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 1H, 13C, and 31P NMR spectroscopy and negative ion fast atom bombardment mass spectroscopy, the structure of the carbohydrate-containing part of the molecule is determined as:

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2-AEP

Galβ1→3 Manpα1→2 Manpα1→2 Manpα1→6 Manpα1→4 GlcNH2-Inositol-O-P-O-CER
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**STRUCTURE I**

There is uncertainty as to which 2-O-substituted α-D-Manp unit is attached the side chain or whether it is distributed between the two units. Some of the structures lack the Galβ 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 Yamashita, 1969). 13C 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-Galf linked (1→3) to α-D-Manp. Another structural feature was up to 3 consecutive (1→2)-linked α-L-Manp residues (Gorin et al., 1981). Such crude extract arises principally from an electrophoretically homogenous glycoconjugate, called LPPG (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., 1992). LPPG is antigenic; the β-D-Gaf(1→3)-α-D-Manp structure being involved in its antigenicity (Mendonça-Previato et al., 1983).

Methylation studies on LPPG showed that 2-O-, 3-O-, and 6-O-, and 2,6-di-O-substituted Manp units were present and the Galf units were linked (1→3) to 2-O-substituted Manp. 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). Glycosylinositolphosphoglycans anchored to the cell membrane have been found in several eucaryotic cells (Ferguson and Williams, 1988). Anchor structures may contain diacylglycerols, alkylacylglycerols, lysolgalaktylglycerols, 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 glycosylinositolphosphoglycan anchors prompted us to investigate further its fine structure. This report describes that LPPG contains two nonreducing end-units of Galf linked to a mannnotetraose 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...
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 centrifuged, 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 x 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.

**Conversion of LPPG—**Chromatography of the LPPG on Bio-Gel P-100 (2 x 100 cm) resulted in the isolation of three fractions of LPPG. The main peak was eluted in the void volume (V0) of the column and the effluent was collected and concentrated. The concentrated solution was applied to a column (2 x 100 cm) of Bio-Gel P-100 and eluted with 0.1 M phosphate buffer, pH 7.0. The main peak was collected and concentrated. The concentrated solution was applied to a column (2 x 100 cm) of Bio-Gel P-100 and eluted with 0.1 M phosphate buffer, pH 7.0. The main peak was collected and concentrated.

**Identification of Long-chain Sphingosine Bases in LPPG—**After chromatography on Bio-Gel P-100, the main peak was lyophilized and eluted with 0.1 M phosphate buffer, pH 7.0. The main peak was collected and concentrated.

**Analysis of Neutral Sugar Content—**The samples were hydrolyzed with 2 M HCl for 18 h at 80 °C. The hydrolysates were extracted with diethyl ether and the aqueous phase was concentrated to dryness. The resulting residue was dissolved in acetic acid and the solution was applied to a thin-layer chromatography plate. The plate was developed with a solvent system of ethyl acetate/pyridine/water (4:1). The bands were visualized by spraying with alcian blue and the sugars were identified by their Rf values.

**Analysis of Amino Acid Content—**The samples were hydrolyzed with 6 M HCl for 24 h at 120 °C. The hydrolysates were extracted with diethyl ether and the aqueous phase was concentrated to dryness. The resulting residue was dissolved in water and the solution was applied to a thin-layer chromatography plate. The plate was developed with a solvent system of ethyl acetate/pyridine/water (4:1). The bands were visualized by spraying with alcian blue and the amino acids were identified by their Rf values.

**Analysis of Phosphorus Content—**The samples were hydrolyzed with 6 M HCl for 24 h at 120 °C. The hydrolysates were extracted with diethyl ether and the aqueous phase was concentrated to dryness. The resulting residue was dissolved in water and the solution was applied to a thin-layer chromatography plate. The plate was developed with a solvent system of ethyl acetate/pyridine/water (4:1). The bands were visualized by spraying with alcian blue and the phosphoruscontaining compounds were identified by their Rf values.

**Analysis of Lipid Content—**The samples were hydrolyzed with 2 M HCl for 18 h at 80 °C. The hydrolysates were extracted with diethyl ether and the aqueous phase was concentrated to dryness. The resulting residue was dissolved in acetic acid and the solution was applied to a thin-layer chromatography plate. The plate was developed with a solvent system of ethyl acetate/pyridine/water (4:1). The bands were visualized by spraying with alcian blue and the lipids were identified by their Rf values.

