Branched lipid chains to prepare cationic amphiphiles producing hexagonal aggregates: supramolecular behavior and application to gene delivery.

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Summary

1. Synthesis and NMR Spectra

Synthesis of compound 2: 2-hexyl-1-decanol (5 g, 20.6 mmol) and diphenyl phosphite (2.3 g; 9.82 mmol) were mixed and heated at 130 °C in a Kugelroch distillation under reduced pressure ($4.2\ 10^{-2}$ mbar) for 3 h to remove phenol by distillation. The residue product (5.2 g) corresponds to compound **2**.

Yield 100 %: ^{1}H NMR (400 MHz, $CDCl_3$): δ=0.85 (t, J = 6.8 Hz, 12H), 1.24 (m, 50H), 1.6 (m, 2H), 3.25-3.57 (m, 4H), 5.90-7.63 (d, $^{1}J_{H\text{-}P}$ =692 Hz, 1H). $^{13}C\{^{1}H\}$ NMR (75 MHz, $CDCl_3$): 13.5 (CH_3). 22.1- 31.5 (CH_2, fatty chain), 38.2 (d, $J_{C\text{-}P}$ = 7.5 Hz, CH), 67.6 (d, $J_{C\text{-}P}$ = 6.0 Hz, CH_2-O-P), $^{31}P\{^{1}H\}$ NMR (162 MHz, $CDCl_3$): δ= 8.9.

Synthesis of compound 3: To a solution of compound **2** (3 g, 5.65 mmol) in 20 mL CH_2Cl_2 was added *N*,*N*-dimethylethylenediamine (6.21 mmol), DIPEA (6.21 mmol) and $BrCCl_3$ (6.21 mmol) at 0°C. The reaction was stirred for 15 min. at 0°C then for 4 h. at 20°C. The solvent was evaporated and 30 mL of diethylether were added and the mixture was stirred for 10 min. then filtered, and the solvent evaporated. The residue was then dissolved in 20 mL of CH_2Cl_2, washed with water, dried over $MgSO_4$ filtered and concentrated. The compound was purified by silica gel chromatography using CH_2Cl_2/MeOH: 90/10 (v/v) as eluent to produce a pale yellow oil (2.6 g).

Yield 75%: ^{1}H NMR (400 MHz, $CDCl_3$): δ=0.82 (t, J = 6.8 Hz, 12H), 1.21 (m, 50H), 1.56 (m, 2H), 2.19 (s, 6H), 2.37 (t, J= 5.8, 2H), 2.89-2.96 (m, 2H), 3.51-3.54 (m, 1H), 3.79-3.86 (m, 4H). $^{31}P\{^{1}H\}$ NMR (162 MHz, $CDCl_3$): δ= 10.1. $^{13}C\{^{1}H\}$ NMR (75 MHz, $CDCl_3$): δ=13.9 (CH_3), 21.4-31.3 (CH_2, fatty chain), 38.1 (CH_2-NH), 38.2 (d, $J_{C\text{-}P}$ = 7.5 Hz, CH), 44.5 ($^{+}N(CH_3)_2$), 59.0 (d, $J_{C\text{-}P}$ = 7.5 Hz, CH_2-$^{+}N(CH_3)_3$), 68.2 (d, $J_{C\text{-}P}$ = 6.0 Hz, CH_2-O-P).

Synthesis of compound 4: To a solution of compound **2** (2 g, 3.76 mmol) in 20 mL CH_2Cl_2 was added 2-Bromoethylamine hydrobromide (15 mmol) DIPEA (15 mmol) and $BrCCl_3$ (15 mmol) at 0°C. The reaction was stirred for 15 min. at 0°C then for 18 h at rt. The solvent was evaporated and 30 mL of diethylether were added and the mixture was stirred for 10 min. then filtered, and the solvent evaporated. The residue was then dissolved in 20 mL of CH_2Cl_2, washed with water, dried over $MgSO_4$ filtered and concentrated. The compound was purified by silica gel chromatography using CH_2Cl_2/MeOH: 90/10 (v/v) as eluent to produce a colorless oil (1.4 g).

Yield 60%: ^{1}H NMR (400 MHz, $CDCl_3$): δ=0.83 (t, J = 6.6 Hz, 12H), 1.21 (m, 50H), 1.56 (m, 2H), 3.22-3.26 (m, 2H), 3.38 (t, J= 6.0 Hz, 2H), 3.81-3.87 (m, 4H). $^{13}C\{^{1}H\}$ NMR (75 MHz, $CDCl_3$): δ=13.9 (CH_3), 21.4-31.2 (CH_2, fatty chain), 33.2 (CH_2-NH), 38.2 (d, J_{C-P} = 7.5 Hz, CH), 42.6 (CH_2-Br), 68.2 (d, J_{C-P} = 6.0 Hz, CH_2-O-P). $^{31}P\{^{1}H\}$ NMR (162 MHz, $CDCl_3$): δ= 9.1.

