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RAPID GAS CHROMATOGRAPHIC ANALYSIS OF PARTIALLY METHYLATED ALDOSES AS TRIMETHYLSILYLATED DIETHYL DITHIOACETALS

SUSUMU HONDA*, MARI NAGATA and KAZUAKI KAKEHI
Faculty of Pharmaceutical Sciences, Kinki University, Kowakae, Higashi-osaka (Japan)
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SUMMARY

A number of partially methylated aldoses were derivatized to their trimethylsilylated diethyl dithioacetals, and the products were analyzed by gas chromatography. The use of a capillary column coated with silicone SF-96 allowed good resolution and quantification of these sugar derivatives. Gas chromatography–mass spectrometry was useful for the identification of peaks.

INTRODUCTION

The gas chromatographic (GC) analysis of partially methylated sugars constitutes an important step of methylation analysis, which is very useful for the elucidation of the structures of oligo- and polysaccharides. This analysis is often done on the methylglycoside forms, which are the products of methanolysis of permethylated carbohydrates[1]. However, the analysis is complicated because of peak splitting due to the presence of anomers. On the other hand, although partially methylated alditol acetates give single peaks because of the lack of an anomeric centre, the derivatization procedure requires a number of steps, including treatment with sodium borohydride, desalting, evaporation and acetylation[2].

Recently we developed a method to analyze a variety of aldehydes related to carbohydrates, such as aldoses[3], uronic acids[3], hexosamines[4] and products of periodate oxidation of carbohydrates[5], as their trimethylsilylated diethyl dithioacetals. The procedure is simpler than the alditol acetate method, and each aldehyde sample gives a single peak of its derivative. This paper describes the results of the application of this method to the analysis of partially methylated sugars.

EXPERIMENTAL

Materials

The samples of oligo- and polysaccharides, as well as plant oligosaccharide glycosides, were purchased from Tokyo Kasei Kogyo (Nihonbashi, Tokyo, Japan). Ethanethiol and chlorotrimethylsilane were from the same source, but trifluoroacetic

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acid, hexamethyldisilazane and 3-O-methyl-D-glucose were from Wako (Osaka, Japan). Other chemicals and solvents were also obtained from commercial sources. All these samples, solvents and chemicals were of the highest grade available. Pyridine and dimethyl sulphoxide were dehydrated with sodium hydroxide and molecular sieves, respectively, and distilled before use.

2,3,4,6-Tetra- and 2,3,4-tri-O-methyl-D-glucoses were prepared by permethylation of gentiobiose by the Hakomori method[6] and subsequent hydrolysis of the product, followed by fractionation by preparative thin-layer chromatography (TLC) on precoated silica gel plates (E. Merck, Darmstadt, G.F.R.) with chloroform–acetone (1:1) as solvent. The purified samples were obtained in crystalline state. Samples of 2,3,6-, 2,4,6- and 3,4,6-tri-O-methyl-D-glucoses were obtained in a similar manner from cellobiose, laminarabiose and sophorose, respectively. 2,3,4,6-Tetra- and 2,3,4-tri-O-methyl-D-galactoses were prepared from stachyose, and 2,3,6- and 2,4,6-tri-O-methyl-D-galactoses were obtained from digitonin. The samples of 2,3,4,6-tetra-O-methyl-D-mannose, as well as 2,4,6- and 3,4,6-tri-O-methyl-D-mannoses, were isolated from the hydrolysate of permethylated yeast mannan and that of 2,3,6-tri-O-methyl-D-mannose was obtained from the hydrolysate of permethylated Guar gum. 2,3,4-Tri-O-methyl-D-mannose was prepared by sequential reactions of selective 6-O-tritylation[7], methylation by the method of Kuhn *et al.*[8] and hydrolysis on methyl α-D-mannopyranoside. The samples of 2,3,4-tri-O-methyl-L-rhamnose and -D-xylose were obtained by methylation of the corresponding methylglycosides, followed by hydrolysis.

