Supporting Information

Lipid chain-driven interaction of a lipidated Src-family kinase Lyn with the bilayer membrane

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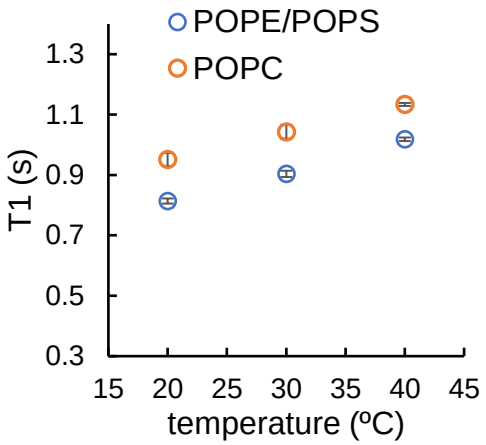


Fig.S1. Temperature-dependent changes of the ^{31}P-NMR $T1$ relaxation times of the POPC/POPE/POPS 50:25:25 membrane without Lyn peptides.

The $T1$ relaxation time relates to the following equation expressed with the rotational correlation time (τ_C) and the frequency (ϖ).

$$\frac{1}{T_1} \propto \frac{\tau_C}{1 + \varpi^2 \tau_C^2}$$

$T1$ can be minimum when $\varpi\tau_C \approx 1$. Here, membrane phospholipid motion relating to τ_C should increase according to increasing temperatures, and the POPC and POPE/POPS signals showed a temperature-dependent increase in the $T1$ relaxation times. Therefore, the decrease of $T1$ relaxation time in the presence of Lyn peptides means restriction of the phospholipid motion.

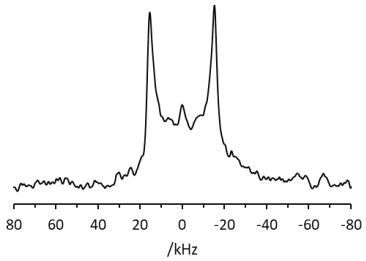


Fig.S2. ^{2}H NMR spectrum of the unitary 10′,10′-d_2-POPC membrane at 30°C, showing 19.1 kHz.

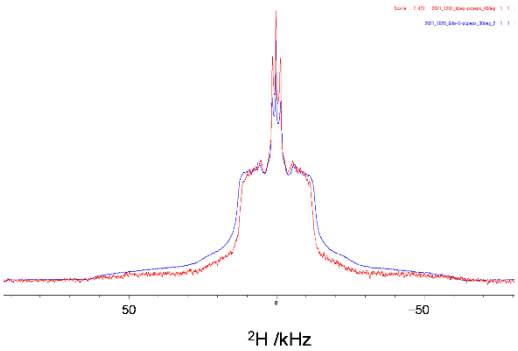


Fig.S3. ^{2}H NMR spectra of M_d-Lyn in POPC/POPE/POPS membranes at 30°C (blue trace) and 40°C (red trace). The spectrum at 30°C is also shown in Fig 2D, while the line broadening factor here is larger (150 Hz). The scan number for the blue trace was ~1,200k, and the red trace was ~450k, respectively.

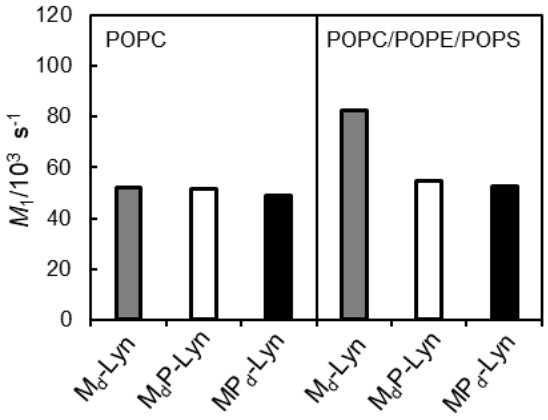


Fig.S4. First moments of the d_{27}-myristoyl chain or d_{31}-palmitoyl chain of Lyn-peptides in the POPC and POPC/POPE/POPS membranes at 30°C.

MATERIALS AND METHODS

Materials

CLEAR-Amide Resin (100-200 mesh), Fmoc protected amino acids; Fmoc-Gly, Fmoc-Lys(Boc), Fmoc-Ser(Bu^t), Fmoc-Ile, Fmoc-Cys(Trt), and Fmoc-Cys(dmt), and coupling reagents for solid-state peptide synthesis were purchased from Peptide Institute (Osaka, Japan). Palmitic acid-d_{31} was purchased from Cayman Chemical (MI, U.S.) Myristic acid-d_{27} and deuterium depleted water were obtained from Sigma-Aldrich (MO, U.S.). 10',10'-d_2-POPC was synthesized as described below. POPC, POPS, and POPE were purchased from NOF CORPORATION (Tokyo, Japan) and Avanti Polar Lipids, inc. (AL, U.S.). Other chemicals and solvents were obtained from Nacalai Tesque (Kyoto, Japan) and FujiFilm Wako Pure Chemical (Osaka, Japan). The ESI-FT-MS spectra were collected by Orbitrap XL (Thermo Fisher Scientific, MA, U.S.). Solution NMR was acquired by ECX-400 (JEOL, Tokyo, Japan) and AVANCE neo-700 (Bruker, U.S.).

Vesicle preparation and ^2H NMR

Multilamellar vesicles (MLVs) for the NMR sample were prepared according to a reported procedure. [1] First, the solution of lipids (20 mg in total) in methanol-chloroform (1:3) was prepared. The organic solvent was removed by an argon gas flow, and the obtained lipid film was further dried for at least 12 h under a high vacuum. The resulting lipid film was hydrated with Milli-Q water (30 times for the weight of the dried lipids) and vortexed at 65°C. The sample was then freeze-thawed several times to give MLVs, and extruded through a 0.1 μm membrane using an extruder (Avanti) to give large unilamellar vesicles (LUVs). The LUVs suspension added an aqueous solution of the peptide (10 mol% of total lipids) was incubated overnight at 30°C, freeze-thawed (10 times), and lyophilized. The lipid film was rehydrated with deuterium-depleted water to be 50% moisture (w/w), and then freeze-thawed several times. The sample was then transferred into a Kel-F insert tube for 4-mm Bruker MAS rotors, and the screw cap of the tube was sealed tight with epoxy glue.

Solid-state ^{2}H-NMR spectra were collected with a 400 MHz spectrometer (AVANCEIII 400, Bruker, U.S.) equipped with a ^{2}H static probe. To observe the ^{2}H quadrupole splitting pattern under the static conditions, a solid-echo pulse sequence was used with a 90° pulse width set to 4.65 μs; the solid-echo delays were set to 30 μs and 24 μs, and the recycle delay was set to 0.5 s, respectively. The sweep width was set at 250 kHz with 4k data points, and the number of scans was ~1,200,000. FID data was Fourier transformed upon exponential multiplication.

