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**RESEARCH ARTICLE**

Characterization of Hydroxyphthioceranoic and Phthioceranoic Acids by Charge-Switch Derivatization and CID Tandem Mass Spectrometry

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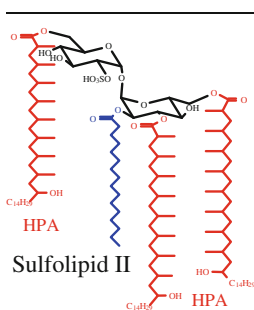
Abstract. Hydroxyphthioceranoic (HPA) and phthioceranoic (PA) acids are polymethylated long chain fatty acids with and without a hydroxyl group attached to the carbon next to the terminal methyl-branched carbon distal to the carboxylic end of the long-chain fatty acid, respectively. They are the major components of the sulfolipids found in the cell wall of *Mycobacterium tuberculosis* (*M. tuberculosis*) strain H37Rv. In this report, I describe CID linear ion-trap MS^n mass spectrometric approaches combined with charge-reverse derivatization strategy toward characterization of these complex lipids, which were released from sulfolipids by alkaline hydrolysis and sequentially derivatized to the N-(4-aminomethylphenyl) pyridinium (AMPP) derivatives. This method affords complete characterization of HPA and PA, including the location of the hydroxyl group and the multiple methyl side chains. The study also led to the notion that the hydroxyphthioceranoic acid in sulfolipid consists of two (for hC_{24}) to 12 (for hC_{52}) methyl branches, and among them 2,4,6,8,10,12,14,16-octamethyl-17-hydroxydotriacontanoic acid (hC_{40}) is the most prominent, while phthioceranoic acids are the minor constituents. These results confirm our previous findings that sulfolipid II, a family of homologous 2-stearoyl(palmitoyl)-3,6,6′-tris(hydroxyphthioceranoy1)-trehalose 2′-sulfates is the predominant species, and sulfolipid I, a family of homologous 2-stearoyl(palmitoyl)-3-phthioceranoyl-6,6′-bis(hydroxyphthioceranoy1)-trehalose 2′-sulfates is the minor species in the cell wall of *M. tuberculosis*.

Keywords: HCD, Charge-remote fragmentation, Microbial lipids, Lipidomics, Linear ion-trap, Charge reversed derivatization, *Mycobacterium tuberculosis*

Sulfolipid II

HPA

HPA

Introduction

The family of sulfated acyl trehaloses defined as sulfolipids (SLs) were characterized by Goren and coworkers in their early studies on *M. tuberculosis* H37Rv [1–4]. The principal SLs were thought to be sulfolipid-I (SL-I), which is a homologous mixture of 2,3,6,6′-tetraacyl-α,α′-D-trehalose-2′-sulfate consisting of a pair of hydroxyphthioceranoic acid (HPA) located at 6 and 6′-position, and a nonhydroxylated phthioceranoic acids (PA) and a saturated fatty acid (16:0 or 18:0) located at the 3- and 2-position of the trehalose skeleton,

respectively (Scheme 1). In addition to the major SL-I, minor species that were termed as SL-II (2-palmitoyl/stearoyl-3,6,6'-tris-hydroxyphthioceranyl-2'-sulfate), SL-I' (2-palmitoyl/stearoyl-3,6-bis-phthioceranyl-6'-hydroxyphthioceranyl-2'-sulfate), and SL-II' (2-palmitoyl/stearoyl-4,6,6'-tris-hydroxyphthioceranyl-2'-sulfate) were also reported [1–4]. However, recent studies with mass spectrometry including high resolution electrospray ionization (ESI) linear ion-trap MS^n and MALDI-TOF [5, 6] confirmed that the principal sulfolipid family is sulfolipid II, rather than sulfolipid I reported by Goren.

Both hydroxyphthioceranoic and phthioceranoic acids in sulfolipids are multiple methyl-branched long chain fatty acids. The traditional methods to define the structure require NMR, IR, and GC/MS analysis, following alkaline solvolyses of the purified sulfolipid to the free acids, which were then derivatized to methyl esters [3]. Rhoades et al. applied mmultiple stage mass spectrometric approach to locate the

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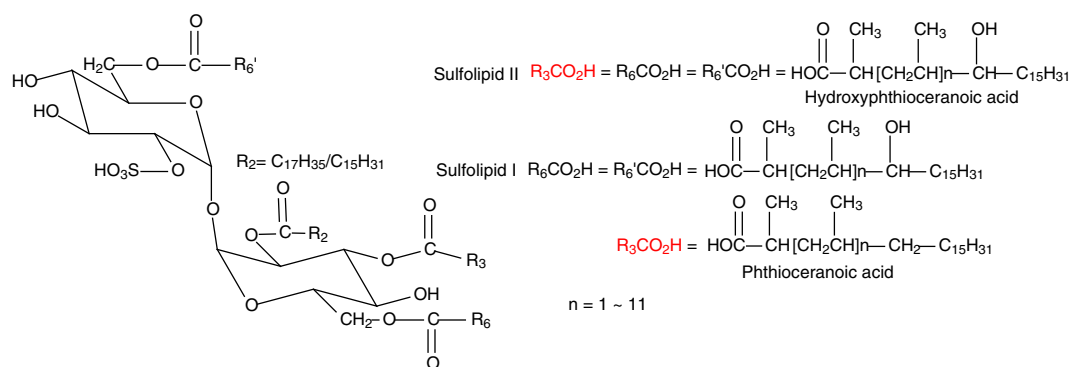
Scheme 1. Structures of sulfolipid I and II

hydroxyl side chain of the hydroxyphthioceranoic acids, which were detected as [M – H]⁻ ions formed by skimmer CAD on the intact sulfolipids. However, the location of the methyl side chains along the hydroxyphthioceranoic and phthioceranoic acids could not be assigned [5].

Towards sensitive quantitation and characterization of long chain fatty acid by ESI tandem quadrupole mass spectrometry, conversion of the free fatty acid to the *N*-(4-aminomethylphenyl) pyridinium (AMPP) derivative and detected as M⁺ ions was first described by Bollinger et al., followed by several groups [7–10]. This charge-reversed strategy also has been successfully applied to locate the methyl side chain of iso- and anteiso-long chain fatty acids in *Listeria monocytogen* cells [11]. In this report, similar charge-reversed strategy was used to convert HPA and PA to their AMPP derivatives. This is followed by ESI linear ion-trap (LIT) MSⁿ analysis of the derivatives to locate the hydroxyl and methyl side chains for unambiguous structural assignment of these complex long-chain fatty acids.

Materials and Methods

Materials

AMP+ Mass Spectrometry Kit (50 tests) containing AMPP derivatizing reagent, n-butanol (HOBt), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), acetonitrile/DMF solution, was purchased from Cayman Chemical Co. (Ann Harbor, MI, USA). All other solvents (spectroscopic grade) and chemicals (ACS grade) were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Sample Preparation

M. tuberculosis strain H37Rv were grown and sulfolipids were extracted and isolated as previously described [5]. To the dry sulfolipid extract (200 ug), 500 µL methanol and 500 µL tetrabutylammonium hydroxide (40 wt% solution in water) were added. The solution was heated at 75 °C for 2 h, cooled to room temperature, and 2 mL water and 2 mL hexane were added, vortexed for 1 min, and centrifuged at 1200 × g for 2 min. The top layer containing hydroxyphthioceranoic and phthioceranoic acids was transferred to a centrifuge tube, dried

under a stream of nitrogen, and AMPP derivative was made with the AMP+ Mass Spectrometry Kit, according to the manufacturer's instruction. Briefly, the dried sample was re-suspended in 20 µL ice-cold acetonitrile/DMF (4:1, v/v), and 20 µL of ice-cold 1 M EDCI (3-(dimethylamino)propyl)ethyl carbodiimide hydrochloride) in water was added. The vial was briefly mixed on a vortex mixer and placed on ice. To the vial, 10 µL of 5 mM *N*-hydroxybenzotriazole (HOAt) solution and 30 µL solution of 15 mM AMPP (in distilled acetonitrile) were added, mixed, and heated at 65 °C for 30 min. After cooling to room temperature, 1 mL water and 1 mL n-butanol were added. The final solution was vortexed for 1 min, centrifuged at 1200 × g for 3 min, and the organic layer was transferred to another vial.

