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Supporting Information for

Monolayer Properties of 1,3-Diamidophospholipids

lllya A. Fedotenko, Cristina Stefaniu, Gerald Brezesinski,* Andreas Zumbuehl*

General Remarks

Starting compounds and solvents were purchased from Sigma-Aldrich/Fluka or Acros and were used without further purification. Pad-PE-Pad and it's homologs were synthesized according to published protocols.[1]

Column chromatographic separation was carried out using 40-63 and 63-200 μm silica gels and 63-200 μm activated basic aluminium oxide. TLC plates were developed either with potassium permanganate mixture (1 g of $KMnO_4$, 2 g of Na_2CO_3, 100 mL of H_2O) or ethanolic solution of phosphomolybdic acid. ^1H,^{13}C and ^{31}P NMR spectra were recorded (as indicated) on either a Bruker 300 MHz, 400 MHz or 500 MHz spectrometer and are reported as chemical shifts (δ) in ppm relative to TMS (δ = 0). Spin multiplicities are reported as a singlet (s) or triplet (t) with coupling constants (J) given in Hz, or a multiplet (m). ESI-MS for the characterization of new compounds was performed on an ESI API 150EX and are reported as mass-per-charge ratio *m/z*. IR spectra were recorded on a Perkin Elmer Spectrum One FT-IR spectrometer (ATR, Golden Gate). Melting points are uncorrected.

Normally the obtained amidophosphocholines, even after the first purification, contain a small amount of an impurity with a singlet peak in ^1H NMR at 3.7 ppm. To remove the impurity, the phospholipids (20-50 mg) were purified by gel filtration chromatography (Sephadex-LH20) using a 1 meter long column, d=1.5 cm and a mobile phase 75% CH_2Cl_2, 22% MeOH, 3% aq. NH_4OH (25%). Yield 75-95%. We acknowledge Dr. Daniel Varon Silva for pointing out this procedure to us.

Cryo-TEM (transmission electron microscopy) was carried out for LUVET100 using a Tecnai F20 TEM (FEI, USA) at the Electron Microscopy Center, ETH Zurich.

