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Structural analysis of a glycosylphosphatidylinositol glycolipid of *Leishmania donovani*

DANIEL SEVLEVER[1,2], PETER PÅHLSSON[3], GRACIELA ROSEN[1]*, BO NILSSON[4] and MAURICIO V. LONDNER[1]

[1] *Department of Parasitology, The Kuvin Centre for the Study of Infectious and Tropical Diseases, Hebrew University—Hadassah Medical School, P.O. Box 1172, Jerusalem 91010, Israel*

[2] *Present address: Department of Pharmacology, School of Medicine, Case Western Reserve University, Cleveland, OH 44106, USA*

[3] *Department of Carbohydrate Chemistry, University of Lund, Solvegatan 41, S-22370 Lund, Sweden*

[4] *Biocarb Technology AB, S-22370, Lund, Sweden*

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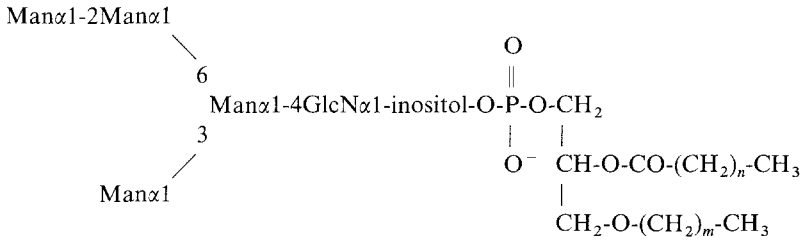
A glycosylphosphatidylinositol (GPI) glycolipid antigen recognized by sera from patients with visceral leishmaniasis was isolated from *Leishmania donovani* promastigotes. The carbohydrate moiety was cleaved from the lipid part by digestion with specific phosphatidylinositol phospholipase C. After separation, structural analysis was carried out on the phosphorylated inositol oligosaccharide and the alkylacyl glycerol. The following major structures were found:



n = 16, 18, 20, 22, 24, 26 m = 16, 17, 18, 21

The presence of the conserved sequence Manα1-2Manα1-6Manα1-4GlcN-PI of glycosyl phosphatidylinositol protein anchors in this antigen may be consistent with a precursor role of *Leishmania* glycosyl phosphatidylinositol anchored proteins for this glycolipid.

Keywords: antigen, glycolipid, glycosyl phosphatidylinositol, *Leishmania donovani*, protein anchor

The parasitic protozoan *Leishmania* relies heavily on glycosylated phosphatidylinositol (GPI) for anchoring its cell surface molecules. The abundant glycoconjugates of the parasite membrane, the gp63 protease and the lipophosphoglycan (LPG), are GPI-containing molecules [1–4]; LPG and gp63 are believed to play important roles during the parasite life cycle [5, 6], which includes an intracellular amastigote stage in macrophages of a susceptible mammalian host and an extracellular promastigote stage in the gut of the sandfly vector.

In promastigotes of *Leishmania major* (*L. major*), the causative agent of cutaneous leishmaniasis, we found three glycosyl phosphatidylinositol glycolipids resembling the pharmacologically active glycosylphosphatidylinositol glycans present in many mammalian tissues [7–9]. GPIs A, B and C are cell surface-located and are recognized by sera from patients with cutaneous leishmaniasis [10]. The chemical structure of the GPIs was recently reported as $(Gal)_n\alpha$1-3Gal$f\alpha$1-3Manα1-3Manα1-4GlcN-alkylacyl PI where n = 1, 2 and 0 for A, B and C [11–13]. A family of glycoinositol phospholipids from *L. major* that contains GPI A, B and C was also described by McConville *et al.* [14, 15]. The common structure of one of the *L. major* GPIs and the phosphoglycan core of the LPG, together with kinetic studies [16] and the preferentially intracellular localization of these glycolipids [17] strongly support their role as

* To whom correspondence should be addressed.

precursors of LPG. A glycolipid with properties consistent with a precursor role for the variant surface glycoprotein GPI anchor was recently reported for *Trypanosoma brucei* [18, 19]. The biosynthetic pathways involved in its synthesis have been partially described [20, 21]. However, in *Leishmania*, despite the known importance of gp63, neither the precursors nor the biosynthetic pathways leading to its formation have been elucidated. The importance of determining a precursor of gp63 is due to the enzyme's putative role in protecting intracellular survival of the parasite [22], among other functions.

Thy-1, variant surface glycoprotein (VSG) and gp63, possessing distinct biochemical properties and with different evolutionary origins, are anchored to the membrane by GPIs, which all share the conserved minimal Manα1-2Manα1-6Manα1-4GlcN1-6Ins structure in the glycan core [23–25].

In this study, structural analysis of GPI-III, the most antigenic among the *Leishmania donovani* (*L. donovani*) GPIs, was carried out. Its glycan has a GPI-like anchor structure linked to 1-alkyl-2-acyl phosphatidylinositol (PI) through a non-*N*-acetylated glucosamine. The structure of GPI-III may be consistent with a precursor role for *Leishmania* GPI-anchored proteins.

Materials and methods

Parasites and growth conditions

L. donovani (LRC-52) from a human case of visceral leishmaniasis (Kala Azar) was obtained from the World Health Organization's International Reference Centre for Leishmaniasis, Jerusalem, cloned by limited dilution and named clone 9. Promastigotes were grown in Schneider's medium [26] containing 5% fetal calf serum.

Sera

Sera were obtained from patients with active kala azar from India (*L. donovani donovani*).

