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Fabrication of Hydrolase Responsive Diglycerol Based Gemini Amphiphiles for Dermal Drug Delivery Applications

Supplementary Information

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Figure S15. cLSM images from HeLa cells after 24 h incubation of Nile red encapsulated amphiphiles **AM-C_{15}-P_{750}** (a-c) and **AM-C_{15}-P_{1000}** (d-f).

3. References

1. Experimental section

1.1 Materials

All the solvents and chemicals were purchased from Spectrochem Pvt. Ltd. (India), Sigma Aldrich Chemicals (USA), Sisco Research Laboratories (SRL) Pvt. Ltd. (India), and TCI. Immobilized *Candida antarctica* lipase was obtained from Julich Chiral Solutions GmbH (Julich, Germany). All the dyes and drugs were purchased from Fluka Chemie GmbH, (Buchs, Switzerland) and Sigma-Aldrich Chemicals, USA. All the solvents used in the reactions were dried and distilled prior to use. Silica gel (100-200 mesh) was used for the column chromatography. Pre-coated TLC plate (Merck silica gel $60F_{254}$) was used to monitor the progress of the reaction and for visualisation of the spots ceric stain was used. HPLC grade water was used for preparing samples for physiochemical characterization and transport analysis, was purchased from Sigma Aldrich Chemicals, USA.

1.2 Methods and Instrumentation

1.2.1 Infrared-red (IR) spectrum was recorded using Perkin-Elmer FT-IR model 9 and BRUKER ALPHA ATR. The ^{1}H & ^{13}C NMR spectra were recorded on JEOL 400 MHz spectrometer and referencing done using solvent residual peak. The chemical shift values are located on δ scale and coupling constant is in Hertz (Hz). HRMS data were recorded on Q-TOF LCMS-Agilent Technology-6530 and HPLC/MS- Agilent 6210 (Agilent Technologies).

1.2.2 Gel permeation chromatography

The molecular weight M_w, M_n and M_z of the nano-carriers were determined using an Agilent GPC system having PLgel based columns and Agilent 1100 pump. The flow rate was set at 1.2 mL min^{-1} and polystyrene standards were used for weight calibration and THF used as an eluent.

1.2.3 Critical aggregation concentration (CAC) measurements

The CAC of the synthesised nano-carriers was recorded using fluorescence technique and 'Nile red' was used as a model dye. Stock solution was prepared for the dye at a concentration of 3.14 x 13^{-3} M by taking 1 mg of dye in 1 mL HPLC grade THF. 20 µL of stock solution was transferred to each of ten sample vials and a thin film of dye at the bottom of the vial was formed by the evaporation of THF. Simultaneously, 2 ml aqueous solution of amphiphile was prepared at a concentration of 5mg mL^{-1}. Two fold serial dilution of amphiphilic solution was

done to prepare solution of different concentrations and added to the vials having thin film of Nile red, which were allowed for overnight stirring. The solution was filtered through 0.45 μm PTFE filter to remove the trace amount of dye that remains insoluble. The fluorescence was then recorded for all the samples using Cary Eclipse Fluorescence spectrophotometer. Critical aggregation concentration was determined by drawing a plot of fluorescence intensity vs. log[amphiphile conc.].

1.2.4 Dynamic light scattering (DLS) and Transmission Electron Microscopy (TEM) measurements

Size of the nanoparticles was determined by dynamic light scattering method using Malvern Zetasizer Nano ZS analyzer consisting of thermostated sample chamber of 4 mW He-Ne laser, λ = 633 nm, using back scattering detection (scattering angle $\theta = 173^{o}$) with an avalanche photodiode as a detector. Amphiphilic aqueous solution at a concentration of 5 mg mL^{-1}, well above their CAC's, was prepared by constant stirring for 24 h followed by filtration using PTFE filter and DLS measurement was recorded in disposable micro BRAND UV-Cuvettes. Measurements were obtained in triplicate with 10 runs for each measurement and the mean of the recorded values was used. The morphology of nanostructures was further analyzed by TEM using a TECNAI G2-30 U-TWIN TEM instrument (FEI, Eindhoven, The Netherlands) with an acceleration voltage of 200 kV. The aqueous solution of amphiphile was sonicated and was drop-coated on the formvar-coated 200 mesh copper grids (Ted Pella, USA) and then the sample was analysed by TEM.

1.2.5 Drug/Dye encapsulation and quantification

Nile red, nimodipine and curcumin encapsulation was studied by the thin film method, using UV-Vis and fluorescence spectrometer. All the amphiphilic solutions were prepared at a concentration of 5 mg mL^{-1} by stirring for 1-2 h and taking either 0.12 mg of Nile red/1 mg of nimodipine or curcumin. The required amount of drug/dye was first dissolved in THF followed by the solvent evaporation to form a thin layer and then 1 mL of an aqueous solution of amphiphile was added to it. All the samples were kept in overnight, stirring at room temperature, so as to allow encapsulation of dye/drug into amphiphiles and the non-encapsulated drug/dye was filtered through 0.45 μm PTFE filter. For quantification, 200 μL of the Nile red encapsulated samples and 400 μL of drug samples were taken in sample vials were lyophilized and redissolved in HPLC grade methanol (for Nile red) and HPLC grade ethanol (for nimodipine/curcumin). The absorbtion (200-800 nm) was recorded on a Cary-300 series

UV-Vis (Agilent Technologies) spectrophotometer using standard quartz cuvettes with a path length of 1 cm. Fluorescence measurement (450-800 nm) was carried on a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies) with a slit width of 5 nm for Nile red. Origin 8 software was used for data analysis.

1.2.6 Cytotoxicity study

Cytotoxicity of amphiphiles was analysed at a final concentration of 0.5, 0.1 and 0.05 mg mL^{-1} using the cell counting kit 8 (CCK-8) from Sigma Aldrich according to manufacturer's instructions and HeLa (DSMZ no.: ACC 57) as well as MCF7 (DSMZ no.: ACC 115) cells. Cells were routinely propagated in supplemented DMEM (Dulbecco′s Modified Eagle′s Medium supplemented with 1% penicillin/ampicillin, 10% fetal bovine serum, 1% glutamine and 4.5mg/ml glucose all from Gibco) and subcultured twice a week when 70% to 90% confluent. For assessing cytotoxicity, pre-dilutions of the amphiphiles were prepared in MilliQ water and 4.500 cells in 90 µl DMEM were seeded in a 96-well plate and cultured overnight at 37 °C and 5% CO_2. After 24h, 10 µl of the amphiphile prediltuions or control solutions (the solvent MilliQ water, 10% SDS or supplemented DMEM) were added in triplicates to the cells in the 96- well plate. Additional samples were also added to 1 well containing no cells for later on background correction. Cells were incubated for another 24 h at 37 °C and then 10 µL of CCK-8 solution was added. After 3 h absorbance was recorded at a measurement wavelength of 490 nm and a reference wavelength of 630 nm using a Tecan plate reader (Infinite Pro200, TECAN-reader Tecan Group Ltd., Mannedorf, Switzerland). The assays was repeated in three consecutive weeks. The cell viability was calculated by setting the non-treated control to 100 % and the non-cell control to 0 % after subtracting the background using Microsoft Excel. GraphPad Prism was used for data visualization. In the graph, the mean of the three repetitions with standard deviation is shown.

