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IDENTIFICATION OF A TRISACCHARIDE REPEATING-UNIT IN THE ENTEROBACTERIAL COMMON-ANTIGEN

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ABSTRACT

In addition to the previously reported 2-acetamido-2-deoxy-D-glucose and 2-acetamido-2-deoxy-D-mannuronic acid, 4-acetamido-4,6-dideoxy-D-galactose (D-Fuc4NAc) is a major component of the enterobacterial common-antigen. The trisaccharide repeating-unit →4)-β-D-Man*p*NAcA-(1→4)-α-D-Glc*p*NAc-(1→3)-D-Fuc*p*4NAc-(1→ constitutes 70% or more of this antigen.

INTRODUCTION

The enterobacterial common-antigen (ECA) is a polysaccharide antigen elaborated by most wild-type strains of Enterobacteriaceae[1]. It is present as a hapten in these bacteria. In some bacteria, namely, R-mutants containing the R1 or R4 core, ECA is chemically linked to this core and, as part of the lipopolysaccharide, becomes immunogenic. Until recently, structural information on ECA was scarce. In 1978, however, it was demonstrated[2,3] that 2-acetamido-2-deoxy-D-glucose and 2-acetamido-2-deoxy-D-mannuronic acid were main constituents of ECA. It was further demonstrated, by methylation analysis, that both these sugars are linked through the 4-position[2]. We now report further structural studies of ECA.

RESULTS AND DISCUSSION

ECA was prepared from *Shigella sonnei* phase I and purified as previously described[3]. A sample was treated with methanolic hydrogen chloride, and the resulting methyl glycosides were re-*N*-acetylated and fractionated by t.l.c. In addition to the expected glycosides of 2-acetamido-2-deoxy-D-glucose and 2-acetamido-2-deoxy-D-mannuronic acid (as the methyl esters), two further glycosides were obtained. One of these gave signals in the ^{13}C-n.m.r. spectrum at δ 105.3, 73.0, 72.1, 71.2, 58.7, 54.8, 23.1, and 16.7, indicating that it was a methyl glycopyranoside

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(with MeO-1 equatorial) of a 6-deoxyhexose containing an acetamido group. The signal expected in the carbonyl region was obscured by the noise.

The glycosides were acetylated and further purified by column chromatography on silica gel. Analysis of the 400-MHz, ^{1}H-n.m.r. spectra (Table I) showed that one of the substances (that discussed above) was a methyl 4-acetamido-4,6-dideoxy-β-galactopyranoside and the other the corresponding α-glycoside. The *galacto* configuration was evident from the coupling constants of the ring protons. The large coupling-constants of H-4 to N-H, 9.6 and 9.8 Hz, respectively, demonstrated that the acetamido group is linked to C-4. The values for optical rotations, $[\alpha]^{22}_{578}$ +38° for the α-glycoside and −12° for the β-glycoside (both in chloroform), are not very accurate because of the small amounts available, but nevertheless demonstrate that the sugar has the D configuration. 4-Acetamido-4,6-dideoxy-D-galactose is consequently a component of ECA. This sugar has been found[4] in the O-antigen of *Escherichia coli* O 10.

4-Amino-4,6-dideoxy-D-galactose, which should be formed on hydrolysis of ECA with acid, is readily decomposed *via* pyrrole derivatives. This may explain why it was overlooked in previous investigations. However, the 4-acetamido derivative should be considerably more stable. Hydrolysis of glycosidic linkages without *N*-deacylation may be effected on solvolysis with liquid hydrogen fluoride[5], followed by hydrolysis with acid under mild conditions. The glycosyl fluorides formed are probably hydrolysed during the work-up, and it is questionable if the treatment with aqueous acetic acid used in the present study is actually necessary. This method, which was used[6] in studies of the *Vibrio cholerae* O-antigen, was consequently used in the further studies of ECA.

A sample of ECA was *O*-deacetylated by treatment with base, any free amino groups were reacetylated, and the product was carboxyl-reduced[7]. After three consecutive reductions, essentially all of the carboxyl groups had been reduced, as evident from the sugar analysis and from the ^{13}C-n.m.r. spectrum. The resulting polymer was purified by chromatography on Sephadex G-25, and then had $[\alpha]^{27}_{578}$ +102° (water).

