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Novel Methods of Chromatography and Mass Spectrometry for Quantitative Investigation of Bacteria-derived Lipopeptides and Their Relationship with Human Disease

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Reza Nemati Josheghani, Ph.D.
University of Connecticut, 2016

Abstract

Diseases such as periodontal disease, chronic inflammatory bowel disease and autoimmune disease are reported to be associated with changes in the bacteria populations in specific locations of the body, particularly the gastrointestinal tract and the oral cavity. Because oral and gastrointestinal microbiomes do not typically invade tissues in the host with chronic inflammatory disease, considerable work has instead focused on virulence factors produced by these organisms and their role in triggering innate immune responses that may promote chronic inflammatory diseases. Toll-like receptors (TLRs) are innate immune receptors that recognize specific structural moieties shared among microbes of different classes. My work has concentrated on developing mass spectrometry (MS)-based technologies to study the structure-function characteristics of a subclass of complex lipids called lipopeptides that engage TLR2. A significant structure-activity relationship is controlling TLR2 binding for lipopeptides and the resulting activation of immune responses. Separation power of chiral liquid chromatography (cLC) and a diastereomeric mixture of isotopically labeled internal standards were utilized to develop a novel method of cLC stable

isotope dilution multiple reaction monitoring MS (cLC-SID-MRM MS) to simultaneously determine structure and amount of stereoisomers of lipopeptides. In the second part of the study, similar MS-based approaches were applied to study the relationship between enzymatic hydrolysis of these lipopeptides and human disease. This work identified a novel function for phospholipase A2 (PLA2) enzymes. The combination of ^{18}O-labeling and MS was used to investigate the hydrolysis reaction. Furthermore, cLC-MRM MS was utilized to investigate stereoselectivity of PLA2 enzymes for the hydrolysis of a specific class of lipopeptides. Overall, this work provides novel methods for structure elucidation and quantitation of bacteria-derived lipopeptides as well as their expression in specific human inflammatory diseases.

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Novel Methods of Chromatography and Mass Spectrometry for Quantitative Investigation of Bacteria-derived Lipopeptides and Their Relationship with Human Disease

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Table of Contents

Chromatographic and Mass Spectrometric-Based Approaches to Study Microbial-Associated Lipopeptides and Their Relationship with Human Disease

List of Figures

List of Tables

List of Schemes

Chapter 1

1 Introduction

1.1 Human microbiome in health and disease

Human body consists of many interacting systems that functionally connect organs, tissues and cellular communities. However, more than 90% of the cells in and on the human body are microbial cells. As a result, the human microbiome must be considered in human biology.(Pflughoeft, Versalovic 2012) The US National Institutes of Health (NIH) launched the Human Microbiome Project as one its Roadmap Initiatives, which cost US$140 million for its completion.(Turnbaugh, Ley et al. 2007)The main aims of the project were to investigate the diversity of human microbiome and to evaluate the relationship between human microbiome and human health and disease.(Blaser, Falkow 2009)

An individual's health status, diet, hygiene and genotype can change the microbiome diversity.(Pflughoeft, Versalovic 2012) Also, it was suggested that the microbiome can be helpful for the human body by preventing the invasion of pathogens,(Medzhitov 2001) shaping our immune response(Medzhitov 2001) and providing nutrients for our body.(Kau, Ahern et al. 2011) On the other hand, they can cause acute or chronic diseases.(Pflughoeft, Versalovic 2012, Wade 2013, Bennett, Dolin et al. 2014) Therefore considerable work has focused on understanding how changes in the human microbiome can be correlated with changes in human health.(Pflughoeft, Versalovic 2012)

Bacteria are the most studied group of human microbiome. Human-associated bacteria are categorized into four phyla, the "big-four": *Actinobacteria, Firmicutes, Proteobacteria* and

Bacteroidetes.(Bik 2009) It is important to understand how the diversity and complexity of human microbiome changes with age. We are born without oral, intestinal and genitourinary bacteria. However, bacterial communities increase their complexity to an adult-like complexity by the end of first year of life.(Pflughoeft, Versalovic 2012) Localized disease such as periodontal disease and also systemic disorders such as autoimmune disease are shown to be related to changes in the bacterial populations found in specific locations in the body.(Tanzer, Livingston et al. 2001, Pflughoeft, Versalovic 2012, Darveau 2010, Scannapieco 2013, Curtis, Zenobia et al. 2011, Honda, Littman 2016) Infectious bacterial pathogens can invade and initiate an immune response, which can lead to disease or can be controlled by our immune system. In addition, microbial pathogens or commensal bacteria can produce virulence factors that interact with the host without direct invasion, formation of invading colonies and/or without elicting a host immune system.(Medzhitov 2001)

1.2 Toll-like receptors

Understanding how the body detects the presence of infectious agents without destroying self-tissues has been an area of intensive investigation that has led to the discovery of toll-like receptors (TLRs).(Chtarbanova, Imler 2011, Medzhitov 2001) TLRs are a class of pattern recognition receptors (PRRs) that can detect constitutive and conserved products of microbes, including lipopolysaccharide, lipoproteins, flagella, RNA and DNA.(Medzhitov 2001) Other pattern recognition receptors are known to exist in host immune cells including NOD receptors and numerous others. The microbial products recognized by PRRs are called pathogen-associated molecular patterns (PAMPs). Innate immunity can differentiate self from non-self when PAMPs are detected by TLRs. The following are characteristic features of the innate immune system. PAMPs are unique to microorganisms and are not expressed by host cells. Some of the metabolic

pathways that produce PAMPs are essential for the microorganism survival and are conserved across many types of organisms. As a result, various species, genera, and phyla of bacteria produce similar products with conserved structural components such as the lipid A in lipopolysaccharide (LPS) produced by all Gram negative bacteria. In contrast, the O-antigen portion of LPS is variable and unique among all species of Gram negative organisms.(Medzhitov 2001) Molecules such as LPS are called molecular signatures of the microbes. In 2011, the Nobel Prize in physiology for medicine was awarded to Bruce A. Beutler and Jules A. Hoffmann for their work on TLRs as the key components in recognition of microbial pathogens by the immune system and to Ralph M. Steinman for his discovery of the dendritic cell engagement of TLRs and how this bridges between innate and adaptive immunity.(Volchenkov, Sprater et al. 2012)

1.2.1 TLRs structure

TLRs are type I transmembrane receptors. They are characterized by an extracellular leucine-rich repeat (LRR) domain and an intracellular Toll/IL-1 receptor (TIR) domain.(Rock, Hardiman et al. 1998) The LRR domain is responsible for molecular recognition. Similarly, a diverse set of proteins that play a role in TLR molecular recognition and signal transduction possess LRR domains.(Kobe, Deisenhofer 1995) The intracellular TIR domain, on the other hand, is the conserved signaling domain of the TLRs, which shares homology with signaling domain of IL-1R family members.(O'Neill, Bowie 2007) The LRR domain is separated from TIR domain by the transmembrane (TM) domain, which spans the cell membrane (Figure 1).(Medzhitov 2001)

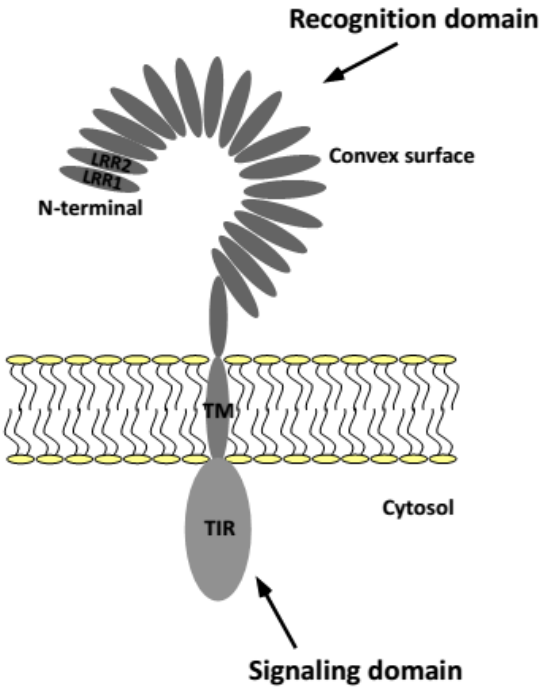

Figure 1. Structure of TLRs. TLRs have three domains: LRR domain that is the recognition domain, the transmembrane domain spanning the cell membrane and the TIR domain that is responsible for intracellular signal transduction.

1.3 Lipopeptides

Lipopeptides are amphiphilic molecules which contain a peptide head-group attached to one or more lipid chains. The peptide head-group of lipopeptides can be cyclic or linear.(Hamley 2015) Natural lipopeptides are commonly produced by bacteria and can be recognized by toll-like receptors (TLRs) that are located on the cell surface of immune cells.(Schenk, Belisle et al. 2009) TLR2 is usually responsible for lipopeptide recognition. When recognizing lipopeptides, TLR2 heterodimerizes with either TLR1 or TLR6 depending on the number of acyl chains within the lipopeptide structure.(Jin, Kim et al. 2007, Kang, Nan et al. 2009) For example, binding of the tri-acylated lipopeptide, Pam3CSK4, mediates the heterodimerization of TLR1 and TLR2 but not

TLR6 (Figure 2.).(Kang, Nan et al. 2009) On the other hand, di-acylated lipopeptides engage TLR6 and TLR2. Due to immunological activity of lipopeptides, there is great interest in using these molecules in vaccine development and antibiotics.

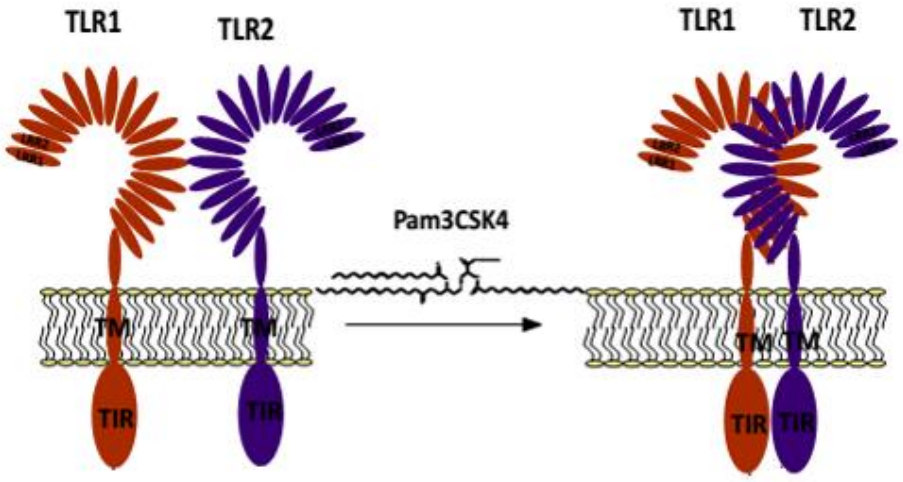

Figure 2. Heterodimerization of TLR1 and TLR2 upon recognition of Pam3CSK4 lipopeptide. Adopted from Jin et al 2009.

1.3.1 Lipopeptides in vaccine development and antibiotics

Bacterial or synthetic lipopeptides constitute very potent and nontoxic vaccine adjuvants and they are replacing more traditional adjuvants such as alum salts as there are concerns regarding alum toxicity.(Moyle, Toth 2008, Ghaffar, Marasini et al. 2016) More importantly, lipopeptides can produce widespread immune responses without using adjuvants. As a result, lipopeptides can offer a new class of self-adjuvant vaccines.(Moyle, Toth 2008, BenMohamed, Wechsler et al. 2002) In fact, lipopeptides as self-adjuvanting lipopeptide vaccine have been tested in human clinical trials.(Moyle, Toth 2008) Furthermore, lipopeptides can act as a new class of antibiotics against drug-resistant bacteria.(Laurens, Bruin 2014, Grau-Campistany, Manresa et al. 2016,

Deshpande, Hurless et al. 2016, Cochrane, Vederas 2016) In summary, lipopeptides are becoming a very important class of peptides for pharmaceutical investigations. Therefore the design, synthesis, and quantitation of these molecules in complex biological matrices is of great importance.

1.3.2 Lipopeptides in activating TLRs

Significant structure-activity relationships dictate the TLR binding following stimulation of innate immune responses by lipopeptides.(Buwitt-Beckmann, Heine et al. 2005, Spohn, Buwitt-Beckmann et al. 2004, Gisch, Kohler et al. 2013, Seyberth, Voss et al. 2006) More importantly, it is suggested that the recognition of lipopeptides by TLRs can be stereoselective.(Takeuchi, Kaufmann et al. 2000, Wu, Li et al. 2010, Muhlradt, Kiess et al. 1998, De Zoysa, Cameron et al. 2015, Chang, Varamini et al. 2015, Konno, Abumi et al. 2015, Yin, Li et al. 2015) As a result, determination of the absolute configurations of the chiral building blocks of the stereoisomers of lipopeptides as well as separation and quantitation of stereoisomers of lipopeptides is required in order to identify new agonists for TLRs, investigate cellular and molecular mechanisms involved in their immunogenicity, and/or to design and synthesize new antibiotics and self-adjuvanting vaccines.(BenMohamed, Wechsler et al. 2002, Takeuchi, Kaufmann et al. 2000, De Camp 1993)

1.4 Mass spectrometry-based approaches for structure elucidation and quantitation of lipopeptides

Mass spectrometry (MS) along with nuclear magnetic resonance (NMR) are the most frequently utilized technologies to determine the structure of lipopeptides.(Iwasaki, Ohno et al. 2015, Gill, Berrué et al. 2014, Naing, Lee et al. 2015, Ma, Hu et al. 2014a, Gisch, Kohler et al. 2013) Tandem mass spectrometry (MS/MS) analysis of lipopeptides provides structural information complementary to NMR results needed for structure elucidation of lipopeptides.

Structure elucidation of lipopeptides generally requires high resolution MS/MS spectra acquired using high resolution MS platforms such as matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF), electrospray ionization-Fourier transform ion cyclotron resonance (ESI-FTICR) or orbitrap mass analyzers.(Vater, Niu et al. 2015, Mnif, Grau-Campistany et al. 2016, Ma, Hu 2014, HÖLTZEL, Schmid et al. 2002, Jiang, Gao et al. 2016, Sharma, Mandal et al. 2014, Ma, Hu et al. 2014b) On the other hand, MS-based approaches for quantitation of lipids and lipopeptides usually utilize ESI-multiple reaction monitoring (MRM) MS using triple quadrupole (QqQ) instruments.(Tsai, Sun et al. 2013, Rubio, Moore et al. 2012, Eom, Kang et al. 2016, Qi, Morena et al. 2015, Li, Hu et al. 2013)

1.4.1 Multiple reaction monitoring for targeted analysis of lipids and lipopeptides

MRM or selected reaction monitoring (SRM) is the gold standard for targeted quantitation of peptides and proteins.(Lange, Picotti et al. 2008, Picotti, Aebersold 2012) MRM was originally applied to the measurement of small molecules and metabolites and upon emergence of the field of lipidomics, MS platforms and data analysis software solutions allow for targeted quantitation of lipids as well.(Lam, Shui 2013, Peng, Ahrends 2015, Guo, Chen et al. 2012, Zweigenbaum, Henion 2000) MRM is particularly useful when a set of known analytes are available and a precise, sensitive and multiplex method can be applied to multiple samples.(Picotti, Aebersold 2012)

MRM utilizes the capability of QqQ mass spectrometer for targeted analysis. The first and third quadrupoles select a specific predefined *m/z* value for the lipid precursor and its corresponding product ions, respectively, and the second quadrupole acts as a collision cell. Each set of precursor-fragment ions is called a transition. In a MRM experiment, several transitions are monitored over time and the number of fragment ions reaching the detectors versus time is recorded (Figure 3).(Lange, Picotti et al. 2008) In order to increase confidence in the specificity

of MRM detection, two to four transitions are monitored for each analyte.(Lange, Picotti et al. 2008) MRM also demonstrates excellent sensitivity due to the non-scanning nature of MRM experiments compared to conventional full scanning methods. The most important advantage of MRM is the capability of monitoring hundreds of transitions in a single experiment. This is particularly important in biomarker discovery pipeline where the biomarker candidates need to be validated with a highly multiplexed method.(Picotti, Aebersold 2012)

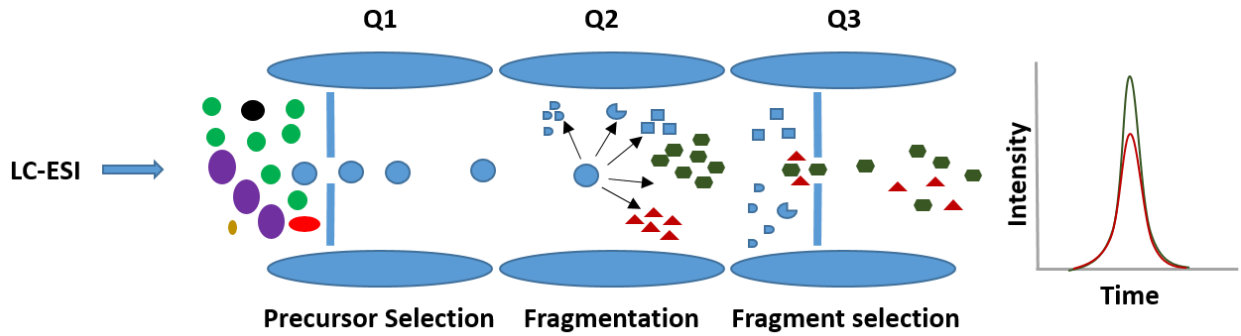

Figure 3. Multiple reaction monitoring technique. Precursor ions that are generated in the ion source enter the first quadrupole. Specific lipid precursor is selected in Q1 and is fragmented in Q2. When two transitions are monitored, only two predefined fragment ions pass through Q3 alternately and hit the detector. Intensity of the two transitions is monitored over the course of an MRM experiment.

1.4.2 Stable isotope dilution for accurate quantitation

MS is not intrinsically quantitative and quantitation of lipids and lipopeptides in complex biological samples requires accurate, sensitive, precise, and reproducible measurements. Several approaches can be employed for relative or absolute quantitation of lipid and lipopeptide molecules. Stable isotope dilution (SID) is the method of choice when accurate measurement is required.(Picotti, Aebersold 2012) LC-SID-MRM MS has shown great promise for targeted quantitation of lipid and lipopeptide molecules in complex biological matrices.(Verdier, Bentué-

Ferrer et al. 2011, Tsai, Sun et al. 2013, Levison, Zhang et al. 2013, Cheng, Liu et al. 2010, Jansson, Karvanen et al. 2009) An isotopically labeled internal standard (IS) provides an almost identical response factor in MS for the analyte of interest and the same retention time in LC, which are required for accurate quantitation.(Yang, Han 2011) IS must be added to the samples as early as possible to correct for experimental and instrumental variabilities. Isotope-labeled IS of known quantity is added into biological samples and quantification is achieved by comparing ion signals from isotope-labeled IS and native lipids or lipopeptides in samples with high specificity and precision.(Keshishian, Addona et al. 2007)

1.4.3 Limitations of mass spectrometry-based approaches for the study of lipid and lipopeptide stereoisomers

Understanding the mechanisms for biological activation by lipids and lipid-modified molecules such as lipopeptides have proven to be difficult as lipids and lipopeptides are unique biological molecules in that they are not typically comprised of smaller subunits as seen with proteins, nucleic acids, or carbohydrates.(Kyle, Zhang et al. 2016) Moreover, lipid and lipopeptide stereoisomers result from small variations in their structure, which lead to unique roles in lipid homeostasis and pathology.(Kyle, Zhang et al. 2016) Therefore, one must be able to differentiate these stereoisomers in order to investigate the physiological roles of these lipids and lipopeptides.(Hullin-Matsuda, Luquain-Costaz et al. 2009) On the other hand, conventional MS, MS/MS or MRM analyses are not capable of differentiating stereoisomers of lipids or lipopeptides. These isomers produce the same MS/MS spectra. Therefore, additional separation based on orthogonal molecular properties to those used in conventional MS and LC is needed to address this analytical challenge.

1.5 Separation of stereoisomers of lipopeptides

1.5.1 Challenges in separation of stereoisomers

Separation of stereoisomers of lipids and lipopeptides with multiple chiral centers is challenging. Reversed phase (RP) and normal phase (NP) LC-MS methods are not able to separate certain stereoisomers.(BenMohamed, Wechsler et al. 2002, Han, Yang et al. 2012). One possibility is the gas-phase separation based on differential ion mobility of stereoisomers inside mass spectrometers.(Jónasdóttir, Papan et al. 2015, Shvartsburg, Isaac et al. 2011, Kyle, Zhang et al. 2016) Another approach is to exploit the chiral property of stereoisomers of lipopeptides and utilize chiral liquid chromatography.(Chen, Yamamoto et al. 2007) Chiral liquid chromatography (cLC) utilizes chiral stationary phases (CSPs) with excellent chiral recognition abilities to separate stereoisomers.

1.5.2 Methods for separation of stereoisomers of lipopeptide

Several methods are available to achieve separation of stereoisomers such as enzymatic kinetic resolution,(Kamal, Azhar et al. 2008, Pellissier 2008) crystallization,(Kinbara 2005, Peng, He et al. 2012) ion mobility mass spectrometry(Jónasdóttir, Papan et al. 2015) and chromatographic separation.(Jibuti, Mskhiladze et al. 2012) Ion mobility mass spectrometry or gas phase separation and chromatographic separation methods are described in details in the next section as these methods were implemented to separate stereoisomers of lipopeptides.

1.5.2.1 Gas-phase separation: Ion mobility mass spectrometry

Ion mobility (IM) MS is an emerging technology that has been applied to lipidomics recently.(Paglia, Kliman et al. 2015) Three contemporary IM techniques which have shown great promise for separation of lipids prior to MS detection include (a) conventional drift time ion

mobility in which separation of ions is based on differential drift down a continuous declining potential field upon introduction into a gas-filled chamber, (b) traveling wave ion mobility whereby ions are separated in a gas-filled chamber using a dynamic traveling potential hill field, and (c) asymmetric field ion mobility or differential mobility spectrometry which separates ions based on their different ion migration orthogonal to the direction of a sweeping gas.(Kliman, May et al. 2011, Paglia, Kliman et al. 2015) In terms of instrumentation, ion mobility is added as a feature to mass spectrometers. In other words, it shares an ion source, mass analyzer and detector within the mass spectrometer. However, contrary to mass spectrometers that function only with high internal vacuum, ion mobility needs a pressurized chamber typically held between 1 and 760 Torr. Separation of ions in IM is related to ions' physical size or so called the ion collision cross section. In an ideal experiment, IM can provide gas-phase separation of ions, which is orthogonal to LC-MS. Therefore, IM data can be considered as a new dimension in LC-MS analysis. Commercially IM-MS instruments are available although they are relatively expensive. SelexION technology or differential mobility spectrometry (DMS) are available in QTRAP 5500 and 6500 series instruments from Sciex, MA3100 system available in Thermo mass spectrometers and SYNAPT G2-Si and Vion IMS QTOF available in the latest versions of IM MS instruments from Waters.

