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β-Elimination of glucosyluronic residues during methylation of an acidic polysaccharide from *Erwinia chrysanthemi* CU 643

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Abstract

The *Erwinia chrysanthemi* CU643 EPS has a linear hexasaccharide repeating unit in which a 4-linked uronic acid residue is present. The EPS was methylated by either the NaOH–Me_2SO–MeI or Li-dimsyl procedure. MALDI-TOF MS analysis of the methylated products indicates that the β-eliminative degradation occurs during the methylation, as characterized by serial fragments of the hexasaccharide repeating units. The degradation was clearly defined from the methylation of a glucosyluronic-containing pyruvated pentasaccharide.

Keywords: Methylation; β-Elimination; Acidic extracellular polysaccharide

1. Introduction

Per-*O*-methylated sugar derivatives are readily prepared by the modified NaOH–Me_2SO–MeI procedure,[1] which has been used successfully in our studies for the linkage analyses of sugar residues in bacterial polysaccharides,[2–6] glycoproteins and glycopeptides,[7,8] even though the 'dimsyl' method has some advantage for the methylation of glycans containing an inorganic substituent such as phosphate or sulfate.[9,10] However, using the NaOH–Me_2SO–MeI procedure, relatively low recoveries of glucosyluronic residues have been observed in the methylation analysis of oligosaccharides containing 4-linked, or non-reducing terminal, glucuronic acid residues.[2,3] In such studies, the problem of β-eliminative degradation is always present, together with the subsequent complex reactions that occur when the resulting unsaturated glucuronate residues are subjected to acid hydrolysis, during which the β-eliminative degradation product or its enolic isomer rearranges to form volatile furan derivatives[11] that are not accounted for in the subsequent analyses.

β-Elimination of hexuronic acid derivatives and glycuronans has been investigated[12–14] and its application to the sequence of the glucosyluronic residues in the polysaccharides has been illustrated.[11] Under alkaline conditions, 4-substituted glycopyranuronosylic residues may be degraded with the release of the 4-substituent and the subsequent formation of a 4-deoxy-hex-4-enoglycuronate. A 'peeling' reaction[15] from a reducing terminus may also occur with the formation of a sac-

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charinic acid and concomitant release of any substituent at positions 2, 3, or 4, which, if a reducing sugar, will continue to 'peel' until it reacts to form a saccharinic acid without further elimination of its substituent. Since glycosyl or methoxyl residues are good leaving groups in these alkaline degradation reactions, it is of interest to determine their extent in methylation reactions where there is a short exposure of the carbohydrate (less than a minute for the NaOH–Me_2SO–MeI procedure and 1 h for the dimsyl procedure) to alkali or base before introduction of the methyl groups. In the NaOH–Me_2SO–MeI procedure, the alkali is neutralized by the excess methyl iodide in a few minutes at room temperature.

This communication evaluates the extent of β-eliminative degradations concomitant with the methylation of an oligo- and a polysaccharide containing 4-linked glucosyluronic residues.

2. Experimental

Analytical and general methods.—Methods used for methylation analysis, gas liquid chromatography with flame ionization detector (GLC–FID) or mass selective detector (GLC–MS), matrix assisted laser desorption–ionization time of flight mass spectrometric analysis (MALDI-TOF MS) of per-*O*-methylated derivatives have been described previously.[2,3]

Methylation analysis.—The freeze-dried material of extracellular polysaccharide (EPS, 1.6 mg) **1** of *Erwinia chrysanthemi* CU643[3] and a pyruvated pentasaccharide **2** from *E. chrysanthemi* Ech6 (100 μg)[16] were methylated by the NaOH–Me_2SO–MeI procedure. Per-*O*-methylated products were fractionated on a Sephadex LH-20 column (1 × 23 cm, Pharmacia LKB, Uppsala, Sweden). The column was eluted with 50% $CHCl_3$ in MeOH for the methylation products of polysaccharide **1** and MeOH for the methylation products of **2**. The elution was followed by phenol–H_2SO_4 analysis[17] and/or by MALDI-TOF MS (Voyager-DE STR BioSpectrometry, PerSeptive Biosystems, Framingham, MA).

