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4-*O*-(1-Carboxyethyl)-D-galactose

A new acidic sugar from the extracellular polysaccharide produced by *Butyrivibrio fibrisolvens* strain 49

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The structure of a new acidic sugar from the extracellular polysaccharide of *Butyrivibrio fibrisolvens* strain 49 was determined as 4-*O*-(1-carboxyethyl)-D-galactose on the basis of ^{13}C-n.m.r. and ^{1}H-n.m.r. spectroscopy, m.s. and chemical degradation studies.

INTRODUCTION

Butyrivibrio fibrisolvens is a strictly anaerobic bacterial species commonly isolated from caecal or ruminal contents of mammals. The neutral sugar composition of extracellular polysaccharides (EPS) produced by over 30 isolates of *B. fibrisolvens* was recently reported in a study by Stack [1]. Although considerable heterogeneity in EPS composition was observed, the isolates appeared to fall into two major taxonomic groups categorized by the presence or absence of an unusual acidic sugar of unknown structure. This sugar was detected as its acetylated lactone during g.l.c. of alditol acetates prepared from EPS hydrolysates. The present study describes the isolation and characterization of derivatives of this compound and identification of the original acidic sugar as 4-*O*-(1-carboxyethyl)-D-galactose. This unusual sugar has not previously been reported to occur in nature.

MATERIALS AND METHODS

Organism, growth conditions and EPS purification

Butyrivibrio fibrisolvens strain 49, used in all studies, was grown under anaerobic conditions on the defined medium of Cotta & Hespell [2]. Cells were removed from stationary-phase cultures by centrifugation, and crude EPS was phenol-extracted and dialysed as previously described [3] to yield purified EPS preparations.

Carboxyl reduction of acidic sugars in purified EPS

Carboxyl groups in purified EPS samples were 'activated' by reaction with the water-soluble di-imide 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide (EDC), and then reduced with either sodium borohydride ($NaBH_4$) or sodium borodeuteride (NaB^2H_4) by means of the procedure described by Taylor & Conrad [4]. Following extensive dialysis against water and lyophilization, these samples were designed as EPS-EDC/BH_4 (or EPS-EDC/B^2H_4). These preparations were subsequently hydrolysed at 20 mg/ml in trifluoroacetic acid (TFA, 2 M, 1 h, 110 °C), blown down with a stream of N_2 gas, and stored dry at −80 °C.

Purification of the neutral sugar (NS-A) derived from the unknown acidic sugar (AS-A)

Hydrolysates of EPS-EDC/BH_4 (50–300 mg) were dissolved in water (0.6 ml) and applied to the top of a small 'guard' column (1 cm × 1.5 cm) of Dowex 50W-X4 in the calcium form prepared as described by Angyal *et al.* [5]. The monosaccharides were eluted with water (~ 30 ml), lyophilized, and reconstituted in 0.6 ml of water. This sample was then applied to the top of a larger column (97 cm × 1.5 cm) of the same resin and eluted with water at a flow rate of 0.3 ml/min. The eluate was monitored by a Hewlett-Packard Model 1037A Refractive Index Monitor and 2.4 ml fractions were collected. Carbohydrate content of appropriate fractions was determined by g.l.c. following the conversion of small aliquots (25–200 μl) to alditol acetate derivatives. The tubes which contained the neutral sugar derived from the unknown acidic sugar and no other components, were pooled, lyophilized, and designated as NS-A. Similarly, purified samples of NS-A obtained from preparations of EPS-EDC/B^2H_4 were designated as *d*-NS-A, reflecting the deuterium incorporated into the carboxyl group of AS-A from NaB^2H_4.

N.m.r. spectroscopy

^{13}C- and ^{1}H-n.m.r. spectra of NS-A and *d*-NS-A were obtained with a Bruker WM-300 WB spectrometer utilizing a 5 mm dual (^{1}H/^{13}C) probe and deuterium oxide as the solvent.

Mass spectroscopy

Electron impact (70 eV) and chemical ionization (isobutane, 40 Pa) mass spectra were obtained on various alditol acetate samples by means of a Finnigan 4535 TSQ g.l.c./m.s. equipped with a DB-225 capillary column (0.25 mm × 30 m; J & W Scientific, Rancho Cordova, CA, U.S.A.) run isothermally at 210 °C.

