Supporting Information for

Enzyme-Regulated Supramolecular Assemblies of Cholesterol Conjugates against Drug-Resistant Ovarian Cancer Cells

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Table S1. The IC_{50} value of **1a** against different cell lines at 48 h or 72 h.

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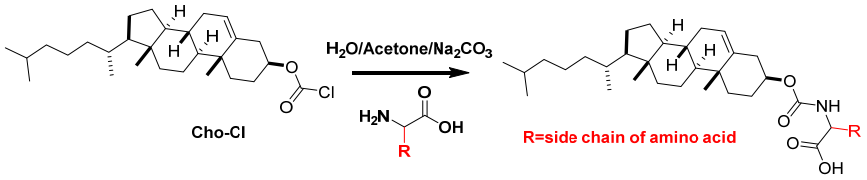
S1. Experiment materials and instruments

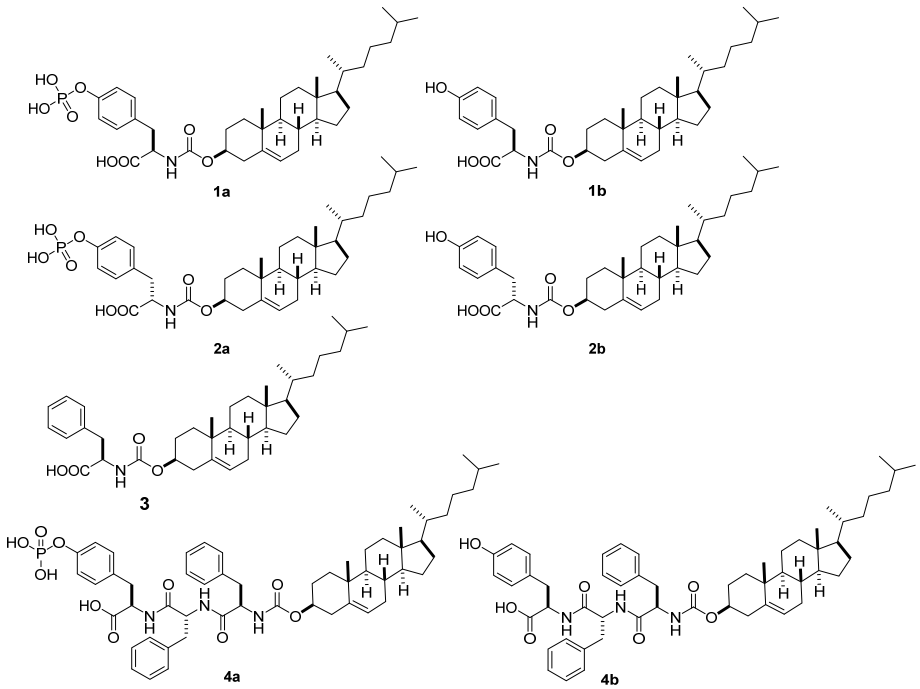
2-Cl-trityl chloride resin (1.0-1.2 mmol/g), Fmoc-OSu and other Fmoc-amino acids were obtained from GL Biochem (Shanghai, China). Other chemical reagents and solvents were obtained from Fisher Scientific; all chemical reagents and solvents were used as received from commercial sources without further purification; alkaline phosphatase was purchased from Biomatik. Dulbecco's modified Eagle's medium (DMEM), McCoy's 5a Medium and 1640 Medium were purchased from ATCC and fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Gibco by life technologies. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from ACROS Organics. All precursors were purified with Water Delta600 HPLC system, equipped with an XTerra C18 RP column. LC-MS spectrum was obtained on Waters Acquity Ultra Performance LC with Waters MICROMASS detector, and ^{1}H-NMR spectra on Varian Unity Inova 400, and TEM images on Morgagni 268 transmission electron microscope and CM12 transmission electron microscope. MTT assay for cell toxicity test on DTX880 Multimode Detector.

S2. Synthesis and characterization of the precursors

The synthetic process of Fmoc-Tyr(PO_3H_2)-OH is according to the previous work.[1] The synthetic route of conjugation of cholesterol and amino acid is straightforward. Briefly, 1 mM of cholesteryl chloroformate dissolved in 100 mL of acetone, amino acid dissolved in water with pH about 8 was added dropwise to above solution, after stirring at room temperature for 24 h, acetone was removed and HCl was added to adjust the pH to 3, followed by filtration. We used HPLC to purify the upper portion for the precursor, and used flash column chromatography to purify the conjugate without phosphate. The yield of all precursors is more than 70%.



Scheme1: Synthetic route of cholesterol derivatives.



Scheme S2. Molecular structures of different cholesterol derivatives.

S3. TEM sample preparation

1. Place sample solution on the grid (5 μL, sufficient to cover the grid surface).
2. Rinsing: ~10 sec later, place a large drop of the ddH_2O on parafilm and let the grid touch the water drop, with the sample-loaded surface facing the parafilm. Tilt the grid and gently absorb water from the edge of the grid using a filter paper sliver. (3 times)
3. Staining (immediately after rinsing): place a large drop of the UA (uranyl acetate) stain solution on parafilm and let the grid touch the stain solution drop, with the sample-loaded surface facing the parafilm. Tilt the grid and gently absorb the stain solution from the edge of the grid using a filter paper sliver.
4. Allow the grid to dry in air and examine the grid as soon as possible.

S4. Cell culture and MTT assay

Cell culture: HeLa, HS-5, and A2780 cells were purchased from American-type Culture Collection (ATCC, USA), A2780cis cell is purchased from Sigma. HeLa cells were cultured in MEM Medium supplemented with 10% v/v fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin; HS-5 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% v fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin; A2780cis cells were cultured in RPMI 1640 Medium supplemented with 10% v/v fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin (cisplatin only necessary every 2-3 passages). All cells were at 37 °C in a humidified atmosphere of 5% CO_2.

MTT assay: We used MTT assay to test the cytotoxicity. All different cell lines were seeded in 96-well plates at 1×10^5 cells/well for 24 h followed by culture medium removal and subsequently addition of culture medium containing different of the precursors. At designated time (24/48/72

hours), 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-HCl solution was added to stop the reduction reaction and dissolve the purple formazan. The absorbance of each well at 595 nm was measured by a multimode microplate reader. The cytotoxicity assay was performed three times and the average value of the three measurements was taken. To ensure the reproducibility for determining of the IC_{50} of **1a**, we did six separate experiments and average all the experiments.

S5. Laurdan staining

Cells in exponential growth phase were seeded in confocal dish (3.5 cm) at 2×10^5 cells per dish. After 24 h, the medium was removed, and 2 mL of **1a** (12.5 μM) with a fresh medium was added. After putting the dish in incubator for 12 h, we removed culture medium, and washed by FBS fresh medium for 3 times, and added FBS fresh medium containing laurdan (10 μM) for 30 minutes. After that, we removed the medium and use PBS to wash the cells for three times. After fixing by 4% paraformaldehyde for 15 minutes, the cells were washed three times with PBS buffer before imaging.

