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Glycophosphoceramides from Plants

PURIFICATION AND CHARACTERIZATION OF A NOVEL TETRASACCHARIDE DERIVED FROM TOBACCO LEAF GLYCOLIPIDS*

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A glycophosphoceramide concentrate prepared from tobacco leaves was shown to contain a mixture of related lipids (Kaul, K., and Lester, R. L. (1975) *Plant Physiol.* 55, 120-129; Kaul, K., and Lester, R. L. (1978) *Biochemistry* 17, 3569-3575) with the simplest and most abundant components having the structure GlcN(±Ac)(α1 → 4)GlcUA(α1 → 2)myoinositol-1-*O*-phosphorylceramide (Hsieh, T. C.-Y., Kaul, K., Laine, R. A., and Lester, R. L. (1978) *Biochemistry* 17, 3575-3579). To determine the structure of the more complex members of this series, a mixture of oligosaccharides was prepared from a carboxyl-reduced glycophosphoceramide concentrate by alkali-catalyzed hydrolysis and alkaline phosphatase treatment. A combination of reverse-phase high pressure liquid chromatography (Wells, G. B., and Lester, R. L. (1979) *Anal. Biochem.* 97, 184-190), normal-phase high pressure liquid chromatography, and thin layer chromatography were used to resolve several oligosaccharides as acetylated derivatives. Products of methylation analysis, CrO_3 oxidation, and deacetylation-deamination were identified using chemical ionization mass spectrometry to give the following novel structures. A major tetrasaccharide was completely characterized as Gal(α1 → 4)GlcNAc(α1 → 4)GlcUA(α1 → 2)myoinositol. An additional structure of a minor tetrasaccharide was partially characterized as GlcNAc(α1 → 4)GlcUA(α1 → ?)myoinositol(? ← 1α)Man. These are representatives of a class of acidic glycolipids from plants, possibly analogous to the acidic gangliosides found in animal cell membranes.

Glycophosphoceramides, as inositol-containing acidic glycolipids, occur widely in plants and fungi (1-3), but few examples of perhaps 20 or more existing compounds have been reported as chemically characterized (4, 5). This has been due to the extreme difficulty in isolation of chemically analyzable amounts of individual members from glycophosphoceramide mixtures. Pioneering work in this area was performed by Carter *et al.* (1, 4). Recently, we reported the complete structure of a ceramide-phosphotrisaccharide which comprises 20% of the glycophosphoceramide concentrate prepared from tobacco leaves (5). This structure, GlcNAc(α1 → 4)GlcUA(α1 → 2)inositol-1-*O*-phosphorylceramide, may be a basic core structure of the tobacco glycophosphoceramides. More complex glycolipids in the concentrate were shown to contain galactose and arabinose in addition to the trisaccharide constituents (5).

We now describe the preparation of an oligosaccharide concentrate from which we have prepared adequate quantities to characterize completely the major tetrasaccharide phosphoceramide. The concentrate was obtained by first removing the phosphoceramide component from the carboxyl-reduced glycophosphoceramide mixture and then acetylating the resulting intact oligosaccharides prior to normal and reversed-phase chromatography. Fractions were further purified by thin layer chromatography to yield a trisaccharide and two tetrasaccharide fractions in pure form.

EXPERIMENTAL PROCEDURES

A glycophosphoceramide concentrate from tobacco leaves was prepared by Kaul and Lester (6) with a molar ratio of hexuronic acid:glucose:phosphorus of (1.5:0.04:1.0). Hexuronic acid was assayed according to Blumenkrantz and Asboe-Hansen (7). Glucose was determined by gas-liquid chromatography of glucitol acetate prepared after hydrolysis in 2 N trifluoroacetic acid at 120 °C for 90 min (8). Phosphorus was determined after perchloric acid digestion according to Bartlett (9).

The myoinositol-containing disaccharide galactinol (1-L-1-*O*-α-D-galactopyranosylmyoinositol) was a gift from Dr. Clinton Ballou, University of California, Berkeley. Unless otherwise indicated all high pressure liquid chromatography was carried out with a pair of Model 6000A pumps controlled by a Model 660 programmer (Waters Associates, Milford, MA) and detection was performed with a moving-wire flame-ionization detector (Model LCM2, Pye Unicam Ltd., Cambridge, England).

N-Acetylation—About 50% of the glycophosphoceramide from tobacco leaves contain glucosamine without the *N*-acetyl group (3, 6). *N*-Acetylation was carried out according to Roseman and Daffner (10) on 5 g of the glycophosphoceramide concentrate. Since *N*-acetylated hexosamines are more easily liberated by acid hydrolysis than the unacetylated hexosamine (11), the ratio (hexosamine hydrolyzed in 2 N HCl at 100 °C for 1 h:hexosamine hydrolyzed in 6 N HCl at 100 °C for 18 h) was used to monitor the extent of the *N*-acetylation of the glycophosphosphingolipid concentrate. The colorimetric procedure for hexosamine after hydrolysis was according to Gatt and Berman (12). The ratio of 2 N HCl-releasable hexosamine doubled after one addition of acetic anhydride and remained the same after a second addition. Therefore, the *N*-acetylation was considered essentially complete.

The *N*-acetylated concentrate was lyophilized and resuspended in 66 ml of water and passed through a 200-ml column of cation exchange resin (AG 50W-X8, H^+ form, 200-400 mesh, Bio-Rad). A total of 1200 ml of eluate in water was collected and lyophilized.

Carboxyl Reduction to Convert Glucuronic Acid to Glucose in the Glycophosphoceramide Concentrate—The carboxyl-reduction procedure was adapted from that of Taylor *et al.* (13), yielding a product

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with a uronic acid:glucose:phosphorus ratio of 0.0:1.3:1.0. The sample was then dialyzed and lyophilized. Recovery based on phosphorous was 94%.

Removal of Phosphoceramide—An aliquot of the carboxyl-reduced glycophosphoceramide concentrate containing 0.41 mmol of total phosphate was dissolved in 60 ml of 0.5 N KOH and heated at 100 °C for 8 h. The sample was cooled to room temperature, 2.8 ml of glacial acetic acid were added, and the pH was adjusted to 3 with 12 ml of 4 N HCl. A total of 60 ml of chloroform was used to extract lipids from the sample. The aqueous layers were combined and pH was adjusted to 8 with 6 ml of 15 N NH_4OH and the remaining chloroform was removed by rotary evaporation. The pH was readjusted to 8 and the white precipitate formed was removed by centrifugation. The mixture of oligosaccharide and phospholigosaccharides obtained at this stage was treated with several 20 unit doses of *Escherichia coli* alkaline phosphatase (Sigma P-4377) to remove the phosphate. After 56 h, 82% of total phosphate was released as P_i. The sample was applied to a 200-ml column of Dowex 1-X2 resin (bicarbonate form, 200–400 mesh) to isolate the dephosphorylated oligosaccharides free of phosphate and phosphorylated impurities. The yield at this step, based on pentose, was 76%. Since the alkaline hydrolysis also *N*-deacylated the glucosamine, the procedure of Roseman and Daffner (10) was again used to *N*-acetylate the oligosaccharide mixture. The sample was then passed through a 210-ml column of AG 50W-X8 (H^+ form, 200–400 mesh). Unbound fractions were collected in 3 column volumes of water. Rotary evaporation at 35 °C gave a syrup-like sample. Absolute ethanol was added in 2-ml quantities six times during further rotary evaporation to remove water. Five ml of redistilled pyridine/acetic anhydride (1:1) were added to the oligosaccharide mixture. Peracetylation was carried out at 100°C for 1.5 h. Redistilled toluene was used to remove acetic anhydride as an azeotrope at 40 °C under nitrogen. The peracetylated oligosaccharide mixture was extracted with 5 ml of redistilled chloroform three times against 5 ml of water. The chloroform layers were combined.

