THE CHARACTERIZATION BY MEANS OF NMR SPECTROSCOPY OF PARTIALLY METHYLATED MONOSACCHARIDES OBTAINED IN PERMETHYLATION ANALYSIS

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SUMMARY

A method has been developed for identification of partially methylated monosaccharides using PMR and CMR spectroscopy. The free hydroxyl groups of a partially methylated monosaccharide are blocked with deuterium or carbon-13 labelled methyl groups. By comparison of the PMR or CMR spectra of the formed permethyl derivatives with those of standard permethyl monosaccharides the nature of the monomer and the positions of the methoxyl groups can be determined. The PMR and CMR spectra of the α- and β- anomers of D-glucopyranose, D-galactopyranose and D-mannopyranose are investigated ; the spectral data of the methoxyl groups and also those of the ring atoms are characteristic for each of the monomers.

The configuration of glycosidic linkages in oligo- or polysaccharides can be determined from the chemical shifts and the coupling constants of the anomeric protons or from the chemical shifts of the anomeric carbons in the spectra of the intact permethylated oligo- or polymers.

On the basis of the proton coupling constants, which were obtained from 220 MHz PMR spectra, the conformation of the permethylated glucopyranoses was established by using a modified Karplus equation, to be $^4C_1$ (D).

RESUME

CARACTERISATION PAR RMN DES MONOSACCHARIDES PARTIELLEMENT METHYLES OBTENUS PAR PERMETHYLATION DES GLUCIDES

La résonance magnétique nucléaire (RMN) du 1-H et du 13-C a été utilisée pour le développement d'une méthode d'identification des monosaccharides partiellement méthylés. Les groupes hydroxyles libres des monosaccharides ont été marqués par des groupes $-CD_3$ ou par $-^{13}CH_3$. Par comparaison des spectres de RMN des dérivés perméthylés formés et ceux des monosaccharides perméthylés standards, la nature des monomères et la position des groupes méthoxyles peuvent être déterminées. Les RMN du 1-H et du 13-C des α- et des β- anomères de D-glucopyranose, de D-galactopyranose et de D-mannopyranose ont été étudiées. Les pics des groupes méthoxyles des spectres de RMN et aussi ceux des carbones du cycle glycosidique sont caractéristiques pour chaque monomère.

La configuration des liaisons glycosidiques des oligo- et des polysaccharides peut être déterminée à partir des déplacements chimiques et des constantes de couplages des protons anomères ou des déplacements chimiques des carbones anomères des spectres des oligo- ou polymères intacts perméthylés. La conformation des glucopyranoses perméthylés a été établie par les constantes de couplages des protons des spectres de RMN du 1-H à 220 MHz en utilisant une modification de l'équation de Karplus : $^4C_1$ (D).

INTRODUCTION

One of the most useful techniques in the structural analysis of oligo- and polysaccharides is the preparation of the complete methylated compounds, followed by cleavage of the glycosidic bonds, yielding methylated monosaccharides. The suitability of the permethylation analysis depends on the availability of appropriate techniques for the separation and identification of the (partially) methylated monosaccharides. Several approaches are in use for this analysis. For the separation of the sugar derivatives as such or after chemical modification and blocking of the free hydroxyl groups, the mixture is often subjected to paper-, thin-layer-, column- or gas-liquid chromatography. The identity of the methylated monomers or of suitable derivatives can be determined by comparison with reference compounds. However due to the little differences in structure the identification of methylated monosaccharides is difficult and a vast amount of model compounds is indispensable. Analytical techniques which are frequently used are the following : optical rotation measurement, melting point determination and chromatographical methods eventually in combination with mass spectrometric detection.

Only little attention has been paid to the potencies of nuclear magnetic resonance spectroscopy in the identification of partially methylated monosaccharides (1-3). Proton magnetic resonance spectral data of most of the partially methylated galactopyranoses and galactopyranosides are published by Rathbone et al. (2). The various methoxyl groups can be brought into resonance completely separate from each other. The assignment of these signals has been carried out by comparison of the resonance positions in the spectra of galactopyranoses with methoxyl groups in different positions. The chemical shifts of the methoxyl resonances appear to vary to some extent if the spectra of the various methylated galactopyranoses are compared. Hence the identification of partially methylated monosaccharides can be unequivocally performed if the parent monosaccharide is known and if reference data of compounds with exactly the same structure are available.

