Supporting Information for

Enzymatic self-assembly of an immunoreceptor tyrosine-based inhibitory motif (ITIM)

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Contents

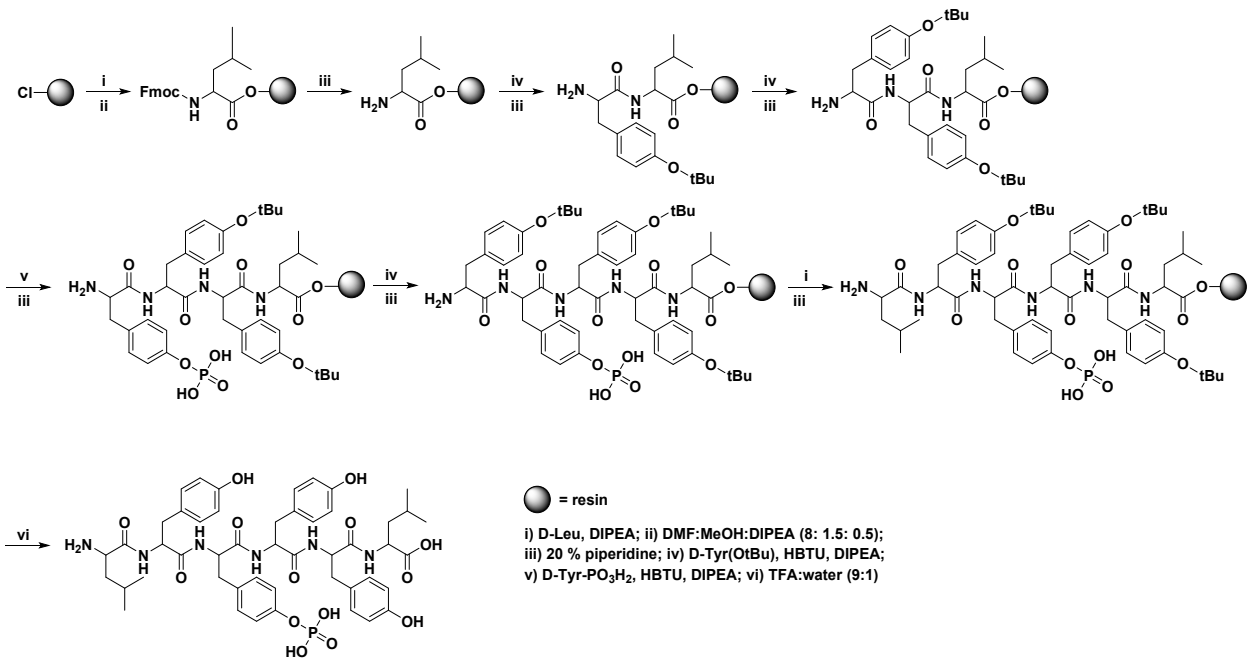
S1. Experimental materials and instruments

Alkaline phosphatase (ALP) was purchased from Biomatik USA, 2-naphthylacetic acid from Alfa Aesar, N,Ndiisopropylethylamine (DIPEA), O-benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) from Acros Organics USA, and all amino acid derivatives from GL Biochem (Shanghai) Ltd. All the solvents and chemical reagents were used directly as received from the commercial sources without further purification. All products (L precursor, D precursor, retro inverso precursor, L control and D control) were purified with Water Delta600 HPLC system, equipped with an XTerra C18 RP column and an in-line diode array UV detector. ^{1}H-NMR spectra were obtained on Varian Unity Inova 400, LC-MS spectra on a Waters Acquity ultra performance LC with Waters MICRO-MASS detector, rheological data on TA ARES G2 rheometer with 25 mm cone plate, TEM images on Morgagni 268 transmission electron microscope, confocal microscopy images on Leica TCS SP2 spectral confocal microscope.

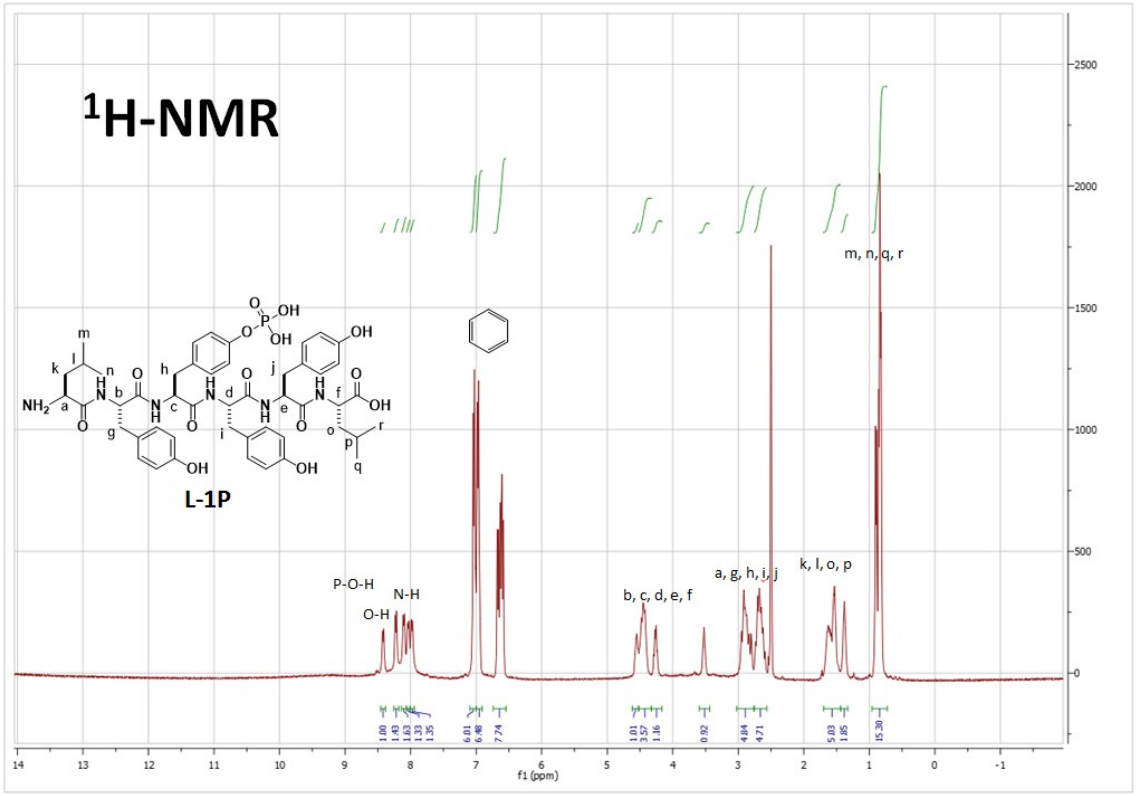
S2. Peptide synthesis, purification and characterization

We prepared the precursors and hydrogelators by solid phase peptide synthesis (SPPS) in fair yields (70-80%). The standard SPPS uses 2-chlorotriyl chloride resin (100-200 mesh and 0.3-0.8 mmol/g) and N-Fmoc-protected amino acids with side chains properly protected. We first placed 1 g of resin in a peptide synthesis reactor, swelled the resin with sufficient dry dichloromethane (DCM) with continuous nitrogen gas (N_2) bubbling for 30 min, and washed the resin with dry N,N-dimethylformamide (DMF, 3 x 8 mL). Then we anchored the first amino acid on the resin by adding the solution of Fmoc-D-Leu-OH (0.884 g, 2.5 mmol) in DMF (8 mL) and