Hi-Flosil Liquid Chromatography of the Peracetylated Oligosaccharide Mixture—The peracetylated oligosaccharide mixture was chromatographed on a column (2.54 × 100 cm) of silica gel (Hi-Flosil, 60/200 mesh, Applied Sciences, Ltd.). In each pilot run, a sample containing 21.4 μmol of hexosamine was applied to the column. Specific solvent compositions are described in the legend for Fig. 1. In a preparative run, a sample containing about 130 μmol of hexosamine was applied to the same column and chromatographed under similar conditions.

Gradient Reverse-phase High Pressure Liquid Chromatography of Hi-Flosil Fraction 3 from Fig. 1—The reverse-phase HPLC[1] conditions used to fractionate the peracetylated oligosaccharide mixture were adapted from those of Wells and Lester (14). In each analytical run, an aliquot of Hi-Flosil Fraction 3 containing 2.5 μmol of hexosamine was dissolved in 50 μl of acetonitrile and chromatographed on an octadecyl reverse-phase column (0.46 × 25 cm, Partisil 5-ODS, Whatman) (Fig. 2). In preparative runs 30 μmol of Fraction 3 were chromatographed on a column (0.45 × 200 cm) of C_{18}-Corasil (37/50 μm, Waters Associates) kept at 65 °C (Fig. 3). Fraction C of Fig. 2 is comparable to Fraction 4 of Fig. 3.

Isocratic Reverse-phase High Pressure Liquid Chromatography of Oligosaccharide Fraction 5 from Gradient HPLC in Fig. 3—Oligosaccharide Fraction 5 (Fig. 3) from the reverse-phase HPLC gradient chromatography was chromatographed on the C_{18}-Corasil reverse-phase column isocratically in acetonitrile water (3:7) (see Fig. 4).

Thin Layer Chromatography—Silica Gel G plates with 250 μm thickness or high efficiency Silica Gel G plates (HETLC, Analtech, Inc.) were used. For acetylated sugars the TLC solvent was chloroform/methanol ranging in composition from 50:1 to 17:1. For unacetylated sugars, an acetonitrile water solvent system in proportions of 3:1 to 5:1 was used. Sugar spots were visualized by spraying the plate first with a solution of orcinol (200 mg of orcinol/100 ml of 95% ethanol in water) and then with 75% sulfuric acid in water followed by heating at 100 °C for 5 to 10 min (Fig. 5).

Normal-phase High Pressure Liquid Chromatography—Further separation of the major components in the gradient HPLC oligosaccharide Fraction 5 (Fig. 3) was achieved by normal-phase high pressure liquid chromatography on a column (0.39 × 30 cm) of μPorasil (Waters Associates) eluted isocratically with chloroform:2-propanol (98.5:1.5). The flow rate was 1 ml/min (Fig. 6). Thin layer chromatography from fractions of this run is shown in Figs. 7 and 8.

Determination of Carbohydrate Composition and Anomeric Configuration of Tetrasaccharides A and B—To an aliquot containing 40 nmol of the major tetrasaccharide (B) (*Fractions 9–17* in Fig. 6) or the minor tetrasaccharide (A) (*Fractions 5–7* in Fig. 6) was added 50 nmol of xylitol (as internal standard). The sample was dried under nitrogen and acetylated in 1 ml of pyridine/acetic anhydride (1:1, v/v) at 100 °C for 1 h. One-half of the sample was then evaporated to dryness, mixed with 0.5 ml of glacial acetic acid and 50 mg of CrO_3, and kept for 15–20 min at 40 °C in an ultrasonic bath. The mixture was then diluted with 3 ml of water and extracted with 2 ml of chloroform three times. The combined chloroform layers were extracted with 1.5 ml of water before evaporation to dryness. The above procedures were adapted from those of Hoffman *et al.* (15) and Laine and Renkonen (16). The sample was then hydrolyzed at 120 °C for 90 min in 0.5 ml of 2 N trifluoroacetic acid. After evaporation to dryness under nitrogen, the sample was reduced in 0.5 ml of 1 N NH_4OH containing 20 mg of $NaBH_4$ at room temperature for 17 h. The sample was acidified with glacial acetic acid to destroy $NaBH_4$ and borate was removed by codistillation with methanol repeated five times (17). After incubation at 100 °C for 1 h in 1 ml of pyridine/acetic anhydride (1:1), the sample was evaporated to dryness several times with addition of 0.5 ml of redistilled toluene each time. After addition of 2 ml of water, the mixture was extracted with 2 ml of chloroform four times. The chloroform layers were then combined and evaporated under nitrogen to dryness. The alditol and myoinositol acetates thus obtained were dissolved in 25–50 μl of redistilled acetone and analyzed by GC as described below.

The other half of the acetylated oligosaccharide was processed by the procedure described above except that CrO_3 was omitted. This sample was used to determine the carbohydrate composition which, on comparison with the composition obtained after treatment, would give evidence for the anomeric configuration of the glycosidic linkages.

The alditol and myoinositol acetates were chromatographed on a glass column (150 cm × 20 mm inside diameter) packed with 3% OV-275 on 100/200 Chromosorb WAW (Supelco, Inc.) with nitrogen as carrier gas at a flow rate of 40 ml/min in a Perkin-Elmer gas chromatograph (Model 3920) equipped with a flame-ionization detector. Column temperature was programmed from 210–250 °C at a linear rate of 4 °C/min. Peak area was calculated by triangulation.

Methylation Analyses of Oligosaccharides by GC/MS—Aliquots from the oligosaccharide fractions at various stages of isolation were taken to prepare permethylated oligosaccharides using methylsulfinyl-carbanion (18) and were subsequently derivatized to partially methylated alditol and myoinositol acetates according to Bjorndal *et al.* (19, 20) and Stellner *et al.* (21) with deuterium labeling at the C-1 position of the alditols by use of sodium borodeuteride. The acetates were chromatographed and analyzed in a Finnigan gas chromatograph/mass spectrometer/data system (Model 3300/6110), using both electron impact and chemical ionization mass spectrometry. Detailed EI or CI GC/MS conditions are described in the figure legends.

Carbohydrate Sequence Determination of the Major Tetrasaccharide (Deacetylation-Deamination)—Approximately 600 nmol of peracetylated major tetrasaccharide (*Fractions 9–17* in Fig. 7) was *O*- and *N*-deacetylated by heating at 100 °C for 12 h in 1.0 ml of 0.5 N KOH in water. The pH was adjusted to 6 with glacial acetic acid. The mixture was then desalted on a column (115 × 1.4 cm) of Bio-Gel P-2 (Bio-Rad) in 15% acetic acid. A small aliquot from each fraction was spotted on a Silica Gel G thin layer plate and the sugar was visualized by orcinol/H_2SO_4 spray. Orcinol-positive fractions were pooled and evaporated to dryness under reduced pressure. For deamination, 2.0 ml of the nitrous acid reagent, prepared according to Bayard and Roux (22), were added to the sample. After 18 h at room temperature, the deamination mixture was applied to a 10-ml column of AG 50W-X4 (H^+ form, 50–100 mesh, Bio-Rad) and eluted with 35 ml of water. The eluate was evaporated to dryness under reduced pressure, taken up in 1 ml of water, applied to a 10-ml column of AG 1-X8 (acetate form, 200–400 mesh, Bio-Rad), eluted with 35 ml of water, and evaporated to dryness. One-half ml of 1 N NH_4OH containing 15 mg of $NaBD_4$ was used at room temperature for 16 h to reduce the deamination products. After acidification with glacial acetic acid to destroy $NaBD_4$, the sample was evaporated with methanol at 40 °C under nitrogen to remove borate. The mixture was passed through both cation and anion exchange resins again as described above. The eluate was evaporated to dryness and taken up

[1] The abbreviations used are: HPLC, high pressure liquid chromatography; Inos, myoinositol; GC/MS, gas chromatography/mass spectrometry; CI, chemical ionization; EI, electron impact; TLC, thin layer chromatography. PSL I and II, glycophosphosphingolipids I and II.

in 0.5 ml. An aliquot was dried and permethylated using methylsulfinyl-carbanion (18) and subjected to CI GC/MS analysis. For detailed GC/MS conditions see legend for Fig. 10.