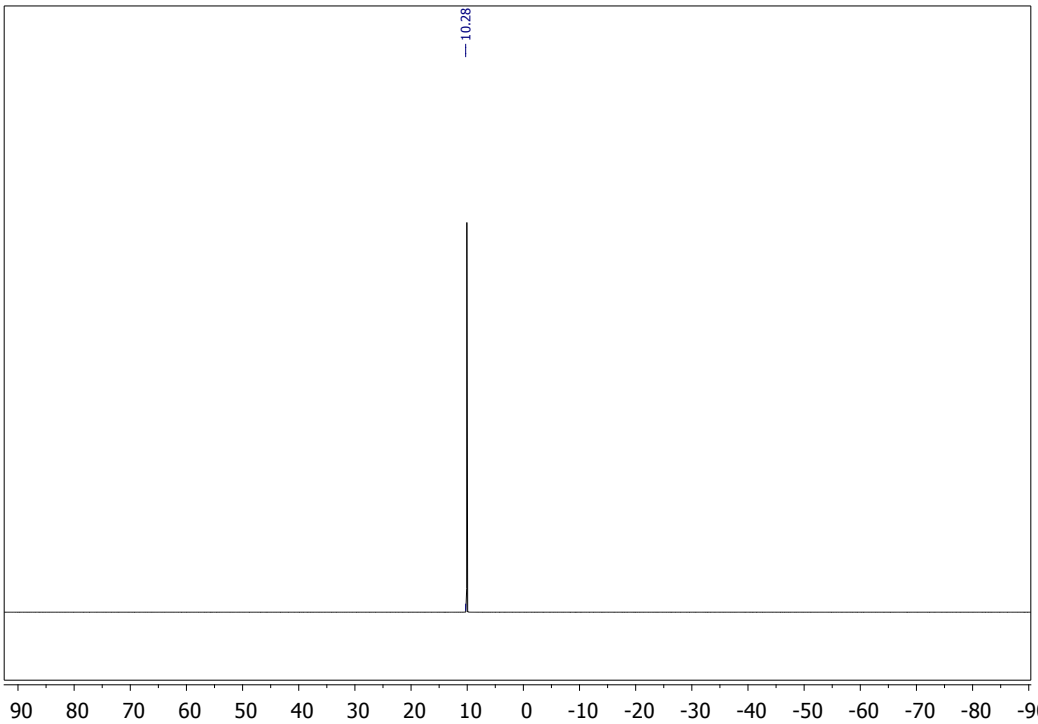


Figure SI-1 : $^{31}P\{^{1}H\}$ NMR ($CDCl_3$) of compound **3**

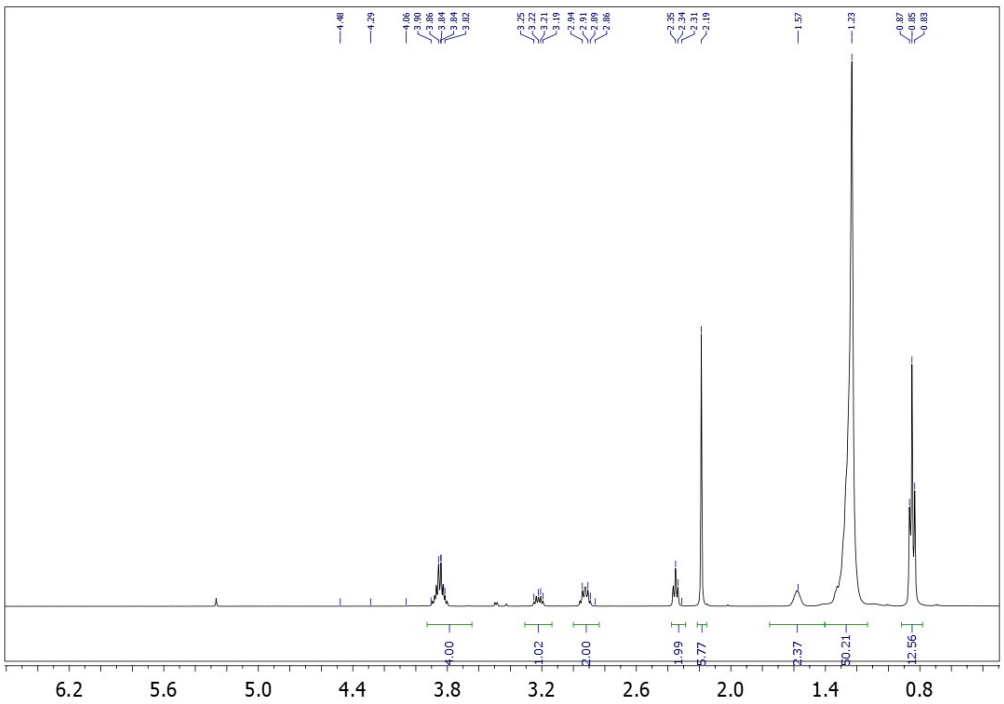


Figure SI-2 : ^{1}H NMR ($CDCl_3$) of compound **3**

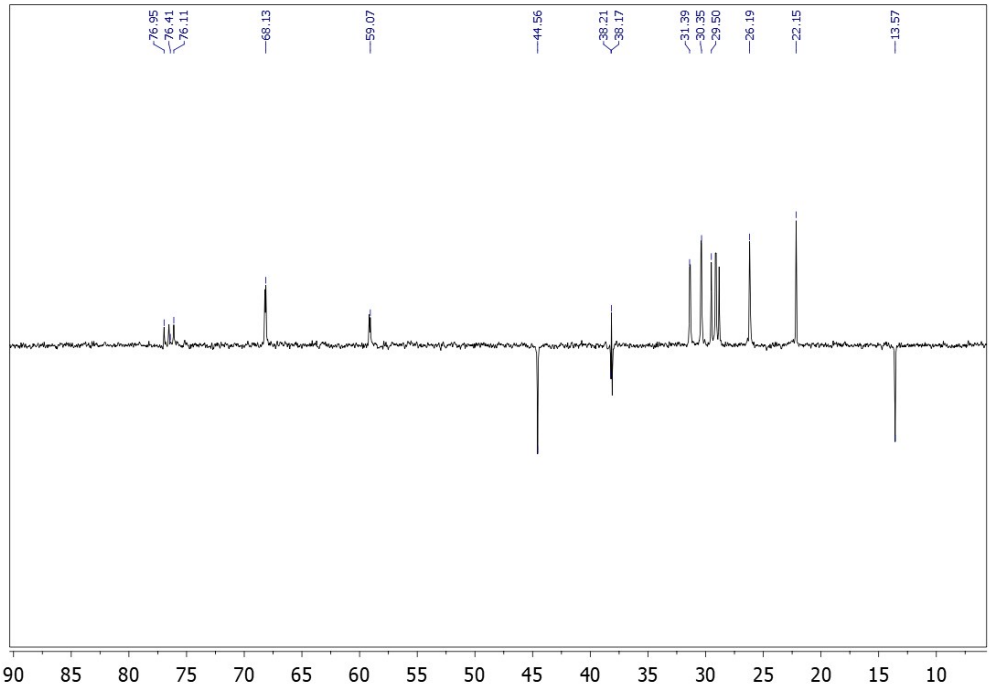


Figure SI-3 : $^{13}C\ \{^{1}H\}$ NMR ($CDCl_3$) of compound **3**

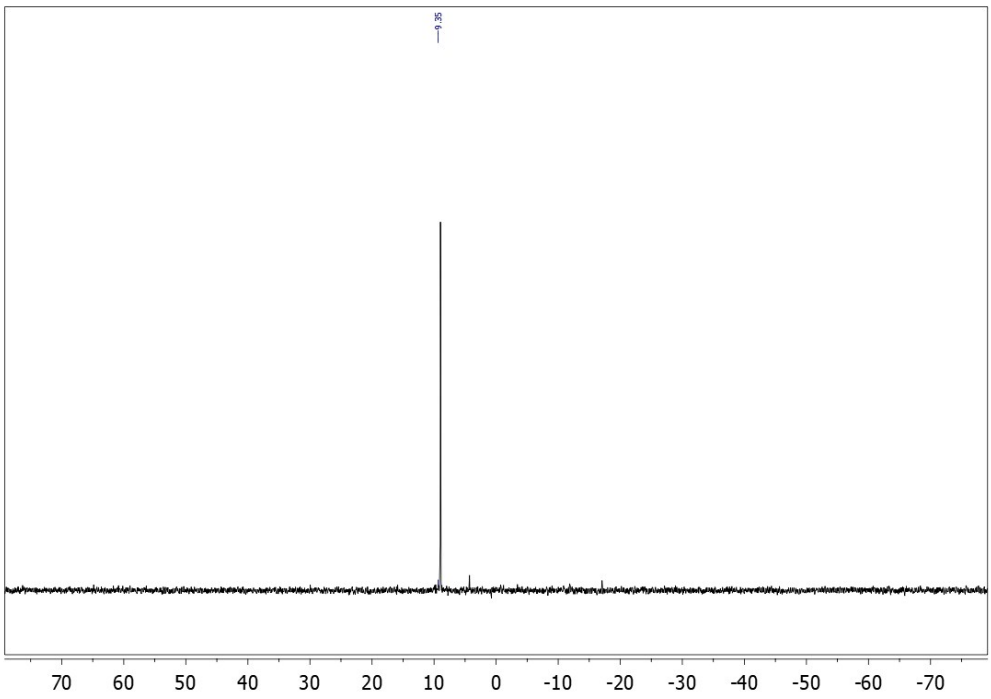


Figure SI-4 : $^{31}P\{^{1}H\}$ NMR ($CDCl_3$) of compound **4**

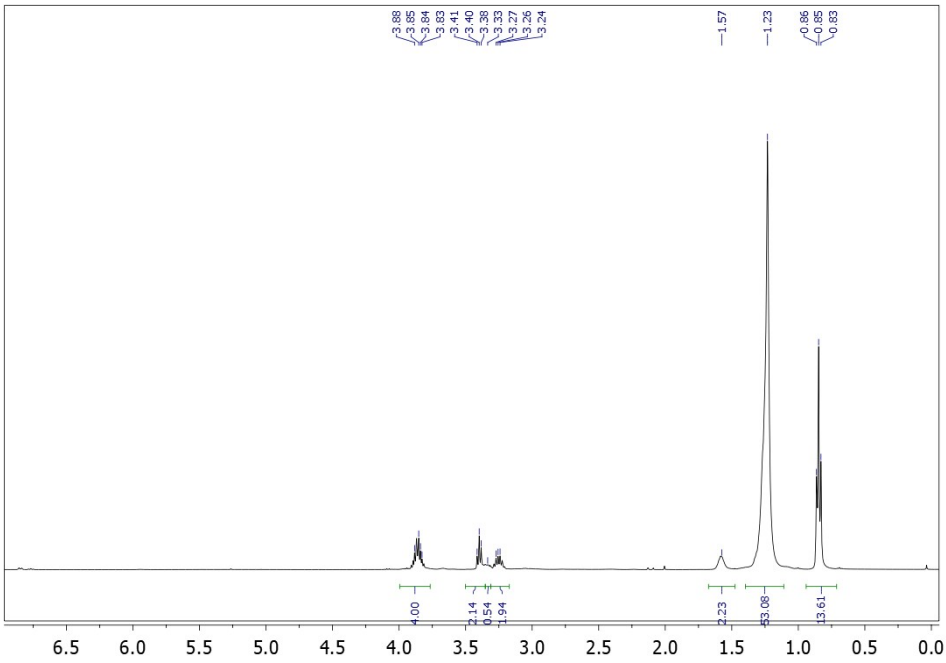


Figure SI-5 : ^{1}H NMR ($CDCl_3$) of compound **4**

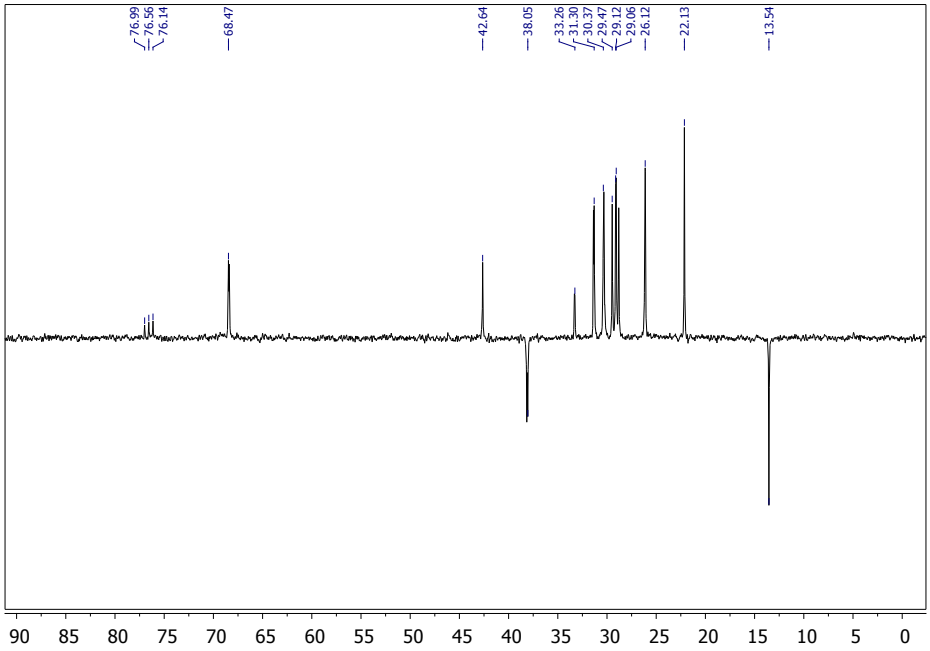


Figure SI-6 : $^{13}C\ \{^{1}H\}$ NMR ($CDCl_3$) of compound **4**

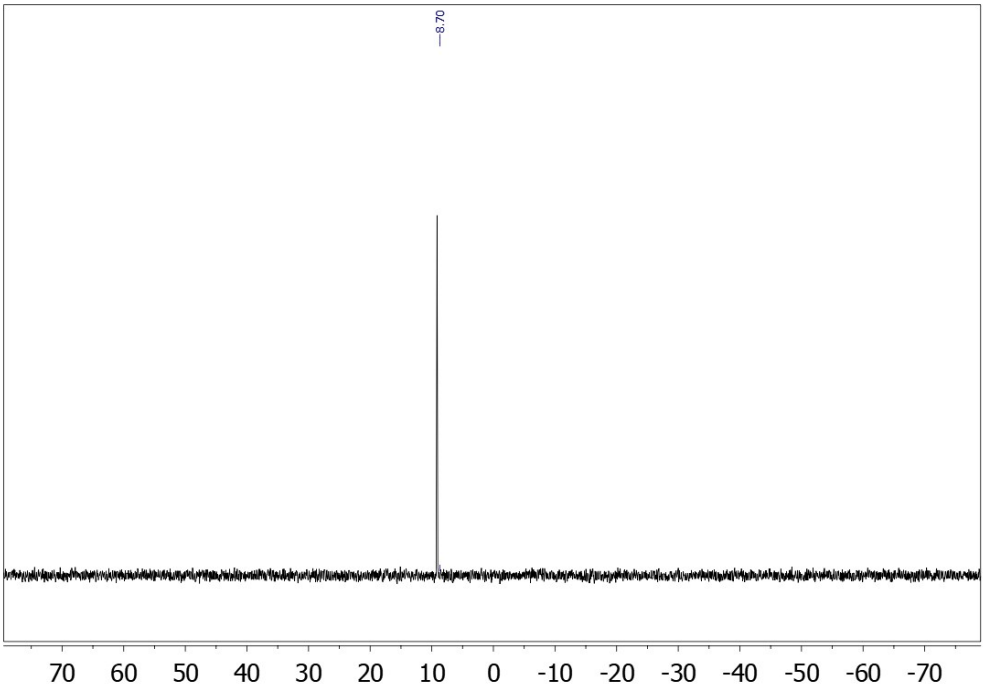


Figure SI-7 : $^{31}P\{^{1}H\}$ NMR ($CDCl_3$) of compound **5**

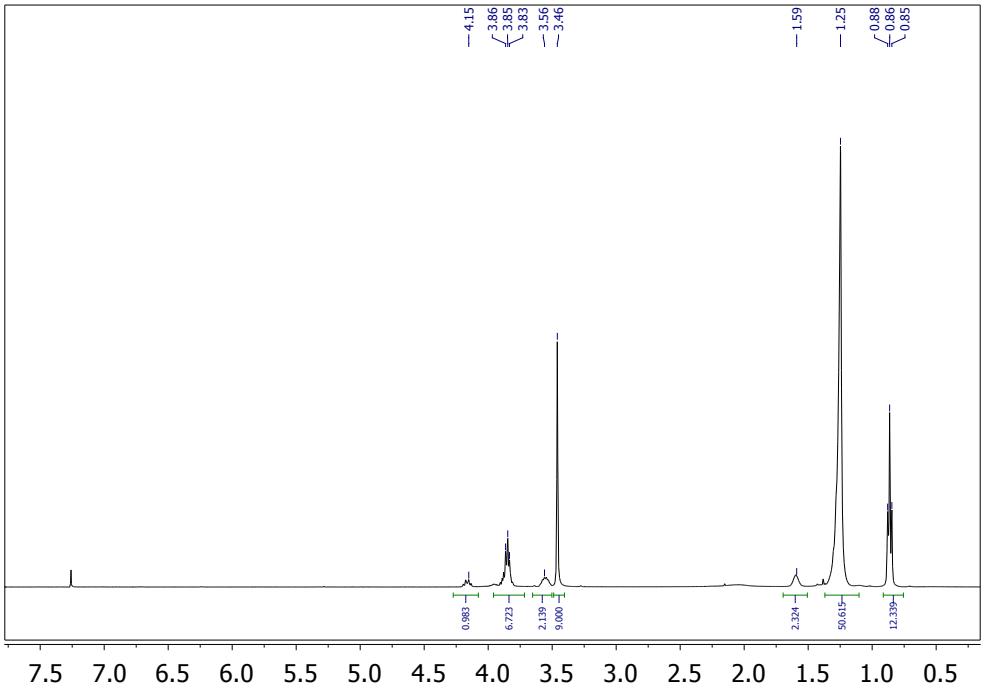


Figure SI-8 : ^{1}H NMR ($CDCl_3$) of compound **5**

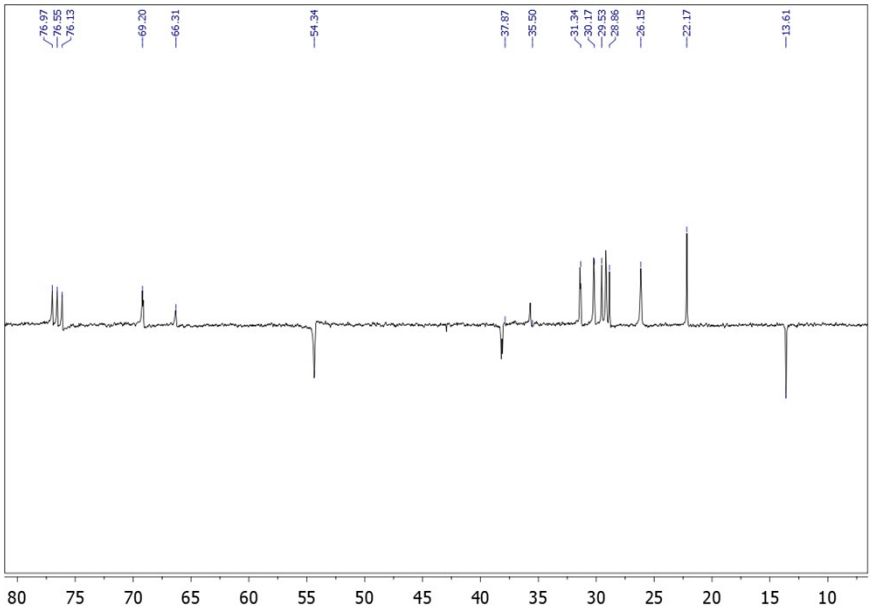


Figure SI-9 : ^{13}C {^{1}H} NMR ($CDCl_3$) of compound **5**

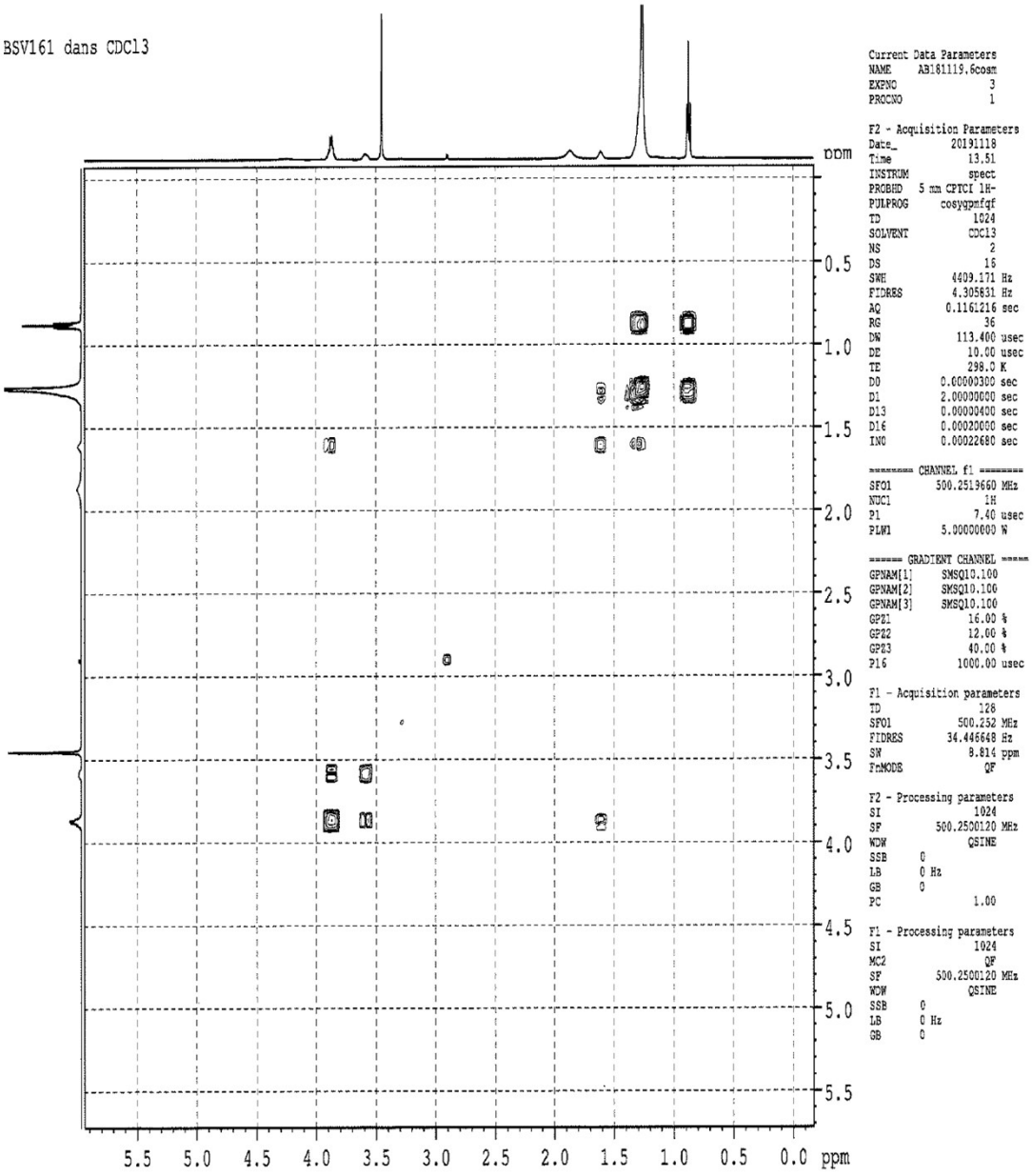


Figure SI-10 : COSY NMR ($CDCl_3$) of compound **5**

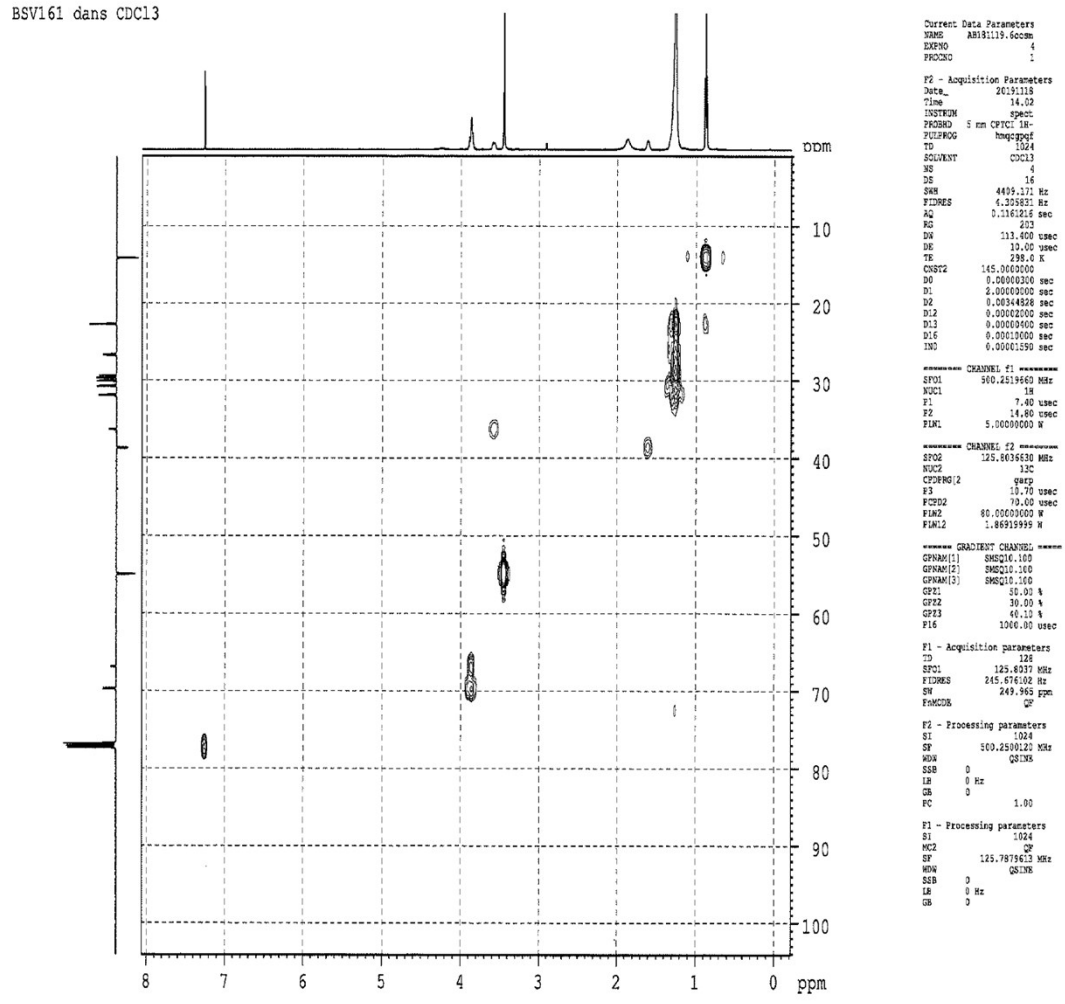


Figure SI-11: HMQC NMR ($CDCl_3$) of compound **5**

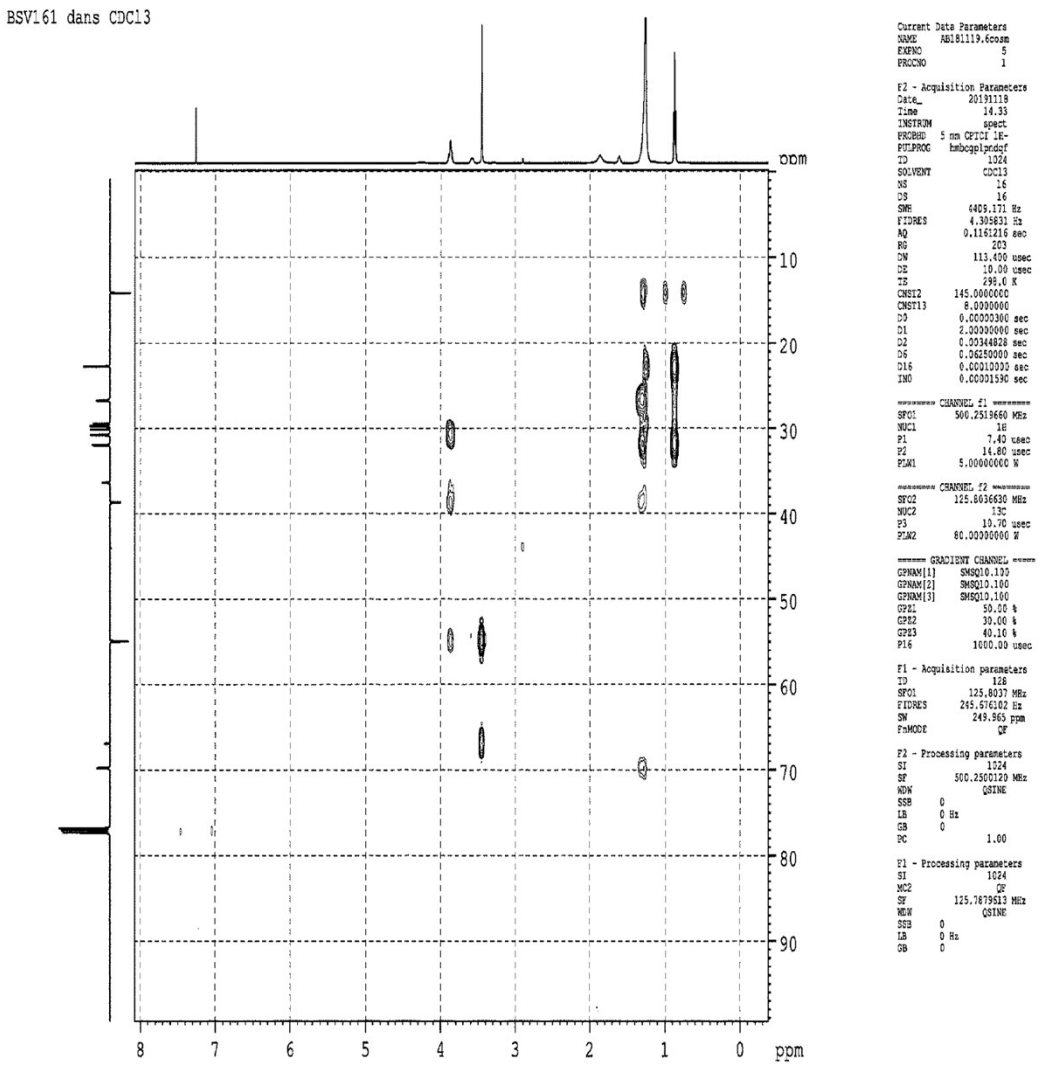


Figure SI-12: HMBC NMR ($CDCl_3$) of compound **5**

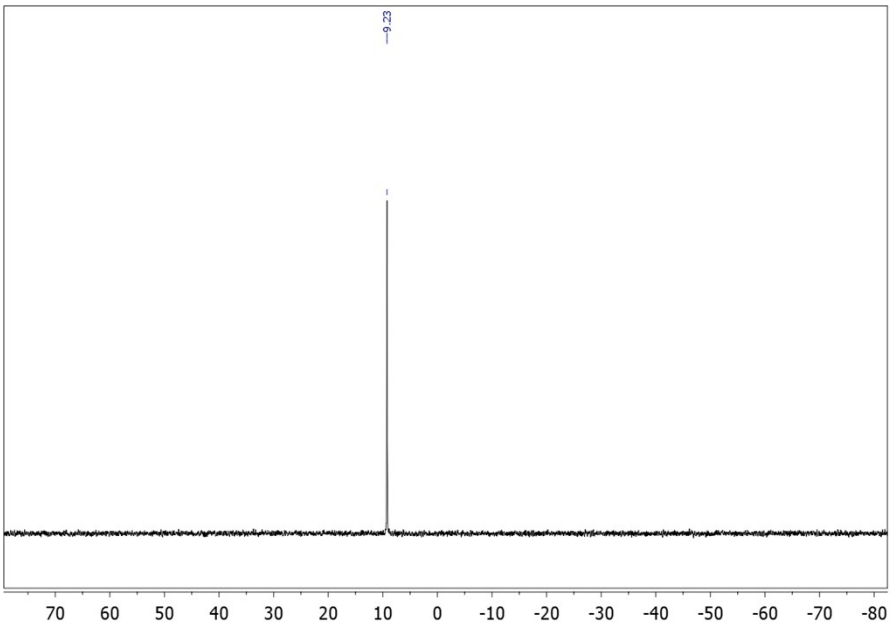