The main use for IM in lipid analysis is to provide complementary analysis in MS for major lipid classes according to their chemical structures in shotgun lipidomics.(Paglia, Kliman et al. 2015, Kliman, May et al. 2011) IM separates ions in the gas phase based on charge, molecular shape, and size, so when coupled with MS, three major benefits to traditional lipidomic approaches can be obtained. First, IM-MS is capable of determining the collision cross section (CCS), which is related to the conformational structure of lipid ions. Therefore, CCS enhances the confidence in

structure elucidation of lipid isomers. Second, IM-MS makes it possible to combine collision-induced dissociation (CID) fragmentation with IM separation to improve the specificity of MS/MS. Third, IM-MS enhances the peak capacity and also signal-to-noise ratio of conventional analytical approaches.(Paglia, Kliman et al. 2015, Basit, Pontis et al. 2016) IM MS can be also exploited for separation of stereoisomers.(Dwivedi, Wu et al. 2006, Giles, Wildgoose et al. 2010, Schneider, Covey et al. 2010, Dong, Shion et al. 2010) Gas phase separation of stereoisomers using conventional drift time ion mobility requires the presence of chiral modifier in the drift tube.(Dwivedi, Wu et al. 2006) However, DMS has been utilized to separate stereoisomers of leukotrienes and protectins without the use of chiral modifier.(Jónasdóttir, Papan et al. 2015) Although IM MS is a promising approach to separate stereoisomers, the accessibility of these instruments is limited due to the high cost of purchase and maintenance. Also, analytical merits of these instruments for quantitation of lipids and lipopeptides in complex biological matrices is yet to be determined.

1.5.2.2 Chiral liquid chromatography separation of stereoisomers

Direct chiral separation using chiral stationary phases (CSPs) for high-performance liquid chromatography (HPLC) is recognized as the most reliable tool for separation of stereoisomers, which will be discussed in detail below.(Aboul-Enein, Ali 2003, Ribeiro, Maia et al. 2014, Nageswara Rao, Guru Prasad 2015) Although chiral separations can be achieved using chiral mobile phases for HPLC, the application of chiral mobile phases is limited.(Hare, Gil-Av 1979) Direct chiral separation using chiral mobile phases has some disadvantage: a) chiral additives are usually expensive or have to be synthesized, b) the instrument operation can be complicated and c) downstream sample preparation is required as the chiral additives must be removed after separation. In addition to HPLC, supercritical fluid chromatography (SFC) has received increasing

attention.(Kalíková, Šlechtová et al. 2014) SFC has advantages of high resolution, low organic solvent consumption and short analysis time.(Abbott, Veenstra et al. 2008, Petersson, Markides 1994) SFC has been used recently for chiral separation of stereoisomers of pharmaceutical and natural products.(Qiao, An et al. 2014, Miller 2012) Due to its high efficiency, speed and success rate in chiral separation, SFC surpasses HPLC in some drug development projects.(Abbott, Veenstra et al. 2008)

The separation mechanism for CSPs is the formation of diastereomeric complexes with different energies on the CSPs (the three-point interaction model). Hydrogen bonding, π-π interactions, dipole-dipole interactions, steric hindrance, hydrophobic interactions, and electrostatic interactions are the main interactions utilized in CSPs in order to separate stereoisomers.(Ikai, Okamoto 2009) Depending on the type of CSPs used for HPLC separations, one or more of interactions mentioned above play a role in chiral recognition and separation.

Stereoisomer separation ability of CSP can be quantitatively evaluated using three factors: retention factor (k), separation factor (R), and resolution factor (Rs), defined as follows:

$$k1 = \frac{(t1-t0)}{t0} \qquad (1)$$

$$k2 = \frac{t2-t0}{t0} \qquad (2)$$

$$\alpha = \frac{k2}{k1} \qquad (3)$$

$$Rs = \frac{2\times(t2-t1)}{W2+W2} \qquad (4)$$

Where t_0, t_1 and t_2 are retention times for the non-retained compound, first-, and second-eluted stereoisomers respectively, and k_1 and k_2 are retention factors. W_1 and W_2 are peak widths at the

bases and R_s is the chromatographic resolution. Retention factor k manifests the degree of interaction between stereoisomers and the CSP and R_s corresponds to the recognition ability of a CSP and theoretical plate number of a column. The R_s value is related to the energy difference ($\Delta\Delta G$) in the interactions between the CSP and an individual stereoisomer and the relation is defined as follows:

$$(\Delta\Delta G) = -RT \ln R_s \qquad (5)$$

A suitable CSP should provide a sufficient $\Delta\Delta G$ so that a successful separation is achieved. Usually, the baseline separation of stereoisomers corresponds to an R = 1.20, which requires ($\Delta\Delta G$) = -0.46 kJ/mol.(Ikai, Okamoto 2009)

CSPs for HPLC can be prepared using a wide variety of compounds and polymers. One type of CSPs that use small molecules: amino acids,(Davankov 2003, Bocian, Skoczylas et al. 2016) cyclodextrins,(Muderawan, Ong et al. 2006, Li, Wei et al. 2016, Xiao, Ng et al. 2012, Hyun 2016) crown ethers,(Hyun 2005, Hyun 2016) π-basic or π-acidic aromatic compounds,(Pirkle, Finn et al. 1981, Kacprzak, Lindner 2011) macrocyclic glycopeptide,(Ilisz, Berkecz et al. 2006, Barhate, Wahab et al. 2015, Ilisz, Grecsó et al. 2015) and cinchona alkaloid.(Hoffmann, Laemmerhofer et al. 2007, Lajkó, Orosz et al. 2016) The other type is polymer-based CSPs that use either synthetic or natural chiral polymers. Examples of synthetic polymers are polymethacrylates,(Okamoto, Honda et al. 1981, Dong, Zheng et al. 2015) molecularly imprinted polymers,(Maier, Lindner 2007, Blaschke 1986, Dong, Zheng et al. 2015) polyacrylamide,(Blaschke 1986, Ciogli, D'Acquarica et al. 2010) and poly(maleimide).(Gao, Isobe et al. 2007) Proteins(Millot 2003, Bocian, Skoczylas et al. 2016, Liang, Wang et al. 2014) and polysaccharides(Ikai, Okamoto 2009, Shen, Ikai et al. 2014) are also natural polymer CSPs. These

polymers are usually coated on silica gel to enhance their mechanical strength and resolution efficiency.(Ikai, Okamoto 2009)

1.5.2.2.1 Polysaccharide chiral stationary phases

Polysaccharide CSPs are the most popular CSPs for HPLC.(Chen, Yamamoto et al. 2007, Chankvetadze 2012) In order to improve chiral recognition of polysaccharides, extensive studies on the modification of these CSPs started in 1980s.(Chankvetadze 2012, Francotte, Wolf et al. 1985, Koller, Rimböck et al. 1983) Modified cellulose and amylose CSPs have shown particularly exceptional chiral recognition ability.(Chen, Yamamoto et al. 2007, Ikai, Okamoto 2009) Among the various polysaccharide derivatives, esters and carbamates have been studied intensively and shown excellent chiral recognition.(Chankvetadze 2012) For example, cellulose and amylose phenylcarbamate derivatives coated on silica are commercially available chiral selectors (Figure 4).

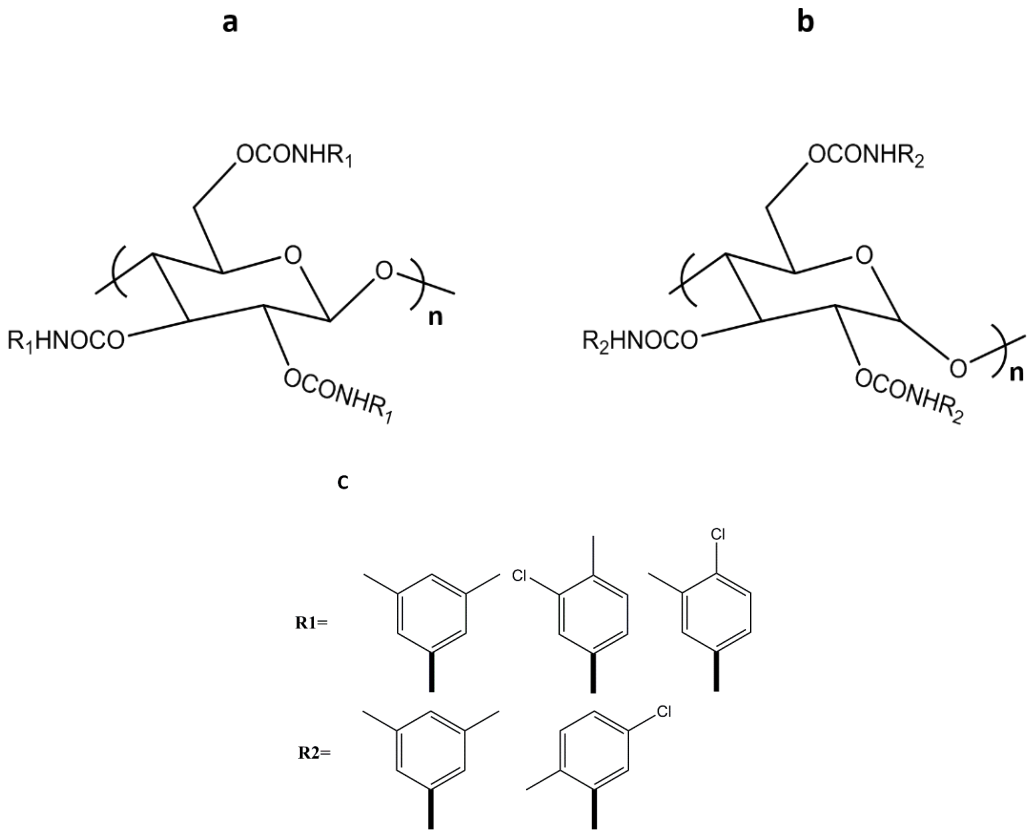

Figure 4. Structures of phenylcarbamate derivatives of polysaccharides. Structure of (a) Cellulose and (b) amylose carbamate derivatives. (c) R1 and R2 are phenyl groups that are modified with either methyl or chlorine groups. Thicker lines indicate bonds where phenyl groups are attached to carbamate group of CSP.

Recognition ability of phenylcarbamate polysaccharide CSPs changes with substituents on the phenyl group. Cellulose phenylcarbamate (Figure 4a) with electron-withdrawing substituents, such as halogens, or electron-donating substituents, such as alkyl groups, show better chiral recognitions compared with the non-substituted cellulose derivatives. These substituents perturb the electronic property of the carbamate group via inductive effect. Therefore, the interaction mode between cellulose derivatives and chiral analytes is affected. For example, the acidity of the NH group of the carbamate group increases when electron-withdrawing substituents are introduced

and the retention time of acetone increases.(Ikai, Okamoto 2009) This is because the hydrogen-bonding interaction between acetone and NH group increases. Chiral recognition of phenylcarbamate derivatives also depends on the position of substituents on the phenyl group.(Chankvetadze, Yashima et al. 1994) Phenylcarbamate derivatives with high recognition ability form lyotropic liquid crystallinity in highly concentrated solution under a polarizing microscope when they are cast from a solution. However, some positions for substituents do not form such a liquid crystalline phase, which is believed to be important for efficient chiral recognition.(Okamoto, Kawashima et al. 1986)

For amylose phenylcarbamates (Figure 4b), similar to cellulose phenylcarbamates, introduction of electron-donating or electron-withdrawing substituents such as methyl or chlorine on the phenyl groups improves chiral recognition ability of the corresponding amylose CSPs.(Ikai, Okamoto 2009) Differences in chiral recognition based on the nature and position of substituents, however, are observed between cellulose and amylose phenylcarbamates. For example, 5-chloro-2-methylphenylcarbamate, ortho position of the substituents, exhibits a relatively higher recognition ability for amylose phenylcarbamates than cellulose phenylcarbamates.(Chen, Yamamoto et al. 2007)

1.5.2.2.2 Chiral recognition mechanism of polysaccharide derivatives CSPs

NMR spectroscopy, x-ray and computational methods have been used to elucidate the mechanism of chiral recognition by polysaccharide CSPs.(Ikai, Okamoto 2009) High-ordered helical structures of the phenylcarbamates of cellulose and amylose is the primary factor determining chiral recognition. It is suggested that phenylcarbamates of cellulose and amylose have the left-handed 3/2 and 4/3 helical conformations, respectively.(Steinmeier, Zugenmaier 1987, Vogt, Zugenmaier 1983) The different substituent effects on their chiral recognition may

also be partially due to differences in their helical structures. Glucose residues are regularly arranged along the helical axis, and polar carbamate groups surround a chiral helical groove, which exists along the main chain of polysaccharide. Polar carbamate groups are residing inside whereas hydrophobic aromatic groups are extending outside of the polymer chain.(Ikai, Okamoto 2009) Hydrogen bonding interactions between the racemate and NH or C=O groups of phenylcarbamate or π–π interactions between phenyl groups of phenylcarbamates and an aromatic group of a racemate on the chiral groove of CSP may play a role in chiral discrimination.(Chen, Yamamoto et al. 2007)

1.6 Dissertation objectives

1.6.1 Development of a method of cLC-SID-MRM MS for separating and quantifying stereoisomers of lipopeptides in biological samples

This investigation presents a novel LC-MS method for separation and quantitation of stereoisomers of lipopeptides. Synthetic (3R,S)-(15-methyl-3-((13-methyltetradecanoyl)oxy)hexadecanoyl)glycyl-L-serine) stereoisomers abbreviated as (R,S)-Lipid 654 (Figure 5), an agonist for TLR2, are used as model compounds for the development of a novel cLC-SID-MRM MRM method. The cLC method and column selection are carried out using a functional group index based on hundreds of chiral compound screens. Three cellulose and amylose chiral polysaccharide columns are chosen. A synthetic deuterated form of (R,S)-Lipid 654 named as (R,S)-Lipid 654-D9 is used as IS for quantification of Lipid 654 stereoisomers in bacterial samples. MRM transitions for Lipid 654 and IS are selected based on their MS/MS spectra using high resolution mass spectrometers. CE and DP voltages for the transitions are optimized. (R,S)-Lipid 654 stereoisomers are separated with Lux-4 column, and are quantified

simultaneously using separated Lipid 654-D9. Calibration curves are generated for both isoforms of Lipid 654 using serially diluted IS added to bacterial lipid extracts. Total lipid extract of nine bacteria strains are spiked with a known amount of IS and analyzed using the developed cLC-SID-MRM MS. These results are compared with RP LC-SID-MRM MS analysis of the same samples in order to validate the results obtained by the cLC-SID-MRM MS method.

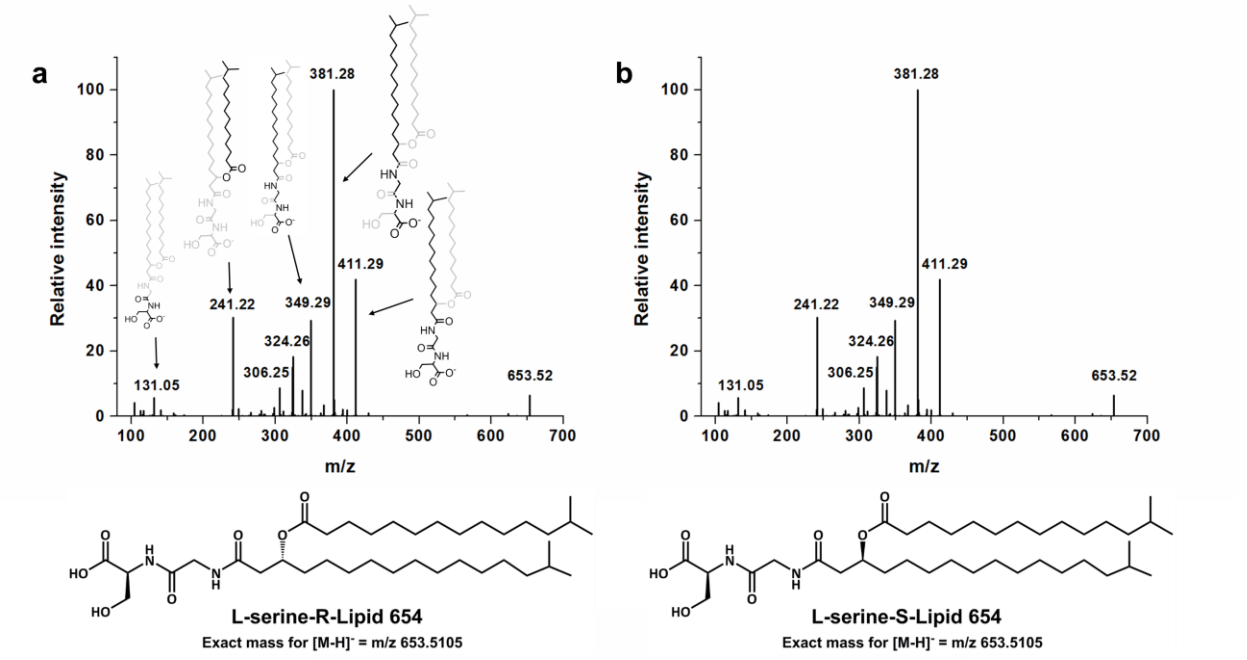

Figure 5. Structure of synthetic R- and S-Lipid 654 stereoisomers and their MS/MS spectra. (a) Both isoforms of Lipid 654 have L-serine, but the other chiral center on the fatty acid moiety (circled in red) can have R or S configurations. (b) MS/MS spectra for R- and S-Lipid 654 stereoisomers; CE -40 V was used for both isoforms.

It has been shown that the recognition of lipopeptides by TLRs can be stereoselective. Therefore, there is a need to develop analytical methods to determine the absolute configurations of chiral constituents of lipopeptide stereoisomers. Furthermore, separation and accurate, precise

and sensitive quantification of these stereoisomers can accelerate the identification of new agonists for TLRs, investigation of cellular and molecular mechanisms involved in immunogenicity, or design of new antibiotics, vaccine adjuvant or self-adjuvanting vaccines. The method of cLC-SID-MRM MS offers several advantages. Using a racemic mixture of isotopically labeled ISs of Lipid 654 and the separation power of cLC, we simultaneously determined the stereochemistry and accurately quantified the amount of Lipid 654 stereoisomers in biological matrices. Also, MRM MS analysis offered multiplexed and sensitive analysis of stereoisomers when coupled with cLC-SID. This robust method doesn't require costly and high maintenance instrumentation of IM-MS.

1.6.2 Investigation of the relationship of human disease with Lipid 654 and Lipid 430

Preliminary data from NP LC-MRM MS analysis of bacteria samples as well as human serum and tissue samples suggests the presence of another lipopeptide molecule related to Lipid 654. Mass of the new lipopeptide is 430 and thus the new molecule is accordingly named as Lipid 430 (Figure 6). Lipid 430 has a similar structure as Lipid 654, but lacks the ester-linked $C_{15:0}$ fatty acid. We aim to compare the level of Lipid 430 and Lipid 654 in bacteria samples as well as in various biological samples LC-SID-MRM MS.

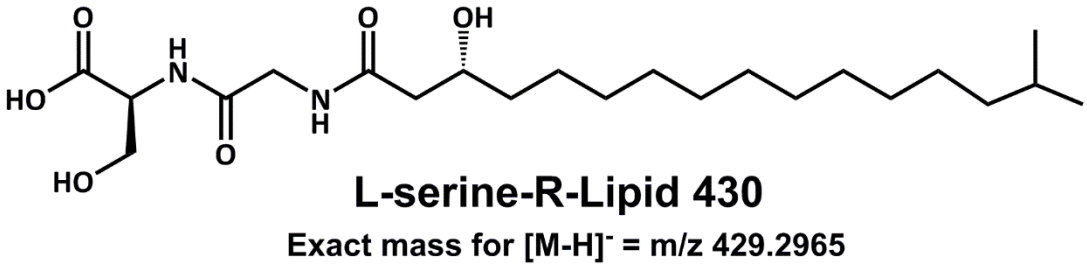

Figure 6. Structure of Lipid 430. Lipid 430 lacks ester-bound $C_{15:0}$ fatty acid compared to Lipid 654.

Previous results also showed that the level of Lipid 430 compared to Lipid 640 varies in bacteria and tissue samples. We also investigated whether changing relative levels of Lipid 654 and Lipid 430 in tissue samples can be due to metabolic conversion (hydrolysis) of Lipid 654 to Lipid 430 and whether this hydrolysis is due to increased activity of specific esterase enzymes in disease tissues. Lastly, racemic Lipid 430 and Lipid 654 are synthesized and cLC-MRM MS is utilized to evaluate stereoselectivity of PLA2 hydrolysis of Lipid 654.

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Chapter 2

2. Chiral liquid chromatography and a mixture of isotopic diastereomer internal standards for simultaneous determination of the stereochemistry and quantitation of stereoisomers of lipopeptides in biological samples

2.1 Introduction

Recent advances in mass spectrometry (MS) platforms and data analysis have guided researchers towards new breakthroughs in the regulation of cellular metabolism and in a better understanding of etiology of human disease by using metabolomics and lipidomics approaches.(DeBerardinis, Thompson 2012, Kolovou, Kolovou et al. 2015) Coupling MS to chromatographic separation can reduce matrix effects of complex biological samples. Liquid chromatography-MS (LC-MS) and gas chromatography-MS (GC-MS) are the most applied chromatography-MS tools for analysis of non-volatile and volatile metabolites respectively.(Cajka, Fiehn 2015, Wolfender, Marti et al. 2015) Lipidomics is an important subcategory of metabolomics and can be classified into untargeted or shotgun and targeted approaches.(Xu, Valenzuela et al. 2013, Wang, Wang et al. 2016) Untargeted lipidomics centers on the analysis of all the detectable lipids in biological samples whereas targeted lipidomics is the analysis of predefined groups of lipids. Both untargeted and targeted approaches have their own advantages and limitations.(Cajka, Fiehn 2015) In an untargeted lipidomics analysis more lipid molecules are monitored or quantified, however, more reliable and accurate measurements are achieved using targeted approaches. For example, the ideal approach in quantitative lipidomics is

using isotopically labeled internal standard (IS) of the analyte, which is not practical when untargeted lipidomics is performed due to the excessive number of analytes to be monitored.(Cajka, Fiehn 2015) This is because isotopically labeled ISs are expensive or not available for all the analytes. Also, we deal with unknown lipids as well in untargeted lipidomics.

Lipopeptides can be considered as special subclass of complex lipids or peptides because they contain a cyclic or linear peptide head-group attached to one or more lipid chains.(Hamley 2015, Schneider, Müller et al. 2014, Patel, Ahmed et al. 2015, Mandal, Barbosa et al. 2013, Deleu, Crowet et al. 2014) Natural lipopeptides, commonly produced by bacteria, and synthetic lipopeptides can be recognized by toll-like receptors (TLRs) that are located on the cell surface of immune cells as well as different cell types of periodontium.(Schenk, Belisle et al. 2009, Khakpour, Wilhelmsen et al. 2015, Basto, Leitao 2014, Li, Lee et al. 2013, Mahanonda, Pichyangkul 2007) Bacterial or synthetic lipopeptides constitute very potent vaccine adjuvants without showing the toxicity of more traditional adjuvants such as alum salts,(Moyle, Toth 2008, Ghaffar, Marasini et al. 2016, Kelesidis, Falagas 2015, Ortwine, Sutton et al. 2015) More importantly, lipopeptides can lead to general systemic immune responses without using adjuvants.(Moyle, Toth 2008, BenMohamed, Wechsler et al. 2002, Basto, Leitao 2014) Lipopeptides may also act as a new class of antibiotics against drug-resistant bacteria.(Laurens, Bruin 2014, Baltz 2012, Patel, Ahmed et al. 2015) Hence, lipopeptides are becoming a very important target for pharmaceutical investigations and accurate quantitation of these molecules in complex biological matrices is of a great importance.

