Electronic Supplementary Information

Enzyme-instructed self-assembly of taxol promotes axonal branching

Bin Mei,[a,b,§] Qingqing Miao,[a,§] Anming Tang,[a] and Gaolin Liang*[,a]

[a]CAS Key Laboratory of Soft Matter Chemistry, Departments of Chemistry, 96 Jinzhai Road, Hefei, Anhui 230026, China

[b]Department of Biology, College of Life Science, Anhui Medical University, Hefei, Anhui 230032, China

*** Corresponding author:**

E-mail: gliang@ustc.edu.cn (G.-L. L.).

Contents:

1. Materials and methods

1.1 Materials

All the starting materials were obtained from Adamas or Sangon Biotech. Commercially available reagents were used without further purification, unless noted otherwise. All other chemicals were reagent grade or better.

1.2 Methods

1.2.1 General methods

HPLC analyses were performed on a Shimadzu UFLC system equipped with two LC-20AP pumps and an SPD-20A UV-vis detector using a Shimadzu PRC-ODS column, or on an Agilent 1200 HPLC system equipped with a G1322A pump and an in-line diode array UV detector using an Agilent Zorbax 300SB-C18 RP column, with CH_3CN (0.1% of TFA) and ultrapure water (0.1% of TFA) as the eluent. The spectra of electrospray ionization-mass spectrometry (ESI-MS) were recorded on a LCQ Advantage MAX ion trap mass spectrometer (Thermo Fisher). ^1H NMR spectrum was obtained on a Bruker AV 300. Cryo transmission electron microscopy (Cryo-TEM) images were obtained on a Tecnai F20 Transmission Electron Microscope from FEI Company, operating at 200 kV. The cryo samples were prepared as following: a special copper grid coated with carbon was put into Gatan SOLARUSTM plasma-cleaning system to remove hydrocarbon contamination on the sample holder, and then the sample was dropped on the copper grid in FEI Vitrobot sample plunger. The sample preparation was completed in the plunger.

1.2.2 Synthesis of Fmoc-Phe-Phe-Lys (taxol)-Tyr(H_2PO_4)-OH (1)

1.2.2.1 Synthesis of Fmoc-Phe-Phe-Lys-Tyr(H_2PO_4)-OH (E)

Tetrapeptide Fmoc-Phe-Phe-Lys(Boc)-Tyr(H_2PO_4)-OH (**D**) was prepared with solid phase peptide synthesis (SPPS). The Boc protecting group was cleaved with 95% TFA in DCM for 3 h at room temperature to yield Fmoc-Phe-Phe-Lys-Tyr(H_2PO_4)-OH (**E**) after HPLC purification (Scheme S1). Mass of **E**: calc. for $C_{48}H_{53}N_5O_{11}P$, [$(M+H)^+$]: 906.35, obsvd. ESI/MS: *m*/*z* 906.34 (Figure S1).

1.2.2.2 Synthesis of Suc-taxol (B)

Taxol (341 mg, 0.4 mmol) was added to succinic anhydride (140 mg, 1.4 mmol) in the presence of 4-dimethylamino-pyridine (86 mg, 0.7 mmol) which was previously dried under vacuum for 2 h. Then 8 mL of dry pyridine was added and the solution was stirred for 3 h at room temperature. The 2'-succinyl-taxol was purified by extraction according to following procedure: After 20 mL of dry dichloromethane (DCM) was added into the reaction mixture, the organic phase was washed using 1 M HCl solution (20 mL × 3) and water (20 mL × 3). Water phase was extracted by DCM (10 mL × 3). The organic phase was combined and washed by brine (10 mL × 3) and dried over Na_2SO_4. The filtrate was concentrated on rotary evaporator and the crude product suc-taxol was used without further purification (Scheme S1).

1.2.2.3 Synthesis of NHS-suc-Taxol (C)

Suc-taxol (**B**, 190.6 mg, 0.2 mmol) was mixed with N-hydroxysuccinimide (23.0 mg, 0.2 mmol), then 10 mL of $CHCl_3$ was added to obtain a well-dispersed solution. After N, N'-dicylcohexylcarbodiimide (41.2 mg, 0.2 mmol) was added into the mixture, the solution was stirred for 4 h at room temperature. The

NHS-suc-Taxol (**C**) was purified by chromatography with chloroform-methanol as the eluent (19:1) (Scheme S1).