1.2.7 Cellular uptake study

Confocal laser scanning fluorescence microscopy was used to analyse the cellular uptake of Nile red encapsulated amphiphiles in HeLa cells. For experiment, HeLa cells cultured as mentioned above were seeded in 8-well ibidi µ-slides in DMEM and incubated at 37 °C and 5% CO_2 before amphiphiles were added at a test concentration of 0.5 mg mL^{-1}. After 4 h and 24 h incubation, respectively, cells were washed and cell nuclei were stained with 1 µg mL^{-1} Hoechst 33342 and images were taken with an inverted confocal laser scanning microscope Leica DMI6000CSB SP8 (Germany).

1.2.8 Skin penetration study

The skin penetration of amphiphile **AM-C_{15}-P_{750}** (0.5 mg/mL) loaded with Nile red (loading: 0.01 w/w %) was performed on a freshly excised human skin. Skin obtained from cosmetic surgeries with informed consent of donors and ethic approval of the Charité – Universitätsmedizin Berlin (approval EA1/135/06, renewed in November 2019). Commercially available base cream containing (0.01 w/w %) Nile red served as a control. Briefly, skin from three different donors was cut in pieces of 1.5 × 1.5 cm and fixed on the surface of Styrofoam blocks using needles. The prepared samples were kept in a box with wet towels to preserve humidity of the skin. Subsequently, 40 µL of the compound **AM-C_{15}-P_{750}** and cream (according to infinite dose approach) were applied on 1 cm^2 skin area. After 18 h incubation at 37 °C, 5% CO_2, and 95% humidity, the surface of the skin was cleaned with cotton swabs. Untreated skin areas were removed, skin was frozen in liquid nitrogen, and stored at −20 °C. For cryosectioning, skin samples were embedded in the tissue freezing medium (Leica Microsystems, Germany) and sections of 8 µm thickness were obtained using a microtome (2800 Frigocut-N, Reichert-Jung, Heidelberg, Germany). Skin samples were subjected to confocal laser microscope (LSM 700, Zeiss, Germany). Pictures of at least 15 sections per donor were taken with a charge coupled device (CCD) camera always using the same settings. The mean fluorescence intensity (MFI) of Nil red was analysed using ImageJ software (version 1.47, National Institute of Health, Bethesda, MD, USA) for areas of stratum corneum and viable epidermis. The averages of at least 15 MFI values for each sample and controls from the three donors were calculated. Averages were plotted in diagrams using Microsoft Excel (Microsoft Corp. Redmond, WA, USA).

1.2.9 Enzyme triggered release study

The enzyme-responsive release of the encapsulated guest from the synthesised amphiphiles was studied using fluorescence measurements (Cary eclipse spectrophotometer, Agilent Technologies) with the Nile red as a model dye. The sample was prepared by the similar procedure as followed in quantification. After encapsulation of dye, non-encapsulated dye was removed through 0.45 µm PTFE filter, few drops of *n*-butanol and 200 wt. % of the enzyme (immobilized *Candida antarctica* lipase *i.e.* Novozym 435) were added. The solution was incubated at 37 °C and 200 rpm under dark conditions. Fluorescence data were recorded in an interval of time till all the dye will come out.

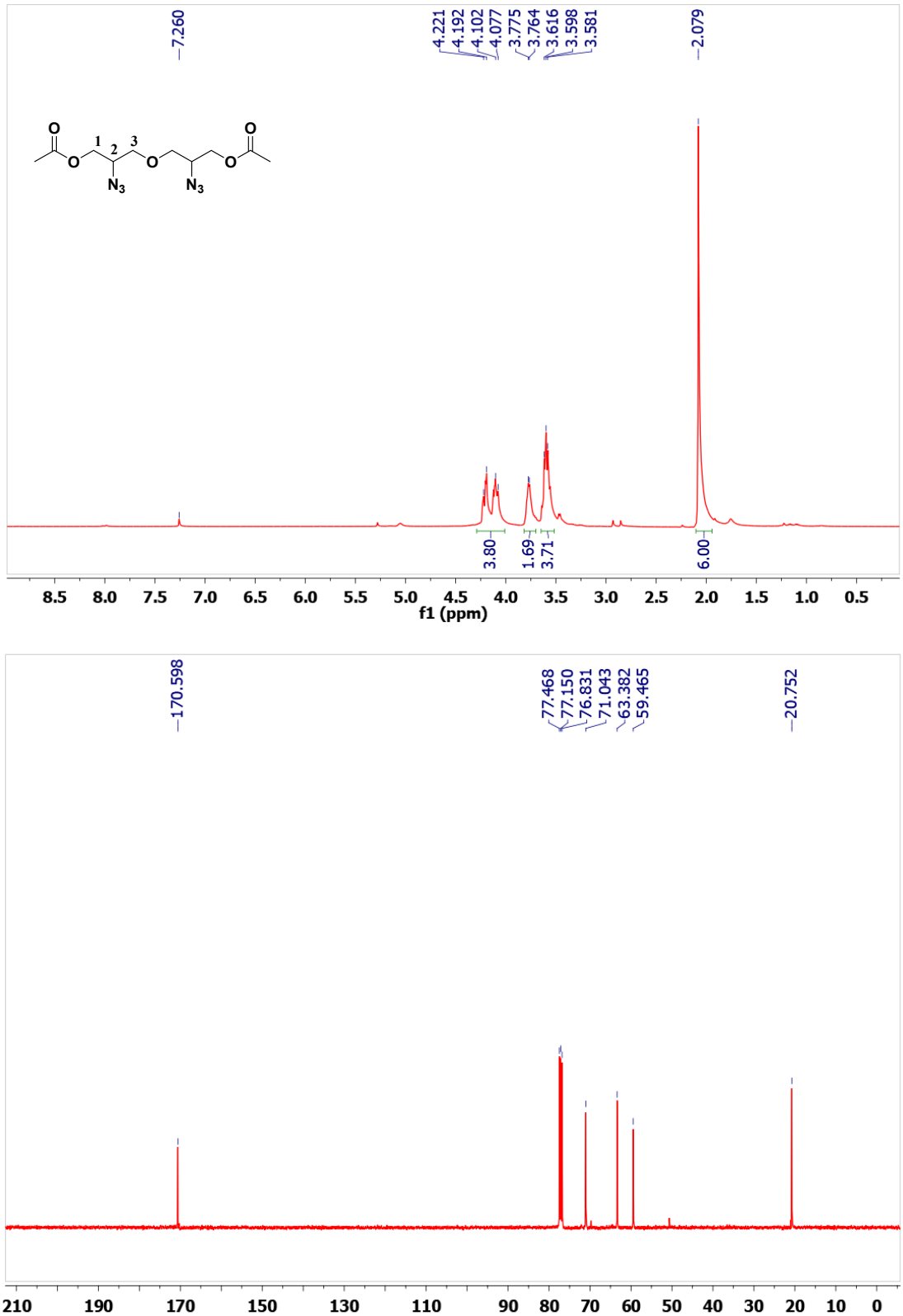

Figure S1. ^{1}H & ^{13}C NMR spectra of oxybis(2-azidopropane-1,3-diyl) diacetate (**4**) in $CDCl_3$.

