PhD THESIS

University of Pécs

Doctoral School of Chemistry

Electrospray ionization tandem mass spectrometry-based structure elucidation of lipid A molecules

Ibrahim Aissa

Supervisor
Dr. Ágnes Dörnyei

Co-supervisor
Dr. Anikó Kilár

Head of the Doctoral School
Prof. Dr. Attila Felinger

PÉCS, 2023

1. Introduction

Lipid A is a portion of the lipopolysaccharide (LPS; also called endotoxin) layer, which is the main component of the outer membrane of Gram-negative bacteria. It triggers an immune response in mammalian cells.

Over the decades, more and more lipid A variants have been successfully extracted from a variety of bacterial strains to perform structure elucidation and bioactivity profiling. One main reason for this is that understanding the structure and function of the lipid A moiety is a key strategic point in the development of new and innovative drugs, such as vaccine adjuvants or anticancer agents. Several synthetic lipid A standards, such as PHAD, PHAD-504, 3D-PHAD, and 3D-(6-acyl)-PHAD) (Fig. 1) are used as adjuvants in many commercialized human vaccines.

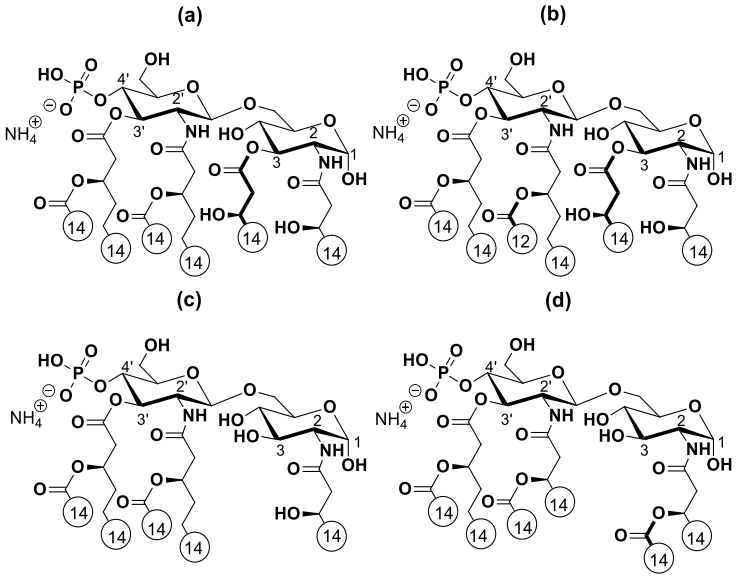

Figure 1. Structures of the four synthetic lipid A standards: (a) PHAD, (b) PHAD-504, (c) 3D-PHAD, and (d) 3D(6-acyl)PHAD. The bold structural parts indicate the differences between the four structures.

The lipid A portion is mostly composed of a disaccharide containing D-glucosamines phosphorylated at position C-1 and/or C-4′ and acylated at positions C-2, C-3, C-2′, and C-3′. Four or less acyl chains attached directly to the glucosamine sugars are beta hydroxy acyl

chains usually between 10 and 16 carbons in length. Additional acyl chains are often attached to the beta hydroxy group.

Nowadays, tandem mass spectrometry techniques are recognized as being among the most potent methods for the sensitive and comprehensive characterization of lipid A. Depending on the experimental conditions used during electrospray ionization mass spectrometry experiments, lipid A can be detected as various molecular species (e.g., deprotonated, protonated and sodium adducts) when analyzed in both negative and positive ionization modes *via* direct introduction or hyphenated mass spectrometry-based methods. Fragmentation rules, described for both negatively and positively charged lipid A molecules, greatly assist in the interpretation of the observed fragment ions. Moreover, energy resolved mass spectrometry may provide an opportunity to differentiate isobaric lipid A species.

Knowing the precise location of phosphate groups and fatty acyl chains in a lipid A molecule is important for understanding immunological properties related to fine structural modifications of this endotoxic substance. However, the complexity of characterizing lipid A isomers from natural mixtures with only negative ESI mode MS/MS necessitate further development of mass spectrometric strategies that can provide more comprehensive and predictable structural information. The negative ion approaches cannot discern phosphorylation isomers and identify chimera mass spectra, which may for example arise during a shotgun mass spectrometry analysis or from co-elution of phosphoisomers during an LC−MS separation.