Mass Spectrometry

Both high-resolution (R = 100,000 at *m/z* 400) higher energy collision induced dissociation (HCD) and low-energy CID tandem mass spectrometric experiments were conducted on a Thermo Scientific (San Jose, CA, USA) LTQ Orbitrap Velos mass spectrometer with Xcalibur operating system. Samples in methanol were infused (1.5 µL/min; ~1 pmol/µL) to the ESI source, where the skimmer was set at ground potential, the electrospray needle was set at 4.0 kV, and temperature of the heated capillary was 300 °C. The automatic gain control of the ion trap was set to 5 × 10⁴, with a maximum injection time of 100 ms. Helium was used as the buffer and collision gas at a pressure of 1 × 10⁻³ mbar (0.75 mTorr). The MSⁿ experiments were carried out with an optimized relative collision energy ranging from 55%–70%, with an activation q value at 0.25, and the activation time at 10 ms to leave a minimal residual abundance of precursor ion (around 20%). For HCD experiments, the collision energy was set at 60%–70% and mass scanned from *m/z* 100 to the upper *m/z* value that covers the M⁺ ions. The mass selection window for the precursor ions was set at 1 Da wide to admit the monoisotopic ion to the ion-trap for collision-induced dissociation (CID) for unit resolution detection in the ion-trap or high resolution accurate mass detection in the Orbitrap mass analyzer. Mass spectra were accumulated in the profile mode, typically for 3 to 10 min for MSⁿ spectra (n = 2, 3, 4). MALDI-TOF spectrum of the same

AMPP derivative of the hydroxyphthioceranoic and phthioceranoic acids was also obtained by an Applied Biosystem (Foster City, CA) Voyager DE-STR instrument using α-cyano 4-hydroxycinnamic acid as matrix.

Nomenclature

To facilitate data interpretation, the following abbreviations as previously described were adopted [5, 12]. The abbreviation of the nonhydroxylated multiple methyl-branched phthioceranoic acids, for example, the 2,4,6,8,10,12,14,16-octamethyl-dotriacontanoic acid is designated as C_{40}-acid to reflect the fact that the structure represents a saturated C_{40} fatty acid with multiple methyl branches. For hydroxydotriacontanoic acids, e.g., 2,4,6,8,10,12,14,16-octamethyl-17-hydroxydotriacontanoic acid is designated as hC_{40}-acid to reflect the fact that the compound is a saturated C_{40} fatty acid with multiple methyl side chains and one hydroxyl group attached at C-17. Therefore, the principal SL-II species (the position of the substituents on the trehalose backbone is adopted from the definition by Goren [13], which is a 2-stearoyl-3,6,6'-tris-2,4,6,8,10,12,14,16-octamethyl-17-hydroxydotriacontanoyl-α,α'-D-trehalose-2'-sulfate) is designated as (18:0, hC_{40}, hC_{40}, hC_{40})-SL, signifying that the compound consists of one stearoyl and three 2,4,6,8,10,12,14,16-octamethyl-17-hydroxydotriacontanoyl groups located at 2-, 3-, 6-, and 6'-position of the trehalose backbone, respectively, whereas SL-I molecule such as 2-palmitoyl-3-2,4,6,8,10-Pentamethyl-pentaeicosanoyl-6.6'-bis-2,4,6,8,10,12,14,16-octamethyl-17-
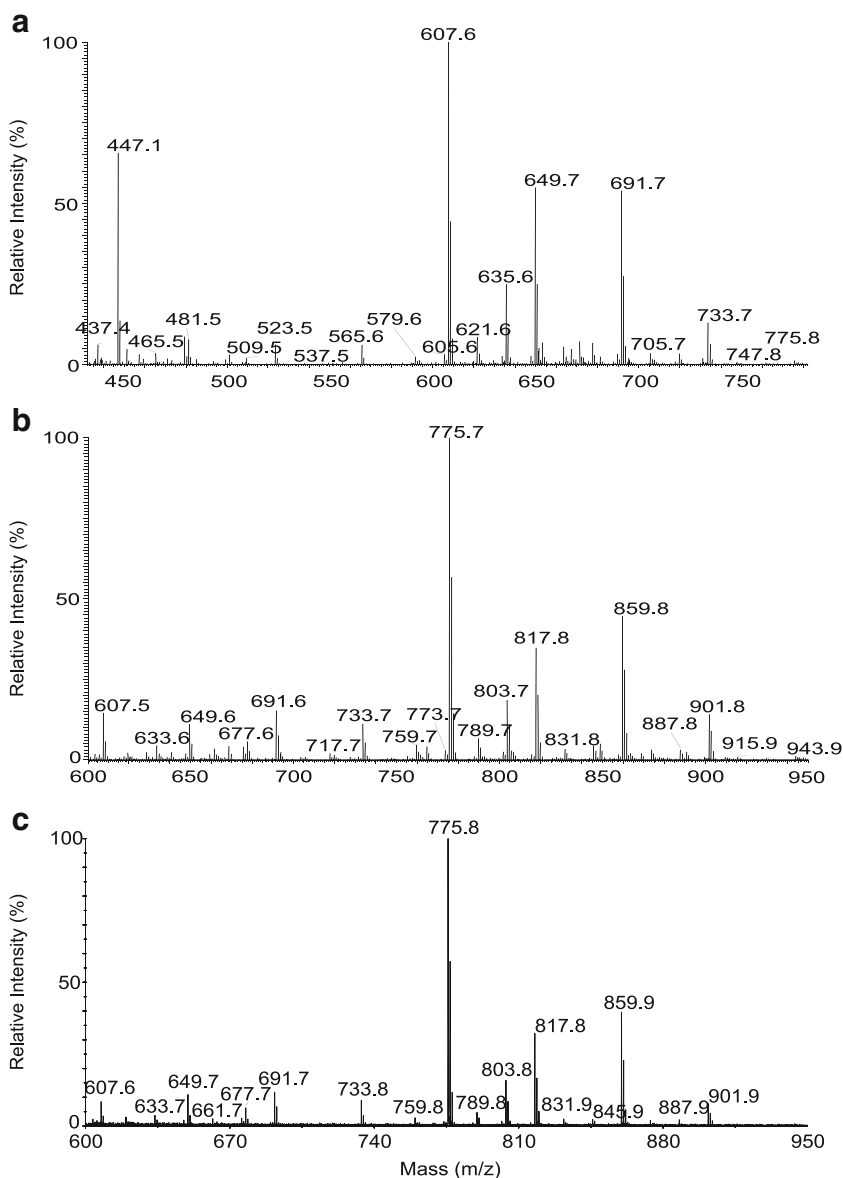
Figure 1. The full scan ESI mass spectrum of the hydroxyphthioceranoic and phthioceranoic acids released from alkaline hydrolysis of sulfolipids seen as the [M – H]⁻ ions in the negative-ion mode **(a)**, as the [M]⁺ ions of the AMPP derivative in positive-ion mode **(b)**, and the MALDI-TOF spectrum of the same AMPP derivative **(c)**

hydroxydotriacontanoyl-α,α'-D-trehalose-2'-sulfate is designated as (16:0, C_{30}, hC_{40}, hC_{40})-SL.

