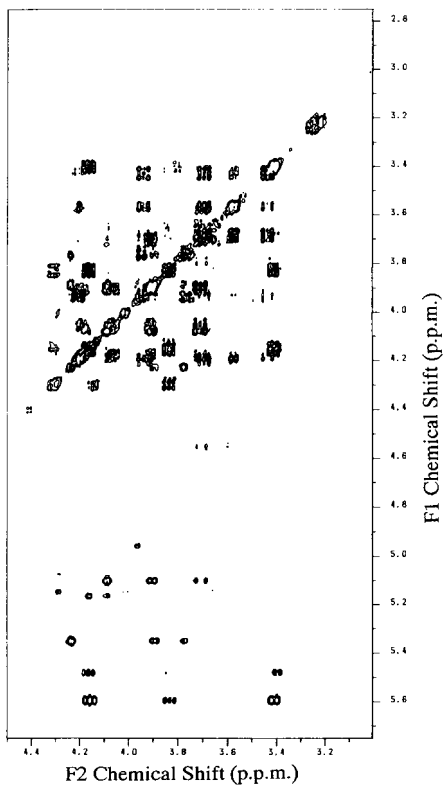

FIG. 3. **Partial 500 MHz triple-relayed COSY spectrum of PI-oligosaccharide A obtained at 30 °C.** Fixed delays were tuned for inter-proton coupling constants of 6 and 8 Hz.

using data from the carbon-proton correlation spectrum, comparison with PI-oligosaccharide B and literature data. No cross-peaks were observed for Man H-4/H-5 in either residue and the 2-AEP groups were located from mass spectrometric evidence (see below). The anomeric configuration of the Man

TABLE IV

Assignments for the proton and carbon NMR spectra of PI-oligosaccharide B

Numbers in parentheses indicate ^{13}C assignment tentative, based on chemical shift arguments. ND indicates not determined.

$OPO_2CH_2CH_2NH_3^+$ (6) and $OPO_2CH_2CH_2NH_3^+$ (6)

β-Gal*f*-(1→3)-α-Man*p*(1→3)-α-Man*p*-(1→4)-α-Glc*p*NH_2-(1→6)-Ino-1-OPO_3

		(2)	(1)				
				major	minor	major	minor
H-1	5.149	5.129	5.334	5.582	5.464	4.18	
H-2	4.140	4.274	4.224	3.389	3.334	4.179	
H-3	4.06	3.989	3.888	4.139	4.149	3.549	
H-4	4.055[a]	3.789[a]	3.760[a]	3.814	n.d	3.673	
H-5	3.819[a]	3.945[b]	3.763[a]	4.28	n.d	3.410	
H-6	3.649[a]		3.938[a]	4.12	n.d	3.925	3.768
H-6'	3.680[a]		3.736[a]		n.d		
C-1	105.42	103.26	101.81	95.89	97.18	77.05*	
C-2	82.20	67.74	70.63	54.87	55.42	72.38	
C-3	(77.89)	76.16	79.57	(71.40)		71.33	
C-4	(83.90)	(65.81)	66.77	(75.37)		73.13	
C-5	(71.70)	(73.78)	74.72	(70.86)		73.51	
C-6	63.65	64.45*	61.85*	63.66		78.31*	

[a] Proton chemical shifts from C-H correlation spectrum.
[b] Tentative assignment based on NOE to α-Man(1)H-2.
*, shows coupling to phosphorus (about 5 Hz).

residues was established from chemical shift arguments, primarily the low field position of the H-3 and H-5 resonances.

