SUPPLEMENTARY INFORMATION for

Structure-function relationship of phase-separated liposomes containing diacylglycerol analogues

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Supplementary figures

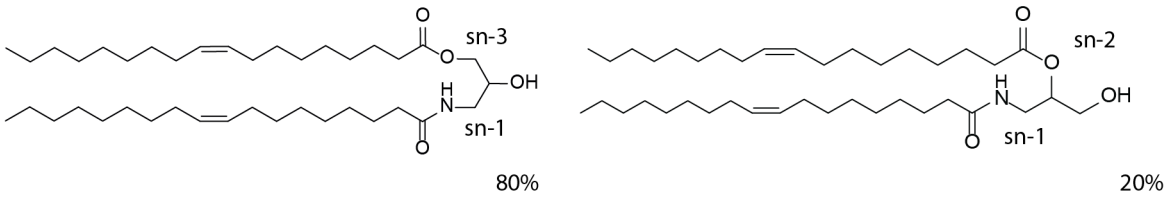
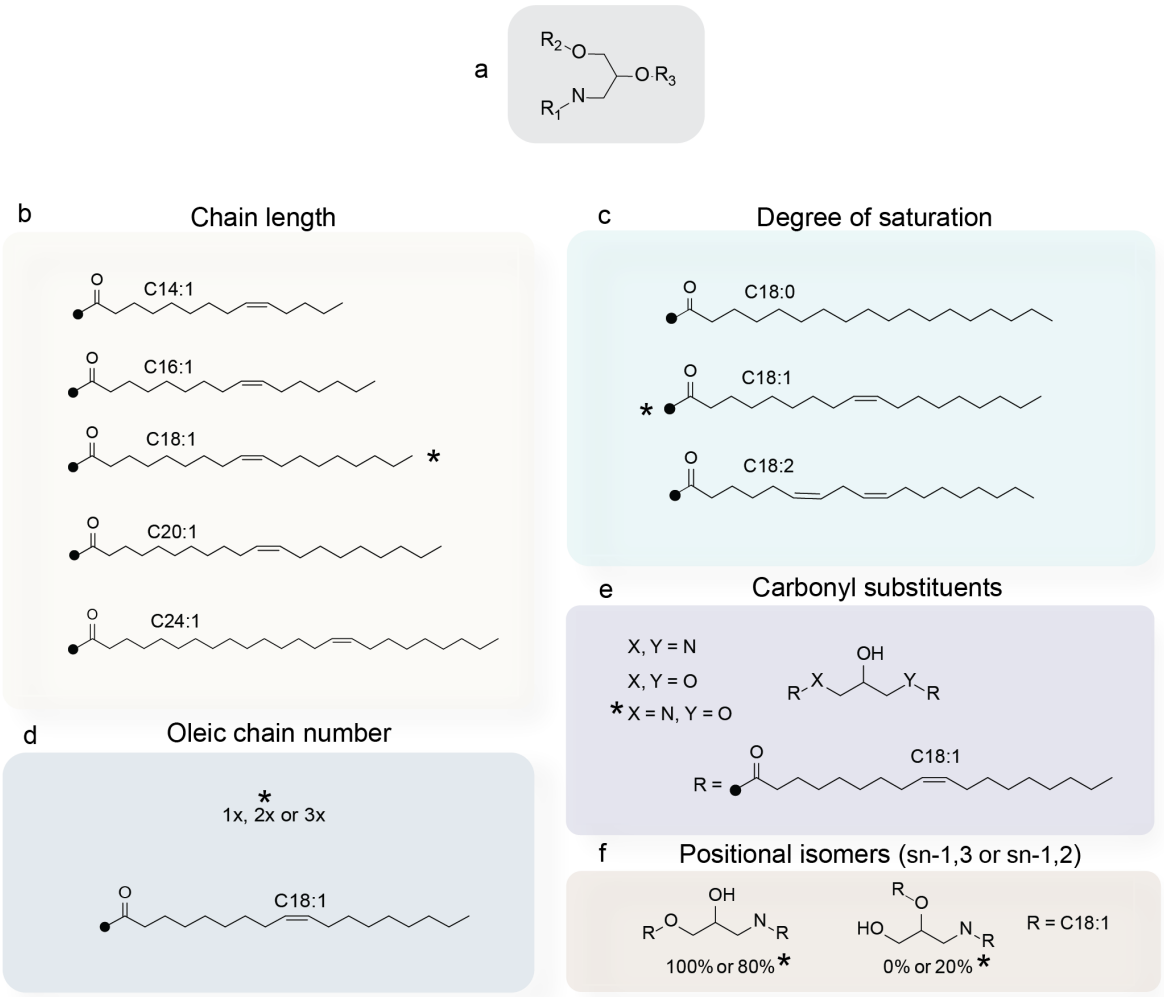


Figure S1. Positional isomers found as a mixture in DOaG lipid. Fatty acid tails substituting the *sn*-1,3 position (80%, majority) and *sn*-1,2 (20%). All lipids are also a racemic mixture.



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No.	Code	R_1	R_2	R_3	No.	Code	R_1	R_2	R_3
1	**C14:1**	C14:1	C14:1	H	**8**	**C18:2**	C18:2	C18:2	H
2	**C16:1**	C16:1	C16:1	H					
***3**	**C18:1**	C18:1	C18:1	H	**9**	**Monoacyl**	C18:1	H	H
4	**C20:1**	C20:1	C20:1	H	**10**	**Triacyl**	C18:1	C18:1	C18:1
5	**C24:1**	C24:1	C24:1	H	**11**	**N-N**	C18:1	C18:1	H
6	**C18:0**	C18:0	C18:0	H	**12**	**DOG**	C18:1	C18:1	H
7	**C18:0-C18:1**	C18:0	C18:1	H	**13**	***sn*-1,3**	C18:1	H	C18:1

Figure S2. Library of DOaG analogues. a) Glycerol-like DOaG backbone. DOaG analogues varying **b)** chain length, **c)** degree of saturation, **d)** number of fatty acid tails, **e)** carbonyl substituents of the backbone, **f)** positional isomers. **g)** table depicting the number and code of each lipid and the substitution of each *sn* position of the DOaG backbone. DOaG is indicated with *.

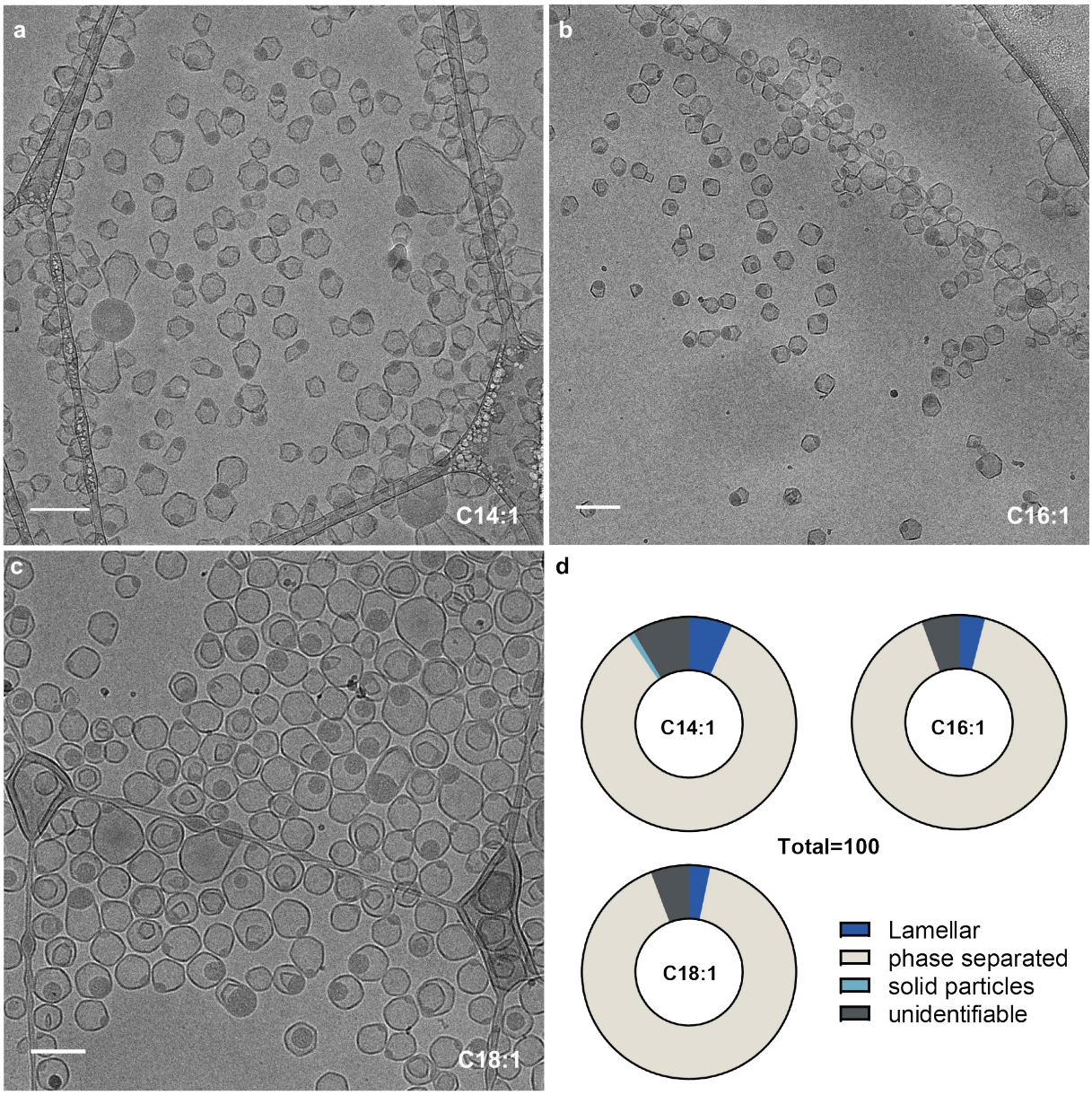


Figure S3. Cryo-TEM images of liposomes containing 1:1 molar ratio of DSPC and a) C14:1, **b)** C16:1, **c)** 18:1 (DOaG). **d)** Quantification of all populations in liposomal formulations containing 1:1 molar ratio of DSPC and C14:1, C16:1 or 18:1 (DOaG). Quantification based on cryo-TEM images a-c (N=100). Scale bars: 200 nm.

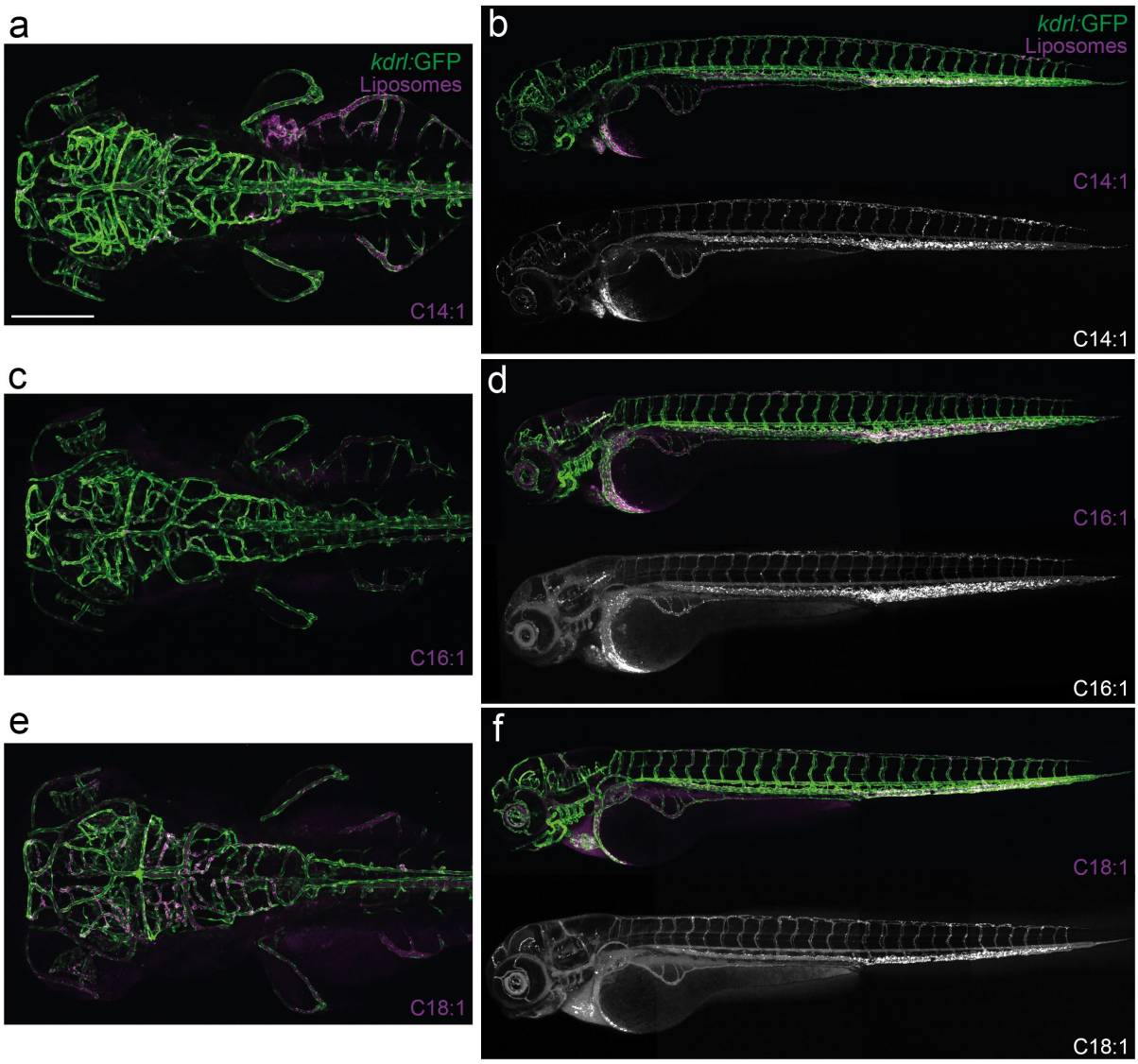


Figure S4. Biodistribution of liposomes containing 1:1 molar ratio of DSPC and **a, b)** C14:1, **c, d)** C16:1 and **e, f)** C18:1 (PAP3 liposomes) in Tg(kdrl:GPF) zebrafish embryos, expressing GFP in all endothelial cells, in dorsal (10x magnification) and lateral view, 1.5 hours post injection (hpi), at 78 days post fertilization (dpf). Liposomes in grey/magenta at 5mM total lipid concentration containing 0.2% DOPE-LR (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[lissamine rhodamine B sulfonyl]) for visualization. Vasculature in green. Scale bar for a, c, e: 200 μm.

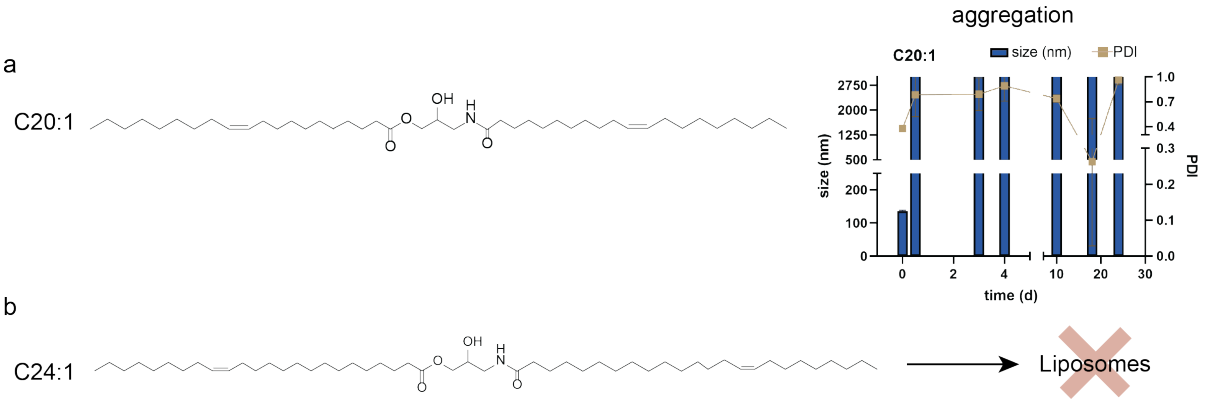


Figure S5. DOaG variants resulting in unstable formulations. a) Liposomes composed with DSPC and C20:1 variant (1:1 molar ratio) aggregate rapidly within ~6 hours as seen by size and polydispersity index (PDI) measurements (size and PDI limit of a formulation relevant for *in vivo* application = 200nm and 0.2, respectively). **b)** A mixture of DSPC and C24:1 (1:1 molar ratio) cannot be formulated into liposomes as the lipid film hydration step failed.

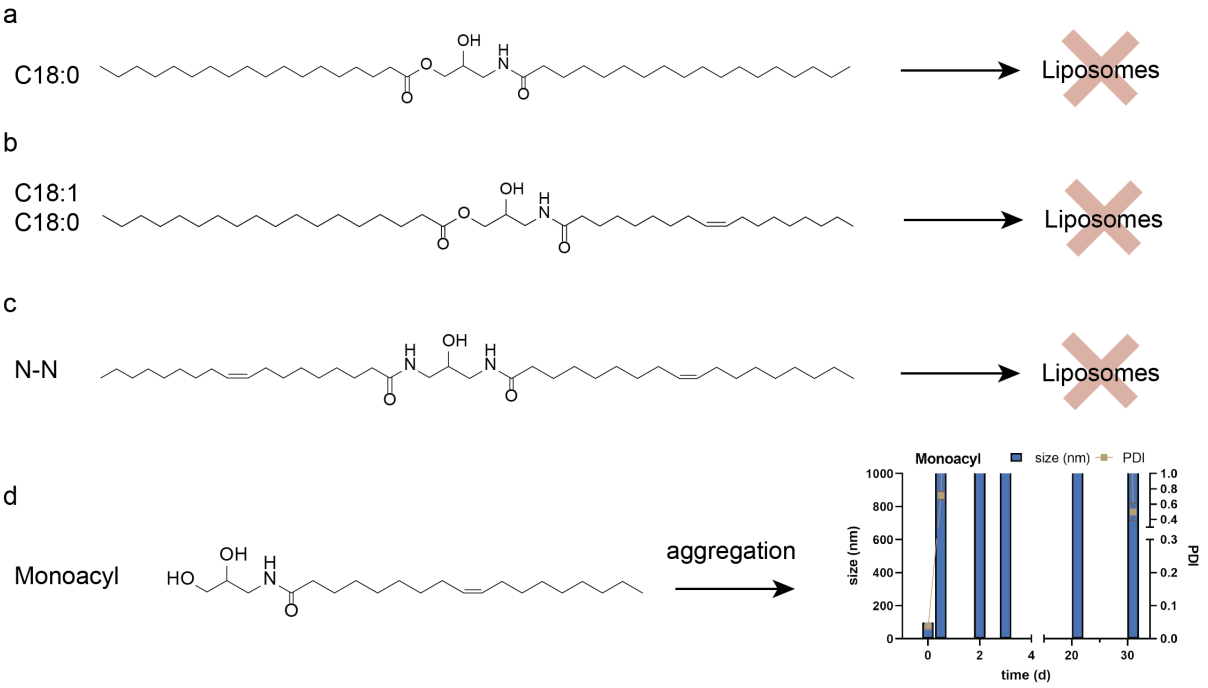


Figure S6. DOaG variants resulting in unstable formulations. Mixture of DSPC and **a)** C18:0, **b)** C18:0-C18:1 or **c)** N-N (in 1:1 molar ratio) cannot be formulated into liposomes as the lipid film hydration step failed. **d)** Liposomes composed with DSPC and monoacyl variant (1:1 ratio) aggregate rapidly within ~6 hours, as seen by size and PDI measurements (size and PDI limit of a formulation relevant for *in vivo* application = 200nm and 0.2, respectively).

