Fluorocarbon Oligonucleotide Conjugates for Nucleic Acids Delivery

Supporting information

Guilhem Godeau,[†,¶,] Hélène Arnion, [†,¶,] Christophe Brun[a,b], Cathy Staedel[†,¶,] and Philippe Barthélémy[†,¶,*]

[†]Inserm, U869, Bordeaux, F-33076 France. [¶]Université Victor Segalen Bordeaux 2, F-33076, Bordeaux, France

philippe.barthelemy@inserm.fr

Contents of supporting information :

I. Experimental section

II. Synthesis

III. Additional data (^{31}P NMR from compound **2**; HPLC profile for compound **4**, **5a** and **5b**; HRMS for compound **5a** and **5b)**

I. Experimental section

Materials. Instruments.

Unless noted otherwise, all starting materials were obtained from commercial suppliers and were used without further purification. The solvents were commercial dry quality. All compounds were characterized using standard analytical and spectroscopic data such as ^{1}H, ^{13}C and ^{31}P NMR spectroscopy (apparatus BRUKER Advance DPX-300, ^{1}H at 300.13 MHz, ^{13}C at 75.46 MHz and ^{31}P at 121.49 MHz) and mass spectrometry (Instrument JEOL SX 102, NBA matrix). The NMR chemical shifts are reported in ppm relative to tetramethylsilane using the deuterium signal of the solvent ($CDCl_3$ or DMSO-d6) as a heteronuclear reference for ^{1}H, ^{13}C, ^{31}P. The 1H NMR coupling constant J are reported in Hz. Merck RP-18 F254S plates were used for analytical thin layer chromatography. Silica gel 60 (particule size: 40 – 60 μm) was employed for flash chromatography. MASHEREY – NAGEL prepacked HPLC column cc 250-4 Nucleosil 120-5 C4 and cc 250-4.6 nucleodur 100-5 C18 ec were used for analytic and preparative HPLC. The oligonucleotide quantifications by UV-absorbance at 260 nm were performed on a NanoDrop® ND-1000 spectrophotometer. The microwave assisted reactions were carried out using a CEM discover apparatus.

Abbreviation: DCM, methylene chloride; DCU, dicyclohexylurea; DIEA, diisopropyl-ethylamine; DMF, dimethylformamide; FON, Fluorocarbon oligonucleotide; ONA, oligonucleotide amphiphile; TEA, triethylamine; THF, tetrahydrofurane; DNA, deoxyribonucleic acid; RNA, ribonucleic acid.

HPLC proceed for FONs purification

The FONs were purified on RP-HPLC using column cc 250-4 Nucleosil 120-5 C4. The elution gradient was 0% to 80% of solvent B in A with 30 minutes of elution.

Solvent A: Triethylammonium acetate Buffer 0.1 M with 5% of acetonitrile

Solvent B: 20% of solvent A with 80% of acetonitrile

Cell culture

All culture reagents were purchased from Invitrogen.

Huh-7 (human hepatoma): cells were grown in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine and 1% non-essential amino acids, at 37 °C in a 5% CO_2 atmosphere. 10^5 cells per well were seeded into a 24-well plate.

NCI: cells were grown in RPMI supplemented with 10% fetal calf serum, 2 mM L-glutamine and 1% non-essential amino acids, at 37 °C in a 5% CO_2 atmosphere. 3.10^5 cells per well were seeded into a 24-well plate.

Hek: cells were grown in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine and 1% non-essential amino acids, at 37 °C in a 5% CO_2 atmosphere. 10^5 cells per well were seeded into a 24-well plate.

Cell survival

2.10^3 Huh7 cells per well were seeded into a 96-well plate and incubated the following day with increasing concentrations of FON^{17F}* in complete growth medium. After 4 days in the continuous presence of the FON^{17F}*, the living cells were quantified by the colorimetric CellTiter Aqueous One Solution Cell Proliferation Assay (Promega), as recommended.

Flow Cytometry

Huh-7: 10^5 cells per well were seeded into a 24-well plate. The following day, they were incubated for 4 or 24 h with 0.1 or 0.5 µM fluorescein-labeled ON or FON^{17F}* in complete growth medium. The cell layers were then rinsed once with PBS and treated for 1 min at 37 °C with 100 µL of 0.05 % (*w/v*) trypsin to detach the cells, which were then suspended in 400 µL of ice cold PBS. The percentage of fluorescent cells as well as the mean fluorescent intensity was analyzed in a COULTER EPICS® XL flow cytometer.

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Fluorescence microscopy

Huh-7: 10^5 cells per well were seeded into a 24-Labteck®. The following day, they were incubated for 4 and 24 h with 0.5 µM fluorescein-labeled ON or FON^{17F*} in complete growth medium. The cell layers were then rinsed once with PBS, fixed in 4 % formaldehyde in PBS, washed three time with PBS, treated 5 minutes by Dapi (1 µg/ml in PBS) and finally washed once with PBS. The cells are then mounted on glass slides slow fade (invitrogene). The slides were observed with an Axiovert 200 fluorescent microscope (Zeiss) and confocal microscope (Leica SP5).

