Elucidation and identification of amino acid-containing membrane lipids using liquid chromatography-high resolution mass spectrometry

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ABSTRACT

RATIONALE: Intact polar lipids (IPLs) are the building blocks of cell membranes, and amino acid-containing IPLs have been observed to be involved in response to changing environmental conditions in various species of bacteria. High performance liquid chromatography-mass spectrometry (HPLC/MS) has become the primary method for analysis of IPLs. Many glycerol-free amino acid-containing membrane lipids (AA-IPLs), which are structurally different than abundant aminophospholipids, have not been characterized using HPLC/MS. This results in many lipids remaining unrecognized in IPL analysis of microbial cultures and environmental samples, hampering the study of their occurrence and functionality.

METHODS: We analyzed the amino acid-containing IPLs of a number of bacteria (i.e. *Gluconobacter cerinus*, *Cyclobacterium marinus*, *Rhodobacter sphaeroides*, and *Pedobacter heparinus*) in order to decipher fragmentation pathways, and explore potential novel lipid structures using HPLC/electrospray ionization-ion trap-MS (HPLC/ESI-IT-MS) and HPLC/high resolution-MS (HPLC/HRMS).

RESULTS: We report differentiation between glutamine and lysine lipids with the same nominal masses, novel MS fragmentation pathways of cytolipin, the lipopeptides cerilipin and flavolipin, head group hydroxylated ornithine lipids, and the novel identification of cerilipin with a hydroxylated fatty acid.

CONCLUSIONS: Non-glycerol AA lipids can be readily recognized as their fragmentation follows a clear pattern with initial dehydration or other loss from the head group, followed by

22 fatty acid losses resulting in a diagnostic fragment ion. Higher level MS^n and HRMS are

23 valuable tools in characterizing AA lipid head group structural components.

INTRODUCTION

Intact polar lipids (IPLs) are the building blocks of cell membranes, typically consisting of a polar head group (phosphatidylcholine, digalactosyldiacylglycerol, etc.) and an apolar core, typically a diglyceride or diether, and are considered to represent living biomass since polar head groups are rapidly lost following cell lysis.[1,2] The structures of IPLs can be taxonomically and environmentally specific, making them potentially useful biomarker molecules.[3,4] Some glycerol-based IPLs contain amino acids in the polar head group, such as phosphatidylserine or homoserine-containing betaine lipids.

A special group of membrane lipids is the glycerol-free amino acid IPLs (AA-IPLs), which are different than aminophospholipids, such as the common phosphatidylethanolamine and phosphatidylcholine. In these AA-IPLs the headgroup consists of an amino acid which is linked via an amide-bond to a β-hydroxy fatty acid, esterified to a fatty acid (Fig. 1). Ornithine lipids (OL) contain the amino acid ornithine as the headgroup and are common phosphorus-free membrane lipids among bacteria, but up to date OLs have not been observed in eukaryotes or archaea. Approximately 50% of bacterial species whose genomes have been sequenced are predicted to have the capacity to form OLs based on the presence of either the *Ols*BA or *Ols*F genes coding for enzymes involved in the OL biosynthetic pathway.[5-8] In addition, it has been speculated that OLs are important for Gram-negative bacteria outer membrane stability because of their zwitterionic character.[9] In certain bacteria, the production of OLs increases under phosphorus limitation,[10,11] and in other microbes OL fatty acids are hydroxylated under temperature or pH stress.[12-14] These studies show that OLs are used by certain bacteria to modify their membrane in response to changing environmental conditions.

Other AA-IPLs have been identified in various microbes, including ornithine-taurine lipid (cerilipin, CL), glycine lipid (cytolipin, CYL), serine-glycine lipid (flavolipin, FL), and

glutamine lipid (GL; Fig. 1).[15-19] Recently, novel mono-, di-, and trimethylornithine (MMO, DMO, TMO) IPLs and hydroxylysine-containing IPLs (lysine lipids hydroxylated on the fatty acid and/or lysine headgroup) were characterized from northern wetland planctomycetes and soil bacteria, respectively, and it was suggested that these modifications were related to stress conditions (Fig. 1).[20,21] It therefore seems that modification of both fatty acid composition and amino acid headgroup occur frequently, perhaps in response to changing environmental conditions, making AA-IPLs an interesting class of compounds to study.

In the last decade high performance liquid chromatography/electrospray ionization-mass spectrometry (HPLC/ESI-MS) has become the primary analytical approach for IPL analysis.[3,4,22-24] However, HPLC/MS characterization has only been accomplished for a limited number of AA-IPLs, such as OLs or betaines (diacylglyceryl-N,N,N-trimethylhomoserine; DGTS, DGTA, DGCC).[6] The AA-IPLs described above (CL, CYL, FL, TL, GL) were identified using combinations of thin layer chromatography, infrared spectrometry, mass spectrometry, or ^{1}H nuclear magnetic resonance (NMR). MS fragmentation knowledge for these and other AA-IPLs will advance their investigation in complex biological and environmental samples and potentially open new lines of research into microbial environmental stress response.

Here we report the identification of modified and novel AA-IPLs in the bacteria *Gluconobacter cerinus*, *Cyclobacterium marinus*, *Rhodobacter sphaeroides*, and *Pedobacter heparinus*, using HPLC/ESI-ion trap-MS (ESI-IT-MS) multi-stage fragmentation and high resolution accurate mass/mass spectrometry (HRMS) in order to improve our analytical capabilities for studying this interesting class of lipids.

EXPERIMENTAL

Cultures and lipid extractions – *R. sphaeroides* was grown in liquid medium LB Broth containing (g per liter of distilled water): peptone from casein, 10.0; yeast extract, 5.0; NaCl, 10.0; pH 7.0. *G. cerinus* was grown in liquid sorbitol medium (g per liter of distilled water): D-sorbitol, 50.0; peptone, 10.0; yeast extract, 10.0. *C. marinus* was grown in marine broth liquid medium containing (g per liter of distilled water): marine broth, 37.4. *P. heparinus* was grown as previously described.[25] Culture biomass was collected by centrifugation, freeze dried, and lipids were extracted by a modified Bligh & Dyer method.[26,27] The dried residue was dissolved in hexane:2-propanol:H_2O (718:271:10, v/v/v) injection solvent, and filtered through a 0.45 μm, 4 mm diameter True™ Regenerated Cellulose syringe filter (Grace Davison) prior to injection.

HPLC/ESI-IT-MS and HPLC/HRMS – IPLs were analyzed by HPLC/ESI-IT-MS according to Sturt et al.[3] with some modifications.[20,30] An Agilent 1200 series high performance liquid chromatograph, with thermostated autoinjector, was coupled to a Thermo LTQ XL linear ion trap mass spectrometer with an Ion Max source and ESI probe (Thermo Scientific, Waltham, MA). The typical lipid extract injection concentration was 2 mg/ml, and an injection volume of 10 μl. Chromatographic separation was accomplished on a Lichrosphere diol column (250 mm by 2.1 mm; 5-μm particles; Grace Alltech Associates Inc.) at 25°C. Elution was achieved with hexane–2-propanol–formic acid–14.8 M aqueous NH_3 (79:20:0.12:0.04 [vol/vol/vol/vol]) (A) and 2-propanol–water–formic acid–14.8 M aqueous NH_3 (88:10:0.12:0.04 [vol/vol/vol/vol]) (B) mobile phases starting at 10% B, followed by a linear increase to 30% B in 10 min, followed by a 20-min hold and a further increase to 65% B at 45 min. The flow rate was 0.2 ml min^{-1}, and the total run time was 60 min, followed by a 20-min re-equilibration period.

