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Note

Formation of 3,4-di-*O*-acetyl-1,6-anhydro-2,7-di-*O*-methyl-L-*glycero*-D-*manno*-heptopyranose during methylation analysis of lipopolysaccharide cores representative of the *Vibrionaceae* family*

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Aeromonas hydrophila and *A. salmonicida* are Gram-negative bacteria belonging to the family *Vibrionaçeae*, and are common inhabitants of freshwater lakes and streams[1,2]. In freshwater fish, they are serious pathogens responsible for frequent fish kills; *Vibrio ordalii*, on the other hand, is an important pathogen of marine and estuarine fish, causing the hemorrhagic septicemia vibriosis[3].

Interest in the precise molecular structure of the lipopolysaccharides of the different species of the genus *Aeromonas* has increased, in an attempt to define the different specificities of the antigenic determinants of the cell surface polysaccharides[4–7].

During methylation analysis of various core oligosaccharides of *Aeromonas* species[4–6] and *Vibrio ordalii* by the Hakomori method, followed by hydrolysis, reduction, acetylation, and identification by g.c.–m.s., we have noticed the formation of an unknown compound **1**, having a retention time of 2.6 (on Silar 7CP), or 1.19 (on CP-Sil 5), relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol as unity.

The aforementioned core oligosaccharides have, in common, a doubly branched L-*glycero*-D-*manno*-heptose unit substituted by three residues linked through O-3, O-4, and O-6, which, after methylation analysis, yielded 1,3,4,5,6-penta-*O*-acetyl-2,7-di-*O*-methyl-L-*glycero*-D-*manno*-heptitol (**2**). Comparative studies on the hydrolysis products of the respective permethylated core oligosaccharides showed that reasonable stoichiometry for the 2,7-di-*O*-methylheptitol **2** was found only when 2M trifluoroacetic acid was used to cleave the permethylated core, instead of such other methods of hydrolysis, as acetolysis[8], and 90% formic

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TABLE I

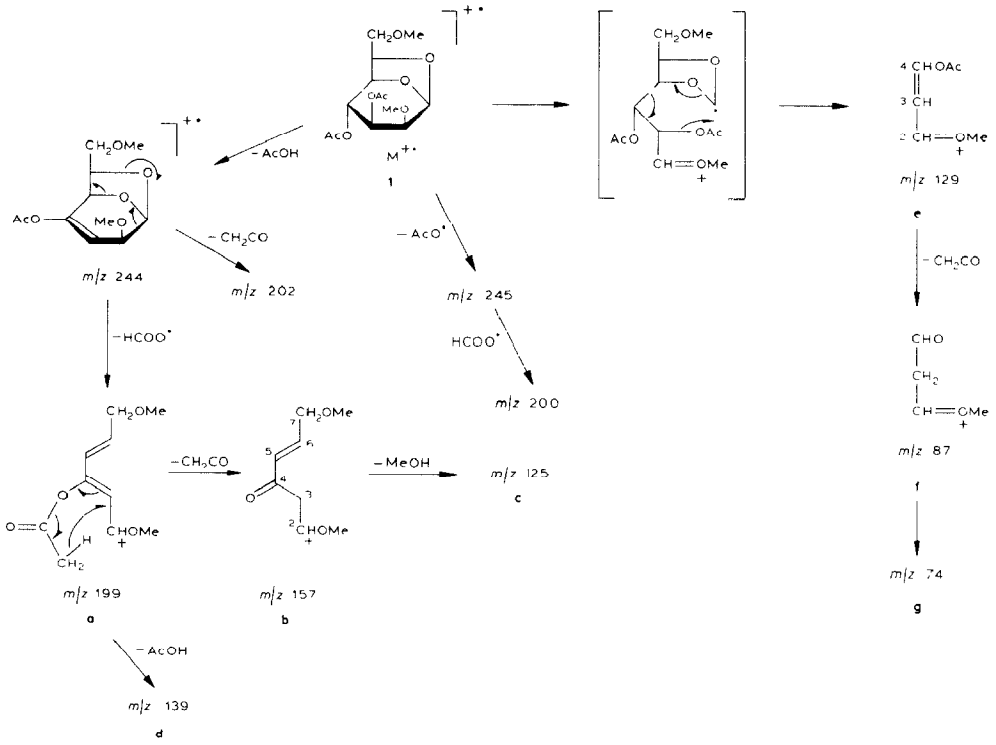
COMPARATIVE HYDROLYSIS OF A METHYLATED CORE OLIGOSACCHARIDE FROM *A. salmonicida* (MOL % OF RECOVERED METHYL ETHERS)