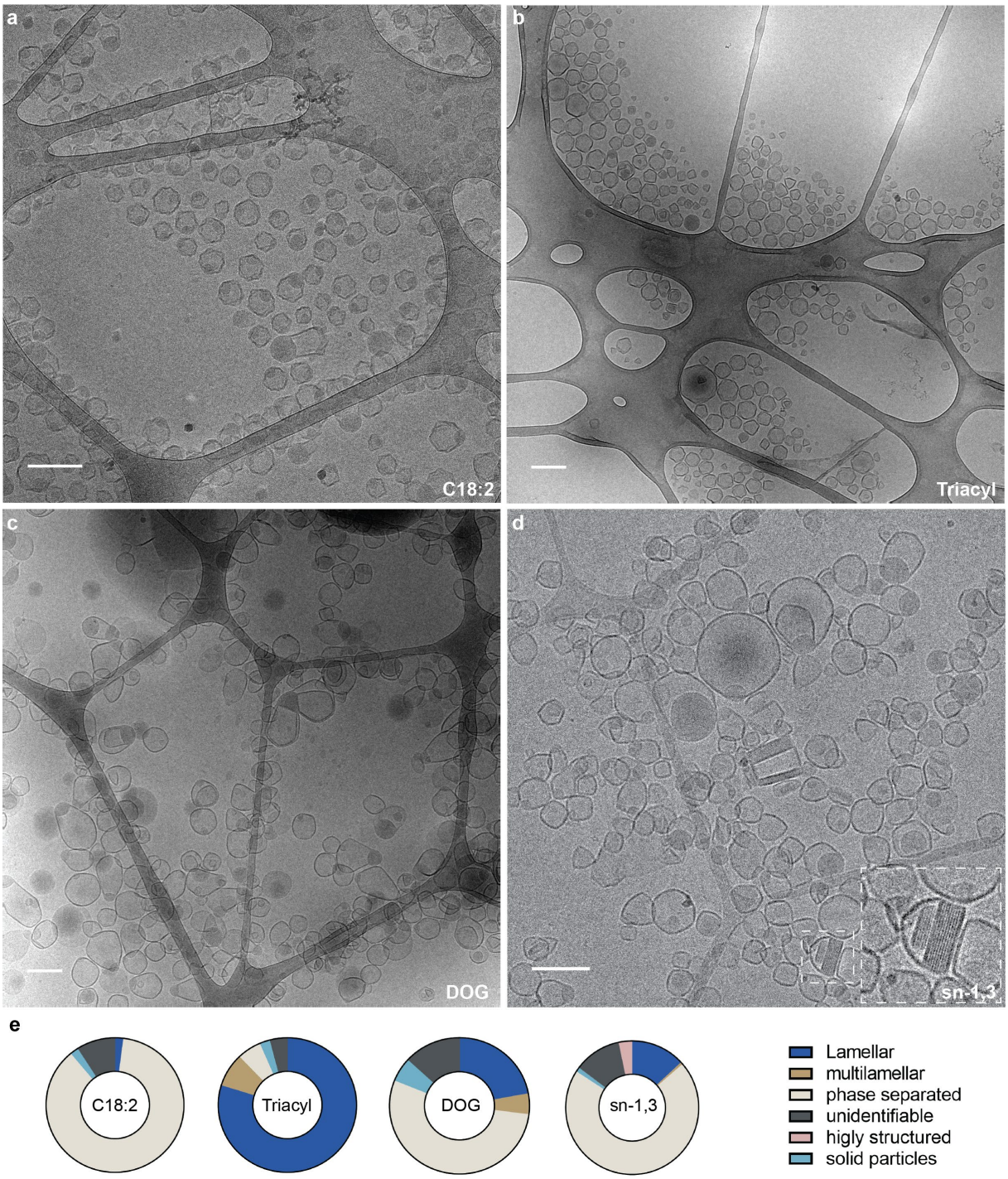


Figure S7. Cryo-TEM images of liposomes containing 1:1 molar ratio of DSPC and a) C18:2, **b)** triacyl, **c)** DOG or **d)** *sn*-1,3. **e**) Quantification of all populations found in liposomal formulations containing 1:1 molar ratio of DSPC and C18:2, triacyl, DOG or *sn*-1,3. Inset in d depicts highly structured, crystalline assemblies present in the formulation. Quantification based on cryo-TEM images a-d (N=100). Scale bars: 200 nm.

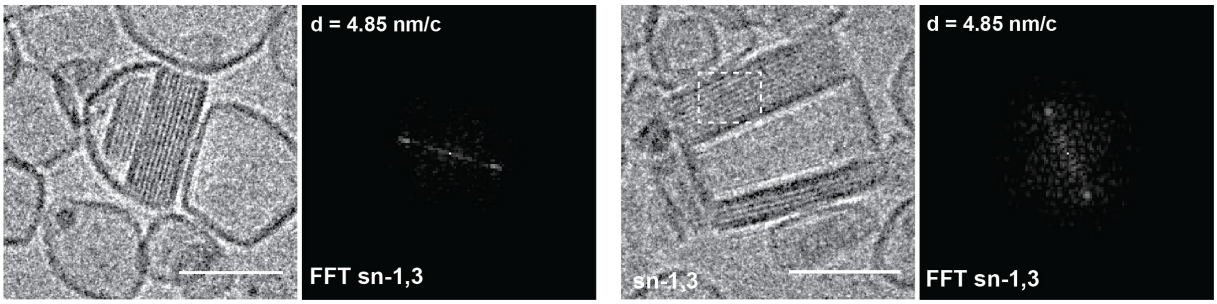


Figure S8. Liquid-crystalline assemblies as seen in *sn*-1,3 containing liposomes. Cryo-TEM images of liposomes containing the liquid-crystalline phases and average repeating distance of the lattice as indicated by FFT (nm per repeating cycle). Scale bars: 100 nm.

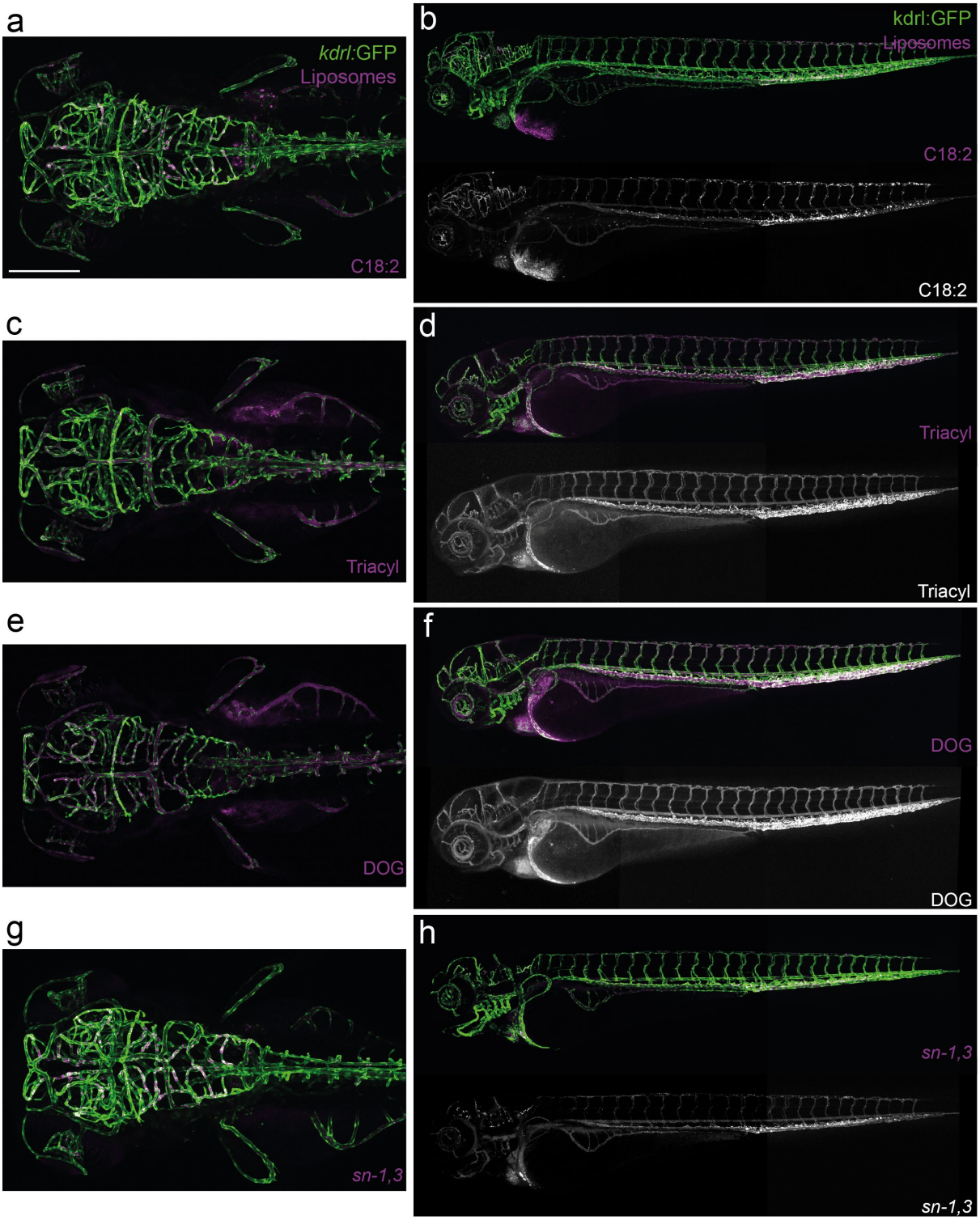


Figure S9. Biodistribution of liposomes containing 1:1 molar ratio of DSPC and **a, b)** C18:2, **c, d)** Triacyl, **e, f)** DOG and **g, h)** *sn*-1,3 in Tg(kdrl:GPF) zebrafish embryos, expressing GFP in all endothelial cells, in dorsal (10x magnification) and lateral view, 1.5 hpi, at 78 dpf. Liposomes in grey/magenta at 5mM total lipid concentration containing 0.2% DOPE-LR for visualization. Vasculature in green. Scale bar for a, c, e, g: 200 µm.

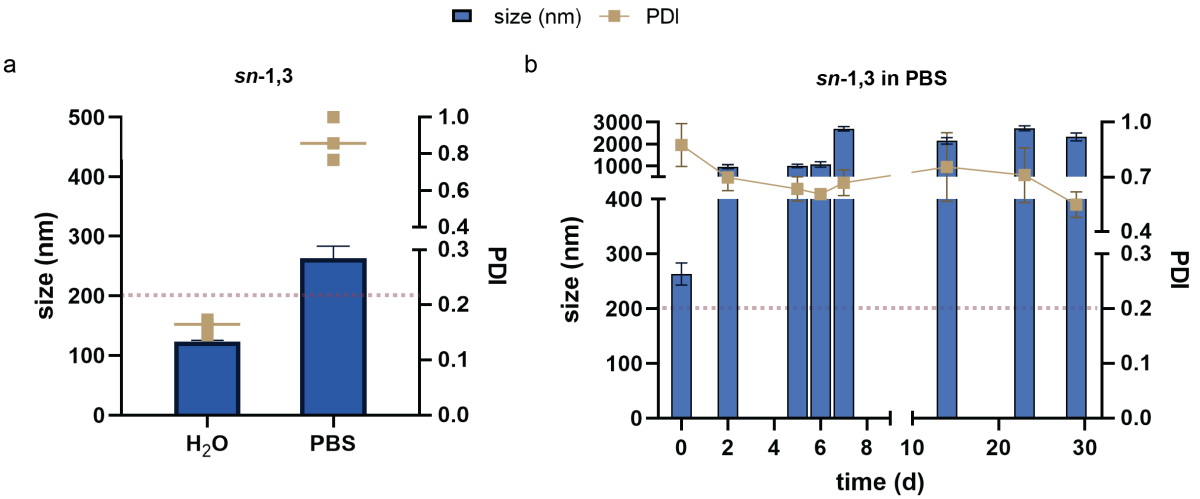


Figure S10. Stability of liposomes containing DSPC and *sn*-1,3 (1:1) made in ddH_2O and PBS. a) Size (nm) and PDI measurement of liposomes containing DSPC and *sn*-1,3 (1:1) made in ddH_2O and PBS. b) Size and PDI of liposomes containing DSPC and *sn*-1,3 (1:1) made in PBS over a period of ~30 days. Red dashed line indicates the threshold of size and PDI relevant for *in vivo* use.

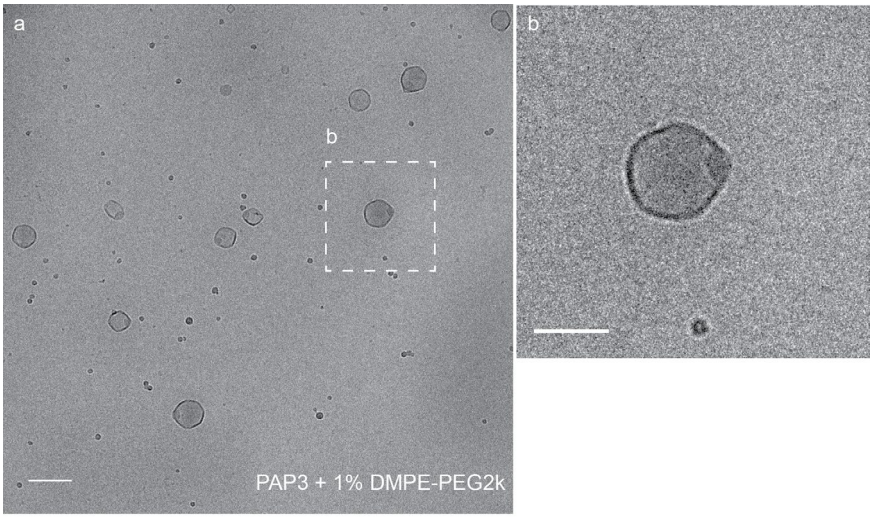


Figure S11. Cryo-TEM images of PAP3 liposomes containing 1% mol DMPE-PEG2k. a) Low magnification image and b) inset from **a** (white box). Scale bar = 200 nm for (a) and 100 nm for (b).

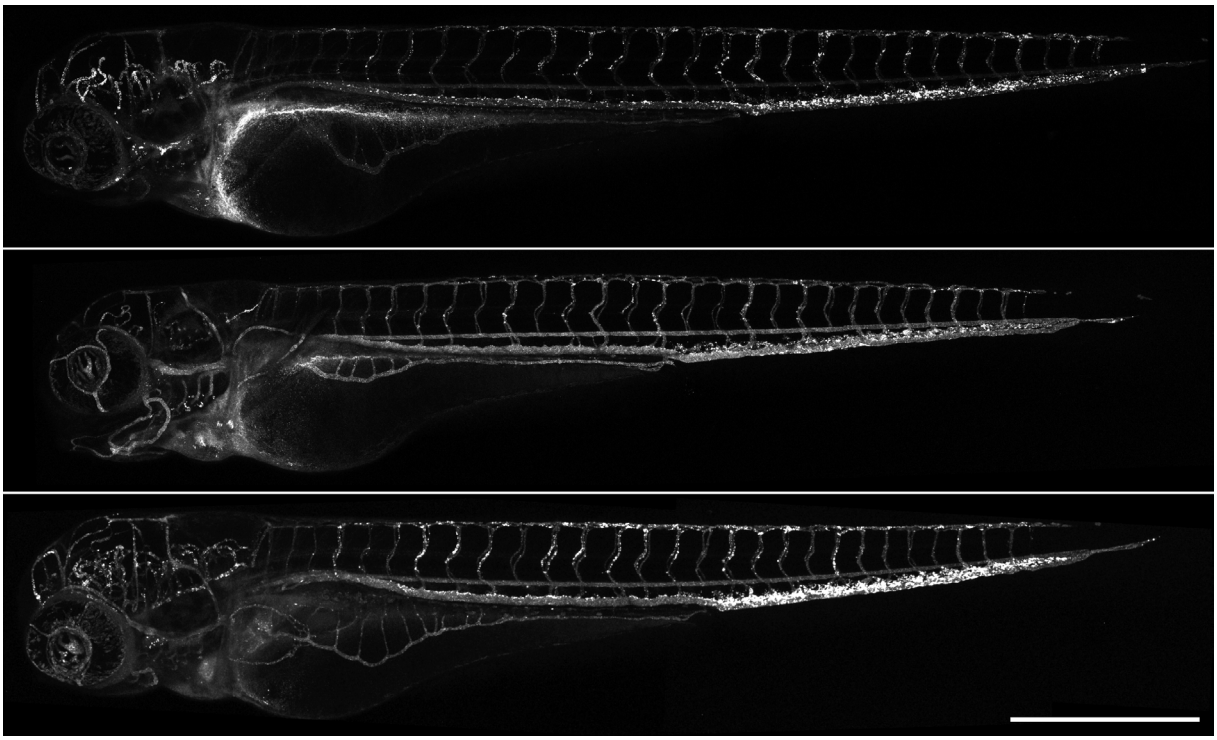


Figure S12. Biodistribution of PAP3 liposomes coated with 1 % mol DMPE-PEG2k in AB/TL zebrafish embryos (wild type), in lateral view, 2.5 hpi at 78 dpf. Liposomes in grey at 5mM total lipid concentration containing 0.2% DOPE-LR for visualization. Scale bar: 500 µm.

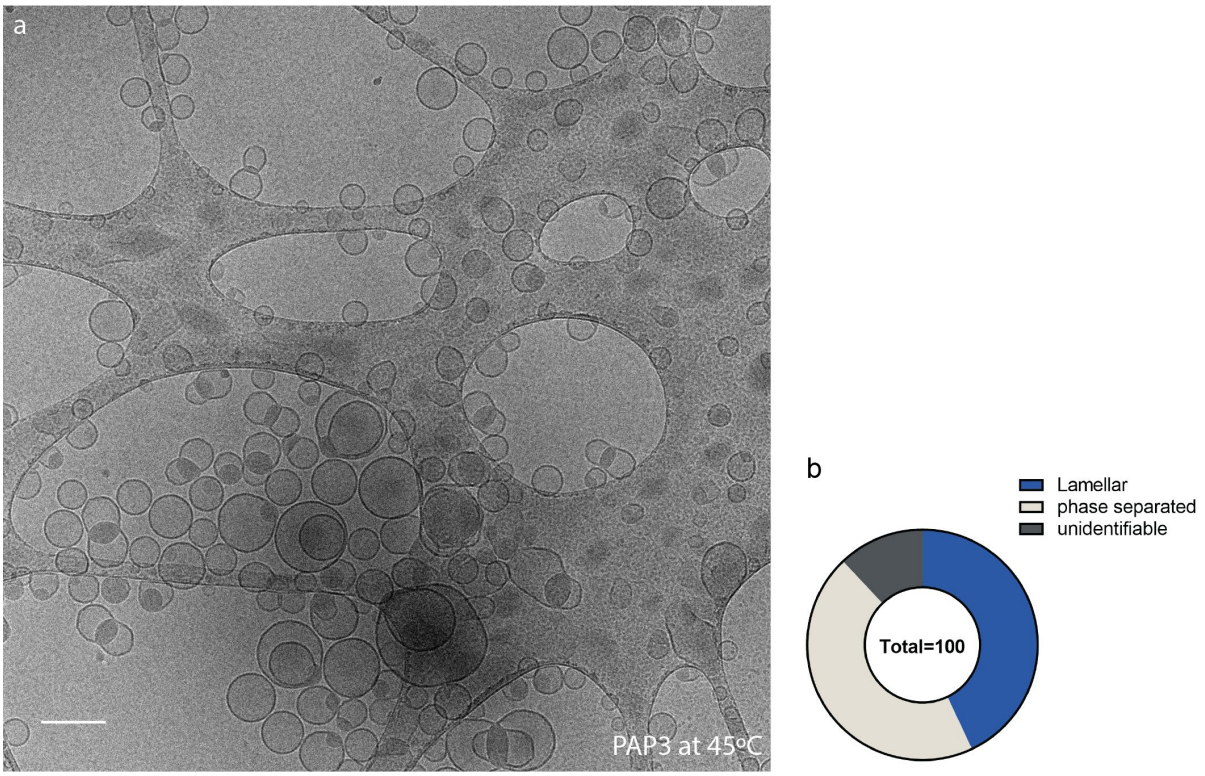


Figure S13. PAP3 liposomes at 45 < T < 65 ºC. a) Cryo-TEM image of PAP3 liposomes cooling to 45 ºC, immediately after formation at 65 ºC. **b)** Quantification of all populations found in the formulation. Quantification based on cryo-TEM image a (N=100). Scale bar = 200 nm.

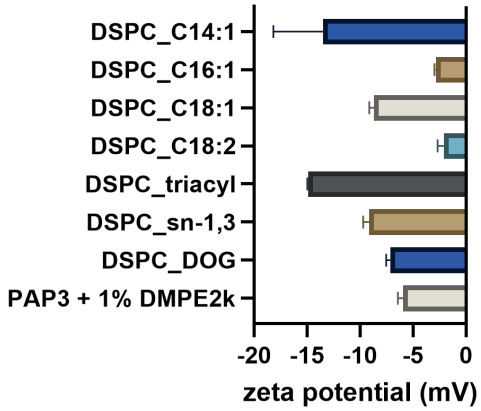


Figure S14. Zeta potential (mV) of stable formulations containing DSPC and DOaG analogues (1:1).

