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Structures of Glycophosphosphingolipids of *Tritrichomonas foetus:* A Novel Glycophosphosphingolipid

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The glycophosphosphingolipids of *Tritrichomonas foetus*, an aerotolerant parasite of the urogenital tract of cattle, have been characterized by a combination of metabolic labeling, chromatography, and tandem mass spectrometry. The acidic glycolipid fraction of *T. foetus* obtained by DEAE Sephadex A-25 column chromatography was subfractionated by high performance thin layer chromatography and the component lipids were purified by high performance liquid chromatography. Two nonsaponifiable lipid fractions, designated TF_1 and TF_2, could be metabolically labeled with [^{3}H]*myo*-inositol and [^{32}P]orthophosphate. [^{3}H]Fucose and [^{14}C]ethanolamine were preferentially incorporated into the TF_1 fraction. TF_1 was partially hydrolyzed by α-fucosidase. Both TF_1 and TF_2 contain ceramides, the most abundant having either sphinganine or sphingosine and a 16:0 *N*-acyl group. TF_2 contains inositolphosphoceramides. TF_1, on the other hand, contains three closely related components, in each of which fucose is linked to inositol diphosphate with one of the phosphates linked to the ceramide moiety and the other phosphate either free or linked to ethanolamine or *N*-acetylethanolamine. TF_1 appears to be a novel class of glycophosphosphingolipid which shows some structural similarities to the glycosylphosphatidylinositol anchors of eukaryotic membrane proteins. © 1991 Academic Press, Inc.

Bovine trichomoniasis is an infection of the genitourinary tract caused by the flagellated protozoan *Tritrichomonas foetus*. *T. foetus* is an important pathogen since infection in cattle results in spontaneous abortion. Destruction of the placental attachments is accompanied by abortion of the fetus and by destruction of the fetal membranes that are retained in the uterus. The cow may reconceive after the loss of the fetus; however, frequently the cow becomes permanently sterile as the result of a chronic endometritis. Trichomonads live on the surface of the epithelium of the urogenital tract where they derive their energy through anaerobic metabolism and produce molecular hydrogen as one of their waste products. How these parasites survive in the hostile immune environment of the host is a most intriguing problem. Several studies have shown that the surface membranes of parasitic protozoans contain a variety of carbohydrate-rich molecules and these surface glycoconjugates may play important roles in the survival of the parasite (1, 2). The carbohydrate moieties of the glycoconjugates are often immunogenic and in some cases may induce protective immunity by the host.

Identification and characterization of surface antigens of parasitic protozoans is fundamental to understanding host–parasite relationships. In recent years, glycosylated phosphatidylinositol glycolipids have been detected as membrane anchors for a wide variety of eukaryotic cell proteins (3, 4). There is some evidence that certain protozoans have glycoconjugates anchored by a ceramide moiety and this was recently suggested for *Dictyostelium* adhesion proteins (5) and also lipopeptidophosphoglycan (LPPG)[2] (6) and lipophosphoglycan from *Acanthamoeba castellani* (7). In an effort to identify potential membrane anchors for trichomonad glycoconjugates, or intermediates in the metabolism of membrane anchors, we have used a combination of metabolic labeling and chromatographic and mass spectrometric techniques to characterize the glycophosphosphingolipids of *T. foetus*. In the present

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[2] Abbreviations used: LPPG, lipopeptidophosphoglycan; GPI, glycosylphosphatidylinositol; HPTLC, high performance thin layer chromatography; FABMS, fast atom bombardment mass spectrometry; MS/MS, tandem mass spectrometry; CID, collision-induced decomposition; *B*, magnetic field; *E*, electric sector field, *B*/*E*, ratio of mass spectrometer magnetic and electric sector fields.

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study we took advantage of the sensitivity and specificity of high performance tandem mass spectrometry to analyze minute amounts of the glycolipid fractions in order to elucidate the structural features of individual components present in the mixture of phosphoinositolsphingolipids.

MATERIALS AND METHODS

Cell culture and radiolabeling. *T. foetus* strain KV_1 cells (ATCC 30924) were grown in Diamond's medium (8) with 10% heat-inactivated fetal calf serum (Hyclone Labs, Inc.) at 37°C in screw-capped 100-ml and 500-ml serum bottles in 100-ml to 500-ml amounts. The initial pH was 7.2. The inoculum was 10^6/ml and each of the following radiolabeled precursors was added at the time of inoculation or at 12 h postinoculation: [^{32}P]orthophosphoric acid (carrier-free, 1 μCi/ml, New England Nuclear) and [2-^{14}C]ethanolamine HCl (43 Ci/mmol, 1 μCi/ml, Amersham Corp.); *myo*-[2-^{3}H]inositol (19 Ci/mmol, 1–2 μCi/ml, Amersham Corp.); L-[5,6-^{3}H]fucose (60 Ci/mol, 2.075 μCi/ml, American Radiolabeled Chem., Inc.). Organisms were counted at 24 h (Coulter Counter, Model Z_F, Coulter Electronics), harvested by centrifugation (6000*g*) and washed twice in cold, phosphate-buffered saline.

Glycolipid extraction and purification. Cells were extracted sequentially with 20 vol $CHCl_3/CH_3OH/H_2O$ (30/60/8) followed by $CHCl_3/CH_3OH/H_2O$ (88/44/7), $CHCl_3/CH_3OH$ (2/1 and 1/1), and $CHCl_3/CH_3OH$ (1/2 with 5% H_2O) twice as described earlier (9) with slight modifications. Samples were sonicated (20–30 s) in the first extraction solvent mixture with a high intensity ultrasonic processor equipped with a tapered microtip (Model VC500, Sonics and Materials Inc.). The extracts were pooled and the solvents were removed by flash evaporation. The extracted lipid mixture in some experiments was saponified with 0.35 M KOH in methanol and neutralized as described earlier (9) prior to Sephadex G-25 column chromatography. The remainder of the lipid extract was purified without base hydrolysis to check the stability of the components.

