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Analysis of Amino Sugar-Containing Glycosphingolipids by Combined Gas-Liquid Chromatography and Mass Spectrometry

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Summary: A modification of the original method of Corey *et al.* for quantitative methylation of both hydroxyl groups and of *N*-acyl groups of amino sugars and amino sugar-containing glycosphingolipids on a preparative and on a microanalytical scale has been developed. The completeness of the reaction has been tested by thin-layer chromatography, IR, NMR and mass spectroscopy. The corresponding partially methylated alditol acetates and *N*-methylacetamido alditol acetates were analyzed by combined gas-liquid chromatography and mass spectrometry. Mass spectra of the partially methylated amino sugars as their alditol acetates exhibited the characteristic fragments due to the 2-*N*-methylacetamido configuration. The advantages of the methylation technique described here are a high yield and a simple procedure using analytical amounts down to 250 µg. Due to the quantitative methylation of all OH- and NH-protons of amide linkages, the technique yields homogeneous products. Consequently a mixture of glycosphingolipids can be methylated in one step and separated by thin-layer chromatography. It has been demonstrated that the separating efficiency of silica gel chromatography is far greater for methylated than for non-methylated glycosphingolipids. The techniques described here were tested and exemplified with the tetrahexosylceramide (Gal-GalNAc-Gal-Glc-ceramide) derived from ganglioside A_2 (Klenk) and applied to the structural determination of the ceramide trihexoside isolated from pig erythrocytes. Its structure proved to be Gal1→4Gal1→4Glc1→1ceramide. It is identical with the porcine spleen trihexosyl ceramide.

Analyse von Aminozucker-haltigen Glykosphingolipiden mittels kombinierter Gaschromatographie und Massenspektrometrie

Zusammenfassung: Zur quantitativen Methylierung sowohl der Hydroxyl- als auch der *N*-Acyl-Gruppen von Aminozuckern und Aminozucker-haltigen Glykosphingolipiden im präparativen wie im mikroanalytischen Maßstab wurde eine Modifikation der ursprünglichen Methode von Corey et al. ausgearbeitet. Die quantitative Methylierung wurde mittels Dünnschichtchromatographie, IR-, NMR- und Massenspektrometrie kontrolliert. Außerdem werden besonders im Hinblick auf die *N*-methylierten Aminozucker Bedingungen für die quantitative Spaltung der glykosidischen Bindungen der permethylierten Glykosphingolipide ohne wesentliche Zersetzung und Demethylierung im Mikromaßstab beschrieben. Die entsprechenden partiell methylierten Alditolacetate und *N*-Methylacetamidoalditolacetate wurden mittels Gaschromatographie in Kombination mit der Massenspektrometrie untersucht. Für die gaschromatographischen Untersuchungen wurden stationäre Phasen hinsichtlich

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Abbreviations: ECNSS-M = mixed polymer of ethylenglycol succinate and cyanoethylsilicone (Applied Science Laboratories Inc.); OV-225 (Methyl silicone with 25% phenyl and 25% cyanopropyl groups; Applied Science Laboratories Inc.).

ihrer Trennfähigkeit gegenüber den erwähnten Zuckerderivaten beschrieben. Die massenspektroskopische Analyse der partiell methylierten Aminozucker in Form ihrer Alditolacetate erbrachte die für die 2-*N*-Methylacetamido-Gruppe typischen Fragmente sowie solche der entsprechenden neutralen Zucker. Die Vorteile der hier beschriebenen Methylierungstechnik sind die hohe Ausbeute und die einfache Durchführung ohne nötige Wiederholung der Methylierung auch für Mengen von 250 µg. Da alle Hydroxyl- und Amido-Gruppen quantitativ methyliert werden, erhält man ein homogenes Produkt. Es ist dadurch möglich, ein Gemisch von Glykosphingolipiden in einem Schritt zu methylieren und erst anschließend auf Grund der hohen Trennleistung der Kieselgel-Dünnschichtchromatographie für diese methylierten Glykosphingolipide die Auftrennung des Gemischs für die anschließende Komponenten-Analyse vorzunehmen.

Die beschriebene Methodik wurde an dem Tetrahexosylceramid Gal-GalNAc-Gal-Glc-Ceramid, das durch Abspaltung von *N*-Acetylneuraminsäure aus Gangliosid A_2 (Klenk) gewonnen wurde, und am in der glykosidischen Verknüpfung mit dem aus Schweinemilz isolierten Ceramidtrihexosid identischen Schweineerythrocyten-Ceramidtrihexosid exemplifiziert. Die Struktur des letzteren wurde als Gal1→4Gal1→4Glc1→1Ceramid bestimmt.

The occurrence of glycolipids and glycoproteins on the surface of the plasma membrane and subcellular particles introduces new factors of chemical morphology of these membranes. The number and nature of the carbohydrate residues, the sequence, glycosidic linkages and their anomeric forms provide features affecting their specificity for a number of functions. Antigenicity of glycosphingolipids is, so far, the only well known property. Methodical studies are required to establish their often rather complex structures on a microscale, since in general only minute amounts of these compounds are available for analysis from biological sources. Efforts in this laboratory are aimed at making available improved methods for the analysis of these compounds. We would like to report the results of studies on the glycosidic linkages of glycosphingolipids, particularly those containing hexosamines.

The generally applied method to establish glycosidic linkages of free or bound oligo- and polysaccharides is the complete methylation followed by complete or partial hydrolysis and the identification of the methylated sugar residues in one or the other form.