**Identification of Sphingosine Bases—**The samples were hydrolyzed with 2 M HCl for 18 h at 80 °C. The hydrolysates were extracted with diethyl ether and the aqueous phase was concentrated to dryness. The resulting residue was dissolved in acetic acid and the solution was applied to a thin-layer chromatography plate. The plate was developed with a solvent system of ethyl acetate/pyridine/water (4:1). The bands were visualized by spraying with alcian blue and the sphingosine bases were identified by their Rf values.

**Identification of Neutral Lipids—**The samples were hydrolyzed with 2 M HCl for 18 h at 80 °C. The hydrolysates were extracted with diethyl ether and the aqueous phase was concentrated to dryness. The resulting residue was dissolved in acetic acid and the solution was applied to a thin-layer chromatography plate. The plate was developed with a solvent system of ethyl acetate/pyridine/water (4:1). The bands were visualized by spraying with alcian blue and the neutral lipids were identified by their Rf values.

**Identification of Amino Acids—**The samples were hydrolyzed with 6 M HCl for 24 h at 120 °C. The hydrolysates were extracted with diethyl ether and the aqueous phase was concentrated to dryness. The resulting residue was dissolved in water and the solution was applied to a thin-layer chromatography plate. The plate was developed with a solvent system of ethyl acetate/pyridine/water (4:1). The bands were visualized by spraying with alcian blue and the amino acids were identified by their Rf values.

**Identification of Phosphorus-containing Compounds—**The samples were hydrolyzed with 6 M HCl for 24 h at 120 °C. The hydrolysates were extracted with diethyl ether and the aqueous phase was concentrated to dryness. The resulting residue was dissolved in water and the solution was applied to a thin-layer chromatography plate. The plate was developed with a solvent system of ethyl acetate/pyridine/water (4:1). The bands were visualized by spraying with alcian blue and the phosphorus-containing compounds were identified by their Rf values.

**Identification of Lipids—**The samples were hydrolyzed with 2 M HCl for 18 h at 80 °C. The hydrolysates were extracted with diethyl ether and the aqueous phase was concentrated to dryness. The resulting residue was dissolved in acetic acid and the solution was applied to a thin-layer chromatography plate. The plate was developed with a solvent system of ethyl acetate/pyridine/water (4:1). The bands were visualized by spraying with alcian blue and the lipids were identified by their Rf values.

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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 Aqueous Alkaline Hydrolysis of LPPG and FAB-MS Examination—The LPPG (20 mg) was treated with 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 V0 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 (V0) and hydrolyzed with 5 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 glucosaminol detected.

Mild Aqueous Alkaline Treatment of LPPG—LPPG (30 mg) was treated with KOH (6 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 V0 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/acet 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-propanol acetic acid/pyridine/water 1:1:1, v/v/v). The sheets were dried and sprayed with 0.5% orcinol in M ethanolic H2SO4 (105 °C for 10 min) reagent for detection of sugars, 0.2% ninhydrin in 1-butanol (100 °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 (1958) methods. The method of Lauter and Trams (1982) was used for the quantitative analysis of the long chain bases, after acidic methanalysis of LPPG. Sphingosine was used as standard.

RESULTS

Approximately 1012 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-200. 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 (Mendonga Previato et al., 1983).

In spite 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. 1A) used for separation of glycosphinolipid of up to 8 sugar units. Only a very small proportion migrates from the origin with the solvent b (Fig. 1B), employed for the separation of Leishmania major glycosphingolipids 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. 1C).

The 13C 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-
Lipopeptidophosphoglycan from T. cruzi

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-Galp units linked (1→3) to two differently situated α-D-Manp units (Gorin et al., 1981). Typical of β-D-Galp 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 methylaldosides, 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

<table>
<thead>
<tr>
<th>Analysis</th>
<th>% by weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutral sugars*</td>
<td>54</td>
</tr>
<tr>
<td>Mannose*</td>
<td>36</td>
</tr>
<tr>
<td>Galactose*</td>
<td>18</td>
</tr>
<tr>
<td>Glucosamine*</td>
<td>5.8</td>
</tr>
<tr>
<td>Inositol*</td>
<td>5.7</td>
</tr>
<tr>
<td>Sphingolipid bases*</td>
<td>8.8</td>
</tr>
<tr>
<td>2-AEP*</td>
<td>4.0</td>
</tr>
<tr>
<td>Phosphate</td>
<td></td>
</tr>
<tr>
<td>(Ames’ method)</td>
<td>7.3</td>
</tr>
<tr>
<td>(Bartlett’s method)</td>
<td>4.3</td>
</tr>
<tr>
<td>Protein*</td>
<td>3.3</td>
</tr>
</tbody>
</table>

* Phenol-sulfuric acid assay.
* GLC as alditol acetates.
* Amino acid analysis.
* Lauter and Trams’ method.

