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5 1 *Top down tandem mass spectrometric analysis of a chemically*
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8 2 *modified rough-type lipopolysaccharide vaccine candidate*
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11 3 Running Title: Top down LPS vaccine analysis
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21 **Abstract**

22 Recent advances in lipopolysaccharide (LPS) biology have led to its use in drug discovery pipelines,
23 including vaccine and vaccine adjuvant discovery. Desirable characteristics for LPS vaccine candidates
24 include both the ability to produce a specific antibody titer in patients and a minimal host inflammatory
25 response directed by the innate immune system. However, in-depth chemical characterization of most
26 LPS extracts has not been performed; hence, biological activities of these extracts are unpredictable.
27 Additionally, the most widely adopted workflow for LPS structure elucidation includes nonspecific
28 chemical decomposition steps before analyses, making structures inferred and not necessarily biologically
29 relevant. In this work, several different mass spectrometry workflows that have not been previously
30 explored were employed to show proof-of-principle for top down LPS primary structure elucidation,
31 specifically for a rough-type mutant (J5) *E. coli*-derived LPS component of a vaccine candidate. First, ion
32 mobility filtered precursor ions were subjected to collision induced dissociation (CID) to define
33 differences in native J5 LPS v. chemically detoxified J5 LPS (dLPS). Next, ultra-high mass resolving
34 power, accurate mass spectrometry was employed for unequivocal precursor and product ion empirical
35 formulae generation. Finally, MS³ analyses in an ion trap instrument showed that previous knowledge
36 about dissociation of LPS components can be used to reconstruct and sequence LPS in a top down
37 fashion. A structural rationale is also explained for differential inflammatory dose-response curves, *in*
38 *vitro*, when HEK-Blue hTLR4 cells were administered increasing concentrations of native J5 LPS v.
39 dLPS, which will be useful in future drug discovery efforts.

40 Introduction

41 Lipopolysaccharide (LPS), also known as endotoxin, is a major component of the outer leaflet of most
42 Gram-negative bacterial outer cell membranes [1, 2]. It is amphipathic, allowing it to interact with a wide
43 range of ions and molecules to maintain cell membrane integrity [1], participate in cell-cell interactions
44 [3–5], contribute to pathogenicity [6], and protect the bacterium from exogenous threats [7–10]. LPS is
45 composed of three parts (listed in order from the membrane to the extracellular space): 1.) a lipophilic,
46 multiply acylated diglucosamine membrane anchor that produces the canonical biologic activity of Gram-
47 negative bacterial infection (lipid A), 2.) a non-repeating oligosaccharide core (core OS), and 3.) a
48 polysaccharide, composed of repeating oligosaccharide units, that produces an immunodominant antigen
49 responsible for the O serotype (O-antigen). LPS exists as a mixture of biosynthetic products that can be
50 broadly classified into two groups: rough-type LPS (R-LPS) or lipooligosaccharide (LOS), and smooth-
51 type LPS (S-LPS). S-LPS is a complete LPS molecule, comprising all three aforementioned parts, while
52 R-LPS lacks the O-antigen portion, usually resulting in a loss of virulence. The biosynthesis pathway
53 enzymes for making lipid A and core OS in Gram-negative bacteria are relatively well-conserved.
54 However, due to differences in abundance or structure of LPS modifying enzymes, the resultant products
55 from these syntheses can vary greatly, both in structure and function, even within one species [11]. Many
56 non-stoichiometric substitutions of phosphate groups, sugars, amino acids, amines, and other R-groups
57 are also observed in LPS extracts, making accurate structural analyses challenging.

58 The dramatic increase in antibiotic resistance has left clinicians with fewer options to treat Gram-negative
59 bacterial infections. Vaccines have proven to be one of the most efficient strategies to prevent infectious
60 disease-related mortality and morbidity [12]. Although some of the structural features of LPS resulting in
61 specific illnesses like sepsis have been established (*e.g.* lipid A-TLR4 ligand-receptor binding induced
62 cytokine storm), there is no FDA-approved drug or vaccine against Gram-negative bacteria-induced
63 sepsis in large part because anti-lipid A antibodies have not shown much promise in clinical settings [13–
64 16]. Lipid A three dimensional (3D) structure is usually quite flexible and varies greatly between species

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4 65 of *Enterobacteriaceae*; however, the 3D structure of core OS remains similar due to conservation of
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6 66 biosynthetic enzymes [1]. In the past, studies have drawn a correlation between survival after Gram-
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8 67 negative bacterial sepsis and measured levels of circulating anti-core endotoxin antibodies in patient sera
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10 68 [17, 18]. Consequently, one feasible strategy for preventing sepsis is modification of LPS to generate
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12 69 antibodies to conserved epitopes in the core OS without eliciting a strong innate immune response.
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14 70 Passive infusion of immune sera after immunization of human volunteers with a vaccine composed of a
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16 71 heat-killed mutant of *E. coli* O111 which lacked the O polysaccharide (J5, or Rc chemotype mutant)
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18 72 resulted in protection against Gram-negative bacterial sepsis. A subsequent vaccine was developed using
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20 73 the purified J5 R-LPS that was alkali-treated to reduce the lipid A-induced toxicity and make the vaccine
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22 74 less reactogenic. This detoxified J5 LPS (J5 dLPS) was non-covalently complexed with group B
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24 75 meningococcal outer membrane protein (OMP) to form a hydrophobic complex. The hydrophilic portion
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26 76 on the outer surface enhanced its solubility and delivery. When administered to rodents, it improved
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28 77 survival from polymicrobial sepsis [19–21] and was well tolerated in humans [22] where it elicited a 37–
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30 78 142-fold increase in anti-core LPS antibody titer post-vaccination [21]. Interestingly, anti-core LPS
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32 79 vaccines (*e.g.* J5 Bacterin®) have been successful in treating bovine mastitis and decreasing Gram-
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34 80 negative bacterial sepsis incidence in animals [23, 24]. Additional vaccines have been developed against
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36 81 core OS but have not progressed to clinical trial [18, 25].
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43 82 The core glycolipid-carrier protein complex or liposome-associated preparations have been developed to
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45 83 facilitate vaccine delivery and immunization processes as lipid A in liposomes imparts significantly
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47 84 reduced toxicity [26] and liposome-encapsulated TLR4 ligands produce higher antibody response [27].
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49 85 Since LPS is highly reactogenic and can impart severe toxicity, chemical modification of LPS vaccines,
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51 86 such as the J5 vaccine, to reduce lipid A toxicity while retaining core OS immunogenicity has been
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53 87 proposed [21, 24]. In addition, the potent pro-inflammatory activity of the lipid A portion of the LPS has
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55 88 been modified to safely provide adjuvant activity for many vaccines. In fact, a chemically modified LOS,
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57 89 monophosphoryl lipid A (MPL®), that has diminished reactogenicity but potent adjuvanticity [28, 29] is
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90 used as a vaccine adjuvant in GlaxoSmithKline’s hepatitis B vaccine Fendrix® and human
91 papillomavirus (HPV) vaccine Cervarix®. Recently, rationally designed lipid A-based Toll-like receptor
92 4 (TLR4) ligands have been reported for vaccine adjuvant discovery and modulating the innate immune
93 response [30, 31]. Given both the potential use of core OS as a vaccine and a modified lipid A as a
94 vaccine adjuvant, LPS structure elucidation capability to support these efforts is critical.

95 In the past, LPS has been mostly analyzed after hydrolysis into its three distinct components by mass
96 spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy, or most commonly, a combination
97 of both techniques, and then reconstructed by inference to demonstrate “representative” LPS structures
98 for a species or strain. The results from these experiments can be misleading for several reasons
99 mentioned below; this list is not exhaustive. First – and most importantly – LPS extracts are always a
100 mixture of very similar compounds, sometimes producing exactly isobaric ions, making exact data
101 interpretation very difficult. This problem is compounded by relatively nonspecific methods like
102 hydrolysis or solvolysis for dissociation. Mixture complexity inevitably increases when these methods are
103 employed. Second, it is impossible, using this sub-component based structure definition approach, to infer
104 which candidate LPS structures are actually present in the cell membrane and are consequently of
105 biological relevance. Third, biological activities cannot be positively attributed to specific structures
106 because the complete structures are unknown. Attribution of structure to activity, or structure-activity
107 relationships (SAR), are also impossible to infer from mixtures of biologically active molecules unless the
108 relative activities of each of the components, as well as any interactions they may have with one another,
109 are known. Indeed, it has been shown that pure, chemically synthesized lipid As corresponding to
110 molecular formulae found together in extracted *E. coli* lipid A mixtures cause markedly different cytokine
111 responses in murine macrophages [32]. Therefore, even though the hypothesis has not been exhaustively
112 tested, it is reasonable to work on the assumption that differences in activity between LPS extracts are
113 cumulative, since any mixture of LPS molecules likely contains full agonists, partial agonists, and
114 antagonists of TLR4.