The lipid extracts were analyzed by scanning a mass range of *m/z* 400 to 2,000 in positive-ion mode, followed by data-dependent, dual-stage tandem MS (MS^2), in which the four most abundant masses in the mass spectrum were fragmented successively (source conditions: capillary temperature, 275ºC; sheath gas (N_2) pressure, 25 arbitrary units (AU); auxiliary gas (N_2) pressure, 15 AU; source voltage, 4.5 kV; tube lens, 120 V; normalized collision energy, 25; isolation width, 5.0; activation Q, 0.175). Each MS^2 was followed by data-dependent, triple-stage tandem MS (MS^3), where the base peak of the MS^2 spectrum was fragmented under identical fragmentation conditions to those described for MS^2. In the analysis of *G. cerinus* quadruple-stage tandem MS (MS^4) was used, in which the base peak of the MS^3 spectrum was fragmented under identical fragmentation conditions as previously described for MS^2 and MS^3. Multistage fragmentation conditions were selected in order to optimize the number of fragment ions observed. Additional ESI-IT-MS analysis of *P. heparinus* was performed in negative ionization mode with MS^2 fragmentation, in which the four most abundant masses in the mass spectrum were fragmented successively (source conditions: capillary temperature, 275ºC; sheath gas (N_2) pressure, 25 AU; auxiliary gas (N_2) pressure, 15 AU; source voltage, 5.5 kV; tube lens, -128.82 V; normalized collision energy, 50; isolation width, 5.0; activation Q, 0.175), and MS^3 fragmentation where the base beak of the MS^2 spectrum was fragmented under identical conditions to those described in negative-ion MS^2. The ion trap MS was calibrated using the Thermo Scientific LTQ ESI Positive Ion Calibration Solution (containing a mixture of caffeine, methionine-arginine-phenylalanine-alanine (MRFA), and Ultramark 1621 in an acetonitrile-methanol-acetic solution). The performance of HPLC/ESI-IT-MS was monitored by regular injections of platelet-activating factor (PAF) standard (1-*O*-hexadecyl-2-acetyl-*sn*glycero-3-phosphocholine).

To acquire exact mass information on the lipid extracts HPLC/high resolution accurate mass-orbitrap-mass spectrometry (HRMS) analysis was performed on a 3000 UltiMate series

LC, with thermostatted auto-injector, coupled to a Q Exactive mass spectrometer (Thermo Scientific). The chromatographic conditions were the same as described above for HPLC/ESI-IT-MS, but with the use of a YMC diol column (250 mm × 2.1 mm, 5 μm particles; YMC America, Inc.). The positive ion ESI settings were as follows: capillary temperature, 275°C; sheath gas (N_2) pressure, 35 AU; auxiliary gas (N_2) pressure, 10 AU; spray voltage, 4.0 kV; probe heater temperature, 300°C; S-lens, 50 V. Target lipids were analyzed with a mass range of *m/z* 400 to 1,000 (resolution, 70,000), followed by data dependent MS^2 (resolution, 17,500), in which the five most abundant masses in the mass spectrum were fragmented (normalized collision energy 35; isolation width 1.0). The Q Exactive was calibrated within a mass accuracy range of 1 ppm using the Pierce LTQ Velos ESI Positive Ion Calibration Solution (containing a mixture of caffeine, MRFA, Ultramark 1621, and *N*-butylamine in an acetonitrilemethanol-acetic solution; Thermo Scientific).

RESULTS AND DISCUSSION

The extracts of the four bacteria analyzed contained many well described IPLs such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylglycerol (PG), as well as many types of AA-IPLs (Fig. 2). Here, we will focus on the HPLC/MS characterization of known and novel AA-IPLs.

General MS fragmentation characteristic of AA-IPLs – OLs are the most commonly occurring AA-IPLs and were detected in all cultures in this study except for *C. marinus*. The MS fragmentation pattern of OLs can be used as a guide for the identification of other AA-IPLs. Characteristic multi-stage MS fragmentation of OLs includes the sequential losses of H_2O from the head group, the ester-linked fatty acid, and the amide-linked β–hydroxy-fatty acid resulting in a diagnostic *m/z* 115 fragment, representing the cyclized protonated dehydrated ornithine amino acid (Fig 3.).[6,19,22,23] Like OLs, the diagnostic MS fragment ions of other previously described AA-IPLs, typically contain the complete AA head group or smaller AA head group fragment ions.[6,15-21,28,29] These fragment ions of AA head groups are an important aid in AA IPL identification.

Differentiation between glutamine and lysine IPLs – HPLC/ESI-IT-MS analysis of the *R. sphaeroides* lipid extract revealed two low abundance IPLs with AA-IPL like fragmentation eluting at retention times 15.89 min and 26.74 min (Fig. 2A). These AA-IPLs were both characterized by a precursor protonated molecule at *m/z* 719 and fragment ions at *m/z* 129, 130, and 147. Zhang *et al*.[19] previously reported a glutamine-containing IPL (e.g. Fig. 1) in *R. sphaeroides*, generating fragment ions at *m/z* 129, 130, and 147, representing the glutamine head group. However, the Zhang et al.[19] study was done using direct infusion of the total extract and hence it was impossible to discriminate different IPLs with the same *m/z* from each other. Here we detected two peaks producing fragments indicative of glutamine

lipids, however, with HPLC/ESI-IT-MS analysis it remained unclear which of these two peaks represented the glutamine IPL and what the identity of the other peak was.

HRMS analysis yielded an assigned elemental composition to the *m/z* 147 fragment ion from the IPL eluting at 15.89 min (Fig. 4A, Table 1) of $C_5H_{11}N_2O_3$. This assignment matches the elemental composition of protonated glutamine. The elemental composition of the *m/z* 130 ($C_5H_8NO_3$) and *m/z* 129 ($C_5H_9N_2O_2$) fragment ions are consistent with a glutamine portion after losses of NH_3 and H_2O, respectively, further confirming its identity as a glutamine-containing IPL (GL)[19] (Fig. 4A, Table 1). Neutral losses representing the loss of $C_{18:1}$ and βOH-$C_{20:1}$ fatty acids were also observed after HRMS fragmentation of the GL. In contrast to the GL, HRMS analysis of the IPL eluting at 26.74 min revealed an elemental composition of the *m/z* 147 fragment ion of $C_6H_{15}N_2O_2$, matching the elemental composition of protonated lysine. The elemental composition of the *m/z* 130 ($C_6H_{12}NO_2$) and *m/z* 129 ($C_6H_{13}N_2O$) fragment ions are consistent with a lysine portion minus NH_3 and H_2O, respectively (Fig. 4B; Table 9). HRMS analysis also confirmed the presence of $C_{18:1}$ and βOH-$C_{20:1}$ fatty acids after fragmentation of the lysine lipid. This identification is in good agreement with the fragmentation of lysine IPL (LL; Fig. 1) as described by Moore et al.[21] Due to their nearly identical fragmentation spectra when using nominal mass resolution MS, lysine and glutamine lipid could not be distinguished from each other by direct infusion analysis as performed by Zhang et al.[19] Only after LC separation and/or HRMS can these lipids be distinguished from each other. This is the first detection of LL in *R. sphaeroides* and demonstrates the importance of HPLC separation when characterizing IPLs with similar MS fragmentation and diagnostic fragment ions.

Cerilipin – HPLC/ESI-IT-MS analysis of the *G. cerinus* extract showed the presence of three low abundance IPLs of *m/z* 772, 788 and 748 eluting at retention time 28.71 min, 34.15 min, and 34.37 min, respectively (Fig. 2B), with unique MS fragmentation (Fig. 5;

Table 1). The MS^2 spectrum of the *m/z* 772 IPL showed a loss of *m/z* 125 (Fig. 5B) followed by a subsequent loss of a fatty acid (Fig. 5C). *G. cerinus* is known to produce cerilipin (CL), an IPL containing an ornithine-taurine head group originally characterized by Tahara et al.[15] using thin layer chromatography (TLC), infrared (IR) spectrophotometry, gas chromatography/mass spectrometry (GC/MS), and amino acid analysis. The initial neutral loss of *m/z* 125 is expected for taurine based on typical peptide MS^2 fragmentation amino acid neutral losses.[31-33] The loss of the taurine portion results in the formation of an ornithine lipid, which as described earlier should produce the *m/z* 115 diagnostic fragment ion upon further fragmentation. Indeed, the loss of a fatty acid in MS^3 fragmentation resulted in a fragment ion at *m/z* 351 (Fig. 5C), which after MS^4 fragmentation and further loss of the β-OH fatty acid yielded the ornithine *m/z* 115 diagnostic fragment ion (Fig. 5D).[19,34] The elemental composition of the *m/z* 115 fragment ($C_5H_{11}N_2O$) was confirmed by HRMS (Table 1), however, the initial *m/z* 125 loss was not observed during HRMS fragmentation. The combined analyses confirmed that the IPL of *m/z* 772 is CL with $C_{19:1}$ and $\beta OH\text{-}C_{16:0}$ fatty acids (Table 1).

The IPLs of *m/z* 788 and 748 displayed the same multistage HPLC/ESI-IT-MS fragmentation loss pattern and HRMS *m/z* 115 fragment ion elemental composition as the IPL of *m/z* 772 also confirming their identity as CL (Table 1, 2). The fatty acid loss of the *m/z* 788 CL was *m/z* 16 greater than the fatty acid loss from *m/z* 772 CL. Together with the observed increased retention time and HRMS results (Table 1), this suggests that the IPL of *m/z* 788 is a CL, but with a hydroxylated fatty acid (CL_{HFA}; $OH\text{-}C_{19:1}$, $\beta OH\text{-}C_{16:0}$; Table 1). The *m/z* 748 is also a CL_{HFA}, but with a shorter ester-linked fatty acid ($OH\text{-}C_{16:0}$, $\beta OH\text{-}C_{16:0}$) than the *m/z* 788 CL_{HFA}. This is the first characterization of CLs by HPLC/ESI-IT-MS multistage fragmentation and the novel identification of CLs with a hydroxylated fatty acid. The

identification of these CLs illustrates the effectiveness of MS^4, and higher level MS^n, combined with HRMS in identifying AA-IPL headgroup structural components.