It was essentially the methylation procedure of Corey *et al.*[1] using the sodium methylsulfinylcarbanion, which helped to overcome the tedious complete methylation in one step, as also demonstrated by Hakomori[2] and Sandford and Conrad[3] for polyol compounds. Only a few studies have been concerned with the linkage analysis of amino sugar containing saccharide chains by methylation, hydrolysis and sugar identification[4–10], although a number of completely or partially *O*-methylated amino sugars has been described for identification purposes, and their gas-liquid chromatographic and mass spectroscopic data have been reported[11]. One obstacle in Corey's methylation procedures is the variable degree of methylation of the acetamido group of hexosamines, which complicates the analysis of the hydrolysis products. Further problems may occur during the hydrolysis of oligosaccharides containing methylated hexosamines, such as methyl ether cleavage, loss due to the volatility of these sugar derivatives, and severe

[1] Corey, E. J. & Chaykovsky, M. (1962) *J. Amer. Chem. Soc.* **84**, 866–868.

[2] Hakomori, S. I. (1964) *J. Biochem.* **55**, 205–208.

[3] Sandford, P. A. & Conrad, H. E. (1966) *Biochemistry* **5**, 1508–1517.

[4] Kuhn, R., Trischmann, H. & Löw, I. (1955) *Angew. Chem.* **67**, 32.

[5] Kobata, A. & Ginsburg, V. (1969) *J. Biol. Chem.* **244**, 5496–5502.

[6] Etzler, M. E., Anderson, B., Beychock, S., Gruezo, F., Lloyd, K. O., Richardson, N. G. & Kabat, E. A. (1970) *Arch. Biochem. Biophys.* **141**, 588–601.

[7] Anderson, B., Kabat, E. A., Beychock, S. & Gruezo, F. (1971) *Arch. Biochem. Biophys.* **145**, 490–504.

[8] Adams, E. P. & Gray, G. M. (1967) *Chem. Phys. Lipids* **1**, 368–375.

[9] Adams, E. P. & Gray, G. M. (1968) *Chem. Phys. Lipids* **2**, 147–155.

[10] Yang, H. J. & Hakomori, S. I. (1971) *J. Biol. Chem.* **246**, 1192–1200.

[11] Björndal, H., Hellerqvist, C. G., Lindberg, B. & Svensson, S. (1970) *Angew. Chem.* **82**, 643–674.

degradation, as pointed out by Croon[12]. For the microanalysis of the partially methylated monosaccharide residues, gas-liquid chromatography is the method of choice. Three different derivatives of the methylated sugars have been used for gas-liquid chromatographyic identification and quantitative analysis, the methylglycosides, the trimethylsilyl ethers and the alditol acetates after reduction of the carbonyl to the alcohol group with subsequent acetylation (for review see Clamp *et al.*[13]). The first two derivatives give complex chromatograms, due to their anomeric structures. One peak for each neutral and amino sugar in gas-liquid chromatography would facilitate their identification and quantitative determination. This can be achieved with the method last mentioned.

In order to overcome the aforementioned analytical problems, we used the following conditions for the quantitative *O*- and *N*-methylation of neutral and amino sugars of oligosaccharide chains. This methylation procedure can also be applied to mixtures of glycosphingolipids, which can subsequently be separated nicely by preparative thin-layer chromatography. Furthermore, optimized conditions for the hydrolysis of permethylated carbohydrate chains containing amino sugars are described. The alditol acetates of the latter were studied by combined gas-liquid chromatography and mass spectroscopy.

Although the methods were elaborated for glycosphingolipids as model compounds, they may likewise be applied for quantitative studies on the structure of glycopeptides and oligosaccharides.

Results and Discussion

Methylation procedure

The reaction conditions described by Sandford and Conrad[3] were used for the methylation of hexoses, lactose and *N*-acetylglucosamine. Under the same conditions (see Experimental), *N*-acetylgalactosamine and neuraminic acid-free tetrahexosylceramide from ganglioside A_2 were methylated on a preparative scale (500 mg), and globoside and trihexosylceramide from pig erythrocytes on a microanalytical scale (900–1000 µg). Completeness of *O*- and *N*-methylation was checked by thin-layer chromatography: the anomeric mixtures of permethylated hexoses and lactose showed one anthrone-positive double band together with small amounts of other anthrone negative contaminants. *N*-Acetylglucosamine, when methylated under the conditions most frequently used[2], exhibited two equally intensive bands on charring, with very different R_F-values. They proved to be due to methyl 2-acetamido- ($R_F = 0.26$) and 2-*N*-methylacetamido-2-deoxy-3,4,6-tri-*O*-methylglucoside ($R_F = 0.53$). *N*-Acetylgalactosamine after permethylation according to Sandford *et al.*[3] and described in detail under Experimental showed only one double band with an R_F-value similar to that of corresponding *N*-acetyl-*N*-methylglucosamine. The latter substances gave a faint, yellow-green anthrone reaction. All glycosphingolipids permethylated in this way appeared as one homogeneous anthrone-positive spot on thin-layer chromatography. Small amounts of by-products detected by charring ran near the front (probably fatty acid methyl esters). Tetrahexosylceramide, globoside and trihexosylceramide had R_F-values of 0.17, 0.22 and 0.34 before methylation (solvent system: chloroform/methanol/water 65:25:4) and 0.22, 0.43 and 0.68 after methylation (solvent system: chloroform/methanol 95:5). The methylation of tetrahexosylceramide, after purification by silicic acid column chromatography, gave a yield of 92%. On the microscale the yield of permethylated glycosphingolipids was about 80%.