Dealkylation of NS-A

The alkyl group substituent of purified NS-A was removed by treatment with the ether-cleaving reagent

Abbreviations used: EPS, extracellular polysaccharides; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide; TFA, trifluoroacetic acid; c.i.m.s., chemical-ionization m.s.; e.i.m.s., electron-impact m.s.; DEPT, driven equilibrium pulse transfer.

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boron tribromide (BBr_3), using a modification of the procedure described by Hough & Theobald [6]. NS-A (0.2 mg) was dissolved/suspended under N_2 in CH_2Cl_2 (0.75 ml), cooled to −70 °C, and 16% BBr_3 in CH_2Cl_2 (v/v, 0.25 ml, −70 °C) prepared under N_2 was added. The mixture was allowed to react in a Teflon-capped sealed tube for 30 min at −70 °C, and then allowed to gradually regain room temperature over 10–16 h. The solvent and excess reagents were evaporated with a stream of N_2, the residue was redissolved in methanol (0.3 ml), and then dried with a stream of N_2. The washed, dried residue was treated with 10% TFA (v/v, 0.25 ml, 100 °C, 1 h), to afford a hexose that was identified by g.l.c. and g.l.c./m.s. of its alditol acetate derivative.

Determination of the configuration of the hexose obtained from dealkylated NS-A

The absolute configuration of the hexose obtained from dealkylated NS-A was determined by g.l.c. analysis of the acetylated diastereomeric glycosides prepared from (−)-2-octanol by the basic procedure described by Leontein *et al.* [7], as modified by Stack *et al.* [8].

Alditol acetate analyses

Alditol acetates were prepared by hydrolysis, reduction, and acetylation of EPS samples, column fractions, and various preparations as appropriate using the basic method described by Albersheim *et al.* [9].

RESULTS

The chromatogram obtained from g.l.c. analysis of the alditol acetates prepared from the EPS of *B. fibrisolvens* strain 49 is shown in Fig. 1(*a*). Peaks corresponding to rhamnose, galactose, glucose, and inositol (internal standard) were identified, but other peaks not corresponding to any known carbohydrate standards were also present. Those derived from the unknown AS-A were designated as A-1 and A-2, while the presence of a second unknown acidic sugar (AS-B) was indicated by peaks B-1, B-2, B-3, and B-4. Since the unknown AS-B was found in the EPS made by only *B. fibrisolvens* strains 49 and H17c [1], we have attached a lower priority to its structural determination. In contrast, the two g.l.c. peaks corresponding to AS-A were identified in the EPS made by over 20 strains of this organism [1]. Neutral sugar analysis of EPS-EDC/BH_4 gave the results shown in Fig. 1(*b*). The peak designated as A-2 essentially disappeared and a concomitant rise in peak A-1 occurred. Similarly, peaks B-1, B-2, and B-3 also disappeared with a concomitant rise in peak B-4. These results suggested that peaks A-2, B-1, B-2, and B-3 might correspond to acetylated lactones derived from the unknowns AS-A and AS-B. The relative monosaccharide composition of these EPS preparations was inferred from the integrated peak areas detected by a flame ionization detector. These data are presented in Table 1.

The molecular masses of the acetylated compounds represented by peaks A-1 and A-2 were 492 and 404 Da respectively, as determined by c.i.m.s. This again suggested that peak A-1 represented an acetylated alditol of the neutral sugar derived from AS-A, and that peak A-2 represented an acetylated lactone of unknown structure also derived from AS-A.

NS-A and *d*-NS-A were purified from EPS-EDC/BH_4 and EPS-EDC/B^2H_4 hydrolysates by column