S6. Actin staining

Cells in exponential growth phase were seeded in confocal dish (3.5 cm) at 2×10^5 cells per dish. After putting the dish in incubator for 24 h, we removed culture medium, and added fresh medium containing **1a** (12.5 μM for A2780cis, 25 μM for HeLa). After 12 h, we removed the medium and use PBS to wash the cells for three times. After fixing by 4% paraformaldehyde for 15 minutes, we added 1 mL of 0.1% Triton X-100 in PBS buffer for 30 minutes. After washing the cells three times by PBS, we added 1 mL of 0.1% BSA in PBS for 30 minutes, and then washed the cells by PBS for three times. 1 mL of PBS containing 5 unit of Alexa 633 was added to the cells for 1 h. After removing the buffer and washing the cells three times by PBS, we added 1 mL of Hoechst (1 μg/mL) for 10 minutes. Then, the cells were washed three times with PBS buffer before imaging.

S7. Tubulin staining

We used the procedure recommended by Molecular Probes™ (Thermo Fisher Scientific) for tubulin staining, briefly:

1. The cells were seeded in confocal dish (3.5 cm) at 2×10^5 cells per dish;
2. After putting the dish in incubator for 24 h, we removed culture medium, and added fresh medium containing **1a** (12.5 μM) for 12 h;
3. After removing the medium and used PBS to wash the cells for three times, we then added sufficient amount of the final staining solution (100 nM) for 30 minutes;
4. After removing the staining solution and washing the cells three times by PBS, we added 1 mL of Hoechst (1 μg/mL) for 10 minutes. Then, the cells were washed three times with PBS buffer before imaging.

S8. Antibody staining

We used the procedure recommended by ABCAM for antibody staining, as following:

1. The cells were seeded (100,000-200,000 cell/3.5cm confocal dish) and allowed to attach (24 hours);
2. The cells were incubated with 12.5 μM of **1a** for 12 h or 24 h;
3. The cells were washed by PBS buffer for three times and fixed by 4% formaldehyde for 15

minutes;

4. The cells were washed by PBS buffer for three times and incubated in 1.0 %BSA / 10% normal goat serum / 0.3M glycine in 0.1% PBS-Tween for 1 h to be permeabilized and for blocking non-specific protein-protein interactions.
5. The cells were washed by PBS buffer for three times and incubated with the antibody (1/100) overnight at 4°C.
6. After washed by PBS buffer for three times, we added the secondary antibody (green) ab150077 Alexa Fluor® 488 goat anti-rabbit IgG (H+L) at 2 μg/ml (1/1000) for 1 h.
7. Hoechst 33342 was used to stain the cell nuclei (blue).
8. The cells were washed by PBS buffer for three times and mounted for imaging.

S9. Solid-state NMR spectroscopy

Solid-state NMR experiments were performed on a Bruker (Billerica, MA) DSX-400 spectrometer at a resonance frequency of 400 MHz for ^{1}H and 162 MHz for ^{31}P, using a magic-angle-spinning (MAS) probe in double-resonance mode. Samples were packed into Kel-F 22 μL inserts for 4 mm rotors. MAS experiments were carried out without sample rotation ("static") or at a spinning frequency of 10 kHz. Typical ^{31}P and ^{1}H 90° pulse-lengths were 4 μs and 6 μs, respectively. ^{31}P spectra were obtained with a recycle delay of 2-4 s and two-pulse phase modulation (TPPM) decoupling. ^{31}P chemical shifts were referenced to 85% phosphoric acid at 0 ppm, using the resonance of calcium hydroxyapatite (National Institute of Standards and Technology) at 2.73 ppm as an external secondary reference.

The MAS direct-polarization (DP) ^{31}P NMR spectrum of **1a** in water is dominated by a single resonance at -3.4 ppm. After treatment with phosphatases, two peaks are resolvable in the MAS/DP spectrum, the peak at -3.4 ppm (56 %) is assigned to un-cleaved **1a** and the peak at 0.4 ppm (~ 44 %) is assigned to phosphate ions.

At the high concentration used for the solid-state NMR measurement, static DP spectra of **1a** reveal a large (>80 ppm) broadening due to chemical shift anisotropy (CSA). This large CSA is consistent with limited molecular re-orientation on the time scale of ~10 μs, indicative of relatively immobile molecular segments in large (> 50 nm) particles. Not all **1a** phosphorus sites have a large CSA, as reveled by the narrower spectral component that can be selected by a 0.5-s $T_{1,P}$ relaxation filter. The mostly large CSA is consistent with the TEM image of **1a** at the same concentration (Figure S5), which shows large (192 nm wide and 200-1450 nm in length) sheets with rectangular morphologies.

In the static DP ^{31}P NMR spectra of **1a** after treatment with alkali phosphatase, both peaks are only slightly broadened, so neither phosphate type has a large CSA. The peak with an isotropic chemical shift of -3.4 ppm has a CSA that is scaled down significantly due to large-amplitude motions on the <10-μs time scale, with a segmental dynamic order parameter of S ~ 0.05. The line shape of the signal (specifically the biaxiality parameter $\eta \sim 0.8$) indicates a non-uniaxial phase, unlike liquid-crystalline bilayer lipids, where $\eta = 0$. The residual CSA clearly indicates that the local environment is distinct from isolated and solvated molecules in solution. The peak at +0.4 ppm has a more Lorentzian line shape, consistent with the phosphate-ion assignment. The TEM image of **1a** after treatment shows small spherical particles (18 nm), consistent with the NMR results: Through

diffusion of molecules in such particles, or by whole-body rotation, the chemical-shift anisotropy is motionally averaged on the NMR time-scale and a small dynamic order parameter is obtained.

S10. Supplemental figures

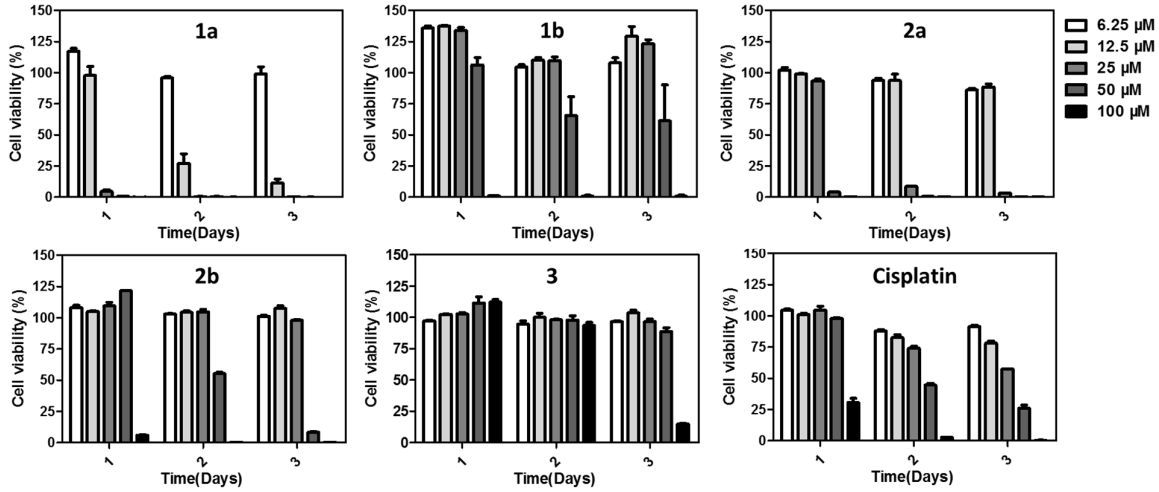


Figure S1. Cell viability of A2780cis cells treated with **1a**, **1b**, **2a**, **2b**, **3** and cisplatin at 24, 48 and 72 h.