Materials and Methods

General reagents

1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (DOPE-LR), were purchased from Avanti Polar Lipids (Alabaster, AL, US). Additional DSPC was purchased from Lipoid GmbH. All other chemical reagents were purchased at the highest grade available from Sigma Aldrich and used without further purification. All solvents were purchased from Biosolve Ltd. Ultrapure MilliQ® water, purified by a H_2O Advantage A10 water purification system from MilliPore, was used throughout.

Cryo-TEM Quantification

Software Fiji (ImageJ) was used for image processing and quantification. One or more low magnification images were used to visualize at least 100 nanoparticles. Particles were counted and divided into categories (lamellar, multilamellar, phase separated, solid particles), according to their morphology. Liposomes whose morphology was not able to be identified were marked as "unidentifiable" and the number obtained was used as standard deviation for the rest of population. Liposomes that were observed to be on top or in close contact with the copper grid or overlapping with each other were excluded from the quantification.

Zeta potential measurement

Zeta potential of each formulation was measured at 500 µM total lipid concentration, using a dip-cell electrode (Malvern), at room temperature. For liposomes formulated in water, aq. NaCl was added to the liposome solution prior to the measurement, to a final concentration of 10 mM. Total NaCl concentration was <20 mM for all measurements.

Synthesis and characterization of DAG analogues

Column Chromatography was performed using silica gel (40-63 µm, 60 Å, Screening Devices, The Netherlands). TLC analysis was performed on Merck silica gel 60/Kieselguhr F254, 0.25 mm TLC plates and compounds were visualized using a $KMnO_4$ stain (10 mg/ml $KMnO_4$ and

75 mg/ml K_2CO_3 in H_2O). ^1H spectra were recorded on a Bruker AV 400 MHz spectrometer. Chemical shifts are reported in ppm (δ), relative to the deuterated solvent as internal standard. Data are reported as follows: chemical shifts (δ), multiplets (s = singlet, d = doublet, dd = doublet of doublets, td = triplet of doublets, t = triplet, q = quartet, m = multiplet), coupling constants (J) reported in Hz.

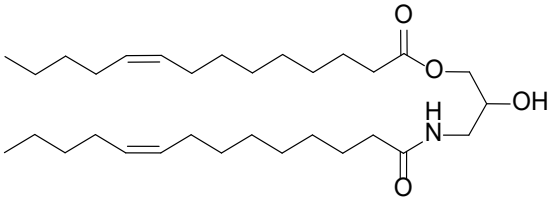
High resolution mass spectra were recorded by direct injection (2 µL of a 1 µM solution in methanol) using a mass spectrometer (Q-Exactive HF Orbitrap) with an electrospray ion source (ESI) run in positive mode (source voltage 3.5 kV, capillary temperature 275 ºC, no sheath gas), and with a resolution R = 240000 at m/z = 400 (mass range m/z = 160-2000). Eluents used: MeCN:H_2O (1:1 v/v) supplemented with 0,1% formic acid. For lipids **1** and **16,** high resolution mass spectra were recorded by direct injection (2 µL of a 2 µM solution in methanol) using a mass spectrometer (Synapt G2-Si [Waters]) with an electrospray ion source (ESI-TOF) run in positive mode, with LeuEnk(m/z=556.2771) as "lock mass". Source voltage of 3.5 kV, 275 ºC as temperature. Mass range m/z = 160-2000. The high-resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan).

Synthesis of DOaG and DOG lipids

DOaG and DOG lipids were synthesized as previously reported.[1]

All lipids (except lipids **7**, **9**, **10**, **12** and **21**) were isolated as regioisomeric mixtures: 80% isomer where acyl chains substituting the *sn*-1,3 positions of the backbone and 20% isomer where acyl chains substituting the *sn*-1,2 positions of the backbone as determined by ^1H NMR.

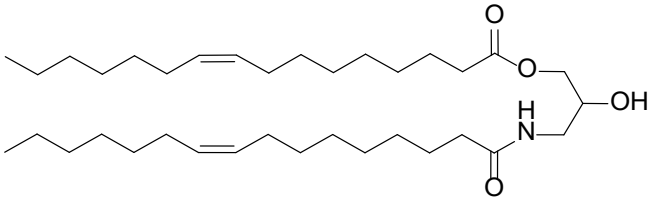
(1) C14:1; 2-hydroxy-3-((Z)-tetradec-9-enamido)propyl (Z)-tetradec-9-enoate



In a round bottom flask, (±)-3-amino-1,2-propane diol (10.59 mg, 0.12 mmoL), DMAP (35.51 mg, 0.29 mmoL), DIPEA (37.49 mg, 0.29 mmoL) and EDC (45.00 mg, 0.29 mmoL) were dissolved in CH_2CL_2 (10 mL). Myristoleic acid (50.00 mg, 0.22 mmoL) was added to the solution and the reaction mixture was allowed to stir overnight. The reaction mixture was washed with sat. NH_4Cl (10 mL), followed by dd. H_2O (3 x 20 mL), brine (20 mL) and subsequently was dried (with Na_2SO_4), filtered and concentrated *in vacuo*. Purification by Column Chromatography (EtOAc: hexane; graduate elution from 0:100 to 20:80) yielded compound **1** as a white solid (26.96 mg, 0.053 mmoL).

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.00 (t, J = 6.0 Hz, 1H), 5.43 – 5.27 (m, 4H), 4.15 (dd, J = 11.5, 5.1 Hz, 1H), 4.05 (dd, J = 11.5, 5.8 Hz, 1H), 3.93 (qd, J = 5.8, 3.4 Hz, 1H), 3.65 – 3.46 (m, 1H), 3.23 (m, 1H), 2.33 (td, J = 7.7, 5.6 Hz, 2H), 2.27 – 2.17 (m, 2H), 2.08 – 1.96 (m, 8H), 1.62 (q, J = 7.1 Hz, 4H), 1.30 (d, J = 4.0 Hz, 24H), 0.94 – 0.81 (t, J = 8 Hz, 6H); **ESI-HRMS** (m/z) [M+H] +: calcd. for $C_{31}H_{57}NO_4$, 507.4360; found 507.4367, delta =1.4 ppm.

(2) C16:1; 3-((Z)-hexadec-9-enamido)-2-hydroxypropyl (Z)-hexadec-9-enoate

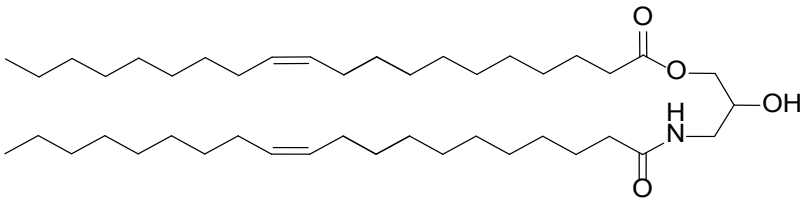


In a round bottom flask, (±)-3-amino-1,2-propane diol (100.0 mg, 1.10 mmoL), DMAP (336.0 mg, 2.75 mmoL), DIPEA (479 µL, 2.75 mmoL) and EDC (427.0 mg, 2.75 mmoL) were dissolved in CH_2Cl_2 (10 mL). Palmitoleic acid (530.5 mg, 2.10 mmoL) was added to the solution and the reaction mixture was allowed to stir overnight. The reaction mixture was washed with sat. NH_4Cl solution (10 mL), followed by dd. H_2O (3 x 10 mL), brine (10 mL) and subsequently was dried (Na_2SO_4), filtered and concentrated *in vacuo*. Purification by Column

Chromatography (EtOAc : CH_2CL_2; 10:90 to 20:80) yielded compound **2** as a white solid (247.0 mg, 0.44 mmoL).

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.40 (t, J = 5.8 Hz, 1H), 5.36 – 5.23 (m, 4H), 4.10 – 4.00 (m, 2H), 3.93 – 3.81 (m, 1H), 3.64 – 3.37 (m, 1H), 3.21 (dt, J = 14.1, 6.1 Hz, 1H), 2.34 – 2.24 (m, 2H), 2.17 (dd, J = 9.5, 5.8 Hz, 2H), 1.97 (q, J = 6.3 Hz, 8H), 1.58 (t, J = 7.4 Hz, 4H), 1.25 (d, J = 9.3 Hz, 32H), 0.88 – 0.80 (t, J = 8 hz, 6H). **ESI-HRMS** (m/z) $[M+H]^+$: calcd. for $C_{35}H_{65}NO_4$, 563.4986; found 563.4988, delta =0.4 ppm.

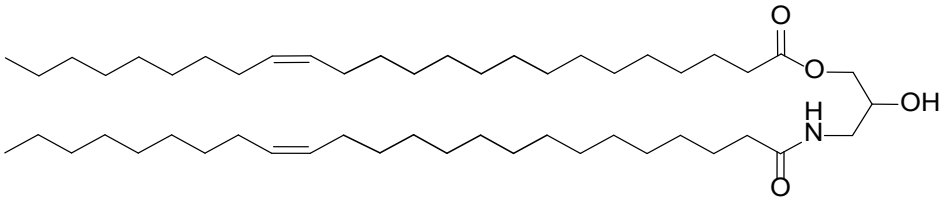
(4) C20:1; 2-hydroxy-3-((Z)-icos-11-enamido)propyl (Z)-icos-11-enoate



In a round bottom flask, (±)-3-amino-1,2-propane diol (7.70 mg, 0.08 mmoL), DMAP (25.66 mg, 0.21 mmoL), DIPEA (27.14mg, 0.21 mmoL) and EDC (32.60 mg, 0.21 mmoL) were dissolved in CH_2Cl_2 (10 mL). Eicosenoic acid (50 mg, 0.16 mmoL) was added to the solution and the reaction mixture was allowed to stir overnight. The reaction mixture was washed with sat. NH_4Cl solution (10 mL), followed by dd. H_2O (3 x 10 mL), brine (10 mL) and subsequently was dried (Na_2SO_4), filtered and concentrated *in vacuo*. Purification by Column Chromatography (EtOAc : CH_2CL_2; 0:100 to 20:80) yielded compound **4** as a white solid (12.00 mg, 0.02 mmoL).

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.98 (t, J = 5.9 Hz, 1H), 5.39 – 5.28 (m, 4H), 4.16 (dd, J = 11.5, 5.1 Hz, 1H), 4.05 (dd, J = 11.5, 5.9 Hz, 1H), 3.94 (qd, J = 5.9, 3.4 Hz, 1H), 3.65 – 3.46 (m, 1H), 3.24 (dt, J = 14.3, 6.0 Hz, 1H), 2.40 – 2.28 (m, 2H), 2.23 (td, J = 7.6, 4.9 Hz, 2H), 2.01 (q, J = 6.0 Hz, 8H), 1.62 (q, J = 6.5, 4.9 Hz, 4H), 1.26 (d, J = 4.4 Hz, 48H), 0.90 – 0.85 (t, J = 8 hz, 6H). **ESI-HRMS** (m/z) [M+H] +: calcd. for $C_{43}H_{81}NO_4$, 676.6238; found 676.6231, delta =1.0 ppm.

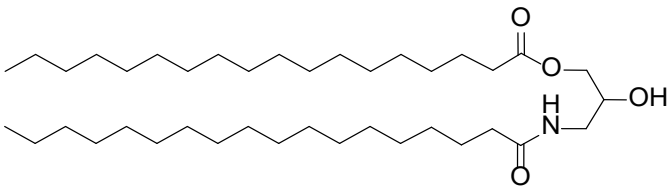
(5) C24:1; 2-hydroxy-3-((Z)-tetracos-15-enamido)propyl (Z)-tetracos-15-enoate



In a round bottom flask containing stirred solution of (±)-3-amino-1,2-propanediol (3.90 mg, 0.04 mmoL) in 10 mL CH_2Cl_2, nervonic acid (31.40 mg, 0.09 mmoL), DMAP (19.80 mg, 0.16 mmoL), DIPEA (13.30 mg, 0.10 mmoL) and EDC (21.84 mg, 0.14 mmoL) were added. To this solution 2 mL THF were added. The reaction mixture was allowed to stir overnight at room temperature and subsequently the mixture was washed with sat. NH_4Cl solution (10 mL), followed by dd. H_2O (3 x 10 mL), brine (10 mL). Next, the mixtrure was dried (Na_2SO_4), filtered and concentrated *in vacuo*. Purification by Column Chromatography (CH_2Cl_2 to 20% EtOAc in CH_2Cl_2), yielded the target compound **5** (17.30 mg, 0.02 mmoL).

TLC (CH_2Cl_2:EtOAc, 8:2 v/v) : Rf = 0.26 ; **^{1}H NMR** (400 MHz, $CDCl_3$) δ 5.95 (t, J = 10.2 Hz 1H), 5.41 – 5.28 (m, 4H), 4.16 (dd, J = 11.5, 5.2 Hz, 1H), 4.05 (dd, J = 11.5, 5.9 Hz, 1H), 3.93 (dt, J = 9.1, 5.7 Hz, 1H), 3.66 – 3.48 (m, 1H), 3.27 – 3.19 (m, 1H), 2.34 (dq, J = 7.8, 5.7, 5.0 Hz, 2H), 2.22 (td, J = 7.7, 5.7 Hz, 2H), 2.01 (q, J = 6.5 Hz, 8H), 1.62 (d, J = 7.4 Hz, 4H), 1.26 (d, J = 4.0 Hz, 64H), 0.93 – 0.84 (t, J = 8 Hz, 6H). **ESI-HRMS** (m/z) [M+H]+: calcd. for $C_{51}H_{97}NO_4$, 788.7490; found 788.7485, delta = 0.7 ppm.

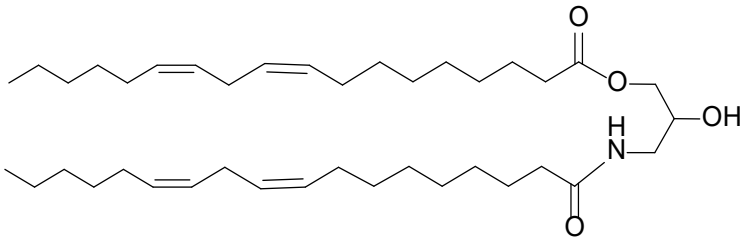
(6) C18:0; 2-hydroxy-3-stearamidopropyl stearate



In a round bottom flask containing stirred solution of (±)-3-Amino-1,2-propanediol (100.0 mg, 1.10 mmoL) in THF (~15 mL), stearic acid (594.5 mg, 2.10 mmoL), EDCI (426.9 mg, 2.75 mmoL), DMAP (336.0 mg, 2.75 mmoL) and DIPEA (355.0 mg, 2.75 mmoL) were added. After overnight stirring at RT and subsequent evaporation of THF, the reaction mixture was diluted with $CHCl_3$, washed with sat. NH_4Cl (~15 mL) and brine (~15 mL) and subsequently was dried (Na_2SO_4), filtered *in vacuo* and concentrated. Purification by Column Chromatography (CH_2Cl_2 to 20% EtOAC in CH_2Cl_2) yielded the target material **6**.

Rf: 0.4 (CH_2Cl_2: EtOAc_8:2), **^{1}H NMR** (400 MHz, $CDCl_3$) δ 5.94 (t, J = 8 Hz 1H), 4.16 (dd, J = 11.5, 5.2 Hz, 1H), 4.05 (dd, J = 11.5, 5.8 Hz, 1H), 3.93 (qd, J = 5.7, 3.3 Hz, 1H), 3.56 (m, 1H), 3.23 (dt, J = 14.3, 5.9 Hz, 1H), 2.34 (t, J = 7.6 Hz, 2H), 2.26 – 2.18 (m, 2H), 1.62 (s, 4H), 1.25 (s, 56H), 0.91 – 0.84 (t, J = 8 Hz, 6H).

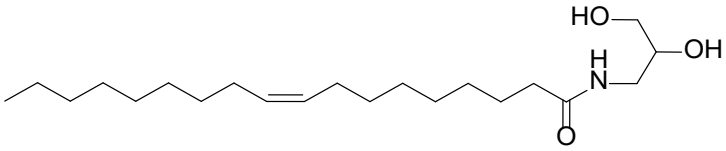
(8) C18:2; 2-hydroxy-3-((9Z,12Z)-octadeca-9,12-dienamido)propyl (9Z,12Z)-octadeca-9,12-dienoate



In a round bottom flask containing stirred solution of (+-)-3-amino-1,2-propanediol (54.50 mg, 0.60 mmoL) in 50 mL CH_2Cl_2, linoleic acid (336.5 mg, 1.20 mmoL), DMAP (205.1 mg, 1.68 mmoL), DIPEA (191.3 mg, 1.48 mmoL) and EDC (280.2 mg, 1.80 mmoL) were added. Next, 10 mL THF was added to the solution so that the solvent mixture was in a CH_2Cl_2:THF (5:1) ratio. The reaction mixture was stirred overnight at RT, then it was washed with saturated NH_4Cl solution and brine, and the organic phase was dried with Na_2SO_4, filtered *in vacuo*, and concentrated under reduced pressure. Purification by Column Chromatography (CH_2Cl_2 to 40% EtOAc in CH_2Cl_2) yielded the target compound **8** as a white solid (29.40 mg, 0.05 mmoL).

TLC (CH_2Cl_2:EtOAc, 8:2 v/v) : Rf = 0.45 ; **^{1}H NMR** (400 MHz, $CDCl_3$) δ 6.09 (t, J = 6 Hz, 1H), 5.43 – 5.25 (m, 8H), 4.13 (dd, J = 11.5, 5.1 Hz, 1H), 4.05 (dd, J = 11.5, 5.7 Hz, 1H), 3.92 (qd, J = 5.7, 3.3 Hz, 1H), 3.65 – 3.46 (m, 1H), 3.23 (dt, J = 14.2, 5.9 Hz, 1H), 2.76 (t, J = 6.6 Hz, 4H), 2.37 – 2.27 (m, 2H), 2.21 (td, J = 7.6, 4.8 Hz, 2H), 2.04 (q, J = 6.9 Hz, 8H), 1.61 (dq, J = 11.2, 7.1, 6.3 Hz, 4H), 1.38 – 1.22 (m, 28H), 0.90 – 0.85 (t, J = 8 Hz, 6H) ; **ESI-HRMS** (m/z) [M+H]+: calcd. for $C_{39}H_{69}NO_4$, 616.5299; found 616.5299, delta = 0.1 ppm.

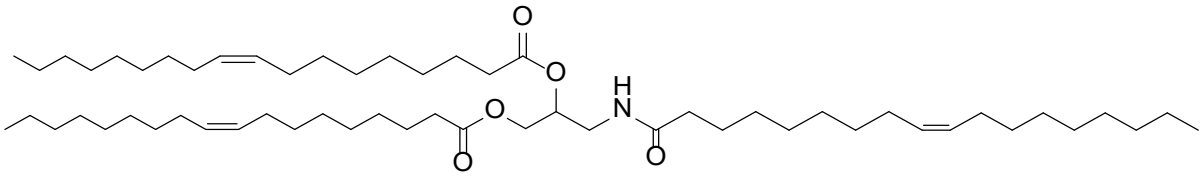
(9) monoacyl; N-(2,3-dihydroxypropyl)oleamide



In a round bottom flask containing stirred solution of (±)-3-Amino-1,2-propanediol (200.0 mg, 2.20 mmoL) in CH_2Cl_2:THF (5:1) (50 mL), oleic acid (496.0 mg, 1.76 mmoL), EDC (402.0 mg, 2.60 mmoL), DMAP (268.0 mg, 2.20 mmoL) and DIPEA (284.0 mg, 2.20 mmoL) were added. After overnight stirring at RT and subsequent evaporation of CH_2Cl_2 and THF, the reaction mixture was diluted with $CHCl_3$, washed with sat. NH_4Cl (~25 mL) and brine (~25 mL) and subsequently was dried (Na_2SO_4), filtered in vacuo and concentrated, so the crude compound was obtained. Purification by Column Chromatography (CH_2Cl_2 to 50% EtOAc in CH_2Cl_2) yielded the target material **9** as a white solid (469.3 mg, 1.32 mmoL).