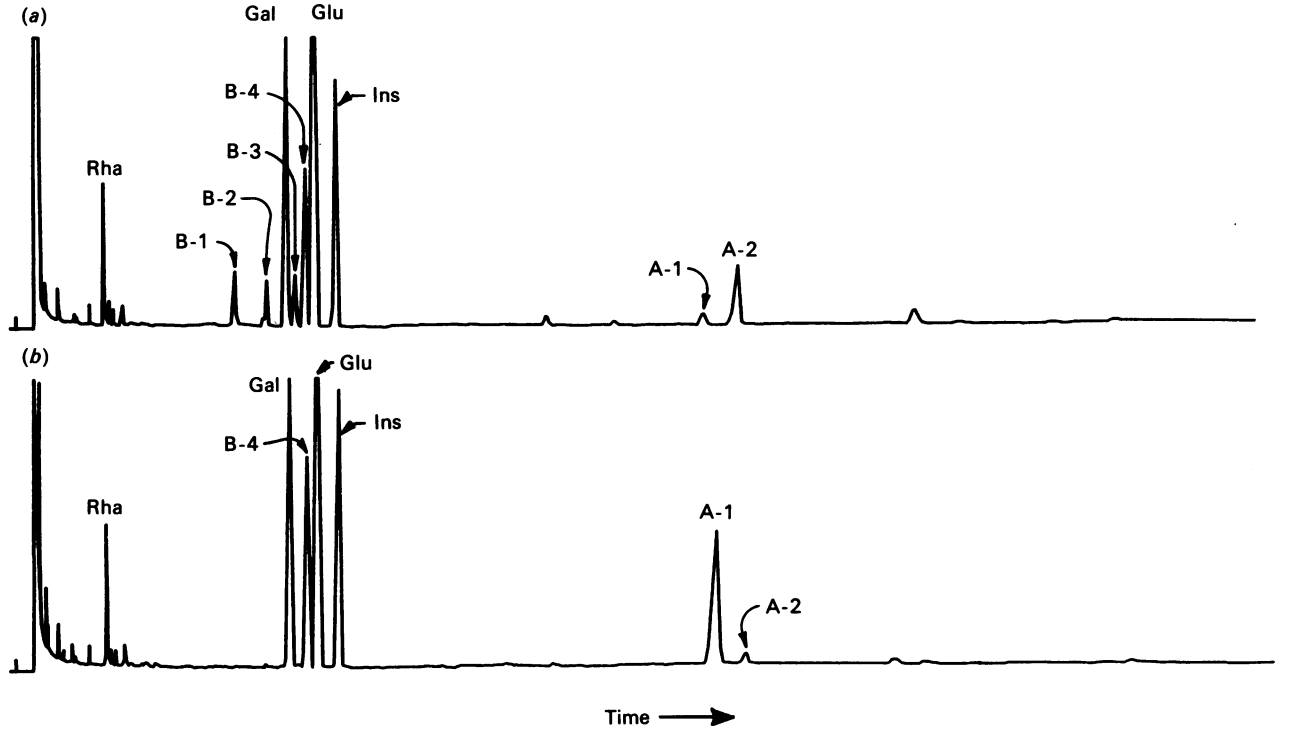

Fig. 1. G.l.c. analyses of alditol acetates prepared from the EPS of *B. fibrisolvens* strain 49 (*a*), or the carboxyl-reduced EPS fraction designated as EPS-EDC/BH_4 (*b*)

Rhamnose (Rha), galactose (Gal) and glucose (Glu) are indicated. Inositol (Ins) was added as an internal standard. Peak A-1 corresponded to the acetylated alditol of the neutral sugar derived from 4-*O*-(1-carboxyethyl)-D-galactose, while peak A-2 was identified as an acetylated lactone of this compound.

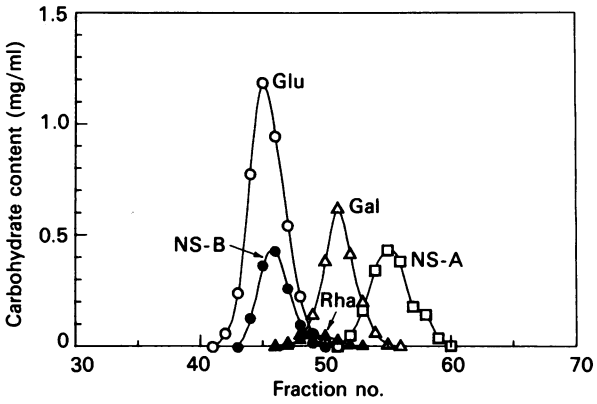

Fig. 2. Elution profile of monosaccharides from a hydrolysate of EPS-EDC/BH_4 following chromatography on Dowex 50W-X4 (Ca^{2+} form)

The elutions of Rhamnose (Rha), glucose (Glu), galactose (Gal), NS-A and NS-B are indicated. Fractions 55–59 were pooled and designated as pure NS-A. NS-B designates the neutral sugar derived from the other unknown acidic component (AS-B) in this EPS, the structure of which is still under investigation.

chromatography as shown in Fig. 2. Fractions 55–59 contained only NS-A (or *d*-NS-A) and no other components, as determined by g.l.c. analysis of alditol acetates prepared from small aliquots of these fractions. The remainder of fractions 55–59 was pooled, lyophilized, and utilized for further studies. The $[\alpha]_D^{20}$ of purified preparations of NS-A and *d*-NS-A ranged between +33 and +38°.

