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Structure of a hexasaccharide proximal to the hydrophobic region of lipopolysaccharides present in *Bordetella pertussis* endotoxin preparations

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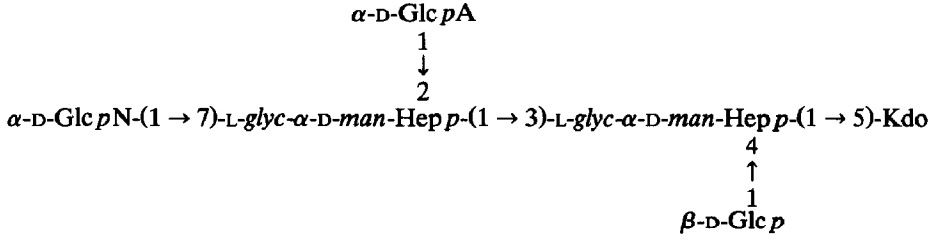
Abstract

A branched-chain hexasaccharide containing 3-deoxy-D-*manno*-oct-2-ulosonic acid was released by detergent-promoted hydrolysis from *Bordetella pertussis* endotoxin preparations that were first dephosphorylated with aqueous HF and then treated with nitrous acid. Its structure (**2**)



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was determined by chemical and physical methods. This hexasaccharide is present in all four lipopolysaccharides that make up the *B. pertussis* strain 1414 (phase 1) endotoxin preparations analysed, and is situated near to the hydrophobic domains. An analogous structure reported previously (ref 7) is erroneous and should be disregarded.

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1. Introduction

Endotoxin preparations obtained from *Bordetella pertussis* strain 1414 (phase 1) cells are made up of two major and two minor lipopolysaccharides [1]. Repeating mono- or oligo-saccharide units, like those often encountered in enterobacterial endotoxin preparations, are not present in either of them. In one of the major lipopolysaccharides (LPS-2), which gives a negative response in the thiobarbiturate test, a disubstituted 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) residue is present; O-5 of this glycose is substituted by the polysaccharide chain and O-4 by a phosphate group; the latter substituent is not present in the other major lipopolysaccharide (LPS-1) [2]. When isolated from endotoxin dephosphorylated by treatment with aqueous hydrofluoric acid, the isolated polysaccharide chains of the two major lipopolysaccharides are identical dodecasaccharides. Their molecular mass, determined by ^{252}Cf plasma desorption mass spectrometry, is 2311 Daltons, and the reducing terminal glycose is a Kdo residue. Isolated polysaccharide chains of the two minor lipopolysaccharides differ from those of the two major ones only in that three sugars, 2-acetamido-2-deoxy-D-glucose (*N*-acetylglucosamine), 2,6-di-deoxy-2-(*N*-methylacetamido)galactose (*N*-acetyl-*N*-methylfucosamine), and 2,3-di-acetamido-2,3-dideoxymannuronic acid, present in the major pair, are absent from the minor pair [1]. The absolute configurations of the fucosamine and mannuronic acid derivatives have not been established. Glycose chains of the *B. pertussis* lipopolysaccharides are very resistant to hydrolysis [3]. So far, structural analyses have only been performed on one di- [4], two tri- [5,6], and one tetra-saccharide [7] fragment obtained in very small amounts by acid hydrolysis. The complete structure of a hexasaccharide that was released from dephosphorylated endotoxin preparations by treatment with nitrous acid followed by mild (pH 4.5, 100°C, 1 h) detergent-promoted (1% SDS) hydrolysis [8] is reported in the present study. Since the reducing terminal sugar of this hexasaccharide is a 3-deoxy-D-*manno*-oct-2-ulosonic acid, it represents, for all four lipopolysaccharides of the endotoxin preparation analysed, a region of the hydrophilic domain near to the hydrophobic domain (Lipid A).

2. Materials and methods

Endotoxin.—*B. pertussis* strain 1414 (phase 1, vaccinal; Institut Mérieux, Lyon, France) cells were grown in Cohen and Wheeler's liquid medium [9]; the endotoxin was isolated by the phenol–water method as previously described [10] and extracted with 60:30:5 $CHCl_3$–MeOH–H_2O before use.

NMR spectra.—^1H NMR spectra were obtained at 250, 400, and 500 MHz; chemical shifts (δ) are given in ppm relative to sodium 3-(trimethylsilyl)-1-propanesulfonate (DSS). For pentasaccharide **HF-B**, partial ^1H and ^{13}C assignments — pertinent to the molecular species that had 3-deoxy-α-D-*manno*-oct-2-ulopyranosonic acid as reducing terminal sugar — were made on the basis of double

quantum-filtered, pure-phase COSY, single, double, and triple homonuclear RELAY and HETCOR experiments (Fig. 6; Tables 1 and 2).

GLC–MS.—Acetylated and methylated glycose derivatives were analysed using a fused capillary column (0.32 × 25 m) coated with SE30 bonded phase (150 to 240°C, 2°C min^{-1}) coupled to a Delsi–Nermag Spectral 30 quadrupole mass spectrometer. Retention times were established using a fused-silica WCOT-type column (0.32 × 25 m) coated with BP10 [dimethyl siloxane containing 14% of (cyanopropyl)phenysiloxane; thickness: 0.5 μm] bonded phase (temperature gradient: 160 to 240°C, 4°C min^{-1}). Detection: flame ionisation.

Table 1
^{1}H NMR data for pentasaccharide **HF-B** determined at 400 MHz in D_2O at room temperature. Data refer to the preponderant α-Kdo isomer; values in parentheses are those of the β isomer

Unit		δ	J (Hz)					
			1,2	2,3	3,4	3*e*,4	3*a*,4	3*e*,3*a*
Kdo	H-3*e*	1.92 (2.44)				4.5	12.0	12.0
	H-3*a*	2.08 (1.84)				(4.6)	(12.5)	(12.5)
	H-4	4.12 (3.87)						
	H-5							
	H-6							
	H-7							
	H-8a, b	3.65–3.75						
Hep-1	H-1	5.07 (5.00)	1.8 ($<1_{1,2}$)					
	H-2	4.14						
	H-3	3.62						
	H-4							
	H-5							
	H-6							
	H-7a, b	3.65–3.75						
Hep-2	H-1	5.52 (5.57)	~1.5 (~$1.5_{1,2}$)					
	H-2	4.14						
	H-3	3.96						
	H-4	3.65						
	H-5							
	H-6							
	H-7a, b	3.65–3.75						
GlcA	H-1	5.09	3.7					
	H-2	3.60		10.0				
	H-3	3.76			10.0			
	H-4	3.44						
	H-5	4.10						
Glc	H-1	4.48	7.8					
	H-2	3.31		10.0				
	H-3	3.41			~10.0			
	H-4	3.72						
	H-5							
	H-6a, b							

Colorimetric methods.—Kdo (standard: Kdo ammonium salt, monohydrate), neutral sugars and hexoses (standard for both: D-glucose), heptoses (standard: D-*glycero*-L-*manno*-heptose), hexuronic acids (standard: sodium D-glucuronate), 2-amino-2-deoxyhexoses (standard: D-glucosamine · HCl), and nitrogen (standard: D-glucosamine · HCl) were estimated by methods used previously [11]. Periodate was estimated according to Avigad [12] and formaldehyde according to Nash [13]. Primary amines were estimated according to Moore and Stein [14]. D-Glucose was estimated enzymatically either with D-glucose oxidase (Biotrol) [15], or with the hexokinase–D-glucose 6-phosphate dehydrogenase system (Boehringer).