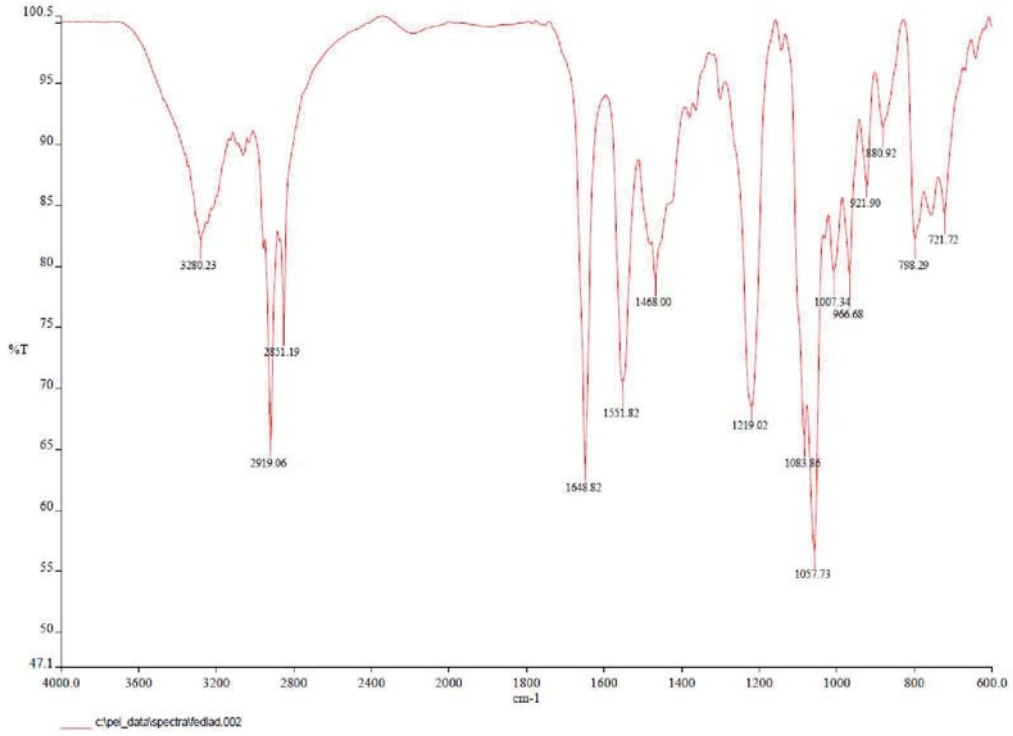
Production of vesicles

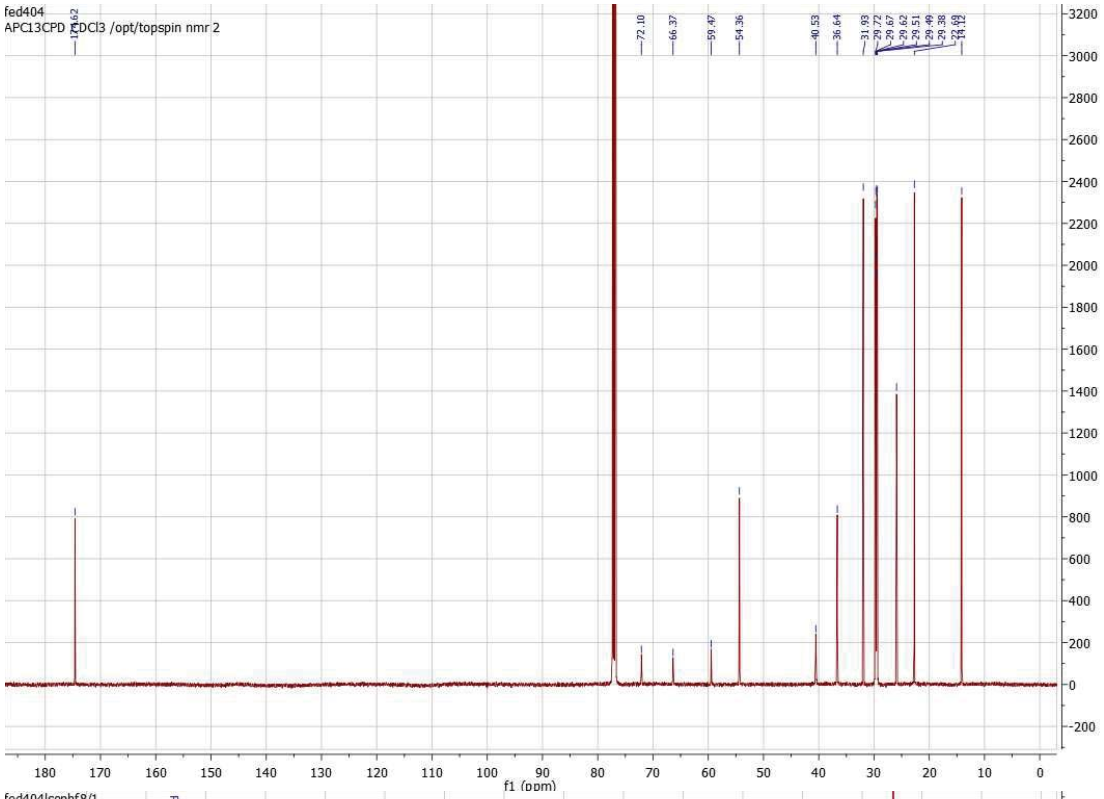
Preparation of vesicles

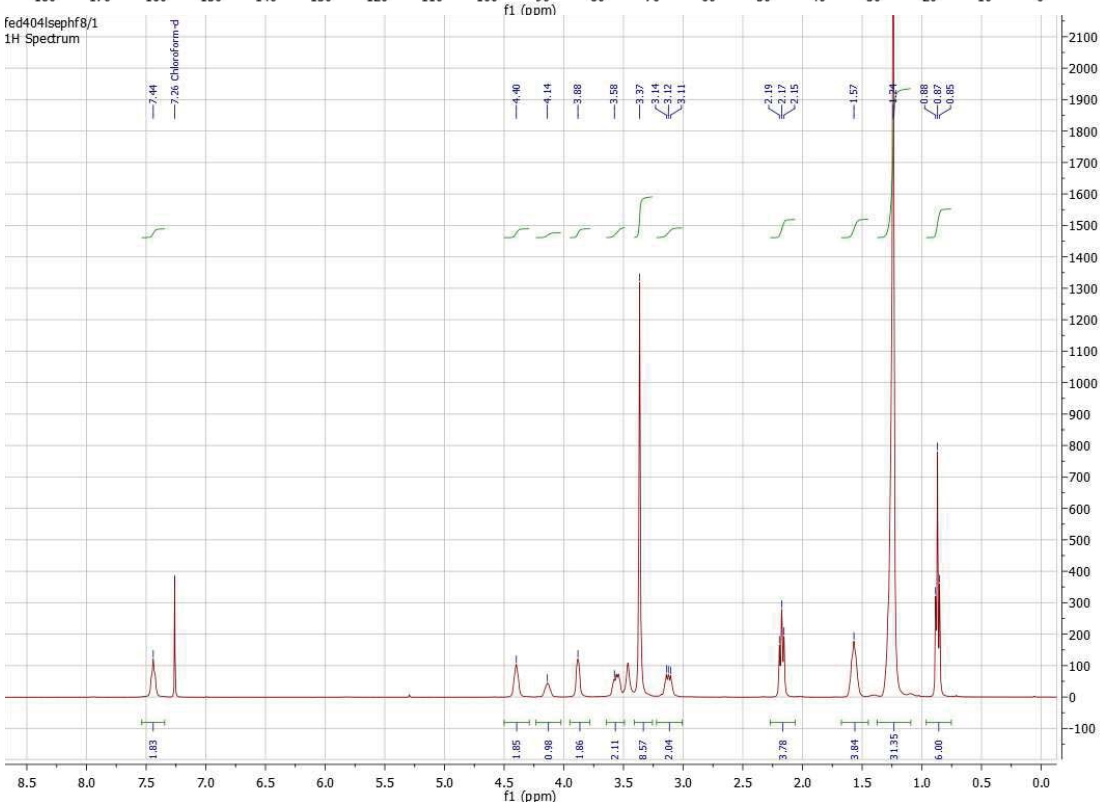
All compounds are purchased from Sigma-Aldrich or Avanti Polar Lipids and used without further purification unless otherwise stated. Liposome formulation is based on the techniques described in: Olson et al.[2]

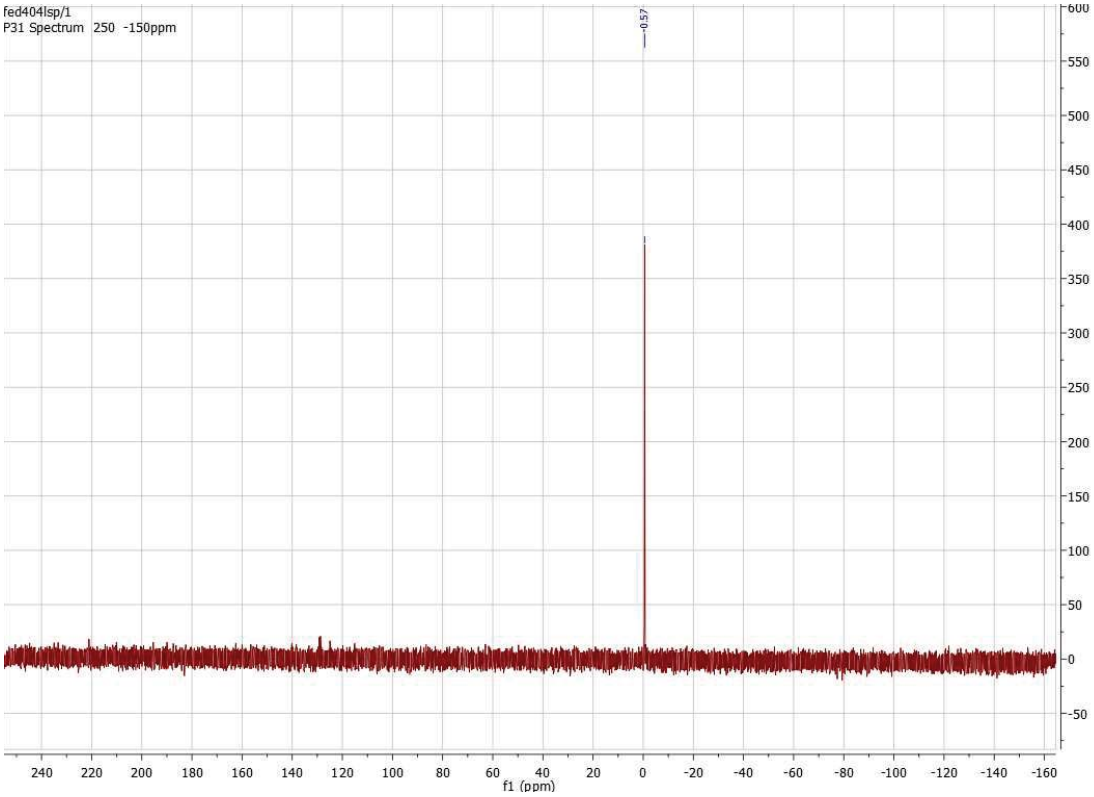
Large unilamellar vesicles by extrusion technique (LUVET100) were prepared as follows: 30 μmol Sad-PC-Sad (**4**) was weighed into a 25 mL round bottomed flask and dissolved in 1 mL chloroform. After evaporation to dryness, the film was dried for 12 h under high vacuum. 1 mL of buffer (107 mM NaCl, 10 mM HEPES dissolved in ultra pure water, pH=7.4 (NaOH)) was added to the flask to hydrate the film for 30 min. The suspension was sequentially frozen (liquid nitrogen bath) and melted (water bath at 60 °C) five times, then extruded 11 times using a mini-extruder (Avanti Polar Lipids) and 100 nm filter-size (Whatman). Vesicles were stored at 5 °C in the dark until use. All experiments were conducted within 24 h of vesicle formulation.

Synthesis of 1,3-dilauramidopropan-2-yl 2-(trimethylammonio)ethyl phosphate (Lad-PC-Lad, 1)
0.80 g (1.38 mmol) of Lad-PE-Lad (non-methylated phosphoethanolamine) was mixed with 100 mL of methanol. 1.30 mL (1.74 g, 18.5 mmol) of dimethylsulfate was added shortly after that. The mixture was warmed up to 40 °C and then a solution of 1.91 g (18.5 mmol) of potassium carbonate in 20 mL water was added in one minute, while the mixture was strongly stirring. It was then stirred further 30 min at 40 °C and then cooled down to 20 °C. Methanol-water was removed under reduced pressure. To the solid residue was added methanol and the solvent was again removed under reduced pressure. Then the solid was mixed with 10 mL of a solution containing by volume 75% CH_2Cl_2, 22% MeOH, 3% aq. NH_4OH (25%) and purified on a silica gel column using the mobile phase of the same composition. Obtained was 0.26 g of the product (0.42 mmol, 30%).
***R*f**= 0.47(75% CH_2Cl_2, 22% MeOH, 3% aq. NH_4OH (25%))
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.44 (s, 2H), 4.40 (s, 2H), 4.14 (s, 1H), 3.88 (s, 2H), 3.37 (s, 9H), 3.12 (s, 2H), 2.17 (t, *J* = 7.7 Hz, 4H), 1.57 (s, 4H), 1.24 (s, 31.3H), 0.87 (t, *J* = 6.8 Hz, 6H).
^{13}C NMR (126 MHz, $CDCl_3$) δ 174.62, 72.10, 66.37, 59.47, 54.36, 40.53, 36.64, 31.93, 29.72, 29.67, 29.62, 29.51, 29.49, 29.38, 25.92, 22.69, 14.12.
^{31}P NMR (121 MHz, $CDCl_3$) δ -0.6.
HRMS (ESI+)*m/z* calcd $[M+H]^+$ 620.4762, obs. 620.4775
FTIR (cm^{-1}): 3280, 2919, 2851, 1648, 1551, 1468, 1219, 1084, 1058, 966, 798.
mp=145-150°C









Synthesis of 1,3-dimyristamidopropan-2-yl 2-(trimethylammonio)ethyl phosphate (Mad-PC-Mad, 2)

0.82 g (1.29 mmol) of Mad-PE-Mad (non-methylated phosphoethanolamine) was mixed with 100 mL of methanol. 2 mL (2.66 g, 21.1 mmol) of dimethylsulfate was added shortly after that. The mixture was warmed up to 40 °C and then a solution of 2.92 g (21.1 mmol) of potassium carbonate in 20 mL water was added in one minute, while the mixture was strongly stirring. It was then stirred further 30 min at 40 °C and then cooled down to 20 °C. Methanol-water was removed under reduced pressure. To the solid residue was added methanol and the solvent was again removed under reduced pressure. Then the solid was partially mixed with 10 mL of a solution containing by volume 75% CH_2Cl_2, 22% MeOH, 3% aq. NH_4OH (25%) and purified over a silica gel column using the mobile phase of the same composition. Obtained was 0.56 g of the product (0.83 mmol, 63%).

