Electronic Supplementary Information

In situ self-assembled peptide nanoparticles improves anti-hepatic fibrosis effect

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1. General Methods

Experimental materials and instruments

All the starting materials were obtained from Aladdin, Sigma or Sangon Biotech. Commercially available reagents were used without further purification, unless noted otherwise. All other chemicals were reagents grade or better. Calf intestinal alkaline phosphatase (ALP) was obtained from Takara Bio Inc. (Beijing, China). HPLC analyses were performed on an Agilent 1260 HPLC system equipped with a G7111A pump and an in-line diode array UV detector using an Agilent Zorbax 300SB-C18 RP column, with CH3CN (0.1% of TFA) and ultrapure water (0.1% of TFA) as the eluent. Electrospray ionization (ESI) mass spectra were obtained on a Finnigan LCQ Advantage ion trap mass spectrometer (ThermoFisher Corporation). ^{1}H NMR spectrum was obtained on a JNM-ECZ400S. ^{13}C NMR spectrum was obtained on a JNM-ECZ600R.

Enzyme kinetic study

Firstly, the standard curves of peak area of a series of **NapFFYpLSKL** solutions at different concentrations (50, 100, 200, 400, 600, 1200, 1600, 2000, 2400 and 2800 μM) were obtained by HPLC. Secondly, the initial velocity of different concentrations of **NapFFYpLSKL** solutions were obtained after incubation with 0.05 U μL^{-1} ALP in PB for 8 min at 37 ℃. Finally, the initial velocity was plotted against the concentration of **NapFFYpLSKL** and fitted to the Michaelis-Menten model.

Cell viability assay

(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was employed to evaluate the cytotoxicity. Cells were seeded into a 96-well plate at a density of 5×10^3/well. The cells were cultured overnight at 37 ℃ under 5% CO_2. For normal LO2 cells, two groups of cells were treated with 0, 10, 20, 40 μM **NapFFYpLSKL** or **LSKL** for 48 h, respectively. Besides, normal LO2 cells were treated with 0, 50, 100, 150, 200 mM alcohol for 48 h when the density reached about 70% in the 96-well plate, which was used to establish the damaged liver cell model. For damaged LO2 cells, four groups of normal LO2 cells were treated with 150 mM

alcohol for 24 h and further added with **NapFFYpLSKL** (0-40 μM), **LSKL** (0-40 μM), **NapFFYpLSKL** (0-40 μM) + 10 mM L-phenylalanine (L-F) and KRFK (0-40 μM) + 40 μM **NapFFYpLSKL** for further 24 h, respectively. MTT (5 mg/mL, 20 μL) was added to each well and incubated with cells for another 4 h. Next, 200 μL/well dimethylsulfoxide (DMSO) was added to dissolve the formazan. The absorption at 490 nm was detected using an ELISA reader (Varioskan Flash). The viability of cell growth was calculated: viability (%) = (mean of absorbance value of treatment group/mean of absorbance value of control) × 100.

Evaluation of TGF-β_1 expression level in LO2 cells

LO2 cells were divided into six groups: (1) the group blank without treatment; 2. the group Alc with incubation of 150 mM alcohol for 48 h; 3. the group **Alc + NapFFYpLSKL** with incubation of 150 mM alcohol for 24 h and with addition of 40 μM **NapFFYpLSKL** for further 24 h; 4. the group **Alc + LSKL** with incubation of 150 mM alcohol for 24 h and with addition of 40 μM **LSKL** for further 24 h; 5. the group **Alc + NapFFYpLSKL + L-F** with incubation of 150 mM alcohol for 24 h and with addition of 40 μM **NapFFYPLSKL** and 10 mM L-F for further 24 h; 6. the group **Alc + NapFFYpLSKL + KRFK** with incubation of 150 mM alcohol for 24 h and with addition of 40 μM **NapFFYpLSKL** and 40 μM KRFK for further 24 h. Then the TGF-β_1 level inside cells was detected according to the kit instructions.