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4 115 Qureshi *et al.* showed that R-LPS could be purified based on the number of acyl chains present and
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6 116 analyzed offline by plasma desorption mass spectrometry in 1988 [33]. Recently, O'Brien *et al.* published
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8 117 a top down liquid chromatography-tandem MS approach to determining primary structural features of R-
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10 118 LPS from *E. coli* laboratory strains using collision induced dissociation (CID) and ultraviolet
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12 119 photodissociation (UVPD) [34]. Although it has been feasible to perform top down tandem MS
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14 120 experiments on R-LPS since the 1980's, most researchers have opted for the divide-and-conquer approach
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16 121 of wet chemistry followed by MS analysis of the separate components.
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20 122 With advances in MS instrument hardware, software, and electronics have come substantial increases in
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22 123 mass spectrometer capabilities since the heyday of plasma desorption. Modern instruments have become
23
24 124 more sensitive, can analyze many more samples in the same amount of time, and possess greater mass
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26 125 resolving powers and mass accuracies than older mass spectrometers. These benefits allow operators to
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28 126 separate, detect, and identify many ions solely in the gas phase. This approach was applied to the
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30 127 following research, using several different MS instruments for confirmation of previous results and to add
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32 128 complementary data for strengthened structural conclusions. Since the primary chemical structure of the
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34 129 J5 vaccine's LPS component has never been evaluated, and to investigate the hypothesis that the
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36 130 detoxified R-LPS vaccine from the J5 strain of *E. coli* derives its decreased inflammatory potential, while
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38 131 maintaining therapeutic value, from complete O-deacylation of the lipid A moiety, the following research
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40 132 was conducted:
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45 133 **Experimental**

46 134 *Materials*

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49 135 Purified (low protein and nucleic acid content) and lyophilized LPS from *E. coli* J5 strain was
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51 136 purchased from both List Biological Laboratories, Inc. (Campbell, CA) and Sigma Aldrich (St. Louis,
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53 137 MO). Note: LPS can be toxic if ingested or inhaled; proper personal protective equipment should be
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4 138 worn at all times. All solvents and water used throughout all experiments were Fisher (Waltham, MA)
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6 139 Optima LC/MS grade.
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9 140 *Detoxification of R-LPS and preparation of vaccine*
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12 141 To prepare a detoxified *E. coli* J5 LPS (J5 dLPS), purified LPS from List Biological Laboratories was
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14 142 re-suspended in water (4 mg mL⁻¹) and an equal volume of 0.2 M NaOH solution was added slowly
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16 143 with gentle stirring, followed by heating in a water bath at 65 °C for 2 hours. The mixture was shaken
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18 144 every 5 minutes for the first hour and every 10 minutes thereafter. The solution was then neutralized
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20 145 with 1 M acetic acid, ethanol precipitated, and lyophilized to isolate the dry product. To prepare J5
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22 146 dLPS/group B meningococcal outer membrane protein (OMP) complex vaccine, OMP extracted from
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24 147 phenol-killed bacteria was mixed with J5 dLPS as described elsewhere [19, 22]. Briefly, OMP was
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26 148 extracted from phenol-killed bacteria by Empigen BB (Huntsman Corporation, The Woodlands, TX;
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28 149 licensed to Sigma Aldrich for sale) detergent. Extracted OMP was mixed with J5 dLPS (in 0.9%
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30 150 NaCl) at a ratio of 1.2:1 (w/w) in Tris-EDTA buffer, pH 8.0 containing 0.1% Empigen BB. Extensive
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32 151 dialysis was achieved to remove detergent and the vaccine was further filter sterilized and stored at 5
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34 152 °C.
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40 153 *Cell culture and cytokine reporter assay*
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43 154 HEK-Blue hTLR4 cells (Invitrogen, Waltham, MA) were cultured in Dulbecco's Modified Eagle
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45 155 Medium (DMEM; Gibco, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine
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47 156 serum (FBS; Sigma Aldrich, St. Louis, MO), 100 IU mL⁻¹ penicillin, 100 µg mL⁻¹ streptomycin, 1
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49 157 mM sodium pyruvate, and 200 mM L-glutamine in a humidified incubator at 37 °C, 5% CO₂. For cell
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51 158 stimulation, lyophilized LPS was reconstituted in sterile, endotoxin-free water at a concentration of 1
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53 159 mg mL⁻¹. This stock was serially diluted in DMEM before addition to the cell culture for the
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55 160 stimulation experiment. Post-stimulation (16 h), supernatants were collected from HEK-Blue cells,
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57 161 and the production of SEAP reporter was detected using Quanti-Blue (Invitrogen) according to the
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4 162 manufacturer's instructions. NF- κ B reporter cell line stimulation data were plotted as the mean (\pm
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6 163 SD) from biological duplicates using GraphPad Prism 7.0 (La Jolla, CA).
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9 164 *Defining mixture composition differences between intact and detoxified LPS samples*
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12 165 Both intact J5 LPS and J5 dLPS samples from List Biological Laboratories, Inc. were dissolved in a
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14 166 solution, composed of 50% (v/v) 2-propanol and 50% water, and directly infused by syringe pump (5
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16 167 $\mu\text{L min}^{-1}$, at an estimated concentration of $20 \mu\text{g mL}^{-1}$) into the source of a Waters (Milford, MA)
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18 168 Synapt G2 HDMS quadrupole-ion mobility separation-orthogonal acceleration time of flight mass
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20 169 spectrometer, equipped with a 4 kDa quadrupole, and operated with negative polarity electrospray
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22 170 ionization (ESI) and in "Resolution" mode. Traveling wave ion mobility separation (TWIMS) was
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24 171 employed to isolate ions with similar size, shape, and charge and to consequently simplify mass
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26 172 spectra. TWIMS was also used for gas-phase separation after quadrupole precursor isolation and prior
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28 173 to tandem MS experiments. The ion mobility separations were all performed using N_2 as buffer gas at
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30 174 a flow rate of 90 mL min^{-1} , with a wave velocity of 650 m s^{-1} and a wave height of 40.0 V. The ESI
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32 175 source was operated with a capillary potential of 3.00 kV, source temperature of $100 \text{ }^\circ\text{C}$, sampling
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34 176 cone at 40.0 V, source offset at 40.0 V, source gas (N_2) flow at 0.0 mL min^{-1} , desolvation temperature
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36 177 of $400 \text{ }^\circ\text{C}$, cone gas flow at 25 L hr^{-1} , desolvation gas flow at 400 L hr^{-1} , and nebulizer gas pressure at
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38 178 5.0 bar. Collision induced dissociation (CID) tandem MS experiments were performed using ultra-
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40 179 pure argon (Airgas, Radnor Township, PA) as collision gas, with manual collision energy ramping
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42 180 from 0 V to 100 V in increments of 10 V to produce comprehensive product ion spectra. All other
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44 181 instrument parameters are available upon request.
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51 182 *Ultra-high mass resolving power, high mass accuracy FT-ICR MS to determine empirical formulae*
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53 183 *and define J5 LPS primary structure*
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56 184 Intact *E. coli* J5 LPS from Sigma Aldrich (St. Louis, MO), dissolved in a solution of 50% 2-propanol
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58 185 and 50% water at an estimated total LPS concentration of $50 \mu\text{g mL}^{-1}$, was directly infused through a
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4 186 home-built nano-electrospray ionization (nESI) source at a flow rate of 1 $\mu\text{L min}^{-1}$ into a hybrid linear
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6 187 ion trap – 21 Tesla Fourier transform-ion cyclotron resonance (FT-ICR) mass spectrometer, described
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8 188 in detail in a previous publication [35]. The ion spray was visually optimized for each experiment by
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11 189 adjusting capillary voltage and position while monitoring for constant signal as well as observation of
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13 190 a uniform electrospray plume. Multiple tandem MS experiments were performed, including trap CID,
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15 191 beam-type collisionally activated dissociation (beam CAD), and in-cell ultraviolet photodissociation
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17 192 (UVPD). Trap CID and beam CAD were performed with stepped collision energy. UVPD was carried
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20 193 out similarly to a previous publication [36] using a Coherent (Santa Clara, CA) Excistar XS ArF
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22 194 excimer laser operated at 193 nm wavelength and 522 μJ per pulse. The laser was previously aligned
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24 195 through a window in the rear of the ICR magnet housing on-axis with the ICR cell.
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27 196 *Multi-stage MS (MS^n) to confirm structural inferences*

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30 197 Intact *E. coli* J5 LPS from both List Biological Laboratories, Inc. (Campbell, CA) and Sigma Aldrich
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32 198 (St. Louis, MO) were directly infused in a solution composed of 50% 2-propanol and 50% water at a
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35 199 flow rate of 2 $\mu\text{L min}^{-1}$ into a home-built nESI source of a linear ion trap (linear trapping quadrupole;
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37 200 LTQ) mass spectrometer (Thermo Finnigan, San Jose, CA) with post-production ion funnel optics
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39 201 added for increased ion transmission efficiency. The mass spectrometer was operated in negative
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41 202 ionization mode with a capillary potential of 2.3 kV. Tandem MS experiments were performed in the
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43 203 ion trap with ultra-pure helium as collision gas. MS^3 was carried out on product ions from LPS,
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45 204 corresponding to lipid A and core OS, at stepped normalized collision energies to evaluate its utility
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48 205 for top down sequencing.
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51 206 *Data analysis*

