Supporting Information

Self-assembly-Directed Cancer Cell Membrane Insertion of Synthetic Analogues for Permeability Alteration

Enming Du, Xunwu Hu, Guanying Li, Shijin Zhang, Dingze Mang, Sona Roy, Toshio Sasaki, and Ye Zhang*

Table of Contents

Experimental Procedures

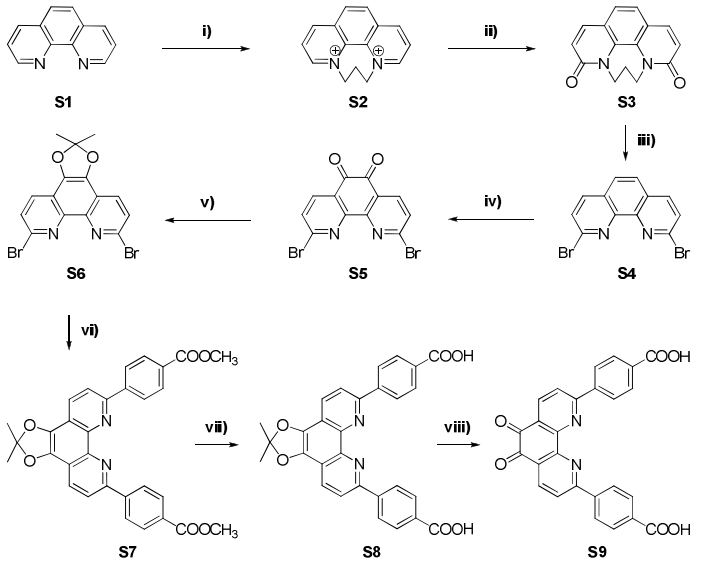
Methods

Materials and Instruments

Ammonium acetate was purchased from Nacalai Tesque, INC. and purified by sublimation before usage. Other chemical reagents were purchased from Sigma, Nacalai and Wako and used without further purification.

Mass spectra were recorded using a Thermo LTQ-ETD mass spectrometer (ESI) and high resolution mass spectra were measured with a Thermo LTQ-Orbitrap Classic mass spectrometer (ESI). ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded on a Bruker Ascend 400 (400, 100 and 162 MHz, respectively) spectrometer. Time-dependent ^{31}P NMR for dephosphorylation process was recorded on a JEOL 600 MHz NMR (243 MHz). TEM micrographs were obtained on a JEM-1230R Transmission Electron Microscope. SEM micrographs were measured on a FEI Quanta 250 FEG Scanning Electron Microscope. Confocal images were obtained on a Zeiss LSM780 Confocal Microscope. The fluorescence intensity of PI was detected using imaging flow cytometer (ImageStream X Mark, Germany). LDH release was determined using a Tecan Infinite M1000 PRO microplate reader.

Synthesis



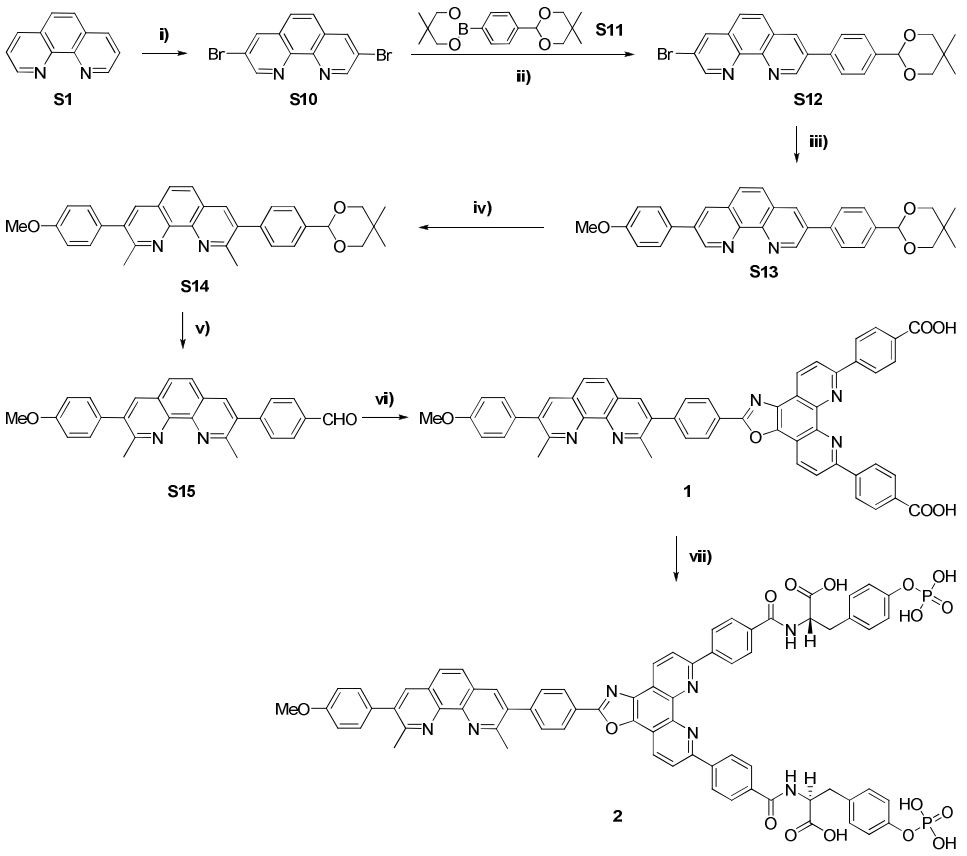
Scheme S1. Synthesis of compound **S9**. Reagents and conditions: i) 1,3-dibromopropane, chlorobenzene, 120°C, 4 h; ii) *t*-BuOK, *t*-BuOH, 40°C, 4 h; iii) $POBr_3$, PBr_5, nitrogen atmosphere, 110°C, 8 h; iv) H_2SO_4, HNO_3, KBr, 100°C, 4 h; v) 2-nitropropane, Na_2CO_3, CH_3CN/H_2O (1:1), reflux, 8 h; vi) methyl 4-(5,5-dimethyl-1,3,2-dioxaborinan-2-yl)- benzoate, $Pd(PPh_3)_4$, Na_2CO_3, toluene/H_2O (2:1), 106°C, 24 h; vii) LiOH, THF/H_2O (1:1), 50°C, 24 h; viii) TFA/H_2O (2:1); 50°C, 20 h.

Compound S4. This compound was prepared by a published procedure.[1] MS (ESI), *m/z*: 339.55 ($[M+H]^+$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.18 (d, J = 2.4 Hz, 2H), 8.40 (d, J = 2.4 Hz, 2H), 7.75 (s, 2H).

Compound S7.[2] Compound **S6** (0.50 g, 1.22 mmol), methyl 4-(5,5-dimethyl-1,3,2-dioxaborinan-2-yl)-benzoate (0.88 g, 3.55 mmol) and Na_2CO_3 (2 M in H_2O, 12.2 mL, 24.4 mmol) were added to toluene (25 mL) successively. The solution was degassed and then $Pd(PPh_3)_4$ (200 mg, 0.17 mmol) was rapidly added under a flow of nitrogen. The mixture was kept stirring at 105°C for 24 h. It was then allowed to cool to room temperature and extracted with CH_2Cl_2. The combined organic layer was washed with brine, dried over Na_2SO_4, filtered, and concentrated under reduced pressure to give the crude product, which was purified by chromatography on silica gel to obtain the compound **S7** (0.53 g, 83.6%). MS (ESI), *m/z*: 521.36 ($[M+H]^+$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.52 (d, J = 8.4 Hz, 4H), 8.37 (d, J = 8.4 Hz, 2H), 8.26 (d, J = 8.4 Hz, 4H), 8.20 (d, J = 8.4 Hz, 2H), 3.99 (s, 6H), 1.92 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.0, 153.2, 143.3, 137.0, 130.6, 130.2 (2C), 129.2, 127.3 (2C), 121.2, 120.2, 117.8, 52.2 (2C), 26.2 (2C).

Compound S8. Compound **S7** (0.70 g, 1.35 mmol) and LiOH (1.13 g, 26.92 mmol) were added to a solution of THF and H_2O (1:1, 20 mL). The reaction mixture was stirred at 50°C for 48 h. After the reaction was complete, THF was removed under reduced pressure, and then adjust pH to 2 with 1 N HCl followed by filtration and washing with CH_3OH and CH_2Cl_2 to obtain the yellow solid **S8** (610 mg, 92.1%). MS (ESI), *m/z*: 493.36 ($[M+H]^+$); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.65 (d, J = 8.8 Hz, 4H), 8.51 (d, J = 8.8 Hz, 2H), 8.44 (d, J = 8.8 Hz, 2H), 8.21 (d, J = 8.8 Hz, 4H), 1.92 (s, 6H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 167.1, 152.1, 142.5, 141.7, 136.4, 131.3, 130.1 (2C), 129.1, 127.1 (2C), 121.5, 120.5, 117.2, 25.7 (2C).

