

# Structural elucidation of Gram-negative bacterial endotoxins by tandem mass spectrometry

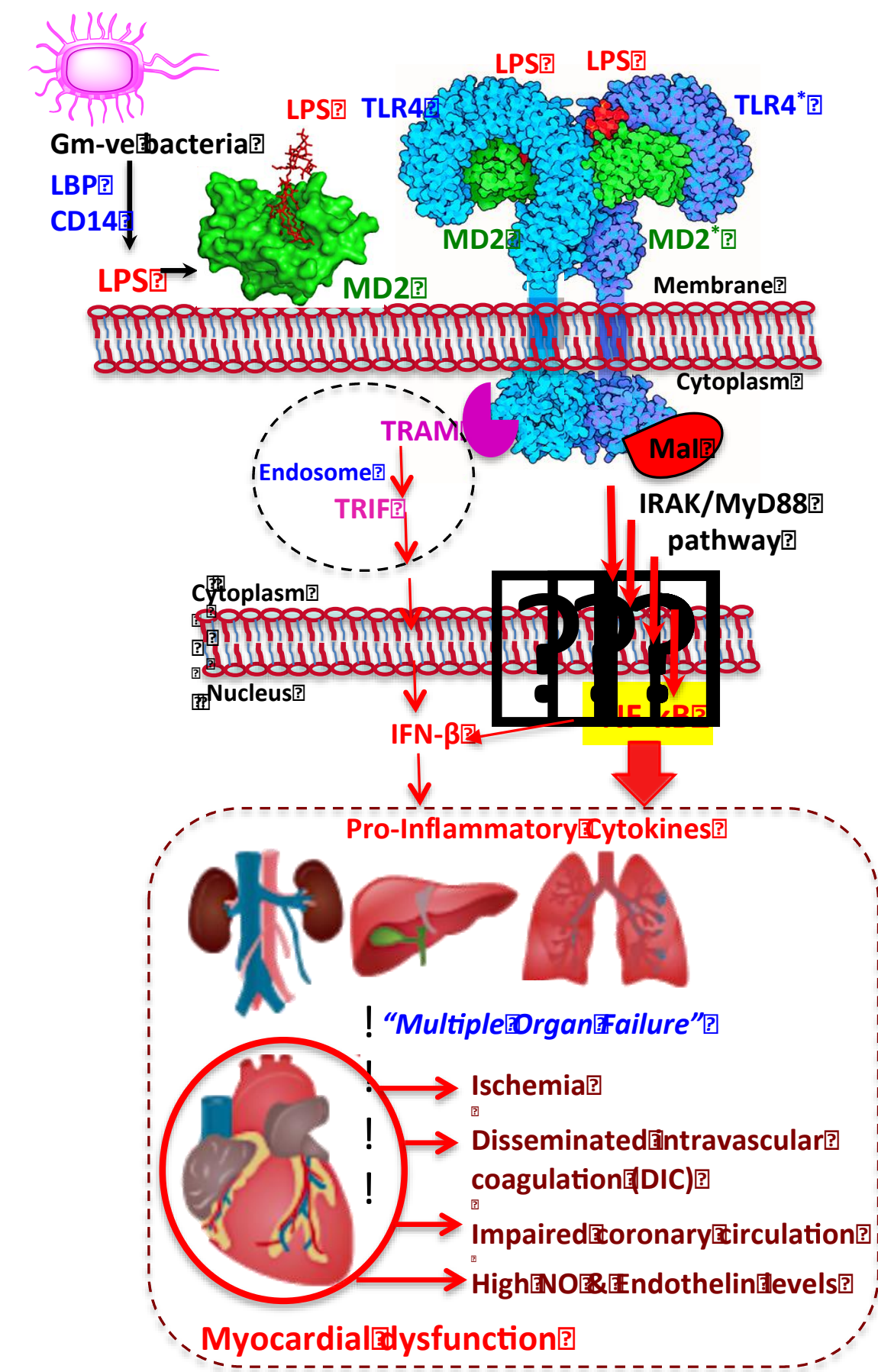
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## Introduction:

Lipopolysaccharide (LPS) or endotoxin is the major component of the outer leaflet of the outer cell membrane in Gram-negative bacteria, which is specifically recognized by host Myeloid differentiation protein 2/Toll like receptor-4 (MD2/TLR4) complex and results in pro-inflammatory cytokine production, which in turn helps with bacterial clearance. However, if their production is dysregulated, it can also lead to a septic shock (**Figure 1**) which is frequently fatal. Structurally, LPS consists of a distal O-antigen and lipooligosaccharide (LOS), which is composed of membrane-anchored lipid A and core sugars. Lipid A consists of a disaccharide backbone decorated with phosphate groups and fatty acyl chains, whose number and composition varies among bacteria. There is evidence that the number and composition of FA groups accounts, in part, for the fact that different bacteria produce different endotoxin potentials because the minimum determinant for TLR4 stimulation resides in lipid A. In earlier studies, it was observed that a detoxified Escherichia coli O111, Rc chemotype J5 lipopolysaccharide (J5dLPS)/group B meningococcal outer membrane protein (OMP) vaccine protected animals from experimental lethal sepsis and was safe and well-tolerated in human subjects. In the present study, we have applied tandem mass spectrometry (MS/MS) strategies, utilizing a Waters SYNAPT G2-S mass spectrometer (MS) in order to characterize J5LPS and its hypo-acylated form.



**Figure 1. Endotoxin-mediated septic shock.** Gram-negative bacterial lipopolysaccharide (LPS; endotoxin) can over-activate pro-inflammatory cascades via MD2/TLR4-receptor activation, leading to cytokine storm.

