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OCCURRENCE OF A GALACTOFURANOSE DISACCHARIDE IN IMMUNOADJUVANT FRACTIONS OF *MYCOBACTERIUM TUBERCULOSIS* (CELL WALLS AND WAX D)

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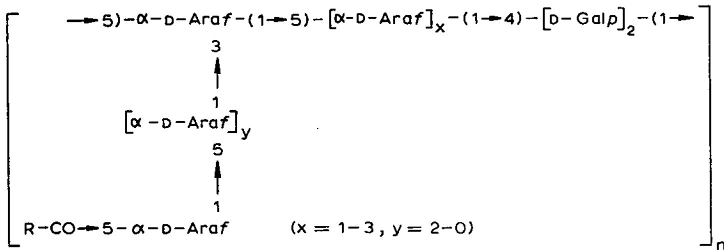
SUMMARY

By partial acid hydrolysis a digalactoside was obtained from the arabinogalactan of the cell walls and Wax D of *Mycobacterium tuberculosis*. By using instrumental and chemical analysis methods (gas–liquid chromatography, mass spectrometry, PMR spectroscopy, periodate oxidation, permethylation) the saccharide was identified as 6-*O*-*β*-D-galactofuranosyl-D-galactose.

INTRODUCTION

The mycobacterial cell walls contain mycolates of an arabinogalactan linked to the typical peptidoglycan probably by a phosphodiester bridge[1]. Wax D which seems to be a product of partial cell wall autolysis has a similar composition[2]. Azuma *et al.*[3] have shown the immunological identity of the arabinogalactan of cell walls and of Wax D. Misaki *et al.*[4] have proposed the following structure for the arabinogalactan of mycobacterial cell walls:



D-Ara*f* = D-arabinofuranose, D-Gal*p* = D-galactopyranose,
R–CO = mycolic acid residue

Abbreviation: TMS, trimethylsilyl.

This structure was consistent with our first results obtained with the human strain of *Mycobacterium tuberculosis* Peurois[5] and $H_{37}Rv$[6]. Especially methylation and periodate oxidation data seemed to be in agreement with a structure containing 1 → 4-linked galactopyranose units.

Recently, we isolated among the products of partial acid hydrolysis of cell walls and of Wax D from Peurois and $H_{37}Ra$ strains a disaccharide which contains a D-galactofuranose residue[7]. This paper presents the results of studies on this digalactoside. The 1 → 4 linkage between two galactopyranose units is definitely excluded. The digalactoside is identified as 6-*O*-β-D-galactofuranosyl-D-galactose.

MATERIALS AND METHODS

The cell walls were prepared from *M. tuberculosis* var. hominis $H_{37}Ra$ as described previously[8]. The microorganisms were grown for 15 days at 37 °C on Sauton's medium in the Department of Microbiology of Institut de Chimie des Substances Naturelles, 91190 Gif-sur-Yvette. Wax D used in this study was isolated from *M. tuberculosis* var. hominis strain Peurois, a gift from Dr Augier, Institut Pasteur, Paris (for its preparation see ref. 9). 5-*O*-β-D-Galactofuranosylgalactitol was a gift from Dr P. A. J. Gorin; 6-*O*-β-D-galactofuranosyl-D-galactose was kindly supplied by Dr S. H. Buttery.

Chromatography

Paper chromatography was carried out on Whatman No. 1. The following solvent systems were used: (A) ethyl acetate–pyridine–water (2 : 1 : 2, v/v/v); (B) butan-1-ol–ethanol–water (10 : 1 : 2, v/v/v); (C) butan-1-ol–ethanol–water (5 : 1 : 4, v/v/v); (D) ethyl acetate–acetic acid–water (9 : 2 : 2, v/v/v); (E) butan-1-ol–pyridine–water (6 : 4 : 3, v/v/v); and (F) butan-1-ol–acetic acid–water (4 : 1 : 5, v/v/v).

For thin-layer chromatography on Schleicher and Schüll silica gel (F 1500 LS 254) the solvent system acetone–ammonia–water (250 : 1.5 : 3, v/v/v) was used. Reducing sugars and polyols were detected with alkaline $AgNO_3$[10] and benzidine–HIO_4[11]; reducing sugars with aniline hydrogen phthalate and with aniline–diphenylamine–phosphoric acid[12].