The completeness of *N*- and *O*-methylation was checked by IR spectroscopy[14]. For comparison the IR spectra of methyl 2-*N*-methylacetamido-2-deoxy-3,4,6-tri-*O*-methylglucoside and methyl 2-acetamido-2-deoxy-3,4,6-tri-*O*-methylglucoside are shown in Fig. 1. The spectra of the permethylated tetrahexosylceramide and *N*-methylacetamidogalactose consistently show absorption peaks at 1560 cm^{-1} in addition to those shown in Fig. 1, indicating the occurrence of a carbonyl group attached to amino-nitrogen as in ceramide or amino sugars. Absorption by secondary amide bonds at 3300 cm^{-1}, 3100 cm^{-1} and 1560 cm^{-1} was not apparent. Therefore the methylation of all OH-groups and of the *N*-acetamido group must have been complete. On the other hand, absorption bands of the *O*-methylated *N*-acetylglucosamine were

[12] Croon, J., Herrström, G., Kull, G. & Lindberg, B. (1960) *Acta Chem. Scand.* **14**, 1338–1342.

[13] Clamp, J. R., Bhatti, T. & Chambers, E. (1971) *Methods Biochem. Anal.* **19**, 229–344.

[14] Wallenfels, K., Bechtler, G., Kuhn, R., Trischmann, H. & Egge, H. (1963) *Angew. Chem.* **75**, 1014–1022.

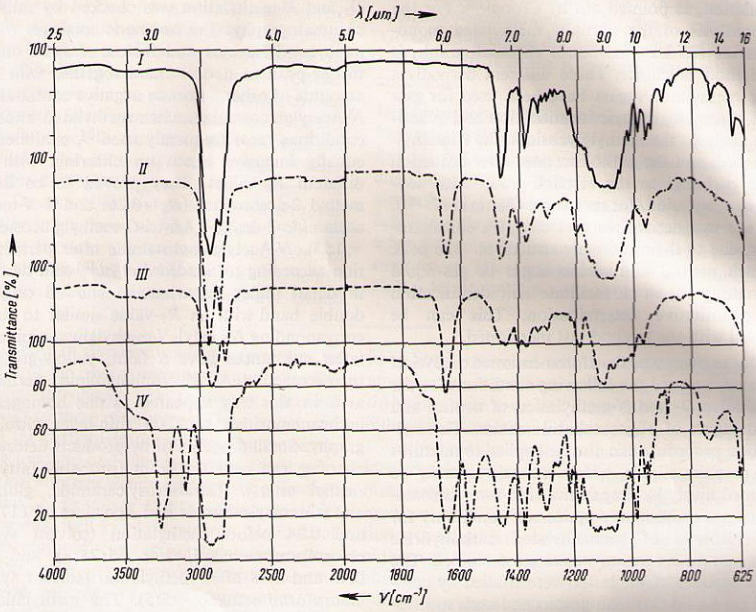


Fig. 1. IR spectra of permethylated lactose (I), per-*O*- and *N*-methylated tetrahexosylceramide (II), per-*O*- and *N*-methylated *N*-acetylgalactosamine (III) and per-*O*- methylated *N*-acetylglucosamine (IV).

visible at 3300 cm^{-1}, 3100 cm^{-1} and 1560 cm^{-1}, indicating the non-methylation of the *N*-acetyl group. Comparative NMR and mass spectra of *O*-methylated *N*-acetylglucosamine and *N*-methylacetamidogalactose and their alditol acetates gave further proof of complete methylation. The NMR spectrum of methyl 2-*N*-methylacetamido-2-deoxy-3,4,6-tri-*O*-methylgalactoside (Fig. 2) can be interpreted as follows. The doublet at 4.8 and 4.72 ppm is derived from the two protons of the C-1 and C-2 atoms, the signal at 3.03 ppm from the three protons of the *N*-methyl group and the singlet at 2.05 ppm from the three protons of the acetyl group, whereas the remaining protons are expressed in a doublet at 3.5 and 3.35 ppm. The integral supports this interpretation.

Also the mass spectrum of this fully methylated compound differs from the corresponding non-*N*-methylated amino sugar derivative (see p. 27). The fragmentation of its alditol acetate is almost identical with that of 2-*N*-methylacetamido-1,5-di-*O*-acetyl-2-deoxy-3,4,6-tri-*O*-methylglucose described by Björndal *et al.*[11].

In systematic studies the following conditions for the cleavage of methyl glycosides, disaccharides and ceramide oligosaccharides proved to be optimal: All neutral methyl glycosides and disaccharides were completely hydrolyzed in 1N aqueous H_2SO_4 when heated in a sealed tube for 12 h at 100°C. Also 80% aqueous acetic acid led to almost no degradation or demethylation when 80 °C was not exceeded. 0.7N sulfuric acid in 80% aqueous acetic acid at 80°C completely solubilized and cleaved all water-insoluble permethylated compounds with discoloration. Fig. 3a and b demonstrate the results of the gas-liquid chromatographic

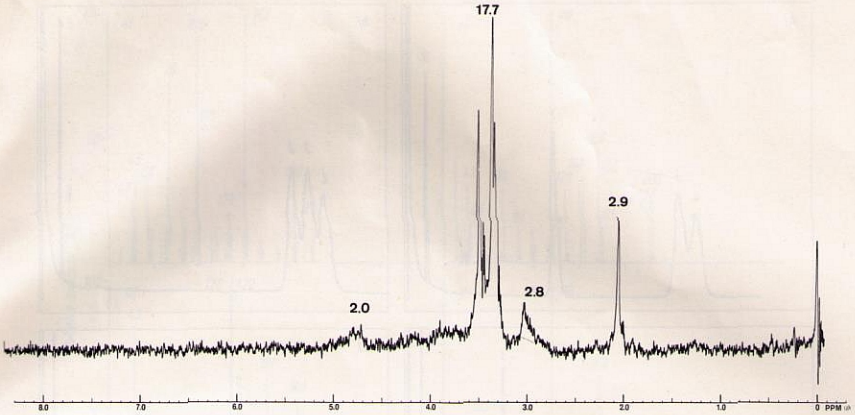


Fig. 2. NMR spectrum of methyl 2-*N*-methylacetamido-2-deoxy-3,4,6-tri-*O*-methylgalactoside in CCl_4 with tetramethylsilane as an internal reference.