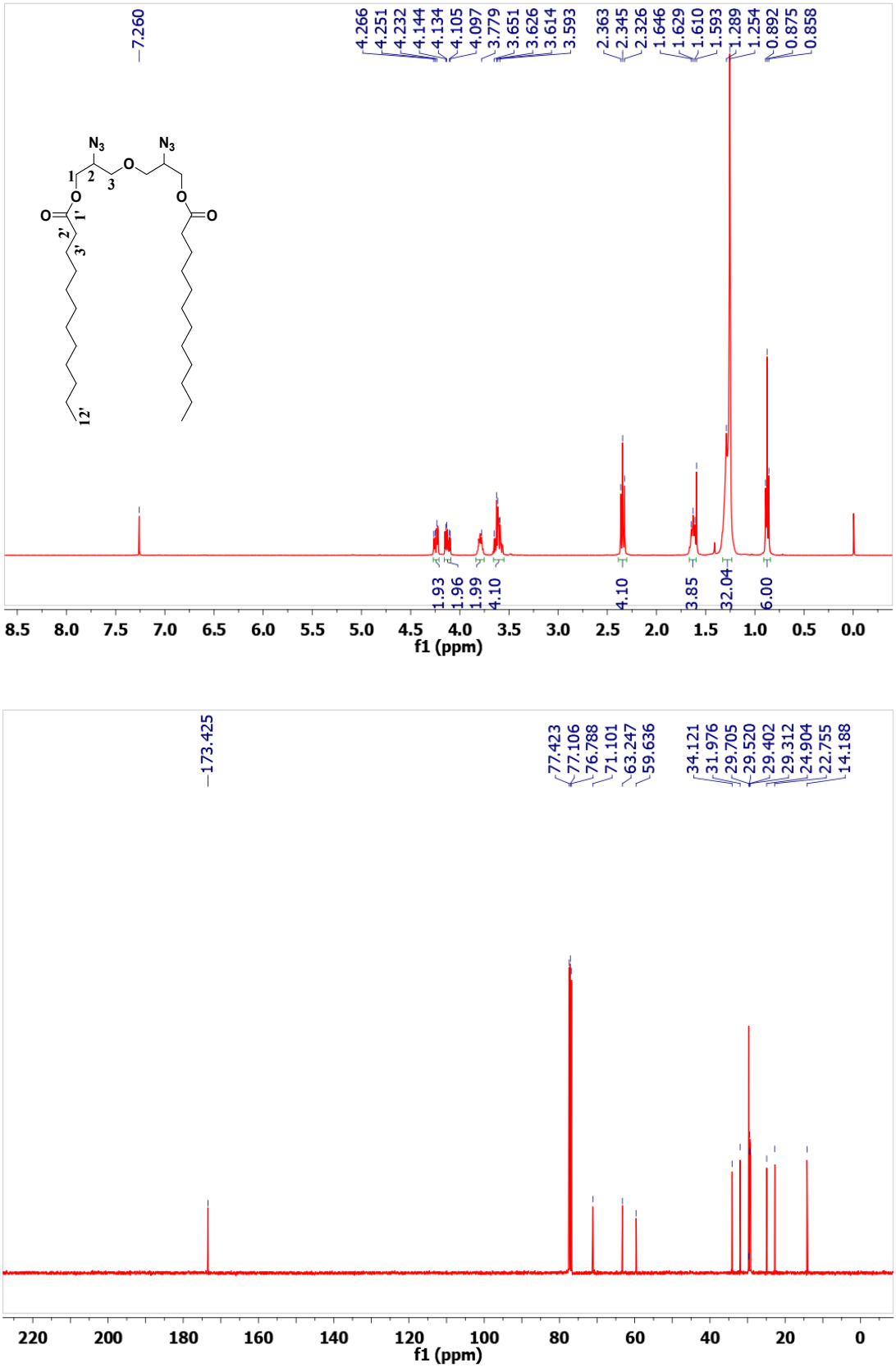

Figure S2. ^{1}H & ^{13}C NMR spectra of oxybis(2-azidopropane-1,3-diyl) didodecanoate (**6**) in $CDCl_3$.

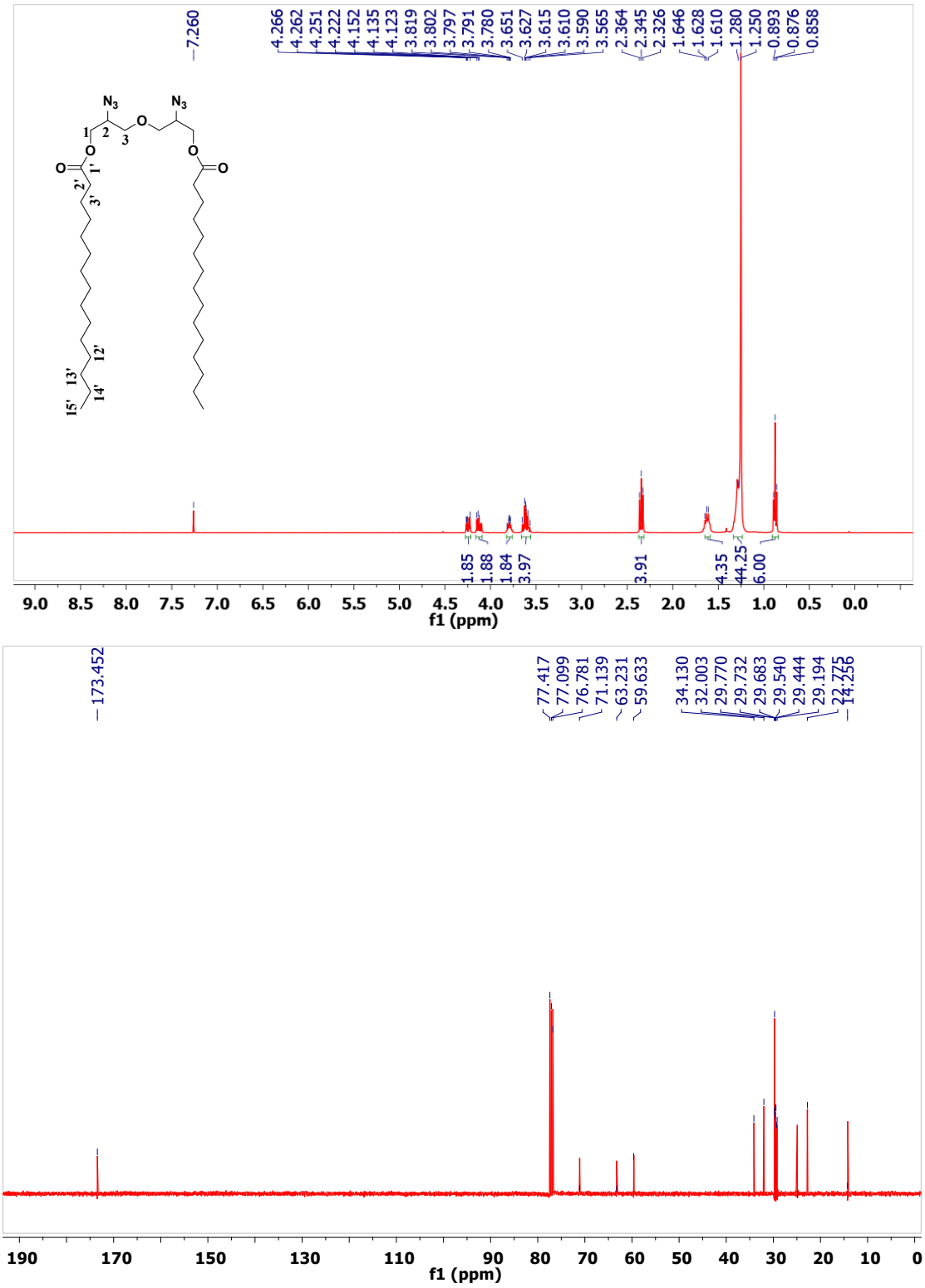

Figure S3. ^{1}H & ^{13}C NMR spectra oxybis(2-azidopropane-1,3-diyl) dipentadecanoate (**7**) in $CDCl_3$.

