SUPPLEMENTARY INFORMATION

N-Acyl-O-Phosphocholineserines are a Novel Class of Lipids that Serve as Biomarkers for Niemann-Pick C1 Disease

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Chemicals and reagents

L-serine tert-butyl ester hydrochloride, 2-chloro-5,5-dimethyl-1,3,2-dioxaphosphorinane 2-oxide, and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) was obtained from VWR (West Chester, PA). Methanol, isopropanol, and acetonitrile were high performance liquid chromatography (HPLC) grade and were purchased from EMD Chemicals (Gibbstown, NJ). Milli-Q ultrapure water was prepared in-house with a Milli-Q Integral Water Purification System (Billerica, MA). All other reagents were obtained from Sigma–Aldrich (St. Louis, MO). The choline tetraphenylborate was prepared according to a previously reported method (33).

Profiling of lysoSM-509 (APCS) in human plasma and dried blood spot

Plasma samples (50 µL) were aliquoted into 2 mL polypropylene tubes (VWR, West Chester, PA). To each tube methanol (200 µL) was added. The sample was vortexed for 3 min, centrifuged for 10 min at 3000 g, and supernatant transferred to 1.2 mL glass inserts (VWR, West Chester, PA) for liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay.

A 3 mm disc was punched from each dried blood spot using a Harris Micro-Punch (Thermo Fisher Scientific, Waltham, MA). Each punch was transferred to a clean 2 mL polypropylene tube (VWR, West Chester, PA). Fifty µL of water was added to each punch and the mixture was vortexed for 10 min. To each tube methanol (200 µL) was added. The sample was vortexed for 3 min, centrifuged for 10 min at 9400 g, and supernatant transferred to 1.2 mL glass inserts for LC-MS/MS assay of lysoSM-509 (PPCS).

The LC-MS/MS analysis was conducted on a Shimadzu Prominence HPLC system (Columbia, MD) coupled with an Applied Biosystems/MDS Sciex 4000QTRAP mass spectrometer (Ontario, Canada). The PPCS was separated on a XBridge C8 analytical column (50 × 3 mm, 3.5 µm) (Waters, Milford, MA) protected with a SecurityGuard C8 guard column (4 x 3 mm) (Phenomenex, Torrance, CA).

The mobile phases consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in methanol-acetonitrile (4:1) (solvent B), and the flow rate was 0.6 mL/min. The gradient was as follows: 0-3 min, 70 to 100% solvent B; 3-3.5 min, 100% solvent B; 3.5-3.6 min, 100 to 70% solvent B; 3.6-5 min, 70% solvent B. The eluate was directed into the mass spectrometer for data acquisition from 2.0 – 3.5 min; elsewhere, eluate was sent to waste to minimize source contamination. The injection volume was 5 μL, and the total run-time was 5 min. The electrospray ionization (ESI) source temperature was 500 ºC; the ESI needle was 5000 V; the declustering potential was 80 V; the collision energy was 31 eV; the entrance potential was 10 V; and the collision cell exit potential was 10 V. The collision and curtain gas were set at medium and 20, respectively. Both desolvation gas and nebulizing gas were set at 35 L/min. Positive multiple reaction monitoring (MRM) transition of *m/z* 509.3 to 184 was used to detect PPCS, and the dwell time was set at 50 ms. Data were acquired and analyzed by the Analyst software (version 1.5.2). In a separate LC run, fractions containing the PPCS were collected from plasma samples for further structure analysis.

The measurement of lysoSM-509 (PPCS) in dried blood spot was also carried out with ultra performance liquid chromatography (UPLC)-MS/MS on a Waters Xevo-TQ MS/MS instrument coupled to a Waters Aquity binary solvent system. To each 3 mm DBS in the well of a 96-well microtiter plate 150 μL 30 nM d_3-PPCS was added in methanol for extraction. The plate was sealed with a silicone sealing mat. After extraction at 37 °C for 2 hours with shaking on an orbital platform (~200 rpm), the plate was centrifuged at 3000 x g for 5 min at room temperature. A 100 μL aliquot was transferred to a new well. The sample was then subjected to UPLC-MS/MS analysis. The UPLC column (HSS T3 column, 1.8 μm, 2.1 x 50 mm) and guard column were from Waters (#186003538and #186003976) and were held at 40 °C in the column manager. The flow rate was 0.5 mL/min. Solvent A was 50/50 water/acetonitrile with 0.1% formic acid (v/v/v), and solvent B was 80/20 isopropanol/acetonitrile with 0.1% formic acid (v/v/v). The weak and strong needle wash for the autosampler were 50/25/25 water/methanol/isopropanol (v/v/v) and 47.5/47.5/5