Information on the structure and unique modification of lipid A is of profound significance for understanding the survival and virulence of pathogens, such as *Pseudomonas aeruginosa*. Analysis of bacterial cell wall constituents for the development of effective antibacterial strategies is crucial.

2. Aims of the study

The topic of my PhD thesis is the in-depth structural analysis of natural and synthetic lipid A using the traditional collision-induced dissociation methodology with different mass spectrometers. We focused on three new areas of interest as follow:

1- The investigation of positively charged lipid A ions for the identification of chimera mass spectra of lipid A isomers by shot-gun mass spectrometry.
   - Study the fragmentation of three positively charged lipid A ions: $[M + H]^+$ (protonated molecule), $[M + Na]^+$ (monosodium adduct), and $[M – H + 2Na]^+$ (disodium adduct) using three mass spectrometers: ESI-Q-TOF, ESI-IT, and ESI-QqQ.
   - Compare results with the commonly detected negatively charged lipid A ions: $[M – H]^–$ (deprotonated molecule).

2- Making suggestion of several dissociation mechanisms of deprotonated, protonated and sodiated forms to understand the fragmentation behavior of lipid A under low-energy collision-induced dissociation.
   - Interpret the formation of fragment ions using possible mechanisms consistent with the principles of reactions described in organic chemistry.

3- Characterization of lipid A isomers from *P. aeruginosa* PAO1 by non-aqueous capillary electrophoresis coupled to mass spectrometry.
   - Demonstrate the confident identification of structurally very similar, isobaric lipid A species in a bacterial extract.

3. Materials and Methods

3.1. Synthetic lipid A standards

Synthetic monophosphoryl lipid A standards were bought from Sigma-Aldrich, the exclusive Avanti Polar Lipids provider (Alabaster, AL, USA) in Hungary. The standards were PHAD, PHAD-504,3D-PHAD,and 3D(6-acyl)-PHAD (Fig. 1).

3.2. Bacterial strains and culture conditions

Bacterial strains of *Escherichia coli* O83 and *Pseudomonas aeruginosa* PAO1 strains were cultured at 37 and 25 °C, respectively, in a laboratory fermentor on a Mueller-Hinton broth at pH 7.2, until they reached the late logarithmic phase (about 10 h). The bacterial cells were collected by centrifugation and dried with acetone.

3.3. Lipopolysaccharide and lipid A isolation

Cell-wall lipopolysaccharides were extracted from the acetone-dried organisms by the hot phenol/water procedure, and were lyophilized. The lipid A part was released from each LPS by mild acid hydrolysis with 1% (v/v) AcOH (pH 3.9) at 100 °C for 1 h. Then, the solution was centrifuged. The sediment, containing lipid A, was washed four times with distilled water and lyophilized.

3.4. Lipid A sample preparation

Approximately 0.1 mg of the synthetic lipid A standards and lipid A extracted from *E. coli* was dissolved in 1 mL of a MeOH:DCM (70:30, v/v) mixture. Next, 5 mg of ammonium formate was added, and the sample was vortexed and then placed in an ultrasonic bath for 5 min. Subsequently, 300 μL of the sample and 700 μL of methanol was pipetted into a sealed glass vial. A small amount of NaCl (about 0.5 mg) was added to promote sodium adduct formation. After vortexing for 1 min, the sample was ready for injection. The lipid A (0.1 mg)

extracted from *P. aeruginosa* PAO1 was dissolved in 200 µL of a MeOH:$CHCl_3$ (50:50, v/v) mixture, vortexed and centrifuged.

3.5. Mass spectrometric analysis

Mass spectra of the four lipid A standards and *E. coli* lipid A were recorded in both negative and positive ion modes using a 6530 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) mass spectrometer (Agilent Technologies, Singapore) equipped with an electrospray ionization interface. The lipid A samples were injected using a UHPLC autosampler (Agilent Technologies, Waldbronn, Germany).

To investigate the genealogy of the fragments from precursor ion to fragments through all fragment generations, ESI-ion trap MS^n analyses were conducted in both ionization modes using an MSD Trap XCT Plus mass spectrometer (Agilent Technologies, Germany) equipped with an electrospray ionization (ESI) interface. A syringe pump was used to inject the samples directly into the ion source.

