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Primary Structure of the Oligosaccharide Chain of Lipopeptidophosphoglycan of Epimastigote Forms of *Trypanosoma cruzi**

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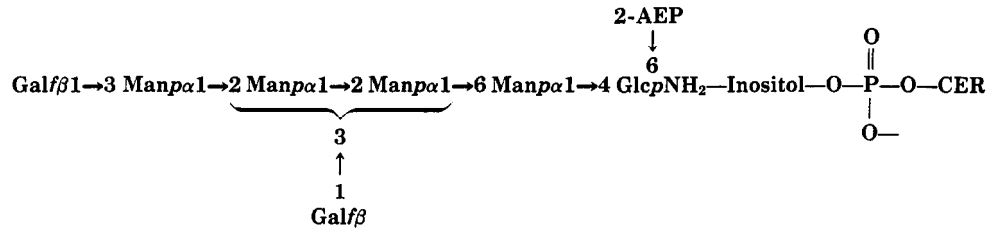
The lipopeptidophosphoglycan of epimastigote forms of *Trypanosoma cruzi* is composed of a glycan linked through a non-*N*-acetylated glucosamine residue to an inositol phosphorylceramide. Using conventional analysis techniques, including ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy and negative ion fast atom bombardment mass spectroscopy, the structure of the carbohydrate-containing part of the molecule is determined as:



STRUCTURE I

There is uncertainty as to which 2-*O*-substituted α-D-Man*p* unit is attached the side chain or whether it is distributed between the two units. Some of the structures lack the Gal*f* side chain. The inositol unit is linked to ceramide via a phosphodiester bridge. The major aliphatic components of the ceramide portion were lignoceric acid and sphinganine.

The surface glycoconjugates of *Trypanosoma cruzi*, the causative agent of Chagas' disease, are of interest by virtue of their association with host cell penetration and immunogenic properties. Aqueous chloral extraction of epimastigote forms of *T. cruzi* provided a crude antigenic preparation, containing galactose, glucose, mannose, glucosamine, and xylose (Gonçalves and Yamaha, 1969). ^{13}C NMR examination of a hot aqueous alkali extract of epimastigote forms of *T. cruzi*, containing units of galactose, mannose, and inositol, indicated the presence of β-D-Gal*f* linked (1→3) to α-D-Man*p*. Another structural feature was up to 3 consecutive (1→2)-linked α-D-Man*p* residues (Gorin *et al.*, 1981). Such crude extract arises principally from an electrophoretically homogenous glycoconjugate, called LPPG[1] (lipopeptidophosphoglycan), isolated via aqueous phenol extraction, followed by solubilization of the residue, obtained from the aqueous layer, in chloroform/methanol/water. It contained galactose, mannose, and glucose (35:22:1 molar ratio), 0.8% of glucosamine (Lederkremer *et al.*, 1976), and phosphodiester bonds between sugar residues (Lederkremer *et al.*, 1985a). 2-Aminoethyl phosphonic acid (2-AEP) was shown to be present in LPPG (Ferguson *et al.*, 1982; Mendonça-Previato *et al.*, 1983; Lederkremer *et al.*, 1985a). LPPG is antigenic; the β-D-Gal*f*-(1→3)-α-D-Man*p* structure being involved in its antigenicity (Mendonça-Previato *et al.*, 1983).

Methylation studies on LPPG showed that 2-*O*-, 3-*O*-, 6-*O*-, and 2,6-di-*O*-substituted Man*p* units were present and the Gal*f* units were linked (1→3) to 2-*O*-substituted Man*p*. It was also proposed that 3-*O*-substituted Man units were part of an external chain and that ribose was a component of the molecule (Lederkremer *et al.*, 1985b). Glycosylinositolphospholipids anchored to the cell membrane have been found in several eucaryotic cells (Ferguson and Williams, 1988). Anchor structures may contain diacylglycerols, alkylacylglycerols, lysoalkylglycerols, and ceramides and are linked to enzymes (Roberts *et al.*, 1988), adhesion molecules (Stadler *et al.*, 1989), antigenic proteins (Ferguson *et al.*, 1988) or polysaccharides (Turco *et al.*, 1989). The analogy of the LPPG component from *T. cruzi* with the glycosylinositolphospholipid anchors prompted us to investigate further its fine structure. This report describes that LPPG contains two nonreducing end-units of Gal*f* linked to a mannotetraose main-chain, whose sequence of linkages is determined and is linked (1→4) to a glucosaminyl unit substituted at *O*-6 by a phosphate ester of

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[1] The abbreviations used are: LPPG, lipopeptidophosphoglycan; 2-AEP, 2-aminoethylphosphonic acid; CER, ceramide; FAB-MS, fast atom bombardment mass spectroscopy; Gal*f*, galactofuranose; GC-MS, gas chromatography-mass spectroscopy; GLC, gas liquid chromatography; Man*p*, mannopyranose; TLC, thin layer chromatography.

2-AEP. This is then linked glycosidically to an inositol-phosphorylceramide (see formula in Summary).

EXPERIMENTAL PROCEDURES

Growth of Parasites—Epimastigotes of *T. cruzi* (Y strain) were grown in brain heart infusion medium supplemented with 10 mg/liter hemin (BHI-hemin), containing 5% (v/v) fetal calf serum. For inoculum, 15 ml of a fresh culture of *T. cruzi* in BHI-hemin with 5% fetal calf serum were transferred to flasks containing 200 ml of the same medium and incubation was at 26 °C with shaking (80 rpm) for 5 days. This was used to inoculate three liter flasks containing 1 liter of the same medium. After 5 days at 26 °C, with shaking, the cells were harvested by centrifugation, washed three times with 0.9% aqueous NaCl, and frozen at −20 °C.

Isolation and Purification of Lipopeptidophosphoglycan (LPPG)—Frozen cells were thawed and extracted three times with cold water. The residue, remaining after the last centrifugation, was extracted with 45% (v/v) aqueous phenol at 75 °C (Osborn, 1966). The aqueous layer was dialyzed, freeze-dried, dissolved in water, and applied to a column (2 × 100 cm), of Bio-Gel P-100. The excluded material was lyophilized and the dry residue shaken several times with chloroform/methanol/water (10:10:3) for extraction of LPPG. The extracts were evaporated to dryness, under reduced pressure; the residue was dissolved in water and precipitated overnight at −20 °C by addition of 5 volumes of methanol.

^{13}C NMR Spectroscopy—The spectra of most of the samples were recorded with a Bruker Am-360-WB spectrometer (Fourier Transform mode) using D_2O solutions (2 ml) in tubes of 10-mm diameter, maintained at 33 °C. The spectral width (SW) was 20 KHz, the acquisition time (AQ) 0.44 s with a delay of 0.44 s, the pulse width (PW) 90°, and the number of transients (NS) up to 100,000, depending on the sample size. The ^{13}C NMR spectrum of LPPG was obtained with a Bruker Am-400 NMR machine using 40 mg of material dissolved in D_2O (0.5 ml) maintained at 80 °C (5-mm diameter tube). Spectral details: SW 25 KHz, AQ 0.65 s, PW 30°, NS 54,000.