Solvent designations

Solvents used were as follows: solvent A, hexane:isopropanol, 3:2 by vol; solvent B, butanol:acetic acid:water, 6:2:2 by vol; solvent C, methanol:acetic acid, 7:0.02 by vol; solvent D, chloroform:methanol:4.2 N ammonium hydroxide, 45:35:10 by vol; solvent E, chloroform:methanol:0.25 M KCl, 50:50:15 by vol; solvent F, ethyl acetate:methanol:acetic acid:water, 60:15:15:10 by vol.

Metabolic labelling of promastigotes

1.5×10^8 promastigotes in late exponential phase were washed three times with 0.15 M NaCl. The labelling was performed in 12 ml DMEM phosphate free medium supplemented with 20% dialyzed fetal calf serum, 500 μCi [^{32}P] orthophosphate (Amersham, 200 μCi mmol^{-1}), xanthine (0.05 mM), adenosine (0.05 mM), hemin (5 mg l^{-1}) and biotin (1 mg ml^{-1}), for 4 h at 28°C. [^{3}H]Inositol and [^{3}H]myristic acid incorporations were as previously described [10, 11].

Purification of the glycolipids

L. donovani promastigotes were harvested by centrifugation and washed three times with PBS, pH 7.2. The packed cells containing 1×10^{10} parasites were extracted five times (5, 4, 3, 3 and 3 ml) with solvent A. The lipid extract was dried by a stream of nitrogen and resuspended by sonication in 3 ml water. The water phase was washed twice with 1.5 ml hexane, dialyzed for 48 h against distilled water and lyophilized. Glycolipids contained in the fraction were separated by TLC (Silica Gel 60, Merck) in solvent B. [^{32}P]Glycolipids extracted from [^{32}P]orthophosphate labelled promastigotes and purified under the same conditions were used for their identification by autoradiography of the plates. The glycolipids were eluted from the silica with solvent C and further purified from inorganic salts by LH-20 chromatography. The efficiency of the purification procedure was assessed by TLC in solvent D followed by solvent E in the same dimension. The plates were sprayed with orcinol/H_2SO_4 reagents for detection of sugars and exposed to iodine vapor for detection of lipids. ^{32}P-labelled, [^{3}H]myristic acid labelled and [^{3}H]inositol labelled GPI III migrated to a single spot in two dimensional high performance thin layer chromatography (HPTLC) using solvents B and F for the first and second direction, respectively. The glycolipids were quantified by phosphate determination [27]. The yield of GPI-III was approximately 0.16 mg per 2.4×10^{11} cells.

Chemical and enzymatic treatments

Nitrous acid deamination of [^{3}H]inositol labelled GPI-III was performed by sonicating the glycolipid in 1 ml 0.2 M sodium acetate, pH 4.0, containing 0.25 M sodium nitrate (BDH), and incubated for 16 h at 37°C. The products of the reaction were extracted to an organic phase and analyzed in TLC with appropriate standards as described elsewhere [11]. PI-PLC hydrolysis was done in 15 mM Hepes, pH 7.4, containing 10 μg ml^{-1} purified PI-PLC from *S. aureus*, a generous gift from Dr. Martin Low (Columbia University), 4 h at 37°C. The percentage of cleavage was quantified by paper chromatography as described previously [11].

The susceptibility of GPI-III to phospholipase A_2 (PLA_2) (Sigma) was determined by incubating the [^{3}H]myristic acid labelled glycolipid in 100 μl 125 mM Tris-HCl, 0.1% deoxycholate, 11 mM $CaCl_2$, pH 7.4, containing 500 units ml^{-1} of the enzyme for 3 h at 37°C. The reaction was stopped by the addition of 900 μl water (adjusted to pH 1 with HCl) and 3 ml chloroform:methanol, 2:1 by vol. The organic phase was applied to HPTLC and developed twice in solvent B. Quantification was performed by scraping 0.5 cm silica gel segments and counted by liquid scintillation spectrometry.

Analysis of GPI-III lipid moiety

GPI-III purified from 1.25×10^{12} promastigotes was resuspended by sonication in 200 µl 15 mM Hepes, pH 7.4, containing 10 µg ml^{-1} of phosphatidylinositol-specific phospholipase C (PI-PLC) from *Staphylococcus aureus* and incubated for 24 h. The hydrolysis was followed by spotting 1 µl of the reaction mixture on HPTLC, developed in butanol:acetic acid:water (solvent B) and stained with orcinol. After 24 h the orcinol-positive band with R_F of 0.23 disappeared and the origin became orcinol-positive indicating that the GPI-III was totally hydrolyzed. The lipid was separated from the glycan by partitioning in chloroform and water (1:1, by vol). Methanolysis of the lipid was performed with 0.5 ml conc. HCl:H_2O:methanol, 8.6:9.4:82 by vol) at 70°C for 18 h [28]). After concentration the products were acetylated and analyzed by gas liquid chromatography/mass spectrometry (GLC/MS).

Analysis of GPI-III carbohydrate moiety

Sugar analysis was performed after acid hydrolysis in 4 M trifluoroacetic acid at 100°C for 4 h. The monosaccharides were converted into their corresponding alditol acetates and quantified by GLC [29]. Peracetylation in 1 ml acetic anhydride containing 100 µl pyridine was carried out overnight at room temperature. Methylation analysis was carried out as previously described [30]. Permethylation was performed according to the method of Hakomori [31]. Permethylated oligosaccharides were purified on a Sep-Pak C18 cartridge (Waters) according to Waeghe *et al.* [32]. Nitrous acid deamination was carried out in 0.2 M sodium acetate/0.25 M sodium nitrite, pH 4.0, for 16 h at 25°C. The pH was set to 8.0 and reduction was carried out with sodium borodeuteride for 2 h at room temperature.