The order parameter (S_{CD}) is highly related to the observed value of the quadrupolar coupling widths ($\Delta\nu$), as in the following equation:[2-3]

$$\Delta\nu = \frac{3}{4}\frac{e^2qQ}{h}|S_{CD}| \quad (1)$$

where e^2qQ/h is the value of the quadrupolar coupling constant (167 kHz for ^{2}H in a C-^{2}H segment), and S_{CD} corresponds to the chain order parameter for the deuterated methylene positions relative to the principal ordering axis. The S_{CD} ranging ±0.5 is due to the rotation averaging around the ordering axis

that intersects the C-D bond vector of the methylene segments at θ = 90° through scaling by $(3\cos^2\theta-1)/2$. The S_{CD} value of the hydrocarbon chains in the membrane is assumed to have a minus sign.

The ^{2}H NMR spectra originating from perdeuterated lipid chains were de-Paked.[4]

The spectral first moment (M_1) was obtained according to

$$M_1 = \frac{\int_0^\infty |\omega| f(\omega) d\omega}{\int_0^\infty f(\omega) d\omega} \qquad (2)$$

where $\omega = 2\pi\nu$, ν is the resonance frequency in Hz, and $f(\omega)$ indicates the spectral intensity at ω. M_1 relates proportionally to the averaged order parameters of the deuterated lipid chains.[5]

Acy chain extent obtained from S_{CD}

The average area per chain (A_C) and membranes' volumetric thickness (D_C) were obtained using the maximum $|S_{CD}|$ value of the deuterated chain (S_{CD}^{max}) according to Kinnun et al.,[6]

$$A_C \cong \frac{2V_{CH2}}{D_M}\left(\frac{11}{6} - \frac{1}{2}\sqrt{3(-8S_{CD}^{max}-1)} - \frac{4}{3}S_{CD}^{max}\right) \qquad (3)$$

$$D_C = \frac{n_c V_{CH2}}{A_C} \qquad (4)$$

where V_{CH2} is a standard chain volumic area of methylene used 27.5 Å^2 [7-8], n_c is a carbon number, 16 for palmitoyl chain, by considering $V_{CH3} \approx 2V_{CH2}$ [9], and D_M is the maximum length of C_{i-1} and C_{i+1} used in 2.54 Å. The acyl chain extents (L_C) can be obtained by a total extent of the methylenes and terminal methyl $\langle L_C^i \rangle$ obtained from S_{CD}^i as follows, according to Vogel et al.[10].

$$\langle L_C^i \rangle = \frac{1}{2} D_M \langle cos\beta_i \rangle = \frac{1}{2} D_M \left(\coth\left(\frac{-U_i}{k_B T}\right) + \frac{k_B T}{U_i}\right) \qquad (5)$$

Where $D_M\langle cos\beta\rangle$ is the average travel of the methylene segment i along the bilayer normal and U_i is the first-order mean-torque parameter of each methylene and methyl segment. Since $\langle cos^2\beta\rangle^{1/2}$ is not

<*cosβ*>, <$cos^2\beta$> is obtained from the following equations.

$$\langle cos^2\beta_i \rangle = 1 + 2\left(\frac{-k_BT}{U_i}\right)^2 + \frac{2k_BT}{U_i}\coth\left(\frac{-U_i}{k_BT}\right) \quad (6)$$

$$\langle cos^2\beta_i \rangle = \frac{1 - 4S_{\mathrm{CD}}^i}{3} \quad (7)$$

The $S_{\mathrm{CD}}^{\mathrm{i}}$ of the methylene group was directly obtained from the ^{2}H NMR spectrum, while the terminal methyl group has a three-fold symmetric rotation along with the C-CD_3 bond, leading to $S_{\mathrm{CD}}^{\mathrm{i}} = -3S_{\mathrm{CD3}}$, along with the cosine of the rotating corn angle in 109.5° [3]. In the approximation of $\coth(-U/k_BT) \approx 1$ when $S_{\mathrm{CD}}^{\mathrm{i}}$ is small enough (here used <−0.14), <*cosβ*> can be obtained as follows.

$$\langle cos\beta_i \rangle = \frac{1}{2}\left(1 + \sqrt{\frac{-8S_{\mathrm{CD}}^i - 1}{3}}\right) \quad (8)$$

Solid-state ^{31}P NMR

The solid-state ^{31}P-NMR spectra were collected with a 400 MHz spectrometer. The static ^{31}P-NMR spectra were collected using a 4.0 μs single 90° pulse with a 4.0 s recycle delay. 70 kHz ^{1}H decoupling field was applied with a spinal64 scheme. 1024 scans were taken, and the free induction decay was processed with 100 Hz line-broadening. The spectral width was set to 400 ppm with 4k points. For the spin-lattice (T1) relaxation analysis, the saturation recovery experiment with a 4 kHz magic angle spinning (MAS) rate was employed. Saturation was achieved by a train of 90°-pulse-20 ms-delay for 30 times. The 12 recovery delay points from 5 ms to 5 s were collected with 128 scans. Ammonium dihydrogenphosphate (δ=1.0 ppm with respect to phosphoric acid (85%):δ= 0 ppm) was used as the secondary external reference. The data processing and *T*1 analysis were performed using Topspin 4.1.3 (Bruker).

Synthetic Procedures

Myr-Gly-Cys-Ile-Lys-Ser-Lys-Gly-NH_2 **1**

CLEAR-Amide Resin (208 mg, 0.48 mmol/g) was swelled with DMF (2 mL) for 30 min, and then treated with 20% piperidine/DMF (2 mL) for 5 min and followed 15 min-treatment with the fresh 20% piperidine/DMF (2 mL) to remove the Fmoc group. After rinsing the resin, peptide elongation was achieved in the repetition of the following steps. The coupling cocktail of 0.45 M HBTU in DMF (1.0 mL), including DIEA (0.174 mL) and Fmoc amino acid or myristic acid (0.50 mmol) was added to the resin, and the suspension was vortexed for 60 min at room temperature. After the reaction, the resin was repeatedly rinsed with DMF. The myristoylated peptide on the resin was repeatedly washed with CH_2Cl_2 and ether, and then dried under reduced pressure overnight to give Myr-Gly-Cys(Trt)-Ile-Lys(Boc)-Ser(Bu^t)-Lys(Boc)-Gly-NH-Resin (219 mg). A part of the dried resin was treated with TFA cocktail (TFA/TIS/H_2O = 1900 μL: 50 μL: 50 μL) for 90 min at room temperature. After filtration, TFA was removed by a nitrogen stream, and ether was added to form a precipitate. The precipitate was washed three times with ether and dried in vacuo. The crude peptide was purified by reverse phase HPLC (column; 5C4-AR-300, 4.6 mmI.D.×150 mm) to give the peptide **1** (15.0 mg, 16.6 μmol, 16%). ESI-MS; m/z calcd $C_{42}H_{80}N_{10}O_9S$ for $[M+H]^+$ 901.5909, found 901.5902.

