Supporting Information

Mg^{2+}-driven selection of natural phosphatidic acids in primitive membranes

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Materials and Methods. Reagents and solvents were bought from Sigma-Aldrich, Fisher Scientific, Acros Organics, and TCI Europe and were used as received, *N*-decanoyl imidazole (NDI) was prepared by the method of Bonfio et al (*J. Am. Chem Soc.* 2019, *141*, 3934-3939), ammonium decanoate was prepared by adapting the method of Eichberg et al (*J. Mol. Evol.* 1977, *10*, 221-230), choline tetraphenylborate was prepared by the method of Harbison and Griffin (*J. Lipid Res.* 1984, *25*, 1140-1142), and 1-decanoyl-*rac*-glycerol-2,3-cyclic phosphate sodium salt (**MDG>P**) was prepared by the method of Bonfio et al (*Chem. Sci*. 2020, *11*, 10688-10697). Effective molecular weights of the hydrated sodium salts of *rac*-glycerol-1-phosphate (**G1P**, Sigma-Aldrich) and glycerol-2-phosphate (**G2P**, Sigma-Aldrich) were determined by ^{31}P NMR in H_2O/D_2O (9:1, v/v) using dibasic sodium phosphate as an internal standard. pH monitoring was performed using a Mettler Toledo FiveEasy pH Meter and adjustments were made with aqueous solutions of NaOH or HCl as appropriate. Thin layer chromatography (TLC) was performed on aluminium plates coated with silica gel 60 F_{254} (Merck). Spots were visualized with molybdenum blue, prepared by the method of Ryu and MacCoss (*J. Lipid. Res.* 1979, *20*, 561-563), ceric ammonium molybdate, or UV light. Retention factors (R_fs) are approximate. Column chromatography was performed using silica gel 60 (VWR, 40-63 μm). ^{1}H and ^{31}P NMR spectra were acquired using a Bruker Ascend AVANCE NEO operating at 500 MHz and 202.4 MHz, respectively. Chemical shifts (δ) are given in ppm, the notations s, d, t, q, quint, and m represent the multiplicities of singlet, doublet, triplet, quartet, quintet, and multiplet signals, respectively, and coupling constants (*J*) are given in Hertz (Hz). ^{1}H NMR spectra acquired in pure solvents are referenced to the residual non-deuterated solvent signal using the chemical shifts reported by Fulmer et al (*Organometallics*, 2010, *29*, 2176-2179), and those in mixtures of solvents (e.g. $CDCl_3/CD_3OD$, 2:1, v/v) are referenced to tetramethylsilane (TMS; δ_H 0). Yields of conversion were determined by relative integration of the signals in the ^{31}P NMR spectra. Data analysis was performed using MestReNova (version 14.3.0) and GraphPad Prism (version 9.5.1). Critical aggregation concentrations (CACs) for lipids dispersed in buffered aqueous solutions were determined by the method of Bonfio et al (*Chem. Sci*. 2020, *11*, 10688-10697) using a BMG Labtech CLARIOstar^{plus} and merocyanine 540 as a solvatochromic probe. Generalized polarisation (GP) measurements were made using the same instrument and Laurdan as fluorescent probe. Dynamic Light Scattering (DLS) and membrane Zeta (ζ)-potential measurements were made using a Malvern Panalytical Zetasizer Advance Ultra (Red). Liposomes were imaged using a Nikon Eclipse TS2 inverted epifluorescence microscope equipped with a Moment A21K635003 camera and a x100 oil objective. Images were processed using Fiji (http://rsb.info.nih.gov/ij/). The data shown are representative of distinct samples, n = 3 replicates.

Potentially prebiotic synthesis of phospholipids

Synthesis of phosphatidic acids by reaction of glycerol-1-phosphate with N-decanoyl imidazole in solution. 20 μL of a 1.0 M solution of glycerol-1-phosphate sodium salt in water (**G1P**; 0.02 mmol) was diluted into water (0.78 mL) and D_2O (0.2 mL) in a plastic Eppendorf tube. The pH of the solution was adjusted to 7.4. Separately, *N*-decanoyl imidazole (NDI; 44.4 mg, 0.20 mmol; 10 eq.) was dissolved in acetonitrile (1.0 mL) in a glass test tube equipped with a magnetic stirrer bead and capped with a rubber septum. The solution of **G1P** was added dropwise (by pipette) to the solution of NDI and the mixture was stirred vigorously (800 rpm) at room temperature. {Note: Mixing is endothermic and the drop in temperature causes some

of the NDI to precipitate; it does, however, dissolve on stirring}. The reaction was monitored by ^{31}P NMR (D_2O), withdrawing aliquots after 24 h, 48 h, 96 h, and 8 d, and also by TLC, with plates developed in chloroform/methanol/water (65:25:4, v/v/v) and spots visualized by molybdenum blue. The presence (or absence) of NDI, decanoic acid, mixed anhydrides, and phospholipids (as observed by NMR) was confirmed at each time point. {Note: By TLC, NDI, R_f 0.9, and decanoic acid, R_f 0.8, show as white spots. Mixed anhydrides, R_f 0.6, phosphatidic acid, R_f 0.3, and lysophosphatidic acids, R_f 0.1, show as blue spots. The various mixed anhydrides and isomeric lysophosphatidic acids are not resolved. G1P is not stained by molybdenum blue}. By 96 h, NDI was completely consumed (TLC) and by 8 d mixed anhydrides were no longer visible (NMR). The mixture obtained after 8 d consisted of residual **G1P** (76%), 2-hydroxy-3-decanoyl-glycerol-1-phosphate and 2-decanoyl-3-hydroxy-glycerol-1-phosphate (lysophosphatidic acids; 20% total), and 2,3-didecanoyl-glycerol-1-phosphate (phosphatidic acid; 4%).

G1P:
^{31}P NMR (D_2O, 202.4 MHz): δ_P 2.18 (t, J = 6.6 Hz);
^{31}P{^{1}H} NMR (D_2O, 202.4 MHz): δ_P 2.18 (s).

2-hydroxy-3-decanoyl-glycerol-1-phosphate, **MDG1PA** (major):
^{31}P NMR (D_2O, 202.4 MHz): δ_P 1.68 (t, J = 6.8 Hz);
^{31}P{^{1}H} NMR (D_2O, 202.4 MHz): δ_P 1.68 (s).

2-decanoyl-3-hydroxy-glycerol-1-phosphate, **MDG1PA** (minor):
^{31}P NMR (D_2O, 202.4 MHz): δ_P 1.59 (t, J = 6.6 Hz);
^{31}P{^{1}H} NMR (D_2O, 202.4 MHz): δ_P 1.59 (s).

2,3-didecanoyl-glycerol-1-phosphate, **DDG1PA**:
^{31}P NMR (D_2O, 202.4 MHz): δ_P 0.89 (t, J = 6.7 Hz);
^{31}P{^{1}H} NMR (D_2O, 202.4 MHz): δ_P 0.89 (s).

Four mixed anhydrides were visible by ^{31}P NMR between 24 and 96 h. These species appeared at -7.23, -7.62, -7.72, and -8.19 ppm respectively.

Synthesis of phosphatidic acids by reaction of glycerol-2-phosphate with N-decanoyl imidazole in solution. 20 µL of a 1.0 M solution of glycerol-2-phosphate sodium salt in water (**G2P**; 0.02 mmol) was diluted into water (0.78 mL) and D_2O (0.2 mL) in a plastic Eppendorf tube. The pH of the solution was adjusted to 7.4. Separately, *N*-decanoyl imidazole (44.4 mg, 0.2 mmol) was dissolved in acetonitrile (1.0 mL) in a glass test tube equipped with a magnetic stirrer bead and capped with a rubber septum. The two solutions were combined as described above and the mixture was stirred vigorously for 8 d. The progress of the reaction was monitored by ^{31}P NMR (D_2O) and TLC after 24 h, 48 h, 96 h, and 8 d, as described above. The mixture obtained after 8 d consisted of 1-decanoyl-glycerol-2,3-cyclic phosphate (1%), residual **G2P** (12%), 1-decanoyl-3-hydroxy-glycerol-2-phosphate (43% total), 1,3-didecanoyl-glycerol-2-phosphate (44%). {Note: By TLC, phosphatidic acids derived from **G2P** run faster than phosphatidic acids derived from **G1P**. The difference in chromatographic behaviour is slight, but is most apparent when isomeric phospholipids are spotted on the same plate. Mixed anhydrides, R_f 0.65, 1,3-didecanoyl-glycerol-2-phosphate, R_f 0.35, and 1-decanoyl-3-hydroxy-

glycerol-2-phosphate, R_f 0.15, show as blue spots. The various mixed anhydrides are not resolved by TLC. G2P is not stained by molybdenum blue.}

1-decanoyl-glycerol-2,3-cyclic phosphate, **MDG>P**:
^{31}P NMR (D_2O, 202.4 MHz): δ_P 17.67 (m);
$^{31}P\{^1H\}$ NMR (D_2O, 202.4 MHz): δ_P 17.67 (s).

G2P:
^{31}P NMR (D_2O, 202.4 MHz): δ_P 1.92 (d, J = 8.2 Hz);
$^{31}P\{^1H\}$ NMR (D_2O, 202.4 MHz): δ_P 1.92 (s).

1-decanoyl-3-hydroxy-glycerol-2-phosphate, **MDG2PA**:
^{31}P NMR (D_2O, 202.4 MHz): δ_P 1.05 (d, J = 8.9 Hz);
$^{31}P\{^1H\}$ NMR (D_2O, 202.4 MHz): δ_P 1.05 (s).

1,3-didecanoyl-glycerol-2-phosphate, **DDG2PA**:
^{31}P NMR (D_2O, 202.4 MHz): δ_P 0.47 (d, J = 9.7 Hz);
$^{31}P\{^1H\}$ NMR (D_2O, 202.4 MHz): δ_P 0.47 (s).

Three mixed anhydrides were visible by ^{31}P NMR between 24 and 96 h. These species appeared at -7.81, -8.47, and -9.08 ppm respectively.

Synthesis of phosphatidic acids by reaction of a mixture of glycerol-1-phosphate and glycerol-2-phosphate with N-decanoyl imidazole in solution. 10 µL of a 1.0 M solution of glycerol-1-phosphate sodium salt in water (**G1P**; 0.01 mmol) and 10 µL of a 1.0 M solution of glycerol-2-phosphate sodium salt in water (**G2P**; 0.01 mmol) were diluted into water (0.78 mL) and D_2O (0.2 mL) in a plastic Eppendorf tube. The pH of the mixture was adjusted to 7.4. Separately, *N*-decanoyl imidazole (NDI; 44.4 mg, 0.2 mmol, 10 eq.) was dissolved in acetonitrile (1.0 mL) in a glass test tube equipped with a magnetic stirrer bead and capped with a rubber septum. The two solutions were combined as described above and the mixture was stirred vigorously for 8 d. The progress of the reaction was monitored by ^{31}P NMR (D_2O) and TLC after 24 h, 48 h, 96 h, and 8 d, as described above. The mixture obtained after 8 d consisted of 1-decanoyl-glycerol-2,3-cyclic phosphate (1%), residual **G1P** (39%), residual **G2P** (6%), 2-hydroxy-3-decanoyl-glycerol-1-phosphate and 2-decanoyl-3-hydroxy-glycerol-1-phosphate (lysophosphatidic acids; 10% total), 1-decanoyl-3-hydroxy-glycerol-2-phosphate (20%), 2,3-didecanoyl-glycerol-1-phosphate (phosphatidic acid; 3%), and 1,3-didecanoyl-glycerol-2-phosphate (21%). {Note: The positional isomers of the various phospholipids are not resolved by TLC; the phosphatidic acids, R_f ~0.35, and lysophosphosphatidic acids, R_f ~0.15 show as single diffuse blue spots.} NMR data as above.

Synthesis of phospholipids by drying out and heating a mixture of glycerol-1-phosphate, ammonium decanoate, imidazole, and cyanamide. 10 µL of a 1.0 M solution of glycerol-1-phosphate sodium salt in water (**G1P**; 0.01 mmol) was added to an opalescent solution of ammonium decanoate (18.9 mg, 0.1 mmol; 10 eq.) and imidazole (6.8 mg, 0.1 mmol; 10 eq.) in water (1.0 mL) at pH 7.4 in a plastic Eppendorf tube. To initiate the reaction, cyanamide (4.2 mg, 0.1 mmol; 10 eq.) was added and the mixture was briefly vortexed. The mixture was

then poured into a 50 mm diameter glass Petri dish and allowed to evaporate (open) in a fume hood at room temperature for 4 h. The dried (partially crystalline) mixture was then transferred to an oven and heated at 65°C for 18 h. The reaction was brought to room temperature and the mixture was extracted with acetonitrile/water/D_2O (5:4:1, v/v/v; 1.0 mL). {Note: The pH of the extract was ~4 by pH paper.} The composition of the extract was determined by ^{31}P NMR (D_2O) and the presence of phospholipids was confirmed by TLC, as described above. The extract consisted of glycerol-2,3-cyclic phosphate (**G>P**; 16%), 1-decanoyl-glycerol-2,3-cyclic phosphate (6%), residual **G1P** (52%), 2-hydroxy-3-decanoyl-glycerol-1-phosphate and 2-decanoyl-3-hydroxy-glycerol-1-phosphate (lysophosphatidic acids; 15% total), **G2P** (2%), 2,3-didecanoyl-glycerol-1-phosphate (phosphatidic acid; 4%), inorganic phosphate (P_i; 1%), 1-decanoyl-3-hydroxy-glycerol-2-phosphate (2%), 1,3-didecanoyl-glycerol-2-phosphate (1%), and two species tentatively assigned as glycerol-1,3-cyclic phosphate (major) and 2-decanoyl-glycerol-1,3-cyclic phosphate (1% total). Total phospholipids (29%).

G>P:
^{31}P NMR (D_2O, 202.4 MHz): δ_P 18.37 (m);
$^{31}P\{^1H\}$ NMR (D_2O, 202.4 MHz): δ_P 18.37 (s).

1-decanoyl-glycerol-2,3-cyclic phosphate, **MDG>P**:
^{31}P NMR (D_2O, 202.4 MHz): δ_P 17.68 (m);
$^{31}P\{^1H\}$ NMR (D_2O, 202.4 MHz): δ_P 17.68 (s).

G1P:
^{31}P NMR (D_2O, 202.4 MHz): δ_P 0.87 (t, *J* = 6.3 Hz);
$^{31}P\{^1H\}$ NMR (D_2O, 202.4 MHz): δ_P 0.87 (s).

2-hydroxy-3-decanoyl-glycerol-1-phosphate, **MDG1PA** (major):
^{31}P NMR (D_2O, 202.4 MHz): δ_P 0.67 (t, *J* = 6.6 Hz);
$^{31}P\{^1H\}$ NMR (D_2O, 202.4 MHz): δ_P 0.67 (s);

2-decanoyl-3-hydroxy-glycerol-1-phosphate, **MDG1PA** (minor):
^{31}P NMR (D_2O, 202.4 MHz): δ_P 0.54 (t, *J* = 6.3 Hz);
$^{31}P\{^1H\}$ NMR (D_2O, 202.4 MHz): δ_P 0.54 (s);

G2P:
^{31}P NMR (D_2O, 202.4 MHz): δ_P 0.46 (d, *J* = 8.3 Hz);
$^{31}P\{^1H\}$ NMR (D_2O, 202.4 MHz): δ_P 0.46 (s).

2,3-didecanoyl-glycerol-1-phosphate, **DDG1PA**:
^{31}P NMR (D_2O, 202.4 MHz): δ_P 0.30 (t, *J* = 6.3 Hz);
$^{31}P\{^1H\}$ NMR (D_2O, 202.4 MHz): δ_P 0.30 (s).

P_i:
^{31}P NMR (D_2O, 202.4 MHz): δ_P 0.16 (s, overlapped with the signal of the following species);
$^{31}P\{^1H\}$ NMR (D_2O, 202.4 MHz): δ_P 0.16 (s).

1-decanoyl-3-hydroxy-glycerol-2-phosphate, **MDG2PA**:
^{31}P NMR (D_2O, 202.4 MHz): δ_P 0.08 (d, *J* = 9.6 Hz);

$^{31}P\{^{1}H\}$ NMR (D_2O, 202.4 MHz): δ_P 0.08 (s).

1,3-didecanoyl-glycerol-2-phosphate, **DDG2PA**:
^{31}P NMR (D_2O, 202.4 MHz): δ_P -0.30 (d, J = 9.4 Hz);
$^{31}P\{^{1}H\}$ NMR (D_2O, 202.4 MHz): δ_P -0.30 (s).

glycerol-1,3-cyclic phosphate:
^{31}P NMR (D_2O, 202.4 MHz): δ_P -3.56 (m);
$^{31}P\{^{1}H\}$ NMR (D_2O, 202.4 MHz): δ_P -3.56 (s).

2-decanoyl-glycerol-1,3-cyclic phosphate:
^{31}P NMR (D_2O, 202.4 MHz): not observed;
$^{31}P\{^{1}H\}$ NMR (D_2O, 202.4 MHz): δ_P -4.26 (s).

Synthesis of phospholipids by drying out and heating a mixture of glycerol-2-phosphate, ammonium decanoate, imidazole, and cyanamide. 10 µL of a 1.0 M solution of glycerol-2-phosphate sodium salt in water (**G2P**; 0.01 mmol) was added to an opalescent solution of ammonium decanoate (18.9 mg, 0.1 mmol; 10 eq.) and imidazole (6.8 mg, 0.1 mmol; 10 eq.) in water (1.0 mL) at pH 7.4 in a plastic Eppendorf tube. To initiate the reaction, cyanamide (4.2 mg, 0.1 mmol; 10 eq.) was added and the mixture was briefly vortexed. The mixture was then poured into a 50 mm diameter glass Petri dish and allowed to evaporate (open) in a fume hood at room temperature for 4 h. The dried (partially crystalline) mixture was then transferred to an oven and heated at 65°C for 18 h. The reaction was brought to room temperature and the mixture was extracted with acetonitrile/water/D_2O (5:4:1, v/v/v; 1.0 mL). The composition of the extract was determined by ^{31}P NMR (D_2O) and the presence of phospholipids was confirmed by TLC as described above. The extract consisted of glycerol-2,3-cyclic phosphate (**G>P**; 22%), 1-decanoyl-glycerol-2,3-cyclic phosphate (11%), **G1P** (2%), 2-hydroxy-3-decanoyl-glycerol-1-phosphate (major lysophosphatidic acid; 2% total), residual **G2P** (34%), 2,3-didecanoyl-glycerol-1-phosphate (phosphatidic acid; 1%), inorganic phosphate (P_i; 1%), 1-decanoyl-3-hydroxy-glycerol-2-phosphate (14%), and 1,3-didecanoyl-glycerol-2-phosphate (4%). Total phospholipids (31%). Total unknowns (9%). NMR data as above.

Synthesis of phospholipids by drying out and heating a mixture of glycerol-1-phosphate, glycerol-2-phosphate, ammonium decanoate, imidazole, and cyanamide. 5 µL of a 1.0 M solution of glycerol-1-phosphate sodium salt in water (**G1P**; 0.005 mmol) and 5 µL of a 1.0 M solution of glycerol-2-phosphate sodium salt in water (**G2P**; 0.005 mmol) were added to an opalescent solution of ammonium decanoate (18.9 mg, 0.1 mmol; 10 eq.) and imidazole (6.8 mg, 0.1 mmol; 10 eq.) in water (1.0 mL) at pH 7.4 in a plastic Eppendorf tube. To initiate the reaction, cyanamide (4.2 mg, 0.1 mmol; 10 eq.) was added and the mixture was briefly vortexed. The mixture was then poured into a 50 mm diameter glass Petri dish and allowed to evaporate (open) in a fume hood at room temperature for 4 h. The dried (partially crystalline) mixture was then transferred to an oven and heated at 65°C for 18 h. The reaction was brought to room temperature and the mixture was extracted with acetonitrile/water/D_2O (5:4:1, v/v/v; 1.0 mL). The composition of the extract was determined by ^{31}P NMR (D_2O) and the presence of phospholipids was confirmed by TLC as described above. The extract consisted of glycerol-2,3-cyclic phosphate (**G>P**; 19%), 1-decanoyl-glycerol-2,3-cyclic phosphate (12%), residual

G1P (23%), 2-hydroxy-3-decanoyl-glycerol-1-phosphate (major) and 2-decanoyl-3-hydroxy-glycerol-1-phosphate (minor) (lysophosphatidic acids; 10% total), residual **G2P** (15%), 2,3-didecanoyl-glycerol-1-phosphate (phosphatidic acid; 3%), inorganic phosphate (P_i; 1%), 1-decanoyl-3-hydroxy-glycerol-2-phosphate (7%), 1,3-didecanoyl-glycerol-2-phosphate (3%), and two species tentatively assigned as glycerol-1,3-cyclic phosphate (major) and 2-decanoyl-glycerol-1,3-cyclic phosphate (1% total). Total phospholipids (35%). Total unknowns (6%). NMR data as above.

Synthesis of phosphatidic acids from glycerol phosphates

These compounds were prepared by adapting the method of Gupta et al (*Proc. Natl. Acad. Sci. USA*. 1977, *74*, 4315-4319); The appropriate glycerol phosphate sodium salt (either *rac*-glycerol-1-phosphate or glycerol-2-phosphate; 1.0 mmol) was dissolved in water (~25 mL) and the solution was passed through a 2 x 14 cm column of pyridinium AmberChrom 50WX2 200-400 Mesh ion exchange resin (Alfa Aesar; prepared from the hydrogen form by washing with three column volumes of 1.0 M aqueous pyridine). The glycerol phosphate was eluted under gravity with three column volumes of water. The aqueous pyridine solution was evaporated under reduced pressure. {Note: The concentrated solution was transferred to a 100 mL round bottom flask before the last of the solvent was evaporated.} The gummy pyridinium salt was dried azeotropically by dissolving it in dry pyridine (~1 mL) and evaporating under reduced pressure. This process was repeated three times before a magnetic stirrer bead was added to the flask, it was capped with a rubber septum, and the pyridinium salt was dried under high vacuum overnight.

The appropriate glycerol phosphate pyridinium salt was placed under an atmosphere of nitrogen and suspended in dry chloroform (50 mL) before 4-dimethylaminopyridine (DMAP; 4.0 mmol; 4 eq.) was added followed by the appropriate fatty acid anhydride (either caprylic, decanoic, or lauric anhydride; 3.0 mmol; 3 eq.). The mixture was stirred vigorously (800 rpm) at room temperature for 4 days. {Note: TLC, with plates developed in chloroform/methanol/water (65:25:4, v/v/v) and spots visualized by molybdenum blue, showed mixtures of fatty acids, R_f 0.8, phospholipid mixed anhydrides, R_f ~0.6, cyclophospholipids (traces), R_f ~0.4, phosphatidic acids, R_f ~0.3, and lysophosphatidic acids (traces), R_f ~0.1.} The solvent was evaporated under reduced pressure and the oily residue was dissolved in methanol/chloroform/pyridine/water (2:1:1:1, v/v/v/v; 50 mL) and stirred at room temperature overnight. {TLC as described above showed that the mixed anhydrides had been hydrolysed.} The solvent was evaporated under reduced pressure and the residue was dried under high vacuum overnight to remove traces of water. The mixture of products was fractionated by column chromatography, eluting first with chloroform/methanol (9:1, v/v) to remove the fatty acid, then chloroform/methanol (1:1, v/v) to give the phosphatidic acid DMAP salt as a white solid in 50-75% yield. {Note: Best results were obtained using fresh fatty acid anhydrides. Traces of cyclophopholipids were produced in these reactions when aged anhydrides were used. These species eluted ahead of the main phosphatidic acid fraction, R_f ~0.4, and were identified by the staining behaviour. The initially deep blue cyclophospholipid spots faded rapidly and developed a white halo while phosphatidic acid spots remained deep blue.}

The phosphatidic acid DMAP salts obtained by this method were stable for six months, stored at -20°C.