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-Galp units to form fraction A. Its specific rotation of +65° indicated that the predominant n-Manp 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 13C NMR spectrum (Fig. 4A) 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, 102.5, and 100.0 are similar to those of the linear mannan of *Hansenula capsulata*, which has repeating Structure 1 (Gorin and Spencer, 1972). Confirmation was obtained by partial acetylation 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 methylaldosides, which indicated the presence of nonreducing end-units of

![Fig. 3. GLC of O-acetylated partially O-methylated methylaldosides of native LPPG. Peaks were identified by retention time and GC-MS.](image)

![Fig. 4. 360 MHz 13C 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 D2O.](image)
mannopyranose (21%), 2-O- (56%), and 6-O-substituted manno-
pyranosyl residues (23%). The C-1 signal at δ 102.5 should
arise, by process of elimination, from a mannoyl unit linked
to glucosamine. The 13C NMR spectrum contains a signal at
δ 62.6 of unsubstituted C-6 of hexopyranosyl units. Compari-
son of C-1 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, J1P,31P 133 Hz and a typical low-
field signal in its 31P NMR spectrum and was not accompanied
by a phospho monooester 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 sub-
jected to 1.6 M trifluoroacetic acid at 100 °C for 2 h, which is
strong enough to cleave the glycosidic linkages of mannopy-
ranose, 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
triasaccharide 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

\[
\text{Gal} \quad \text{Man} \quad \text{Man} \\
\text{Gal}-(1-3) \quad \text{Man}-(1-2) \quad \text{Man}-(1-2) \\
1499 \quad 1337 \quad 851 \quad 688 \quad 527
\]


different structures containing α-glucosamine units. The com-
bined 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, boro-
hydride 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.

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, sphingan-
inine and sphingosine, were found in a 4.2:1 molar ratio. The
fatty acid composition consisted mainly of lignoceric acid
(78%). Palmitic acid (14%), stearic acid (7%), oleic acid (9%),
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.

\begin{align*}
\text{H}_{2}N-\text{CH}_{2}-\text{CH}_{2}-\text{P}-\text{O}-\text{CH}_{3} \\
\text{HO} \\
\text{O-}\text{Inositol} \\
\text{NH}_{2}
\end{align*}

\text{Gal} \quad \text{1} \\
\text{3}

\text{STRUCTURE II}

showing that the 2-AEP ester of glucosamine is glycosidically
linked to myo-inositol (Structure II). The partial 'H NMR
spectrum (Fig. 5A) 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
CH2P complex centered at δ 1.93.

A preparation, related to fraction B, was obtained by sub-
mmiting the oligosaccharide isolated from LPPG by hot alka-
line 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 character-
istics were also similar to that of a hexose trisaccharide.
Mannose was absent since the phenol-sulfuric acid test was
negative, but positive ninhydrin and molibdate reactions were
obtained. The 13C NMR spectrum (Fig. 4C) contained gluco-
samine signals at δ 98.5 (C-1), 56.3 (C-2), and 64.7 (C-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 'H NMR spectrum of fraction C,
however, differed from that of fraction B. Three H-1 signals
were detected at 5.544 (J 4.0 Hz), 5.364 (J 3.6 Hz), and 5.31
(J 3.8 Hz) in a ratio of 5:7:8 (Fig. 5B), indicating three
different structures containing α-glucosamine units. The com-
bined area of three signals, when compared with those of 2-
AEP, showed a glucosamine to 2-AEP ratio of 1:0.8.