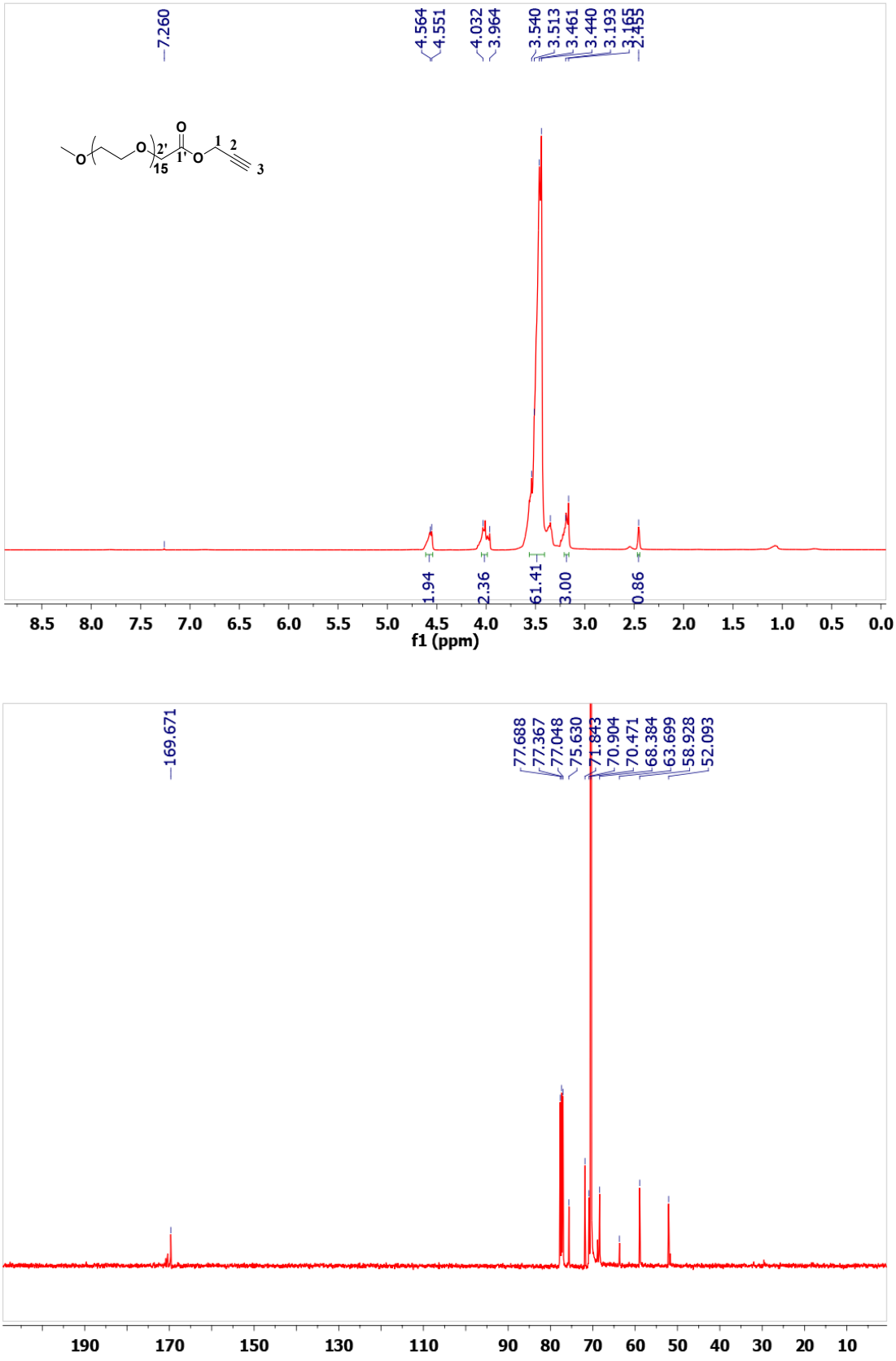

Figure S4. ^{1}H & ^{13}C NMR spectra compound **12** (mPEG-750 alkyne) in $CDCl_3$.

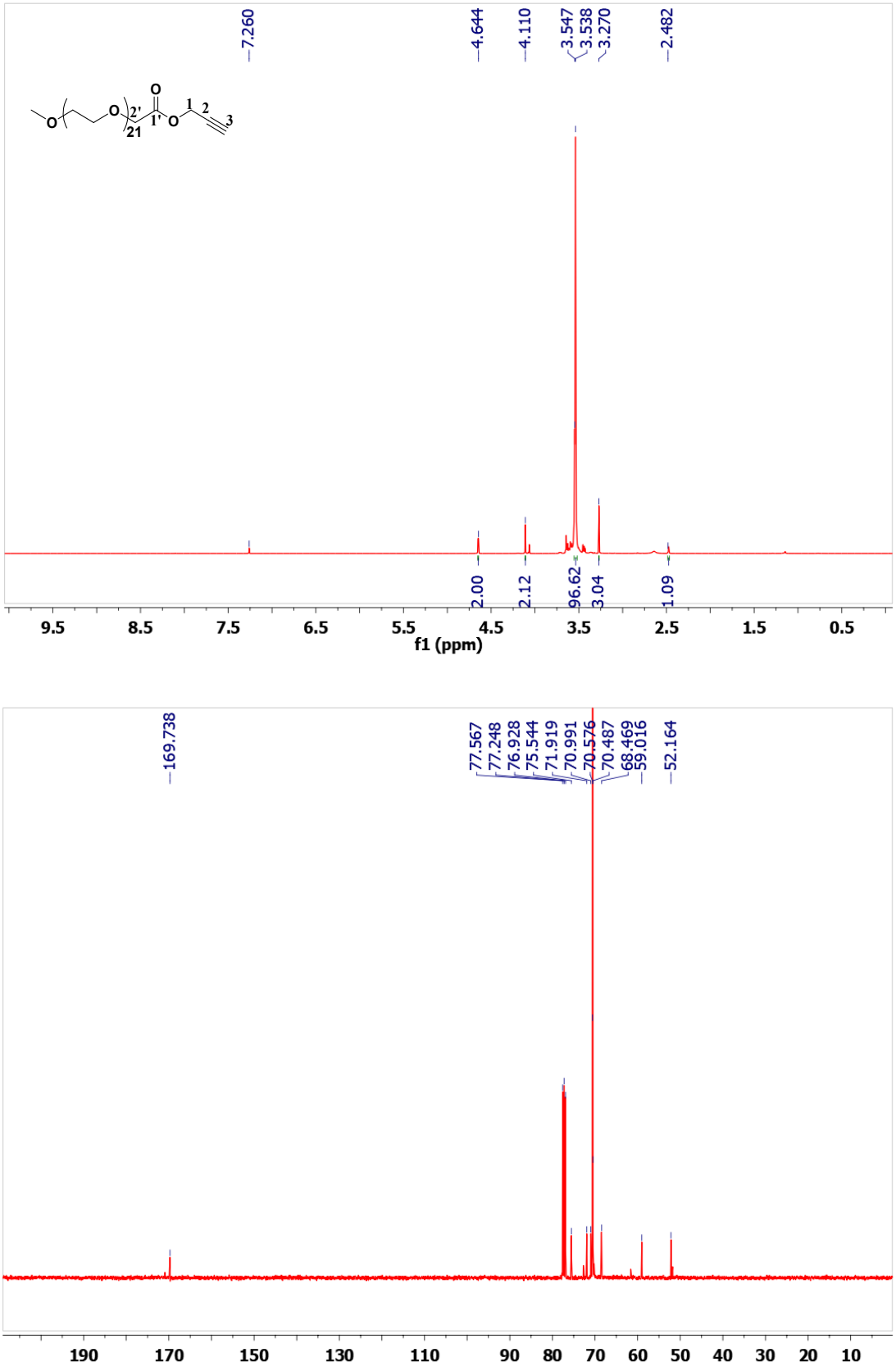

Figure S5. ^{1}H & ^{13}C NMR spectra of compound **13** (mPEG-1000 alkyne) in $CDCl_3$.