Mass spectrometry

Fast atom bombardment mass spectrometry (FAB-MS) was carried out in the positive ion mode on a VG ZAB SE instrument. Samples were dissolved in thioglycerol (1-thio-2,3-propanediol) and loaded on a stainless steel target which was bombarded with xenon atoms with a kinetic energy of 8 keV. GLC/MS in electron ionization mode, was performed on a VG 12-250 quadrupole instrument fitted with SE-30 capillary columns. For analysis of partially methylated alditol acetates and lipid derivatives a 30 m × 0.2 mm column was used and the ion source temperature was 200°C. Deaminated oligosaccharides were analyzed as permethylated derivatives on a 7 m × 0.2 mm column with an ion source temperature of 350°C. Spectra were recorded at 70 eV.

NMR spectrometry

^{1}H-NMR spectra were recorded on a Bruker AM-500 FT spectrometer. Oligosaccharides obtained after PI-PLC cleavage of the GPI-III were exchanged with ^2H_2O, lyophilized and dissolved in high purity ^2H_2O (99.95%). As a complement to the 1D spectrum, 2D ^{1}H–^{1}H correlation experiments COSY [33] and relay COSY [34] were performed. The chemical shifts are expressed relative to internal sodium 4,4-dimethyl-4-silapentane, but were actually measured relative to internal acetate, the CH_3 signal set to 1.908 ppm downfield from sodium 4,4-dimethyl-4-sila-pentane. The spectra were recorded at 27°C with a p^{2}H of approximately 7.

ELISA

Glycolipids 0 to VII (Fig. 1) were resuspended in water by sonication, and 160 µg (100 µl per well) were dispensed to a 96 well microtiter plate and incubated for 1 h at 37°C. The resuspended glycolipids were re-used at least fifteen times without significant loss of activity. More passages were not pursued. After washing twice with 0.2% Tween-20 PBS (T-PBS), the plates were blocked during 2 h with the same buffer. 100 µl of immune human sera, diluted 1:100 in PBS supplemented with 10% fetal calf serum (FCS-PBS), were added to each well. After 1 h at room temperature, the wells were washed three times with 0.05% T-PBS, and 100 µl per well of horseradish peroxidase conjugated goat anti-human IgG (BioMakor) (diluted 1:1000 in 10% FCS-PBS)

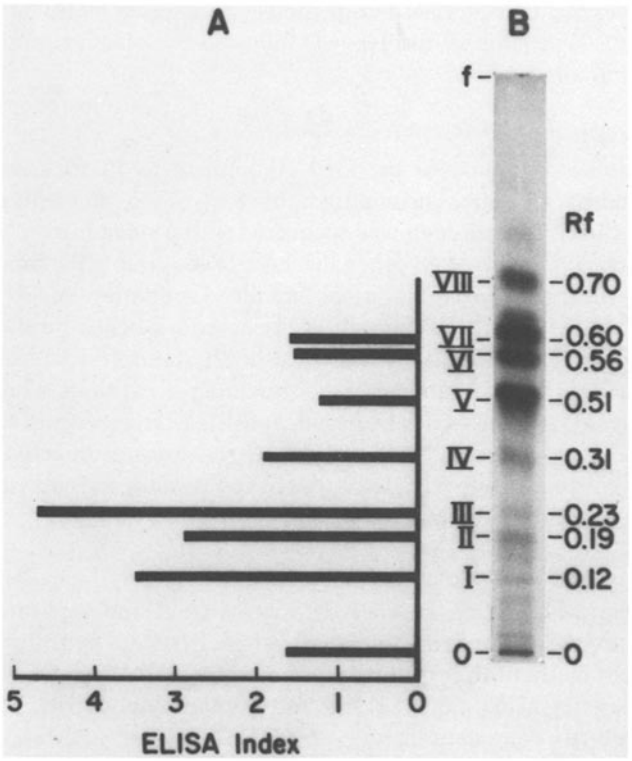


Figure 1. A. ELISA of each glycolipid after elution from the TLC plates using anti *L. donovani* immune sera. The results are expressed as: index = OD immune sera/OD normal sera. B. Autoradiographic TLC pattern of ^{32}P-labelled CLF developed in solvent B.

were added and incubated for 1 h at room temperature. After three washes with 0.05% T-PBS, 100 µl per well of ABTS (Calbiochem) (2,2′-azino-di-3-ethyl benzthiazoline-6-sulphonic acid) 1.8 M containing 0.1 mmol H_2O_2 were added, and after 15 min the absorbancy values were read at 420 nm. For each antigen, the optimal conditions for an antigen/serum combination were determined by a checkerboard titration. The results were expressed as an index: optical density (OD) of immune sera/OD of the normal controls.

Results

Previous studies revealed that a hexane/isopropanol extraction from *L. donovani* promastigotes contained a carbohydrate lipid fraction (CLF) able to bind antibodies from patients with VL [35]. The glycolipids contained in the CLF were separated by TLC and detected by autoradiography of the ^{32}P-labelled CLF (Fig. 1B). The three glycolipids responsible for the immunological activity of the CLF were I, II and III, with an R_F of 0.12, 0.19 and 0.23, respectively. They were identified in ELISA using VL sera after their elution from the plates and gel chromatography on a LH-20 column (Fig. 1A). Structural analysis was further performed on GPI-III ($R_F = 0.23$), the glycolipid with the higher ELISA index. The efficiency of the purification protocol as described in Materials and methods was assessed by HPTLC (Fig. 2) developed in solvent D followed by solvent E in the same dimension.

