Carbohydrate Research, 123 (1983) 117–122
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

HYDROLYTIC RELEASE, AND IDENTIFICATION BY G.L.C.–M.S., OF 3-DEOXY-D-*manno*-2-OCTULOSONIC ACID IN THE LIPOPOLYSACCHARIDES ISOLATED FROM BACTERIA OF THE *Vibrionaceae*

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(Received May 18th, 1983; accepted for publication, June 13th, 1983)

ABSTRACT

The identification of the peracetylated methyl glycosides of 3-deoxy-D-*manno*-2-octulosonic acid (KDO) methyl esters was achieved by g.l.c.–m.s. These peracetylated methyl glycoside methyl esters were obtained from fully acetylated lipopolysaccharides and core oligosaccharides of representative strains of the *Vibrionaceae* family by the following sequence of mild reactions: acetolysis, methanolysis, and acetylation. KDO was shown to be present in all of the lipopolysaccharides (LPS), a result in direct contrast to the generally accepted view of the absence of this compound in LPS from this family of bacteria.

INTRODUCTION

3-Deoxy-D-*manno*-2-octulosonic acid (KDO) is regarded as a ubiquitous constituent of the inner core-region of the lipopolysaccharide of Gram-negative bacteria[1], and it is also contained in several exopolysaccharides from *Escherichia coli*[2] and from *Neisseria*[3] species.

In almost all lipopolysaccharides investigated to date, the inner core-region consists of an oligosaccharide of the core specific sugars L-*glycero*-D-*manno*-heptose and KDO, each forming a trisaccharide[4]. The reducing end of the KDO trisaccharide, glycosidically attaches the polysaccharide, in a relative acid-labile, β-D-(2→3) ketosidic linkage, to the nonreducing 2-amino-2-deoxy-D-glucose unit[4] of the lipid A. The exact linkages and the connecting fashion of the constituent units of the KDO trisaccharide have not yet been completely established, and may vary for different families of bacteria[1].

The incorporation of KDO appears to be a vital step in lipopolysaccharide biosynthesis and in the growth of most bacteria[5,6]. KDO does, however, appear to be absent from the lipopolysaccharides of a few Gram-negative bacteria, such as some species of *Pseudomonas*[7], *Bacteroides*[8], *Anabaene variabilis*[9], and most of the *Vibrionaceae* family, including all of its constituting genera[10], *i.e.*, *Vibrio, Aeromonas, Photobacterium*, and *Lucibacterium*.

0008-6215/83/$ 03.00

In the present investigation, we report the presence of KDO in the *Vibrionaceae* family, and a rapid, g.l.c.–m.s. method for the identification of this component, using very mildly acidic conditions.

RESULTS AND DISCUSSION

The estimation of KDO during structural investigations of the bacterial lipopolysaccharides is based on the lability of the ketosidic linkage to mild hydrolysis with acid. Treatment of any enterobacterial lipopolysaccharide with dilute acid generally cleaves the ketosidic linkage between KDO and lipid A, with concomitant release of free KDO, which may be estimated by the colorimetric methods currently used, namely, the thiobarbiturate test[11], the semicarbazide reaction[12], and the diphenylamine test[13]. Although these methods can be used only for the estimation of free KDO (that is, neither glycosidically bound nor substituted), none of them are suitable for determination of the true content of KDO from bacterial lipopolysaccharide[14].

Considerable differences in the stability of the glycosidic linkage of KDO have been observed, which may be due to the nature and the position of the KDO substituents. In effect, free 3-deoxy-D-*manno*-2-octulosonic acid is only released by mild hydrolysis with acid when it is present either in a lateral position or substituted by a similar residue[15,16]. However, in LPS where the KDO units are substituted by phosphonoaminoethanol and phosphorylated groups, or in the LPS containing two distinct KDO residues[17,18], the release of free KDO can only be achieved by hydrolysis with strong acid (M HCl at 100°), causing considerable degradation of the KDO molecule (which may lead to erroneous results during quantitative estimation).

Identification, and determination, of KDO by g.l.c. has been infrequently reported in the literature, and it is not a method currently in vogue. Free KDO has been analyzed by g.l.c. as the alditol acetates[19], or by g.l.c.–m.s. as the peracetylated methyl esters[20]. Identification of KDO from LPS by g.l.c.–m.s. may be achieved by methanolysis and per(trimethylsilyl)ation[21] or by methanolysis and per(trifluoroacetyl)ation[22].

It was reasonable to expect that methanolysis of KDO-containing lipopolysaccharides with 2M methanolic HCl during 16 h at 85°, followed by acetylation, would give peracetylated methyl glycosides readily identifiable by g.l.c.–m.s. In effect, methanolysis of the lipopolysaccharide of *Vibrio ordalli* with 2M HCl for 16 h at 85° yielded identifiable peaks, but, unfortunately, overlapping peaks were observed for the peracetylated methyl glycosides of 3-deoxy-D-*manno*-2-octulosonic acid methyl esters and the peracetylated methyl heptosides. The mass spectra of these mixtures gave fragmentation patterns that contained the respective, diagnostic primary-ions *m/z* 375 ($M^+ - 59$) for the KDO, and *m/z* 403 ($M^{+\cdot} - 31$) for the heptose, together with their derived, secondary ions (see Fig. 1), which accorded with the results of Charon and Szabó[20]. Decomposition products, arising from considerable degradation of the free KDO released during methanolysis at 85°, were also noticed.