methanol/isopropanol/water (v/v/v), respectively. Elution started with 50% mobile phase B, and increased linearly to 95% mobile phase B over 1 min, then increased linearly to 100% mobile phase B from 1 min to 1.5 min. The gradient composition was switched back to 50% mobile phase B at 1.5 min, and held for another 0.5 min. The source and desolvation temperatures were 150 ºC and 500 ºC, respectively; the capillary voltage and extractor were 3500 V and 3.0 V, respectively; the cone gas flow and desolvation gas flow were 30 L/hr and 1000 L/hr, respectiely. The PPCS, d_3-PPCS, and d_5-PPCS were detected in positive MRM transitions *m/z* 509.3 to 184, *m/z* 512.3 to 184, and *m/z* 514.3 to 184, respectively. The cone voltage and collision energy were 28 V and 26 V, respectively, for PPCS, d_3-PPCS, and d_5-PPCS.

LysoSM-509 structure identification

Liquid chromatography-high resolution mass spectrometry (LC-HRMS) of endogenous lysoSM-509 and synthetic PPCS

The endogenous lysoSM-509 isolated from plasma was reconstituted in methanol, and further chromatographic separation was performed on a XBridge C8 analytical column (50 × 3 mm, 3.5 μm) protected with a SecurityGuard C8 guard column (4 x 3 mm) using a Shimadzu Prominence HPLC system (Columbia, MD). The mobile phases consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in methanol-acetonitrile (4:1) (solvent B), and the flow rate was 0.6 mL/min. The gradient was as follows: 0-3 min, 70 to 100% solvent B; 3-3.5 min, 100% solvent B; 3.5-3.6 min, 100 to 70% solvent B; 3.6-5 min, 70% solvent B. The eluate was directed into the mass spectrometer for data acquisition from 2.0 – 3.5 min; elsewhere, eluate was sent to waste to minimize source contamination. The injection volume was 5 μL, and the total run-time was 5 min. The HPLC system was coupled to a Q-Exactive Orbitrap MS (Thermo Fisher Scientific, Waltham, MA) operating with a heated electrospray interface (HESI-II) in electrospray ionization. The vaporizer temperature, the capillary temperature, spray voltage, sheath gas flow rate, auxiliary gas flow rate, ion sweep gas pressure, and S-lens RF level were set to 300 °C, 270 °C, 3.5kV (positive ion mode) or -3.5kV

(negative ion mode), 60, 30, 2, and 55, respectively. The mass spectra were acquired with full mass scan mode followed by data dependent analysis product ion scan. The full mass scan spectra were acquired at a resolution of 70,000 within a mass range of *m/z* 500–600 in positive and negative ion modes. Ion accumulation was set at 3.0×10^6 of Automatic Gain Control (AGC) and a maximum injection time of 100 ms. After ion activation/dissociation, the 10 most abundant peaks (Top 10 method) were measured with higher energy collisional dissociation (HCD) at a normalized collision energy of 30%, and AGC target was 1×10^5 (maximum inject time 25 ms). The analyses were performed without lock mass. Data were processed using Xcalibur™ version 2.2.1 (Thermo Fisher Scientific, Waltham, MA).

Hydrogen/deuterium (H/D) exchange experiment

The H/D exchange experiment was conducted on a Shimadzu Prominence HPLC system coupled with the Applied Biosystems/MDS Sciex 4000QTRAP mass spectrometer operating in positive MRM. The mobile phases consisted of 0.1% formic acid in deuterium oxide (solvent A) and acetonitrile (solvent B), and flow rate was 0.6 mL/min. The gradient was as follows: 0-3 min, 50 to 95% solvent B; 3-3.5 min, 95% solvent B; 3.5-3.6 min, 100 to 50% solvent B; 3.6-5 min, 50% solvent B. The eluate was directed into the mass spectrometer for data acquisition from 1.0 – 3.0 min; elsewhere, eluate was sent to waste to minimize source contamination. The injection volume was 5 µL and the total run-time was 5 min. The ESI source temperature was 500 ºC; the electrospray voltage was 5000 V; the declustering potential was 80 V; the collision energy was 31 eV; the entrance potential was 10 V; and the collision cell exit potential was 10 V. The collision and curtain gas were set at medium and 20, respectively. Both desolvation gas and nebulizing gas were set at 35 L/min. The MRM transitions are listed in **Supplemental Table S1**. The dwell time was set at 50 ms for each of MRM transition. Data were acquired and analyzed by Analyst software (version 1.5.2).

