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Novel mono-, di-, and trimethylornithine membrane lipids in northern wetland planctomycetes

Eli K. Moore[1*], Ellen C. Hopmans[1], W. Irene C. Rijpstra[1], Laura Villanueva[1], Svetlana N. Dedysh[2], Irina S. Kulichevskaya[2], Hans Wienk[3], Frans Schoutsen[4], Jaap S. Sinninghe Damsté[1]

1. *Royal Netherlands Institute for Sea Research, Department of Marine Organic Biogeochemistry, P.O. Box 59, 1790 AB Den Burg, Texel, The Netherlands*
2. *S. N. Winogradsky Institute of Microbiology, Russian Academy of Sciences, Prospect 60-letya Octyabrya 7/2, Moscow 117312, Russia*
3. *Bijvoet Center for Biomolecular Research, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands*
4. *Thermo Fisher Scientific, Takkebijsters 1, 4817 BL Breda, The Netherlands*

*corresponding author: Elisha.Moore@nioz.nl

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Abstract

Northern peatlands represent a significant global carbon store and commonly originate from *Sphagnum*-moss dominated wetlands. These ombrotrophic ecosystems are rain-fed, resulting in nutrient-poor, acidic conditions. Members of the bacterial phylum *Planctomycetes* are highly abundant and appear to play an important role in the decomposition of *Sphagnum*-derived litter in these ecosystems. High performance liquid chromatography coupled to high resolution accurate mass/mass spectrometry (HPLC-HRAM/MS) analysis of lipid extracts of four isolated planctomycetes from wetlands of European North Russia revealed novel ornithine membrane lipids (OLs) that are mono-, di-, and trimethylated at the ε–nitrogen position of the ornithine head group. Nuclear magnetic resonance (NMR) analysis of the isolated trimethylornithine lipid confirmed the structural identification. Similar fatty acid distribution between mono-, di-, and trimethylornithine lipids suggests that the three lipid classes are biosynthetically linked, as in the sequential methylation of the terminal nitrogen in phosphatidylethanolamine to produce phosphatidylcholine. The mono-, di-, and trimethylornithine lipids described here represent the first report of methylation of the ornithine head groups in biological membranes. Various bacteria are known to produce OLs under phosphorus limitation, or fatty acid hydroxylated OLs under thermal or acid stress. The sequential methylation of OLs leading to a charged choline-like moiety in the trimethylornithine lipid head group, may be an adaptation to provide membrane stability in acidic conditions without the use of scarce phosphate in nutrient-poor ombrotrophic wetlands.

INTRODUCTION

Northern peatlands are a significant global carbon store accounting for approximately one third of global soil organic carbon, despite only making up 3% of the world's land area (1-3). Areas with peatland development correspond closely to the distribution of *Sphagnum* moss-dominated bogs (4). Ombrotrophic, nutrient-poor, acidic conditions of these wetlands, along with low temperatures, and the presence of decay resistant phenolic compounds and waxes in *Sphagnum* tissues, lead to low decomposition rates of *Sphagnum*-derived litter (5-9). Consequently, carbon sequestration due to net primary production in *Sphagnum*-dominated bogs is greater than the amount of carbon lost to the atmosphere by decomposition of dead organic matter, making northern wetlands an important global carbon sink (10-12).

The composition of the bacterial community in *Sphagnum* peat bogs is fundamentally different from the community that decomposes plant debris in eutrophic systems at neutral pH (13-15). It has been recently discovered that *Planctomycetes* make up an important part of the bacterial population responsible for *Sphagnum* decomposition, accounting for up to 14% of total bacterial cells (14, 16-17). Cell numbers of planctomycetes have been observed to increase in the late stages of *Sphagnum* debris degradation (14). All currently described peat-inhabiting planctomycetes are capable of degrading various heteropolysaccharides, while only one of them, *Telmatocola sphagniphila*, possesses weak cellulolytic potential (18-22). The addition of available nitrogen to cellulose-amended *Sphagnum* peat degradation experiments resulted in a decrease of the relative abundance of *Planctomycetes* in the total microbial community (15). This suggests that planctomycetes in ombrotrophic wetlands are highly adapted to nutrient-poor conditions and contribute mainly to final stages of plant litter decomposition.

Various studies have predicted that northern peatlands will change from atmospheric carbon sink to source in future climate warming scenarios further accelerating global warming

(23-24). High temperature sensitivity of soil respiration at low temperatures (25-26) makes these ecosystems especially susceptible to climate impacts. It has been observed that 1°C temperature increase for *in situ* subarctic peatland climate manipulation experiments accelerated total ecosystem respiration rates by 50 to 60% depending on the season (27). Heterotroph-dominated subsurface peat respiration accounted for at least 70% of the increased respiration rates, demonstrating that climate change may have a major impact on the microbial recycling of stored organic carbon in northern wetlands. Therefore, it is important to study the microbial communities involved in the degradation of *Sphagnum*-derived material and the mechanisms of their adaptation to ombrotrophic conditions including *Planctomycetes*, of which little is known.

Cell membranes are built out of intact polar lipids (IPLs), which consist of a polar head group connected to hydrophobic carbon chain tail groups. IPLs can be useful biomarker molecules because their molecular structures can be taxonomically and environmentally specific, and are thought to represent living biomass (28-29). Given the unique environmental niche which these *Sphagnum*-bog *Planctomycetes* occupy, their membranes may be adapted to these conditions and contain unique biomarker lipids that could be used to further investigate the role of *Planctomycetes* in this globally important ecosystem, and indicate how they may respond to environmental change. Indeed, all planctomycete strains that have been isolated from *Sphagnum* wetlands of Northern Russia contained polar membrane lipids of unknown structure (19, 21-22), which might have biomarker potential. Here we describe the isolation and characterization of several of these unidentified polar membrane lipids. High resolution accurate mass orbitrap mass spectrometry (HRAM/OT/MS) combined with nuclear magnetic resonance (NMR) revealed a series of ornithine lipids with increasing head group methylation. These lipids appear to be common in planctomycetes from this unique environment.

MATERIALS AND METHODS

Strains and culture conditions. The planctomycetes *S. acidiphila* MOB10^T (DSM 18658^T), *S. rosea* S26^T (DSM 23044^T), *T. sphagniphila* SP2^T and OB3 (DSM 23888^T), and *Gemmata*-like strain SP5 were isolated from the upper oxic layer (0-10 cm) of acidic peat collected from wetlands of European North Russia and described by Kulichevskaya *et al*. (19, 21-22, Supplementary Material – Appendix I). *S. acidiphila* MOB10^T and *S. rosea* S26^T were grown in liquid medium M31 (modification of medium 31 described by Staley *et al*. (30) containing (g per liter of distilled water): KH_2PO_4, 0.1; Hutner's basal salts, 20 ml; N-acetylglucosamine, 1.0; peptone, 0.1; yeast extract, 0.1; pH 5.8. *T. sphagniphila* SP2^T and OB3 as well as *Gemmata*-like strain SP5 were grown in liquid medium M1N of the following composition (gram per liter of distilled water): KH_2PO_4, 0.1; $(NH_4)_2SO_4$, 0.1; $MgSO_4$ x $7H_2O$, 0.1; $CaCl_2$ x $2H_2O$, 0.02; yeast extract, 0.1; glucose, 0.25; 1 ml of trace element solution '44' and 1 ml Staley's vitamin solution (30); pH 5.5. Cultivation was done in 500-ml flasks containing 100 ml medium. Cultures were incubated at 22°C on a shaker and harvested in the late exponential growth phase. Biomass was collected and freeze dried for extraction and analysis.

