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PYRUVIC ACID-CONTAINING MONO- AND OLIGO-SACCHARIDES FROM *Rhizobium trifolii* BART A*

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ABSTRACT

After partial, acid hydrolysis of the extracellular, acid polysaccharide from *Rh. trifolii* Bart A, the following products were isolated and characterized: 3,4-*O*-(1-carboxyethylidene)-D-galactose, 4,6-*O*-(1-carboxyethylidene)-D-galactose, 3-*O*-[3,4-*O*-(1-carboxyethylidene)-β-D-galactopyranosyl]-D-glucose, 3-*O*-[4,6-*O*-(1-carboxyethylidene)-β-D-galactopyranosyl]-D-glucose, *O*-[3,4-*O*-(1-carboxyethylidene)-β-D-galactopyranosyl]-(1→3)-*O*-β-D-glucopyranosyl-(1→4)-D-glucose, and *O*-[4,6-*O*-(1-carboxyethylidene)-β-D-galactopyranosyl]-(1→3)-*O*-β-D-glucopyranosyl-(1→4)-D-glucose. The presence of pyruvic acid linked either to O-3 and O-4 or to O-4 and O-6 of the D-galactopyranosyl group of these saccharides indicates that both structures may be present in the original polysaccharide.

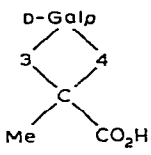
INTRODUCTION

Pyruvate was first detected as a constituent of a polysaccharide in agar[1] and subsequently in capsular polysaccharides from *Xanthomonas campestris*[2]. Pyruvate occurs widely as a constituent of exopolysaccharides, such as those produced by different strains of *Rhizobium*[3]. The pyruvate moiety is an immunological determinant[3,4]. The polysaccharides of fast-growing strains of *Rhizobium* contain D-galactose, D-glucose, D-glucuronic acid, *O*-acetyl groups, and acetal-bound pyruvic acid. Methylation analysis established that pyruvic acid is linked to positions 4 and 6 of galactose and glucose residues[5–7]. Pyruvic acid linked to positions 2 and 3 of galactose residues has been found in a *Pneumococcus* polysaccharide[8], and to positions 3 and 4 of galactose in *Salmonella*[9] and *Klebsiella*[10] polysaccharides.

Pyruvate is usually found in polysaccharides that also contain *O*-acetyl groups, and partial hydrolysis with acid may yield pyruvylated mono- and oligo-saccharides[11,12]. *Rhizobium* polysaccharides have been submitted to partial hydrolysis with acid[5,6,13–15], but no acidic oligosaccharides have been isolated except three glucuronic acid-containing oligomers[15].

*Fragmentation Analysis of Extracellular Acid Polysaccharides from Seven *Rhizobium* Strains, Part II. For Part I, see ref. 15.

We now report on the identification of the pyruvylated components **1–6**.



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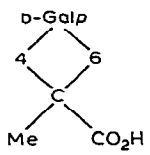

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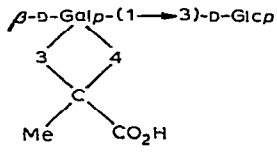

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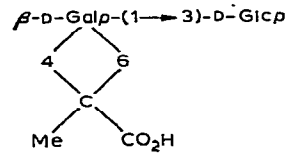

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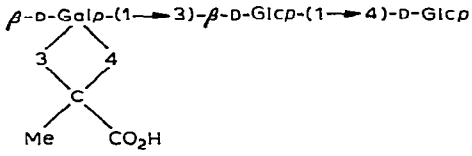

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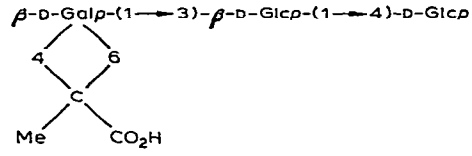

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RESULTS AND DISCUSSION

The polysaccharide (PS) was partially hydrolysed with acid and the products were fractionated as described in the Experimental section. The acidic fraction contained pyruvic acid and glucuronic acid, the latter indicating some breakage of the PS backbone. The components with the highest paper-chromatographic (p.c.) mobilities consisted of one of the glucuronic acid-containing oligosaccharides formerly identified[15], cellobiouronic acid, and the components **1–6** having pyruvic acid as the acidic constituent (see Table I).

