^{1}H and ^{13}C NMR Assignments of a Lipopolysaccharide Obtained from the Deep Rough Mutant of *Escherichia coli* D31m4[†]

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ABSTRACT: The hexamethyl derivative of lipopolysaccharide (LPS), obtained by diazomethane methylation of LPS extracted from a deep mutant of *Escherichia coli* D31m4, followed by high-performance liquid chromatographic purification, was subjected to homo- and heteronuclear two-dimensional NMR spectroscopy (DQF-COSY, HOHAHA, NOESY, HMQC and HMBC) to achieve ^{1}H and ^{13}C NMR assignments, particularly of the sugar backbone. These studies confirmed two each of glucosamine (GlcN), 3-deoxy-D-*manno*-octulosonic acid (Kdo), phosphate, β-hydroxymyristate and β-acyloxymyristate and the structure of the tetrasaccharide as α-Kdo$_P$-(2 → 4)-α-Kdo$_P$-(2 → 6)-β-D-Glc$_P$N-(1 → 6)α-D-Glc$_P$N, bisphosphorylated at the 1 and 4′ positions. © 1998 John Wiley & Sons Ltd.

KEYWORDS: NMR; ^{1}H NMR; ^{13}C NMR; rough LPS; *E. coli*

INTRODUCTION

The lipopolysaccharide (LPS),[‡] amphillic macromolecules located in the outer surface of the outer membrane of Gram-negative bacteria, are highly potent stimulators of the immune system. A variety of responses, both beneficial and harmful, can be elicited by LPS. Most of the biological activities of LPS reside in a relatively small portion of the molecule, that is, the terminal disaccharide phospholipid subunit known as lipid A, which is the hydrophobic anchor substance holding a more complex carbohydrate chain to the cell wall.[1,2] The *O*-polysaccharide chain represents the major heat-stable antigen whereas lipid A plays a crucial role in the barrier function of the outer membrane, e.g. to certain antibiotics, but is also responsible for the endotoxicity of Gram-negative bacteria.

The structure of the intact Re-type of LPS (Re-LPS), obtained from heptoseless mutants of *Escherichia coli*, has been the subject of several studies including the ^{13}C and ^{31}P NMR spectroscopic investigation of intact LPS,[3] analysis of free lipid A,[4] the *O*-deacylated Re-LPS and lipid A[5] and liberated KDO units.[6] The complete structure of Re-LPS obtained from the deep rough mutant of *E. coli* D31m4 has recently been determined by combined use of chemical analysis, plasma desorption mass spectrometry and NMR spectroscopy of its hexamethylated derivative.[7] It contains two each of GlcN, Kdo and phosphate, in addition to four β-hydroxymyristic acyl residues (3-OH, 14:0) amide and ester-linked to positions 2, 2′, 3 and 3′ of the β-D-Glc$_P$N-(1 → 6)-α-D-Glc$_P$N disaccharide moiety and ester linked lauric (12:0) and myristic (14:0) residues to the distal GlcN residue at 2′ and 3′ positions respectively. The 6′-hydroxyl group of the distal GlcN residue is α-linked to the α-D-(2 → 4)-interlinked Kdo disaccharide. The structure of the tetrasaccharide backbone was thus proposed as α-D-Kdo$_P$-(2 → 4)-α-D-Kdo$_P$-(2 → 6)-β-D-Glc$_P$N-(1 → 6)-α-D-Glc$_P$N bisphosphorylated at 1 and 4′ positions of the reducing and non-reducing GlcN residues, which was the same as found in Re-mutant *E. coli* F 515[5c] and Re-mutant *Salmonella minnesota* R595.[6b]

The ^{13}C NMR spectrum of hexamethylated Re-LPS, obtained from heptoseless mutants of *E. coli* D31m4, however, exhibits anomeric resonances at δ 98.2, 99.1, 101.4 and 102.2 and resonances at δ 4.30 and 4.62 in its ^{1}H NMR spectrum.[7] The analysis of the DEPT spectrum infers the non-protonated nature of the resonances