Flavolipin – Two moderately abundant IPLs with *m/z* 655 and *m/z* 653, eluting at retention times of 20.90 min and 21.16 min, respectively, were observed in the *C. marinus* extract using HPLC/ESI-IT-MS analysis (Fig. 2C). MS fragmentation of both the *m/z* 655 and 653 IPLs resulted in consecutive losses of H_2O, a fatty acid, βOH-fatty acid, and an *m/z* 145 fragment ion (Table 1). *C. marinus* is known to produce the serine-glycine lipopeptide flavolipin (FL).[35] The nominal mass of a protonated serine-glycine dipeptide is 163 Da. If the initial H_2O loss from the *m/z* 655 and 653 IPLs represents the dehydration of the FL serine-glycine dipeptide headgroup, then subsequent fatty acid losses would yield the *m/z* 145 fragment ion representing the dehydrated serine-glycine dipeptide head group.

HRMS analysis was used to confirm if the IPLs with *m/z* 655 and 653 were indeed FLs. The HRMS fragmentation of the *m/z* 655 and 653 IPLs included multiple combinations of head group losses and fatty acid losses to produce four fragment ions of *m/z* 163, 145, 106, and 60 (Fig. 6; Table 1). The assigned elemental composition of *m/z* 163 ($C_5H_{11}N_2O_4$) is the same as a protonated serine-glycine dipeptide, and we propose that the elemental composition of *m/z* 145 ($C_5H_9N_2O_3$) results from water loss of the serine-glycine head group forming a cyclic structure (Fig. 6) as suggested in previous studies of MS fragmentation of other AA-IPLs.[19-21] The *m/z* 106 fragment ion has the elemental composition of protonated serine ($C_3H_8NO_3$), while that (C_2H_6NO) of the *m/z* 60 fragment ion corresponds to protonated glycine minus an oxygen (Fig. 6). Fragmentation also results in the neutral loss of serine followed by a fatty acid loss and dehydration yielding *m/z* 550, 326, and 308 fragment ions, respectively (Fig. 6). Fatty acid carbocations with *m/z* 251 and 233 are also produced during MS^2 fragmentation. Given the observed fragmentation and elemental compositions of losses and diagnostic fragment ions, the *m/z* 655 IPL eluting at 20.90 min and the *m/z* 653 IPL at

retention time 21.16 min were both confirmed to be FLs. The *m/z* 653 FL contained a monounsaturated ester-linked fatty acid. FL was originally identified as a serine containing lipid in *Flavobacterium meningosepticum* by Kawai et al.[36] using GC/MS, TLC, electrophoresis, and IR spectrophotometry, and the structure was later revised to include glycine by Shiozaki et al.[17] using ^{1}H NMR, IR spectrophotometry, fast atom bombardment/mass spectrometry (FAB-MS), and TLC. This represents the first characterization of FL by HPLC/MS.

Cytolipin – Along with FL, two other low abundance IPLs with AA-IPL-like fragmentation of *m/z* 568 and 566 were observed in the *C. marinus* extract by HPLC/ESI-IT-MS analysis eluting at retention times 6.36 min and 6.54 min, respectively (Fig. 2C). Fragmentation of both the *m/z* 568 and 566 IPLs resulted in fatty acid losses and an *m/z* 76 fragment ion (Table 1). *C. marinus* is known to produce the glycine-containing lipid cytolipin.[18] The nominal mass of protonated glycine is 76, which would match with the *m/z* 76 fragment ion of the *m/z* 568 and 566 IPLs. HRMS analysis confirmed the fatty acid losses of the *m/z* 568 ($C_{15:0}$, βOH-$C_{17:0}$) and 566 ($C_{15:1}$, βOH-$C_{17:0}$) IPLs. HRMS analysis also revealed that the *m/z* 76 fragment ion has an elemental composition of $C_2H_6NO_2$ (Fig. 7), which is identical to that of protonated glycine. The *m/z* 566 cytolipin (CYL) contains a monounsaturated ester-linked fatty acid. CYLs were identified in *P. heparinus* as well (Fig. 2D; Table 1). This is the first characterization of CYL by HPLC/MS.

Headgroup- and fatty acid-hydroxylated OLs – Along with the above mentioned AA-IPLs identified in the *P. heparinus* extract, there were two additional unknown IPLs with AA-IPL-like fragmentation eluting at retention times 27.74 (IPL with molecular ion of *m/z* 641; I) and 32.75 min (IPL with molecular ion of *m/z* 657; I') (Fig. 2D). Fragmentation of each of the unknown IPLs resulted in subsequent fatty acid losses and an *m/z* 131 fragment ion. IPL I' produced the same fragment ion as the IPL I, but the fatty acid loss of I' was *m/z* 16 greater

than I, indicating that I' is a fatty acid hydroxylated version of I (Table 1). The distribution of fatty acids contained in the structures of I and I' were also the same as the fatty acid chain lengths of the abundant OL and hydroxylated fatty acid OL (OL_{HFA}) IPLs (Table 1).

The HRMS fragmentation of *P. heparinus* IPLs I and I' confirmed the same fatty acid losses as the OL and OL_{HFA}, respectively, and revealed the elemental composition of the *m/z* 131 fragment ion to be $C_5H_{11}N_2O_2$ (Fig. 8, Table 1). This is the same elemental composition of the diagnostic OL *m/z* 115 fragment ion ($C_5H_{11}N_2O$) with an additional oxygen, suggesting that the headgroups of AA-IPLs I and I' are hydroxylated ornithines. Further fragmentation yielded an *m/z* 114 fragment ion with elemental composition of $C_5H_8NO_2$, resulting from removal of NH_3 from the *m/z* 131 $C_5H_{11}N_2O_2$ fragment ion. We propose that the *m/z* 131 and 114 fragment ion are formed in the same way that the *m/z* 115 fragment ion is formed in OL fragmentation: loss of H_2O from the hydroxyornithine head group results in a cyclic headgroup structure, followed by sequential fatty acid and βOH-fatty acid losses yielding the six membered ring structure $C_5H_{11}N_2O_2$ and NH_3 loss yielding the six membered ring structure $C_5H_8NO_2$ (Fig. 8B). OLs with ornithine headgroup hydroxylation (HOL), and OLs with ornithine headgroup hydroxylation and fatty acid hydroxylation (HOL_{HFA}) have been previously identified in *Rhizobium tropici* using ESI-MS in negative ionization mode.[14] For comparison the *P. heparinus* extract was analyzed by HPLC/ESI-IT-MS in negative ion mode as well. Here we observed the same characteristic *m/z* 147 fragment ion upon fragmentation as was reported in the analysis of *R. tropici* by Vences-Guzmán et al.,[14] further confirming the hydroxylated ornithine headgroup structure in both species.

CONCLUSIONS

Along with other recently described AA-IPLs, the GL, LL, CL, CL_{HFA}, FL, HOL, HOL_{HFA} membrane lipid structures, novel fragmentation pathways, and methods described in this paper can be used to continue to expand the knowledge of the distribution and function of amino acid-containing membrane lipid structures in microbial cultures and the environment. We have summarized AA-IPL fragment ions in Table 2, which is based on data reported here and in the literature. Amino acid-containing lipid fragment ions reflect headgroup structures, often after dehydration or other head group losses, which we propose commonly yields a cyclic fragment ion (Figs. 3, 5, 6, 8). Higher level MS^n, such as MS^4 used in this study, can be effective in identifying IPL head group structural components. Exact mass elemental composition of fragment ions is extremely valuable for confirming lipid structural composition, and LC separation is important for distinguishing between compounds with the same nominal masses or similar fragment ions. As more glycerol-free amino acid-containing membrane lipid structural knowledge is obtained these lipids can be readily identified in the environment giving greater understanding to microbial responses and adaptation.

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TABLES

Table 1: AA-IPLs analyzed by HRMS in *Rhodobacter sphaeroides*, *Gluconobacter cerinus*, *Cyclobacterium marinus*, *Pedobacter heparinus* in this study. The observed exact masses of precursor ions, characteristic fragment ions and associated elemental compositions are reported. RT = retention time, EC = elemental composition, Δ mmu = difference between calculated and observed *m/z* (mmu).