Figure SI-10 : $^{31}P\{^{1}H\}$ NMR ($CDCl_3$) of compound **6**

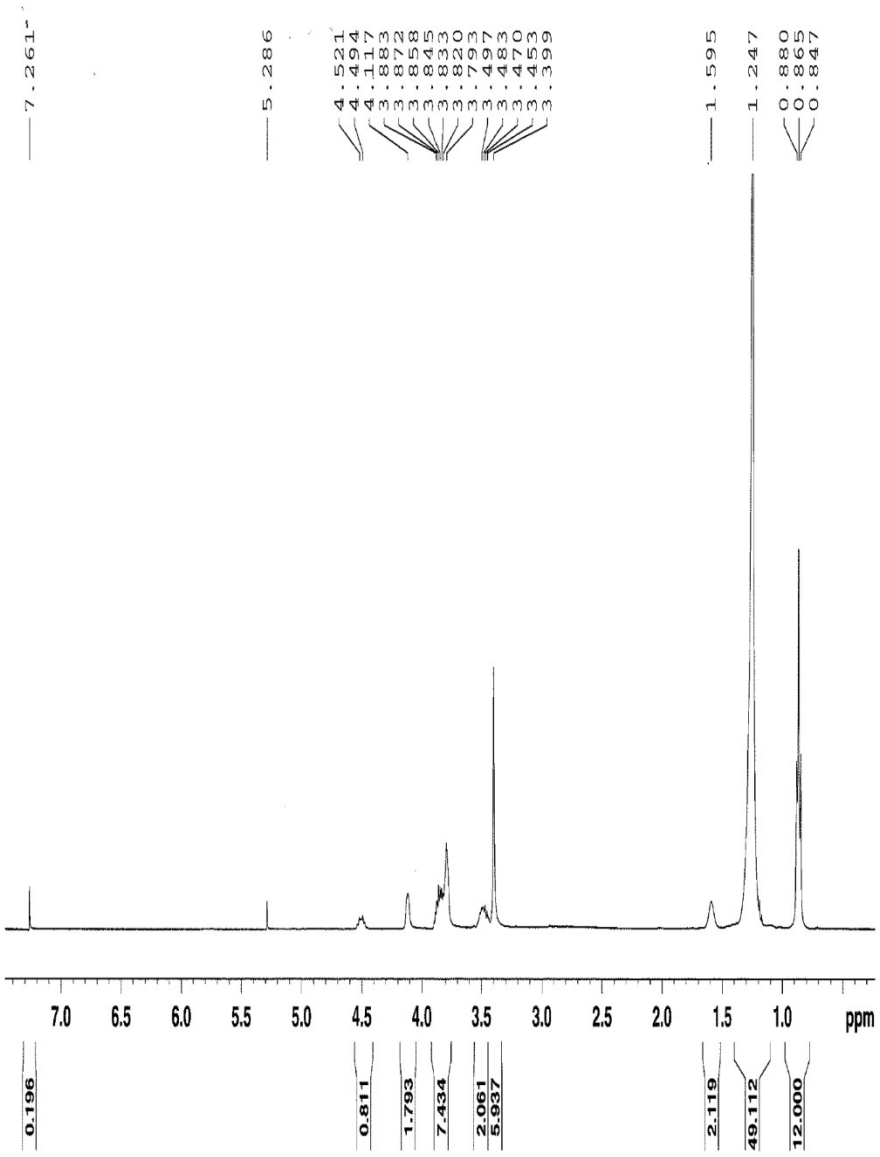


Figure SI-11 : ^{1}H NMR ($CDCl_3$) of compound **6**

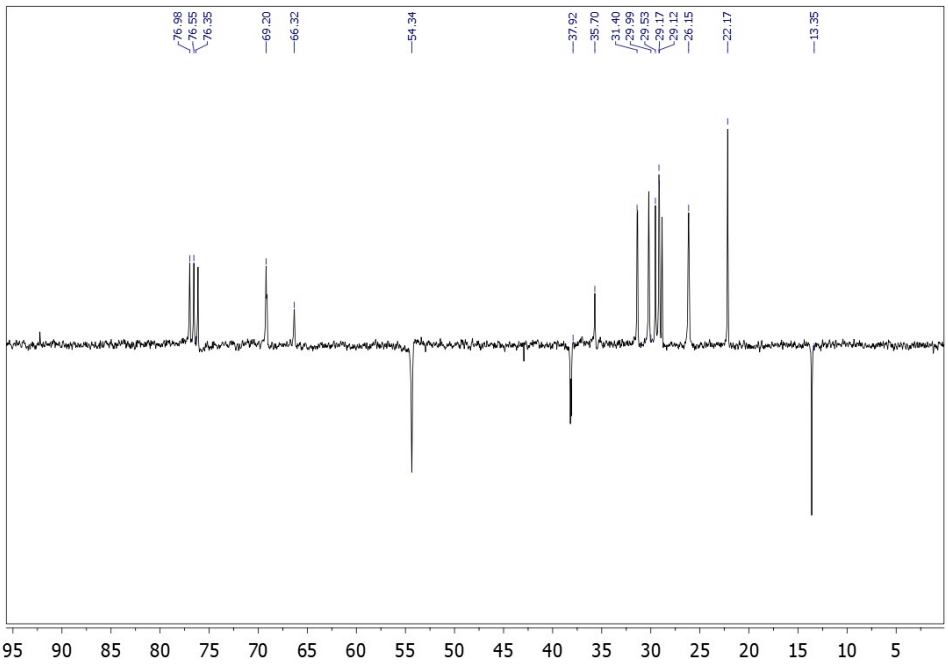


Figure SI-12 : $^{13}C\ \{^{1}H\}$ NMR ($CDCl_3$) of compound **6**

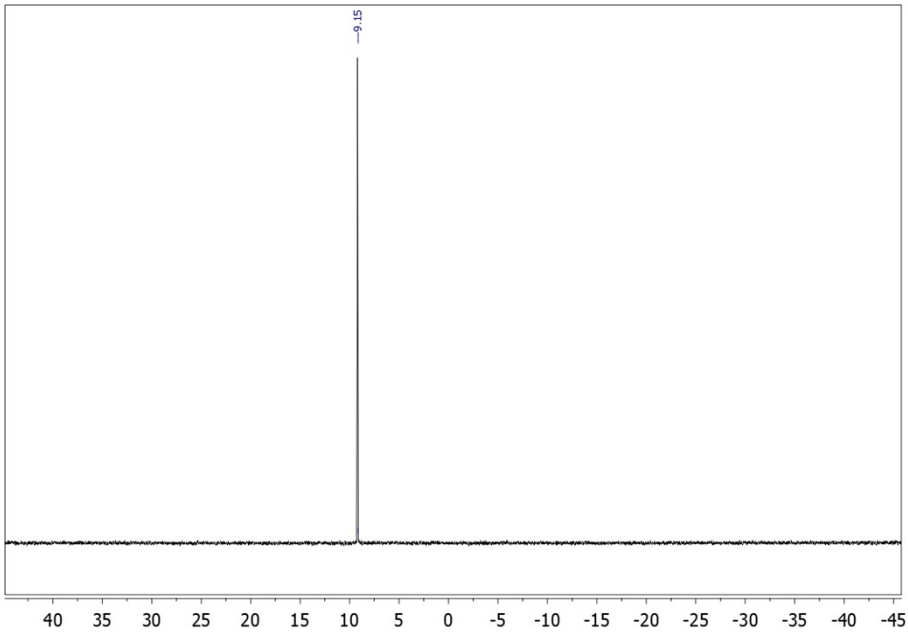


Figure SI-13 : $^{31}P\{^{1}H\}$ NMR ($CDCl_3$) of compound **7**

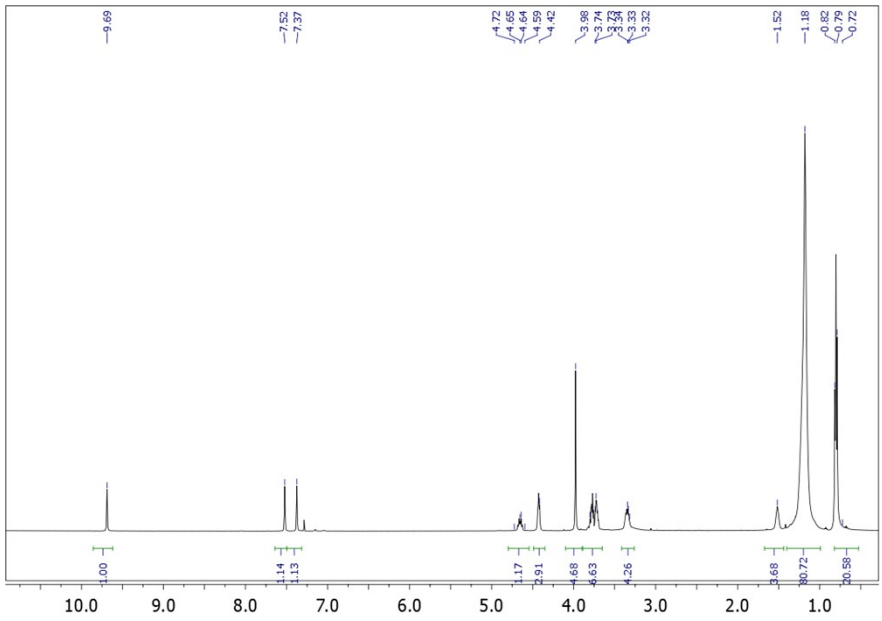


Figure SI-14 : ^{1}H NMR ($CDCl_3$) of compound **7**

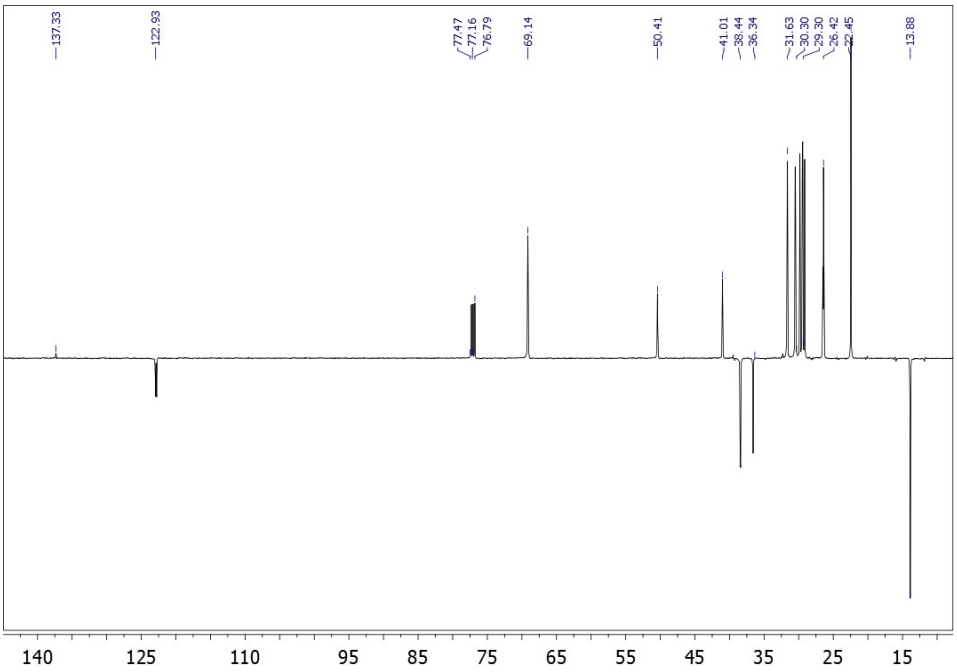


Figure SI-15 : ^{13}C {^{1}H} NMR ($CDCl_3$) of compound **7**

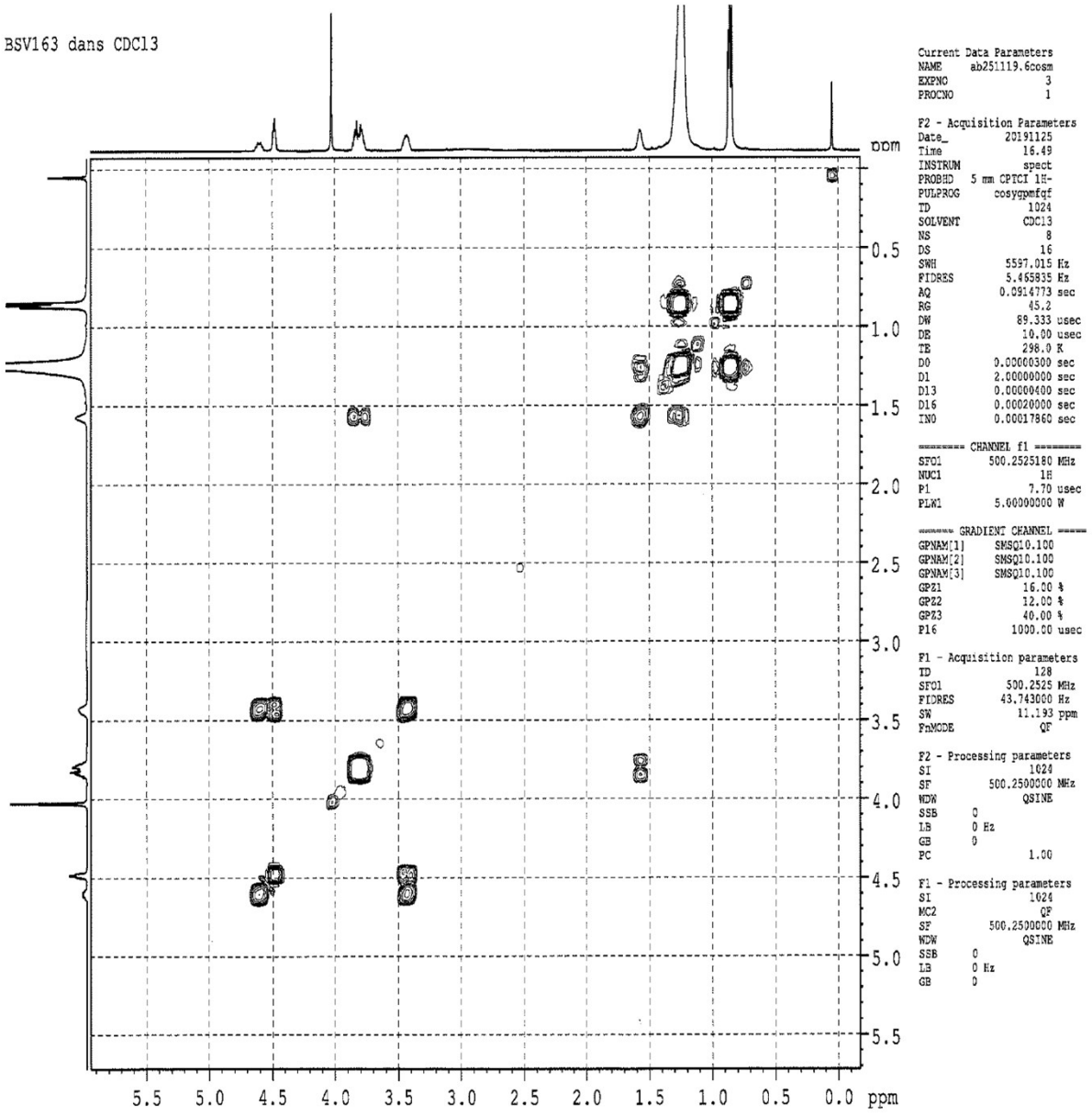


Figure SI-19: COSY NMR ($CDCl_3$) of compound **7**

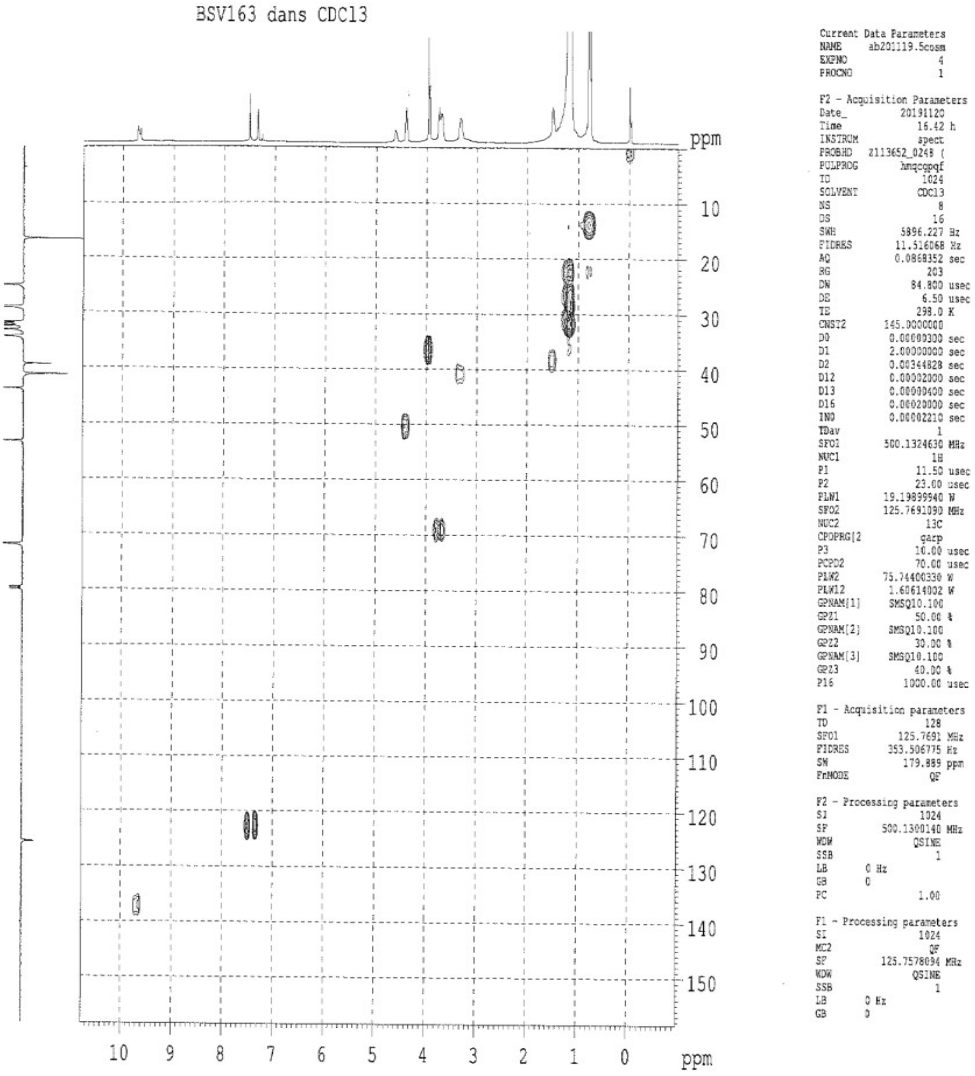


Figure SI- 20 : HMQC NMR ($CDCl_3$) of compound **7**

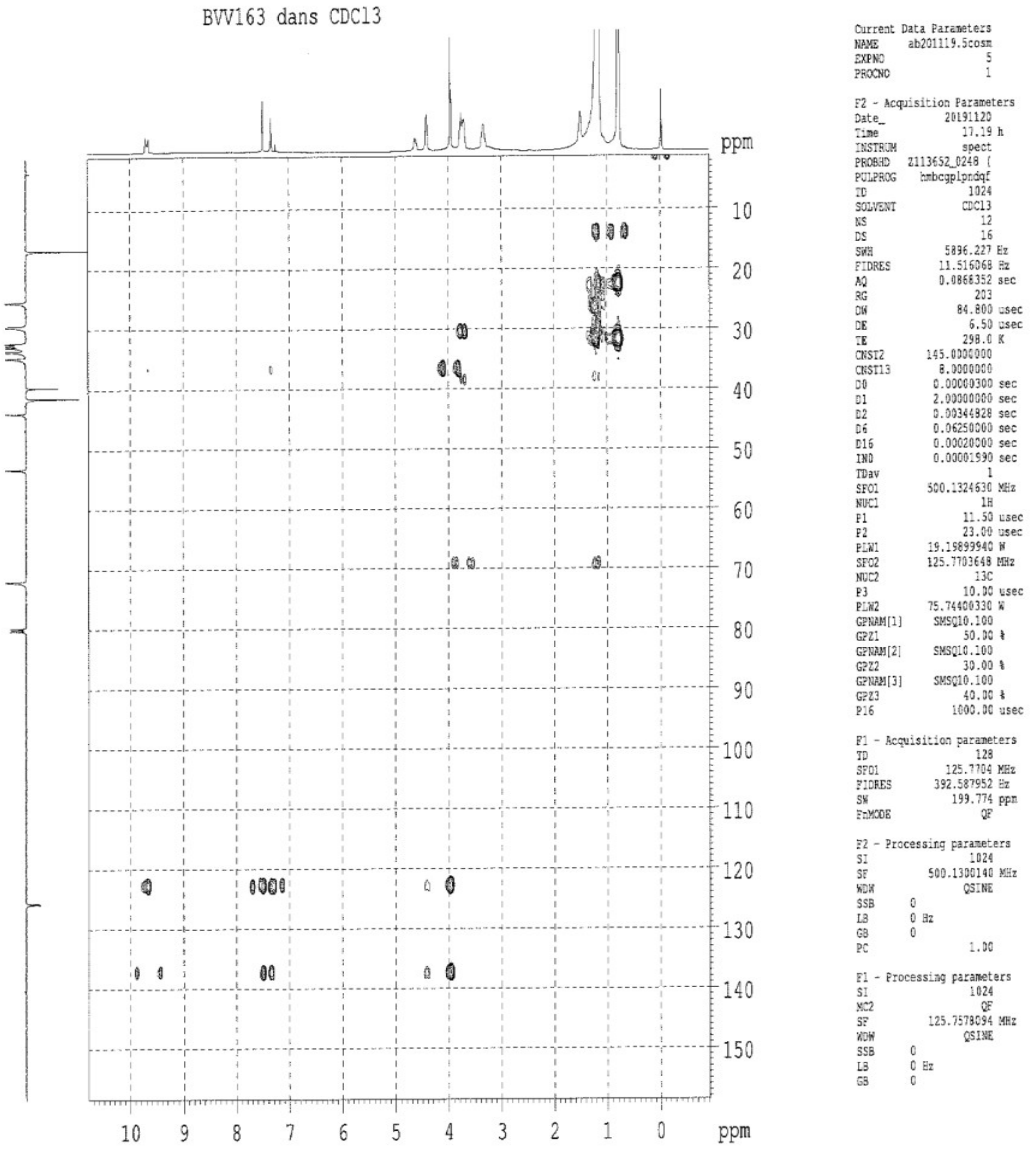


Figure SI-21: HMBC NMR ($CDCl_3$) of compound **7**

2. Formulations – DLS and zeta potential.

An aliquot of a cationic amphiphile in chloroform was placed in a glass tube. Then the solvent was evaporated (4 h in vacuum) to get a thin lipid film to which 2 mL of sterile water was added and the film was hydrated for 18 h to 3 days at 4°C. The solution was then allowed to go back to rt, vortexed for 30 s and sonicated for 30 min in an ultrasonic bath at 80°C.