Furthermore, significant structure-activity relationships dictate TLR binding of synthetic lipopeptides and subsequent stimulation of innate immune responses.(Buwitt‐Beckmann, Heine

et al. 2005, Spohn, Buwitt-Beckmann et al. 2004, Gisch, Kohler et al. 2013, Seyberth, Voss et al. 2006) It has been shown that the recognition of lipopeptides by TLRs can be stereoselective.(Takeuchi, Kaufmann et al. 2000, Wu, Li et al. 2010, Muhlradt, Kiess et al. 1998) Takeuchi et al. showed that preferentially R-stereoisomer of the mycoplasmal lipopeptide macrophage-activating lipopeptide-2 (MALP-2) activates immune cells through a TLR2- and MyD88-dependent pathway.(Takeuchi, Kaufmann et al. 2000) Also, Wu et al. synthesized analogues of MALP-2 and they showed (R)-diacylthioglycerol analogues are maximally active in reporter gene assays using human TLR2.(Wu, Li et al. 2010) Their results indicated that the stereochemistry of the peptide component of the synthetic lipopeptides is not a critical determinant of the activity. On the other hand, the stereochemistry of lipid moiety of lipopeptides as well as the acyl chain length of ester bound fatty acids turned to be critical for their activity. A minimum of eight carbons for the ester-bound fatty acids was found to be essential, with shorter acyl analogues being inactive. Therefore, developing tools to determine the absolute structure of and quantity of lipopeptide stereoisomers in biological samples is extremely important. These findings are therefore relevant to the identification of new agonists for TLRs, to the investigation of the cellular and molecular mechanisms involved in their immunogenicity, as well as to help design and synthesize new self-adjuvanting vaccines.(BenMohamed, Wechsler et al. 2002, De Camp 1993, Takeuchi, Kaufmann et al. 2000)

Elucidation of complete structure of lipopeptides requires determination of absolute configuration of all chiral building blocks; chiral amino acids and if present, 3-hydroxyalkanoic acid.(Biniarz, Łukaszewicz et al. 2016, Barbaro, Zangrando et al. 2014, Ma, Hu 2014) Lipopeptides can be hydrolyzed into amino acids and fatty acids. Analysis of amino acids can use indirect methods including pre-column derivatization coupled with either GC-MS or LC-

MS.(Huang, Hu et al. 2013, Müller, Fonseca et al. 2014) Chiral LC-MS (LC-MS) and enantiopure standards have been utilized to directly determine the configuration of amino acids(Barbaro, Zangrando et al. 2014, Horak, Gerhardt et al. 2014, Ianni, Pataj et al. 2014, Tsai, Sun et al. 2013) as well as stereochemistry of 3-hydroxyfatty acids that are released upon hydrolysis of lipopeptides.(Gerhardt, Sievers-Engler et al. 2016, Ianni, Pataj et al. 2014) Nuclear magnetic resonance (NMR) spectrometry and MS/MS analysis using high resolution mass spectrometers are complementary techniques for comprehensive elucidation of the absolute structure of lipopeptides.(Gerhardt, Sievers-Engler et al. 2016, Iwasaki, Ohno et al. 2015) While hydrolysis of lipopeptides into amino acids and lipids could be time-consuming, fast and sensitive methods to simultaneously determine stereochemistry of lipopeptides and quantify them is absent in the literature.

Quantitation of stereoisomers of lipopeptides in complex biological samples require accurate, sensitive, precise, and reproducible measurements. Stable isotope dilution multiple reaction monitoring (SID-MRM) using a triple quadrupole mass spectrometer is the gold standard for targeted omics analysis and a promising approach for quantitation of lipids and lipopeptides in complex biological matrices.(Picotti, Aebersold 2012, Keshishian, Addona et al. 2007, Levison, Zhang et al. 2013, Gelhaus, Mesaros et al. 2011, Rønning, Madslien et al. 2015, Tsai, Sun et al. 2013, Verdier, Bentué-Ferrer et al. 2011, Cheng, Liu et al. 2010, Jansson, Karvanen et al. 2009) As mentioned previously, an isotopically labeled IS is a preferred reference for accurate MS quantitation.(Yang, Han 2011) A known quantity of isotope-labeled IS is added to biological samples (a process called stable isotope diluation [SID]) and quantification with high specificity, multiplexing capability, and precision is achieved by comparing MS signals from isotope-labeled and native lipopeptides.(Keshishian, Addona et al. 2007) However, direct application of SID-

MRM MS of stereoisomers of lipopeptides requires prior knowledge of stereochemistry of the target molecules and preparation of stereochemically-pure isotopic IS. These analysis challenges can be overcome by hyphenating appropriate front-end separation with SID-MRM MS.

Separation of stereoisomers of lipopeptides with multiple chiral centers is challenging when using conventional reversed-phase (RP) and normal phase (NP) LC methods.(Laurens, Bruin 2014, Han, Yang et al. 2012) Instead, separation of chiral isomers utilizing different molecular properties from those for RP and NP LC are needed. One possibility is the use of gas-phase separation based on differential ion mobility of stereoisomers.(Shvartsburg, Isaac et al. 2011, Jónasdóttir, Papan et al. 2015a) Utilizing the separation power of ion mobility mass spectrometry (IM-MS) requires having access to these platforms that are not available in most laboratories due to their high cost. Another approach exploits the chiral properties of stereoisomers of lipopeptides and chiral LC.(Chen, Yamamoto et al. 2007) cLC uses chiral stationary phases (CSPs) and can achieve excellent separation of stereoisomers.(Chen, Yamamoto et al. 2007) Many CSPs have been developed based on proteins,(Haginaka 2008, Juvancz, Szejtli 2002) oligosaccharides(Juvancz, Szejtli 2002) and polysaccharides,(Chen, Yamamoto et al. 2007) and optically-active synthetic polymers.(Nakano 2001) for chromatographic separation of stereoisomers.

Polysaccharide CSPs are widely used for chiral separation. In particular, phenylcarbamate derivatives of cellulose and amylose immobilized on silica gel have shown excellent chiral recognition abilities.(Ikai, Okamoto 2009) High-ordered helical structures of cellulose and amylose provide a molecular basis for chiral recognition.(Chen, Yamamoto et al. 2007) Depending on substituents and their positions on the phenyl groups, recognition selectivity of these derivatives can significantly change. Electron-withdrawing and electron-donating substituents such as

chlorine and methyl groups, respectively, exhibit different chiral recognition by changing the electronic property of the carbamate group, which in turn affects the interactions of CSP and stereoisomers.(Ikai, Okamoto 2009) Combining the separation power of cLC and SID-MRM MS, enabled us to develop a robust method to accurately quantify stereoisomers of lipopeptides.

Herein, a novel method of chiral LC-SID-MRM MS, coined as cLC-SID-MRM MS, is presented to simultaneously determine stereochemistry of and quantify the amount of stereoisomers of lipopeptides in biological samples. (3R,3S)-(15-methyl-3-((13-methyltetradecanoyl)oxy)hexadecanoyl)glycyl-L-serine) lipopeptide stereoisomers abbreviated as (R,S)-Lipid 654, which belong to a new class of TLR2 ligands,(Clark, Cervantes et al. 2013) were used as model compounds for method development (Figure 1a). Using in-vitro and in-vivo studies, Clark et al. showed that Lipid 654 isolated from total lipid extract of *P. gingivalis* bacteria engages both human and mouse TLR2. In-vitro experiments showed that Lipid 654 activate TLR2-expressing HEK cells, and this activation was inhibited by anti-TLR2 neutralizing antibody. Also, administration of Lipid 654 to wild type (WT) mice resulted in a significant increase of chemokine CCL2. However, TLR2-deficient ($TLR2^{-/-}$) mice showed no increase upon intravenous administration of Lipid 654.(Clark, Cervantes et al. 2013) These findings were followed by an assessment of Lipid 654 in the human systemic circulation. Farrokhi et al. showed that Lipid 654 routinely gain access to blood circulation. More importantly, the level of Lipid 654 was recovered at significantly lower levels in the serum of patients with MS compared with both healthy individuals and patients with Alzheimer's disease. As a result, Lipid 654 may be an important blood biomarker for multiple sclerosis.(Farrokhi, Nemati et al. 2013) Furthermore, Wang et al. showed that Lipid 654 can promote TLR2-dependent bone loss as is reported in experimental periodontitis following oral infection with *P. gingivalis*.(Wang, Nemati et al. 2015) A diastereomeric mixture of (R,S)-Lipid

662-D_9 was used as quantitation references to simultaneously determine the absolute configuration of the lipid moiety and quantify bacterial L-serine-Lipid 654. This approach is not only cost effective as there is no need to obtain stereoisomerically pure ISs, but also allows for accurate quantitation of both bacterial (R,S)-Lipid 654 stereoisomers in biological samples. cLC-SID-MRM, to our knowledge, is utilized for the first time to quantify stereoisomers of lipopeptides.

2.2 cLC-SID-MRM MS analysis of Lipid 654 stereoisomers

2.2.1 Experimental

2.2.1.1 Research Facilities

All sample preparations were performed in a Biosafety Level 2 laboratory within the Department of Chemistry (R409 and R411, Xudong Yao Lab, Chemistry Building). LC-TOF-MS will be performed using 10ADvp system (Shimadzu) and quadrupole time-of-flight (QTOF) mass spectrometer (QSTAR Elite, Sciex). LC-MRM-MS analysis was performed using the 10ADvp system described above and 4000 QTRAP triple quadrupole mass spectrometer (Sciex). The mass spectrometers are located in the MS facility of the Department of Chemistry (R403).

2.2.1.2 Material and Reagents

KCl, K_2HPO_4 and HPLC solvents were obtained from Thermo Fisher Scientific (Waltham, MA). Tris base, CaCl2, glacial acetic acid, honey bee venom HBV-PLA2 (Apis mellifera, 600-2400 units/mg protein) and diethylamine (DEA) were obtained from Sigma-Aldrich (St. Louis, MO). Direct-Q3 water system (Millipore, Billerica, MA) was used to purify deionized water. Samples were dried using SpeedVac (Savant, Farmingdale, NY), vacuum oven or ultrasonic

cleaner (Fisher Scientific, Waltham, MA), or lyophilizer (Labconco, Kansas City, MO). Incubation of samples was performed on a Thermomixer R (Eppendorf, Hauppauge, NY).

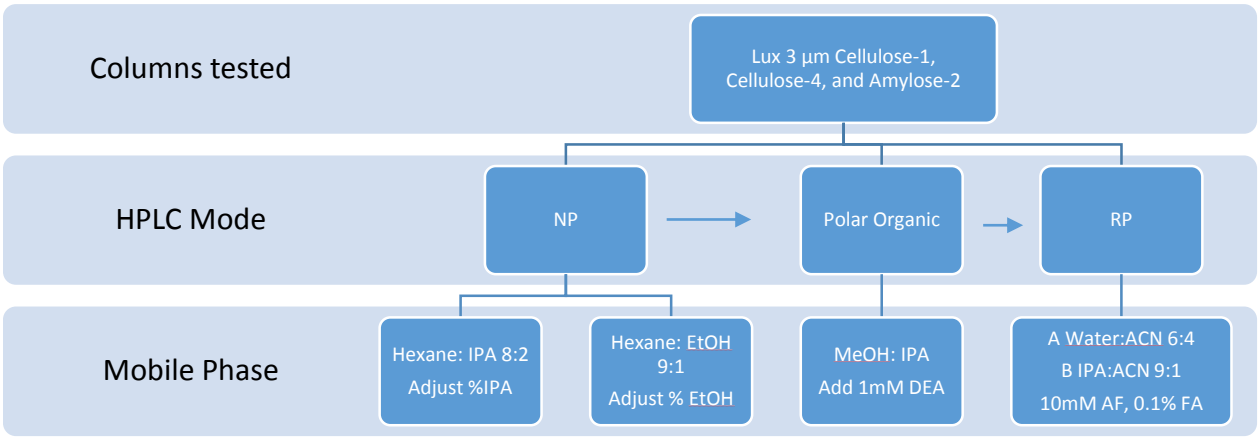
2.2.1.3 ESI-MS/MS and MRM analysis

Tandem MS (MS/MS) spectra of synthetic Lipid 654, Lipid 430 and Lipid 662-D_9 IS (synthesis method to be reported elsewhere) were obtained by direct infusion of lipids at a flow rate of 5 μL/min into a QTOF mass spectrometer, QSTAR Elite from Sciex (Framingham, MA), in negative mode. Key instrument parameters were IS -4500 V, GS1 20, DP -80 V, CE -40 V, FP -380 V, DP2 -10 V, and CAD 5. MRM transitions were selected based on known MS/MS spectra of bacterial lipids, respectively. Lipid samples were directly infused into a triple quadrupole mass spectrometer, 4000 QTrap from Sciex, for CE and DP optimization. MRM negative ion transitions for Lipid 654 were *m/z* 653.5/306.2, 653.5/349.3 and 653.5/381.4, transitions for Lipid 430 were *m/z* 430.2/400.3, 430.3/173.1 and 430.3/382.3. Transitions for IS were *m/z* 662.5/390.5, 662.5/358.4 and 662.5/315.3. Negative ion ESI-MRM MS analysis was carried out at IS −4500 V, FP −350 V, EP −10 V using optimized values of CE and DP.

2.2.1.4 Method development of cLC

cLC method development was carried out as shown in Scheme 1. The method and column selection were performed using a database comprising information of hundreds of chiral compounds and methods that have been used for their separations (For more information one can visit http://www.phenomenex.com). Three columns were tested for separation of stereoisomers of synthetic (R,S)-Lipid 654; Lux Cellulose-4, Lux Amylose-2 and Lux Cellulose-1. For each column, method development was started by trying NP solvent system (Scheme 1). If separation

of Lipid 654 stereoisomers was not achieved, the polar phase and eventually RP solvent systems were tested. For polar organic solvent system, diethylamine (DEA) was used as an additive for mobile phase.



Scheme 1. cLC method development pipeline. Three columns were tested for separation of Lipid 654 stereoisomers. For each column, method development started with NP solvent system. If separation of Lipid 654 stereoisomers was not achieved, polar and RP modes were tested. IPA; 2-propanol, EtOH: ethanol, MeOH: methanol, ACN: acetonitrile, DEA: diethylamine and FA: formic acid.

It is extremely important to condition the column before changing from NP to polar or from polar to RP chromatography. When solvent switching was from NP to polar organic or from polar organic to RP, columns were flushed by 10 time column volume of 2-propanol. When columns were transferred from polar organic to NP, columns were flushed by 10 times column volume of

methanol: ethanol 9:1, v/v. When not being used, columns were stored in hexane:2-propanol 9:1, v/v.

2.2.1.5 cLC of Lipid 654

Synthetic L-serine-(R,S)-Lipid 654 as well as enriched L-serine-R-Lipid 654 and L-serine-S-Lipid 654 diastereomers were dissolved in methanol:2-propanol (9:1, v/v) solvent separately. A HPLC (10ADVP, Shimadzu, Columbia, MD) was used to isocratically separate stereoisomers of L-serine-Lipid 654 using a chiral Lux Cellulose-4 50x 20 mmxmm, 5 μm (Phenomenex, Torrance, CA) at 30°C. Mobile phase was composed of methanol:2-propanol (9:1, v/v) and 1 mM of DEA and the solvent flow rate was 20 μL/min. The column was conditioned with methanol:2-propanol (9:1, v/v) at a flow rate of 0.2 mL/min for at least 20 min before running separations and was kept in hexane:2-propanol (9:10, v/v) after use.

To calculate t_0 (retention time for un-retained eluents) while performing MRM experiments, Qtrap 4000 capability of performing multiple experiments in one run was utilized. Two experiments were defined as below: experiment 1:Q1MS between 100-1200 Da, and experiment 2: MRM for Lipid 654 transitions. LC-MS parameters are similar to the cLC-MRM MS experiment described above.

2.2.1.6 Characterization of (R,S)-Lipid 662-D_9 IS

Solutions of 6.0 pmol/μL of synthetic (R,S)-Lipid 654 and 2.0 pmol/μL of diastereomeric, isotopic ISs in methanol:2-propanol (9:1, v/v) were prepared for analysis using cLC-SID MRM MS, with the same conditions described above.

2.2.1.7 RP LC-SID-MRM MS analysis of bacteria Lipid 654

One hundred micrograms of total lipid extract of nine bacteria strains were dissolved in acetonitrile:2-propanol:water (65:30:5, v/v/v) and were spiked with ISs to a final concentration of 600 fmol/μL. A RP column (XSELECT CSH C18 column 2.1 x 100 mm, 2.5 μm, Waters, Milford MA) was used to separate the total lipid extracts under gradient conditions. Mobile-phase flow rate was 160 μL/min. Solvent A was composed of 60% (v/v) water, 40% (v/v) acetonitrile, 0.1 % (v/v) formic acid and 10 mM of ammonium formate. Solvent B was composed of 90% (v/v) 2-propanol, 10% (v/v) acetonitrile, 0.1% (v/v) formic acid and 10 mM of ammonium formate. A typical running gradient was 35% B at 0 min ➔ 75% B at 4 min ➔ 97% B at 11 min ➔ 35% B at 12.1 min with a 2-minute column equilibration. MRM transitions for both L-serine-Lipid 654 and IS were monitored.

2.2.1.8 Quantitation of bacteria samples using cLC-SID-MRM MS

The same bacteria samples as described in the previous section were dissolved in methanol:2-propanol (90:10, v/v). A chiral column (Lux Cellulose-4 50 x 20 mm, 3 μm) was used to isocratically separate extracted lipids with a run time of 8 minutes. Mobile phase was composed of methanol:2-propanol (90:10, v/v) and 1 mM DEA. Solvent flow rate was 50 μL/min and oven temperature was 30°C. MRM transitions for L-serine-Lipid 654 and ISs were monitored using similar parameters.

2.3 Results and Discussion

2.3.1 Synthesis and MS/MS of stereoisomers of Lipid 654 and stable isotopic stereoisomers as internal standards

All the syntheses mentioned in this section were carrid out by Christopher Dietz in the Dr. Smith's lab at the Chemistry Department of University of Connecticut.(Dietz, Hart et al. 2016) Structure elucidation of bacterial Lipid 654 showed the presence of two chiral centers: one is on the serine amino acid and the other one is on the lipid moiety of Lipid 654 lipopeptide.(Clark, Cervantes et al. 2013) Since chiral GC-MS analysis of acid hydrolyzed Lipid 654 preparations from bacteria revealed the presence of only L-serine, as reported previously,(Muhlradt, Kiess et al. 1998, Clark, Cervantes et al. 2013) only L-serine was used for the synthesis of Lipid 654 and Lipid 662-D_9 IS. For the second chiral center on the fatty acid moiety, racemic 3-OH iso C17:0 was successfully used to prepare synthetic (R,S)-Lipid 654 and (R,S)-Lipid 662-D_9 IS.(Dietz, Hart et al. 2016) The only structural difference that (R,S)-Lipid 662-D_9 has compared to (R,S)-Lipid 654 is that the deuterated ISs have one fatty acid with straight chain and nine deuterium atoms (Figure 1c). Nevertheless, L-serine-Lipid 662-D9 IS shows the same MS/MS spectrum as L-serine-Lipid 654, allowing for accurate quantitation of L-serine-Lipid 654 stereoisomers (Figure 1c). Also, enantioenriched R- and S-Lipid 654 were synthesized utilizing a stereoselective reduction as the key step,(Shioiri, Terao et al. 1998) using a ruthenium catalyst along with (R)-BINAP and (S)-BINAP as chiral auxiliaries, respectively, in 98% ee.(Dietz, Hart et al. 2016) Chemical structure of R- and S-Lipid 654 are shown in Figure 1c and 1d. MS/MS spectra of the synthetic R- and S-Lipid 654 confirmed successful synthesis of the lipid molecules (Figure 1a and 1b). Both R- and S-Lipid 654 show the same MS/MS spectra. Therefore, there are no isoform-

specific precursor-to-fragment transitions available for differential quantitation the R and S isoforms.

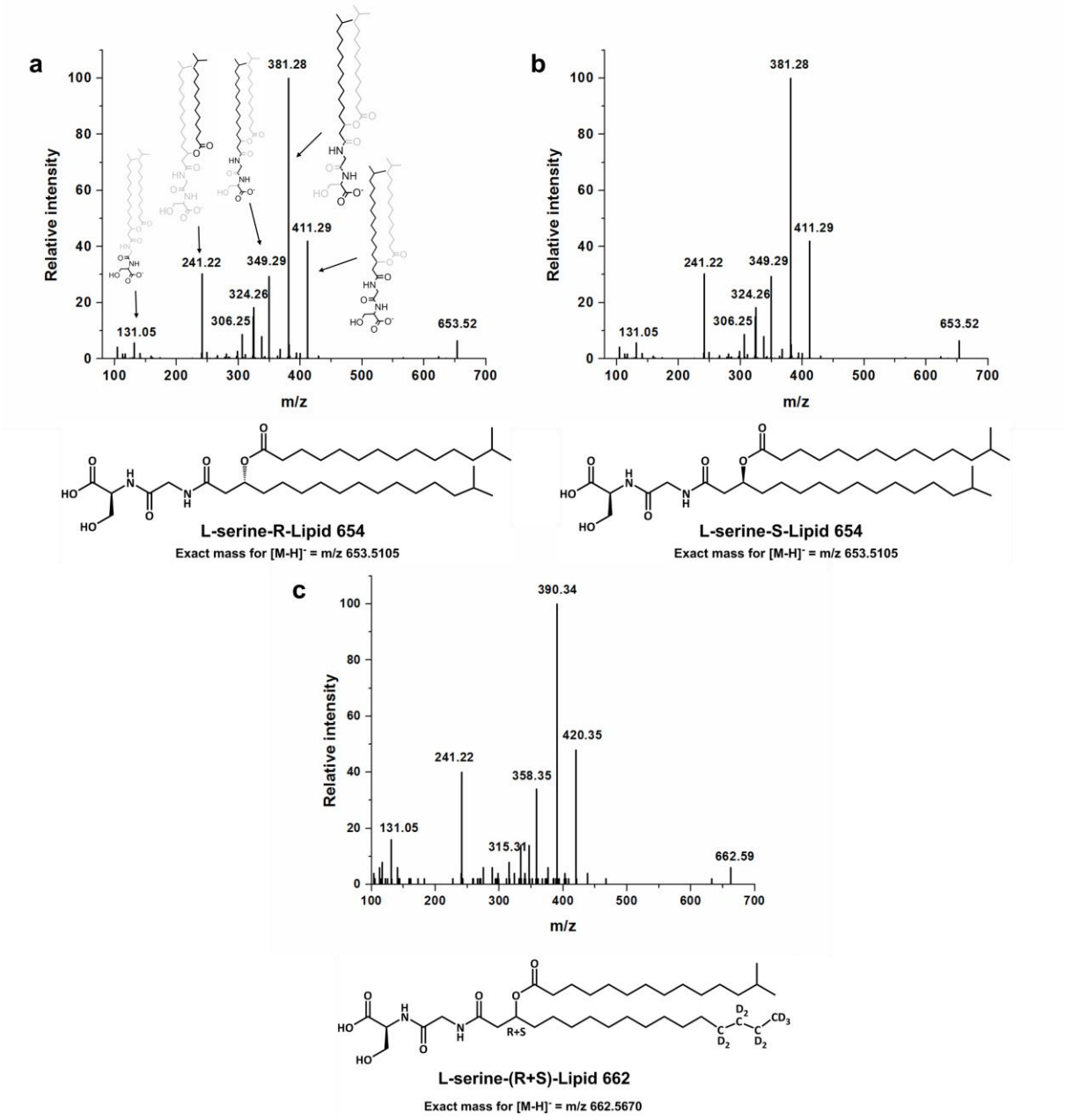


Figure 1. Structure of synthetic R- and S-Lipid 654 and (R,S)-Lipid 662 D_9-IS stereoisomers and their MS/MS spectra. (a) MS/MS spectrum and structure of R-Lipid 654. Structure of the fragments that are used for MRM are shown as well. (b) MS/MS spectrum and structure of S-Lipid 654. (c) MS/MS spectrum

and structure of (R,S)-Lipid 662-D_9-IS. IS also shows a complementary MS/MS pattern. Four fragments with 9 Da mass shifts are observed which corresponds to the fragments that carry deuteriums within the fatty acid.