For characterisation by NMR and use in experiments involving liposomes (see below), the phosphatidic acid DMAP salts were converted to their protonated acid forms by Bligh and Dyer

extraction (*Can*. *J*. *Biochem*. *Physiol*. 1959, *37*, 911-917) with aqueous acid; Briefly, the DMAP salt (20 mg) was dissolved in 20 mL of chloroform/methanol (2:1, v/v), the solution was transferred to a 50 mL glass separatory funnel and washed with 1.0 M HCl (3 x 6.5 mL). Successive phase separations were aided by adding methanol (~6 mL) to bring about an instant change in appearance of the otherwise milky phases. The composition of the chloroform phase was checked by TLC, as described above, to ensure that no lysophosphosphatidic acid was present. The solvent was then evaporated under reduced pressure and the product dried under high vacuum overnight to give the (protonated) phosphatidic acid as a colourless glassy solid.

2,3-dicapryloyl-*rac*-glycerol-1-phosphatidic acid, **DCG1PA**:
^{1}H NMR ($CDCl_3$/CD_3OD, 2:1 v/v, with 0.05% TMS, 500 MHz): δ_H 5.15 (1H, m), 4.30 (1H, dd, *J* = 12.0, 3.7 Hz), 4.11 (1H, dd, *J* = 12.0, 6.3 Hz), 4.02 (2H, dd, *J* = 6.4, 5.6 Hz), 2.25 (4H, app q, *J* = 7.4 Hz), 1.53 (4H, m), 1.30-1.10 (16H, m), 0.80 (6H, app t, *J* = 7.0);
^{31}P{^{1}H} NMR ($CDCl_3$/CD_3OD, 2:1 v/v, 202.4 MHz): δ_P -0.24 (s).

{Note: By ^{31}P NMR, the signal corresponding to the 1-phosphatidic acid lies downfield of the signal corresponding to the isomeric 2-phosphatidic acid, see below. This trend is also conserved for 1- and 2-phosphatidic acids of equal chain length in aqueous solution at a given pH, see above.}

1,3-dicapryloyl-glycerol-2-phosphatidic acid, **DCG2PA**:
^{1}H NMR ($CDCl_3$/CD_3OD, 2:1 v/v, with 0.05% TMS, 500 MHz): δ_H 4.56 (1H, app dq, *J* = 14.8, 4.8 Hz), 4.19 (4H, ddd, *J* = 16.2, 11.8, 5.0 Hz), 2.27 (4H, t, *J* = 7.6 Hz), 1.54 (4H, app quint, *J* = 7.5 Hz), 1.30-1.10 (16H, m), 0.80 (6H, t, *J* = 7.0 Hz);
^{31}P{^{1}H} NMR ($CDCl_3$/CD_3OD, 2:1 v/v, 202.4 MHz): δ_P -0.74 (s).

2,3-didecanoyl-*rac*-glycerol-1-phosphatidic acid, **DDG1PA**:
^{1}H NMR ($CDCl_3$/CD_3OD, 2:1 v/v, with 0.05% TMS, 500 MHz): δ_H 5.15 (1H, m), 4.30 (1H, dd, *J* = 12.0, 3.7 Hz), 4.11 (1H, dd, *J* = 12.1, 6.5 Hz), 4.02 (2H, dd, *J* = 6.6, 5.4 Hz), 2.25 (4H, app q, *J* = 7.5 Hz), 1.53 (4H, m), 1.30-1.10 (24H, m), 0.80 (6H, app t, *J* = 7.0);
^{31}P{^{1}H} NMR ($CDCl_3$/CD_3OD, 2:1 v/v, 202.4 MHz): δ_P -0.20 (s).

1,3-didecanoyl-glycerol-2-phosphatidic acid, **DDG2PA**:
^{1}H NMR ($CDCl_3$/CD_3OD, 2:1 v/v, with 0.05% TMS, 500 MHz): δ_H 4.54 (1H, app dq, *J* = 14.8, 5.1 Hz), 4.18 (4H, ddd, *J* = 15.7, 11.8, 5.0 Hz), 2.26 (4H, t, *J* = 7.4 Hz), 1.53 (4H, app quint, *J* = 7.2 Hz), 1.30-1.10 (24H, m), 0.80 (6H, t, *J* = 7.0 Hz);
^{31}P{^{1}H} NMR ($CDCl_3$/CD_3OD, 2:1 v/v, 202.4 MHz): δ_P -0.54 (s).

2,3-dilauroyl-*rac*-glycerol-1-phosphatidic acid, **DLG1PA**:
^{1}H NMR ($CDCl_3$/CD_3OD, 2:1 v/v, with 0.05% TMS, 500 MHz): δ_H 5.15 (1H, m), 4.30 (1H, dd, *J* = 12.4, 3.5 Hz, partially obscured by the HOD signal), 4.11 (1H, dd, *J* = 12.0, 6.3 Hz), 4.01 (2H, dd, *J* = 6.6, 5.4 Hz), 2.25 (4H, m), 1.30-1.10 (32H, m), 0.80 (6H, app t, *J* = 7.1 Hz);
^{31}P{^{1}H} NMR ($CDCl_3$/CD_3OD, 2:1 v/v, 202.4 MHz): δ_P -0.22 (s).

1,3-dilauroyl-glycerol-2-phosphatidic acid, **DDG2PA**:

^{1}H NMR ($CDCl_3$/CD_3OD, 2:1 v/v, with 0.05% TMS, 500 MHz): δ_H 4.56 (1H, dq, *J* = 14.6, 5.0 Hz), 4.19 (4H, ddd, *J* = 16.3, 11.8, 5.0 Hz), 2.27 (4H, t, *J* = 7.6), 1.53 (4H, app quint, *J* = 7.4 Hz), 1.30-1.10 (32H, m), 0.80 (6H, t, *J* = 7.0 Hz);
^{31}P{^{1}H} NMR ($CDCl_3$/CD_3OD, 2:1 v/v, 202.4 MHz): δ_P -0.73 (s).

Synthesis of phosphatidylcholines from phosphatidic acids

These compounds were prepared by adapting the method of Harbison and Griffin (*J. Lipid Res.* 1984, *25*, 1140-1142); The appropriate phosphatidic acid DMAP salt (either 2,3-didecanoyl-*rac*-glycerol-1-phosphatidic acid DMAP salt or 1,3-didecanoyl-glycerol-2-phosphatidic acid DMAP salt; 0.1 mmol) was dried under high vacuum overnight in a 10 mL round bottom flask equipped with a magnetic stirrer bead and capped with a rubber septum. The dried phosphatidic acid DMAP salt was placed under an atmosphere of nitrogen and dissolved in dry pyridine (2 mL) before choline tetraphenylborate (87 mg, 0.2 mmol; 2 eq.) was added followed by 2,4,6-triisopropylbenzenesulfonyl chloride (91 mg, 0.3 mmol; 3 eq.). The mixture was stirred at room temperature for 20 h. The reaction was quenched with water (0.1 mL) and the solvent was evaporated under reduced pressure. The residue was extracted with diethyl ether (5 mL), the extract was filtered and the solids washed with diethyl ether. The ether extracts were combined and evaporated under reduced pressure. The mixture of products obtained was fractionated by column chromatography, eluting with chloroform/methanol (9:1, v/v) to remove traces of sulfonic acid, chloroform/methanol (1:1, v/v) to remove traces of unreacted phosphatidic acid, and finally chloroform/methanol/water (10:10:1, v/v/v) to give the phosphatidylcholine as a white solid in 10-12% yield.

2,3-didecanoyl-*rac*-glycerol-1-phosphocholine, **DDG1PC**:
^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 5.18 (1H, m), 4.38 (1H, dd, *J* = 4.4, 2.8 Hz), 4.28 (2H, m), 4.11 (1H, dd, *J* = 12.1, 7.5 Hz), 3.91 (2H, m), 3.75 (2H, m), 3.32 (9H, s, overlapped with the HOD signal), 2.28 (4H, app q, *J* = 8.3 Hz), 1.57 (4H, m), 1.38-1.16 (24H, m), 0.87 (6H, app t, *J* = 7.0 Hz);
^{31}P{^{1}H} NMR ($CDCl_3$, 202.4 MHz): δ_P -0.93 (s).

1,3-didecanoyl-glycerol-2-phosphocholine, **DDG2PC**:
^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 4.52 (1H, dq, *J* = 14.9, 5.1 Hz), 4.33 (2H, m), 4.25 (4H, ddd, *J* = 12.0, 11.7, 5.5 Hz), 3.79 (2H, m), 3.37 (9H, s), 2.30 (4H, t, *J* = 7.3 Hz), 1.58 (4H, app quint, *J* = 7.3 Hz), 1.38-1.16 (24H, m), 0.88 (6H, t, *J* = 7.2 Hz);
^{31}P{^{1}H} NMR ($CDCl_3$, 202.4 MHz): δ_P -1.27 (s).

Hydrolysis of phosphatidic acids

The appropriate amount of either 2,3-didecanoyl-*rac*-glycerol-1-phosphatidic acid (**DDG1PA**) or 1,3-didecanoyl-glycerol-2-phosphatidic acid (**DDG2PA**), prepared as described above, was dispersed in the appropriate quantity of 50 mM buffer (HEPES, pH 8.0; or sodium carbonate, pH 10.0) in water/D_2O (6:4 v/v) to give a 5 mM dispersion of liposomes. The pH of each dispersion was adjusted to that of the buffer, as specified. The dispersion of liposomes was then divided into two; one half was retained to study the hydrolysis of phosphatidic acids in liposomes and the other half was diluted with acetonitrile (1:1, v/v) to give a 2.5 mM solution

of (monomeric) phosphatidic acid. The extent of hydrolysis of the phosphatidic acid in solution was determined periodically by ^{31}P NMR (D_2O). For comparison, when the extent of hydrolysis of the (monomeric) phosphatidic acid in solution reached 50%, an aliquot was removed from the dispersion of phosphatidic acid liposomes. This aliquot was diluted with acetonitrile (1:1, v/v) to break the liposomes or other supramolecular aggregates and the extent of hydrolysis was determined by ^{31}P NMR (D_2O). This process was repeated when hydrolysis of the phosphatidic acid in solution was complete.

Preparation and investigation of phospholipid liposomes

Phospholipid and mixed fatty acid/alcohol/phospholipid liposomes were prepared by the method of Zhu et al (*Methods Enzymol.* 2013, *533*, 267-274); The appropriate quantity of a solution of phospholipid and/or fatty acid/alcohol in chloroform was evaporated onto the surface of a glass mass spectrometry vial under a stream of nitrogen. The lipid film was then dried under high vacuum overnight. {Note: Phosphatidic acids, prepared as described above, were dissolved in chloroform immediately before use. Phosphatidic acids slowly degrade in chloroform, even when stored at -20°C. Phosphatidylcholines are, by contrast, stable in chloroform for months when stored at -20°C.} The dried phospholipid was dispersed in the appropriate volume of aqueous buffer solution (see below), the dispersion was heated at 60°C for 10 min, vortexed for 1 min, and tumbled for 30 min* at room temperature. {Note: Sodium hydroxide (1.5 eq. from a 5.0 M solution in water, with respect to the total amount of lipid) was added to each vial containing phosphatidic acid prior to dispersion in buffered aqueous solution. *Phosphatidylcholine dispersions were sonicated for 1 h prior to tumbling.}

Critical aggregation concentrations (CACs) of phospholipids, mixtures of phospholipids, or mixtures of phospholipids and fatty acids/alcohols dispersed in aqueous buffers were determined by the method of Maurer and Nguyen (*Orig. Life Evol. Biosph*. 2016, *46*, 215-222), using merocyanine 540 as solvatochromic probe and following changes in the absorbance ratio A_{565nm}/A_{525nm}; Three μL of a 0.5 mM solution of merocyanine 540 in ethanol was added to each required well to be used of a transparent flat-bottomed 96-well plate (Thermo Fisher) and the solvent was allowed to evaporate at room temperature over 1 h. The lipid dispersion was serially diluted across 10 empty wells and aliquots (50 μL) of each dispersion were then added to the wells containing the dried probe. The serially diluted dispersions were mixed thoroughly with the probe before absorbance spectra were recorded. CACs were calculated from the inflection point of a plot of the absorbance ratio A_{565nm}/A_{525nm} vs total lipid concentration.

For microscopy, giant unilamellar liposomes were prepared from 25 mM dispersions of phosphatidic acids containing 1 mol% NBD-PE by gentle hydration of lipid films; The dried phospholipid was first dispersed in half of the required total volume of Milli-Q water and the dispersion was heated at 40°C for 30 min. Then, half of the required total volume of 0.1 M sodium phosphate buffer, pH 7.5, was added to each dispersion to give a final buffer concentration of 50 mM, the dispersion was heated for a further 30 min at 40°C, then briefly vortexed.

For generalised polarization and fluorescence anisotropy, liposomes were prepared from 5 mM dispersions of phosphatidic acids containing 0.1 mol% Laurdan or 1,6-diphenyl-1,3,5-hexatriene DPH, respectively, in 50mM phosphate buffer at pH 7.5, unless otherwise stated.

Membrane Zeta (ζ)-potentials were measured in a high concentration quartz cuvette using 200 μL aliquots of 5 mM dispersions of phosphatidic acids in HEPES buffer at pH 7.5, prepared as described above. Liposomes were extruded by 11 passages through a 100 nm pore membrane (Whatman) using a Mini-Extruder (Avanti Polar Lipids).

Precipitation of phospholipids from liposomes on addition of Mg^{2+}, Ca^{2+} or Zn^{2+}

Liposomes composed of **DDG1PA** (5 mM), **DDG2PA** (5 mM), or a 1:1 mixture of **DDG1PA** and **DDG2PA** (5 mM each) were prepared by gentle hydration; Half the required volume of Milli-Q water/D_2O (1:1, v/v) was added, the dispersion was heated at 40°C for 30 min, then half the required volume of 0.1 M sodium phosphate buffer, pH 7.5, in Milli-Q water/D_2O (1:1, v/v) to give a final buffer concentration of 50 mM phosphate. The pH was readjusted to 7.5 and the dispersion was tumbled for 30 min. To induce precipitation, the required amount of a 0.2 M solution of $MgCl_2$ (or $CaCl_2$ or $ZnCl_2$) was added to 400 μL of the liposome solution, the mixture was vortexed for ~30 seconds, and any precipitate was removed by filtration through a plug of cotton wool in a plastic P1000 pipette tip. Subsequently, 250 μL of the filtrate was mixed with 250 μL of a solution of 0.2 M bicine in water at pH 8.5* containing 0.2 M Triton X-100. The phospholipid composition of the filtrate was determined by ^{31}P NMR (D_2O). {*This pH change was made because at pH 7.5 the signal corresponding to 2,3-didecanoyl-*rac*-glycerol-1-phosphatidic acid (**DDG1PA**) overlaps with the that of inorganic phosphate.}

Precipitation of phospholipids by addition of Mg^{2+} to liposomes composed of mixtures of phosphatidic acids obtained by prebiotic synthesis

Mixtures of phosphatidic acids were prepared by acylation of either **G1P** (10 mM), **G2P** (10 mm), or a 1:1 mixture of **G1P** and **G2P** (5 mM of each), with NDI (100 mM) in acetonitrile/water/D_2O (5:4:1, v/v/v), as described above. Each reaction was allowed to run for 8 d before the composition of the mixture was determined by ^{31}P NMR (D_2O) and the mixture of products was lyophilised. Liposomes composed of each mixture of phospholipids were then prepared by dispersion in 1 mL of water/D_2O (1:1, v/v) using the gentle hydration method described above, but no additional buffer was added as the imidazole released on reaction served this purpose. The pH was adjusted to 7.0 before the dispersion was tumbled for 30 min. To induce precipitation, the required amount of a 1.0 M solution of $MgCl_2$ was added to 400 μL of the liposome solution, the mixture was vortexed for ~30 seconds, and any precipitate was removed by filtration through a plug of cotton wool in a plastic P1000 pipette tip. Subsequently, 250 μL of the filtrate was mixed with 250 μL of a solution of 0.2 M Triton X-100 in water/D_2O (1:1, v/v). The phospholipid composition of the filtrate was determined by ^{31}P NMR (D_2O).

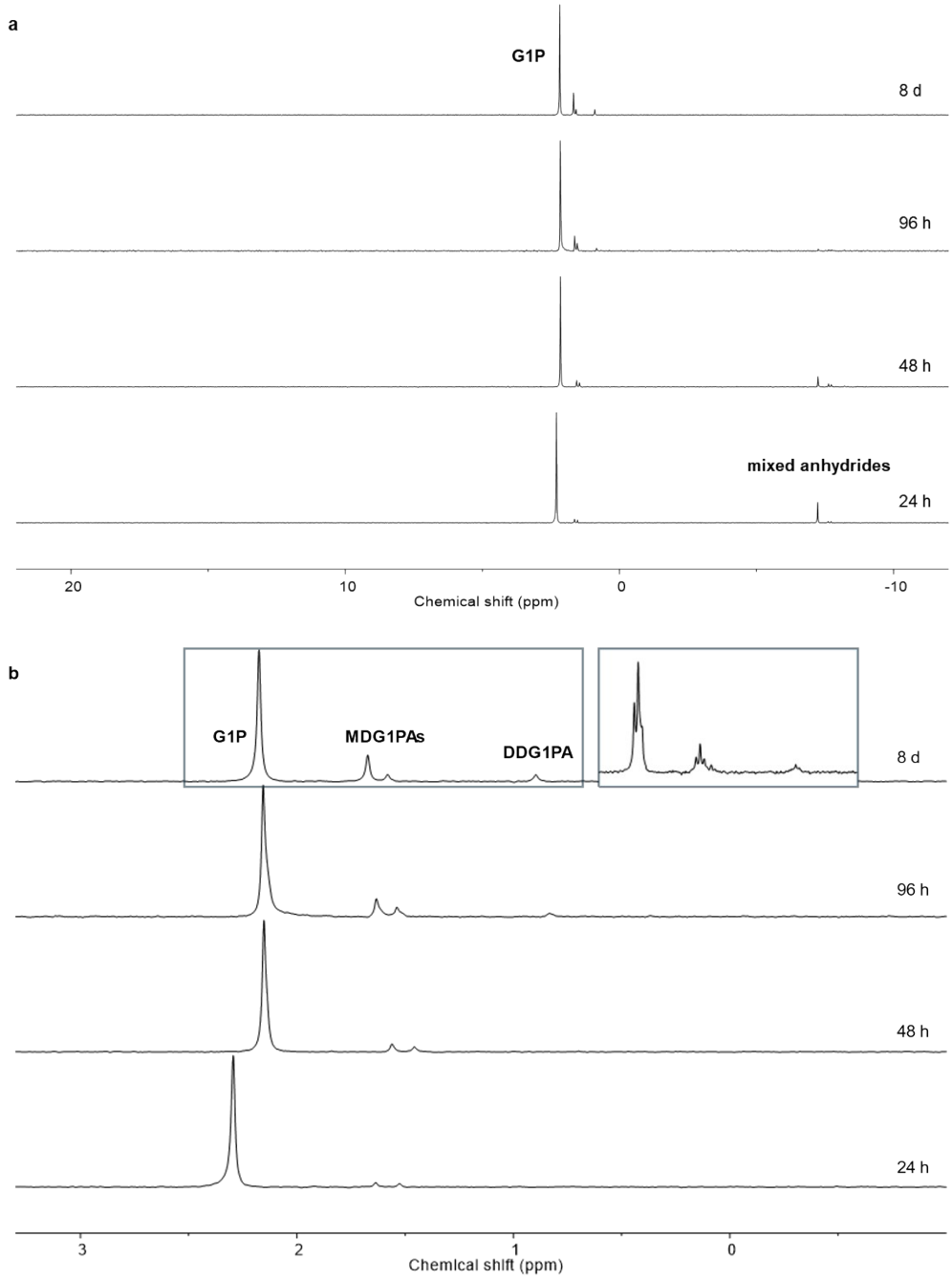


Figure S1. a) Stack of $^{31}P\{^{1}H\}$ NMR spectra showing the time course of the reaction of glycerol-1-phosphate (**G1P**; 10 mM) with *N*-decanoyl imidazole (NDI; 100 mM) in acetonitrile/water/D_2O (5:4:1, v/v/v), pH 7.4. b) Expansion of the +4.0 to 0 ppm region of the stack of $^{31}P\{^{1}H\}$ NMR spectra shown in a). The insert shows the ^{1}H-coupled ^{31}P NMR spectrum of the highlighted region.

