Electronic Supplementary Information

Tumor targeted micellar nanocarriers assembled from Epipodophyllotoxin-based amphiphiles

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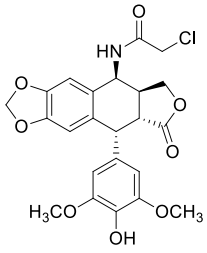
CHEMISTRY

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-PEG amphiphile (1)

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

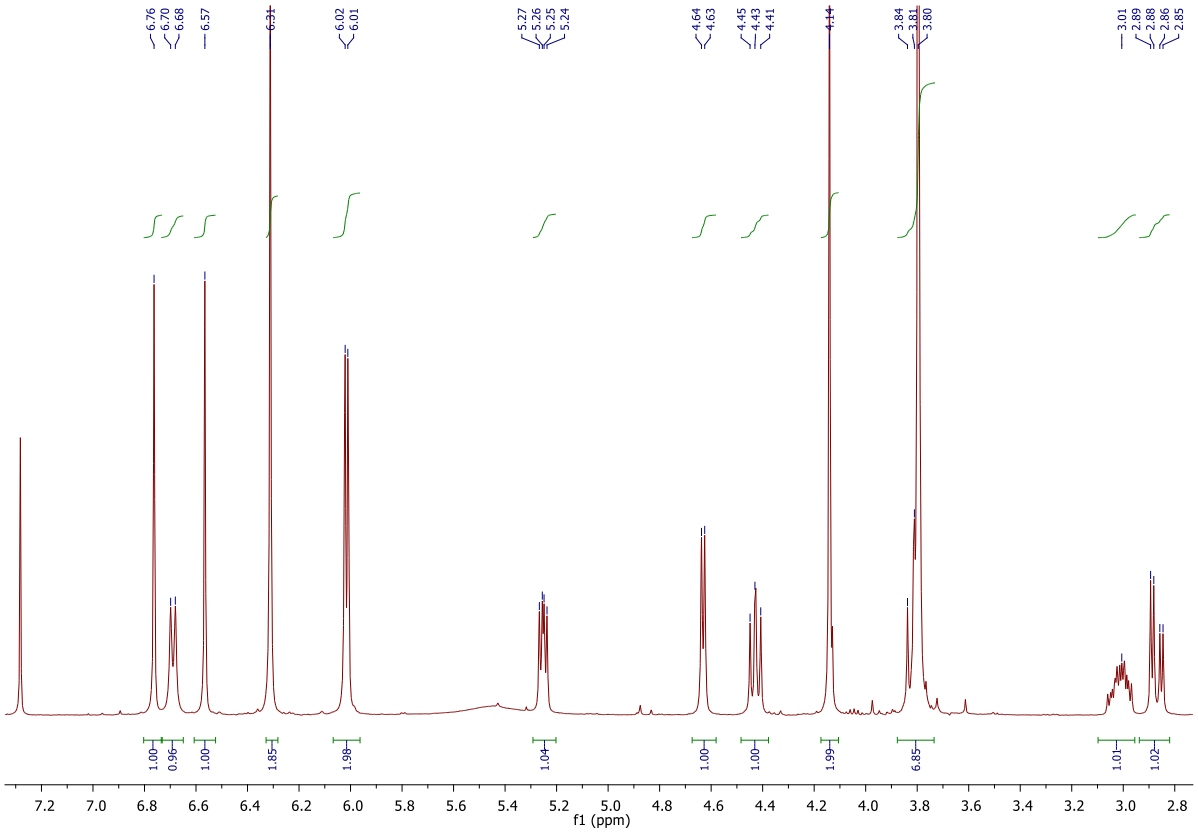


To a suspension of 4'-demethylepipodophyllotoxin (2.0 g, 5.0 mmol) in 5 mL of chloroacetonitrile were added three drops of 98% H_2SO_4. The suspension was stirred for 1 h at room temperature before 25 mL *i*PrOH were added. The suspension was further stirred for 1 h and filtered. The solid was washed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

