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Substitution pattern of 3-deoxy-D-*manno*-oct-2-ulosonic acid in bacterial lipopolysaccharides investigated by methylation analysis of whole LPS

Jacek Rybka, Katarzyna Zielińska-Kuźniarz, Agnieszka Korzeniowska-Kowal, Aneta Sondej, Andrzej Gamian*

Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Weigla 12, PL-53 114 Wrocław, Poland

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Abstract

3-Deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) is a constituent of the inner core part of bacterial lipopolysaccharides (LPS). This sugar may contribute to biological activities of the LPS, the type of substitution of Kdo is thus of importance and this work is aimed at the evaluation of a method for monitoring the substitution of Kdo in LPS. The procedure consists of three steps, namely permethylation of the lipopolysaccharide, with iodomethane and sodium methylsulfinylmethanide or NaOH in Me_2SO, or with methyl triflate, then the product is methanolysed with HCl in MeOH and acetylated with acetic anhydride in pyridine. The resulting partially methylated acetates of Kdo methyl glycosides were analyzed by gas-liquid chromatography–electron impact ionization mass spectrometry (GLC–MS). For several derivatives of Kdo, specific GLC retention times and MS fragmentation patterns were determined. Lipopolysaccharides from several bacterial strains were isolated and analyzed with three different methods of methylation. The complete solubilization of the LPS in the acid form allows diminishing possible undermethylation. Sodium methylsulfinylmethanide is the most efficient agent in the permethylation of the whole LPS, of all the tested procedures. Methylation with methyl triflate allows the detection of base labile substituents on Kdo residues.

Keywords: Lipopolysaccharide; Endotoxin; LPS; 3-Deoxy-D-*manno*-oct-2-ulosonic acid; Kdo

1. Introduction

The lipopolysaccharide (LPS) is an integral component of the outer membrane of the cell wall of Gram-negative bacteria. Lipopolysaccharides exhibit a wide variety of endotoxic activities, playing a prominent role in the host during infections, e.g. sepsis and septic shock. The LPS molecule can generally be divided into three regions differing in genetic determination, biosynthesis, structure and also functions, namely the O-specific polysaccharide, core oligosaccharide region and lipid A.[1] Sometimes, in the LPS core two regions can be distinguished, an outer core and an inner core, the latter composed mainly of heptose and 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo). In the inner part of the core, Kdo spans the carbohydrate portion with lipid A. Generally one to four Kdo residues occur in the LPS; recently it has also been found in other regions than the inner core.[2–4] Although lipid A was assigned to be responsible for a variety of biological and immunological activities of LPS, the influence of Kdo on these functions has been reported, like induction of release of interleukin-1 and leukotriene C4.[5–9] The Kdo-containing inner core has been considered a cellular target for a protective antibody.[1] Kdo is an inherent constituent of LPS and plays a vital role for the bacterial cell. Thus, synthetic antibiotics with antibacterial activity could be obtained, based on Kdo derivatives. Kdo mediates binding of divalent Ca^{2+} and Mg^{2+} cations at the bacterial cell

Abbreviations: LPS, lipopolysaccharide; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; CPS, capsular polysaccharide.

* Corresponding author. Tel.: +48-71-337-1172; fax: +48-71-337-1382.
E-mail address: gamian@immuno.iitd.pan.wroc.pl (A. Gamian).

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surface and may also carry a great variety of substituents [Ref. 1 and refs. cited therein], however the role of these substitutions in the Kdo region for the biological activities of the LPS is not well understood. Among these substituents are e.g., Kdo, phosphate, uronic acids, Hep, Man, Rha, Glc, Gal, L-Ara4N, Gly.[1,2,10–12] The lability of Kdo and its ketosidic linkages, especially in acid hydrolysis, complicates structural investigations; also specific degradations like deacylation can result in the loss of base labile substituents. In spite of these problems the structure of the Kdo region, the structural variability and the array of substituents in the Kdo region of LPS have been investigated across a wide spectrum of bacteria, reviewed recently by Holst.[2,3] Several approaches have been undertaken in the studies of the Kdo region of LPS and its structure was investigated using isolated Kdo-containing disaccharides,[13–15] isolated phosphorylated Kdo compounds,[16,17] or by methylation analysis of the LPS [Refs. 18,19 and refs. cited in 2,3]. Further understanding of the influence of the Kdo region on the immunological properties of LPS and of a role that the Kdo region of the LPS may play in infection might be facilitated by a simplified procedure for the determination of Kdo substitution in the isolated LPS. This prompted us to evaluate the existing methods towards the optimization of conditions for more routine analysis.