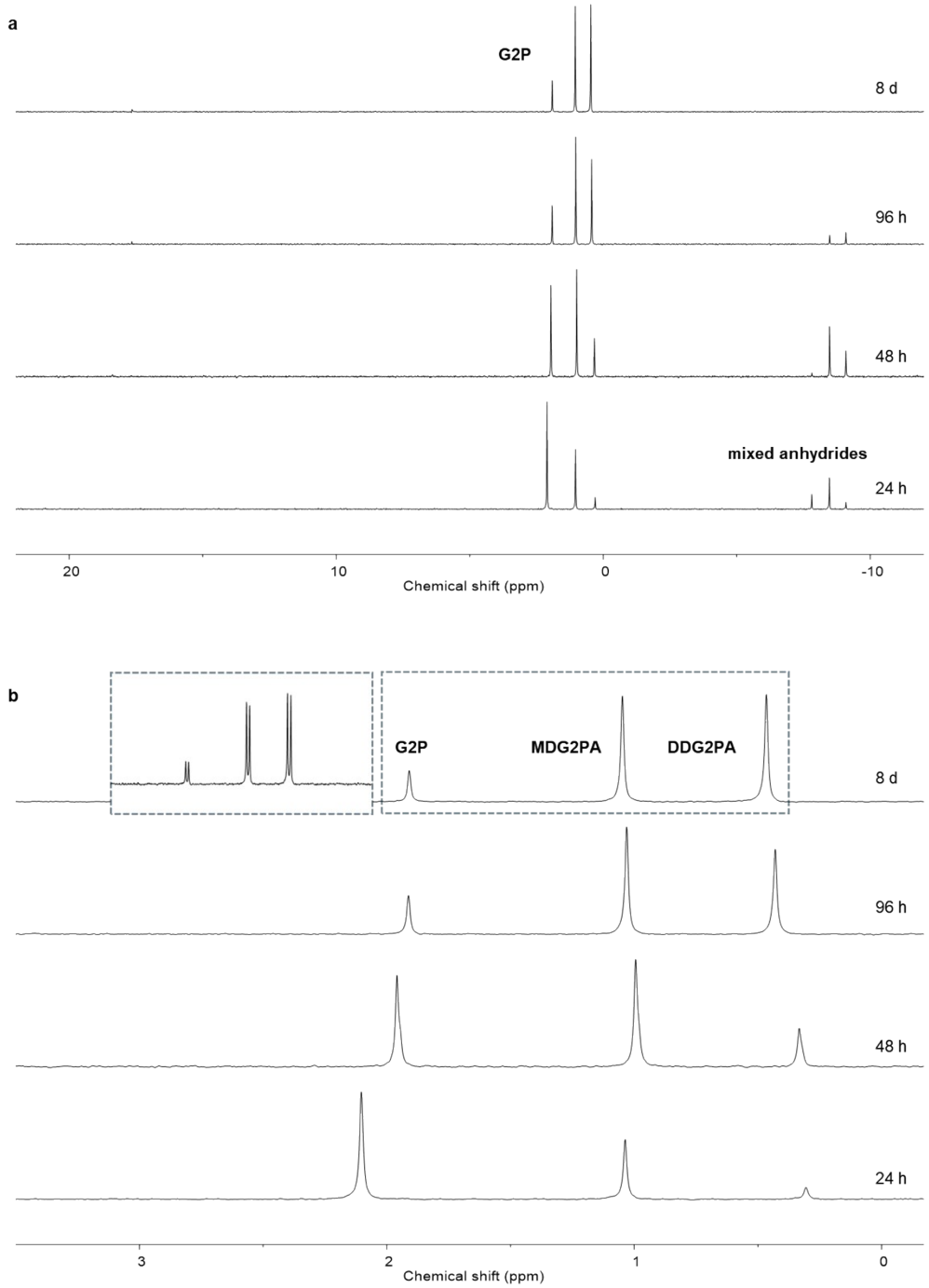


Figure S2. a) Stack of $^{31}P\{^{1}H\}$ NMR spectra showing the time course of the reaction of glycerol-2-phosphate (**G2P**; 10 mM) with *N*-decanoyl imidazole (NDI; 100 mM) in acetonitrile/water/D_2O (5:4:1, v/v/v), pH 7.4. b) Expansion of the +3.0 to 0 ppm region of the stack of $^{31}P\{^{1}H\}$ NMR spectra shown in a). The insert shows the ^{1}H-coupled ^{31}P NMR spectrum of the highlighted region.

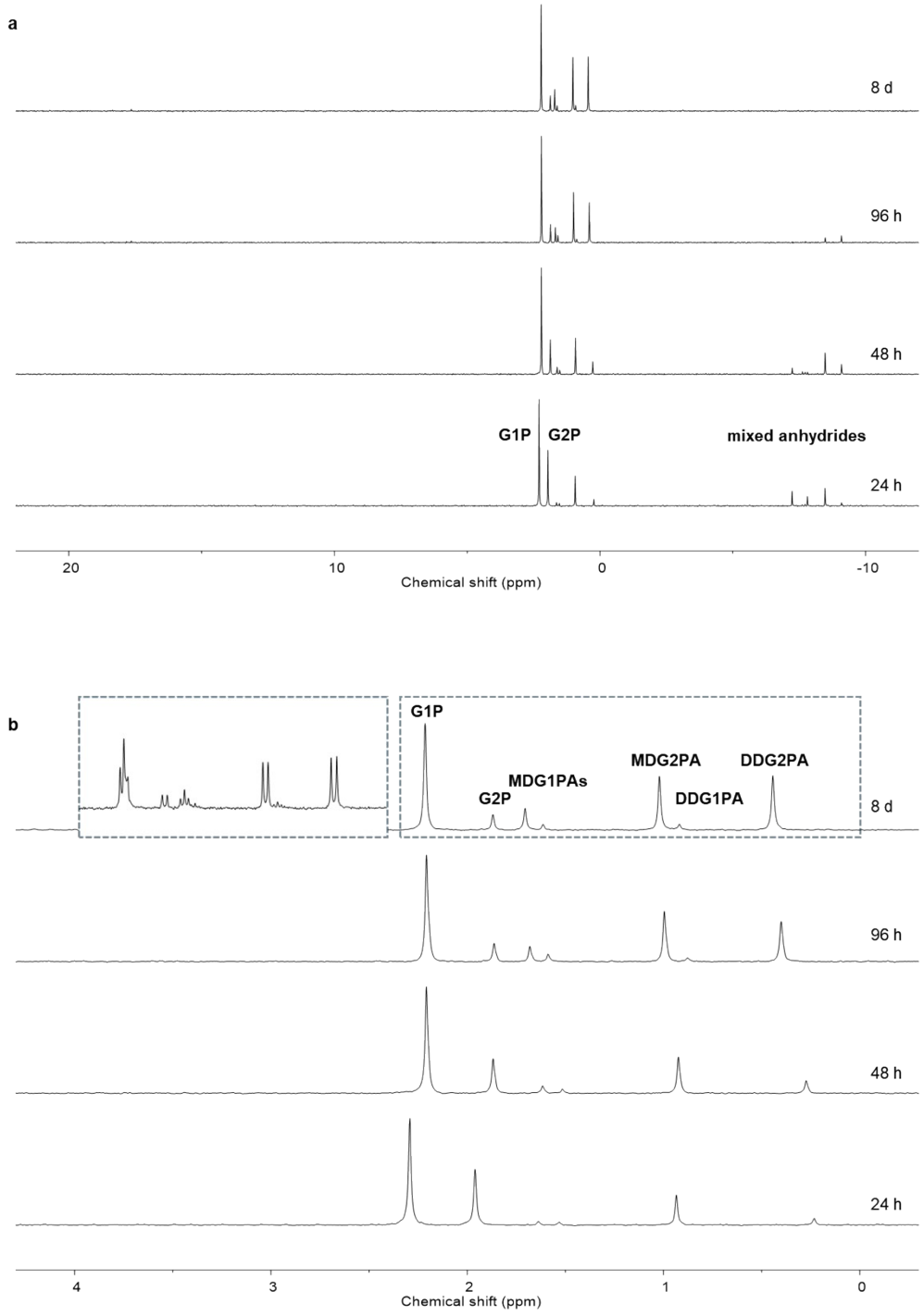


Figure S3. a) Stack of $^{31}P\{^{1}H\}$ NMR spectra showing the time course of the reaction of a 1:1 mixture of glycerol-1-phosphate (**G1P**; 5 mM) and glycerol-2-phosphate (**G2P**; 5 mM) with *N*-decanoyl imidazole (NDI; 100 mM) in acetonitrile/water/D_2O (5:4:1, v/v/v), pH 7.4. b) Expansion of the +4.0 to 0 ppm region of the stack of $^{31}P\{^{1}H\}$ NMR spectra shown in a). The insert shows the ^{1}H-coupled ^{31}P NMR spectrum of the highlighted region.

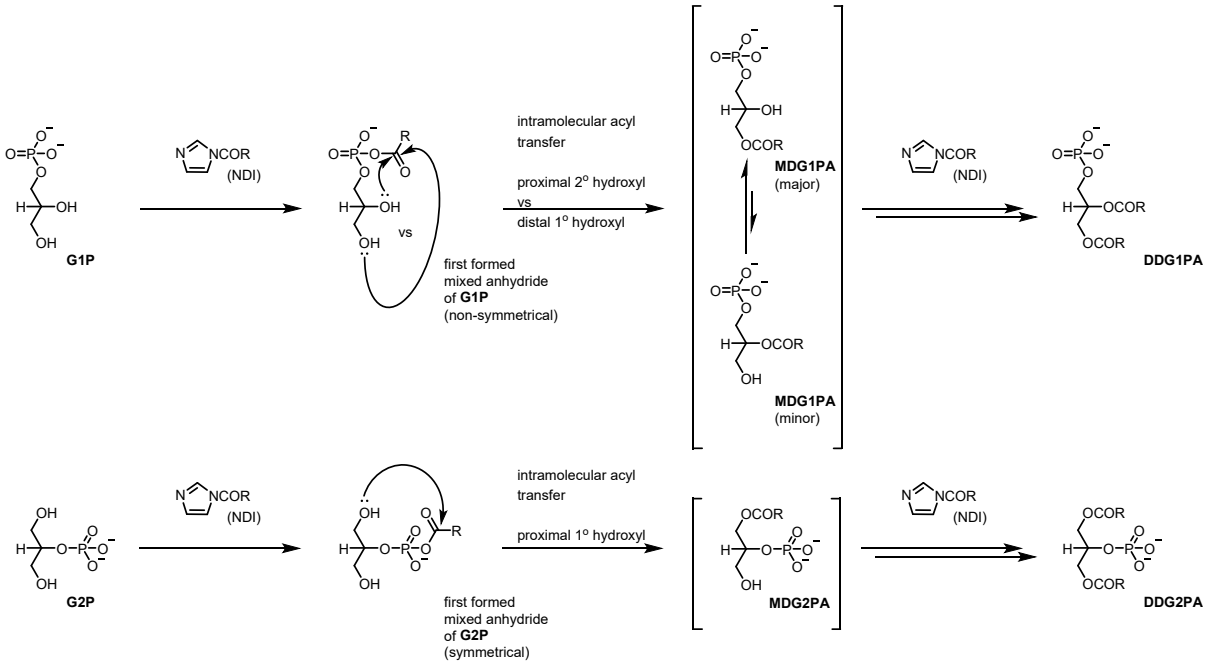


Figure S4. On reaction with *N*-decanoyl imidazole (NDI) in aqueous media, the acylation of each hydroxyl group of each glycerol phosphate takes place via a two-step phosphate-assisted mechanism, as described by Bonfio et al (*J. Am. Chem Soc.* 2019*, 141,* 3934-3939). The first step involves acylation of the phosphate group to give a mixed phosphoric-carboxylic acid anhydride. The second step involves the transfer of the acyl group from the phosphate group to a neighbouring hydroxyl group. Each mixed anhydrides is susceptible to hydrolysis and so intramolecular acyl transfer must to some extent outcompete intermolecular attack of water for acylation to be efficient. The acylation of **G2P** is a higher yielding process because each acyl transfer step involves a proximal primary hydroxyl group (7-membered cyclic transition state). By contrast, in the case of **G1P**, acyl transfer to either the proximal yet sterically hindered secondary hydroxyl group (7-membered transition state) or the distal primary hydroxyl group (8-membered transition state) occurs less favourably, and the mixed anhydride is mostly lost to hydrolysis.

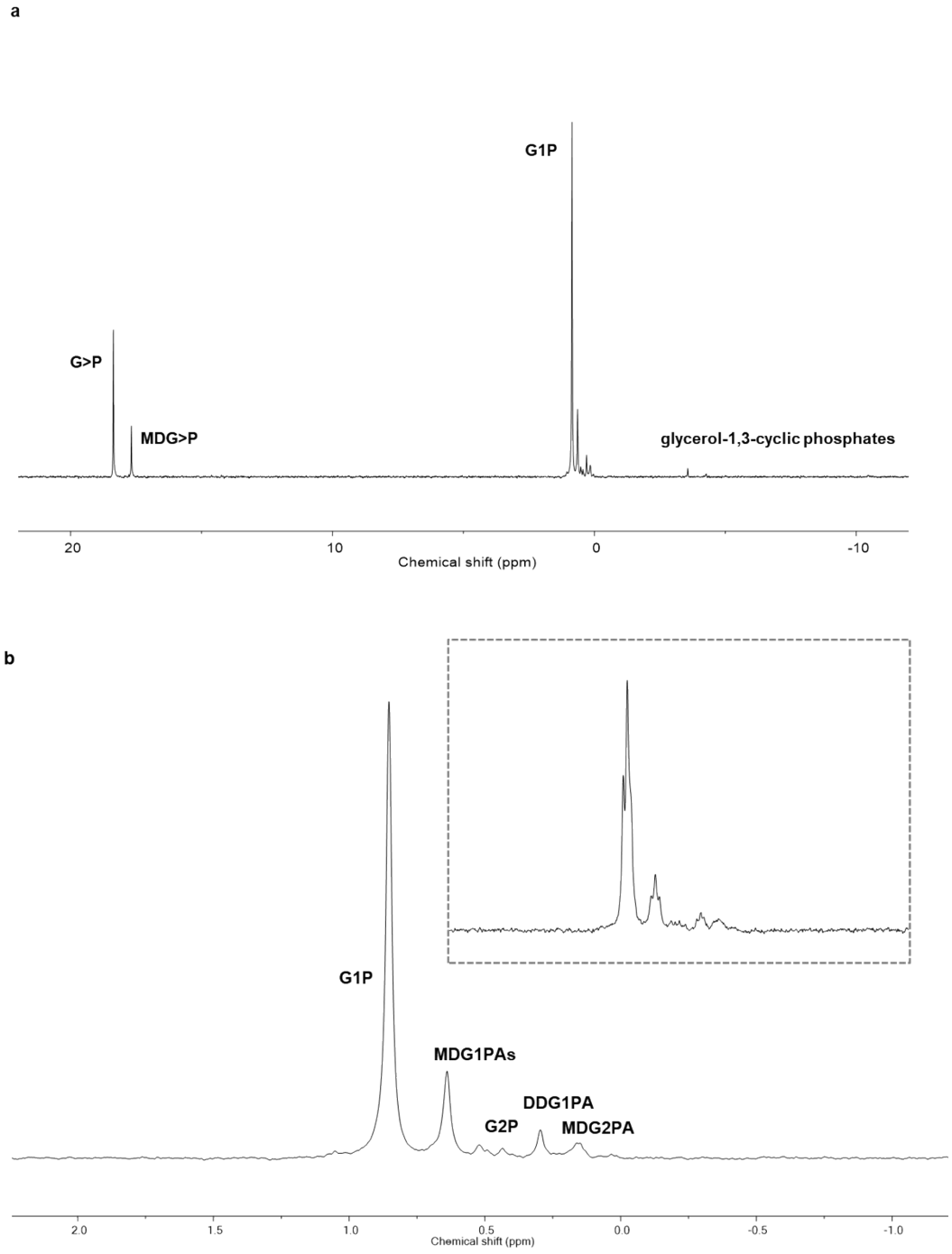


Figure S5. a) $^{31}P\{^{1}H\}$ NMR spectrum showing the mixture of products obtained on drying out a mixture of glycerol-1-phosphate (**G1P**; 10 mM), ammonium decanoate (100 mM), cyanamide (100 mM), and imidazole (100 mM), pH 7.4, in a glass Petri dish and heating the dried (partially crystalline) mixture at 65 °C for 18 h. {Note: The NMR spectrum was obtained after extracting the mixture of products into acetonitrile/water/D_2O (5:4:1, v/v/v).} b) Expansion of the +2.0 to -1.0 ppm region of the stack of $^{31}P\{^{1}H\}$ NMR spectra shown in a). The insert shows the ^{1}H-coupled ^{31}P NMR spectrum of the expanded region.

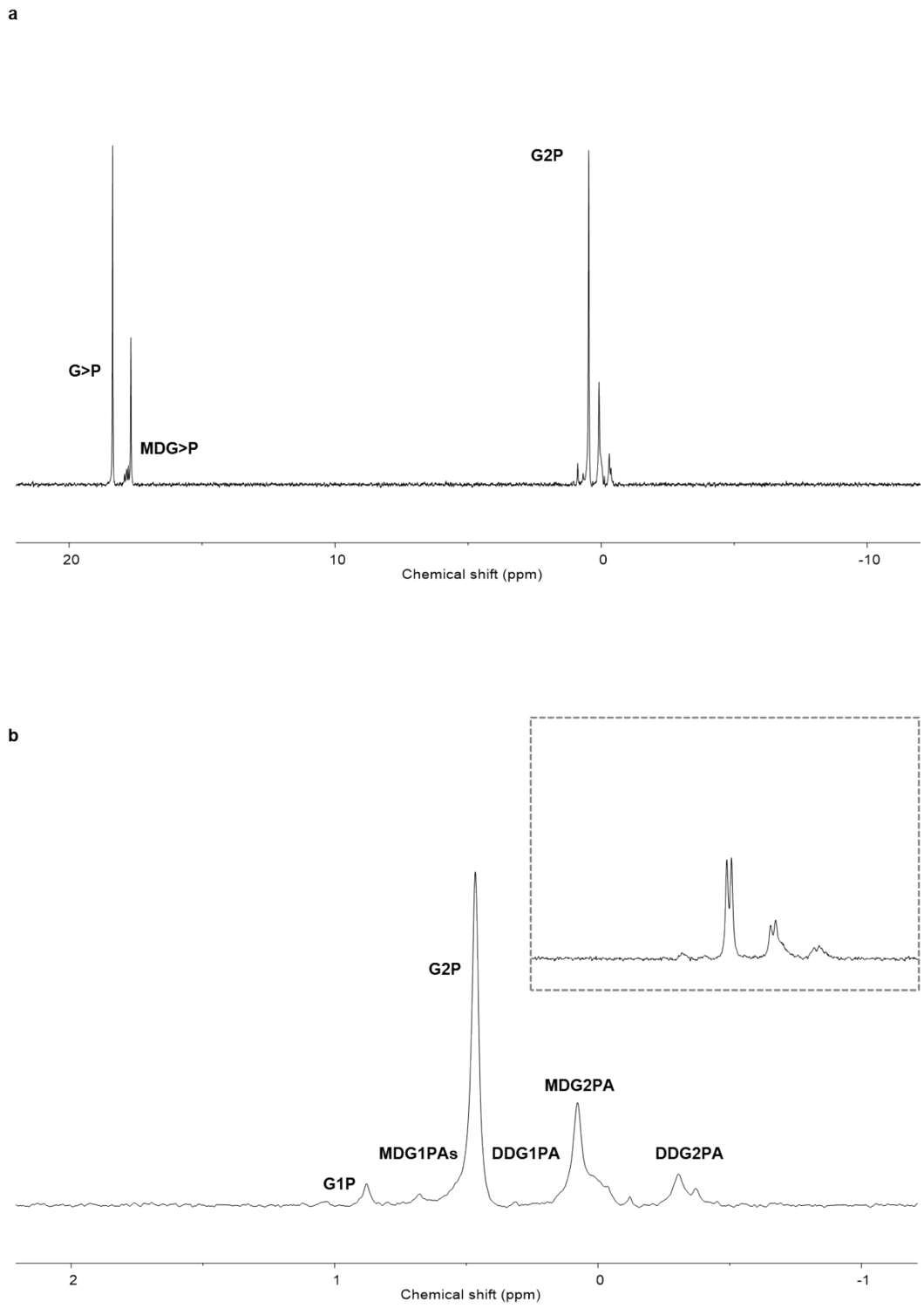


Figure S6. a) $^{31}P\{^{1}H\}$ NMR spectrum showing the mixture of products obtained on drying out a mixture of glycerol-2-phosphate (**G2P**; 10 mM), ammonium decanoate (100 mM), cyanamide (100 mM), and imidazole (100 mM), pH 7.4, in a glass Petri dish and heating the dried (partially crystalline) mixture at 65 °C for 18 h. {Note: The NMR spectrum was obtained after extracting the mixture of products into acetonitrile/water/D_2O (5:4:1, v/v/v).} b) Expansion of the +2.0 to -1.0 ppm region of the $^{31}P\{^{1}H\}$ NMR spectrum shown in a). The insert shows the ^{1}H-coupled ^{31}P NMR spectrum of the expanded region.

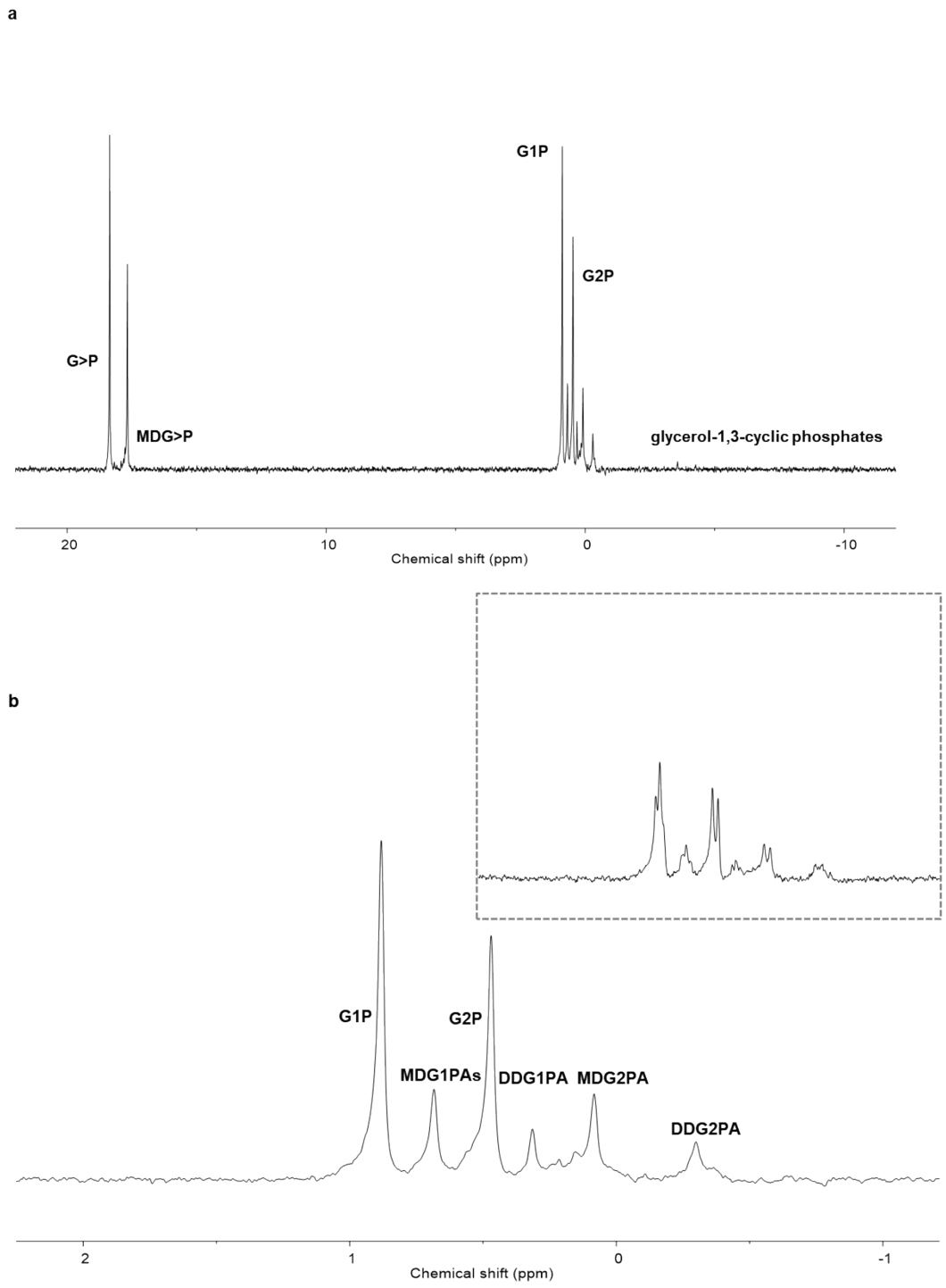


Figure S7. a) $^{31}P\{^{1}H\}$ NMR spectrum showing the mixture of products obtained on drying out a mixture of glycerol-1-phosphate (**G1P**; 5 mM), glycerol-2-phosphate (**G2P**; 5 mM), ammonium decanoate (100 mM), cyanamide (100 mM), and imidazole (100 mM), pH 7.4, in a glass Petri dish and heating the dried (partially crystalline) mixture at 65 °C for 18 h. {Note: The NMR spectrum was obtained after extracting the mixture of products into acetonitrile/water/D_2O (5:4:1, v/v/v).} b) Expansion of the +2.0 to -1.0 ppm region of the $^{31}P\{^{1}H\}$ NMR spectrum shown in a). The insert shows the ^{1}H-coupled ^{31}P NMR spectrum of the expanded region.

