Characterization of Bacterial Lipooligosaccharides by Delayed Extraction Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry

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Matrix-assisted laser desorption ionization (MALDI) with a time-of-flight analyzer has been used to analyze bacterial lipooligosaccharides (LOS). Crude LOS preparations from pathogenic strains of *Haemophilus influenzae* and *Haemophilus ducreyi* and a commercial preparation of lipopolysaccharide from *Salmonella typhimurium* were treated with hydrazine to remove *O*-linked fatty acids on the lipid A moiety. The resulting *O*-deacylated LOS forms were water soluble and more amenable to cocrystallization with standard MALDI matrices such as 2,5-dihydroxybenzoic acid and 1-hydroxyisoquinoline. Under continuous extraction conditions, *O*-deacylated LOS yielded broad peaks with abundant salt adducts as well as forming prompt fragments through β-elimination of phosphoric acid, that is, $[M\text{-}H_3PO_4\text{-}H]$. However, when a time delay was used between ionization and extraction ("delayed extraction") a significant improvement was seen in both mass resolution and the stability of the molecular ions against β-elimination of phosphoric acid, especially in the negative-ion mode. Both an external two-point calibration and an internal single-point calibration were used to assign masses, the latter of which provided the highest degree of accuracy (better than 0.01% in most cases). At higher laser powers, the LOS molecules cleave readily between the oligosaccharide and lipid A moieties yielding a number of prompt fragments. Postsource decay (PSD) analysis of selected molecular ions provided a set of fragments similar to those seen in the linear spectra, although they were more limited in number because they were derived from a single LOS-glycoform. Both the prompt and PSD fragments provided important structural information, especially in assigning the phosphate and phosphoethanolamine substitution pattern of the lipid A and oligosaccharide portions of LOS. Last, with the addition of ethylenediaminetetraacetic acid followed by pulsed sonication, the relatively insoluble (and impure) LOS preparations yielded MALDI spectra similar to the *O*-deacylated LOS, although these intact LOS preparations required higher laser powers to ionize and were generally more affected by competing impurities. (J Am Soc Mass Spectrom 1997, 8, 645–658) © 1997 American Society for Mass Spectrometry

The outer membrane of pathogenic Gram-negative bacteria contain proteins, polysaccharides, and glycolipids that play critical roles in the organism's ability to colonize human tissue and evade host defenses. To investigate the molecular mechanism off bacterial pathogenesis, our laboratory has been working on methods for the structural and functional characterization of the bacterial surface glycolipids or lipooligosaccharides (LOS) from *Haemophilus* and *Neisseria* species (see for example, [1–3]). The LOS from these pathogenic organisms are highly complex and anionic in character, and contain both neutral and acid sugars, as well as phosphate and phosphoethanolamine (PEA) (see Figure 1).

The LOS molecule can be thought of as consisting of two parts: a hydrophobic and anionic lipid A moiety, and a complex oligosaccharide that is linked to the lipid A moiety through the acidic sugar 2-keto-3-deoxyoctulosonic acid (Kdo). The lipid A moiety itself consists of two 1-6-linked glucosamine sugars substituted with two phosphate groups and up to six (and sometimes more) *N*- and *O*-linked fatty acids. The fatty acid groups of lipid A from the outermost bilayer of the outer membrane and are thought to provide a formidable barrier by forming salt bridges through

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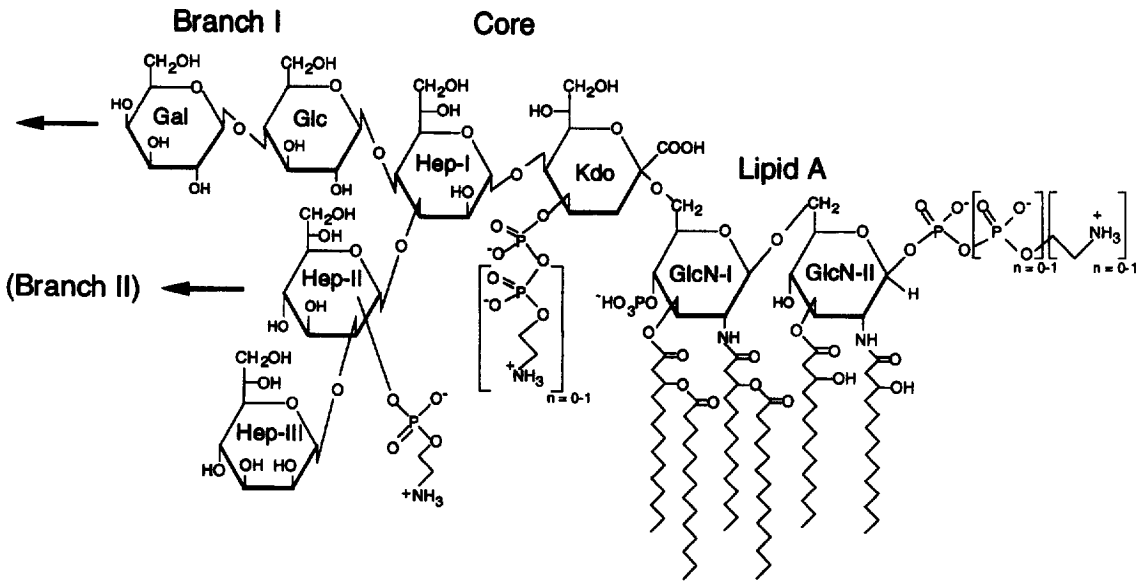


Figure 1. Structure model for *Haemophilus* lipooligosaccharides. Arrows indicate positions of further oligosaccharide branch extensions seen in many wild-type strains. Substitutions by PEA on the phosphate group of phospho-Kdo and phosphate and/or PEA on the reducing terminal phosphate of lipid A are structural modifications elucidated in this work.

their phosphate groups with divalent cations [4]. The oligosaccharide regions of LOS extend out into the external environment and are therefore most likely to interact with host macromolecules and cell surfaces. These LOS-oligosaccharides are relatively short but highly branched, and are most often substituted with phosphate and PEA moieties (see Figure 1). LOS are highly heterogeneous, primarily in the oligosaccharide regions, although variations in lipid A structure have also been reported [5,6]. Overall, the presence of lipophilic, polar, and ionic moieties in LOS makes these glycoconjugates highly amphipathic and largely insoluble in water and most organic solvents. Together, these properties make the analysis and characterization of LOS extremely challenging.

In an effort to find ways to better characterize LOS, our laboratory has developed analytical methods for the characterization of modified or partially degraded LOS and oligosaccharides by using liquid secondary ionization mass spectrometry [1] and electrospray ionization mass spectrometry (ESI-MS) [2]. Other groups have used plasma desorption mass spectrometry for the analysis of intact rough-type lipopolysaccharides that are of similar size to LOS [7].

LOS are known to vary their structure during growth, a phenomenon termed "phase variation," presumably through alternate biosynthetic pathways in their oligosaccharide constituents [8]. It is generally thought that the ability to vary these external oligosaccharide structures in response to external stimuli underlies the successful adaptation of these organisms to the changing and hostile host environment. To date, LOS analysis and structural characterization studies have been carried out almost exclusively on relatively large scale preparations ($\geq$ 10 mg) isolated from passaged organisms grown on solid or liquid cultured media. However, to truly understand the specific roles of individual LOS-glycoforms in the disease process, methods are required that are capable of analyzing LOS obtained from human sources without external multiplication or passage. Such a source might be bacteria isolated directly from humans, such as patients exhibiting primary chancroid ulcers or gonococcal lesions. The amount of LOS that can be realistically obtained from these latter sources is still in question, but our own estimates suggest it to be in the subpicomole range or lower.

Matrix-assisted laser desorption ionization (MALDI) mass spectrometry with a time-of-flight (TOF) analyzer has been shown to have exceedingly high sensitivities for peptides and proteins [9,10], as well as some other classes of biological compounds such as oligonucleotides and oligosaccharides [11–14]. Given this high sensitivity, we initiated a program to find conditions that might be suitable for the analysis of LOS that would ultimately allow us to design strategies for the analysis of LOS from clinical sources. In this report, we present our first data on the analysis of bacterial LOS by using delayed extraction MALDI-TOF in both the linear, reflectron, and postsource decay modes of operation.

Experimental

Materials

Rough type lipooligosaccharide from *Salmonella typhimurium* type Ra was purchased directly from Sigma Chemical Co. (St. Louis, Mo). All lipooligosaccharide

preparations were isolated by a modified phenol–water extraction method from bacteria grown on cultured media according to procedures described in detail elsewhere [1, 15]. The MALDI matrices 2,5-dihyroxybenzoic acid (DHB) and 1-hydroxyisoquinoline (HIC) were purchased from Aldrich Chemical Co. (Milwaukee, WI) and DHB was recrystallized from deionized water prior to use. Acetonitrile and water were obtained as the highest high-performance liquid chromatography Fisher Scientific (Fairlawn, NJ).

Sample Preparation

O-deacylated LOS was prepared from the crude LOS preparation (≈ 0.5–1 mg) by treatment with hydrazine at 37 °C for 30 min. Afterward, the *O*-deacylated LOS was precipitated with cold acetone, washed with water, and lyophized as described in detail elsewhere [1].