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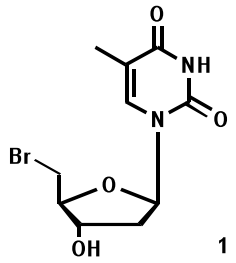
Syntheses:

5'-Bromo-5'-deoxythymidine (1)

Dried pyridine (40 mL) was added to thymidine (1 g, 4.1 mmoles). The mixture was cooled to 0 °C and methanesulfonyl chloride (496.5 mg, 1.05 eq, 4.3 mmoles) was added drop wise. The mixture was stirred at room temperature for 4 hours. The solvent was evaporated under reduced pressure and the residual compound was used directly without further purification in the following step.

DMF (80 mL) and potassium bromide (2 g, 16.4 mmol, 4 eq) were added. The reaction mixture was maintained at 80 °C under stirring for 4 hours. The DMF was removed under reduced pressure. The residual solid was dissolved in 100 mL of ethyl acetate and was washed successively twice with $NaHCO_3$ 5% in water (20 mL) and once with brine (20 mL). The organic phase was dried on Na_2SO_4 and evaporated under reduced pressure. 820 mg of product was isolated after precipitation (methanol/acetonitrile), (Yield: 65%).

R_f: 0.58 (ethyl acetate/methanol, 9/1).



1

NMR ^1H (300.13 MHz, DMSO d_6) : 1.79 (s, 3H), 2.10 (m, 1H), 2.11 (m, 1H), 2.25 (m, 1H), 3.75 (m, 2H), 3.92 (s, 1H), 4.23 (s, 1H), 5.50 (s, 1H), 6.23 (t, J = 3 Hz, 1H), 7.52 (s, 1H), 11.36 (s, 1H).

NMR ^{13}C (75.46 MHz, DMSO d_6): 12.7, 34.2, 38.4, 45.2, 71.6, 72.4, 84.3, 85.6, 85.8, 110.3, 136.5, 150.9, 164.1.

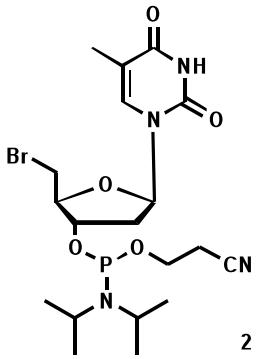
HRMS $[M+Na]^+$: Calcd for 326.9956, found 326.9941

5'-Bromo-5'-deoxy-3'-O(2-cyanoethoxy(diisopropylamino)-phosphino)thymidine (2)

Under argon, freshly distillated DIEA (381 mg, 2.94 mmol, 2 eq), 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite (523 mg, 2.2 mmol, 1.5 eq) were added to 5'-Bromo-5'-deoxythymidine **(1)** (450 mg, 1.47 mmol) in anhydrous 30 mL of DCM. The reaction mixture was maintained at room temperature under stirring for 6 hours. The mixture was successively washed by $NaHCO_3$ 5 % in water (2 X 5 mL) and brine (5 mL). The organic layer was then dried on $NaSO_4$ and evaporated under reduce pressure.

524 mg of product was isolated after chromatography (Hexane/ethyl acetate 15/85), (Yield: 70 %).

R_f: 0.7 (ethyl acetate/methanol 3/7)



2

NMR ^{31}P (121.49 MHz, $CDCl_3$): 152.3

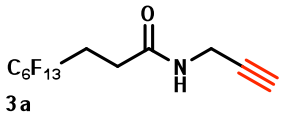
HRMS $[M+H]^+$: Calcd for 527.1035, found 527.10

N-propargyl-1H,1H,2H,2H-perfluorononoyl amide (3a)

Anhydrous DCM (10 mL) was added under nitrogen to 1H,1H,2H,2H-perfluorononoic acid (200 mg, 0.5 mmol, 1 eq). The DCC (206 mg, 1 mmol, 2 eq) and DMAP (122 mg, 1 mmol, 2 eq) were added. Propargyl amine (55 mg, 1 mmol, 2 eq) was then added at room temperature. The reaction was stirred over the night. The mixter was then filtred to remove the DCU. 50 mL of DCM were added and the mixture was washed successively twice with saturated $NaHCO_3$ aqueous solution (20 mL) and once with brine (20 mL). The organic phase was

dried on Na_2SO_4 and evaporated under reduced pressure. 0.2 g of product was isolated after chromatography (hexane/ethyl acetate, starting from 8/2), (Yield: 93 %).

R_f: 0.2 (hexane/ethyl acetate, 8/2).



3a

NMR ^1H (300.13 MHz, $CDCl_3$): 2.24 (s, 1H), 2.52 (s, 4H), 4.07 (s, 2H).

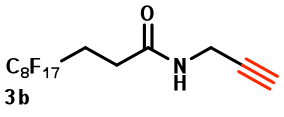
NMR ^{13}C (75.46 MHz, $CDCl_3$): 24.9, 29.3, 71.6, 79.2, 169.7.

HRMS $[MH]^+$: Calcd for 452.0296, found 452,0277

N-propargyl-1H,1H,2H,2H-perfluoroundecanoyl amide (3b)

Anhydrous DCM (40 mL) was added under nitrogen to 1H,1H,2H,2H-perfluoroundecanoic acid (1 g, 2 mmol, 1 eq). The DCC (825 mg, 4 mmol, 2 eq) and DMAP (489 mg, 4 mmol, 2 eq) were added. Propargyl amine (220 mg, 4 mmol, 2 eq) was then added at room temperature. The reaction was stirred over the night. The mixter was then filtred to remove the DCU. 50 mL of DCM were added and the mixture was washed successively twice with saturated $NaHCO_3$ aqueous solution (20 mL) and once with brine (20 mL). The organic phase was dried on Na_2SO_4 and evaporated under reduced pressure. 0.89 g of product was isolated after chromatography (hexane/ethyl acetate, starting from 9/1 to 8/2), (Yield: 84 %).