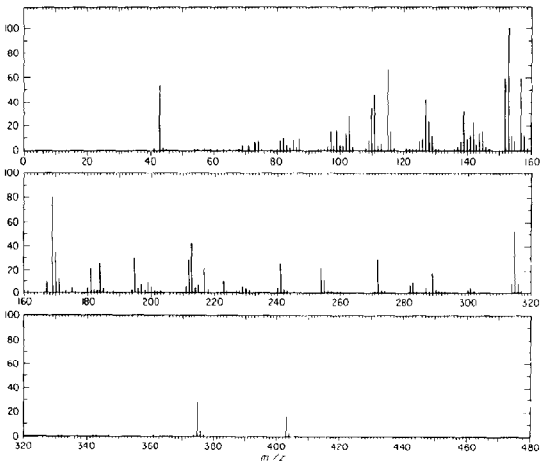

Fig. 1. Mass spectrum of the mixture of peracetylated methyl glycosides of 3-deoxy-D-*manno*-2-octulosonic acid methyl esters and peracetylated methyl D-heptosides.

To avoid the formation of the degradation products, we now propose a simple, mild procedure to release KDO, and identify it by g.l.c.–m.s. This modification consists of the complete peracetylation of the LPS or the core oligosaccharide with 1:1:1 acetic anhydride–pyridine–*N,N*-dimethylformamide, followed by acetolysis with 1:1:0.01 acetic acid–acetic anhydride–sulfuric acid for 16 h at room temperature, and methanolysis with 2M methanolic HCl for 16 h at room temperature, followed by neutralization and acetylation. G.l.c.–m.s. of the peracetylated methyl glycosides is conducted under temperature-programmed conditions, and several well resolved peaks, identified by their mass-spectral fragmentation-patterns, are obtained for the various forms of each monosaccharide.

This modification was applied to lipopolysaccharides isolated from representative strains of several members of the *Vibrionaceae* family, from which KDO had been claimed to be absent[10]. Thus, when acetolysis and methanolysis were successively applied to the peracetylated lipopolysaccharides of *Aeromonas hydrophila* Chemotype I and of *Vibrio ordalli*, and to the peracetylated core oligosaccharides of *Aeromonas salmonicida*, *Aeromonas hydrophila* Chemotype I and Chemotype II, and *Vibrio ordalli*, KDO was released, and was identified as the peracetylated methyl glycosides of 3-deoxy-D-*manno*-2-octulosonic acid methyl esters by g.l.c.–m.s.[20].

The mass spectra of the peracetylated methyl glycosides of KDO obtained from the aforementioned LPS and core oligosaccharides were identical.

Important features associated with this method of release, and identification,

of KDO from LPS and core oligosaccharides are that all of the reactions are simple operations conducted under very mild conditions; g.l.c.–m.s. of the peracetylated methyl glycosides affords a rapid method for the identification of the methyl esters of KDO; and glycosidic linkages of amino sugars, normally resistant to hydrolysis, are cleaved. The fragile constituents of LPS, such as dideoxy sugars, which are normally degraded during methanolysis with 2M methanolic HCl during 16 h at 85°, would survive the gentle treatment described herein.

Although phosphorus is known to be a structural component of the lipid A of lipopolysaccharides, recent work has shown that this element is absent from the core oligosaccharide[23] of *Aeromonas hydrophila* Chemotype II. Unpublished work from our laboratory indicates the same lack of phosphate in the core oligosaccharides of *Aeromonas salmonicida*, *Aeromonas hydrophila* Chemotype I, and *Vibrio ordalli*. Thus, it is not possible that substitution of KDO by phosphate is the cause for the apparent lack of KDO erroneously indicated on using the conventional hydrolytic and colorimetric methods of analysis. Determination of KDO by the thiobarbiturate-based, colorimetric assays seemed to indicate that it was essentially absent from the aforementioned LPS and core oligosaccharides representative of the *Vibrionaceae* family. The absence of measurable free KDO clearly indicates the lack of a lateral KDO residue and of a nonphosphorylated KDO disaccharide residue. Our results suggest that, in a molecule of the LPS of the *Vibrionaceae* family, there is indeed at least one molecule of KDO. The cleavage of these LPS with mild acid to yield lipid A and a polysaccharide containing one KDO residue as an intrinsic part would also indicate a direct linkage between the KDO and lipid A. Strittmatter *et al.*[24] have established the presence of a single KDO unit in the LPS of *Rhodopseudomonas sphaeroides*, ATCC 17023, and Brade *et al.*[25] demonstrated the presence of one KDO residue in a molecule of *Vibrio cholerae* lipopolysaccharide by using strong hydrolysis conditions.

