References

1. Martin-Lomas M.; Chapman D. Chem. Phys. Lipids 10, 152 (1973).
2. Falk, K. E.; Karlsson, K. A.; Samuelsson, B. E. Arch. Biochem. Biophys. 192, 164 (1979); ibid. 191, 177 (1979); ibid. 192, 191 (1979).
3. Dabrowski, J.; Egge, H.; Hanfland P. Chem. Phys. Lipids, submitted.
4. Hanfland, P.; Egge, H.; Dabrowski, J.; Roelke, D., in Glycoconjugates, R. Schauer, P. Boer, E. Buddeke, M. F. Kramer, J. F. G. Vliegenthart, A. Wiegandt eds., J. Thieme Verlag Stuttgart 520 (1979).
5. Dorland, L.; Haverkamp, J.; Vliegenthart, J. F. G.; Strecker, G.; Michalski, J. -C.; Fournet, B.; Spik, G.; Montreuil, J. Eur. J. Biochem. 87, 323 (1978).
6. De Bruyn, A.; van Beeumen, J.; Anteunis A.; Verhegge, G. Bull. Soc. Chim. Belg. 84, 799 (1975); A. De Bruyn (1979) Dissertation, Rijksuniversiteit Gent, Belgium.

RECEIVED December 10, 1979.

5

Glycophosphoceramides from Plants

ROGER A. LAINE, THOMAS C.-Y. HSIEH[1], and ROBERT L. LESTER

Department of Biochemistry, University of Kentucky College of Medicine, Lexington, KY 40536

Glycophosphoceramides contain a phosphodiester linkage between the carbohydrate moiety and the ceramide. They occur in plants and fungi (1,2,3) and have not been reported in animals. These negatively charged as well as ubiquitous glycophosphoceramides in plants may be analogous to, and rival in complexity, the sialic acid-containing glycosphingolipids in animal cell membranes, which have not been reported to occur in plants.

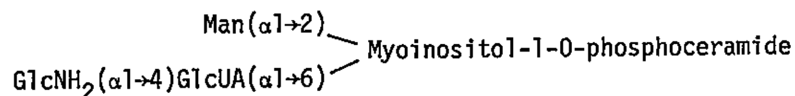
Carter and his co-workers (1) reported the preparation of major phytosphingosine-containing glycolipids from soybean, corn, flaxseed, peanut, sunflower seed, cotton seed, and wheat phospholipids. These materials were obtained by an alkaline saponification procedure (1 N KOH at 37°C for 24 h) which was designed to hydrolyze the esters of glycerol-containing lipids. They reported that these materials, comprising about 5% of the total crude phospholipids, were obtained as white amorphous powders of similar composition, optical activity, and solubility properties from various plant sources, and were named "phytoglycolipids". Composition analyses of these substances indicated the presence of phytosphingosine, fatty acids, phosphate, inositol, glucosamine, hexuronic acid, galactose, arabinose, and mannose. A preparation of oligosaccharides from corn phytoglycolipids (4) was obtained by barium hydroxide treatment, which presumably would not hydrolyse the glycosidic linkages of the oligosaccharide chain. The first indication of the heterogeneity of Carter's oligosaccharide preparation was provided by paper chromatography (4). They reported that all efforts to obtain separate discrete spots from the sample failed. However, partial fractionation was achieved on carbon-Celite columns eluted with increasing concentrations of aqueous ethanol. Further separation was obtained by anion ex-

[1]Current address: R & D, Brown & Williamson Tobacco Corp., 1600 W. Hill St., Louisville, KY 40232

change chromatography. These workers concluded that the oligosaccharide mixture obtained by alkaline hydrolysis of the "purified" corn phytoglycolipids had the following approximate distribution:

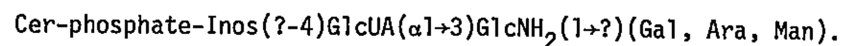
Fraction		%
A	$GlcNH_2$-GlcUA-Inositol	9
B	Tetrasaccharide	41
C	Pentasaccharide	10
D	Hexasaccharide	10
E	Heptasaccharide	14
F	Octa- and higher oligosaccharides	8