The same EPS of CU643 (1.6 mg) was methylated by the lithium 'dimsyl' procedure as described elsewhere[18] and analyzed in the same way as described above.

→3)-β-D-Gal*p*-(1→2)-α-L-Rha*p*-(1→4)-β-D-GlcA*p*-(1→2)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→) **1**

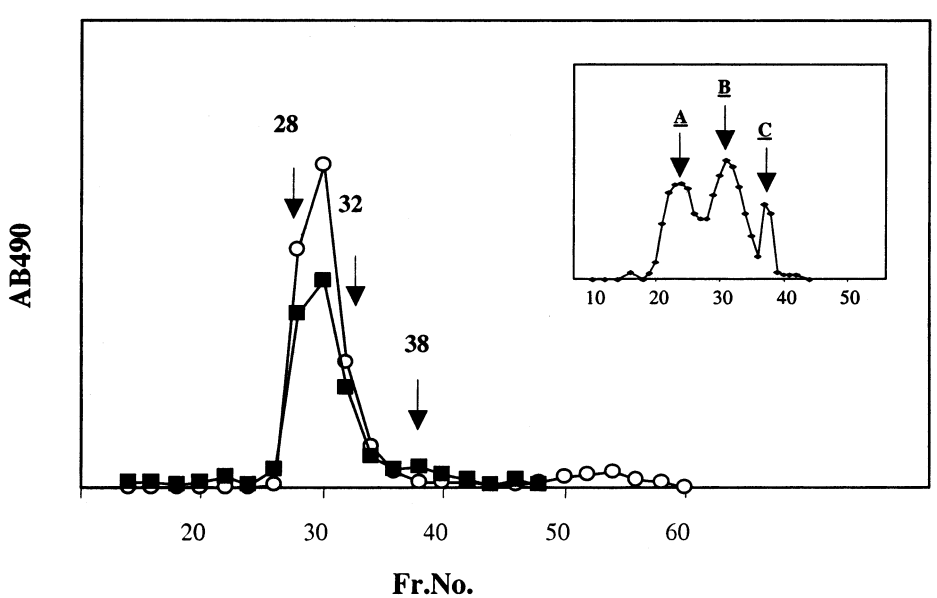

Fig. 1. Chromatographic separation of per-O-methylated *E. chrysanthemi* CU643 EPS. The native EPS was methylated with either NaOH–Me_2SO–MeI (■) or lithium dimsyl (○) and fractionated on a Sephadex LH-20 column, eluting with 50% $CHCl_3$ in MeOH. Remethylation products of methylated EPS were fractionated on the same column eluting with MeOH as shown in inset.

4,6 - *O* - (1 - carboxyethylidene) - α - D - Gal*p*-(1→4)-β-D-GlcA*p*-(1→3)-α-D-Gal*p*-(1→3)-L-[β-D-Glc*p*-(1→4)]-Fuc*p* **2**

3. Results and discussion

The EPS of *E. chrysanthemi* CU643 has a linear hexasaccharide repeating unit (**1**), in which a 4-linked uronic acid residue is present. It presents a good polysaccharide to study β-eliminative degradation resulting from methylation. Per-*O*-methylation of the native EPS was performed using either the NaOH–Me_2SO–MeI or Li-dimsyl procedures. The subsequent methylated derivatives were fractionated by chromatography on a Sephadex LH-20 column (Fig. 1) and then analyzed across the peaks by MALDI-TOF MS (Fig. 2).

The methylation of native EPS results in a spectrum of per-*O*-methyl polymers, characterized by MALDI-TOF MS analyses, with mass increments (*m*/*z* 1119) of the per-*O*-

