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The 'click' synthesis of new cytotoxic conjugate based on *meso*-arylporphyrin and Erlotinib

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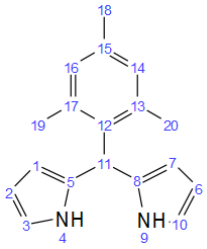
Materials and methods

2,4,6-Trimethylbenzaldehyde (Sigma–Aldrich), pyrrole (Chimmed), *p*-acetoamidobenzaldehyde (Sigma–Aldrich), TFA (Acros Organics), boron trifluoride etherate (Sigma–Aldrich), 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) (Acros Organics), hydrochloric acid (Aldosa), sodium bicarbonate (Aldosa), sodium nitrite (Chimmed), sodium azide (Sigma–Aldrich), zinc acetate dehydrate (Chimmed), sodium L-ascorbate (Sigma-Aldrich), copper sulfate pentahydrate (Chimmed), erlotinib hydrochloride (Sigma-Aldrich), sodium chloride (Chimmed), pyridine (Aldosa) were used for the synthesis. All commercially available reagents were applied without additional purification. Solvents were purified according to the standard procedures. Dichloromethane was distilled over phosphorus pentoxide; pyrrole and THF over calcium hydride. The individuality of the obtained compounds was determined by TLC on TLC silica gel 60 F254 plates (Merck, Rahway, NJ, USA). Silica gel G60 0.04–0.064 mm/230–400 mesh ASTM (Macherey-Nagel GmbH & Co. KG, Düren, Germany) was used for column chromatography. Electronic absorption spectra of compounds were recorded using a HACH DR-4000V (Hach-Lange, Ames, IA, USA) spectrophotometer operating in 320–800 nm spectral range with 10 mm quartz cells at room temperature. Stationary fluorescence spectra were recorded using a Cary Eclipse Fluorescence Spectrometer (Agilent Technologies Bayan Lepas Free, Penang, Malaysia) under similar conditions at a monochromator slit width of 5 nm and an excitation wavelength corresponding to the Soret band maximum. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker MSL-300 pulsed Fourier spectrometer (FRG) with an operating frequency of 300 MHz; measurements were performed on the δ scale using tetramethylsilane or boron trifluoride etherate as an internal standard and $CDCl_3$ or DMSO-d_6 as a solvent. MALDI mass spectra were registered on a Bruker autoflex speed time-of-flight (TOF) mass spectrometer (Bruker Daltonics Inc., Bremen, Germany) equipped with a solid-state UV laser with λ = 355 nm (frequency of 1 kHz, 1000 pulses for each sample) and a reflectron in the mode of positively charged ions registration. IR spectra (4000–400 cm^{-1}) were received on Infralum FT 08 Fourier spectrometer (Lumex) with a resolution of 1 cm^{-1}. SEM imaging was performed using TESCAN AMBER GMH Electron Microscope (Czech Republic).

Synthesis and ^{1}H-, ^{13}C-NMR, mass and IR spectral data



(2,4,6-Trimethylphenyl)(dipyrrolyl)methane **1***:* Pyrrole (100 mL, 1.4 mol) and 2,4,6-trimethylbenzaldehyde (4.3 g, 29 mmol) were mixed, and the reaction mixture was stirred under argon atmosphere for 15 minutes. Then, TFA (215 μL, 2.9 mmol) was added, and this was stirred for another 1 hour under inert atmosphere at room temperature. The excess pyrrole was removed under vacuum. The target product was purified by column chromatography on G60 silica gel, which was eluted with a CH_2Cl_2–hexane, 2:1 (v/v). The gray powder **1** was triturated with cyclohexane, filtered and dried *in vacuo* over P_2O_5. The yield was 62%. R_f 0.4 (CH_2Cl_2–hexane, 2:1). ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C) *δ*: 2.08 (s, 6H, *o*-mesityl-CH_3), 2.30 (s, 3H, *p*-mesityl-CH_3), 5.94 (s, 1H, meso-H), 6.01–6.04 (m, 2H, β-pyrrole), 6.19 (m, 2H, β-pyrrole), 6.68 (dd, *J* = 2.7, 1.6 Hz, 2H, α-pyrrole), 6.88 (s, 2H, *m*-mesityl-H), 7.96 (bs, 2H, NH). ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C) *δ*: 138.15 (C15), 136.02 (C13, C17), 134.93 (C5, C8), 133.94 (C12), 126.36 (C16, C14), 125.88 (C1, C7), 119.63 (C3, C10), 107.36 (C2, C6), 50.85 (C11), 21.94 (C19, C20), 20.77 (C18).

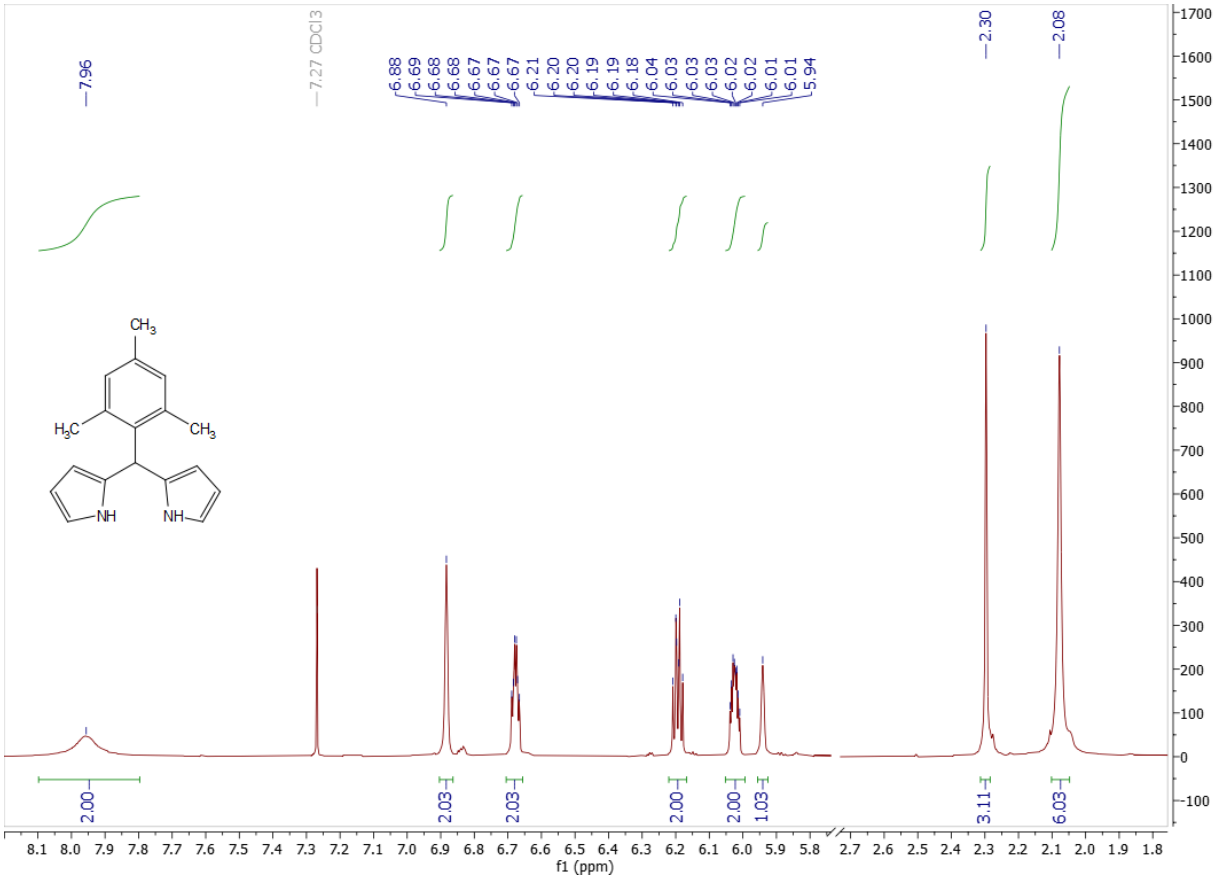


Figure S1 ^{1}H NMR spectrum of compound **1** in $CDCl_3$ at 300 MHz.