The dried lipid extract was dissolved in $CHCl_3/CH_3OH/H_2O$ (120/60/9) and lipids were separated from nonlipid contaminants and salt by Sephadex G-25 (Pharmacia) column chromatography (10). The lipid mixture was dried, redissolved in $CHCl_3/CH_3OH/H_2O$ (30/60/8), and separated into neutral and acidic fractions by DEAE Sephadex A-25 (Pharmacia) column chromatography (11). The acidic lipids were then eluted with $CHCl_3/CH_3OH$/0.8 M sodium acetate (30/60/8), taken to dryness, and incubated with 0.1 N NaOH in methanol at 40°C for 2 h. The mixture was dried by rotary evaporation, dissolved in distilled H_2O/0.4 M EDTA (tetrasodium salt) (9/1), and dialyzed through Spectropor tubing (molecular weight cutoff, 2000; Spectrum Medical Ind.) against changes of distilled water for 60 h at 4°C. The nondialyzable lipids were lyophilized. In some experiments, samples with small amounts of lipids (100- to 300-ml cell cultures) were desalted on C_{18} Sep-Pak cartridges (Waters) as described by Ledeen and Yu (11) instead of dialysis. The acidic lipid fraction obtained from either C_{18} Sep-Pak or dialysis was dissolved in $CHCl_3/CH_3OH$ (85/15) and applied to an Iatrobead (6RS-8060; Iatron Lab) silicic acid column (12) to separate inositol-phosphate-containing nonsaponifiable lipids as described (9). Inositol-phosphate-containing glycolipids were eluted with $CHCl_3/CH_3OH$ (1/2) as judged by monitoring of the column eluate by HPTLC and TLC-autoradiography on precoated Silica Gel 60 plates (200 μm thick, E. Merck). Thin layer chromatography was performed using the following solvent systems: A, $CHCl_3/CH_3OH$/0.05% $CaCl_2$ (55/40/10); and B, $CHCl_3/CH_3OH/H_2O$ (60/35/8). Inositol-phosphate-glycolipids were detected on HPTLC by spray reagents: Dittmer–Lester for phosphate, orcinol-H_2SO_4 for sugars, and ninhydrin for free amino groups. Lipids radiolabeled with ^{14}C and ^{32}P were detected by TLC autoradiography on Kodak X-Omat AR film directly exposed to TLC plates. Tritiated components were detected by fluorography at −75°C, with the film exposed to the plates presprayed with EN^{3}HANCE (New England Nuclear). Partial purification of lipid components was performed on preparative HPTLC in solvent system A. Individual separated glycolipid bands were recovered from silica gel by scraping and extracted by sonication with $CHCl_3/CH_3OH$ (1:1 with 10% H_2O) followed by $CHCl_3/CH_3OH/H_2O$ (88/44/7) twice prior to HPLC.

Purification of inositol-phosphate-containing glycolipids by HPLC. The inositol-phosphate-containing glycolipids eluted from Iatrobead silicic acid column followed by HPTLC were subfractionated by HPLC (Waters) employing a 4.6 mm × 150 mm Iatrobead column (6RS-8010, Iatron Lab, Tokyo). The column was equilibrated with $CHCl_3/CH_3OH/H_2O$ (75/24/1) and the lipids were eluted using a linear gradient of $CHCl_3/CH_3OH/H_2O$ (74/24/1 → 50/42/8) programmed for 40 min. The flow rate was 1.5 ml/min and fractions were collected at 2-min intervals. Each fraction was analyzed by HPTLC (solvent system A) as described above. Fractions containing similar components were pooled and analyzed by HPTLC. The pooled fractions containing similar components were named TF_2 (fractions 3–6) and TF_1 (fractions 13–22).

Enzymatic hydrolysis of TF_1. The TF_1 glycolipid labeled with [^{3}H]fucose (~350 μg, 45,000 dpm) was dissolved in 0.6 ml of $CHCl_3/CH_3OH$ (1/1) containing sodium deoxycholate (1 mg/ml, Calbiochem Ultrapure). The mixture was dried under N_2 and incubated with 0.2 units of α-L-fucosidase from bovine epididymis (Sigma) in 0.1 M sodium citrate buffer, pH 6.0, containing 0.02% sodium azide (13) at 37°C, in a total volume of 0.6 ml, with constant stirring for 100 h. A control sample was incubated in the same way, except devoid of enzyme. The reaction was stopped by the addition of 6 ml of CH_3OH followed by 4 ml of 0.1 N NaCl. The reaction mixture was desalted and purified on a C_{18} Sep-Pak cartridge as described earlier (11, 14). The nonhydrolyzed glycolipid was eluted with methanol (3 ml) followed by $CHCl_3/CH_3OH$ (1/1, 10 ml). The resultant glycolipid fraction was dried and its radioactivity was determined by liquid scintillation counting.

GLC analysis of alditol acetates. Dried samples of purified TF_1 and TF_2 (200–300 μg) were suspended in 0.5 ml of 2.5 N trifluoroacetic acid (Sequential Grade, Pierce Chemical Co.) for 5 h at 100°C under N_2. The reaction mixture was cooled and dried under N_2 with 2–3 ml of CH_3OH and resuspended in 0.5 ml of an aqueous solution of sodium borohyride (10 mg) for reduction. Reduction was stopped by the addition of 2–3 drops of glacial acetic acid and the mixture was extracted 3× with hexane. The aqueous phase was dried under N_2 with repeated addition of methanol. The dried sample was acetylated with pyridine/acetic anhydride (1/1) for 80 min at 100°C (15) and analyzed by gas–liquid chromatography (GLC) [Hewlett–Packard HP 5790A, fused silica capillary column SP 2330 (Supelco Inc.) 20 m × 0.32 mm, temperature-programmed 220–240°C at 4°C/min, He carrier gas flow rate 1 ml/min].

Inositol analysis by GLC. HPLC-purified glycolipid samples (200–350 μg) were hydrolyzed for 16 h at 110°C in 1.2 ml 6 M HCl. Tris–HCl (3 μM) was also added at the beginning of hydrolysis to improve inositol recovery (16). The samples were dried under N_2 and derivatized with *N,O*-bis(trimethylsilyl) trifluoroacetamide/trimethylchlorosilane/pyridine (10/1/10) for 2 h at room temperature. Samples were dried under N_2 and dissolved in hexane. Aliquots of the mixture were analyzed on a Hewlett–Packard Model HP 5790A GC equipped with a 30 m × 0.32 mm SPB-1 column (Supelco, Inc.), 175–260°C programmed temperature at 4°C/min, flame ionization detector and a Hewlett–Packard Model 3390A integrator.

Analysis of ceramide moiety. Glycolipid samples (200–350 μg) were hydrolyzed at 75°C for 18 h in aqueous methanolic HCl reagent (6/27/3). Liberated fatty acid methyl esters and long-chain bases were separated and analyzed by GLC as described previously (17).

Dansylation of TF_1 glycolipid. [^{3}H]Fucose-labeled, HPLC-purified TF_1, along with unlabeled HPLC-purified TF_1 (100–300 μg) was dissolved in 30 μl of 0.2 M sodium carbonate buffer, pH 9.8, with sonication. To this, 50 μl of dansyl chloride solution (Sequential Grade, Pierce Chemical Co., 20 mg/ml in acetone; 200 μl of this stock solution was mixed with 200 μl of H_2O and centrifuged; 50 μl of this mixture was used for the reaction) was added, mixed, and incubated for 1 h at 37°C in the dark.