During the last years some CMR spectral data of carbohydrates and carbohydrate derivatives have been reported (4-14). A few partially methylated glycoses and methyl glycosides were studied. The CMR chemical shift data of the methoxyl signals are also useful in identification studies.

METHODS AND RESULTS

DETERMINATION OF POSITIONS OF METHOXYL GROUPS IN MONOSACCHARIDE DERIVATIVES

In this report a technique is described to determine the nature of the monomer and the positions of methoxyl groups in a partially methylated monosaccharide by PMR and CMR spectroscopy. The free hydroxyl functions of a partially methylated monosaccharide are blocked with deuterium or carbon-13 labelled methyl groups and the NMR spectra of the formed permethyl monosaccharide anomers are studied. The results of this investigation show that by means of the chemical shift values and the relative intensities of the methoxyl resonances in the PMR and CMR spectra of permethyl monosaccharides the location of trideuteromethoxyl groups or of methoxyl groups enriched in carbon-13 can be established unambiguously. In this

way a partially methylated glycose or glycoside which is obtained from the degradation of a permethylated oligo- or polymer can be characterized and therefore the position(s) of glycosidic bond(s). The number of reference compounds necessary for identification is reduced to a maximum of four : the permethyl pyranoses and furanoses of each monosaccharide.

The methoxyl resonance data from PMR and CMR spectra of various permethyl monosaccharides differ significantly from each other. From the most important aldohexoses : glucose, galactose and mannose the permethyl pyranose forms are investigated. Their PMR spectra, as well as their CMR spectra show five distinct methoxyl singlets if recorded in acetonitrile $-d_3$ as solvent. The chemical shift data are given in Table I.

TABLE I - PMR AND CMR CHEMICAL SHIFTS (a) OF METHOXYL GROUPS IN SOME PERMETHYLATED ALDOHEXOPYRANOSES

Permethyl ether of	PMR					CMR				
α-D-Gp (b)	3.49	3.43	3.37	3.30	3.29	60.71	60.55	59.23	58.40	55.27
β-D-Gp (b)	3.51	3.45	3.43	3.41	3.31	60.74	60.48	60.39	59.30	56.96
α-D-Galp (c)	3.43	3.39	3.35	3.30	3.28	61.31	59.19	58.52	58.11	55.30
β-D-Galp (c)	3.43	3.42	3.40	3.39	3.30	61.26	60.64	59.18	58.27	56.82
α-D-Manp	3.40	3.37	3.36	3.30	3.27	60.51	59.15	59.09	57.48	55.11 (d)
β-D-Manp	3.44	3.39	3.38	3.37	3.31	61.26	60.64	59.29	57.36	57.17

(a) Determined at 100 MHz (PMR) and at 25.2 MHz (CMR) for solutions in acetonitrile-d_3. δ-values are given in ppm relative to TMS.
(b) For the assignments of these resonances see Table II.
(c) These resonances are assigned to the following positions (mentioned consecutively from low to high field) :
4, 3, 2, 6, 1 in the PMR spectrum of the α anomer
2, 4, 3, 1, 6 in " " " " " β "
4, 6, 2, 3, 1 in the CMR " " " α "
4, 2, 6, 3, 1 in " " " " " β "
(d) This resonance is assigned to 1-OMe.

The unequivocal assignment of the methoxyl resonances in the glucose and galactose derivatives was carried out by specific introduction of deuterium or carbon-13 labelled methoxyl groups. For the permethyl glucopyranose anomers the assignment was effected by comparison of the methoxyl chemical shifts in the spectra of the deuterium or carbon-13 labelled permethylated compounds, mentioned in Table II.

