

A novel GlcNAc α 1-HPO₃-6Gal(1-1)ceramide antigen and alkylated inositol-phosphoglycerolipids expressed by the liver fluke *Fasciola hepatica*

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The acidic (glyco)lipids of the parasitic liver fluke *Fasciola hepatica* exhibited two different phosphate-containing species, designated AL-I and AL-II, which were analyzed by MALDI-TOF MS, ESI MS, NMR, methylation analysis, and combined GC-MS in conjunction with HF treatment. AL-I was structurally determined as 1-*O*-hexadecyl-*sn*-glycerol-3-phosphoinositol, an ether bond variant of lysophosphatidylinositol. The structure of AL-II was shown to be GlcNAc α 1-HPO₃-6Gal(1-1)ceramide. Ceramide analysis revealed as major components 2-hydroxyoctadecanoic acid [18:0(2-OH)] together with C₁₈- and C₂₀-phytosphingosines. AL-II was apparently highly antigenic and strongly recognized by both animal- and human-*F. hepatica* infection sera. Furthermore, inhibition ELISAs revealed that the unusual antigenic determinant GlcNAc α 1-HPO₃- phosphate might have a potential in the serodiagnosis of *F. hepatica* infections.

Key words: electrospray mass spectrometry/liver fluke glycolipids/oligosaccharide structural analysis/parasitic trematode

Introduction

Fascioliasis, liver rot, is a major disease of domestic animals, sheep, and cattle, the etiological agent of which is the trematode liver fluke *Fasciola hepatica*. In addition, *F. hepatica* is emerging as a significant pathogen of humans (Mas-Coma *et al.*, 1999). The disease is a zoonosis distinguished by both domestic and wild animal host reservoirs (Mas-Coma *et al.*, 1999; Menard *et al.*, 2000; Valero *et al.*, 2001). Within human populations, endemic foci exist (for example, in the Bolivian Altiplano), which are characterized by high prevalence and intensity of the disease (Esteban *et al.*, 1997; O'Neill *et al.*, 1998). Infection of the definitive host is caused by the ingestion of metacercariae.

The excysted larval flukes pass through the duodenal wall, across the peritoneal cavity, into the liver parenchyma, and then enter the bile ducts. Within 3–4 months after infection the worms start laying eggs. In the acute phase of the disease, the liver exhibits parenchymal tissue destruction, hemorrhage, eosinophil-dependent inflammation, and fibrosis, and chronic phase pathology of the bile ducts is defined by inflammatory and fibrotic responses (Roberts and Janovy, 2000). Immunomodulation of the resultant infection-induced humoral and cellular immune responses is specified by a down-regulation of the resistant type 1 and an up-regulation of the susceptible type 2 immune responses (O'Neill *et al.*, 2000; Paz *et al.*, 1998).

F. hepatica displays antigenic cross-reactivity with the trematodes *Schistosoma mansoni* and *S. bovis* as well as the nematode *Trichinella spiralis* (Aronstein *et al.*, 1986; Hillyer, 1984; Rodriguez-Osorio *et al.*, 1999). In addition, cross-protection has been demonstrated for *F. hepatica* and *S. mansoni* infections by heterologous challenges with *S. mansoni* cercariae and *F. hepatica* metacercariae, respectively (Hillyer, 1984). Glycoconjugates were believed to be the reason for these phenomena (Hillyer, 1984), and some of the molecules responsible for the observed cross-reactivity between *F. hepatica* and *S. mansoni* have been identified as glycoproteins (Aronstein *et al.*, 1985a,b, 1986). The molecular basis of this phenomenon can be attributed at least in part to a shared fucose-containing glycanic determinant present on acidic glycoproteins of various tissues and organs, including the tegument glycocalyx of the adult *F. hepatica* fluke as well as other life-cycle developmental stages (Abdul-Salam and Mansour, 2000).

In contrast to the glycoproteins, neutral glycolipids of *F. hepatica* apparently displayed no serological cross-reactivity with those of *S. mansoni* (Dennis *et al.*, 1996). *F. hepatica* expresses the globo-series of glycosphingolipids (Gal(α 1-4)Gal(β 1-4)Glc(β 1-1)Cer; Wuhrer *et al.*, 2001), whereas *S. mansoni* exhibits the schisto-series of glycosphingolipids based on GalNAc(β 1-4)Glc(β 1-1)Cer (Makaaru *et al.*, 1992) with the dominant structural determinants Fuc(α 1-3)GalNAc-, Gal(β 1-4)[Fuc(α 1-3)] GlcNAc- (Lewis X), GalNAc(β 1-4)GlcNAc-, and -4[Fuc(α 1-2)Fuc(α 1-3)] GlcNAc- (Khoo *et al.*, 1997; Wuhrer *et al.*, 2000b, 2002). Of particular interest are the charged glycolipids of parasitic helminths, as exemplified by the structurally conserved zwitterionic phosphocholine-containing glycolipids of both parasitic and free-living nematodes (Gerdts *et al.*, 1999; Lochnit *et al.*, 1998; Wuhrer *et al.*, 2000c), which are active in stimulating the release of proinflammatory cytokines from peripheral blood mononuclear cells (Lochnit *et al.*, 1998). We present here the structural characterization of *F. hepatica* acidic (glyco) lipids, one of which exhibits

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unique structural features, such as GlcNAc linked via a phosphodiester to ceramide monohexoside (CMH), and is apparently active in evoking a strong humoral immune response in both human and animal liver fluke infections.

