Supporting Information for

Intercellular Instructed-Assembly Mimics Protein Dynamics to Induce Cell Spheroids

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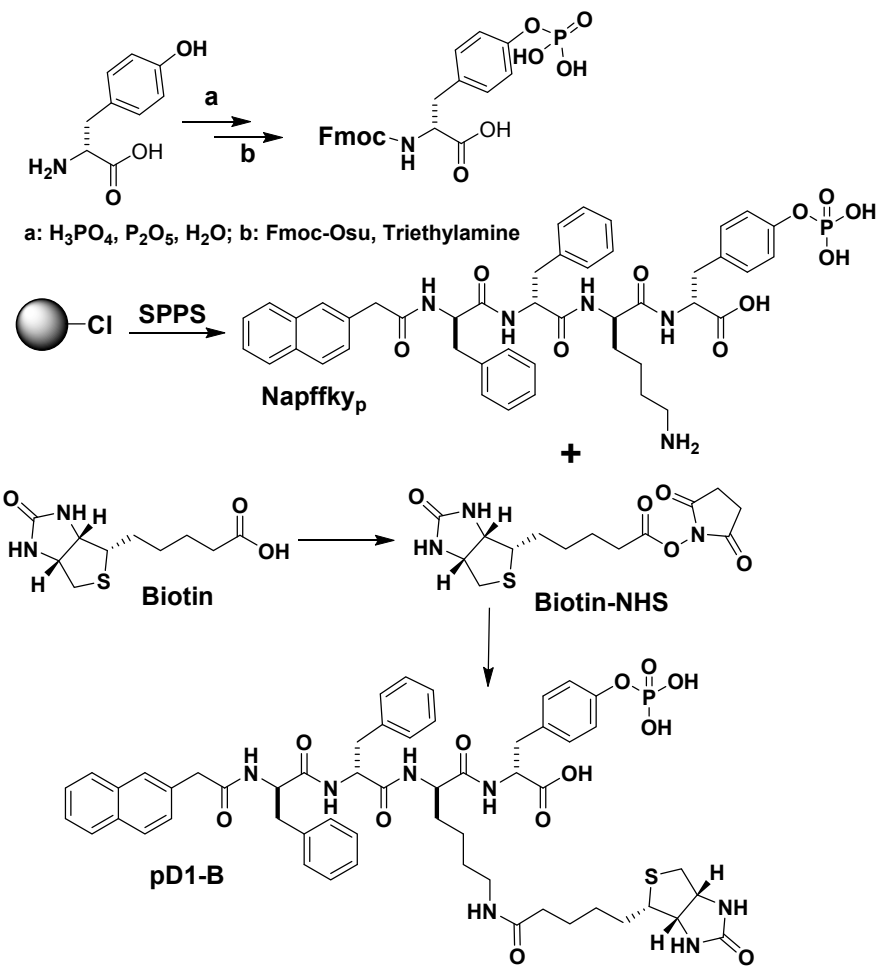
Content

S1. Experiment materials and instruments

Fmoc protected amino acid, HBTU, Fmoc-OSu and 2-Cl-trityl chloride resin (0.6 mmol/g), were obtained from GL Biochem (Shanghai, China). N, N-diisopropylethylamine (DIPEA) and other chemical reagents and solvents were obtained from Fisher Scientific. Alkaline phosphatase was purchased from Biomatik (Cat. No. A1130, alkaline phosphatase [ALP], >1300U/MG, in 50% glycerol.). All the chemical reagents and solvents were used as received from commercial sources without further purification. Dulbecco's Modified Eagle's medium (DMEM) and McCoy's 5a Medium obtained from ATCC. Fetal bovine serum (FBS) and penicillin/streptomycin from Gibco by Life Technologies. All precursors were purified using a reverse phase HPLC (Agilent 1100 Series) with HPLC grade acetonitrile (0.1% TFA) and HPLC grade water (0.1% TFA) as the eluents, and LC-MS spectra using a Waters Acquity Ultra Performance LC with Waters MICROMASS detector. TEM images on Morgagni 268 transmission electron microscope, and the ^{1}H-NMR and ^{31}P-NMR spectra of compounds were obtained using Varian Unity Inova 400MHz .

S2. Synthesis and characterization of the precursors

Synthesis of pD1-B



Scheme S1. Synthetic procedure of **pD1-B**

Synthesis of Fmoc-pY

Briefly, put fresh phosphorus pentoxide (10.0 g) and 85% of phosphoric acid (13.0 g) into a two-necked round bottle under the protection of nitrogen gas. 3.22 g of tyrosine was added and mixed with the aid of stirring. The reaction mixture was heated to 80 °C for 24 h. The stirring was continued with further 30 min after addition of 30 mL water. The reaction mixture was cooled to room temperature and diluted with n-butanol (650 mL) and kept at 4 °C overnight. Collected the final white precipitate *via* filtration and washed with iced water, ethanol, and ether. The final white powder was used for the next step (yield ~87%).

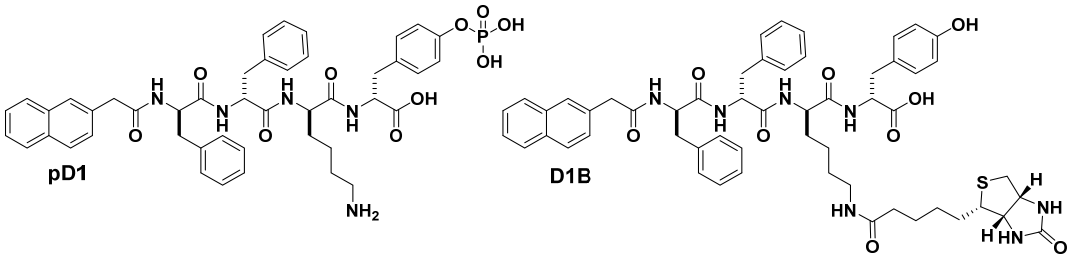
To introduce the Fmoc group, the white power (2.5 g) of phosphotyrosine, Fmoc-OSu (0.4 g) and triethylamine (adjust final pH to 8~9) were suspended in a mixture of water (25 mL) and acetonitrile (25 mL). After 2 h stirred at room temperature, used vacuo rotary to remove organic solvent and added 300 mL of ethyl acetate with same volume of water. The mixture was acidified to pH ~2 with HCl, the organic phase was taken, while water phase was extracted twice by ethyl acetate. Organic phase was washed with 1N HCl, H_2O and saturated aqueous NaCl. After dried by $MgSO_4$, the solvent was removed by vacuo, the product of Fmoc-pY was obtained as a white power (yield 83%).

Peptide synthesis

After synthesis of Fmoc protected phosphotyrosine through two steps, we first synthesized Napffky$_p$ by standard Fmoc solid phase peptide synthesis using 2-chlorotrityl chloride resin and the corresponding Fmoc-protected amino acids with side chains properly protected. Briefly, the first amino acid was loaded onto the resin at about 0.6 mmol/g of resin. After loading the first amino acid to the resin, the capping regent (DCM: MeOH: DIPEA = 17: 2: 1) was used to ensure that all the active sites of the resin were protected. 20% piperidine in DMF was used to remove the Fmoc group, the next Fmoc-protected amino acid was coupled to the free amino group using HBTU as the coupling reagent. The growth of the peptide chain followed the established Fmoc SPPS protocol. The crude peptides were collected using TFA-mediated cleavage method: The peptide derivative was cleaved using 95% TFA, 2.5% TIS, and 2.5% H_2O for 0.5 h. After removing the solvent, 20 mL of ice-cold ethyl ether/per gram of resin was then added to above solution. After discarding most of the supernatant, the resulting precipitate was filtered and dried by a lyophilizer.

