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DETERMINATION OF POSITION OF SUBSTITUTION ON 2-ACETAMIDO-2-DEOXY-D-GALACTOSYL RESIDUES IN GLYCOLIPIDS

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ABSTRACT

The substitution site on 2-acetamido-2-deoxy-D-galactosyl residues in oligosaccharide chains of glycolipids was determined by permethylation of the glycolipid with methyl iodide in the presence of dimethylsulfinyl carbanion, methanolysis of the permethylated product under mild conditions, acetylation with acetic anhydride–pyridine, and identification of the resulting substituted methyl glycosides of 2-deoxy-2-(*N*-methylacetamido)-D-galactose by g.l.c. The method was applied to glycolipids of known structure, including normal brain ganglioside, Tay–Sachs ganglioside, and Forssman glycolipid.

INTRODUCTION

One of the most widely used approaches to the determination of complex carbohydrate structures has been permethylation of free hydroxyl groups, followed by acid cleavage, and identification of the partially methylated monosaccharide units. An important advance in this methodology was the introduction of a procedure by Hakomori[1] that utilizes methyl iodide and dimethylsulfinyl carbanion, the latter being a more powerful nucleophile than the bases previously used. This reagent leads to rapid and complete methylation of all free hydroxyl groups as well as *N*-methylation of the acetamido group in hexosamine residues without loss of *N*-acetyl groups[2]. Techniques have been elaborated by Lindberg and associates for the hydrolysis of such permethylated derivatives, conversion of the substituted monosaccharides into partially methylated alditol acetates, and identification of the latter through g.l.c.[3] or g.l.c.–m.s.[4,5].

This method has proved quite effective for the determination of methylated neutral sugars obtained from a variety of complex carbohydrates[6–9], but its usefulness

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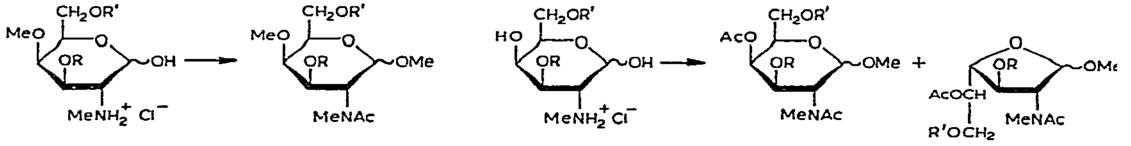
with hexosamine residues has been limited. One reason was the difficulty in obtaining adequate yields of methylated hexosamine alditol acetates, although a recent modification reportedly gave improved yield[10]. In attempting to determine the structures of various 2-amino-2-deoxy-D-galactose-containing glycolipids, we have explored an alternative procedure: formation of substituted methyl glycosides of 2-acetamido-2-deoxy-D-galactose by methanolysis of the fully methylated glycolipid. Recent synthesis of the various *O*-methyl derivatives of 2-deoxy-2-(*N*-methylamino)-D-galactose[11] afforded an opportunity to prepare standards consisting of the various methyl glycosides of 2-amino-2-deoxy-D-galactose derivatives that would result from methanolysis of permethylated glycolipids containing this hexosamine. The present report describes the identification of these methyl derivatives by g.l.c., and demonstrates the usefulness of the method by determination of the substitution positions in known glycolipids.

RESULTS AND DISCUSSION

In the conversion of the various synthetic *O*-methyl derivatives of 2-deoxy-2-(*N*-methylamino)-D-galactose into the corresponding methyl glycosides, acetylation of the methylamino group (in addition to free hydroxyl groups) improved the yield of the subsequent glycosidation reaction. This reaction was performed under relatively mild conditions and gave mixtures containing at least two and, in some cases, three or four methyl glycosides. The *O*-acetyl groups removed by methanolysis were replaced by subsequent *O*-reacetylation prior to g.l.c. Compounds **1**–**4** could form only pyranoside derivatives (**5**–**8**), whereas **9**–**12** could form both pyranosyl (**13**–**16**) and furanosyl (**17**–**20**) derivatives.



1 $R = R' = Me$
2 $R = Me, R' = H$
3 $R = H, R' = Me$
4 $R = R' = H$

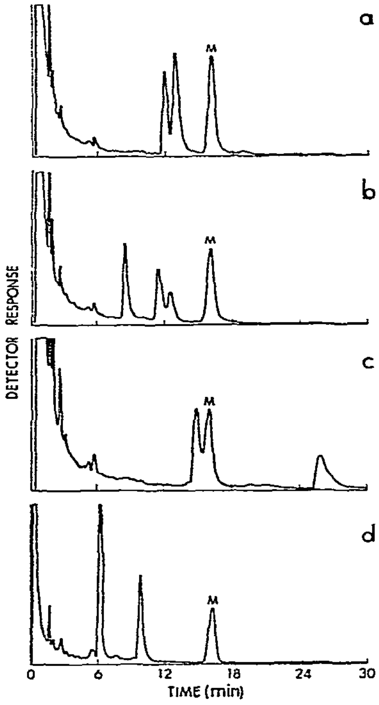
5 $R = R' = Me$
6 $R = Me, R' = Ac$
7 $R = Ac, R' = Me$
8 $R = R' = Ac$