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54 207 Data acquired on the Synapt G2 HDMS instrument were initially processed in Driftscope version 2.7
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56 208 and MassLynx version 4.1 software (Waters, Milford, MA). When necessary, data were converted to
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58 209 mzML format using the ProteoWizard msconvert utility. Automated peak picking was performed
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210 using mMass version 5.5 (www.mmass.org) [37]. Data acquired on the FT-ICR instrument were
211 converted from magnitude mode to absorption mode and processed (including peak picking and
212 absorption mode isotopic modeling) using AutoVectis (Spectroswiss Sàrl, Lausanne, Switzerland)
213 [38–42]. For rapid visualization and spot checking of data, ICR mass spectra were saved as Thermo
214 .raw files and manipulated in Thermo (San Jose, CA) Xcalibur version 3.0.63. Data acquired on the
215 LTQ instrument were processed in Xcalibur version 3.0.63, and when necessary, were converted to
216 mzML format for use in open source software suites. Peak picking for LTQ data was also performed
217 using mMass version 5.5. Data were plotted using QtiPlot version 0.9.8.9 (www.qtiplot.com) and
218 Figures were generated in Inkscape version 0.91 (<https://inkscape.org>) and when necessary, modified
219 to an acceptable format using GIMP version 2.8.18 (www.gimp.org).

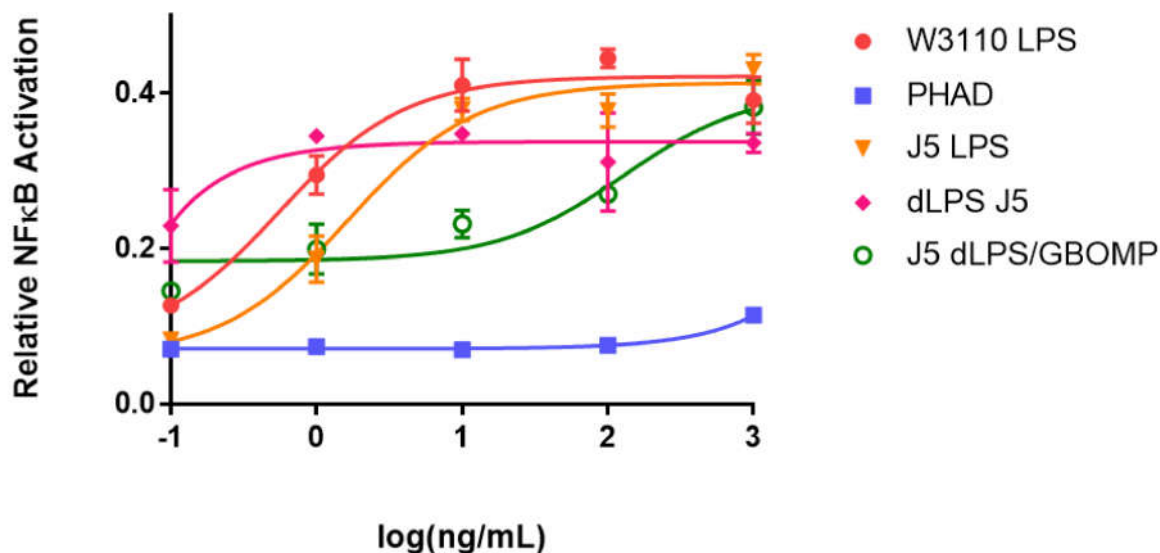
220 **Results and Discussion**

221 *Cell culture and cytokine reporter assay*

222 To test the pro-inflammatory capacity of various LPS described in this manuscript, LPS were
223 incubated with the HEK-Blue hTLR4 reporter cell line from which NFκB activation can be directly
224 measured (Fig. 1). Both *E. coli* strains tested had similar stimulation profiles with J5 LPS reaching
225 maximum stimulation at a slightly higher concentration than the W3110 strain. The J5 dLPS reached
226 a lower maximum signaling level at a lower concentration, indicating that it is less inflammatory but
227 maintained a strong binding affinity to the activated signaling pathway. The J5 dLPS/GBOMP
228 formulation reached a similar maximal signaling level as the J5 dLPS but activation could be titrated
229 down at a much higher concentration. PHAD, an adjuvant molecule already in use, stimulated cells at
230 a much lower level than all other molecules tested, only rising above baseline at the highest
231 concentration tested. At low concentrations J5 dLPS/GBOMP is capable of maintaining NF-κB
232 stimulation while PHAD, a known adjuvant molecule, is not. This novel method of detoxifying LPS
233 could be used to create TLR4 agonists that are not toxic and still capable of stimulating the immune

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234 system to a desirable level for adjuvant use.



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236 **Fig. 1** Agonists were cultured with HEK-Blue hTLR4 cells over a 5-log dose range from 0.1-1000
237 mL⁻¹. W3110 *E. coli* LPS (red), J5 *E. coli* LPS (orange), J5 dLPS (pink), J5 dLPS/GBOMP (green),
238 or PHAD (blue) were incubated for 16 hours. Then NF-κB activation was measured by quantification
239 of SEAP in the supernatant. Mean ± SD of duplicate samples and an associated 4-parameter non-
240 linear regression are shown

241 *Defining mixture composition differences between intact and detoxified LPS samples*

242 Direct infusion of J5 LPS and dLPS into the Synapt G2 HDMS produced many ions attributed to
243 intact LPS and fragments thereof (Sup. Fig. 1). For the sake of simplicity, ions which could be easily
244 associated with the “canonical” structures for *E. coli* LPS were isolated in the quadrupole, separated
245 from isobars by ion mobility (Sup. Fig. 2), and subjected to CID in the “Transfer” region of the
246 collision cell by ramping collision energy and averaging the resultant tandem mass spectra. The
247 precursor ions used for direct comparison (m/z 1071 for LPS and m/z 789 for dLPS) happened to have
248 the largest complete LPS peak amplitudes and represented $[M-3H]^{3-}$ ions.

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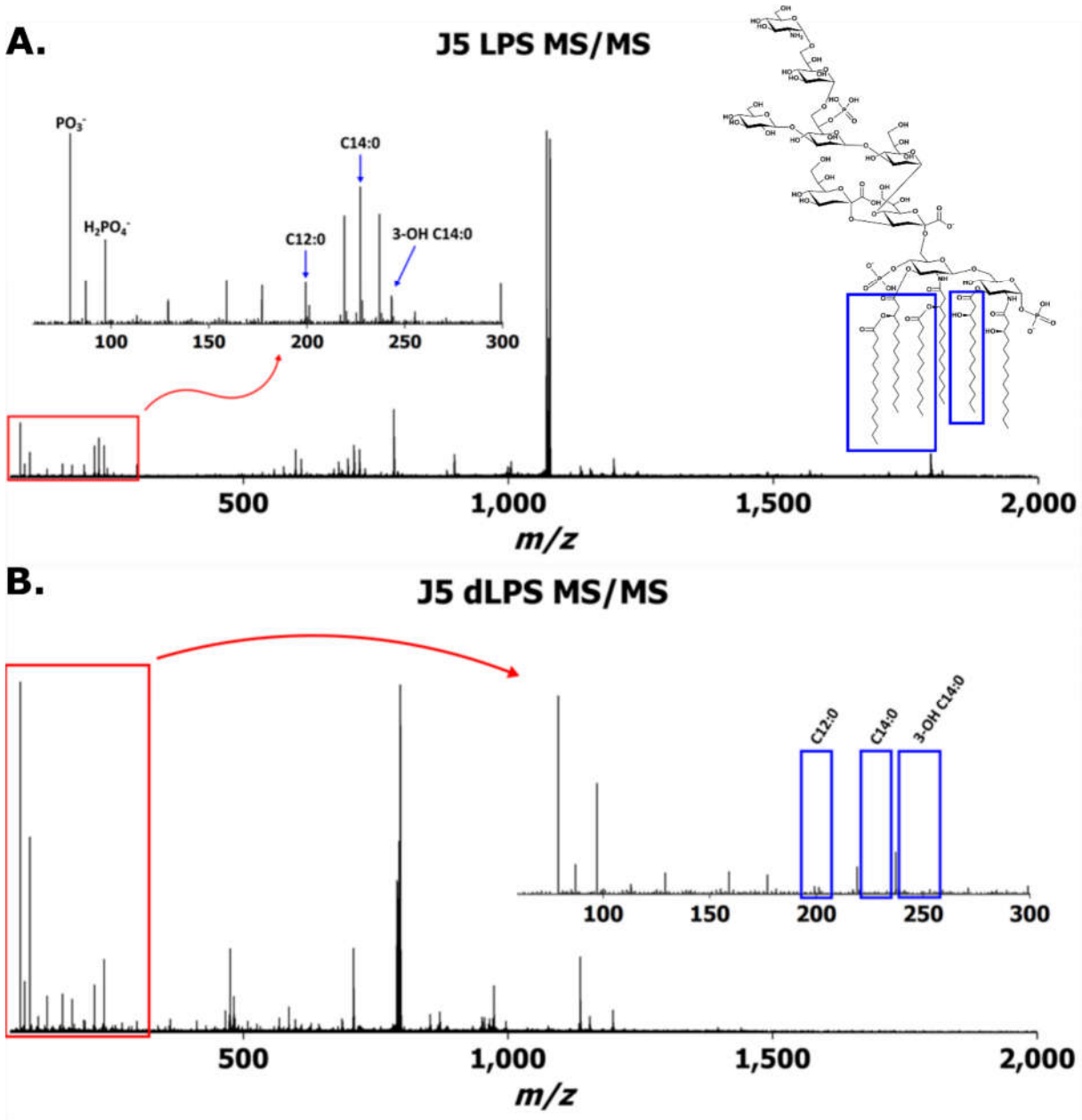
249 In the single stage mass spectrum of dLPS, there were no detectable ions corresponding to the
250 complete R-LPS structure. This was interpreted to mean that the detoxification chemistry had
251 proceeded to completion. After dissociation of J5 LPS and dLPS, one difference in the tandem mass
252 spectra was immediately obvious: Liberated O-linked fatty acids were not present in dLPS product
253 ion spectra (Fig. 2). The observation of low m/z product ions, including deprotonated fatty acids and
254 phosphorous-containing ions, is a particular advantage to using a beam-type mass spectrometer for
255 LPS tandem MS experiments rather than any quadrupolar ion trap mass analyzer because the 1/3
256 cutoff rule, meaning product ions less than ~30% the m/z of the precursor ion are always unstable
257 under trapping conditions, does not apply. These product ions can be directly diagnostic to structural
258 changes in LPSs for which some structural information is already known. In aggregate, they can
259 inform the analyst about which class of compound has been detected in an unknown.