Mass Spectrometry

For all mass spectrometry experiments, either a Voyager Elite or XL Voyager Elite matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) instrument (PerSeptive Biosystems, Framingham, MA) were used. Both instruments were equipped with a nitrogen laser (337 nm) and, except where noted, all experiments were carried out by using delayed extraction conditions as described in detail elsewhere [16]: 200–350-time delay with a grid voltage of 92–94% of full acceleration voltage (20–30 kV). Spectra were mass assigned by using an external two-point external calibration or, in the case of the *O*-deacylated LOS preparation from *Haemophilus ducreyi* 35000 a known peak in the spectrum was used as a one-point internal calibrant to correct the external calibration as available in version 3.5 of the manufacturer's software (PerSeptive Biosystems). (Any known peak can be used; the slope value of the linearized calibration equation is modified and the intercept from a previous external calibration is retained.)

All intact LOS and *O*-deacylated LOS samples were run in recrystallized 2,5-dihydroxybenzoic acid (DHB) [17] or in a mixture of 100-mM DHB solution containing 33-mM 1-hydroxyisoquinoline as originally suggested for underivatized oligosaccharides by Mohr et al. [18]. In most cases, small aliquots of *O*-deacylated LOS samples containing a total of 0.1–0.2 $\mu g/\mu L$ were mixed together with the matrix solution (total volume ≈ 5–6 μL) and desalted with cation exchange beads (Dowex 50X, NH_4^+ from prior to crystallization on the MALDI plates [19]. In this cleanup step, a 5-μL aliquot of LOS–matrix mixture was prepared and transferred as a single droplet onto the piece of Parafilm® (American Can Co., Greenwich, CT) which had a dispersed layer of cation-exchange resin beads. By using the tip of the microtip dispenser, the droplet was moved so as to be exposed to about 5–10 beads in a 15-s period and then transferred to the MALDI plate (≈ 1 μL per well). To prepare a soluble form of the intact LOS, 5-mM ethylenediaminetetraacetic acid (EDTA) was added to the insoluble LOS followed by several 2–3 pulses of ultrasonication (microtip with maximum 50-W output; Fisher Scientific). This LOS–EDTA mixture was added directly to an equal volume of 100-mM DHB in water and then transferred to the MALDI plate.

Results and Discussion

Relative Abundances and Molecular Masses of LOS-Glycoforms

In our first attempts at analyzing LOS by MALDI-TOF, the first priority was to find suitable conditions for the determination of the relative abundances and molecular weights of the LOS-glycoforms present in various LOS preparations. For most of these experiments, chemically degraded forms of LOS were used that had undergone removal of *O*-acyl groups from the lipid A moiety by mild hydrazine treatment. The rationale for working with *O*-deacylated LOS preparation was threefold. First, removal of *O*-linked fatty acids on the lipid A moiety of LOS by mild hydrazine treatment renders the resulting *O*-deacylated LOS water soluble, and therefore in a less aggregated state and more likely to cocrystallize with standard MALDI matrices such as DHB. Second, removal of *O*-linked fatty acids from the lipid A moiety reduces the heterogeneity of LOS and enables one to concentrate on the more variable oligosaccharide regions of the LOS molecule. Third, LOS preparations by themselves are contaminated with impurities that often ionize much better than LOS. The mild hydrazine treatment includes an acetone precipitation step that removes many of these competing impurities.

In the first set of experiments, a commercial preparation of *S. typhimurium* RA LPS was used because it was known to be relatively free of most non-LOS impurities and its structure has been published [20]. Moreover, this mutant *Salmonella* strain makes an LPS that lacks a repeating polysaccharide component and whose remaining core oligosaccharide region is similar in size to the oligosaccharides found in the LOS of wild-type strains of pathogenic *Haemophilus* and *Neisseria*. As seen in the bottom panel of Figure 2, the positive-ion MALDI spectrum from this *O*-deacylated. *S. typhimurium* Ra LOS preparation obtained under continuous extraction conditions was complex, with very poor peak resolution among the various LOS-glycoforms. Indeed, the poor resolution and broad peak shapes of these LOS components resemble these of oligonucleotides, where the combination of metastable decay and salt adducts has been shown to greatly reduce the quality of MALDI spectra [13]. However, under delayed extraction conditions, the resulting spectra were dramatically improved. As shown in the

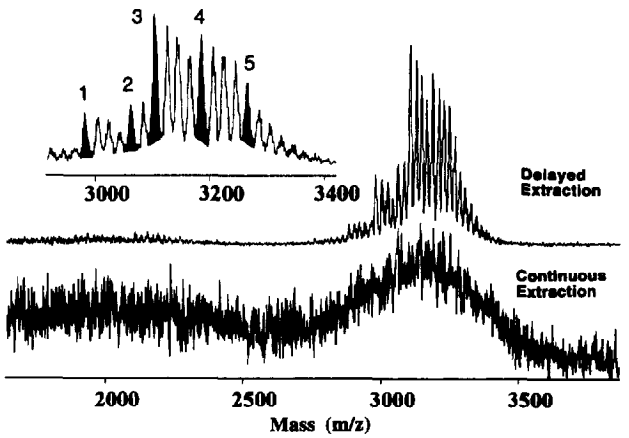
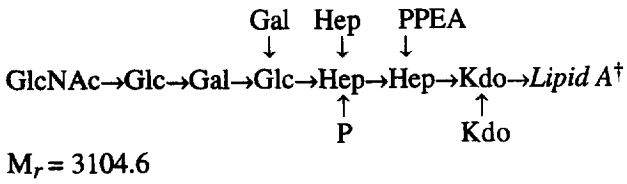


Figure 2. Positive-ion MALDI-TOF spectrum of *S. typhimurium* Ra strain *O*-deacylated LOS run under continuous extraction (bottom panel) and by delayed extraction (top panel) conditions. The inset at the top right is an expansion of the m/z 3000–3400 region of the delayed extraction spectrum. At least five separate LOS-glycoforms whose singly deprotonated molecular ions $[M - H]^-$ are shown as filled-in peaks, each with several salt adducts including sodium and/or potassium, can be observed in this latter spectrum with M_rs at 2982, 3062, 3105, 3185, and 3265 (see text for explanation).

top panel of Figure 2, multiple LOS-glycoforms and their salt adducts were readily resolved and much better peak shapes were obtained. However, even under delayed extraction conditions, interpretation of the molecular ion region was difficult due the large number of salt adducts present for each LOS-glycoform, for example, $[M + Na]^+$, $[M + K]^+$, $[M - H + 2Na]^+$, and so forth.

To reduce the number of cationized molecular ions, a rapid desalting step of the analyte–MALDI matrix was carried out by using Dowex resin on Parafilm prior to the MALDI crystallization. As shown in the top spectrum of Figure 3, this simple procedure dramatically improved the overall MALDI spectrum and greatly reduced the number of cationized molecular ions. The LOS-glycoforms and their masses could now be assigned with much greater certainty because they were present predominantly as their protonated forms. In fact, the heterogeneity of the *Salmonella* LOS-glycoforms could be identified as arising through differences in the number of phosphate (P, ΔM = 80u) and/or phosphoethanolamine (PEA, ΔM = 123u) moieties they contained. For example, from the base $[M + H]^+$ peak at m/z 3005.3, nearby peaks are seen at m/z 2882.7 and 3085.1 that differ by approximately 123 (loss of PEA) and 80u (addition of P), respectively. However, when we attempted to assign compositions based on the observed masses of these molecular ion species, a discrepancy of ~ 98u was encountered compared with their expected masses based on prior ESI mass spectrometry (data not shown) [2] and/or chemical and NMR studies [20]. Indeed, the expected masses are either not present at all or present at very low abundances. For example, the *major* *O*-deacylated LOS has a predicted M_r of 3104.6, yet the $[M + H]^+$ peak corresponding to this LOS-glycoform is one-tenth as abundant as the peak 98u lower in mass at m/z 3005.3:



This mass difference suggested that these LOS undergo facile loss of phosphoric acid from the molecular ions to form dehydro-LOS species $[M - H_3PO_4 + H]^+$. This loss may explain why MALDI spectra obtained under continuous extraction conditions provided such low resolution and poor peak shapes for these highly phosphorylated LOS molecules, as one would expect the metastable loss of phosphoric acid to contribute to peak tailing and loss of mass resolution. Moreover, the loss of phosphate groups through a presumed β-elimination of phosphoric acid (−98 u, H_3PO_4) adds an additional (and undesirable) level of complexity to the interpretation of the molecular ion region. Follow-up studies on a number of different LOS preparations have shown that this loss is generally observed in the positive-ion MALDI spectra of *O*-deacylated LOS. The loss of phosphoric acid leads to the formation of prompt fragments that are 18 u lower in mass than what one would expect from composition assignment of an LOS containing *one less* phosphate group that is, $[M + H - 98]^+$ for prompt loss of H_3PO_4, compared to the $[M + H - 80]^+$ peak observed for the loss of the phosphate group HPO_3.