The reaction mixture was dried under N_2 and resuspended in 20 μl $CHCl_3/CH_3OH$ (2:1) and separated on an HPTLC plate in the $CHCl_3/CH_3OH$/ 0.05% $CaCl_2$ (55/40/10) solvent system. The HPTLC plate was sprayed with Dittmer–Lester reagent (18) and orcinol reagent to locate the TF_1 glycolipid. Radiolabeled dansylated derivatives on HPTLC were subjected to autoradiography as described earlier. The resultant dansylated TF_1 component was scraped off the HPTLC plate, eluted with $CHCl_3/CH_3OH$ (2:1), and further purified by HPLC for mass spectral analysis as described earlier.

FABMS and FABMS/MS. Samples for mass spectral analysis were dissolved (5 μg/μl) in $CHCl_3/CH_3OH$ (2/1) and this solution was mixed 1/1 with the matrix (glycerol for positive ion spectra and triethanolamine for negative ion spectra). About 0.3 μl of the mixture was applied to the FAB probe tip for each measurement. FABMS was carried out on the first (MS-1) of the two mass spectrometers of a tandem high resolution mass spectrometer (JEOL HX110/HX110) at ±10-kV accelerating voltage, and 1:1500 resolution, with ±18-kV postacceleration at the detector. The JEOL FAB gun was operated at 6 kV to produce the neutral xenon beam. FABMS/MS was performed using all four sectors of the HX110/HX110 spectrometer, an instrument of E_1B_1–E_2B_2 configuration. Collision-induced decomposition (CID) took place in the third field-free region (between MS-1 and MS-2), in a cell floated at 3 kV from ground potential, so that the collision energy was 7 keV unless noted otherwise. Helium was used as the collision gas, at a pressure sufficient to reduce the precursor ion signal to 20% of its initial value. The FABMS/MS scans were recorded during linked scans of MS-2 at a constant B/E ratio. Resolution of MS-2 was 1:1000. Mass assignment accuracy was within 0.3 um of the calculated values.

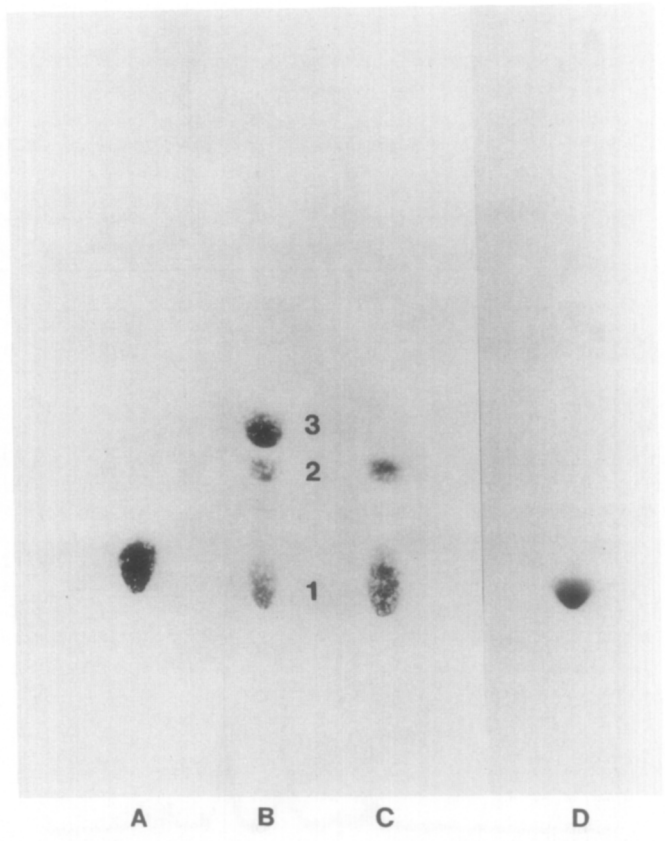


FIG. 1. HPTLC/fluorography of metabolically radiolabeled acidic glycolipid fraction of *T. foetus*. A, [^{3}H]fucose; B and C, [^{3}H]*myo*-inositol; and D, [^{14}C]ethanolamine. The chromatogram was developed in solvent system: $CHCl_3/CH_3OH$/0.05% $CaCl_2$ (55/40/10). [^{3}H]*myo*-inositol labeled lipid fraction (B) was not treated with base; A, C, and D lipid fractions were treated with base. 1, TF_1; 2, TF_2 3, phosphatidylinositol.

TABLE I

Effect of α-Fucosidase on [^{3}H]Fucose-Labeled TF_1 Glycolipid[a]

		Products	
	Initial material TF_1 (dpm)	H_2O soluble (dpm)	Lipid soluble (dpm)
Control	45,000	3,000	35,000
+ α-Fucosidase	45,000	32,000	6,300

[a] Purified [^{3}H]TF_1 glycolipid was subjected to treatment with α-fucosidase and the products separated on a C_{18} Sep-Pak cartridge as described under Materials and Methods.

RESULTS

Analysis of the nonsaponifiable lipid fraction of *T. foetus* obtained upon DEAE-Sephadex A-25, followed by Iatrobead column chromatography, revealed two major phosphate-containing components, designated TF_1 (slow migrating) and TF_2 (fast migrating). These glycolipids constituted approximately 4% of the total crude lipid fraction. The yield of pure acidic glycolipid components TF_1 and TF_2 (isolated by HPTLC followed by HPLC) was approximately 0.7–1.0 mg and 0.5–0.7 mg, respectively, from 1.2 g of crude total lipid (11.64×10^{10}; 6-liter culture)). [^{3}H]*myo*-Inositol and ^{32}P were incorporated into TF_1 and TF_2 and both compounds have chromatographic properties distinct from phosphatidylinositol (Fig. 1). [^{14}C]Ethanolamine and [^{3}H]fucose were incorporated into fraction TF_1 but not TF_2 (Fig. 1). TF_1 gave a positive reaction with orcinol spray reagent. Both lipid fractions gave positive reactions with Dittmer–Lester spray reagent, indicating the presence of phosphates. TF_1 also gave a positive reaction with ninhydrin spray reagent, which may indicate the presence of a free amino group. TF_1 was partially hydrolyzed by α-L-fucosidase (Table I).