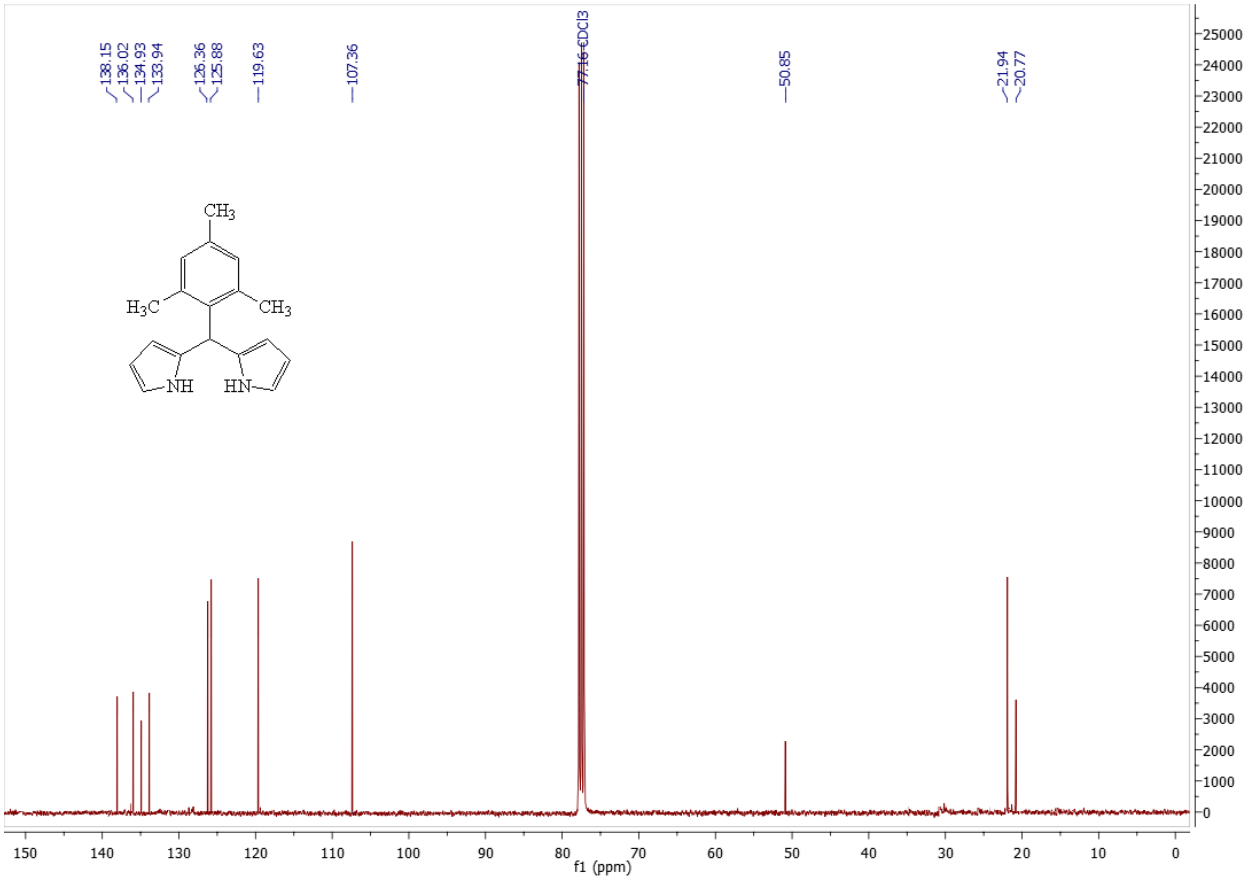
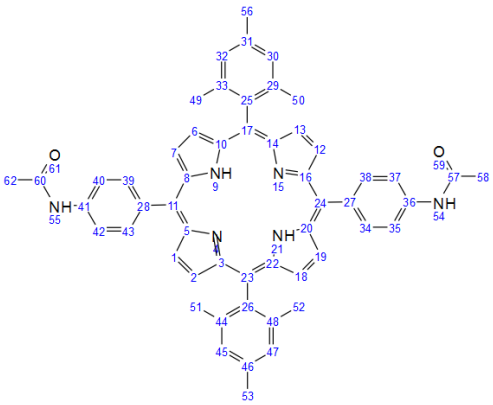


Figure S2 ^{13}C NMR spectrum of compound **1** in $CDCl_3$ at 75 MHz.



5,15-Bis(4-acetamidophenyl)-10,20-bis(2,4,6-trimethylphenyl)porphyrin **2:** 4-Acetamidobenzaldehyde (407.0 mg, 2.5 mmol) and compound **1** (660.0 mg, 2.5 mmol) were dissolved in 250 mL of chloroform, and this was stirred under argon flow for 20 min at room temperature. Then, $BF_3{\cdot}OEt_2$ (100 µL) was added, and the mixture was stirred for 1 h. DDQ (430 mg, 1.9 mmol) was added, and stirring was continued for another 2 h. The target product was purified by column chromatography on G60 silica gel, which was eluted with a methylene chloride-ethanol, 97:3 *(v/v)*. The purple powder was dried in vacuo over P_2O_5. The yield was 25%. R_f 0.4 (methylene chloride-ethanol, 97:3). UV ($CHCl_3$, λ/nm): 424 (5.82), 519 (4.53), 558 (3.44), 594 (3.26), 651 (3.23). ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C) δ: -2.62 (s, 2H, pyrrole-NH), 1.84 (s, 12H, *o*-mesityl-CH_3), 2.36 (s, 6H, $COCH_3$), 2.64 (s, 6H, *p*-mesityl-CH_3), 7.29 (s, 4H, *m*-mesityl-H), 7.70 (s, 2H, *p*-acetamidophenyl-NH), 7.90 (d, J = 8.2 Hz, 4H, *m*-acetamidophenyl-H), 8.17 (d, J = 8.3 Hz, 4H, *o*-acetamidophenyl-H), 8.70 (d, J = 4.8 Hz, 4H, β-pyrrole), 8.82 (d, J = 4.8 Hz, 4H, β-pyrrole). MS (MALDI-TOF), *m/z*: 813.193 $[M]^+$, (calc. for $C_{54}H_{48}N_6O_2$, *m/z*: 813.02).

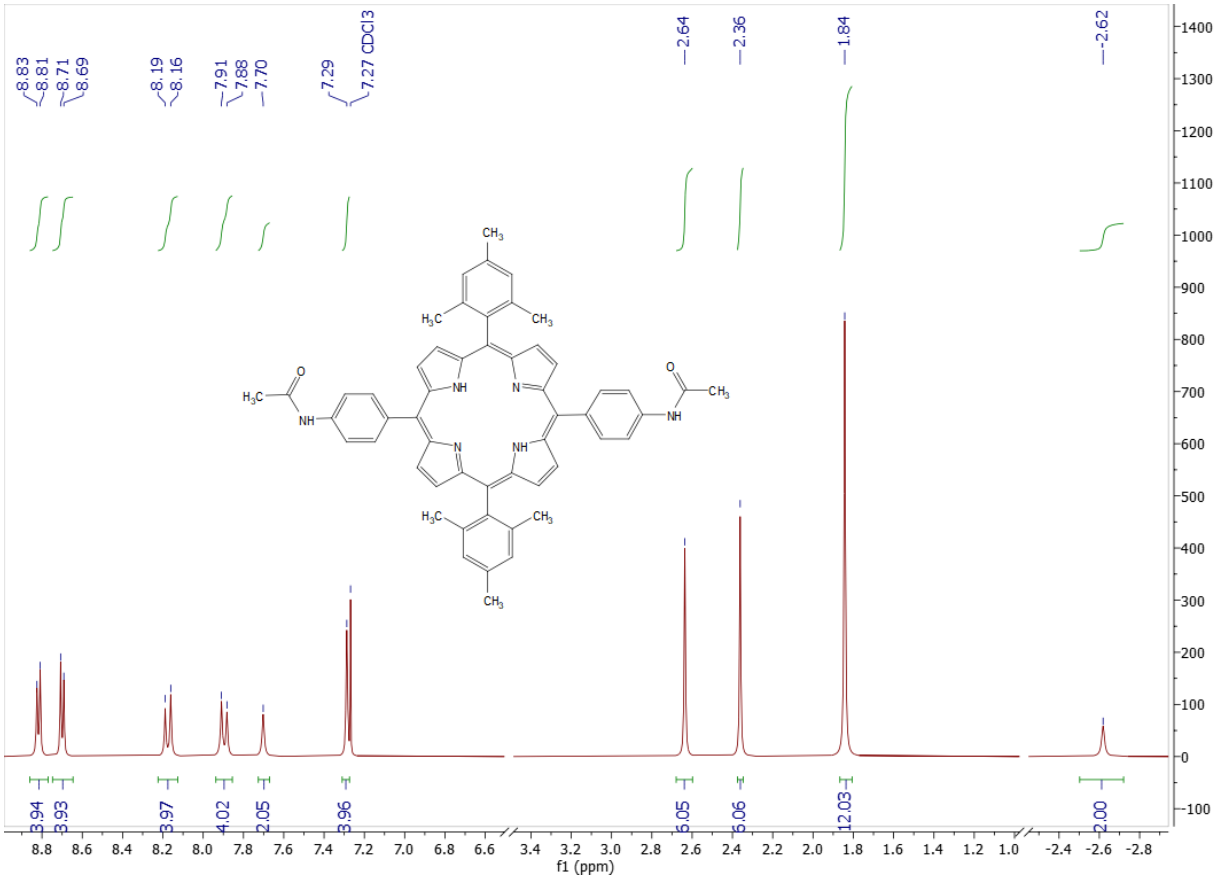


Figure S3 ^{1}H NMR spectrum of compound **2** in $CDCl_3$ at 300 MHz.