Fragmentation patterns of the lipid A standards and the natural-sourced *E. coli* lipid A were also studied in the negative-ion mode with a SCIEX Triple Quad TM 6500+ mass spectrometer (AB Sciex LP, Concord, ON, Canada) equipped with an electrospray ionization (ESI) interface and an integrated syringe pump.

3.6. NACE–ESI-Q-TOF MS/MS

Measurements of the *P. aeruginosa* PAO1 lipid A sample were performed with the non-aqueous capillary electrophoresis (NACE) method using a 7100 CE system (Agilent Technologies, Waldbronn, Germany). The CE instrument was connected to the 6530 Q-TOF mass spectrometer.

4. Results and discussion

4.1. Advantage of CID fragmentation pathways of positively charged precursor ions for the complete structural elucidation of monophosphorylated lipid A

By means of pure substances with known structures (Fig. 1), and a well-characterized biological sample from *E. coli*, we explored the advantages of positive ESI mode MS/MS of sodiated adducts ($[M + Na]^+$, and $[M - H + 2Na]^+$) and the protonated molecule in providing structural information of diagnostic importance for monophosporylated lipid A mixtures. The combined analysis of three positively charged precursor ions results in complementary structural information. Typically, cleavages of the protonated lipid A gives information on the acyl linkages at the C-2 primary and C-2/C-2′ secondary positions, and cleavages of sodiated lipid As identifies fatty acids at the C-3′ secondary and C-3/C-3′ primary positions. In addition, assignment of the phosphorylation site (i.e., C-1 or C-4′) is highly facilitated by the fragmentation pattern of $[M + H]^+$ and/or $[M + Na]^+$. Particularly, the B_2 ion is of great importance, as it directly points out the position of the phosphate group in lipid A, while such a distinctive ion is absent in the common negative mode MS/MS mass spectrum of deprotonated lipid A. We found that the CID fragmentation pattern of disodiated lipid A is quite similar to that of the well-characterized deprotonated lipid A molecule (detected in the negative ion mode), while the fragmentation pattern of monosodiated lipid A contains common ion types with those of both protonated and deprotonated lipid A molecules. We have proposed guidelines for the structure elucidation of unknown 4’-monophosphorylated lipid A by the combined analysis of MS/MS mass spectra of positively charged precursor ions**.**

Altogether, the described method may be useful for qualitative analysis of native, heterogeneous lipid A samples using a single (positive) ESI ionization mode in an LC− or CE−MS/MS workflow.

4.2. Mechanistic study on the CID fragmentation pathways of monophosphoryl lipid A

Despite the fragmentation behavior of lipid A is reproducible and predictable, the general descriptions of the underlying mechanisms and dependence of fragmentation on specific linkages are rarely described or addressed. Mechanistic investigation, regarding the fragment ion formation by MS^n, is essential to a better understanding of the ion chemistry and to facilitate the identification of specific product ions, such as cross-ring fragments or those formed by fatty acid eliminations. We have determined the multitude of ions formed during the fragmentation of four synthetic lipid A compounds with structural similarities using tandem in space and tandem in time mass spectrometry with electrospray ionization (ESI), to discover more details about the specific dissociation events and fragmentation routes in both ionization modes.

The MS/MS and MS^n analyses of deprotonated, protonated and some sodiated 4'-monophosphoryl lipid A led to proposals of several alternative dissociation routes that have not been reported previously. Specifically, the hypothesized mechanisms are: (i) cleavage of the C-3 primary fatty acid (as an acid) leaves behind an epoxide group attached to the reducing sugar; (ii) cleavage of the C-3' primary fatty acid (as an acid) generates a cyclic phosphate connected to the nonreducing sugar; (iii) cleavage of the C-2' secondary fatty acid occurs both in acid and ketene forms; (iv) the C-2 and C-2' primary fatty acids are eliminated as an amide and ketene, respectively; (v) the $^{0,2}A_2$ cross-ring fragment contains a four-membered ring (oxetanose); (vi) the $^{0,4}A_2$ ion is consecutively formed from the $^{0,2}A_2$ ion by retro-aldol, retro-cycloaddition, and transesterification; (vii) formations of $H_2PO_4^-$ and PO_3^- are associated with the formation of sugar epoxide; and (viii) formation of the B_1 ion is associated with the formation of a bicyclic sugar derivative. An understanding of the relation between $^{0,2}A_2$ and $^{0,4}A_2$-type sugar fragments and the different cleavage mechanisms of the two ester-linked primary fatty acids is invaluable for distinguishing lipid A isomers with