Compound S9. The protected diketone **S8** (500 mg, 1.02 mmol) was dissolved in a mixture of TFA and H_2O (2:1, 30 mL). Under air condition, the mixture was stirred for 12 h at 50°C. After the reaction was complete, the solvents were evaporated under reduced pressure and the residue was neutralized with 1 N $NaHCO_3$ followed by filtration. The filtrate was adjusted to pH 2.0 with 1 N HCl followed by filtration and washing with CH_3OH and CH_2Cl_2 to give the yellow solid **S9** (360 mg, 78.4%). MS (ESI), *m/z*: 453.27 ($[M+H]^+$); ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.07 (brs, 2H), 9.65 (s, 1H), 8.70 (d, J = 8.8 Hz, 1H), 8.66-8.61 (m, 2H), 8.58 (d, J = 8.4 Hz, 1H), 8.52 (d, J = 8.4 Hz, 1H), 8.46 (d, J = 8.8 Hz, 1H), 8.40 (d, J = 8.4 Hz, 1H), 8.24-8.13 (m, 5H).



Scheme S2. Synthesis of compound **2**. Reagents and conditions: i) Br_2, S_2Cl_2, pyridine, 1-chlorobutane, 90°C, 12 h; ii) $Pd(PPh_3)_4$, Na_2CO_3, toluene/H_2O, 120°C, 24 h; iii) 4-methoxyphenylboronic acid, $Pd(PPh_3)_4$, Na_2CO_3, toluene/H_2O, 120°C, 24 h; iv) 1) MeLi, toluene, 2) MnO_2, 3) MeLi, toluene, 4) MnO_2; v) HCl, dioxane/H_2O, 80°C, 20 h; vi) **S9**, NH_4OAc, DMF, nitrogen atmosphere, 105°C; vii) 1) NHS, DIC, DMF, 2) **Yp**, DIEA, CH_3CN/DMF.

Compound S10. This compound was prepared by a published procedure.[3] MS (ESI), *m/z*: 339.64 ($[M+H]^+$); ^1H NMR (400 MHz, $CDCl_3$) δ 9.18 (d, J = 2.0 Hz, 2H), 8.41 (m, 2H), 7.76 (s, 2H).

Compound S15. This compound was prepared by a published procedure.[4] MS (ESI), *m/z*: 419.91 ($[M+H]^+$); ^1H NMR (400 MHz, $CDCl_3$) δ 10.13 (s, 1H), 8.08 (s, 1H), 8.04 (s, 1H), 8.03 (d, J = 8.4 Hz, 2H), 7.77 (dd, J = 11.2, 8.8 Hz, 2H), 7.66 (d, J = 8.4 Hz, 2H), 7.41 (d, J = 8.4 Hz, 2H), 7.05 (d, J = 8.4 Hz, 2H), 3.90 (s, 3H), 2.91 (s, 3H), 2.90 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.8, 159.4, 158.1, 156.8, 146.1, 137.3, 136.7, 136.5, 135.9, 135.7, 131.8, 130.4 (2C), 130.0 (2C), 129.9 (2C), 127.5, 126.9, 126.2, 125.7, 114.0 (2C), 55.4, 25.0.

Compound 1. Compound **S9** (40.0 mg, 0.0885 mmol), compound **S15** (60.0 mg, 0.1435 mmol) and ammonium acetate (170.0 mg, 2.2078 mmol, freshly purified by sublimation) were dissolved in anhydrous DMF (5.0 mL) in a sealed tube filled with N_2. The reaction mixture was stirred at 105°C for 24 h and then allowed to cool down to room temperature followed by filtration. The obtained yellow solid was washed with a small amount of MeOH and CH_2Cl_2 and dried in vacuum (50.0 mg, 66.5%). HRMS calcd for $C_{54}H_{36}N_5O_6$ ($[M+H]^+$): 850.2660, found 850.2675; ^1H NMR (400 MHz, DMSO-d_6) δ 8.90 (m, 2H, H-4' and 7'), 8.70-8.53 (m, 6H, H-o', 3' and 8'), 8.46 (d, J = 8.0 Hz, 2H, H-m_1), 8.36 (s, 1H, H-7), 8.25-8.10 (m, 5H, H-m' and 4), 7.95-7.80 (m, 4H, H-o_1, 5 and 6), 7.50 (d, J = 8.0 Hz, 2H, H-o_2), 7.09 (d, J = 8.0 Hz, 2H, H-m_2), 3.85 (s, 3H, OMe), 2.83 (s, 3H, H-g), 2.73 (s, 3H, H-h).

Yp (*o*-Phospho-L-tyrosine). This compound was prepared by a published procedure.[5] MS (ESI), *m/z*: 262.09 ($[M+H]^+$); ^1H NMR (400 MHz, D_2O) δ 7.16 (dd, J = 19.2, 8.8 Hz, 4H), 3.47 (dd, J = 7.6, 5.6 Hz, 1H), 2.96 (dd, J = 13.6, 5.6 Hz, 1H), 2.78 (dd, J = 13.6, 7.6 Hz, 1H); ^{31}P NMR (162 MHz, D_2O) δ 0.267.

Compound 2. NHS (50.0 mg, 0.4348 mmol) and DIC (100 µL, 0.6468 mmol) were added to a solution of compound **1** (15.0 mg, 0.0177 mmol) in anhydrous DMF (2.0 mL) at 0°C. The mixture was stirred at 0°C for 30 min, then at 40°C for 48 h. After the reaction was complete, the reaction mixture was allowed to cool down to 0°C and then added water (2 mL) followed by filtration. The obtained yellow solid was washed with a small amount of water, which was used for the next step without further purification. DIEA (500 µL, 2.876 mmol) was added to a solution of the obtained yellow solid and **Yp** (27.6 mg, 0.1057 mmol) in dry DMF (4 mL) at 0°C. The mixture was stirred at 0°C for 30 min, then at 35°C for 48 h. After the reaction was complete, the solvent was blow-dried with nitrogen. The residue was added 1 N HCl (2 mL) followed by filtration. The obtained yellow product **2** was sequentially washed with a small amount of 1 N HCl, methanol and dichloromethane (6.0 mg, 25.4%). MS (ESI), calculated *m/z*: 1335.32 (100.0%), 1336.32 (77.9%),

1337.32 (29.9%), 1338.33 (6.7%), 1337.32 (3.3%), 1336.32 (2.6%), 1338.33 (2.6%), 1337.32 (2.0%), 1339.33 (1.4%); observed *m/z*: 1334.36 ([M-H]$^-$); ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.04 (d, J = 8.4 Hz, 1H, H-7'), 9.00 (d, J = 8.4 Hz, 1H, H-4'), 8.80-8.73 (m, 2H, H-3' and 8'), 8.72-8.62 (m, 4H, H-o'), 8.56 (d, J = 8.4 Hz, 2H, H-m$_1$), 8.46 (s, 1H, H-7), 8.29 (s, 1H, H-4), 8.20-8.08 (m, 4H, H-m'), 8.04-7.88 (m, 4H, H-o$_1$, 5 and 6), 7.53 (d, J = 8.0 Hz, 2H, H-o$_2$), 7.32 (m, 4H, H-o$_3$), 7.12 (m, 6H, H-m$_2$ and m$_3$), 4.72 (m, 2H, CH), 3.87 (s, 3H, OMe), 3.24 (dd, J = 14.0, 4.4 Hz, 2H, H-α), 3.14 (dd, J = 14.0, 10.0 Hz, 2H, H-β), 2.88 (s, 3H, H-g), 2.80 (s, 3H, H-h); ^{31}P NMR (162 MHz, DMSO-d_6) δ -6.075, -6.205.

Absorption and emission

UV-vis absorptions were measured by using Thermo Nanodrop 2000C spectrophotometer. The path length of the cuvette was 1 cm. The detection range was set to 200-600 nm and the spectral resolution to 1.0 nm. All emission spectra were measured using a Hitachi F-7000 fluorescence spectrometer. The excitation wavelength was set to 320 nm and emission collection range from 350 to 900 nm.

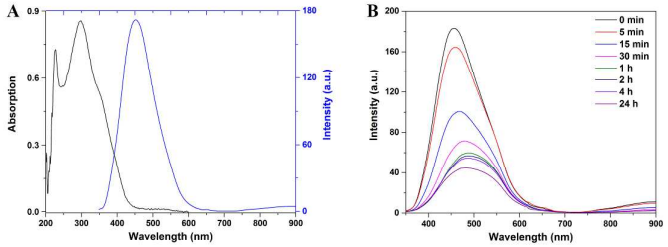


Figure S1. (A) UV-vis absorption (black) and emission (blue, λ_{ex} = 320 nm) spectra of compound **2** (15 μM) in PBS buffer; (B) Time-lapse emission spectra of dephosphorylation of compound **2** (100 μM) in PBS buffer (λ_{ex} = 320 nm) incubated with ALP (1 UmL^{-1}) at room temperature.s

TEM images

Aliquots (10 μL) of sample solution were added into a glow discharge copper grid (400 mesh) coated with thin carbon film and incubated for 30 s at room temperature. After removing excess solution, the grid was washed with deionized water three times and then stained with 2.0% (w/v) uranyl acetate (UA) by exposing the grid in three drops of UA solution for 30 s. TEM images were captured at high vacuum on transmission electron microscope JEM-1230R (JEOL, Japan).

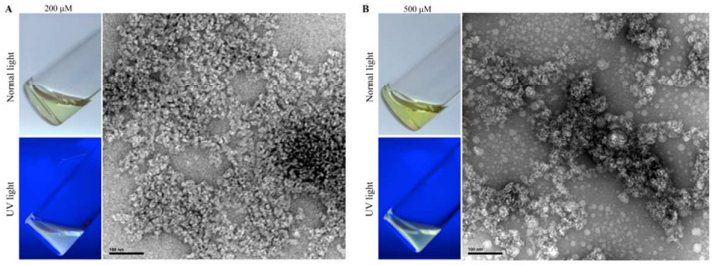


Figure S2. Optical images (under normal and UV (365 nm) light) and TEM images of compound **2** at different concentrations, after incubation with ALP (1 UmL^{-1}) at room temperature for 24 h in PBS buffer.

