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ISOLATION AND CHARACTERIZATION OF THE TETRASACCHARIDE (BIS)PHOSPHATE FROM THE GLYCOSYL BACKBONE OF *SALMONELLA MINNESOTA* AND *ESCHERICHIA COLI* RE-MUTANT LIPOPOLYSACCHARIDES

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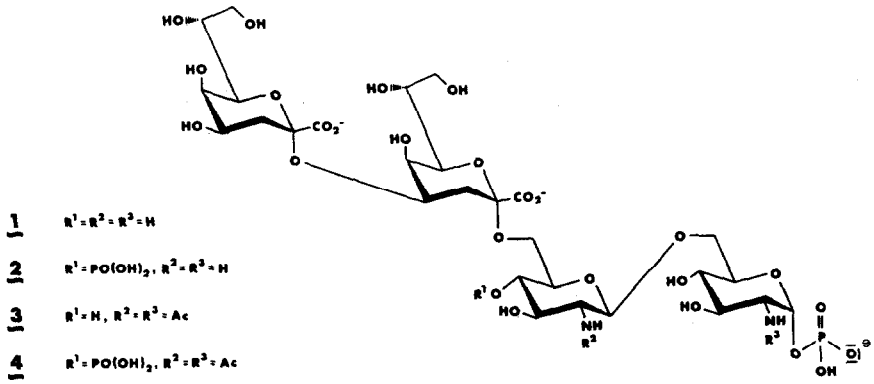
Summary: Intact tetrasaccharide mono- (1) and bisphosphates (2) were isolated from deep rough mutant strains of *Salmonella minnesota* (mutant R595) and *Escherichia coli* (mutant F515) and their chemical structure was unequivocally characterized by ^{1}H-, ^{13}C- and ^{31}P-NMR spectrometry and FAB-MS and chemical analysis as 1 and 2



INTRODUCTION

Chemical degradation procedures towards oligosaccharides derived from de-O- and de-N-acylated lipopolysaccharides by strong hydrazinolysis[1] hamper the disadvantage of poor yields and partly dephosphorylated oligosaccharides. We here describe a new approach towards the isolation and purification of lipopolysaccharide (LPS)-derived oligosaccharide phosphates in acceptable yields. Their structures, including the position of the phosphate(s), could be unequivocally determined by means of ^{1}H-, ^{13}C-, ^{31}P-NMR, and FAB-mass spectrometry.

