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IDENTIFICATION OF PYRUVATED MONOSACCHARIDES IN POLYSACCHARIDES BY GAS–LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

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ABSTRACT

Twelve bacterial polysaccharides of known structure containing a representative range of pyruvated monosaccharides, were methanolysed, trimethylsilylated, and analysed by g.l.c. and g.l.c.–m.s. Except for 3,4-*O*-(1-carboxyethylidene)-L-rhamnose, which was unusually labile, the pyruvic acid substituents were largely retained during methanolysis and the Me_3Si derivatives of the resulting pyruvated methyl glycosides gave distinctive g.l.c. peaks with characteristic mass spectra. The pyranose rings of 4,6-*O*-(1-carboxyethylidene)-D-glucose, 4,6-*O*-(1-carboxyethylidene)-D-mannose, 4,6-*O*-(1-carboxyethylidene)-D-galactose, and 3,4-*O*-(1-carboxyethylidene)-D-galactose survived the methanolysis, but that of 2,3-*O*-(1-carboxyethylidene)-D-glucuronic acid was cleaved to give the methyl ester of 2,3-*O*-(1-carboxyethylidene)-*aldehydo*-D-glucuronic acid dimethyl acetal. In the case of 2,3-*O*-(1-carboxyethylidene)-D-galactose, cleavage of the pyranose ring was less complete; under the conditions used in these experiments two-thirds of the pyranose rings were intact while one-third were cleaved to give the methyl ester of 2,3-*O*-(1-carboxyethylidene)-*aldehydo*-D-galactose dimethyl acetal. A very small amount of 3,4-*O*-(1-carboxyethylidene)-L-rhamnose from one polysaccharide retained its pyruvic acid substituent after gentle methanolysis to give the methyl ester of 3,4-*O*-(1-carboxyethylidene)-*aldehydo*-L-rhamnose dimethyl acetal. Susceptibility to cleavage of the pyranose ring during methanolysis appears to be a property of pyruvated monosaccharides with *trans*-fused 1,3-dioxolane rings.

INTRODUCTION

Pyruvic acid has been found as an acetal-linked substituent in the extracellular polysaccharides of many bacteria, especially among those of *Klebsiella* and *Rhizobium* species[1,2]. The presence of a pyruvic acid substituent may be readily detected and quantified by colorimetric[3], enzymic[4], and ^{1}H-n.m.r. methods[5], and the size of the acetal ring and the configuration of the carbon atoms in it established

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by ^{1}H- and ^{13}C-n.m.r. methods[6–8]. Further structural elucidation is complicated by the fact that the pyruvic acid may be attached to any one of the monosaccharides in the repeating unit of the polysaccharide by one of several alternative pairs of hydroxyl groups. Additional complexities arise when polysaccharides are substituted with two pyruvic acid groups per structural repeating unit[9–11].

Present methods for determining the location of a pyruvic acid substituent in a polysaccharide either involve partial hydrolysis and fractionation to isolate the pyruvated monosaccharide which is then identified by further hydrolysis, or involve comparison of the results of methylation analyses of the polysaccharide in its native state and after it has been carefully hydrolysed in dilute acid to remove the pyruvic acid without hydrolysing the glycosidic linkages.

When we examined the extracellular polysaccharides of *Rhizobium meliloti*, *R. trifolii*, *R. phaseoli*, and *R. leguminosarum* strains by methanolysis, formation of the Me_3Si derivatives and analysis by g.l.c., we confirmed an earlier observation that pyruvic acid substituents remain attached during methanolysis[12] and we have sought to develop a general procedure based on methanolysis for identifying pyruvated monosaccharides. This paper describes validation of the resulting g.l.c. method by applying it to the analysis of twelve polysaccharides of established structure, containing between them seven of the nine different pyruvated monosaccharides known at the present time. The trimethylsilylated methanolysates of the polysaccharides were examined by g.l.c. and g.l.c.–mass spectrometry. In one case, the pyruvated monosaccharide moiety was isolated to confirm its structure by mass spectrometry using different modes of ionisation.

RESULTS AND DISCUSSION

Preliminary experiments with extracellular polysaccharides of *Rhizobium* strains containing pyruvic acid showed that gas–liquid chromatograms of trimethylsilylated methanolysates exhibited additional peaks with retention times different from those of the monosaccharides known to be present in the polysaccharide (Fig. 1, a). However, when such polysaccharides were first hydrolysed with aqueous acid to remove the pyruvic acid substituents and then methanolysed, these unidentified peaks were absent; also, peaks of known monosaccharides were enlarged and sometimes peaks of known monosaccharides appeared only after this treatment (Fig. 1, b). These results suggested that the additional peaks from the methanolysed samples were due to the pyruvated monosaccharides and that the parent monosaccharide would be identified if its g.l.c. peak was relatively enlarged in samples that were hydrolysed before methanolysis than in those that were methanolysed directly.

To test this hypothesis, samples of pyruvated polysaccharides of known structure (Table I) were analysed by g.l.c. after methanolysis and conversion into Me_3Si derivatives. Additional peaks, which did not correspond with any of the monosaccharides known to be in the polysaccharides, were present in the gas–liquid

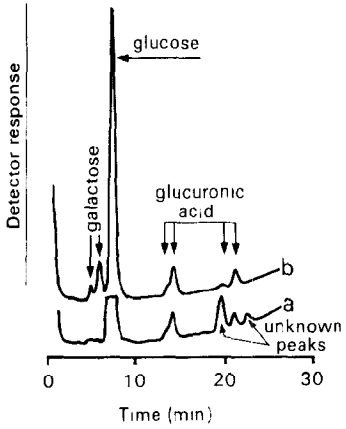


Fig. 1. Analysis of *R. phaseoli* 127K44 polysaccharide, containing D-glucose, D-galactose, D-glucuronic acid, and pyruvic acid. *a*, G.l.c. trace of a sample methanolysed and trimethylsilylated; and *b*, G.l.c. trace of a sample first hydrolysed and then methanolysed and trimethylsilylated. Note the presence of unknown peaks and the absence of galactose peaks in *a*, and the absence of unknown peaks and the presence of galactose peaks in *b*.

chromatograms of the methanolysates of all the polysaccharides (Fig. 2). These mixtures were subsequently analysed by g.l.c.–m.s. in order to establish the nature of the unknown peaks, especially to distinguish between pyruvated monosaccharides and possible disaccharides of comparable retention times (between 1.5 and 3.0 relative to that of glucose). In all the g.l.c. traces shown in Fig. 2, only one peak (**h**) was found to represent a disaccharide: it was in the methanolysate of the polysaccharide of *Klebsiella* K70 (see later).