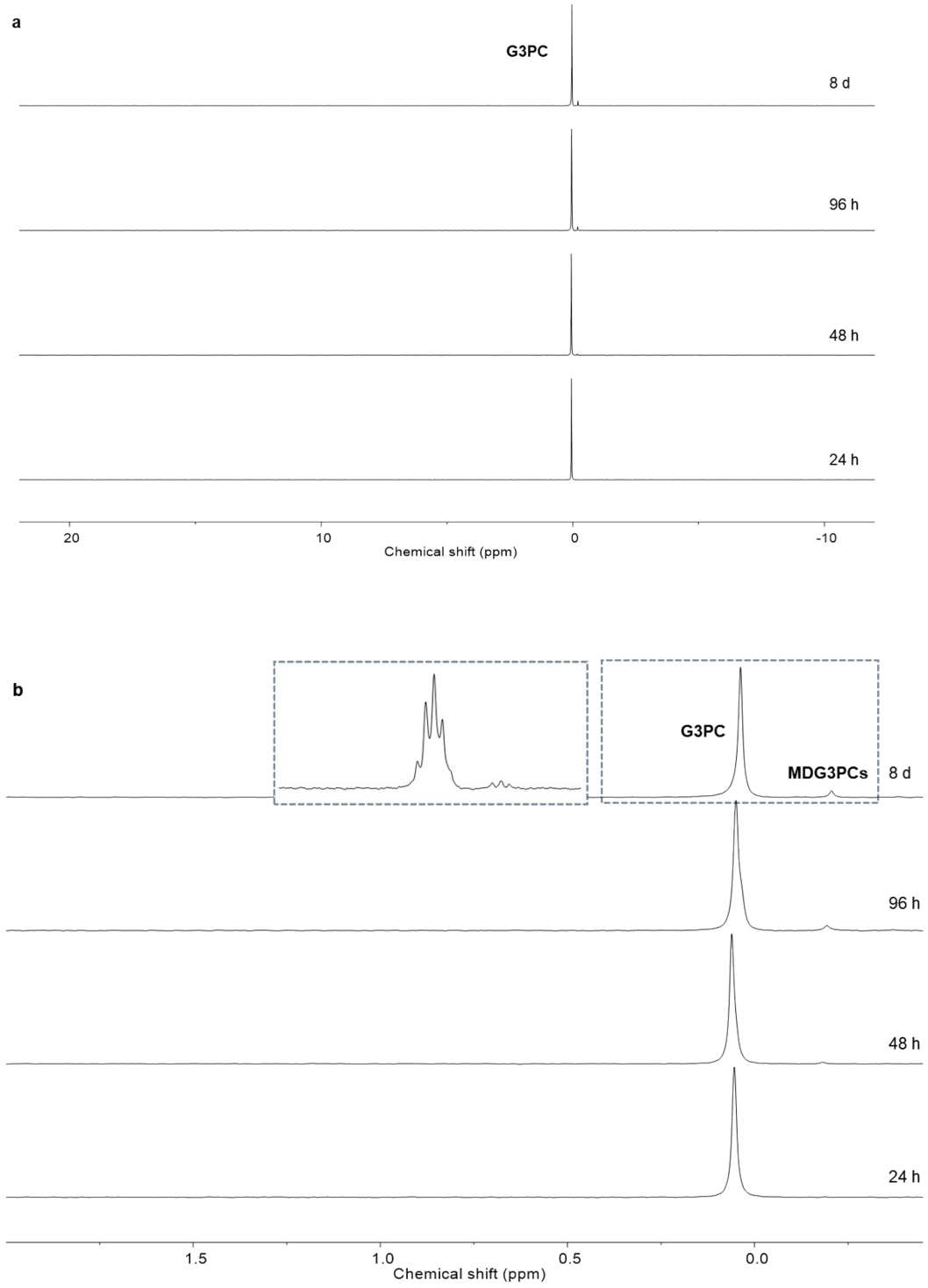


Figure S8. a) Stack of $^{31}P\{^{1}H\}$ NMR spectra showing the time course of the reaction of sn-glycero-3-phosphocholine (**G3PC**; 10 mM) with *N*-decanoyl imidazole (100 mM) in acetonitrile/water/D_2O (5:4:1, v/v/v), pH 7.4. b) Expansion of the +1.5 to -0.5 ppm region of the stack of $^{31}P\{^{1}H\}$ NMR spectra shown in a). The insert shows the ^{1}H-coupled ^{31}P NMR spectrum of the highlighted region.

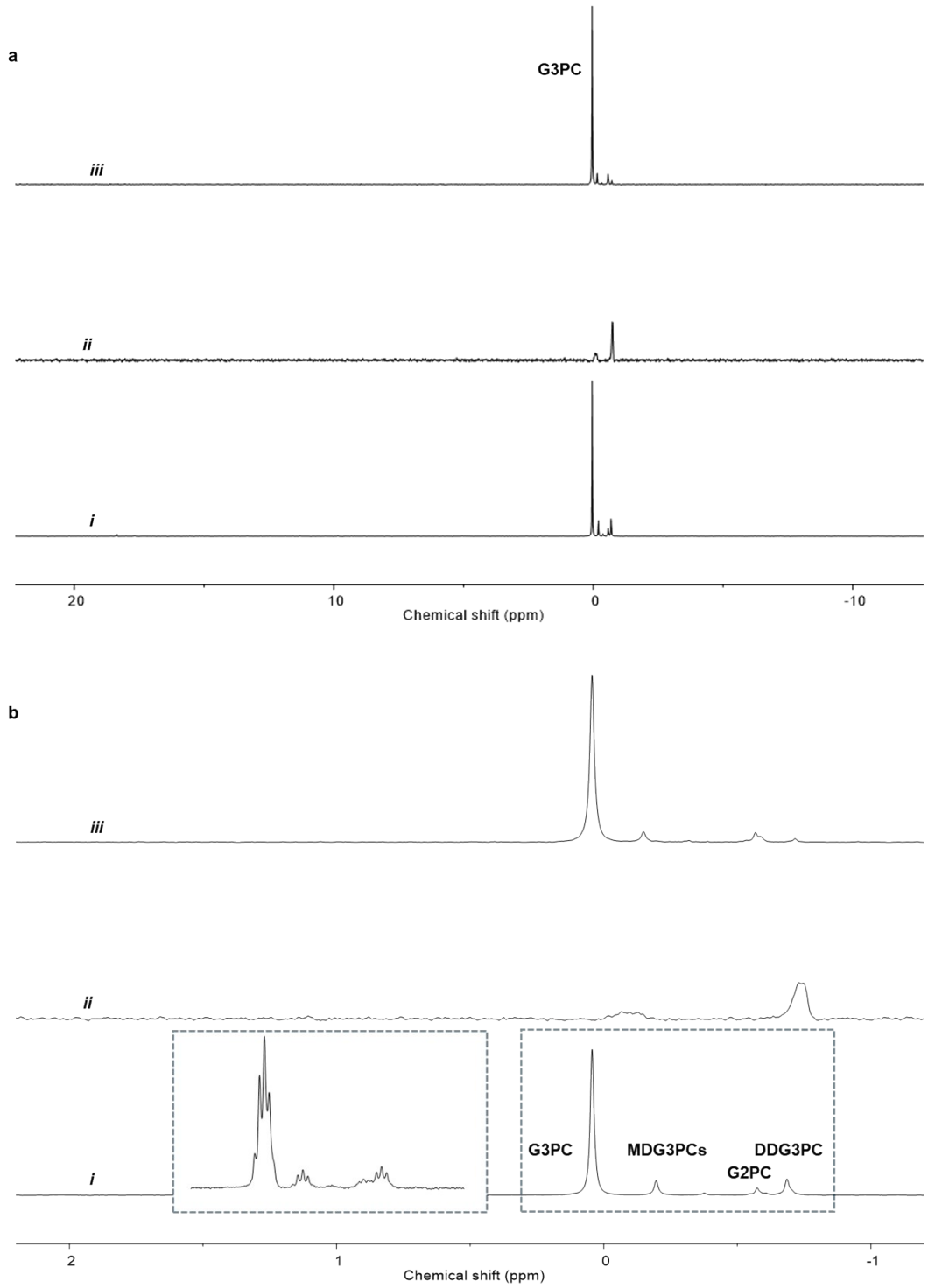


Figure S9. a) Stack of $^{31}P\{^{1}H\}$ NMR spectra showing: i) The mixture of products obtained on drying out a mixture of sn-glycero-3-phosphocholine (**G3PC**; 10 mM), ammonium decanoate (100 mM), cyanamide (100 mM), and imidazole (100 mM), pH 7.4, in a glass Petri dish and heating the dried (partially crystalline) mixture at 65 °C for 18 h. {Note: The NMR spectrum was obtained after extracting the mixture of products into acetonitrile/water/D_2O (5:4:1, v/v/v).}; ii) The composition of the $CDCl_3$ phase after Bligh and Dyer extraction of the lyophilised mixture of products; and iii) The composition of the CD_3OD/water phase after Bligh and Dyer extraction of the lyophilised mixture of products. b) Expansion of the +2.0 to -1.0 ppm region

of the stack of $^{31}P\{^{1}H\}$ NMR spectra shown in a). The insert shows the ^{1}H-coupled ^{31}P NMR spectrum of the highlighted region.

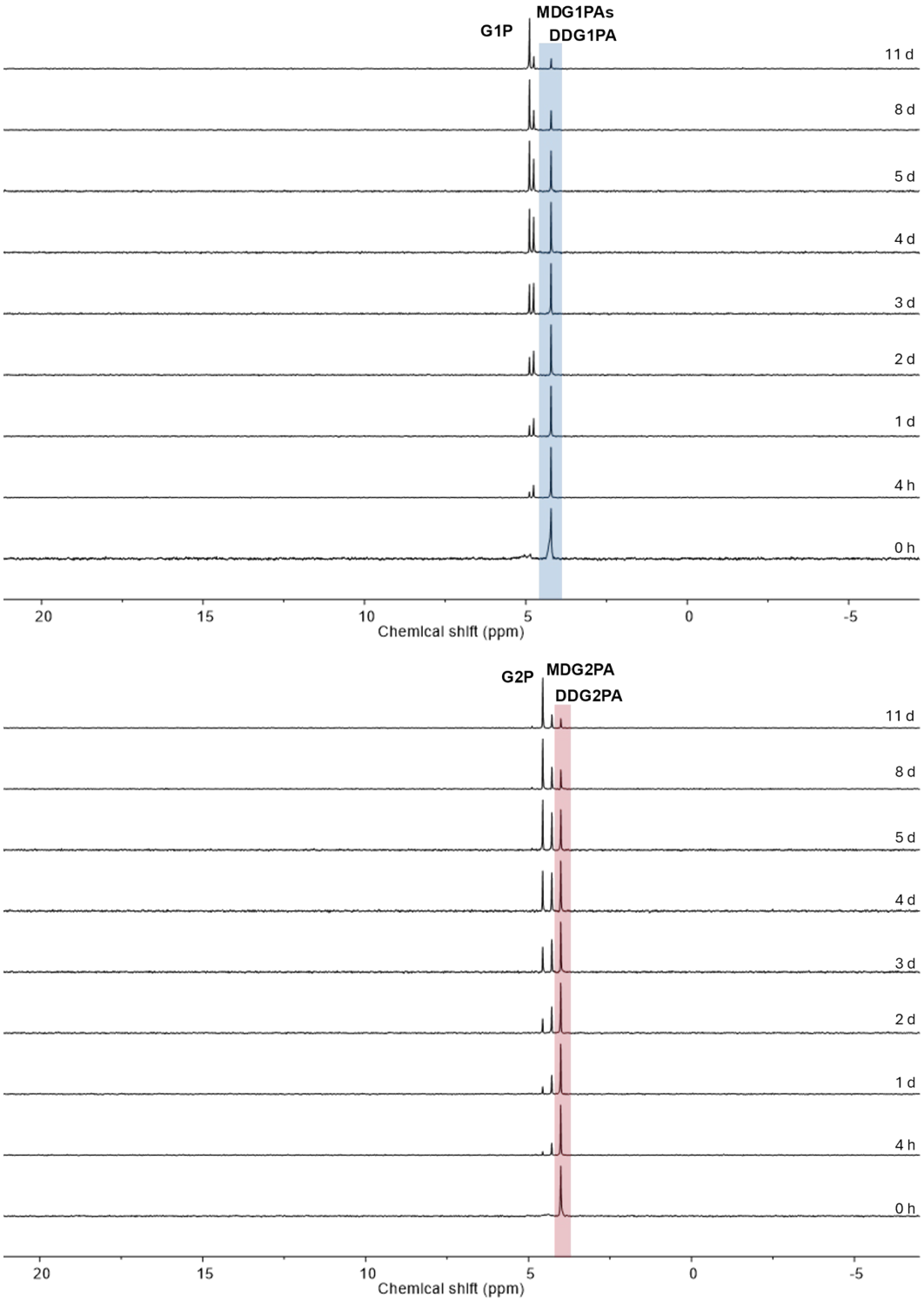


Figure S10. a) Stack of $^{31}P\{^{1}H\}$ NMR spectra showing the time course of the hydrolysis of **DDG1PA** (5 mM) in acetonitrile/carbonate buffer (1:1, v/v, total buffer concentration 50 mM, pH 10.0). b) Stack of $^{31}P\{^{1}H\}$ NMR spectra showing the time course of the hydrolysis of **DDG2PA** (5 mM) in acetonitrile/carbonate buffer (1:1, v/v, total buffer concentration 50 mM, pH 10.0).

Figure S10 continues overleaf.

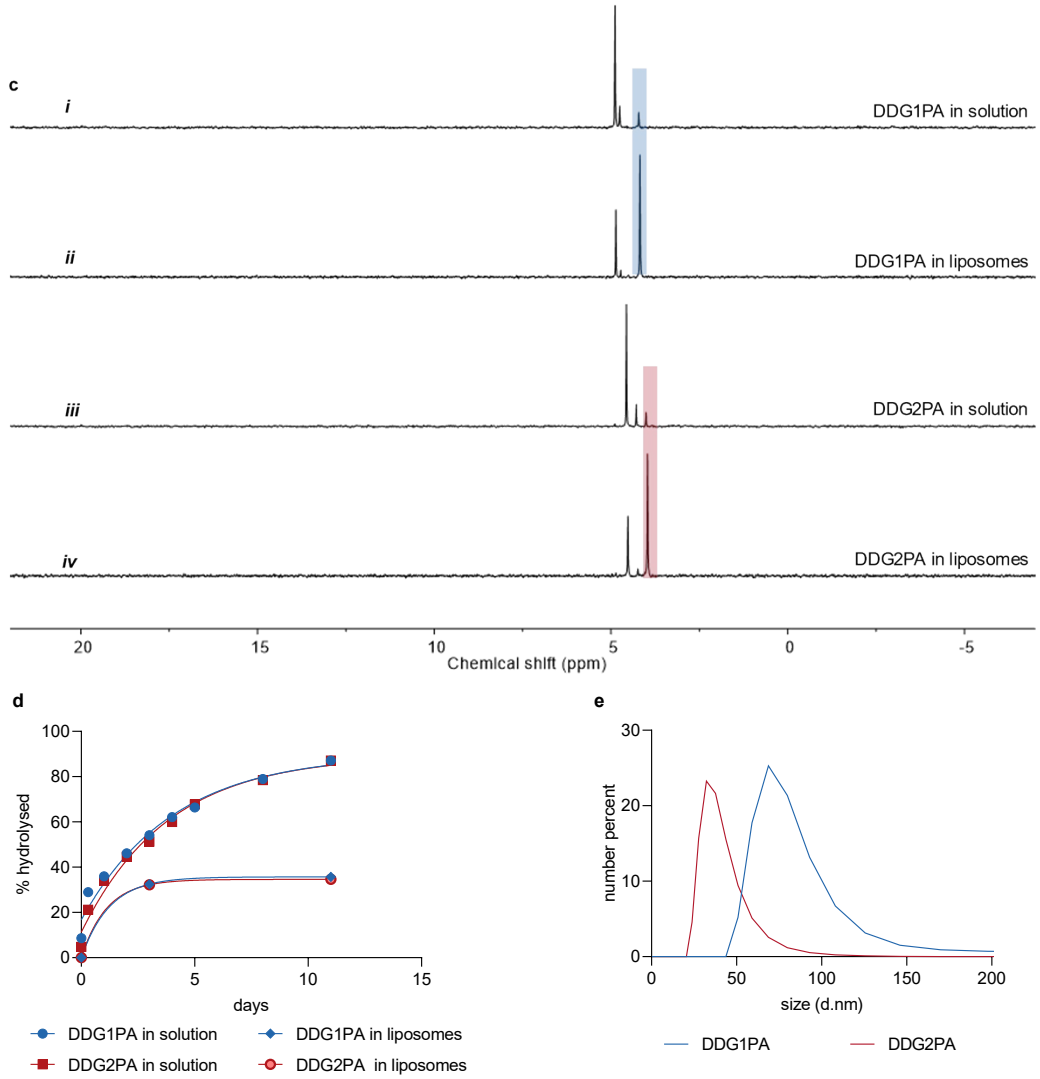


Figure S10 (continued). c) Stack of $^{31}P\{^{1}H\}$ NMR spectra showing the extent of hydrolysis after 11 d of *i*) **DDG1PA** dissolved in acetonitrile/carbonate buffer (1:1, v/v, total buffer concentration 50 mM, pH 10.0); *ii*) **DDG1PA** liposomes in carbonate buffer (50 mM, pH 10); *iii*) **DDG2PA** dissolved in acetonitrile/carbonate buffer (1:1, v/v, total concentration 50 mM, pH 10.0); *iv*) **DDG1PA** liposomes in carbonate buffer (50 mM, pH 10). d) Graph showing the time course of the hydrolysis of **DDG1PA** and **DDG2PA** (5 mM) either dissolved in acetonitrile/carbonate buffer (1:1, v/v, total buffer concentration 50 mM, pH 10.0) or as liposomes in carbonate buffer (50 mM, pH 10.0). The rate of hydrolysis of each (monomeric) lipid in solution is greater than its the rate of hydrolysis in liposomes. However, in each set of experiments, the rates of hydrolysis of **DDG1PA** and **DDG2PA** are approximately equal. e) DLS profiles showing the presence of **DDG1PA** and **DDG2PA** (5 mM) liposomes in carbonate buffer (50 mM, pH 10.0).

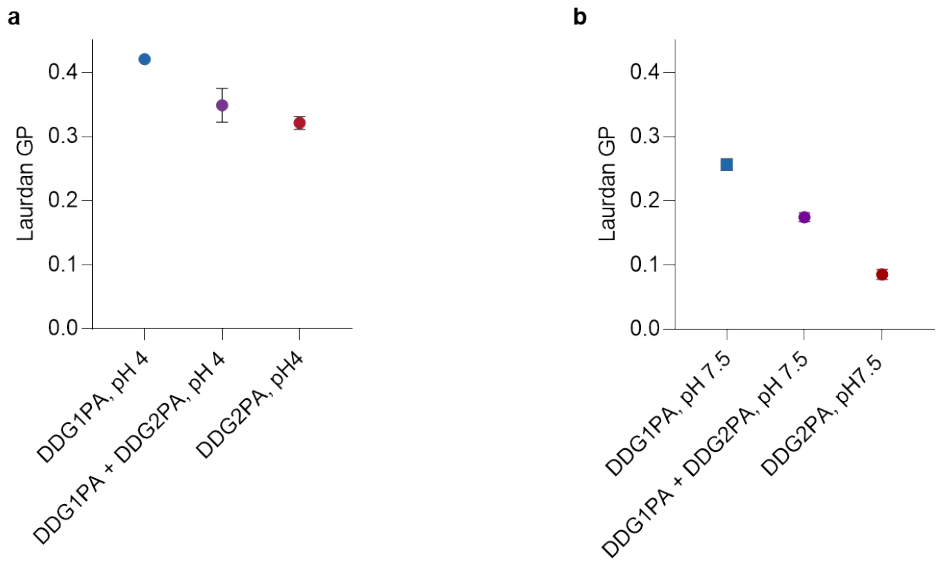


Figure S11. a) Laurdan generalized polarization (GP) of **DDG1PA** and **DDG2PA** membranes at pH 4.0 in 50mM phosphate buffer. b) Laurdan generalized polarization (GP) of **DDG1PA** and **DDG2PA** membranes at pH 7.5 in 50mM phosphate buffer.