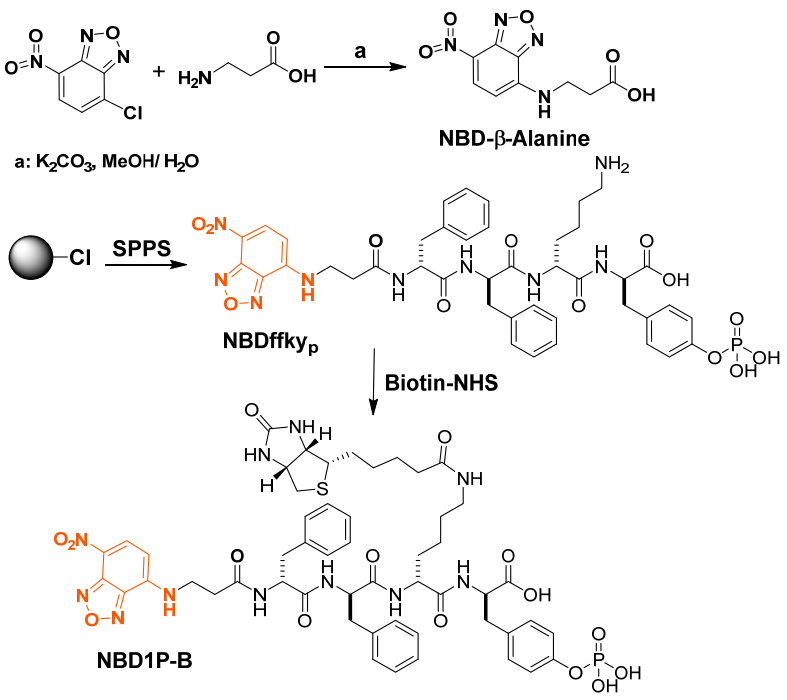
Synthesis of pD1-B

50 mg of biotin was dissolved in 10 mL of dichloromethane (DCM), then 1.1 equivalent of N-Hydroxysuccinimide (NHS) and 1.2 equivalent of N,N'-dicyclohexylcarbodiimide (DCC) with catalytic amount of 4-dimethylamiopryidine (0.01 equivalent) were added. After being stirred at room temperature for 3 h, the solution was filtered by a filter paper to remove precipitates. The filtrate was evaporated under reduced pressure to yield a white powder, which was used directly for the next step. After the white powder obtained in above steps, we dissolved biotin-NHS in 5 mL of N,N-dimethylformamide, 1.3 equivalent of corresponding peptide was then added with 3 equivalent of N-diisopropylethylamine (DIPEA). The resulting reaction mixture was stirred overnight and the title products were purified by reverse phase HPLC.



Scheme S2. Molecular structure of **pD1 and D1B.**

Synthesis of NBD1P-B



Scheme S3. Synthetic procedure of **NBD1P-B.**

Synthesis of NBD-β-Alanine:

To a 10 mL water solution of β-Alanine (5.5 mmol, 490 mg) and potassium carbonate (16.5 mmol, 2.07g), NBD-Cl (5 mmol, 1g) in 20 mL of MeOH was added to the above solution dropwise with stirring in the nitrogen gas atmosphere (low yields if without nitrogen protection). After stirring at room temperature for 5 h, we removed methanol by a rotary evaporator and acidified the remained solution to pH 3 by HCl (2 N). The acidic aqueous solution was then extracted by diethyl ether. The combined organic solution was dried over anhydrous magnesium sulfate, and then concentrated by a rotary evaporator. The resulting yellow powder (NBD-β-Alanine, in some case the color is dark) was directly used for solid phase peptide synthesis.

After obtained NBD-ffky$_p$ by SPPS, we synthesized **NBD1P-B** according to the similar procedure as **pD1-B**. Briefly, NBD-ffky$_p$ (0.05 mmol, 46.0 mg) was dissolved in anhydrous DMF with addition of DIPEA (final pH~8.0), biotin-NHS (0.055 mmol, 18.8 mg) in DMF (5 mL) was added to the above solution dropwise with stirring. After the reaction mixture was stirred overnight, we used vacuo rotary to remove organic solvent and washed the remaining product by ethyl ether for twice. The final crude product was dissolved in methanol and purified by HPLC.

S3. Cell culture

HS-5 and Saos-2 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), HS-5 cells were propagated in DMEM medium with 10% FBS and 1% P/S (100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin, Invitrogen Life Technologies). Saos-2 cells were cultured in McCoy's 5A with 15% FBS and 1% P/S. The incubation conditions for all the cells are at 37 °C in a humidified atmosphere of 5% CO_2. To induce the formation of cell spheroid, HS-5 cell lines were seeded in 96-well cell plates at 3×10^4 cells/well for 24 h followed by culture medium removal and subsequently addition of fresh culture medium containing different of the precursors. At designated time, we use microscopy to capture the morphology of cells with the 10× lens.

S4. Determination of dephosphorylation rates in vitro and in HS-5 cells

To determine the dephosphorylation rates in vitro, we put peptide **pD1-B** at the concentration of 200 μM (Tris-Cl, pH=7.4) at 37°C with different amount of ALP, at the designated time, we added same volume of methanol to eliminate the activity of ALP and used HPLC to analysis the result. To determine the dephosphorylation rates of **pD1-B** in HS-5 cells, we first co-incubate **pD1-B** with HS-5 at different times and then collect the medium and cells. After dissolving all the compounds in methanol, we used LC-MS to analyze the degradation rate and the components in cells.

S5. TEM experiments

1. First place sample solution by pipettor on the carbon coated grid (5 μL, sufficient to cover the grid surface).

2. After 30 seconds, put the grid with the face of sample to a large drop of the ddH_2O on parafilm and let the grid touch the water drop for 5 seconds, tilt the grid and gently absorb water from the edge of the grid using a filter paper sliver. This process repeated for 3 times.

3. Staining (immediately after rinsing): place a large drop of the UA (uranyl acetate, 2% v/v) 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 by Morgagni 268 transmission electron microscope at the HV of 80 KV with filament of 2.

S6. Immunofluorescence

After incubating HS-5 cell lines (5×10^5) in 3.5 cm confocal dish for 24 h at 37 °C in a humidified atmosphere of 5% CO_2, we added the precursor (200 μM) for 48 h. After three time wash by PBS buffer, HS-5 cells are fixed by 4% formaldehyde for 15 min at 37 °C and then incubated in 1% BSA/10% normal goat serum/0.3M glycine in 0.1% PBS-Tween for 1 h to permeate the cells and block non-specific protein-protein interactions. After another three time wash by PBS buffer, the cells are treated with primary antibodies overnight at 4 °C. The secondary antibody is (Alexa Fluor® 647 goat anti-rabbit (or mouse) IgG (H+L) used at the concentration of 2 μg/mL for 1 h. Finally, we used Hochest 33342 to stain cell nucleus.

S8. Supplementary Figures

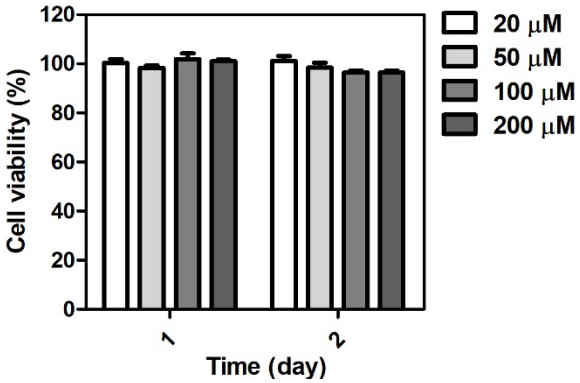


Figure S1. Cytotoxicity of **pD1-B** against HS-5 cells for 1 and 2 days.