The application of the chemical shift method to gluco-oligomers is demonstrated in the determination of the $4\text{-}OCH_3$ and $6\text{-}OCH_3$ resonances in the permethyl glucopyranoses. Methanolysis of permethyl maltose α-D-Gp(1→4)D-Gp and permethyl gentiobiose β-D-Gp(1→4)D-Gp yields methyl 2,3,6-tri-O-methyl-glucosides and methyl 2,3,4-tri-O-methyl-glucosides respectively, besides the methyl 2,3,4,6-tetra-O-methyl-glucosides. The permethylated glucoses were separated from the partially methylated products by preparative TLC. The former compounds we-

TABLE II - PMR AND CMR CHEMICAL SHIFTS OF METHOXYL GROUPS FOR METHYL 2,3,4,6-TETRA-O-METHYL-α- AND β-D-GLUCOPYRANOSIDES

Starting Compound	Permethylation (a)	Permethyl ether with labelled methoxyl groups in position(s)	PMR (b, c) 1	2	3	4	6	CMR (d, e) 1	2	3	4	6
α-D-Gp			3.29	3.36	3.49	3.43	3.30					
Me α-D-Gp		2 3 4 6	3.29	- d	-	-	-	55.27	58.36[e]	60.69	60.52	59.24
3-O-Me—α-D-Gp		1 2 4 6	-	-	3.48	-	-	55.28	58.43	60.75	60.60	59.22
2,3-di-O-Me—α-D-Gp		1 4 6	-	3.37	3.49	-	-	55.27	58.41	60.71	60.55	59.24
Me 2,3,6-tri-O-Me—α-D-Gp		4	3.29	3.37	3.49	-	3.30	55.31	58.41	60.72	60.53	59.25
Me 2,3,4-tri-O-Me—α-D-Gp		6	3.29	3.37	3.49	3.44	-	55.24	58.38	60.68	60.49	59.22
β-D-Gp			3.41	3.45	3.51	3.43	3.31					
Me β-D-Gp		2 3 4 6	3.41	-	-	-	-	56.97	60.47	60.78	60.57	59.30
3-O-Me- β-D-Gp		1 2 4 6	-	-	3.51	-	-	57.02	60.42	60.78	60.51	59.34
2,3-di-O-Me-β-D-Gp		1 4 6	-	3.45	3.51	-	-	56.91	60.34	60.69	60.41	59.30
Me 2,3,6-tri-O-Me-β-D-Gp		4	3.41	3.45	3.51	-	3.31	56.96	60.36	60.72	60.45	59.34
Me 2,3,4-tri-O-Me-β-D-Gp		6	3.41	3.45	3.51	3.43	-	56.96	60.37	60.72	60.44	59.35

(a) For PMR spectroscopy α-D-Gp and β-D-Gp were permethylated, the other compounds were pertrideuteromethylated. For CMR spectroscopy the compounds, except α-D-Gp and β-D-Gp, were permethylated with 5 or 10% 13-CH_3I. (b) Determined at 100 MHz for solutions in acetonitrile-d_3, δ-values are given in ppm relative to TMS. (c) - : Signal is missing as a result of the introduction of OCD_3 at this position. (d) Determined at 25.2 MHz for solutions in acetonitrile-d_3, δ-values are given in ppm relative to TMS. (e) The resonances of the methoxyl groups enriched in 13-C are underlined.

re identified as such by GLC and NMR spectroscopy. Remethylation of the partially methylated compounds with CH_3I, containing 5% carbon-13 gave permethyl α- and β-D-glucopyranoses which were separated by TLC. A simple model compound, cotton wool cellulose was permethylated and methanolysed. After remethylation with CD_3I the permethyl α- and β-D-glucopyranoses were separated by preparative TLC. In the PMR spectra as well as in the CMR spectra the 4-methoxyl signal was absent indicating the (1→4) glycosidic linkages in the polymer.

From these results it is to be expected that this method is a powerful replenishment in permethylation analysis.