Results

Isolation of *F. hepatica* acidic (glyco)lipids

Acidic (glyco)lipids of the liver-fluke *F. hepatica* as obtained by anion-exchange chromatography comprised a mixture of two major compounds as evidenced by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS; Figure 1A). The two species were separated by reverse-phase (RP) chromatography yielding acidic lipid I (AL-I; Figure 1B) and acidic glycolipid II (AL-II; Figure 1C). AL-I and AL-II were resolved by high-performance thin-layer chromatography (HPTLC) and stained with iodine or orcinol/H₂SO₄, thus visualizing AL-I (Figure 1D) and AL-II (Figure 1E), respectively. Both MALDI-TOF MS (Figure 1C) and HPTLC (Figure 1E) revealed several AL-II species, which is indicative of heterogeneities in the lipid part. Immunostaining with both human- and rabbit-*F. hepatica* infection sera (Figure 1F, G) detected neutral glycolipids larger than ceramide tetrahexoside (CTetH) as well as AL-II, but not AL-I. Acidic (glyco)lipids were structurally analyzed by various chromatographic and mass spectrometric methods, as well as nuclear magnetic resonance spectroscopy (NMR).

Characterization of the AL-II glycan moiety

MALDI-TOF mass spectra in the negative-ion mode (Figure 1C) and electrospray ionization (ESI) MS data (Figure 2A) of acidic glycolipid AL-II were dominated by signals at *m/z* 1027, 1043, 1057, and 1071. ESI-MS² showed a loss of 221 Da (increment for *N*-acetylhexosamine) from the precursor molecules at *m/z* 1027 (Figure 2B) and 1043 (Figure 2C), resulting in Z₃ ions at *m/z* 806 and 822, respectively. An additional fragment ion at *m/z* 300 (Figure 2B, C) could be explained as HexNAc-O-HPO₃⁻, as further fragmentation resulted in a loss of HexNAc (H₂PO₄⁻ at *m/z* 97; Figure 2D). Carbohydrate constituent analysis of AL-II using trifluoroacetic acid (TFA) hydrolysis revealed Gal and GlcNAc, whereas hydrofluoric acid (HF) treatment released solely GlcNAc from AL-II (Table I) in agreement with a phosphodiester bridge between GlcNAc and the galactose-containing hydrophobic moiety of the molecule.

AL-II was further characterized by ESI MS in the positive-ion mode, and fragmentation of the major pseudomolecular ion [M-H + 2Na]⁺ at *m/z* 1073 (Figure 2F) resulted in the loss of HexNAc (Y₃ ion at *m/z* 870) or loss of HexNAc-PO₃Na (Y₂ ion at *m/z* 768), in addition to a Z₃ fragment at *m/z* 852 (Figure 2G). Preparative release of the GlcNAc-phosphate moiety by HF treatment converted AL-II to CMH, which resembled native *F. hepatica* CMH (Figure 3A, lane 2, and 3D) in both HPTLC (Figure 3A, lane 1) and MALDI-TOF MS (Figure 3C). To determine the linkage position of the GlcNAc-phosphate moiety to CMH, AL-II was subjected to linkage analysis by sequential permethylation, HF treatment, TFA hydrolysis, reduction, and

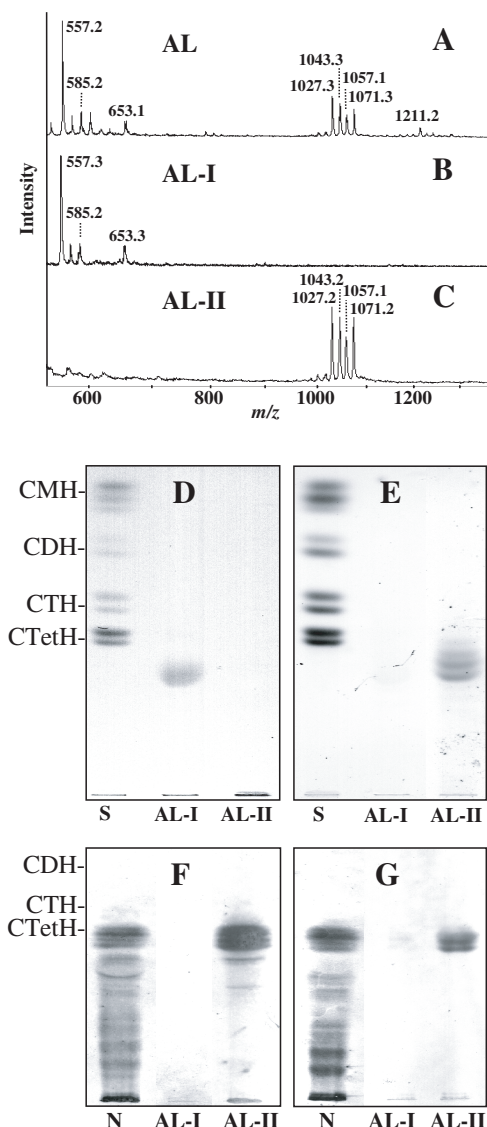


Fig. 1. MALDI-TOF MS and HPTLC of *F. hepatica* acidic (glyco)lipids. Both total acidic (glyco)lipids (AL, A) and isolated AL-I (B) and AL-II (C) species obtained after RP separation were monitored as [M-H]⁻ ions by MALDI-TOF MS in the negative-ion mode. AL-I, AL-II, and *F. hepatica* neutral glycolipids (N; containing in addition some acidic (glyco)lipids) were resolved by HPTLC using chloroform: methanol: 0.2% aqueous CaCl₂ (60:35:8, by volume; D and E) or chloroform: methanol: 0.25% aqueous KCl (50:40:10, by volume; F and G) and visualized by either iodine staining (D), orcinol/H₂SO₄ staining (E), or immunostaining using *F. hepatica* infection sera from rabbit (F) or humans (G). The standard (S) of CMH-CTetH corresponded to globo-series ceramide mono-, di-, tri-, and tetrahexosides, respectively.