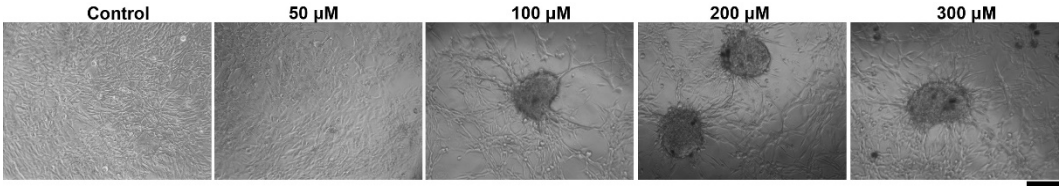


Figure S2. Optical images of HS-5 cells co-incubated with **pD1-B** at different concentrations for 24 h. Scale bar is 150 μm.

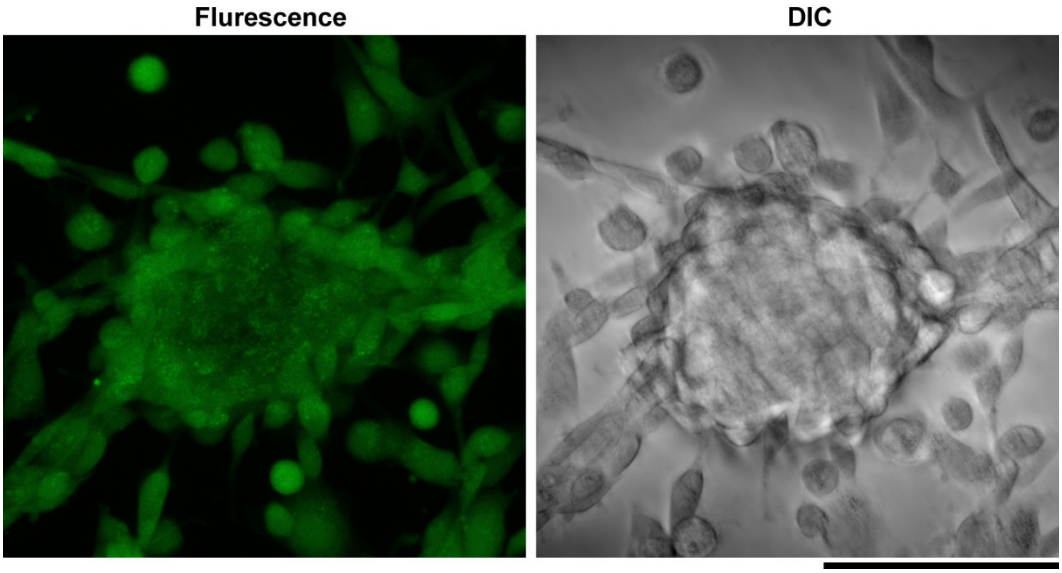


Figure S3. 3D construction of CLSM image of HS-5 cells co-incubated with **pD1-B** (200 μM) for 24 h. Scale bar is 100 μm. We used live-dead assay to stain cells (green indicates live cells and red indicates dead cells)

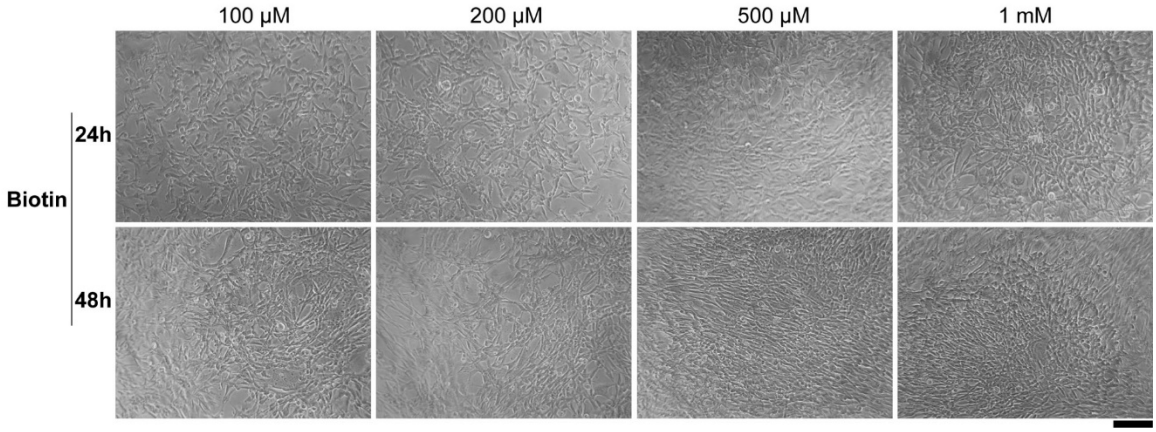


Figure S4. Optical images of HS-5 cells treated with biotin at the concentration from 100 μM to 1 mM for 24 h and 48 h. Scale bar is 150 μm.

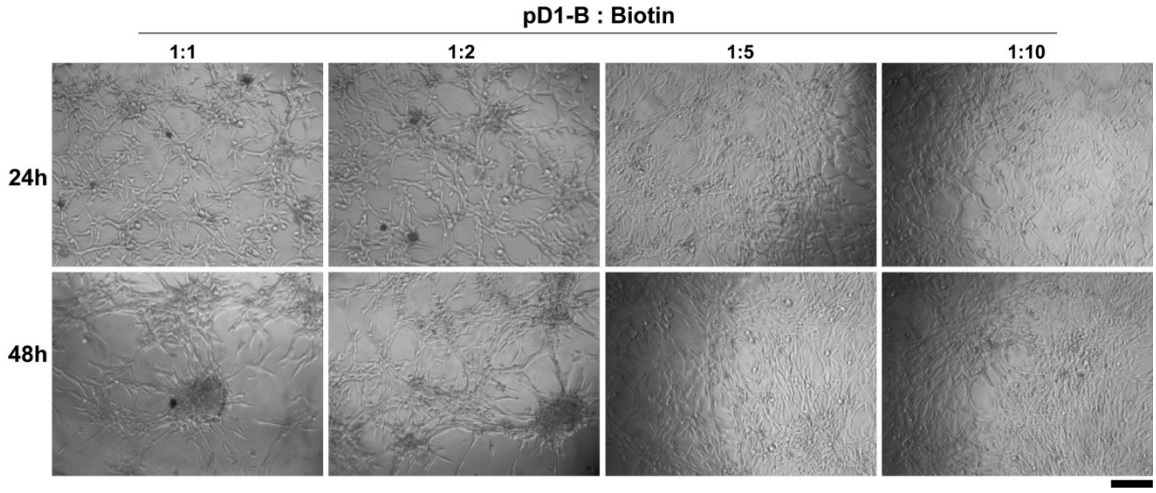


Figure S5. Optical images of HS-5 cells treated with **pD1-B** (100 μM) with different concentration of biotin (molar ratio) for 24 h and 48 h. Scale bar is 150 μm.

