

## *Supporting Information*

### **Enzyme-responsive double-locked amonafide prodrug for the treatment of glioblastoma with low side effects**

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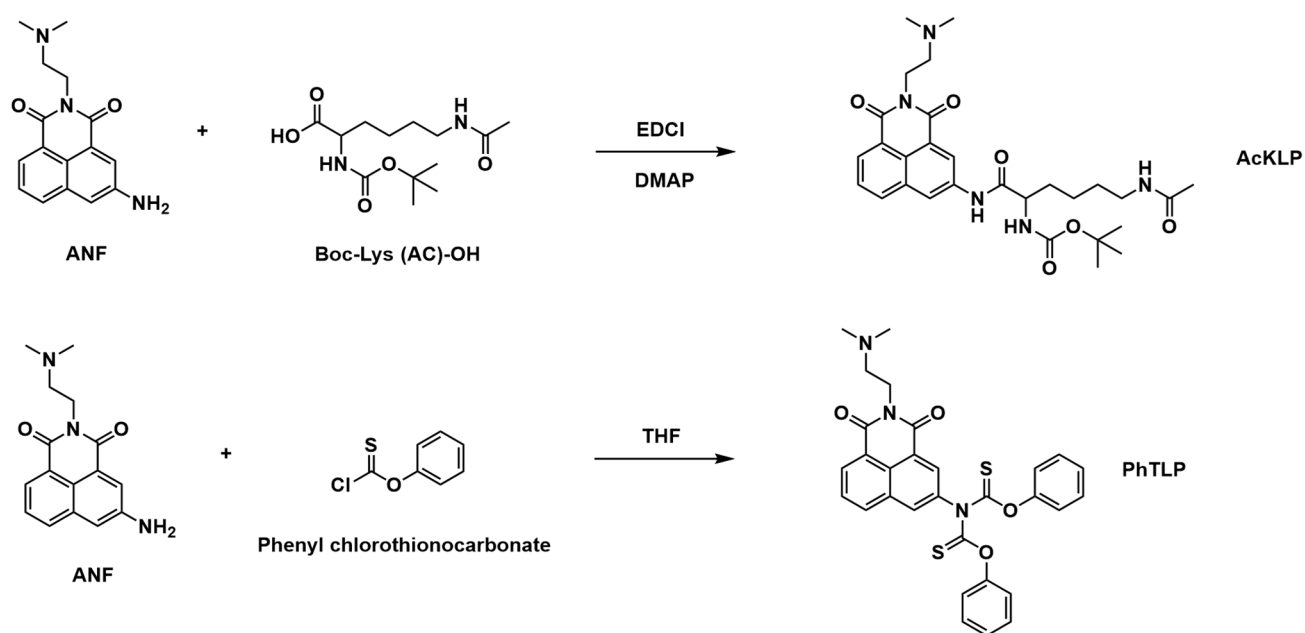
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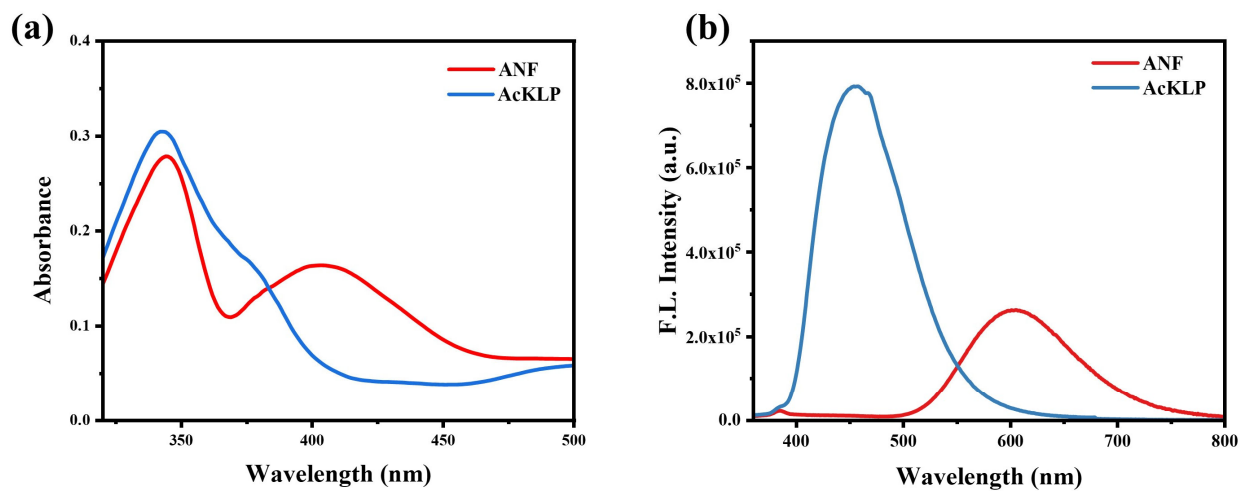
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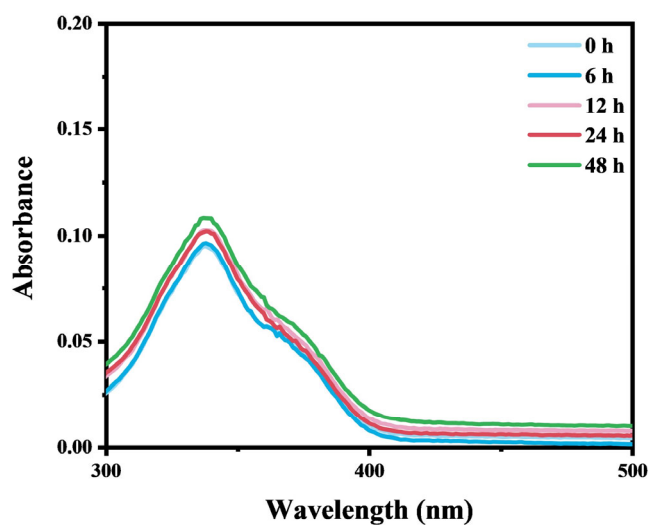
## 1. Supplemental Figures



