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The two major classes of complex lipids in biological materials are glycerolipids and sphingolipids, which are substituted derivatives of glycerol and of sphingosine or a related long-chain base respectively. We have developed approaches involving predominantly electrospray ionization (ESI) and tandem mass spectrometry (MS²) on tandem quadrupole and quadrupole linear ion trap instruments to characterize both glycerolipids and sphingolipids, and among the biomedically important areas to which we have applied these approaches is the characterization of the complex lipids of pathogenic microorganisms, particularly with respect to differences in lipid synthetic pathways in such organisms compared to mammals in order to identify potential target processes for antimicrobial drug development.

**Mass Spectrometry of Glycerolipids.** The simplest glycerolipids contain only fatty acid substituents esterified to the glycerol backbone, and we have developed positive ion ESI/MS/MS methods for charactering both neutral acyl glycerols and fatty acids as Li⁺ adducts, which yield a much more informative fragmentation pattern than the protonated species or adducts with other metal ions. Glycerophospholipids contain fatty acids esterified to the sn-1, sn-2, or both of those carbon atoms and a phosphodiester moiety linked to a polar head-group in the sn-3 position, such as choline, ethanolamine, inositol, serine, or glycerol. Glycerophosphocholine lipids are most

![Figure 1. Triacylglycerol. R₁, R₂, and R₃ denote aliphatic chains.](image)

![Figure 2. Glycerophospholipids. The head-group X defines the phospholipid class.](image)
readily characterized by ESI/MS$^n$ as Li$^+$ adducts in positive ion mode, which yield more informative tandem spectra than do species that are protonated or adducted with other examined ions. Glycerolipids with ethanolamine, serine, inositol, or glycerol head-groups are in general more readily characterized by negative ion ESI/MS$n$ by collisionally activated dissociation (CAD) of [M – H]- ions, although positive ion spectra of metal adducts of these compounds may also be informative in some cases. We have described approaches for structural characterization of all major glycerolipid classes by ESI/MS$n$ and have prepared a recent of these studies. We have also recently published a description of a computer algorithm for automatic identification of glycerophospholipid molecular species from raw electronic mass spectrometric data.

**Mass Spectrometry of Sphingolipids.** Ceramide is a relatively simple sphingolipid that contains a fatty acid in an amide linkage to the amine moiety of the long-chain base. Ceramide molecular species differ in the identity of the fatty acid substituent and of the long-chain base and can be characterized a Li$^+$ adducts by positive ion ESI/MS$^n$ or by negative ion analyses. We

![Figure 3. Sphingolipid Structural Classes.](image-url)
have also developed ESI/MS" approaches for structural characterization of the sphingolipids sphingomyelin, sulfatide, and glycosphingolipids, all of which are substituted ceramides. In sphingomyelin phosphocholine is linked via a phosphodiester group to the hydroxyl moiety on the long-chain base of serine, and in glycosphingolipids, various carbohydrates are linked to that hydroxyl moiety. Sulfatide is a glycosphingolipid in which the carbohydrate substituent is galactose substituted with a sulfate group. We have also prepared recent reviews describing our approaches to the structural characterization of sphingolipid molecular species.

Mass Spectrometry of Complex Lipids from Pathogenic Microorganisms. We have applied the ESI/MSn methods that we have developed to the characterization of complex lipids in pathogenic microorganisms including *Leishmania major*, the etiologic agent in visceral and cutaneous Leishmaniasis; pathogenic bacteria, including the gram-negative organisms *Salmonella typhimurium* and *Escherichia coli* and the gram-positive organism *Streptococcus pyogenes*; and mycobacterial species, including *Mycobacterium tuberculosis*.

Visceral Leishmaniasis is a fatal illness and cutaneous Leishmaniasis frequently afflicts U.S. soldiers in Iraq as the so-called “Baghdad boil.” We have identified a novel pathway for of sphingolipid metabolism involved in ethanolamine biosynthesis in these organisms that is not used in mammals, and it therefore represents a potential target for development of therapeutic drugs with antimicrobial activity toward *Leishmania major*.

Among gram-negative bacteria, *Salmonella* species are frequent causes of gastrointestinal illnesses, and *E. coli* is the organism that most commonly causes urinary tract infections in humans. We have characterized mechanisms underlying resistance to antimicrobial agents in *Salmonella* that involve modifications of complex lipids in the cell envelope. Our findings could facilitate the design of more effective antibiotics against gram-negative bacteria, such as *Salmonella* and *E. coli*.

We have also characterized a novel enzyme involved in the synthesis of phosphatidylinositol mannosides and have developed MS methods to structurally characterize such molecules in the cell wall of *Mycobacterium tuberculosis*, the causative organism in human tuberculosis, that might also represent a target for development of antimycobacterial drugs, which is an area of intense current interest in view of the emergence of multi-drug resistant strains of this organism.

Reviews. Our MS approaches to characterization of glycerolipids and sphingolipids have recently been reviewed in the following book chapters:


II.) Core Technologic Research and Development: Mass Spectrometry of Glycerolipids

A.) Neutral Acylglycerols and Free Fatty Acids by ESI/MS/MS

A.1.) Structural characterization of triacylglycerols as lithiated adducts by electrospray ionization mass spectrometry using low-energy collisionally activated dissociation on a triple stage quadrupole instrument. Tandem mass spectra of lithiated adducts of triacylglycerol (TAG) species obtained by electrospray ionization mass spectrometry (MS) with low-energy collisionally activated dissociation (CAD) on a triple stage quadrupole instrument have been obtained and found to distinguish isomeric triacylglycerol species and permit assignment of the mass of each fatty acid substituent and positions on the glycerol backbone to which substituents are esterified (1). Source CAD-MS\(^2\) experiments permit assignment of double bond locations in polyunsaturated fatty acid substituents. The ESI/MS/MS spectra contain \([M + Li - (R_n CO_2H)]^+\), \([M + Li - (R_n CO_2Li)]^+\), and \(R_n CO^+\) ions, among others, that permit assignment of the masses of fatty acid substituents. Relative abundances of these ions reflect positions on the glycerol backbone to which substituents are esterified. The tandem spectra also contain ions reflecting combined elimination of two adjacent fatty acid residues, one of which is eliminated as a free fatty acid and the other as an alpha, beta-unsaturated fatty acid. Such combined losses always involve the \(sn-2\) substituent, and this feature provides a robust means to identify that substituent. Fragment ions reflecting combined losses of both \(sn-1\) and \(sn-3\) substituents without loss of the \(sn-2\) substituent are not observed. Schemes are proposed to rationalize formation of major fragment ions in tandem mass spectra of lithiated TAG that are supported by studies with deuterium-labeled TAG and by source CAD-MS\(^2\) experiments. These schemes involve initial elimination of a free fatty acid in concert with a hydrogen atom abstracted from the alpha-methylene group of an adjacent fatty acid, followed by formation of a cyclic intermediate that decomposes to yield other characteristic fragment ions. Determination of double bond location in polyunsaturated fatty acid substituents of TAG is achieved by source CAD experiments in which dilithiated adducts of fatty acid substituents are produced in the ion source and subjected to CAD.
in the collision cell. Product ions are analyzed in the final quadrupole to yield information on double bond location (1).

![Tandem mass spectrum of the dilithiated adduct of arachidonic acid upon ESI/MS/MS](image)

**Figure 2. Tandem mass spectrum of the dilithiated adduct of arachidonic acid upon ESI/MS/MS**

**A.2. Distinction among isomeric unsaturated fatty acids as lithiated adducts by electrospray ionization mass spectrometry using low energy collisionally activated dissociation on a triple stage quadrupole instrument.** Features of tandem mass spectra of dilithiated adduct ions of unsaturated fatty acids obtained by electrospray ionization mass spectrometry with low-energy collisionally activated dissociation (CAD) on a triple stage quadrupole instrument have been found to distinguish among isomeric unsaturated fatty acids and permit assignment of double-bond location (2). Informative fragment ions reflect cleavage of bonds remote from the charge site on the dilithiated carboxylate moiety. The spectra contain radical cations reflecting cleavage of bonds between the first and second and between the second and third carbon atoms in the fatty acid chain. These ions are followed by a closed-shell ion series with members separated by 14 m/z units that reflect cleavage of bonds between the third and fourth and then between subsequent adjacent pairs of carbon atoms. This ion series terminates at the member reflecting cleavage of the carbon-carbon single bond vinylic to the first carbon-carbon double bond. Ions reflecting cleavages of bonds distal to the double bond are rarely observed for monounsaturated fatty acids and are not abundant when they occur. For polyunsaturated fatty acids that contain double bonds separated by a single methylene group, ions reflecting cleavage of carbon-carbon single bonds between double bonds are abundant, but ions reflecting cleavages distal to the final double bond are not. Cleavages between double bonds observed in these spectra can be rationalized by a scheme involving a six-membered transition state and subsequent rearrangement of a bis-allylic hydrogen atom to yield a terminally unsaturated charge-carrying fragment and elimination of a neutral alkene. The location of the beta-hydroxy-alkene moiety in ricinoleic acid can be demonstrated by similar methods. These observations offer the opportunity for laboratories that have tandem quadrupole instruments but do not have instruments with high energy CAD capabilities to assign double bond location in unsaturated free fatty acids by mass spectrometric methods without derivatization (2).
B.) Glycero-phosphocholine Lipids by ESI/MS/MS

B.1.) Formation of lithiated adducts of glycerophosphocholine lipids facilitates their identification by electrospray ionization tandem mass spectrometry. Electrospray ionization (ESI) tandem mass spectrometry (MS) has simplified analysis of phospholipid mixtures, and, in negative ion mode, permits structural identification of picomole amounts of phospholipid species. Collisionally activated dissociation (CAD) of phospholipid anions yields negative ion tandem mass spectra that contain fragment ions representing the fatty acid substituents as carboxylate anions. Glycerophosphocholine (GPC) lipids contain a quaternary nitrogen moiety and more readily form cationic adducts than anionic species, and positive ion tandem mass spectra of protonated GPC species contain no abundant ions that identify fatty acid substituents.