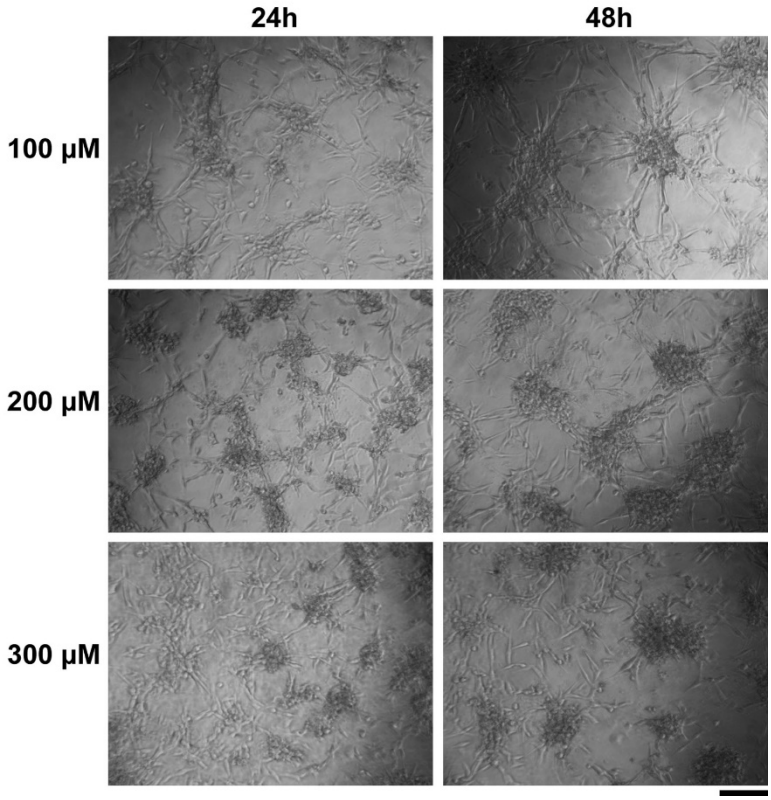


Figure S6. Optical images of HS-5 cells treated with **pL1-B** at concentration from 100 μM to 300 μM for 24 h and 48 h. Scale bar is 150 μm.

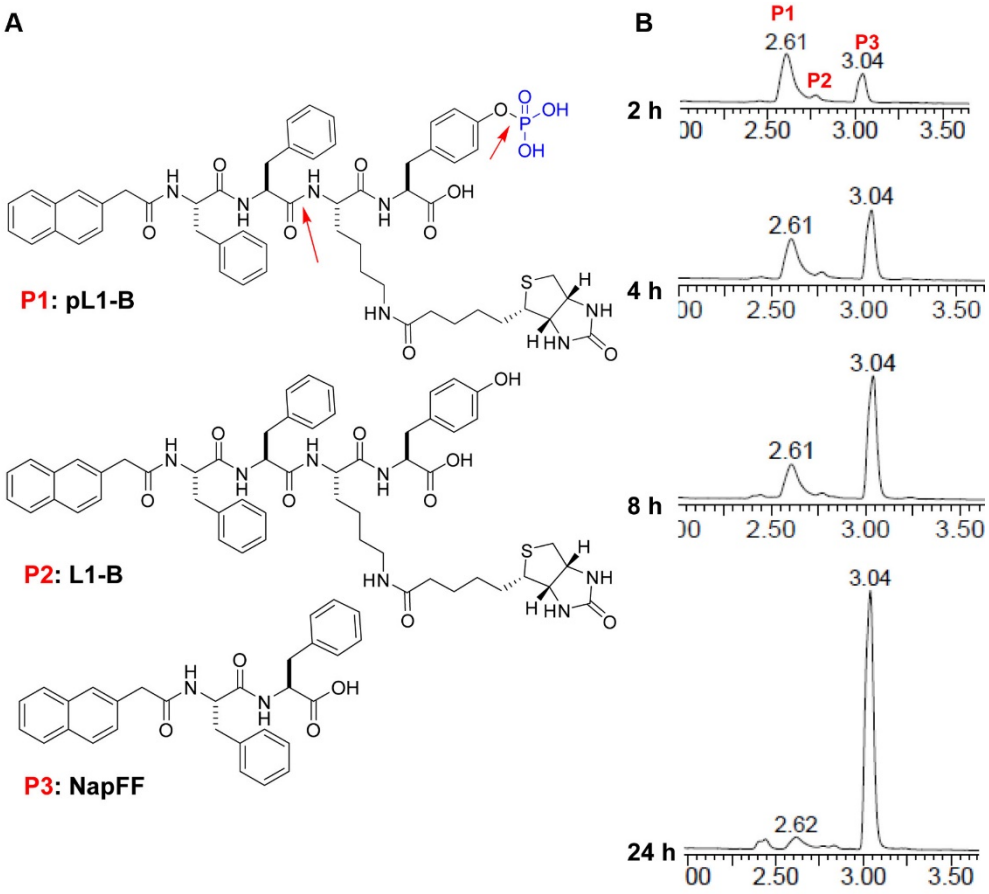


Figure S7. Results of degradation of **pL1-B** in the presence of HS-5 cells. A) Molecular structures of remaining peptide exist in cell condition. Arrows indicate the cleavage sites. B) HPLC tracking of **pL1-B** treated with HS-5 cells at different times.

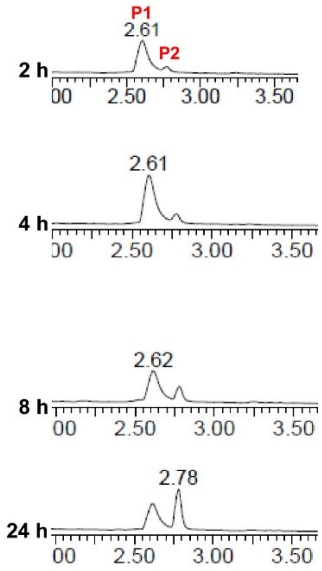


Figure S8. HPLC tracking of **pD1-B** treated with HS-5 cells at different times. Peak of P1 is **pD1-B**, and peak of P2 is **D1-B**.

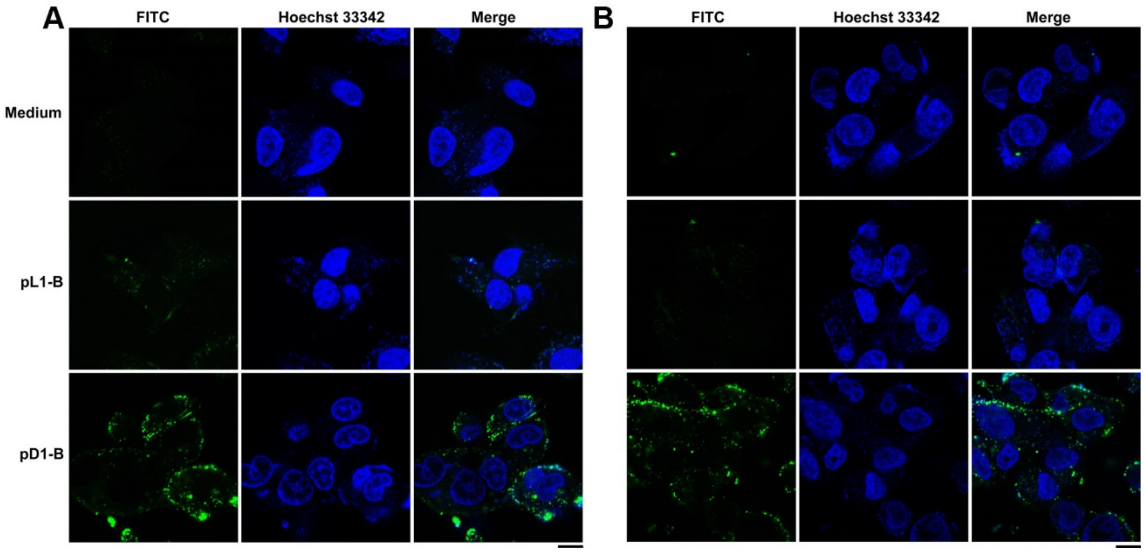


Figure S9. HS-5 cells treated with **pL1-B** or **pD1-B** (200 μM) for 24 h and then stained with FITC-avidin for (A) 1 h; (B) 2 h. Scale bar is 10 μm.