9 $R = R' = Me$
10 $R = Me, R' = H$
11 $R = H, R' = Me$
12 $R = R' = H$

13 $R = R' = Me$
14 $R = Me, R' = Ac$
15 $R = Ac, R' = Me$
16 $R = R' = Ac$

17 $R = R' = Me$
18 $R = Me, R' = Ac$
19 $R = Ac, R' = Me$
20 $R = R' = Ac$

Among the g.l.c. patterns depicted in Fig. 1, only compound (**9**) gave three products (**13** and **17**); the other di-*O*-methyl (**2** and **3**) and the tri-*O*-methyl (**1**) compounds yielded only two peaks, which is consistent with a substituent on the hydroxyl group at C-4 preventing furanosyl glycoside formation. The g.l.c. patterns of these derivatives, obtained on both OV-1 and OV-225 columns, are quite distinctive, but the latter column is more useful because of virtual absence of overlap among individual peaks. Although the smallest of the three peaks produced by the 3,6-di-*O*-methyl derivatives (**13** and **17**) overlaps to some extent with the two peaks of the



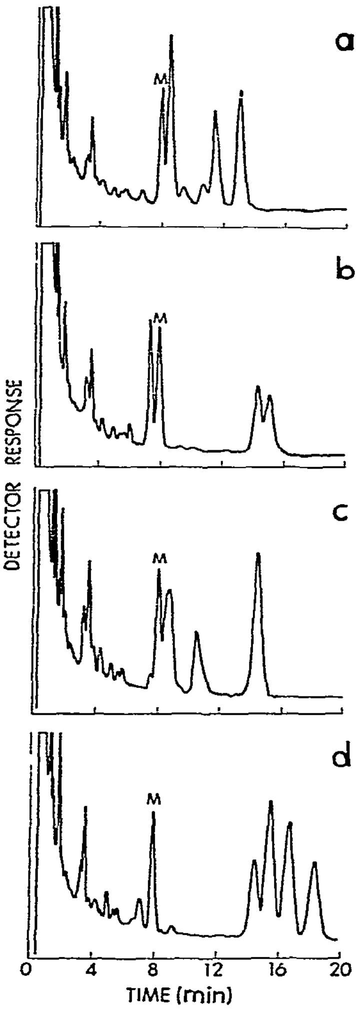


Fig. 1. Gas–liquid chromatograms of methyl glycosides of 2-deoxy-2-(*N*-methylacetamido)-D-galactose with various *O*-acetyl and *O*-methyl substituents; the products in this series contain either two or three *O*-methyl groups. The OV-225 column was operated isothermally at 180°: M, D-mannitol hexaacetate used as standard; a, methyl 3-*O*-acetyl-2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-galactopyranosides (7); b, methyl 4(and 5)-*O*-acetyl-2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-galactosides (13 and 17); c, methyl 6-*O*-acetyl-2-deoxy-3,4-di-*O*-methyl-2-(*N*-methylacetamido)-D-galactopyranosides (6); and d, methyl 2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-D-galactopyranosides (5).

Fig. 2. Gas–liquid chromatograms of methyl glycosides of 2-deoxy-2-(*N*-methylacetamido)-D-galactose with various *O*-acetyl and *O*-methyl substituents. The products in this series contain either one or zero *O*-methyl groups. The OV-225 column was operated isothermally at 215°: M, D-mannitol hexaacetate used as standard; a, methyl 4,6(and 5,6)-di-*O*-acetyl-2-deoxy-3-*O*-methyl-2-(*N*-methylacetamido)-D-galactosides (14 and 18); b, methyl 3,6-di-*O*-acetyl-2-deoxy-4-*O*-methyl-2-(*N*-methylacetamido)-D-galactopyranosides (8); c, methyl 3,4(and 3,5)-di-*O*-acetyl-2-deoxy-6-*O*-methyl 2-(*N*-methylacetamido)-D-galactosides (15 and 19); and d, methyl 3,4,6(and 3,5,6)-tri-*O*-acetyl-2-deoxy-2-(*N*-methylacetamido)-D-galactosides (16 and 20).

4,6-di-*O*-methyl derivatives (7), differences in the total pattern readily distinguish the two isomers. Retention times relative to that of D-mannitol hexaacetate, obtained on both OV-1 and OV-225 columns, are reported in Table I.

TABLE I

G.L.C. CHARACTERISTICS OF METHYL GLYCOSIDES OF *O*-ACETYL-*O*-METHYL-SUBSTITUTED 2-DEOXY-2-(*N*-METHYLACETAMIDO)-D-GALACTOSE