100 µL of the liposomal solution was then diluted in 900 µL of sterile water then the size and the Zeta potential were measured.

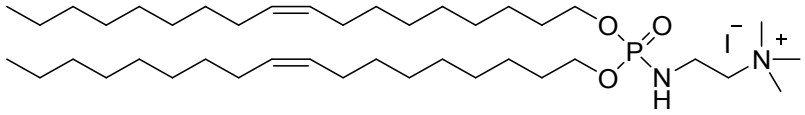
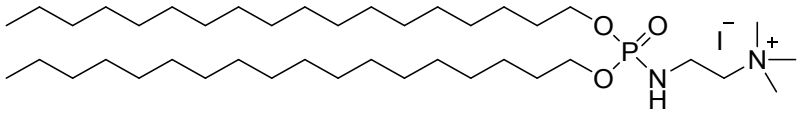
3. Compression isotherm

The Langmuir trough was cleaned with ethanol and water then it was filled with the sub-phase (ultrapure water). A good base line in the (π–A) and (Δ-A) isotherms indicated the cleanliness of the interface. The cationic lipid in $CHCl_3$/MeOH (2/1) mixture (25 µL) was slowly spread at the air-water interface using a Hamilton micro syringe. After waiting 10 minutes till the solvents evaporate, the isotherm of the lipid monolayer was recorded by compressing the barriers at the rate of 5 cm^2/min. The surface pressure was measured using a filter paper held by a Wilhelmy balance connected to a microelectronic feedback system (Nima technology). The experiment is stopped once the collapse is reached.

For each compound, the measurement is repeated at least two times. If the two obtained isotherms do not overlap (surface pressure variation more than 0.25mN/m), a third experiment is done in order to have a better overlap between at least two of the registered isotherms.