Unlike the positive-ion spectra, however, the analogous negative-ion MALDI spectrum of this same *S. typhimurium* *O*-deacylated LOS sample contained abundant deprotonated molecular ions $[M - H]^-$ that agreed well with the expected masses for these LOS-glycoforms (see bottom spectrum of Figure 3 and Table 1). For example, two of the most abundant peaks at m/z 3102.8 and 3182.7 agree to within ±1 u of the expected masses for these LOS-glycoforms. The β-elimination of phosphoric acid from the deprotonated molecular ion species was considerably less than in positive-ion mode and virtually absent when the laser power was at or near threshold levels (data not shown). This effect makes the negative-ion spectra much more suitable for the characterization of the LOS-glycoform populations.

Given the clear advantages of analyzing *O*-deacylated LOS mixtures under the conditions of delayed extraction and in the negative-ion mode, this experi-

† In this case and others, lipid A is present in its *O*-deacylated form, that is, two glucosamines containing two N-linked β-hydroxymyristic acids and two phosphates, one at the 4′ position [GlcN(II)] and the other at the reducing terminus [GlcN(I)].

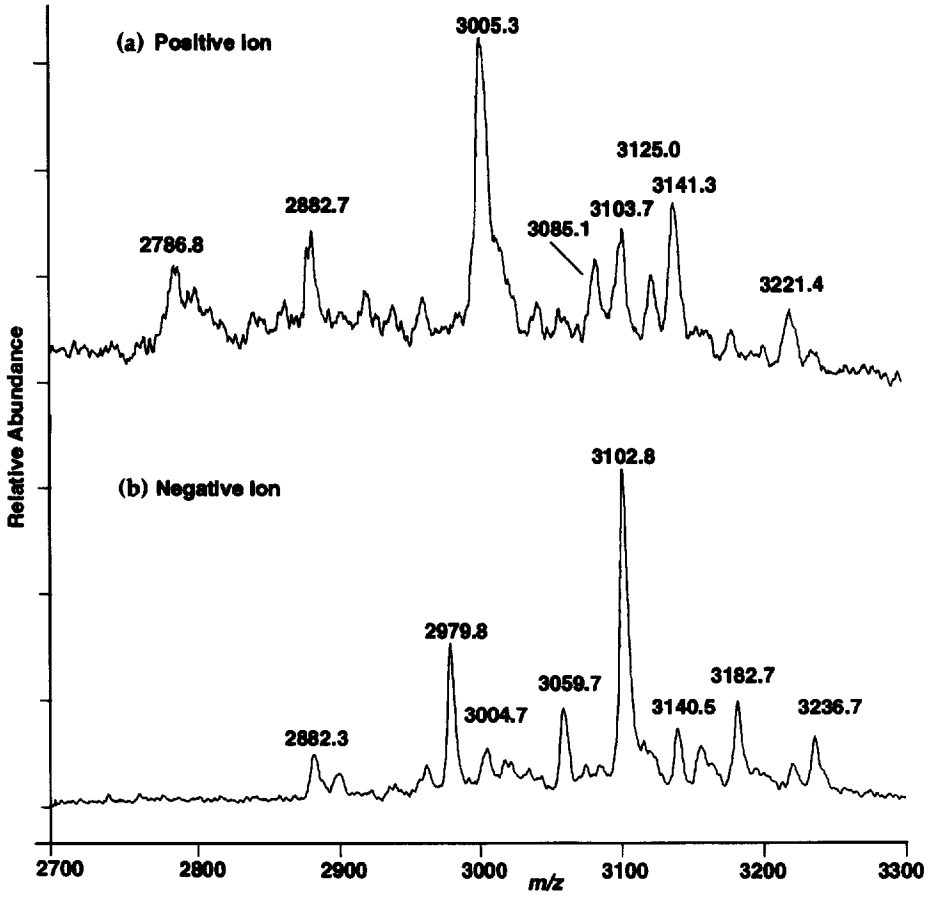


Figure 3. Molecular ion regions of the (a) positive-ion and (b) negative-ion MALDI-TOF spectra of *O*-deacylated LOS obtained under delayed extraction conditions from *S. typhimurium* Ra strain.

mental protocol was used exclusively in the remaining experiments. Even when more complex mixtures of LOS-glycoforms were analyzed, such as that shown in Figure 4 for the *O*-deacylated LOS from a wild-type strain of *H. ducreyi* (strain 35000), well-resolved molecular ion species were obtained that allowed for straightforward mass assignments. For example, the heterogeneity in this *H. ducreyi* LOS preparation can be unambiguously assigned to differences in both phosphorylation (phosphate and PEA) and carbohydrate moieties (Gal, GlcNAc, and NeuAc) (see Table 1). However, unlike the first LOS preparation in which an *external* calibration was used as the calibrant for mass assignments, a single-point *internal* calibration marker was used in this example to modify the initial external calibration. For this purpose, the major LOS-glycoforms with a calculated M_r of 2711.5 was used because its molecular weight and composition had been determined in a previous study [21]:

Gal→GlcNAc→Gal→Hep→Glc→Hep→Kdo(P)→*Lipid A*†
↑
Hep
↑
Hep

$M_r = 2711.5$

As seen in this MALDI spectrum, a series of peaks is present between m/z 3200–2100, with the base peak at an experimental value of m/z 2710. When the internal calibration was used, mass assignments were obtained for the remaining LOS-glycoforms that were in excellent agreement with the calculated masses for these LOS-glycoforms, with typical mass accuracies of better than ± 0.01% (see Table 1). An inspection of this and other MALDI data obtained in these experiments (see Table 1) suggests that the reason for this marked improvement in mass accuracy is primarily due to the high degree of mass precision, where the mass differences between the experimentally determined and expected masses in any single spectrum almost always disagree in one direction only, that is, they are either all to high or all too low in mass.

Prompt Fragmentation

In addition to the abundant molecular ion region observed in the linear MALDI spectrum of *O*-deacylated LOS preparations, significant fragment ions were also observed at lower masses. These prompt fragments were almost always present and were sometimes observed under continuous extraction conditions as well, although they appeared as sharp well-defined peaks only under delayed extraction conditions and their

Table 1. Structures and molecular weights of *O*-deacylated LOS

Bacterial Strain Major *O*-Deacylated LOS Structure[a]	Expt. M_r	Calc +/– Moiety[b]	Calc. M_r	ΔM
S. typhimurium Ra *O*-deacylated LPS				
Gal P ↓ | GlcNAc → Glc → Gal → Glc → Hep → Hep → $(Kdo)_2$ → *lipid A** ↑ | Hep PPEA	2980.8	–PEA	2981.6	–0.8
	3060.7	–EA	3061.6	–0.9
	3103.8	—	**3104.6**	–0.8
	3141.5	+EA	3141.5	0.0
	3183.7	+P	3184.6	–0.9
	3237.7	+P(Fe^{III})?	(3237.4)	+ 0.3
	3263.9	+2P	3264.6	–0.7
H. influenzae 276.4 *O*-deacylated LOS				
Glc → Glc → Hep → Kdo(P) → *lipid A** ↑ Glc → Glc → Hep - - PEA ↑ Hep	2277.9	–2 Glc	2277.0	+0.9
	2440.2	–1 Glc	2439.1	+1.1
	2602.6	—	**2601.3**	+1.3
	2967.8	+Gal-GlcNAc	2966.6	+0.8
H. ducreyi 35000 *O*-deacylated LOS				
Gal → GlcNAc → Gal → Hep → Glc → Hep → Kdo(P) → *lipid A** ↑ Hep ↑ Hep	2184.3	–2Gal-GlcNAc	2184.0	–0.3
	2346.5	–Gal-GlcNAc	2346.1	–0.4
	2549.4	–Gal	2549.3	–0.1
	2711.5	—	**2711.5**	—
	2834.6	+PEA	2834.5	–0.1
	3002.8	+NeuAc	3002.7	–0.1
	3125.8	+NeuAc-PEA	3125.8	0.0
H. ducreyi 1741 *O*-deacylated LOS				
Glc → Hep → Kdo(P) → *lipid A** ↑ Hep ↑ Hep	N.D.	—	**1991.8**	—
	2113.9	+PEA	2114.9	–1.0
	2166.9	+PEA, (Fe^{III})	2167.7	–0.8
	2236.9	+2PEA	2237.9	–1.0
	2290.0	+2PEA, (Fe^{III})	2290.7	–0.7

[a] *Lipid A** is *O*-deacylated and, as referred to herein, contains two N-linked β-hydroxymyristic acids and two phosphates (M_r = 953.0, average). Abbreviations: P, phosphate; PEA, phosphoethanolamine; Gal, galactose; Glc, glucose; glcNAc, N-acetylglucosamine; Hep, heptose; Kdo, 2-keto-3-deoxyoctulosonic acid; NeuAc, N-acetylneuraminic acid or sialic acid; N.D., none detected.
[b] Mass differences are relative to major LOS structure drawn in the far left column and whose molecular weight is listed in bold type.