RESULTS

Hi-Flosil Liquid Chromatography of the Peracetylated Oligosaccharide Mixture—The peracetylated oligosaccharide mixture obtained from the carboxyl-reduced glycophosphosphingolipid concentrate was chromatographed in a Hi-Flosil silica gel column as shown in Fig. 1. The major fraction, Fraction 3 (Fig. 1), contained a mixture of oligosaccharides further resolved by reverse-phase HPLC as described below. Higher number fractions (Fractions 4–11) contained higher oligomer oligosaccharides and remain to be investigated.

Analytical Reverse-phase High Performance Liquid Chromatography of the Major Hi-Flosil Fraction (Fraction 3) on Partisil-ODS—Hi-Flosil Fraction 3 (Fig. 1) was chromatographed on an analytical reverse-phase column as shown in Fig. 2. The major fraction (Fraction C) in Fig. 2 has the same

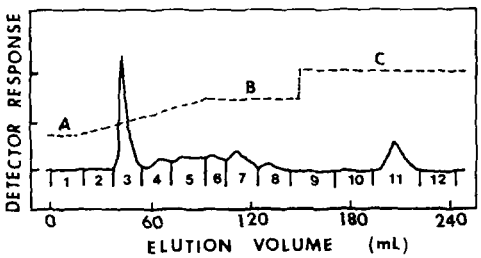


FIG. 1. **Hi-Flosil liquid chromatography of peracetylated oligosaccharide mixture.** *Solvent A* is chloroform/methanol (60:1). *Solvent B* is chloroform/methanol (1:1). *Solvent C* is chloroform/methanol/water (16:16:5). Flow rate was 9.9 ml/min. Other details are described under "Experimental Procedures." Fractions pooled are indicated by *vertical lines* and *numbers* under the chromatogram. The major fraction (*Fraction 3*) was utilized for further anlaysis by reverse-phase high pressure liquid chromatography.

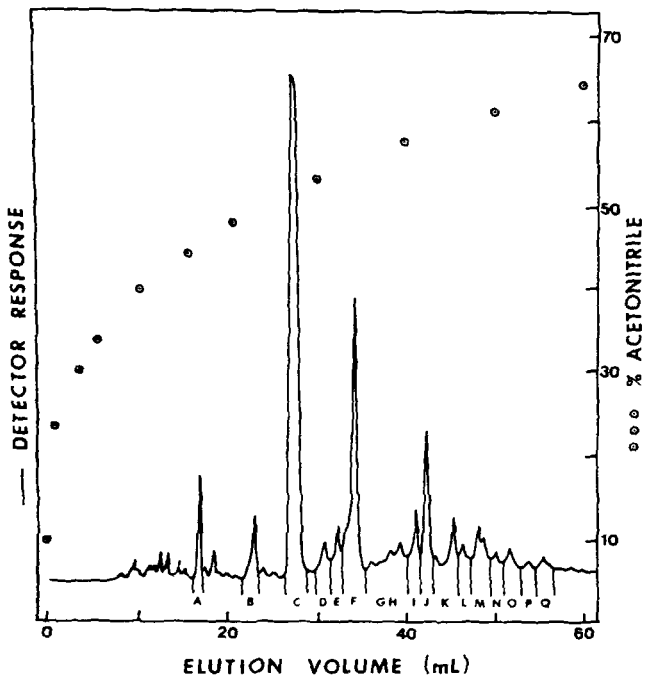


FIG. 2. **Analytical reverse-phase high pressure liquid chromatogram of the major Hi-Flosil fraction on Partisil-ODS (Whatman).** A nonlinear solvent gradient was used as indicated by ⊙ from acetonitrile/water (1:9–7:3) at a flow rate of 1 ml/min. Pooled fractions are indicated by *vertical lines* below the tracing and labeled *A–Q*.

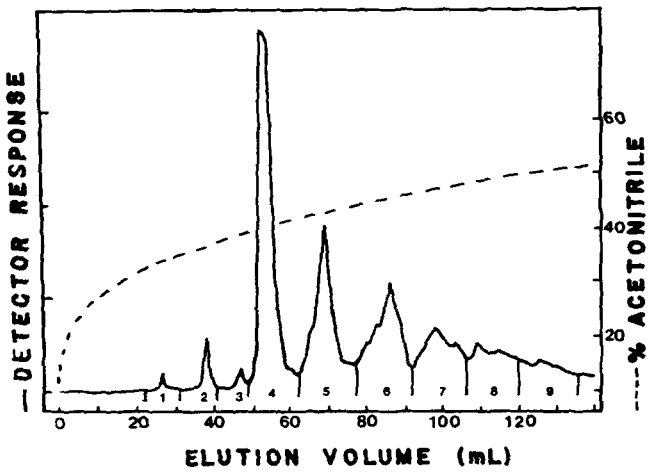


FIG. 3. **Preparative reverse-phase chromatography of the major Hi-Flosil fraction (*Fraction 3* in Fig. 1) of peracetylated oligosaccharides.** A nonlinear solvent gradient from acetonitrile/water (1:9–6:4) was used for elution. Flow rate was 2 ml/min. For other chromatographic conditions, see "Experimental Procedures." Fractions pooled are indicated by *numbers* under the *peaks*.

TABLE I

Hi-Flosil chromatography yields

Fraction	1	2	3	4	5	6	7	8	9
Weight (mg)	1.7	6.2	3.5	92.4	47.7	56.9	32.2	20.6	18.7

elution volume as the carboxyl-reduced and acetylated trisaccharide derived from PSL I (5): GlcNAc*p*(α1 → 4)Glc*p*(α1 → 2)myoinositol. The later peaks, *D–Q* (Fig. 2), are higher oligomers containing galactose and/or arabinose.

Methylation Linkage Analyses of Partisil-ODS Fractions from Fig. 2—For preliminary knowledge about the kinds of glycosidic linkages present in analytical fractions from the Partisil-ODS chromatography, methylation analyses were carried out. The major fraction, Fraction C in Fig. 2, had the same linkage type as those of the PSL I carboxyl-reduced trisaccharide, terminal *N*-acetylglucosamine, 4-linked glucose, and monosubstituted myoinositol (5), and is therefore considered to have the same structure as indicated above. Further characterization of the fraction as the PSL I carboxyl-reduced trisaccharide will be discussed below. Other fractions (Fractions D–Q, Fig. 2) had substitutions on the saccharides indicating the presence of terminal arabinose, terminal galactose, 3,6-branched galactose, 4-linked *N*-acetylglucosamine, and combinations of these. These methylation data as well as results from thin layer chromatography (not shown) indicated that the most abundant oligosaccharide in the mixture (Fraction C in Fig. 2) was a trisaccharide which most likely arose from the major glycophosphosphingolipids (PSL I and PSL II) in the concentrate.