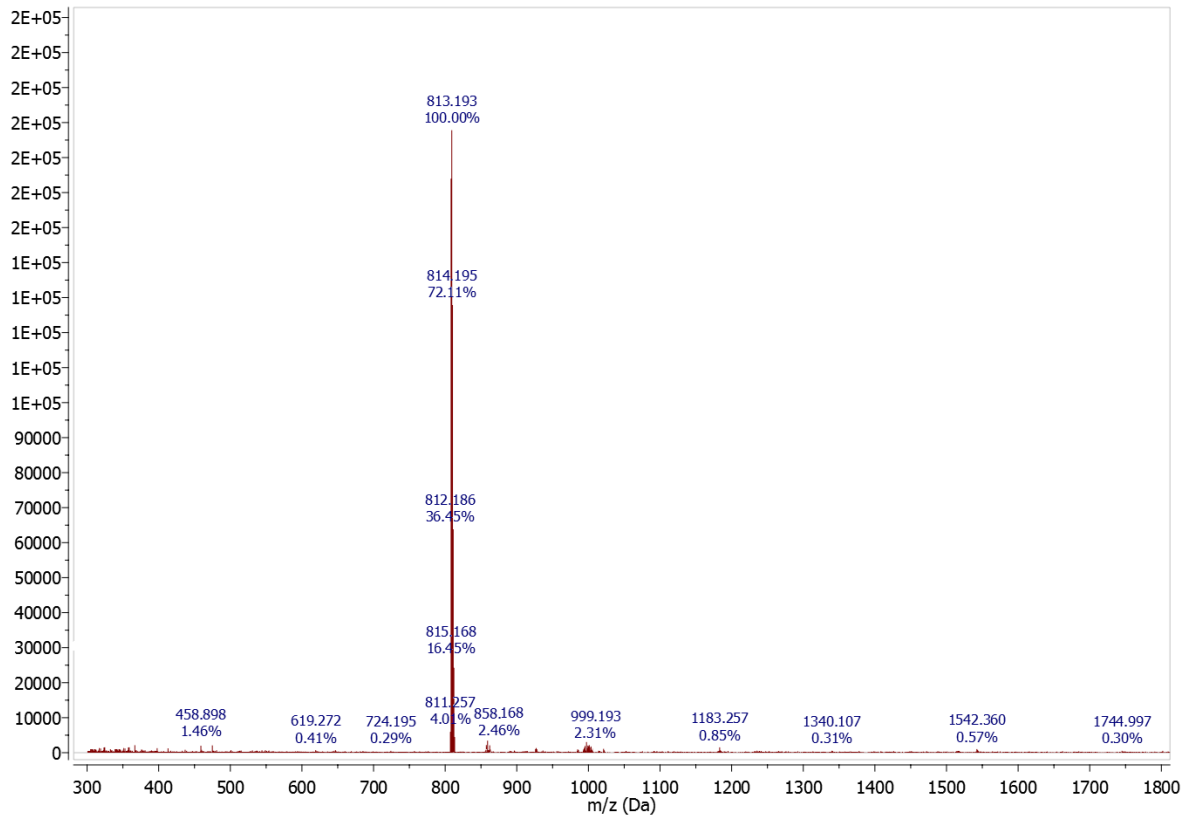
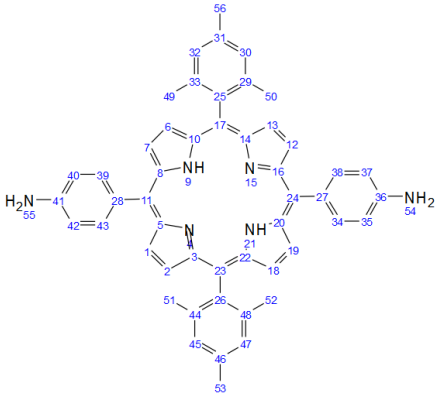


Figure S4 MS (MALDI-TOF) spectrum of compound **2**.



5,15-Bis(4-aminophenyl)-10,20-bis(2,4,6-trimethylphenyl)-porphyrin **3***:* Compound **2** (200.0 mg, 246 µmol) was dissolved in 160 mL of ethanol/hydrochloric acid mixture (3:2) and boiled at 78 °C for 48 h. The reaction mixture was neutralized by the addition of saturated $NaHCO_3$ solution until the color changed from green to violet. The organic layer was extracted with chloroform and washed with saturated $NaHCO_3$ solution. The target product was purified by column chromatography on silica gel G60, which was eluted with chloroform. The purple powder **3** was dried *in vacuo* over P_2O_5. The yield was 90 %. R_f 0.5 (chloroform). UV ($CHCl_3$, λ/nm): 425 (5.84), 520 (4.42), 561 (3.43), 597 (3.22), 650 (3.19). ^{1}H NMR (300 MHz, $CDCl_3$, 25°C) δ*:* -2.56 (s, 2H, pyrrole-NH), 1.86 (s, 12H, *o*-mesityl-CH_3), 2.65 (s, 6H, *p*-mesityl-CH_3), 3.99 (s, 4H, *p*-aminophenyl-NH_2), 7.06 (d, J = 8.3 Hz, 4H, *m*-aminophenyl-H), 7.30 (s, 4H, *m*-mesityl-H), 8.01 (d, J = 8.3 Hz, 4H, *o*-aminophenyl-H), 8.69 (d, J = 4.8 Hz, 4H, β-pyrrole), 8.89 (d, J = 4.8 Hz, 4H, β-pyrrole). ^{13}C NMR (75 MHz, $CDCl_3$, 25°C) δ: 145.92 (C36, C41), 139.46 (C3, C5, C8, C10, C14, C16, C20, C22), 138.71 (C29, C33, C44, C48), 137.60 (C25, C26), 135.59 (C1, C2, C6, C7, C12, C13, C18, C19), 132.31 (C27, C28), 127.72 (C30, C32, C45, C47), 119.62 (C11, C24), 117.91 (C17, C23), 113.50 (C35, C37, C40, C42), 21.65 (C49, C50, C51, C52), 21.50 (C53, C56).

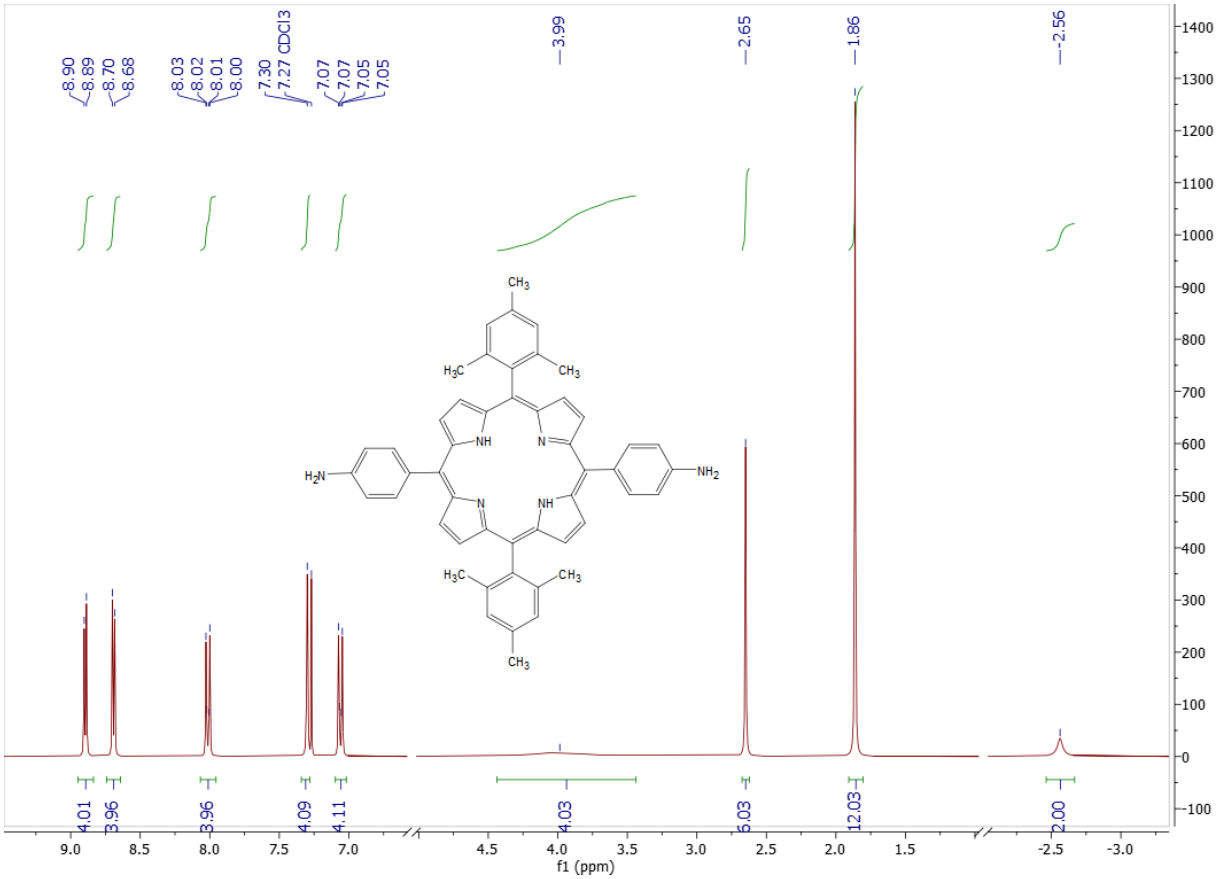


Figure S5 ^{1}H NMR spectrum of compound **3** in $CDCl_3$ at 300 MHz.