N,Ndiisopropylethylamine (DIEA, 0.87 mL, 5 mmol), and agitated the resin with N_2 for 1 hr. Later we washed the resin with DMF (5 x 8 mL). We then block the un-reacted sites of the resin with DCM/methanol (MeOH)/DIEA = 16/3/1 (20 mL, 30 min). After washing the resin with DMF (5 x 8 mL), we removed the N-protected Fmoc group by 20 % piperidine in DMF (10 mL, 30 min). Then we washed the resin with DMF (5 x 8 mL) again, added the second amino acid solution of Fmoc-D-Tyr(tBu)-OH (1.15 g, 2.5 mmol) in DMF (8 mL) with coupling reagent O-benzotriazole-N,N,N',N'- tetramethyl-uronium hexafluoro-phosphate (HBTU, 0.948 g, 2.5 mmol) and DIEA (0.87 mL, 5 mmol), and agitated the resin with N_2 for 1 hr. Here, before adding the solution to the reactor, we sonicated it to make HBTU dissolve faster. To elongate the peptide chain, we repeated the washing, deprotection and coupling steps. After connecting the last amino acid to the resin, we washed the resin successively with DMF (5 x 8 mL), DCM (5 x 8 mL), MeOH (5 x 8 mL), and hexane (5 x 8 mL). As the final step, the resin-bound peptide was cleaved using a cocktail of TFA/water (9:1) for 2 hr under N_2, after which we washed the resin twice using acetone, and collected the filtrate. Crude product was obtained after the addition of cold diethyl ether into concentrated filtrate. Fmoc-$_P$Tyr-OH was synthesized using a combination of established procedures by Alewood.[1] Figure S1 illustrates the synthetic procedure of **D-1P**. The synthetic route of others is the same with that of **L-1P**, **RI-1P**, **L-1**, and **D-1**. The crude product was purified by reverse phase high performance liquid chromatography (HPLC) using a semi-preparatory C18 column. HPLC solvents consisted of solvent A (0.1% TFA in water) and solvent B (0.1% TFA in acetonitrile). The resulting peptide solution was frozen by liquid nitrogen and lyophilized to afford purified compounds in about 60% yield after purification.