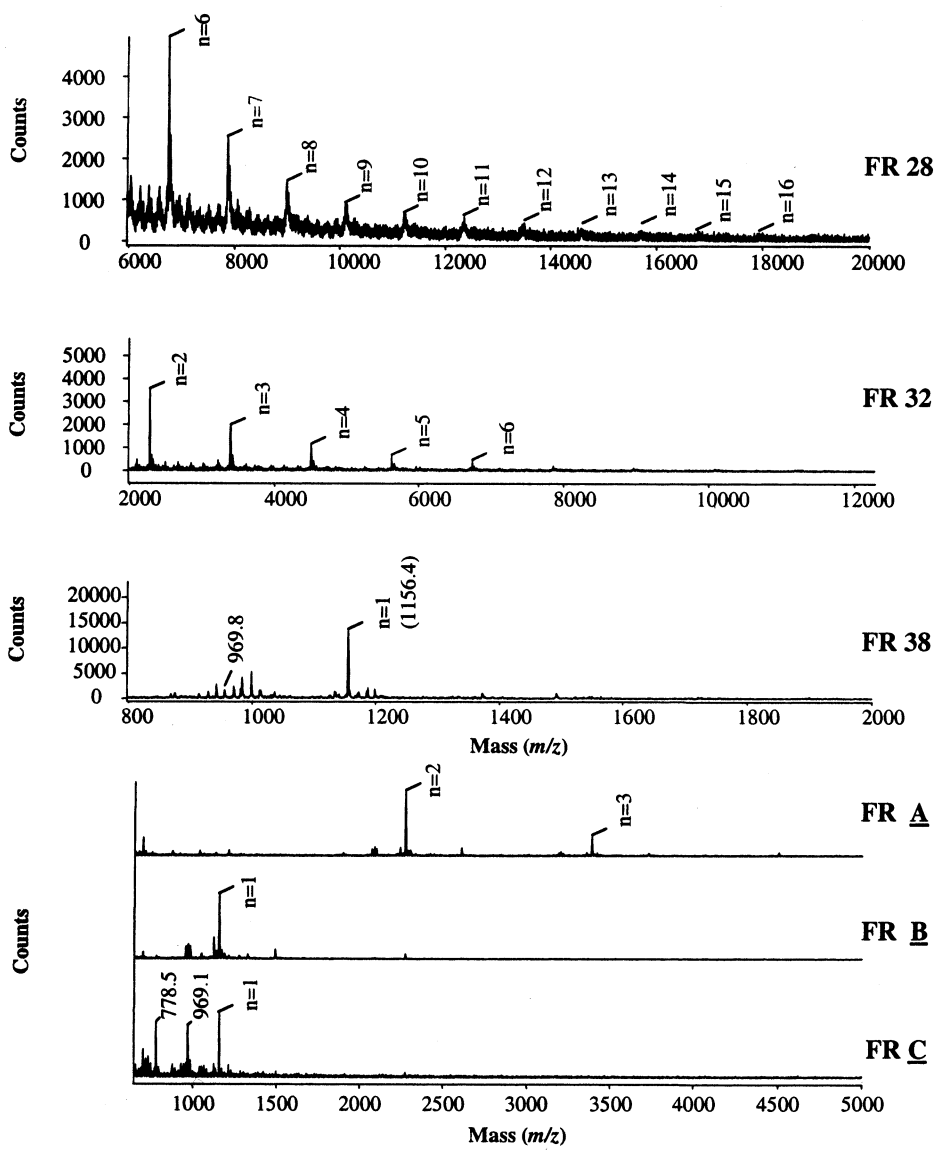

Fig. 2. MALDI-TOF MS of per-*O*-methylated *E. chrysanthemi* CU643 EPS and remethylated products of methylated EPS. Fraction no. refers to the corresponding fraction in Fig. 1. A series of β-eliminative degradation products of per-*O*-methylated hexasaccharide repeating unit, $n = 1$–16, are represented by ΔGlcA–Rha–Rha–Rha–Gal–Rha–(GlcA–Rha–Rha–Rha–Gal–Rha)$_{n-1}$, where ΔGlcA is unsaturated GlcA (4-deoxy-hex-4-enoglucuronate derivative). Fractions **A** and **B**, from remethylated products, show as principal components two repeating unit ($n = 2$) and one repeating unit ($n = 1$) of hexasaccharide, respectively. Fraction **C** contains a mixture of unknown products (*m*/*z*, 778.5 and 969.1, see text) and one repeating unit.

