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N- and *O*-Alkylation of Glycoconjugates and Polysaccharides by Solid Base in Dimethyl Sulphoxide/Alkyl Iodide

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_O_-Methylation of simple neutral oligosaccharides is readily accomplished in dimethyl sulphoxide containing solid sodium hydroxide and methyl iodide [Cincanu I, Kerek F (1984) Carbohydr Res 131:209-17]. This procedure has been extended to 2-acetamido-2-deoxy sugars and sialic acid-containing oligosaccharides. Complete _O_- and _N_-methylation was in most cases achieved in 15 min. Esterification of carboxylic groups in uronic acids was fast and resulted in concomitant β-elimination. The method is also suitable for methylation of glycoproteins and glycosphingolipids. Polysaccharides can also be methylated by this technique. Analysis of the products by gas-liquid chromatography and mass spectrometry showed no degradation products.

Methylation is a common procedure to derivatize carbohydrates for analysis by GLC and gas-liquid chromatography-mass spectrometry (GLC-MS). Earlier methods of Purdie [1] and Haworth [2] and developments of these [3, 4] have the limitation of incomplete methylation especially of polysaccharides and acetamido sugars. Today the Hakomori method [5] is considered to be the most efficient procedure but still there are complications in methylation of large polysaccharides and acetamido sugars.

The methylation method based on the use of solid bases in dimethyl sulphoxide (DMSO) and methyl iodide (MeI) showed complete *O*-methylation of neutral oligosaccharides [6] and by changing the solvent system polysaccharides are methylated in one step [7]. This investigation reports complete *O*- and *N*-methylation of oligosaccharides, glycoproteins, glycosphingolipids and polysaccharides by treatment with solid NaOH in DMSO/MeI.

Abbreviations: lacto-*N*-tetraose, $LcOse_4$, Galβ3GlcNAcβ3Galβ4Glc; lacto-*N*-fucopentaose III, III^3Fuc-$nLcOse_4$, Galβ4[Fucα3]GlcNAcβ3Galβ4Glc; trihexosylceramide, $GbOse_3Cer$, Galα4Galβ4Glcβ1-1Cer; globoside, $GbOse_4Cer$, GalNAcβ3Galα4Galβ4Glcβ1-1Cer; FAB-MS, fast atom bombardment mass spectrometry.

Materials and Methods

Materials

Methyl 2-acetamido-2-deoxy-α-D-glucopyranoside (Me α-GlcNAc) was kindly provided by Dr. Bo Nilsson (BioCarb AB, Lund, Sweden). D-Glucopyranosiduronic acid (GlcA) and D-galactopyranosiduronic acid (GalA) were purchased from Janssen Chimica (Beerse, Belgium). Methyl α-D-glucopyranosiduronic acid (Me α-GlcA) was prepared by catalytic oxidation (Pt/O_2) of methyl α-D-glucopyranoside [8]. Nigerose (α-D-Glc-(1-3)-D-Glc) was purchased from Sigma (St. Louis, MO, USA). Chitobiose, isomaltotriose, 3-fucosyllactose, 3'-sialyllactose, lacto-*N*-tetraose, lacto-*N*-fucopentaose III, trihexosylceramide and globoside were obtained from BioCarb Chemicals (Lund, Sweden). Gangliosides (G_{M3}, G_{M1}, G_{D3}, G_{D1a} and G_{D1b}) were isolated from calf brain [9]. Fetuin was prepared from fetal calf serum [10] and desialylated by mild acid hydrolysis [11]. Native dextran and dextran 158 (Mol wt 158 000) were from Pharmacia (Uppsala, Sweden) and native glycogen was purchased from Merck (Darmstadt, W. Germany). All solvents were of HPLC grade and all other chemicals and reagents were of analytical grade or better.

Methylation

Microvials (Supelco Inc. PA, USA) with magnetic stirrers were used. A solution of carbohydrate (0.5-1.0 mg) in DMSO (100-200 μl) was added to finely powdered NaOH (8-16 mg) in a microvial. MeI (12.5-25 μl) was added to the solution and it was left with stirring for 15-60 min at room temperature. The reaction mixture was neutralized with 1 M acetic acid (200-400 μl) then water (100-200 μl) was added and the product was extracted into chloroform (3 × 0.5 ml). The combined chloroform phases were washed three times with water (5 ml). Methylation of glycoproteins and glycosphingolipids was performed in the same way. Glycoproteins were dialysed against distilled water after neutralization with acetic acid and removal of excess methyl iodide. Glycosphingolipids were neutralized with acetic acid and then purified on SEP-PAK C_{18} cartridges (Waters Associates AB, Partille, Sweden) [12, 13]. The cartridges were consecutively prewashed with chloroform (20 ml), methanol (10 ml), acetonitrile (2 × 2 ml) and finally water (2 × 2 ml). The sample was applied to the cartridge with a syringe and pushed through the cartridge bed (1-2 drops per second) until the liquid level was just above the resin bed. The microvial was rinsed with DMSO/water, 1/1 by vol (0.5 ml), and the solution was applied to the cartridge in the same manner as the sample solution. The more polar contaminants of the methylation procedure were eluted from the cartridge with five 5 ml flushes of water and then two 2 ml flushes of acetonitrile. The methylated glycosphingolipid was removed from the cartridge by elution with 2 ml of methanol and then two 2 ml flushes of chloroform. Smaller glycosphingolipids were eluted with the methanol and larger ones with the chloroform.