^{1}H NMR Spectroscopy—^{1}H NMR spectra were obtained from D_2O solutions at 33 °C using a 360 MHz spectrometer with SW 3.6 KHz, AQ 3.2 s, PW 45°, NS ~ 3000.

The ^{13}C and ^{1}H spectra were obtained using calibration with an external reference of tetramethylsilane (Me_4Si) in a coaxial tube. This calibration was done for each of the different temperatures at which the spectra were obtained since the susceptibility of the correction is slightly temperature-dependent.

^{31}P NMR Spectroscopy—Spectra were obtained at 32.4 MHz with a Bruker HX-80 spectrometer in Fourier-transform mode. Samples (~15 mg) were dissolved in D_2O (2.0 ml) maintained at 30 °C with spectral parameters: SW 4 KHz, AQ 1.02 s, PW 30°, NS 50,000. Chemical shifts are based on those of 85% aqueous orthophosphoric acid, determined in a separate experiment.

Analysis of Neutral Sugars—After hydrolysis of the samples with 2 M trifluoroacetic acid for 4 h at 100 °C, the neutral sugars were converted to their corresponding alditol acetates (Albersheim *et al.*, 1967) and analyzed by GLC on a Silicone OV-101 capillary column (25 m × 0.2 mm inner diameter) 120 °C (2 °C/min, then hold).

Analysis of Inositol and Glucosamine—The samples were treated with 3 M HCl in methanol for 18 h at 80 °C. The methanolysates were dried under a stream of N_2, the resulting residue was dissolved in 1.0 ml of aqueous 6 M HCl and heated for 18 h at 105 °C. After hexane extraction, to remove fatty acids, the aqueous layer was lyophilized, and internal standard xylitol added. The mixture was reduced with sodium borohydride, acetylated with acetic anhydride-pyridine (Albersheim *et al.*, 1967) and the products analyzed by GLC as described for neutral sugar analysis.

GC-MS—Capillary GC-MS was carried out with a Riber-Mag R10-10 (Rueil Malmainson, France) gas chromatograph using a DB-1 capillary column (0.2 mm inner diameter × 30 m). For analysis of fatty acid methyl esters, and acetylated, partly *O*-methylated methyl glucosides, a program of 100 to 240 °C (5 °C/min, then hold) was used. For GC-MS of trimethylsilyl derivatives of *N*-acetylated sphingosine bases, it was 190 to 280 °C (5 °C/min, then hold). Compounds were characterized by electron impact or chemical ionization (ammonia) mass spectroscopy. Electron impact mass fragmentation was performed as previously described (Fournet *et al.*, 1981).

Methylation Analysis—Permethylation was carried out in dimethyl sulfoxide solution using sodium hydroxide and methyl iodide as reagents (Ciucanu and Kerek, 1984). Samples were lyophilized and kept under argon throughout the methylation procedure. To a solution of native or modified LPPG (50 μg) in dimethyl sulfoxide (150 μl) was added finely powdered NaOH (1.0 mg) and methyl iodide (200 μl); the mixture was sonicated for 40 min. Saturated aqueous sodium thiosulfate (2.0 ml) was then added and the methylated product extracted with chloroform (3.0 ml). The chloroform extract was washed 10 times with equal volume of water and evaporated under a stream of N_2. The product was methanolyzed with 0.5 M HCl in methanol (1 ml) for 18 h at 80 °C, the solvent removed with a stream of N_2, and the residue treated with acetic anhydride-pyridine (50 μl, 9:1) for 24 h at 18 °C. The resulting mixture of acetylated partly *O*-methylated methyl glycosides was examined by GLC with a silicone OV-101 capillary column (25 m × 0.2 mm inner diameter) 120 to 180 °C (2 °C/min, then hold), using helium at 0.05 bar as carrier. The resulting peaks were identified by their typical retention times and GC-MS and quantitated by their areas.

Fatty Acid Analysis of LPPG—LPPG (100 μg) was methanolyzed in 0.5 M HCl in methanol (1 ml) for 18 h at 80 °C, the solution extracted with hexane, and the resulting mixture of fatty acid methyl esters analyzed by GLC on a capillary column of OV-101 at 120 to 240 °C (2 °C/min, then hold) and GC-MS.

Identification of Long-chain Sphingosine Bases in LPPG—Methanolysis of native LPPG (100 μg) was carried out using 1 M methanol-HCl made 10 M with respect to water (Carter and Gaver, 1967) for 18 h at 80 °C. The solution was adjusted to pH 11 with aqueous NaOH, extracted three times with two volumes of diethyl ether. The combined extracts dehydrated with sodium sulfate, dried under nitrogen, dissolved in 1 ml of methanol and *N*-acetylated with acetic anhydride (Gaver and Sweeley, 1966). The conversion of the *N*-acetyl derivatives of sphingolipids bases into *O*-trimethylsilyl ethers was carried out by dissolving a dried sample with pyridine/bis-(trimethylsilyl)trifluoroacetamide (1:1, v/v) for 4 h at room temperature. The product was examined by GLC on a capillary column of OV-101 (190 to 280 °C at 4 °C/min, then hold) and GC-MS.

Partial Acetolysis of LPPG—LPPG (10 mg) was partly acetolyzed in acetic anhydride-acetic acid-sulfuric acid. The products were de-*O*-acetylated with methanolic sodium methoxide (Lee and Ballou, 1965), and the resulting mixture examined by paper chromatography (solvent: ethyl acetate/pyridine/water 5:3:2, spray: *p*-anisidine hydrochloride).

Mild Acid Hydrolysis of LPPG—LPPG (200 mg) was hydrolyzed with 0.02 M trifluoroacetic acid (50 ml) for 2 h at 100 °C. The solution was evaporated and applied to a column (1.5 × 100 cm) of Bio-Gel P-2. Fractions of 2.0 ml were collected and assayed with phenol-sulfuric acid (Dubois *et al.*, 1956), ninhydrin (Toennies and Kolb, 1951), and molybdate (Burrows *et al.*, 1952) reagents for detection of carbohydrate, nitrogen, and phosphorus-containing compounds, respectively. Eluted in the void volume (V_0) was fraction A, containing the partly hydrolyzed LPPG.

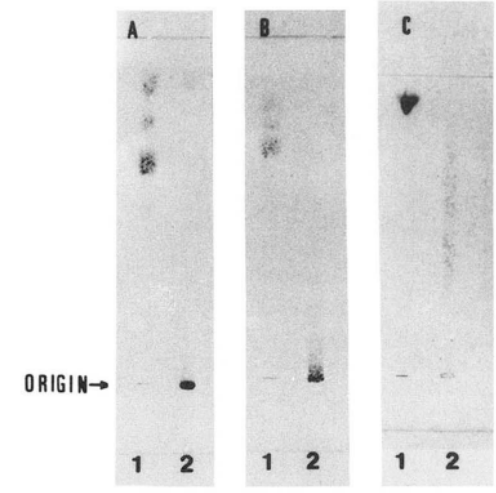


FIG. 1. **Thin layer chromatography of LPPG.** The TLC plates were developed in solvent a (*A*), solvent b (*B*), and solvent c (*C*). *Lanes 1*, glycolipids obtained from blood group O erythrocytes: ceramide dihexoside, ceramide trihexoside, globoside; *lanes 2*, LPPG. The plates were dried and sprayed with 0.5% orcinol in M ethanolic H_2SO_4 (105 °C for 10 min).