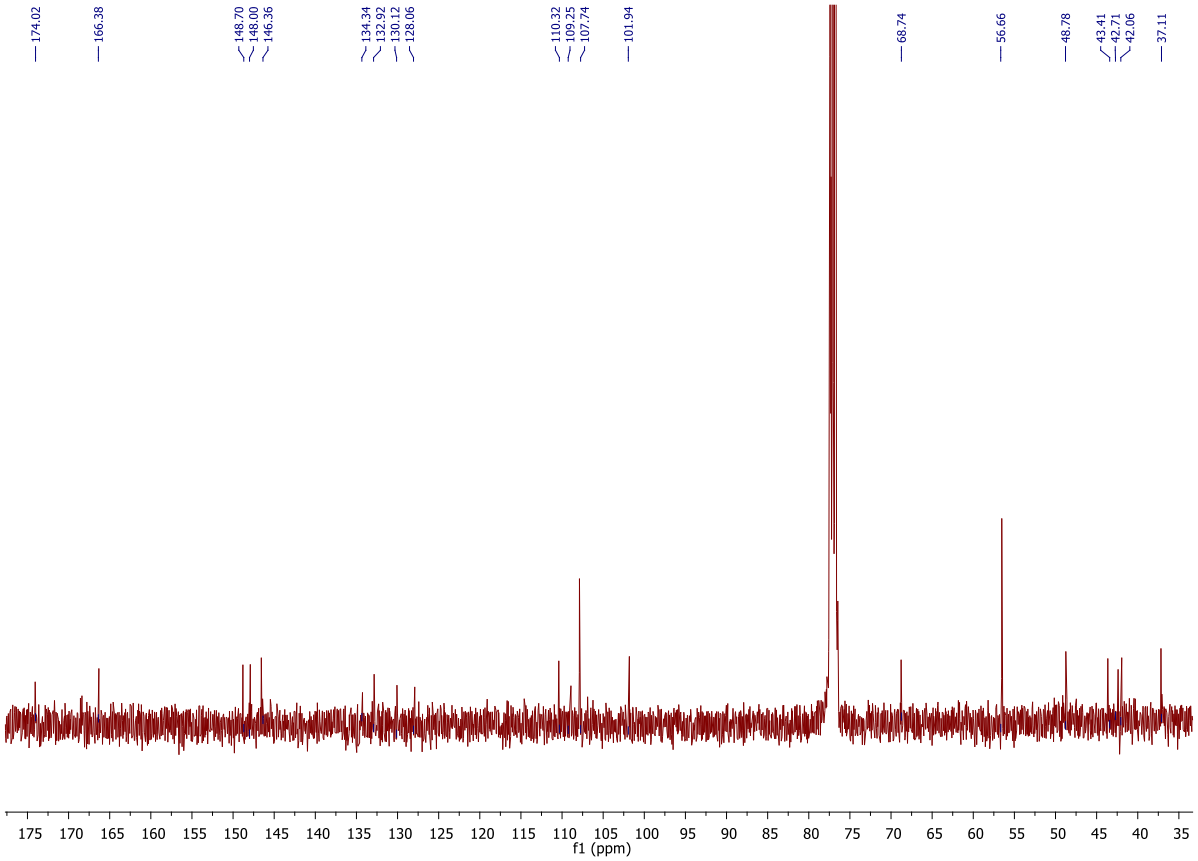
IR 3454, 3371, 1775, 1508 cm^{-1}.

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

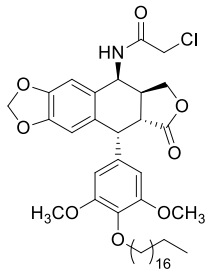
^{1}H NMR (400 MHz, $CDCl_3$) δ 6.76 (s, 1H), 6.69 (d, J = 7.2 Hz, 1H), 6.57 (s, 1H), 6.31 (s, 2H), 6.02 (s, 1H), 6.01 (s, 1H), 5.25 (dd, J = 7.2 Hz, J = 4.4 Hz, 1H), 4.63 (d, J = 5.0 Hz, 1H), 4.43 (dd, J = 9.6 Hz, J = 7.6 Hz, 1H), 4.14 (s, 2H), 3.84–3.77 (m, 7H), 3.09–2.95 (m, 1H), 2.87 (dd, J = 14.4, 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 (**3**)

