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A Galactofuranose Disaccharide from the Galactan of *Mycoplasma mycoides*

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Mycoplasma mycoides var. *mycoides*, the causal organism of contagious bovine pleuropneumonia, forms an immunologically specific polysaccharide, extractable with warm aqueous phenol (Buttery & Plackett, 1960). Although heterogeneous in immunodiffusion tests and in the ultracentrifuge, the preparations contained 90% by weight of anhydrogalactose in acid-labile combination. Periodate-oxidation data were consistent with a structure composed mainly of (1→4)-linked galactopyranose units, or of (1→5)- or (1→6)-linked galactofuranose units. The optical rotation, $[\alpha]_D - 140^\circ$, indicated a high proportion of β -linkages. We have obtained further evidence for (1→6)-galactofuranose linkages by isolating the corresponding disaccharide from a partial acid hydrolysate.

EXPERIMENTAL

Materials

Mycoplasma mycoides galactan was prepared by extraction with warm aq. phenol (phenol-water, 6:1, w/w), and treated with Dowex 1 to remove RNA, as described by Buttery & Plackett (1960). A crude preparation of *Penicillium charlesii* polysaccharides was obtained from the extracellular fluid of cultures grown in Raulin-Thom medium (Haworth, Raistrick & Stacey, 1937). Lactitol and melibiitol were obtained by reduction of the corresponding disaccharides with NaBH_4 in aqueous solution. 5-O-D-Galactofuranosylgalactitol was a gift from Dr P. A. J. Gorin.

Methods

Melting points are corrected.

Analytical methods. Total carbohydrate was determined by the anthrone method (Trevelyan & Harrison, 1952), and reducing sugar by the colorimetric procedure of Somogyi (1952), galactose being used as the standard.

Periodate consumption was determined spectrophotometrically at 230 m μ , freshly prepared solutions of NaIO_4 and NaIO_3 being used as standards.

For the determination of 1,2-glycols, samples (0.05–0.15 μ mole in 0.25 ml.) were mixed with sodium metaperiodate (0.15 ml. of 0.1M solution) and kept at 30° for 7–8 min. Sodium metabisulphite (6%, w/v, in $\text{N-H}_2\text{SO}_4$), 0.25 ml., was added to destroy excess of periodate, after which formaldehyde was determined with chromotropic acid reagent according to Hanahan & Olley (1958). Erythritol was used as a standard. Blanks, in which the metabisulphite reagent was added before the periodate, were also run. Many carbohydrate substances gave appreciable colour under these conditions. The blank extinctions were subtracted from the values obtained after periodate oxidation.

Paper chromatography. Whatman no. 1 paper was used with the following solvent systems: (A) butan-1-ol-pyridine-water (6:4:3, by vol.); (B) butan-1-ol-ethanol-water (40:11:19, by vol.); (C) butan-1-ol-acetic acid-water (5:2:3, by vol.); (D) water-saturated phenol (in the presence of aq. NH_3 soln. and KCN).

Reducing sugars and polyols were detected with AgNO_3 (Dedonder, 1952), reducing sugars with aniline hydrogen phthalate (Cummins & Harris, 1956) and with aniline-diphenylamine-phosphoric acid (Bailey & Bourne, 1960).

Isolation of the galactobiose

Paper chromatograms of the partly hydrolysed galactan (for conditions see below) showed the presence of a number of reducing oligosaccharides. A plot of $\log(1 - R_f)/R_f$ versus fragment number is shown in Fig. 1. The linear relationship indicates that the fragments belong to an homologous series, of which free galactose is a member (French, 1954). Also plotted are data for partial-hydrolysis products of galactocarlose, obtained by hydrolysis of the *Penicillium charlesii* polysaccharides at pH 2.2 and 75° for 18 hr. (Gorin & Spencer, 1959). In the latter case the line does not pass through the point corresponding to galactose, presumably because the (1→5)-linkage prevents the formation of a pyranose ring in the reducing terminal residues.