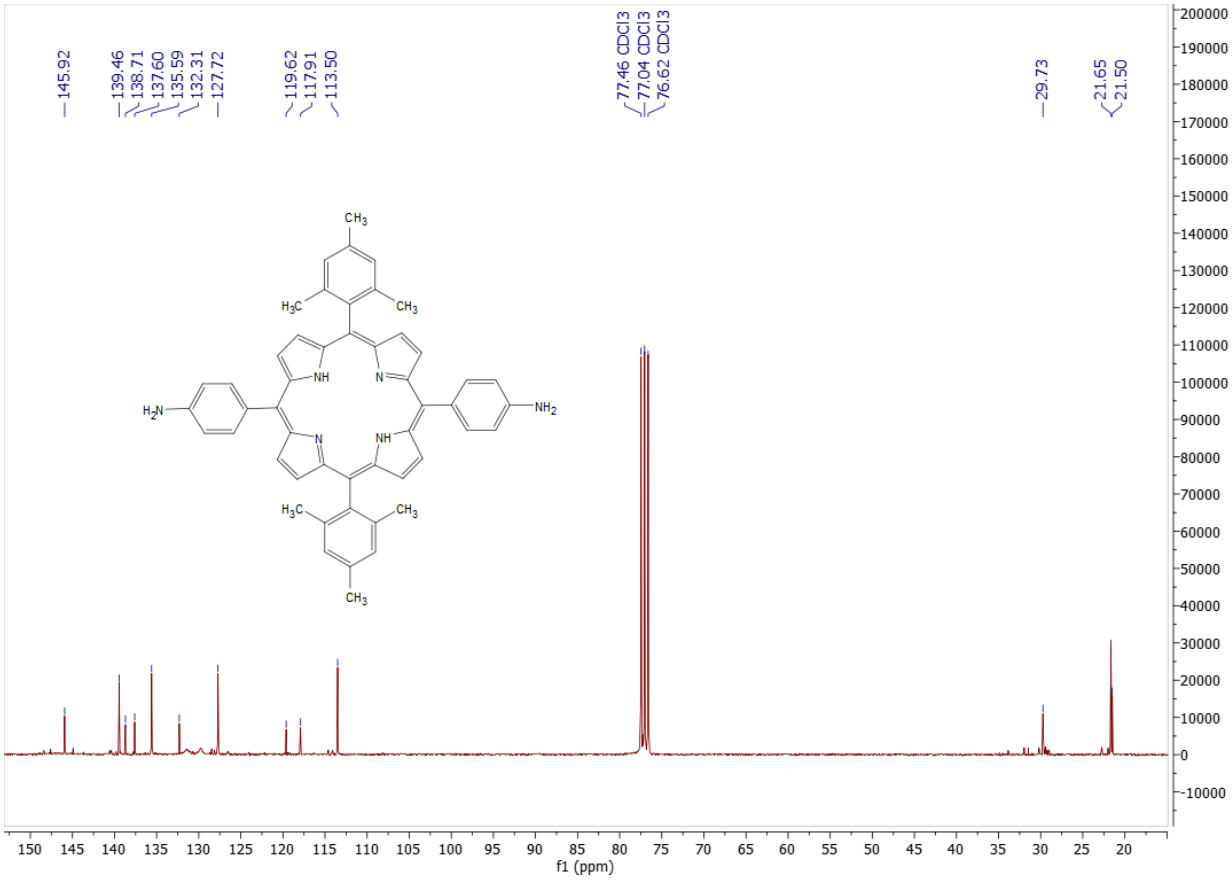
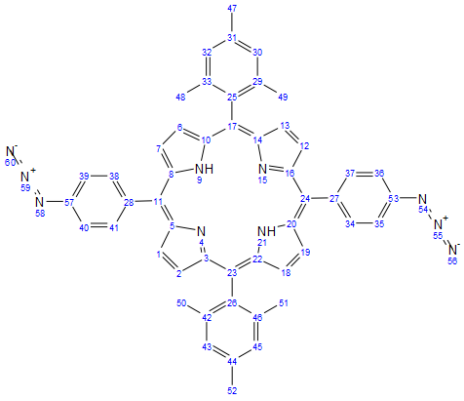


Figure S6 ^{13}C NMR spectrum of compound **3** in $CDCl_3$ at 75 MHz.



5,15-Bis(4-azidophenyl)-10,20-bis(2,4,6-trimethylphenyl) porphyrin **4***:* The amino derivative of *meso*-arylporphyrin **3** (90.0 mg, 124 μmol) was dissolved in TFA (10 mL). A solution of sodium nitrite (20.6 mg, 298 μmol) in 5 mL of water was added dropwise to the resulting solution for 15 min. The resulting mixture was stirred at 0 °C for 30 min, then a solution of sodium azide (48.4 mg, 744 μmol) in 5 mL of water was added dropwise for 15 min. The second step was carried out at room temperature for 2 h. After completion of the reaction, the mixture was extracted in a CH_2Cl_2/water system with an aqueous ammonia solution until the organic layer changed color from green to burgundy. The organic layer was evaporated using a rotary evaporator. The target product was purified by column chromatography on silica gel G60, which was eluted with a CH_2Cl_2–hexane, 8:1 (v/v). The compound **4** was eluted with the first fraction. The purple powder **4** was dried in vacuo over P_2O_5. The yield was 80%. R_f 0.3 (CH_2Cl_2–hexane, 8:1). UV ($CHCl_3$, λ/nm): 426 (5.81), 522 (4.40), 560 (3.41), 599 (3.30), 653 (3.18). ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C) δ: -2.60 (s, 2H, pyrrole-NH), 1.85 (s, 12H, *o*-mesityl-CH_3) 2.64 (s, 6H, *p*-mesityl-CH_3), 7.30 (s, 4H, *m*-mesityl-H), 7.45 (d, J = 8.1 Hz, 4H, *m*-azidophenyl-H), 8.24 (d, J = 8.1 Hz, 4H, *o*-azidophenyl-H), 8.72 (d, J = 4.8 Hz, 4H, β-pyrrole), 8.76 (d, J = 4.8 Hz, 4H, β-pyrrole). IR (ν/cm^{-1}): 2948, 2919, 2855, 2122, 2085 (-N_3), 1811, 1581, 1337, 1287, 1270 (-N_3), 1205, 1180, 1130, 1109, 1065, 1033, 959, 797, 724.

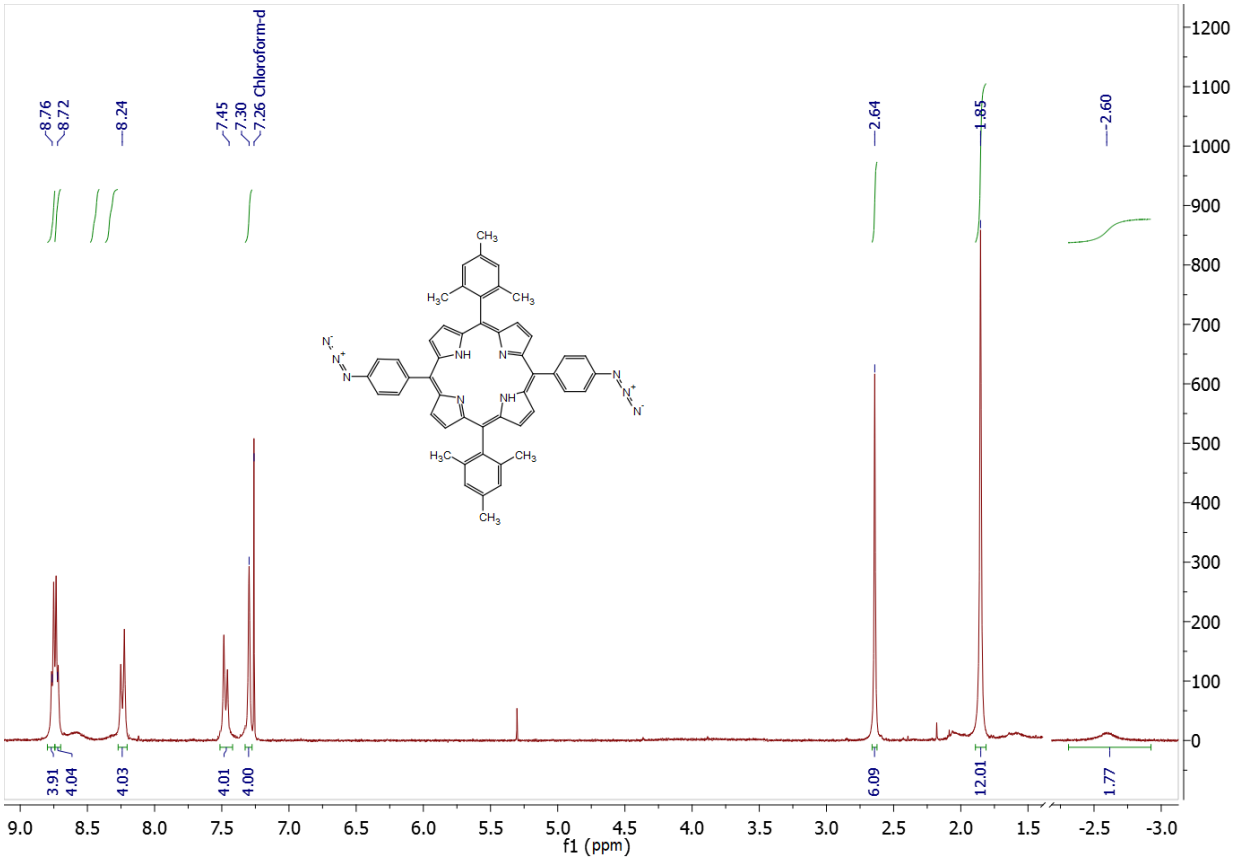


Figure S7 ^{1}H NMR spectrum of compound **4** in $CDCl_3$ at 300 MHz.