Chemical and enzymatic analysis

The susceptibility of the GPI-III antigen to PI-PLC was followed by paper chromatography. 85% of the radioactivity of the treated antigen was recovered in the solvent front as expected for a free glycan, while 84% of the counts remained at the origin in the control sample. Incubation of [^{3}H] inositol-GPI with sodium nitrite released a labelled product migrating on TLC together with the PI standard (data not shown). Mild alkaline hydrolysis, under conditions where only acyl chains are hydrolyzed, and PLA2 treatment of the [^{3}H]myristic acid labelled GPI-III, released radioactivity migrating with myristate (unpublished results) and myristic acid standard, respectively (Fig. 3).

Structure of the lipid portion of GPI-III

The lipid portion of GPI-III was isolated and separated from the carbohydrate moiety after PI-PLC hydrolysis. Acid methanolysis of the lipid moiety of GPI-III generated free fatty acids methyl esters and monoalkylglycerols. The products were characterized by GLCMS after acetylation. Fatty acids and alkylglycerol ethers give characteristic mass spectra from which molecular weight, chain length, number of unsaturations and hydroxyl groups present in the chain can be deduced. Six different fatty acids were identified: C26:0, C24:0, C22:0, C20:0, C18:0, and C16:0, in a ratio

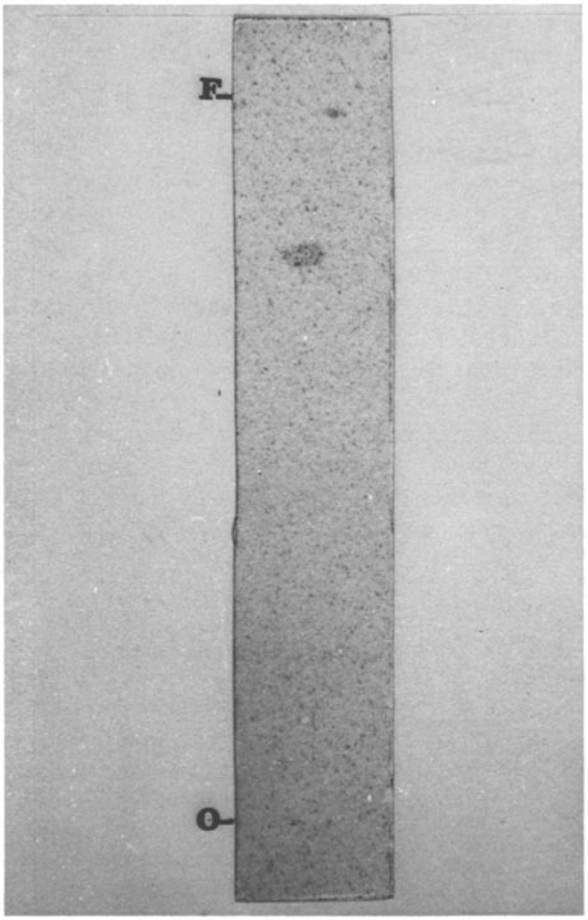


Figure 2. Thin layer chromatography of purified GPI-III. The glycolipid was extracted and purified as described in the Materials and methods section. The TLC was developed in solvent D followed by solvent E in the same dimension. The plate was stained with orcinol/H_2SO_4.

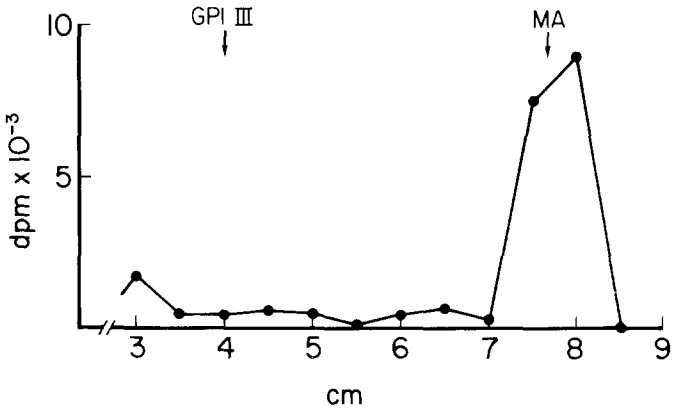


Figure 3. TLC radiochromatogram of [^{3}H]myristic acid labelled products released from GPI-III by treatment with 500 units ml^{-1} of PLA2 for 3 h at 37°C.