It was estimated, by visual comparison of spots on paper chromatograms, that the maximum yield of disaccharide from the *M. mycoides* galactan was obtained after about 100 min. in 0.02N-H₂SO₄ at 100°. Accordingly, 410 mg. of the material was hydrolysed under these conditions in a volume of 33 ml. Turbidity developed after heating for about 60 min. and increased as hydrolysis continued. The hydrolysate was cooled in ice and extracted with light petroleum (b.p. 58–62°) (2 × 15 ml.) and with CHCl₃ (4 × 15 ml.) to remove the lipid component. The aqueous layer was neutralized with BaCO₃. After removal of BaSO₄, the solution was concentrated and chromatographed on a Chromax cellulose column (size no. 1; LKB Produkter, Stockholm, Sweden). The column was eluted with solvent (B) at a flow rate of about 0.3 ml./min. Fractions (9.3 ml.) were collected and examined by paper chromatography. Six main peaks were eluted. The corresponding fractions were concentrated *in vacuo*. Water was added to assist removal of butanol. The amounts of total carbohydrate recovered are shown in Table 1. Paper chromatography of the concentrated solutions showed the presence, in each of peaks 2–5, of at least one minor component, migrating more slowly than the major component in solvent (A). It has not been determined whether these are derived from branch points in the galactan structure.

The aqueous concentrate from the second peak (3.6 ml., containing carbohydrate equivalent to 78 mg. of galactose) was mixed with ethanol (2.2 ml.) and butan-1-ol (16 ml.). After concentration *in vacuo* to about 6 ml., a quantity of amorphous solid was deposited. This was dissolved in hot 90% (v/v) ethanol (8 ml.). On cooling, small irregular bar-shaped crystals formed. They were recovered by centrifuging, washed with ethanol (1 ml.), and dried *in vacuo* over H₂SO₄ and NaOH at room temperature. The product (55 mg.) had m.p. 171–174° (decomp.). The supernatant and

washings were combined with the butanolic supernatant from the previous step and treated to yield a second crop (6 mg.; m.p. 171–174°, decomp.).

The galactobiose had $[\alpha]_D^{24} = 28.2 \pm 1.3^\circ$ (c 4.5 in water). The estimated error is given as the s.d. of 12 pairs of readings.

Chromatographic mobilities relative to lactose (R_{Lac}) in solvents (A), (B), (C) and (D) were respectively 1.33, 1.14, 1.07 and 0.89. Traces of the minor component of peak 2 were still detectable on heavily loaded chromatograms, but the amount present was judged to be well below that which might interfere with structural studies. With the aniline hydrogen phthalate reagent the galactobiose gave yellow-brown spots, indistinguishable from those of galactose, melibiose and lactose. It also behaved like melibiose and galactose with the

Fig. 1. Chromatographic mobility of oligogalactosides in solvent (A): O, fragments of *M. mycoides* galactan; ●, fragments of galactocarlose.

Table 1. Cellulose-column chromatography of oligosaccharides

Carbohydrate was determined by the anthrone method with galactose as the standard. Recoveries are calculated as a percentage of the sample applied to the column.

Peak no.	Tube nos.	Carbohydrate (mg.)	Recovery (%)
1	35–45	100	24
(Galactose)			
2	47–63	79	19
3	66–89	66	16
4	93–117	48	11
5	135–165	31	7
6	203–232	18	4

aniline-diphenylamine-phosphoric acid reagent. The spots were greyish green after brief (3–5 min.) heating at 80°. Under these conditions lactose gave blue spots. All the spots subsequently turned grey and the difference between lactose and the other sugars diminished. The oligosaccharides from galactocarolose behaved quite differently with this reagent. After brief heating they gave vivid blue-green spots, which later changed to brownish green.

The yield of reducing sugars after acid hydrolysis of the galactobiose was 1.96 moles/342 g. The reducing equivalent of the unhydrolysed material was 1.18 moles (as galactose). After periodate oxidation 0.9 mole of formaldehyde was found. In a control experiment, neither lactose nor laminaribiose gave measurable yields of formaldehyde. The infrared spectrum of the galactobiose was determined by Dr H. G. Higgins. The presence of bands at 916, 883 and 806 cm^{-1} was consistent with a furanose structure (Barker & Stephens, 1954).

Reduction of the galactobiose

To 33.5 mg. (99 μmoles) of the galactobiose in 2.0 ml. of water was added 20.7 mg. (550 μmoles) of NaBH_4 . After 4 hr. at 20–22°, 0.03 ml. of acetic acid was added with shaking. The acidified solution was passed rapidly through a column of Dowex 50 (H^+ form) and freeze-dried. The residue was dissolved in methanol and evaporated to dryness six times. It was dissolved in hot methanol-ethanol. On cooling, an amorphous precipitate was formed. The supernatant was removed and the solid dried *in vacuo* over H_2SO_4 and NaOH . Yield: 21.8 mg.