Preparative Reverse-phase Gradient High Pressure Liquid Chromatography of the Major Hi-Flosil Fraction—Hi-Flosil Fraction 3 (Fig. 1) was chromatographed on a preparative reverse-phase column as shown in Fig. 3. Clusters of peaks were analogous to those oserved in analytical runs on the Partisil-ODS column (Fig. 2), except that resolution was somewhat sacrificed within each cluster in these preparative runs. Fractions were pooled and evaporated to dryness under reduced pressure. The weight of each fraction (as peracetylated derivatives) is shown in Table I. These numbers represented the yield of each fraction derived from a total of 3.5 g of the glycophosphosphingolipid crude concentrate used to prepare this batch of the carboxyl-reduced oligosaccharide mixture. It is not excluded that small amounts of noncarbohydrate impurities could still be present in these fractions.

Preparative Isocratic Reverse-phase High Pressure Liquid Chromatography of the Tetrasaccharide Fraction—Pooled Fraction 5 (Fig. 3, tetrasaccharide fraction) from the gradient preparative reverse-phase C_{18}-Corasil column was chromatographed again on the same column isocratically with acetonitrile/water (3:7) further to resolve the individual oligosaccharides as shown in Fig. 4; Fractions 13 and 14 represented material eluted with 100% acetonitrile.

Pooled fractions from isocratic reverse-phase chromatography (Fig. 4) were dried under reduced pressure and aliquots were analyzed by TLC in chloroform/methanol (40:1), as shown in Fig. 5. Fractions 5–8 from the isocratic reverse-phase step (Fig. 4) showed only single spots. In Fractions 9–12 from Fig. 4, the major component seen on TLC (characterized as tetrasaccharide B and discussed below) was well separated from the minor component, tetrasaccharide A, which had a higher R_F (partially characterized as the minor tetrasaccharide and discussed below). Since the chromatography principles involved in silica gel TLC under the conditions used are similar to those of normal-phase HPLC on silica gel columns, the latter was used to separate these two oligosaccharides. As shown in Fig. 4, Fractions 6–8 were combined (*Pool X*) and Fractions 9–12 were combined (*Pool Y*).

Normal-phase High Pressure Liquid Chromatography of the Tetrasaccharide Fraction—Pool X and *Pool Y* (Fig. 4) were chromatographed on μPorasil (Waters Associates) with chloroform:2-propanol (98.5:1.5). Fig. 6 shows the normal-phase HPLC chromatogram of *Pool Y*. The pooled fractions were evaporated to dryness under nitrogen and taken up in 100 μl of chloroform. An aliquot from each fraction was chromatographed on TLC in chloroform/methanol (40:1) as shown in Fig. 7. Fractions 3–7 of the μPorasil HPLC (Fig. 6) contained a single spot on TLC (tetrasaccharide A) which migrated with an R_F less than that of the PSL I trisaccharide. The major oligosaccharide component (tetrasaccharide B), which distributed mainly in Fractions 8–17 of the μPorasil chromatogram (Fig. 6), had an R_F less than that of tetrasaccharide A.

Pool X material from the isocratic reverse-phase HPLC (Fractions 6–8, Fig. 4) was also chromatographed on the μPorasil column under identical conditions. The chromatogram (not shown) was similar to Fig. 5 except that the minor peak (tetrasaccharide A) was not present.

Pooling of Oligosaccharides A and B from μPorasil Liquid Chromatography—The μPorasil fractions corresponding to the minor peak (*Fractions 5* and *6*, Fig. 5) from several repetitive runs were combined. The fractions contained one of the minor tetrasaccharides, referred to as tetrasaccharide A.

The μPorasil fractions corresponding to the major peak (*Fractions 8–11*, Fig. 5) were combined. The major component, referred to as tetrasaccharide B, is the major tetrasaccharide isolated from the carboxyl-reduced glycophosphosphingolipid concentrate of tobacco leaves.

Aliquots of the purified tetrasaccharides were chromato-

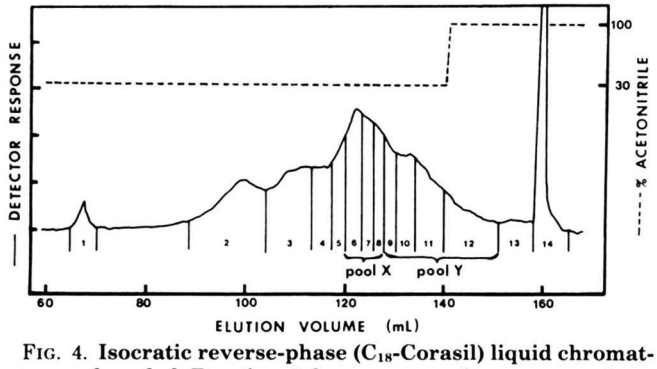


FIG. 4. **Isocratic reverse-phase (C_{18}-Corasil) liquid chromatogram of pooled *Fraction 5* from preparative reverse-phase liquid chromatography (Fig. 3).** The column was eluted with acetonitrile/water (3:7) isocratically until *Fraction 12* was obtained (150 ml). *Fractions 13* and *14* were eluted with 100% acetonitrile. The flow rate was 2 ml/min. For chromatography details, see "Experimental Procedures." Fractions pooled were indicated by *numbers* below the chromatogram. *Fractions 6–8* were combined into *Pool X*. *Fractions 9–12* were combined into *Pool Y*.

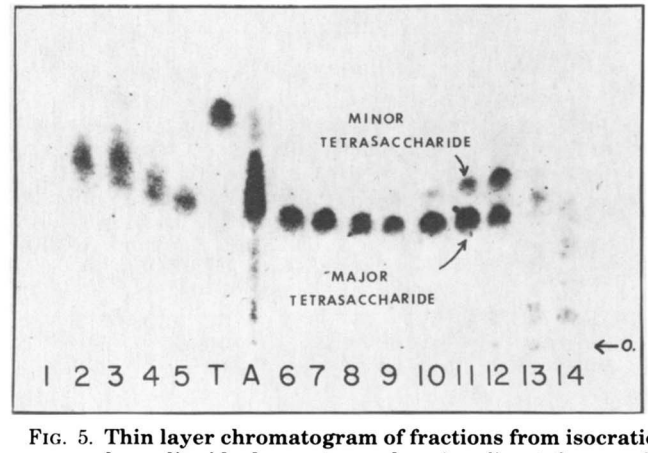


FIG. 5. **Thin layer chromatogram of fractions from isocratic reverse-phase liquid chromatography.** An aliquot from each pooled fraction was spotted and labeled by numbers from *1* through *14* (see Fig. 4). *Lane T* was an aliquot of the carboxy-reduced and peracetylated PSL I trisaccharide. *Lane A* was an aliquot of the total sample applied to the column. The *arrow* points to the origin. Chloroform/methanol (40:1) was used as developing solvent. Sugar spots were visualized by orcinol/H_2SO_4 spray as described under "Experimental Procedures." Gradual increase in R_F values observed in samples chromatographed on lanes toward both sides of the TLC plate was due to solvent edge effect.

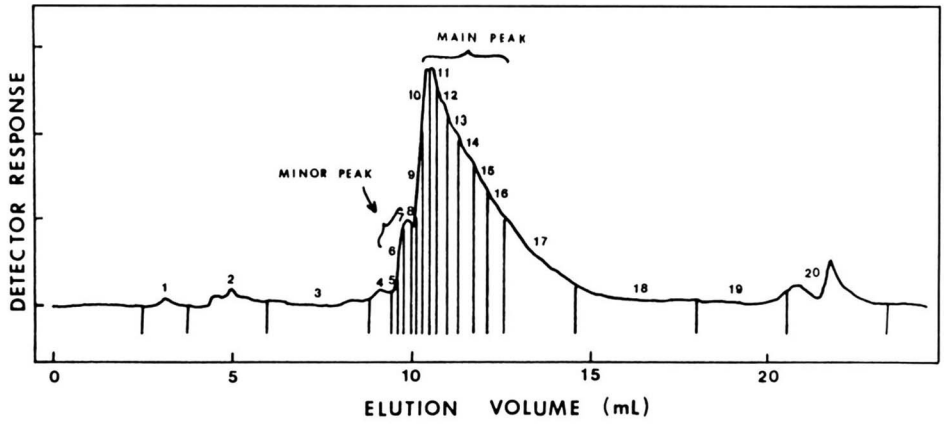


FIG. 6. **Normal-phase liquid chromatogram of the tetrasaccharide fraction (*Pool Y*, Fig. 4) on a μPorasil column.** Fractions pooled are indicated by *vertical lines* below the chromatogram and *numbers* above. Chromatography of *Pool X* under identical conditions gave a similar broad peak except the minor peak (*Fractions 5–7*) was absent (not shown). See "Experimental Procedures" for details.

