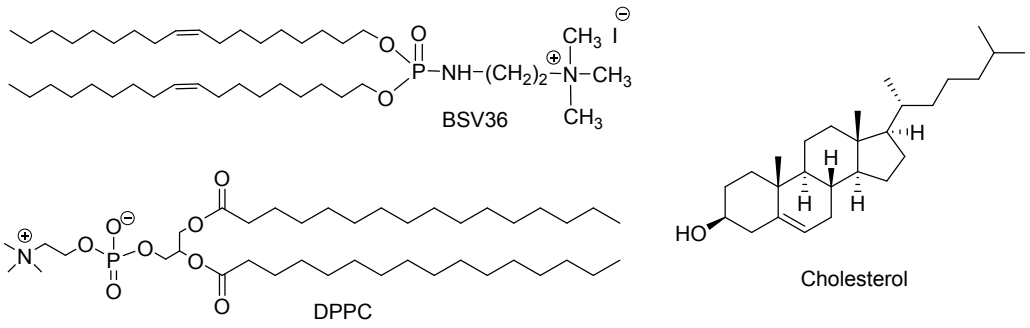
Phosphonodithioester-Amine Coupling in water: A fast reaction to modify the surface of liposomes.

Montassar Khalil, Dany Jeanne Dit Fouque, Mathieu Berchel, Aurore Fraix, Aurélien Dupont, Salvatore Sortino, Antony Memboeuf, Paul-Alain Jaffrès

Summary

1- General

Materials: All reagents were purchased from commercial sources (Aldrich, TCI) and used as received. Solvents were dried with a MBRAUN solvent purification system and kept under molecular sieve. Water milli Q was used as a solvent in all formulations. **BSV36,**[1] **5**[2] and **7**[3] were synthesized as previously reported. For compound **5** we found a molar extinction coefficient equal to 12300 $M^{-1}cm^{-1}$ at 436 nm which is consistent with the value of the literature (**ε**= 12800 $M^{-1}cm^{-1}$at 437 nm).[4]



Scheme S1: Chemical structure of BSV36, DPPC and Cholesterol

NMR Spectra: For NMR spectroscopy, Bruker Avance 400 (^{1}H: 400.1 MHz, ^{13}C: 100.6 MHz, T = 293 K) or Bruker Avance 500 (^{1}H: 500.1 MHz, ^{13}C: 125 MHz, T = 293 K) were used. All chemical shifts are reported in δ [ppm] (multiplicity, coupling constant *J*, number of protons) relative to the solvent's residual peaks as the internal standard ($CDCl_3$: ^{1}H: δ = 7.26 ppm, ^{13}C: δ = 77.16 ppm). The coupling constants are given in Hertz [Hz]. Abbreviations used for signal multiplicity: ^{1}H-NMR: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet.

Mass Spectra:

Characterization of organic compounds: Mass spectra were recorded with the autoflex III Brucker MALDI-TOF mass spectrometer (α-Cyano-4-hydroxycinnamic acid (HCCA) was used as matrix) and with a SYNAPT XS High Resolution Mass Spectrometer (ESI-qTOF).

Analysis of liposomal solutions: Mass spectrometry experiments were carried out using a QqQ Ultima Platinium (Waters) mass spectrometer equipped with a Z-spray ionization source and operated in the positive ion mode.

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[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.

[2] J-A. Hong, N-E. Choi, Y-K. La, H.Y. Nam, J. Seo and J. Lee, *Org. Biomol. Chem*., 2017, **15**, 8018.

[3] H. Chen, C. Cai, S. Li, Z. Zhu, Y. Ma and S. Luozhong, *Chem. Eur. J*. 2017, **23**, 4730 – 4735.

[4] Z. Ou, Y. Qian, Y. Gao, Y. Wang, G. Yang, Y. Li, K. Jiang and X. Wang, *RSC Adv*, 2016, **6**, 36923.

Dynamic Light Scattering (DLS): The mean particle diameter and zeta potential (ξ) were measured using a Malvern Zetasizer Nano ZS at 25 °C using 1 cm disposable polystyrene cuvettes.

Purity: the synthesized compounds were analyzed (after their purification by a chromatography) either by a Grace REVELERIS® flash chromatography equipped with UV and EDLS detectors or by HPLC. The analytic HPLC was performed on a Prominence Shimadzu HPLC/LCMS-2020 equipped with a UV SPD-20 A detector. The chromatographic system employs analytic HPLC Uptisphere strategy 100A 5µm C18HQ (250 × 4.6 mm) with water methanol (v/v) as eluents [isocratic 20 % MeOH (4 min), linear gradient from 20 to 80 % MeOH (4 min), isocratic 80 % MeOH (6 min), linear gradient from 80 to 20 % MeOH (2 min), isocratic 20 % MeOH (6 min)] at a flow rate of 1 mL/min and UV detection at 280 nm.

Dialysis: Dialysis step was performed with Cellu-Sep regenerated cellulose tubular membranes with a molecular weight cut off (MWCO) of 6-8 KDa.

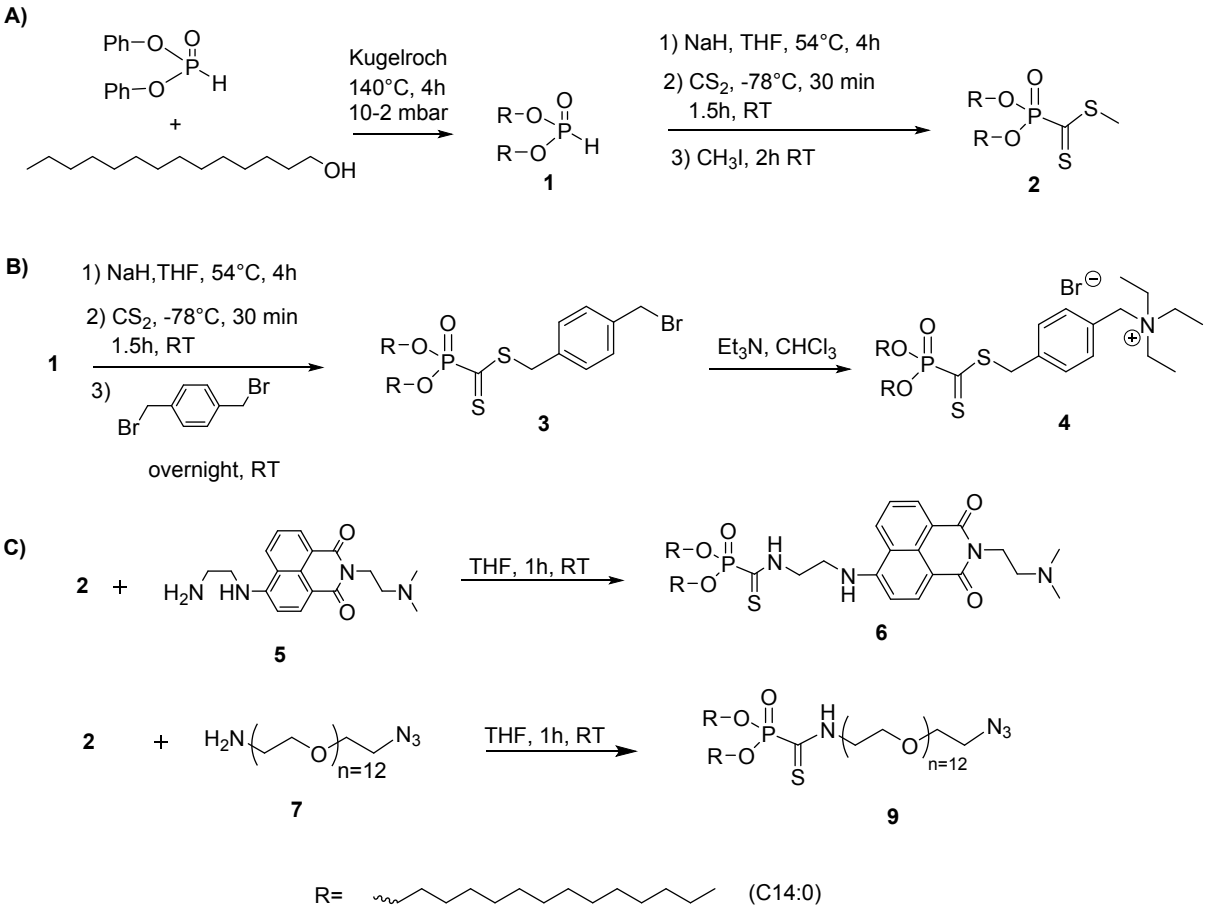
Absorbance measurements: Absorbance spectra were recorded with JASCO V-760 spectrometer using quartz cells with a path length of 1 cm.

Fluorescence measurements: Fluorescence emission spectra were recorded with a Spex Fluorolog-2 (mod. F-111) spectrofluorimeter using quartz cells with a path length of 1 cm. Fluorescence lifetimes were recorded with the same fluorimeter equipped with a TCSPC Triple Illuminator.

Cryo-TEM: Vitrification of liposomal solutions was performed using an automatic plunge freezer (EM GP, Leica) under controlled humidity and temperature.[5] The samples were deposited to glow-discharged electron microscope grids followed by blotting and vitrification by rapid freezing into liquid ethane. Grids were transferred to a single-axis cryo-holder (model 626, Gatan) and were observed using a 200kV electron microscope (Tecnai G^2 T20 Sphera, FEI) equipped with a 4k × 4k CCD camera (model USC 4000, Gatan). Micrographs were acquired under low electron doses using the camera in binning mode 1 and at a nominal magnification of 29.000x.

[5] J. Dubochet and A.W. McDowall, *J. Microscopy*, 1981, **124**, RP3-RP4

2- Synthesis



Scheme S2 : A) Synthesis of methyl (bis(tetradecyloxy)phosphoryl)methanedithioate **2**. B) Synthesis of *N*-(4-((((bis(tetradecyloxy)phosphoryl)carbonothioyl)thio)methyl)benzyl)-N,N,N-triethylammonium bromide **4**. C) Click reaction involving the phosphonodithioester **2** and 6-((2-aminoethyl)amino)-2-(2-(dimethylamino)ethyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione **5** or PEG-600 NH_2/N_3 **7.**

Ditetradecylphosphite **1**

To a solution of diphenylphosphite (2 g, 8.54 mmol) was added 1-tetradecanol (3.84 g, 17.83 mmol). The mixture was placed in Kugelroch distillation apparatus at 140°C under reduced pressure (4.10^{-2}mbar) for 4h. Phenol is removed by distillation and the undistilled compound corresponds to the desired product (4 g; 98% yield). Spectroscopic data (below) are consistent with the literature.[6]

1 (4.2 g; Yield 98%): **^{1}H NMR** (400 MHz, $CDCl_3$) : δ= 6.76 (d, $^1J_{HP}$ =692 Hz ; 1H), 4.03 (m, 4H), 1.64 (m, 4H), 1.39 – 1.14 (m, 44H), 0.84 (t, $^3J_{HH}$ = 6.7 Hz, 6H) ; **^{31}P{^{1}H} NMR** (162 MHz, $CDCl_3$) : δ= 8.46 ; **^{13}C{^{1}H}**

[6] Q. Xiao, Y. Ju, and Y. Zhao, *Heteroatom Chem*., 2003, **14**, 208-210.

NMR (125 MHz, $CDCl_3$) : δ=65.87 (d, $^3J_{CP}$= 5.85, CH_2-O-P), 31.93, 30.44, 30.39, 29.66, 29.56, 29.50, 29.36, 29.12, 25.50, 22.69, 14.11; HRMS (ESI–qTOF): m/z calcd for $C_{28}H_{59}O_3PNa$ $[M+Na]^+$= 497.4099; observed $[M+Na]^+$=497.4095.

Methyl (bis(tetradecyloxy)phosphoryl)methanedithioate **2**

To a mixture of NaH (262 mg, 10.92 mmol) in 40 mL of anhydrous tetrahydrofuran placed in a schlenk flask under nitrogen, a solution of phosphite **1** (4g, 8.4 mmol) in 10 mL of THF is added at room temperature. After the addition, the mixture is heated at 54°C for 4h (leading to a limpid solution) (monitoring by ^{31}P NMR with D_2O as an internal standard in a sealed capillary, a peak at +151 ppm is observed). Then, the solution was cooled up to -78°C and carbon disulfide was added (3.83 g, 3 mL, 50.4 mmol 6eq) for 30 min. The mixture is stirred for 1.5h at room temperature and methyl iodide (1.3 g, 0.57 mL, 9.2 mmol, 1.1eq) is subsequently added. After stirring for an additional 2h, the mixture is washed with a saturated aqueous solution of ammonium chloride and is extracted twice with 60 mL of ethyl acetate. The organic phase is washed with a saturated solution of sodium chloride, dried over anhydrous sodium sulfate, filtered and concentrated in vacuum. The crude product was purified by chromatography on silica gel (eluent: n-hexane/ethyl acetate: from 100:0 to 70/30 in volume). Compound **2** was isolated as a purple solid (1.5 g; 32% yield).

Rf (0.42 n-hexane : ethyl acetate 90 : 10): ; **^{1}H NMR** (500 MHz, $CDCl_3$) : δ= 4.23 – 4.15 (m, 4H), 2.71 (s, 3H), 1.75 – 1.69 (m, 4H), 1.39 – 1.27 (m, 49H), 0.89 (t, $^3J_{HH}$ = 6.8 Hz, 6H) ; **^{31}P{^{1}H} NMR** (162 MHz, $CDCl_3$) : δ= -1.71, **^{13}C{^{1}H} NMR** (125 MHz, $CDCl_3$) : δ= 229.63 (d, $^1J_{CP}$ = 176.6 Hz, C=S), 68.61 (d, $^3J_{CP}$= 6.98, CH_2-O-P), 31.93, 29.69, 29.66, 29.57, 29.50, 29.37, 29.11, 25.41, 22.69, 19.22, 14.12 , MALDI- TOF (matrix: UTL HCCA): m/z calcd for $C_{30}H_{61}O_3PS_2$+K $[M+Na]^+$= 587.369; observed $[M+Na]^+$= 587.478. HRMS (ESI-qTOF): m/z calcd for $C_{30}H_{61}O_3PS_2$ $[M+H]^+$=565.3878 ; observed $[M+H]^+$=565.3878, purity (Flash chromatography – detection EDLS) : >95%.

4-(Bromomethyl)benzyl (bis(tetradecyloxy)phosphoryl)methanedithioate **3**

To a mixture of NaH (197 mg, 8.21 mmol 1.3 eq) in 30 mL of anhydrous THF, a solution of phosphite **1** (3 g, 6.32 mmol) in 7.5 mL of THF is added under stirring at room temperature. At the end of the addition the mixture is heated to 54°C for 4h, then cooled down to -78°C and treated with anhydrous carbon disulfide (2.89 g, 37.92 mmol, 2.29 mL, 6 eq). Then, the solution is stirred for 1h at room temperature and dibromo-para-xylene (5 g, 18.96 mmol, 3 eq) in 15 mL of anhydrous DMF and 10 mL of anhydrous THF under heat is subsequently added. After stirring at 25°C for an additional night, the mixture is filtrated and then concentrated in vacuum. The crude product is precipitated, filtered and evaporated several times in hexane. The precipitation aims to remove most of the excess of para-xylene. Finally, the mixture is evaporated a last time and chromatographed on silica gel by using a mixture of n-

hexane/ethyl acetate 10:0.5 as eluent. The final product is obtained with a 24% yield with a 1.1 g of pure product **3**.

Rf (0.2 Hexane : ethyl acetate 10 : 0.5): **^{1}H NMR** (400 MHz, $CDCl_3$) : δ= 7.38 – 7.36 (d, J=8.08, 2H), 7.31 – 7.30 (d, J=8.08, 2H), 4.49 (s, 4H), 4.24 – 4.16 (m, 4H), 1.74 – 1.64 (m, 4H), 1.38 – 1.27 (m, 49H), 0.90 (t, J = 7.3 Hz, 6H) ; **^{31}P{^{1}H} NMR** (162 MHz, $CDCl_3$) : δ= -1.28 ; **^{13}C{^{1}H} NMR** (125 MHz, $CDCl_3$) : δ= 228.14 (d, $^{1}J_{CP}$= 175.46 Hz, C=S), 137.68, 134.12, 129.75, 129.49, 129.36, 68.76 (d, $^{3}J_{CP}$=7.2Hz, 6H, CH_2-O-P), 40.00, 32.80, 31.94, 30.40, 30.36, 29.69, 29.67, 29.58, 29.51, 29.37, 29.12, 25.41, 22.70, 14.13.