When chromatographed in solvent (A) the product (R_{gal} 0.80) was readily distinguished from 5-O-D-galactofuranosyl-D-galactitol (R_{gal} 0.92). It migrated much more rapidly than the galactopyranosylglucitols lactitol (R_{gal} 0.47) and melibitol (R_{gal} 0.48). It was completely hydrolysed to galactose and galactitol after 2 hr. in 0.1N- H_2SO_4 at 100°. The yield of reducing sugar after acid hydrolysis was 0.96 mole/344 g. Yields of formaldehyde (for determination, see Analytical methods) from the periodate oxidation of the galactobi-itol and related polyols are (moles/mole): galactobi-itol 1.8, melibi-itol 0.9, lactitol 1.8, erythritol 2.0, dulcitol 1.9. The time-course of oxidant consumption in dilute periodate solution at pH 4.6 is shown in Table 2.

Degradation of the galactobi-itol

A portion (8 mg.; 23 μmoles) of the galactobi-itol and 140 μmoles (6.1 mol.prop.) of NaIO_4 in 0.9 ml. of unbuffered aqueous solution, were kept at 21° for 4 min. The solution was filtered through a mixed-bed column of Dowex 50 (H^+ form) and Dowex 3 (OH^- form) containing 0.2 g. of each resin.

Table 2. Oxidant consumption in dilute periodate solution

Reaction mixtures contained 0.4 mM-sodium metaperiodate and 0.6 mM-potassium acetate buffer (pH 4.6). They were kept in the dark at about 25°. Samples were withdrawn at intervals and diluted for measurement of E_{230} .

Substrate	Concn. (μM)	Oxidant consumed (moles/mole) after			
		20 min.	60 min.	360 min.	1360 min.
Galactobi-itol	27	3.8	—	4.9	5.3
	54	—	4.3	4.8	5.0
Melibi-itol	39	3.4	—	4.3	4.9
	52	—	3.6	4.2	4.7
Lactitol	62	1.9	—	3.7	5.0
Dulcitol	54	—	4.2	4.5	4.8
Erythritol	56	3.0	—	3.0	3.2
	62	—	3.0	3.0	3.1

Table 3. Products of sequential oxidation and reduction of the galactobi-itol

The unhydrolysed product gave brown spots with silver nitrate. Product (a) and arabinose gave red spots with aniline hydrogen phthalate. Product (b) and ethylene glycol gave black spots with silver nitrate.

	R_{gal} in solvent			
	(A)	(B)	(C)	(D)
Unhydrolysed product	1.99	2.39	1.88	—
After acid hydrolysis (a)	1.30	1.33	1.25	1.21
After acid hydrolysis (b)	2.07	3.02	2.25	—
Arabinose	1.30	1.33	1.23	1.21
Ethylene glycol	2.05	3.04	2.27	—

Sodium tetrahydroborate (325 μmoles) was added to the effluent and the solution was kept at room temperature for 4 hr. Acetic acid (10%, v/v; 0.15 ml.) was added with shaking, after which the solution was passed through a column of Dowex 50 (H^+ form) and freeze-dried. The residue was dissolved in 0.5 ml. of methanol and evaporated to dryness six times. Samples were chromatographed before and after hydrolysis in 0.1N- H_2SO_4 at 100° for 2 hr. Mobilities relative to galactose are recorded in Table 3. Traces of material migrating like galactose and glycerol were also observed on heavily loaded chromatograms of the hydrolysate. When melibi-itol was degraded under the same conditions, substantial amounts of glycerol, as well as ethylene glycol and galactose (but no arabinose), were found in the hydrolysate.

DISCUSSION

The operations used to obtain and degrade the galactobiose are summarized in Fig. 2. The same sequence of reactions was used by Gorin & Spencer

(1959) to characterize 5-*O*- β -D-galactofuranosyl-D-galactose from galactocarlose. They obtained 2-*O*- α -L-arabinofuranosylglycerol by degradation of the galactobi-itol. Although we did not isolate the presumed arabinoside of ethylene glycol (III), chromatographic identification of the hydrolysis products is evidence for a (1 $\rightarrow$ 6)-linkage in the galactobiose (I) from *M. mycoides*. The formation of considerable amounts of glycerol, in addition to galactose and ethylene glycol, observed when melibi-itol was degraded by this procedure, probably resulted from oxidation of the galactopyranose ring, which would take place preferentially at the *cis*-hydroxyl pair (C-3,4). In the galactofuranosyl-galactitol (II) on the other hand, the *trans* pair (C-2,3) in the five-membered ring would be relatively resistant to oxidation by periodate (Kjølberg, 1960).