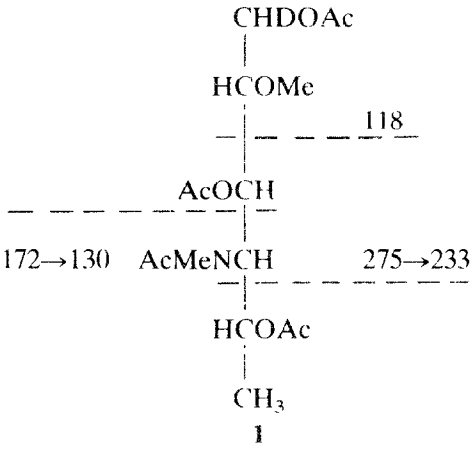
ECA was carboxyl-reduced, methylated (Hakomori), and hydrolysed as discussed above, and the resulting mixture of methylated sugars was reduced with sodium borodeuteride and then acetylated. The alditol acetates were analysed by g.l.c.–m.s.; comparable amounts of the alditols derived from 4,6-dideoxy-2-*O*-methyl-4-*N*-methylacetamido-D-galactose, 2-deoxy-3,6-di-*O*-methyl-2-*N*-methylacetamido-D-glucose, and 2-deoxy-3,6-di-*O*-methyl-2-*N*-methylacetamido-D-mannose were obtained. When the carboxyl-reduction was performed with sodium borodeuteride, the expected labelling in the 2-amino-2-deoxy-D-mannose derivative was observed. As generally observed in methylation analyses of products containing *N*-acetylamino sugars, the acetamido derivatives, with shorter retention-times than the corresponding *N*-methylated products, were also obtained. The components were identified from their mass spectra. Some typical fragments from the 4,6-dideoxy-2-*O*-methyl-4-*N*-methylacetamido-D-galactose derivative (**1**) are indicated in the formula.

TABLE I

^{1}H-N M R DATA FOR METHYL 4-ACETAMIDO-2,3-DI-*O*-ACETYL-4,6-DIDEOXY-α- AND -β-D-GALACTOSIDE

Compound	*Chemical shifts (δ)*										*Coupling constants (Hz)*[c]					
	H-1	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*	*NH*	*OMe*	*OAc*	*OAc*	*NAc*	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6}$	$J_{H,NH}$
α-Glycoside	4.91[a]	4 89[a]	5.257[b]	4 518	4.215	1 156	5 664	3.383	2.101	2.073	1 996	3 9	12.2	3.9	1.5	6.5	9.6
β-Glycoside	4.322	4 99[a]	4.95[a]	4.487	3 806	1 235	5 715	3 516	2.074	2.058	1.993	7 6	10.5	3 4	1 2	6 3	9.8

[a]The chemical shifts are approximate only, because of overlapping signals and higher order spectra. [b]This signal was complex, because of virtual coupling caused by the small chemical-shift difference for H-1 and H-2. [c]Determined with ±0.2 Hz accuracy



1

The results therefore confirm that the 2-acetamido-2-deoxy-D-glucosyl and 2-acetamido-2-deoxy-D-mannosyluronic acid residues are linked through O-4, and demonstrate that the 4-acetamido-4,6-dideoxy-D-galactosyl residue is linked through O-3.

In order to gain further insight into its structure, ECA was subjected to partial hydrolysis, using the two-step procedure discussed above. In one experiment, the treatment with hydrogen fluoride was performed at room temperature for 15 min, and the product was fractionated by chromatography on Sephadex G-25. The main oligomer after this treatment was a disaccharide that gave an alditol having $[\alpha]^{27}_{578}$ +60°. The substances were identified as disaccharide **2** and its alditol from their ^{1}H- and ^{13}C-n.m.r. spectra (Tables II and III).

α-D-Glc*p*NAc-(1→3)-D-Fuc4NAc

2

When the treatment with liquid hydrogen fluoride was performed at −20° for 15 min and the product was worked-up as above, a trisaccharide, which was characterised by ^{1}H-n.m.r. spectroscopy, was the main product. The yield of this trisaccharide was 70%, of which 40% was pure and 30% (obtained from an adjacent fraction) was almost pure according to n.m.r. evidence. ^{1}H-N.m.r. spectroscopy also demonstrated that some 40% of the trisaccharide was *O*-acetylated, which influenced the chemical shift of H-1 of the 2-acetamido-2-deoxy-β-D-mannopyranosyluronic acid residue (Table II). As discussed below, 70% of the trisaccharide units in ECA are *O*-acetylated, and >50% of the *O*-acetyl groups thus survived on solvolysis. Reduction of the trisaccharide gave an alditol having $[\alpha]^{27}_{578}$ +20°. Analyses of the spectra (Tables II and III) demonstrated that the alditol was derived from trisaccharide **3**. Units of this trisaccharide consequently make up a considerable part of ECA.