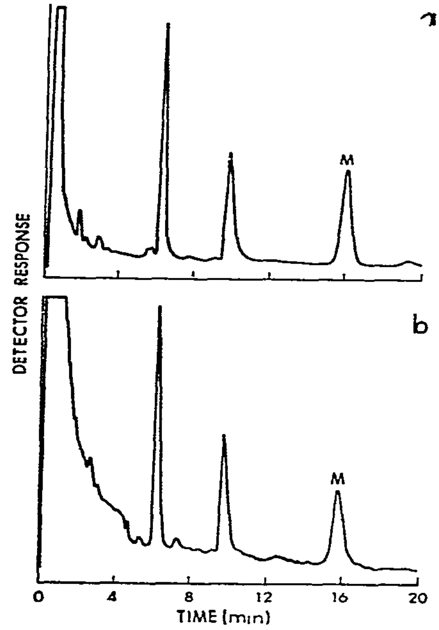
Positions of substituents			*Starting compound*	*Retention times*[a] *of peaks on columns of*			
Methyl	*Acetyl*			*OV-225*		*OV-1*	
				Major	*Minor*	*Major*	*Minor*
3,4,6		(5)	1	0.39 (60, β)	0.61 (40, α)	0.36 (59, β)	0.57 (41, α)
3,4	6	(6)	2	0.92 (55, β)	1.62 (45, α)	0.65 (65, β)	1.08 (35, α)
4,6	3	(7)	3	0.80 (61, β)	0.74 (39, α)	0.62 (59, β)	0.69 (41, α)
3,6	4	(13, 17)	9	0.53 (43)	0.71 (38) 0.77 (19)	0.44 (39)	0.59 (27) 0.68 (34)
3	4,6	(14, 18)	10	1.06 (37) 1.63 (36)	1.43 (27)	1.17 (34)	0.75 (28) 1.00 (20) 1.82 (18)
4	3,6	(8)	4	0.92 (35) 1.80 (34)	1.90 (31)	0.79 (41)	1.12 (27) 1.26 (32)
6	3,4	(15, 19)	11	1.78 (47)	1.09 (33) 1.31 (20)	0.96 (42)	0.84 (32) 0.87 (15) 1.45 (11)
	3,4,6	(16, 20)	12	1.95 (33) 2.10 (29)	1.82 (18) 2.32 (20)	1.37 (68)	1.16 (14) 1.27 (18)

[a] Retention times are relative to D-mannitol hexaacetate. Percentages in parentheses are relative peak areas of glycosides resulting from methanolysis in 0.1M methanolic hydrogen chloride for 18 h at 80°. G.l.c. was performed isothermally at 180° and 215° with the OV-225 column, and at 170° and 180° with the OV-1 column (see Experimental part).

The g.l.c. patterns (Fig. 2), obtained for the compounds resulting from the treatment of the three mono-*O*-methyl ethers (**4**, **10**, **11**) and the unsubstituted 2-deoxy-2-(*N*-methylamino)-D-galactose (**12**), are generally more complex than those illustrated in Fig. 1, and indicate the formation of both furanosyl and pyranosyl glycosides. The relative retention times of these derivatives on OV-1 and OV-225 columns are reported in Table I.

Most naturally-occurring glycolipids having a 2-amino-2-deoxy-D-galactose residue would be expected to give g.l.c. patterns of the type depicted in Fig. 1, since the hexosamine residue is generally in terminal position or substituted by only one residue. In addition, the presence in most of the known glycolipids of a single hexosamine residue would favor the production of simple chromatograms. The Forssman glycolipid, which contains two 2-amino-2-deoxy-D-galactose residues, gives a four-peak pattern which is readily intrepreted with the help of the standards (see subsequent paragraphs).

Application of the procedure to the Tay–Sachs ganglioside, a glycolipid of known structure having a terminal 2-acetamido-2-deoxy-D-galactosyl group[12,15], gave two peaks (Fig. 3) which correspond to methyl 2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-α- and β-D-galactopyranoside (5), thus confirming the presence of the terminal group. Application of the procedure to asialoganglioside G_4*, prepared from the major monosialoganglioside of brain (G_4), produced two major peaks (Fig. 4) with retention times identical to those of methyl 2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)-α- and β-D-galactopyranoside (7). This establishes the structure D-Gal-(1→3)-D-GalNAc-(1→ as terminal disaccharide unit. Brain monosialoganglioside G_4, from which the asialo derivative was prepared, gave similar results, as did the two major disialogangliosides of this organ. The presence of a (1→3) linkage between the two terminal sugar units of brain ganglioside has been



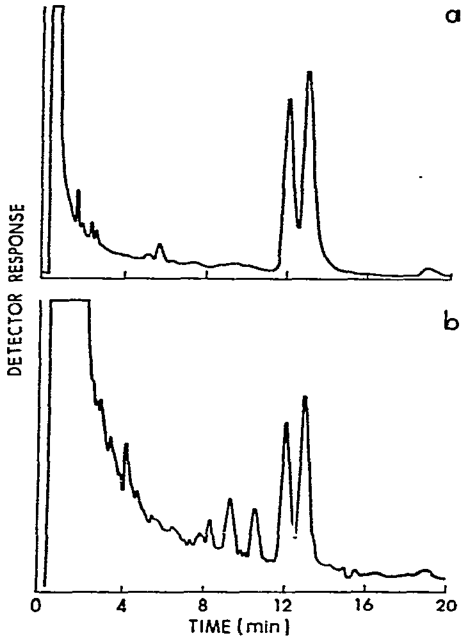


Fig. 3. Comparison of the methyl glycosides obtained from permethylated Tay–Sachs ganglioside with standards: a, methyl 2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-D-galactopyranosides (5); b, corresponding methyl glycosides obtained by methanolysis of permethylated Tay–Sachs ganglioside; M, D-mannitol hexaacetate used as standard. G.l.c. was performed with an OV-225 column operated isothermally at 180°.