The complete structure of a tetrasaccharide, the major oligosaccharide of corn phytoglycolipids, was reported by Carter, et al. (5). The N-acetylated carboxyl-reduced tetrasaccharide was oxidized by periodate and the products were reduced with sodium borohydride and then hydrolyzed with acid. Isolation of D-arabitol as one of the polyol products showed that in the tetrasaccharide the inositol was 2,6-disubstituted. Proton magnetic resonance studies on the derived glycosylinositol and N-acetylated carboxyl-reduced trisaccharide suggested that glucuronic acid moiety was attached to the C-6 position of inositol. The mannose, therefore, according to these workers was attached to the C-2 position of inositol in the tetrasaccharide (5). All -α anomeric configurations were also deduced from proton magnetic resonance spectra. Although the intact phytoglycolipid preparation was a mixture of members with varying carbohydrate chain lengths, they carried out another periodate experiment on this mixture. The major polyol isolated was a tetritol fraction which was shown by paper chromatography to be a mixture of erythritol-threitol (8:1). D-arabitol was isolated (tetritol:pentitol, approximately 11:1) and a small amount of hexitol was also isolated. The weight of evidence suggested to these workers that in mild acid hydrolysis (reflux in 2 N formic acid for 3 h) of phosphorylated oligosaccharide (from corn and flax), inostiol-1-phosphate was detected as the major product. Thus these workers proposed the complete structure of the major member of phytoglycolipids from corn seeds as follows.



No further work was reported on characterization of the more complex members in this series of phytoglycolipids from plants.

Wagner, et al. (6) reported to have isolated from peanuts a phytoglycolipid-like material for which a tentative structure was proposed as follows:



Carter and Koob (70 isolated a phytoglycolipid fraction from bean leaves (*Phaseolus vulgaris*). They extracted these glycophosphoceramides by refluxing in hot 70% ethanol (0.1 N in HCl) for 20 min. This acidic extraction procedure may have caused partial breakdown of these complex compounds. Wagner, et al. (8) reported isolation of a glycophosphoceramide similar to phytoglycolipids from the green alga *Scenedesmus obliquus*, but the only carbohydrates detected were glucose and glucuronic acid. This was also the first indication that algae synthesize sphingosine or sphingolipids. Carter and Kisic (9) reported partial characterization of another class of glycophosphoceramides from crude inositol lipids of plant seeds, which was related to corn phytoglycolipids but contained no amino sugars.

Kaul and Lester (3) developed a mild extraction procedure to obtain a crude concentrate of glycophosphoceramides from fresh mature tobacco leaves. Thin layer chromatography of this concentrate indicated the presence of a dozen or more polar lipids containing inositol, phosphate, and carbohydrate. Two of the major members were purified by chromatography on porous silica gel beads and partially characterized as GlcNac-GlcUA-Inositol-phosphoceramide (termed PSL-I) and $GlcNH_2$-GlcUA-Inositol-phosphoceramide (termed PSL-II). Although not fully characterized, the other members in the concentrate were reported to be inositol-containing glycophosphoceramides with a higher carbohydrate content (10). The reported amount of glycophosphoceramide concentrate (about 100 μmol per Kg fresh weight) was of comparable magnitude to the estimate of phytoglycolipids present in the crude extract from bean leaves (0.1% of dry weight) (7) with leaf moisture taken into consideration. The proposed structure of PSL-I and PSL-II, as well as the properties of the other glycophosphoceramides in the tobacco leaf concentrate indicated their close similarity to the phytoglycolipids studied by Carter and his group. Kaul and Lester (3) reported that the trisaccharide-containing PSL-I and PSL-II constituted a total of approximately 40% of the tobacco glycophosphoceramides, in contrast to the report by Carter, et al. (4) that trisaccharides constituted only about 9% of corn phytoglycolipid.

PSL-I

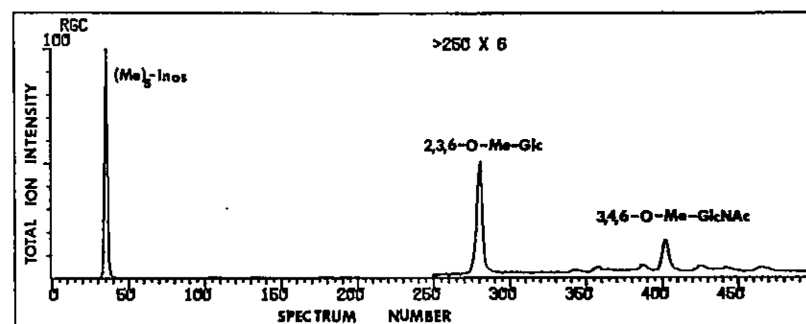
Kaul and Lester (10) reported the preparation of six novel glycophosphoceramide fractions from the above crude concentrate from tobacco leaves. The crude concentrate was first resolved into two groups by column chromatography on diethylaminoethyl-cellulose. The first group contained no acetyl residues, whereas the second group contained one N-acetyl per phosphorus. Three lipid fractions from each group were further resolved by chromatography on Porasil columns. The chemical composition and the percent of the total P in the crude concentrate of these lipid fractions obtained are as follows:

PSL-IA:	PSL-I-$(Ara)_2(Gal)_2$	0.96%
PSL-IB:	PSL-I-$(Ara)_3(Gal)_2$	0.27%
PSL-IC:	PSL-I-$(Ara)_4(Gal)_2$	1.56%
PSL-IIA:	PSL-II-$(Ara)_3$Gal	0.75%
PSL-IIB:	PSL-II-$(Ara)_{2-3}(Gal)_2$	3.90%
PSL-IIC:	PSL-II-$(Ara)_2(Gal)_2$Man	0.85%

Apparently, these glycophosphoceramide fractions were related to but much less abundant than the major members PSL-I and PSL-II in the concentrate.

PSL-I: The Major Glycophosphoceramide from Tobacco Leaves

For characterization of PSL-I, the major glycophosphoceramide previously isolated from the tobacco glycophosphoceramide concentrate by Kaul and Lester (3), the carboxyl-reduced (11) trisaccharide moiety was first obtained by alkaline degradation of the carboxyl-reduced PSL-I, followed by alkaline phosphatase treatment on the resulting trisaccharide and phospho-trisaccharide mixture (12). Methylation linkage analyses (13,14,15,16) were performed on the trisaccharide by combined gas chromatography/mass spectrometry in both electron-impact and chemical ionization modes (17, 18) and the data (Figure 1) suggested a partial structure GlcNAcp(1→4)Glcp(1→?)Inos for the carboxyl-reduced PSL-I trisaccharide (12). Carbohydrate composition and CrO_3 oxidation products for anomeric configuration on the trisaccharide were analyzed by gas chromatography (19,20,21). The data suggested the structure GlcNAcp(α 1→4)Glcp(α 1→?)Inos for the PSL-I carboxyl-reduced trisaccharide. Periodate oxidation experiments to determine the linkage between glucuronic acid and myoinositol were carried out on the intact PSL-I (12). The phospho-alcohol product from myoinositol was separated from other products by anion exchange chromatography and the final derivative examined by chemical ionization mode of gas chromatography/mass spectrometry was shown to be erythritol, indicating that the glucuronic acid was attached to the C-2 position of the myoinositol ring (Figures 2,3a,3b). This completed the characterization of PSL-I as



PSL-I Carboxyl-Reduced Trisaccharide:

GlcNAcp(1→4)Glcp(1→?)Inos

Figure 1. Methylation linkage analysis of PSL-I by GC/MS: total ion chromatogram of partially methylated alditol and myoinositol acetates (PMAA) from PSL-I carboxyl-reduced trisaccharide by gas chromatography/mass spectrometry in electron-impact mode.

Peaks identified: penta-O-methyl-mono-O-acetylmyoinositol derived from mono-linked myoinositol, 2,3,6-tri-O-methyl-1,4,5-tri-O-acetylglucitol derived from a 4-linked glucose, and 3,4,6-tri-O-methyl-1,5,di-O-acetyl-2-acetamido-2-N-methylglucitol derived from a terminal N-acetylglucosamine. The PMAA sample was chromatographed on a 1.5 m × 2 mm ID column packed with 3% OV-210 in a Finnigan automated GC/MS model 3300/6110. Temperature program: 150° to 215°C at 6°C/min.

