Location of *O*-Methyl Sugars in Antigenic (Lipo-)Polysaccharides of Photosynthetic Bacteria and Cyanobacteria

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(Received 30 August 1977)

An attempt was made to localize a number of *O*-methyl sugars in lipopolysaccharides and antigenic polysaccharides isolated from photosynthetic bacteria and from cyanobacteria. Methylation analysis with [$^2\text{H}_3$]methyl iodide as methylating agent was the method of choice. One has to differentiate between (lipo-)polysaccharides having only trace amounts (less than 1% of polymer dry weight) of *O*-methyl sugars and those having them in larger amounts (more than 4% of polymer dry weight). In the former case *O*-methyl sugars occupy either non-reducing or reducing terminals. When present in larger quantities they may be present as part of each repeating unit either in chain-linked or in terminal positions or in both. A possible role of *O*-methyl sugars in biosynthesis of *O*-chains, and their contribution to the lipophilic character of the cell surface are discussed.

Application of more refined techniques [gas-liquid chromatography-mass spectrometry (g.l.c.-m.s.), mass fragmentography] to polysaccharide analysis led to the recent discovery of a number of naturally occurring *O*-methyl sugars. These lipophilic constituents are reported to occur in lipopolysaccharides (*O*-antigens) of Gram-negative bacteria (Mayer *et al.*, 1974), in mycosides of mycobacteria (MacLennan, 1962), in *O*-antigens of cyanobacteria (Weckesser *et al.*, 1974) as well as in fungal (Porter *et al.*, 1971) and algal (Fareed & Percival, 1977) polysaccharides, in plants (Reichstein & Weiss, 1962) and in haemocyanins of snails (Hall *et al.*, 1977) to name only a few.

O-Methyl sugars are especially frequent in *O*-antigens of photosynthetic prokaryotes [photosynthetic bacteria and cyanobacteria (formerly blue-green algae)] in contrast with their rare occurrence in Enterobacteriaceae. In the latter, lipophilic sugars are mostly deoxy or dideoxy sugars ('deoxy-principle' versus 'methoxy-principle') (Westphal & Lüderitz, 1960). The 3,6-dideoxy hexoses are located exclusively at the non-reducing terminal of the branched repeating units of *O*-chains (Lüderitz *et al.*, 1971) and, are thus of eminent importance for the serological specificity of the strains.

Only a few reports deal with the location of *O*-methyl sugars in lipopolysaccharide. When present as minor constituents they seem to occupy predominantly non-reducing terminals, as demonstrated with 3-*O*-methyl-L-rhamnose in *Klebsiella* K73:010 (Björndal *et al.*, 1970) and 3-*O*-methyl-D-mannose in *Klebsiella* 05 (K75:05 and K57:05) (Lindberg *et al.*,

1972). 6-*O*-Methyl-*N*-acetyl-D-glucosamine in *Rhodopseudomonas palustris*, however, being present in a larger amount, is probably chain-linked.

O-Methyl sugars in lipopolysaccharides of photosynthetic prokaryotes are either present as main constituents or, in other cases, in only trace amounts. In the present paper a comparative study on the location of a number of *O*-methyl sugars in various *O*-antigens of photosynthetic bacteria and cyanobacteria as well as in two low-molecular-weight polysaccharides of *Rhodopseudomonas gelatinosa* is reported.

Materials and Methods

Cultivation of photosynthetic bacteria and cyanobacteria and the isolation of lipopolysaccharide and polysaccharide are described elsewhere (Weckesser *et al.*, 1970, 1974). Sugar analysis of polysaccharide hydrolysates was carried out, after reduction with either NaBH_4 or NaB^2H_4 (in $^2\text{H}_2\text{O}$) and a following peracetylation, by g.l.c. of the resulting mixture of alditol acetates. All evaporations were done under reduced pressure and below 40°C. For g.l.c.-m.s. a Finnigan quadrupole model 3200 system coupled to a model 6000 data and graphic output system was used (Finnigan Corp., Sunnyvale, CA, U.S.A.). Separation of partially methylated alditol acetates was performed on U-shaped glass columns (0.2 cm × 152 cm) filled with ECNSS-M phase (3% on Gaschrom Q, 100–200 mesh; Applied Science Laboratories, State College, PA, U.S.A.) at a column temperature of 170°C. The spectra were taken at 70 eV

in the mass range of 35–400 with an integration time of 7 ms/mass.