peracetylation; the resulting partially methylated alditol acetates were analyzed by combined gas chromatography/mass spectrometry (GC-MS). Obtained results revealed terminal GlcNAc and 6-substituted Gal (Figure 4A). HF treatment prior to permethylation converted 6-substituted galactose into terminal Gal (Figure 4B). The ¹H NMR spectrum of AL-II contained inter alia signals for fatty acids present in the glycolipid (1.18 ppm, -CH₂-; 0.79 ppm, -CH₃), as well as one prominent signal assignable to

the anomeric proton of GlcNAc (H-1, δ 5.37 ppm, dd), which showed two characteristic coupling constants, $J_{1,2}$ 3.8 Hz and $J_{H-1,P}$ 6.5 Hz, indicative of a GlcNAc α 1-HPO₃ linkage similar to that of the GlcN1-HPO₃ moiety in lipid A expressing an identical structural feature (Ribeiro *et al.*, 1999).

Serological recognition of the AL-II glycan moiety

Both human- and rabbit-*F. hepatica* infection sera were shown to recognize AL-II strongly (Figure 1F, G). The latter was further tested for its recognition of AL-II in

enzyme-linked immunosorbent assay (ELISA). To elucidate the specificity of antibody binding, various monosaccharide derivatives were tested for their potential to inhibit serological reactivity (Figure 5). Glucose- α -methyl glycoside and free GlcNAc did not interfere with binding, but GlcNAc α 1-phosphate, UDP-GlcNAc, and GlcNAc-methyl glycoside (the majority of which may be assumed to comprise α -anomeric configuration; Kamerling *et al.*, 1975) diminished AL-II recognition by polyclonal antibodies at concentrations of approximately 1 mM.

Structural analysis of the AL-II ceramide moiety

Characterization of the ceramide moiety was performed by GC-MS analysis of fatty acids as their corresponding, *O*-acetylated methyl esters (Figure 4C) and of sphingoid bases as their pentafluoropropionyl derivatives (Figure 4D). The major fatty acids were 18:0, 18:0(2-OH), 22:1, and 30:0(2-OH) (Figure 4C). Sphingoid bases were dominated by C₁₈-phytosphingosine (t18:0) and C₂₀-phytosphingosine (t20:0) (Figure 4D). In addition, as described for

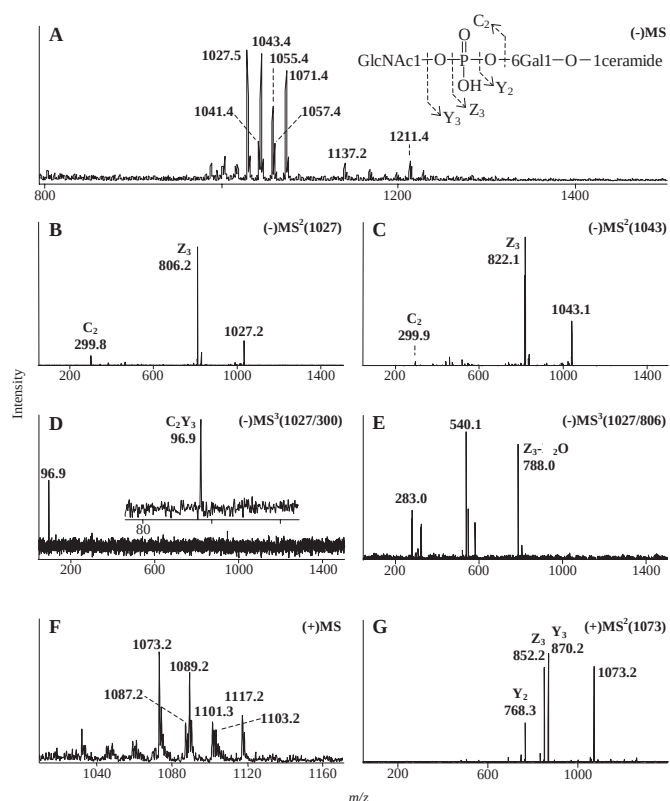


Fig. 2. Nano-ESI MSⁿ analysis of native AL-II. Measurements were performed in the negative-ion mode with registration of [M-H]⁻ ions (A-E) as well as in the positive-ion mode leading to the detection of [M-H+2Na]⁺ ions for species containing phosphate and [M+Na]⁺ ions after loss of phosphate (F and G). Designation of fragment ions is analogous to that of Domon and Costello (1988).

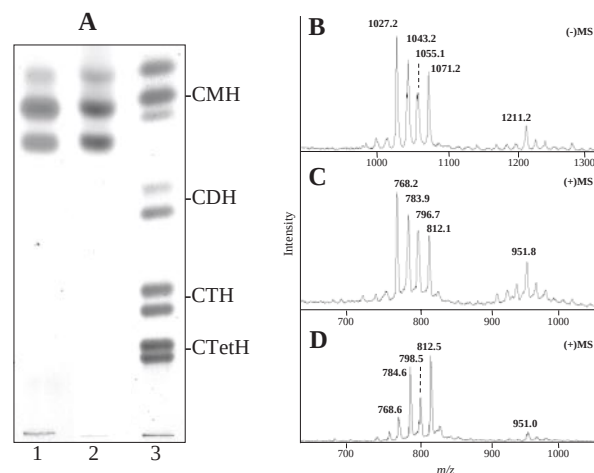


Fig. 3. HPTLC and MALDI-TOF MS analyses of AL-II after HF treatment. (A) AL-II after HF treatment (1 μ g galactose; lane 1), *F. hepatica* CMH (1 μ g galactose; lane 2), and a globo-series standard (lane 3) were compared by HPTLC using the running solvent chloroform:methanol:water (65:25:4, by volume) and orcinol/H₂SO₄ staining. AL-II samples were compared by MALDI-TOF MS before ([M-H]⁻; B) and after ([M+Na]⁺; C) HF treatment with *F. hepatica* CMH ([M+Na]⁺; D). CMH-CTetH corresponded to globo-series ceramide mono-, di-, tri-, and tetrahexosides, respectively.