Derivatization

Methyl esterification of lysoSM-509

Acetyl chloride (50 μL) was added to methanol (1 mL) to give a hydrogen chloride - methanol solution. The lysoSM-509 isolated from plasma was dissolved in hydrogen chloride - methanol solution (50 μL), and heated at 50 ^{o}C for 1 hour. The mixture was evaporated to dryness with nitrogen flow at 50 ^{o}C, and the residue was reconstituted in methanol for LC-MS/MS assay.

Acetylation of lysoSM-509

The lysoSM-509 isolated from plasma was dissolved in a mixture of acetic anhydride (50 μL) and pyridine (50 μL), and heated at 50 ^{o}C for 2 hours. The mixture was evaporated to dryness with nitrogen flow at 50 ^{o}C, and residue was reconstituted in methanol for LC-MS/MS assay.

Ozonolysis of lysoSM-509

The PPCS isolated from plasma was dissolved in 0.4 mL of methanol, and ozone flow was passed through the PPCS solution at -78 ^{o}C for 15 min. The excess ozone was removed by flow of oxygen. To the solution was added 2 drops of dimethyl sulfide, and the mixture was kept at room temperature for 24 hours. The mixture was evaporated to dryness with nitrogen flow at 50 ^{o}C, and the residue was reconstituted in methanol for LC-MS/MS assay.

Alkaline degradation of endogenous lysoSM-509 and synthetic PPCS

The lysoSM-509 isolated from plasma was dissolved in 0.1 mL of 0.33 M NaOH in methanol-water (95:5), and the mixture left at room temperature for 60 hours. The reaction was quenched with 35 μL of 10% formic acid in acetonitrile. The mixture was evaporated to dry with nitrogen flow at 50 ^{o}C, and residue was reconstituted in methanol for LC-MS/MS assay.

Alkaline hydrolysis of (S)-N-palmitoyl-O-phophocholine-serine methyl ester (**Figure 4**, compound **5a**) (1.4 mmol) at room temperature for 12 hours in 5 mL of 0.33 M NaOH in methanol-water (95:5) generated synthetic PPCS, which was confirmed by LC-MS/MS. However, the conversion of compound **5a** to PPCS was not complete at 12 hours as indicated by LC-MS/MS. The synthetic PPCS was completely degraded under the same condition for 60 hours, and the reaction was quenched with 1.75 mL of 10% formic acid in acetonitrile. The mixture was evaporated to dryness with nitrogen flow at 50 ^{o}C, and the residue was reconstituted in methanol for LC-MS assay.

LC-MS/MS analysis of derivatized endogenous lysoSM-509 and synthetic PPCS

The LC-MS/MS analysis of derivatized endogenous lysoSM-509 and synthetic PPCS was conducted on a Shimadzu Prominence HPLC system coupled with an Applied Biosystems/MDS Sciex (Ontario, Canada) 4000QTRAP mass spectrometer or TSQ Quantum Ultra mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The products of methyl esterification of lysoSM-509 (PPCS), acetylation of lysoSM-509, ozonolysis of lysoSM-509 were separated on a XBridge C8 analytical column (50 × 3 mm, 3.5 μm) protected with a SecurityGuard C8 guard column (4 x 3 mm) (Phenomenex, Torrance, CA). The mobile phases consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in methanol-acetonitrile (4:1) (solvent B), and the flow rate was 0.6 mL/min. The gradient was as follows: 0-3 min, 70 to 100% solvent B; 3-3.5 min, 100% solvent B; 3.5-3.6 min, 100 to 70% solvent B; and 3.6-5 min, 70% solvent B. The eluate was directed into the mass spectrometer for data acquisition from 2.0 – 3.5 min; elsewhere, eluate was sent to waste to minimize source contamination. The injection volume was 5 μL, and the total run-time was 5 min. The products of acetylation of lysoSM-509 and ozonolysis of lysoSM-509 were detected with positive enhanced MS scan (EMS) on a 4000QTRAP mass spectrometer. The ESI source temperature was 500 ºC; the electrospray voltage was 5000 V; and the declustering potential was 50 V. The collision and curtain gases were set at high and 20, respectively. Both desolvation gas and nebulizing gas were set at 35 L/min. The collision energy for EMS scan was 10 eV; the mass scan range was *m/z* 250 – 700; scan speed was 1000 u/s; and dynamic fill time was