*G.l.c.–m.s. analysis of polysaccharides containing 4,6-O-(1-carboxyethylidene)-*D*-glucose, 4,6-O-(1-carboxyethylidene)-*D*-mannose, 4,6-O-(1-carboxyethylidene)-*D*-galactose, and 3,4-O-(1-carboxyethylidene)-*D*-galactose.* — The unknown peaks in the gas–liquid chromatograms of the trimethylsilylated methanolysates of polysaccharides containing the pyruvated monosaccharides listed above (from *R. phaseoli* 127K36, 127K38, 127K44 and 127K87, *E. coli* K12, and *Klebsiella* K3 and K30) gave similar 70 eV electron ionisation (e.i.) mass spectra (Fig. 3). The fragmentation patterns show features common to those previously reported for *O*-isopropylidene and *O*-carboxyethylidene derivatives[12–14].

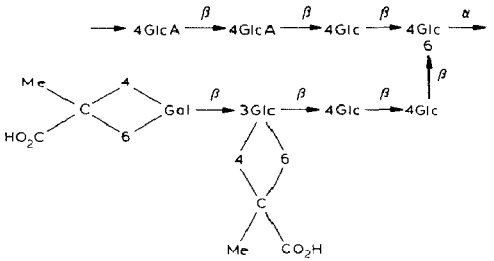
The e.i. mass spectra of the isomers all showed a prominent ion of *m/z* 363, corresponding to $(M - CO_2Me)^+$ formed by alpha cleavage from the carboxyethylidene ring and consistent with a molecular mass of 422, the predicted value for methyl *O*-(1-carboxyethylidene)hexopyranoside methyl ester di-Me_3Si ethers. The g.l.c. peaks were therefore assigned to the Me_3Si derivatives of the methyl glycoside methyl esters of the pyruvated monosaccharides previously established by classical structural methods to be present in these polysaccharides.

The double peaks given by 4,6-*O*-(1-carboxyethylidene)-D-galactose and 3,4-

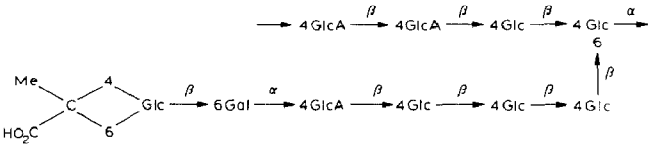
TABLE I

STRUCTURES OF REPEATING UNITS OF PYRUVATED POLYSACCHARIDES USED IN THE PRESENT STUDY ALL MONOSACCHARIDES ARE D EXCEPT WHERE L IS SHOWN

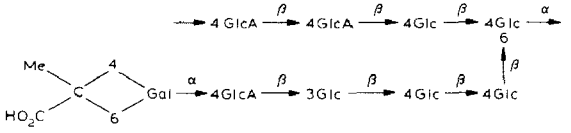
A[a] *Rhizobium phaseoli* 127K36 (Ref 11)



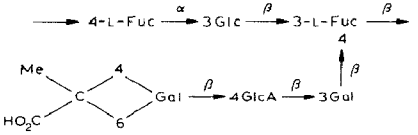
B *Rhizobium phaseoli* 127K38 (Ref 30)



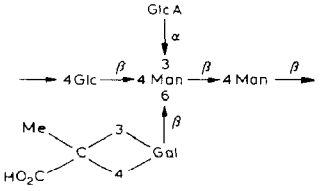
C *Rhizobium phaseoli* 127K44 (Ref 31)



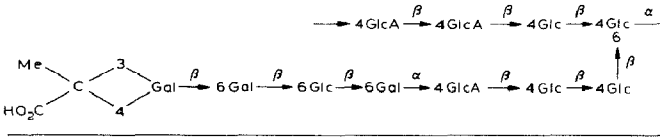
D *Escherichia coli* K12 (S53) (Ref 32)



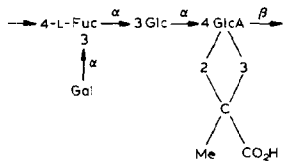
E *Klebsiella* K30 (Ref 33)



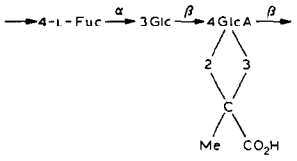
F *Rhizobium phaseoli* 127K87 (Ref 34)



G *Klebsiella* K58 (Ref 35)

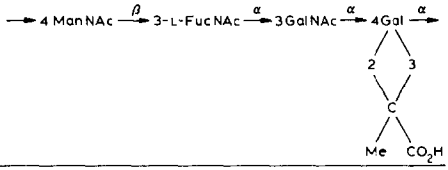


H *Klebsiella* K1 (Ref 36)

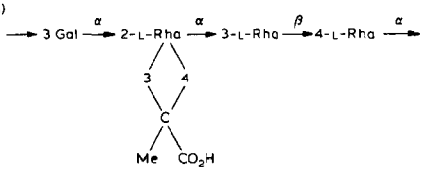


I *Klebsiella* K3 Contains Gal, Man, GalA, and pyruvic acid (10%) (ref. 2) but structure not determined. 4,6-*O*-(1-Carboxyethylidene)-D-mannose known to be present (personal communication, G. G. S. Dutton, 1982).

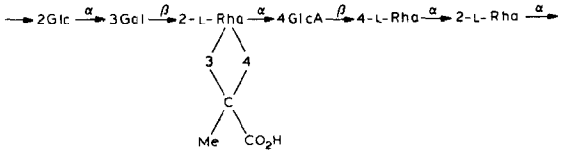
J *Streptococcus pneumoniae* type IV (Ref 37)



K *Klebsiella* K32 (Ref 38)



L *Klebsiella* K70 (Ref 39)



(Only 50% of the repeating units of this polysaccharide are pyruvated).