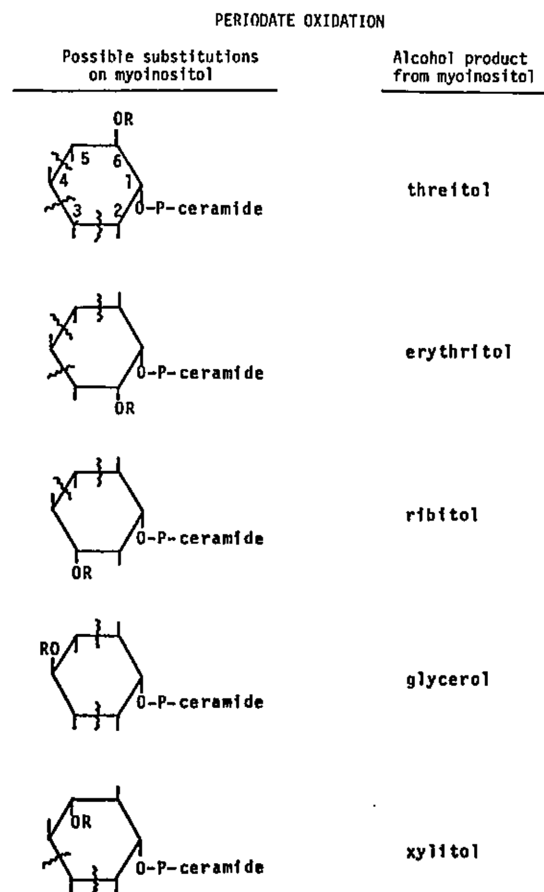


Figure 2. Possible substitutions on myoinositol by glucuronic acid. Shown are the bonds susceptible to periodate oxidation (wavy lines) and the predicted corresponding final myoinositol-derived alcohol products after periodate oxidation, followed by $NaBD_4$ reduction, hydrolysis, anion exchange chromatography and dephosphorylation (12).

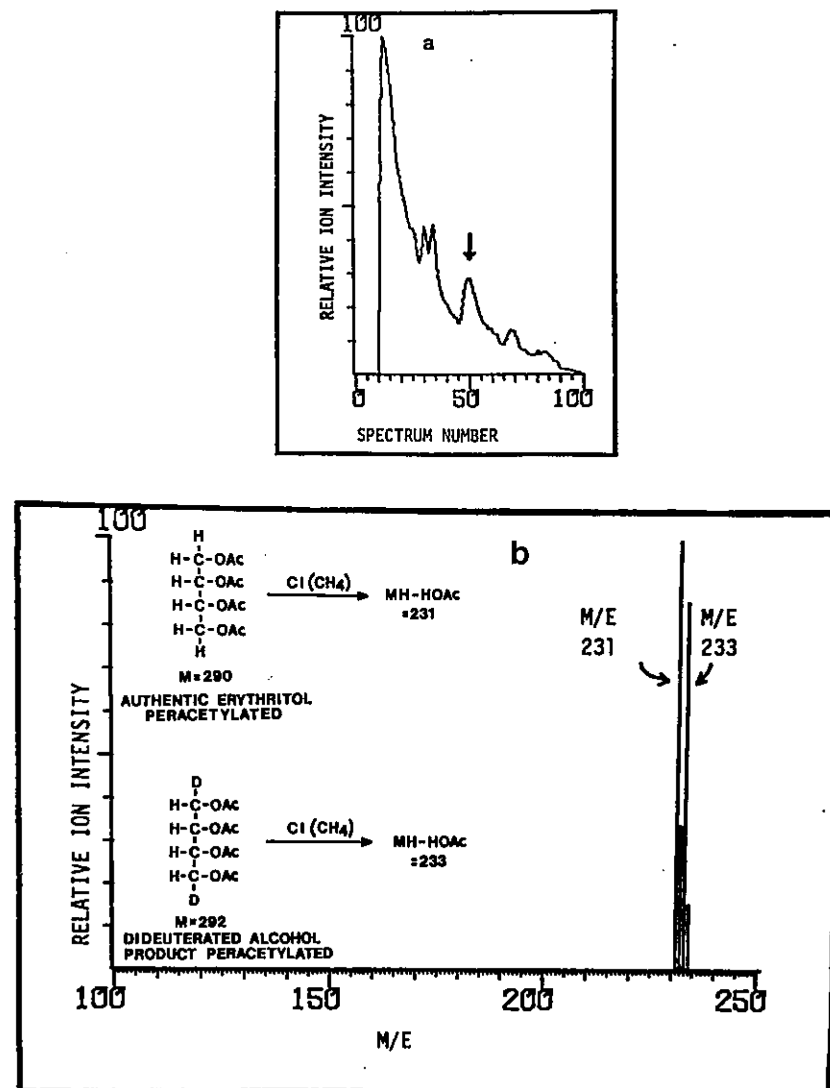


Figure 3. Chemical ionization (methane) GC/MS of the acetylated final product derived from periodate oxidation of the myoinositol ring in PSL-I. (a): Total ion chromatogram of co-injected mixture of the unknown dideuterated alcohol product and the authentic erythritol. (b): Chemical ionization spectrum of peak indicated by an arrow in (a). Inset diagrams depict the fragmentation.