Results and Discussion

Mass Spectrometry of HPA and PA and Their AMPP Derivatives

The full scan mass spectra of the released HPA and PA after hydrolysis are shown in Figure 1, in which panel a represents the [M − H]⁻ ions of the free acids and panel b represent the [M]⁺ ions of corresponding AMPP derivative of the acids (panel b) obtained by ESI. The profile of the MALDI-TOF spectrum of the acid-AMPP derivative (panel c) is similar to that shown in panel b, demonstrating the utility of fatty acid-AMPP derivative for sensitive and fast analysis by MALDI-TOF mass spectrometry. High resolution mass measurements on the [M − H]⁻ ions (Table 1) indicate that two ion series were formed. The principal ion series belong to the hydroxyphthioceranoic acid family consisting of homologous ions from m/z 383 (hC_{24}) to m/z 775 (hC_{52}), with 2–12 methyl branches and a hydroxyl group attached to the carbon next to the C15, C16, or C17 alkyl chain terminal, whereas the minor ion series ranging from m/z 381 (C_{25}) to m/z 675 (C_{46}) belong to the phthioceranoic acid family with no hydroxyl group (Table 1). High resolution mass measurements on the M⁺ ions

Table 1. High Resolution Mass Measurement of Hydroxyphthioceranoic and Phthioceranoic Acids and their AMPP Derivatives of Sulfolipid Hydrolysate

Free acid				AMPP derivatives				Terminal alkyl chain length	Number of methyl side chain	Structure type	Rel. Int. (%)
Measured m/z	Theo. mass Da	Deviat. (mDa)	Elemental composition	Measured m/z	Theo. mass Da	Deviat. (mDa)	Elemental composition				
383.3527	383.3531	-0.34	C24 H47 O3	551.4563	551.4571	-0.79	C36 H59 O2 N2	17	2	hC24	1.96
397.3684	397.3687	-0.33	C25 H49 O3	565.4722	565.4728	-0.57	C37 H61 O2 N2	15	3	hC25	1.97
409.4048	409.4051	-0.32	C27 H53 O2	577.5082	577.5091	-0.96	C39 H65 O N2	15	4	C27	2.64
411.3840	411.3844	-0.33	C26 H51 O3	579.4877	579.4884	-0.73	C38 H63 O2 N2	16	3	hC26	0.91
423.4204	423.4208	-0.35	C28 H55 O2	591.5240	591.5248	-0.83	C40 H67 O N2	15	4	C28	2.55
425.3997	425.4000	-0.31	C27 H53 O3	593.5033	593.5041	-0.8	C39 H65 O2 N2	17	3	hC27	5.3
437.4361	437.4364	-0.27	C29 H57 O2	605.5397	605.5404	-0.73	C41 H69 O N2	16	4	C29	7.08
439.4154	439.4157	-0.29	C28 H55 O3	607.5190	607.5197	-0.72	C40 H67 O2 N2	15	4	hC28	1.31
451.4517	451.4521	-0.37	C30 H59 O2	619.5555	619.5561	-0.6	C42 H71 O N2	17	4	C30	5.33
453.4310	453.4313	-0.36	C29 H57 O3	621.5348	621.5354	-0.55	C41 H69 O2 N2	16	4	hC29	0.46
465.4674	465.4677	-0.35	C31 H61 O2	633.5713	633.5717	-0.47	C43 H73 O N2	15	5	C31	2.4
467.4466	467.4470	-0.36	C30 H59 O3	635.5505	635.5510	-0.53	C42 H71 O2 N2	17	4	hC30	0.88
479.4830	479.4834	-0.32	C32 H63 O2	647.5869	647.5874	-0.48	C44 H75 O N2	16	5	C32	9.93
481.4623	481.4626	-0.35	C31 H61 O3	649.5662	649.5667	-0.45	C43 H73 O2 N2	15	5	hC31	7.54
493.4986	493.4990	-0.44	C33 H65 O2	661.6026	661.6030	-0.48	C45 H77 O N2	17	5	C33	1.01
495.4778	495.4783	-0.46	C32 H63 O3	663.5819	663.5823	-0.41	C44 H75 O2 N2	16	5	hC32	0.67
507.5143	507.5147	-0.4	C34 H67 O2	675.6184	675.6187	-0.29	C46 H79 O N2	15	6	C34	0.8
509.4935	509.4939	-0.39	C33 H65 O3	677.5977	677.5980	-0.25	C45 H77 O2 N2	17	5	hC33	1.91
523.5092	523.5096	-0.35	C34 H67 O3	691.6134	691.6136	-0.24	C46 H79 O2 N2	15	6	hC34	6.16
535.5458	535.5460	-0.2	C36 H71 O2	703.6497	703.6500	-0.27	C48 H83 O N2			C36	0.8
537.5247	537.5252	-0.47	C35 H69 O3	705.6290	705.6293	-0.3	C47 H81 O2 N2	16	6	hC35	0.35
549.5612	549.5616	-0.43	C37 H73 O2	717.6655	717.6656	-0.15	C49 H85 O N2	15	7	C37	0.64
551.5404	551.5409	-0.45	C36 H71 O3	719.6447	719.6449	-0.17	C48 H83 O2 N2	17	6	hC36	0.68
565.5561	565.5565	-0.45	C37 H73 O3	733.6604	733.6606	-0.11	C49 H85 O2 N2	15	7	hC37	6.09
577.5924	577.5929	-0.5	C39 H77 O2	745.6966	745.6969	-0.28	C51 H89 O N2	17	7	C39	0.35
579.5716	579.5722	-0.53	C38 H75 O3	747.6761	747.6762	-0.15	C50 H87 O2 N2	16	7	hC38	0.53
591.6081	591.6086	-0.45	C40 H79 O2	759.7127	759.7126	0.11	C52 H91 O N2	15	8	C40	2.63
593.5873	593.5878	-0.48	C39 H77 O3	761.6919	761.6919	0.17	C51 H89 O2 N2	17	7	hC39	1.39
605.6237	605.6242	-0.48	C41 H81 O2	773.6920	773.7282	0.16	C53 H93 O N2	16	8	C41	0.45
607.6029	607.6035	-0.62	C40 H79 O3	775.7076	775.7075	0.06	C52 H91 O2 N2	15	8	hC40	100
619.6392	619.6399	-0.67	C42 H83 O2	787.7440	787.7439	0.14	C54 H95 O N2	17	8	C42	1.14
621.6185	621.6191	-0.59	C41 H81 O3	789.7232	789.7232	0.05	C53 H93 O2 N2	16	8	hC41	8.56
633.6549	633.6555	-0.58	C43 H85 O2	801.7597	801.7595	0.11	C55 H97 O N2	15	9	C43	2.82
635.6342	635.6348	-0.57	C42 H83 O3	803.7390	803.7388	0.16	C54 H95 O2 N2	17	8	hC42	25.17
647.6705	647.6712	-0.64	C44 H87 O2	815.7900	815.7752	0.15	C56 H99 O N2	16	9	C44	0.54
649.6497	649.6504	-0.69	C43 H85 O3	817.7546	817.7545	0.16	C55 H97 O2 N2	15	9	hC43	55.51
661.6861	661.6868	-0.73	C45 H89 O2	829.7910	829.7908	0.11	C57 H101 O N2	17	9	C45	1.07
663.6654	663.6661	-0.7	C44 H87 O3	831.7702	831.7701	0.1	C56 H99 O2 N2	16	9	hC44	5.52
675.7017	675.7025	-0.8	C46 H91 O2	843.8059	843.8065	-0.6	C58 H103 O N2	15	10	C46	0.73
677.6810	677.6817	-0.75	C45 H89 O3	845.7852	845.7858	-0.52	C57 H101 O2 N2	17	9	hC45	6.53
691.6965	691.6974	-0.84	C46 H91 O3	859.8008	859.8014	-0.61	C58 H103 O2 N2	15	10	hC46	53.32
705.7122	705.7130	-0.85	C47 H93 O3	873.8165	873.7807	-0.61	C59 H105 O2 N2	16	10	hC47	3.3
719.7278	719.7287	-0.83	C48 H95 O3	887.8320	887.8327	-0.67	C60 H109 O2 N2	17	10	hC48	3.21
733.7435	733.7443	-0.82	C49 H97 O3	901.8478	901.8484	-0.6	C61 H111 O2 N2	15	11	hC49	12.3
747.7591	747.7600	-0.92	C50 H99 O3	915.8634	915.8640	-0.63	C62 H111 O2 N2	16	11	hC50	0.82
761.7747	761.7756	-0.94	C51 H101 O3	929.8791	929.8797	-0.55	C63 H113 O2 N2	17	11	hC51	0.49
775.7903	775.7913	-0.97	C52 H103 O3	943.8938	943.8953	-1.52	C64 H115 O2 N2	15	12	hC52	1.15

