O-Methyl Sugars in Lipopolysaccharides of Rhodospirillaceae

IDENTIFICATION OF 3-O-METHYL-D-MANNOSE IN *RHODOPSEUDOMONAS VIRIDIS* AND OF 4-O-METHYL-D-XYLOSE AND 3-O-METHYL-6-DEOXY-D-TALOSE IN *RHODOPSEUDOMONAS PALUSTRIS* RESPECTIVELY

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1. This paper deals with the identification of three *O*-methyl sugars in lipopolysaccharides isolated from strains of the Gram-negative photosynthetic family Rhodospirillaceae. In addition to the previously described 3-*O*-methyl-L-xylose, a second *O*-methyl sugar was encountered in the lipopolysaccharide of *Rhodopseudomonas viridis* F, namely 3-*O*-methyl-D-mannose. The lipopolysaccharides of two strains of *Rhodopseudomonas palustris* (strain 1e5 and 8/1) contain two *O*-methylsugars, 4-*O*-methyl-D-xylose and 3-*O*-methyl-6-deoxy-D-talose (D-acovenose). 4-*O*-Methyl-D-xylose, but not 3-*O*-methyl-6-deoxy-D-talose, could be identified in the lipopolysaccharides of the strains K/1 and 2/2 of the same species. 2. The *O*-methyl sugars described in this communication were isolated by paper chromatography and identified by g.l.c., paper chromatography, high-voltage electrophoresis and mass spectrometry. Besides the genuine sugars, their alditol acetates and their demethylated (parental) forms were investigated. Optical rotation measurements and, in one case, enzymic reactions were used to establish the optical configuration of the sugars under investigation.

Extensive immunochemical investigations on lipopolysaccharides of Gram-negative bacteria have revealed that the chemical make-up of the lipopolysaccharides (*O*-antigens) is responsible for the serological *O*-specificity of bacterial strains (for a review see Lüderitz *et al.*, 1966; Lüderitz *et al.*, 1971). A close correlation was found between the qualitative sugar composition of lipopolysaccharide, the so-called chemotype, and the serological grouping of the strains, the so-called serotype, as described in the Kauffmann-White scheme of the *Salmonella* species (Kauffmann, 1966). However, the serological typing is of a higher degree of specificity than the chemical grouping, which means that quite a few different serotypes may belong to the same chemotype (Lüderitz *et al.*, 1966). The diversity of serotypes is paralleled by a diversity of sugar components and their linkages in the *O*-specific chains of the lipopolysaccharide. Many sugars with unusual structures are found only in bacterial *O*-antigens, e.g. some 3,6-dideoxyhexoses, 4-deoxyhexoses and certain amino sugars (Lüderitz *et al.*, 1971; Jann & Jann, 1967; Volk *et al.*, 1970). In many cases these unusual sugars play the determinative role in the serological specifi-

city of the respective strains, and have thus been designated as 'immunodominant sugars' (Lüderitz *et al.*, 1966).

Most previous chemical analyses of the lipopolysaccharide sugars have been carried out with enterobacterial lipopolysaccharides, other Gram-negative families being only occasionally investigated. We are presently investigating the sugar and fatty acid composition of the Gram-negative photosynthetic family Rhodospirillaceae. Chemotyping of the lipopolysaccharides of strains of this family will certainly provide a basis for a serological scheme. The chemical characterization of *O*-methyl sugars isolated from lipopolysaccharides of this family has been reported, namely 3-*O*-methyl-L-rhamnose in lipopolysaccharide of *Rhodopseudomonas capsulata* 37b4 (Weckesser *et al.*, 1970) and 3-*O*-methyl-L-xylose in lipopolysaccharide of *Rhodopseudomonas viridis* F (Weckesser *et al.*, 1971). In the present communication the identification of three additional *O*-methyl sugars from lipopolysaccharides of strains of Rhodospirillaceae is described. 4-*O*-Methyl-D-xylose and 3-*O*-methyl-6-deoxy-D-talose, not hitherto reported as lipopolysaccharide constituents, could be isolated

from lipopolysaccharides of *Rhodopseudomonas palustris* strains, whereas 3-*O*-methyl-D-mannose occurs in *R. viridis* F.