260 A total of 179 monoisotopic product ions were detected by the Synapt G2 HDMS above a signal to
261 noise ratio (S:N) of 10:1 in the J5 LPS IMS-CID spectrum of m/z 1071; 221 were detected in the
262 dLPS IMS-CID spectrum of m/z 789. Singly and doubly deprotonated product ions representing the
263 full-length core OS (m/z 1418 and 708, respectively) were detected in both LPS and dLPS sample
264 mass spectra, while the product ions representing the *bis*-phosphorylated, hexa-acylated *E. coli* lipid
265 A (m/z 1796 and 897) were only present in the native LPS mass spectrum. Product ions assigned to
266 the detoxified, di-acylated lipid A (m/z 951 and 475) were detected in the dLPS mass spectrum. These
267 data further confirmed that the vaccine detoxification chemistry had proceeded as hypothesized.

268 It is important to emphasize that both the LPS and dLPS samples produced heterogeneous mass
269 spectra, even after crude IMS filtering, presumably due to many different LPS structures in the
270 samples. Other analyses of R-LPS by both ESI-MS and MALDI-MS have demonstrated similar
271 results [34, 43]. This means that the activity data shown in the previous section are cumulative and
272 cannot be attributed to a particular R-LPS structure. However, the alkaline hydrolysis that was
273 performed produced an entirely different mass spectrum than the commercial product. Since the mass

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274 spectra are complex, quality control and quality assurance protocol development will be necessary to
275 maximize the vaccine's robustness. One example to illustrate this need can be seen in Sup Fig 1.
276 Second and third envelopes of ions were observed in the dLPS spectrum corresponding to differences
277 in the number of core OS sugars. It is unclear from this study whether these differences were part of
278 the original LPS mixture or if they were a result of the sample processing.



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4 **280** **Fig. 2** Averaged IMS-CID tandem mass spectra of (a) J5 LPS m/z 1071 and (b) J5 dLPS m/z 789 after
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6 **281** collision energy ramping. Insets show deprotonated fatty acid product ions' presence in (a) and
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9 **282** absence in (b)

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11 **283** *Ultra-high mass resolving power, high mass accuracy FT-ICR MS to determine empirical formulae*
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14 **284** *and define J5 LPS primary structure*

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17 **285** In total, 252 unique, unambiguous monoisotopic masses were observed above a S:N of 10:1 in the
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19 **286** Sigma J5 LPS sample with masses greater than the monoisotopic mass of KDO₂-lipid_{IV} A (J5 lipid A
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21 **287** attached to two 2 – 4 linked 3-Deoxy-D-manno-oct-2-ulosonic acid residues through a 2 – 6'
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23 **288** glycosidic bond, [M] = 1844.971 Da), excluding redundant masses from additional charge states; 3-,
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25 **289** 4-, and 5- ions were observed for intact R-LPS (Sup. Table 1). One hundred ninety-six of these
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28 **290** masses were larger than the mass of the canonical *E. coli*, hexa-acylated lipid A attached to five core
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30 **291** OS sugars. Interestingly, thirty-six monoisotopic masses observed were greater than the mass of the
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32 **292** intact, canonical J5 *E. coli* R-LPS. Some of these ions corresponded to empirical formulae for *E. coli*
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34 **293** R-LPS with known substitutions such as phosphoethanolamine and phosphate/pyrophosphate. A few
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37 **294** were inferred to be cation adducts of multiply deprotonated ions. The vast majority, however, were
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39 **295** unable to be inferred using accurate mass alone. In short, the R-LPS mixture was very heterogeneous,
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41 **296** including ions not previously described in the literature for *E. coli* R-LPS. One example of the
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43 **297** heterogeneity observed in only a 4 m/z window is shown in Fig. 3. In this window, at least eight
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46 **298** different monoisotopic masses were observed, at a mass resolving power of ~300,000 FWHM, in
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48 **299** absorption mode. There was also not a single dominant species in the mixture as has sometimes been
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50 **300** reported.

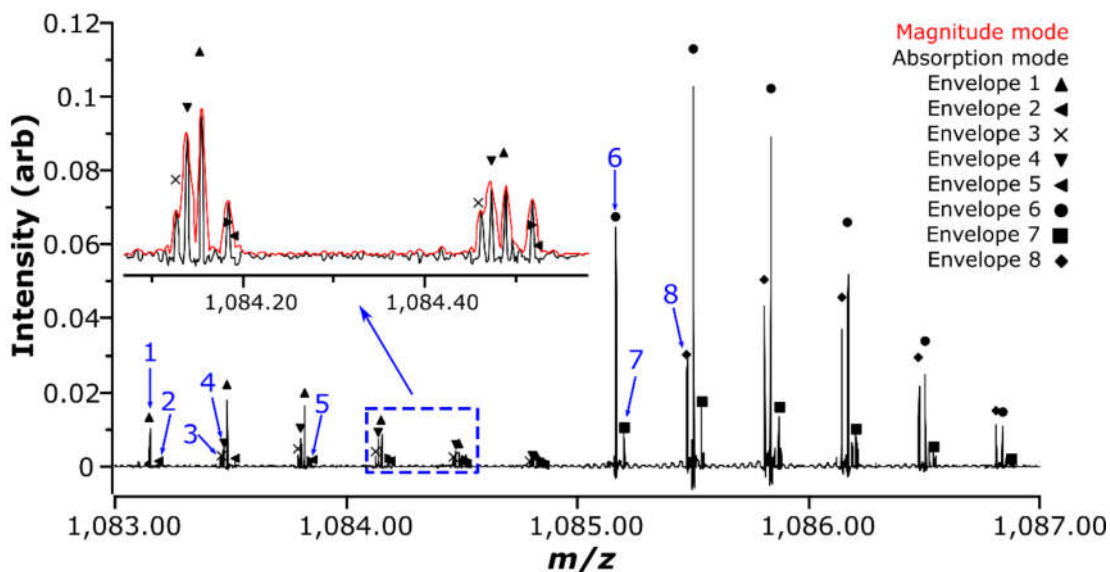


Fig. 3 Zoomed negative mode FT-ICR mass spectrum ($R \sim 300,000$ FWHM, in absorption mode) after direct infusion of J5 LPS. Eight potential isotopic distribution envelopes can be identified in absorption mode in this 4 m/z window; these are denoted, at the m/z of their respective monoisotopic ions, with blue arrows. (inset) Magnified portion of the spectrum showing fine detail (including the magnitude mode and the proposed overlap between isotopologues from envelopes 2 and 5)

The highest intensity complete R-LPS monoisotopic mass in the J5 LPS spectrum, as in the previous experiment, was m/z 1071.1976. This mass corresponded to a similar structure to that reported in [44, 45] (Empirical formula: $[C_{143}H_{257}N_3O_{69}P_3]^{3-}$, $\Delta = 0.139$ ppm). The only differences observed were an addition of a phosphate moiety on the second heptose and a terminal glucosamine (GlcN) rather than an N-acetylglucosamine (GlcNAc). An ion was also observed in the spectrum at m/z 1085.2009, corresponding to the previously published structure with an added phosphate group (Empirical formula: $[C_{145}H_{259}N_3O_{70}P_3]^{3-}$, $\Delta = -0.381$ ppm). An isolation of m/z 1071 prior to CID experiments resulted in an error of 0.064 ppm, in magnitude mode, for a single 0.767 second transient.

