Published in final edited form as:

Analyst. 2018 June 25; 143(13): 3091–3099. doi:10.1039/c8an00561c.

Mapping Phosphate Modifications of Substituted Lipid A via a Targeted MS³ CID/UVPD Strategy

Christopher M. Crittenden[†], Carmen M. Herrera[‡], Peggy E. Williams[†], Dante P. Ricci[§], Lee R. Swem[§], M. Stephen Trent[‡], and Jennifer S. Brodbelt^{*,†}

[†]Department of Chemistry, University of Texas at Austin, Austin, Texas 78712, USA

[‡]Department of Infectious Diseases, University of Georgia, Athens, Georgia 30602, USA

[§]Achaogen, Inc., 1 Tower Place, South San Francisco, CA, 94080, USA

Abstract

Structural characterization of lipid A from Gram-negative bacteria remains a significant challenge, especially with respect to localizing modifications of the phosphate groups typically found on the reducing and non-reducing ends of the β -1',6-linked glucosamine disaccharide backbone of lipid A. As reported here, combining traditional collisional activated dissociation (CAD) and ultraviolet photodissociation (UVPD) in a hybrid MS³ approach facilitates identification and localization of substituents of the phosphate groups. The focus is on rapid identification and characterization of substituted lipid A species with specific emphasis on the modifications on the 1 and 4' phosphate moieties. Mapping these modifications, typically ones that modify the surface charges of lipopolysaccharides, is particularly important owing to the impact of these types of modifications on antibiotic resistance. The presence of phosphoethanolamine, aminoarabinose, and galactosamine moieties in hexaacylated and heptaacylated lipid A species, including ones from *Enterobacter cloacae* and *Acinetobacter baumannii*, are characterized using a targeted MS³ strategy to identify glycosidic product ions (^{1,5}X₁ and ^{0,4}A₂, typically) which allow localization of the substituents.

Graphical abstract

*Phone: (512) 471-0028, jbrodbelt@cm.utexas.edu.

Introduction

Lipopolysaccharides (LPS) are complex phosphoglycolipids that comprise a major portion of the outer membrane of Gram-negative bacteria.^{1–3} LPS consist of a hydrophilic polyglycan region which includes both the O-antigen and the core oligosaccharide domain and the hydrophobic lipid A moiety. This latter portion is responsible for the toxicity associated with LPS and is a powerful activator of the mammalian immune system.¹ Lipid A contains phosphate groups, which contribute negative charges to the outer membrane, that mediate interactions with other charged species, such as metal ions or antimicrobial peptides. Modification of the structure of lipid A may occur in response to environmental and external stress factors, thus altering the bacterial surface and virulence of the organism.^{1,4,5} For example, in the presence of cationic antimicrobial peptides (CAMPs) substituents that reduce the negative charge of LPS increase bacterial fitness by diminishing the electrostatic-based association of these positively charged peptides.^{4,6–10} Attachment of amine-containing sugars (e.g. aminoarabinose, glucosamine) or ethanolamine to the phosphate groups decreases the negative charge character of lipid A, altering the overall charge of the surface of the bacterium.^{4,9–11} In *Campylobacter jejuni*, an important diarrheal pathogen, loss of phosphoethanolamine modification of lipid A results in greater sensitivity to the CAMP polymyxin B.¹² Along similar lines, addition of glycine or diglycine residues to lipid A of LPS results in resistance of *Vibrio cholera* to polymyxin.¹³ These past and newly emerging studies indicate that structural modification of lipid A plays a significant role in virulence, immune system recognition, and resistance to antimicrobial agents, thus motivating new strategies for deciphering biological pathways that modify lipid A and characterizing the resulting structural modifications.

Mass spectrometry has long been utilized for the analysis of lipid A^{14,15} and recent developments of elegant MSⁿ strategies and new activation techniques have facilitated characterization of key structural features like acyl chain modifications, and substitutions on phosphate groups.^{16–23} While conventional collision induced dissociation (CID) techniques remain among the most popular activation methods, CID promotes preferential cleavage of the most labile bonds of lipid A, an outcome that limits localization of modifications.^{24–26} More informative fragment ions, such as those generated by cleavage of the β -1',6-linked glucosamine disaccharide backbone, are often absent upon CID. Fragment ions arising from these backbone cleavages are diagnostic for the localization of primary and secondary acyl chains and their connectivities as well as determining whether modifications occur on the reducing or non-reducing glucosamine. Multi-stage MSⁿ methods provide more extensive step-by-step information about the lipid A structures but are less well-adapted for automated high-throughput data collection.²⁷ The development of a data processing algorithm, HitMS, has advanced the interpretation of hierarchical MS/MS data for lipid A based on integration of candidate structures, neutral losses in MSⁿ spectra, and assignment of diagnostic fragment ions unique to lipid A structures.^{28,29} An alternative ion activation method, 193 nm ultraviolet photodissociation (UVPD), has proven to yield richer fragmentation patterns than conventional CID for lipid A, thus allowing additional structural details to be deciphered.^{18–20,23,30–32} Specifically, UVPD cleaves the glycosidic (inter- and intra-ring) bonds of the lipid A backbone as well as promoting the characteristic neutral losses of acyl chains also

typically observed upon collisional activation.^{18,19} Further, CID and UVPD provide complementary information, with collisional activation typically producing fewer but more abundant fragment ions, and UVPD affording more details via unique diagnostic ions.³³ Hybrid CID/UVPD methods, where CID and UVPD are employed in tandem, offer another strategy which facilitates elucidation of the linkages and lengths of fatty acyl chains of lipid A and the double bond positional isomers of phosphatidylcholine lipids.^{23,34}