Rf=0.51 (75% CH_2Cl_2, 22% MeOH, 3% aq. NH_4OH (25%)).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.52 (s, 2H), 4.37 (s, 2H), 4.19 – 4.05 (m, 1H), 3.85 (s, 2H), 3.55 (s, 2H), 3.35 (s, 9H), 3.20 – 2.98 (m, 2H), 2.17 (t, J = 7.3 Hz, 4H), 1.57 (s, 4H), 1.24 (s, 39H), 0.87 (t, J = 6.8 Hz, 6H).

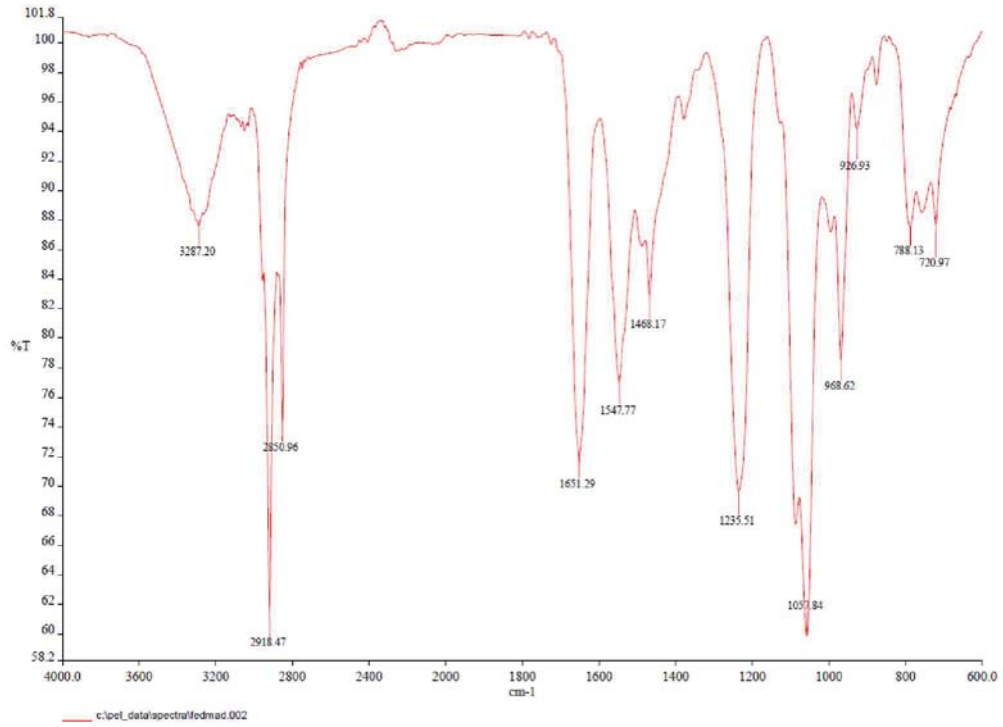
^{13}C NMR (126 MHz, $CDCl_3$) δ 174.61, 72.10, 66.36, 66.31, 59.47, 54.34, 40.52, 36.64, 31.94, 31.80, 29.74, 29.69, 29.64, 29.53, 29.50, 29.39, 25.92, 22.83, 22.70, 22.55, 14.12.

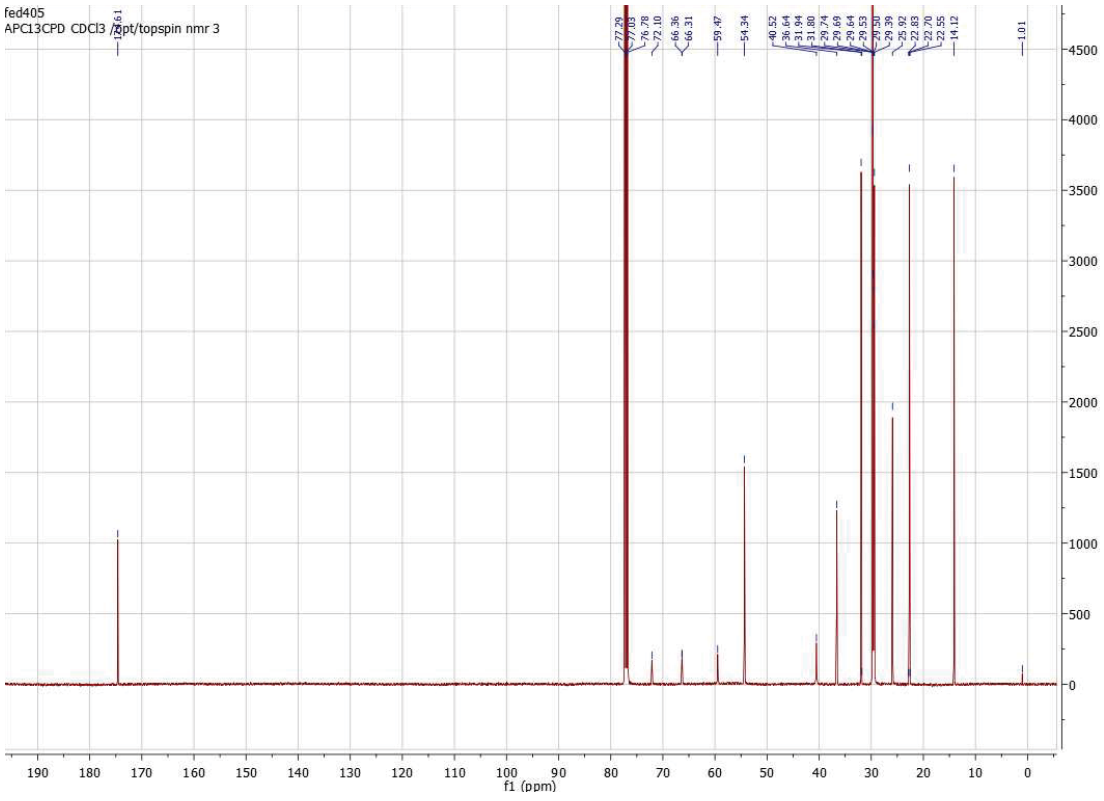
^{31}P NMR (121 MHz, $CDCl_3$) δ -2.9.

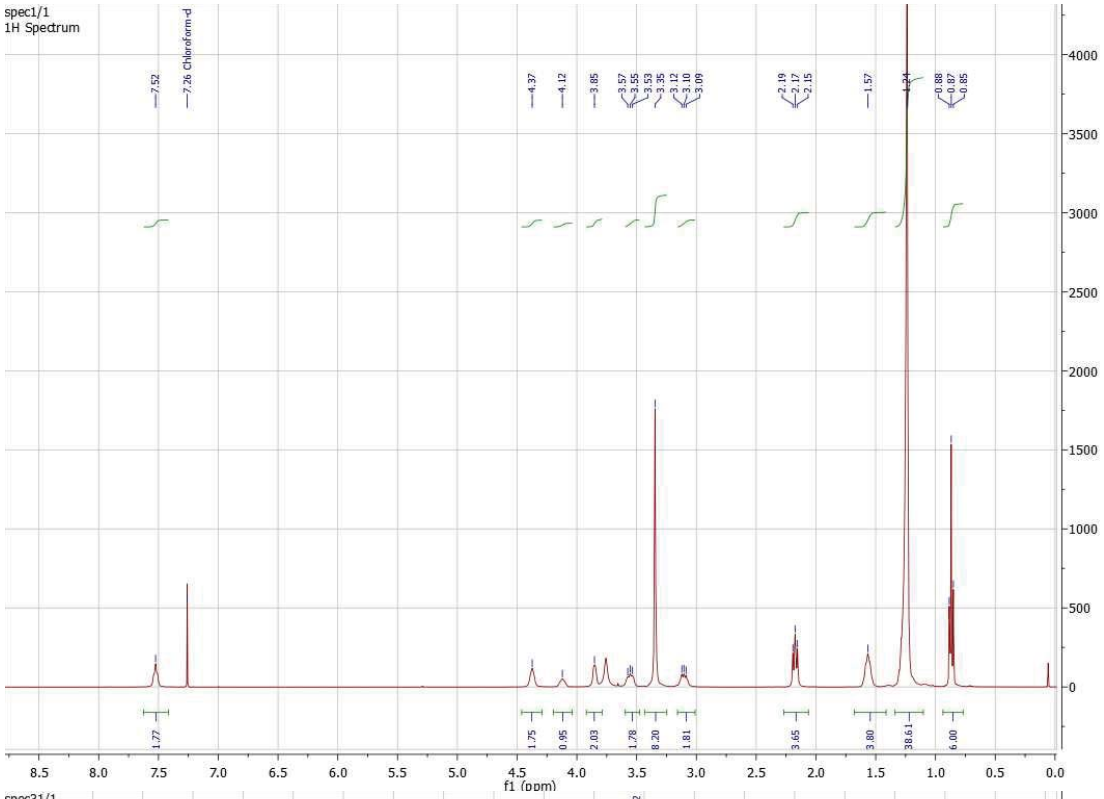
HRMS (ESI+)*m/z calc.* $[M+H]^+$ 676.5388, obs. 676.5385.