High-voltage paper electrophoresis.—This was carried out using Whatman 3MM paper (75 cm length) and 0.2 M pyridinium acetate (11.4 : 16 : 972 AcOH–pyridine–H_2O, pH 5; ~ 50 V cm^{-1} in a flat-plate apparatus. On pherograms, Kdo was detected with the thiobarbiturate spray [16], glycoses with $AgNO_3$–NaOH [17] and NH_2 with ninhydrin.

Dephosphorylation of the B. pertussis *endotoxin.*—The endotoxin (600 mg) was dispersed in aq HF (50% HF w/v, 40 mL) and the stirred mixture was kept at +4°C for 48 h. The mixture was then placed in a polypropylene dish in a polypropylene desiccator containing KOH pellets in a Petri dish. Water and HF were removed by evaporation at room temperature under oil pump vacuum and trapped at −78°C, the pump being protected by a glass turret containing KOH pellets. During the later stages, when evaporation becomes very slow, the dish containing the endotoxin was placed just over the Petri dish containing the KOH pellets (but not in contact with it) so that the heat evolved by the pellets kept the

Table 2
^{13}C NMR data for pentasaccharide **HF-B** determined at 100 MHz for a solution in D_2O at room temperature

δ	Assignment [a]	δ	Assignment [a]
176.34	Kdo-1 *	72.45	
176.11	GlcA-6 *	72.10	GlcA-2
102.64	Glc-1	71.70 (2×)	
101.15	(Hep-1)-1	71.39	
100.97	GlcA-1	70.94	
99.74	(Hep-2)-1	70.48 (2×)	
96.46	Kdo-2	69.28	
79.71		68.88	
76.50	Glc-5	68.48	
76.13	Glc-3	66.78	
74.54		66.09	
73.77		63.41	Kdo-8 **[b]
73.60	Glc-2	63.31	(Hep-1)-7 **[b]
73.48		63.13	(Hep-2)-7 **[b]
73.32		61.73	Glc-6 [b]
73.10		34.45	Kdo-3

[a] The data presented are those of the preponderant α-Kdo anomer; * and ** indicate mutually interchangeable assignments. [b] Tentative assignments based on data obtained for model compounds.

material at room temperature. If this technique is not followed, complete removal of the liquid may last for a day or more. Some water was then added to, and evaporated from, the dry residue which was then taken up in water (150 mL), and the dephosphorylated endotoxin was sedimented by centrifugation (1 h, 200 000*g*). The material was suspended in water (150 mL) and centrifuged again, and the sediment was dried by lyophilisation.

Cleavage of the glycose chains by deamination.—Aqueous acetic acid (30%, v/v; 36 mL) and aq $NaNO_2$ (5%, w/v; 36 mL) were added at room temperature to a stirred dispersion of dephosphorylated endotoxin (600 mg) in water (36 mL). Stirring was continued for 4 h, thereafter the mixture was centrifuged (30 min, 200 000*g*), and the sediment was suspended in water, centrifuged again, and dried by lyophilisation. Completeness of the reaction was monitored by TLC (Silica Gel 60, 0.2 mm, on aluminium foil (E. Merck); 5:3 isobutyric acid–M NH_4OH [18]).

Detergent-promoted hydrolysis [8] of dephosphorylated endotoxin after deaminative cleavage of the glycose chains.—The material to be hydrolysed (200 mg) was dispersed by sonication in NaOAc buffer (pH 4.5, 20 mM, 50 mL) containing sodium dodecyl sulfate (0.5 g) and the stirred mixture was kept at 100°C for 2 h. The residue remaining after lyophilisation of the hydrolysate was dispersed in EtOH (10 mL) containing HCl (2 M, 50 μL) and the mixture was centrifuged (1500*g*, 5 min); this operation was repeated twice with pure EtOH. Residual solvent was removed in vacuo, the dry residue was taken up in water (50 mL), and the mixture was centrifuged (200 000*g*, 90 min). The supernatant solution containing the oligosaccharides **HF-A** and **HF-B** was lyophilised. Conditions for chromatographic separation and purification of these are given in Fig. 1.

Identification of glycose constituents.—*(a) As alditol acetates.* Samples (0.5 mg) of the oligosaccharides in HCl (2 M,0.5 mL) were kept in sealed tubes at 100°C for 2 h. The mixture was brought to dryness, and water was evaporated from the residue until it became neutral. Aqueous $NaBH_4$ or NaB^2H_4 (1 mg/mL, 1 mL) was then added, followed, 16 h later, by dil AcOH (final pH 3–4). Solvents were removed, and acidified (AcOH) MeOH (3 × 1 mL) was added to, and evaporated from, the residue which was then dissolved in Ac_2O (1 mL) and sealed. After 2 h at 100°C, solvents were removed, toluene was evaporated from the residue which was then dissolved in EtOAc (50 mL), and samples of the solution were used for GLC (column: OV-1 on GasChrom Q, 2 m, Ø3.2 mm; temperature gradient, 170°C to 200°C, 4°C min^{-1}).

(b) As (R)-(−)-2-butyl glycosides. Hydrolysis of the oligosaccharides was carried out as described above. The residue remaining after the removal of water was treated with 1:4 CF_3CO_2H–(*R*)-(−)-2-butanol according to ref 34 and analysed by GLC on a column of BP10 [160°C (1 min) to 220°C, 5°C min^{-1}].

Periodate oxidations: Removal of extracyclic diols from polysaccharides prepared from HF-treated B. pertussis *endotoxin.*—*(a) Kinetics (as determined by using the* B. pertussis *endotoxin).* Aqueous $NaIO_4$ (7×10^{-2} M, 5μL) was added to samples (100 μL) of a homogeneous dispersion of *B. pertussis* endotoxin (6 mg) in water (1 mL) and the well-mixed samples were kept for 5, 10, 20, 30, and 40 min. At the times indicated, 2 aliquots (50 μL) were taken from each sample, and periodate

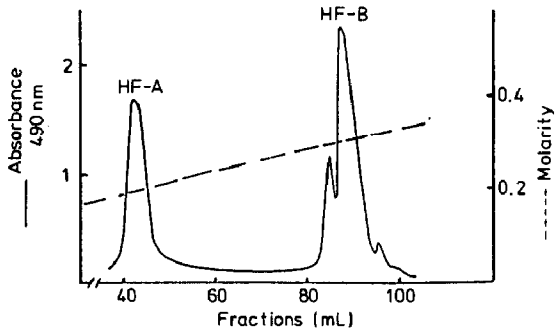


Fig. 1. Separation of oligosaccharides **HF-A** and **HF-B** (cf. Scheme 1) on a DEAE-cellulose column (1.8×12 cm) with pyridinium acetate (pH 5.5, 0.1 to 0.5 M; fractions: 0.2 mL; flow rate: 0.2 mL min^{-1}). Detection: neutral sugars with phenol–sulfuric acid.

consumption [12] (directly) and formaldehyde production [after addition of $NaAsO_2$ (as in ref 13)] were estimated (Fig. 2).