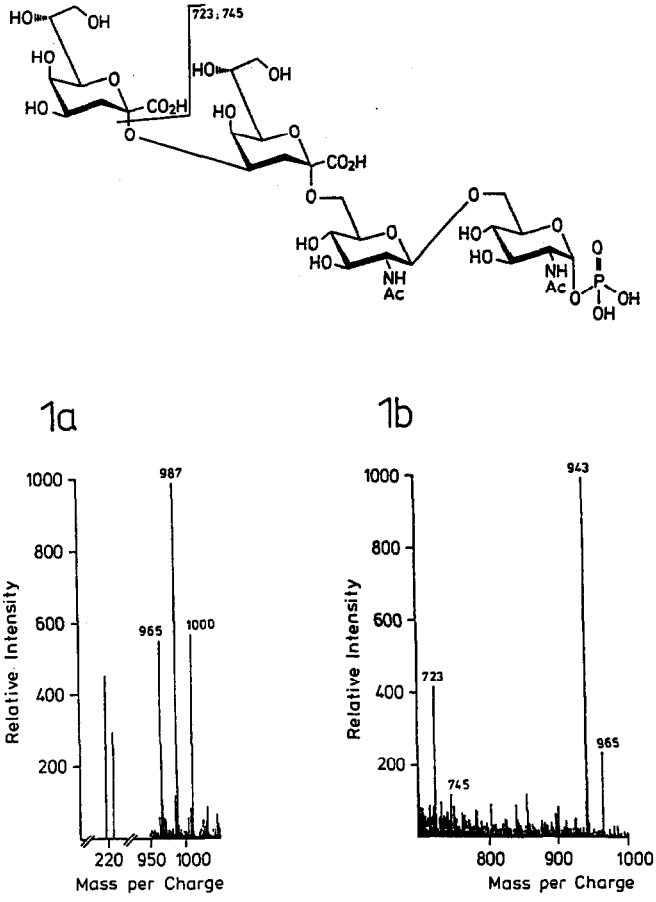


Fig. 1 Negative ion FAB-MS and chemical structure of the *N*-acetylated tetrasaccharide monophosphate **3**. (1a) The native sample revealed mainly pseudomolecular mass peaks with 1 Na^+ (*m/z* 965), 2 Na^+ (*m/z* 987) and 3 Na^+ (*m/z* 1009) attached. (1b) Addition of 2M HCl to the matrix supressed attachment of Na^+ giving rise to the molecular ion *m/z* 943 [$(M-H^+)^-$] as the predominant peak.

RESULTS AND DISCUSSION

LPS from the deep rough (Re) mutant strain R595 of *Salmonella minnesota*, after de-O-acylation and de-*N*-acylation, and revealed upon ion-exchange HPLC on CarboPac PA1, revealed two main peaks containing about 75% of the total thiobarbituric acid (TBA) reactive material. The first peak (0.3 M NaOAc, t_R = 61 min) contained D-glucosamine (GlcN), 3-deoxy-D-*manno*-2-octulosonic acid (Kdo), and phosphate (*P*) in a molar ratio of 2:2:1. This compound was later identified as **1**. The second peak (0.55 M NaOAc, t_R = 81 min) contained the same components in a molar ratio of 2:2:2 and was identified as **2**. In contrast analysis of the LPS of *Escherichia coli* Re mutant strain F515 yielded only **2** as no monophosphate was identified in the HPLC eluate.

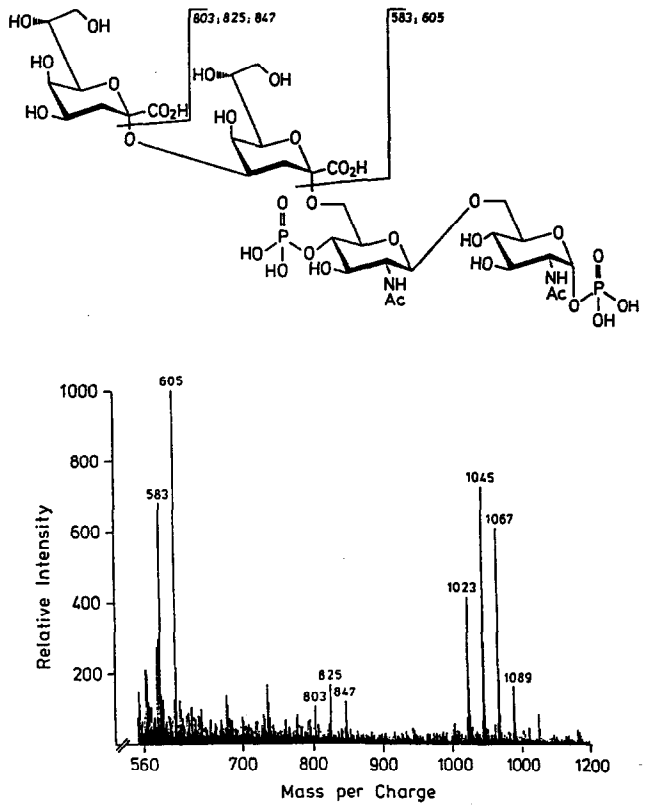


Fig. 2 Negative ion FAB-MS and fragmentation pattern of the *N*-acetylated tetrasaccharide bisphosphate **4** after addition of 2M HCl to the matrix.

FAB mass spectrometry.