It is important to note that, contrary to a published report[10], the absence of KDO as supposedly established by the thiobarbiturate-based, colorimetric assay method cannot be used as a taxonomical characteristic for the classification of the *Vibrionaceae*.

EXPERIMENTAL

Chemicals. — Analytical-grade solvents were distilled before use. Methanolic HCl (2M) was prepared by adding acetyl chloride (15.6 g) to a cooled solution of dry methanol (184 mL). The acetolysis mixture was prepared by adding conc. sulfuric acid (1 mL) to a cooled solution of glacial acetic acid (100 mL) and acetic anhydride (100 mL).

Bacterial culture. — *Aeromonas hydrophila* Chemotype I, strain No. SJ-55, and Chemotype II, strain No. SJ-26, and *Aeromonas salmonicida*, strain No. SJ-15, were obtained from the Northwest Atlantic Fisheries Centre collection; *Vibrio ordalli*, strain No. SJ-42, was isolated from experimental sockeye salmon, and was supplied by Dr. T. P. Evelyn, Pacific Biological Station, Nanaimo, B.C.

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[26]. Lipopolysaccharide was extracted from the wet cell-cake by the aqueous phenol method of Westphal and Jann[27]. Production of core oligosaccharide devoid of O-polysaccharide and lipid A was achieved by hydrolysis of the LPS in 1% aqueous acetic acid during 90 min at 100°, followed by gel chromatography on Sephadex G50, as previously described[26].

Chromatographic methods. — Thin-layer chromatography of the methyl glycosides was conducted on plates precoated with Merck silica gel 60F-254 in a solvent system of 10:3:1 chloroform–methanol–water or 20:5:1 chloroform–methanol–acetic acid; detection was achieved by charring after spraying with 5% sulfuric acid in ethanol.

High-voltage paper-electrophoresis was conducted for 90 min in a Shandon flat-bed electrophoresis-apparatus, using a buffer of 5:2:43 (v/v) pyridine–acetic acid–water, at pH 5.4, 2 kV, and 170 mA. Free KDO was detected by spraying with the thiobarbiturate reagent, or the alkaline silver nitrate reagent.

Gas–liquid chromatography of the peracetylated methyl glycosides was performed in columns (183 × 2 mm i.d.) packed with 1.5% of Silar 7 CP on Gas Chrom Q (100–120 mesh) in a Perkin–Elmer model 3920 gas chromatograph equipped with a hydrogen-flame detector and a model 3380A electronic integrator, employing a temperature program starting at 180° for 32 min, and then increased to 270° at 8°/min (final temperature held for 64 min). G.l.c. was also performed in a capillary column (25 m) of W.C.O.T. CP-Sil 5 (0.25 mm film thickness; Chrompack, the Netherlands), using the same temperature program.

Combined g.l.c.–m.s. was performed in a Hewlett–Packard model 5981A 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.

Peracetylation of the lipopolysaccharide or core oligosaccharide. — The polysaccharide (20 mg) was dissolved in *N,N*-dimethylformamide (1.5 mL), and acetic anhydride (1.5 mL) and pyridine (1.5 mL) were added. The solution was stirred for 16 h at room temperature, diluted with water (10 mL), and extracted with chloroform (10 mL). The extract was dried (anhydrous sodium sulfate), and evaporated under diminished pressure. The residue was reacetylated with 1:1 acetic anhydride–pyridine during 1 h at 100°, and the solution was evaporated to dryness to afford the peracetylated product.

Acetolysis. — A solution of the peracetylated product (10 mg) in the acetolysis mixture (1 mL) was kept for 16 h at room temperature, poured into crushed ice (10 mL), and extracted with chloroform (10 mL). The extract was successively washed with a saturated solution of sodium hydrogencarbonate (5 mL) and water (5 mL), dried (anhydrous sodium sulfate), and evaporated to dryness, to afford a residue composed of the peracetylated glycose components of the LPS or core oligosaccharides.

Methanolysis and acetylation. — A solution of the foregoing residue in 2M methanolic HCl was stirred for 6 h at room temperature, and evaporated under diminished pressure. The residue was redissolved in methanol, and the solution reevaporated (to remove traces of HCl). T.l.c. of the residue containing the methyl glycosides obtained from the different polysaccharides indicated the presence of the methyl glycosides of 3-deoxy-D-*manno*-2-octulosonic acid methyl esters[20]. Hydrolysis of these methyl glycosides with 125mM sulfuric acid for 8 min at 100°, followed by high-voltage electrophoresis, indicated the presence of free KDO. The residue (containing the methyl glycosides) was acetylated with 1:1 acetic anhydride–pyridine during 1 h at 100°, and the solution was evaporated to dryness.

ACKNOWLEDGMENTS

The authors express their thanks to Dr. R. Roy (N.R.C., Ottawa) for constructive criticism, to M. J. Squires for recording the mass spectra, and to Mr. H. J. Hodder for excellent technical assistance.

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