Apparatus

Isothermal GC was performed on a Shimadzu 4BMPF instrument equipped with a hydrogen flame ionization detector (FID) (240°C). A sodium chloride-treated open tubular glass capillary column (50 × 0.8 mm I.D.) coated with silicone SF-96 was used at 225°C throughout the work. This column was supplied by Gasukuro Kogyo (Shinjuku, Tokyo, Japan), and had 180,000 theoretical plates for *n*-tridecane. The flow-rate of the carrier gas (nitrogen) was regulated at 1.5–2.0 ml/min by use of a splitting ratio of 100:1. The eluate was continuously mixed with the scavenger gas (nitrogen), 50 ml/min, and the mixture was introduced to the detector. Peaks were integrated by a Shimadzu E1A integrator.

Mass spectrometric (MS) detection was carried out by connecting the column to a Hitachi M-70 spectrometer through a molecular separator. The temperature of the ion source was 200°C, and the spectra were recorded at 70 eV.

Permethylation of carbohydrate materials

In analytical scale experiments, carbohydrate samples were permethylated by the Hakomori method as follows. For oligosaccharides and glycosides, a sample (0.5–1 mg) was dissolved in dimethyl sulphoxide (200 μl) at less than 50°C with sonification under nitrogen for a few minutes. A solution of methylsulphinyl carbanion (100 μl) was added and the mixture was kept at 25°C for 3 h. Methyl iodide (100 μl) was then added and the solution was maintained at 25°C for another 2 h. Chloroform (300 μl) and water (300 μl) were added, and the mixture was shaken vigorously. The aqueous layer was discarded, and the chloroform layer was washed five times with water (300 μl). The chloroform layer was evaporated to dryness to give a syrupy residue. For the

samples of polysaccharides, the residue was methylated twice more by the procedure described above. The chloroform solution finally obtained from each sample was transferred to a small reaction tube (10 × 0.7 cm I.D.) and the solvent was evaporated under reduced pressure.

Hydrolysis of methylation products

The permethylation product obtained above was dissolved in 2 *M* trifluoroacetic acid (200 μl). The tube was then flushed with nitrogen for a few minutes, sealed and heated for 6 h on a bath of boiling water. After cooling, the tube was opened and the hydrolysate was evaporated to dryness under reduced pressure in a desiccator containing sodium hydroxide. The residue was dissolved in a small volume of aqueous acetone (1:1), then transferred to another reaction tube (5 × 0.5 cm I.D.) and evaporated to dryness. The partially methylated aldoses in the product were analyzed as described below.

Analysis of partially methylated aldoses

The procedure for derivatization of partially methylated aldoses to their trimethylsilylated diethyl dithioacetals was essentially the same as that described previously[3]. An aqueous solution (100 μl) of 10^{-3} *M* 3-O-methyl-D-glucose (internal standard) was added to an authentic sample (10^{-8}–10^{-6} mole) or to the hydrolysate of the permethylation product, contained in a small reaction tube, and the mixture was evaporated to dryness under reduced pressure in a desiccator containing phosphorus pentoxide. A mixture of ethanethiol and trifluoroacetic acid (2:1), (20 μl) was added, the reaction tube was tightly closed with a polyethylene stopper and the residue was dissolved with gentle swirling. The resultant solution was kept at 25°C for 10 min, and pyridine (50 μl) was added, followed by hexamethyldisilazane (100 μl) and chlorotrimethylsilane (50 μl). The mixture was incubated at 50°C for 30 min with occasional shaking, and then centrifuged. The partially methylated aldoses were analyzed by injecting a 1–10 μl sample of the supernatant into the GC column.