used. Data were acquired and analyzed by Analyst software (version 1.5.2). The product ion scans of methyl esters of endogenous lysoSM-509 and synthetic PPCS at *m/z* 514.3 were acquired on TSQ Quantum Ultra mass spectrometer operating with HESI-II in positive electrospray ionization. The vaporizer temperature, the capillary temperature, spray voltage, sheath gas flow rate, auxiliary gas flow rate, ion sweep gas pressure, lens offset, collision gas pressure, collision energy, the mass scan range, and scan time were set to 250 °C, 270 °C, 4 kV, 60, 20, 2, 110, 1.5 mTorr, 20 eV, *m/z* 50–550, and 1 second, respectively. Data were acquired and analyzed by Xcalibur (version 2.0.7).

The products of alkaline degradation of lysoSM-509 (PPCS) were detected with a 4000QTRAP mass spectrometer and separated on a ACE Excel Super C18 analytical column (50 × 4.6 mm, 3 μm) (MAC-MOD, Chadds Ford, PA) protected with a SecurityGuard Gemini C18 guard column (4 x 3 mm). The mobile phases consisted of 2.9 mM diethylamine and 20 mM hexafluoro-2-propanol in water (solvent A) and methanol-acetonitrile (4:1) (solvent B). The gradient was as follows: 0-3 min, 70 to 100% solvent B; 3-4 min, 100% solvent B; 4 -4.1 min, 100 to 70% solvent B; and 4.1-6.5 min, 70% solvent B. The eluate was directed into the mass spectrometer for data acquisition from 2.0 – 4 min; elsewhere, eluate was sent to waste to minimize source contamination. The injection volume was 5 μL, and the total run-time was 6 min. The ESI source temperature was 600 ºC; the electrospray voltage was -4500 V; and the declustering potential was -60 V. The collision and curtain gases were set at medium and 20, respectively. To identify the structures of products from alkaline degradation of synthetic PPCS, negative Q1 and product ion spectra were acquired. The mass range for negative Q1 scan was *m/z* 250 – 700 with scan time of 1 second. The negative product ion scans of N-palmitoylserine (decomposition product 1) and N-palmitoyl-O-methylserine (decomposition product 2) at *m/z* 342.2 and 356.2 were acquired at collision energy of 30 eV and in a mass range of *m/z* 50 – 400. To compare decomposition products 1 and 2 generated from synthetic PPCS and endogenous lysoSM-509, MRM detection was used, and MRM transitions and collision energies are given in **Supplemental Table S2**. The entrance

potential was -10 V and the collision cell exit potential was -10 V. The dwell time was set at 20 ms for each of MRM transition. Data were acquired and analyzed by Analyst software (version 1.5.2).

Synthesis of potential precursors for PPCS bioanalysis

N-d_5-palmitoyl-L-serine (6)

The **2c** (20 mg, 0.05 mmol, 1 eq) in trifluoroacetic acid (0.25 mL) and chloroform (0.25 mL) was stirred at room temperature for 1 day, and evaporated to dry. The residue was chromatographed on C18 silica (methanol-water, 7/3) to give N-d_5-palmitoyl-L-serine (**6**) (15.5 mg, 90%). MS (ESI^-) for $[M-H]^-$; calculated: 347.3, found: 347.3.

N-d_5-palmitoyl 1,2-distearoyl-sn-glycero-3-phospho-L-serine sodium salt (7)

A solution of 1,2-distearoyl-sn-glycero-3-phospho-L-serine sodium salt (Avanti Polar Lipids, Cat. No. 840029P) (10 mg, 0.012 mmol, 1 eq), N-succinimidyl d5-palmitate (8.8 mg, 0.025 mmol, 2 eq) in chloroform (0.5 mL) was stirred at room temperature for overnight. The solution was loaded to silica column and purified with chromatography (chloroform-methanol-water (50:49:1)) to give N-d_5-palmitoyl 1,2-distearoyl-sn-glycero-3-phospho-L-serine sodium salt (**7**) (5.8 mg, 45%). MS (ESI^-) for $[M-H]^-$; calculated: 1032.8, found: 1032.8.