Species/IPL	RT	Precursor Ion (*m/z*)	EC	Δ mmu	Characteristic Fragment Ion (*m/z*)	EC	Δ mmu
Rhodobacter sphaeroides							
OL - C18:1, βOH-C20:1	25.99	705.6140	$C_{43}H_{81}N_2O_5^+$	0.0	115.0869	$C_5H_{11}N_2O^+$	0.3
GL - C18:1, βOH-C20:1	15.89	719.5924	$C_{43}H_{79}N_2O_6^+$	0.9	147.0766	$C_5H_{11}N_2O_3^+$	0.2
					130.0501	$C_5H_8NO_3^+$	0.2
					129.0660	$C_5H_9N_2O_2^+$	0.1
					291.2684	$C_{20}H_{35}O^+$	0.2
LL - C18:1, βOH-C20:1	26.74	719.6307	$C_{44}H_{83}N_2O_5^+$	1.0	147.1129	$C_6H_{15}N_2O_2^+$	0.1
					130.0865	$C_6H_{12}NO_2^+$	0.2
					129.1024	$C_6H_{13}N_2O^+$	0.2
					291.2684	$C_{20}H_{35}O^+$	0.1
Gluconobacter cerinus							
OL - C20:0, βOH-C16:0	29.54	681.5782	$C_{41}H_{81}N_2O_5^+$	35.8[b]	115.0870	$C_5H_{11}N_2O^+$	0.4
CL - C19:1, βOH-C16:0	28.79	772.5851	$C_{42}H_{82}N_3SO_7^+$	1.7	115.0870	$C_5H_{11}N_2O^+$	0.4
CL_{HFA} - OH-C19:1, βOH-C16:0	34.69	788.5820	$C_{42}H_{82}N_3SO_8^+$	0.3	115.0870	$C_5H_{11}N_2O^+$	0.4
CL_{HFA} - OH-C16:0, βOH-C16:0	35.30	748.5513	$C_{39}H_{78}N_3SO_8^+$	0.9	115.0870	$C_5H_{11}N_2O^+$	0.4
Cyclobacterium marinus							
CYL - C15:0, βOH-C17:0	6.36	568.4925	$C_{34}H_{66}NO_5^+$	1.1	76.0399	$C_2H_6NO_2^+$	0.6
CYL - C15:1, βOH-C17:0	6.54	566.4772	$C_{34}H_{64}NO_5^+$	0.7	76.0399	$C_2H_6NO_2^+$	0.6
FL - C15:0, βOH-C17:0	20.90	655.5245	$C_{37}H_{71}N_2O_7^+$	1.1	163.0712	$C_5H_{11}N_2O_4^+$	0.2
					145.0606	$C_5H_9N_2O_3^+$	0.1
					106.0502	$C_3H_8NO_3^+$	0.3
					60.0452	$C_2H_6NO^+$	0.8
FL - C15:1, βOH-C17:0	21.16	653.5092	$C_{37}H_{69}N_2O_7^+$	0.7	163.0710	$C_5H_{11}N_2O_4^+$	0.3
					145.0606	$C_5H_9N_2O_3^+$	0.2
					106.0502	$C_3H_8NO_3^+$	0.3
					60.0452	$C_2H_6NO^+$	0.8
Pedobacter heparinus							
CYL - C17:0, βOH-C17:0	6.19	596.5236	$C_{36}H_{70}NO_5^+$	1.3	76.0399	$C_2H_6NO_2^+$	0.6

CYL - C16:0, βOH-C17:0	6.30	582.5078	$C_{35}H_{68}NO_5^+$	1.4	76.0399	$C_2H_6NO_2^+$	0.6
CYL - C15:0, βOH-C17:0	6.53	568.4924	$C_{34}H_{66}NO_5^+$	1.2	76.0399	$C_2H_6NO_2^+$	0.6
FL - C15:0, βOH-C17:0	18.00	655.5246	$C_{37}H_{71}N_2O_7^+$	1.0	163.0709	$C_5H_{11}N_2O_4^+$	0.4
					145.0606	$C_5H_9N_2O_3^+$	0.2
					106.0502	$C_3H_8NO_3^+$	0.3
					60.0452	$C_2H_6NO^+$	0.8
OL - C15:0, βOH-C17:0	27.25	625.5499	$C_{37}H_{73}N_2O_5^+$	1.5	115.0868	$C_5H_{11}N_2O^+$	0.2
OL_{HFA} - OH-C15:0, βOH-C17:0	30.79	641.5455	$C_{37}H_{73}N_2O_6^+$	0.8	115.0868	$C_5H_{11}N_2O^+$	0.2
OL_{HFA} - OH-C15:0, βOH-C15:0	31.46	613.5154	$C_{35}H_{69}N_2O_6^+$	0.4	115.0868	$C_5H_{11}N_2O^+$	0.2
I - C15:0, βOH-C17:0	27.74	641.5456	$C_{37}H_{73}N_2O_6^+$	0.7	131.0815	$C_5H_{11}N_2O_2^+$	0.0
					114.0552	$C_5H_8NO_2^+$	0.2
I' - OH-15C:0, βOH-C17:0	32.75	657.5396	$C_{37}H_{73}N_2O_7^+$	1.6	131.0815	$C_5H_{11}N_2O_2^+$	0.0
					114.0552	$C_5H_8NO_2^+$	0.2
I' - OH-15C:0, βOH-15C:0	34.42	629.5100	$C_{35}H_{69}N_2O_7^+$	0.1	131.0815	$C_5H_{11}N_2O_2^+$	0.0
					114.0552	$C_5H_8NO_2^+$	0.2

[a]OL = ornithine lipid, GL = glutamine lipid, LL = lysine lipid, CL = cerilipin, CL_{HFA} = fatty acid hydroxylated cerilipin, CYL = cytolipin, FL = flavolipin, OL_{HFA} = fatty acid hydroxylated ornithine lipid, I = proposed head group hydroxylated ornithine lipid (HOL), I' = proposed head group hydroxylated and fatty acid hydroxylated ornithine lipid (HOL_{HFA}).

[b] = apparent co-elution with other compound resulting in higher Δ mmu value.

*In general the 0.5 Δ mmu (millimass unit) range was used as a measure of very high confidence molecular formula assignments and the 1.0 Δ mmu range was used as a measure of good confidence molecular formula assignments.[37-39]

Table 2: Overview of nominal and calculated exact mass, and elemental composition (EC) of typical diagnostic fragment ions and fragmentation losses (**M**-) (in order of decreasing *m/z* horizontally) from AA-IPLs (see Figure 1 for structures) observed in this and other studies. IPLs are listed in order of high performance liquid chromatography (HPLC) relative retention time vertically. Fragment and loss masses observed in this and/or other studies using HRMS have exact masses listed, fragment and loss masses observed in this and/or other studies by ESI-IT-MS have nominal mass listed.