(b) Smith degradation of the polysaccharide chain prepared from HF-treated B. pertussis *endotoxin. (i) Removal of extracyclic diols.* The polysaccharide (150 mg) dissolved in water (15 mL) was added to aq $NaIO_4$ (7×10^{-3} M, 33 mL) at room temperature and the mixture was stirred for 20 min, ethylene glycol (M, 0.5 mL) and, 15 min later, $NaBH_4$ (solid, 100 mg) were added, and stirring was continued overnight. The pH was brought to 4–5 with dilute AcOH and the solution containing the modified polysaccharide was desalted by filtration (Diaflow YC 05 membrane, Amicon Corp., Lewington, MA, USA). The modified polysaccharide (140 mg) was recovered from the retentate by lyophilisation.

(ii) Smith degradation. Aqueous $NaIO_4$ (7×10^{-2} M, 33 mL) was added to the polysaccharide material (140 mg) obtained under (*i*) and the stirred mixture was kept at room temperature for 48 h. Analysis (GLC, alditol acetates) of a sample desalted by filtration and hydrolysed with acid (2 M HCl, 100°C, 2 h) showed, besides mannose, the presence of undegraded glucose. Accordingly, the treatment with periodate was repeated whereafter no more glucose was detected. The material was desalted and submitted to column chromatography (Biogel P-2, H_2O). Pooled fractions containing mannose (GLC of alditol acetates) were lyophilised and analysed by ^1H NMR; a single signal appeared in the anomeric region at δ 5.08 ($J_{C,H}$ 175 Hz).

Methylation analysis [19].—This was carried out on 1-mg samples, but with material peracetylated as described above. Before methylation, the acetylated samples were dried in vacuo at 30°C for 2 h. Sodium methylsulfinylmethanide solution (2 M, 500 μL, in Me_2SO) was added at room temperature to stirred samples dissolved in Me_2SO (250 μL) followed, 16 h later, at 0°C, by MeI (150 μL). The stirred mixture was allowed to reach room temperature; 1 h later, the sample was diluted with CH_2Cl_2 (1 mL) and washed with water (3 × 8 mL), and the solvent was removed. Formic acid (90%, 1 mL) was added and the mixture was kept in a capped tube at 100°C for 2 h. After removal of the formic acid, H_2SO_4

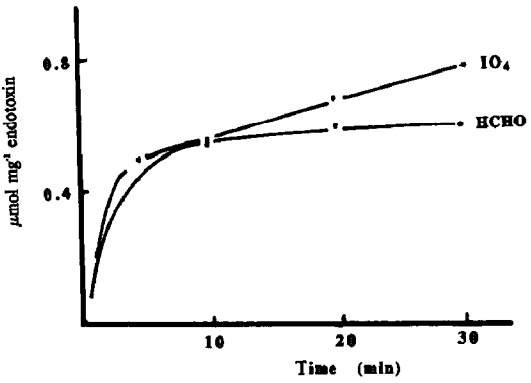


Fig. 2. Kinetics of periodate-reduction and production of formaldehyde upon treatment of the *B. pertussis* endotoxin with sodium periodate (for conditions, see text).

(0.25 M, 1 mL) was added to the sample and the mixture was kept at 100°C for 16 h. The cooled solution was neutralised with $BaCO_3$ and centrifuged. The sediment was extracted with 1:1 MeOH–H_2O (3 × 1 mL) and NaB^2H_4 (1 mg) was added to the pooled supernatant solutions. After 24 h, the mixture was slightly acidified with dil AcOH, solvents were removed, and acidified MeOH was evaporated several times from the dry residue which was finally acetylated as above and the product extracted into EtOAc. Aliquots were used for GLC–MS (column: CPSIL 5, 25 m, Ø 0.32 mm; temperature gradient: 150 to 220°C, 6°C min^{-1}).

Identification of D-glucose as the 4-O-substituent of the 3,4-di-O-substituted heptose.—$NaIO_4$ (7×10^{-3} M, 400 μL) was added to the pentasaccharide **HF-B** (400 μg, solid) at room temperature followed, 20 min later, by ethylene glycol (1.5 μequiv mol^{-1} of periodate added). The pH was adjusted (M NaOH) to 9.5 and, 1 h later, samples (~20, 200, and 170 μg of pentasaccharide, respectively) were removed for analysis by TLC, hexokinase–D-glucose oxidase [15] and GLC (alditol acetate).

3. Results and discussion

During previous studies, it was recognised that several sugar units in the polysaccharide chain of the *B. pertussis* endotoxin had free amino groups [7]. Consequently, specific cleavage of the polysaccharide chain was attempted by deamination with nitrous acid [20], which is known to cleave the glycosidic bonds of 2-amino-2-deoxy sugars that have equatorially oriented NH_2 functions. In this reaction, α-glucosaminides are cleaved much more slowly than β-glucosaminides [21]. The molecular composition of *B. pertussis* endotoxin preparations was considerably altered by treatment with nitrous acid; after deamination, the ratio of hexuronic acid to 2-amino-2-deoxyhexose rose from 0.16 to 0.5 (colorimetry). Detergent-promoted hydrolysis [8] of such modified endotoxin preparations afforded, upon column chromatography on Biogel P-2 (H_2O), one main peak

containing neutral sugars. Upon high-voltage paper electrophoresis at pH 5, this material was separated into 3 anionic compounds [R_{Kdo} 0.29 (**A**), 0.64 (**B'**), and 0.79 (**B**)]. **B'** was a minor component present in amounts insufficient to be analysed. **A**, but not **B**, gave a positive reaction with ninhydrin. **A** and **B** were isolated by preparative paper electrophoresis, and further purified by column chromatography on Biogel P-2: **A** (15 mg solid) was eluted as a single peak; **B** was separated into two components **B-1** (12 mg, solid) and **B-2** (7 mg, liquid) (Fig. 3a, b). Since, under the conditions of hydrolysis used, 400 mg of undeaminated endotoxin afforded ca. 100 mg of oligosaccharide material, and since the isolated polysaccharide chains are dodecasaccharides (unpublished), the total amount of isolated oligosaccharide material (34 mg) recovered from deaminated endotoxin was nearly that theoretically obtainable. It follows that the procedure used was valid to produce representative fragments of the polysaccharide chains. According to colorimetric analyses, **B-1** and **B-2** were pentasaccharides composed of heptose (2), hexose (1), uronic acid (1), and, presumably, Kdo, while **A** was a hexasaccharide containing the same components and one additional hexosamine unit (Table 3). Once the presence of a hexosamine unit in **A** but not in **B** was established, the two components could be separated from the crude mixture by column chromatography on DEAE-cellulose. This (Fig. 3c) and examination by ^{1}H NMR spectra of all fractions (not shown) of both **A** and **B** revealed that neither was homogeneous, although colorimetric estimation suggested that they comprised the same sugars.