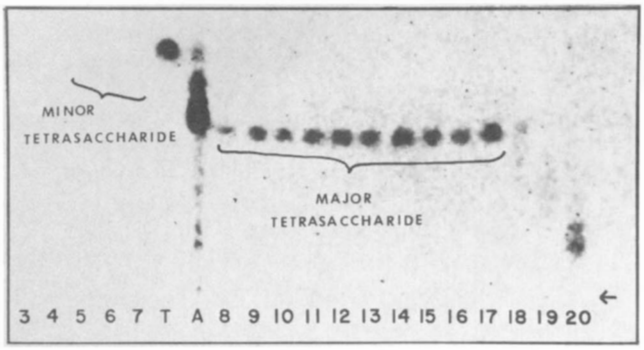


FIG. 7. **Thin layer chromatogram of μPorasil fractions of *Pool Y* (see Fig. 5).** An aliquot of 1 μl out of 100 μl of each μPorasil fraction was spotted on a silica gel plate. *Numbers* correspond to fraction numbers from the μPorasil column (Fig. 6). *Lane T,* an aliquot of carboxyl-reduced and peracetylated trisaccharide of PSL I. *Lane A,* an aliquot of *Fraction 5* from C_{18}-Corasil chromatography (Fig. 3). The *arrow* indicates the origin. Chloroform/methanol (40:1) was used for development and the sugar spots were visualized by orcinol/H_2SO_4 spray.

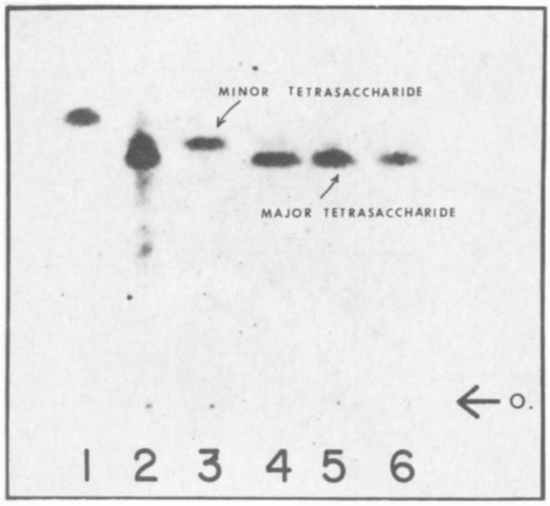


FIG. 8. **Thin layer chromatogram of pooled acetylated tetrasaccharide fractions on high efficiency silica gel (HETLC, Analtech, Inc.).** Origin is marked by *arrow*. The plate was developed in chloroform/methanol (35:1) twice. Sugar spots were visualized by orcinol/H_2SO_4 spray. *Lane 1,* PSL I trisaccharide; *Lane 2, Fraction 5* from C_{18}-Corasil (Fig. 3) chromatography; *Lane 3,* tetrasaccharide A; *Lanes 4–6,* tetrasaccharide B.

graphed on a high efficiency silica gel thin layer plate in chloroform/methanol (35:1). The chromatogram visualized by orcinol/H_2SO_4 spray is shown in Fig. 8. The minor tetrasaccharide, A, migrated as a single band in *Lane 3* (Fig. 8). The major tetrasaccharide, B, from various pooled fractions is shown in *Lanes 4–6* (Fig. 8). The R_F of the PSL I carboxyl-reduced and peracetylated trisaccharide (*Lane 1*) is higher than the R_F of peracetylated tetrasaccharide A (*Lane 3*), both being higher than that of tetrasaccharide B (*Lanes 4–6*) (see Fig. 8). The TLC suggests that tetrasaccharide B is the major component in Fraction 5 from the C_{18}-Corasil chromatograhy (Fig. 8, *Lane 2*) while tetrasaccharide A occurs in smaller amounts in the same C_{18}-Corasil fractions. Net weights (peracetylated) of these pooled fractions after solvent evaporation were as follows: 4.8 mg of minor tetrasaccharide A, 18.5 mg of major tetrasaccharide B.

Determination of Carbohydrate Composition and Anomeric Configuration of Tetrasaccharides A and B—Alditol and myoinositol acetates, prepared from the tetrasaccharides A and B before and after CrO_3 oxidation, were analyzed by gas chromatography. Molar quantities of each sugar were calculated per mol of myoinositol. Table II shows the data with the molar ratio and anomeric configurations assigned. The composition data suggest that the minor tetrasaccharide A contains equimolar amounts of Man, Glc, GlcNAc, and Inos, while the major tetrasaccharide B contains equimolar amounts of Gal, Glc, GlcNAc, and Inos. The assumption that these components are tetrasaccharides rests on their retention on a reversed phase column (14) and on the fact that glycosidic assembly of the four components can only lead to a tetrasaccharide.

According to the studies on Angyal and James (23), Hoffman *et al.* (15), and Laine and Renkonen (16), treatment of peracetylated hexopyranosides with CrO_3 in acetic acid, in which the aglycone occupies an equatorial position, gives acetylated 5-hexulosonates. The corresponding anomers in which the aglycone occupies an axial position are oxidized only slowly. Thus, the β-glycosidic D-hexopyranoside units of the carbohydrate chain should be oxidized, whereas α-glycosidic units should not. The corresponding α- and β-furanosides are readily oxidized, yielding 4-hexulosonates.

As shown in Table II, all the sugar residues in tetrasaccharide A survived CrO_3 treatment and, hence, all three glycosidic linkages were assigned the α configuration. In tetrasaccharide B, both Glc and GlcNAc survived more than 80% and were also assigned the α configuration, whereas the galactosidic linkage was assigned β configuration since only 29% of Gal survived the CrO_3 test. The latter value was a little higher than expected for an ideally selective oxidation; however, Laine and Renkonen (16) observed similar resistance of β-galactose units.

Methylation Linkage Analysis of the Major Tetrasaccharide—Tetrasaccharide B (*Fraction 14*, Figs. 6 and 7) was processed for linkage analysis as described under "Experimental Procedures." Fig. 9 shows the total ion chromatograms obtained under identical EI GC/MS conditions from the partially methylated alditol acetates of the following samples: *I,* μPorasil Fraction 14 (Fig. 6) (major tetrasaccharide B); *II,* the carboxyl-reduced trisaccharide of PSL I (GalNAc(α1 → 4)Glc(α1 → 2)inositol); *III,* lactose; and *IV,* galactinol (Gal(α1 → 1)inositol). EI mass spectra from the four major peaks in the gas chromatogram represented by Fig. 9*I* were identical with those from authentic standards (not shown). *Peak C* (Fig. 9*I*) was assigned as 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetylgalactitol from its mass spectrum. This indicates the presence of a terminal galactose in the major tetrasaccharide.