The inositol system lacks an anomeric proton from which to begin the assignment, and so the starting point was fixed as follows: the 150-ms ROESY spectrum of PI-oligosaccharide B (see below) showed an NOE between the α$GlcNH_2$ H-1 and a 10-Hz triplet at 3.91 p.p.m., which was assigned as inositol H-6 because in all previously reported structures the α$GlcNH_2$-inositol linkage is (1→6). Two low field ^{13}C resonances at 77.85 and 77.10 p.p.m. showed phosphorus couplings of about 5 Hz, and in the C-H correlation spectra of PI-oligosaccharide A (see below) these were linked to protons at 3.91 and 4.19 p.p.m., respectively. These two proton resonances were correlated in the COSY spectrum, establishing assignments for H-1 and H-6, and the other assignments were made from the correlation experiments. The interproton coupling constants (estimated from the COSY cross-peak fine structure) around the inositol ring confirmed the *myo*-configuration: thus the H-6, H-5, and H-4 resonances are 10 Hz triplets. The H-3 resonance appears as a double doublet (10 Hz and small), consistent with an axial H-2, and in agreement with the low field position of the H-2 resonance. H-4 also resonates at low field (3.69 p.p.m.) because of a 1,3-diaxial interaction with O-2 (*cf.* H-5 which lacks this interaction and resonates at 3.43 p.p.m.). The H-1/H-6 cross-peak is overlapped so its fine structure is difficult to interpret accurately. The H-1/H-2 cross-peak was likewise not observable.

Fragment ions in the FAB spectra provided evidence for the location of the AEP substituents, and further confirmation of the sequence of residues. The major cleavages produced "reducing-terminal" containing ions of the X, Y, and Z series, and nonreducing-terminal containing ions of the B and C series. Their tentative assignments in the positive ion case are indicated in Fig. 4. The labeling system used is that introduced by Domon and Costello (1988). The fragmentation

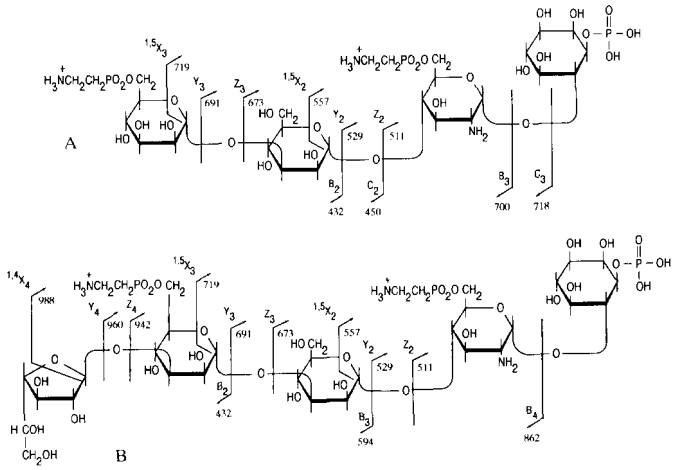

FIG. 4. **FAB-MS fragmentation diagram of PI-oligosaccharides A and B.**

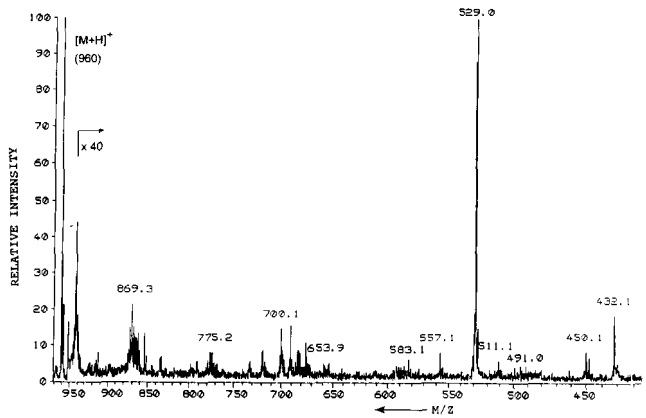

FIG. 5. **Positive ion collisional activation FAB mass spectrum of oligosaccharide A.** The protonated molecule (*m/z* 960) was fragmented by collision with helium in the field free region between the ion source and the electrostatic analyzer, and the product ions were analyzed by means of a linked scan at constant B/E. The origins of the fragment ions are indicated in Fig. 4*A*.