Table I. Carbohydrate constituent analysis of AL-II

Monosaccharide	HF treatment + TFA hydrolysis	TFA hydrolysis	H ₂ SO ₄ hydrolysis	HF treatment RP flow-through	HF treatment RP eluate + TFA hydrolysis
Gal	1.1	0.15	0.15	—	1.0
GlcNAc	1.0	1.0	1.0	1.0	0.05

Samples were hydrolyzed with TFA, H₂SO₄, and/or HF; reduced; acetylated; and analyzed by GC using flame-ionization detection. Alternatively, samples were fractionated on an RP cartridge after HF treatment, and monosaccharides were determined in the eluate as well as the flow-through. The relative molar ratios of monosaccharides are given for each analysis.

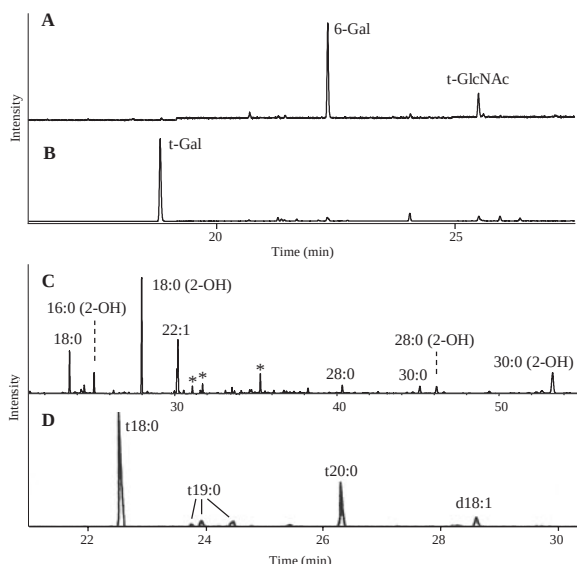


Fig. 4. GC analysis of AL-II-derived compounds using flame-ionization or mass spectrometric detection. Linkage analysis of AL-II was performed with HF treatment before (B) or after (A) permethylation procedure by GC-MS using single-ion monitoring after chemical ionization with ammonia. 6-Gal, 6-substituted galactose; t-GlcNAc, terminal GlcNAc, t-Gal, terminal galactose. (C) Analysis of fatty acids as their acetylated methyl esters using flame-ionization detection. (D) Analysis of sphingoid bases as their pentafluoropropionic acid derivatives by capillary GC-MS using electron-impact ionization. The chromatogram was recorded by selected-ion monitoring of characteristic fragment ions (m/z 188 and 240). *, contaminant.

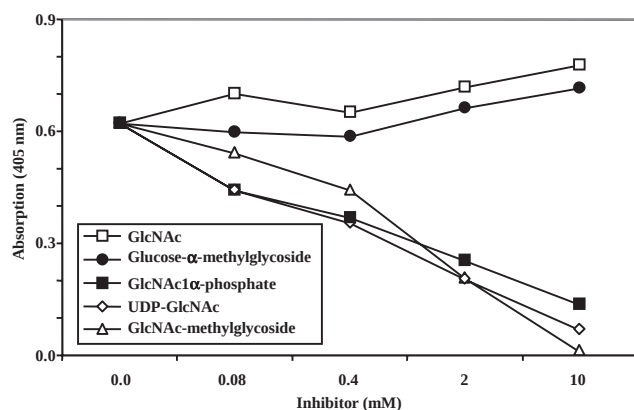


Fig. 5. Inhibition-ELISA of AL-II. Rabbit *F. hepatica* infection serum (diluted 1:1000) was assayed for its recognition of AL-II (20 ng adsorbed per well). Various monosaccharide derivatives indicated in the box were tested for their ability to inhibit this interaction.

F. hepatica CMH (Wuhrer *et al.*, 2001), AL-II exhibited three species of C_{19} -phytosphingosine (t19:0) as well as small amounts of C_{18} -sphingosine (d18:1). From these data, the ceramide compositions for the major AL-II species may be deduced (Table II). For all AL-II species, the ceramide compositions were independently inferred from their ESI MS³ spectra (see, for example, Figure 2E), as fragment ions were detected that corresponded to

octadecanoic acid (m/z 283), 2-hydroxyoctadecanoic acid (m/z 299), or a 2-hydroxy fatty acid with 30 carbon atoms (m/z 467) and lyso-glycosphingolipids with C_{18-20} phytosphingosines (m/z 540, 554, and 568; see Table II). Taken together, AL-II was shown to have the structure GlcNAc α 1-HPO₃-6Gal(1-1)ceramide.