Analytical Procedure

A Perkin-Elmer Sigma 1 gas chromatograph equipped with a flame ionisation detector, a splitless injector and a DB-1 fused silica capillary column (30 m × 0.25 mm) was used. GLC-MS was performed on a VG MassLab 1250 quadropole instrument linked to a Hewlett-Packard 5790 gas chromatograph equipped with splitless injection and appropriate column. The spectra were recorded at 70 eV with an ion source temperature

Table 1. Methylation of carbohydrates by NaOH-DMSO-MeI, according to the conditions described in the Materials and Methods section.

Compound	Yield (%)[a]
Me α-GlcNAc	90[b]
Me α-GlcNAc	110
Chitobiitol	70[b]
Chitobiitol	130
3-Fucosyllactose	100
3-Sialyllactose	60
3-Sialyllactose	110[c]
Lacto-*N*-tetraose	110

[a] Compared to Hakomori methylation (set to 100%). Determined by GLC-MS versus internal standard (perseitol or isomaltotriose).
[b] Conditions as in [6].
[c] Reaction time 30 min.

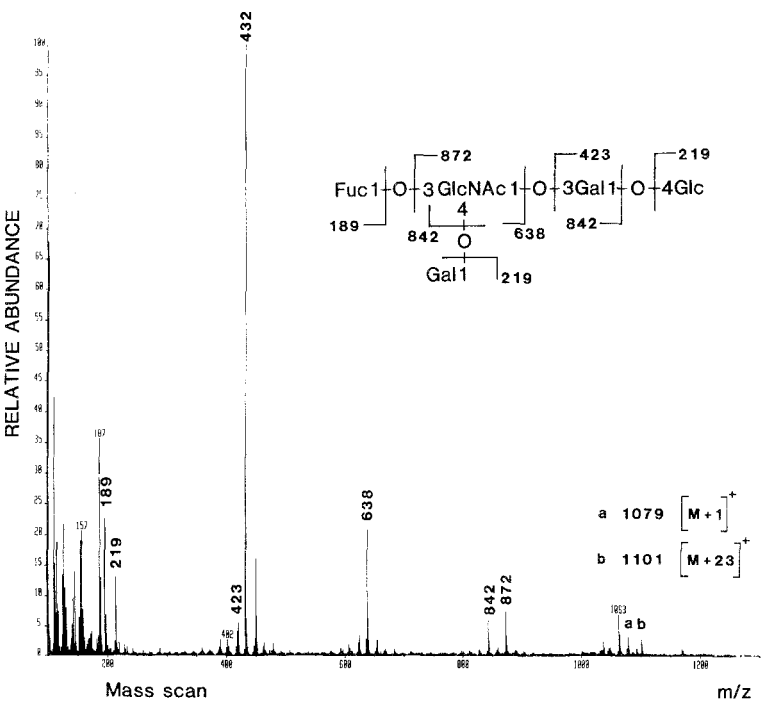

Figure 1. FAB Mass spectrum of lacto-*N*-fucopentaose III (LNF III) after permethylation.

Table 2. Methylation analysis of polysaccharides performed with NaOH-DMSO-MeI (A) or the Hakomori procedure (B)

Partially methylated alditol acetates	Weight (%) of found carbohydrates					
	Dextran 158		Dextran		Glycogen	
	A	B	A	B	A	B
2,3,4,6-Me-Glc	5	4	5	4	8	9
2,3,4-Me-Glc	91	93	87	90	-	-
2,3,6-Me-Glc	-	-	-	-	81	80
2,4-Me-Glc	3	2	6	4	-	-
2,6-Me-Glc	-	-	-	-	+[a]	1
3,6-Me-Glc	-	-	-	-	+	+
2,3-Me-Glc	+	+	+	+	9	9
2-Me-Glc	-	-	+	+	-	+
1,2,3,4,5,6-Ac-Glc	+	1	1	1	+	+

[a] trace amounts (< 1%).