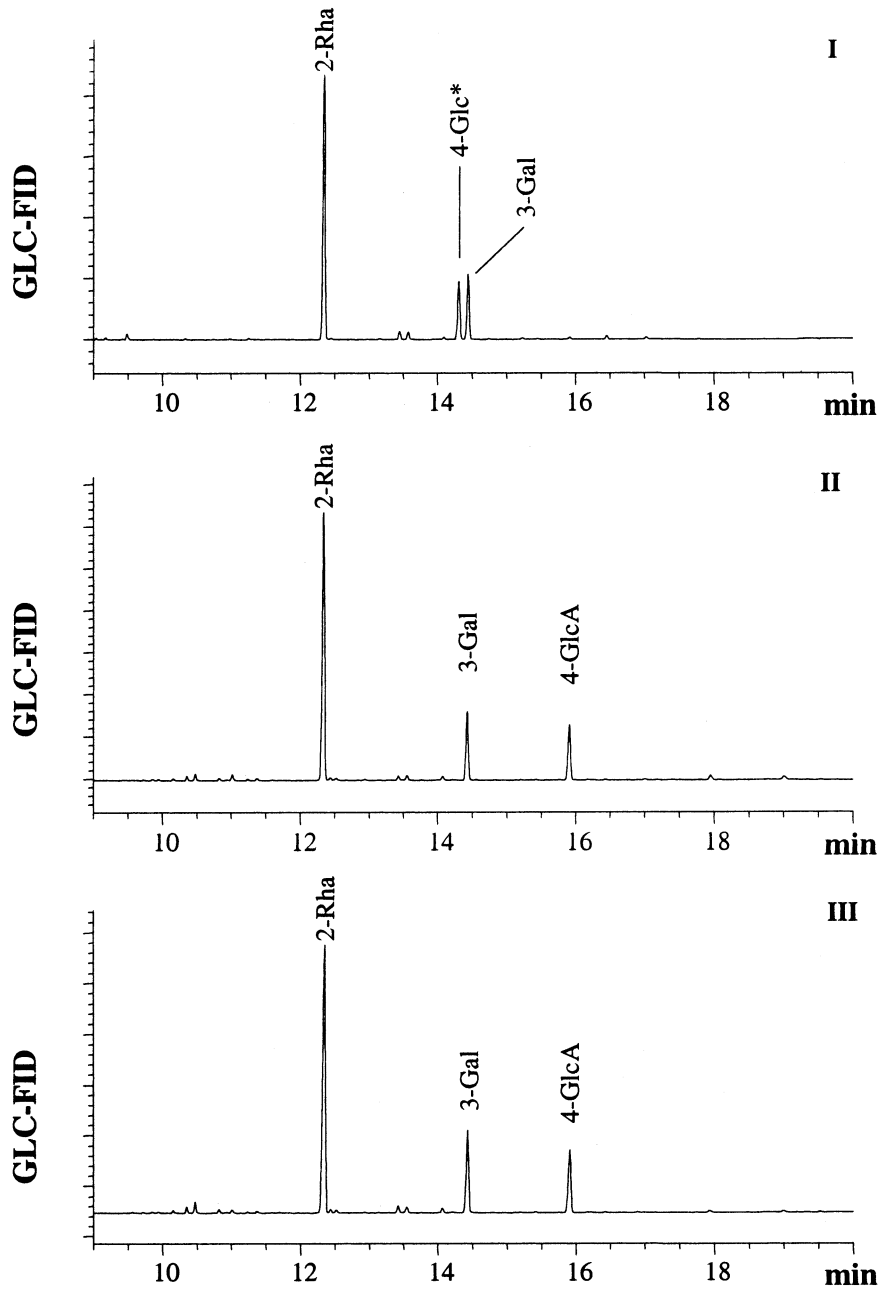

Fig. 3. GLC analyses of the methylation products of CU643 EPS. (I) Carboxyl-reduced EPS methylated by NaOH–Me_2SO–MeI. (II) Native EPS methylated by NaOH–Me_2SO–MeI. (III) Native EPS methylated by lithium dimsyl. Linkage analyses, summarized in Table 1, are shown as the deduced linkages in EPS. Glc* derived from methylation of carboxyl-reduced EPS.