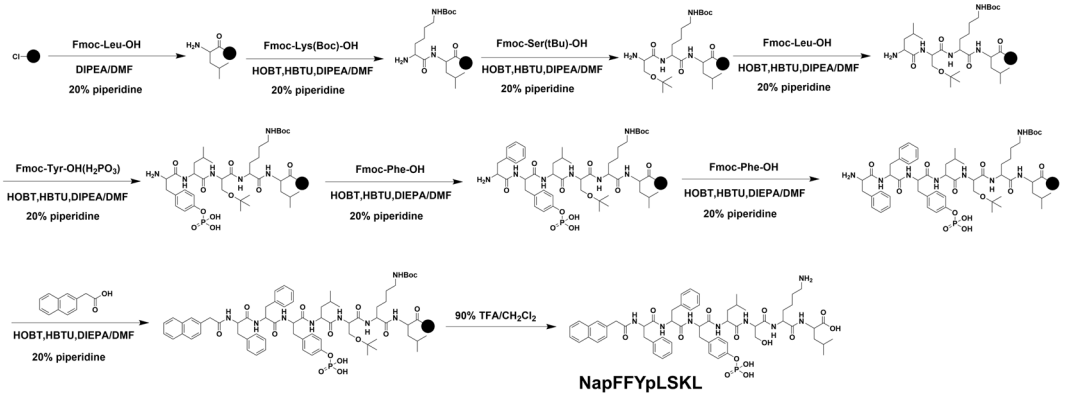
Treatment of liver fibrosis in *vivo*

All C57BL/6 male mice received care according to the guidelines of the Care and Use of Laboratory Animals. The procedures were approved by the Anhui University Animal Care and Use Committee (IACUC(AHU)-2024-031). 6-week C57BL/6 mice were randomly divided into 6 groups (n = 5): (1) mice in group **Blank** without treatment; (2) mice in group **Saline** were intraperitoneally (i.p.) injected with 10% CCl_4/olive oil (200 μL) three times a week at 1-4 weeks and i.p. injected with saline three times a week at 2-4 weeks; (3) mice in group **NapFFYpLSKL** were i.p. injected with 10% CCl_4/olive oil three times a week at 1-4 weeks and i.p. injected with **NapFFYpLSKL** (6.5 mM, 100 μL) three times a week at 2-4 weeks; (4) mice in group **LSKL** were i.p.

injected with 10% CCl_4/olive oil three times a week at 1-4 weeks and i.p. injected with **LSKL** (6.5 mM, 100 μL) three times a week at 2-4 weeks; (5) mice in group **NapFFYpLSKL + L-F** were i.p. injected with 10% CCl_4/olive oil three times a week at 1-4 weeks and i.p. injected with a mixture (6.5 mM **NapFFYpLSKL** and 15 mM L-F, 100 μL) three times a week at 2-4 weeks; (5) mice in group **NapFFYpLSKL + KRFK** were i.p. injected with 10% CCl_4/olive oil three times a week at 1-4 weeks and i.p. injected with a mixture **NapFFYpLSKL** (6.5 mM **NapFFYpLSKL** and 6.5 mM KRFK, 100 μL) three times a week at 2-4 weeks. At the end of the experiment, the mice were euthanized and blood and liver organs were collected. The blood was kept at room temperature for 2 h and then centrifuged at 3000 RPM for 10 min to obtain clear serum. The levels of ALT, AST, ALP and TGF-β_1 in serum were determined by standard method. Liver tissues were stained with H&E and Sirius red for further analysis.

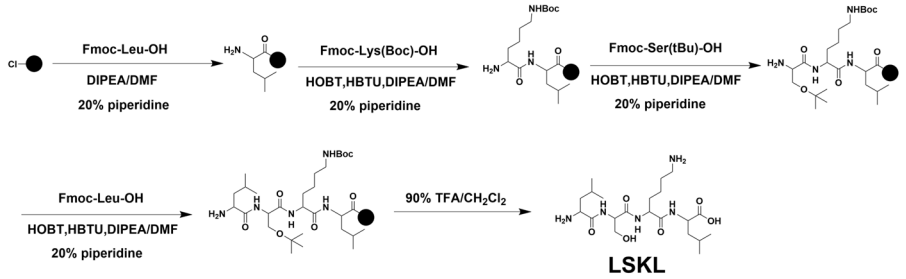
2. Syntheses and Characterizations

Scheme S1. The synthetic route for **NapFFYpLSKL**.



