# Structural Studies on the M-Antigen Produced by *Salmonella typhimurium* 395MRO-M

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The M-antigen is a capsular polysaccharide, produced by different *Enterobacteriaceae*, grown under appropriate conditions. Earlier studies on this antigen are summarised in Ref. 1. The present communication reports structural studies on an acidic, extracellular polysaccharide produced by *Salmonella typhimurium* 395MRO−M, which seems to be similar to the M-antigens previously investigated.

On acid hydrolysis, the polysaccharide, which showed $[\alpha]_{578}$ +24° (water), yielded D-glucose, D-galactose, L-fucose, and D-glucuronic acid. No O-acetyl groups were present, as demonstrated by the absence of IR absorption in the 1735−1750 cm$^{-1}$ region. The polysaccharide was methylated by the Hakomori

procedure.[2] Part of the product was hydrolysed and the mixture of methylated sugars in the hydrolysate analysed, as their alditol acetates, by GLC[3]-mass spectrometry.[4] The other part was subjected to methanolysis and reduction with lithium aluminium hydride, and was then treated as above. The results of these analyses are given in Table 1.

In addition to the five methylated sugars found in both analyses, 2,3-di-O-methyl-D-glucose was obtained from the methylated and carboxyl-reduced polysaccharide, and consequently derives from D-glucuronic acid residues. The methylation analysis further indicates that the polysaccharide contains D-glucose, D-galactose, L-fucose and D-glucuronic acid residues in the relative proportions 1:2:2:1, and is probably composed of hexasaccharide repeating units.

The presence of 2-O-methyl-L-fucose and 2,6-di-O-methyl-D-galactose indicates a highly branched structure but, intriguingly, there were no methyl ethers indicative of terminal sugar residues. This suggested that some sugar residues in the polysaccharide were substituted with a group that was stable during the methylation but was removed during the subsequent acid hydrolysis. An acetal or ketal grouping would meet these requirements. Pyruvic acid, ketalically linked to D-galactose [5] or D-glucose [6] residues in the 4- and 6-positions has been found in other polysaccharides.

The polymeric material obtained after treatment of the polysaccharide with 0.025 M sulphuric acid for 1 h at 100° was subjected to methylation analysis, without reduction of carboxyl groups. The results are given in Table 2. The appearance of 2,3,4,6-tetra-O-methyl-D-galactose and the corresponding

*Table 1.* Methyl ethers from the hydrolysates of methylated polysaccharide.

| Sugars | $T^a$ | %$^b$ | %$^c$ |
|---|---|---|---|
| 2,3-Di-O-methyl-L-fucose | 1.18 | 19 | 15 |
| 2-O-Methyl-L-fucose | 1.67 | 23 | 19 |
| 2,4,6-Tri-O-methyl-D-glucose | 1.95 | 22 | 19 |
| 2,4,6-Tri-O-methyl-D-galactose | 2.28 | 18 | 16 |
| 2,6-Di-O-methyl-D-galactose | 3.65 | 18 | 18 |
| 2,3-Di-O-methyl-D-glucose | 5.39 | — | 13 |

*$^a$ Retention times of the corresponding alditol acetates on the ECNSS-M column relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol.

*$^b$ Not carboxyl-reduced.

*$^c$ Carboxyl-reduced.

*Table 2.* Methyl ethers from the hydrolysate of methylated, partially degraded polysaccharide.

| Sugars | $T^a$ | %$^b$ |
|---|---|---|
| 2,3,4-Tri-O-methyl-L-fucose | 0.65 | 2 |
| 2,3,4,6-Tetra-O-methyl-D-glucose | 1.00 | 4 |
| 2,3-Di-O-methyl-L-fucose | 1.18 | 17 |
| 2,3,4,6-Tetra-O-methyl-D-galactose | 1.25 | 10 |
| 2-O-Methyl-L-fucose | 1.67 | 24 |
| 2,4,6-Tri-O-methyl-D-glucose | 1.95 | 18 |
| 2,4,6-Tri-O-methyl-D-galactose | 2.28 | 16 |
| 2,6-Di-O-methyl-D-galactose | 3.65 | 9 |

For $^a$ and $^b$, see Table 1.

decrease of 2,6-di-O-methyl-D-galactose demonstrate that one of the D-galactose residues is terminal and contains an acid labile substituent, blocking the 3- and 4-positions. This substituent is not pyruvic acid, as an ether extract of the hydrolysate gave negative colour reactions [7] for that substance. The only methylated sugar derived from a branching point is 2-O-methyl-L-fucose, and the terminal D-galactose residue is consequently linked to a L-fucose residue, in the 3- or 4-position.