RESULTS AND DISCUSSION

Under the conditions used, a difference of 0.003 in relative retention time (RRT) was sufficient for complete resolution of peaks. Table I gives the RRT values of the trimethylsilylated diethyl dithioacetals of various partially methylated aldoses, relative to that of 3-O-methyl-D-glucose (36.4 min). The values for 2,3,4,6-tetra-O-methyl-D-galactose, -glucose and -mannose were 0.594, 0.602 and 0.615, respectively, and these compounds were well resolved. The order of elution, Gal, Glc, Man, was different from that of the derivatives of free aldoses, *i.e.*, Glc, Man, Gal[3]. Similar behaviour was also observed for 2,3,4- and 2,4,6-trimethylhexoses, which had RRT values ranging from *ca.* 0.7 to 0.8. Trimethylhexoses substituted at the 2, 3 and 6 positions were exceptions, being eluted in the order Gal, Man, Glc in the RRT range of 0.67–0.70. On the other hand, comparison of the RRT values of the positional isomers of each trimethylhexose indicated that whereas the 3,4,6-trisubstituted derivative eluted quickly; the 2,3,4-trisubstituted derivative was retarded.

Although a complete generalization was not possible, because one specimen of D-galactose was unavailable, it is obvious that steric factors including configuration

TABLE I

RELATIVE RETENTION TIMES OF THE TRIMETHYLSILYLATED DIETHYL DITHIOACETALS OF PARTIALLY METHYLATED ALDOSES

Ara = L-Arabinose; Xyl = D-xylose; Fuc = L-fucose; Rha = L-rhamnose; Gal = D-galactose; Glc = D-glucose; Man = D-mannose.

Position(s) of substitution by the methyl group	*Relative retention time*						
	Ara	*Xyl*	*Fuc*	*Rha*	*Gal*	*Glc*	*Man*
3						1	
2, 3		0.493				0.876	0.879
2, 4					0.889		
3, 4		0.559					0.930
4, 6						0.800	
2, 3, 4		0.432	0.488	0.474	0.750	0.770	0.791
2, 3, 6	0.460				0.671	0.700	0.686
2, 4, 6					0.690	0.697	0.702
3, 4, 6						0.683	0.674
2, 3, 4, 6					0.594	0.602	0.615

and position of substitution have important roles in gas–liquid partition. Under the conditions used, all the trimethylhexoses, except for the pair 2,3,6-tri-O-methyl-D-glucose and 2,4,6-tri-O-methyl-D-mannose, were satisfactorily resolved and identification of peaks was possible on the basis of RRT values.

Table I also lists the RRT data for some dimethylhexoses. The separation of 2,3-di-O-methyl-D-glucose and -mannose was incomplete, but the derivatives of other dimethylhexoses were well resolved. The derivatives of trimethyl-6-deoxyhexoses were eluted before those of trimethylhexoses, but after those of trimethylpentoses. The derivatives of dimethylpentoses had slightly higher RRT values than those of trimethyl-6-deoxyhexoses.

Table II gives the relative molar response factors of the derivatives of partially methylated aldoses. All the tetramethylhexoses gave the same molar response, which was the highest observed. Similarly, all the trimethylhexoses except the 2,3,4-trimethyl isomers had the same response factor, which was slightly lower than that of tetramethylhexoses. All the samples of 2,3,4-trimethylhexoses had slightly higher FID responses regardless of configuration, the magnitude being the same as that of the tetramethylhexoses. The molar responses of trimethyl-pentoses and -6-

TABLE II

RELATIVE MOLAR RESPONSES OF THE TRIMETHYLSILYLATED DIETHYL DITHIOACETALS OF PARTIALLY METHYLATED ALDOSES

Dithioacetal derivatives of	*Relative molar response factor*
Tetramethylhexoses	1
Trimethylhexoses	
2,3,4-Trisubstituted	1.00
Others	0.93
Trimethyl-6-deoxyhexoses	0.91
Trimethylpentoses	0.90

TABLE III

METHYLATION ANALYSIS OF SELECTED CARBOHYDRATES BY COMBINATION OF THE HAKOMORI METHOD AND THE TRIMETHYLSILYLATED DITHIOACETAL METHOD
3,4,6-Trimethyl-Glc = 3,4,6-Tri-O-methyl-D-glucose, etc.