of the corresponding AMPP derivatives (with a terminal $C_5H_5N^+$-C_6H_4-CH_2NH- substituent) confirm the findings (Table 1). These results are consistent with the recent reports that sulfolipid II, which consists of three hydroxyphthioceranoyl substituents is the predominate sulfolipid family found in *M. tuberculosis* H37Rv, whereas sulfolipid I that possesses one phthioceranoyl and two hydroxyphthioceranoyl substituents is the minor species [5, 6], a reversal to the earlier findings by Goren [1–3]. The CID and HCD LIT MS^n mass spectrometric approaches toward complete structural characterization of these hydrophthioceranoic and phthioceranoic acids as AMPP derivatives are described below.

Characterization of Hydroxyphthioceranoic Acid-AMPP Derivatives

Both CID LIT MS^n and the unique HCD MS^2 feature of an Orbitrap were employed in these structural studies. As shown in Figure 2a, the HCD MS^2 spectrum of the M^+ ion of *m/z* 775 contained prominent ions at *m/z* 169 and 183, together with *m/z* 211 that are characteristic ions for the fatty acid-AMPP derivatives [7–9]. The spectrum also contained the ion series of *m/z* 239, 281, 323, 365, 407, 449, 491, and 533 arising from cleavages of the $CH(CH_3)$–CH_2 bonds, together with the ion series of 253, 295, 337, 379, 421, 463, and 505 arising from cleavage of CH_2–$CH(CH_3)$ bonds along the acid-AMPP chain
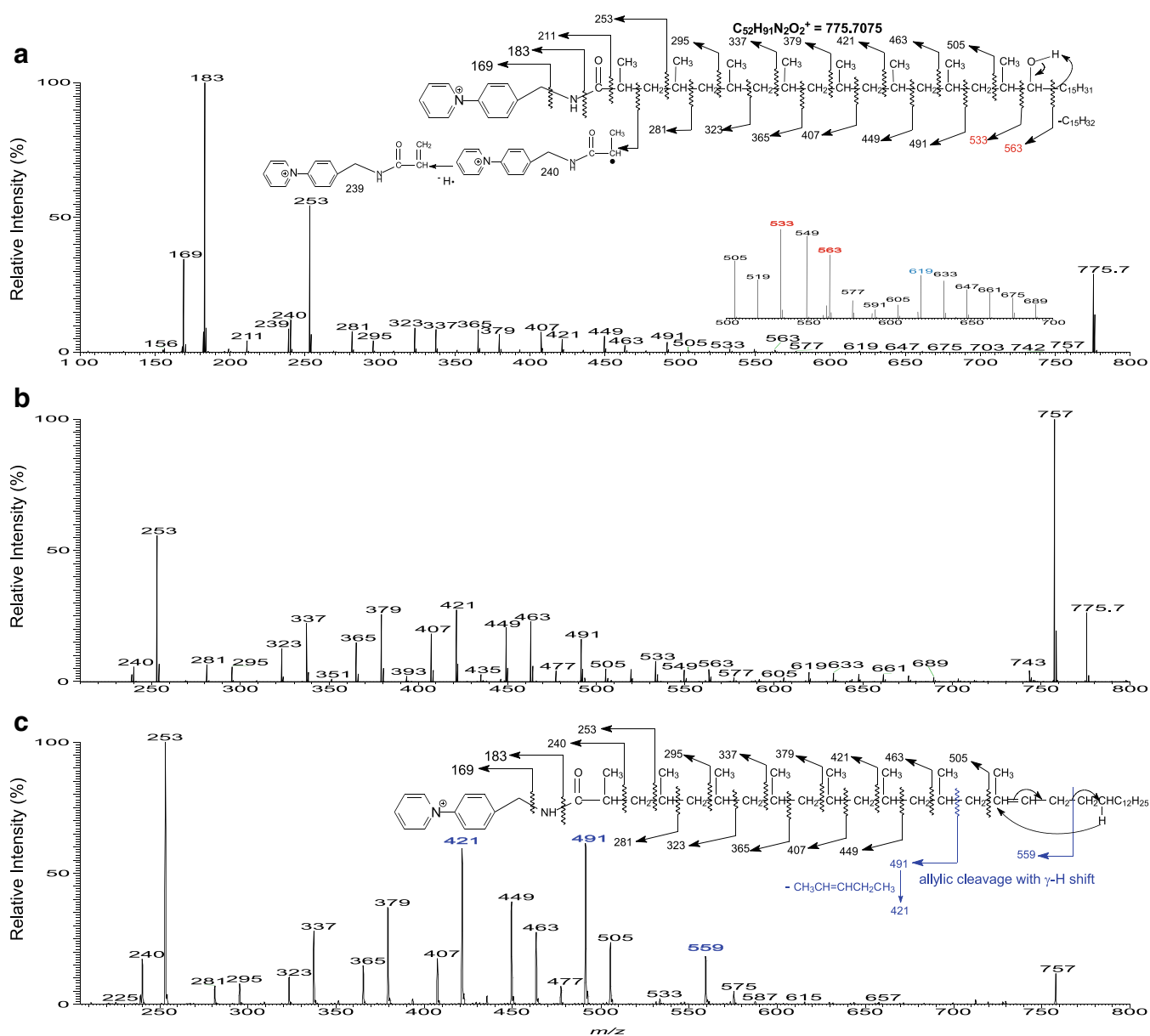
Figure 2. The MS^2 spectra of the $[M]^+$ ion of the AMPP derivative of *m/z* 775 obtained with higher collision energy (HCD) **(a)**, with low energy CID **(b)**, and its MS^3 spectrum of the ion of *m/z* 757 (775 → 757) **(c)**

via charge-remote fragmentation processes, indicating the presence of the multiple methyl groups at 2, 4, 6, 8, 10, 12, 14, and 16 of the fatty acid chain (Figure 2a, inset).

In addition to the above ions locating the methyl groups, ions at m/z 563 arising from cleavage of $CH(OH)–C_{15}H_{31}$ bond are also present. This ion is 30 Da (CH_2O) heavier than the ion of m/z 533 that possesses the terminal methyl side chain, indicating that the hydroxyl side chain is attached to C-17 (Scheme 2a). These results point to the structure of 2,4,6,8,10,12,14,16-octamethyl-17-hydroxydotriacontanoic acid (hC$_{40}$), consistent with that reported by Goren [1–4]. In contrast, the CID MS^2 spectrum of the ion of m/z 775 (Figure 2b) is dominated by the ion of m/z 757 arising from loss of H_2O, together with the ion series that locate the methyl side chains at 2, 4, 6, 8, 10, 12, 14, and 16, as well as the hydroxyl group at C-17 as seen in Figure 2a.