^{1}H NMR (700 MHz, CD_3OD) δ 8.40 (dd, 1H, *J* = 5.8, 5.8 Hz, GlyNH), 8.19-8.12 (m, 5H, Gly7NH, LysNH×2, CysNH, IleNH), 8.05 (d, 1H, SerNH), 7.48 (brs, 1H, $GlyCONH_2$a), 7.14 (brs, 1H, $GlyCONH_2$b), 4.56 (br, 1H, CysCαH), 4.38-4.31 (m, 3H, LysCαH×2, SerCαH), 4.12 (dd, 1H, *J* = 7.0, 7.0 Hz, IleCαH), 3.96-3.82 (m, 6H, $GlyC\alpha H_2$×2, Ser $C\beta H_2$), 2.98-2.94 (m, 6H, $CysC\beta H_2$, $LysC\varepsilon H_2$×2), 2.31 (m, 2H, $MyrCH_2CO$), 2.02-1.77 (m, 5H, IleCβH, $LysC\beta H_2$×2), 1.71 (m, 4H, $LysC\delta H_2$×2), 1.67-1.47 (m, 7H, $MyrCH_2$, IleCγHa, $LysC\gamma H_2$×2), 1.40-1.25 (m, 21H, Myr, IleCγHb), 0.99 (d, 3H, *J* = 6.9 Hz, IleCγ'Me), 0.96 (dd, 3H, *J* = 7.6, 7.6 Hz, IleCδMe), 0.93 (t, 3H, *J* = 7.1 Hz, Myr-Me).

^{13}C NMR (175 MHz, CD_3OD) δ177.5 (Myr), 174.9, 174.7019, 174.6, 174.4, 173.3, 173.2, 172.7

(GlyCONH$_2$), 63.0 (Ser Cβ), 61.0 (Ile Cα) , 57.8 (Ser Cα), 57.6 (Cys Cα), 55.5(^{a}Lys Cα), 55.2 (^{b}Lys Cα), 44.5 (^{a}Gly Cα), 43.4 (^{b}Gly Cα), 41.0 (^{a}Lys Cε, Lys7 Cε), 37.3 (Ile Cβ), 37.0 (myr C2), 33.2, 32.0 (^{a}Lys Cβ), 31.8 (^{b}Lys Cβ), 30.96, 30.95, 30.93, 30.8, 30.7, 30.64, 30.57, 28.3 (^{a}Lys Cδ), 28.25 (^{b}Lys Cδ), 27.0 (Cys Cβ), 26.9, 26.7, (Ile Cβ), 24.0, 23.9 (^{a}Lys Cγ), 23.8 (^{b}Lys Cγ), 16.2 (Ile Cγ'), 14.6, 11.5 (Ile Cδ).

d_{27}Myr-Gly-Cys-Ile-Lys-Ser-Lys-Gly-NH$_2$ (**2**)

Peptide **2** was synthesized on the same scale in a similar manner as **1,** except for using d_{27}-myristic acid. The crude peptide was purified by HPLC to give **2** (24.9 mg, 26.8 μmol, 27%). ESI-MS; m/z calcd C42H53D27N10O9S for $[M+H]^+$ 928.7603, found 929.7596.

Myr-Gly-Cys(Pal)-Ile-Lys-Ser-Lys-Gly- NH$_2$ (**3**)

Myr-Gly-Cys(Mmt)-Ile-Lys(Boc)-Ser(Bu^t)-Lys(Boc)-Gly-NH-Resin was similarly prepared on the same scale as the peptide **1** except for using Fmoc-Cys(Mmt). After washing several times with CH_2Cl_2, The Mmt group was removed by a cocktail of 5%TIS/1%TFA/CH_2Cl_2 (1 mL). After repeating cycles of the 3-min treatment and DMF wash until the orange-yellow color disappeared (required about 10 cycles), the coupling cocktail of 0.45 M HATU in DMF (10 mL), including DIEA (0.174 mL) and palmitic acid (128 mg, 0.50 mmol), was added and the reaction mixture was vortexed overnight at room temperature. After the reaction, the resin was washed with CH_2Cl_2 and ether, and dried overnight under reduced pressure to give Myr-Gly-Cys(Pal)-Ile-Lys(Boc)-Ser(Bu^t)-Lys(Boc)-Gly-OH-Resin (302 mg). The dried resin was treated with TFA cocktail (TFA/TIS/H_2O = 2850 μL: 75 μL: 75 μL) for 90 min at room temperature. After filtration, TFA was removed by a nitrogen stream, and ether was added to form a precipitate. The precipitate was washed three times with ether and dried in vacuo. The crude peptide was purified by reverse phase HPLC (column; 5C4-AR-300, 4.6 mmI.D.×150 mm) to give the peptide **3** (5.0 mg, 4.38 μmol, 9.5%). ESI-MS; m/z calcd C58H110N10O10S for $[M+H]^+$

1139.8205, found 1139.8206.

^{1}H NMR (700 MHz, CD_3OD) δ 8.45 (dd, 1H, *J* = 6.2, 6.2 Hz, Gly2NH), 8.22 (brd, 1H, *J* = 6.5 Hz, CysNH), 8.19 (brdd, 1H, *J* = 6.2, 6.2 Hz, GlyNH), 8.16-8.13 (m, 2H, LysNH ×2), 8.02 (brd, 1H, *J* = 6.4 Hz, IleNH), 7.99 (brd, 1H, *J* = 7.6 Hz, IleNH), 7.48 (brs, 1H, $GlyCONH_2$a), 7.12 (brs, 1H, $GlyCONH_2$b), 4.54 (m, 1H, CysCαH), 4.39-4.33 (m, 3H, LysCαH×2, SerCαH), 4.11 (brd, 1H, *J* = 8.05 Hz, IleCαH), 3.96-3.82 (m. 6H, $GlyC\alpha H_2$×2, Ser $C\beta H_2$), 3.43 (dd, 1H, *J* = 14.6, 3.7 Hz, $CysC\beta H_2$a), 3.27 (dd, 1H, *J* = 14.6, 9.0 Hz, $CysC\beta H_2$a), 2.98(m, 4H, $LysC\varepsilon H_2$×2), 2.66 (dd, 2H, *J* = 7.4, 7.4 Hz, $S\text{-}COCH_2$), 2.35 (dd, 2H, *J* = 7.6, 7.6 Hz, $NHCOCH_2$), 2.03-1.91 (m. 5H, IleCβH, $LysC\beta H_2$×2), 1.72 (m, 4H, $LysC\delta H_2$×2), 1.69-1.48 (m, 9H, $MyrCH_2$, $PalCH_2$, IleCγHa, $LysC\gamma H_2$×2), 1.42-1.24 (m, 45H, Myr, Pal, IleCγHb), 0.99 (d, 3H, *J* = 6.5 Hz, IleCγ'Me), 0.96 (dd, 3H, *J* = 7.5, 7.5 Hz, IleCδMe), 0.94 (dd, 6H, *J* = 7.5, 7.5 Hz, Myr-Me, Pal-Me).