1.2.2.4 Synthesis of Fmoc-Phe-Phe-Lys(taxol)-Tyr(H_2PO_4) (1)

Fmoc-Phe-Phe-Lys-Tyr(H_2PO_4) (**E**, 54.4 mg, 0.06 mmol) was dissolved in 5 mL water, and the pH of the solution was adjusted to 8.5 with sodium carbonate. NHS-suc-taxol (**C**, 50 mg, 0.0476 mmol) was dissolved in 3 mL acetone, and then added into the water solution drop wise. The ratio of water/acetone was adjusted to keep the reaction mixture clear. The mixture was stirred at room temperature for 12 h. The reaction mixture was subjected to HPLC purification to yield **1** (Scheme S1). Mass of **1**: calc. for $C_{99}H_{104}N_6O_{27}P$, [$(M-H)^-$]: 1839.67, obsvd. ESI-MS: *m*/*z* 1839.92 (Figure S2). ^{1}H NMR of **1** (d_6-DMSO, 300 MHz, Figure S3) δ (ppm): 9.21 (d, J = 6.0 Hz, 1 H), 8.18 (d, J = 6.0 Hz, 1 H), 8.08 (t, 2 H), 7.97 (d, J = 9.0 Hz, 1 H), 7.86 (t, 6 H), 7.76-7.47 (br, 10 H), 7.44 (d, J = 3.0 Hz, 4 H), 7.29-7.11 (br, 16 H), 7.07 (d, J = 6.0 Hz, 2 H), 6.29 (s, 1 H), 5.82 (t, 1 H), 5.53 (t, J = 9.0 Hz, 1 H), 5.41 (d, J = 6.0 Hz, 1 H), 5.33 (d, J = 9.0 Hz, 1 H), 4.90 (d, J = 9.0 Hz, 1 H), 4.62 (m, 2 H), 4.41 (m, 1 H), 4.30-4.00 (br, 7 H), 3.08-2.53 (br, 14 H), 2.36 (m, 2 H), 2.23 (s, 3 H), 2.08 (s, 3 H), 1.76 (s, 3 H), 1.49 (s, 4 H), 1.23 (br, 6 H), 1.06-0.96 (br, 6 H).

1.2.3 MTT assay

The cytotoxicity was measured using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay with HeLa cells. Cells growing in log phase were seeded into 96-well cell-culture plate at 3 × 10^3/well. The cells were incubated for 12 h at 37 °C under 5% CO_2. The solutions of **1** or taxol (100 μL/well) at concentrations of 1 nM, 10 nM, 100 nM, 1 μM, or 10 μM in 100 μL medium were added to the wells, respectively. The cells were incubated for 24 and 48 h at 37 °C under 5% CO_2. A solution of 5

mg/mL MTT dissolved in phosphate buffered saline (PBS) (pH 7.4) (10 μL /well) was added to each well of the 96-well plate. A solution of 10% SDS dissolved in 0.01 M HCl (100 μL/well) was added to dissolve the formazan after an additional 4 h-incubation. The data were obtained using an ELISA reader (VARIOSKAN FLASH) to detect its absorption at 570/680 nm. The following formula was used to calculate the viability of cell growth: Viability (%) = (mean of Absorbance value of treatment group / mean of Absorbance value of control) ×100.

1.2.4 Microtubule imaging assay

Transfected cherry-tubulin HeLa cell lines was offered by Laboratory of Cellular Dynamics in University of Science and Technology of China. HeLa cells were cultured in Dulbecco's modified eagle medium (DMEM, GIBCO) supplemented with 10% fetal bovine serum (FBS, GIBCO) and streptomycin (100 μg/mL) and kept in a humid atmosphere of 5% CO_2 at 37 °C. The HeLa cells were plated on 3.5 cm cell culture dish at 50% cell density in the next day. Then the HeLa cells were washed for three times with phosphate buffered saline (PBS) and incubated with 10 μM **1** or taxol in serum-free medium at 37 °C for 2 h in a CO_2 incubator. Then, the cells were washed with PBS for another three times and fixed by 4% paraformaldehyde for 30 min prior to imaging.