Gas–liquid chromatography of methylated derivatives was carried out with an Intersmat gas chromatograph provided with a flame ionization detector on a copper column (2 m × 6.4 mm) containing 15 % butanediol succinate on celite, 80–100 mesh. The injection port temperature was 240 °C and the column oven temperature 170 °C. The gas flow rate for N_2 was 60 ml/min. The retention times (R_T) are given relative to that of methyl 2,3,4,6-α-tetra-*O*-methyl glucoside. Gas–liquid chromatography of pertrimethylsilyl (TMS) derivatives[13] was performed on an F and M gas chromatograph Model 700, equipped with a dual flame ionization detector and coiled stainless steel columns (2.70 m × 3.2 mm) on 3 % OV-17 on Chromosorb W (HP), 80–100 mesh (Pierce Chemicals Company). The injection port temperature was 290 °C, the detector temperature 310 °C and the column oven temperature 227 °C. The gas flow rates for H_2, air and N_2 were 45, 375 and 18 ml/min, respectively. The retention times (R_S) are given relative to that of TMS-sucrose.

Permethylation

Methylation of the digalactoside (4 mg) was performed by the procedure de-

scribed by Hakomori[14]. The products obtained after methanolysis of the permethylated saccharide (refluxing with 3 % methanolic HCl for 8 h) were examined by gas–liquid chromatography; the products obtained after hydrolysis of the permethylated saccharide (1.5 M HCl at 100 °C for 5 h in a sealed tube) were examined by thin-layer chromatography.

Peracetylation

Acetylation of the digalactoside (0.5 mg) was performed with acetic anhydride in pyridine at room temperature for 48 h.

Pertrimethylsilylation

For the investigation of the saccharides (anomerized in water for 48 h) and their corresponding alditols, by gas–liquid chromatography, mass spectrometry and Fourier Transform PMR spectroscopy, 0.5 mg or less was pertrimethylsilylated[15] in each case.

Mass spectrometry

Mass spectrometry was performed with an AEI MS9 mass spectrometer at an ion chamber temperature of about 220 °C for the peracetylated products and of about 100–120 °C for the pertrimethylsilyl derivatives (Department of Institut de Chimie des Substances Naturelles, 91190 Gif-sur-Yvette, Dr B. C. Das (acetyl derivatives) and Laboratory of Analytical Chemistry, State University, Utrecht, Dr J. Vink (TMS derivatives)).

PMR spectroscopy

PMR spectroscopy was performed with a Varian HA 100-FT Spectro system 100 spectrometer with tetramethylsilane as internal standard (Dr D. J. Frost, Unilever Research Laboratories, Vlaardingen, The Netherlands).

Isolation of the digalactoside

1 g of Wax D (or cell walls) was hydrolysed by heating with 10 % trichloroacetic acid at 40 °C for 22 h with mechanic stirring. Then the acidic solution was extracted with chloroform and ether to remove the lipid component and freeze-dried. The trichloroacetic acid was removed by repeated ether extraction. Paper chromatography of the concentrated solution showed the presence of a number of reducing oligosaccharides. The disaccharide was obtained by preparative paper chromatography in Solvents B ($R_{Trehalose} = 0.82$) and E ($R_{Galactose} = 0.77$). The amorphous white powder, obtained after freeze-drying, has an optical rotation $[\alpha]_D = -26 \pm 2$ ° ($c = 0.5$; water) and a melting point of about 170–175 °C (decomposition). Total acid hydrolysis reveals the presence of D-galactose only ($[\alpha]_D = +80$ °). The chromatographic mobilities relative to lactose (R_{Lac}) of the digalactoside in the Solvents D and E are 1.3 and 1.19, respectively.

Partial acid hydrolysis of the digalactoside (ref. 16)

2 mg of the digalactoside were hydrolysed as a 1 % solution in 5 mM H_2SO_4 at 85 °C for 90 min. After hydrolysis, by paper chromatography two spots could be detected: galactose ($R_F = 0.16$ in Solvent F) and digalactoside ($R_{Gal} = 0.8$ in Solvent E).

Reduction of the digalactoside (*ref.* 17)

5 mg of the digalactoside (15 μmoles) were reduced with 2 mg of $NaBH_4$ in water (0.3 ml) for 4 h. The solution was neutralized with a few drops of acetic acid and Dowex 50(H^+) and subsequently freeze-dried. The residue was dissolved in methanol and evaporated to dryness five times. The product was chromatographed in the Solvents D and E ($R_{Gal} = 0.33$ and 0.83, respectively).

Degradation of the reduced digalactoside (*ref.*17)

3 mg (8.6 μmoles) of the digalactoside alditol were treated with 55 μmoles of $NaIO_4$ (0.55 ml of 0.1 M solution) at 22 °C for 4 min. The solution was then deionized by Amberlite MB 3 and reduced with $NaBH_4$ as described above. After hydrolysis with 1.5 M HCl at 100 °C for 3 h the product was chromatographed in Solvents E and F. The mobilities are summarized in Table I.