TABLE II

Determination of carbohydrate compositions and anomeric configurations of tetrasaccharides A and B

	Moles per mol of myoinositol detected				
	Man	Gal	Glc	GlcNAc	Inos
Tetrasaccharide A					
Before CrO_3	0.67		0.81	0.69	1.00
After CrO_3	0.65		0.86	0.69	1.00
% survival	97%		106%	100%	100%
Molar composition	1		1	1	1
Anomeric configuration	α		α	α	
Tetrasaccharide B					
Before CrO_3		0.94	0.91	0.68	1.00
After CrO_3		0.27	0.74	0.60	1.00
% survival		29%	81%	88%	100%
Molar composition		1	1	1	1
Anomeric configuration		β	α	α	

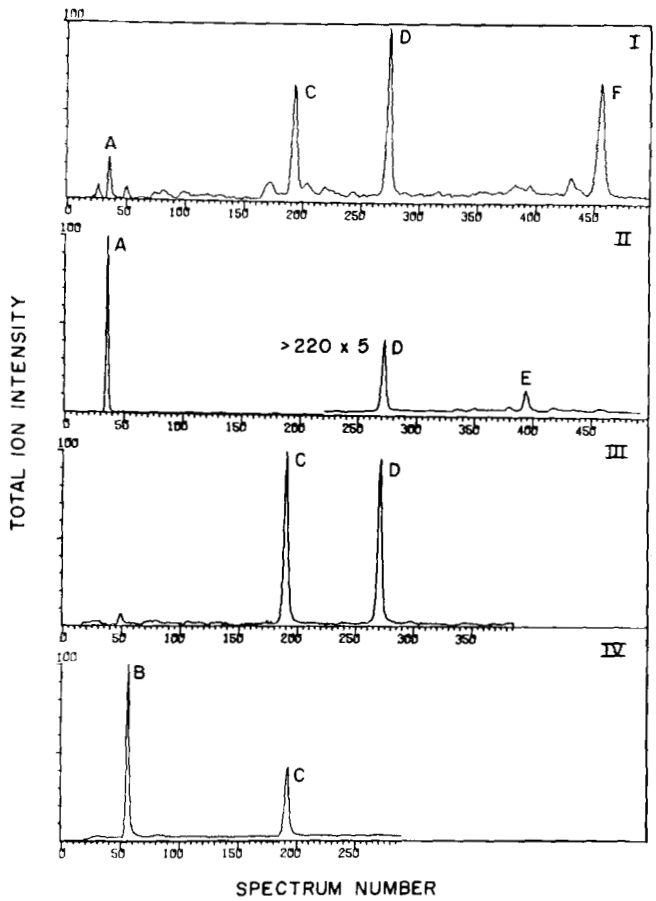


FIG. 9. **Total ion chromatograms of partially methylated alditol acetates of: *I*, μPorasil *Fraction 14* (Fig. 6); *II*, PSL I carboxyl-reduced trisaccharide; *III*, lactose; *IV*, galactinol.** Each of the partially methylated alditol acetates mixture was chromatographed on a glass column (150 cm × 2 mm inside diameter) packed with 3% OV-210 (Supelco, Inc.). Flow rate of the helium carrier gas was 30 min/min. Column temperature was programmed from 150–210 °C at 6 °C/min. Separator and transfer line temperatures were 220 °C. MS analyzer temperature was 60 °C. Electron-impact ion source parameters were: lens, 120 V; collector, 35 V; extractor, 2.7 V; ion energy, 2.5 eV (programmed); electron energy, 70 eV; ion volume, 0.54 mA; emission current, 0.60 mA. Mass spectral scans of GC effluent were recorded at a rate of 2 s/spectrum from m/z 40 to m/z 250. GC retention time in seconds can be calculated by multiplying the spectrum number (*abscissa*) by 2.

Peak C in Fig. 9*I* had the same GC retention time of 6.4 min and the same mass spectrum as those of non-reducing terminal galactose (*Peak C*) from lactose in Fig. 9*III*, and terminal galactose (*Peak C*) from galactinol (Fig. 9*IV*).

Peak D in Fig. 9*I* was assigned as 2,5,6-tri-*O*-methyl-2,4,5-tri-*O*-acetyl-glucitol indicating the presence of a 4-linked glucose in the tetrasaccharide from μPorasil Fraction 14 (Fig. 6). This peak (Fig. 9*I*) had the same GC retention time of 9.1 min as those of *Peak D* from the PSL I carboxyl-reduced trisaccharide (4-linked glucose) (Fig. 9*II*) and *Peak D* from lactose (4-linked glucose) (Fig. 9*III*). In addition, the EI spectrum showed features characteristic for the assignment of a 4-linked glucose.

Peak F in Fig. 9*I*, identified as 3,6-di-*O*-methyl-1,4,5-tri-*O*-acetyl-2-deoxy-*N*-methylacetamidoglucitol, arises from a 4-linked GlcNAc in the tetrasaccharide of μPorasil Fraction 14 (Fig. 6).

Peak A in Fig. 9*I* was postulated to be 1,3,4,5,6-penta-*O*-methyl-2-*O*-acetylmyoinositol derived from a 2-linked myoinositol. This peak had the same retention time (1.2 min) as *Peak A* in Fig. 9*II*, which was derived from the 2-linked myoinositol in the PSL I trisaccharide as characterized by periodate oxidation experiments (5). The 1-*O*-acetyl isomer from galactinol had a different retention time of 1.9 min under these conditions, shown as *Peak B* in Fig. 9*IV*.

These methylation analyses indicated that the oligosaccharide in μPorasil Fraction 14 (Fig. 6, the major tetrasaccharide, or tetrasaccharide B) contained the following types of sugar linkages: terminal Gal, 4-linked GlcNAc, 4-linked Glc, and 2-linked myoinositol.

In a separate experiment a partially methylated alditol acetate sample prepared from the gradient Partisil-ODS Fraction C (Fig. 2) contained a trisaccharide which was the most abundant oligosaccharide in the oligosaccharide concentrate (Fig. 9*II*). This result indicated that the major trisaccharide (Partisil ODS, Fraction D) shared the same structure as the PSL I carboxyl-reduced trisaccharide GlcNAc*p*(α1 → 4)Glc*p*(α1 → 2)Inos, as already reported (5).

Methylation Linkage Analyses of the Major Tetrasaccharide and the Minor Tetrasaccharide by CI Mass Fragmentography—To verify the linkage analyses of tetrasaccharide B (μPorasil Fraction 14) and to obtain linkage information on the small amount of tetrasaccharide A isolated, a more sensitive approach, chemical ionization mass fragmentography, was used to analyze the partially methylated alditol acetates prepared from tetrasaccharides A and B.

The distribution of the ion fragment at m/z 201 (*MH*-acetic acid-methanol, M = 292) which is a characteristic ion for

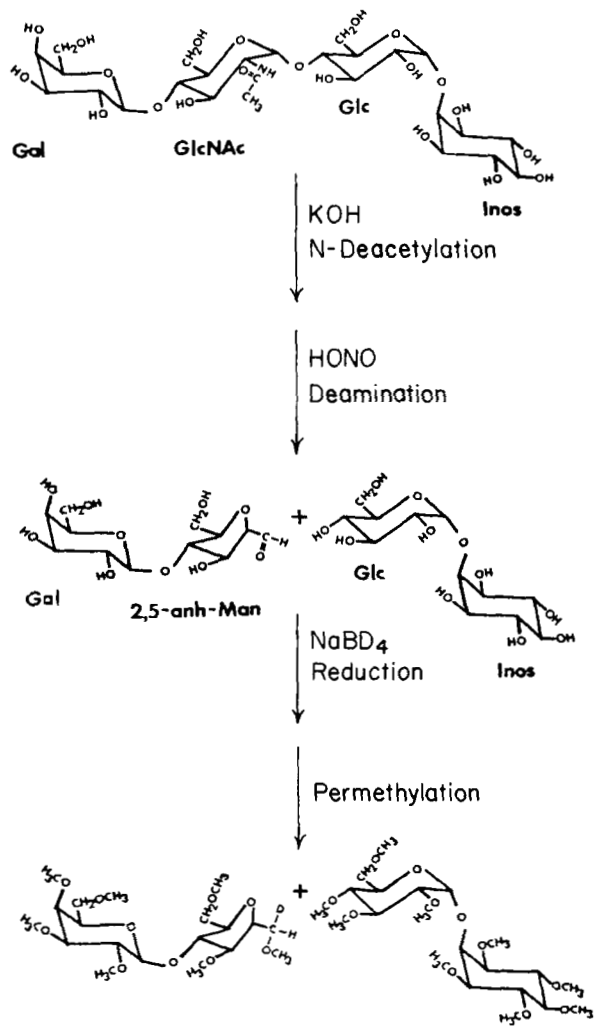


FIG. 10. **Scheme for deaminative cleavage of tetrasaccharide B, followed by reduction of the products.**