The ^1H- and ^{13}C-n.m.r. spectra of purified NS-A and *d*-NS-A were then obtained, with relevant portions of the ^{13}C spectra of NS-A shown in Fig. 3. Assignment of the ^{13}C-resonance signals was aided by a driven equilibrium pulse transfer (DEPT) experiment [10]. The $\beta:\alpha$ ratio approximated 2:1 in the mutarotated sample, and therefore the ^{13}C resonance signal at 68.9 p.p.m. must represent the sum of two signals from a methine carbon and a methylene carbon of the α anomer (see Fig. 3). Methylene resonances at 65.1 p.p.m. (β) and 65.2 p.p.m. (α) were absent from the ^{13}C spectrum of *d*-NS-A (results not shown), allowing these resonance signals to be assigned to the methylene carbon of the alkyl group substituent (equivalent to the carboxyl carbon in AS-A). Complete assignment of the ^{13}C chemical shifts for NS-A deduced from these data is

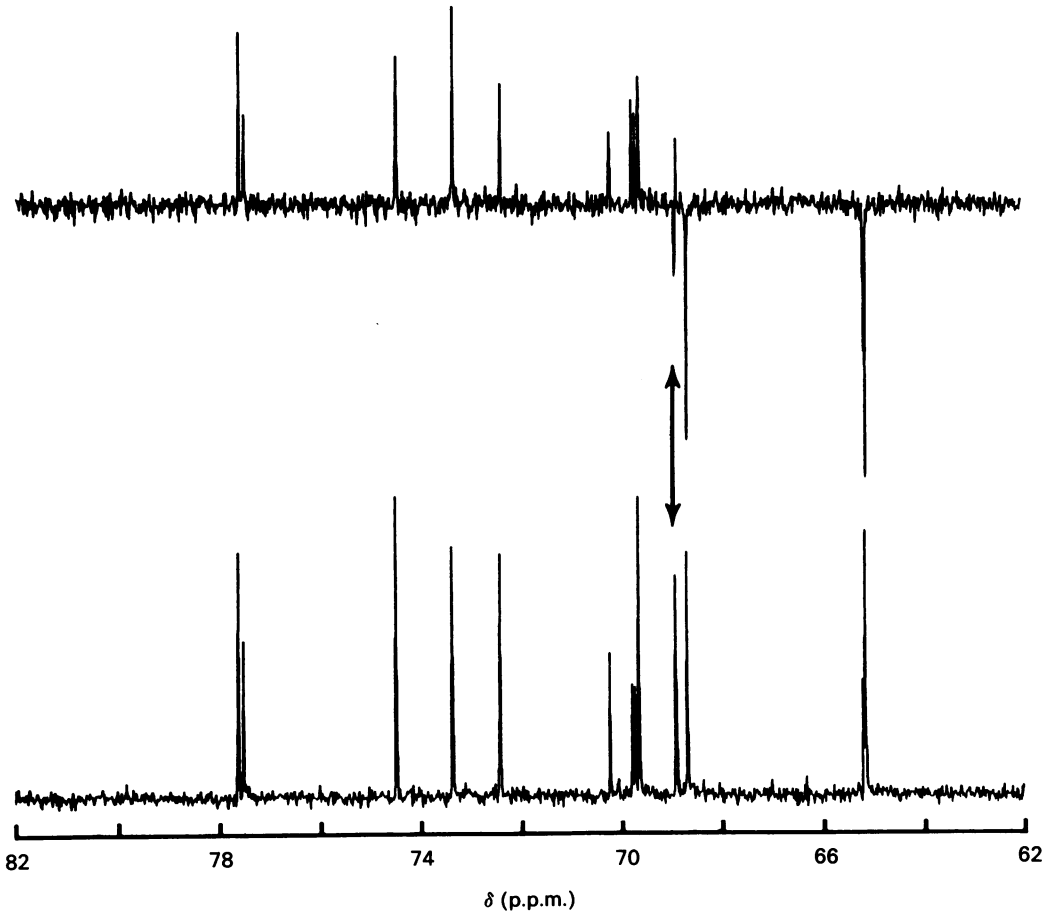

Fig. 3. A portion of the ^{13}C-n.m.r. (lower) and DEPT (upper) spectra of purified NS-A showing the peak which contains both a methine and a methylene resonance (double arrow) of the α anomer

The peaks at 65.1 and 65.2 ppm were absent from the ^{13}C-n.m.r. spectrum of purified *d*-NS-A (results not shown).

Table 1. Relative composition of the EPS and EPS-EDC/BH_4 from *B. fibrisolvens* strain 49, as deduced from alditol acetate data

Tr., trace, defined as less than 0.1 relative to galactitol hexa-acetate.

Sugar or peak	EPS	EPS-EDC/BH_4
1. Rhamnose	0.1	0.1
2. B-1*	0.1	–
3. B-2*	0.1	–
4. Galactose	1.0	1.0
5. B-3*	0.1	–
6. B-4*	0.5	0.7
7. Glucose	2.1	2.1
8. A-1†	Tr.	0.8
9. A-2†	0.6	Tr.

* Peaks B-1, B-2, and B-3 correspond to acetylated lactones of the unknown acidic sugar designated as AS-B. The lactone(s) of AS-B are partially reducible by $NaBH_4$, even without prior carbodi-imide activation to yield the acetylated compound designated as peak B-4 (R. Stack, unpublished results).