GLC analysis of the alditol acetates of the sugars indicated the presence of fucose and inositol in TF_1 and only inositol in TF_2 (data not shown). Hydrolysis of fraction 1 in 2.5 M trifluoroacetic acid for 5 h at 100°C generated mainly fucose and trace amounts of *myo*-inositol (inositol glycosidic linkages are only partially released by milder conditions). However, hydrolysis of TF_1 and TF_2 with 6 M HCl for 16 h at 110°C provided mainly *myo*-inositol when analyzed by GLC as their alditol acetates or TMS derivatives (fucose is destroyed completely by these conditions). GLC analysis of the *N*-acetyl-*O*-TMS derivatives of the long chain bases of TF_1 and TF_2 showed mainly sphinganine and some sphingosine [(4*E*)-sphin-

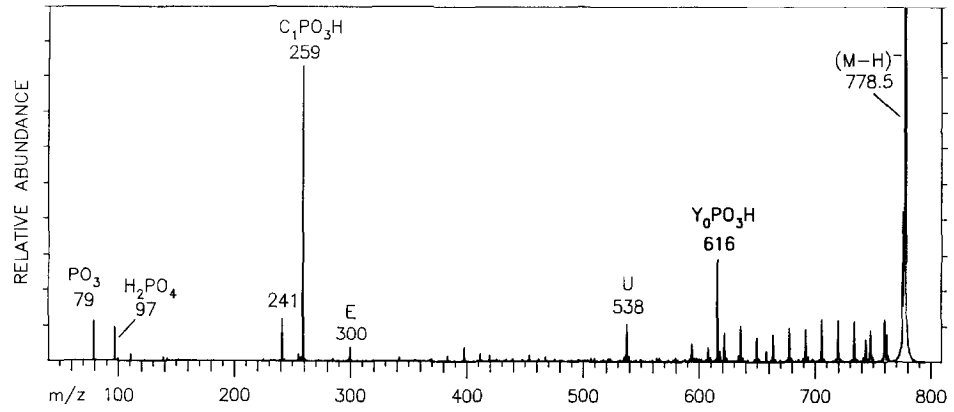


FIG. 2. CID mass spectrum of the $(M - H)^-$ m/z 778.5 in the negative ion FAB mass spectrum of *T. foetus* nonsaponifiable lipid fraction TF_2. Fragment ion assignments are given in Scheme 1.

genine]. In both glycophosphosphingolipids, the major fatty acids were palmitic acid (65–70%), stearic acid (20%), and oleic acid (15%) as determined by GLC of the fatty acid methyl esters.

Mass spectra obtained in both the positive and the negative ion mode provided molecular weight information and reflected the samples' heterogeneity. MS/MS analysis by CID of the molecular ions in the FAB mass spectra of TF_1 and TF_2 confirmed the assignments of long chain bases and fatty acids.

The positive ion FAB mass spectrum of TF_2 had peaks at m/z 780.5 and 782.5 $(M + H)^+$, and more abundant peaks at m/z 802.5 and 804.5 $(M + Na)^+$. Fragment ions at m/z 520.5 and 522.5 corresponded to the ceramides. The negative ion FAB mass spectrum had $(M - H)^-$ at m/z 778.5 and 780.5. The base peak of the negative ion spectrum was m/z 259.1, which has been reported as characteristic for inositol phosphates (19). CID of the positive and negative molecular ions and the ceramide fragments were used to assign the base and fatty acyl components (20). The CID MS/MS spectrum of the $(M - H)^-$ m/z 778.5 is shown in Fig. 2 and discussed below.

The molecular ion regions of the negative and positive ion FAB mass spectra of TF_1 are shown in Figs. 3A and 3B. The negative ion spectrum had molecular ions corresponding to three components, $(M_1 - H)^-$ m/z 1004.6 and 1006.6, $(M_2 - H)^-$ m/z 1047.6 and 1049.6, and $(M_3 - H)^-$ m/z 1089.6 and 1091.6. The base peak was the phosphoceramide fragment at m/z 616.6 and 618.6. CID mass spectra were obtained for the molecular ions and selected fragments. The positive ion spectrum had $(M + H)^+$ peaks at m/z 1049.6 and 1051.6, $(M + Na)^+$ m/z 1071.6 and 1073.6, $(M + K)^+$ m/z 1087.6 and 1089.6, and a ceramide fragment at m/z 520.5 and 522.5. The CID MS/MS spectra of $(M - H)^-$ m/z 1047.6, $(M + H)^+$ m/z 1049.6, and the positive ion fragment at m/z 384.0 are shown in Figs. 4A, 4B and 5, respectively.

In order to characterize the unique ninhydrin-positive substituent of the TF_1 fraction, TF_1 glycolipid was dansylated. The dansylated derivative of the TF_1 fraction migrated slightly faster than native TF_1 upon HPTLC in

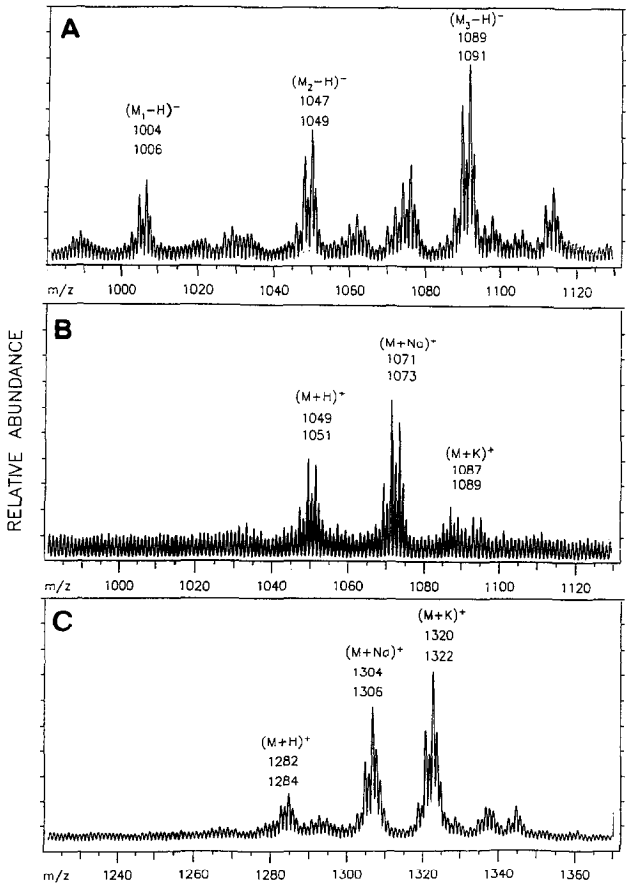


FIG. 3. Molecular ion regions of FAB mass spectra of *T. foetus* nonsaponifiable lipid fraction TF_1. (A) Negative ion spectrum of the native material. (B) Positive ion spectrum of the native material. (C) Positive ion spectrum obtained after dansylation.