While colorimetric estimation for glycoses were coherent, values obtained for Kdo by semicarbazide [22] differed considerably from those given by thiobarbiturate [10]. The latter were much too low and the absorption band was not characteristic for Kdo ($\lambda_{max} \neq 449$ and $A_{449/433} \neq 0.5$). In the ^{1}H NMR spectrum, signals appearing in the anomeric region (δ 4.5–5.5 ppm) indicated heterogeneity and the signals characteristic [23] for the pyranose form of Kdo (α anomer: H-3a,δ

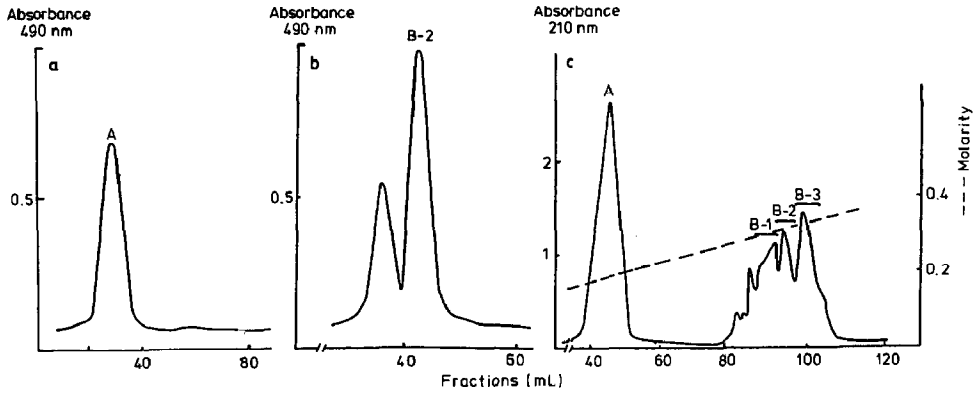


Fig. 3. Elution profiles from Biogel P-2 columns of oligosaccharides **A** (a) and **B** (b) recovered by preparative paper electrophoresis from *B. pertussis* endotoxin that had been treated with nitrous acid and hydrolysed. Elution profile from a DEAE-cellulose column of the mixture of oligosaccharides **A** and **B** prepared as indicated in the text (c).

1.97; H-3*e*,1.87; β anomer: H-3*a*,δ 1.74; H-3*e*,2.38) were blurred and very small compared to those of the anomeric protons. Moreover, unexpected ethylenic proton signals (δ 6-7) were clearly visible, which disappeared upon hydrogenation (catalyst: 10% Pd-on-C) and gave rise to methylene proton signals (δ ca. 1.5). The strong ultraviolet absorption band (λ_{max} 250 nm) — observed for all of the oligosaccharides and not accounted for by the identified components — also disappeared upon hydrogenation (Fig. 4). These data suggested that the elimination reaction observed with synthetic 4-*O*-phosphorylated 3-deoxyald-2-ulosonic acids [24] took place during the hydrolysis used to prepare the polysaccharide chains and that, O-5 of the Kdo unit probably being substituted, the heterogeneity of both **A** and **B** was due to degradation products of Kdo shown to be formed from synthetic 5-*O*-substituted Kdo-4-phosphate upon treatment with mild acid [25]. This conclusion was confirmed when the sequence of manipulations leading to **A** and **B** was applied to *B. pertussis* endotoxin previously dephosphorylated by treatment with aqueous hydrofluoric acid (Scheme 1). The hexa- (**HF-A**) and the penta-saccharide (**HF-B**) isolated by column chromatography (Fig. 1) from such material were homogeneous by both ^{1}H NMR (Fig. 5) and mass spectrometry, and gave the expected values for Kdo content in the thiobarbiturate test (λmax 449 nm, $A_{449}/A_{433} = 0.5$), while the analytical figures obtained for the other constituents remained unaltered (Table 3). Thus, *only* the Kdo component of the oligosaccharides had been affected by the treatment with aqueous HF. **HF-A** but not **HF-B** gave a positive reaction with ninhydrin. At pH 5, both oligosaccharides migrated as anions.

In D_2O, the pentasaccharide **HF-B** is present as a 3:1 α, β-mixture due to the

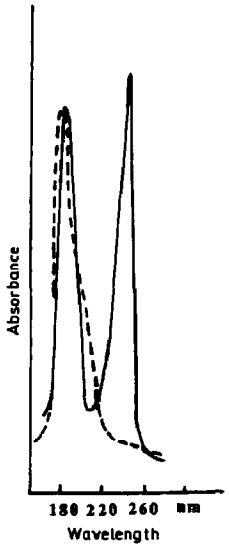
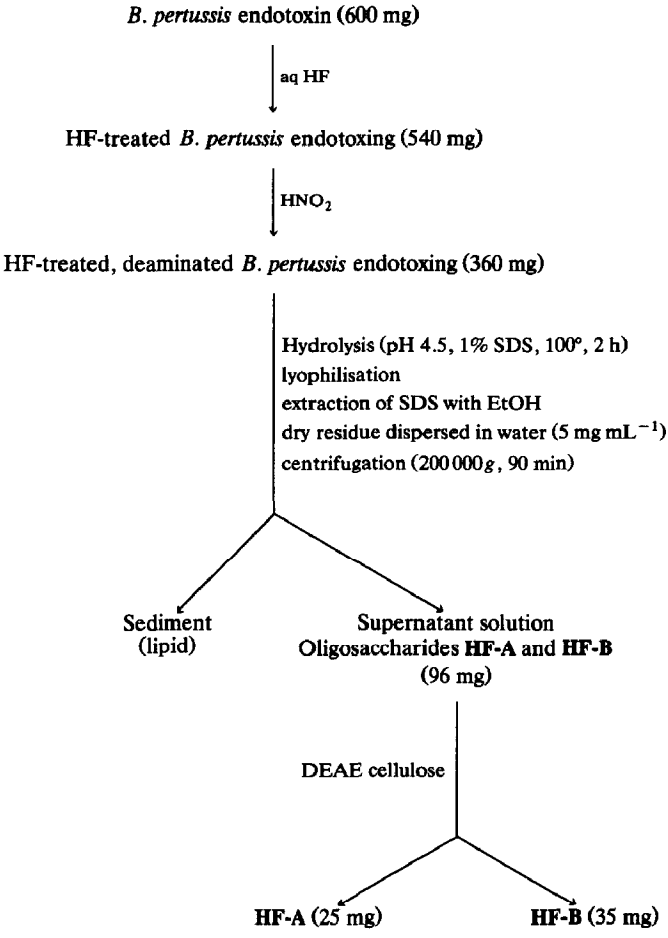


Fig. 4. Absorption spectrum of fragment **B-2** (2 mg mL^{-1}) before (———) and after (- - - - -) catalytic (Pd) hydrogenation.

reducing terminal Kdo (Fig. 5, bottom). In the ^{1}H NMR spectrum (400 MHz, room temperature) (Table 1 and Fig. 5, bottom) of the pentasaccharide **HF-B**, four anomeric protons [δ 4.48 ($J_{1,2}$ 7.8 Hz), 5.07 ($J_{1,2}$ 1.8 Hz), 5.09 ($J_{1,2}$ 3.7 Hz), 5.52 ($J_{1,2}$ 1.5 Hz)] were seen, while in that (500 MHz, room temperature) of the hexasaccharide **HF-A** (Fig. 5, top), five anomeric protons [δ 4.49 ($J_{1,2}$ 7.5 Hz), 5.10 ($J_{1,2}$ 1.8 Hz), 5.14 ($J_{1,2}$ 3.5 Hz), 5.25 ($J_{1,2}$ 3.8 Hz), 5.46 ($J_{1,2}$ 1.5 Hz)] were visible. Signals due to H-3*e* and H-3*a* of Kdo units were present in both **HF-A** and **HF-B**; their intensities were commensurate with those due to the anomeric protons and corresponded to an α/β ratio of 3 : 1. The same ratio was also reflected by some of the anomeric signals.