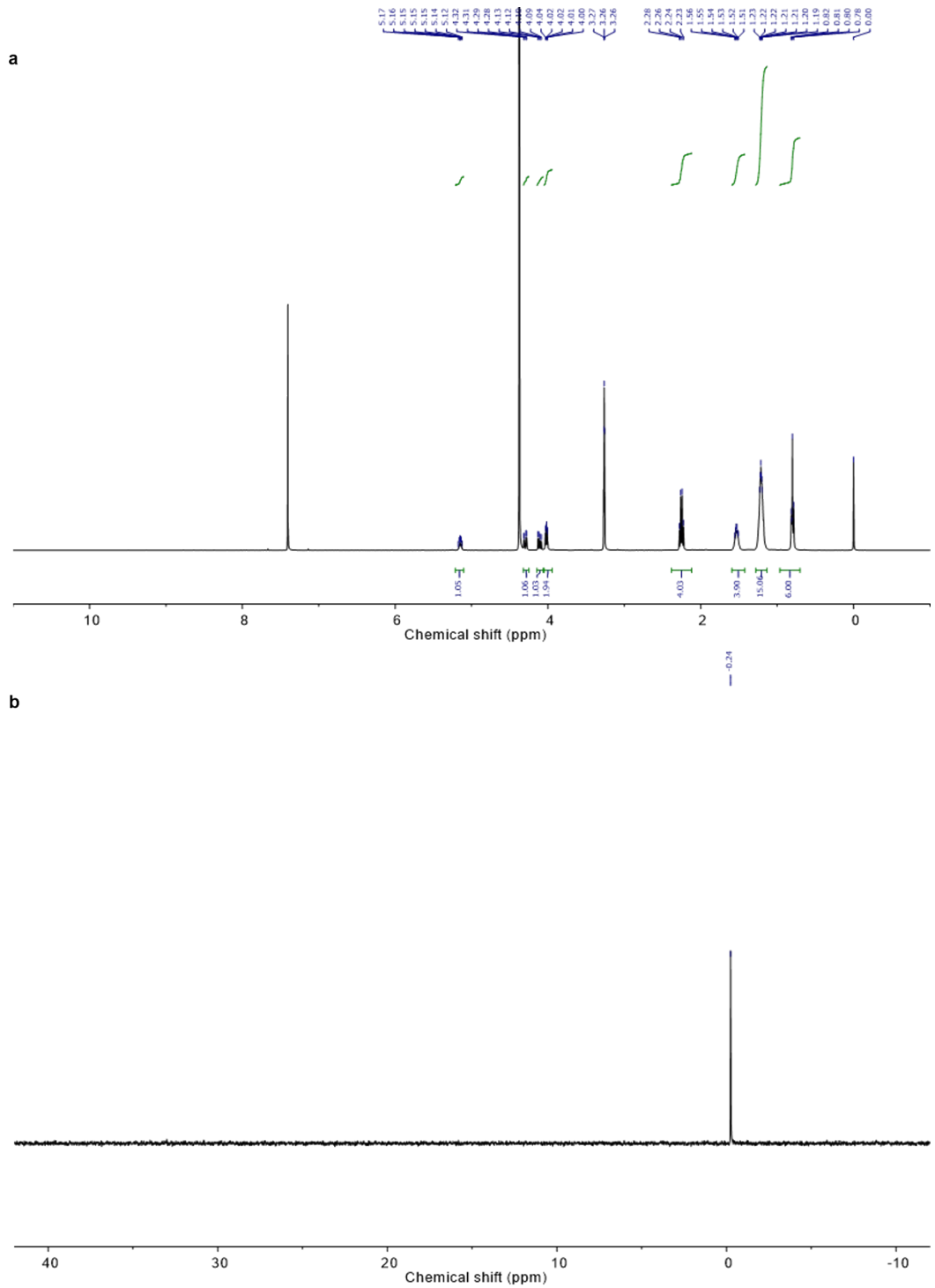


Figure S12. a) ^{1}H NMR spectrum of 2,3-dicapryloyl-*rac*-glycerol-1-phosphatidic acid (**DCG1PA**; $CDCl_3/CD_3OD$, 2:1 v/v, with 0.05% TMS, 500 MHz); b) $^{31}P\{^{1}H\}$ NMR spectrum of 2,3-dicapryloyl-*rac*-glycerol-1-phosphatidic acid (**DCG1PA**; $CDCl_3/CD_3OD$, 2:1 v/v, with 0.05% TMS, 202.4 MHz).

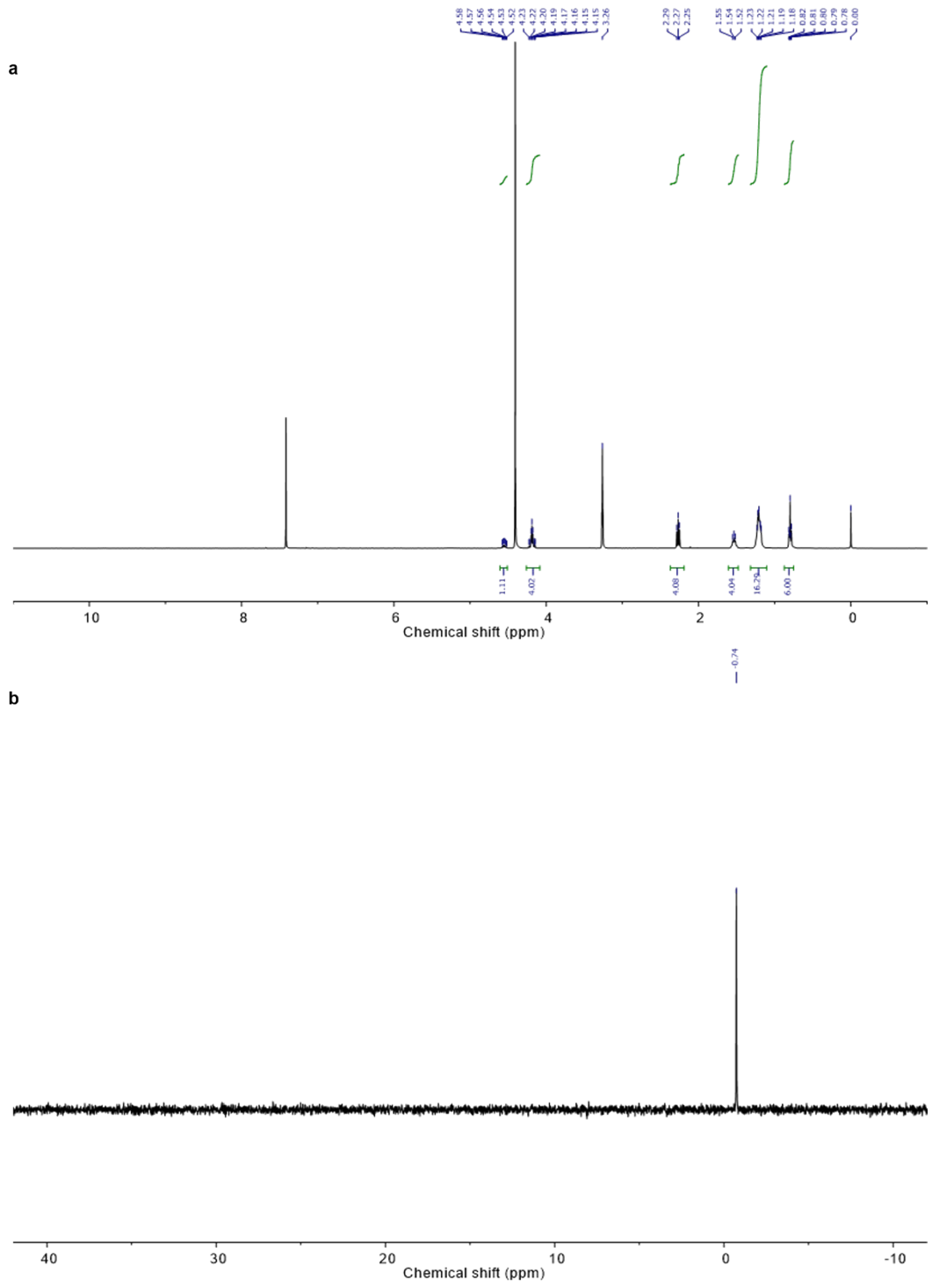


Figure S13. a) ^{1}H NMR spectrum of 1,3-dicapryloyl-glycerol-2-phosphatidic acid (**DCG2PA**; $CDCl_3/CD_3OD$, 2:1 v/v, with 0.05% TMS, 500 MHz); b) $^{31}P\{^{1}H\}$ NMR spectrum of 1,3-dicapryloyl-glycerol-2-phosphatidic acid (**DCG2PA**; $CDCl_3/CD_3OD$, 2:1 v/v, with 0.05% TMS, 202.4 MHz).

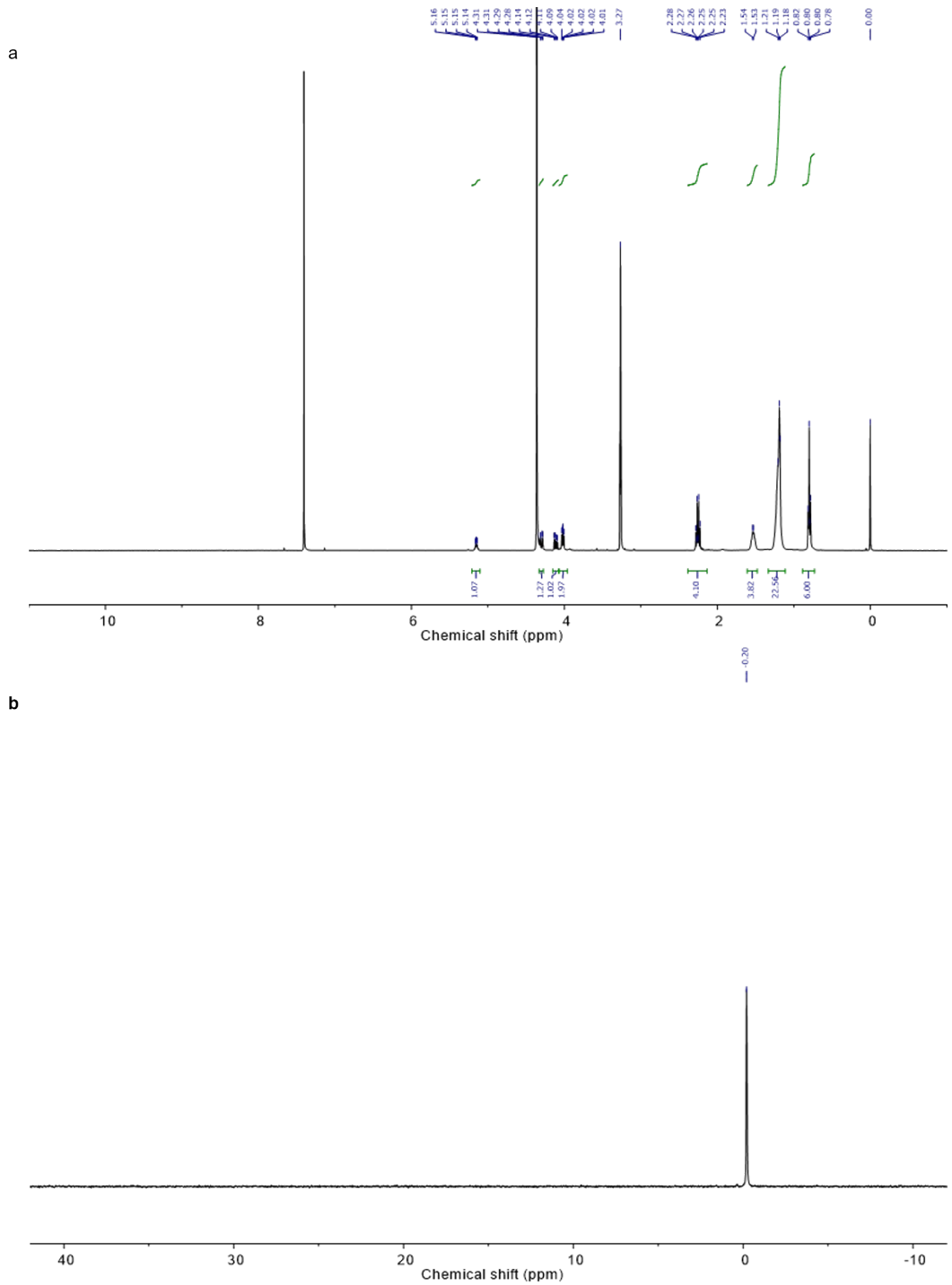


Figure S14. a) ^{1}H NMR spectrum of 2,3-didecanoyl-*rac*-glycerol-1-phosphatidic acid (**DDG1PA**; $CDCl_3/CD_3OD$, 2:1 v/v, with 0.05% TMS, 500 MHz); b) $^{31}P\{^{1}H\}$ NMR spectrum of 2,3-didecanoyl-*rac*-glycerol-1-phosphatidic acid (**DDG1PA**; $CDCl_3/CD_3OD$, 2:1 v/v, with 0.05% TMS, 202.4 MHz).

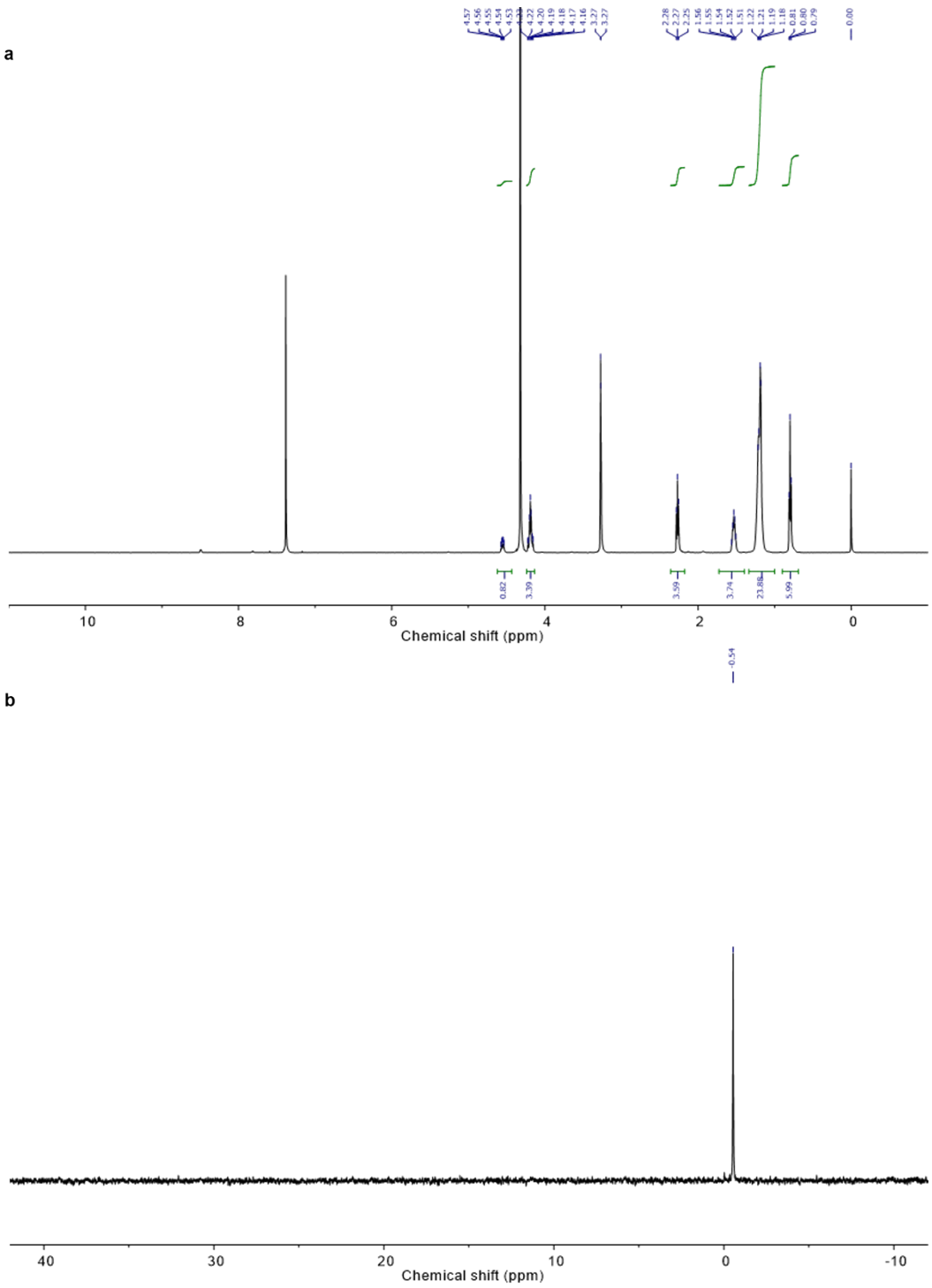
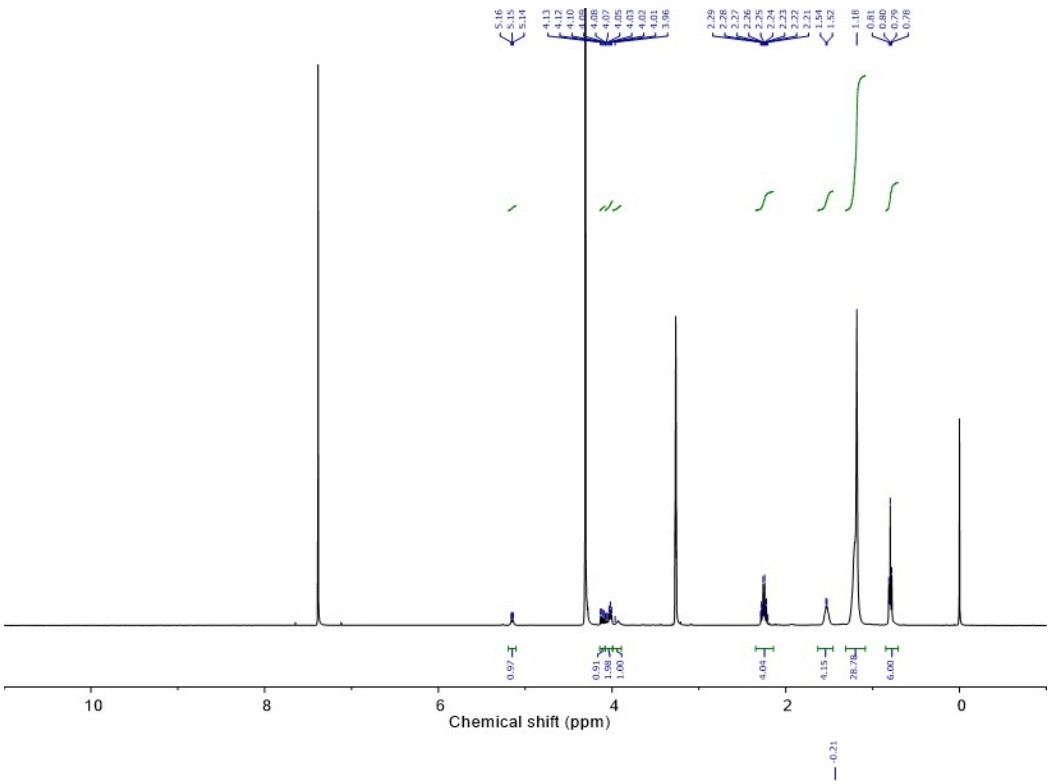


Figure S15. a) ^{1}H NMR spectrum of 1,3-didecanoyl-glycerol-2-phosphatidic acid (**DDG2PA**; $CDCl_3/CD_3OD$, 2:1 v/v, with 0.05% TMS, 500 MHz); b) $^{31}P\{^{1}H\}$ NMR spectrum of 1,3-didecanoyl-glycerol-2-phosphatidic acid (**DDG2PA**; $CDCl_3/CD_3OD$, 2:1 v/v, with 0.05% TMS, 202.4 MHz).



b

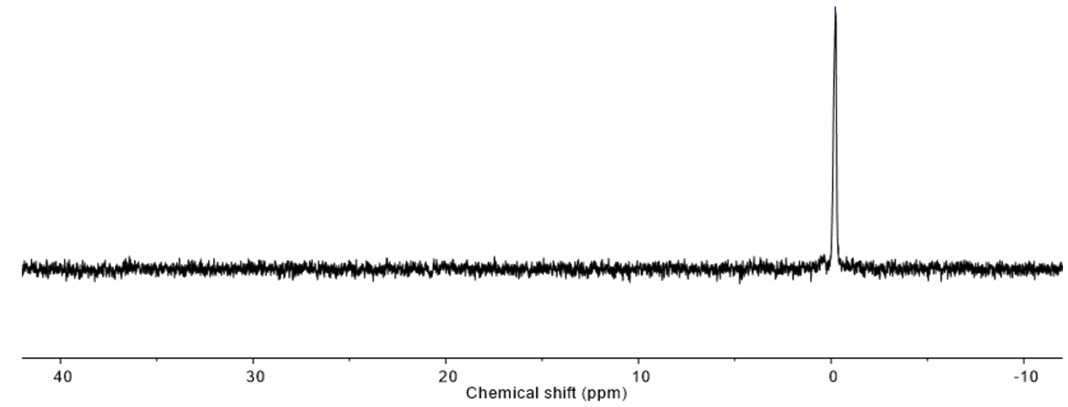


Figure S16. a) ^{1}H NMR spectrum of 2,3-dilauroyl-*rac*-glycerol-1-phosphatidic acid (**DLG1PA**; $CDCl_3/CD_3OD$, 2:1 v/v, with 0.05% TMS, 500 MHz); b) $^{31}P\{^{1}H\}$ NMR spectrum of 2,3-dilauroyl-*rac*-glycerol-1-phosphatidic acid (**DLG1PA**; $CDCl_3/CD_3OD$, 2:1 v/v, with 0.05% TMS, 202.4 MHz).

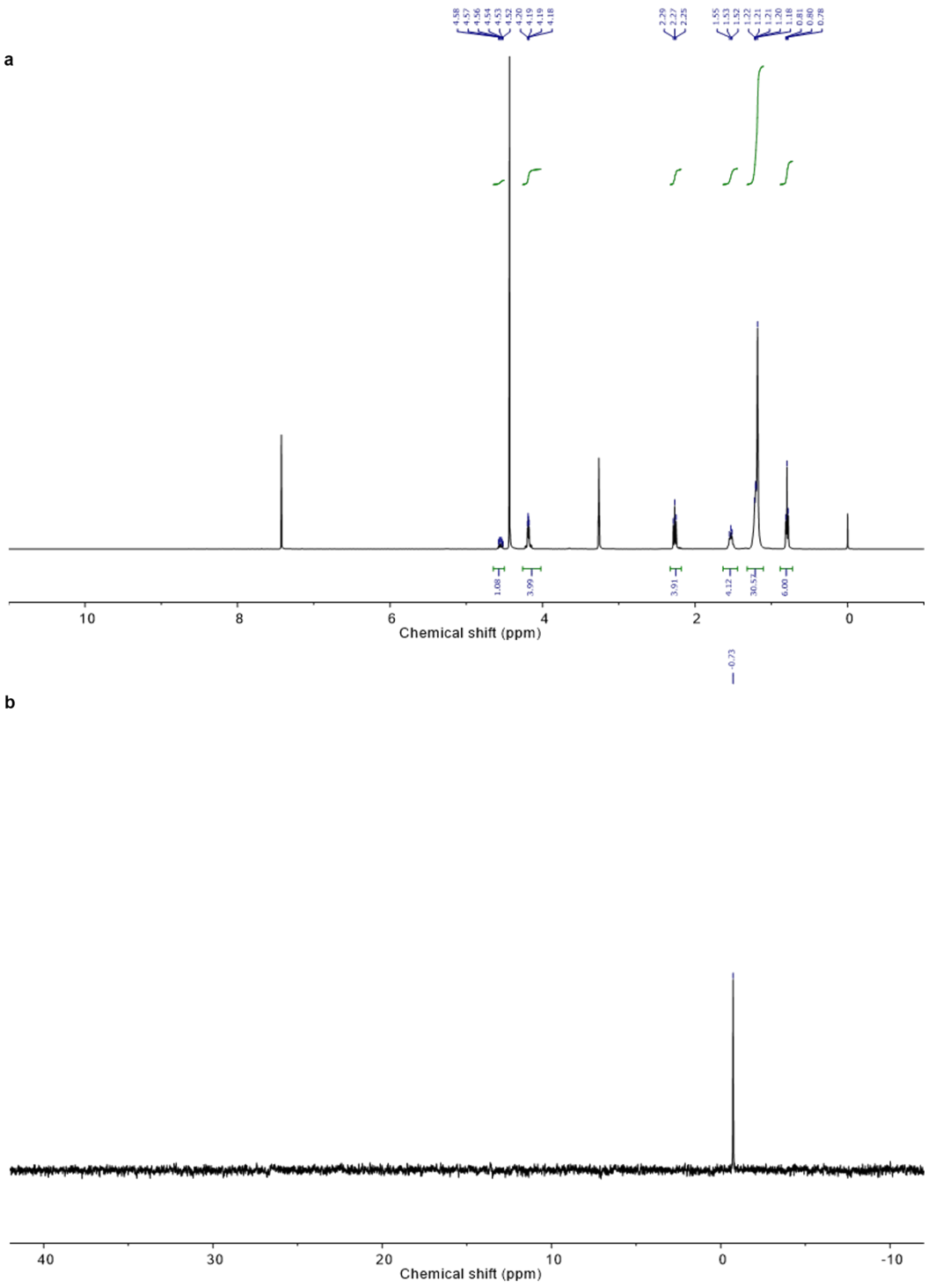


Figure S17. a) ^{1}H NMR spectrum of 1,3-dilauroyl-glycerol-2-phosphatidic acid (**DLG2PA**; $CDCl_3/CD_3OD$, 2:1 v/v, with 0.05% TMS, 500 MHz); b) $^{31}P\{^{1}H\}$ NMR spectrum of 1,3-dilauroyl-glycerol-2-phosphatidic acid (**DLG2PA**; $CDCl_3/CD_3OD$, 2:1 v/v, with 0.05% TMS, 202.4 MHz).