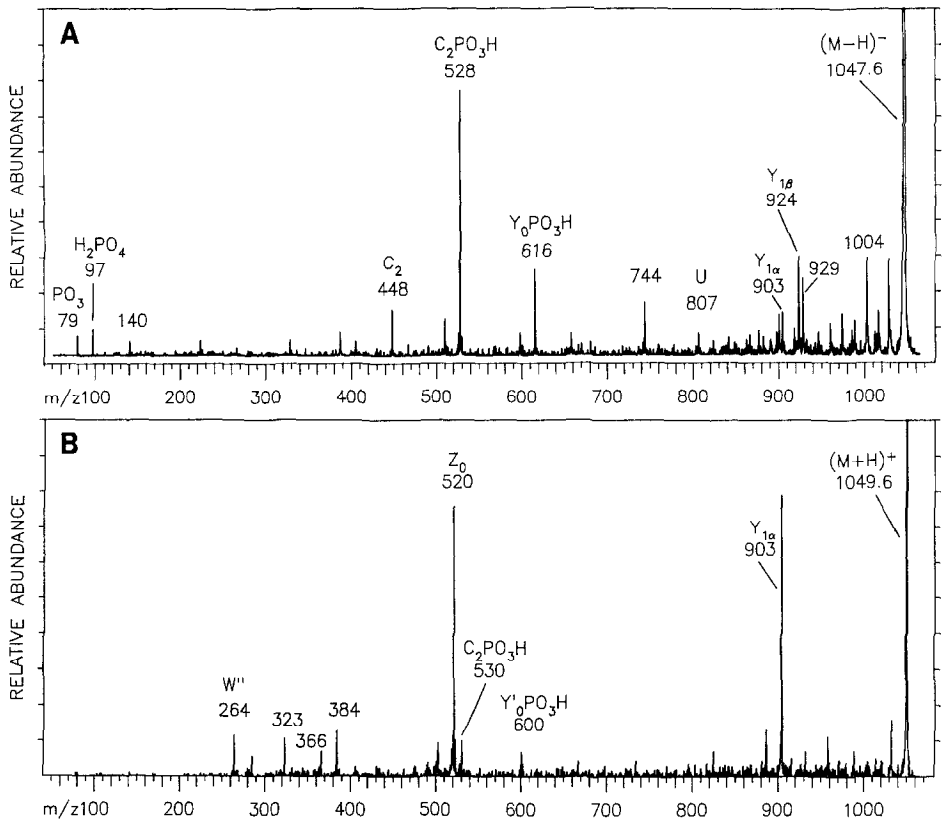


FIG. 4. CID mass spectra of *T. foetus* nonsaponifiable lipid fraction TF_1. (A) $(M_2 - H)^-$ *m/z* 1047.6. Fragment ion assignments are given in Scheme 3. (B) $(M + H)^+$ *m/z* 1049.6; collision energy 10 keV. Fragment ions assignments are given in Scheme 4.

solvent systems A and B and gave intense fluorescence when examined under uv light (Fig. 6). Dansylated derivatives of TF_1 upon HPTLC gave positive reactions with orcinol as well as Dittmer-Lester spray reagents. This dansylated derivative of TF_1 was scraped from the HPTLC silica gel and further purified on HPLC. The purified fraction was again examined on HPTLC for homogeneity and further analyzed by FABMS. The molec-

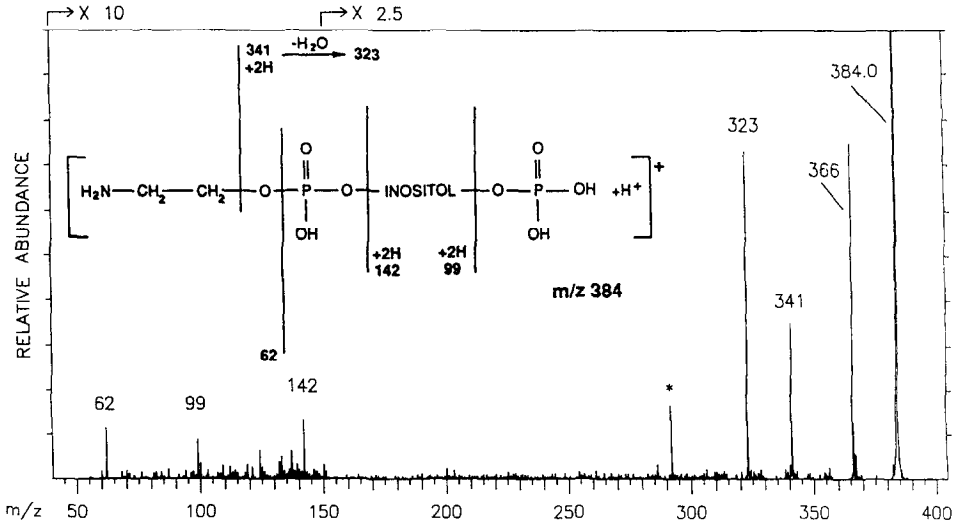


FIG. 5. CID mass spectrum of the fragment at *m/z* 384.0 in the positive ion FAB mass spectrum of *T. foetus* nonsaponifiable lipid fraction TF_1.

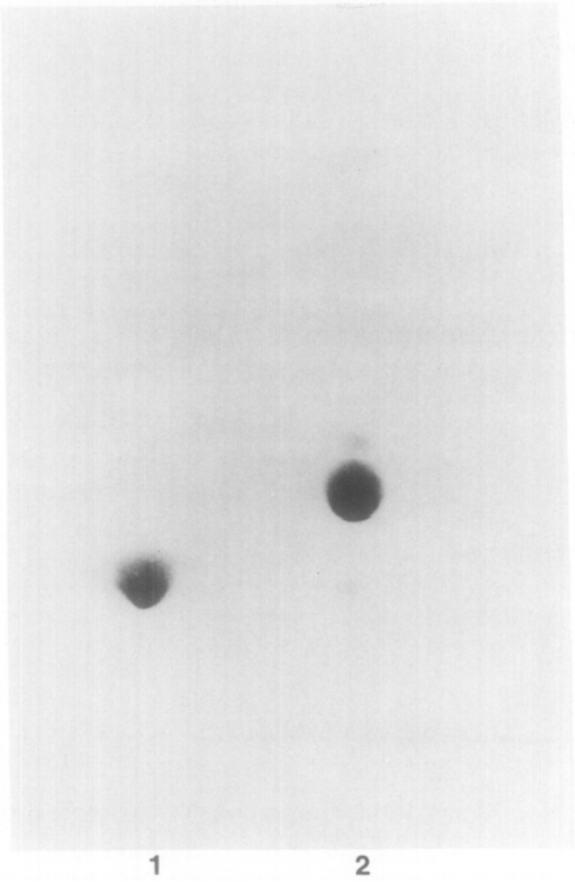


FIG. 6. HPTLC/fluorography of metabolically radiolabeled [^{3}H]fucose glycophosphosphingolipid fraction TF_1 of *T. foetus* (1) and its dansylated derivative (2). The chromatogram was developed in the solvent system $CHCl_3/CH_3OH/0.05\%$ $CaCl_2$ (55/40/10). Glycophosphosphingolipid fraction TF_1 obtained by HPTLC followed by HPLC.

ular ion region of the positive ion FAB mass spectrum of dansylated TF_1 is shown in Fig. 3C. The CID mass spectrum of the $(M + H)^+$ m/z 1284.6 is shown in Fig. 7.