TABLE I

PRODUCTS OBTAINED AFTER THE DEGRADATION OF THE DIGALACTOSIDE ALDITOL

	E	*F*
Arabinose	0.4*	0.24*
Ethylene glycol	0.72	0.63
Glycerol	0.55	0.50

* A pink spot characteristic of pentose with aniline hydrogen phthalate.

RESULTS AND DISCUSSION

By partial acid hydrolysis of cell walls and Wax D of *M. tuberculosis* a number of reducing oligosaccharides were obtained. From a purified Wax D fraction a digalactoside could be isolated in 1–2 % yield. Its chromatographic mobility in Solvent system C is identical to that of $\alpha(1 \rightarrow 4)$ digalactopyranoside, but it migrates more rapidly in Solvent systems D and E. In Solvent system A it migrates more rapidly than the $\beta(1 \rightarrow 4)$ digalactopyranoside. The R_{Gal} values in Solvent systems D and E are almost identical to that of 6-*O*-β-D-galactofuranosyl-D-galactose described by Plackett and Buttery[18] (see Table II).

The optical rotation of the digalactoside from *M. tuberculosis* ($[\alpha]_D = -26 \pm 2°$)

TABLE II

R_{Gal} VALUES OF MISCELLANEOUS DIGALACTOSIDES

	Solvent systems:			
	A	*C*	*D*	*E*
Digalactoside of *M. tuberculosis*	0.77	0.42	0.38	0.80
Gal*f*-β(1 → 6)-Gal			0.37	0.77
Gal*p*-α(1 → 4)-Gal[19]		0.41	0.31	0.65
Gal*p*-β(1 → 4)-Gal[19]	0.59			

differs from that of 4-*O*-β-D-galactopyranosyl-D-galactopyranose ($[\alpha]_D = +67°$) and from that of its α isomer ($[\alpha]_D = +186 \rightarrow +173°$)[19], but resembles that of 6-*O*-β-D-galactofuranosyl-D-galactose ($[\alpha]_D = -28.2 \pm 1.3°$)[18]. The tests with α and β galactosidase were negative. This suggests that the non-reducing unit could be a galactofuranoside. The furanose non-reducing end unit was also indicated by the rapid hydrolysis in very weak acid medium[16].

With the aniline hydrogen phthalate reagent the digalactoside gives a yellow brown spot, just as galactose, lactose and maltose. With the aniline–diphenylamine–phosphoric acid the digalactoside gives a greyish-green colour, which is not in agreement with the colour characteristic for $1 \rightarrow 4$ aldohexosylaldohexoses[12] (lactose and maltose give blue spots).

These results seem to exclude the structure 4-*O*-galactopyranosylgalactose for the disaccharide of cell walls and Wax D.

Periodate oxidation

The chemical degradation of the digalactoside is summarized in Fig. 1. The same sequence of reactions was used by Gorin and Spencer[17] to characterize 5-*O*-β-D-galactofuranosyl-D-galactose from galactocaralose and by Plackett and Buttery[18] to study 6-*O*-β-D-galactofuranosyl-D-galactose from *Mycoplasma mycoides*. The only reducing sugar which could be detected was arabinose, but both ethylene glycol and glycerol were formed. It is clear that arabinose is formed by oxidation of galactofuranose between C-5 and C-6. The formation of ethylene glycol suggests a $1 \rightarrow 6$ linkage. The occurrence of glycerol has to be considered as an incomplete oxidation of the galactitol unit.

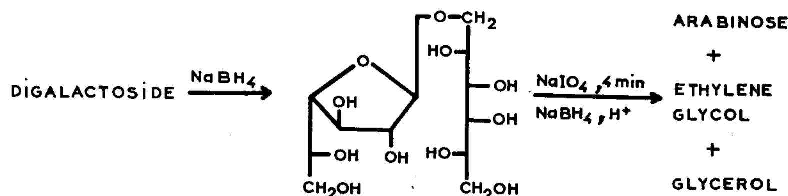


Fig. 1. Degradation of the digalactoside.

Permethylation

Gas–liquid chromatography of the components obtained after methanolysis of the permethylated digalactoside indicated the presence of methyl 2,3,5,6-tetra-*O*-methyl galactofuranoside ($R_T = 1.1$), methyl 2,3,5-tri-*O*-methyl galactofuranoside ($R_T = 2.8$) and methyl 2,3,4-tri-*O*-methyl galactopyranoside ($R_T = 4.5$). Thin-layer chromatography gave the same results; three spots were observed, *viz.* 2,3,5,6-tetra-*O*-methylgalactofuranose ($R_{Glc} = 1.01$), 2,3,5-tri-*O*-methylgalactofuranose ($R_{Glc} = 0.86$) and 2,3,4,-tri-*O*-methylgalactopyranose ($R_{Glc} = 0.59$).