1.2.5 Neuronal morphology assay

1.2.5.1 Neurons culture

Primary cultured cortical neurons were obtained from 18-day-old C57BL/6 mouse embryos. Briefly, the cerebral cortex was dissected and incubated with 0.25% trypsin at 37 °C for 15 minutes. Cells were then mechanically dissociated using a Pasteur pipette with a fire-narrowed tip in culture medium and plated at a

low density of 2×10^4 cells/ml on the coverslips pre-coated with poly-D-lysine (10 μg/ml). Cells were maintained in neurobasal/B27 medium containing 0.5 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified environment of 5% CO_2/95% air at 37 °C. Half-changes of medium were done twice weekly.

1.2.5.2 Immunofluorescence

Neurons (days in vitro 5, DIV5) were incubated with **1** or taxol at 10 nM for 20 h, then fixed with 4% paraformaldehyde for 20 min at room temperature. Subsequently, fixed neurons were washed for 3 times with PBS, followed by blocking with 5% bovine serum albumin in PBS for 1 h at room temperature. After blocking, cells were incubated with monoclonal anti-MAP2 antibody (1:500, Millipore) overnight at 4 °C. After washing for three times with PBS, cells were incubated with Alexa Fluor 488 (green)-conjugated antimouse secondary antibody for 1 h at 37 °C. After washing with PBS for three times and distilled water once, the final samples were mounted on a glass slide with VECTASHIELD mounting medium. Images were acquired using Carl Zeiss microscope.

1.2.5.3 Neuron morphology analysis

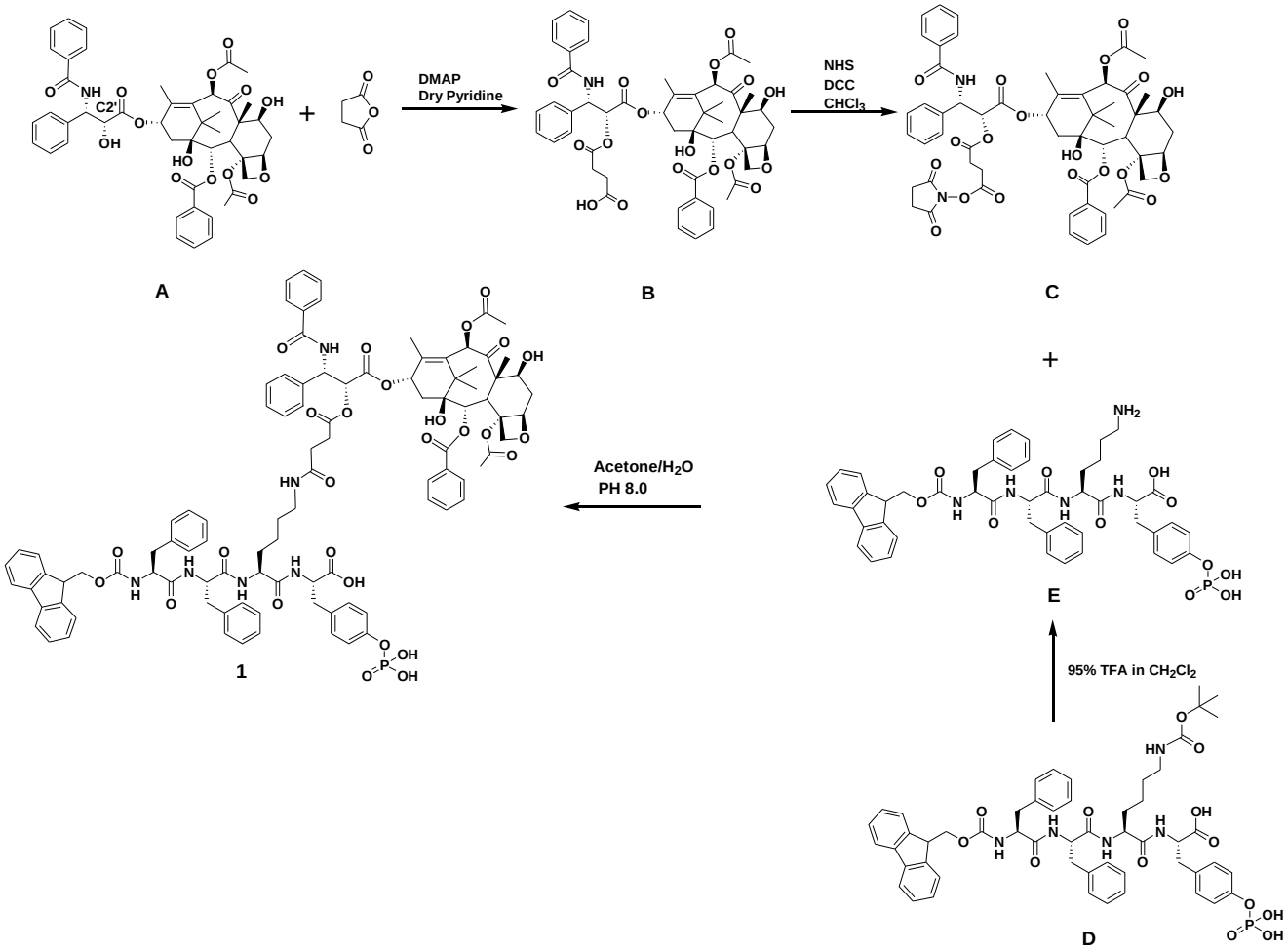
ImageJ (National Institute of Health) with Neuron J plugin were respectively used for the reconstruction of the neurons' morphology and for the quantitative analysis.