^{13}C NMR (175 MHz, CD_3OD) δ 202.2 (SC(=O)-Pal), 177.7 (NH C(=O)-Myr), 175.0, 174.6 (×2), 174.5, 173.2 (×2), 173.1, 62.9 (Ser Cβ), 60.9 (Ile Cα), 57.7 (Ser Cα), 56.2 (Cys Cα), 55.4 (^{a}Lys Cα), 55.1 (^{b}Lys Cα), 45.1 ($SCOCH_2$-), 44.7(^{a}Gly Cα),43.4 (^{b}Gly Cα), 40.8 (LysCε × 2), 37.4 (Ile Cβ), 37.1 (myr C2), 33.3, 32.0 (^{a}Lys Cβ), 31.8 (^{b}Lys Cβ), 31.1 (Cys Cβ), 31.0, 30.9, 30.8, 30.75, 30.71, 30.3, 28.2 (^{a}LysCδ), 28.1(^{b}Lys Cδ), 27.0, 26.8 (Ile Cβ), 24.0, 23.97 (^{a}Lys Cγ), 23.94 (^{b}Lys Cγ), 16.3 (Ile Cγ'), 14.7 (myr), 11.6 (Ile Cδ).

d_{27}Myr-Gly-Cys(Pal)-Ile-Lys-Ser-Lys-Gly- NH_2 (**4**)

The peptide **4** was synthesized on the same scale in the similar manner as **3**, except for using d_{27}-myristic acid. The crude peptide was purified by HPLC to give **4** (6.7 mg, 5.74 μmol, 5.7%). ESI-MS; m/z calcd $C_{58}H_{83}D_{27}N_{10}O_{10}S$ for $[M+H]^+$ 1166.9900, found 1166.99.

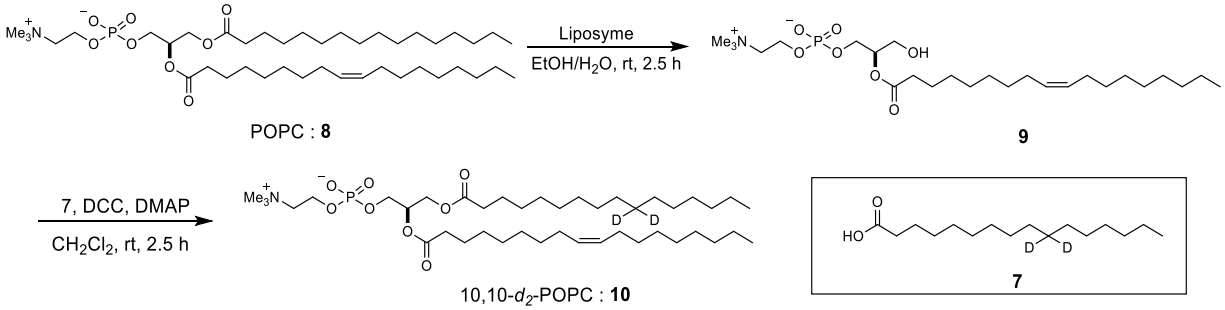
Myr-Gly-Cys(d_{31}Pal)-Ile-Lys-Ser-Lys-Gly- NH_2 (**5**)

The peptide **5** was synthesized on the same scale in a similar manner as **3**, except for using d_{31}-palmitic

acid. The crude peptide was purified by HPLC to give **5** (6.8 mg, 5.80 μmol, 5.8%). ESI-MS; m/z calcd C58H79D31N10O10S for $[M+H]^+$ 1171.0151, found 1172.0143.

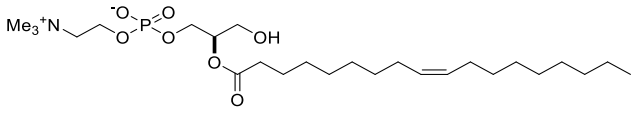
Synthesis of 10′,10′-d_2-POPC

10,10-d_2-palmitic acid **7** was synthesized from sebacic acid monomethyl ester in a similar procedure as reported previously.[11] As shown in Scheme 1, Lyso-OPC **9** was prepared from commercial POPC **8** by selective hydrolyzation using Lipozyme® (*Mucor miehei*) in ethanol/H_2O solution[12] (Scheme 2). Following esterification of **9** with the 10,10-d_2-palmitic acid **7** produced 10′,10′-d_2-POPC **10**.



Scheme 1. Synthesis of 10′,10′-d_2-POPC

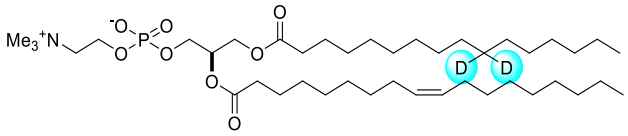
2-Oleoyl-sn-glycero-3-phosphocholine **9**



To a solution of POPC (72.3 mg, 0.095 mmol) in ethanol (0.9 mL) and water (0.1 mL) added Lipozyme (Sigma-Aldrich: 134 mg) was stirred for 2.5 h at room temperature while keeping the pH between 5 to 7. The mixture was then filtered and evaporated. The residue was purified with SiO_2 chromatography (chloroform/methanol/H_2O 65:25:4) to give lyso-OPC **9** (36.4 mg, 0.070 mmol, 73%) as a white solid. **9**: colorless oil; R_f= 0.01 (silica gel, chloroform/methanol/water = 65/25/4); ^{1}H NMR (500 MHz,

$CDCl_3$) δ 5.33 (2H, m), 4.90 (1H, m), 4.30 (2H, br), 4.01 (1H, br), 3.94 (1H, br), 3.80 (2H, br), 3.66 (2H, br), 3.34 (9H, s), 2.28 (2H, t, *J* = 7.8 Hz), 2.00 (4H, m), 1.55 (2H, m), 1.25 (20H, m), 0.87 (3H, t, *J* = 7.0 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.2, 129.9, 129.6, 73.2, 66.0, 62.9, 59.6, 59.2, 54.1, 34.2, 31.8, 29.6, 29.4, 29.2, 29.1, 27.1, 24.8, 22.5, 14.0; ESI-MS (positive mode); *m/z* calcd for $[C_{26}H_{52}NO_7P + Na]^+$ 544.3481, found 544.3376.

1-O-(10,10-d_2-palmitoyl)-2-O-oleoyl-sn-glycero-3-phosphocholine **10**



To a solution of lyso-OPC **9** (22.9 mg, 0.044 mmol) and 10,10-d_2-palmitic acid[11] (27.5 mg, 0.107 mmol) in dry CH_2Cl_2 (0.5 mL) added DCC (32.4 mg, 0.157 mmol) and a catalytic amount of DMAP was stirred for 2.5 h at room temperature, and then the solvent was evaporated. The residue was purified with SiO_2 chromatography (chloroform/methanol/H_2O 65:25:4) to give 10′,10′-d_2-POPC **10** (23.6 mg, 0.031 mmol, 70%).