N-(4-((((bis(tetradecyloxy)phosphoryl)carbonothioyl)thio)methyl)benzyl)-N,N,N-triethylammonium bromide **4**

To a solution of phosphonodithioester **3** (200 mg, 0.27 mmol) in 5 mL of $CHCl_3$ we added 2 equivalents of triethylamine (73 µL) and the mixture was stirred at room temperature. The formation of the final product was monitored by ^{1}H NMR and ^{31}P NMR. The reaction was stopped after 24h and the crude product was purified by chromatography on silica gel (eluent: methylene chloride/methanol: 100/5 in volume). The final product is obtained with a quantitative yield (204 mg).

Rf (0.4 DCM: MeOH 90: 10): **^{1}H NMR** (400 MHz, $CDCl_3$): δ= 7.58 (d,$^{3}J_{HH}$= 8.1 Hz, 2H), 7.48 (dd, $^{3}J_{HH}$= 8.1 Hz, 4H), 4.85 (s, 2H), 4.51 (s, 2H), 4.23 – 4.16 (m, 4H), 3.44 – 3.40 (m, 6H), 1.73 – 1.69 (m, 4H), 1.48 – 1.45 (m, 4H), 1.38 – 1.36 (m, 12H), (m, 54H), 0.88 (t, J = 6.8 Hz, 19H). **^{31}P{^{1}H} NMR** (162 MHz, $CDCl_3$): δ= -1.42; **^{13}C{^{1}H} NMR** (125 MHz, $CDCl_3$): δ= 227.80 (d, $^{1}J_{CP}$= 175.31 Hz, C=S), 137.11, 133.11, 132.91, 130.29, 126.97, 68.76 (d, $^{3}J_{CP}$=7.06 Hz, CH_2-O-P, 4H), 61.01, 53.18, 39.26, 31.92, 30.42, 30.37, 29.69, 29.66, 29.58, 29.51, 29.36, 29.12, 25.41, 22.68, 14.12, 8.62; MALDI-TOF (matrix: UTL HCCA): m/z calcd for $C_{43}H_{81}NO_3PS_2[M]^+$= 754.539; observed $[M]^+$=754.641. HRMS (ESI-qTOF): m/z calcd for $C_{43}H_{81}NO_3PS_2$ $[M]^+$ = 754.5396; observed $[M]^+$=754.5397; Purity (Flash chromatography – detection EDLS)> 95%

Ditetradecyl((2-((2-(2-(dimethylamino)ethyl)-1,3-dioxo-2,3-dihydro-1H-benzo[de]isoquinolin-6-yl)amino)ethyl)carbamothioyl)phosphonate **6**

5 (58 mg, 0.177 mmol) in 5 mL of THF was added dropwise at 20°C to a solution of compound **2** (100 mg, 0.177 mmol) in 5 mL of anhydrous tetrahydrofuran. The purple color of the reaction turned instantly into yellow color. Then, the solvent is evaporated and the crude product was purified by chromatography silica gel (methylene chloride/methanol: from 95/5 to 80/20 in volume). Compound **6** was isolated as an orange wax (150 mg; 74%).

Rf (0.36 DCM : MeOH 95 : 5): **^{1}H NMR** (400 MHz, $CDCl_3$) : δ= 8.42 (d, $^{3}J_{HH}$ = 7.2 Hz, 1H), 8.27 (d, $^{3}J_{HH}$ = 8.4 Hz, 1H), 8.08 (d, $^{3}J_{HH}$ = 8.4 Hz, 1H), 7.43 (t, $^{3}J_{HH}$ = 7.9 Hz, 1H), 6.49 (d, $^{3}J_{HH}$ = 8.5 Hz, 1H), 6.36 (s, 1H), 4.34 – 4.26 (m, 2H), 4.26 – 4.18 (m, 2H), 4.13 (m, 4H), 3.66 (m, 2H), 2.70 (m, 2H), 2.40 (s, 6H), 1.66 – 1.52 (m,

4H), 1.18 (m, 45H), 0.85 (t, J = 6.9 Hz, 6H). **$^{31}P\{^{1}H\}$ NMR** (162 MHz, $CDCl_3$) : δ= -1.10, **$^{13}C\{^{1}H\}$ NMR** (125 MHz, $CDCl_3$) : δ= 197.05 (d, $^{1}J_{CP}$= 180.83 Hz, C=S), 167.24 (C=O), 167.60 (C=O), 151.97 (C_{quat}-NH), 136.85, 133.72, 132.32, 129.53, 127.28, 125.49, 122.90, 113.18, 106.50, 72.54 (d, $^{3}J_{CP}$=7.36, CH2-O-P), 59.62, 48.23 ($N(CH_3)_2$), 45.94 (d, $^{1}J_{C-H}$=8.65, NH-CH_2-CH_2-NH), 45.52, 40.31, 34.63, 32.99, 32.38, 32.26, 32.17, 32.07, 31.17, 28.04, 25.40, 16.83. (MALDI-TOF (matrix: UTL HCCA): m/z calcd for $C_{47}H_{79}N_4O_5PS$+H $[M+H]^+$=843.550; observed $[M+H]^+$=843.811; HRMS (ESI-qTOF): m/z calcd for $C_{47}H_{79}N_4O_5PS$+H $[M+H]^+$ = 843.5587 ; observed $[M+H]^+$=843.5588; λ_{abs}= 430 nm in $CHCl_3$. Purity (HPLC) > 98%.

Synthesis of compound **9**

A solution of PEG600-amine/N_3 **7** (240 mg, 0.389 mmol) in 5 mL of THF was added slowly to the solution of compound **2** (200 mg, 0.354 mmol) in 5 mL of tetrahydrofuran. The purple color of the solution turned instantly into yellow color. Then, the solvent is evaporated and the residue is purified by chromatography on silica gel (eluent: methylene chloride/methanol: from 90/10 to 60/40 ratio). Compound **9** was isolated as a yellow wax (295 mg; 74% yield).

Rf (0.62 DCM : MeOH 95 : 5); **^{1}H NMR** (400 MHz, $CDCl_3$) : δ= 4.07 – 4.16 (m, 4H), 3.85 – 3.84 (m, 2H), 3.70-3.62 (m, 52H), 3.33-3.36 (m, 2H), 1.64-1.67 (m, 4H), 1.21 – 1.26 (m, 45H), 0.82-0.85 (t, $^{3}J_{HH}$ = 6.9 Hz, 6H). **$^{31}P\{^{1}H\}$ NMR** (162 MHz, $CDCl_3$) : δ= -1.15, **$^{13}C\{H\}$ NMR** (125 MHz, $CDCl_3$) : δ= 193.3 (d, $^{1}J_{CP}$ = 181.3 Hz, C=S), 70.4(m), 69.89, 68.8 (d, $^{3}J_{CP}$= 6.91, CH2-O-P, 4H), 67.5, 50.5, 44.7, 31.7, 29.5, 25.23, 23.9, 13.97, MALDI-TOF (matrix: UTL HCCA): m/z calcd for $C_{55}H_{111}N_4O_{15}PS$+K $[M+K]^+$ = 1169.714 ; observed $[M+K]^+$=1169.742 ; HRMS (ESI-TOF): m/z calcd for $C_{55}H_{111}N_4O_{15}PSK$ $[M+K]^+$ = 1169.7141 ; observed $[M+K]^+$=1169.7159;