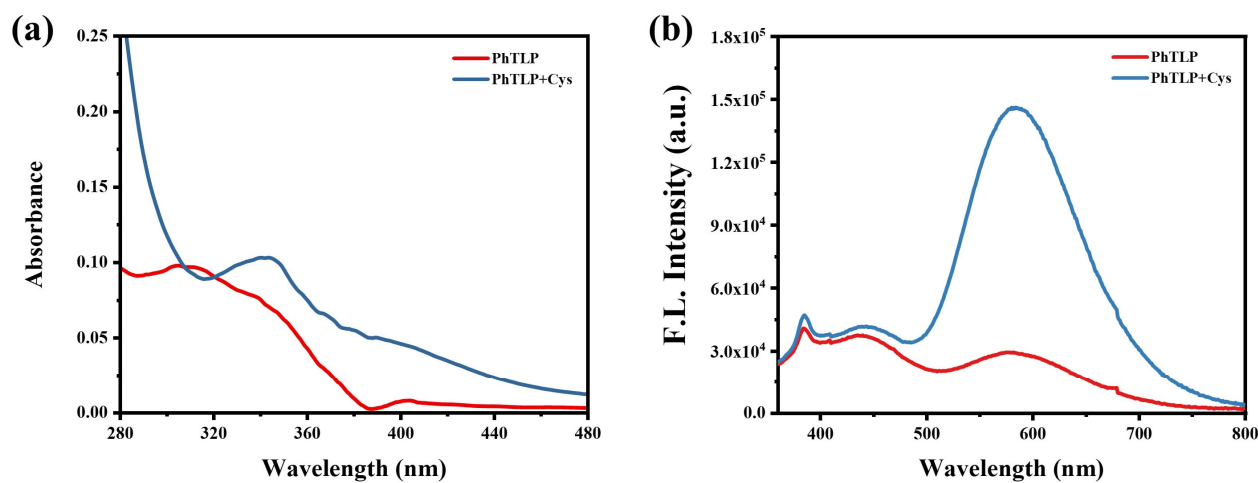
**Scheme S1.** Synthesis routes of **AcKLP** and **PhTLP**.



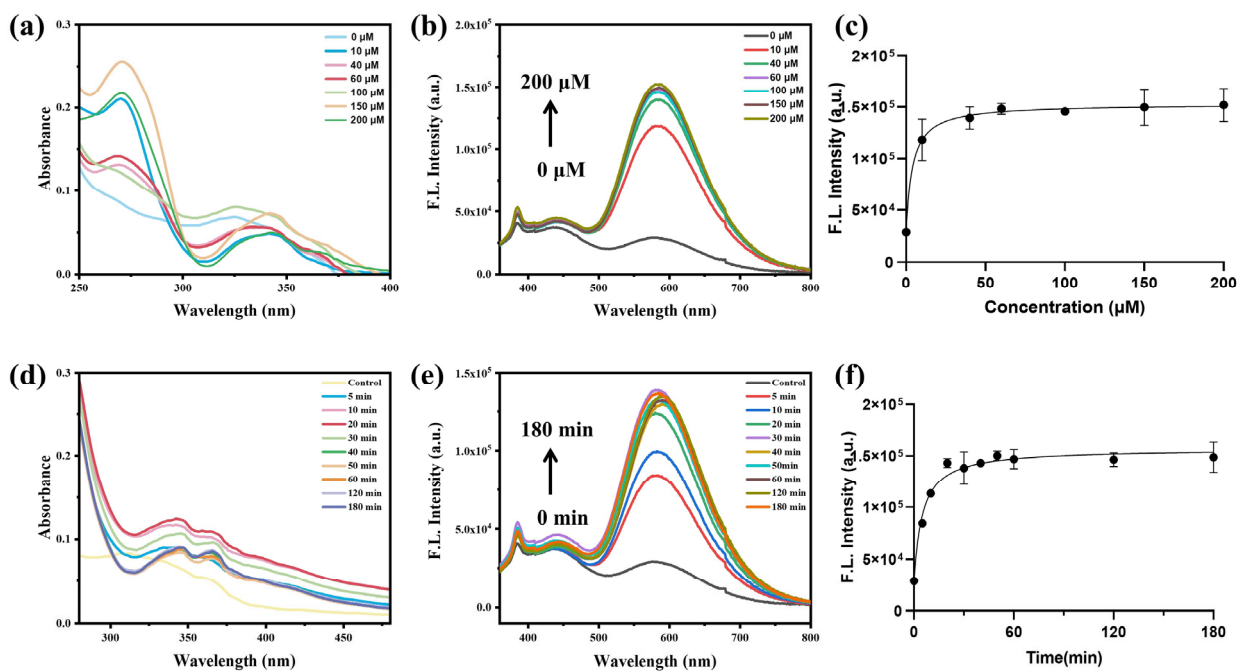
**Fig. S1.** Absorption (a) and fluorescence emission (b) spectra of **AcKLP** and ANF (10  $\mu$ M) in a PBS (10 mM, pH 7.4, 1%DMSO) buffer (AcKLP,  $\lambda_{ex}/\lambda_{em}$  = 340 nm/485 nm).



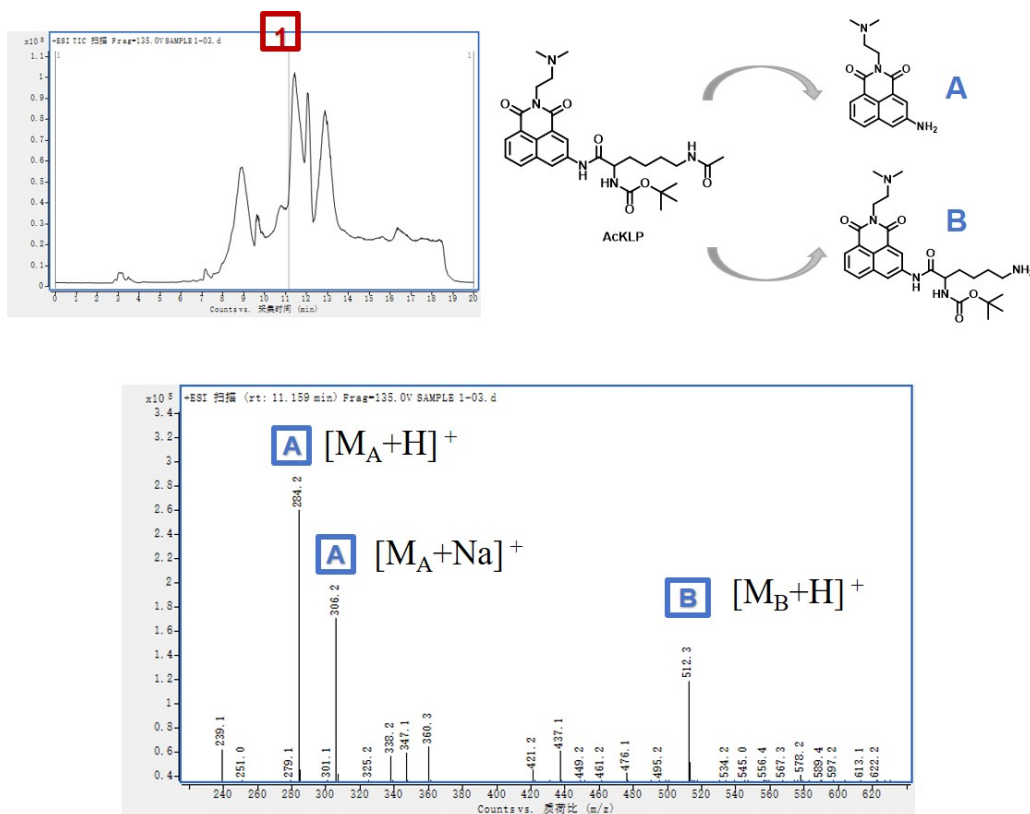
**Fig. S2.** UV-vis spectra of **AcKLP** (10  $\mu$ M) in a PBS (10 mM, pH 7.4, 1% DMSO) buffer recorded for a period of 48 h at 298 K.