‡ Abbreviations used: LPS, lipopolysaccharide; Re-LPS, deep rough chemotype LPS; Kdo, 3-deoxy-D-*manno*-octulosonic acid; GlcN, glucosamine; MM, myristoxymyristoyl; LM, lauroxymyristoyl; COSY, two-dimensional ^{1}H–^{1}H correlation spectroscopy, HOHAHA, homonuclear Hartmann Hahn spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; HMQC, heteronuclear multiple-quantum correlation spectroscopy; HMBC, heteronuclear multiple-bond correlation spectroscopy; α, β and γ refer to carbons and hydrogens of positions 2, 3, 4, respectively, of the β-acyloxymyristoyl group, and also to lauric and myristic acid groups.

at δ 98.2 and 99.1 and the monoprotonated nature of the resonances at δ 101.4 and 102.2. Therefore, the former pair correspond to C-2 of Kdo and the latter pair to C-1 of GlcN residues.[7] However, there were diagnostic differences in the observed ^{1}H and ^{13}C NMR data, particularly for the reducing end GlcN residue for underivatized Re-LPS[3] and reported literature values and for related lipid A having a bisphosphorylated β-D-Glc$_p$N-(1 $\rightarrow$ 6)-α-D-Glc$_p$N disaccharide backbone. In all of these cases,[8] H-1 of the reducing end Glc$_p$N residue appears as a doublet of doublets ($^3J_{1,2}$ = 3.5 Hz, $^3J_{1,P}$ = 6–7.5 Hz) at δ 5.2–5.9 and the respective anomeric carbon appears at δ 94–95. These ambiguities initiated our interest in reinvestigating ^{1}H and ^{13}C NMR spectroscopic data for hexamethylated Re-LPS, obtained by methylation of LPS from the deep rough mutant of *E. coli* D31m4,[7] because it has been reported that methylation of negatively charged phosphate groups is essential for obtaining clear and well resolved NMR and because it does not alter the anomeric configurations.[9]

EXPERIMENTAL

Hexamethylated Re-LPS (**1**), available from earlier studies,[7] was exchanged three times in D$_2$O (99.9 atom% D) followed by lyophilization. The final solution was prepared by dissolving **1** in high purity (99.96 atom% D) methanol. The 1D and 2D experiments were carried out at 25 °C on a GE GN-500 spectrometer under standard experimental conditions as reported earlier.[10] Chemical shifts are referenced to TMS. Processing of the NMR data was carried out on a VAX station 3200 and on a personal Iris computer, using the FTNMR program (Hare Research, Woodinville, WA, USA).