1.3 Statistical analysis

All the data are analyzed using Origin 8.0. All the bars in figures are represented as mean ± sd. The data was compared and analyzed using the one-way analysis of variance test (ANOVA). MTT assay result was analyzed using pair sample t-test. Results were considered significant at $P < 0.05$.

2. Synthetic route for compound 1

Scheme S1. Synthetic route for compound **1**.



3. Supporting figures

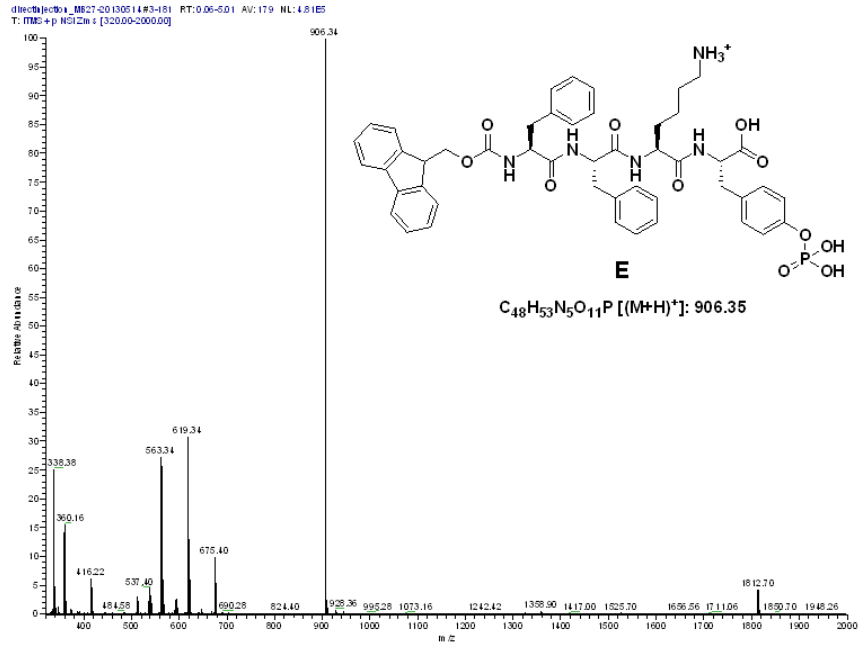


Figure S1. ESI-MS spectrum of **E**.