10 : pale yellow oil; R_f= 0.20 (silica gel, chloroform/methanol/water = 65/25/4); ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.33 (2H, m), 5.20 (1H, br), 4.49 (1H, dd, *J* = 11.5 Hz, 2.8 Hz), 4.31 (2H, br), 4.12 (1H, dd, *J* = 12.0 Hz, 7.3 Hz), 3.94 (2H, m), 3.82 (2H, br), 3.38 (9H, s), 2.27 (4H, m, H2), 2.00 (4H, m), 1.56 (4H, m), 1.25 (42H, m), 0.87 (3H, t, *J* = 6.8 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.4, 173.1, 129.8, 129.5, 70.3, 66.2, 63.3, 62.9, 59.3, 54.2, 34.2, 34.0, 31.8, 29.6-29.4, 29.2- 29.0, 27.1, 24.8, 22.5, 14.0; ESI-MS (positive mode); *m/z* calcd for $[C_{42}H_{80}D_2NO_8P + Na]^+$ 784.5904, found 784.5795.

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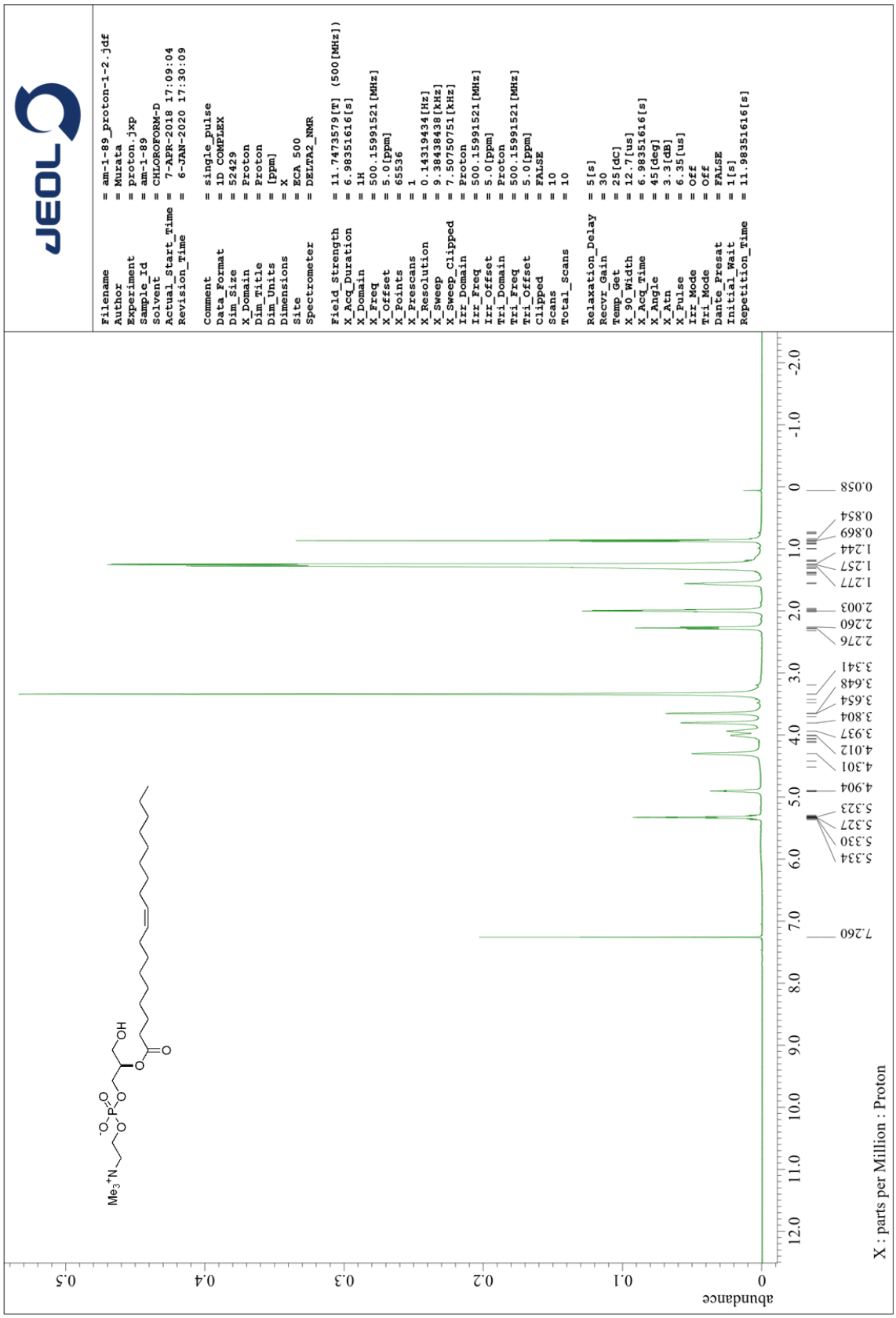
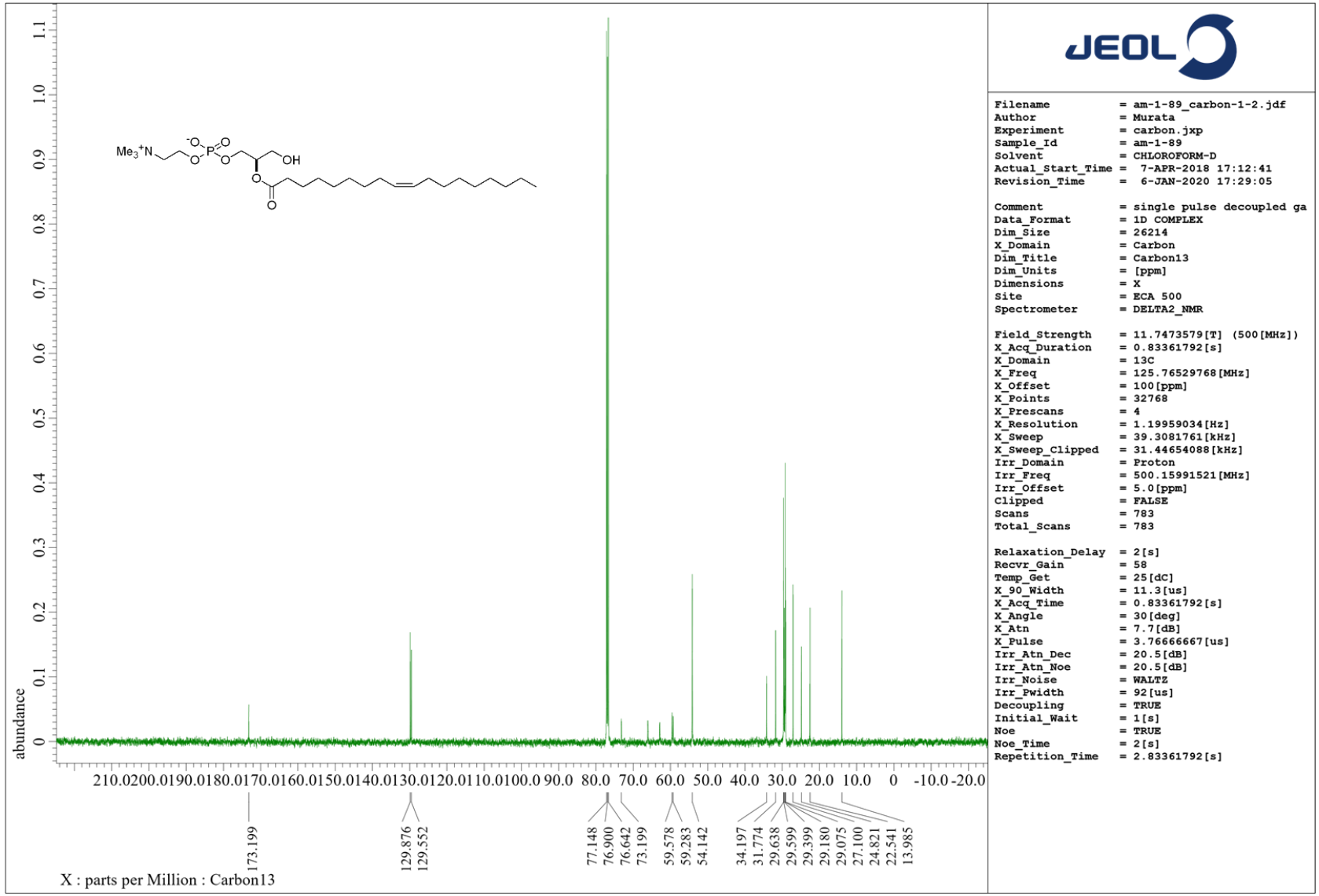
^{1}H and ^{13}C NMR spectra