R_f: 0.33 (hexane/ethyl acetate, 8/2).



3b

NMR ^1H (300.13 MHz, $CDCl_3$): 2.24 (s, 1H), 2.52 (s, 4H), 4.07 (s, 2H).

NMR ^{13}C (75.46 MHz, $CDCl_3$): 26.8, 29.5, 71.9, 79.0, 169.5.

TOF MS ES+ $[M+Na]^+$: 552.00

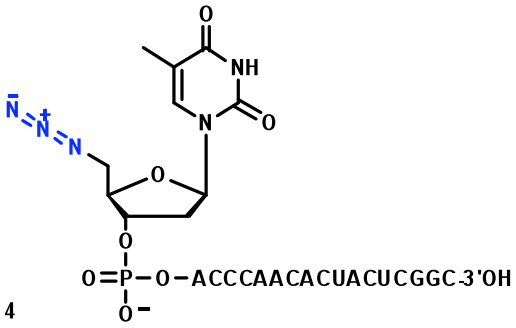
Oligonucleotides

The sequences of the oligonucleotides are shown in Figure SI1. The antisense 2'-O-methyloligonucleotides and the 5'-bromo antisense 2'-O-methyloligonucleotides were synthesized on an expedite 8909 synthesizer on a solid phase (GPC support, Glen research) from base-protected 1-(2-O-methyl-3-O-(2-cyanoethoxy(diisopropylamino)-phosphino)-5-(4,4'dimethoxytrityl)-α-D-nucleoside and 5-bromo-5-deoxy-1-(3-O(2-cyanoethoxy(diisopropylamino)-phosphino)-α-D-thymidine (**2**) by using 1H-tetrazole as activator. The ODN complementary was purchased from Eurogentec.

5'-azido-oligonucleotide: ON^{N3} (4)

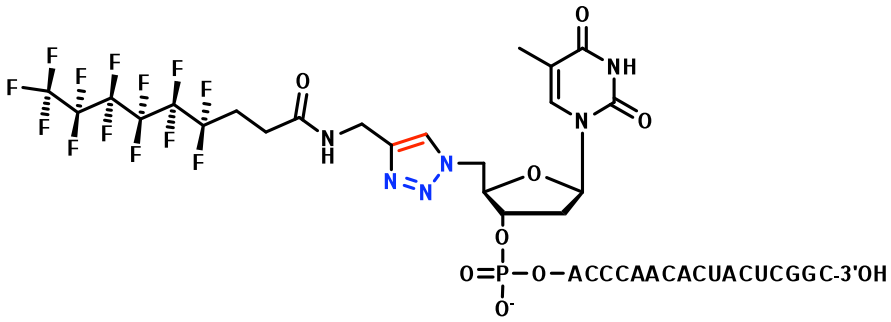
1 µmol of protected 5'-bromo-ON on support was suspended in 1 mL of DMF with sodium iodide (100 eq, 100 µmol, 15 mg) and sodium azide (100 eq, 100 µmol, 6.5 mg). The mixture was then stirred 80 min at 70 °C. After cooling at room temperature, the mixture was washed twice with 1 mL of DMF and twice with 1 mL of water. 1/5 of the supported ON was deprotected in aqueous ammonia 6 hours at 55 °C to evaluate the conversion yield. The reaction appeared to be quantitative after observation on HPLC profile.

ON^{N3} (**4**): $(M-H)^-$= 5896.68



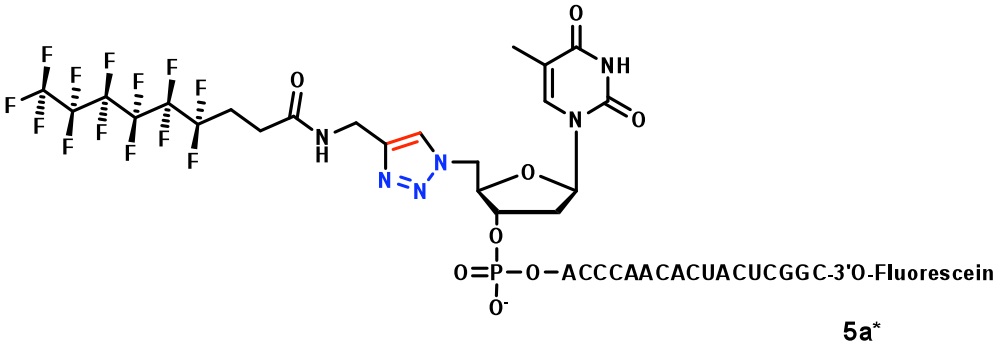
Oligonucleotide amphiphile: FON^{13F} (5a) and FON ^{13F} 3'fluorescein (5a*)

In a 2 mL vial, 0.4 µmol of protected 5'-azido-ON on support (**4**) was suspended in 1 mL of *ter*-butanol/water (50/50) mixture with $CuSO_4$ (50 eq, 20 µmol, 3.2 mg), sodium ascorbate (50 eq, 20 µmol, 4 mg) and N-propargyl-1H,1H,2H,2H-perfluorononoyl amide (**3a**) (50 eq, 20 µmol, 9 mg). The suspension was irradied 35 min at 65 °C (100 Watt). After cooling, the protected FON^{13F} on support was washed with 1 mL of DCM, twice with 2 mL of EDTA saturated aqueous solution and once with 1 mL of water. The FON^{13F} was deprotected in aqueous ammonia 6 hours at 55 °C.