Lipid extraction. Lipids were extracted from freeze dried biomass of each culture by a modified Bligh & Dyer method (31). For structural comparison, lipids were also extracted from freeze dried biomass of the Bacteriodete *Flavobacterium johnsoniae*, known to contain high concentration of ornithine lipids. Freeze dried biomass of each culture was fully submerged and extracted three times in methanol/dichloromethane/phosphate-buffer (MeOH/DCM/P-buffer, 2/1/0.8, v/v/v) extraction solvent for 10 min in an ultrasonic bath (P-buffer: 8.7 g K_2PO_4 L^{-1} bi-distilled water adjusted to pH 7–8 with 1 N HCl). Extracts were centrifuged for 2 min at 1400 g to separate DCM phase from the MeOH/P-buffer phase, and the lower DCM layer was pipetted into a separate vial. The MeOH/P-buffer layer was

96 washed twice more with DCM, centrifuged, and the resulting DCM layers were pipetted into
97 the same vial as the original DCM layer. DCM was removed under a stream of nitrogen, the
98 residue was dissolved in injection solvent (hexane:2-propanol:H_2O, 718:271:10, v/v/v), and
99 filtered through a 0.45 µm, 4 mm diameter True^{TM} Regenerated Cellulose syringe filter
100 (Grace Davison) prior to injection. Extracts were stored at -80°C until analysis.

101 **HPLC-MS analysis.** Intact polar lipids were analyzed by high performance liquid
102 chromatography-electrospray ionization/ion trap mass spectrometry (HPLC-ESI/IT/MS)
103 according to Sturt *et al*. (28) with some modifications (32). Briefly, an Agilent 1200 series
104 HPLC, with thermostatted auto-injector, was coupled to a Thermo LTQ XL linear ion trap
105 mass spectrometer with Ion Max source and ESI probe (Thermo Scientific, Waltham, MA).
106 Typical lipid extract injection concentration was 2 mg/ml, and the injection volume was 10 µl.
107 Chromatographic separation was performed on a Lichrosphere diol column (250 mm × 2.1
108 mm, 5 µm particles; Grace Alltech Associates Inc.). Elution was achieved with (A) hexane-2-
109 propanol-formic acid-14.8 M aqueous NH_3 (79:20:0.12:0.04, v/v/v/v) and (B) 2-propanol-
110 water-formic acid-14.8 M aqueous NH_3 (88:10:0.12:0.04, v/v/v/v) starting at 10% B, followed
111 by a linear increase to 30% B in 10 min, followed by a 20 min hold, and a further increase to
112 65% B at 45 min. Flow rate was 0.2 ml min^{-1}, total run time was 60 min, followed by a 20
113 min re-equilibration period. The lipid extracts were analyzed by scanning a mass range of *m/z*
114 400-2000 in positive ion mode, followed by data-dependent, dual stage tandem MS (MS^2), in
115 which the four most abundant masses in the mass spectrum were fragmented successively
116 (normalized collision energy, 25; isolation width, 5.0; activation Q, 0.175). Each MS^2 was
117 followed by data dependent, triple-stage tandem MS (MS^3), where the base peak of the MS^2
118 spectrum was fragmented under identical fragmentation conditions as described for MS^2. The
119 ion trap MS was calibrated using the Thermo Scientific LTQ ESI Positive Ion Calibration
120 Solution (contains mixture of caffeine, MRFA, and Ultramark 1621 in an acetonitrile/

methanol/acetic solution). Performance of the HPLC-ESI/IT/MS was monitored by regular injections of platelet-activating factor (PAF) standard (1-O-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine). Relative differences in ornithine and methylornithine IPL ionization and instrument response were corrected for by use of external standards (Avanti Polar Lipids, Inc.: 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-methyl; 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N,N-dimethyl; 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine).

HPLC-HRAM/OT/MS analysis of the *T. sphagniphila* extract was accomplished on a 3000 UltiMate series LC with thermostated auto-injector coupled to a Q Exactive high resolution accurate mass Orbitrap mass spectrometer (Thermo Scientific). Chromatographic conditions and column were the same as described above for HPLC-ESI/IT/MS. Positive ion ESI settings were: capillary temperature, 275°C; sheath gas (N_2) pressure, 35 arbitrary units (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* 400–1000 (resolution 70,000), followed by data-dependent tandem MS (MS^2; resolution 17,500), in which the five most abundant masses in the mass spectrum were fragmented successively (normalized collision energy, 35; isolation width, 1.0). The Q Exactive was calibrated within a mass accuracy range of 1 ppm using the Thermo Scientific Pierce LTQ Velos ESI Positive Ion Calibration Solution (contains mixture of caffeine, MRFA, Ultramark 1621, and n-butylamine in an acetonitrile/methanol/acetic solution).

Fatty acid analysis. An aliquot of *T. sphagniphila* Bligh & Dyer extract was hydrolyzed with 1.5 N HCl in MeOH by reflux for 3 hr. The hydrolysate was adjusted to pH 4 with 2 N KOH-MeOH (1:1, v/v). Water was added to give a final ratio of 1:1 H_2O-MeOH, and then extracted three times with DCM. The DCM fractions were collected and dried over sodium sulfate. The extract was then methylated with diazomethane (33), followed by

silylation in pyridine with N,O-bis(trimethylsilyl)trifluoro-acetamide (BSTFA) at 60°C for 20 min. Methylated-silylated extracts were then dissolved in ethyl acetate for gas chromatography/mass spectrometry (GC/MS) analysis.

GC and GC-MS. GC was performed using a Hewlett-Packard (HP6890) gas chromatograph equipped with an on-column injector and a flame ionization detector. Chromatography was accomplished using a fused silica capillary column (25 m × 0.32 mm) coated with CP Sil-5 CB (film thickness, 0.12 µm) with helium as the carrier gas. The samples were injected at 70°C, and the oven temperature was ramped to 130°C at 20°C/min and then 4°C/min to 320°C, at which it was held for 10 min. GC-MS was performed on a Finnigan Trace Ultra gas chromatograph interfaced with a Finnigan Trace DSQ mass spectrometer operated at 70 eV with a mass range of *m/z* 40 to 800 and a cycle time of 1.7 s (resolution, 1,000). The gas chromatograph employed a fused silica capillary column as described for GC, and also used helium as the carrier gas. The same temperature program was used as with GC.

