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Structure of the *Bordetella pertussis* 1414 endotoxin[1]

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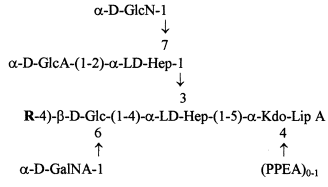
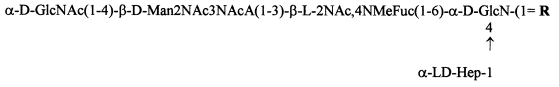
Abstract The endotoxin (lipopolysaccharide) of *Bordetella pertussis*, the agent of whooping cough, consists of a lipid A linked to a highly branched dodecasaccharide containing several acid and amino sugars. The elucidation of the polysaccharide structure was accomplished by first analyzing the structures of fragments obtained by hydrolysis and nitrous deamination and then piecing the fragments together. The fine structure of the antigenic distal pentasaccharide, presented here, was determined by chemical analyses as well as by high-resolution nuclear magnetic resonance and mass spectrometry. The complete structure was reconstituted and confirmed by matrix-assisted laser desorption/ionization mass spectrometry. The following structure was derived from the combined experimental data:





The detailed structure combined with previously reported serological data now allows the synthesis of its epitopes for potential vaccines.

Key words: Endotoxin; Structure; Matrix-assisted laser desorption/ionization mass spectrometry; Nuclear magnetic resonance; *Bordetella pertussis*

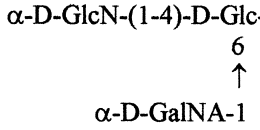
1. Introduction

Endotoxins are powerful immunomodulators from the outer membrane of Gram-negative bacteria. They consist of complex mixtures of related lipopolysaccharides (LPS) of which the lipid region is covalently linked to a polysaccharide (PS) comprising a core region to which may be linked an *O*-chain. Since they are amphipathic and form heterogeneous micelles, intact LPSs have posed problems for their analysis. This was especially true when mass spectrometry was introduced into the analytical process. To obtain spectra it was necessary to diminish either hydrophobicity or hydrophilicity by deacylation, dephosphorylation, methylation, or separation of the lipid and PS moieties. It was found that after decationization and disruption of the micelles, native LPS of masses up to 4 kDa gave usable spectra by plasma desorption mass spectrometry (PDMS) [1].

Bordetella pertussis is a Gram-negative bacterium responsible for whooping cough. Its LPSs have no *O*-chains, unlike most other species of this genus [2,3]. Recently, evidence that *B. pertussis* lacks the genes for *O*-chain synthesis was reported [4]. It has been reported that the pertussis endotoxin may be involved in the whooping cough syndrome by inducing NO production and release by tracheal cells, thereby poisoning the activity of adjacent ciliated cells [5]. Comparison of LPS structures from different members of the genus for their capacity to help induce this cytokine in respiratory cells will advance our understanding of the process.

The lipid A part of the molecules has been fully characterized [6]. The PS moiety is heterogeneous, resulting in two bands on SDS–PAGE analysis of LPS. Band A, the more abundant, slower-migrating species corresponds to an LPS with a dodecasaccharide core and band B, to another species lacking the three distal sugars [7]. The LPS of some strains of *B. pertussis* (e.g. A100 and 134 [7]) also lack these sugars.

Previous studies showed that a trisaccharide isolated after acidic hydrolysis was a glucose (Glc) residue substituted at C-6 and C-4 by galactosaminuronic acid (GalNA) and glucosamine, respectively (Scheme 1) [8]. Nitrous deamination of the intact LPS produced two oligosaccharides, one of which was soluble and the other insoluble because of the attached lipid A moiety. The latter contained a hexasaccharide branched structure (Scheme 2). The analysis of its fine structure minus the location of its phosphate group has been reported [9].



Scheme 1.

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[1] This paper is dedicated to Dr. Ladislas Szabo (Orsay, France) who initiated the subject.