5a

FON^{13F} (**5a**): Maldi (-) $[M-H]^-$: 6327.50

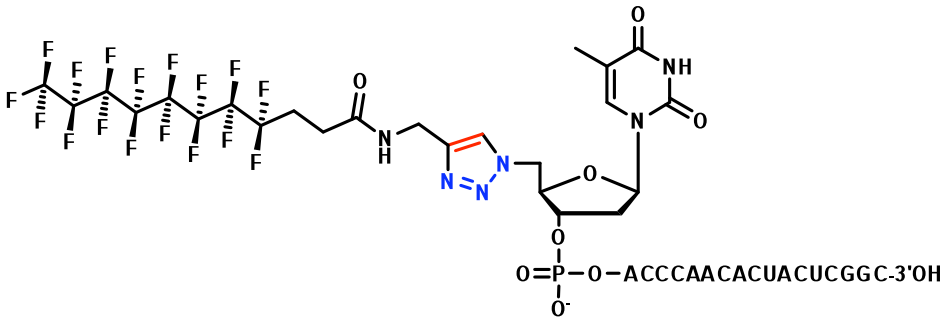


5a*

FON^{13F} 3'Fluorescein (**5a***): Maldi (-) $[M-H]^-$: 6894.29

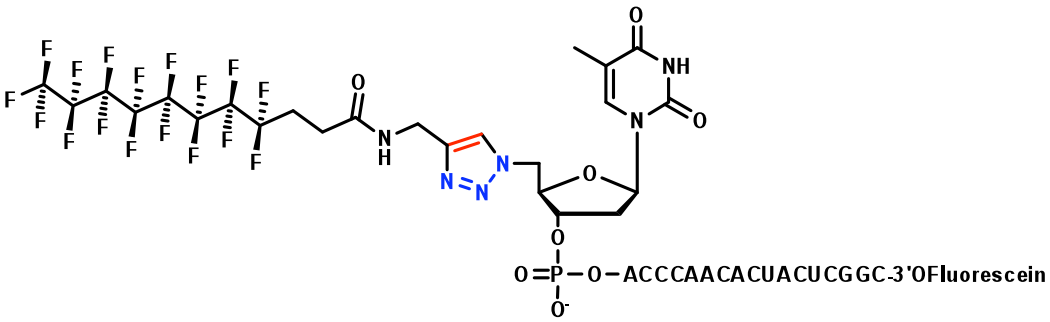
Oligonucleotide amphiphile: FON^{17F} (5b) and FON ^{17F} 3'fluorescein (5b*)

In a 2 mL vial, 0.4 µmol of protected 5'-azido-ON on support was suspended in 1 mL of *ter*-butanol/water (50/50) mixture with $CuSO_4$ (50 eq, 20 µmol, 3.2 mg), sodium ascorbate (50 eq, 20 µmol, 4 mg) and N-propargyl-1H,1H,2H,2H-perfluoroundecanoyl amide (**3b**) (50 eq, 20 µmol, 9 mg). The suspension was irradied 35 min at 65 °C (100 Watt). After cooling, the protected FON^{17F} on support was washed with 1 mL of DCM, twice with 2 mL of EDTA saturated aqueous solution and once with 1 mL of water. The FON^{17F} was deprotected in aqueous ammonia 6 hours at 55 °C.