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.22 (t, J = 6.1 Hz, 1H), 5.42 – 5.26 (m, 2H), 3.75 (t, J = 4.9 Hz, 1H), 3.64 – 3.49 (m, 4H), 3.39 (m, 2H), 2.25 – 2.17 (m, 2H), 2.00 (q, J = 6.4 Hz, 4H), 1.61 (t, J = 7.4 Hz, 2H), 1.27 (d, J = 12.0 Hz, 20H), 0.94 – 0.83 (t, J = 8.0 Hz, 3H). **ESI-HRMS** (m/z) [M+H]+: calcd. for $C_{21}H_{41}NO_3$, 355.3159; found 355.3158, delta = 0.3 ppm.

(10) triacyl; 3-oleamidopropane-1,2-diyl dioleate

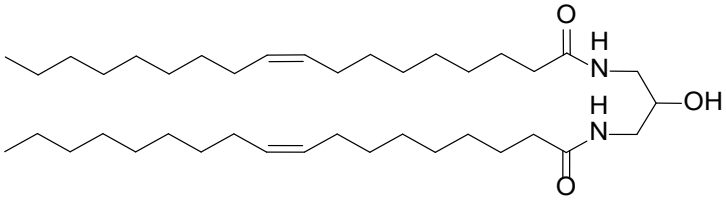


In a round bottom flask containing stirred solution of (±)-3-Amino-1,2-propanediol (92.00 mg, 1.01 mmoL) in CH_2Cl_2 (25 mL), oleic acid (1.0 g, 3.54 mmoL), HCTU (2.0 g, 5.26 mmoL) and DIPEA (1.3 g, 10.23 mmoL) were added. After overnight stirring at RT, the reaction mixture was diluted with CH_2CL_2, washed with sat. NH_4Cl (~30 mL) and brine (~30 mL) and subsequently was dried (Na_2SO_4), filtered *in vacuo* and concentrated. Purification by Column Chromatography (CH_2Cl_2 to 2% EtOAC in CH_2Cl_2) yielded the target material **10** as a white solid (193.0 mg, 0.22 mmoL).

TLC (CH_2Cl_2: EtOAc_8:2) : R_f = 0.9, **^{1}H-NMR** ($CDCl_3$, 400MHz) δ 5.73 (t, J = 5.8 Hz, 1H), 5.39 – 5.27 (m, 6H), 5.09 (m, 1H), 4.25 (dd, J = 12.0, 4.1 Hz, 1H), 4.13 (dd, J = 12.0, 5.7 Hz, 1H), 3.48 (m, 2H), 2.31 (td, J = 7.6, 2.2 Hz, 4H), 2.16 (t, J = 7.6 Hz, 2H), 2.00 (q, J = 6.3 Hz,

12H), 1.60 (s, 6H), 1.28 (d, J = 10.4 Hz, 62H), 0.88 (t, J = 6.8 Hz, 9H); **ESI-HRMS** (m/z) [M+H]+: calcd. for $C_{57}H_{105}NO_5$, 884.8066; found 884.8062, delta = 0.5 ppm.

(12) N-N; (9Z,9'Z)-1,1'-((2-hydroxypropane-1,3-diyl)bis(l2-azanediyl))bis(octadec-9-en-1-one)



In a round bottom flask containing a stirred solution of 1,3-diamino-2-propanol (102.4 mg, 1.12 mmoL) and oleic acid (595.2 mg, 2.11 mmoL), DMAP (356.9 mg, 2.92 mmoL), EDC (437.1 mg, 2.82 mmoL) and DIPEA (356.7 mg, 2.76 mmoL) were added. The mixture was diluted with 10 mL of CH_2Cl_2 and 3 mL of THF. After stirring overnight at RT, the reaction mixture was diluted with CH_2Cl_2 before washing with saturated NH_4Cl (2x ~15 mL), and brine (~15 mL). Subsequently, the mixture was dried with Na_2SO_4 and filtered *in vacuo* and concentrated. Purification by Column Chromatography (CH_2Cl_2 to 50% EtOAc in CH_2Cl_2), yielded the target material **12** as a white solid (274.5 mg, 0.44 mmoL).

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.79 (t, J = 6.3 Hz, 2H), 5.38 – 5.30 (m, 4H), 3.74 (m, 1H), 3.38 (m, 2H), 3.21 (dt, J = 14.0, 5.5 Hz, 2H), 2.24 – 2.15 (m, 4H), 1.99 (q, J = 6.6 Hz, 8H), 1.60 (m, 4H), 1.26 (d, J = 13.1 Hz, 40H), 0.90 – 0.82 (t, J = 8 Hz, 6H).
ESI-HRMS (m/z) $[M+H]^+$: calcd. for $C_{39}H_{69}NO_4$, 619.5772; found 619.5771, delta = 0.2 ppm.

Synthetic route for lipids 7 and 21. Each *sn*-position of the amino-propanediol was first coupled to a protecting group (PG) in 3 synthetic steps, leading to a full protected amino-propanediol with PG1, PG2 and PG3 protecting the -NH_2 of *sn*-1 position, -OH of the *sn*-3 position and the -OH of the *sn*-2 position, respectively (Figure S15). All protecting groups were orthogonal with each other. Synthesis steps: 1) Deprotection of the NH group, 2) subsequent coupling with fatty acid (oleic acid) in *sn*-1 position, 3) deprotection of OH group in *sn*-3 position, 4) subsequent coupling with fatty acid (stearic acid for **7** or oleic acid for **21**) in *sn*-3 position, 5) deprotection of OH group in *sn*-2 position.

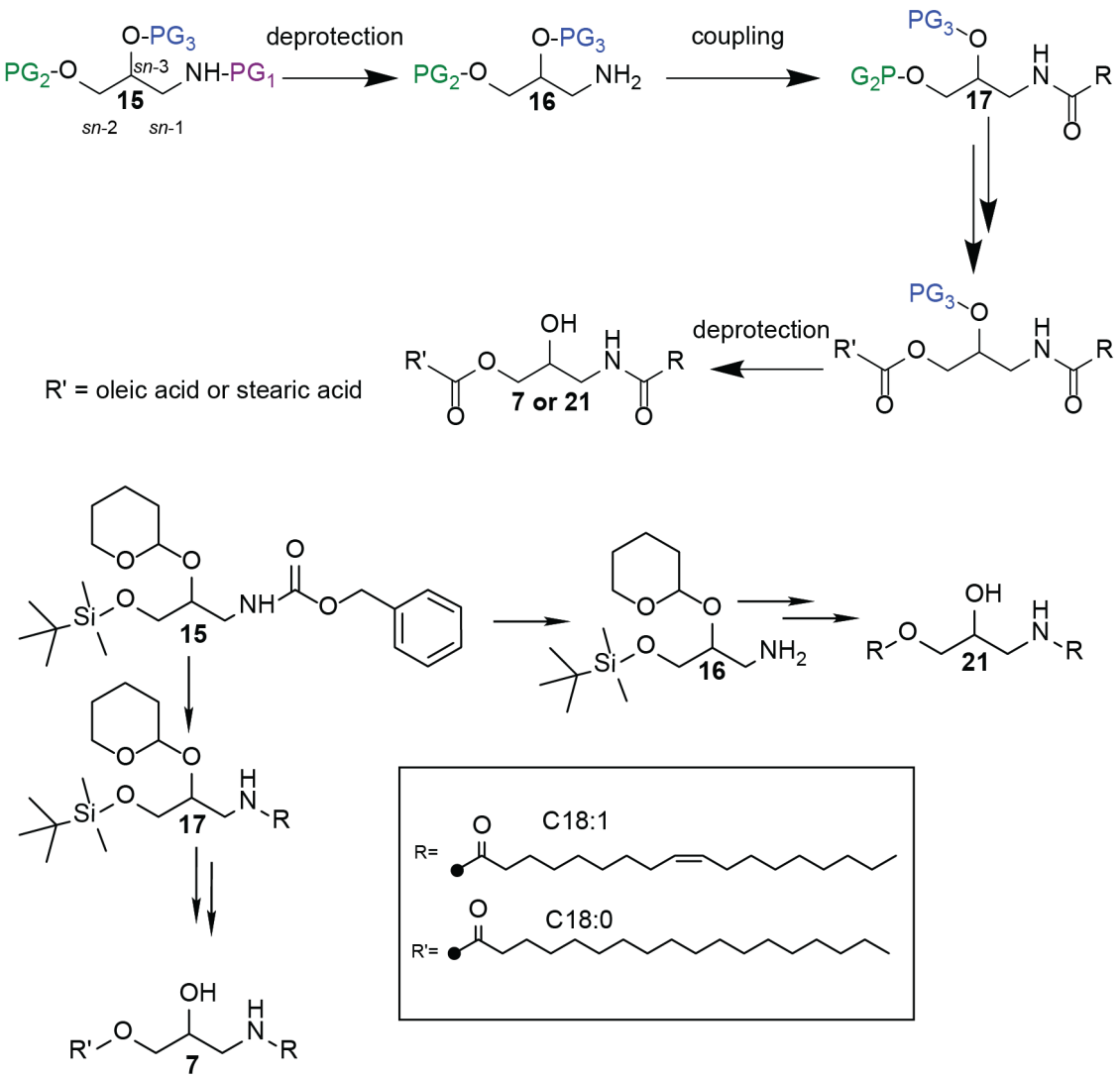
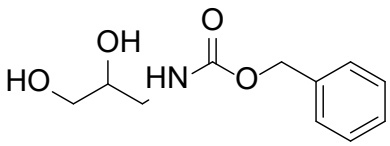


Figure S15. Schematic of synthetic route for lipids 7 and 21.

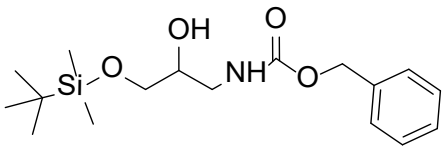
(13) Benzyl (2,3-dihydroxypropyl) carbamate



In a round bottom flask, 3-amino-1,2-propane diol (1.5 g, 16.6 mmol) was dissolved in THF (50 mL). Water (25 mL) and potassium carbonate (6.9 g; 49.7 mmol) were added to the solution and the reaction mixture was cooled down to 0 °C. Benzyl chloroformate (3.8 g, 16.5 mmol) was added dropwise over 2 hours and afterwards was allowed to warm to room temperature and left stirring overnight. The reaction mixture was extracted with EtOAc (3 x 25 mL), the combined organic layers were washed with brine (20 mL) and subsequently was dried (Na_2SO_4), filtered and concentrated *in vacuo*. Purification by Column Chromatography (MeOH: CH_2Cl_2; graduate elution from 0:100 to 6:94 in steps of 2%) yielded the title compound **13** as a white solid (3.47 g, 15.4 mmol).

^{1}H NMR (400 MHz, MeOD) δ 7.37 – 7.23 (m, 5H), 5.06 (s, 2H), 3.66 (dq, J = 7.1, 5.1 Hz, 1H), 3.47 (tt, J = 11.4, 6.3 Hz, 2H), 3.29 – 3.21 (m, 1H), 3.12 (dd, J = 13.8, 6.8 Hz, 1H).

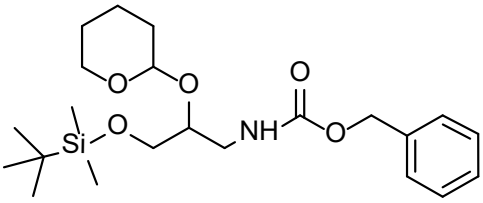
(14) Benzyl (3-((tert-butyldimethylsilyl)oxy)-2-hydroxypropyl)carbamate



In a 500 mL round bottom flask compound **13** (3.1 g; 13.9 mmol) was dissolved in CH_2Cl_2 (30 mL). Imidazole (2.4 g, 34.7 mmol) was added to the solution and the reaction mixture was cooled down to 0 °C. TBDMS-Cl (3.1 g, 20.8 mmol) was added and the reaction mixture was allowed to stir for 2 hours while maintaining 0 °C. The reaction mixture was quenched with sat. $NaHCO_3$ solution (25 mL) and washed with dd. H_2O (3 x 10 mL) and subsequently was dried (Na_2SO_4), filtered and concentrated *in vacuo*. Purification by Column Chromatography (EtOAc: petroleum ether; 10:90 to 25:75) yielded the title compound **14** as a colorless oil (3.83 g, 11.0 mmol).

^{1}H NMR (400 MHz, MeOD) δ 7.39 – 7.24 (m, 5H), 6.85 (d, J = 5.9 Hz, 1H), 5.07 (s, 2H), 3.70 (dt, J = 10.6, 5.1 Hz, 1H), 3.65 – 3.54 (m, 2H), 3.40 – 3.29 (m, 1H), 3.20 – 3.08 (m, 1H), 0.92 (s, 9H), 0.08 (s, 6H).

(15) Benzyl (3-((tert-butyldimethylsilyl)oxy)-2-((tetrahydro-2H-pyran-2-yl)oxy)propyl) carbamate

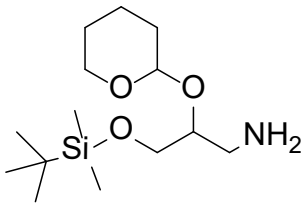


In a round bottom flask, compound **14** (2.9 g, 8.65 mmoL) was dissolved in CH_2Cl_2 alongside pyridinium p-toluenesulfonate (0.22 g, 0.884 mmoL). 3,4-dihydro-pyran (2.2 g, 25.95 mmoL) was added and the reaction was allowed to stir for 3 hours. The reaction mixture was washed with sat. $NaHCO_3$ solution (25 mL), followed by H_2O (2 x 25 mL) and brine (25 mL) and subsequently was dried (Na_2SO_4), filtered and concentrated *in vacuo*. Purification by Column Chromatography (EtOAc: hexane; graduate elution from 1:99 to 10:90 in steps of 1%, followed by a flush of 50:50) yielded the title compound **15** as a colorless oil (3.14 g, 7.40 mmoL).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42 – 6.99 (m, 5H), 5.80 – 5.45 (m, 1H), 5.03 (s, 2H), 4.71 – 4.52 (m, 1H), 3.84 (dt, J = 10.5, 4.7 Hz, 1H), 3.73 (dq, J = 16.1, 5.3, 4.9 Hz, 1H), 3.57 (dd, J =

12.9, 4.5 Hz, 1H), 3.50 – 3.33 (m, 2H), 3.29 – 3.08 (m, 1H), 1.83 – 1.56 (m, 2H), 1.51 – 1.36 (m, 4H), 0.86 (s, 9H), 0.02 (d, J = 6.1 Hz, 6H).

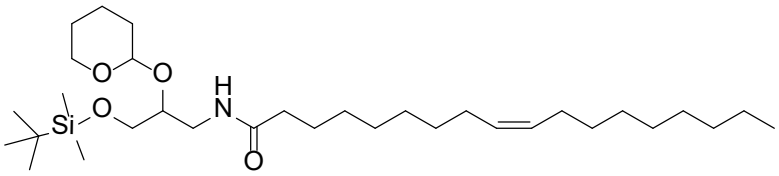
(16) 3-((tert-butyldimethylsilyl)oxy)-2-((tetrahydro-2H-pyran-2-yl)oxy)propan-1-amine



Compound **15** (1.9 g, 4.50 mmoL) was dried in a round bottom flask before use in a vacuum oven (25 °C) for at least 2 hours. Palladium on carbon (191.0 mg) was added and the round bottom flask was flushed with nitrogen gas for 10 minutes. Dry MeOH was added, and to the stirred solution with addition of a nitrogen balloon, triethylsilane (7.9 g, 67.6 mmoL) was added dropwise. The reaction mixture was allowed to stir for 4 hours and then was filtered over Celite, cleansed with MeOH and concentrated *in vacuo*. Excess of triethylsilane was removed under airflow for 30 min. Purification by Column Chromatography (CH_2Cl_2 + 0,5 % isopropylamine with graduate elution to 2.5 % EtOH in CH_2Cl_2 + 0.5 % isopropylamine in steps of 0.5% EtOH) yielded the title compound **16** as a colorless oil (1.16 g, 4.00 mmoL).

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.61 (td, J = 5.7, 5.1, 2.7 Hz, 1H), 3.83 (m, 1H), 3.77 – 3.70 (m, 1H), 3.63 – 3.45 (m, 2H), 3.44 – 3.34 (m, 1H), 2.80 (m, 1H), 2.65 (m, 1H), 1.80 – 1.57 (m, 2H), 1.53 – 1.36 (m, 4H), 1.28 (s, 2H), 0.79 (s, 9H), -0.05 (d, J = 3.2 Hz, 6H). **ESI-HRMS** (m/z) [M+H]+: calcd. for $C_{14}H_{31}NO_3Si$, 289.2146; found 289.2154, delta=2.8 ppm.

(17)N-(3-((tert-butyldimethylsilyl)oxy)-2-((tetrahydro-2H-pyran-2-yl)oxy)propyl) oleamide

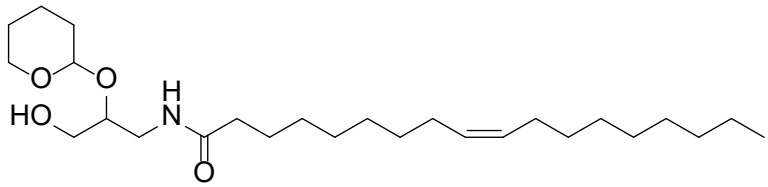


In a round bottom flask, compound **16** (915.3 mg, 3.16 mmoL), DMAP (1.0 g, 8.2 mmoL), DIPEA (1.1 g, 8.2 mmoL) and EDC (1.3 g, 8.2 mmoL) were dissolved in CH_2Cl_2 (20 mL). Oleic acid (1.3 g, 4.74 mmoL) was added to the solution and the reaction mixture was allowed to stir at RT overnight. The reaction mixture was washed with sat. NH_4Cl solution (20 mL), followed by H_2O (3 x 20 mL), brine (20 mL) and subsequently was dried (Na_2SO_4), filtered and concentrated *in vacuo*. Purification by Column Chromatography (EtOAc: hexane; graduate

elution from 0:100 to 20:80 in steps of 10%) yielded the title compound **17** as a colorless oil (1.30 g, 2.35 mmoL).

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.67 – 6.01 (m, 1H), 5.38 – 5.25 (m, 2H), 4.62 (m, 1H), 4.00 – 3.83 (m, 1H), 3.83 – 3.67 (m, 2H), 3.64 (dd, J = 9.6, 6.0 Hz, 1H), 3.61 – 3.53 (m, 2H), 3.48 (m, 1H), 3.40 – 2.96 (m, 1H), 2.14 (td, J = 8.3, 6.9 Hz, 2H), 2.05 – 1.94 (m, 4H), 1.88 – 1.67 (m, 2H), 1.64 – 1.55 (m, 2H), 1.54 – 1.46 (m, 4H), 1.26 (d, J = 13.0 Hz, 20H), 0.88 (s, 9H), 0.87 – 0.82 (t, J = 4 Hz, 3H), 0.04 (dd, J = 9.8, 1.4 Hz, 6H).

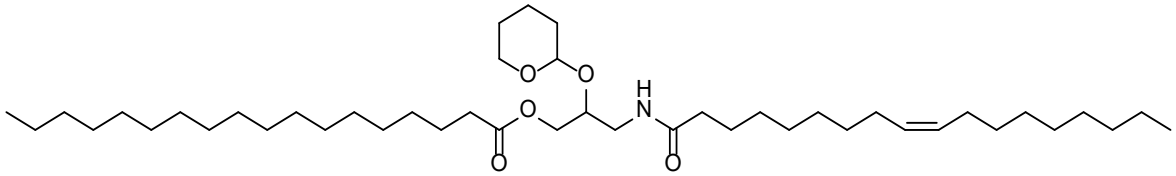
(18) N-(3-hydroxy-2-((tetrahydro-2H-pyran-2-yl)oxy)propyl)oleamide



In a round bottom flask, compound **17** (1.2 g; 2.09 mmoL) was dissolved in THF (30 mL) and the reaction mixture was cooled down to 0 °C. TBAF·3 H_2O (2.0 g, 6.27 mmoL) was dissolved in a small amount of THF and was added dropwise. The mixture was allowed to stir for 1 hour. Afterwards the reaction mixture was diluted with H_2O (30 mL) extracted with EtOAc (3 x30 mL) and subsequently was dried (Na_2SO_4), filtered and concentrated *in vacuo*. Purification by Column Chromatography (EtOAc: hexane; elution from 0:100 to 50:50 in steps of 10%) yielded the title compound **18** as a colorless oil (0.78 g, 1.77 mmoL).

