Triggering bilayer to inverted-hexagonal nanostructures by thiol-ene click chemistry on cationic lipids: consequences on gene transfection.

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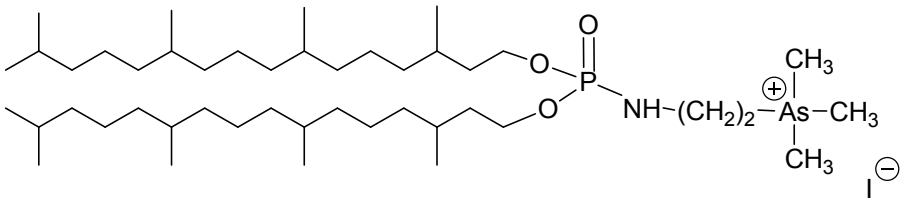
Summary

1. Chemical structure of BSV18 2
2. Protocol for synthesis 2
3. ^{31}P solid state NMR 4
4. ^{2}H solid state NMR 9
5. Liposomal solution: Size, Zeta 11
6. DNA Condensation experiments 11
7. In vitro transfection assays of compounds 1-5 12
8. Cell toxicity of compounds 1-5 13
9. Transfection with DOTMA and compound 6 14
10. Toxicity of DOTMA and compound 6 15
11. Liquid-state NMR spectra of compounds 2-6 16

1. Chemical structure of BSV18



Scheme S1: Chemical structure of BSV18.[1]

2. Protocol for synthesis

General

Solvents were dried with a solvent purification system MBraun-SPS (Et_2O, CH_2Cl_2). All compounds were fully characterized by liquid state NMR: ^{1}H (500.13 or 400.133 or 300.135 MHz), ^{13}C (125.773 or 75.480 MHz) and ^{31}P (161.970 or 121.498 MHz) with Bruker spectrometers such as AC 300, Avance DRX 400 and Avance DRX 500. NMR spectra on liposomal solution were performed at Brest (Service Commun de RMN, Avance DRX 400) or at Bordeaux (Large-scale research infrastructure IR-RMN FR3050, IECB-CBMN platform on a Bruker Avance III 800 SB and on Bruker DPX 400 SB spectrometers. The 800MHz and 400MHz spectrometers were equipped with a CP-MAS dual 4-mm 1H/X probe and a QNP 5mm $^{1}H/^{31}P$-^{13}C-^{19}F/lock probe, respectively. The lipids were purified by flash chromatography (GRACE REVELERIS Flash Chromatography System) equipped with UV and DLS detectors allowing to attest the high purity (> 95%) of the purified compounds. Coupling constants J are given in Hertz. The following abbreviations were used: s for singlet, d doublet, t triplet, q quadruplet, qt quintuplet, m for multiplet and dt for doublet of triplets. When needed, ^{13}C and ^{31}P heteronuclear HMQC and HMBC were used to unambiguously establish molecular structures. Mass spectroscopy analyses were performed at Brest (service commun de spectrometrie de masse) on a Bruker Autoflex MALDI TOF-TOF III LRF200 CID. Commercial compounds [Oleyl alcohol 85 %, phosphorus oxychloride, unsym-dimethylethylenediamine, triethylamine, methyl iodide, all thioalcanes and 2,2-dimethoxy-2-phenylacetophenone] were used as received. Compound **1** was synthesized following a method describe by Le Corre S. S. et al[2]. The thiol-ene reaction was performed under UV-light (Phillips TL-D 18W/08 BLB, λ = 365 nm). The mean particle diameter and zeta potential (ξ) of the liposomes were measured using a Zetazier Nano (Malvern Instruments) at 25 °C.

<u>General procedure for thiol-ene click reaction</u>

[1] M. Lindberg, N. Carmoy, T. Le Gall, A. Fraix, M. Berchel, C. Lorilleux, H. Couthon-Gourvès, P. Bellaud, A. Fautrel, P.A. Jaffrès, P. Lehn and T. Montier, *Biomaterials* 2012, **33**, 6240-6253.

[2] S.S. Le Corre, M. Berchel, N. Belmadi, C. Denis, J.P. Haelters, T. Le Gall, P. Lehn, T. Montier and P.A. Jaffrès, *Org. Biomol. Chem*., 2014, **12**, 1463-1474.

Compound **1** (1 eq.) and alkane-thiol (3.5 eq.) were mixed in a glass tube and placed in an ultrasonic bath until total dissolution (10 minutes to 20 minutes). Then, the mixture was degassed with argon for 2 minutes before the addition of 2,2-dimethoxy-2-phenylacetophenone (10% wt.). The solution was placed under UV for 4 h at room temperature. The product was purified by flash chromatography on silica gel (CH_2Cl_2/methanol: 0 to 15) to obtain yellow wax with 40 to 70 % yield.

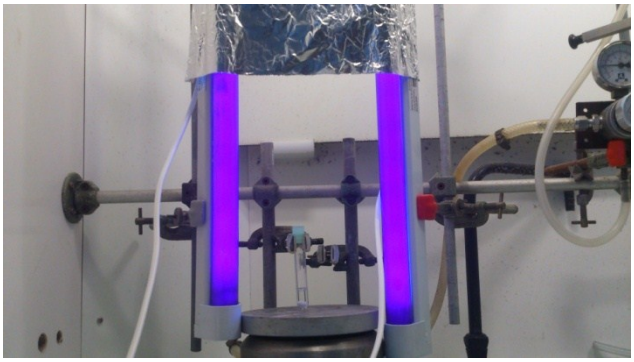


Figure S1: "thiol-ene" reaction system

Compound **2**

Yield: 51 % (133 mg); **^{31}P NMR:** δ (ppm, ref 85% H_3PO_4: 0 ppm in $CDCl_3$) = 8.6; **^{1}H NMR:** δ (ppm, ref TMS: 0 ppm in $CDCl_3$) = 0.86 (m, 6H, C**H$_3$**-CH_2), 1.24 to 1.90 (m, H, fatty chain) ; 2.60 (m, 4H, C**H**-S-C**H**), 3.53 (s, 9H, $^{+}$N(C**H$_3$**)$_3$), 3.57 (m, 2H, C**H$_2$**-NH), 3.84 (m, 2H, C**H$_2$**-$^{+}$N$(CH_3)_3$), 3.97 (m, 4H, C**H$_2$**-O-P), 4.35 (brs, 1H, N**H**); **^{13}C NMR:** δ (ppm, ref TMS: 0 ppm in $CDCl_3$) = 14.1 (C**H$_3$**-CH_2), 22.6 to 31.9 (**C**H_2 fatty chain), 34.3 (**C**H_2 cyclohexyle), 35.5 ((**C**H_2)$_2$-CH-S), 36.2 (**C**H_2-NH), 42.6 (**C**H cyclohexyle), 44.2 ((CH_2)$_2$-**C**H-S), 54.9 ($^{+}$N(**C**H_3)$_3$), 66.7 (**C**H_2-$^{+}$N$(CH_3)_3$), 67.1 (P-O-**C**H_2); **MALDI-TOF:** $[M]^+$ calculated for $C_{53}H_{108}N_2O_3PS_2$ = 915.753 ; $[M]^+_{measured}$ = 915.741; **IR(ν, cm^{-1})**: 1234 (P=O), 2851 to 2922 (C-H), 3208 (N-H); **TLC** [CH_2Cl_2 / MeOH – 90/10]: r_f= 0.35

Compound **3**

Yield: 58 % (150 mg) ; **^{31}P NMR:** δ(ppm, ref 85% H_3PO_4: 0 ppm in $CDCl_3$) = 8.7; **^{1}H NMR:** δ (ppm, ref TMS: 0 ppm in $CDCl_3$) = 0.89 (m, 6H, C**H$_3$**-CH_2), 1.25 to 1.68 (m, 60H, fatty chain), 2.48 (qt, 2H, (CH_2)$_2$C**H**-S, J_{H-H}= 6,4Hz), 3.43 (s, 9H, $^{+}$N(C**H$_3$**)$_3$), 3.56 (m, 2H, C**H$_2$**-NH), 3.68 (s, 4H, C**H$_2$**-Ph), 3.84 (m, 2H, C**H$_2$**-$^{+}$N$(CH_3)_3$), 3.98 (m, 4H, C**H$_2$**-O-P), 4.40 (brs, 1H, N**H**), 7.19 à 7,33 (m, 10H, C**H** aromatic); **^{13}C NMR:** δ (ppm, ref TMS: 0 ppm in $CDCl_3$) = 14.1 (**C**H_3-CH_2), 22.6 to 31.8 (**C**H_2 fatty chain), 34.5 ((CH_2)$_2$-CH-S), 35.0 (**C**H_2-Ph), 45.3 ((CH_2)$_2$-**C**H-S), 54.8 ((**C**H_3)$_3$N+), 66.6 (**C**H_2-$^{+}$N$(CH_3)_3$), 67.1 (d, **C**H_2-O-P, $^{2}J_{P-C}$= 5,3ppm), 126.7 (**C**H aromatic), 128.3 à 129.0 (**C**H aromatic), 138.9 (quaternary **C**); **MALDI-TOF:** $[M]^+$ calculated for $C_{55}H_{100}N_2O_3PS_2$ = 931.691 ; $[M]^+_{measured}$= 931.686; **IR (ν, cm^{-1})**: 1233 (P=O), 696 and 764 (C-H aromatic), 2851 and 2922 (C-H), 3211 (N-H); **TLC** [CH_2Cl_2 / MeOH – 90/10]: r_f= 0.39.

Compound **4**

Yield: 42 % (120 mg); **^{31}P NMR:** δ (ppm, ref 85% H_3PO_4: 0 ppm in $CDCl_3$) = 8.6; **^{1}H NMR:** δ (ppm, ref TMS: 0 ppm in $CDCl_3$) = 0.86 (m, 12H, C**H$_3$**-CH_2), 1.30 to 1.66 (m, 76H, **C**H_2 fatty chain), 2.45 (t, 4H, C**H$_2$**-S, J_{H-H}= 8.0Hz), 2.52 ppm (qt, 2H, (CH_2)$_2$C**H**-S, J_{H-H}= 6Hz), 3.45 (s, 9H, $^{+}$N(C**H$_3$**)$_3$), 3.55 (m, 2H, C**H$_2$**-NH), 3.85 (m, 2H, C**H$_2$**-$^{+}$N$(CH_3)_3$), 3.96 ppm (m, 4H, C**H$_2$**-O-P), 4.40ppm (brs, 1H, N**H**); **^{13}C NMR:** δ (ppm, ref TMS: 0 ppm in

$CDCl_3$) = 13.8 (C**H**$_3$-CH_2), 22.3 to 31.6 (**C**H_2 fatty chain), 34.6 (**C**H_2-CH-**C**H_2), 35.9 (**C**H_2-NH), 45.6 (**C**H-S), 54.5 ($^+$N(**C**H_3)$_3$), 66.4 (**C**H_2-$^+$N$(CH_3)_3$), 66.8ppm (d, **C**H_2-O-P, $^2J_{C\text{-}P}$= 5,2Hz); **MALDI-TOF:** $[M]^+$ calculated for $C_{53}H_{112}N_2O_3PS_2$= 919.785, $[M]^+_{measured}$= 919.775; **IR (ν, cm^{-1}):** 1231 (P=O), 2852 and 2922 (C-H), 3210 (N-H); **TLC [CH_2Cl_2 / MeOH – 90/10]:** r_f= 0.34

Compound **5**

Yield: 65 % (150 mg) ; **^{31}P NMR:** δ (ppm, ref 85% H_3PO_4: 0 ppm in $CDCl_3$) = 8.6; **^{1}H NMR:** δ (ppm, ref TMS: 0 ppm in $CDCl_3$) = 0.85 (m, 12H, C**H**$_3$-CH_2), 1.25 to 1.67 (m, 100H, fatty chain), 2.45 (t, 4H, C**H**$_2$-S, $J_{H\text{-}H}$= 8.0Hz), 2.54 (qt, 2H, $(CH_2)_2$C**H**-S, $J_{H\text{-}H}$= 8.0Hz), 3.45 (s, 9H, $^+$N(C**H**$_3$)$_3$), 3.58 (m, 2H, C**H**$_2$-NH), 3.85 (m, 2H, C**H**$_2$-$^+$N$(CH_3)_3$), 3.97 (m, 4H, C**H**$_2$-O-P), 4.40 (brs, 1H, N**H**); **^{13}C NMR:** δ (ppm, ref TMS: 0 ppm in $CDCl_3$) = 13.9 (**C**H_3-CH_2), 22.5 ppm à 31.8 (**C**H_2 fatty chain), 34.8 (**C**H_2-CH-**C**H_2), 36.0 (**C**H_2-NH), 45.8 (**C**H-S), 54.7 ($^+$N(**C**H_3)$_3$), 66.6 (**C**H_2-$^+$N$(CH_3)_3$), 66.9 (d, **C**H_2-O-P, $^2J_{C\text{-}P}$= 5.3Hz); **MALDI-TOF:** $[M]^+$ calculated for $C_{65}H_{136}N_2O_3PS_2$= 1087,972 ; $[M]^+_{measured}$= 1087.975. **IR (ν, cm^{-1})**: 1234 (P=O), 2851 and 2921 (C-H), 3200 (N-H); **TLC** [CH_2Cl_2 / MeOH – 90/10]: r_f= 0.42.