[a]The letters A–L relate these polysaccharides to the gas–liquid chromatograms in Fig. 2.

O-(1-carboxyethylidene)-D-galactose during g.l.c. (Fig. 2, peaks **b**,**b'**,**c**,**c'**) may be attributed to their respective anomers. The mass spectra of the anomers of the 4,6-acetal (**b**,**b'**) were similar to each other but differed with respect to the relative intensities of the daughter ions of *m/z* 273, 243 and 133 (Fig. 3, i, ii), whereas the

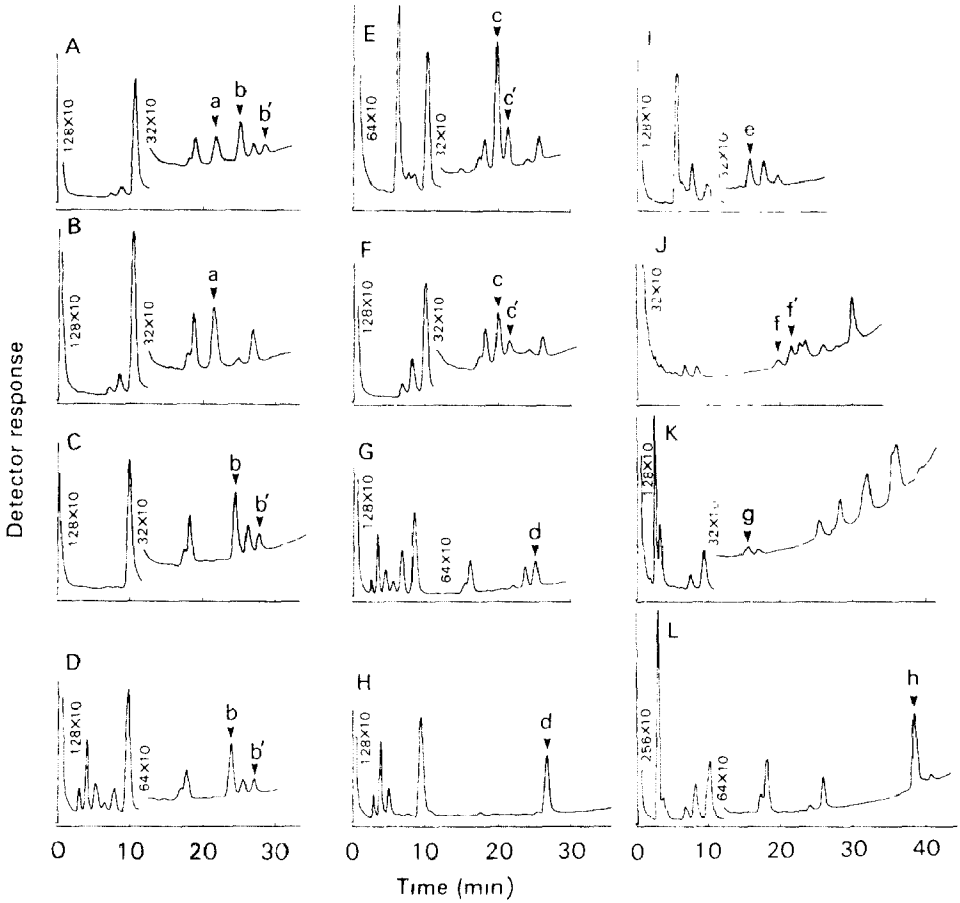
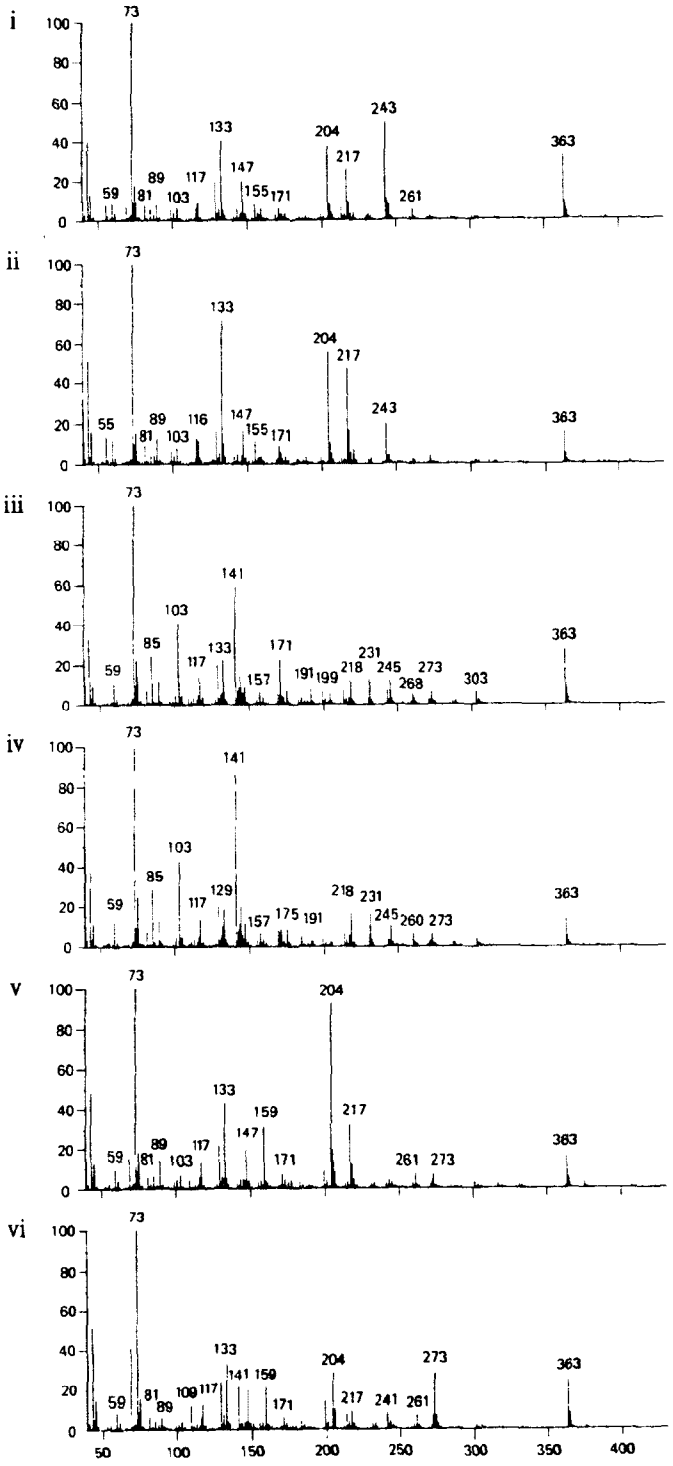


