Carbohydrate Research, 149 (1986) 279–291
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

METHYLATION ANALYSIS OF THE HEPTOSE/3-DEOXY-D-*manno*-2-OCTULOSONIC ACID REGION (INNER CORE) OF THE LIPOPOLYSACCHARIDE FROM *Salmonella minnesota* ROUGH MUTANTS*

ANGELIKA TACKEN, ERNST T. RIETSCHEL, AND HELMUT BRADE**

Forschungsinstitut Borstel, Institut für Experimentelle Biologie und Medizin, Parkallee 1-40, D-2061 Borstel (Federal Republic of Germany)

(Received September 18th, 1985; accepted for publication, November 20th, 1985)

ABSTRACT

A modified methylation analysis is described which allows the elucidation of the structure of the inner core region [heptose/3-deoxy-D-*manno*-2-octulosonic acid (KDO)] of enterobacterial lipopolysaccharides (LPS) of *Salmonella minnesota* rough mutants (Re, strain R595; and Rd_2P^-, strain R4). Methylation, carboxyl-reduction, remethylation, hydrolysis, carbonyl-reduction, and acetylation of the Re-mutant LPS yielded the 2,6-di-*O*-acetyl and 2,4,6-tri-*O*-acetyl derivatives of partially methylated 3-deoxyoctitol in equimolar amounts, indicating the presence of a terminal and a 4-linked pyranosidic KDO residue. For Rd_2P^- LPS, the hydrolysis step involved 0.1M trifluoroacetic acid at 100° for 1 h which cleaved ketosidic linkages, and the final products included the foregoing acetyl derivatives in the molar ratio of 1:0.2 and a partially methylated and acetylated 3-deoxyoctitol derivative which was substituted at O-5 by a methylated heptopyranosyl residue. Trideuteriomethylation of the latter product followed by methanolysis and acetylation gave 5-*O*-acetyl-3-deoxy-1,7,8-tri-*O*-methyl-2,4,6-tri-*O*-trideuteriomethyl-D-*glycero*-D-*talo*/*galacto*-octitol and 1,5-di-*O*-acetyl-2,3,4,6,7-penta-*O*-methyl-L-*glycero*-D-*manno*-heptitol. These results prove the presence of a (2→4)-linked KDO disaccharide in Re LPS and show that the core region of Rd_2P^- LPS contains a terminal α-L-*glycero*-D-*manno*-heptopyranosyl group and a non-substituted, a 4-*O*-, and a 4,5-di-*O*-substituted pyranosidic KDO residue in the molar ratios 1:1:0.2:1.

INTRODUCTION

Lipopolysaccharides (LPS) are common constituents of the cell walls of Gram-negative bacteria and comprise a heteropolysaccharide covalently linked to a lipid portion (lipid A)[1,2]. Whereas lipid A is the endotoxic principle of LPS, the

*Presented at the Third European Symposium on Carbohydrates, Grenoble, September 16–20, 1985.
**To whom correspondence should be addressed.

0008-6215/86/$ 03.50

polysaccharide acts as an antigen (O-antigens and core antigens)[3] and as a receptor for certain bacteriophages[4]. The polysaccharide portion can be subdivided from a genetic, biosynthetic, and chemical point of view into the O-specific chain and the core oligosaccharide[3,4].

The core oligosaccharide has attracted our interest since its structure is identical or similar in many different LPS[5–7], and since it has peculiar immunogenic and antigenic properties[3,8,9]. In addition, we have shown recently that the core oligosaccharide of various LPS binds specifically to a serum protein of mammalians[10]. Although numerous structural investigations have been carried out on the core oligosaccharide, the detailed structure is not known and few data are available on the structure of the inner core region which consists of KDO and heptoses[5,6]. This lack of data is mainly due to the difficulties encountered in the structural analysis of KDO. We have reported on an α-(2→4)-linked KDO disaccharide as a common constituent of bacterial LPS, which was isolated from enterobacterial rough mutants[7,11]. From mutants other than Re, the KDO disaccharide was isolated without cleaving the main polysaccharide chain; moreover, one KDO residue was found to be substituted at O-5 by a heptosyl residue. Thus, a new structure was proposed for the inner core region[7], the main characteristic feature of which is a linear rather than a branched KDO trisaccharide[5,6] in which all residues are α-(2→4)-linked, the first KDO residue being additionally substituted at O-5 by heptose[12]. Accordingly, one KDO residue in the polysaccharide main chain is substituted at O-5 by heptose and at O-4 by an α-(2→4)-linked KDO disaccharide; in Re mutants (where heptose or other neutral sugars are absent), only two KDO residues are found which are also α-(2→4)-linked.

Although our previous data accorded with this proposal, direct proof was lacking. Following the development of a modified methylation-analysis procedure[13], the structure of the inner core region of enterobacterial LPS has been investigated and the results now reported show that the structure recently proposed is correct.

EXPERIMENTAL

Bacterial lipopolysaccharides (LPS). — LPS was obtained[14] from *S. minnesota* rough mutants, chemotypes Re (strain R595) and Rd_2P^- (strain R4), and converted into the free acid form (LPS-H^+) by electrodialysis[15]. *O*-Deacylated LPS (LPS-OH) was prepared by treatment[16] with methanolic 0.25M sodium methoxide (60°, 1 h).