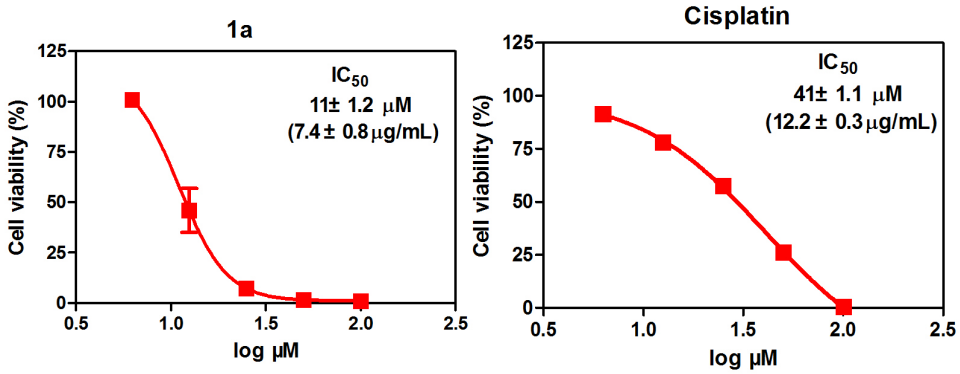


Figure S2. The dosage curves of **1a** (obtained by average six separate cell viability experiments) and cisplatin against A2780cis cells at 72h.

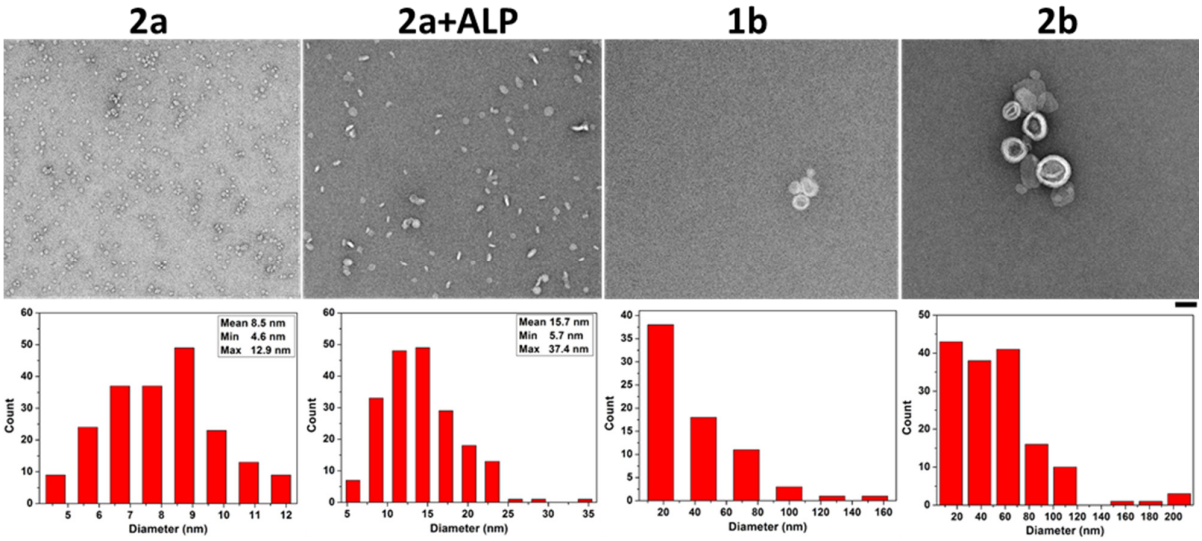


Figure S3. TEM images of 100 µM of **2a**, **2a** treated with 1U/mL of ALP, **1b** and **2b** in PBS buffer (pH=7.4) and their relative size distribution. Scale bar = 50 nm.

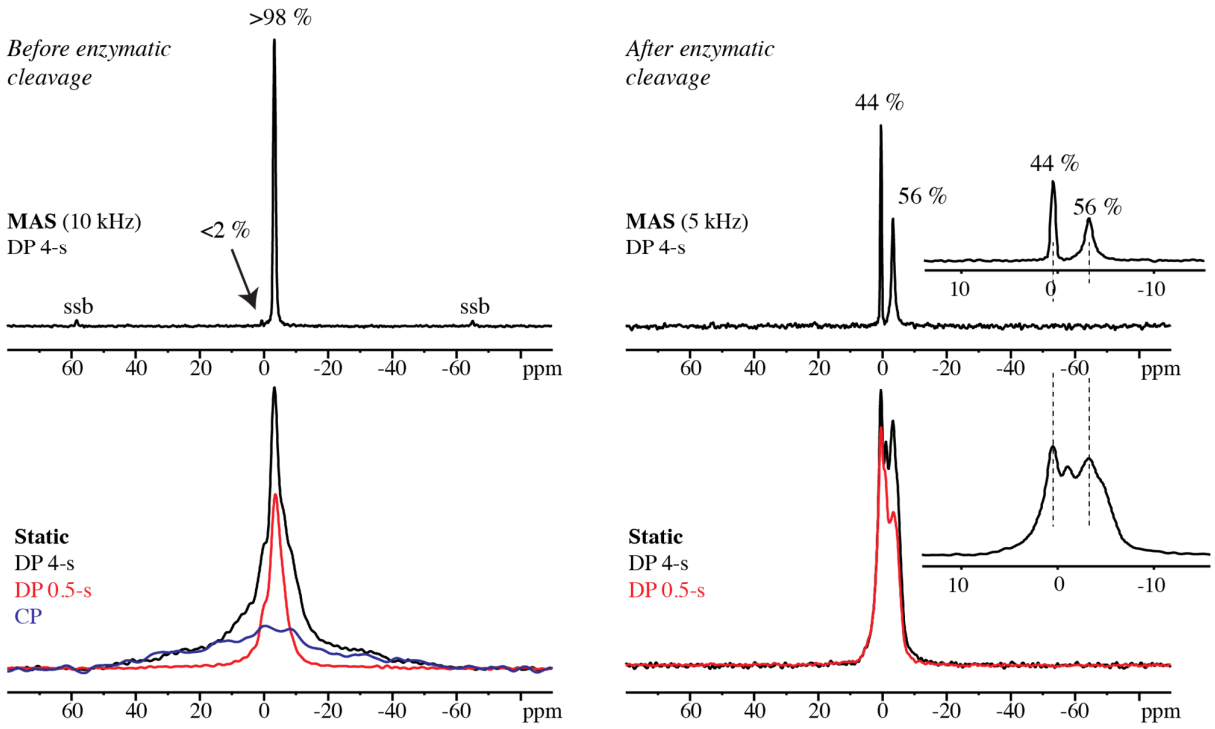


Figure S4. Magic-angle-spinning (MAS) direct-polarization (DP) ^{31}P NMR spectrum (top row) and static ^{31}P NMR spectra (bottom row) of **1a** (40 wt%) (left column) and of **1a** treated with ALP (1 U/mL) (right column).