In this study, substituted lipid A molecules (SLAM) are analyzed via a hybrid, hierarchical MS³ approach in which CID is used in the first stage of fragmentation to identify substituted species and confirm the length of the most labile acyl chain. 193 nm UVPD is used in the second stage of fragmentation to localize the substitutions, namely whether they are located at the 4'-phosphate position on the non-reducing end of the molecule or the 1-phosphate position on the reducing end of lipid A. Relative to the growing landscape of hybrid MS/MS and MSⁿ strategies developed for lipids,^{18–20,23,31,32} the current method provides several advances. The current CID-UVPD MS³ approach affirms the identity of the substitutions based on mass confirmation. Additionally, the first collisional activation step targets a key acyl chain loss, thus providing a 'clean-up' by filtering out isobaric non-lipid A contaminants that are commonly present in these crude samples. Performing UVPD in the MS³ step after a second stage of ion isolation corresponding affords rich fragmentation with high signal-to-noise unimpaired by competing isobaric contaminants. CID-UVPD also generates diagnostic cross-ring fragments with greater abundance than UVPD alone, as the diagnostic ions are not competing with the labile acyl chain losses prevalent in an MS² approach. In addition, utilizing CID as the first step of a hybrid MS³ scheme funnels a majority of the ion current into one prominent pathway (loss of a labile acyl chain) with high efficiency, enhancing the signal-to-noise of the fragment ions generated in the subsequent UVPD event. An MS³ method based on multiple stages of UVPD suffers from lower MS/MS efficiency and signal-to-noise, and an analogous method based on multiple stages of CID does not yield the diagnostic cross-ring fragmentation critical for localization of the substituted moiety to either the reducing or non-reducing end of the lipid A molecule. The modifications of interest are: phosphoethanolamine (PETN; +124.02 Da), aminoarabinose (AARA; +132.07 Da), and galactosamine (HEXN, +162.08 Da), each which may be covalently attached via an ester bond to the phosphate group on either the reducing or non-reducing end of lipid A. The structures of the modifications of hexaacylated lipid A are shown in Figure S1. The combination of CID and HCD in an MSⁿ workflow allows an effective means for localization of acyl chains and phosphate substitutions, as well as alleviating the contamination of the MS/MS spectra with fragment ions originating from co-isolated isobaric ions inherent in the direct analysis of complex biological samples. Finally, the effect of antimicrobial peptide interaction with *E. cloacae* is explored as a function of lipid A structure. The hybrid CID-UVPD technique allows the complete characterization of the molecule with respect to acyl chain content and substitutions.

Experimental

E. coli strain WD *arnT* (SLAM 1),³⁵ *E. coli* strain WD *eptA* (SLAM 2),³⁵ and an *A. baumannii* clinical isolate, strain AABA1205 (SLAM 3) were cultured in lysogeny broth (LB). For each bacterial strain, single colonies were isolated by streaking from a glycerol

stock onto an agar plate and left to grow at 37 C. *E. coli* and *A. baumannii* strains were grown on lysogeny broth (LB) agar plates, whereas single colonies of *E. cloacae* were isolated on Muller Hinton agar. Single colonies were then used to inoculate an overnight culture and the following day fresh cultures started at an OD 600nm of 0.05. *E. coli* strain WD *arnT* (SLAM 1),³⁵ *E. coli* strain WD *eptA* (SLAM 2),³⁵ and an *A. baumannii* clinical isolate, strain AABA1205 (SLAM 3) were cultured in LB. For *E. cloacae* R/S,³⁶ bacteria were grown in Muller Hinton broth in the presence (SLAM 4 100 PMB) or absence (SLAM 4 0 PMB) of 100 µg/ml of polymyxin B. All cultures were harvested at an absorbance of 1.0 at 600 nm and the cell pellets washed in phosphate buffered saline (PBS). Lipid A was released from all bacterial strains and purified as previously described.^{37,38} Briefly, cell pellets were resuspended in a single-phase Bligh/Dyer mixture consisting of chloroform/methanol/water (1:2:0.8, v/v). The insoluble material, which contains the lipid A covalently linked to the lipopolysaccharide core, was release by mild-acid hydrolysis (pH 4.5) at 100 C as previously described.^{36–38} The lipid A species were then recovered using a two-phase Bligh/Dyer extraction consisting of chloroform/methanol/water (2:2:1.8, v/v), dried under a stream of nitrogen, and analyzed by mass spectrometry.