Target lipid isolation. Target lipids were isolated from extracts using an Agilent Technologies (Santa Clara, CA) 1100 series LC equipped with an auto-injector, and a fraction collector (Foxy Jr., Isco, Inc., Lincoln, NE). A first isolation was achieved on a semi-preparative Lichrosphere diol column (10 × 250 mm, 5 µm; Grace Alltech Associates Inc.) according to Boumann et al. (34) with the same gradient program as described above for HPLC-ESI/IT/MS, but at a flow rate of 3 ml min^{-1}. Typical injection volume was 200 µL containing up to 2 mg material. Column effluent was collected in 1 min fractions, which were screened for the presence of target lipids by flow injection analysis cf. Smittenberg *et al.* (35) using the ESI/IT/MS (5 µl injection of each fraction, ESI source settings same as described above for HPLC-ESI/IT/MS with a scan range of *m/z* 400–2000). Fractions containing target lipids were pooled and further purified on a second Lichrosphere diol column (4.6 × 250 mm,

5 µm; Grace Alltech Associates Inc.). Typical injection volumes were 65 µl containing up to 0.65 mg of material. Lipids were eluted using the identical gradient program and conditions as described above for HPLC-ESI/IT/MS, but with slightly modified mobile phases such that neither mobile phases A or B contained NH_3 or formic acid, at a flow rate of 1 ml min^{-1}. Column effluent was collected in 15 sec fractions and screened as described above. Fractions containing target lipids were again combined and elution solvent was removed under a stream of nitrogen. Purity was assessed by HPLC-ESI/IT/MS as described above for IPL analysis. Isolated lipids were stored at -80°C prior to further analysis.

Nuclear Magnetic Resonance. Purified lipids were dissolved in 99.9% $CDCl_3$ at a concentration of 1.5 µmol ml^{-1}, and NMR experiments were performed at 298K on Bruker 600 MHz and 750 MHz Avance spectrometers, equipped with triple resonance TXI probes, and running under TOPSPIN 2.1. The 600 MHz 1D ^{1}H, and ^{1}H-decoupled 1D ^{13}C and DEPT-135 experiments were recorded with spectral widths/offsets of 24 ppm/6 ppm, 200 ppm/100 ppm, and 200 ppm/100 ppm, and with 16k, 1k and 1k complex points, respectively. The 14 × 14 ppm 2D COSY, TOCSY and NOESY experiments were performed at 750 MHz with 512 × 512 complex points and offset frequency of 6 ppm. Mixing times were 60 ms and 250 ms for TOCSY and NOESY, respectively. The (^{1}H, ^{13}C)-HSQC and (^{1}H, ^{13}C)-HMBC experiments were recorded at 600 MHz with spectral widths/offset 14/6 ppm for proton and 200/100 ppm for carbon-13. Spectra were calibrated with respect to internal residually protonated $CHCl_3$ at 7.24 ppm (^{1}H) and 77.0 ppm (^{13}C).

RESULTS

Unknown IPLs detected by HPLC-ESI/IT/MS. Intact polar lipid analysis of four recently described planctomycete strains from Russian peatlands revealed known and unknown membrane lipid structures. In addition to the commonly observed lipid classes phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidic acid (PA), phosphatidylethanolamine (PE), monomethylphosphatidylethanolamine (MMPE), dimethylphosphatidylethanolamine (DMPE), and amino acid-containing ornithine lipids (Fig. 1; OL, phosphorus free membrane lipids found only in bacteria), three groups of unknown lipids (I-III) were observed in the lipid extracts from each of the four analyzed planctomycete species by HPLC-ESI/IT/MS polar lipid analysis in agreement with previously reported results by Kulichevskaya *et al.* (19, 21-22) (Fig. 2). Group I, II, and III lipids made up the majority of IPLs in *T. sphagniphila* and *Gemmata*-like SP5 extracts, and a large portion of IPLs in *S. rosea* and *S. acidiphila* (Table 1). The lipid extract from *T. sphagniphila* contained the greatest amount of these unknown lipids and the identification efforts, therefore, focused on this species. In the *T. sphagniphila* extract, each of the three unknown lipid clusters showed 5 dominant mass signals separated by 14 Th. The main five observed masses in group I (*m/z* 637, 651, 665, 679, and 693) were each 14 Th lighter than the five masses in group II (*m/z* 651, 665, 679, 693, and 707), and the masses in group II were 14 Th lighter than the masses in group III (*m/z* 665, 679, 693, 707, and 721).

Each of the unknown lipid groups displayed ornithine-like MS fragmentation but different fragmentation products. Typical ornithine lipid MS^2 fragmentation involves the loss of a water molecule and a fatty acyl chain ($R_2CH=C=O$), followed by the loss of the second fatty acid as a ketene ($R_1CH=C=C=O$) during MS^3 fragmentation resulting in a diagnostic product at *m/z* 115 ($C_5H_{11}N_2O$) representing the ornithine head group (36). Ion trap fragmentation of lipid groups I, II, and III followed a similar pattern, but resulted in different

fragmentation products in MS^3, suggesting a similar general IPL structure to ornithine lipids but with a different polar head group. Compounds in group I yielded a product of *m/z* 129, after MS^3 fragmentation. Group II was characterized by multiple low abundance MS^3 fragmentation products at *m/z* 100, 115, 144, 161, and 185. Lipids in cluster III displayed slightly different fragmentation behavior with the apparent loss of a fatty acid, followed by the additional loss of *m/z* 59 during MS^2 fragmentation. MS^3 fragmentation of the latter fragment resulted in the apparent loss of another fatty acid which generated an *m/z* 116 product.

HRAM/OT/MS analysis of unidentified IPL group III. The lipid extract of *T. sphagniphila* was subjected to HRAM/OT/MS analysis to elucidate the elemental composition of the lipid head groups of the three unknown lipid clusters. Group III was the most abundant IPL and therefore analyzed first. As was the case in IT/MS^2 fragmentation, the initial loss observed for the molecular ion of *m/z* 665.5824 from group III, the apparently most abundant unidentified IPL group in *T. sphagniphila*, is of a C16:1 fatty acid (Fig. 3A). Subsequent loss of C_3H_9N generates *m/z* 352.2847, the base peak in the mass spectrum. The presence of C_3H_9N, potentially a trimethylated nitrogen, is a common molecular feature in other membrane lipid head groups such as PCs and betaines. From the base peak, a loss of a C16:0 ketene, results in a product at *m/z* 116.0709 with a molecular composition of $C_5H_{10}O_2N$. The observed fragmentation for group III molecular ion *m/z* 665.5824 appears to represent an ornithine-like lipid that is trimethylated on the ε-nitrogen position (Fig. 3B). This fragmentation pattern was observed for all molecular ions in group III lipids (Tables 2, 3). Proposed gas phase fragmentation includes the formation of a cyclic head group structure after loss of a fatty acid, the trimethylated nitrogen, and β-OH fatty acid. This fragmentation is similar to the formation of the cyclic *m/z* 115 diagnostic product, formed by loss of H_2O in the head group, observed during MS fragmentation of ornithine lipids (36). In total there are

six main lipids in group III with different fatty acid compositions (*m/z* 693 consists of two co-eluting isobaric lipids), which follow the proposed fragmentation pattern for the trimethylornithine (TMO) structure (Tables 2, 3). Analysis by GC and GC/MS of the *T. sphagniphila* extract confirmed that C18:1ω5c and C16:1ω5c fatty acids were the most abundant core lipids, followed by C16:0, C18:0, βOH-C16, βOH-C18, and C17:0 fatty acids. This is in agreement with previous lipid analysis reported by Kulichevskaya *et al*. (22).