IPL[a] (Reference)	Characteristic fragment ions and losses (*m/z*) and their elemental composition (EC)											
	Nominal	Exact	EC	Nominal	Exact	EC	Nominal	Exact	EC	Nominal	Exact	EC
CYL*[16]	76	76.0393	$C_2H_6NO_2^+$	-	-	-	-	-	-	-	-	-
GL*[19]	147	147.0764	$C_5H_{11}N_2O_3^+$	130	130.0499	$C_5H_8NO_3^+$	129	129.0659	$C_5H_9N_2O_2^+$	-	-	-
FL*[17]		**M**-105.0420	$C_3H_7NO_3$		163.0713	$C_5H_{11}N_2O_4^+$	145	145.0608	$C_5H_9N_2O_3^+$		106.0499	$C_3H_8NO_3^+$
		60.0444	$C_2H_6NO^+$	-	-	-	-	-	-	-	-	-
CL*[15]	**M**-125	-	$C_2H_7NSO_3$	115	115.0866	$C_5H_{11}N_2O^+$	-	-	-	-	-	-
OL*[19,28,29]	115	115.0866	$C_5H_{11}N_2O^+$	-	-	-	-	-	-	-	-	-
LL*[21,40]	147	147.1128	$C_6H_{15}N_2O_2^+$	130	130.0863	$C_6H_{12}NO_2^+$	129	129.1022	$C_6H_{13}N_2O^+$	-	-	-
MMO[20]	**M**-31	**M**-31.0412	CH_5N		173.0921	$C_7H_{13}N_2O_3^+$		147.1128	$C_6H_{15}N_2O_2^+$	129	129.1022	$C_6H_{13}N_2O^+$
		116.0706	$C_5H_{10}NO_2^+$	-	-	-	-	-	-	-	-	-
DMO[20]	**M**-45	**M**-45.0573	C_2H_7N		187.1077	$C_8H_{15}N_2O_3^+$	161	161.1285	$C_7H_{17}N_2O_2^+$	144	144.1019	$C_7H_{14}NO_2^+$
	116	116.0706	$C_5H_{10}NO_2^+$	-	-	-	-	-	-	-	-	-
HOL*[14]	131	131.0815	$C_5H_{11}N_2O_2^+$		114.0550	$C_5H_8NO_2^+$	-	-	-	-	-	-
HLL[21]		163.1077	$C_6H_{15}N_2O_3^+$	145	145.0972	$C_6H_{13}N_2O_2^+$	128	128.0706	$C_6H_{10}NO_2^+$	100	100.0757	$C_5H_{10}NO^+$
CL_{HFA}*	**M**-125	-	$C_2H_7NSO_3$	115	115.0866	$C_5H_{11}N_2O^+$	-	-	-	-	-	-
OL_{HFA}*[14]	115	115.0866	$C_5H_{11}N_2O^+$	-	-	-	-	-	-	-	-	-
LL_{HFA}[21]	147	147.1128	$C_6H_{15}N_2O_2^+$	130	130.0863	$C_6H_{12}NO_2^+$	129	129.1022	$C_6H_{13}N_2O^+$	-	-	-
HOL_{HFA}*[14]	131	131.0815	$C_5H_{11}N_2O_2^+$		114.0550	$C_5H_8NO_2^+$	-	-	-	-	-	-
HLL_{HFA}[21]		163.1077	$C_6H_{15}N_2O_3^+$	145	145.0972	$C_6H_{13}N_2O_2^+$	128	128.0706	$C_6H_{10}NO_2^+$	100	100.0757	$C_5H_{10}NO^+$
TMO[20]	**M**-59	59.0730	C_3H_9N	116	116.0706	$C_5H_{10}NO_2^+$	-	-	-	-	-	-

[a] CYL = cytolipin; GL = glutamine lipid; FL = flavolipin; CL = cerilipin; OL = ornithine lipid; LL = lysine lipid; MMO = monomethylornithine lipid; DMO = dimethyl-ornithine lipid; HOL = head group hydroxylated ornithine lipid; HLL = head group hydroxylated lysine lipid; CLHFA = fatty acid hydroxylated cerilipin; OLHFA = fatty acid hydroxylated ornithine lipid; LLHFA = fatty acid hydroxylated lysine lipid; HOLHFA = head group hydroxylated and fatty acid hydroxylated ornithine lipid; HLLHFA = head group hydroxylated and fatty acid hydroxylated lysine lipid; TMO = trimethylornithine lipid.

*This study.

FIGURE CAPTIONS

Figure 1: Amino acid intact polar lipid (AA-IPL) structures, based on fatty acid and βOH-fatty acid core lipids, and a variety of headgroups (R1). R2 and R3 are alkyl chains.

Figure 2: High pressure liquid chromatography-electrospray ionization/ion trap/mass spectrometry (HPLC/ESI-IT-MS) base peak chromatograms of (A) *Rhodobacter sphaeroides*; (B) *Gluconobacter cerinus*; (C) *Cyclobacterium marinus*; (D) *Pedobacter heparinus*. Key: PE = phosphatidylethanolamine; PG = phosphatidylglycerol; DMPE = dimethylposphatidyl-ethanolamine; CL = cerilipin; OL = ornithine lipid; PC = phosphatidylcholine; PC_{DE} = diether-PC; CL_{HFA} = cerilipin with hydroxylated fatty acid; FL = flavolipin; GL = glutamine lipid; LL = lysine lipid; CYL = cytolipin; OL_{HFA} = ornithine lipid with hydroxylated fatty acid; PI = phosphatidylinositol; I = proposed headgroup hydroxylated ornithine lipid (HOL), I' = proposed headgroup hydroxylated and fatty acid hydroxylated ornithine lipid (HOL_{HFA}).

Figure 3: Typical electrospray ionization-ion trap-mass spectrometry (ESI-IT-MS) fragmentation of ornithine lipid (OL). R1 and R2 are alkyl chains.

Figure 4: High Resolution Accurate Mass-Mass Spectrometry (HRMS) mass spectra of (A) *Rhodobacter sphaeroides* glutamine lipid (GL) at retention time 15.89 min, the relative abundance of the *m/z* 400-450 range is magnified 10x; (B) *R. sphaeroides* lysine lipid (LL) at 26.74 min, the relative abundance of the *m/z* 410-450 range is magnified 5x. The elemental composition of the fragment ions and losses are indicated.

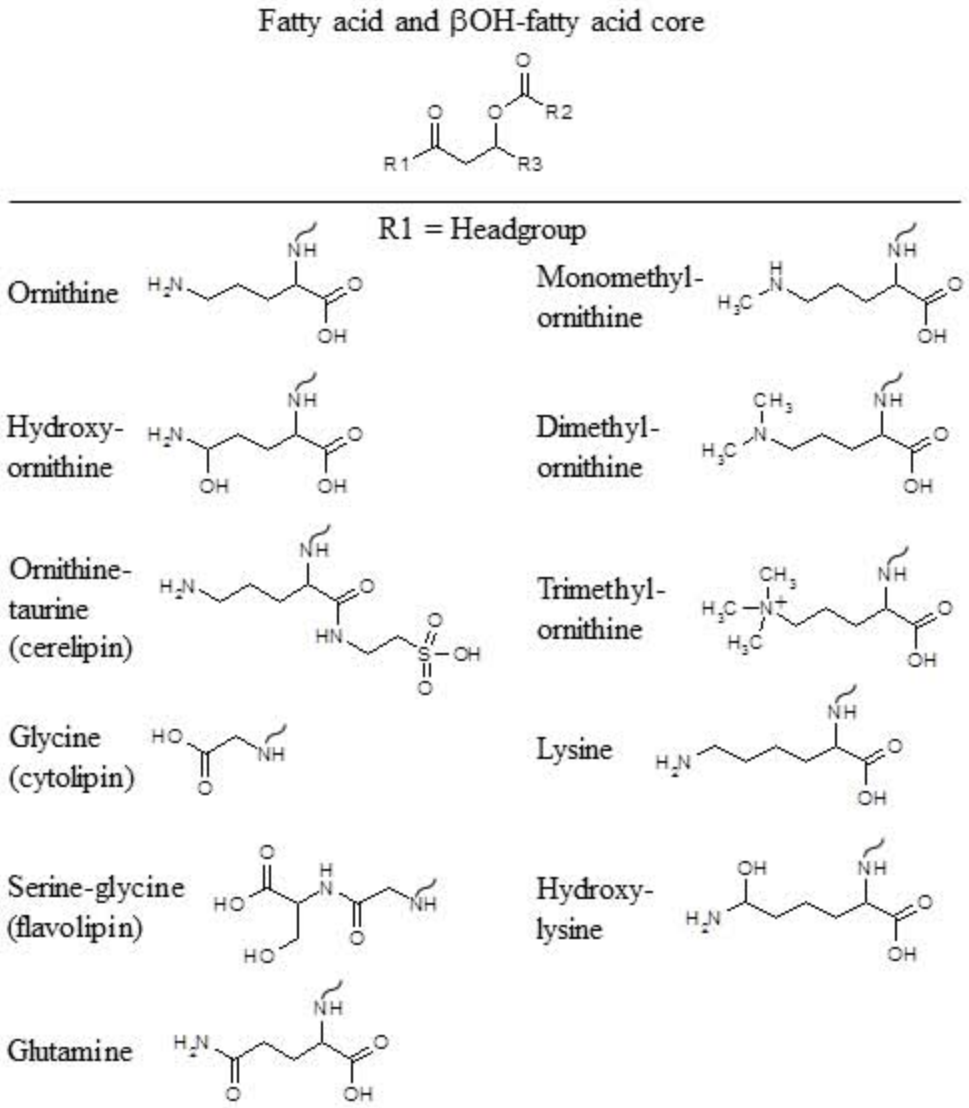
Figure 5: Electrospray Ionization-Ion Trap-Mass Spectrometry (ESI-IT-MS) multistage fragmentation of *Gluconobacter cerinus* ornithine-taurine lipid (cerilipin; CL) at 28.75 min.

(A) MS^1; (B) MS^2; (C) MS^3; (D) MS^4; (E) Proposed fragmentation of *G. cerinus* ornithine-taurine lipid. The *m/z* 494 and 476 fragment ions represent losses of fatty acids from the precursor ion.