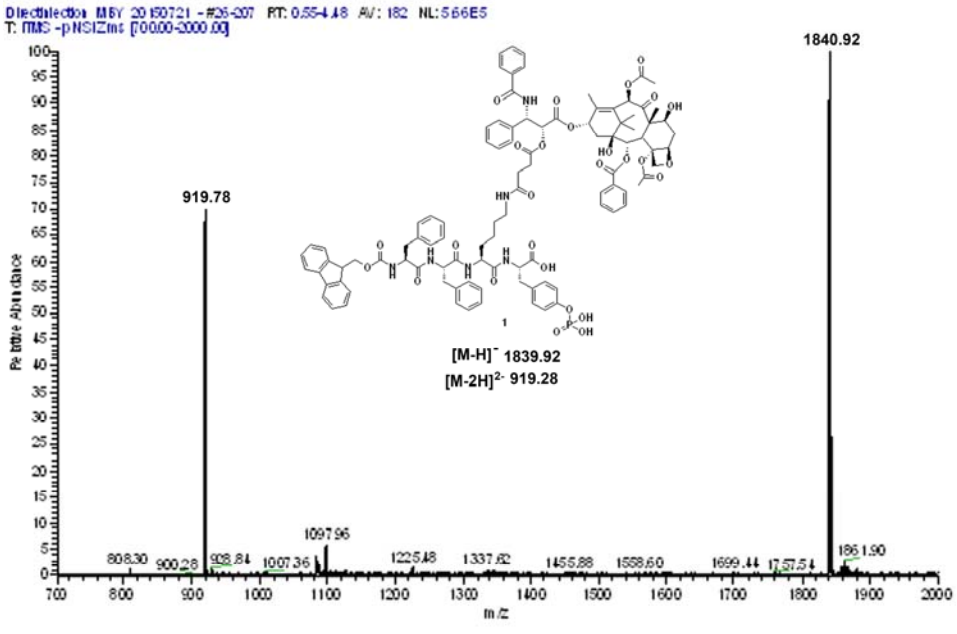


Figure S2. ESI-MS spectrum of **1**.