3- NMR spectra

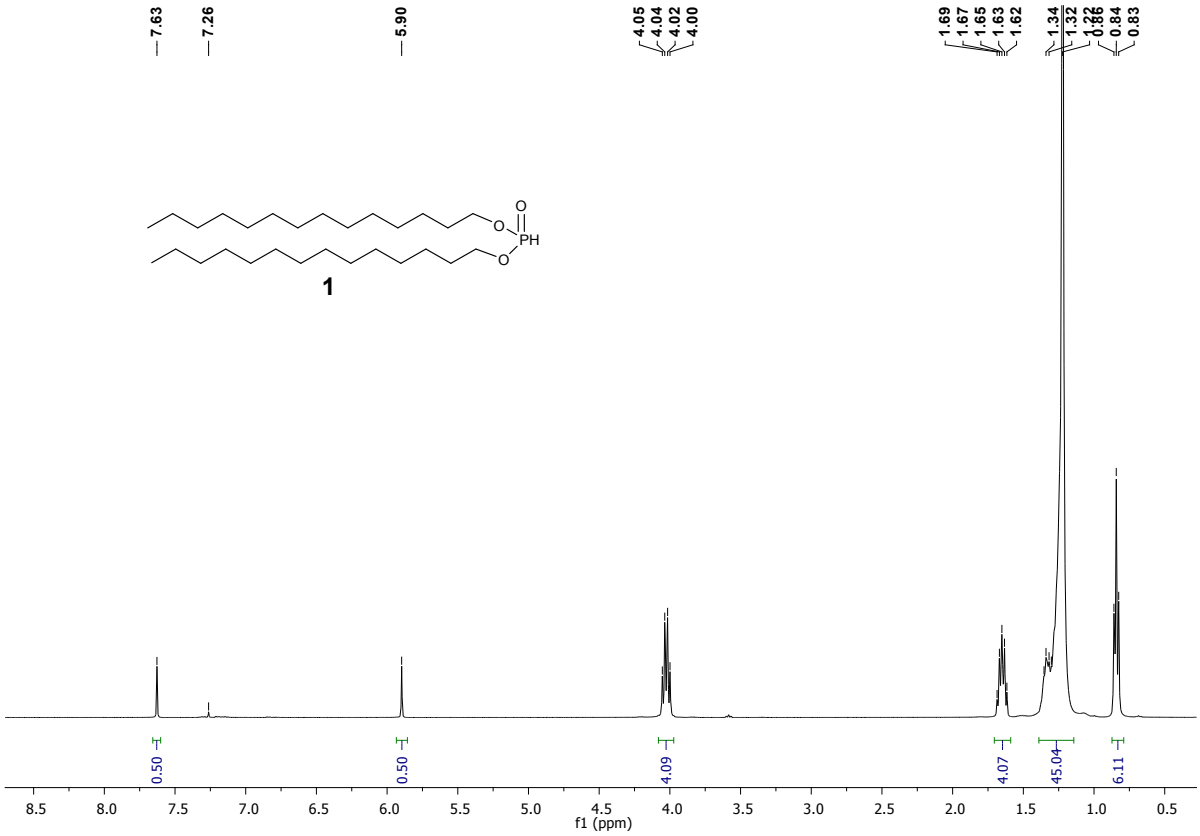


Figure S1 ^{1}H-NMR ($CDCl_3$) of **1**

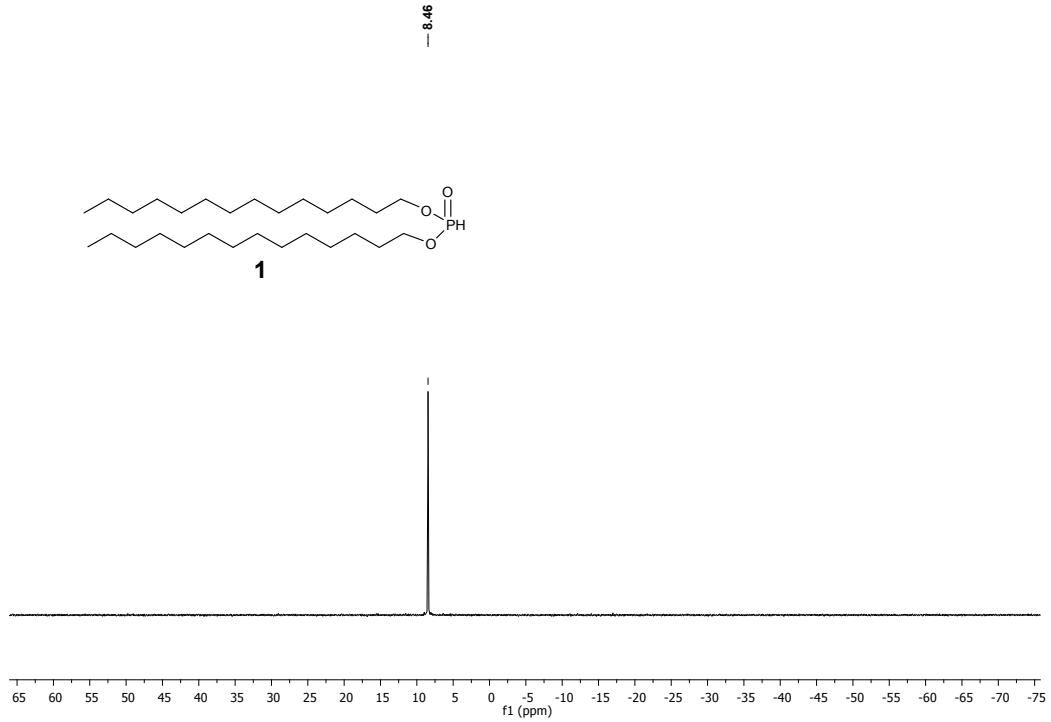


Figure S2: $^{31}P\{^{1}H\}$ NMR ($CDCl_3$) of **1**

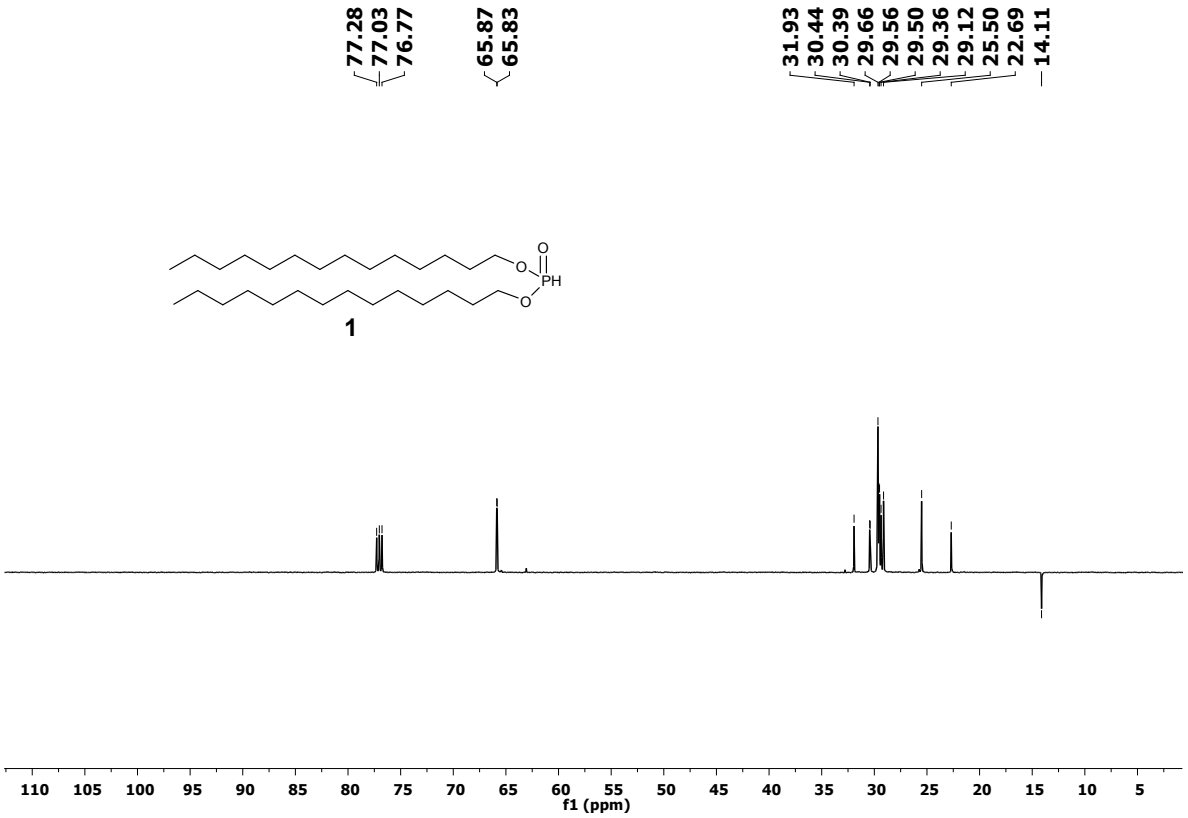


Figure S3: $^{13}C\{^{1}H\}$ NMR JMOD ($CDCl_3$) of **1**

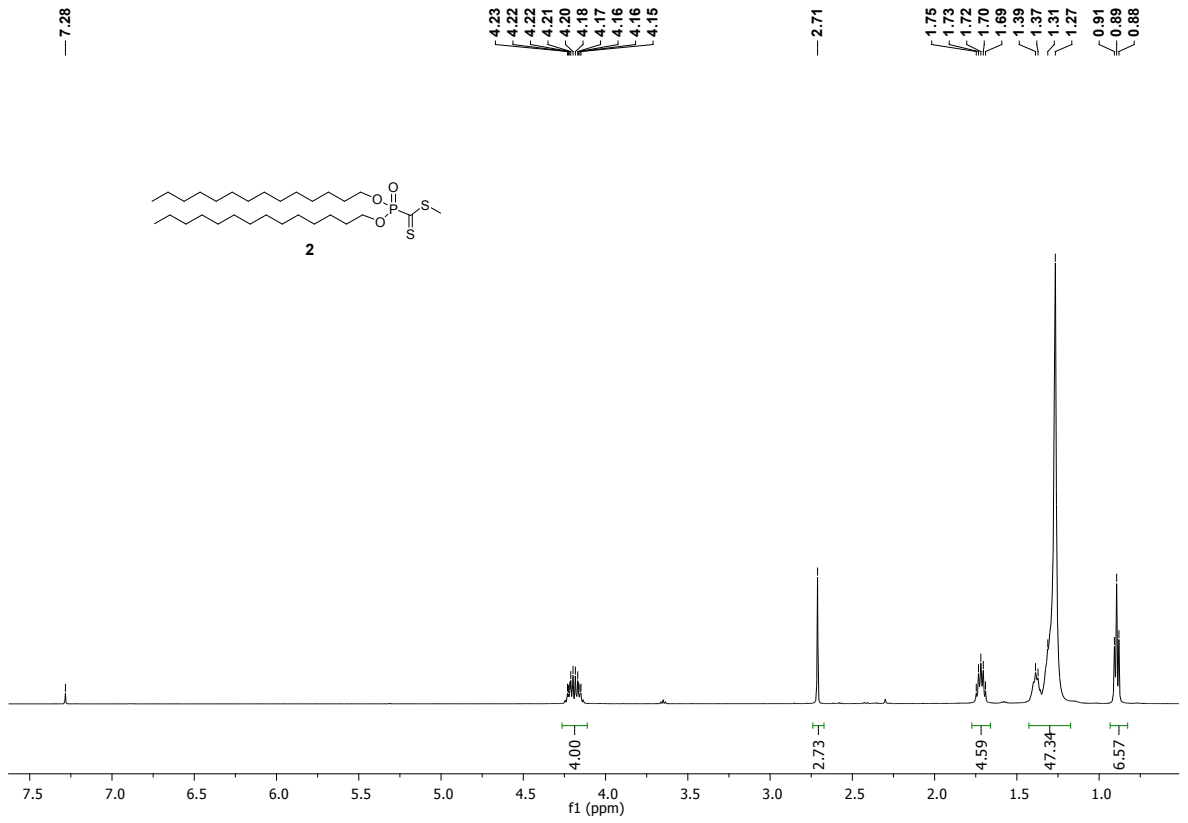


Figure S4: ^{1}H-NMR ($CDCl_3$) of **2**

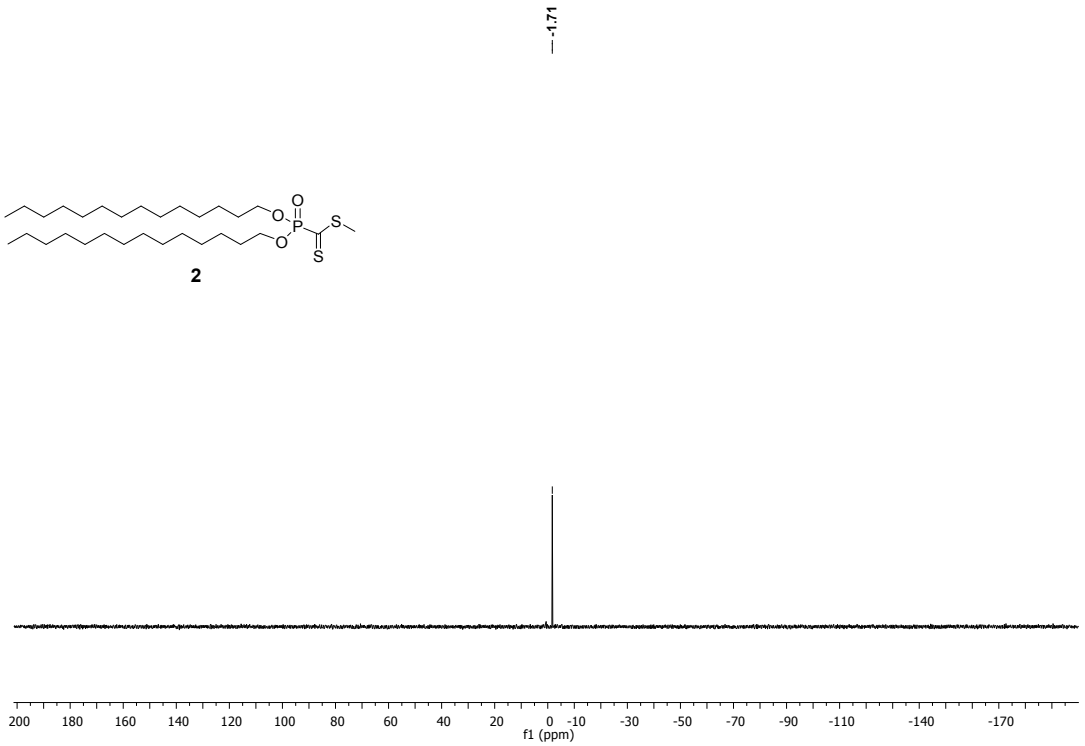


Figure S5: $^{31}P\{^{1}H\}$ NMR ($CDCl_3$) of **2**

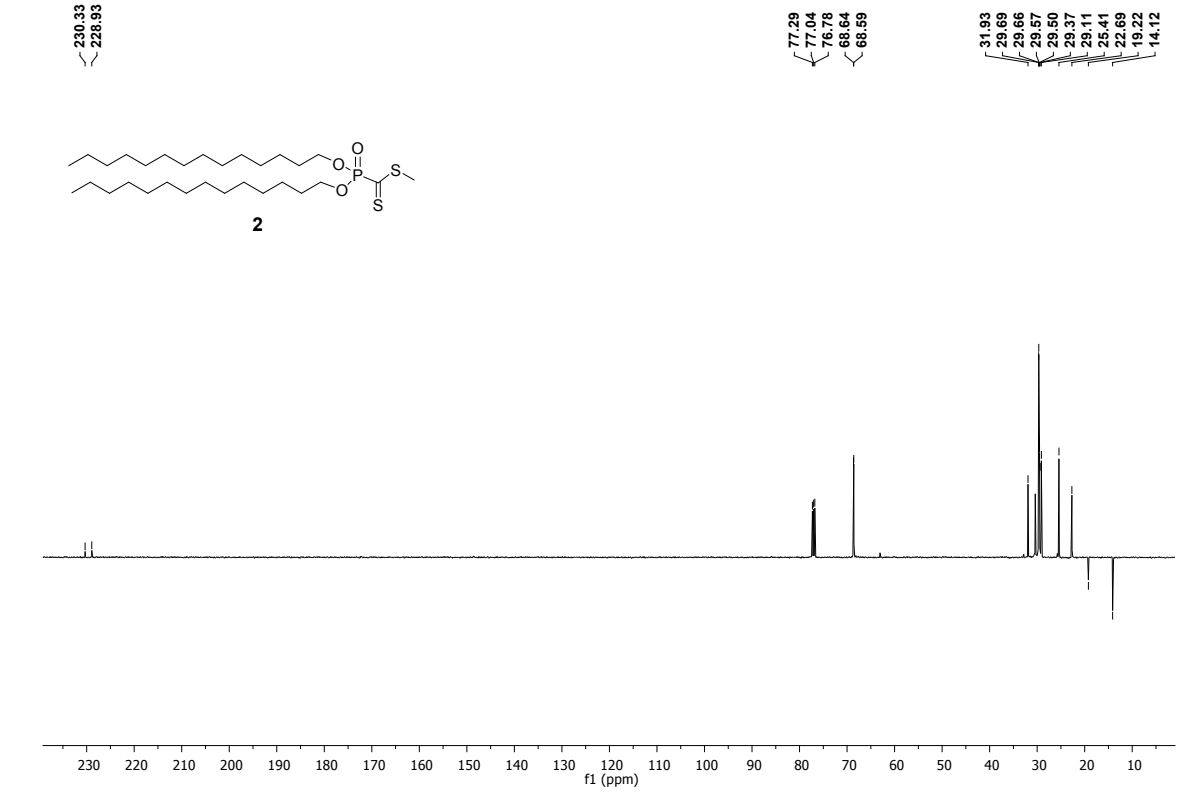


Figure S6: $^{13}C\{^{1}H\}$ NMR JMOD ($CDCl_3$) of **2**

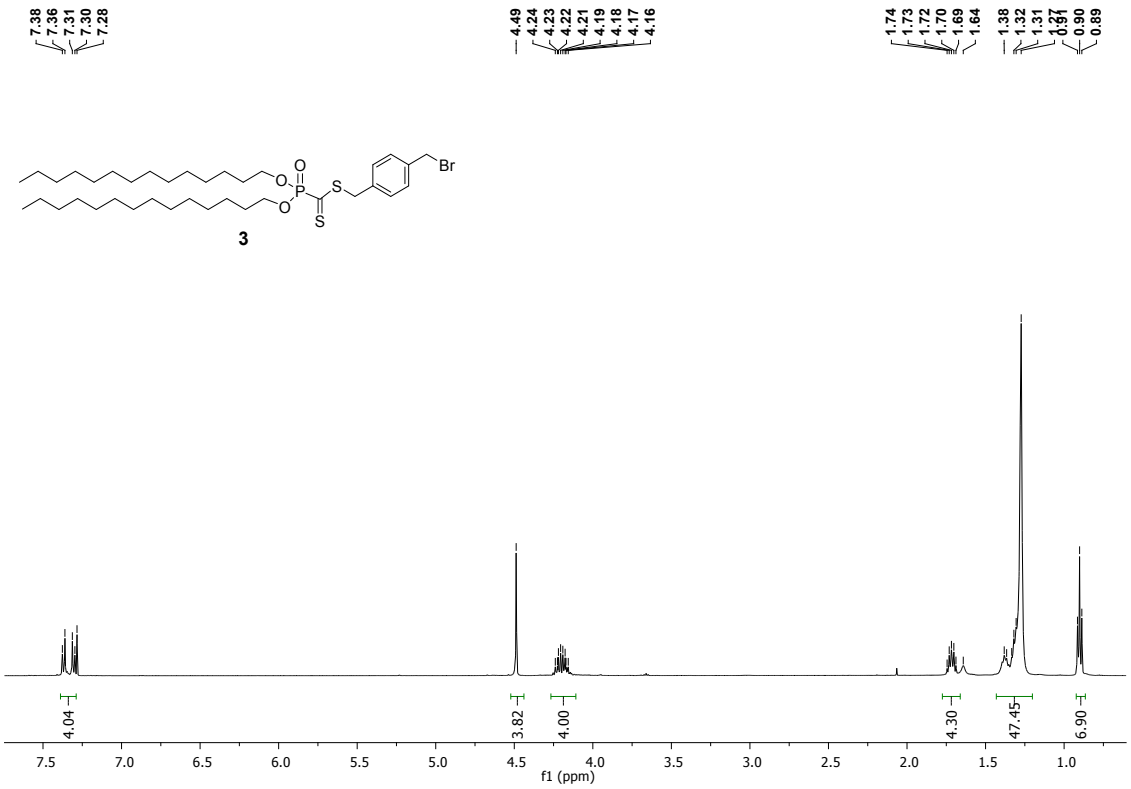


Figure S7: ^{1}H-NMR ($CDCl_3$) of **3**

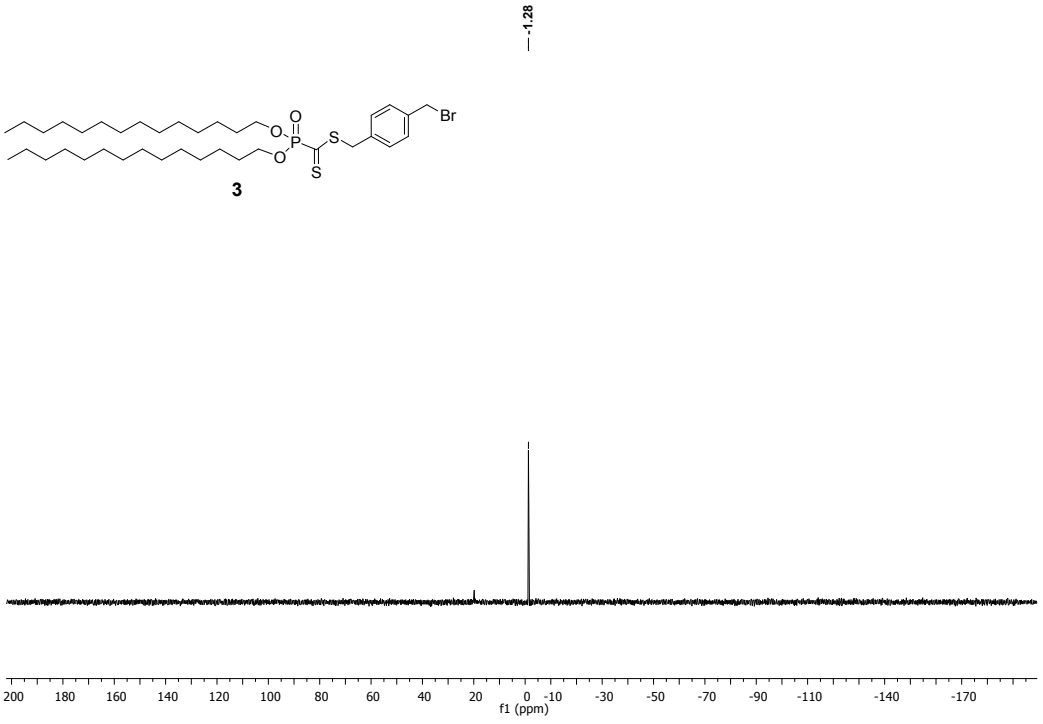


Figure S8: $^{31}P\{^{1}H\}$ NMR ($CDCl_3$) of **3**

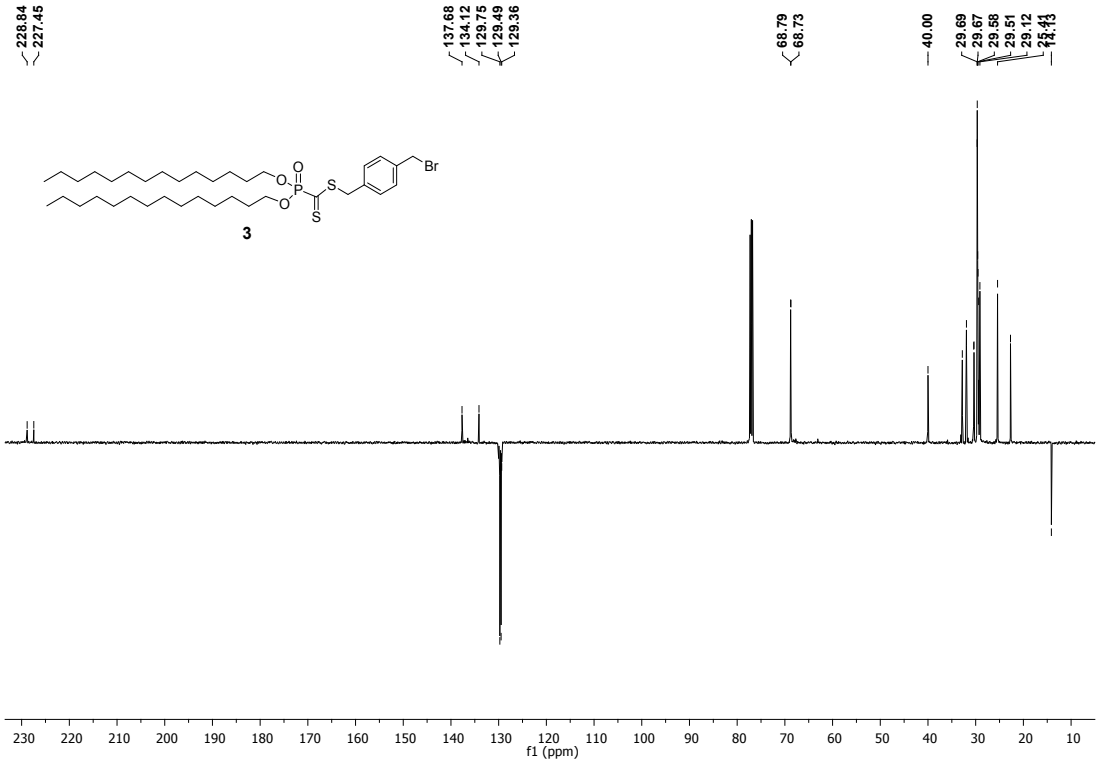


Figure S9: $^{13}C\{^{1}H\}$ NMR JMOD ($CDCl_3$) of **3**

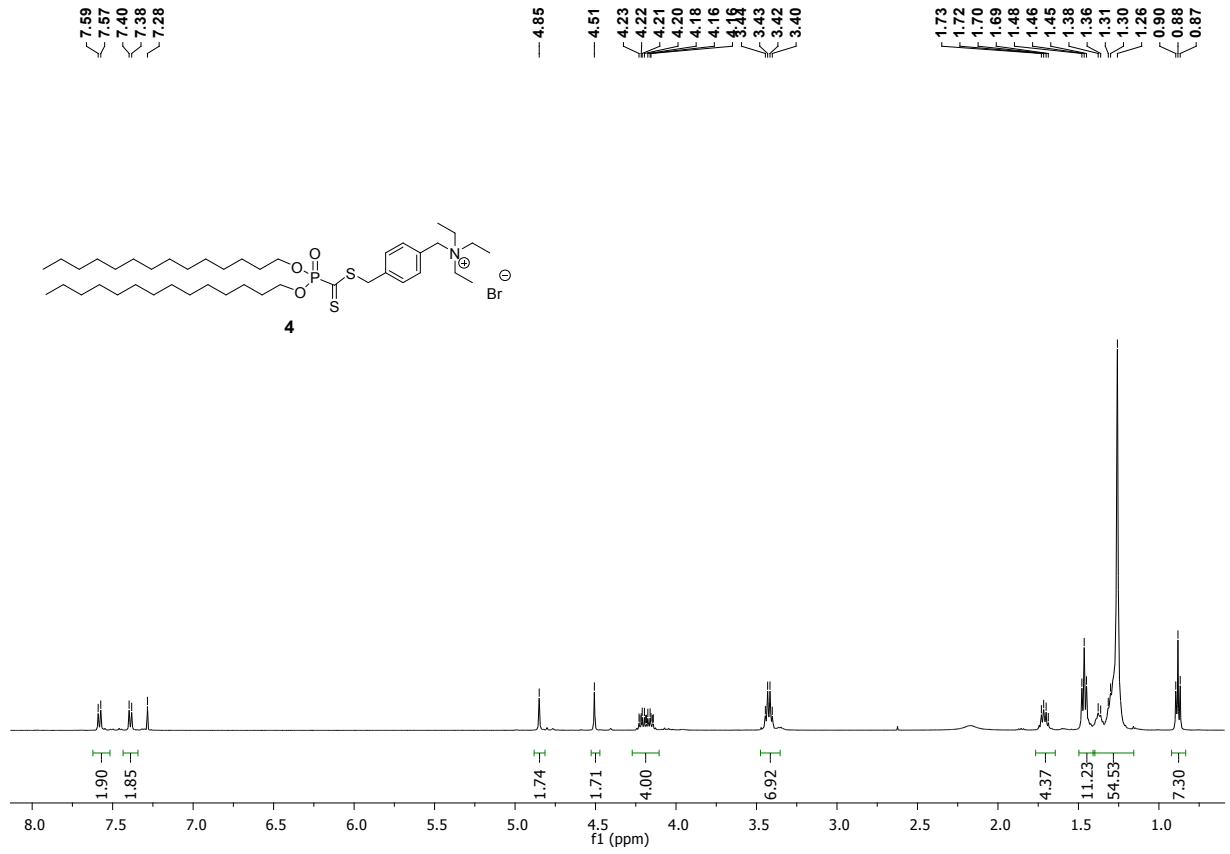


Figure S10: ^{1}H-NMR ($CDCl_3$) of **4**

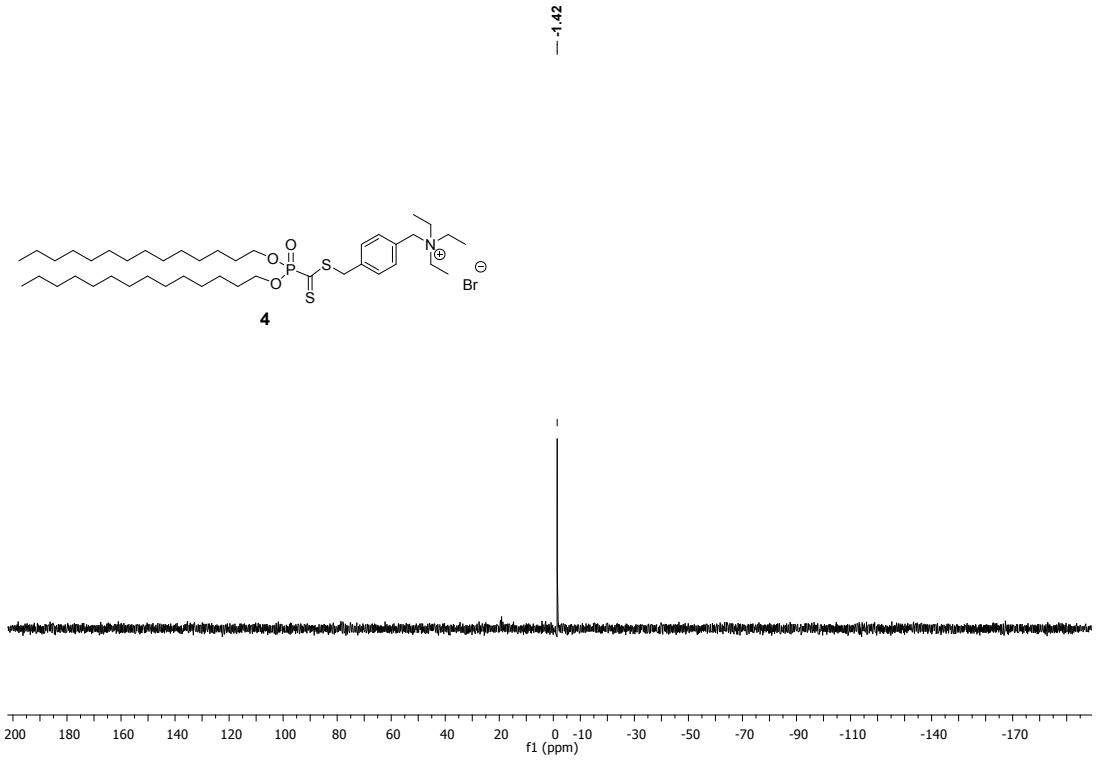


Figure S11: $^{31}P\{^{1}H\}$ NMR ($CDCl_3$) of **4**

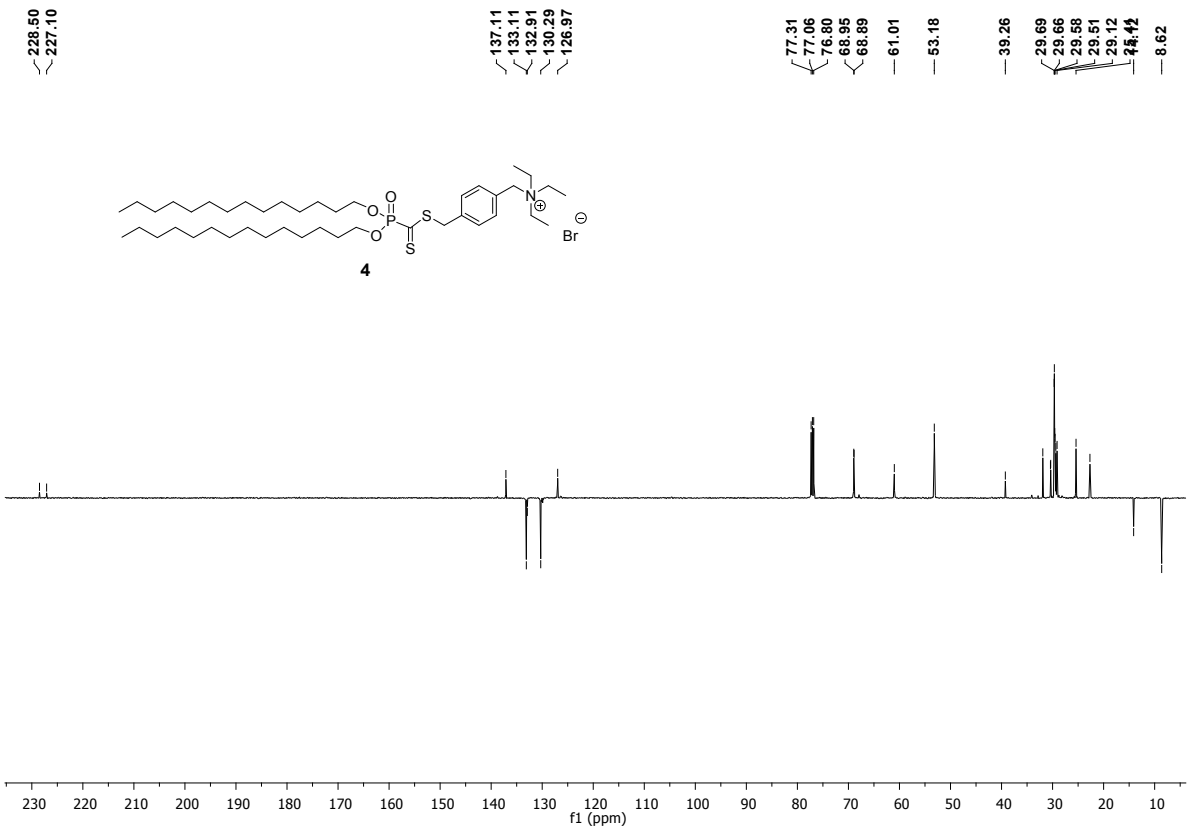


Figure S12: $^{13}C\{^{1}H\}$ NMR JMOD ($CDCl_3$) of **4**

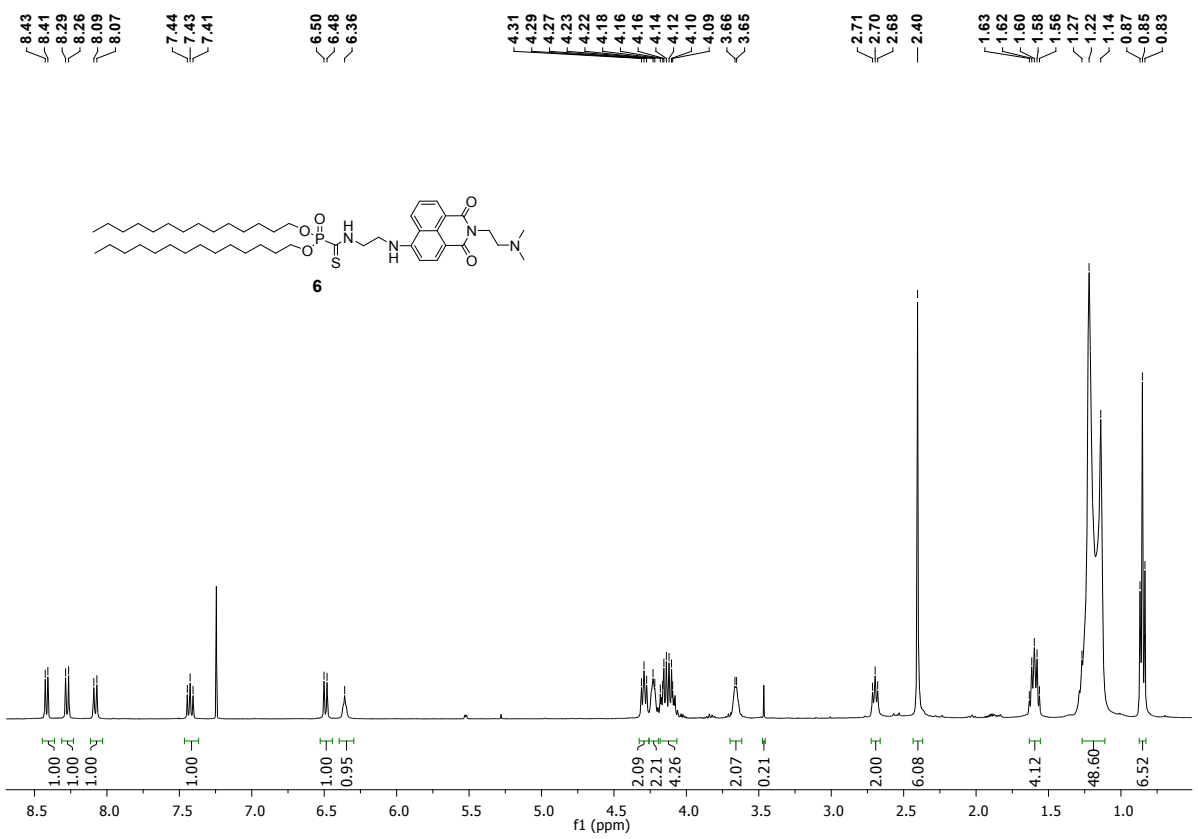


Figure S13 ^{1}H-NMR ($CDCl_3$) of **6**

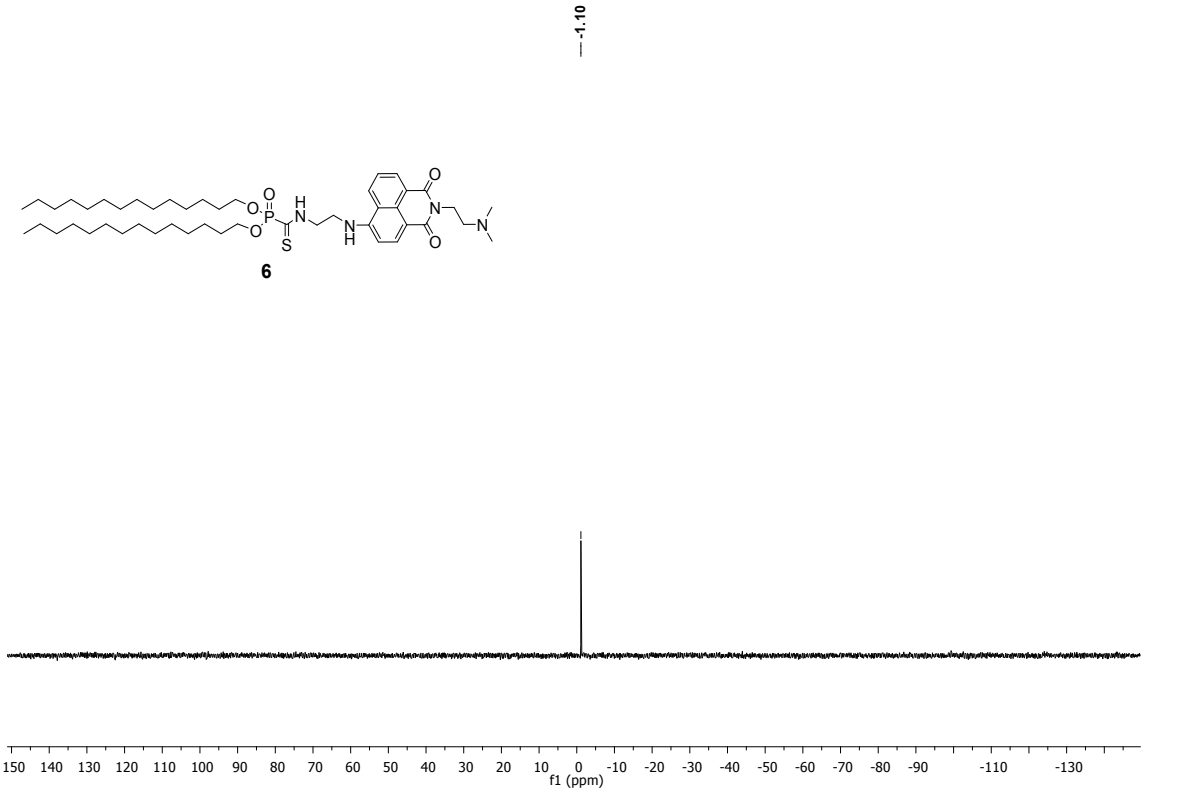


Figure S14: $^{31}P\{^{1}H\}$ NMR ($CDCl_3$) of **6**

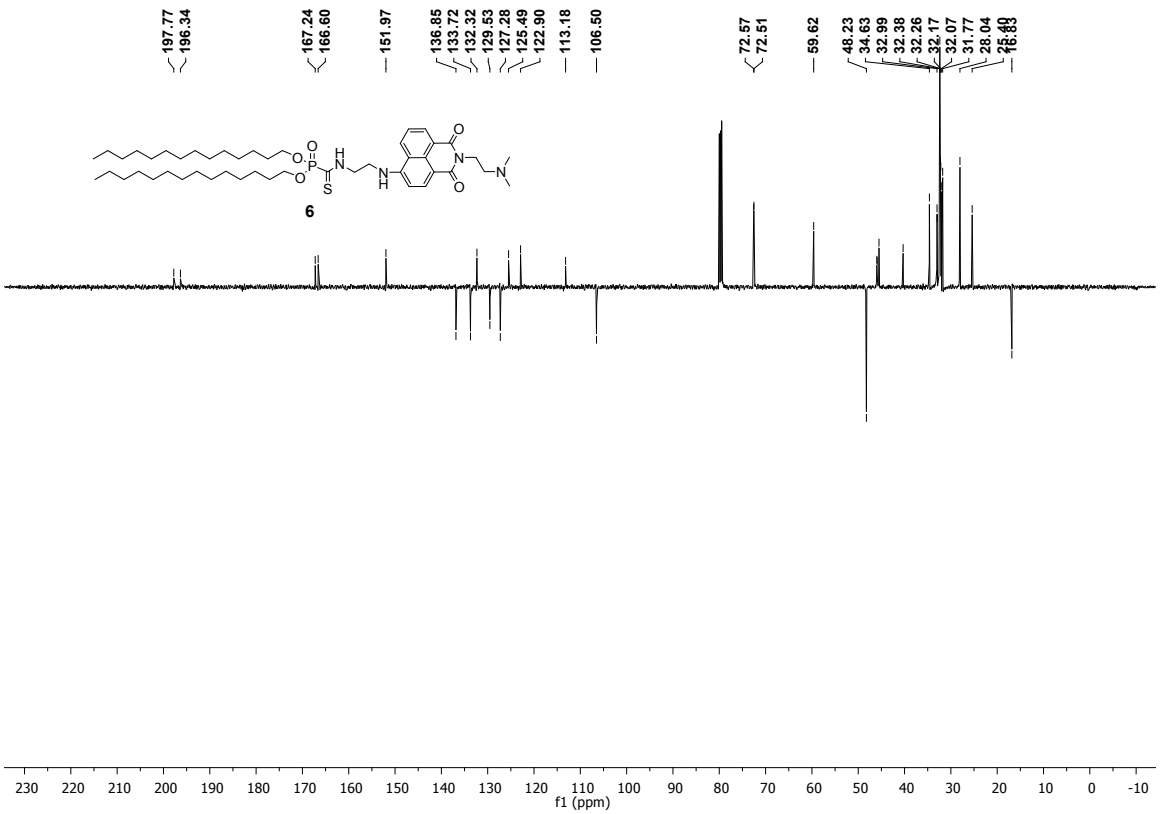


Figure S15: $^{13}C\{^{1}H\}$ NMR JMOD ($CDCl_3$) of **6**

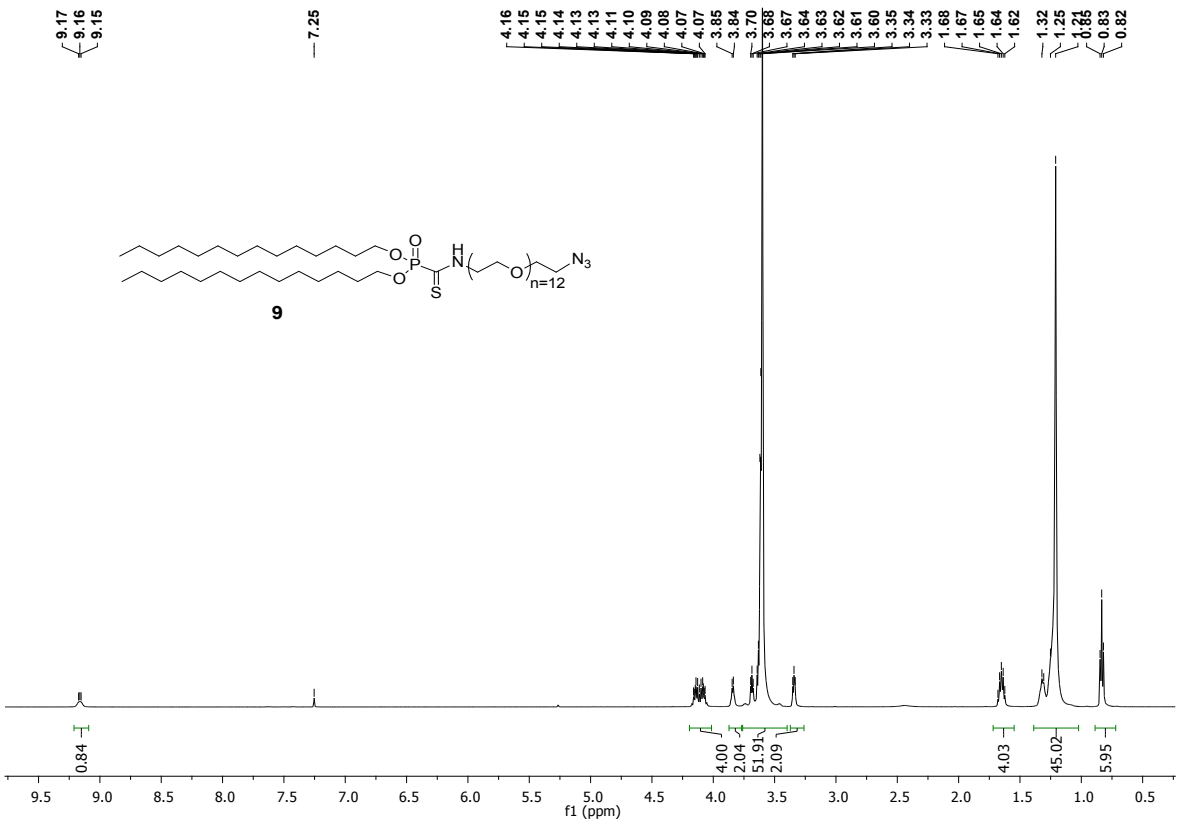


Figure S16: ^{1}H-NMR ($CDCl_3$) of **9**

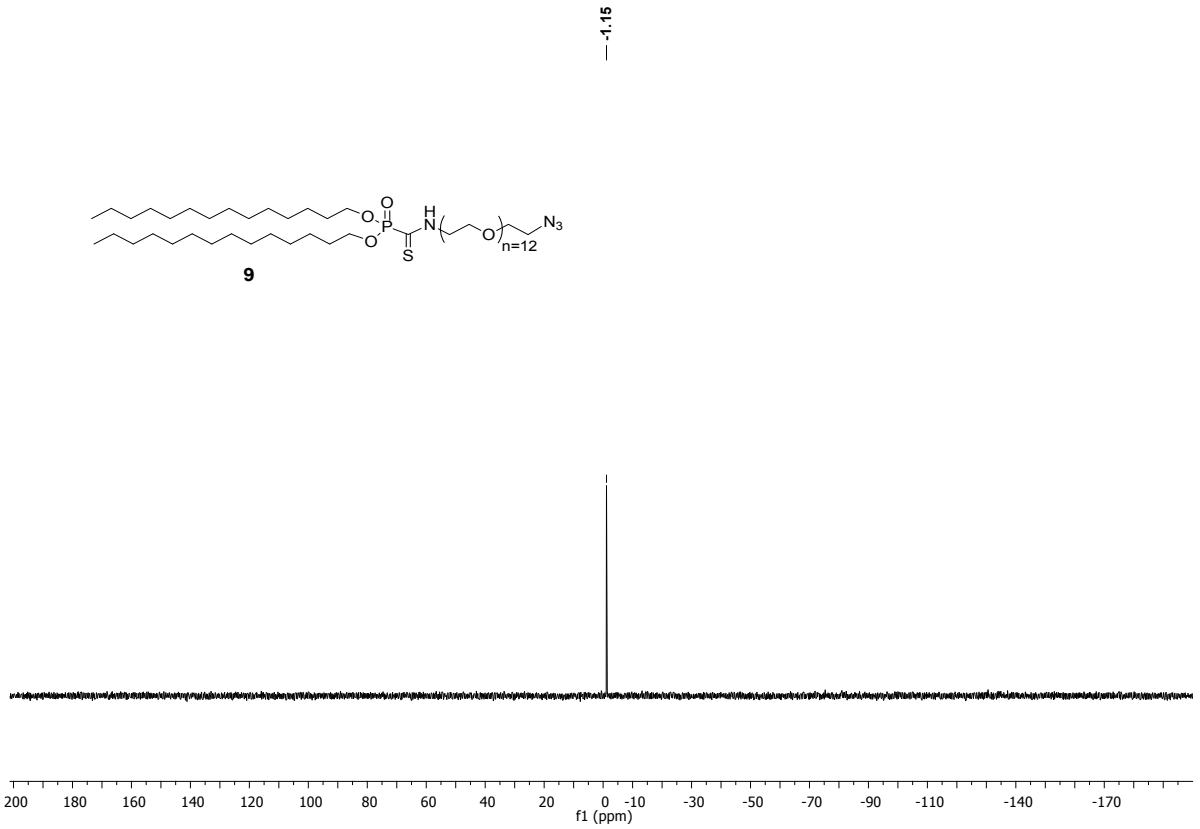


Figure S17 $^{31}P\{^{1}H\}$ NMR ($CDCl_3$) of **9**

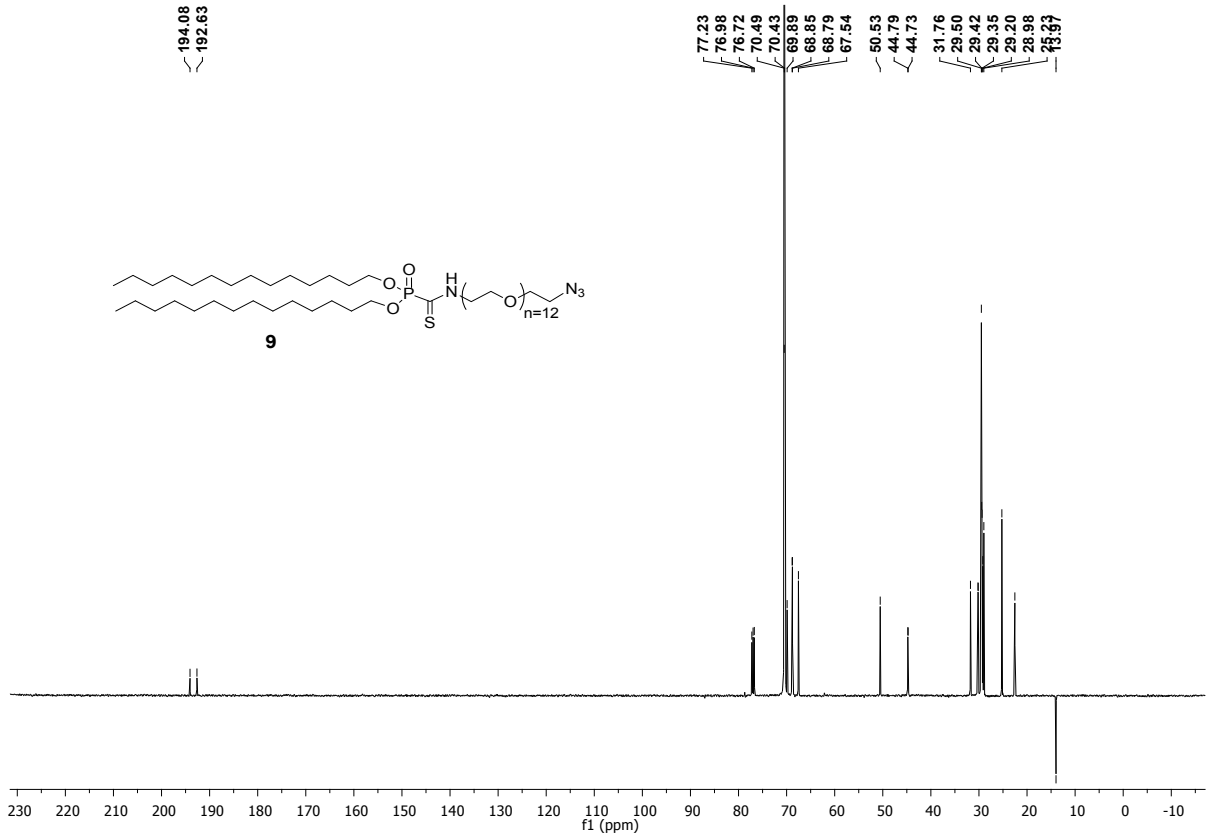


Figure S18: $^{13}C\{^{1}H\}$ NMR JMOD ($CDCl_3$) of **9**

4- Preparation of liposomal solutions

4-1 Formulations