N-d_5-palmitoyl-phytosphingosine phosphocholine (8)

To a solution of phytosphingosine phosphocholine (Avanti Polar Lipids, Cat. No. 860603) (1 mg, 2.07 μmol), triethylamine (7 μL), *d_5*-palmitic acid (CDN isotopes, Cat. No. D5397) (4 mg, 15.3 μmol) and hydroxybenzotriazole (5 mg, 0.037 mmol) in THF (0.6 mL), chloroform (0.4 mL) and methanol (0.4 mL), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (6 mg, 31.4 μmol) was added and left to stir at room temperature for 16 hours. The reaction mixture was concentrated and re-dissolved in methanol (2 mL) and subjected to preparative HPLC purification. The HPLC was done using a C4 reversed-phase column with water as solvent A and acetonitrile as solvent B (gradient of

20% to 100% solvent B over 25 minutes). HPLC fractions with the desired compound were combined and concentrated to yield *N-d_5*-palmitoyl-phytosphingosine phosphocholine (**8**) (0.775 mg, 50%). MS (ESI^+) for $[M+H]^+$; calculated: 726.6, found: 727.0.

N-d_5-palmitoyl-phosphoserine (9)

To *d_5*-palmitic acid (CDN isotopes, Cat. No. D5397) (15 mg, 57.4 μmol), thionyl chloride (1.5 mL) and DMF (2 μL) was added and left to stir at 75°C for 2 hours. The reaction mixture was cooled to room temperature and concentrated to dryness. The resulting residue was dissolved in anhydrous dichloromethane (1 mL) and was dropwise added to an ice-cold solution of phosphoserine (50 mg, 0.270 mmol) and triethylamine (100 μL) in methanol (1 mL). The reaction mixture was warmed to room temperature and left to stir for 6 hours. This mixture was then concentrated on a rotovap and subjected to desalting using a C18 reverse-phase (1 g) SPE column. The residue was loaded on the SPE column and first washed with 0.01 N HCl solution then followed by DI water. The desired compound was then eluted with 100% methanol. The methanol fractions were concentrated to get *N-d5*-palmitoyl-phosphoserine (**9**). MS (ESI^+) for $[M+H]^+$; calculated: 429.3, found: 429.5.

O-phosphocholine-d_3-L-serine (10)

To a solution of L-serine (2,3,3-d_3) (350 mg, 3.24 mmol) in saturated sodium bicarbonate (4.5 mL) and water (4.5 mL), sodium carbonate (343 mg, 3.23 mmol) was added and cooled to 0°C. To this a solution of di-*tert*-butyl dicarbonate (848 mg, 3.88 mmol) in dioxane (1 mL) was added dropwise and warmed to room temperature. Upon stirring for 24 hours the organic solvent was removed in the rotovap. To the resulting solution ethyl acetate was added and acidified to pH 2, using 1 N HCl solution. Upon extraction the combined organic layers were washed with brine, then dried with anhydrous sodium sulfate and concentrated on a rotovap. The resulting residue was suspended in anhydrous dichloromethane and 2-*tert*-butyl-1,3-diisopropylisourea (1.2 mL) was added dropwise and refluxed for 2 hours. At 2 hours, another portion of 2-*tert*-butyl-1,3-diisopropylisourea (0.46 mL) was

added to the refluxing reaction mixture. Again at 4 hours, another portion of 2-*tert*-butyl-1,3-diisopropylisourea (0.46 mL) was added to the refluxing reaction mixture. At the end of 6 hours the reaction was cooled to room temperature and concentrated under vacuum. The residue was dissolved in ethyl acetate and passed through a short silica bed. The filtrate was concentrated and subjected to silica flash column chromatography with 20% ethyl acetate in hexanes mixture for elution. *t*-Butyl (*t*-butoxycarbonyl)-L-serinate-2,3,3-d_3 (446 mg, 52%) was obtained by combining the fractions with desired compound (identified by staining TLC with phosphomolybdic acid solution) and concentration under reduced pressure. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.38 (br s, 1H), 1.48 (s, 9H), 1.45 (s, 9H). MS (ESI^+) for $[M+Na]^+$; calculated: 287.2, found: 287.4.