Structural identification of trimethylornithine lipids by NMR. *T. sphagniphila* group III lipids appeared to be the most abundant unknown lipid cluster among the four analyzed planctomycetes. This lipid cluster was purified using semi-preparatory HPLC for further analysis. In addition, ornithine lipids were purified from *F. johnsoniae* (Fig. 2) to be used as a reference compound for structural comparison to the *T. sphagniphila* group III lipids. Approximately 2 mg of group III lipid was isolated from the *T. sphagniphila* lipid extract and 5 mg of ornithine lipid was isolated from *F. johnsoniae* lipid extract for NMR analysis.

The ^{1}H-NMR, COSY, and ^{13}C-NMR spectra of the purified ornithine lipid agreed well with previously reported NMR structural characterization of ornithine lipids (37, 38). There were many similarities between the ^{1}H-NMR and ^{13}C-NMR signals of the purified group III lipids and ornithine lipids, particularly for the aliphatic chains (Table 4). The main differences between the TMO lipid and ornithine ^{1}H-NMR spectra were the intense signal at 3.27 ppm in the TMO lipid spectra, the absence of a ε-NH_2 signal at 5.71 ppm, and the increase in chemical shift of the α-NH and head group δ-position signals. Both the ^{1}H-NMR and ^{13}C-NMR chemical shifts of the unknown signal (^{1}H 3.27 ppm, ^{13}C 54 ppm) and the head group δ-position (^{1}H 3.70, ^{13}C 66 ppm) in the TMO lipids match with the chemical shifts of the trimethylamine ($(CH_3)_3N^+$) group and adjacent carbon position (CH_2) reported for the choline group of PC lipids in previous studies ($(CH_3)_3N^+$: ^{1}H 3.25, ^{13}C 54.2; CH_2: ^{1}H 3.70, ^{13}C

66.1 – (39); $(CH_3)_3N^+$: ^{13}C 54.3; CH_2: ^{13}C 66.65 – (40); $(CH_3)_3N^+$: ^1H 3.30, ^{13}C 54.6 – (41)). (^1H, ^{13}C)-Heteronuclear single quantum correlation spectroscopy (HSQC) analysis revealed that the apparent trimethylamine proton at 3.27 ppm was directly bound to the carbon atom at 54 ppm. The (^1H, ^{13}C)-HSQC analysis also showed that the head group δ-position ^1H chemical shift was bound to an increased ^{13}C-NMR chemical shift as well compared to the head group δ-position signal in the ornithine lipid spectra.

Further confirmation of structural assignments was given by heteronuclear multiple bond correlation spectroscopy (HMBC) which showed a connectivity between the apparent trimethylamine signal and head group δ-position, and nuclear Overhauser effect spectroscopy (NOESY), which confirmed a short distance between the trimethylamine signal and head group δ-protons as well as the other head group and fatty acid chain protons. The presence of a highly charged quaternary amine in the lipid head group explains the absence of the δ-NH_2 signal in the proton spectrum, since no protons are attached directly to the nitrogen. It may also be expected that that this functional group increases the chemical shift of the α-NH compared to the ornithine lipid as observed. Alkene chemical shifts in the TMO NMR spectra (Table 4) were expected to be detected, as observed in the MS results. Therefore, the NMR results confirm the structural identification of the lipids in group III as a series of ornithine lipids that are trimethylated on the ε-nitrogen position (Fig. 3B, Table 2, 3, 4).

Structural elucidation of IPL groups II and I. The HRAM/OT/MS fragmentation of *T. sphagniphila* group II molecular ion *m/z* 651.5666 and group I *m/z* 637.5519 produced the same initial losses of a C16:1 fatty acid as observed for the TMO *m/z* 665.5824 lipid (Figs. 4, 5; Table 2). The thus formed group II *m/z* 379.3429 ion then loses C_2H_7N, while group I formed *m/z* 383.3429 loses CH_5N (Figs. 4, 5, Table 3). Analogues to the loss of C_3H_9N for trimethylornithine, the loss of C_2H_7N and CH_5N appears to indicate dimethylated and monomethylated nitrogen structures, respectively (Figs. 4, 5). After the methylated nitrogen

losses, the formed *m/z* 352.2847 ions for both group II *m/z* 651.5666 and group I *m/z* 637.5519 lose a C16:0 fatty acid, identical to what has been observed for the TMOs (Table 2), resulting in the same *m/z* 116.0710 $C_5H_{10}NO_2$ product (Table 3; Figs. 4, 5). For group II, fragmentation also resulted in formation of *m/z* 144.1021, *m/z* 161.1285, and *m/z* 187.1079 ions, and for group I, fragmentation resulted in *m/z* 129.1024, *m/z* 147.1129, and *m/z* 173.0921 products (Table 3, Fig. 4, 5). These fragmentation patterns were the same for all group II and group I lipids, respectively (Table 2).

Based on the fragmentation behavior and formula assignments of key losses and products we propose that the unknown lipids in cluster II represent a series of ornithine lipids that are dimethylated on the ε-nitrogen position, i.e. dimethylornithine (DMO, Fig. 4B), while unknown lipids in cluster I represent ornithine lipids that are monomethylated on the ε-nitrogen position, i.e. monomethylornithine (MMO, Fig. 5B). As with the TMO cluster, there were five main masses in both the DMO and MMO clusters that represent six lipids (Tables 2, 3). The fatty acid distribution was identical among the MMO, DMO, and TMO lipid groups and the relative abundance of the various lipids within each group was similar (Table 2). Under the chromatographic conditions used here, the elution order of ornithine, MMO, DMO, and TMO was the same as observed for PE, MMPE, DMPE, and PC lipids identified in the *Gemmata*-like SP5 lipid extract (Fig 2D), displaying an increasing retention time with increasing methylation of the ε-nitrogen.

Distribution of MMO, DMO, and TMO lipids in planctomycete strains. The trimethylornithine lipids appeared to be the most abundant of the three classes of methylornithines in each of the four planctomycetes (Fig 2). The MMO, and DMO lipids were both most abundant in *T. sphagniphila*, followed by *Gemmata*-like strain SP5, *S. rosea*, and *S. acidiphila*. The dominant core lipids observed in MMO, DMO, and TMOs were C18:1 and C16:1 for *T. sphagniphila*, C18:1 and C18:0 for *S. rosea*, C18:1, C18:0 for *S. acidiphila*,

316 and C20:1 and C16:0 for the *Gemmata*-like strain SP5. This difference in fatty acid
317 distribution observed between the lipid extracts of the four planctomycete species based on
318 HPLC/ESI/IT/MS fragmentation among the MMO, DMO, and TMO lipids resulted in slightly
319 different retention times of the three lipid classes for each species in the base peak
320 chromatograms (Fig 2).

DISCUSSION

This study describes the identification of a series of novel OLs with increasing methylation on the terminal ε-nitrogen position, resulting in mono-, di- and trimethylated ornithine head groups. OLs are common phosphorus-free membrane lipids in bacteria, but absent in Eukaryotes and Archaea (42-43). Roughly 25% of bacteria whose genomes have been sequenced have the predicted capability to produce OLs (44). It has been proposed that OLs are important for outer membrane stability in Gram-negative bacteria due to their zwitterionic nature (45). In some bacteria OLs are produced under phosphorus limitation (46-47), or hydroxylated under thermal or acid stress (48-50). Other amino acid-containing lipids have been identified in various bacteria including glycine lipids (51-52), a lysine lipid (53), and an ornithine-taurine linked lipid (54), although they are not nearly as widespread as OLs. While the exact function of OL modifications is not yet fully described, characterization of mutants that are deficient in ornithine lipid biosynthesis and modification genes has further supported a mechanism of bacterial stress response to changing environmental conditions (44, 49-50). The unique environmental settings from which the planctomycetes in this study were isolated, and the adaptable nature of ornithine lipids in various bacteria, suggest that the synthesis of methylated ornithine lipids by these microbes may be a response to the ombrotrophic conditions of northern wetlands.