The observation of two tri-*O*-methyl derivatives suggests that the reducing unit exists in the pyranose as well as in the furanose ring form. It can not be excluded that a mixture of ring forms is formed during the permethylation. The results lead to the structure 6-*O*-galactofuranosylgalactose.

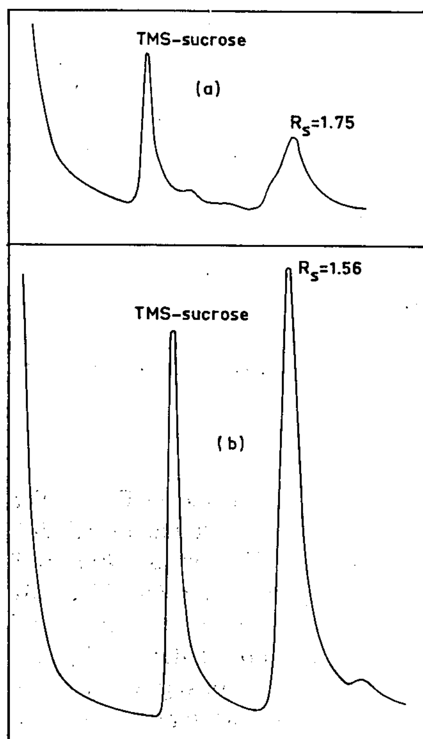


Fig. 2. (a) Gas chromatogram of the TMS-digalactoside. (b) Gas chromatogram of the TMS-digalactoside alditol.

Gas–liquid chromatography

In Fig. 2 the gas chromatograms of the TMS-digalactoside and its TMS-alditol are given with TMS-sucrose as internal standard. The chromatogram of the TMS-galactosylgalactose shows a main peak with a shoulder at $R_S = 1.75$ and a small peak at $R_S = 1.22$. Probably the shoulder belongs to one of the anomeric forms of the disaccharide. The chromatogram of the TMS-galactosylgalactitol shows a main peak at $R_S = 1.56$ and two small peaks at $R_S = 1.00$ and $R_S = 1.91$. It is likely one can make the assumption that the very small peaks are due to impurities.

The gas chromatograms of the TMS-6-*O*-β-D-galactofuranosyl-D-galactose and its TMS-alditol give the same R_S values as described for the digalactoside and its alditol, respectively (co-chromatography). The chromatogram of TMS-5-*O*-β-D-galactofuranosyl-D-galactitol shows a peak at $R_S = 1.18$.

Mass spectrometry

(a) The mass spectrum of the acetyl derivative of the digalactoside ($M = 678$) shows as the first detectable fragment ion in the high mass range a peak at *m/e* 619 ($M^{+\cdot}$ *minus* ·OAc) (Fig. 3). The strong peak at *m/e* 533 corresponds to $M^{+\cdot}$ *minus* CH_2OAc-$\dot{C}HOAc$, indicating the presence of a non-reducing furanose ring.

