Polyamine transport system-targeted nanometric micelles assembled from epipodophyllotoxin-amphiphiles

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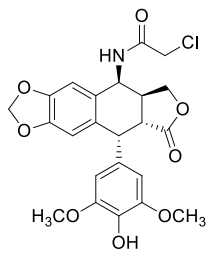
A. Chemistry

1. General

Unless otherwise specified, chemicals were purchased from Sigma–Aldrich and used without further purification. 4'-Demethylepipodophyllotoxin was obtained from the "Pierre Fabre research Institute". Reactions were carried out under nitrogen using dry solvents, unless otherwise stated. Flash chromatography was carried out on Kieselgel 60 (230–240 mesh, Merck) and analytical TLC was performed on Merck precoated silica gel (60 F254). NMR spectra were recorded on a Bruker AVANCE DPX 400 spectrometer. ^{1}H NMR spectra were recorded at 400 MHz and data are reported as follows: chemical shift in ppm from tetramethylsilane as internal standard; multiplicity: singlet (s), doublet (d), triplet (t), quartet (q), quintuplet (quint.), multiplet or complex signals (m); coupling constant (*J*) in Hz; integration. ^{13}C NMR spectra were recorded at 100 MHz. Mass spectra were recorded using a Waters Micromass ZQ 2000 ESI spectrometer. IR-spectra were recorded using a Perkin-Elmer 2000 FT-IR, wavenumbers are given in cm^{-1} at their maximum intensity. Dynamic Light Scattering size measurements were performed on a VascoFlex instrument from Cordouan Technologies equipped with a 450 nm laser diode. Zeta potential measurements were performed on a Wallis instrument from Cordouan Technologies equipped with a 635 nm laser diode.

2. Synthesis of C_{18}-ePT-Sper amphiphile (3)

2.1. Synthesis of 4-Chloroacetamide-4'-demethylpodophyllotoxin (**1**)

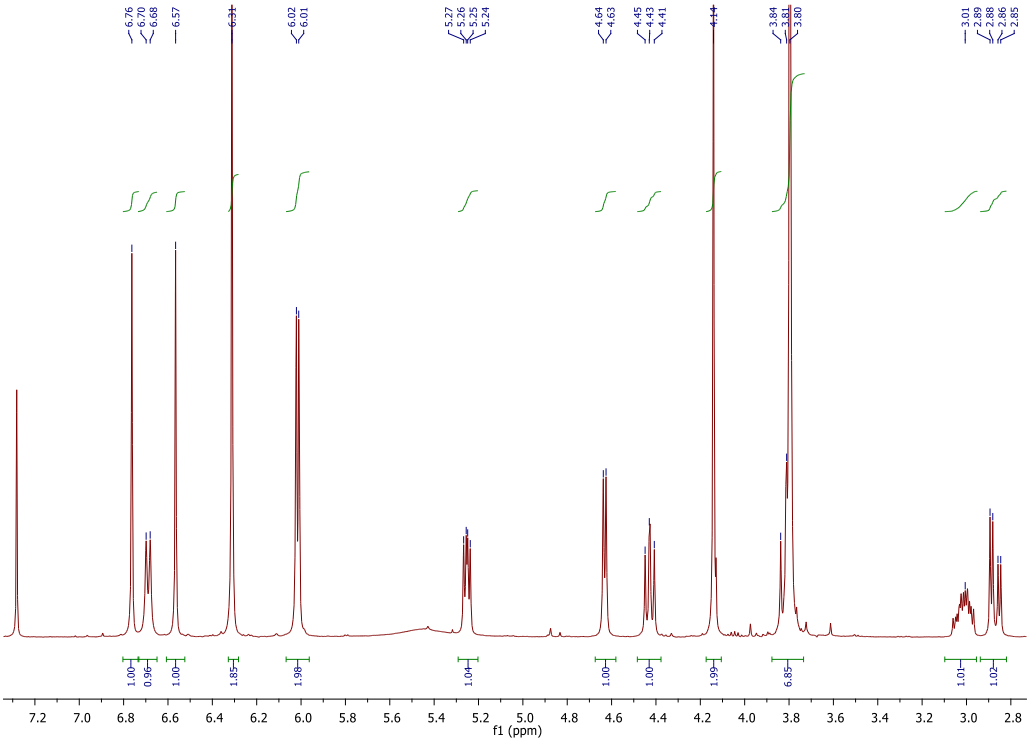


To a suspension of 4'-demethylepipodophyllotoxin (2.0 g, 5.0 mmol) in 5 mL of chloroacetonitrile were added three drops of 98% H_2SO_4. The suspension was stirred for 1 h at room temperature before 25 mL *i*PrOH were added. The suspension was further stirred for 1 h and filtered. The solid was washed with cold *i*PrOH (2 × 5 mL). After drying, **1** was isolated as a white solid (2.3 g, 96%). NMR data of **1** matched those of the literature (Imbert *et al.*, *Synthetic Commun*. **2012**, *42*, 2780–2789).