Figure 6: (A) High Resolution Accurate Mass-Mass Spectrometry (HRMS) mass spectra of *Cyclobacterium marinus* serine-glycine lipid (flavolipin; FL) at retention time 20.90 min; (B) Proposed fragmentation of *C. marinus* flavolipin. The elemental composition of fragment ions is indicated. R1 = R2 = $C_{14}H_{29}$; R3 = $C_{11}H_{23}$.

Figure 7: High Resolution Accurate Mass-Mass Spectrometry (HRMS) mass spectra with elemental composition of (A) *Cyclobacterium marinus* glycine lipid (cytolipin, CL) at retention time 6.82 min; (B) Proposed fragmentation of *C. marinus* cytolipin.

Figure 8: (A) High Resolution Accurate Mass-Mass Spectrometry (HRMS) mass spectra with elemental composition of the *Pedobacter heparinus m/z* 641 IPL at retention time 27.74 min; (B) Proposed fragmentation scheme of *P. heparinus* headgroup hydroxylated ornithine lipid (HOL).



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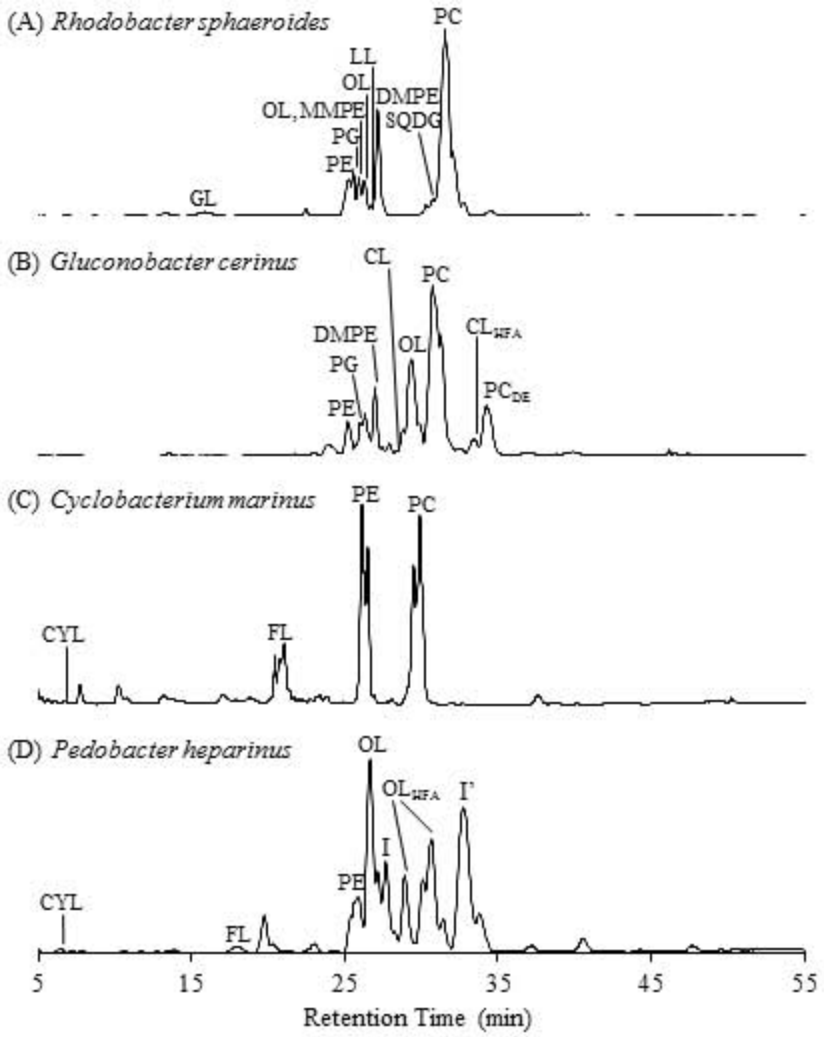

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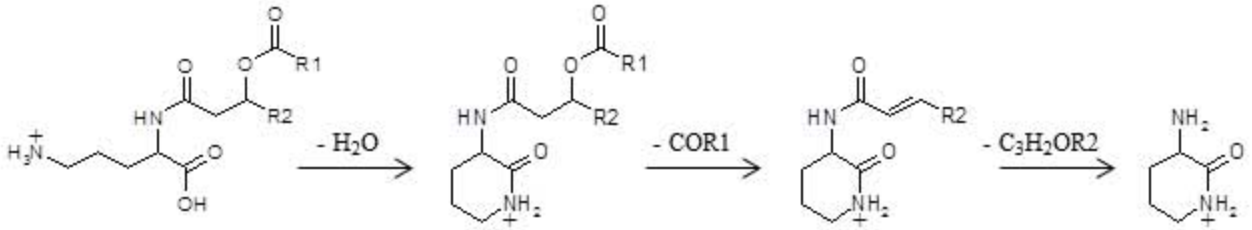
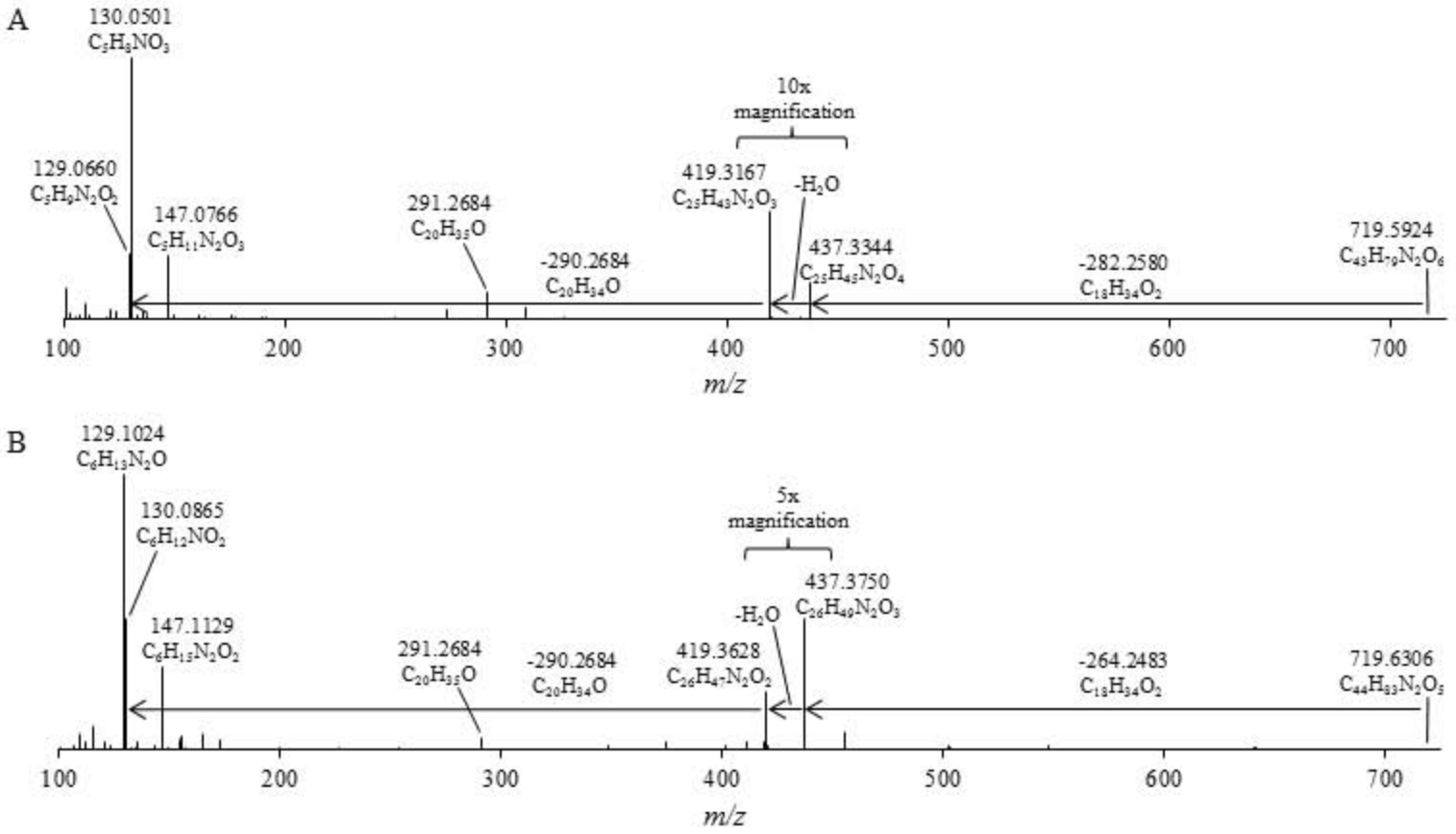


Figure 3: Typical electrospray ionization-ion trap-mass spectrometry (ESI-IT-MS) fragmentation of ornithine lipid (OL). R1 and R2 are alkyl chains.