Formulations with BSV 36 (LF1, LF2, LF3, LF4): A mixture of lipids (or BSV36 alone) including BSV36 (40.56 mg; 50 µmol; 1 eq), **2** (2.82 mg; 5 µmol; 0.1 equivalent) or **4** (3.76 mg; 5 µmol; 0.1 eq) were homogeneously dissolved in $CHCl_3$ (1 mL). The solvent was evaporated under vacuum for 5h. Next, the lipid film was hydrated for 1 night at 4°C with 2mL of milli-Q water (BSV36 concentration = 25 mM). Then, the samples were vortexed resulting in a turbid suspension and sonicated for 1 h until obtaining a clear solution. The composition of the final Liposomal Formulations (LF) is presented in Table S1. The size of the particles was measured by DLS (Table S2).

Formulations with DPPC (LF5, LF6) A lipid mixture consisting of DPPC (18.4 mg, 25µmol; 2 eq), with Cholesterol (4.83 mg, 12.5µmol; 1 eq) and **2** (1.41 mg, 2.5µmol, 0.2 eq) or **4** (1.89 mg, 2.5µmol, 0.2 eq), were homogeneously dissolved in $CHCl_3$. The solvent was evaporated under gentle vacuum for 5h. Next, the lipid film was hydrated for 1 night at 4°C with milli-Q water (DPPC concentration = 12.5 mM). Then, the samples were vortexed resulting in a turbid suspension and sonicated for 1 h until obtaining a clear solution. The composition of the final Liposomal Formulations (LF) is presented in Table S1. The size of the particles was measured by DLS (Table S2).

Liposomal Formulations (LF)	Composition of the formulation; Volume 2 mL
LF1	**BSV36** (25 mM)
LF2	**BSV36** (25 mM), **2** (2.5 mM)
LF3	**BSV36** (25 mM), **4** (2.5 mM)
LF4	**BSV36** (25 mM), **6** (2.5 mM)
LF5	**DPPC** (12.5mM), **Chol** (6.25 mM), **2** (2.5 mM)
LF6	**DPPC** (12.5mM), **Chol** (6.25 mM), **4** (2.5 mM)

Table S1: The composition of the Liposomal Formulations LF1-6.

4-2 Size and zeta measurements

10 µL of the liposomal Formulations LF were diluted in 990 µL of water milli Q and then, charged all the volume into a MALVERN DTS1060 cuvette to register the measurements.