Abbreviations: COSY, correlated spectroscopy; TOCSY, total correlated spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy; HSQC, heteronuclear single quantum coherence; HMBC, heteronuclear multiple bond connectivity; LPS, lipopolysaccharide; MALDI, matrix-assisted laser desorption/ionization mass spectrometry; PPEA, pyrophosphorylethanolamine; Fuc2NAc4NMe, 2-acetamido-4-*N*-methyl-2,4,6-deoxy-galactose; FucNAcMe, 2-acetamido-2-*N*-methyl-2,6-deoxy-galactose; Glc, glucose; GlcA, glucuronic acid; GlcNAc, *N*-acetyl glucosamine; Hep, heptose; Kdo, 3-deoxy-D-*manno*-octulopyranosonic acid; Man2NAc3NAcA, 2-acetamido-3-acetamido-2,3-dideoxy-mannuronic acid; d-ManH, dehydromannitol

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α-D-GlcN-1
↓
7
α-D-GlcA-(1-2)-α-LD-Hep-1
↓
3
β-D-Glc-(1-4)-α-LD-Hep-(1-5)-Kdo-

Scheme 2.

We have communicated the sugar sequence of the core, as a putative structure, in a serological context [10] and as a personal communication from us in a review [11]. However, they contained an error in a fucosamine derivative. The present report (with the correction) gives the structural analysis of the distal soluble oligosaccharide with the help of high resolution nuclear magnetic resonance (NMR), the location of the phosphate group, and combines the various components to give the structure of the complete LPS and its confirmation by mass spectrometry.

2. Materials and methods

2.1. Bacterial endotoxins

B. pertussis strain 1414 was grown and extracted as previously described [7]. The lipid moieties were removed by mild acid hydrolysis [12] and centrifugation. The soluble PS moieties were fractionated on a Sephadex G-50 column (in 0.05 M pyridine-acetate pH 5). PS fractions were collected after TLC migration [13] and lyophilized.

2.2. Nitrous deamination

Nitrous deamination of the LPS (5 mg/ml) was done as described previously [7]. This cleaved the LPS oligosaccharide chain to give the soluble distal pentasaccharide, the insoluble lipid A bound to the proximal oligosaccharide plus free anhydro-sugars. The reaction mixture was centrifuged at 200 000 × *g* for 2 h to separate these two fractions.

2.3. Preparation of the distal pentasaccharide

The above supernatant was adjusted to pH 4–5 with 1 M NaOH, taken to dryness under reduced pressure, redissolved in 15 ml water, centrifuged (3000 × *g*, 10 min), diluted with 40 ml water, and dialyzed in a Diaflo cell under N_2 pressure with a UM05 membrane (Amicon Corp.) to remove the salt. The sample in 3 ml water was applied to a P2 column (80 cm × 2 cm, 100–200 mesh, Biogel) and washed through with water. Fractions of 1 ml/tube were collected. Fractions containing carbohydrates were identified by spotting on silica-gel plates and charring. TLC in the solvent butanol/pyridine/water (6:4:3) identified the pentasaccharide-containing peaks ($R_f = 0.57$). These fractions were combined and lyophilized.

2.4. Reduction

Reduction was done at room temperature with excess sodium tetrahydroborate. The reagent was destroyed with acetic acid, the mixture taken to dryness under reduced pressure.

2.5. Peracetylation

Peracetylation was done with acetic anhydride-dry sodium acetate in sealed tubes at 100°C for 1 h. After drying (50°C, reduced pressure), the product was extracted three times with ethyl acetate.

2.6. Gas chromatography (GC)

Alditol acetates were analyzed by GC (Carlo Erba) on a BP10 (SGA)WCOT vitreous silica capillary column (0.5 μ, 25 m) at 0.4 kPa using the program 160–240° at 4°/min.

2.7. Heptose (Hep)

Ring type was identified by subjecting the pentasaccharide (1.1 mg) to 2.2 μmol of sodium periodate in 0.2 ml water at room temperature for 20 min, stopping the reaction with ethylene glycol 15 min, and reducing with sodium tetrahydroborate. After 2 h the solution was neutralized by glacial acetic acid and lyophilized. The product was then hydrolyzed in 2N HCl for 2 h at 100°C and the acid removed by evaporation under reduced pressure. The sugars were converted to alditol acetates and analyzed by GC. The heptitol acetate had the same retention time on GC as LD-heptitol acetate.

2.8. The D or L configurations

The D or L configurations of *N*-acetyl glucosamine (GlcNAc) and Hep were determined according to the method of Gerwig et al. [14] after hydrolysis of the pentasaccharide (2 N HCl, 2 h) and *N*-reacetylation in the case of GlcN.

2.9. Esterification of uronic acid

Freshly prepared diazomethane in ether (few drops) was added to 12 mg pentasaccharide plus a few drops of methanol, then dried in vacuo. This was repeated once. The solvent was removed under reduced pressure, and the residue dissolved in 0.6 ml water and fractionated on a P2 column (0.6 × 30 cm).