Fig. 2. Gas–liquid chromatograms of Me_3Si derivatives of methanolysis products of the polysaccharides listed A–L in Table I. The peaks labelled **a**–**h** were subsequently identified by mass spectrometry as the Me_3Si derivatives of the methyl esters of the following compounds: **a**, methyl 4,6-*O*-(1-carboxyethylidene)-D-glucopyranoside; **b**,**b'**, methyl 4,6-*O*-(1-carboxyethylidene)-D-galactopyranoside; **c**,**c'**, methyl 3,4-*O*-(1-carboxyethylidene)-D-galactopyranoside; **d**, 2,3-*O*-(1-carboxyethylidene)-*aldehydo*-D-glucuronic acid dimethyl acetal; **e**, methyl 4,6-*O*-(1-carboxyethylidene)-D-mannopyranoside; **f**, 2,3-*O*-(1-carboxyethylidene)-*aldehydo*-D-galactose dimethyl acetal; **f'**, methyl 2,3-*O*-(1-carboxyethylidene)-D-galactopyranoside; **g**, 3,4-*O*-(1-carboxyethylidene)-*aldehydo*-L-rhamnose dimethyl acetal; and **h**, methyl glycoside of the disaccharide β-D-Glc*p*A-(1→4)-L-Rha*p*.

Fig. 3. E.i. mass spectra (70 eV) of the Me_3Si derivatives of the methyl esters of. (i) methyl 4,6-*O*-(1-carboxyethylidene)-D-galactopyranoside, first anomer (Fig. 2, peak **b**); (ii) methyl 4,6-*O*-(1-carboxyethylidene)-D-galactopyranoside, second anomer (Fig. 2, peak **b'**); (iii) methyl 3,4-*O*-(1-carboxyethylidene)-D-galactopyranoside, first anomer (Fig. 2, peak **c**), (iv) methyl 3,4-*O*-(1-carboxyethylidene)-D-galactopyranoside, second anomer (Fig. 2, peak **c'**); (v) methyl 4,6-*O*-(1-carboxyethylidene)-D-glucopyranoside (Fig. 2, peak **a**), and (vi) methyl 4,6-*O*-(1-carboxyethylidene)-D-mannopyranoside (Fig. 2, peak **e**) Molecular mass of all derivatives, 422

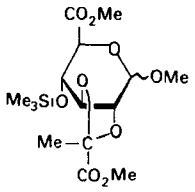

i
ii
iii
iv
v
vi
100
80
60
40
20
0
50
100
150
200
250
300
350
400

mass spectra of the anomers of the 3,4-acetal (**c**,**c'**) were almost identical with each other (Fig. 3, iii, iv). Although 4,6-*O*-(1-carboxyethylidene)-D-glucose gave only a single peak during g.l.c. (Fig. 2, peak **a**) gradual changes in the mass spectrum during the elution of the peak indicated that it was present as two anomers which had failed to be separated on the column used. However, analysis of the polysaccharide of *R. phaseoli* 127K38 by ethanolysis instead of methanolysis yielded two peaks (with relative areas 2:1) for the ethyl glycoside ethyl ester of 4,6-*O*-(1-carboxyethylidene)-D-glucose (results not shown). 4,6-*O*-(1-Carboxyethylidene)-D-mannose yielded only a single peak irrespective of whether the polysaccharide of *Klebsiella* K3 was depolymerised by methanolysis (Fig. 2, peak **e**) or ethanolysis (results not shown); this is in accord with the tendency of D-mannose to form the α anomer predominantly when it is glycosidated to equilibrium[15].

Certain features diagnostic of stereochemistry were evident in the e.i. mass spectra of each of these pyruvated derivatives. The prominent daughter ion of *m/z* 243 in the spectra of the 4,6-*O*-(1-carboxyethylidene)-D-galactose anomers was absent from those of the corresponding glucose and mannose derivatives. Conversely, the ion of *m/z* 159 was prominent in the spectra of 4,6-*O*-(1-carboxyethylidene)-D-glucose and 4,6-*O*-(1-carboxyethylidene)-D-mannose but not in the spectra of either of the 4,6-galactose anomers. A significant ion of *m/z* 273 for the mannose derivative distinguishes it from the glucose analogue. The prominent ions of *m/z* 204 and 217 in the spectra of the 4,6-linked derivatives were insignificant in the spectra of the 3,4-*O*-(1-carboxyethylidene)-D-galactose anomers, as is to be expected in the absence of adjacent Me_3Si groups in this compound[16].

G.l.c.–m.s. analysis of polysaccharides containing 2,3-O-(1-carboxyethylidene)-D-glucuronic acid, 2,3-O-(1-carboxyethylidene)-D-galactose and 3,4-O-(1-carboxyethylidene)-L-rhamnose. — A molecular mass of 378 would be expected from the methanolysates of polysaccharides known to contain 2,3-*O*-(1-carboxyethylidene)-D-glucuronic acid (*Klebsiella* K1 and K58), if the unknown peak **d** (Fig. 2) represented the methyl glycoside methyl ester of 2,3-*O*-(1-carboxyethylidene)-4-*O*-trimethylsilyl-D-glucopyranosiduronic acid, **1**. In fact, the mass spectrum of the derivative in peak **d** (compound I) showed a significant peak at *m/z* 423 (Fig. 4, i) which would imply a molecular mass of 482 on the basis of loss of $\cdot CO_2Me$ by alpha cleavage. Further, the base peak was at *m/z* 75 rather than *m/z* 73 as previously observed with the 4,6-*O*- and 3,4-*O*-(1-carboxyethylidene) analogues (Fig. 3) and with other Me_3Si derivatives[16]. To elucidate the structure of compound I, it was isolated from the methanolysate of *Klebsiella* K1 polysaccharide by column chromatography on Bio-Gel P-2 (Fig. 5) and analysed by mass spectrometry.