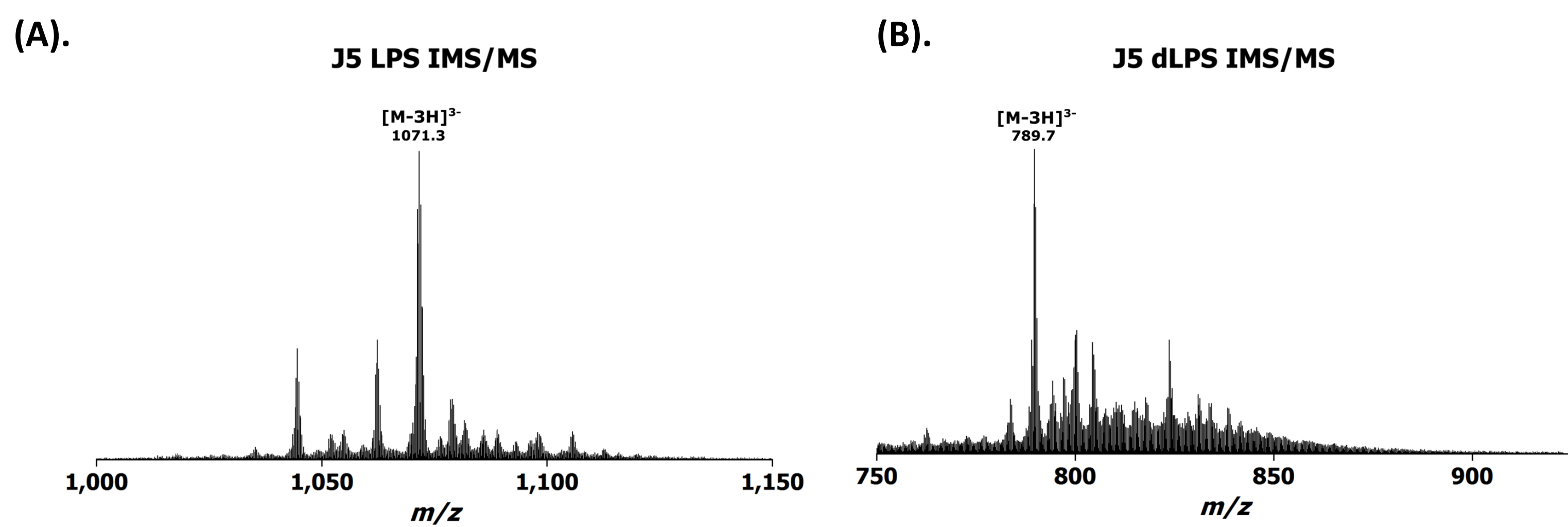
## Methods

Native LPS standards were obtained from Biological Labs, Inc., Campbell, California. To prepare a hypo-acylated standard, the purified LPS was resuspended in water (4 mg/ml) and an equal volume of 0.2M NaOH solution was added slowly with gentle stirring, followed by heating in a water bath at 65°C for 2 hours. The mixture was shaken every 5 minutes for the first