β-D-Man*p*NAcA-(1→4)-α-D-Glc*p*NAc-(1→3)-D-Fuc4NAc

3

TABLE II

PERTINENT SIGNALS IN THE ^{1}H-N M R. SPECTRA OF OLIGOSACCHARIDES AND OLIGOSACCHARIDE-ALDITOLS FROM ECA (COUPLING CONSTANTS[a] IN PARENTHESES)

Compound	*Anomeric protons ManNAcA or ManNAc*	*GlcNAc*	*Fuc4NAc*	*C-CH$_3$*
α-**2** (1/4)		4.97(3.5)	5.24(3.9)	1.08(6.3)
β-**2** (3/4)		4.97(3.5)	4.61(7.3)	1.12(6.3)
Alditol of **2**		5.05(3.4)		1.17(6.3)
α-**3** (1/4)[b]	4.87(1.7)[c] 4.83(1.7)[d]	4.97(broad)	5.24(3.9)	1.08(6.3)
β-**3** (3/4)[b]	4.87(1.7)[c] 4.83(1.7)[d]	4.97(broad)	4.61(7.3)	1.12(6.3)
Alditol of **3**[e]	4.95(1.7)	5.03(3.4)		1.16(6.4)
α-**4** (1/4)	4.87(1.4)	4.97(3.5)	5.24(3.9)	1.08(6.3)
β-**4** (3/4)	4.87(1.4)	4.97(3.5)	4.61(7.3)	1.12(6.3)
Alditol of **4**[e]	4.87(1.4)	5.03(3.9)		1.17(6.3)

[a]Determined with ±0.3 Hz accuracy. [b]A signal at δ 2.16 from OAc was also obtained. [c]0.6 H. [d]0.4 H. [e]Signals at δ 4.58 (**3**) and 4.53 ($J_{2,3}$ 3.9 Hz) (**4**) for H-2 of D-ManNAcA and D-ManNAc, respectively, were also obtained.

In another experiment, carboxyl-reduced ECA was subjected to partial hydrolysis, the treatment with liquid hydrogen fluoride being performed at 0° for 15 min. A trisaccharide-alditol was obtained after reduction, which, according to n.m.r. evidence (Tables II and III), was derived from trisaccharide **4**.

β-D-Man*p*NAc-(1→4)-α-D-Glc*p*NAc-(1→3)-D-Fuc4NAc

4

In the ^{13}C-n.m.r. spectrum of ECA, two signals for hydroxymethyl groups (δ 64.5 and 61.7) and signals for *O*-acetyl groups (δ 21.6 and 175.2) were observed. In the ^{13}C-n.m.r. spectrum of deacetylated ECA, only the signal at δ 61.7 remained. From this evidence, and from the relative intensities of the signals, it is concluded that some 70% of the 2-acetamido-2-deoxy-α-D-glucopyranosyl residues are *O*-acetylated at C-6.

From the combined evidence discussed above, it is evident that 70% or more of ECA is composed of trisaccharide residues having the structure **5**, in which the anomeric nature of the 4-acetamido-4,6-dideoxy-D-galactopyranosyl residue is not determined. The high, positive value of the optical rotation for carboxyl-reduced ECA, however, indicates that this residue is α-linked.

→4)-β-D-Man*p*NAcA-(1→4)-α-D-Glc*p*NAc-(1→3)-D-Fuc4NAc-(1→

6

⋮

OAc

5

TABLE III

^{13}C-CHEMICAL SHIFTS OF OLIGOSACCHARIDES OBTAINED FROM ECA AND OF SOME MODEL SUBSTANCES[a]

	ManNAcA or ManNAc						*GlcNAc*						*Fuc-4-NAc-ol*					
	C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
Reduced **2**							96.1	54.8	72.6	70.9	73.8	61.6	64.1	72.2	74.2	54.6	67.3	21.0
Reduced **3**	100.2	54.0	72.5	69.4	76.0	173.5	96.1	54.9	70.6	80.1	72.7	61.0	63.9	72.2	74.6	54.4	67.2	21.0
Reduced **4**	100.5	54.4	73.1	67.7	77.7	61.5	96.2	55.0	70.8	80.0	72.5	61.0	64.0	72.2	74.6	54.4	67.2	21.0
β-D-ManNAcA[12]	94.3	54.9	72.8	69.3	76.2	—												
β-D-ManNAc[13]	94.3	55.3	73.2	67.8	77.5	61.7												
Methyl α-D-Glc*p*NAc[14]							99.5	54.9	72.9	71.2	72.4	61.8						