† Peak A-2 corresponds to the acetylated lactone of the unknown sugar AS-A, while peak A-1 corresponds to the fully-reduced acetylated alditol of AS-A.

Table 2. ^{13}C chemical shifts for the neutral sugar 4-*O*-(1-hydroxypropyl)-D-galactose (NS-A) compared with published data for D-galactose

	D-Galactose*		4-*O*-(1-Hydroxypropyl)-D-galactose	
Carbon	α	β	α	β
Hexose				
1	93.2	97.3	92.9	97.0
2	69.4	72.9	68.9	72.4
3	70.2	73.8	69.8	73.4
4	70.3	69.7	77.5	77.6
5	71.4	76.0	70.2	74.5
6	62.2	62.0	68.9	68.7
Side-chain†				
1′	–	–	69.8	69.6
2′	–	–	16.0	16.0
3′	–	–	65.2	65.1

* Data from ref. [11].

† Side-chain numbering of carbons is as follows: C-1′ corresponds to -CH; C-2′ to $-CH_3$; and C-3′ to $-CH_2OH$ (derived from the carboxyl group in the original AS-A).

shown in Table 2; these are compared with those previously reported for D-galactose [11].

Comprehensive e.i.m.s. and c.i.m.s. studies of four deuterium-labelled alditol acetates prepared from NS-A and *d*-NS-A (using $NaBH_4$ and NaB^2H_4 for aldehyde reductions) yielded much structural information. These data are summarized in Table 3, and the structures of both 4-*O*-(1-carboxyethyl)-D-galactose and its corresponding fully-deuterated alditol acetate are shown in Fig. 4.

Treatment of NS-A with BBr_3, followed by reduction and acetylation, yielded galactitol hexa-acetate in approximate yield of 40%. NS-A was also treated with

Table 3. Key fragments detected by e.i.m.s. of alditol acetates prepared from NS-A and *d*-NS-A

Sample	Molecular mass*	Key fragments, *m/z*			
NS-A-BH_4†	492	101	73	145	275
NS-A-B^2H_4‡	493	101	73, 74	145, 146	275
d-NS-A-BH_4†	494	103	73	145	277
d-NS-A-B^2H_4‡	495	103	73, 74	145, 146	277

* Molecular masses determined by c.i.m.s., as described in the Materials and methods section.

† Aldehyde group reduced with $NaBH_4$ prior to acetylation.

‡ Aldehyde group reduced with NaB^2H_4 prior to acetylation.