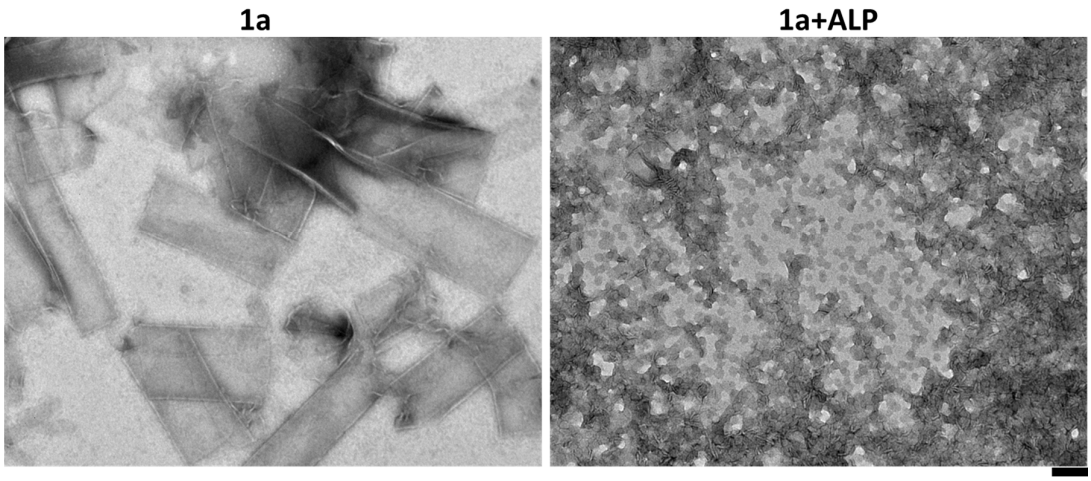


Figure S5. TEM images of **1a** (at a high concentration of 40 wt%) before (left) and after (right) treatment with ALP (1 U/mL). The TEM images were taken after solid-state NMR. Scale bar: 100 nm.

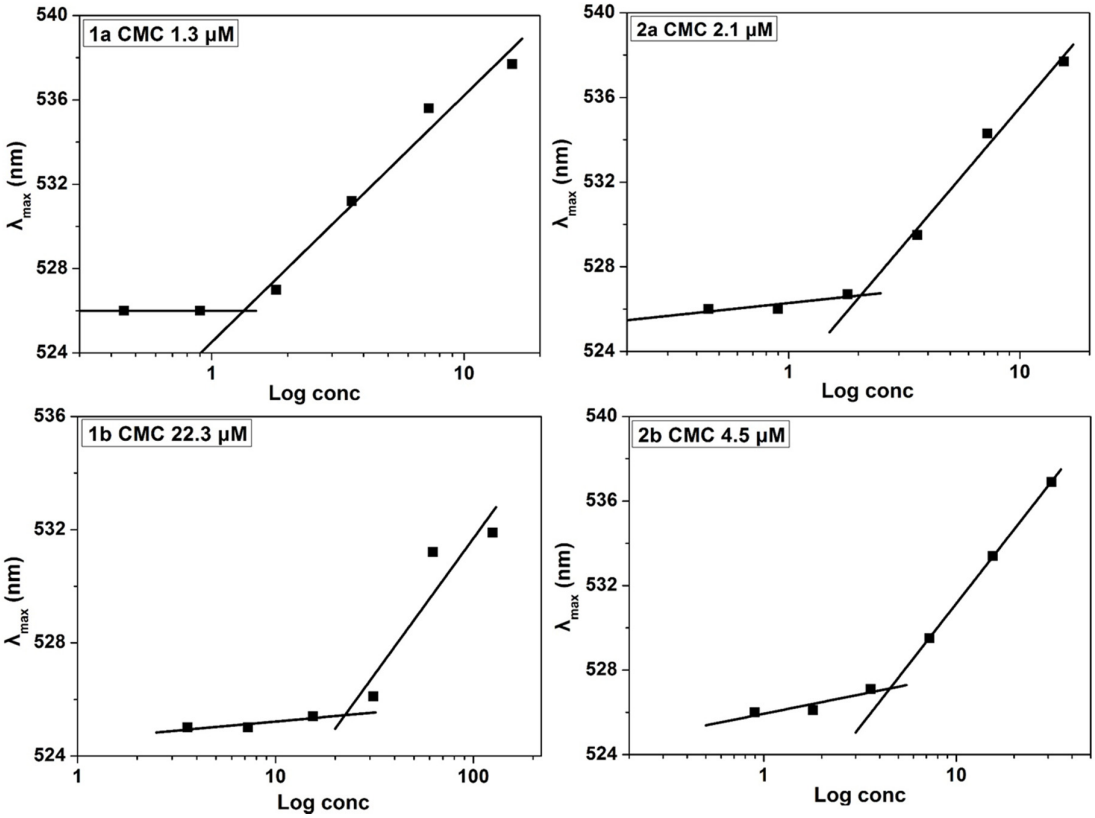


Figure S6. CMC-determination for **1a**, **2a**, **1b** and **2b** (by using Rhodamine 6G).[2]

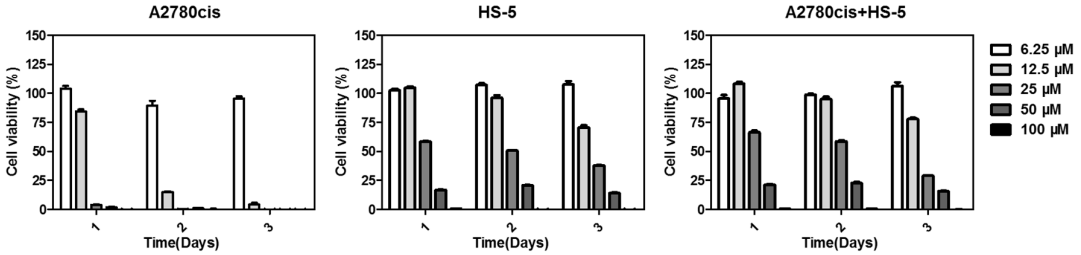


Figure S7. Viability of the co-cultured cells (A2780 cis and HS-5 cells) incubated with **1a** at 24, 48 and 72 h.