RESULTS

Assignment of the ^{1}H NMR spectra

The ^{1}H NMR spectrum of **1** shows one doublet ($^3J_{1,2}$ = 7.8 Hz) at δ 4.62, a broad doublet at δ 4.33 for the anomeric protons, a broad signal at δ 1.20 and a triplet-like signal at δ 0.81, characteristic of chain methylene and terminal methyl groups of lipid residues. The process of assigning the ^{1}H NMR resonances was performed by following a standard procedure,[11] i.e. COSY and TOCSY spectra (Fig. 1) were first examined in order to detect scaler coupled protons belonging to same monosacchride residue. These allow the anomeric resonances appearing at δ 4.33 and 4.62 to be assigned to H-1 of the GlcN residues designated as residues A and B, respectively, and also the assignment of most of the spin systems belonging to the individual residues. The chemical shifts of H-2 and H-3 of GlcN residues suggest the presence of acylamino and acyloxy substituents at these positions. The appearance of H-4 at δ 3.46 in residue A and at δ 4.13 in residue B demonstrates that OH-4 is unsubstituted in the former whereas it is phosphorylated in the latter. The β-anomeric configuration of residue A is evident: (a) by the appearance of H-1 as a broad doublet (J = 8.6 Hz) in a 1D ^{1}H NMR spectrum, due to unresolved $^3J_{1,2}$ and $^3J_{1,P}$ cuplings; (b) intra-residual NOE connectivities between H-1 and H-3 and H-5 observed in the 2D NOESY spectrum and (c) the anomeric ^{13}C chemical shift of δ 102.2 from the HMQC spectrum. The analysis of the DQF-COSY spectrum: (a) The H2/H1 cross peak in the DQF-COSY spectrum shows $^3J_{1,2}$ = 7.5 Hz as an active and $^3J_{1,P}$ = 2.7 Hz as a passive coupling; and (b) the H1/H2 cross peak exhibits splitting of an anti-phase component ($^3J_{1,2}$ = 7.5 Hz) via passive components ($^3J_{2,3}$ = 12.7 Hz, $^3J_{2,P}$ = 2.6 Hz), in agreement
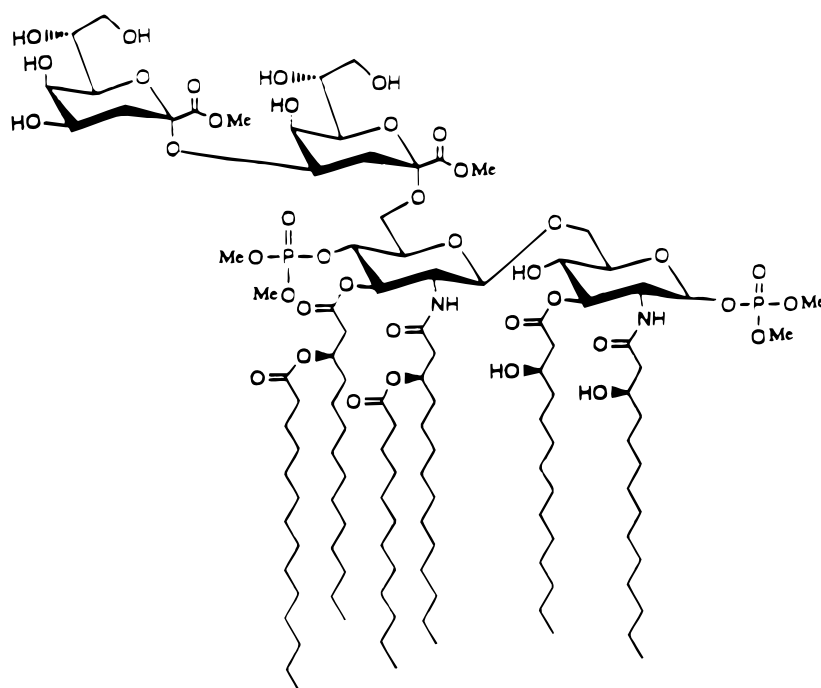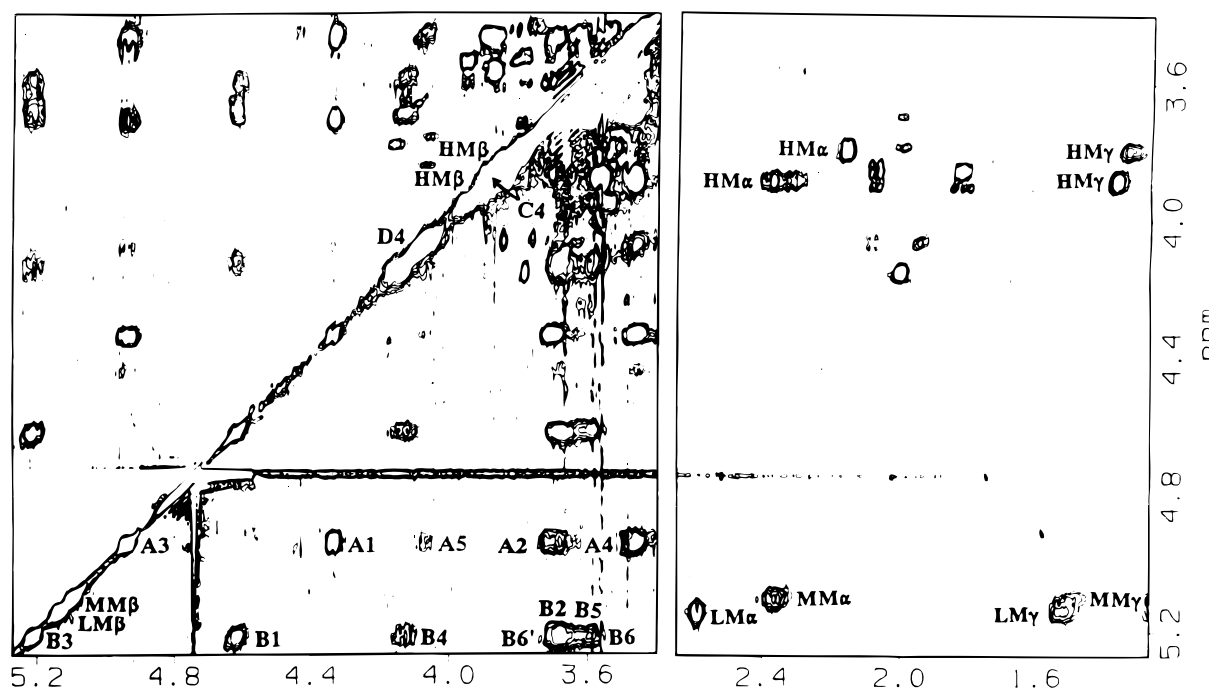
Figure 1. TOCSY spectrum of hexamethylated Re-LPS of E. coli.