Colorimetric estimations. — The thiobarbituric acid (TBA) assay[17,18] was performed[19] with ammonium 3-deoxy-D-*manno*-2-octulosonate (KDO) or 2-deoxy-D-*arabino*-hexose (2-deoxygalactose) as a standard to determine β-formylpyruvate[18] (549 nm) and malonaldehyde[17] (532 nm), respectively. Hexosamines were determined by the Morgan–Elson reaction as modified by Strominger[20], and by an amino acid analyser. Ester groups were determined[21] as hydroxamates, using

methyl palmitate as the standard, which yielded the same calibration curve as KDO methyl ester (data not shown).

Methylation analysis (general procedure) and chromatographic procedure. — The general experimental conditions (reduction, methylation, acetylation, and acid hydrolysis) and the conditions for g.l.c.–m.s. have been reported in detail[13]. The particular conditions used with bacterial LPS are described below. Methylated and partially methylated and acetylated mono- and oligo-saccharide derivatives were separated by reversed-phase chromatography on Silica C_{18}-cartridges[22] (SEP-PAK, Waters). Each cartridge was conditioned successively with water, methanol, chloroform, methanol, and water (5 mL each), charged with the sample in Me_2SO–water (1:1), purged with water (20 mL), and eluted stepwise with acetonitrile–water (1 + 9, 3 + 17, and 1 + 4, 10 mL each). The first eluate contained monosaccharide derivatives, and oligosaccharides were found in the third fraction. The second fraction contained a mixture of <5% of the total amount of mono- and oligo-saccharides. T.l.c. was performed on silica gel 60F (Merck), using chloroform–methanol–water (40:40:12).

Carboxyl-methylation[23] *of KDO in Re LPS of* S. minnesota *R595.* — LPS-OH (H^+ form, 50 mg), dried over phosphorus pentaoxide, was suspended in anhydrous methanol (10 mL), and freshly regenerated AG 50W-X8 (H^+) resin (1 g, Bio-Rad) was added. The vial was sealed with a Teflon-lined cap and the contents were stirred at room temperature for 48 h. The resin was then collected and washed twice with methanol, the combined filtrate and washings were taken to dryness, and the residue (LPS-OH methyl ester) was dialysed against water (3 × 10 mL). The dialysate contained 820 nmol of KDO (TBA assay), which was identified as the methyl ester by t.l.c. (R_F 0.67) in comparison with authentic standards. The amount corresponded to 1.2% of the total amount of KDO (69 μmol).

The lyophilised retentate gave LPS-OH methyl ester (30.7 mg; the low yield, which accounts for ~60% of the starting material, was due to partial absorption of LPS on the ion-exchange resin). The material was analysed for KDO by the TBA assay after mild [0.1M acetate buffer (pH 4.4), 100°, 1 h] and strong acid hydrolysis (M HCl, 100°, 2 h), for 2-amino-2-deoxy-D-glucose, and for ester groups[4].

Carboxyl-reduction. — The degree of carboxyl-reduction in LPS methyl esters was quantified by the TBA assay used: (*a*) KDO yields a chromophore[18] with λ_{max} 549 nm, which was shown to be identical to that of KDO methyl ester; (*b*) 2- and 3-deoxyaldoses yield a chromophore with λ_{max} 532 nm due to the formation of malonaldehyde upon periodate oxidation[17]; (*c*) carbonyl-reduced KDO (3-deoxyoctonic acid) does not give a chromophore; and (*d*) 3-deoxy-2-octulose (the compound expected after carboxyl-reduction and hydrolysis) does not give a chromophore at 532 nm unless the carbonyl group has also been reduced. A solution of LPS-OH methyl ester (10 mg) in methanol–water 1:1 (2.0 mL) was reduced at 0° with sodium borohydride (2 × 25 mg, each for 24 h). The carboxyl-reduced LPS-OH was dialysed against water and recovered from the retentate by lyophilisation. It was hydrolysed in 0.1M acetate buffer (pH 4.4; 2 mL, 100°, 1 h) followed by

dialysis against water (3 × 10 mL). The combined dialysates (assumed to contain 3-deoxy-2-octulose) were neutralised with sodium hydroxide and concentrated to dryness. One aliquot was carbonyl-reduced with sodium borohydride and analysed by the TBA assay in comparison to a second aliquot which had not been carbonyl-reduced. The absorption spectrum was recorded between 500 and 600 nm for each sample and the amount of KDO (λ_{max} 549 nm) and 3-deoxyoctitol (λ_{max} 532 nm) was calculated relative to authentic KDO and 2-deoxygalactose, respectively. To exclude a mixture of the two chromophores, the ratio of the absorption values at 532 and 549 nm was determined and compared to the values obtained with defined mixtures of KDO and 2-deoxygalactose.

Methylation analysis of LPS. — Carboxyl-reduced LPS-OH was methylated twice, then dialysed against water, recovered from the retentate by freeze-drying, and hydrolysed with 0.1M trifluoroacetic acid (100°, 1 h). A precipitate (partially methylated lipid A-OH) was removed by centrifugation, the supernatant solution was freed from trifluoroacetic acid by evaporation with several additions of water, carbonyl-reduced, acetylated, and analysed by g.l.c.–m.s. In a second experiment, a sample of LPS-OH was methylated twice and carboxyl-reduced; one part was then methylated, hydrolysed, carbonyl-reduced, and acetylated, and another part hydrolysed, carbonyl-reduced, and acetylated.