Scheme S1. Synthesis route of **D-1p**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P-O-H
O-H
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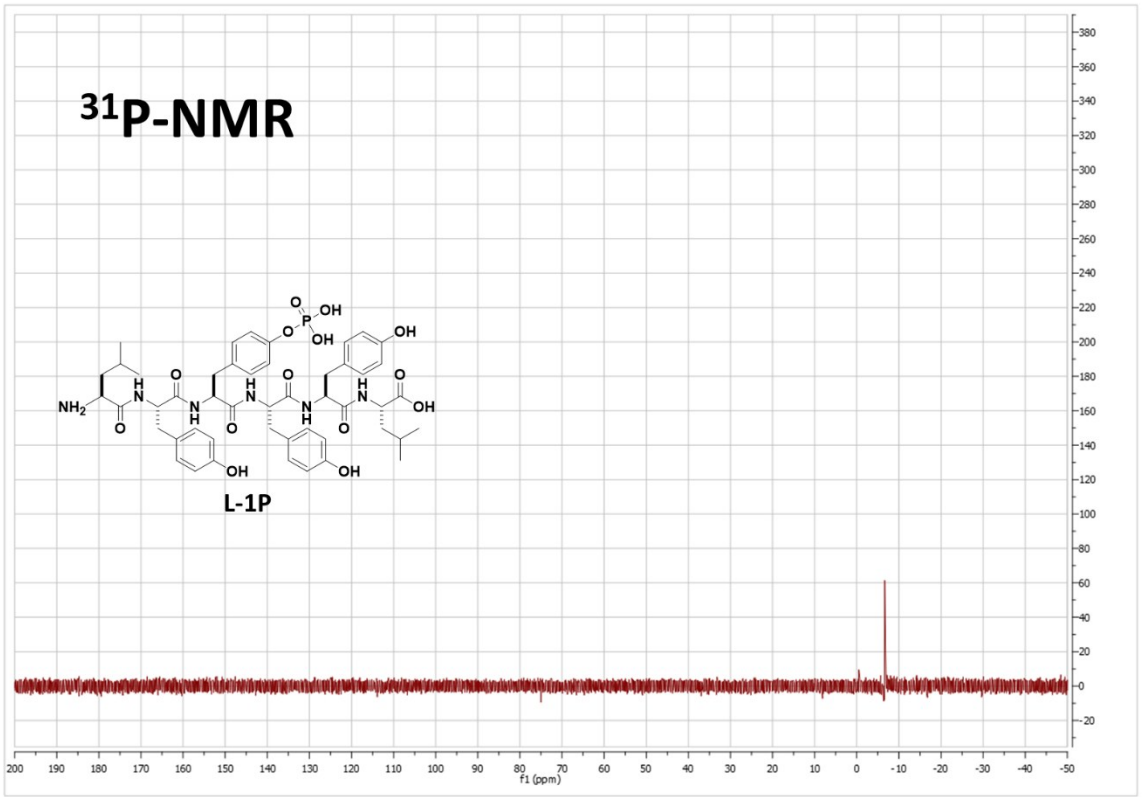
a, g, h, i, j
k, l, o, p
m, n, q, r
f1 (ppm)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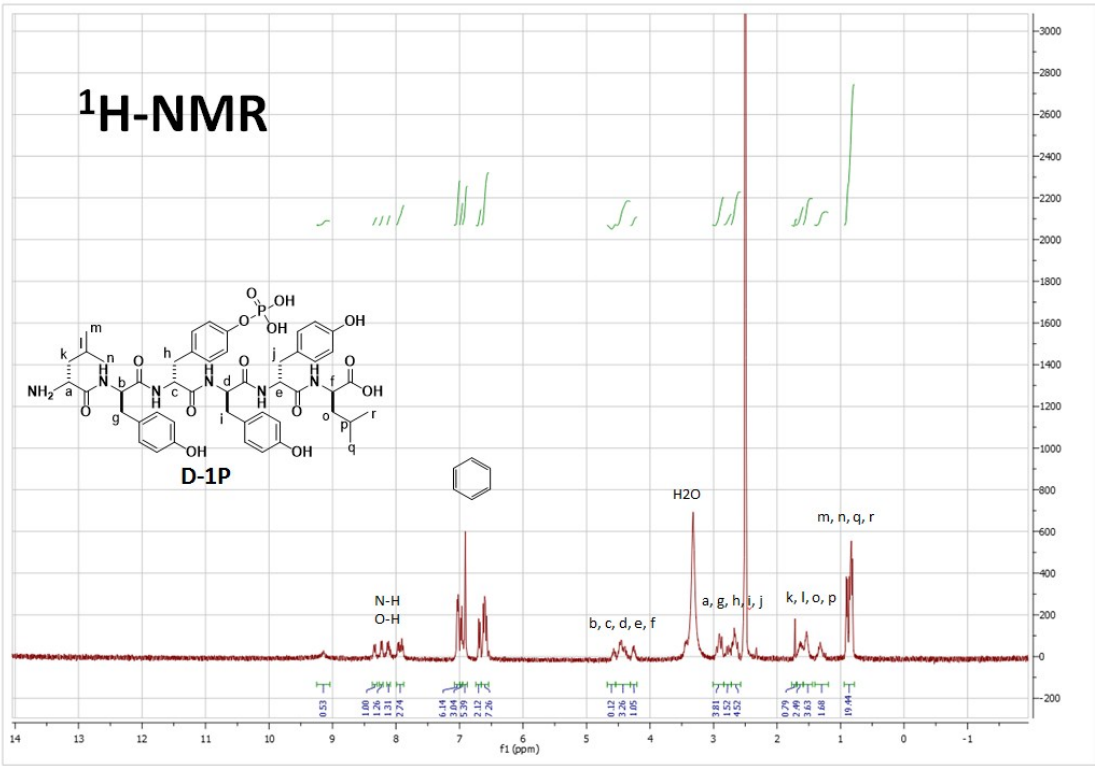