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.09 (dt, J = 196.7, 6.1 Hz, 1H), 5.40 – 5.26 (m, 2H), 4.56 (dq, J = 6.2, 2.8 Hz, 1H), 3.95 (dq, J = 13.0, 3.6, 2.9 Hz, 1H), 3.76 (dp, J = 11.0, 5.4 Hz, 1H), 3.58 – 3.33 (m, 5H), 2.19 (q, J = 7.1 Hz, 2H), 1.99 (q, J = 6.4 Hz, 4H), 1.90 – 1.71 (m, 2H), 1.61 (t, J = 7.2 Hz, 2H), 1.58 – 1.46 (m, 4H), 1.27 (d, J = 14.6 Hz, 20H), 0.87 (t, J = 6.7 Hz, 3H).

(19) 3-oleamido-2-((tetrahydro-2H-pyran-2-yl)oxy)propyl stearate

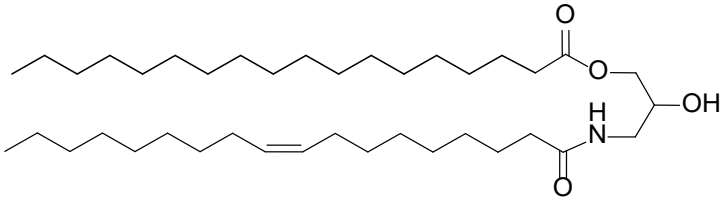


In a round bottom flask, compound **18** (322.7 mg, 0.73 mmoL), DMAP (224.2 mg, 1.83 mmoL), DIPEA (236.5 mg, 1.83 mmoL) and EDC (284.1 mg, 1.83 mmoL) were dissolved in CH_2Cl_2 (10 mL). Stearic acid (31.93 mg, 0.11 mmoL) was added to the solution and the reaction mixture

was allowed to stir overnight at RT. The reaction mixture was washed with sat. NH_4Cl solution (10 mL), followed by H_2O (3 x 10 mL), brine (20 mL) and subsequently was dried (Na_2SO_4), filtered and concentrated *in vacuo*. Purification by Column Chromatography (Acetone : CH_2Cl_2, 5:95) yielded the title compound **19** as a white solid (247.2 mg, 0.35 mmoL).

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.38 – 5.27 (m, 2H), 4.74 – 4.47 (m, 1H), 4.28 – 4.05 (m, 2H), 3.99 – 3.83 (m, 2H), 3.65 – 3.42 (m, 3H), 3.35 – 3.08 (m, 1H), 2.31 (q, J = 7.9 Hz, 3H), 2.21 – 2.12 (m, 2H), 1.99 (q, J = 6.8 Hz, 4H), 1.88 – 1.67 (m, 2H), 1.60 (td, J = 7.3, 3.9 Hz, 4H), 1.56 – 1.46 (m, 3H), 1.27 (d, J = 19.3 Hz, 34H), 0.90 – 0.84 (t, J = 8 Hz, 6H).

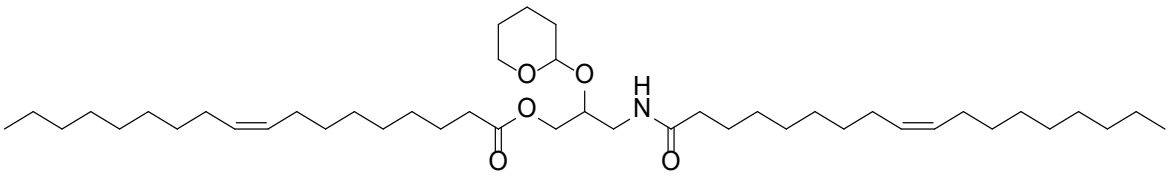
(7) C18:0-C18:1; 2-hydroxy-3-oleamidopropyl stearate



In a round bottom flask, compound **19** (223.0 mg, 0.32 mmoL) and pyridinium p-toluene sulfonate (8.04 mg, 0.032 mmoL) were dissolved in MeOH (5 mL) and the reaction mixture was allowed to stir overnight at 50 °C. The reaction mixture was quenched with sat. NaHCO3 solution (10 mL) and was extracted with EtOAc (3 x 10 mL) and subsequently was dried (Na_2SO_4), filtered and concentrated *in vacuo*. Purification by Column Chromatography (EtOAc:CH_2Cl_2, graduate elution from 0:100 to 30:70) yielded the final compound **7** as a white solid (168.0 mg, 0.27 mmoL).

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.00 (t, J = 5.9 Hz, 1H), 5.39 – 5.28 (m, 2H), 4.14 (dd, J = 11.5, 5.2 Hz, 1H), 4.05 (dd, J = 11.5, 5.8 Hz, 1H), 3.93 (qd, J = 5.7, 3.3 Hz, 1H), 3.53 (m, 1H), 3.23 (dt, J = 14.3, 6.0 Hz, 1H), 2.33 (t, J = 7.6 Hz, 2H), 2.26 – 2.17 (m, 2H), 2.00 (q, J = 6.5 Hz, 4H), 1.62 (m, 4H), 1.27 (d, J = 19.7 Hz, 48H), 0.91 – 0.83 (m, 6H); **ESI-HRMS** (m/z) [M+H]+: calcd. for $C_{39}H_{75}NO_4$, 622.5769; found 622.5767, delta = 0.5 ppm.

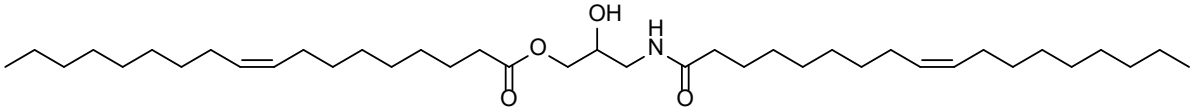
(20) 3-oleamido-2-((tetrahydro-2H-pyran-2-yl)oxy)propyl oleate



In a round bottom flask, compound **18** (295.1 mg, 0.67 mmoL), DMAP (205.0 mg, 1.68 mmoL), DIPEA (217.1 mg, 1.68 mmoL) and EDC (260.8 mg, 1.68 mmoL) were dissolved in CH_2Cl_2 (10 mL). Oleic acid (282.5 mg, 1.0 mmoL) was added to the solution and the reaction mixture was allowed to stir overnight at RT. The reaction mixture was washed with sat. NH_4Cl solution (10 mL), followed by H_2O (3 x 10 mL), brine (20 mL) and subsequently was dried (Na_2SO_4), filtered and concentrated *in vacuo*. Purification by Column Chromatography (Acetone: CH_2Cl_2, 5:95) yielded the title compound **20** as a colorless oil (308.5 mg, 0.44 mmoL).

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.61 – 6.09 (m, 1H), 5.31 – 5.18 (m, 4H), 4.70 – 4.45 (m, 1H), 4.10 (m, 1H), 4.01 (dd, J = 5.5, 2.5 Hz, 1H), 3.91 – 3.76 (m, 2H), 3.54 – 3.32 (m, 2H), 3.28 – 2.95 (m, 1H), 2.23 (q, J = 7.2 Hz, 2H), 2.09 (q, J = 7.4 Hz, 2H), 1.92 (q, J = 6.4 Hz, 8H), 1.79 – 1.59 (m, 2H), 1.59 – 1.38 (m, 4H), 1.45 (dq, J = 8.6, 5.5, 5.1 Hz, 4H), 1.20 (d, J = 13.5 Hz, 40H), 0.83 – 0.75 (t, J = 8 Hz, 6H).

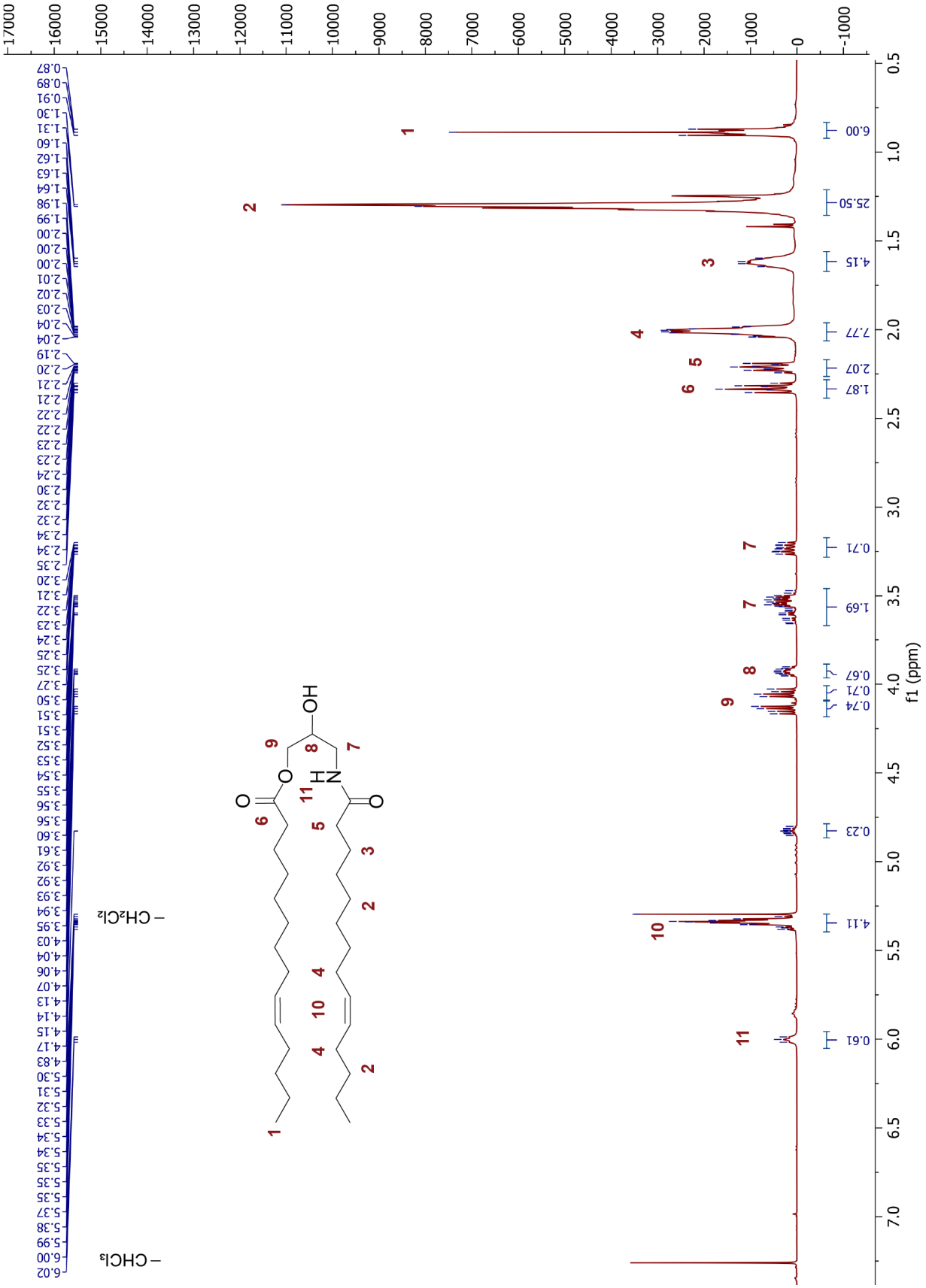
(21) *sn*-1,3; 2-hydroxy-3-oleamidopropyl oleate



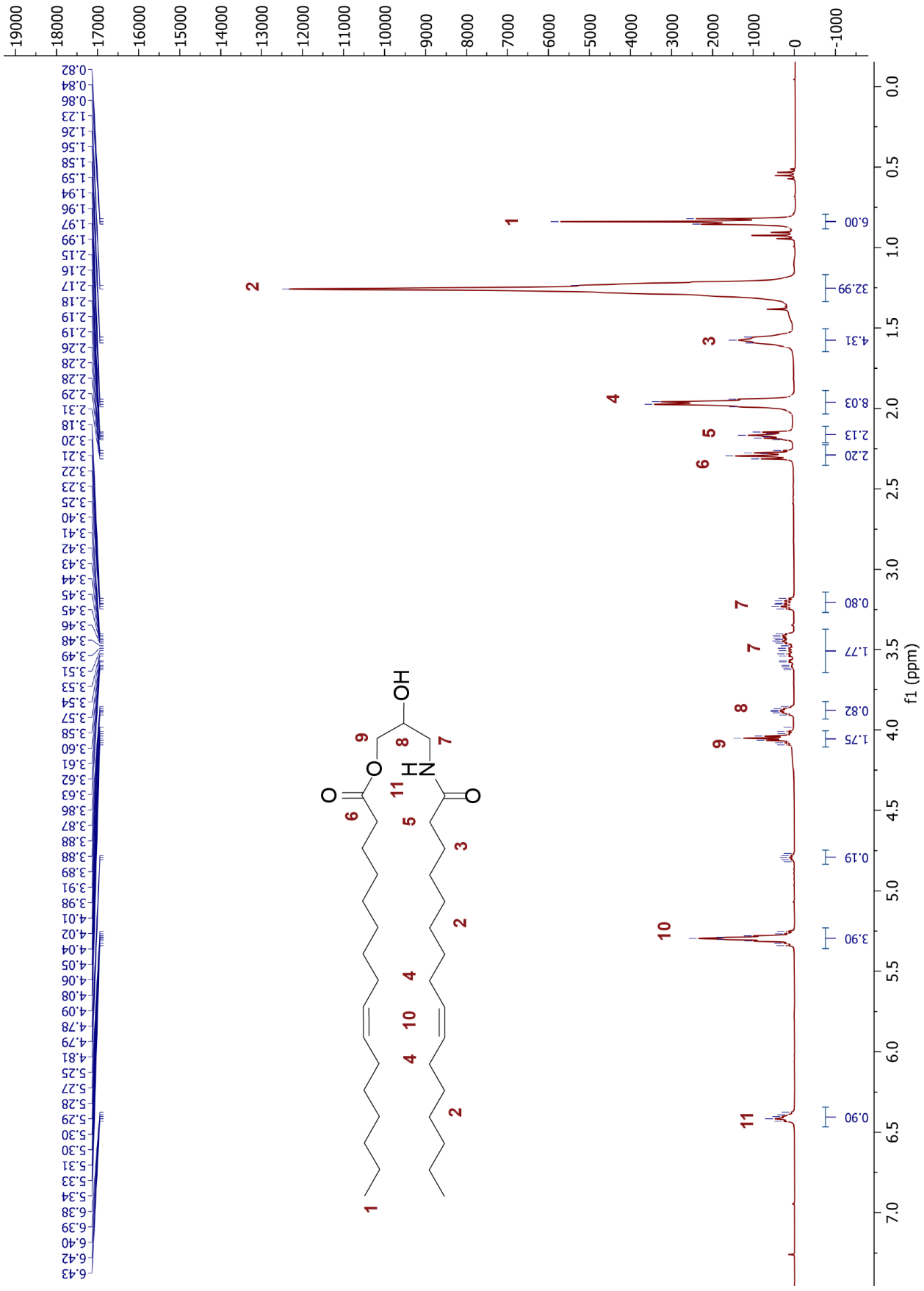
In a round bottom flask, compound **20** (269.3 mg, 0.38 mmoL) and pyridinium p-toluene sulfonate (9.50 mg, 0.038 mmoL) were dissolved in MeOH (5 mL) and the reaction mixture was allowed to stir overnight at 50 °C. The reaction mixture was quenched with sat. NaHCO3 solution (10 mL) and was extracted with EtOAc (3 x 10 mL) and subsequently was dried (Na_2SO_4), filtered and concentrated *in vacuo*. Purification by Column Chromatography (EtOAc:CH_2Cl_2, graduate elution from 0:100 to 30:70) yielded the final compound **21** as a white solid (200.2 mg, 0.32 mmoL).

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.98 (t, J = 6.0 Hz, 1H), 5.40 – 5.27 (m, 4H), 4.15 (dd, J = 11.4, 5.1 Hz, 1H), 4.05 (dd, J = 11.5, 5.8 Hz, 1H), 3.93 (qd, J = 5.8, 3.3 Hz, 1H), 3.53 (m, 1H), 3.23 (dt, J = 14.2, 6.0 Hz, 1H), 2.33 (t, J = 7.6 Hz, 2H), 2.24 – 2.18 (m, 2H), 2.00 (q, J = 6.5 Hz, 8H), 1.62 (m, 1H), 1.28 (d, J = 14.3 Hz, 40H), 0.91 – 0.83 (t, J = 8 Hz, 6H); **ESI-HRMS** (m/z) [M+H]+: calcd. for $C_{39}H_{73}NO_4$, 619.5612; found 619.5610, delta = 0.3 ppm.