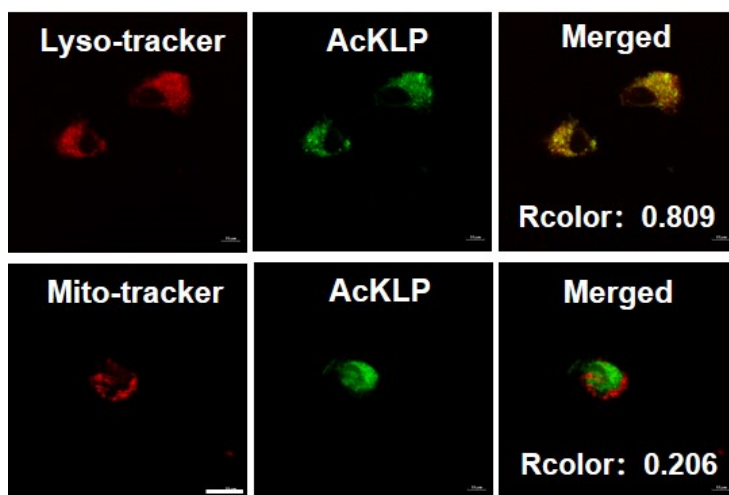
**Fig. S3.** Absorption (a) and fluorescence emission (b) spectra of **PhTLP** (10  $\mu$ M) before and after addition of Cys (100  $\mu$ M) to PBS (10 mM, pH 7.4, 1% DMSO) and 1 mM CTAB buffer (**PhTLP**,  $\lambda_{ex}/\lambda_{em}$  = 340 nm/585 nm).



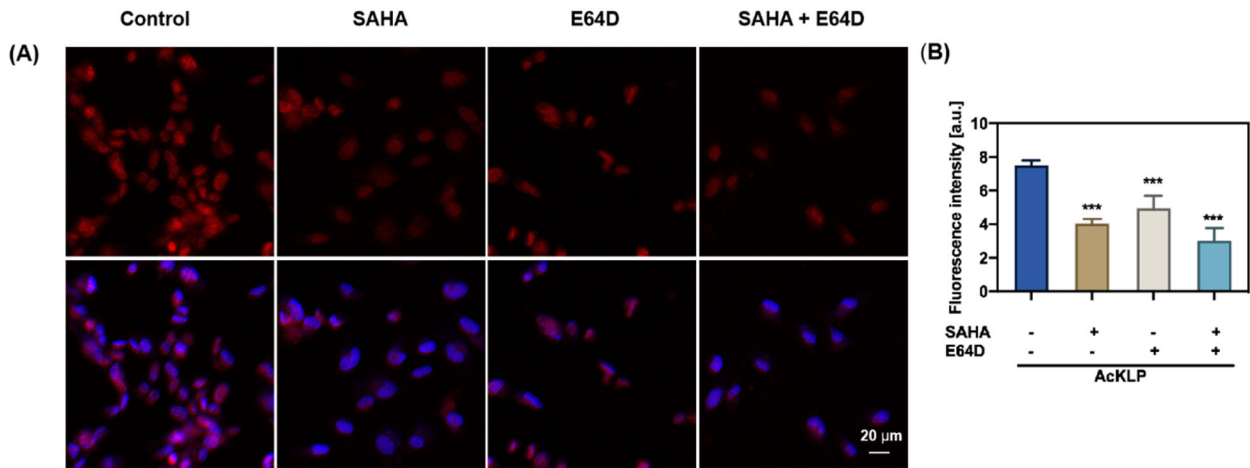
**Fig. S4.** The change of **PhTLP** (10  $\mu\text{M}$ ) absorption (a) and fluorescence intensity (b) with time (0-180 min) in the presence of **Cys** (100  $\mu\text{M}$ ). (c) The time-dependent fluorescence intensity of **PhTLP** (10  $\mu\text{M}$ ) at 585 nm in the presence of **Cys** (100  $\mu\text{M}$ ). Absorption (d) and fluorescence intensity (e) of **PhTLP** (10  $\mu\text{M}$ ) incubated with different concentrations of **Cys** (1-200  $\mu\text{M}$ ).



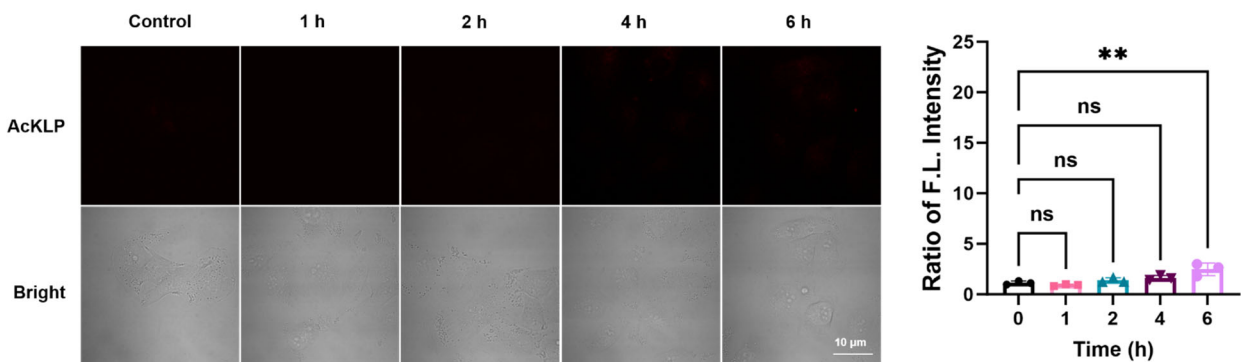
**Fig. S5.** The release behavior of intracellular AcKLP was analyzed by LC-MS. U87 cells were treated with AcKLP (5  $\mu$ M) for 48 h. Cells were then lysed with cell lysis solution and the supernatant was extracted. Followed by the addition of 0.5 mL of methanol to the supernatant to precipitate the protein, the supernatant was taken for LC-MS analysis.