Strong Acid Hydrolysis of Fraction A—Fraction A (50 mg) was treated with 1.6 M trifluoroacetic acid (7.5 ml) for 2 h at 100 °C, the solution evaporated, and the residue chromatographed on a column (1.0 × 100 cm) of Bio-Gel P-2. Fractions of 1.5 ml were collected and assayed for carbohydrate, nitrogen, and phosphorus as described for fraction A. A fraction with elution characteristics, resembling that of a hexose trisaccharide, (fraction B) was isolated.

Strong Acid Hydrolysis of Oligosaccharide Obtained from LPPG via Treatment with Hot Aqueous $NaBH_4$-NaOH—This material (40 mg), obtained as described by Mendonça-Previato *et al.* (1983) was treated with 2 M trifluoroacetic acid (7.5 ml) for 2 h at 100 °C and the product chromatographed on a column (1.0 × 100 cm) of Bio-Gel P-2. Fractions of 1.5 ml were collected and assayed for carbohydrate, nitrogen, and phosphorus as described for fraction A. A fraction C was isolated with elution characteristics resembling that of a hexose trisaccharide.

Strong Aqueous Alkaline Hydrolysis of LPPG and FAB-MS Examination—The LPPG (20 mg) was treated with M KOH (3 ml) for 6 h at 100 °C. After neutralization with acetic acid the product was fractionated on a column (1.0 × 80 cm) of Bio-Gel P-2. The oligosaccharide (fraction D) moiety was obtained in the V_0 and examined by FAB-MS in the negative ion mode from a glycerol matrix.

Smith Degradation of Fraction D—Fraction D (4 mg) was oxidized in 0.1 M sodium metaperiodate (2 ml) for 72 h at 4 °C in the dark. Oxidized product was isolated by elution from a column (1 × 50 cm) of Bio-Gel P-2 (V_0) and hydrolyzed with 6 M HCl for 8 h at 110 °C. The hydrolysate was reduced with sodium borohydride, acetylated with acetic anhydride-pyridine, and the product examined by GLC with a capillary column of OV-101 (25 m × 0.2 mm inner diameter) at 120 to 240 °C (2 °C/min, then hold). A blank run was carried out in which unoxidized oligosaccharide was submitted to the same procedure and the acetate of glucosaminitol detected.

Mild Aqueous Alkaline Treatment of LPPG—LPPG (30 mg) was treated with M KOH (5 ml) for 18 h at 37 °C (Smith and Lester, 1974). The mixture was neutralized with acetic acid and non-polar material extracted with chloroform. The aqueous layer was desalted on a Bio-Gel P-2 column (1.0 × 100 cm), and the material eluted in the V_0 was analyzed, before and after treatment with alkaline phosphatase (Horton *et al.*, 1981) for neutral sugars, hexosamine, inositol, phosphorus, and lipids.

Amino Acid Analysis—The LPPG was hydrolyzed in 5 N HCl for 24 h at 110 °C and the amino acids, glucosamine, and 2-AEP were analyzed in an autoanalyzer (type 119 CL; Beckman Instruments, Fullerton, CA) by the method of Fauconnet and Rochemont (1978).

TLC—Thin layer chromatography was performed on 20 × 20 cm precoated TLC sheets Silica Gel 60 F-254 (Merck). Development was either with solvent a (chloroform/methanol/water/acetic acid 55:45:5:5, v/v/v/v); solvent b (chloroform, methanol, 0.2% KCl 5:5:1.5, v/v/v); or solvent c (*n*-propyl alcohol/pyridin/water 1:1:1, v/v/v). The sheets were dried and sprayed with 0.5% orcinol in M ethanolic H_2SO_4 (105 °C for 10 min) reagent for detection of sugars, 0.2% ninhydrin in 1-butanol (105 °C for 10 min) reagent for detection of nitrogen-containing compounds, and exposed to iodine vapor for detection of lipids.

Other Analytical Methods—Phosphorus was assayed by the Ames (1966) and Bartlett (1959) methods. The method of Lauter and Trams (1962) was used for the quantitative analysis of the long chain bases, after acidic methanolysis of LPPG. Sphingosine was used as standard.

RESULTS

Approximately 10^{12} cells of *T. cruzi* were extracted exhaustively with water at 4 °C. The remaining debris were treated with phenol-water. The aqueous phase of the hot phenol-water extraction was fractionated on a column of Bio-Gel P-100. The carbohydrate-containing material present in the void volume gave two Schiff-positive bands at 20–30 and 40–45 kDa when analyzed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as reported earlier by Previato *et al.*, 1985. The former component, that corresponds to the LPPG, was purified to apparent homogeneity by selective solubilization in chloroform/methanol/water (10:10:3 v/v/v). This compound showed on 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, a single coincident broad band at 20–30 kDa when stained with PAS, Coomassie Blue, and Sudan Black (Mendonça Previato *et al.*, 1983).

In despite of LPPG behavior, as a broad band on SDS-PAGE, attempts to fractionate it either by solubilization in chloroform/methanol (2:1 and 1:1, v/v), or by ion-exchange and gel-filtration chromatography were unsuccessful. Furthermore, LPPG was analyzed by TLC and remains at the origin with solvent a (Fig. 1*A*) used for separation of glycosphingolipids of up to 8 sugar units. Only a very small proportion migrates from the origin with the solvent b (Fig. 1*B*), employed for the separation of *Leishmania major* glycoinositol phospholipids in the 2-kDa range (McConville and Bacic, 1989), and with solvent c, used for analysis of LPG of *Leishmania donovani*, LPPG migrates as a broad band (Fig. 1*C*).

The ^{13}C NMR spectrum of LPPG (Fig. 2) shows that the resonance signals are in the typical regions of glycolipids. In a qualitative examination, it was clear that LPPG contains double bonds (δ 129.9–134.0), different sugar components (C-1 signals at δ 96.8 to 106.8 and others in the region δ 55.7 to 84.8) and a high proportion of aliphatic chain (δ 31.1 to 39.0). Various other small signals at δ 12.1 to 46.3 are consistent with those of protein. All of these observations are in agreement with the chemical composition of LPPG (Table I).