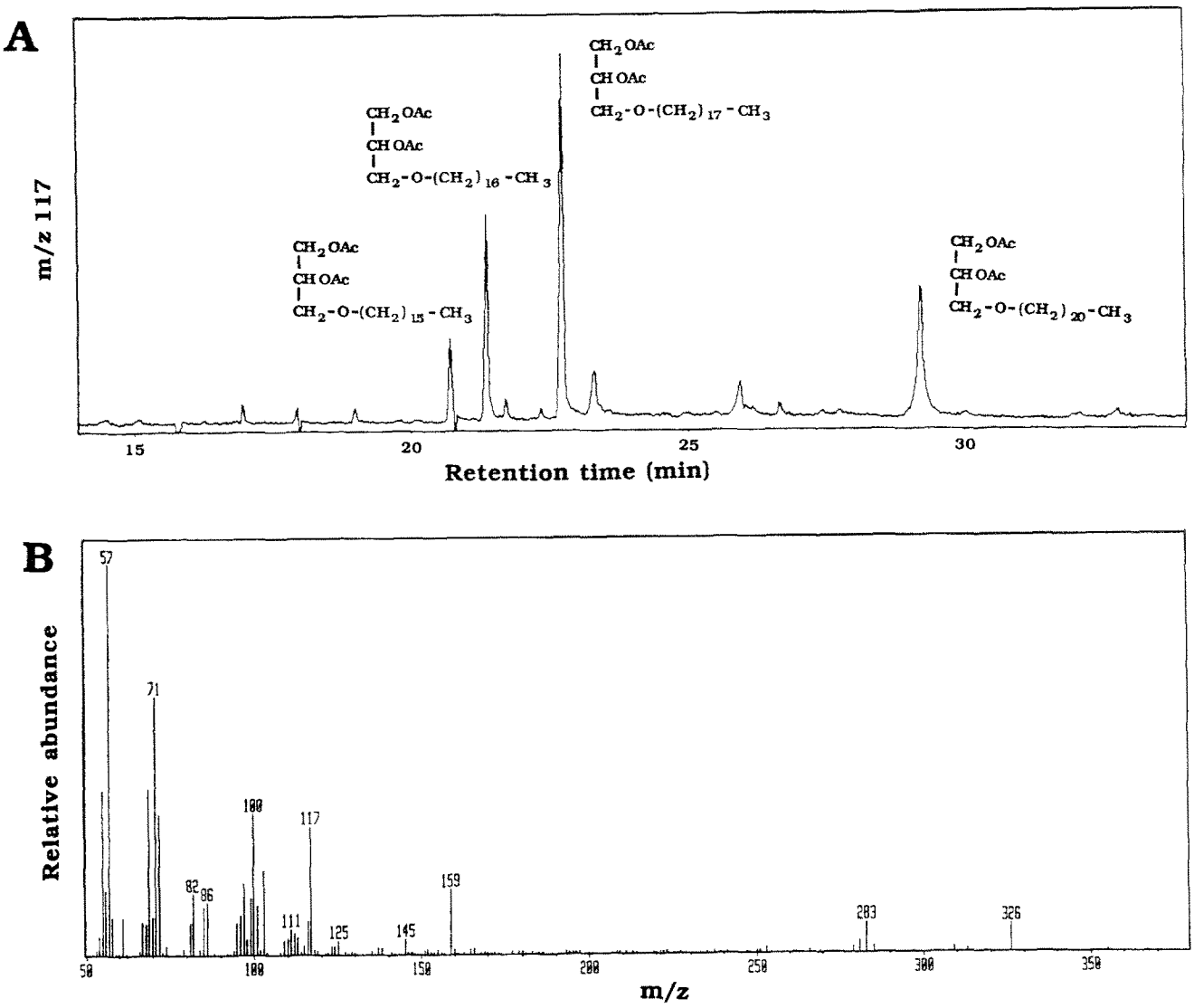


Figure 4. Selected-ion monitoring of the 1-*O*-alkyl glycerols of GPI-III, as acetylated derivatives. A. The ion of *m/z* 117 shows four 1-*O*-alkyl glycerols with C16:0, C17:0, C18:0, and C21:0 alkyl chains, respectively. B. Mass spectrum (electron ionization) of 1-*O*-octadecanyl glycerol as acetylated derivative.

of 1:1:1:1:3:1. No hydroxy or unsaturated fatty acids were detected.

All mass spectra obtained from the alkylglycerol ethers as acetylated derivatives showed characteristic ions of *m/z* 159 and 117. An ion chromatogram using the ion *m/z* 117 is shown in Fig. 4A. The alkyl chain length was determined from the spectra by the $[M-102]^+$ ions, formed from the molecular ions (M) after elimination of acetic acid followed by elimination of ketene (represented by the ion *m/z* 326 in Fig. 4B) and the ions formed by cleavage of the bond between carbon-1 and carbon-2 of the glycerol moieties. The presence of the latter ions, $[CHO\text{-}(CH_2)_n\text{-}CH_3]^+$, which is represented by *m/z* 283 for $n = 17$ in Fig. 4B, supports that the alkyl chain is linked to the 1-position of the glycerol. The different alkyl chains observed were C16:0, C17:0, C18:0, and C21:0 (Fig. 4A).

Structure of the carbohydrate portion of GPI-III

GPI-III could be cleaved by nitrous acid, releasing the glycan from the PI, thus indicating the presence of a non-*N*-acetylated glucosamine. In order to obtain a quantitative recovery of the hexosamine, *N*-acetylation is necessary. Since glycosidic linkages to a nonacetylated hexosamine would be stable to hydrolysis, the carbohydrate of GPI-III was acetylated prior to sugar and methylation analyses.

Sugar analysis showed a monosaccharide composition of mannose and *N*-acetylglucosamine in a ratio of 4:1. Inositol and galactose were detected in trace amounts. The low recovery of inositol is due to the difficulties of hydrolyzing inositol phosphate in 4 M trifluoroacetic acid.

Table 1. Methylation analysis data of GPI-III of *L. donovani*[a].

2,3,4,6-OMe-Man	2.3
3,4,6-OMe-Man	1.0[b]
2,4,6-OMe-Man	0.7
2,4-OMe-Man	1.2
3,6-OMe-GlcN(Me)Ac	0.9

[a] The values were obtained by GLC using a flame ionization detector.
[b] 3,4,6-OMe-Man set to 1.0.

Methylation analysis showed mannose derivatives corresponding to nonreducing terminal, internal 2- and 3-substituted residues and a branched 3,6-disubstituted residue (Table 1). Furthermore, the *N*-acetylglucosamine residue was determined to be 4-substituted. No galactose derivatives were observed.