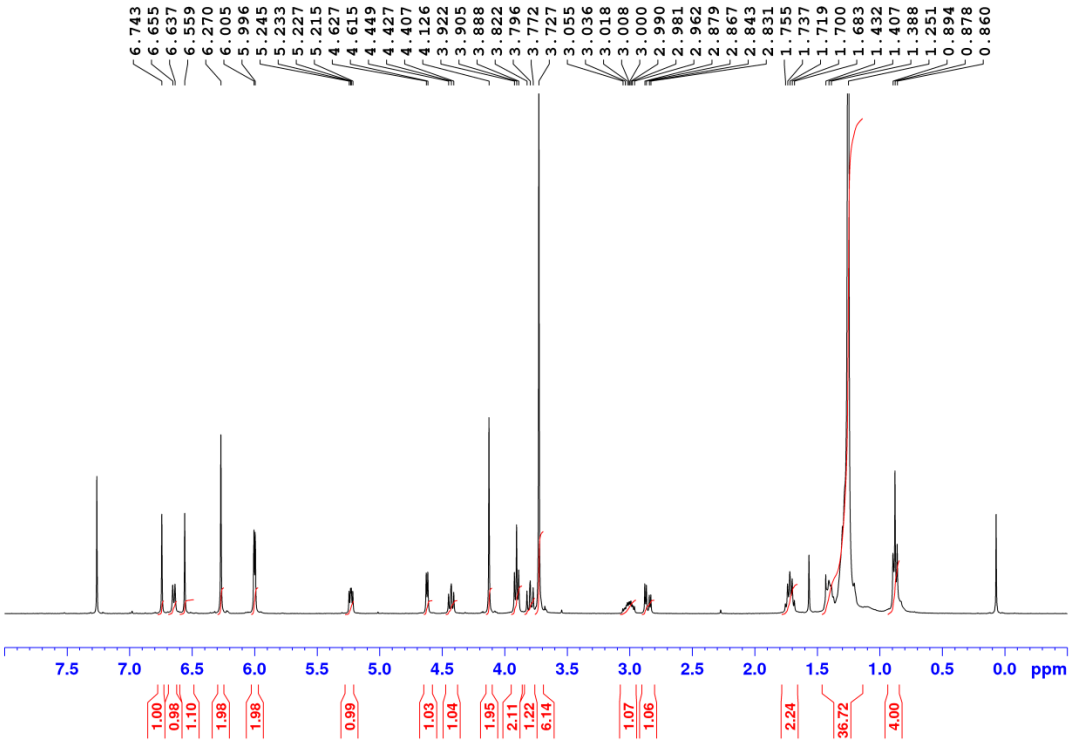


Under N_2, PPh_3 (0.65 g, 1.2 equiv.), 1-octadecanol (0.68 g, 1.2 equiv.) and DIAD (0.49 mL, 1.2 equiv.) were added to a suspension of compound **2** (1.0 g, 2.1 mmol, 1 equiv.) in 5 mL of dry CH_2Cl_2. The reaction mixture was stirred at room temp for 1.5 h and quenched with 5 mL of 0.1 N HCl. The aqueous 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 **3** (1.2 g, 78%) as a white solid.

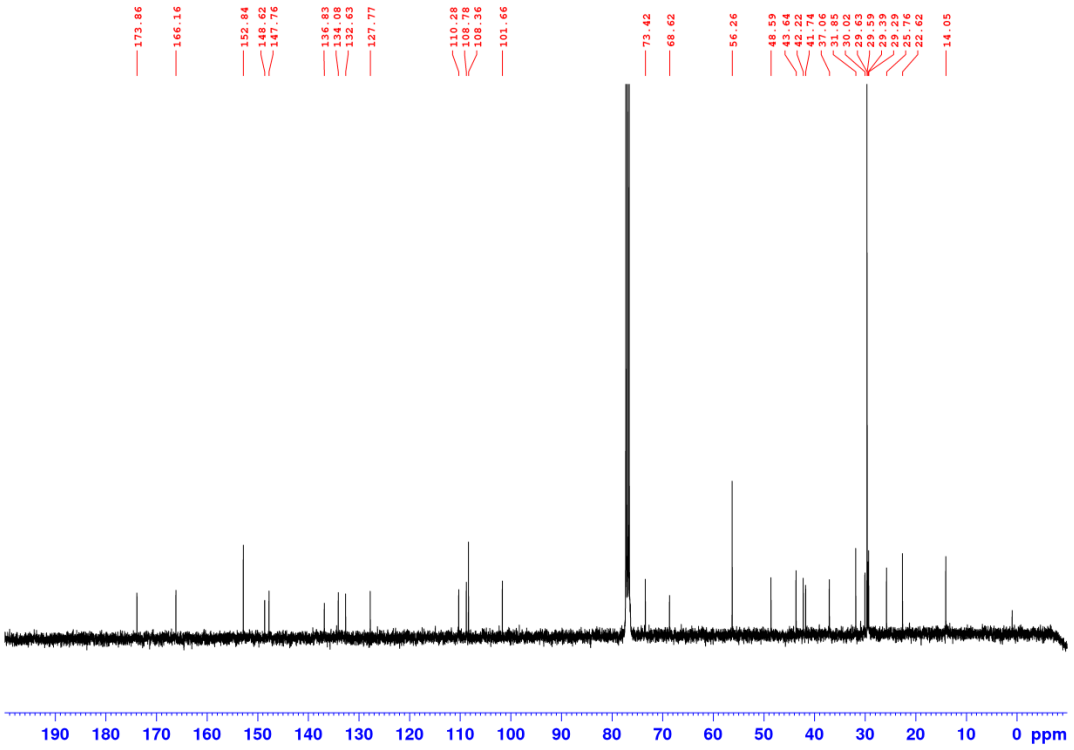
IR 2924, 1776, 1484 cm^{-1}.