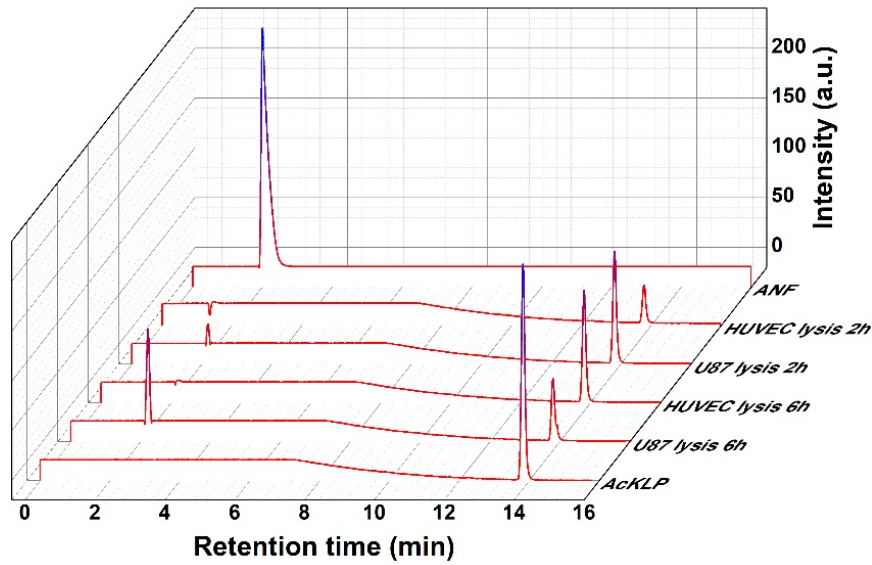
**Fig. S6.** Confocal microscopy images of U87 cells treated with AcKLP for 6 h, respectively. AcKLP (5  $\mu$ M,  $\lambda_{ex}$  = 405 nm,  $\lambda_{em}$  range 550–650 nm); MitoTracker Red (0.3  $\mu$ M,  $\lambda_{ex}$  = 561 nm,  $\lambda_{em}$  range 570–620 nm); LysoTracker Green (2  $\mu$ M,  $\lambda_{ex}$  = 488 nm,  $\lambda_{em}$  range 500–550 nm). Scar bar: 20  $\mu$ m.



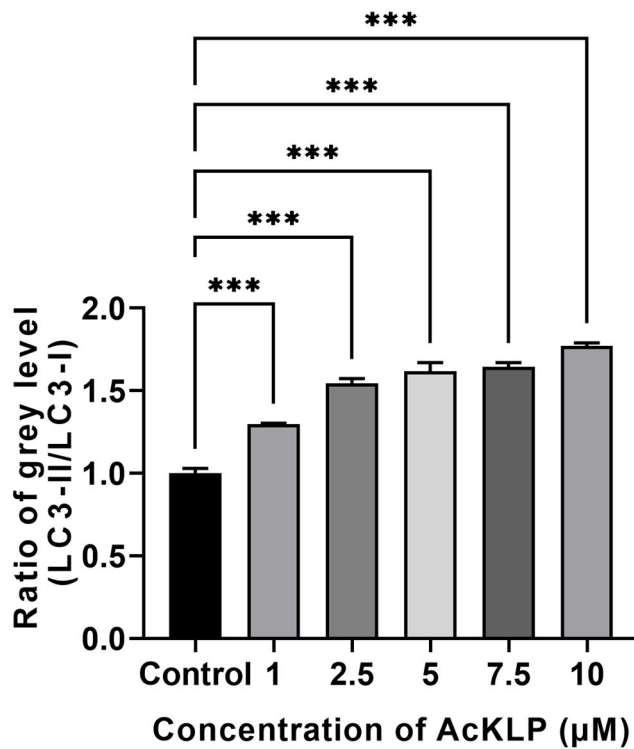
**Fig. S7.** (A) U87 cells were pre-incubated with 50 μM histone deacetylases inhibitor SAHA (2 h) or 50 μM cathepsin L inhibitor E64D (0.5 h), followed by treatment with 5 μM **AcKLP** for 6 h. Cell images were obtained using confocal fluorescence microscopy (scale bar = 20 μm). (B) Quantification of relative fluorescence intensities in (A). Data are presented as mean ± S.D., n = 3. \*\*\* *p*-value < 0.001.



**Fig. S8.** Confocal microscopic images of HUVEC cells treated with 5 μM **AcKLP** for different times ( $\lambda_{ex} = 405$  nm,  $\lambda_{em} = 550-650$  nm). All the data represent the average of three independent experiments with an error bar of ± standard deviation (SD).

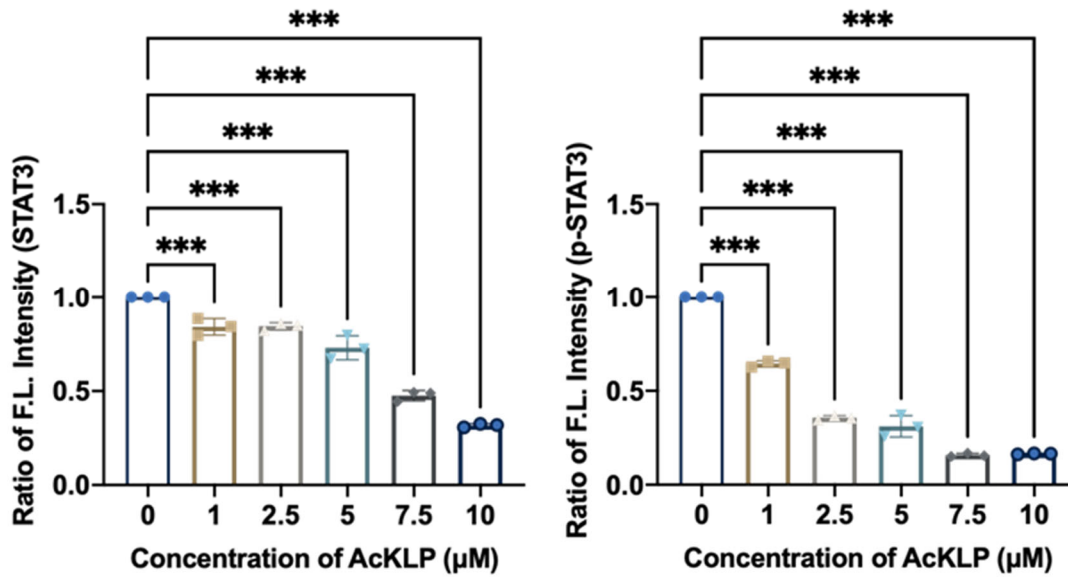


**Fig. S9.** U87 or HUVEC cells, incubated with 10  $\mu\text{M}$  AcKLP for 2 and 6 h, were lysed, and the supernatant was removed for analysis by HPLC (detection at 340 nm).