Since Lipid 654 stereoisomers show identical MS/MS spectra, front-end separation is required to quantify R and S isoforms by MS approaches. Ion mobility spectrometry (IMS) is an emerging technology to separate lipid isomers in the gas phase prior to MS analysis.(Kyle, Zhang et al. 2016, Jónasdóttir, Papan et al. 2015b, Damen, Isaac et al. 2014, Milic, Kipping et al. 2015, Shvartsburg, Isaac et al. 2011) Lipid IMS-MS studies include classification of lipid molecules and distinguishing double bond positions.(Damen, Isaac et al. 2014, Groessl, Graf et al. 2015, Paglia, Kliman et al. 2015) Furthermore, coupling LC and IMS prior to MS analysis is advantageous due to orthogonality of IMS to LC separation. Therefore, IMS increases the overall peak capacity in LC separations.(Kyle, Zhang et al. 2016) LC-IMS MS has been recently exploited for separating stereoisomers of lipids.(Kyle, Zhang et al. 2016, Jónasdóttir, Papan et al. 2015b)

2.3.2 cLC separation of (R,S)-Lipid 654 isoforms

Another possibility for separating stereoisomers of Lipid 654 is to use specific molecular interactions afforded by the stationary phase of cLC. Column selection was based on matching functional groups of Lipid 654 with functional group indices available on the Phenomenex website. These indices are based on hundreds of chiral compounds that are screened. Using this information, selection of candidate columns were narrowed down to three columns: Lux Cellulose-1 with the stationary phase of cellulose tris(3,5 dimethylphenylcarbamate), Lux Amylose-2 with the stationary phase of amylose tris(5-chloro-2-methylphenylcarbamate) and Lux Cellulose-4

with the stationary phase of cellulose tris(4-chloro-3-methylphenylcarbamate) all from Phenomenex, Torrance CA (Figure 2).

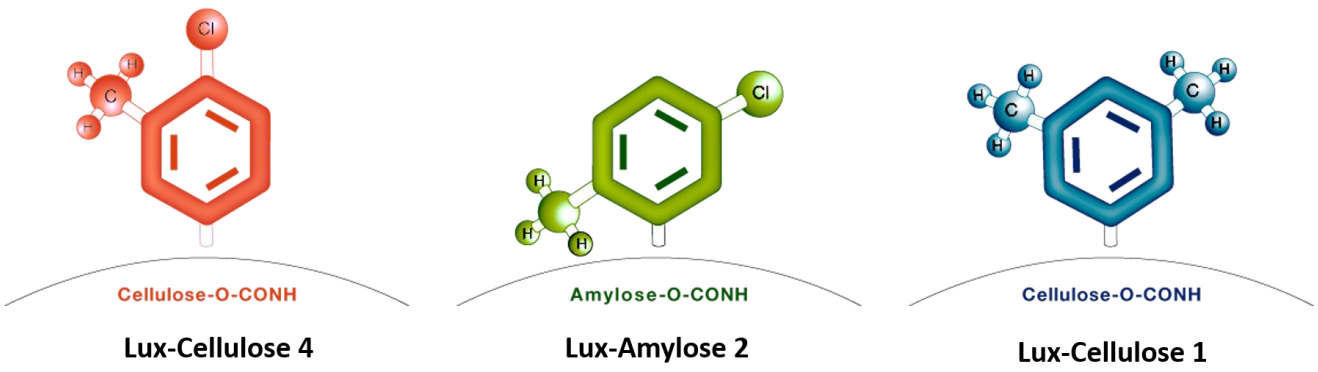


Figure 2. Phenylcarbamate derivatives of cellulose and amylose CSPs used for (R,S)-Lipid 654. (a) Lux Cellulose-4, (b) Lux Amylose-2 and (c) Lux Cellulose-1. (Adopted from Phenomenex website)

Since the mechanism of the recognition is not fully understood, all three columns were tested for (R,S)-Lipid 654 as described in experimental section. Lipid 654 stereoisomers were successfully separated using a Lux Cellulose-4 column (50 x 20 mm, 5 µm) under isocratic conditions. Mobile phase was composed of methanol:2-propanol (9:1,v/v) and 1mM DEA and the mobile phase flow rate was 20 µL/min. The oven temperature was set at 30°C with (Figure 3c).

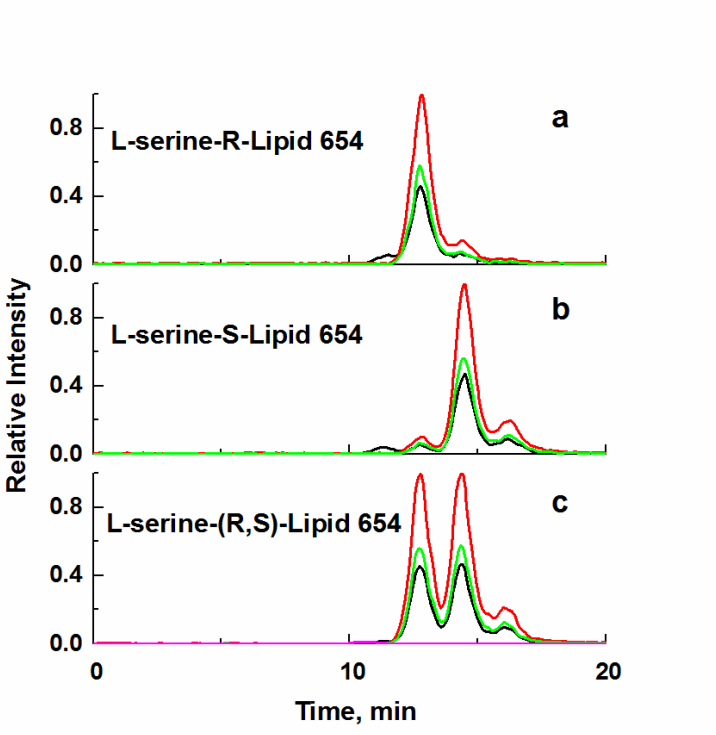


Figure 3. cLC separation of Lipid 654 stereoisomers. cLC-MRM MS analysis of (a) synthetic enantioenriched S-Lipid 654, (b) synthetic enantioenriched R-Lipid (c) synthetic (R,S)-Lipid 654 diastereomeric mixture.

Separation of stereoisomers using selected columns is based on differential interactions of isomers with cellulose and amylose phenylcarbamate derivatives. Chiral recognition for these CSPs significantly changes with type and position of substituents on the phenyl group.(Chen, Yamamoto et al. 2007) Only the Lux Cellulose-4 column achieved successful separation of (R,S)-Lipid 654 stereoisomers and the molecular mechanism behind this separation capacity still requires further investigation. MRM MS was used to detect eluted R and S isoforms. Based the retention times of pure R- and S-Lipid 654 isoforms, the first peak was assigned as R-Lipid 654 and the second peak was assigned as S-Lipid 654 (Figure 3a, 3b and 3c).

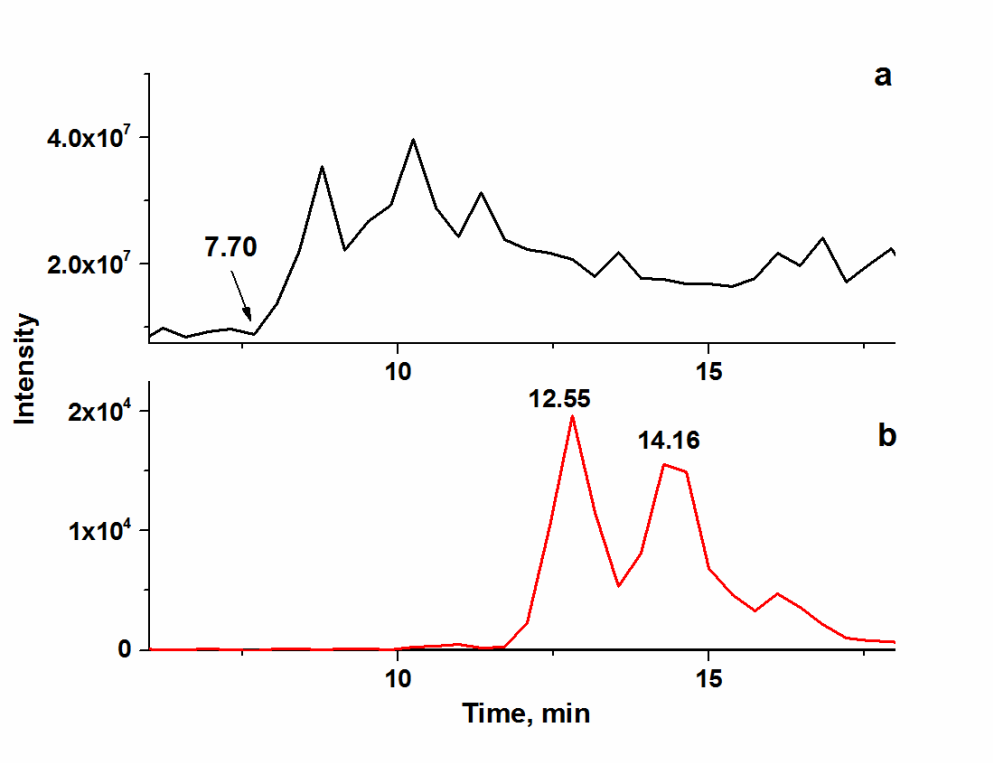


Figure 4. Simultaneous Q1MS and MRM MS experiments using QTrap 4000 upon cLC separation of (R,S)-Lipid 654. (a) Q1MS experiment to calculate t_0 shown by an arrow. (b) MRM experiment monitoring Lipid 654 transitions. Only one transition is shown.

Utilizing simultaneous cLC Q1MS and MRM experiments in one run as described in experimental section t_0 was calculated, which is needed to calculate separation parameters in cLC-MRM MS experiments (Figure 4). Separation parameters of retention time (t_R), retention factor (k), selectivity factor (α) and asymmetric factor of the analytes were thus determined and summarized in Table 1. Analyses were carried out in triplicate.

Peak (3 replicates)	t_R (min)	A	w (min)	k	α	A_s
R-Lipid 654	12.8 (±0.1)	1.7e6 (± 0.1 e6)	1.9 (±0.1)	0.7 (±0.1)	1.3 (±0.1)	1.7 (±0.1)
S-Lipid 654	14.4 (±0.1)	1.6e6 (±0.1e6)	1.9 (±0.1)	0.9 (±0.1)	1.3 (±0.1)	1.6 (±0.1)

Table 1. cLC separation of (R,S)-Lipid 654. T_R is retention, A is peak area, k is retention factor, α is selectivity factor and A_s is the asymmetry factor for each peak.

Both R- and S-Lipid 654 have small amount of the other stereoisomer as the synthesis method resulted primarily in enantioenriched R- and S-Lipid 654 stereoisomers (Figure 2b and 2c). Also, there is a minor peak that eluted from the cLC column after the S isoform. This peak corresponds to the presence of a small amount of anteiso-$C_{15:0}$ fatty acid when Lipid 654 or IS are synthesized. Anteiso-$C_{15:0}$ provides a third chiral center in the molecule and thus the retention time of this stereoisomer is different from (R,S)-Lipid 654 stereoisomers (Figure 3c).

2.3.3 Characterization of (R,S)-Lipid 662-D_9 IS for cLC-SID-MRM MS analysis of (R,S)-Lipid 654

In order to assess the suitability of L-serine-(R,S)-Lipid 662-D9 as a diastereomeric mixture of ISs to accurately quantify stereoisomers of L-serine-Lipid 654, LC-MS/MS characteristics of synthetic L-serine-(R,S)-Lipid 654 and ISs were compared. Accurate quantitation can be achieved provided that LC-MS/MS characteristics of isotopically labeled ISs, including retention times and fragmentation patterns, are similar. As described previously the MS/MS fragmentation pattern of (R,S)-Lipid 662-D_9 is similar to that of Lipid 654 (Figure 1). A

9 Da m/z shift was observed for the fragment ions that contain 3-hydroxy C17:0 heavy fatty acid with nine deuterium atoms. To assess the chromatographic behavior of (R,S)-Lipid 654 and (R,S)-Lipid 662-D_9 IS, LC-MRM MS of synthetic (R,S)-Lipid 654 that was spiked with (R,S)-Lipid 662-D_9 IS was carried out as described in the experimental section. Our cLC-MRM MS resulted similar chromatographic separation, shown in extracted ion chromatogram (XIC), for (R,S)-Lipid 654 and (R,S)-Lipid 662-D_9 (Figure 5 and Table 2). Separation power of cLC and quantitation capabilities of MRM experiments allowed for quantifying both stereoisomers of synthetic Lipid 654 simultaneously.

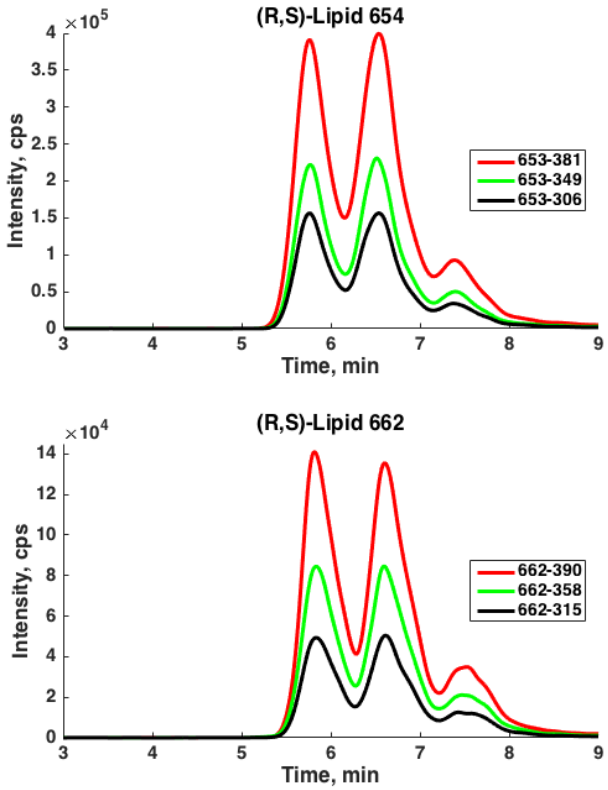


Figure 5. cLC-MRM MS analysis of synthetic (R,S)-Lipid 654 and (R,S)-Lipid 662-D_9.

Percentage of R- and S-isoforms of the IS were calculated and were compared with corresponding synthetic Lipid 654 (Table 2). Utilizing a diastereomeric mixture of ISs and cLC separation of these stereoisomers using Lux Cellulose-4 column, enabled the development of a novel method of cLC-SID-MRM MS method to identify the stereochemistry of bacterial Lipid 654 and quantify its stereoisomers simultaneously if both isoforms are present.

Sample	R-isoform t_R	S-isoform t_R	R-isoform %	S-isoform %
Lipid 662-D_9 IS	5.8 (±0.1)	6.5 (±0.1)	29 (±1)	30 (±1)
Lipid 654	5.9 (±0.1)	6.6 (±0.1)	31 (±1)	29 (±1)

Table 2. Characterization of R-Lipid 662-D_9 as IS for quantitation of (R,S)-Lipid 654. Retention time (t_R) and percentage of each isoform in total lipid calculated using XIC of each Lipid in cLC-MRM MS.

Accuracy and precision for quantitation of both R- and S-Lipid 654 using cLC-SID-MRM MS were calculated and summarized in (Table 3). Solutions of synthetic (R,S)-Lipid 654 with the concentration of 6.0 pmol/μL were spiked with (R,S)-Lipid 662-D_9 with the concentration of 2.0 pmol/μL. Hence, the expected ratio for Lipid 654 to IS was 3. cLC-SID-MRM MS was utilized to quantify R- and S-Lipid 654. Quantitation using R- or S-isoforms of Lipid 654 and Lipid 662-D_9 resulted in very good accuracy and precision, similar to quantitation using the whole peak area of (R,S)-Lipid 654 and (R,S)-Lipid 662-D_9. As a result, cLC-SID-MRM MS can be utilized for quantitation of stereoisomers of lipopeptides with a good accuracy and precision as long as a racemic or diastereomeric mixture of isotopically labeled IS of lipopeptides is available.

Sample	R-654/R-662	S-654/R-662	Lipid 654/Lipid 662
Calculated ratio	2.79	3.03	2.94
% Accuracy	92.9	100.9	97.9
% CV	1.4	1.7	1.0

Table 3. Precision and accuracy of cLC-SID-MRM MS for quantitation of R- and S-Lipid 654.

2.3.4 cLC-SID-MRM MS analysis of bacterial total lipid extracts

The developed cLC-SID-MRM MS method was used to analyze the total lipid extract of bacteria from nine different strains. Three transitions for both Lipid 654 isomers and IS were monitored: 653.5/306.2, 653.5/349.3 and 653.5/381.4 m/z for Lipid 654 and 662.5/390.5, 662.5/358.4 and 662.5/315.3 for IS. All of bacterial strains showed a single peak in XIC of Lipid 654, which co-eluted with the R isoform of IS, indicating the bacteria strains only produce R isoform of Lipid 654 (Figure 6a). As a result, R percentage of IS was used to quantify R-Lipid 654 in bacterial samples using Equation 1.

$$C_{R654} = \frac{\text{Peak area for bacterial R Lipid 654}}{(\text{Peak area for R IS})} \times C_{IS} \times X_{R\,IS} \qquad \text{Equation 1}$$

$$X_{R\,IS} = \frac{\text{Peak area for R IS}}{\text{peak area for IS}} \qquad \text{Equation 2}$$

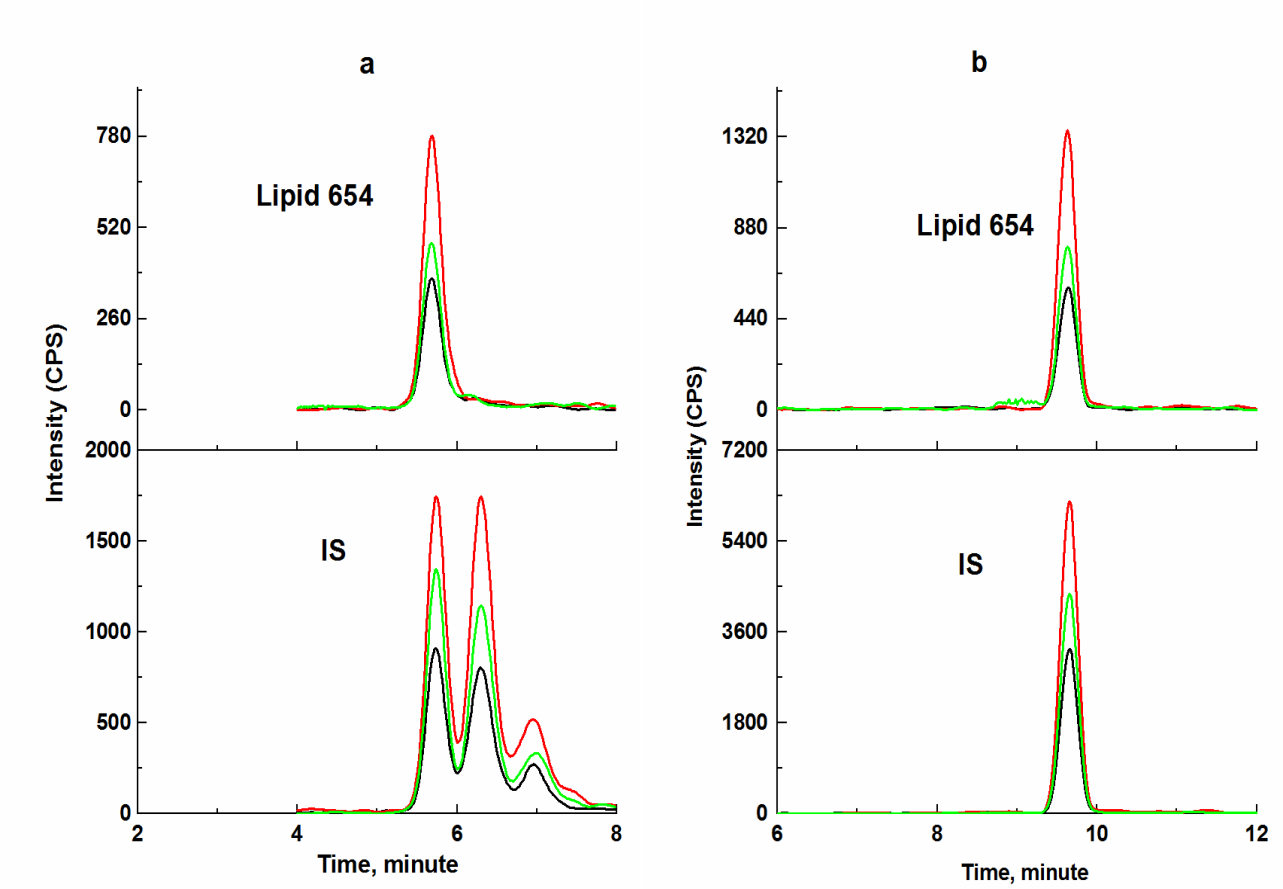


Figure 6. RP and cLC-SID-MRM MS quantitation of Lipid 654 in bacterial samples. XIC for Lipid 654 and IS using. Lower graphs show the XIC of IS and upper graph for Lipid 654 using (a) cLC-SID-MRM MS and (b) RP LC-SID-MRM MS.

$C_{R\ 654}$ is the concentration of bacterial R-Lipid 654 and C_{IS} is concentration of IS spiked in bacterial samples. To further validate cLC-SID-MRM MS quantitation of bacterial R-Lipid 654, these results were compared with those obtained using RP LC-SID-MRM MS (Table 3). (R,S)-Lipid 662-D_9 stereoisomers eluted from the C18 column as a single peak, co-eluting with Lipid 654, indicating no separation of Lipid 654 or IS stereoisomers (Figure 6b). Comparison between the two methods was done by dividing the ratios of Lipid 654 and IS obtained from cLC-SID-MRM MS and RP LC-SID-MRM MS methods (Table 4). Chiral/RP ratios for Lipid 654 were

calculated, giving an average ratio of 1.0 (Table 4) confirming that all of the analyzed bacteria produce only R isoform of Lipid 654. Both methods show somewhat similar precision and accuracy in quantitation of bacterial Lipid 654. However, coupling separation power of cLC and using diastereomeric mixture of IS provides additional information that is not accessible by RP LC-SID-MRM MS; i.e., simultaneous determination of stereochemistry and amount of Lipid 654. Another advantage of using a diastereomeric mixture of ISs, as opposed to using pure stereoisomers, is the synthesis of racemic or diastereomeric mixtures are easier and more cost effective. This cost advantage further augments when expensive isotopes are used to produce ISs.

Sample	(Chiral) $\frac{\text{R Lipid 654}}{\text{R IS}} \times X_{\text{R Lipid 654}}$	Coefficient of variation (CV)	(RP) Lipid 654/IS	Coefficient of variation (CV)	Chiral/RP ratio
T. denticola	0.19	3.43	0.19	7.33	1.0
P. endodontalis	110	11.2	120	10.1	0.96
FD 0450	35	3.12	37	4.26	0.94
FD 0580	0.25	13.1	0.23	7.48	1.1
P. gingivalis	100	10.3	89	12.5	1.2
P. intermedia	33	4.84	31	7.12	1.0
B. forsythia	12	4.47	11	4.14	1.1
P. distasonis	2.8	5.71	2.6	6.23	1.1
W83 *P. gingivalis*	70	3.29	68	6.77	1.0

Table 4. RP and cLC-MRM MS quantitation of Lipid 654 in bacterial samples.