processes are similar in both the positive and negative mode, except that ions Y_2, B_3, and C_3 were weaker in the negative spectra. In collision spectra ion Y_2 was particularly intense (Fig. 5). This ion (*m/z* 529/527 +ve/−ve) indicates that one AEP substituent is located on either the glucosamine or the inositol phosphate. An analogous fragment has been observed in the negative FAB spectrum of an oligosaccharide obtained from the lipopeptidophosphoglycan of *Trypanosoma cruzi*, in which the glucosamine is substituted with AEP (Previato *et al.*, 1990). In the present case the AEP substituent is clearly located on glucosamine rather than inositol, since ion B_3 (*m/z* 700) is formed by the elimination of a neutral fragment of mass 260 which is attributable to inositol phosphate, whereas the mass difference of 268 between ions B_3 and B_2 (and C_2 and C_3) corresponds to the loss of glucosamine plus AEP. The other AEP substituent is located on the terminal residue, since ions X_3, Y_3, and Z_3 originate by the elimination of the mass of the terminal hexose plus 107.

Characterization of the PI-oligosaccharide B—Positive and negative ion FAB mass spectra of the PI-oligosaccharide B contained protonated and deprotonated molecular ions at *m/z* 1122/1120 (+ve/−ve), consistent with a composition of 3 hexose, 1 hexosamine, 1 inositol phosphate residues, and two 2-aminoethylphosphonate substituents (calculated M_r = 1121.3). Sequence related fragment ions belonging to the X, Y, Z, and B series were present in both the positive and negative ion spectra, and their assignments are shown (for the positive ion case) in Fig. 4. The masses of fragments Y_2, Z_2, and B_4 imply that one AEP group is located on glucosamine, as in PI-oligosaccharide A. However, elimination of the residue mass of hexose to form fragments X_4, Y_4, and Z_4 (*m/z* 988, 960, and 942, respectively), demonstrates that the terminal sugar does not bear a substituent, the other AEP being located on the penultimate hexose, as shown by the 269 mass difference between Y_4 and Y_3. Although the ion *m/z* 960 is the same mass as the $[M+H]^+$ ion of PI-oligosaccharide A and could be attributed to cross-contamination, it is correctly assigned as a Y_4 fragment since it is accompanied by an X_4 ion *m/z* 988, which cannot originate from PI-oligosaccharide A. Furthermore, *m/z* 960 is also prominent in the product ion spectrum resulting from a B/E scan of the collision products of *m/z* 1122. The enhanced intensity of Y_4 and Y_2 over the other glycosidic cleavage ions in the spectrum is presumably the result of the proximity of an AEP substituent in each case, which can readily accommodate the positive charge.

Compared to that of PI-oligosaccharide A, the 50 MHz one-dimensional ^{13}C spectrum of PI-oligosaccharide B contained additional anomeric and CH_2 resonances at 105.42 and 63.65 p.p.m., respectively, and several others (generally at low field) attributable to ring carbons consistent with the presence of 1 extra residue. The ^1H and ^{13}C NMR spectra were assigned in a similar manner to those of oligosaccharide A. The spin system of the additional residue was heavily overlapped, again complicating the interpretation, but was assigned as β-galactofuranose on the basis of the low field positions of the ^{13}C ring carbon resonances (Previato *et al.*, 1990) and by comparison with model systems (Bock and Pedersen, 1983; Ritchie *et al.*, 1975). The assignments are summarized in Table IV.

The sequence and linkages between the sugars were determined from a 600 MHz ROESY experiment obtained with a 150-ms mixing time (Fig. 6). This was run with high digital resolution in f2 (0.58 Hz/point) so that coupling patterns of enhanced peaks could be determined. The important inter-residue NOEs are identified in Fig. 6. The linkage positions deduced from these are consistent with the carbon NMR assignments.