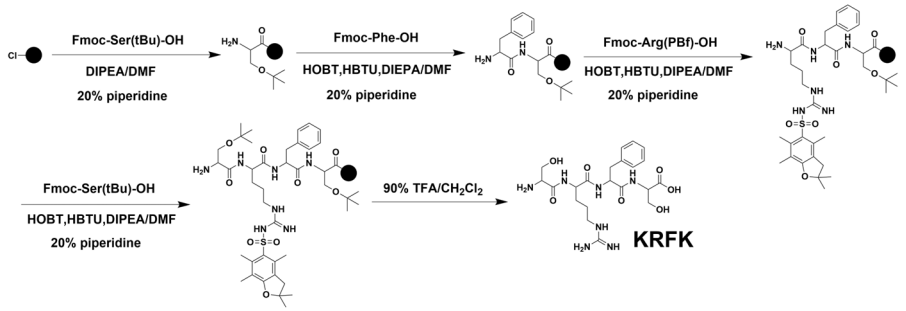
*Synthesis of **NapFFYpLSKL***: Peptide **NapFFYpLSKL** was synthesized by solid phase peptide synthesis (SPPS) using 2-chlorotrityl chloride resin (800 mg, 0.88 mmol) and the corresponding Fmoc-protected amino acids with side chains properly protected. The solution of 20% piperidine in N, N -Dimethylformamide (DMF) was used to remove the Fmoc group. The growth of the peptide chain followed the established Fmoc SPPS protocol. Compound **NapFFYpLSKL** (303 mg, yield 30.5%) was obtained after HPLC purification. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.26 (d, J = 8.6 Hz, 1 H), 8.16 – 8.03 (m, 4 H), 8.02 – 7.82 (m, 3 H), 7.74 (dd, J = 16.1, 8.2 Hz, 2 H), 7.64 (s, 3 H), 7.56 (s, 1 H), 7.50 – 7.42 (m, 2 H), 7.19 – 7.09 (m, 10 H), 7.04 (d, J = 8.1 Hz, 2 H), 6.64 (d, J = 8.0 Hz, 2 H), 4.51 (s, 3 H), 4.42 – 4.14 (m, 4 H), 3.54 (m, 5 H), 3.01 – 2.87 (m, 3 H), 2.70 (m, 5 H), 1.78 – 1.57 (m, 4 H), 1.56 – 1.39 (m, 7 H), 1.37 – 1.19 (m, 3 H), 0.95 – 0.74 (m, 12 H) (Figure S1). ^{13}C NMR (150 MHz, DMSO-d_6) δ (ppm): 179. 10 (1 C), 177.33 (1 C), 176.66 (1 C), 176.38 (1 C), 176.19 (1 C), 176.02 (1 C), 175.10 (2 C), 155.61 (1 C), 143.05 (1 C), 142.84 (2 C), 139.26 (1 C), 138.19 (1 C), 137.00 (2 C), 135.46 (2 C), 134.52 (4 C), 133.27 (2 C), 133.17 (1 C), 132.85 (1 C), 132.71 (3 C), 132.64 (1 C), 132.51 (1 C), 131.25 (2 C), 130.72 (1 C), 124.94 (2 C), 66.98 (1 C), 60.21 (1 C), 58.99 (3 C), 57.38 (1 C), 55.57 (2 C), 47.47 (1 C), 46.14 (1 C), 42.68 (2 C), 36.45 (3 C), 31.88 (1 C), 29.52 (1 C), 28.40 (1 C), 28.12 (2 C), 27.27 (1 C), 26.84 (1 C), 26.58 (2 C) (Figure S2). MS: calc. $[M]^+$ = 1165.5351, obsvd. ESI-MS: m/z = 1165.5358 (Figure S3).

Scheme S2. The synthetic route for **LSKL**.



*Synthesis of **LSKL***: Peptide **LSKL** was synthesized by solid phase peptide synthesis (SPPS) using 2-chlorotrityl chloride resin (800 mg, 0.88 mmol) and the corresponding Fmoc-protected amino acids with side chains properly protected. The solution of 20% piperidine in N, N -Dimethylformamide (DMF) was used to remove the Fmoc group. The growth of the peptide chain followed the established Fmoc SPPS protocol. Compound **LSKL** (535 mg, yield 52.8%) was obtained after HPLC purification. ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 8.68 (s, 1 H), 8.12 – 8.02 (m, 4 H), 7.71 (s, 3 H), 4.44 – 4.38 (m, 1 H), 4.19 (m, 2 H), 3.82 (d, J = 8.1 Hz, 1 H), 3.68 – 3.46 (m, 3 H), 2.70 (q, J = 8.1 Hz, 2 H), 1.73 – 1.42 (m, 9 H), 1.28 (p, J = 8.1 Hz, 2 H), 0.93 – 0.78 (m, 12 H) (Figure S6). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 174.34 (1 C), 172.10 (1 C), 171.91 (1 C), 169.91 (1 C), 62.26 (1 C), 55.53 (1 C), 52.76 (1 C), 51.25 (1 C), 50.79 (1 C), 31.71 (1 C), 27.15 (2 C), 24.75 (1 C), 24.10 (1 C), 24.01 (1 C), 23.32 (1 C), 23.15 (1 C), 22.55 (1 C), 22.39 (1 C), 21.85 (1 C), 21.75 (1 C) (Figure S7). MS: calc. $[M]^+$ = 460.3130, obsvd. ESI-MS: m/z = 460.3135 (Figure S8).

Scheme S3. The synthetic route for **KRFK**.



*Synthesis of **KRFK***: Peptide **KRFK** was synthesized by solid phase peptide synthesis (SPPS) using 2-chlorotrityl chloride resin (800 mg, 0.88 mmol) and the corresponding Fmoc-protected amino acids with side chains properly protected. The solution of 20%