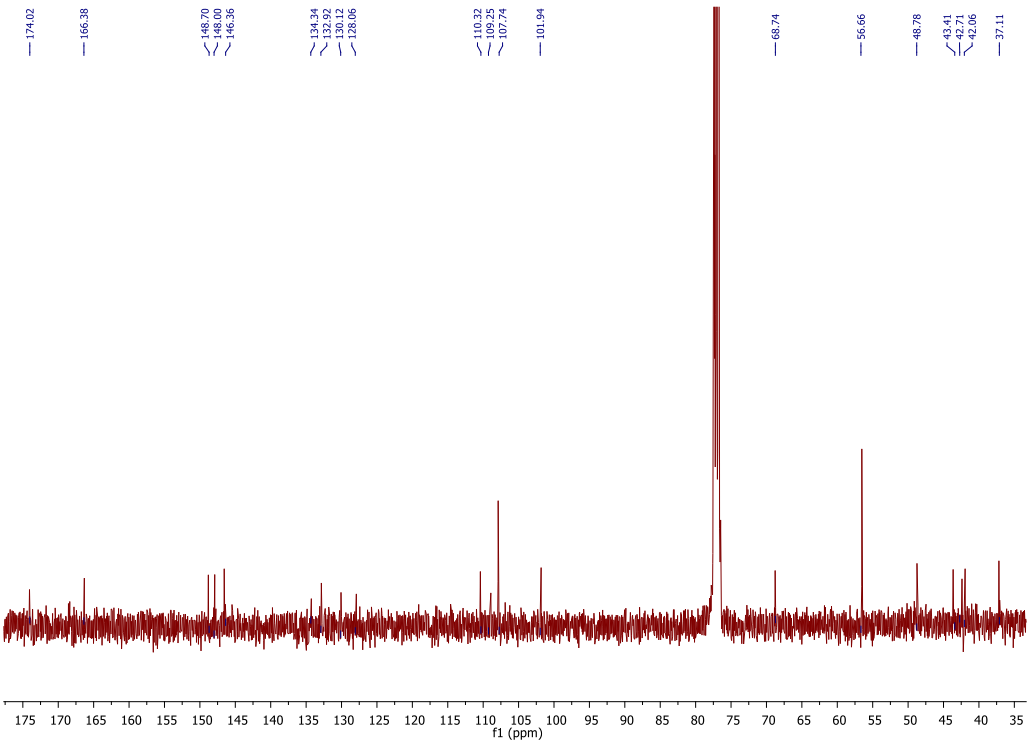
IR 3454, 3371, 1775, 1508 cm^{-1}.

ESI-MS (ES^+) 476 $[M+H]^+$, 478 $[M+H]^+$.

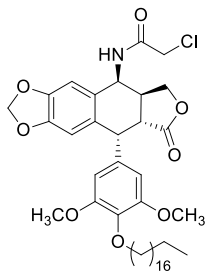
^{1}H NMR (400 MHz, $CDCl_3$) δ 6.76 (s, 1H), 6.69 (d, J = 7.2 Hz, 1H), 6.57 (s, 1H), 6.31 (s, 2H), 6.02 (s, 1H), 6.01 (s, 1H), 5.25 (dd, J = 7.2 Hz, J = 4.4 Hz, 1H), 4.63 (d, J = 5.0 Hz, 1H), 4.43 (dd, J = 9.6 Hz, J = 7.6 Hz, 1H), 4.14 (s, 2H), 3.84–3.77 (m, 7H), 3.09–2.95 (m, 1H), 2.87 (dd, J = 14.4, 5.0 Hz, 1H) ppm.



^{13}C NMR (100 MHz, $CDCl_3$) δ 174.0, 166.4, 148.7, 148.0, 146.4 (2C), 134.3, 132.9, 130.1, 128.1, 110.3, 109.3, 107.7 (2C), 101.9, 68.7, 56.7 (2C), 48.8, 43.4, 42.7, 42.1, 37.1 ppm.