with the assigned β-anomeric configuration. All of these features led us to infer the identification of the residue A as 2,3-diacylated β-D-glucosaminopyranose having a glycosidic phosphate. In a similar manner, analysis of the DQF-COSY and NOESY spectra led to the establishment of β-anomeric configuration of the other GlcN residue and subsequently residue B was identified as 2,3-diacylated-4-phosphorylated-β-D-glucosamino-pyranose.

A survey of the literature[8] reveals that the chemical shifts of the H-2 for reducing and non-reducing GlcN residues in the case of lipid A having β-D-GlcpN-(1 → 6)-α-D-GlcpN disaccharide bisphosphorylated at the 1 and 4' positions as the parent skeleton differ remarkably and H-2 of the reducing α-GlcN residue appears to low field by 0.3–0.5 ppm relative to the chemical shift of the non-reducing β-GlcN residue. The H-2 of both GlcN residues was observed at 3.695 ± 0.005 ppm in the present studies, which further supported the assigned β-anomeric configuration.

The distinctive peaks in the region 1.8–2.3 ppm, where deoxy protons (H$_2$-3) of Kdo would be expected to resonate,[12] could not be assigned directly from the 1D ^{1}H NMR spectrum owing to overlapping of these resonances with the α-methylene resonances of β-hydroxymyristoyl, lauroyl and myristoyl groups. In the COSY spectrum, the resonances at δ 2.08 and 1.82 exhibit correlations with the resonances at 3.86 ppm. The cross-section taken along the chemical shift of the lower field resonances at δ 2.08 reveals geminal coupling ($^2J_{3a, 3e} = -13.0$ Hz) and a small passive coupling ($^3J_{3e, 4} = 4.8$ Hz). Therefore, it could be assigned to H-3e of the Kdo residue. Cross-sections taken along the chemical shifts of H-3e and H-3a in 2D HOHAHA spectrum, in addition to the cross peaks to H-4, show a

cross peak to H-5 at δ 3.90 at a lower contour level. Connectivities beyond the H-5 resonance were not readily visible owing to small $J_{5, 6}$ (>1 Hz) coupling.[13] The assignment of the resonances at δ 2.08, 1.95, 4.09 and 3.77 to H-3e, H-3a, H-4 and H-5 of the other Kdo residue was also mapped out in an analogous manner. The consideration of the vicinal coupling constants $^3J_{3a, 4}$, $^3J_{3e, 4}$ and $^3J_{4, 5}$ and the chemical shifts of H-3e, H-3a, H-4 and H-5 imply a chair conformation of the pyranoside ring of both of the Kdo residues.[12,13] The chemical shift difference between methylene protons ($\Delta\delta_{3e-3a}$) was 0.25 and 0.14 ppm, which was characteristic of an α-anomeric configuration.[12,13] Further connectivity between H-6 to H-8 could not be traced owing to strong coupling and ^{1}H NMR assignments for the rest of the resonances were achieved by assigning ^{13}C resonances in analogy with the reported literature values,[10,12,13a] following the analysis of the HMQC spectrum. Therefore, these could be considered as tentative. The ^{1}H NMR data for GlcN and Kdo residues are summarized in Table 1.