TABLE I

YIELDS AND P.C. AND ELECTROPHORETIC MOBILITIES OF **1–6**

	1	**2**	**3**	**4**	**5**	**6**
mg/g of PS	2	9	3	3	2	6
R_{Glc}[a]	0.86	0.55	0.68	0.42	0.42	0.29
R_{Glc}[b]	3.11	1.96	1.25	0.70	0.37	0.16
M_{GlcA}[c]	0.90	0.92	0.63	0.62	0.46	0.46

[a]Ethyl acetate–acetic acid–pyridine–water (5:1:5:3). [b]Ethyl acetate–acetic acid–formic acid–water (18:3:1:4). [c]Pyridine–acetic acid–water (5:2:43, pH 5.3).

Component **1** was 3,4-*O*-(1-carboxyethylidene)-D-galactose. Acid hydrolysis gave D-galactose and pyruvic acid. Reduction of **1** followed by hydrolysis gave galactitol, which was identified by g.l.c. after trimethylsilylation. The reduction of **1** required relatively vigorous conditions. Conventional methylation analysis (methylation, hydrolysis, reduction, acetylation, and g.l.c.–m.s.) of **1** gave 2,6-di-*O*-methyl-D-galactitol tetra-acetate and 1,2,5,6-tetra-*O*-methyl-D-galactitol diacetate (**7**). When

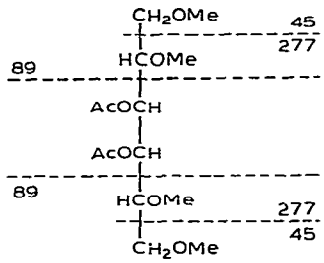
TABLE II

G.L.C. AND M.S. DATA FOR ACETATES OF METHYLATED ALDITOLS

Methylated sugar[a]	T[b]	*Relative intensities (%)* m/e	*249*	*205*	*185*	*177*	*175*	*173*	*161*	*157*	*145*	*133*	*129*	*117*	*113*	*102*	*101*	*99*	*89*	*87*	*75*	*71*	*59*	*45*	*43*
1,3,4,5,6-Glc	0.51		—	29	—	—	—	—	51	—	60	16	72	—	10	20	61	—	36	26	16	29	21	100	64
1,2,4,5,6-Glc	0.54		12	48	8	—	5	9	—	14	5	21	8	10	17	7	69	—	95	11	22	13	35	60	36
1,2,3,5,6-Glc	0.60		7	57	6	—	3	10	—	11	5	15	8	8	23	5	69	10	100	11	23	11	33	56	33
1,2,3,4,5-Glc	0.61		—	—	—	12	—	6	23	—	26	16	—	76	1	10	100	9	57	13	27	16	19	62	86
		m/e	*277*	*233*	*205*	*185*	*175*	*161*	*159*	*145*	*143*	*133*	*129*	*127*	*117*	*111*	*101*	*89*	*87*	*85*	*75*	*71*	*59*	*58*	*45*	*43*
1,2,5,6-Gal	0.89		33	—	—	31	18	—	—	—	51	—	39	—	10	15	10	53	51	13	24	12	34	13	39	100
1,2,3,5-Gal	1.03		2	11	—	3	1	—	11	—	9	8	4	9	51	2	71	16	7	11	13	8	16	7	42	100
2,3,4,6-Glc	1.00		—	—	12	—	—	38	—	33	—	—	34	—	41	1	67	8	18	3	8	13	3	3	34	100
2,3,4,6-Gal	1.09																									

[a]1,3,4,5,6-Glc = 2-*O*-acetyl-1,3,4,5,6-penta-*O*-methyl-D-glucitol, *etc.* [b]Retention time of the corresponding alditol acetate relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on an OV-225 column programmed at 2°/min from 160→200°.

prolonged reduction preceded methylation, **7** was the sole product of methylation analysis. The secondary fragments given in Table II can be elucidated from the primary fragments of **7**.