2.2. Synthesis of 4-Chloroacetamide-4'-octadecyl-4'-demethylpodophyllotoxin (**2**)

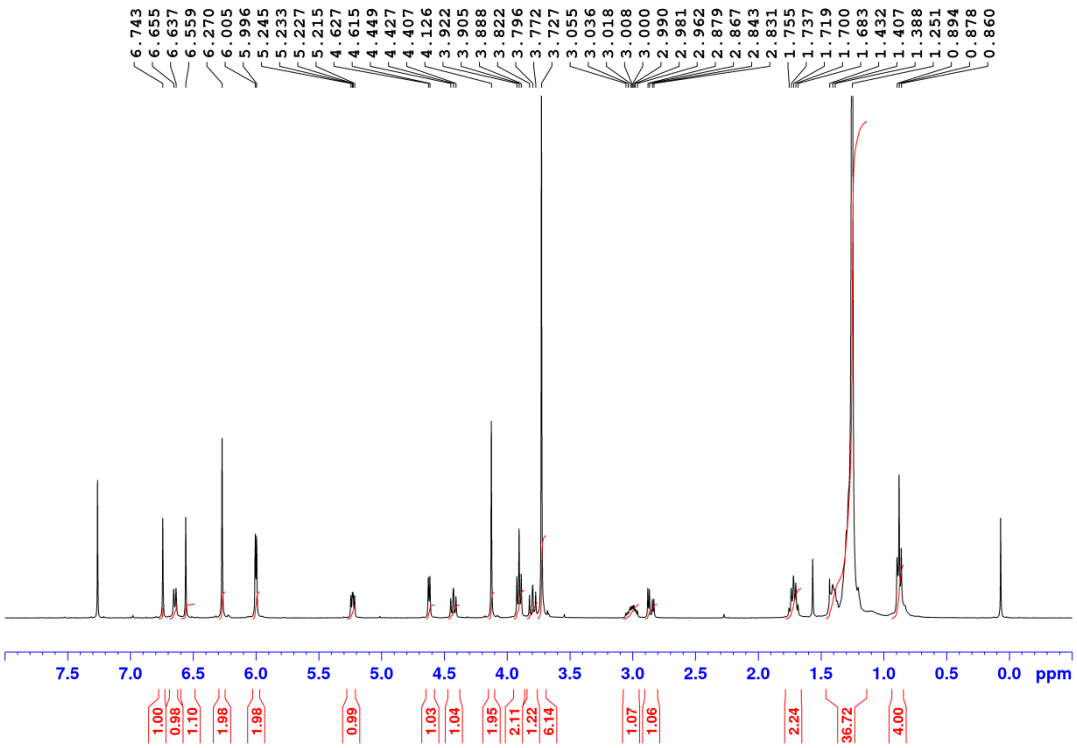


Under N_2, PPh_3 (0.65 g, 1.2 equiv.), 1-octadecanol (0.68 g, 1.2 equiv.) and DIAD (0.49 mL, 1.2 equiv.) were added to a suspension of compound **1** (1.0 g, 2.1 mmol, 1 equiv.) in 5 mL of dry CH_2Cl_2. The reaction mixture was stirred at room temp for 1.5 h and quenched with 5 mL of 0.1 N HCl. The aqueous phase was extracted with Et_2O (3 × 5 mL) and the combined organic layers were washed with brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure. Flash chromatography (Et_2O/pentane 1:1 to 1:0) afforded **2** (1.2 g, 78%) as a white solid.

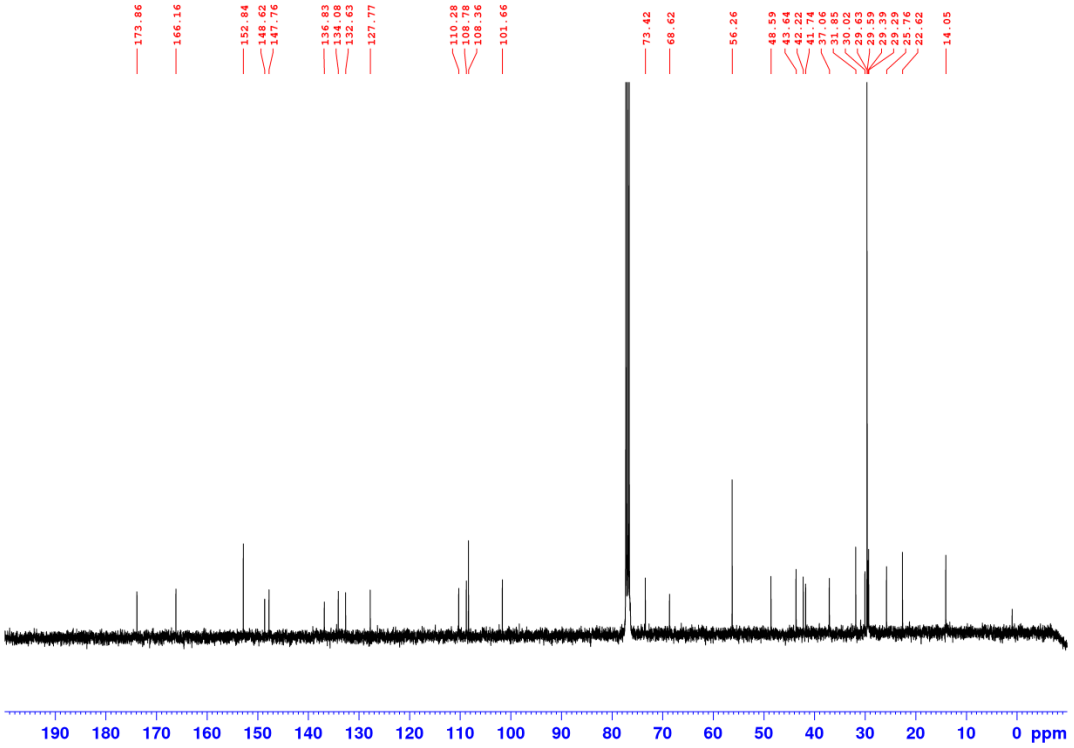
IR 2924, 1776, 1484 cm^{-1}.

ESI-MS (ES$^+$) 728 $[M+H]^+$, 730 $[M+H]^+$.

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.74 (s, 1H), 6.64 (d, *J* = 7.3 Hz, 1H), 6.56 (s, 1H), 6.27 (s, 2H), 6.01 (s, 1H), 6.00 (s, 1H), 5.23 (dd, *J* = 7.4 Hz, *J* = 5.1 Hz, 1H), 4.62 (d, *J* = 4.7 Hz, 1H), 4.42 (t, *J* = 8.1 Hz, 1H), 4.12 (s, 2H), 3.91 (t, *J* = 7.5 Hz, 2H), 3.82 (t, *J* = 8.1 Hz, 1H), 3.73 (s, 6H), 3.04–3.00 (m, 1H), 2.856 (dd, *J* = 14.4 Hz, *J* = 4.8 Hz, 1H), 1.74 (quint., *J* = 7.4 Hz, 2H), 1.45–1.43 (m, 2H), 1.27–1.23 (m, 28H), 0.88 (t, *J* = 7.4 Hz, 3H) ppm.

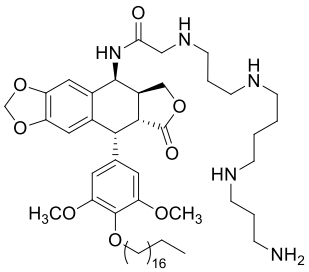


^{13}C NMR (100 MHz, $CDCl_3$) δ 173.9, 166.2, 152.8 (2C), 148.6, 147.8, 136.8, 134.1, 132.6, 127.8, 110.3, 108.8, 108.4 (2C), 101.7, 73.4, 68.6, 56.3 (2C), 48.6, 43.6, 42.2, 41.7, 37.1, 31.9, 30.0, 29.6 (10C), 29.5, 29.4, 29.3, 25.8, 22.6, 14.0 ppm.