	O ‖ *C*	*O* ‖ *C*	*O* ‖ *C*	CH_3	CH_3	CH_3	OCH_3
Reduced **2**		175.3	175.3	23.5		23.1	
Reduced **3**	176.6	175.3	175.1	23.4	23.1	23.1	
Reduced **4**	176.6	175.3	175.1	23.4	23.1	23.1	
β-D-ManNAcA[12]	176.8				23.2		
β-D-ManNAc[13]	176.8				23.2		
Methyl α-D-Glc*p*NAc[14]			175.5			23.2	56.4

[a] The assignments of some chemical shifts with similar δ values are tentative

It seems reasonable to assume that these, as oligosaccharide repeating-units, are combined into a linear part of the antigen. Small proportions of other components, mainly fatty acids, have been found in preparations of ECA, but, at present, it cannot be decided if these are part of ECA. Further studies are needed before a complete structure can be proposed.

EXPERIMENTAL

General methods. — Concentrations were performed under diminished pressure at bath temperatures below 40°. G.l.c.–m.s. was performed with a JEOL JMS-D300 instrument, with an INCOS 2400 data-system, using an SE-52 glass-capillary column. The n.m.r. spectra were recorded for solutions in D_2O and $CDCl_3$ with JEOL FX-100 and GX-400 instruments, using external tetramethylsilane (^{13}C), internal tetramethylsilane (^1H, $CDCl_3$), and internal sodium 3-trimethylsilylpropionate-d_4 (^1H, D_2O) as references. Gel filtrations were monitored by differential refractometry.

ECA was prepared and purified as previously described[3].

Methanolysis[8] *of ECA.* — ECA (20 mg) was treated with M methanolic hydrogen chloride (2 mL) at 80° for 24 h. The mixture was neutralised with silver carbonate, the products were re-*N*-acetylated, and the mixture was filtered and concentrated to dryness. The glycosides formed were fractionated by t.l.c. on silica gel, using chloroform–methanol (3:1). The two components having the higher R_F-values (1.5 mg and 1 mg, respectively) were isolated, and acetylated by treatment with acetic anhydride (0.5 mL) in pyridine (0.5 mL) at 80° for 1 h, and further purified by chromatography on a column (15 × 0.6 cm) of silica gel irrigated with ethyl acetate–toluene (2:1).

O-*Deacylation.* — A solution of ECA (100 mg) in 0.25M aqueous sodium hydroxide (2 mL) was kept at 60° for 1 h, neutralised with M hydrochloric acid, and concentrated, and the product was purified by chromatography on a column (90 × 2.6 cm) of Sephadex G-25 irrigated with water. The main component (77 mg) was eluted in the void volume and recovered by freeze-drying.

Carboxyl-reduction. — ECA (100 mg) was deacylated as described above with M sodium hydroxide at 60° for 2 h. The product, in M sodium hydrogencarbonate (10 mL), was re-*N*-acetylated by treatment with acetic anhydride (0.3 mL), and purified by gel-permeation chromatography as described above. The product was carboxyl-reduced according to Taylor *et al.*[7]. Uronic acids in the reduced product were determined by g.l.c. as the 1,5-dideoxy-1,5-imino-D-mannitol derivative according to Leontein *et al.*[9], and three consecutive reductions were needed for complete reaction. Carboxyl-reduced ECA (40 mg), $[\alpha]_{578}^{27}$ +102° (*c* 1, water), was obtained after purification on Sephadex G-25. A small sample was reduced as described above, but using sodium borodeuteride as the reducing agent.