Figure 3. Initial loss of trimethylamine from [M + Li]$^+$ of phosphatidylcholine species.

We have found that lithiated adducts of GPC species are readily formed by adding lithium hydroxide to the solution in which phospholipid mixtures are infused into the ESI source (3). CAD of [MLi]$^+$ ions of GPC species yields tandem mass spectra that contain prominent ions representing losses of the fatty acid substituents. These ions and their relative abundances can be used to assign the identities and positions of the fatty acid substituents of GPC species. Tandem mass spectrometric scans monitoring neutral losses of the head-group or of fatty acid substituents from lithiated adducts can be used to identify GPC species in tissue phospholipid mixtures. Similar scans monitoring parents of specific product ions can also be used to identify the fatty acid substituents of GPC species, and this facilitates identification of distinct isobaric contributors to ions observed in the ESI/MS total ion current (3).

B.2.) Electrospray ionization/tandem quadrupole mass spectrometric studies on phosphatidylcholines: the fragmentation processes. Low-energy collisionally activated dissociation (CAD) tandem quadrupole mass spectrometry was used to study the fragmentation pathways of the [M + H]$^+$ and [M + Li]$^+$ ions of phosphatidylcholine (PC), generated by electrospray ionization (ESI) (4). Fragmentation pathways leading to loss of the polar head group and of the fatty acid substituents do not involve the hydrogen atoms attached to the glycerol
backbone, in contrast to previous suggestions. The pathway for formation of the major ion of m/z 184 by loss of the polar head group from the [M + H]^+ precursor of a diacyl PC involves the participation of the α-hydrogen of the fatty acyl substituents, whereas the H^+ participates in the loss of fatty acid moieties. The α-hydrogen atoms of the fatty acid substituents also participate in other major fragmentation processes, including formation of [M + Li-R_xCO_2H]^+ and [M + Li-59-R_xCO_2H]^+ ions for the [M + Li]^+ ions of diacyl PCs, when subjected to low-energy CAD. These fragmentation processes are deterred by substitution of the fatty acyl moieties with alkyl, alkenyl, or hydroxyl groups and consequentially result in a distinct product-ion spectrum for various PC, including diacyl-, plasmanyl- plasmenyl-, and lyso-PC isomers. The α-hydrogen atoms of the sn-2 fatty acyl substituents are more labile than those at sn-1. This is reflected by preferential loss of the R_xCO_2H over the R_yCO_2H observed for the [M + Li]^+ ions of diacyl PCs. Ions that result from such preferential losses permit identification and assignment of the fatty acid moieties on the glycerol backbone. New fragmentation pathways are established by tandem and source CAD tandem mass spectra of various PC molecules and their deuterium-labeled analogs, and these pathways clarify the mechanisms underlying ion formation that permits structural characterization of PC molecular species (4).

**Figure 4. Fragmentation of [M + Li]^+ ions of phosphatidylcholine species after initial loss of trimethylamine.**

Atoms that result from such preferential losses permit identification and assignment of the fatty acid moieties on the glycerol backbone. New fragmentation pathways are established by tandem and source CAD tandem mass spectra of various PC molecules and their deuterium-labeled analogs, and these pathways clarify the mechanisms underlying ion formation that permits structural characterization of PC molecular species (4).

**B.3.) Characterization of alkylacyl, alk-1- enylacyl and lyso subclasses of glycerophosphocholine by tandem quadrupole mass spectrometry with electrospray ionization.**

Positive ion tandem quadrupole mass spectrometric methods have been established for structural characterization of subclasses of sn-glycero-3-phosphocholine (GPC) lipids, including diacyl- (phosphatidyl), alkylacyl- (plasmenyl), and alk-1-enylacyl- (plasmenyl) GPC lipids and lysophosphatidylcholine (LPC) (5). Following collisionally activated dissociation, the [M + Li]^+ ions generated by electrospray ionization yield abundant informative fragment ions that permit structural determination and distinction among LPC regioisomers. In contrast, structurally
informative ions arising from \([M + H]^+\) or \([M + Na]^+\) ions are less prominent. The most abundant ion observed in the product-ion spectra of the \([M + Li]^+\) ions of plasmenyl- and plasmanyl-GPC lipid species and of LPC arises from loss of \(N(CH_3)_3\) \(([M + Li - 59]^+)\). This feature permits their distinction from a product-ion spectrum arising from diacyl-GPC lipid species, in which the \([M + Li - 183]^+\) ion reflecting loss of phosphocholine is the most prominent. These fragmentation processes have been exploited to identify various subclasses of GPC lipids in biological extracts by constant neutral loss scanning (5).

C. Glycerophosphoethanolamine Lipids by ESI/MS/MS

Figure 6. Fragmentation of \([M + Li]^+\) ions of phosphatidylethanolamine species upon ESI/MS/MS.

C.1.) Characterization of phosphatidylethanolamine as a lithiated adduct by triple quadrupole tandem mass spectrometry with electrospray ionization: positive ions. Structural characterization glycerophosphoethanolamine (GPE) species can be structurally characterized as
lithiated adducts by collisionally activated dissociation (CAD) tandem mass spectrometry with electrospray ionization (6). Abundant fragment ions reflecting the polar head group and fatty acid constituents were observed in GPE [M + Li]$^+$ product ion spectra that permit unambiguous structural assignments that include the positions of the fatty acyl substituents on the glycerol backbone. Proposed fragmentation pathways are supported by tandem mass spectra of various deuterium-labeled analogs and source CAD of GPE followed by CAD tandem mass spectrometry. GPE molecular species and specific GPE subclasses in biological mixtures can be identified by tandem mass spectrometry and constant neutral loss scanning (6).

C.2. Charge-remote and charge-driven fragmentation processes in diacyl glycerophosphoethanolamine upon low-energy collisional activation: negative ions. A mechanistic study of diacyl glycerophosphoethanolamine fragmentation under low energy collision-activated dissociation with electrospray ionization tandem mass spectrometry reveals fragmentation pathways that result in formation of carboxylate anions (R$_x$CO$_2^-$) ($x = 1$ or $2$) and of ions representing neutral losses of the fatty acids as ketenes ([M - H - R$_x$'CH=C=O]$^-$) (7). These are charge-driven processes governed by gas-phase basicity and steric configuration. Formation of [M - H - R$_x$'CO$_2$H]$^-$ ions is a charge-remote process that involves participation of the glycerol C-1 and C-2 hydrogen atoms to yield [M - H - R$_x$CO$_2$H]$^-$ > [M - H - R$_x$'CH=C=O]$^-$ (7). The observed relative abundances R$_2$CO$_2^-$ > R$_1$CO$_2^-$, and of [M - H - R$_x$'CH=C=O]$^-$ > [M - H - R$_y$'CH=C=O]$^-$ are attributable to the steric favorability of charge-driven processes at sn-2. The

![Figure 7. Fragmentation of [M - H]$^-$ ions of phosphatidylethanolamine species upon ESI/MS/MS.](image)

observed relative abundance [M - H - R$_x$'CH=C=O]$^-$ > [M - H - R$_y$CO$_2$H]$^-$ is attributed to the fact that the [M - H]$^-$ ions of GPE are basic precursor ions, which eliminate fatty acid substituents as a ketene more readily than as a free acid. The dominant route to R$_x$CO$_2^-$ ions involves
nucleophilic attack of the anionic phosphate on C-1 or C-2 and charge transfer. Steric proximity results in the observed relative abundance $R_2CO_2^- > R_1CO_2^-$. These features permit assignment of the positions of the fatty acid substituents of GPE on the glycerol backbone (7).