*2.3. Synthesis of C_{18}-ePT-Sper amphiphile (**3**)*

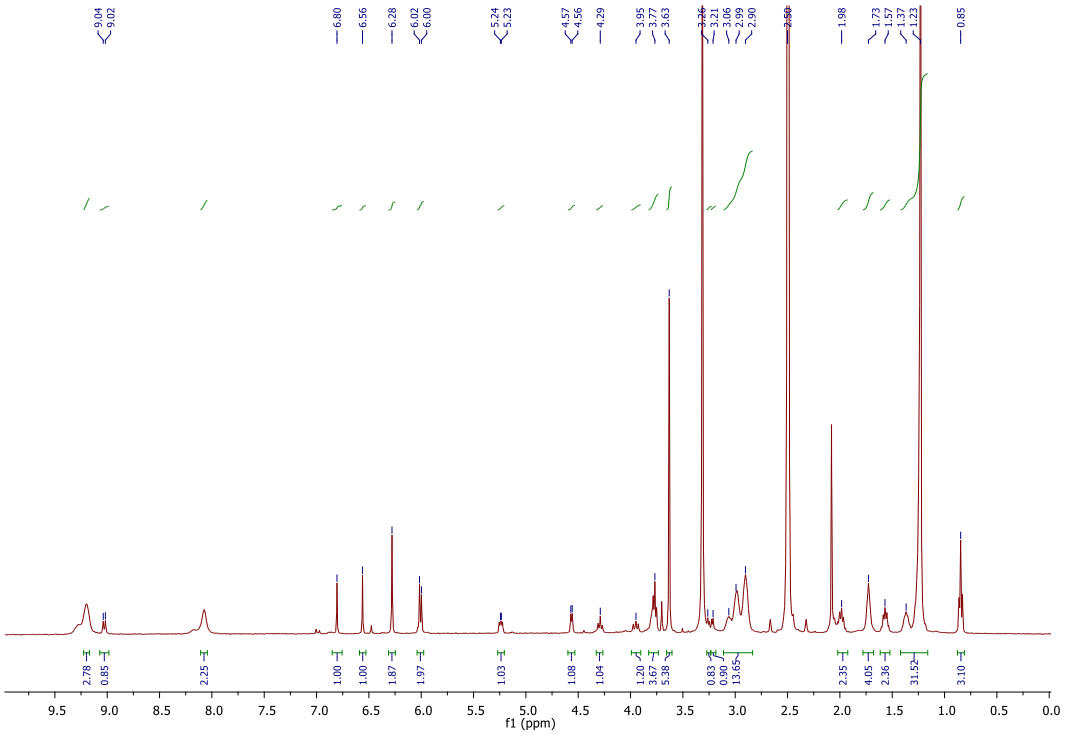
Spermine (465 mg, 10 equiv.) was added to a solution of compound **2** (170 mg, 0.23 mmol, 1 equiv.) in 2 mL of DMF. The reaction mixture was stirred, at room temperature for 1 h and 2.5 mL of 3 M HCl in isopropanol was then added dropwise. The solid was recovered by filtration and washed with isopropanol (2 × 2 mL). The crude mixture was purified by reverse phase chromatography (5 mM HCl/acetonitrile, 95:5 to 30:70). The collected fractions were lyophilized and **3** was recovered as a white solid (193 mg, 81%).



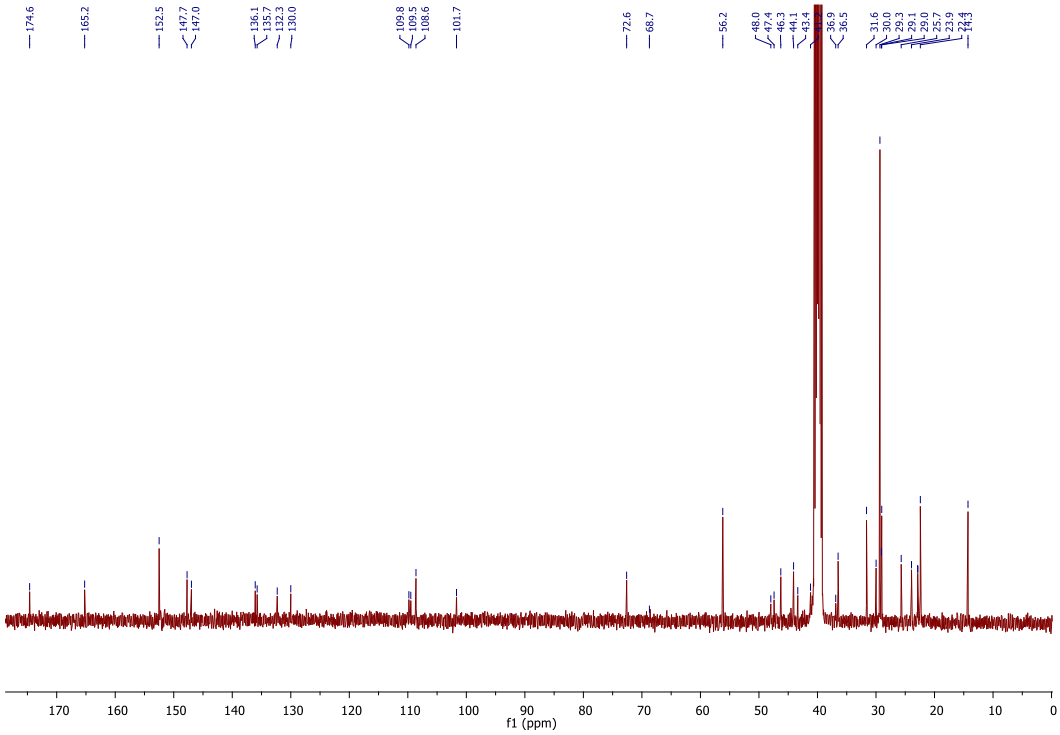
IR 3424, 2920, 1773, 1678 cm^{-1}.