Polysaccharides were permethylated as described by Stellner *et al.* (1973) by using methylsulphonyl carbanion/[$^2\text{H}_3$]methyl iodide in dimethyl sulphoxide. The degree of methylation was ascertained from the absence of a significant hydroxyl absorption ($3600\text{--}3200\text{cm}^{-1}$) by i.r. spectrometry.

Results and Discussion

Previous reports on the occurrence of *O*-methyl sugars in *O*-antigens of photosynthetic prokaryotes concerned only their chemical identification. Little or nothing was known about their location in the individual *O*-antigens. The only report available is based on a periodate-oxidation study on the lipopolysaccharide of *Rhodopseudomonas palustris* (Mayer *et al.*, 1974). 6-*O*-Methyl-*N*-acetyl-D-glucosamine, present in a fairly large amount in the *O*-antigen of strains of the chemotype III (Weckesser *et al.*, 1973), is periodate-stable and therefore probably chain-linked and might thus well be part of the repeating units of the *O*-chain. In the present study on the location of a number of additional *O*-methyl sugars in photosynthetic prokaryotes, we used methylation analysis with [$^2\text{H}_3$]methyl iodide to discriminate between naturally occurring CH_3 groups and artificially introduced C^2H_3 groups. The resulting partially methylated ethers, obtained on hydrolysis of the methylated products, were examined as alditol acetate derivatives by g.l.c.–m.s. and by the more sensitive single-ion-mass fragmentography. For clear-cut interpretations of the mass spectra, the partially methylated sugars were reduced to alditols with NaB^2H_4 (in $^2\text{H}_2\text{O}$) before their acetylation.

It is obvious from Table 1 that the *O*-methyl sugars under investigation can be separated into two distinct groups, depending on their amount relative to the heteropolymer dry weight. Those from group I are

present in amounts of less than 1%, whereas *O*-methyl sugars of group II represent main constituents (amounting to more than 4%) of the respective heteropolymers.

O-Methyl sugars of group I

3-*O*-Methyl-D-mannose and 4-*O*-methyl-D-mannose in *Anacystis nidulans* KM. Small amounts of 3-*O*-methyl-D-mannose and of 4-*O*-methyl-D-mannose were identified in the lipopolysaccharide of the cyanobacterium *A. nidulans* (Katz *et al.*, 1977). The polysaccharide moiety was found to consist predominantly of a polymannan with 1→3 and 1→4 linkages in a gross ratio of 3:1. Lindberg *et al.* (1972) have reported a (1→2)- and (1→3)-linked mannan chain containing a small amount of 3-*O*-methyl-mannose in the lipopolysaccharide of *Klebsiella* 05 (K75:05 and K57:05). By methylation analysis they could further show that the non-reducing ends of *O*-chains are terminated by 3-*O*-methyl-D-mannose. In the light of this report, it was decided to see whether the *O*-methyl ethers of *A. nidulans* occupy similar terminal positions in this *O*-antigen. 3-*O*-Methyl-D-mannose was indeed one of the terminal sugars (Table 2). The mass spectrum showed among others prominent primary fragments at *m/e* 165 and 211 in accordance with the expected fragmentation of 2,4,6-tri-*O*-[$^2\text{H}_3$]methyl-3-*O*-methyl-mannitol 1,5-diacetate (I). For 4-*O*-methyl-D-mannose, however, a 2,6-di-*O*-[$^2\text{H}_3$]methyl-4-*O*-methyl-mannitol 1,3,5-triacetate (II) was obtained as indicated by primary fragments at *m/e* 164 and 237 (see also Table 2). Mass-fragmentographic analysis of ions of *m/e* 165 (I) and 164 (II) were of comparable intensities. Since the alditol acetates of the two sugars, without permethylation of the polymer, also yielded a 1:1 ratio of the fragments *m/e* 190/191 and *m/e* 261/262, it is evident that 3- and 4-*O*-methyl-D-mannoses occur in different linkages (terminal for 3-*O*-methyl-D-mannose and 1→3-linked for 4-*O*-methyl-D-mannose).

Table 1. *O*-Methyl sugars and their parental sugars in lipopolysaccharides (and polysaccharides) of photosynthetic bacteria and cyanobacteria

Additional sugar constituents (neutral and amino sugars) are present in each polymer listed. Their identification and approximate quantification are given in previous papers (for references see the text).