5b

FON^{17F} (**5b**): Maldi (-) $[M-H]^-$: 6425.51



5b*

FON^{17F} 3' Fluorescein (**5b***): Maldi (-) $[M-H]^-$: 6994.46

II. Additional data

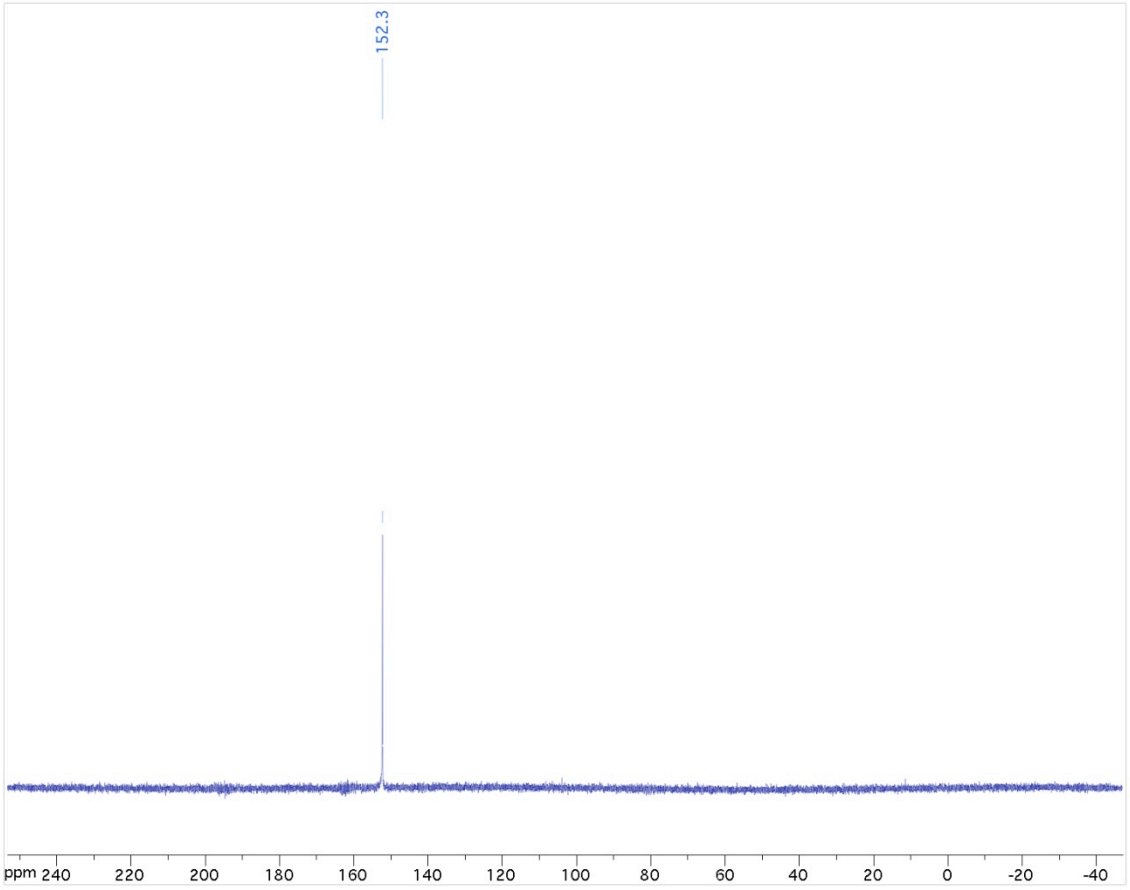


Figure SI1: Example of ^{31}P NMR spectrum of compound **2**

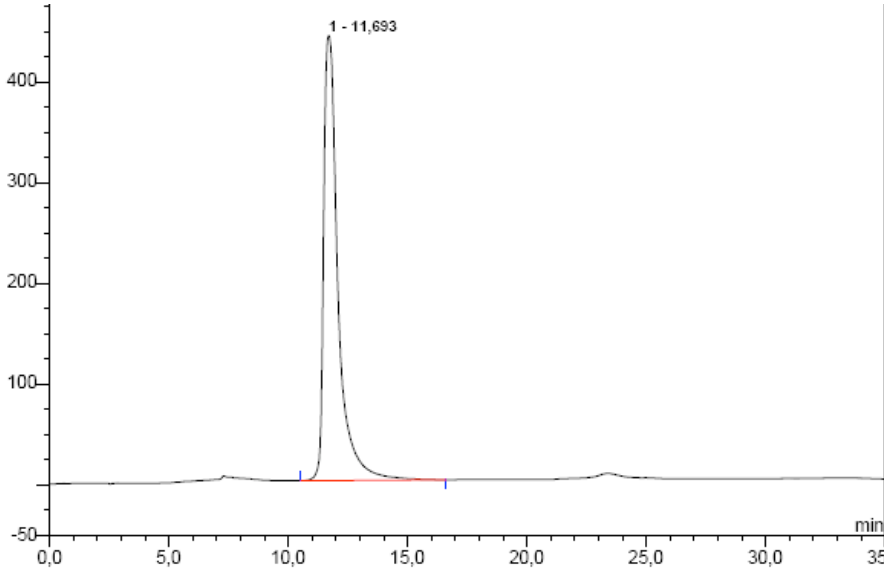


Figure SI2: Example of HPLC profile for compound **4**

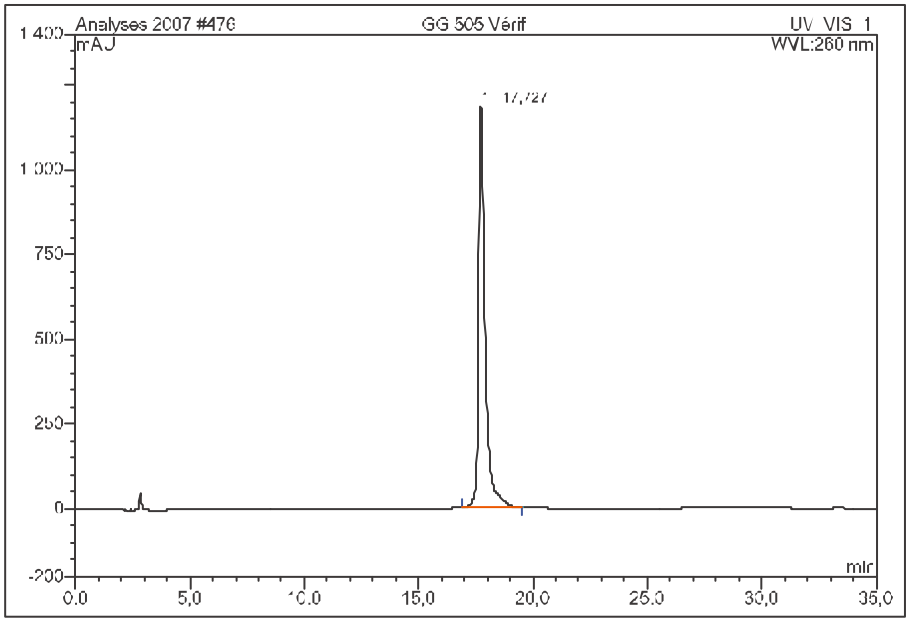