2.10. β-Elimination

The esterified pentasaccharide (3.7 mg), after being vacuum dried at 40°C, was dissolved in 1 ml dry sodium methanolate at room temperature. The reaction was followed spectrometrically (232–254 nm) for 2 h with occasional stirring. One tenth of the reaction mixture was taken to identify the released residue as its alditol acetate. The rest of the above reaction mixture was neutralized with 50% acetic acid, desalted on pyridinium Dowex 50W-X8, and lyophilized. Dissolved in 1.4 ml 1% acetic acid, it was hydrolyzed at 100°C for 1 h, then fractionated on a P2 column (0.9 × 13 cm). Fractions containing the main component were collected, lyophilized, and deuterium-substituted for NMR.

2.11. Smith degradation

Smith degradation of 250 mg of core was carried out as described [7]. The fractions obtained by chromatography on a Biogel P2 column were collected and analyzed by NMR.

2.12. Methylation

Methylation was done by the method of Hakamori [15].

2.13. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI/MS)

MALDI/MS was done on a Perseptive Voyager-DE STR model (PE Biosystems, France) time-of-flight mass spectrometer in linear mode with delayed extraction. Gentisic acid (2,5-dihydroxybenzoic acid) (Sigma Chemical Co., St. Louis, MO, USA) was used as a matrix. 1 μl of a solution of LPS in water (1 mg/ml) desalted with few grains of Dowex 50W-X8, was deposited on the target and mixed with 1 μl of the matrix suspended at 10 mg/ml in a 0.1 M solution of citric acid (unpublished results). Analyte ions were desorbed from the matrix with pulses from a 337 nm nitrogen laser. Spectra were obtained in the negative ion-mode and represent an average of 64 pulses. Acceleration voltage was −20 kV. Lipid A peaks were used as internal standards. The masses are average masses.

2.14. NMR spectroscopy

^{1}H- and ^{13}C-NMR spectra were obtained with a Varian Inova 600 MHz spectrometer using standard pulse sequences, Varian software, and a triple resonance probe. The observed ^{1}H and ^{13}C chemical shifts are reported relative to the methyl group of the internal acetone (δ_H 2.225 ppm, δ_C 31.07 ppm). The sample (6 mg) was dissolved in 0.6 ml H_2O and the pH adjusted to 7.0 with 10 mM NaOH. After lyophilization with D_2O, it was re-dissolved in 0.6 ml of D_2O. For the detection of NH protons, spectra were recorded at 25°C in 90% H_2O/10% D_2O. Acquisition and processing of respective 2D experiments in D_2O (correlated spectroscopy (COSY), total correlated spectroscopy (TOCSY), nuclear Overhauser enhancement spectroscopy (NOESY), heteronuclear single quantum coherence (HSQC), HSQC-TOCSY and

heteronuclear multiple bond connectivity (HMBC)) and in 90% H_2O/10% D_2O (TOCSY and NOESY) were done as described previously [16,17]. Spectra were recorded at 25°C. 1D-NOESY experiments were also done at 15°C. For experiments in H_2O, water suppression was done using the WET sequence [18].

2.15. Molecular modeling

The conformational analysis was done using Metropolis Monte Carlo calculations as previously described [19]. All calculations were performed using the minimized coordinates for the methyl glycosides obtained from molecular mechanics calculations using MM3(92) (QCPE) [20].

3. Results

3.1. Structural analysis of a soluble oligosaccharide obtained by nitrous deamination of B. pertussis 1414 LPS

NMR showed that the oligosaccharide had four anomers, four *N*-acetate groups, an *N*-methyl, the C-6-methyl of a fucosamine, a diaminomannuronic acid substituted at C-4 by another sugar and at C-2 and C-3 by acetamido groups, and was linked to the C-3 of the fucosamine. GC/MS of the alditol acetates established the presence of GlcN, Hep, and 2,5-anhydromannose, the deamination derivative of a GlcN. This established that the derivative was the reducing sugar of the pentasaccharide. In addition, it was known by methylation analyses [7] that there was terminal Hep and GlcN, and that the other GlcN (2,5-anhydromannose in the pentasaccharide) was substituted at C-4 and C-6 in the dodecasaccharide.

Nitrous deamination performed on strain A100 LPS, which lacks the three terminal sugars, gave a disaccharide in which the glucosamine derivative was substituted at C-4 by the terminal L-*glycero*-D-*manno*-Hep (Section 2).