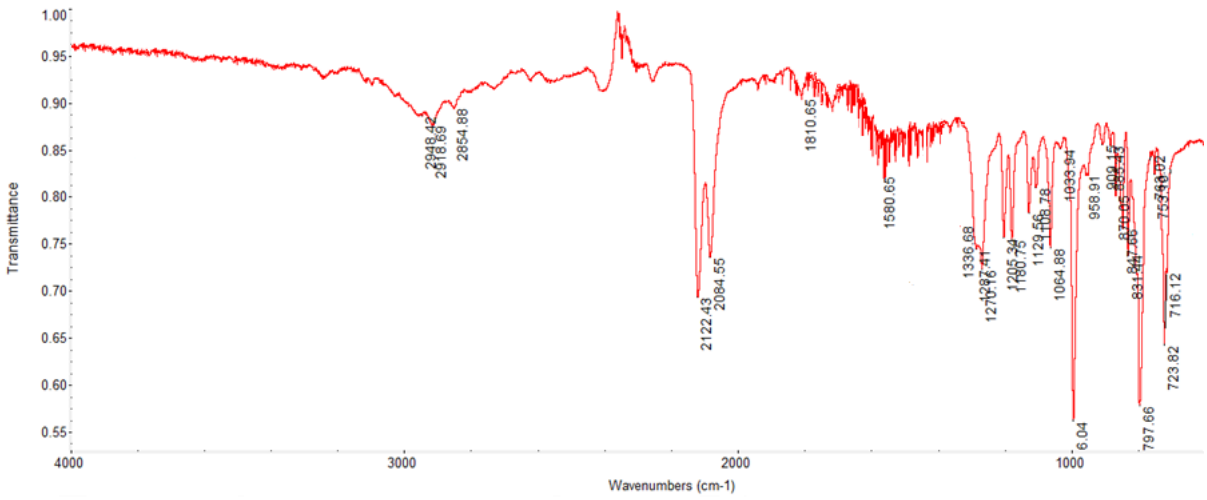
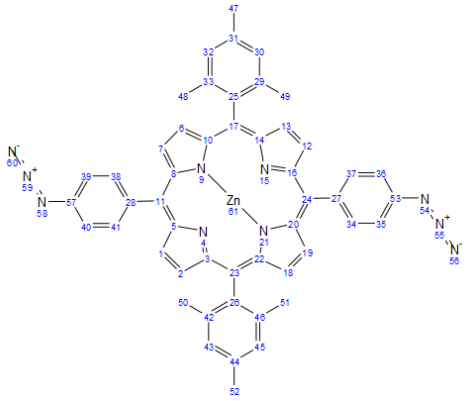


Figure S8 IR spectrum of compound **4**.



Zinc 5,15-bis(4-azidophenyl)-10,20-bis(2,4,6-trimethyl-phenyl)porphyrinate **5***:* A solution of zinc acetate dihydrate (112.6 mg, 510 µmol) in 5 mL of methanol was added to a solution of compound **4** (40.0 mg, 51 µmol) in CH_2Cl_2 (10 mL); then, the mixture was stirred for 5 h at room temperature. The mixture was extracted into a CH_2Cl_2/water system. The organic layer was separated and evaporated on a rotary evaporator. The purple solid was purified by column chromatography on G60 silica gel using the following solvent system: CH_2Cl_2–hexane, 15:1 (v/v). The target compound was eluted with the first fraction. The product was recrystallized from methanol. The purple powder **5** was dried *in vacuo* over P_2O_5. The yield was 95%. R_f 0.4 (methylene chloride–hexane, 15:1). UV ($CHCl_3$, λ/nm): 426 (5.72), 562 (4.16), 603 (3.76). ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C) δ: 1.83 (s, 12H, *o*-mesityl-CH_3) 2.64 (s, 6H, *p*-mesityl-CH_3), 7.28 (s, 4H, *m*-mesityl-H), 7.40 (d, J = 8.4 Hz, 4H, *m*-azidophenyl-H), 8.21 (d, J = 8.5 Hz, 4H, *o*-azidophenyl-H), 8.77 (d, J = 4.7 Hz, 4H, β-pyrrole), 8.85 (d, J = 4.6 Hz, 4H, β-pyrrole). ^{13}C NMR (101 MHz, $CDCl_3$, 25 °C) δ: 150.11 (C5, C8, C16, C20), 150.05 (C3, C10, C14, C22), 139.85 (C53, C57), 139.35 (C29, C33, C42, C46), 137.61 (C31, C44), 135.88 (C25, C44), 135.70 (C2, C6, C13, C18), 132.16 (C1, C7, C12, C19), 130.98 (C34, C37, C38, C41), 128.39 (C27, C28), 125.68 (C30, C32, C43, C45), 119.43 (C11, C24), 119.11 (C17, C23), 117.34 (C35, C36, C39, C40), 21.82 (C48, C49, C50, C51), 21.62 (C47, C52).

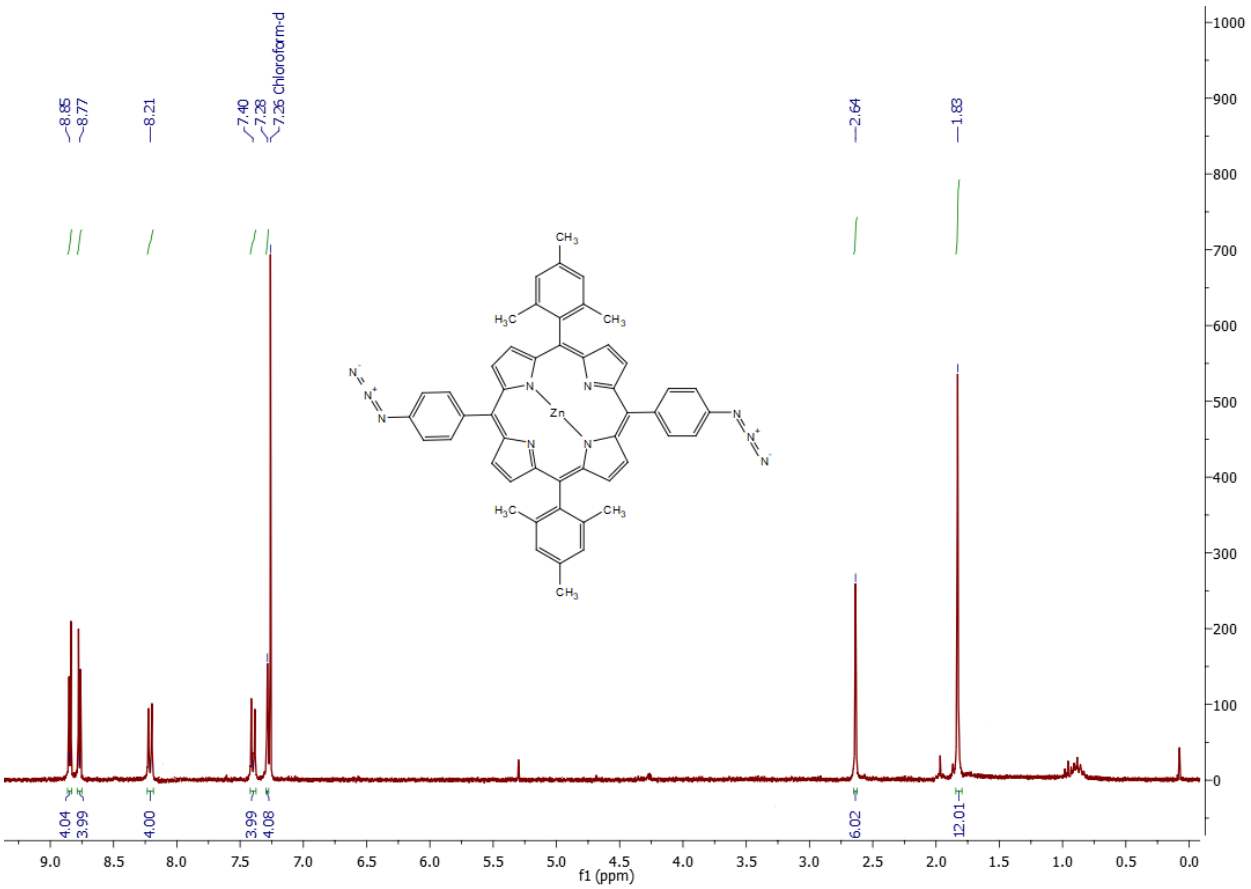


Figure S9 ^{1}H NMR spectrum of compound **5** in $CDCl_3$ at 300 MHz.