relative abundances were substantially lower when sampling near the threshold laser power. As seen in the MALDI spectrum shown in Figure 5 of the *O*-deacylated preparation from *S. typhimurium* Ra, two regions of prompt fragment ions can be defined. The first region is centered ~1000 u lower in mass than the molecular ion regions, between m/z 2300–1700, whereas a second set of fragments is centered at m/z 952. Both sets of these LOS fragments originate from cleavage at the glycosidic bond between the acidic Kdo sugar and lipid A, yielding peaks that are related to either the oligosaccharide or the lipid A portion of the LOS molecules. For example, the peaks in the higher mass region can be assigned as B-type oligosaccharide fragments, according to the nomenclature of Domon and Costello [22], where the glycosidic oxygen stays with the reducing terminal lipid A moiety (see Scheme I[‡]). These oligosaccharide fragments undergo additional neutral losses, primarily through the loss of one or two CO_2 groups (44 u) from the terminal Kdo sugars or the loss of the Kdo sugars themselves (220 u). For example, the oligosaccharide fragment at m/z 2151.4 presumably arises from the molecular ion at m/z 3103.6, as would be expected from the neutral loss of lipid A (–953 u) with the formation of oligosaccharide fragments with the composition HexNAc, Hex_5, Hep_3, PPEA, P, Kdo[Kdo – H_2O], where the reducing terminal Kdo has lost water to form the B-type ion [22]. This fragment then undergoes the additional loss of one or two CO_2 moieties to form the peaks at m/z 2107.3 and 2064.0, respectively. Alternatively, one or both Kdo sugars can be cleaved from the reducing terminus to yield the peaks at m/z 1931.3 and 1710.7, respectively. In the low mass region, an abundant peak was seen that corresponds to the diphosphoryl diacyl lipid A moiety at m/z 952.7 (Y ion), which also loses 98 u to form the peak at m/z 854.7, presumably by β-elimination of phosphoric acid from one of the two phosphate groups. It is interesting to note that a peak at m/z 1032.6 is also observed in

[‡] Calculated masses of molecular ions and fragments for LOS are listed in Schemes I–III. Experimental masses are listed in the text, figures, and tables to within 0.1 u.

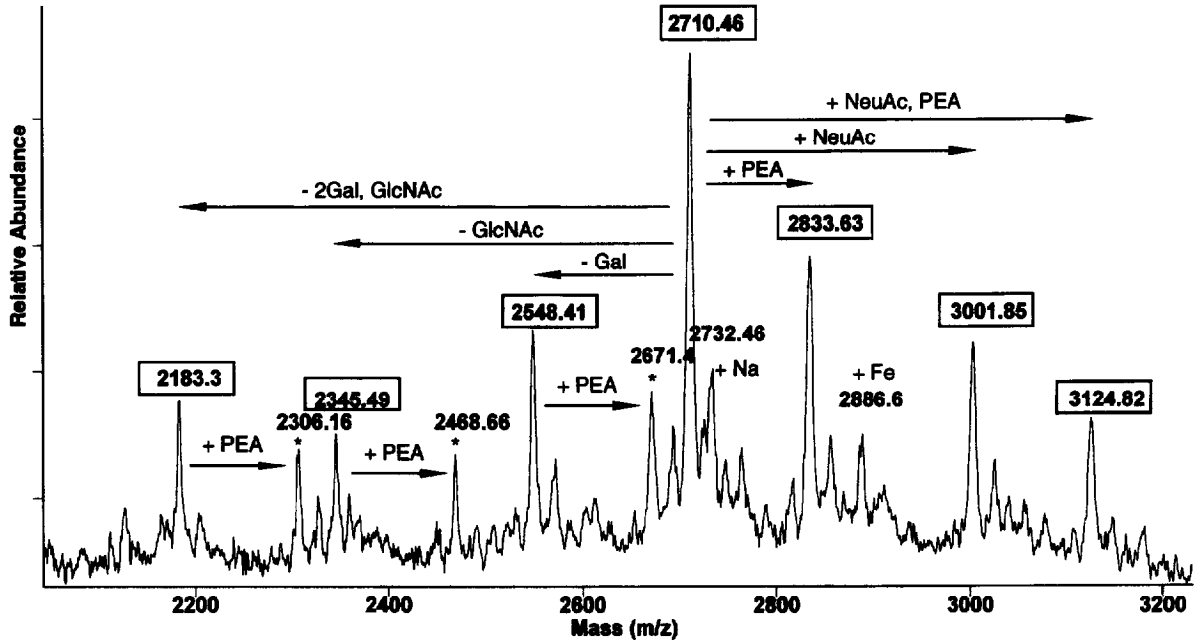


Figure 4. Single-point calibration of the negative-ion MALDI-TOF spectrum under delayed extraction conditions of *O*-deacylated LOS from *H. ducreyi* strain 35000. The peak at *m/z* 2710.46 was used as the internal calibrant and corresponds to the singly deprotonated structure shown in the text with an average M_r of 2711.46. The experimental masses of the other LOS species are shown above each peak, and a comparison of the expected and calculated masses for the additional LOS-glycoforms are listed in Table 1. The initial externally calibrated spectrum had assigned this base peak at *m/z* 2709.5, within experimental error.

this low mass region, 80 u higher in mass than the diphosphoryl lipid A moiety. This 80-u mass shift suggests that the lipid A moiety contains a *third* phosphate group, most likely substituted at one of the two existing phosphate groups on either the 4′ or 1 position to form a terminal pyrophosphate moiety. Therefore, the heterogeneity in these *Salmonella* LOS-glycoforms arises in part out of lipid A phosphorylation differences, and not just phosphorylation differences in the oligosaccharide region.

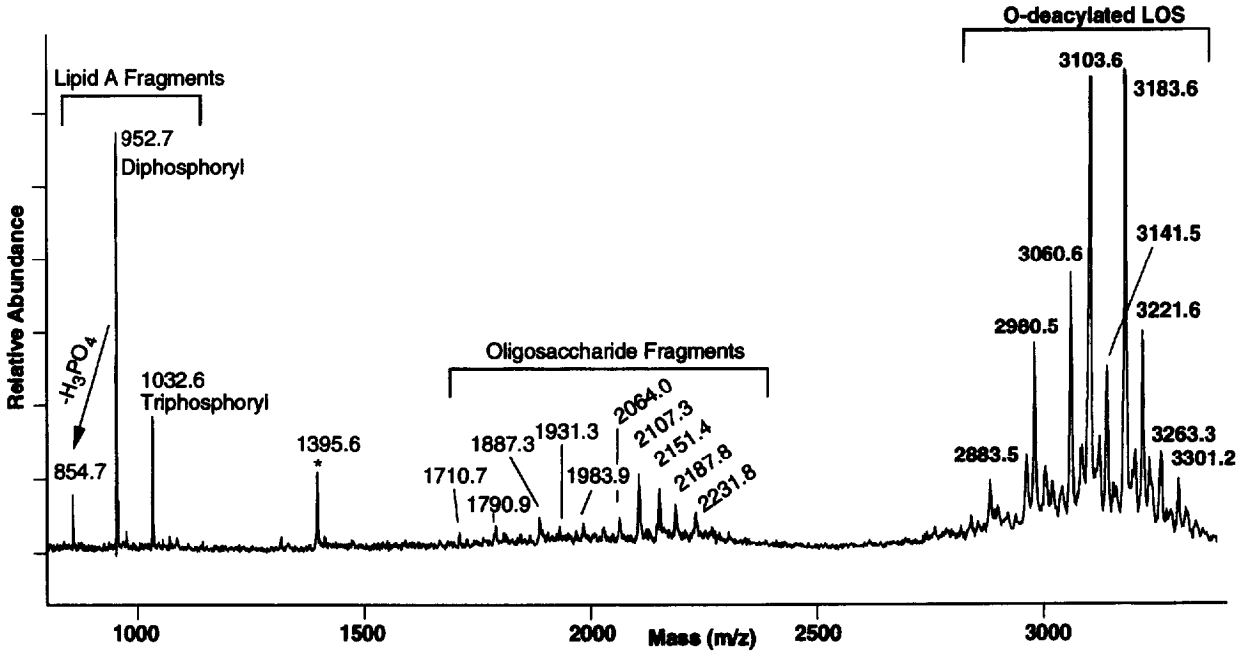
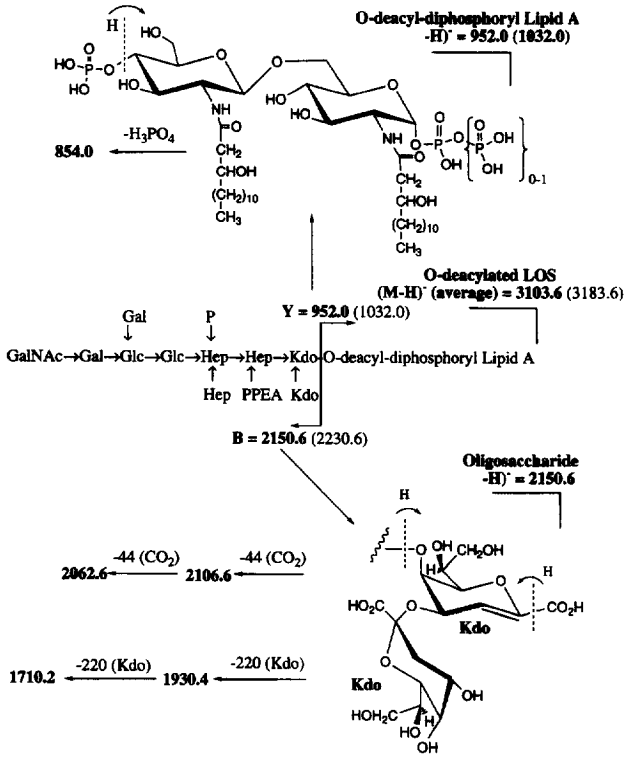


Figure 5. Linear MALDI-TOF spectrum of *O*-deacylated LOS from *S. typhimurium* Ra strain in the negative-ion mode under delayed extraction conditions showing extensive prompt fragmentation. The impurity marked with an asterisk is also present in the MALDI spectrum of the intact LOS preparation shown in Figure 9 (see text and Scheme I for details).