The choice of OCD_3- or $O^{13}CH_3$ label depends on the type of problem to be solved and the preference for one of these labels will often be determined by the amount of material available for analysis. In the proton-noise decoupled CMR spectrum the carbon-13 label increases the intensity of the methoxyl signal whereas deuterium labelling decreases the intensity of the carbon resonance due to ^{13}C-D spin-spin couplings. If the carbon-13 label is taken for remethylation the degree of ^{13}C labelling must be high enough to overcome the variations in resonance intensities arising from differences in relaxation times and NOE contributions. We found 5-10% labelling to be sufficient. The resonances of the methoxyl groups at different positions in the molecule but at the same isotopic abundance have almost identical intensities under the conditions used.

The extension with Fourier Transform in both PMR and CMR spectroscopy has lowered the amount of material necessary for analysis. For PMR spectroscopy less than one mg. of a monosaccharide derivative is sufficient. For CMR about 10 mg. is necessary to detect the resonances of the carbon-13 atoms in natural abundance. The carbon-13 labelled methoxyl groups are already detectable in a smaller sample, however this gives loss of information about the carbon skeleton.

SPECTRAL DATA OF THE RING ATOMS

Besides the NMR parameters of the methoxyl groups also those of the ring protons and ring carbons give valuable information and this can be used for the characterization of monomers (It has to be kept in mind that there are also other methods available to identify permethylated monosaccharides).

In the PMR spectrum of permethyl monosaccharides the resonances of the skeleton protons coincide with those of the methoxyl groups with the exception of the anomeric protons. Therefore only the chemical shifts and the coupling constants of the latter protons can be applied for the identification of the monosaccharide.

In the CMR spectra the skeleton carbon resonances are quite characteristic for the monosaccharide permethyl ethers as illustrated in Table III for the pyranose forms of glucose, galactose and mannose.

DETERMINATION OF THE CONFIGURATION OF GLYCOSIDIC LINKAGES IN OLIGO- AND POLYMERS

Permethylation analysis as such gives no indication about the stereo-chemistry of the glycosidic bonds. However the configuration of these linkages can be directly inferred

TABLE III - CMR CHEMICAL SHIFTS (a) OF THE SKELETON CARBONS OF SOME PERMETHYLATED ALDOHEXOPYRANOSES

Permethyl ether of	CMR					
α-D-Gp (b)	98.16	84.28	82.58	80.61	72.41	70.98
β-D-Gp (b)	105.00	87.21	84.58	80.48	75.38	72.36
α-D-Galp	98.80 (c)	80.97	78.78	77.25	72.34 (d)	69.88
β-D-Galp	105.26 (c)	84.65	81.50	76.10	73.86	72.01 (d)
α-D-Manp	99.33 (c)	82.34	77.71	77.12	72.58	72.21
β-D-Manp	103.42 (c)	84.78	77.73	77.18	76.13	72.64 (d)

(a) δ-values are given in ppm relative to TMS. (b) These resonances are assigned to the following carbons (mentioned consecutively from low to high field): C_1, C_3, C_2, C_4, C_6, C_5 in the α anomer and C_1, C_3, C_2, C_4, C_5, C_6 in the β anomer (26). (c) This resonance is assigned to C_1. (d) This resonance is assigned to C_6.

from the coupling constants and the chemical shifts of the inter-sugar anomeric protons in the PMR spectra of the permethylated oligo- and polysaccharides (15, 16). As we described for TMS ethers, this method has some restrictions (17). The chemical shifts of inter-sugar anomeric protons in α-glycosidic linkages (doublet $J_{1,2}\sim 3{,}5$ Hz) occur in a lower field region (δ 5.0-5.7 ppm) than the chemical shifts of protons in β-glycosidic linkages (doublet $J_{1,2}\sim$ 7.0 Hz ; δ 4.0-5.0 ppm)*. These chemical shifts are only slightly influenced by the configuration of the methyl glycosidic group.