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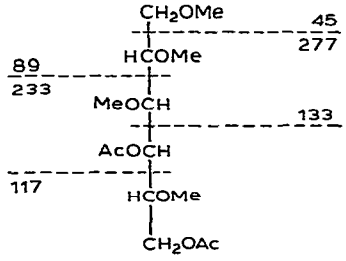

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Component **2** was 4,6-*O*-(1-carboxyethylidene)-D-galactose. Acid hydrolysis gave D-galactose and pyruvic acid, and reduction followed by hydrolysis gave only galactitol, identified by g.l.c. of the trimethylsilylated derivative. Component **2** was easily reduced with sodium borohydride. Methylation analysis of **2** gave 2,3-di-*O*-methyl-D-galactitol tetra-acetate as the main product. When the methylation analysis was preceded by reduction, the product was 1,2,3,5-tetra-*O*-methyl-D-galactitol diacetate (**8**). The secondary fragments given in Table II can be elucidated from the primary fragments of **8**.

Component **3** was 3-*O*-[3,4-*O*-(1-carboxyethylidene)-β-D-galactopyranosyl]-D-glucose. Acid hydrolysis gave D-galactose, D-glucose, and pyruvic acid. The molar ratio of hexose and pyruvic acid was 2:1. G.l.c.–m.s. of the products obtained when **3** was reduced, hydrolysed, and acetylated showed that glucose was the reducing moiety. Reduction of **3** followed by methylation analysis gave equal amounts of 2,6-di-*O*-methyl-D-galactitol tetra-acetate and 1,2,4,5,6-penta-*O*-methyl-D-glucitol acetate.

Component **4** was 3-*O*-[4,6-*O*-(1-carboxyethylidene)-β-D-galactopyranosyl]-D-glucose. Total, acid hydrolysis gave D-galactose, D-glucose, and pyruvic acid. The molar ratio of hexose and pyruvic acid was 2:1. G.l.c.–m.s. of the products obtained when **4** was reduced, hydrolysed, and acetylated showed that glucose was the reducing moiety. Reduction of **4** followed by methylation analysis gave equal amounts of 2,3-di-*O*-methyl-D-galactitol tetra-acetate and 1,2,4,5,6-penta-*O*-methyl-D-glucitol acetate.

Component **5** was *O*-[3,4-*O*-(1-carboxyethylidene)-β-D-galactopyranosyl]-(1→3)-*O*-β-D-glucopyranosyl-(1→4)-D-glucose. Acid hydrolysis gave D-galactose, D-glucose, and pyruvic acid. The molar ratio of hexose and pyruvic acid was 3:1. G.l.c.–m.s. of the products obtained when **5** was reduced, hydrolysed, and acetylated showed that glucose was the reducing moiety. Reduction of **5** followed by methylation analysis gave equal amounts of 2,6-di-*O*-methyl-D-galactitol tetra-acetate, 2,4,6-tri-*O*-methyl-D-glucitol triacetate, and 1,2,3,5,6-penta-*O*-methyl-D-glucitol acetate.

Component **6** was *O*-[4,6-*O*-(1-carboxyethylidene)-β-D-galactopyranosyl]-

(1→3)-*O*-β-D-glucopyranosyl-(1→4)-D-glucose. Acid hydrolysis gave D-galactose, D-glucose, and pyruvic acid. The molar ratio of hexose and pyruvic acid was 3:1. G.l.c.–m.s. of the products formed when **6** was reduced, hydrolysed, and acetylated showed that glucose was the reducing moiety. Reduction of **6** followed by methylation analysis gave equal amounts of 2,3-di-*O*-methyl-D-galactitol tetra-acetate, 2,4,6-tri-*O*-methyl-D-glucitol triacetate, and 1,2,3,5,6-penta-*O*-methyl-D-glucitol acetate.