The oxymethine resonances at δ 5.13 and 5.09 exhibit cross peaks at δ 2.58 and 1.53 and at 2.36 and 1.51, respectively, in the COSY spectrum. The cross-peak connectivities between the resonances at δ 2.58 and 2.36 with those at δ 5.13 and 5.09 led to the assignment of the former pair of resonances to β-methine resonances of the acyloxyacyl LM and MM residues acylated at the 2 and 3 positions of residue B. In an analogous manner, the cross-peak correlation allows the assignment of the resonances at δ 1.53 and 1.51 to the methylene resonances at the γ position. It is known from mass spectral fragmentation[7] and chemical degradation studies[5c] that lauroyl (12:0) and myristoyl (14:0) groups are the acyloxy substituents acylated at the β-hydroxyl group

Table 1. NMR chemical shifts of the tetrasaccharide of LPS obtained from deep rough mutant of *E. coli* D31m4

	Residue			
Assignment	β-GlcN A	β-GlcN B	α-Kdo C	α-Kdo D
[1]H:				
H-1	4.33	4.62	—	—
H-2	3.70	3.69	—	—
H-3	4.94	5.21	2.08	2.08
H-3	—	—	1.82	1.95
H-4	3.46	4.13	3.86	4.09
H-5	4.06	3.69	3.90	3.77
H-6	3.84	3.59	3.46[a]	3.48[a]
H-6′	3.89	3.71	—	—
H-7	—	—	3.58[b]	3.67[b]
H-8	—	—	3.79[c]	3.70[c]
H-8	—	—	3.79[c]	3.90[c]
[13]C:				
C-1	102.27	101.48	175.5	176.7
C-2	52.27	54.81	98.6	99.5
C-3	76.25	73.18	34.6	33.3
C-4	68.70	75.53	70.5	69.3
C-5	69.39	73.31	66.8	68.9
C-6	68.58	62.84	74.38[a]	75.68[a]
C-7	—	—	71.58[b]	73.21[b]
C-8	—	—	64.78[c]	64.91[c]

[a,b,c] Assignments bearing the same superscript can be exchanged in a row.

of β-hydroxymyrsitoyl residues and are substituted at positions 2 and 3 of residue B. The presence of lauric and myristic acid (2 mol of saturated acid) can be deduced from the cross-peak connectivities observed between the regions 2.0–2.3 and 1.48–1.52 ppm in the COSY spectrum, which in turn were correlated with the methylene protons at δ 1.23–1.25. Therefore, these correspond to the methylene resonances of the α, β and γ positions of these saturated fatty acid residues forming the acyl portion of the acyloxyacyl (LM and MM) groups. A similar approach has been used to identify α,

β and γ resonances of the β-hydroxymyristic acid [14:0, 3-OH (HM)] residues esterified at the 2 and 3 positions of the reducing end GlcN reside A. Table 2 presents ^{1}H NMR assignments of the lipid residues. Further assignment of proton resonances of the long chain is hindered because of the large number of overlapping resonances.