RESULTS

Determination of the degree of carboxyl-methylation and carboxyl-reduction of KDO in LPS. — The LPS-OH of *S. minnesota* R595 was analysed in comparison to a sample of LPS-OH methyl ester for KDO, 2-amino-2-deoxy-D-glucose, and ester groups. The results are shown in Table I. In LPS-OH, KDO and 2-amino-2-deoxy-D-glucose were present in the molar ratio 1:1; comparable amounts of KDO were obtained after mild and strong acid hydrolysis. Ester groups were not detected. LPS-OH methyl ester contained KDO, 2-amino-2-deoxy-D-glucose, and ester groups in the molar ratios 1:1:1. Thus, carboxyl-methylation of KDO was

TABLE I

CHEMICAL ANALYSIS OF LPS-OH (H^+) AND LPS-OH METHYL ESTER FROM *S. minnesota* R595

Sample	*KDO*		*Glucosamine*[a]	*Ester groups*[b]
	(nmol/mg)			
LPS-OH (H^+)	1380[c]	1300[d]	1400	n.d.[e]
LPS-OH methyl ester	705	1530	1360	1655

[a]Determined as hexosamine by the Morgan–Elson reaction; identified as 2-amino-2-deoxy-D-glucose (amino acid analyser). [b]Determined colorimetrically as hydroxamates. [c]Determined by the TBA assay after hydrolysis in acetate buffer [0.1M, pH 4.4; 100°, 1 h). [d]Determined by the TBA assay after hydrolysis in HCl (M, 100°, 2 h). [e]Not detected.

TABLE II

RATIO OF THE ABSORPTION VALUES AT 532 AND 549 nm OBTAINED FROM KDO, 2-DEOXYGALACTOSE, AND CARBOXYL-REDUCED LPS-OH IN THE TBA ASSAY

KDO + 2-deoxygalactose					*Carboxyl-reduced and hydrolysed LPS-OH*[a]	
10 + 0	*9 + 1*	*5 + 5*	*1 + 9*	*0 + 10*	*Without carbonyl-reduction*	*With carbonyl-reduction*
A_{532}/A_{549}						
0.5	0.69	1.1	1.7	2.3	0.5	2.3

[a]Carboxyl-reduced LPS-OH was hydrolysed in 0.1M acetate buffer (pH 4.4; 100°, 1 h) and dialysed. The outer dialysate was subjected to the TBA assay with or without carbonyl-reduction.

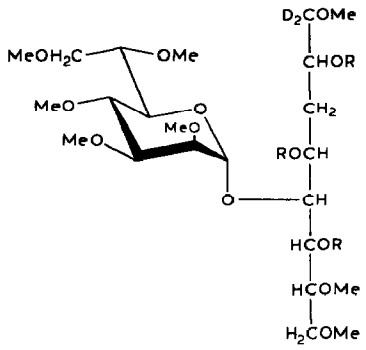
quantitative. The higher amount of KDO determined after hydrolysis with HCl compared to that for hydrolysis in acetate buffer confirmed the stabilisation of the ketosidic bond by esterification.

The degree of carboxyl-reduction, determined by the TBA assay for KDO and 3-deoxyoctulose, was ~80%. As shown in Table II, no mixture of the two chromophores (λ_{max} 532 and 549) was present before or after carbonyl-reduction.

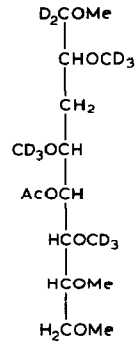
Methylation analysis of S. minnesota *R595 LPS-OH.* — In g.l.c., peaks for two pairs of products were observed, two of which exhibited retention times and e.i.- and c.i.-mass spectra identical to those of D-*glycero*-D-*talo* and D-*glycero*-D-*galacto* isomers of 2,6-di-*O*-acetyl-3-deoxy-1,4,5,7,8-penta-*O*-methyloctitol and 2,4,6-tri-*O*-acetyl-3-deoxy-1,5,7,8-tetra-*O*-methyloctitol, in the molar ratio 1:1.

Application of the sequence of carboxyl-reduction, methylation, carbonyl-reduction, and acetylation to methylated LPS-OH led to the same result. If, however, the second methylation step was omitted, the corresponding 1-acetates were obtained. In g.l.c.–m.s., two pairs of peaks were obtained with retention times and molecular weights (c.i.-m.s.) indistinguishable from those of 1,2,6-tri-*O*-acetyl-3-deoxy-4,5,7,8-tetra-*O*-methyl-D-*glycero*-D-*talo*/*galacto*-octitol and 1,2,4,6-tetra-*O*-acetyl-3-deoxy-5,7,8-tri-*O*-methyl-D-*glycero*-D-*talo*/*galacto*-octitol. The molar ratio of these compounds was 1:1.