Components **1–6**, which form two series of saccharides involving pyruvic acid linked to galactose through positions 4 and 6 (**2,4,6**) and 3 and 4 (**1,3,5**), are not artifacts. When a mixture of the monomer constituents of the polysaccharide in the correct molecular proportions was heated with trifluoroacetic acid, none of the products **1–6** was formed.

The identification of **2**, **4**, and **6** confirms the results[16] obtained on methylation analysis of the polysaccharide, which revealed that the pyruvic acid was linked as an acetal to positions 4 and 6 of terminal D-galactopyranosyl groups.

Terminal D-galactopyranosyl groups having a pyruvic acid acetal at positions 3 and 4 were extensively decomposed when a capsular polysaccharide from *Klebsiella* type 33 was submitted[10] to uronic acid degradation (methylation, treatment with base, and mild hydrolysis with acid.)

If a related degradation occurs during methylation studies of the *Rhizobium* polysaccharide, it might explain why pyruvic acid acetal-linked to positions 3 and 4 of galactose is a product of the partial hydrolysis, whereas the corresponding 2,6-di-*O*-

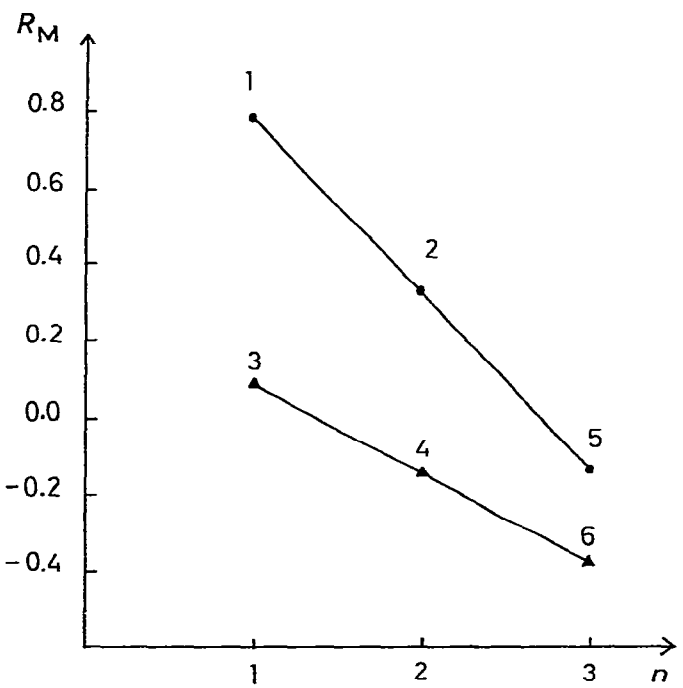

Fig. 1. Relation between R_M {log[R_{Glc}/(1 − R_{Glc})]} and chain-length (*n*).

methyl-D-galactose was not detected[16] as a product of methylation analysis of the polysaccharide.

The possibility of 4,6→3,4-acetal migration during the partial hydrolysis with acid should not be neglected. However no 4,6→3,4-acetal migration was observed in a study of 4,6-*O*-isopropylidene-D-galactopyranose[17].

Neither α-D-galactosidase nor β-D-galactosidase affected the pyruvylated galactose residue in **3**–**6**. The glycosidic linkages of the corresponding neutral fragments and fragments containing glucose only, isolated[16] simultaneously from the polysaccharide, were cleaved by β-D-galactosidase and β-D-glucosidase.

Electrophoresis of **1**–**6** revealed that the pyruvyl group had no influence on mobility. However, the p.c. mobilities of **1**, **3**, and **5** were much higher than those of **2**, **4**, and **6**. Similar behaviour has been found for the 3,4- and 4,6-*O*-isopropylidene-D-galactopyranoses[18].

The relationship between the two series **1**, **3**, and **5** and **2**, **4**, and **6** was supported by plots of $\log[R_{Glc}/(1 - R_{Glc})]$ *versus* the chain-length *n*, which were linear (Fig. 1, solvent *A*)[19].