**D. Glycerophosphatidic Acid by ESI/MS/MS**

**D.1. Charge-driven fragmentation processes in diacyl glycerophosphatidic acids upon low-energy collisional activation.** Mechanistic studies of diacyl glycerophosphatidic acid (GPA) under low energy collisionally activated decomposition (CAD) with electrospray ionization tandem mass spectrometry reveal fragmentation pathways that result in formation of carboxylate anions $[R_xCO_2^-]$, $(x=1$ or $2)$ and ions representing neutral losses of the fatty acid substituents as a free acid $(M-H-R_xCO_2H)$ or as a ketene $(M-H-R'_xCH=CH=O)$ $(R_x=R'_xCH_2) (8)$. These are charge-driven processes governed by the gas-phase basicity and steric configuration of the molecules. The observed relative abundances of ions reflecting losses of the fatty acid substituents are $(M-H-R_xCO_2H) > (M-H-R_1CO_2H)$ and $(M-H-R'_xCH=CH=O) > (M-H-R'_1CH=CH=O)$. This is attributable to the fact that losses of the $sn$-2 fatty acid substituent as a free acid and or as a ketene is sterically favorable. The observed relative abundances of losses of a given fatty acid substituent as a free acid or ketene is $(M-H-R_xCO_2H) > (M-H-R'_xCH=CH=O)$. This is attributable to gas phase acidity of the GPA $(M-H)$ ion, which undergoes neutral loss of the free acid more readily than the ketene. The dominant route to the $R_xCO_2^-$ ion under low energy CAD involves neutral loss of 136 from $(M-H-R_xCO_2H)$ and results in a relative

![Figure 7. Fragmentation of $[M - H]$ ions of phosphatidic acid species upon ESI/MS/MS.](image-url)
The abundance of $R_1CO_2 > R_2CO_2^-$. The differential formation of these carboxylate anions permits assignment of the positions of the fatty acid substituents of GPA on the glycerol backbone (8).

E.) **Glycerophosphoinositol Lipids and Their Metabolites by ESI/MS/MS**

![Figure 8. Structure of phosphoinositide species.](image)

**E.1. Characterization of phosphatidylinositol, phosphatidylinositol-4-phosphate, and phosphatidylinositol-4,5-bisphosphate by electrospray ionization tandem mass spectrometry: a mechanistic study.** Structural characterization of phosphatidylinositol (PI), phosphatidylinositol-4-phosphate (PI-4P), and phosphatidylinositol-4,5-bisphosphate (PI-4,5-P₂) has been achieved by collisionally activated dissociation (CAD) tandem mass spectrometry with electrospray ionization (9). In negative ion mode, the major fragmentation pathways under low energy CAD for PI arise from neutral loss of free fatty acid substituents ([M - H - R\textsubscript{1}CO\textsubscript{2}H]) and neutral loss of the corresponding ketenes ([M - H - R\textsubscript{1}'CH=C=O]), followed by loss of the inositol head group. The intensities of the ions arising from neutral loss of the sn-2 substituent as a free fatty acid ([M - H - R\textsubscript{2}CO\textsubscript{2}H]) or as a ketene ([M - H - R\textsubscript{2}'CH=C=O\textsuperscript{+}]) are greater than those of ions reflecting corresponding losses of the sn-1 substituent. This is consistent with our demonstration elsewhere that ions reflecting those losses arise from charge-driven processes that occur preferentially at the sn-2 position. These features permit assignment of the positions of the fatty acid substituents on the glycerol backbone. Nucleophilic attack of the anionic phosphate on glycerol C-1 or C-1 to which the fatty acids are attached results in formation of the sn-1 (R\textsubscript{1}CO\textsubscript{2}⁻) or sn-2 (R\textsubscript{2}CO\textsubscript{2}⁻) carboxylate anion, respectively, and this is sterically more favorable at sn-2 than at sn-1. Alternate routes to R\textsubscript{1}CO\textsubscript{2}⁻ ions include decomposition of [M - H - R\textsubscript{1}CO\textsubscript{2}H - inositol], [M - H - R\textsubscript{1}'CO\textsubscript{2}H], or [M - H - R\textsubscript{1}CH=C=O]) ions, and formation by these routes varies with collision energy. The relative intensities of R\textsubscript{1}CO\textsubscript{2}⁻ ions thus do not reliably reflect the positions of the fatty acid substituents on the glycerol backbone. The spectra also contain the ion series m/z 315, 279, 259, 241, and 223 that reflect the inositol head group. The last three of those ions are also observed in tandem spectra of [M - H]⁻ ions of phosphatidylinositol monophosphate (PI-P) and phosphatidylinositol bisphosphate (PI-P₂). Tandem spectra of PI-P and PI-P₂ [M - H]⁻ ions also contain ions of m/z 321 and 303 that reflect doubly phosphorylated inositol ions. The PI-P₂ spectrum also contains unique ions of m/z 401 and 383 that reflect triply phosphorylated inositol ions. [M - H]⁻ ions of PI-P and PI-P₂ undergo fragmentation similar to that of PI upon CAD, but
the doubly charged ([M - 2H]^2+) ions undergo fragmentation typical of glycerophosphoethanolamine [M - H] ions, which are basic. These results suggest that the [M - 2H]^2+ ions of PI-P and PI-P_2 are also basic in the gas phase (9).

E.2.) **Structural distinction among inositol phosphate isomers using high-energy and low-energy collisional-activated dissociation tandem mass spectrometry with electrospray ionization.** Electrospray (ESI) collisionally activated dissociation (CAD) tandem mass spectrometric methods for the structural characterization of inositol phosphates (InsPs) using both quadrupole and sector mass spectrometers have been established (10). Under low-energy CAD, [M + H]^+ ions of the positional isomers of inositol phosphates, including inositol mono-, bis- and trisphosphates, yield distinguishable product-ion spectra, but these isomers are less readily distinguishable by product-ion spectra from high-energy CAD (2 keV collision energy, floating at 50%) tandem sector mass spectrometers. Differences in the product-ion spectrum profiles among the InsP isomers do permit differentiation of positional isomers when the collision energy is reduced to 1 keV (floating at 75%). These results demonstrate that the applied collision energy plays a pivotal role in fragmentation upon CAD. Product-ion spectra are similar among positional isomers of inositol tetrakisphosphates and of inositol pentakisphosphates. Thus, isomeric distinction for these two inositol polyphosphate classes could not be achieved by tandem mass spectrometric methods that do distinguish less highly phosphorylated inositol phosphate classes. Under both high- and low-energy CAD, [M + H]^+ ions of all InsPs undergo similar fragmentation pathways, which are dominated by the consecutive losses of \( \text{H}_2\text{O}, \text{HPO}_3 \) and \( \text{H}_3\text{PO}_4 \) (10).

![Figure 9. Tandem mass spectra of inositol trisphosphates isomers upon ESI/MS/MS.](image)
F.) Cardiolipin (Diphosphatidyglycerol) by ESI/MS

Figure 10. Structure of cardiolipin species.

F.1.) Structural characterization of cardiolipin by tandem quadrupole and multiple-stage quadrupole ion-trap negative ion mass spectrometry with electrospray ionization. Negative-ion electrospray tandem mass spectrometric methods for structural characterization of cardiolipin (CL) have been established (11). CL is a four-acyl-chain phospholipid that contains two distinct phosphatidyl moieties. Structural assignment of the fatty acid residues attached to the glycerol backbones performed by low-energy CAD tandem mass spectrometry had not been previously described. The low-energy MS²-spectra of the [M - H]⁻ and [M - 2H]⁻ ions obtained with ion-trap or with tandem quadrupole instruments combined with ion-trap MS³-spectra or with source CAD product-ion spectra permit detailed structural characterization of CL. MS²-spectra of [M - H]⁻ ions contain two sets of prominent fragment ions that include a phosphatidate, a phosphatidylglycerol dehydration product, and a (phosphatidic acid + 136) anion. Substantial differences in abundances of the two distinct phosphatidate anions in the MS²-spectra of the [M - H]⁻ ions permit assignment of the phosphatidyl moieties attached to the 1' or 3' position of the central glycerol. Upon further collisional dissociation, MS³-spectra of the phosphatidate anions identify the fatty acyl substituents and their positions on the glycerol backbone. MS²-spectra of the [M - 2H]⁻ ions obtained with a triple stage quadrupole or an ion trap mass spectrometer contain complementary information that confirms structural assignments. These methods have been used to differentiate cardiolipin isomers and to identify complex cardiolipin mixtures that include multiple molecular species (11).

F.2.) Characterization of cardiolipins as sodiated adducts by positive-ion electrospray ionization with multiple stage quadrupole ion-trap mass spectrometry. Multiple-stage ion-trap (IT) mass spectrometric methods for the structural characterization of cardiolipin (CL) were developed (12). CL is 1,3-bisphosphatidyl-sn-glycerol and contains four fatty acyl substituents and three glycerol moieties (designated A, B, and central glycerol, respectively). CL species were analyzed as sodiated adducts in positive-ion mode. Upon collisionally activated dissociation (CAD), the [M - 2H + 3Na]⁺ ions of CL yield two prominent fragment ion pairs that consist of the phosphatidyl moieties attached to the 1'- and 3'-position of the central glycerol, respectively, that arise from differential losses of diacylglycerol moieties containing A and B glycerol, respectively. The results are consistent with those described above (5) for [M - H]⁻ and
[M - 2H + Na]+ ions in negative-ion mode and permit assignment of the two phosphatidyl moieties attached to the 1'- or 3'-position of the central glycerol. Identities of the fatty acyl substituents and their positions on the glycerol backbones (glycerol A and B) are deduced from further decomposition of these ion pairs to yield fragment ions that reflect the fatty acid substituents at the sn-1 (or sn-1') and sn-2 (or sn-2') positions. Ions that arise from losses of fatty acid substituents at sn-1 and sn-1', respectively, are prominent, but the analogous ions from losses of the fatty acid substituents at sn-2 and sn-2', respectively, are of low abundance in MS² product-ion spectra. This feature confirms assignment of positions of fatty acid substituents. Similar IT multiple-stage MS approaches involving CAD of [M + Na]+ and the [M - H + 2Na]+ ions and MS² and MS³ can also be used for structural characterization of CL, but the abundance of [M + Na]+ and the [M - H + 2Na]+ ions of CL is variable and not readily predictable (12).