ESI-MS (ES^+) 894 $[M+H]^+$.

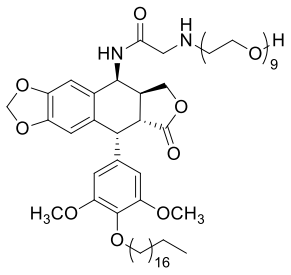
^{1}H NMR (400 MHz, DMSO-*d6*): *δ* 9.02 (d, *J* = 8.0 Hz, 1H), 6.80 (s, 1H), 6.56 (s, 1H), 6.28 (s, 2H), 6.02 (s, 1H), 6.00 (s, 1H), 5.23 (dd, *J* = 8.0 Hz, *J* = 4.4 Hz, 1H), 4.56 (d, *J* = 5.0 Hz, 1H), 4.29 (t, *J* = 10.4 Hz, 1H), 3.95 (t, *J* = 10.4 Hz, 1H), 3.78-3.70 (m, 4H), 3.63 (s, 6H), 3.24 (dd, *J* = 14.6 Hz, *J* = 5.2 Hz, 1H), 3.09-2.86 (m, 13H), 2.09-2.05 (m, 2H, H), 1.99-1.95 (m, 2H), 1.73 (br. s, 4H), 1.57 (quint., *J* = 6.8 Hz, 2H), 1.37 (quint., *J* = 6.8 Hz, 4H), 1.30-1.17 (m, 26H), 0.85 (t, *J* = 7.0 Hz, 3H) ppm.



^{13}C NMR (100 MHz, DMSO-*d6*): δ 174.6, 162.2, 152.5 (2C), 147.7, 147.0, 136.1, 135.7, 132.3, 130.0, 109.8, 109.5, 108.6 (2C), 101.7, 72.6, 68.7, 56.2 (2C), 48.0, 47.4, 46.3 (2C), 44.1 (2C), 43.4, 41.2, 41.1, 36.9, 36.5, 31.6 (2C), 30.0, 29.3 (10C), 29.1, 29.0 (2C), 25.7, 23.9, 22.9, 22.8, 22.6 (2C), 14.3 ppm.



3. Synthesis of C_{18}-ePT-PEG amphiphile (4)

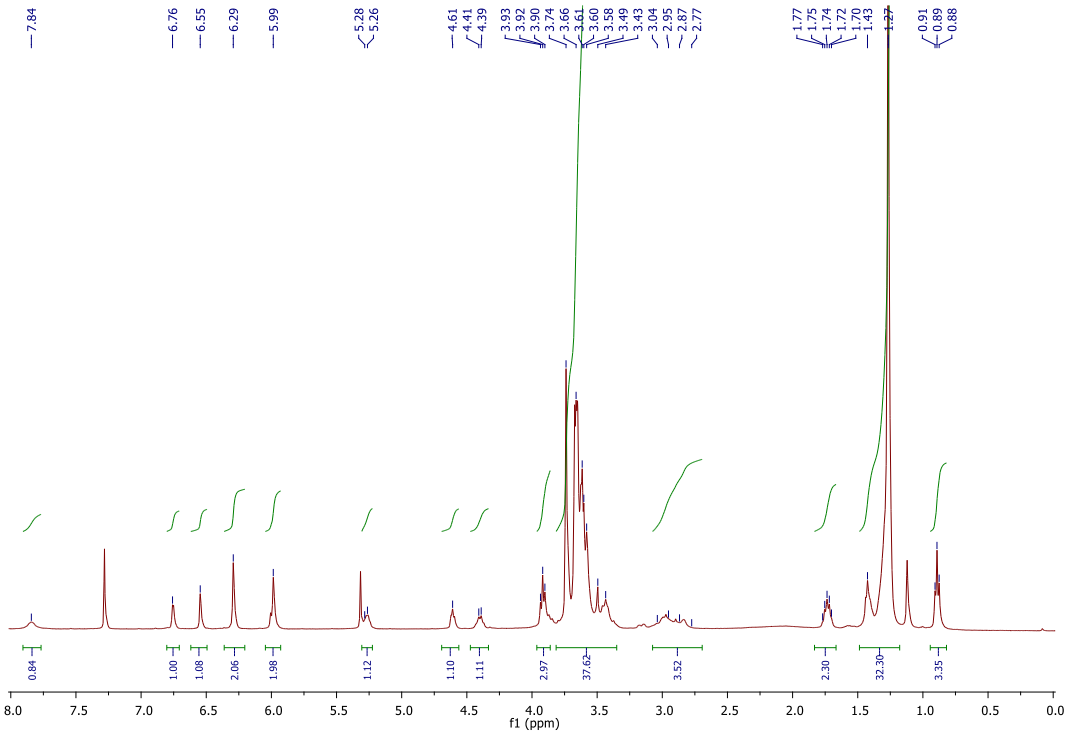


NEt_3 (80.0 µL, 1.5 equiv.) and HO-PEG_{400}-NH_2 (190.0 mg, 1.2 equiv.) were sequentially added to a solution of compound **2** (274.7 mg, 0.38 mmol, 1 equiv.) in 4 mL of dry DMF. The solution was heated to 100 °C for 5 h, cooled to room temperature, quenched with 5 mL of 2 N HCl, and extracted with CH_2Cl_2 (5 × 10 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure. Flash chromatography (CH_2Cl_2/MeOH 1:0 to 97:3, column pretreated with NH_4OH) afforded **1** (287 mg, 68 %) as a yellow oil.