	Size (nm)	PDI	Zeta potential (mV)
LF1	168.7±9	0.24	+58±0.05
LF2	150±25	0.41	+57±0.7
LF3	127±2	0.22	+60±0.5
LF4	219±10	0.35	+63±3
LF5	107±7	0.24	-36±0.6
LF6	138±4	0.26	+57±1

Table S2: Sizes and zeta potentials of Liposomal Formulations (LF).

5- Functionalization of liposomes by Phosphonodithioester-Amine Coupling (PAC) reaction

5-1 PAC reaction on liposomal solution with compound 5.

To a Liposomal Formulation (LF2, LF3, LF5 or LF6; 2 mL) containing 5µmole or 2.5µmole of either **2** or **4** was added the fluorescent probe **5** (4.86 mg, 15 µmol) previously dissolved in 1mL of milli-Q water. The mixture was stirred for 4h at room temperature. After, the samples were dialyzed (2x300 mL of water). The dialysate was changed every 3 hours. The final liposomal solutions were analyzed by UV-Visible and by ESI-MS.

5-2 DLS characterization before and after PAC reaction

	Before reaction with 5			After reaction with 5		
	Size (nm)	PDI	Zeta potential (mV)	Size (nm)	PDI	Zeta potential (mV)
LF2	150±25	0.41	+57±0.7	263±16	0.38	+61±0.4
LF3	127±2	0.22	+60±0.5	193±25	0.32	+79±4
LF5	107±7	0.24	-36±0.6	200±41	0.43	+46±0.3
LF6	138±4	0.26	+57±1	129±3	0.19	+53±1

Table S3: Size and zeta measurements for the liposomal solution LF2, LF3, LF5 and LF6 before and after PAC reaction with **5** and the dialysis step.

5-3 UV-vis characterization after PAC reaction and validation of the dialysis protocol

The fluorescent amine **5** (4.86 mg, 15 µmol) previously dissolved in 1 mL of water was added to the liposomal formulation LF1, LF2 or LF3 (2 mL). The mixture was stirred for 4h at room temperature. Then, the samples were dialyzed (2*300 mL of water). The dialysate was changed every 3 hours. The final liposomal solutions were analyzed by UV-Visible and compare to LF4 (Figure S19).