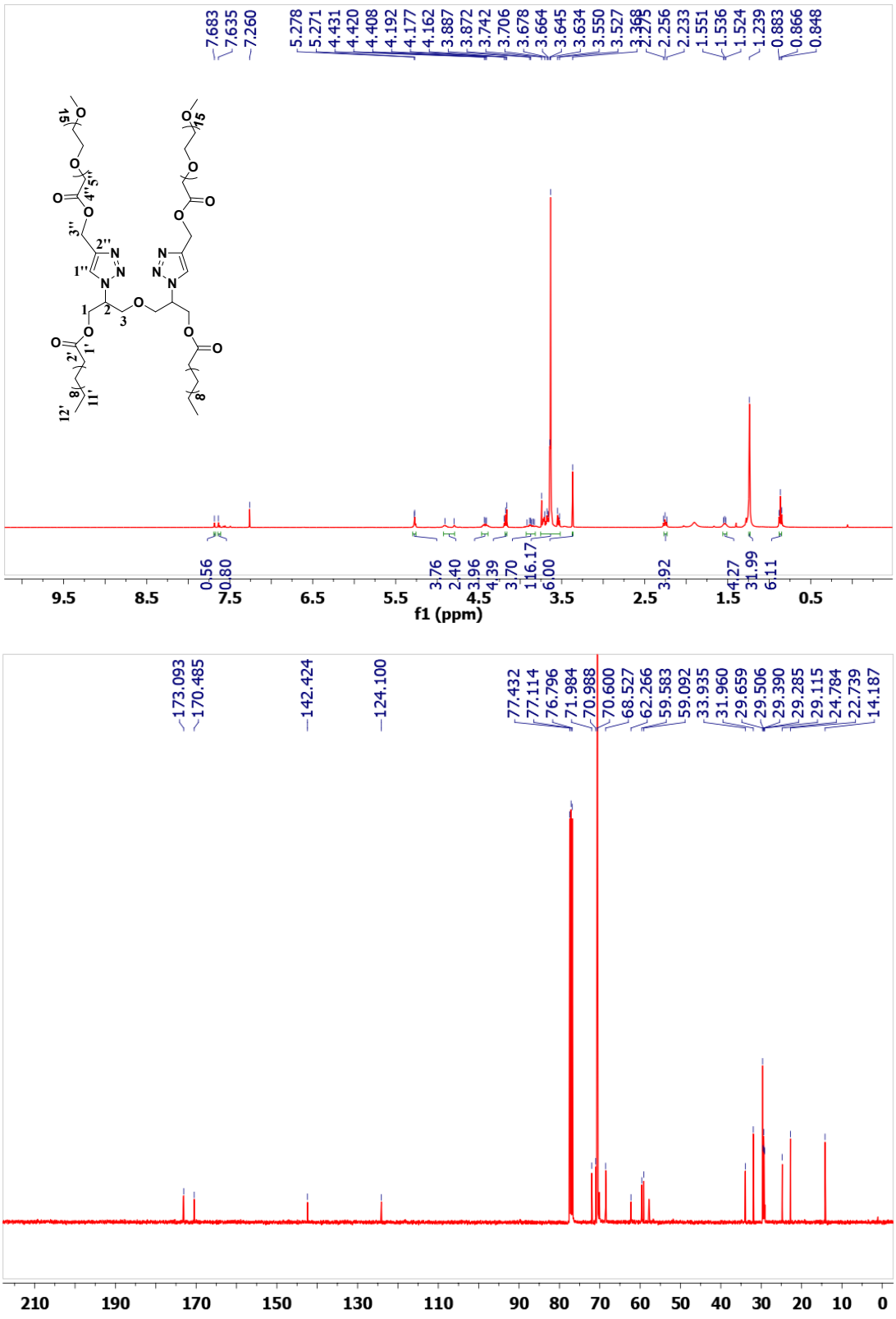

Figure S6. ^{1}H & ^{13}C NMR spectra of amphiphile **AM-C_{12}-P_{750}** in $CDCl_3$.