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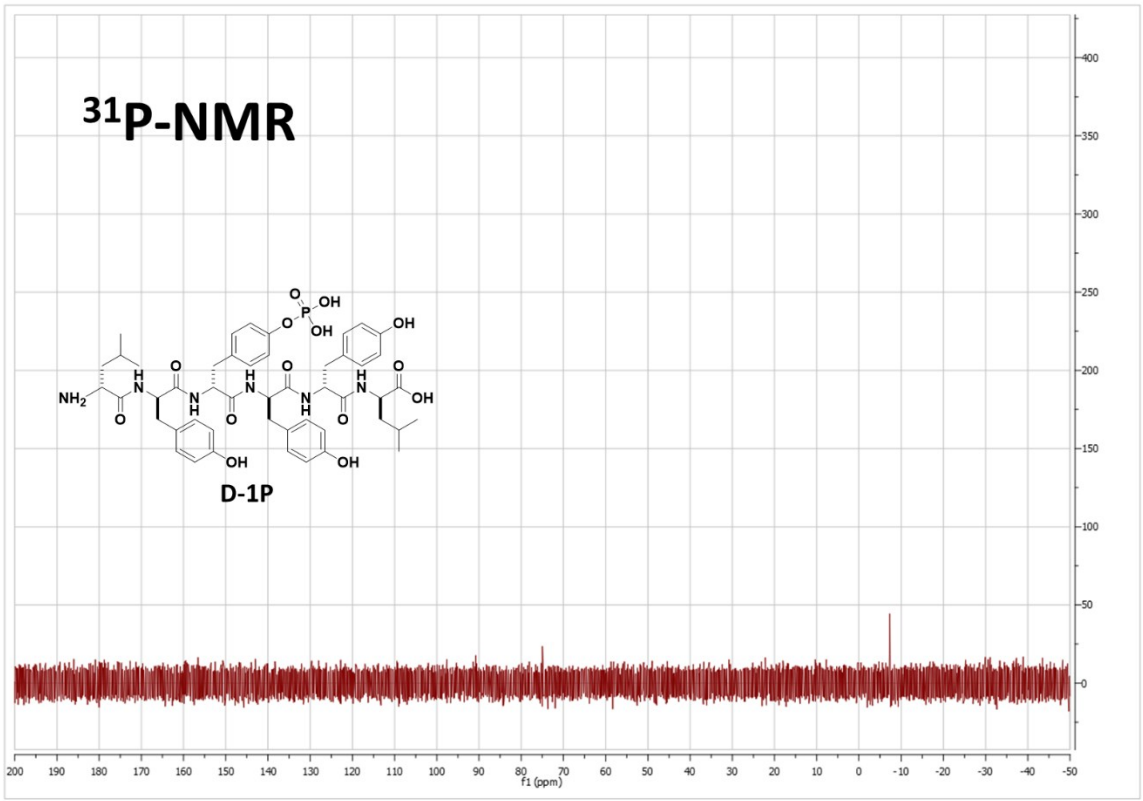
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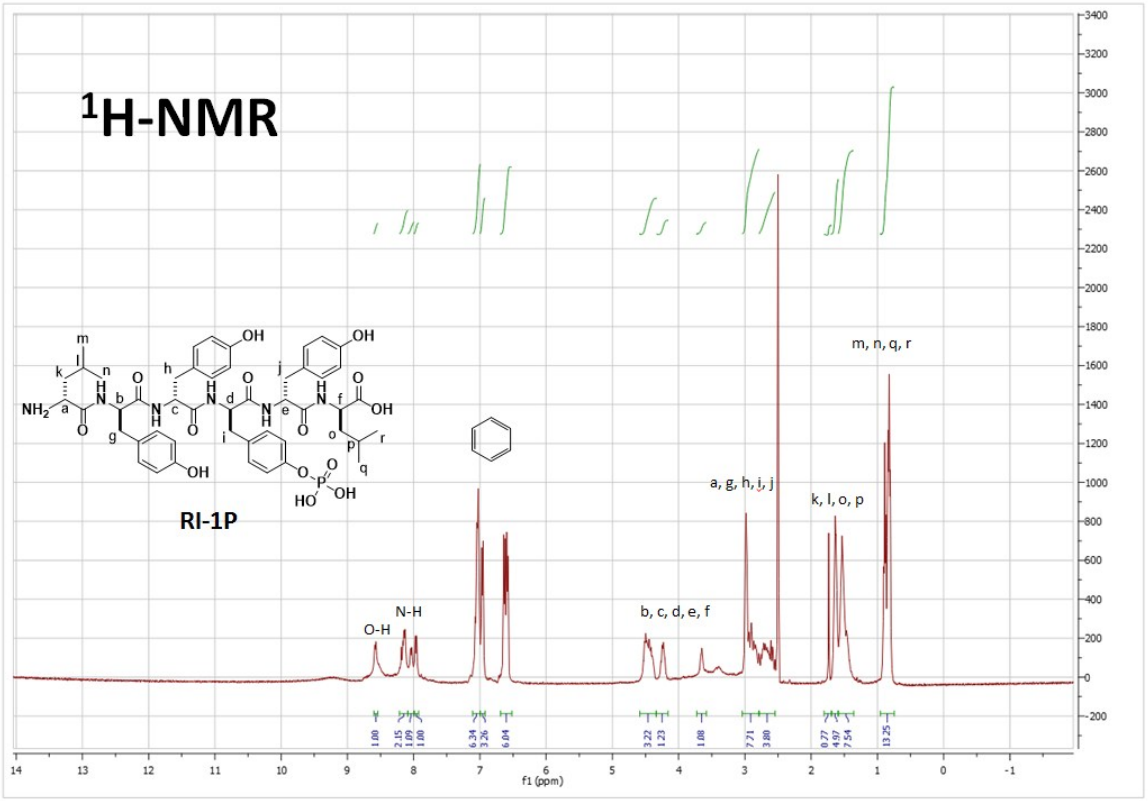
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O-H
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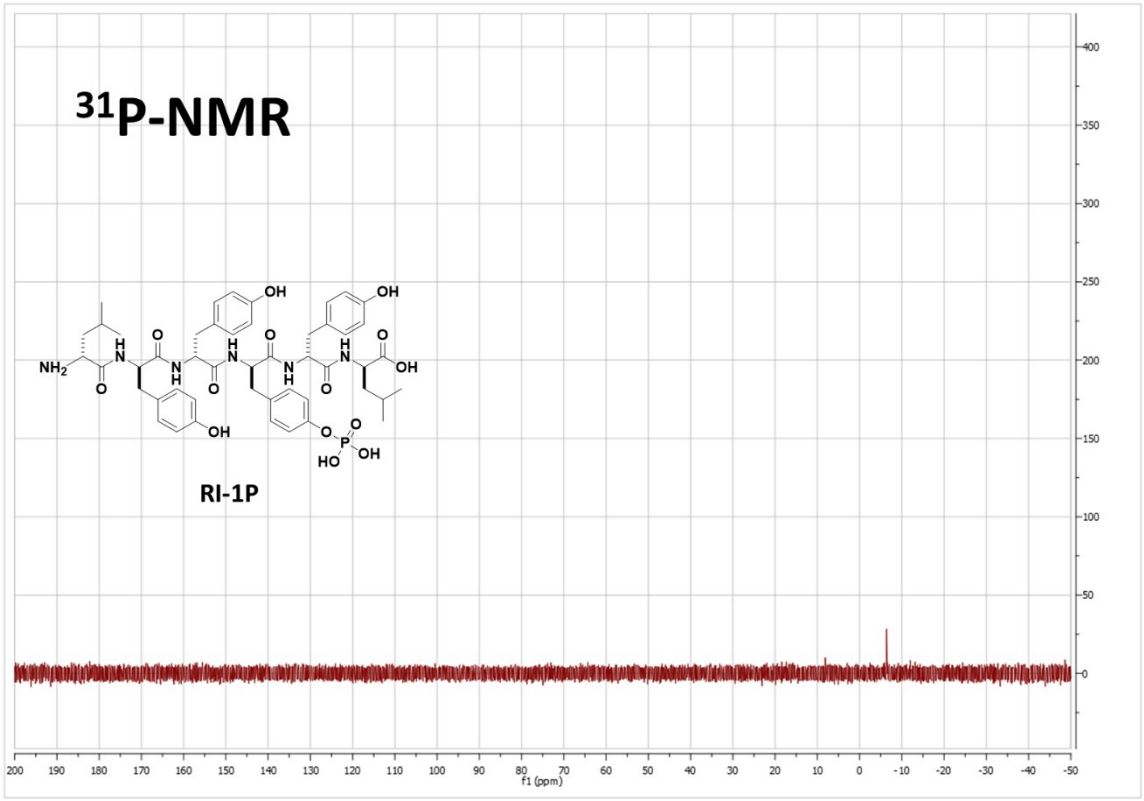