The *N*-acetylated tetrasaccharides monophosphate **3** revealed a clear negative ion FAB mass spectum (Fig. 1) with pseudomolecular ions at $m/z = 965$ ($[M+Na^+-2H^+]^-$), 987 ($[M+2Na^+-3H^+]^-$), and 1009 ($[M+3Na^+-4H^+]^-$) for the native compound (Fig. 1a). Exchange of cations for protons [after addition of 1 μl of 2M HCl to the liquid matrix (thioglycerol)] gave rise to a molecular ion at $m/z = 943$ ($[M-H^+]^-$)(Fig. 1b). Under these conditions the fragment ion at $m/z = 723$ and 745, arising from the loss of one Kdo residue were also present. A similar observation was made after addition of HCl to the tetrasaccharide bisphosphate **4**, whereafter $m/z = 1023$ ($[M-H^+]^-$), 1045 ($[M+Na^+-2H^+]^-$) and 1067 ($[M+3Na^+-4H^+]^-$) and 1089 ($[M+4Na^+-5H^+]^-$) were observed (Fig. 2). In contrast, the native tetrasaccharides **1** and **2** containing free NH_2-groups revealed a number of ions in the molecular ion area, arising from the presence of sodium and potassium counter ions (data not shown).

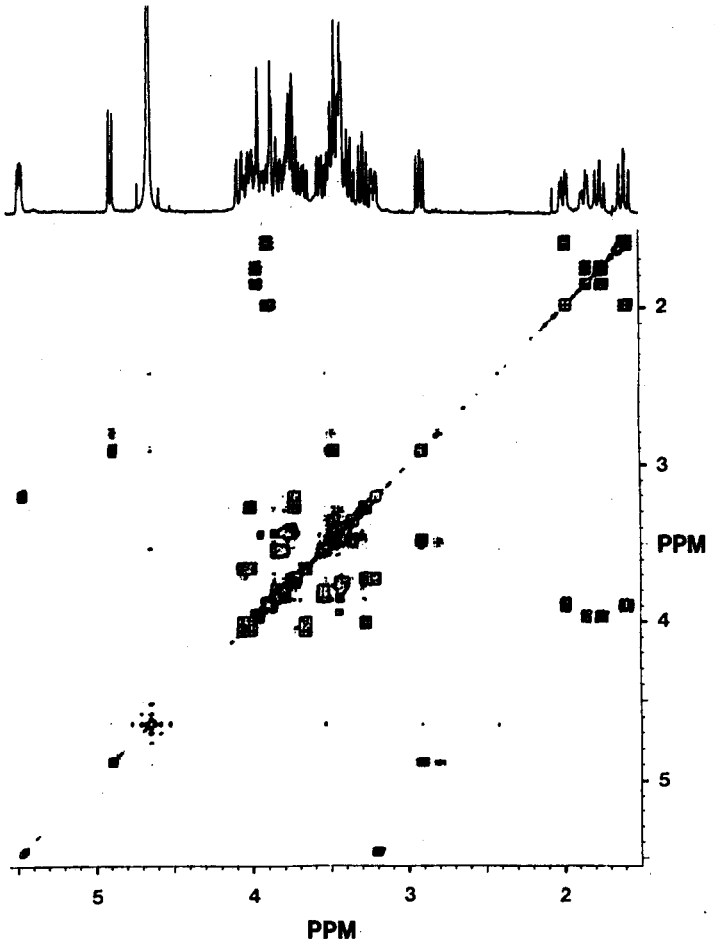


Fig. 3 Nuclear magnetic resonance homonuclear $^1H,^1H$-COSY spectrum (400 MHz) (contour plot) of tetrasaccharide monophosphate 1.

NMR spectroscopy.