Seven distinct signals, ranging from δ 96.8 to 106.8 were present, arising from C-1s of sugar units. The highest-field signal at δ 96.8 is typical of 2-amino (or 2-acetamido)-2-deoxy-

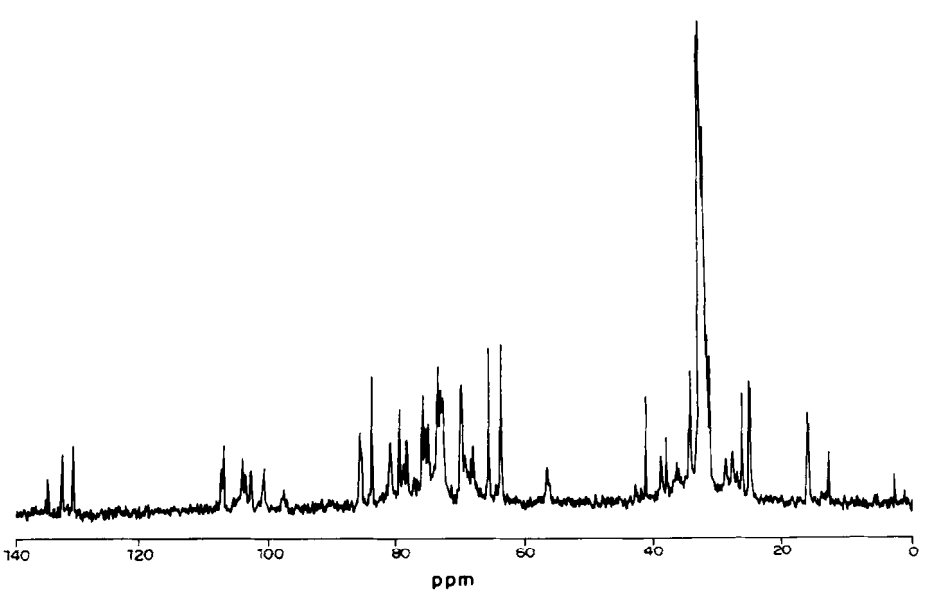


FIG. 2. **400 MHz ^{13}C NMR spectrum of native LPPG.** The spectrum was obtained using a 5-mm tube (outer diameter) containing 40 mg of LPPG dissolved in 0.5 ml of D_2O.

D-glucopyranosyl (glucosamine) units with the α-configuration and while a corresponding C-2 signal was present at δ 55.7 (Bock and Pedersen, 1983), it was relatively large due to superimposition on the resonance of carbons linked to nitrogen of ceramide (Sillerud *et al.*, 1978). The low-field C-1 signals at δ 106.6 and 106.8 are from β-D-Gal*f* units linked (1→3) to two differently situated α-D-Man*p* units (Gorin *et al.*, 1981). Typical of β-D-Gal*f* residues is a C-6 signal at δ 64.6, but since it is larger than those at δ 106.6 and 106.8 combined, superimposition on other signal(s) must occur.

LPPG was methylated and the product converted to *O*-acetylated, partly *O*-methylated methyl aldosides, which were analyzed by GLC and GLC-MS. Peaks were detected (Fig. 3), corresponding to nonreducing end-units of galactofuranose (32%) and from 2-*O*- (15%), 3-*O*- (18%), 6-*O*- (17%), and 2,3-di-*O*-substituted (17%) mannopyranosyl residues, consistent with a structure containing 6 hexose units. A derivative arising from glucosamine units was not detected.

TABLE I

Analytical data of lipopeptidophosphoglycan from epimastigote forms of T. cruzi

Analysis	% by weight
Neutral sugars[a]	54
Mannose[b]	36
Galactose[b]	18
Glucosamine[c]	5.8
Inositol[b]	5.7
Sphingolipid bases[d]	8.8
2-AEP[c]	4.0
Phosphate	
(Ames' method)	7.3
(Bartlett's method)	4.3
Protein[c]	3.3

[a] Phenol-sulfuric acid assay.
[b] GLC as alditol acetates.
[c] Amino acid analysis.
[d] Lauter and Trams' method.

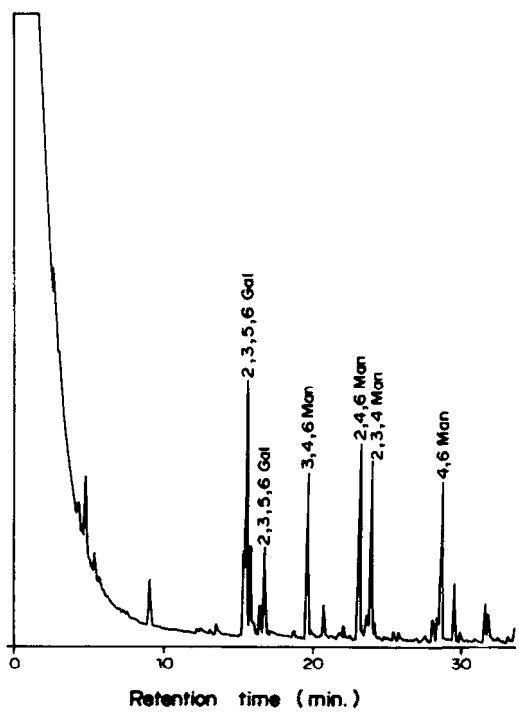


FIG. 3. **GLC of *O*-acetylated partially *O*-methylated methyl aldosides of native LPPG.** Peaks were identified by retention time and GC-MS.

LPPG was partly hydrolyzed under mild acidic conditions with 0.02 M trifluoroacetic acid for 2 h at 100 °C which selectively removed β-D-Gal*f* units to form fraction A. Its specific rotation of +65° indicated that the predominant D-Man*p* units have the α-configuration. Analysis showed that fraction A contains mannose, glucosamine, inositol, and 2-AEP in the ratio of 4:1:0.8:1. Its ^{13}C NMR spectrum (Fig. 4*A*) contains signals at δ 96.8 and 55.3, similar to those of the LPPG (Fig. 2) and which are assigned, respectively, to C-1 and C-2 units of 2-amino-2-deoxy-α-glucopyranose. The spectrum does not contain an *N*-acetyl signal at δ 23.0. Three C-1 signals at δ 103.7, 102.3, and 100.0 are similar to those of the linear mannan of *Hansenula capsulata*, which has repeating Structure I (Gorin and Spencer, 1972). Confirmation was obtained by partial acetolysis of LPPG, followed by deacetylation and paper chromatography, spots being detected with *p*-anisidine hydrochloride with order of intensity: galactose > mannotriose > mannobiose > mannose. The oligosaccharide had mobilities identical to those with α-(1→2) structures (Gorin and Perlin, 1957). Methylation analysis of fraction A provided *O*-acetylated partly *O*-methylated methyl aldosides, which indicated the presence of nonreducing end-units of

$$[-\alpha\text{-D-Man}p\text{-}(1\rightarrow2)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow2)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow6)-]_n$$