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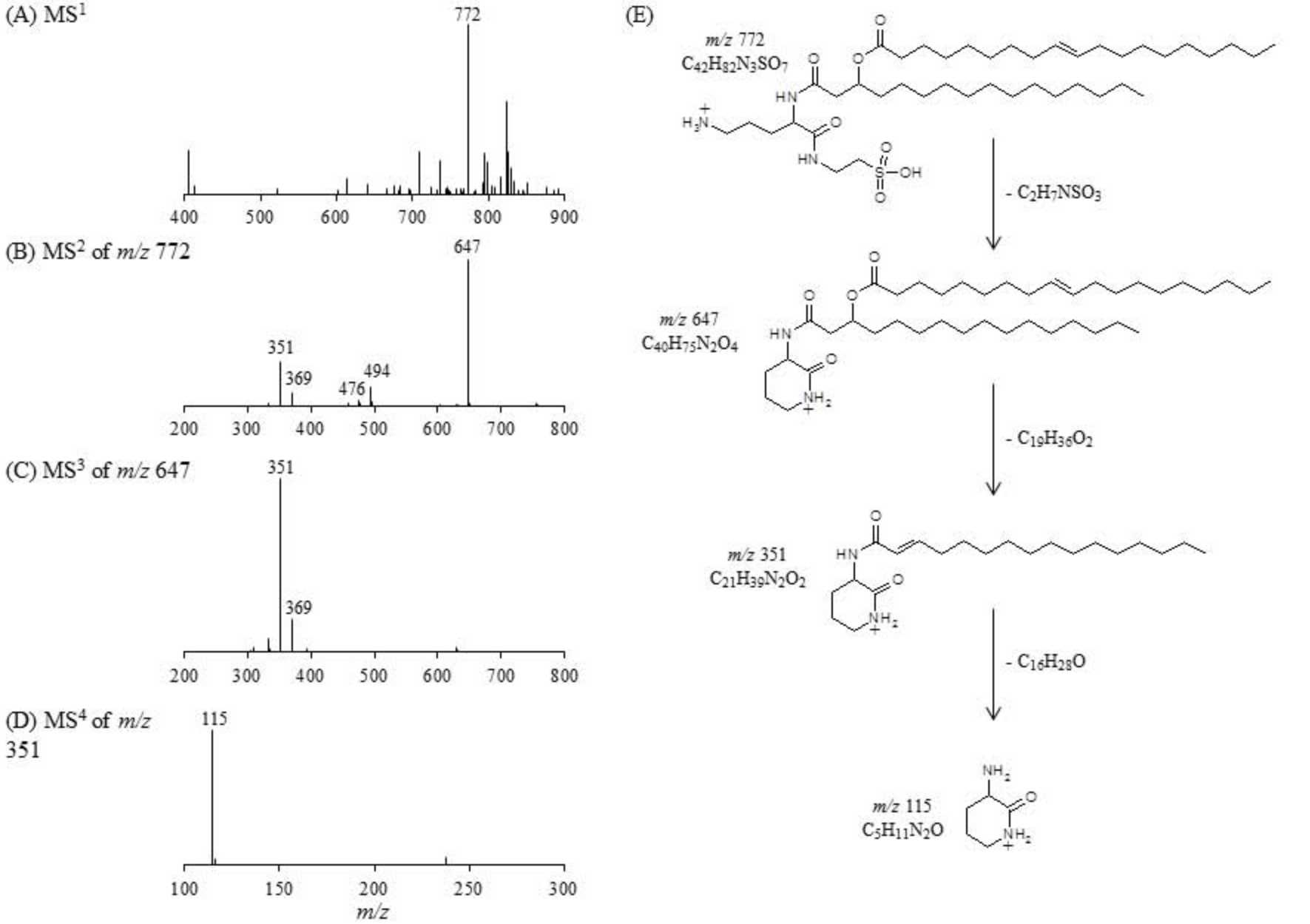

Figure 5: Electrospray Ionization-Ion Trap-Mass Spectrometry (ESI-IT-MS) multistage fragmentation of *Gluconobacter cerinus* ornithine-taurine lipid (cerilipin; CL) at 28.75 min. (A) MS^1; (B) MS^2; (C) MS^3; (D) MS^4; (E) Proposed fragmentation of *G. cerinus* ornithine-taurine lipid. The *m/z* 494 and 476 fragment ions represent losses of fatty acids from the precursor ion.

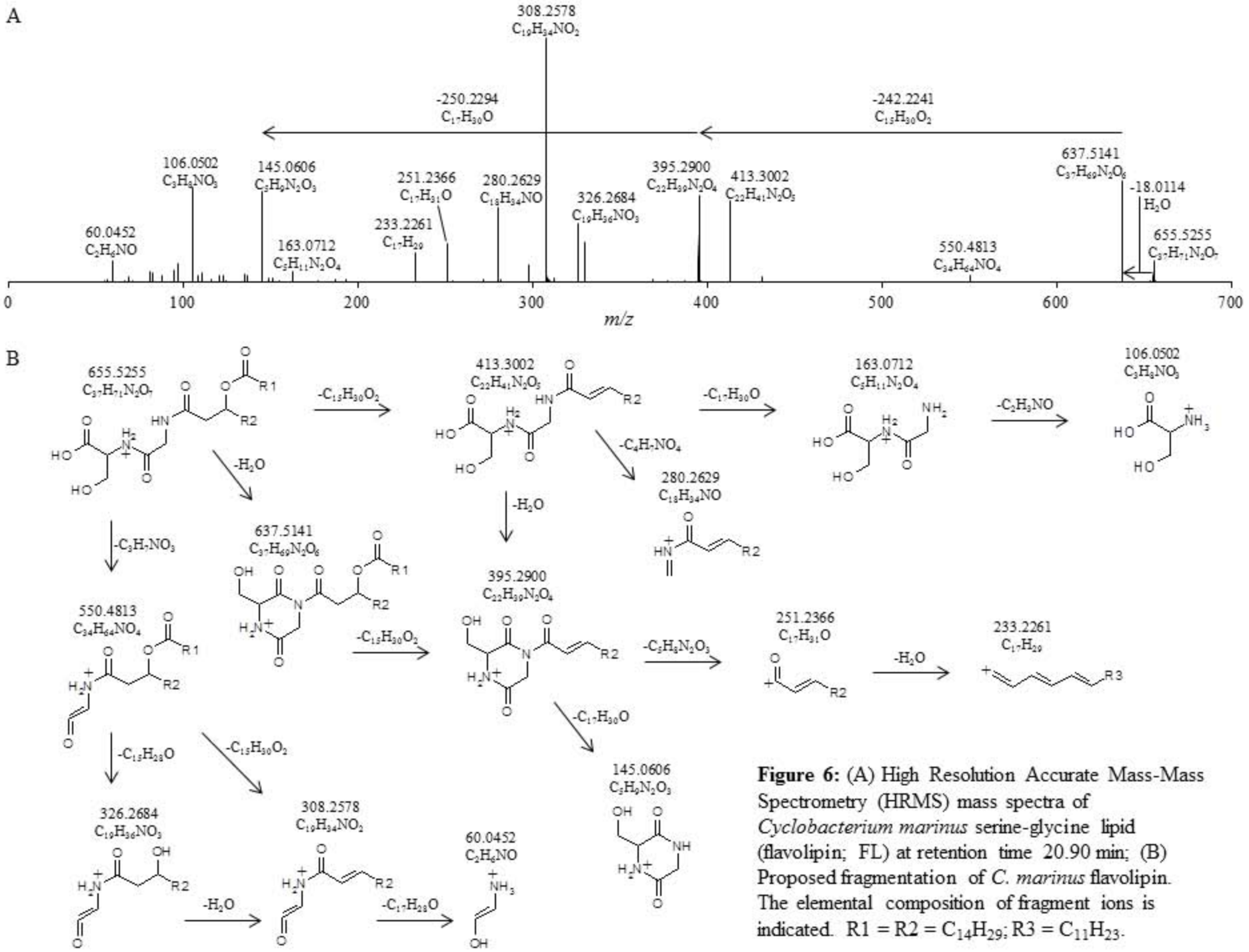

Figure 6: (A) High Resolution Accurate Mass-Mass Spectrometry (HRMS) mass spectra of *Cyclobacterium marinus* serine-glycine lipid (flavolipin; FL) at retention time 20.90 min; (B) Proposed fragmentation of *C. marinus* flavolipin. The elemental composition of fragment ions is indicated. R1 = R2 = $C_{14}H_{29}$; R3 = $C_{11}H_{23}$.