piperidine in N, N -Dimethylformamide (DMF) was used to remove the Fmoc group. The growth of the peptide chain followed the established Fmoc SPPS protocol. Compound **KRFK** (621 mg, yield 62.5%) was obtained after HPLC purification. ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 8.51 (d, J = 7.6 Hz, 1 H), 8.29 (d, J = 7.9 Hz, 1 H), 8.21 – 8.05 (m, 4 H), 7.95 – 7.68 (m, 8 H), 7.54 – 7.14 (m, 6 H), 4.55 (m, 1 H), 4.29 (p, J = 8.3 Hz, 1 H), 4.12 (q, J = 7.4 Hz, 1 H), 3.73 (s, 1 H), 3.03 (d, J = 5.7 Hz, 2 H), 2.77 – 2.65 (m, 6 H), 1.69 (m, 1 H), 1.59 (q, J = 6.8 Hz, 4 H), 1.48 (m, 6 H), 1.31 (t, J = 7.8 Hz, 1 H), 1.26 – 1.19 (m, 2 H) (Figure S10). ^{13}C NMR (150 MHz, DMSO-d_6) δ (ppm): 173.86 (1 C), 171.53 (1 C), 168.87(1 C), 159.49 (1 C), 157.53 (1 C), 138.02 (1 C), 129.74 (2 C), 128.49 (2 C), 127.68 (1 C), 52.88 (1 C), 52.43 (1 C), 52.37 (1 C), 40.93 (1 C), 39.11 (1 C), 38.98 (1 C), 31.03 (2 C), 29.86 (1 C), 27.17 (2 C), 26.98 (1 C), 25.48 (2 C), 22.90 (2 C) (Figure S11). MS: calc. $[M]^+$ = 578.3919, obsvd. ESI-MS: m/z = 578.3917 (Figure S12).

3. Supporting Figures and Tables

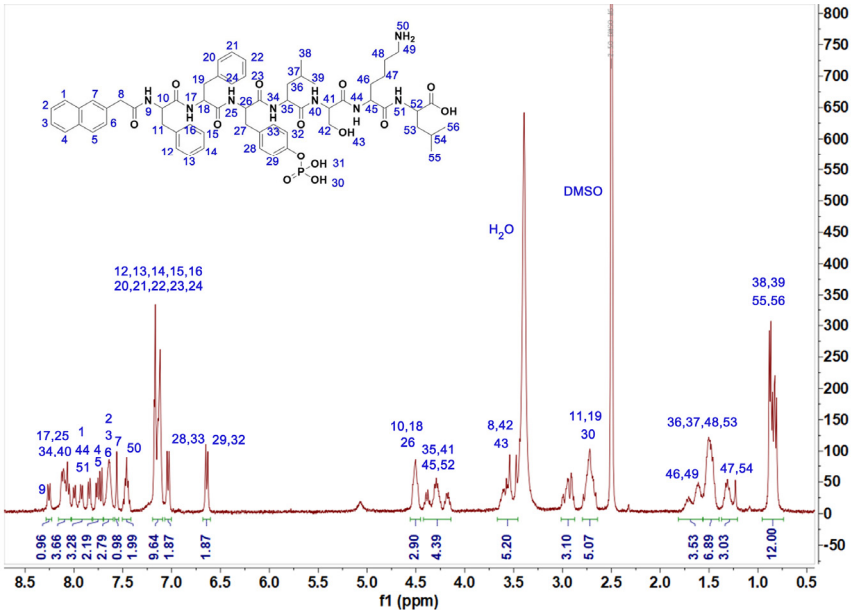


Figure S1. ^{1}H NMR spectrum of **NapFFYpLSKL**.

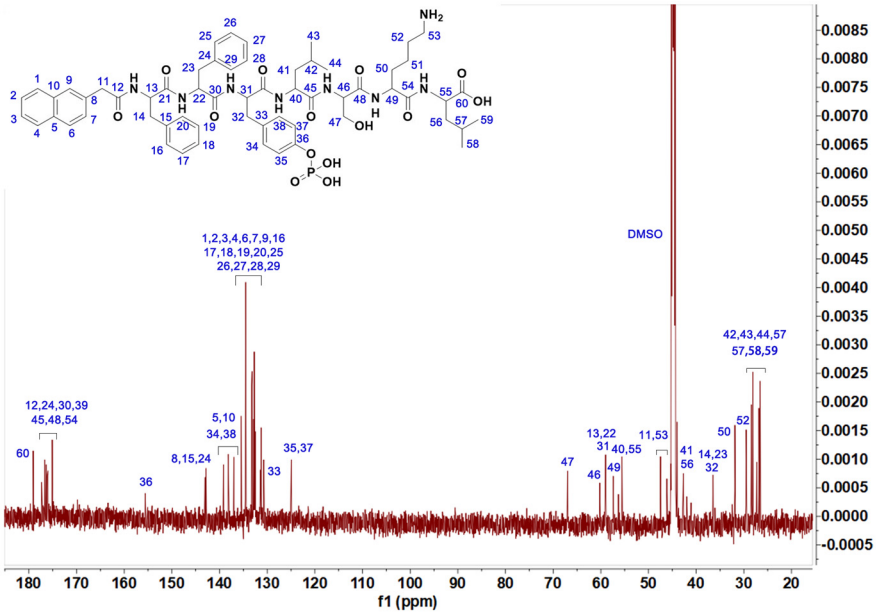


Figure S2. ^{13}C NMR spectrum of **NapFFYpLSKL**.