Scheme I

Haemophilus influenzae 276.4, a stable mutant strain whose LOS structures have only been partially determined [23], produces four major LOS -glycoforms after neuraminidase treatment. As seen in Figure 6, four major molecular ion species were found in the high mass region of the *O*-deacylated LOS preparation between m/z 2277 and 2967, with the base peak at m/z 2601.6. This latter mass corresponds to the previously determined LOS structure shown below:

Glc→Glc→Hep→Kdo(P)→*Lipid A*†
↑
Glc→Glc→Hep--PEA
↑
Hep

M_r calc.= 2601.3

Unlike the LOS from *Salmonella*, these *Haemophilus* LOS-glycoforms differ primarily in the monosaccharide components hexose (162 u) and N-acetylhexosamine (203 u), and not in their phosphorylation state (see Table 1). Nevertheless, several prompt fragments are clearly visible for each major peak, corresponding to the losses of H_3PO_4 (−98 u) and HPO_3 (−80 u).

Although one might be tempted to assign the peaks 80 u lower as separate LOS-glycoforms lacking phosphate, examination of both the lipid A and oligosaccharide-related prompt fragments does not support this interpretation (see Scheme II). Rather, the facile losses of phosphoric acid (and to a lesser extent HPO_3) from the molecular ion species appear to be due to the presence of phosphorylated Kdo as opposed to the usual unsubstituted Kdo at this position. The substitution of phosphate at the 4 position of Kdo is known to make this group highly susceptible to β-elimination. For example, the lipid A peak at m/z 952.2 has no

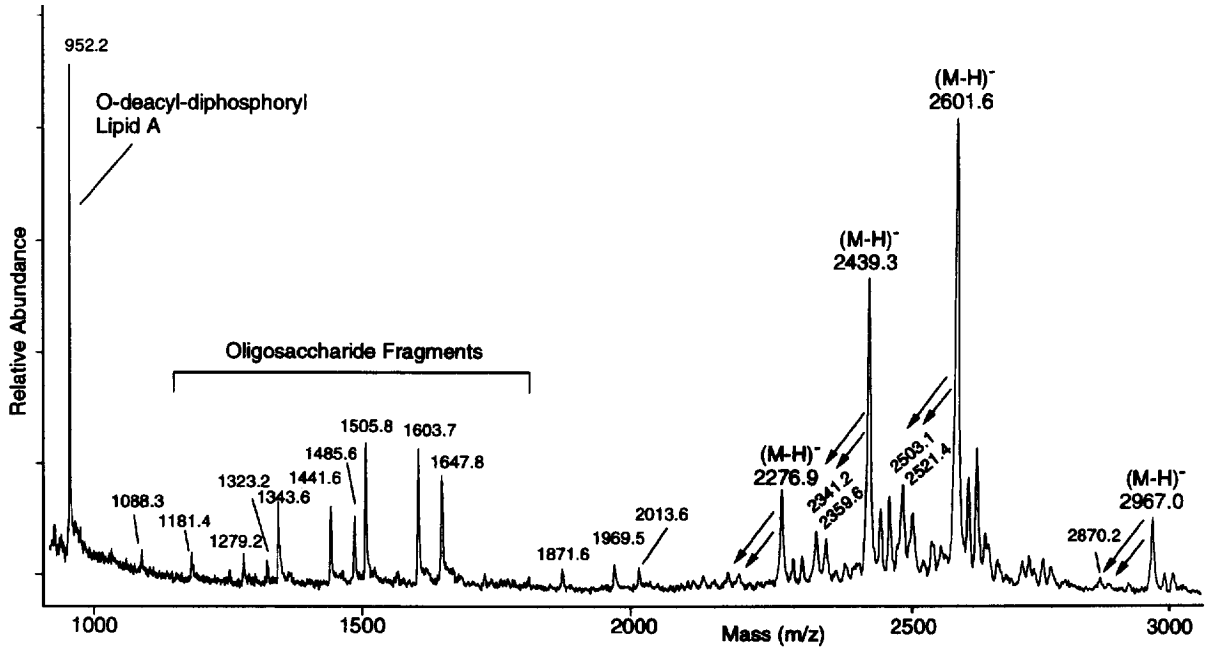
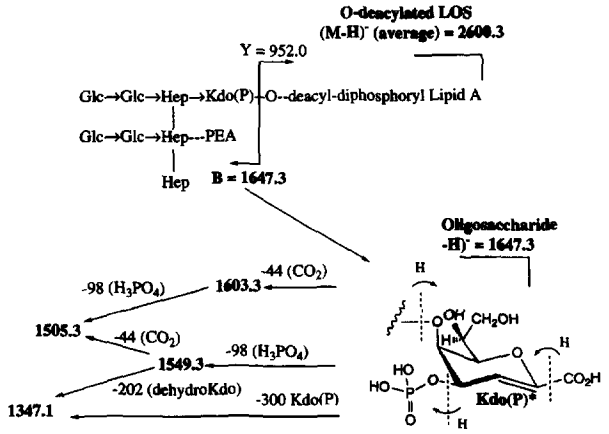


Figure 6. Linear prompt fragmentation MALDI-TOF spectrum of *H. influenzae* strain 276.4 taken in the negative-ion mode under delayed extraction conditions. Molecular ions for the *O*-deacylated LOS are present in the range of m/z 2200–2700 and differ in mass by 162 u, the mass increment for hexose (Hex). Arrows indicate prompt losses of phosphate (loss of HPO_3 and H_3PO_4, respectively) from the major molecular ions. Peaks at lower mass ($m/z \leq 2200$) are additional prompt fragments (see text and Scheme II for details).



Scheme II

other peaks associated with it such as one would expect if there were a mono- or triphosphorylated lipid A analog (i.e., m/z 872 or 1032). Likewise, the oligosaccharide prompt fragments arising from the neutral loss of the lipid A moiety from the four LOS species m/z 2013.6, 1647.8, 1485.6, and 1323.2 (B-type ions) do not have any associated peaks that differ by 80 u. Instead, additional neutral losses are seen for these oligosaccharides fragments corresponding to the loss of CO_2 (m/z 1969.5, 1603.7, 1441.6, and 1279.2, respectively) followed by the loss of phosphoric acid (m/z 1871.6, 1505.8, 1343.6, and 1181.4). At lower abundances, one can also see some evidence for the loss of the entire reducing terminal phospho-Kdo moiety (−300 u), although these peaks are partially obscured by other more abundant fragments.

Post-Source Decay

One difficulty encountered in the interpretation of the prompt fragments in some linear MALDI spectra is the ambiguity concerning which LOS-glycoform they originate from. Interpretation of the prompt fragment ions is particularly difficult in the assignment of phosphorylation differences, because phosphate heterogeneity has been shown to exist in both the lipid A and oligosaccharide portions of LOS. One obvious way around this problem is to select individual LOS-glycoforms for subsequent postsource decay (PSD) analysis [24]. Under PSD conditions, one can select a molecular ion and then examine its decomposition fragments formed in the field free region of the TOF analyzer. Figure 7 shows the PSD spectrum of the major molecular (precursor) ion at m/z 2601.6 that appears in the linear spectrum shown previously in Figure 6. Comparison of these two spectra clearly shows that all ions in the PSD spectrum were in fact contained in the linear spectrum of the total LOS mixture, leading one to conclude that these decomposition reactions occur throughout the accessible time frame of the experiment. However, one can now clearly see that this particular ion is made up of a single lipid A moiety at m/z 952 and a single oligosaccharide moiety at m/z 1646.7. Together, these two ions account for the precursor mass even though no (or very little) precursor ion itself survives intact in the reflector spectrum. Last, one also sees further fragmentation of the oligosaccharide moieties analogous to those described previously for prompt fragmentation. These fragments show that the oligosaccharide moiety contains phosphate that is, m/z 1548.8, 1505.2, and 1347 correspond to the loss of H_3PO_4, $H_3PO_4 + CO_2$, and phospho-Kdo, respectively, from the m/z 1646.7 oligosaccharide fragment (see Scheme II). The low abundance of the lipid A peak at m/z 952, however, appears to preclude the observation of the additional fragments for this moiety, such as those observed in the corresponding linear spectrum as prompt fragments, m/z 936 ($-H_2O$) and 854 ($-H_3PO_4$).