Compound **6**

Yield: 61 % (199 mg); **^{1}H NMR:** δ (ppm, ref TMS: 0 ppm in $CDCl_3$) = 0.83 to 0.87 (t, 12H, $^3J_{H\text{-}H}$= 6.6 Hz), 1.23 to 1.24 (m, 73H, C**H**$_2$ fatty chains), 1.35 to 1.37 (m, 12H, C**H**$_2$ fatty chain), 1.46 to 1.54 (m, 16H, C**H**$_2$-CH_2-S, C**H**$_2$-CH-S and C**H**$_2$ fatty chains), 2.41 to 2.45 (t, 4H, C**H**$_2$-S, $^3J_{H\text{-}H}$= 7.4 Hz), 2.48 to 2.55 (qt, 2H, (CH_2)-C**H**-S, $^3J_{H\text{-}H}$= 7,3 Hz), 3.37 to 3.43 (m, 2H, C**H**$_2$ aminoglycerol skeleton), 3.43 to 3.56 (m, 10H, C**H**$_2$ aminoglycerol skeleton and $^+$N(C**H**$_3$)$_3$), 3.65 to 3.69 (m, 2H, C**H**$_2$ aminoglycerol skeleton), 3.95 to 3.99 (dm, 1H, C**H**$_2$ aminoglycerol skeleton, $^2J_{H\text{-}H}$= 13.2 Hz), 4.02 (brs, 1H, $(CH_2)_2$-C**H**-O).**^{13}C NMR:** δ (ppm, ref TMS: 0 ppm in $CDCl_3$) = 14.12 (**C**H_3-CH_2), 22.68 (**C**H_2 fatty chains), 26.03 (**C**H_2 fatty chains), 26.23 (**C**H_2 fatty chains), 26.81 (**C**H_2 fatty chains), 29.07 to 29.95 (**C**H_2 fatty chains), 30.35 (**C**H_2-S-CH), 31.90 (**C**H_2 fatty chains), 34.91 (**C**H_2 fatty chains), 46.88 ($(CH_2)_2$-**C**H-S), 54.79 ($^+$N(**C**H_3)$_3$), 67.93 (**C**H_2 aminoglycerol skeleton), 68.38 (**C**H_2 aminoglycerol skeleton), 69.31 (**C**H_2 aminoglycerol skeleton), 72.01 (**C**H_2 aminoglycerol skeleton), 73.63 ($(CH_2)_2$-**C**H-O). **IR :** ν (cm^{-1}) = **TLC [CH_2Cl_2 / MeOH – 90/10]:** r_f = 0.60

3. ^{31}P solid state NMR

Sample preparation: A mother solution of a selected cationic amphiphile was prepared in chloroform. A volume corresponding to a mass of cationic lipid ranging from 50 to 100 mg, was pipetted in a glass tube and chloroform was evaporated under N_2 flux to obtain a lipid film. Then, 500 µL of water were added and the mixture was sonicated until the dispersion of the film. This mixture was lyophilized overnight to get a nice and fluffy powder (we observed that this first hydration step had deeply facilitated the subsequent hydration step)[3]. The needed volume of distilled water was added to obtain a final lipid concentration of 100 mg.mL^{-1} In order to ensure sample equilibrium, the samples were then submitted to three freeze-thaw-shaking cycles that consisted of placing them at -198°C (liquid nitrogen) for 5 minutes, shaking at room temperature and then going up to at 50°C (water bath) for 30 minutes and shaking in a vortex mixer for 10 minutes.

[3] Beck, J. G.; Mathieu, D.; Loudet, C.; Buchoux, S. ; Dufourc, E. J. *Faseb Journal* **2007**, *21*, 1714-1723.

NMR conditions: ^{31}P spectra were acquired by means of a Hahn echo sequence (90°–τ–180°–τ–acq).[4] The acquisition parameters were as follow: spectral window of 200 KHz , π/2 pulse width of 4.88µs, recycle delay of 5s, and echo delay of 40µs. The number of acquisitions was dependent on sample volume and temperature, and ranged from 128 to 1700 scans. Samples were allowed to equilibrate for 30 min at a given temperature before the NMR signal was acquired. Experimental temperature were 243 K, 253 K, 263 K, 268 K, 273 K, 298 K, 303 K, 308 K, 313 K, 318 K, 320 K, 322 K and 328 K. Lorentzian noise filtering with a width of 200Hz was applied prior Fourier transformation from the top of echo signal.

Calculation of second spectral moments: Second moments were calculated using a python home made routine (Buchoux S.,unpublished) to which Bruker NMR data were exported using a proper subroutine.

Simulation of ^{31}P-NMR Spectra: Lineshapes were calculated to obtain chemical shielding anisotropies with a better accuracy. A user-friendly software interface was developed by Arnaud Grélard using computer programs written in FORTRAN by Erick Dufourc.

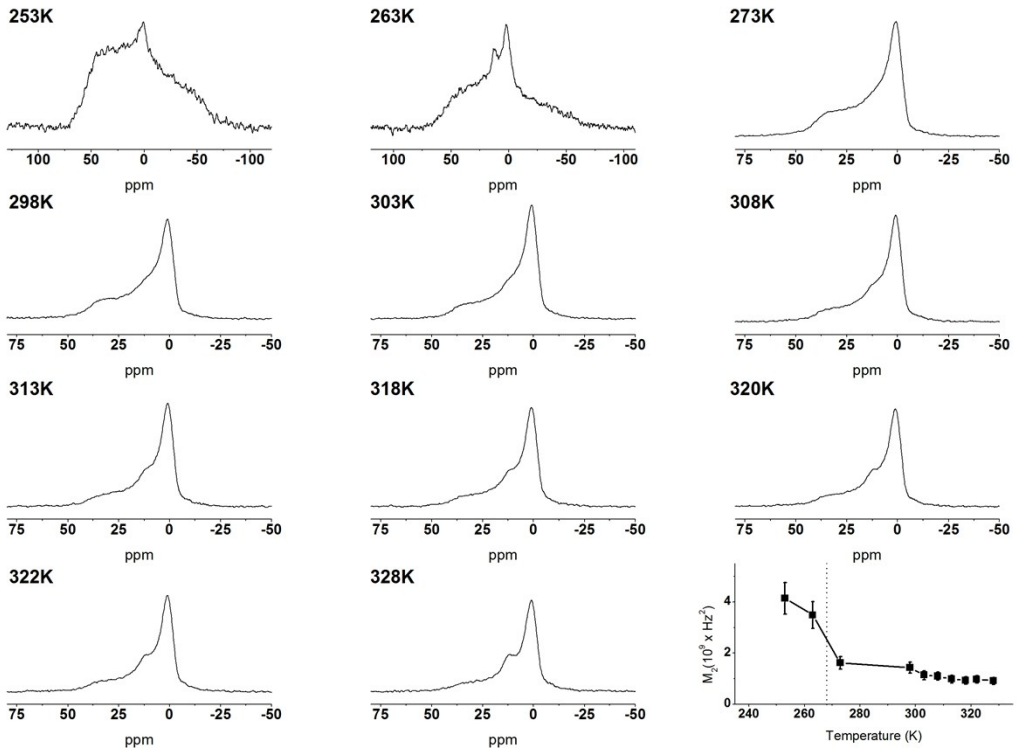


Figure S2-1: ^{31}P solid-state NMR spectra of compound **1**, fully hydrated, in the temperature range 253K to 328K. Right bottom panel, calculation of the second moment, M_2, as a function of temperature. The transition from gel-solid ordered phase to fluid-liquid disordered phase occurs at ca. 268 ± 5K (-5 ± 5°C). ^{31}P Chemical shifts are expressed relative to H_3PO_4 (0 ppm).

[4] Rance, M.; Byrd, R.A. *J. Magnet. Res*. **1983**, *52*, 221-240.

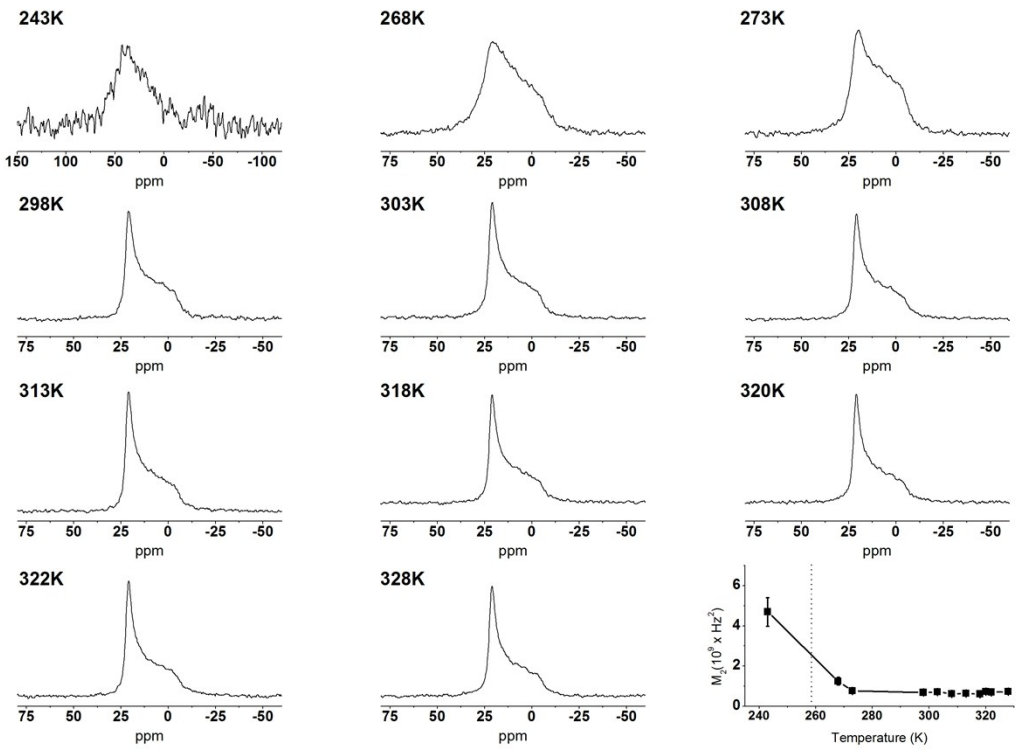


Figure S2-2: ^{31}P solid-state NMR spectra of compound **3**, fully hydrated, in the temperature range 243k to 328K. Right bottom panel, calculation of the second moment as a function of temperature. The transition from gel-ordered phase to hexagonal fluid phase occurs at ca. 258 ± 5K (-15 ± 5°C). ^{31}P Chemical shifts are expressed relative to H_3PO_4 (0 ppm).