	<i>O</i> -Methyl sugar (% of polymer dry wt.)	Parental sugar (% of polymer dry wt.)
Group I Lipopolysaccharide		
<i>A. nidulans</i> KM	3-Mono- <i>O</i> -Me-D-Man (<1) 4-Mono- <i>O</i> -Me-D-Man (<1)	D-Mannose (40) D-Mannose (40)
<i>R. capsulata</i> 37b4	3-Mono- <i>O</i> -Me-L-Rha (<1)	L-Rhamnose (14)
Group II		
<i>A. variabilis</i>	3-Mono- <i>O</i> -Me-L-Rha (20)	L-Rhamnose (20)
<i>C. vinosum</i>	3-Mono- <i>O</i> -Me-D-Rib (6)	D-Ribose (4)
Polysaccharide		
<i>R. gelatinosa</i> P18f3.1	2-Mono- <i>O</i> -Me-D-Gal (4)	D-Galactose (12)
<i>R. gelatinosa</i> 2150	2,3-Di- <i>O</i> -Me-D-Gal (12)	D-Galactose (24)

Table 2. Methyl ethers obtained on methylation analysis, and location of the naturally occurring O-methyl sugars in the respective polymers

The numbers in parentheses refer to the naturally occurring O-methyl groups. Abbreviation: 2,3,4,6-Man, 2,3,4,6-tetra-O-methyl-D-mannose etc. Only neutral sugars are listed. See also the legend to Table 1.

Source	Naturally occurring O-methyl ether		Other neutral sugars, after permethylation
	Identification	Location	Identification
Group I			
Lipopolysaccharide			
<i>A. nidulans</i> KM	2,(3),4,6-Tetra-O-Me-Man 2,(4),6-Tri-O-Me-Man	Terminal Chain-linked	2,3,4,6-Man/Glc; 2,4,6-Man; 2,3,6-Man
<i>R. capsulata</i> 37b4	2,(3),4-Tri-O-Me-Rha	Terminal	3,4-Rha; 2,3,4,6-Glc; 2,3,4,6-Gal; 2,3,6-Glc
Group II			
<i>A. variabilis</i>	2,(3),4-Tri-O- and (3),4-Di-O-Me-Rha	Terminal and chain-linked	2,3,4-Rha; 3,4-Rha; 2,3,4,6-Glc/Man; 2,3,4,6-Gal; 3,4,6-Glc; 2,3,6-Glc; 3,4-Glc
<i>C. vinosum</i>	2,(3)-Di-O-Me-Rib	Chain-linked	2,3,4-Rib; 3,5-Rib; 2,4-Ara; 2,3,6-Glc; 2,3-Glc
Polysaccharide			
<i>R. gelatinosa</i> P18f3.1	(2),4,6-Tri-O-Me-Gal	Chain-linked	2,4-Fuc; 2,3,6-Man; 2,3,6-Gal; 2,3,6-Glc
<i>R. gelatinosa</i> 2150	(2),(3),4,6-Tetra-O-Me-Gal	Terminal	2,3,4,6-Man; 2,3,6-Man; 2,3,6-Gal; 4,6-Gal; 2,3-Man

As the O-specific chains of lipopolysaccharides are generally composed of oligosaccharide repeating units, it is improbable that these O-methyl ethers of mannose, present in very small amounts, are part of the repeating units. Thus, from the identification of 3-O-methylmannose at the non-reducing terminal it can be assumed that the 4-O-methylmannose may occupy the reducing end of the polymer chain, which is unbranched (Table 2). Attempts to identify the latter O-methyl ether by reducing the polymer with NaBH₄, however, were not successful.

If the following tentative structure is assumed for the mannan

it may be argued that the O-methyl ethers might possibly act as a start and/or stop signal in the biosynthesis of the O-chain, and thus, play a role in control of the polymer growth. Further studies are needed to investigate whether this biological methylation is really directional.

The 2,(3),4,6-tetra-O-methylmannose peak (Table 2; where parentheses indicate positions of natural O-methyl groups) contained comparable amounts of ethers derived from D-mannose and/or D-glucose besides that from 3-O-methyl-D-mannose. Thus it is obvious that in addition to 3-O-methyl-D-mannose, one of these sugars occupies other non-reducing terminals, probably indicating that unfinished or growing O-chains are present.