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

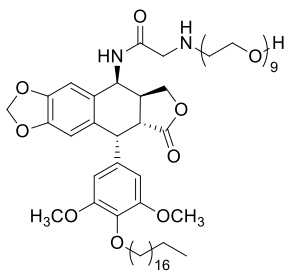
^{1}H NMR (400 MHz, $CDCl_3$) δ 6.74 (s, 1H), 6.64 (d, *J* = 7.3 Hz, 1H), 6.56 (s, 1H), 6.27 (s, 2H), 6.01 (s, 1H), 6.00 (s, 1H), 5.23 (dd, *J* = 7.4 Hz, *J* = 5.1 Hz, 1H), 4.62 (d, *J* = 4.7 Hz, 1H), 4.42 (t, *J* = 8.1 Hz, 1H), 4.12 (s, 2H), 3.91 (t, *J* = 7.5 Hz, 2H), 3.82 (t, *J* = 8.1 Hz, 1H), 3.73 (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-PEG (**1**)

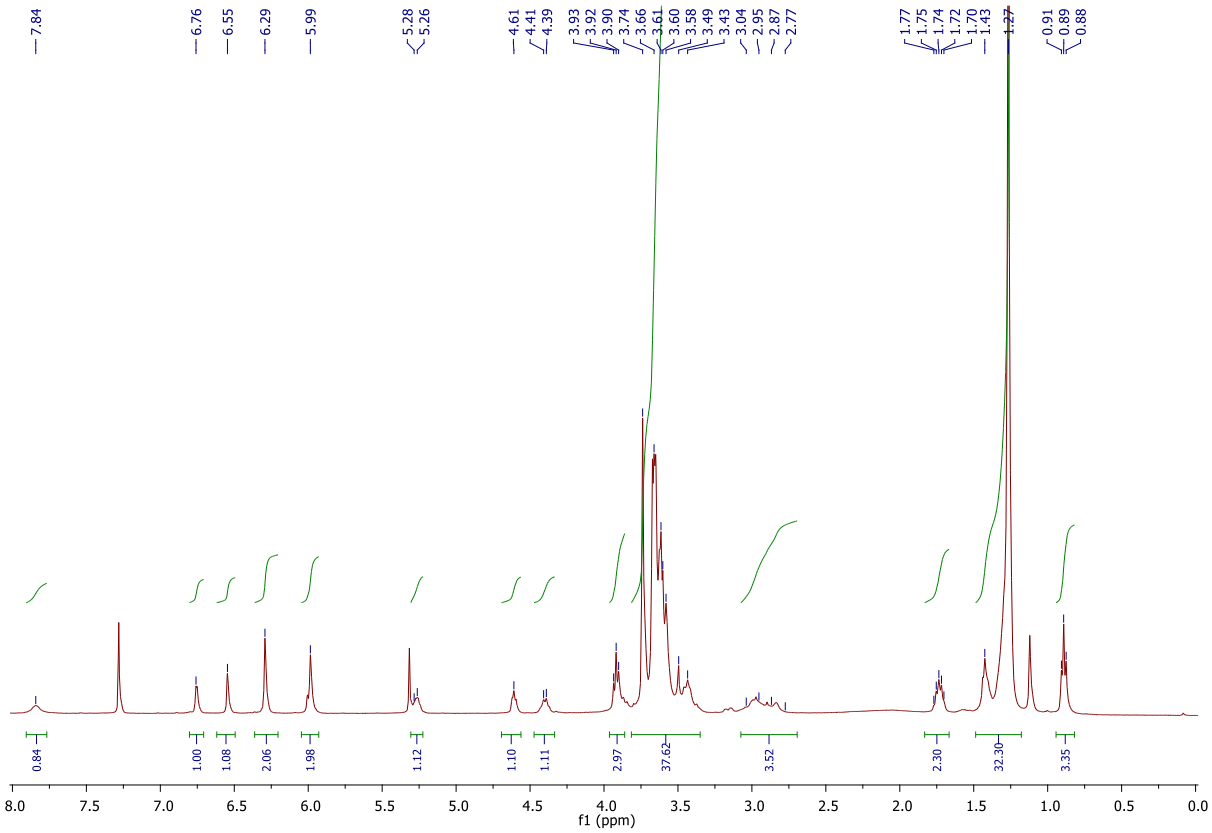


NEt_3 (80.0 µL, 1.5 equiv.) and HO-PEG_{400}-NH_2 (190.0 mg, 1.2 equiv.) were sequentially added to a solution of compound **3** (274.7 mg, 0.38 mmol, 1 equiv.) in 4 mL of dry DMF. The solution was heated to 100 °C for 5 h, cooled to room temperature, quenched with 5 mL of 2 N HCl, and extracted with 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.

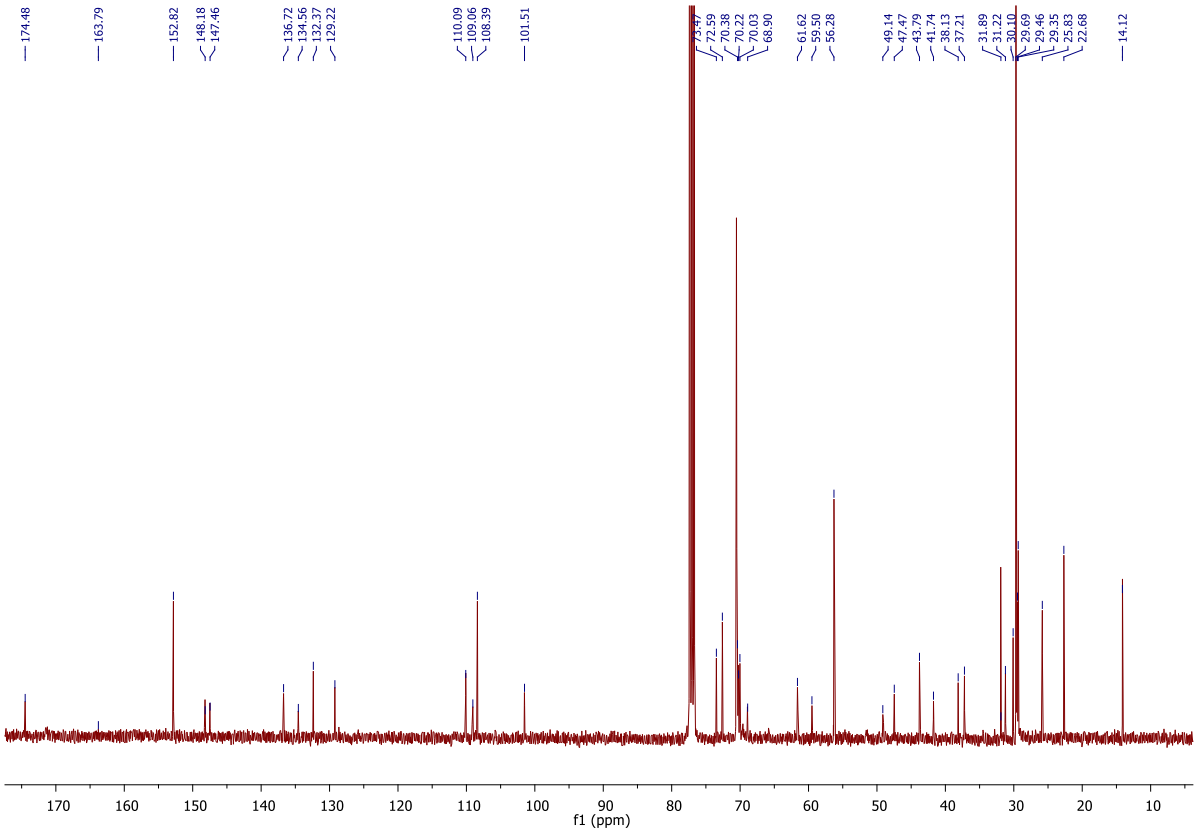
IR 3425, 2931, 1770, 1682 cm^{-1}.