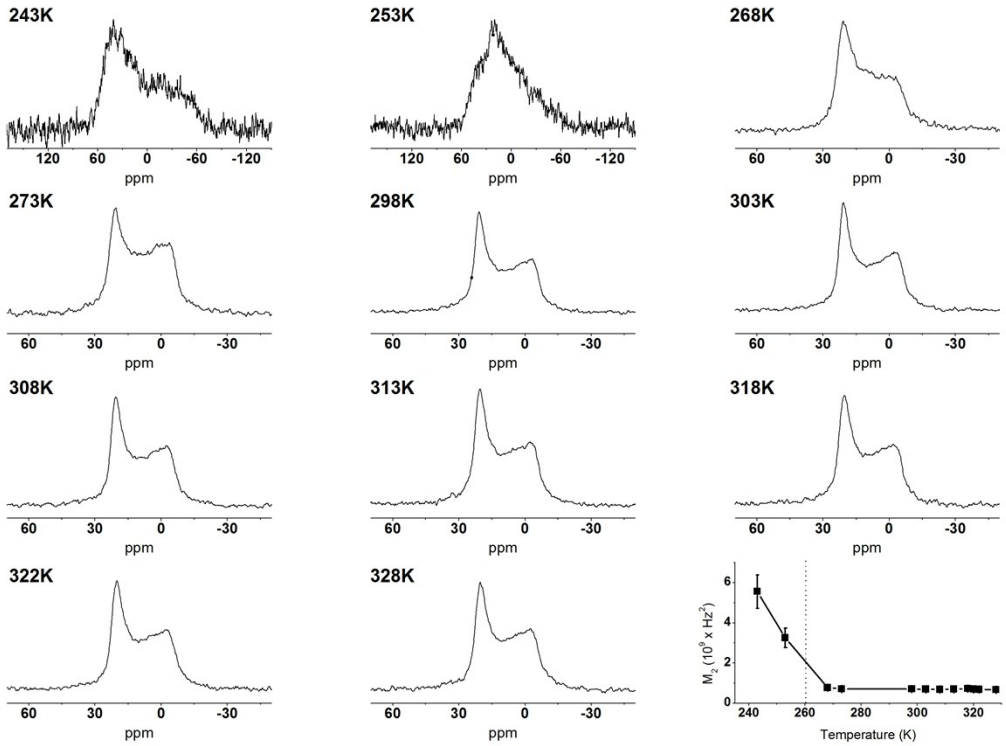


Figure S2-3: ^{31}P solid-state NMR spectra of compound **5**, fully hydrated, in the temperature range 243k to 328K. Right bottom panel, calculation of the second moment as a function of temperature. The transition from gel-ordered phase to a hexagonal fluid phase occurs at ca. 260 ± 5K (-8 ± 5°C). ^{31}P Chemical shifts are expressed relative to H_3PO_4 (0 ppm). It is noteworthy that use of a strong magnetic field (18.8 Tesla) affects the spectral lineshape suggesting a slight alignment of hexagonal phases in the magnetic field at high temperature.

4. ^{2}H solid state NMR

Sample preparation: First, 40 mg of compound **5** and 0.5 mg of DOPC-D31 (2 % mol) were mixed in chloroform. Chloroform was evaporated under N_2 flux to obtain a lipid film. Then, 500 µL of water were added and the mixture was sonicated until the dispersion of the film. This mixture was lyophilized overnight (we observed that this first hydration step had deeply facilitated the subsequent hydration step). 100 µL of depleted (2-3ppm ^{2}H content) water was added. Then, the sample was submitted to three thermal cycles that consisted to placed it at -198°C and then at 50°C.

NMR conditions: solid state ^{2}H-NMR spectra were acquired by means of a quadrupolar echo pulse sequence ($90°-\tau-90°-\tau-$acq)[5]. The acquisition parameters were as follow: spectral window of 500 KHz, $\pi/2$ pulse width of 4.88µs, recycle delay of 1.5s, and echo delay of 40µs, number of scans 7K. Samples were allowed to equilibrate for 30 min at a given temperature before the NMR signal was acquired. Experimental temperature were 268 K, 273 K, 298 K, 303 K, 308 K, 313 K, 318 K, 320 K and 325 K. Lorentzian noise filtering with a width of 50Hz was applied prior Fourier transformation from the top of echo signal.

Spectral simulations: A user-friendly software interface was developed by Arnaud Grélard using computer programs written in FORTRAN by Erick Dufourc: the time domain trace composed of the weighed sum of signals corresponding to each quadrupolar splitting (most intense doublet of the Pake pattern) of individual C-D bonds of the entire lipid chain is calculated, and a Fourier transformation is performed, leading to spectra as in Figure SI3. Adjustable parameters are the individual quadrupolar splittings and their intrinsic line width. All measurable quadrupolar splittings are taken as input parameters, the weight of each splitting being set according to the molecular structure. Comparison between experimental spectra and calculated is made until a satisfactory superimposition is obtained.

[5] Davis, J. H. The description of membrane lipid conformation, order and dynamics by 2H-NMR. BBA - Reviews on Biomembranes, **1983**, 737, 117-171

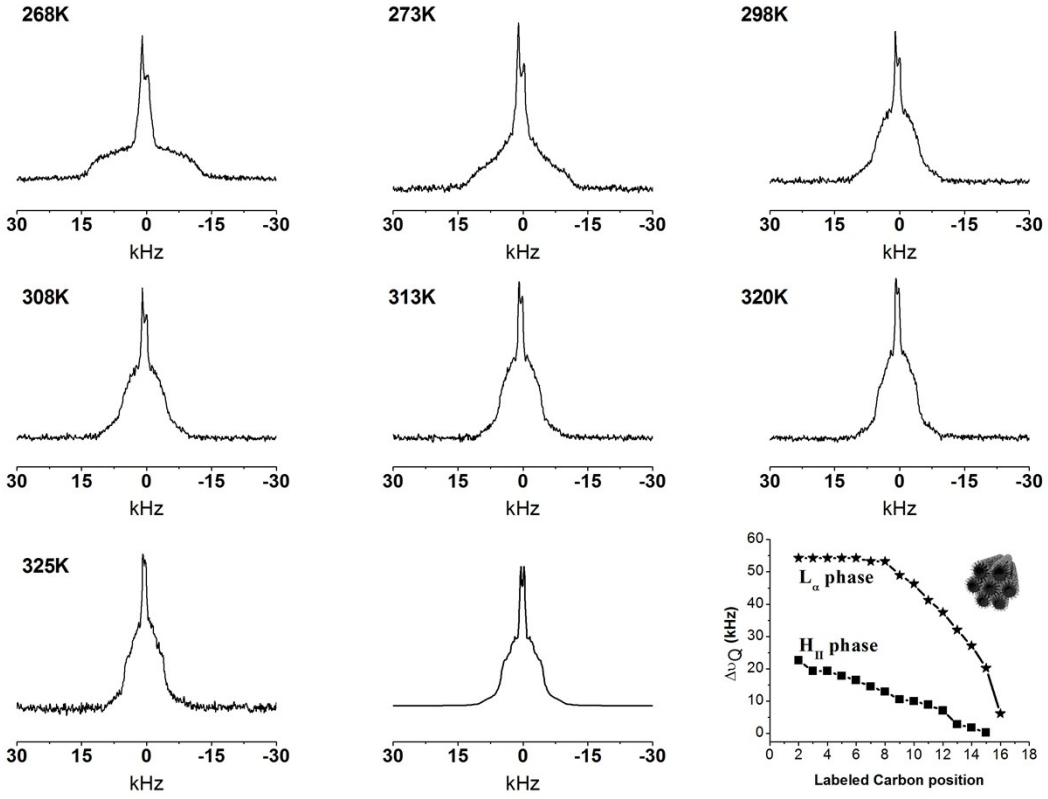


Figure S3: ^{2}H solid-state NMR spectra of compound **5**, fully hydrated, in the temperatures range 268K to 328K. 2% of chain deuterated POPC ($^2H_{31}$-POPC) has been inserted in the lipid dispersion. Bottom row: simulated spectrum at 313K (center, corresponding experimental spectra is on the middle row above) and plot of the individual C-D bond quadrupolar splitting, $\Delta\nu_Q$, as a function of labeled carbon position along the chain (right, filled squares). Position 2 is near the glycerol backbone and position 16 is at the bilayer center. Quadrupolar splittings are directly proportional to C-D bond order parameters that report on the internal packing of lipid chains in the lipid assembly. The quasi-linear decrease of quadrupolar splittings indicates an hexagonal packing of type II (the chains pointing outside the hexagonal cylinders as shown in the inserted sketch. As a comparison the quadrupolar splitting profile of POPC multi-lamellar vesicles in Lα phase, with the well-known "plateau" of quadrupolar splittings up to carbon 8 characteristic of tight chain packing, is also shown on this figure (filled stars). Quadrupolar splittings are directly proportional to segment order parameters, S_{CD} ($\Delta\upsilon_Q^L = \frac{3}{4}A_Q S_{CD}$ for lamellar phases and $\Delta\upsilon_Q^H = \frac{3}{8}A_Q S_{CD}$ for hexagonal phases, A_Q is the static quadrupolar coupling constant, 167 kHz).[2]

5. Liposomal solution: Size, Zeta

Preparation of liposomal solution: The cationic lipids were formulated as liposomal solution by using the hydration of a lipid film. First, an aliquot of a concentrated solution of a desired lipid in chloroform was placed in a glass tube and the solvent was evaporated (12h in vacuum) to produce a thin lipid film. Then 1 mL of water was added on this film and the film was hydrated for 3 days at 4°C. The solution was vortexed (1 min) and sonicated (30 to 60 minutes) at 45 kHz using a VWR ultrasonic bath. 150 µL of the liposomal solution were taken and dissolved in 1,5 mL of sterile water. After filtration (200 µm) the size and the zeta potential were determined.

Name	Time of sonication (min)	Size (nm)	PdI	Zeta (mV)	Δ Zeta (mV)
BSV 36 (**1**)	30	94	0.26	+ 54	4
BSV 102 (**4**)	60	99	0.26	+ 33	7
BSV 103 (**5**)	60	148	0.15	+ 37	5
BSV 104 (**2**)	60	187	0.36	+ 44	5
BSV 105 (**3**)	30	137	0.19	+ 50	7

Tableau SI1: Size and zeta potential (Result of size reported in volume)

6. DNA condensation experiments

Liposomal solutions were mixed with pDNA (3,7kb, pCMV-Luc) as follows: to 1 µg of pDNA in Optimem (Gibco) were added cationic lipids at concentrations corresponding to +/- charge ratios (CR) ranging from 1 to 8. These mixtures were incubated for 30 min at RT. The complexes were subjected to electrophoresis in 0.8% agarose gel at 100 V, 90 mA. The gel was stained with ethidium bromide (10 mg/mL) in order to be thereafter visualized using an UV transilluminator (Fischer Bioblock).

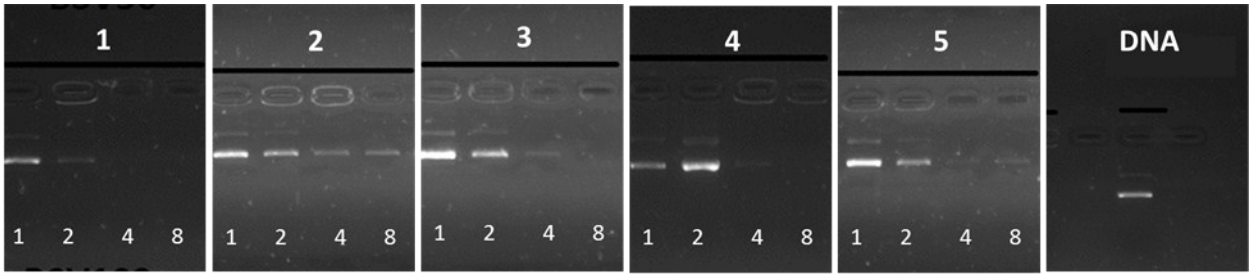


Figure S5: DNA condensation experiments images with compounds 1, 2, 3, 4, 5 and free DNA at different charge ratio (1, 2, 4 and 8)

7. *In vitro* transfection assays of compounds 1-5

Cells were grown in EMEM (16HBE, CFBE and SKMel28), or DMEM (A549), supplemented with 10% bovine fetal serum, 1% antibiotic or 0.4% for the SKMel28, and 1% L-Glutamine, in a humidified incubator with 5% CO_2 at 37°C.

The protocol of the experiments was similar to those previously reported.[6] Shortly, cells were seeded 24 h before transfection onto a 24-well plate at a density of 100,000 cells per well and incubated overnight in a humidified 5% CO_2 atmosphere at 37 ◦C. About 200 µL of a solution of lipoplex was added to each well. Lipofectamine (LFM) was used as a positive control. After 2h30 of incubation at 37 ◦C, 20 µL of the surrounding was picked up to evaluate the toxicity of each formulation (see below). The culture medium was removed to be replaced with fresh. Following a further 48 h incubation at 37 ◦C, the cells were lysed in order to be assayed for luciferase expression using a chemiluminescent assay (Luciferase Assay System, Promega). The total protein content of the cell lysate was determined using the BC assay kit (Uptima). Data are expressed as relative light units (RLU) per milligram of total proteins (mean ± SD with n = 3).

