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STRUCTURE OF DE-O-ACYLATED LIPOPOLYSACCHARIDE FROM THE ESCHERICHIA COLI RE MUTANT STRAIN F 515

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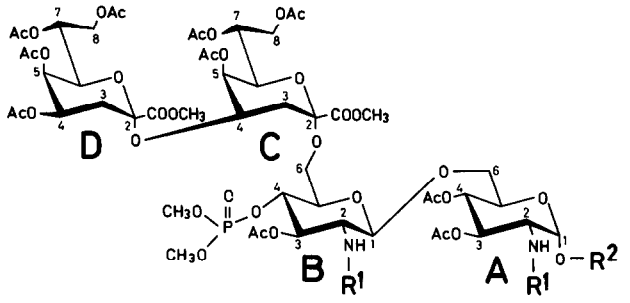
SUMMARY: The structure of the oligosaccharide portion of an E.coli Re lipopolysaccharide was determined as α-KDO-(2→4)-α-KDO-(2→6)-β-GlcN-(1→6)-α-GlcN, bisphosphorylated at positions 1 and 4'. Taking into account the previous determination of the acylation pattern of the GlcN disaccharide, the total structure of E.coli Re LPS was thus established.

The lipophilic part (lipid A) of bacterial lipopolysaccharide (LPS) is known to be responsible for the endotoxic activities of LPS.[1] The structure of lipid A from an Re mutant of E.coli has been elucidated by different analytical approaches [2-5] and finally confirmed by total synthesis. The structure of the hydrophilic oligosaccharide portion has been reported to comprise an α(2→4) linked disaccharide of 3-deoxy-D-manno-2-octulosonic acid (KDO).[8] However, the structural features of the linkage region between KDO and lipid A were not known. Since the KDO-containing region of LPS is of considerable biomedical interest, e.g. as an antigenic structure common to many gram-negative bacteria [9] we have investigated its structure, mainly by NMR spectroscopy. In this communication we present evidence to establish the complete and intact oligosaccharide structure of de-O-acylated LPS from the E.coli Re mutant F 515.

For structural studies the isolation of the homogeneous main component of the E.coli LPS was attempted. Thus, in order to eliminate possible heterogeneity due to incomplete acylation, ester-bound fatty acids were first removed by treating LPS (1.1g) with hydrazine (37°C, 15 min) to give a product designated LPS-OH (0.88g). LPS-OH was, in turn, acetylated (Ac_2O-pyridine-DMAP, room temperature, 24 hrs) to yield acetylated LPS-OH (0.79g), which was methylated with diazomethane to afford the acetylated LPS-OH methyl ester (0.58g). The acetylation procedure had to be repeated after methyl esterification [10] and the product was purified by two steps of silica-gel chromatography [11] followed by two additional semi-preparative HPLC runs.[12] Two homogeneous compounds of the derivatized LPS were obtained (1 35 mg and 2 16 mg). Material 1 corresponds to the main component of the derivatized LPS as confirmed in several TLC test systems.[13] The low yield is mainly due to loss of material during multi-step purification. Chemical analysis indicated that 1 is composed of D-glucosamine (GlcN), KDO, phosphate and (R)-3-hydroxytetradecanoic acid in a molar ratio of 2:2:2:2.[14,15] Since the lipid A backbone consists of a β(1→6)-D-glucosamine disaccharide 1,4'-bis(phosphate), 1 proved to be the expected compound containing two mole KDO in addition. Since very mild conditions were employed for derivatization and isolation, 1 can be assumed to possess the intact sugar skeleton of the original LPS. The two mole of (R)-3-hydroxytetradecanoic acid are likely to be bound to the amino groups of the two GlcN residues. Compound 2, on the other hand, contains the same components as 1 but in a ratio of 2:2:1:2, i.e., in comparison to 1 it lacks one phosphoryl group.[16]



$R^1 = \overset{O}{\overset{\|}{C}}-CH_2-\underset{OAc}{\underset{|}{CH}}-(CH_2)_{10}-CH_3$ $\quad R^2 = \overset{O}{\overset{\|}{P}}(OCH_3)_2$ **1**