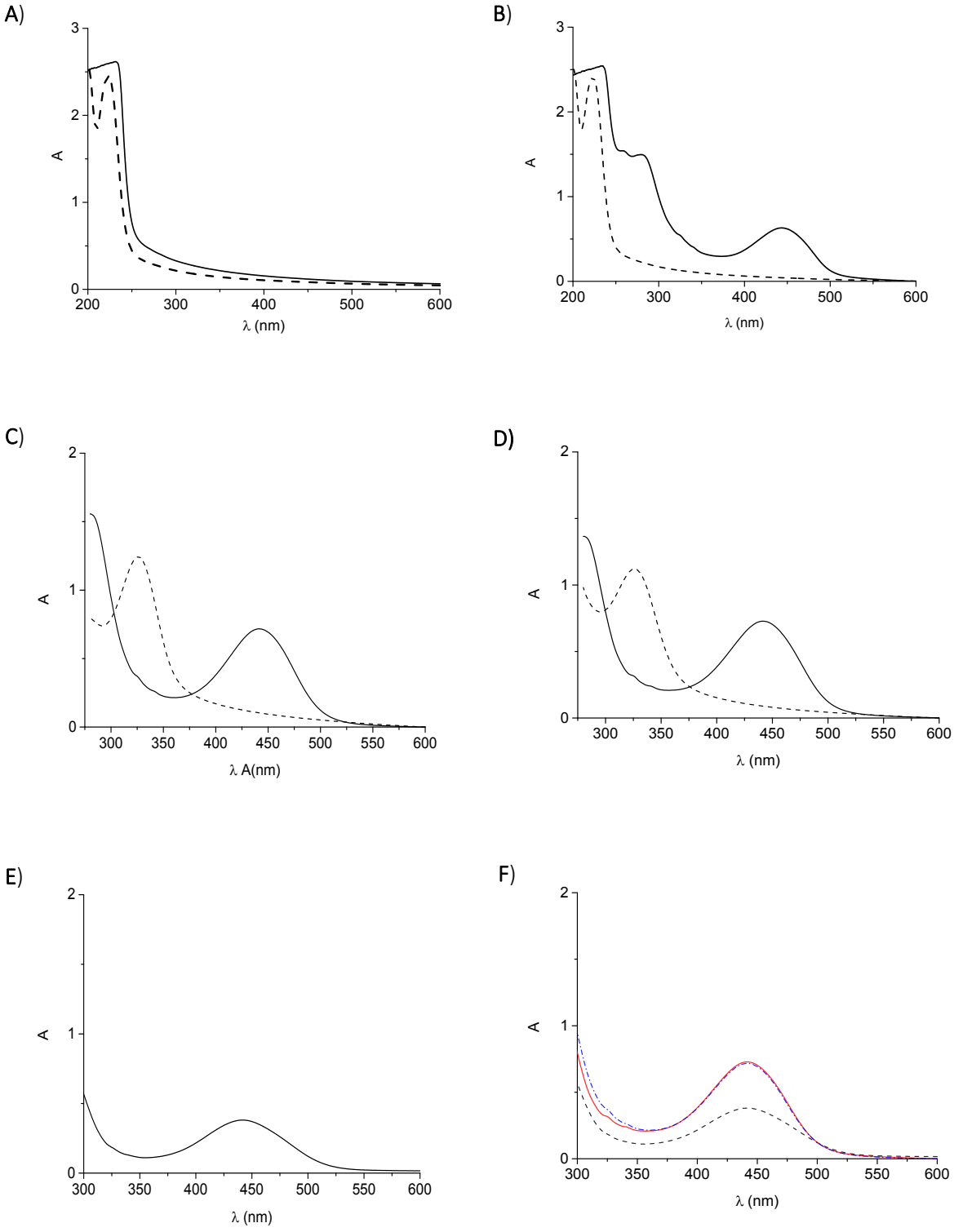


Figure S19: UV-vis spectra of liposomal solutions A) LF1 (solid line), and LF1 treated with **5** and purified by dialysis (dashed line), B) LF2 treated with **5** and purified by dialysis (solid line) and LF1 with the same treatment (dashed line), C) LF2 (dashed line) and LF2 treated with **5** and purified by dialysis (solid line) 1.25×10^{-3}M, D) LF3 (dashed line) and LF3 treated with **5** and purified by dialysis (solid line) 1.25×10^{-3} M,

E) LF4 5x10^{-4}M, F) LF2 (blue dashed line) and LF3 (red solid line) after reaction with **5** and purified by dialysis. LF4 (dark dashed line).

5-4 General protocol for the quantification of the amount of fluorescent probe immobilized at the surface of the liposomes.

After the PAC reaction using **5** as fluorescent amine, **5** recovered in dialysate was quantified by UV-vis leading to determine n(**5** released). Then, the quantity of **5** that reacted with liposomes n(reacted **5**) was calculated with equation (1).

$$n(reacted\ 5) = n(total\ 5) - n(5\ released) \quad (1)$$

n(reacted **5**) : number of mole of **5** immobilized in the liposomal solution
n(total **5**): total number of mole of **5** added for the PAC reaction
n(**5** released): number of mole of **5** recovered during the dialysis step.

The conversion rate of the phosphonodithioester **2** or **4** was then determined by equation (2):

$$\%(conversion) = \frac{n(reacted\ 5)}{n(phosphonodithioester)} * 100 \quad (2)$$

n(reacted **5**) : number of mole of **5** immobilized in the liposomal solution
n(Phosphonodithioester): number of moles of phosphonodithioester (**2** or **4**) included in the liposome solution.

5-5 ESI-MS analyses of liposomal solutions before and after PAC reaction.

The samples studied were dissolved to obtain a final concentration at $1x10^{-5}$ M in water. Eventually a 7:3 mixture of H_2O/MeOH with HCOOH 0.1% v/v was used to improve the intensity of protonated adducts.

Mass spectrometry experiments were carried out using a QqQ Quattro Ultima Platinum mass spectrometer (Micromass, Waters Inc.) equipped with a Z-spray ionization source and operated in the positive ion mode. The solutions were introduced by direct injection and electrosprayed *via* a syringe pump at 75 µL.min^{-1} flow rate. The temperature of the source was set at 90°C, nitrogen was used as desolvation gas at a flow rate of 450 L.h^{-1} and a temperature of 90°C. The experiments were performed with capillary at 3.33 kV and with a sample cone at 35 V. The first and the second quadrupole analyzers were used with Low-Mass (LM) and High-Mass (HM) resolutions set to 16 for all the samples without fluorescent probe and LM=HM=14 with the fluorescent probe. The MassLynx software 4.0 (Waters, Manchester, UK) was used for data acquisition and mass spectra processing.

a) ESI-MS of liposomal solution of BSV36 alone

LF1 was diluted in water to obtain a final concentration at $1x10^{-5}$ M in **BSV36**. The ESI MS of LF1 is depicted on Figure S20.

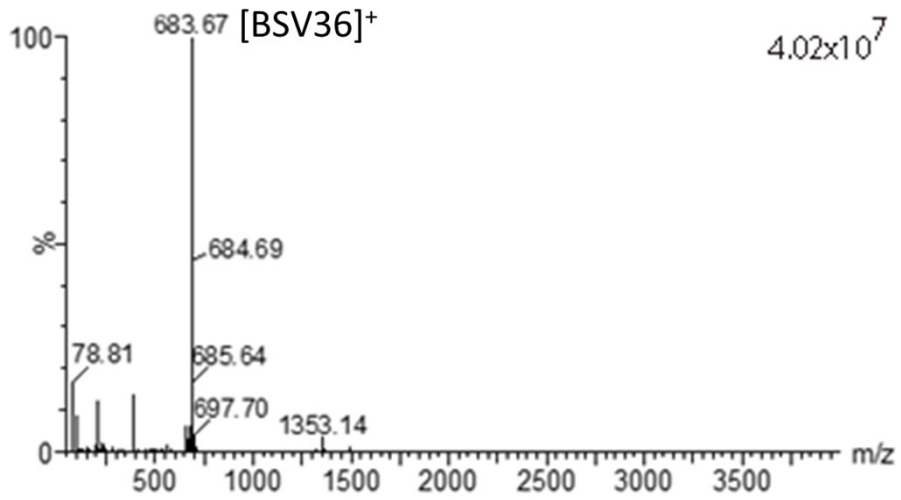


Figure S20: ESI-MS of LF1 diluted in water.

b) Reaction between LF2 and the amine **5**.

LF2 (**BSV36** / **2:** 10 / 1) was functionalized with the fluorescent amine **5** as detailed above. After dialysis, the sample was dissolved in water (final **BSV36** concentration: $1x10^{-5}$ M) and analyzed. ESI MS spectra are presented in Figure 2 and a summary is reported in Table S4.

LF2	**BSV36** m/z 683.87	Dithioester **2** m/z 565.38	Phosphonothioamide **6** m/z 843.74
Before PAC reaction	present	not detectable	absent
After PAC reaction	present	not detectable	present

Table S4: Summary of the ESI-MS spectra (main peaks) before and after the reaction of LF2 with the amine **5** and dialysis.

c) Reaction between LF3 and the amine **5**

LF3 (BSV36/**4**: 10/1) was functionalized with the fluorescent amine **5** as detailed above. After dialysis, the sample was dissolved in water (final concentration in **BSV36**: $1x10^{-5}$ M). ESI MS spectra are presented in Figure 2 and a summary is reported in Table S5.

LF3	**BSV36** m/z 683.87	Dithioester **4** m/z 754.72	Phosphonothioamide **6** m/z 843.74
Before PAC reaction	present	present	absent
After PAC reaction	present	absent	present

Table S5: Summary of the ESI-MS spectra (main peaks) before and after the reaction of LF3 with the amine **5** and dialysis.

d) Reaction of LF5 and LF6 with **5**.

LF5 (DPPC/Chol/**2**: 2/1/0.2) or LF6 (DPPC/Chol/**4**: 2/1/0.2) were functionalized with the fluorescent amine **5** as detailed above. After dialysis, the samples were dissolved in water (final concentration in **DPPC**: 10^{-5} M). ESI MS spectra are presented in Figure S21.

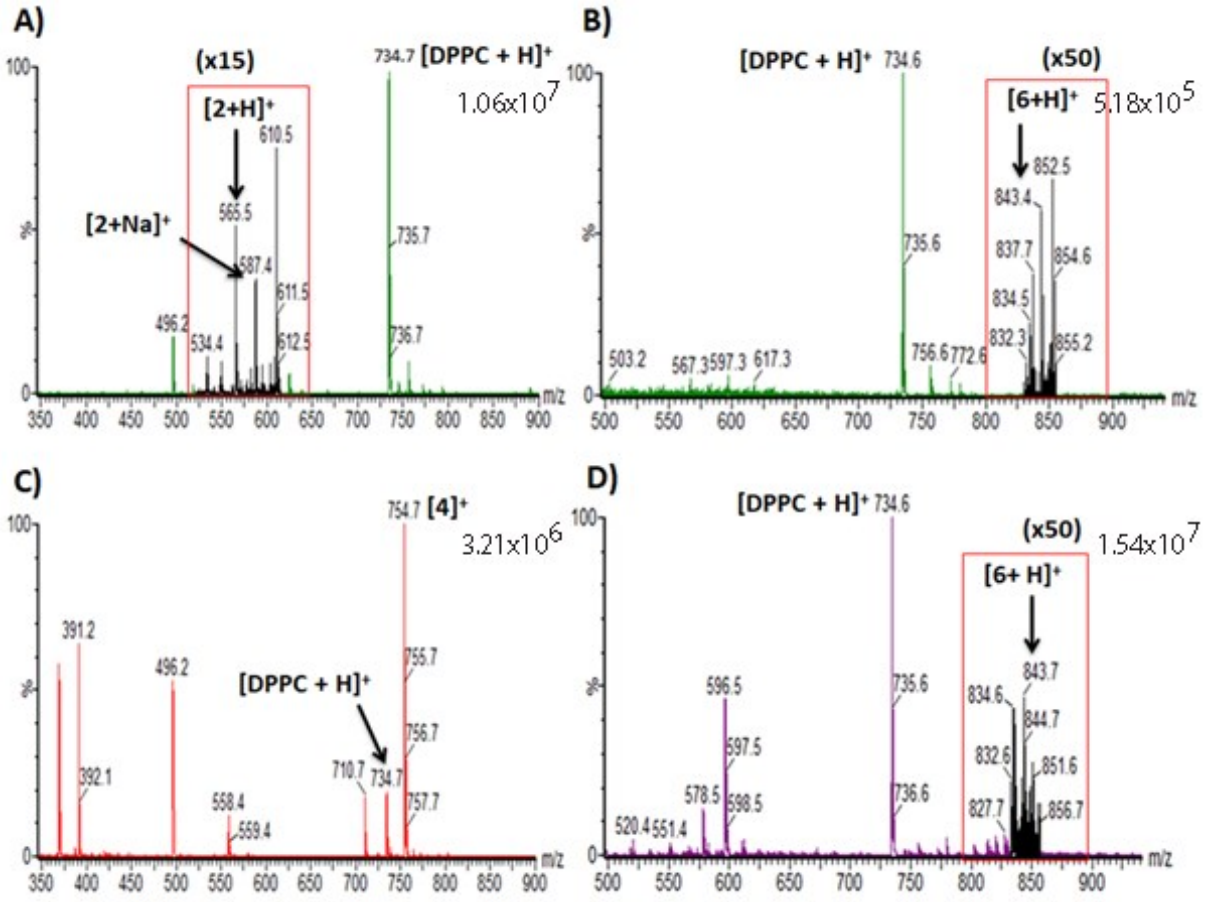


Figure S21: ESI-MS spectra: A) LF5 (DPPC/Chol/**2**: 2:1:0.2) before PAC reaction (the dilution was achieved with water/methanol 7:3), B) LF5 after PAC reaction with **5** and dialysis in water, C) LF6 (DPPC/Chol/**4**: 2:1:0.2) before PAC reaction D) LF6 after PAC reaction with **5** and dialysis in water. The solutions were diluted with water (final concentration in **DPPC**: $1x10^{-5}$M). The red boxes correspond to a magnification factor of 50.

e) Reaction between LF6 and the PEG-amine **7**

LF6 (DPPC / CHOL/**4**: 2/1/0.2) reacted with PEG-amine **7** following the general protocol (In this protocol compound **5** was replaced by the PEG-amine **7**). After dialysis, the sample was dissolved in water (final concentration in **DPPC**: $1x10^{-5}$M) and analyzed by ESI-MS (Figure S22).

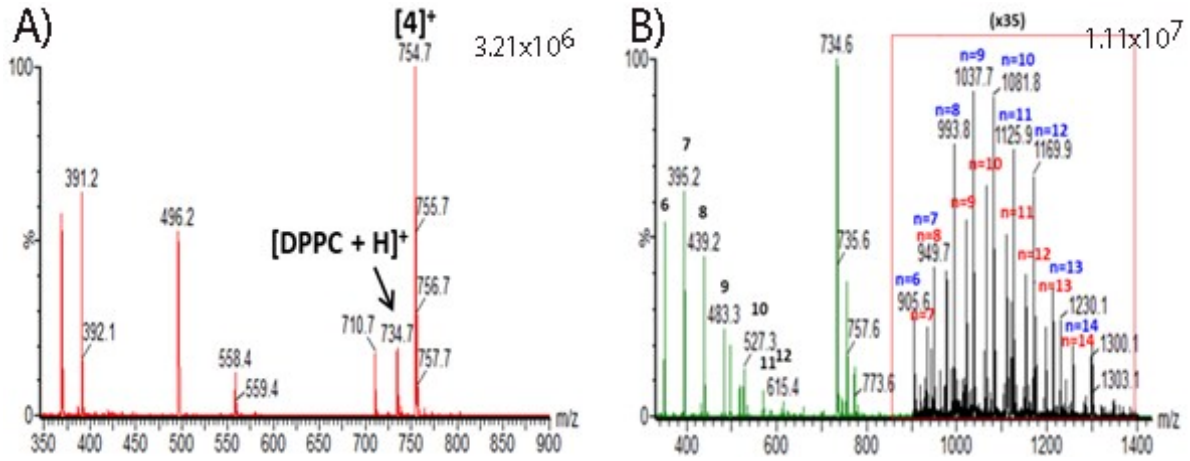


Figure S22: ESI MS spectra of: A) LF6 (DPPC/CHOL/**4**), B) LF6 after the addition of **7** and dialysis. The solutions were diluted with water (final concentration in **DPPC**: $1x10^{-5}$M). The red box corresponds to a magnification factor of 35.

6- Determination of the life time and the quenching of fluorescent experiments.

Fluorescence was recorded on a Spex Fluorolog-2 (mod. F-111) spectrofluorimeter. Fluorescence life times were recorded with the same fluorimeter equipped with a TCSPC Triple illuminator.