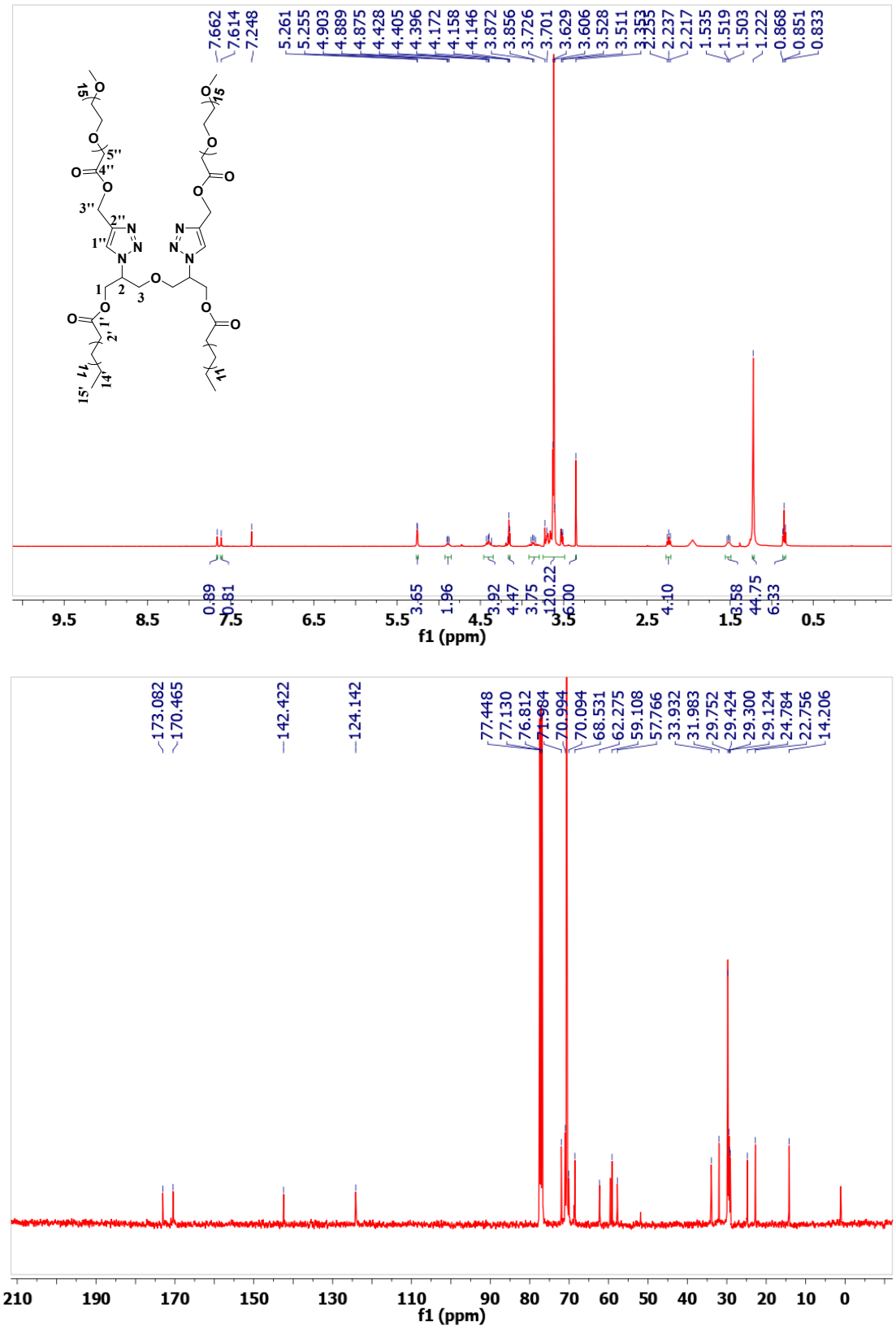

Figure S7. ^{1}H & ^{13}C NMR spectra of amphiphile **AM-C_{15}-P_{750}** in $CDCl_3$.

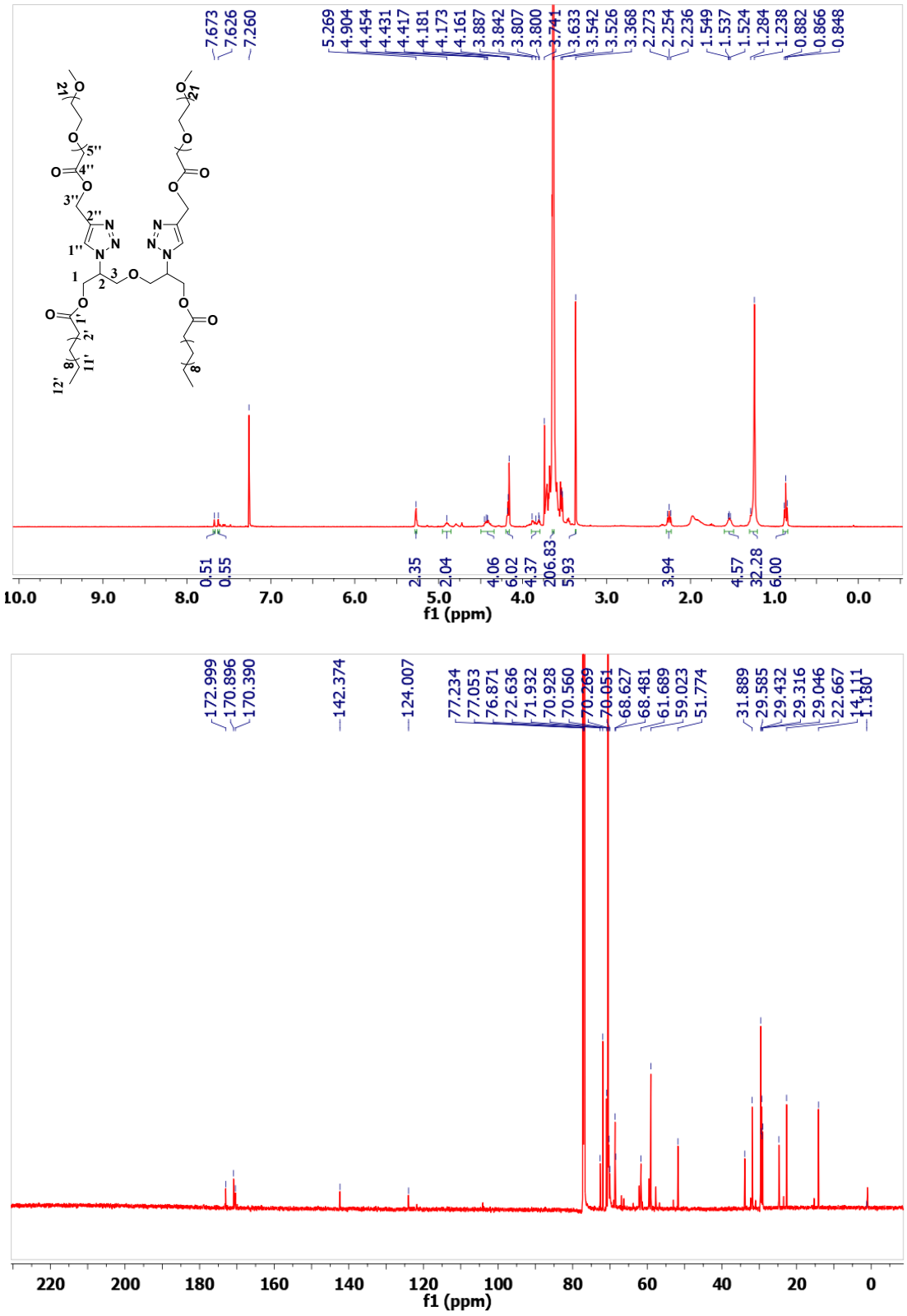

Figure S8. ^{1}H & ^{13}C NMR spectra of amphiphile **AM-C_{12}-P_{1000}** in $CDCl_3$.