G.) Glycerophosphoserine Lipids by ESI/MS/MS

![Diagram of fragmentation of [M - H] ions of phosphatidylserine species upon ESI/MS/MS.](image)

Figure 11. Fragmentation of [M - H] ions of phosphatidylserine species upon ESI/MS/MS.

G.1.) Studies on phosphatidylserine by tandem quadrupole and multiple stage quadrupole ion-trap mass spectrometry with electrospray ionization: structural characterization and the fragmentation processes. Low-energy CAD product-ion spectra of various molecular species of phosphatidylserine (PS) as [M-H] and [M-2H+Alk] ions in negative-ion mode and as [M+H]+, [M+Alk]⁺, [M-H+2Alk]⁺, and [M-2H+3Alk]⁺ (where Alk = Li or Na) in positive-ion mode contain abundant fragment ions that permit detailed structural assignments (13). Upon CAD, the PS [M-H] ions eliminate the serine moiety (loss of C₃H₅NO₂) to yield an [M-H-87]⁺ ion, which is equivalent to the [M-H]⁺ ion of the corresponding phosphatidate (PA) species, and the MS³-spectrum of the [M-H-87]⁺ ion is identical to the MS²-spectrum of PA [M-H]. CAD of the PS [M-2H+Alk]⁺ ion induces elimination of the serine moiety and yields an [M-2H+Alk-87]⁺ ion, which then eliminates the fatty acid substituents as free acids (RₓCO₂H, x = 1 or 2) or as alkali salts (e.g., RₓCO₂Li, x = 1 or 2). The resultant [M-2H+Alk-87-RₓCO₂H]⁺ ion is more abundant than [M-2H+Alk-87-RₓCO₂Li]⁺ ion, and the [M-2H+Alk-87-RₓCO₂Li]⁺ ion is more abundant than the [M-2H+Alk-87-RₓCO₂Li]⁺ ion. This permits assignment of the positions of the fatty acid substituents. Further dissociation of [M-2H+Alk-87-Rₓ(or Rᵧ)CO₂Li]⁺ ions results in preferential
formation of the \( sn-1 \) carboxylate anion \((R_1\text{CO}_2^-) \) relative to \((R_2\text{CO}_2^-) \). Another major fragmentation process involves differential loss of the fatty acid substituents as ketenes (loss of \( Rx'\text{CH}=\text{CO} \), \( x = 1 \) or \( 2 \)). This results in greater abundance of the \([M-2\text{H}+\text{Alk}-R_2'\text{CH}=\text{CO}]^+ \) ion relative to the \([M-2\text{H}+\text{Alk}-R_1'\text{CH}=\text{CO}]^+ \) ion. Structurally informative fragment ions are not abundant in the MS²-spectra of PS \([M+\text{H}]^+ \) or \([M+\text{Alk}]^+ \) ions, but such ions are abundant in MS³-spectra. The MS²-spectrum of the PS \([M+\text{Alk}]^+ \) ion contains a unique ion that corresponds to internal loss of the phosphate moiety probably via rearrangement. CAD of the PS \([M-H+2\text{Alk}]^+ \) ion yields an abundant \([M-H+2\text{Alk}-87]^+ \) ion, which is equivalent to an alkaline adduct of a monoalkaline PA salt. That ion then eliminates the fatty acid substituents to yield an intense \([M-H+2\text{Alk}-87-R_1'\text{CO}_2\text{H}]^+ \) ion and a less abundant \([M-H+2\text{Alk}-87-R_2'\text{CO}_2\text{H}]^+ \) ion. Similarly, CAD of the PS \([M-2\text{H}+3\text{Alk}]^+ \) ion yields a prominent \([M-2\text{H}+3\text{Alk}-87]^+ \) ion that differentially eliminates the two fatty acyl substituents. All of these tandem mass spectra contain several sets of ion pairs that reflect differential losses of the fatty acid substituents as ketenes or as free fatty acids, and this permits identification of the fatty acyl substituents and their positions on the glycerol backbone (13).

H.) **Glycerophosphoglycerol Lipids by ESI/MS/MS**

H.1.) *Electrospray ionization multiple stage quadrupole ion-trap and tandem quadrupole mass spectrometric studies on phosphatidylglycerol from Arabidopsis leaves.* Phosphatidylglycerol (PG) is the major phospholipid of plant chloroplasts. PG from *Arabidopsis thaliana* has an unusual fatty acyl chain, 3-trans-hexadecenoyl \((\Delta^3-16:1)\) in the \( sn-2 \) position of the major \( 18:3/(\Delta^3-16:1) \)-PG species and in \( 18:2/(\Delta^3-16:1) \)-PG and \( 16:0/(\Delta^3-16:1) \)-PG (14). Upon low-energy collisionally activated dissociation (CAD) in a tandem quadrupole or an ion-trap mass spectrometer, PG \([M - H]^+ \) ions that contain \( \Delta^3-16:1 \) yields product ion spectra that are
readily distinguishable from those of PG molecules without the Δ³-16:1 substituent (8). The (Δ³-
16:1)-containing PG species yield MS² product-ion spectra that contain predominant [M - H -
236]¹ ions that reflect loss of Δ³-16:1 as a ketene. This is attributable to the fact that the α-
hydrogen atom of Δ³-16:1 involved in the ketene loss is a labile allylic hydrogen atom. This
results preferential neutral loss of 236 relative to neutral loss of 254 (i.e., loss of Δ³-16:1 as a free
fatty acid). These features identify (Δ³-16:1)-containing PG species. Neutral loss of 236 scanning
provides a sensitive tandem quadrupole mass spectrometric means to identify (Δ³-16:1)-
containing PG species in lipid mixtures. This low-energy tandem mass spectrometric approach
also permits structural identification of Arabidopsis PG isomers (14).

I. Distinction Among Plasmenyl-, Plasmanyl-, and Phosphatidyl Glycerophospholipids.

I.1. Differentiation of 1-O-alk-1’-enyl-2-acyl and 1-O-alkyl-2-acyl glycerophospholipids by
multiple-stage linear ion-trap mass spectrometry with electrospray ionization. We developed
electrospray ionization/linear ion-trap mass spectrometric approaches involving MS³ and MS⁴

Figure 13. Fragmentation of [M - H] ions of plasmenyl- (A) and plasmenyl- (B) ethanolamine species on
ESI/MS/MS.

studies in negative ion mode for structural characterization of 1-O-alk-1’-enyl-2-acyl-
(plasmenyl-), 1-O-alkyl-2-acyl- (plasmanyl-), and diacyl- (phosphatidyl-) glycerophospholipids
(GPL) of various head-group classes (15). [M – H]¹ ions of such GPL yield [M – H – R₂CO₂H –
polar head group] ions upon CAD, and fragmentation of those ions upon further CAD provides
structural information about the sn-1 radyl group. The MS³ (or MS⁴) spectra from CAD of the [M
– H – R₂CO₂H – polar head group] ion from plasmenyl-GPL are dominated by an alkenoxide anion that represents the sn-1 radyl moiety, while such spectra for plasmanyl-GPL are dominated by an ion of m/z 135 that arises from neutral loss of the 1-O-alkyl moiety as an alcohol. The optimal collision energy to obtain such spectra is significantly lower for plasmenyl- than for plasmanyl-GPL. These approaches permitted us to distinguish among isomeric compounds from various GPL classes in biological mixtures. Because [M – H]⁺ ions are readily formed from several GPL head-group classes (e.g., -ethanolamine, -inositol, and -serine), this strategy should have broad applicability in the structural identification of GPLs and represents a means to distinguish among plasmenyl-, plasmanyl-, and phosphatidyl-GPL species that does not depend on non-mass spectrometric criteria that require off-line manipulation, such as determination of acid-lability (15).

J.) Glycerophospholipid Analyses by MALDI/TOF/MS

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Table 1. Ionic-liquid matrices for analysis of phospholipids by MALDI-TOF mass spectrometry.