The positive-ion chemical ionisation (p.c.i.) mass spectrum, using ammonia as reagent gas, of the Me_3Si derivative (I) exhibited a prominent $(M + NH_4)^+$ ion of *m/z* 500, in agreement with the molecular mass of 482 inferred from the e.i. spectrum (Fig. 4, i). The e.i. spectrum of the derivative that had not been trimethylsilylated (II) (Fig. 4, ii) exhibited a prominent ion at *m/z* 279, while the p.c.i.



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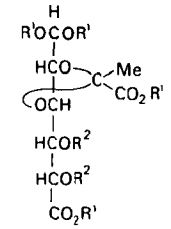
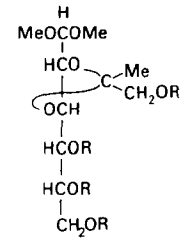


2 R^1=Me, R^2=H, (Compound II)

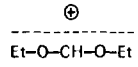
3 R^1=Me, R^2=Me_3Si, (Compound I)

4 R^1=Et, R^2=Me_3Si, (Compound III)



5 R =H (Compound IV)

6 R =Me_3Si (Compound V)



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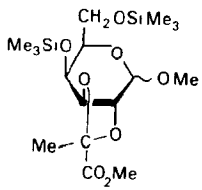

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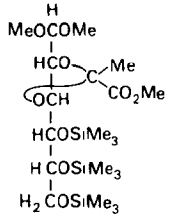

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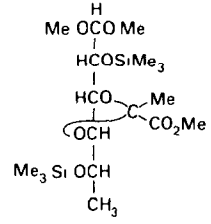

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(NH_3) spectrum of II was dominated by the $(M + NH_4)^+$ ion at *m/z* 356. The inferred molecular mass of 338 for II reveals that I contains *two* Me_3Si groups.

In order to assess the number of alkyl groups introduced into the molecule by the alcoholysis, a compound III analogous to I was prepared by ethanolysis followed by trimethylsilylation. The e.i. spectrum of III (Fig. 4, iii) exhibited a prominent ion at *m/z* 465, while its p.c.i. (NH_3) spectrum revealed an $(M + NH_4)^+$ ion of *m/z* 556. The inferred molecular mass of 538 for III is consistent with the introduction of *four* alkyl groups into the molecules by alcoholysis. Further, the base peak at *m/z* 75 in the e.i. spectra of I and II is shifted to *m/z* 103 in the spectrum of III, demonstrating that the base peak is due to ions containing *two* alkyl groups from alcoholysis.

To confirm the number of ester groups present after alcoholysis, the ester groups in II were reduced with sodium borohydride to yield a compound IV. The p.c.i. (NH_3) spectrum of IV exhibited an $(M + NH_4)^+$ ion of *m/z* 300, demonstrating that II contained *two* methoxycarbonyl groups. This conclusion was substantiated by the observation that the e.i. spectrum of compound V, formed by complete trimethylsilylation of IV, showed a significant ion of *m/z* 467 (Fig. 4, iv),

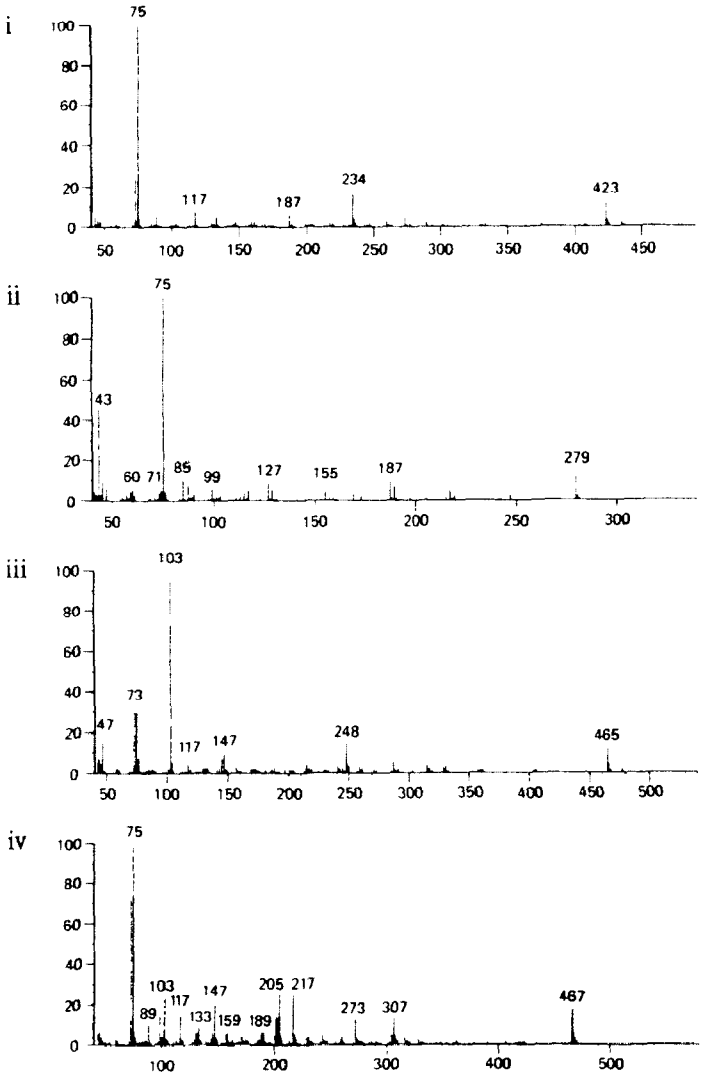

Fig. 4. E.i. mass spectra (70 eV) of derivatives of 2,3-*O*-(1-carboxyethylidene)-D-glucuronic acid: (i) after methanolysis, as the Me_3Si derivative (compound I) **3**, molecular mass 482; (ii) after methanolysis, without trimethylsilylation (compound II) **2**, molecular mass 338; (iii) after ethanolysis, as the Me_3Si derivative (compound III) **4**, molecular mass 538; and (iv) after methanolysis and sodium borohydride reduction, as the Me_3Si derivative (compound V) **6**, molecular mass 526

consistent with $(M - CH_2OSiMe_3)^+$ and demonstrating that V contained *four* Me_3Si groups.