Methylated alditol	*TFA*[a]	*Acetolysis*	*Formic acid–H_2SO_4*
2,3,4,6-Me_4Gal	17.7	15.1	13.8
2,7-Me_2anhydroHep (**1**)	tr[b]	11.0	10.3
2,7-Me_2Hep (**3**)	11.9	3.7	5.5
3,6-Me_2GalN	6.6	6.7	6.7
Other methyl ethers	63.8	63.5	63.7

[a]TFA, trifluoroacetic acid. [b]tr, trace.

acid followed by 0.5M sulfuric acid[9]. When the last hydrolysis conditions were used, we noticed a decrease in the percentage of the 2,7-di-*O*-methylheptitol **2**, together with a sharp increase in the formation of the unknown compound **1**.

A comparison between the different methods of hydrolysis is made in Table I, where the permethylated core oligosaccharide of *A. salmonicida* was chosen as representative of the genus *Aeromonas*.



Scheme 1. Fragmentation pattern of 3,4-di-*O*-acetyl-1,6-anhydro-2,7-di-*O*-methyl-L-*glycero*-D-*manno*-heptopyranose (**1**).

The identity of the unknown compound **1** has been established by electron-impact and chemical-ionization (isobutane) mass spectrometry as 3,4-di-*O*-acetyl-1,6-anhydro-2,7-di-*O*-methyl-L-*glycero*-D-*manno*-heptopyranose. The following rationale is offered for the identification and formation of the 1,6-anhydroheptose derivative **1**.

The e.i. mass spectrum of **1** gave, *inter alia*, peaks at the following *m/z* values: 43, 45, 74, 87, 97, 111, 125 (base peak), 129, 139, 157, 199, 200, 202, 244, and 245. A summary of the tentative modes of formation, and suggested structures, of the specific ions formed during the breakdown of the molecular radical-ion is given in Scheme 1.

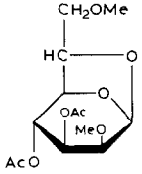
It may be seen that one of the important initial cleavages is dominated by elimination of the 3-acetoxyl group[10] by two distinct routes which involve loss of a molecule of acetic acid and of an acetoxyl radical to afford the primary fragment ions at *m/z* 244 and 245, respectively.

The primary fragment ion at *m/z* 244 loses the HCOO· radical, to form the secondary fragment ion at *m/z* 199, to which is tentatively assigned structure **a**, and it appears to be mainly composed of C-2, C-3, C-4, C-5, C-6, and C-7. Elimination of one molecule of ketene from ion **a** produces the intense ion **b**, at *m/z* 157, which, in turn, loses a molecule of methanol to yield the ion **c** at *m/z* 125 (base peak).

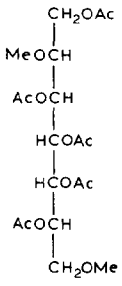
The alternative, heterolytic cleavage of the C-1–C-2 bond of the sugar molecule logically leads to the primary fragment ion at *m/z* 129, to which is assigned structure **e**, and it appears to be mainly composed of C-2, C-3, and C-4. Elimination of one molecule of ketene from ion **e** gives the ion at *m/z* 87, assigned structure **f**.

The breakdown processes leading to the production of the aforementioned fragment ions were investigated by using a deuterium labeling procedure[11]. In effect, when the methylated alditol mixture obtained after hydrolysis of the respective core oligosaccharide and reduction of the products was (trideuterio)acetylated, there was obtained, as one of the products, the corresponding 1,6-anhydro-2,7-di-*O*-methyl-3,4-di-*O*-(trideuterioacetyl)-L-*glycero*-D-*manno*-heptopyranose (**3**).

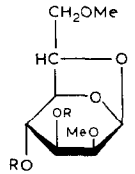
The e.i. mass spectrum of the (trideuterio)acetylated derivative **3** gave, *inter alia*, peaks at the following *m/z* values: 45, 46, 75, 88, 97, 126, 132, 139, 158, 202,



1



2



3

R = D_3CCO

and 203. These peaks are in agreement with the fragmentation pattern expected of this (trideuterio)acetylated derivative.

Thus, the primary fragment ions **a** and **e** at *m/z* 202 and 132, respectively, have indeed shifted to three a.m.u. higher than the corresponding ions obtained in the e.i. mass spectrum of the 1,6-anhydroheptose derivative **1**. Similarly, the secondary fragment ions **b, c, f,** and **g**, at *m/z* 158, 126, 88, and 75, respectively, have shifted to one a.m.u. higher.