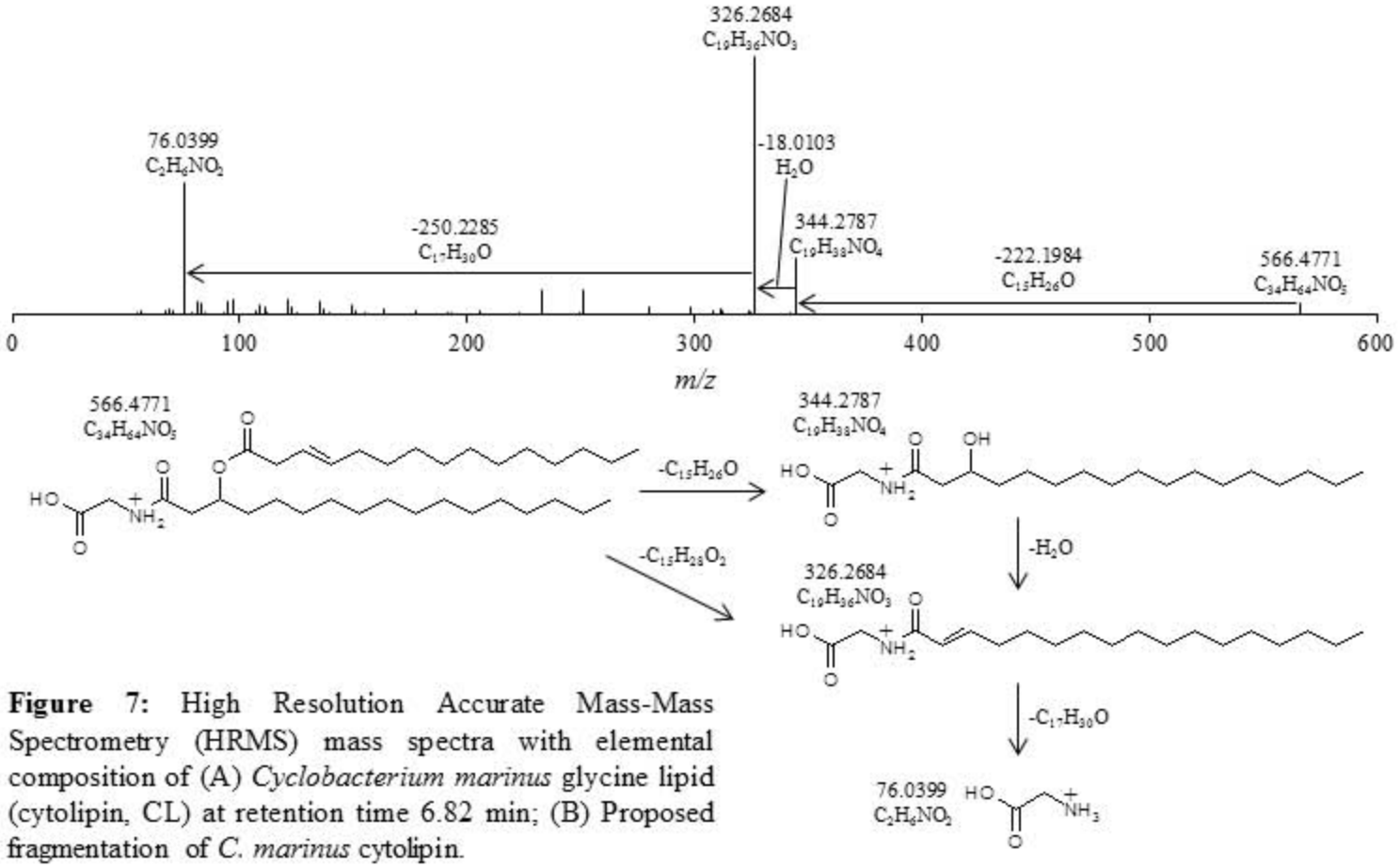

Figure 7: High Resolution Accurate Mass-Mass Spectrometry (HRMS) mass spectra with elemental composition of (A) *Cyclobacterium marinus* glycine lipid (cytolipin, CL) at retention time 6.82 min; (B) Proposed fragmentation of *C. marinus* cytolipin.

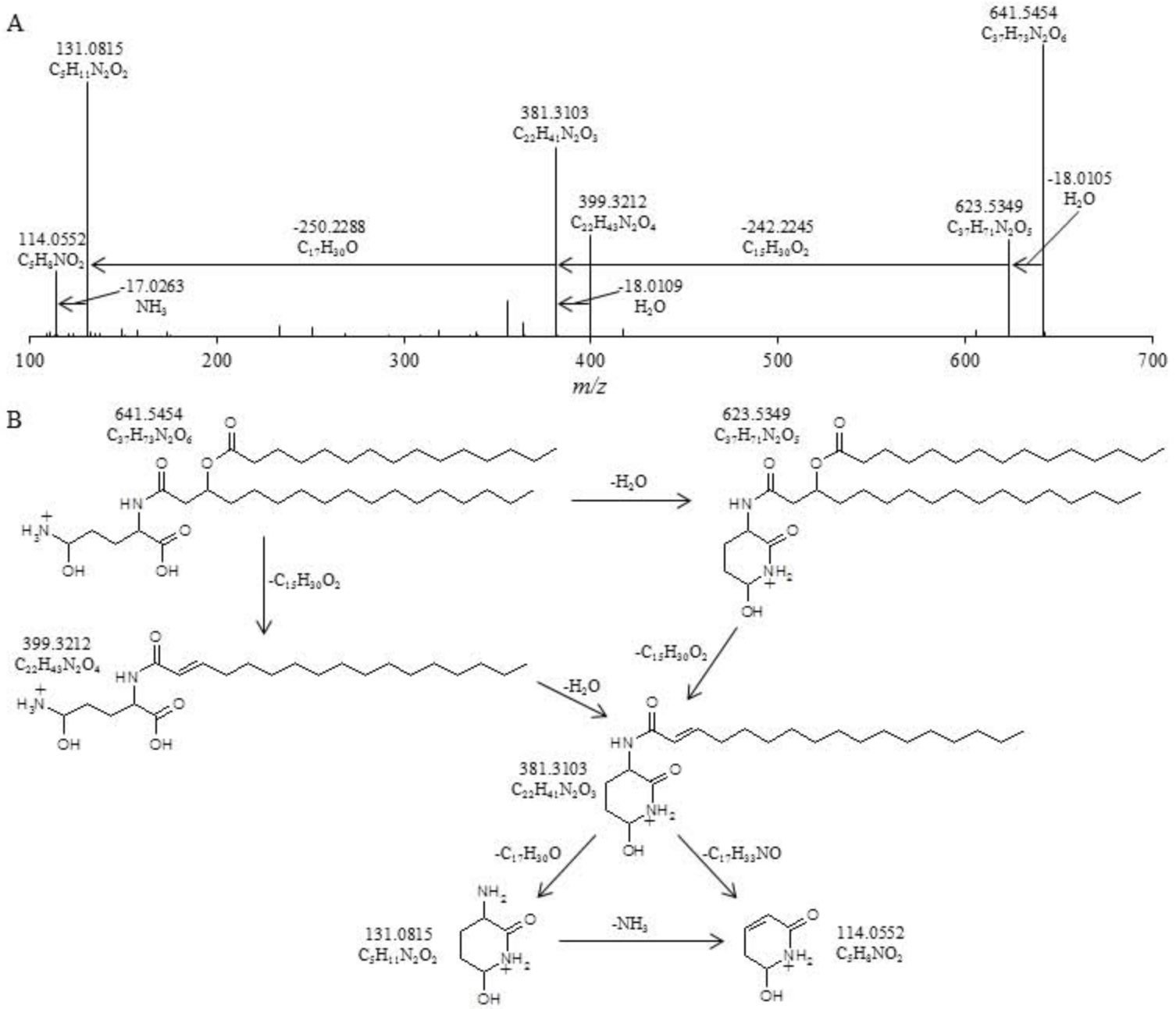

Figure 8: (A) High Resolution Accurate Mass-Orbitrap-Mass Spectrometry (HRMS) mass spectra with elemental composition of the *Pedobacter heparinus m/z* 641 IPL at retention time 27.74 min; (B) Proposed fragmentation scheme of *P. heparinus* headgroup hydroxylated ornithine lipid (HOL).