Samples were dissolved in 200 µL of 74:23:3 chloroform:methanol:water followed by analysis on a Thermo Scientific Orbitrap Fusion Lumos mass spectrometer (San Jose, CA, USA). The Orbitrap Fusion Lumos was equipped with a 193 nm Coherent Existar excimer laser (Santa Clara, CA, USA) to perform photodissociation in the low pressure linear ion trap as previously described.^{32,39} Approximately 10 µL of solution was loaded into a Au/Pd-coated pulled-tip borosilicate glass emitter (1.2 mm o.d. pulled to a tip of less than 1 µm o.d.), and a voltage of 1100–1300 V was applied to generate stable electrospray in the negative ion mode. Ions observed in the MS¹ spectra were isolated in the quadrupole (isolation width of 5 m/z) prior to CID (in the high pressure linear ion trap) or UVPD (in the low pressure linear ion trap). For automation of the hybrid CID-UVPD workflow, the following parameters were used: MS¹ spectra (2 microscans) were collected at an Orbitrap resolution of 60K (at m/z 400). For data dependent MS² (CID), a targeted mass list of the various lipid A species was input for selection, isolation, and activation, with a tolerance of ± 0.5 m/z. CID was performed following quadrupole isolation (isolation width of 5 m/z) with a normalized collision energy (NCE %) of 28, and 5 microscans were averaged. Data dependent MS³ was performed for targeted loss inclusion where the mass of the 3' e acyl chain of lipid A was input for the targeted loss of interest, with a tolerance of ± 1.0 m/z. UVPD (3 pulses at 3.5 mJ per pulse) was performed for the MS³ stage on the ion corresponding to the loss of the 3' e species after ion isolation. Five microscans were collected for the MS³ spectra, and an identification tolerance was set for 50 ppm in the high mass negative ion mode with Orbitrap detection. Calibration of the Orbitrap mass analyzer in the negative high mass mode is based on the duplication of parameters from the calibration in the positive high mass mode, a process which results in greater ppm errors in the negative mode. Nomenclature for acyl chain and glycosidic fragmentation is adapted from Domon and Costello⁴⁰ and Morrison *et al.*²³

Results and Discussion

The general experimental method, depicted in Figure 1, is based on an MS³ workflow that utilizes CID as the first fragmentation stage and 193 nm UVPD as the second fragmentation stage. The rationalization for this sequence of events is based on the fact that CID provides a relatively simple fragmentation pattern with high S/N including a dominant fragmentation pathway via cleavage of the 3' ϵ acyl chain that is prevalent for all lipid A species that possess a secondary acyl chain at the 3' position. UVPD as the second step affords a rich fragmentation pattern with numerous diagnostic fragment ions, specifically glycosidic fragmentation which allows localization of substituents to the non-reducing and/or reducing end of the molecule. The two-stage workflow offers two special benefits that are particularly useful for screening lipid A species in biological samples. First, MS³ enhances selectivity by establishing criteria for selection and analysis of species in complex samples and in many cases eases the need for high resolution chromatographic separations often employed to reduce the spectral congestion caused by co-eluting molecules. Second, MS³ methods are well-suited for targeted screening applications in which strategically selected neutral losses (such as the easily calculated masses of common acyl chains of lipid A) can be used to guide an automated workflow for a particular class of molecules (like lipid A). For the workflow shown in Figure 1, mixtures of lipid A species were isolated from Gram-negative bacteria prior to introduction by direct infusion. The extraction procedure is effective for general isolation of lipid A species, but the samples are complex and typically contain numerous lipid A candidates as well as an array of other constituents and residual surfactants used in the cell lysing process. Although on-line chromatography also provides a means to decongest the mixtures, this direct infusion shotgun approach facilitates rapid, high throughput screening for targeted assessment of samples prior to more elaborate LCMS analysis.

Figure S2 displays an example of an MS¹ spectrum for one of the biological extracts, SLAM 1. The spectrum is congested below m/z 800, and many of these ions are expected to be ones related to detergents used during the growth and extraction process. Examples of the key lipid A region (m/z 800 to 1300) of the MS¹ spectra obtained upon electrospray ionization of three of the lipid A extracts are shown in Figure 2. SLAM 1 contains hexaacylated lipid A extensively modified with phosphoethanolamine (Figure 2a). The core unmodified hexaacylated lipid A is observed as a doubly deprotonated ion of m/z 898; the addition of one or two PETN modifications result in the abundant ions of m/z 960 and 1021, respectively. Low abundances of heptaacylated lipid A modified with one or two phosphoethanolamines are observed in the 2- charge state at m/z 1078 and 1140, respectively. In Figure 2b, the MS¹ spectrum for the SLAM 2 extract is shown. The most dominant ion corresponds to unsubstituted lipid A (m/z 898, -2), and there are significant ion peaks consistent with the incorporation of one or two aminoarabinose modifications (m/z 963 and 1028). Contributions from heptaacylated lipid A are also observed in the spectrum: unsubstituted heptaacylated lipid A at m/z 1017 and one with a single AARA modification (m/z 1082). The lipid A species in the third extract, SLAM 3, were primarily heptaacylated (Figure 2c). The lipid A containing two PETN modifications produces the ion of m/z 1079, and the other prominent ion of m/z 1098 corresponds to incorporation of one PETN and one