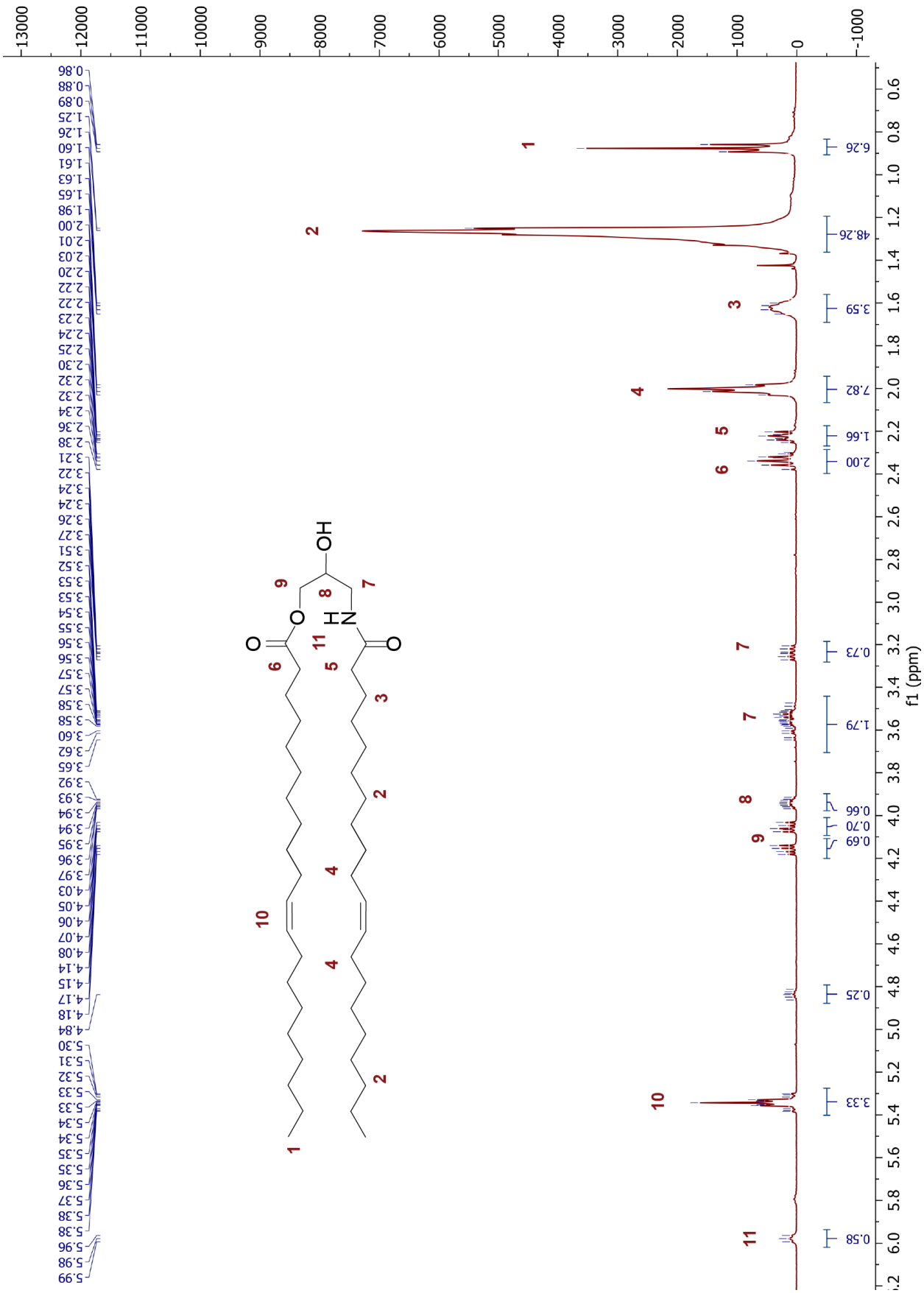
APPENDIX



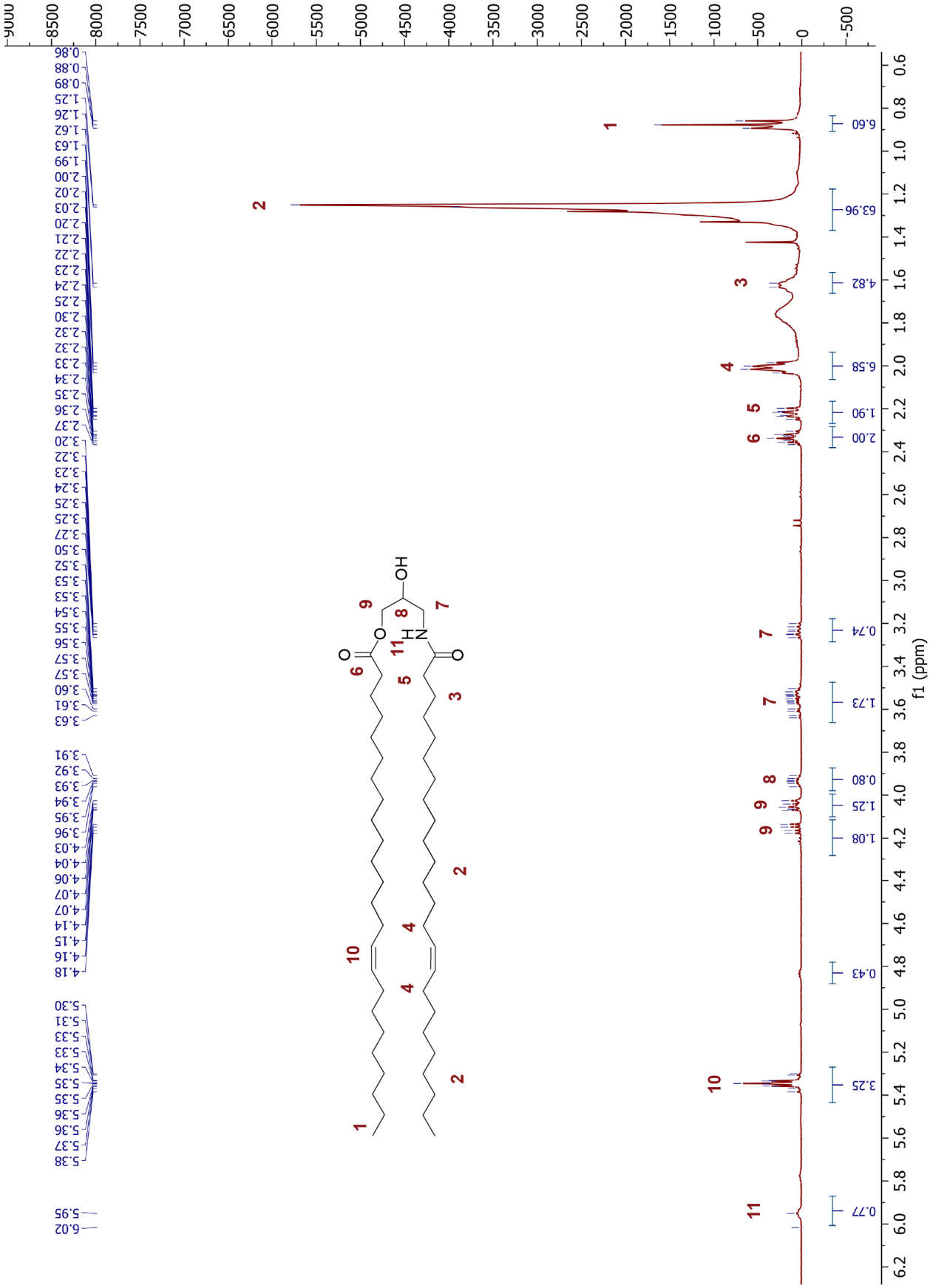
^{1}H-NMR of **1** in $CDCl_3$



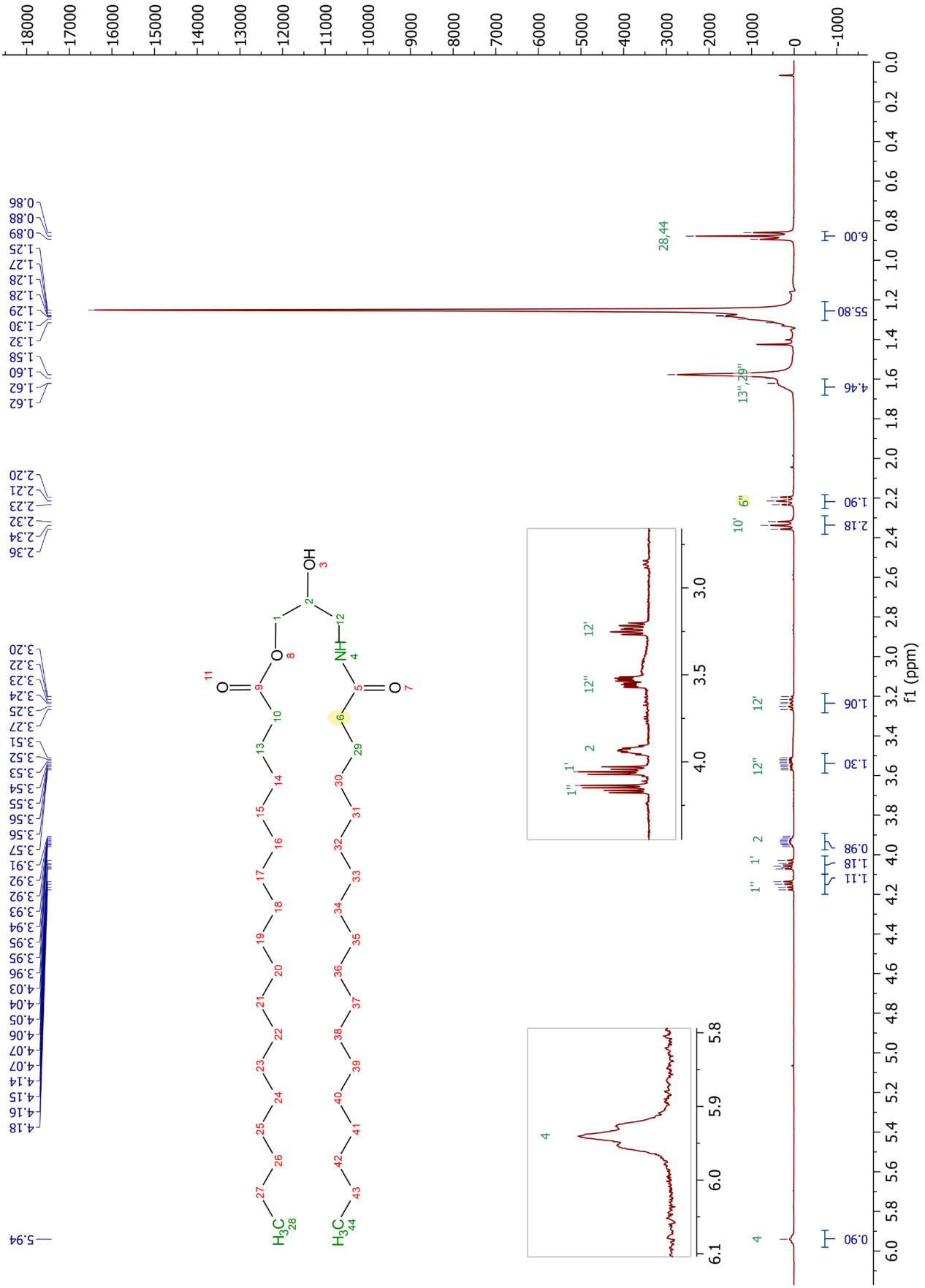
^{1}H-NMR of **2** in $CDCl_3$



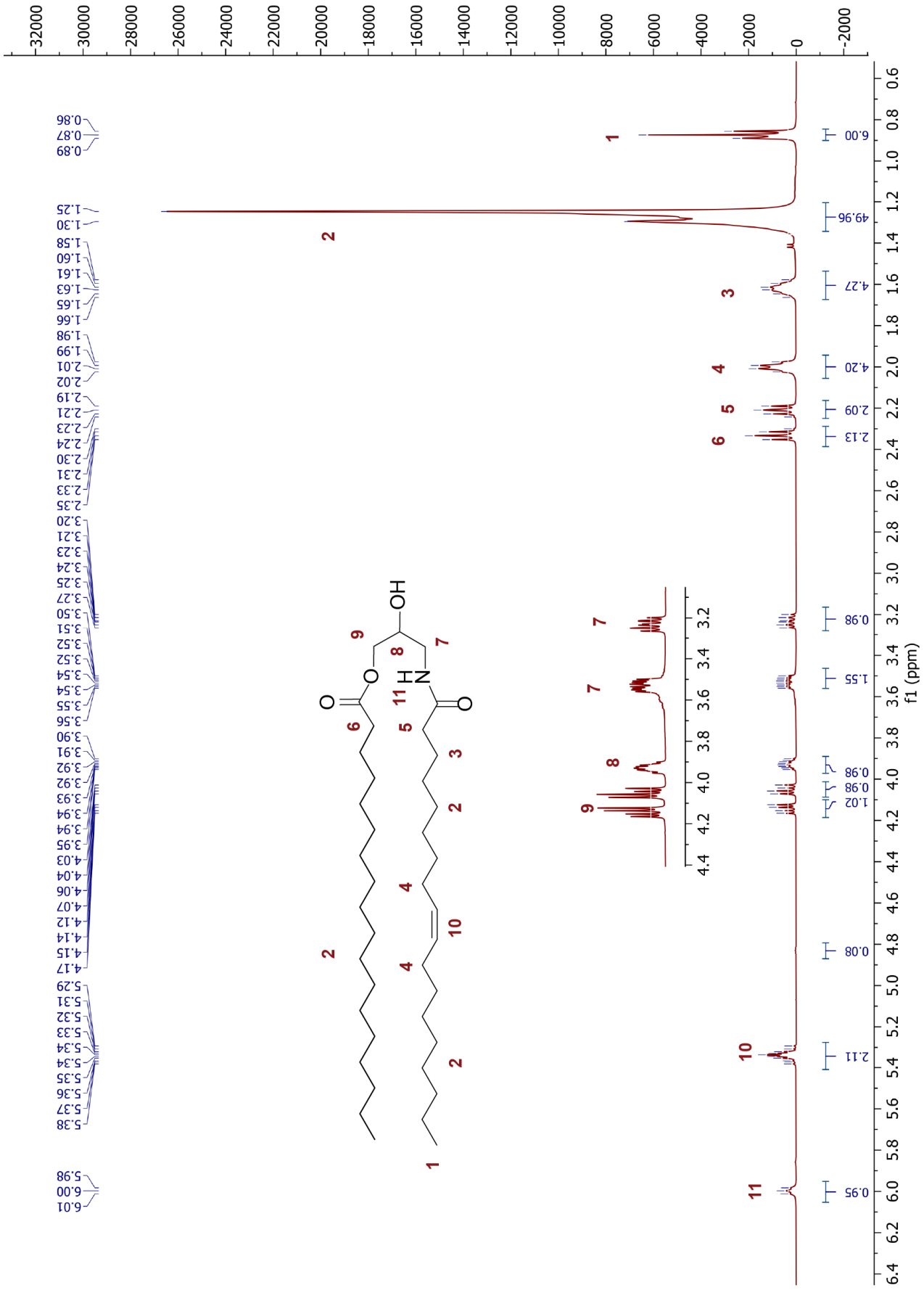
^{1}H-NMR of **4** in $CDCl_3$



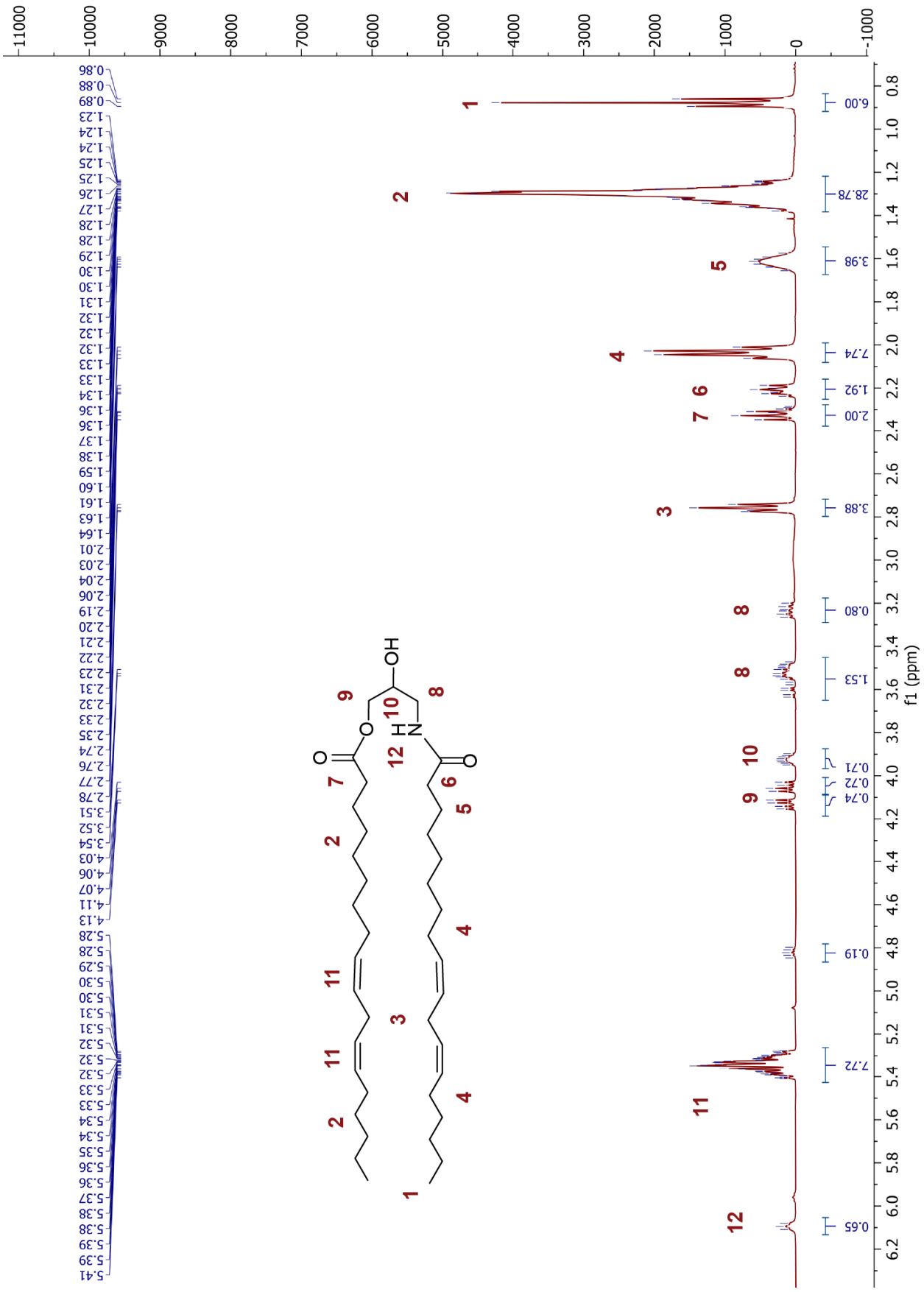
^{1}H-NMR of **5** in $CDCl_3$



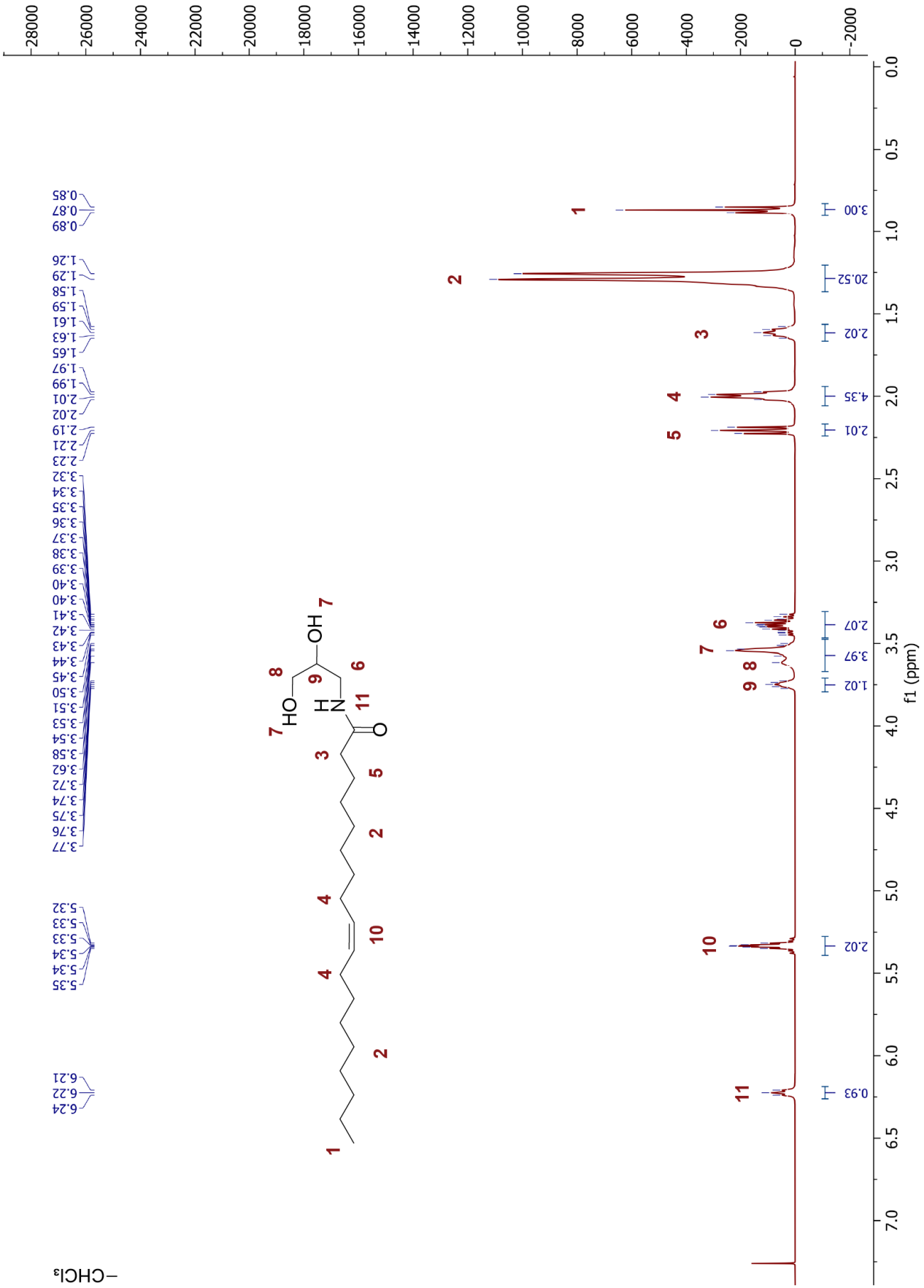
^{1}H-NMR of **6** in $CDCl_3$



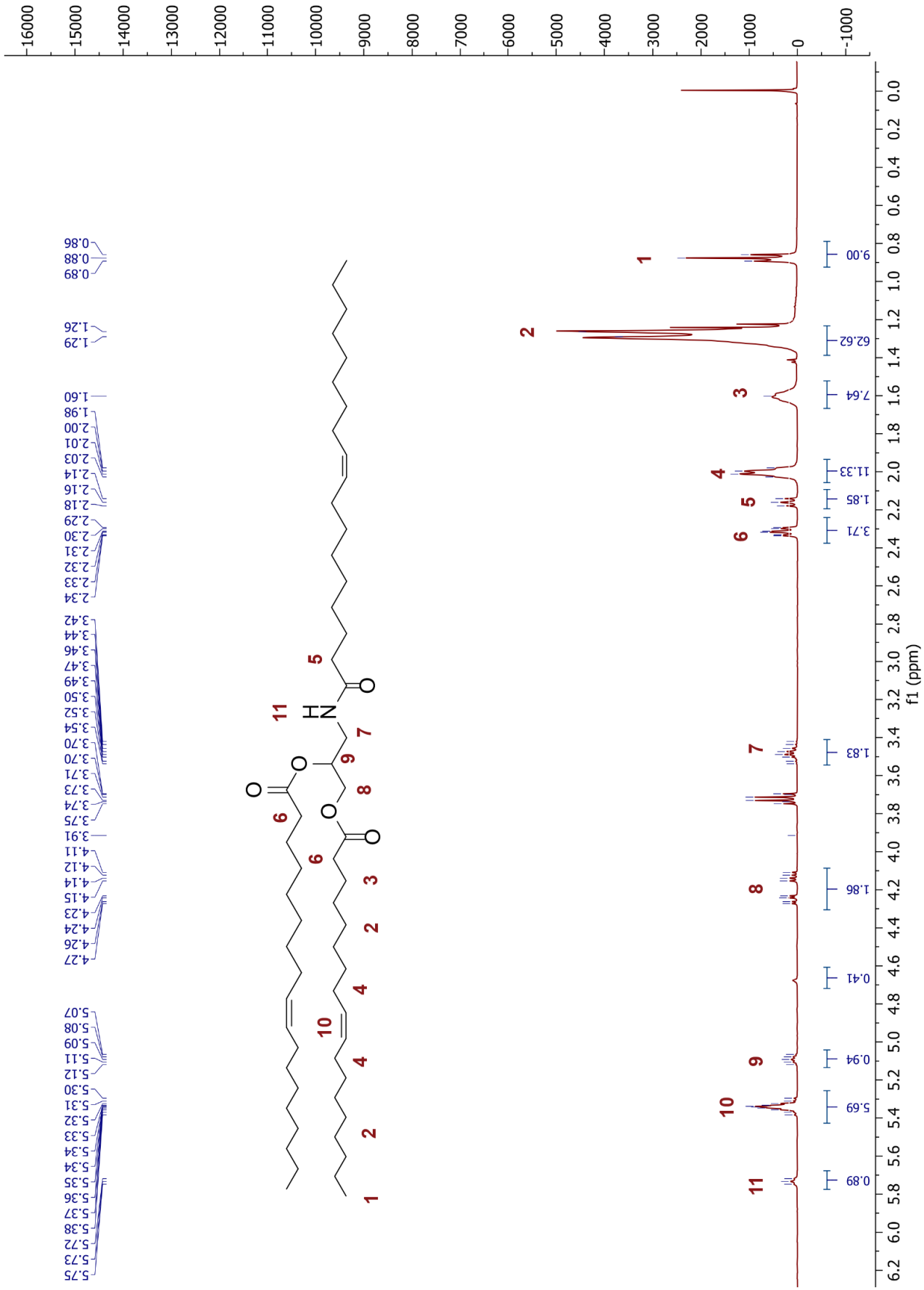
^{1}H-NMR of **7** in $CDCl_3$



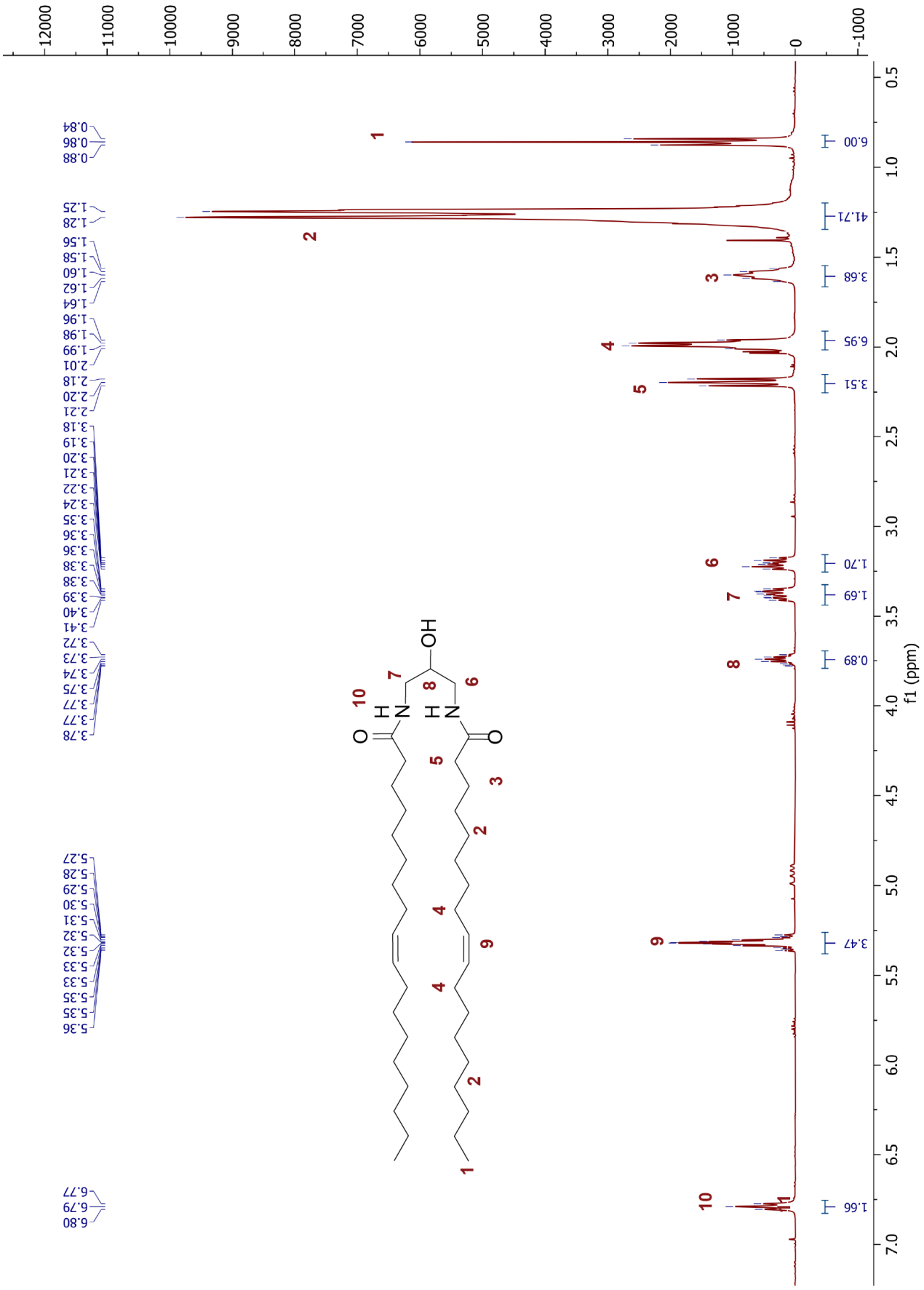
^{1}H-NMR of **8** in $CDCl_3$



