

## A New Method for Specific Degradation of Polysaccharides

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A new method for the specific degradation of polysaccharides is presented. It involves the preparation of a methylated polysaccharide containing a limited number of free hydroxyl groups, at defined positions, oxidation of these to carbonyl groups by ruthenium tetroxide and subsequent  $\beta$ -elimination by treatment with base. Analysis of the degraded product provides evidence concerning the sequence of sugar residues in the original polysaccharide. The utility of the method is demonstrated with two polysaccharides of known structures, the lipopolysaccharide from *Salmonella typhimurium* LT2 and the capsular polysaccharide from *Klebsiella* type 47.

It is possible to prepare methylated polysaccharides with a limited number of free hydroxyl groups, by using suitable selective procedures. Suitable methods are the acid hydrolysis of furanosidic or other acid labile linkages, sulphone degradation<sup>1</sup> or base-catalysed elimination of pyranosidouronate residues.<sup>2,3</sup> Oxidation with ruthenium tetroxide<sup>4</sup> would then give carbonyl groups, and methoxyl or glycosyl residues in  $\beta$ -positions to these would be eliminated on treatment with base. A carbonyl group at the 3-position would lead to cleavage of the glycosidic linkage. Analysis of the degraded product should give information on the sequence of sugar residues in the original polysaccharide. We have recently studied the  $\beta$ -elimination with model substances of low-molecular weight<sup>5-7</sup> and demonstrated that the elimination goes to completion. We now report the application of this degradation method to two bacterial polysaccharides of known structure.

### RESULTS AND DISCUSSION

The O-specific side chains in the lipopolysaccharide (LPS) from *Salmonella typhimurium* LT2 are composed of repeating units with the structure I. The D-glucose connected with a dotted arrow is only present in some of the repeating units. Some of the abequose residues are acetylated at C-2. This structure has been demonstrated by chemical<sup>8,9</sup> and biosynthetic<sup>10</sup> studies. The LPS was fully methylated by the Hakomori procedure.<sup>11</sup> Part of the product was

hydrolysed and the mixture of partially methylated sugars was analysed by GLC-MS<sup>12</sup> (Table 1, column A). This analysis was in accordance with that of previous investigations.<sup>8</sup> The methylated LPS was subjected to mild acid hydrolysis and the polysaccharide, free from lipid and components of low-molecular weight, was recovered by gel filtration. Part of this product was methylated using trideuteriomethyl iodide, hydrolysed and analysed (Table 1, column B). Most of the 4,6-di-*O*-methyl-D-mannose now appeared as 3,4,6-tri-*O*-methyl-D-mannose, with a trideuteriomethyl group at the 3-position, demonstrating that most of the abequose residues had been hydrolysed off. The only free hydroxyl groups in the recovered polysaccharide are consequently those at the 3-position of the mannose residue. The product was oxidised with ruthenium tetroxide, thus transforming the secondary alcohol groups into keto groups.

The oxidised polysaccharide was treated with sodium ethoxide in ethanol-dichloromethane and part of the product was hydrolysed and analysed (Table 1, column C). This analysis indicated that the oxidation was virtually complete, as the percentage of 4,6-di-*O*-methyl-D-mannose was approximately the same as after trideuteriomethylation of the mild acid degraded polysaccharide (Table 1, column B). Another portion of the degraded material was trideuteriomethylated and hydrolysed. Analysis of this material (Table 1, column D) showed that most of the 2,3-di-*O*-methyl-L-rhamnose now appeared as 4-*O*-trideuteriomethyl-2,3-di-*O*-methyl-L-rhamnose. This demonstrates that, in

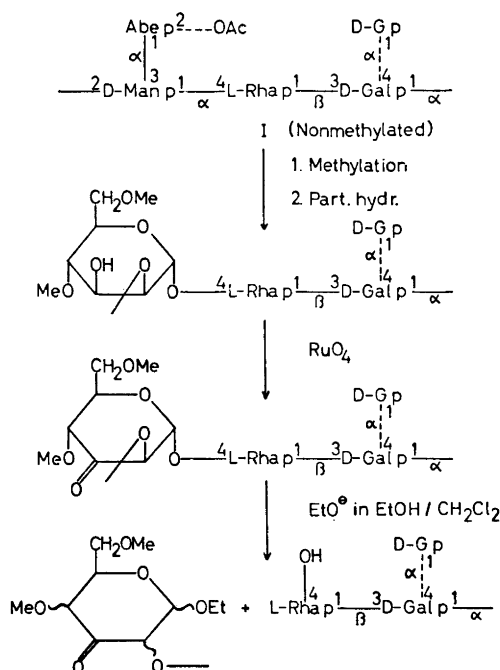
Table 1. Methylation analyses of the original and modified *S. typhimurium* LT2 polysaccharides.