This work presents the results of monitoring of the Kdo substitutions in LPS molecules of various bacterial strains. We have evaluated three different methods of methylation, namely according to Hakomori,[20] Ciukanu and Kerek,[21] and Prehm.[22] Special attention has been paid to the *Hafnia alvei* lipopolysaccharides. In several strains of that species, rare Kdo substitutions have previously been reported.[23,24]

2. Experimental

2.1. Bacterial strains and isolation of LPS and capsular polysaccharides

Bacterial strains were obtained from the Polish Collection of Microorganisms (PCM) at the Institute of Immunology and Experimental Therapy (Wrocław, Poland): *Citrobacter youngae* O2a,1b:5,6 (PCM 1507), *C. freundii* O23 (PCM 2352), *C. braakii* O37 (PCM 2346), *Escherichia coli* O56 (PCM 2372), O24 (PCM 195), O25:K19 (PCM 196), and O6:K13 (PCM 374), *H. alvei* strains PCM 537 (ATCC13337), 1185, 1186, 1188, 1189, 1190, 1196 and 1216, *Klebsiella oxytoca* O3:K58$^-$, *Shigella sonnei* Ph I (PCM 2336) and *Sh. sonnei* Ph II (PCM 1984) and *Salmonella enterica* subsp. *enterica* ser. Typhimurium (*S.* Typhimurium) mutant Re (PCM 2266). Bacteria were cultivated in Davis broth supplemented with casein hydrolysate and yeast extract (Difco) with aeration at 37 °C for 24 h, harvested and freeze-dried.[24] The LPS from S-type strains was isolated by phenol–water extraction of dry cell mass and purified by ultracentrifugation[25] and the LPS from R-type strains was isolated by extraction with phenol–chloroform–light petroleum.[26] Purification of LPS was also accomplished by gel filtration on a Sepharose 2B column.[27] LPS of *Salmonella* Minnesota mutant Re 595 was from Sigma (L9764). Capsular polysaccharides (CPS) were isolated according to the published procedure[28] modified as follows: The culture supernatant was concentrated on an Amicon device with a YM-1 membrane (cutoff 1000 Da), the capsular polysaccharide was then precipitated with cetyl pyridinium chloride and centrifuged (10,000 × g, 4 °C, 20 min). The precipitate was extracted with cold 1 M $CaCl_2$ at 4 °C for 2 h with stirring, centrifuged and the CPS was precipitated from the supernatant with cold EtOH. The centrifuged precipitate was dissolved in 0.2 M phosphate buffer pH 6.98, then extracted with the same volume of 90% phenol at 65 °C. The phenol phase was extracted for a second time and the combined water phases were diluted six times with buffer, and washed on the Amicon device with a YM-1 membrane. The polysaccharide preparation was treated with DNAse, RNAse and Pronase, washed again on an Amicon device with the same membrane and freeze-dried.

2.2. Methylation of LPS

The acid (H^+) form of electrodialyzed[29] (2000 V, 7.5 h) lipopolysaccharides was used in all the experiments. Reactions were performed on LPS (5 mg) dried over P_2O_5, in tightly capped serum bottles flushed with Ar. Sonications were carried out on an ultrasonic water bath (Branson 2210). In the Hakomori methylation[20] the LPS was suspended in dry Me_2SO (1 mL) and after 30 min of sonication sodium methylsulfinylmethanide (Me_2SONa, dimsyl base, 1 mL) was added. After 2 h of sonication and freezing at −20 °C for 10 min, methyl iodide (1.5 mL) was added followed by 2 h of sonication. The excess of methyl iodide was evaporated on a rotary evaporator with the addition of MeOH. For the methylation according to procedure of Ciukanu and Kerek,[21] LPS was dissolved in dry Me_2SO (0.5 mL) and a small amount of dry powdered NaOH (ca. 5mg) stored under heptane was added. After 30 min of sonication and addition of 2 vol of methyl iodide, samples were sonicated for 2 h. After evaporation of the excess of methyl iodide with addition of MeOH on the rotary evaporator, samples were dialysed against deionized water. The Prehm methylation[22] was performed on LPS samples dissolved in dry trimethyl phosphate (1 mL). After 30 min of sonication, 2,6-di-*tert*-butylpyridine (150 μL) and methyl trifluoromethanesulfonate (methyl triflate, 150 μL) were added and samples were

sonicated at 50 °C for 2 h. After the methylation, excess methyl triflate was evaporated. In all methods, samples were dialysed against deionized water after methylation, solutions were concentrated to dryness, dissolved in MeOH and filtered on a Sephadex LH-20 column (0.6 × 15 cm), where the high-molecular mass fraction was collected and dried over P_2O_5. The CPS from *Actinobacillus pleuropneumoniae* types 5a and 5b (gift from E. Altman) and meningococcal group 29e polysaccharide (gift from H. J. Jennings) were used as reference samples.