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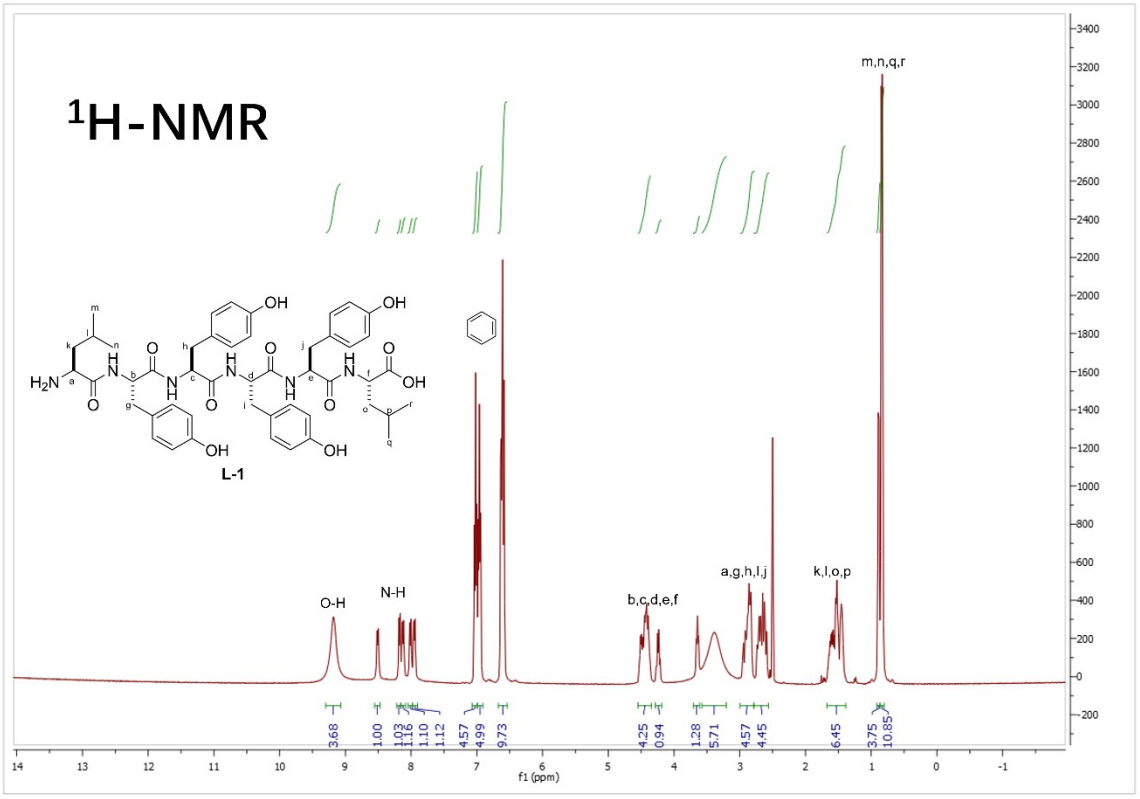
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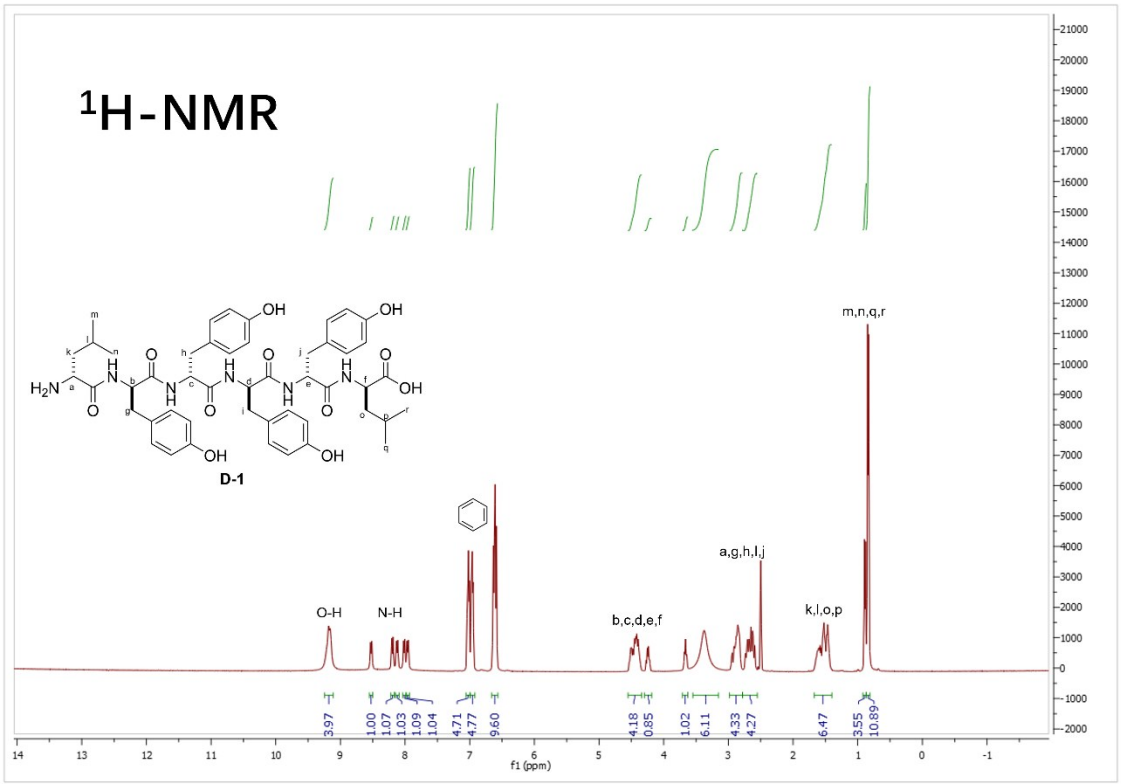
f1 (ppm)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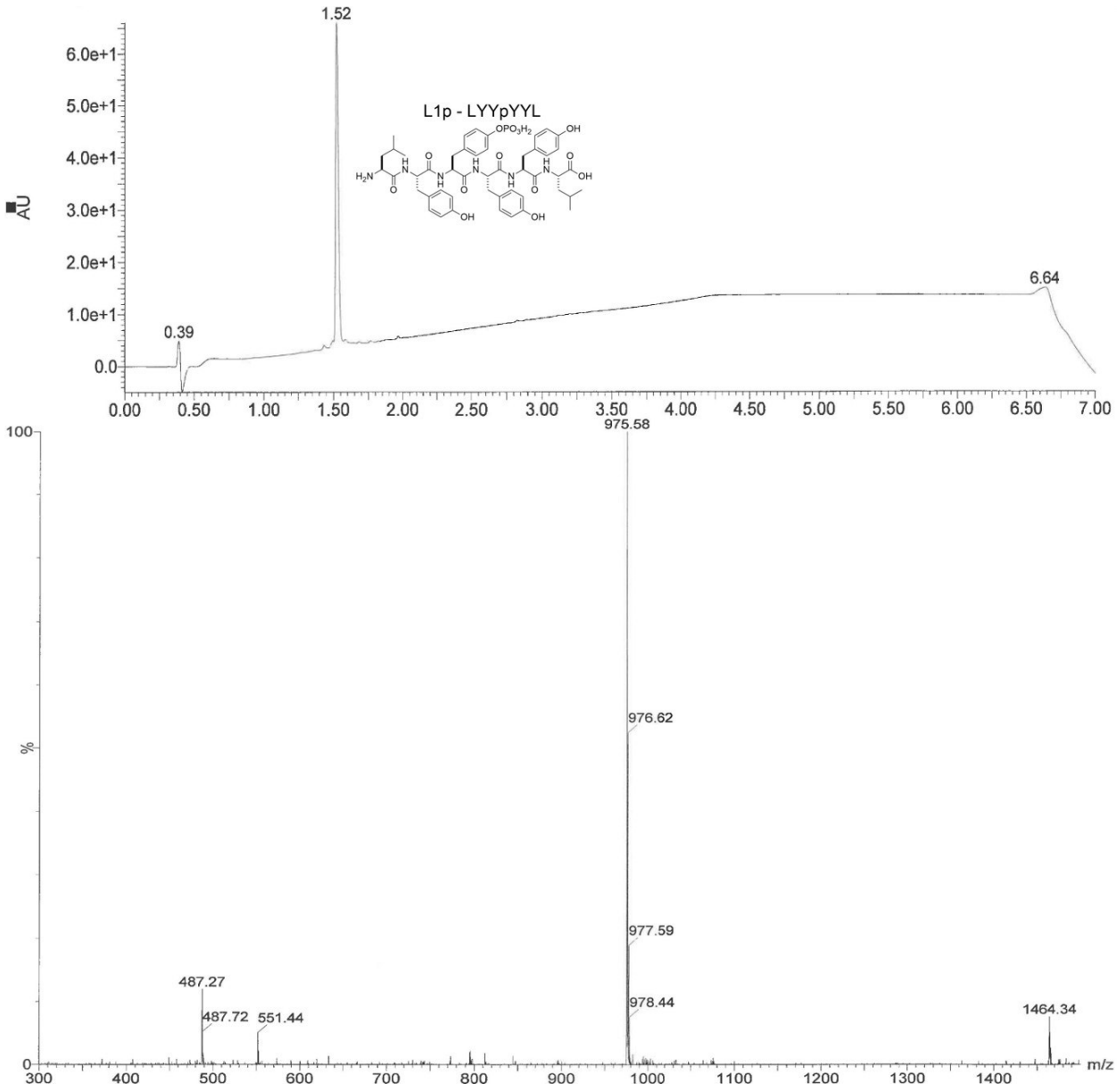
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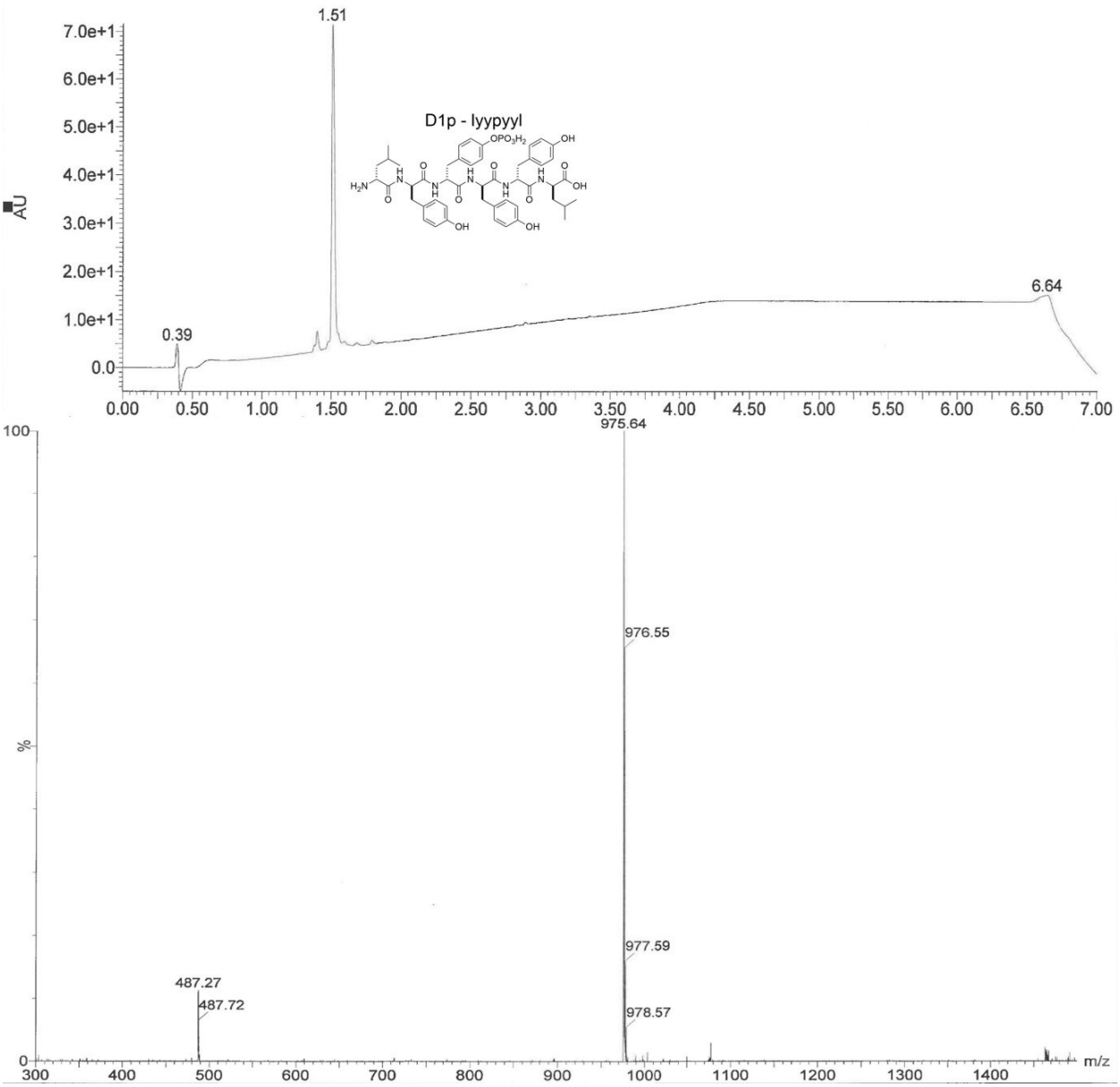