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.



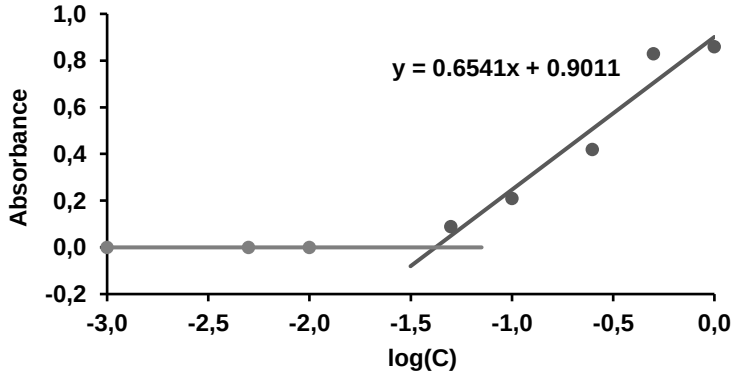
^{13}C NMR (100 MHz, $CDCl_3$) δ 174.5, 163.8, 152.8 (2C), 148.2, 147.5, 136.7, 134.6, 132.4, 129.2, 110.1, 109.1, 108.4 (2C), 101.5, 73.5, 72.6, 70.5 (multiple C), 70.2, 70.1, 70.0, 68.9, 61.6, 59.5, 56.3 (2C), 49.1, 47.5, 43.8, 41.7, 38.1, 37.2, 31.9, 31.2, 30.1, 29.7 (multiple C), 29.5, 29.4, 25.8,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



3. Assembly of the micelle

3.1. Measurement of the critical micelle concentration (CMC)

A concentration range of C_{18}-ePT-PEG amphiphile **1** in deionized water was prepared form 1 mg mL^{-1} to 0.0001 mg mL^{-1} (typically 1, 0.5, 0.25, 0.1, 0.05, 0.01, 0.005, 0.001, 0.0005 and 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).



3.2. Preparation of DiR-loaded micelles

25 mg of C_{18}-ePT-PEG amphiphile **1** was dispersed in 2.5 mL of deionized water before 10 µL of a 25 mg mL^{-1} solution of the fluorescent dye DiR ($\lambda_{ex}/\lambda_{em}$: 750 nm/780 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.

To confirm the amount of DiR encapsulated in the micelles and accurately measure the dye concentration, 10 µL of the DIR-micelle colloid was diluted in 990 µL of MeOH (d100), so as to release the dye from the micelles. Absorbance at 750 nm was then recorded and compared to that of a calibration curve obtained from pure DiR in MeOH.

The 10 mg/mL colloidal suspension of the micelles in pure water is stable at room temperature for more than 3 months, as we observed neither alteration nor aggregation of the nanometric system.

4. References

1) Y. Guminski, M. Grousseaud, S. Cugnasse and T. Imbert, *Synthetic Commun*. **2012**, *42*, 2780-2789.

BIOLOGY

1. Animal model

Female nude mice were purchased at JANVIER LABS (Saint-Berthevin, France). They were housed under standard conditions with food and water *ad libitum* until they weighted *ca.* 25 gr. MDA-MB231 cancer cells were purchased from ATCC (Manassas, VA). 10^6 cells were subcutaneously implanted in the dorsal fat pad in a mixture of 100 µL of PBS (Phosphate Buffer Saline) and 100 µL of Matrigel (BD Bioscience, Le Pont de Claix, France) at 0 °C.

2. *In vivo* Planar near infra-red (NIR) fluorescence imaging

Anesthetized mice were injected intravenously in the tail vein with 150 µL of a solution of DiR-labeled micelles (100 µM for DiR and 10 mg mL^{-1} for the monomer unit of the considered micelles). Whole body planar NIR fluorescence images of dorsal, left lateral and ventral side were obtained before and after the i.v. injection using the planar imaging option of the TomoFluo3D fluorescent tomographic system (developed by CEA/LETI and Cyberstar).[1] Measurements were acquired at different time points (1 min, 10 min, 30 min, 60 min, 3 h, 1 day and 2 days post injection). For the semi-quantitative analysis of fluorescence planar images, region of interest (ROIs) of the desired zone (tumor and leg) were drawn manually using the ImageJ software (http://rsbweb.nih.gov/ij/). For each image, each ROI value was corrected by subtracting the background ROI's value (at the same zone but before injection) and dividing it by the exposure time.