(b) The mass spectrum of the TMS derivative of the digalactoside shows a

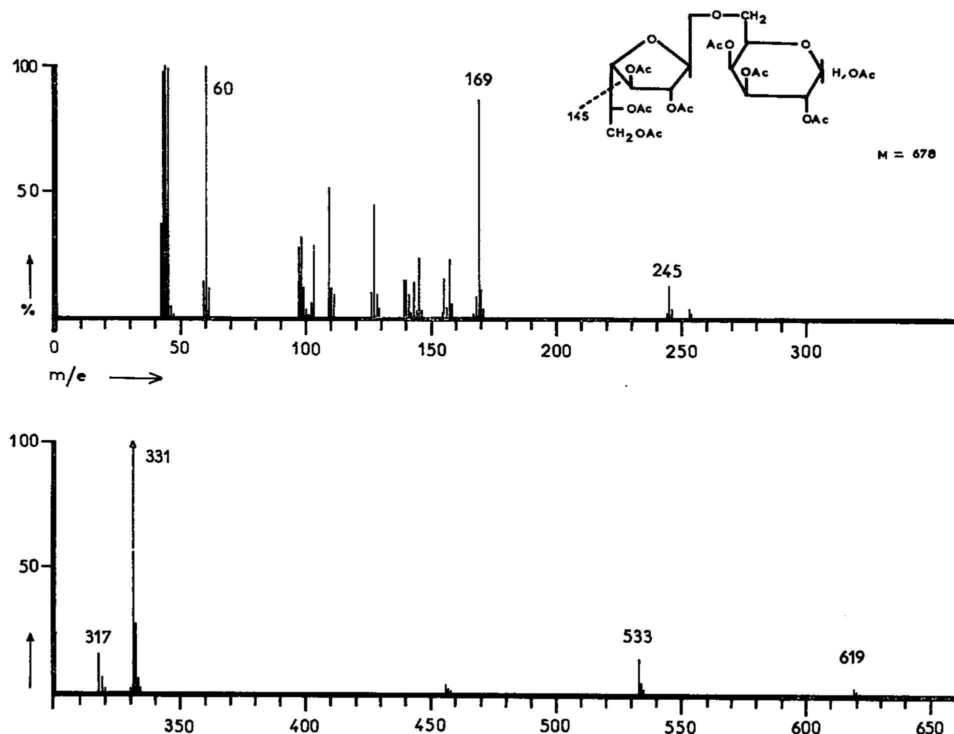


Fig. 3. 70 eV mass spectrum of the acetyl digalactoside.

molecular ion peak at *m/e* 918 (Fig. 4). For the interpretation of the peaks, refer to Kamerling *et al.*[15]. The presence of a very intense peak at *m/e* 583 demonstrates the occurrence of a 1 → 5 or 1 → 6 glycosidic linkage. The ratio of the intensities of the peaks at *m/e* 217 and *m/e* 204 (217/204 = 1.5) suggests the presence of at least one furanose ring. The peak at *m/e* 623 ($M^{+\cdot}$ *minus* TMSOH *minus* CH_2OTMS–$\dot{C}HOTMS$) is, in the case of a 1 → 5 or 1 → 6 link, indicative for the presence of a non-reducing furanose ring. This observation is supported by the ratio of the intensities of the peaks at *m/e* 205 and *m/e* 204, which points to the presence of a non-reducing furanose ring with a CH_2OTMS–CHOTMS side chain (measured ratio 205/204 = 0.42; calculated isotopic ratio 205/204 = 0.20).

The mass spectrum of the TMS derivative of the disaccharide alditol (M = 992) shows as the first detectable fıagment ion in the high mass range a peak at *m/e* 977 ($M^{+\cdot}$ *minus* $^{\cdot}CH_3$) (Fig. 5). For the interpretation of the peaks, refer to Kärkkäinen[20]. This author demonstrated further that, in the case of TMS-aldohexopyranosylaldohexitols, the ratios 217/204 and 205/204 are both considerably smaller than unity. The peak at *m/e* 205 is hardly formed in the alditol unit of these disaccharide alditols. Therefore the ratios of the intensities of the peaks at *m/e* 217 and *m/e* 204 (217/204 = 8.0) and at *m/e* 205 and *m/e* 204 (205/204 = 2.2) from the mass spectrum of the TMS-galactosyl-galactitol point to a galactofuranose unit.

The mass spectra of TMS-6-*O*-*β*-D-galactofuranosyl-D-galactose and its TMS-

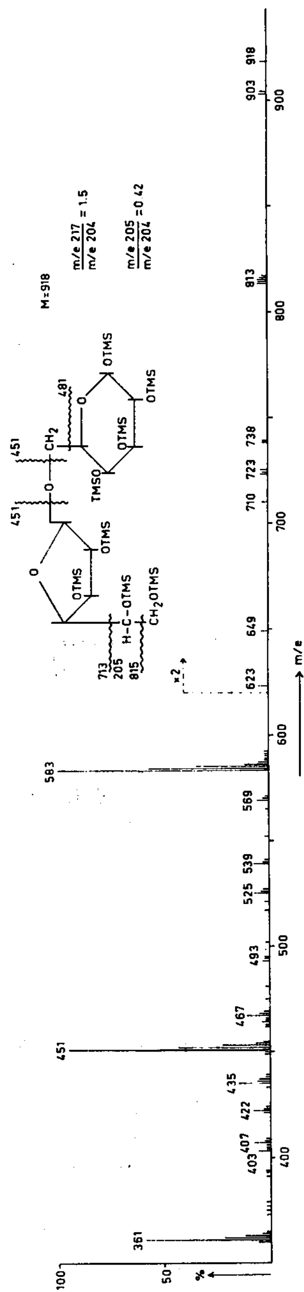


Fig. 4. 70 eV mass spectrum of the TMS-digalactoside. Only values > *m/e* 360 are given.