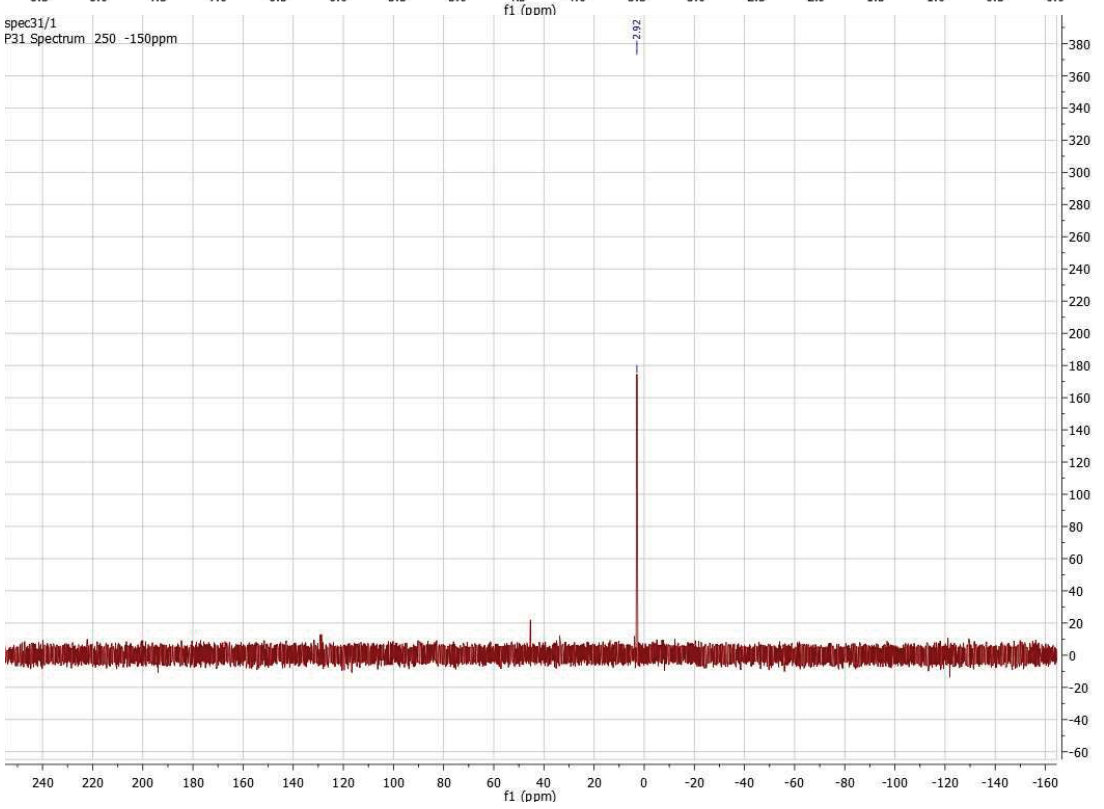
FTIR (cm^{-1}): 3287, 2918, 2851, 1651, 1547, 1468, 1235, 1058, 969, 788.

mp=189-193°C









Improved synthesis of 1,3-distearamidopropan-2-yl 2-(trimethylammonio)ethyl phosphate (Pad-PC-Pad 3)

1 g (1.45 mmol) of Pad-PE-Pad (non-methylated phosphoethanolamine) was mixed with 100 mL of methanol. 1 mL (1.33 g, 10.5 mmol) of dimethylsulfate was added shortly after that. The mixture was warmed up to 40 °C and then a solution of 1.45 g (10.5 mmol) of potassium carbonate in 20 mL water was added in one minute, while the mixture was strongly stirring. It was then stirred further 30 min at 40 °C and then cooled down to 20 °C. Methanol-water was removed under reduced pressure. To the solid residue was added methanol and the solvent was again removed under reduced pressure. Then the solid was partially mixed with 10 mL of a solution containing by volume 75% CH_2Cl_2, 22% MeOH, 3% aq. NH_4OH (25%) and purified on an alumina column using the mobile phase of the same composition. The second time it was purified over a silica gel column. Obtained were 0.56 g of the product (0.81 mmol, 53%).

R*f* = 0.56 (75% CH_2Cl_2, 22% MeOH, 3% aq. NH_4OH (25%)).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.58 (s, 2H), 4.39 (s, 2H), 4.12 (s, 1H), 3.89 (s, 2H), 3.36 (s, 9H), 3.12 (s, 2H), 3.25 – 3.02 (m, 2H), 2.18 (t, *J* = 7.3 Hz, 4H), 1.56 (s, 4H), 1.24 (s, 48H), 0.87 (t, *J* = 6.7 Hz, 6H).

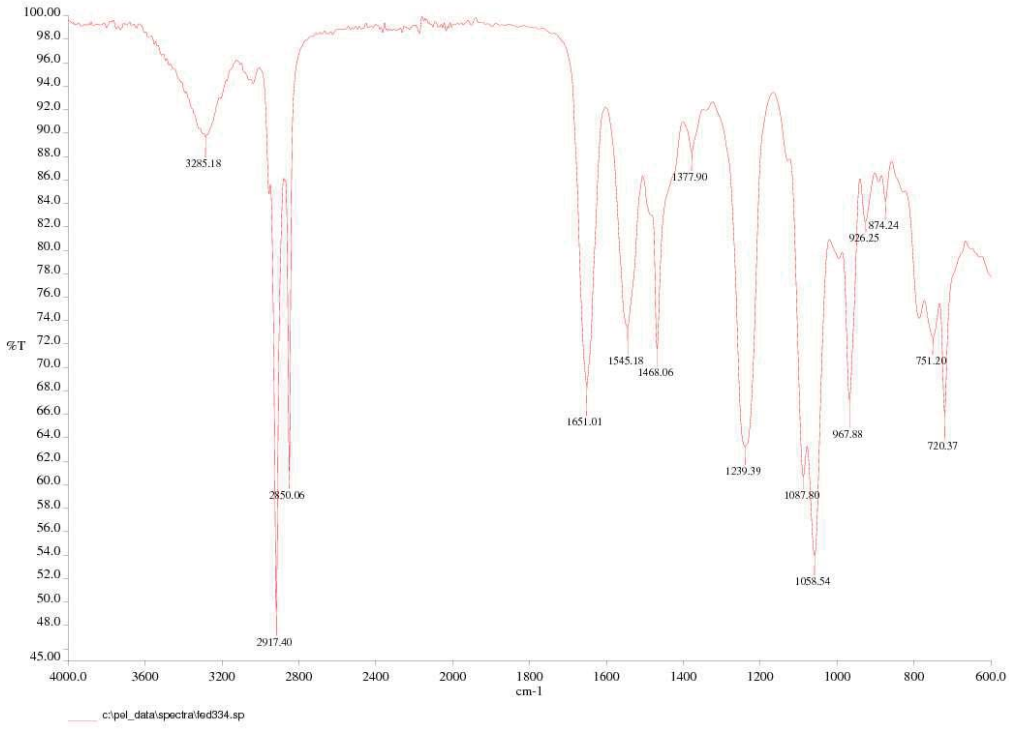
^{13}C NMR (101 MHz, $CDCl_3$) δ 174.7 (C=O), 72.2 (CH), 66.7, 59.6, 54.7(CH3s at the headgroup), 41.0, 36.9, 32.1, 29.89, 29.84, 29.72, 29.70, 29.59, 26.2, 22.9, 14.3 (CH3s of the tails).