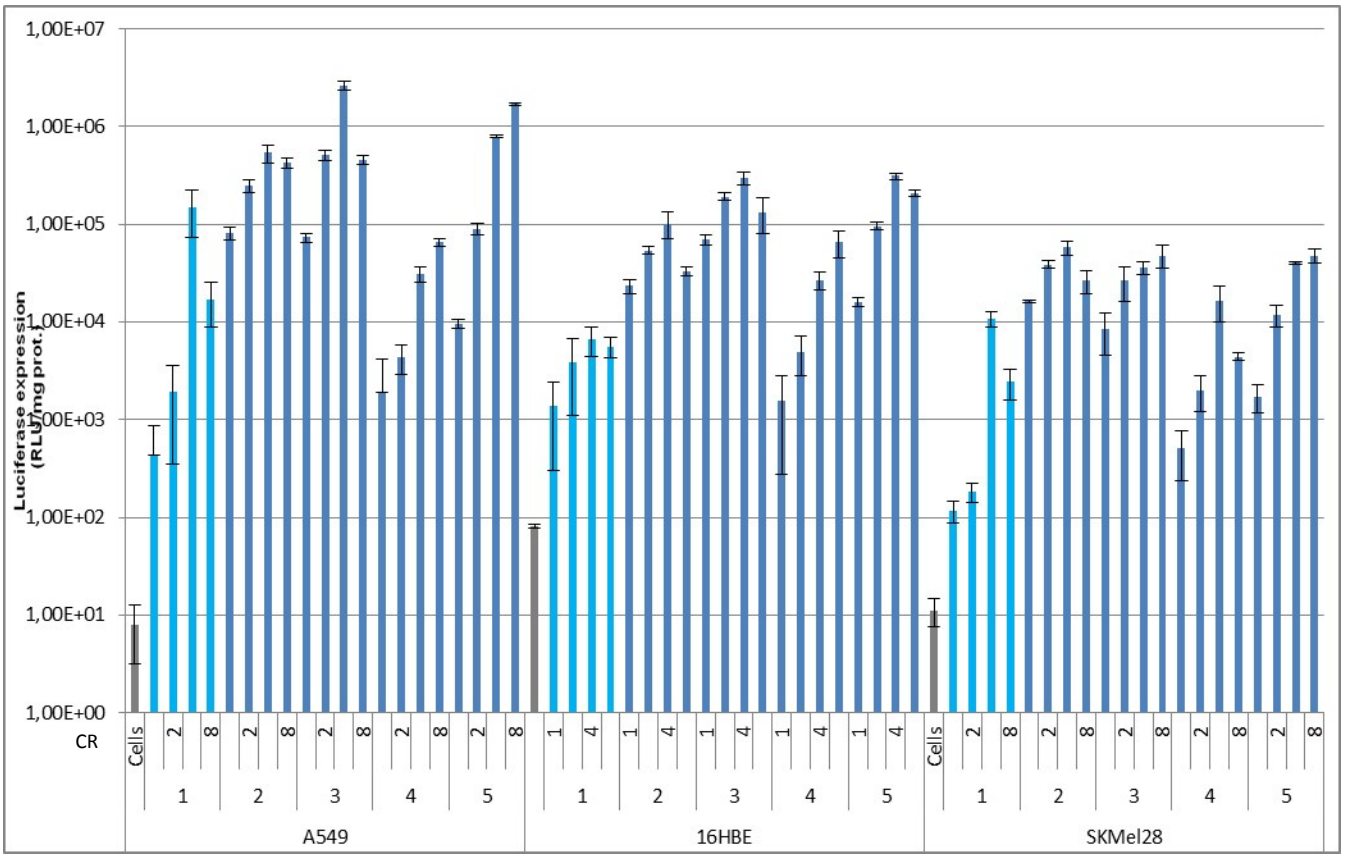


Figure S6: transfection efficiencies with compounds **1** to **5** at different charge ratio (1, 2, 4 and 8) on three cell lines: a) A549; b) 16HBE; c) SKMEL28.

[6] A. Fraix, T. Montier, N. Carmoy, D. Loizeau, L. Burel-Deschamps, T. Le Gall, P. Giamarchi, H. Couthon-Gourvès, J.P. Haelters, P. Lehn and P.A. Jaffrès, *Org. Biomol. Chem*., **2011**, *9*, 2422-2432.

8. Cell toxicity of compounds 1-5

Experimental part

The early toxicity of the different lipid/DNA complexes was determined by using a chemiluminescent assay (Toxilight - Cambrex, Liège, Belgium). Briefly, this test consists of the measurements of the release of a normally cytoplasmic enzyme (adenylate kinase, AK) from damaged cells into the surrounding medium. The reaction involves two steps. The first one requires the addition of ADP as a substrate for AK. In the presence of AK, the ADP is converted to ATP for assay by bioluminescence. Then, the bioluminescence method utilizes an enzyme Luciferase, which catalyzes the emission of photons from ATP and luciferin. By combining both reactions, the emitted light intensity is directly related to the AK concentration. The relative light units (RLU) are conversely proportional to the intensity of damages induced to the cells consequently of their exposure with the complexes studied. Untransfected cells were used to evaluate the AK background activity indicative of an absence of toxicity.

Results

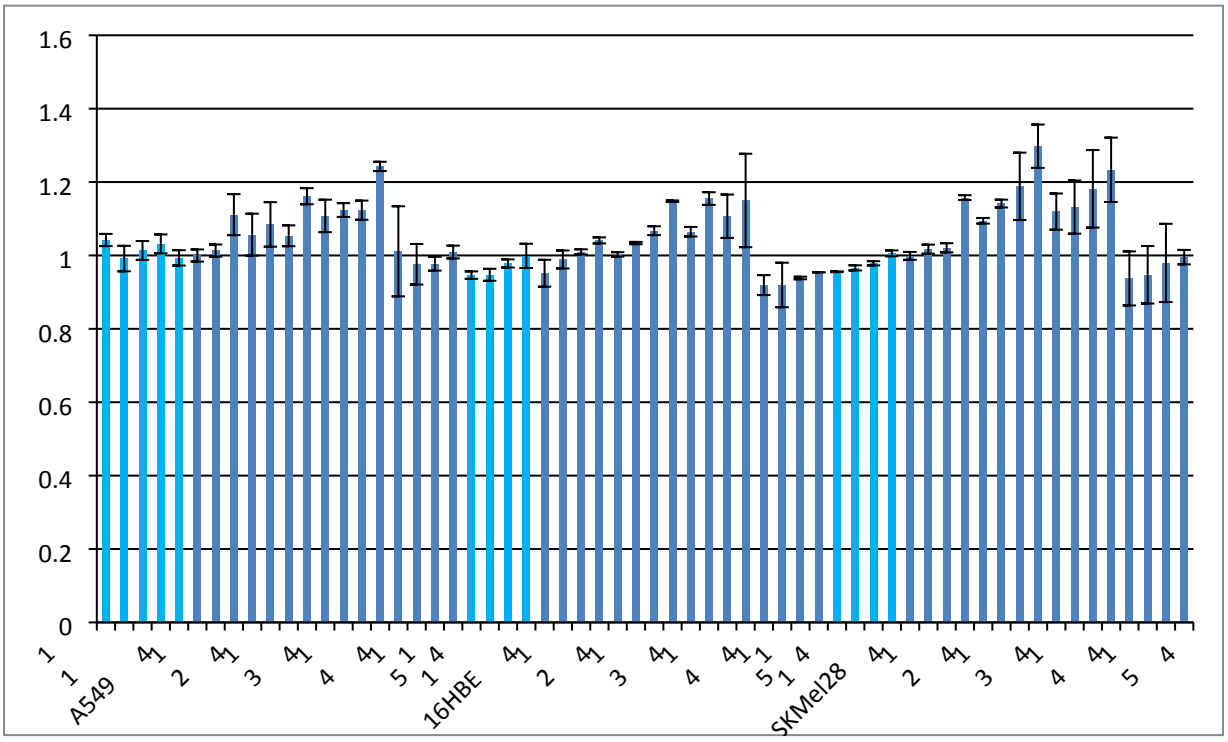
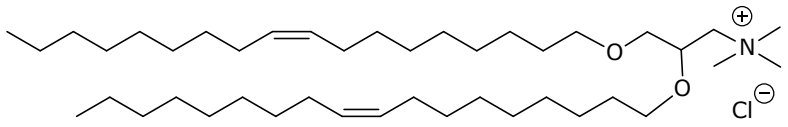
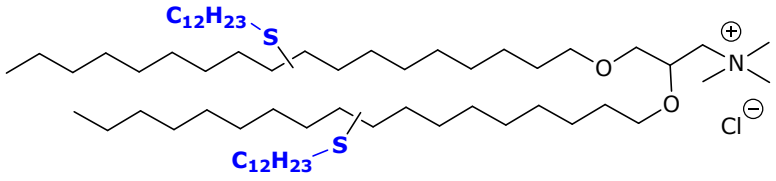


Figure S7: relative cell toxicity of compound **1** to **5** formulated as lipoplexes at different CR (CR= 1, 2, 4 or 8) (ratio of transfection efficacies of a selected compound / untransfected cell)

9. Transfection with DOTMA and compound 6



DOTMA



6 (BSV 139)

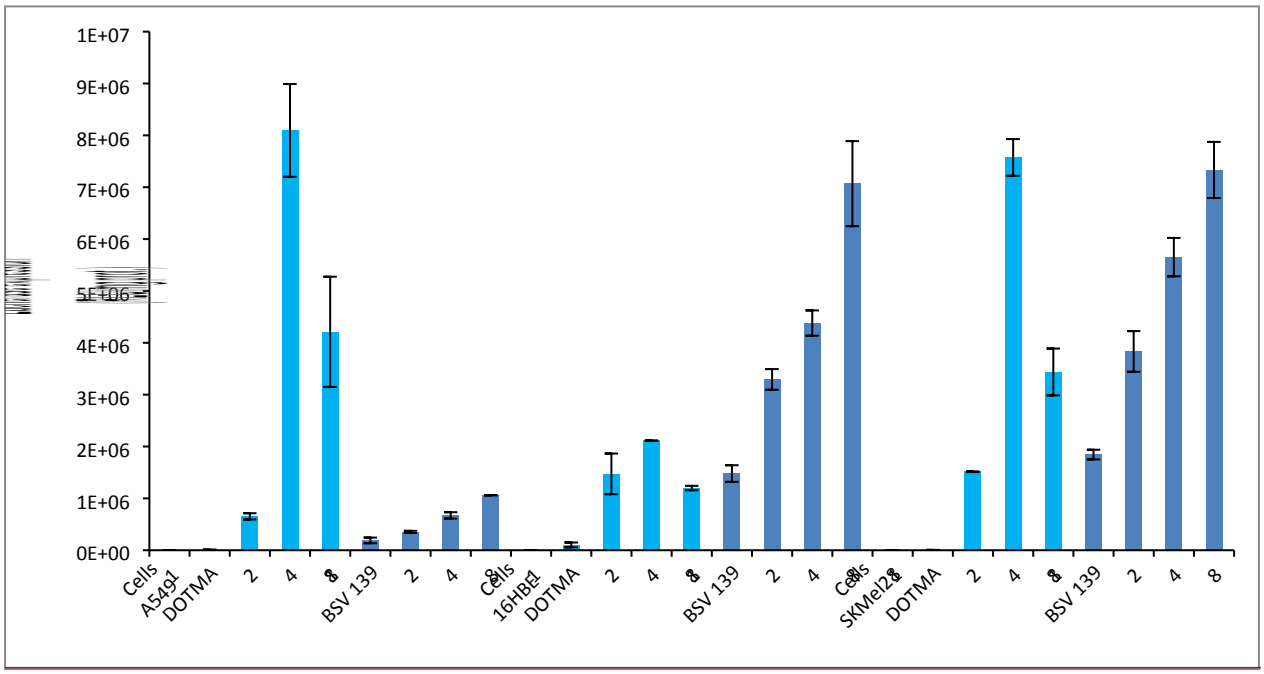
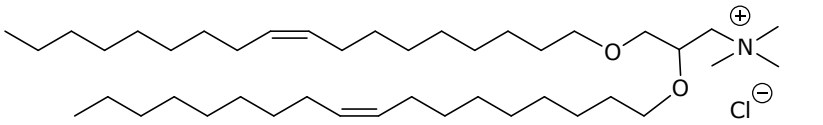


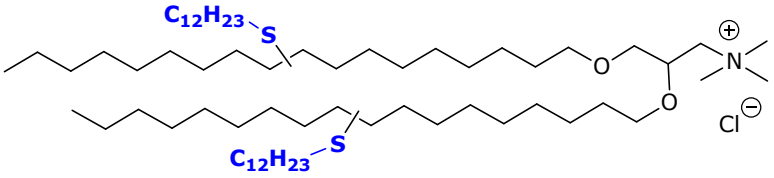
Figure S8: transfection efficiencies with DOTMA and compound **6** (BSV139) on three cell lines: a) A549; b) 16HBE; c) SKMEL28**.**

10. Toxicity of DOTMA and compound 6

A cell viability assay (ViaLight™ Plus Cell Proliferation and Cytotoxicity) furnished by Lonza was used here. This test consists of measuring the ATP content of the cells using a chemiluminescent detection method. The ATP content is an informative marker of the cell density. Cells that were not exposed to any reagent were used to assess the higher viability (100% cell viability). A lower cell density can result from a lower proliferation and/or a higher mortality, both reflecting in a lower cell viability as compared to that of untreated cells and in a proportional way.