2.3.5 Linearity of cLC-SID-MRM MS quantitation

L-serine-(R,S)-Lipid 662-D9 showed two groups of peaks that co-elute with the corresponding peaks from synthetic L-serine-(R,S)-Lipid 654. To investigate the linearity of the method of cLC-SID-MRM MS, calibration curves were generated for both L-serine-R- and L-serine-S-Lipid 654. Serially diluted L-serine-Lipid 662-D9 were added to vials containing a pooled total lipid extract. Ratios of peak areas of L-serine-R- and L-serine-S-Lipid 662-D9 to the peak area of endogenous bacterial Lipid-654 were plotted separately versus concentration of spiked ISs. R2 values of 0.9992 and 0.9998 were obtained for L-serine-R and L-serine-S Lipid 662-D9, respectively. Bacterial samples containing L-serine-(R,S)-Lipid 662-D9 in a range of 0.1 to 100 nmol/mL were quantified by cLC-SID-MRM MS. Limits of detection were calculated to be 10 pmol/mL of L-serine-Lipid 662-D9 for both L-serine-R- and L-serine-S-isoforms of Lipid 662-D9 (Figure 7 and 8).

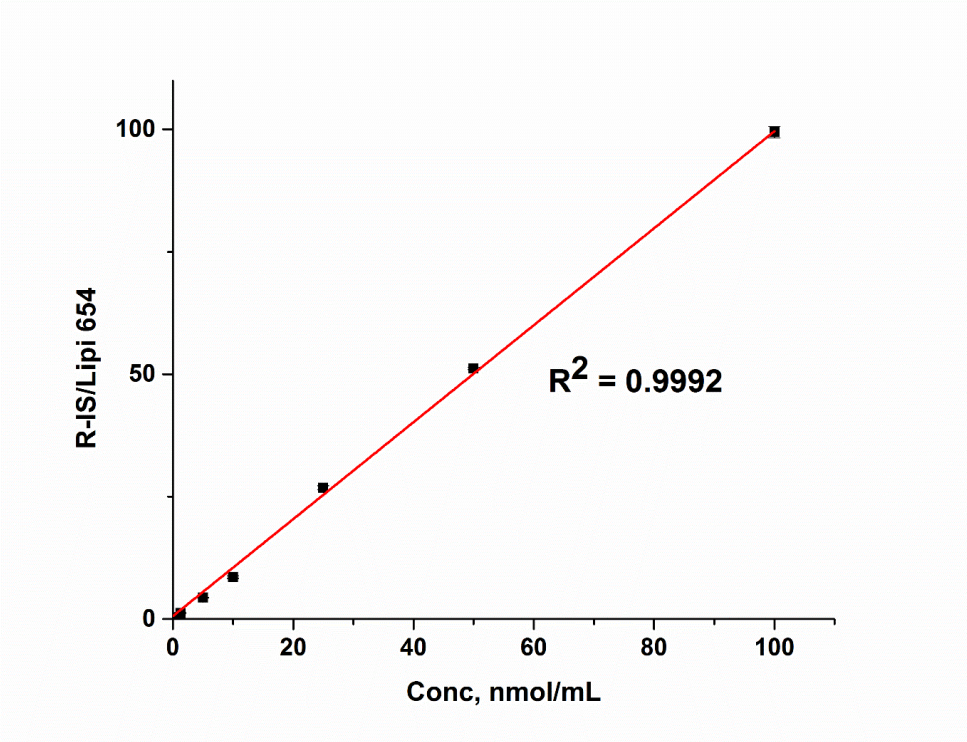


Figure 7. Calibration curve for R-Lipid 654 using serial dilution of (R,S)-Lipid 654-D_9 spiked to a pooled bacterial total lipid extract. Ratio of R-Lipid 654-D_9 to bacterial Lipid 654 is plotted against concentration of IS spiked in each sample**.**

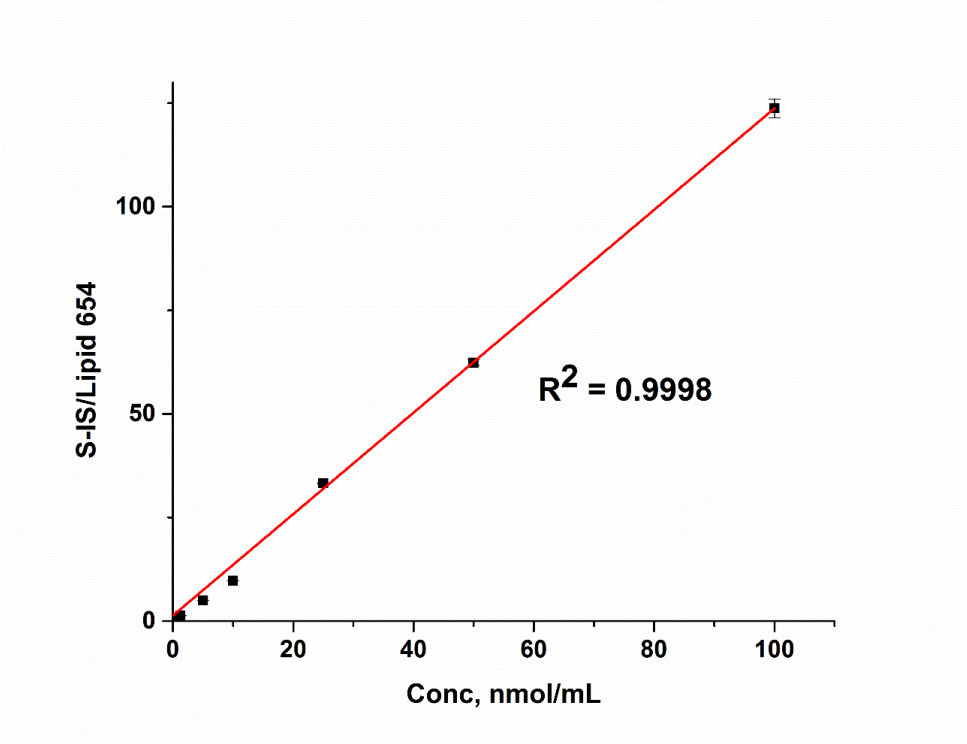


Figure 8. Calibration curve for S-Lipid 654 using serial dilution of (R,S)-Lipid 654-D_9 spiked to a pooled bacterial total lipid extract. Ratio of S-Lipid 654-D_9 to bacterial Lipid 654 is plotted against concentration of IS spiked in each sample.

2.4 Conclusions

Lipopeptides have recently emerged as promising vaccines, antibiotics and anticancer agents. Due to the presence of multiple chiral centers in their structure, lipopeptides usually exert their biological activity via stereoselective interactions with immune receptors such as TLRs. Therefore, determination of the absolute configuration of the chiral building blocks of lipopeptides is of great importance. So is the accurate quantitation of lipopeptide stereoisomers for assessing the safety and efficacy of synthetic lipopeptide vaccines or antibiotics or to identify novel lipopeptide

biomarkers for human disease. Combining the separation power of cLC and use of a diastereomeric mixture of isotopic ISs with the clinically-proven quantitation potential of MRM MS, a novel and robust method of cLC-SID-MRM MS is developed to simultaneously determine the stereochemistry and quantify the amount of bacteria-derived L-serine-Lipid 654 in total lipid extract of complex samples. This method is demonstrated for analysis of bacteria samples and also applied to study a novel enzymatic conversion of L-serine-Lipid 654, a potential disease biomarker. cLC-SID-MRM MS presents a sensitive and powerful platform for quantitative investigation of stereoselective chemical or biological processes.

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3 Mass spectrometry-based study of enzymatic hydrolysis of Lipid 654: relationship between hydrolysis of Lipid 654 and human disease

3.1 Introduction

Phospholipases A2 (PLA2s) enzymes catalyze the hydrolysis of the sn-2 fatty acyl ester of phosphoglyceride releasing a free fatty acid, predominantly arachidonic acid and a lysophospholipid (Figure 1).(Burke, Dennis 2009a) PLA2s comprise a large family of proteins found in various mammalian cells and tissues as well as in the venoms of snakes, scorpions and bees.(Six, Dennis 2000) PLA2s are divided into 16 groups based on their source, catalytic activity, amino acid sequence, chain length, and disulfide bond pattern.(Samel, Vija et al. 2013) The first type of PLA2s discovered, were secreted or secretory PLA2s (sPLA2). All sPLA2s have low molecular weight (13–19 kDa), histidine in the catalytic site, Ca^{2+}-bound in the active site, and six conserved disulfide bonds with one or two additional variable disulfide bonds.(Burke, Dennis 2009a) Mechanism of all sPLA2-catalyzed hydrolysis reactions includes abstraction of a proton from a water molecule followed by a nucleophilic addition on the carbonyl of the sn-2 bond. Histidine/aspartic acid dyad in the active site of sPLA2s activates the water molecule in a Ca^{2+}-dependent manner.(Scott, White et al. 1990) It has been shown, using biochemical analyses of purified sPLA2 preparations, that individual sPLA2s exhibit substrate selectivity towards either the phospholipid polar head groups or the sn-2 fatty acid constituent.(Murakami, Sato et al. 2015) For example, sPLA2-IIA shows higher activity for phosphatidylethanolamine (PE) or phosphatidylserine (PS) compared with phosphatidylcholine (PC). However, sPLA2-X has much higher affinity for PC. It has also been suggested that the substrate selectivity is partly attributable

to structural differences of sPLA2s.(Murakami, Sato et al. 2015) Furthermore, sPLA2s have very important biological and physiological functions.(Murakami, Sato et al. 2015, Lambeau, Gelb 2008)

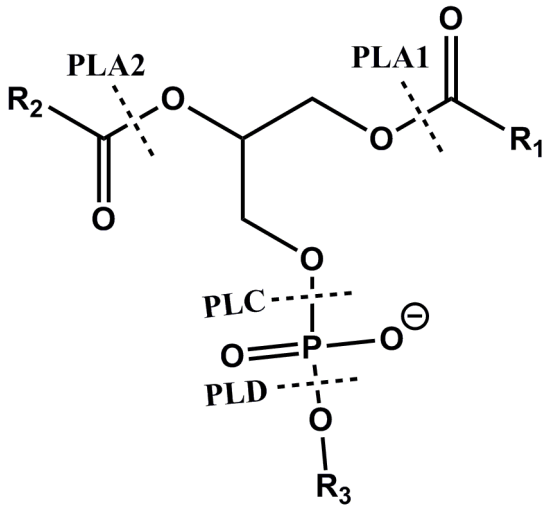


Figure 1. Phospholipase cleavage sites. Phospholipase A1 cleaves the sn-1 acyl chain, phospholipase A2 cleaves the sn-2 acyl chain and releases predominantly arachidonic acid, phospholipase C cleaves proximal to the phosphate head group and releases diacylglycerol as well as a phosphate-containing head group and phospholipase D cleaves distal to the phosphate releasing phosphatidic acid and an alcohol.

Biochemistry and physiology of mammalian sPLA2s have been studied extensively.(Lambeau, Gelb 2008, Boyanovsky, Webb 2009, Murakami, Sato et al. 2015) Well-established functions of sPLA2 are their roles in digestion of dietary phospholipids and in innate immunity against bacterial infections.(Richmond, Boileau et al. 2001, Buckland, Wilton 2000, Movert, Wu et al. 2013, Weiss 2015) Furthermore, several studies indicated critical functions of sPLA2s in arachidonic acid release and eicosanoid generation as well as in atherosclerosis.(Ghosh, Tucker et al. 2006, Hurt-Camejo, Camejo et al. 2001, Bonnefont-Rousselot 2016, Kimak,

Strycharz-Dudziak et al. 2015, Murakami, Sato et al. 2015) The role of sPLA2s in inflammation leading to atherosclerosis and eicosanoid biosynthesis is one of the important areas of ongoing PLA2 research.(Lambeau, Gelb 2008, Murakami, Sato et al. 2015)

The association between sPLA2 activity and atherosclerosis as well as between periodontal conditions and vascular disease have been investigated separately in large-scale studies names Coronary Event and Periodontal Disease (CORODONT) and Oral Infection and Vascular Disease Epidemiology Study (INVEST).(Mallat, Benessiano et al. 2007, Desvarieux, Demmer et al. 2003, Desvarieux, Demmer et al. 2005, Spahr, Klein et al. 2006) As a part of INVEST project, Boillot et al. investigated the relationship of periodontal microbiota and PLA2s in order to study the link between periodontal infections and cardiovascular disease such as atherosclerosis.(Boillot, Demmer et al. 2015) They suggested that increased activity of sPLA2s might provide a mechanistic explanatory link for the relationship between dysbiotic periodontal microbiota and vascular diseases.(Boillot, Demmer et al. 2015)

Periodontal or gum diseases include a group of oral inflammatory infections that are initiated by oral pathogens residing on the teeth or in the gingival sulcus surrounding the teeth.(Costalonga, Herzberg 2014) Periodontal diseases can also lead to the destruction of periodontal tissues by promoting loss of attachment of connective tissue and supporting bone for teeth.(Wang, Nemati et al. 2015) The spectrum of periodontal diseases spans from reversible chronic inflammation of gingiva to chronic inflammatory disease that results in destruction of connective tissues and bone, which eventually can lead to the loss of teeth.(How, Song et al. 2016) There is a significant difference between the subgingival periodontal microbiota observed in gingival health versus chronic inflammatory periodontal disease.(Hajishengallis, Darveau et al. 2012, Colombo, Magalhães et al. 2016, Costalonga, Herzberg 2014) Hajishengallis et al. suggested

that there is a “keystone-pathogen” that although is present in low abundance, can lead to inflammatory disease by shifting a normally symbiotic microbiota into a dysbiotic one.(Hajishengallis, Darveau et al. 2012) They indicated that *Porphyromonas gingivalis* (*P. gingivalis*), which is considered a major periodontal pathogen, could represent as the keystone-pathogen; *P. gingivalis* impairs innate immunity by evading Toll like receptor (TLR) recognition. As a result, *P. gingivalis* changes the growth and development of the entire biofilm and triggers a destructive change in the normally homeostatic host–microbiota interaction in the periodontium.(Hajishengallis, Darveau et al. 2012, Darveau 2009)

Nichols and his collaborators previously reported that the total lipid extract of *P. gingivalis* promotes activation of mouse dendritic cells and inhibits osteoblast-mediated bone deposition through engagement of TLR2.(Wang, Jiang et al. 2010, Nichols, Housley et al. 2009) Accordingly, Liquid chromatography-mass spectrometry (LC-MS) methods were developed to identify biological active components of *P. gingivalis* responsible for the observed biological activities. Following structure elucidation of an active component using MS/MS and NMR spectroscopy, it was concluded that a serine dipeptide lipid, called Lipid 654, is a primary component of *P. gingivalis* lipids responsible for activating TLR2.(Clark, Cervantes et al. 2013) It was suggested that Lipid 654 engages TLR2 co-receptors, TLR6 and CD-14, in a manner similar to known TLR2 activator ligands such as diacylated lipopeptide Pam2Cys (Figure 2).

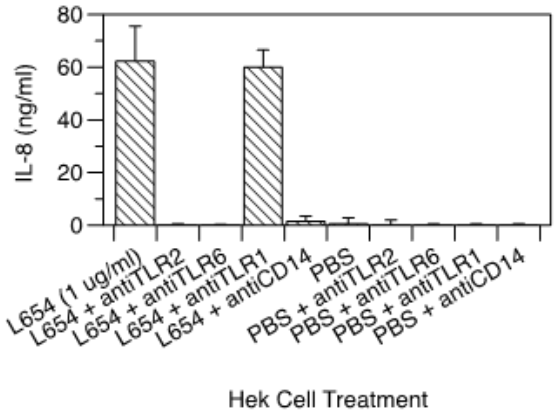


Figure 2. Bacterial Lipid 654 effects on HEK cells stably transfected to express human TLR2. Cells were treated with either PBS (Controls) or *P. gingivalis*-derived Lipid 654 (1 ug/ml) with or without the indicated neutralizing antibodies. This experiment was completed together with Emily Anstadt at the Dr. Robert Clark lab from Department of Immunology at UCONN Health Center.

LC-MS analysis of total lipid extract of *P. gingivalis* also indicated the presence of another serine dipeptide lipid molecule called Lipid 430 based on its mass. Structure of Lipid 430 is similar to Lipid 654, but lacks the ester-bound $C_{15:0}$ fatty acid (Figure 3). Lipid 430 was also isolated and biological activity of the isolates were investigated by Dr. Nichols lab.

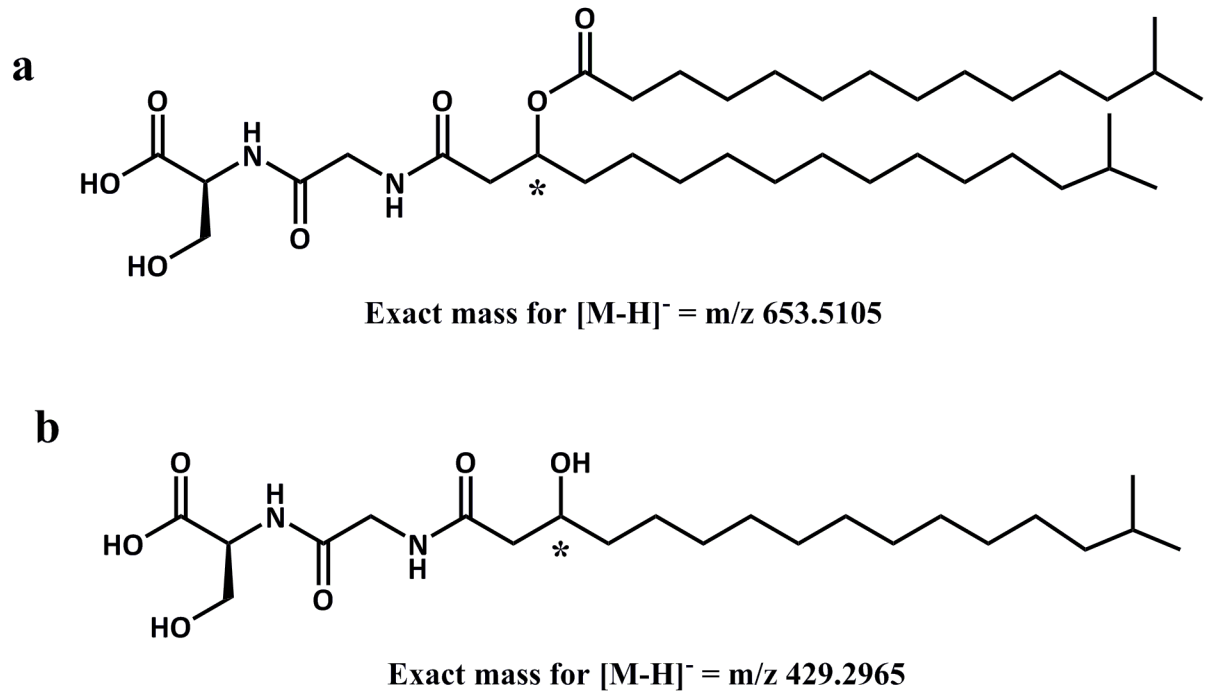


Figure 3. Structure of a) Lipid 654 and b) Lipid 430. Lipid 430 has structure similar to Lipid 654, but lacks the ester-linked $C_{15:0}$ fatty acid.

Bacterial Lipid 430 also significantly inhibited osteoblast gene expression and differentiation, and mineral nodule formation.(Wang, Nemati et al. 2015) However, these effects were only minimally contributed to engagement of TLR2. Osteoblasts are the primary cells responsible for bone formation. More importantly, Lipid 430 stimulated tumor necrosis factor alpha (TNF-α) and gene expression of receptor activator of nuclear factor kappa-B ligand (RANKL) in wild type bone marrow cells, but not in TLR2 knockout cells. In searching for the relationship between serine dipeptide lipids of *P. gingivalis* and human diseases such as chronic periodontitis and vascular diseases, a LC-MS method was developed to quantify the amount of these lipids in various biological matrices. Results showed that the level of Lipid 430 in bacteria samples is very low relative to Lipid 654. However, the relative amount of Lipid 430 compared to Lipid 654 is significantly higher in gingivalis tissue and carotid atheroma lipid extracts. Therefore,

it was hypothesized that there is a metabolic conversion of Lipid 654 to Lipid 430 in the diseased tissue. Commercially available esterase enzymes were screened for their capability for hydrolyzing Lipid 654 to Lipid 430 under physiological pH and ionic strength. Of the esterase enzymes evaluated, only PLA2 enzymes were observed to hydrolyze Lipid 654. The goal of the present research is to exploit MS-based approaches to study PLA2-mediated hydrolysis of Lipid 654 to Lipid 430 in order to better clarify a potentially very important relationship among PLA2 activity, periodontal pathogens and human diseases including chronic periodontitis and atherosclerosis.

3.2 Quantitation of Lipid 654 and Lipid 430 in bacteria and human tissue samples using normal phase (NP) LC-MRM MS

3.2.1 Experimental

3.2.1.1 Research Facilities

Sample preparations were performed in a Biosafety Level 2 laboratory within the Department of Chemistry (R409 and R411, the Xudong Yao Lab, Chemistry Building) at University of Connecticut and Department of Periodontology (room L1061, the Frank Nichols lab) at University of Connecticut Health Center. LC-TOF-MS will be performed using a 10ADvp Shimadzu HPLC (Columbia, MD) and quadrupole time-of-flight (QTOF) mass spectrometer (QSTAR Elite, Sciex). LC-MRM-MS analysis was performed using a 10ADvp Shimadzu HPLC and a QTRAP 4000 triple quadrupole mass spectrometer (Sciex, Framingham MA). The mass spectrometers are located in the MS facility of the Department of Chemistry (R403). ELISA assay was performed at the Department of Immunology (room ARB, the Robert Clark lab) at University of Connecticut Heath Center.

3.2.1.2 Material and Reagents

Acetyl chloride, Chloroform, and diisopropylethylamine (DIEA) were obtained from Sigma (St. Louis, MO). Pentafluorobenzyl bromide, N,O-bis(trimethylsilyl)triflouroacetamide (BSTFA), trypsin (0.25%) in EDTA (1X), and HPLC solvents were obtained from Thermo Fisher Scientific (Waltham, MA). Lipoteichoic acid was obtained from InvivoGen (San Diego, CA). Direct-Q3 water system (Millipore, Billerica, MA) was used to purify deionized water. Samples were dried using SpeedVac (Savant, Farmingdale, NY), vacuum oven (Fisher Scientific, Waltham, MA), or lyophilizer (Labconco, Kansas City, MO). Incubation of samples was performed on a Hula Mixer (Invitrogen, Waltham, MA) or a Thermomixer R (Eppendorf, Hauppauge, NY).

3.2.1.3 Lipid extraction and LC fractionation (by Dr. Nichols lab)

Lipid extraction and LC fractionation of extracted lipids were done by the Dr. Nichols' lab. Lipids were extracted from lyophilized bacterial pellets and human samples using the method of Bligh and Dyer as modified by Garbus.(GARBUS, DELUCA et al. 1963) Bacterial samples were dissolved in chloroform:methanol:water (1.33:2.67,1, v/v/v, 4 ml). The mixture was vortexed at 15 min intervals for 2 h and the mixture was supplemented with 0.75 ml of chloroform and 0.75 ml of (2M KCl + 0.5M K_2HPO_4). The mixture was then vortexed and centrifuged (2000 g) at 20°C for 4 h. The lower organic phase was removed and dried under nitrogen. Carotid endarterectomy samples were recovered at the time of surgical excision, a portion of each atheroma was removed for pathological evaluation and the remainder of the sample was frozen until processing. Control carotid arteries from young adults were recovered at necropsy as fresh frozen samples as processed at the National Disease Research Interchange. Frozen samples were thawed and minced into small pieces. Lipid extraction from tissue samples was performed using the same procedure as bacterial

samples. The dried artery samples were dissolved in chloroform and the total lipid content was determined. A known amount of synthetic Lipid 662-D_9 internal standard (IS) ([M-H] m/z 662.5) was added to each lipid sample in order to quantify the level of Lipid 654 in the artery samples.

