*Carbohydrate Research*, 133 (1984) 95–104
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

# THE ENTEROBACTERIAL COMMON-ANTIGEN, A CYCLIC POLYSACCHARIDE

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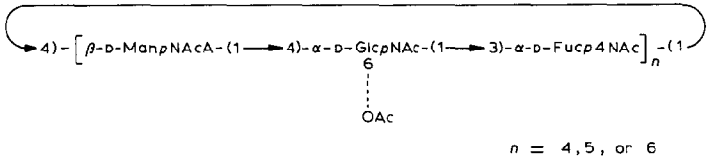
(Received March 12th, 1984; accepted for publication, March 29th, 1984)

ABSTRACT

Structural studies of the enterobacterial common-antigen, using chemical methods and fast-atom-bombardment mass spectrometry, indicate that it is a cyclic polysaccharide, composed of four, five, and, to a smaller extent, six trisaccharide repeating-units. In the structure of the antigen, given below, D-Fuc4NAc stands for 4-acetamido-4,6-dideoxy-D-galactose.




## INTRODUCTION

The enterobacterial common-antigen (ECA) occurs both as a hapten and, in some bacteria, linked to a core as part of a lipopolysaccharide[1]. We recently demonstrated that the trisaccharide repeating-unit **1** constitutes 70% or more of the ECA hapten[2]. The anomeric configuration of the 4-acetamido-4,6-dideoxy-D-galactosyl residue (D-Fuc4NAc) was, however, not determined. The ECA preparation also contained small proportions of fatty acids and phosphoric acid, and it was not determined if these were contaminants or were covalently linked to the antigen. Finally, the regions for anomeric protons and carbon atoms in the $^{1}H$- and $^{13}C$-n.m.r. spectra (Figs. 1 and 2) were more complicated than expected for a polysaccharide composed of trisaccharide repeating-units, even after hydrolysis of the non-

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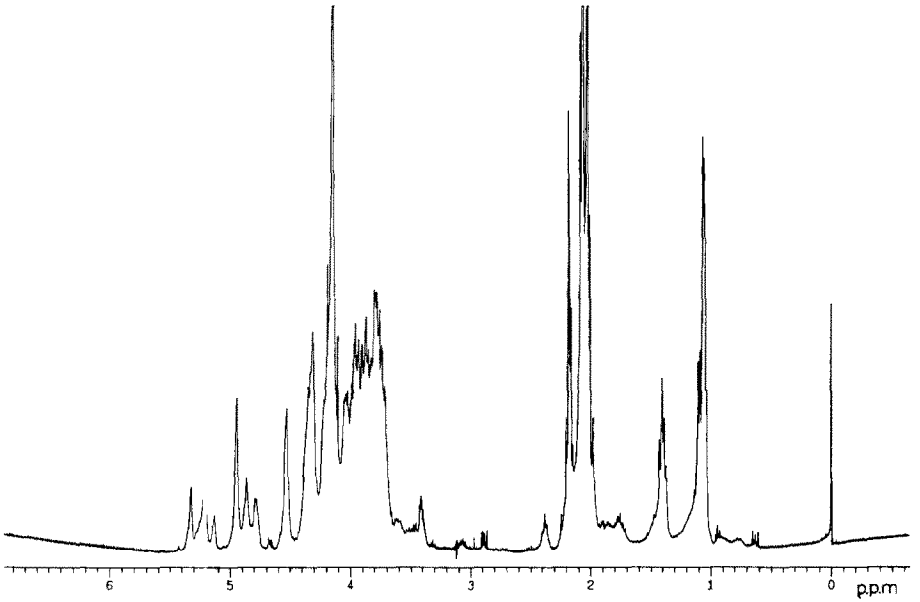



Fig. 1. $^1$H-N.m.r. spectrum of ECA.