Methylated sugar <sup>a</sup>	<i>T</i> <sup>b</sup>	Mol % <sup>c</sup>			
		A	B	C	D
2,4-Abe <sup>d</sup>	0.32	8	1	1	< 1
2,3,4 CD <sub>3</sub> -Rha <sup>d</sup>	0.46	—	—	—	32
2,3-Rha <sup>e</sup>	0.98	20	22	34	2
2,3,4,6-G <sup>e</sup>	1.00	14	17	20	33
2,3,4,6-Gal	1.25	2	3	2	3
3 CD <sub>3</sub> , 4,6-Man	1.95	—	23	—	—
2,4,6-Man	2.09	2	—	—	—
2,4,6-Gal	2.28	12	14	15	10
3,4,6-Gal	2.50	2	1	2	1
4,6-Man	3.29	22	2	3	< 1
2,6-Gal	3.62	15	15	21	17
3,6-G	4.30	1	1	1	1
2,4-G	5.10	3	1	2	1

<sup>a</sup> 2,4-Abe = 2,4-di-*O*-methyl-abequose, 2,3,4 CD<sub>3</sub>-Rha = 2,3-di-*O*-methyl-4-mono-*O*-trideuterio-methyl-L-rhamnose, etc. <sup>b</sup> Retention time of the corresponding alditol acetate relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on an ECNSS-M column at 170°. <sup>c</sup> A: original polysaccharide; B: methylated, acid degraded and trideuteriomethylated polysaccharide (see text); C: methylated, acid degraded, oxidised and alkali treated polysaccharide (see text); D: methylated, acid degraded, oxidised, alkali treated and trideuteriomethylated polysaccharide (see text).

<sup>d</sup> Part of this volatile ether and its corresponding alditol acetate was probably lost during the work up procedure. <sup>e</sup> These components were separated on an OV-225 SCOT column, on which they showed *T* 0.92 and 1.00, respectively.

the original polysaccharide, the D-mannose residue was linked to the L-rhamnose at position 4. The stoichiometry of the latter analysis (Table 1, column D) is not very good. This may be due to non-sugar components and to losses of volatile L-rhamnose derivatives. This, however, does not affect the interpretation of the results, which are in agreement with previous results,<sup>10</sup> obtained by other methods. The different steps in the degradation are depicted in Scheme 1. It is assumed, in this scheme, that all positions in the sugar residues are methylated unless otherwise indicated.



Scheme 1.

The capsular polysaccharide elaborated by *Klebsiella* type 47 is composed of tetrasaccharide repeating units, the structure of which (II) has recently been determined.<sup>13</sup> On treatment of the fully methylated polysaccharide with base followed by mild acid hydrolysis, the side chains are eliminated and a linear, methylated polysaccharide with free hydroxyls at position 3 in the L-rhamnose residues can be recovered.<sup>2</sup> Methylation analysis of the original and degraded polysaccharide (Table 2, column A and B) demonstrated that the degradation was essentially complete. The degraded polysaccharide was oxidised with ruthenium tetroxide and treated with sodium ethoxide in ethanol-dichloromethane. Part of the product was hydrolysed and analysed (Table 2, column C). The presence of some 2-O-methyl-L-rhamnose in the hydrolysate of this material indicates that some 20 % of the L-rhamnose

Table 2. Methylation analyses of the original and modified *Klebsiella* type 47 polysaccharides.