hour and every 10 minutes thereafter. The solution was then neutralized with 1M acetic acid, ethanol precipitated, and lyophilized to isolate hypo-acylated LPS. The native LPS standards and prepared hypo-acylated LPS were resuspended in water (20 µg/mL) and directly infused into a Waters SYNAPT G2-S MS instrument for data acquisition.

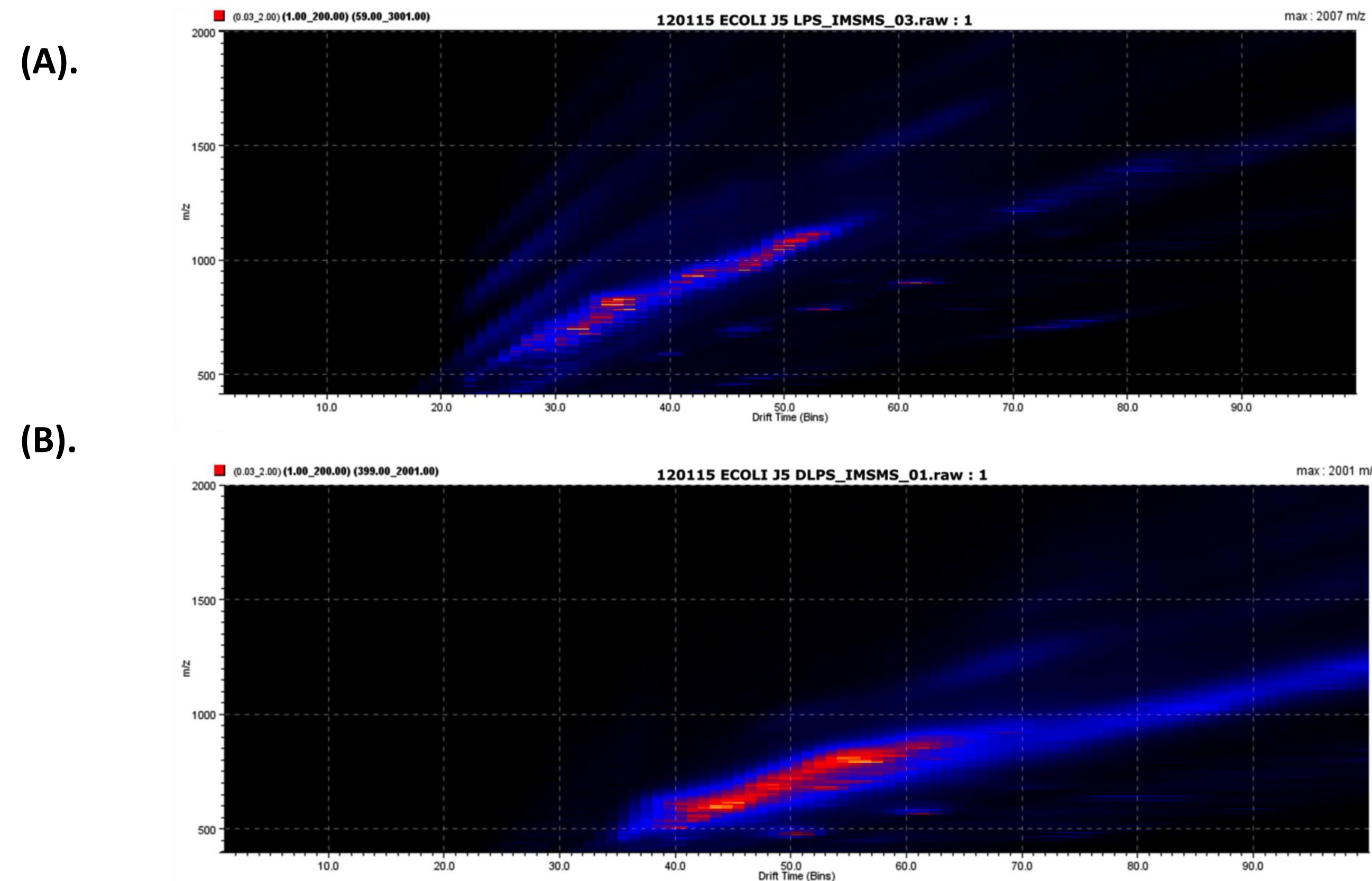
## Experimental Design and Data Analyses