Molecular dynamics simulations and polymorph prediction

Molecular mechanics calculations were performed using Materials Studio. As the crystal structure prediction method uses a rigid body approximation in the initial search for crystal packing alternatives, it is necessary to perform an analysis to determine low energy geometry to be used as input for the packing calculations. The molecules were drawn and geometrical energy minimization scans were performed using Forcite module of Materials Studio. The optimized low energy conformations were used as the starting points for crystal structure prediction using the Materials Studio Polymorph Predictor (PP).

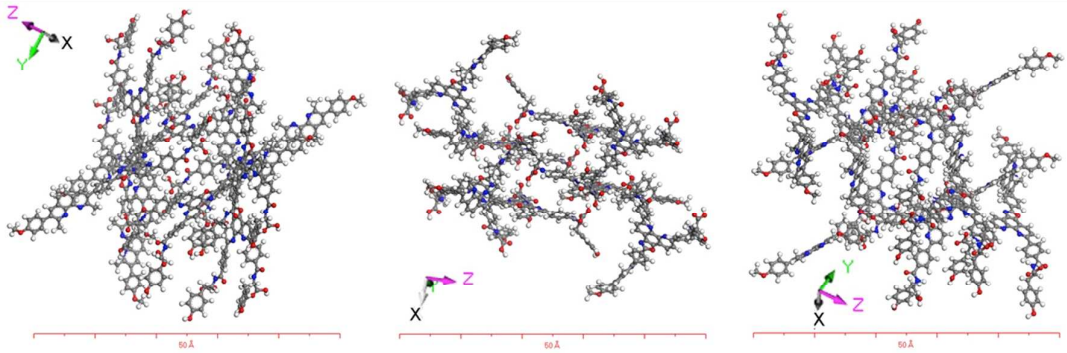


Figure S3. Predicted molecular packings by Polymorph Predictor visualized at different angles showing strong π-π interactions.

Cell culture

All cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, USA). HeLa cells were propagated in DMEM medium with 10% FBS containing 1% antibiotic solution. HS-5 cells, A375 cells were propagated in DMEM medium with 10% FBS without antibiotics. OVCAR-3 cells were propagated in RPMI-1640 medium with 0.01 mg/mL bovine insulin and 20% FBS. All cell lines were maintained in a fully humidified incubator (HERACELL 150i, Thermo Fisher Scientific, USA) containing 5% CO_2 at 37 °C.

Table S1. ALP isozyme expression profile of four cell lines.

Cell line	Overexpression of PLAP	Overexpression of TNAP	Exogenous ALP[a] (FBS concentration)
HS-5	-	-	10%
HeLa	+	+	10%
A375	-	+	10%
OVCAR-3	+	+	20%

[a] FBS contains ALP. The concentrations of FBS in culture medium for cell culture indicate the difference of exogenous ALP among the cell lines.

Cell viability assay

Cells in exponential growth phase were seeded in a 96 well plate at a concentration of 7×10^3 cells/well for HeLa, A375, and 1×10^4 cells/well for HS5, OVCAR3, cell lines. The cells were allowed to attach to the wells for 12 h at 37ºC, 5% CO_2. The culture medium was removed followed by addition of 100 µL culture medium containing different concentrations (0.1, 1, 10, 100 and 200 µM) of compound **2** (immediately diluted from 10 mM stock solution in PBS buffer). After the desired time of exposure, 10 µL MTT solution (5 mg/mL) was added to each well and incubated at 37°C for another 4 h, and then 100 µL of SDS solution (10% in Milli-Q water) was added to stop the reduction reaction and dissolve the purple formazan. The absorbance of each well at 570 nm was measured by a Tecan microplate reader. All experiments were conducted triplicate. The results were calculated as means, which are expressed as cell viability (%).

Cell imaging

Molecular probes were purchased from Life Technologies (Thermo Fisher Scientific, USA). Cells in exponential growth phase were seeded into a glass bottomed culture dish at 2×10^5 cells per dish. The cells were allowed for attachment for 24 h at 37 °C under 5% CO_2. Culture medium was removed and fresh medium containing different concentrations of compounds was added. After incubation for desired time, cells were washed with live cell imaging solution for three times, and further stained with commercial cell labels. Cells were then washed two times with fresh live-cell imaging solution and visualized by laser confocal microscopy (LSM 780, Carl Zeiss) immediately (λ_{ex}: 405 nm for compound **2**, 488 nm for Lysotracker Green DND-26, 561 nm for Actinred 555 and Alexa Fluor 555, 633 nm for CellMask deep red; λ_{em}: 420-480 nm for compound **2**, 508-570 nm for Lysotracker Green DND-26, 580-720 nm for Actinred 555, 570-606 nm for Alexa Fluor 555, 650-750 nm for CellMask deep red).

SEM

After taking confocal images, the samples were washed with PBS and then the cells were cross-linked with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 10 min. The samples were washed with 0.1 M cacodylate buffer for 5 min (×3) and then further fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer for 30 min, followed by washing with water for 5 min (×3) and then progressive dehydration in a graded series of ethanols (70%, 80%, 90%, 95% and twice in 100%, 3 minutes at each concentration). The cells were rinsed with *t*-butanol for 3 min (×3) and then dried by freeze-dryer overnight. SEM images of the dried samples were captured before platinum coating.

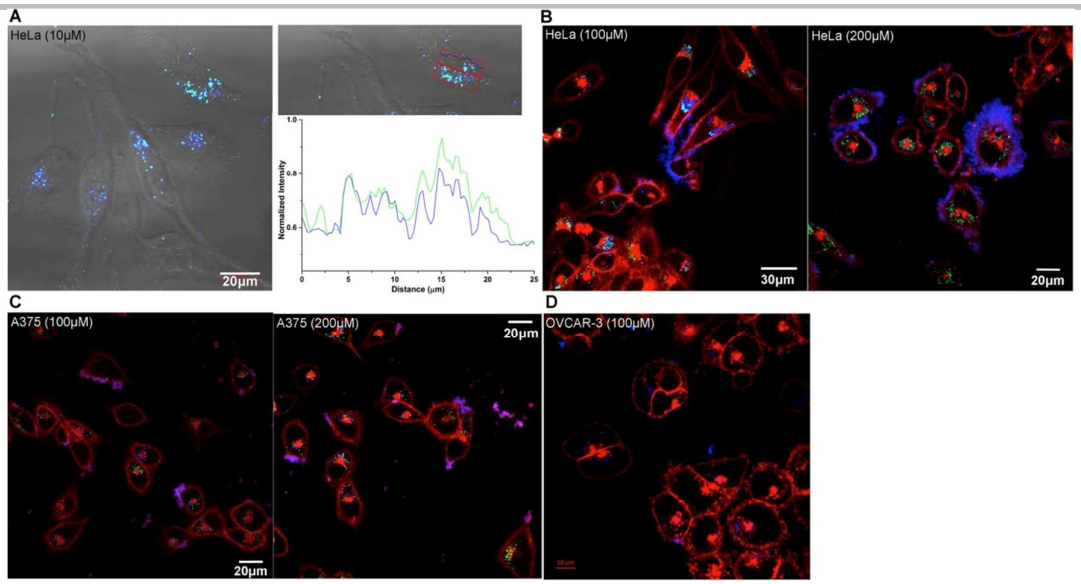


Figure S4. (A) Merged fluorescent image and DIC image of HeLa cells treated with precursor **2** (10 μM) for 24 h, and labeled with LysoTracker (green). The red frame in the inserted image indicates the interested area for intensity profile. The fluorescent intensity profile indicates the co-localization of self-assemblies (blue) in lysosomes (green). Fluorescent images of HeLa cells (B), A375 cells (C) and OVCAR-3 cells (D) treated with precursor **2** at 100 or 200 μM for 24 h, labeld with CellMask (red) and LysoTracker (for HeLa cells) (green).

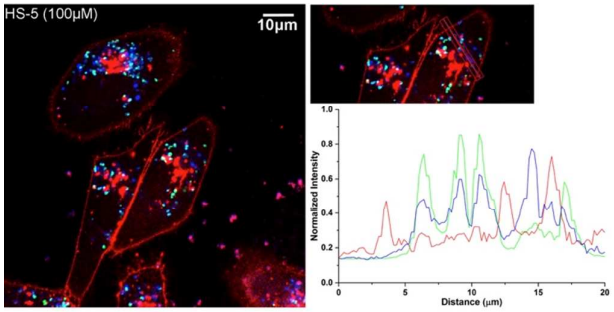


Figure S5. Fluorescent image of HS-5 cells treated with precursor **2** (100 μM) for 24 h, and labeled with CellMmask (red) and lysoTracker (green). The red frame in the inserted image indicates the interested area for intensity profile. The fluorescent intensity profile indicates the co-localization of self-assemblies (blue) in lysosomes (green).