103.7 102.3 100.3

STRUCTURE I

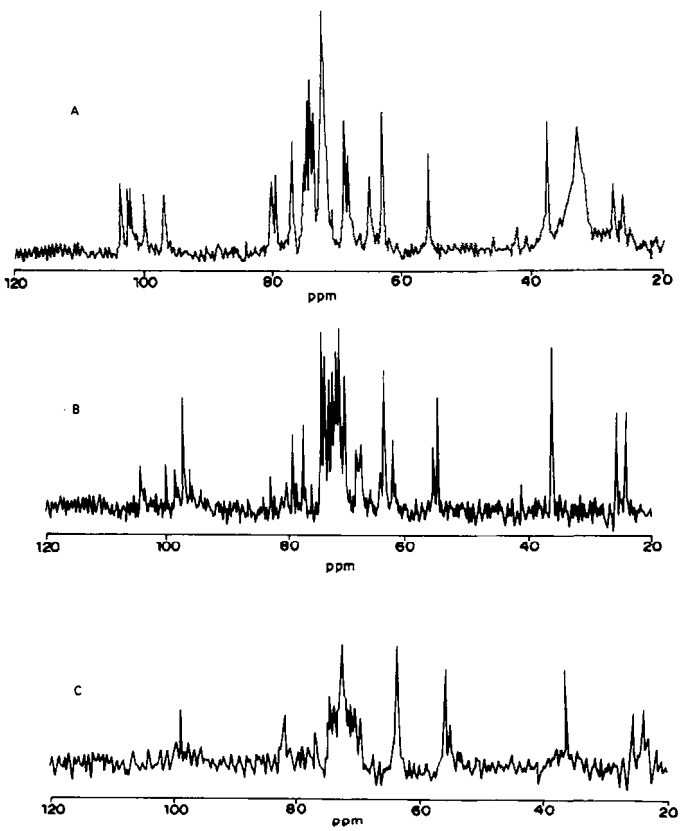
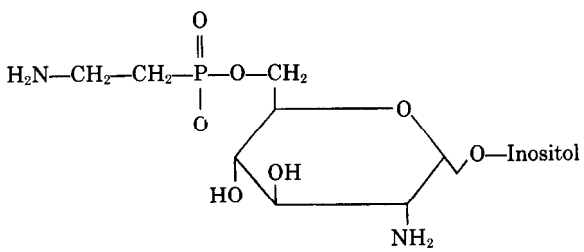


FIG. 4. **360 MHz ^{13}C NMR spectra of fractions obtained from LPPG.** *A*, fraction A (35 mg) obtained from LPPG by mild acid hydrolysis (0.02 M trifluoroacetic acid for 2 h at 100 °C); *B*, fraction B (14 mg) obtained from fraction A by strong acid hydrolysis (1.6 M trifluoroacetic acid for 2 h at 100 °C); *C*, fraction C (6 mg) obtained via strong acid hydrolysis (2 M trifluoroacetic acid for 3 h at 100 °C) from oligosaccharide moiety, which was in turn prepared from LPPG by reductive alkaline cleavage. The spectra were obtained using a 10-mm tube (outer diameter) containing the fractions dissolved in 2 ml of D_2O.

mannopyranose (21%), 2-*O*- (56%), and 6-*O*-substituted mannopyranosyl residues (23%). The C-1 signal at δ 102.5 should arise, by process of elimination, from a mannosyl unit linked to glucosamine. The ^{13}C NMR spectrum contains a signal at δ 62.6 of unsubstituted C-6 of hexopyranosyl units. Comparison of C-6 shifts of methyl α-D-mannopyranoside and methyl 2-amino-2-deoxy-α-D-glucopyranoside (Bock and Pedersen, 1983) shows that unsubstituted C-6 of units of 2-amino-2-deoxy-α-D-glucopyranoside would give a resonance at 1.5 ppm higher field. As none was detected, C-6 is substituted and it follows that the signal at δ 64.7 arises from a downfield α-phosphorylation shift. Since 2-AEP is evidenced by typical signals at δ 37.1 and 26.1, $^1J_{^{13}C,^{31}P}$ 133 Hz and a typical low-field signal in its ^{31}P NMR spectrum and was not accompanied by a phosphate monoester signal, 2-AEP is linked to 0–6 of the glucosamine units as a phosphate ester.

With the object of obtaining a preparation with a higher proportion of glucosamine and 2-AEP, fraction A was subjected to 1.6 M trifluoroacetic acid at 100 °C for 2 h, which is strong enough to cleave the glycosidic linkages of mannopyranose, but not those of highly resistant 2-amino-2-deoxy-α-glucopyranose (Ferrier and Collins, 1972) or phosphate esters of 2-AEP. Chromatography of the hydrolyzate, on Bio-Gel P-2, provided material with elution characteristics of a hexose trisaccharide which was called fraction B. Hydrolysis with 3 M HCl in methanol for 18 h at 80 °C followed by treatment with 6 M HCl for 18 h at 105 °C provided mainly glucosamine and *myo*-inositol, characterized by GLC following sodium borohydride reduction and acetylation. However, hydrolysis of fraction B in 2 M trifluoroacetic acid for 4 h at 100 °C provided mannose and trace amounts of *myo*-inositol. This is consistent with the presence of non-*N*-acetylated glucosamine which forms glycosidic linkages, that are stable, under the hydrolysis conditions employed (2 M trifluoroacetic acid for 4 h at 100 °C). The presence in the ^{13}C NMR spectrum (Fig. 4*B*) of a predominant C-1 signal at δ 97.2 indicates that one isolated unit of glucosamine is present and linked to an aglycone. Other signals of equivalent size are at δ 64.4 (C-6), δ 55.7 (C-2), and at δ 37.2 and 26.1, J 134 Hz (2-AEP),

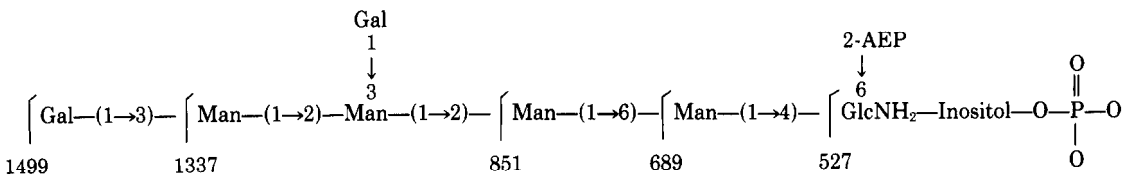


STRUCTURE II

showing that the 2-AEP ester of glucosamine is glycosidically linked to *myo*-inositol (Structure II). The partial ^1H NMR spectrum (Fig. 5*A*) is in agreement with this structure with the largest H-1 signal at δ 5.51, J 4.0 Hz, the coupling value agreeing with those of N-substituted derivatives of 2-amino-2-deoxy-α-D-glucopyranose (Bhacca and Ludowieg, 1969). The area of this signal is approximately equal to that of the CH_2P complex centered at δ 1.93.