Figures above signals refer to number of protons calculated from the integral.

analysis of the hydrolysis products of the neuraminic acid-free tetrahexosylceramide derived from ganglioside A_2. The products were 2,3,4,6-tetramethylgalactose, 2,3,6-trimethylgalactose and 2,3,6-trimethylglucose with a ratio of the peak areas of 0.97:1.03:1 when separated on 3% OV-225. Contaminations derived from demethylation and degradation did not exceed 1–2%. The results were similar for globoside (1.03:1.09:1 for 2,4,6-trimethylgalactose, 2,3,6-trimethylgalactose and 2,3,6-trimethylglucose, respectively). Again the degree of demethylation and degradation was as small as that shown for tetrahexosylceramide. Trihexosylceramide from pig erythrocytes has no amino sugars. The analysis made of this trihexosylceramide completely agrees with the established nature of its glycosidic linkages. The results of the gas-liquid chromatographic separation using 3% OV-225 as stationary phase were very similar to those obtained for the neutral sugars of tetrahexosylceramide. The peak area ratios were 0.99:1.05:1 for 2,3,4,6-tetramethylgalactose, 2,3,6-trimethylgalactose and 2,3,6-trimethylglucose, respectively. Therefore the structure of the trihexoside is: Gal1→4Gal1→4Glc1→1ceramide. The degradation and demethylation was found to be 1.25%.

The partially methylated alditol acetate of galactosamine was recovered in a 55% yield from tetrahexosylceramide (Gal→GalNAc→Gal→Glc-ceramide) and a 45% yield from globoside, calculated on a molar basis and related to the neutral sugars (Fig. 3a, b). These results are fully sufficient for linkage analysis since no other peaks were present besides those of the amino sugars. The peaks in the amino sugar area can be identified without difficulty by gas-liquid chromatography and by mass spectroscopy (see below). With these optimized conditions for the cleavage at hand, all analyses were performed with 250 μg of permethylated material. The quality of the gas-liquid chromatographic analyses were unchanged.

Gas-liquid chromatography

The separation of 2,3,6-trimethylglucose, 2,3,6-trimethylgalactose and 2,4,6-trimethylgalactose as their alditol acetates on 3% ECNSS-M was insufficient for the quantitative analysis, even if longer columns (2–3 m) were used. This combination of the three sugar units, however occurs very often in glycosphingolipids. Ebert *et al.*[15] and Lönngren

[15] Ebert, W., Metz, J., Weicker, H. & Roelcke, D. (1971) *this J.* **352**, 1309–1318.

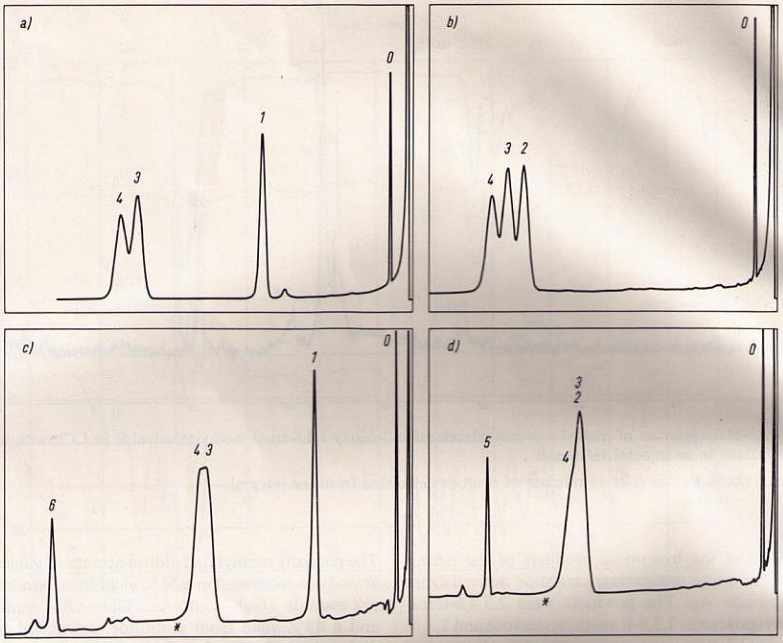


Fig. 3. Gas-liquid chromatograms of alditol acetates of partially methylated neutral and amino sugars released from permethylated glycosphingolipids.

a, c) tetrahexosylceramide derived from ganglioside A_2; b, d) globoside; 0 = artefact (probably arises from the acetylating reagent); 1 = 2,3,4,6-tetra-*O*-methylgalactose; 2 = 2,4,6-tri-*O*-methyl-galactose; 3 = 2,3,6-tri-*O*-methyl-galactose; 4 = 2,3,6-tri-*O*-methylglucose; 5 = 2-*N*-methylacetamido-2-deoxy-3,4,6-tri-*O*-methylgalactose (chromatograms a and b on 3% OV-225, c and d on 3% ECNSS-M); * = temperature increase to 210°C. Further conditions see Experimental.

and Pilotti[16] applied 3% OV-225 as stationary phase for the separation of alditol acetates. This stationary phase gave a satisfactory separation of the structurally very similar sugar derivatives 2,4,6-trimethylgalactose, 2,3,6-trimethylglucose and 2,3,4,6-tetramethylglucose (all methyl sugars as their alditol acetates) with retention times of 2.10:2.03:2.32 relative to 2,3,4,6-tra-*O*-methylglucose 1.0.

