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CHEMICAL STRUCTURE OF E. COLI LIPID A: LINKAGE SITE OF ACYL GROUPS IN THE DISACCHARIDE BACKBONE

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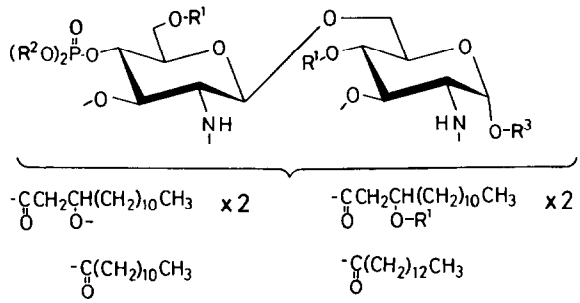
Summary : The structure of the lipid A component of E. coli lipopolysaccharide was determined by means of chemical and 2D-NMR methods unequivocally to be a glucosamine β(1'-6)-disaccharide 1,4'-diphosphate acylated at the two hydroxyl (positions C-3 and -3') and the two amino groups.

Lipid A, which represents the lipophilic portion of bacterial lipopolysaccharides (LPS), is responsible for most of the endotoxic activities of LPS. The chemical structure of lipid A from Salmonella, E. coli and other species was proposed to be the 1,4'-diphosphate of an O,N-polyacylated β(1'-6)glucosamine disaccharide.[1,2] In order to confirm the proposed structure, we prepared 1- and/or 4'-phosphates of the disaccharide which are acylated at two amino as well as the 3,4,6'-hydroxyl groups with various combinations of fatty acids.[3] Although some of the synthetic compounds exhibited typical endotoxin effects, their activity was significantly lower than that of natural lipid A.[4] This fact prompted us to reinvestigate the structure of the natural product. By means of chemical analysis and application of a recently developed NMR technique, we now succeeded to elucidate the structure of E. coli lipid A as 1.



1 $R^1 = R^2 = H$, $R^3 = PO(OH)_2$

2 $R^1 = R^3 = H$, $R^2 = CH_3$

4 $R^1 = R^3 = CH_3CO$, $R^2 = CH_3$

For structural studies, we first attempted to isolate a homogeneous fraction of lipid A. As the starting material, free lipid A from the LPS of an E. coli 08 K27 Re mutant (strain F515) was used. In order to facilitate chromatographic separation, the labile 1-phosphate was removed and the remaining 4'-phosphate esterified. Blocking of the ionic phosphate moiety was also expected to improve the resolution of NMR signals and their assignment.[5]

Free lipid A which was prepared from E. coli Re LPS as described previously[6] showed four major spots on silica gel TLC ($CHCl_3$-MeOH-H_2O-Et_3N, 30:12:2:0.1). Two polar compounds (Rf 0.20 and 0.15) corresponded to 1,4'-diphosphates (positive reaction to Dittmer-Lester reagent[7] but negative to the triphenyltetrazolium chloride reagent[8]); the two less polar compounds (Rf 0.54 and 0.49) were 4'-monophosphates (positive to both reagents).[9] On heating of this mixture in 1% aqueous acetic acid (at 95°C for 15 min), the diphosphates were converted almost completely into the 4'-monophosphates, which were then treated with diazomethane to give the corresponding methyl esters 2 and 3 (Rf 0.45 and 0.40 on silica gel TLC with $CHCl_3$-MeOH, 9:1). They were isolated on a silica gel column ($CHCl_3$-MeOH, 15:1 ; 159 mg of 2 and 85 mg of 3 from 694 mg of the original free lipid A preparation). The result of the component analysis (Table 1) shows that 2 is a monophosphate (dimethyl ester) of a glucosamine disaccharide which contains approximately 4 moles of 3-hydroxytetradecanoic, 1 mole of dodecanoic, and 1 mole of tetradecanoic acid.[10] Compound 3, the composition of which is also shown in Table 1, was not further investiaged because it gave a badly resolved NMR spectrum.

Compound 2 was subjected to NMR analysis. Its ^{1}H-NMR spectrum was much improved as compared to that of the unfractionated free lipid A and several signals were separated at 360MHz. The presence of two amide protons near δ 6.7 ppm indicated that both amino groups of the disaccharide are acylated. The signals however which were still rather broad, many of them overlapping each other, could not be assigned by a simple decoupling method. To overcome this problem, the two-dimensional NMR technique was applied. The J-correlated 2D-NMR spectrum[11] of compound 2 at 360MHz is shown in Fig. 1.[12] Assignment of the proton signals of the reducing glucosamine residue was performed starting from the NHCO proton at δ 6.72 ppm. As shown in Fig. 1, signals of H-2, H-1, H-3, and H-4 could be successively assigned. The signals of the non-reducing glucosamine unit could be correlated from the signal of H-1' to H-5' as indicated. Since the signals of H-4 and H-5' are located at the same position, further correlation of signals to H-5, H-6 and H-6' was not possible. The chemical shift values of protons thus obtained are listed in Table 2. From these values it can be concluded that the hydroxyl groups on positions 3 and 3' are certainly acylated but that the hydroxyl group on the 4-position is not.