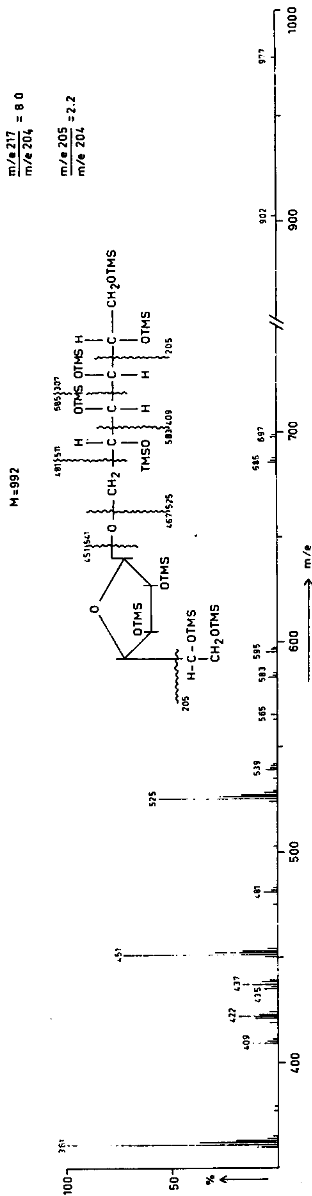


Fig. 5. 70 eV mass spectrum of the TMS-digalactoside alditol. Only values > *m/e* 360 are given.

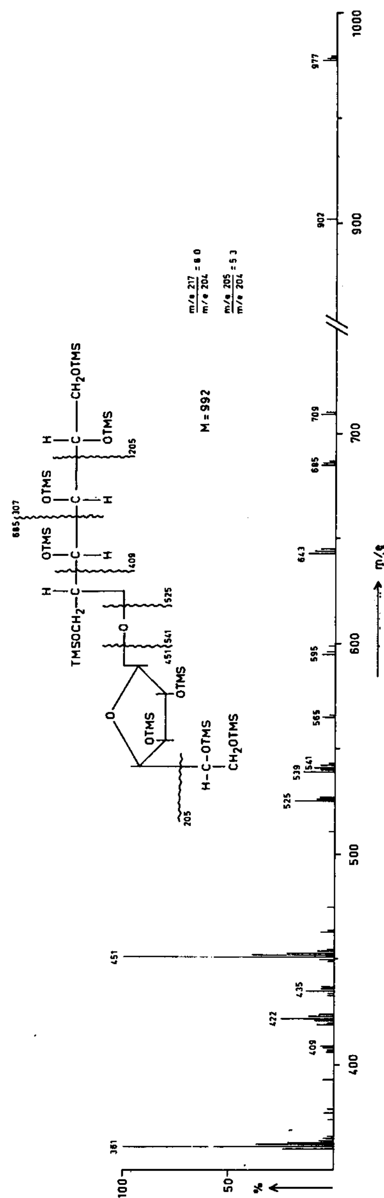


Fig. 6. 70 eV mass spectrum of TMS-5-*O*-β-D-galactofuranosyl-D-galactitol. Only values > *m/e* 360 are given.

alditol show only minor differences compared with the spectra described above. The mass spectrum of TMS-5-*O*-β-D-galactofuranosyl-D-galactitol (Fig. 6) shows definite differences compared with the spectra of the other TMS-alditols.

PMR spectroscopy

The PMR spectrum of the TMS derivative of the digalactoside, dissolved in [$^2H_6$]acetone, shows in the anomeric region three doublets with a small coupling constant at $\delta = 5.02$ ppm ($J_{1,2} \cong 2.0$ Hz), $\delta = 4.80$ ppm ($J_{1,2} \cong 2.9$ Hz) and $\delta = 4.77$ ppm ($J_{1,2} \cong 2.9$ Hz) and one doublet with a large coupling constant at $\delta = 4.53$ ppm ($J_{1,2} \cong 6.8$ Hz). Previously it has been described that, for $1 \rightarrow 6$ aldohexosylglucoses, the δ values of the anomeric protons of the reducing unit in both anomeric forms are hardly influenced by this type of linkage; they have nearly the same δ values as TMS-α- and β-D-glucopyranose, respectively (Kamerling *et al.*[21]). For TMS-α- and β-D-galactopyranose these values are 5.02 ppm ($J_{1,2} = 2.2$ Hz) and 4.52 ppm ($J_{1,2} \doteq 6.9$ Hz), respectively. Therefore, it is obvious that one can corre-