The carbohydrate portion was further analyzed by FAB-MS. In order to obtain more structural information from the mass spectrum, the oligosaccharides were derivatized prior to analysis. Permethylation will enhance the formation of fragment ions and thereby give additional information about the structure [36]. FAB mass spectrometry of permethylated oligosaccharides gives information about monosaccharide sequence, molecular weight and some binding positions. Permethylated *N*-acetylhexosamine-containing carbohydrates are characterized by intense ions formed by cleavage of the 2-acetamido-2-deoxyhexosyl linkage. The FAB mass spectrum of the permethylated and *N*-acetylated GPI-III oligosaccharide is shown in Fig. 5. The primary ion *m/z* 1077 is characteristic for a Hex_4-HexNAc sequence. The presence of a secondary ion of *m/z* 1045, formed after elimination of methanol from *m/z* 1077, indicates that the *N*-acetylhexosamine is substituted in the 4-position, confirming the methylation analysis data [37]. A protonated molecular ion $[M+1]^+$ of *m/z* 1421 is consistent with a Hex_4-HexNAc sequence linked to an inositol phosphate.

Present in the spectrum also are ions derived from other, minor components. The sequence ion of *m/z* 872 with the corresponding $[M+1]^+$ ion of *m/z* 1216 indicates a Hex_3-HexNAc-inositol phosphate structure and the sequence ion of *m/z* 668 with the corresponding $[M+1]^+$ ion of *m/z* 1012 indicates a Hex_2-HexNAc-inositol phosphate structure.

^{1}H-NMR of the GPI-III carbohydrate showed major signals in the anomeric region of δ 5.225, 5.147, 5.118 and 5.038 ppm, with coupling constants $J_{1,2}$ in the range 1.8–1.9 Hz, consistent with four α-linked mannoses (Fig. 6). A signal at δ 5.698 ppm with a coupling constant $J_{1,2}$ of 4.1 Hz is characteristic for a α-linked non-*N*-acetylated glucosamine [23]. Using COSY and double relayed COSY 2D experiments, the chemical shifts and coupling pattern for the H2 and H3 protons for the mannoses, and H2, H3 and H4 protons for the glucosamine, could be determined (Fig. 6). Comparing these shifts with what was previously found for similar structural elements [23, 28], the following assignments could be made: two nonreducing terminal mannoses, Manα1-3 and Manα1-2, one -2Manα1-6 residue, one branched -3,6Manα1-4 residue, and one -4GlcNα1 residue (Table 2). Weak anomeric signals from other components are also seen in the spectrum. However, the major structure constitutes at least 75% of the mixture, as judged by integration of the anomeric region.

From the ^{1}H-NMR data it was concluded that the Manα1-2Man sequence is linked to the 6-position of the 3,6-disubstituted mannose residue, and not to the 3-position. If this would have been the case, a characteristic anomeric signal from an α(1-6)-linked terminal mannose would appear in the region of δ 4.90 ppm. In Fig. 6 only a weak signal is seen at this shift, excluding the possibility that it derives from the major component in the mixture. However, this signal implies that some of the minor compounds contain a terminal α(1-6)-linked mannose. The chemical shift from H1 and H2 of Manα1-2 at δ 5.038 ppm and δ 4.075 ppm, respectively, is characteristic for a Manα1-2Manα1-6 sequence [38]. For a Manα1-3Manα1-6 sequence the corresponding shift for H1 would appear in the region δ 5.10–5.13 ppm. From these data, it could be concluded that the 2,4,6-OMe-mannose found in the methylation analysis, indicating the presence of a 3-substituted mannose, is derived from the minor components in the mixture.

To further confirm these results, the GPI-III carbohydrates were deaminated and analyzed by GLCMS as permethylated derivatives. Nitrous acid deamination results in the conversion of the 2-amino-2-deoxyglucose residues to 2,5-anhydromannose and cleavage of the hexosaminidic linkages. The products were reduced with sodium borodeuteride and analyzed by GLCMS after permethylation. One trisaccharide alditol, two tetrasaccharide alditols and one pentasaccharide alditol were observed (Fig. 7). The pentasaccharide alditol being the major component. The mass spectrum of the pentasaccharide alditol showed primary and secondary fragments of *m/z* 219, 187 and 155, characteristic for a nonreducing hexose (spectrum not shown). Ions of *m/z* 190 and 158 indicated a 2,5-anhydrohexitol-1-*d*. A primary ion of *m/z* 423 giving a secondary ion of *m/z* 391 after elimination of methanol, indicated a Hex-Hex- sequence. The absence of *m/z* 627, which excludes a linear trisaccharide sequence, indicated a branched structure. These data are in agreement with the postulated structure for the major compound of GPI-III.

The mass spectrum of one of the tetrasaccharide alditols showed the primary and secondary ions *m/z* 423 and 391. These ions are indicative of a linear structure. The mass spectrum of the second tetrasaccharide was devoid of these ions, indicating a branched structure. Further characterization of the minor components, however, was not possible due to the small amount of material available.