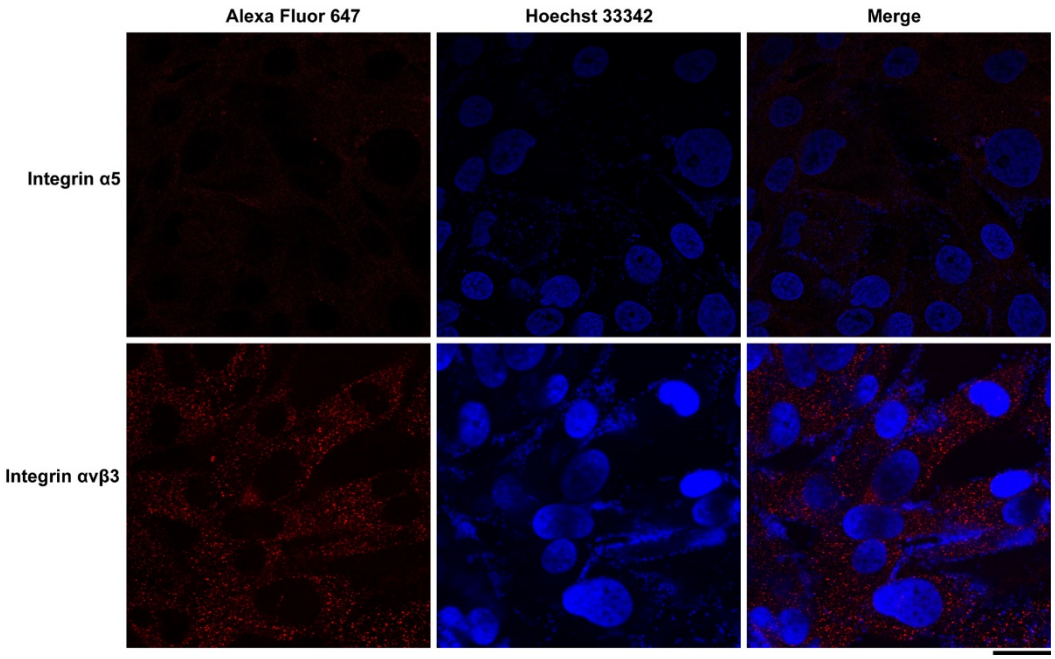


Figure S10. CLSM image of immunofluorescence for integrin α5 and integrin αvβ3 without any treatment. Red represents the fluorescence of antibodies and blue is nucleus. Scale bar is 20 μm.

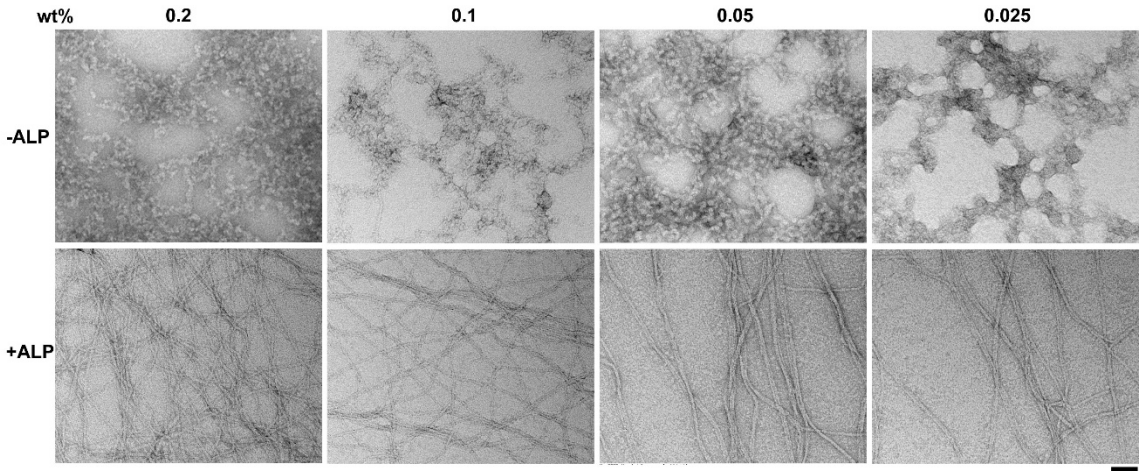


Figure S11. TEM images of **pL1-B** at concentration from 0.025 wt% to 0.2 wt% in Tris-Cl buffer (pH = 7.4) without or with the treatment of ALP (1 U/mL). Scale bar is 50 nm.

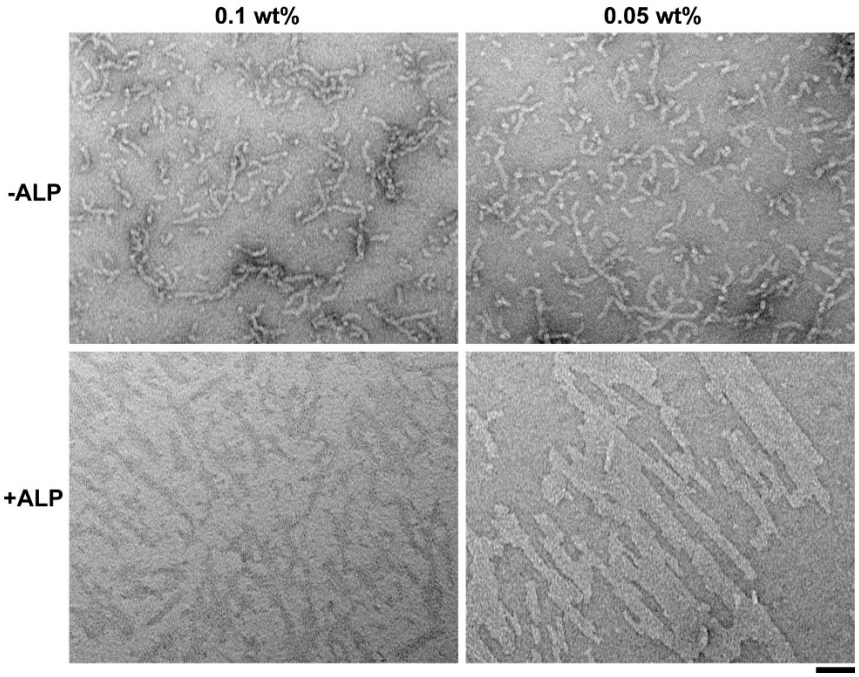


Figure S12. TEM images of **pD1** at concentration of 0.1 wt% or 0.05 wt% in Tris-Cl buffer (pH = 7.4) without or with the treatment of ALP (1 U/mL). Scale bar is 50 nm.