The dried extract was reconstituted in 24 mL of HPLC grade solvent (hexane:2-propanol:water, 6:8:0.75, v/v/v) and vortexed for 30 seconds. The samples were centrifuged at 2500 g for 10 minutes and the supernatant removed for HPLC analysis. Semipreparative HPLC fractionation was accomplished using a Shimadzu HPLC system equipped with dual pumps (LC-10ADvp), automated controller (SCL-10Avp) and in line UV detector (SPD-10Avp). Lipids were fractionated using a NP silica column (Ascentis®Si, 25 cm x 10 mm, 5 μm, Supelco, St. Louis MO) under isocratic conditions with a solvent flow rate of 1.8 mL/min and eluents were collected in 1 min fractions. The eluates were monitored at 205 nm. For pooled lipid extracts of carotid atheroma samples, replicate fractionations were pooled and dried under nitrogen. The dried samples were reconstituted in the HPLC solvent for mass spectrometric analysis as described below. Fractions containing Lipid 654 were pooled and dried before additional HPLC fractionation. HPLC fractions containing Lipid 654 or Lipid 430 were further purified for Lipid by injecting the fractions over the same HPLC column at 1.8 mL/min, but using HPLC solvent supplemented with 0.1% acetic acid. The purity of Lipid 654 and Lipid 430 fractions was verified by direct infusion of the HPLC fractions into Qstar Elite as described previously.

3.2.1.4 NP LC-MRM MS analysis of Lipid 654 and Lipid 430 in bacteria and tissue samples

A 10ADvp Shimadzu HPLC was used to isocratically separate total lipid extract of bacteria and tissue samples using an Ascentis column, 2.1 x 50 mm, 3 μm (Supelco, St. Louis MO) packed with NP silica gel. Shimadzu HPLC was interfaced with a QTrap. Mobile phase was hexane:2-

propanol:water (6:8:0.75, v/v/v) and flow rate for HPLC separation was 0.12 mL/min. Negative ion ESI-MRM experiments were carried out at -4,500 V, with a declustering potential (DP) of -90 V, focusing potential (FP) of -350 V, and entrance potential (EP) of -10 V. The ion source temperature was maintained at 300°C. Multiple reaction monitoring (MRM) negative ion transitions for Lipid 654 were m/z 653.5/131.1, 653.5/306.2, 653.5/349.3 and 653.5/381.4 and for transitions for Lipid 430 were m/z 430.2/140.9, 430.3/173.1 and 430.3/382.3. CE values were optimized for each transition. Lipid 430/Lipid 654 ratios were calculated using only the m/z 430.3/382.3 and 653.5/381.4 transitions. The synthetic IS was monitored using m/z 662.5/390.5, 662.5/358.4 and 392.5/315.3. Lipid 654 amount per mg of total lipid extract was determined using the ratio of endogenous Lipid 654 to deuterated IS from integrated transitions peaks using Analyst 1.6 software.

3.2.1.5 Statistical analysis

Normally distributed data sets are expressed as the mean ± standard deviation, for which statistical paired Student's t-test was applied. For data sets that did not satisfy a normal distribution, the results were depicted as Box Whisker plots and Kruskal-Wallis test or Mann-Whitney U-test were applied. A p value of <0.05 was considered to be significant.

3.2.2 Results and discussion

LC-MS approaches were used to evaluate the recovery of Lipid 654 and Lipid 430 in lipid extracts of human carotid atheroma samples. Lipid extracts from carotid atheroma samples (n=12) were pooled and fractionated first by NP HPLC as previously described. The fractions containing Lipid 654 were pooled and re-fractionated over the same column but using HPLC solvent supplemented with 0.1% acetic acid. Lipid 654 was recovered primarily in one fraction (acidic

HPLC fraction 13) of carotid atheroma fractionated lipids and Figure 4A shows the MS spectrum indicating the characteristic 3 negative ions (m/z 654.2, 640.3 and 626.1) for the Lipid 654 class. Lipids of *P. gingivalis* were fractionated using the same method and the maximum abundance of the Lipid 654 lipid class was observed in HPLC fraction 14 (Figure 4B). MS/MS analysis of the most abundant negative ion species of the Lipid 654 class (m/z 653.5) recovered in fraction 13 of pooled endarterectomy lipids is shown in Figure 4C. Also, MS/MS spectrum of the dominant Lipid 654 species recovered in HPLC fraction 14 of *P. gingivalis* lipids is shown in Figure 4D. Lipid 654 isolated from *P. gingivalis* and atheroma samples showed identical MS/MS fragmentation patterns indicating that lipid extracts from carotid atheroma samples contained bacterial Lipid 654. This finding is of great significance in that it could reveal a potential link between periodontal pathogens, especially *P. gingivalis*, and atherosclerosis.

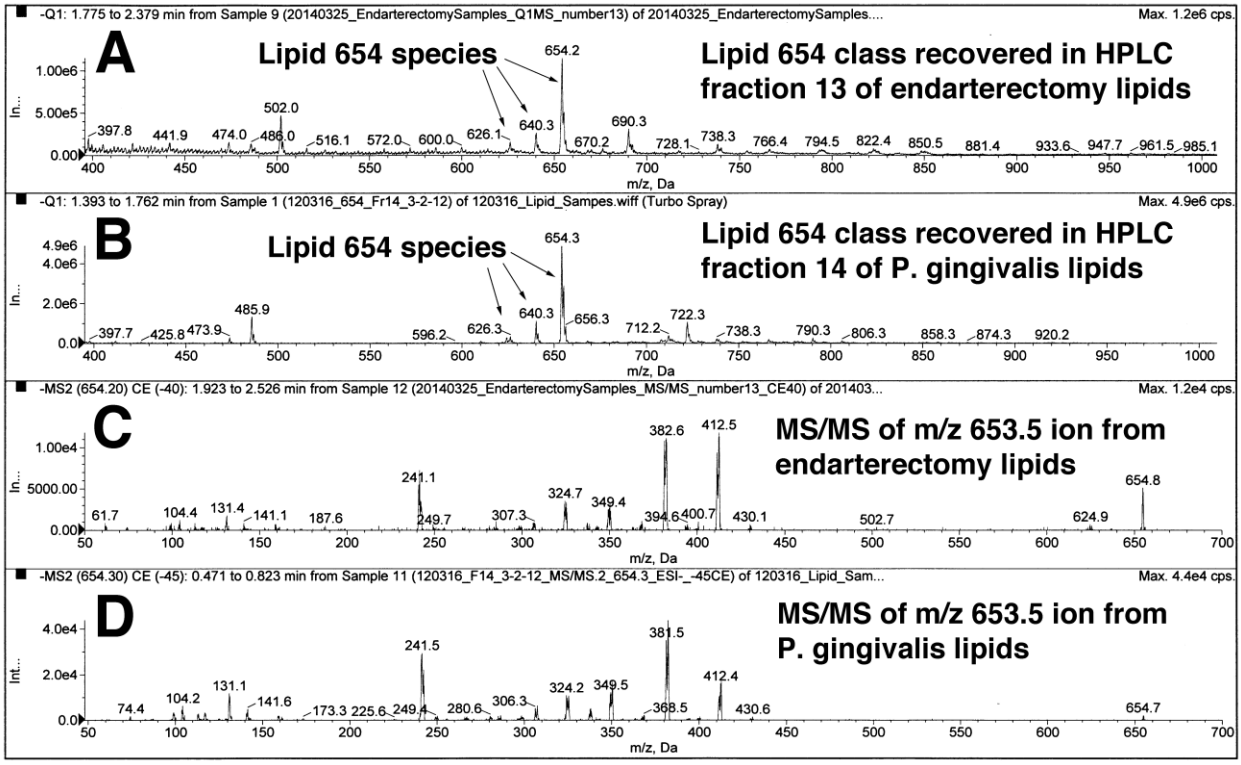


Figure 4. Lipid 654 recovered in pooled carotid atheroma lipid extracts. (A) ESI-MS spectrum of a Lipid 654-enriched fraction from a pooled carotid atheroma samples. Lipid extracts from 12 carotid atheroma samples were pooled and fractionated first by using a neutral HPLC solvent fractionation followed by pooling fractions containing Lipid 654 and re-fractionating with acidic HPLC solvent. (B) ESI-MS spectrum of a Lipid 654-enriched fraction from *P. gingivalis*. (C) MS/MS spectrum of Lipid 654 extracted from atheroma samples. (D) MS/MS spectrum of Lipid 654 extracted from *P. gingivalis*.

Total lipid extracts from fourteen oral Bacteroidetes species and four intestinal Bacteroidetes species were analyzed for Lipid 430 and Lipid 654 as described in the Methods section. LC-MRM MS showed that bacteria produce significantly less Lipid 430 than Lipid 654. The Lipid 430/Lipid 654 ratios ranged from 0.001 to 0.006 (Figure 5A) and these ratios were averaged for all bacteria analyzed. Furthermore, NP LC-MRM MS was utilized to quantify the amount of Lipid 654 and Lipid 430 in lipid extracts of individual carotid endarterectomy samples

(n=12) and carotid artery samples of healthy individuals between the age of 16 and 36 (control artery samples obtained from the National Disease Research Interchange, Philadelphia, PA, n=6 samples) (see Figure 5B). All carotid artery lipid extracts contained detectable levels of Lipid 654 and Lipid 430. Figure 5C shows the average level of Lipid 654 relative to a fixed amount of Lipid 662-D_9 IS normalized by weight of total lipid analyzed for carotid endarterectomy and control artery samples. The average level of Lipid 654 per milligram of total lipid extract was four-fold less in carotid atheroma lipid samples compared with the control artery samples. Reduced levels of Lipid 654 in total lipid extract of carotid endarterectomy samples could result from dilution of Lipid 654 due to the accumulation of cholesterol and other atherogenic lipids or metabolic de-esterification of Lipid 654 in diseased artery walls. The median Lipid 430/Lipid 654 ratio observed in carotid atheroma samples was almost five-fold higher than that observed in the control artery samples, supporting the possibility of more hydrolysis of Lipid 654 to Lipid 430 in diseased arteries. Therefore, if cholesterol accumulation causes a net decrease in the relative amount of Lipid 654 in diseased artery walls, it is not reflected in a similar dilution of Lipid 430 in the same samples.

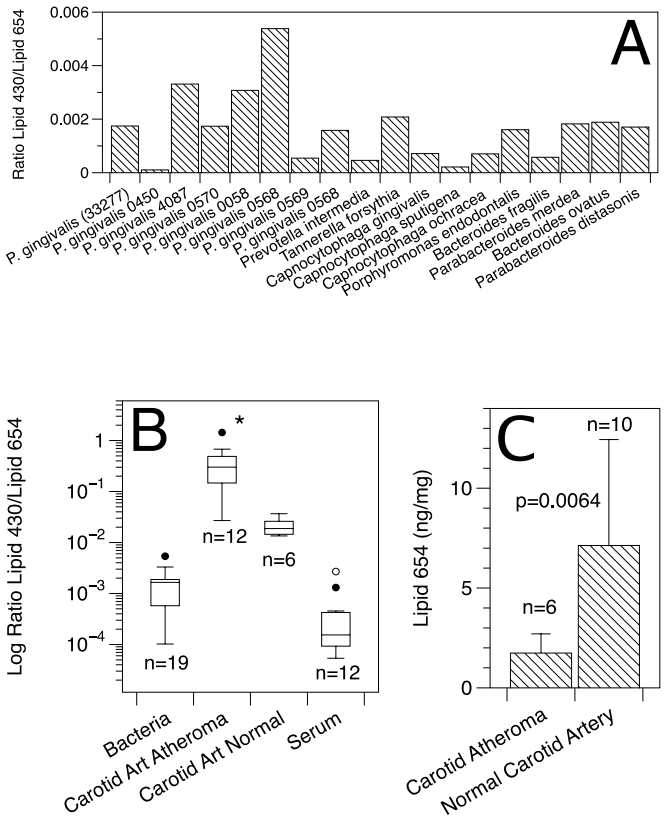


Figure 5. Recovery of Lipid 430 and Lipid 654 in common Bacteroidetes species, carotid artery samples and human serum samples analyzed by LC-MRM MS. (A) Level of Lipid 654 and Lipid 430 in total lipids extract of 14 bacteria strains (oral and gastrointestinal Bacteroidetes) were analyzed using NP LC-ESI-MRM MS. Lipid 430/Lipid 654 ratios were calculated by dividing the peak area of the most abundant transitions for Lipid 430 and Lipid 654 (430.3/382.3 for Lipid 430 and 653.5/381.4 for Lipid 654) are shown for each bacterial total lipid extract. (B) Lipid 430/Lipid 654 ratios are shown for carotid atheroma samples, carotid normal artery samples, total lipid extract of different strains of bacteria and serum from healthy adult subjects. Lipid samples were analyzed using ESI-MRM MS. The asterisk indicates a significant difference from all other sample categories by one way ANOVA on ranks test. (C) Quantification of Lipid 654 levels in carotid artery total lipid extracts using NP LC-SID-MRM MS. Lipid 654 per milligram of total lipid was determined using the ratio of endogenous Lipid 654 to deuterated IS.

Results are depicted as the mean ± standard deviation and the significance level was determined by paired Student's t-test.

It was interesting to compare relative amounts of Lipid 430 and Lipid 654 in bacteria samples and tissue samples were compared. The median ratios of Lipid 430 to Lipid 654 for carotid atheroma and control carotid artery samples were compared to bacterial lipid extracts (Figure 5). The carotid atheroma samples showed a median Lipid 430/Lipid 654 that is about 180 times greater than that observed in Bacteroidetes species, and 16 times greater than that observed in carotid artery samples from healthy young subjects. The differences among these sample categories are statistically significant. Because the median Lipid 430/Lipid 654 ratio in carotid atheroma samples is significantly higher than that observed in cultured Bacteroidetes species, these results do not support the notion that live Bacteroidetes are prevalent in carotid atheroma.

Although all human carotid artery samples tested were contaminated with detectable levels of Lipid 654 and Lipid 430, the median ratio of Lipid 430/Lipid 654 recovered in Bacteroidetes species is inconsistent with the distribution of the serine dipeptide lipids in carotid artery samples. Therefore, direct invasion of the artery walls by live bacteria is not likely to account for the serine lipid recovery in carotid artery samples. In addition, the levels of these lipids in serum samples suggests that Lipid 654 rather than Lipid 430 is preferentially transported in blood or blood cells and the elevated recovery of Lipid 430 in diseased carotid arteries probably resulted from the conversion of Lipid 654 to 430 in cells located on or in the diseased artery wall.

The present study demonstrated that serine dipeptide lipids derived from organisms of the oral and intestinal human microbiome are also present in human carotid artery samples. Though we have not established that all Bacteroidetes species are capable of synthesizing Lipid 654 and Lipid 430, a survey of common oral and intestinal species showed that only Bacteroidetes species

produce substantial amounts of these serine dipeptide lipids (Figure 5A). The HPLC fractionation of pooled carotid atheroma lipids and LC-MS analysis confirmed that Lipid 654 is present in substantial amounts in lipid extracts of carotid atheroma samples. However, the absolute amount of Lipid 654 as a percentage of the total lipid present was 4-folds lower in the total lipid extracts of carotid atheroma samples compared with the control carotid artery samples (Figure 5C). This observation could be related to the substantial accumulation of cholesterol, triglycerides, and other atherogenic lipids in the carotid atheroma samples, which essentially dilute the amount of Lipid 654 in the total lipid extract, or it could represent the accumulation of Lipid 654 before mammalian atherogenic lipids begin to accumulate in substantial amounts. If atherogenic lipids and Lipid 654 accumulate together in carotid atheroma and the Lipid 654 is not metabolically broken down, the Lipid 654 levels would remain essentially unchanged as a percentage of the total lipid present. However, because the median Lipid 430/Lipid 654 ratio is substantially different from that in bacteria and serum samples, one explanation could be de-esterification of Lipid 654 to Lipid 430, which could explain the lower levels of Lipid 654 in atheroma versus healthy carotid artery.

3.3 MS-based study of enzymatic hydrolysis of Lipid 654 to Lipid 430

3.3.1 Experimental

3.3.1.1 Research Facilities

Sample preparations were performed in a Biosafety Level 2 laboratory within the Department of Chemistry (R409 and R411, the Xudong Yao Lab, Chemistry Building) at University of Connecticut and Department of Periodontology (room L1061, the Frank Nichols lab) at University of Connecticut Health Center. LC-TOF-MS was performed using a 10ADvp Shimadzu HPLC and a QSTAR Elite. LC-MRM-MS analysis was performed using a 10ADvp

Shimadzu HPLC and a QTRAP 4000. The mass spectrometers are in the MS facility of the Department of Chemistry (R403).

3.3.1.2 Material and Reagents

Tris-buffered saline (TBS) and trifluoroacetic acid (TFA) were purchased from Fisher Scientific (Waltham, MA). 1,2-dimyristoyl-sn-glycero-3-phosphocholine (14:0 PC) was purchased from Avanti (Alabaster, AL). HPLC grade chloroform, Lyophilized cobra venom factor (CVF) (Ophiophagus Hannah, King Cobra), porcine pancreatic PLA2 (PP PLA2, >600 units/mg protein), honey bee venom PLA2 (HBV-PLA2) (Apis mellifera, 600-2400 units/mg protein), phospholipase A1 (PLA1) (Thermomyces lanuginosus), phospholipase C (PLC) (Clostridium perfringens, 10-50 units/mg protein), phospholipase C (PLD) (Arachis hypogaea, 300-700 units/mg protein), lipoprotein lipase (LL) (Pseudomonas sp., >50,000 units/mg protein), Pentafluorobenzyl bromide and N,O-bis(trimethylsilyl)triflouroacetamide (BSTFA) were obtained from Sigma (St. Louis, MO). Direct-Q3 water system (Millipore, Billerica, MA) was used to purify deionized water. Samples were dried using SpeedVac (Savant, Farmingdale, NY), or lyophilizer (Labconco, Kansas City, MO). Incubation of samples was performed on a Hula Mixer (Invitrogen, Waltham, MA) or a Thermomixer R (Eppendorf, Hauppauge, NY). Sonication was performed using ultrasonic cleaner (Fisher Scientific, Waltham, MA) or ..(Dr. Nichols lab).

3.3.1.3 Enzymatic hydrolysis of *P. gingivalis*-derived Lipid 654. Screening of various esterase enzymes

Purified samples from *P. gingivalis* were dissolved in chloroform and aliquoted at 10 μg into 5 ml conical reaction vials and dried under nitrogen. One milliliter of TBS buffer (10 mM, pH 7.4) with 10 mM $CaCl_2$ was added to each vial. The samples were sonicated for 20 seconds using

an ultrasonic sonicator and approximately 100U of HBV-PLA2 and PP-PLA2 and approximately 400U of lyophilized LL, CVF, PLA1, PLC and PLD were added to each vial. To quench the reaction at each time point, samples were acidified with glacial acetic acid (25 μL). Then, 1mL of chloroform was added for lipid extraction and the mixture was vortexed vigorously. Upon separation of the two organic and aqueous phases, the lower phase (chloroform) was transferred to a new tube. The upper layer was extracted with 1mL of chloroform for two more times. All three extractions were pooled and dried under nitrogen. For LC-MS analysis, samples were dissolved in hexane:2-propanol:water, (6:8:0.75, v/v/v).

3.3.1.4 ^{18}O-labeling investigation of the enzymatic hydrolysis reaction and MS analysis of hydrolysis products

One thousand and eight hundred microliters of a buffer solution (10 mM TBS and 2.5 mM $CaCl_2$, pH=7.4) was lyophilized. A total volume of 1800 μL of ^{18}O-water was then added to the lyophilized buffer to form our ^{18}O-Buffer. Fifty microliters of ^{18}O-Buffer was added to each aliquot (10 μg) of lipid substrate. Substrates include Lipid 654, iso-$C_{15:0}$ fatty acid, and 14:0 PC. Solutions were incubated at room temperature for 10 minutes and then sonicated for 5 minutes in an ultrasonic cleaner. Another 50 μL of ^{18}O-Buffer was added to each aliquot of enzyme (50 μg of HBV-PLA2 and PP-PLA2). Solutions were incubated at room temperature for 10 minutes and mixed for another 10 minutes at room temperature and 800 rpm. Enzyme and substrate solutions were mixed and briefly vortexed to let the enzymatic hydrolysis reaction start. For control samples instead of enzyme solutions, another 50 μL of ^{18}O-Buffer was added to the substrate solutions. Five aliquots of Lipid 654 samples were used for a time-course experiment and were mixed with 5 aliquots of PP-PLA2. Incubations were stopped at 3. 5, 6 h, 16 h, 48 h and 75 h. Another set of experiments included Lipid 654 with HBV-PLA2, iso-$C_{15:0}$ fatty acid with both PP-PLA2 and

HBV-PLA2, as well as control samples (control experiments are the lipid samples including Lipid 654, iso-$C_{15:0}$ fatty acid or anteiso-$C_{15:0}$ fatty acid without PLA2 enzyme) were stopped after 75 h of incubation. At the conclusion of hydrolysis reactions, 5 μL of glacial acetic acid was added to the reaction mixtures to quench the reaction and 500 μL of chloroform was added to extract the lipids after the reaction. The mixture were vortexed vigorously. Upon separation of the two organic and aqueous phases, lower phase (chloroform) was transferred to a new tube. To the upper layer, another 500 μL of chloroform was added for the second extraction. The latter step was repeated one more time. All three extractions were pooled and dried under nitrogen. Samples were analyzed for fatty acid release using both GC-MS and NP LC-ESI MS.

3.3.1.5 Fatty acid analysis (Performed by the Dr. Nichols lab)

Fatty acids that are released after PLA2 catalyzed hydrolysis of Lipid 654 in ^{18}O-Buffer were converted to pentafluorobenzyl ester derivatives by reacting fatty acid extracts in a mixture of acetonitrile, DIEA, pentafluorobenzyl bromide (36:10:4, v/v/v, 50 μl) for 20 min at 50°C.(Nichols 1994) Derivatized fatty acid samples were analyzed using a 5975C gas chromatography-MS (GC-MS) instrument from Agilent Technologies (Santa Clara, CA) equipped with an Agilent HP-5M nonpolar column with a helium flow rate of 1 mL/min for both positive and negative ionization conditions. The column was heated from 100 to 290°C. The injection block and transfer line were maintained at 280° and 290°C, respectively. Methyl ester-MTPA derivatives were run in the negative chemical ionization modes. Fatty acid quantification was accomplished by integration of selected ion chromatograms of each fatty acid ion (including iso- and anteiso-$C_{15:0}$ fatty acids and their corresponding ^{18}O- and $^{18}O_2$-labeled ones).

For LC-ESI MS analysis of fatty acids produced after hydrolysis reaction in ^{18}O-Buffer, samples were dissolved in hexane:2-propanol:water (6:8:0.75, v/v/v). A Shimadzu LC (10 AD-VP) was used to isocratically separate lipid samples on an Ascentis column (2.1 x 50 mm, 3 μm, Supelco, St. Louis MO) with a NP silica gel . A 10ADvp Shimadzu HPLC was interfaced with a QTRAP 4000. Mobile phase was hexane:2-propanol:water (6:8:0.75, v/v/v) and flow rate of HPLC solvent was 0.12 mL/min. Negative ion ESI-Q1MS experiments were carried out at -4,500 V, with a declustering potential (DP) of -90 V, focusing potential (FP) of -350 V, and entrance potential (EP) of -10 V. The ion source temperature was maintained at 300°C. Extracted ion chromatograms (XIC) of each fatty acid and their corresponding ^{18}O-labeled species were monitored.