A preparation, related to fraction B, was obtained by submitting the oligosaccharide isolated from LPPG by hot alkaline borohydride treatment, with 2 M trifluoroacetic acid for 3 h at 100 °C. Fractionation of the hydrolyzate, on a column of Bio-Gel P-2, provided fraction C, whose elution characteristics were also similar to that of a hexose trisaccharide. Mannose was absent since the phenol-sulfuric acid test was negative, but positive ninhydrin and molybdate reactions were obtained. The ^{13}C NMR spectrum (Fig. 4*C*) contained glucosamine signals at δ 98.5 (C-1), 56.3 (C-2), and 64.7 (*O*-6 substituted) along with those of 2-AEP (δ 37.2 and 26.1, J 135 Hz). The signal at δ 64.7 indicates that migration of the 2-AEP ester to other positions had not taken place under alkaline conditions. The ^1H NMR spectrum of fraction C, however, differed from that of fraction B. Three H-1 signals were detected at δ 5.54 (J 4.0 Hz), 5.36 (J 3.6 Hz), and 5.31 (J 3.8 Hz) in a ratio of 5:7:8 (Fig. 5*B*), indicating three different structures containing α-glucosamine units. The combined area of these signals, when compared with those of 2-AEP, showed a glucosamine to 2-AEP ratio of 1:0.8.

Elucidation of Structure III of the oligosaccharide complex was carried out using material obtained from LPPG by hot aqueous alkali treatment (fraction D). Oxidation with sodium periodate of this fraction D showed that the glucosaminyl unit was attacked, as successive strong acid hydrolysis, borohydride reduction, acetylation, and GLC examination showed the absence of the acetate of glucosaminitol. Examination of the fraction D by negative ion FAB-MS, in a glycerol matrix, gave rise to a series of ion peaks from 527 to 1499, separated at intervals of 162 (Fig. 6) corresponding to the depicted breakdown.



STRUCTURE III

The structure of the lipid moiety of LPPG was analyzed by GLC and GC-MS after acidic methanolysis as described under "Experimental Procedures." Two long-chain bases, sphinganine and sphingosine, were found in a 4.2:1 molar ratio. The fatty acid composition consisted mainly of lignoceric acid (76%). Palmitic acid (14%), stearic acid (7%), oleic acid (3%), and trace amounts of myristic acid were also present. The profile of the fatty acids and sphingolipid bases released from LPPG by acid methanolysis is shown in Figs. 7 and 8.

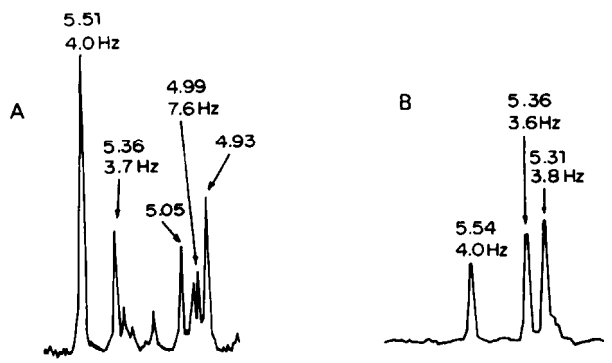


FIG. 5. **H-1 region of the 360 MHz ^1H NMR spectra of fractions obtained from LPPG.** *A*, fraction B (14 mg); *B*, fraction C (6 mg). Both spectra were obtained using a 10-mm tube (outer diameter) containing the fractions dissolved in 2 ml of D_2O.

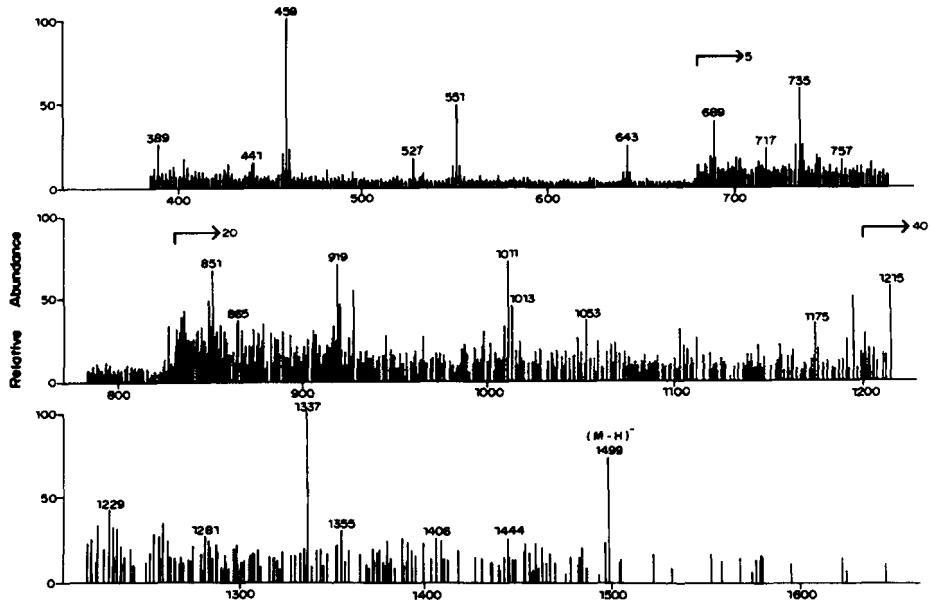


FIG. 6. **FAB-MS of fraction D obtained from LPPG by hot aqueous alkali treatment (1 M KOH for 6 h at 100 °C).** The mass spectrum was recorded in the negative ion mode using glycerol as a matrix. Ions arising from the matrix are *m/z*: 459, 551, 643, 735, 919, 1011, 1113, and 1195.

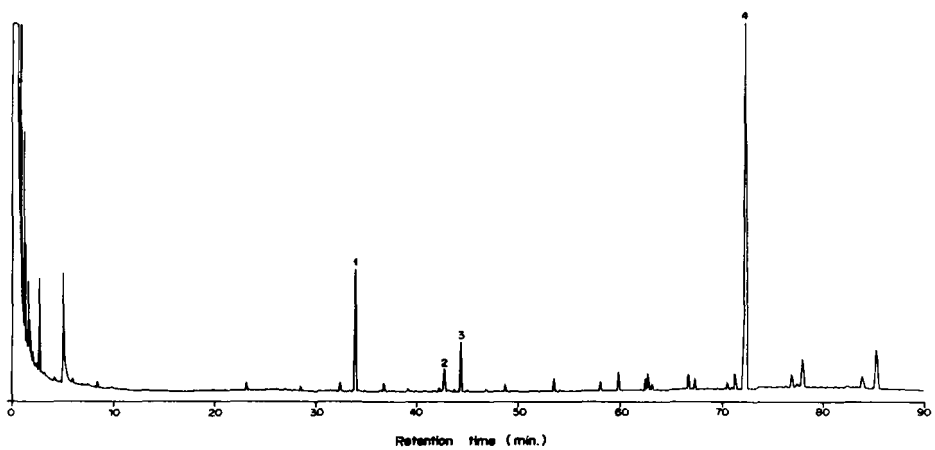


FIG. 7. **GLC of fatty acids methyl esters from LPPG.** *1*, methyl palmitate; *2*, methyl oleate; *3*, methyl stearate; *4*, methyl lignocerate. Peaks were identified by retention time and GC-MS.

In order to establish the linkage of the phosphoinositol group to the ceramide, LPPG was treated with 1 M KOH for 18 h at 37 °C, conditions that would hydrolyze inositol phosphoceramide, with formation of a cyclic inositol phosphate as intermediate, producing an inositol monophosphate and ceramide.