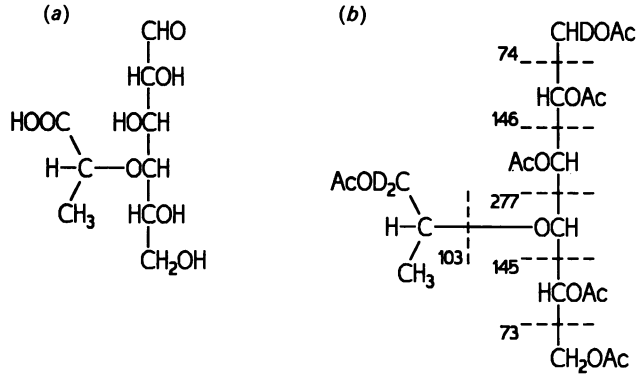

Fig. 4. The structure of (*a*) 4-*O*-(1-carboxyethyl)-D-galactose and (*b*) its fully reduced, acetylated, and deuterated analogue (*d*-NS-A-B^2H_4) showing the fragmentation pattern observed by e.i.m.s.

BBr_3, reacted with (−)-2-octanol and acetylated. G.l.c. analysis of these acetylated glycosides yielded a chromatogram nearly identical with that obtained after similar treatment of standard D-galactose. Two additional peaks, identified by g.l.c./m.s. as brominated acetylated compounds of unknown structure, were obtained from the BBr_3-treated NS-A, but not from the standard D-galactose (results not shown).

DISCUSSION

The EPS of *B. fibrisolvens* strain 49 contains glucose and galactose in an approximately 2:1 ratio, and two additional unidentified acidic components which we have designated as AS-A and AS-B. The identity of AS-A is established here as 4-*O*-(1-carboxyethyl)-D-galactose (Fig. 4), an unusual sugar not previously reported in nature. The identity of AS-B has not yet been established, but it also appears to be a compound with a 1-carboxyethyl side-chain. AS-B is a constituent of EPS made by only two strains of *B. fibrisolvens*, while 4-*O*-(1-carboxyethyl)-D-galactose appears in the EPS from over 20 strains of this organism [1].

The structural elucidation of AS-A as 4-*O*-(1-carboxyethyl)-D-galactose is deduced from the following observations and data. Neutral sugar analysis of the EPS from strain 49 yielded an acetylated lactone (Fig. 1, peak A-2) and a small amount of the corresponding acetylated alditol (peak A-1), which was incidentally generated by $NaBH_4$ during the aldehyde reduction step of the alditol

acetate procedure. That none of the unknown in the original EPS was present as the neutral sugar was confirmed by the complete absence of a *m/e* 101 fragment in the e.i. mass spectra of the alditol acetates prepared from *d*-NS-A (Table 3). Essentially complete conversion of the unknown AS-A to the corresponding NS-A was achieved by EDC activation of the carboxyl groups in EPS prior to their reduction with $NaBH_4$. Subsequent neutral sugar analyses of these EPS-EDC/BH_4 preparations showed that peak A-2 (corresponding to the acetylated lactone) disappeared, while peak A-1 (corresponding to the acetylated alditol) had risen (Fig. 1*b*). G.l.c./m.s. studies showed the molecular masses of these two compounds to be 404 and 492 Da respectively, with the gain of 88 Da attributable to the reduction of the lactone with acetylation of both resultant hydroxyl groups.