3. *Ex vivo* Planar near infra-red (NIR) fluorescence imaging of organs

48 h post i.v. injection of DiR-labeled micelles, mice were euthanized and organ resection was performed. Planar fluorescence imaging of organs was performed as described above for *in vivo* imaging. For each organ, the mean of fluorescence signal was subtracted by its autofluorescence that corresponds to the mean of fluorescence signal measured for the same organ recovered from a mouse that was not injected.

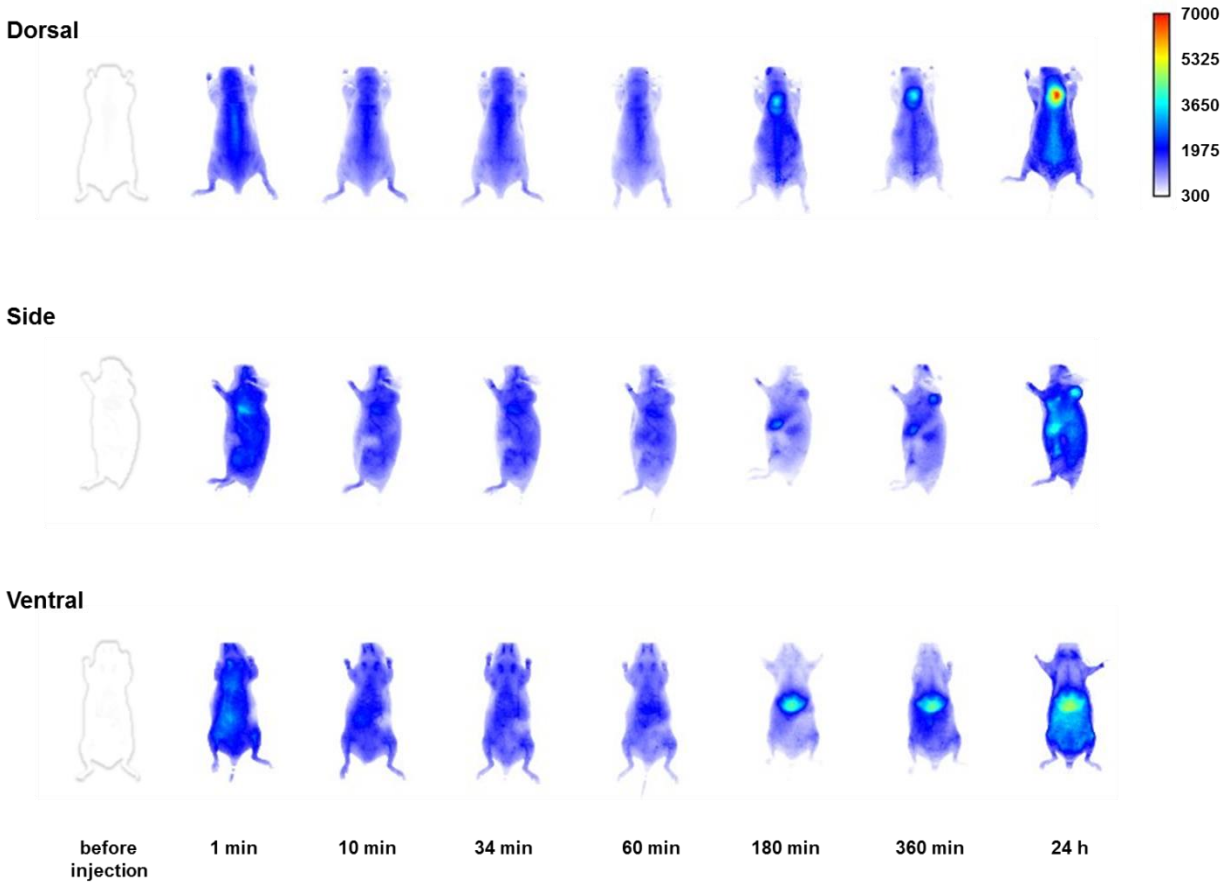


Figure S1. Imaging of the biodistribution of C_{18}-ePT-PEG micelles in MDA-MB-231 xenografted mice at different time points.