Structural analysis of AL-I

AL-I was dominated by molecular species, which were detected as $[M-H]^-$ ions at about m/z 557 in MALDI-TOF MS (Figure 1B) and ESI MS (Figure 6A). Constituent analysis revealed the presence of a terminal inositol residue linked via a phosphodiester bridge to the hydrophobic moiety of the molecule (release of inositol by HF treatment; data not shown). This correlated with the loss of a 180-Da fragment (indicative of either hexose or inositol) in ESI MS² of the precursor ion at m/z 557, resulting in a Z_2 fragment at m/z 377 (Figure 6B). An additional B_2 fragment at about m/z 241 (Figure 6B) turned out to be an inositol-1,2-cyclic-phosphate ion, as an identical fragment ion at m/z 241 has been detected in the ESI MS analysis of phosphatidylinositol species from various sources (Treumann *et al.*, 1998). Further fragmentation resulted in loss of inositol (PO_3^- at m/z 79; Figure 6E). Fragmentation of the ion at m/z 585 gave similar results (Figure 6C). A 241-Da fragment was also obtained on fragmentation of the ion at m/z 653, indicating that this compound similarly contained the $InoPO_3^-$ unit (data not shown). Positive-ion mode ESI MSⁿ experiments exhibited corresponding fragment ions for the major AL-I species (Figure 6F,G). Native AL-I was registered as $[M-H+2Na]^+$, and fragmentation of the major ion at m/z 603 (Figure 6F) resulted in a loss of inositol (Z_2 ion at m/z 423, Figure 6G) and detection of $[InoPO_3+2Na]^+$ at m/z 287. After permethylation, the major compound was detected as $[M+Na]^+$ and $[M+Li]^+$ at m/z 679 and 663, respectively (Figure 7A). The latter adducts are due to the use of butyl lithium in the permethylation procedure. Subsequent MS/MS experiments yielded the corresponding adducts of permethylated inositol with or without methylated phosphate (fragment ions C_1 and C_2 in Figure 7B).

As to the chemical nature of the hydrophobic part of this phosphoinositol-containing compound, ceramide could be ruled out due to the low molecular mass of the lipid moiety. Putative diacyl-phosphatidylinositol species would have been destroyed by the saponification steps included in the purification procedure and therefore could be also precluded. Instead, the ESI MS data indicated the hydrophobic moiety to be an alkylated glycerol structurally related to platelet activating factor (PAF). To scrutinize this assumption, the fragment ion at m/z 377 (Figure 6B), representing the phosphorylated lipid moiety, was subjected to further fragmentation, resulting in the elimination of an alkyl chain (mass difference of 242 Da, corresponding to hexadecanol) and formation of a dehydrated glycerophosphate ion at m/z 135 (Figure 6D). This interpretation was supported by ESI MS of permethylated AL-I, which revealed the total incorporation of seven methyl groups, with five CH_3 -groups linked to inositol, one to the phosphate, and one to glycerol (*sn*-2 position; Figure 7B, C). For the AL-I species at m/z 585 (Figure 6A), ESI MS² of its native (Figure 6C) and

Table II. Ceramide compositions of the major AL-II species

AL-II species		Fragment ions obtained		Composition	
Native $[M-H]^-$	Native $[M-H+2Na]^+$	MS^2	MS^3	Sphingoid base	Fatty acid
1027.5	1073.2	806.2	283.0 ^b /540.1 ^c (522.0)	C ₁₈ -phytosphingosine	18:0
1041.4	1087.2	820.3	283.0 ^b /554.1 ^c (536.1)	C ₁₉ -phytosphingosine	18:0
1043.4	1089.2	822.3	299.8 ^b /540.1 ^c (522.1)	C ₁₈ -phytosphingosine	18:0(2-OH)
1055.4	1101.3	834.4	283.1 ^b /568.0 ^c (550.0)	C ₂₀ -phytosphingosine	18:0
1057.4	1103.2	836.2	299.5 ^b /554.3 ^c (536.0)	C ₁₉ -phytosphingosine	18:0(2-OH)
1071.4	1117.2	850.4	299.0 ^b /568.0 ^c (550.3)	C ₂₀ -phytosphingosine	18:0(2-OH)
1211.4	1257.2 ^a	990.4	467.3 ^b /540.0 ^c (522.1)	C ₁₈ -phytosphingosine	30:0(2-OH)

The structures were deduced from the analytical results obtained by ESI MS, fatty acid and sphingoid base analyses. $[M-H]^-$ and $[M-H+2Na]^+$ ions were determined by ESI MS. Ceramide compositions were deduced by ESI MS subjecting corresponding Z_3 ions obtained in the MS^2 experiments (cf. Figure 2B) to further fragmentation (MS^3 ; cf Figure 2E).

^aNot visible in Figure 2F.

^bIons corresponding to fatty acids.

^cIons reflecting lyso-glycosphingolipid moieties; ions obtained after loss of water are given in parentheses.

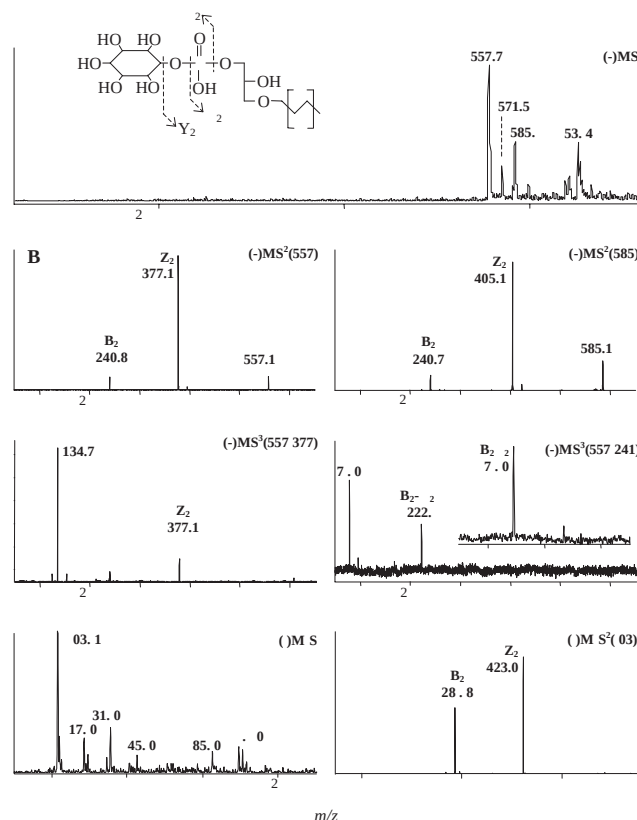


Fig. 6. Nano-ESI MS^n analysis of native AL-I. Measurements were performed in the negative- or positive-ion mode with registration of $[M-H]^-$ (A–E) or $[M-H+2Na]^+$ ions (F and G), respectively. Designation of fragment ions is analogous to that of Domon and Costello (1988).