Materials and Methods

Bacterial strains and lipopolysaccharides

R. viridis (strain F) and *R. palustris* (strains 1e5, 8/1, 2/2 and K/1) were obtained from the strain collection of the Institute of Biology II, Lehrstuhl für Mikrobiologie der Universität Freiburg i. Br., Germany. Bacteria were cultivated under anaerobic conditions and in the light (2000lx) in a malate (0.25%)–yeast extract (0.1%) medium (Drews, 1965). The cells were harvested at the end of the exponential growth phase, and extracted by the hot phenol–water procedure of Westphal *et al.* (1952). The lipopolysaccharide of *R. viridis* was present in good yield in the aqueous phase. The lipopolysaccharides of *R. palustris*, however, were found in the phenol phase, and only a trace amount could be isolated from the aqueous layer. The lipopolysaccharide-containing phases were freed from phenol by extensive dialysis against running tap water. The insoluble material was removed by centrifugation at 2500g for 30 min and the supernatants were freeze-dried. The lipopolysaccharide was dissolved in water and purified by sedimentation in a Spinco ultracentrifuge (three times at 105 000g for 4 h).

Reference sugars

Authentic standards of 3-*O*-methyl-D-mannose, 3-*O*-methyl-6-deoxy-L-talose (L-acovenose) and 2-*O*-methyl-D-xylose were very kindly provided by Dr. R. W. Wheat, Durham (N.C., U.S.A.), Professor Dr. T. Reichstein, Basel (Switzerland), and Dr. Ch. Pedersen, Lyngby (Denmark).

Hydrolysis of lipopolysaccharides and chromatographic procedures

Lipopolysaccharides were hydrolysed with 0.5M-H₂SO₄ at 100°C for 4 h in sealed glass tubes and then neutralized with a saturated solution of Ba(OH)₂. Analytical and preparative paper chromatography was carried out in pyridine–butan-1-ol–water (4:6:3, by vol.). For high-voltage electrophoresis the vertical chamber system of Kickhöfen & Warth (1968), and a borate buffer of pH 10.4 (Kaufmann *et al.*, 1967), were used. Tetra-*O*-methylglucose provided a marker for endosmosis. Sugars were stained either with aniline hydrogen phthalate (Partridge, 1949) or by the method of Trevelyan *et al.* (1950). G.l.c. was carried out with alditol acetate derivatives of the respective sugars (Sawardeker *et al.*, 1967) in a Varian Aerograph model 1520B fitted with an ECNSS-M glass column at 155–165°C. Xylose was added to the

hydrolysates as an internal standard before reduction and acetylation.

Mass spectrometry

Because all three unknown sugars could be obtained by paper chromatography in satisfactory purity, the direct inlet system was used to insert the alditol acetates into the mass spectrometer (Perkin–Elmer 270B, Honevewell Visicorder 3508). The reduction of the sugars was done with NaB²H₄ in ²H₂O to have a marker for C-1. Mass spectra were usually taken at an electron energy of 70 eV and an ionization current of 100 µA.

Demethylation

The isolated *O*-methyl sugars were demethylated in amounts of 1–2 mg with boron tribromide by the method of McOmie *et al.* (1968).

Oxidation with D-glucose oxidase

Xylose, obtained by demethylation and authentic standards of D- and L-xylose were incubated in amounts of 0.1 µmol with glucose oxidase from *Penicillium* in the presence of peroxidase (0.5 mg of glucose oxidase and 0.04 mg of peroxidase in 100 µl of 0.1 M-sodium phosphate buffer, pH 7.0). The enzymes were purchased from Boehringer G.m.b.H., Mannheim (Germany). After incubation at 25°C for 12 h the lactones formed by the enzymic treatment were converted into D-xylonic acid by adjusting the pH to 9.0 with 0.1 M-NaOH. High-voltage electrophoresis in pyridine–acetate buffer (pyridine–acetic acid–water, 10:4:86, by vol.), pH 5.3, was used to separate xylonic acid from uncharged material.

Results

Identification of 3-*O*-methyl-D-mannose

The presence of mannose, galactose, glucose and 3-*O*-methyl-L-xylose in the neutral sugar fraction of the lipopolysaccharide of *R. viridis* F has been reported (Weckesser *et al.*, 1971). An additional sugar was observed, which migrates like rhamnose on paper chromatography in the solvent system used. However, unlike rhamnose, this sugar shows an intense yellow fluorescence in u.v. light after staining with aniline hydrogen phthalate. It stains very poorly with AgNO₃–NaOH, whereas rhamnose reduces AgNO₃ readily. G.l.c. of the neutral sugar fraction (as alditol acetate derivatives) revealed the absence of rhamnose and the presence of an unknown peak which forms a shoulder at the ascending part of the hexa-acetylmannitol peak.