A solution of *t*-butyl (*t*-butoxycarbonyl)-L-serinate-2,3,3-d_3 (246 mg, 0.930 mmol) and triethylamine (94 mg, 0.930 mmol) in anhydrous toluene (8 mL) was cooled to 0°C. To the cold solution 2-chloro-1,3,2-dioxaphospholane 2-oxide (133 mg, 0.933 mmol) was added dropwise and kept at 0°C for 30 minutes and then warmed to room temperature and left to stir for 5 hours. The reaction mixture was filtered using a syringe filter (0.2 μm, PTFE) and the filtrate was concentrated under vacuum. The resulting residue was re-dissolved in anhydrous acetonitrile (7 mL) and transferred to a nitrogen flushed sealed tube. The solution was cooled to -35°C and anhydrous trimethylamine gas was bubbled through it. The addition of trimethylamine was continued till the reaction volume increased by 3 mL. The sealed tube was then securely capped, leak proof, and heated to 80°C for 16 hours. The sealed tube was then cooled on an ice bath and slowly open to release the pressure. The resulting mixture was concentrated under vacuum and subjected to reverse phased HPLC purification with water and acetonitrile as solvent A and B respectively. The residue obtained by concentrating the desired fractions was then dissolved in formic acid (88%) (1 mL) and heated to 90°C for 1 hour. The reaction mixture was then concentrated under reduced pressure to yield *O*-phosphocholine-d_3-L-serine (**10**). MS (ESI^+) for $[M+H]^+$; calculated: 274.1, found: 274.2.

Generation of PPCS in heathy human blood and quantification of PPCS in tissues from NPC animal models

The calibration standards (0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 100, 500 ng/mL) were prepared by serial dilution of PPCS (1 mg/mL) stock solution in methanol with 2% CHAPS solution.

Freshly collected adult blood (20 μL), red blood cells (20 μL), white blood cells prepared from 3 mL of blood and suspended in 20 μL of phosphate-buffered saline, and plasma (20 μL) were transferred to 2-mL polypropylene tubes (VWR, West Chester, PA) followed by addition of water (30 μL) to each tube. Freshly collected adult blood (20 μL), red blood cells (20 μL), white blood cells prepared from 3 mL of blood and suspended in 20 μL of phosphate-buffered saline, and plasma (20 μL) were spotted onto Whatman 903® newborn screening cards and then dried for 3 hours at room temperature to yield dried blood spots, dried red blood cell spots, dried white blood cell spots, dried plasma spots, respectively. The whole dried blood spot, dried red blood cell spot, dried white blood cell spot, and dried plasma spot were punched from newborn screening cards, and transferred to 2-mL polypropylene tubes followed by addition of water (50 μL) to each punch. The calibration standards (20 μL) and water (30 μL) were added to 2-mL polypropylene tubes. The above samples were vortexed for 10 min. To each tube d_5-PPCS (25 ng/mL) in isopropanol (200 μL) was added. The samples were vortexed for 3 min and centrifuged for 10 min at 9400 g. The supernatants were transferred to 1.2 mL glass inserts.

The brains and livers from mice and cats were homogenized in 2% CHAPS solution (4 mL/g tissue). The calibration standards (50 μL), mouse plasma, cat serum, mouse and cat brain and liver homogenates were transferred to 2-mL polypropylene tubes. To each tube d_5-PPCS (25 ng/mL) in isopropanol (200 μL) was added. The samples were vortexed for 3 min and centrifuged for 10 min at 9400 g. The supernatants were transferred to 1.2 mL glass inserts.

LC–MS/MS analysis was conducted on the Shimadzu Prominence HPLC system coupled with the Applied Biosystems/MDS Sciex 4000QTRAP mass spectrometer using MRM. The PPCS and d_5-PPCS were separated on a Halo HILIC (100 × 4.6 mm, 2.7 μm) protected with a SecurityGuard HILIC guard column (4 x 3 mm) at a flow rate of 1 mL/min. The mobile phases consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The gradient was as follows: 0-2 min, 85 to 80% solvent B; 2-3.5 min, 80% solvent B; 3.5-3.6 min, 80 to 10% solvent B; 3.6-4.5 min, 10% solvent B; 4.5-4.6 min, 10 to 85% solvent B; 4.6-7 min, 85% solvent B. The eluate was directed into the mass spectrometer for data acquisition from 4.0 - 5.2 min; elsewhere, eluate was sent to waste to minimize source contamination. The injection volume was 5 μL and the total run-time was 7 min. The ESI source temperature was 500 ºC; the electrospray voltage was 5000 V; the declustering potential, collision energy, entrance potential, and collision cell exit potential were 80 V, 31 eV, 10 V, and 10 V, respectively, for both PPCS and d_5-PPCS. The collision and curtain gas were set at medium and 20, respectively. Both desolvation gas and nebulizing gas were set at 35 L/min. For MRM, the dwell time was set at 50 ms for the transition of *m/z* 509.3 to 184 (PPCS) and *m/z* 514.3 to 184 (d_5-PPCS), respectively. Data were acquired and analyzed by Analyst software (version 1.5.2).