2.3. Methanolysis and acetylation

The permethylated LPS was dissolved in a methanolic solution of HCl (1 M, 1 mL). Methanolysis was carried out at 80 °C for 4 h, then the sample was evaporated to dryness with a stream of dry N_2 with two additions of MeOH. The sample was finally acetylated with 1:1 Ac_2O–pyridine at 100 °C for 30 min. In some experiments, MeOH was replaced by deuterated MeOH (CD_3OD).

2.4. Gas-liquid-chromatography–mass spectrometry (GLC–MS) and analytical methods

GLC–MS was performed on a Hewlett-Packard HP 5971A apparatus using an HP-1 fused-silica capillary column (0.22 mm × 12 m) with helium as the carrier gas. Temperature was programmed to hold at 150 °C for 5 min, which was followed by an 8 °C/min rise to 270 °C. Quantification of Kdo was performed with the periodate–thiobarbituric acid assay.[30]

3. Results and discussion

3.1. Mass spectrometric analysis of partially methylated Kdo acetates

The capsular polysaccharides (CPS) containing Kdo of known structures from strains of *E. coli* K13 and K19 were used as standards for Kdo glycosylated at positions 7 and 4, respectively. The CPS from *Neisseria meningitidis* group 29e and from *Actinobacillus pleuropneumoniae* types 5a and 5b were standards for Kdo substituted at positions 7 and 5 and 4,5, respectively. These polysaccharides were subjected to permethylation by the method with dimsyl base and after methanolysis the resulting O-methylated Kdo carboxymethyl ester methyl glycosides were acetylated at the free hydroxyl groups prior to the GLC-MS analysis. This procedure of methylation analysis was then used to determine terminal and substituted Kdo residues in lipopolysaccharides. The LPS from *Hafnia alvei* 1186 (identical with 2386) was considered as another standard because it contained 4,7- and 7,8-disubstituted Kdo residues in oligosaccharides of structures determined before.[31] The EIMS spectra of Kdo derivatives were interpreted in accordance with data in the literature[13,15,32–34] and by using deuterium-labelling techniques. The experiments with deuterium labelling using CD_3OD also allowed differentiating similar fragments from methyl glycosides of Kdo and heptose both present in LPS. The GLC retention times for Kdo derivatives are given in Table 1. The nomenclature of the major fragment ions shown in Fig. 1 was adopted from that used for sialic acid.[33] Permethylated methyl ester methyl glycoside of Kdo, which derived from terminal 3-deoxy-octulosonic acid (4,5,7,8-tetra-*O*-methyl-3-deoxyoctonate methyl ester methyl glycoside) was present in all lipopolysaccharides isolated from different enterobacterial species. The mass spectrum of Kdo-4,5,7,8-Me_4 methyl ester methyl glycoside derived from terminal Kdo (Fig. 2A), contains characteristic fragment ions at *m/z* 263 (type B fragment, M-$COOCH_3$), 277 (type C fragment, M-C_8) and 88 (type G fragment M-C_4-C_5). The mass spectra of derivatives of terminal Kdo and 4,5-disubstituted Kdo were identical with those obtained from the *Moraxella catarrhalis* LPS.[19] The peracetylated derivative, with typical fragment ions at *m/z* 158, 181, 213 as well as 153 and 375,[35] was found only in trace amounts, as a result of complete undermethylation, the largest amount found after methyl triflate treatment. Furanose derivatives, characterized by the presence of a fragment ion at *m/z* 157,[32] were only found in trace amounts and are not considered here. Among the intense marker ions were B-type fragments formed after cleavage of the carboxymethyl residue, at *m/z* 263, 291, 319, 347 and 375 for derivatives of terminal, mono-, di-, tri- and tetra-substituted Kdo, respectively (Figs. 2–4). In the region of high-molecular-mass ions, the additional prominent fragment ion A′ (M − OCH_3) occurs at *m/z* 319 in the spectrum of Kdo-4,5,7-$Me_3$8Ac methyl ester methyl glycoside (Fig. 2D) and fragment ion C from cleavage of a C_8 fragment, at *m/z* 305, in the spectrum of the derivative of 5-substituted Kdo (Fig. 2B), at *m/z* 333 in derivatives of 4,5-di-substituted (Fig. 3B) and 4,5,8-tri-substituted Kdo (Fig. 4C). Indicative are also fragment ions of type G involving fragment C_4–C_5 with two methyl groups (at *m/z* 88 in spectra of terminal, 7-, 8- and 7,8-substituted Kdo derivatives) or methyl and acetyl groups (*m/z* 116 for 4-, 4,7-, 4,8- and 4,7,8-substituted Kdo derivatives). These ions are less intense for 5- and 5,7,8-substituted Kdo derivatives. The prominent ion at *m/z* 117 of fragment type F comprising the C_7–C_8 fragment is found in 8- and 4,5,8-substituted Kdo derivatives. The fragment ion at *m/z* 167 in the spectrum of the 4,5,7-substituted Kdo derivative (Fig. 4A) was assigned the structure M-$COOCH_3$–3xAcOH. Interestingly, the fragment ion at *m/z* 153, formed from the B ion by sequential elimination of acetic acid and