Single stage mass spectra with traveling wave ion mobility separation were first acquired by direct infusion using the Waters SYNAPT G2-S HDMS, which consists of the following geometry: hybrid *quadrupole-ion mobility separation-orthogonal acceleration time-of-flight* (Q-IMS-oaTOF) MS. Intact LPS forms were detected as mostly [M-3H]<sup>3+</sup> ions (**Figure 2**) and easily separated from other species in the sample by drift time (**Figure 3**). Subsequent tandem MS experiments were performed with collision induced dissociation (CID) occurring both prior to and after ion mobility separation (**Figure 4**). In all experiments, collision energy was ramped from 5 to 100 eV to simulate MS<sup>n</sup> data acquisition. Marked differences between the single stage mass spectra from parent LPS and their hypo-acylated forms were readily observed. As expected for a sample treated by mild base hydrolysis, an ~840 Da mass shift corresponding to the loss of four O-linked fatty acyl chains was detected. In addition, the untreated LPS tandem mass spectra showed product ions corresponding to fatty acids, while the hypo-acylated LPS tandem mass spectra did not. In both cases, a mixture of LPS molecules was observed, as evidenced by several ion envelopes differing by 14, 16, or 18 Da surrounding the base peak.

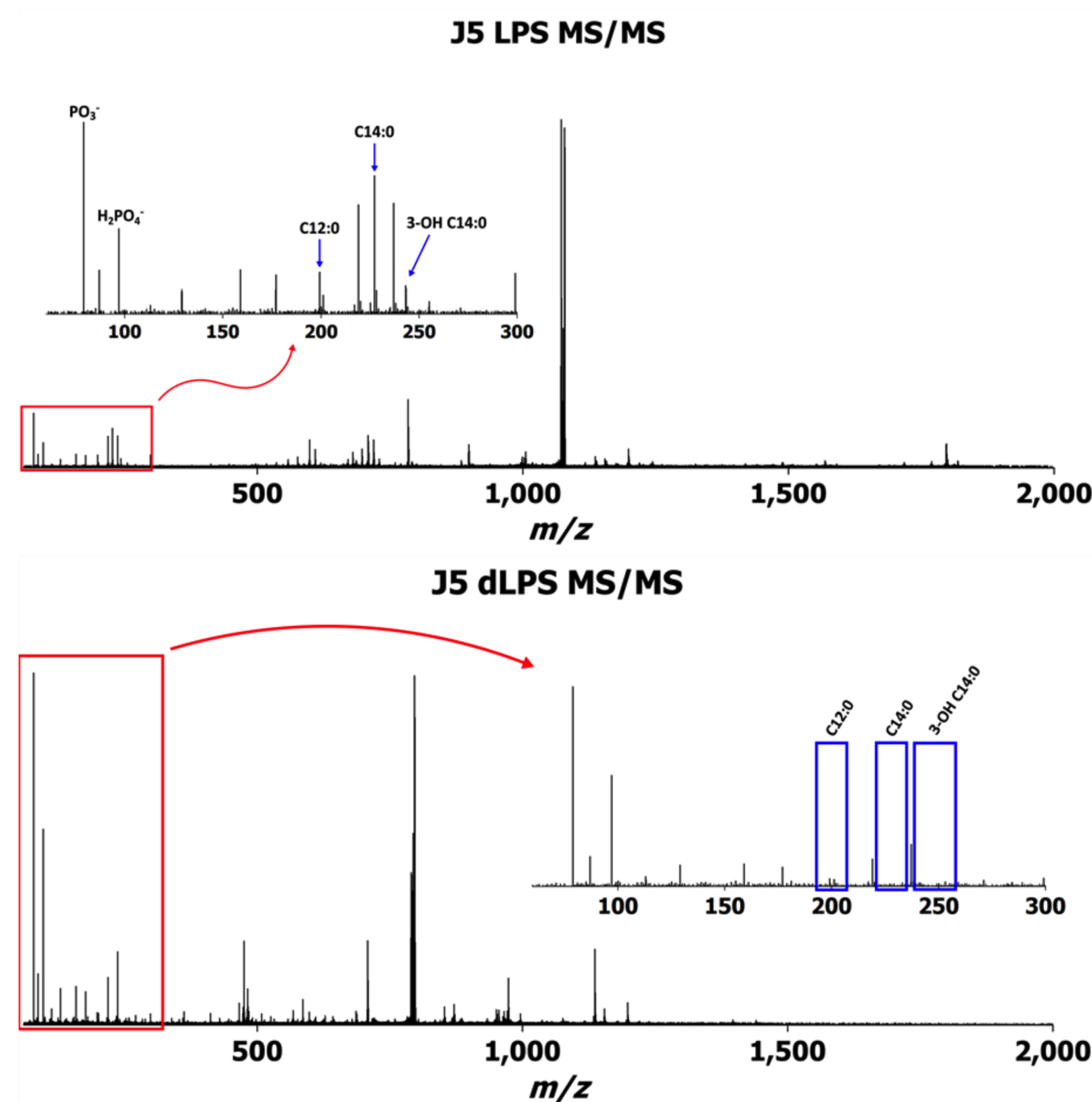
## Results:



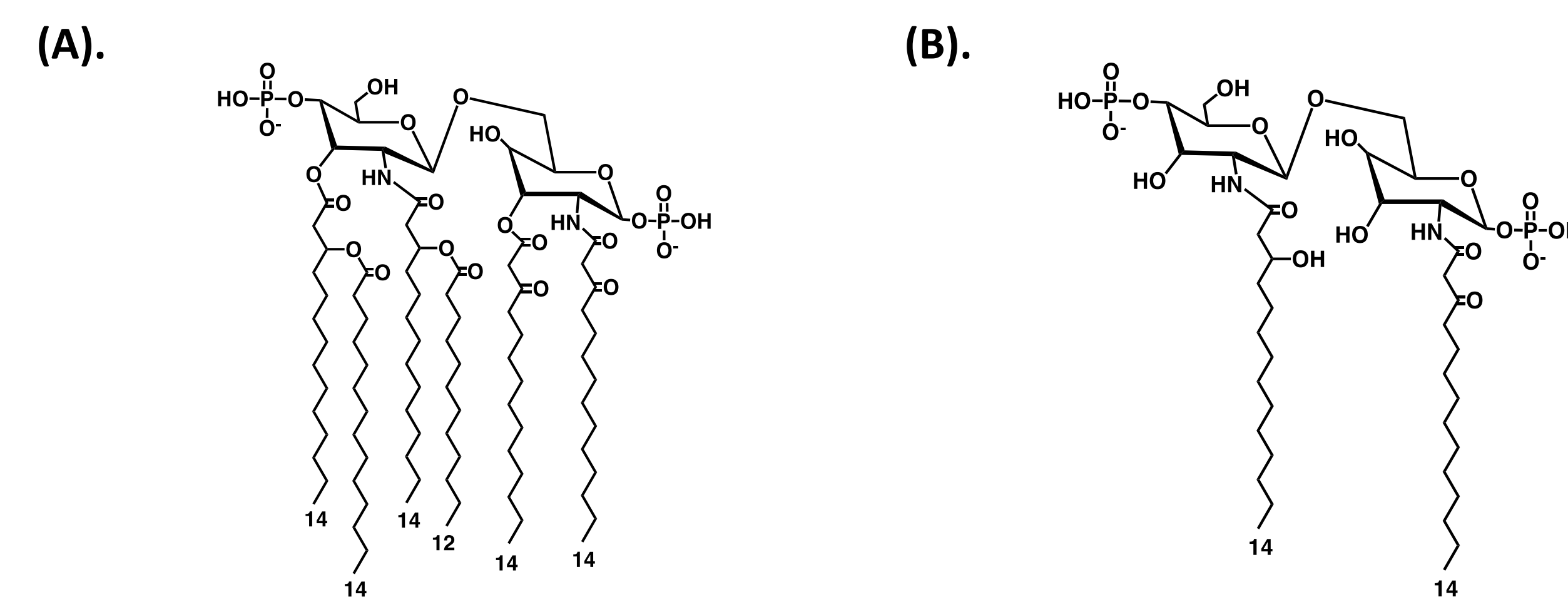
**Figure 2. Spectra obtained for (A) J5LPS, and (B) J5dLPS a Q-IMS-oaTOF MS**



**Figure 3. Multidimensional Plots of ESI-IMS/MS traveling wave ion mobility spectrometry (TWIMS) data readily highlight distinct ion profiles for (A) J5LPS and (B) J5dLPS.**



**Figure 4. Tandem mass spectrometric analyses of J5 LPS and J5dLPS**



**Figure 5. Chemical structures of lipid A part of E. coli J5 endotoxin: (A) Hexa-acylated wild-type strain lipid A and (B) detoxified endotoxin with the four O-linked alkyl chains removed.**

## Summary:

Here, we derive the structure of the dLPS produced by alkaline treatment. The reason for the deacylation was to reduce the reactogenicity (side effects) of the native LPS. This structural change results in loss of the ability to elicit endotoxicity (reactogenicity), as seen by J5LPS. However, the dLPS retains the ability to induce the B-cell production of IgG antibodies that are as protective as the IgG produced by the whole-boiled bacterial vaccine. This change in function is due to loss of the four O-linked fatty acids in the lipid A scaffold of J5LPS while the polysaccharide core remains intact. While the structure of J5dLPS had been speculated based on known chemical reactions of base with lipid A, the actual structure had not been derived by tandem MS previously.

## References:

1. Cross AS, Opal SM, Palardy JE, Drabick JJ, Warren HS, Huber C, Cook P, Bhattacharjee AK. Vaccine. 2003; 21(31):4576-87.
2. Cross AS, Opal SM, Warren HS, Palardy JE, Glaser K, Parejo NA, Bhattacharjee AK.. J Infect Dis. 2001;183(7):1079-86.
3. Jones JW, Cohen IE, Tureček F, Goodlett DR, Ernst RK.. J Am Soc Mass Spectrom. 2010; (5): 785-99.

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