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129.552
77.148
76.900
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59.578
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34.197
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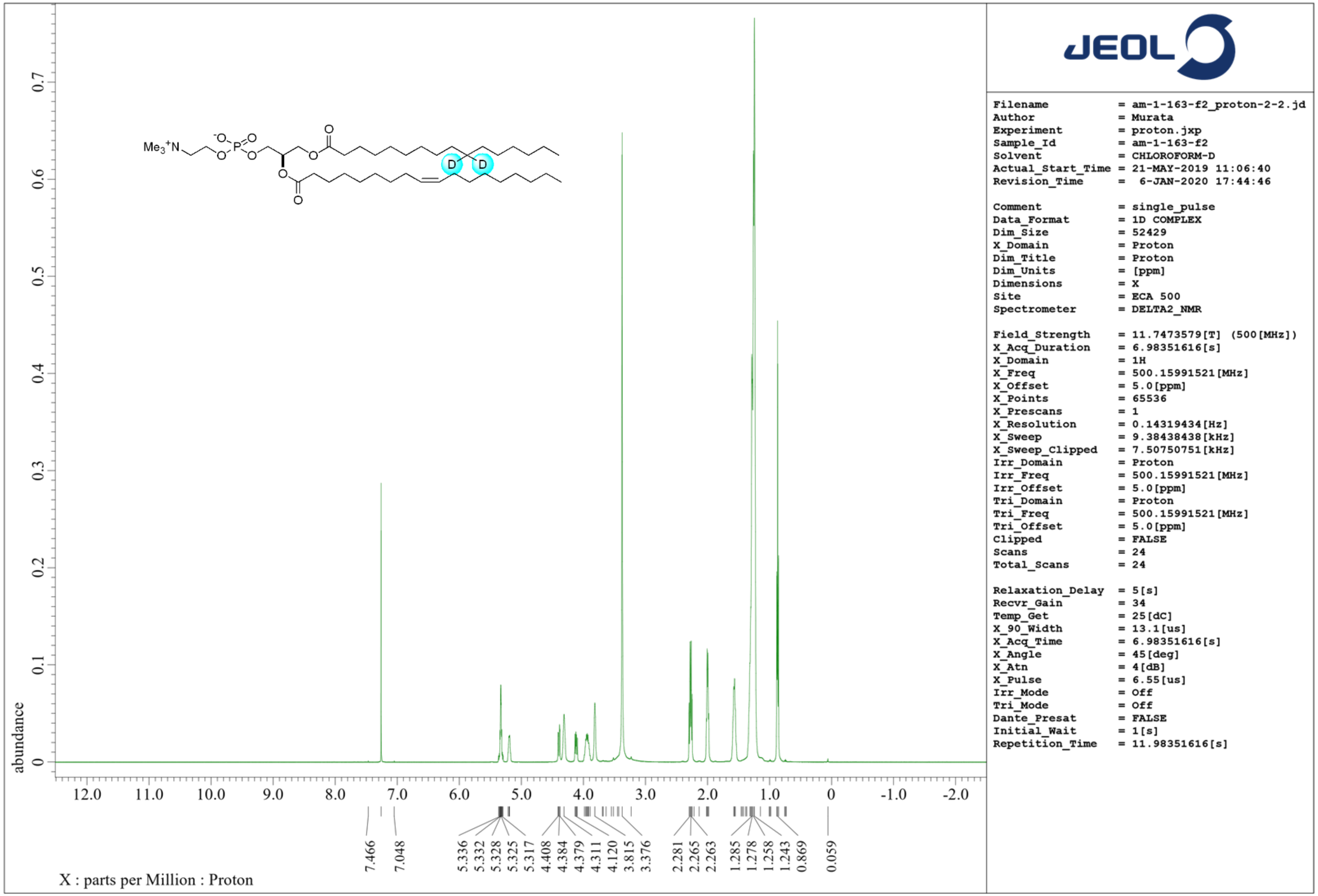
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O
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D