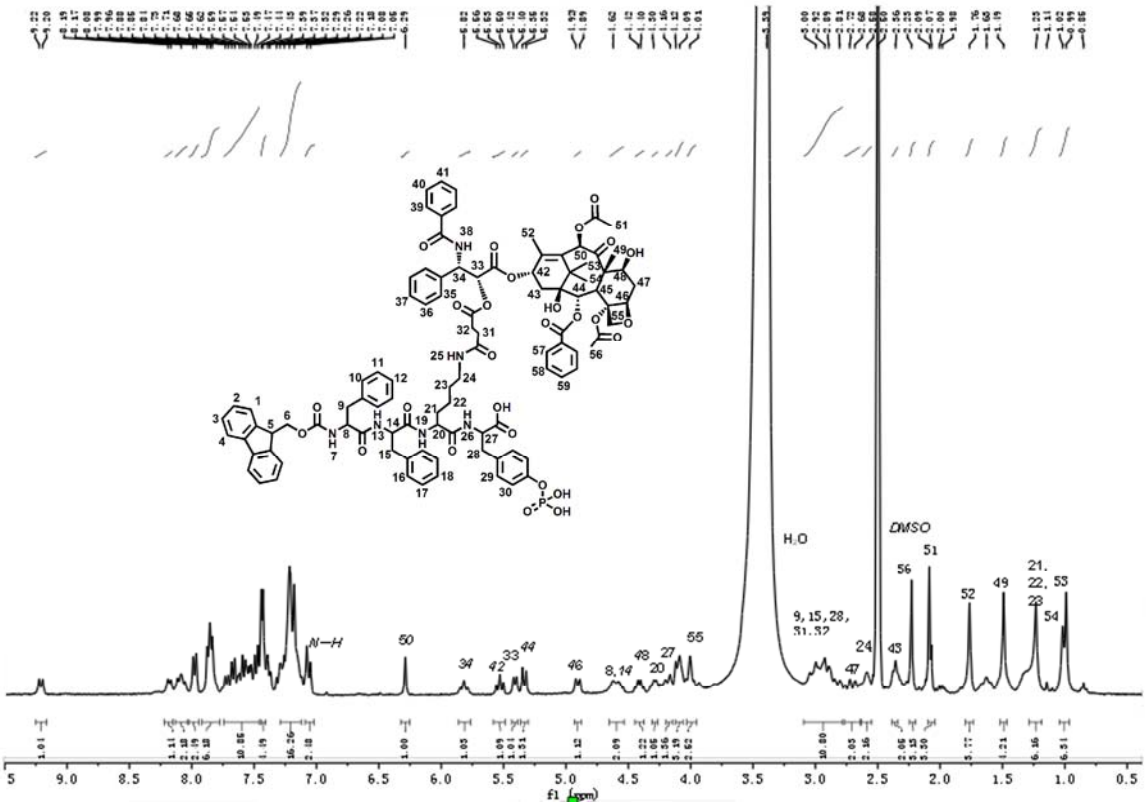


Figure S3. ^{1}H NMR spectrum of **1**.

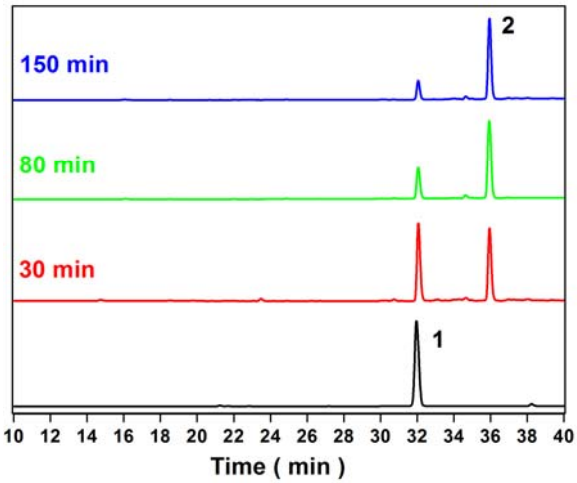


Figure S4. HPLC trace of **1** (black), and time course HPLC traces of the solution containing 1 wt% **1** incubated with 300 U/mL alkali phosphatase at 37 °C at pH 8.5. Wavelength for detection: 300 nm.

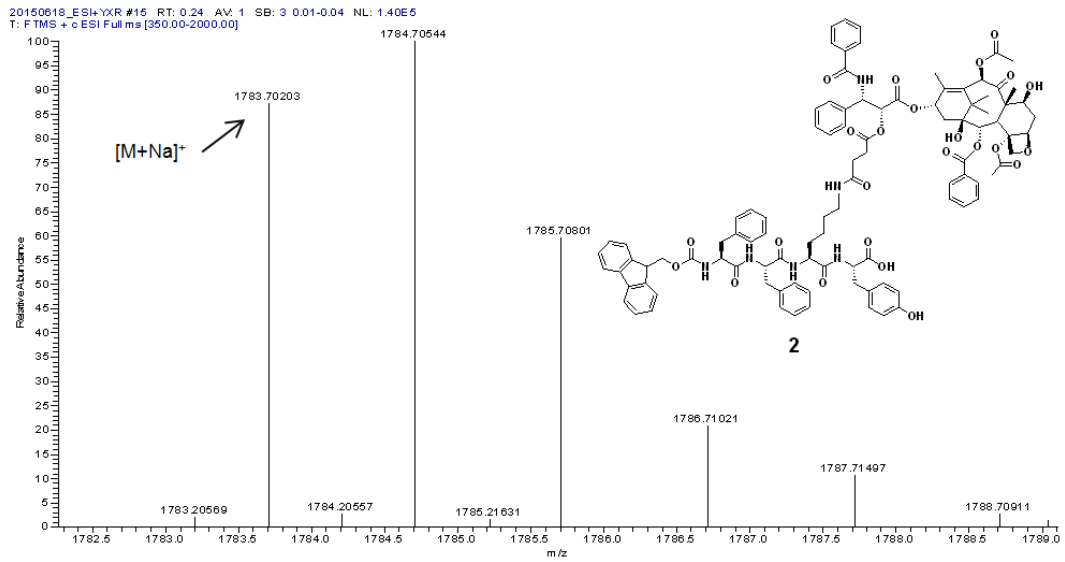


Figure S5. HR-ESI/MS spectrum of **2**.