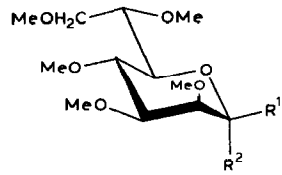
Methylation analysis of the LPS from S. minnesota Rd_2P^- *mutant (strain R4).* — Methylated LPS-H^+ was carboxyl-reduced with sodium borodeuteride, remethylated, hydrolysed, carbonyl-reduced, and acetylated as described for the Re LPS. On g.l.c.–m.s., the same derivatives of 3-deoxyoctitol as obtained from the Re LPS were identified as the 1,1-dideuterio derivatives. The ratio of the 2,6-di-*O*-acetyl and 2,4,6-tri-*O*-acetyl derivatives was 1:0.2. In addition, there was a peak with a retention time of 27.9 min which was identified (g.l.c.–m.s.) as 2,4,6-tri-*O*-acetyl-3-deoxy-1,7,8-tri-*O*-methyl-5-*O*-(2,3,4,6,7-penta-*O*-methyl-L-*glycero*-D-*manno*-heptopyranosyl)-D-*glycero*-D-*talo*/*galacto*-(1,1-$^2H_2$)octitol (**1**). Upon c.i.-(ammonia)-m.s., the molecular weight was found to be 658 (*m/z* 676 for



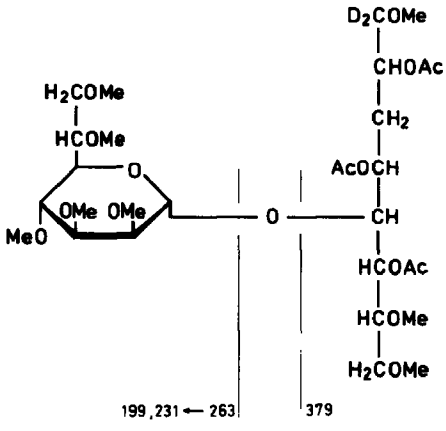
1 R = Ac
2 R = CD_3



3



4a R^1 = H, R^2 = OMe
4b R^1 = OMe, R^2 = H



M = 658

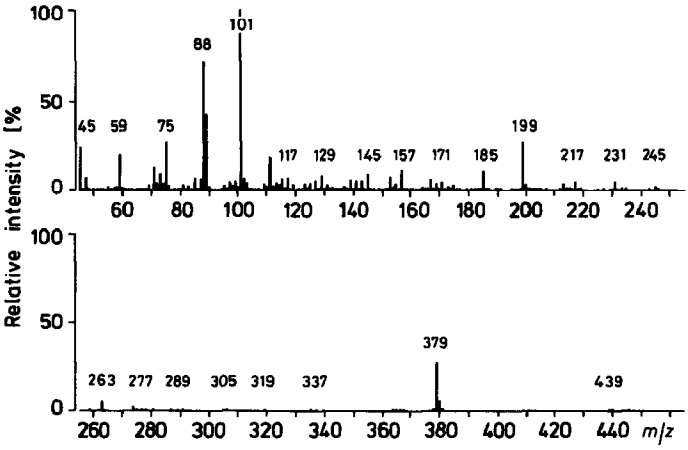


Fig. 1. Fragmentation pattern and e.i.-mass spectrum of 2,4,6-tri-*O*-acetyl-3-deoxy-1,7,8-tri-*O*-methyl-5-*O*-(2,3,4,6,7-penta-*O*-methyl-α-L-*glycero*-D-*manno*-heptopyranosyl)-D-*glycero*-D-*talo*/*galacto*-(1,1-$^2H_2$)octitol (**1**).

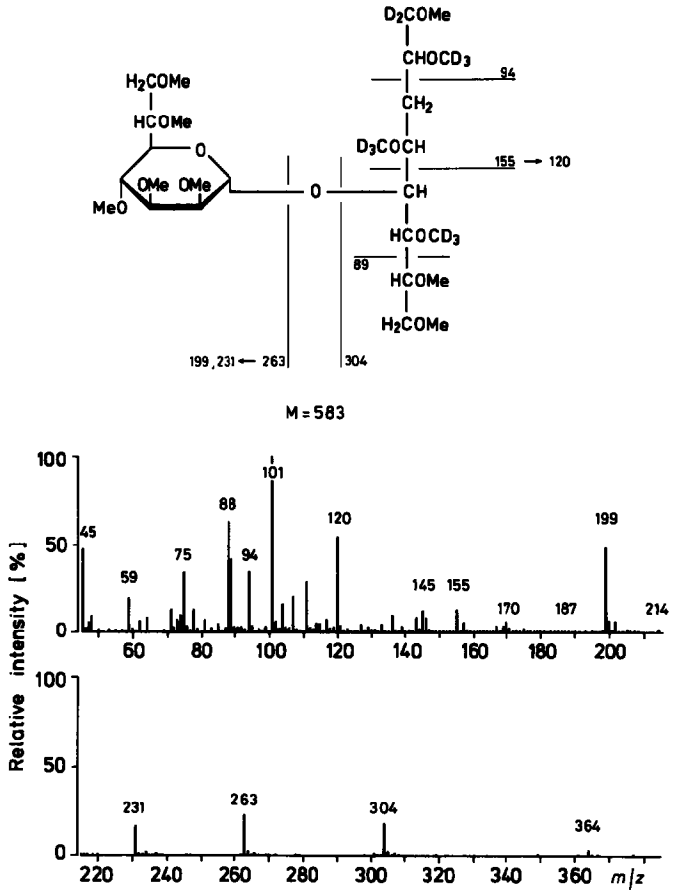


Fig. 2. Fragmentation pattern and e.i.-mass spectrum of 3-deoxy-1,7,8-tri-*O*-methyl-5-*O*-(2,3,4,6,7-penta-*O*-methyl-α-L-*glycero*-D-*manno*-heptopyranosyl)-2,4,6-tri-*O*-trideuteriomethyl-D-*glycero*-D-*talo*/*galacto*-(1,1-$^2H_2$)octitol (**2**). Note the fragment ions at *m/z* 155 and 120 and at *m/z* 94, comprising the C-1/4 and C-1/2 moieties, respectively.