different locations of a single ester-linked fatty acid (i.e., at C-3 or C-3'). Thus, in addition to a better comprehension of lipid A fragmentation processes in mass spectrometers, our observations can be applied for a more precise elucidation of naturally occurring lipid A structures.

4.3. Structural characterization of isomeric lipid A species from *P. aeruginosa* PAO1 by NACE–ESI-MS/MS method.

We have demonstrated that the NACE method coupled to positive and negative ion (CID)MS/MS is a powerful alternative strategy regarding the site of phosphorylation of the lipid A structures, which makes it a good orthogonal technique to chromatographic characterizations of the bacterial lipid A composition, where the separation is mainly based on acylation differences of the species. Overall, the results of this electrophoretic approach revealed hitherto unreported isomeric monophosphorylated PAO1 lipid A constituents, including both phosphate and acyl chain positional isomers. The parallel fragmentation in the complementary positive and negative ion modes enabled the unequivocal assignment of the phosphorylation site and position of acyl chains in lipid A compounds of three acylation families ranging from tetra- to hexa-acylation. Moreover, we have identified C1-monophosphorylated lipid A species in *P. aeruginosa* for the first time. We have proved that the ions formed in the positive and negative ion CID experiments have complementary roles in the structural elucidation. In summary, NACE followed by the joint application of both positive and negative ion mode CID experiments provided extended structural information of the acylated families of PAO1 lipid A, with regard to the identification of C1 phosphorylated species. In the wider context of the biological significance of lipid A modifications, it is possible that many other Gram-negative bacteria also produce phosphorylation isomers.

5. Thesis points

1. We conclude that the combined analysis with low-energy CID of the $[M + H]^+$, $[M + Na]^+$ and $[M – H + 2Na]^+$ precursor ions allow for the full structural characterization of 4’-monophosphorylated lipid A compounds in natural mixtures of bacterial lipid A. Our approach is capable to distinguishing phosphorylation isomers and identifying chimera mass spectra.

2. There is a high degree of similarity between the fragmentation pattern of lipid A as a disodium adduct and as a deprotonated molecule; meanwhile, the fragmentation pattern of monosodiated lipid A shows similarity with the protonated and deprotonated molecule, as well.

3. New alternative mechanisms explaining the gas-phase dissociation routes of deprotonated, protonated and sodiated 4'-monophosphoryl lipid A species have been suggested, regarding the release of the different ester- or amide-linked fatty acyl chains and the phosphate group, as well as ring cleavages. By applying low-energy CID conditions, mainly charge-induced processes were considered.

4. The NACE method coupled with positive and negative ion (CID)MS/MS revealed hitherto unknown C-1 phosphate positional isomeric compounds among three acylation families (tetra-, penta-, and hexa-acylated species), and thus, it expanded the structural information obtained by chromatographic characterization of the lipid A composition of *Pseudomonas aeruginosa* PAO1 bacterium. Therefore, the NACE–MS/MS strategy using CID fragmentation in the complementary positive and negative ion modes can replace commonly used HPLC–MS/MS approaches for monitoring bacterial lipid A compositions.

6. Acknowledgements

First, I sincerely appreciate **Almighty God** to bless me with the opportunity to pursue my studies in a peaceful and charming country "**Hungary**".

Additionally, I am deeply grateful to my supervisor **Dr. Ágnes Dörnyei**, for her permanent support, scientific guidance, constant optimism and encouragement throughout this long walk with all its “ups and downs”. I consider myself very fortunate for being receiving kindness and respect from her. Without her acceptance to be my supervisor, I would not be able to finish my studies in **Hungary**. Thus, I’m highly indebted to her.