methyl hexasaccharide repeating units. One repeating unit (m/z 1156.4, Calcd 1155.5) of per-*O*-methyl hexasaccharide comprising of terminal unsaturated glucuronate (4-deoxy-hex-4-enoglucuronate) was observed at the tailing edge of the peak and up to approximately 16 repeating units ($n = 16$) of hexasaccharide observed at the leading edge. The β-eliminative degradation occurred during the methylation of native EPS by either method, as noted in Fig. 2. The extent of degradation in the EPS was difficult to quantify by MALDI-TOF MS since the smaller molecules in the mixed products suppress the ionization of larger molecules in MALDI-TOF MS,[19] many of which would consequently not be detected in the analysis.

Significant β-eliminative degradation of the per-*O*-methyl EPS down to one, two and three repeating units ($n = 1$, 2 and 3) of per-*O*-

methyl hexasaccharide can be achieved by re-methylation with the NaOH–Me_2SO–MeI procedure (inset in Figs. 1 and 2). The fraction **C** contains two extra signals, of which the ion of m/z 778.5 is equivalent to the mass of a sodiated per-*O*-methyl tetrasaccharide (Calcd m/z 777.4) composed of one unsaturated glucuronic acid and three rhamnose residues and the ion of m/z 969.1 to that of sodiated per-*O*-methyl pentasaccharide (Calcd m/z 969.5) composed of one galactose and four rhamnose residues. These oligosaccharides, the structures of which are not proven, were present as very minor components during the initial methylation (see Figs. 1 and 2, FR 38), then increased upon the second methylation as shown in Figs. 1 and 2 (FR C). The per-*O*-methyl tetrasaccharide could arise from the release of a hexasaccharide repeat unit from the β-eliminative degradation of the glycuronic residues followed by a sequential 'peeling' of the rhamnosyl and galactosyl residues. The product of the ion m/z 969.5 would need to arise from the elimination of the unsaturated glucuronic acid residues from the hexasaccharide by some other mechanism.

The pooled fractions across the peak (Fig. 1) of per-*O*-methylated EPS were reduced with Superdeuteride ($Li(Et)_3BD$) and hydrolyzed. The resulting hydrolyzate was analyzed as the alditol acetates by GLC–FID (Fig. 3 and Table 1). Both results are compared with the result obtained from the methylation of the carboxyl-reduced[20] and thus neutral EPS, which avoids the β-eliminative degradations. As noted in Table 1, 0.80 or 0.85 mol of glucuronate residue per mole of galactose were obtained from methylation of native EPS versus 0.92 mol from methylation of carboxyl-reduced EPS. The approximately 10% loss of the glucosyluronic residues due to the degradative reactions would probably not be very significant in the methylation analyses of a polysaccharide, but does relate to the frequent low recovery of glucuronic acid residue in the linkage analysis of polysaccharides.

The complexity of the side reactions in NaOH–Me_2SO–MeI methylation is reflected in the MALDI-TOF analyses of the lower molecular weight components (Fig. 2, FR 38). Several minute but significant signals around m/z 750–1200 represent losses of sugar residues in methylation linkage analysis. Such minor losses are also seen in Fig. 4(top panel) from the methylation of **2**. The products m/z 867.4 and 1159.2 are not seen following the methylation of the reduced pentasaccharidiitol, suggesting that they result from reactions involving the reducing terminal fucose residue.

MALDI-TOF MS analyses of per-*O*-methylated pentasaccharide derivative showed three signals, m/z, 1129.3, 837.5, and 329.8 (Fig. 4). The signal of m/z 1129.3 represents the sodiated parent molecule (Calcd m/z, 1129.5). The other two signals, m/z 837.5, and 329.8, reflect the degradation products of the parent molecule by β-elimination, corresponding to the per-*O*-methylated derivative (Calcd m/z, 837.4) of a tetrasaccharide containing unsaturated glucuronate (4-deoxy-hex-4-enoglucuronate) and the per-*O*-methylated derivative of pyruvated-galactose (Calcd m/z, 329.1), respectively. The same degradation products, but with an increase of 16 amu for the reduced terminal residue, were obtained by MALDI-TOF MS analyses of per-*O*-methylated pentasaccharidiitol derivative, i.e., m/z 1145.6, 853.5 and 329.6.