Assignment of the ^{13}C NMR spectra

Since we have achieved unambiguous assignments for most of the ^{1}H NMR resonances, ^{13}C resonances are assigned in a straightforward manner by the analysis of the HMQC spectrum. It is important to mention that none of the anomeric ^{1}H NMR resonances exhibit a single-bond correlation with a ^{13}C resonance at δ 93–95, which would be expected if residue A possessed an α-glycosidic phosphate,[8] but instead the ^{1}H NMR resonances at δ 4.33 and 4.62 exhibit a single-bond correlation with the ^{13}C resonances at δ 102.27 and 101.48, respectively. The H-3 of GlcN residues at δ 5.21 (residue A) and 4.94 (residue B) exhibit a correlation with the ^{13}C resonances at δ 73.18 and 76.25, which is in conformity with the fact that these positions are O-acylated. The unsubstituted position at C-4 of residue A is evident from the correlation of H-4 (δ 3.46) with the ^{13}C resonance at δ 68.70, and correlation of H-4 of residue B at δ 4.13 with the ^{13}C resonance at δ 75.53 is in agreement with the phosphorylation at C-4 position of residue B.[8a] The correlation of H_2-6s of residue A with the ^{13}C resonance at δ 68.58 demonstrates its involvement in $(1 \rightarrow 6)$-interglycosidic linkage with GlcN residue B due to the α-effect of glycosylation by an aldopyranosyl residue.[14] The correlation of H_2-6s of residue B with the ^{13}C resonance at δ 62.84 reflects the presence of an α-$(2 \rightarrow 6)$-interglycosidic linkage between the proximal Kdo (residue C) and the non-reducing GlcN residue (residue B), as glycosylation by a ketosidic sugar exhibits a negligible glycosylation-induced α-effect.[8a] The confirmatory evidence for the α-$(2 \rightarrow 6)$-

Table 2. NMR chemical shifts of the lipid residues of LPS obtained from deep rough mutant of *E. coli* D31m4

	Residue[a]					
	LM-2		MM-3			
Assignment	L	M	M	M	HM-2	HM-3
[1]H:						
Hα	2.20	2.58	2.25	2.36	2.31	2.16
Hβ	1.52	5.13	1.51	5.09	3.87	3.78
Hγ	1.23–1.25	1.53	1.23–1.25	1.51	1.36	1.33
[13]C:						
Cα	34.7	38.68	34.6	40.02	42.57	44.12
Cβ	25.5	70.14	25.5	70.91	68.58	68.88
Cγ	30.1	34.4	30.1	34.4	37.4	37.4

[a] Abbreviations: LM = lauroxymyristoyl (L = lauroyl substituent of LM, M = β-acyloxymyristic substituent of LM); MM = myristoxymyristoyl (M = myristoyl substituent of MM, M = β-acyloxymyristic substituent of MM); HM = β-hydroxymyristoyl.

interglycosidic linkage was provided by HMBC data, as discussed below.

The ^{13}C resonance at δ 14.1 is assigned to terminal methyl groups due to its correlation with the ^{1}H resonance at δ 0.81. In analogy with the reported ^{13}C NMR shielding data for aliphatic compounds,[15] the ^{13}C resonances at 22.9 and 32.3 ppm were assigned to β and γ methylene resonances with respect to the terminal methyl group whereas the ^{13}C resonance at δ 30.1 corresponds to most of the chain methylene carbons of all of the fatty acid residues and correlates with the ^{1}H NMR resonance at δ 1.20 in HMQC spectrum. The ^{13}C resonances at δ 70.14, 70.91, 68.58 and 68.88 were ascribed for β-methine carbon resonances of MM, LM and both HM residues due to their correlation with the ^{1}H resonances at δ 5.12, 5.09, 3.87 and 3.78, respectively. Thus, acylation of the β-hydroxyl group of the HM residue causes a downfield shift of the methine resonance by 1.05 ± 0.06 ppm in the ^{1}H NMR and 1.41 ± 0.5 ppm in the ^{13}C NMR spectrum. A comparison of the ^{13}C NMR chemical shifts of the α and γ methylene resonances of LM and MM residues esterified at the 2 and 3 positions of residue B with the ^{13}C NMR chemical shifts of HM residues esterified at the 2 and 3 positions of residue A reflects 4.0 ± 0.1 and 3.0 ppm upfield shifts. These effects were consistent with the reported values for esterification-induced shifts.[16] A similar comparison of the ^{1}H NMR chemical shifts of the α and γ methylene resonances reveals that esterification causes downfield shifts of these resonances by 0.15–0.34 ppm. An understanding of these effects may be of significance for determining the number of acyloxyacyl and hydroxyacyl residues in lipid A.