To identify the sugar distal to the uronic acid, the uronic carboxyl was esterified by diazomethane and the distal sugar β-eliminated (see Section 2) and identified as D-glucosamine. It was found to have one of the *N*-acetyl groups. The uronic derivative obtained after β-elimination was easily split off by mild acetic acid hydrolysis leaving a trisaccharide analyzed by NMR. It was shown that the *N*-methyl was still present and thus had to be on the fucosamine, which was the substituent at the C-6 position of the glucosamine (2,5-anhydromannose in the pentasaccharide).

3.2. NMR of the oligosaccharides

The reduced distal pentasaccharide **(OS)** obtained by nitrous deamination of the LPS and the disaccharide obtained by Smith degradation of the core were analyzed by NMR.

The ^1H (Fig. 1) and HSQC spectra of the **OS** indicated the presence of four anomeric carbons, five nitrogen-bearing carbons, an *N*-methyl group ($\underline{C}H_3$ at 38.2 ppm, $C\underline{H}_3$ at 3.25 ppm), four *N*-acetyl groups (methyl proton signals at 1.9–2.10 ppm, carbon signals around 23 ppm), and one methyl group (^1H and ^{13}C chemical shifts at 1.18 ppm and 16.6 ppm, respectively). Absence of a ^{13}C resonance corresponding to methylene carbons (60–65 ppm) indicated that one of the sugars was a uronic acid.

The ^1H- and ^{13}C-NMR spectra of the **OS** were fully assigned by 2D homonuclear and heteronuclear correlation techniques, in conjunction with 1D TOCSY experiments (Table 1). In order to locate the nitrogen containing groups, NMR experiments were performed in 90% H_20/10% D_2O (Fig. 1). The N–H resonances of the NAc groups were assigned on the basis of their large coupling constants with the ring protons via TOCSY experiments. The methyl signals of the NAc groups were assigned on the basis of their strong NOE with the corresponding NH protons within the same *N*-acetyl group (Fig. 1).

The sugar residues in the repeating unit of the **OS** were designated **FN** for the fucosyl residue, **GN** for the GlcNAc residue, **MN** for the mannuronic acid residue, **HEP** for the Hep residue, and **AM** for the anhydromannitol residue.

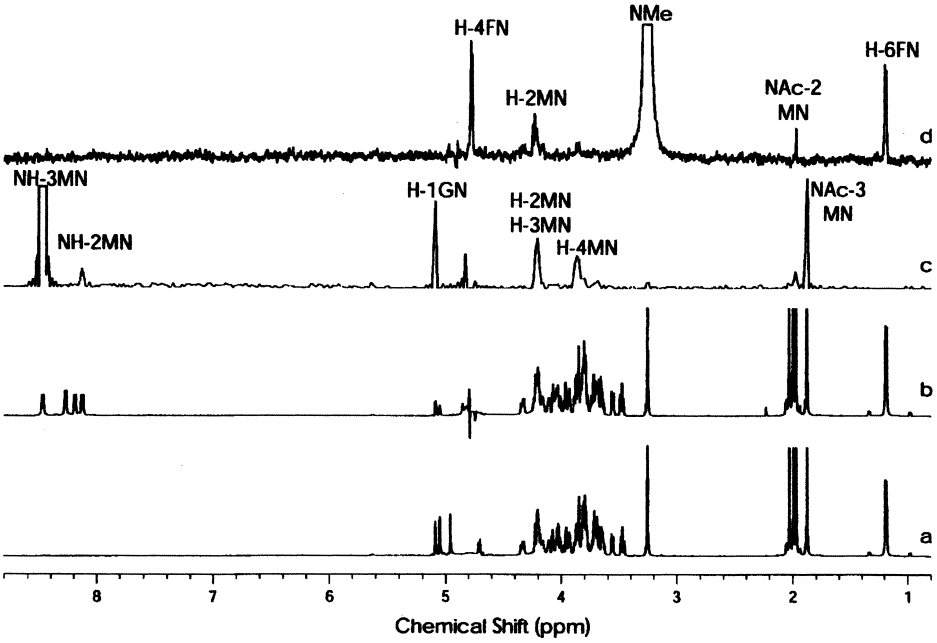

Fig. 1. Proton 600 MHz NMR spectra of the pentasaccharide from *B. pertussis* in D_2O (a) and 90% H_2O (b). Trace for the NH-3 **MN** resonance from the 2D-NOESY experiment in 90% H_2O (c). Selective 1D NOESY spectrum for the NMe resonance (d).