It was possible to isolate the unknown sugar by preparative paper chromatography. Mass spectro-

metry of the sugar in its deuterium-reduced and peracetylated form was chosen for identification of the sugar class to which the unknown sugar belongs. The main fragments were obtained at m/e 43, 88, 99, 130, 190, 201 and 261. The comparison of the fragments expected for a deuterium-reduced and peracetylated 3-*O*-methylhexose (Fig. 1) with the fragments obtained, indicates that the sugar under investigation is a 3-*O*-methylhexose. Primary fragments at m/e 190 and 261 are partly converted into secondary fragments by subsequent elimination of acetic acid ($M = 60$) or keten ($M = 42$) or of both.

Demethylation of the isolated sugar produced a hexose that was identified as mannose by paper chromatography and g.l.c. A standard of authentic 3-*O*-methyl-D-mannose and the unknown sugar from *R. viridis* had the same chromatographic and staining properties in paper chromatography and their alditol acetates gave a single peak on the ECNSS-M column on g.l.c.

The optical rotation of the isolated sugar (2mg) showed $[\alpha]_D^{25} = +4^\circ$. Aspinall & Zweifel (1957) found $[\alpha]_D^{25} = +14^\circ \rightarrow +5^\circ$ for authentic 3-*O*-methyl-D-mannose. From all the data it is concluded that the unknown sugar in the lipopolysaccharide from *R. viridis* F is 3-*O*-methyl-D-mannose. This sugar makes up about 2–3% (dry wt.) of the lipopolysaccharide material.

Identification of 4-*O*-methyl-D-xylose

A comparative analysis of the lipopolysaccharides from several strains of *R. palustris* revealed that strains K/1 and 2/2 contain galactose, glucose and L-glycero-D-mannoheptose in the neutral sugar fraction, the strains 8/1 and 1e5 contain, in addition, xylose and 6-deoxytalose (J. Weckesser, G. Drews, I. Fromme & H. Mayer, unpublished results). All these strains contain an additional sugar, which moves quickly on paper chromatography ($R_{xylose} = 1.26$) in the solvent system used and which stains red with aniline hydrogen phthalate and reacts also with $AgNO_3 - NaOH$. Strain K/1 was chosen for the isolation of the sugar since the lipopolysaccharide of this strain contains no rhamnose and no 6-deoxytalose, both of which show migration properties similar to the unknown sugar on paper chromatography.

The sugar was isolated by preparative paper chromatography. A sample was reduced with NaB^2H_4 (in 2H_2O) and peracetylated for characterization by mass spectrometry. The spectrum showed, *inter alia*, main fragments at m/e 43, 117 and 262. The latter two fragments are those expected for a 4-*O*-methylpentose alditol acetate derivative (Fig. 2a). An authentic standard of deuterium-reduced and peracetylated 2-*O*-methylxylose, which forms the same alditol acetate, produced major fragments at m/e 43, 118 and 261 (Fig. 2b). The difference in the fragment patterns is as predicted from the difference in the deuterium labelling of the respective fragments. Both samples had the largest fragment at m/e 276 (about 1% of the intensity of the base peak, m/e 43), which arises from *M*-59 and which is usually the largest fragment observed with alditol acetates (Kochetkov & Chizhov, 1966). The mass-spectrometric analysis thus allows the classification of the unknown sugar as a 4-*O*-methylpentose.

The parental pentose was gained by demethylation of the isolated sugar by BBr_3 . Paper chromatography and g.l.c. revealed that xylose was formed. It is therefore obvious that the sugar under examination is 4-*O*-methylxylose. A suitable comparative standard for 4-*O*-methylxylose is 2-*O*-methyl-D-xylose, since the two sugars form the same alditol acetate. After mixing, the alditol acetates of authentic 2-*O*-methyl-D-xylose and of the unknown sugar gave only a single peak at the ECNSS-M column with a relative retention time $T = 0.54$ respectively to xylitol pentaacetate. Paper chromatography of authentic 2-*O*- and 3-*O*-methyl-D-xylose and of 4-*O*-methylxylose from *R. palustris* in pyridine–butan-1-ol–water (4:6:3, by vol.) allows the discrimination of the three mono-*O*-methylethers of xylose, the R_{xylose} values being 1.32 for 2-*O*-methyl-D-xylose, 1.36 for 3-*O*-methyl-D-xylose and 1.26 for 4-*O*-methyl-D-xylose respectively. The optical rotation of 4-*O*-methyl-D-xylose was reported by Hough & Jones (1952) to be $[\alpha]_D^{25} = +9$ (H_2O). Owing to the small amount of the sugar available, only a small positive rotation could be measured, indicating the D configuration. Confirmation was gained by identification of the optical configuration of the sugar in its demethylated (parental) form by enzymic reaction. In this experiment the product of demethylation, xylose, was incubated with glucose oxidase in the presence of peroxidase. The xylonolactone formed was identified by high-voltage electrophoresis after ring-opening by treatment with alkali. Since the enzyme oxidizes only D-xylose and not L-xylose, the D configuration of the xylose and its 4-*O*-methyl ether must be considered proven (see also Weckesser *et al.*, 1971).