permethylated form (Figure 7C) revealed a similar composition in the lipid part with hexadecanol being replaced by octadecanol. The deduced alkylglycerophosphate structure was further corroborated by GC analysis of the

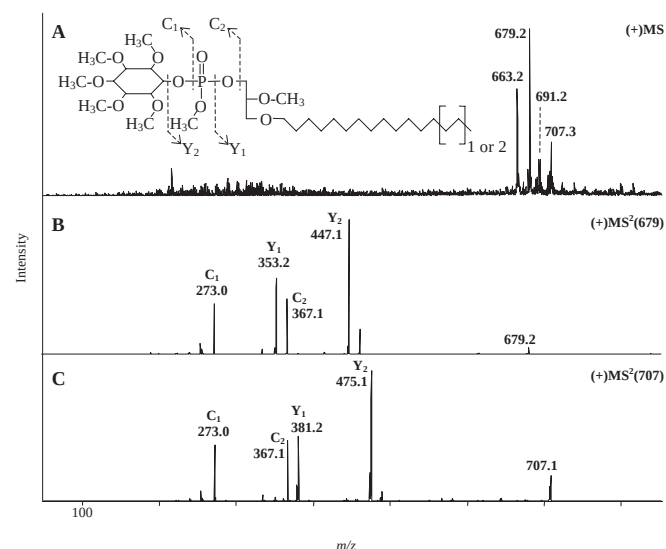


Fig. 7. Positive-ion mode nano-ESI MS^n of permethylated AL-I. Due to the use of butyl lithium in the permethylation procedure, $[M+Li]^+$ adducts were detected in addition to $[M+Na]^+$ ions. Designation of fragment ions is analogous to that of Domon and Costello (1988).

HF-released peracetylated hexadecylglycerol using flame ionization and electron impact ionization detection (Figure 8A, C). As a comparative standard, synthetic PAF with a hexadecyl chain in position 1 of the glycerol was used (Figure 8B). Resultant mass spectra after electron impact ionization and retention times on two different columns were found to be identical with the analytical data for AL-I. Taken together, the structure of the major species of AL-I is 1-*O*-hexadecyl-*sn*-glycerol-3-phosphoinositol. Corresponding derivatives with octadecyl chains represented minor compounds (Figures 6C and 7C).

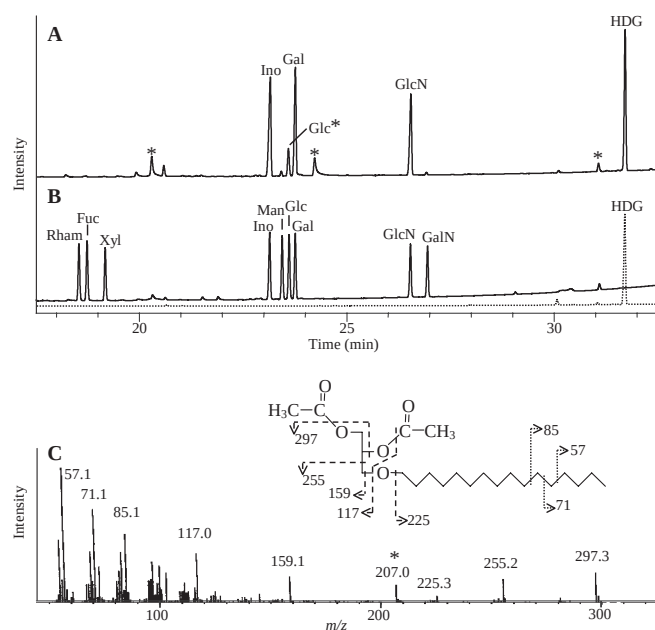


Fig. 8. GC identification of the hexadecylglycerol moiety occurring in AL-I using flame-ionization or mass spectrometric detection. (A) GC constituent analysis of *F. hepatica* acidic (glyco)lipids after sequential HF treatment, TFA hydrolysis, reduction, and peracetylation using flame-ionization detection as compared with a monosaccharide standard (continuous line in B) and hexadecylglycerol (HDG; dashed line in B). (C) Electron-impact mass spectrum of the hexadecylglycerol from (A). *, contaminants.

Discussion

F. hepatica ceramide monohexosides, that is galactosylceramide (90%) and glucosylceramide (10%) (Wuhrer *et al.*, 2001), would appear to be differentially used as precursors for the synthesis of acidic and larger neutral glycosphingolipids. Whereas both *F. hepatica* lactosyl- and globotriaosylceramide are based on glucosylceramide (Wuhrer *et al.*, 2001), the herein described AL-II derives from galactosylceramide. Ceramide compositions of *F. hepatica* CMH, globo-series CTH, and AL-II are similar and characterized by phytosphingosines and α -hydroxylated fatty acids, which can contain up to 30 carbon atoms. In this context, it is interesting to note that *S. mansoni* glycosphingolipids exhibit a similar ceramide composition with mainly C₂₀-phytosphingosine and large amounts of α -hydroxylated fatty acids (Khoo *et al.*, 1997; Wuhrer *et al.*, 2000a). In schistosomes, however, there is no analog to the finding of an acidic glycosphingolipid in *F. hepatica* because only neutral glycosphingolipids have been described there (Khoo *et al.*, 1997; Wuhrer *et al.*, 2000b, 2002).