Further dissociation of the ion of m/z 757 (775 → 757; Figure 2c) gave rise to the ion series of m/z 281, 323, 365, 407, 449, and 491, indicating the presence of the methyl side chains at 2, 4, 6, 8, 10, 12, and 14; however, the ions at m/z 533 and 563, previously observed in Figure 2a and b are absent. The results indicate that the m/z 757 ion arising from a water loss likely involves the participation of the hydrogen located at C-16 to form a 2, 4, 6, 8, 10, 12, 14-heptamethyl dotriacont-16-enoic acid (C$_{40:1}$). The support of this proposed structure is recognized by the presence of the ion of m/z 559 (Figure 2c), arising from cleavage of the allylic bond distal from the cationic pyridinium charge site, via charge remote fragmentation with γ-H rearrangement as shown in Scheme 2b. Similar fragmentation process arising from cleavage of the allylic bond proximal to the charge site also results in the formation of the prominent 1-alkene ion at m/z 491, which undergoes further CRF with γ-H shift to yield the prominent ion of m/z 421 via loss of a $CH_3CH=CHCH_2CH_3$ residue (Scheme 2b) [14, 15].

A distonic ion at m/z 240 and an abundant ion at m/z 253 are observed in the spectra shown in Figure 2a, b, and c. The former ion most likely derives from homolytic cleavage of the C2–C3 bond, whereas the latter ion may arise from cleavage of the C3–C4 bond to form a stable 2-methyl prop-2-enamide cation (Scheme 2a). The assignments of these ions are consistent with the observation of the analogous distonic ion of m/z 226 and the prop-2-enamide cation at m/z 239 in the MS^2 spectra of the palmitate-AMPP derivative released from the 2-palmitoyl substituent of HPA and PA (Figure S1 of Supplementary Material), and of iso- and ante-iso fatty acid-AMPP derivatives previously reported [11], and were confirmed by high resolution mass measurements (Table S1, Supplemental Material). Notably, this distonic ion of m/z 226 was not previously reported in the similar product-ion spectra obtained with a tandem quadrupole instrument [7]. The observation of m/z 240, analogous to m/z 226, also points to the notion that a methyl group is attached to C2, consistent with the assigned structure of 2,4,6,8,10,12,14,16-octamethyl-17-hydroxydotriacontanoic acid.