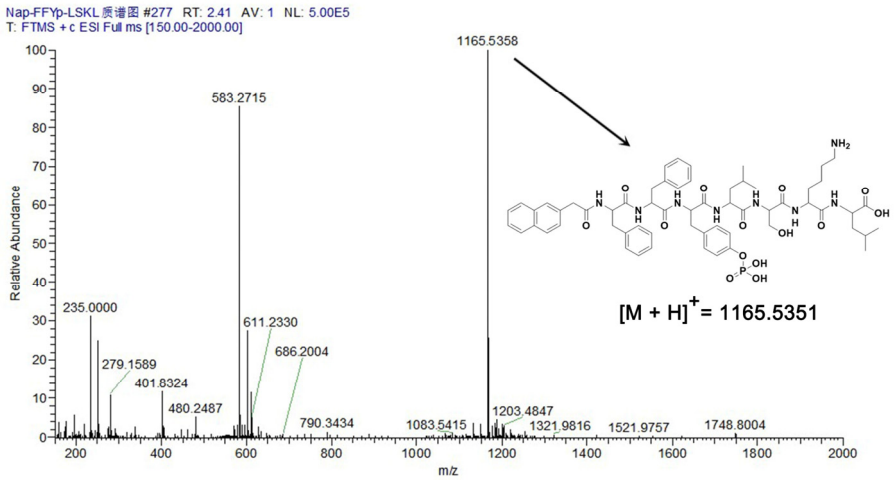


Figure S3. ESI-MS spectrum of **NapFFYpLSKL**.

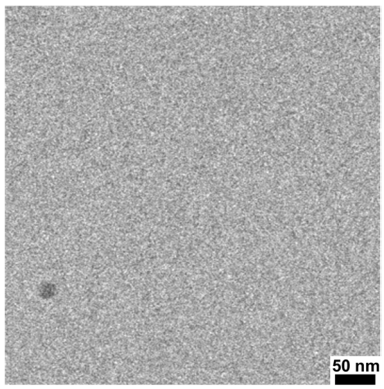


Figure S4. TEM image of 200 μM **NapFFYpLSKL** without addition of ALP.

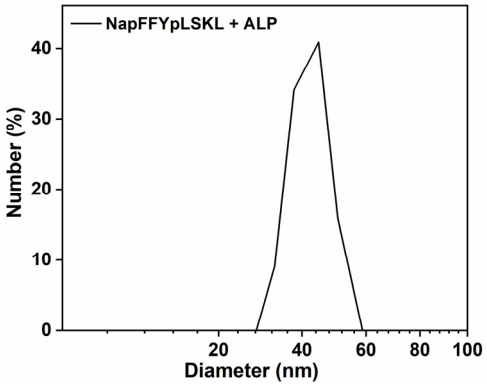


Figure S5. DLS measurement of 200 μM **NapFFYpLSKL** upon incubation with 1 U/μL ALP for 8 h.

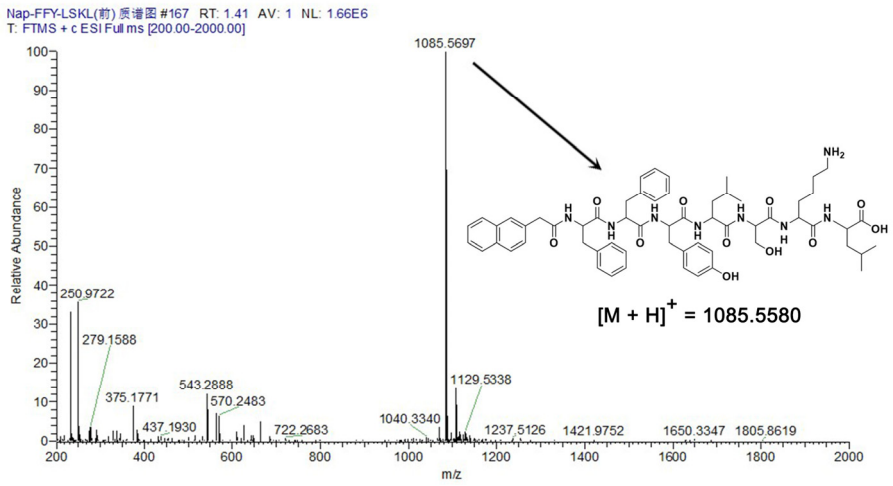


Figure S6. ESI-MS spectrum of **NaFFYLSKL**.

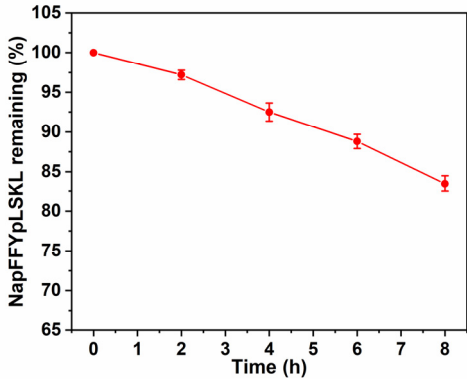


Figure S7. Stability of **NapFFYpLSKL** under incubation with protease K.