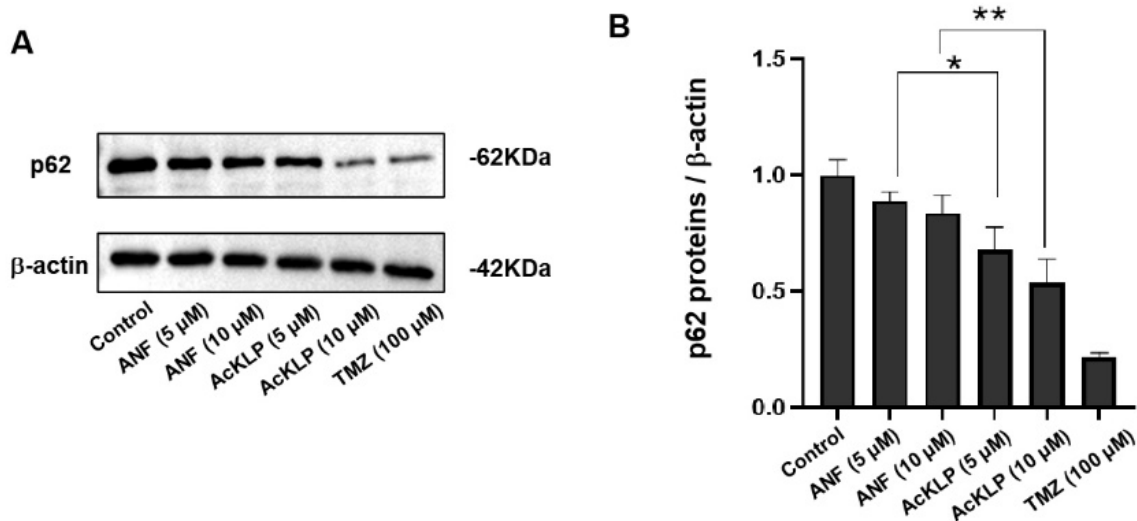


**Fig. S10.** Relative grey intensity of LC-3 is based on the result of Fig 3C. Error bars are  $\pm$  S.D. (n = 3).

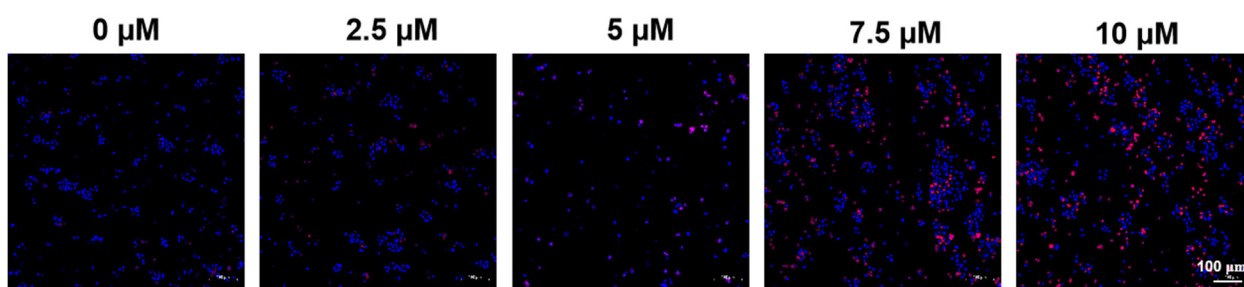




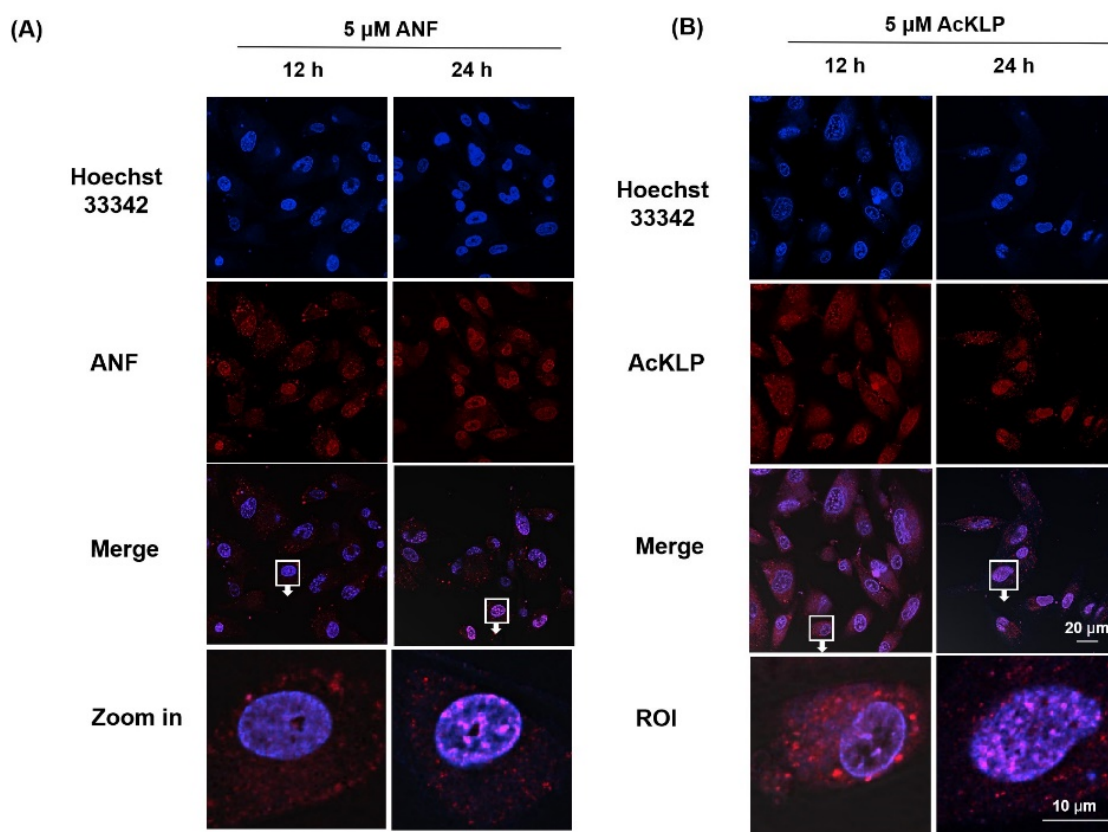
**Fig. S11.** The relative ratio of the grey levels of STAT3 and p-STAT3 is based on the result of Fig 3D. The grey level of 0  $\mu\text{M}$  AcKLP-treated cells was set as 1, respectively. Statistical analyses were performed using two-way ANOVA with multiple comparisons, \*\*\* $P < 0.001$  vs. 0  $\mu\text{M}$  AcKLP group. Error bars are  $\pm$  S.D. ( $n = 3$ ).