DOTMA



6 (BSV 139)

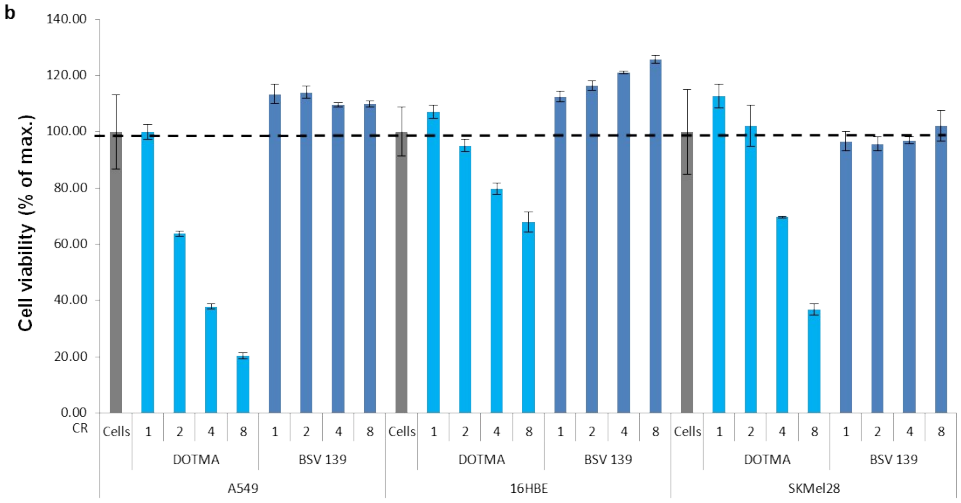


Figure S9: cell toxicity expressed as % of cell viability of DOTMA and compound **6** (BSV139) formulated as lipoplexes at different CR (CR= 1, 2, 4 or 8).

11. Liquid-state NMR spectra of compounds 2-6

Compound **2**

^{1}H NMR

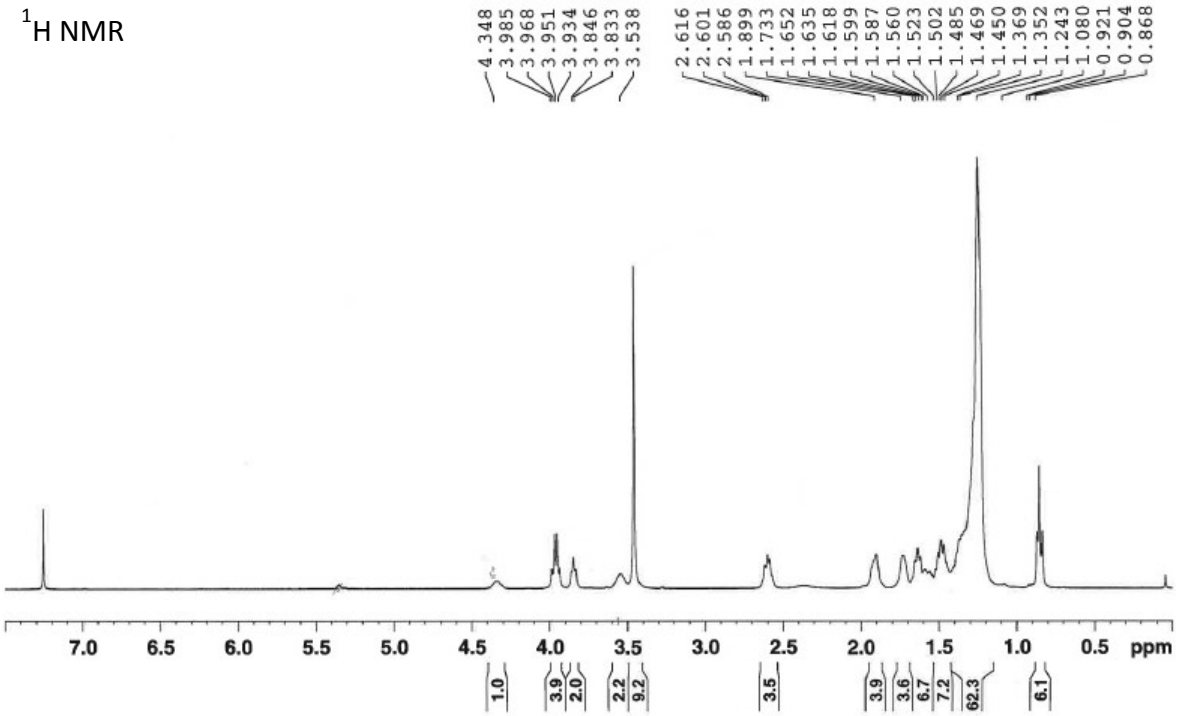


Figure S10-1: ^{1}H NMR of compound **2** (in $CDCl_3$)

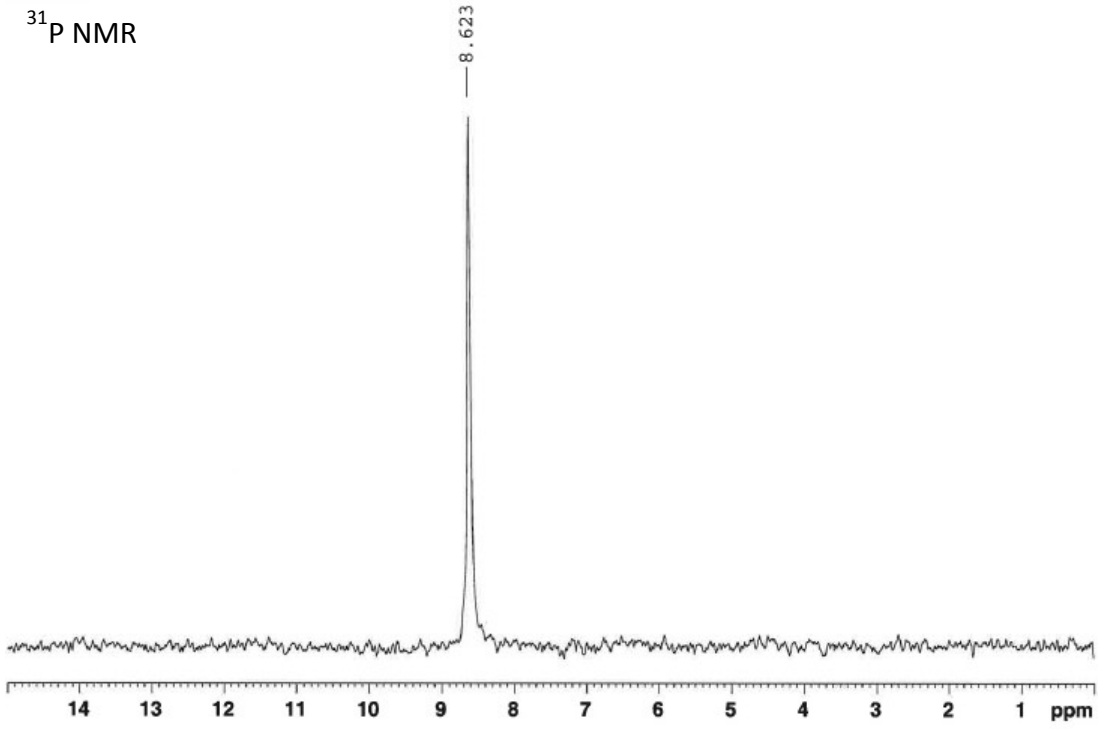


Figure S10-2: $^{31}P\{^{1}H\}$ NMR of compound **2** (in $CDCl_3$)

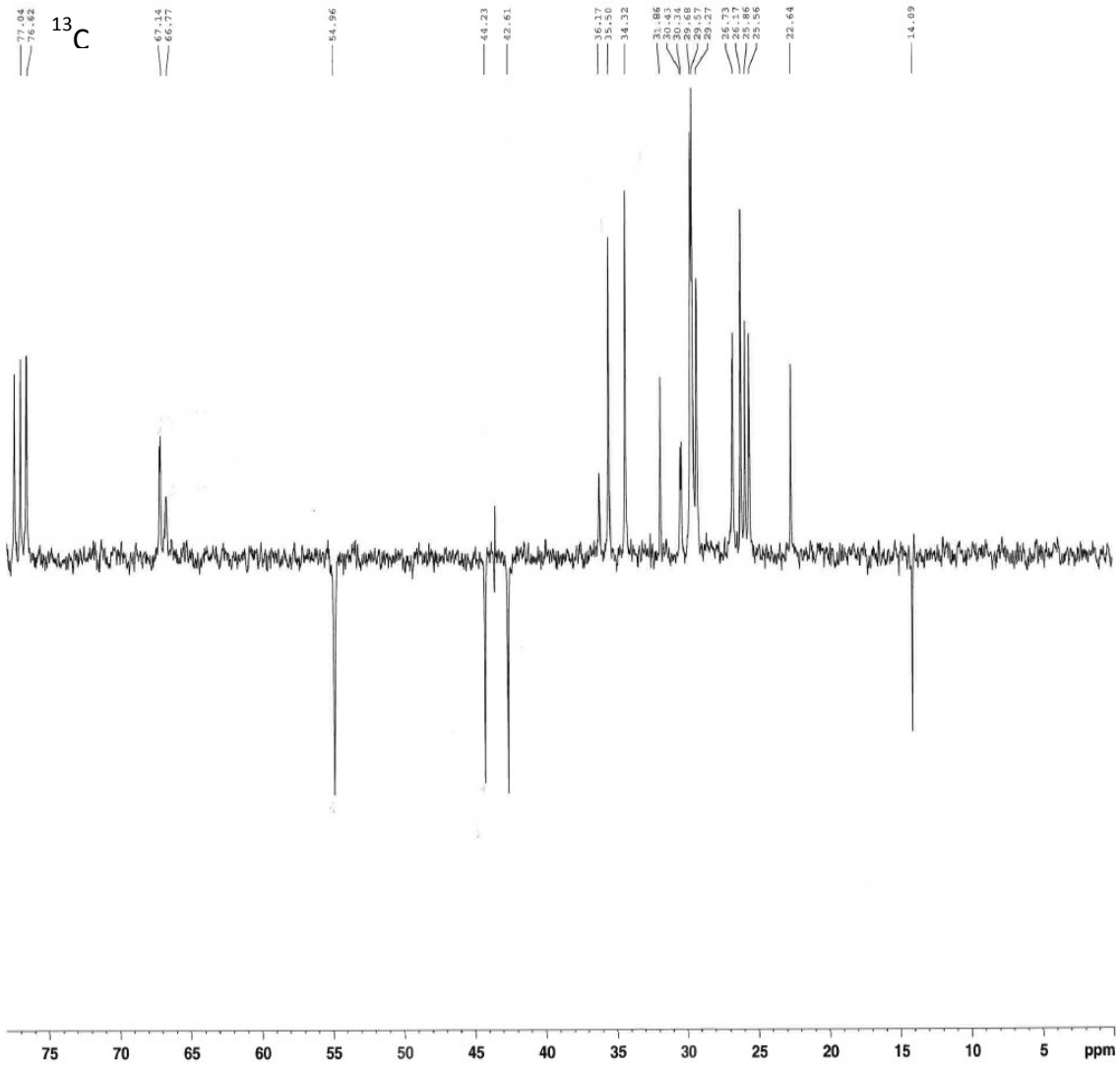


Figure S10-3: ^{13}C NMR (Jmod) of compound **2** (in $CDCl_3$)