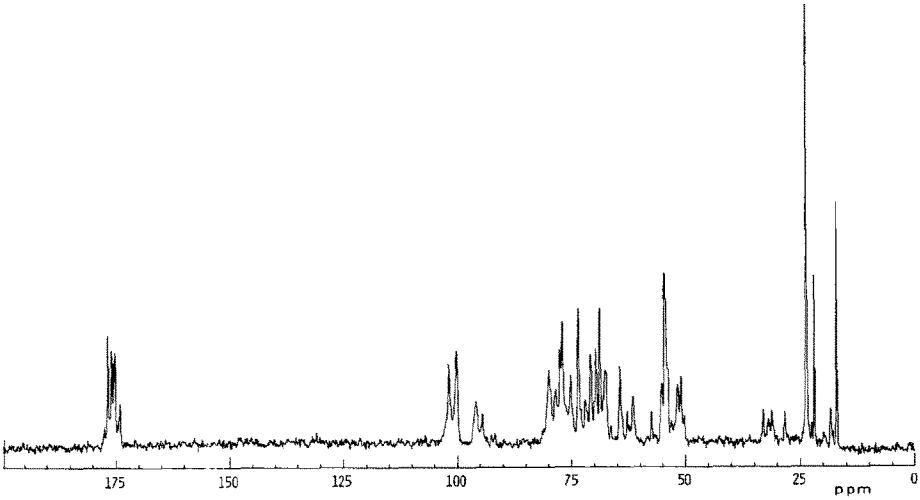



Fig. 2. $^{13}$C-N.m.r. spectrum of ECA.

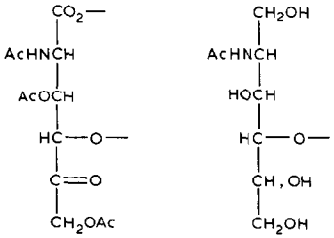
stoichiometrical *O*-acetyl groups. We now report further studies of this antigen.

→4)-β-D-Man*p*-NAcA-(1→4)-α-D-Glc*p*NAc-(1→3)-α-D-Fuc*p*4NAc-(1→
6
⋮
OAc

**1**

RESULTS AND DISCUSSION

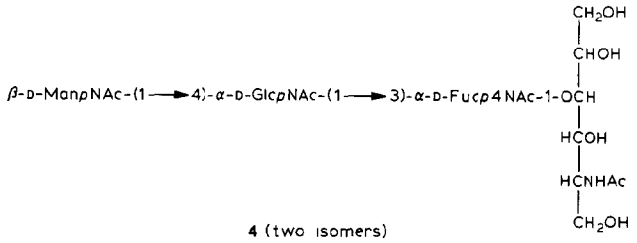
The high specific rotation of carboxyl-reduced ECA, $[\alpha]_{578}^{22}$ +100° (in water), indicated that the 4-acetamido-4,6-dideoxy-D-galactopyranosyl residue should be α-linked. In agreement with this inference, no signals having coupling constants of 7–8 Hz were observed in the anomeric region of the $^1$H-n.m.r. spectrum. In order to obtain further information, a sample of carboxyl-reduced ECA was acetylated and treated with chromium trioxide in acetic acid. Sugar analysis of the oxidised product gave 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-mannose, and 4-acetamido-4,6-dideoxy-D-galactose in the proportions 1:0.55:0.52. Ideally, only sugar residues in which the aglycon is equatorially disposed should be oxidised[3], and no definite conclusions could be drawn from this result. The product was therefore treated with sodium borohydride, in order to reduce the oxidised residues, which should be esters of 5-glyculosonic acids, with simultaneous saponification of *O*-acetyl groups, as indicated below (**2**→**3**).