Tandem MS experiments on m/z 1071 (3.5 m/z isolation) using trap CID, beam CAD, and UVPD all yielded feature-rich tandem mass spectra. Trap CID at normalized collision energy (NCE) of 30%

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317 yielded 201 monoisotopic product ions above a S:N of 4:1. Six pairs of product ions (twelve ions
318 total) were found to be duplications due to multiple charge states. Beam CAD at 35 V yielded 152
319 product ions, with three pairs of duplicate identifications due to multiple charge states, using the same
320 peak picking parameters. For many ions charge was not automatically identified, probably due to
321 insufficient isotopic ion abundance. A wider precursor ion selection window and/or spectral
322 averaging or summing would improve this statistic. Ninety-nine ions were detected in both trap CID
323 and beam CAD experiments; either set of product ions was sufficient to confirm a hypothesized
324 structure for the precursor ion (Fig. 4), when taking into account the cumulative effect of measuring
325 every product ion at low ppb mass accuracy. These product ions corresponded to acyl chain
326 cleavages, glycosidic bond cleavages, cross-ring cleavages, neutral losses of modifications, and
327 combinations thereof. UVPD yielded ninety product ions, twenty-five of which were common to the
328 CID and/or beam CAD experiments. This demonstrates, as in O'Brien *et al.*'s publication [34], a
329 complementary set of product ions to those obtained through collisional activation. However, the
330 efficiency of the UVPD process in this experiment was very low, so its value added would only be
331 noticed with respect to very specific structural questions in this particular configuration and for this
332 application (*e.g.* Are there hydroxylated fatty acyl chains in the lipid A moiety?). These types of
333 questions are beyond the scope of this work.

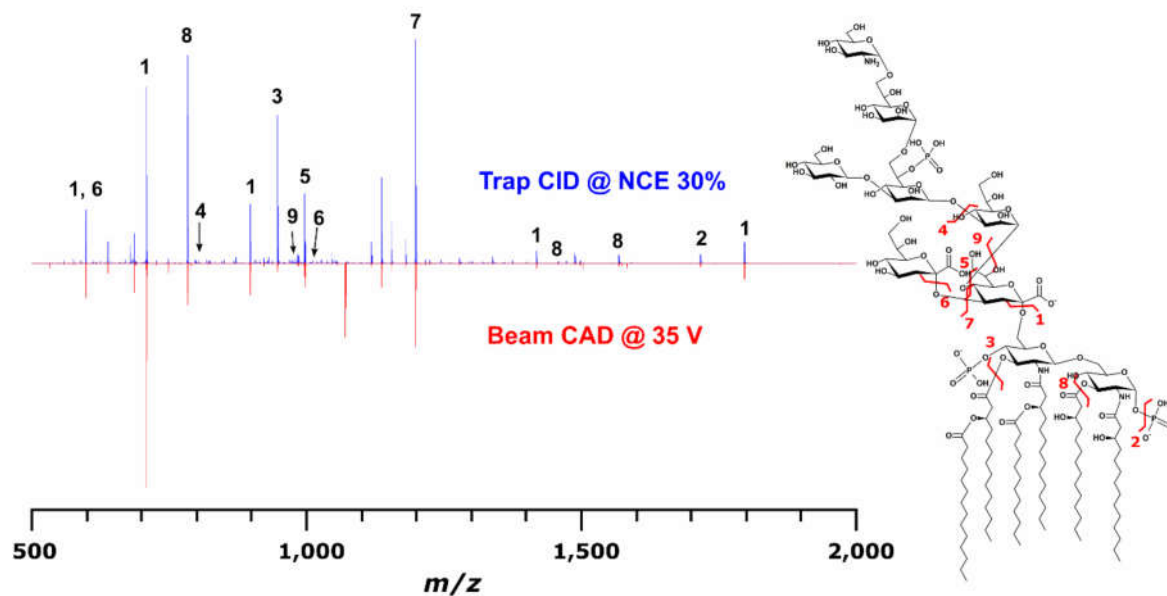


Fig. 4 Comparison of trap CID (blue) and beam CAD (red) for the same precursor ion at m/z 1071.

Ninety-nine monoisotopic product ions common to both experiments were observed, fifteen of which are annotated in the CID mass spectrum with corresponding bond cleavages in the structure on the right. All product ion m/z were measured with less than 100 ppb error

Multi-stage MS (MS^n) to confirm structural inferences

After conducting multiple product ion scans on intact R-LPS precursor ions, it became apparent that abundant product ions were always formed which corresponded to both the lipid A and core OS moieties. Since there have been many published papers describing dissociation phenomena for both chemically isolated lipid A [46–51] and oligosaccharides [52–56], it seemed that the logical next step would be to dissociate these products to determine whether MS^3 product ions would be formed according to these well-established rules. If so, top down sequencing data interpretation and deconvolution for R-LPS would become much less challenging, and could be performed with confidence on nominal mass accuracy, low resolving power ion trap instruments.

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348 Fig. 5 shows an MS³ experiment for both J5 lipid A (*m/z* 1796) and core OS (*m/z* 1418) ions formed
349 in the ion trap after dissociation from intact R-LPS (*m/z* = 1071). Characteristic tandem mass spectra
350 for both lipid A and core OS were obtained, suggesting the feasibility of simplified top down
351 sequencing for both moieties and attribution of these to a specific LPS precursor. These data
352 demonstrate that gas phase decomposition chemistry proceeds similarly to the widely adopted
353 approach of solution phase decomposition followed by analyses of the reaction products separately.
354 The primary benefit of the gas phase decomposition approach is that both lipid A and core OS can be
355 directly attributed to a R-LPS structure present in the sample precluding the need for inference when
356 analyzed separately. For lipid A, product ions indicative of neutral losses of ester-linked fatty acyl
357 chains and metaphosphoric acid were the most abundant features in the mass spectra. For core OS, B-
358 and Y- ions, as described by Domon and Costello [52], corresponding to glycosidic bond cleavages
359 were the most abundant product ions, with minor product ions corresponding to cross-ring cleavages.
360 It is likely that any MS³-capable trapping instrument, as well as beam-type instruments outfitted with
361 post-CID ion mobility separation capability and subsequent secondary CID, will be able to perform a
362 similar experiment. As in our previous work with lipid A [57], stepped or ramped collision energy at
363 the MS³ level was able to simulate subsequent levels of MSⁿ, with qualitatively minimal ion losses, to
364 provide more complete dissociation and primary structure coverage for lipid A and core OS (data not
365 shown).