GlcNAcp(α1→4)GlcUAp(α1→2)Inos-1-O-phosphoceramide (Figure 4)(12).

Major Oligosaccharides Prepared from the Carboxyl-Reduced Concentrate of Glycophosphoceramides of Tobacco Leaves

For the remaining components in the concentrate, Hsieh (22) prepared a mixture of oligosaccharides from the carboxyl-reduced (23) glycophosphoceramide concentrate. A large number of chromatographic conditions were examined for optimal fractionation. A series of closely related oligosaccharides with increasing complexity and in decreasing abundance were observed on reverse-phase high pressure liquid chromatography as the peracetylated derivatives [procedure adapted from those of Wells and Lester (24)]. Combinations of both reverse-phase and normal-phase columns were used under various solvent conditions to achieve isolation of the major oligosaccharides.

Methylation Analyses

Methylation linkage analysis of the partially methylated alditol acetates gave the following derivatives:

Major trisaccharide:

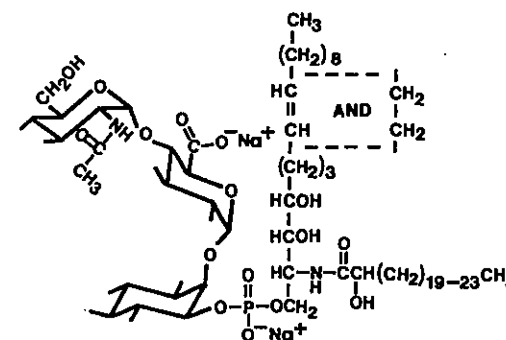
3,4,6-tri-O-methyl-2-deoxy-2-methylaminoglucitol
2,3,6-tri-O-methylglucitol
1,3,4,5,6-penta-O-methylinositol

Major tetrasaccharide: (Figure 5)

2,3,4,6-tetra-O-methylgalactitol
3,6-di-O-methyl-2-deoxy-2-methylaminoglucitol
2,3,6-tri-O-methylglucitol
1,3,4,5,6-penta-O-methylinositol

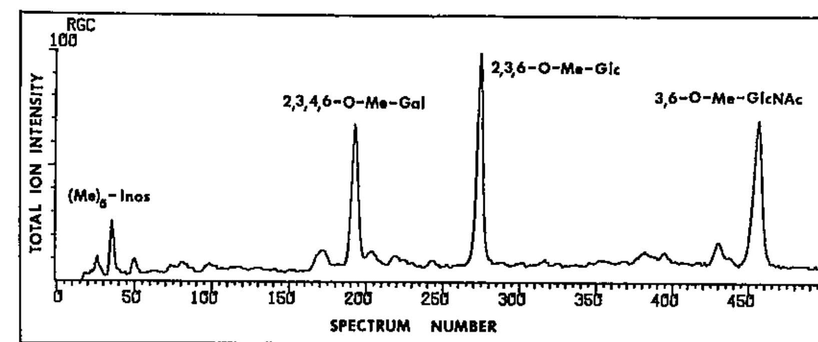
Minor tetrasaccharide:

2,3,4,6-tetra-O-methylmannitol
3,4,6-tri-O-methyl-2-deoxy-2-methylaminoglucitol
2,3,6-tri-O-methylglucitol
tetra-O-methylinositol



Biochemistry

Figure 4. Proposed structure of PSL-I: GlcNAcp(α1→4)GlcUAp(α1→2 myoinositol-1-O-phosphoceramide (12)

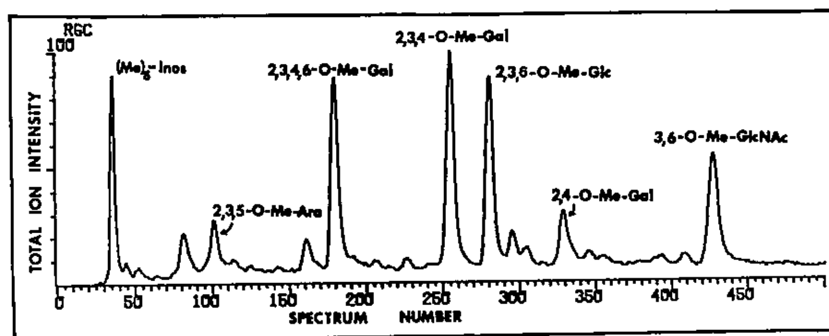


Major Tetrasaccharide:

Galp(1→4)GlcNAcp(1→4)Glcp(1→2)Inos

Figure 5. Methylation linkage analysis of the major tetrasaccharide from tobacco glycophosphoceramide concentrate

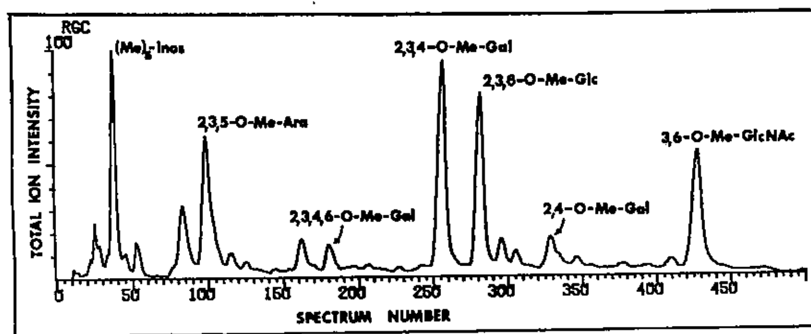
Total ion chromatogram: penta-O-methyl-mono-O-acetylmyoinositol derived from monolinked myoinositol, 2,3,4,6-tetra-O-methyl-1,5-di-O-acetylgalactitol derived from a terminal galactose, 2,3,6-tri-O-methyl-1,4,5-tri-O-acetylglucitol derived from a 4-linked glucitol, and 3,6-di-O-methyl-1,4,5-tri-O-acetyl-2-acetamido-2-N-methylglucitol from a 4-linked N-acetylglucosamine.



Major component: Galp(1→6)Galp(1→4)<u>GlcNAcp(1→4)Glcp(1→2)Inos</u>

Minor component: Galp(1→6) / Araf(1→3) > Galp(1→4)<u>GlcNAcp(1→4)Glcp(1→2)Inos</u>

Figure 6. Preliminary methylation linkage analysis of the major pentasaccharide from tobacco glycophosphoceramide concentrate



Major component: Araf(1→6)Galp(1→4)<u>GlcNAcp(1→4)Glcp(1→2)Inos</u>

Minor component: Galp(1→6) / Araf(1→3) > Galp(1→4)<u>GlcNAcp(1→4)Glcp(1→2)Inos</u>

Figure 7. Preliminary methylation linkage analysis of the minor pentasaccharide from tobacco glycophosphoceramide concentrate

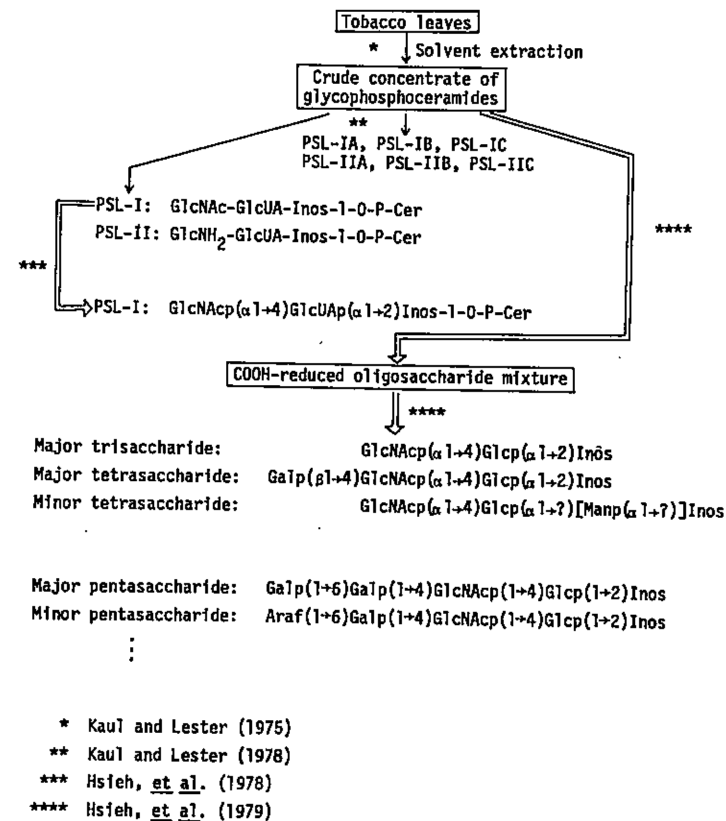


Figure 8. Summary of structural characterization of glycophosphoceramides from tobacco leaves