J.1.) Ionic-liquid matrices for improved analysis of phospholipids by MALDI-TOF mass spectrometry. The use of ionic liquid matrices (ILMs) for MALDI/TOF/MS of phospholipids (PLs) affords higher signal intensity, smaller spot size, improved spot homogeneity, better signal reproducibility, and comparable or better detection limits compared to that of the crystalline matrix 2,5-dihydroxybenzoic acid (2,5-DHB) (16). Ionization products are comparable to those with 2,5-DHB although the use of ILMs gives a stronger tendency to produce alkali-metal-ion adducts and a lower extent of prompt fragmentation (16).
Computer Algorithm to Identify Glycerolipid Species in Mixtures from MS Data

**Algorithm for Processing Raw Mass Spectrometric Data to Identify and Quantitate Complex Lipid Molecular Species in Mixtures by Data-Dependent Scanning and Fragment Ion Database Searching.** A Lipid Qualitative/Quantitative Analysis (LipidQA) software platform has been developed to identify and quantitate complex lipid molecular species in biological mixtures (17). LipidQA can process raw electronic data files from the TSQ-7000 triple stage quadrupole and LTQ linear ion trap mass spectrometers from Thermo-Finnigan and the Q-TOF hybrid quadrupole/time-of-flight instrument from Waters-Micromass and could readily be modified to accommodate data from others. The program processes multiple spectra in a few seconds and includes a deisotoping algorithm that increases the accuracy of structural identification and quantitation. Identification is achieved by comparing MS^2 spectra obtained in a data-dependent manner to a library of reference spectra of complex lipids that have been acquired or constructed from established fragmentation rules. The current form of the algorithm can process data acquired in negative or positive ion mode for glycerophospholipid species of all major head-group classes (17). To identify glycerolipid species from an observed spectrum, the algorithm compares that spectrum to a library of reference spectra of glycerolipids that we have acquired from standard materials or constructed from fragmentation rules for glycerolipid molecular species that we have recently reviewed (18).

**Glycerophospholipid Mass Spectrometry Literature Cited:**


2. **Hsu FF & Turk J.** Distinction among isomeric unsaturated fatty acids as lithiated adducts by electrospray ionization mass spectrometry using low energy collisionally


III.) Core Technologic Research and Development: Mass Spectrometry of Sphingolipids

A.) Ceramides by ESI/MS/MS

A.1.) Characterization of ceramides by low energy collisionally activated dissociation tandem mass spectrometry with electrospray ionization in negative ion mode. Negative-ion electrospray ionization tandem quadrupole mass spectrometry is useful for structural characterization of ceramides (1). Fragment ions that reflect the identities of the fatty acid substituent and of the long chain base of these molecules are abundant and permit structural identification ceramides. An novel fragmentation pathway results in formation of fatty acid carboxylate anions (RCO$_2^-$) and is initiated by cleavage of the LCB C2-C3 bond to yield a N-acylaminoethanol anion ([RCONHCH$_2$CH$_2$O]$^-$) that rearranges to form a carboxyethylamine ([RCO$_2$CH$_2$CH$_2$NH]$^-$) intermediate, which dissociates to yield RCO$_2^-$. The proposed pathway is supported by CAD tandem mass spectra of the synthetic N-acylaminoethanol standard and of deuterated ceramide analogs obtained by H/D exchange. The RCO$_2^-$ ion unambiguously identifies the ceramide fatty acyl moiety. Structural isomers of ceramides can be identified by product ion scanning, and specific ceramide subclasses can be identified in biological mixtures with constant neutral loss scanning approaches (1).

A.2.) Structural studies on ceramides as lithiated adducts by low energy collisionally activated dissociation tandem mass spectrometry with electrospray ionization in positive ion mode. Fragmentation pathways for more than 30 ceramide molecular species in nine structural subclasses have been studied with Electrospray ionization (ESI) tandem quadrupole mass spectrometry and low energy collisionally-activated dissociation (CAD) (2). Product-ion spectra of ceramide [M + Li]$^+$ ions contain abundant fragment ions that identify the fatty acyl substituent and the long-chain base (LCB) of the molecules, and this permits the structures of ceramide species to be assigned. Fragment ions specific for each ceramide subclass are also observed. Ions arising from classical C-C bond cleavages described for fast-atom bombardment (FAB)-high energy tandem mass spectra are not observed, but product-ion spectra contain multiple structurally informative fragment ions that distinguish among isomers. Tandem mass spectra of fragment ions generated by in-source CAD (pseudo-MS$^3$) and of deuterium-labeled species obtained by H/D exchange support structural assignments and the fragmentation pathways proposed to explain them (2).
B.) **Sphingomyelin by ESI/MS/MS**

Figure 1. Sphingomyelin.

B.1.) **Structural determination of sphingomyelin species as alkali metal adducts by tandem mass spectrometry with electrospray ionization in positive ion mode.** Alkali metal adduct ions of sphingomyelin can be formed by electrospray ionization in positive ion mode (3). Under low energy collisionally activated dissociation (CAD), the product ion spectra yield abundant fragment ions that identify both the long chain base and fatty acid substituent, and this permits unequivocal structural assignments. Constant neutral loss scanning permits identification of sphingomyelin classes and species that contain specific long chain bases in mixtures. Proposed fragmentation pathways under CAD are supported by source CAD tandem mass spectrometric analyses. Such methods have been used to analyze sphingomyelin mixtures from bovine brain, bovine erythrocytes, and chicken egg yolk (3).

C.) **Glycosphingolipids by ESI/MS/MS**
C.1.) Structural determination of glycosphingolipids as lithiated adducts by electrospray ionization mass spectrometry using low energy collisionally activated dissociation on a triple stage quadrupole instrument in positive ion mode. Glycosphingolipids can be structurally characterized as Li$^+$ adducts using low-energy collisional-activated dissociation (CAD) tandem mass spectrometry with electrospray ionization (ESI) (4). The tandem mass spectra contain abundant fragment ions reflecting the long chain base (LCB), fatty acid, and the sugar constituent of the molecule and permit unequivocal identification of cerebrosides, di- and tri-hexosyl ceramides, and globosides. Major fragmentation pathways involve loss of the sugar moiety to yield a lithiated ceramide ion, which undergoes further fragmentation to form multiple fragment ions that confirm the structures of the fatty acid and LCB. Mechanisms for ion formation and the probable configurations of fragment ions resulting from CAD of monoglycosylceramide [M + Li]$^+$ ions have been established and are supported by CAD and source CAD tandem mass spectra of various cerebrosides and of analogs prepared by H-D exchange. Constant neutral loss and precursor ion scans can be used to identify galactosylceramides with sphingosine or sphinganine LCB moieties and those with specific N-2-hydroxyl fatty acid substituents in mixtures (4).

D.) Sulfatides by ESI/MS/MS

![Sulfatide (I)](image)

D.1.) Electrospray ionization tandem mass spectrometric analysis of sulfatide in negative ion mode on a triple quadrupole instrument: Determination of fragmentation patterns and characterization of molecular species expressed in brain and in pancreatic islets. The sphingolipid sulfatide is a component of myelin and some non-neuronal cells. Antibodies to sulfatide occur in some patients with autoimmune neuropathies and in patients with insulin-dependent diabetes mellitus (IDDM) caused by immunologic destruction of insulin-secreting pancreatic islet beta-cells. Distinct sulfatide molecular species may differ in immunogenicity, and facile means to identify sulfatide species in islets and other tissues obtainable in only small amounts could be useful. Electrospray ionization mass spectrometry (ESI/MS) permits structural determination of small quantities of phospholipids and is applied here to sulfatide analysis. We find that sulfatide standards are readily analyzed by negative ion ESI/MS, and tandem mass
spectra of individual species exhibit some ions common to all species and other ions that reflect distinct fatty acid substituents in different sulfatide molecules (5). A signature ion cluster resulting from cleavage directed by the alpha-hydroxy group of sulfatide species with an hydroxylated fatty acid substituent identifies such species. Sulfatide profiles in tissue lipid extracts can be obtained by ESI/MS/MS scanning for common sulfatide ions and for ions reflecting fatty acid substituents. Islets are demonstrated to contain sulfatide and to exhibit a profile of species different from that of brain (5).

D.2.) Studies on sulfatides by quadrupole ion-trap mass spectrometry with electrospray ionization in negative ion mode: Structural characterization and fragmentation processes. Structural characterization of sulfatides by collisional-activated dissociation (CAD) quadrupole ion-trap tandem mass spectrometric methods with electrospray ionization has been achieved (6). CAD of sulfatide [M - H] ions yield abundant structurally informative ions that permit unequivocal assignments of the long-chain base (LCB) and fatty acid substituent, including double bond location. The location of the fatty acyl substituent double bond can be determined from an ion series that arises from classical charge-remote fragmentation in a manner similar to that observed with high-energy CAD and tandem quadrupole mass spectrometry. An unusual internal galactose residue loss that occurs via rearrangement is also observed. Sulfatide [M - H] ions also dissociate to a ceramide anion, which subsequently fragments to yield ions that identify the ceramide moiety and distinguish sphingosine and sphinganine LCB. MS² spectra of sulfatides with a sphingosine LCB and an α-hydroxy fatty acyl substituent (d18:1/hFA-sulfatide) contain prominent sets of ions of m/z 568, 550, 540, and 522 that arise from primary cleavage of the fatty acyl CO-CH(OH) bond. Such ions are of low abundance with sulfatides with a sphingosine LCB and a non-hydroxy-fatty acid substituent (d18:1/nFA-sulfatide), and this permits distinction between sulfatides with hFA and nFA fatty acid substituents. Proposed pathways of sulfatide fragmentation under low-energy CAD are supported by MS² and MS³ spectra of multiple standard sulfatide molecular species and of their analogs prepared by H-D exchange (6).