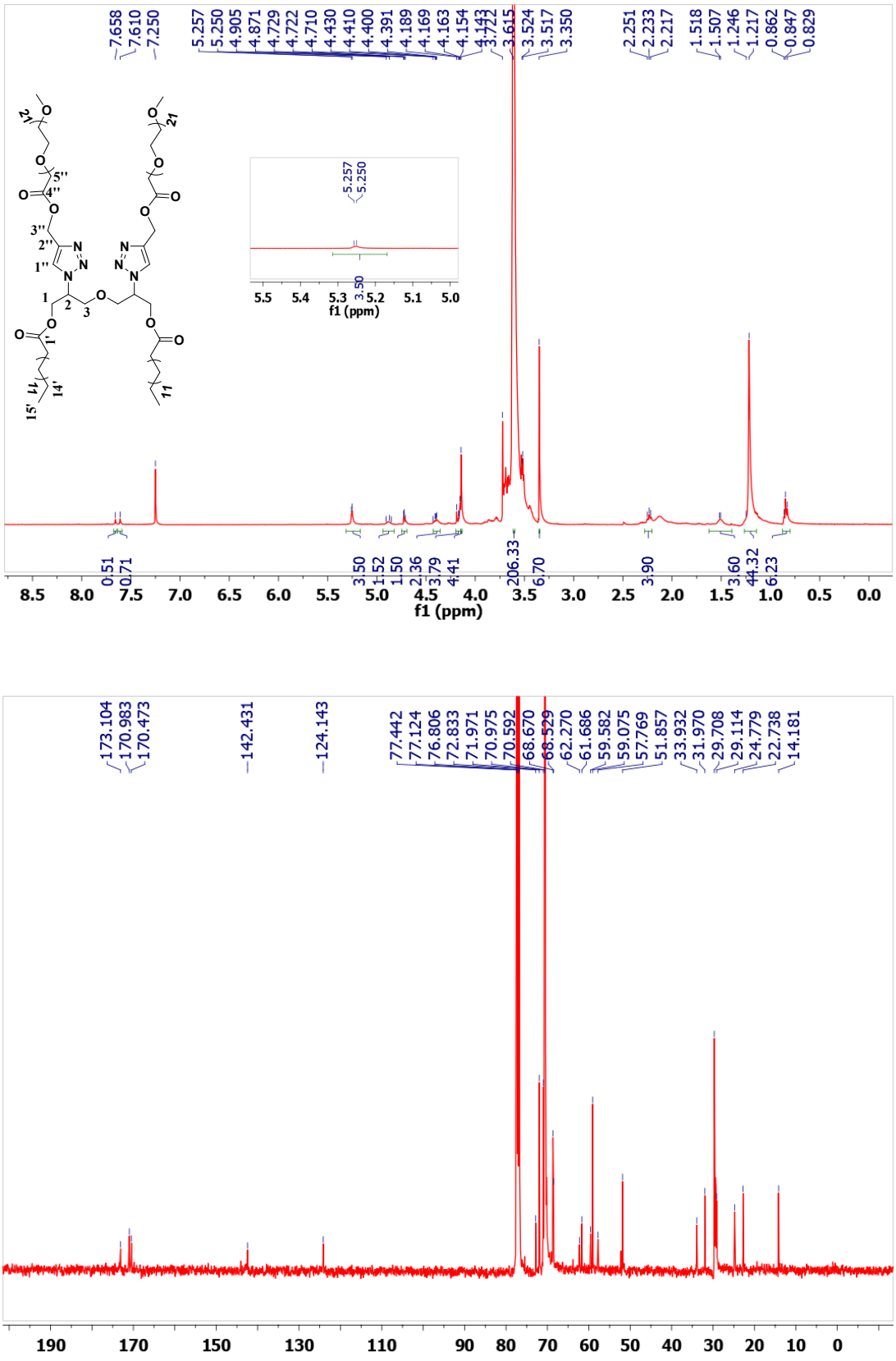

Figure S9. ^{1}H & ^{13}C NMR spectra of amphiphile **AM-C_{15}-P_{1000}** in $CDCl_3$.

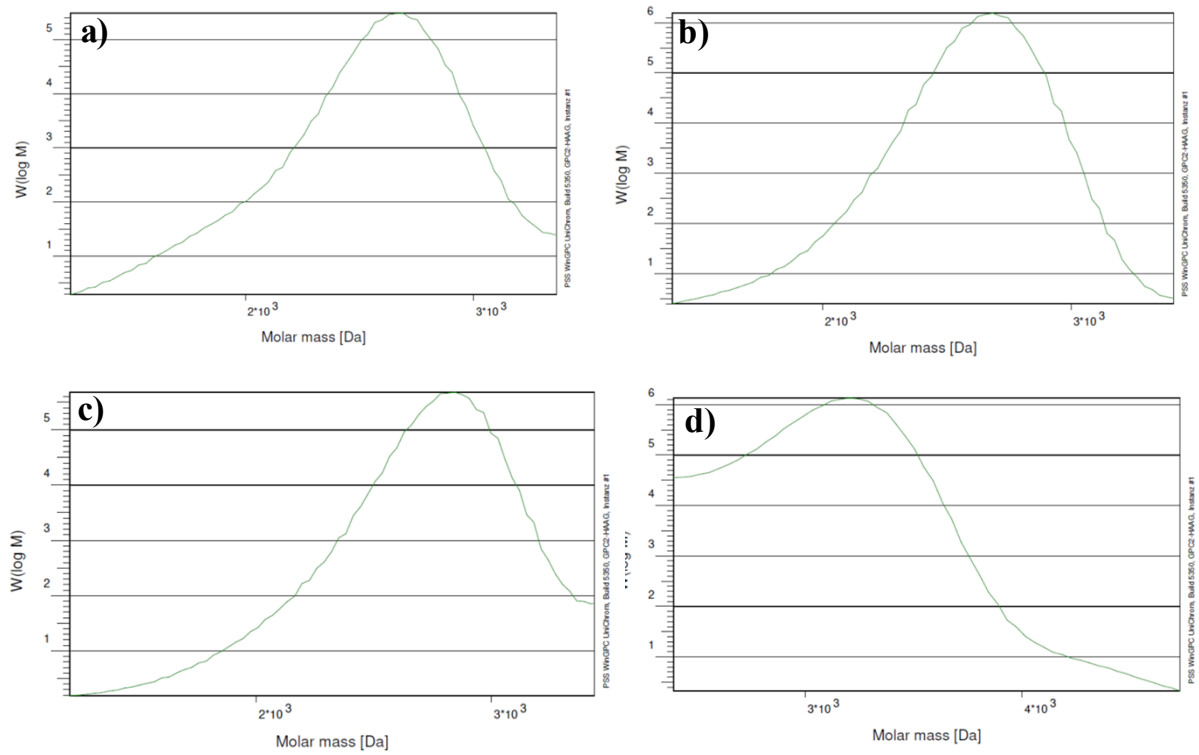

Figure S10. Gel permeation chromatogram of synthesised nanocarriers (a) **AM-C_{12}-P_{750}**, (b) **AM-C_{15}-P_{750}**, (c) **AM-C_{12}-P_{1000}** (d) **AM-C_{15}-P_{1000}**.

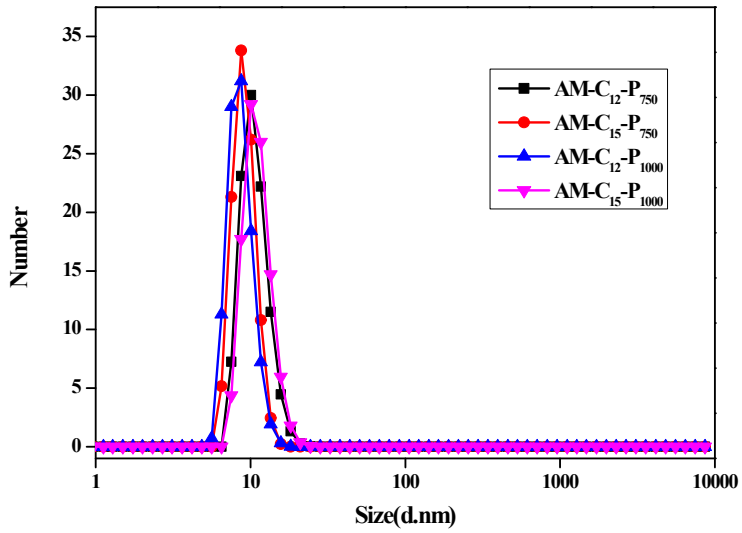

Figure S11. DLS number profile of all amphiphiles.