The configuration of the inter-sugar linkages can also be revealed from the chemical shift values of the anomeric carbons in the CMR spectra. This is shown in figure 1 for the permethylated maltose and lactose anomers. The anomeric carbons resonate in the range δ=96-106 ppm relative to TMS. Axial substituted carbons in a pyranose ring are more shielded than equatorial substituted ones (12). C_1 in permethyl α-D-glucopyranose resonates at higher field than C_1 in the β-anomer. In permethyl maltose almost the same chemical shift values for $C'_{1\alpha}$ and $C'_{1\beta}$ are observed as in the corresponding permethyl glucosides. The resonance at about 96.7 ppm is assigned to C_1. The spectrum of the anomeric mixture of permethyl maltose shows three resonances in the range of the axial substituted anomeric carbons (∿96-100 ppm) and only one resonance in the range of the equatorial substituted carbons (∿102-106 ppm). Therefore the glycosidic bond has α-configuration. The CMR spectrum of the anomeric mixture of per-

* These regions are given by Minnikin (15) for spectra of permethylated gluco- and galactodisaccharides, recorded in C_6D_6 or $CDCl_3$ solutions. The spectra of permethylated glucose or galactose containing disaccharides recorded in CD_3CN show corresponding resonances with identical coupling constants in the same δ-regions. Because of the little difference between the chemical shift regions of the protons in α and β glycosidic linkages it is advisable to handle these ranges with care.

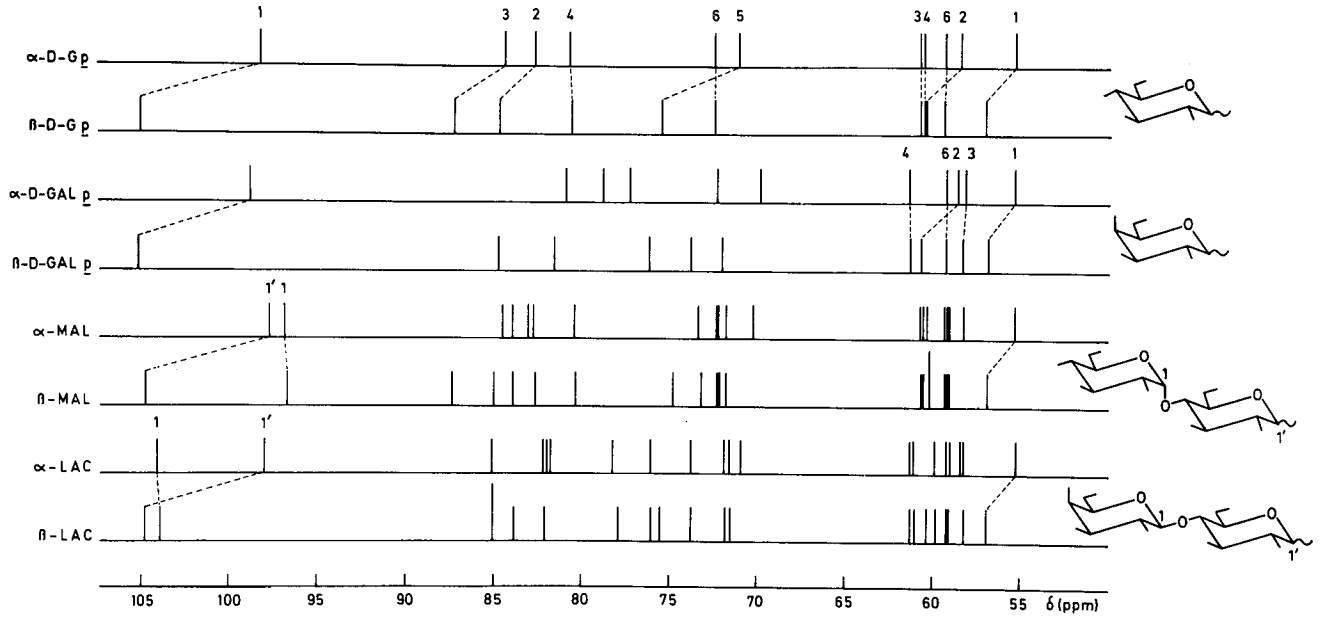

Fig. I - CMR SPECTRA OF PERMETHYLATED α- AND β-ANOMERS OF D-GLUCOPYRANOSE D-GALACTOPYRANOSE MALTOSE AND LACTOSE.

methyl lactose shows three resonances in the range of β anomeric carbons and one in the α range which is in accordance with a β glycosidic bond. It is notable that the inter-sugar anomeric carbons resonate (∿1 ppm) at higher field than the methyl glycosidic anomeric carbons. This observation may be useful for spectrum interpretation. From permethyl cellulose no CMR spectrum could be obtained because of the low solubility of the compound.