Table 1
Proportions (in %) of substituted Kdo residues in enterobacterial lipopolysaccharides analyzed by the method with dimsyl base (1), NaOH in Me_2SO (2) and methyl triflate (3)

No.	Kdo substitution	R_f	*H. alvei* 1216			*H. alvei* 1185			*H. alvei* 1186			*H. alvei* 1188			*H. alvei* 1189			*H. alvei* 1190			*H. alvei* 1196			*K. oxytoca* O3		
			1	2	3	1	2	3	1	1	2	3	2	3	1	2	3	1	2	3	1	2	3	1	2	3
1	**terminal-Kdo**	1.00	20	37	7	42	46	14	30	36	57	21	37	15	48	51	25	32	50	11	41	47	13	36	57	21
2	**5-Kdo**	1.14	1		4	8	2	2	3	3	3	6		8	3	3	3	3		4	1		8	3	3	6
3	**7-Kdo**	1.22	4		3	15	17	5	4	4		5	3	5	3	4	5	5	1	3	5	2	4	4		5
4	**8-Kdo**	1.29	3	8	6	5	6	11	4	15	5	9		19	5	6	8	6	7	18	12	14	24	15	5	9
5	**4-Kdo**	1.32	21	21	5	9	9	4	23	12	11	5	27	5	19	22	9	22	16	5	22	23	5	12	11	5
6	**4,5-Kdo**	1.41	19	20	20	11	12	18	7	16	24	34	5	29	4	5	9	8	12	19	5	3	22	16	24	34
7	**7,8-Kdo**	1.47	9	12	21	3	2	10	2	3		3	8	11	10	9	15	9	9	17	6	2	9	3		3
8	**4,7-Kdo**	1.49	3		8	3		5	16	2		6	12	3	2		3	3	2	4	2	5		2		6
9	**4,5,7-Kdo**	1.52	7		5	3	5	17	5	5		9			2		10	1	1	6	1	2	7	5		9
10	**4,8-Kdo**	1.60	0		3	0	0	1		1			2	1	2		3	3	0	1	1	1	3	1		
11	**4,5,8-Kdo**	1.66	3		4	1		6	1	2		1	2		1		4	2	1	2	0	1	2	2		1
12	**4,7,8-Kdo**	1.73	4	2	4	1		5	2				3	3	1		5	3	0	4	2	1	3			
13	**4,5,7,8-Kdo**	1.75	6		10	1	1	2	3				2	1	1		2	4	1	5	2	1	1			

No.	Kdo substitution	R_f	*E. coli* O24			*E. coli* O56			*S. sonnei* PhI			*S. sonnei* PhII			*C. freundii* O23			*C. braakii* O37			*C. youngae* O2		
			1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
1	**terminal-Kdo**	1.00	55	40	20	41	30	26	55	81	30	55	48	37	61	47	14	27	39	16	45	60	26
2	**5-Kdo**	1.14	2		11	5	6	4	3		4	3	8	5	3		16	4		12	2		
3	**7-Kdo**	1.22	4	4	5	2	2	3	4	2	6	4	5	5	3	5	3	5	6	5	4		6
4	**8-Kdo**	1.29	8	6	11	26	26	21	7	5	11	6	6	10	7	4	9	20	13	10	11	11	9
5	**4-Kdo**	1.32	10	14	3	9	9	4	11	6	7	14	17	6	8	12	4	14	11	4	9	6	7
6	**4,5-Kdo**	1.41	9	8	29	10	11	25	6	4	15	9	6	26	9	12	38	16	12	37	16	14	31
7	**7,8-Kdo**	1.47	3	3		2	3	5	3		10	2	2	11	3	2	6	3	2		2		5
8	**4,7-Kdo**	1.49	2	7	10	2	3	2	2		2	3	4		1	4	2	2	5	8	3	3	3
9	**4,5,7-Kdo**	1.52	3	5	5	1	6	9	3		9				3	6	5	4	5	8	7		11
10	**4,8-Kdo**	1.60	2	2		1	1		1		2	1	2		1	3		1	1				
11	**4,5,8-Kdo**	1.66	1	3	3	1	2		1		1	1			1	2	1	3	1		1		2
12	**4,7,8-Kdo**	1.73		3				1	1	1	2				1	1			1			7	
13	**4,5,7,8-Kdo**	1.75	2	5	1	1	1	1	3	1	2	2	8		1	3	1	1	4	1	2		2