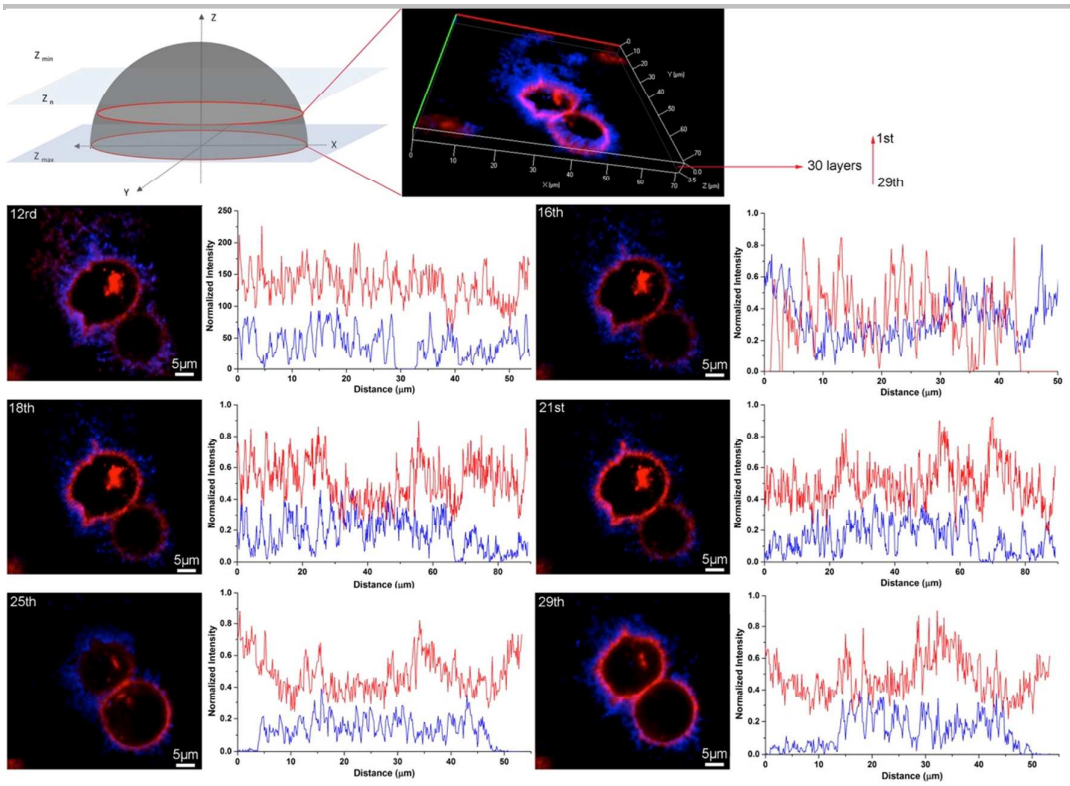


Figure S6. Fluorescent images of Hela cell and the fluorescence intensity profiles of the cell edges along the z-axis. We selected 6 layers among the 30 sections close to the bottom of the cell for exhibition and intensity profile plotting, and all the results indicate membrane insertion of self-assemblies.

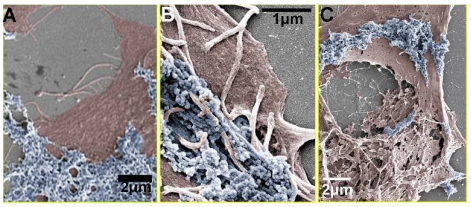


Figure S7. SEM images of HeLa cells (A), A375 cells (B), and OVCAR-3 cells (C) treated with precursor **2** (100 μM, 24 h). Cell membranes are highlighted in light pink, and self-assemblies are in light blue.

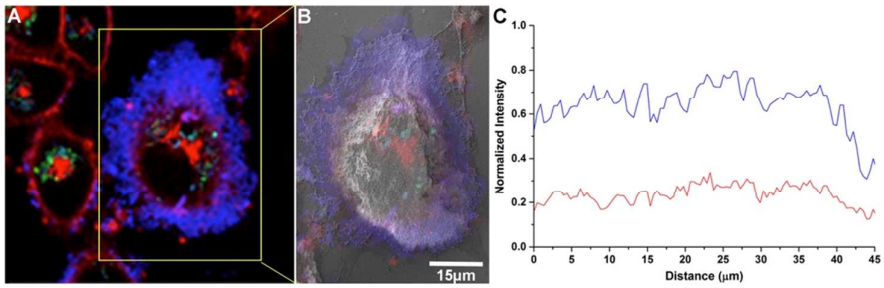


Figure S8. (A) Fluorescent image of HeLa cells treated with precursor **2** (200 μM) for 24 h, and labeled with CellMask (red) and lysoTracker (green). (B) Merged fluorescent microscopy correlated SEM image of HeLa cell highlighted by the yellow frame in figure A. (C) The fluorescent intensity profile indicates the co-localization of self-assemblies (blue) and cell membrane (red).

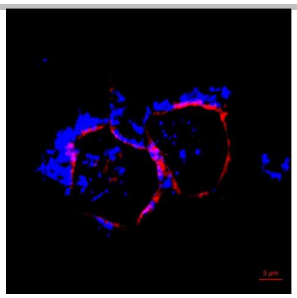


Figure S9. Fluorescent image of HeLa cells treated with PLAP inhibitor and incubated with **2** (100 μM) for 12h, strained with CellMask far red.

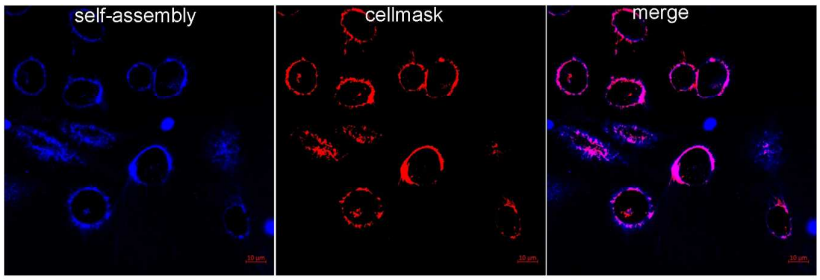


Figure S10. Fluorescent images of HeLa cells incubated with precursor **2** for 4 hours without FBS in the culture medium co-stained with CellMask far red.

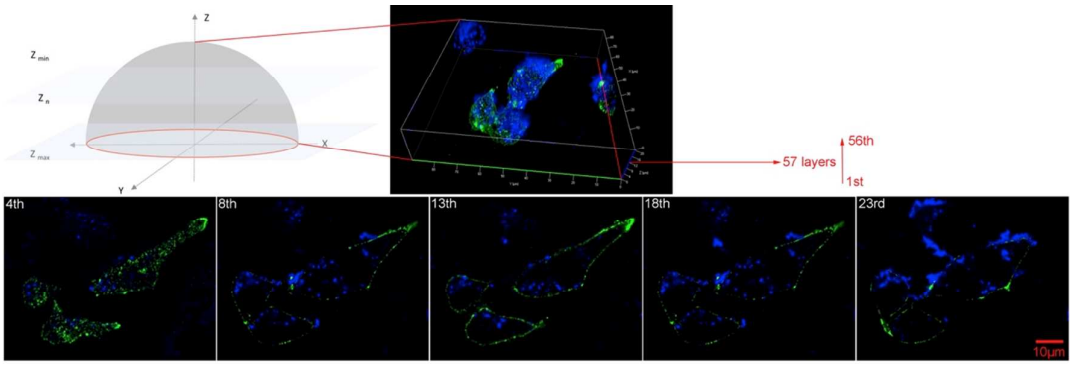


Figure S11. Fluorescent images of HeLa cells along the z-axis. We selected 5 layers among the 57 sections.

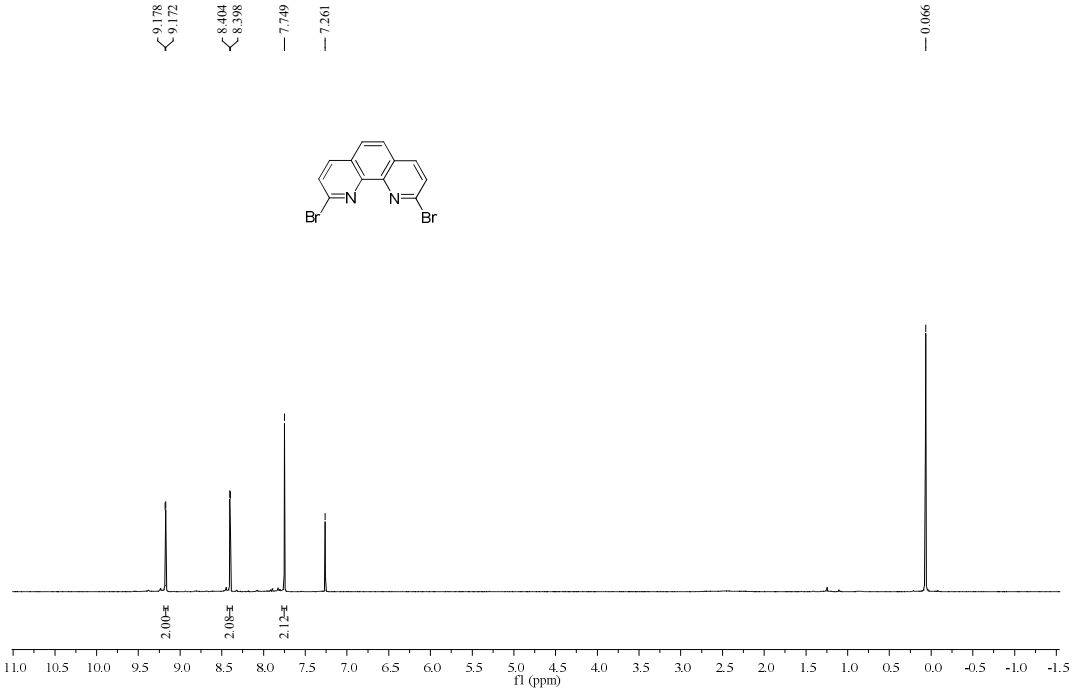
LDH release

LDH release activity was measured by pierce LDH cytotoxicity assay kit (Thermo Scientific) according to the manufacturers' instructions. HeLa, A375 and OVCAR3 cells were seeded at 1.5 ×10^4 cells/well in 100 μL of medium in a 96 well plate, respectively. After incubation with compound **2** (10, 50, 100 μM) for 12 h, the release of LDH in the supernatant was measured with a microplate reader at 490 nm. Cells treated with 10 μL of water (12 h) or Lysis buffer (10X, 45 min) was used as negative and positive controls, respectively. All experiments were carried out in triplicates. LDH activity was calculated as follows:

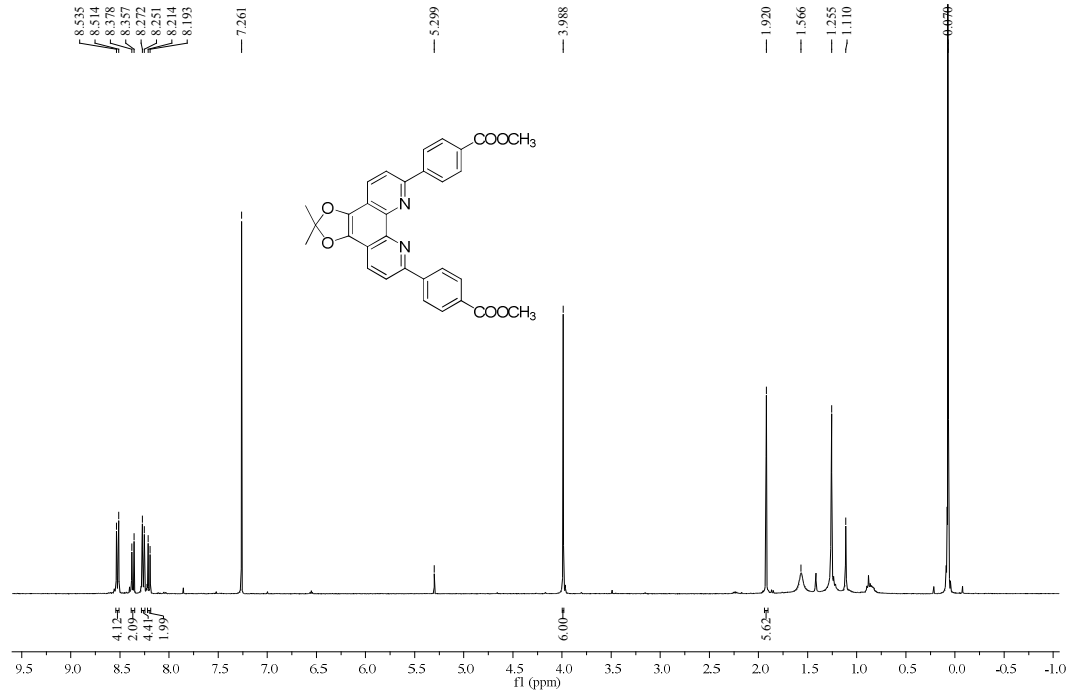
$$\text{LDH release (\%)} = \frac{\text{Compound treated activity} - \text{Negative activity}}{\text{Positive activity} - \text{Negative activity}} \times 100$$

Appendix

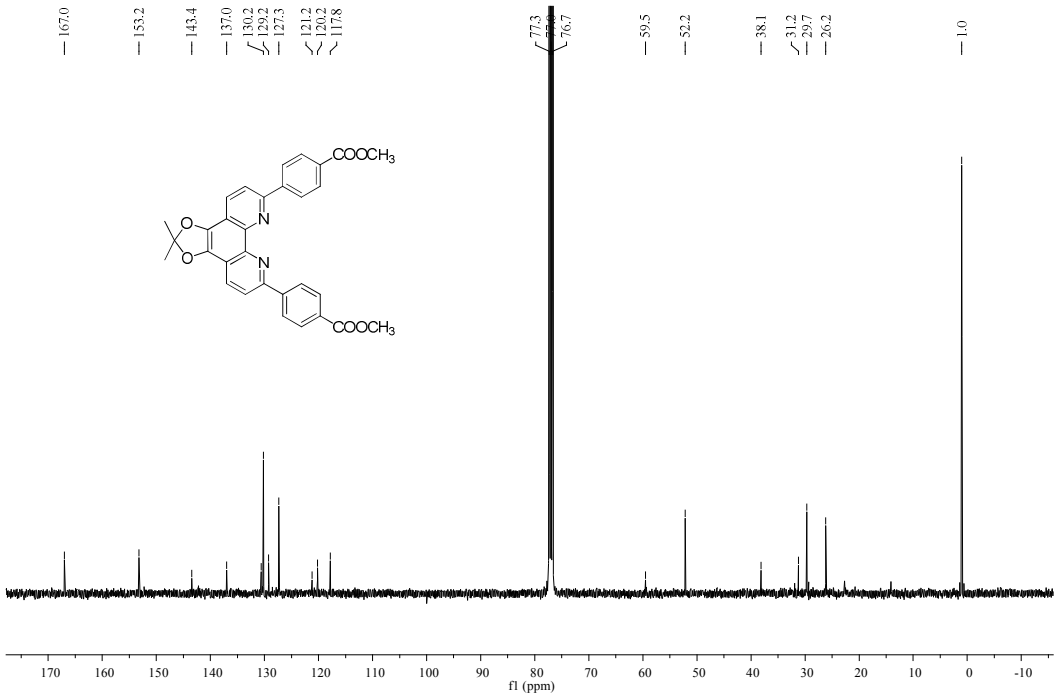
^{1}H NMR spectrum ($CDCl_3$) of compound **S4.**



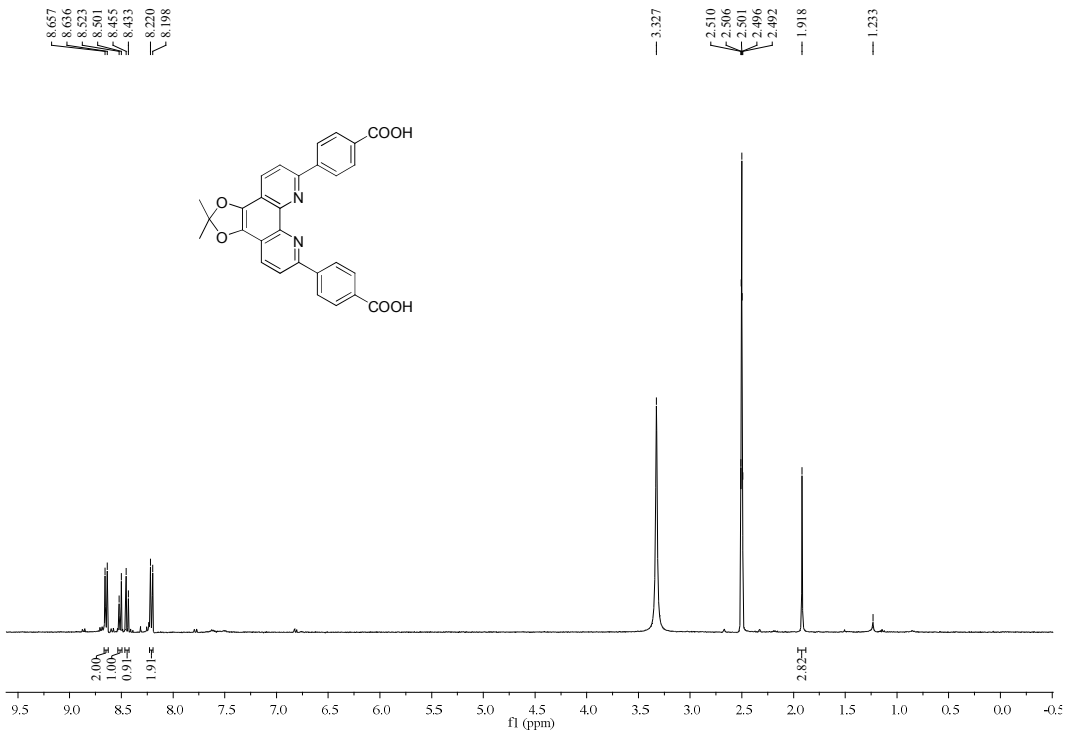
^{1}H NMR spectrum ($CDCl_3$) of compound **S7.**