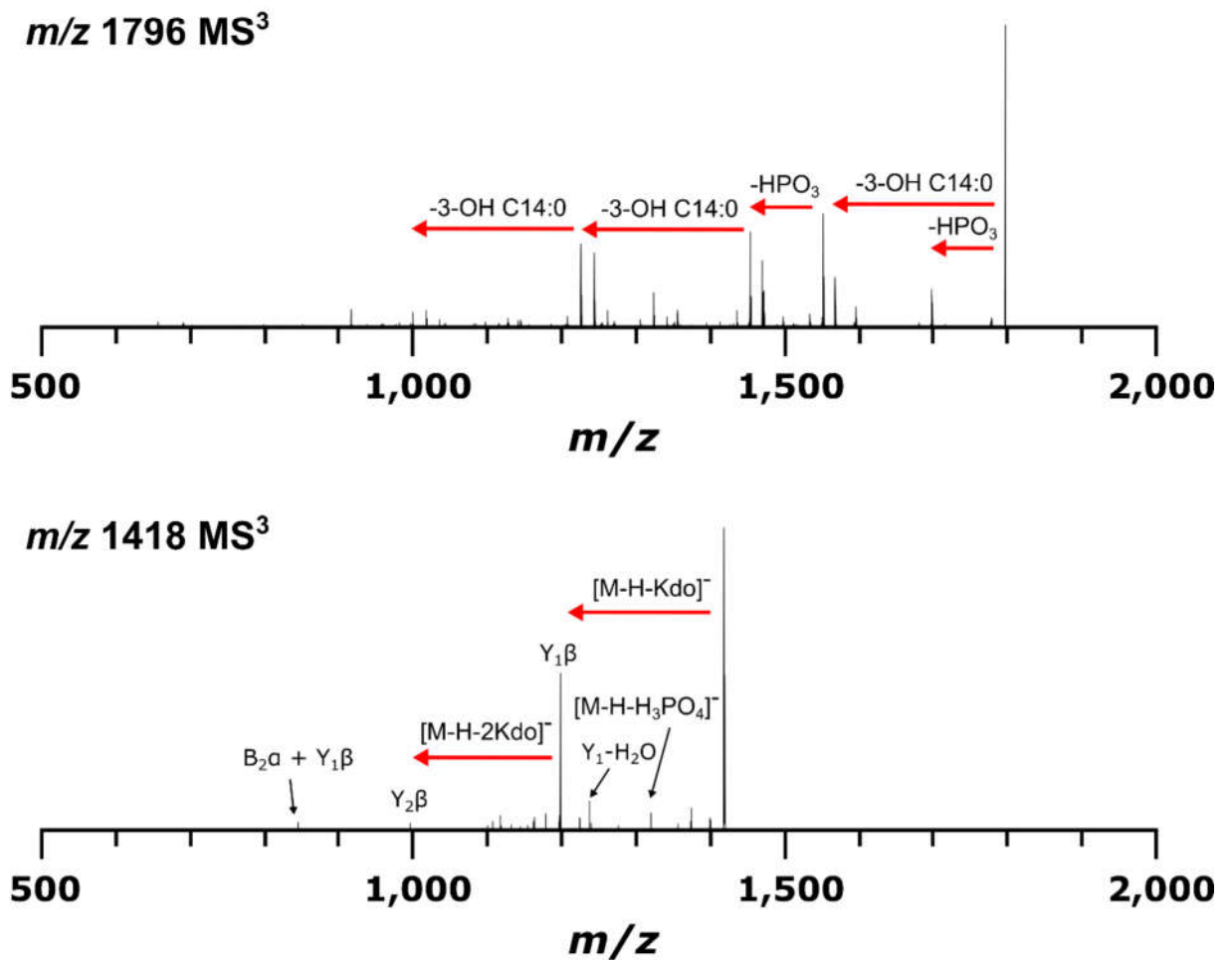


Fig. 5 MS³ CID mass spectra from MS² product ions representing J5 *E. coli* lipid A at *m/z* 1796 (top) and core OS at *m/z* 1418 (bottom). Similar dissociation phenomena were observed as in MS² experiments for chemically isolated lipid A and oligosaccharides, indicating feasibility of LPS top down sequencing in this manner

Conclusions

As with all biologically active molecules, LPS activity is directly related to LPS structure. In this work, a chemically modified R-LPS vaccine candidate's reduced innate immunogenicity was shown to be the result of O-deacylation of its lipid A moiety. Several different approaches were employed to more completely define structural features in commercially available, heterogeneous R-LPS mixtures. This

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376 study has shown that R-LPS can be analyzed on three different mass spectrometers with similar but
377 complementary results. Success of LPS-based drugs in the clinic will be partially dependent on well-
378 defined compositions of LPS extracts, if purification of single compounds or total chemical synthesis
379 prove to be unfeasible, and batch-to-batch reproducibility of LPS production. This will inevitably lead to
380 a better understanding of off-target effects and decrease probability of drug attrition. Reproducibility of
381 immunological studies will also be improved through these efforts by improving quality control and
382 quality assurance guidelines. In the past, this has been an onerous undertaking, but with improved data
383 acquisition efficiency and the ability to acquire more complete data sets, the current rate limiting step is
384 user-friendly, automated software development.

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4 **582 Legends for Figures**

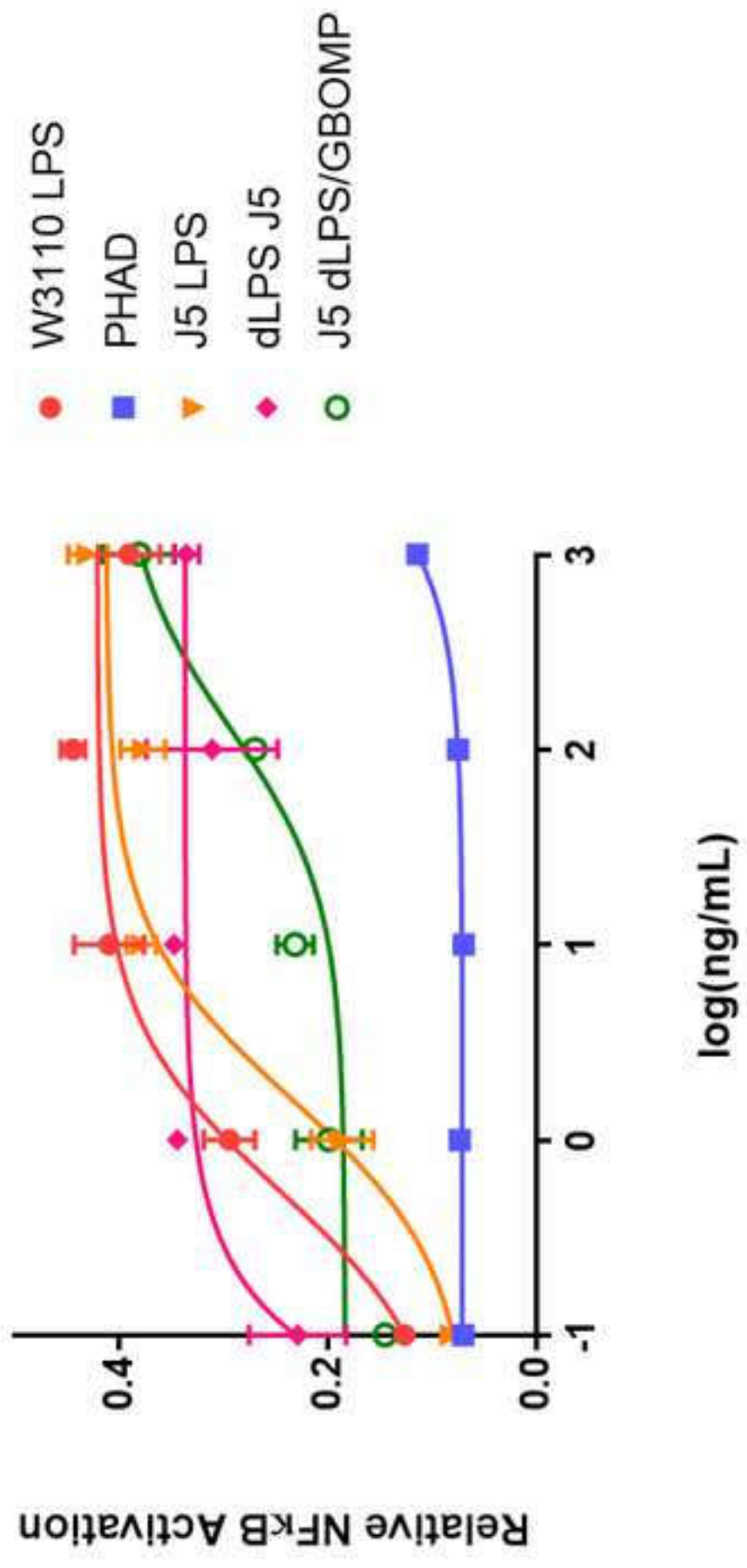
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7 **583 Fig. 1** Agonists were cultured with HEK-Blue hTLR4 cells over a 5-log dose range from 0.1-1000 ng mL⁻¹.
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9 **584** ¹. W3110 *E. coli* LPS (red), J5 *E. coli* LPS (orange), J5 dLPS (pink), J5 dLPS/GBOMP (green), or PHAD
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11 **585** (blue) were incubated for 16 hours. Then NF-κB activation was measured by quantification of SEAP in
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13 **586** the supernatant. Mean ± SD of duplicate samples and an associated 4-parameter non-linear regression are
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16 **587** shown

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19 **588 Fig. 2** Averaged IMS-CID tandem mass spectra of (a) J5 LPS *m/z* 1071 and (b) J5 dLPS *m/z* 789 after
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21 **589** collision energy ramping. Insets show deprotonated fatty acid product ions' presence in (a) and absence in
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23 **590** (b)

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26 **591 Fig. 3** Zoomed negative mode FT-ICR mass spectrum ($R \sim 300,000$ FWHM, in absorption mode) after
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28 **592** direct infusion of J5 LPS. Eight potential isotopic distribution envelopes can be identified in absorption
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30 **593** mode in this 4 *m/z* window; these are denoted, at the *m/z* of their respective monoisotopic ions, with blue
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32 **594** arrows. (inset) Magnified portion of the spectrum showing fine detail (including the magnitude mode and
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34 **595** the proposed overlap between isotopologues from envelopes 2 and 5)

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38 **596 Fig. 4** Comparison of trap CID (blue) and beam CAD (red) for the same precursor ion at *m/z* 1071.
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40 **597** Ninety-nine monoisotopic product ions common to both experiments were observed, fifteen of which are
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42 **598** annotated in the CID mass spectrum with corresponding bond cleavages in the structure on the right. All
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44 **599** product ion *m/z* were measured with less than 100 ppb error