HEXN group. Other ions are sodium-adducted and hydroxylated species, and no hexaacylated lipid A species were observed for this sample. There is also a series of polymeric ions that congest the spectrum; likely originating from polypropylene glycol (PPG) that is pervasive in the processing of bacteria for extraction of the lipids. It is exactly these types of difficult-to-remove polymeric contaminants that motivate the use of MS³ strategies for filtering spectra and enhancing specificity in the analytical workflow.

Examples of the MS² (CID) spectra and MS³ (CID/UVPD) spectra are shown in Figures 3 – 5, along with key cleavages highlighted on the lipid A structures. In addition, summaries of the diagnostic fragment ions identified for each lipid A species are presented in Tables S1–S5. As displayed in the CID spectra shown in Figure 3a, 4a, and 5c, cleavage of the labile 3'ε acyl chain is predominant, in addition to cleavages of the substituents appended to the phosphate groups. Additional minor pathways commonly observed upon CID are cleavages of acyl chains at the 3'α and 3α positions and a combination of the 3'ε acyl chain in conjunction with elimination of the phosphate substituents. In each case, isolation and subsequent UVPD of the ion originating from the neutral loss of the 3'ε acyl chain results in the rich MS³ spectra shown in Figures 3b, 4b and 5d that allow nearly complete structural characterization of the lipid A molecule, including localization of substituents via glycosidic backbone cleavages (^{1,5}X₁ and ^{0,4}A₂ cross-ring cleavages) and also production of C₁, Y₁, and Z₁ ions. The identities of the substituents appended on the phosphate are confirmed in the CID spectra based on the masses of the observed neutral losses from the selected precursor, and further confirmed in the UVPD (MS³) spectra acquired after the 3'ε acyl chain loss.

At the crux of the MS³ strategy is confirmation and localization of substituents on the phosphate groups of lipid A. Two benchmark examples are illustrated in Figures 3 and 4. For the first example, heptaacylated lipid A with a single PETN modification was subjected to the experimental workflow (Figure 3). CID was undertaken on the ion of *m/z* 960 (2- charge state), revealing that the most labile acyl chain corresponded to a 14 carbon chain evident by the abundant product ion of *m/z* 845 (mass loss of 230 Da). Another important piece of information that is gained through this first stage of analysis is the identity of the substituent, if present. For this example, a neutral loss corresponding to characteristic loss of a PETN moiety is observed. UVPD (Figure 3b) is used to interrogate the dominant product ion resulting from loss of the 3'ε acyl chain, providing diagnostic cleavages of the glycosidic backbone that allow the location(s) of substituent(s) on the phosphate groups to be pinpointed. In this case, the diagnostic ions highlighting the single PETN modification were the ^{1,5}X₁, Y₁, Z₁, ^{1,4}A₂, and ^{0,4}A₂ ions, all of which confirm the location of the PETN modification on the 1-phosphate position on the reducing end of the molecule. (The ^{1,4}A₂ ion specifically localizes the PETN modification to the 1-phosphate position and not a reducing end acyl chain, which has demonstrated the propensity to undergo modifications at the ε-position).⁴¹ A complete map of the key fragment ions for this substituted lipid A molecule is shown in Figure 3c.

An MS³ strategy based on two stages of CID was also considered, and a typical outcome is illustrated in Figure S3 for the singly PETN-modified lipid A from SLAM 1 (the same lipid A used for the example presented in Figure 3). In this case, the prominent product (*m/z* 845)

generated upon cleavage of 3' ϵ acyl chain from the PETN-modified lipid A (precursor m/z 960) was subjected to a second stage of CID, resulting in the spectrum shown in Figure S3. The MS³ spectrum is dominated by acyl chain cleavages and does not display any products from glycosidic bond cleavages that allow localization of the PETN substituent. This type of behavior is consistently observed for the two-stage CID/CID. The potential to promote glycosidic bond cleavages via a two-step CID/CID strategy was explored by varying the collision energy applied in the MS³ step (Figure S4). Variation of the applied collision energy depletes the selected precursor (loss of 3' ϵ , m/z 845), and the resulting fragments arise from combinatorial losses of primary acyl chains as well as neutral losses of PO₃ and phosphoethanolamine. However, cleavage of glycosidic bonds does not occur, prohibiting the confident localization of substituents. HCD, a method known to afford higher internal energy deposition compared to conventional low energy CID, was also evaluated as the second step in the MS³ strategy (Figure S4b). The only notable difference in the distribution of products from CID/HCD is the more extensive combinations of acyl chain losses and PO₃ and phosphoethanolamine neutral losses. This outcome is consistent with a previous report on energy variable mass spectrometry of lipid A molecules.²⁰ A single-stage UVPD method was also evaluated, and the outcome is shown in Figure S5 for the same PETN-modified lipid A described above. The UVPD spectrum produces some of the same glycosidic cleavage products as observed for CID/UVPD, such as the key ^{0,4}A₂ product ion which allows localization of the PETN on the reducing end of the molecule. However, more of the ion current was distributed among simple acyl chain cleavages, and the benefit of the MS³ selectivity was lost.