Table 1
NMR chemical shifts for the pentasaccharide isolated from *B. pertussis* endotoxin

Residue	H or C	^{1}H (ppm)	^{13}C (ppm)
AM	1	3.71, 3.64	63.7
	2	3.92	84.2
	3	4.18	76.3
	4	4.07	83.6
	5	4.11	81.6
	6	3.79, 4.00	70.5
FN	1	4.70	102.9
	2	4.20	52.9
	3	4.33	78.6
	4	4.77	67.7
	5	4.16	70.8
	6	1.18	16.6
	CH_3–NAc-2	2.02	23.0
	C=O–NAc-2		175.4
	NMe-4	3.25	38.2
	NH-2	8.19	
MN	1	4.95	100.8
	2	4.20	52.4
	3	4.21	54.1
	4	3.87	71.0
	5	3.84	79.5
	6		175.9
	CH_3–NAc-2	1.96	22.6
	CH_3–NAc-3	1.87	22.6
	C=O–NAc-2		175.6
	C=O–NAc-3		174.6
	NH-2	8.12	
	NH-3	8.47	
GN	1	5.08	97.4
	2	3.80	54.3
	3	3.67	71.2
	4	3.47	70.4
	5	3.70	72.5
	6	3.78, 3.78	60.7
	CH_3–NAc-2	1.99	22.8
	C=O–NAc-2		174.9
	NH-2	8.27	
HEP	1	5.05	100.2
	2	3.95	71.0
	3	3.81	71.3
	4	3.86	66.9
	5	3.56	72.7
	6	4.03	69.6
	7	3.71, 3.64	63.7

Obtained from HSQC and HMBC spectra in D_2O and proton spectrum in 90% H_2O. The error is ±0.02 ppm for ^{1}H chemical shifts and ±0.2 ppm for ^{13}C chemical shifts.

3.3. Residue FN

From selective 1D-TOCSYs on the anomeric resonance at 4.70 ppm and the CH_3 resonance at 1.18 ppm, the H-1 to H-6 resonances of this sugar could be assigned. The small $J_{4,5}$ value of 1 Hz was typical of the *galacto*-configuration. The value of 8.4 Hz for $J_{1,2}$ and the NOE between H-1 to both H-3 and H-5 confirmed the β-configuration. The location of the NMe at C-4 was determined by the strong NOE from NMe to H-4 and H-6, and the (NMe, C-4) HMBC correlation. The NAc group at C-2 was assigned from the (NH-2, COCH3) NOE and the (COC$\underline{H}_3$, CO) HMBC correlations. The NH-2 resonance was assigned from a 1D-TOCSY experiment on the NH resonances to detect the (NH-2, H-2) correlation. The NH resonance of the NMe group cannot be observed because of fast exchange with solvent. Residue **FN** was thus identified as 2-acetamido-4-*N*-methyl-2,4,6-deoxy-galactose (β-Fuc2NAc4NMe).

3.4. Residue GN

From selective 1D-TOCSY experiments on the anomeric resonance at 5.08 ppm, the H-1 to H-5 resonances of this sugar could be assigned. The H-6 resonances were assigned from the COSY spectrum and 1D-TOCSY on the isolated H-4 resonance at 3.47 ppm. The large $J_{H,H}$ values were typical of the *gluco*-configuration. The value of 3.6 Hz for $J_{1,2}$ and the NOE between H-1 and H-2 and not to H-3 and H-5 confirmed the α-configuration. The NAc group at C-2 was assigned from the (NH-2, COCH3) NOE and the (COC$\underline{H}_3$, CO) HMBC correlations. The NH-2 resonance was assigned from a 1D-TOCSY experiment on the NH resonances to detect the (NH-2, H-2) correlation. Residue **GN** was thus identified as α-GlcNAc.

3.5. Residue MN

From a selective 1D-TOCSY experiment on the anomeric resonance at 4.95 ppm, signals were observed at 4.2 and 3.87 ppm. Then, from an HSQC and HSQC-TOCSY experiments, the H-1 to H-5 resonances of this sugar could be assigned. The small $J_{1,2}$ value of <1 Hz was typical of the *manno*-configuration. The NOE H-1 to both H-3 and H-5 confirmed the β-configuration. The NAc groups at C-2 and C-3 were assigned from the (NH, COCH3) NOE and the (COC$\underline{H}_3$, CO) HMBC correlations. The NH resonances were assigned from 1D-TOCSY experiments on the NH resonances to detect the (NH-2, H-2) and (NH-2, H-3) correlations. The location of the carboxyl group at C-5 was confirmed by the presence of the (H-5, COO) HMBC correlation. Residue **MN** was thus identified as β-2-acetamido-3-acetamido-2,3-dideoxy-mannuronic acid (Man2NAc3NAcA).