In addition, the 2D-spectrum yielded information on the substitution of the 3-hydroxytetradecanoic acid residues in compound 2. Among α-methylene signals of acyl moieties located between δ 2.2 and 2.62 ppm, two signals couple with those at δ 3.99 and 3.88 ppm, respectively, due to β-methines of 3-hydroxy acids, while the other two couple with β-methines of 3-acyloxy

Table 1. Chemical composition of compounds 2 and 3 isolated from E. coli free lipid A.

Compd		Fatty acids *					P	GlcN
		12:0	14:0	16:0	2-OH-14:0	3-OH-14:0		
2	nmol/mg	484	470	10	44	1802	508	1110
	(mol.ratio)	(0.95)	(0.93)			(3.55)	(1.00)	(2.18)
3	nmol/mg	440	126	9	86	1792	640	1170
	(mol.ratio)	(0.69)	(0.19)			(2.80)	(1.00)	(1.82)

* The symbol 12:0 stands for a normal fatty acid with twelve carbon atoms containing no double bond, i.e., dodecanoic acid, 3-OH-14:0 for 3-hydroxytetradecanoic acid, and so on.

acids at δ 5.19 and 5.10 ppm. Consequently, 2 moles of 3-hydroxytetradecanoic acid are further acylated at their hydroxyl groups, i.e., only 4 out of 6 moles of the fatty acids present in the molecule are directly bound to the disaccharide skeleton. The two hydroxyl (on positions 3 and 3') and the two amino groups of the disaccharide are acylated as described above. Therefore, besides the hydroxyl group on C-4 which was already shown to be not acylated, the remaining one on C-6' must be free as well.

Fig. 1. ^{1}H-Homonuclear J-correlated 2D-NMR spectrum of 2 (360MHz in $CDCl_3$).

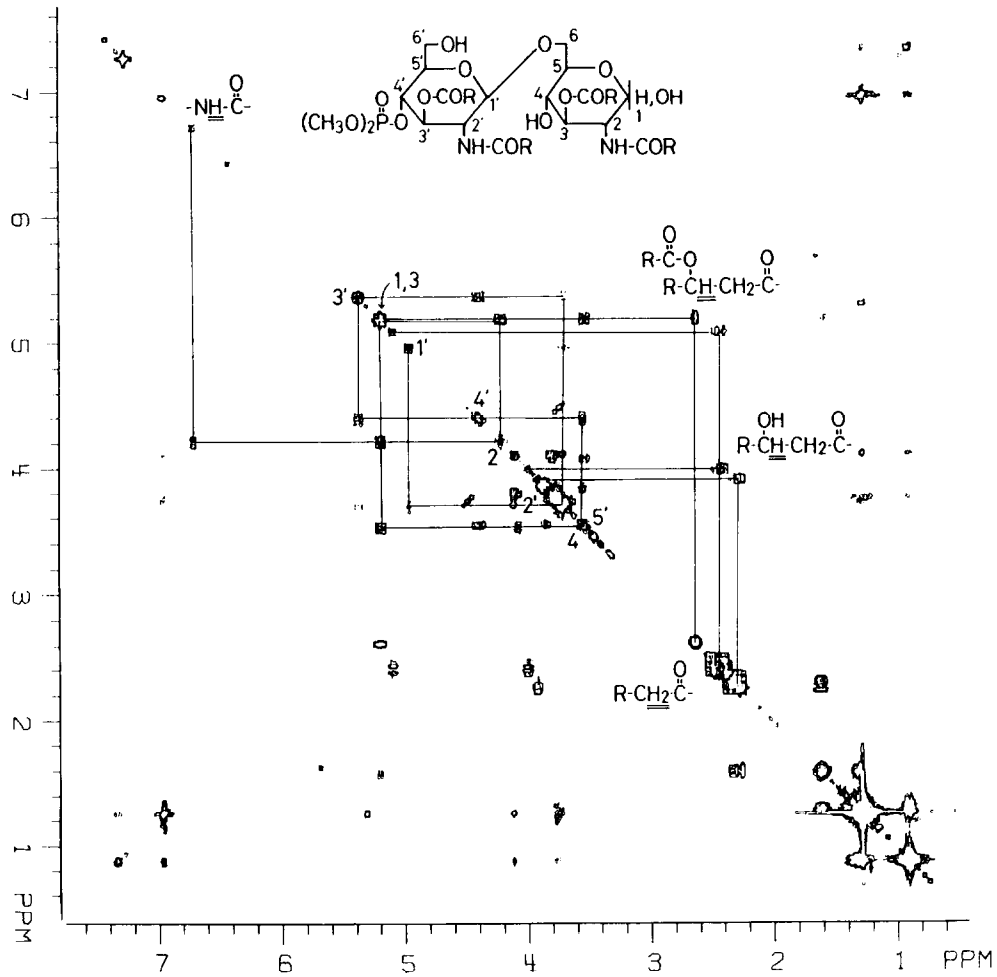


Table 2. Chemical shift values of sugar protons of compounds 2 and 4 (ppm from TMS in $CDCl_3$)