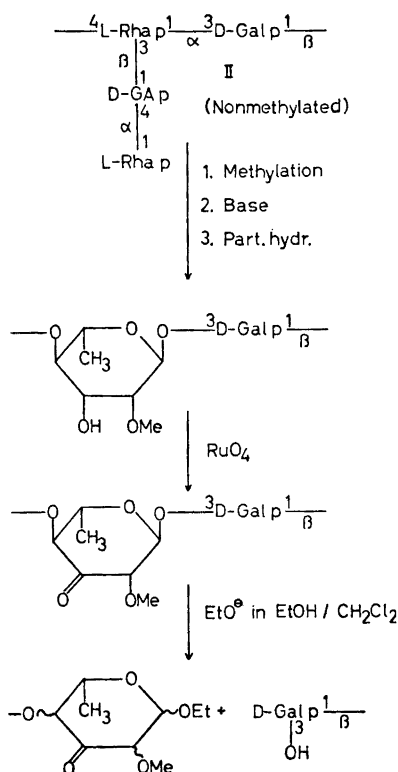
Methylated sugar <sup>a</sup>	<i>T</i> <sup>b</sup>	Mol % <sup>c</sup>			
		A	B	C	D
2,3,4-Rha <sup>d</sup>	0.46	19	3	4	1
2,3 CD <sub>3</sub> -Rha	0.98	—	51	—	—
2,3 Et-Rha	0.92	—	—	—	11
2,3 Et, 4,6-Gal	1.23	—	—	—	77
2-Rha	1.52	32	2	17	—
2,4,6-Gal	2.28	27	44	80	11
2,3-G	5.39	22	—	—	—

<sup>a</sup> See Table 1, note *a*. <sup>b</sup> See Table 1, note *b*. <sup>c</sup> A: original polysaccharide, carboxyl-reduced after methylation; B: methylated polysaccharide, degraded at the uronic acid residue and tri-deuteriomethylated (see text and Ref. 2); C: methylated, degraded, oxidised and alkali treated polysaccharide (see text); D: methylated, degraded, oxidised, alkali treated and ethylated polysaccharide (see text). <sup>d</sup> See Table 1, note *d*.

residues were not oxidised. On prolonged oxidation, some methoxyls were converted into formate ester groups. Another portion of the product was ethylated, hydrolysed and analysed (Table 2, column D). The presence of 3-*O*-ethyl-2-*O*-methyl-L-rhamnose, and a comparable amount of 2,4,6-tri-*O*-methyl-D-galactose, supports the assumption that some L-rhamnose residues had not been oxidised. The main product, however, was 3-*O*-ethyl-2,4,6-tri-*O*-methyl-D-galactose, demonstrating that the branching L-rhamnose residues are linked to the 3-position of the D-galactose residues in the original polysaccharide. The consecutive degradations depicted in Scheme 2, leaving essentially only the D-galactose residue intact, afford chemical evidence for the presence of a repeating unit in this polysaccharide. It is assumed, in this scheme, that all positions in the sugar residues are methylated unless otherwise indicated. Similar evidence for other capsular polysaccharides has previously been obtained by biosynthetic studies<sup>14</sup> and by hydrolysis with phage-induced enzymes.<sup>15,16</sup>

In the examples discussed above, a free hydroxyl was generated at C-3 in a sugar residue of a polysaccharide. The glycosidic linkage of that residue was then cleaved by oxidation, followed by base-catalysed elimination. Secondary alcoholic hydroxyls may, however, also be liberated in 2- or 4-positions in glycopyranosides. By the same sequence of reactions, the substituent at C-4 or C-2, respectively, would be eliminated. If a methylated sugar residue is eliminated, the reducing sugar thus formed can be subjected to a second elimination,<sup>17</sup> thus providing further structural evidence. Additional information may be obtained by degradation of unsaturated carbonyl sugar by mild acid hydrolysis with concomitant release of substituents other than those in the  $\beta$ -position to the carbonyl group.<sup>18</sup>

The oxidation with ruthenium tetroxide was performed in a two-phase system, with the methylated polysaccharide in dichloromethane and ruthenium dioxide-periodate in water.<sup>4</sup> The latter system generates ruthenium tetra-



Scheme 2.

oxide, which dissolved in the organic phase. During the oxidation, black ruthenium dioxide is formed. When the rate of oxidation decreases, both phases turn yellow, as almost all ruthenium is present as ruthenium tetraoxide. Prolonged oxidation should be avoided as methoxyl groups may be oxidised into formate ester groups.<sup>18</sup> We have also found that acetylated  $\beta$ -glucopyranosides may be oxidised into 5-hexosulosonates,<sup>19</sup> as has been observed with chromic trioxide in acetic acid<sup>20</sup> and with ozone.<sup>21</sup>

The two-phase oxidation has another advantage as it prevents the formation of ruthenium dioxide on to which the polysaccharide may be adsorbed, thereby preventing complete oxidation.