3.3.1.6 Synthesis of Lipid 430 stereoisomers

Lipid 430 as mentioned previously has two chiral centers. GC-MS analysis of bacterial Lipid 430 showed that bacteria produce only L-serine.(Clark, Cervantes et al. 2013) As a result, only L-serine but (R,S)-3-OH $C_{17:0}$ fatty acids were used to synthetize a diastereomeric mixtures of L-serine-(3R,3S)-Lipid 430, abbreviated as (R,S)-Lipid 430. (R,S)-Lipid 430 were synthesized by Christopher Dietz in the Michael Smith lab at the Chemistry Department of University of Connecticut.(Dietz, Hart et al. 2016)

3.3.1.7 Chiral LC separation of synthetic (R,S)-Lipid 430 and Lipid 430 produced after hydrolysis of bacteria Lipid 654

Synthetic diastereomeric mixtures of (R,S)-Lipid 430 and Lipid 430 that is produced after hydrolysis of *P. gingivalis*-derived Lipid 654 were analyzed using chiral LC-MS. a Chiralpak®OD chiral column (250 x 4.6 mm, 10 μm, Daicel Corporation, Tokyo Japan) under isocratic conditions was utilized to separate stereoisomers of Lipid 430. Mobile phase was hexane, 2-propanol (9:1,

v/v) and 0.1% TFA at a flow rate of 0.6 ml/min. A UV-visible detector was used to monitor the eluents at the wavelength of 205 nm.

3.3.1.8 ESI-MS/MS analysis of synthetic (R,S)-Lipid 430 and bacterial-derived Lipid 430

Tandem MS (MS/MS) spectra of synthetic (R,S)-Lipid 430, *P. gingivalis* Lipid 430 and Lipid 430 from hydrolysis of Lipid 654 were obtained by direct infusion of lipids at a flow rate of 5 μL/min into a QSTAR Elite, in negative ion mode. Key instrument parameters were IS -4500 V, GS1 20, DP -80 V, CE -30 V, FP -380 V, DP2 -10 V, and CAD 5.

3.3.1.9 Chiral LC-MRM MS analysis of PLA2 hydrolysis of (R,S)-Lipid 654

A diastereomeric mixture of synthetic (R,S)-Lipid 654 was dissolved in chloroform, transferred into glass vials in 10 μg aliquots and dried under nitrogen. To the vials were added 100 μL of TBS (10 mM, pH 7.4) and 10 mM $CaCl_2$. Resulting solutions were incubated for 10 minutes without shaking at room temperature (RT) and then sonicated for 5 minutes in an ultrasonic cleaner. Fifty microliters of the same buffer was added to vials containing 50 μg aliquots of lyophilized HBV-PLA2 enzyme. Resulting solutions were incubated at RT for 10 minutes without shaking and another 10 minutes on thermomixer at 1200 rpm. Then, enzyme solutions were transferred to the vials containing Lipid 654 solutions except for the control samples. To quench the hydrolysis reaction, reaction mixtures were acidified with 5 μL of glacial acetic acid and extracted with chloroform (500 μL each for three times). The extracts were pooled and dried under nitrogen and then dissolved in 100 μL of methanol: 2-propanol (9:1,v/v) for cLC-SID-MRM MS. Three transitions (m/z 430.2/400.3, 430.3/173.1 and 430.3/382.3), in addition to the transitions for Lipid 654 (m/z 653.5/131.1, 653.5/306.2, 653.5/349.3 and 653.5/381.4) were monitored to measure the amount of Lipid 430 produced from hydrolysis of Lipid 654. A Shimadzu LC (10

AD-VP) was used to isocratically separate the re-constitutes reaction products on a chiral column (Lux Cellulose-4 50 x 20 mm, 5 μm, Phenomenex, Torrance CA). Mobile phase was composed of methanol:2-propanol: diethylamine (DEA) (90:10:0.1). Solvent flow rate was 20 μL/min and oven temperature was set at 30°C. A QTRAP 4000 was used to monitor MRM transitions for (R,S)-Lipid 654 and Lipid 430.

3.3.2 Results and discussion

3.3.2.1 Identification of esterase enzymes catalyzing hydrolysis of Lipid 654

Of all the esterases tested, only HBV-PLA2 (a group III PLA2)(Burke, Dennis 2009a) and PP-PLA2 (secretory PLA2 and group IB PLA2)(Murakami, Taketomi et al. 2011) enzyme preparations hydrolyzed Lipid 654 to the Lipid 430 (Scheme 1). Other enzymes including PLA1, PLC, PLD, LL and CVF showed minimal hydrolysis similar to the control sample without enzyme (Figure 6). Also, PLA2 enzymes didn't hydrolyze Lipid 654 in solution without Ca^{2+} ions.

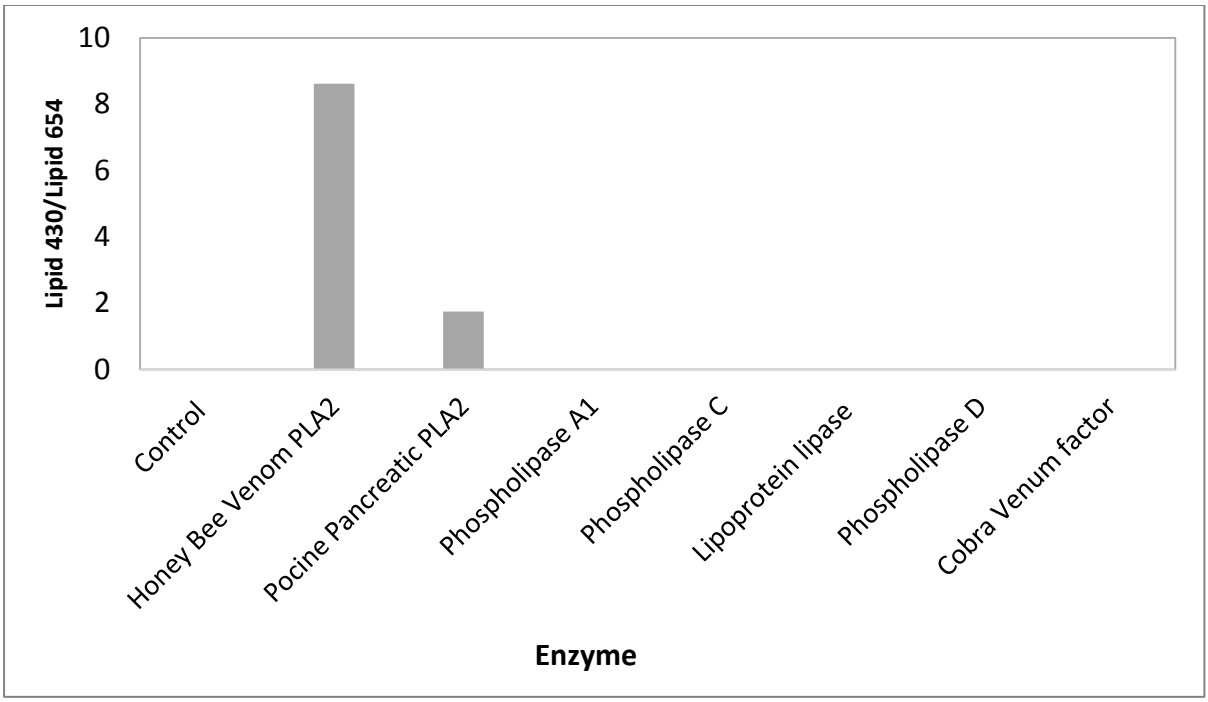


Figure 6. Hydrolysis of Lipid 654 by various esterase enzyme preparations. Ratios of peak area for Lipid 430 to peak area for Lipid 654 are shown. Most intense transitions (430.3/382.3 for Lipid 430 and 653.5/381.4 for Lipid 654) were used to calculate the peak area for each lipid.

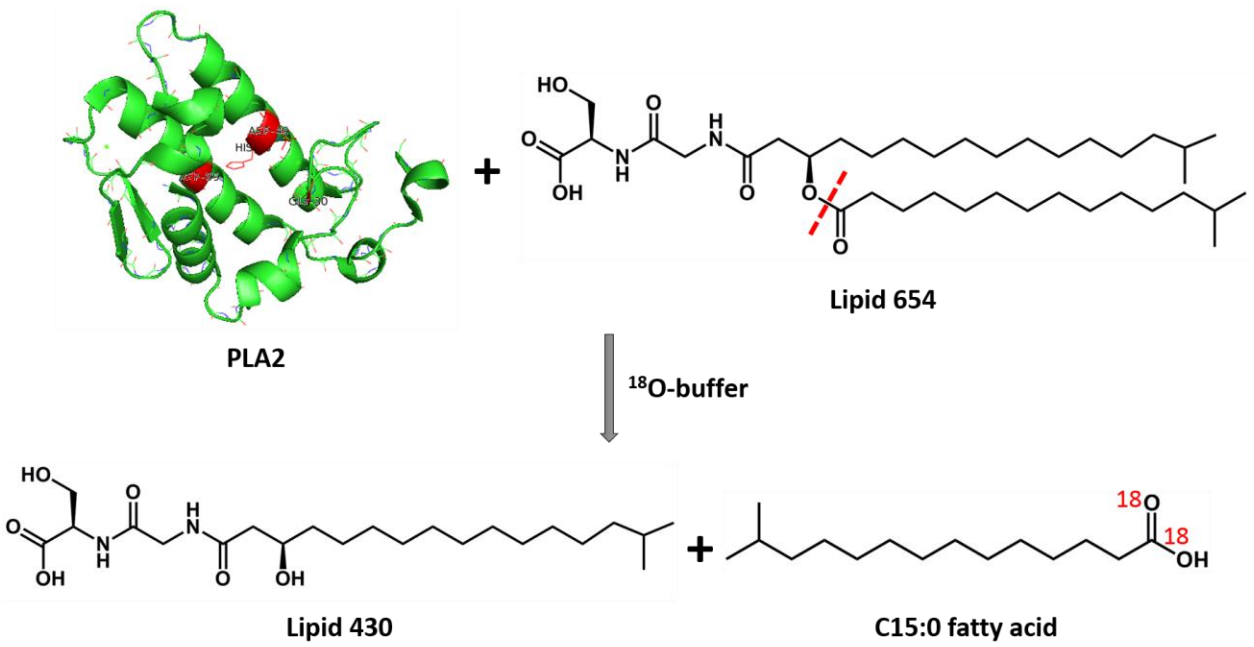
The samples shown in Figure 6 were treated in neutral 10 mM TBS buffer (pH 7.4), but PLA2 hydrolysis of the Lipid 654 was repeated in phosphate buffered saline (PBS) buffer and physiological pH and produced the same results. Also, neither of PLA2 preparations contained Lipid 654 or Lipid 430. PLA2 enzymes hydrolyze the ester bond formed on the second hydroxyl of glycerol-based phospholipids. PLA2 levels are characteristically elevated at sites of chronic inflammatory responses and result in the release of polyunsaturated fatty acids, predominantly arachidonic acid. Arachidonic acid release is prerequisite for elevated prostaglandin synthesis. To the best of our knowledge, there are no reports describing PLA2 hydrolysis of lipids other than glycerol-based phospholipids. Lipid 654 is not a glycerol-based phospholipid but evidence provided here suggests that both HBV-PLA2 and PP-PLA2 enzymes can de-esterify *P. gingivalis*

Lipid 654 to Lipid 430 through a calcium dependent mechanism. Other esterases evaluated in this investigation showed essentially no capacity to catalyze the hydrolysis of bacterial Lipid 654. Therefore, the enzymatic de-esterification of bacterial Lipid 654 appears to be limited either to specific enzymes of the PLA2 class. Porcine pancreatic PLA2 is a group IB enzyme and is structurally distinct from honey bee venom PLA2 which is a group III enzyme.

3.3.2.2 ^{18}O-labeling to investigate the enzymatic hydrolysis reaction and MS analysis of hydrolysis products

As indicated previously, preparations of HBV-PLA2 and PP-PLA2 catalyze the hydrolysis reaction of Lipid 654 to Lipid 430 and $C_{15:0}$ fatty acid (Scheme 1). In order to confirm the enzymatic hydrolysis, determine the breakage bond and obtain more mechanistic information, ^{18}O-lebaling and MS were exploited. LC-ESI MS analysis of hydrolysis products strongly suggested that upon enzymatic hydrolysis of Lipid 654 with PP-PLA2 and HBV-PLA2 in ^{18}O-Buffer, one or two ^{18}O atoms reside on the cleaved $C_{15:0}$ fatty acid. However, minimal incorporation of ^{18}O atoms was observed on cleaved Lipid 430 (Table 1). Fatty acid analysis using GC-MS further validated LC-MS results in that $C_{15:0}$ fatty acid incorporate one or two ^{18}O atoms (Table 1 and 2).

Consequently, these results suggested that HBV-PLA2 and PP-PLA2 facilitate cleavage of the bond between acyl group and oxygen atom on ester-bound $C_{15:0}$ fatty acid (Scheme 1). In order to more mechanistically investigate the enzymatic hydrolysis of Lipid 654, we designed a time-course experiment, in which products of PP-PLA2 hydrolysis of Lipid 654 in ^{18}O-Buffer were monitored after 3.5, 6, 16, 48 and 75 h as described in experimental section.



Scheme 1. Enzymatic hydrolysis of Lipid 654 with PLA2 enzymes in ^{18}O-Buffer. One or two ^{18}O atoms are added to $C_{15:0}$ fatty acid upon hydrolysis of Lipid 654. Cleavage site is shown by a red dashed line.

In addition to the time-course experiment, incorporation of ^{18}O by standard synthetic iso-$C_{15:0}$ was also monitored (with and without the presence of PLA2 in ^{18}O-Buffer); synthetic iso-$C_{15:0}$ was our control experiment for incorporation of ^{18}O by $C_{15:0}$ fatty acid released upon hydrolysis of Lipid 654. Furthermore, 14:0 PC (Figure 7), a standard substrate for PLA2 enzymes, was also treated with HBV-PLA2 and PP-PLA2 in ^{18}O-Buffer for 75 h under similar conditions. Later experiment was designed to compare the PLA2-facilitated hydrolysis of Lipid 654 with a known PLA2 substrate. LC-ESI MS and GC-MS were utilized to analyze all the ^{18}O-labeling experiments and the results of these analyses are shown in Table 1 and 2, respectively.

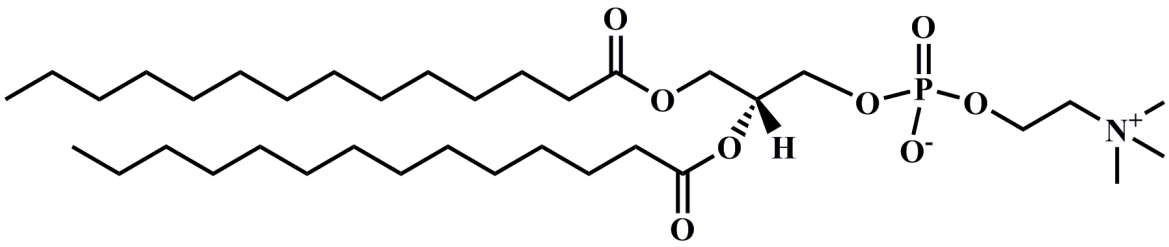


Figure 7. Structure of $C_{14:0}$ PC, a standard substrate for PLA2s. PLA2s catalyze the hydrolysis of the sn-2 fatty acyl ester of $C_{14:0}$ PC releasing a free $C_{14:0}$ fatty acid.

^{18}O-lebaling clearly showed that PP-PLA2 and HBV-PLA2 catalyze a direct attack of water on the carbonyl of $C_{15:0}$ acyl group of Lipid 654, presumably by general base catalysis by histidine as shown previously for their standard substrates.(Lombardo, Fanni et al. 1986, Burke, Dennis 2009b, Yu, Dennis 1991) Also, our control experiments including incubation of synthetic iso-$C_{15:0}$ fatty acid in ^{18}O-Buffer with and without PLA2s for 75 h showed rather a minimal incorporation of ^{18}O at similar conditions (Table 1 and 2). Lombardo et al. also reported no incorporation of ^{18}O when a standard $C_{16:0}$ fatty acid ([1-^{13}C] palmitic acid) was reacted with PLA2 in ^{18}O-water buffer (Tris-HCl pH 7.9) for 24 h at 40 ^{o}C.(Lombardo, Fanni et al. 1986) Although the presence of PLA2 increased the incorporation of ^{18}O from approximately 4 to 8% (based on LC-MS data) or 2 to 7% (based on GC-MS data), these incorporations cannot explain the significant amount of second ^{18}O incorporated by cleaved $C_{15:0}$ fatty acid upon hydrolysis of Lipid 654 (Table 1 and 2). These results suggested that ^{18}O-labeling of free fatty acids through the exchange of $H_2{}^{18}O$ is not possible in these conditions. Also, PLA2 cannot efficiently catalyze the exchange of $H_2{}^{18}O$ of free fatty acids under similar conditions. PLA2 hydrolysis of $C_{14:0}$ PC (a known substrate for PLA2s) also showed cleavage of sn-2 position as expected and incorporation of one ^{18}O to the released $C_{14:0}$ fatty acid. This latter result is also in agreement with the work of Lombardo et al. in that released fatty acid

incorporated one ^{18}O and doesn't exchange the second oxygen.(Lombardo, Fanni et al. 1986) In striking contrast, $C_{15:0}$ fatty acid that is released upon hydrolysis of Lipid 654, incorporates the second ^{18}O atom and the percentage of $^{18}O_2$-$C_{15:0}$ fatty acid increases while the percentage of ^{18}O-$C_{15:0}$ fatty acid decreases between 3.5 and 75 h time points (Table 1 and 2). On the other hand, the percentages of released Lipid 430 and ^{18}O-Lipid 430 didn't change significantly after 3.5 h (Figure 8).

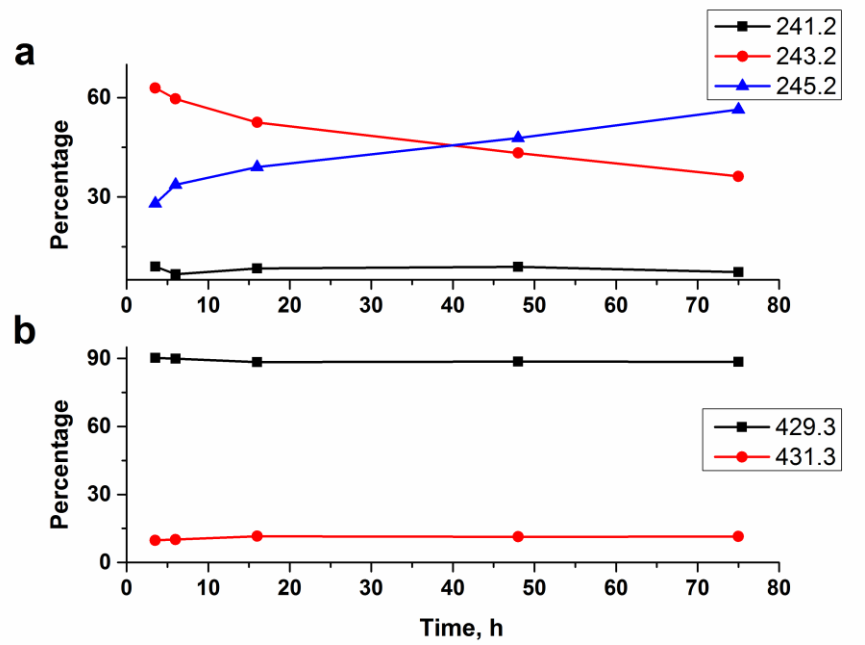


Figure 8. LC-ESI MS analysis of products of PP-PLA2 hydrolysis of Lipid 654 (a time-course experiment). a) Percentage of ^{16}O-$C_{15:0}$ fatty acid, ^{18}O-$C_{15:0}$ fatty acid and $^{18}O_2$-$C_{15:0}$ fatty acid calculated after 3.5, 6, 16, 48 and 75 h. b) Percentage of ^{16}O-Lipid 430 and ^{18}O-Lipid 430 calculated after the time points mentioned above.

These results suggest that PLA2-Lipid 654 complex is different from PLA2-PC complex in that cleaved $C_{15:0}$ fatty acid is retained longer or released from active site slower in such a way that incorporation of the second ^{18}O is possible; this is most likely due to the presence of high

energy transition state after breakdown of tetrahedral intermediate (such as fatty acid release or conformational change of the enzyme's active site).(Lombardo, Fanni et al. 1986, Burke, Dennis 2009b) Nevertheless, further investigation is required to further test these hypotheses.

LC-ESI MS					
Source, Lipid, Enzyme, Incubation time\Monitored ion	^{16}O-lipid	^{18}O-Lipid	$^{18}O_2$-Lipid	^{18}O-Lipid %	$^{18}O_2$-Lipid %
Standard synthetic, iso-$C_{15:0}$, Ctrl, 75 hr	7.7E+08	3.1E+07	0	3.9	0
Standard synthetic, iso-$C_{15:0}$, PP-PLA2, 75 hr	8.6E+08	7.2E+07	4.2E+06	7.8	0.4
Cleaved, iso-$C_{15:0}$, PP-PLA2, 3.5 hr	3.1E+07	2.2E+08	1.0E+08	62.8	28.5
Cleaved, iso-$C_{15:0}$, PP-PLA2, 6 hr	2.5E+07	2.5E+08	1.4E+08	59.6	34.4
Cleaved, iso-$C_{15:0}$, PP-PLA2, 16 hr	3.1E+07	2.2E+08	1.6E+08	53.2	39.5
Cleaved, iso-$C_{15:0}$, PP-PLA2, 48 hr	3.2E+07	1.8E+08	1.9E+08	44.9	47.1
Cleaved, iso-$C_{15:0}$, PP-PLA2, 75 hr	2.5E+07	1.4E+08	2.0E+08	38.8	54.2
Cleaved, iso-$C_{15:0}$, HBV-PLA2, 75 hr	6.4E+07	2.4E+08	2.1E+08	46.7	40.7
Cleaved, $C_{14:0}$, PP-PLA2, 75 hr	0	3.4E+08	4.6E+08	42.4	57.7
Cleaved, $C_{14:0}$, HBV-PLA2, 75 hr	0	3.7E+08	4.8E+08	43.8	56.2
Cleaved, Lipid 430, PP-PLA2, 3.5 hr	6.3E+08	6.8E+07	0	9.8	0
Cleaved, Lipid 430, PP-PLA2, 6 hr	7.1E+08	8.0E+07	0	10.1	0
Cleaved, Lipid 430, PP-PLA2, 16 hr	9.3E+08	1.2E+08	0	11.6	0
Cleaved, Lipid 430, PP-PLA2, 48 hr	9.6E+08	1.2E+08	0	11.4	0
Cleaved, Lipid 430, PP-PLA2, 75 hr	6.5E+08	8.4E+07	0	11.5	0

Table 1. NP LC-ESI MS analysis of products of PLA2 hydrolysis of Lipid 654 and $C_{14:0}$ PC in ^{18}O-Buffer. Products of hydrolysis reactions were extracted and analyzed by NP LC-ESI MS. Peak area of XIC for each hydrolytic lipid product (endogenous and ^{18}O-labeled) are shown, except the first two row that show the incubation of standard iso-$C_{15:0}$ fatty acid without and with PP-PLA2, respectively. Shown are

also the percentage of ^{18}O- and $^{18}O_2$-labeled Lipid products. Note: Peak area of XICs, for which integration was not possible is reported as zero.