E.) Sphingolipid Mass Spectrometry Reviews and Biomedical Applications: We have reviewed our studies on tandem mass spectrometric characterization of sulfatides and of other sphingolipids (7, 8) and have applied these methods to determine the structures of complex sphingolipids from pathogenic microorganisms (9, 10).

F.) Sphingolipid Mass Spectrometry Literature Cited:


4.) **Hsu FF & Turk J.** Structural determination of glycosphingolipids as lithiated adducts by electrospray ionization mass spectrometry using low-energy collisional-activated


IV.) Core Technologic Research and Development: Mass Spectrometry of Complex Lipids from Pathogenic Microorganisms

A.) Leishmania major sphingolipids and ethanolamine lipids

A.1.) Sphingolipids are essential for differentiation but not growth in Leishmania. Sphingolipids (SLs) play critical roles in eukaryotic cells in the formation of lipid rafts, membrane trafficking, and signal transduction. We created a SL-null mutant in the protozoan parasite Leishmania major through targeted deletion of the key de novo biosynthetic enzyme serine palmitoyltransferase subunit 2 (SPT2) (1). Although SLs are typically essential, spt2-Leishmania were viable, yet were completely deficient in de novo sphingolipid synthesis, and lacked inositol phosphorylceramides and other SLs. Remarkably, spt2- parasites maintained

![Figure 1. Negative ion ESI/MS spectra of total lipids purified from log phase WT (A) and spt2- (B) cells. The assigned identities of peaks are indicated. Abbreviations: p18:0/18:2-PE, 1-O-octdec-1’-enyl-2-octadecadienoyl-sn-glycero-3-phosphethanolamine (plasmalogen); p18:0/18:1-PE, 1-O-octadec-1’-enyl 2-octadecenoyl-sn-glycerophosphoethanolamine; d16:1/18:0-Pl-Cer, phosphorylinositol N-stearoyl-hexadecesphing-4-enine (IPC-d16:1); d18:1/18:0-Pl-Cer, phosphorylinositol N-stearoylsphingosine (IPC-d18:1); 18:0/18:1-Pl, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoinositol; 16/18:1-Pl, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoinositol; a18:0/18:1-Pl, 1-octadecanoyl-2-octadecenoyl-sn-glycero-3-phosphoinositol (plasmanyl inositol).]
'lipid rafts' as defined by Triton X-100 detergent resistant membrane formation. Upon entry to stationary phase spt2- failed to differentiate to infective metacyclic parasites and died instead. Death occurred not by apoptosis or changes in metacyclic gene expression, but from catastrophic accumulation of small vesicles characteristic of the multivesicular body/multivesicular tubule network. Stage specificity may reflect changes in membrane structure as well as elevated demands in vesicular trafficking required for parasite remodeling during differentiation. SL-deficient Leishmania provide a useful biological setting for tests of essential SL enzymes in other organisms where SL perturbation is lethal (1).

A.2.) **Leishmania salvage and remodelling of host sphingolipids in amastigote survival and acidocalcisome biogenesis.** Although sphingolipids (SLs) are essential in most eukaryotes, the

![Image](image-url)

**Figure 2. Amastigotes contain abundant parasite-specific IPCs.** Lipids from log phase WT (A) or spt2- (B) promastigotes, from foot pads of uninfected mice (C), or amastigotes purified from mice infected with WT (D) or spt2- (E) parasites, were extracted and analyzed by negative-ion ESI/MS using a precursor ion scan of m/z 241 (specific for IPCs and PIs). Before lipid extraction, a PI standard (16:0/16:0-PI at m/z 809.8) was added as internal standard.
A.3.) **Characterization of inositol phosphorylceramides from Leishmania major by tandem mass spectrometry with electrospray ionization.** We have developed tandem mass spectrometric approaches, including multiple stage ion-trap and source collisionally activated dissociation (CAD) tandem mass spectrometry with electrospray ionization (ESI) to characterize inositol phosphorylceramide (IPC) species as [M - H]⁻ and [M - 2H + Li]⁻ ions in negative-ion mode and as [M + H]⁺, [M + Li]⁺, and [M - H + 2Li]⁺ ions in positive-ion mode (3). Following CAD in an ion-trap or a triple-stage quadrupole instrument, IPC [M - H]⁻ ions yield fragment ions reflecting only the inositol and the fatty acyl substituent. In contrast, spectra from MS² of [M - H - Inositol]⁻ ions contain abundant ions that identify the fatty acid and long-chain base (LCB) moieties. Product-ion spectra from MS² and MS³ of [M - 2H + Alk]⁺, [M + H]⁺, [M + Alk]⁺, and [M - H + 2Alk]⁺ ions also contain fragment ions that permit unambiguous assignment of the fatty acyl substituent and the LCB, but the abundance of those precursor ions is about ten-fold lower than that of [M - H]⁻ ions, which limits sensitivity. In addition to the major fragmentation pathways involving elimination of the inositol or inositol monophosphate moiety, several structurally informative ions result from rearrangement processes, and the fragmentation processes are similar to those we previously reported for ceramides. Although the MS⁵ (n = 2, 3) tandem mass spectrometry permits detailed structural assignment of *Leishmania major* IPC species, including isomer discrimination, constant neutral loss scans provide a simple method for detecting IPC species in biological mixtures (3).

![Fragmentation of Li⁺ adducts of inositol-phosphorylceramides upon ESI/MS/MS.](image)

A.4.) **Redirection of sphingolipid metabolism toward de novo synthesis of ethanolamine in Leishmania.** In most eukaryotes, sphingolipids (SLs) are critical membrane components and signaling molecules, but mutants of the trypanosomatid protozoan *Leishmania* lacking serine palmitoyltransferase (spt2-) and SLs grow well but are defective in stationary phase differentiation and virulence. Similar phenotypes are observed in sphingolipid (SL) mutant lacking the degradatory enzyme sphingosine 1-phosphate lyase (spl-). This epistatic interaction implicates a metabolite downstream of SLs, we have shown that, unlike other organisms, the *Leishmania* SL pathway has evolved to be the major route for ethanolamine (EtN) synthesis, as EtN supplementation completely reversed the viability and differentiation defects of both mutants (4). Thus, *Leishmania* has undergone two major metabolic shifts: first in de-emphasizing the metabolic roles of SLs themselves in growth, signaling, and maintenance of membrane structures, then in shifting to the de novo synthesis of EtN.
Figure 4. Synthesis of Sphingolipids and Phosphatidylethanolamine in Eukaryotes. Open block arrows represent pathways not present in Leishmania. Filled block arrows represent dominant metabolic routes in Leishmania promastigotes. ADS1 = 1-alkyl dihydroxyacetone phosphate synthase; SDC = serine decarboxylase*; PSS = phosphatidylserine synthase*; BE = base exchange enzyme (phosphatidylserine synthase 2 or PSS2); PSD = phosphatidylserine decarboxylase; Smy = sphingomyelin* GSL = glycosphingolipid; DAG = diacylglycerol; DHAP = dihydroxyacetone phosphate; * denotes absent in Leishmania.

Microdomains, which may arise from the unique combination of abundant parasite lipids; Second, freed of typical SL functional constraints and a lack of alternative routes to produce EtN, Leishmania redirected SL metabolism toward bulk EtN synthesis. These results thus reveal a striking example of remodeling of the SL metabolic pathway in Leishmania (4).

B.) Complex Lipids of Pathogenic Bacteria

B.1.) Gram-Negative Bacteria: Salmonella species

B.1.a.) Characterization of acylphosphatidylglycerols from Salmonella typhimurium by tandem mass spectrometry with electrospray ionization. Acylphosphatidylglycerol (Acyl-PG), a polar lipid that contains three fatty acyl groups, has been isolated from Salmonella bacteria and characterized by tandem quadrupole and quadrupole ion-trap mass spectrometry with electrospray ionization. For acyl-PG species with various acyl groups (A-B/C-PG, where A, B, and C differ from each other) (R<sub>2</sub>CO<sub>2</sub>−) that arise from sn-2 (R<sub>3</sub>CO<sub>2</sub>−) are more abundant than those that arise from sn-3' (R<sub>3</sub>CO<sub>2</sub>−). In addition, R<sub>3</sub>CO<sub>2</sub>− is much more abundant than R<sub>1</sub>CO<sub>2</sub>−) (5). This permits assignment of the positives of the fatty acyl substituents. In addition, the
Figure 5. Structure of acylphosphatidylglycerols in Salmonella.