Lipopolysaccharides with mannan-like O-chains from *Escherichia coli* strains 08:K27⁺, 08:K27⁻ and 08:K42 were also investigated by mass-fragmentographic monitoring for the presence of O-methyl-

mannoses. 3-O-Methyl-D-mannose was found in all of them, which agrees with a report by Nimmich (1970). The absence of 4-O-methylmannose possibly indicates a biosynthetic pathway different from that in *A. nidulans*. An investigation by Flemming (1977) on the biosynthesis of mannans in *E. coli* 08 and 09 has shown that in *E. coli* 09, in contrast with the situation in *Salmonella*, the O-chain is growing at the non-reducing end. In addition, no lipid-bound intermediates were found in these strains. On the other hand, the mannan-like O-chains of *Salmonella montevideo* (group C₁) and *E. coli* 09 contained neither 3- nor 4-O-methylmannose, as no significant peaks at *m/e* 190/189 and *m/e* 261/262 were detectable. This clearly shows that, in contrast with the discussed similarities existing between the polymannose chains of *A. nidulans*, *E. coli* 08 and *Klebsiella* 05 lipopolysaccharides, not all mannan chains in O-specific polysaccharides follow the same building concept.

3-O-Methyl-L-rhamnose (L-acofriose) in *Rhodospseudomonas capsulata* 37b4. The lipopolysaccharide of the photosynthetic bacterium *R. capsulata* 37b4 (Rhodospirillaceae) has been shown to contain small amounts of L-acofriose in addition to a large quantity of L-rhamnose (Weckesser *et al.*, 1970) (Table 1). Permethylation studies revealed the L-acofriose to be at a non-reducing terminal position (Table 2). A similar situation is reported from *Klebsiella* K73:010. On the basis of methylation analysis also with [²H₃]methyl iodide, Björndal *et al.* (1970) proposed a structure for these O-chains, consisting of approximately 12 repeating units, the 3-linked L-rhamnose in the terminal repeating unit being replaced by an L-acofriose residue.

O-Methyl sugars of group II

3-O-Methyl-L-rhamnose in *Anabaena variabilis*. The lipopolysaccharide of the cyanobacterium *A. variabilis* is characterized by the presence of a large percentage of L-acofriose (Weckesser *et al.*, 1974) (Table 1). The methylation analysis clearly demonstrates that L-acofriose is located both at terminal and chain-linked positions (Table 2). This is evident from the identification of 2,4-di-O-[$^2\text{H}_3$]methyl-3-O-methyl-L-rhamnitol acetate and of 4-O-[$^2\text{H}_3$]methyl-3-O-methyl-L-rhamnitol acetate respectively, by monitoring single ions of m/e 165 and 190. The

origins of these fragments, derived from acofriose, are illustrated as follows (terminal position, III, chain-linked position, IV).

Mass-fragmentographic monitoring of the ions m/e 168 and 193 reveal also that the hydrolysate contains in addition about equal amounts of ethers derived from terminal and chain-linked L-rhamnose residues [the respective fragments of m/e 168 (V) and 193 (VI) being of comparable intensity]. The ratio of terminal to chain-linked acofriose was also about 1:1 on the basis of a comparison of the intensities of the respective fragments. As the proportions of L-

acofriose/L-rhamnose/D-glucose in the lipopolysaccharide is about 1:1:2, the simplest repeating unit conceivable would contain a minimum of four sugar residues. However, the identification of tetramethyl derivatives of glucose and/or mannose and of galactose along with other derivatives of these sugars (Table 2) suggests a branched structure for the polymer or the existence of a R-core oligosaccharide. The concomitant occurrence of L-acofriose at different locations in the same molecule is important from the point of view of biological methylation.

3-*O*-Methyl-D-ribose in *Chromatium vinosum*. 3-*O*-Methyl-D-ribose is one of the main constituents of the lipopolysaccharide of the photosynthetic bacterium *C. vinosum* (Chromatiaceae) (Hurlbert *et al.*, 1976). From the methylation analysis it is inferred that all 3-*O*-methyl-D-ribose residues are chain-linked (identification of 2-*O*-[$^2\text{H}_3$]methyl-3-*O*-methyl-D-ribitol acetate (VII). Further, from Table 2 it is evident that the terminally linked ribose as well as the chain-linked arabinose residues are pyranosidic, whereas the chain-linked ribose is furanosidic. It seems reasonable to assume that the chain-linked 3-*O*-methyl-D-ribose may also be furanosidic, although this is not deducible from the mass spectrum. The dual presence of the different ring forms of the same sugar (D-ribose) in the polymer is conspicuous.