**Fig. S12.** (A) Effects of ANF and AcKLP on the protein expression of p62 in U87 cells after 24 h treatment. (B) The relative grey intensity of p62 is based on the result of (A). The fluorescence intensity of cells treated with 0  $\mu\text{M}$  AcKLP was set as 1. Data are presented as mean  $\pm$  S.D.,  $n = 3$ . \*\* $p$ -value  $< 0.01$ , \*\*\* $p$ -value  $< 0.001$ . Error bars are  $\pm$  S.D. ( $n = 3$ ).



**Fig. S13.** U87 cells stained by Hoechst 33342 and PI after being treated with different concentrations of **AcKLP** for 24 h. The blue color (Hoechst 33342,  $\lambda_{\text{ex}} = 405 \text{ nm}$ ,  $\lambda_{\text{em}} = 420\text{--}500 \text{ nm}$ ) indicates healthy cells, and the red color (propidium iodide,  $\lambda_{\text{ex}} = 561 \text{ nm}$ ,  $\lambda_{\text{em}} = 600\text{--}640 \text{ nm}$ ) indicates dead cells. Scale bar, 100  $\mu\text{m}$ .



**Fig. S14.** U87 cells were treated with ANF and **AcKLP** (5  $\mu\text{M}$ ) for 12 and 24 h. The cells were imaged and analyzed after incubation with Hoechst 33342 and Lyso-Tracker Red DND-99. ANF,  $\lambda_{\text{ex}}/\lambda_{\text{em}} = 405/550\text{--}650\text{nm}$ ; Hoechst 33342,  $\lambda_{\text{ex}}/\lambda_{\text{em}} = 405/425\text{--}475 \text{ nm}$ . Lyso-Tracker Red DND-99,  $\lambda_{\text{ex}}/\lambda_{\text{em}} = 577/590 \text{ nm}$ . Scale bar = 25  $\mu\text{m}$ .

## 2. NMR and MS Spectra

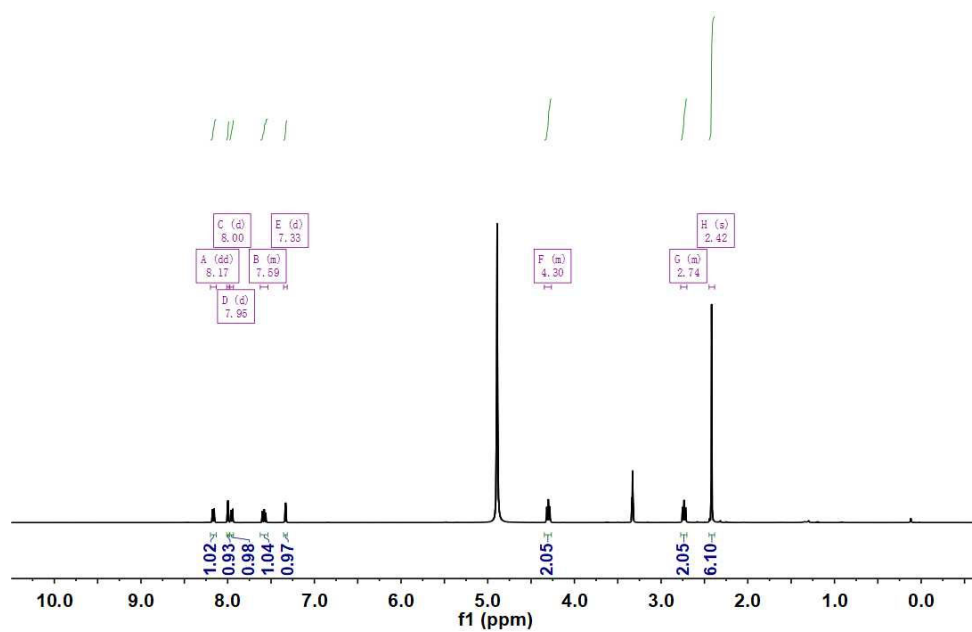


Fig. S15.  $^1\text{H}$  NMR of compound ANF.