To study the biosynthesis of PPCS, potential precursors including d_5-palmitatic acid, N-d_5-palmitoylserine, N-d_5-palmitoylphosphatidylserine, N-d_5-palmitoyl-phytosphingosine phosphocholine, N-d5-palmitoyl-phosphoserine, and O-phosphocholine-d_3-L-serine were spiked into whole blood from healthy adults to final concentration of 0, 10 μM and 100 μM. A 5 or 20 μL aliquot of the spiked whole blood was spotted onto Whatman 903® newborn screening cards or protein saver cards (Whatman #10534612), and the cards were allowed to dry at room temperature for at least three hours. The extraction and detection of d_5-PPCS and d_3-PPCS were performed as described as above using LC-MS/MS or UPLC-MS/MS.

Profiling of APCS in plasma

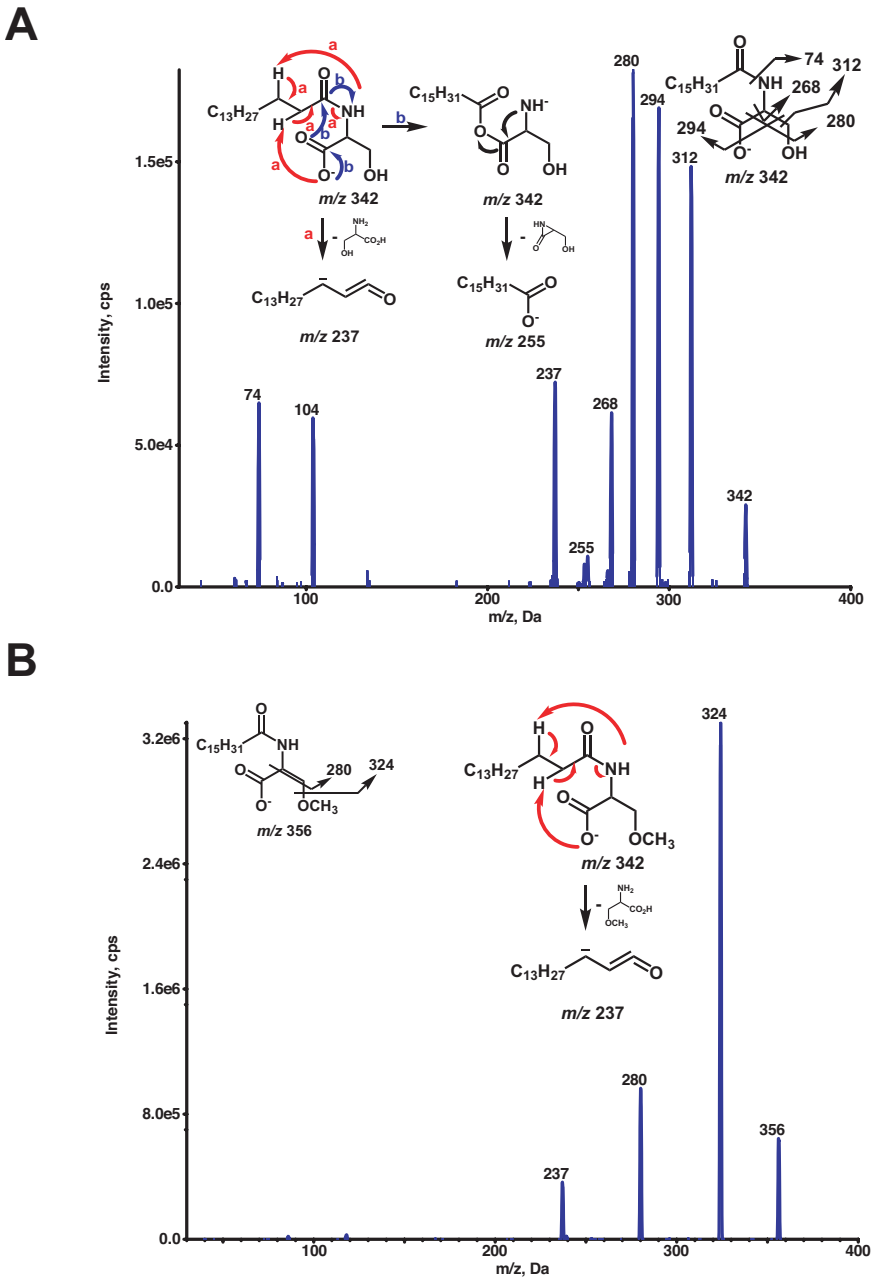
A volume of 50 µL of plasma samples and 200 µL of methanol were added to a 2-mL polypropylene tube. The sample were vortexed for 3 min and centrifuged at 9400 g for 10 min. The supernatants were transferred to 1.2 mL glass inserts. LC–MS/MS analysis was conducted on the Shimadzu Prominence HPLC system coupled with the Applied Biosystems/MDS Sciex 4000QTRAP mass spectrometer using MRM. Separation of APCS was carried out using a Gemini C18 analytical column (4.6 x 100 mm, 3 µm) (Phenomenex, Torrance, CA) protected with a SecurityGuard C18 guard column (4 x 3 mm) at a flow rate of 1 mL/min. The mobile phase consisted of 0.1% formic acid in water (solvent A), and 0.1% formic acid in acetonitrile-methanol (1:4) (solvent B). The gradient was as follows: 0-3 min, 80 to 100% solvent B; 3-6 min, 100% solvent B; 6-8 min, 80% solvent B. The eluate was directed into the mass spectrometer for data acquisition from 2.5 – 6.5 min; elsewhere, eluate was sent to waste to minimize source contamination. The injection volume was 5 µL, and the total run-time was 8 min. The ESI source temperature was 650 ºC; the electrospray voltage was 5000 V; the entrance potential was 10 V; and the collision cell exit potential was 10 V. The collision and curtain gas were set at medium and 20, respectively. Both desolvation gas and nebulizing gas were set at 35 L/min. The MRM transitions for APCS are listed in **Table 1**. The dwell time was set at 20 ms for each of MRM transition. Data were acquired and analyzed by Analyst software (version 1.5.2).