Carbohydrate	*Partially methylated aldoses found (molar proportion)*
Sophorose	3,4,6-Trimethyl-Glc (1.01), 2,3,4,6-tetramethyl-Glc (1)
Laminarabiose	2,4,6-Trimethyl-Glc (1.12), 2,3,4,6-tetramethyl-Glc (1)
Cellobiose	2,3,6-Trimethyl-Glc (1.15), 2,3,4,6-tetramethyl-Glc (1)
Gentiobiose	2,3,4-Trimethyl-Glc (1.05), 2,3,4,6-tetramethyl-Glc (1)
Amylopectin (potato)	2,3-Dimethyl-Glc (1.79), 2,3,6-trimethyl-Glc (37.6), 2,3,4,6-tetramethyl-Glc (1)
Glycogen (rabbit liver)	2,3-Dimethyl-Glc (1.93), 2,3,6-trimethyl-Glc (23.5), 2,3,4,6-tetramethyl-Glc (1)
Mannan (baker's yeast)	3,4-Dimethyl-Man (0.95), 2,3,4-trimethyl-Man (0.03), 2,4,6-trimethyl-Man (0.70), 3,4,6-trimethyl-Man (0.49), 2,3,4,6-tetramethyl-Man (1)
Digitonin	4,6-Dimethyl-Glc (1.19), 2,3,6-trimethyl-Gal (0.83), 2,4,6-trimethyl-Gal (1.21), 2,3,4-trimethyl-Xyl (1.00), 2,3,4,6-tetramethyl-Glc (1)
Tomatine	4,6-Dimethyl-Glc (1.04), 2,3,6-trimethyl-Gal (1.06), 2,3,4-trimethyl-Xyl (0.99), 2,3,4,6-tetramethyl-Glc (1)

deoxyhexoses were lower than those of tetra- and trimethylhexoses (*ca.* 90 % of that of tetramethylhexoses). It is advantageous for the quantification of partially methylated aldoses that all the molar responses are not affected by configuration.

On the basis of these results some oligo- and polysaccharides, as well as oligosaccharide glycosides, were permethylated and the products were hydrolysed with