DISCUSSION

T. foetus contains two major glycophosphosphingolipids, designated TF_1 and TF_2, which can be metabolically labeled with [^{3}H]*myo*-inositol and $H_3{}^{32}PO_4$. TF_1 and TF_2 are the only major glycophosphosphingolipids found in the acidic glycolipid fractions of *T. foetus*. These organisms do contain several neutral glycosphingolipids found in the neutral glycolipid fractions which will be the subject of a subsequent publication. *T. foetus* labeled with [^{3}H]fucose and [^{14}C]ethanolamine preferentially incorporated radioactivity into the TF_1 fraction of acidic glycolipids. Both these lipids contain ceramides. The major ceramide has the base sphinganine or sphingosine and the C16:0 *N*-acyl group.

The lipid fraction TF_2 contains inositol, phosphate, sphinganine (C18:0), or sphingosine (C18:1), with the major fatty acid C16:0, and appears to be a mixture of inositolphosphoceramides, as determined by chemical degradation, GLC and FABMS. The CID mass spectrum of the $(M - H)^-$ m/z 778.5 of the most abundant component, M_r 779 (c18:1, n16:0), is shown in Fig. 2, and the fragment assignments are shown in Scheme 1. The assignments of the fragment ions (and the nomenclature system, Scheme 2) indicated in Scheme 1 are based on interpretations of the CID mass spectra of a large number of glycosphingolipids (20, 21). The ion at m/z 96.9 corresponds to the phosphate anion $(H_2PO_4)^-$. Water loss from this ion leads to the peak observed at m/z 78.9. The most abundant fragment, designated C_1PO_3H, m/z 259.1, could be assigned to inositol phosphate, since GLC analysis of alditol acetates had determined that inositol was present. Observation of this fragment in negative ion FAB mass spectra of inositol phosphate-containing lipids has been reported previously (19). Water loss from this species gives rise to the peak at m/z 241. Cleavage of the C(2)–C(3) bond in the ceramide base accompanied by hydrogen transfer leads to the U ion at m/z 538. This same bond cleavage, when accompanied by loss of the fatty acyl group, yields the E ion at m/z 300. All these fragments retain the inositol moiety. Cleavage of the inositol–phosphate bond with charge retention on the phosphate results in elimination of dehydroinositol to yield the fragment Y_0PO_3H at m/z 616.5. Observation of this fragment verifies that the inositol and the ceramide are linked through the phosphate group.

Chemical degradation, GLC, and FABMS studies indicate that the polar component TF_1 contains fucose, inositol, two phosphate groups, ethanolamine, and ceramide (sphingosine or sphinganine and primarily the 16:0 *N*-acyl group). Chemical degradation and GLC analysis (not shown) of the alditol acetates showed that the only M_r 180 sugar present was inositol. This information made possible the specific mass spectral assignment of the increment that corresponds to a $C_6H_{10}O_5$ residue as inositol, rather than one of the hexose isomers. These experiments showed that fucose (M_r 162) was also present. The negative ion FAB mass spectrum (Fig. 3A) indicated that three distinct components, each having some heterogeneity in the base, were present in this fraction. Only one of the three components was observed in the positive ion FAB mass spectrum (Fig. 3B), probably because their ionization efficiencies were dissimilar. CID mass spectra of the molecular and fragment ions enabled assignment of the carbohydrate sequence and the ceramide structures. They showed that three components contain fucose linked to inositol diphosphate; one of the phosphates is then linked to the ceramide, while the other phosphate is either free or linked to ethanolamine or to *N*-acetylethanolamine. These assignments are discussed below and some of the CID mass spectra are shown in Figs. 4 and 5 as illustrations of this approach to structural elucidation.

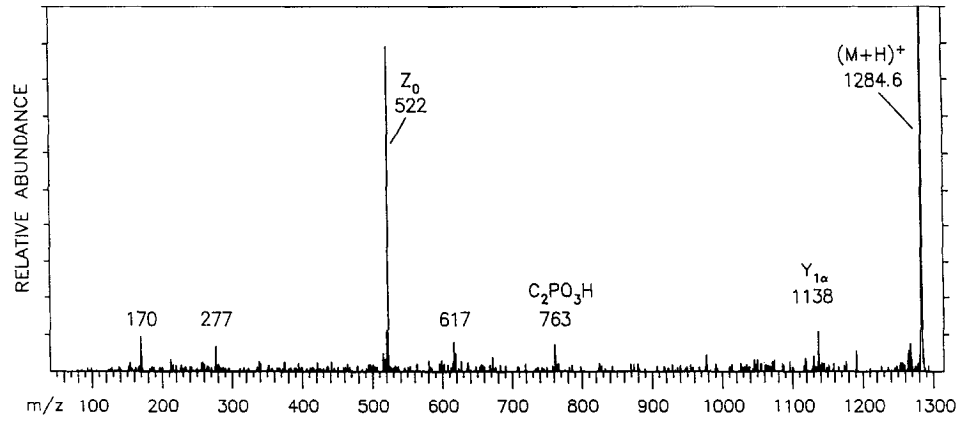
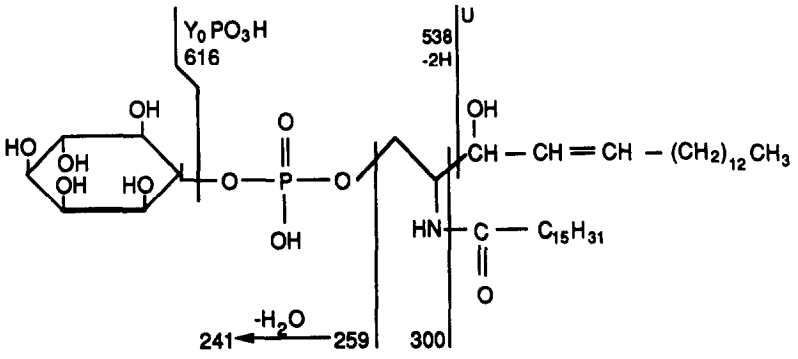