1,2-diacyl-sn-glycero-3-phospho-(3'-acyl)-1'-sn-glycerol (I)

fragment ion reflecting loss of the sn-2 fatty acid substituent as a ketene is more abundant than the ion reflecting loss of that substituent as a free acid (i.e., [M - H - R₂CH=CO] > [M - H – R₃CO₂H]). In contrast, the sn-1 or sn-3' fatty acid substituents are eliminated more readily as a free acid than as a ketene (i.e., [M - H - R₃CO₂H] > [M - H - R₁CH=CO]; [M - H - R₂CO₂H] > [M - H –R₃CH=CO]). The identity of the sn-3' acyl moiety is also reflected by an acyl-glycerophosphate anion in the product-ion spectrum from a triple-stage quadrupole (TSQ) instrument but not in that from an ion-trap mass spectrometer (ITMS). However, the MS²-spectrum obtained with an ITMS is featured by the ion series that abundances of [M - H – R₂CH=CO – R₃CO₂H - 74] > [M - H - R₁CH=CO – R₃CO₂H - 74]; [M - H - R₁(or3')CH=CO – R₃(or 1)CO₂H - 74]. Structural identification of acyl-PG species with two identical fatty acyl substituents at sn-1, sn-2, or sn-3' or of members of mixtures that contain isomers can also be achieved with this approach (5).

B.1.b.) The PmrA-regulated pmrC gene mediates phosphoethanolamine modification of lipid A and polymyxin resistance in Salmonella enterica. The PmrA/PmrB regulatory system of Salmonella enterica controls the modification of lipid A with aminoarabinose and phosphoethanolamine. The aminoarabinose modification is required for resistance to the antibiotic polymyxin B, as mutations of the PmrA-activated pbg operon or ugd gene result in strains that lack aminoarabinose in their lipid A molecules and are more susceptible to polymyxin B. Additional PmrA-regulated genes appear to participate in polymyxin B resistance, as pbgP and ugd mutants are not as sensitive to polymyxin B as a pmrA mutant. Moreover, the role that the phosphoethanolamine modification of lipid A plays in the resistance to polymyxin B has remained unknown. We addressed both of these questions by establishing that the PmrA-activated pmrC gene encodes an inner membrane protein that is required for the incorporation of phosphoethanolamine into lipid A and for polymyxin B resistance (6). The PmrC protein consists of an N-terminal region with five transmembrane domains followed by a large periplasmic region harboring the putative enzymatic domain. A pbgP pmrC double mutant resembled a pmrA
Figure 6. Lipid A species profiles from wild-type (14028s) (A), pbgP (EG9241) (B), ΔpmrC1.1 (EG14590) (C), ΔpmrC1.1/ppmrC (EG14595) (D), and ΔpmrC1.1/vector (EG14656) (E) strains grown to logarithmic phase in N-minimal medium, pH 5.8, with 10 µM MgCl₂, and analyzed by negative-ion-mode MALDI-TOF mass spectrometry. These profiles show that the pmrC mutant lacks lipid A species modified with phosphoethanolamine.
mutant both in its lipid A profile and in its susceptibility to polymyxin B, indicating that the PmrA-dependent modification of lipid A with aminoarabinose and phosphoethanolamine is responsible for PmrA-regulated polymyxin B resistance (6).

B.1.c.) **PhoP-regulated Salmonella resistance to the antimicrobial peptides magainin 2 and polymyxin B.** In *Salmonella enterica*, the PhoP-PhoQ two-component system governs resistance to structurally different antimicrobial peptides including the alpha-helical magainin 2, the beta-sheet defensins and the cyclic lipopeptide polymyxin B. To identify the PhoP-regulated determinants mediating peptide resistance, we prepared a plasmid library from a phoP mutant, introduced it into a phoP mutant and selected for magainin-resistant clones (7). One of the clones

![Figure 7. PhoP-regulated lipid A modifications associated with resistance to antimicrobial peptides.](image)

The formation of monophosphorylated lipid A is mediated by the UgtL protein, which is required for resistance to both magainin 2 and polymyxin B. Although UgtL is shown as acting at the 1 position of lipid A, it is presently unclear whether it affects the 1 and/or 4’ positions. The PagP-mediated transfer of a palmitate from phospholipids to lipid A is required for resistance to magainin 2 but not to polymyxin B. PmrA-controlled loci encode enzymes involved in the biosynthesis and incorporation into lipid A of 4-aminoarabinose, which is necessary for resistance to polymyxin B but not magainin 2. Although the 4-aminoarabinose modification is shown as acting at the 4’ position of lipid A, it is presently unclear whether it affects the 1 and/or 4’ positions.
harboured the PhoP-activated ugtL gene, deletion of which rendered *Salmonella* susceptible to magainin 2 and polymyxin B, but not defensin HNP-1. We established that ugtL encodes an inner membrane protein that promotes the formation of monophosphorylated lipid A in the lipopolysaccharide. Inactivation of both ugtL and the regulatory gene pmrA, which controls lipid A modifications required for resistance to polymyxin B (but not to magainin 2) and is post-translationally activated by the PhoP-PhoQ system, resulted in a strain that was as susceptible to polymyxin B as a phoP mutant. The most frequently recovered clone harboured the yqjA gene, which we show is PhoP regulated and required for resistance to magainin 2 but not to polymyxin B or defensin HNP-1. Our results indicate that different PhoP-mediated modifications in lipid A are necessary for resistance to different antimicrobial peptides (7).

**B.1.d.) Identification of the lipopolysaccharide modifications controlled by the *Salmonella* PmrA/PmrB system mediating resistance to Fe(III) and Al(III).** Iron is an essential metal but

![Figure 8. PmrG is a Phosphatase in *Salmonella* that Targets the Hep(II) Phosphate of Lipopolysaccharide (LPS) and Localizes to the Periplasm.](image)

Schematic representation of LPS phosphates targeted by Fe(III) resistance determinants. PmrC protein mediates modification of lipid A phosphates with phosphoethanolamine whereas Ugd and PbgPE proteins modify lipid A phosphates with 4-aminoarabinose. PmrG protein removes phosphate from Hep(II) in the LPA inner core region normally introduced by RfaY protein.
can be toxic in excess. While several homeostatic mechanisms prevent oxygen-dependent killing promoted by Fe(II), little is known about how cells cope with Fe(III), which kills by oxygen-independent means. Several Gram-negative bacterial species harbour a regulatory system - termed PmrA/PmrB - that is activated by and required for resistance to Fe(III). We now report the identification of the PmrA-regulated determinants mediating resistance to Fe(III) and Al(III) in Salmonella enterica serovar Typhimurium. We establish that these determinants remodel two regions of the lipopolysaccharide, decreasing the negative charge of this major constituent of the outer membrane (8). Remodeling entails the covalent modification of the two phosphates in the lipid A region with phosphoethanolamine and 4-aminoarabinose, which has been previously implicated in resistance to polymyxin B, as well as dephosphorylation of the Hep(II) phosphate in the core region by the PmrG protein. A mutant lacking the PmrA-regulated Fe(III) resistance genes bound more Fe(III) than the wild-type strain and was defective for survival in soil, suggesting that these PmrA-regulated lipopolysaccharide modifications aid Salmonella's survival and spread in non-host environments (8).

B.2.) Gram-Negative Bacteria: Escherichia coli

B.2.a.) Characterization of cardiolipin from Escherichia coli by electrospray ionization with multiple stage quadrupole ion-trap mass spectrometric analysis of [M - 2H + Na]+ ions. We have developed a multiple-stage ion-trap (IT) mass spectrometric approach with electrospray ionization (ESI) for structural characterization of cardiolipin (CL) species by collisionally activated dissociation (CAD) of their [M - 2H + Na]+ ions (9). CL is a 1,3-bisphosphatidyl-sn-glycerol that consists of four fatty acyl chains and three glycerol moieties designated A, B, and central glycerol, respectively. Upon CAD, CL [M - 2H + Na]- ions yield two prominent...
fragment ions that arise from the differential losses of the diacylglycerol (DAG) moieties containing A or B glycerol, respectively. Ions that arise from loss of the DAG moiety containing glycerol B are more abundant than those arising from DAG that contains glycerol A. This permits assignment of the two phosphatidyl moieties attached to the 1' or 3'-position of the central glycerol. The structures of those two ions, including the identities of the fatty acyl substituents and their positions on the glycerol A and B backbones, are determined by MS³ experiments. This approach permits assignment of the structures of CL species, including isomers, in a mixture isolated from Escherichia coli (9).

B.3.) Gram-Positive Bacteria: Streptococcus pyogenes

B.3.a.) Anionic lipids enriched at the ExPortal of Streptococcus pyogenes. The ExPortal of Streptococcus pyogenes is a membrane microdomain dedicated to the secretion and folding of

![Figure 10. Lipid profile of streptococcal membranes.](image)

The electrospray ionization/mass spectrometry spectra of the lipid extracts arising from the [M-H]⁻ ions of cardiolipin from Cls⁻ cells (panel A) and from the WT (panel B). Panels C and D show the [M-H]⁻ ions of phosphatidylglycerol species from the extracts shown in panels A and B, respectively. Ions from the (12:0)₄-cardiolipin internal standard seen at m/z 1239.8 (panels A and B) are [M-H]⁻ ions, while the ions seen at m/z 619.7 (panels C and D) are [M-2H]⁻ ions. Both the [M-H]⁻ (panel A) and [M-2H]⁻ (panel C) ions of cardiolipin are absent in the lipid extract from Cls⁻ cells but are abundant in the lipid extract from WT cells (panels B and D). In contrast, phosphatidylglycerol is abundant from Cls⁻ cells (panel C) and is of relatively low abundance in the WT (panel D).
proteins. We investigated the lipid composition of the ExPortal by examining the distribution of anionic membrane phospholipids (10). Staining with 10-N-nonyl-acridine orange revealed a single microdomain enriched with an anionic phospholipid whose staining characteristics and behavior in a cardiolipin-deficient mutant were characteristic of phosphatidylglycerol. Furthermore, the location of the microdomain corresponded to the site of active protein secretion at the ExPortal. These results indicate that the ExPortal is an asymmetric lipid microdomain, whose enriched content of anionic phospholipids may play an important role in ExPortal organization and protein trafficking (10).