OV-225 was less suitable for the separation of methylated amino sugar alditol acetates. Their mass peaks were much smaller compared with those of the neutral sugars. Short 3% ECNSS-M columns (1 − 1.20 m) which had been conditioned for several days at 200°C allowed an acceptable quantitative gas-liquid chromatographic analysis of the partially methylated amino sugars as their alditol acetates. Conditioning for a shorter period of time reduced the yield of the amino sugars. The reduced yield compared with the neutral sugars therefore is probably not due to degradation during cleavage. Column temperatures higher than 210°C were not chosen because of the instability of ECNSS-M at 210°C.

The relative retention times of the partially methylated aminohexitol acetates on 3% ECNSS-M at 200°C are summarized in Table 1.

[16] Lönngren, J. & Pilotti, Å. (1971) *Acta Chem. Scand.* **25**, 1144 − 1145.

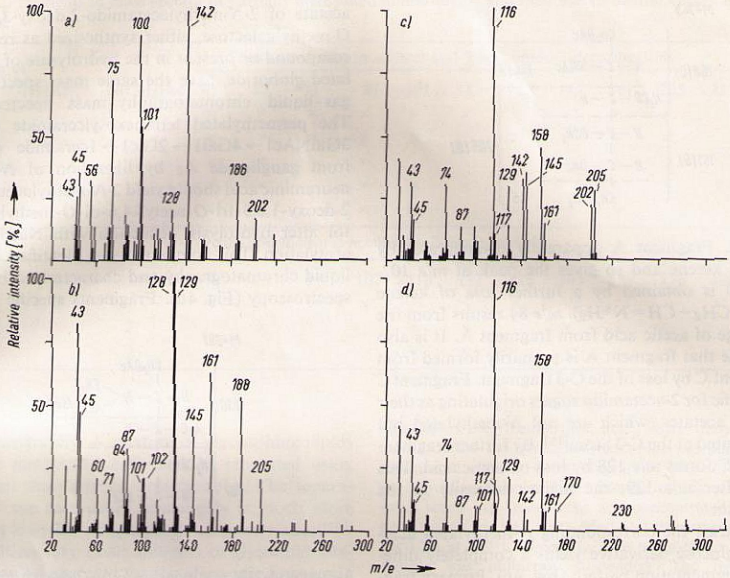


Fig. 4. Mass spectra of methylated amino sugars.

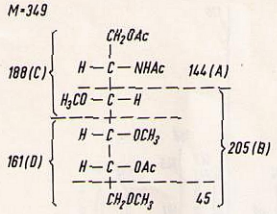
a) Methyl 2-acetamido-2-deoxy-3,4,6-tri-*O*-methylgalactoside; alditol acetates of b) 2-acetamido-2-deoxy-3,4,6-tri-*O*-methylglucose, c) 2-*N*-methylacetamido-2-deoxy-3,4,6-tri-*O*-methylglucose and d) 2-*N*-methylacetamido-2-deoxy-4,6-di-*O*-methylgalactose.

Table 1. Retention times (R_t) of alditol acetates derived from partially methylated hexosamines relative to 2,3,4,6-tetramethyl-1,5-diacetylglucitol on 3% ECNSS-M.

Alditol acetates derived from	R_t
2,3,4,6-tetra-*O*-methylglucose	1.00
2-*N*-acetamido-2-deoxy-3,4,6-tri-*O*-methylglucose	8.39
2-*N*-methylacetamido-2-deoxy-3,4,6-tri-*O*-methylglucose	7.50
2-*N*-methylacetamido-2-deoxy-3,4,6-tri-*O*-methylgalactose	7.41
2-*N*-methylacetamido-2-deoxy-4,6-di-*O*-methylgalactose	20.39

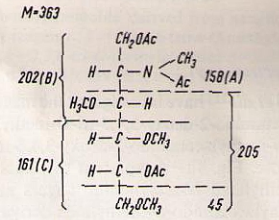
Mass spectrometry

Björndal *et al.*[11] have investigated the mass spectra of 2-acetamido-2-deoxy-3,4,5-tri-*O*-methylglucose and 2-*N*-methylacetamido-2-deoxy-3,4,6-tri-*O*-methylglucose. Fig. 4a–d represent the mass spectra of *O*-methylated *N*-acetylamino sugars and their 2-*N*-methylacetamido-*O*-methylated derivatives; of the methyl glycoside (4a) or of the alditol acetates (4b–d). In the spectrum of the non-*N*-methylated alditol acetate of 2-acetamido-2-deoxy-3,4,6-tri-*O*-methylglucose (Fig. 4b) the well known fragment at *m/e* 145 typical for the alditol acetates of 2,3,4,6-tetramethylhexoses is present. It is derived from fragment B (see fragmentation scheme) by loss of acetic acid. Further fragmentation of fragments B and D proceeds in the same way as for the corresponding neutral sugars and can also be found for the corresponding *N*-methylacetamido sugar deri-

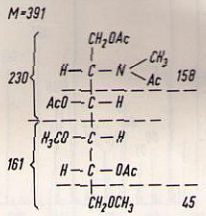


vatives. Fragment A apparently disintegrates by loss of ketene and so gives the peak at *m/e* 102. *m/e* 60 is obtained by a further loss of ketene ($HO-CH_2-CH=N^{\oplus}H_2$). *m/e* 84 results from the cleavage of acetic acid from fragment A. It is also possible that fragment A is primarily formed from fragment C by loss of the C-3 fragment. Fragment C is specific for 2-acetamido sugars originating as their alditol acetates, which are not *N*-methylated but methylated at the C-3 atom[11]. By further fragmentation C forms *m/e* 128 by loss of acetic acid. This has, after *m/e* 129, the highest intensity in the spectrum.