I would like to express my gratitude and appreciation to my co-supervisor **Dr. Anikó Kilár** who made this work possible, she continuously provided encouragement and was always willing and enthusiastic to assist in any way she could throughout the research project. Her positive outlook, cheerful mood and support in my research inspired me and gave me confidence. Her careful editing contributed enormously to the production of the papers.

I am thankful to my colleague **Dr. Viktor Sándor** for his help and contribution in research. I am especially thankful to him for the excellent training as well as to share with me his expertise and competences. We really had a great time during the measurements full of jokes and spectacular moments that we will remember forever.

I am thankful to the former head of our doctoral school **Prof. Dr. Ferenc Kilár** and the head of our department **Prof. Dr. Attila Felinger** for their invaluable advice, continuous support during my PhD study. Their immense knowledge and plentiful experience have encouraged me in all the time of my academic research and daily life.

I would like to express my deepest gratitude to **Prof. Bela Kocsis** for preparing natural lipid A samples.

I also grab the opportunity to thank my best friend **Dr. Sami Chniti** who is one of the few people in my life who has helped me become who I am today. I'm so thankful for this friendship.

I must not forget my friend **Péter Noveczky**, who helped me a lot at the beginning of my journey. I wish him the best for his career and life.

Finally, I appreciate the **Stipendium Hungaricum Scholarship Programme** for funding my doctoral study.

Ibrahim

7. List of publications

Publications related to the PhD thesis

1. Study on the CID Fragmentation Pathways of Deprotonated 4'-Monophosphoryl Lipid A.
   Ibrahim Aissa, Anikó Kilár, Ágnes Dörnyei.
   Molecules **2021**, 26, 5961. (IF: 4.924, Q1)

2. Complete Structural Elucidation of Monophosphorylated Lipid A by CID Fragmentation of Protonated Molecule and Singly-Charged Sodiated Adducts.
   Ibrahim Aissa, Ágnes Dörnyei, Viktor Sándor, Anikó Kilár.
   Journal of the American Society for Mass Spectrometry **2022**, 34(1), 92–100. (IF: 3.262, Q2)

3. Characterization of Isomeric Lipid A Species from *Pseudomonas aeruginosa* PAO1 by Non-Aqueous Capillary Electrophoresis with Positive and Negative Ion Electrospray Tandem Mass Spectrometry.
   Viktor Sándor, Bettina Űrmös**, Ibrahim Aissa**, Ágnes Dörnyei, Anikó Kilár.
   Arabian Journal of Chemistry **2023**, 16(8), 104944 (IF: 6.212, Q1)

Publications not related to this thesis

4. Isocostic Acid, a Promising Bioactive Agent from The Essential Oil of *Inula Viscosa* (L): Insights from Molecular Docking and SAR Analysis.
   Ibrahim Aissa, Vijaykumar D Nimbarte, Afifa Zardi-Bergaoui, Mansour Znati, Guido Flamini, Roberta Ascrizzi, Hichem Ben Jannet.
   Chemistry & biodiversity **2019**, 16(4), e1800648 (IF: 2.03, Q3)

5. GC, GC-MS, and NMR Spectroscopy Integrated Analyses and *In Vitro* Antibacterial, Anticholinesterase, Anti-tyrosinase and Anti-5 lipoxygenase, Potential of *inula viscosa* (L) Root Fractionated Essential Oil.
   Ibrahim Aissa, Mansour Znati, Afifa Zardi-Bergaoui, Guido Flamini, Roberta Ascrizzi, Hichem Ben Jannet.
   South Africa Journal of Botany **2019**, 125, 386-392 (IF: 1.79, Q2)

Oral presentations related to the PhD thesis

1. Strategy for Structural Elucidation of Lipid A: Elucidation of Protonated and Disodiated Molecules using CID based MS/MS and MS^n Methods.
   Ibrahim Aissa, Ágnes Dörnyei, Viktór Sándor, Anikó Kilár.
   33rd International Symposium on Chromatography
   Budapest, Hungary, 2022.

2. Étude des Lipides A Monophosphorylés par Spectrométrie de Masse en Tandem (MS/MS et MS^n) et Exploitation de Nouveaux Processus de Fragmentation par des Calculs de Chimie Quantique Standards.
   Ibrahim Aissa, Anikó Kilár, Ágnes Dörnyei.
   REncontres de Chimie Organique Biologique 18^{éme} edition
   Aussois, France, 2022.