Figure SI3: Example of HPLC profile for Compound **5b**

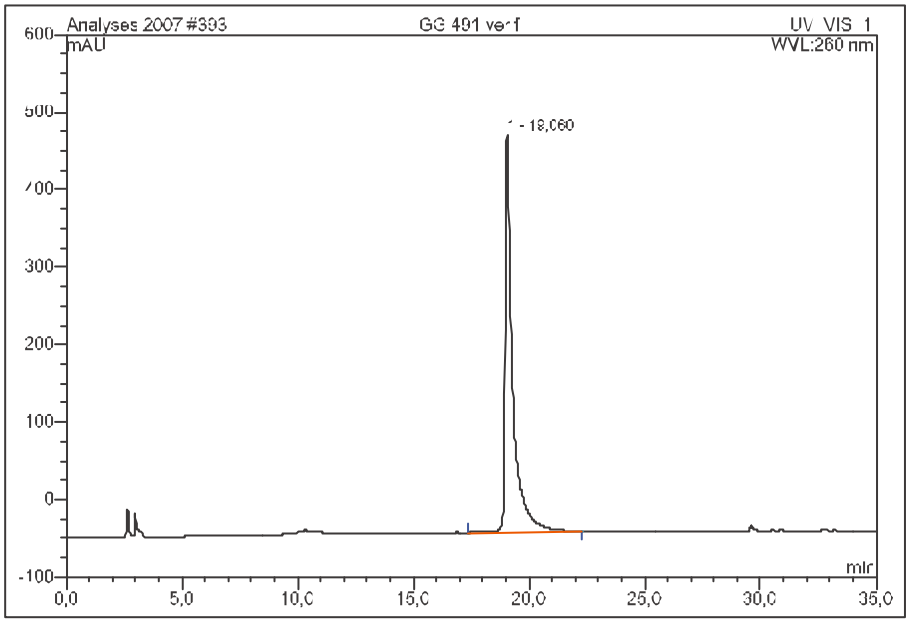


Figure SI4: Example of HPLC profile for Compound **5b***

Figure SI5: Example of MS profile for compound **5b**

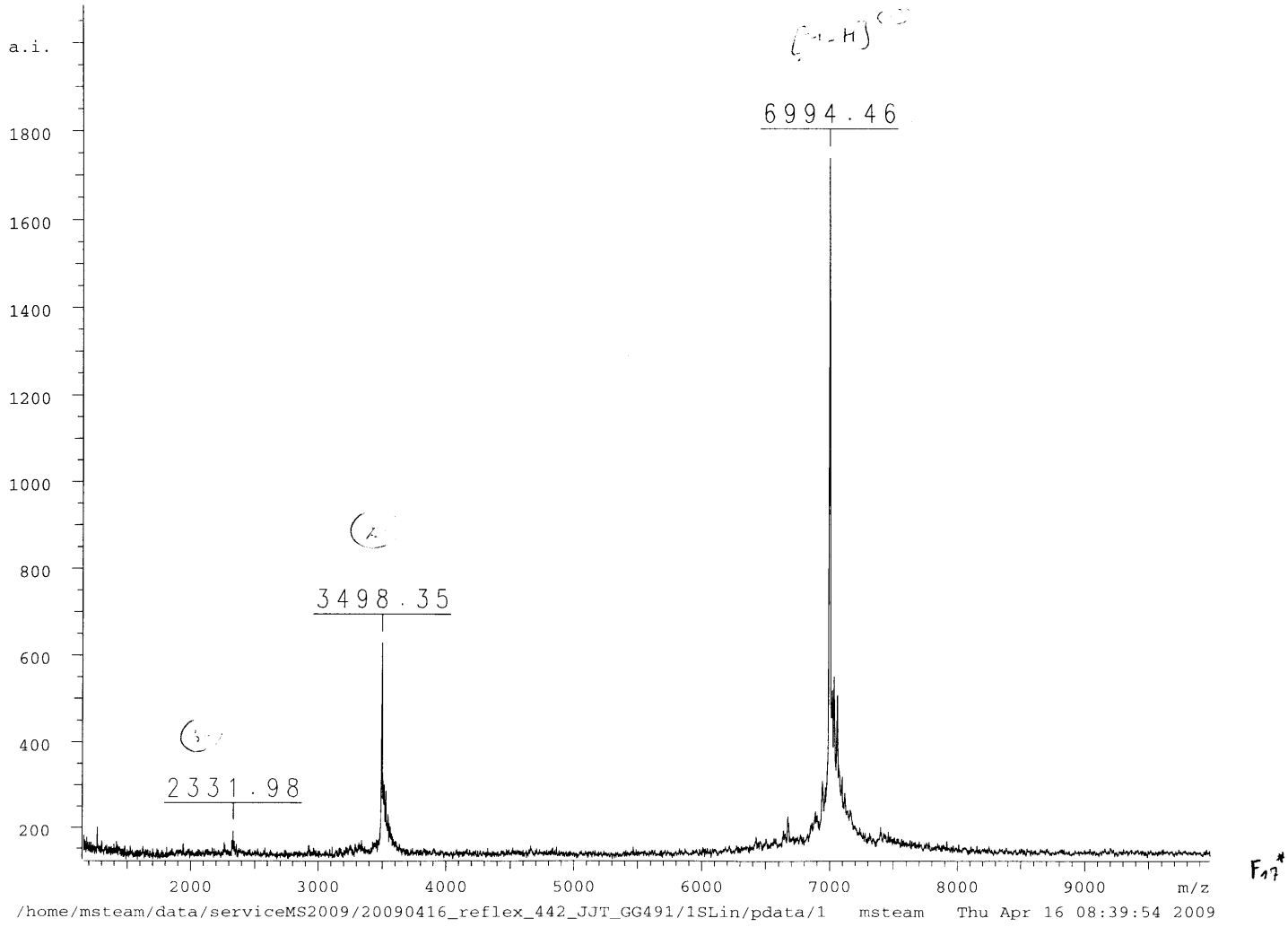


Figure SI6: Example of MS profile for compound **5b***

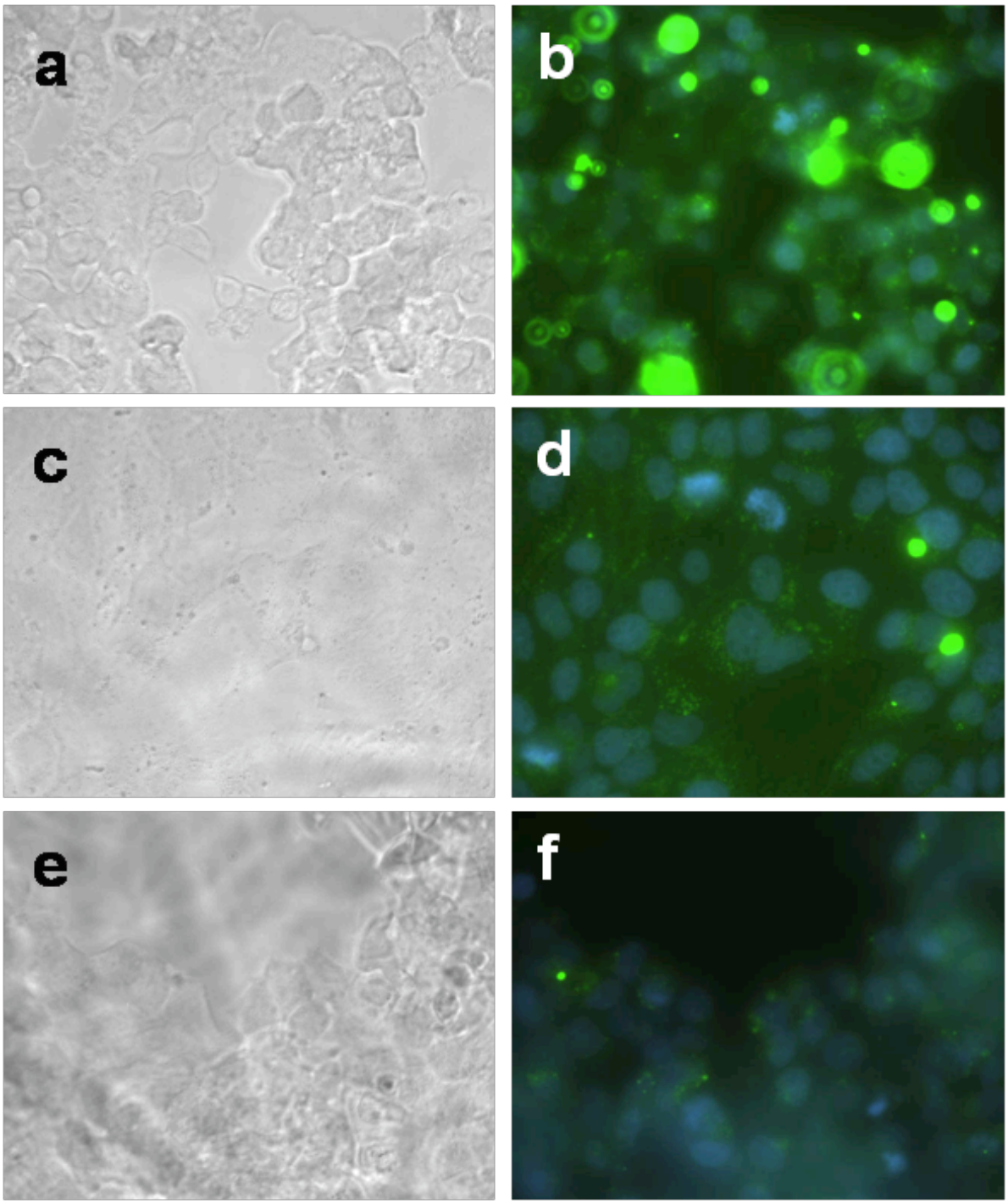


Figure SI7: Example of fluorescence microscopy for FON^{17F}* (0.5 µM, 24 H) internalisation. FON^{17F}* is presented in green, nucleus in blue. a: light microscopy on NCI cells, b: fluorescence microscopy on NCI cells, c: light microscopy on Huh-7 cells, d: fluorescence microscopy on Huh-7 cells, e: light microscopy on Hek cells, f: fluorescence microscopy on Hek cells.