$[M + NH_4]^+$, spectrum not shown). The e.i.-mass spectrum is shown in Fig. 1. Characteristic fragment ions at *m/z* 263, 231, and 199 are attributed to the methylated heptosyl residue. The peaks at *m/z* 379 and 439 correspond to the alditol chain and the J_1 fragment[24].

Compound **1** was purified by reversed-phase chromatography and trideuteriomethylated to give 3-deoxy-1,7,8-tri-*O*-methyl-5-*O*-(2,3,4,6,7-penta-*O*-methyl-α-L-*glycero*-D-*manno*-heptopyranosyl)-2,4,6-tri-*O*-trideuteriomethyl-D-*glycero*-D-*talo*/*galacto*-octitol (**2**), which was identified by g.l.c.–m.s. C.i.-(ammonia)-m.s. yielded a peak for $[M + NH_4]^+$ at *m/z* 601, indicating a molecular weight of 583. The e.i.-mass spectrum of **2** is shown in Fig. 2. The fragment ions derived from the non-reducing end (*m/z* 263, 231, and 199) were unchanged. The peaks at *m/z* 304 and 364 represent the alditol chain and the J_1 fragment[24]. Peaks

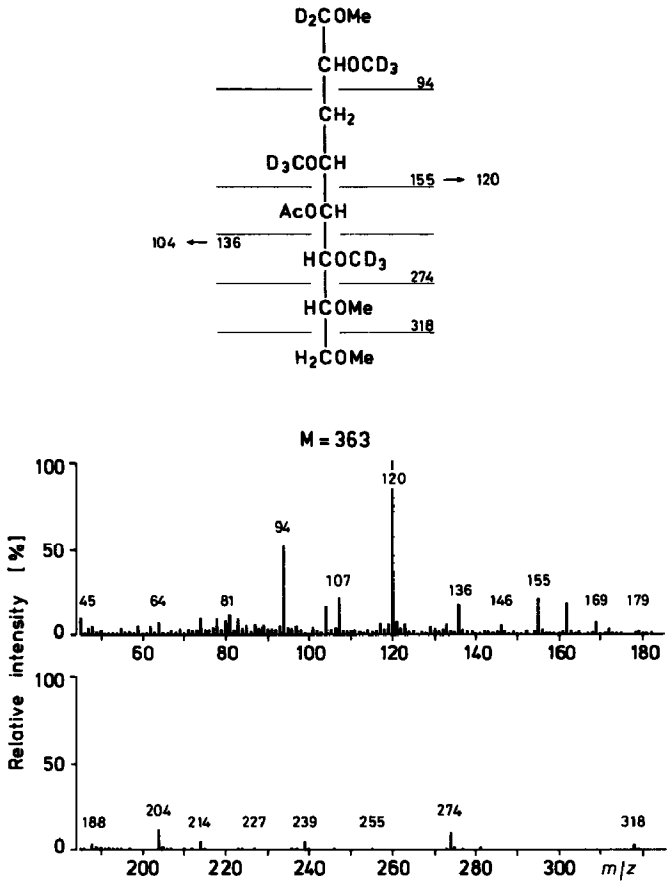
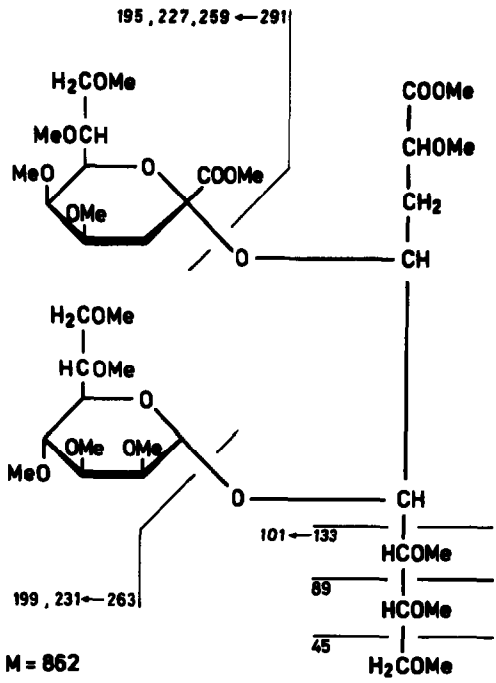


Fig. 3. Fragmentation pattern and e.i.-mass spectrum of 5-*O*-acetyl-3-deoxy-1,7,8-tri-*O*-methyl-2,4,6-tri-*O*-trideuteriomethyl-D-*glycero*-D-*talo*/*galacto*-(1,1-$^2H_2$)octitol (**3**).

of high intensity were observed at *m/z* 155 and 120 (155 − 35), comprising the C-1/4 moiety of the alditol chain and subsequent loss of trideuteriomethanol, and at *m/z* 94, representing the C-1/2 moiety. Fragments corresponding to these moieties were not formed from **1**, further confirming the rules[13] for the fragmentation of partially methylated and acetylated derivatives of 3-deoxyoctitols, namely, (*1*) a C-1/2 fragment is only observed when C-2 carries an *O*-methyl group, and (*2*) a C-1/4 fragment is found with high abundance when C-4 is *O*-methylated (*cf.* Figs. 1 and 2).