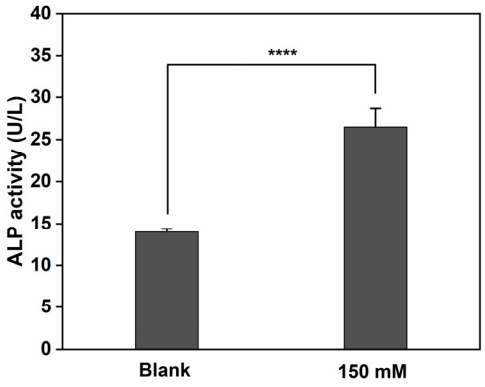


Figure S8. ALP activity in LO2 cells without and with 150 mM alcohol induction.

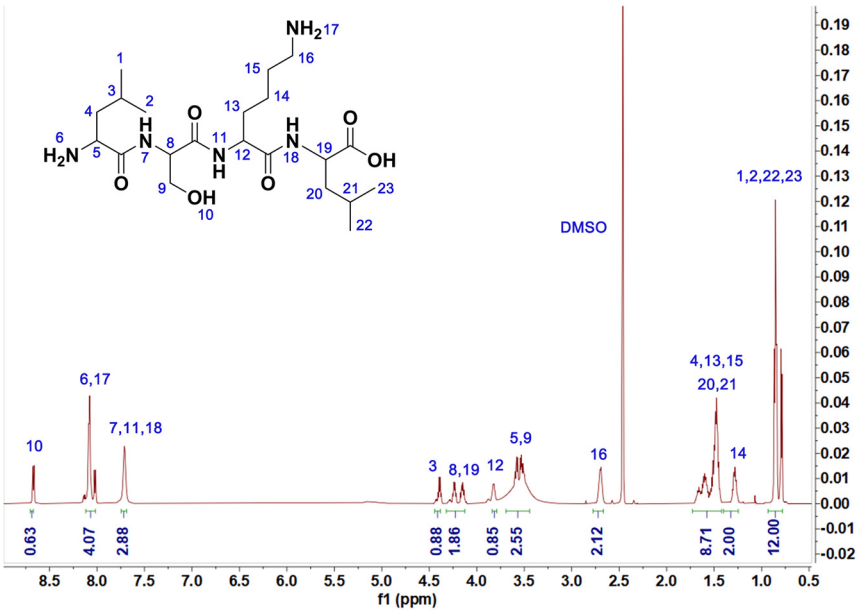


Figure S9. ^{1}H NMR spectrum of **LSKL**.

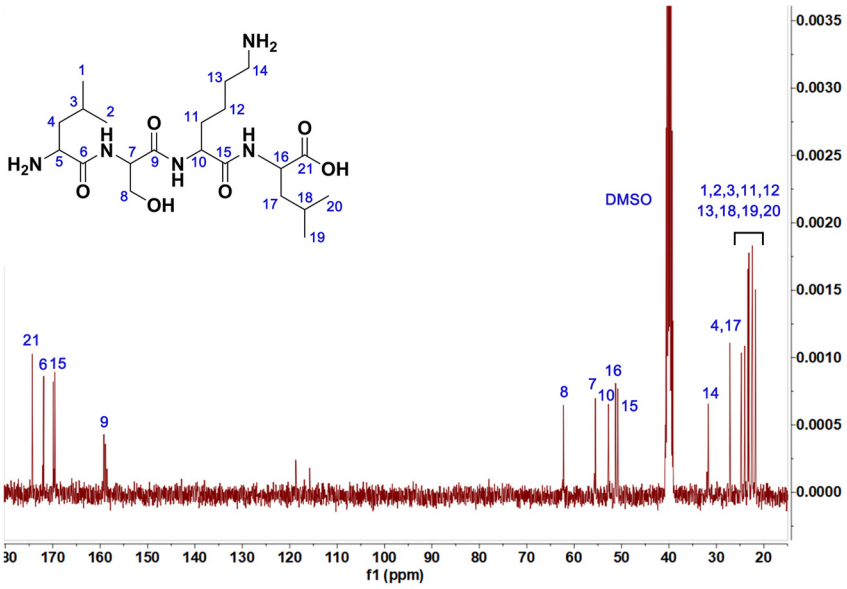


Figure S10. ^{13}C NMR spectrum of **LSKL**.