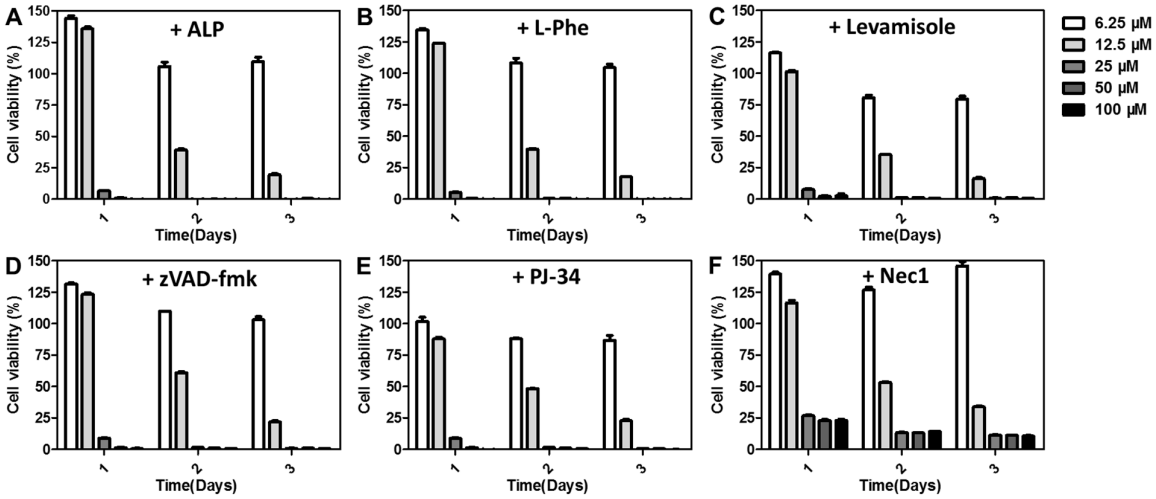


Figure S8. Cell viability of A2780cis cells treated with **1a** in the presence of A) ALP (1 U/mL); B) L-Phe (1 mM); C) leavamisole (1 mM); D) zVAD-fmk (45 μM); E) PJ-34 (1 μM) and F) Nec-1 (50 μM) at time point of 24, 48 and 72 h.

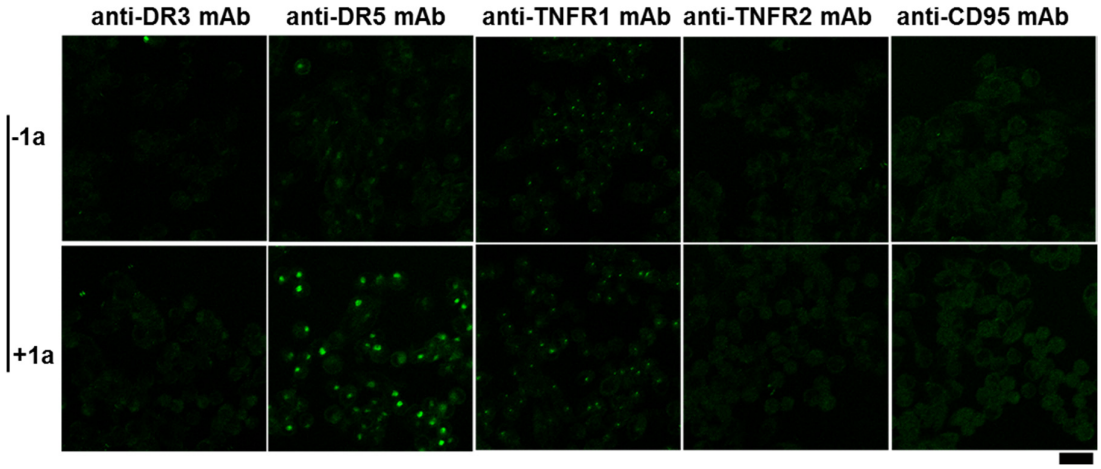


Figure S9. CLSM images of cell death receptors without or with the treatment of **1a**. The

concentration of **1a** is 12.5 μM and incubated with A2780cis cell for 24 h (**-1a** represents the cells only treat with culture medium). Scale bar in A is 30 μm (×40 oil lens).

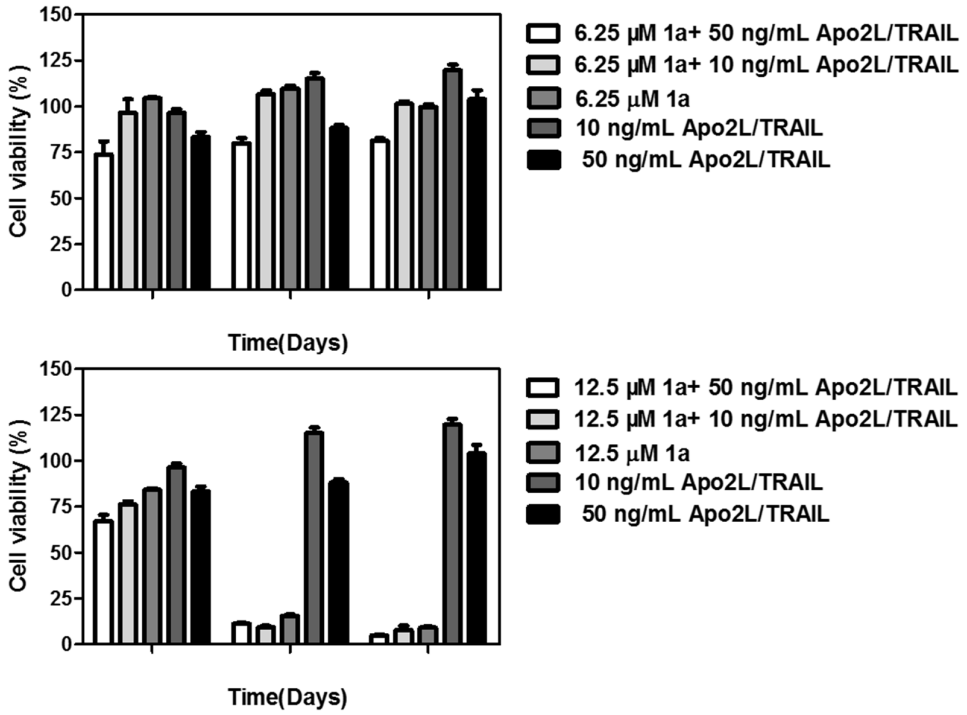


Figure S10. Cell viability of A2780cis cell in the presence of Apo2L/TRAIL (10 ng/mL or 50 ng/mL) at 24, 48 and 72 h.