EXPERIMENTAL

General methods. — The *Rhizobium* strain was *Rh. trifolii* Bart A. The cultivation technique and the preparation of the PS were as reported earlier[20]. Washed Whatman No. 1 or 3MM paper was used for preparative p.c. and preparative, high-voltage electrophoresis. The electrophoresis was performed with pyridine–acetic acid–water (5:2:43, pH 5.3) and strips (22 × 47 cm) of paper at 1.6 kV for 3 h (No. 1) and 0.8 kV for 6 h (No. 3MM). P.c. was performed by the descending method with *A*, ethyl acetate–acetic acid–pyridine–water (5:1:5:3); or *B*, ethyl acetate–acetic acid–formic acid–water (18:3:1:4); and detection with *p*-anisidine hydrochloride and aniline hydrogenphthalate. Pyruvic acid was detected with ethanolic *o*-phenylenediamine–trichloroacetic acid[21]. The total carbohydrate was determined by the phenol–sulphuric acid method[22]. G.l.c. of the alditol acetates and trimethylsilyl derivatives was performed on a Perkin–Elmer F11 gas chromatograph, equipped with a flame-ionisation detector and a glass column (180 × 0.15 cm) filled with 3% of OV-225 on 100/120 Supelcoport, programmed at 2°/min from 160→200° for the alditol acetates and 130→150° for the trimethylsilyl derivatives. The nitrogen flow-rate was 20 ml/min. Mass spectra (70 eV) were recorded on a Micromass 12 F instrument. Pyruvic acid was determined with lactate dehydrogenase (Sigma test kit No. 726) after liberation[23] of the acid with M HCl at 100° for 3 h. Acid hydrolysis was effected with M trifluoroacetic acid at 120° for 2 h. All evaporations were performed under diminished pressure below 40°. Trimethylsilylation of products (0.5–1 mg) was effected with pyridine (0.5 ml), hexamethyldisilazane (0.1 ml), and chlorotrimethylsilane (0.05 ml) at 60° for 30 min. The solvents were evaporated and a solution of the residue in 2,2,4-trimethylpentane was subjected to g.l.c.

Graded hydrolysis of the PS. — The PS (2 g) was soaked in water (300 ml)

overnight, and trifluoroacetic acid was then added to give a 0.5% solution in 0.5M acid, which was heated under reflux for 2 h, cooled, filtered, and concentrated. A solution of the residue in water (100 ml) was passed through an Amicon type PM-10 membrane (10,000 mol. wt. cut-off), and the products of low molecular weight were adsorbed on a column (30 × 3 cm) of DEAE-Sephadex A-25 ($HCOO^-$ form). Neutral sugars were eluted with water (500 ml), and the acid sugars with 0.5M formic acid (750 ml) followed by M formic acid (500 ml). The elution was monitored by the phenol–sulphuric acid method. Preparative p.c. (solvent *A*) of the acidic fraction gave one slow- and several fast-moving components. The latter components were further purified by preparative p.c. and high-voltage electrophoresis.

Methylation analysis. — Each oligosaccharide (0.5–1 mg) was reduced with $NaBH_4$ (5 mg) for 2 h, and the solution was acidified to pH 3 with Dowex-50W X8 (H^+) resin (1.2 g, 100–200 mesh), filtered, and concentrated, and methanol (3 × 5 ml) was distilled from the residue. The dry residue was methylated conventionally by the Hakomori method[24]. The methylated oligosaccharide was treated with 90% formic acid (3 ml) at 100° for 3 h, the hydrolysate was concentrated, and the residue was hydrolysed with 0.5M sulphuric acid at 100° for 16 h. The acid was neutralised with Dowex-1 (HCO_3^-) resin, the sugars were reduced as described above, and the products were treated with acetic anhydride in pyridine (1:1, 2 ml) at 100° for 1 h. The partially methylated alditol acetates were dissolved in dichloromethane and subjected to g.l.c.–m.s.

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