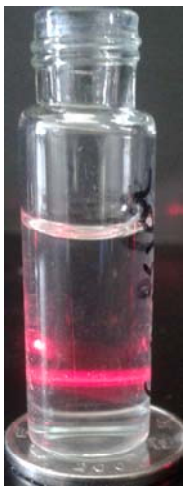


Figure S6. Tyndall effect of the 4-fold diluted incubation mixture of **1** at 1 wt% after 12 h incubation with 300 U/mL alkali phosphatase at 37 °C and pH 8.5 excited by laser light.

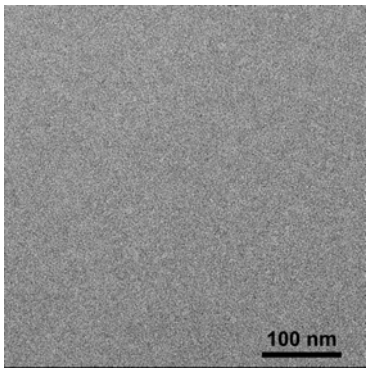


Figure S7. Cryo-TEM image of the solution of 1 wt% **1** at pH 8.5 (adjusted with Na_2CO_3) and 37 °C after 12 h stand.

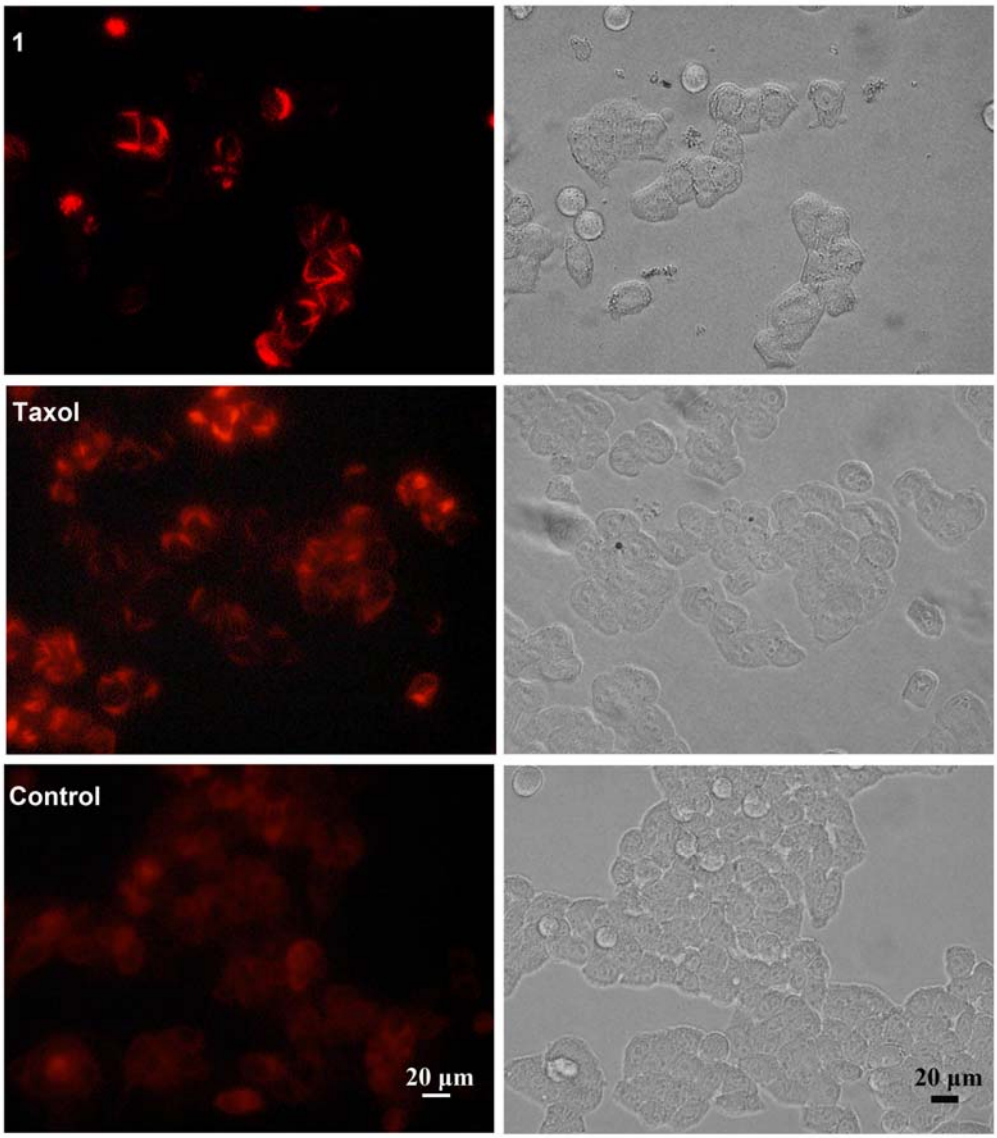


Figure S8. Fluorescence images (left) and differential interference contrast (DIC) images (right) of HeLa cells in the absence (bottom row) or presence of 10 μM of **1** (top row) or taxol (middle