3. Structural characterization of isomeric lipid A species by NACE–ESI-MS/MS method.
   Ibrahim Aissa, Viktor Sándor, Bettina Űrmös, Anikó Kilár, Ágnes Dörnyei.
   Anakon
   Vienna, Austria, 2023.

Poster presentations related to the PhD thesis

4. Lipid-A Foszforilációs- és Acilációs Izomereinek Elválasztása és Szerkezeti Jellemzése NACE-ESI-MS/MS Módszerrel.
   Anikó Kilár, Viktor Sándor, Ágnes Dörnyei, **Ibrahim Aissa**, Béla Kocsis, Ferenc Kilár.
   „METT25" A Magyar Elválasztástudományi Társaság jubileumi konferenciája
   Egerszalók, Hungary, 2021.

5. Prediction of the Fragmentation Pathways of Deprotonated 4'-Monophosphoryl Lipid A by Using low Energy Collision Induced Dissociation (CID) Mass Spectrometry.
   Ibrahim Aissa, Anikó Kilár, Ágnes Dörnyei
   REncontres de Chimie Organique Biologique 18^{éme} edition
   Aussois, France, 2022.

6. Workflow for Structural Elucidation of Protonated and Disodiated Lipid A Molecules using Low Energy CID Tandem Mass Spectrometry.
   Ibrahim Aissa, Ágnes Dörnyei, Viktor Sándor, Anikó Kilár.
   20th International Symposium and Summer School on Bioanalysis
   Pécs, Hungary, 2022.

7. Characterization of Isomeric Lipid-A Species from *Pseudomonas Aeruginosa* by non-Aqueous CE-MS/MS with Collision-Induced Dissociation.
   Viktor Sándor, Anikó Kilár, Bettina Űrmös, **Ibrahim Aissa**, Ágnes Dörnyei.
   20th International Symposium and Summer School on Bioanalysis
   Pécs, Hungary, 2022.

8. NACE-ESI-MS/MS Method for the Separation and Characterization of Phosphate and Acyl Chain Positional Isomers of Bacterial Lipid A.
   Anikó Kilár, Ágnes Dörnyei, **Ibrahim Aissa**, Viktor Sándor.
   20th International Symposium and Summer School on Bioanalysis
   Pécs, Hungary, 2022.

9. NACE-ESI-MS/MS Method for the Separation and Characterization of Phosphate and Acyl Chain Positional Isomers of Bacterial Lipid A.
   Anikó Kilár, Ágnes Dörnyei, **Ibrahim Aissa**, Viktor Sándor.
   APCE-CECE ITP-IUPAC
   Siem Reap (Angkor Wat), Cambodia, 2022.

Poster presentations not related to the PhD thesis

10. Etude de la Composition Chimique et de l'Activité Antibactérienne de l'Huile Essentielle et de ses Fractions Extraite des Feuilles Fraîches d'*Inula viscosa*.
    Ibrahim Aissa, Mansour Znati and Hichem Ben Jannet
    International Gongress Of Applied Chemistry & Environment
    Sousse, Tunisia, 2018.

11. Etude Chromatographique de l'Huile Essentielle des Feuilles d'*Inula viscosa* (L.): Accès à l'Acide Isocostique et sa Valorisation Biologique
    Ibrahim Aissa and Hichem Ben Jannet.
    The Second International Symposium: Medicinal Plants and Materials
    Tozeur, Tunisia, 2019.

12. Etude de la Composition Chimique et des Activités Antibactérienne, Anticholinestérase, Anti-tyrosinase et Anti-5-lipoxygénase de l'Huile Essentielle et de ses Fractions Extraites des Racines d'*Inula viscosa* (L.).
    Ibrahim Aissa and Hichem Ben Jannet.
    The Second International Symposium: Medicinal Plants and Materials
    Tozeur, Tunisia, 2019.

13. Novel 1,2,3-Triazoles Linked Isocostic Acid: Design, Microwave Assisted Synthesis and Evaluation as Antibacterial Agent.
    Ibrahim Aissa and Hichem Ben Jannet.
    Troisième Colloque Maghrébin sur la Chimie Hétérocyclique Monastir, Tunisia, 2019.