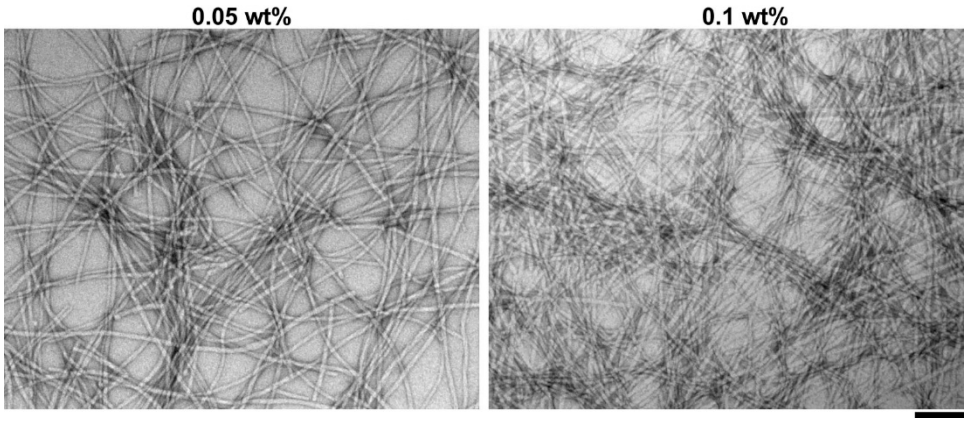


Figure S13. TEM images of **D1-B** in Tris-Cl buffer (pH=7.4). Scale bar is 100 nm.

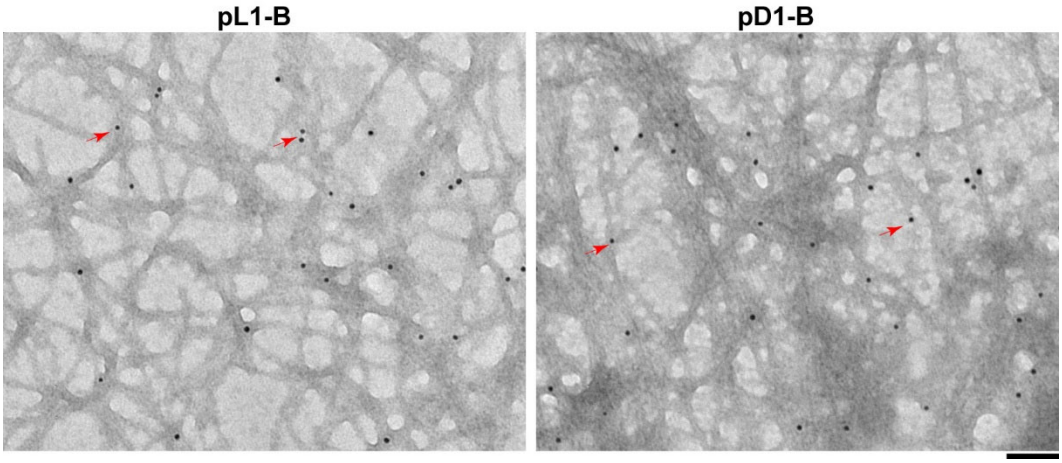


Figure S14. TEM of gold-avidin (10 nm) stained fibers, red arrows point to the gold nanoparticle. Scale bar is 100 nm

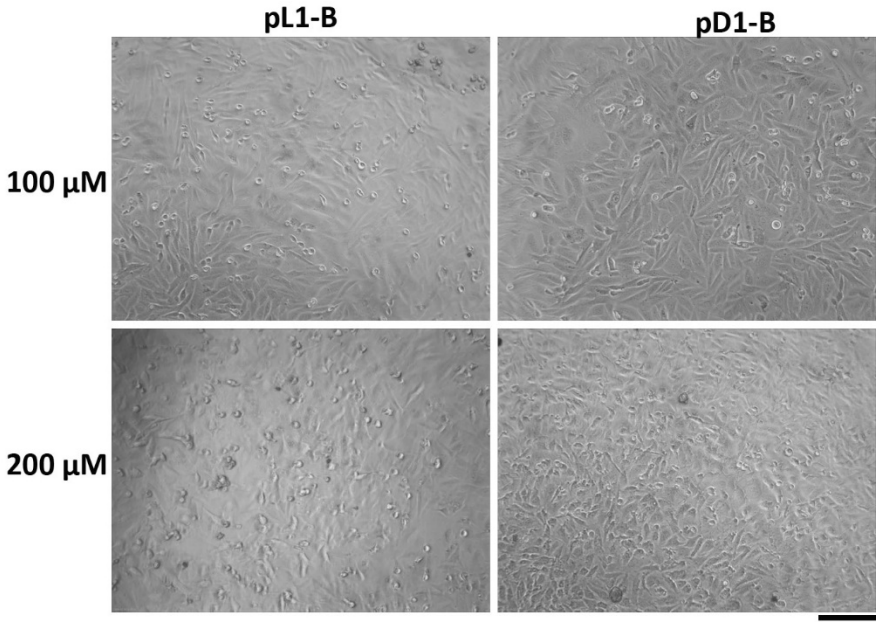


Figure S15. Optical images of Saos-2 cells treated with **pL1-B** or **pD1-B** for 24 h. Scale bar is 150 µm.