These results support the conclusion that the pyranose ring of the glucuronic acid is cleaved during methanolysis of the polysaccharide to give an acetal of structure **2** (compound II). Trimethylsilylation leads to structure **3** (compound I). Compound III, obtained by ethanolysis and trimethylsilylation is assigned the

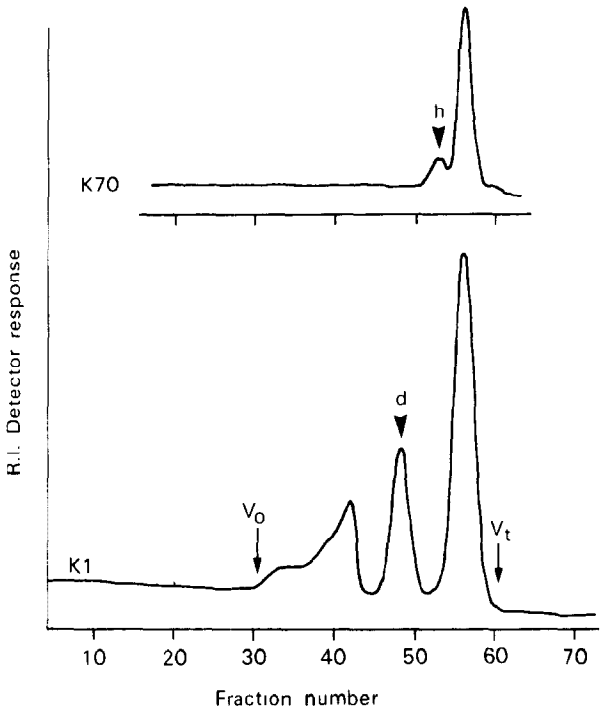

Fig. 5. Gel-filtration chromatography on Bio-Gel P-2 of neutralised methanolysates of polysaccharides of *Klebsiella* K1 and *Klebsiella* K70 (5 mg and 2 mg samples applied to the column, respectively). **d**, Methyl ester of 2,3-*O*-(1-carboxyethylidene)-*aldehydo*-D-glucuronic acid dimethyl acetal; and **h**, methyl glycoside methyl ester of the disaccharide β-D-Glc*p*A-(1→4)-L-Rha*p*.

analogous structure **4**, while compound IV, obtained by borohydride reduction of II, and compound V, the trimethylsilyl derivative of compound IV, are assigned the structures **5** and **6**, respectively.

The ion of *m/z* 103 in the e.i. spectrum of compound III has an identical collision-induced dissociation spectrum to the $(M - OEt)^+$ ion from ethyl orthoformate, which is consistent with common ion-structures (**7**). The homologous stable ions of *m/z* 75 and 103 can be readily derived from the proposed structures **2–6** and it is not surprising that they dominate the e.i. spectra.

Peaks due to daughter ions of *m/z* 234 and 248 in the spectra of the trimethylsilylated esters (Fig. 4, i, iii) are diagnostic of the $ROOC.CHOSiMe_3.CHOSiMe_3$ substituents (R = Me, Et) of the 1,3-dioxolane ring (structures **3** and **4**). Six-centred rearrangement of a Me_3Si group to the carbonyl group would lead to these daughter ions, as has been described previously for analogous functional groups[17].

2,3-*O*-(1-Carboxyethylidene)-D-glucuronic acid from the polysaccharides of *Klebsiella* K1 and K58 was detected only as **3** after methanolysis and the analogue with an intact pyranose ring was not observed. In contrast, g.l.c.–m.s. analysis of the trimethylsilylated methanolysate of the polysaccharide of *S. pneumoniae* type

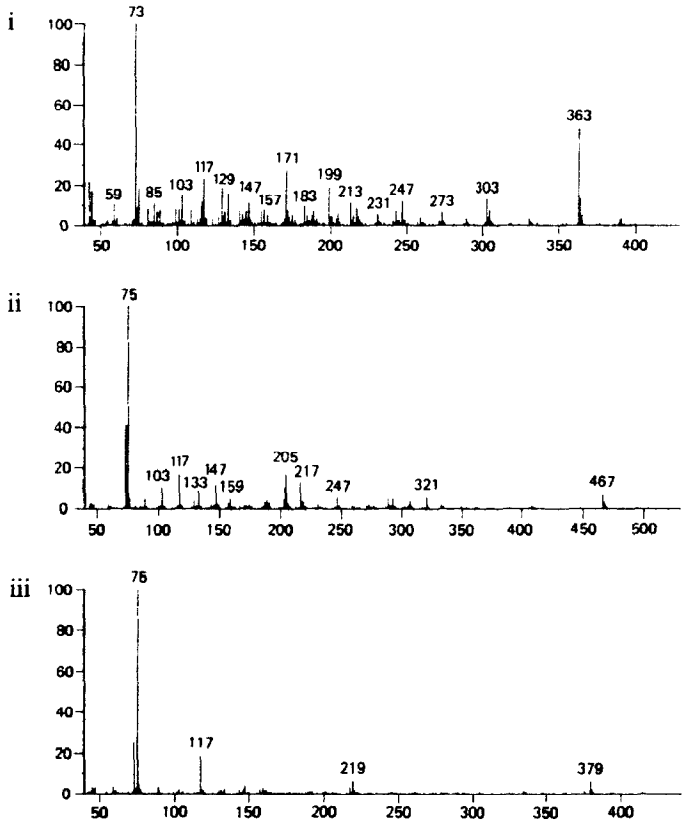

Fig. 6. E.i. mass spectra (70 eV) of the Me_3Si derivatives of the methyl esters of (i) methyl 2,3-*O*-(1-carboxyethylidene)-D-galactopyranoside (Fig. 2, peak **f'**) **8**, molecular mass 422; (ii) 2,3-*O*-(1-carboxyethylidene)-*aldehydo*-D-galactose dimethyl acetal (Fig. 2, peak **f**) **9**, molecular mass 526; and (iii) 3,4-*O*-(1-carboxyethylidene)-*aldehydo*-L-rhamnose dimethyl acetal (Fig. 2, peak **g**) **10**, molecular mass 438.