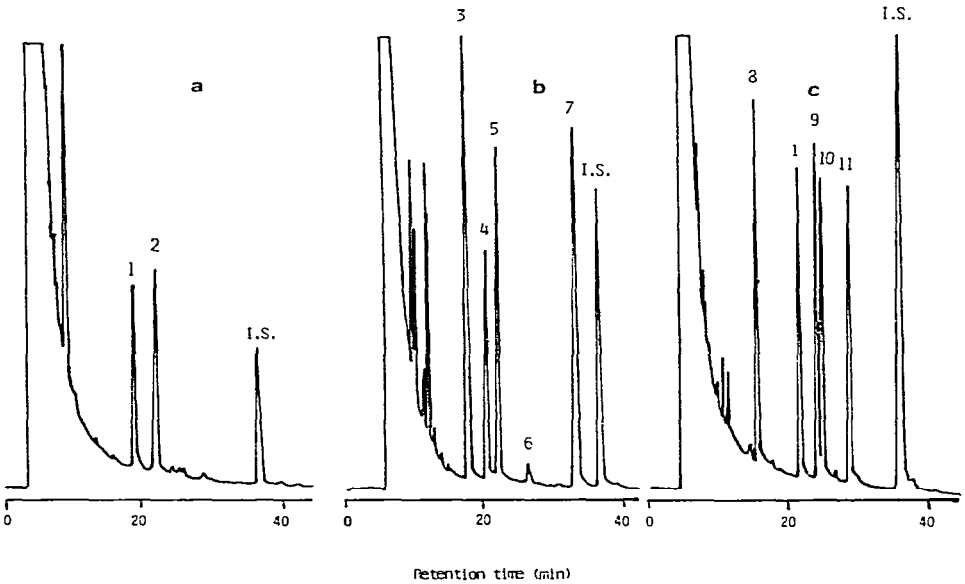

Fig. 1. Gas chromatograms for the hydrolysate of permethylated laminarabiose (a), yeast mannan (b) and digitonin (c). Peaks: 1 = 2,3,4,6-tetra-O-methyl-D-glucose; 2 = 2,4,6-tri-O-methyl-D-glucose; 3 = 2,3,4,6-tetra-O-methyl-D-mannose; 4 = 3,4,6-tri-O-methyl-D-mannose; 5 = 2,4,6-tri-O-methyl-D-mannose; 6 = 2,3,4-tri-O-methyl-D-mannose; 7 = 3,4-di-O-methyl-D-mannose; 8 = 2,3,4-tri-O-methyl-D-xylose; 9 = 2,3,6-tri-O-methyl-D-galactose; 10 = 2,4,6-tri-O-methyl-D-galactose; 11 = 4,6-di-O-methyl-D-glucose; I.S. = 3-O-methyl-D-glucose (internal standard).

trifluoroacetic acid. The results are summarized in Table III. With all the glucobioses examined, the molar ratios of trimethylglucoses relative to 2,3,4,6-tetra-O-methyl-D-glucose were in good agreement with the theoretical value of 1. The chromatogram for laminarabiose is shown in Fig. 1a as an example of those obtained for glucobioses.

The results for potato amylopectin indicated that there was one 1 → 6 linked branch per 23 1 → 4 linked D-glucose residues. Similarly, the sugar chain of rabbit liver glycogen had approximately one branch per 14 D-glucose residues. These results are consistent with those reported in the literature[9].

The analysis of the hydrolysate of permethylated mannan gave 2,3,4,6-tetra-, 2,4,6-tri-, 3,4,6-tri- and 3,4-di-O-methyl-D-mannoses in a molar proportion of 1:0.70:0.49:0.95, together with a trace amount of 2,3,4-tri-O-methyl-D-mannose, as shown in Fig. 1b and Table III. The amount of 2,4,6-tri-O-methyl-D-mannose was larger than that reported by Peat *et al.*[10], but that for 2,3,4-tri-O-methyl-D-mannose was considerably smaller.

The commercial sample of digitonin gave a molar proportion of 1.19:0.83:1.21:1.00:1 for 4,6-di-O-methyl-D-glucose, 2,3,6-tri-O-methyl-D-galactose, 2,4,6-tri-O-methyl-D-galactose, 2,3,4-tri-O-methyl-D-xylose and 2,3,4,6-tetra-O-methyl-D-glucose, respectively, which deviates slightly from the theoretical value (1:1:1:1:1) probably due to contamination, as evidenced by the presence of minor spots on a thin-layer silica gel plate, developed with chloroform–methanol (1:1). The chromatogram for digitonin is shown in Fig. 1c as an example of those obtained for glycosides.