Figure 3 shows the homonuclear ^1H-COSY contour plot of compound 1 and table 1 summarizes the ^1H-NMR data of 1 and 2[2]. Two anomeric signals assigned to H-1^{II} in 1 (5.03 ppm, $J_{1,2}$ 8.2 Hz) and 2 (4.874 ppm and $J_{1,2}$ 8.7 Hz) as well as to H-1^{I} in 1 (5.61 ppm, $J_{1,2}$ 3.5 Hz)], and 2 (5.61 ppm, $J_{1,2}$ 3.5 Hz) proved the structure of the lipid A backbone [ß-D-GlcpN-(1→6)-α-D-GlcpN-*P*]. All ^1H- and ^{13}C-NMR signals of both Kdo-residues were found to be in excellent agreement with previous findings[3]. The assignment of the two phosphate groups in 1 and 2 was based on the $^3J_{H\text{-}1,P}$ (≈ 6.7 Hz) and $^4J_{H\text{-}2,P}$ (1.8 Hz) which indicated a glycosidic phosphate at GlcN^{I}. The assignment of the second phosphate to position 4 of GlcN^{II} (2) was made by ^{13}C-NMR whereby carbon C-4^{II} expressed a diagnostic downfield shift (70.53 vs. 73.96) with a characteristic coupling constant $^2J_{C\text{-}4,P}$ of 5.0 Hz and $^3J_{C\text{-}5,P}$ of 8.7 Hz. Small downfield shifts ($\Delta\delta \approx 0.2$ ppm) of H-3^{II}, H-4^{II} and H-5^{II} were also observed by ^1H-NMR when 1 and 2 were compared. The *N*-acetylated tetrasaccharide mono- (3) and bisphosphate (4) expressed identical chemical shift values as compared with

their NH_2-free counterparts. The only difference in the ^{13}C- NMR signals were the observed downfield shifts of approx. ≈1 ppm for C-3^{I} and C-3^{II}, respectively when NH_2-free (1 and 3) and *N*-acetylated (2 and 4) oligosaccharides were compared. These results were also in agreement with ^{31}P-NMR data, where one (glycosidic) phosphate signal was observed in 1 (-2.21 ppm) whereas in 2 two signals were obtained at -1.0 (1^{I}-*P*) and -3.82 ppm (4^{II}-*P*).

The observation that the *S. minnesota* LPS revealed mono- and bisphosphorylated oligosaccharides 1 and 2 in a ratio of approx. 2 : 1 (by weight), whereas *E. coli* LPS gave only 2 is likely to be due to the fact that, in the case of *S. minnesota*, under alkaline conditions, L-Ara*p*N-1-*P* at position C-4^{II} is liberated[4].

EXPERIMENTAL

Degradation (de-acylation) of lipopolysaccharides

Dried LPS of the Re mutants of *S.minnesota* R595 (1.07 g) or *E. coli* F515 (0.9 g) was kept in water-free hydrazine (50 ml, 37°C, 30 min) and the de-*O*-acylated LPS precipitated from chilled iso-butanol (350 ml) by centrifugation (-15°C, 40.000 x *g*, 30 min.) [yields *S. minnesota* 472 mg (43%, by weight) and *E. coli* 388 mg (43%, by weight)]. The de-*O*-acylated LPS (460 mg) and (308 mg) was dissolved in 8 ml 4 M KOH and kept at 100°C for 5 h. After cooling, neutralization [Amberlite IR 120 (H^+-form)] and extraction (chloroform), the crude oligosaccharide preparation was lyophilized. [yield *S.minnesota*, 250 mg (23%, by weight), *E. coli*, 250 mg (27%, by weight)].

For the preparation of the *N*-acetylated oligosaccharide phosphate derivatives 3 and 4 the same degradation procedure with a similar amount of LPS for *S. minnesota* (0.98 g) and *E. coli* (1.0 g) was carried out as described above with the following modification: After treatment with strong alkali (4M KOH) the oligosaccharides were *N*-acetylated at 0°C using acetic anhydride (Ac_2O) until pH neutral was achieved. The crude oligosaccharide phosphates were desalted (Sephadex G-10) and lyophilized to give 348 mg (35%, by weight, *S.minnesota*), and 315 mg (32%, by weight, *E. coli*) of crude *N*-acetylated oligosaccharide phosphate(s).

HPLC of oligosaccharide phosphates

HPLC ion-exchange chromatography [CarboPac PA1 column (9 x 250mm, Dionex, Sunnyvale, CA, USA) at 40°C (3.5 ml/min) using a linear salt gradient (Solvent A: 0.1 M NaOH, and solvent B: 0.75 M NaOAc in 0.1 M NaOH) starting with 5% B (by vol.) for 10 min, then raising to 50% B (60 min) and 100% B in 15 min, remaining there for additional 15 min. Aliquots of 20-25 mg of crude oligosaccharide phosphate were applied for each HPLC run. Two main compounds eluting at t_R 61 min (0.32 M NaOAc, 1) and t_R 81 min (0.59 M NaOAc, 2) were obtained from *S. minnesota* LPS containing Kdo, GlcN, or phosphate in a molar ratio of 2.0 : 1.8 : 1.0 (1) and 2.0 : 1.9 : 2.0 (2). The purified oligosaccharide phosphates were desalted (Sephadex G-10) and lyophilized to give 13 mg (1) and 7 mg (2) for *S. minnesota* (yield 1.9% by weight) and 20 mg of 2 (*E.coli*, 2.2% by weight).