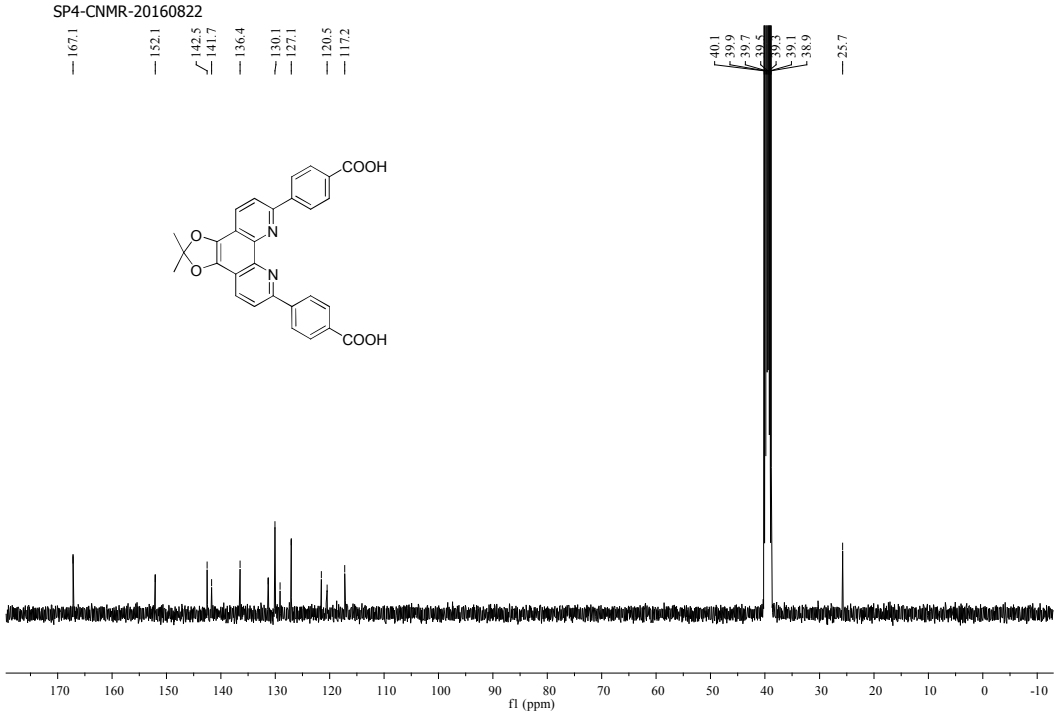
^{13}C NMR spectrum ($CDCl_3$) of compound **S7.**



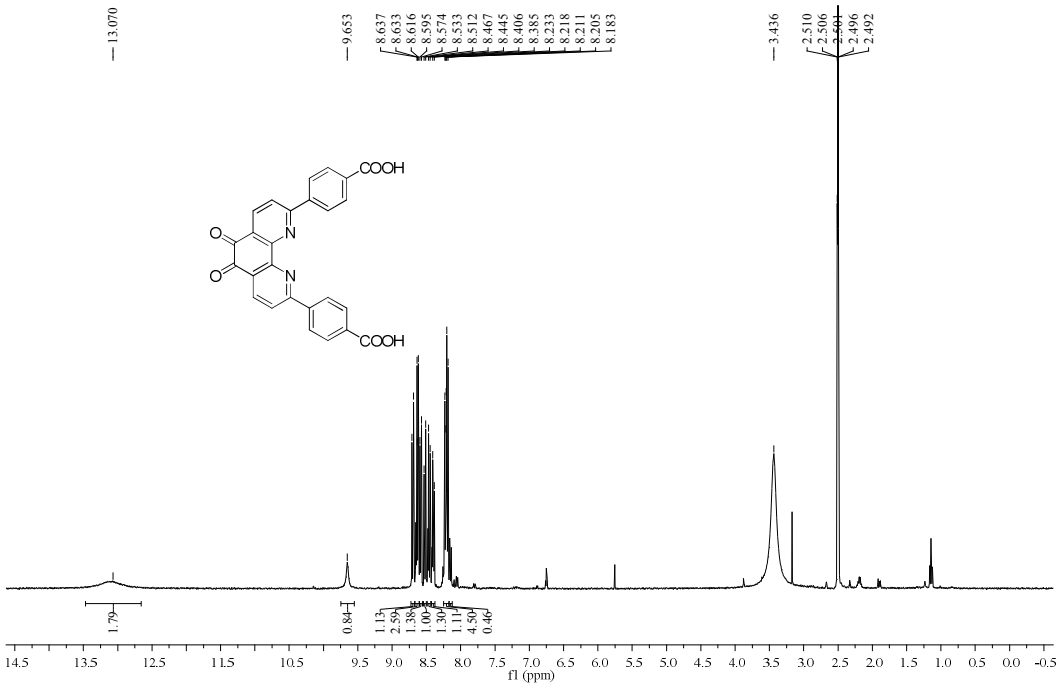
^{1}H NMR spectrum (DMSO) of compound **S8.**



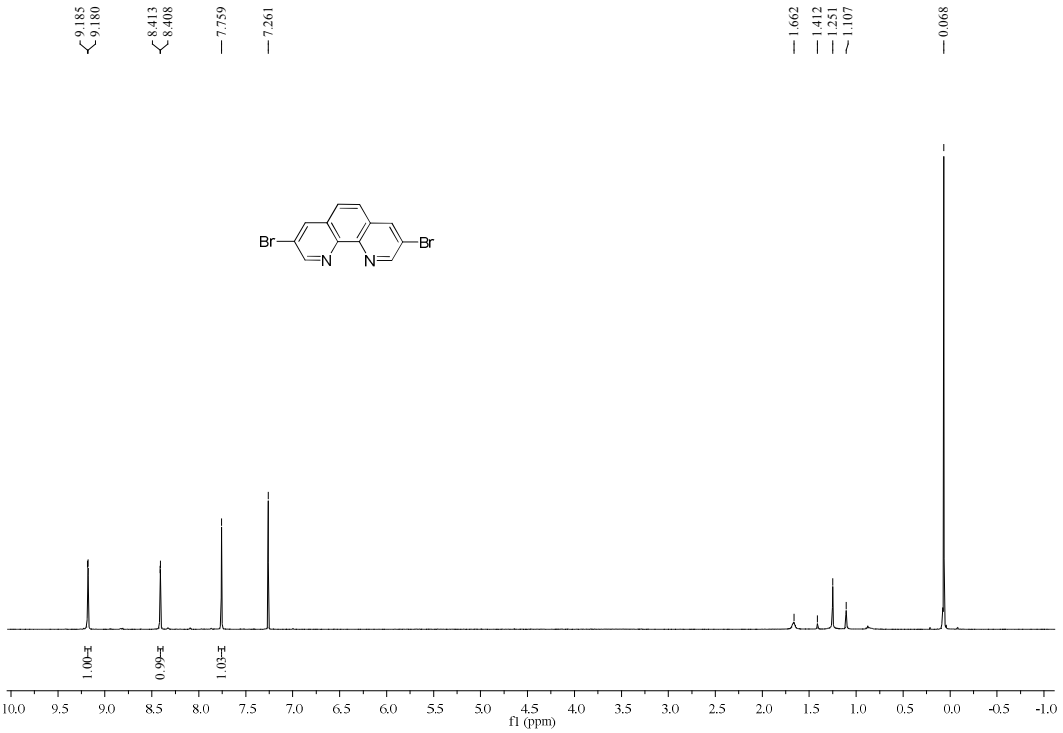
^{13}C NMR spectrum (DMSO) of compound **S8.**



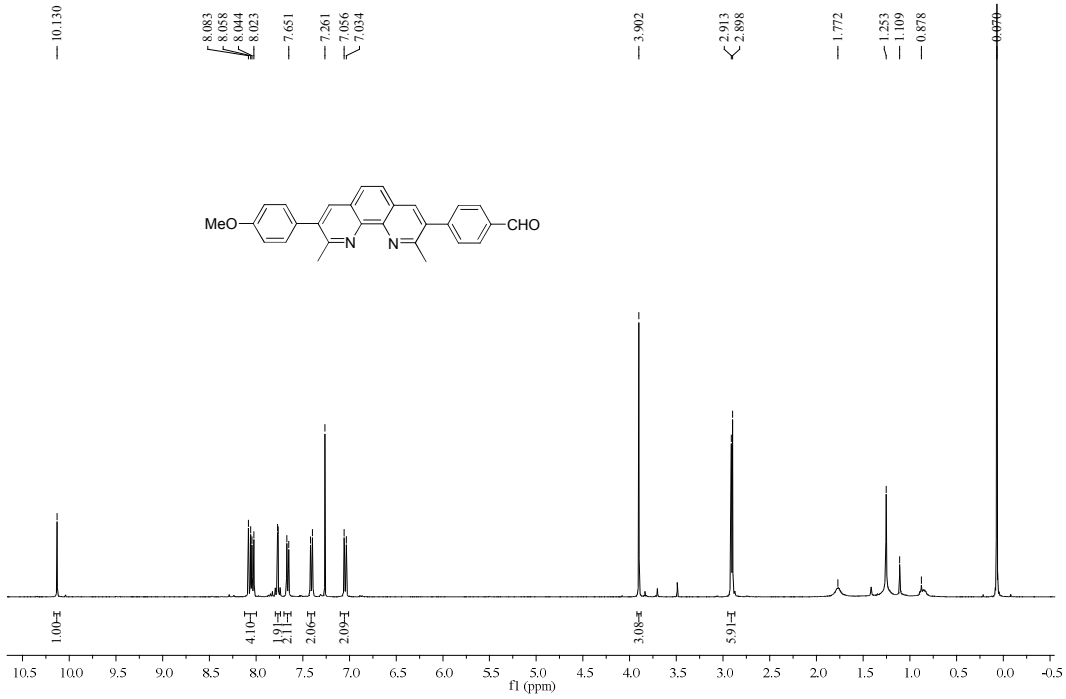
^{1}H NMR spectrum (DMSO) of compound **S9.**