A further example of the usefulness of PSD analysis to interpret the molecular ion region can be seen in the MALDI spectral of LOS obtained from *H. ducreyi* strain 1741. This strain was recently constructed from the parent strain *H. ducreyi* 35000, whose major LOS structure has been determined by mass spectrometry and NMR [21, 25]. Although the precise function of the target knockout gene has not yet been determined, recent work by using transposon mutagenesis [26]

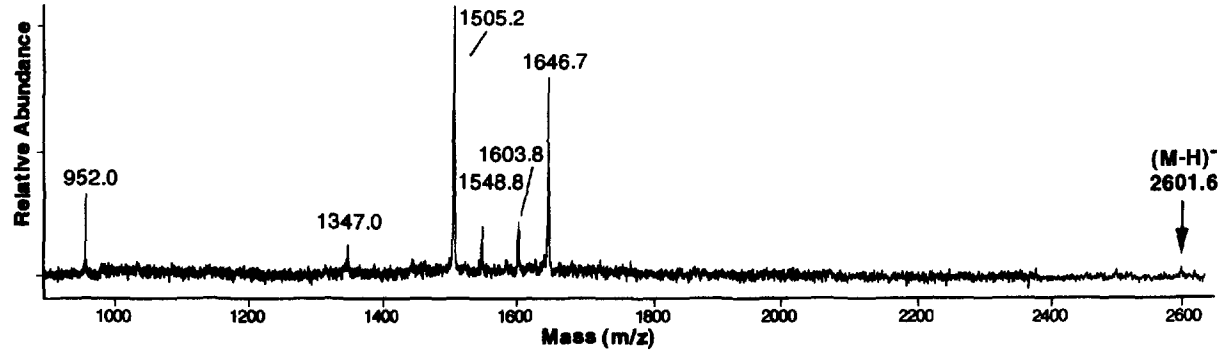


Figure 7. PSD spectrum of *O*-deacylated LOS from *H. influenzae* strain 276.4 taken in the negative-ion mode under delayed extraction conditions. Timed ion selection of the m/z 2601 precursor ion is shown (see Figure 6 and text).

suggests that this gene encodes a *D*-glycero-*D*-*manno*-heptosyltransferase. A defect in this glycosyltransferase would be predicted to cause an LOS terminating in a single glucose just prior to the addition of the heptose:

Glc→Hep→Kdo(P)→*Lipid A*†
↑
Hep
↑
Hep

M_r = 1991.8

When this LOS preparation was analyzed by linear MALDI (see Figure 8a), several discrepancies were found between the expected structure (shown above) and the experimental masses (see Table 1). First, instead of a single peak corresponding to the mass at $[M - H]^-$ = 1990.8, there were at least four major peaks observed at masses approximately 100–350 u higher. Two of the more abundant unexpected masses at m/z 2114.2 and 2237.1 can be readily identified as arising from one to two PEA substitutions of the expected M_r = 1991.8 structure, respectively. Second, although these latter two molecular ions also show the usual sodiated adducts $[M - 2H + Na]^-$, a more unusual pattern of iron adducts, that is, $[M - 4H + Fe^{III}]^-$ at m/z 2167.2 and 2290.2 or iron and sodium, that is $[M - 5H + Na + Fe^{III}]^-$ at m/z 2189.2 and 2312.2 was present. (The iron likely originated from the stainless steel MALDI target.) In the LOS-glycoform containing two PEAs, one even observes a molecular ion corresponding to the addition of two iron ions $[M - 7H + 2Fe^{III}]^-$ at m/z 2344.1. Previous experience with other phosphorylated biomolecules that form significant iron adducts upon desorption [27] suggests that these LOS species may contain an internal pyrophosphate diester linkage, most likely formed by the substitution of phosphate with phosphoethanolamine to yield phosphorylphosphoethanolamine (PPEA). Indeed, pyrophosphate has been shown in other systems to bind well to a variety of mono-, di-, and trivalent cations, especially iron(III) [27]. Third, although the wild-type parental strain is known to be partially substituted with PEA (~ 20%, based on relative ion abundance [21]), no LOS species were found that contained two PEAs. (For example, no $[M - H]^-$ ion at m/z 2956 was observed in the linear spectrum of the *O*-deacylated LOS from the parental *H. ducreyi* strain shown in Figure 4, as would be predicted for an analog of the major LOS-glycoform containing two PEA moieties.)

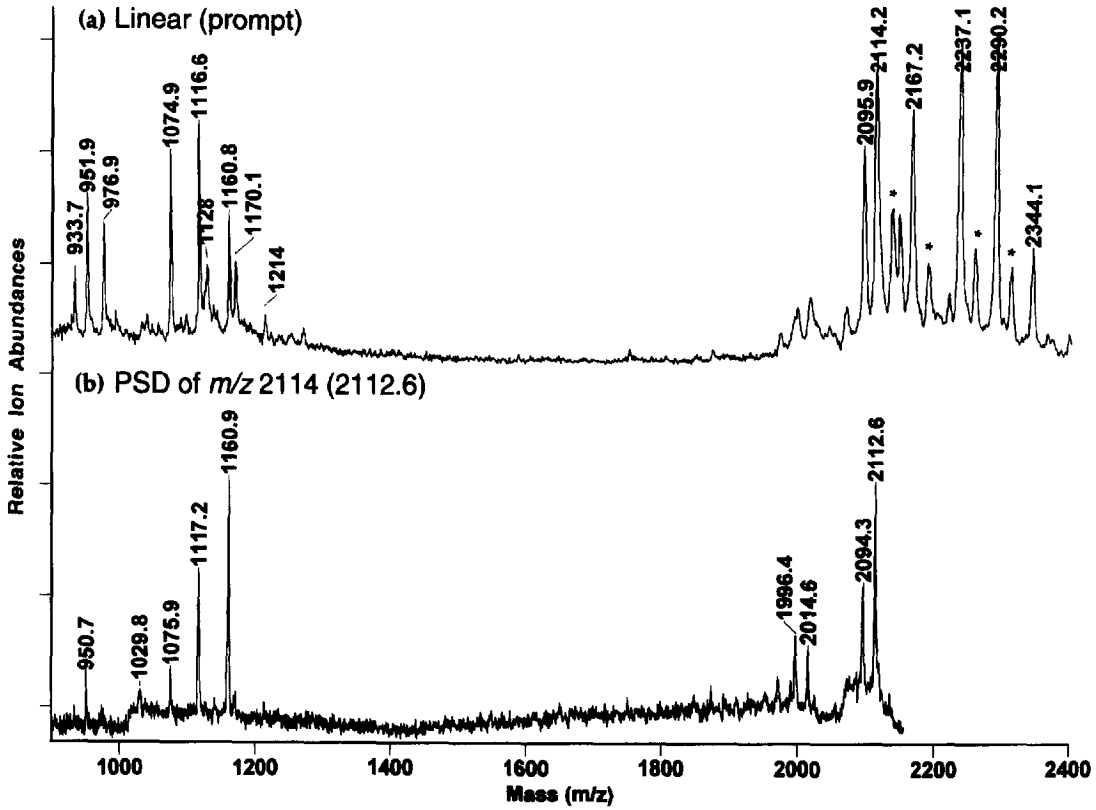


Figure 8. Negative-ion MALDI-TOF spectra of *H. ducreyi* LOS strain 1741 in the linear mode with prompt fragmentation and under PSD conditions with timed ion selection of m/z 2114.2 (now appearing at m/z 2112.6). The asterisks in the top linear spectrum refer to sodiated forms of the peaks directly to their left (see text).

Moreover, it was also assumed that the PEA substitution in the wild-type *H. ducreyi* strain 35000 was located on one of the core heptose saccharides based on its similarity to *H. influenzae* LOS, where the PEA has been shown to be on the second core heptose [28]. Closer inspection of the linear MALDI spectrum suggests that this assumption was incorrect. Instead, the PEA substitution appears to be on *both* the lipid A and oligosaccharide moieties, and fragments contained in the linear and PSD spectra suggests that the PEA in the oligosaccharide is not substituted on the presumed heptose core region of the LOS molecule.

In support of this conclusion, prompt fragments in this linear spectrum were observed at both m/z 951.9 and 1074.9 for the *O*-deacylated lipid A moiety, showing that lipid A exists as the expected diphosphoryalted form as well as a previously unidentified form that is partially substituted with PEA (+123 u). Likewise, the oligosaccharide fragments in this spectrum are present at masses that are consistent with their containing PEA substituents. For example, the major oligosaccharide fragments at m/z 1160.8 and 1116.6 correspond to the B-type oligosaccharide fragments consisting of Glc-Hep_3-Kdo(P)PEA with and without the neutral loss of CO_2 from Kdo, respectively. In addition, all ions presumed to have this additional PEA group also have associated Fe(III) adducts. When one now examines the PSD spectrum of the singly substituted PEA LOS-glycoform at m/z 2114.2 (Figure 8), the two B-type oligosaccharide fragments consistent with stoichiometric PEA substitution clearly dominate the spectrum, and no fragment ions are found at masses predicted for an oligosaccharide without a PEA moiety (i.e., m/z 1037.8 and 993.7, calculated). This leads one to conclude that all LOS-glycoforms of this mutant contain a PEA substituted on the oligosaccharide portion, whereas only some LOS contain an additional second PEA group on the lipid A.