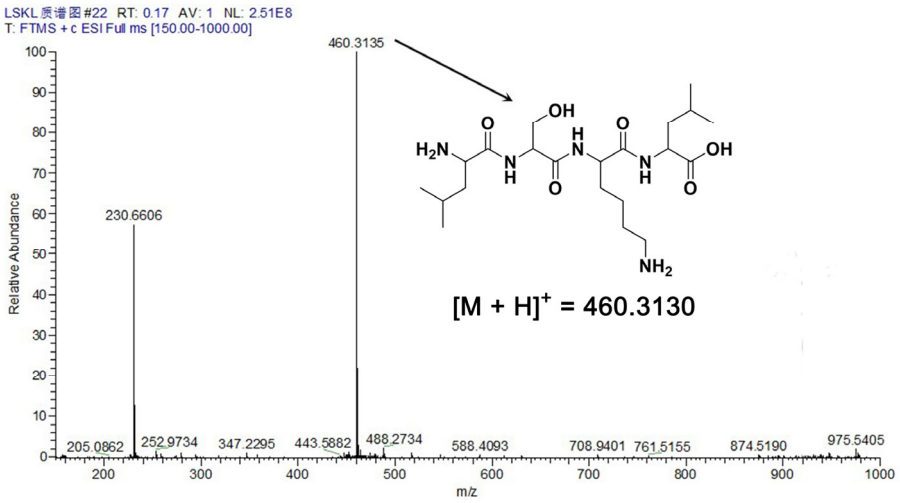


Figure S11. ESI-MS spectrum of **LSKL**.

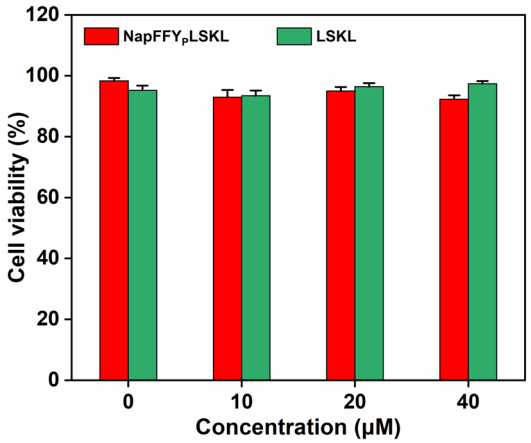


Figure S12. Cell viability of LO2 cells with different concentration **NapFFYpLSKL** and **LSKL** for 24 h.

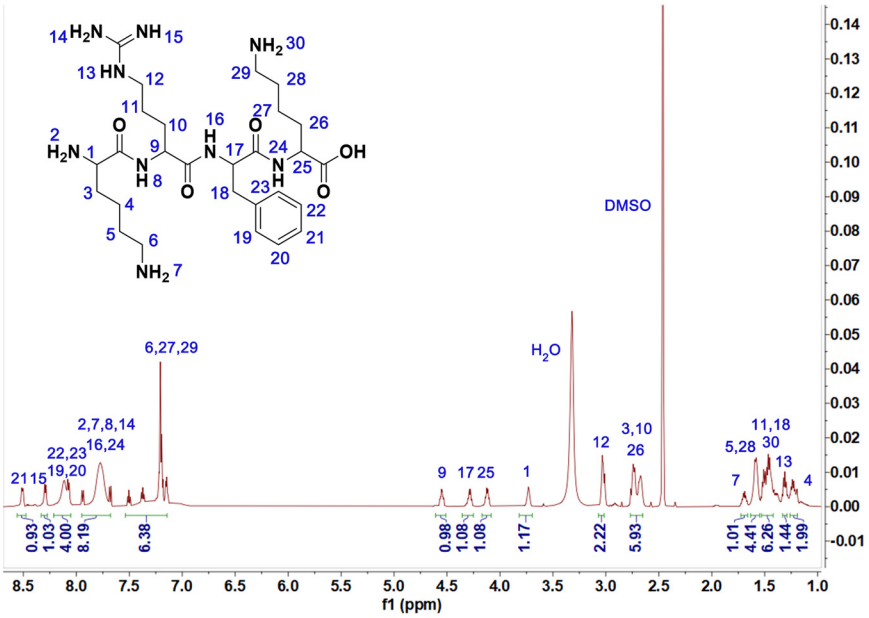


Figure S13. ^{1}H NMR spectrum of **KRFK**.