IV revealed the presence of products of 2,3-*O*-(1-carboxyethylidene)-D-galactose in two forms. The e.i. spectrum of one product (Fig. 6, i) was consistent with structure **8** with the pyranose ring intact (molecular mass 422), while the spectrum of the other (Fig. 6, ii) was consistent with structure **9** generated by pyranose ring cleavage (molecular mass 526). This suggests that with 2,3-*O*-(1-carboxyethylidene)-D-galactose the relative amounts of these two methanolysis products could be varied depending on conditions of methanolysis. Under the conditions in the present investigation the pyranose and open-chain products were formed in the proportions of ~2:1 (Fig. 2, peaks **f'** and **f**, respectively).

3,4-*O*-(1-Carboxyethylidene)-L-rhamnose was the most labile of all the pyruvated monosaccharides examined here. When the polysaccharides of *Klebsiella* K32 and K70 were methanolysed under the standard conditions used with all the other polysaccharides in this study, no material resembling a carboxy-

ethylidene derivative was detected. A prominent peak with a relatively long retention time was present in gas–liquid chromatograms of the trimethylsilylated methanolysate of the polysaccharide of *Klebsiella* K70 (Fig. 2, peak **h**) which could have been mistaken for the 1-carboxyethylidene derivative of L-rhamnose. The material represented by peak **h** was isolated from the methanolysate of the polysaccharide of *Klebsiella* K70 (Fig. 5) and trimethylsilylated; its p.c.i. (NH_3) spectrum showed an $(M + NH_4)^+$ ion of *m/z* 746, consistent with it being the Me_3Si derivative of the methyl glycoside methyl ester of the disaccharide β-D-GlcA-(1→4)-L-Rha (molecular mass 728).

Methanolysis of the polysaccharide of *Klebsiella* K70 under milder conditions (0.01M hydrogen chloride in methanol at 82, 50, and 25° for periods of 1, 2 and 4 h) did not result in the appearance of any additional peaks in gas–liquid chromatograms. However, examination by g.l.c.–m.s. of the Me_3Si derivatives of a methanolysate of the polysaccharide of *Klebsiella* K32 treated under relatively mild conditions (0.01M hydrogen chloride in methanol, 1 h, 82°) showed a complex chromatogram in which one small peak (Fig. 2, peak **g**) exhibited an e.i. spectrum (Fig. 6, iii) consistent with it being the pyranose ring cleavage-product expected from 3,4-*O*-(1-carboxyethylidene)-L-rhamnose, of structure **10**. Our failure to detect peak **g** in methanolysates of the polysaccharide of *Klebsiella* K70 may have been the result of its presence in much smaller proportions in this polysaccharide; 3,4-*O*-(1-carboxyethylidene)-L-rhamnose represents one-quarter of the repeating unit of the *Klebsiella* K32 polysaccharide but only one-twelfth of that of the K70 polysaccharide (cf K and L in Table I).

The pyruvated polysaccharides found here to be susceptible to cleavage of their pyranose rings and formation of open-chain dialkyl acetals [*i.e.*, 2,3-*O*-(1-carboxyethylidene)-D-glucuronic acid, 2,3-*O*-(1-carboxyethylidene)-D-galactose, and 3,4-*O*-(1-carboxyethylidene)-L-rhamnose] share the common feature of having their 1-carboxyethylidene substituents attached to vicinal *trans* hydroxyl groups, resulting in *trans*-fused 1,3-dioxolane rings. It might be argued that the formation of such rings would introduce strain on the pyranose chair[18]. Such strain might facilitate cleavage of the pyranose rings and the formation of open-chain dimethyl acetals by methanolysis, as has been found with 3,6-anhydrogalactose and 3,6-anhydroglucose[19,20]. However, examination by n.m.r. spectrometry[21] of the recently synthesised methyl 2,3:4,6-di-*O*-isopropylidene-α- and -β-D-glucopyranosides and of 2,3:4,6-di-*O*-isopropylidene-5-thio-α-D-glucopyranose by X-ray crystallography[22] do not support major distortion of the pyranose $^4C_1$ ring in structures with *trans*-fused 1,3-dioxolane rings. The reason for the susceptibility of the aforementioned pyruvated monosaccharide residues of bacterial polysaccharides to ring cleavage during methanolysis is unclear and it will be instructive to learn how the methyl 2,3:4,6-di-*O*-isopropylidene-D-glucopyranosides behave when subjected to similar methanolysis. It is of interest to note that when forming *O*-isopropylidene derivatives by treating aldohexopyranoses with 2,2-dialkoxypropane and *p*-toluenesulphonic acid, it is possible to obtain alternative derivatives,

with the pyranose rings intact, or cleaved (resulting in open-chain dialkyl acetals) by using *N,N*-dimethylformamide or 1,4-dioxane, respectively, as solvents[23].

The present g.l.c. method based on methanolysis of the polysaccharide can be used to identify pyruvated monosaccharide constituents with the possible exception of the unusually labile rhamnose derivatives. The relative retention times and patterns of peaks of the Me_3Si derivatives of the methyl glycoside methyl esters of the carboxyethylidene products of methanolysis are sufficiently distinctive for direct assignment of the known pyruvated monosaccharides from the gas–liquid chromatograms, but g.l.c.–m.s. would be required for confirmation if peaks in the relevant regions of chromatograms were to overlap. Once the monosaccharide composition of a pyruvated polysaccharide is known, it should be relatively straightforward to establish which of the sugars is pyruvated, unless the pyruvic acid substituent is attached in a manner not yet reported or was omitted from this study. The present investigation did not include the polysaccharides of *S. pneumoniae* type XXVII and *Hyphomicrobium* sp JTS-811, which contain 2-acetamido-4,6-*O*-(1-carboxyethylidene)-2-deoxy-D-glucose[24] and 4,6-*O*-(1-carboxyethylidene)-2-*O*-methyl-D-mannose[25], respectively.