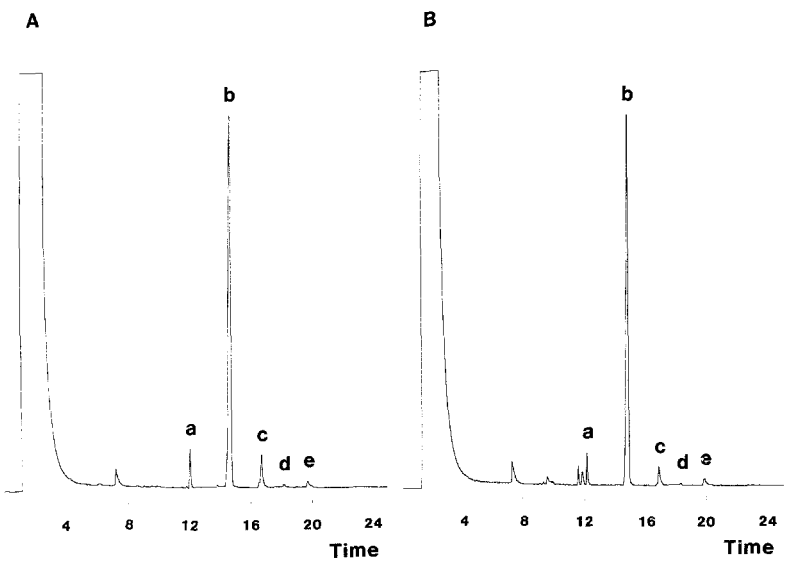

Figure 2. Gas-liquid chromatogram of partially methylated alditol acetates from native dextran after (A), methylation with NaOH-DMSO-MeI; and (B) with the Hakomori procedure followed by hydrolysis, reduction and acetylation.
a; 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucitol
b; 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-glucitol
c; 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-glucitol
d; 1,3,4,5,6-penta-*O*-acetyl-2-*O*-methyl-glucitol
e; 1,2,3,4,5,6-hexa-*O*-acetyl-glucitol

of 250°C and processed by an on-line computer (PDP 11/23, DEC, USA). Fast atom bombardment-mass spectrometry (FAB-MS) was performed on a VG ZAB-SE mass spectrometer. The spectra were processed on an on-line computer system (PDP 11/24, DEC, USA).

Results and Discussion

Methylation of Oligosaccharides

When Me α-GlcNAc was methylated according to the method of Cincanu and Kerek [6] almost complete *O*- and *N*-methylation was achieved after 15 min at room temperature. Reduced chitobiose gave under the same conditions complete *O*-methylation but only 70% *N*-methylation. By extending the reaction time to 45 min complete *O*- and *N*-methylation was accomplished. It was observed that the molar proportion between MeI and replaceable protons was very critical for optimal methylation. The amount of MeI was increased to 20-30 equivalents per mol replaceable protons compared to the 3-7 equivalents recommended by Cincanu and Kerek [6]. Chitobiitol was completely *O*- and *N*-methylated after 15 min at room temperature using the higher amount of MeI. The procedure was then tested on higher oligosaccharides (reduced and unreduced) and complete *O*- and *N*-methylation was achieved after 15-30 min (Table 1). FAB-MS of permethylated lacto-*N*-fucopentaose III showed complete *O*- and *N*-methylation seen by the presence of m/z 432, 638 and 872 and by the lack of these m/z-14 (Fig. 1). No degradation of glycosidic linkages could be seen in the oligosaccharides tested. Therefore nigerose, labile to alkaline conditions, was treated in the same way for 90 min and only trace amounts (< 1%) of degradation products were seen.

When applied to unreduced and reduced glucuronic and galacturonic acids fast esterification and complete *O*-methylation occurred with concomitant β-elimination. Glucuronic acid was degraded to a greater extent than galacturonic acid and appeared on GLC-MS among other things as the permethylated 4-deoxy derivative. This is in contrast to reports that the corresponding anomeric acetylated galactopyranuronate is more sensitive to β-elimination than the respective glucopyranuronate [14].

Methylation of Polysaccharides, Glycosphingolipids and Glycoproteins

The polysaccharides dextran 158, dextran and glycogen were dissolved in DMSO and methylated for 60 min. After dialysis, hydrolysis, reduction and acetylation the polysaccharides showed by GLC-MS the expected structural elements in correct proportions (Table 2). Native dextran and glycogen showed minor undermethylation, shown by the existence of peracetylated glucitol, performed with both methylation procedures. The amount of hexa-acetate was less in the polysaccharides methylated by the described method than by the Hakomori method (Table 2, Fig. 2). This indicates that the NaOH-DMSO-MeI methylation is as good as the Hakomori procedure in methylating polysaccharides.