**2** **3**

Fractionation of the reduced material on Biogel P-2 gave five distinct peaks in the region for penta- to mono-saccharides. The material in one of these peaks, in the tetrasaccharide region, was a pure component, as indicated by its $^1$H-n.m.r. spectrum. This contained, *inter alia*, signals at δ 1.07 (3 H, $J_{5,6}$ 6.5 Hz, H-6 of a 6-deoxyhexose), 1.99–2.05 (12 H, protons of *N*-acetyl groups), 4.87 (1 H, not resolved), 4.95 (1 H, $J_{1,2}$ 3.5 Hz), and 5.29 (1 H, $J_{1,2}$ 3.5 Hz). From these and previous[2] results, it was concluded that the substance is the alditol (**4**) of a tetrasaccharide. The signals at δ 4.87 and 4.95 could be assigned to the 2-acetamido-2-deoxy-β-D-mannopyranosyl and 2-acetamido-2-deoxy-α-D-glucopyranosyl residues, respectively, and the signal at δ 5.27, therefore, to the 4-acetamido-4,6-dideoxy-D-galactopyranosyl residue, which should be α-linked.

A reducing terminal in ECA was looked for after reduction with sodium borodeuteride followed by a search for a deuterated product in sugar and methylation analyses, but no such product was detected. Further, no non-reducing terminal could be detected on methylation analysis of carboxyl-reduced ECA. As evident from the structure of the repeating unit (**1**), only a non-reducing terminal in ECA should be oxidised by periodate, but no oxidation of carboxyl-reduced ECA could




4 (two isomers)

be observed by methylation analysis after periodate oxidation and either treatment with base or Smith degradation. More positive evidence concerning the nature of the reducing and non-reducing ends of ECA was obtained when ECA and modified ECA were investigated by fast-atom-bombardment (f.a.b.) mass spectrometry. In the discussions below, the trisaccharide ManNAcA–GlcNAc–Fuc4NAc is abbreviated as ABC. Spectra of ECA run in the positive mode gave one group of molecular ions $(M + H)^+$ at *m/z* 3036, 3078, 3120, 3162, and 3204, and another group at *m/z* 2429, 2471, 2513, and 2555. These molecular ions are each 18 mass units less than those expected for oligosaccharides of compositions $(ABC)_5$ and $(ABC)_4$, without or with 1–4 and 1–3 *O*-acetyl groups, respectively. In agreement with this, the spectra run in the negative mode showed molecular ions $(M - H)^-$ at *m/z* 3034, 3076, 3118, 3160, and 3202, and at *m/z* 2427, 2469, 2511, and 2553.

These results indicate that ECA is a mixture of the cyclic species $\overbrace{(ABC)_4}$ and $\overbrace{(ABC)_5}$, with different degrees of *O*-acetylation. In agreement with this inference, no changes in the f.a.b. spectra were observed after treatment of ECA with sodium borodeuteride, except that all of the *O*-acetyl groups were lost. An ion corresponding to $\overbrace{(ABC)_5} - 42$ was also observed, indicating some *N*-deacetylation. Another explanation would be that, in some molecules, an amino group is not acetylated, which, due to *O*-acetylation, was not detected in the spectra of original ECA.

On treatment with base or periodate followed by base, all of the *O*-acetyl groups and a small proportion of the *N*-acetyl groups were hydrolysed, but no other modifications were indicated by the f.a.b. spectra.

In order to obtain further evidence for the cyclic structure, *O*-deacetylated ECA, containing the presumed $\overbrace{(ABC)_4}$ $[(M + H)^+ 2429]$ and $\overbrace{(ABC)_5}$ $[(M + H)^+ 3036]$ and some material devoid of one *N*-acetyl group, was treated with aqueous 6M hydrochloric acid at 60° and aliquots from the reaction mixture were monitored at frequent intervals by f.a.b.–m.s. in the 3200 to 2900 mass-range. After 11 min, the spectrum had changed considerably and signals corresponding to ring-opened species $(ABC)_5$ $[(M + H)^+ 3054]$ and the corresponding *N*-deacetylated species $[(M + H)^+ 3012]$ were as abundant as signals derived from the cyclic molecules. After 33 min, signals were no longer present above 3000 mass units, and a scan of the complete mass range revealed that extensive hydrolysis had taken place. All signals in the f.a.b. spectrum corresponded to molecular ions of reducing oligosaccharides, $(ABC)_n$ with $n = 1$–4. The results therefore indicate that one

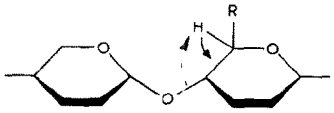
type of linkage, almost certainly that of the 4-acetamido-4,6-dideoxy-α-D-galactopyranosyl residues, is preferentially hydrolysed. This linkage was also considerably more labile than the others on solvolysis of ECA with liquid hydrogen fluoride[2].