When pentasaccharides **B** or **HF-B** were hydrolysed with mineral acid, and the hydrolysate was treated with borodeuteride and then acetylated, GLC–MS revealed the presence of per-*O*-acetylated hexitol (glucitol by retention time), heptitol (a single peak that had the retention time of per-*O*-acetyl-D-*glycero*-L-*manno*-heptitol), and 1,6-anhydroheptose [26] in the ratios ca. 1 : 1.5 : 0.2. Per-*O*-acetylated



Scheme 1.

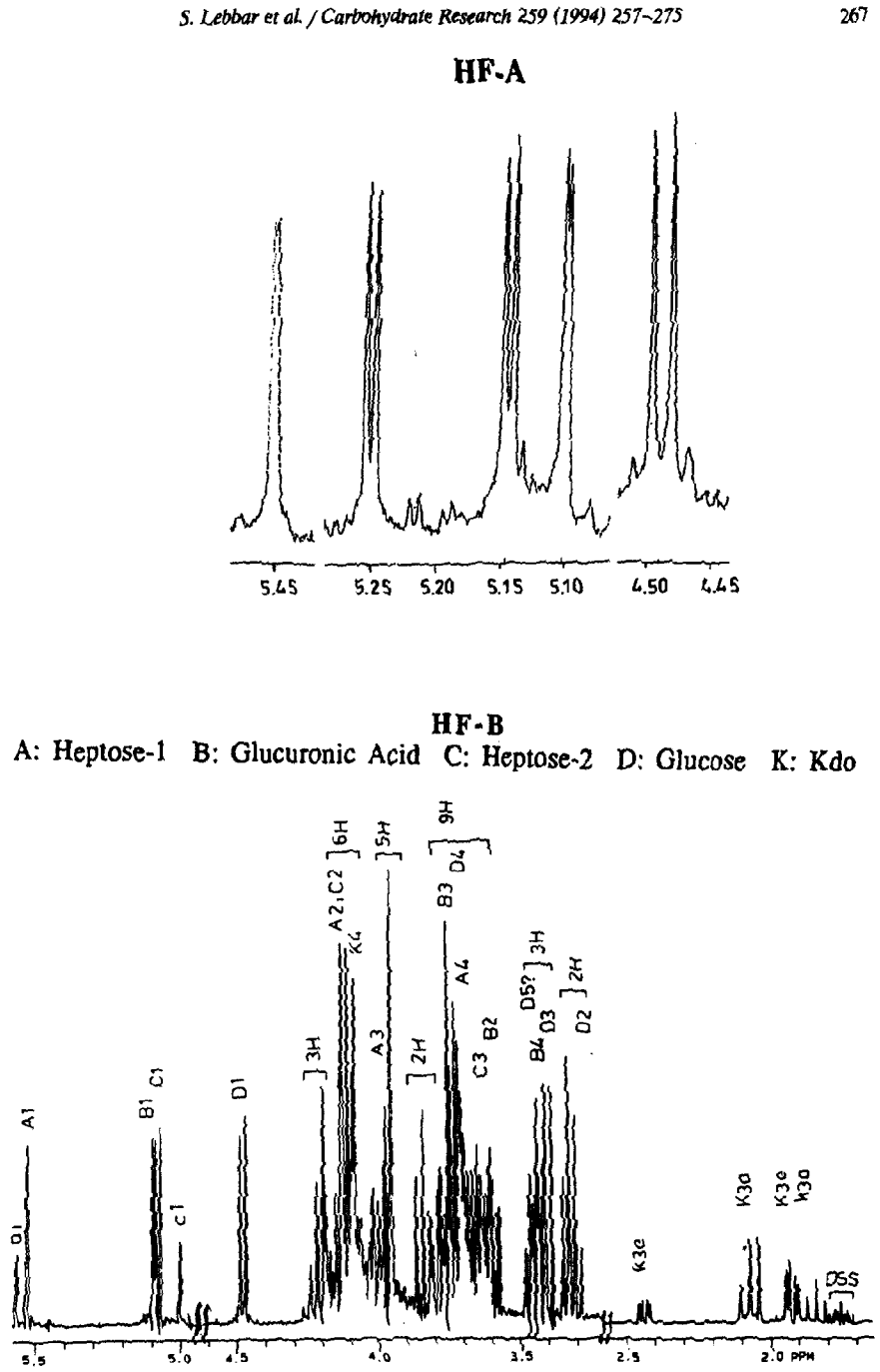


Fig. 5. Selected regions of the ^{1}H NMR spectra of hexasaccharide **HF-A** (500 MHz; anomeric protons) (top) and pentasaccharide **HF-B** (400 MHz; anomeric protons, and H-3*a* and H-3*e* of the reducing terminal Kdo unit) (bottom).