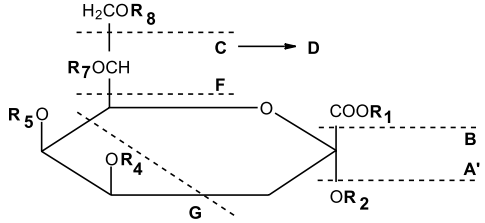

Fig. 1. Structure of methyl ester methyl glycoside derivatives of 3-deoxy-D-*manno*-oct-2-ulosonate and the pattern of main EIMS fragment ions

methanol residues, still retained the deuteriomethyl group as the increase of 3 amu after deuteriomethylation persisted, as shown also previously,[32] is observed as an intensive ion in the derivatives of 4,5-, 4,5,7-, 4,5,8- and 4,5,7,8-substituted Kdo. Important fragment ions at *m*/*z* 232 and 172, resulting from initial cleavage of the three carbon fragment CH_2OR_8-$CHOR_7$-CHO, are formed in acetylated derivatives in the series of 4-substituted Kdo (4-, 4,7- and 4,7,8-substituted Kdo, Fig. 3A, D, Fig. 4D), which allows the differentiation from Kdo derivatives substituted at position 5, respectively. In the latter derivatives acetylated at position 5, like 5-, 4,5- and 4,5,8-substituted Kdo, the formation of the fragment M-CH_2OR_8-$CHOR_7$-CHO is hindered, but instead formation of fragment ion C is favoured, then facilitating fragmentation to *m*/*z* 129 without 232 and 172. The derivatives of 5,7-, 5,8- and 5,7,8-substituted Kdo were found only in trace amounts in the analyzed preparations (data not shown) and were not considered in this study.

3.2. Analysis of Kdo in lipopolysaccharides

The LPS from deep rough strains of *Salmonella* Minnesota Re595 and *S.* Typhimurium mutant Re yielded two major peaks (not shown) of derivatives from terminal Kdo and of 4-substituted Kdo. These results are in accord with published data, because in these strains the LPS has an α-(2→4)-linked Kdo disaccharide connected to the lipid A region.[2,13] This pattern was improved after remethylation when low amounts of other derivatives disappeared.

The analysis of smooth lipopolysaccharide from *Klebsiella oxytoca* O3 gave a pattern with three major derivatives, from terminal Kdo, from 4-substituted and from 4,5-disubstituted Kdo (Table 1). These results indicate the presence of an additional, 4-substituted residue of Kdo in the smooth LPS of *Klebsiella oxytoca*,