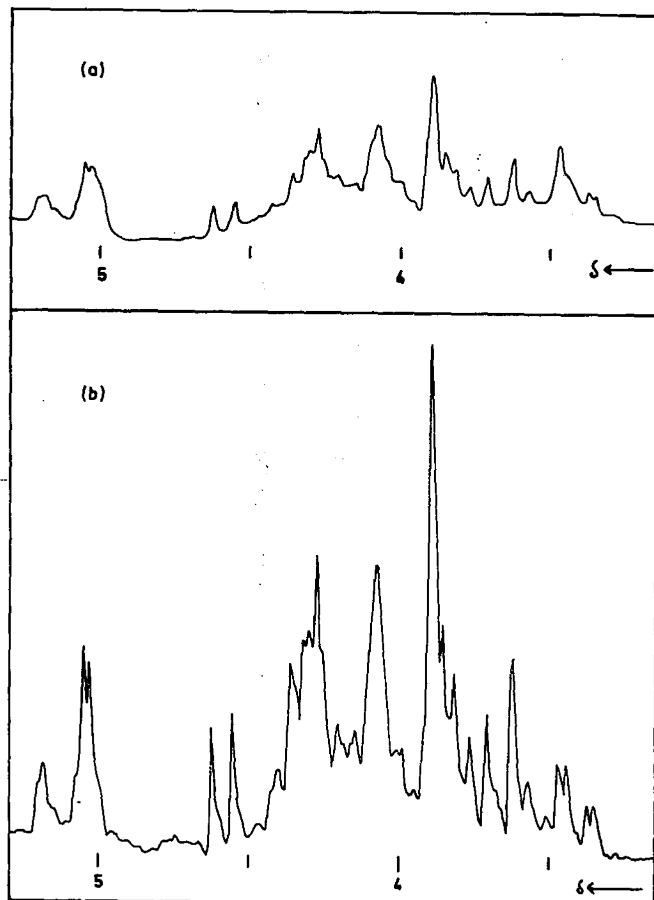


Fig. 7. (a) Fourier Transform PMR spectrum of the TMS-digalactoside (105 000 transients). (b) Fourier Transform PMR spectrum of TMS-6-*O*-β-D-galactofuranosyl-D-galactose (53 750 transients).

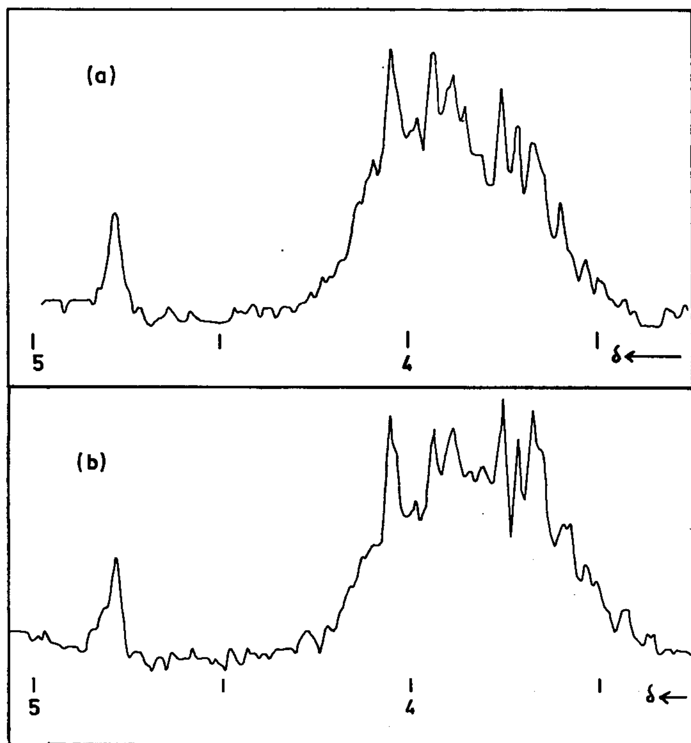


Fig. 8. (a) Fourier Transform PMR spectrum of the TMS-digalactoside alditol (1000 transients). (b) Fourier Transform PMR spectrum of TMS-6-*O*-β-D-galactofuranosyl-D-galactitol (1000 transients).

late the value at $\delta = 5.02$ ppm with H^{α} and that at $\delta = 4.53$ ppm with H^{β}. These results also demonstrate the presence of a reducing pyranose ring in this derivative, which eliminates the possibility of a 1 → 5 glycosidic bond (see section *Mass spectrometry*). Lemieux *et al.*[22] described that the coupling constant $J_{1,2}$ in furanose ring forms can vary between 3.5 and 8 Hz (dihedral angle 0–45°) for *cis* H_1–H_2 couplings and between 0 and 8 Hz (dihedral angle 75–165°) for *trans* H_1–H_2 couplings. D. G. Streefkerk (personal communications) has found that $J_{1,2}$ amounts to 2.9 Hz for TMS-β-D-galactofuranose (*trans* coupling) and to 4.5 Hz for TMS-α-D-galactofuranose (*cis* coupling) ([$^2H_6$]acetone; see also Acree *et al.*[23]). Therefore, in the case of a non-reducing furanose ring, the other two doublets with coupling constants of 2.9 Hz point to β configuration of the glycosidic bond.