Compound **2** was methanolysed (0.5M acid, 85°, 16 h) followed by acetylation to give compounds with the retention times of 5-*O*-acetyl-3-deoxy-1,7,8-tri-*O*-methyl-2,4,6-tri-*O*-trideuteriomethyl-D-*glycero*-D-*talo*/*galacto*-(1,1-$^2H_2$)octitol (**3**) and methyl 2,3,4,6,7-penta-*O*-methyl-α- (**4a**) and -β-L-*glycero*-D-*manno*-heptosides (**4b**). Hydrolysis of **4** (M trifluoroacetic acid, 100°, 1 h), followed by reduction and acetylation, yielded 1,5-di-*O*-acetyl-2,3,4,6,7-penta-*O*-methyl-L-*glycero*-D-*manno*-



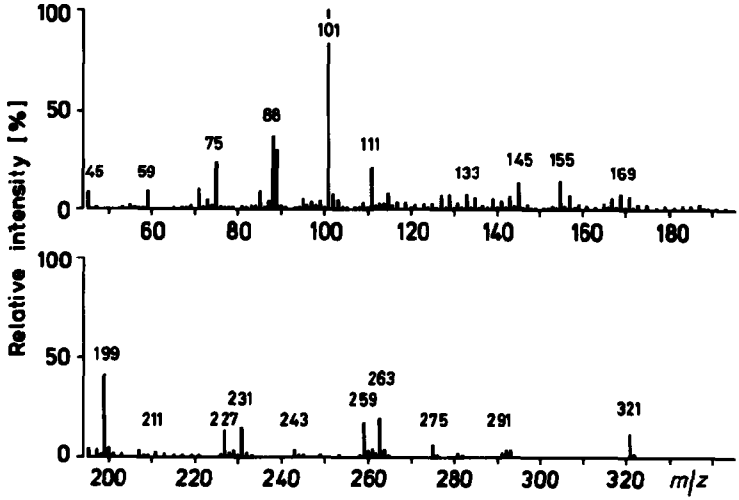


Fig. 4. Fragmentation pattern and e.i.-mass spectrum of methyl 3-deoxy-2,6,7,8-tetra-*O*-methyl-4-*O*-(methyl 3-deoxy-4,5,7,8-tetra-*O*-methyl-α-D-*manno*-2-octulopyranosylonate)-5-*O*-(2,3,4,6,7-penta-*O*-methyl-α-L-*glycero*-D-*manno*-heptopyranosyl)-D-*glycero*-D-*talo*/*galacto*-octonate.

heptitol. Compound **3** was further identified by g.l.c.–m.s. The molecular weight of 363 was confirmed by c.i.(ammonia)-m.s. which gave a peak at *m/z* 381 for $[M + NH_4]^+$ (spectrum not shown). The e.i.-mass spectrum is shown in Fig. 3. Fragment ions at *m/z* 94, 155, 274, 318, and 136 correspond to the C-1/2, C-1/4, C-1/6, C-1/7, and the C-6/8 moieties, respectively, from which the other fragments are derived by loss of trideuteriomethanol or acetic acid. The base peak (*m/z* 120) was derived from the C-1/4 fragment after loss of trideuteriomethanol (155 − 35). These results show that **3** was derived from a pyranosidic KDO residue which, in LPS, was substituted at O-4 by an acid-labile substituent and at O-5 by heptose.

Analysis of carbonyl-reduced and methylated oligosaccharides obtained from Rd_2P^-R4 LPS after mild acid hydrolysis. — LPS was hydrolysed in 20mM acetate buffer (pH 4.4; 70°, 1 h), and the resulting mono- and oligo-saccharides were carbonyl-reduced and methylated[12]. On g.l.c.–m.s., KDO, α-KDO*p*-(2→4)-KDO, and α-L-*glycero*-D-*manno*-heptopyranosyl-(1→5)-KDO were identified, the mass spectra of which have been published[7,11,12]. In addition, a pair of peaks was observed (retention times, 37.44 and 37.59 min) which yielded identical c.i.- and e.i.-mass spectra. C.i.(ammonia)-m.s. gave a peak for $[M + NH_4]^+$ at *m/z* 880 indicating a molecular weight of 862. The e.i. spectrum is shown in Fig. 4. Fragment ions at *m/z* 291, 259, and 227 are attributed to a non-reducing methylated KDO residue, whereas fragment ions at *m/z* 263, 231, and 199 are derived from a non-reducing, methylated heptosyl residue and subsequent loss of methanol. The fragment ions at *m/z* 275, 243, and 211 correspond to a di-*O*-substituted, methylated methyl 3-deoxyoctonate chain. Together with the data of the methylation analysis and our previous results[12] on this LPS, the compound is proposed to be methyl 3-deoxy-2,6,7,8-tetra-*O*-methyl-4-*O*-(methyl 3-deoxy-4,5,7,8-tetra-*O*-methyl-α-D-*manno*-2-octulopyranosylonate)-5-*O*-(2,3,4,6,7-penta-*O*-methyl-α-L-*glycero*-D-*manno*-heptopyranosyl)-D-*glycero*-D-*talo*/*galacto*-octonate.