penta-*O*-methyl-mono-*O*-acetylmyoinositol gave a gas-chromatographic peak (not shown) derived from a monosubstituted myoinositol in the tetrasaccharide (5). Neutral losses of acetic acid and methanol from the ions are common in fragmentation under the methane CI MS conditions used. Another peak showed the distribution of an ion at m/z 264 (*MH*-acetic acid, M = 323) which is characteristic for tetra-*O*-methyl-di-*O*-acetyl-1-*deutero*-galactitol derived from a terminal Gal in the tetrasaccharide. A third peak showed the profile of ions at m/z 292 (*MH*-acetic acid, M = 351) which is characteristic for tri-*O*-methyl-tri-*O*-acetyl-1-*deutero*-glucitol derived from a 4-linked Glc in the tetrasaccharide. A final peak showed the distribution of an ion at m/z 393 (*MH*, M = 392) which is characteristic for di-*O*-methyl-tri-*O*-acetyl-2-*N*-methyl-2-acetamido-2-deoxy-1-*deutero*-glucitol derived from a 4-linked GlcNAc in the tetrasaccharide. The above data from CI mass fragmentography is consistent with the data from EI GC/MS for the major tetrasaccharide B. Scans of appropriate ions showed the absence of branching structures in oligosaccharide B.

Another CI mass fragmentogram of PMAA prepared from tetrasaccharide A gave a peak showing the distribution of an ion at m/z 264 (*MH*-acetic acid, M = 323) which is a characteristic ion for tetra-*O*-methyl-di-*O*-acetyl-1-*deutero*-mannitol derived from a terminal Man. A second peak showed the distribution of an ion at m/z 292 (*MH*-acetic acid, M = 351) which is a characteristic ion for tri-*O*-methyl-tri-*O*-acetyl-1-*deutero*-glucitol in this case derived from a 4-linked glucose. Because of the terminal GlcNAc, 4-linked Glc, and terminal Man, a branched (or disubstituted) structure, was proposed for the myoinositol residue in tetrasaccharide A. A well defined peak for the myoinositol derivative was not apparent because of the small amount of material available. A partial structure is proposed as follows for the minor tetrasaccharide: GlcNAc*p*(α1 → 4)Glc*p*(α1 → ?)Inos(? ← 1α)Man.

Carbohydrate Sequence Determination of the Major Tetrasaccharide B—The sugar composition and the methylation data suggest the following two possible sequences since the mono-substituted myoinositol (*e.g.* 2-linked myoinositol) must be at the "reducing end" of the saccharide chain and a terminal galactose must be at the nonreducing end:

$$\text{Gal}p(\beta1 \rightarrow 4)\text{GlcNAc}p(\alpha1 \rightarrow 4)\text{Glc}p(\alpha1 \rightarrow 2)\text{Inos} \quad \text{(I)}$$

$$\text{Gal}p(\beta1 \rightarrow 4)\text{Glc}p(\alpha(1 \rightarrow 4)\text{GlcNAc}p(\alpha1 \rightarrow 2)\text{Inos}. \quad \text{(II)}$$

Sequence I was proved to be correct for this major tetrasaccharide by nitrous acid deamination experiments. Deamination of the glucosamine residue specifically cleaves the glucosaminic linkage in the tetrasaccharide, which would give a mixture of Gal(1 → 4)2,5-anhydromannitol and Glc(1 → 2)Inos, if the sequence were Gal(1 → 4)GlcNAc(1 → 4)Glc(1 → 2)Inos, or a mixture of Gal(1 → 4)Glc(1 → 4)2,5-anhydromannitol and myoinositol, if the sequence were Gal(1 → 4)Glc(1 → 4)GlcNAc(1 → 2)Inos.

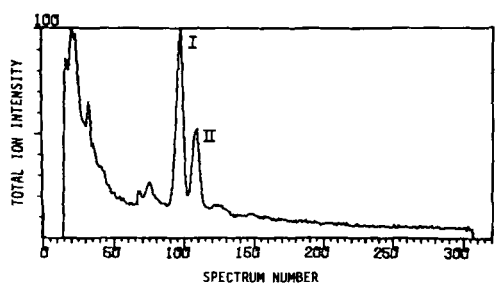


FIG. 11. **Carbohydrate sequence determination of the major tetrasaccharide by CI GC/MS of deamination products.** Total ion chromatogram of reduced and permethylated products of deaminative cleavage of *N*-deacylated tetrasaccharide B. *Peak I* is permethylated Glc*p*(1 → 2)inositol and *Peak II* is permethylated Gal*p*(1-4)2,5-anhydromannitol. GC/MS conditions: column, 150 × 0.2 cm packed with 3% OV on Supelcoport 100/120 with methane as a carrier and reagent gas supplying 1 Torr pressure in the ion source; column temperature, 190 °C; injector and transfer lines, 225 °C; ionizing electron energy, 150 eV; ion source temperature, 60 °C; scan rate, 4.2 s/complete spectrum.

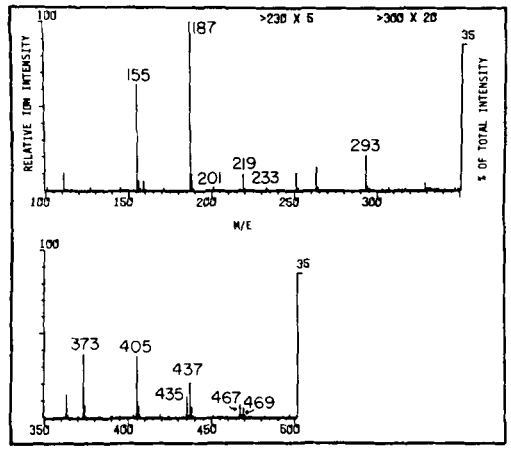
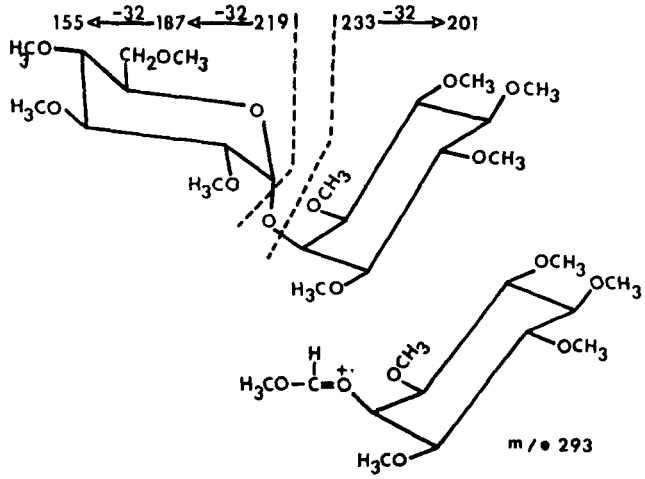


FIG. 12. **Methane chemical ionization mass spectrum of *Peak I* in Fig. 11.** Conditions were as described in the legend to Fig. 11.