$R^2 = Ac$ **2**

Laser desorption mass spectra of 1 and 2 (after adding CsI) revealed quasimolecular peaks $(M+Cs)^+$ at m/z 2116 and m/z 2049 corresponding to molecular weights of 1983 and 1916, respectively. These values are in agreement with data of the above chemical analysis indicating 1 to be the hexamethyl ester of the N,N'-bisacylated dodeca-O-acetyl tetrasaccharide bis(phosphate). Since 2 appeared to correspond to the acetylated product of a monophosphoryl derivative of 1, it was considered as an artifact formed during derivatization or isolation procedures.[16]

Positions and stereochemistry of the linkages between the component sugars in 1 were investigated next. As in the case of a previous study on E.coli lipid A [3] careful analysis of 2D-^{1}H NMR (COSY) at 360 MHz gave quite satisfactory results. The multiple proton signals in the 2D spectrum allowed an almost complete and clear-cut assignment which is summarized in Table 1.[17]

Table 1. Chemical shift values of characteristic sugar protons of 1 (360 MHz, ppm from TMS)

Proton	KDO(D)	KDO(C)	Proton	GlcN(B)	GlcN(A)
3ax	2.02	2.02*	1	4.79	5.65
3eq	2.18	2.02*	2	4.00	4.24
4	5.21	4.72	3	5.08	5.21
5	5.36	5.08	4	4.07	5.43
6	4.17	4.18	5	3.68	4.22
7	5.16	5.08	6	3.75+	3.75+
8	4.52	4.64	6'	3.75+	3.75+

*narrow multiplet, +could not be precisely assigned.

As recognized from the structure of lipid A [3], one KDO residue must be linked to the hydroxyl group on either position 4 of GlcN(A) or position 6' of GlcN(B). The δ-value of H-4A clearly shows that the hydroxyl group in position 4 is acetylated in 1. This leads to the conclusion that KDO is linked to position 6' of GlcN(B)[7] which is further supported by the fact that all methylene signals on H-A6 and H-6B are located around 3.75 ppm indicating the hydroxyl group on B6 not to be acetylated in 1. The chemical shifts of C-6A and C-6B in the ^{13}C NMR spectrum (δ 66.56 and 62.92 ppm, Table 2) further support this interpretation. Signals at 96.81 and

98.54 ppm are assigned to the α-anomeric carbon atoms (C-2C and C-2D) of KDO residues (whereas the signal at δ 100.07 ppm has been attributed to the ß-glycosidic linkage of C-2B). Comparison of the ^{1}H NMR data of 1 in Table 1 with those of model ketosides [18] shows that both KDO residues in 1 adopt the pyranoside form. Their α-ketosidic configuration follows clearly from the ^{13}C NMR line pattern of carbons 4 and 6 [20] (Table 2). Also, small differences in the chemicals shift values of 3ax and 3eq protons and large differences between those of 8-methylene protons in the individual KDO residues are known to be characteristic of α-ketosides [18] further supporting the structure of the KDO-region as shown in 1.

Concerning the determination of the position of the inter-KDO linkage the signal of proton H-4C resonates at obviously higher field (4.72 ppm) than that of H-4D (5.21 ppm) and other model tetra-O-acetyl methyl ketosides.[18] This allows to conclude that the 4-hydroxyl group of KDO(C) participates in the inter-KDO-linkage.[19]

Table 2. ^{13}C NMR chemical shift values of the tetrasaccharide carbons in 1 ($CDCl_3$, 62.9MHz, δ $CDCl_3$ = 77.00 ppm).

Carbon-Atom	KDO(D)	KDO(C)	GlcN(B)	GlcN(A)
1	167.39 h	167.17 h	100.07	96.19 a
2	98.54 f	96.81 f	53.22 c,i	51.50 b
3	31.88 f,g	31.28 f	73.63 d	70.41
4	65.74 h	66.46 h	73.56 e,i	68.10
5	64.26 h	65.16 h	74.31 f	73.56 f
6	68.96 h	69.07 h	62.92	66.56
7	67.50 f,h	67.71 f,h	-	-
8	61.98	61.61	-	-

a) ^{2}J C,P=6.7Hz; b) ^{3}J C,P=8.3Hz; c) ^{3}J C,P≈6Hz; d) ^{2}J C,P≈2Hz; e) ^{3}J C,P=5.8Hz; f) shown with spin echo [21]; g) superimposed by the ω-2 signals of the (R)-3-acetoxytetradecanoic acid; h) assignment of carbons from KDO(C) and KDO(D) in the same line are exchangable; i) assignments exchangable.