THE CONFORMATION OF THE PYRANOSE RINGS

The ring conformation of saccharides can be deduced from the PMR parameters of the protons attached to the ring. The $^4C_1$ (D) (18) conformation was demonstrated for permethyl glucopyranose anomers on the basis of the coupling constants obtained from the 220 MHz PMR spectra of pertrideuteromethylated α- and β-D-glucopyranose in acetonitrile-d_3. The experimental coupling constants were refined by spectrum simulation. For the calculation of the vicinal coupling constants $J_{1,2}$, $J_{2,3}$, $J_{3,4}$ and $J_{4,5}$ in dependence of the dihedral angle ϕ between H and H' the following modified Karplus equation was used (19).

$$J_{H,H'} = (6.6 - 1.0 \cos \phi + 5.6 \cos 2\phi)(1-\sum_{i=1}^{4} f_i \Delta X_i)$$

The experimental coupling constants were compared with those for ideal conformations obtained by calculation from the dihedral angles in Dreiding molecular models. The electronegativity factor f_i was taken 0.15 for $\Theta>90°$ and 0.05 for $\Theta<90°$ (Θ is the dihedral angle between the substituent R and proton H in the system R-C-C-H) the electronegativity X of the substituents is $X_{or} = 3.3$; $X_{-C-O-} = 2.5$; $X_{OMe} = 3.3$ and $X_H = 2.1$. The values for coupling

constants and chemical shifts obtained are given in Table IV. The experimental coupling constants agree well with those calculated for $^4C_1$ (D) conformations (Table V).

TABLE IV - PMR PARAMETERS (a) OF THE SKELETON PROTONS OF METHYL-d_3 2,3,4,6-TETRA-O-METHYL-d_3-α- AND -β-D-GLUCOPYRANOSIDE

anomer	H_1	H_2	H_3	H_4	H_5	H_6	$H_{6'}$	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6}$	$J_{5,6'}$	$J_{6,6'}$
α	4.76	3.09	3.29	3.01	3.47	3.48	3.48	3.5	9.6	8.8	9.8	3.4	3.4	- (b)
β	4.12	2.83	3.09	3.01	3.24	3.47	3.54	7.8	8.9	9.0	9.6	4.9	2.1	-10.8

(a) Determined at 220 MHz for solutions in acetonitrile-d_3. δ-values are given in ppm relative to TMS, coupling constants are given in Hz. (b) no $J_{6,6'}$ is observed.

TABLE V - COUPLING CONSTANTS $J_{H,H'}$ OBSERVED AND CALCULATED FOR IDEAL $^4C_1$ (D) CONFORMATIONS, AND THE CALCULATED DIHEDRAL ANGLES $\phi_{H,H'}$

Permethyl ether of	Observed values				Calculated values							
	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$\phi_{1,2}$	$\phi_{2,3}$	$\phi_{3,4}$	$\phi_{4,5}$
α-D-Gp	3.5	9.6	8.8	9.8	2.1	11.1	11.1	11.1	47	157	151	159
β-D-Gp	7.8	8.9	9.0	9.6	10.6	11.1	11.1	11.1	147	152	152	157