3.6. Residue HEP

From a selective 1D-TOCSY experiment on the anomeric resonance at 5.05 ppm and the isolated resonance at 3.56 ppm, the H-1 to H-6 resonances of this sugar could be assigned. From COSY experiments, the H-7 and H-7′ resonances were then assigned. The small $J_{1,2}$ value of <1 Hz was typical of the *manno*-configuration. The NOE H-1 to H-2 and not to H-3 and H-5 confirmed the α-configuration. Residue **HEP** was thus identified as α-Hep.

3.7. Residue AM

From COSY experiments, HSQC and HSQC-TOCSY experiments, the remaining eight crosspeaks in the HSQC spectrum could be easily assigned. From knowledge of the chemical modification necessary to prepare this pentasaccharide, this residue was identified as anhydromannitol.

3.8. Sequence of sugars

The sugar sequence was determined from the transglycosidic NOEs between anomeric and aglyconic protons in adjacent glycosyl residues and from $^{3}J_{C,H}$ HMBC correlations across the glycosidic bond. The occurrence of the major transglycosidic NOEs (H-1 **GN**, H-4 and H-5 **MN**), (H-1 **MN**, H-2 and H-3 **FN**), (H-1**FN,** H-6 and H-6′ **AM**), (H-1 **HEP**, H-3 and H-4 **AM**), along with the HMBC correlations (C-1 **GN**, H-4 **MN**), (H-1 **MN**, C-3 **FN**), (C-1 **MN**, H-3 **FN**), (C-1 **FN**, H-6 **AM**), (H-1 **HEP**, C-4 **AM**), (C-1 **HEP**, H-4 **AM**) were indicative of the proposed sequence for the pentasaccharide **GN**-^{4}**MN**-^{3}**FN**-^{6}[**HEP**-^{4}]**AM**.

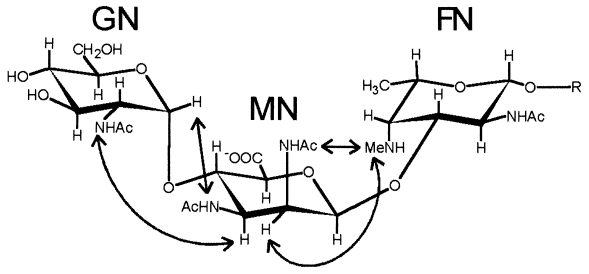

Fig. 2. Structure of the trisaccharide present in the distal pentasaccharide from *B. pertussis*. Some short interproton distances that could be observed in the NOE experiments and used to determine the absolute configuration of the sugars are shown.

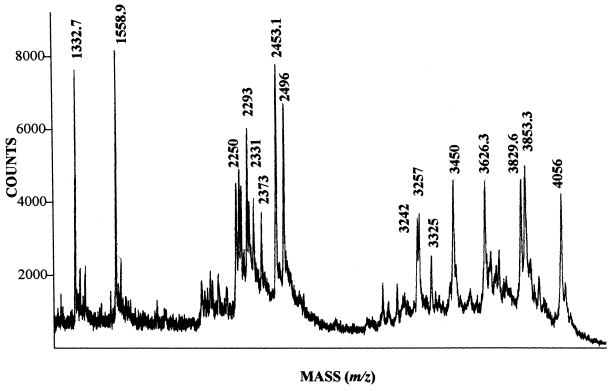

Fig. 3. MALDI negative-ion spectrum of the *B. pertussis* 1414 endotoxin.

3.9. Absolute configuration of monosaccharides

Only the absolute configuration of the residue **GN** and **HEP** could be determined by GC–MS. The absolute configurations of residues **MN** and **FN** were determined using potential energy calculations in combination with NOE measurements. This method has been previously used to establish the absolute configuration of constituent monosaccharide residues in poly- and oligo-saccharides in cases where conventional methods were not amenable [21–23]. The approach has been particularly successful for the monosaccharide residues with a number of pendant groups around the pyranose ring, as is the case here, since they are more constrained in their rotation around the glycosidic bond [16,17].