Purification of NS-A (or *d*-NS-A) from EPS-EDC/BH_4 preparations (or EPS-EDC/B^2H_4) was accomplished by chromatography of hydrolysates on Dowex 50W-X4 (Fig. 2). Several lines of evidence allowed the unambiguous deduction of the carbon skeleton of the unknown: a large and prominent *m/z* 101 fragment was observed in the e.i. mass spectra of the alditol acetates prepared from NS-A (regardless of whether $NaBH_4$ or NaB^2H_4 was used to reduce the aldehyde); a similar *m/z* 103 fragment was noted in the e.i. mass spectra of the alditol acetates prepared from *d*-NS-A; ^{1}H-n.m.r. studies of purified NS-A or *d*-NS-A showed the presence of a $-CH_3$ group split into a doublet at 1.15 p.p.m. (results not shown); and dealkylation of NS-A with the ether-cleaving reagent BBr_3 yielded a hexose identified by g.l.c. as D-galactose. These data and other n.m.r. and m.s. data shown in Table 3 suggested that AS-A was a hexose with an ether-linked three-carbon alkyl side-chain comprised of one carboxyl group, one methyl group, and one -CH group. A 1-carboxyethyl group represents the only way to arrange these moieties. The *m/z* 101 fragment observed in the e.i. mass spectra of the alditol acetates prepared from NS-A (noted above) must be the acetylated 1-hydroxypropyl group derived from the reduced 1-carboxyethyl substituent of AS-A. Similarly, the *m/z* 103 fragment noted in the e.i. spectra of *d*-NS-A represents the splitting off of the same group containing two deuterium atoms. The linkage of the 1-carboxyethyl substituent to the 4 position of D-galactose was established by e.i. mass spectral studies of samples deuterated in various positions (Table 3). The shifts in the *m/z* 73 and 145 fragments to 74 and 146, which occurred when the aldehyde of either NS-A or *d*-NS-A was reduced with NaB^2H_4 established that the original 1-carboxyethyl group was not linked through the 1, 2, 5 or 6 positions. The shift of the *m/z* 275 fragment to 277 occurred only in alditol acetates prepared from *d*-NS-A, establishing the linkage to the 4 position of D-galactose.

Comparison of the ^{13}C chemical shifts of D-galactose with the isolated neutral sugar 4-*O*-(1-hydroxypropyl)-D-galactose (Table 2) also suggested that the linkage of the alkyl group is through the 4 position. The published resonance signals for C-4 of galactose at 70.3 p.p.m. (α) and 69.7 p.p.m. (β) are shifted downfield to 77.5 and 77.6 p.p.m., respectively, in the isolated compound. With the exception of C-6, the remaining carbon resonances of 4-*O*-(1-hydroxypropyl)-D-galactose are quite similar to those reported for D-galactose [11]. The relative downfield shift of C-6 observed in the isolated neutral sugar may be due to interaction of this carbon with the alkyl group substituent, as suggested by molecular models constructed of this compound.

With the exception of muramic acid [3-*O*-(1-carboxyethyl)-D-glucosamine], 1-carboxyethyl substituted sugars are not commonly encountered in nature. A few examples which have been previously reported include 3-*O*-(1-carboxyethyl)-L-rhamnose and 4-*O*-(1-carboxyethyl)-D-glucose in the lipopolysaccharide of *Shigella*, 4-*O*-(1-carboxyethyl)-D-glucose in the extracellular polysaccharide of *Aerococcus*, and 4-*O*-(1-carboxyethyl)-D-mannose in the capsular polysaccharide of *Mycobacterium* [12]. A few additional 1-carboxyethyl-substituted amino sugars and uronic acids have also been reported [12].

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REFERENCES

1. Stack, R. J. (1988) Appl. Environ. Microbiol. **54**, 878–883
2. Cotta, M. A. & Hespell, R. B. (1986) Appl. Environ. Microbiol. **52**, 51–58
3. Stack, R. J. (1987) FEMS Microbiol. Lett. **48**, 83–87
4. Taylor, R. L. & Conrad, H. E. (1972) Biochemistry **11**, 1383–1388
5. Angyal, S. J., Bethell, G. S. & Beveridge, R. J. (1979) Carbohydr. Res. **73**, 9–18
6. Hough, L. & Theobald, R. S. (1965) Methods Carbohydr. Chem. **2**, 203–206
7. Leontein, K., Lindberg, B. & Lohngren, J. (1978) Carbohydr. Res. **62**, 359–362
8. Stack, R. J., Plattner, R. D. & Cote, G. L. (1988) FEMS Microbiol. Lett. **51**, 1–6
9. Albersheim, P., Nevins, D. J., English, P. D. & Karr, A. (1967) Carbohydr. Res. **5**, 340–345
10. Doddrell, D. M., Pegg, D. T. & Bendall, M. R. (1982) J. Magn. Reson. **48**, 323–327
11. Bock, K. & Thogersen, H. (1980) Annu. Rep. Nucl. Magn. Reson. Spectros. **13**, 1–57
12. Kenne, L. & Lindberg, B. (1983) in The Polysaccharides (Aspinall, G. O., ed.), vol. 2, pp. 287–363, Academic Press, New York

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