The GlcNAc α 1-HPO₃ unit of AL-II is paralleled in various biomolecules. The lipid A structure of Gram-negative bacteria exhibits a similar -6GlcNAc1-O-H₂PO₃ motif with acyl chains in 2- and 3-position of GlcN (Alexander and Zähringer, 2002). It may be speculated as to whether this structural similarity might provide a basis for a related biological activity of *F. hepatica* AL-II and bacterial lipid

A, of which the latter is specifically recognized by cell-surface toll-like receptors (TLR4 and TLR2) of various host cells and leads to the production of bioactive compounds as tumor necrosis factor α , various interleukins, oxygen radicals, and bioactive lipids (Alexander and Zähringer, 2002; Ulmer *et al.*, 2002). GlcNAc α 1-O-HPO₃- has also been described as O-linked to a serine residue of the lysosomal proteinase I of *Dictyostelium discoideum*, where it might be involved in lysosomal targeting of the proteinase and influence its substrate specificity (Gustafson and Gander, 1984). In addition to GlcNAc α 1-O-HPO₃-, the β -anomeric variant has recently been described to occur on glycosylphosphatidylinositol (GPI) anchors from various vertebrates (Fukushima *et al.*, 2001).

Concerning the biosynthesis of AL-II, one might postulate an enzymatic transfer of GlcNAc α 1-O-HPO₃- to the 6-position of galactosylceramide in analogy to the first step of the biosynthesis of the mannose-6-phosphate marker on N-glycans of lysosomal hydrolases which targets them to the lysosome. So far, however, the responsible enzyme, GlcNAc-phosphotransferase, has been only characterized from bovine origin (Bao *et al.*, 1996a,b).

Though there are structural similarities of AL-II to other glycoconjugates as detailed, the GlcNAc α 1-HPO₃-unit has not been described as an autonomous antigenic determinant in other infectious diseases. In fascioliasis, however, AL-II is a major target of the host humoral immune response against glycolipids, as evidenced by its intense recognition in HPTLC overlay (Figure 1). Anti-AL-II antibodies present in infection sera recognize the GlcNAc α 1-HPO₃-determinant (Figure 5) and might provide the basis for the usage of AL-II or structurally related neoglycoconjugates in the serodiagnosis of fascioliasis.

The second acidic lipid compound analyzed in this study, AL-I, has been structurally determined as 1-O-hexadecyl- (or octadecyl)-*sn*-glycerol-3-phosphoinositol, that is, the ether bond variants of lysophosphatidylinositol. These species might be derived from 1-alkyl-2-acyl-phosphatidylinositol after loss of the ester-linked fatty acid on saponification, which had been included in the work-up procedure. 1-Alkyl-2-acyl-phosphatidylinositol compounds are present in glycoinositol phospholipids of protozoan parasites as well as in many eukaryotic protein GPI anchors (Campbell, 2001; Treumann *et al.*, 1998). As for trematodes, in particular, GPIs have been shown to anchor various *S. mansoni* proteins in the parasite tegument, for example, acetylcholinesterase (Arnon *et al.*, 1999; Pearce and Sher, 1989; Sauma and Strand, 1990). In addition, GPI-specific phospholipase activities have been described for *F. hepatica* and *S. mansoni* adult worms and could provide an enzymatic mechanism for the release of GPI-anchored proteins (Hawn and Strand, 1993).

Materials and methods

Isolation and purification of acidic (glyco)lipids

F. hepatica adult worms were collected from infected sheep at abattoirs in Muscat and Salalah, Sultanate of Oman, and stored in 10% formaldehyde at room temperature until use. (Glyco)lipids were isolated by consecutive extractions as

described previously (Wuhrer *et al.*, 1999). Raw extracts were saponified in 50 ml methanolic 0.1 M NaOH for 2 h at 37°C. Salt and hydrophilic contaminants were removed using an RP cartridge (Chromabond C₁₈ec, Macherey & Nagel, Düren, Germany) as described (Dennis *et al.*, 1998). (Glyco)lipids were fractionated on a DEAE-Sephadex-A25 column (Dennis *et al.*, 1998). Acidic species were eluted with chloroform:methanol:0.8 M aqueous sodium acetate (30:60:8, by volume) and further purified by Florisil and subsequent silica-gel cartridge chromatography (Waters, Eschborn, Germany; Dennis *et al.*, 1995). Fractions that were positive on HPTLC orcinol/H₂SO₄ staining were collected, and the two species of acidic (glyco)lipids (AL-I and AL-II) were separated on an RP cartridge (500 mg Chromabond C₁₈ec, Macherey & Nagel) by step-wise elutions with 10 ml of the following solvent mixtures: water, methanol:water (30:70, 50:50, and 70:30, by volume), and chloroform:methanol:water (2:70:28, 5:70:25, and 20:70:10, by volume). AL-I was retrieved with methanol:water (70:30), whereas AL-II was eluted with chloroform:methanol:water (20:70:10).