Methylation analysis. — Carboxyl-reduced ECA (7 mg) was methylated according to Hakomori[10]. The reaction mixture (2 mL) was diluted with water (4 mL)

and extracted with chloroform (4 × 4 mL). The chloroform phase was concentrated and applied to a column (10 × 0.6 cm) of Sephadex LH-20 which was irrigated with acetone–chloroform (2:1). The fractionation was monitored by t.l.c., using chloroform–methanol (3:1) as irrigant. A homogeneous fraction (3 mg) was dried under reduced pressure and dissolved in anhydrous, liquid hydrogen fluoride (8 mL), and the solution was kept at room temperature for 2.5 h. The hydrogen fluoride was pumped off at room temperature, the residue was treated with 20% aqueous acetic acid at 100° for 2 h, and the solution was concentrated to dryness. A solution of the product in water (1 mL) was treated with sodium borodeuteride (30 mg) at room temperature, neutralised with Dowex 50 (H^+) resin, and concentrated to dryness, and boric acid was removed by codistillation with methanol. A solution of the product in acetic anhydride–pyridine (1:1, 2 mL) was kept at 100° for 1 h and then concentrated, and the last traces of acetylating agent were removed by codistillation with toluene (3 × 2 mL). The resulting, partially methylated alditol acetates were analysed by g.l.c.–m.s.

Partial solvolysis. — (*a*) A solution of ECA (23 mg) in anhydrous hydrogen fluoride (4 mL) was kept at room temperature for 15 min. The hydrogen fluoride was pumped off, and the residue was treated with M acetic acid (2 mL) at 100° for 1 h. The solution was freeze-dried, and the residue was fractionated on a column (80 × 2.6 cm) of Sephadex G-25. The main oligomer was a disaccharide, which was reduced with sodium borohydride to give the alditol (4.3 mg), $[\alpha]_{578}^{27}$ +60° (*c* 0.4, water).

(*b*) A solution of ECA (20 mg) in anhydrous hydrogen fluoride was kept at −20° for 15 min. The mixture was neutralised and worked-up as described by Mort and Bauer[11]. The product was fractionated on a column (90 × 1.6 cm) of Bio-Gel P-2 irrigated with water. The first fraction (8 mg) consisted of pure **3**, and the immediately following fraction (6 mg) contained **3** as the main component. The subsequent fractions (2.4 mg) contained compounds of lower molecular mass.

(*c*) A solution of carboxyl-reduced ECA (15 mg) in anhydrous hydrogen fluoride (4 mL) was kept at 0° for 15 min and then worked-up as described under (*a*). The product was fractionated on a column of Sephadex G-25, and the main oligosaccharide (**4**) was rechromatographed on a column (90 × 1.6 cm) of Biogel P-2. The trisaccharide **4** was reduced to give the alditol (3 mg), $[\alpha]_{578}^{27}$ +25° (*c* 0.3, water), as described above.

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REFERENCES

1 H. MAYER AND G. SCHMIDT, *Curr. Top. Microbiol. Immunobiol.*, 85 (1979) 99–153.
2 D. MANNEL AND H. MAYER, *Eur. J Biochem.*, 86 (1978) 361–370.
3 C. LUGOWSKI AND E. ROMANOWSKA, *Eur. J. Biochem.*, 91 (1978) 89–97.
4 B. JANN AND K. JANN, *Eur. J. Biochem.*, 2 (1967) 26–31.
5 A. J. MORT AND D. T. A. LAMPORT, *Anal. Biochem.*, 82 (1977) 289–309.
6 L. KENNE, B. LINDBERG, P. UNGER, B. GUSTAVSSON, AND T. HOLME, *Carbohydr. Res.*, 100 (1982) 341–349.
7 R. L. TAYLOR, J. E. SHIVELY, AND H. E. CONRAD, *Methods Carbohydr. Chem.*, 7 (1976) 149–151.
8 R. E. CHAMBERS AND J. R. CLAMP, *Biochem. J.*, 125 (1971) 1009–1018.
9 K. LEONTEIN, B. LINDBERG, AND J. LONNGREN, *Acta Chem. Scand., Ser B*, 36 (1982) 515–518.
10 S. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
11 A. J. MORT AND W. D BAUER, *J. Biol. Chem.*, 257 (1982) 1870–1875.
12 P. BRANEFORS-HELANDER, L. KENNE, B. LINDBERG, K. PETERSSON, AND P. UNGER, *Carbohydr. Res.*, 88 (1981) 77–84.
13 D. R. BUNDLE, H. J. JENNINGS, AND I. C. P. SMITH, *Can. J. Chem.*, 51 (1973) 3812–3819.
14 S. J. PERKINS, L. N. JOHNSON, D. C. PHILLIPS, AND R. A. DWEK, *Carbohydr. Res.*, 59 (1977) 19–34.