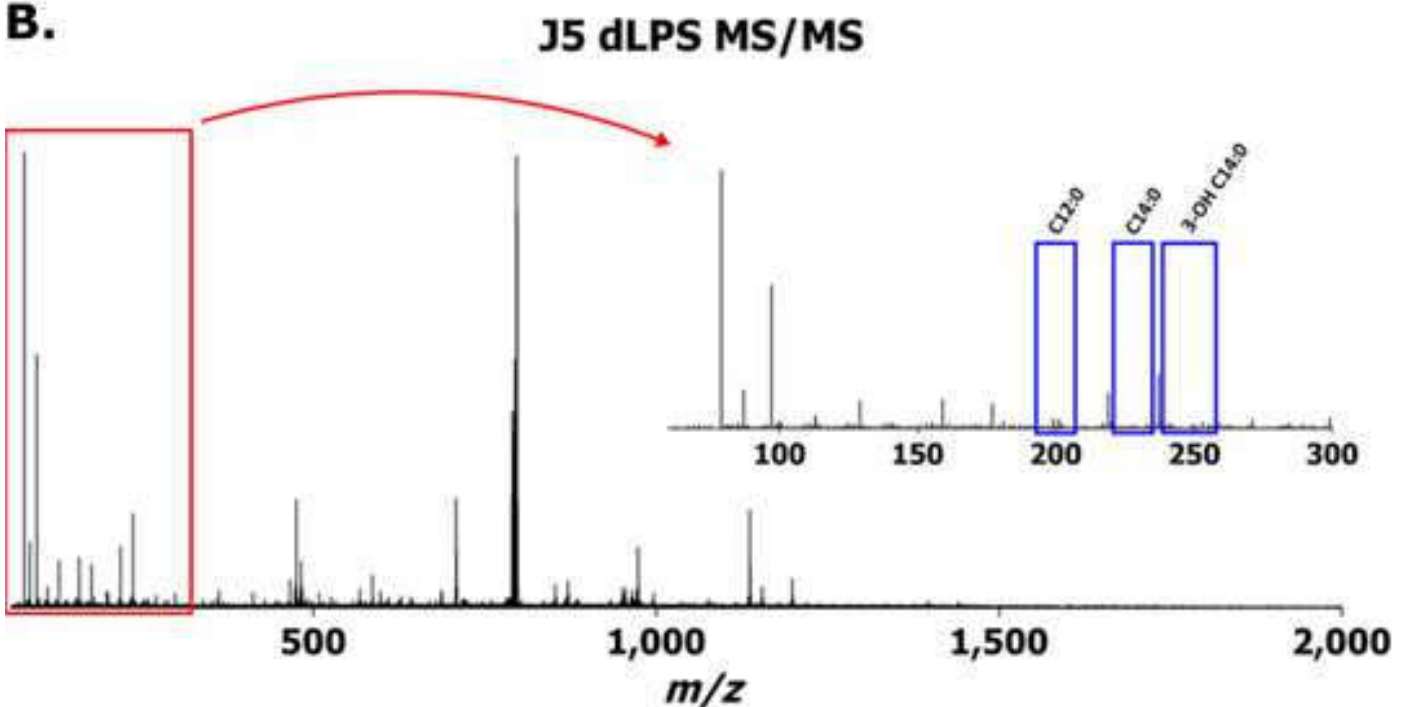
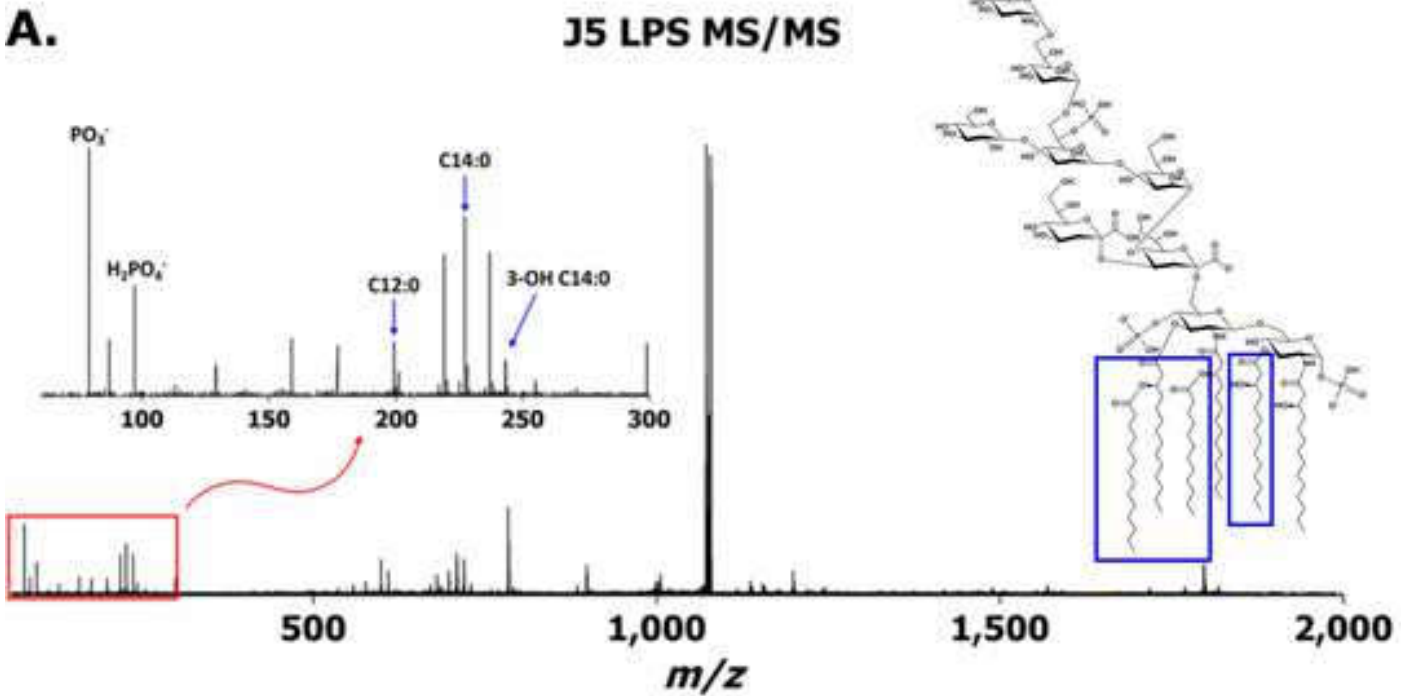
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48 **600 Fig. 5** MS³ CID mass spectra from MS² product ions representing J5 *E. coli* lipid A at *m/z* 1796 (top) and
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50 **601** core OS at *m/z* 1418 (bottom). Similar dissociation phenomena were observed as in MS² experiments for
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52 **602** chemically isolated lipid A and oligosaccharides, indicating feasibility of LPS top down sequencing in
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54 **603** this manner



Relative NFκB Activation

log(ng/mL)