As discussed earlier, the MS¹ spectrum for SLAM 1 revealed a prominent bis-modified lipid A ion (Figure 2a), corresponding to incorporation of two PETN groups. The MS³ CID/UVPD workflow was implemented starting with the precursor m/z 1021 (2- charge state) selected for CID, and the modifications were confirmed at the 4'-phosphate and the 1-phosphate positions based on the subsequent stage of UVPD (precursor m/z 906, 2- charge state) (see Figure S6). For this lipid A, diagnostic backbone fragments supporting the locations of the two PETN substituents were the ^{1,5}X₁, Y₁, C₁, ^{1,4}A₂, and ^{0,4}A₂ ions. UVPD was also evaluated for the alternative product ion originating from the neutral loss of one of the substitutions for the same bis-modified lipid A containing two PETN groups. In the case, the MS³ CID/UVPD workflow entailed isolation of the product from the loss of one of the PETN groups (m/z 960) produced in the CID spectrum (primary precursor m/z 1021), then UVPD of the ion of m/z 960, as shown in Figure S7. UVPD of this secondary product ion results in a series of acyl chain losses, however, diagnostic glycosidic or cross-ring cleavages were not observed, likely owing to insufficient ion current to allow confident identification (low S/N).

To localize AARA modifications in the sample SLAM 2, the hybrid MS³ approach was utilized (Figure S8). CID of the singly modified hexaacylated lipid A resulted in dominant loss of the 3' ϵ acyl chain (m/z 849) and to a lesser extent, loss of the 2' ϵ , 3 α , and the 3' ϵ acyl chain in tandem with the AARA modification, as demonstrated in Figure S8a. Subsequent UVPD on the loss of the 3' ϵ acyl chain at m/z 849 results in a diverse array of product ions, many of which allow for the positional localization of the AARA modification (Figure S8b). Specifically, glycosidic product ions were observed in the 1- charge state at the

$^{1,5}X_1$, Y_1 , Z_1 , confirming that the AARA moiety was not localized to the reducing end of the molecule. The C_1 and $^{0,4}A_2$ ions localized the AARA moiety to the 4'-phosphate position, as did the unique $^{1,5}X_2$ ion, which is a cross-ring fragment ion associated with breaking the 1- and 5-bond of the AARA moiety (note: this X-ion would not be detected if the AARA was positioned at the 1-phosphate position). The identified product ions are labeled on the structure of this substituted lipid A molecule in Figure S8c.

This same type of MS³ method also offers the ability to confirm the identities and localize the positions of different modifications on the same lipid A molecule. As shown in Figure 4, a heptaacylated lipid A from SLAM 3 contained both phosphoethanolamine and galactosamine modifications (MS¹ of SLAM 3 is shown in Figure 2c). CID (Figure 4a) of the SLAM 3 precursor ion (m/z 1098, 2- charge state) reveals that most dominant acyl chain cleavage corresponds to a C12 chain via the neutral loss of 202 Da. Additionally, neutral losses corresponding to the HEXN and the PETN moieties in the CID spectrum confirm the identities of the two substituents present, but not their locations. The CID spectrum in Figure 4a is rather cluttered with a number of unassigned product ions; an outcome attributed to the co-isolation of lipid A with an isobaric PPG contaminant of the same m/z value. Targeting the dominant 3'e acyl chain loss product in Figure 4a for subsequent UVPD alleviates the impact of the PPG interference (Figure 4b). UVPD results in product ions that allow localization of both of the substituents: the $^{0,4}A_2$ cross-ring cleavage ion confirms that the phosphoethanolamine modification is found on the 4'-phosphate on the non-reducing end, and the $^{1,5}X_1$ product ion indicates that the galactosamine modification is located on the 1-phosphate on the reducing end of the heptaacylated lipid A. A complete fragmentation map of the substituted heptaacylated lipid A based on the UVPD spectrum is shown in Figure 4c.