The polysaccharide was subjected to the same mild hydrolytic treatment as above, but in a sealed tube and in the presence of 2,4-dinitrophenylhydrazine. Extraction of the mixture with chloroform yielded a product which was crystallised from ethanol. 0.8 mg of crystals were obtained from 40 mg of polysaccharide. TLC of this product (silica gel, isopropyl ether) gave two spots, with the same mobilities as the 2,4-dinitrophenylhydrazones of acetaldehyde and acetone, respectively. Also by GLC (3 % OV 1 methyl silicone on Chromosorb W) two components, in the proportions 8:1, were revealed with the same retention times as the reference substances mentioned above. The identity of these components with the 2,4-dinitrophenylhydrazones of acetaldehyde and acetone was confirmed by mass spectrometry. GLC of the hydrolysate [direct injection of the aqueous solution on a Carbowax column (10 % Carbowax polyglycol on Chromosorb W)] also demonstrated the presence of acetaldehyde and acetone. None of these components could be detected when authentic pyruvic acid was subjected to the same hydrolytic treatment.

The 3- and 4-positions of the terminal D-galactose residue are therefore protected by acetal formation with acetaldehyde. The smaller amount of acetone found may indicate that some of the galactose residues are protected, in the same positions, as an isopropylidene derivative, but the acetone may also be an artefact. This question remains to be investigated. This is, to our knowledge, the first observation of an ethylidene acetal in Nature.

The polysaccharide was hydrolysed overnight with 0.25 M sulphuric acid at 100°. An aldobiouronic acid, $[\alpha]_{578}$ +1° (water), was isolated from the hydrolysate, and on further hydrolysis yielded D-glucuronic acid and D-galactose. This, in conjunction with the methylation analysis, demonstrates that the aldobiouronic acid is 3-O-β-D-glucuronopyranosyl-D-galactose, previously isolated from other M-antigens.[1]

Two minor components, 2,3,4-tri-O-methyl-L-fucose and 2,3,4,6-tetra-O-methyl-D-glucose, were obtained in the methylation analysis of the partially hydrolysed polysaccharide but not in the methylation analysis of the original polysaccharide. It seems reasonable to assume that the formation of these ethers is due to hydrolysis of a small portion of the L-fucosidic linkages, which are the most readily hydrolysed glycosidic linkages in this polysaccharide. The results therefore indicate that a disubstituted L-fucose residue is linked to a second L-fucose residue, which in its turn, is linked to a D-glucose residue.

The structure given in Fig. 1 is proposed for the repeating unit of the polysaccharide. In this structure, the sugar residues are pyranosidic, as was demonstrated by methylation analysis, except for the disubstituted L-fucose residue, whose ring structure could not be determined by this method. However, if this L-fucose residue were furanosidic, it would have been more readily hydrolysed than was actually observed. The structure must be regarded as tentative only. In addition to the alternative arrangements shown in Fig. 1, the assignment of other features must await further studies.
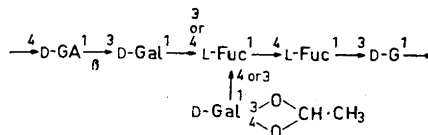


*Fig. 1.* Proposed structure for the repeating units of the *Salmonella typhimurium* 395MRO-M (M-antigen).

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# Note on the Crystal Structure of MoO₃·2H₂O

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The main features of the crystal structure of the yellow molybdic acid $MoO_3 \cdot 2H_2O$ were reported in 1950 by Lindqvist.[1] It was stated that the structure is built up of $MoO_6$ octahedra which are joined by corners to form approximately square nets of composition $MoO_4$. The layers are stacked on top of each other, the remaining oxygen atoms occupying positions between the layers. In a later X-ray study [2] systems of weak reflections were observed which correspond to a much larger unit cell than the one of the structure described above. No attempt was made at that time to solve the details of the superstructure.

The present investigation, suggested by Professor Lindqvist, was undertaken in order to elucidate the role of the hydrogen atoms in the bonding system of the structure. Three-dimensional X-ray data have been collected with a PALLRED diffractometer in equi-inclination setting using LiF monochromatized $MoK\alpha$ radiation ($2\theta \leq 100°$).

It was observed that reflections $hkl$ ($h+l \neq 2n$) (in the notation of Ref. 2) of significant intensities were very few and also of very low intensities. Special investigations of these reflections by slight missetting of the inclination angle ($\mu$) demonstrated that these are actually caused by accidental multiple diffraction. Systematically occurring multiple diffraction was avoided by rotating the crystal around an axis parallel to [100] (in the notation of Ref. 2). The actual unit cell is thus half the one reported in Ref. 2. The relations between the new unit-cell vectors and those of the larger unit cell, the latter being primed, is:

$$\mathbf{a} = \tfrac{1}{2}(\mathbf{a}' + \mathbf{c}')$$
$$\mathbf{b} = \mathbf{b}'$$
$$\mathbf{c} = \tfrac{1}{2}(-\mathbf{a}' + \mathbf{c}')$$

The values of the cell parameters as obtained from Guinier-Hägg powder photographs taken with $CuK\alpha_1$ radiation and potassium chloride ($a = 6.2919$ Å) [3] added to the specimen as an internal standard are (20°C):

$$a = 10.474 \pm 1 \text{ Å}$$
$$b = 13.825 \pm 2 \text{ Å}$$
$$c = 10.608 \pm 1 \text{ Å}$$
$$\beta = 91.59 \pm .01°$$

The analysis of the superstructure is in progress.

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