- W3110 LPS
- PHAD
- J5 LPS
- dLPS J5
- J5 dLPS/GBOMP



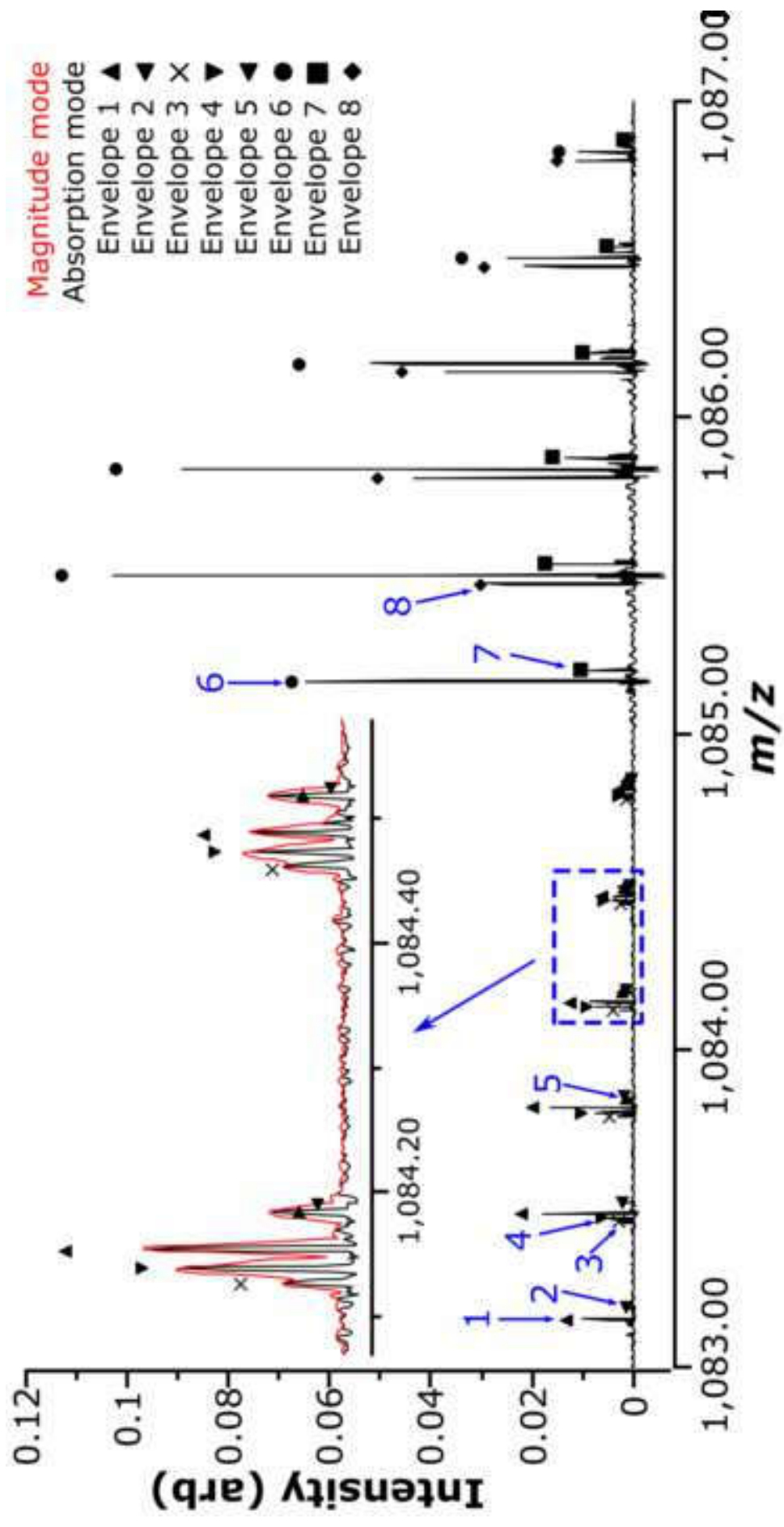
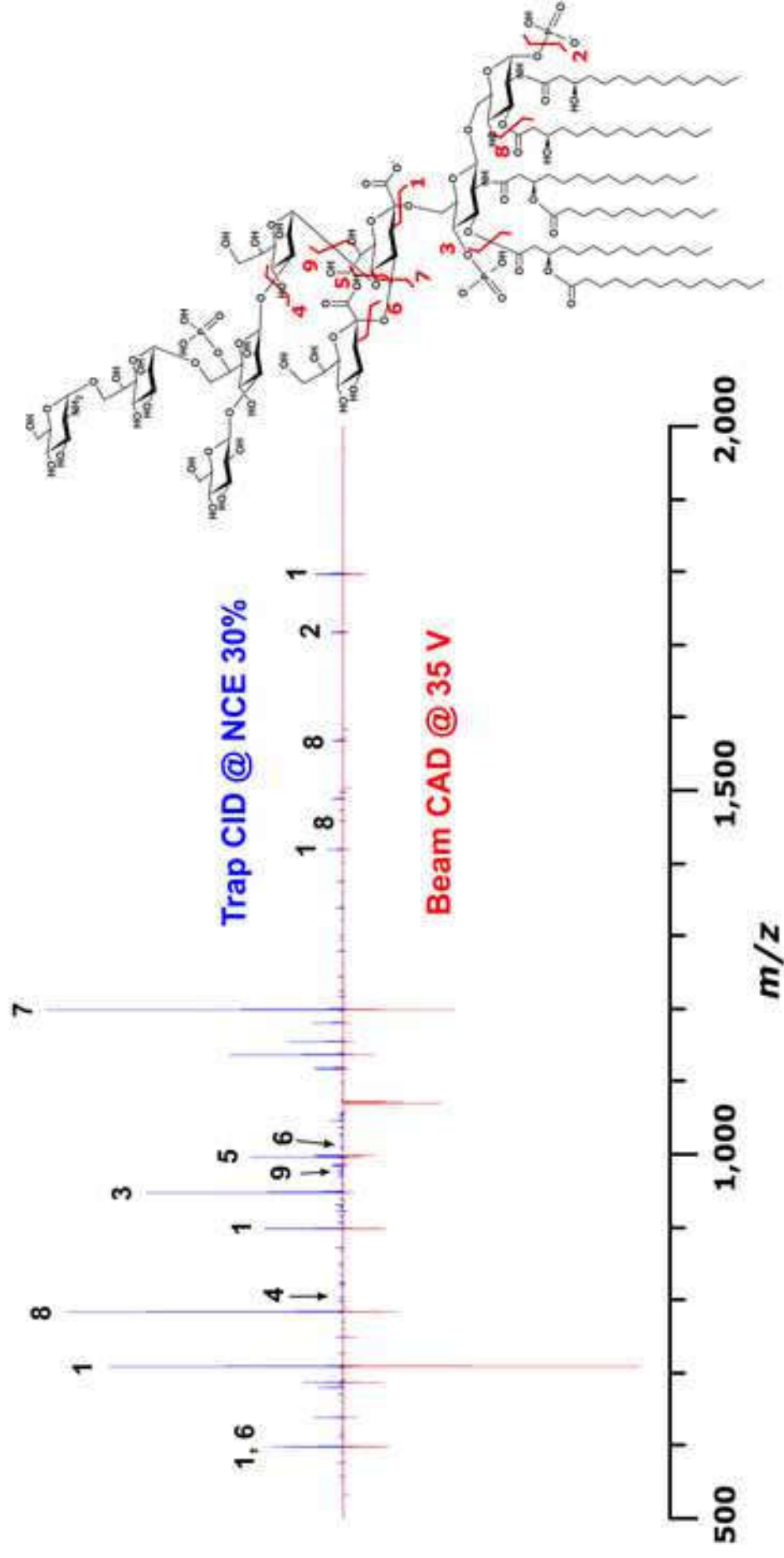


Figure 3



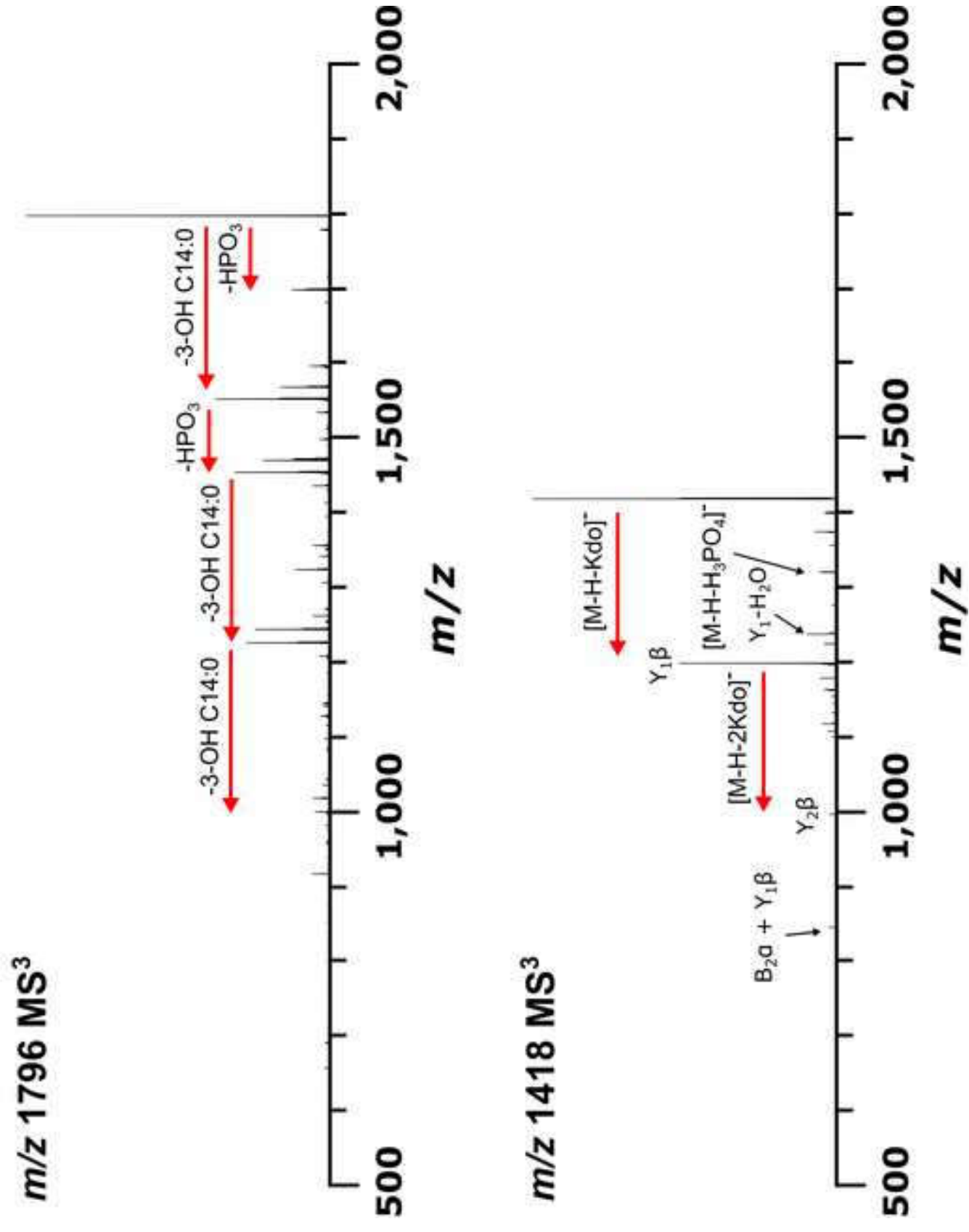


Figure 5