In contrast the corresponding *N*-methylated acetamidoglucose derivative yields a completely different fragmentation pattern (Fig. 4c). Primary fragment A (*m/e* 158) disintegrates by loss of ketene to *m/e* 116, which forms the peak with strongest intensity in the spectrum. The peak at *m/e* 117 may be



either interpreted as fragment A minus ketene with protonated nitrogen, or as resulting from fragmentation between C-4 and C-5, which does not occur in neutral sugar derivatives. Further loss of ketene from *m/e* 116 results in *m/e* 74. Fragment B is characteristic for all *N*-methylated acetamido sugar derivatives, present as their alditol acetates, which are methylated at the C-3 atom. Other fragments are exactly the same as for the corresponding neutral sugar derivatives. The alditol acetate of 2-*N*-methylacetamido-2-deoxy-3,4,6-tri-*O*-methylgalactose, either synthesized as reference compound or present in the hydrolysate of methylated globoside, gave the same mass spectrum in gas-liquid chromatography-mass spectroscopy. The permethylated tetrahexosylceramide Gal1→3GalNAc1→4Gal1→2Glc1→1ceramide derived from ganglioside A_2 by liberation of *N*-acetylneuraminic acid should yield 2-*N*-methylacetamido-2-deoxy-1,3,5-tri-*O*-acetyl-4,6-di-*O*-methylgalactitol after hydrolysis, reduction with $NaBH_4$ and acetylation. This compound was identified by gas-liquid chromatography and characterized by mass spectroscopy (Fig. 4d). Fragments specific for the



1-*O*-acetyl-2-*N*-methylacetamido-2-deoxy group at *m/e* 158, 116 and 74 were detected, but no fragment was found at *m/e* 202. The latter could only be found for those derivatives which were methylated at C-3. The corresponding peak at *m/e* 230, which resulted from fragmentation between C-3 and C-4, was very small. *m/e* 170 resulted from a loss of acetic acid from *m/e* 230. Further fragmentation was very similar to that of the corresponding neutral sugar derivative. The main fragments of the partially methylated alditol acetates of amino sugars mentioned here are listed in Table 2 and compared with those from the corresponding neutral sugar derivatives.

The partially methylated alditol acetates arising from the neutral sugars of the hydrolysates of the aforementioned permethylated tetrahexosylceramide, globoside and trihexosylceramide showed exactly the same fragmentation patterns as the corresponding alditol acetates synthesized in this laboratory as reference compounds. The results were in good agreement with those obtained by Björndal *et al.*[11].

The complete *N*- and *O*-methylation of glycosphingolipids has many advantages, especially the homogeneous behavior of the products in thin-layer

Table 2. Fragments in mass spectra of partially methylated amino sugars and their parent neutral sugars as alditol acetates.

Position of			Peaks occurring in neutral and 2-acetamido-2-deoxyhexitols											
$-CH_3$	-NHAc	$-N(CH_3)Ac$	43	45	71	87	101	117	129	145	161	205	233	
2,3,4,6			×	×	×	×	×	×	×	×	×	×		
3,4,6	2		×	×	×	×	×		×	×	×	×		
3,4,6		2	×	×		×			×	×	×	×		
2,4,6			×	×		×	×	×	×		×		×	
4,6		2	×	×		×	×		×		×			
			Peaks specific for 2-acetamido-2-deoxyhexitols											
			60	74	84	102	116	117	128	142	158	170	188	202
3,4,6	2		×		×	×			×				×	
3,4,6		2		×			×	×		×	×			×
4,6		2		×			×	×		×	×	×		

chromatography. A mixture of glycosphingolipids can be methylated and then be separated using silica gel thin-layer chromatography. The separation of the methylated glycolipids is much more efficient than that achieved following acetylation[17]. In addition very small amounts of glycolipid mixtures can be analyzed for the glycosidic linkages in the individual compounds when the reaction sequence permethylation, hydrolysis, $NaBH_4$-reduction, acetylation is followed.

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Experimental

Preparation of Gal1→3GalNAc1→4Gal1→4Glc-ceramide (neuraminic acid free ganglioside A_2)

A crude human brain ganglioside fraction was isolated according to Klenk *et al.*[18]. It was further purified by the Folch procedure[19]. Repetitive mild acid hydrolysis performed with 0.01N aqueous HCl for 1 h at 80°C according to Klenk *et al.*[20–22] and Gielen[23] released *N*-acetylneuraminic acid. After dialysis and lyophilization the tetrahexosylceramide was purified by silicic acid column chromatography. 100–150 µg of an individual glycolipid was transferred into a tapered small screw cap sealed tube and concentrated to dryness. 0.3 ml 1N HCl was added and the sample heated for 12 h at 100°C under N_2, care being taken that the sample was uniformly suspended. The hydrolysis mixture was then extracted three times with 0.3 ml hexane and three times with chloroform. The combined hexane/chloroform phases were stored for the fatty acid analysis.