This procedure should be useful in screening pyruvated polysaccharides produced by related strains of bacteria, *e.g.*, various strains of a *Rhizobium* species, to determine whether or not the pyruvic acid substituent is linked in the same way to the same monosaccharide in all of them. It should also be useful in monitoring the nature of the variation in pyruvic acid content of polysaccharides, especially those containing two pyruvic acid substituents per repeating unit, during the course of growth of bacterial cultures[26,27].

EXPERIMENTAL

Polysaccharides. — The *Rhizobium* polysaccharides were prepared here as described previously[11]. The other polysaccharides were gifts from the investigators who established their respective structures (see Table I) and were used as received.

Methanolysis. — Samples (~5 mg) of the polysaccharides were methanolysed with M hydrogen chloride in anhydrous methanol (1 mL) under nitrogen, in screw-capped tubes closed with Teflon-lined caps, for 16 h at 82°. The solutions were made neutral with silver carbonate and glacial acetic acid (0.05 mL) was added to assist desorption of materials from the silver carbonate[28]; the solutions were centrifuged and the supernatant solutions transferred to clean tubes. In the case of the polysaccharide of *S. pneumoniae* type IV, known to contain GalNAc, ManNAc, and FucNAc, acetic anhydride (0.05 mL) was added (instead of acetic acid) to re-*N*-acetylate the methyl 2-amino-2-deoxyhexopyranosides, and the solutions kept for 16 h at room temperature before proceeding with the subsequent steps as already described. After evaporation to dryness in a stream of nitrogen, the samples were stored over phosphorus pentaoxide and solid sodium hydroxide in a vacuum desiccator for 16 h.

The residues were trimethylsilylated by adding 1 mL of a freshly prepared mixture of pyridine, hexamethyldisilazane, and chlorotrimethylsilane (10:2:1, v/v). After ~1 h at room temperature, the solutions were evaporated to dryness at 35° in a stream of nitrogen and the residues dissolved in hexane (0.1–0.5 mL) for analysis by g.l.c. and g.l.c.–m.s.

Hydrolysis prior to methanolysis. — Polysaccharide samples (~5 mg) were hydrolysed in M sulphuric acid (1 mL) under nitrogen in screw-capped tubes for 3 h at 100°. The solutions were made neutral with barium carbonate, centrifuged, and the clear supernatant liquids transferred to clean tubes. The solutions were evaporated to dryness in a stream of nitrogen and the residues dried further over phosphorus pentaoxide. The dried residues were methanolysed as already described.

Gas-liquid chromatography. — G.l.c. was performed on a Pye model 104 series 64 instrument with flame-ionisation detectors, using a steel column (3 m × 2 mm i.d.) packed with 3% OV-225 on Gas-Chrom Q (80–100 mesh) and nitrogen carrier gas at 54 mL/min. The temperature programme consisted of an initial isothermal stage (5 min, 160°) followed by a temperature rise at 2°/min to 230°.

Mass spectrometry. — Mass spectra were recorded using a VG Micromass 70-70 mass spectrometer interfaced to a Varian 1400 gas chromatograph and a VG 11-250 data system. Trimethylsilylated compounds were determined by g.l.c.–m.s. using a packed glass column (1.8 m × 6 mm i.d.; 5% OV-225; helium carrier gas at 25 mL/min) and a temperature programme similar to that already described. The direct insertion probe was used for non-trimethylsilylated compounds. Electron ionisation (e.i.) spectra were recorded using an ionisation energy of 70 eV and trap currents of 100 μA. Positive-ion chemical ionisation (p.c.i.) spectra were obtained at 50 eV ionisation energy and 200 μA emission current, using ammonia as a reagent gas at estimated source pressures of 40–60 Pa. Source temperatures were 180°. Collision-induced dissociation spectra for selected parent ions were obtained at a collision cell pressure (helium) of 0.04 Pa by linked scanning of the magnetic field B and electric sector voltage E at constant B/E. The collision cell pressure was sufficient to reduce the height of the main-beam parent ion peak to 30% of that in the absence of the gas. Collision-induced dissociation spectrum for parent ions of m/z 103: m/z (I%) 88(2), 75(100), 60(2), 47(24), 45(2), 29(2).

Column chromatography on Bio-Gel P2. — The column (83 × 1.6 cm i.d.) packed with Bio-Rad Bio-Gel P2 (200–400 mesh) was eluted at 28 mL/h with distilled water de-aerated by passage through a de-gassing unit. Solutes in the liquid from the column were monitored by differential refractometry. Chromatography at 65° has been recommended[29] for separations on Bio-Gel columns but as no differences could be found in preliminary experiments between results obtained with methanolysates of polysaccharides eluted at room temperature (~25°) with those at the higher temperature, all column chromatography was performed at room temperature to minimise the risk of de-esterifying the methyl esters of the carboxyethylidene substituents.

Before application to the column, the samples of methanolysed polysaccharides were evaporated to dryness to remove the methanol in which they were stored, dissolved in water (1 mL) and filtered through Millipore membranes (0.45 μm) to remove particles. Fractions (2 mL) were collected, pooled as required and analysed after methanolysis by g.l.c. as described earlier.

Methanolysis product of 2,3-O-(1-carboxyethylidene)-D-glucuronic acid. — A sample of the methanolysis product of this compound was isolated from the polysaccharide of *Klebsiella* K1 (16 mg) by methanolysis and chromatography on Bio-Gel P-2 (see Fig. 5). An aliquot (~one-third of the total) was converted into the ethyl glycoside ethyl ester derivative by treating the dried sample with 0.7M hydrogen chloride in anhydrous ethanol (1 mL) for 3 h at 82°. G.l.c. analysis of the Me_3Si derivative gave a single peak, free of the starting material, showing that transesterification and transglycosylation were complete.

Another aliquot of the methanolysis product (~one-third of the total) was reduced in ethanol (1 mL) with sodium borohydride (~100 mg) for 72 h at room temperature. The reaction mixture was acidified with acetic acid [10% (v/v) in methanol] and evaporated to dryness. This was followed by three cycles of addition of acetic acid in methanol and evaporation to dryness, and then three further cycles of addition of methanol and evaporation to dryness. The final residue was dissolved in water and treated with Zeokarb 225(H^+) ion-exchange resin to remove sodium ions. The solution was evaporated to dryness and converted into the Me_3Si derivative for mass spectrometry.

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