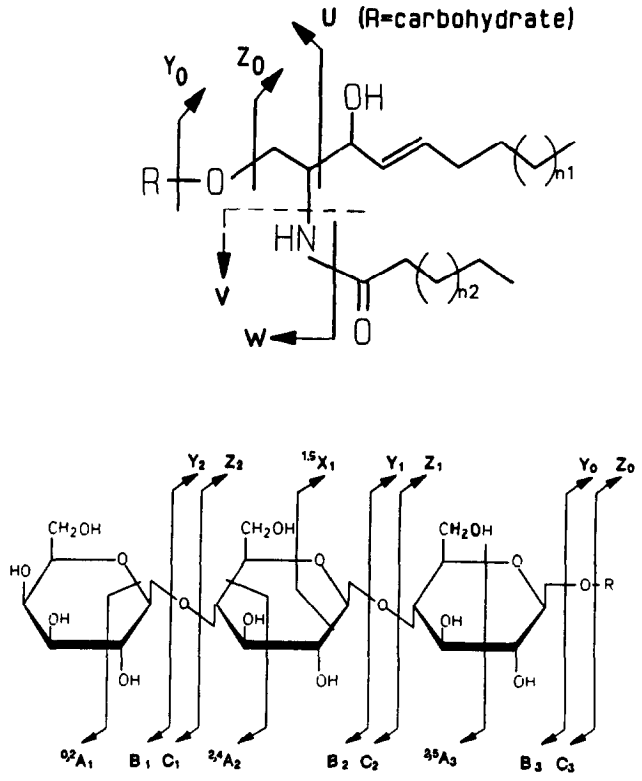
FIG. 7. CID mass spectrum of the $(M + H)^+$ *m/z* 1284.6 in the FAB mass spectrum obtained after dansylation of *T. foetus* unsaponifiable lipid fraction TF_1. Fragment ion assignments are given in Scheme 5.

The CID mass spectrum of the M_r 1048 component, which has $(M - H)^-$ *m/z* 1047.6 is shown in Fig. 4A and the assignments are indicated in Scheme 3. The Y_0PO_3H fragment at *m/z* 616.5, as discussed above for the CID spectrum of *m/z* 778.5 from fraction TF_2, arises from cleavage of the inositol–phosphate bond, with charge retention on the phosphoceramide. Cleavage of the phosphate–ceramide bond, with charge retention on the substituted phosphoinositol leads to the abundant C_2PO_3H fragment at *m/z* 528.3. The presence of fucose linked at C-1 is indicated by the fragments at *m/z* 921.5 and 901.5 that arise from the losses of 118.1 u (cleavage of the fucose C(1)–C(2) and C(5)–O bonds, $^{1,5}X_{1\alpha}$) and 146.1 u (loss of dehydrofucose, $Y_{1\alpha}$). Losses of ethanolamine and phosphoethanolamine, accompanied by hydrogen transfer, lead to the fragments at *m/z* 1004.5 and 924.5, respectively. Since either fucose or phosphoethanolamine is lost directly from the molecular ion, it can be concluded that both of these groups occupy terminal positions. Loss of both fucose and phosphoethanolamine leads to the fragment at *m/z* 744.5. The fragment ions at low mass can be assigned to the phosphate group (*m/z* 78.9 and 96.9, as discussed above) and to the phosphoethanolamine anion, *m/z* 140.0.

In the CID mass spectrum of the $(M + H)^+$ *m/z* 1049.6, which corresponds to the same M_r 1048 component (Fig. 4B), the 146.0 u loss of dehydrofucose to produce the $Y_{1\alpha}$ fragment at *m/z* 903.6 is readily apparent (Scheme 4). The most abundant ion, Z_0 at *m/z* 520.5, corresponds to the protonated, dehydrated ceramide. The W′ and W″ ions at *m/z* 282.2 and 264.2, which arise from this species by cleavage of the *N*-acyl group and subsequent dehydration, respectively, allow assignment of the base as sphingenine and the fatty acyl group as palmitoyl. The ions at *m/z* 323.0, 366.0, and 384.0 could not be assigned using the scheme developed for interpretation of the CID spectra of glycosphingolipids (20, 21). However, since a fragment ion at *m/z* 384 was observed in the normal FAB mass spectrum, it was possible to obtain its CID mass spectrum as an aid for the interpretation of this ion series. The CID mass spectrum of the fragment ion *m/z* 384.0 is shown in Fig. 5. From this spectrum, it can be seen that the ions at *m/z* 366.0 and 323.0 derive from the ion at *m/z* 384.0, probably resulting from losses of water and ethanolamine,



SCHEME 1



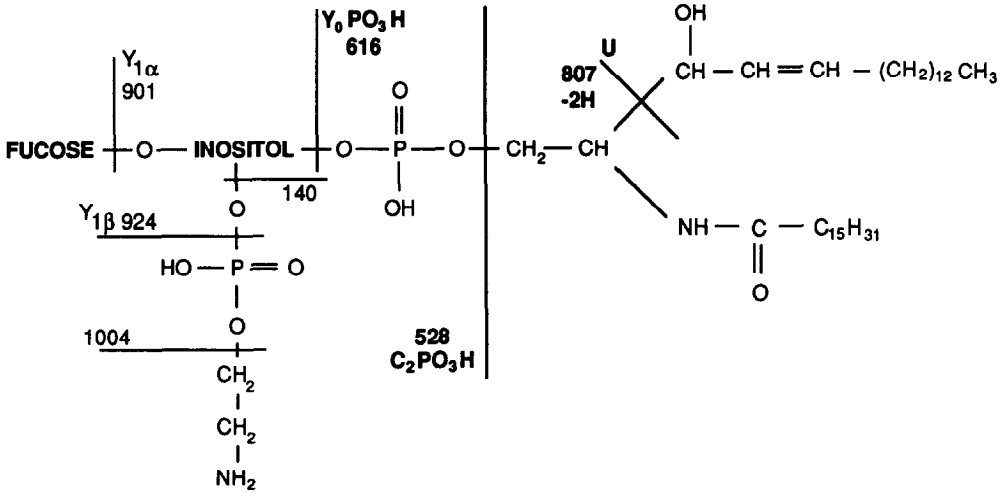
SCHEME 2

as indicated in the scheme which appears on the figure. The ions at m/z 62 and m/z 142 are appropriate for protonated ethanolamine and phosphoethanolamine, respectively, and the ion at m/z 99 for protonated phosphoric acid. These constitute strong evidence for the assignment of inositol diphosphate, with one of the phosphate groups esterified by ethanolamine, as a substructure of TF_1.