The samples were irradiated by a pulsed diode excitation source Nanoled at 455 nm. The kinetics were monitored at 530 nm and each solution itself was used to register the prompt at 455 nm except for solution of **5** for which ethanol was used as prompt. The system allowed measurement of fluorescence lifetimes from 200 ps. The multiexponential fit of the fluorescence decay was obtained using the following equation:

$$I(t) = \Sigma\alpha_i \exp(^{-t/\tau i})$$

6-1 Life time determination

In this part, two formulations were prepared at the concentration of 10 mM: 1) LF2 after the PAC reaction with **5** (Liposomal formulation by post-functionalization). 2) LF4 including BSV36 and the phosphonothioamide **6** in a 10/1 molar ratio (Liposomal formulation by pre-functionalization).

Then, the free fluorescent probe, LF2 and LF4 were diluted at the same concentration (0.75 mM related to BSV36) and placed in a cuvette (volume of 2mL). The samples were illuminated by a pulsed diode excitation source Nanoled at 455 nm, followed by measuring the emission intensity against time at 530

nm and the prompt was registered at 455 nm for each solution. The fluorescence life times obtained are summarized in (Table S6).

Samples	τ1 (ns)	% of contribution	τ2 (ns)	% of contribution	χ^2
Free probe 5	4.42				3.41
LF2+5	1,12	44%	4.82	56%	1.63
LF4	1.06	60%	3.57	40%	1.81

Table S6: The life times of free probe, LF2 +5 and LF4

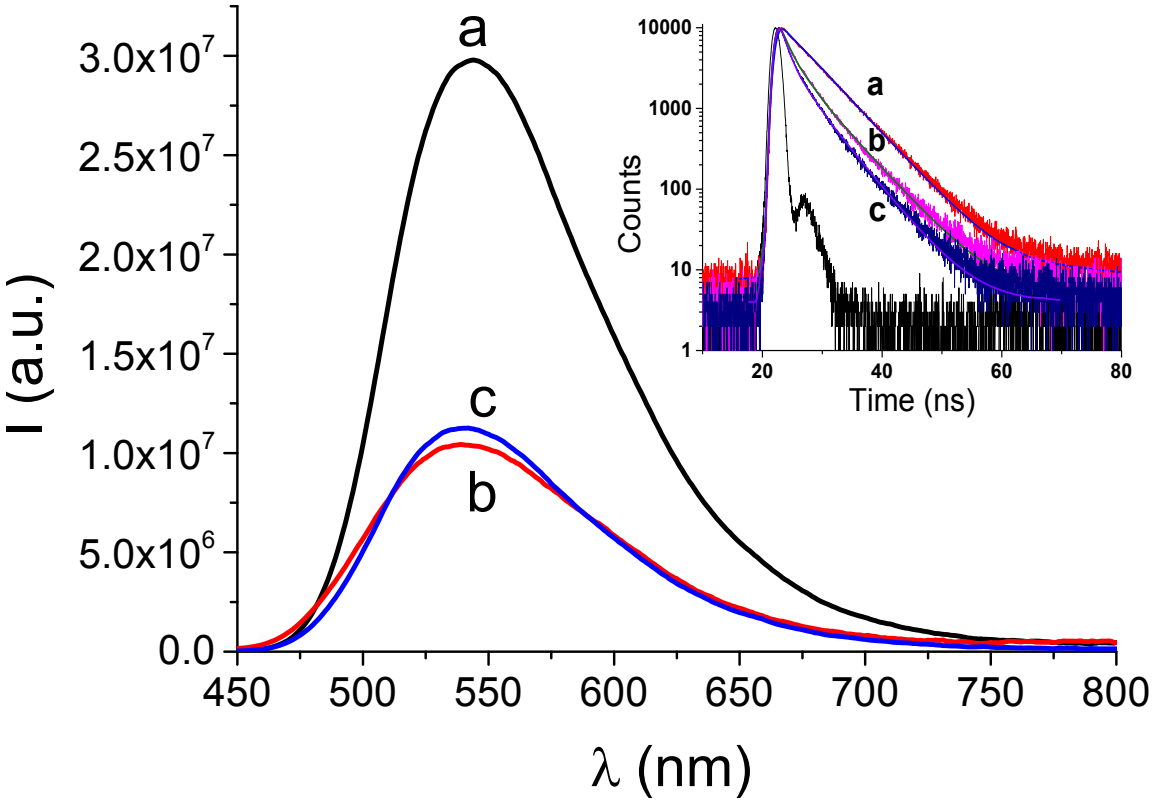


Figure S23: Fluorescence emission of optically matched aqueous solutions of **5** (a), **LF 4** (b) and **LF2+5** (c), λexc = 430 nm. The inset shows the fluorescence decay and the related fitting of the same solution recorded at λexc = 455 nm and λem = 530 nm.

6-2 Quenching of fluorescence

Aliquots of I^- were added to the 3 solutions (free probe in water, **LF2 +5** and **LF4**) at 0.75 mM of BSV36. The concentration of [I^-] was increased after every measurement.

7- Kinetics model

The kinetics of the Phosphonodithioester-Amine Coupling (PAC) reaction was determined for the reaction between LF2 (**BSV36** (25 mM), **2** (2.5 mM)) and the amine **5** (1 equivalent irrespective to the phosphonodithioester **2** present in LF2).

The chemical equation for the reaction between **2** (**B**) and **5** (**C**) leading to the coupling product **6** (**D**) may be written as:

	B	+	C	➔	D
t = 0	βn_C		n_C		0
t	$n_C(\beta-\xi)$		$n_C(1-\xi)$		$n_C\,\xi$
t→ +∞	$n_C(\beta-1)$		0		n_C

With: $\beta = \frac{[B](t=0)}{[C](t=0)} = 1$ the ratio of reactant concentrations at the starting of reaction,

$0 \leq \xi \leq 1$ the fraction of C compounds that have reacted,

$n_C = n_C(t=0)$ the initial molar amount of C.

We anticipate the studied reaction to follow a second-order rate law, as it can reasonably be thought of as a bimolecular elementary reaction process. One may thus write:

$$\frac{d[C](t)}{dt} = -k[B](t)[C](t) = -k[C]^2(t)$$

that leads to:

$$C_0\frac{d(1-\xi(t))}{dt} = -kC_0^2(1-\xi(t))^2 \Rightarrow \frac{d\xi(t)}{dt} = kC_0(1-\xi(t))^2$$

$$\frac{d\xi(t)}{(1-\xi(t))^2} = kC_0dt$$

which, after integration on a time-interval [0, t], becomes:

$$\frac{1}{1-\xi(t)} = 1 - kC_0t \quad \text{or} \quad \xi(t) = 1 - \frac{1}{1-kC_0t} (Eq.1)$$

7-1 Spectroscopic analysis:

UV-visible spectrum was obtained on a large range of wavelengths to identify the working conditions for the kinetic analysis.

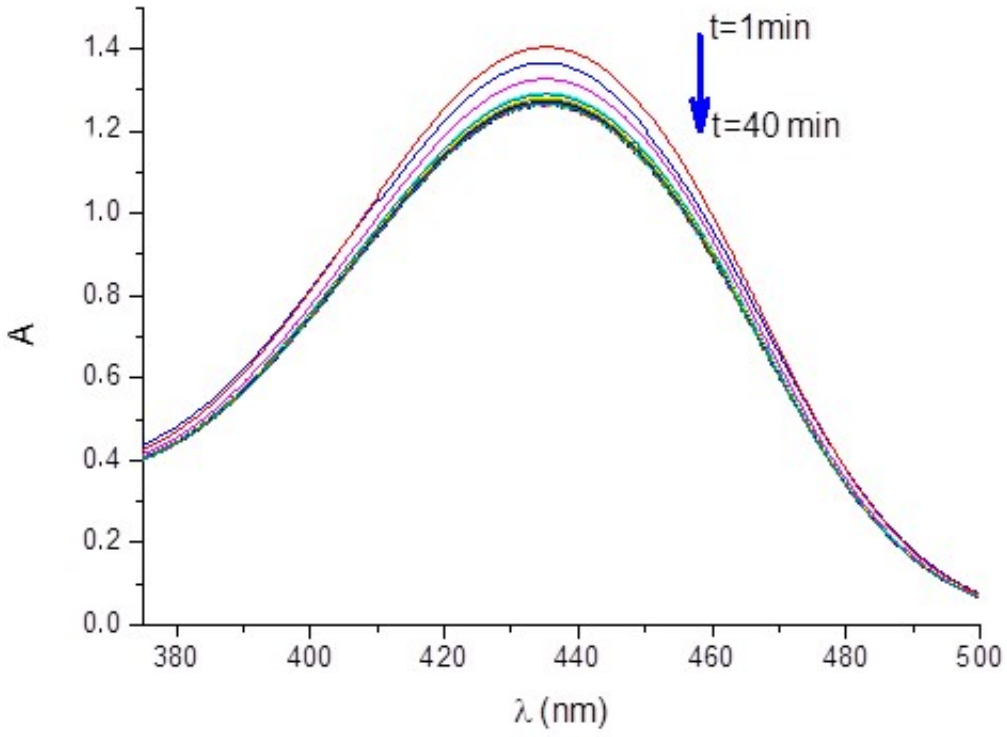


Figure S24: Evolution of the UV-vis spectrum (370-500 nm) during the reaction of LF2 with **5**.

According to the spectrum above, the maximum and most specific wavelength for the absorbance of fluorescent probe C is determined at λ=436 nm. Generally, the absorbance of a compound C may be written as a function of its concentration:

$$A_C(t,\lambda) = \epsilon_C(\lambda,[C](t)).[C](t)$$

with $\epsilon_C(\lambda,[C](t))$ the molar attenuation coefficient for C depending both on concentration and wavelength only (all other experimental parameters were maintained constant: e.g., T=25°C, unique vial). According to the Beer-Lambert law, a calibration curve was measured at λ=436 nm. The value of the molar attenuation coefficient ε_c= 12292 +/- 80 $M^{-1}cm^{-1}$ was obtained by linear fitting on the range of concentrations studied [0; C_0] (solution in ultra-pure water) and uncertainty determined by using t-distribution (12 degrees of freedom, 98% confidence). This value is consistent with the value of the literature (ε= 12800 $M^{-1}cm^{-1}$at 437 nm).[4]

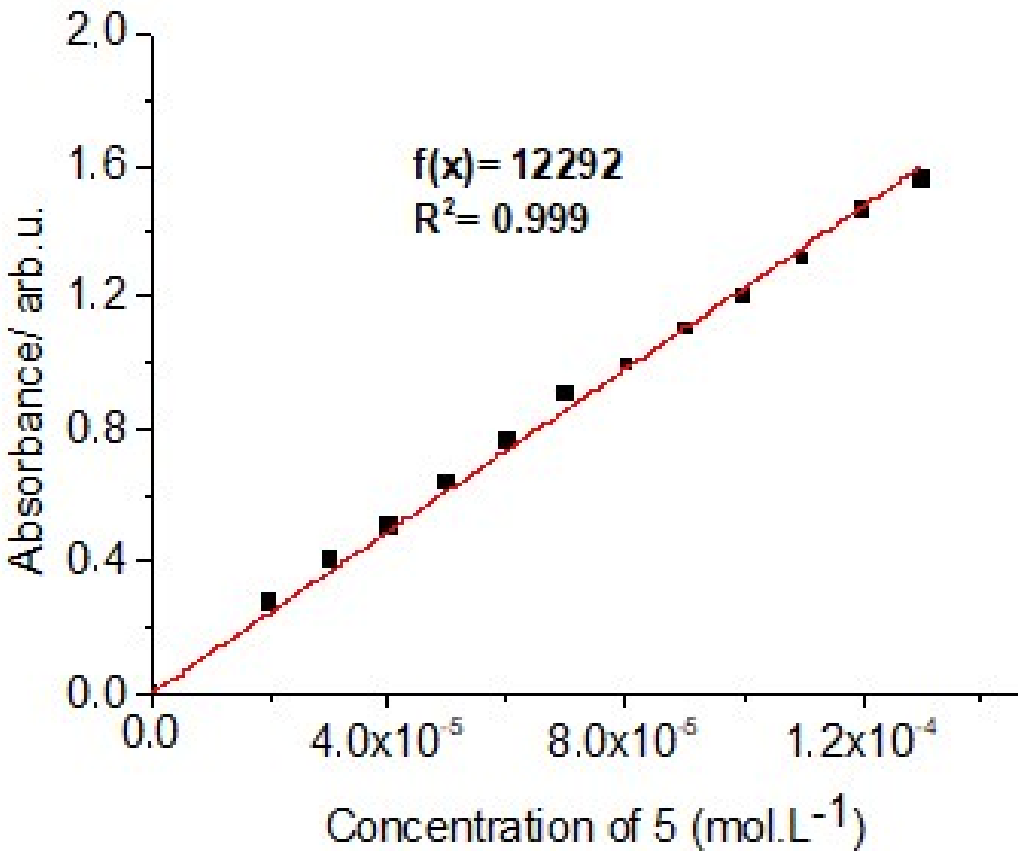


Figure S25: Calibration curves to determine the molar attenuation coefficient ε of 5.

However, it appears that both the reactant C and product D absorb the radiation at this wavelength, although with different efficiencies. The absorbance $A(\lambda = 436nm,t) = A_{436}(t)$ must then be written as a sum of contributions from the two components:

$$A_{436}(t) = \epsilon_C.[C](t) + \epsilon_D.[D](t) \qquad (Eq.2)$$

7-2 Time-evolution of absorbance:

By introducing ξ(t), the fraction of compounds that have reacted, one obtains the time-evolution of absorbance:

$$A_{436}(t) = \epsilon_C \frac{(1-\xi(t))n_C}{V} + \epsilon_D \frac{\xi(t)n_C}{V}$$

after reorganizing:

$$A_{436}(t) = (\epsilon_D - \epsilon_C)\xi(t)C_0 + \epsilon_C C_0$$

or:

$$A_{436}(t) = a\xi(t) + b \qquad (Eq.3)$$

with $C_0 = n_C/V$ the initial concentration of compound C and, hypothesizing ϵ_C and ϵ_D are constant on the range of concentrations of interest, $a = (\epsilon_D - \epsilon_C)C_0$ and $b = \epsilon_C C_0$ must be constants as well.

by injecting (Eq. 1) into (Eq. 3), this leads to the following three-parameters expression:

$$A_{436}(t) = \frac{-a}{1 - kC_0 t} + (b + a)(Eq.4)$$

with $a = (\epsilon_D - \epsilon_C)C_0$, $b = \epsilon_C C_0$ and k the rate constant to be determined.

Non-linear fitting of time-dependent spectroscopic data:

A mixture of reactants was prepared at $C_0 = 8.3x10^{-5}M$ and submitted to UV-visible spectroscopic analysis. Data were collected at λ=436 nm every 20 seconds during 3000 seconds and plotted with the LibreOffice 7.0 suite of programs.

The 3-parameter function (Eq.4) was used as a fitting function to model the kinetic of reaction and to extract an estimate of the rate constant k. To this end, the values of the 3 parameters a, b and k were necessary to be determined simultaneously accounting for the unknowns ϵ_D and k, as well as inaccuracies in the values of ϵ_C and C_0. The solver algorithm (intrinsic function of the LibreOffice program) was used to minimize the sum of squared of residuals between experimental data and (Eq. 4).

The asymptotic behavior of A_{436} toward large time was obtained by averaging the absorbance measured during the last 400 seconds and, the a and b parameters determined according to the reasoning below:

$$\lim_{t\to\infty} A_{436}(t) = \lim_{t\to\infty}\left(\frac{-a}{1 - kC_0 t} + (b + a)\right) = (a + b) = \frac{\sum_{i=(N-19)}^{N} A_{436}(t_i)}{20}$$
$$a = A_{436}(t\to +\infty) - \epsilon_C C_0$$

Due to the initial inhomogeneity of the reaction pot, the reaction was considered to start with 30 seconds delay (experimental data were then shifted by 30 seconds). Experimental data were then fitted (see Figure 7-3 below) leaving the following variables to be modified with bracketed values to account for experimental inaccuracies: k > 0, $6x10^{-5}$M < C_0 < $1x10^{-4}$ M and $15\,000 < \epsilon_C < 20\,000$ M^{-1}.

With the conditions listed above, the second order rate law lead to k = 842 $M^{-1}.s^{-1}$. Optimal parameters were found at: C_0 = $6.0x10^{-5}$M and $\epsilon_C = 15\,000$ M^{-1} with the sum of residuals $5x10^{-3}$ with 147 datapoints used and a maximum of 2.5% absolute deviation from experimental data (at t=50 s on the modified kinetic data).