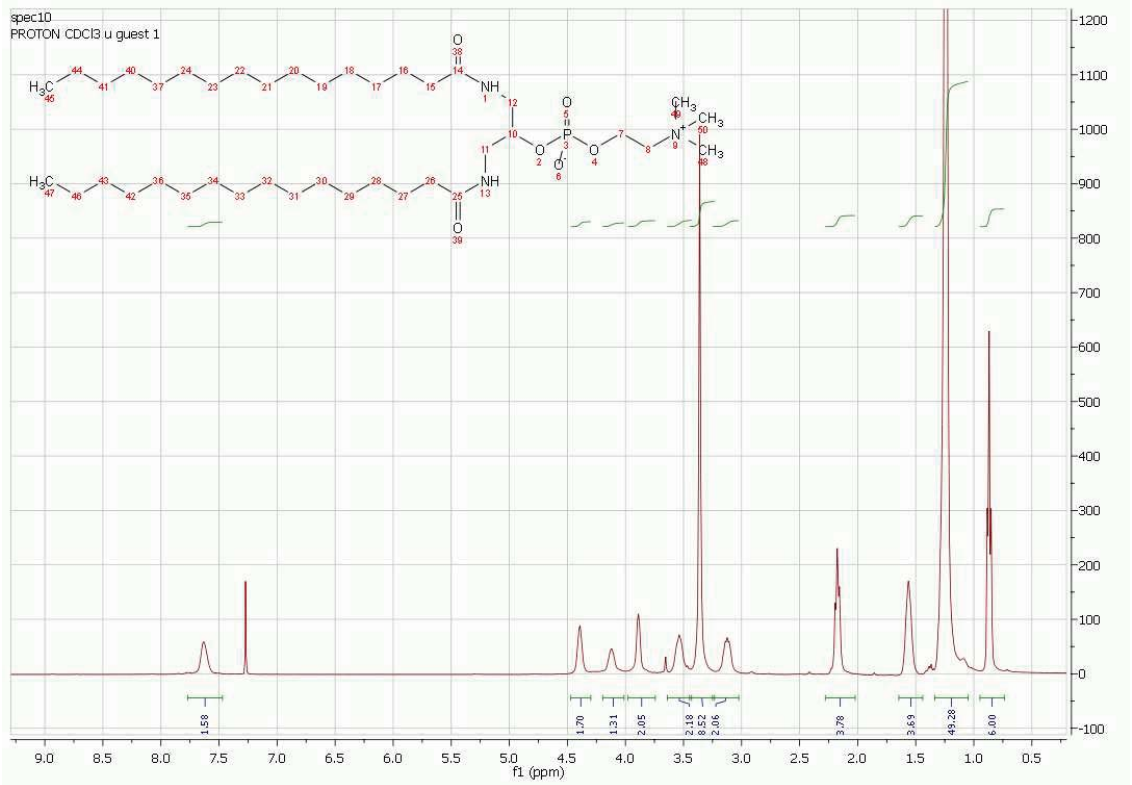
^{31}P NMR (121 MHz, $CDCl_3$) δ 2.97.

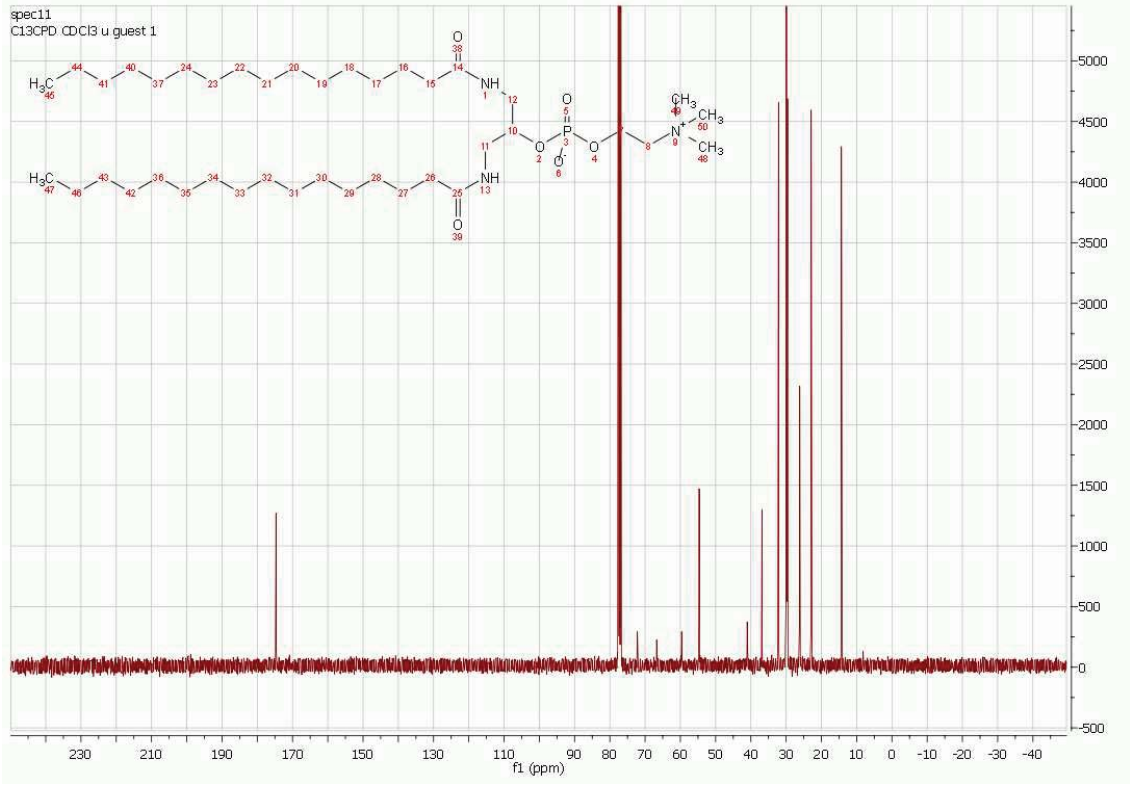
HRMS (ESI+)*m/z* calcd for $C_{40}H_{83}N_3O_6P$ $[M+H]^+$ 732.6, found 732.7.

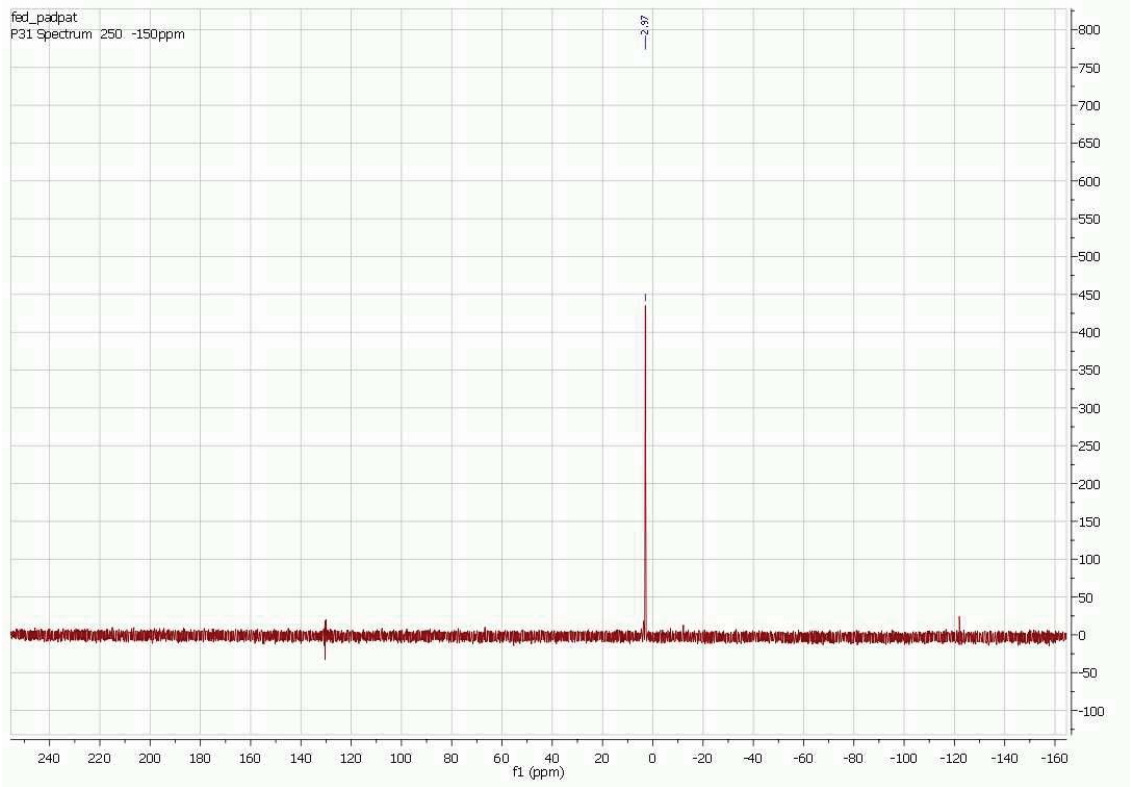
FTIR (cm^{-1}): 3285, 2917, 2850, 1651, 1545, 1468, 1239, 1088, 1058, 967, 720.

mp 202-204°C.