Supplemental Table S1 MRM transitions for H/D exchange experiment

MRM transition	Precursor ion		Product ion	
	LysoSM-509	m/z	Choline	m/z
1	$[M+H]^+$	509.3	$[M+H]^+$	184
2	$[M-H+D]^+$	510.3	$[M+H]^+$	184
3	$[M-H+D]^+$	510.3	$[M+D]^+$	185
4	$[M-H+2D]^+$	511.3	$[M+H]^+$	184
5	$[M-H+2D]^+$	511.3	$[M+D]^+$	185
6	$[M-H+2D]^+$	511.3	$[M-H+2D]^+$	186
7	$[M-2H+3D]^+$	512.3	$[M+H]^+$	184
8	$[M-2H+3D]^+$	512.3	$[M+D]^+$	185
9	$[M-2H+3D]^+$	512.3	$[M-H+2D]^+$	186
10	$[M-3H+4D]^+$	513.3	$[M+H]^+$	184
11	$[M-3H+4D]^+$	513.3	$[M+D]^+$	185
12	$[M-3H+4D]^+$	513.3	$[M-H+2D]^+$	186
13	$[M-4H+5D]^+$	514.3	$[M+H]^+$	184
14	$[M-4H+5D]^+$	514.3	$[M+D]^+$	185
15	$[M-4H+5D]^+$	514.3	$[M-H+2D]^+$	186

Supplemental Table S2 MRM transitions for alkaline degradation products of PPCS

MRM transition	Decomposition product	Precursor ion (m/z)	Product ion (m/z)	Collision energy (eV)
1	N-palmitoylserine (decomposition product 1)	342.2	74	-50
2	N-palmitoylserine (decomposition product 1)	342.2	104.1	-35
3	N-palmitoylserine (decomposition product 1)	342.2	237.2	-35
4	N-palmitoylserine (decomposition product 1)	342.2	268.2	-28
5	N-palmitoylserine (decomposition product 1)	342.2	280.2	-30
6	N-palmitoylserine (decomposition product 1)	342.2	294.2	-32
7	N-palmitoylserine (decomposition product 1)	342.2	312.2	-25
8	N-palmitoyl-O-methylserine (decomposition product 2)	356.2	237.2	-34
9	N-palmitoyl-O-methylserine (decomposition product 2)	356.2	280.2	-27
10	N-palmitoyl-O-methylserine (decomposition product 2)	356.2	324.2	-17



Supplemental Figure S1. Product ion spectra of alkaline decomposition products of PPCS. (A) Product ion spectrum of alkaline decomposition product 1 (N-palmitoylserine) of PPCS. (B) Product ion spectrum of alkaline decomposition product 2 (N-palmitoyl-O-methylserine) of PPCS.