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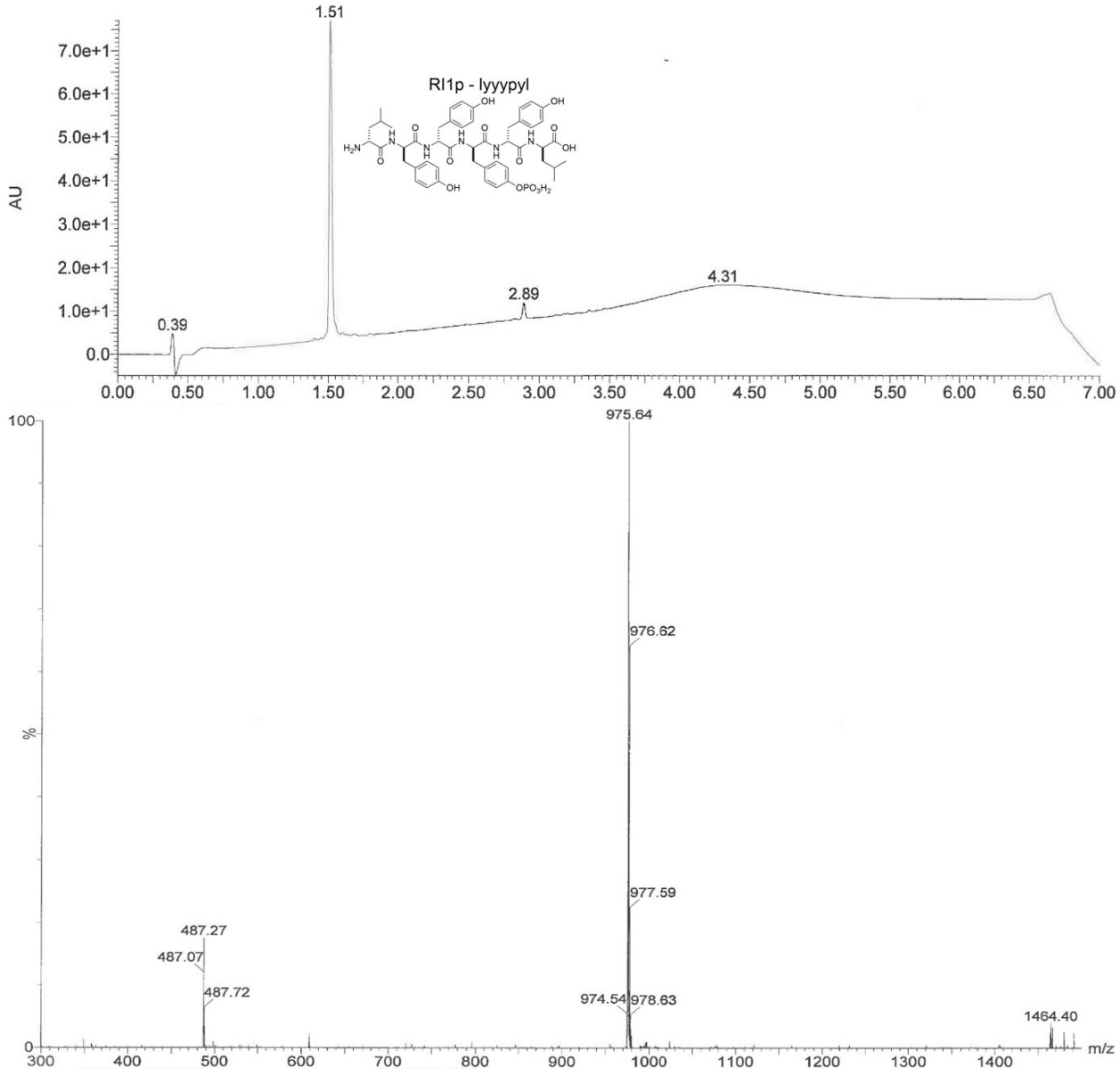
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0.0
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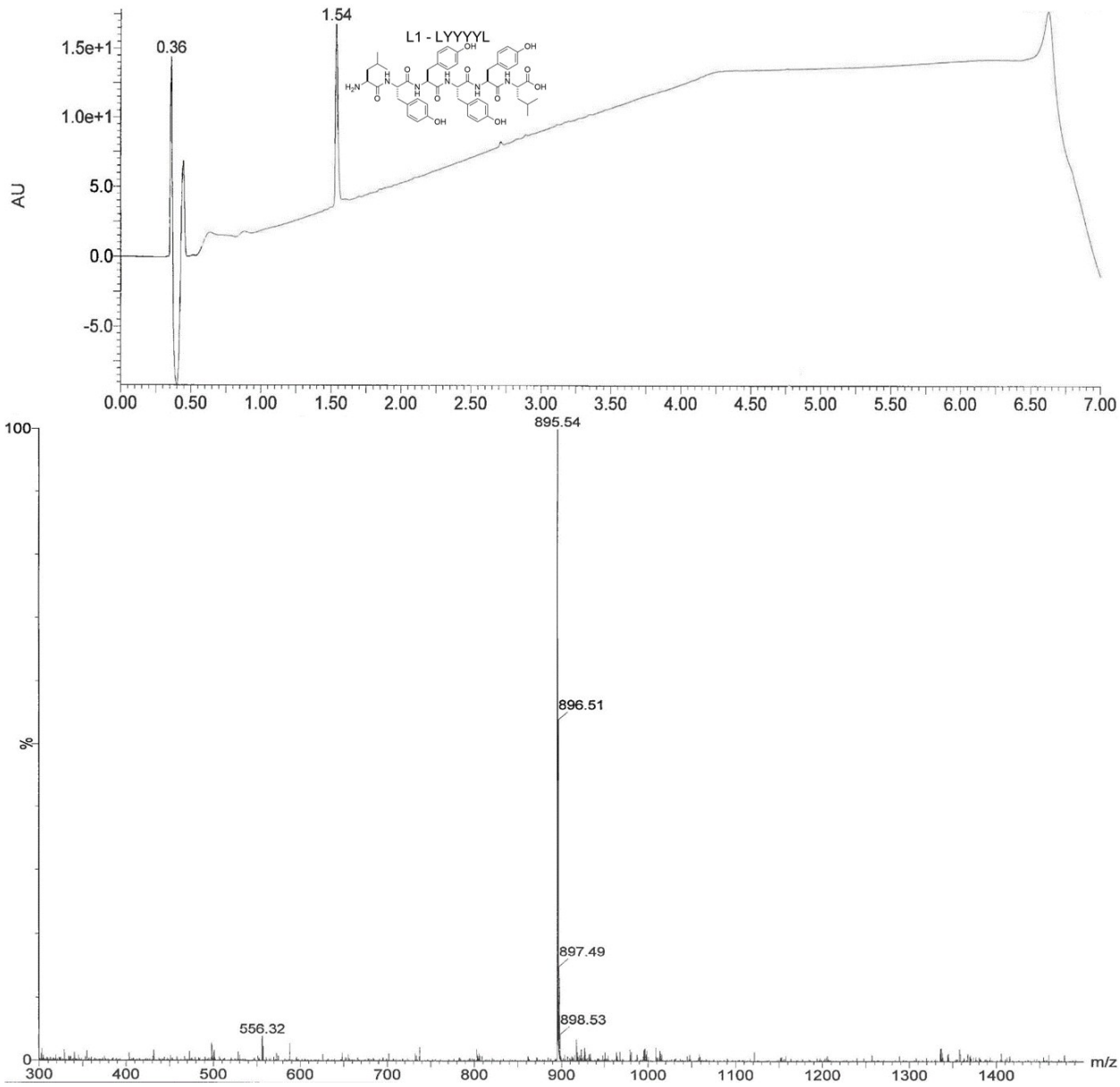
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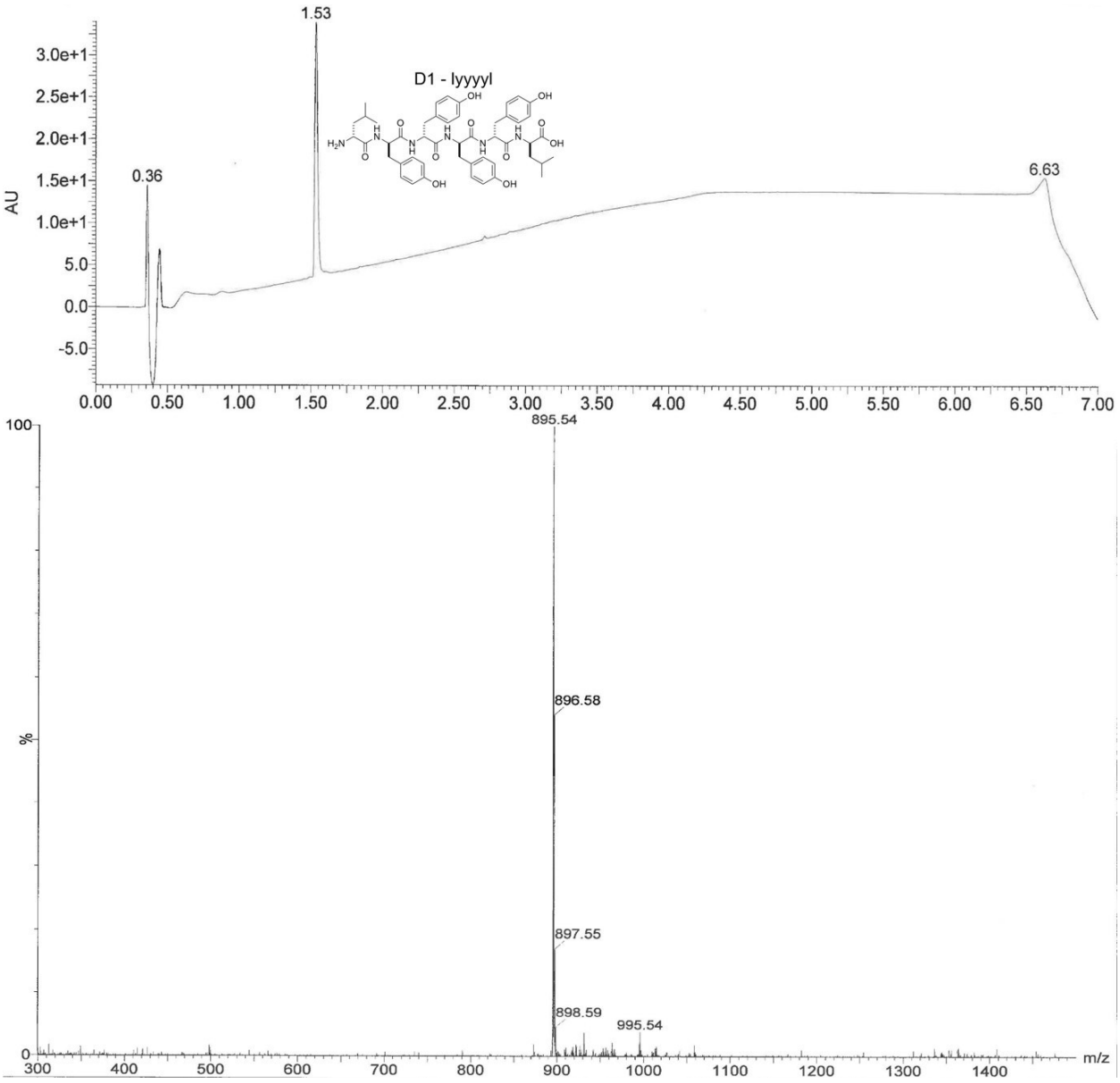