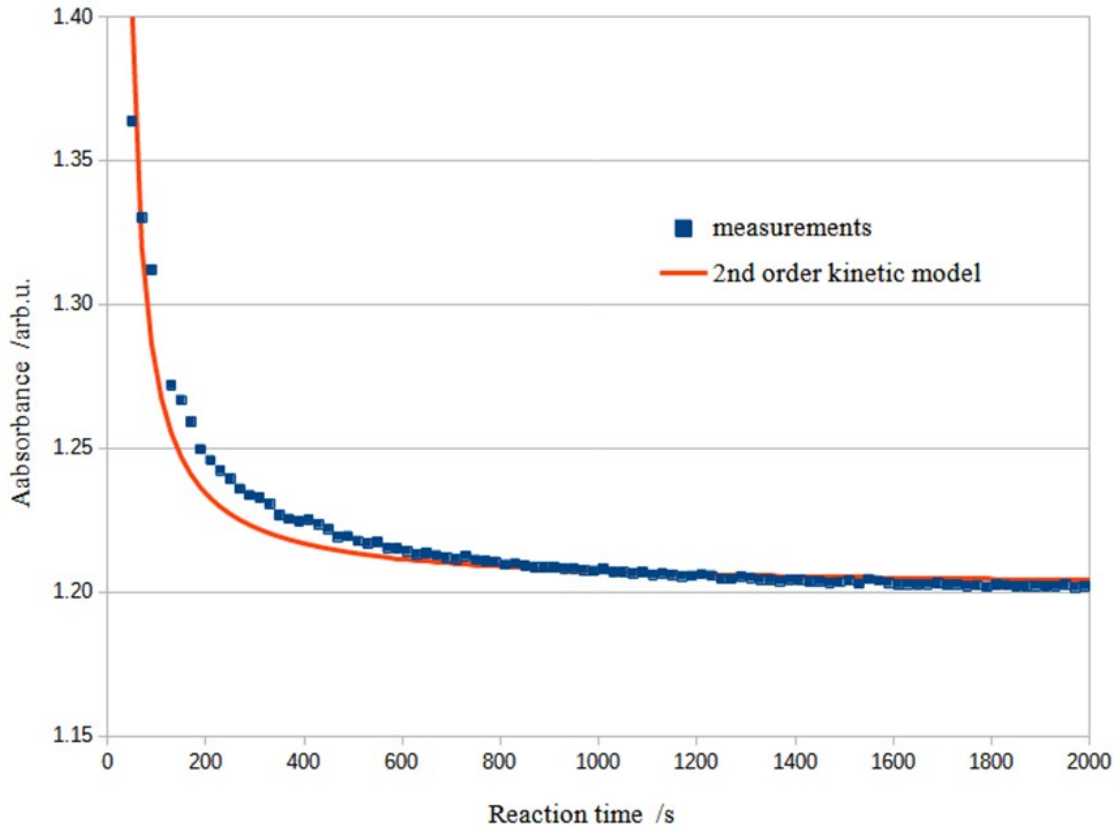


Figure S26: spectroscopic data points (blue squares) and 2nd order rate law fitting model (red curve).

A test of robustness of the model was done to evaluate a reasonable range of variation of the rate constant according to possible inaccuracies in the initial conditions or values of parameters. "**Experimental**" corresponds to data obtained based on the hypothesis of exact measurements, while "**Best fit**" does not assume any constrain."**Initial**" accounts for the absorbance measured initially (the absorbance of the probe is modified within the reaction mixture). "**Chosen fit**" reports the compromise made between experimental data and inaccuracies (transient mixing, attenuation coefficient in the mixture, preparation of the mixture).

		Experimental	Best fit	Initial	Chosen fit
Rate constant k	$/M^{-1}.s^{-1}$	1 150	945	766	842
Attenuat. coefficient ε_c	$/M^{-1}$	12 292 (calibration)	11 565	15 500 (initial)	15 000
Initial concentration	/M	$8.3x10^{-5}$ (prepared)	$6.0x10^{-5}$	$5.4x10^{-5}$	$6.0x10^{-5}$
Initial time shift	/s	0	49.1	55.9	30
Sum of residuals		$2.8x10^{-2}$	$1.7x10^{-3}$	$1.7x10^{-3}$	$5.0x10^{-3}$

8- Cryo-TEM

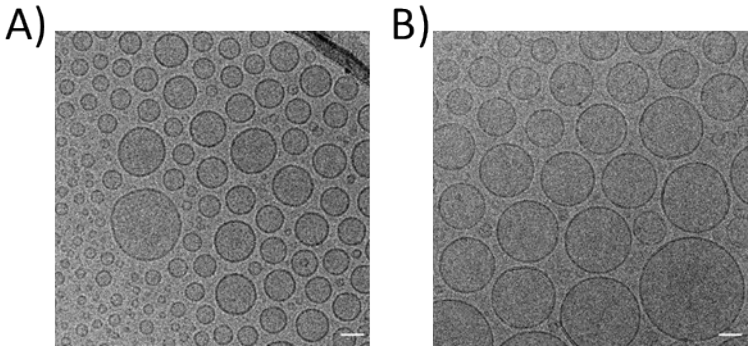


Figure S27: Cryo-TEM analysis of A) LF1 (BSV36 alone) (25 mM), B) LF2 (**BSV36/2** 10/1 molar ratio) after PAC reaction with $PEG_{600}NH_2/N_3$ **7** and dialysis. Scale bar: 50 nm

9- PAC reaction with PEG

b) PAC reaction with $PEG_{5000}NH_2$

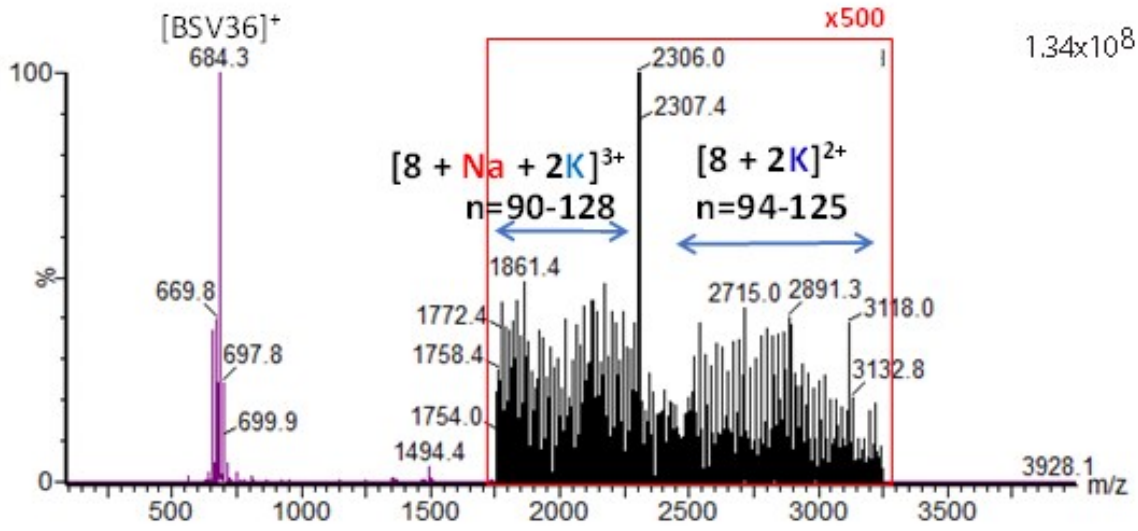


Figure S28: ESI-MS analysis of LF3 after PAC reaction with $PEG_{5000}NH_2$ and dialysis. The red box corresponds to a magnification factor of 500.

b) PAC reaction with $PEG_{600}NH_2/N_3$ **7**.

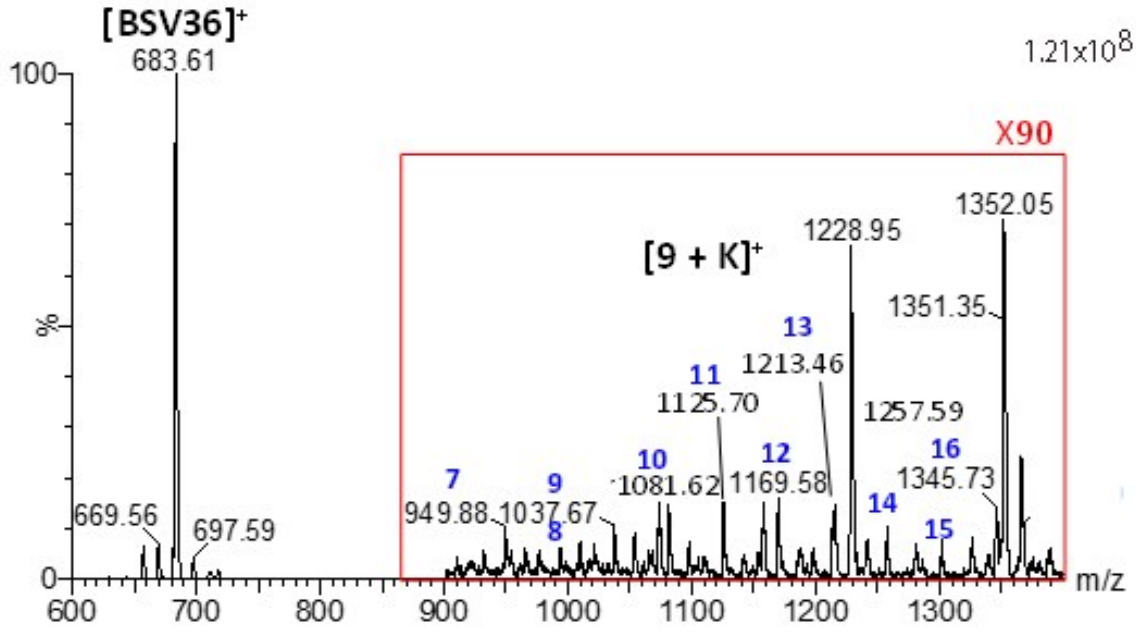
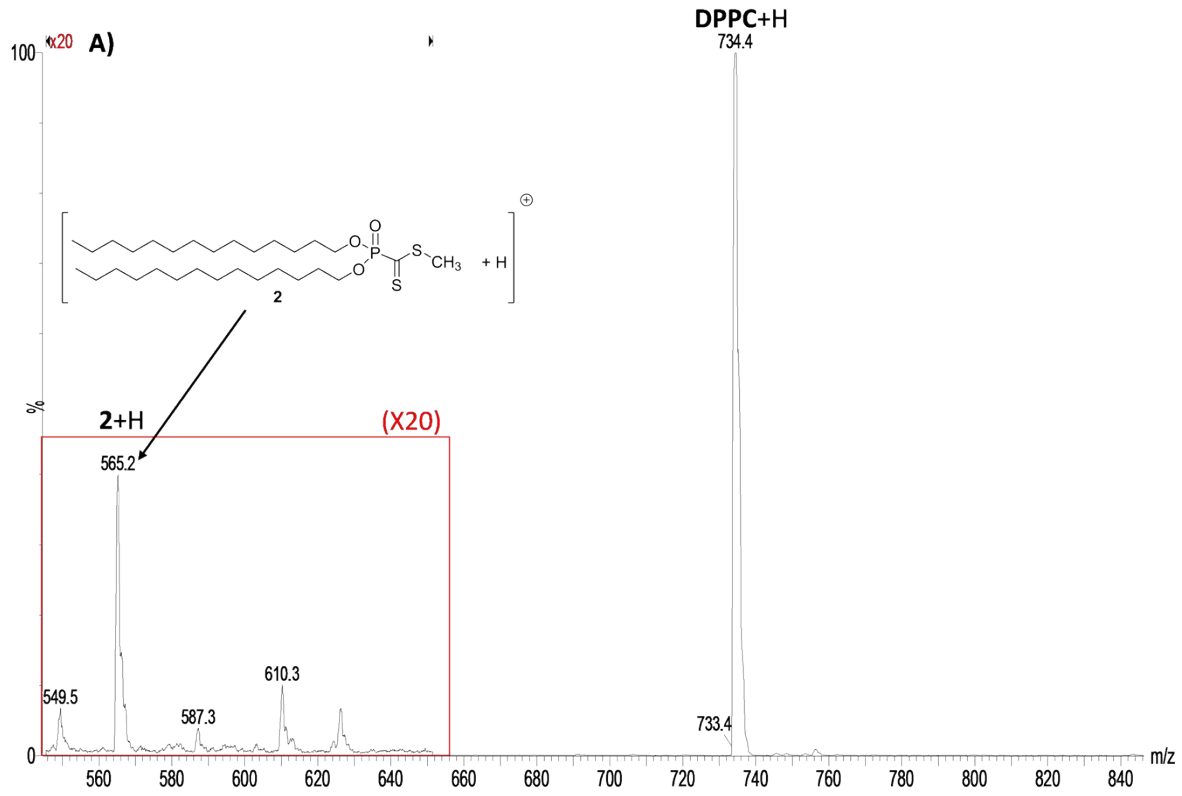


Figure S29: ESI-MS analysis of LF3 after PAC reaction with **7** and dialysis. The red box corresponds to a magnification factor of 90.

10- PAC reaction protocol without dialysis

LF5 (DPPC/Chol/**2**: 2/1/0.2) 1 mL ([dppc]= 12.5 mol/L) was functionalized with 0.5 equivalent of the fluorescent amine **5** (6.25×10^{-4} mol) at 40°C for 30 min. Then, the functionalized solution was quenched with an excess of 2-aminoethanol (7.4 µL, 125 mmol) and reacted for an additional 30 min. Then, the liposomal solution was analyzed by ESI-MS. Formic acid was added in 0.1% in volume for the analysis in order to facilitate the protonation of molecular species. The measurement was recorded after diluting an aliquot of 10µL of the solution with a 0.1% of HCOOH concentrated in a total volume of water equal to 1 mL. ESI MS spectra of LF5 before PAC reaction (Figure S29A) and after PAC reaction with **5** and dialysis (Figure S29B) are presented below.



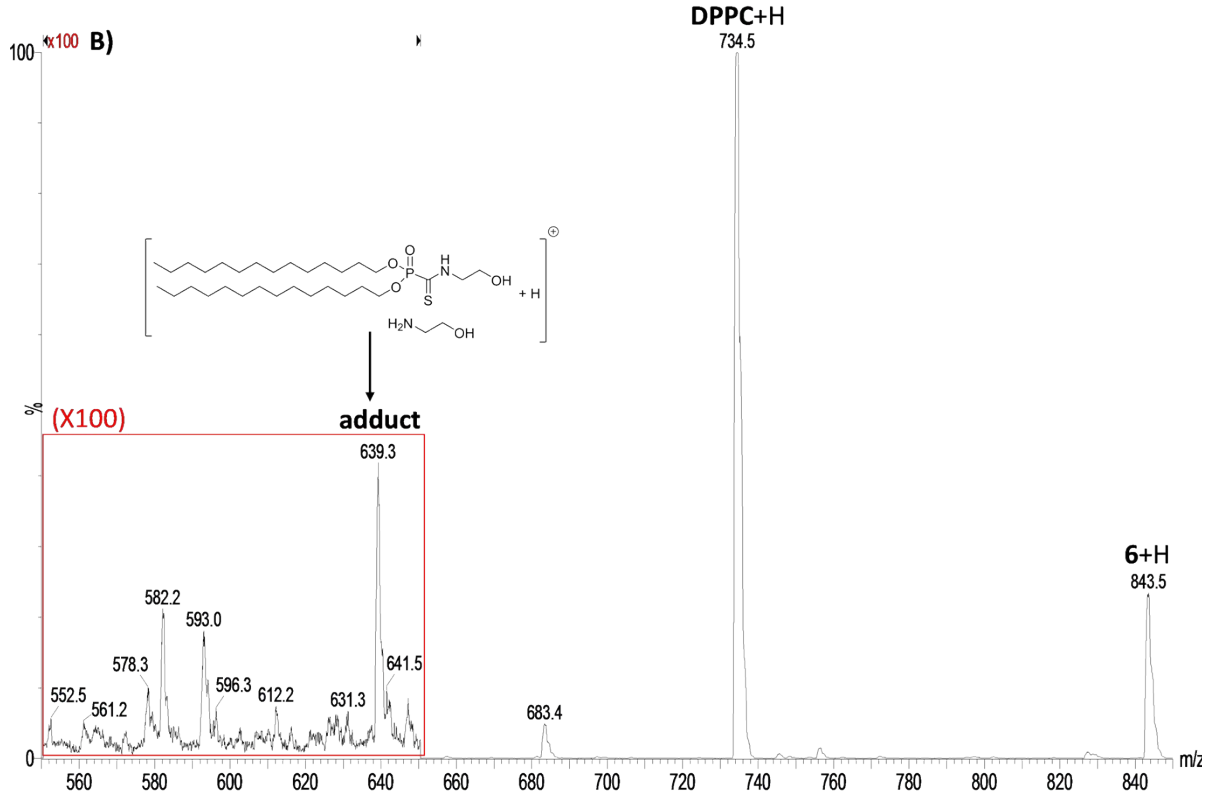


Figure S30. ESI-MS analysis of A) **LF5** before PAC reaction, B) **LF5** after PAC reaction with **5** and with an excess of 2-aminoethanol without dialysis. The red box corresponds to a magnification factor of either 20 or 100.