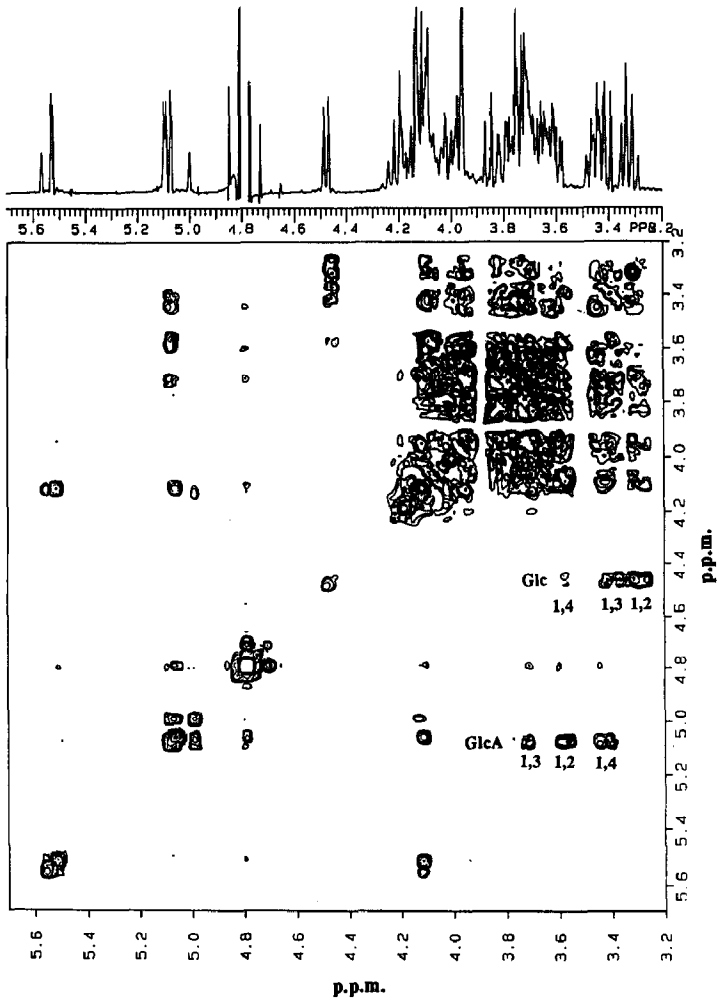


Fig. 6. 400-MHz ^{1}H double-relay NMR spectrum of pentasaccharide **HF-B** in D_2O at 25°C. COSY (1, 2), single-relay (1, 3), and double-relay (1, 4) cross-peaks of the anomeric protons of the glucuronic acid and glucose residues.

methyl hexopyranoside, methyl (methyl hexopyranosid)uronate [retention time: methyl (methyl α-D-glucopyranosid)uronate], and methyl heptoside were detected by GLC–MS as the main components of methanolysates (data not shown).

Methylation analysis [19] of pentasaccharide **B** and **HF-B**, carried out on samples that were first treated with borohydride to reduce the carbonyl function of the presumably terminal Kdo unit and then peracetylated [27], revealed the presence of unsubstituted hexopyranose (1), 3,4-di-*O*-substituted heptopyranose (1), and 2-*O*-substituted heptopyranose (1). If, before acid hydrolysis and acetylation, methylated material was treated with borohydride [28] (to transform carboxyl groups into primary alcohols), the amount of unsubstituted hexopyranose (retention time: 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol) detected by GLC–MS in-

Table 3
Colorimetric analyses [a] of the *B. pertussis* endotoxin and of its fragments **A**, **B-1**, **B-2**, **HF-A**, and **HF-B**

	Endotoxin	A	B-1	B-1	HF-A	HF-B
Heptose	1.4	1.15	1.82	1.78	1.3	1.59
Uronic acid	0.6	0.86	1.15	0.99	0.7	0.80
Hexose	0.5	0.93	0.93	0.85	0.67	0.73
Hexosamine	1.3	0.90	0	0	0.7	0
NH_2	n.d.	1.0	0	0	n.d.	n.d.
Kdo [22]	n.d.	0.9	1.1 [b]	1.2 [b]	0.7	0.8
HCHO [c]	n.d.	1.26	3.14	1.7	1.39	2.3
HCHO/Uronic acid	n.d.	1.46	2.73	1.79	1.98	2.87

[a] Results are expressed as μmol mg^{-1}; n.d.: not determined. [b] Intrinsic absorption of **B-1** and **B-2** (at 250 nm) causes overestimation in this test. [c] Estimated after treatment with periodate (15 min).

creased ca. two-fold. Furthermore, in gas liquid chromatograms of **HF-B** thus treated, one additional peak was seen which was not observed in material derived from pentasaccharide **B**. According to its mass spectrum, it was due to a 1,5-di-*O*-acetyl-3-deoxy-2,4,6,7,8-penta-*O*-methyloctitol. This established that the reducing terminal sugar of pentasaccharide **HF-B** was a 5-*O*-substituted 3-deoxyoct-2-ulopyranosonic acid. The absence of this peak from chromatograms of pentasaccharide **B** is in agreement with the postulated [2] elimination of phosphate from, and subsequent transformation of, the terminal Kdo unit. Methylation analysis of the *N*-acetylated hexasaccharide **HF-A** revealed, in addition to the components identified in **HF-B**, the presence of a 1,5-di-*O*-acetyl-2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)hexitol (retention time and mass spectrum identical to that of 1,5-di-*O*-acetyl-2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-D-glucitol). This established that the 2-amino-2-deoxyhexose detected in **HF-A** by colorimetry and by GLC–MS as alditol acetate was an unsubstituted 2-amino-2-deoxyhexopyranose. These data, and the molecular masses measured for **HF-B** (M_r 960 amu) and **HF-A** (M_r 1121 amu) confirmed the compositions indicated by the destructive analytical methods.

Neither untreated nor HF-treated endotoxin preparations could be deaminated completely. A mixture of penta- and hexa-saccharides was isolated in all experiments, even if the treatment was repeated several times. However, when the deaminative step was carried out in 1% SDS solution instead of water, the ratio of isolated **HF-A**/**HF-B** decreased from 3:2 to 1:3. Incomplete deamination was therefore due, at least in part, to the presence of aggregates or micelles having "buried" amino groups.

Identities, ring forms, anomeric configurations, and substitution patterns of the component glycoses.—3-Deoxy-oct-2-ulosonic acid. 3-Deoxy-D-*manno*-oct-2-ulosonic acid, being an apparently obligatory [29,30] component of all endotoxic lipopolysaccharides of bacterial origin, the 3-deoxyoctulosonic acid present in both oligosaccharides has been characterised by its reaction with periodate–thiobarbiturate (λ_{max} at 449 nm), by the appearance of the expected [23] signals in the ^1H NMR spectrum of pentasacharide **HF-B** (Table 1, Fig. 5, bottom), and by the methylated and acetylated 3-deoxyoctitols detected by methylation analysis. The

latter established unambiguously that only O-5 of Kdo was substituted. The observation (see below) that three molar equivalents of formaldehyde were produced upon brief treatment of **HF-B** with periodate afforded confirmatory evidence for this point. Kdo units substituted on O-7 (by substituents other than phosphate or substituted phosphate which, in the present case, would have been removed by the treatment with HF) have been detected in endotoxins [31,32]. It has been shown previously [33] that both of the 3-deoxyhexitols derived from the 3-deoxyoct-2-ulosonic acid present in *B. pertussis* endotoxin preparations, and representing carbon atoms C-1 to C-6 of that acid, had the configuration expected for the degradation product of 3-deoxy-D-*manno*-oct-2-ulosonic acid.

Hexose. Pentasaccharide **HF-B** that had been pretreated with borohydride in order to reduce the carbonyl group of the terminal Kdo unit produced three molar equivalents of formaldehyde within 10–15 min when oxidised with periodate; the rate of periodate reduction then diminished sharply. According to the data of the methylation analysis, formaldehyde could be produced from pentasaccharide **HF-B** only by cleavage of the extracyclic diols of the terminal Kdo and of the two heptoses; the 3,4-di-*O*-substituted heptose was thus transformed into a 3,4-di-*O*-substituted 6-*aldehydo*-hexopyranose. As expected, exposure to pH 9.5 at room temperature of the material treated with periodate led to rapid and quantitative elimination of the 4-*O*-substituent of this *aldehydo*-hexopyranose: it proved to be the hexose residue which was identified as glucose by GLC (retention time of its alditol acetate), and as D-glucose by its reaction with hexokinase–/D-glucose 6-phosphate dehydrogenase. The methylation analysis, which established that the hexose was unsubstituted, concurred. In the ^{1}H NMR spectra of the penta- and hexa-saccharides, the signals of the anomeric proton of the glucose residue appeared at 4.48 ppm and had a coupling constant of 7.5–7.8 Hz. It follows that, within the oligosaccharides, its anomeric configuration was β.