LC-MS (ESI):

L-1p (m/z): $C_{48}H_{61}N_6O_{14}P$, calc. 976.40; observed (M-1)- 975.58.

D-1p (m/z): $C_{48}H_{61}N_6O_{14}P$, calc. 976.40; observed (M-1)- 975.64.

RI-1p (m/z): $C_{48}H_{61}N_6O_{14}P$, calc. 976.40; observed (M-1)- 975.64.

L-1 (m/z): $C_{48}H_{60}N_6O_{11}$, calc. 896.43; observed (M-1)- 895.54.

D-1 (m/z): $C_{48}H_{60}N_6O_{11}$, calc. 896.43; observed (M-1)- 895.54.

S3. General procedures for hydrogel preparation

Enzymatic gelation: **L-1P**, **D-1P**, or **RI-1P** (2.5 mg) was dissolved in either distilled water or PBS buffer (500 μL), and the pH of the solution was adjusted to pH 7.4 by carefully adding 1 M NaOH, monitored by pH paper. Following the addition of alkaline phosphatase (ALP, 10 U/mL), enough distilled water or PBS buffer was added to make the final concentration of 0.5 wt%.

Acidic gelation: **L-1** or **D-1** (2.5 mg) was dissolved in either distilled water or PBS buffer (500 μL). 0.1 M HCl was carefully added until the mixture reached their gelation point; phase change was monitored by eye. The final pH was measured using pH paper. Since **L-1** and **D-1** lack phosphate groups, no alkaline phosphatase was added to the mixture.

S4. TEM sample preparation

A negative staining technique was used to study the TEM images. The 400 mesh copper grids coated with continuous thick carbon film (~ 35 nm) were first glow-discharged prior to use to increase the hydrophilicity. After loading samples (4 μL) on the grid, the grid was rinsed with dd-water three times. Immediately after rinsing, the grid containing the sample was stained with 2.0 % w/v uranyl acetate three times. Afterwards, the grid was dried in air to be placed inside the electron microscope.

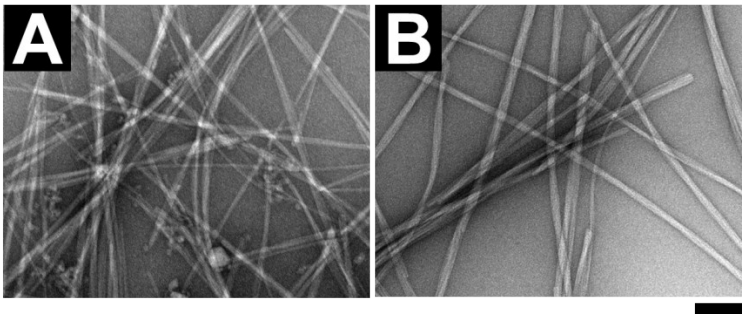

Figure S1. TEM images of hydrogels formed by the control hexapeptides A) **L-1** and B) **D-1** at the concentration of 0.5 wt %. The two compounds dissolve in PBS buffer and form gels at pH 4.5 and 4.0, respectively, by careful addition of hydrochloric acid (0.1 M). Scale bar is 100 nm.

S5. Rheological measurement

Rheological tests were conducted on a TA ARES-G2 rheometer, having a parallel-plate geometry with an upper plate diameter of 25 mm and a 0.4 mm gap. During the measurement, the stage temperature was maintained at 25 °C by Peltier heating/cooling system. The hydrogel was loaded on the stage with a spatula, and then we performed the dynamic strain (0.1−100%) at 6.28 rad/s; the strain for maximum G′ in the linear range of strain sweep test was picked for frequency sweep test (0.1−200 rad/s).

S6. Circular Dichroism (CD) measurement

CD spectra were recorded (185−300 nm) using a JASCO 810 spectrometer equipped with nitrogen. The hydrogel (0.5% wt., 400 μL) was placed carefully in a 1 mm thick quartz cuvette and scanned at a 0.5 nm interval three times for three times per measurement. The CD spectra in

the Results and Discussion section confirm the chirality of enantiomeric pairs of **L-1P**, **D-1P**, and **RI-1P**.

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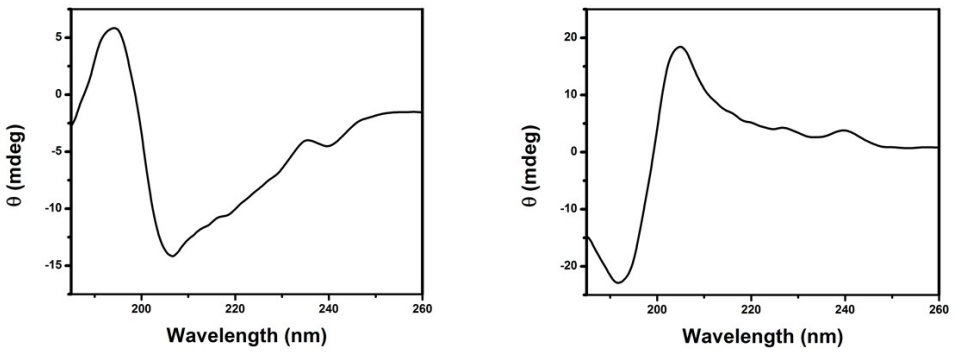

Figure S2. CD analysis of hydrogels formed by control peptides **L-1** (left) and **D-1** (right), formed upon addition of hydrochloric acid (0.1 M). **L-1** forms a gel at pH 4.5, while **D-1** forms a gel at pH 4.0.

S7. General procedure for the digestion experiment

3 mL of solution for each compound dissolved in PBS buffer (500 μM, pH = 7.4) were treated with proteinase K (4.0 U/mL) at 37 °C. A 200 μL aliquot of each sample was taken out at the desired time and mixed with 200 μL of methanol. Each of the obtained samples was injected into and analyzed by analytical HPLC to determine the amount of compound remaining in solution.