The c.i. mass spectra of the 1,6-anhydroheptose compound **1** and its corresponding (trideuterio)acetylated derivative **3** showed the protonated molecular ion $[MH]^+$ at *m/z* 305 and 311, respectively. The secondary fragment ions, at *m/z* 245 and 248 (base peaks, respectively) were generated by the loss of one molecule of acetic acid and one molecule of trideuterioacetic acid from the respective, protonated molecular-ions $[MH]^+$ at *m/z* 305 and 311.

It is important to note that the acetolysis method[10] widely used for cleavage of methylated polysaccharides, which was originally introduced in order to facilitate the estimation of amino sugars, will grossly underestimate the 3,4,6-tri-*O*-substituted heptose **2**. It is interesting that the use of trifluoroacetic acid for hydrolysis adequately permits estimation of the 2,7-di-*O*-methylheptitol **2**, without excessive formation of the 1,6-anhydroheptose derivative **1**.

We therefore propose that the formation of the 1,6-anhydroheptose derivative **1** occurs during the hydrolysis of the permethylated core-oligosaccharide with acid, and that its rate of formation depends on the hydrolytic conditions used. Chaby and Szabó[12] reported the formation of 1,6-anhydro-L-*glycero*-D-*manno*-heptopyranose during acid hydrolysis and methanolysis of a non-methylated, L-*glycero*-D-*manno*-heptose-containing lipopolysaccharide from *Bordetella pertussis*[12], and it is obvious that a similar phenomenon occurs in methylated polysaccharides containing a heptose residue substituted at O-3, O-4, and O-6.

EXPERIMENTAL

Bacterial culture. — *A. hydrophila* Chemotype I, strain No. SJ-55; Chemotype II, strain No. SJ-26; Chemotype III, strain No. SJ-25; *A. salmonicida*, strain No. SJ-15; and *V. ordalii*, strain No. SJ-42 were obtained from the Northwest Atlantic Fisheries Centre collection.

Extraction of lipopolysaccharide. — All strains were grown in Trypticase Soy Broth (25 L) (Baltimore Biological Laboratories) for 24 h at 25° with aeration at 12 L/min, as previously described[4]. Lipopolysaccharide was extracted from the wet cell cake by the aqueous phenol method of Westphal and Jann[13]. Production of core oligosaccharide, devoid of *O*-polysaccharide and lipid A, was achieved by hydrolysis of the LPS in 1% aqueous acetic acid for 90 min at 100°, followed by gel chromatography on Sephadex G50, as previously described[4].

Methylation analysis. — The core oligosaccharides were methylated by the Hakomori method[14], and then purified on Sephadex LH-20 (Pharmacia). The

methylated oligosaccharides were hydrolyzed with 2M trifluoroacetic acid for 12 h at 100°, by the acetolysis method[10] or by formic acid–sulfuric acid[11]. The resulting partially methylated sugars were reduced with sodium borohydride, the alditols acetylated, and the alditol acetate derivatives analyzed by g.c.–m.s.

(Trideuterio)acetylation was performed by treating the resulting permethylated alditols with trideuterioacetic anhydride in pyridine for 1 h at 100° (ref. 11).

Gas-liquid chromatography–mass spectrometry. — Gas-liquid chromatography of the partially methylated alditol acetates was performed on packed columns (183 cm × 2 mm i.d.) of 1.5% of Silar 7 CP on Gas Chrom Q (100–120 mesh) in a Perkin–Elmer model 3920 gas chromatograph, with a hydrogen-flame detector and a temperature program starting at 180° for 32 min, and then increasing to 270° at 8°/min (final temperature held for 64 min). G.l.c. was also performed on a 25 m, WCOT CP-Sil 5 (0.25 μm film thickness) capillary column (Chrompack, The Netherlands) using the same temperature program.

Combined g.l.c.–e.i. mass spectrometry was performed on a Hewlett–Packard model 5980A GC/MS instrument controlled by a 5934A data system, with a membrane separator, a source temperature of 160°, and an ionizing voltage of 70 eV, using the same temperature program. Combined g.l.c.–c.i. (isobutane) mass spectrometry was performed on a Hewlett–Packard model 5985 GC/MS/DS instrument equipped with a dual e.i./c.i. source. Spectra were recorded at a source pressure of 120 Pa, using isobutane as the reagent gas and the carrier, a source temperature of 150°, and an ionization voltage of 230 eV; and using a packed glass column of 2% of OV-101 on Chromosorb W(H.P.) (80–100 mesh) with the same temperature program.

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