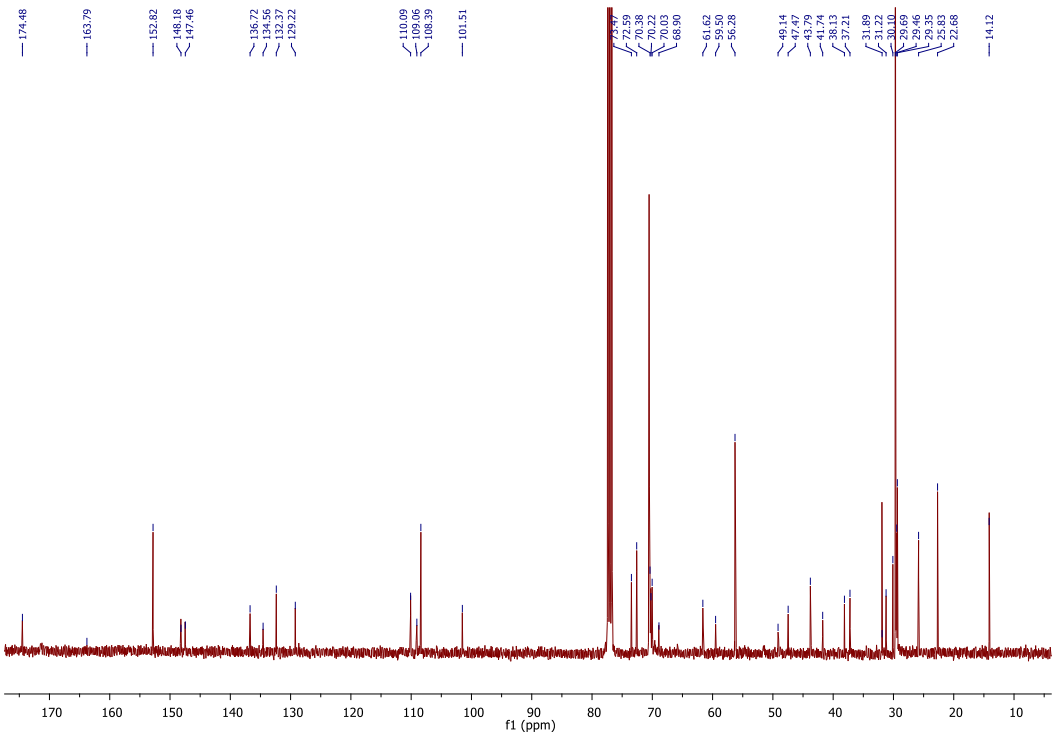
IR 3425, 2931, 1770, 1682 cm^{-1}.

ESI-MS (ES^+) 1106 $[M+H]^+$.

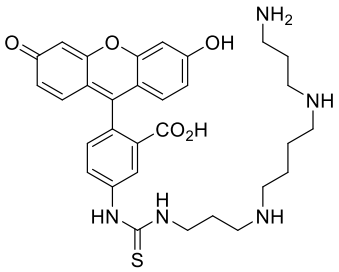
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84 (d, J = 8.5 Hz, 1H), 6.76 (s, 1H), 6.55 (s, 1H), 6.30 (s, 2H), 5.99 (s, 2H), 5.27 (d, J = 7.5 Hz, 1H), 4.61 (t, J = 4.7 Hz, 1H), 4.40 (d, J = 8.1 Hz, 1H), 3.92 (t, J = 6.8 Hz, 2H), 3.88–3.83 (m, 1H), 3.74 (s, 6H), 3.73–3.50 (m, 38H), 3.02–2.98 (m, 2H), 2.81–2.77 (m, 2H), 1.74 (quint., J = 7.4 Hz, 2H), 1.45–1.43 (m, 2H), 1.27–1.23 (m, 28H), 0.90 (t, J = 7.4 Hz, 3H) ppm.



^{13}C NMR (100 MHz, $CDCl_3$) δ 174.5, 163.8, 152.8 (2C), 148.2, 147.5, 136.7, 134.6, 132.4, 129.2, 110.1, 109.1, 108.4 (2C), 101.5, 73.5, 72.6, 70.5 (multiple C), 70.2, 70.1, 70.0, 68.9, 61.6, 59.5, 56.3 (2C), 49.1, 47.5, 43.8, 41.7, 38.1, 37.2, 31.9, 31.2, 30.1, 29.7 (multiple C), 29.5, 29.4, 25.8, 22.7, 14.1 ppm.