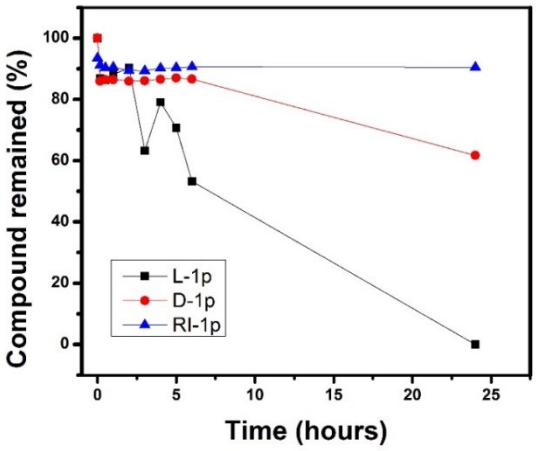

Figure S3. Digestion curve of three precursors **L-1P**, **D-1P**, and **RI-1P** upon treatment with proteinase K (4 U/mL) for 24 h. All compounds are at the concentration of 0.5 wt. %, pH 7.4.

S8. Dephosphorylation assay

6 mL of precursor solution in PBS buffer (500 μM, pH = 7.4) was treated with ALP (1 U/mL) at 37 °C. 500 μL of sample was taken out at the desired time, and mixed with 500 μL of methanol. Each of the obtained samples was injected into and analyzed by HPLC to determine the ratio of precursor and hydrogelator in each sample.

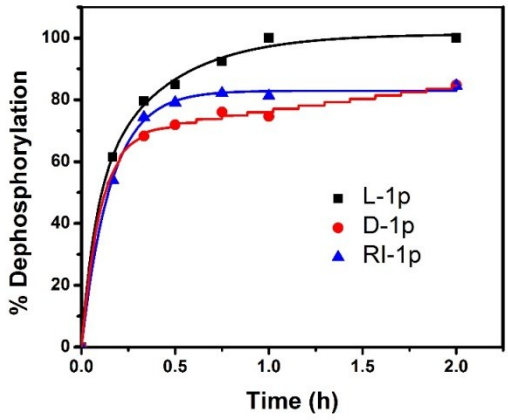

Figure S5. Dephosphorylation assay of L-1P, D-1P, and RI-1P upon incubation with ALP (1 U/mL) at 37 °C for 2h. The precursors dissolve in PBS buffer at the concentration of 500 μM (pH 7.4).

S9. Cell culture

All cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The HeLa cells were propagated in Minimum Essential Media (MEM), supplemented with 10% fetal bovine serum (FBS) and antibiotics, in a fully humidified incubator containing 5% CO_2 at 37°C. The Saos-2 cells were propagated in McCoy's 5A, supplemented with 15% FBS and antibiotics, in a fully humidified incubator containing 5% CO_2 at 37°C. The HS-5 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with FBS to a final concentration of 10% and antibiotics, in a fully humidified incubator containing 5% CO_2 at 37°C.

S10. MTT cell viability assay

We followed the protocol available on the ThermoFisher Scientific website. Cells in exponential growth phase were seeded in a 96 well plate at a concentration of 1×10^4 cell/well. The cells were allowed to attach to the wells for 24 h at 37 ºC, 5% CO_2. The culture medium was removed and 100 μL culture medium containing compounds (immediately diluted from freshly prepared stock solution of 10 mM) at gradient concentrations (0 μM as the control) was placed into each well. After culturing at 37 ºC, 5% CO_2 for 48 h, to each well was added 10 μL of 5 mg/mL MTT ((3-(4, 5- DimethylthiazoL-2-yl)-2, 5-diphenyltetrazolium bromide). The plated cells were incubated in the dark for 4 h. 100 μL of 10% SDS with 0.01M HCl was added to each well to

stop the reduction reaction and to dissolve the purple dye. After incubation of the cells at 37 ºC overnight, the OD at 595 nm of the solution was measured in a microplate reader.

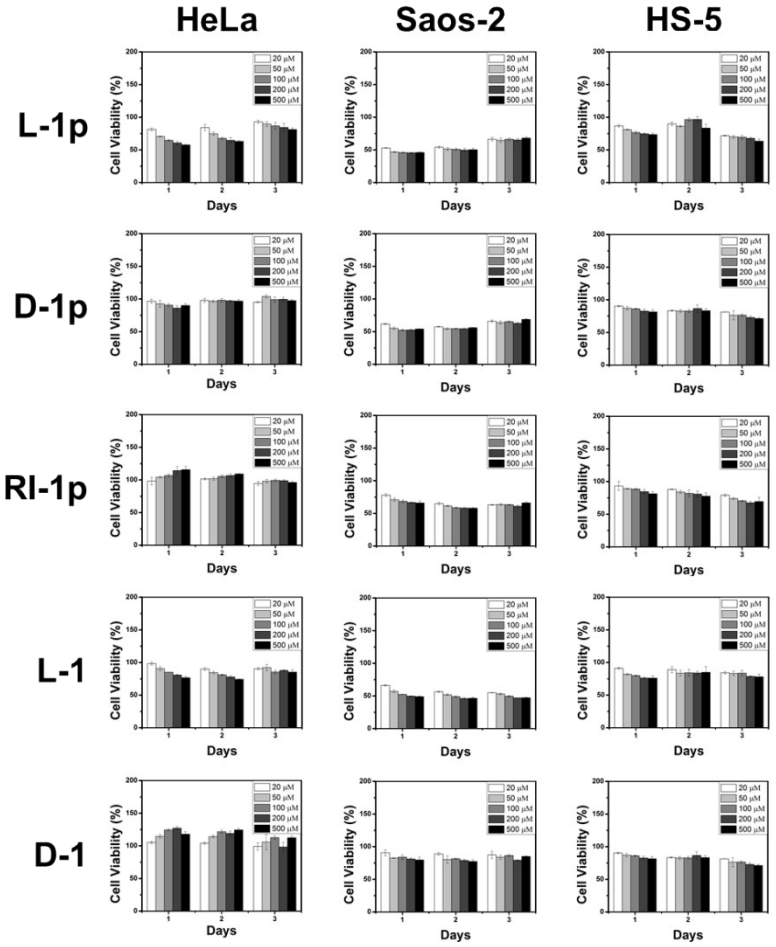

Figure S5. Cell viability (measured by MTT assay) of HeLa, Saos-2 and HS-5 cells treated with **L-1p**, **D-1p**, **RI-1p**, **L-1**, and **D-1** (pH 7.4) at concentrations from 20 μM to 500 μM for 3 days.

S11. Live/Dead cell viability/cytotoxicity assay (2D and 3D)

We followed the protocol available on the back labels of the LIVE/DEAD® Viability/Cytotoxicity Kit and on the ThermoFisher Scientific website. In a 35 mm disposable petri dish, prior to the assay, we washed the HeLa cells gently with 500–1,000 volumes of

Dulbecco's phosphate-buffered saline (D-PBS). Upon dilution of the calcein AM dye to 1 μM and EthD-1 dye to 2 μM, we added 100–150 μL of the combined LIVE/DEAD® assay reagents to the surface of a 22 mm square coverslip, so that all cells are covered with solution. The cells were incubated in a covered 35 mm disposable petri dish for 30–45 minutes at room temperature. After incubation, we added approximately 10 μL of the fresh LIVE/DEAD® reagent solution/D-PBS to a clean microscope slide and viewed the labeled cells under a fluorescence microscope. Images taken after staining cells with 5 μL of FITC annexin V and 1 μL of the 100 μg/mL propidium iodide working solution to each 100 μL of cell suspension for 15 minutes according to the FITC Annexin V/Dead Cell Apoptosis Kit protocol (ThermoFisher Scientific).

1. P. F. Alewood, R. B. Johns, R. M. Valerio and B. E. Kemp, *Synthesis-Stuttgart*, 1983, 2.