Increasing acidity ultimately impedes microorganism growth, causing a range of physiological problems, including outer membrane damage (55). Various microbes have developed different strategies to respond to pH stress (56-58). The hydroxylation of ornithine lipids in *Rhizobium tropici* under acidic conditions increases the potential for hydrogen bonding among individual polar lipid molecules resulting in greater membrane stability (49-50). Like PEs, MMPEs, DMPEs, and PCs, the addition of each subsequent methyl group to the ε-NH_2 ornithine head group in MMOs, DMOs, and TMOs appears to increase the polarity

of the lipid based on LC retention time. This is especially true in the TMO lipids where the presence of a quaternary amine yields a charged choline-like moiety similar to that of PCs. Due to their polarity and relatively cylindrical shape, PCs spontaneously form bilayers, whereas the cone shape of PEs (non-methylated equivalent to PC) causes it to assemble into inverted hexagonal phases. Perhaps, the additional three methyl groups in the head group of TMO also give it a more cylindrical shape and greater polarity compared to non-modified ornithine lipids, and thus result in greater bilayer stability. The ombrotrophic wetlands, where these planctomycetes were isolated from, are acidic and nutrient-poor ecosystems, and all the four species in which the TMO lipid were observed are moderately acidophilic (19, 21-22). Increased membrane stability provided by a polar quaternary amine in lipid head groups would be beneficial to these organisms in their native environment. Furthermore, because these ecosystems are nutrient limited, the synthesis of TMO lipids could provide membrane stability without relying on limited phosphorus. It has been shown that marine microbes in phosphorus limited conditions will switch from producing phosphorus containing membrane lipids such as PCs and PGs to non-phosphorus containing lipids such as betaines and sulfoquinovosyl diacylglycerols (SQDGs) to maintain growth and survival (59). We hypothesize that the increased polarity and possible cylindrical shape of the trimethylornithine head group provides membrane stability without using scarce phosphorus and is thus, an adaptation to the acidic, nutrient-poor conditions of *Sphagnum*-dominated European North Russian wetlands.

The distributions of core lipid chains among MMO, DMO, TMO lipids in *T. sphagniphila* are identical among the three lipid classes (Table 2). There are also many similarities in core lipid distribution among MMO, DMO, and TMO lipids within the extracts of *S. rosea*, *S. acidiphila*, and *Gemmata*-like strain SP5. The similar fatty acid distribution among the three lipid classes within each species suggests a biosynthetic link between these

classes. This sequence of lipids with increasing methylation is not unprecedented in membrane lipids. One of the pathways for biosynthesis of PC involves sequential methylation of PE by the enzyme phosphatidylethanolamine N-methyltransferase producing MMPE and DMPE as intermediates (60-62). A similar methylation process of the ornithine lipid head group may be involved in the biosynthesis of methylornithine lipids.

Pyrosequencing of bacterial 16S rRNA gene fragments was performed by Kulichevskaya *et al*. (22) for the peat sample from which *T. sphagniphila* was originally isolated in order to assess importance of this species to the *Planctomycete* community. The study revealed that *T. sphagniphila* accounted for 10% of the planctomycete-related reads obtained from the peat sample, therefore suggesting that *T. sphagniphila* is a typical representative of *Planctomycetes* in peat bogs (22). *Singulisphaera*-like planctomycetes were also abundant in this peat sample. However, the majority (61%) of all planctomycete-related 16S rRNA gene sequences retrieved from this sample could not be assigned to taxonomically described organisms, leaving open the possibility that other species at this location produce TMO lipids as well. *Planctomycetes* have been observed to account for up to 14% of total bacterial cells in the upper, oxic peat layers of acidic northern wetlands and were mostly represented by members of the *Isosphaera-Singulisphaera* group (17). This suggests that the TMOs may be an important membrane constituent among the *Planctomycete* community in peat bogs, and may serve as an important environmental adaptation towards this group's function as slow acting decomposers of plant-derived organic matter. Further research is needed to investigate the specific function of these lipids, the mono-, di-, and trimethylated ornithine lipid biosynthetic pathway, and the importance of methylornithine lipids in ombrotrophic environments.

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555 **TABLES**

556 **Table 1:** Contribution of group I, II and III lipids (%) to the total lipid signal as determined
557 from the ESI/IT/MS base peak chromatogram of *T. sphagniphila*, *S. rosea*, *S. acidiphila*, and
558 *Gemmata*-like SP5 strain lipid extracts.

	I	II	III
T. sphagniphila	24.7	13.9	45.3
S. rosea	<1.0	3.6	44.4
S. acidiphila	<1.0	<1.0	37.2
SP5	<1.0	4.8	49.9

559 **Table 2:** The *m/z* of the molecular species, fatty acid losses, elemental composition, and
560 accuracy values (Δ mmu**) that occur during MS fragmentation of each mass in lipid groups
561 III (TMO), II (DMO), and I (MMO). R2-COOH and R1-CH=C=C=O losses result from
562 fragmentation to fatty acid (FA) and βOH-fatty acid (βOH-FA) respectively in the structure
563 below. For TMO: R3 = R4 = R5 = CH_3; DMO: R3 = R4 = CH_3, R5 = H; MMO: R3 = CH_3,
564 R4 = R5 = H.
565 *Relative abundance (Rel. Abun.) indicates the peak area percentage of each lipid within its
566 respective group.
567 **In general the 0.5 Δ mmu (milli mass unit) range was used as a measure of very high
568 confidence molecular formula assignments and the 1.0 Δ mmu range was used as a measure
569 of good confidence molecular formula assignments (Kostiainen et al., 1997; Sakayanagi et al.,
570 2006; Calza et al., 2012).

571 **Table 3:** The *m/z*, HRAM/OT/MS assigned molecular formulas, elemental composition, and
572 accuracy values (Δ mmu) of formula assignments of observed losses and products that occur
573 during MS fragmentation (Figure 2, 3, 4) of each mass in lipid groups III (TMO), II (DMO),
574 and I (MMO). Elemental composition shown below Δ mmu values.

575 **Table 4:** ^{13}C- and ^{1}H-NMR signals (ppm) from trimethylornithine (structure below) and

576 ornithine lipids. Positions are shown below.