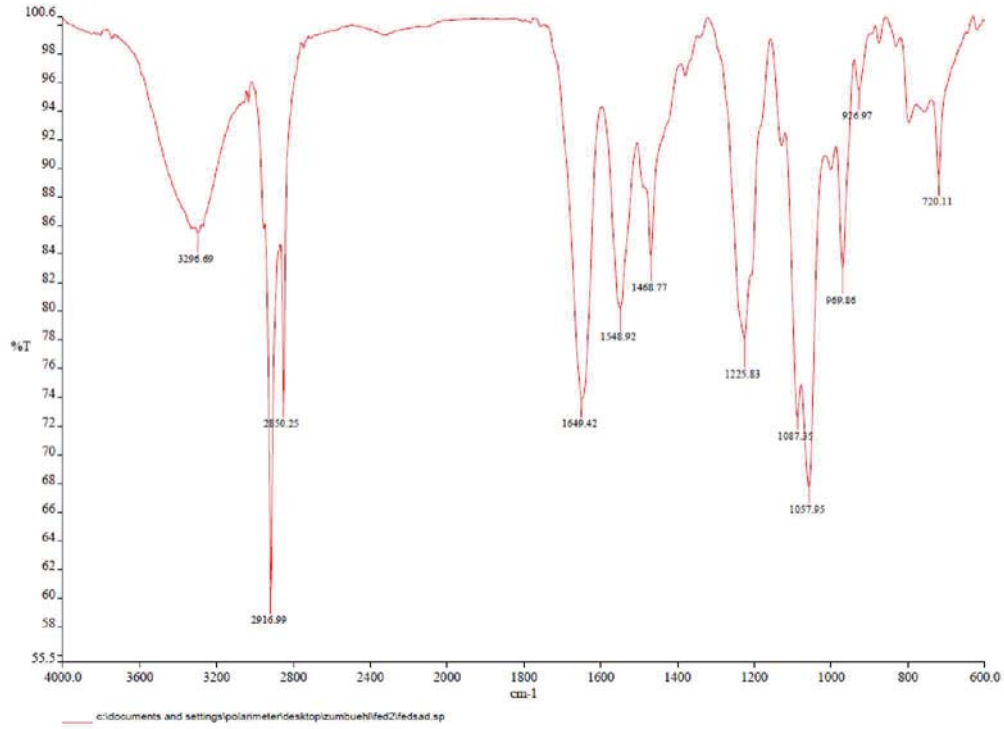


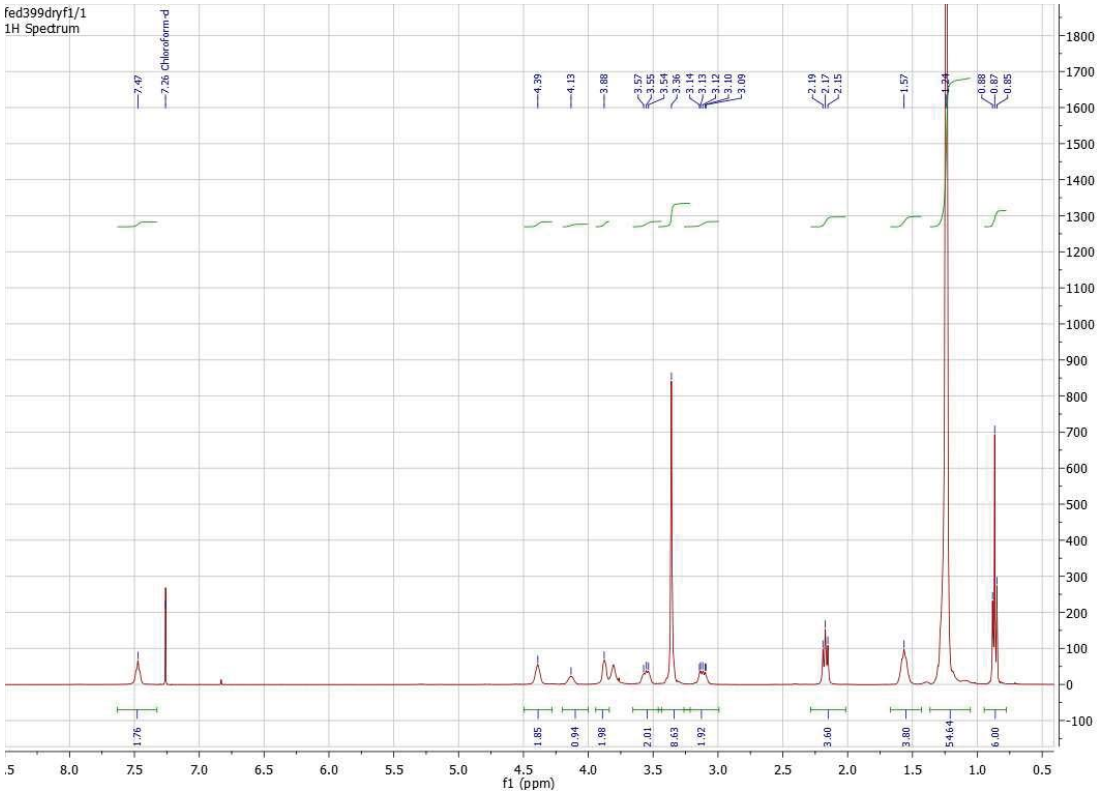


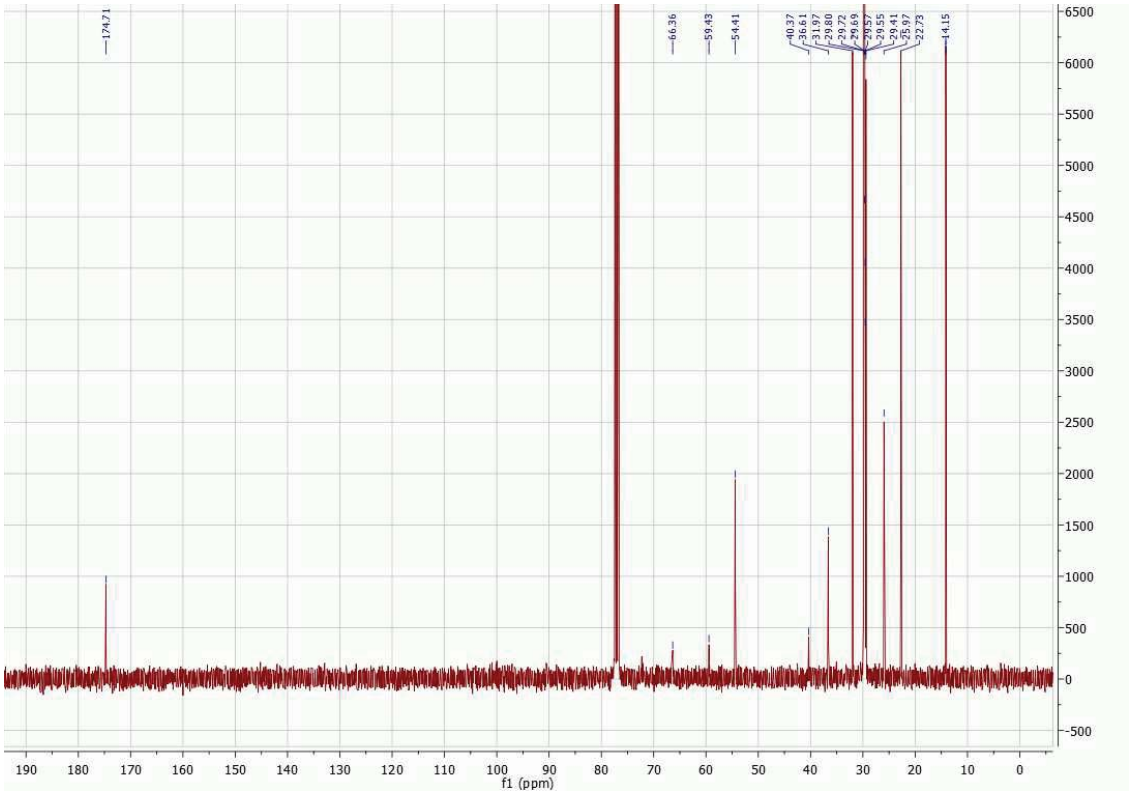


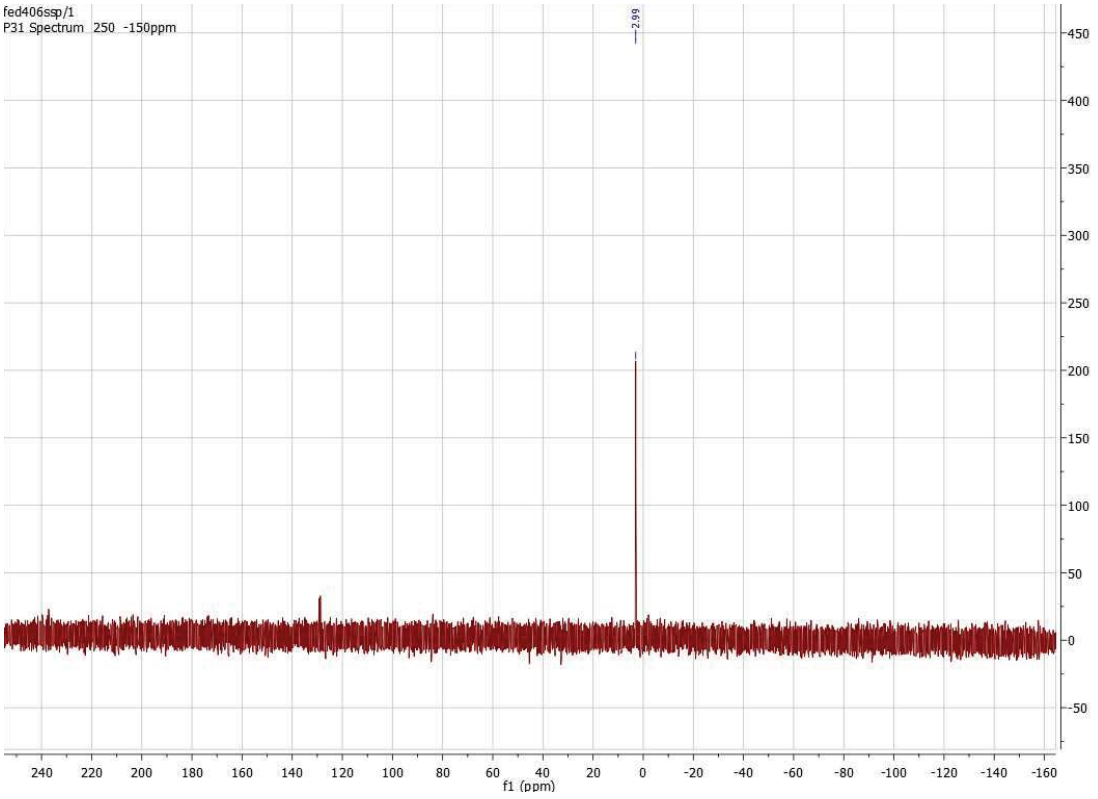


Synthesis of 1,3-distearamidopropan-2-yl 2-(trimethylammonio)ethyl phosphate (Sad-PC-Sad, 4)
116 mg (0.155 mmol) of Sad-PE-Sad (non-methylated phosphoethanolamine) was mixed with 15 mL of methanol. 0.1 mL (133 mg , 1.05 mmol) of dimethylsulfate was added shortly after that. The mixture was warmed up to 40 °C and then a solution of 145 mg (1.05 mmol) of potassium carbonate in 2 mL water was added in one minute, while the mixture was strongly stirring. It was then stirred further 30 min at 40 °C and then cooled down to 20°C. Methanol-water was removed under reduced pressure. To the solid residue was added methanol and the solvent was again removed under reduced pressure. Then the solid was partially mixed with 2 mL of a solution containing by volume 75% CH_2Cl_2, 22% MeOH, 3% aq. NH_4OH (25%) and run over an alumina and a silica gel column using the mobile phase of the same composition. Obtained were 60 mg of the product (0.08mmol, 49%).
Rf= 0.64 (75% CH_2Cl_2, 22% MeOH, 3% aq. NH_4OH (25%)).
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.47 (s, 2H), 4.39 (s, 2H), 4.13 (s, 1H), 3.88 (s, 2H), 3.66 – 3.43 (m, 2H), 3.36 (s, 9H), 3.26 – 2.99 (m, 2H), 2.17 (t, *J* = 7.3 Hz, 4H), 1.57 (s, 4H), 1.24 (s, 55H), 0.87 (t, *J* = 6.8 Hz, 6H).
^{13}C NMR (101 MHz, $CDCl_3$) δ 174.71, 66.36, 59.43, 54.41, 40.37, 36.61, 31.97, 29.80, 29.72, 29.69, 29.57, 29.55, 29.41, 25.97, 22.73, 14.15.
^{31}P NMR (121 MHz, $CDCl_3$) δ 3.0.
HRMS (ESI+)*m/z* calcd $[M+H]^+$ 788.6616, obs. 788.6641
FTIR (cm^{-1}): 3297, 2917, 2850, 1649, 1549, 1469, 1226, 1087, 1058, 970, 720.
mp=222-225°C



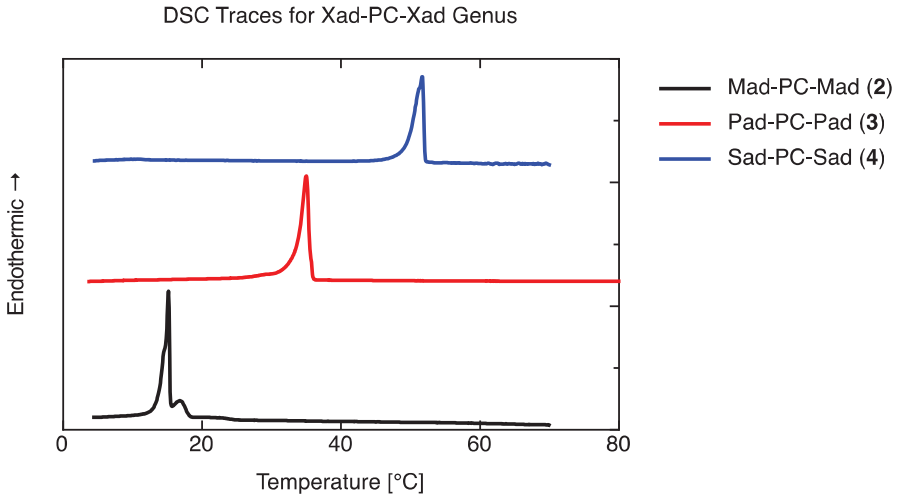






DSC of 1,3-Diamidophospholipids

DSC scans were performed with a VP-DSC instrument (Microcal, Northampton, MA). The vesicle suspensions in HEPES buffer containing ca. 1.5 mg/mL of the phospholipid were injected into the sample chamber (0.7 mL). The reference chamber contained the HEPES buffer. The heating rate was 1-5 °C/min. The measurements were recorded 2 or more times, always with fresh solutions.



Surface Pressure – Area isotherms

The pressure-area isotherms of monolayers at the air/water interface were measured with a Langmuir trough system equipped with one moving barrier. The setup included a surface pressure microbalance with a filter paper Wilhelmy plate. The results were plotted as surface pressure (π) versus area per molecule (A). The bare water surface was proved to be clean by compression before each measurement. The temperature of the Milli-Q Millipore water subphase was maintained by using a circulating water bath. The compression of the film was started 20 min after spreading to ensure the complete evaporation of the solvent and the uniform distribution of the molecules at the interface. Each measurement was repeated at least 2 times to prove the reproducibility of the results. In order to avoid dust contamination of the interface and to ensure a constant humidity, the Langmuir trough was placed in a sealed box.