The broad absorbance in the UV spectrum of the per-*O*-methylated derivative in MeOH

Table 1
Methylation analyses of EPS CU643 [a]

Me sugar [b]	GLC response with FID [e]		
	I	II	III
3,4-Me_2Rha	3.61	3.94	3.59
2,4,6-Me_3Gal	1.00	1.00	1.00
2,3,6-Me_3Glc [c]	0.92		
2,3-Me_2GlcA [d]		0.85	0.80

[a] Native EPS was methylated by either the NaOH–Me_2SO–MeI (II) or the Li-dimsyl (III) procedure. Pre-reduced EPS (I) was methylated by the NaOH–Me_2SO–MeI procedure.

[b] 3,4-Me_2Rha = 1,2,5-tri-*O*-acetyl-1-deuterio-3,4-di-*O*-methyl rhamnitol.

[c] Derived from methylation of carboxyl-reduced EPS CU643.

[d] Observed as 1,4,5,6-tetra-*O*-acetyl-1,6,6′-trideuterio-2,3-di-*O*-methylglucitol derived from the reduction of the methyl ester of 2,3-Me_2GlcA with Superdeuteride.

[e] Expressed relative to the peak area of 2,4,6-Me_3Gal residue.

at around 230 nm indicates the presence of the α,β-unsaturated carboxyl group of the degradation products (data not shown).

The degradation products from the methylation of oligosaccharide **2** were fractionated on a Sephadex LH-20 column and followed by MALDI-TOF MS. The fractions containing methylated pyruvated-galactose was directly analyzed by GC–MS and identified as the per-*O*-methylated glycosides of the α and β anomeric mixtures (Fig. 4(a)). The methylated tetrasaccharide eluted very close to the