Fig. 1. Fragmentation scheme of a 2H_2 -reduced and peracetylated 3-*O*-methylhexose

Fig. 2. Fragmentation scheme of a $^2\text{H}_2$ -reduced and peracetylated (a) 4-*O*-methylpentose and (b) 2-*O*-methylpentose

The above data show that the unknown sugar present in the lipopolysaccharides of four strains of *R. palustris* is 4-*O*-methyl-D-xylose. The sugar is present in about 1 % dry wt. of the respective lipopolysaccharides.

Identification of 3-*O*-methyl-6-deoxy-D-talose (D-acovenose)

Two strains of *R. palustris* (1e5 and 8/1) were found to contain in large amounts (more than 5 % of the lipopolysaccharide dry wt.) an additional sugar. It moves very quickly on paper chromatograms ($R_{\text{xylose}} = 1.63$) in pyridine-butan-1-ol-water, and stains brown with aniline hydrogen phthalate, but only weakly with alkaline AgNO_3 . Because of its high R_F , the sugar could easily be obtained in a pure form by paper chromatography.

Fragmentation at 70 eV in mass spectrometry afforded main fragments at m/e 43, 88, 101, 130, 143, 190 and 203 and in low intensity (less than 1 % of the base peak, m/e 43) at m/e 290. These fragments were recently observed for deuterium-reduced and peracetylated L-acofriose (Weckesser *et al.*, 1970), which belongs to the class of 3-*O*-methyl-6-deoxyhexoses. The differentiation of all stereoisomeric 3-*O*-methyl-6-deoxyhexoses and of their parental 6-deoxyhexoses by high-voltage electrophoretic separation in borate buffer of pH 10.4 and by paper chromatography has been described by Kaufmann *et al.* (1967). We observed the following migration values in high-voltage electrophoretograms with the same borate buffer: $M_{\text{rhamnose}} = 1.06$ for the unknown sugar, and 1.10 for its demethylated form. These two values can only be compared with the values obtained by Kaufmann *et al.* (1967) for 3-*O*-methyl-6-deoxytalose ($M_{\text{rhamnose}} = 1.03$) and for 6-deoxytalose ($M_{\text{rhamnose}} = 1.13$) respectively. All other sugar pairs (*O*-methyl sugar and its parental demethylated form) differ markedly at least in one value. An authentic standard of 3-*O*-methyl-6-deoxy-D-talose (D-acovenose) was available and showed the same paper-chromatographic and staining properties as the sugar under investigation. The respective alditol acetates gave only a single peak after mixing at the ECNSS-M

column at $T = 0.31$ (respectively to xylitol pentaacetate). In addition, the demethylated sugar and authentic 6-deoxy-D-talose also afforded a single peak at $T = 0.41$ in their reduced and peracetylated form.

The optical rotation of 3-*O*-methyl-6-deoxytalose, isolated from the lipopolysaccharide of *R. palustris*, was measured to be positive ($[\alpha]_D^{25} = +16.5^\circ$). L-Acovenose isolated from plant material was reported by Reichstein & Weiss (1962) to have $[\alpha]_D^{25} = -19.4$. From all these data it can be concluded that the unknown sugar isolated from lipopolysaccharides of *R. palustris* strains 1e5 and 8/1 is 3-*O*-methyl-6-deoxy-D-talose (D-acovenose).