X-ray Diffraction Measurements (GIXD)

The lattice structure in condensed monolayers was investigated using the liquid surface diffractometer at the undulator beamline BW1 (HASYLAB, DESY, Hamburg, Germany) by grazing incidence X-ray diffraction (GIXD) measurements. At BW1, a Langmuir film balance equipped with a Wilhelmy plate was positioned in a hermetically closed container flushed with helium. A monochromatic synchrotron X-ray beam (λ = 1.304 Å) was adjusted to strike the helium/water interface at a grazing incidence angle α_i = 0.85α_c (α_c = 0.13°) lighting up roughly 2×50 mm^2 of the surface. The trough was laterally moved during the measurements to prevent any sample damage by the strong X-ray beam. For the measurement of the diffracted signal a MYTHEN detector system (PSI, Villigen, Switzerland) was rotated to scan the in-plane Q_{xy} component values of the scattering vector. The vertical strips of the MYTHEN measured the out-of-plane Q_z component of the scattering vector between 0.0 and 0.75 Å^{-1}. The diffraction data consisted of Bragg peaks at diagnostic Q_{xy} values. The diffracted intensity normal to the interface was integrated over the Q_{xy} window of the diffraction peak to calculate the corresponding Bragg rod. The thickness of the monolayer is estimated from the *FWHM* of the Bragg rod using $0.9(2\pi)/FWHM\ (Q_z)$.
The in-plane component (i. e. the position of maximum Bragg peak intensity, Q_{xt}^{hk}) provides information about the lattice spacing d_{hk} (h and k are the Miller indices): $d_{hk}=2\pi/Q_{xy}$. The in-plane coherence length L_{xy}, was approximated from the full-width at half maximum (*FWHM*) of the Bragg peak using $L_{xy}\sim 0.9(2\pi)/FWHM\ (Q_{xy})$. The out-of-plane component can provide information about the polar tilt angle t and the chain tilt direction Ψ (azimuth Ψ)

$$Q_z^{Ax} = Q_{xy}^{Ax} \cos\Psi_{Ax} \tan t$$

Using the obtained tilt angle t, the cross-sectional area per chain A_0 can be calculated from the area per chain in the water plane:

$$A_0 = A_{xy} \cdot \cos t$$

Experimental details have been described in the literature.[3-6]

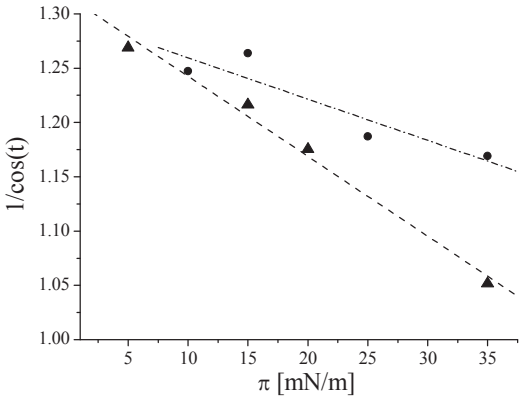


Figure S1. 1/cos(t) as function of the lateral pressure π at different temperatures (● : 20 °C, ▲ : 5 °C). t is the tilt angle. The linear extrapolation towards zero tilt angle (1/cos(t) = 1) yields the pressure of the tilting transition.