Heptoses. When pentasaccharide **HF-B**, which had been treated first with borohydride and then with limiting amounts of periodate (see above), was re-treated with borohydride to reduce the newly formed aldehyde groups, colorimetric analyses revealed the complete disappearance of heptose and the appearance of an equivalent amount of hexose. This proved to be mannose according to analysis of the alditol acetates by GLC–MS in a system in which all the hexitol hexa-acetates were completely separated. Consequently, both heptoses had the *glycero-manno* configuration. A single heptitol peracetate was detected after acid hydrolysis, borohydride reduction, and acetylation of a sample of **HF-B** that had been pretreated with borohydride to reduce the carbonyl groups of the terminal Kdo unit; it had the same retention time as authentic L-*glycero*-D-*manno*-heptitol peracetate and was completely separated from D-*glycero*-D-*manno*-heptitol peracetate. It follows that the heptoses present in **HF-B** were either L-*glycero*-D-*manno*- or D-*glycero*-L-*manno*-heptose. To distinguish between these, the heptosyl residues of **HF-B** were transformed into mannosyl residues as described above, and their enantiomeric form established [34] following acid hydrolysis. Only derivatives of D-mannose (and D-glucose) were identified. The anomeric protons of the two heptoses were found at δ 5.52 and 5.07. Attempts to determine the configuration

of the anomeric carbons of the heptoses by the values for $J_{C\text{-}1,H\text{-}1}$ (ref 35) failed because of overlapping signals. It could, however, be concluded from the data of the methylation analysis that an exhaustive Smith degradation of the polysaccharide chain would produce the disaccharide 2-deoxy-4-*O*-(α- or β-D-mannopyranosyl)-L-*erythro*-pentonic acid. In this, the configuration of the anomeric carbon would be the same as that of the 3,4-substituted heptose in the pentasaccharide. Even small amounts of this simple material were expected to be sufficient to determine the anomeric configuration of the aldose by NMR spectroscopy. Accordingly, *B. pertussis* polysaccharide, prepared from dephosphorylated endotoxin, was treated first with limiting amounts of periodate and then with an excess of borohydride. The polysaccharide thus modified was submitted to two successive Smith degradations and material containing mannose was isolated by column chromatography. In the anomeric region of the ^{1}H NMR spectrum, determined at 500 MHz, a single signal appeared at δ 5.08; $J_{H\text{-}1,C\text{-}1}$ was found to be 175 Hz. The anomeric proton of synthetic [36] 2-deoxy-4-*O*-α-D-mannopyranosyl-L-*erythro*-pentonic acid appeared at δ 5.06, while the corresponding signal of the β anomer was found at δ 4.81. These data firmly established that the anomeric configuration of the 3,4-di-*O*-substituted heptose attached to O-5 of Kdo was α (axial). 5-*O*-(L-*glycero*-α-D-*manno*-Heptopyranosyl)-Kdo [37] and 5-*O*-(α-L-rhamnopyranosyl)-Kdo [38] have been found in the core region of enterobacterial lipopolysaccharides.

For hexasaccharide **HF-A**, the signal of the anomeric proton of the 2,7-di-*O*-substituted heptosyl residue appeared at δ 5.46. For pentasaccharide **HF-B**, the anomeric signal of the 2-*O*-substituted heptose was observed at δ 5.52. These values established that the anomeric configuration of this heptose was α. They are, however, unusually high for aldopyranosides, but compatible with aldofuranosides.

According to results of the methylation analysis, one of the heptose residues present both in **HF-A** and **HF-B** was either a 3,4-di-*O*-substituted pyranose or a 3,5-di-*O*-substituted furanose. Elimination of D-glucose from **HF-A** upon treatment with limited amounts of periodate and exposure to pH 9.5 established, however, that it was a 3,4-substituted heptopyranosyl residue.

The ring form of the 2,7-di-*O*-substituted heptose present in hexasaccharide **HF-A** was also not readily identified by methylation analysis either. Indeed, two primary fragments (*m*/*z* 234 and 189) are specific for the methylated alditol acetate derived from a 2,7-substituted heptopyranose, and two others (*m*/*z* 262 and 161) derived from a heptofuranose (Table 4). Three of these [*m*/*z* 234 (10%), 189 (8.5%), and 161 (5.3%)] were present as minor peaks in the mass spectrum observed (Table 5). The primary fragment *m*/*z* (cleavage: C-4–C-5) and its daughter *m*/*z* 174 (234 − 60; 7%) carry ^{2}H and thus represent carbon atoms 1–4 of the heptitol. As the parent heptoside is known [5] to be substituted on O-2, the ion has the structure [^{2}HHCOAc–HCOAc–HCOMe–HCOMe]$^+$. Since O-4 of the heptitol acetate was methylated, the parent 2,7-*O*-substituted heptose must have been a pyranose. The specific primary ion *m*/*z* 189 (8.5%) corresponds to the sequence C-5–C-7 and has the structure [HCOAc–HCOMe–H_2COAc]$^+$. The presence of the acetate group on O-5 confirms the pyranose structure assigned to the parent heptosyl residue.

Table 4
Expected primary fragments of methylated (1-^{2}H) heptitol acetates derived from a 2,7-di-*O*-substituted heptoside ($M = 423$)

ex Pyranoside			*ex* Furanoside		
423	^{2}HCHOAc	74	74	^{2}HCHOAc	423
349	HCOAc	146	146	HCOAc	349
277	HCOMe	190	190	HCOMe	277
233	HCOMe	**234**	**262**	HCOAc	233
189	HCOAc	306	306	HCOMe	**161**
117	HCOMe	350	350	HCOMe	117
73	H_2COAc	423	423	H_2COAc	73

The heptose that is 2,7-*O*-substituted in hexasaccharide **HF-A** is only 2-*O*-substituted in pentasaccharide **HF-B**. Consequently, identification of a 1,2,5-tri-*O*-acetyl-3,4,6,7-tetra-*O*-methylheptitol upon methylation analysis of the latter, which established that the parent heptose was a pyranose, also proved unequivocally that the 2,7-di-*O*-substituted heptose of the former was a pyranose.