4. Synthesis of the fluorescein-spermine probe

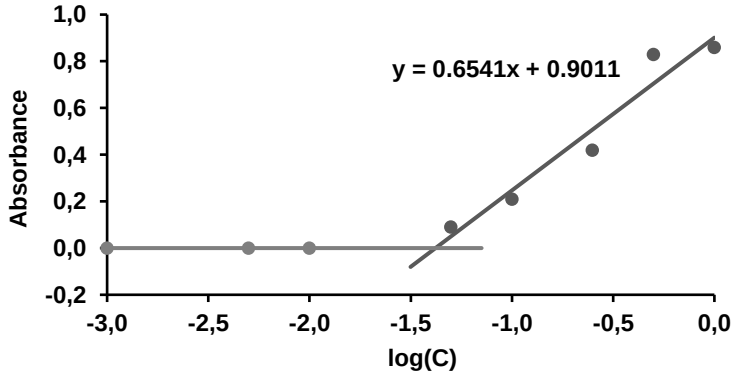


The probe was synthesized according to the procedure reported by Imbert *et al.* (*Bioorg. Med. Chem. Lett.* **2009**, *19*, 2474–2477). Briefly, fluorescein isothiocyanate (FITC, 13.5 mg, 35.1 mmol, 1 equiv.) and spermine (42 mg, 210 mmol, 6 equiv.) were stirred in DMF (0.3 mL) at room temperature for 1 h. The solvent was then removed under vacuum and the residue was purified by reverse phase column chromatography (C_{18} column, 5 mM aq HCl/MeCN 100:00 → 80:20) to yield the desired product (12 mg, 58%). MS, IR, and NMR matched the reported literature data.

5. Assembly of the micelle

5.1. Measurement of the critical micelle concentration (CMC)

A concentration range of C_{18}-ePT-Sper **3** (or C_{18}-ePT-PEG **4**) in deionized water was prepared form 1 mg mL^{-1} to 0.0001 mg mL^{-1}. 20 µL of a 0.1 M solution of pyrene in DMSO was then added to each of the solutions (2 mL) which were further sonicated for 3 min using an ultrasonic probe (Branson Sonifier 450 at 30%). Solutions were filtered on 0.22 µm membranes to remove insoluble pyrene aggregates, and absorbance was measured at 339 nm (absorbance of pyrene). The recorded CMC values are 25 µM (C_{18}-ePT-Sper **3,** see Figure below) and 38 µM (C_{18}-ePT-PEG **4.**)



5.2. Preparation of DiD-loaded micelles

25 mg of C_{18}-ePT-Sper amphiphile **3** (or C_{18}-ePT-PEG **4**) was dispersed in 2.5 mL of deionized water before 10 µL of a 25 mg mL^{-1} solution of the fluorescent dye DiD ($\lambda_{ex}/\lambda_{em}$: 644 nm/665 nm) in chloroform was added. The mixture was sonicated with an ultrasonic probe (Branson Sonifier 450 at 30%) for 10 min yielding a homogenous and limpid solution from which chloroform had evaporated. Micelles were filtered on 0.22 µm membranes to provide the stock micellar solution.

B. Biology

1. Cells

HL-60 and U-937 cells (ATCC, Manassas, VA) were donated by Dr. Daniel Lewandowski (DRF/JACOB/IRCM/SCSR/LRTS at CEA). FaDu cells (ATCC, Manassas, VA) were donated by Dr. Lina Bolotine (Research Centre for Automatic Control (CRAN), Nancy- University, UMR CNRS, France). MCF-7, SKBr3 and A-431 cells were purchased from ATCC (Manassas, VA). HL-60, FaDu and A-431 cells were grown in DMEM medium supplemented with 10% Foetal Bovine Serum (FBS). U-937 and MCF-7 cells were grown in RPMI medium supplemented with 10% FBS. SkBr3 cells were grown in McCoy's 5a medium supplemented with 10% FBS. All cell types were grown at 37 °C in 5% CO_2 atmosphere.

2. Flow cytometry experiments

The cell targeting of control C_{18}-ePT-PEG and targeted C_{18}-ePT-Spermine micelles were evaluated on non-adherent leukemia U-937 or HL-60 cells, which are known to be PTS(−) and PTS(+), respectively. Micelles were loaded with DiD dye as previously described and were incubated with non-adherent leukemia cells (HL-60 or U-937 cells). The fluorescence of cells was measured every hour during 5 h with a BD Accuri C6 flow cytometer (BD biosciences) using a 640 nm laser excitation and a FL4 channel (670 Long pass filter).

To evaluate the PTS activity of cancer cells derived from solid tumors, 24 h before the experiment, 1 million cells were seeded on a 6-well plate in 2.5 mL of their respective medium. Then, fluorescein-labeled spermine was incubated at 37 °C on cells at 1 µM final concentration in absence or presence of 1 mM of unlabeled spermine. After 3 h, the medium was removed and cells were washed twice with 3 mL of PBS before being detached by 200 µL of trypsin (5 min at 37 °C.) 500 µL of PBS was then added to cells and their fluorescence was measured with a BD Accuri C6 flow cytometer (BD biosciences) using a 488 nm laser excitation and a FL2 channel (585/40 nm filter). Forward and side scatter density plots (FSC vs SSC) were used to exclude debris and dead cells from each analysis.