4. Chemical structure of BSV36 and BSV 101

The compounds were synthesized following reported procedures.[1]

BSV36	
BSV101	

[1] 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.

5. Ellipsometry

The ellipsometric measurements were carried out with a home-made ellipsometer associated to the Langmuir trough and the tensiometer. The software developed recorded simultaneously the value of the surface pressure and the value of the ellipsometric angle during the isotherm compression. The principal of the ellipsometric angle measurement is the following: a polarized He–Ne laser beam (λ = 632.8 nm, Melles Griot, Carlsbad, CA) is reflected by the air/water interface. The incidence angle of the light was 1° away from the Brewster angle (53.12° value obtained for an air/water interface). After reflection, the laser light passed through a λ/4 retardation plate, a Glan-Thompson analyzer, and a photomultiplier. Through a computer-controlled feedback loop, the analyzer automatically rotated toward the extinction light position. In this "null ellipsometer" configuration, the analyzer angle, multiplied by 2, yielded the value of the ellipsometric angle (Δ), i.e., the phase difference between parallel and perpendicular polarization of the reflected light, which reflects the thickness of the molecular film adsorbed at the interface. The laser beam probed a surface of 1 mm^2 and a depth of the order of 1 µm. Values of Δ were recorded every 4 s with a precision of ±0.5°.

6. Fluorescence anisotropy.

Fluorescence measurements were recorded on *Agilent* Cary *Eclipse* Fluorescence Spectrophotometer, using automatic polarizers for anisotropy measurements. The fluorescent probe 1,6-diphenylhaxatriene (DPH) was used for anisotropy measurements and was purchased from sigma Aldrich Stock solutions of each lipid and each fluorescent probe were prepared in chloroform/methanol. For each sample, one cationic amphiphile and the probe were then mixed in a glass tube and solvent was removed under reduced pressure. After solvent removal, pure water was added and let at 4°C for 3 nights. Solutions were then introduced in a bath at 60°C, vortexed and then sonicated during 20 min. For anisotropy measurements, the lipids concentration in the final preparation was 2.10^{-4} $mol.L^{-1}$ whereas DPH concentration was 1.10^{-6} $mol.L^{-1}$. The results are presented in figure SI-16.

	Delta min ± 0.2	Delta max ± 0.2	Delta max – Delta min
BSV 161	2	4,3	2,3
BSV 162	2,2	4,9	2,7
BSV 163	2.2	5,0	2,8