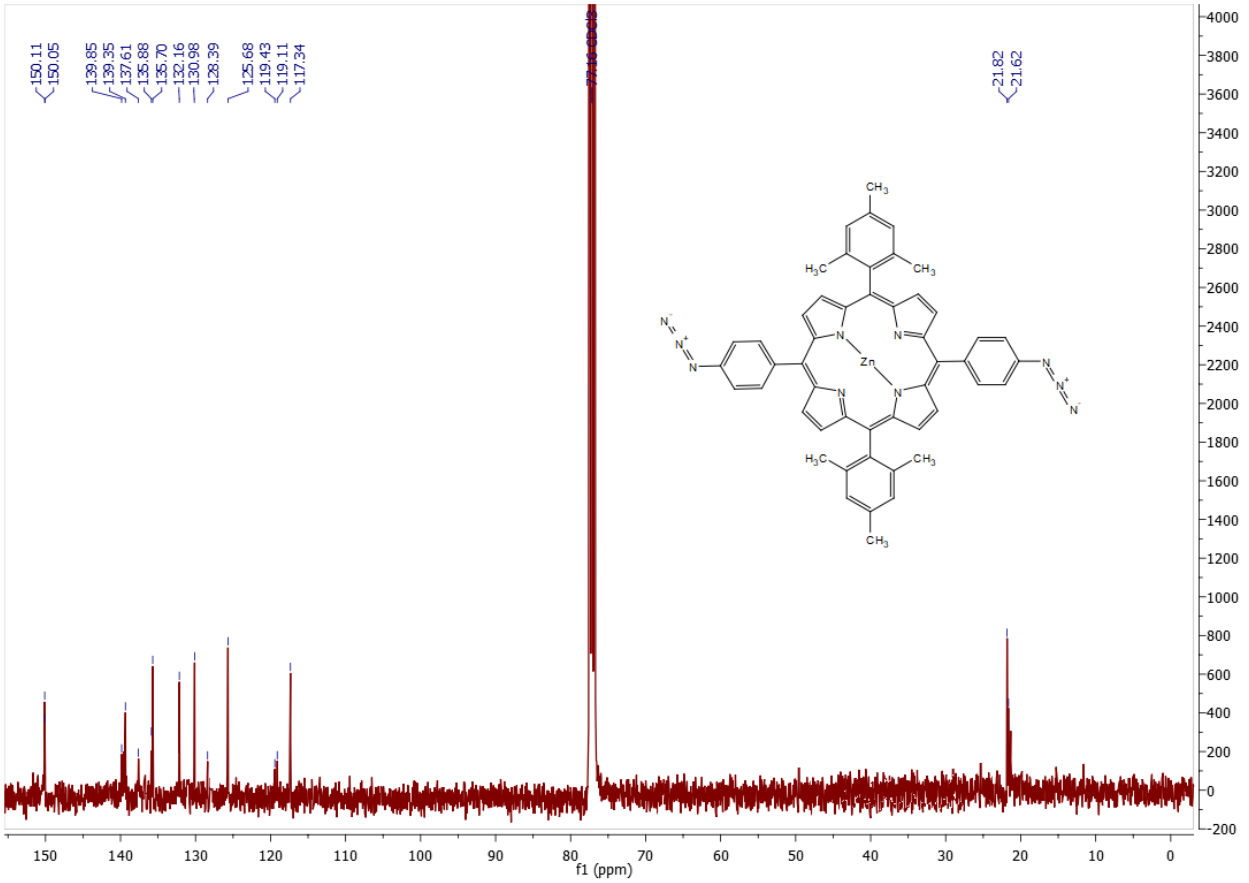
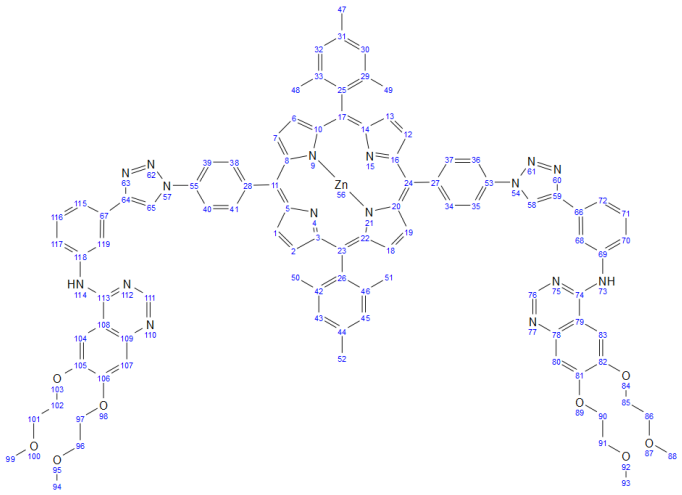


Figure S10 ^{13}C NMR spectrum of compound **5** in $CDCl_3$ at 101 MHz.



*Zinc 5,15-bis[4-(4-{3-[6,7-bis(2-methoxyethoxy)quinazolin-4-yl]aminophenyl}-1*H*-1,2,3-triazol-1-yl)phenyl]-10,20-bis(2,4,6-trimethylphenyl)porphyrinate* **6**: A solution of compound **5** (30 mg, 35 µmol) and Erlotinib ethynyl derivative (33.6 mg, 85 µmol) in 8 mL THF was treated with a solution of copper(II) sulfate pentahydrate (5.3 mg, 21 µmol) and sodium ascorbate (8.3 mg, 42 µmol) in 5 mL water. The reaction mixture was stirred at 64 °C for 6 hours. Then it was extracted into THF/saturated NaCl solution, the organic layer was concentrated under reduced pressure. The purple solid was purified by column chromatography on silica gel G60. A solvent mixture of CH_2Cl_2-ethanol-pyridine, 50:1:0.1 (v/v) was used as the eluent. The target compound was eluted in the first fraction. The product was recrystallized from diethyl ether. The purple powder **6** was dried *in vacuo* over P_2O_5. The yield was 76%. R_f 0.3 (CH_2Cl_2 – ethanol, 50:1). UV (DMSO, λ/nm): 430 (5.74), 562 (4.22), 606 (3.89). ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C) δ: 1.80 (s, 12H, *o*-mesityl-CH_3) 2.58 (s, 6H, *p*-mesityl-CH_3), 3.37 (s, 6H, -OCH_3), 3.40 (s, 6H, -OCH_3), 3.80 (m, 8H, -CH_2O-), 4.36 (m, 8H, -OCH_2O-), 6.87 (s, 2H, phenyl-H), 7.33 (m, 6H, *m*-mesityl-H and phenyl-H), 7.59 (s, 2H, phenyl-H), 7.81 (s, phenyl-H), 8.00 (m, 4H, *m*-phenyl-H-triazol), 8.44 (m, 8H, *o*-phenyl-H-triazol and quinazoline-H), 8.64 (d, J = 4.8 Hz, 4H, β-pyrrole), 8.83 (d, J = 4.8 Hz, 4H, β-pyrrole), 9.64-9.83 (m, 4H, triazol-H and pyrimidine-H). ^{13}C NMR (75 MHz, DMSO-d_6, 25 °C) δ: 169.61 (C74, C113), 149.21 (C5, C8, C16, C20, C59, C64), 149.07 (C3, C10, C14, C22, C82, C105), 148.19 (C81, C106), 147.61 (C76, C111), 143.06 (C78, C109), 140.23 (C29, C33, C42, C46), 139.07 (C69, C118), 138.40 (C53, C55), 136.98 (C31, C44), 136.05 (C66, C67), 135.37 (C2, C6, C13, C18), 131.93 (C27, C28, C58, C65), 130.67 (C1, C7, C12, C19), 130.49 (C71, C116), 129.25 (C30, C32, C43, C45), 127.62 (C11, C24, C25, C26), 122.37 (C17, C23), 120.71 (C72, C115), 119.98 (C70, C117), 119.17 (C68, C119), 118.43 (C35, C36, C39, C40), 118.28 (C80, C107), 118.10 (C83, C104), 103.28 (C79, C108), 70.17 (C86, C91, C96, C101), 68.40 (C85, C90, C97, C102), 58.44 (C88, C93, C94, C99), 21.43 (C46, C49, C50, C51), 21.05 (C47, C52). MS (MALDI-TOF), *m/z*: 1629.807 $[M+H]^+$, (calc. for $C_{94}H_{83}N_{16}O_8Zn$, *m/z*: 1628.59).

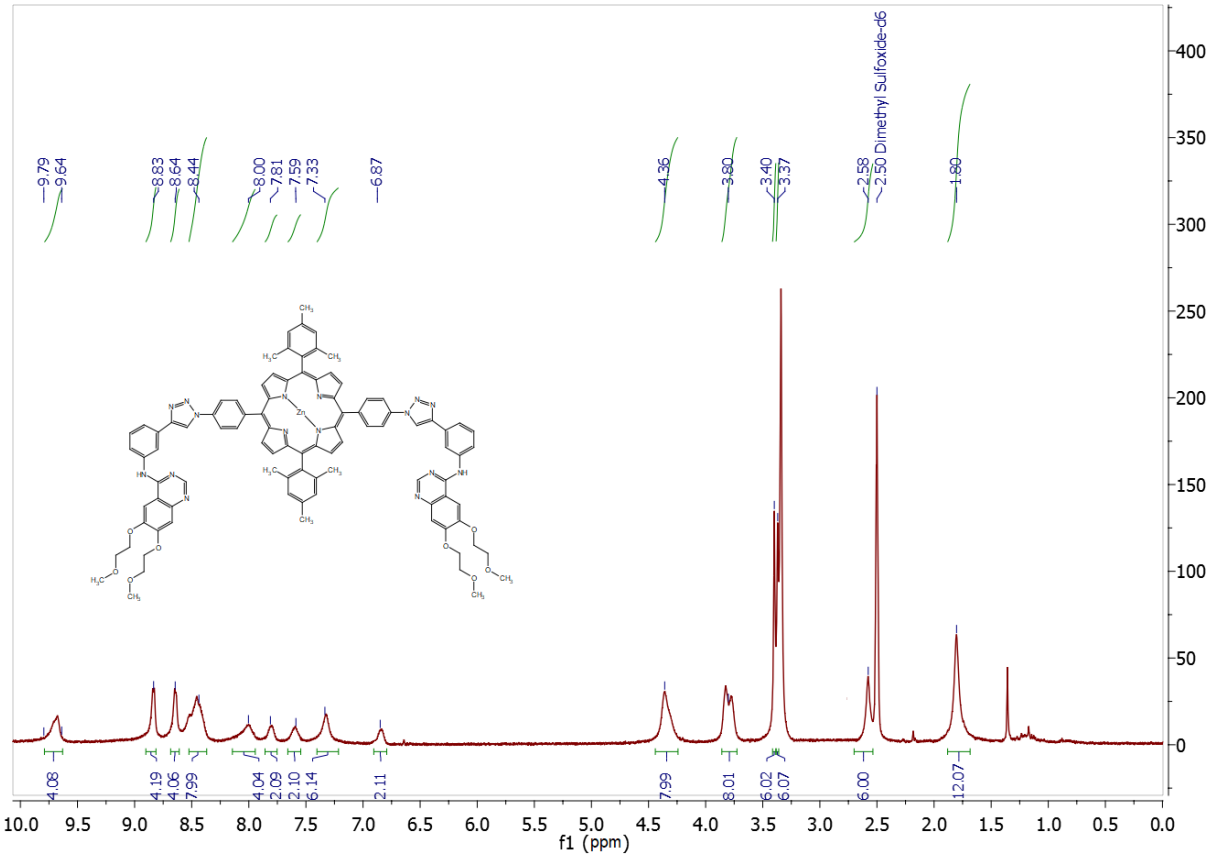


Figure S11 ^{1}H NMR spectrum of compound **6** in DMSO-d_6 at 300 MHz.