The acidic aqueous phase was dried in a stream of nitrogen at 50°C and residual HCl adsorbed on KOH (2 h) under high vacuum. A solution of 100–300 µg $NaBH_4$ (appr. 60- to 100-fold excess) in 0.2 ml water was added to the residue. After 2 h, 2 drops of acetic acid were added for neutralization and the mixture was concentrated at 40°C in a stream of nitrogen. Three portions (100, 50 and 25 µl) of methanol were added at 60°C and the solvent evaporated under N_2. The alditols were then dissolved in 25 µl of pyridine, freshly distilled over BaO, and 25 µl acetic anhydride was added. The capped test tubes were heated for 12 h at 80°C in the dark.

Two drops of water was added and the sample dried in a stream of nitrogen at 60°C. Residual pyridine was evaporated in a high vacuum, the sample repeatedly concentrated with chloroform (3 × 50 µl) and transferred into microtubes for gas-liquid chromatography. Alditol acetates were chromatographed on 3% ECNSS-M on Chromosorb Q, column length 120 cm, temperature 190°C, argon flow rate 30 ml/min.

The molar ratios of Gal:Glc:GalNAc in tetrahexosylceramide was found to be 2:1:1 (see Fig. 3a,c).

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[22] Klenk, E., Hof, L. & Georgias, L. (1967) *this J.* **348**, 149–166.
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Extraction and isolation of globoside and trihexosylceramide from pig erythrocytes

Ghosts were prepared from pig blood according to the method of Dodge *et al.*[24]. Gas-chromatographic analysis of the hexose residues of globoside showed a molar relation identical with that of the tetrahexosylceramide derived from ganglioside A_2. Analysis of trihexosylceramide revealed a molar relation of galactose and glucose of 2:1.

Preparation of N-acetylhexosamines

N-Acetylglucosamine was prepared from glucosamine hydrochloride (purchased from Fa. Schuchardt, München) as described by Roseman and Ludowieg[25]. *N*-Acetylgalactosamine was purchased from Fa. Carl Roth, Karlsruhe. After drying they were used directly for methylation.

Methylation procedure

All solvents used during the methylation procedure and for the preparation of the dimethylsulfinyl carbanion solution were exhaustively dried and distilled immediately before use. This is particularly essential for dimethylsulfoxide. This was refluxed for several hours over finely powdered calcium hydride followed by distillation under reduced pressure. The drying was repeated with highly activated molecular sieve (Union Carbide Corporation type 4 Å). The dry, completely odorless dimethylsulfoxide thus prepared was stored over molecular sieve in the dark until used.

The preparation of the methylsulfinyl anion and the methylation procedure on large scale was similar to the procedure described by Sandford and Conrad[3]. For preparation of the dimethylsulfinyl carbanion 5 g of sodium hydride (50% suspension in mineral oil) was filled into a dry 500 m*l* three necked round-bottom flask containing a magnetic bar, and fitted with a condenser with a closed tap and an inlet tube for dry nitrogen. 200 m*l* of n-pentane was filled through the third neck into the flask, which was closed immediately. Teflon-lined connections were used with the flask to ensure exclusion of moisture. The suspension was magnetically stirred. The pentane supernatant was rapidly decanted, the NaH washed four times with 200 m*l* n-pentane while a stream of dry nitrogen was maintained through the flask. Finally residual pentane was removed in vacuum, the flask regassed with nitrogen and 50 m*l* dimethylsulfoxide added. Nitrogen and hydrogen were allowed to escape through the condenser fitted with a $CaCl_2$-tube. The second neck was fitted with a thermometer. The suspension was magnetically stirred and the temperature was kept at 50°C (oil bath). After 60 to 90 min the hydrogen evolution ceased and the green solution became transparent. The solution was cooled to 4°C, the necks of the flask tightly closed with teflon-lined screw caps and stored at 4°C in the dark until used.

For the methylation stage, the pure glycosphingolipids or sugars were thoroughly dried at 65°C and 10^{-2} to 10^{-3} Torr over P_2O_5. Into a 250 m*l* (500 m*l*) two-necked flask containing 500 mg tetrahexosylceramide (or sugar) and equipped with a thermometer and a screw cap tightly fitted with a rubber septum, 50 m*l* (100 m*l*) dimethylsulfoxide was injected with an all-glass-syringe through the rubber disk. This needle became the inlet for dry N_2 and a second needle was used as the outlet. Glycosphingolipids or sugars were solubilized by magnetically stirring and, if necessary, by slowly raising the temperature up to 50°C. After cooling to room temperature dimethylsulfinyl carbanion was injected into the continuously stirred solution. The amount of dimethylsulfinyl carbanion necessary for the generation of alkoxides was determined by the number of OH-groups and acylamino groups in the glycosphingolipid or sugar. The dimethylsulfinyl carbanion solution was added to the solubilized glycosphingolipid or sugar in about 100% molar excess. After the addition the solutions turned yellow. The temperature was not allowed to rise over 25°C. Stirring was continued for 5–6 h. After cooling the reaction mixture to 20°C, methyl iodide was added slowly to the solution, the temperature being kept below 25°C. Methyl iodide was added in a 100% excess over the dimethylsulfinyl carbanion. The solution became clear and the reaction was completed after appr. 2–3 h. One volume of water was slowly added while the reaction mixture was cooling in an ice bath, so that the temperature did not rise above 30°C. Finally the mixture was neutralized with acetic acid.