Fig. 4. Comparison of the methyl glycosides obtained from permethylated asialo G_4 with standards: a, methyl 3-*O*-acetyl-2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-galactopyranosides (7); b, corresponding methyl glycosides obtained by methanolysis of permethylated asialo G_4; M, D-mannitol hexaacetate used as standard. G.l.c. was performed with an OV-225 column operated isothermally at 180°.

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*This substance is designated in accord with the nomenclature system of Korey and Gonatas[16], and corresponds to G_{M1} of the Svennerholm system[17].

accepted for many years[18–20], the original evidence being the color formation in the Morgan–Elson test[21]. Later, Klenk *et al.*[22] provided more definitive evidence by identifying 2-amino-2-deoxy-4,6-di-*O*-methyl-D-galactose as one of the hydrolysis products of the permethylated ganglioside.

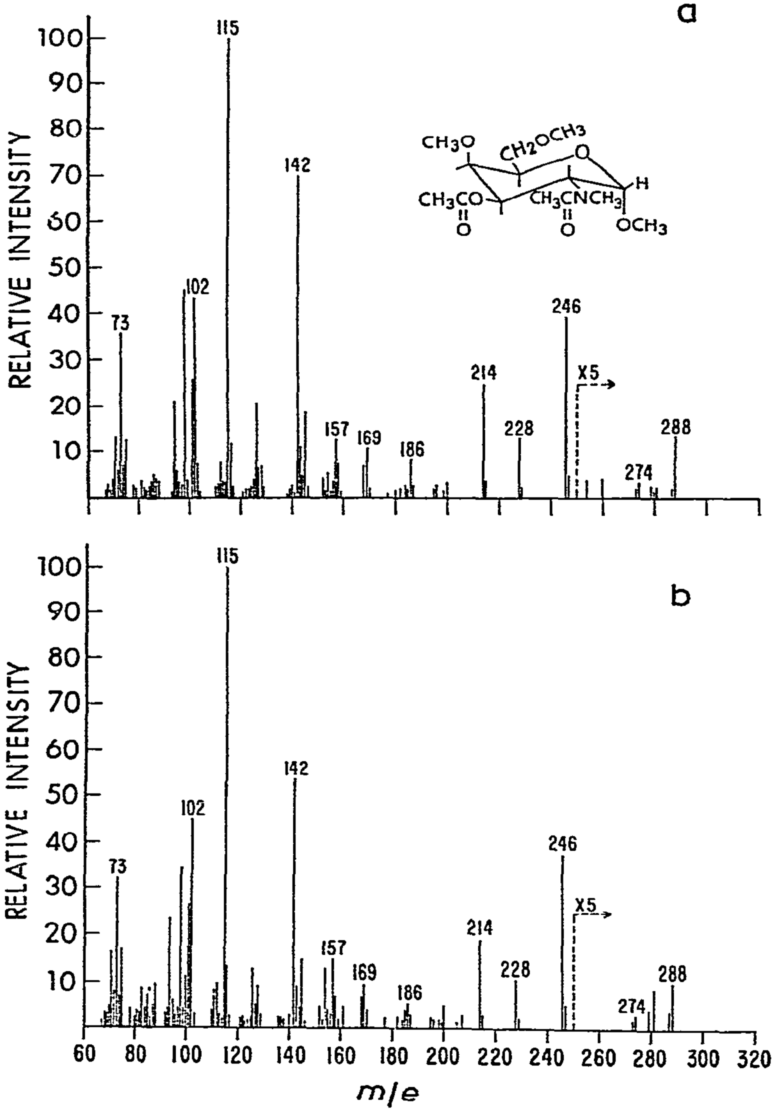

Fig. 5. Comparison of the mass spectrogram of methyl 3-*O*-acetyl-2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)-α-D-galactopyranoside obtained from 7 (a) with that of the corresponding compound resulting from the methanolysis of permethylated asialo G_4, followed by acetylation (b). Both spectra represent the compounds forming the first peak that emerges from an OV-225 column connected to a mass spectrometer and the α configuration was assigned to these compounds.