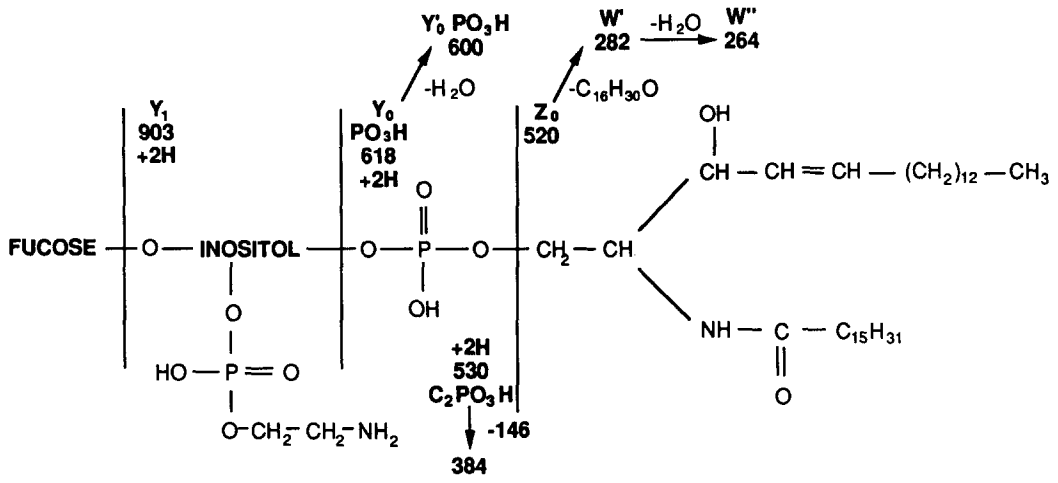
The other components of TF_1 were assigned structures as having a free phosphate group ($(M - H)^-$ m/z 1004, 1006), and having *N*-acetyl-phosphethanolamine ($(M - H)^-$ m/z 1089, 1091), on the basis of appropriate fragment ion shifts in their mass spectra and CID mass spectra (not shown).

Chromatographic and FABMS studies of the dansylated derivative of TF_1 glycolipid provided further evidence for the presence of ethanolamine and its location in the inositol diphosphate ester linkage. The dansylation product was separated from unreacted material by HPTLC, purified by HPLC, and analyzed by mass spectrometry. The molecular ion region of the positive ion FAB mass spectrum of the product is shown in Fig. 3C. The presence of $(M + H)^+$ m/z 1282.6 and 1284.6, $(M + Na)^+$ m/z 1304.6 and 1306.6, and $(M + K)^+$ m/z 1320.6 and 1322.6, a shift of 233 u from the clusters observed in the spectrum of the native material, are appropriate for incorporation of the 5-(dimethylamino)-1-naphthylenesulfonyl group at the primary amine of the ethanolamine phosphate substituent. No dansylation products would be expected for the other components of TF_1. The CID mass spectrum of the $(M + H)^+$ m/z 1284.6 ion was recorded and the fragment ion assignments are shown on Scheme 5. The W′ and W″ fragments which define the base as sphinganine with a 16:0 *N*-acyl substituent do not appear in this CID spectrum of the molecular ion, but were observed in the CID spectrum of the ceramide fragment ion m/z 522.5, along with other diagnostic fragments, V m/z 256.2 (protonated amide) and S m/z 280.2 (amide plus C_2H_2).

To our knowledge, there is no report of a compound having fucose, diphosphate, ethanolamine (or acetylethanolamine), inositol, and a ceramide. A variety of proteins have phosphatidylinositol-containing glycolipid anchors conjugated to their C-terminus through an amide bond



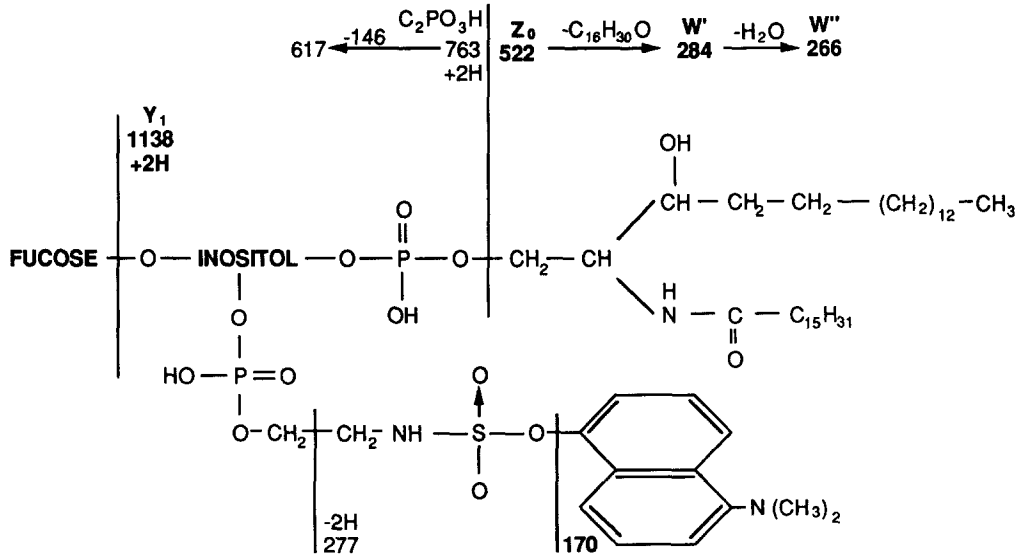
SCHEME 3



SCHEME 4

to ethanolamine which appears to be linked by a phophodiester bond to an oligosaccharide (22, 4). The glycolipids attached to these proteins also contain *myo*-inositol, fatty acids, phosphate, mannose, glucosamine or galactosamine, and glycerol (3). In addition, some phosphatidylinositol glycan anchors contain an additional ethanolamine residue with a free amino group (3). Glycosylated phosphoceramides, not linked to protein, have been reported in plants and yeasts (23, 24) and were also reported to be components of antigen shed by *L. donovani* (25). The presence of three glycosylphosphatidylinositol (GPI) glycolipid antigens which could be precursors of lipophosphoglycan or GPI anchor proteins have been identified from *L. major* (26). We have also reported the presence of inositol-phosphosphingolipids in another parasitic protozoan, *L. mexicana mexicana* (9). There is also some evidence that a contact site A (cs A) glycoprotein of *Dictyostelium discoideum* is anchored by an inositol-linked ceramide-based lipid glycan (5).

In summary, TF_1 appears to be a novel class of glycophosphosphingolipid, which may be an intermediate in the biosynthesis of membrane anchor proteins or other glycoconjugates to trichomonads, although additional work will be needed to establish this point and its biological significance. Because cell surface glycoconjugates anchored by PI-containing glycolipids have been shown to be involved in parasite survival or infectivity, these components are of particular interest since they represent potential targets for the development of chemotherapeutic agents. Elucidation of the structures of these novel gly-



SCHEME 5

cophosphosphingolipids was based largely on data provided by tandem mass spectral analysis of the lipid mixtures, a new approach which should prove extremely useful for such investigations.

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