For microscale preparations (< 1 mg) small tubes (13 × 100 mm) closed with a rubber disk sealed screw cap were used. The tubes each contained a small magnetic stirring bar. One needle served for the introduction of 200 µ*l* dimethylsulfinyl carbanion solution, 75 µ*l* methyl iodide (injected with all-glass syringes) and nitrogen through the rubber and a second as outlet for escaping gases. The time for formation of alkoxide ions and R-N$^{\ominus}$-acyl residues and for the methylation with methyl iodide was exactly the same as required on a preparative scale. A control of the temperature was usually not necessary. Only during addition of methyl iodide were the tubes cooled in ice water for a short time; during dilution with water after the methylation procedure the reaction mixture was also cooled. Neutralization was performed with two drops of acetic acid.

Isolation and purification of permethylated glycosphingolipids

The reaction mixtures were dialyzed against running tap water to remove the dimethylsulfoxide completely.

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Then the permethylated glycosphingolipids were extracted five times with one third of their volume of chloroform. The chloroform phases were evaporated to dryness or, on a microscale, dried in a stream of nitrogen at 40°C. The completeness of methylation was tested by thin-layer chromatography and, in the case of tetrahexosylceramide, by IR spectroscopy. The latter compound was purified by column chromatography (diameter 2.5 cm, 70 g of silicic acid activated for 12 h at 120°C). Chloroform (300 ml) followed by chloroform/methanol 98:2 served as elution solvents. With the latter solvent the permethylated tetrahexosylceramide was eluted from the column as a colourless compound. The glassy residue of the pooled, concentrated fractions was dried over phosphorus pentoxide at 10^{-2} Torr.

The glycosphingolipids methylated on analytical scale were purified by thin-layer chromatography (solvent system: chloroform/methanol 96:4 or 97:3) and the band localized on the plate by spraying with water.

Isolation and purification of permethylated mono- and disaccharides

The reaction mixture was diluted with water, neutralized with acetic acid and extracted 6–10 times with half its volume of chloroform. The combined chloroform extracts were evaporated in a rotary still at 40°C as far as possible. The dimethylsulfoxide of the residue was distilled off at about 70°C and 10^{-2} Torr under continuous stirring with a magnetic bar until a viscous residue remained. This was purified by silicic acid column chromatography. 100 g silicic acid per g of residue was required to remove coloured by-products and the remaining dimethylsulfoxide. Elution was performed first with chloroform until all impurities in the front were removed. The two anomeric forms of pure permethylated sugars appeared in two bands when eluted with chloroform and various amounts of methanol, depending on the nature of the sugar: permethylated hexoses and lactose were eluted with 1% and acetamidohexoses with 2% methanol in chloroform. In this way per-*O*-methylated *N*-methylacetamidoglucose was separated from the corresponding non-*N*-methylated derivative. The purity of the fraction was checked by silica gel thin-layer chromatography.

Microscale preparation of partially methylated alditol acetates

250 µg amounts of pure permethylated glycosphingolipid or permethylated sugar were dissolved in 100 µl of 0.7N sulfuric acid in 80% aqueous acetic acid and heated in small screw capped, tapered tubes at 80°C for 20 h. The hydrolysate was diluted with 300 µl water and slowly filtered through 100 mg of dried Dowex 1X10 (acetate form) packed in a disposable Pasteur pipette. The column was eluted three times with 100 µl methanol. Eluates were collected in a second tapered microtube of the type described above. The sample was concentrated to dryness in a stream of nitrogen at 40°C and dissolved in 100 µl water. In case of glycosphingolipids the released fatty acids were extracted twice with 250 µl redistilled n-hexane. Phases were separated by centrifugation and the upper phase discarded. 500 µg sodium borohydride (in case of amino sugar 1.5 mg) in 50 µl water was added and, after standing overnight at room temperature, the mixture was neutralized with one drop of acetic acid and taken to dryness under a stream of nitrogen at 35°C.

Thin-layer chromatography of the different glycosphingolipids or sugars and their derivatives on an analytical and preparative scale was carried out using the following solvent system:

glycosphingolipids: chloroform/methanol/water 65:25:4 or 60:35:8

permethylated mono- and disaccharides: chloroform/methanol 95:5

permethylated glycosphingolipids: chloroform/methanol 96:4.

Silica gel H chromatoplates were used in all instances. Glycosphingolipids or sugar derivatives were visualized by staining with 50% sulfuric acid in water or with anthrone reagent[26].

IR spectroscopy was carried out on a Perkin Elmer 257 Grating Infrared Spectrometer.

NMR spectroscopy was carried out on a Varian 60 MHZ NMR Spectrometer. The samples of appr. 20 mg were solubilized in carbon tetrachloride with tetramethylsilane as an internal reference.

Mass spectroscopy was performed at 300 µA and 70 eV with the Varian model MAT CH 5 coupled to a Varian Aerograph. Columns and conditions were chosen as described below.

Gas-liquid chromatography: Analyses were performed with a Perkin Elmer F 20 gas chromatograph packed with 3% ECNSS-M on gaschrom Q 100–120 mesh (Applied Science Laboratories Inc.) with a column length of 120 cm for partially methylated neutral and acetamidoalditol acetates at 155°C; after 32 or 34 min the temperature was changed suddenly to 210°C. Partially methylated neutral alditol acetates were separated on 3% OV-225 on gaschrom Q 100–120 mesh (Applied Science Laboratories Inc.) under isothermal conditions at 180°C, with a column length of 200 cm.

Note added in proof: After this paper had been submitted, we got notice of a permethylation study combined with an enzymatic degradation study of cytolipin R[27]. No gas-liquid chromatographic and mass-spectral data regarding the *N*-acetylgalactosamine residue and its linkage were given.

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