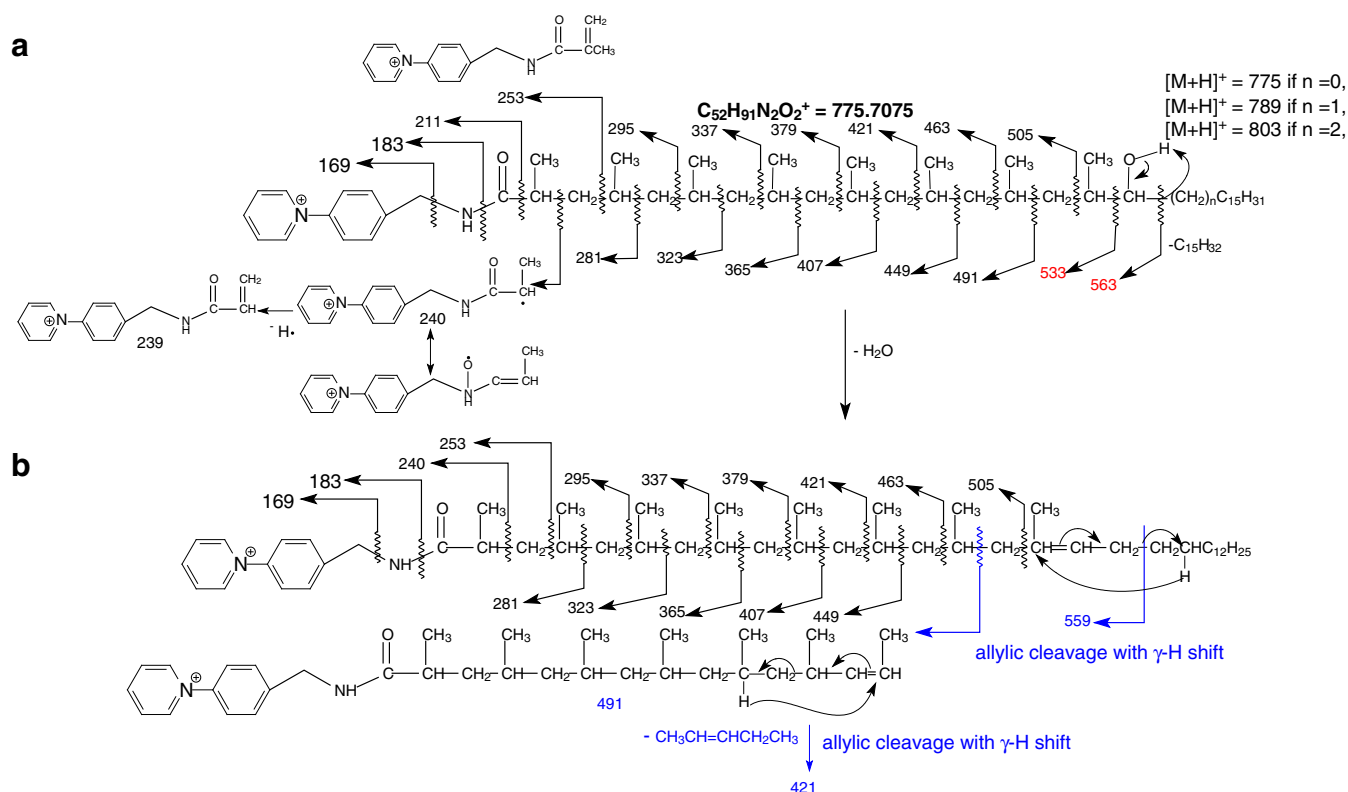
Scheme 2. The fragmentation processes proposed for the C40-hydroxyphthioceranoic acid-AMPP derivative at m/z 775 under HCD **(a)**, CID **(a)** and **(b)**
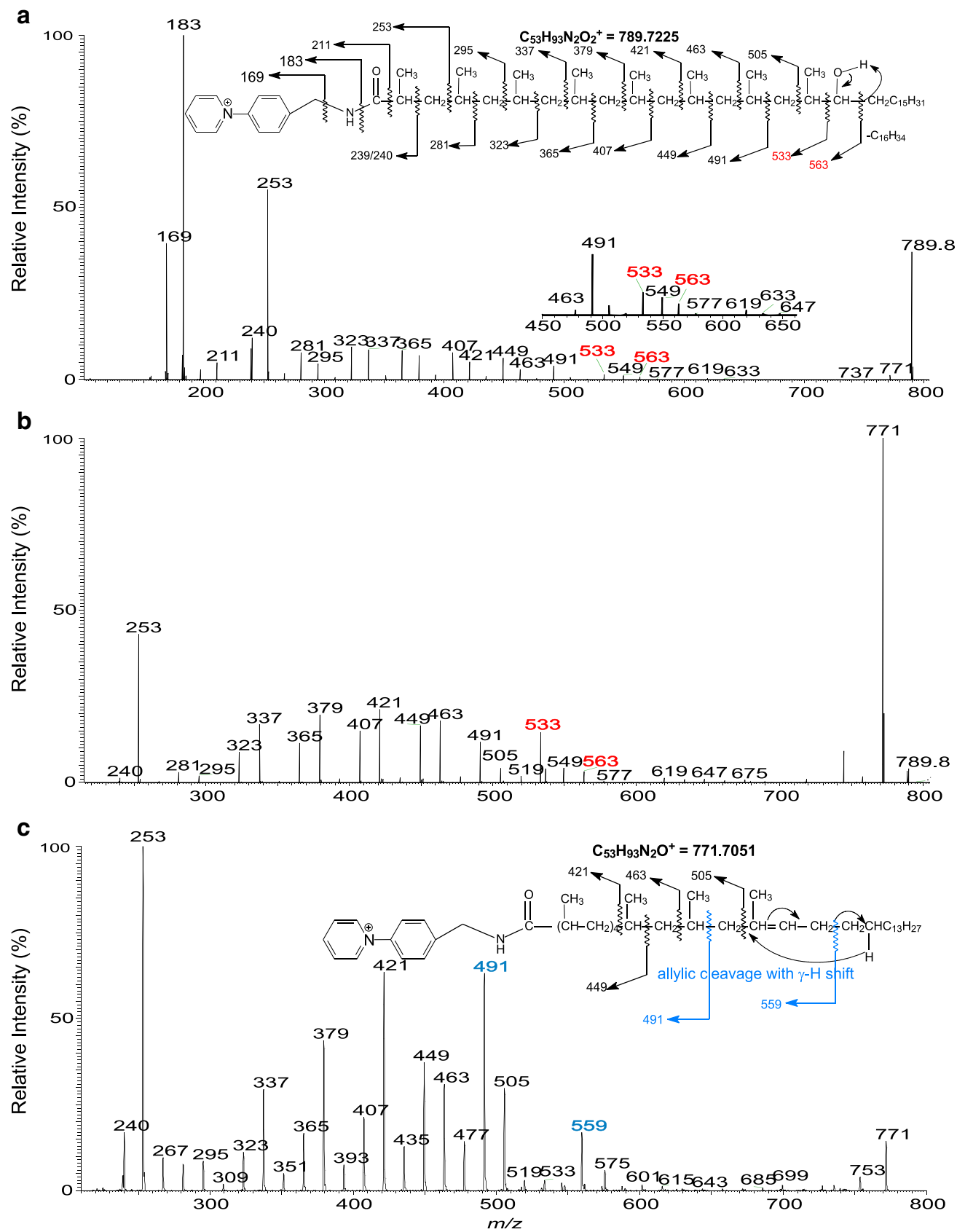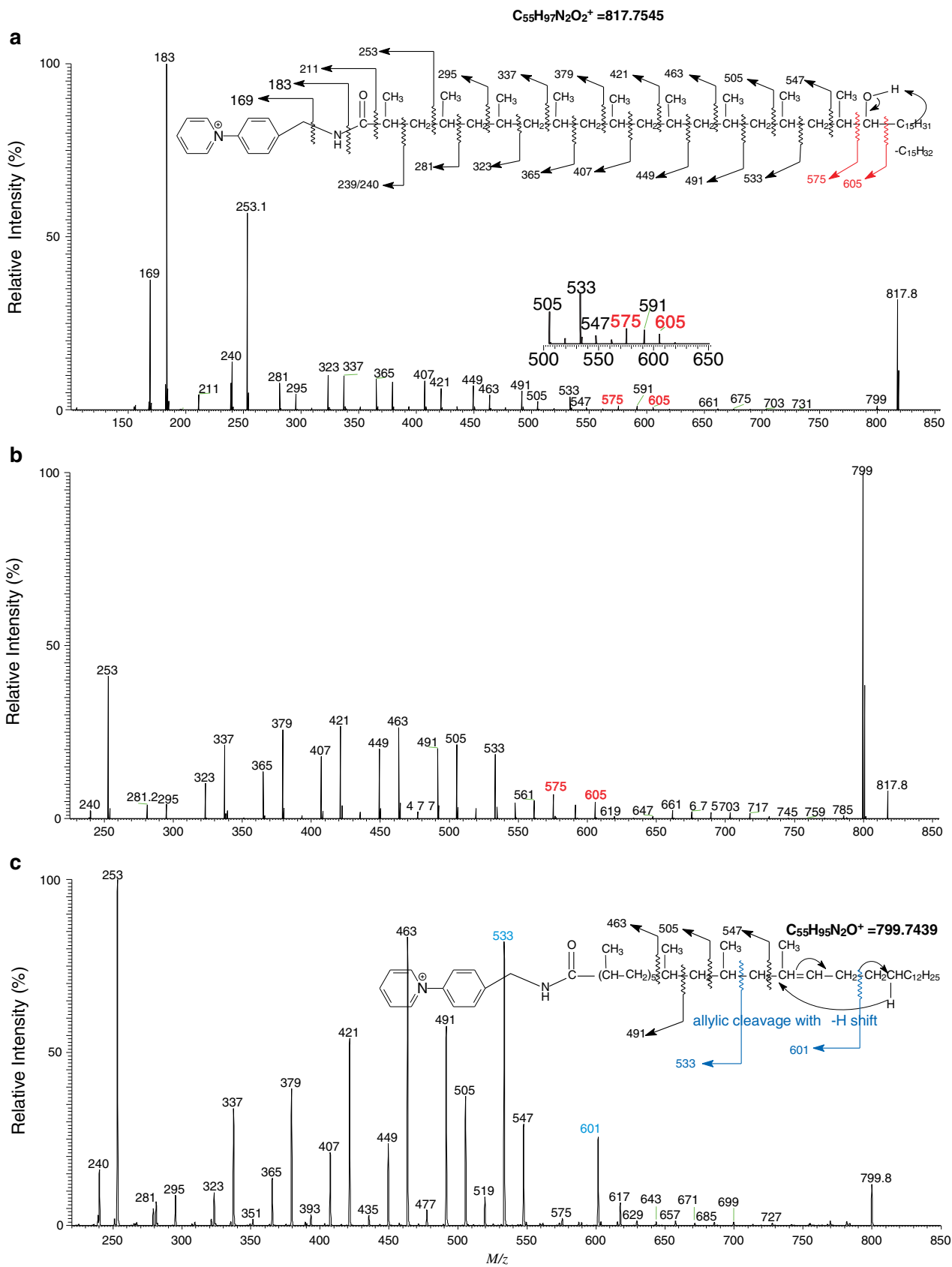
Figure 3. The MS^2 spectra of the [M]$^+$ ion of the AMPP derivative of m/z 789 obtained with HCD **(a)**, with CID **(b)**, and the sequential MS^3 spectrum of m/z 771 (789 → 771) **(c)**

Figure 4. The MS^2 spectra of the ion of m/z 817 obtained with HCD **(a)**, with low energy CAD **(b)**, and its MS^3 spectrum of the ion of m/z 799 (817 → 799) **(c)**

Two pronounced ions at m/z 619 and 549 in the series (Figure 2a and b) are worth mentioning. Elemental compositions derived from high resolution mass measurement indicate that ions at m/z 619.5195 (calculated $C_{41}H_{67}O_2N_2$; 619.5197 Da) and 549.4409 (calculated $C_{36}H_{57}O_2N_2$:549.4415 Da) retain the two oxygen atoms (Supplementary Table S1) of the precursor ion of m/z 775. MS^3 on the ion of m/z 619 (775 → 619; Supplementary Figure S2) yielded the similar ion series of m/z 253, 295, 337, 379, 421, 463, and 505 that define the location of the multiple methyl chains, together with ions at m/z 561, 577 from losses of C_3H_6O and C_3H_6 residues (supported by HR mass measurement; data not shown), respectively. The results point to the notion that the ion may contain a terminal cyclic tetrahydropyran ring, which is likely formed by cyclization and cleavage of a $C_{11}H_{24}$ moiety (see the inset scheme in Supplementary Figure S2 for fragmentation). Similar fragmentation process may also result in the formation of the ion of 549 by loss

of a $C_{16}H_{34}$ residue. This structural information may be an indication of the location of the hydroxyl side chain; however, more studies are required to confirm this finding.