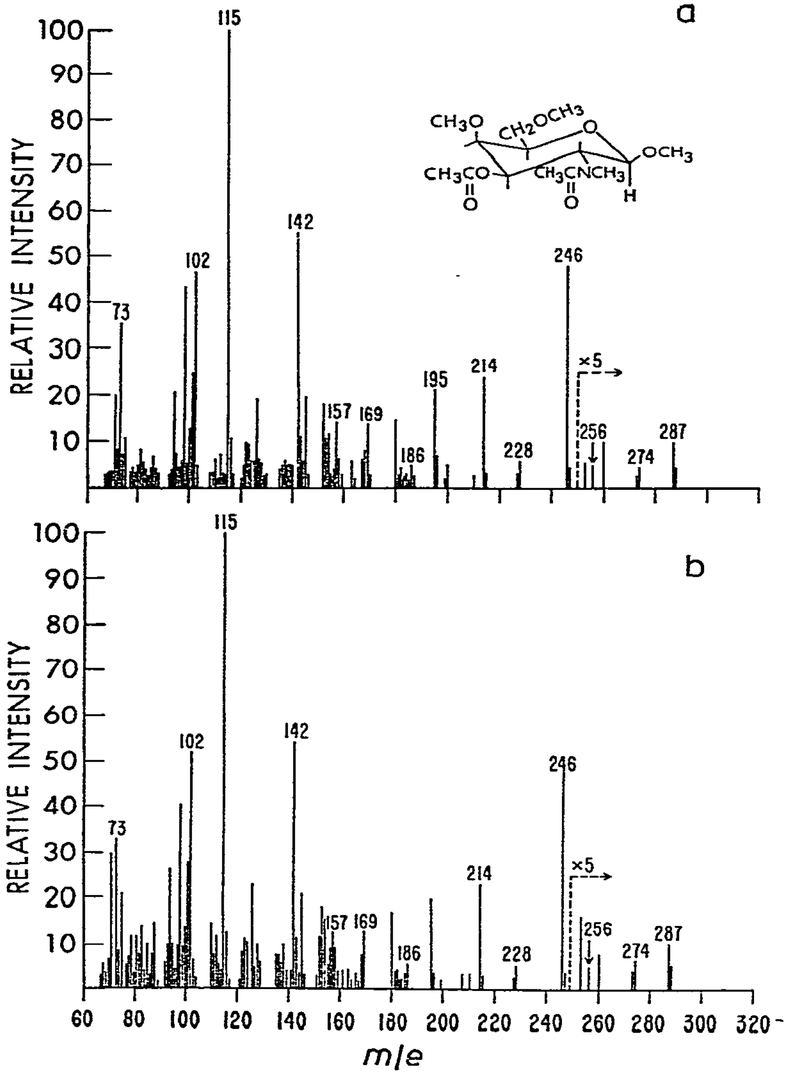

Fig. 6. Comparison of the mass spectrogram of methyl 3-*O*-acetyl-2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)-β-D-galactopyranoside obtained from 7 (a) with that of the corresponding compound resulting from the methanolysis of permethylated asialo G_4, followed by acetylation (b). Both spectra represent the compounds forming the second peak emerging from an OV-225 column connected to a mass spectrometer and the β configuration was assigned to these compounds.

The identity of the two just described g.l.c. peaks produced by asialo G_4 was confirmed by comparison of the g.l.c.–mass spectra with those of standard compounds (Figs. 5 and 6). The α-D configuration* was assigned to the first-eluted peak and the

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*Methyl α- and β-D-glycosides could be rigorously differentiated only for 5, the α anomer in this case being prepared synthetically. Mild methanolysis (heating for 18 h at 80° in 0.1M methanolic

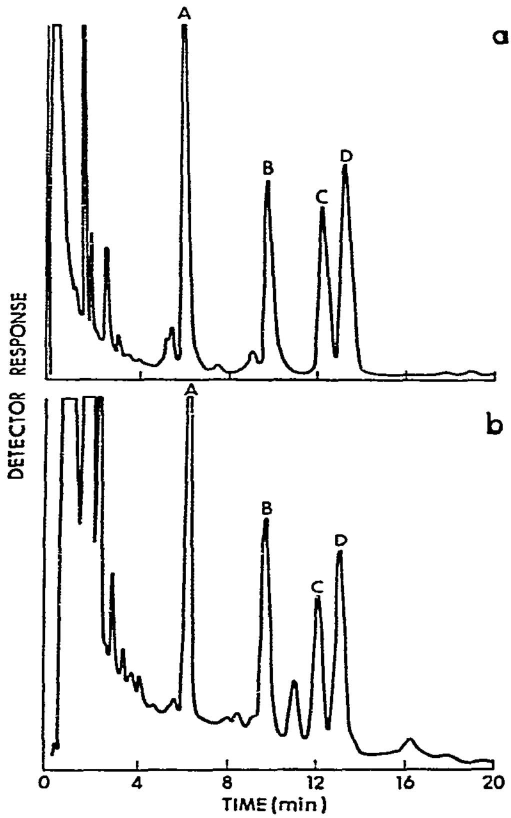

Fig. 7. Comparison of the methyl glycosides obtained from permethylated Forssman glycolipid with standards: a, mixture of methyl 2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-D-galactopyranosides (peaks A and B) and methyl 3-*O*-acetyl-2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-galactopyranosides (peaks C and D); and b, corresponding methyl glycosides obtained by methanolysis of permethylated Forssman glycolipid. G.l.c. was performed with an OV-225 column operated isothermally at 180°.