577 **FIGURE LEGENDS**

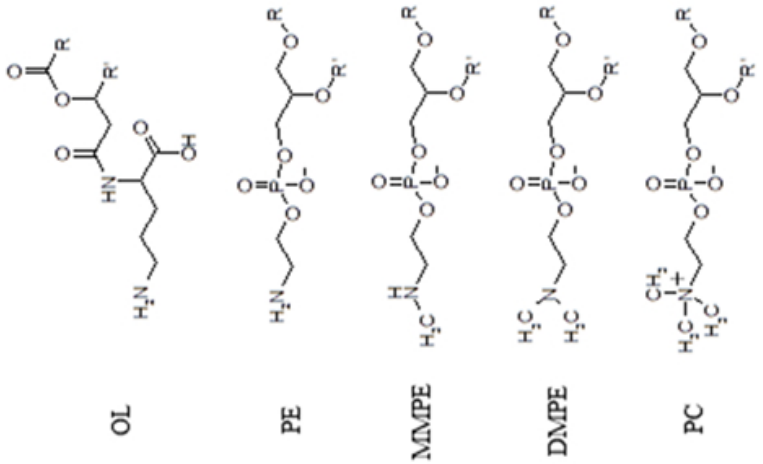
578 **Figure 1:** Molecular structures of ornithine lipid (OL); phosphatidylethanolamine (PE);
579 monomethylphosphatidylethanolamine (MMPE); dimethylphosphatidylethanolamine
580 (DMPE); phosphatidylcholine (PC).

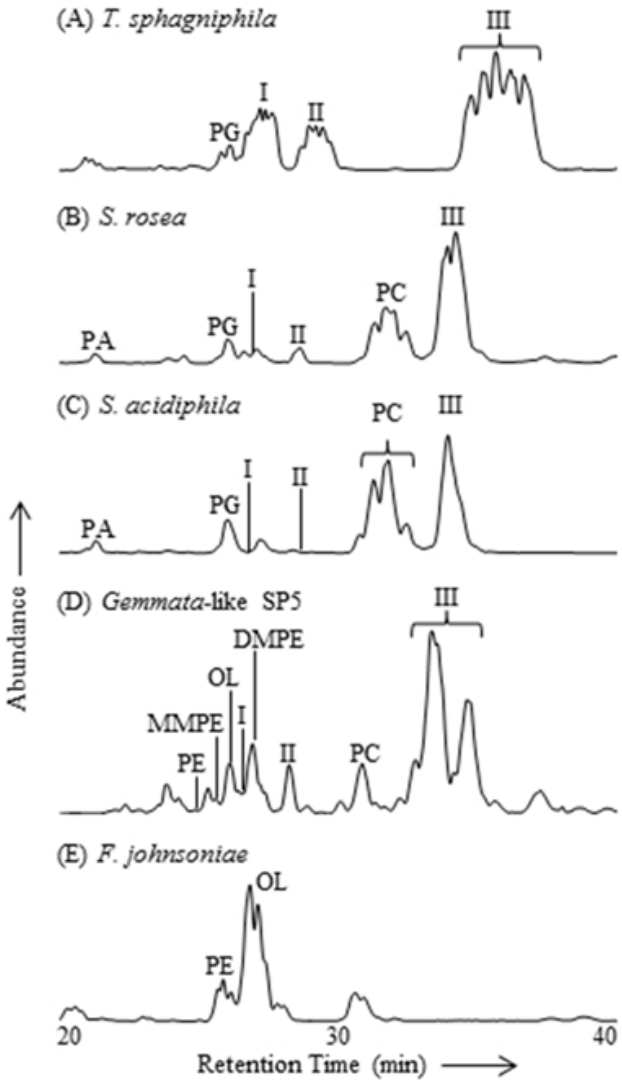
581 **Figure 2:** HPLC-ESI/IT/MS Ion Trap MS^1 base peak chromatograms (*m/z* 400-2000) of
582 Bligh & Dyer lipid extracts from planctomycete cultures: (A) *T. sphagniphila*; (B) *S. rosea*;
583 (C) *S. acidiphila*; (D) *Gemmata*-like SP5 strain; (E) *F. johnsoniae* (bacteriodete). PG =
584 phosphatidylglycerol; PA = phosphatidic acid; PC = phosphatidylcholine; PE =
585 phosphatidylethanolamine; MMPE = monomethylphosphatidylethanolamine; DMPE =
586 dimethylphosphatidylethanolamine; OL = ornithine lipid.

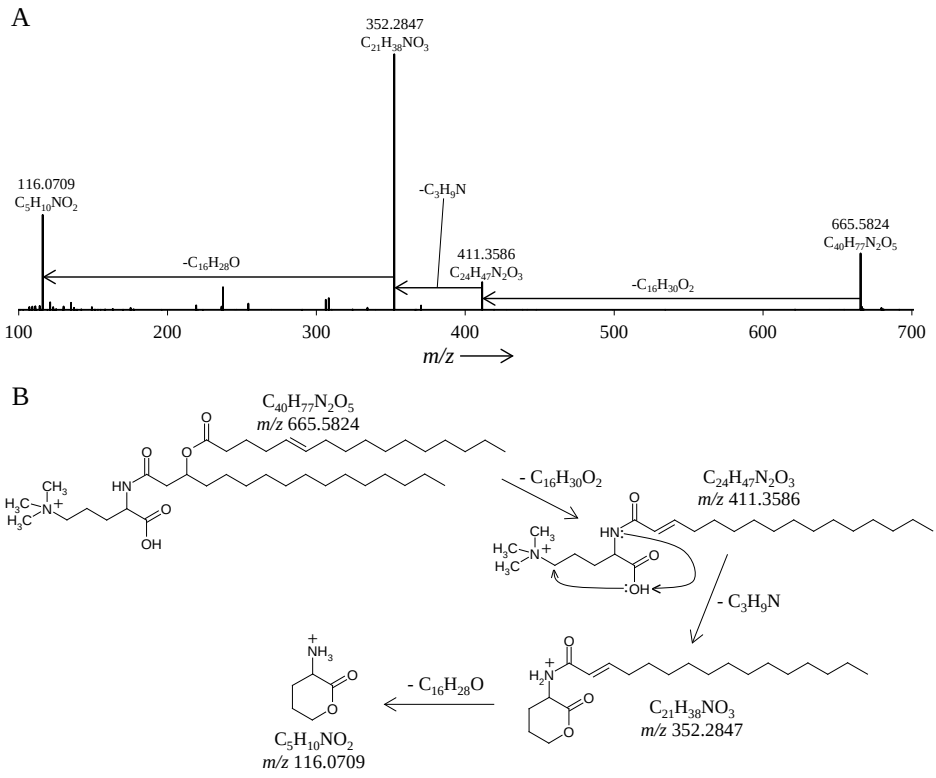
587 **Figure 3:** (A) High resolution accurate mass quadrupole OT MS^2 mass spectrum of *T.*
588 *sphagniphila* lipid extract group III trimethylornithine *m/z* 665.5824 (TMO: C16:1/C16:0).
589 Chemical formulas represent neutral losses and products after MS^2 fragmentation. (B)
590 Proposed gas-phase fragmentation pathway of trimethylornithine *m/z* 665.5824.