As a more challenging application, lipid A was extracted from *Enterobacter cloacae* grown with or without the presence of polymyxin B. Polymyxins are antimicrobial peptides that disrupt the outer and inner membranes of Gram-negative bacteria.⁴² *Enterobacter cloacae* may develop resistance to polymyxin by remodelling its lipopolysaccharides via modification of the phosphate groups which inhibits interaction and binding of positively-charged antibiotics like polymyxin.³⁶ The lipid A profiles of the two *Enterobacter cloacae* samples are shown in Figure 5a (untreated) and 5b (treated). The lipids from the *Enterobacter cloacae* sample grown without the presence of polymyxin are primarily hexaacylated (or heptaacylated to a much lower extent), none with prominent substituents. Each lipid A species appears as a quartet-like cluster owing to sodium adduction and the common presence of an extra hydroxyl group on the secondary acyl chain at the 2'-position. The analogous lipid A species from *Enterobacter cloacae* grown in the presence of polymyxin maintain the same ratio of hexaacylated and heptaacylated forms but all are mass shifted by the addition of two aminoarabinose substituents (Figure 5b). Collisional activation of the doubly-modified hexaacylated lipid A (m/z 1044; 2- charge state) results in the characteristic loss of the 3'e acyl chain (m/z 929) as well as the loss of both aminoarabinose groups (m/z 796) (Figure 5c). These diagnostic ions allow confirmation of the substituents and the length of the most labile acyl chain (C14). UVPD (Figure 5d) of the ion corresponding to the loss of the 3'e acyl chain promotes diagnostic glycosidic cleavages, yielding $^{1,5}X_1$, Y_1 , C_1 , and $^{0,4}A_2$ products that allow localization of the aminoarabinose groups on the 4'-phosphate and the 1-phosphates. Figure 5e displays a comprehensive

fragmentation map for this hexaacylated lipid A molecule with the aminoarabinose substitutions attached to the phosphates on both the non-reducing and reducing ends of the molecule.

The distributions of the lipid A species, based on relative abundances of molecular ions in the MS¹ spectra obtained for the array of lipid A molecules extracted from cell cultures, are shown in Figure 6 with respect to the number of acyl chains (Figure 6a) and type of modifications (Figure 6b). Lipid A from SLAM 1 was approximately 75% hexaacylated and 25% heptaacylated, with approximately 90% of those species substituted with phosphoethanolamine (PETN) and 10% unmodified. Lipid A from SLAM 2 was approximately 90% hexaacylated and 10% heptaacylated, with 40% of the lipid A species containing an aminoarabinose modification. The lipid A profile from SLAM 3 was exclusively heptaacylated, with a distribution of approximately 8% unmodified, 61% solely PETN-modified, and 31% both PETN- and galactosamine (HEXN)-modified. The two profiles for lipid A from the *Enterobacter cloacae* samples (grown in the presence or absence of polymyxin), SLAM 4, displayed similar acyl chain content, each 75–80% hexaacylated and 20–25% heptaacylated. The sample grown without polymyxin showed no modifications, whereas the sample grown in the presence of polymyxin was entirely substituted with AARA. This finding is consistent with the known ability of Gram-negative bacteria like *E. cloacae* to adapt the structure of LPS of its outer membrane in response to exposure to antibiotics.³⁴

Conclusion

In light of the accelerating threat of antibiotic-resistance, new analytical strategies to characterize the structural features that correlate with adaptation and modification of bacteria are critical. The targeted MS³ method presented here represents one approach for mapping phosphate modifications of lipid A of Gram-negative bacteria. The first collisional activation step causes the cleavage of the most labile acyl chain of lipid A, thus establishing a characteristic neutral mass loss that can be used to categorize lipid A structures and filter complex samples contaminated with other constituents. Subsequent UVPD on the acyl chain loss product ion capitalizes on the rich fragmentation promoted by high energy photoactivation to produce an array of diagnostic product ions. UVPD causes cross-ring cleavages, typically ^{1,5}X₁ and ^{0,4}A₂ products, as well as other glycosidic cleavages that confirm the identity and location of the phosphate modifications of the non-reducing and reducing ends of lipid A. Using an MS³ strategy enables a targeted approach that cleans up spectra otherwise congested with isobaric species and is readily automated for high throughput applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding from the NIH (R01 GM103655 to J.S.B.; R01 grants AI064184, AI076322, AI129940 to M.S.T.) and the Welch Foundation (F-1155 to J.S.B.) are gratefully acknowledged. Funding from the UT System for support of the UT System Proteomics Core Facility Network is gratefully acknowledged.