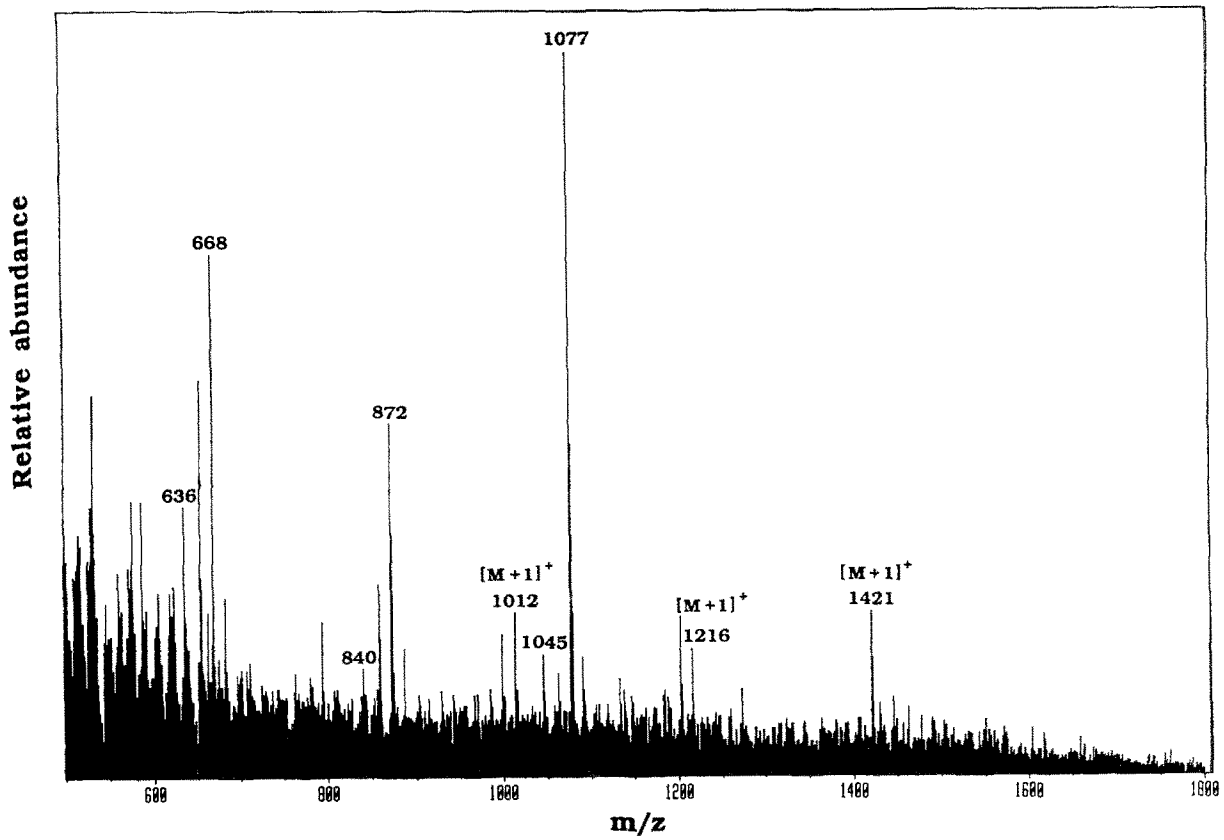
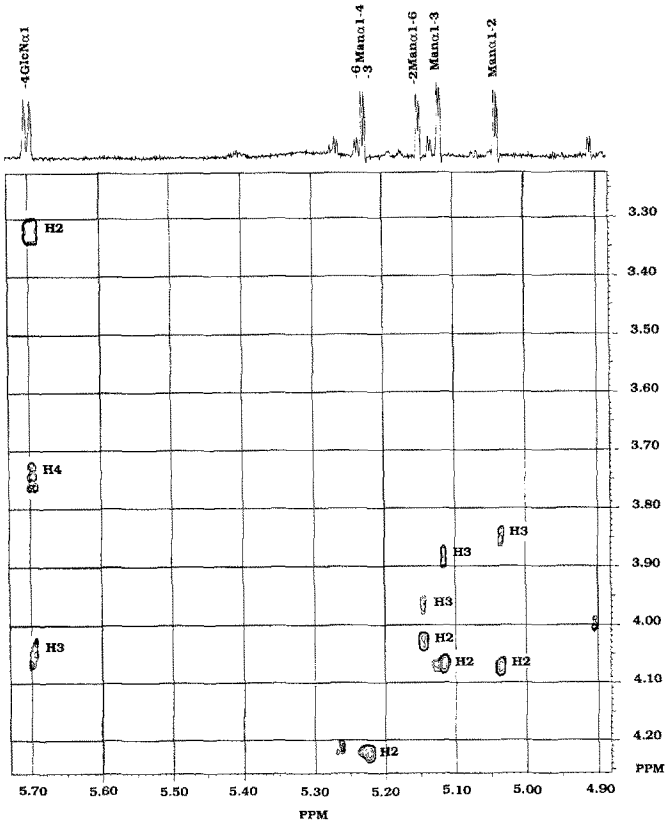


Figure 5. FAB mass spectrum of the carbohydrate moiety of GPI-III after *N*-acetylation and permethylation.



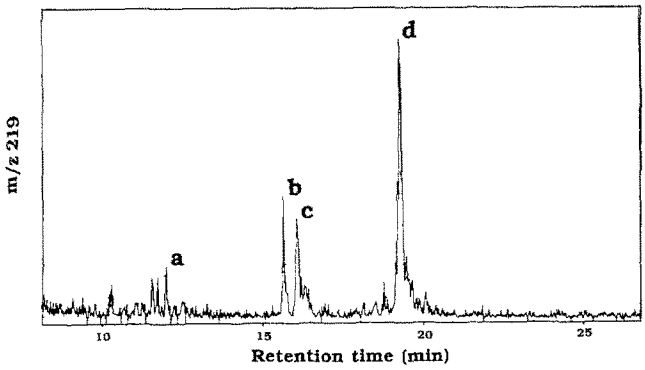


Figure 7. Selected-ion monitoring of the carbohydrate portion of GPI-III after deamination and permethylation. The ion of *m/z* 219, shows one trisaccharide alditol (a), two tetrasaccharide alditols (b, c) and one pentasaccharide alditol (d), as major components. Other peaks in the chromatogram contained no carbohydrate derivatives.