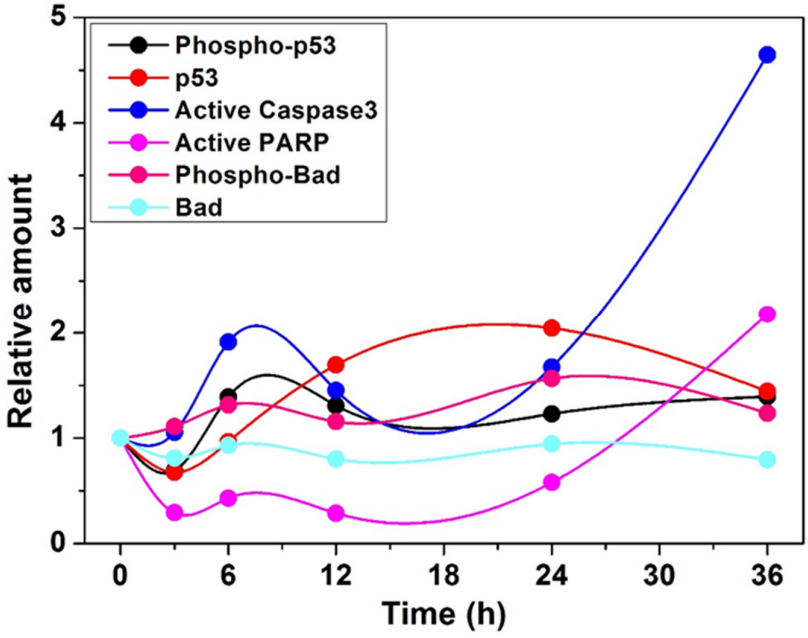


Figure S11. Time-dependent activation of apoptotic proteins of A2780cis cells treated with **1a**.

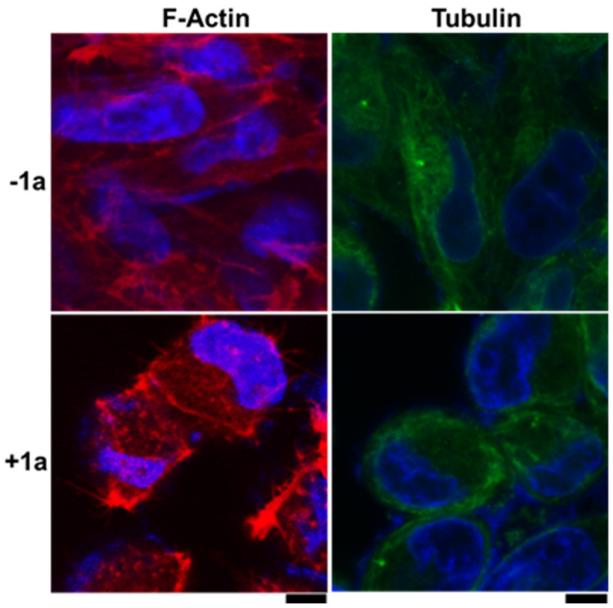


Figure S12. Magnified CLSM images of A2780cis cells stained with Alexa Fluor 633 phalloidin (F-actin, red) and Hoechst (nuclei, blue) or tubulin tracker (green) without or with the addition of **1a** (12.5 μM) for 12 h (-1a represents the cells only treat with culture medium). Scale bar is 5 μm.

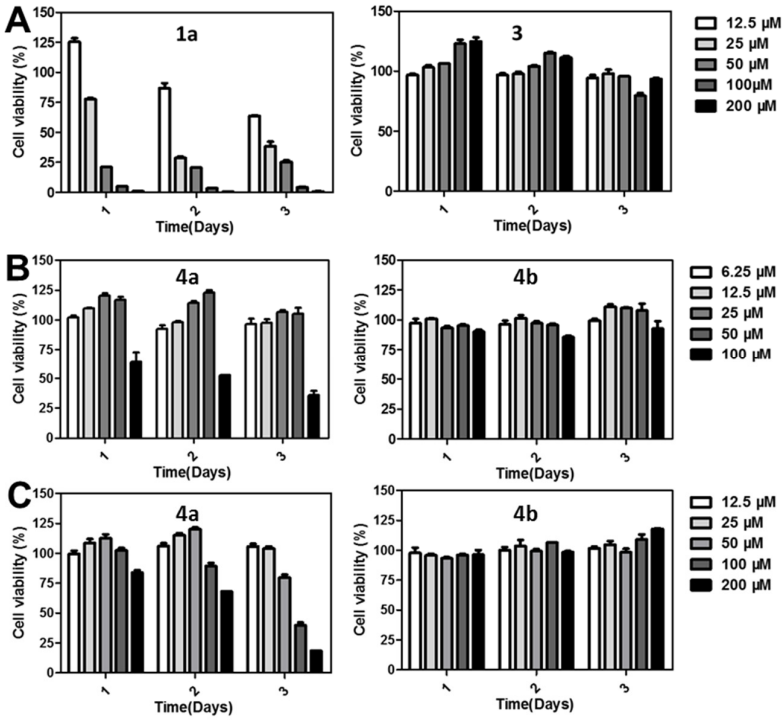


Figure S13. Time-dependent cytotoxicity of (A) Cell viability of HeLa cells treated with **1a** and **3**; (B) cell viability of A2780cis cells treated with **4a** and **4b** and (C) cell viability of HeLa cells treated with **4a** and **4b**.

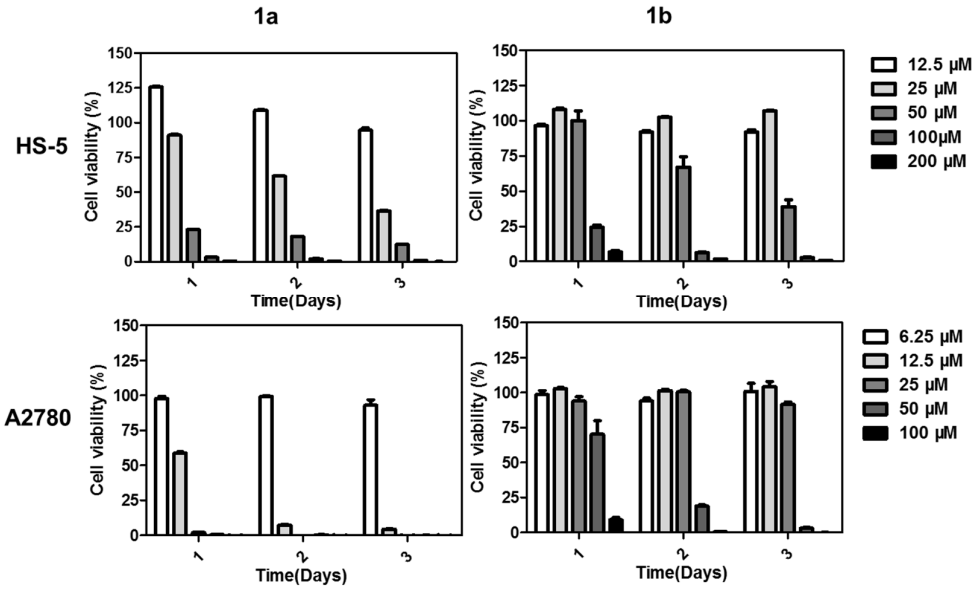


Figure S14. Cell viability of HS-5 and A2780 cells treated with **1a**, **1b** at 24, 48 and 72 h.