Establishment of interglycosidic linkage and sequence

As already discussed, ^{13}C chemical shifts have provided some preliminary information concerning interglycosidic linkages, but sequence specific assignment was based on the NOESY and HMBC data. Residue A represents the reducing end GlcN residue, as is evident from the NOE between the anomeric H at δ 4.33 and the *P*-OMe groups. The inter-residual NOE between the anomeric resonance at δ 4.62 and H_2-6 of residue A reflects the (1 → 6)-interglycosidic linkage between residues B and A. The H_2-3 proton signals of Kdo residues (residues C and D) at δ 2.08 and 1.82 and δ 2.08 and 1.95 exhibit their HMBC connectivity with the anomeric resonances at δ 98.6 and 99.5, respectively. The non-applicability of the HMBC method for the determination of the interglycosidic linkage in Kdo-containing polysaccharides has recently been recognized.[17] In the present study, we could surprisingly observe an HMBC correlation peak between H_2-3 of proximal Kdo to H_2-6 of residue B, but cross peaks relevant to the (2 → 4)-inter Kdo linkage could not be seen in the HMBC and NOESY spectra owing to either unfavorable long-range C–H coupling constants or to the quat-

ernary nature of the anomeric carbon of the Kdo residue. A cross-section taken along the chemical shift of anomeric resonance at δ 98.6 shows HMBC correlations to H_2-6 protons (δ 3.71 and 3.59) of residue B, confirming the identification of residue C as the proximal Kdo residue. The assignment was supported (a) by the HMBC cross peak between the carboxymethyl group at δ 3.34 and the anomeric resonance at δ 98.6 and (b) the HMBC correlations observed between C-3 methylene proton resonances at δ 2.08 and 1.82 with the C-6 resonance of residue B at δ 62.84. Thus the methylene proton resonances at δ 2.08 and 1.95 correspond to H_2-3 of the proximal Kdo residue. The C-4 and C-5 resonances of residue C, observed at 1.2 ppm lower field and 2.1 ppm higher field relative to the chemical shift of C-4 and C-5 of residue D, are in accordance with the (2 → 4)-interglycosidic linkage between the Kdo residues. The ^{13}C NMR assignments for the tetrasaccharide parent skeleton and lipid moieties are presented in Tables 1 and 2, respectively.

DISCUSSION

The initial characterization by plasma desorption mass spectrometry yielded the molecular weight of Re-LPS and permitted confirmation of molar ratios of the subcomponents as determined by conventional chemical analyses. Interestingly, the HMQC data exhibit a correlation of the anomeric ^{1}H resonances with the ^{13}C resonances at the same position as reported previously, which clearly demonstrates that we are investigating the same methylated sample of Re-LPS which has been studied by Qureshi *et al.*[7] We utilized homo- and heteronuclear correlation experiments for the elucidation of the glycosyl and anomeric configurations, interglycosidic linkages and the location of acyl groups and phosphoester substituents. The ^{1}H resonance assignments for both of the GlcN residues were achieved by the analysis of the DQF-COSY and HOHAHA spectral data. The chemical shifts of the H-2 and H-3 of both GlcN residues confirm the previous findings of the presence of acyl substituents at N-2 and O-3 positions. The analysis of the DQF-COSY spectrum also confirms the presence of two hydroxyacyl and two acyloxyacyl substituents, which based upon mass spectrometric evidence have been identified as two HM, an LM and an MM residue. A comparison of the chemical shifts of H-4 of both GlcN residues revealed a downfield shift 0.67 ppm of H-4 of the $GlcN_{II}$ residue, which confirms that OH-4 of the $GlcN_{II}$ residue is phosphorylated whereas OH-4 of the reducing end $GlcN_I$ residue is unsubstituted.

The inter-residual NOE connectivity observed between the anomeric resonance of the $GlcN_{II}$ residue and H_2-6 resonances of the $GlcN_I$ residue, together with the ^{13}C NMR chemical shift of C-6 of $GlcN_I$ at δ 68.58, were in complete agreement with the earlier proposed (1 → 6)-interglycosidic linkage between GlcN residues.