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0
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11.0
10.0
9.0
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7.0
6.0
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2.0
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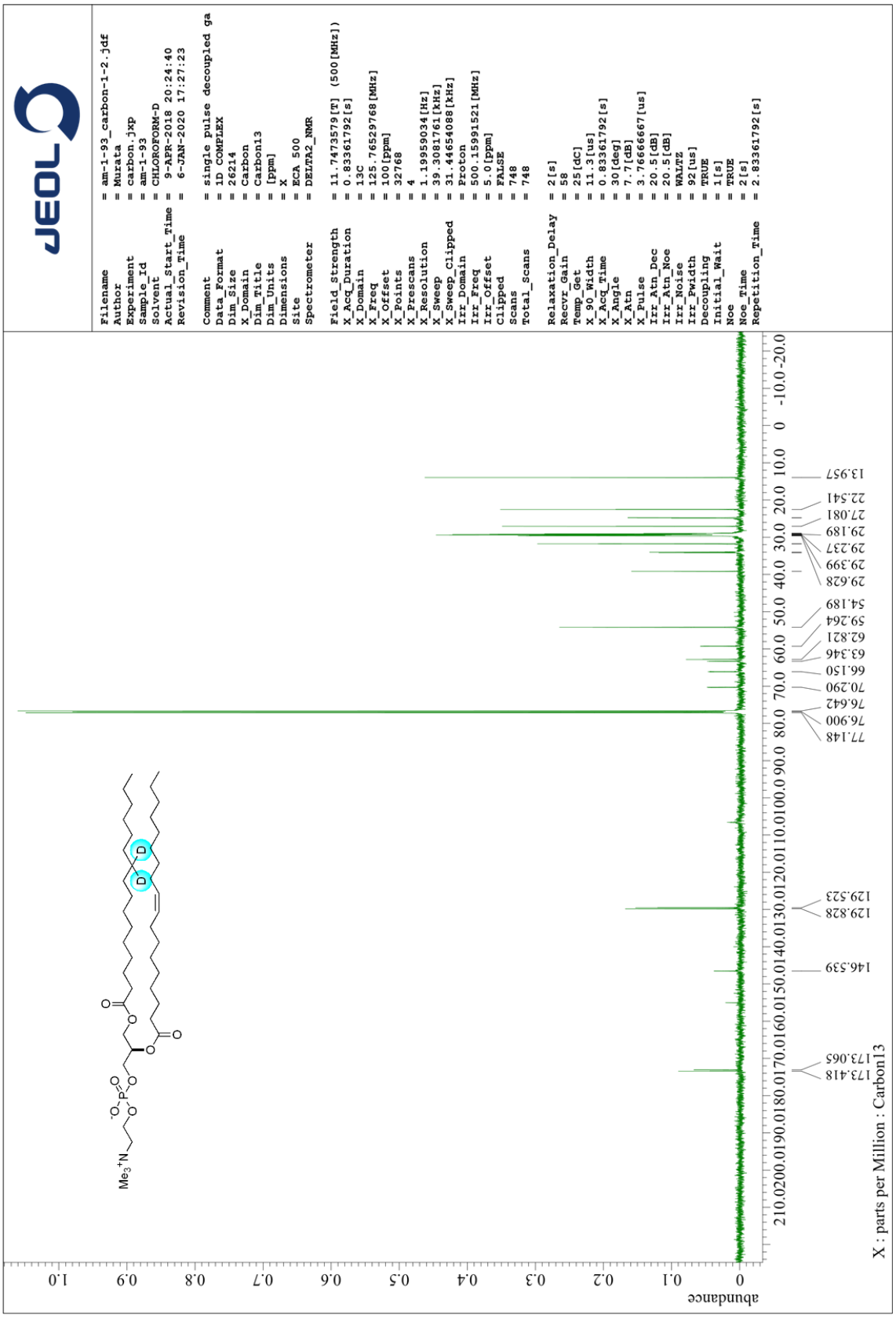
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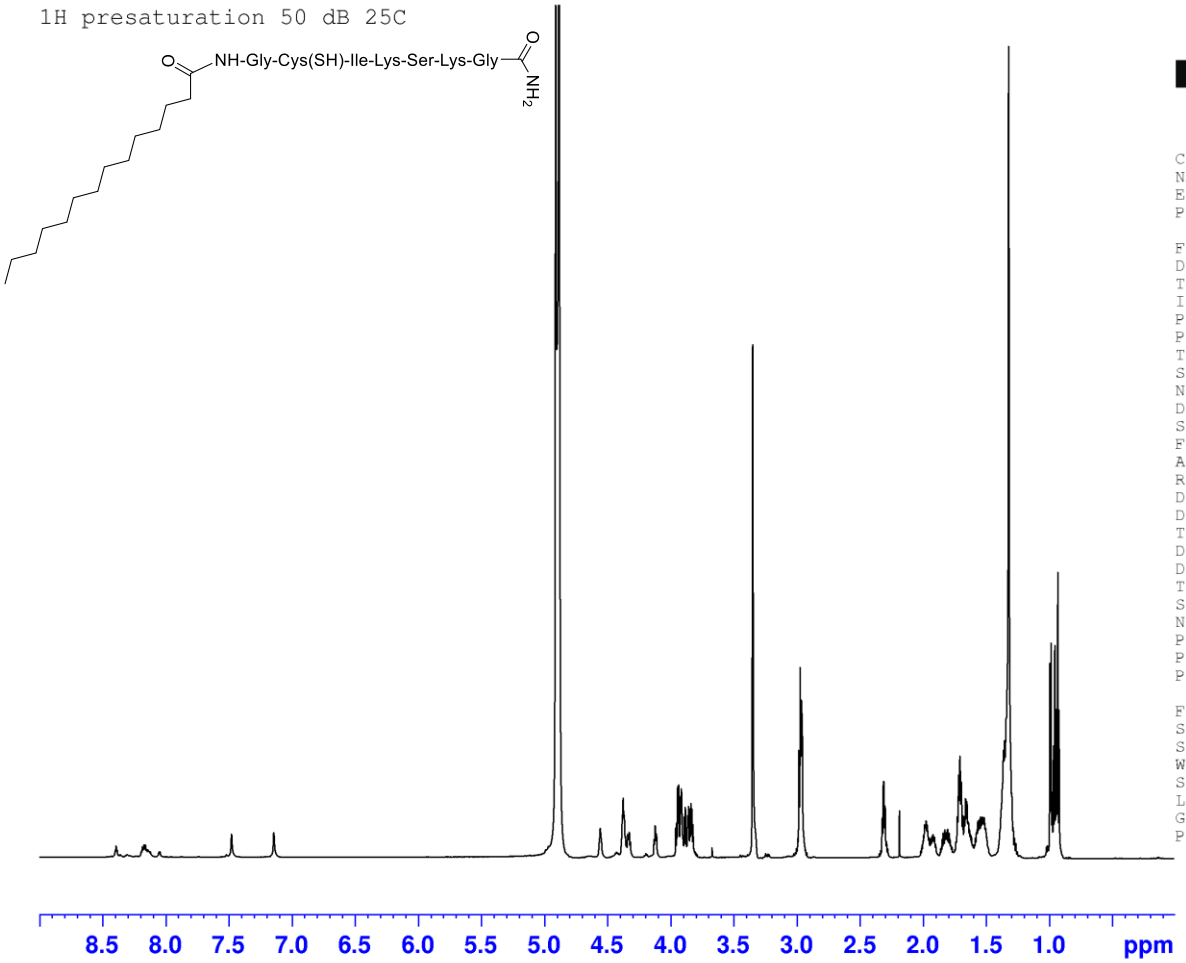