In a similar experiment, ECA (same sample as used above) was treated with methanolic M hydrogen chloride, first at room temperature for 30 min, and then at 37°. After the first 20 min, esterification of ECA was essentially complete, the major ions $\overbrace{(MeABC)_4}$ and $\overbrace{(MeABC)_5}$ being observed at *m/z* 2485 and 3106 (positive mode). After 90 min at 37°, ions corresponding to the linear species $(MeABC)_4OMe$ and $(MeABC)_5OMe$ (*m/z* 2517 and 3138, respectively) and the corresponding species minus one *N*-acetyl group (*m/z* 2475 and 3096) predominated. Ions due to hydrolytic fragmentation were also observed, mainly of the $(MeABC)_nOMe$ type. Ions formed by fission of other glycosidic linkages were present in low abundance only.

Finally, the f.a.b. spectra of fully methylated and fully trideuteriomethylated ECA were investigated. The results were consistent and only the spectra of the trideuteriomethylated samples will be discussed. Significant ions at *m/z* 2973 and 3716 (positive mode) corresponding to trideuteriomethylated $\overbrace{(ABC)_4}$ and $\overbrace{(ABC)_5}$ were observed, but no molecular ions corresponding to trideuteriomethylated open-chain products were present. For each of these ions, a series of lower mass ions separated by 17 m.u. was observed, indicating incomplete methylation. This was not unexpected as, on methylation of substances containing acetamido groups, using the Hakomori procedure[4], *N*-methylation is never complete.

In addition to the clusters around *m/z* 2900 and 3700, a minor cluster around 4400 was observed, indicating the presence of a small percentage of $\overbrace{(ABC)_6}$ in ECA.

Whereas f.a.b.-m.s. of underivatised oligo- and poly-saccharides gives mainly molecular ions, extensive fragmentation has been observed for their methylated products[5–7]. These give predominantly $A_1$-type sequence ions[8], with $A_2$ and $A_3$ ions being produced in some cases. Analogous behaviour was observed in the present study, and all major fragment ions produced by methylated ECA can be rationalised as $A_1$-type oxonium ions which occur as clusters of signals, 17 mass units apart, as a result of the incomplete methylation. These sequence ion-clusters are very complex, because the additional cleavage step necessary for the formation of a fragment ion from ECA can proceed *via* several routes (two separate cleavages must occur in a cyclic molecule to generate fragment ions). The most favoured routes appear to be either β-cleavage or cleavage of the sugar ring, which afford type I or type II structures, respectively, at the non-reducing end of the oligomeric ion (Fig 3). These cleavages have been reported previously for underivatised and partially methylated oligosaccharides and for permethylated cyclic glucans[9–11]. A third series of fragments (type III) is also present at lower abundance and these can arise by loss of water from type I, loss of formic acid from type II, or *via* a glycosidic elimination as shown for partial structure **5**. The major $A_1$ ions belonging to the

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type I, II, and III series present in the spectrum of fully trideuteriomethylated ECA are given in Table I.

To sum up, the only rational explanation of the results discussed above seems to be that the ECA hapten is a mixture of cyclic polysaccharides (**6**), containing four, five, and, to a smaller extent, six trisaccharide repeating-units. No ions in the *m/z* 2000–4000 region, derived from one of these species substituted with a fatty acid or with phosphate, were observed, indicating that these acids, which were found in the ECA preparation, are not chemically linked to the ECA hapten. A fraction of ECA, devoid of fatty acids, was also obtained on silica gel chromatography. As the conformations of the sugar residues and the chemical environments