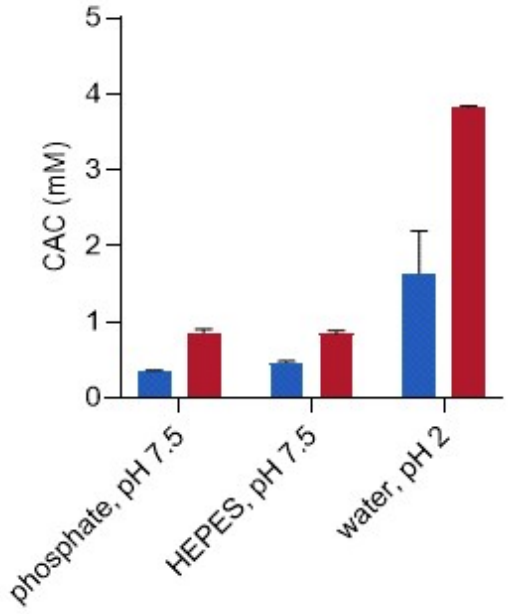


Figure S18. Graph showing the CACs of **DDG1PA** and **DDG2PA** in unbuffered water (pH 2.0, n = 2), phosphate buffer (50 mM, pH 7.5, n = 6), and HEPES buffer (50 mM, pH 7.5, n = 2). The absolute CAC of each lipid varies with the pH and buffer, but, under the same conditions, the CAC of **DDG1PA** is always lower than the CAC of **DDG2PA**. Data show the mean ± SEM.

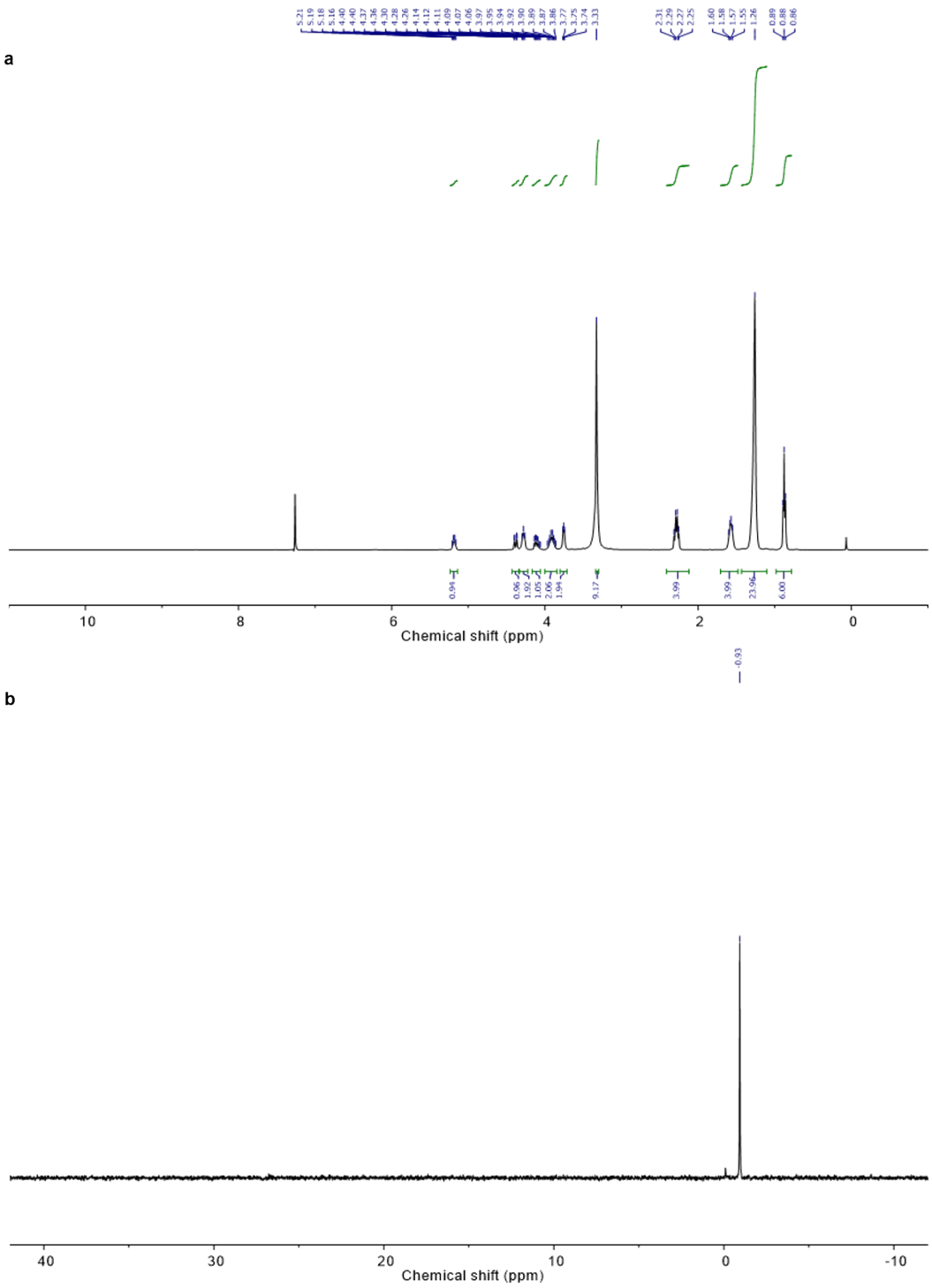


Figure S19. a) ^{1}H NMR spectrum of 2,3-didecanoyl-*rac*-glycerol-1-phosphocholine (**DDG1PC**; $CDCl_3$, 500 MHz); b) $^{31}P\{^{1}H\}$ NMR spectrum of 2,3-didecanoyl-*rac*-glycerol-1-phosphocholine (**DDG1PC**; $CDCl_3$, 202.4 MHz).

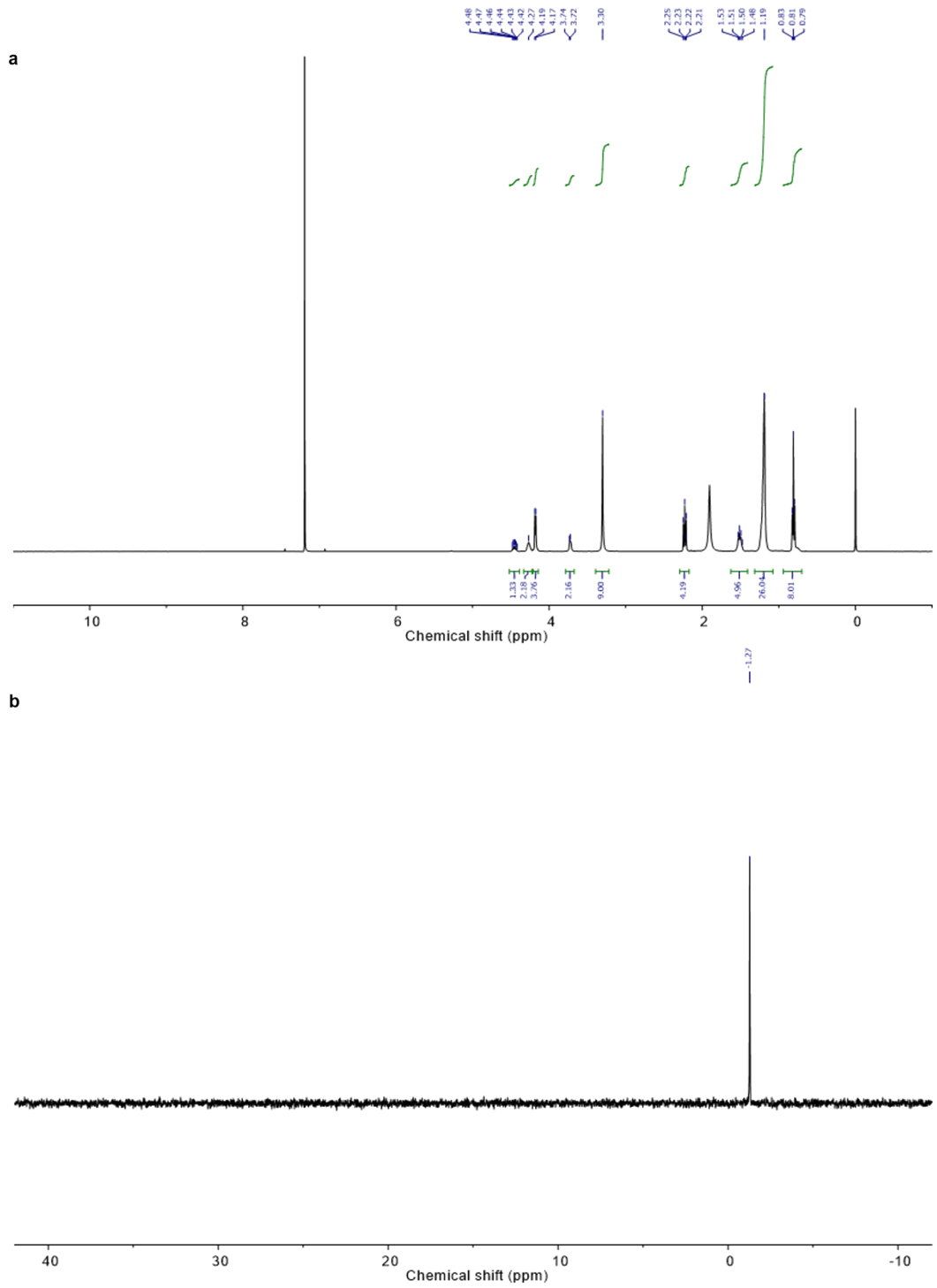


Figure S20. a) ^{1}H NMR spectrum of 1,3-didecanoyl-glycerol-2-phosphocholine (**DDG2PC**; $CDCl_3$, 500 MHz); b) $^{31}P\{^{1}H\}$ NMR spectrum of 1,3-didecanoyl-glycerol-2-phosphocholine (**DDG2PC**; $CDCl_3$, 500 MHz).

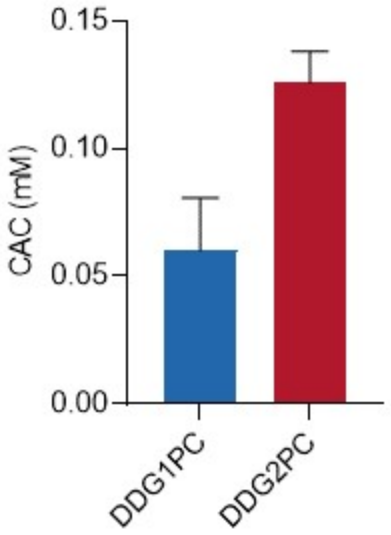


Figure S21. Graph showing the CACs of **DDG1PC** and **DDG2PC** in phosphate buffer (25 mM, pH 7.5). The CAC of **DDG1PC** is lower than the CAC of **DDG2PC**. Data show the mean ± SEM, *n* = 2.

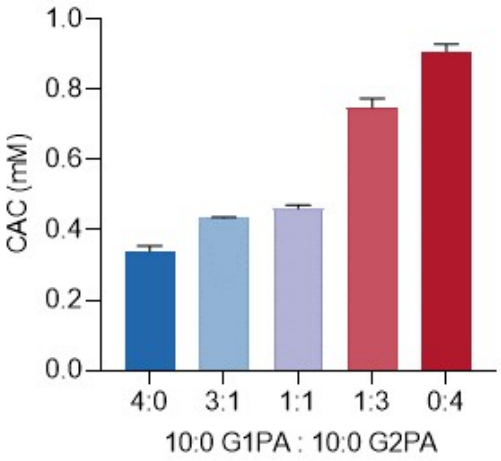


Figure S22. Graph showing the CACs of mixtures of **DDG1PA** and **DDG2PA** in different ratios. The CAC of each mixture is inversely proportional to the amount of **DDG1PA** present. These data show that **DDG1PA** stabilises liposomes composed of **DDG2PA**. Data show the mean ± SEM, *n* ≥ 2.

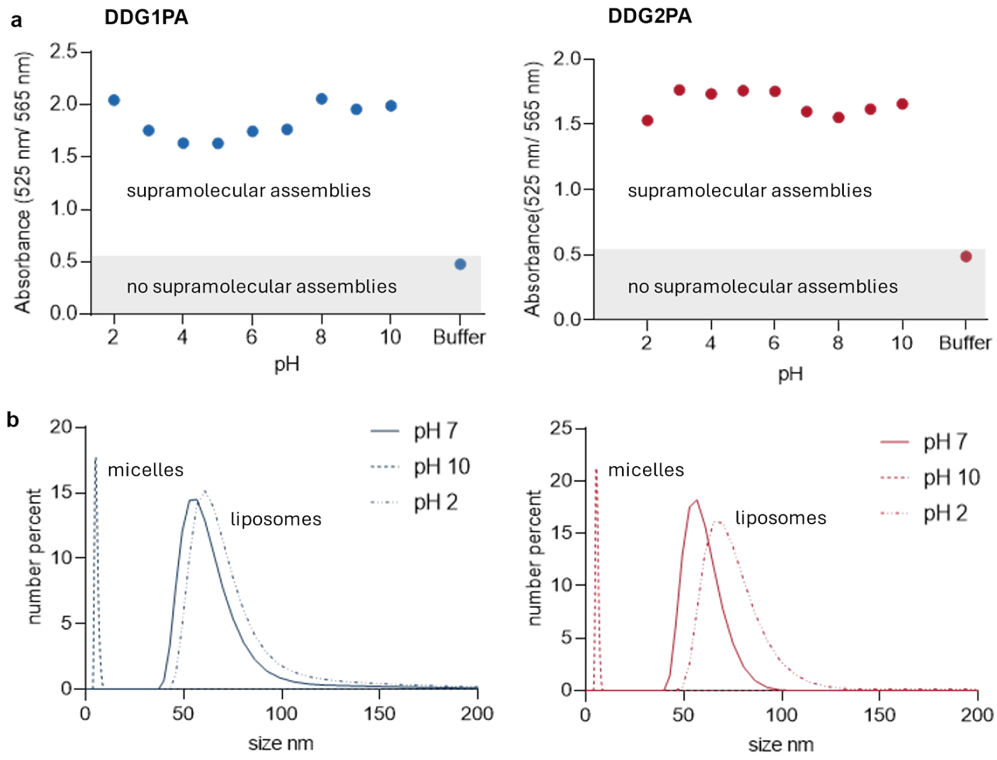


Figure S23. a) Graphs showing the merocyanine 540 absorbance ratio A_{565nm}/A_{525nm}, for dispersions of **DDG1PA** and **DDG2PA** (5 mM) vs pH in phosphate buffer (50 mM). **DDG1PA** and **DDG2PA** form supramolecular assemblies between pH 2 and 11. b) DLS profiles of **DDG1PA** and **DDG2PA** (5 mM) in phosphate buffer (50 mM) at pH 2.0, 7.5, and 10.0. Under these conditions, **DDG1PA** and **DDG2PA** form liposomes at pH 2.0 and 7.5 but form micelles at pH 10.0.

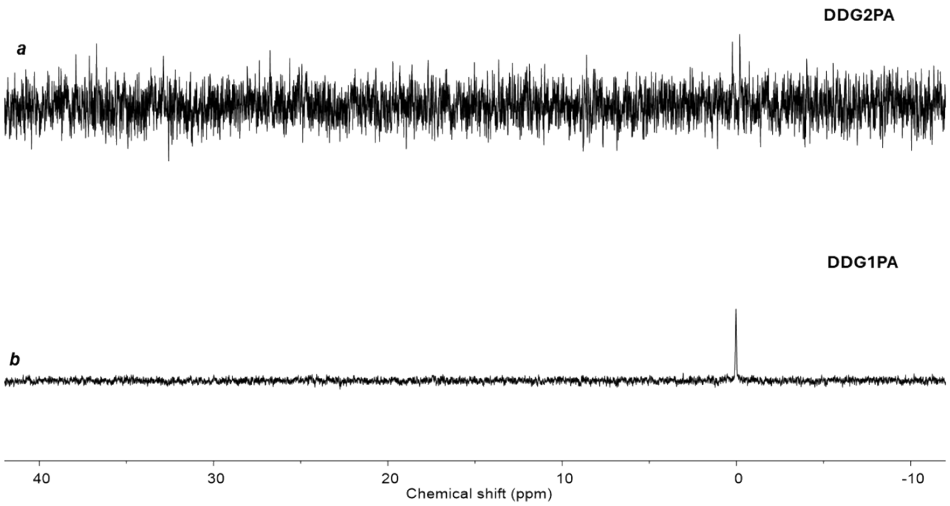


Figure S24. Stack of $^{31}P\{^{1}H\}$ NMR spectra showing a) the complete loss (precipitation) of **DDG2PA** from liposomes (5 mM) in acetate buffer (50 mM, pH 4.0) after incubating overnight at room temperature; and b) the retention (and relative stability) of **DDG1PA** in liposomes (5 mM) in acetate buffer (50 mM, pH 4.0) after incubating overnight at room temperature. Note, precipitated lipid was removed by filtration prior to acquiring the NMR spectra, as described above. For each NMR experiment, 250 μL of the filtrate was mixed with 250 μL of acetonitrile.

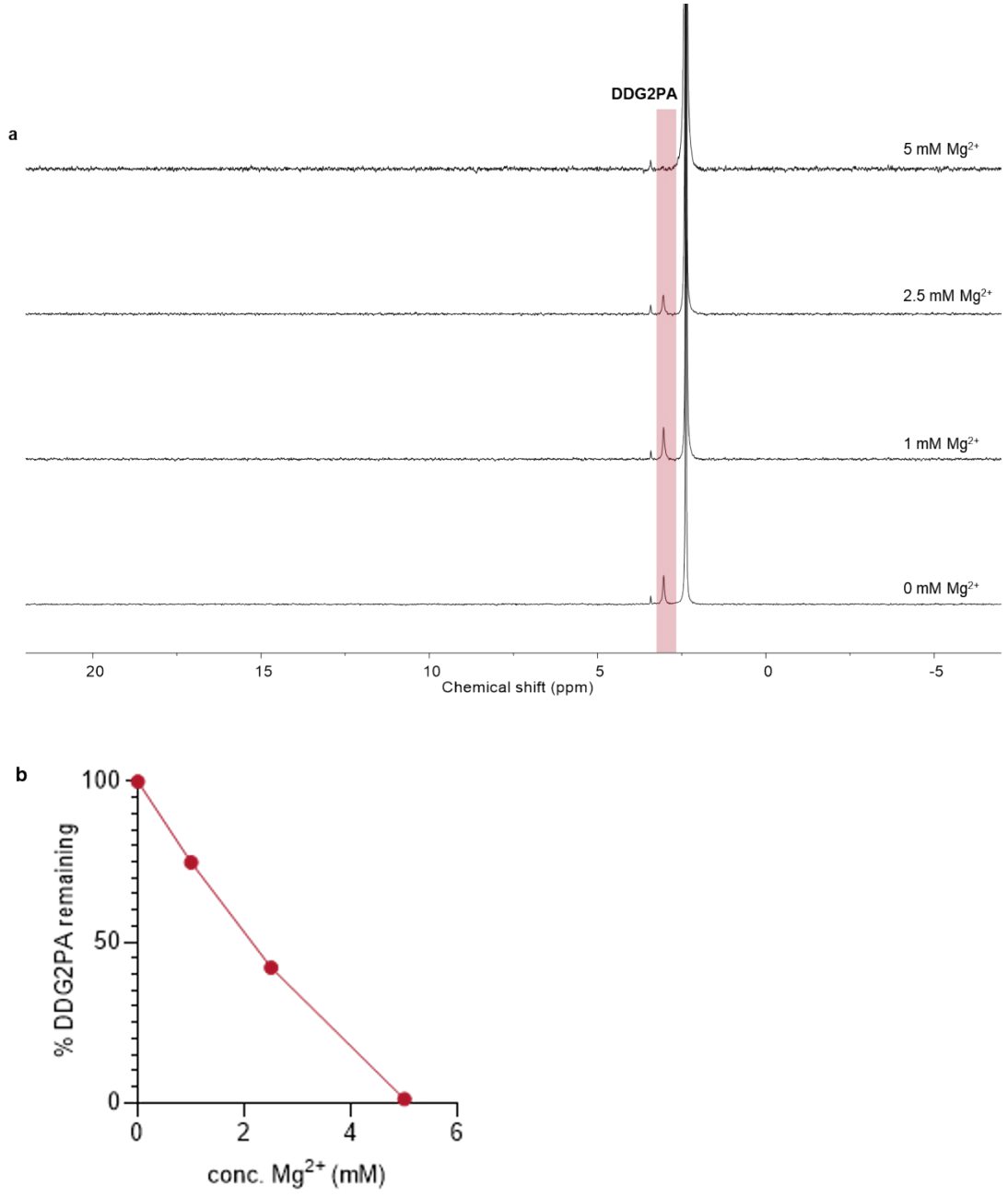
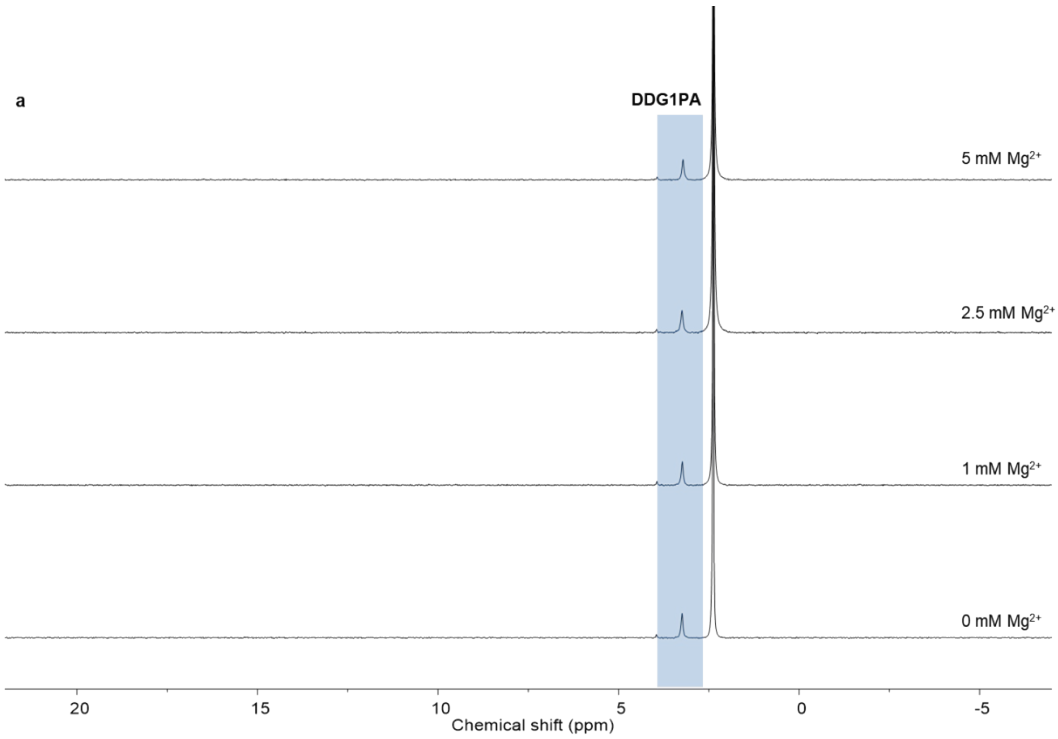


Figure S25. a) Stack of $^{31}P\{^{1}H\}$ NMR spectra showing the loss (precipitation) of phospholipid from liposomes made of **DDG2PA** (5 mM) in phosphate buffer (50 mM, pH 7.5) on addition of 1, 2.5, and 5 mM of $MgCl_2$. **DDG2PA** is particularly sensitive to Mg^{2+} and precipitation of the lipid begins after addition of only 1 mM $MgCl_2$. Precipitation of **DDG2PA** is complete after addition of 5 mM $MgCl_2$. Note, the precipitated lipid was removed by filtration prior to acquiring the NMR spectra, as described above. For each NMR experiment, 250 μL of the filtrate was mixed with 250 μL of a solution of 0.2 M bicine in water at pH 8.5 containing 0.2 M Triton X-100. b) Graph showing the % **DDG2PA** remaining in liposomes vs $MgCl_2$ concentration.