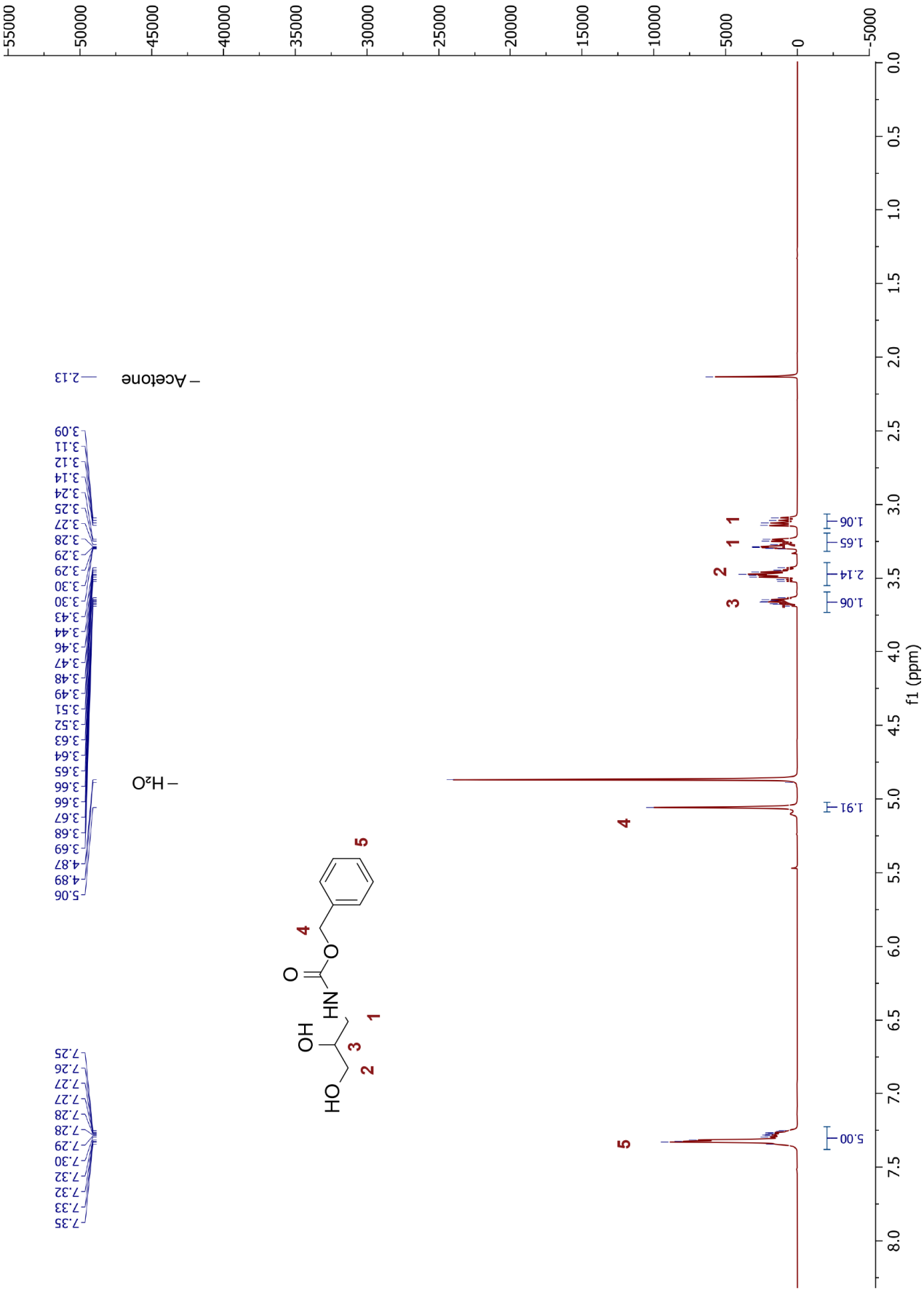
^{1}H-NMR of **9** in $CDCl_3$



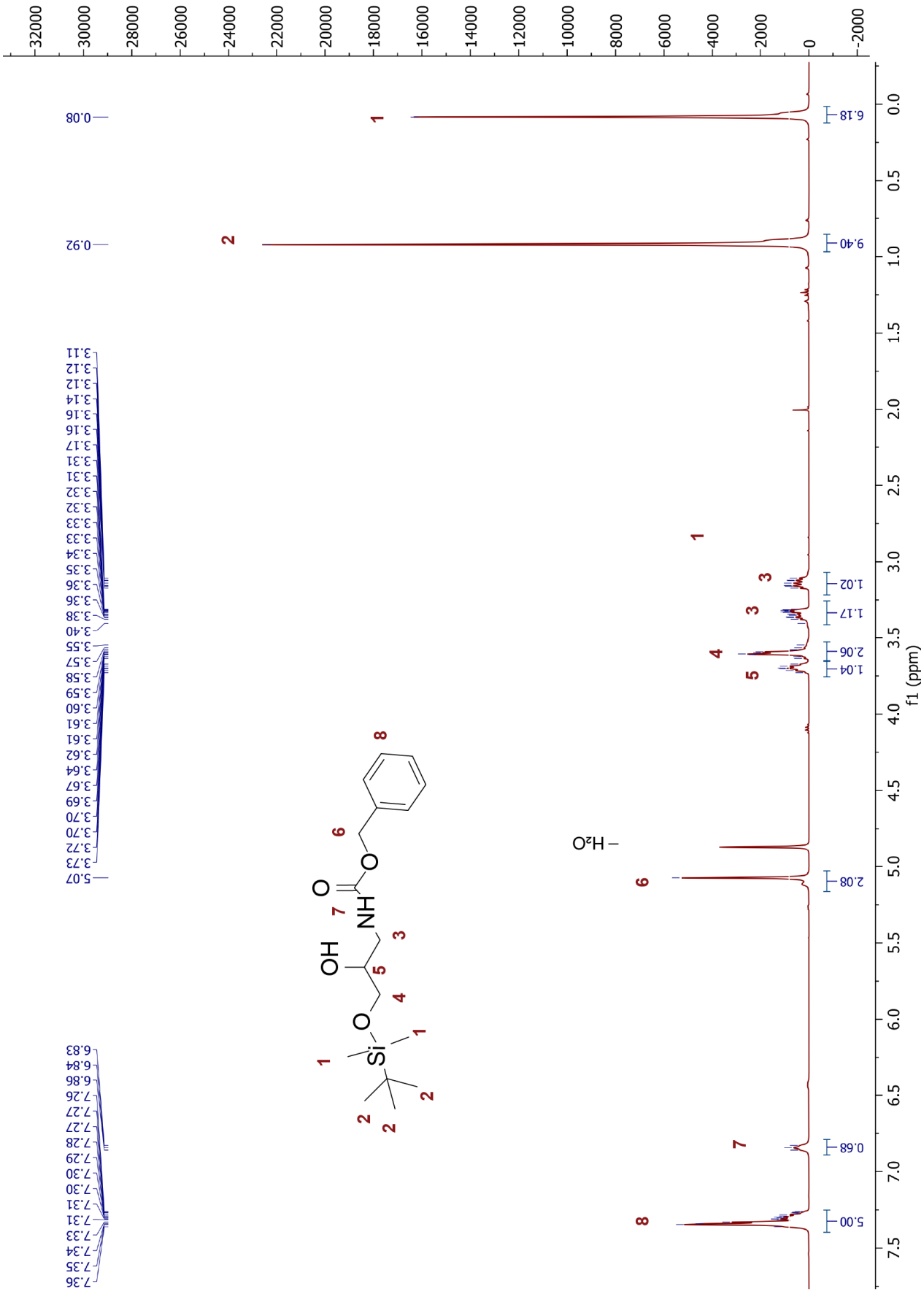
^{1}H-NMR of **10** in $CDCl_3$



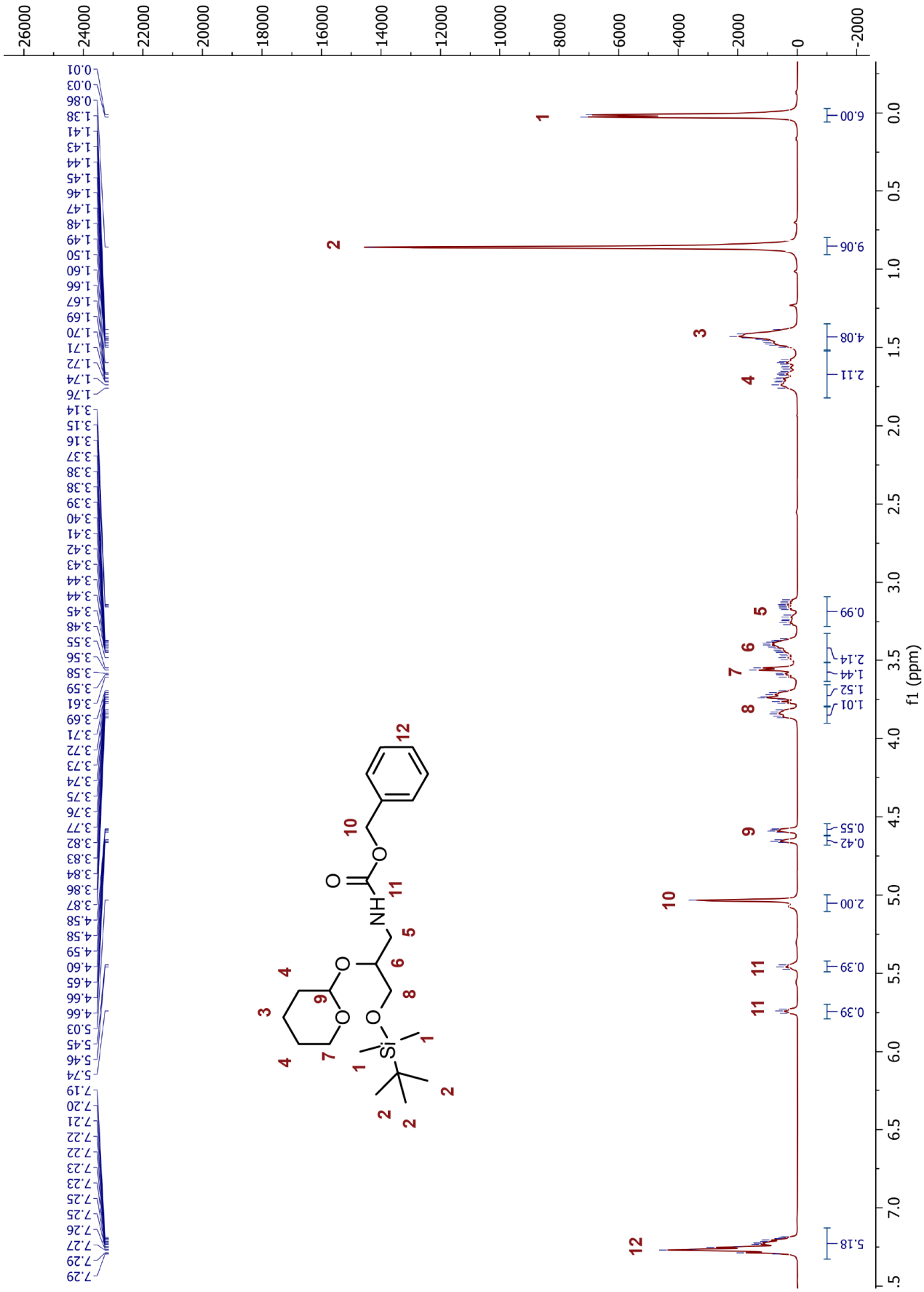
^{1}H-NMR of **12** in $CDCl_3$



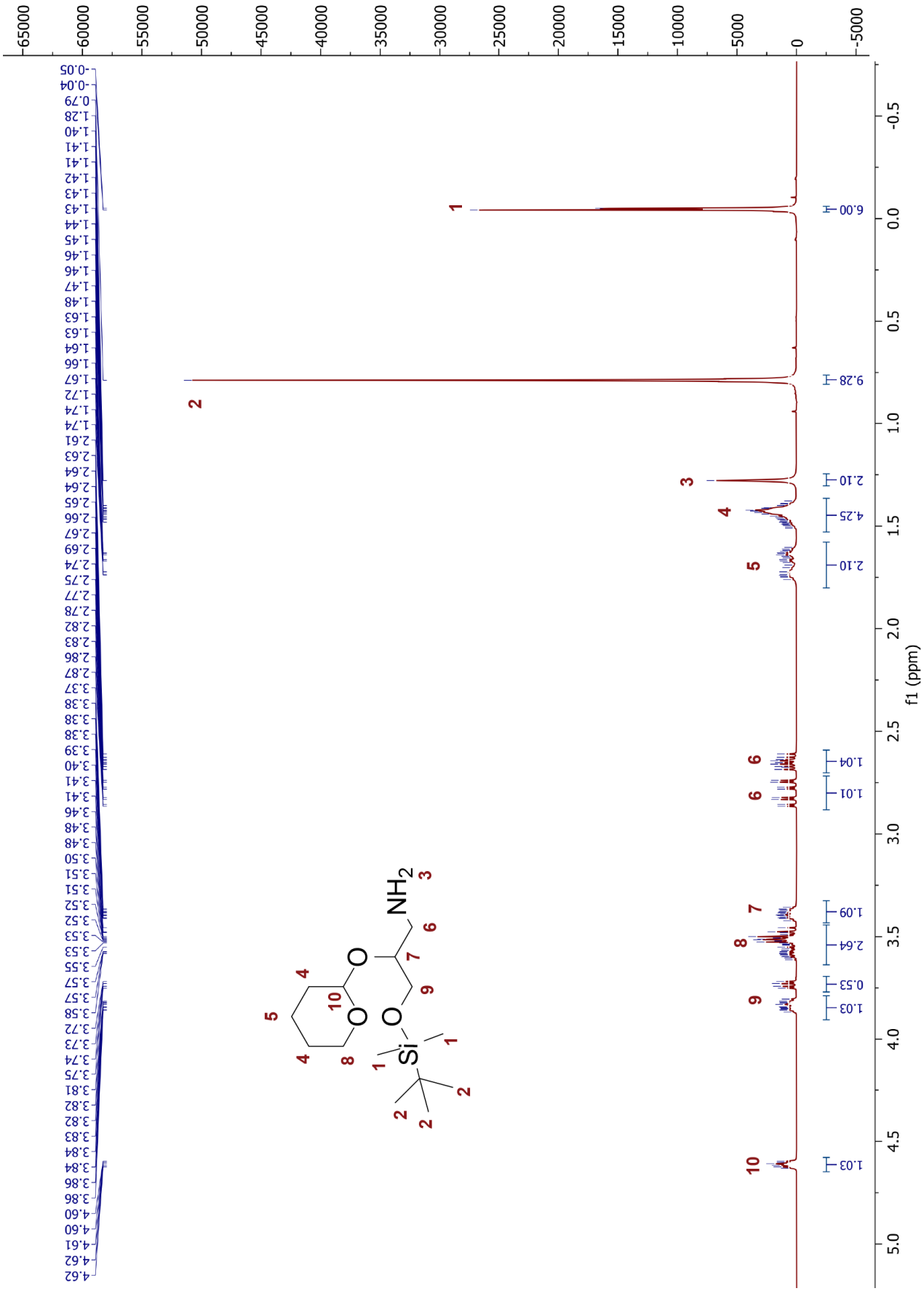
^{1}H-NMR of **13** in MeOD



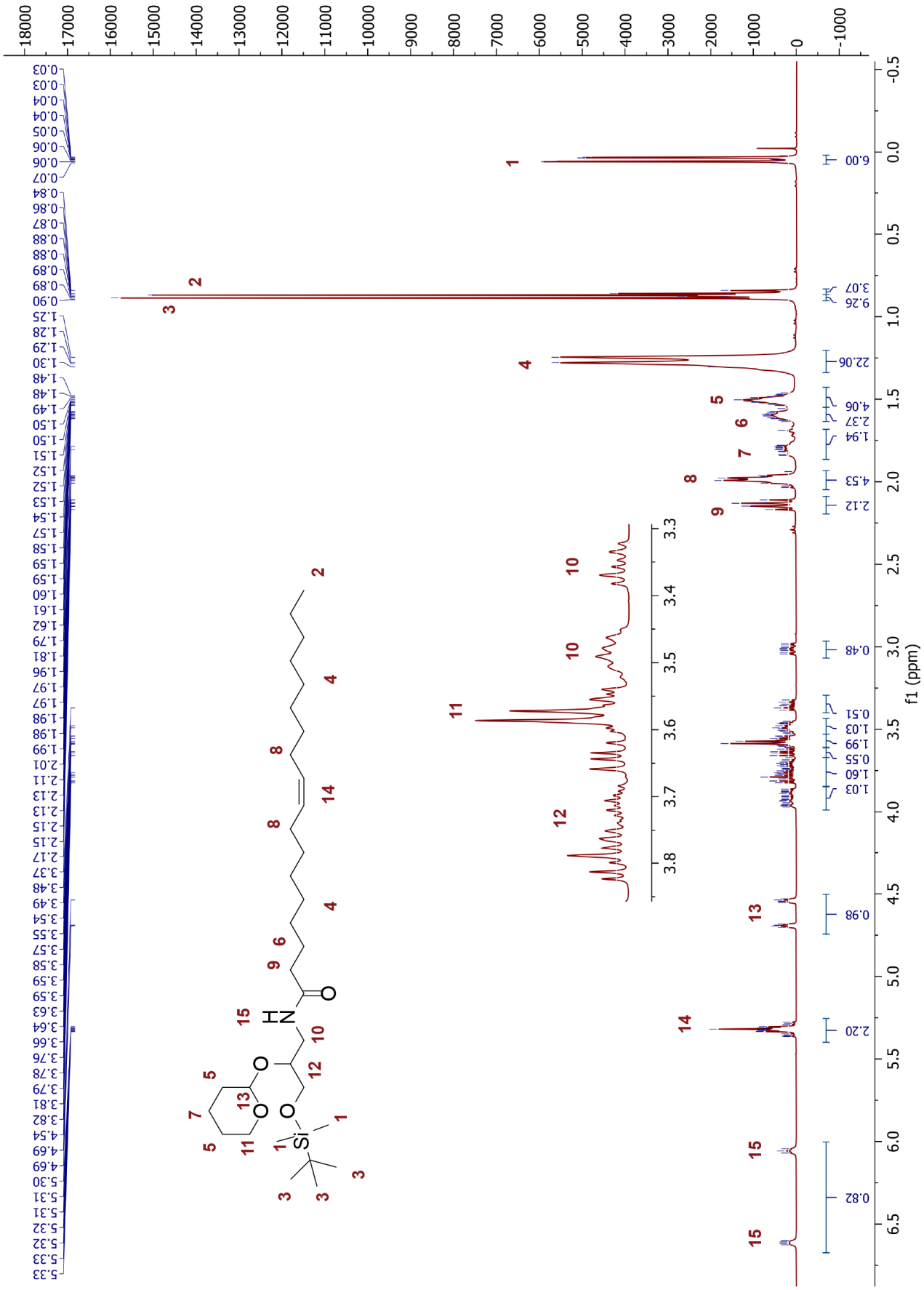
[1]H-NMR of **14** in MeOD



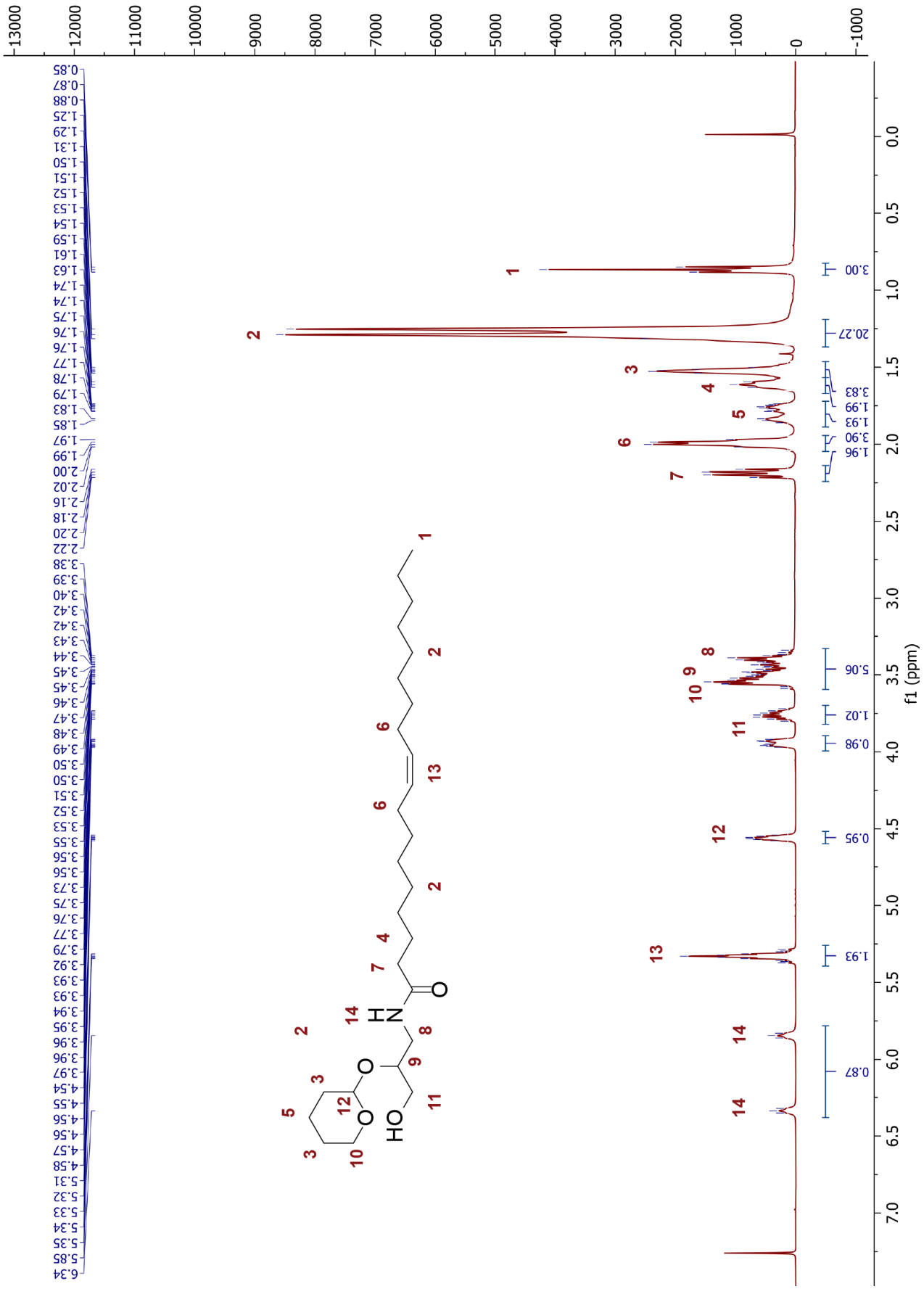
^{1}H-NMR of **15** in $CDCl_3$



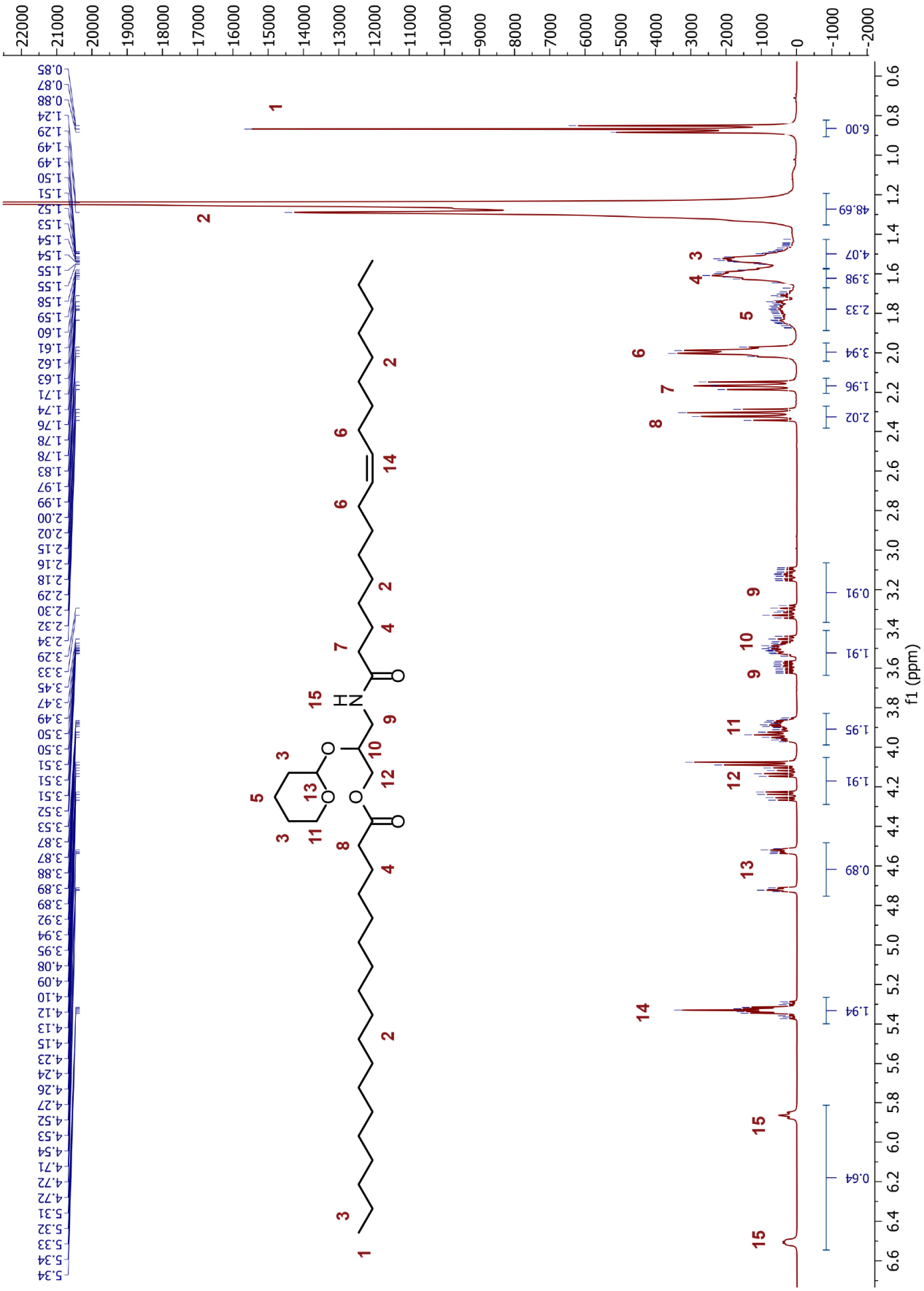
^{1}H-NMR of **16** in $CDCl_3$



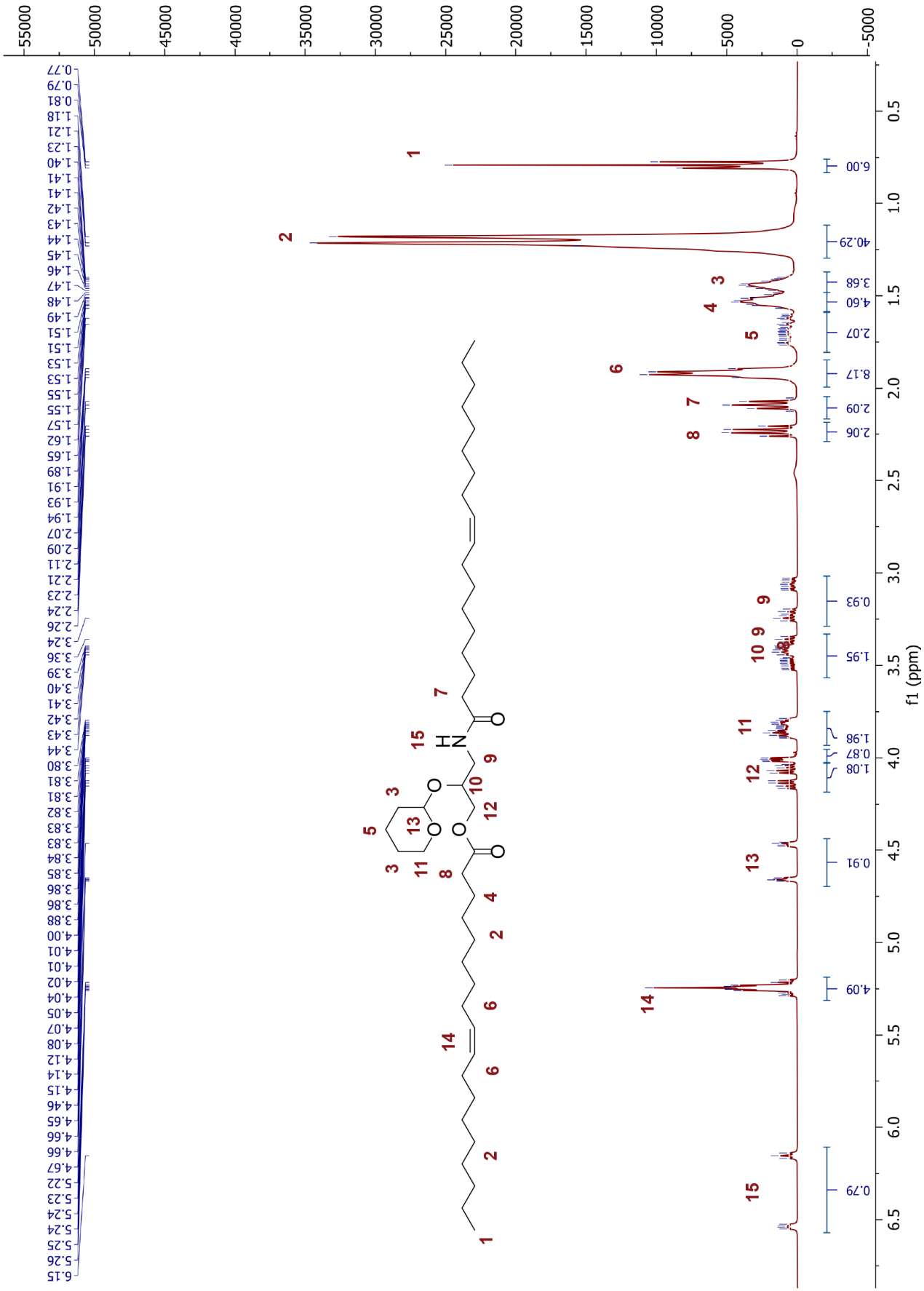