The presented degradation method permits the step-wise elimination of sugar residues in a methylated polysaccharide, thus providing a method which, in combination with methylation analysis, should be valuable for sequencing large structural entities.

## EXPERIMENTAL

*General methods.* Concentrations were performed under diminished pressure, at bath temperatures not exceeding 40°. For GLC, a Perkin-Elmer 900 instrument, fitted with flame-ionisation detectors, was used. Separations were performed on (a) glass-columns (190 × 0.15 cm) containing 3 % ECNSS-M on Gas Chrom Q (100–120 mesh) at 170°, (b) OV-225 SCOT columns (15 m × 0.5 mm) at 190°. For quantitative evaluations of the GLC, a Hewlett Packard 3370 B integrator was used. For GLC–MS a Perkin-Elmer 270 instrument and the same columns as above were used.

Sequential degradation of the LPS from *S. typhimurium* LT 2

*Methylation.* LPS<sup>8</sup> (60 mg) in dimethyl sulphoxide (20 ml) was treated with 2 M methylsulphonyl anion (20 ml) under nitrogen. The resulting solution was agitated ultrasonically for 30 min and then kept for 4 h at room temperature. Methyl iodide (10 ml) was then added dropwise with external cooling. The turbid solution was agitated ultrasonically for 30 min, giving a clear solution. Methyl iodide was then distilled off and the solution was dialysed against running tap water overnight and then against distilled water overnight. The resulting suspension was freeze-dried giving methylated LPS (73 mg). A sample (3 mg) was hydrolysed, transformed into alditol acetates and analysed by GLC–MS<sup>12</sup> (Table 1, column A).

*Partial acid hydrolysis.* Methylated LPS (70 mg) was treated with 50 % aqueous acetic acid (90 ml) for 14 h on a boiling water bath. The solution was evaporated to dryness and the product was fractionated on a Sephadex LH 20 column (28 × 3 cm) using chloroform-acetone (3:1) as irrigant. The separation was monitored polarimetrically. The modified polysaccharide (30 mg) was eluted with the void volume. Part of the product was trideuteriomethylated and analysed as described earlier (Table 1, column B). The partially methylated alditol acetate with a trideuteriomethoxyl group gave a mass spectrum in which fragments containing this group were shifted by three mass units.

*Oxidation.* Methylated partially hydrolysed polysaccharide (10 mg) in dichloromethane (3 ml) was treated with 0.08 M ruthenium tetroxide in dichloromethane (8 ml). After addition of a saturated aqueous solution of sodium metaperiodate the reaction mixture was shaken vigorously until the solution became yellow (≈ 15 min). The aqueous layer was separated and the organic phase was washed with water (4 × 5 ml) and evaporated to dryness.

*Alkaline degradation.* The oxidised material from above was dissolved in dichloromethane (4 ml) and ethanolic 1 M sodium ethoxide (2 ml) was added. The reaction mixture was kept at room temperature for 30 min and then evaporated. The material was divided into two portions. One was hydrolysed and the sugars were analysed as before (Table 1, column C). The other portion was reetherified using trideuteriomethyl iodide, hydrolysed and analysed as before (Table 1, column D).

Sequential degradation of the capsular polysaccharide from *Klebsiella* type 47

*Methylation and elimination of the uronosylic residue and isolation of the degraded polysaccharide* was performed as earlier described<sup>2</sup> (Table 2, column A and B).

*Oxidation* of a 10 mg sample of methylated, degraded polysaccharide was performed as described above.

*Alkaline degradation.* The oxidised material was dissolved in dichloromethane (4 ml) and ethanolic 1 M sodium ethoxide (2 ml) for 30 min, neutralised with acetic acid and evaporated. The material was divided into two portions. One was hydrolysed and the sugars analysed as before (Table 2, column C). The other portion was reetherified using ethyl iodide, hydrolysed and analysed as before (Table 2, column D). The partially methylated and ethylated alditol acetate gave a mass spectrum in which fragments containing an ethoxy group were shifted fourteen mass units.

*Acknowledgements.* The skilled technical assistance of Mrs. Jana Cederstrand and Miss Birgitta Sundberg is acknowledged. The authors are indebted to *Statens Naturvetenskapliga Forskningsråd* for financial support, to Pharmacia AB for a fellowship to one of us (L.K.) and to Professor Bengt Lindberg for his interest.

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Received July 13, 1973.