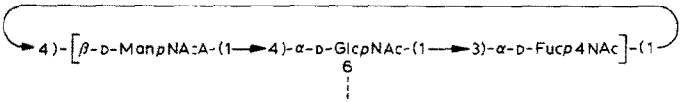


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TABLE I

MAJOR FRAGMENT IONS IN THE SPECTRA OF DEUTERIOPERMETHYLATED ECA

| *Composition* | *Type I* | *Type II* | *Type III* |
|---|---|---|---|
| A | 269 | —[a] | — |
| B | 255 | 283 | 237 |
| C | 222 | 250 | 204 |
| AB | 523 | 551 | 505 |
| BC | 476 | 504 | — |
| AC | — | — | — |
| ABC | 744 | 772 | 726 |
| $A_2BC$ | — | — | 994 |
| $AB_2C$ | 998 | 1026 | 980 |
| $ABC_2$ | 965 | 993 | 947 |
| $A_2B_2C$ | 1266 | 1294 | 1248 |
| $AB_2C_2$ | 1219 | 1247 | — |
| $A_2BC_2$ | — | — | — |
| $A_2B_2C_2$ | 1487 | 1515 | 1469 |
| $A_3B_2C_2$ | — | — | — |
| $A_2B_3C_2$ | 1741 | 1769 | — |
| $A_2B_2C_3$ | 1708 | — | 1690 |
| $A_3B_3C_2$ | — | — | 1991 |
| $A_2B_3C_3$ | — | 1990 | — |
| $A_3B_2C_3$ | — | — | 1958 |
| $A_3B_3C_3$ | 2230 | — | 2212 |

[a]Indicates that the ion having the composition given in the first column was not present in the spectrum.

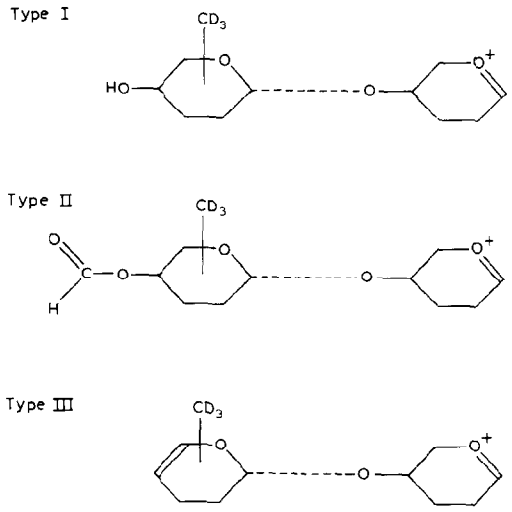


Fig. 3. Fragment ions in f.a.b.-m.s. of methylated ECA.