GC-MS					
Source, Lipid, Enzyme, Incubation time\Monitored ion	Endogenous lipid	^{18}O-Lipid	$^{18}O_2$-Lipid	^{18}O-Lipid %	$^{18}O_2$-Lipid %
Standard synthetic, iso-$C_{15:0}$, Ctrl, 75 hr	1.9E+07	4.1E+05	0	2.1	0
Standard synthetic, iso-$C_{15:0}$, PP-PLA2, 75 hr	2.2E+07	1.7E+06	0	7.0	0
Cleaved, iso-$C_{15:0}$, PP-PLA2, 3.5 hr	1.3E+06	8.3E+06	3.4E+06	63.9	26.3
Cleaved, iso-$C_{15:0}$, PP-PLA2, 6 hr	6.9E+05	9.5E+06	5.1E+06	62.3	33.2
Cleaved, iso-$C_{15:0}$, PP-PLA2, 16 hr	1.4E+06	8.7E+06	5.8E+06	54.6	36.5
Cleaved, iso-$C_{15:0}$, PP-PLA2, 48 hr	8.3E+05	6.6E+06	6.3E+06	48.2	45.7
Cleaved, iso-$C_{15:0}$, PP-PLA2, 75 hr	1.1E+06	6.5E+06	6.8E+06	45.0	47.4
Cleaved, iso-$C_{15:0}$, HBV-PLA2, 75 hr	1.0E+06	6.8E+06	5.3E+06	51.6	40.5
Cleaved, $C_{14:0}$, PP-PLA2, 75 hr	1.3E+06	1.2E+07	1.4E+07	44.3	50.8
Cleaved, $C_{14:0}$, HBV-PLA2, 75 hr	1.8E+06	1.5E+07	1.7E+07	44.1	50.7

Table 2. **GC-MS fatty acid analysis of products of PLA2 hydrolysis of Lipid 654 in ^{18}O-Buffer.** Products of hydrolysis reactions were extracted and were derivatized for GC-MS analysis. Peak area of XIC for each hydrolytic lipid product (endogenous and ^{18}O-labeled) are shown, except the first two row that show the incubation of standard iso-$C_{15:0}$ fatty acid without and with PP-PLA2, respectively.

Shown are also the percentage of ^{18}O- and $^{18}O_2$-labeled Lipid products. Note: Peak area of XICs, for which integration was not possible is reported as zero.

3.3.2.3 Synthesis, separation and LC-MS of Lipid 430 stereoisomers

As discussed in the experimental section, the stereochemistry of serine amino acid of Lipid 654 was already determined to be L-serine by chiral GC-MS analysis, which was done by the Dr. Nichols lab at University of Connecticut Health Center as described previously.(Clark, Cervantes et al. 2013) To determine the absolute structure of Lipid 430 that is produced upon hydrolysis of Lipid 654, L-serine-(3R,3S)-Lipid 430 stereoisomers, abbreviated as (R,S)-Lipid 430, were synthesized by Christopher Dietz in the Dr. Michael Smith's laboratory at the Chemistry Department of University of Connecticut.(Dietz, Hart et al. 2016) An off-line chiral LC-MS method was utilized to separate and validate the synthesis of (R,S)-Lipid 430 as described in the experimental section. Both stereoisomers of synthetic Lipid 430 were successfully separated using a Chiralpak®OD column under isocratic conditions. Chiral HPLC separation of synthetic (R,S)-Lipid 430 eluted as two peaks at 25 and 30 minutes, respectively, giving a selectivity α to be 1.3(Figure 9b). Also, Lipid 430, produced after hydrolysis of Lipid 654 from *P. gingivalis*, was isolated using acidic fractionation of the hydrolysis products with NP HPLC as described in the experimental section. The Lipid 430 fraction was fractionated on the same chiral column that was used to separate synthetic (R,S)-Lipid 430. However, Lipid 430 produced from enzymatic hydrolysis of *P. gingivalis* Lipid 654 yielded only one peak with the same retention time as the first peak observed in chiral separation of synthetic (R,S)-Lipid 430 (Figure 9a), suggesting structure of bacterial Lipid 430 is similar to one of the stereoisomers of synthetic (R,S)-Lipid 430.

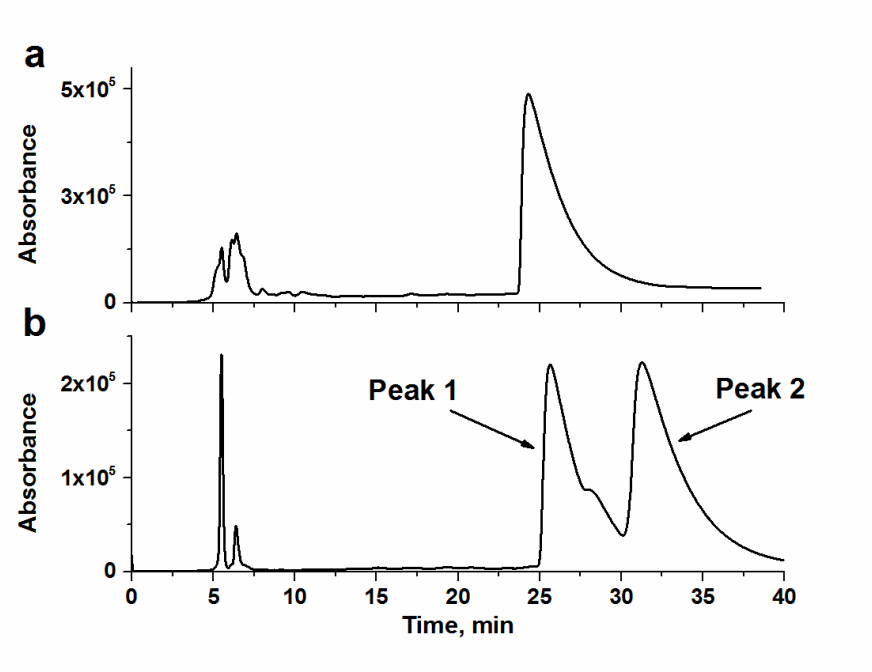


Figure 9. Chiral LC separation of Lipid 430 from hydrolysis of bacteria Lipid 654 and synthetic diastereomeric mixture of (R,S)-Lipid 430. a) Chiral LC isolated Lipid 430 from PLA2 hydrolysis of *P. gingivalis*-derived Lipid 654**.** b) Chiral LC separation of diastereomeric mixture of (R,S)-Lipid 430. Both peaks have the same MS and MS/MS spectra. LC-MS analysis of the fractions was used to assign the peaks. (Done in the Dr. Nichols lab)

In order to further validate the synthesis of (R,S)-Lipid 430 and compare these samples with Lipid 430 produced after PLA2 hydrolysis of Lipid 654, high resolution MS/MS analysis of these samples were performed as described in the experimental section. Synthetic (R,S)-Lipid 430 and Lipid 430 produced by hydrolyzing *P. gingivalis* Lipid 654 showed similar MS/MS spectra indicating the successful synthesis of (R,S)-Lipid 430 (Figure 10).

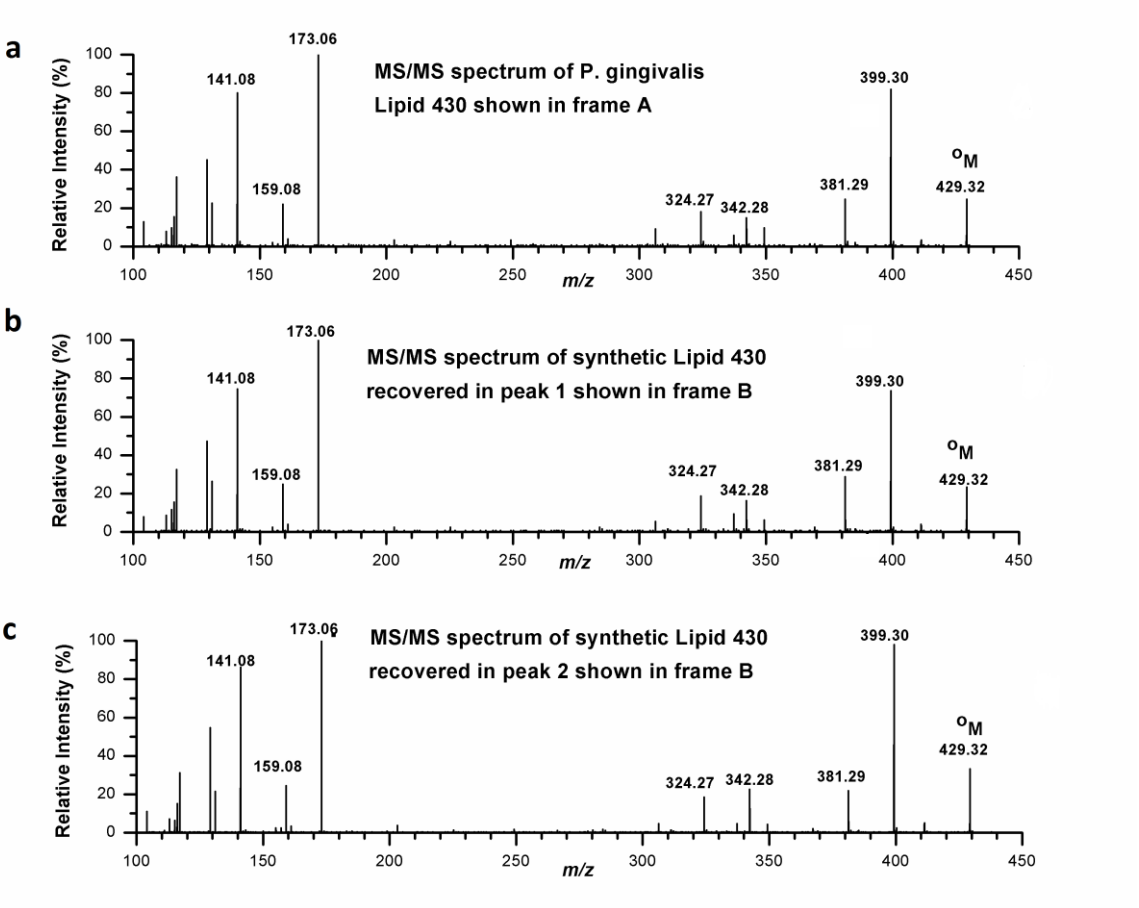


Figure 10. ESI-MS/MS analysis of Lipid 430 preparations. MS/MS spectra of Lipid 430 at CE= -30 V obtained by direct infusion of lipid samples. (a) Lipid 430 isolated after hydrolysis of Lipid 654 with PLA2. (b) First peak in chiral separation of synthetic (R,S)-Lipid 430. (c) Second peak in chiral separation of synthetic (R,S)-Lipid 430.

Chiral LC separation and MS/MS analysis of Lipid 430 from PLA2 hydrolysis of bacterial Lipid 654 and synthetic (R,S)-Lipid 430 indicated that bacterial Lipid 654 molecules produce only one of the Lipid 430 stereoisomers. These results lead to the postulations: 1) bacteria produce only one of the possible L-serine-Lipid 654 stereoisomers, and 2) PLA2 catalyzes the hydrolysis of only one Lipid 654 stereoisomers. Both stereoisomers of Lipid 654 were synthesized in order to

investigate whether the stereoselectivity of PLA2 enzymes or the presence of only one isoform for bacterial Lipid 654 is responsible for production of only one isoform for Lipid 430.

3.3.2.4 Stereoselctivity of PLA2 enzymes: chiral LC-MRM MS analysis of PLA2 hydrolysis of (R,S)-Lipid 654

As described in chapter 2, a diastereomeric mixture of Lipid 654 stereoisomers, (R,S)-Lipid 654 as well as enantioenriched R- and S-Lipid 654 were synthesized by Christopher Dietz in the Dr. Michael Smith lab at the Chemistry Department of the University of Connecticut.(Dietz, Hart et al. 2016) Using synthetic standards of Lipid 654, an online chiral LC-SID-MRM MS method, coined as cLC-SID-MRM MS, was developed. cLC-SID-MRM MS analysis of bacterial samples showed that all the bacteria produce only R-Lipid 654 confirming that hypothesis 1 is correct. Nevertheless, to test the hypothesis 2, cLC-MRM MS was utilized to study stereoselectivity of PLA2 enzymes in catalyzing the hydrolysis of Lipid 654 stereoisomers. The PLA2 enzyme preparation was used to hydrolyze triplicate aliquots of synthetic (R,S)-Lipid 654 as described in the experimental section. For control experiments, triplicate aliquots of synthetic (R,S)-Lipid 654 were incubated in hydrolysis buffer without PLA2 enzyme. Strikingly, cLC-MRM MS clearly showed that PLA2 only hydrolyzes R-Lipid 654, the isoform of Lipid 654 that is produced by bacteria (Figure 11). Control (R,S)-Lipid 654 with no hydrolysis showed two distinct peaks (Figure 11c). The first peak was assigned as R-Lipid 654 and second peak as S-Lipid 654 based the retention time observed for enantioenriched R- and S-Lipid 654 (Figure 11a-b). Furthermore, (R,S)-Lipid 654 aliquots without showed similar results as control samples with incubation and no added enzyme. Stereoselectivity of PLA2 towards R-Lipid 654 is clear in Figure 11d. The peak for R-Lipid 654 was lost after PLA2 hydrolysis (Figure 11d) while S-Lipid 654

showed essentially no change in ion abundance measured as integrated peak area. The Lipid 430 product of PLA2 hydrolysis was detected by monitoring three transitions (Figure 11d).

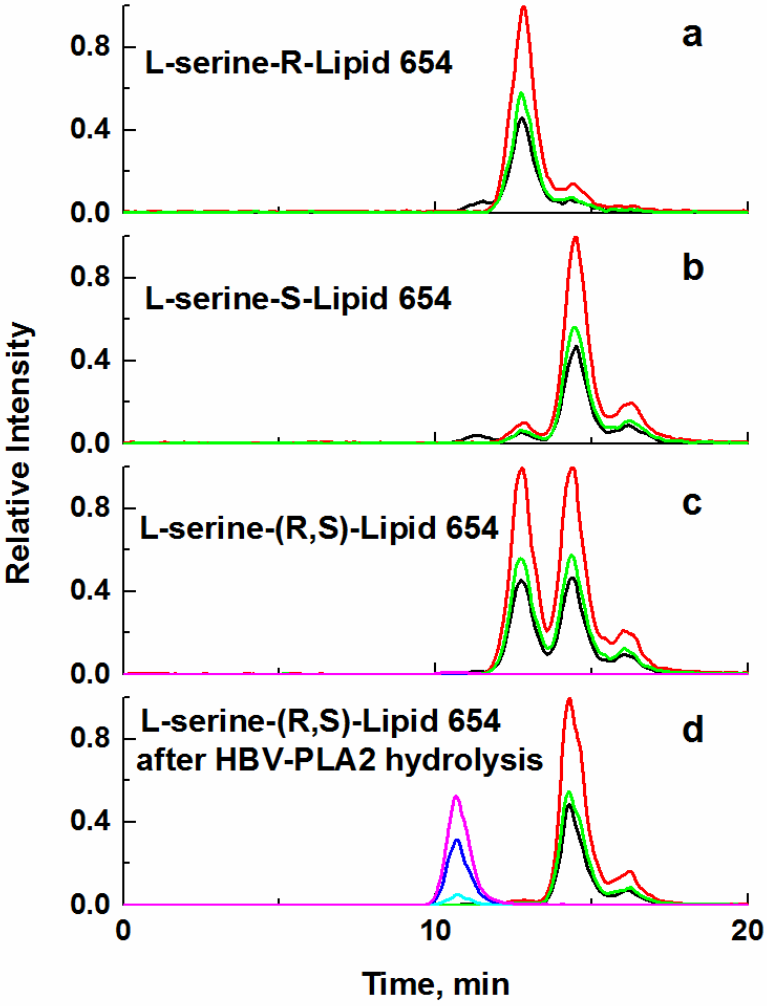


Figure 11. cLC-MRM MS analysis of PLA2 hydrolysis of (R,S)-Lipid 654. XICs of Lipid 654 and Lipid 430 transitions (m/z 430.2/400.3, 430.3/173.1 and 430.3/382.3 653.5/306.2, 653.5/349.3 and 653.5/381.4) for (a) synthetic enantioenriched R-Lipid 654, (b) synthetic enantioenriched S-Lipid 654, (c) (R,S)-Lipid 654 before hydrolysis and (d) products of (R,S)-Lipid 654 hydrolysis with PLA2

cLC-MRM MS established not only that PLA2 enzymes catalyze hydrolysis of Lipid 654, but also that PLA2 enzymatic activity is stereoselective towards R-Lipid 654, which turns out to be the predominant isoform of Lipid 654 produced by bacteria. Quantitative information regarding selectivity of PLA2 in Lipid 654 hydrolysis is summarized in Table 3.

Sample\Peak area	R-Lipid 654	S-Lipid 654	Lipid 430
(R,S)-Lipid 654, control	1.7e6(± 9.4e4) 51%	1.6e6(±1.1e5) 49%	1.4e4(±2.6e3)
(R,S)-Lipid 654, PLA2 treated	3.9e4(±1.5e3) 3%	1.2e6(±1.0e5) 97%	4.1e5(±6.2e4)

Table 3. Peak area of R-Lipid 654, S-Lipid 654 and Lipid 430 before and after enzymatic hydrolysis.

3.4 Conclusions

Using LC-MS approaches, it was shown that commercially available preparations of PLA2 enzymes (PP-PLA2 and HBV-PLA2) catalyze hydrolysis of Lipid 654 to Lipid 430, which is a novel activity for PLA2 enzymes. Also, LC-SID-MRM MS quantitation of Lipid 654 and Lipid 430 in bacteria and tissue samples, showed ratio of Lipid 430/Lipid 654 is increased in diseased tissue samples. It was indicated that PLA2 hydrolysis of Lipid 654 to Lipid 430 can account for lower level of Lipid 430 in diseased (atheroma) samples and may present a potential link between PLA2 activity and atherosclerosis. The Combination of ^{18}O-labeling and MS enabled us to further characterize the hydrolysis reaction of Lipid with PLA2 enzymes. One or two ^{18}O atoms reside on the $C_{15:0}$ fatty acid upon hydrolysis of Lipid 654. Time-course experiment revealed that the incorporation of the second ^{18}O could be due to the slow release of cleaved fatty acid from active

site. cLC-MRM MS also showed preparations of PLA2 enzymes are stereoselective towards hydrolysis of Lipid 654; PLA2 preparations selectively hydrolyze R-Lipid 654 isoform.

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4 Conclusions and perspectives

Changes in the bacterial populations in specific locations in the body, particularly the gastrointestinal tract and the oral cavity, are reported to be associated with localized diseases such as periodontal disease as well as systemic diseases such as autoimmune diseases. Furthermore, relationships between periodontal microbiota and vascular disease are also reported. Hence, a considerable work has centered on characterization of products of human microbiome and their roles in triggering innate immune responses that may promote chronic inflammatory disease. Toll-like receptors (TLRs) are innate immune receptors. These receptors have evolved to recognize conserved products unique to microbial metabolism. Of note, a significant structure-activity relationship controlling TLR2 binding and resulting activation of immune responses.

As discussed in chapter 1, the present work is split into two distinct sections. First part of the present work has focused on developing mass spectrometry (MS)-based technologies to study a subclass of complex lipids called lipopeptides that engage TLR2 and to investigate their relationship with human disease. Notably, recognition of lipopeptides with TLR2 and subsequent immune responses can be stereoselective. Separation power of chiral liquid chromatography (cLC) and diastereomeric mixture of isotopically labeled ISs were utilized to develop a novel method of cLC stable isotope dilution multiple reaction monitoring MS (cLC-SID-MRM MS) to simultaneously determine structure and amount of stereoisomers of bacteria-derived lipopeptides. Elucidation of complete structure of lipopeptides requires determination of absolute configuration of all chiral building blocks; chiral amino acids and 3-hydroxyalkanoic acid. This process can be very time-consuming. Also, enantiopure ISs are required to accurately quantify stereoisomers of

lipopeptides. cLC-SID-MRM MS leverages the need for enantiopure ISs for quantitation of each stereoisomers, which are not always available or costly to make. The present work utilized a diastereomeric mixture of (R,S)-Lipid 654 as model compounds and showed that cLC-SID-MRM MS can be used to simultaneously determine the configuration of 3-hydroxy $C_{17:0}$ moiety of bacterial Lipid 654 and quantify bacterial Lipid 654 in total lipid extract of nine bacteria strains.

In the second part of the presented study, LC-MRM MS approaches were implemented to quantify Lipid 654 and their related lipopeptides in healthy and disease tissue samples and compare these results to those of bacteria samples. It was shown that there is a significant amount of Lipid 654 in these tissue samples and more importantly, the level of Lipid 430, one of hydrolytic products of Lipid 654, is elevated in diseased tissue samples. Therefore, we hypothesized that there is a relationship between hydrolysis of these lipopeptides and human disease such as atherosclerosis. LC-SID-MRM MS showed that hydrolysis of Lipid 654 to Lipid 430 is the most plausible explanation for the observed lower level of Lipid 654 in atheroma samples. The association between sPLA2 activity and atherosclerosis, and between periodontal conditions and vascular disease have been investigated separately in other large-scale studies. Furthermore, it is suggested that increased activity of PLA2s might provide a mechanistic explanatory link for the relationship between dysbiotic periodontal microbiota and vascular diseases. Herein, presented work showed another possible link between increased activity of PLA2 enzymes, periodontal pathogens and atherosclerosis. It was shown, for the first, that preparations of PLA2 enzymes catalyze the hydrolysis reaction of a non-glycerol phospholipid. PLA2s hydrolyze $C_{15:0}$ ester-bound fatty acid of Lipid 654. The combination of ^{18}O-lebaling and MS was successfully utilized to further investigate PLA2 hydrolysis of Lipid 654. It was indicated that the cleavage site PLA2 hydrolysis of Lipid 654 is similar to that of standard substrates of PLA2; the bond between oxygen and

carbonyl group. However, contrary to PLA2 hydrolysis of PC (standard substrate for PLA2), combination of ^{18}O-lebaling and a time-course experiment strongly suggested that there is a slow release of cleaved fatty acid upon PLA2 hydrolysis of Lipid 654, which lead to incorporation of two ^{18}O by cleaved $C_{15:0}$. Finally, cLC-MRM MS was utilized to investigate stereoselectivity of lipopeptide hydrolysis. It was shown that the described PLA2 enzymes predominantly hydrolyze R-isoform of Lipid 654.

Overall, this work utilized various LC and MS methods to study bacteria-derived lipopeptides and proposed a potential link between PLA2 hydrolysis of these lipopeptides and human disease such as atherosclerosis. The ultimate goal of the present work was to use bioanalytical technologies to answer compelling questions in biology and in particular in regards to human disease. However, the research presented in this thesis has raised more questions that it has answered. Of note, further mechanistic investigation of PLA2 hydrolysis of Lipid 654, preferably using recombinant human PLA2, is required to validate the results obtained in this presented research. Also, a chemical description of the action of PLA2 in regards to hydrolysis of Lipid 654 can be inferred with high confidence if one uses a high resolution x-ray crystal structure of PLA2 in a complex with Lipid 654 or its analogues. As for quantitation, collecting more clinically-relevant samples to be analyzed in order to validate the presented results is of great interest. Finally, more biological assays need to be designed to further explore the role of described lipopeptides and their hydrolytic products. New diagnostic biomarkers of human health that include members of the human microbiota and chemical messengers they form, and Pharmaceutical applications based on novel enzymatic activities that are associated with the human microbiota are ultimate goals of the present work.