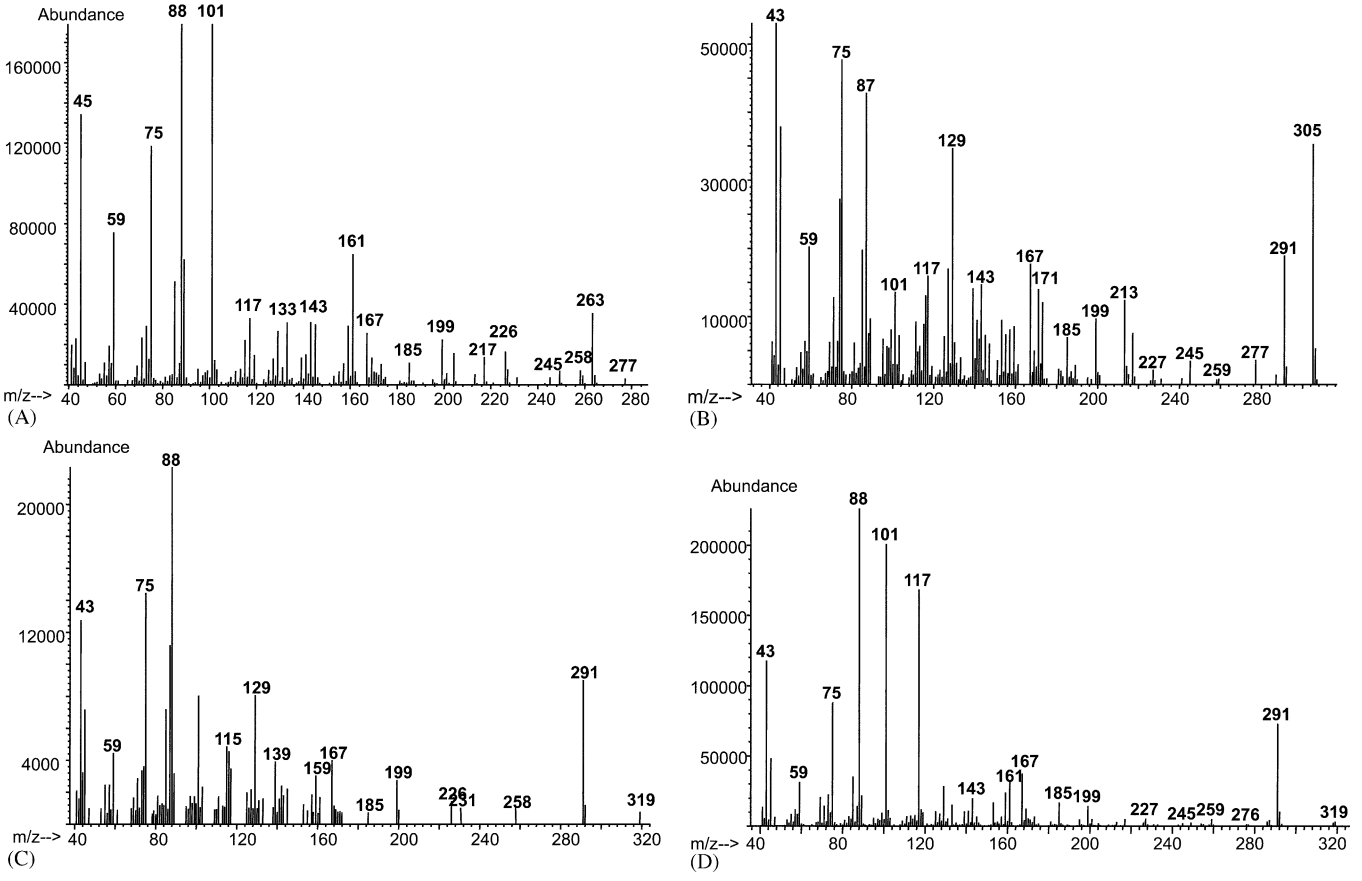

Fig. 2. EI mass spectra of Kdo derivatives 1 (A), 2 (B), 3 (C) and 4 (D)