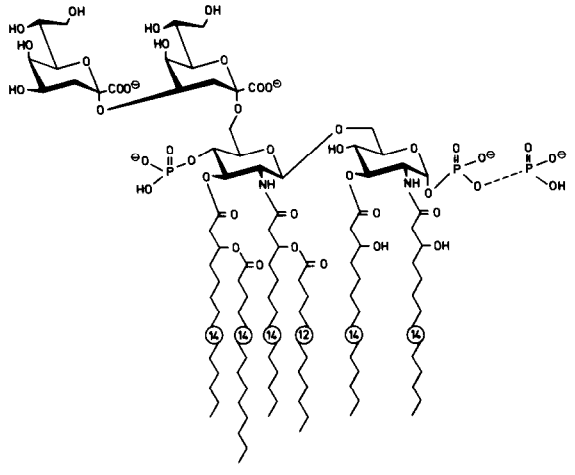


Figure 1. Chemical structure of E.coli Re LPS. Dotted line indicates nonstoichiometric substitution. Numbers in circles refer to the numbers of carbon-atoms in the acyl residue.

These data prove that 1 represents the (derivatized) structure of the main component of LPS derived from the E.coli Re mutant strain F 515 and, together with previous findings [20], they further indicate that 1 represents a common architectural element of enterobacterial Re LPS. The position of acyl and phosphoryl groups in E.coli lipid A (and LPS) has been previously determined. [2-5] Therefore, the complete structure of E.coli Re LPS can now be summarized as shown in Figure 1.

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10 According to TLC analysis ($CHCl_3$-acetone = 2:1) complete O-acetylation of the LPS-OH derivative could only be achieved after the phosphoryl groups had been esterified.

11 i) Column size, 25x4 cm i.d. eluted with $CHCl_3$-MeOH 15:1, ii) Column size, 45x3 cm i.d. eluted with a 1 l linear gradient from CH_2Cl_2-acetone-MeOH 2:1:0 to 15:10:2.5.

12 i) Preparative LC on a Partisil (10 µm) column (500x20.4 mm i.d.) eluted by a 2 l linear gradient from 0 to 56 % (v/v) acetone in $CHCl_3$. ii) Semi-preparative HPLC on a Partisil (5 µm) column (300x6.4 mm i.d.) eluted with EtOAc. The retention times of 1 and 2 were 9.65 and 5.10 min, respectively, at a flow rate of 4.0 ml/min (150 bar).

13 TLC test systems for 1 and 2 on silica-gel plates. Solvent A, $CHCl_3$-MeOH 9:1; Rf=0.55-0.61, solvent B, $CHCl_3$-acetone 2:1, Rf=0.39 and 0.43, solvent C, EtOAc, Rf = 0.31 and 0.49.

14 Compound 1 contained these four components in 0.939, 1.005, 0.934, and 0.959 µmol/mg respectively, while 2 in 0.894, 1.054, 0.506, 1.135 µmol/mg.

15 The presence of two phosphate moities was observed in the ^{31}P NMR spectrum, δ -1.69 and -3.85 ppm from external trimethyl phosphate.

16 The well known acid lability of the glycosidically linked phosphate residue as well as the NMR data of 2 (data not shown) further support this assumption.

17 Chemical shift and coupling constant values of the H-1A (^1H NMR) and C-1A (^{13}C NMR) clearly show the presence of α-glycosyl phosphate on this position. These values are in good agreement with those from the corresponding signals of the 1-α-phosphate monosaccharides, lipid X and lipid Y. The ß-anomeric configuration of GlcN(B) as well as the location of the other phosphate moiety on position 4' was also confirmed by the data.

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19 The present result is in accordance with previous work of H. Brade and E.Th. Rietschel (Ref. 8) but not with that of S. M. Strain et al. (Ref. 7) who proposed an $\alpha(2\rightarrow5)$ linkage between the two KDO residues.

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