Compound **3**

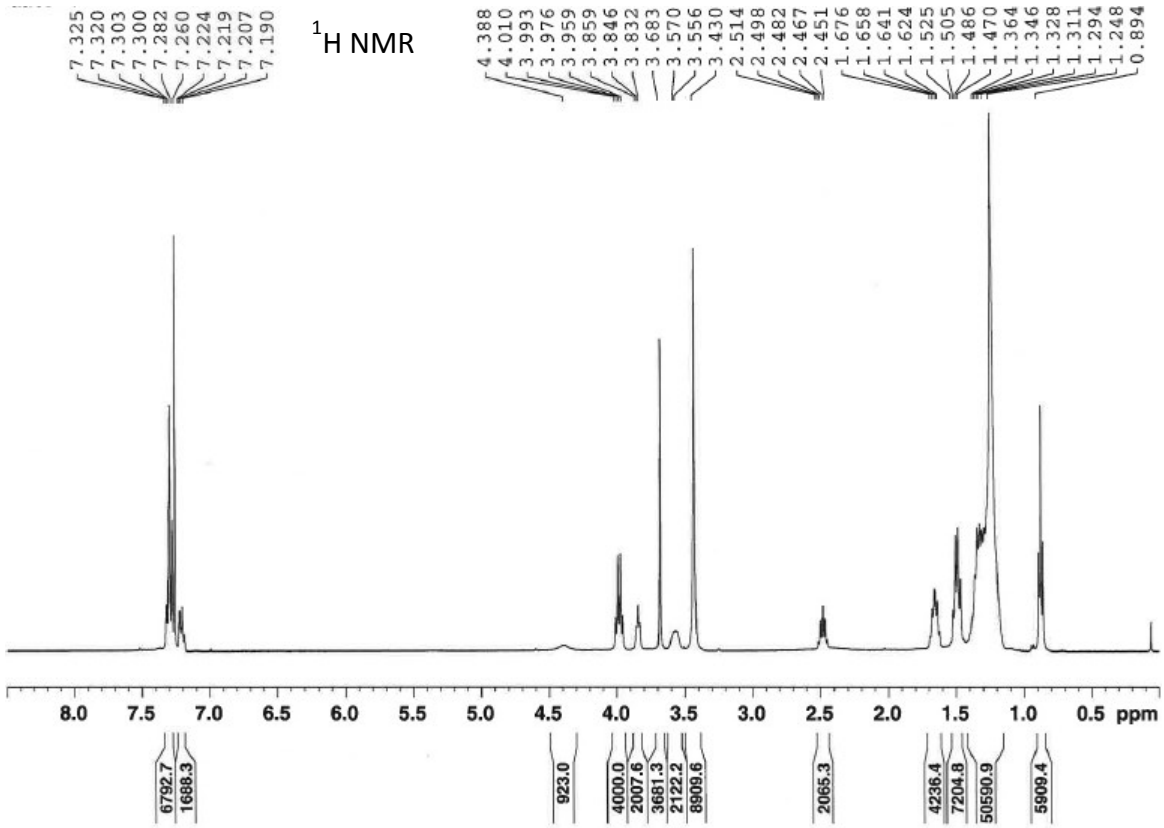


Figure S10-4: ^{1}H NMR of compound **3** (in $CDCl_3$)

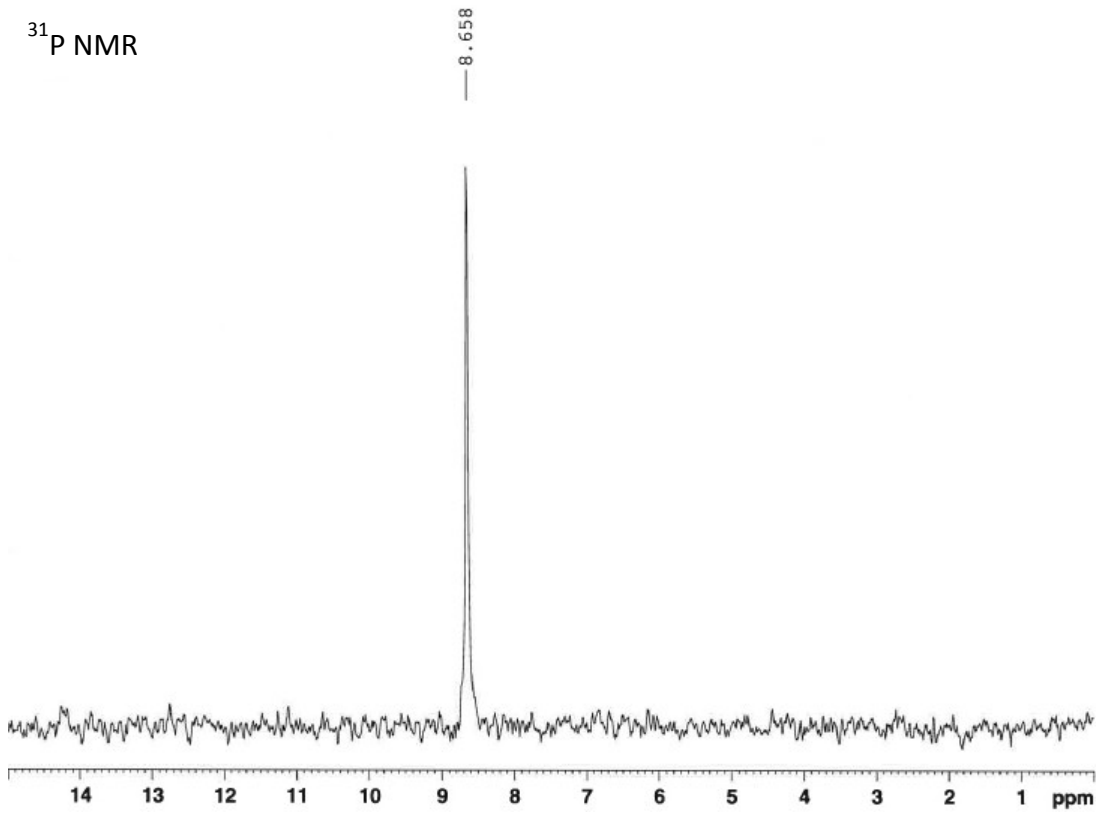


Figure S10-5: $^{31}P\{^{1}H\}$ NMR of compound **3** (in $CDCl_3$)

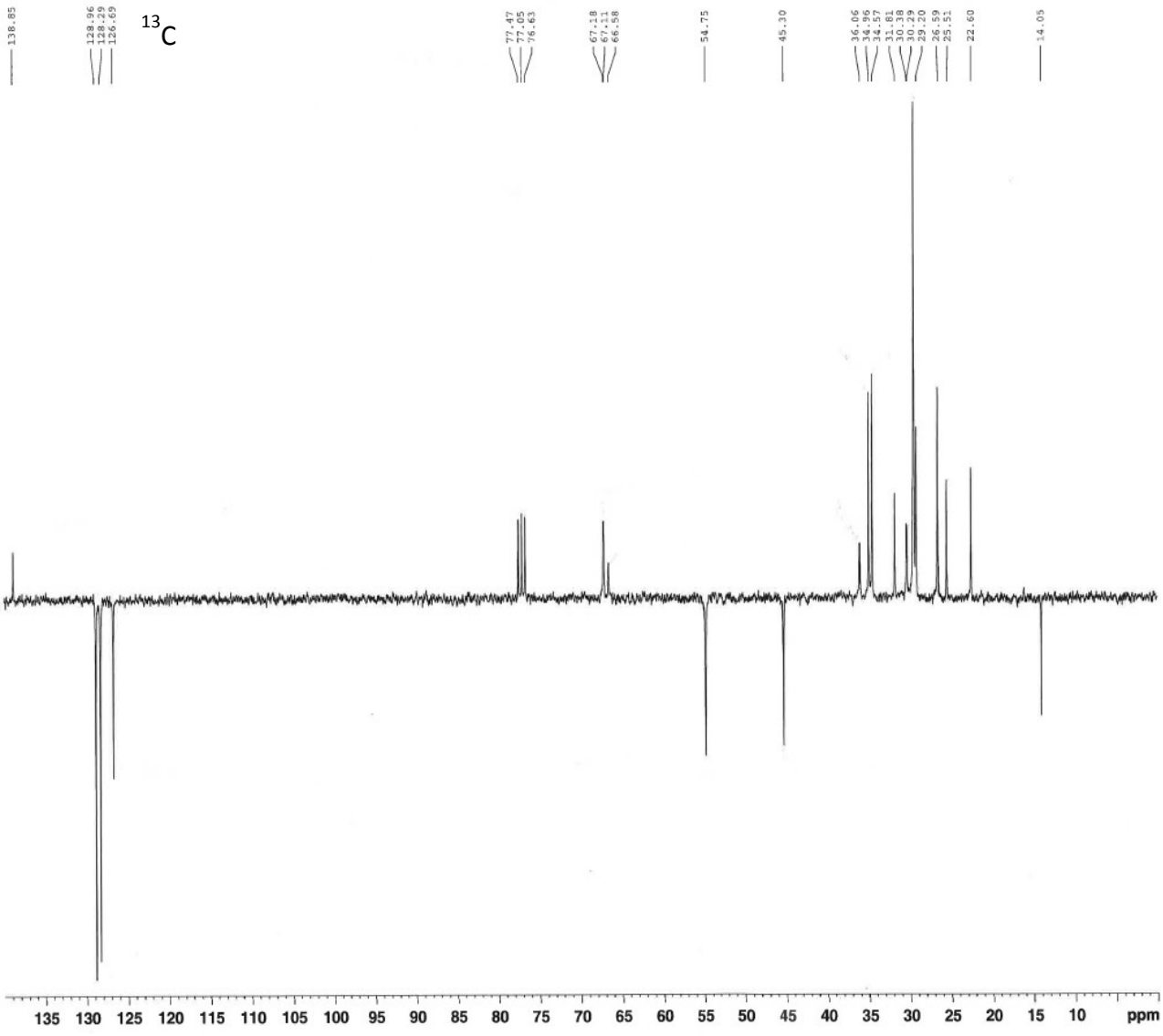


Figure S10-6: ^{13}C NMR (Jmod) of compound **3** (in $CDCl_3$)

Compound 4

^{1}H NMR

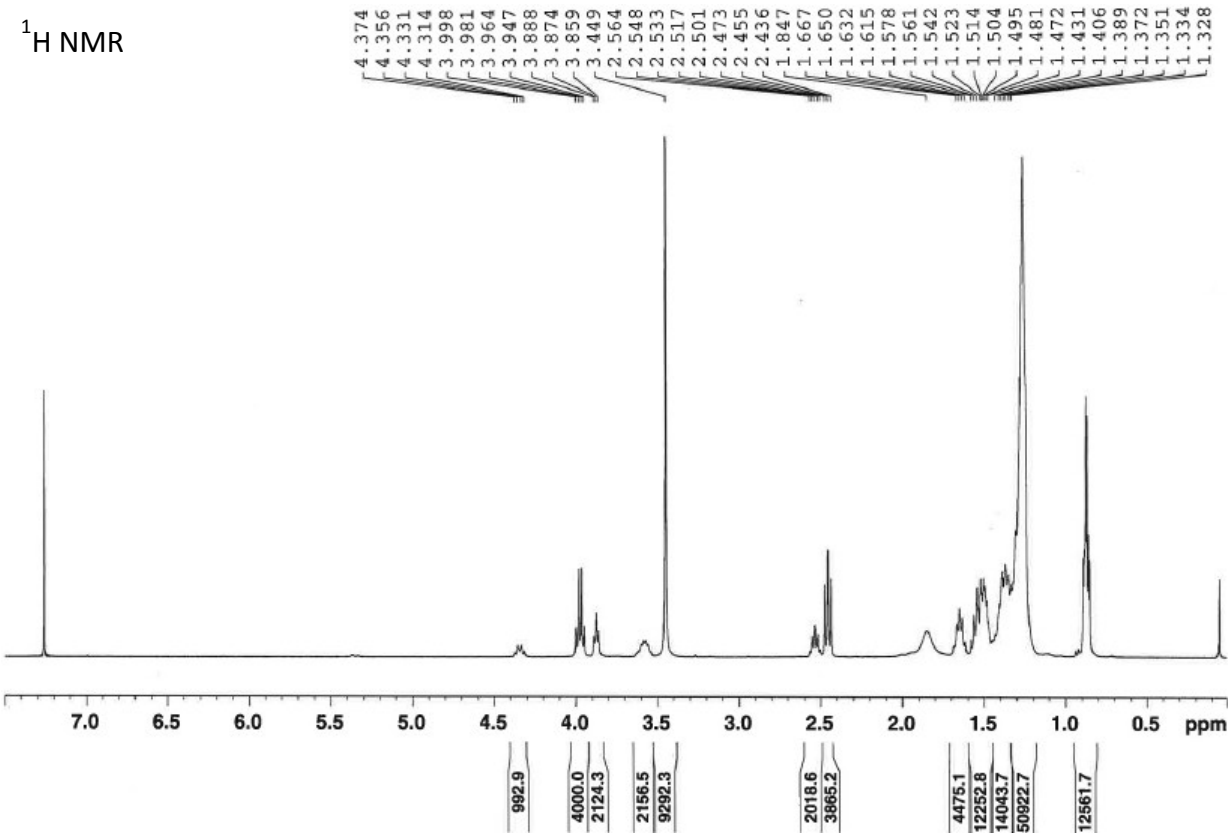


Figure S10-7: ^{1}H NMR of compound **4** (in $CDCl_3$)