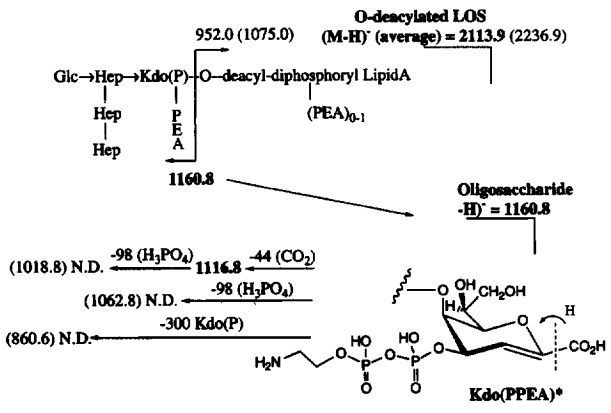
It is interesting to note that despite the stoichiometric substitution of PEA on the oligosaccharide of the M_r = 2115 LOS-glycoform, one still sees a small amount of a lipid A form with a PEA substitution at m/z 1075.9, as well as the expected non-PEA substituted diphosphoryl lipid A fragment at m/z 950.7. Because we had already accounted for the PEA substitution as *exclusively* positioned in the oligosaccharide portion of the M_r = 2115 LOS-glycoform, one can only assume that some amount of the higher mass LOS species containing two PEA moieties was also gated in this PSD experiment. This is indeed possible if one considers that these LOS species undergo prompt losses of both phosphate and phosphoric acid, thus bringing a small amount of the LOS-glycoform containing two PEAs within the window of primary mass selection.

The position of this PEA cannot be directly identified from the data obtained in this spectrum alone, but tandem mass spectrometry data on a four-sector instrument have suggested that it is linked to the phosphate of the phospho-Kdo moiety (Gibson and Melaugh, unpublished data). Moreover, the tendency of the oligosaccharide fragments containing this extra PEA to form Fe(III) adducts also suggests that an internal pyrophosphate diester is present via the formation of a PPEA moiety. Perhaps even more convincing is the lack of oligosaccharide fragments corresponding to the loss of phosphoric acid from the two major oligosaccharide fragments at m/z 1160.9 and 1117.2, that is, calculated fragments of m/z 1160.8 → 1062.8 and/or m/z 1116.8 → 1018.8, as shown in Scheme III. For comparison, recall that the analogous oligosaccharide-related fragments were found to be prominent in the linear and PSD spectra of the *O*-deacylated LOS from *H. influenzae* that also contained a phosphate on the Kdo moiety (see Figures 6 and 7 Scheme II).

Analysis of Intact Salmonella LOS

The dramatic improvement in the delayed extraction spectra of the *O*-deacylated LOS compared to spectra obtained in the continuous extraction mode suggested that it may be possible to find conditions for analyzing crude LOS preparations directly, without first converting them to their water soluble *O*-deacylated forms. LOS by themselves are not very soluble in water and/or organic solvents, but are known to partially disaggregate in the presence of EDTA. Therefore, a solution was prepared of intact LOS in 5-mM EDTA by using pulsed sonication to help solubilize LOS which were then added to the DHB matrix. Figure 9 shows the results obtained from a commercial preparation of *S. typhimurium* LOS in DHB containing EDTA from the same Ra strain used previously to obtain the *O*-deacylated LOS spectra (Figure 3).

The resulting spectrum of this intact LOS preparation contains many of the same LOS-glycoforms seen in the MALDI spectra of the *O*-deacylated preparation, although the intact LOS species are 500–1000 u higher (see Table 2). The shift to higher masses was due to the presence of an additional two to four *O*-linked fatty acids on the lipid A. Indeed, these data show the major LOS-glycoforms consist of two major series of LOS that



Scheme III

Table 2. Structures and molecular weights of intact LOS

Bacterial Strain Major LOS Structure(s)	Expt. M_r	+/− Moiety[a]	Calc. M_r	ΔM
S. typhimurium Ra LPS				
Gal P ↓ | GlcNAc → Glc → Gal → Glc → Hep → Hep → (Kdo)$_2$ → lipid A[b] ↑ | Hep PPEA	*3825.4*	*-P, PEA*	*3827.0*	−1.6
	3906.4	*-PEA*	*3906.9*	−0.5
	3949.5	*-P*	*3950.0*	−0.5
		-EA	*3986.9*	
	4029.4	—	**4030.0**[c]	−0.6
where *lipid A = diphosphorylhexaacyl*	*4068.8*	*+EA*	4066.9	+1.9
	4108.8	*+P*	4110.0	−1.2
where *lipid A = diphosphoryltetraacyl*	*≈ 3470*	*-P*	*3470.2*	≤ 1.0
	≈ 3514	*-EA*	*3513.3*	≤ 1.0
	3590	—	**3593.3**	−0.4

[a] Abbreviations: P, phosphate; PEA, phosphoethanolamine; Gal, galactose; Glc, glucose; GlcNAc, N-acetylglucosamine; Hep, heptose; Kdo, 2-keto-3-deoxyoctulosonic acid.
[b] Lipid A is present primarily in its hexaacyl form with an average M_r of 1798.4. A second major LOS series is also observed with the lipid A in the tetracyl form (see text; average M_r = 1361.7)
[c] The M_rs of the major predicted *O*-deacylated LOS or LOS from each strain are shown in bold type.

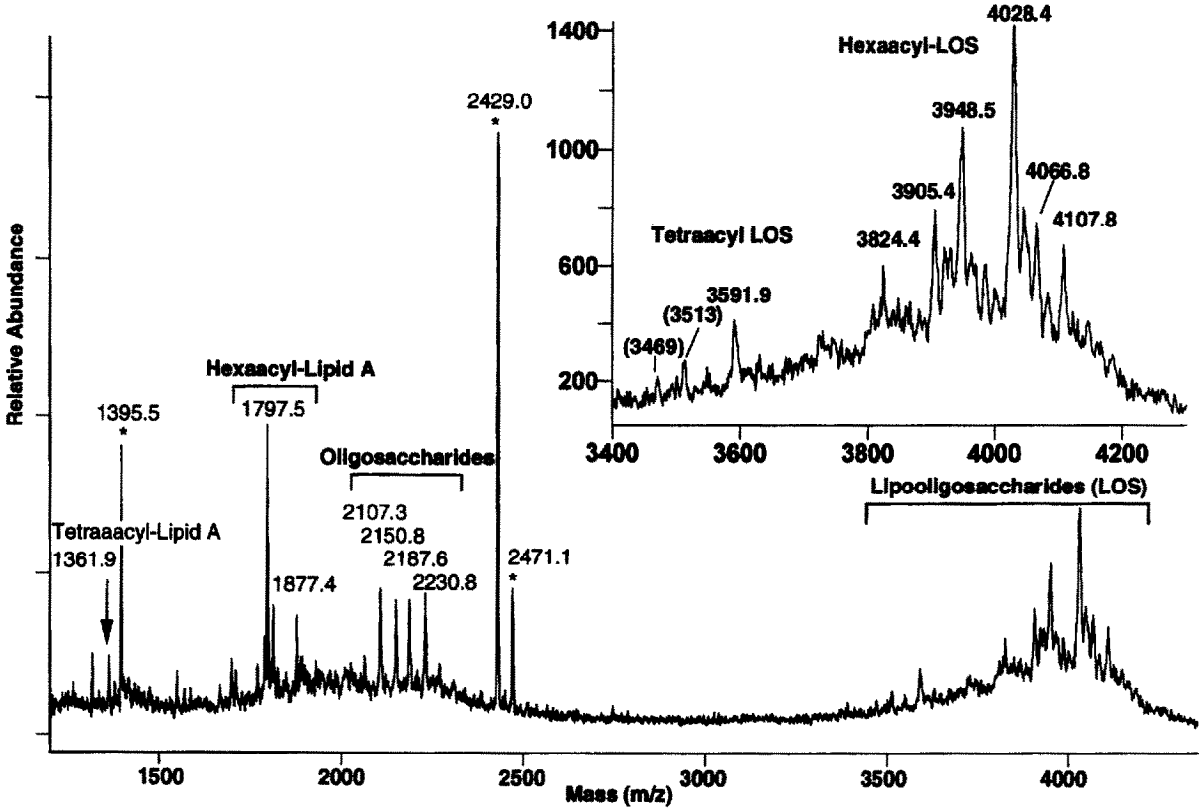


Figure 9. Molecular ion region of *intact* LOS from *S. typhimurium* Ra in the negative-ion mode. Note the dominance of the expected hexaacyl substituted lipid A with the small amount of tetraacyl species at lower masses corresponding to the loss of myristic acid (ΔM = 210.36 u, $C_{14}H_{26}O$) and β-hydroxymyristic acid (ΔM = 226.36 u, $C_{14}H_{26}O_2$), for example -438.7 u, m/z 4028 (hexaacyl LOS) and 3592 (tetraacyl LOS). Impurity peaks are marked with asterisks (see also the corresponding *O*-deacylated LOS spectrum in Figure 5).

are primarily present in their hexaacyl diphosphorylated form, with a second less abundant set containing a tetraacyl lipid A moiety [29, 30]. Superimposed on this lipid A heterogeneity are the phosphorylation differences seen previously in the *O*-deacylated LOS. In fact, this spectrum provides the most direct evidence for the actual fatty acyl substitution state of lipid A while it is *still attached* to the oligosaccharide moiety, and as seen in the *O*-deacylated LOS preparations, prompt fragments are present at the same masses for the oligosaccharide moiety but are shifted upward due to the increased mass of the intact lipid A moiety. The new peaks associated with the intact lipid A species are seen at 1797.5 and 1877.4, corresponding to the hexaacyl species in both a diphosphoryl and triphosphoryl state, and m/z 1361.9, corresponding to the less abundant but analogous diphosphoryl tetraacyl LOS-glycoform.