3. Cytotoxicity evaluation

To evaluate the cytotoxicity of C_{18}-ePT-PEG micelles and of targeted C_{18}-ePT-Spermine micelles, 2×10^3 A-431 cells were seeded in a 96-well plate in 50 µL of DMEM media containing 10% FBS. After 24 h at 37 °C in 5% CO_2 atmosphere, 50 µL of PBS containing micelles at different concentrations (30, 20, 10, 3, 1, 0.3 and 0.1 µM of ePT-derived amphiphile) was added per well. Then after 48 h at 37 °C in a 5% CO_2 atmosphere, the medium was removed and replaced by 100 µL of DMEM. 20 µL of MTS

(tetrazolium compound included in the CellTiter 96 AQueous One Solution cell proliferation assay, Promega) was then added to each well. After 4 h at 37 °C in 5% CO_2 atmosphere, 85 µL of medium was removed and 50 µL of DMSO was added. After 10 min, absorbance at 560 nm was measured using a flx-xenius XC spectrophotometer instrument (SAFAS, Monaco). Experiments were performed in triplicate. IC_{50} were assessed by non-linear fitting of the curves using GraphPad Prism 6 with the "log(inhibitor) *vs.* normalized response -- Variable slope" model. For comparison purposes, MTT tests were also performed on 4'-demethylepipodophyllotoxin (4'-DMEP, central core molecule of the amphiphile).

C_{18}-ePT-PEG micelles IC_{50} = 18.1 ± 1.24 µM.

C_{18}-ePT-Sper micelles IC_{50} = 1.6 ± 1.33 µM.

4'-DMEP IC_{50} = 0.6 ± 1.12 µM.

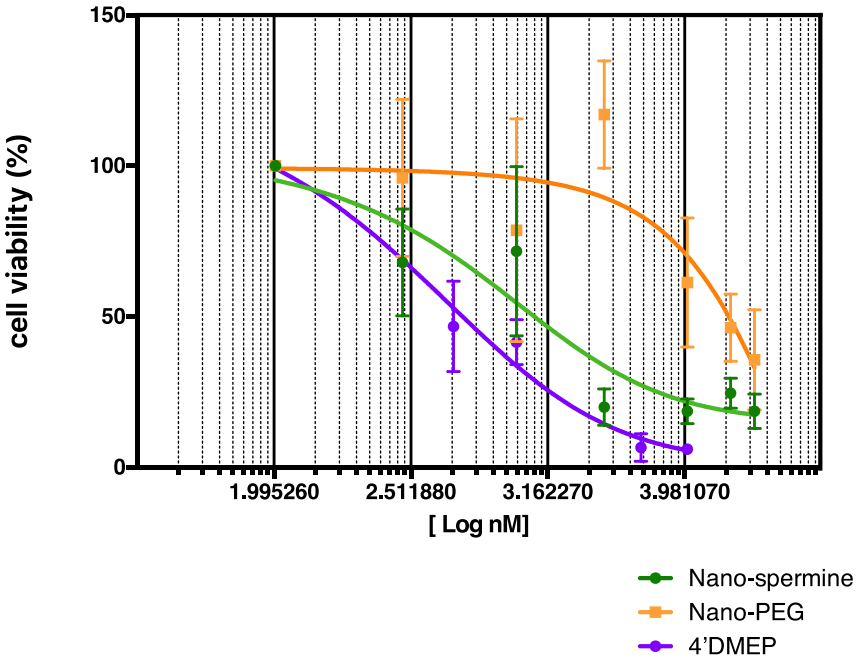


Figure S1. Cytotoxicity studies of C_{18}-ePT-PEG micelles, C_{18}-ePT-Spermine micelles, and 4'-demethylepipodophyllotoxin (4'DMEP).

C. Supplementary figures

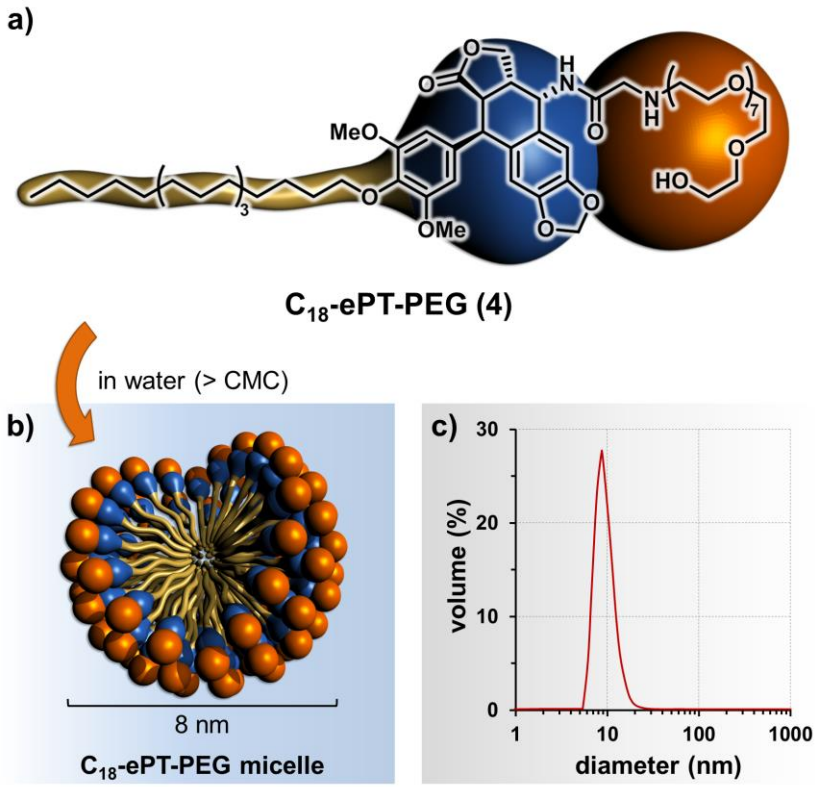


Figure S2. a) Structure of the c18-ePT-PEG amphiphile; b) assembly into micelles; c) DLS analysis.