β-D configuration* to the second peak. Small but reproducible differences were observed between the mass spectra of the α and β anomers of 5: for example, peaks at *m/e* 287 and 256 were detected for the β but not for the α anomer; these 2 peaks are presumably the result of B splitting[24] of the molecule $[M-(CH_3OH+CH_3O)]$. Neither of the pure anomers produced a visible molecular ion (*m/e* 319), although the equilibrium mixture produced a small ion of that weight when injected directly into

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hydrogen chloride) converted the α anomer into a mixture of α and β anomers in the approximate ratio of 2:3, the β anomer being eluted first. The configurational assignments indicated in Table I are postulated on the assumption that the relative peak areas corresponding to the various isomers that yield only pyranosyl glycosides remain unchanged. It may be noted that the elution order of the α and β anomer of the 4,6-di-*O*-methyl derivatives (7) is reversed on the OV-1 column, as compared to the OV-225 column; a similar reversal of the peak was observed on elution of the corresponding derivatives of 2-amino-2-deoxy-D-glucose from these two columns[23].

the mass spectrometer. Another difference was that the peak at *m/e* 304 ($M-CH_3$) was prominent with direct injection but not detectable after g.l.c. The mass spectra of the anomers **7**, as well as that of the equilibrium mixture, are different from that of the equilibrium mixture (**13**, **17**) produced by glycosidation of **9** (compared by direct inlet injection), and these spectra in turn differed from that of methyl 6-*O*-acetyl-2-deoxy-3,4-di-*O*-methyl-2-(*N*-methylacetamido)-α-D-galactopyranoside[2].

When the method was applied to the Forssman glycolipid, a pentaglycosylceramide having the structure[25] α-D-GalNAc-(1→3)-β-D-GalNAc-(1→3)-α-D-Gal-(1→4)-β-D-Gal-(1→4)-β-D-Glc-(1→1)-ceramide, four dinstinct peaks corresponding to 2-amino-2-deoxy-D-galactose derivatives were observed (Fig. 7). They coincided with the peaks produced by a mixture of the 4,6-di-*O*-methyl (**7**) and 3,4,6-tri-*O*-methyl (**5**) derivatives.

The procedure[10] that utilizes g.l.c.–m.s. of the methylated 2-amino-2-deoxyglycosaminitol derivatives gives a single g.l.c. peak in place of the multiple peaks produced here. In many instances, however, the formation of more than one peak for a given *O*-methyl derivative provides a characteristic pattern which enables an improved g.l.c. identification. The "fingerprint" pattern thus obtained may obviate the need for m.s. analysis, and would also be helpful when single peaks of different hexosaminitol derivatives overlap on g.l.c. We have found that the area ratios as well as retention times of the patterns of multiple g.l.c. peaks are quite reproducible, provided experimental conditions are standardized. The procedure outlined here has been applied to 20 μg of glycolipid but could undoubtedly be scaled down.

EXPERIMENTAL

General methods. — For methanolysis, redistilled Baker "Analyzed Reagent Grade" methanol was used. Merck "Reagent Grade" pyridine was distilled in the presence of barium oxide and stored over potassium hydroxide. Fisher "Certified Reagent Grade" acetic anhydride was distilled and stored in a brown bottle. Spectroquality hexane from Matheson, Coleman, and Bell was used without distillation and methyl iodide (Fisher Certified Reagent) was freshly distilled before use. Specially prepared dimethyl sulfoxide (silylation grade) was obtained from Pierce Chemical Co. (Rockford, Ill. 61105) and stored over calcium hydride in the cold. Sodium hydride and calcium hydride were purchased from Ventron Corporation, (Beverly, Mass. 01915). Tay–Sachs ganglioside was isolated from the brain of a patient with Tay–Sachs disease and purified as previously described[15]. Asialoganglioside G_4 was prepared by mild acid hydrolysis of ganglioside G_4 (Ref. 26) and purified by chromatography on activated silicic acid Unisil (Clarkson Chemical Co., Williamsport, Pa. 17701). Forssman glycolipid from caprine erythrocytes was a gift from Dr. M. Naiki. Mild sonication for solubilization was performed with a 100-watt ultrasonic bath (Heat-Systems Ultrasonics, Inc., Plainview, N.Y. 11803).

G.l.c. was performed with a Hewlett–Packard F & M model 402 instrument equipped with a flame-ionization detector. U-shaped glass columns (180 × 0.4 cm) were employed isothermally with two types of packing: OV-225, 3% on Supelcoport

100–120 mesh); and OV-1, 3% on Chromosorb W HP(100–120 mesh)* (Supelco, Inc., Bellefonte, Pa. 16823). Helium carrier-gas was employed at a flow rate of approximately 70 ml/min. Combined g.l.c.–mass spectrometry was performed with a Joel model MSO7 connected to a Varian model 1400 gas chromatograph, equipped with an OV-225 column. The column, injection port, and block were kept at 220°, 260°, and 230°, respectively. The ionizing current was 100 μA, and the ionizing energy 30 eV during mass-spectral scans. For direct inlet-injection, the probe temperature ranged from 50–100° and the block temperature 120–200°. Mass spectrometry was kindly performed by Dr. Francis Hoffman and associates at Columbia University.