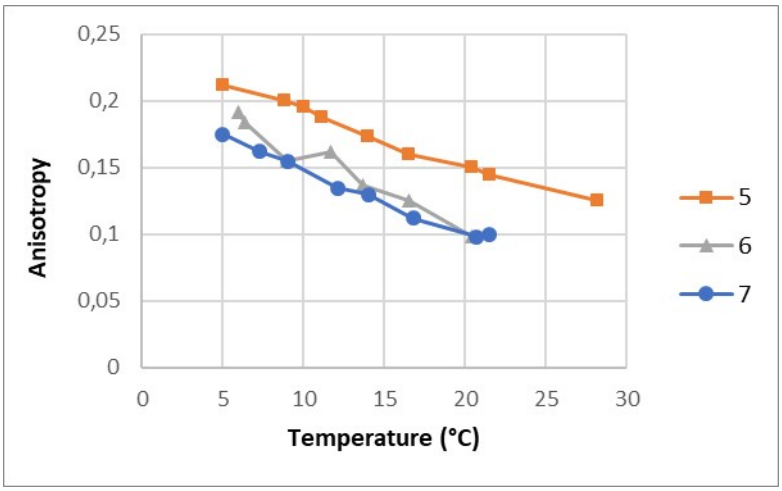


Figure SI-16 : Fluorescence anisotropy of DPH formulated in presence of compounds **5**, **6** and **7** in function of the temperature.

7. Solid state ^{31}P NMR in water.

The samples were prepare at a final concentration of 100 mg / mL and the homogenization was achieved by using 3 freeze-thaw cycles. The ^{31}P NMR spectra were recorded at 20°C with the Hahn echo sequence (90°- τ- 180° - τ - acq.)[2]. The acquisition parameters were: spectral window of 200 KHz , π/2 pulse width of 4.88µs, recycle delay of 5s, and echo delay of 40µs. The number of scans was dependent on sample (340 for **5**, 310 for **6** and 246 for **7**).

Simulation of ^{31}P NMR Spectra were achieved with dmfit software.[3]

δ_{iso} is calculated as $\delta_{iso} = 1/3\ (\sigma_{xx} + \sigma_{yy} + \sigma_{zz})$.

The ^{31}P chemical shielding anisotropy (CSA) was calculated as follow: $\Delta\delta = \sigma_{\square} - \sigma$

Compound **5**.

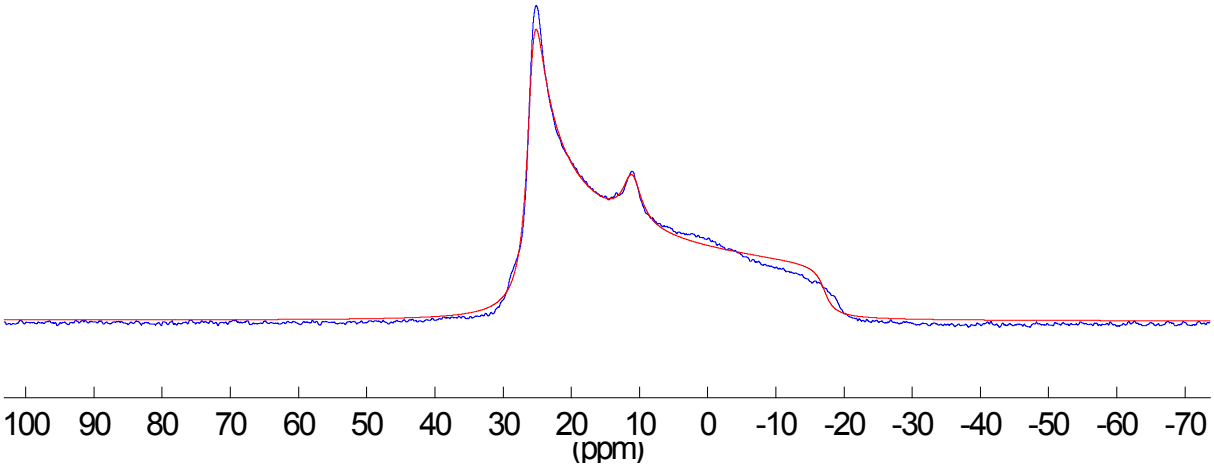


Figure SI-17 : ^{31}P NMR of compound **5**. Blue line: experimental spectrum ; red line calculated spectrum. Main signal (95 % of the integration: δ_{iso} = 11.3 ppm ; CSA = 43 ppm); second signal (5% of the integration) : δ = 11.1 ppm.