Permethylation of glycosphingolipids proceeds smoothly using NaOH-DMSO-MeI methylation as was reported during the processing of this manuscript [15]. In the present work methylation of G_{D1a}-ganglioside showed complete *O*- and *N*-methylation after 30 min as seen by the presence of m/z 344, 376 and 825 and the lack of these m/z-14

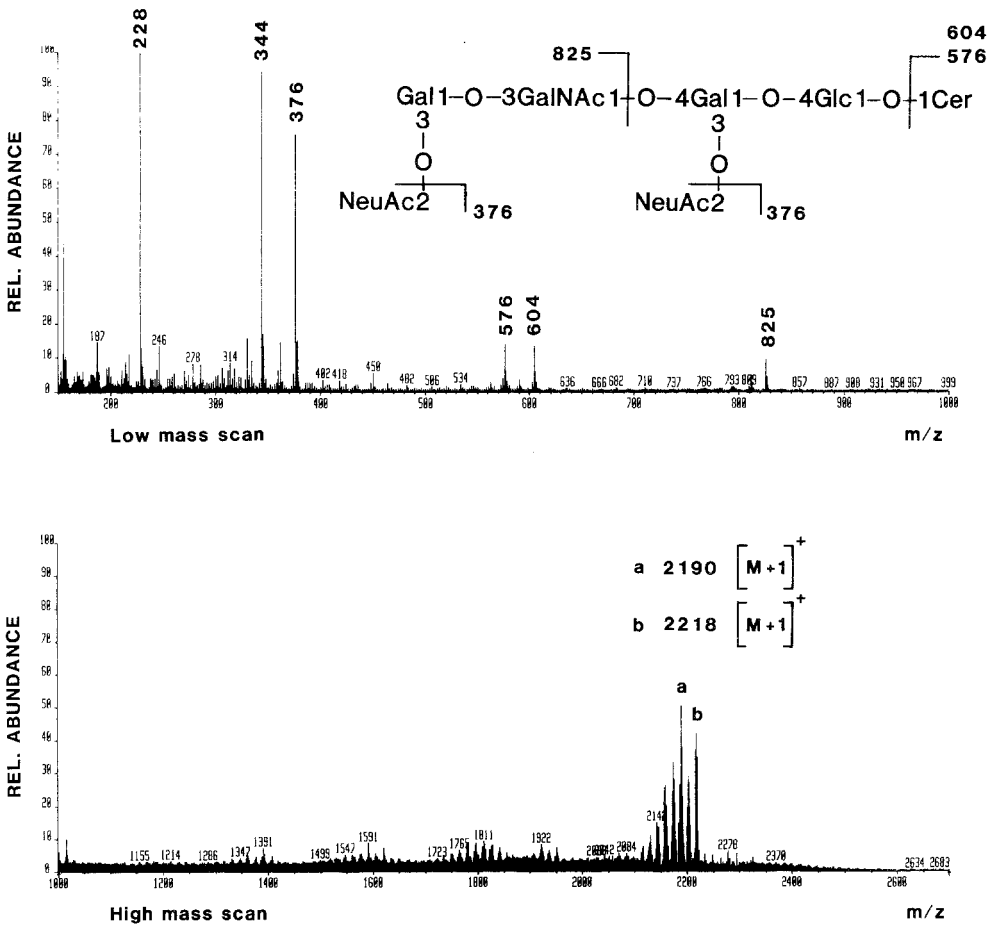

Figure 3. FAB Mass spectrum of G_{D1a}-ganglioside after permethylation.

on FAB-MS analysis (Fig. 3). The existence of m/z 330 and 362 can arise in other ways and is not necessarily an indication of incomplete *N*-methylation [16]. When applied to glycoproteins (fetuin and asialofetuin) methylation for 30 min by the described method gave the same relationship between the individual structural elements as with Hakomori methylation analysed by GLC-MS as partially methylated alditol acetates.

Ethylation and Propylation of Oligosaccharides

O-Ethylation of oligosaccharides could be obtained in the same or higher yield as with the Hakomori method. By extending the reaction time to 1 h *N*-ethylation was quantitative. *N*-propylation could not be achieved quantitatively by either of the two methods but here the Hakomori method gave the best results.

In conclusion, the reaction proceeds rapidly at room temperature without any degradation of base susceptible linkages. The methylation is easy to perform with no need to

prepare any effective basic agent in advance. It yields products suitable for analysing small amounts of carbohydrates by GLC-MS. In general, a 15 min reaction time is sufficient to *O*- and *N*-methylate neutral oligosaccharides and glycosphingolipids. Oligosaccharides and glycoconjugates containing sialic acid residues need a longer reaction time, 30 min, for complete *O*- and *N*-methylation and polysaccharides 60 min due to the lower solubility in the solvent. There is also the possibility of achieving β-elimination from uronic acids for structural studies of polysaccharides in one step instead of the two-step procedure using Hakomori methylation [17]. Methylation of larger amounts of carbohydrate (> 100 mg) has been performed without any complications. Potassium hydroxide can also be used in the same manner instead of sodium hydroxide.

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