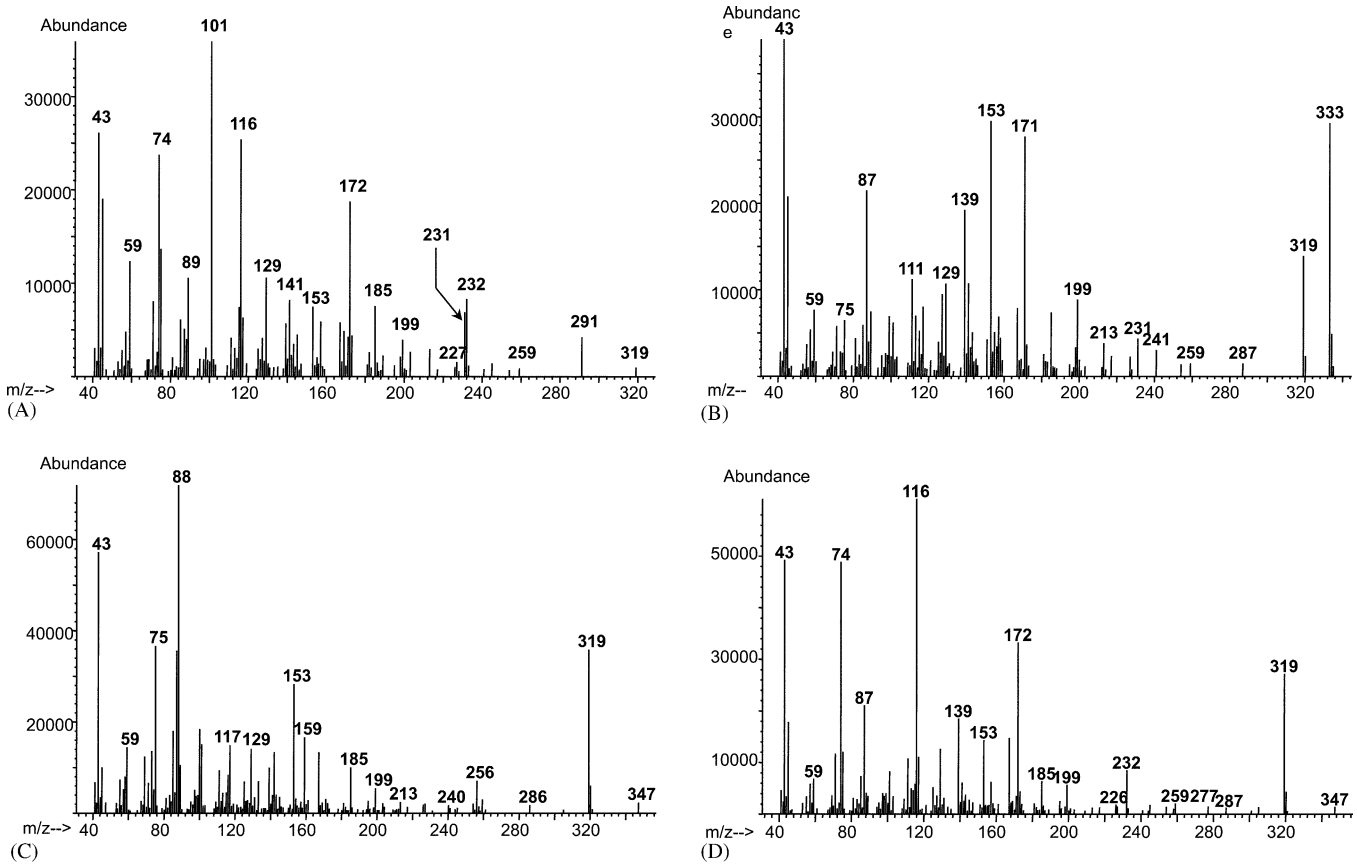

Fig. 3. EI mass spectra of Kdo derivatives 5 (A), 6 (B), 7 (C) and 8 (D)

compared to the presence of only terminal and 4,5-disubstituted Kdo residues in rough LPS of *K. oxytoca*.[36] It could reside either in the inner Kdo region, as in *K. pneumoniae* O1/R20[2] or in the outer part of the LPS core, similarly to a number of serogroups of *Klebsiella pneumoniae*.[3] The 4-substituted Kdo may also originate from a portion of Re-LPS when present in the LPS mixture.

Interestingly, a high proportion of 8-substituted Kdo was observed in the LPS of *E. coli* O56 (Table 1) but also in several other strains including *Citrobacter youngae* O2 and *C. braakii* O37, *Hafnia alvei* PCM 1188, 1190 and 1196. Strains of *H. alvei* display a spectrum of different substitutions with 4- and 4,5-substituted as the major Kdo residues. For example *H. alvei* PCM 1185 also has significant amounts of 7-substituted Kdo, *H. alvei* PCM 1189, 1190 and 1216 contain a high content of 7,8-disubstituted Kdo, *H. alvei* PCM 1186 is rich in 4,7-disubstituted Kdo. These results corroborate previous studies where the presence of such substitutions of Kdo was documented.[23,24,31] Analysis performed on *Shigella sonnei* lipopolysaccharides from a Phase I strain of the S-type and from a Phase II, the spontaneous R-mutant, revealed similar patterns for both the S- and the R-types of LPS (Table 1). A pattern with predominating terminal Kdo was observed in *E. coli* O24 and *C. freundii* O23 LPS.

The Prehm methylation showed higher content of 4,5-substituted Kdo in all the investigated lipopolysaccharides, which might suggest undermethylation. However the presence should not be ruled out of an alkali-labile substituent at position 5 of the 4,5-disubstituted Kdo, or at positions 7 or 8 of terminal Kdo, which is partially cleaved off under the conditions of Hakomori methylation. In case of the presence of a phosphorylated Kdo in LPS, the Kdo derivatives could not be detected in the applied procedure, but instead, the phosphate migration might contribute to the heterogeneity of the patterns of Kdo derivatives. Migration of the phosphate during methylation analysis of Kdo phosphates in LPS was evidenced previously.[17] The methodology adopted in our study did not overcome this problem, despite that the phosphate migration might be avoided by the methylation with methyl triflate.

When comparing results obtained by the three methods of methylation, one could notice that although the method with dimsyl base resulted in the most efficient methylation of LPS, it also produced several low-level derivatives, suggesting degradation or undermethylation. With other methods, the efficiency of methylation was lower but the chromatogram was free