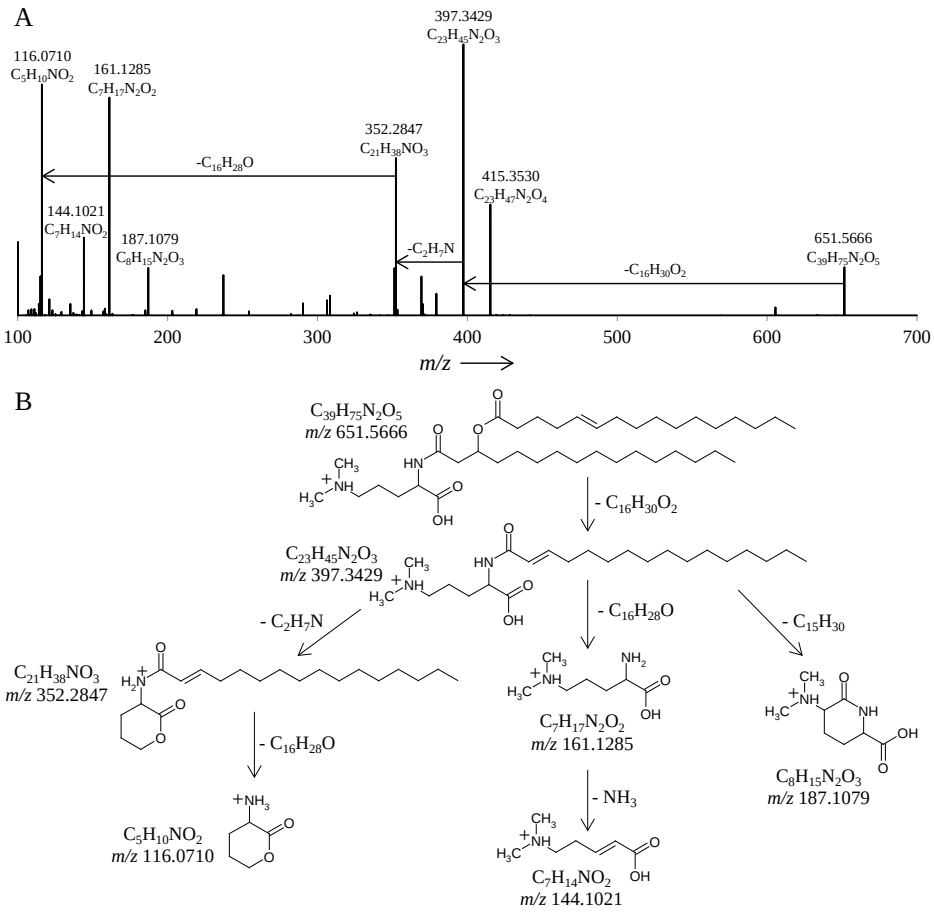
591 **Figure 4:** (A) High resolution accurate mass quadrupole OT MS^2 mass spectrum of *T.*
592 *sphagniphila* lipid extract group II dimethylornithine *m/z* 651.5666. Chemical formulas
593 represent neutral losses and products after MS^2 fragmentation. (B) Proposed gas-phase
594 fragmentation pathway of dimethylornithine *m/z* 651.5666.

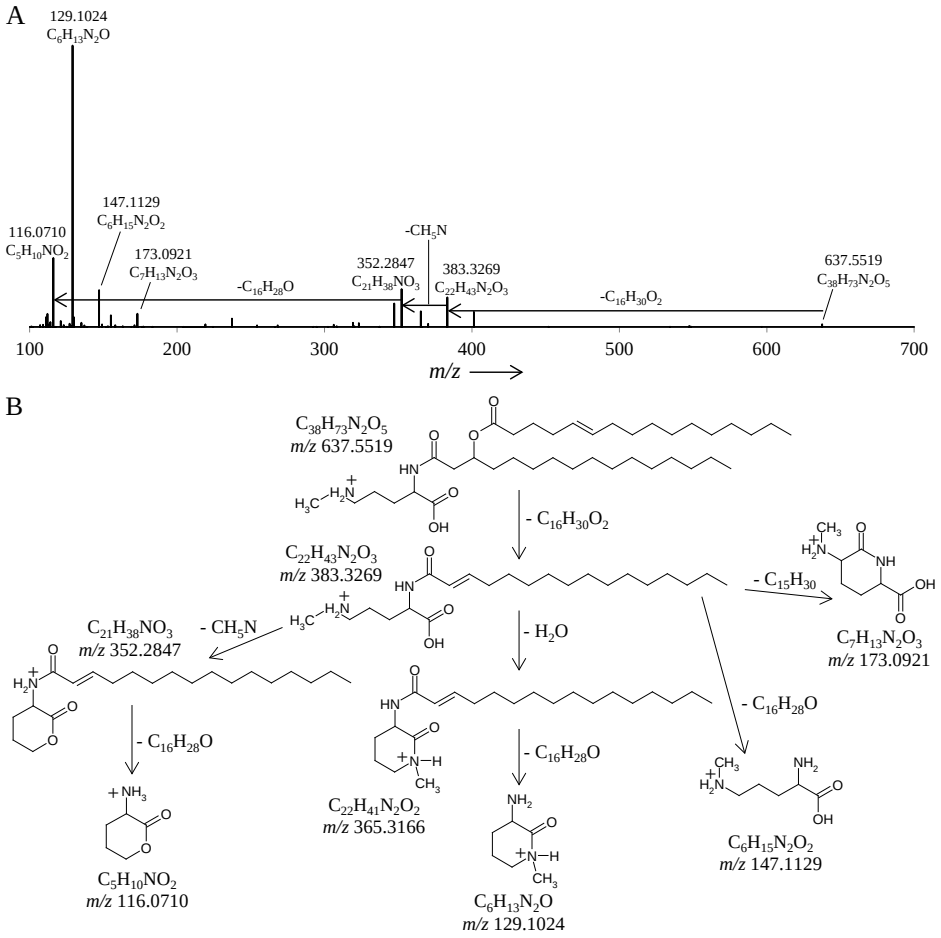
595 **Figure 5:** (A) High resolution accurate mass quadrupole OT MS^2 mass spectrum of *T.*
596 *sphagniphila* lipid extract group I monomethylornithine *m/z* 637.5519. Chemical formulas
597 represent neutral losses and products after MS^2 fragmentation. (B) Proposed gas-phase
598 fragmentation pathway of monomethylornithine *m/z* 637.5519.