Discussion

Recently, the occurrence of two unusual *O*-methyl sugars in *O*-antigens of bacteria belonging to the Gram-negative photosynthetic family Rhodospirillaceae has been described. The sugars were characterized as 3-*O*-methyl-L-rhamnose (L-acofriose) in lipopolysaccharide of *R. capsulata* 37b4 (Weckesser *et al.*, 1970) and as 3-*O*-methyl-L-xylose in lipopolysaccharide of *R. viridis* F (Weckesser *et al.*, 1971). The comparative analysis of further *O*-antigens of strains of this family of bacteria revealed the presence of three additional *O*-methyl sugars. Besides 3-*O*-methyl-L-xylose the lipopolysaccharide of *R. viridis* F contains a second *O*-methyl sugar, namely 3-*O*-methyl-D-mannose. Two *O*-methyl sugars also occur together in the lipopolysaccharides of *R. palustris* strains 1e5 and 8/1. They have been identified as 4-*O*-methyl-D-xylose and 3-*O*-methyl-6-deoxy-D-talose (D-acovenose). The lipopolysaccharides of the strains K/1 and 2/2 contain 4-*O*-methyl-D-xylose, but not D-acovenose.

O-Methyl sugars are fairly widespread in the plant kingdom, especially in cardiac glycosides (Reichstein & Weiss, 1962), but only a few reports deal with their occurrence in *O*-antigens of Gram-negative bacteria. Besides the already-mentioned occurrence of L-acofriose and 3-*O*-methyl-L-xylose in *O*-antigens of *Rhodopseudomonas* strains, L-acofriose was recently reported in *Klebsiella* K73:O10 (Björndal *et al.*, 1970)

and 3-*O*-methyl-D-mannose in *Escherichia coli* O8 and *Klebsiella* O5 (Nimmich, 1970). 3-*O*-Methyl-D-mannose also occurs in polysaccharides isolated from *Streptomyces griseus* (Candy & Baddiley, 1966) and from the fungus *Coccidioides immitis* (Porter *et al.*, 1971). 3-*O*-Methyl-D-xylose was identified in the cell-wall lipopolysaccharide of *Myxococcus fulvus* (Weckesser *et al.*, 1971). However, 4-*O*-methyl-D-xylose and D-acovenose have not previously been encountered in bacteria and, to the best of our knowledge, not from any other natural source. 3-*O*-Methyl-6-deoxy-L-talose (L-acovenose) was recently isolated from mycoside C₂ of *Mycobacterium avium* (Voiland *et al.*, 1971).

The *O*-methyl sugars, previously found in the *O*-antigens of Gram-negative bacteria, except the 3-*O*-methyl-L-xylose in *R. viridis* F, occur only in small amounts compared to the unsubstituted parental sugar. The lipopolysaccharide of *R. capsulata* 37b4 contains about 1% L-acofriose and 13.8% L-rhamnose (Weckesser *et al.*, 1972). L-Acofriose of *Klebsiella* O10 occupies only the terminal repeating units of the *O*-specific chains, whereas the other repeating units contain exclusively L-rhamnose (Björndal *et al.*, 1970). These findings may imply that methylation is a signal either for chain initiation or for termination in the biosynthesis of the *O*-specific chains. D-Acovenose of *R. palustris* strains 8/1 and 1e5, however, is a main constituent of the *O*-antigen and its parent sugar (6-deoxy-D-talose) occurs only in very small amounts in these strains. Likewise, the lipopolysaccharides of *R. palustris* strains 2/2 and K/1 possess 4-*O*-methyl-D-xylose, but not the unsubstituted D-xylose. Structural studies are needed to determine whether the *O*-methyl sugars occupy terminal positions in the *O*-specific chains of these strains, similar to the likewise lipophilic 3,6-dideoxyhexoses in Enterobacteriaceae lipopolysaccharides (Lüderitz *et al.*, 1966). Terminal sugars in branched polysaccharides are of eminent importance for the serological specificity of these substances. Serological studies will show if strains with same *O*-methyl sugars are cross-reacting despite the fact that they may belong to different chemotypes. Recently eight strains of *R. palustris* (42, 15, 11/1, 1f2, 1a1, 16, V₂ and E₅₁) were found to contain 6-*O*-methylglucosamine (H. Mayer, K. Framberg & J. Weckesser, unpublished work). Thus strains of the family Rhodospirillaceae are able to methylate pentoses (D- and L-xylose), hexoses (D-mannose), 6-deoxyhexoses (L-rhamnose and 6-deoxy-D-talose) and hexosamines (glucosamine). Nothing is known about the biosynthesis of the *O*-methyl sugars in the Rhodospirillaceae. Ferguson & Ballou (1970), however, reported the occurrence of a specific methyltransferase from *Mycobacterium phlei*, which methylates suitable α -glycosidically linked glucose polysaccharides in the 6-position in the presence of activated methionine. Since this strain

synthesizes a lipopolysaccharide of a similar structure, this finding is a strong indication that methyltransferases occur in bacteria, which are able to methylate preformed oligo- or poly-saccharides.

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