Table S1: The IC_{50} value of **1a** against different cell lines at 48 h or 72 h

Cell type	HeLa	HS-5	A2780
IC_{50} (μM, 48hrs)	19	32	11
IC_{50} (μM, 72hrs)	16	21	9

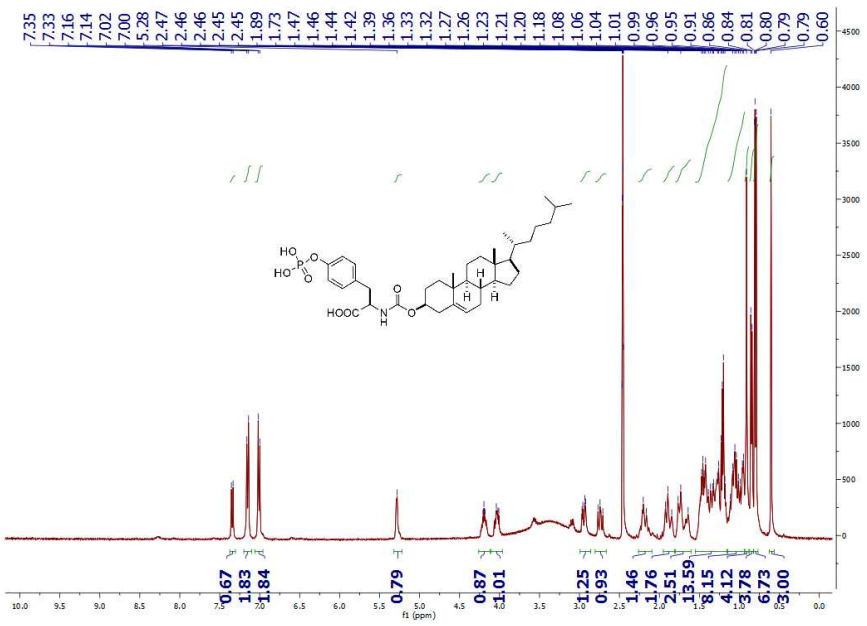


Figure S15. ^{1}H NMR of **1a** in DMSO-d_6.

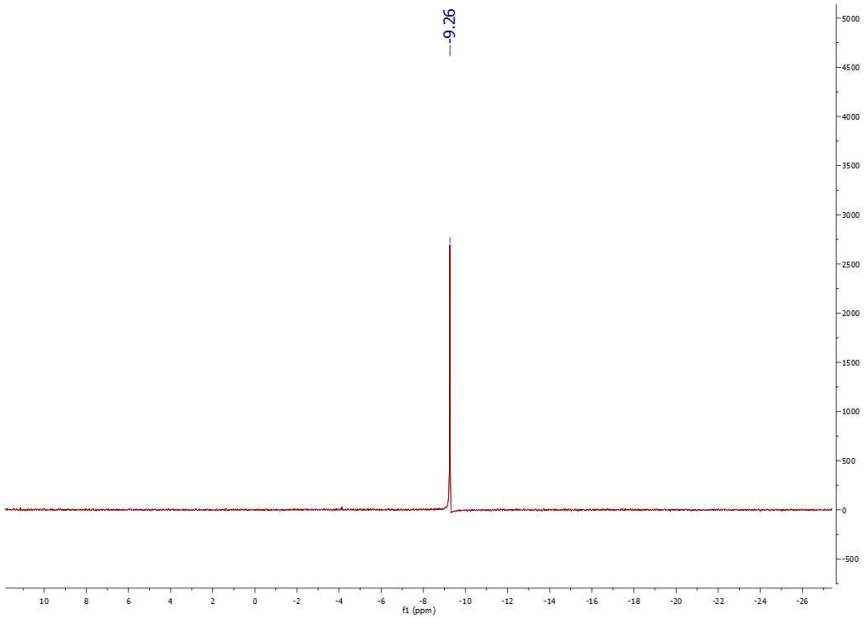


Figure S16. ^{31}P NMR of **1a** in DMSO-d_6.

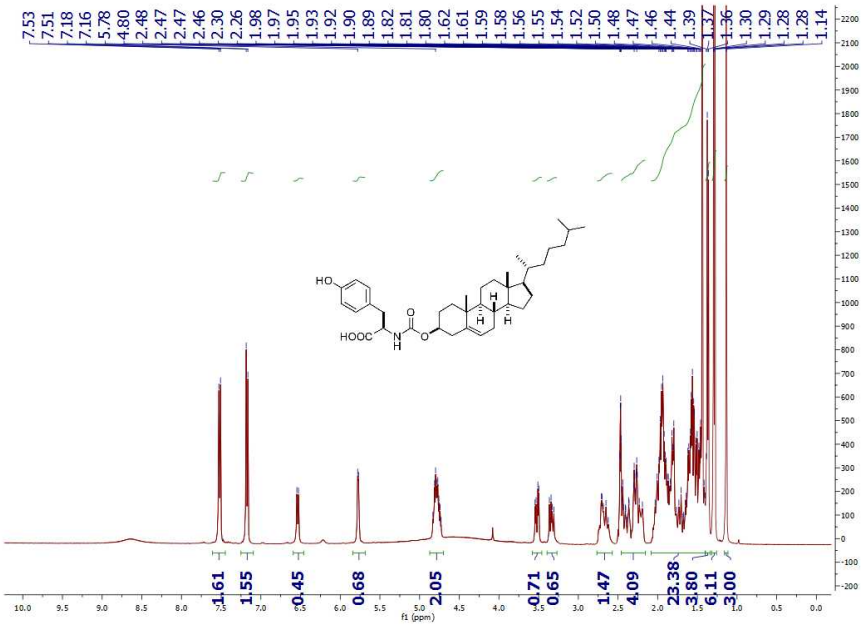


Figure S17. ^{1}H NMR of **1b** in DMSO-d_6.