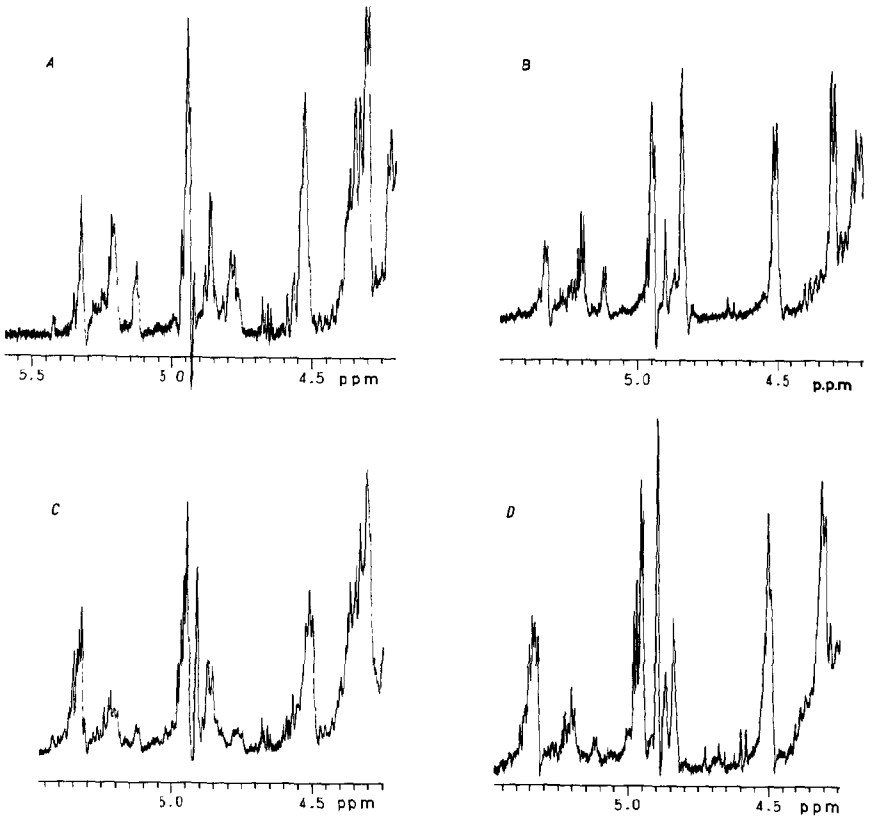


Fig. 4. Low-field region of the resolution-enhanced, $^1$H-n.m.r. spectrum of (*A*) native ECA, (*B*) *O*-deacetylated ECA, (*C*) partially hydrolysed ECA, and (*D*) *O*-deacetylated, partially hydrolysed ECA.

of the hydrogen and carbon atoms will depend on the ring size, it is not unexpected that the $^1$H- and $^{13}$C-n.m.r. spectra of ECA, even after hydrolysis of the non-stoichiometric amount of *O*-acetyl groups, were more complicated than expected for a non-cyclic polymer. After treatment with acid under mild conditions, during which some of the glycosidic linkages were cleaved, the spectra were considerably simplified (Fig. 4). The signals for the anomeric protons of the three sugars appeared at fields close to those observed for the trisaccharide glycoside **4**.

For a long time, the Schardinger dextrins (cyclomalto- hexa-, -hepta-, and -octa-ose) were the only saccharides for which a cyclic structure had been definitely established. Recently, however, the (1→2)-linked β-D-glucan elaborated by different *Agrobacterium* and *Rhizobium* species has been shown to be a mixture of cyclic saccharides containing 17–24 (1→2)-linked β-D-glucopyranosyl residues[11,12]. The ECA hapten is, however, the first heteropolysaccharide for which a cyclic structure has been proposed.

In the biosynthesis of the O-specific side-chains in lipopolysaccharides elaborated by Gram-negative bacteria, the chain, which is composed of oligosaccharide repeating-units and linked to a polyprenyl pyrophosphate, grows by addition to another repeating-unit, also linked to a polyprenyl pyrophosphate. It seems possible that the biosynthesis of ECA is similar but that, when the chain becomes large enough, the terminal glycosyl group comes so close to the glycosyl residue linked to the pyrophosphate that cyclisation occurs instead of chain growth.

## EXPERIMENTAL

*General methods.* — These were the same as previously described[2]. Preparation and purification of ECA, *O*-deacetylation, carboxyl-reduction, and sugar and methylation analysis were performed as previously described. In these analyses, the polymeric material was solvolysed with dry hydrogen fluoride.

*Fractionation of ECA.* — This was attempted by chromatography on a column of silica gel, using ethanol–water (7:3) as irrigant. F.a.b.-m.s., however, revealed that fractionation was only partial, the first eluted fractions having a higher percentage of *O*-acetyl groups. The $^1$H- and $^{13}$C-n.m.r. spectra further revealed that the main fraction did not contain fatty acids.