The *N*-acetylated oligosaccharide phosphates 3 and 4 expressed slightly earlier retention times as compared to 1 and 2, respectively. Both *N*-acetylated oligosaccharides could be monitored for their UV-absorbance at 200 nm eluting at t_R 57 min (0.29 M NaOAc, 3) and t_R 78 min (0.51 M NaOAc, 4). As above mono- and bisphosphorylated tetrasaccharides 3 and 4 were obtained from *S. minnesota* LPS whereas *E. coli* LPS revealed only compound 4. The purified oligosaccharide phosphates were desalted (Sephadex G-10) and lyophilized to give 18 mg of 3 and 7 mg of 4 (*S. minnesota*, 2% by weight) and 24 mg of 4 (*E.coli*, 2% by weight).

Table 1: ^{1}H-NMR data of **1** and **2** (D_2O, 400 MHz)[2]

Structure and Assignment	**1**		**2**		
	δ (ppm)	J (Hz)	δ (ppm)	J (Hz)	
α-Kdo^{IV}(2→					
H-3$_{ax}^{IV}$	1.74	13.0, 12.1	1.797	13.2, 12.1	
H-3$_{eq}^{IV}$	2.13	5.0	2.134	4.8	$^3J_{3eq,5} \approx 1$
H-4^{IV}	4.09		4.070	3.1	
H-5^{IV}	4.05		4.050	1.0	
H-6^{IV}	3.17		3.593	8.9	
H-7^{IV}	3.97		3.978		
H-8a^{IV}	3.46*		3.750		
H-8b^{IV}	3.46*		4.023		
→4)-α-Kdo^{III}-(2→					
H-3$_{ax}^{III}$	1.90	13.0, 12.1	1.976*		
H-3$_{eq}^{III}$	2.00	5.0	1.976*		
H-4^{III}	4.12		4.134	≈1.0	
H-5^{III}	4.01		4.133	8.3	
H-6^{III}	3.58		3.905	7.7	
H-7^{III}	3.97		3.682	12.5	
H-8a^{III}	3.68*	12.1	3.894		
H-8b^{III}	3.97		3.870		
→6)-ß-D-GlcpN^{II}-(1→			→6)-ß-D-GlcpN^{II}-(1→ | 4 P		
H-1^{II}	5.03	8.2	4.874	8.7	
H-2^{II}	3.06	10.6	3.009	10.2	
H-3^{II}	3.48	9.4	3.820		
H-4^{II}	3.50	9.4	3.730		
H-5^{II}	3.58		3.700		
H-6a^{II}	3.50		3.510		
H-6b^{II}	3.50		3.710		
→6)-α-D-GlcpN^{I}-*P*					
H-1^{I}	5.61	3.5, $^3J_{1,P}$ 6.9	5.610	3.5, $^3J_{1,P}$ 6.5	
H-2^{I}	3.36	10.6, $^4J_{2,P}$ 1.8	3.284	10.3, $^4J_{2,P}$ 1.8	
H-3^{I}	3.88	10.2	3.860	10.0	
H-4^{I}	3.42	9.2	3.514	9.1	
H-5^{I}	4.16	8.5	4.150*		
H-6a^{I}	3.81	12.6	3.720	11.9	
H-6b^{I}	4.21	1.4	4.281	1.7	

* non-resolved multiplet.

Table 2: ^{13}C-nmr data[a] of four oligosaccharides obtained from the inner-core/backbone region of LPS of Re mutant strains of *S. minnesota* R595 and *E. coli* F515. Mono- (1,3) and bis-phosphorylated tetrasaccharides (2,4)(D_2O, 90.6 MHz, internal standard acetonitrile 1.70 ppm).