Clancy & Whelan (1959) showed that in dilute (0.4 mM) periodate solution only the hexitol group of the glucopyranosylglucitols was attacked, and that measurements of the rate and extent of oxidant consumption could be used to distinguish between isomaltitol [(1 $\rightarrow$ 6)-linkage], cellobi-itol [(1 $\rightarrow$ 4)-linkage] and laminaribi-itol [(1 $\rightarrow$ 3)-link-

age]. Isomaltitol rapidly consumed the expected 4 mol.prop. of periodate, after which no further reaction took place. Oxidation of the glucitol group in cellobi-itol and laminaribi-itol should consume 3 mol.prop., but it was found that the initial rapid phase of the reaction ceased when about 2 and 1.5 mol.prop. respectively had been consumed. The uptake of oxidant continued at a reduced rate for several hours thereafter. Cyclization of pentose derivatives formed from cellobi-itol and laminaribi-itol may be invoked to explain these results (Cantley, Hough & Pittet, 1959). Angyal & Klavins (1961) concluded, from studies of formate ester production, that aldoses, when produced during periodate oxidation in the *aldehyde* form, are further oxidized predominantly in that form. But they point out that the relative rates of oxidation and cyclization are concentration-dependent, cyclization being a first-order process. The more rapid oxidation of cellobi-itol is probably due to the presence of a free α T-glycol group (Hutson & Weigel, 1961), not present in laminaribi-itol.

Our results for melibi-itol and lactitol are similar to those of Clancy & Whelan (1959) for isomaltitol and cellobi-itol respectively. Slightly higher values

Fig. 2. Degradation of the galactobiose.

for oxidant consumption are expected, since the galactopyranose ring contains a susceptible *cis*-hydroxyl pair. Clancy & Whelan (1959) noted that the methyl galactopyranosides were attacked somewhat faster than the methyl glucopyranosides. The consumption of 5 mol.prop. of periodate, with little or no oxidation of the furanose ring, is consistent with the postulated structure (II) for the galactobitol.

The difference between the molecular rotation of methyl β -galactopyranoside (0°) and methyl β -galactofuranoside (-21700°) (calculated from the data of Augestad & Berner, 1954) is very close to that between the molecular rotation of 6-*O*- β -D-galactopyranosyl-D-galactose ($+11600^\circ$, calculated from data of Elsner, 1935) and the galactobiose from *M. mycoides* (-9600°). We conclude that the latter is 6-*O*- β -D-galactofuranosyl-D-galactose (I).

SUMMARY

1. A galactobiose was isolated from a partial acid hydrolysate of *Mycoplasma mycoides* galactan, and degraded via the corresponding galactobi-itol.
2. The results are consistent with the structure: 6-*O*- β -D-galactofuranosyl-D-galactose.

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Localization of Protein-Bound Radioactive Iodine in Rat Thyroid Glands Labelled with ^{125}I or ^{131}I

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The site of iodination of thyroglobulin has been the subject of a number of radioautographic studies in the past 15 years. Leblond & Gross (1948) found that, in the thyroids of rats on a moderately low-iodine diet, most of the organic ^{131}I was localized in the colloid, even when the animals were killed 2 min. after injection of ^{131}I . Rats whose diet was supplemented with 22 mg. of iodine/day had less active glands, and the presence of organic ^{131}I was demonstrated in the thyroid epithelium of one animal, 1 hr. after injection of the dose. In hypophysectomized rats, the thyroidal radioiodine uptake was low, and organic ^{131}I remained in the epithelial cells up to 24 hr. after injection of the dose.

Doniach & Pelc (1949) also found that organic

^{131}I was detectable only in the follicular lumen of the thyroids of normal rats, within 10 min. of injection of the dose, and that prolonged pre-treatment with thyroxine (to suppress secretion of thyrotrophin; Yamada, Iino & Greer, 1961) restricted organic ^{131}I to the epithelial cells. Nadler & Leblond (1955) confirmed the rapid appearance of organic ^{131}I in the colloid of normal rats; Wollman & Wodinsky (1955) were unable to demonstrate radioactivity in mouse thyroid cells even within a few seconds of injection of ^{131}I .

Mayer, Dimick & Kelly (1956) studied the incorporation of ^{131}I by fresh bovine thyroid slices and by slices from glands that had been stored at -16° for 2 weeks; they found that, whereas the fresh slices formed organic ^{131}I in the colloid,