GLC and GC-MS analysis of the products obtained after treatment of LPPG, with 1 M KOH, 18 h at 37 °C following partition between chloroform and water revealed that all sphingolipid bases (sphinganine and sphingosine), and fatty acids (lignoceric and palmitic acids) were present only in the chloroform layer. These results showed that mild alkaline treatment cleaves the ceramide moiety of LPPG, with release of the corresponding phosphoinositol oligosaccharide. This is consistent with the quantitative analysis, in the LPPG, of sphingolipid bases to inositol in the molar ratio of 0.9:1 (Table I).

Chemical analysis of the phosphoinositol oligosaccharide moiety showed that it contained mannose, galactose, glucosamine, inositol, and phosphorus in a 4:2:1:1:2.4 molar ratio. As expected, phosphorus was measured quantitatively by Ames' method but not by that of Bartlett, indicating that the oligosaccharide contained the following two kinds of phosphorus: one hydrolyzed with acid and the other not, characteristic of 2-AEP. The molar ratio of acid-stable phosphorus to total phosphorus was 0.54. After treatment of the phosphoinositol oligosaccharide with alkaline phosphatase, the ratio of acid stable phosphorus was 0.76.

The ready formation of phosphoinositol oligosaccharide by alkaline hydrolysis, along with the phosphorus determination of this oligosaccharide, before and after treatment with alkaline phosphatase, suggests that the phosphodiester bond, likely, links inositol to ceramide. These results agree with the ^{31}P spectrum of LPPG, which contained two major resonances in the region of phosphonates at δ 22.2 and phosphodiester at δ 0.9 (Fig. 9).

DISCUSSION

The methylation analysis data on LPPG show that there are 2 mol/mol of Gal*f* units and 1 mol/mol each of 2-*O*, 3-*O*, 6-*O*, and 2,3-di-*O*-Man*p* substituted residues, consistent with a 6-unit structure. This differs from a previous report in which these structural components were found but in which Gal*f* and 2,3-di-*O*-substituted Man*p* were detected in much lower proportions (Lederkremer *et al.*, 1985b). We find that there are two different types of Gal*f* units (1→3)-linked to those of Man*p*, by virtue of the ^{13}C NMR spectrum (Fig. 2), which contains two β-Gal*f* C-1 signals at δ 106.6 and 106.8. This agrees with the present methylation analysis of LPPG that was partly acid hydrolyzed (fraction A) to remove Gal*f* units. It is found that the 2,3-di-*O*-substituted Man*p* units were replaced by 2-*O*-substituted ones and that the 3-*O*-substituted Man*p* units were converted to non-reducing

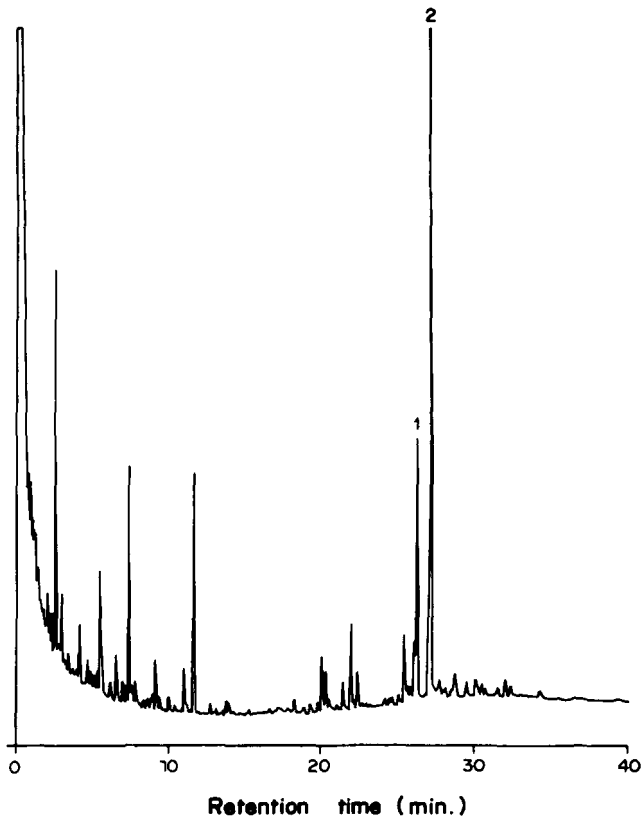


FIG. 8. **GLC of *N*-acetyl-*O*-trimethylsilyl bases from LPPG.** *1*, trimethylsilylether of *N*-acetyl-sphingosine; *2*, trimethylsilylether of sphinganine. Peaks were identified by retention time and GC-MS.

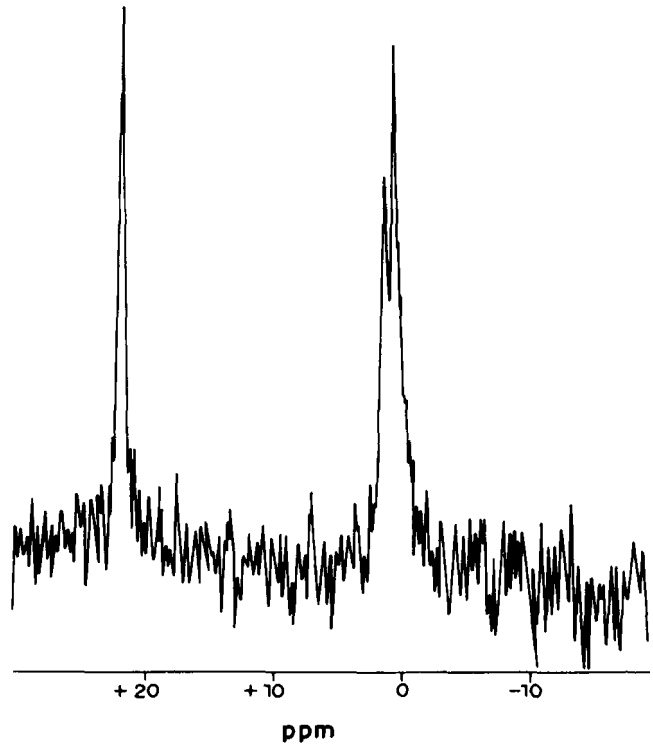


FIG. 9. **^{31}P NMR spectrum of native LPPG.** The spectrum was obtained using a 5-mm tube (outer diameter) containing 15 mg of LPPG dissolved in 2 ml of D_2O.

end-units. These data, combined with those on partial acetolysis on LPPG, which show two consecutive (1→2)-links joining α-D-Man*p* units, and ^{13}C NMR spectroscopy on fraction A, lacking Gal*f* units, indicate that α-D-Man*p* units are consecutively linked (1→2), (1→2), and (1→6) as in Structure IV.