Permethylation procedure. — A stock solution of dimethylsulfinyl carbanion (sodium salt) was prepared from a commercial suspension of sodium hydride (57%) in mineral oil; 200 mg were washed three times with dry hexane (3 ml each) in a 10-ml tube. Dry dimethyl sulfoxide (~5 ml) was added, the tube closed with a Teflon-lined screw-cap after flushing the content well with nitrogen, and the suspension sonicated for 3.5 h during which time it warmed to 50–55°. The resulting pale-yellow solution contained a small amount of insoluble residue, which was centrifuged to give a pellet. The reagent prepared in this manner could be preserved for many months in the frozen state. Its activity was verified by formation of an intense red color with triphenylmethane.

Approximately 100–500 μg of glycolipid was placed in a 10-ml tube with Teflon-lined screw-cap, thoroughly dried in a vacuum desiccator, and dissolved with mild sonication in 0.3–0.5 ml of dry dimethyl sulfoxide. The tube was flushed with nitrogen and the methylsulfinyl carbanion solution (0.5–1.0 ml) was added. The mixture was kept for ~2 h at room temperature with occasional agitation and mild sonication. A droplet was removed to demonstrate color formation with triphenylmethane and, if positive, the mixture was treated with methyl iodide (1.0–1.5 ml). This was kept for ~2 h at room temperature with occasional sonication and then added to water (12 ml), and the mixture extracted with chloroform (12 ml). The organic layer was washed 3 times with water (10 ml) and evaporated to dryness.

The resulting crude permethylated glycolipids were purified by t.l.c. The sample in chloroform–methanol was applied as a streak across a plate (20 × 20 cm) precoated with Silica Gel HR (Analtech, Inc., Newark, Del. 19711). The amount of sample per plate was kept below 350 μg. The plates were developed with benzene–methanol (8:1 or 6:1, v/v), the latter proportion being used for the more polar glycolipids, such as gangliosides. Bands were visualized by lightly spraying the plates with water and placing them in an iodine chamber. It was sometimes necessary to chromatograph permethylated glycolipids of related structure as markers along the edge of the plate, because false bands occasionally appeared in the presence of iodine. The appropriate zone of silica gel was scraped from the plate and eluted with chloroform–methanol (2:1, v/v).

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*The use of Chromosorb W-HP (high performance) as support for OV-1 is essential for the desired resolution.

Methanolysis and gas–liquid chromatography. — The dried sample of permethylated glycolipid (40–80 μg) in a 10-ml tube with Teflon-lined screw-cap was treated with 0.1M hydrogen chloride in methanol (2.5 ml), prepared either by bubbling hydrogen chloride into methanol, or, more simply, by dilution of one part of conc. hydrochloric acid (12M, aqueous) with 119 parts of methanol, the small amount of water not interfering. After being heated for 18 h at 80°, the mixture was cooled in ice and extracted three times in the cold with hexane (3 ml) to remove fatty acid esters*. The methanolic solution was evaporated to dryness with a stream of nitrogen while keeping the tube in ice, and the residue was dried for a few h in a desiccator in the presence of phosphorus pentaoxide and potassium hydroxide (no vacuum). The residue was acetylated by adding acetic anhydride–pyridine (0.5 ml, 1:1, v/v) and heating for 0.5 h at 80°. After evaporation under a stream of nitrogen, the residue was dried for a few h (desiccator, no vacuum) in the presence of phosphorus pentaoxide and potassium hydroxide. The resulting products were directly analyzed by g.l.c. or, alternatively, chromatographed first on Unisil (0.3 g); elution with benzene–chloroform (2:1, v/v, 10 ml) removed contaminants and with benzene–chloroform (1:2, v/v, 10 ml) the products. This step resulted in cleaner chromatograms. D-Mannitol hexaacetate was added as internal standard to the dried residue, and the mixture was dissolved in chloroform (30–60 μl) for g.l.c.

G.l.c. was performed isothermally for the detection of the 2-amino-2-deoxy-D-galactose derivatives. On a OV-225 column, the di- and tri-*O*-methyl ethers were chromatographed at 180°, and the mono-*O*-methyl ethers and the tri-*O*-acetyl derivatives at 215°. Somewhat lower temperatures (170° and 180°, respectively) were used with the OV-1 column. Retention times were measured relative to D-mannitol hexaacetate. Under these conditions, the methylated neutral hexosides were eluted soon after the solvent front, well before the hexosamine derivatives. 2-Amino-2-deoxy-D-glucose derivatives appeared in the same general vicinity as the 2-amino-2-deoxy-D-galactose derivatives, but could be differentiated from the latter compounds[23].

Synthesis of 2-amino-2-deoxy-D-galactose derivatives. — 2-Deoxy-2-(*N*-methylamino)-D-galactose and the 3-, 4-, and 6-mono, 3,4-, 3,6-, and 4,6-di-, and 3,4,6-tri-*O*-methyl derivatives were previously synthesized[11]. These derivatives were each converted into a mixture of methyl glycosides by acetylation and subsequent methanolysis. A solution of the sample (~0.1–1.0 mg) in acetic anhydride–pyridine (0.5 ml, 1:1, v/v) was heated for 1 h at 80°, and the cooled mixture was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 0.1M hydrogen chloride in methanol (3.0 ml), and the solution was heated for 18 h at 80°. The cooled solution was evaporated to dryness, and the residue reacetylated as just described, and dissolved in chloroform for g.l.c. analysis. The 3,4-di-*O*-methyl (2), 4,6-di-*O*-