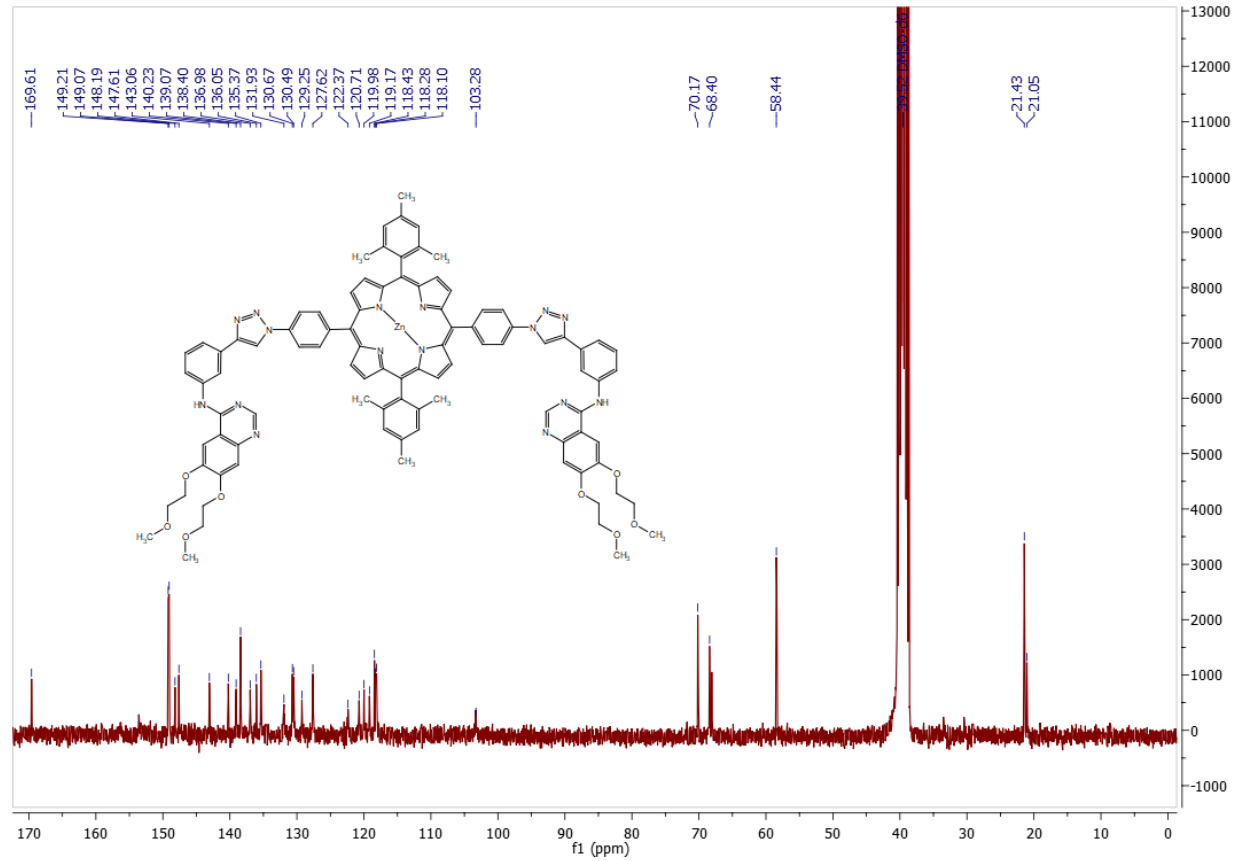


Figure S12 ^{13}C NMR spectrum of compound **6** in DMSO-d_6 at 75 MHz.

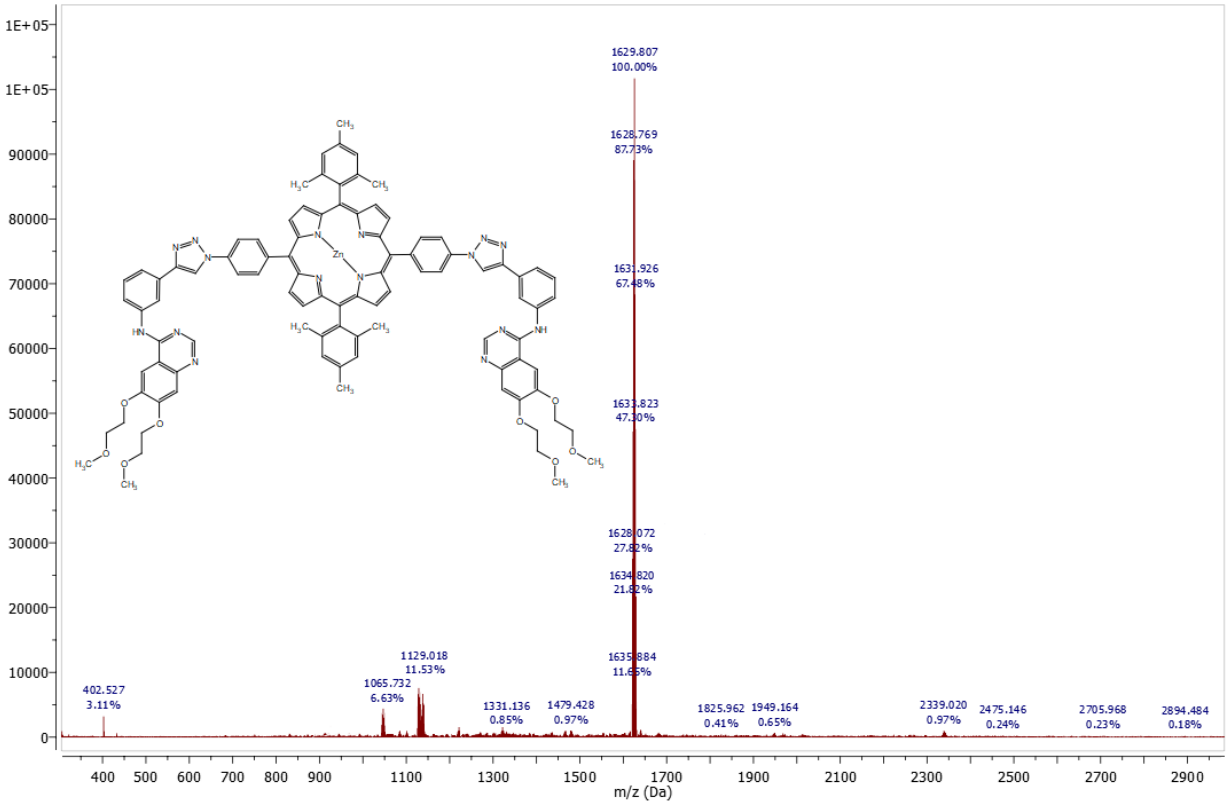


Figure S13 MS (MALDI-TOF) spectrum of compound **6**.

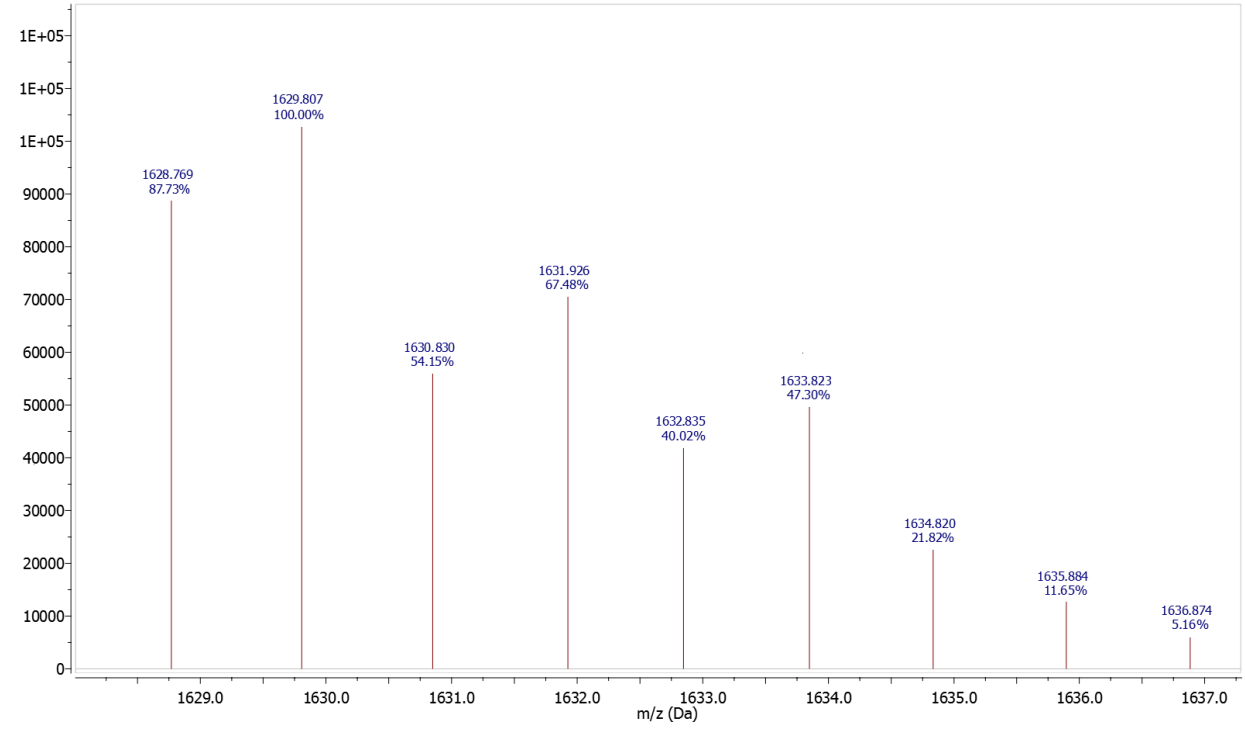


Figure S14 Measured isotopic patterns of the molecular peak of compound **6**.