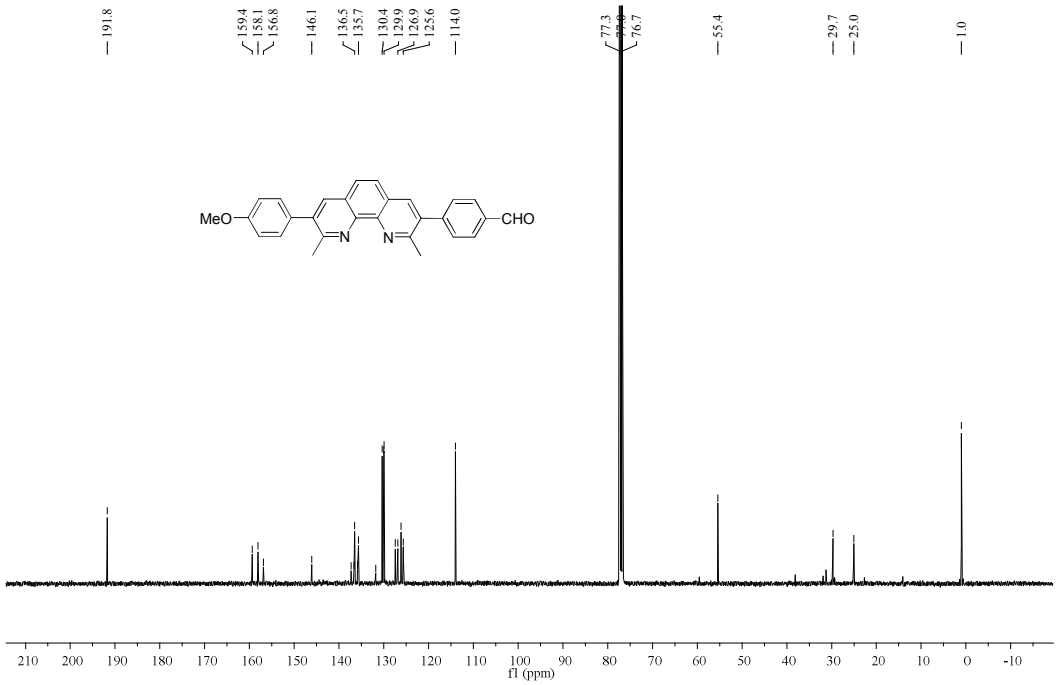
^{1}H NMR spectrum ($CDCl_3$) of compound **S10.**



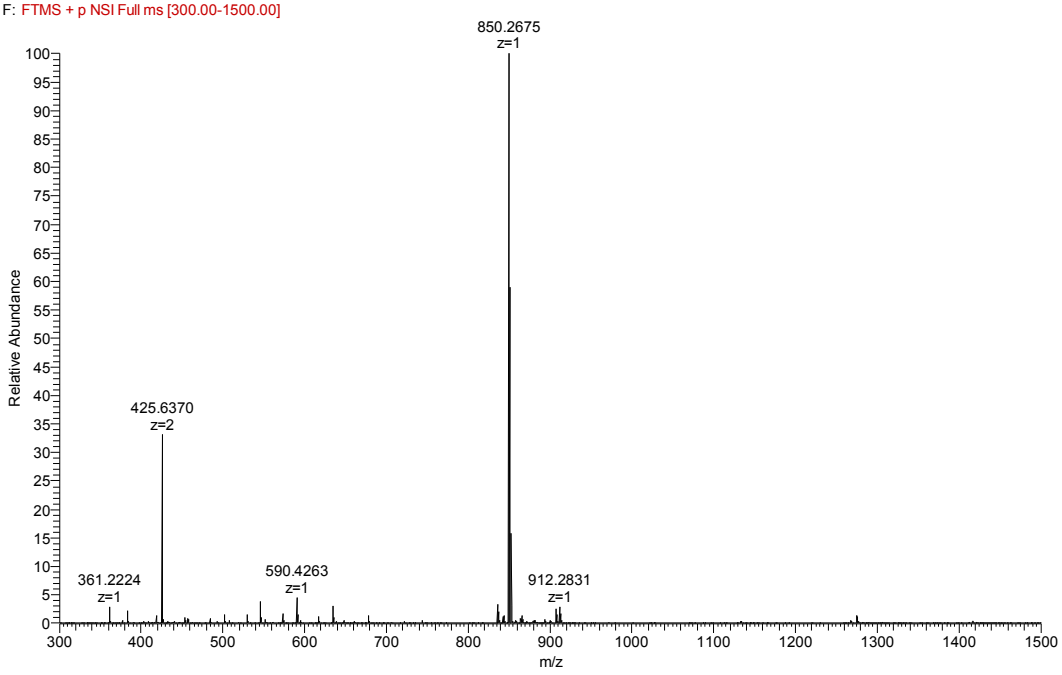
^{1}H NMR spectrum ($CDCl_3$) of compound **S15.**



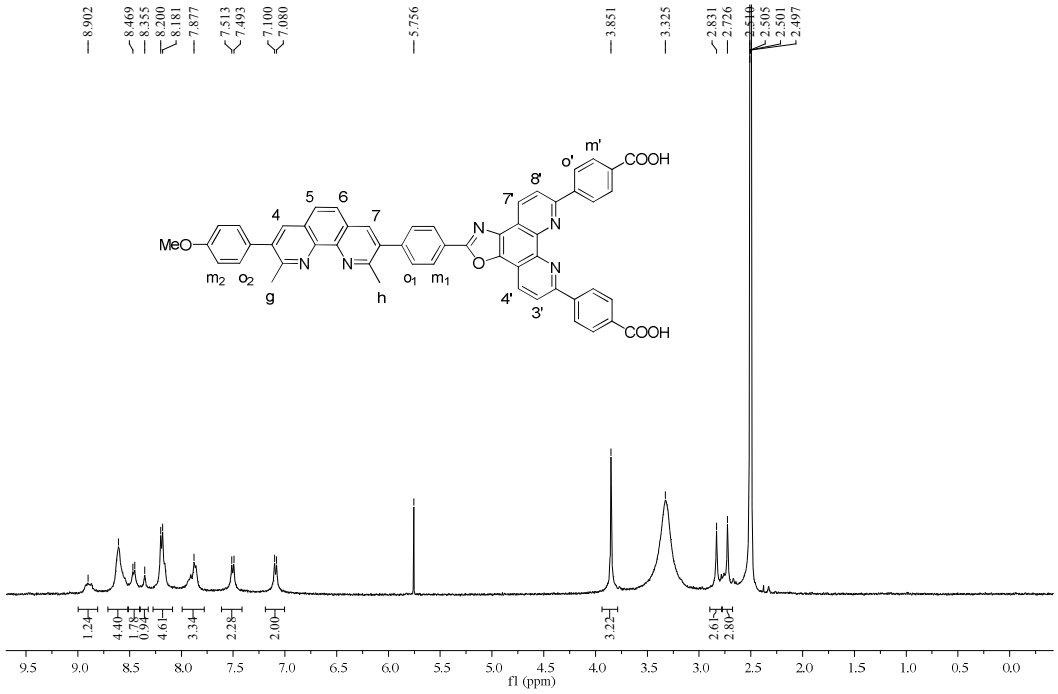
^{13}C NMR spectrum ($CDCl_3$) of compound **S15.**