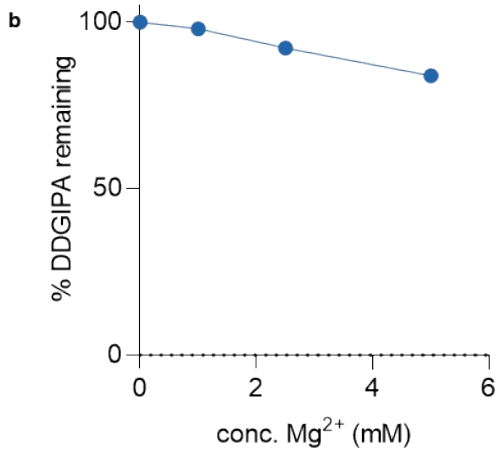


Figure S26. a) Stack of $^{31}P\{^{1}H\}$ NMR spectra showing the slight loss (precipitation) of phospholipid from liposomes made of **DDG1PA** (5 mM) in phosphate buffer (50 mM, pH 7.5) on addition of 1, 2.5, and 5 mM of $MgCl_2$. The bulk of the **DDG1PA** is retained in the liposomes and losses due to precipitation are minimal. Note, the trace quantity of precipitated lipid was removed by filtration prior to acquiring the NMR spectra, as described above. For each NMR experiment, 250 μL of the filtrate was mixed with 250 μL of a solution of 0.2 M bicine in water at pH 8.5* containing 0.2 M Triton X-100. {*This pH change was made because at pH 7.5 the signal corresponding to **DDG1PA** overlaps with the that of inorganic phosphate.} b) Graph showing the % **DDG1PA** remaining in liposomes vs $MgCl_2$ concentration.

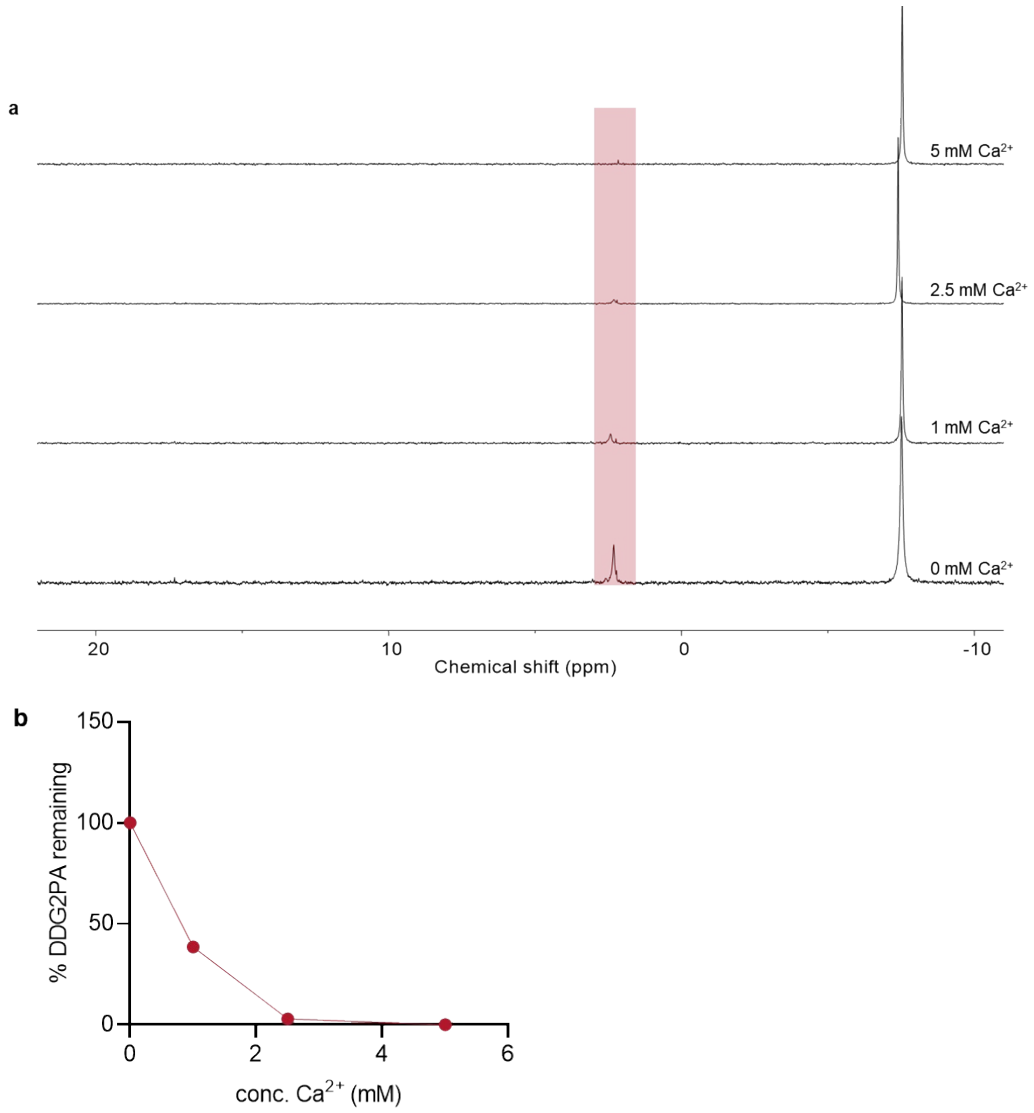


Figure S27. a) Stack of $^{31}P\{^{1}H\}$ NMR spectra showing the loss (precipitation) of phospholipid from liposomes made of **DDG2PA** (5 mM) in HEPES buffer (50 mM, pH 7.5) on addition of 1, 2.5, and 5 mM of $CaCl_2$ and incubating overnight at room temperature. **DDG2PA** is sensitive to Ca^{2+} and precipitation of the lipid begins after addition of only 1 mM $CaCl_2$. Precipitation of **DDG2PA** is complete after addition of 5 mM $CaCl_2$. Note, the precipitated lipid was removed by filtration prior to acquiring the NMR spectra, as described above. For each NMR experiment, 250 μL of the filtrate was mixed with 250 μL of a solution of 0.2 M Triton X-100 in water/D_2O (1:1, v/v) and 10 μL of a solution of 250 mM sodium pyrophosphate in water.

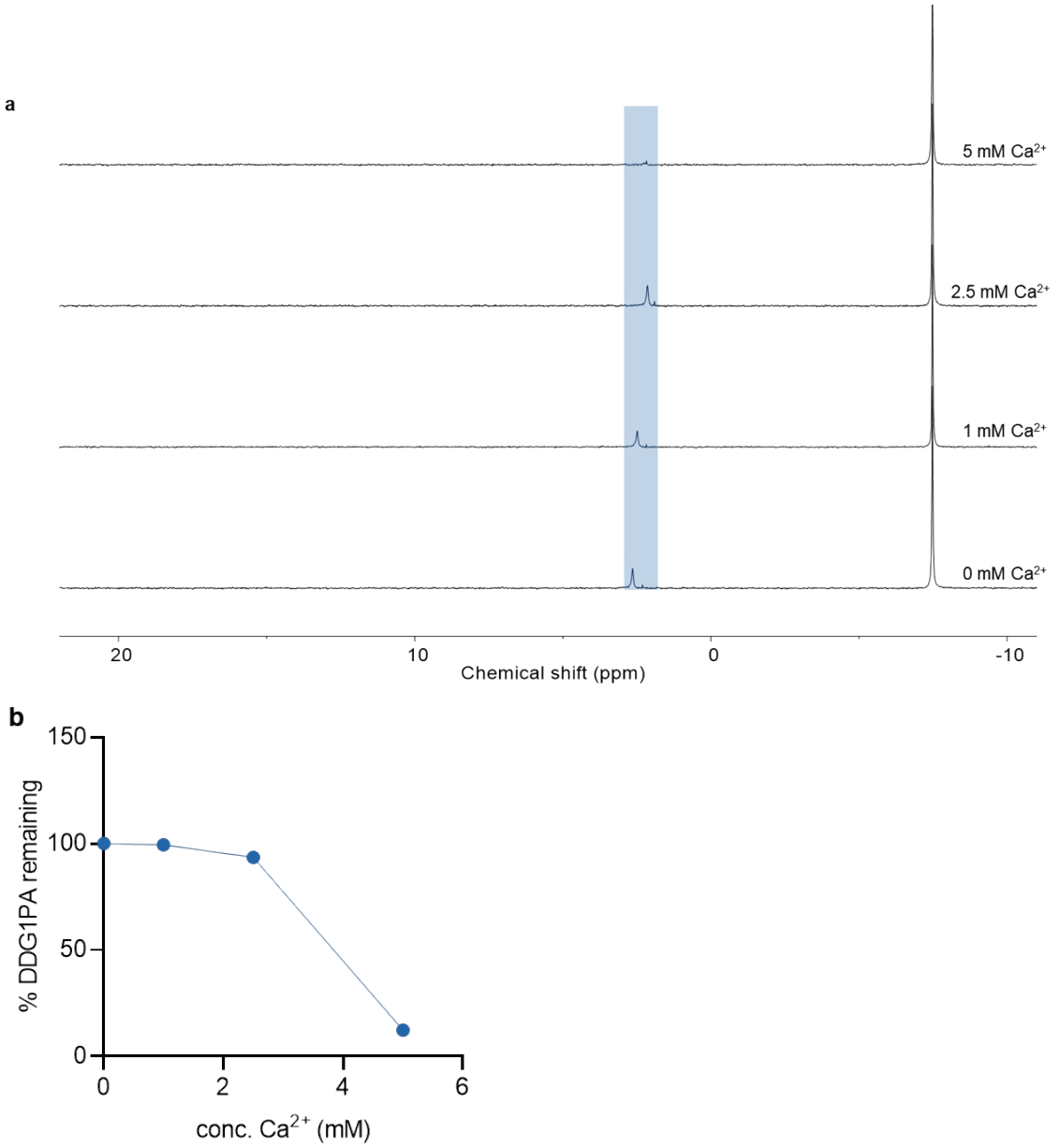


Figure S28. Stack of $^{31}P\{^{1}H\}$ NMR spectra showing the slight loss (precipitation) of phospholipid from liposomes made of **DDG1PA** (5 mM) in HEPES buffer (50 mM, pH 7.5) on addition of 1, 2.5, and 5 mM of $CaCl_2$ and incubating overnight at room temperature. The bulk of the **DDG1PA** is retained in the liposomes and losses due to precipitation are minimal. Note, the trace quantity of precipitated lipid was removed by filtration prior to acquiring the NMR spectra, as described above. For each NMR experiment, 250 μL of the filtrate was mixed with 250 μL 0.2 M Triton X-100 in water/D_2O (1:1, v/v) and 10 μL of a solution of 250 mM sodium pyrophosphate in water.

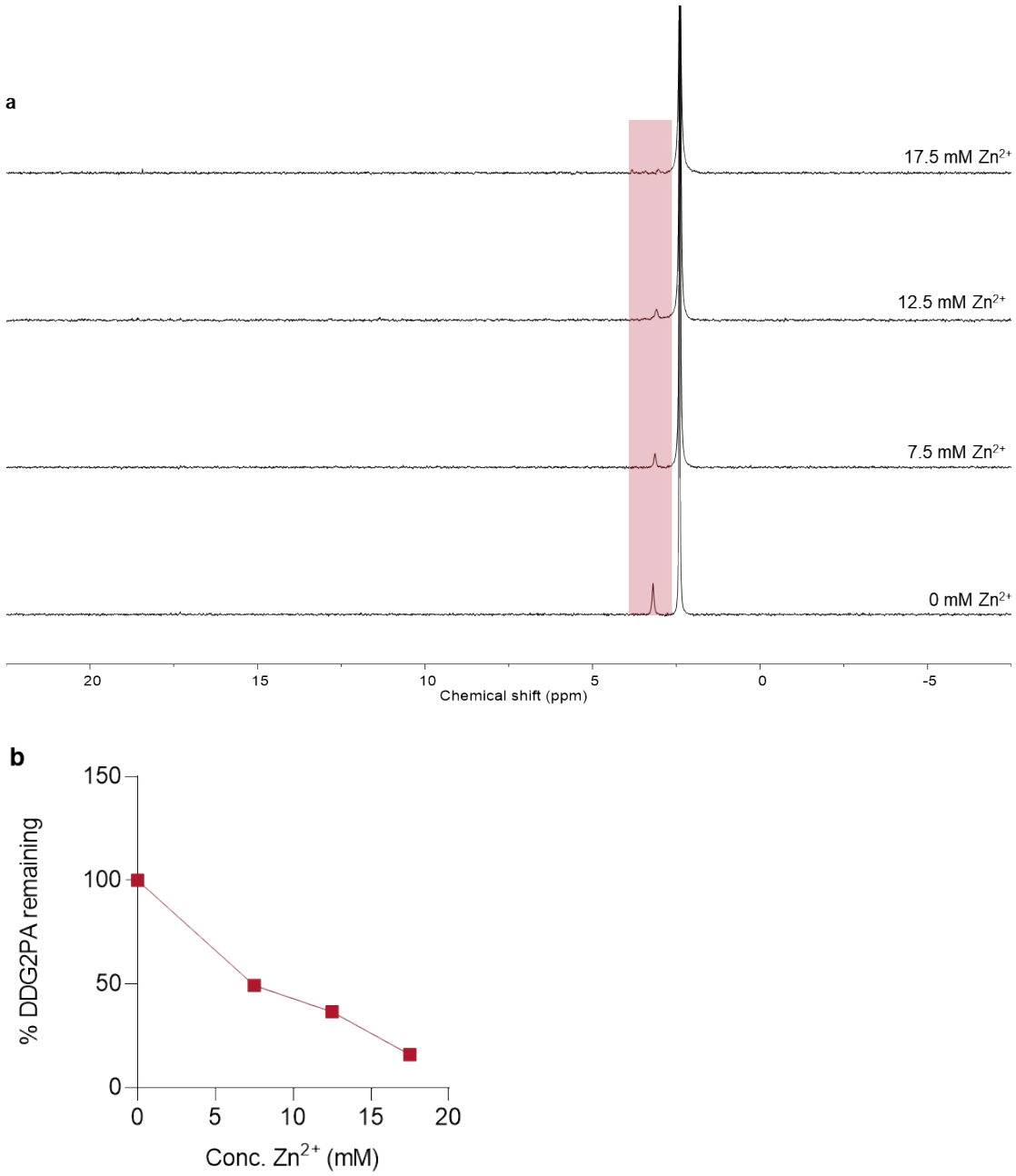


Figure S25. a) Stack of $^{31}P\{^{1}H\}$ NMR spectra showing the loss (precipitation) of phospholipid from liposomes made of **DDG2PA** (5 mM) in phosphate buffer (50 mM, pH 7.5) on addition of 0, 7.5, 12.5, and 17.5 mM of $ZnCl_2$. **DDG2PA** is particularly sensitive to Zn^{2+} and precipitation of the lipid begins after addition of only 7.5 mM $ZnCl_2$. The bulk of **DDG2PA** precipitated after addition of 17.5 mM $ZnCl_2$. Note, the precipitated lipid was removed by filtration prior to acquiring the NMR spectra, as described above. For each NMR experiment, 250 μL of the filtrate was mixed with 250 μL of a solution of 0.2 M bicine in water at pH 8.5 containing 0.2 M Triton X-100. b) Graph showing the % **DDG2PA** remaining in liposomes vs $ZnCl_2$ concentration.

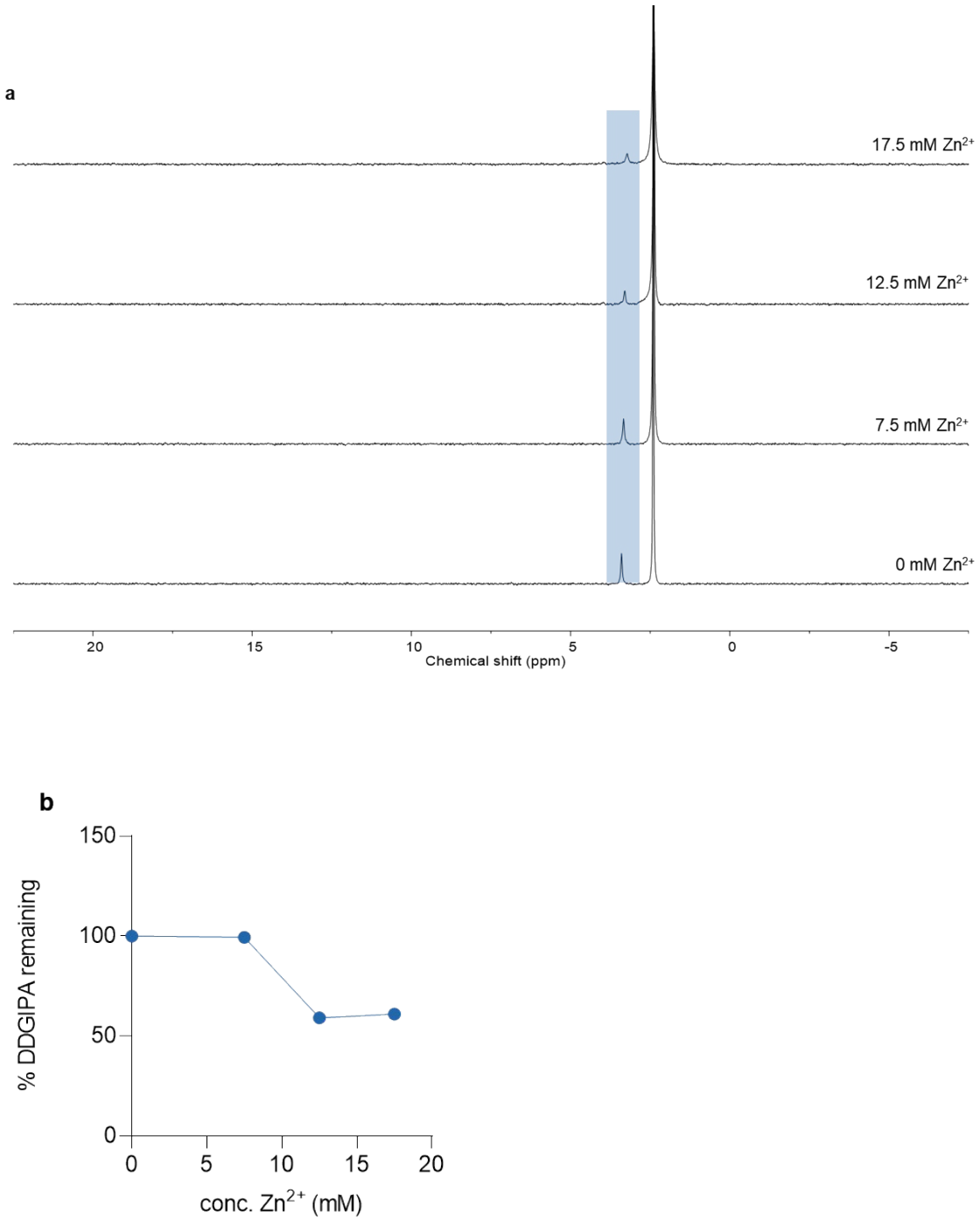


Figure S28. Stack of $^{31}P\{^{1}H\}$ NMR spectra showing the slight loss (precipitation) of phospholipid from liposomes made of **DDG1PA** (5 mM) in phosphate buffer (50 mM, pH 7.5) on addition of 0, 7.5, 12.5, and 17.5 mM of $ZnCl_2$. The bulk of the **DDG1PA** is retained in the liposomes and losses due to precipitation are minimal. Note, the precipitated lipid was removed by filtration prior to acquiring the NMR spectra, as described above. For each NMR experiment, 250 μL of the filtrate was mixed with 250 μL of a solution of 0.2 M bicine in water at pH 8.5 containing 0.2 M Triton X-100. b) Graph showing the % **DDG1PA** remaining in liposomes vs $ZnCl_2$ concentration.

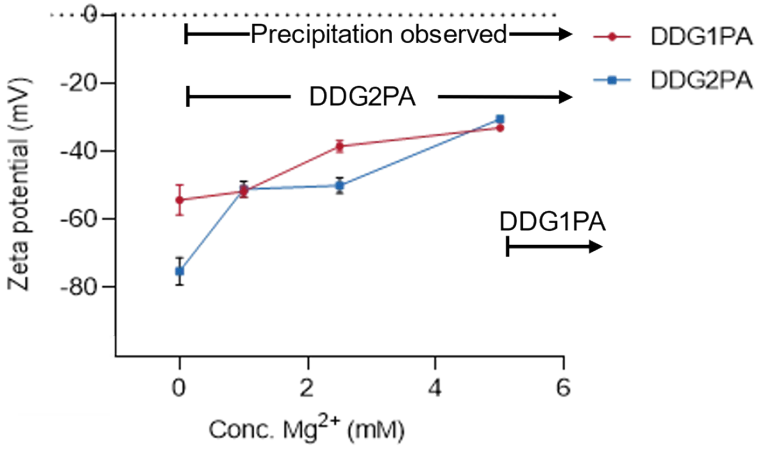


Figure S29. Graph showing the membrane Zeta (ζ)-potential of liposomes composed of **DDG1PA** or **DDG2PA** (5 mM) in HEPES buffer (50 mM, pH 7.5) vs $MgCl_2$ concentration. Arrows indicate the minimum $MgCl_2$ concentration at which precipitation of each lipid is observed.