Electronic absorption and fluorescence spectra

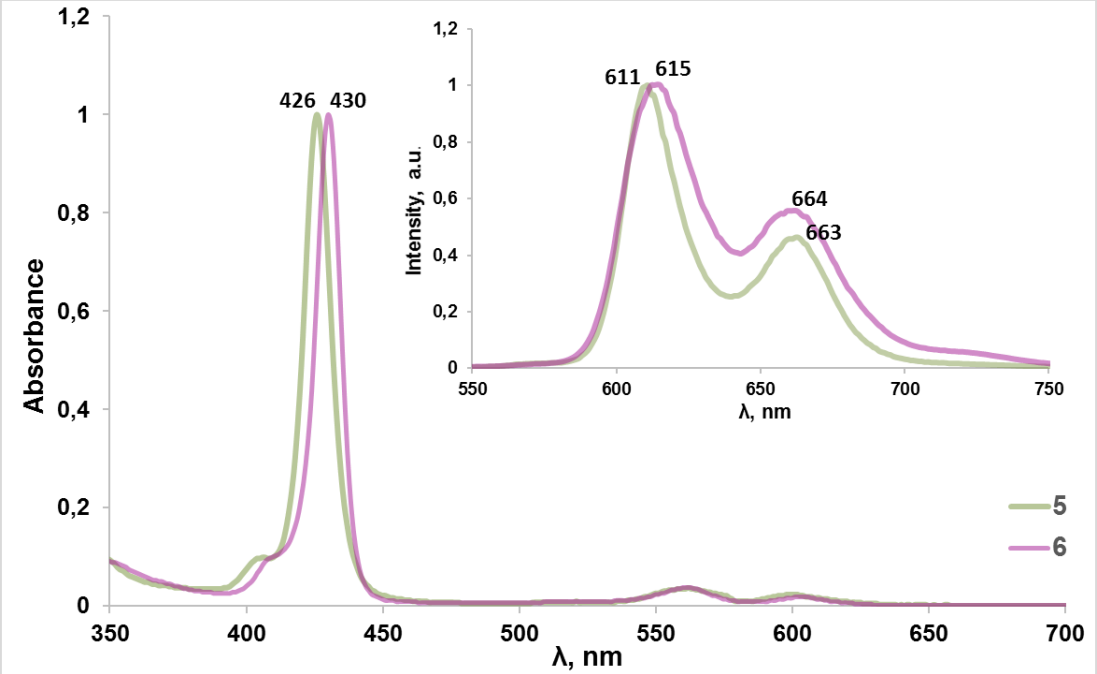


Figure S15. Normalized electronic absorption and fluorescence spectra (inset) for compounds **5** and **6** in DMSO (λ_{ex} = 430 nm).

Encapsulation of compound 6 into Pluronic F-127 micelles.

A solution of compound 6 (1 mg/mL) in THF (15 mL) was added to a solution of Pluronic F127 with a concentration of 20 mg/mL in methanol (15 mL). The resulting mixture was stirred vigorously for 30 min and evaporated using a rotary evaporator. The product was dried in dynamic vacuum. The hydrodynamic radii (R_h) of the received nanoparticles were determined using dynamic light scattering (DLS) by employing Delsa Nano (Beckman Coulter, Inc., Brea, CA, USA).

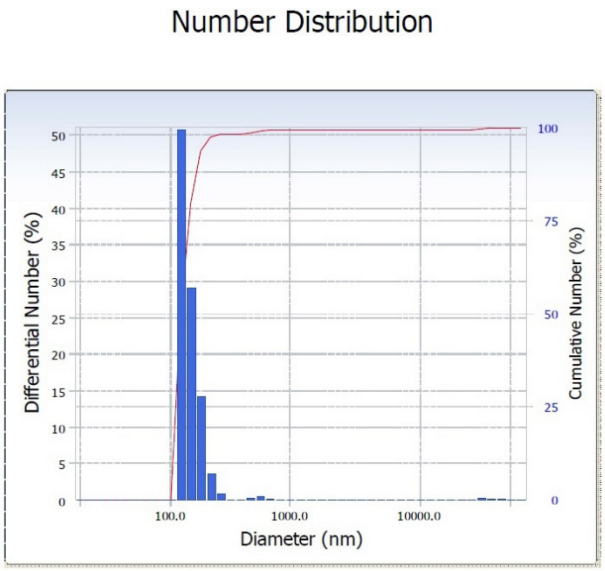


Figure S16. Particle size of conjugate **6** in Pluronic F127 micelles.

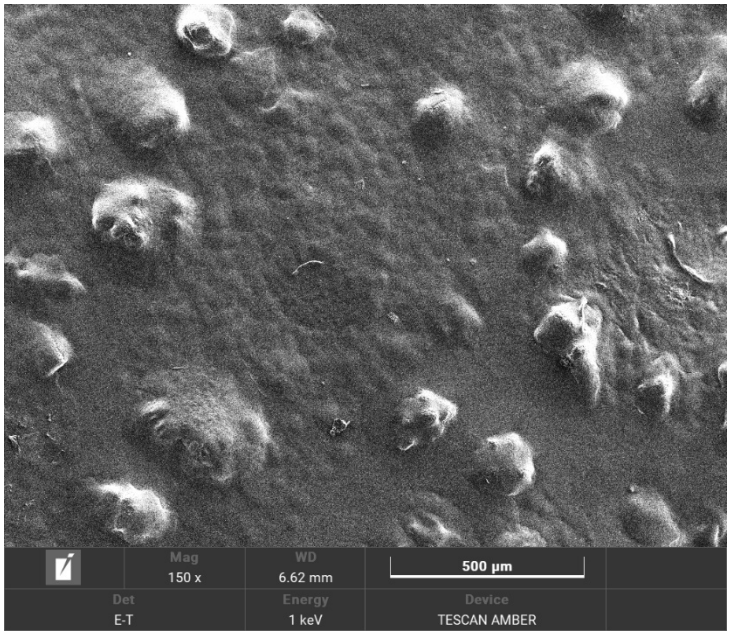


Figure S17. SEM microphotography of obtained particles.

Cell Culture.

The cancer cell lines NKE (human renal tubule epithelium) and A431 (human epidermoid carcinoma) were purchased from the cell bank of N. N. Blokhin National Medical Research Center of Oncology of the Russian Ministry of Health. NKE, and A431 cells were cultivated in DMEM substratum together with the addition of penicillin-streptomycin (50 μg/mL) and 10% fetal bovine serum. Cells were cultivated in plastic cell culture flasks (25 cm^2) at 37 °C in a humidified environment with 5% CO_2. Cells were seeded before reaching 80% fusion using EDTA/trypsin solution.

For the *in vitro* experiments, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) and Mowiol (Sigma–Aldrich); 96% ethanol (Chimmed, Moscow, Russia); fetal bovine serum (FBS) (Gibco, Waltham, MA, USA); Dulbecco's modified Eagle's medium (DMEM) (Gibco); dimethyl sulfoxide (DMSO) (Amreso, Solon, OH, USA); 0.02% EDTA and 0.05% trypsin solutions (Gibco); and penicillin-streptomycin (PanEco, Moscow, Russia) were used. All chemicals were used without further purification. Milli-Q water was used in the experiments.

In Vitro Cell Viability (MTT Test). This experiment was performed using two cell lines: NKE (human renal tubule epithelium) and A431 (human epidermoid carcinoma). In the experiment, cells were seeded into 96-well plates (SPL Lifesciences, Pocheon-si, Korea) at 7×10^3 cells/well in 180 μL of culture medium and incubated for 24 h at 37 °C in 5% CO_2. On the day of the experiment, the medium was replaced with a similar one but which did not contain serum to avoid its interaction with the studied compounds. Serial dilutions of the compound were created in deionized water and added to the cell culture by 20 μL each. The cell culture was irradiated for 90 min using a Medical Therapy Philips TL 20W/52 lamp (wavelength 400–500 nm) with 2.3 mW of power. Subsequently, the plates were placed in an incubator and left for 24 h at 37 °C in 5% CO_2. Parallel incubation of cell culture with compounds without irradiation was performed in a similar manner. After 24 h, 10 μL of MTT reagent solution was added to the cell medium and incubated for another 3.5 h. The formazan forming in the cells was dissolved in DMSO (100 μL). MultiScan MCC 340 spectrophotometer (Labsystems, Boston, MA, USA) was used to determine the optical density of the solution at 540 nm. Experiments were repeated at least 3 times. The titration curves were used to calculate the concentration of the compound that induced 50% of the maximum toxicity effect (IC_{50}). The Excel program package (Microsoft Corporation, Redmond, WA, USA) was utilized for statistical processing of the obtained data.

Table S1. IC_{50} of compound **6** the A431 and NKE cell lines.

Cells/Compounds	Conjugate **6** Light*	Conjugate **6** Dark
A431	0.54±0.011 μM	-
NKE	0.86±0.017 μM	2.5±0.073 μM

*Dose of irradiation 8.073 J/cm^2. Incubation time with the compounds was 2 hours.