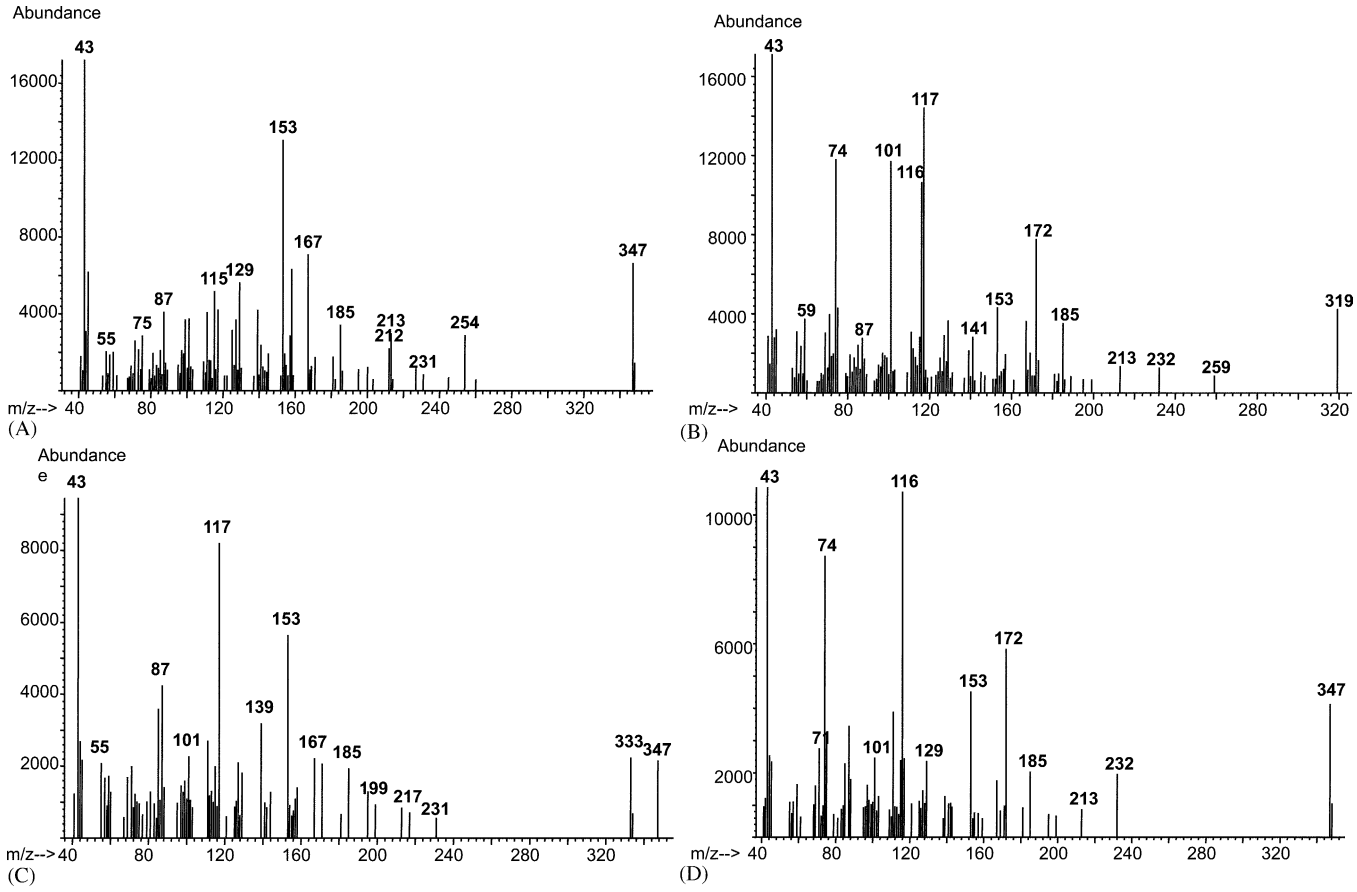

Fig. 4. EI mass spectra of Kdo derivatives 9 (A), 10 (B), 11 (C) and 12 (D)

from other derivatives. Therefore we have performed the analysis of remethylation of an LPS by two procedures, namely with dimsyl base and with methyl triflate. Remethylation of model LPS, *S.* Typhimurium Re, with the first procedure lead to decrease of the low-level derivatives, something that indicates an improvement in methylation efficiency (data not shown). In the case of *E. coli* C600 K12 LPS, we observed a drastic increase of the t-Kdo derivative after remethylation with the dimsyl base procedure. Considering the relatively high level of t-Kdo in most LPS analysis with the dimsyl base procedure, this could suggest a partial degradation of the Kdo region during methylation. Nevertheless, the dimsyl base method is the most efficient in methylation of the whole LPS from all the methods tested.

The evaluated procedure of fast and simple monitoring of Kdo substitutions in bacterial lipopolysaccharides may be useful for structural studies. It might contribute to the studies of structure and function relationship of bacterial lipopolysaccharides. The octulosonic acid (Ko) might be considered in further analyses as well as the synthetic Kdo derivatives to complement the reference mass spectrometric data.

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