2-*O*-Methyl-D-galactose and 2,3-di-*O*-methyl-D-galactose in *Rhodopseudomonas gelatinosa* P18f3.1 and 2150 respectively. Several strains of the photosynthetic bacterium *Rhodopseudomonas gelatinosa* (Rhodospirillaceae) form hapten-like polysaccharides (Weckesser *et al.*, 1975). Two of them contain comparably large quantities of *O*-methyl ethers of D-galactose (see Table 1), i.e. 2-*O*-methyl-D-galactose in strain P18f3.1 and 2,3-di-*O*-methyl-D-galactose in strain 2150, in addition to D-galactose and other sugars. The methylation analysis of the polysaccharide of strain P18f3.1 (Table 2) reveals unambiguously that the 2-*O*-methyl-D-galactose is exclusively chain-linked. The sugar was located as being

1,3-linked from its mass spectrum (4,6-di-*O*-[$^2\text{H}_3$]methyl-2-*O*-methylgalactitol acetate, VIII), all the expected primary and secondary fragments being observed. From the absence of any di-*O*-methyl ethers it may be inferred that the polysaccharide chains are linear.

In the polysaccharide of strain 2150 the amount of 2,3-di-*O*-methyl-D-galactose is half of that of D-galactose. It was therefore assumed that the di-*O*-methyl sugar may be part of the repeating unit. Methylation analysis showed the 2,3-di-*O*-methyl-D-galactose to be located almost exclusively at non-reducing terminals. Mass spectrometry showed prominent fragments *m/e* 118, 162, 151 (211–60), 102 (162–60), 132 (162–30) in accordance with the expected fragmentation of 4,6-di-*O*-[$^2\text{H}_3$]methyl-2,3-di-*O*-methylgalactitol acetate (IX). It is noteworthy that all of the 2,3-di-*O*-methyl-D-galactose, but only a very small amount of mannose and/or glucose residues and no D-galactose residues, occupy the terminal positions in the polysaccharide. The identification of 4,6-di-*O*-[$^2\text{H}_3$]methylgalactose and of a small amount of 2,3-di-*O*-[$^2\text{H}_3$]methylmannose (Table 2) would indicate a branched structure of the polymer, which is terminated by 2,3-di-*O*-methyl-D-galactose.

In conclusion, the foregoing results can be summarized as follows. *O*-Methyl sugars in O-antigens might be terminal-bound or chain-linked or both. When present in trace amounts, they seem to occupy exclusively the non-reducing ends of O-chains, an exception being 4-*O*-methyl-D-mannose in *A. nidulans*, which is probably linked to the reducing end. This assumption is based not only on this study but also on the data obtained by Björndal *et al.* (1970) and Lindberg *et al.* (1972) with *Klebsiella* (see above). It is tempting to speculate from this exclusive location that *O*-methyl sugars may be involved in controlling the biosynthesis of O-chains, e.g. in representing a start signal for a polymerase or in determining the length of polymer chains. *A. nidulans* is especially

useful for studying this question, in that the two different *O*-methyl ethers of D-mannose occurring in equimolar quantities, are located apparently on either end of the *O*-chains.

When *O*-methyl sugars are present as main constituents of *O*-chains, there seems to be no recognizable tendency for a favoured incorporation into terminal or into chain-linked positions of the repeating unit. Both possibilities are realized in the examples presented. Both can be found even simultaneously in the same *O*-antigen, an example being the L-acofriose in *A. variabilis*. In any case *O*-methyl sugars contribute to the lipophilic character of the cell surface, in that a 'methoxy-principle' may be realized here. They may also contribute to the serological *O*-specificity, although data are so far lacking.

No experimental data on the biosynthetic pathway of *O*-methyl sugars in photosynthetic prokaryotes are presently available. In principle, there are two possibilities, either methylation occurs at the sugar-nucleotide level or at the polymer-chain level. Both are probably realized with polysaccharides of bacteria (see Weckesser *et al.*, 1971). It may be easier to assume a methylation on the polymer level in *A. variabilis*, where L-acofriose and L-rhamnase are both terminal and chain-linked and occur in equal amounts. These problems need detailed separate studies for each polymer, considering the variation in amount and location of *O*-methyl sugars in the polymers.

We thank Mr. D. Borowiak for help with mass spectrometry, Dr. G. Schmidt for providing lipopolysaccharides of *E. coli* 08:K27⁺, 08:K27⁻ and 08:K42 and Miss S. Gogowska for skilful technical assistance. This work was partly supported by the Deutsche Forschungsgemeinschaft.

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