DISCUSSION

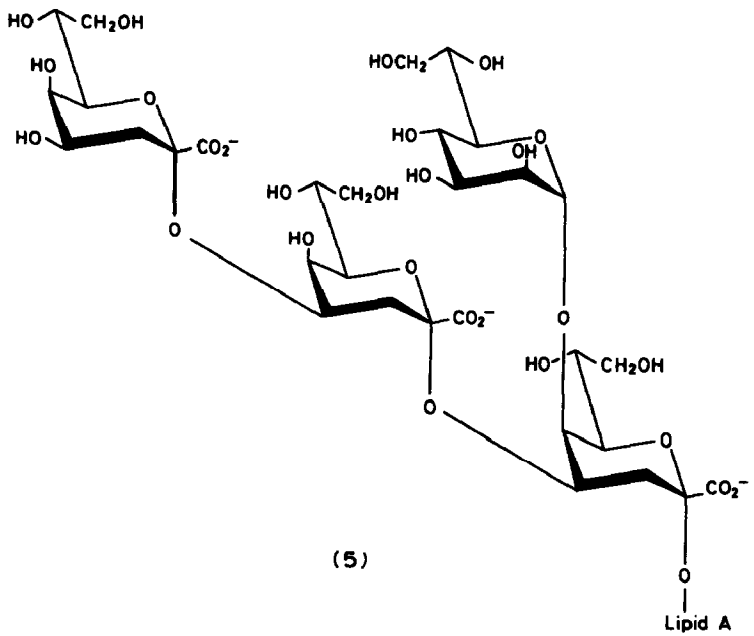
In the present investigation, a modified procedure for methylation analysis has been adopted in order to further elucidate the inner core region of enterobacterial LPS. The procedure was developed[13] using partially methylated and acetylated 3-deoxyoctitol derivatives of defined structure. The most critical points of this modification are the methylation of LPS, the reduction of the carboxyl group of KDO, and the choice of appropriate hydrolysis conditions which do not destroy the KDO moieties. Methylation was performed on the free acid form of LPS and LPS-OH obtained after electrodialysis[15]. LPS and LPS-OH yielded comparable results with respect to the formation of partially methylated and acetylated octitol derivatives. Carboxyl-reduction was performed at 0° on the methyl ester of KDO[23] obtained after carboxyl-methylation or methylation. The usual steps of methylation analysis (*i.e.*, methylation, hydrolysis, carbonyl-reduction, and *O*-acetylation) performed on carboxyl-reduced and methylated LPS-OH of *S. minnesota* R595 gave

2,6-di-*O*-acetyl-3-deoxy-1,4,5,7,8-penta-*O*-methyloctitol and 2,4,6-tri-*O*-acetyl-3-deoxy-1,5,7,8-tetra-*O*-methyloctitol in the molar ratio 1:1. Due to the non-stereospecific reduction of the keto group, the D-*glycero*-D-*talo* and D-*glycero*-D-*galacto* isomers were formed and shown to be present by comparison with authentic compounds. When methylated LPS was carboxyl-reduced, hydrolysed, carbonyl-reduced, and acetylated, 1,2,6-tri-*O*-acetyl-3-deoxy-4,5,7,8-tetra-*O*-methyl-D-*glycero*-D-*talo*/*galacto*-octitol and 1,2,4,6-tetra-*O*-acetyl-3-deoxy-5,7,8-tri-*O*-methyl-D-*glycero*-D-*talo*/*galacto*-octitol were obtained in equimolar amounts. These results indicated a non-substituted (terminal) and a 4-*O*-substituted KDO residue to be present as pyranosides in the molar ratio of 1:1 in the Re LPS, and confirmed our earlier data obtained on an isolated α-KDO*p*-(2→4)-KDO disaccharide[7,11].

When the same procedure was applied to the LPS of the Rd_2P^- mutant (strain R4), the partially methylated octitol acetates corresponding to a terminal and a 4-linked KDO residue were also obtained, but in the molar ratio ~1:0.2. This result also accorded with our earlier investigations, in which it was shown that the two KDO residues in the side chain were not present in stoichiometric amounts reflecting the microheterogeneity in the KDO region.