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*By extracting in the cold, it was possible to avoid the extraction of the methyl 2,3,4,6-tetra-*O*-methyl-D-galactosides with hexane; these compounds are readily identified by g.l.c. and serve to confirm the presence of a galactosyl nonreducing end group when peaks corresponding to a terminal hexosamine group are not observed. However, they are rather volatile and can be lost, if care is not exercised during drying or evaporation under a stream of nitrogen.

methyl (**3**), and 3,4,6-tri-*O*-methyl (**1**) starting compounds gave rise to essentially 2 peaks corresponding to methyl pyranosides (Fig. 1), whereas the other compounds showed 3 or 4 peaks corresponding to both pyranosides and furanosides.

Pure methyl 2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-α-D-galactopyranoside was prepared by permethylation of methyl 2-acetamido-2-deoxy-α-D-galactopyranoside, which in turn was synthesized according to published procedures[27,28]. The methylation product was purified by preparative t.l.c., as just described and found to give a single peak on g.l.c. on a OV-225 column (retention time 0.61, relative to D-mannitol hexaacetate). When this compound was heated for several h at 80° in 0.1M hydrogen chloride in methanol, the single peak gradually diminished and a second peak appeared, at a relative retention time of 0.39, and continued to increase until equilibrium was achieved after approximately 24 h. The latter peak was assumed to correspond to the methyl β-D-glycoside.

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REFERENCES

1 S. Hakomori, *J. Biochem.* (Tokyo), 55 (1964) 205–208.
2 A. Stoffyn, P. J. Stoffyn, and J. C. Orr, *Carbohyd. Res.*, 23 (1972) 251–260.
3 H. Björndal, B. Lindberg, and S. Svensson, *Acta Chem. Scand.*, 21 (1967) 1801–1804.
4 H. Björndal, C. G. Hellerqvist, B. Lindberg, and S. Svensson, *Angew. Chem. Int. Ed. Engl.*, 9 (1970) 610–619.
5 C. G. Hellerqvist, B. Lindberg, A. Pilotti, and A. A. Lindberg, *Carbohyd. Res.*, 16 (1971) 297–302.
6 C. G. Hellerqvist, B. Lindberg, S. Svensson, T. Holme, and A. A. Lindberg, *Carbohyd. Res.*, 8 (1968) 43–55.
7 M. Berst, C. G. Hellerqvist, B. Lindberg, O. Lüderitz, S. Svensson, and O. Westphal, *Eur. J. Biochem.* 11 (1969) 353–359.
8 H. Yang and S. Hakomori, *J. Biol. Chem.*, 246 (1971) 1192–1200.
9 B. Siddiqui, J. Kawanami, Y. T. Li, and S. Hakomori, *J. Lipid Res.*, 13 (1972) 657–662.
10 K. Stellner, H. Saito, and S. Hakomori, *Arch. Biochem. Biophys.*, 155 (1973) 464–472.
11 P. A. J. Gorin, *Carbohyd. Res.*, 18 (1971) 281–288.
12 L. Svennerholm, *Biochem. Biophys. Res. Commun.*, 9 (1962) 436–441.
13 E. Klenk, U. Liedtke, and W. Gielen, *Z. Physiol. Chem.*, 334 (1963) 186–192.
14 A. Makita and T. Yamakawa, *Jap. J. Exp. Med.*, 33 (1963) 361–368.
15 R. Ledeen and K. Salsman, *Biochemistry*, 4 (1965) 2225–2233.
16 S. R. Korey and J. Gonatas, *Life Sci.*, 2 (1963) 296–302.
17 L. Svennerholm, *J. Neurochem.* 10 (1963) 613–623.
18 L. Svennerholm, *J. Lipid Res.*, 5 (1964) 145–155.
19 H. Wiegandt, *Advan. Lipid Res.*, 9 (1971) 249–289.
20 R. W. Ledeen and R. K. Yu, in H. G. Hers and F. Van Hoof (Eds.), *Lysosomes and Storage Diseases*, Academic Press, New York, 1973, pp. 105–145.
21 R. Kuhn and H. Wiegandt, *Chem. Ber.*, 96 (1963) 866–880.
22 E. Klenk, L. Hof, and L. Georgias, *Z. Physiol. Chem.*, 348 (1967) 149–166.
23 S. K. Kundu, R. W. Ledeen, and P. A. J. Gorin, *Carbohyd. Res.*, 39 (1975) 329–334.
24 K. Heyns and D. Muller, *Tetrahedron*, 21 (1965) 3151–3170.

25 B. SIDDIQUI AND S. HAKOMORI, *J. Biol. Chem.*, 246 (1971) 5766–5769.
26 R. LEDEEN, K. SALSMAN, J. GONATAS, AND A. TAGHAVY, *J. Neuropathol. Exp. Neurol.*, 24 (1965) 341–351.
27 M. STACEY, *J. Chem. Soc.*, (1944) 272–274.
28 H. MASAMUNE, M. MAKI, AND N. HIYAMI, *Tohoku J. Exp. Med.*, 54 (1951) 313–317.