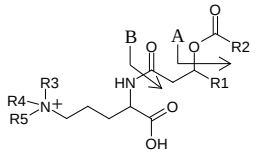




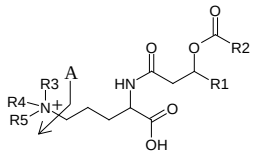




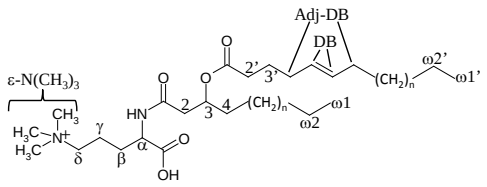




III (TMO)	M^+	Rel. Abun.* (%)	FA	[A]Observed Loss R2-COOH	Assigned Elemental Composition	Δ mmu**	βOH-FA	[B]Observed Loss R1-CH=C=C=O	Assigned Elemental Composition	Δ mmu**
$C_{40}H_{77}N_2O_5$	665	20.4	C16:1	254.2239	$C_{16}H_{30}O_2$	0.7	C16:0	236.2138	$C_{16}H_{28}O$	0.2
$C_{41}H_{79}N_2O_5$	679	29.3	C16:1	254.2243	$C_{16}H_{30}O_2$	0.3	C17:0	250.2291	$C_{17}H_{30}O$	0.6
$C_{42}H_{81}N_2O_5$	693	7.3	C16:1	254.2252	$C_{16}H_{30}O_2$	0.6	C18:0	264.2449	$C_{18}H_{32}O$	0.4
$C_{42}H_{81}N_2O_5$	693	9.8	C18:1	282.2556	$C_{18}H_{34}O_2$	0.3	C16:0	236.2137	$C_{16}H_{28}O$	0.3
$C_{43}H_{83}N_2O_5$	707	23.8	C18:1	282.2556	$C_{18}H_{34}O_2$	0.3	C17:0	250.2293	$C_{17}H_{30}O$	0.4
$C_{44}H_{85}N_2O_5$	721	9.6	C18:1	282.2556	$C_{18}H_{34}O_2$	0.3	C18:0	264.2447	$C_{18}H_{32}O$	0.6
II (DMO)	$[M+H]^+$									
$C_{39}H_{75}N_2O_5$	651	12.1	C16:1	254.2237	$C_{16}H_{30}O_2$	0.9	C16:0	236.2137	$C_{16}H_{28}O$	0.3
$C_{40}H_{77}N_2O_5$	665	27.2	C16:1	254.2251	$C_{16}H_{30}O_2$	0.5	C17:0	250.2290	$C_{17}H_{30}O$	0.7
$C_{41}H_{79}N_2O_5$	679	6.5	C16:1	254.2244	$C_{16}H_{30}O_2$	0.2	C18:0	264.2446	$C_{18}H_{32}O$	0.7
$C_{41}H_{79}N_2O_5$	679	14.2	C18:1	282.2554	$C_{18}H_{34}O_2$	0.5	C16:0	236.2137	$C_{16}H_{28}O$	0.3
$C_{42}H_{81}N_2O_5$	693	34.9	C18:1	282.2546	$C_{18}H_{34}O_2$	1.3	C17:0	250.2291	$C_{17}H_{30}O$	0.6
$C_{43}H_{83}N_2O_5$	707	5.1	C18:1	282.2540	$C_{18}H_{34}O_2$	1.9	C18:0	264.2447	$C_{18}H_{32}O$	0.6
I (MMO)	$[M+H]^+$									
$C_{38}H_{73}N_2O_5$	637	18.3	C16:1	254.2251	$C_{16}H_{30}O_2$	0.5	C16:0	236.2138	$C_{16}H_{28}O$	0.2
$C_{39}H_{75}N_2O_5$	651	25.0	C16:1	254.2255	$C_{16}H_{30}O_2$	0.9	C17:0	250.2294	$C_{17}H_{30}O$	0.3
$C_{40}H_{77}N_2O_5$	665	5.6	C16:1	254.2261	$C_{16}H_{30}O_2$	1.5	C18:0	264.2449	$C_{18}H_{32}O$	0.4
$C_{40}H_{77}N_2O_5$	665	11.7	C18:1	282.2577	$C_{18}H_{34}O_2$	1.8	C16:0	236.2138	$C_{16}H_{28}O$	0.2
$C_{41}H_{79}N_2O_5$	679	27.7	C18:1	282.2559	$C_{18}H_{34}O_2$	>0.1	C17:0	250.2293	$C_{17}H_{30}O$	0.4
$C_{42}H_{81}N_2O_5$	693	11.8	C18:1	282.2567	$C_{18}H_{34}O_2$	0.8	C18:0	264.2448	$C_{18}H_{32}O$	0.5



III (TMO)	[A]Obs. Loss		Obs. Prod.							
M^+	C_3H_9N	Δ mmu	$C_5H_{10}NO_2$	Δ mmu	Obs. Prod.	Δ mmu	Obs. Prod.	Δ mmu	Obs. Prod.	Δ mmu
665	59.0737	0.2	116.0710	0.2	-	-	-	-	-	-
679	59.0731	0.4	116.0710	0.2	-	-	-	-	-	-
693	59.0735	>0.1	116.0709	0.3	-	-	-	-	-	-
693	59.0742	0.7	116.0709	0.3	-	-	-	-	-	-
707	59.0739	0.4	116.0710	0.2	-	-	-	-	-	-
721	59.0733	0.2	116.0710	0.2	-	-	-	-	-	-
II (DMO)										
$[M+H]^+$	C_2H_7N		$C_5H_{10}NO_2$		$C_7H_{14}NO_2$		$C_7H_{17}N_2O_2$		$C_8H_{15}N_2O_3$	
651	45.0582	0.4	116.0710	0.2	144.1020	0.5	161.1285	0.5	187.1079	0.4
665	45.0575	0.3	116.0710	0.2	144.1020	0.5	161.1285	0.5	187.1077	0.6
679	45.0574	0.4	116.0710	0.2	144.1021	0.4	161.1285	0.5	187.1078	0.5
679	45.0574	0.4	116.0710	0.2	144.1021	0.4	161.1285	0.5	187.1078	0.5
693	45.0578	>0.1	116.0710	0.2	144.1021	0.4	161.1285	0.5	187.1078	0.5
707	45.0578	>0.1	116.0710	0.2	144.1021	0.4	161.1285	0.5	187.1078	0.5
I (MMO)										
$[M+H]^+$	CH_5N		$C_5H_{10}NO_2$		$C_6H_{13}N_2O$		$C_6H_{15}N_2O_2$		$C_7H_{13}N_2O_3$	
637	31.0422	>0.1	116.0710	0.2	129.1024	0.4	147.1129	0.5	173.0921	0.5
651	31.0428	0.6	116.0710	0.2	129.1024	0.4	147.1129	0.5	173.0923	0.3
665	31.0422	>0.1	116.0710	0.2	129.1024	0.4	147.1129	0.5	173.0922	0.4
665	31.0417	0.5	116.0710	0.2	129.1024	0.4	147.1129	0.5	173.0922	0.4
679	31.0427	0.5	116.0710	0.2	129.1024	0.4	147.1129	0.5	173.0923	0.3
693	31.0422	>0.1	116.0709	0.3	129.1023	0.5	147.1128	0.6	173.0920	0.6



	Trimethyl Ornithine				Ornithine			
	Carbon Chemical Shift			Proton	Carbon Chemical Shift			Proton
Position	CH_3	CH_2	CH	Chemical Shift	CH_3	CH_2	CH	Chemical Shift
ε-$N(CH_3)_3$		53.8		3.27		-		-
ε-NH_2		-		-		-		5.71
δ		66.1		3.70		41.8		3.37
γ		19.2		1.85		21.9		1.96
β		29.9		1.95		27.9		2.62
β'		29.9		1.80		27.9		1.53
α			53.1	4.25			50.9	4.29
α-NH		-		7.24		-		6.54
2		41.9		2.50		42.0		2.51
3			71.5	5.20			71.3	5.21
4		34.8		1.58		34.1		1.62
5-ω2		29.6		1.29		29.2		1.29
ω1	22.5			0.84	22.5			0.88
2'		34.7		2.28		34.2		2.30
3'		25.1		1.58		25.1		1.62
8'-ω2		29.6		1.29		29.6		1.29
ω1'	22.5			0.84	22.5			0.88
DB		129.5		5.38		-		-
Adj-DB		27.4		2.05		-		-