Tomatine gave a molar proportion of 1.04:1.06:0.99:1 for 4,6-di-O-methyl-D-

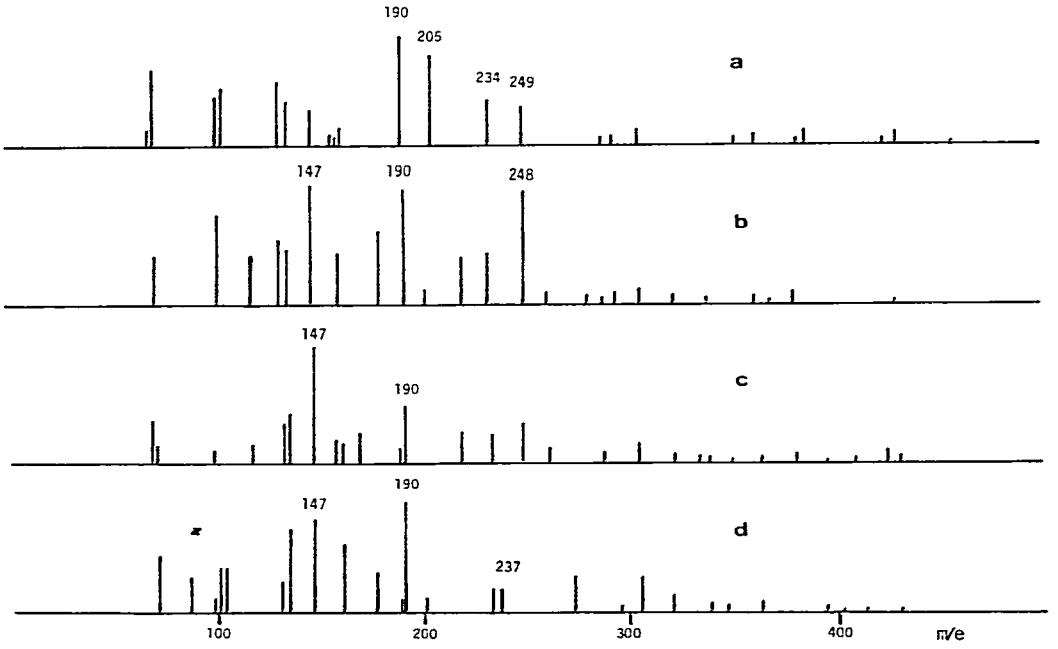

Fig. 2. Mass spectra of the trimethylsilylated diethyl dithioacetals of 2,3,4-tri-O-methyl-D-glucose (a), 2,3,6-tri-O-methyl-D-glucose (b), 2,4,6-tri-O-methyl-D-glucose (c) and 3,4,6-tri-O-methyl-D-glucose (d).

TABLE IV

PROPOSED STRUCTURES OF THE CHARACTERISTIC FRAGMENT IONS

TMS = Trimethylsilyl group. Superscripts refer to carbon numbers.

m/e	*Proposed structure*
147	$[^{5}CH(OCH_3)\text{-}^{6}CH_2(OTMS)]^+$
190	$[^{2}CH(OCH_3)\text{-}^{3}CH(OCH_3)\text{-}^{4}CH(OTMS)]^+$ or $[^{3}CH(OCH_3)\text{-}^{4}CH(OCH_3)\text{-}^{5}CH(OTMS)]^+$
205	$[^{5}CH(OTMS)\text{-}^{6}CH_2(OTMS)]^+$
234	$[^{2}CH(OCH_3)\text{-}^{3}CH(OCH_3)\text{-}^{5}CH(OCH_3)\text{-}^{5}CH(OTMS)]^+$
237	$[^{1}CH(SC_2H_5)_2\text{-}^{2}CH(OTMS)]^+$
248	$[^{3}CH(OCH_3)\text{-}^{4}CH(OTMS)\text{-}^{5}CH(OTMS)]^+$
249	$[^{4}CH(OCH_3)\text{-}^{5}CH(OTMS)\text{-}^{6}CH_2(OTMS)]^+$

glucose, 2,3,6-tri-O-methyl-D-galactose, 2,3,4-tri-O-methyl-D-xylose and 2,3,4,6-tetra-O-methyl-D-glucose, respectively, which is consistent with the predicted value of 1:1:1:1.

Investigation of the mass spectra of some derivatives of partially methylated aldoses demonstrated that they can be used for identification of peaks. Fig. 2 shows the spectra of four positional isomers of trimethylglucoses. All these spectra gave no molecular ions, but showed characteristic fragments. The spectrum of 2,4,6-tri-O-methyl-D-glucose gave a base peak at *m/e* 147, and that of the 2,3,6-isomer showed the presence of three intense peaks with approximately the same intensity at 147, 190 and 248. The spectra obtained for the 2,3,4- and 3,4,6-isomers commonly had the base peak at 190. In addition, the first isomer had characteristic peaks at 205, 234 and 249, and the second isomer had one at 237. The structures proposed for these fragments are given in Table IV. The mass spectra of dimethyl- and monomethylhexoses are now under investigation.

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