References

1. Whitfield C, Trent MS. *Annu. Rev. Biochem.* 2014; 83:99–128. [PubMed: 24580642]
2. Raetz CRH, Whitfield C. *Annu. Rev. Biochem.* 2002; 71:635–700. [PubMed: 12045108]
3. Kahne D, Sherman DJ, Ruiz N, Okuda S, Silhavy TJ. *Nat. Rev. Microbiol.* 2016; 14:337. [PubMed: 27026255]
4. Needham BD, Trent MS. *Nat. Rev. Microbiol.* 2013; 11:467–481. [PubMed: 23748343]
5. Henderson JC, Zimmerman SM, Crofts AA, Boll JM, Kuhns LG, Herrera CM, Trent MS. *Annu. Rev. Microbiol.* 2016; 70:255–278. [PubMed: 27359214]
6. Ernst RK, Guina T, Miller SI. *Microbes Infect.* 2001; 3:1327–1334. [PubMed: 11755422]
7. Pelletier MR, Casella LG, Jones JW, Adams MD, Zurawski DV, Hazlett KRO, Doi Y, Ernst RK. *Antimicrob. Agents Chemother.* 2013; 57:4831–4840. [PubMed: 23877686]
8. Gunn JS. *J Endotoxin Res.* 2001; 7:57–62. [PubMed: 11521084]
9. Delcour AH. *Biochim. Biophys. Acta.* 2009; 1794:808–816. [PubMed: 19100346]
10. Anaya-López JL, López-Meza JE, Ochoa-Zarzosa A. *Crit. Rev. Microbiol.* 2013; 39:180–195. [PubMed: 22799636]
11. Andersson DI, Hughes D, Kubicek-Sutherland JZ. *Drug Resist. Updat. Rev. Comment. Antimicrob. Anticancer Chemother.* 2016; 26:43–57.
12. Cullen TW, Trent MS. *Proc. Natl. Acad. Sci. U. S. A.* 2010; 107:5160–5165. [PubMed: 20194750]
13. Henderson JC, Fage CD, Cannon JR, Brodbelt JS, Keatinge-Clay AT, Trent MS. *ACS Chem. Biol.* 2014; 9:2382–2392. [PubMed: 25068415]
14. Kilár A, Dörnyei Á, Kocsis B. *Mass Spectrom. Rev.* 2013; 32:90–117. [PubMed: 23165926]
15. Banoub JH, El Anead A, Cohen AM, Joly N. *Mass Spectrom. Rev.* 2010; 29:606–650. [PubMed: 20589944]
16. Sforza S, Silipo A, Molinaro A, Marchelli R, Parrilli M, Lanzetta R. *J Mass Spectrom. JMS.* 2004; 39:378–383. [PubMed: 15103651]
17. Shaffer SA, Harvey MD, Goodlett DR, Ernst RK. *J Am. Soc. Mass Spectrom.* 2007; 18:1080–1092. [PubMed: 17446084]
18. Madsen JA, Cullen TW, Trent MS, Brodbelt JS. *Anal. Chem.* 2011; 83:5107–5113. [PubMed: 21595441]
19. O'Brien JP, Needham BD, Henderson JC, Nowicki EM, Trent MS, Brodbelt JS. *Anal. Chem.* 2014; 86:2138–2145. [PubMed: 24446701]
20. Crittenden CM, Akin LD, Morrison LJ, Trent MS, Brodbelt JS. *J Am. Soc. Mass Spectrom.* 2017; 28:1118–1126. [PubMed: 27966172]
21. Jones JW, Cohen IE, Tureček F, Goodlett DR, Ernst RK. *J Am. Soc. Mass Spectrom.* 2010; 21:785–799. [PubMed: 20185334]
22. Sándor V, Dörnyei Á, Makszin L, Kilár F, Péterfi Z, Kocsis B, Kilár A. *J Mass Spectrom.* 2016; 51:1043–1063. [PubMed: 27506631]
23. Morrison LJ, Parker WR, Holden DD, Henderson JC, Boll JM, Trent MS, Brodbelt JS. *Anal. Chem.* 2016; 88:1812–1820. [PubMed: 26728944]
24. Silipo A, De Castro C, Lanzetta R, Molinaro A, Parrilli M, Vago G, Sturiale L, Messina A, Garozzo D. *J Mass Spectrom. JMS.* 2008; 43:478–484. [PubMed: 17975853]
25. Mikhail I, Yildirim HH, Lindahl ECH, Schweda EKH. *Anal. Biochem.* 2005; 340:303–316. [PubMed: 15840504]
26. Jones JW, Shaffer SA, Ernst RK, Goodlett DR, Tureček F. *Proc. Natl. Acad. Sci.* 2008; 105:12742–12747. [PubMed: 18753624]