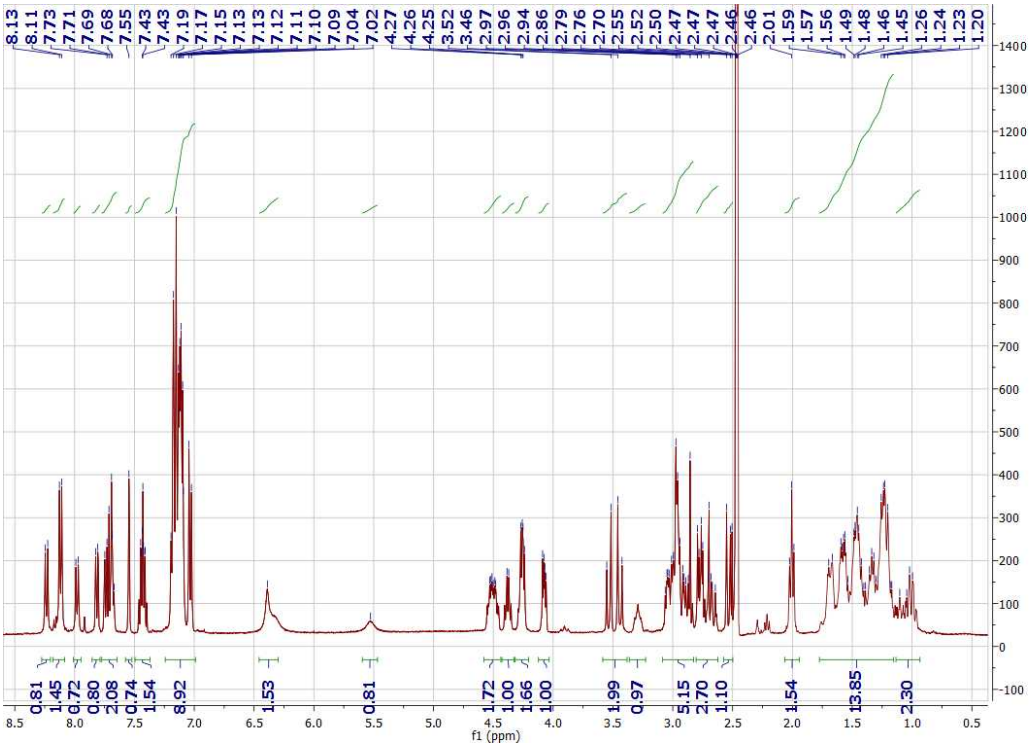


Figure S16. ^{1}H NMR of **pD1-B** in DMSO-d_6

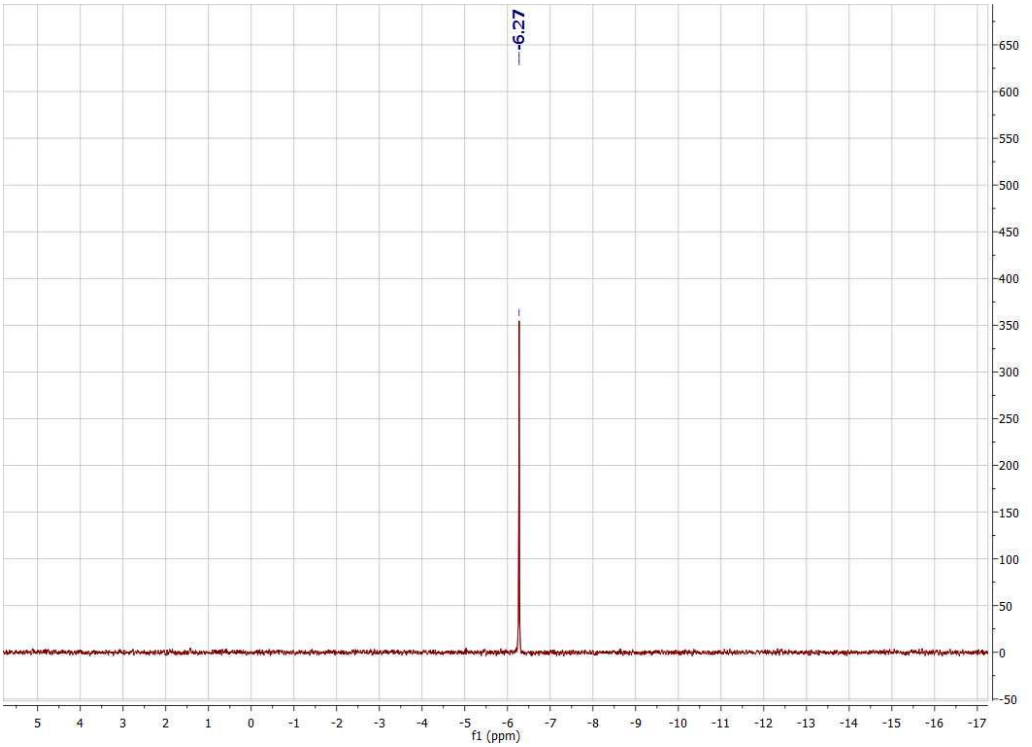


Figure S17. ^{31}P NMR of **pD1-B** in DMSO-d_6

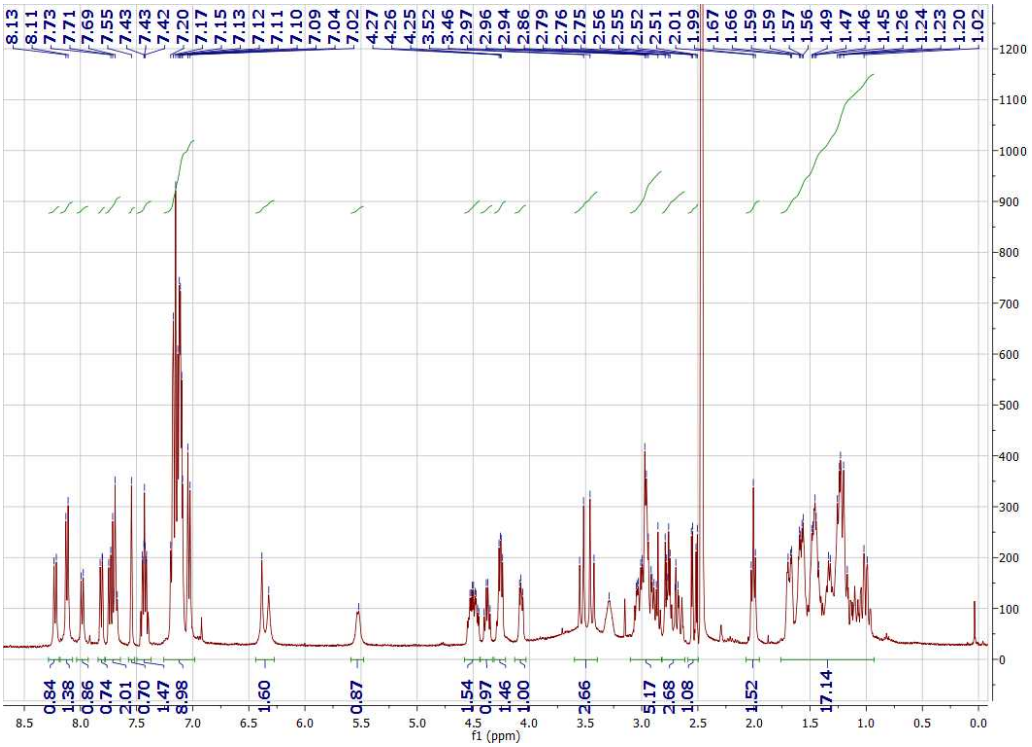


Figure S18. ^{1}H NMR of **pL1-B** in DMSO-*d*$_6$.

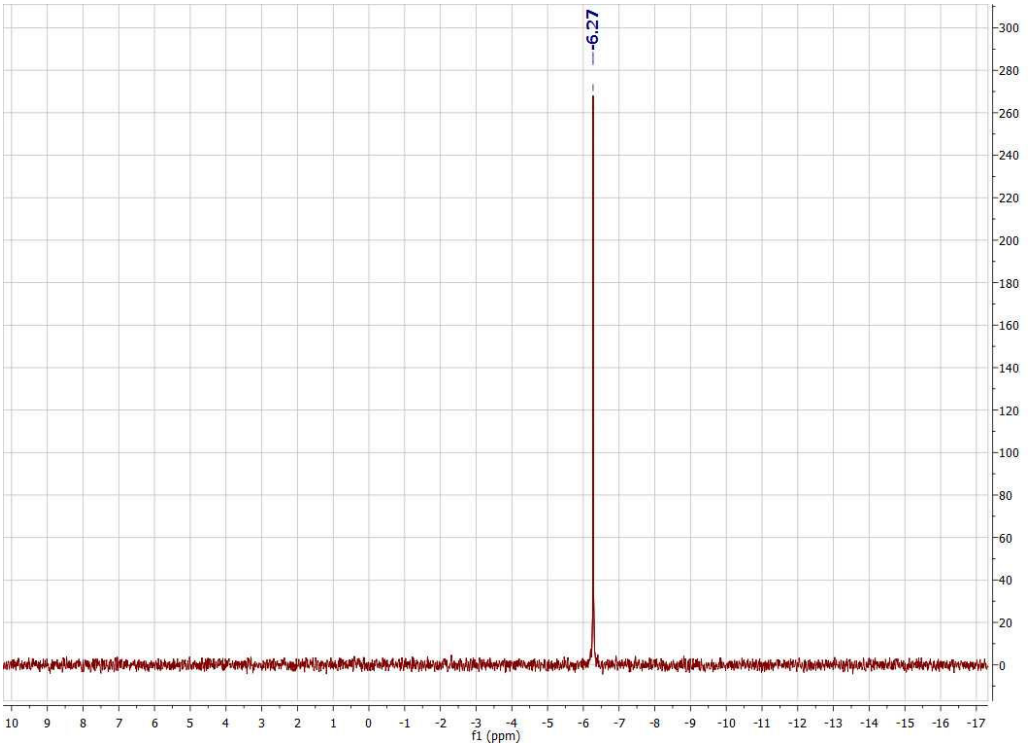


Figure S19. ^{31}P NMR of **pL1-B** in DMSO-*d*$_6$.