The HCD MS^2 spectrum of the ion of m/z 789 (Figure 3a) and the CID MS^2 spectrum of the ion of m/z 789 (Figure 3b) and its subsequent MS^3 spectrum of m/z 771 (Figure 3c) all contained the identical ion series of m/z 239, 281, 323, 365, 407, 449, 491, and 533, as well as of m/z 253, 295, 337, 379, 421, 463, and 505 as seen earlier (Figure 2), defining the methyl side chains at 2, 4, 6, 8, 10, 12, 14, 16, whereas ions at m/z 563 indicate the presence of the hydroxyl group at C-17. This structural information led to the assignment of 2,4,6,8,10,12,14,16-octamethyl-17-hydroxy tritriacontanoic acid (hC_{41}) in which a terminal C16-alkyl chain is attached to the distal (OH)CH-terminal. A series of the homologous ions consisting of a terminal C16-alkyl chain with various methyl side chains were
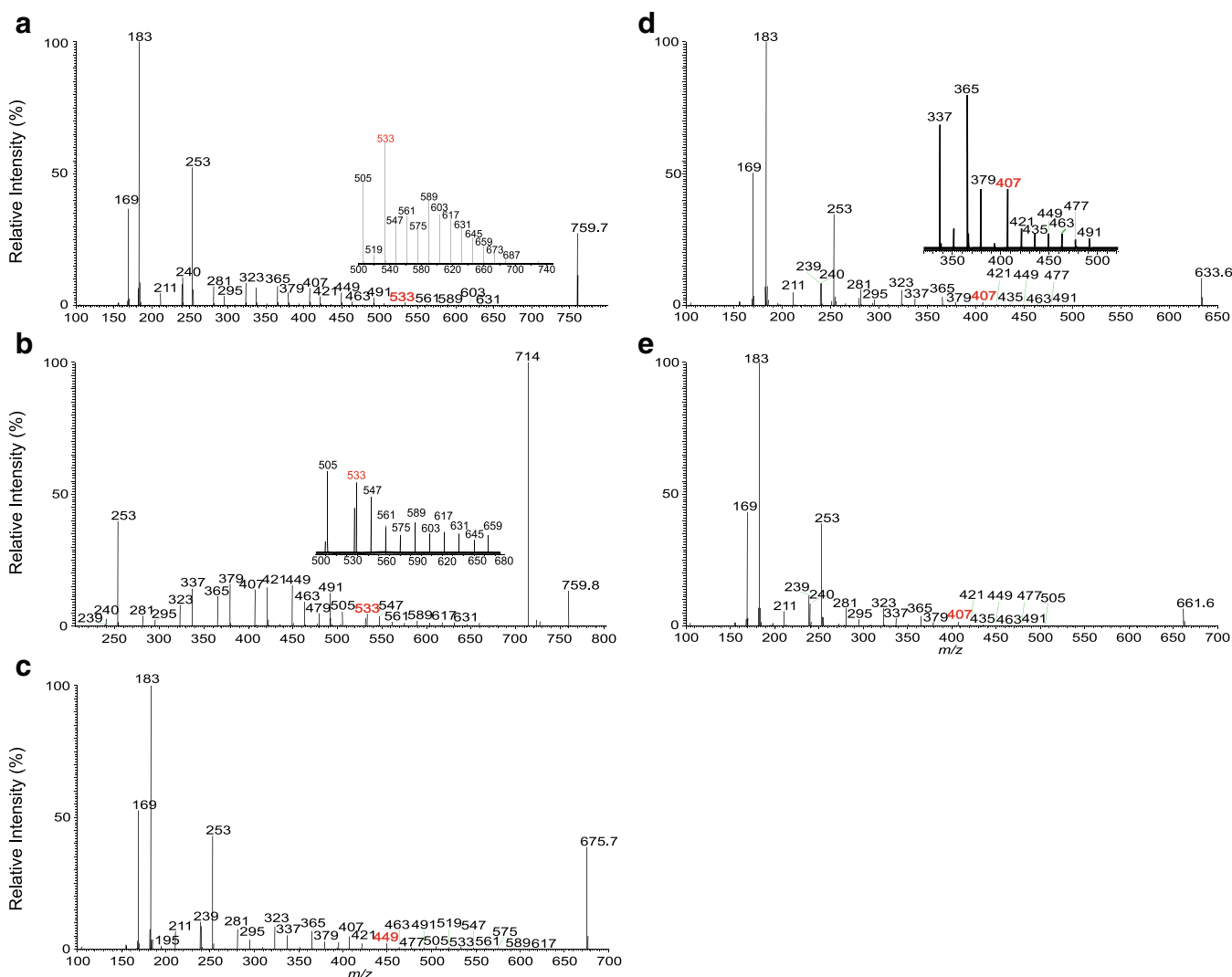

Figure 5. The MS^2 spectra of the ion of m/z 759 obtained with HCD **(a)**, with low energy CAD **(b)**, and the HCD MS^2 spectra of the ions of m/z 675 **(c)**, 633 **(d)**, and of m/z 661 **(e)**

observed at *m/z* 579, 621, 663, 705, 747, 789, 831, and 873 (Table 1). The structures of this minor ion series had not been previously reported by Goren [3].

Assignments of the compounds possessing various methyl side chains are exemplified by the HCD MS^2 spectrum of the ion of *m/z* 817 (Figure 4a), which comprises ions at *m/z* 239, 281, 323, 365, 407, 449, 491, 533, and 575, along with the ion at *m/z* 605. The results indicate the presence of the methyl side chains at 2, 4, 6, 8, 10, 12, 14, 16, 18 and the hydroxyl group at C-19, corresponding to 2,4,6,8,10,12,14,16,18-nonamethyl-19-hydroxytetratriacontanoic acid (inset). This structural assignment is further supported by the CID MS^2 spectrum of *m/z* 817 (Figure 4b), and the MS^3 spectrum of the ion of *m/z* 799 (817 → 799; Figure 4c), arising from loss of water. The spectrum contained the ion series of *m/z* 239, 281, 323, 365, 407, 449, 491, and 533, and of *m/z* 253, 295, 337, 379, 421, 463, 505, and 547 along with the ion of *m/z* 601 and 533 from allylic cleavages with γ-H shift, analogous to those seen in Figure 2c.

### Characterization of Phthioceranoic Acid-AMPP Derivatives

HCD and low energy CID tandem mass spectrometry toward characterization of AMPP derivative of phthioceranoic acid family was exemplified by the ion species at *m/z* 759, which gave rise to the HCD MS^2 spectrum (Figure 5a) with feature ions of *m/z* 169, 183, and 211, along with the ion series of *m/z* 253, 295, 337, 379, 421, 463, and 505, and of *m/z* 239/240, 281, 323, 365, 407, 449, 491, and 533 that locate the multiple methyl side chains at 2, 4, 6, 8, 10, 12, 14, and 16 of the fatty acid backbone (Scheme 3). The spectrum also contained ions at *m/z* 547, 561, 575, 589, 603, 617, 631, 645, 659, …etc. (Figure 5a, subset), arising from CRF cleavage of the C–C bonds of the n-alkyl terminal, indicating the attachment of a terminal n-hexaoctanyl (n-C16) residue. The above information gives assignment of 2,4,6,8,10,12,14,16-octamethyl-dotriacontanoic acid structure (C$_{40}$). Similar ions were also observed in the CID MS^2 spectrum of the ion of *m/z* 759 (Figure 5b and inset); however, the spectrum is dominated by the ion of *m/z* 714, which is absent in Figure 5a. High resolution mass measurement of the ion (measured *m/z*: 714.7506 Da) failed to match an interpretable elemental composition, indicating that the ion may be artificial and the source of this artifact is unclear. Nevertheless, the results readily located the

multiple methyl side chains and gave assignment of the C-40 phthioceranoic acid structure.