β—D—Gal*f*(1→3)—α—D—Man*p*—(1→2)—α—D—Man*p*—(1→2)—α—D—Man*p*—(1→6)—α—D—Man*p*

3
↑
1
β—D—Gal*f*

STRUCTURE IV

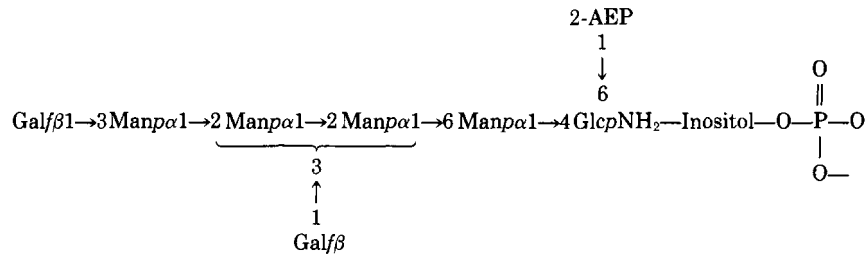
The only doubt rests on which 2-*O*-Man*p* residue is substituted by the Gal*f* single-unit side-chain.

2-AEP, first reported to be a component of *T. cruzi* LPPG (Ferguson *et al.*, 1982), is now found to be attached, as a phosphate ester, to *O*-6 on non-*N*-acetylated glucosaminyl units in fraction A, obtained under mild acid hydrolysis conditions. More vigorous acid hydrolysis of fraction A provided fraction B, which contained a high proportion of a similar moiety attached via an α-glycosidic linkage to *myo*-inositol (Structure 2).

It is remarkable that LPPG contains 2-AEP linked to *O*-6-glucosaminyl units, a feature that has not been reported previously. It has, however, been characterized in the sphingolipid of the sea hare (Araki *et al.*, 1987) and a snail polysaccharide (Fontana *et al.*, 1985) as a substitute on *O*-6-galactopyranosyl units.

Strong acid hydrolysis of the oligosaccharide prepared via hot alkaline borohydride treatment of LPPG (Mendonça-Previato *et al.*, 1983) gave rise to fraction C. Its ^{13}C NMR spectrum (Fig. 4*C*) had similarities to that of fraction B (Fig. 4*B*) with signals typical of α-glucosaminyl units substituted at *O*-6 with 2-AEP and linked glycosidically to inositol (Structure II). In its ^{1}H NMR spectrum (Fig. 5*B*), however, the H-1 signal at δ 5.54, *J* 4.0 Hz, representing this structure, comprised only 25% of the total H-1 region. Others were present at δ 5.36 (*J* 3.6 Hz, 35%) and δ 5.31 (*J* 3.8 Hz, 40%) and likely arise from Structure II substituted with phosphate ester on the inositol, in more than one position, since migration occurred under alkaline conditions.

In the LPPG molecule, the esterified glucosaminyl units are substituted at *O*-4 by α-D-Man*p* units, since the glucosamine residue, in the oligosaccharide moiety isolated from LPPG, using hot aqueous alkali (fraction D), were susceptible to periodate oxidation.



STRUCTURE V

Confirmation of suggested Structure V of fraction D was furnished by negative ion FAB-MS (Fig. 6), which gave rise to a continuous series of peaks from mass 527 to 1499, at intervals of 162. It was not possible to determine the position of the single-unit Gal*f* side-chains on 2-*O*-substituted Man*p* units, since ion peaks of mass 1013 or 1175 were not lacking. Possibly the side chains are distributed between the two Man*p* units. Some of the molecules do not contain the side chain, as the C-1 signal of such units at δ 106.8 is smaller than that of the other Gal*f* unit at δ 106.6 in the ^{13}C NMR spectrum of LPPG (Fig. 2).

Previously, according to ^{31}P NMR spectroscopy, phosphodiester bonds between sugar residues were found to be the predominant phosphorus-containing component in LPPG. This high proportion of phosphodiester to phosphonate linkage, found in LPPG by Lederkremer *et al.* (1985a), could be due to the presence of contaminant RNA, since 7% of ribose was present in their preparation.

In the present study, the ^{31}P NMR spectrum of LPPG (Fig. 9) contains two major signals at δ 22.2 and 0.9, corresponding to 2-AEP and phosphodiester, respectively (Costello *et al.*, 1975). The relative sizes of the signals are in agreement with phosphorus determinations which show a ratio of 1:1.4 of 2-AEP to acid hydrolyzable phosphorus. These data, combined with those obtained by alkaline treatment (1 M KOH, 18 h at 37 °C) of LPPG, suggest that the phosphoroester bridge between inositol and ceramide is the predominant phosphodiester bond in LPPG.

The sphingolipid bases, components of ceramide, were identified as sphinganine and sphingosine (in the ratio of 4.2:1) by their characteristic GLC retention time and electron impact and chemical ionization mass spectra (Fig. 8). These results contrast with a previous report (Lederkremer *et al.*, 1978) that described 17-methyl-sphinganine and sphinganine in a molar ratio of 3:1.

It is of interest to note that LPPG contains a *myo*-inositol head group, substituted by α-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→6)-α-D-Man*p*-(1→4)-α-D-Glc*p*NH_2, which is structurally similar to the core of the glycosylphosphatidyl inositol anchors reported for both the *Trypanosoma brucei* variant surface glycoprotein (Fergunson *et al.*, 1988) and the rat brain Thy-1 glycoprotein (Homans *et al.*, 1988). However, these contain a diacylglycerol moiety and ethanolamine phosphate in place of ceramide and 2-AEP of LPPG. Also, since amino acid units are liberated from LPPG by mild acid treatment, the 3.3% peptide in LPPG is not peptidically linked to the amino group of 2-AEP, unlike ethanolamine residues substituted by a polypeptide chain. This observation agrees with the presence of ester bonds between hydroxyl groups of sugar units and carboxyl groups of amino acids (Lederkremer *et al.*, 1985a).

The core of LPPG component from *T. cruzi*, consisting of the α-Man*p*-(1→4)-α-D-Glc*p*NH_2-inositol structure, resembles that of the lipophosphoglycan of *Leishmania donovani* (Turco *et al.*, 1988) and to the glycoinositolphospholipids isolated from *L. major* (McConville and Bacic, 1989).

Differently of *Leishmania* glycoinositolphospholipid, the LPPG from *T. cruzi*, contains a phosphoceramide as its lipid moiety, a feature also found in the lipophosphoglycan from *Acanthamoeba castellanii* (Dearborn *et al.*, 1976), and in the anchor of a protein in *Dictyostelium discoideum* (Stadler *et al.*, 1989). Furthermore, the LPPG and *A. castellanii* lipophosphonoglycan contain 2-aminoethylphosphonic acid with is absent in the *Leishmania* LPG and glycoinositolphospholipid (Turco *et al.*, 1988; McConville and Bacic, 1989).

The biological significance of the glycophosphosphingolipids in *T. cruzi* is still unknown. These kind of compounds, which have been described mainly in plants, yeast, and fungi may be functionally analogous to the gangliosides found in animal cells (Laine and Hsieh, 1987). Whether the presence of glycophosphoshingolipids in the protozoan has any relation to parasite-vertebrate host interaction needs further investigation.

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