^{1}H-NMR of **17** in $CDCl_3$



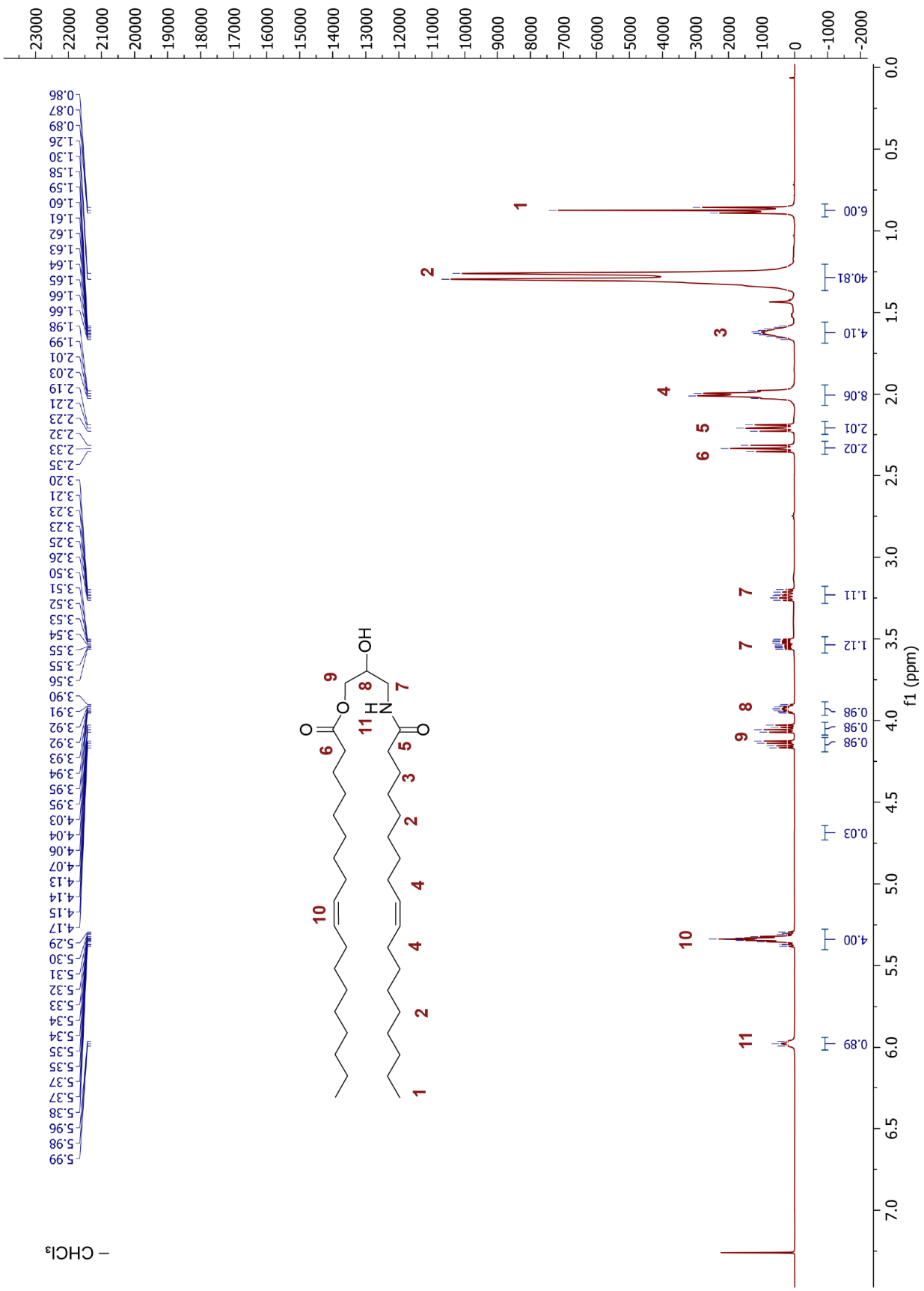
^{1}H-NMR of **18** in $CDCl_3$



^{1}H-NMR of **19** in $CDCl_3$



^{1}H-NMR of **20** in $CDCl_3$



^{1}H-NMR of **21** in $CDCl_3$

References

(1) Arias-Alpizar, G.; Papadopoulou, P.; Rios, X.; Reddy Pulagam, K.; Moradi, M.-A.; Pattipeiluhu, R.; Bussmann, J.; Sommerdijk, N.; Llop, J.; Kros, A.; Campbell, F. Phase-Separated Liposomes Hijack Endogenous Lipoprotein Transport and Metabolism Pathways to Target Subsets of Endothelial Cells in vivo. *Adv Healthc Mater* **2023**, *12*, e2202709.