\begin{align*}
\text{Gal} \quad \text{1} \\
\text{3}
\end{align*}

\text{STRUCTURE III}

\begin{align*}
\text{Gal}-(1-3) \quad \text{Man}-(1-2) \quad \text{Man}-(1-2) \\
1499 \quad 1337 \quad 851 \quad 688 \quad 527
\end{align*}

\begin{align*}
\text{Man}-(1-6) \quad \text{Man}-(1-4) \\
\text{2-AEP} \quad \text{O} \\
\text{O} \\
\text{6}
\end{align*}

\begin{align*}
\text{GlcNH*-Inositol-O-P-O} \\
\text{O}
\end{align*}

\begin{align*}
\text{H}_{2}N-\text{CH-CH2-P-0-CH2} \\
\text{OH} \\
\text{O-}\text{Inositol} \\
\text{NH}_{2}
\end{align*}

\begin{align*}
\text{Gal} \quad \text{1} \\
\text{3}
\end{align*}
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 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 Galf units and 1 mol/mol each of 2-0, 3-0, 6-0, and 2,3-di-O-Manp substituted residues, consistent with a 6-unit structure. This differs from a previous report in which these structural components were found but in which Galf and 2,3-di-O-substituted Manp were detected in much lower proportions (Lederkremer et al., 1985b). We find that there are two different types of Galf units (1→3)-linked to those of Manp, by virtue of the $^{31}$C NMR spectrum (Fig. 2), which contains two β-Galf 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 Galf units. It is found that the 2,3-di-O-substituted Manp units were replaced by 2-O-substituted ones and that the 3-O-substituted Manp units were converted to non-reducing

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

\[ \beta-D-Galf(1\rightarrow3)-\alpha-D-Manp(1\rightarrow2)-\alpha-D-Manp(1\rightarrow2)-\alpha-D-Manp(1\rightarrow6)-\alpha-D-Manp \]

\[ \underline{3} \]

\[ \uparrow \]

\[ \beta-D-Galf \]

**Structure IV**

The only doubt rests on which 2-O-Manp residue is substituted by the Galp 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-Previo et al., 1983) gave rise to fraction C. Its 13C NMR spectrum (Fig. 4C) had similarities to that of fraction B (Fig. 4B) with signals typical of α-glucosaminyl units substituted at O-6 with 2-AEP and linked glycosidically to inositol (Structure II). In its 1H NMR spectrum (Fig. 5B), however, the H-1 signal at δ 5.54, J 4.0 Hz, representing this structure, comprised only 26% 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-Manp units, since the glucosamine residue, in the oligosaccharide moiety isolated from LPPG, using hot aqueous alkali (fraction D), were susceptible to periodate oxidation.

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

**FIG. 9.** 31P 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 D2O.
Lipopeptidophosphoglycan from T. cruzi

2-AEP

Gal(1→6)Manp→1 Manp→1→2 Manp→1→6 Manp→1 GlnpNH₂ · Inositol - O → P - O

3   1

Galβ

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 Galf side-chains on 2-O-substituted Manp units, since ion peaks of mass 1013 or 1175 were not lacking. Possibly the side chains are distributed between the two Manp units. Some of the molecules do not contain the side chain, as the C1 signal of such units at δ 106.8 is smaller than that of the other Galf unit at δ 106.6 in the 13C NMR spectrum of LPPG (Fig. 2).

Previously, according to 31P 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), may be due to the presence of contaminant RNA, since 7% of ribose was present in their preparation.

In the present study, the 31P 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 phosphoester 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 electrophoretic 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-Manp-(1→2)-α-D-Manp-(1→2)-α-D-Manp-(1→6)-α-D-Manp-(1→4)-α-D-GlcpNH₂, which is structurally similar to the core of the glycosylphosphatidylinositol anchors reported for both the Trypanosoma brucei variant surface glycoprotein (Ferguson et al., 1988) and the rat brain Thy-1 glycoprotein (Homans et al., 1988). However, these contain a dicarboxylic 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-Manp→1-GlcpNH₂-inositol structure, resembles that of the lipophosphoglycan of Leishmania donovani (Turco et al., 1958) 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 lipophosphoglycan 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 glycoprophosphoglycolipids 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 glycoprophosphoglycolipids in the protozoan has any relation to parasite-vertebrate host interaction needs further investigation.

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Primary structure of the oligosaccharide chain of lipopeptidophosphoglycan of epimastigote forms of Trypanosoma cruzi.
J O Previato, P A Gorin, M Mazurek, M T Xavier, B Fournet, J M Wieruszesk and L Mendonça-Previato


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