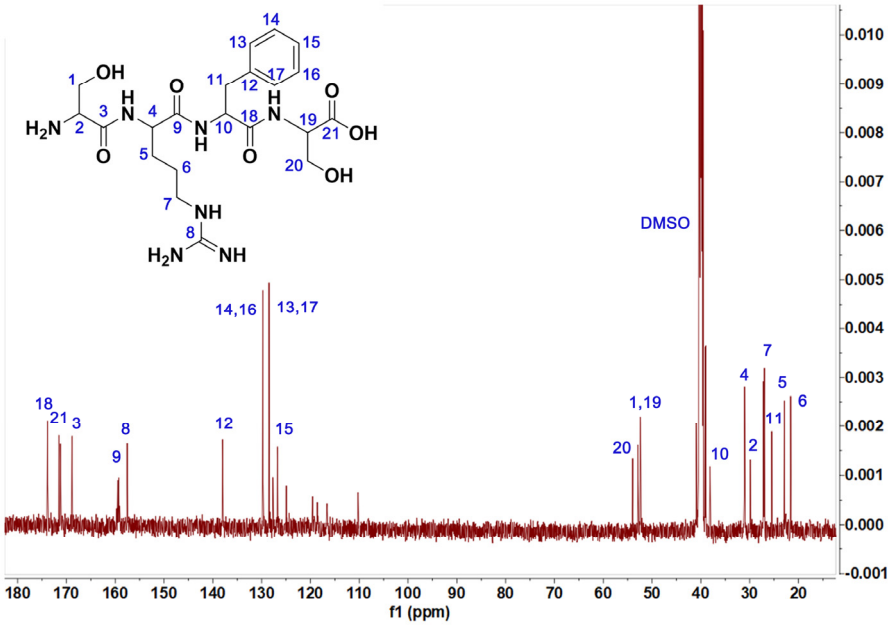


Figure S14. ^{13}C NMR spectrum of **KRFK**.

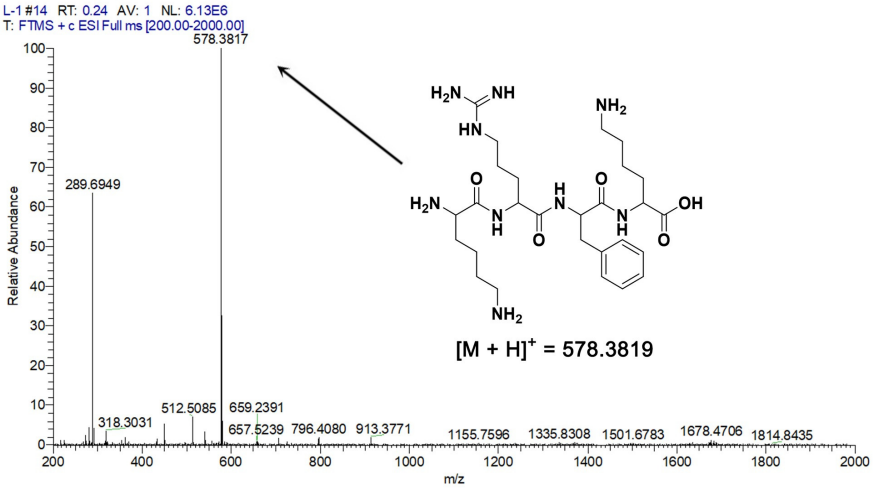


Figure S15. ESI-MS spectrum of **KRFK**.

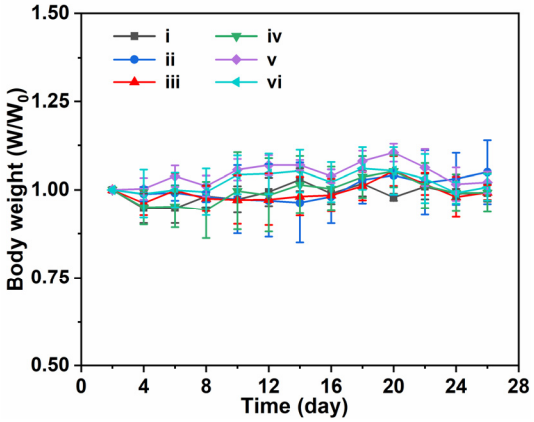


Figure S16. Changes of mice body weight during 4 weeks of treatment. The values are based on the body weight on the second day (W/W_0).

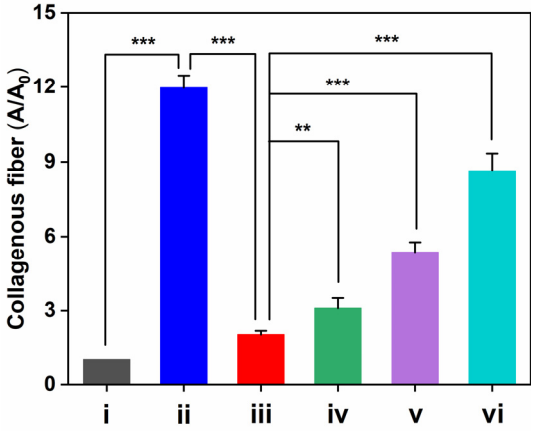


Figure S17. Collagen fiber area in livers of sirius red staining. For the blank group, $A_0 = 1$, the values are based on the blank group (A/A_0).

Table S1. Kinetic parameters for ALP-instructed dephosphorylation of **NapFFYpLSKL**.

	K_m (M)	K_{cat} (s^{-1})	K_{cat}/K_m ($M^{-1}s^{-1}$)
ALP	4.2×10^{-4}	4.08	9.71×10^3

Table S2. HPLC condition of Figure 2c.

Time (min)	Flow (mL/min)	H_2O % (0.1 % TFA)	CH_3CN % (0.1 % TFA)
0	1.0	70	30
3	1.0	70	30
35	1.0	0	100
37	1.0	0	100
39	1.0	70	30
40	1.0	70	30