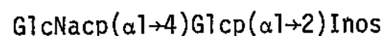
Sequence Analysis

The carbohydrate sequence of the major tetrasaccharide was determined by examining the nitrous acid deamination products (25) as permethylated disaccharides by chemical ionization mode of gas chromatography/mass spectrometry. The products were identified as hexosyl-2,5-anhydromannitol and hexosyl-myoinositol, indicating that the major tetrasaccharide had the sequence Galp(1→4)GlcNAcp(1→4)Glcp(1→2)Inos (Figure 5).

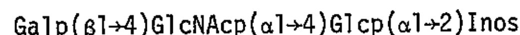
Anomeric Configuration

Additional information on the composition and anomeric configurations were obtained by gas chromatography of alditol acetates prepared from the oligosaccharides with and without CrO_3 oxidation. In the major trisaccharide, and in the minor tetrasaccharide, 80-100% of the sugars survived CrO_3 oxidation indicating all α configuration of the anomeric bonds. In the major tetrasaccharide, however, the yield for galactose was 29% survival, while the other sugars showed 80-100% survival. This data suggested the following structures:

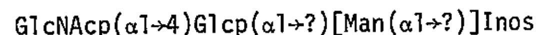
Major trisaccharide:


GlcNacp(α1→4)Glcp(α1→2)Inos

Major tetrasaccharide:


Galp(β1→4)GlcNAcp(α1→4)Glcp(α1→2)Inos

Minor tetrasaccharide


GlcNAcp(α1→4)Glcp(α1→?)[Man(α1→?)]Inos

Thus, the major tri-and tetrasaccharide were completely characterized (Figure 8) (22). The linkage sites on the myoinositol of the minor tetrasaccharide remain undetermined due to the insufficient amount of sample available. Higher oligomers are being fractionated. Preliminary data indicate that a major pentasaccharide has the following structure Galp(1→6)Galp(1→4) GlcNAcp(1→4)Glcp(1→2)Inos and a minor pentasaccharide Araf(1→6) Galp(1→4)GlcNAcp(1→4)Glcp(1→2)Inos (Figures 6, 7). A summary of the results is shown in Figure 8.

Acknowledgements

This investigation was supported in part by Research Grant PCM7609314 from the National Science Foundation , Project KTRB-053 from the Tobacco and Health Research Institute, University of Kentucky, and Grant IROIGM23902 from the National Institutes of Health.

Abstract

Chemical structures of certain glycophosphoceramides from tobacco leaves were studied. The structures which have been characterized to date are as follows:

(1) major glycophosphoceramides PSL-I:
GlcNAcp(α1→4)GlcUAp(α1→2)Inos-1-O-P-Cer

(2) the oligosaccharides isolated from the glycophosphoceramide concentrate after carboxyl-reduction:

(a) major trisaccharide:
GlcNAcp(α1→4)Glcp(α1→2)Inos

(b) major tetrasaccharide:
Galp(β1→4)GlcNAcp(α1→4)Glcp(α1→2)Inos

(c) minor tetrasaccharide:
GlcNAcp(α1→4)Glcp(α1→?)[Manp(α1→?)]Inos

(d) major pentasaccharide:
Galp(1→6)Galp(1→4)GlcNAcp(1→4)Glcp(1→2)Inos

(e) minor pentasaccharide:
Araf(1→6)Galp(1→4)GlcNAcp(1→4)Glcp(1→2)Inos

Literature Cited

1. Carter, H.E., Celmer, W.D., Galanos, D.S., Gigg, R.H., Lands, W.E.M., Law, J.H., Mueller, K.L., Nakayama, T., Tomizawa, H.H., and Weber, E. J. Am. Oil. Chem. Soc., 1958, 35, 335.
2. Lester, R.L., Smith, S.W., Wells, G.B., Rees, D.C., and Angus, W.W. J. Biol. Chem., 1974, 249, 3388.
3. Kaul, K., and Lester, R.L. Plant Physiol., 1975, 55, 120.
4. Carter, H.E., Brooks, S., Gigg, R.H., Strobach, D.R., and Suami, T. J. Biol. Chem., 1964, 239, 743.
5. Carter, H.E., Strobach, D.R., and Hawthorne, J.N. Biochemistry, 1969, 8, 383.
6. Wagner, H., Zofcsik, W., and Heng, I. Z. Naturforsch, 1969, 24, 922.
7. Carter, H.E., and Koob, J.L. J. Lipid Res., 1969, 10, 363.
8. Wagner, H., Pohl, P., and Munzing, A. Z. Naturforsch, 1969, 24, 360.
9. Carter, H.E., and Kisic, A. J. Lipid Res., 1969, 10, 356.
10. Kaul, K., and Lester, R.L. Biochemistry, 1978, 17, 3569.
11. Taylor, R.L., Shively, J.E., Conrad, H.E., and Cifonelli, J.A. Biochemistry, 1973, 12, 3633.
12. Hsieh, T.C.-Y., Kaul, K., Laine, R.A., and Lester, R.L. Biochemistry, 1978, 17, 3575.
13. Björndal, H., Lindberg, B., and Svensson, S. Carbohyd. Res., 5, 433.

14. Björndal, H., Lindberg, B., Pilotti, A., and Svensson, S. Carbohydrate Res., 1970, 15, 339.
15. Hakomori, S. J. Biochem. (Tokyo), 1964, 55, 205.
16. Stellner, K., Saito, H., and Hakomori, S. Arch. Biochem. Biophys., 1973, 155, 464.
17. Hancock, R.A., Marshall, K., and Weigel, H. Carbohyd. Res., 1976, 49, 351.
18. Laine, R.A., Hodges, L.C., and Cary, A.M. J. Supramol. Struct., 1977, 5, Suppl. 1, 31.
19. Hoffman, J., Lindberg, B., and Svensson, S. Acta Chem. Scand., 1972, 26, 661.
20. Laine, R.A., and Renkonen, O. J. Lipid Res., 1975, 16, 102.
21. Albersheim, P., Nevins, D.J., English, P.D., and Karr, A. Carbohyd. Res., 1967, 5, 340.
22. Hsieh, T.C.-Y., Ph.D. dissertation: "Chemical Characterization of Glycophosphosphingolipids from Tobacco"; University of Kentucky: Lexington, Kentucky, 1979.
23. Taylor, R.L., Shively, J.E., and Conrad, H.E. Methods in Carbohyd. Chem., 1976, 7, 149.
24. Wells, G.B., and Lester, R.L. Anal. Biochem., 1979, 97, (in press).
25. Bayard, B., and Roux, D. FEBS Lett., 1975, 55, 206.

RECEIVED December 10, 1979.

6

Glycolipids of Rat Small Intestine with Special Reference to Epithelial Cells in Relation to Differentiation

M. E. BREIMER, G. C. HANSSON, K.-A. KARLSSON, and H. LEFFLER

Department of Medical Biochemistry, University of Göteborg, Göteborg, Sweden

Saccharides may be structurally very complex. In addition to the variation in type and sequence of monomers as for peptide, the heterocyclic carbohydrate monomer may vary in ring size, the glycosidic bond may have both different positions and configurations, and there is often branching of the saccharide chains. A great variability may also mean a rich biochemical language (provided there is specificity of expression) and this is one of the reasons why cell surface carbohydrates are being considered in biological recognition (1, 2).

The membrane-bound carbohydrates exist as glycoproteins and glycolipids. Although the functional importance of these substances is far from proven they appear to be essential parts in phenomena such as cellular adhesion, control of differentiation and cell growth, and the binding by cells of enzymes, hormones and toxins.

One system that we consider of great interest for the study of cell surface glycolipids is the small intestine. Firstly, the epithelial cells lining the intestine exist in a great number on the enlarged surface area and each cell has in itself a large cell surface involved in transport processes and recognition phenomena. Secondly, these cells, arranged as a single columnar layer on the basement membrane, are rapidly renewed (1-3 days) and undergo a successive maturation on their way from the crypt depth to the villus tip (3). Thirdly, these cells are possible to prepare by a gentle washing technique (4), the oldest, less strongly adhered cells (villus tip) being obtained in the first, and the youngest cells (crypt) obtained in the final fractions. Lastly, the concentration of complex glycolipids is high in relation to protein (see 5), which may be explained by a large amount of surface membrane in relation to other membranes.

Our study was divided into two different parts and applied on two separate strains of rat, which were shown to differ in blood groups. In the first stage, following improvement and adaptation of methods, glycolipids were prepared and characterized from pooled whole small intestine of the black and white strain. In the second stage, the knowledge of the general glycolipid