DISCUSSION

The present study has established the covalent structures of two phosphoinositol oligosaccharides derived from the gly-

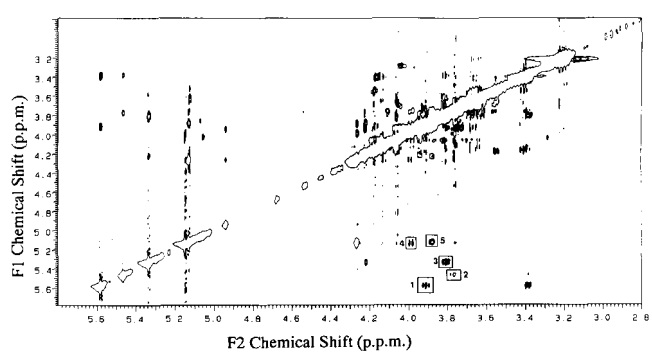

FIG. 6. **600 MHz phase-sensitive ROESY spectrum of PI-oligosaccharide B obtained with a 150-ms mixing time.** ROESY cross-peaks are negative and are plotted in full, whereas only one positive contour is plotted. *Numbered* cross-peaks are inter-residue NOEs which indicate the sequence and linkage: *1*) αGlcNH$_2$ (major) H1/inositol (major) H6; *2*) αGlcNH$_2$ (minor) H1/inositol (minor) H6; *3*) αMan (1) H1/αGlcNH$_2$ (major) H4; *4*) αMan (2) H1/αMan (1) H3; *5*) βGal*f* H1/αMan (2) H3.

cophosphosphingolipids of *L. samueli* promastigotes. These oligosaccharides differ from previously described phosphoinositol containing glycolipids (Laine and Hsich, 1987; Ferguson *et al.*, 1991), in that 2 mol of 2-AEP are present per mol of oligosaccharide. One of these substituents is attached to O-6 of the glucosamine unit, as is also observed in the LPPG of *T. cruzi* (Previato *et al.*, 1990), while the other is linked to O-6 of the second mannose unit distal to inositol, a feature not previously reported, although glucose and galactose residues substituted in position 6 with 2-AEP have been found in a sphingolipid from sea hare (Araki *et al.*, 1987).

These novel glycolipids from *L. samueli* fall within the GPI family, since they contain the core structure Manα(1→4)GlcNH$_2\alpha$(1→6)*myo*-inositol-1-PO$_4$ which is also found in the glycosylphosphatidylinositol anchor of *T. brucei* variant surface glycoprotein (Ferguson and Williams, 1988; Ferguson *et al.*, 1988), *L. major* promastigote surface protease (Schneider *et al.*, 1990), *Leishmania* spp. LPG and GIPLs (McConville, 1991), and *T. cruzi* LPPG (Previato *et al.*, 1990). In the *L. samueli* PI-oligosaccharides this conserved core is connected to the adjacent mannose residue via an α(1→3) linkage rather than by the α(1→6) linkage found in the LPPG of *T. cruzi*, and the protein linked GPI structures. The sequence Manα(1→3)Manα(1→4)GlcNH$_2\alpha$(1→6)*myo*-inositol-1-PO$_4$, indicates that the *L. samueli* glycolipids are more closely related to the non-protein linked GPIs (*i.e.* to the LPGs and GIPLs of *Leishmania* species (Turco *et al.* 1989; McConville *et al.*, 1990a, 1990b; McConville and Blackwell, 1991). Indeed, apart from the presence of the 2AEP substituents, the structure of the PI-oligosaccharide B of *L. samueli* (Gal*f*β(1→3)Manα(1→3)Manα(1→4)GlcNH$_2\alpha$(1→6)*myo*-inositol-1-PO$_4$) is identical to that of GIPL I of *L. major* and is contained in GIPLs II and III (McConville *et al* 1990b) and in the LPG anchor of *Leishmania* spp. (Turco *et al.*, 1989; McConville *et al.*, 1990a). The PI-oligosaccharide A of *L. samueli* is identical to the GIPL of *L. donovani* promastigote (iso-M2), which contains the sequence Manα(1→3)Manα(1→4)GlcNH$_2\alpha$(1→6)*myo*-inositol-1-PO$_4$ (McConville and Blackwell, 1991).