SCHEME 1

The peracetylated major tetrasaccharide was *O*- and *N*-deacylated by KOH treatment. After desalting, the sample was deaminated in acetic acid with nitrous acid. Since 2,5-anhydromannitol is expected to be at the reducing end of one of the oligosaccharide products, the sample was reduced with $NaBD_4$ to give 1-*deutero*-2,5-anhydromannitol. The products were then permethylated by the method of Hakomori (18) and examined by methane CI GC/MS. The scheme for this procedure is shown in Fig. 10.

Fig. 11 shows the total ion gas chromatogram of the reduced and permethylated products from deaminative cleavage of the major tetrasaccharide B showing two major products: *I* and *II*. Fig. 12 is the mass spectrum of *Peak I* in Fig. 11.

The expected fragmentation pattern (Scheme 1) of permethylated Glc*p*(1 → 2)Inos, gives an assignment of all the prominent ions observed in the spectrum. Ions at m/z 219, 187, and 155 are probably fragments of the A ring (glucose), while ions at m/z 233 and 201 are from the B ring (myoinositol). The molecular weight is 468 and both *MH* (m/z 469) and *M*-1 (m/z 467) were observed. Serial losses of methanol from *M*-1, *M*, and *MH* gave ions at m/z 435, 436, 437, 405, and 373. The ion at m/z 293 is probably a fragment from the B ring

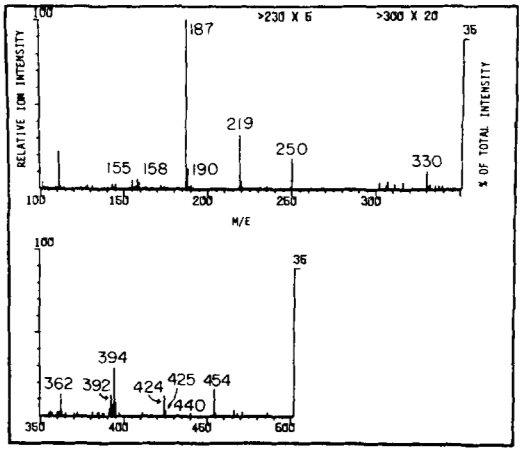
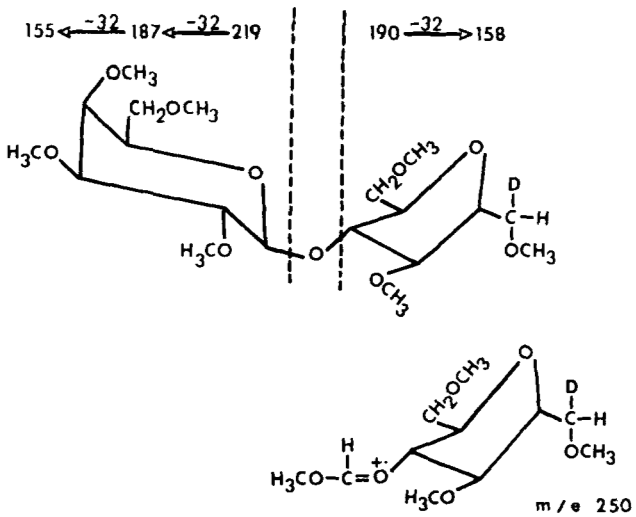


FIG. 13. **Methane chemical ionization mass spectrum of *Peak II* in Fig. 11.** *Permethylated Gal(β1 → 4)2,5-anhydromannitol; instrumental conditions are described in the legend to Fig. 11.*



SCHEME 2

plus the C-1 from the A ring with an additional *O*-methyl group gained from C-2 or C-3 of the A ring through a rearrangement (24, 25). In comparison with the spectrum of permethylated galactinol obtained under identical conditions, all the same prominent ions were present. Observation of ions at *m/z* 293 have also been reported in spectra of permethylated disaccharides containing myoinositol by Naccarato, *et al.* (26) and by Lennartson, *et al.* (27).

Fig. 13 shows the mass spectrum of *Peak II* (Fig. 11).

The fragmentation pattern in Scheme 2 shows permethylated Gal*p*(1 → 4)2,5-anhydromannitol with deuterium at the C-1 position of 2,5-anhydromannitol. All of the prominent ion fragments were interpreted as follows. Ions at *m/z* 219, 187, and 155 are from the A ring (galactose), while the ions at *m/z* 190 and 158 are from the B ring (1-*deutero*-2,5-anhydromannitol). The molecular weight is 425 and both *M* and *M*-1 (*m/z* 424) were detected. The ion at *m/z* 454 corresponds to *M* + C_2H_5. Serial losses of methanol from *M*-1, *M*, and *MH* gave prominent ions at *m/z* 392, 394, 362, and 330. The ion at *m/z* 250 is probably formed (analogous to that of *m/z* 293 in Fig. 9) from the B ring plus the C-1 of the A ring with an additional *O*-methyl group gained from C-2 or C-3 of the A ring through a rearrangement (24, 25) analogous to the rationale for the glucosyl-myoinositol.

In conclusion, the CI GC/MS determination of the two methylated disaccharides, hexosyl-myoinositol and hexosyl-2,5-anhydromannitol, as the major deamination degradation products indicated that the carbohydrate sequence of tetrasaccharide B was Gal*p*(β1 → 4)GlcNAc*p*(α1 → 4)Glc*p*(α1 → 2)Inos.

DISCUSSION

Judging from the closely related structures of the trisaccharide portions of the major glycophosphoceramides. PSL I (GlcNAc:GlcUA:Inos, 1:1:1) and PSL II (GlcNH2:GlcUA:Inos, 1:1:1), to those of the major tri- and tetrasaccharides (GlcNAc:Glc:Inos, 1:1:1; Gal:GlcNAc:Glc:Inos, 1:1:1:1) isolated from the carboxyl-reduced glycophosphoceramide concentrate, as well as the constant presence of glucosamine, glucose, and myoinositol in all the other HPLC fractions (Partisil-ODS or C_{18}-Corasil fractions), the glycophosphoceramide members in this concentrate probably represent a series of complex lipids containing a core trisaccharide moiety, GlcNAc*p* (or GlcNH2*p*) (α1 → 4)GlcUA*p*(α1 → 2)Inos, with additional arabinose, galactose, or mannose attached to the trisaccharide. As the complexity increases, there is concurrent decrease in abundance of the higher oligomers.

The very small amount of glucose (hexuronic acid/glucose, 1:0.03) present before carboxyl reduction indicated that the glucose residues in the oligosaccharides obtained from the carboxyl-reduced concentrate came from glucuronic acid-containing glycophosphoceramide. An entire series of acidic glycolipids is thus present in tobacco leaves, analogous in complexity to the gangliosides of animal tissue.

It should be noted that the glucosamine and glucuronic acid residues in PSL I and PSL II, as well as the glucosamine and glucose residues in the major tri- and tetrasaccharides isolated from the concentrate, have α anomeric configuration. The reported structure of "phytoglycolipid" by Carter and coworkers had this feature also (4). This situation is unlike that found in animal glycosphingolipids where *N*-acetylglucosamine is almost always in the β configuration.

High pressure liquid chromatography played a critically important role in achieving separation and isolation of the major members in this closely related complex mixture. Both normal-phase and reverse-phase columns were employed under various combinations of solvent conditions to separate the peracetylated oligosaccharides (14).

This study has demonstrated the application of the combination of HPLC and GC/MS in isolation and characterization of closely related oligosaccharides in a complex mixture and points to a feasible route to the future examination of the more complex members in the plant glycophosphoceramide family. This structural biochemistry shall hopefully lay ground work for future studies of the function of these ubiquitous plant-derived glycolipids.

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