The hydrolysis conditions by which these derivatives were obtained (0.1M trifluoroacetic acid, 100°, 1 h) cleaved exclusively the ketosidic bonds of KDO. The partially methylated and acetylated pseudo-disaccharide **1** was subsequently isolated and identified by g.l.c.–m.s. Trideuteriomethylation of **1** yielded **2** containing trideuteriomethyl groups at positions 2, 4, and 6, indicating the pyranoid structure and the substitution at C-4 of this KDO residue in LPS. Methanolysis (0.5M acid, 85°, 16 h) was the most convenient method for the cleavage of **2**, and acetylation then yielded **3** and methyl 2,3,4,6,7-penta-*O*-methyl-α- and -β-L-*glycero*-D-*manno*-heptopyranosides. On e.i.-m.s. of **3** in comparison to an authentic sample of 5-*O*-acetyl-3-deoxy-1,2,4,6,7,8-hexa-*O*-methyl-D-*glycero*-D-*talo*/*galacto*-octitol, it was shown that C-2, C-4, and C-6 carried the trideuteriomethyl groups and that C-5 was acetylated. Thus, the formation of **3** from LPS proves the presence of a 4,5-di-*O*-substituted KDO residue in LPS. Attempts to hydrolyse carboxyl-reduced and methylated LPS in one step were not successful, since the conditions needed to cleave the heptosidic linkage destroyed the reducing KDO residue. However, this is an advantage since the two-step hydrolysis yields additional information, namely, the presence of an acid-labile substituent at O-4 and a more stable substituent at O-5. In addition, a trisaccharide was isolated from R4 LPS, in which a reducing KDO residue was substituted at O-4 and O-5 by a KDO and a heptosyl residue, respectively.

The combined results of the present study and earlier findings[7,11,12] show that our recent proposal for the structure of the inner core region of LPS from enterobacterial rough mutants is correct. Thus, the sugar residues of the core oligosaccharide of the LPS from *S. minnesota* Rd_2P^- mutant, strain R4, have the structure **5**.



(5)

ACKNOWLEDGMENTS

We thank Dr. F. M. Unger for fruitful discussion and gifts of KDO, Veronika Susott for technical assistance, Hermann Moll for help with the g.l.c.–m.s., and the Fonds der Chemischen Industrie for financial support.

REFERENCES

1 O. LÜDERITZ, C. GALANOS, AND E. T. RIETSCHEL, *Pharmacol. Ther.*, 15 (1982) 383–402.
2 E. T. RIETSCHEL, H.-W. WOLLENWEBER, R. RUSSA, H. BRADE, AND U. ZÄHRINGER, *Rev. Infect. Dis.*, 6 (1984) 432–438.
3 O. LÜDERITZ, C. GALANOS, H. J. RISSE, E. RUSCHMANN, S. SCHLECHT, G. SCHMIDT, H. SCHULTE-HOLTHAUSEN, R. WHEAT, O. WESTPHAL, AND J. SCHLOSS-HARDT, *Ann. N.Y. Acad. Sci.*, 133 (1966) 349–374.
4 I. SUTHERLAND, *Surface Carbohydrates of the Prokaryotic Cell*, Academic Press, London, 1977.
5 W. DRÖGE, V. LEHMANN, O. LÜDERITZ, AND O. WESTPHAL, *Eur. J. Biochem.*, 14 (1970) 175–184.
6 P. PREHM, S. STIRM, B. JANN, AND K. JANN, *Eur. J. Biochem.*, 56 (1975) 41–55.
7 H. BRADE AND E. T. RIETSCHEL, *Eur. J. Biochem.*, 145 (1984) 231–236.
8 H. BRADE AND C. GALANOS, *J. Med. Microbiol.*, 16 (1983) 203–210.
9 H. BRADE AND C. GALANOS, *Infect. Immun.*, 42 (1983) 250–256.
10 L. BRADE AND H. BRADE, *Infect. Immun.*, 50 (1985) 687–694.
11 H. BRADE, U. ZÄHRINGER, E. T. RIETSCHEL, R. CHRISTIAN, G. SCHULZ, AND F. M. UNGER, *Carbohydr. Res.*, 134 (1984) 157–166.
12 H. BRADE, H. MOLL, AND E. T. RIETSCHEL, *Biomed. Mass Spectrom.*, 12 (1985) 602–609.
13 A. TACKEN, H. BRADE, F. M. UNGER, AND D. CHARON, *Carbohydr. Res.*, 149 (1986) 263–277.
14 C. GALANOS, O. LÜDERITZ, AND O. WESTPHAL, *Eur. J. Biochem.*, 9 (1969) 245–249.
15 C. GALANOS AND O. LÜDERITZ, *Eur. J. Biochem.*, 54 (1975) 603–610.
16 H. W. WOLLENWEBER, K. W. BROADY, O. LÜDERITZ, AND E. T. RIETSCHEL, *Eur. J. Biochem.*, 124 (1982) 191–198.

17 V. S. WARAVDEKAR AND L. D. SASLAW, *J. Biol. Chem.*, 234 (1959) 1945–1950.
18 A. WEISSBACH AND J. HURWITZ, *J. Biol. Chem.*, 234 (1959) 705–709.
19 H. BRADE, C. GALANOS, AND O. LÜDERITZ, *Eur. J. Biochem.*, 131 (1983) 195–200.
20 J. L. STROMINGER, J. T. PARK, AND R. E. THOMPSON, *J. Biol. Chem.*, 234 (1959) 3263–3268.
21 F. SNYDER AND N. STEPHENS, *Biochim. Biophys. Acta*, 34 (1959) 244–245.
22 T. J. WAEGHE, A. G. DARVILL, M. MCNEIL, AND P. ALBERSHEIM, *Carbohydr. Res.*, 123 (1983) 281–304.
23 D. CHARON AND L. SZABO, *Acta Chim. Acad. Sci. Hung.*, 113 (1983) 375–378.
24 J. LÖNNGREN AND S. SVENSSON, *Adv. Carbohydr. Chem. Biochem.*, 29 (1974) 41–106.