Interestingly, *L. samueli* and *Leishmania* species express glycophospholipids with remarkable structural homology in their glycan chains, but exhibit considerable divergence in their lipid moieties. Thus in common with the LPPG of *T. cruzi*, the glycophospholipids of *L. samueli* contain ceramide instead of the glycerol lipids synthesized by *L. donovani* (Orlandi and Turco, (1987) and *L. major* (McConville, 1991). This suggests that the family Trypanosomatidae may have a conserved set of glycosyl transferases which are capable of catalyzing the initial biosynthetic steps of both glycophosphosphingolipids and glycophosphoglycerol lipids.

The presence of glucosamine substituted with 2-AEP in both *L. samueli* and *T. cruzi* is another characteristic feature which may have been conserved during the evolution of certain species of trypanosomatids. The glycophosphosphingolipid structures of *Leptomonas* are therefore intermediate in evolutionary terms between the glycolipids of *T. cruzi* and *Leishmania*, being ceramides which contain 2-AEP substituted glucosamine residues like the former but resembling the latter by the presence of mannose-linked α(1→3) to the GPI core. The α(1→3) Man units are likewise encountered in the GIPLs iso-M2, M3, and M4 of *L. donovani* promastigotes, and in the GIPLs 1, 2, and 3 of *L. major* (McConville and Blackwell, 1991). Common to the glycolipids of all these species is the presence of β(1→3)-linked Gal*f* units and the highly conserved carbohydrate core structure Manα(1→4)GlcNH$_2\alpha$(1→6)*myo*-inositol-1-PO$_4$. Although not conclusive, the presence of these glycolipid structures in *Leptomonas* is consistent with the hypothesis of Baker (1963) that *T. cruzi* and *Leishmania* species evolved from a *Leptomonas*-like ancestor. It is certainly intriguing that this degree of structural homology is found between the glycolipids of a monogenetic protozoan and such diverse digenetic species as *T. cruzi* and *Leishmania* (Baker, 1963; Wallace, 1966). It has been suggested that high levels of GPI protein anchor expression among the ancestors of the kinetoplastid parasites preadapted their descendants for the production of an expanded range of GPI containing glycoconjugates which provided selective advantages initially in monogenetic, and subsequently in digenetic parasitism (Ferguson *et al.*, 1991). This hypothesis needs to take account of the fact that *Leptomonas, Endotrypanum, Herpetomonas,* and *Crithidia*, as well as *T. cruzi* all contain ceramides instead of glycerol lipids, and that with the exception of *T. cruzi* all have phytosphingosine as the long chain base.[3]

Several functions have been ascribed to the GPI-related glycoconjugates of trypanosomatids. The LPG may be important for parasite survival in the digestive tract of the insect vector as well as in the mammalian host. Thus, *L. major* LPG seems to be involved in adhesion of promastigotes to the insect gut epithelium (Davies *et al.*, 1990), and is probably also involved in protecting the parasite surface membrane from complement-mediated lysis (Puentes *et al.*, 1990). GIPLs have been hypothesized to mediate host-parasite cellular interactions (McConville, 1991). The cell surface of *L. samueli* is coated with GPI-related glycophosphosphingolipids, as is also the case in other lower trypanosomatids such as *Crithidia* and *Herpetomonas*. While it is plausible that these glycoconjugates are involved in adhesion and survival in the insect digestive tract, their precise role remains to be defined.

Acknowledgments—We are grateful to Dr. L. R. Travassos for continued advice and to O. A. Agrellos and L. J. Nascimento for skillful technical assistance. We thank the United Kingdom Medical Research Council for access to the NMR spectrometers at the Medical Research Council Biomedical NMR Centre, National Institute for Medical Research, Mill Hill, and Dr. Chris Bauer (NIMR) for help in setting up the ROESY experiment.

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[3] J. Previato and L. Mendonça-Previato, unpublished observations.

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