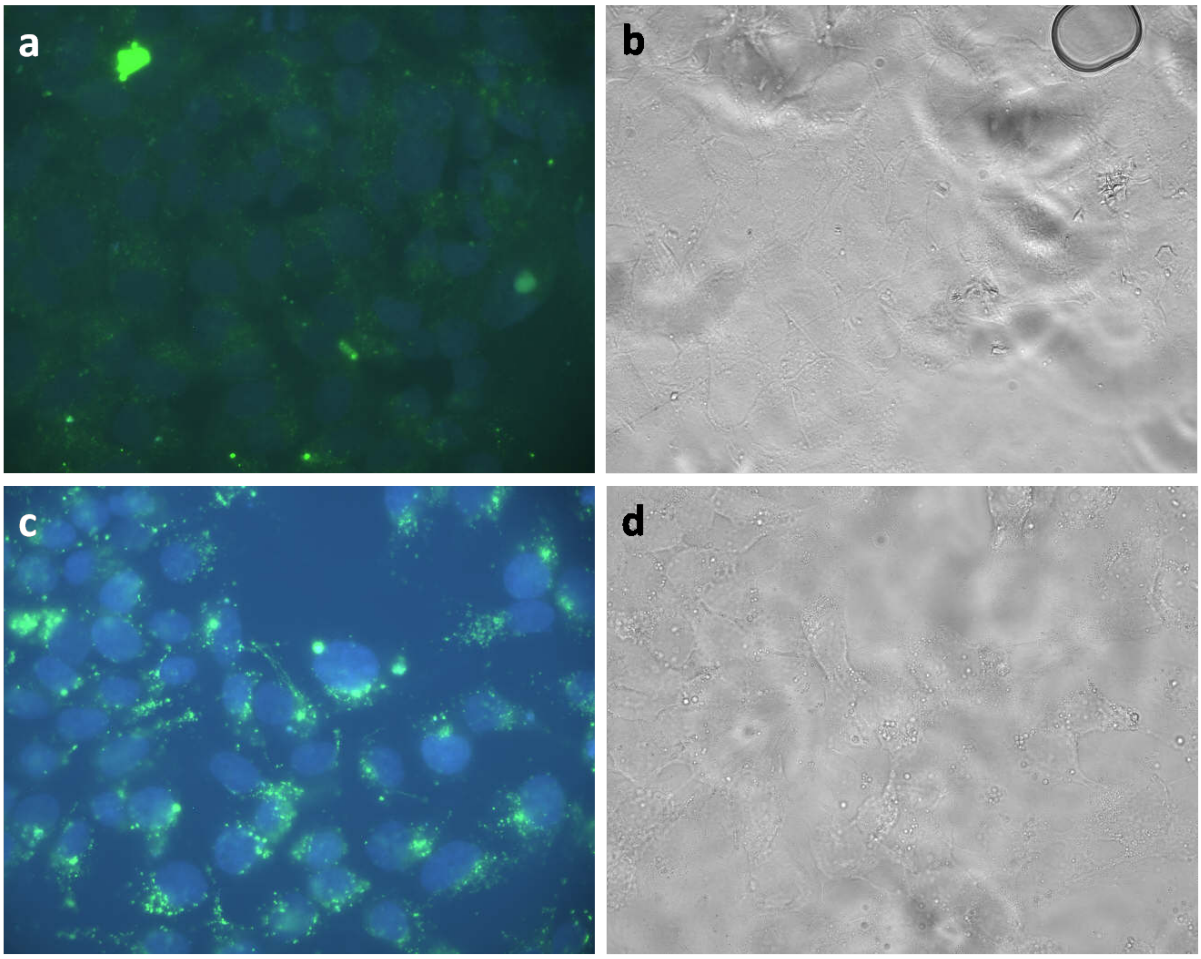


Figure SI8: Example of microscopy images from FON^{17F}* (0,5M) on Huh-7 cells. a: fluorescence microscopy 4 hours of incubation, b: light microscopy 4 hours of incubation, c: fluorescence microscopy 24 hours of incubation, d: light microscopy 24 hours of incubation.

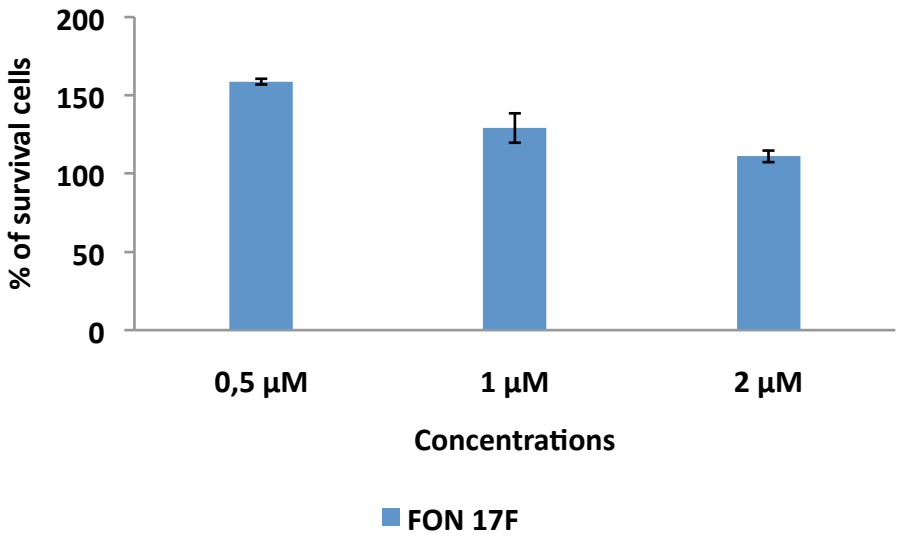


Figure SI9: Cytotoxicity of FON 17F on Huh-7 cells after 5 days of incubation.

Figure SI10: Chemical structure of the labelled FONs