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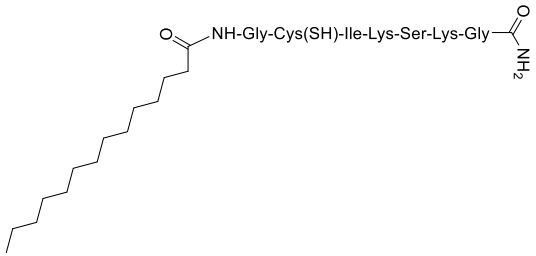


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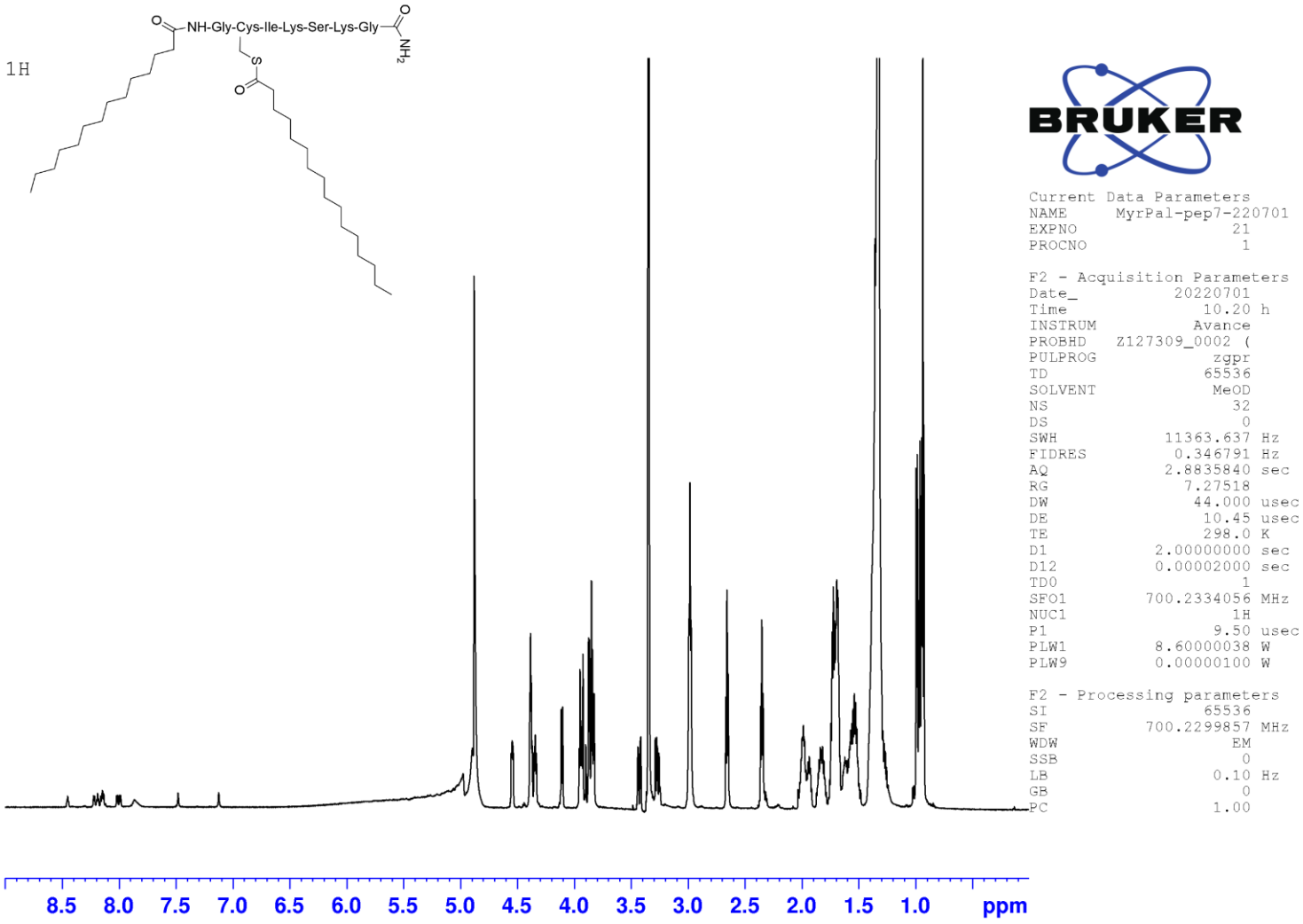


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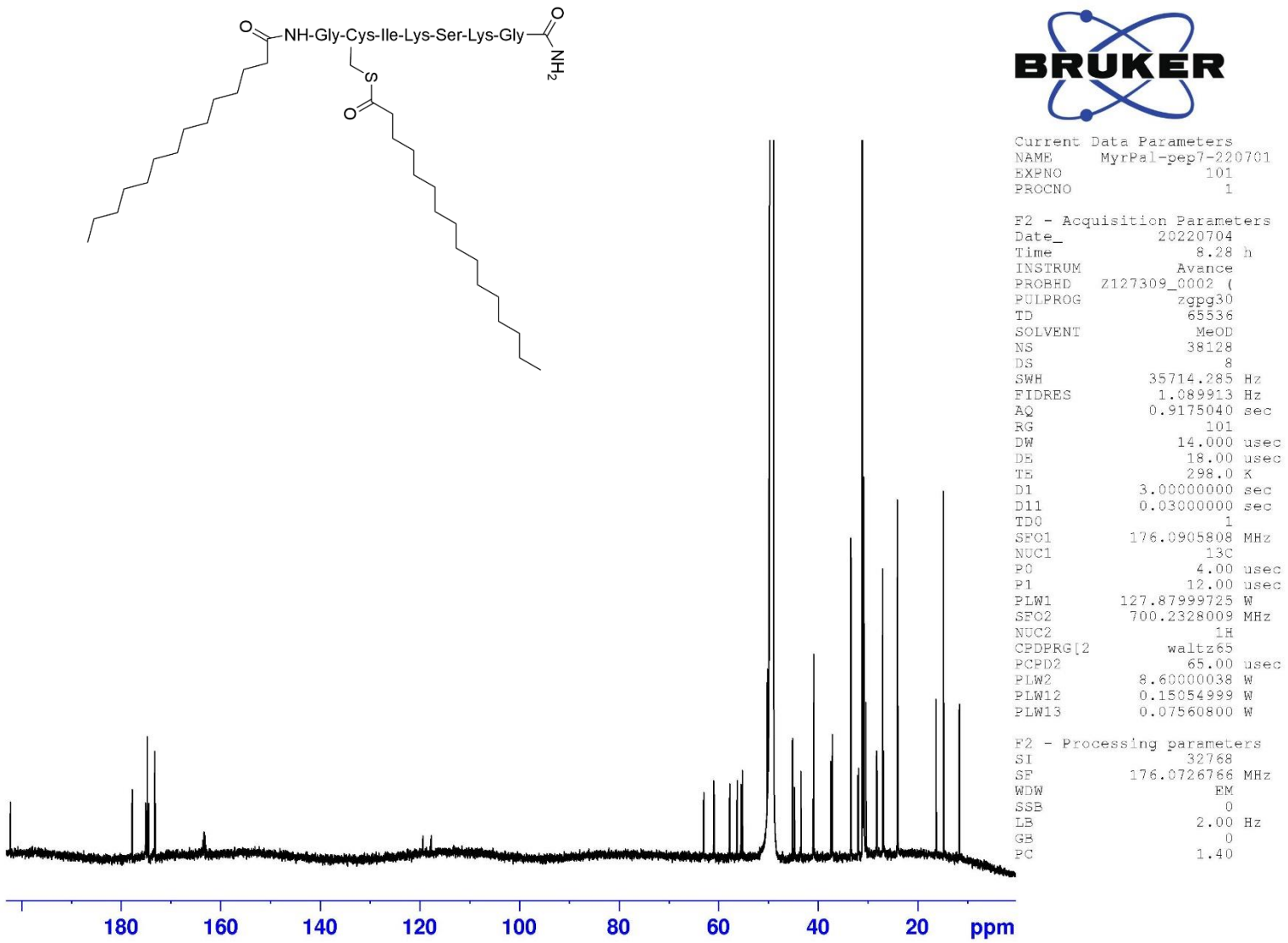

BRUKER

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