Similarly, the HCD mass spectrum of *m/z* 675 (Figure 5c) contained the ion series of *m/z* 239/240, 281, 323, 365, 407, and 449, and of *m/z* 253, 295, 337, 379, and 421 that locate the methyl groups at 2, 4, 6, 8, 10, and 12; together with ions at *m/z* 463, 477, 505, 519, 533, 547, …etc., which arise from cleavages of the terminal n-alkyl C–C bond. The results led to assignment of 2,4,6,8,10,12-hexamethyl-octaeicosanoic acid (C$_{34}$), possessing a terminal n-C16 residue. The HCD mass spectra of *m/z* 633 (Figure 5d) and of 661 (Figure 5e) all contained the ion series of *m/z* 239/240, 281, 323, 365, and 407, along with the ions of 253, 295, 337, and 379 indicating the presence of the methyl side chains at 2, 4, 6, 8, and 10. These ions together with the ions of *m/z* 421, 435, 449, 463, 477, 491, 505, 519, 533, …etc., arising from CRF cleavages of the terminal n-alkyl chain, point to the notion that the former spectrum (Figure 5d) represents a 2,4,6,8,10-pentamethyl-hexaeicosanoic acid (C$_{31}$), whereas the latter represents a 2,4,6,8,10-pentamethyl-octaeicosanoic acid structure (C$_{33}$),in which the n-alkyl terminal is C2 longer (i.e., n-C18 chain).

Conclusions

Both the CID MS^n and HCD tandem mass spectra of the AMPP derivatives of HPA and PA obtained with an Orbitrap provide structural information for complete characterization of their structures. Fragment ions arising from classic charge-remote fragmentations readily recognize the multiple methyl side chains and the hydroxyl groups. Although the sensitivity of the AMPP derivative of the hydroxyphthioceranoic and phthioceranoic acids was not evaluated in this study, a significant improvement in the detection by mass spectrometry was observed compared with that seen as the [M – H]$^-$ ions in the previous studies [5]. Thus, characterization of the minor species becomes feasible, and the structures including the minor phthioceranoic acid family and the low abundance ions such as 2,4,6,8,10,12,14,16-octamethyl-17-hydroxytritriacontanoic acid in the hydroxyphthioceranoic acid family can be determined. This latter species contains a terminal C16-alkyl chain and was not reported previously [1–3]. The observation of near equal abundances of palmitic and stearic acids in the hydrolysate (data not shown) is also consistent with the notion that sulfolipids consist of 2-palmitoyl/stearoyl substituent.
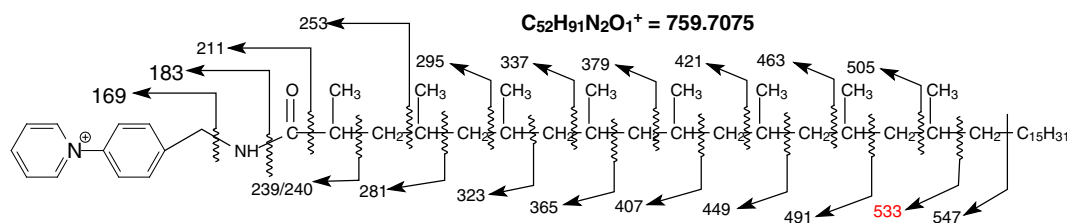
Scheme 3. The fragmentation processes proposed for the C40-phthioceranoic acid-AMPP derivative at m/z 759

Acknowledgments

References

1. Goren, M.B.: Sulfolipid of *Mycobacterium tuberculosis*, strain H37Rv. I. Purification and properties. Biochim. Biophys. Acta **210**, 116–126 (1970)
2. Goren, M.B.: Sulfolipid of *Mycobacterium tuberculosis*, strain H37Rv. II. Structure studies. Biochim. Biophys. Acta **210**, 127–138 (1970)
3. Goren, M.B., Brokl, O., Das, B.C., Lederer, E.: SulfolipidI of *Mycobaterium tuberculosis*, strain H37Rv. Nature of the acyl substituents. Biochemistry **10**, 72–81 (1971)
4. Goren, M.B., Brokl, O., Roller, P., Fales, H.M., Das, B.C.: Sulfatides of *Mycobacterium tuberculosis*: the structure of the principal sulfatide (SL-I). Biochemistry **15**, 2728–2735 (1976)
5. Rhoades, E.R., Streeter, C., Turk, J., Hsu, F.-F.: Characterization of sulfolipids of *Mycobacterium tuberculosis* H37Rv by multiple-stage linear ion-trap high-resolution mass spectrometry with electrospray ionization reveals that the family of sulfolipid II predominates. Biochemistry **50**, 9135–9147 (2011)
6. Layre, E., Cala-De Paepe, D., Larrouy-Maumus, G., Vaubourgeix, J., Mundayoor, S., Lindner, B., Puzo, G., Gilleron, M.: Deciphering sulfoglycolipids of *Mycobacterium tuberculosis*. J. Lipid Res. **52**, 1098–1110 (2011)
7. Yang, K., Dilthey, B.G., Gross, R.W.: Identification and quantitation of fatty acid double bond positional isomers: a shotgun lipidomics approach using charge-switch derivatization. Anal. Chem. **85**, 9742–9750 (2013)
8. Bollinger, J.G., Thompson, W., Lai, Y., Oslund, R.C., Hallstrand, T.S., Sadilek, M., Turecek, F., Gelb, M.H.: Improved sensitivity mass spectrometric detection of eicosanoids by charge reversal derivatization. Anal. Chem. **82**, 6790–6796 (2010)
9. Wang, M., Han, R.H., Han, X.: Fatty acidomics: global analysis of lipid species containing a carboxyl group with a charge-remote fragmentation-assisted approach. Anal. Chem. **85**, 9312–9320 (2013)
10. Bollinger, J.G., Rohan, G., Sadilek, M., Gelb, M.H.: LC/ESI-MS/MS detection of FAs by charge reversal derivatization with more than four orders of magnitude improvement in sensitivity. J. Lipid Res. **54**, 3523–3530 (2013)
11. Tatituri, R.V., Wolf, B., Brenner, M., Turk, J., Hsu, F.-F.: Characterization of polar lipids of Listeria monocytogenes by HCD and low-energy CAD linear ion-trap mass spectrometry with electrospray ionization. Anal. Bioanal. Chem. **407**, 2519–2528 (2015)
12. Merrill Jr., A.H.: Sphingolipids. In: Vance, D.E., Vance, J.E. (eds.) Biochemistry of Lipids, Lipoproteins and Membranes (Fifth Edition), p. 363. Elsevier, San Diego (2008)
13. Goren, M.B., Brokl, O., Das, B.C.: Sulfatides of *Mycobacterium tuberculosis*: the structure of the principal sulfatide (SL-I). Biochemistry **15**, 2728–2735 (1976)
14. Hsu, F.-F., Turk, J.: Elucidation of the double-bond position of long-chain unsaturated fatty acids by multiple-stage linear ion-trap mass spectrometry with electrospray ionization. J. Am. Soc. Mass Spectrom. **19**, 1673–1680 (2008)
15. Hsu, F.-F., Turk, J.: Structural characterization of unsaturated glycerophospholipids by multiple-stage linear ion-trap mass spectrometry with electrospray ionization. J. Am. Soc. Mass Spectrom. **19**, 1681–1691 (2008)