4. Cytotoxicity evaluation

In a 96-well plate, 2 x 10^3 A-431 cells (purchased from ATCC - Manassas, VA) diluted in 50 µL of DMEM media containing 10% FBS were deposited per well. After 24 h in a cell incubator, 50 µL of PBS containing C_{18}-ePT-PEG micelles at different concentrations (30, 20, 10, 3, 1, 0.3 and 0.1 µM of ePT-derived amphiphile) was added. The plate was allowed to stand in a cell incubator for 48 h.

20 µL of MTS (tetrazolium compound included in the CellTiter 96 AQueous One Solution cell proliferation assay, Promega) was then added and the plate was analyzed with a Mithras microplate reader (LB 940, Berthold) at 490 nm after 2 h in a cell incubator. The data were compared to wells containing only 2 x 10^3 cells in 50 µL of culture medium + 50 µL of PBS buffer, and revealed with 20 µL of MTS. The experiments were performed in triplicate. IC50 were assessed by fitting the curve with GraphPad Prism 6 using dose-response - Inhibition model (log(inhibitor) *vs* response - variable slope.

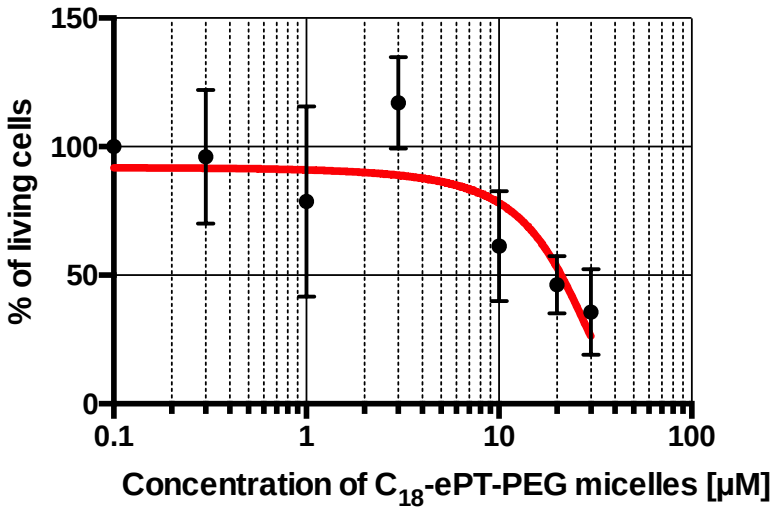


Figure S2. Intrinsic cytotoxicity of C_{18}-ePT-PEG micelles.

For comparison purposes, MTT tests were also performed on 4'-demethylepipodophyllotoxin (4'-DMEP, central core of the micelle) and Etoposide (a commercial formulation based on the 4'-demethylepipodophyllotoxin backbone). 4'-DMEP IC_{50} = 0.78 ± 1.27 µM; Etoposide IC_{50} = 10.77 ± 1.24 µM.

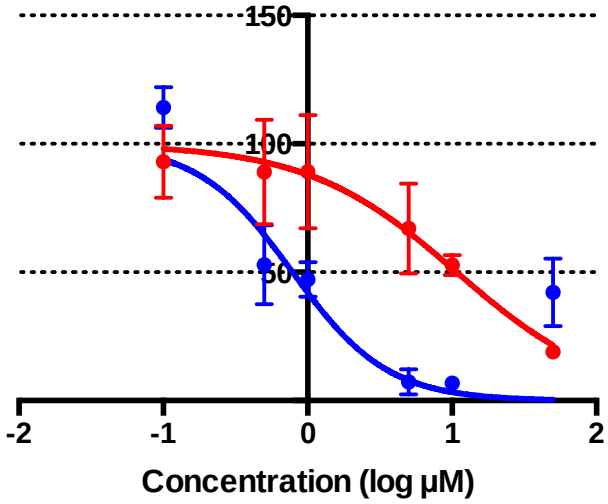


Figure S3. Cytotoxicity of 4'-demethylepipodophyllotoxin (4'DMEP) (–●–) and Etoposide (–●–).

5. References

1) L. Hervé, A. Koenig, A. Da Silva, M. Berger, J. Boutet, J. M. Dinten, P. Peltié and P. Rizo, *Applied Optics*, 2007, **46**, 4896-4906.