Conclusion

In this study, we have demonstrated that MALDI-TOF can be used for the analysis of bacterial lipooligosaccharides. Experiments were successfully carried out on both intact LOS and *O*-deacyalted LOS preparations, although a higher degree of sensitivity was obtained for the *O*-deacylated materials. Under delayed extraction conditions a marked improvement was observed compared to spectra taken with conventional extraction of desorbed ions. In the linear mode, delayed extraction MALDI-TOF provided a remarkable increase in both mass resolution and improved mass accuracy with a concurrent reduction in metastable decay processes. These factors contributed to the generation of much more easily interpretable spectra, which is particularly significant in the case of these bacterial LOS-glycoconjugates because they are highly substituted with many labile and anionic groups such as phosphate, phosphoethanolamine, and acidic sugars. Structural information for identification of individual glycoforms was also obtained without prior separation through the use of higher laser power to generate prompt fragments and by using a reflector to analyze PSD fragments. The ease of fragmentation of the labile groups and linkages enhances these processes, although the preference for these labile group losses appears to be at the expense of glycosidic bond cleavages that might have otherwise provided some carbohydrate sequence information. Nonetheless, important structural information was obtained from these spectra and was especially useful in the identification of phosphate and PEA substitutions of the lipid A and oligosaccharide regions. Indeed, the fragmentation data of the *H. ducreyi* *O*-deacylated LOS allowed us to identify previously unreported modifications of the lipid A and oligosaccharide regions, involving PEA substitution of phosphate at both the lipid A and phospho-Kdo moieties. The importance of the increased PEA substitutions is not known, but it clearly has a significant effect on the ionic character of LOS and may underlie an adaptive mechanism of the organism that partially compensates for a weakened outer membrane structure caused by the truncated LOS structure. We are currently investigating this possibility.

Despite the success of this current study to apply MALDI to the analysis of LOS, the question remains whether this technique can be further improved so as to accommodate the low-level analysis required in clinical sampling experiments. In the work described here, 0.1–0.2 ug of *O*-deacylated LOS was sufficient in most cases to obtain both molecular weight data and some structural information, and $\approx$ 1 ug for the intact LOS preparations. Given that these LOS preparations contain about 5–10 glycoforms each with an average mass of around 3000 u, a sensitivity of analysis on the order of 5–10 pmol per *O*-deacylated LOS species was obtained in the spectra presented here, or a total of 35 pmol for an entire LOS mixture. Although this is a dramatic improvement over previously reported sensitivities for mass spectrometric analysis of LOS, it is likely that at least 2–3 orders of magnitude improvement is still needed to reach the sensitivity needed for the analysis of LOS from clinical specimens. Such an improvement will also require a further scale down in the chemistry of *O*-deacylation unless conditions can be found to better ionize the intact LOS species. Toward this former goal we are examining the possibility of carrying out the hydrazine treatment step through a gas phase reaction on a chemically inert surface, such as on nylon or polyvinylidene difluoride. Recently, Tsai et al. [31] reported on the transfer of *Neisseria meningitidis* LOS from sodium dodecylsulfate–polyacrylamide gel electrophoresis gels to nylon for subsequent lectin binding assays. Such an experimental approach could be adapted in a strategy combining low-level LOS separation with MALDI-TOF analysis. Our laboratory is currently engaged in developing such a protocol.

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References

1. Phillips, N. J.; John, C. M.; Reinders, L. G.; Gibson, B. W.; Apicella, M. A.; Griffiss, J. M. *Biomed. Environ. Mass Spectrom.* **1990**, *19*, 731–745.
2. Gibson, B. W.; Mclaugh, W.; Phillips, N. J.; Apicella, M. A.; Campagnari, A. A.; Griffiss, J. M. *J. Bacteriol.* **1993**, *175*, 2702–2712.

3. Gibson, B. W.; Phillips, N. J.; John, C. M.; Melaugh, W. In *Mass Spectrometry for the Characterization of Microorganisms*; Fenselau, C. Ed.; American Chemistry Society, Washington, DC, 1994; pp 185–202.
4. Galanos, C.; Luderitz, O.; Rietschel, E. T.; Westphal, O. In *International Reviews of Biochemistry*; Goodwin, T. W., Ed.; University Park Press: Baltimore, 1977; pp 239–335
5. Takayama, K.; Qureshi, N.; Hyver, K.; Honovich, J.; Cotter, R. J.; Mascagni, P.; Schneider, H. *J. Biol. Chem.* **1986**, *261*, 10624–10631.
6. Kulshin, B. A.; Zahringer, U.; Lindner, B.; Frasch, C. E.; Tsai, C.-M.; Dmitriev, B. A.; Rietschel, E. T. *J. Bacteriol.* **1992**, *174*, 1793–1800.
7. Caroff, M.; Deprun, C.; Karibian, D. *J. Biol. Chem.* **1993**, *268*, 12321–12424.
8. Weiser, J. N.; Love, J.; Moxon, E. R. *Cell* **1989**, *59*, 657–665.
9. Karas, M.; Hillenkamp, F. *Anal. Chem.* **1988**, *60*, 2299–2301.
10. Beavis, R. C.; Chait, B. T. *Proc. Nat. Acad. Sci. U.S.A.* **1990**, *87*, 6873–6877.
11. Shaler, T. A.; Tan, Y.; Wickham, J. N.; Wu, K.J.; Becker, C. H. *Rapid Commun. Mass Spectrom.* **1995**, *9*, 942–947.
12. Fitzgerald, M. C.; Smith, L. M. *Ann. Rev. Biophys. Biomol. Struct.* **1995**, *24*, 114–140.
13. Juhasz, P.; Roskey, M. T.; Smirnov, I. P.; Haff, L. A.; Vestal, M. L.; Martin, S. A. *Anal. Chem.* **1996**, *68*, 941–946.
14. Harvey, D. J. *J. Chromatogr.* **1996**, *720*, 429–446.
15. Apicella, M. A.; Griffiss, J. M.; Schneider, H. *Methods Enzymol.* **1994**, *235*, 242–252.
16. Vestal, M. L.; Juhasz, P.; Martin, S. A. *Rapid Commun. Mass Spectrom.* **1995**, *9*, 1044–1050.
17. Strupat, K.; Karas, M.; Hillenkamp, F. *Int. J. Mass Spectrom. Ion Processes* **1991**, *111*, 89.
18. Mohr, M. D.; Bornsen, K. O.; Widmer, H. M. *Rapid Commun. Mass Spectrom.* **1995**, *9*, 809–814.
19. Nordhoff, E.; Ingendoh, A.; Cramer, R.; Overberg, A.; Stahl, B.; Karas M.; Hillenkamp, F.; Crain, P. F. *Rapid Commun. Mass Spectrom.* **1992**, *6* 771–776.
20. Luderitz, O.; Westphal, O.; Staub, A. M.; Nikaido, H. In *Microbial Toxins*; Weinbaum, G.; Kadis, S.; Ajl, S. J., Eds.; Academic: New York, 1971; pp 235–263.
21. Melaugh, W.; Phillips, N. J.; Campagnari, A. A.; Tullius, M. V.; Gibson, B. W. *Biochemistry* **1994**, *33*, 13070–13078.
22. Domon, B.; Costello, C. E. *Glycoconjugate J.* **1988**, *5*, 397–409.
23. Phillips, N. J.; McLaughlin, R.; Miller, T. J.; Apicella, M. A.; Gibson, B. W. *Biochemistry* **1996**, *35*, 5937–5947.
24. Kaufmann, R.; Spengler, B.; Lutzenkirchen, F. *Rapid Commun. Mass Spectrom.* **1993**, *7*, 902–910.
25. Melaugh, W.; Phillips, N. J.; Campagnari, A. A.; Karalus, R.; Gibson, B. W. *J. Biol. Chem.* **1992**, *267*, 13434–13439.
26. Gibson, B. W.; Campagnari, A. A.; Wang, J.; Melaugh, W.; Munson, R. S. J. *Abstracts of the 96th General Meeting of the American Society for Microbiology*; New Orleans, LA, 1996; p. 233.
27. Gibson, B. W.; Medzihradszky, D.; Hines, W. M.; Auriola, S.; Kenyon, G. L. *J. Am. Soc. Mass Spectrom.* **1994**, *5*, 443–451.
28. Phillips, N. J.; Apicella, M. A.; Griffiss, J. M.; Gibson, B. W. *Biochemistry* **1992**, *31*, 4515–4526.
29. Takayama, K.; Qureshi, N.; Mascagni, P. *J. Biol. Chem.* **1983**, *258*, 12801–12803.
30. Qureshi, N.; Takayama, K.; Heller, D.; Fenselau, C. *J. Biol. Chem.* **1983**, *258*, 12947–12951.
31. Tsai, C.; Chen, W.; Balakonis, P. *Glycoconjugate J.* **1995**, *12*, 562.