However, our investigations on the anomeric configuration of the glycosidic phosphate are contrary to the disposition of the glycosidic phosphate group reported earlier.[8] It is significant that H-1 of the reducing end GlcN residue appears as a doublet of doublets ($^3J_{1,2}$ = 3.5 Hz, $^3J_{1,P}$ = 6–7.5 Hz) at 5.2–5.9 ppm and the respective anomeric carbons appear at 94–95 ppm in lipid A and other lipopolysaccharides having a 1,4'-bisphosphorylated β-D-GlcpN-(1 → 6)-α-D-GlcpN disaccharide backbone.[10] The intra-residual NOE in the 2D NOESY spectrum and analysis of the DQF-COSY spectrum led us to infer a β-anomeric configuration of the glycosidic phosphate. In earlier studies, it was not possible to assign the anomeric resonances (C-2) of Kdo residues and the assignments were generally based on the chemical shift analogies. However, in the present studies, the two-bond heteronuclear correlations observed between H$_2$-3 and C-2 in the HMBC data provide definitive evidence for the differentiation between the Kdo residues. Despite the fact that the ^{13}C chemical shifts of C-3, C-4 and C-5 of a proximal Kdo residue identify a (2 → 4)-interglycosidic linkage, the HBMC and NOESY data do not show long-range heteronuclear and homonuclear correlation evidence for the inter-Kdo linkage. This suggests the applicability of the glyosylation-induced shift method and a limitation of HMBC and NOESY methods for confirmation of the glycosylation site in Kdo-containing oligosaccharides. The inter-residual HMBC cross peak observed between H$_2$-3 of the proximal Kdo residue and C-6 of the GlcN$_{II}$ residue not only confirmed the (2 → 6)-interglycosidic linkage but also led to the differentiation of the proximal and distal Kdo residues. Hence the structure of the tetrasaccharide backbone for the sample of Re-LPS with which Qureshi *et al.*[7] were dealing could be established as α-Kdo$_P$-(2 → 4)-α-Kdo$_P$-(2 → 6)-β-D-GlcpN-(1 → 6)-β-D-GlcpN, bisphosphorylated at the 1 and 4' positions.

Based upon ^{13}C NMR studies, Qureshi *et al.*[7] suggested the existence of Kdo units as a tautomeric mixture but we could observe correlations consistent with the pyranosidic form of Kdo and could not observe any evidence for the existence of Kdo in furanosidic form. Hence the present investigations suggest that, despite the fact that methylation of the phosphate group has been recommended as a desirable derivatization method for the structural analysis of lipid A by NMR spectroscopy, one must be careful in characterizing the product. However, a question still remains of the stage, either during methylation or purification, at which epimerization of the glycosidic phosphate has occurred.

CONCLUSION

Two-dimensional NMR methods are effective in the complete assignment of carbohydrate and α, β and γ resonances of the lipid chains of Re-LPS at the intact level. They also provide evidence for the determination of acyloxyacyl and hydroxyacyl residues. Since these methods minimize the need for chemical degradation and spectroscopic measurements do not degrade the sample, investigation by homo- and heteronuclear NMR should be considered a first step after the isolation of a homogeneous sample. Since HMQC is a proton-detected technique and Kdo represents the only ketoside found in LPS, a comparison of HMQC and ^{13}C NMR or HMQC and HMBC spectra identifies the number of aldosidic and ketosidic sugar residues. The obvious limitation of NMR methods is that the chain length of the individual fatty acid cannot be determined. Therefore, a combination of NMR and mass spectrometric investigations may be a possible solution to the structural elucidation of lipid A and lipopolysaccharides, at least of the Re type. This is part of the total study of the properties of highly purified Re-LPS obtained from *E. coli* D31m4. Since it was first described by Qureshi *et al.*,[7] the physical properties,[18] the x-ray diffraction pattern at various states of hydration[19] and the biological properties[1,20] have all been determined for this model 'toxic' LPS. This paper represents our attempt to describe further the complete structure based on NMR analysis. We can now state that the Re-LPS from *E. coli* is the most highly characterized LPS known today.

Acknowledgements

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