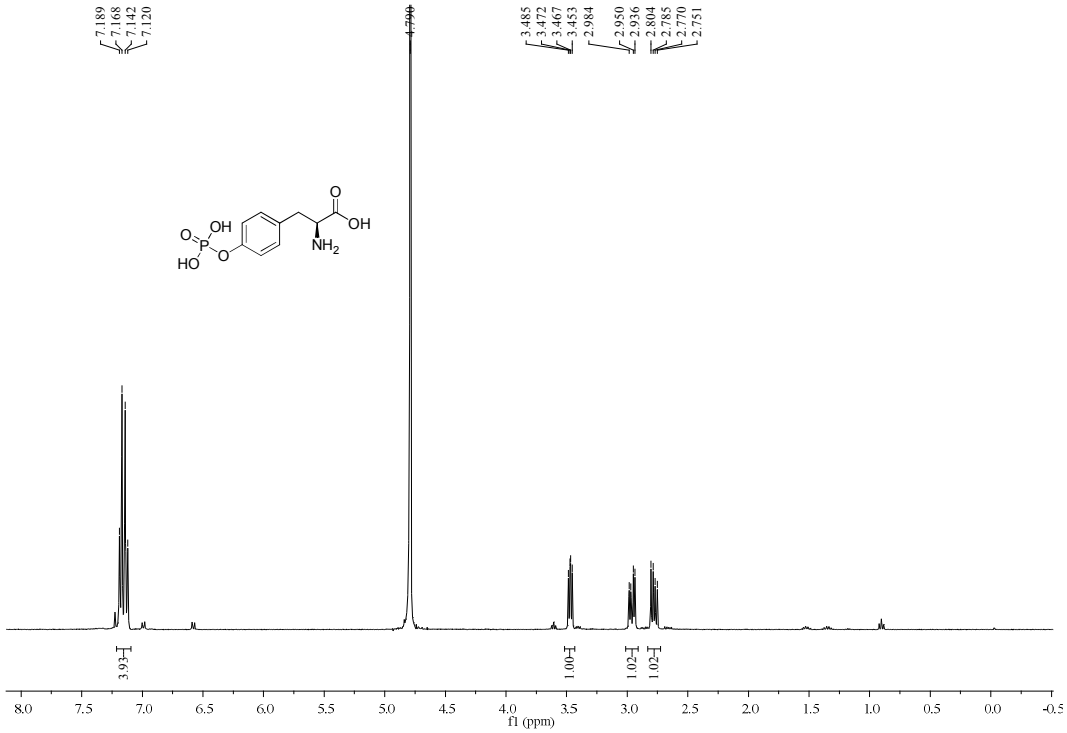
HRMS spectrum of compound **1.**



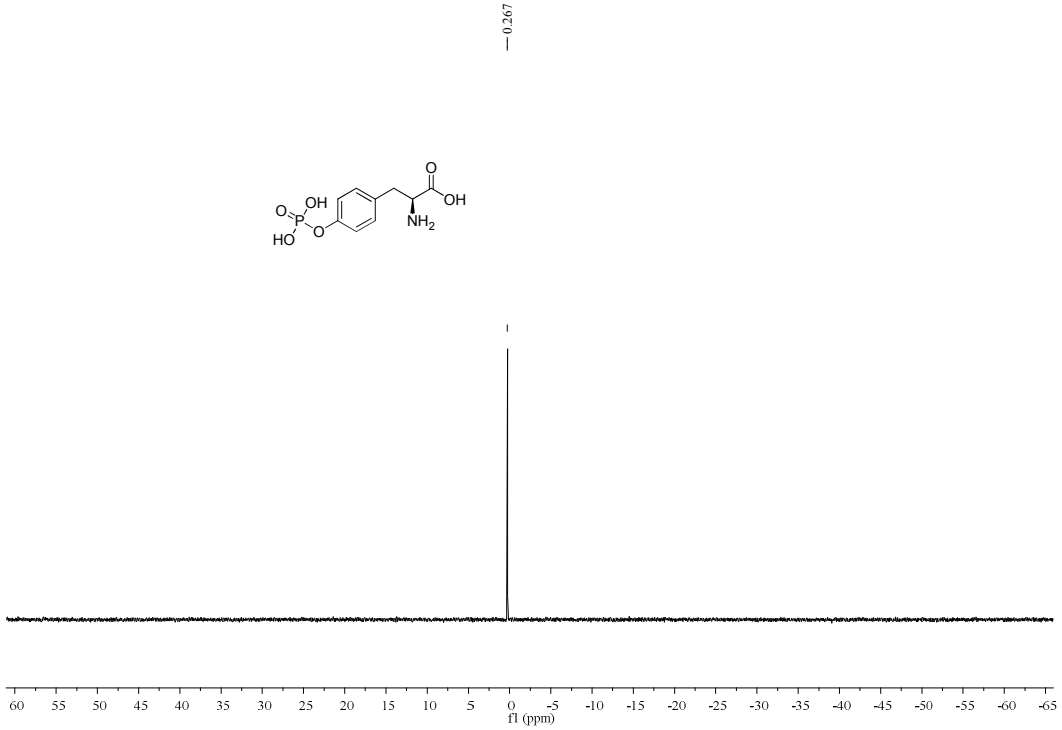
^{1}H NMR spectrum (DMSO) of compound **1.**



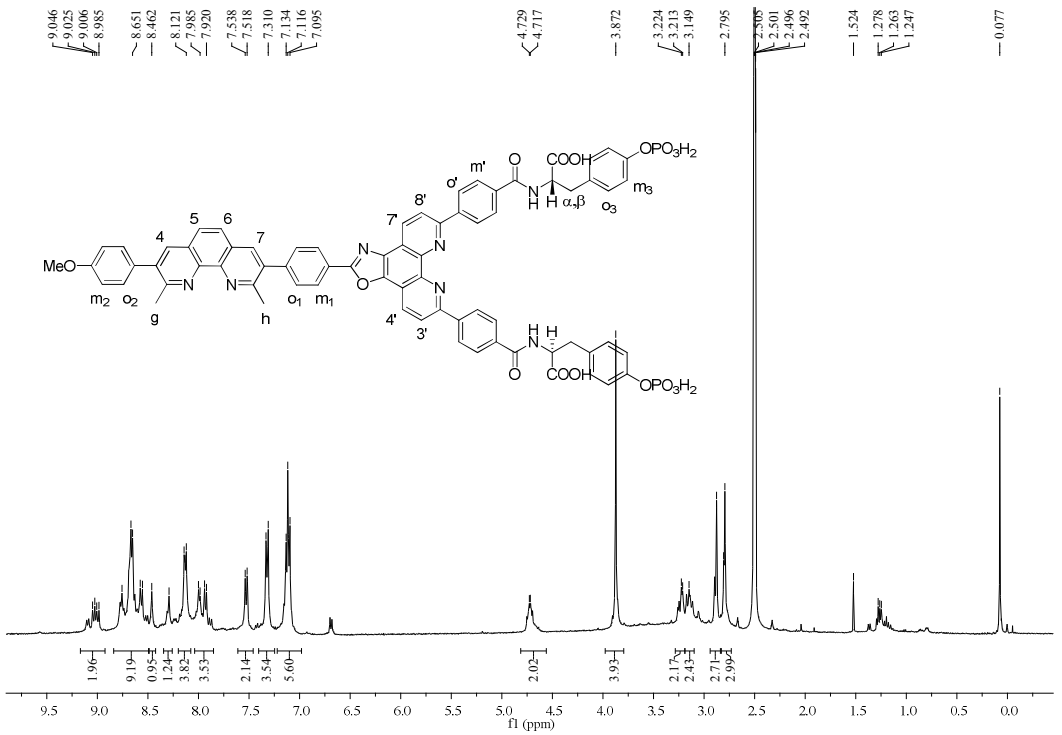
^{1}H NMR spectrum (D_2O) of **Yp.**



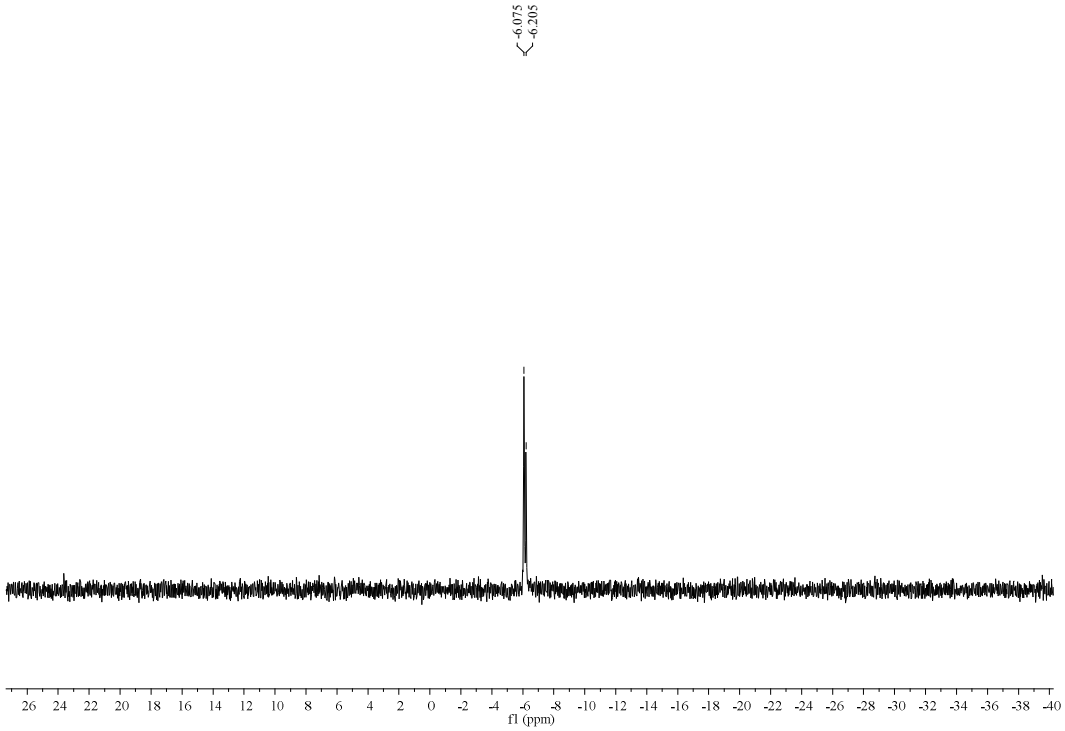
^{31}P NMR spectrum (D_2O) of **Yp.**



^{1}H NMR spectrum (DMSO) of compound **2.**



^{31}P NMR spectrum (DMSO) of compound **2.**



References

[1] H. C. Guo, R. H. Zheng, H. J. Jiang, *Organic Preparations and Procedures International* **2012**, *44*, 392-396.

[2] J. Frey, T. Kraus, V. Heitz, J. P. Sauvage, *Chemistry* **2007**, *13*, 7584-7594.

[3] aY. Saitoh, T.-a. Koizumi, K. Osakada, T. Yamamoto, *Canadian journal of chemistry* **1997**, *75*, 1336-1339; bC. Dietrich-Buchecker, M. C. Jimenez, J. P. Sauvage, *Tetrahedron Letters* **1999**, *40*, 3395-3396.

[4] J. Voignier, J. Frey, T. Kraus, M. Budesinsky, J. Cvacka, V. Heitz, J. P. Sauvage, *Chemistry* **2011**, *17*, 5404-5414.

[5] P. F. Alewood, R. B. Johns, R. M. Valerio, B. E. Kemp, *Synthesis-Stuttgart* **1983**, 30-31.