The PMR spectrum of the TMS derivative of the digalactoside, dissolved in [$^2H_6$]benzene (Fig. 7), leads to the same result, but in this case the anomeric signals of $H_G^{\beta\to\alpha}$ and $H_G^{\beta\to\beta}$ coincide: $\delta H_G^{\beta} = 5.04$ ppm ($J_{1,2} \cong 2$ Hz), $\delta H^{\alpha} = 5.20$ ppm ($J_{1,2} \cong 2$ Hz) and $\delta H^{\beta} = 4.59$ ppm ($J_{1,2} = 7$ Hz).

On the basis of a similar reasoning, the PMR spectrum of the TMS derivative of the digalactoside alditol, dissolved in [$^2H_6$]acetone (Fig. 8), points also to the β configuration for the glycosidic bond ($\delta H_G^{\beta} = 4.78$ ppm; $J_{1,2} < 1$ Hz). The PMR spectra of TMS-6-*O*-β-D-galactofuranosyl-D-galactose and its TMS-alditol (Figs. 7

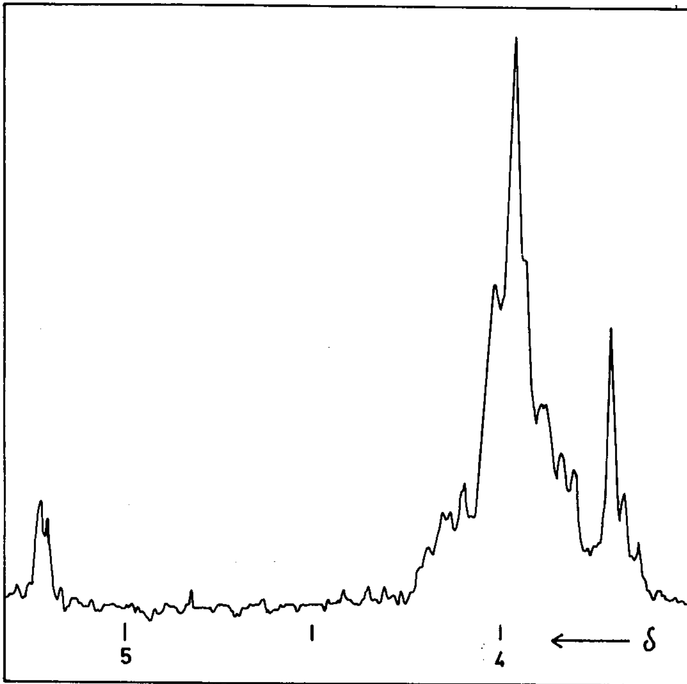


Fig. 9. Fourier Transform PMR spectrum of TMS-5-*O*-β-D-galactofuranosyl-D-galactitol (100 transients).

and 8) are identical with the spectra reported above. The PMR spectrum of TMS-5-*O*-β-D-galactofuranosyl-D-galactitol, dissolved in [$^2H_6$]acetone (Fig. 9) shows a doublet at $\delta = 5.21$ ppm ($J_{1,2} = 2.2$ Hz) which is in agreement with the β configuration of the glycosidic bond.

CONCLUSION

From the results it can be concluded that the digalactoside isolated from the arabinogalactan of cell walls and Wax D is 6-*O*-β-D-galactofuranosyl-D-galactose. The permethylation results suggest the presence of pyranose, as well as furanose ring forms, for the reducing D-galactose unit. On the other hand, the PMR results are in favour of a reducing galactopyranose residue. It is known that, by permethylation of sugars, distortion of the anomeric equilibrium can occur[24]. For the pertrimethylsilylation under the described conditions only a maximal distortion of 5 % has been reported. It can not be excluded that the arabinogalactan itself should contain only galactofuranose units. Then the formation of the pyranose ring would be brought about by the partial hydrolysis of the polysaccharide.

The presence of D-galactofuranosyl units in the arabinogalactan rules out the 1 → 4 linkage between arabinofuranose and galactofuranose. Therefore the structure proposed by Misaki *et al.*[4] has to be modified.

D-Galactofuranosides are not very common in nature. It is noteworthy that

several of the type-specific substances of *Pneumococcus* species contain this unit[25]. Recently, a galactofuranosyl residue has also been found in the T_1-specific chain of a lipopolysaccharide from *Salmonella friedenau*[26]. Other microorganisms known to produce polysaccharides containing D-galactose in the furanose ring form include *M. mycoides*[18], *Gibberella fujikuroi*[27], *Peltigera horizontalis*[28] and *Penicillium charlesii*[29].

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