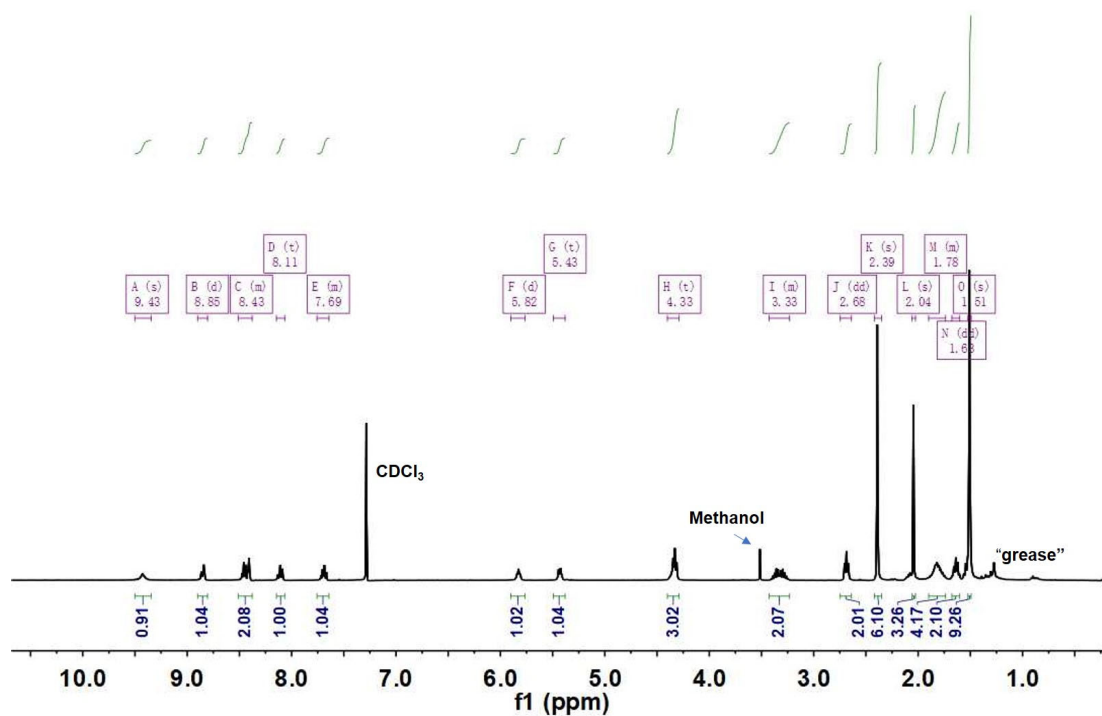
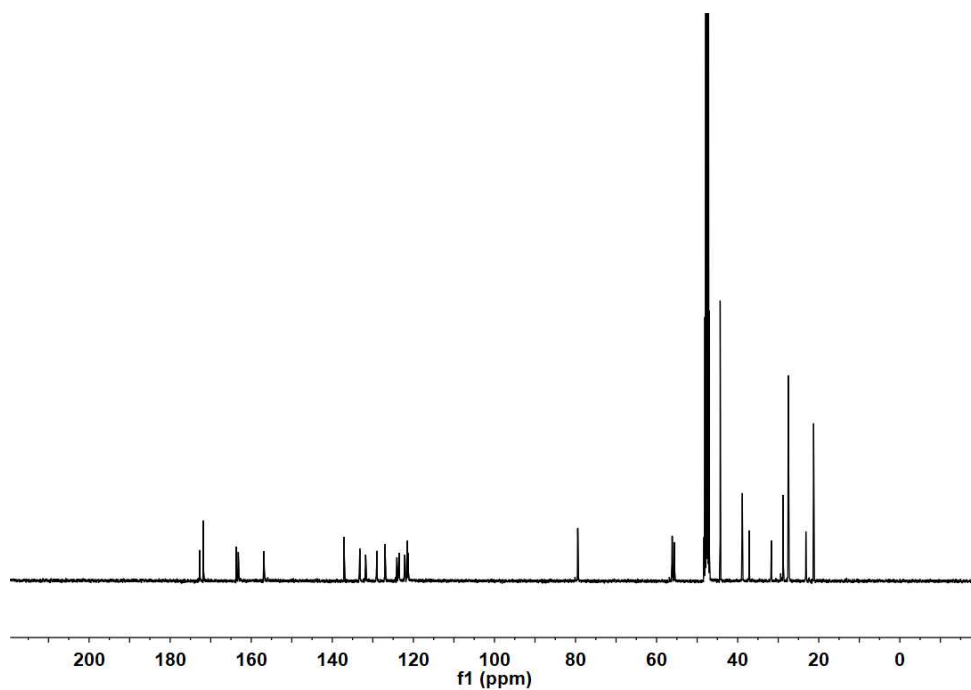
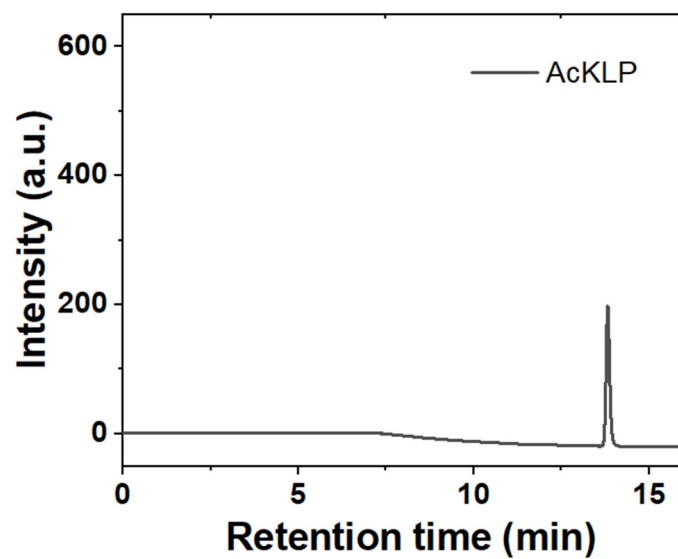


Fig. S16.  $^1\text{H}$  NMR of compound AcKLP.



**Fig. S17.** <sup>13</sup>C NMR of compound AcKLP.



**Fig. S18.** HPLC analyzes the purity of AcKLP.

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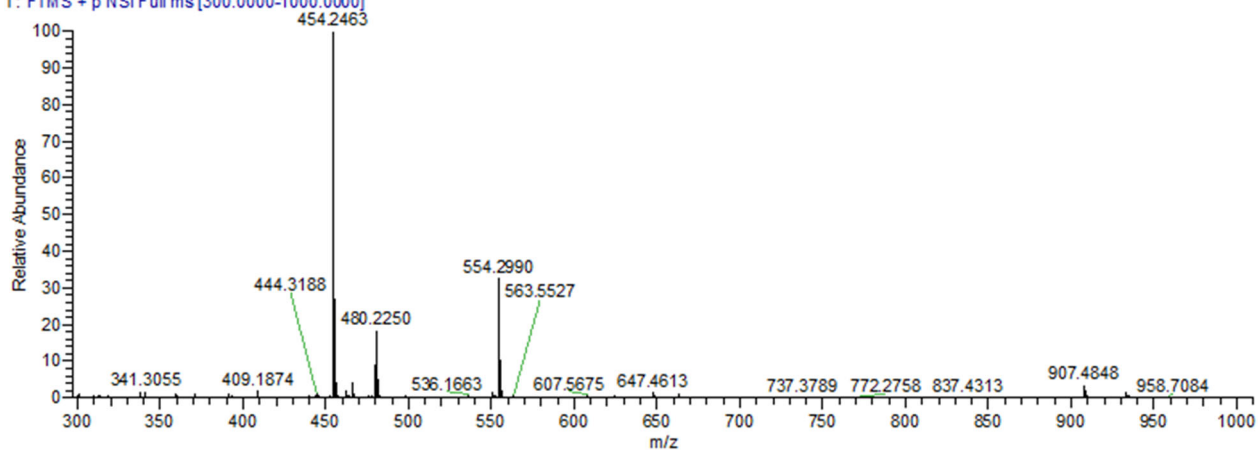


Fig. S19. MS spectrum of compound AcKLP.