*Chromium trioxide oxidation.* — Carboxyl-reduced ECA (30 mg) was dried *in vacuo* over phosphorus pentaoxide and treated with acetic anhydride–pyridine (1:1, 20 mL) at 80° for 5 h. The solution was concentrated under reduced pressure and the last traces of solvent were removed by codistillation with toluene. The acetylated ECA was dissolved in acetic anhydride (7 mL), chromium trioxide (120 mg) was added, and the mixture was sonicated at 50° for 80 min. 2-Propanol (3 mL) was added, followed, after 1 h at room temperature, by chloroform (8 mL) and water (8 mL). The chloroform phase was collected, dried, and concentrated. Part of the residue was taken for sugar analysis. The main part was dissolved in a mixture of 1,4-dioxane (2 mL) and ethanol (2 mL), sodium borohydride (100 mg) was

added, and the mixture was kept at room temperature overnight. Dowex 50 ($H^+$) resin and water (10 mL) were added and, when the pH had decreased to 3, the resin was filtered off. The solution was concentrated and boric acid was removed by codistillation with methanol (5 × 5 mL). The product was fractionated on a column (100 × 1.60 cm) of Biogel P-2 by elution with water. The fractionation was monitored by differential refractometry and six fractions were collected. Four of these, from the elution volumes, contained oligosaccharides and were analysed by $^1$H-n.m.r. spectroscopy. The second fraction contained a pure tetrasaccharide-alditol.

*Periodate oxidation.* — A solution of ECA (20 mg) in 0.1M sodium metaperiodate (5 mL) was kept at 4° for 36 h. The excess of periodate was reduced by addition of ethylene glycol, and the polymer (18 mg) was recovered by chromatography on a column of Sephadex G-25. A solution of part (4 mg) of this product in 0.5M sodium hydroxide (1 mL) was kept at 40° for 18 h and then neutralised, and the product was purified on a column of Sephadex G-25.

Another part (14 mg) was reduced with sodium borohydride (30 mg) in water (1 mL) and, after the usual work-up, treated with 0.5M trifluoroacetic acid (2 mL) at room temperature for 40 h. The product was recovered by freeze-drying. The various products were characterised by methylation analysis, $^1$H-n.m.r. spectroscopy, and f.a.b.-m.s.

*F.a.b. mass spectra.* — These were recorded with a VG Analytical High Field ZAB-1F mass spectrometer at accelerating voltages of 8 (mass range 3300 m.u.), 7 (3770 m.u.), 6 (4400 m.u.), and 5 kV (5280 m.u.) in the positive and negative modes. Xenon was used as the bombarding gas and the atom gun was operated at 10 kV.

F.a.b. mass spectra were obtained by dissolving the sample in aqueous 5% acetic acid (underivatised samples) or methanol (permethylated samples) and loading 1 μL of this solution into a matrix composed of a mixture of glycerol and monothioglycerol (1:1). Aliquots from the time-course methanolysis and acid hydrolysis experiments were loaded into the matrix directly.

Spectra were recorded on oscillographic paper using a 300-s mass-controlled linear scan. Signals were assigned by counting the spectra.

*Acid hydrolysis.* — 6M Hydrochloric acid (20 μL) was added to *O*-deacetylated ECA (100 μg) in a stoppered tube, and the mixture was incubated at 60°. At intervals (3, 7, 11, 15, 20, and 33 min), aliquots (1 μL) were removed for f.a.b.-m.s.

*Methanolysis.* — *O*-Deacetylated ECA (150 μg) was mixed with methanolic M hydrogen chloride (20 μL) and kept at room temperature in a stoppered tube for 30 min. An aliquot (1 μL) was removed after 20 min and analysed directly by f.a.b.-m.s. The remaining mixture was kept at 37°, and further aliquots (1 μL) were removed for f.a.b.-m.s. after 10, 20, 30, 40, 60, and 90 min.

*Partial, acid hydrolysis.* — A solution of native or *O*-deacetylated ECA (5 mg) in water (3 mL) and acetic acid (50 μL) was heated at 100° for 5 h and then freeze-dried, and the residue was investigated by $^1$H-n.m.r. spectroscopy.

ACKNOWLEDGMENTS

This work was supported by grants from the Swedish Medical Research Council (03X-02522), the Swedish Natural Science Research Council, the National Swedish Board for Technical Development, and Stiftelsen Sigurd och Elsa Goljes Minne. A. D. is grateful for financial support from grants awarded to Professor H. Morris by the M.R.C. and S.E.R.C.; J. O. is a recipient of an S.E.R.C. studentship.

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