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

[3] D. Massiot, F. Fayon, M. Capron, I. King, S. Le Calvé, B. Alonso, J.O. Durand, B. Bujoli, Z. Gan and G. Hoatson, *Magn. Reson. Chem*., **2002**, *40*, 70-76.

Compound **6**.

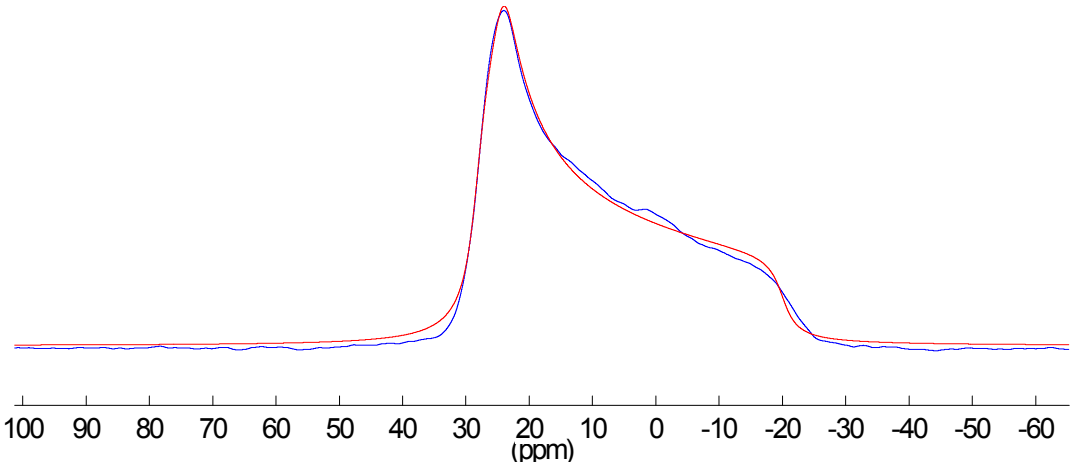


Figure SI-18 : ^{31}P NMR of compound **6**. Blue line: experimental spectrum ; red line calculated spectrum. δ_{iso} = 10.7 ppm ; CSA = 48 ppm

Compound **7**.

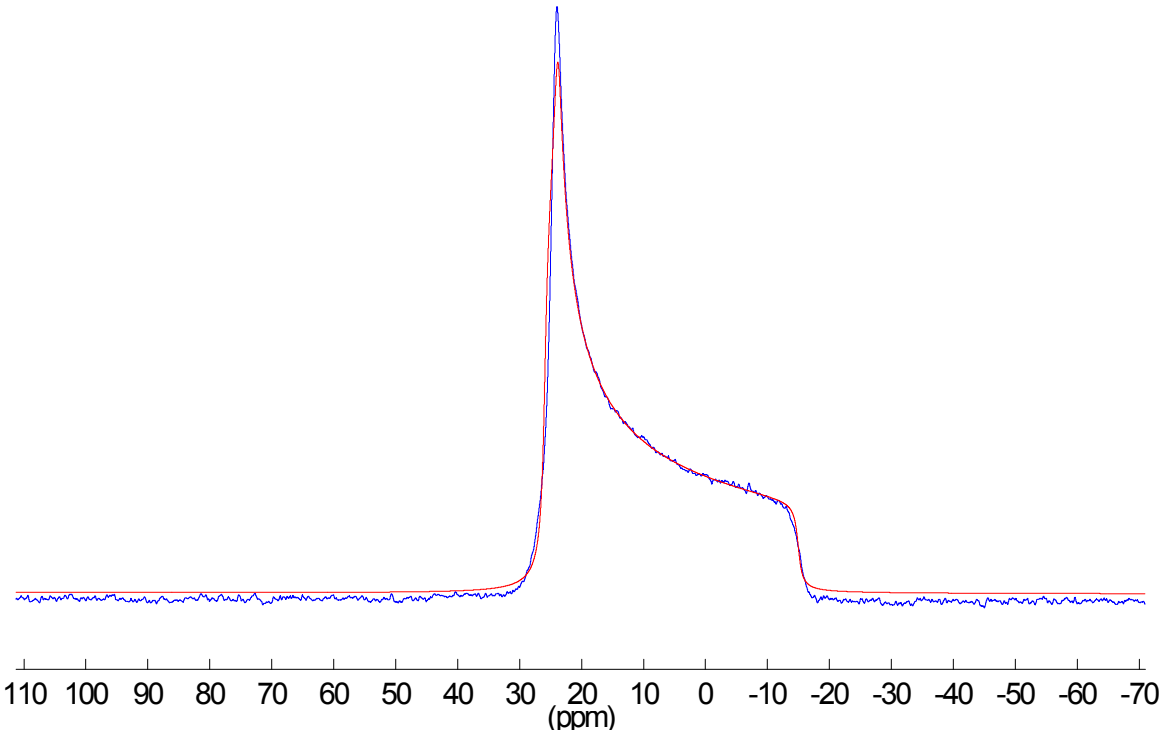


Figure SI-19 : ^{31}P NMR of compound **7**. Blue line: experimental spectrum ; red line calculated spectrum. δ_{iso} = 11.5 ppm ; CSA = 41 ppm

8. Gel electrophoresis of lipoplexes.

Lipoplexes were prepared as follow: pDNA (pGM144 ; 3.7 kb)[4] was added to the liposomal solution at different CR ranging from 0.5 to 8 in water. The resulting mixtures were incubated at room temperature for 30 minutes before being subjected to electrophoresis in a 0.8% agarose gel at 100 V, 90 mA. The gel previously stained with ethidium bromide nuclei acids gel staining (Dominique Deutsher) was visualized using a UV transilluminator (Fisher Bioblock).

9. Transfection.

The three cell lines were grown in either EMEM (16HBE) or DMEM (A549 and HeLa) both supplemented with 10% fetal bovine serum, 1% antibiotic and 1% L-glutamine. All incubations were performed at 37 °C in a humidified atmosphere containing 5% CO_2. One day before transfection, the cells were seeded into a 96-well plate at a density of 20 000 cells per well for A549 and HeLa and 40 000 cells per well for 16HBE. Lipoplexes were prepared in Optimen (Lonza) and then added to each well (20µL/well). The reference compound KLN47 was used as a positive transfection control (CR = 2) whereas naked DNA was used as a negative control. After 24 h at 37 °C, the culture medium was removed and the cells were lysed with Passive Lysis Buffer (Promega) prior to examination via a chemiluminescence assay (Luciferase Assay System, Promega) in order to determine the luciferase expression. The total protein content of each cell lysate was determined using the BC assay kit (Uptima). Finally, data were expressed as relative light units (RLU) per milligram of total proteins (mean ± SD with n = 3).

[4] S.C. Hyde, I.A. Pringle, S. Abdullah, A.E. Lawton, L.A. Davies, A. Varathalingam A, G. Nunez-Alonso, A.M. Green, R.P. Bazzani, S.G. Sumner-Jones, M. Chan, H. Li, N.S. Yew, S.H. Cheng, A.C. Boyd, J.C. Davies, U. Griesenbach, D.J. Porteous, D.N. Sheppard, F.M. Munkonge, E.W. Alton, D.R. Gill, *Nat. Biotechnol*., 2008, **26**, 549-51.

10. Viability.

To estimate the toxicity resulting from the exposure of the cells to the lipoplexes, the ViaLight kit (Lonza) was used to determine the ATP content reflecting the number of living cells (transfected or not) in culture that occurred during 24 h after cells exposure. This assay was used following the manufacturer's recommendation. The results were expressed as percentages relative to the viability of non-transfected cells (control cells) used as the reference (100% cell viability).

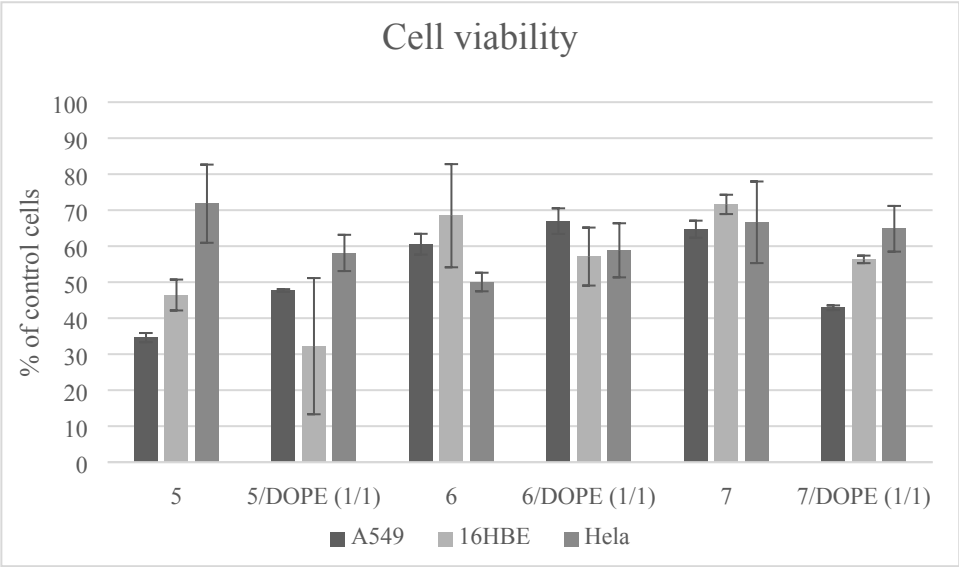


Figure SI-*20* : Cell viability are evaluated at CR2 on 16HBE, A549 and HeLa. Results are expressed as a percentage of the viability determined with non-transfected cells, as the mean ± SD of 3 wells.