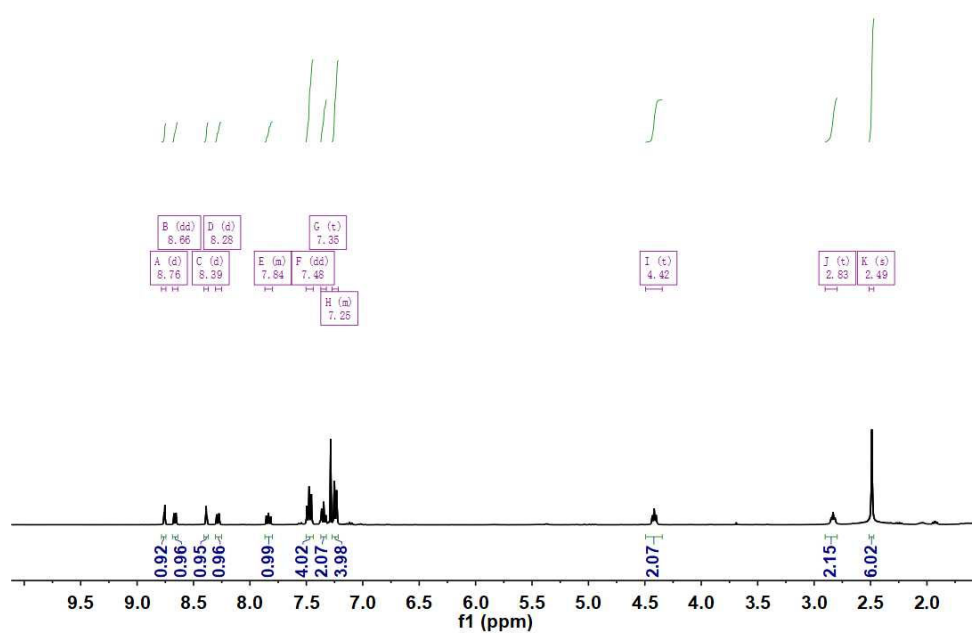
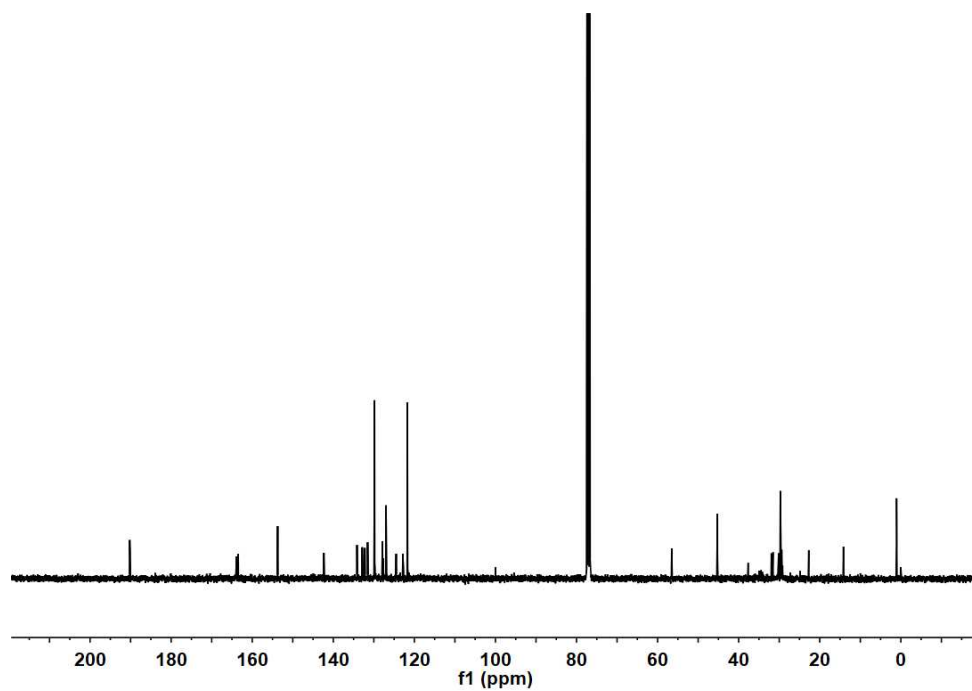
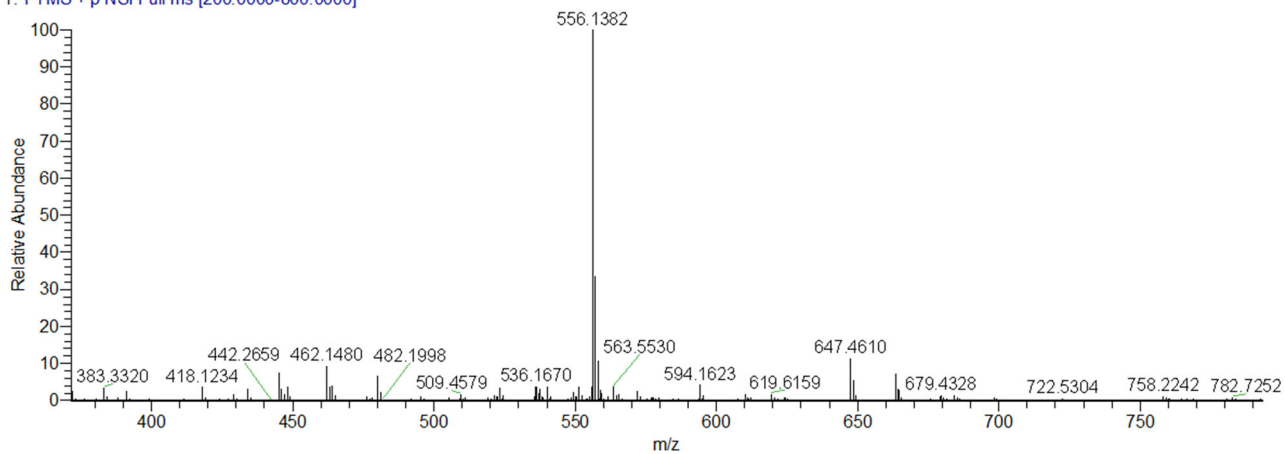


Fig. S20. <sup>1</sup>H NMR of compound PhTLP.



**Fig. S21.**  $^{13}\text{C}$  NMR of compound **PhTLP**.

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**Fig. S22.** HRMS spectrum of **PhTLP**.