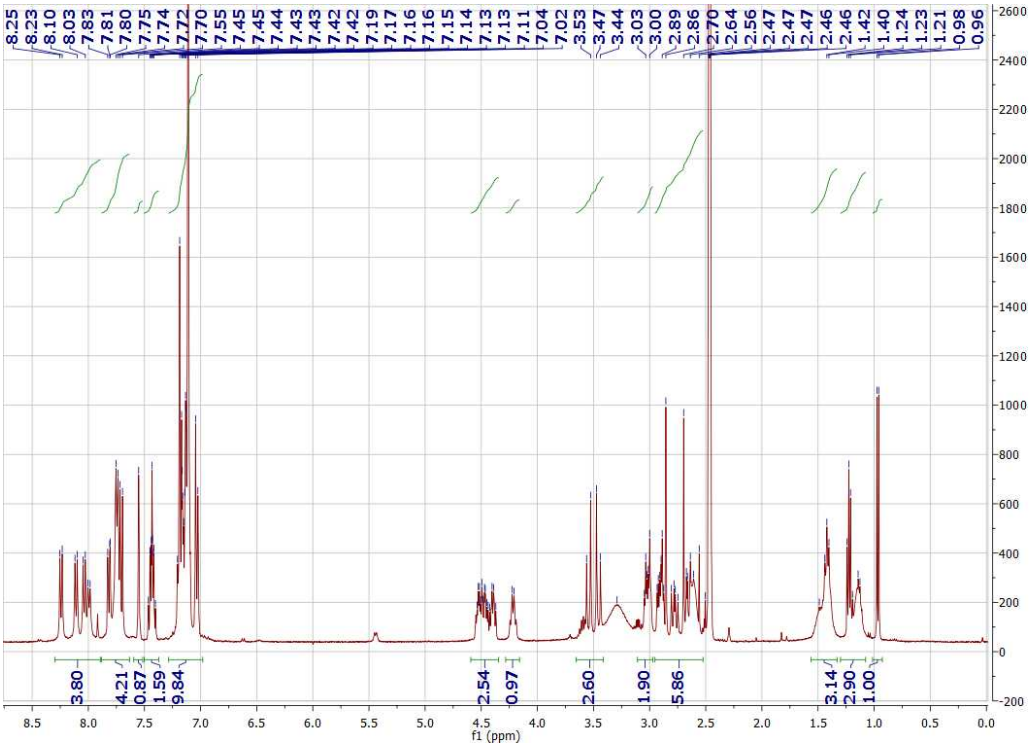


Figure S20. ^{1}H NMR of **pD1** in DMSO-*d*$_6$.

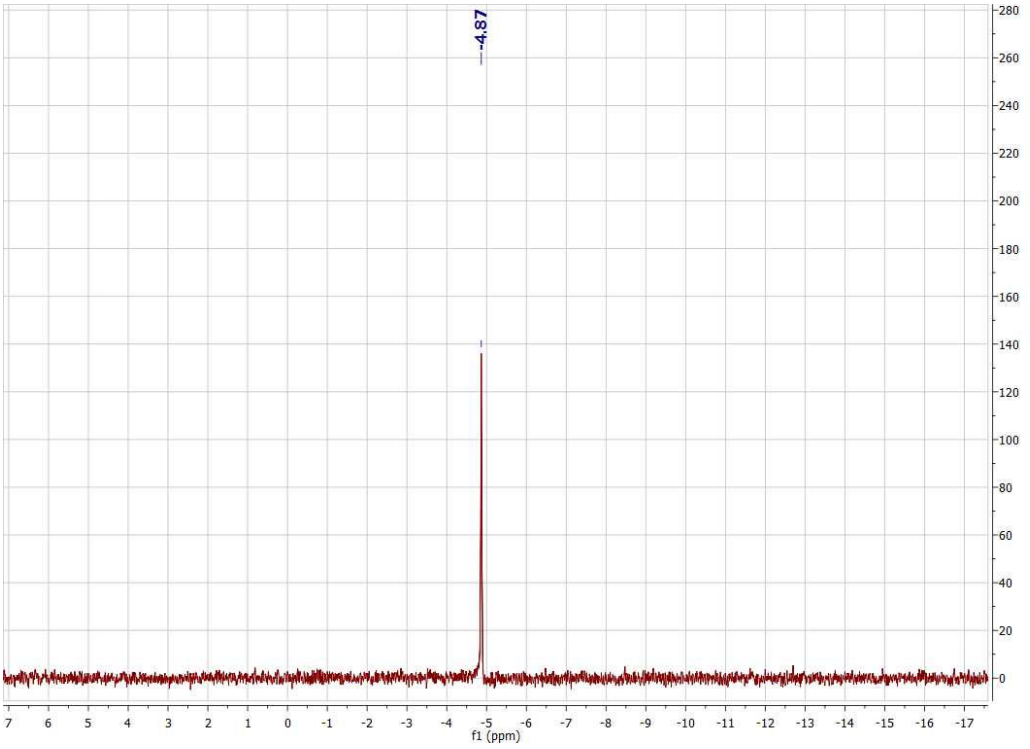


Figure S21. ^{31}P NMR of **pD1** in DMSO-*d*$_6$.

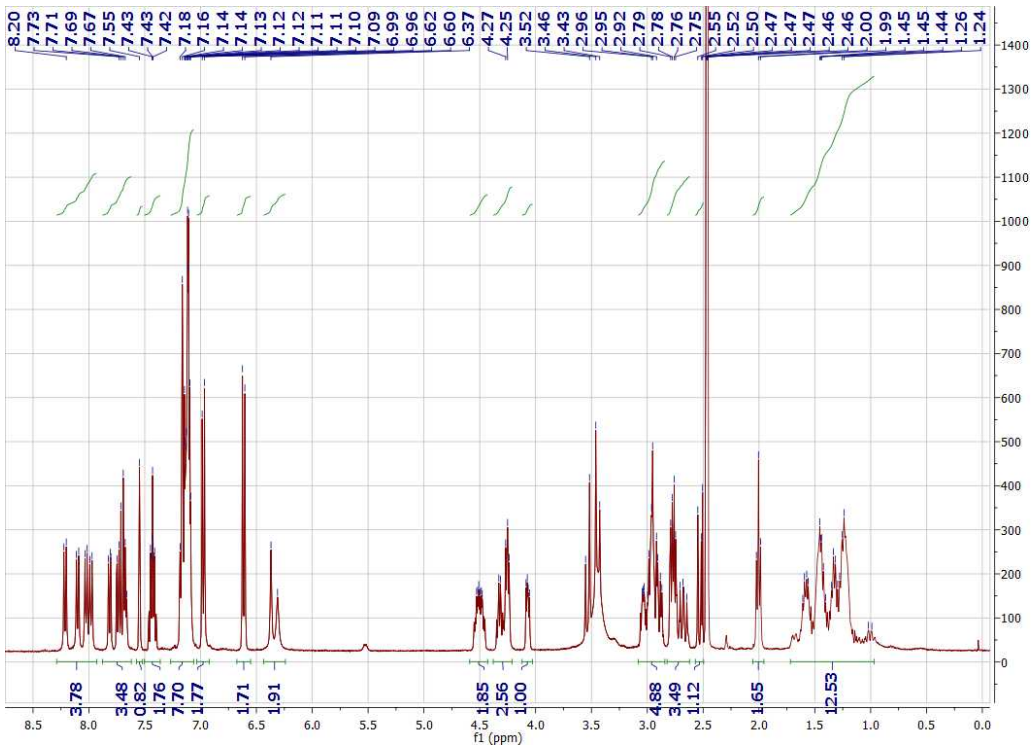


Figure S22. ^{1}H NMR of **D1-B** in DMSO-d_6.