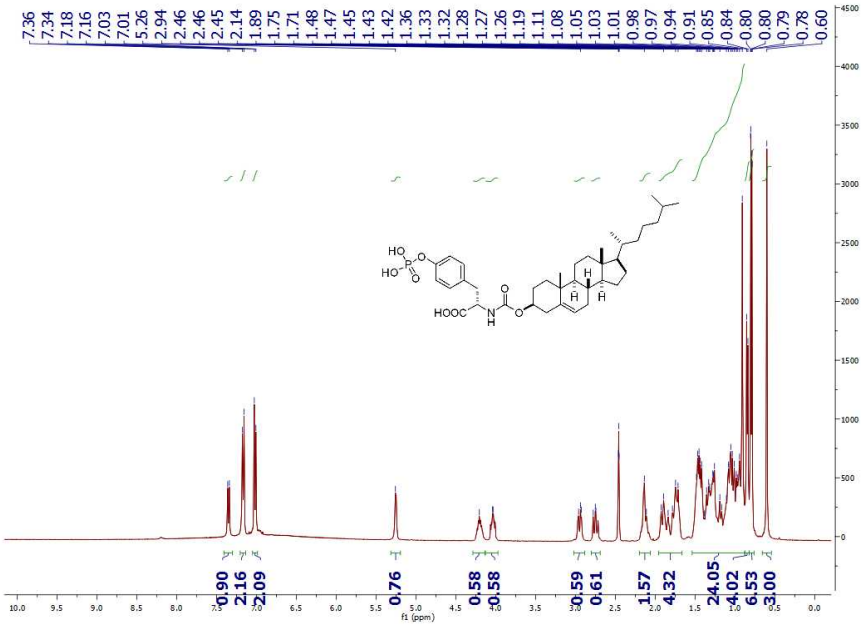


Figure S18. ^{1}H NMR of **2a** in DMSO-d_6.

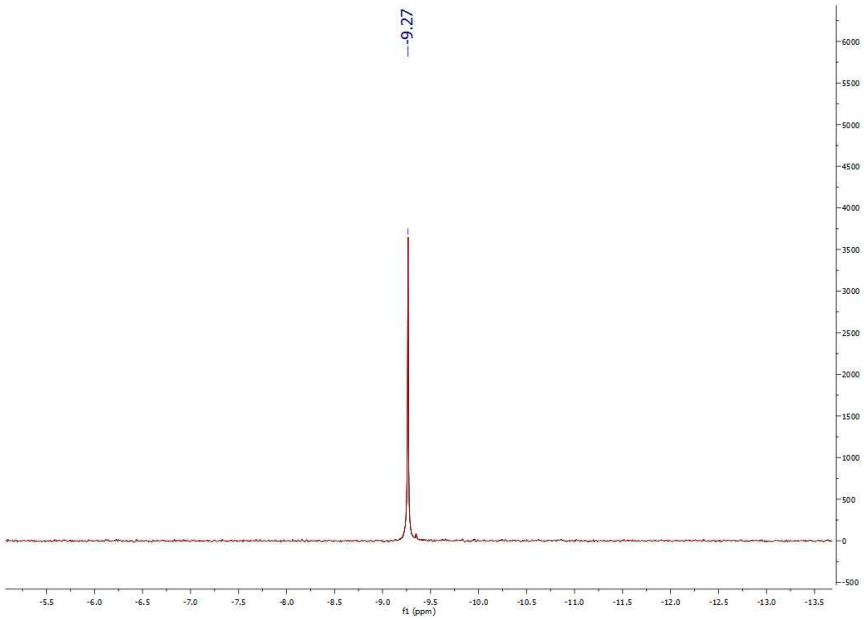


Figure S19. ^{31}P NMR of **2a** in DMSO-d_6.

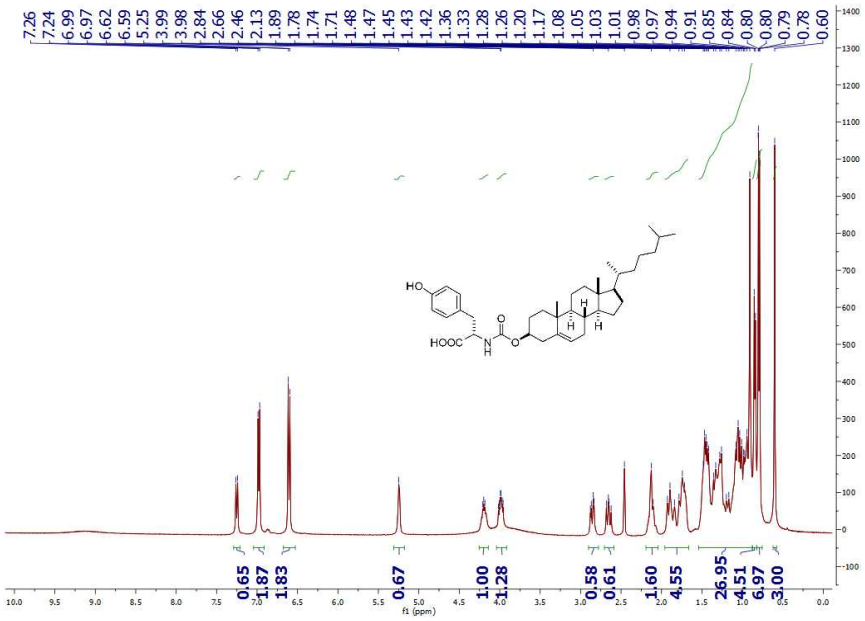


Figure S20. ^{1}H NMR of **2b** in DMSO-d_6.

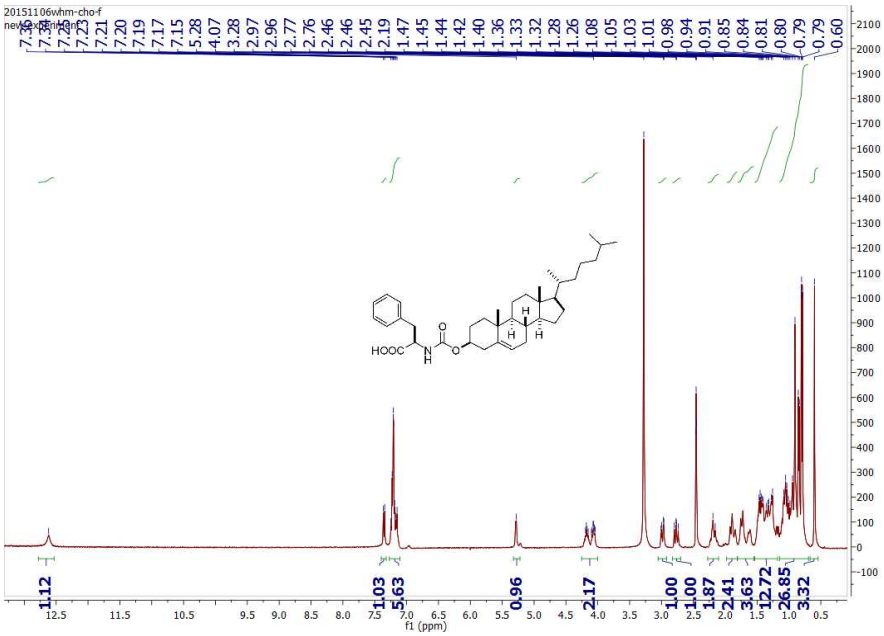


Figure S21. ^{1}H NMR of **3** in DMSO-d_6.

(1) Shi, J.; Du, X.; Yuan, D.; Zhou, J.; Zhou, N.; Huang, Y.; Xu, B. *Biomacromolecules* **2014**, *15*, 3559.

(2) Willemen, H. M.; Vermonden, T.; Koudijs, A.; Marcelis, A. T.; Sudhölter, E. J. *Colloids Surf., A* **2003**, *218*, 59.