C.) **Complex Lipids of *Mycobacterium tuberculosis* and other *Mycobacterial* species**

C.1.) *Identification and macrophage-activating activity of glycolipids released from intracellular Mycobacterium bovis BCG.* Intracellular mycobacteria release cell wall glycolipids into the endosomal network of infected macrophages. We have characterized the glycolipids of

![Figure 11. Mass Spectrometry of culture-derived BCG lipids that co-migrate with intracellularly released lipids.](image)

A and B. ESI mass spectra of the [M-H] ions of 1,2-diacyl-sn-glycero-3-phosphatidylinositol dimannoside (PIM₃) species differing in the degree of additional acylation. The major species of PIM₂ₐ (A) at m/z 1413 (C₁₆:₀-C₁₉:₀-PIM₂) contains and additional C₁₆:₀, whereas the major species of PIM₂₇ (B) at m/z 1693 (C₁₆:₀-C₁₉:₀/C₁₆:₀-PIM₂) contains an additional C₁₆:₀ and C₁₉:₀.
**Mycobacterium bovis** BCG (BCG) that are released into murine bone marrow-derived macrophages (BMMØ) (11). Intracellularly released mycobacterial lipids were harvested from BMMØ that had been infected with \(^{14}\)C-labelled BCG. Released BCG lipids were resolved by thin-layer chromatography, and they migrated similarly to phosphatidylinositol dimannosides (PIM2), mono- and diphosphatidylglycerol, phosphatidylethanolamine, trehalose mono- and dimycolates and the phenolic glycolipid, mycoside B. Culture-derived BCG lipids that co-migrated with the intracellularly released lipids were purified and identified by electrospray ionization mass spectrometry. When delivered on polystyrene microspheres, fluorescently tagged BCG lipids were also released into the BMMØ, in a manner similar to release from viable or heat-killed BCG bacilli. To determine whether the released lipids elicited macrophage responses, BCG lipid-coated microspheres were delivered to interferon gamma-primed macrophages (BMMØ or thioglycollate-elicited peritoneal macrophages), and reactive nitrogen intermediates as well as tumour necrosis factor-alpha and monocyte chemoattractant protein-1 production were induced. When fractionated BCG lipids were delivered on the microspheres, PIM2 species reproduced the macrophage-activating activity of total BCG lipids. These results demonstrate that intracellular mycobacteria release a heterogeneous mix of lipids, some of which elicit the production of proinflammatory cytokines from macrophages that could potentially contribute to the granulomatous response in tuberculous diseases (11).

C.2.) **Mycobacterium tuberculosis** Rv2252 encodes a diacylglycerol kinase involved in the biosynthesis of phosphatidylinositol mannosides (PIMs). Phosphorylated lipids play important roles in biological systems, not only as structural moieties but also as modulators of cellular function. Phospholipids of pathogenic bacteria play roles both as membrane components and as factors that modulate the infectious process. **Mycobacterium tuberculosis** has an extremely
diverse repertoire of biologically active phosphorylated lipids that, in the absence of a specialized protein translocation system, appear to constitute the main means of communication with the host. Many of these lipids are derived from phosphatidylinositol (PI) that is differentially processed to give rise to phosphatidylinositol mannosides (PIMs) or lipoarabinomannan. In preliminary studies on the lipid processing enzymes associated with the bacterial cell wall, a kinase activity was noted that gave rise to a novel lipid species released by the bacterium. It was determined that this kinase activity was encoded by the ORF Rv2252 (12). Rv2252 demonstrates the capacity to phosphorylate various amphipathic lipids of host and bacterial origin, in particular a M. tuberculosis-derived diacylglycerol. Targeted deletion of the rv2252 gene resulted in disruption of the production of certain higher order PIM species, suggesting a role for Rv2252 in the biosynthetic pathway of PI, a PIM precursor (12).

C.3.) **Structural characterization of phosphatidyl-myo-inositol mannosides from Mycobacterium bovis Bacillus Calmette Guérin by multiple-stage quadrupole ion-trap mass spectrometry with electrospray ionization. I. PIMs and lyso-PIMs.** We have developed multiple-stage ion-trap mass spectrometric approaches to characterize structures of phosphatidylinositol and phosphatidyl-myoinositol mannosides (PIMs) in a complex mixture from *Mycobacterium bovis Bacillus Calmette Guérin* (13). Positions of the fatty acyl substituents of PIMs at the glycerol backbone can be assigned are reflected by the fact that ions arising from losses of the sn-2 fatty acid substituent as a free acid or as a ketene, respectively (i.e., [M - H – R₂CO₂H]⁻ and [M - H – R₂CHCO⁻]), are more abundant than ions arising from analogous losses at sn-1 (i.e., [M - H – R₁CO₂H]⁻ and [M - H – R₁CHCO⁻]) in MS³ product-ion spectra of [M - H]⁺ ions produced by electrospray ionization (ESI). [M - H – R₂CO₂H]⁻ and [M - H – R₁CO₂H]⁻ ions decompose to yield a pair of unique ions corresponding to losses of 74 and 56 Da (i.e., [M - H – R₂CO₂H - 56]⁻ and [M - H – R₂CO₂H - 74], x = 1 or 2), respectively. This probably reflects elimination of various glycerol-derived moieties. The relative abundance of members of this ion-pair in the MS³ spectrum of [M - H – R₂CO₂H]⁻ is quite difference from that in the MS³ spectrum of the [M - H – R₁CO₂H]⁻, and this permits assignment of the fatty acid substituents and their positions on the glycerol backbone. Product-ion spectra of [M - H]⁺ ions from 2-lyso-PIM and 1-lyso-PIM are distinguishable and both spectra contain a unique ion that arises from primary loss of the fatty acid substituent, followed by loss of a bicyclic glycerophosphate ester moiety of 136

\[
\begin{align*}
\text{PIM}_x : & \ x = Y = H \\
\text{monoacyl-PIM}_x : & \ x = R_2CO; \ y = H; \\
\text{diacyl-PIM}_x : & \ x = R_2CO; \ y = R_2CO.
\end{align*}
\]

![Figure 13. Structure of phosphatidyl-myo-inositol mannosides from Mycobacterium bovis.](image-url)
Da. Combined information from MS² and MS³ product-ion spectra permit detailed assignments of the structures of PIMs, including isomer discrimination (13).

C.4. Structural characterization of phosphatidyl-myo-inositol mannosides from Mycobacterium bovis Bacillus Calmette Gúerin by multiple-stage quadrupole ion-trap mass spectrometry with electrospray ionization. II. Monoacyl- and diacyl-PIMs. Multiple-stage ion-trap mass spectrometric approaches for characterizing monoacyl-PIM (triacylated PIM) and diacyl-PIM (tetracylated PIM) have been developed (14). Identification of the fatty acid substituents and their positions on the glycerol backbone can be achieved as described above. The identity of the glycoside moiety and its acylation state is reflected by a prominent acylglycoside ion arising from cleavage of the diacylglycerol moiety ([M - H - diacylglycerol]⁻) in the MS²-spectra of monoacyl-PIM and diacyl-PIM. Fatty acid substituents on the 2-O-mannoside (i.e., R₃CO₂H) can be distinguished from those on the inositol (i.e., R₄CO₂H) because the MS³-spectrum of [M - H - diacylglycerol]⁻ contains a prominent ion arising from further loss of the fatty acid substituent on the 2-O-mannoside (i.e., [M - H - diacylglycerol – R₃CO₂H⁻]), while the ion arising from loss of the fatty acid substituent on the inositol moiety (i.e., the [M-H-diacylglycerol–R₄CO₂H⁻] ion) is of low abundance. The fatty acyl substituent on the inositol

Figure 14. Fragmentation of phosphatidyl-myo-inositol mannosides from M. bovis upon ESI/MS/MS.
moiety can be identified by the MS^4 product-ion spectrum of [M–H - diacglycerol–R_CO2H], which contains a prominent ion corresponding to loss of R_CO2H. An [M - H - acylmannose] ion is also observed in the MS^2-spectra and also reveals the identity of the fatty acid substituent on the 2-O-mannoside moiety. Combined information from MS^2, MS^3, and MS^4 multiple-stage product-ion spectra permit identification of monoacyl-PIMs and diacyl-PIMs in a mixture from *M. bovis Bacillus Calmette Guérin*.

**D.) Complex Lipids from Pathogenic Microorganisms Literature Cited:**

13.) **Hsu FF**, **Turk J**, Owens RM, Rhoades ER, & Russell DG. Structural characterization of phosphatidyl-myoinositol mannosides from *Mycobacterium bovis* Bacillus Calmette