X-ray Reflectivity (XR)

X-ray reflectivity measurements were carried out at the same beamline as GIXD experiments. The experimental setup and evaluation procedures are described in detail elsewhere.Jensen[7-9]

The specular X-ray reflectivity (XR) data collection was performed by using a NaI scintillation detector. The X-ray reflectivity was measured with the geometry, $\alpha_i = \alpha_f = \alpha$, where α_i is the vertical incidence angle and α_f is the vertical exit angle of the reflected X-rays. XR data were collected as a function of the incidence angle, α_i, varied in the range of 0.06° - 3.5°, corresponding to a range of 0.01 $Å^{-1}$ - 0.6 $Å^{-1}$ of the vertical scattering vector component Q_z. The background scattering from the subphase was measured at $2\theta_{xy} = 0.7°$ and subtracted from the signal measured at $2\theta_{xy} = 0$. The X-ray footprint area on the sample is inversely proportional to the incident angle of the X-rays.

The electron density profile has been obtained with a model-independent method.[10, 11]

From the experimentally observed reflectivity curve, the corresponding profile correlation function is estimated via indirect Fourier transformation. For this profile correlation function the matching electron density profile is then derived by square-root deconvolution. No a priori assumptions on the shape of the electron-density profile have to be made. Subsequently, the obtained electron density profile was chemically interpreted by applying prior information such as surface area of the molecule. Assuming the tail group to be symmetric and constraining the number of electrons in tails results in a unique electron density distribution for the tails.

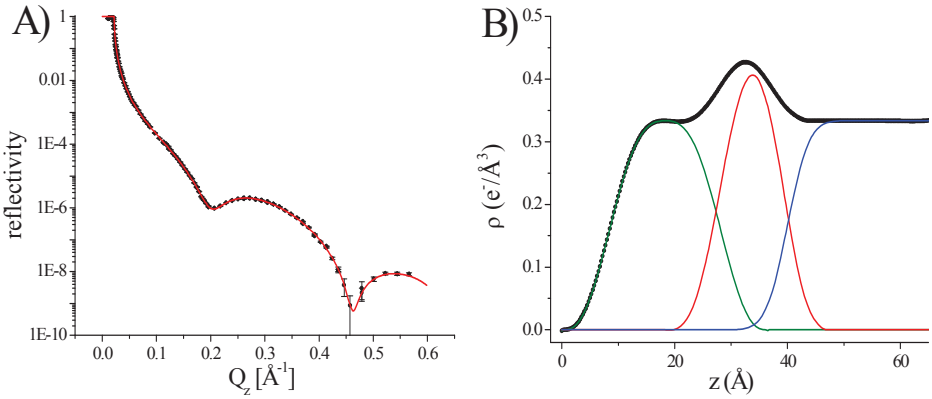


Figure S2. A) Specular X-ray reflectivity data with the corresponding fit (solid red line) of Sad-PC-Sad at the air/water interface at 35 mN/m and 5 °C. B) Electron density profile, obtained from the x-ray reflectivity data shown in A, of Sad-PC-Sad at the air/water interface (black curve). Assuming a symmetrical electron density distribution, the observed electron density is described by the sum of the electron densities of the chains (green curve), of the heads (red curve) and of water with a roughness of 3 Å (blue curve).

IRRAS – description of the method

Infrared reflection-absorption spectra were recorded using the Vertex 70 FT-IR spectrometer (Bruker, Germany), equipped with a liquid-nitrogen cooled MCT detector and coupled to a Langmuir film balance, which was placed in a sealed container (an external air/water reflection unit (XA - 511, Bruker)) to guarantee a constant vapor atmosphere.[12, 13] Using a KRS-5 (thallium bromide and iodide mixed crystal) wire grid polarizer, the IR-beam was polarized parallel (p) or vertical (s) and focused on the fluid subphase at an angle of incidence of 40°.

A computer controlled "trough shuttle system"[14] enables us to choose between the compartment with the sample (subphase with spread layer) and a reference compartment (pure subphase). The single-beam reflectance spectrum from the reference trough was taken as background for the single-beam reflectance spectrum of the monolayer in the sample trough to calculate the reflection absorption spectrum as $-\log(R/R_0)$ in order to eliminate the water vapor signal. FTIR spectra were collected at a resolution of 8 cm^{-1} using 200 scans for s-polarized light and 400 scans for p-polarized light.

References

1. Fedotenko, I.; Zaffalon, P.-L.; Favarger, F.; Zumbuehl, A., The synthesis of 1,3-diamidophospholipids. *Tetrahedron Lett.* **2010,** 51, (41), 5382-5384.
2. Olson, F.; Hunt, C. A.; Szoka, F. C.; J., V. W.; Papahadjopoulos, D., Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes. *Biochim. Biophys. Acta.* **1979,** 557, 9-23.
3. Als-Nielsen, J.; Jaquemain, D.; Kjaer, K.; Lahav, M.; Levellier, F.; Leiserowitz, L., Principles and applications of grazing incidence X-ray and neutron scattering from ordered molecular monolayers at the air-water interface. *Phys. Rep.* **1994,** 246, 251-313.
4. Kjaer, K.; Als-Nielsen, J.; Helm, C. A.; Tippmann-Krayer, P.; Möhwald, H., Synchrotron x-ray diffraction and reflection studies of arachidic acid monolayers at the air-water interface. *J. Phys. Chem.* **1989,** 93, 3200-3206.
5. Rietz, R.; Rettig, W.; Brezesinski, G.; Bouwman, W.; Kjaer, K.; Möhwald, H., Monolayer behaviour of chiral compounds at the air-water interface: 4-hexadecyloxy-butane-1,2-diol. *Thin Solid Films* **1996,** 285, 211-215.
6. Lepere, M.; Chevallard, C.; Brezesinski, G.; Goldmann, M.; Guenoun, P., Crystalline Amyloid Structures at Interfaces. *Angew. Chem. Int. Ed.* **2009,** 48, 5005-5009.
7. Jensen, T. R.; Kjaer, K., Structural Properties and Interactions of Thin Films at the Air-Liquid Interface Explored by Synchrotron X-Ray Scattering. In *Novel Methods to Study Interfacial Layers*, Möbius, D.; Miller, R., Eds. Elsevier: Amsterdam, 2001; Vol. 11, p 205.
8. Kjaer, K.; Als-Nielsen, J.; Helm, C. A.; Tippmann-Krayer, P.; Moehwald, H., Synchrotron x-ray diffraction and reflection studies of arachidic acid monolayers at the air-water interface. *J. Phys. Chem.* **1989,** 93, 3200-3206.
9. Kjaer, K., Some simple ideas on X-ray reflection and grazing-incidence diffraction from thin surfactant films. *Physica B* **1994,** 198, 100-109.

10. Pedersen, J. S., Model-independent determination of the surface scattering-length-density profile from specular reflectivity data. *J. Appl. Crystallogr.* **1992,** 25, 129-145.
11. Pedersen, J. S.; Hamley, I. W., Analysis of neutron and X-ray reflectivity data. II. Constrained least-squares methods. *J. Appl. Crystallogr.* **1994,** 27, 36-49.
12. Maltseva, E.; Brezesinski, G., Adsorption of amyloid beta (1-40) peptide to phosphatidylethanolamine monolayers *ChemPhysChem* **2004,** 5 (8), 1185-1190.
13. Muenter, A. H.; Hentschel, J.; Börner, H. G.; Brezesinski, G., Characterization of Peptide-Guided Polymer Assembly at the Air/Water Interface. *Langmuir* **2008,** 24 (7), 3306-3316.
14. Flach, C. R.; Brauner, J. W.; Mendelsohn, R., *Biophys. J.* **1994,** 66, A373.