27. Madalinski G, Fournier F, Wind F-L, Afonso C, Tabet J-C. *Int. J. Mass Spectrom.* 2006; 249–250:77–92.
28. Ting YS, Shaffer SA, Jones JW, Ng WV, Ernst RK, Goodlett DR. *J Am. Soc. Mass Spectrom.* 2011; 22:856–866. [PubMed: 21472520]
29. Yoon SH, Liang T, Schneider T, Oyler BL, Chandler CE, Ernst RK, Yen GS, Huang Y, Nilsson E, Goodlett DR. *Rapid Commun. Mass Spectrom.* 2016; 30:2555–2560.
30. Hankins JV, Madsen JA, Giles DK, Brodbelt JS, Trent MS. *Proc. Natl. Acad. Sci. U. S. A.* 2012; 109:8722–8727. [PubMed: 22589301]
31. O'Brien JP, Needham BD, Brown DB, Trent MS, Brodbelt JS. *Chem. Sci. R. Soc. Chem.* 2010. 2014; 5:4291–4301.
32. Klein DR, Holden DD, Brodbelt JS. *Anal. Chem.* 2016; 88:1044–1051. [PubMed: 26616388]
33. Hankins JV, Madsen JA, Giles DK, Childers BM, Klose KE, Brodbelt JS, Trent MS. *Mol. Microbiol.* 2011; 81:1313–1329. [PubMed: 21752109]
34. Williams PE, Klein DR, Greer SM, Brodbelt JS. *J Am. Chem. Soc.* 2017; 139:15681–15690. [PubMed: 28988476]
35. Herrera CM, Hankins JV, Trent MS. *Mol. Microbiol.* 2010; 76:1444–1460. [PubMed: 20384697]
36. Band VI, Crispell EK, Napier BA, Herrera CM, Tharp GK, Vavikolanu K, Pohl J, Read TD, Bosinger SE, Trent MS, Burd EM, Weiss DS. *Nat. Microbiol.* 2016; 1:16053. [PubMed: 27572838]
37. Hankins JV, Madsen JA, Needham BD, Brodbelt JS, Trent MS. *Methods Mol. Biol. Clifton NJ.* 2013; 966:239–258.
38. Henderson JC, O'Brien JP, Brodbelt JS, Trent MS. *J Vis. Exp. JoVE.*
39. Madsen JA, Boutz DR, Brodbelt JS. *J Proteome Res.* 2010; 9:4205–4214. [PubMed: 20578723]
40. Domon B, Costello CE. *Glycoconj. J.* 1988; 5:397–409.
41. Casillo A, Ziaco M, Lindner B, Parrilli E, Schwudke D, Holgado A, Verstrepen L, Sannino F, Beyaert R, Lanzetta R, Tutino ML, Corsaro MM. *ChemBioChem.* 2017; 18:1845–1854. [PubMed: 28650563]
42. Poirel L, Jayol A, Nordmann P. *Clin. Microbiol. Rev.* 2017; 30:557–596. [PubMed: 28275006]

Figure 1.

Experimental workflow presented in this manuscript. Collisional activation (CID) of substituted lipid A species occurs in the MS² event, resulting in neutral losses of the substituted species (blue triangle and red square) as well as fragmentation of the most labile acyl chain (orange). Subsequent ultraviolet photodissociation (UVPD) reveals diagnostic glycosidic product ions allowing for the localization of the substituted moieties.

Figure 2. MS¹ spectra (region from *m/z* 800 to 1300) of substituted lipid A species. (a) phosphoethanolamine (PETN) modifications in the sample SLAM 1. (b) aminoarabinose (AARA) modifications in the sample SLAM 2. (c) PETN and galactosamine (HEXN) modifications in the sample SLAM 3, with significant polypropyleneglycol (PPG) contaminants.

Figure 3.

(a) CID of substituted lipid A from SLAM 1 at m/z 960 (2-) reveals labile acyl chain losses as well as a diagnostic neutral loss associated with PETN modifications. (b) UVPD of the ion corresponding to the loss of the 3' e acyl chain (m/z 845, 2-, labeled green) provides diagnostic product ions (shown in blue) that allow localization of the PETN substitution to the 1-phosphate position, as shown in (c), while additional identifications are shown in red. The selected precursor ion in each stage is marked with a star. A detailed list of the fragments identified is provided in Table S1.

(a) CID of PETN/HEXN-substituted lipid A from SLAM 3 (m/z 1098, 2-) reveals loss of the labile 3' e acyl chain as well as a diagnostic neutral loss associated with PETN and HEXN modifications. (b) UVPD of the ion corresponding to the loss of the 3' e acyl chain (m/z 997, 2-, labeled green) provides diagnostic product ions (shown in blue) that allow localization of the PETN substitution to the 4'-phosphate position and the HEXN substitution to the 1-phosphate position, as shown in (c), while additional identifications are shown in red. The selected precursor ion in each stage is marked with a star.

Figure 5.

MS¹ spectra of *Enterobacter cloacae* samples SLAM 4 grown in the presence of 0 µg/mL of polymyxin and 100 µg/mL of polymyxin are shown in (a) and (b), respectively. Hexaacyl lipid A species are denoted as 6A and heptaacyl lipid A species are denoted as 7A. ** represents doubly modified lipid A species containing AARA. (c) CID of substituted lipid A from *Enterobacter cloacae* (m/z 1044, 2-) reveals loss of the labile 3'ε acyl chain (shown in green) as well as neutral loss of two AARA groups. (d) UVPD of the product ion corresponding to the loss of the 3'ε acyl chain (m/z 929, 2-) results in diagnostic glycosidic cleavages (shown in blue) for confirmation and localization of the substituents, mapped in

(e), while additional identifications are shown in red. The selected precursor ion in each stage is marked with a star.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 6.

Summary of the variations in the modifications of lipid A with respect to (a) number of acyl chains and (b) substitution profiles (PETN = phosphoethanolamine, AARA = aminoarabinose). The samples identified as 0 pmb and 100 pmb are those that were grown in the absence or presence of polymyxin B. These percentages were obtained based on abundances of molecular ions in the MS¹ spectra (with identification of the lipid A structures via the MS³ strategy). Ionization efficiencies of lipid A species were assumed to be similar for the different samples, and all samples were analyzed in triplicate.