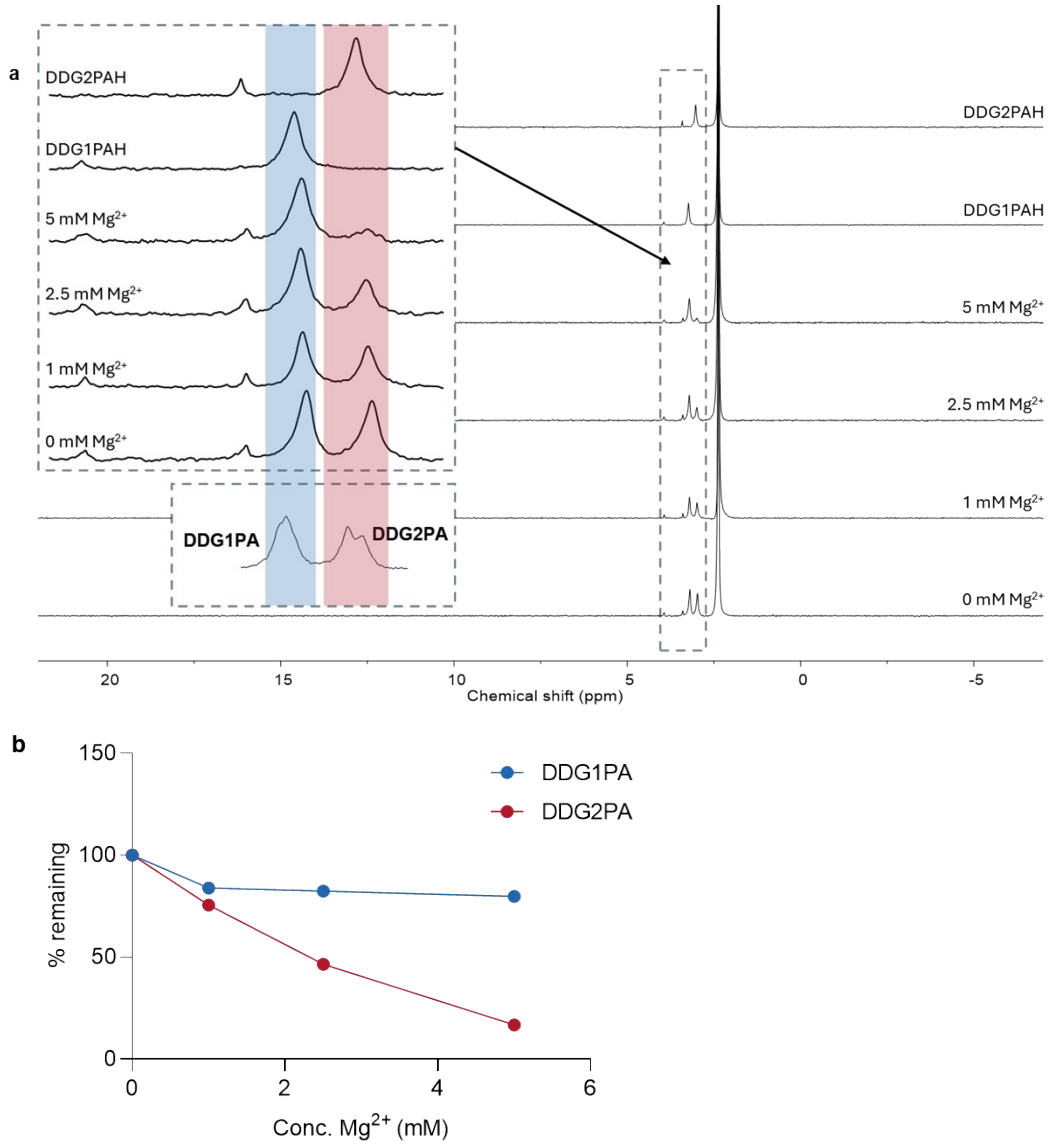


Figure S30. Stack of $^{31}P\{^{1}H\}$ NMR spectra showing the change in phospholipid composition of liposomes made of a 1:1 mixture of **DDG1PA** and **DDG2PA** (5 mM each) in phosphate buffer (50 mM, pH 7.5) on addition of 0, 1, 2.5, and 5 mM of $MgCl_2$. $^{31}P\{^{1}H\}$ NMR spectra of **DDG1PA** and **DDG2PA** in phosphate buffer (pH 7.5) are shown for comparison. **DDG2PA** precipitates on addition of $MgCl_2$ while **DDG1PA** is retained in the liposomes. Note, precipitated lipid was removed by filtration prior to acquiring the NMR spectra, as described above. For each NMR experiment, 250 μL of the filtrate was mixed with 250 μL of a solution of 0.2 M bicine in water at pH 8.5* containing 0.2 M Triton X-100. {*This pH change was made because at pH 7.5 the signal corresponding to **DDG1PA** overlaps with the that of inorganic phosphate.} The lower insert shows the ^{1}H-coupled ^{31}P NMR spectrum of the initial mixture. The upper insert shows an expansion of the highlighted region of the main stack of $^{31}P\{^{1}H\}$ NMR spectra.

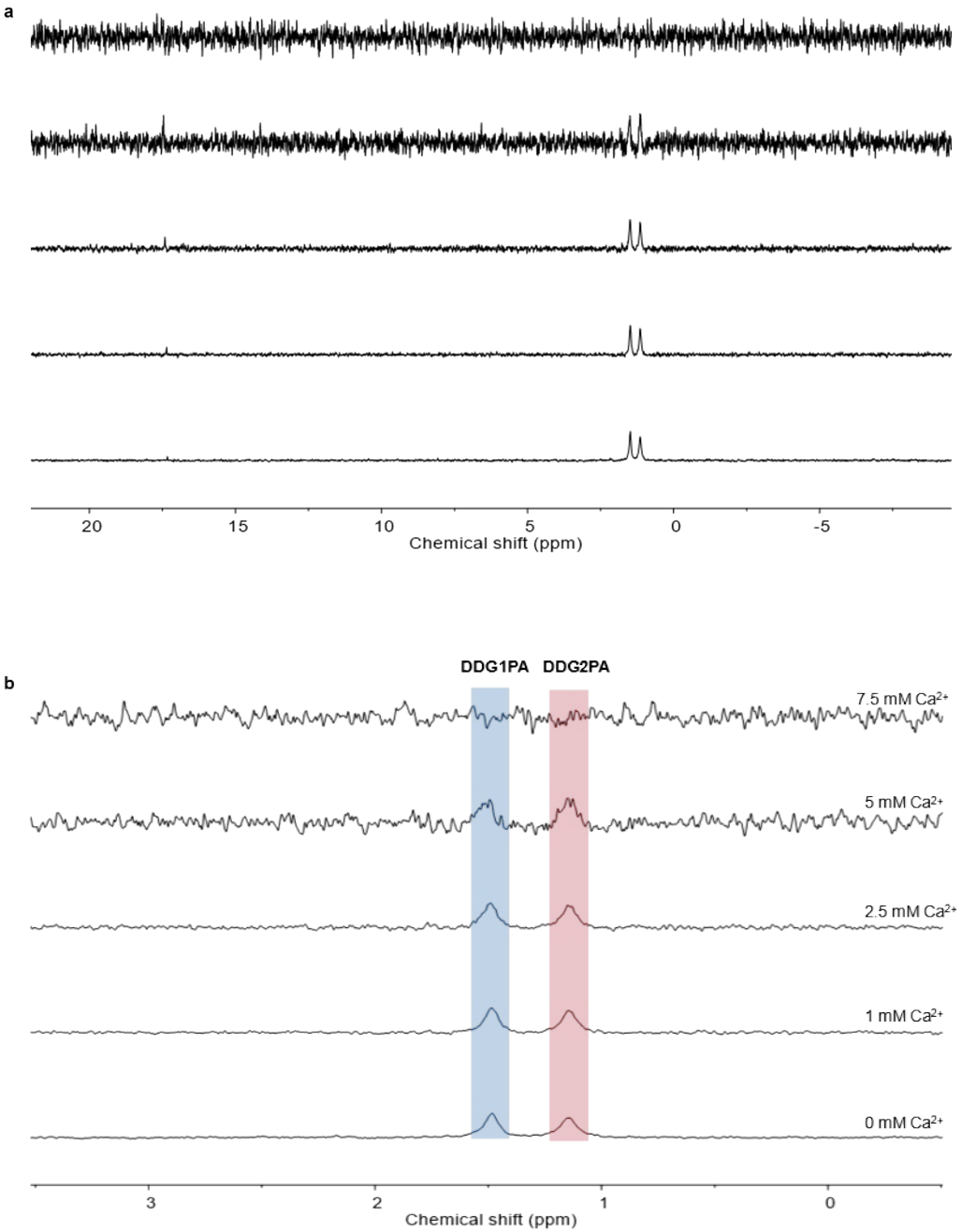


Figure S31. a) Stack of $^{31}P\{^{1}H\}$ NMR spectra showing the change in phospholipid composition of liposomes made of a 1:1 mixture of **DDG1PA** and **DDG2PA** (5 mM each) in HEPES buffer (50 mM, pH 7.5) on addition of 0, 1, 2.5, and 5 mM of $CaCl_2$. b) Expansion of the +3.0 to 0 ppm region of the stack of $^{31}P\{^{1}H\}$ NMR spectra shown in a). Both **DDG2PA** and **DDG1PA** precipitate on addition of $CaCl_2$ to the liposomes and incubating overnight at room temperature. Note, precipitated lipid was removed by filtration prior to acquiring the NMR spectra, as described above. For each NMR experiment, 250 μL of the filtrate was mixed with 250 μL of a solution of 0.2 M Triton X-100 in water/D_2O (1:1, v/v).

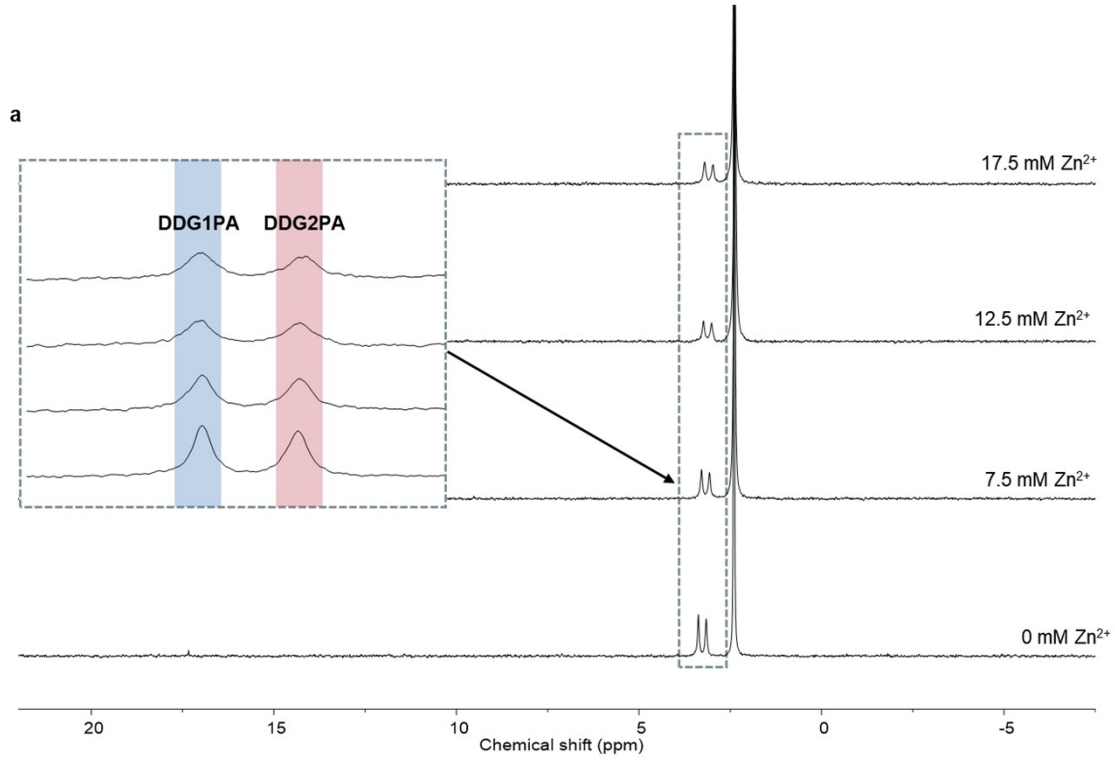


Figure S30. Stack of $^{31}P\{^{1}H\}$ NMR spectra showing the change in phospholipid composition of liposomes made of a 1:1 mixture of **DDG1PA** and **DDG2PA** (5 mM each) in phosphate buffer (50 mM, pH 7.5) on addition of 0, 7.5, 12.5, and 17.5 mM of $ZnCl_2$. Both **DDG2PA** and **DDG1PA** precipitate on addition of $ZnCl_2$ to the liposomes Note, precipitated lipid was removed by filtration prior to acquiring the NMR spectra, as described above. For each NMR experiment, 250 μL of the filtrate was mixed with 250 μL of a solution of 0.2 M bicine in water at pH 8.5* containing 0.2 M Triton X-100. {*This pH change was made because at pH 7.5 the signal corresponding to **DDG1PA** overlaps with the that of inorganic phosphate.}. The insert shows an expansion of the highlighted region of the main stack of $^{31}P\{^{1}H\}$ NMR spectra.

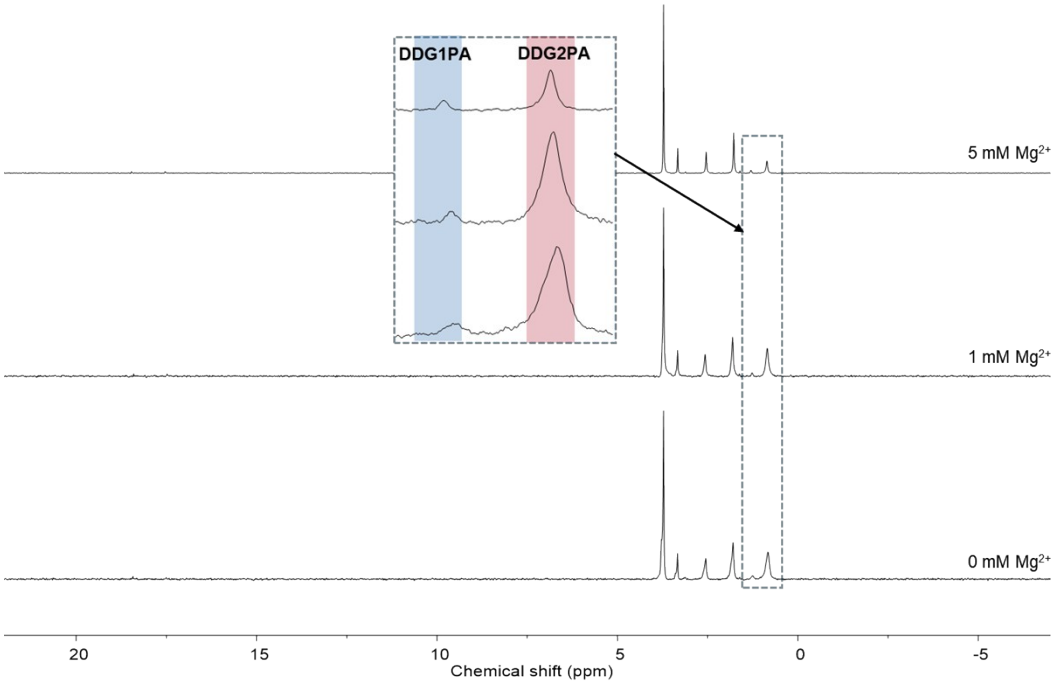


Figure S32. Stack of $^{31}P\{^{1}H\}$ NMR spectra showing the change in phospholipid composition on addition of $MgCl_2$ to liposomes prepared from a lyophilised mixture of **G1PAs** and **G2PAs** obtained by prebiotic synthesis (acylation of a 1:1 mixture of **G1P** and **G2P** (5 mM each) with *N*-decanoyl imidazole (NDI; 100 mM) in acetonitrile/water (1:1, v/v)). The imidazole released during the reaction served as the buffer in these experiments and the pH was adjusted to 7.0 before titration with $MgCl_2$. **DDG2PA** is progressively lost (precipitates) from the liposomes on addition of increasing quantities of $MgCl_2$. By contrast, **DDG1PA** is retained in the liposomes. Note, precipitated lipid was removed by filtration prior to acquiring the NMR spectra, as described above. For each NMR experiment, 250 μL of the filtrate was mixed with 250 μL of a solution of 0.2 M Triton X-100 in water/D_2O (1:1, v/v). The lower insert shows the ^{1}H-coupled $^{31}P\{^{1}H\}$ NMR spectrum of the initial mixture. The upper insert shows an expansion of the highlighted region of the stack of ^{31}P NMR spectra.

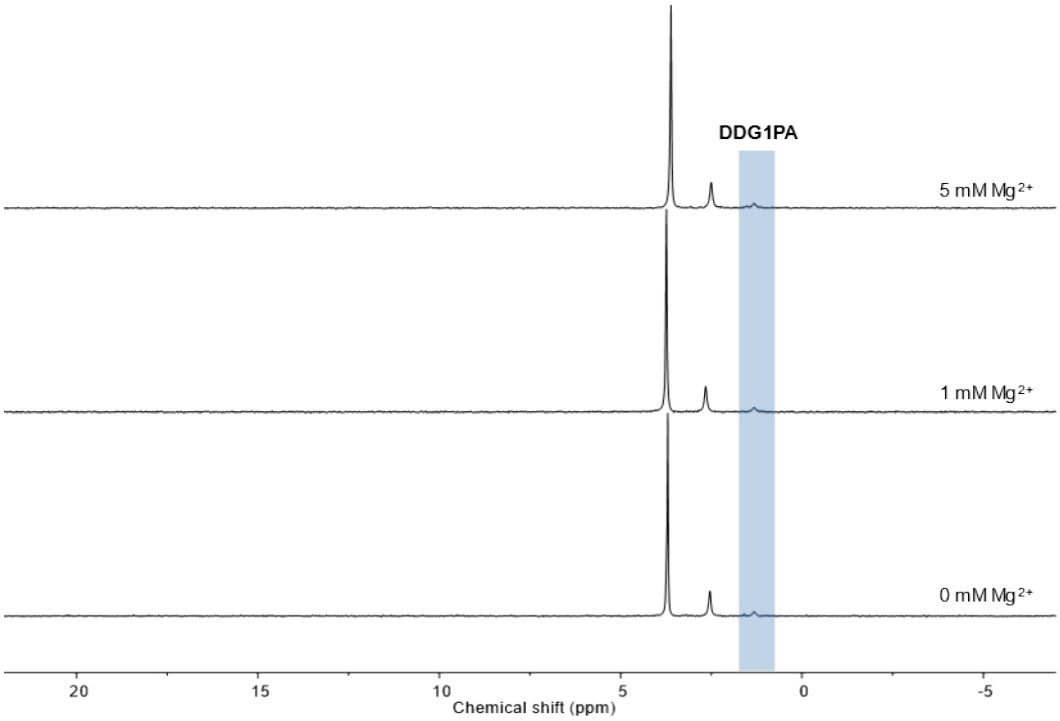


Figure S33. Stack of $^{31}P\{^{1}H\}$ NMR spectra showing the minimal change in phospholipid composition on addition of $MgCl_2$ to liposomes (total phospholipid concentration 5 mM) prepared from a mixture of **G1PAs** obtained by prebiotic synthesis (acylation of **G1P** (10 mM) with *N*-decanoyl imidazole (NDI; 100 mM) in acetonitrile/water (1:1, v/v)). The imidazole released during the reaction served as the buffer in these experiments and the pH was adjusted to 7.0 before titration with $MgCl_2$. **DDG1PA** is retained in the liposomes. Note, any trace of precipitated lipid was removed by filtration prior to acquiring the NMR spectra, as described above. For each NMR experiment, 250 μL of the filtrate was mixed with 250 μL of a solution of 0.2 M Triton X-100 in water/D_2O (1:1, v/v).

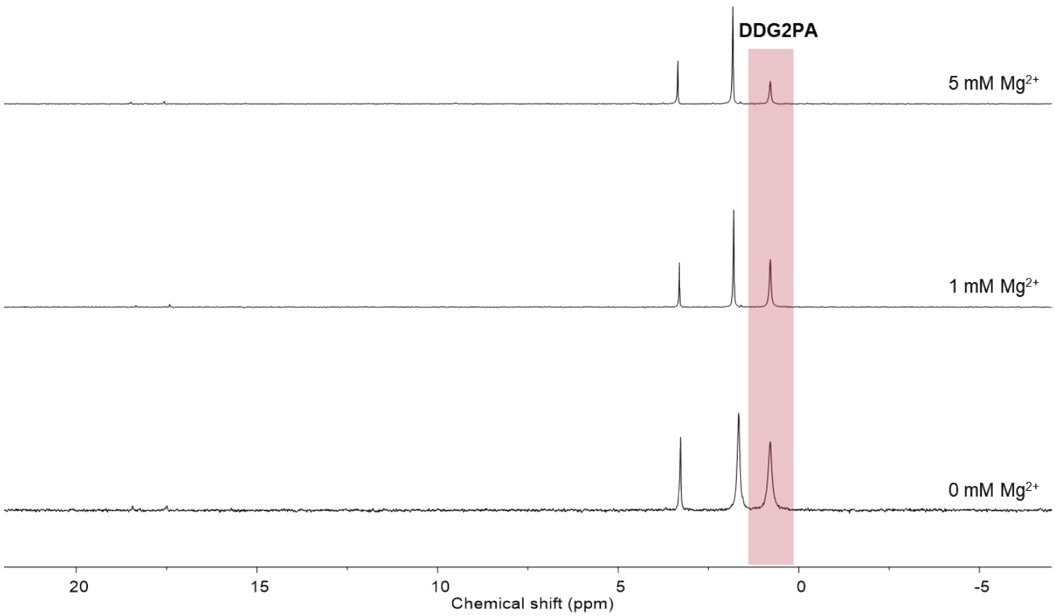


Figure S34. Stack of $^{31}P\{^{1}H\}$ NMR spectra showing the change in phospholipid composition on addition of $MgCl_2$ to liposomes (total phospholipid concentration 5 mM) prepared from a mixture of **G2PAs** obtained by prebiotic synthesis (acylation of **G2P** (10 mM) with *N*-decanoyl imidazole (NDI; 100 mM) in acetonitrile/water (1:1, v/v)). The imidazole released during the reaction served as the buffer in these experiments and the pH was adjusted to 7.0 before titration with $MgCl_2$. **DDG2PA** is progressively lost (precipitated) from the liposomes on addition of increasing quantities of $MgCl_2$. Note, precipitated lipid was removed by filtration prior to acquiring the NMR spectra, as described above. For each NMR experiment, 250 μL of the filtrate was mixed with 250 μL of a solution of 0.2 M Triton X-100 in water/D_2O (1:1, v/v).