Figure 6. Double relayed COSY ^{1}H-NMR spectrum of the carbohydrate moiety of GPI-III. The spectrum was recorded according to standard Bruker Spectro Spin software, at a temperature of 27°C using a sweep width of 1976 Hz and 2048 data points in each dimension. For the t_1 increment 384 transients were added. The length of the coherence step delays was 30 μs.

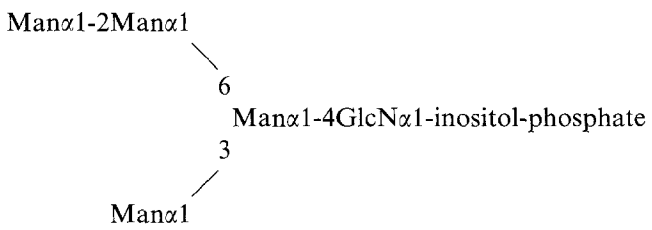
Table 2. ^{1}H-NMR chemical shift table.

Residue	H1	$J_{1,2}$	H2	H3	H4	H5	H6
-4GlcNH$_2$α1-6	5.698	(4.1)	3.326	4.048	3.747	n.d.	n.d.
Manα1-4	5.225	(1.9)	4.225	n.d.	n.d.	n.d.	n.d.
3							
-2Manα1-6	5.147	(1.8)	4.032	3.967	n.d.	n.d.	n.d.
Manα1-3	5.118	(1.8)	4.071	3.886	n.d.	n.d.	n.d.
Manα1-2	5.038	(1.8)	4.075	3.851	n.d.	n.d.	n.d.

Values are expressed in ppm relative to internal acetate CH_3 signal set to 1.908 ppm. Probe temperature 300 K. Coupling constants between H1 and H2 ($J_{1,2}$) are given in Hertz. n.d. = not detected.

Discussion

In the present paper, the major oligosaccharide fragment generated by specific PI-PLC treatment of GPI-III, the most antigenic among the *L. donovani* glycolipids, was determined as



This structure was the major component in the mixture also containing smaller oligosaccharides. Indications in the analyses of terminal α(1-6)-linked mannose and internal 3-substituted mannose were concluded to be derived from the minor components. The lipid portion was characterized by GLCMS as 1-alkyl-2-acylglycerol. The presence of an ester linkage in the C2 of the glycerol was demonstrated by phospholipase A_2 hydrolysis (Fig. 3). The lack of remaining radioactivity in the glycerol backbone after the hydrolysis indicates that the alkyl chains were not labelled with myristic acid and is consistent with our previous results on the *L. major* GPIs [11]. The phosphoinositol glycan is linked via a non-*N*-acetylated glucosamine to 1-alkyl-2-acylglycerol. Heterogeneity was found in both the fatty acids and alkyl chains. Fatty acids ranged from C16:0 to C26:0, and in the alkyl chain from C16:0 to C21:0.

The glycan shows interesting structural features: (a) the absence of galactose, a monosaccharide found in LPG, in the *L. major* GPIs and in the GPI-anchor of the *T. brucei* VSG; (b) the presence of a branched structure reported for the first time in *Leishmania* GPI-containing molecules, and (c) the presence of a Manα1-2Manα1-6Manα1-4GlcN1-InsP sequence, which is the common structure found in all the GPI-anchors of proteins so far studied [39].

The branch on the mannose adjacent to glucosamine is one of the sites of heterogeneity on the GPI-anchor of proteins. For the VSG anchor, glycan chains varying in size and composition were found to be attached to this residue [23, 40]. Phosphoethanolamine and phosphoethanolamine together with *N*-acetylated galactosamine were found attached to the branched mannose in AChE and Thyl anchors, respectively [24, 41]. A terminal Manα1-3 substitutes this site in GPI III.

Alkylacyl PIs were reported in the *L. major* GPIs [12] and in previous lipid analysis of *L. donovani* promastigotes. Stearic acid was the most abundant among the fatty acids in *Leishmania* alkylacyl PI [42, 43], similar to our findings in GPI-III. Alkylacyl glycerols were also found in the GPI-anchor of bovine and human erythrocyte acetylcholinesterase [41, 44] and in the phosphatidylinositol-glycans suggested to be involved in the modulation of many insulin-sensitive enzymes [8]. Alkylacyl substituted glycerols modulate the activity of protein kinase C [45], an enzyme involved in the activation of defensive mechanisms of macrophages and neutrophils [46]. We found that GPIs A, B and C from *L. major*, which contain an alkylacyl glycerol similarly to GPI III, inhibit protein kinase C in an *in vitro* system [47], suggesting a protective role in the survival of the parasite for the GPIs from both species.

Comparing the GPI structures of *L. major* and *L. donovani*, the glucosaminyl alkylacyl PI is conserved, but the structure of the extended glycan differs markedly between species. The linkages on the *L. major* GPIs are exclusively α1-3 and no galactose residues were found in GPI-III. GPI-III may play a role as a precursor of protein GPI anchors due to the common structure of this glycolipid, and the other GPI anchors of protein described until now [23, 24, 41]. The GPI anchor of *Leishmania* gp63 contains the conserved Manα1-2Manα1-6Manα1-4GlcN-sequence linked to an alkylacyl PI similar to the structure found in GPI III [25]. When necessary, the cleavage of the additional Manα1-3 branch present in GPI-III may occur by mannosidase

activity of the parasite. Moreover, kinetic studies on GPI-III are consistent with a role as a metabolic intermediate rather than an end product (unpublished observations). Thus, knowledge of the structure of GPI-III will help to understand the biosynthetic pathways leading to the synthesis of GPI protein anchors in *Leishmania.*

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