Assignment[b]	*(ppm)*			
	1	**2**	**3**	**4**
Kdo^{IV}				
1^{IV}	176.40	176.59	175.51	175.49
2^{IV}	100.39	100.47	100.67	99.88
3^{IV}	35.40	35.30	35.30	35.35
4^{IV}	66.80	66.71	66.77	66.75
5^{IV}	67.11	67.00	66.97	67.02
6^{IV}	73.45	73.47	73.32	73.35
7^{IV}	70.72	70.54	70.55	70.75
8^{IV}	64.30	64.16	64.21	64.09
Kdo^{III}				
1^{III}	175.46	175.77	175.48	175.35
2^{III}	100.30	100.22	100.03	100.68
3^{III}	34.19	34.21	34.08	34.07
4^{III}	69.35	69.31	69.20	69.10
5^{III}	65.23	65.30	65.08	65.04
6^{III}	73.10	72.09	72.38	72.09
7^{III}	70.72	70.54	70.45	70.57
8^{III}	64.30	64.16	64.26	64.15
Glc*p*N^{II}				
1^{II}	100.69	100.82	102.48	102.32
2^{II}	56.55	56.53	56.40	55.94
3^{II}	72.40	73.79	74.89	72.15
4^{II}	70.53	73.96 $^2J_{C,P}$ 5.0	70.16	74.02 $^2J_{C,P}$ 5.1
5^{II}	75.40	75.29 $^3J_{C,P}$ 8.7	75.17	75.40 $^3J_{C,P}$ 8.8
6^{II}	62.18	63.31	62.58	63.90
Glc*p*N^{I}				
1^{I}	91.40 $^2J_{C,P}$ 4.8	92.03 $^2J_{C,P}$ 4.6	94.25 $^2J_{C,P}$ 5.5	93.64 $^2J_{C,P}$ 5.2
2^{I}	55.35 $^3J_{C,P}$ 6.3	55.55 $^3J_{C,P}$ 7.6	54.61 $^3J_{C,P}$ 8.2	55.06 $^3J_{C,P}$ 6.5
3^{I}	70.77	71.20	71.54	72.34
4^{I}	71.01	70.74	71.29	70.46
5^{I}	73.90	73.13	72.70	75.03
6^{I}	69.70	70.39	68.78	68.78

[a] other signals: 3 $NCOCH_3$, 23.08, 22.77, 4 $NCOCH_3$, 23.22, 23.03 ppm.
[b] after ^{1}H,^{13}C-COSY assignment.

Chemical analysis

Aliquots (50 µl) of each HPLC fraction (3.5 ml) were neutralized (50µl 0.1 M H_2SO_4), hydrolyzed (0.2 M H_2SO_4, 30 min, 100°C) and tested for TBA-reactivity[5]. GlcN was estimated after hydrolysis (4 M HCl, 100°C, 16h)[6] and phosphate was determined according to Lowry et al.[7].

FAB-MS Analysis

FAB-Mass spectrometry was done on a VG-ZAB HF mass spectrometer (V.G, Analytical, Manchester, U.K.) equipped with an "Ion Tech" gun[8] in the positive-ion mode. The oligosaccharides were applied to a matrix of thioglycerol on the target and bombarded with xenon atoms at 9 KeV.

NMR spectroscopy

^1H- and ^{13}C-NMR spectra were recorded on a Bruker AM 400 or AM 360 spectrometer [400 MHz (360MHz) for ^1H and 100 MHz (90.6 MHz) for ^{13}C] in D_2O at room temperature with acetonitrile as internal standard [2.07 (^1H) and 1.70 (^{13}C) ppm]. All 2D homo- and heteronuclear correlated spectra were run on pulse sequences using standard Bruker software DISNMR version 89 11 01.0. ^{31}P-NMR spectra were recorded at 145.8 MHz on a Bruker AM 360 spectrometer in D_2O at pD of 8.5. Samples were referenced to a 80% (mass/vol.) solution of H_3PO_4 as external standard (0.00 ppm).

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