Hexuronic acid. When the carboxyl groups of pentasaccharide **HF-B** (in which the carbonyl group of the terminal Kdo was already reduced) were reduced to primary alcohols by treatment with carbodiimide–borohydride [28] and the free aldoses produced upon acid hydrolysis were transformed into acetylated alditol acetates, the amount of glucitol peracetate found upon GLC–MS was almost twice that measured when the reduction of the carboxyl groups was omitted. Under the conditions used for GLC, all hexitol peracetates were eluted separately, and it was concluded that the hexitol was glucitol, and the parent acid glucuronic acid. Data obtained by RELAY and COSY experiments (Fig. 6) [δ_H, 3.44 (H-4,$J_{4,5}$ 10.0 Hz), 3.6 (H-2,$J_{2,3}$ 9.9 Hz), 3.76 (H-3,$J_{3,4}$ 10.0 Hz), 4.10 (H-5,$J_{4,5}$ 10 Hz), 5.10 (H-1,$J_{1,2}$ 3.7 Hz)], concurred and established that the glucuronic acid was α-linked. Analysis of the above-mentioned mixture of the aldoses by the method of Gerwig et al. [34]

Table 5
Observed mass spectrum of the heptitol acetate derived from the 2,7-di-*O*-substituted heptose as obtained by methylation analysis of pentasaccharide **HF-B**

m/z	%	*m/z*	%	*m/z*	%	*m/z*	%	*m/z*	%
277	1.8	191	8.5	145	14.5	100	32	73	10.5
262	[a]	190	50	131	11	99	18	71	19
244	5	189	8.5	130	71	88	25	43	~ 200
234	10	174	7	129	28	87	21		
233	36	173	9	127	23	85	28.5		
207	10	161	5.3	**117**	**100**	75	23		
201	10	159	37.5	101	37.5	74	16		

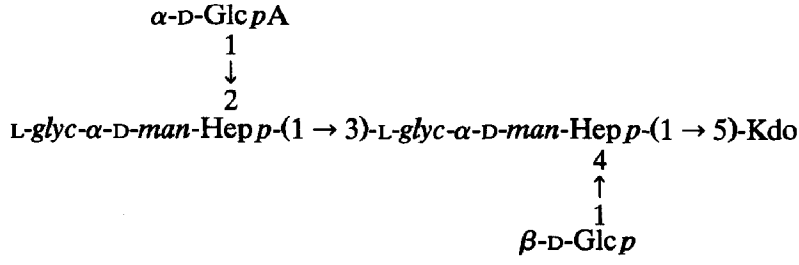
[a] Negligible.

established that the glucose, and consequently the glucuronic acid, was the D enantiomer.

Hexosamine. 2-Amino-2-deoxyhexose was detected in **HF-A** by colorimetric methods. Upon treatment of **HF-A** with nitrous acid, 2,5-anhydromannose was released; its reduction product, 2,5-anhydromannitol, was isolated by column chromatography on Biogel P-2 and identified as its peracetate by GLC–MS. As a corollary, methylation analysis of the peracetylated hexasaccharide established that the hexosamine was a terminal 2-amino-2-deoxyhexopyranose. The retention time and mass spectrum (GLC–MS) of the acetylated hexosaminitol derived from hexasaccharide **HF-A** were indistinguishable from those of 2-acetamido-1,3,4,5,6-penta-*O*-acetyl-2-deoxyglucitol, and the pattern of peaks (GLC) of the acetylated (*R*)-(−)-2-butyl glycoside was identical to that produced from 2-acetamido-2-deoxy-D-glucose. The $J_{1,2}$ value (3.8 Hz) of the signal of an anomeric proton (δ 5.25) present in the ^{1}H NMR spectrum of **HF-A**, but not in that of **HF-B**, then established that the hexosamine unit was the α anomer of glucosamine.

While in keeping with previous observations [21], the very slow and incomplete cleavage of the glycosidic bond of this glucosamine unit upon deamination with nitrous acid, both in the intact polysaccharide chain and in the hexasaccharide **HF-A**, was unexpected, particularly so if compared with the behaviour of the other 2-amino-2-deoxy-α-D-glycopyranosyl residues (2-amino-2-deoxy-α-D-galacturonic acid and a second 2-amino-2-deoxy-α-D-glucopyranose) present in the lipopolysaccharide chains of the *B. pertussis* endotoxin. A similar observation was made [39] when, upon attempted deaminative cleavage of 7-*O*-(2-amino-2-deoxy-α-D-glucopyranosyl)-L-*glycero*-D-*manno*-heptose, only 60% of the disaccharide was cleaved.

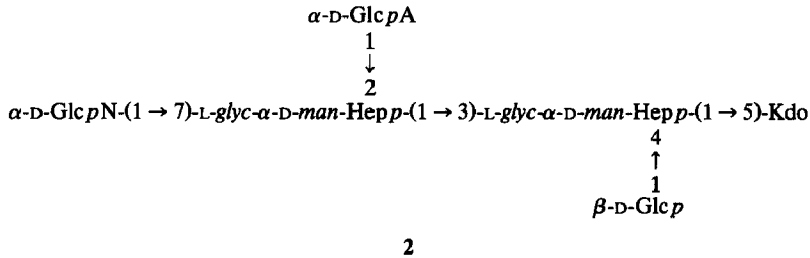
Sequence. The presence of a 3,4-di-*O*-substituted heptose established that pentasaccharide **HF-B** was branched. Elimination of glucose at pH 9.5 following cleavage of the extracyclic diols established that this unit was attached either to O-5 of the Kdo unit or to O-4 of the disubstituted heptose unit. Since exhaustive Smith degradation of the polysaccharide afforded 2-deoxy-4-*O*-α-D-mannopyranosyl-L-*erythro*-pentonic acid, the 3,4-di-*O*-substituted heptose was attached to O-5 of the Kdo unit. It follows that the glucose which had been eliminated was attached to O-4 of the 3,4-di-*O*-substituted heptose. The glucuronic acid being terminal, the 3-*O* substituent of this heptose was the heptose that was 2-*O*-substituted by glucuronic acid. The pentasaccharide **HF-B** thus had the structure **1**.



1

The heptose, which in the pentasaccharide is 2-*O*-substitued, is 2,7-di-*O*-sub-

stituted in the hexasaccharide. It follows that the glucosamine unit must be attached to that position. Accordingly, the hexasaccharide **HF-A** has the structure **2**.



Minute amounts of 7-*O*-(2-amino-2-deoxy-α-D-glucopyranosyl)-L-*glycero*-D-*manno*-heptose [4] and 7-*O*-(2-amino-2-deoxy-α-D-glucopyranosyl)-2-*O*-(D-glycopyranosyluronic acid)-L-*glycero*-D-*manno*-heptose [5] have been ispreviously from *B. pertussis* endotoxin preparations by acid hydrolysis. In the latter work, carried out with radiolabelled material, the β configuration was assigned to the D-glucuronic acid residue because it was cleaved with a commercial β-D-glucuronidase preparation. In the penta- and hexa-saccharides described in this work, an α-D-glucuronic acid residue was identified. Since there is only one glucuronic acid residue in the glycose chains of the *B. pertussis* endotoxin, and since the physical methods used in the present study leave no doubt that the anomeric configuration of this glucuronic acid residue is α, the previously given contrary assignment [5,7] is wrong. In the study [7] in which a tetrasaccharide containing Kdo was prepared by deaminative cleavage of the isolated polysaccharide chains of the *B. pertussis* endotoxin, the 3,4-di-*O*-substituted heptose was not detected. Its present identification, by methylation analysis, in the penta- and hexa-saccharides invalidates the structure proposed in the same paper for the region of the polysaccharide chain adjacent to the 5-*O*-substituted Kdo unit.

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