EXPERIMENTAL

Monosaccharides and their derivatives were permethylated according to Kuhn (20). (CH_3I and Ag_2O in DMF). For PMR and CMR spectroscopy the compounds were methylated with trideuteromethyliodide, whereas for CMR spectroscopy also methylation with 5 or 10% $^{13}CH_3I$ was performed. The anomeric mixtures of permethyl glucopyranoses and mannopyranoses respectively were separated into α- and β-anomers by preparative TLC (1) on 0.5 mm plates of silica G (Merck) in the solvent system benzene : methanol (96:4). Permethyl β-D-mannopyranose is formed only to a very small extent. From partially methylated galactoses generally a mixture of pyranoses and furanoses is obtained. Formation of furanose forms is enhanced by methanolysis of the compound before permethylation. The TLC separation of the galactose permethyl ethers was performed on silica G in the solvent system hexane : acetone (60:40). After spraying with 1% morin in methanol the zones were detected under UV light. After extraction of the silica with chloroform and evaporation of the solvent clear sirups were obtained. Disaccharides were permethylated (20) until the hydroxyl absorptions in the IR spectrum had disappeared. Purification of the permethyl disaccharides was performed by column chromatography on Sephadex LH-20 with the eluant ethanol : chloroform (2:1) (detection of sugars in 0.1 ml aliquots with phenol-sulphuric acid reagent (21). α and β anomers were separated by TLC on Silica G in the solvent system hexane : acetone (60:40). Cotton-wool cellulose was acetylated (22) and methy-

lated with dimethylsulphate and 30% aqueous NaOH in acetone (23).

Methanolysis of mono and oligosaccharide derivatives was performed in 1 M methanolic-HCl at 85° for 24 h and for the cellulose methyl ether at 100°for 48 h. From the methanolysates of permethyl disaccharides the partially methylated components were isolated by TLC as described. GLC of permethyl monosaccharides was carried out on a F & M Model 700 gaschromatograph at a nitrogen flow rate of 28 ml/min. The column (S.S. 2.70 m x 3.2 mm O.D.) contained 3% $^{w}/_{w}$ of ECNSS-M on Chromosorb W-AW-DMCS (80-100 mesh) and was operated at a temperature of 125°.

NMR spectroscopy. PMR spectra of 5-20% solutions of the permethyl carbohydrates in acetonitrile-d_3 were recorded at 100 MHz with a Varian HA-100 spectrometer (Organic Chemical Institue T.N.O., Utrecht) or at 220 MHz with a Varian HR-220 spectrometer (T.N.O. Central Laboratories, Delft). Chemical shifts are given relative to TMS in the δ-scale with an accuracy of about 0.01 ppm, the accuracy of the coupling constants is about 0.2 Hz. Spectrum simulations were run on a 16 k Varian 620 i computer coupled with a Varian XL-100 spectrometer, using a modified SIMEQ spin simulation program (24).

Proton-noise decoupled FT CMR spectra of 5-20% solutions of the carbohydrate derivatives in acetonitrile-d_3 were recorded at 25.2 MHz on a Varian XL-100-15 FT spectrometer, operating in the deutero-lock mode. Chemical shifts are given relative to TMS internal (δ-scale) with an accuracy of 0.04 ppm. The assignment of the ring carbon resonances of the permethyl glucopyranoses is based on a selective heteronuclear spin decoupling procedure (25, 26).

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GENERAL DISCUSSION

N. SHARON - How long does it take to obtain a typical CMR spectrum of a methylated sugar or of the various other derivatives.

J. HAVERKAMP - The CMR spectra were recorded by means of Impuls Fourier Transformation. In the accumulation of interferograms the impuls interval has to be at least of the same magnitude as the longitudinal relaxation times of the various carbon nuclea in the molecule.

In our spectra of the permethylated carbohydrates the puls delay was taken in the range from 2 to 5 seconds, the spectrum acquisition time was about 1 second. Thus the instrumental cycle time is 3 to 6 seconds. For a spectrum of 10 mg of a monosaccharide (derivative) about 30.000 transients is sufficient ; this takes about 16 hours.

E.A. KABAT - What are the possibilities of using smaller amounts of material for your CMR spectra ?

J. HAVERKAMP - If it is sufficient to investigate only the methoxyl groups, the amount of material can considerably be reduced by increasing the enrichment in carbon-13 of the methyl iodide, which is used for methylation.

E.A. KABAT - Have you done anything with the amino sugars ?

J. HAVERKAMP - These compounds are under investigation.