HPTLC

HPTLC, orcinol/H₂SO₄ staining, and immunostaining were performed as described (Wuhrer *et al.*, 1999). Sera from *F. hepatica*-infected humans (kindly provided by Prof. Egbert Geyer, Marburg, Germany) and rabbits (infected with 100 metacercariae, 40 weeks postinfection) were diluted 1:100 and used as primary antibodies. Goat alkaline phosphatase-conjugated antibodies directed against immunoglobulins from rabbit (Sigma, St. Louis, MO) and humans (Dianova, Hamburg) were employed as secondary reagents. Visualization of binding was performed using a 5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium chloride substrate mixture (Wuhrer *et al.*, 1999). Porcine globo-series glycolipids were used as standards (Matreya, Pleasant Gap, PA) and stained by orcinol/H₂SO₄.

MALDI-TOF MS and ESI MS

MALDI-TOF MS was performed on a Vision 2000 (ThermoFinnigan, Egelsbach, Germany) equipped with a UV nitrogen laser (337 nm) using 6-aza-2-thiothymine (Sigma) as matrix. ESI MS was performed with an Esquire 3000 ion-trap mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an off-line nano-ESI source. A 2–5-μl aliquot of native or permethylated (glyco)lipids in chloroform:methanol:water (10:20:3) was loaded into a laboratory-made, gold-coated glass capillary and electrosprayed at 700–1000 V using N₂ as drying gas (100°C, 4 L/min). The skimmer voltage was set to 30 V. For each spectrum 20–100 repetitive scans were recorded and averaged. The accumulation time was between 5 and 50 ms. All MSⁿ experiments were performed with He as collision gas.

HF treatment, constituent and linkage analysis

For cleavage of the phosphodiester linkages, dried samples were treated with 48% HF at 4°C overnight

(Haslam *et al.*, 2000). HF was removed by a stream of nitrogen at room temperature. The CMH generated by HF treatment of AL-II was purified by silica-gel chromatography (Dennis *et al.*, 1995). For constituent analyses, (glyco)lipids were hydrolyzed with 4 M TFA (4 h, 100°C) or 0.5 N H₂SO₄ in 85% aqueous acetic acid (by volume; 16 h, 80°C) and analyzed as alditol acetates by GC or GC-MS using flame-ionization or electron-impact detection, respectively (Geyer *et al.*, 1982). Besides monosaccharide derivatives, PAF (Calbiochem, Schwalbach, Germany) was used as a reference compound. For linkage analysis, (glyco)lipids were permethylated, treated with HF, hydrolyzed (4 M TFA, 4 h, 100°C), reduced (NaBH₄), and peracetylated. The resultant partially methylated alditol acetates were analyzed by GC-MS after electron-impact or chemical ionization (Geyer and Geyer, 1994; Geyer *et al.*, 1982).

NMR spectroscopy

¹H-NMR spectra were recorded on a 600 MHz spectrometer (Bruker Avance DRX 600) in microtubes (3 mm OD, Kontes, Vineland, NJ) with a 5-mm multinuclear probe head. About 25 μg of AL-II were dissolved in 200 μl of CDCl₃-d₁:MeOD-d₄ 7:3 (by volume), and the ¹H NMR spectrum was recorded with 16,000 scans at 300°K with signals referenced to internal tetramethylsilane. Standard Bruker software was used to record and process all NMR data (XWINNMR 2.6).

Inhibition ELISA

Plates (Polysorb; Nunc, Wiesbaden, Germany) were coated with AL-II (20 ng in 20 μl n-propanol per well), air-dried, blocked by a 1-h incubation in 250 μl per well of 0.5% bovine serum albumin in Tris-buffered saline (TBS; 25 mM Tris-HCl, pH 7.5, 100 mM NaCl). For inhibition experiments, GlcNAc (Serva, Heidelberg, Germany), glucose-α-methylglycoside (Serva), GlcNAcα1-phosphate (Sigma), UDP-GlcNAc (ICN, Eschwege, Germany), and GlcNAc-methylglycoside (prepared as described in Kamerling *et al.*, 1975) were used. The inhibitors were first added in 50 μl TTBS-10-B (TBS 1:10 diluted, containing 0.05% Tween 20 and 0.25% bovine serum albumin) per well followed by rabbit *F. hepatica* infection serum in another 50 μl TTBS-10-B. Plates were thoroughly shaken and then incubated for 1 h at 37°C. After multiple washes with TBS, diluted 1:10 and containing 0.05% Tween 20, binding was detected following a 60-min incubation with 100 μl per well of goat alkaline phosphatase-conjugated anti-rabbit Ig (1:1000; Sigma) secondary antibody in TTBS-10-B. Staining was performed with 100 μl per well of 0.1% p-nitrophenylphosphate in 100 mM glycine, 1 mM ZnCl₂, 1 mM MgCl₂. Absorption was measured at 405 nm.

Ceramide analysis

Purified AL-II (after HF treatment and purification by silica-gel chromatography) was treated with 100 μl 1 M HCl and 10 M H₂O in methanol for 16 h at 100°C (Gaver and Sweeley, 1965). Fatty acids and sphingoid bases were sequentially extracted and analyzed as their methyl esters

and pentafluoropropionic acid derivatives by both GC and GC-MS (Wuhler *et al.*, 2001).

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Abbreviations

CMH, ceramide monohexoside; CTetH, ceramide tetrahexoside; ELISA, enzyme-linked immunosorbent assay; ESI, electrospray ionization; GC, gas chromatography; GC-MS, gas chromatography/mass spectrometry; GPI, glycosylphosphatidylinositol; HF, hydrofluoric acid; HPTLC, high-performance thin-layer chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; MSⁿ, repetitive tandem mass spectrometry; NMR, nuclear magnetic resonance; PAF, platelet activating factor; RP, reverse-phase; TBS, Tris-buffered saline; TFA, trifluoroacetic acid.

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