Compd	H-1	H-2	H-3	H-4	H-5	H-6	H-1'	H-2'	H-3'	H-4'	H-5'	H-6'
2	5.19	4.21	5.19	3.55	-	-	4.98	3.72	5.37	4.39	3.55	-
4	6.15	4.37	5.19	5.01	3.95	3.55 3.85	4.88	3.60	5.35	4.41	3.70	4.27 4.35

$J_{1',2'}$ = 8 Hz, $J_{3',4'} = J_{4',5'} = J_{4',P}$ = 10 Hz

Compound 2 was then converted into the peracetate 4 (Ac_2O in $CHCl_3$-pyridine at room temperature). As expected, the presence of five acetyl groups was demonstrated by NMR analysis.[13] In this case, all sugar protons could be assigned by the 2D-technique (Table 2). Comparison of the chemical shift values of the corresponding protons of 2 and 4 further supported the above conclusion as to the positions of acylation.

In addition, the 4'-phosphorylated β(1'-6)disaccharide structure which had been previously proposed[1,2] was confirmed by the NMR data given in Table 2.[14] Consequently, the lipid A component of E. coli LPS possesses the structure 1. Since the 3'-hydroxyl group which was formerly assumed to be the linkage site of the polysaccharide portion was now shown to be acylated, the polysaccharide must be bound to the 6' or 4-hydroxyl group.[15] Although the individual acyl groups bound to the specific positions could not be fixed, the positions of acylation on the disaccharide backbone were clearly demonstrated in this investigation.[16] Approaches are now being undertaken to solve the final structural problem,[17] and synthesis based on the here proposed structure is now in progress in our laboratories.

References and Notes

1) E. Th. Rietschel, C. Galanos, O. Lüderitz, and O. Westphal, Immunopharmacology and the regulation of leucocyte function (ed. by D. Webb), pp. 183, Marcel Dekker Inc., New York and Basel, 1982.
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4) O. Lüderitz, K. Tanamoto et al., Rev. Infect. Des., in press (1983).
5) Because of the tendency to aggregate, natural free lipid A gave poorly resolved NMR spectra, the detailed analysis of which has been so far impossible.
6) C. Galanos and O. Lüderitz, Eur. J. Biochem., 54, 603 (1975).
7) J. C. Dittmer and R. L. Lester, J. Lipid. Res., 5, 126 (1964).
8) F. G. Fischer and H. Dörfel, Hoppe-Seyler's Z. Physiol. Chem., 297, 164 (1954).
9) It is not clear whether the 4'-monophosphate fraction is present in LPS or it is formed artificially from diphosphates during the acid-catalyzed isolation of free lipid A.
10) Elemental analysis of 2 gave the following result. Found : C, 65.08; H, 10.39; N, 1.76 %. Calcd. for $C_{96}H_{181}N_2O_{22}P \cdot H_2O$: C, 65.35; H, 10.45; N, 1.59 %.
11) A. Bax, R. Freeman, and G. Morris, J. Magn. Reson., 42, 164 (1981).
12) The 360MHz 2D-NMR spectra were obtained with Nicolet NT-360 spectrometer.
13) Elemental analysis of 4 also indicated that this compound is a pentaacetate of 2. Found : C, 65.08; H, 9.72; N, 1.37 %. Calcd. for $C_{106}H_{191}N_2O_{27}P$: C, 65.07; H, 9.84; N, 1.43 %.
14) The chemical shifts and the splitting patterns of H-4' of 2 and 4 coincide with those of the corresponding signals of a model compound of 4'-phosphate diester. Cf. M. Inage, H. Chaki, S. Kusumoto, and T. Shiba, Chem. Lett., 1982, 128.
15) This assumption is consistent with the recent demonstration that in Proteus mirabilis and Salmonella minnesota LPS the polysaccharide portion is linked to the 6'-hydroxyl group of lipid A (Z. Sidorczyk, U. Zähringer, and E. Th. Rietschel, Eur. J. Biochem., submitted). It is also in accord with recent findings of Strain et al. (J. Biol. Chem., 258, 2906 (1983).
16) According to previous reports, both amino groups of the disaccharide are acylated with 3-hydroxy fatty acid in E. coli and other lipid A's (ref. 1, 2). However, it is not known yet, whether the 3-hydroxyl group(s) of these hydroxy acids are acylated or not. (see also note 17 below and H.-W. Wollenweber, K. W. Broady, O. Lüderitz, and E. Th. Rietschel, Eur. J. Biochem., 124, 191 (1982).
17) It was recently shown that in E. coli lipid A dodecanoic acid is linked to the 3-hydroxyl group of 3-hydroxytetradecanoic acid amide-bound to the non-reducing glucosamine residue (H.-W. Wollenweber, O. Lüderitz, and E. Th. Rietschel, Eur. J. Biochem., in preparation).

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