Since the NOEs depend on interproton distances (r^{-6}), interresidue NOEs can be highly sensitive to the absolute configuration of a pyranose ring as well as the conformation about the glycosidic bond. Molecular modeling was performed for each disaccharide linkage with two different absolute configurations for one of the component monosaccharides. On the basis of flexibility about the glycosidic bond, the average interproton distances were obtained. Some of the interproton distances across the glycosidic bond were highly dependent on the relative absolute configuration of the monosaccharides and could be used to determine the absolute configuration of the sugars (Fig. 2).

With the absolute configuration of **GN** known to be D by GC–MS, interproton distances for both configurations of **MN** were calculated for the **GN-**^{4}**MN** linkage. The (NH-3 **MN**, H-1 **GN**) NOE (Fig. 1) and (NH-2 **GN**, H-3 **MN**) could be used to determine the absolute configuration of residue **MN**. When residue **MN** has the D-configuration, the (NH-3 **MN**, H-1 **GN**) and (NH-2 **GN**, H-3 **MN**) average distances are 2.2 ± 0.2 Å and 3.1 ± 0.2 Å as opposed to 4.3 ± 0.3 Å and 4.1 ± 0.1 Å for the L-configuration. Hence, the D-configuration for **MN** is consistent with the observed NOEs.

With **MN** having D-configuration, interproton distances for

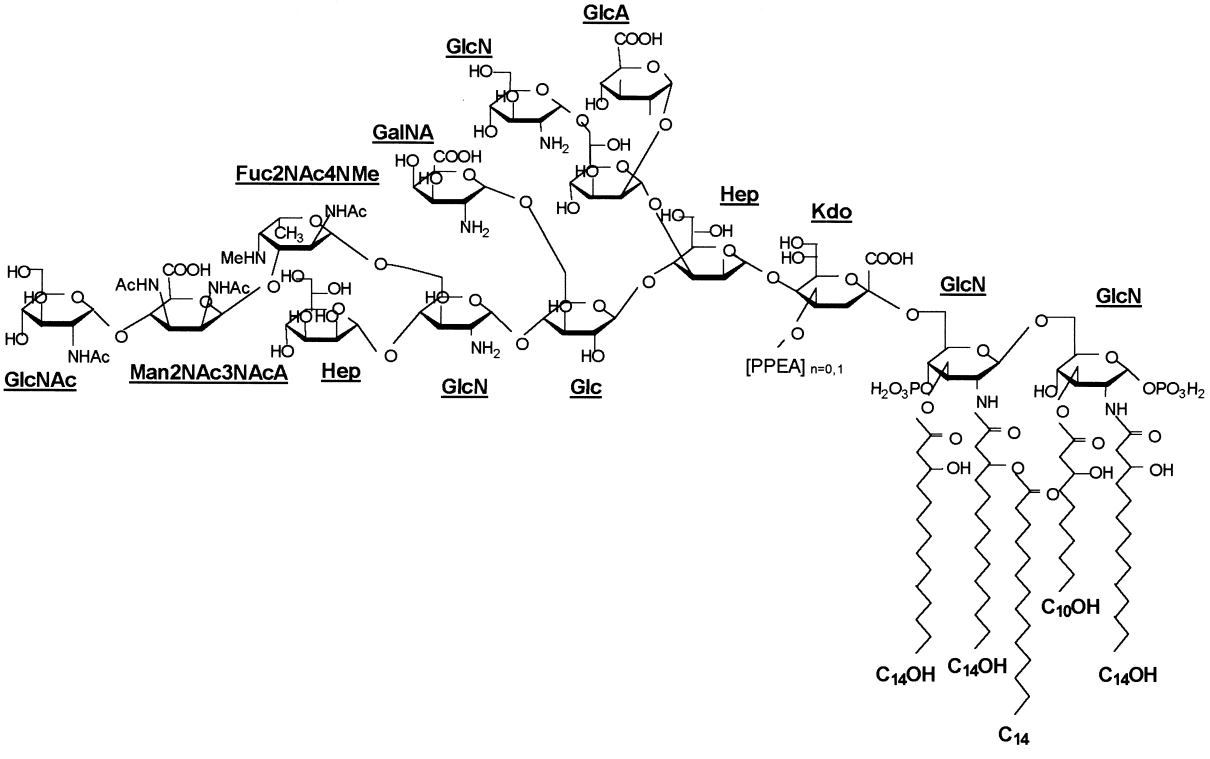

Fig. 4. Structure of the LPS of *B. pertussis* 1414.