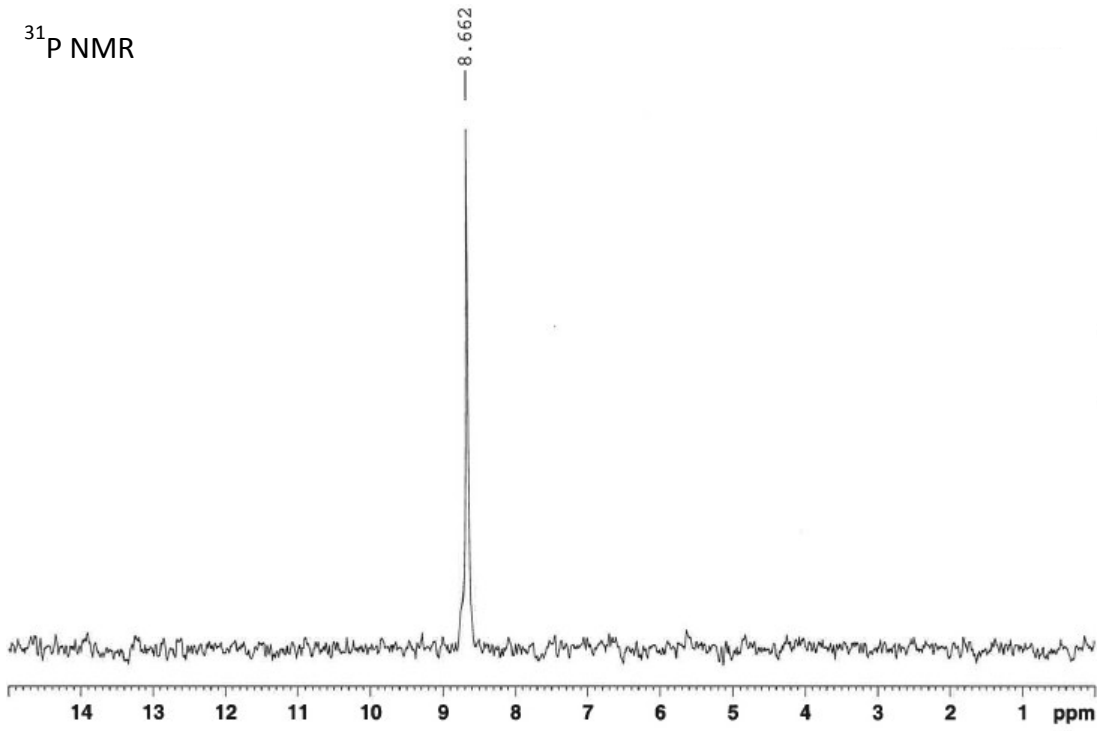


Figure S10-8: $^{31}P\{^{1}H\}$ NMR of compound **4** (in $CDCl_3$)

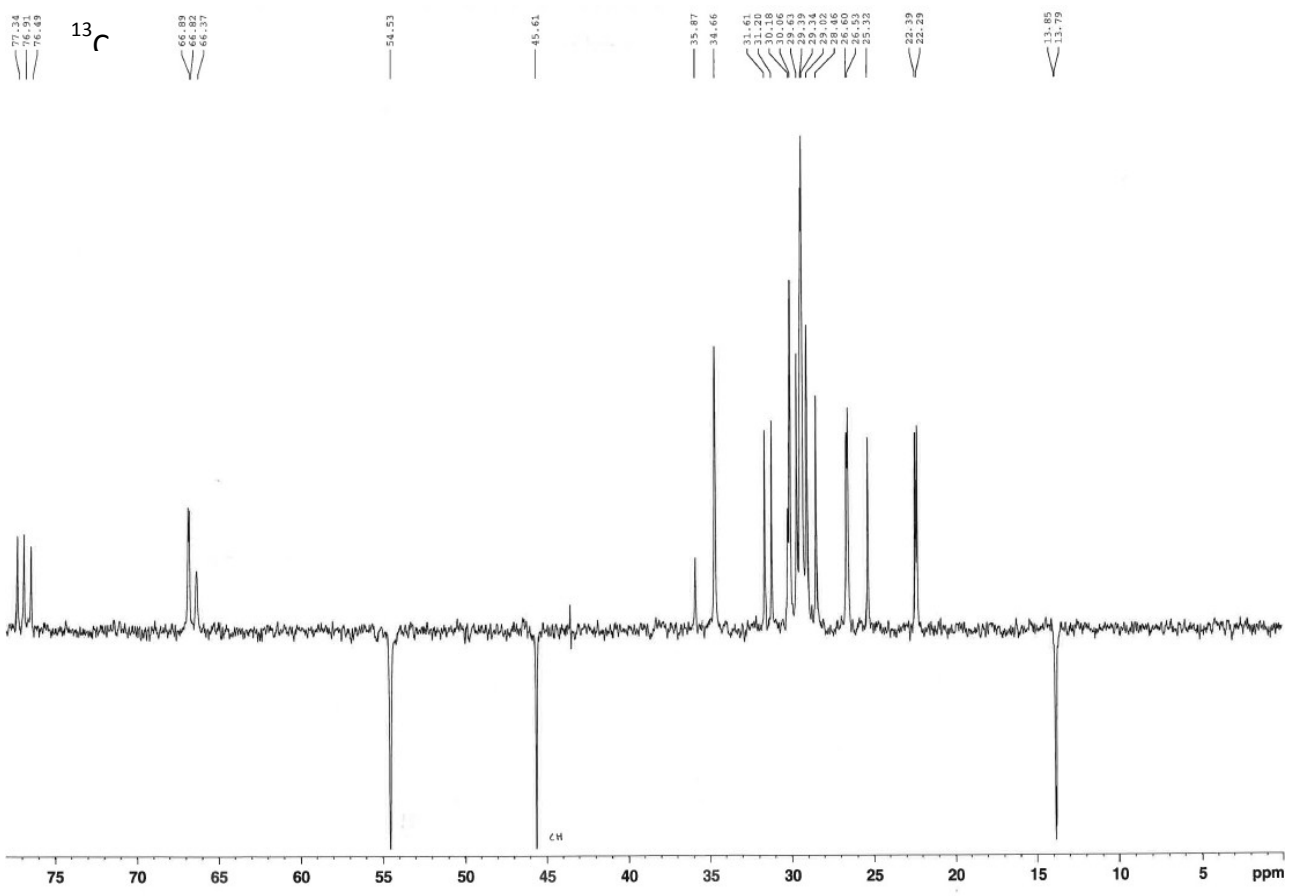


Figure S10-9: ^{13}C NMR (Jmod) of compound **4** (in $CDCl_3$)

Compound 5

^{1}H NMR

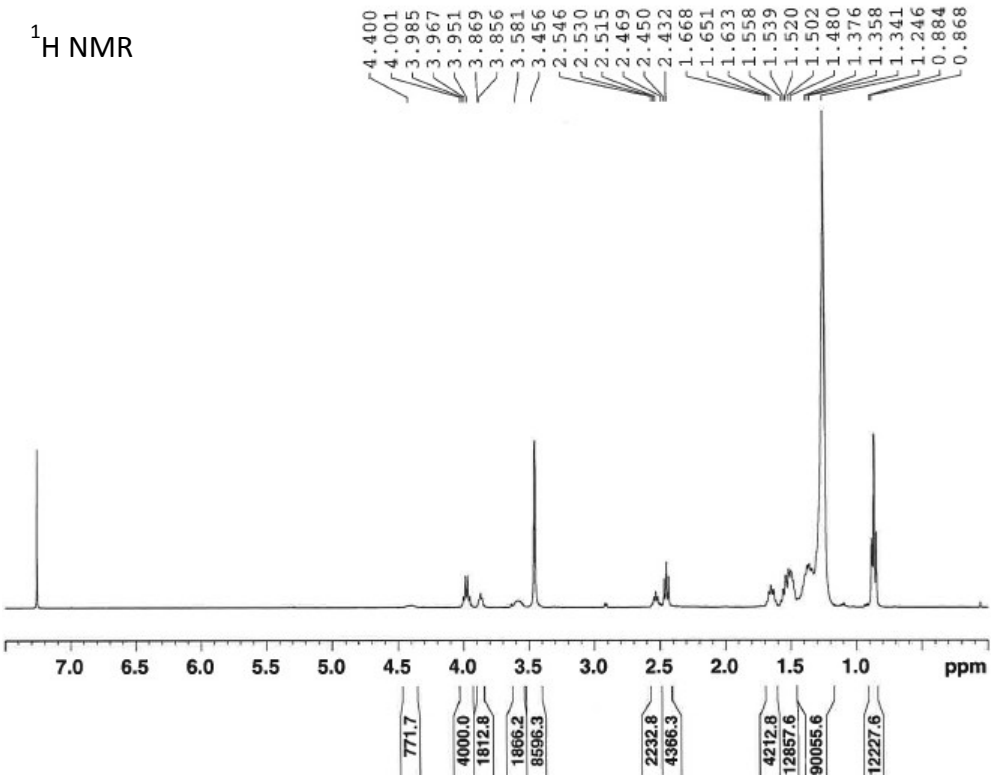


Figure S10-10: ^{1}H NMR of compound **5** (in $CDCl_3$)

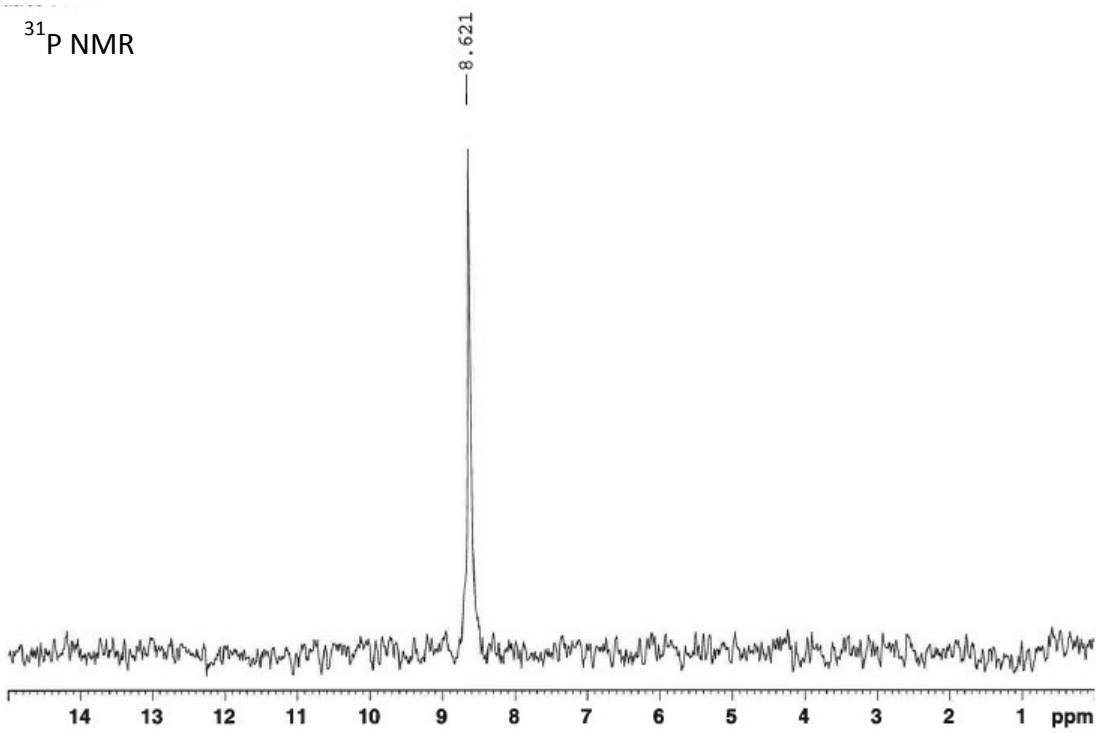


Figure S10-11: $^{31}P\{^{1}H\}$ NMR of compound **5** (in $CDCl_3$)