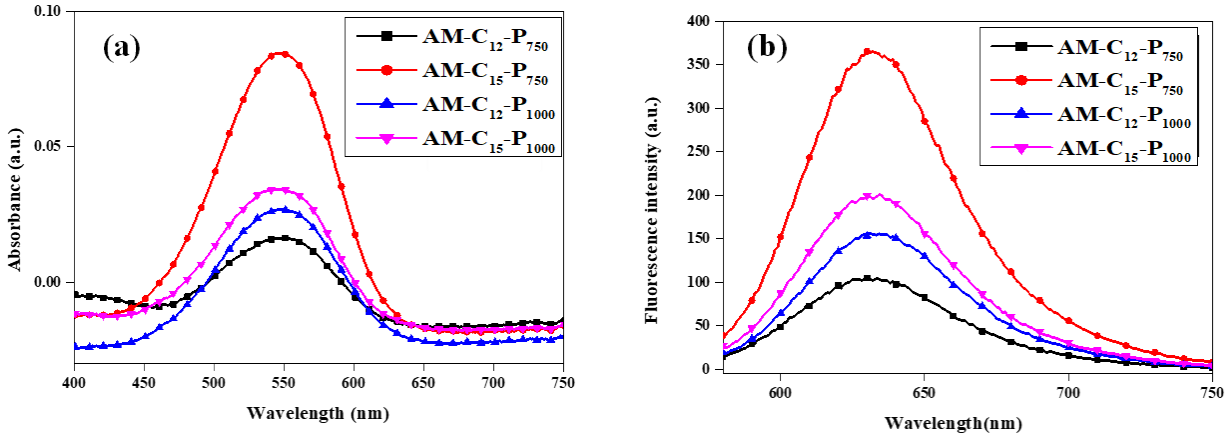

Figure S12. (a) UV absorbance and (b) Fluorescence spectra of Nile red loaded all amphiphiles in methanol.

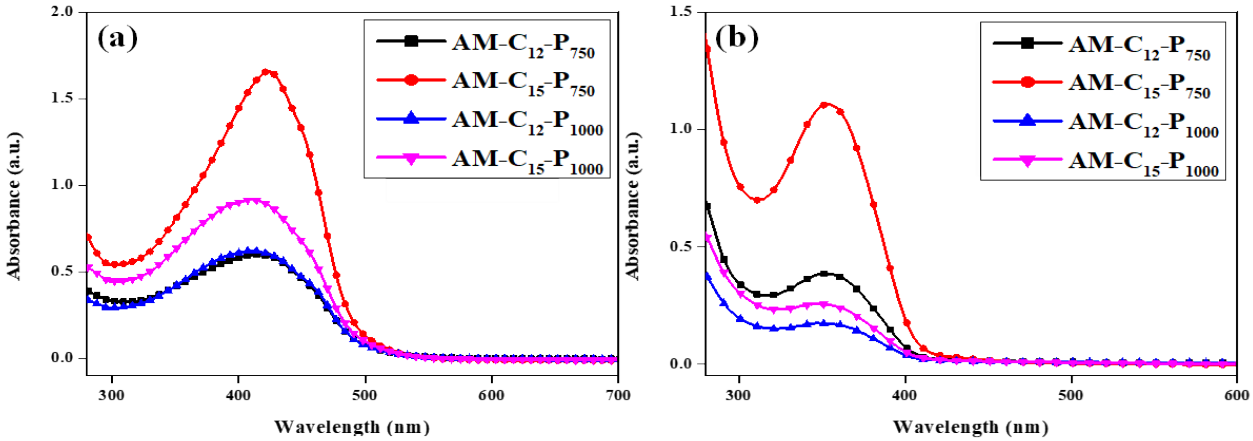

Figure S13. (a) UV absorbance of Curcumin encapsulated of all amphiphiles in methanol; (b) UV absorbance of nimodipine encapsulated of all amphiphiles in ethanol.

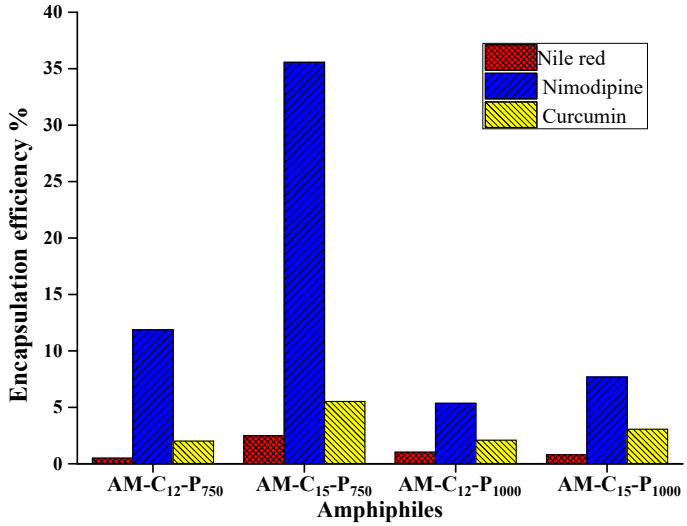

Figure S14. Encapsulation efficiency of amphiphiles for Nile red, nimodipine and curcumin.

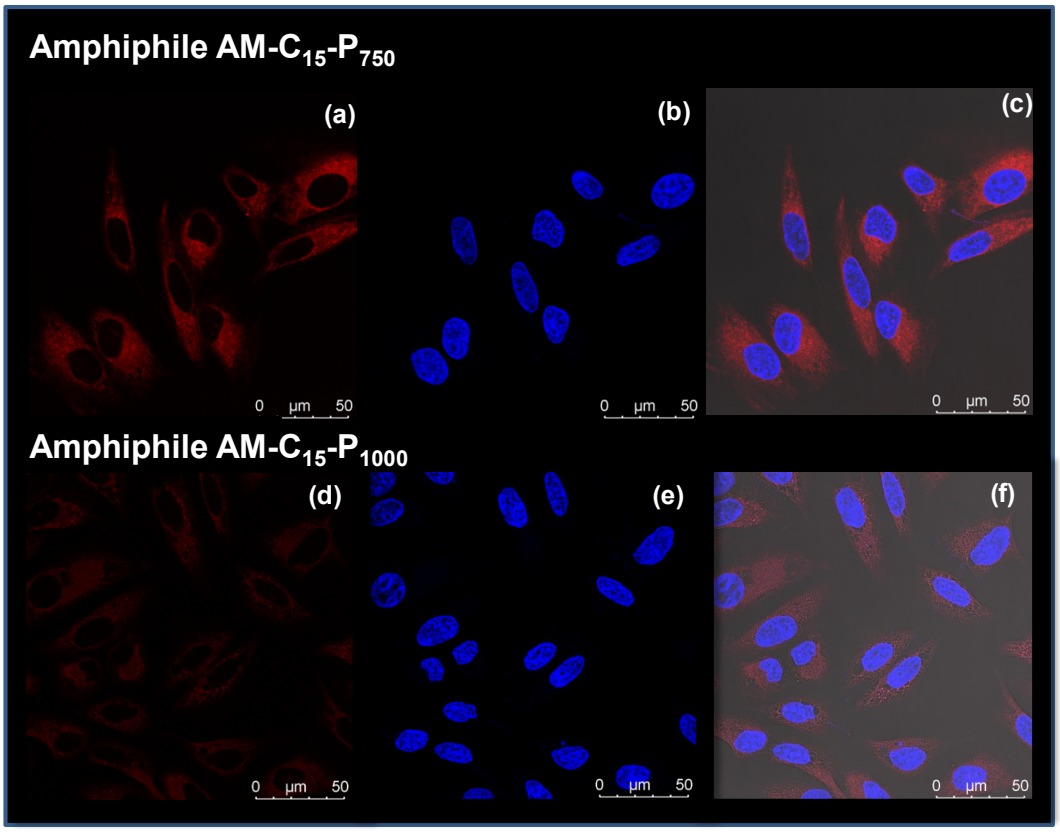

Figure S15. Confocal laser scanning fluorescence microscopy images from HeLa cells after 24 h incubation of Nile red encapsulated in amphiphiles **AM-C_{15}-P_{750}** (a-c) and **AM-C_{15}-P_{1000}** (d-f). In the images Nile red is shown in red colour and the nucleus stained with Hoechst 33342 in blue colour. The scale bar represents 50 µm.