both configurations of **FN** were calculated for the **MN-**^{3}**FN** linkage. The (NMe **FN**, CH3 NAc-2 **MN**) and (NMe **FN**, H-2 **MN**) NOE could be used to determine the absolute configuration of residue **FN** (Fig. 1). When residue **FN** has the L-configuration, the (NMe **FN**, H-2 **MN**) and (NMe **FN**, CH3 NAc-2 **MN**) average distances of closest approach were 3.6 ± 0.8 Å and 5.4 ± 1.4 Å as opposed to 5.9 ± 0.2 Å and 5.8 ± 1 Å for the D-configuration. Hence, the L-configuration for **FN** is consistent with the observed NOEs.

3.10. MALDI/MS analysis

The negative-ion spectrum of the unmodified LPS (Fig. 3) had three zones of signals corresponding to LPS molecular ions, core fragment ions, and lipid A fragment ions. In the lower mass range the two major peaks at *m/z* 1332 and 1558 correspond to the already described tetra- and penta-acylated species of the lipid A [6]. The middle range corresponds to the heterogeneous core moiety. The peak at *m/z* 2293 corresponds to the anhydrododecasaccharide followed by its phosphorylated, pyrophosphorylated, and pyrophosphorylethanolamine (PPEA) forms appearing respectively at *m/z* 2373, 2453, and 2496. In the highest mass range, corresponding to LPS molecular species, the peaks at *m/z* 4056 and 3853 correspond to the penta-acylated LPS with and without PPEA, respectively. The peaks at *m/z* 3829 and 3626 correspond to their tetra-acylated analogs. Peaks at *m/z* 3450 and 3257 correspond to 3626 minus GalNA and the sum (GalNA plus Hep), respectively. The peak at *m/z* 3325 corresponds to *m/z* 3450 minus PEA and *m/z* 3242 to *m/z* 3257 minus a methyl group. Minor heterogeneity due to incomplete acetylation (*m/z* 2250, 2331) or methylation (unlabelled at 15 u below *m/z* 2250) is also observed.

4. Discussion

The analysis of the PS structure of *B. pertussis* was accomplished in three stages as described in Section 1. The structure of the proximal hexasaccharide [9] contained the unique Glc residue found in the trisaccharide. The analysis of the distal pentasaccharide presented here completed the structure. High-resolution NMR made clear that the fucosamine had a second amine group at the C-4 position and that this one held the methyl group. This sugar was thus the unusual β-L-2-acetamido-4-methylamino-fucose, and not *N*-methyl-*N*-acetyl-fucosamine as previously reported [7,10]. The highly antigenic C-PS of *Streptococcus pneumoniae* contains its unmethylated α-D-isomer [24]. The assembled components of the whole LPS had calculated masses identical to those observed by MALDI/MS of the endotoxin. It was earlier shown that 60% of the LPS molecules were phosphorylated on their 3-deoxy-D-*manno*-octulopyranosonic acid (Kdo) [25]. The phosphate group was responsible for the low reactivity of the Kdo in assay systems unless it was previously removed by HF [26,27]. Since the C-7-C-8 diol of Kdo was oxidizable by periodate and the C-5 was occupied by a Hep, the phosphate had to be at the C-4 position. MALDI showed that the Kdo substituent could only be PPEA and not orthophosphate. No other phosphorylated sugar than Kdo was found in the core. PPEA was always eliminated into the supernatant during the mild acid hydrolysis used to separate the PS and lipid components of the LPS [12] with the concurrent formation of an unsaturated derivative [26,27].

Fig. 4 presents the complete fine structure obtained by the synthesis of all the earlier and new data.

With a few exceptions, the basic structure of the *B. pertussis* 1414 LPS core is more 'complete' than those of other strains of this species and of other *Bordetella* species examined ([7,28], unpublished results). The main variations were the absence of Hep and/or GalNA, the *N*-methyl group of Fuc2-NAc4NMe, or the terminal trisaccharide (unpublished results).

The various core fragments (di-, tri-, and pentasaccharide) were coupled with bovine serum albumin to determine the epitopes recognized by monoclonal antibodies prepared against the LPSs for diagnostic purposes [10]. The structures of interest for inclusion in vaccines were identified as the distal tri- and pentasaccharide [10] which can now be synthesized and included in a polyvalent vaccine.

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