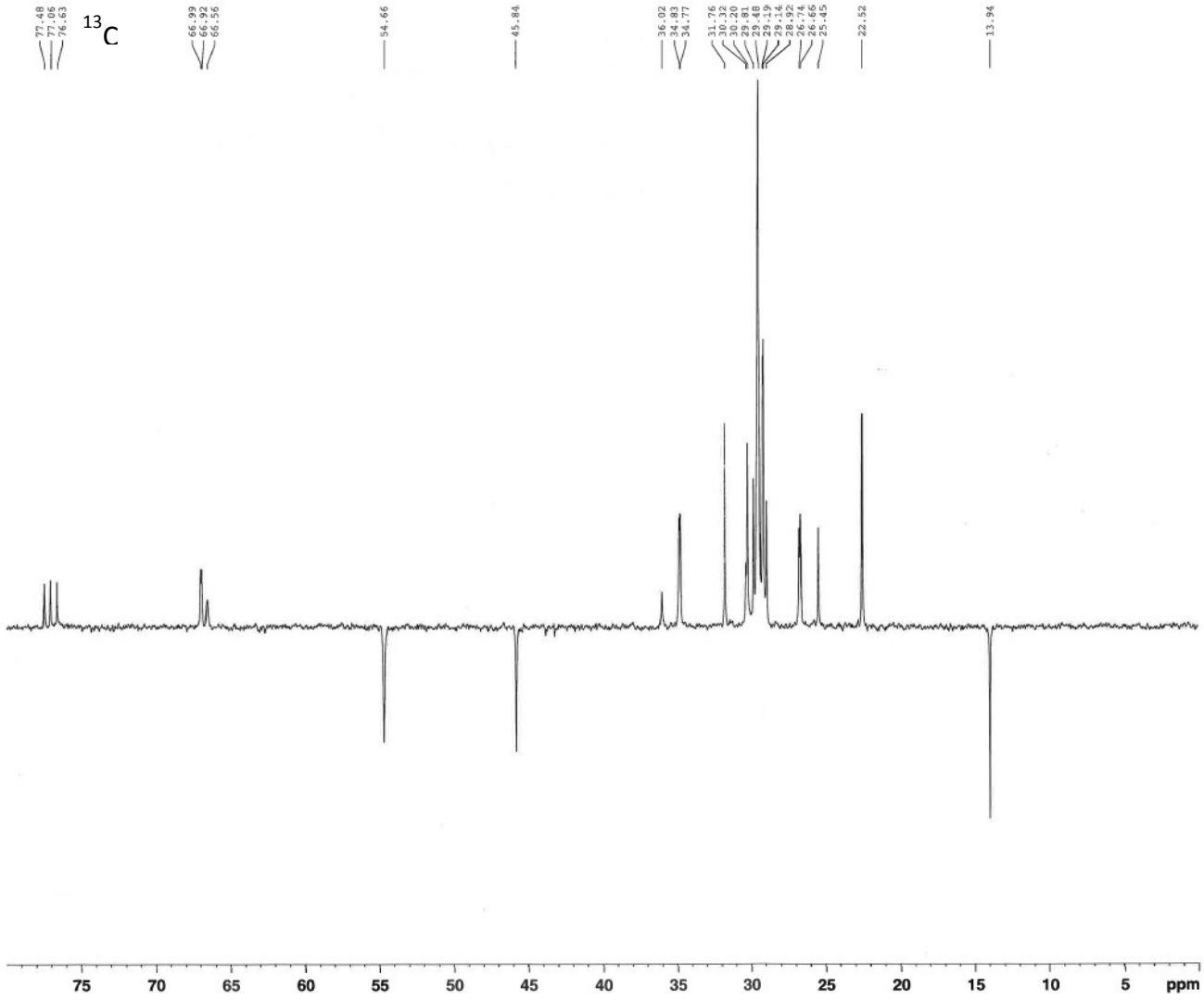


Figure S10-12: ^{13}C NMR (Jmod) of compound **5** (in $CDCl_3$)

Compound **6**

^{1}H NMR

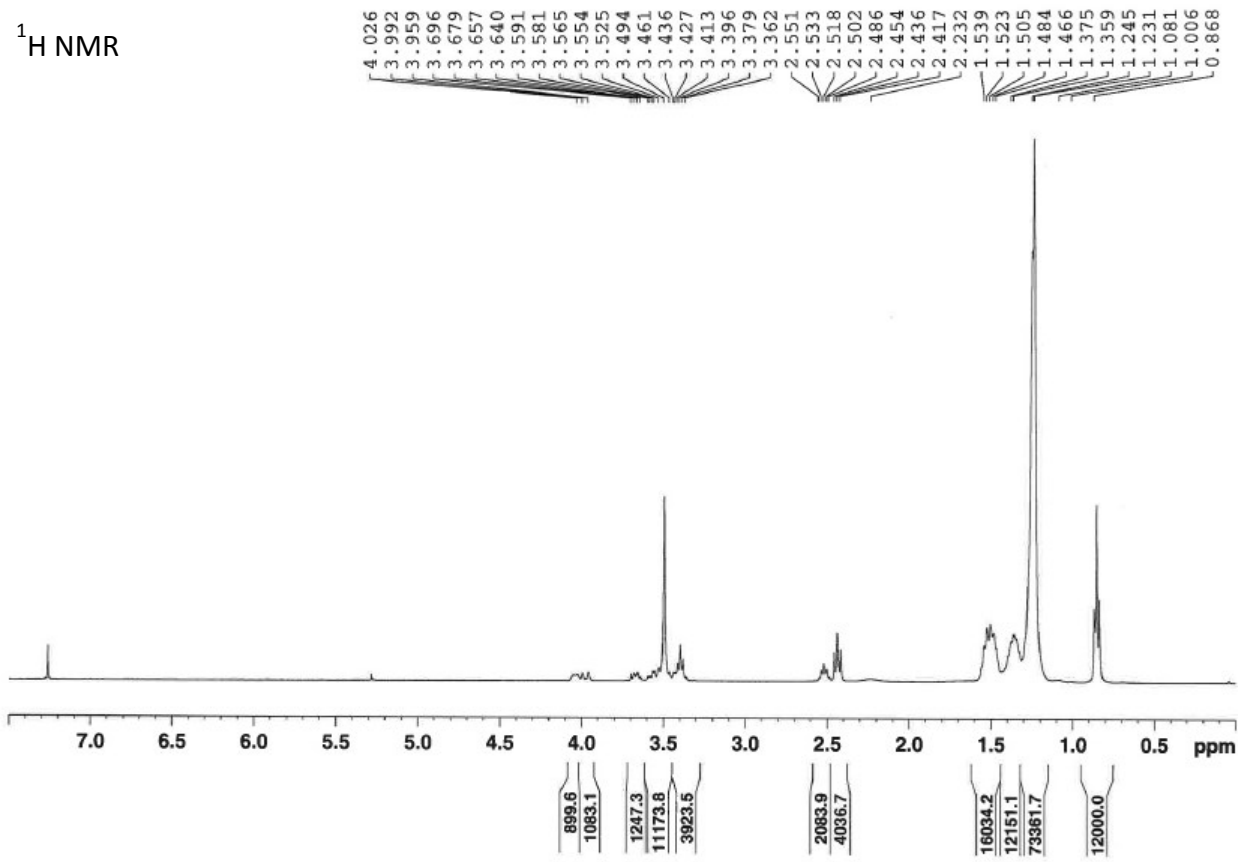


Figure S10-13: ^{1}H NMR of compound **6** (in $CDCl_3$)

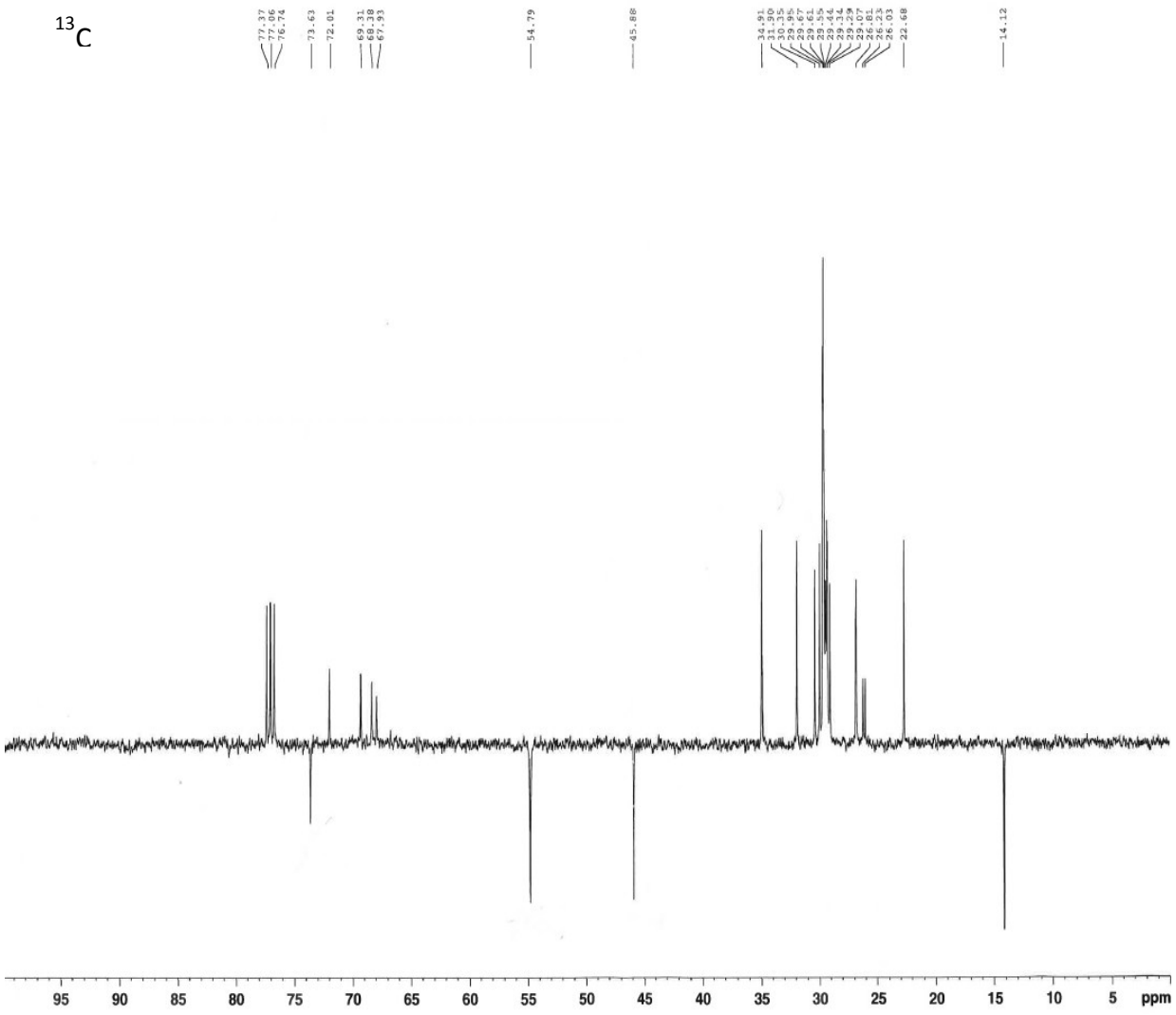


Figure S10-14: ^{13}C NMR (Jmod) of compound **6** (in $CDCl_3$)