row) in serum-free medium at 37 °C for 2 h, washed with PBS for three times prior to imaging, respectively.

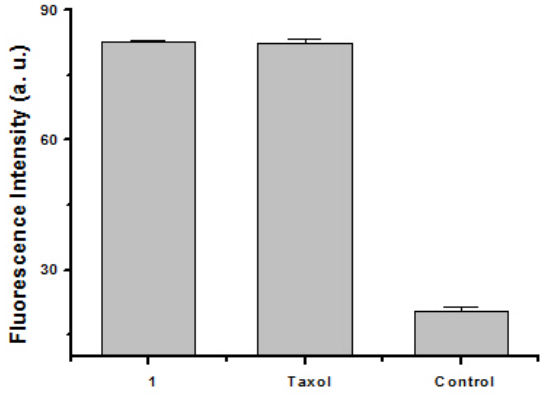


Figure S9. The average fluorescence intensity of microtubules in HeLa cells in Figure S8. There is no difference between **1**-treated and taxol-treated group ($P > 0.05$). But there is obvious difference between treatment groups and control group ($P < 0.001$).

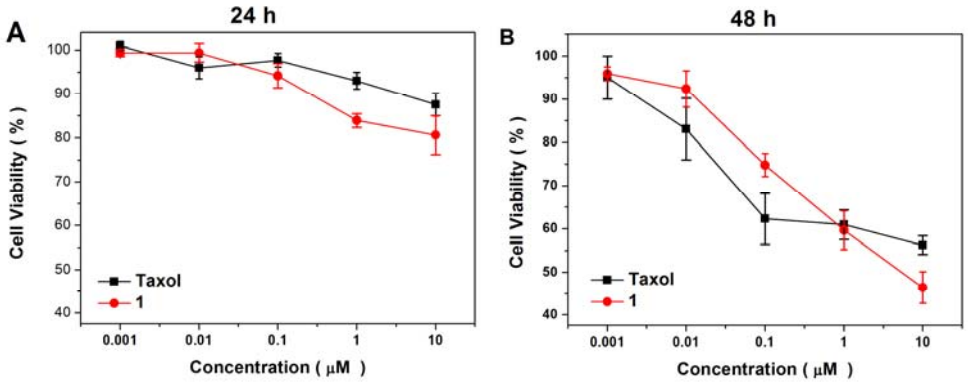


Figure S10. MTT assay of **1** or taxol on HeLa cells. These experiments were performed in triplicate. Error bars represent standard deviations of three independent experiments.