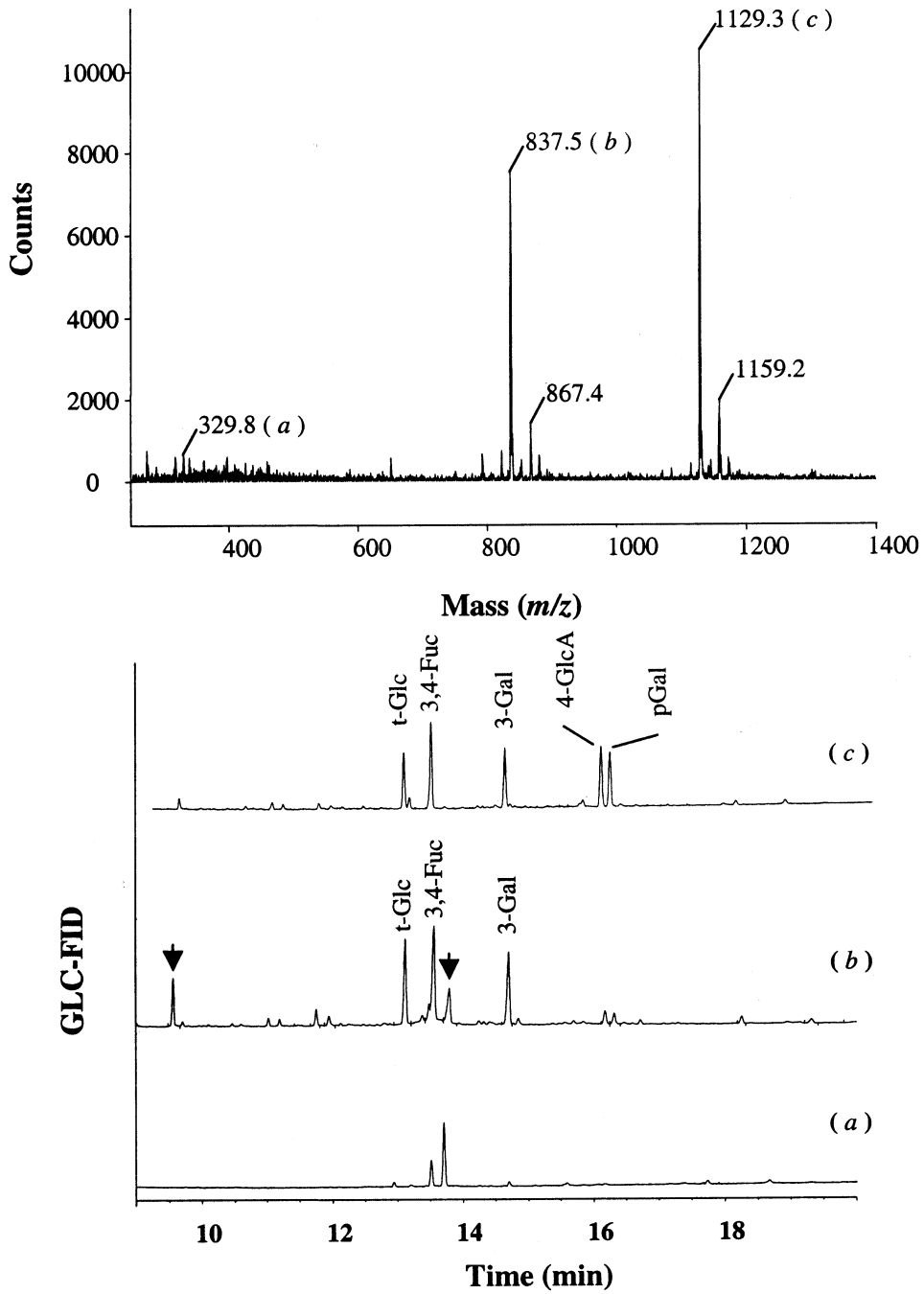

Fig. 4. MALDI-TOF MS (top panel) and GLC analyses of β-eliminative degradation products from the parent pyruvated-pentasaccharide **2**. Methylation was performed by the NaOH–Me_2SO–MeI procedure. The degradation products were separated on a Sephadex LH-20 column. (a) Per-*O*-methylated α- and β-methyl glycosides of pyruvated-galactose; (b) per-*O*-methylated tetrasaccharide comprising unsaturated GlcA, glucose, galactose and fucose residue (unknowns are indicated by arrows); (c) parent per-*O*-methylated pyruvated pentasaccharide. Deduced linkages in the oligosaccharide noted as follows. pGal, terminal pyruvated-Gal; t-Glc, terminal Glc; 3-Gal, 3-linked Gal; 3,4-Fuc, 3,4-linked Fuc; 4-GlcA, 4-linked GlcA.

parent methylated **2**, requiring repetitive chromatographic separations. The purified materials were then reduced with Superdeuteride and MALDI-TOF analyses of the resulting materials gave signals consistent with the masses expected before and after the reduction (data not shown). These reduced materials were hydrolyzed and analyzed as alditol acetate derivatives by GLC and GLC–MS. Quantitative recoveries of five residues were observed from the isolate of the methylated **2** (Fig. 4(c)). However, the tetrasaccharide, comprising of unsaturated glucuronic acid, shows only glucose, galactose and fucose residues and two unknowns as noted in Fig. 4(b). These unknowns are thought to be derived from reactions involving unsaturated glucuronate residues. Upon hydrolysis, the resulting 6,6′-di-deuterio-4-deoxy-hex-5-ulose rearranges into the furan derivatives. Thus the extent of degradation would be reflected by the decreased amounts of the glucuronic acid residue in the methylation analysis of oligosaccharide as observed previously.[2,3]

4. Conclusions

The classical β-elimination reactions occur concomitantly with methylation of uronic acid-containing sugars, since either the glycosidically linked residue or the methoxy substituent at the 4-position of the uronic acid residue are good leaving groups. The degradation of the glycuronans is random and a spectrum of degradation products is produced. However, the extent of degradation seen for the polysaccharide in this study was not such that the methylation linkage analysis was greatly embarrassed unless repeated methylations are made, as was the case in earlier studies when the methylating reagents were not as efficient. It was concluded that the loss of residues by alkaline degradation reactions in the methylation of EPS containing uronic acid residues was not enough to invalidate linkage analyses by methylation.

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