Supporting information

Aminopeptidase N Activatable Fluorescent Probe for Tracking Metastatic Cancer and Image-Guided Surgery via *in Situ* Spraying

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Contents

Experimental section

Recombinant human aminopeptidase (APN) was purchased from R&D Systems China. Nitroreductase, transglutaminase, γ-Glutamyltranspeptidase and Ubenimex were purchased from Sigma-Aldrich. Others reagents used were obtained from commercial suppliers and were used without further purification unless otherwise stated. Twice-distilled purified water used in all experiments was from Milli-Q systems (18 MΩ·cm). All pH measurements were performed using an Ohaus Starter 2100 pH meter. The fluorescence quantum yields for compounds with Absolute PL Quantum Yield Spectrometer (HAMAMATSU C11347). Instruments used in cell imaging tests were carried out on FV1000-IX81 confocal microscopy (Olympus, Japan). Slight pH variations in the solutions were achieved by adding the minimum volumes of HCl or NaOH (1 M). Tumor tissue slices were prepared from freezing microtome (LEICA CM1860 UV). All the interferential reagents were prepared based on published literatures.[1-3]

Determination of the detection limit

The detection limit (DL) was calculation based on the fluorescence titration of YH-APN (10 μM) in the presence of APN (1-9 ng/mL). The fluorescence intensity of YH-APN was measured and standard deviation of the blank measurement was achieved. The detection limit was calculated by the following equation:

$$\text{Detection limit} = 3\sigma/k$$

Where σ is the standard deviation of the blank measurement, k is the slope between the fluorescence intensity (F650 nm) versus various APN concentrations.

Cell incubation

Hepatoma carcinoma cells (HepG-2 cells), mouse melanoma (B16/BL6 cells), 4T1 mammary gland carcinoma cells (4T1 cells), normal liver cells (LO2 cells) and African green monkey kidney fibroblasts (COS-7 cells) were purchased from Institute of Basic Medical Sciences (IBMS) of the Chinese Academy of Medical Sciences. HepG-2 and COS-7 cells treated with Dulbecco's modified Eagle's medium (DMEM, Invitrogen), B16/BL6, 4T1 and LO2 cells were cultured in RPMI medium 1640 supplemented with 10 % fetal bovine serum (Invitrogen). The cells were seeded in confocal culture dishes and then incubated for 24 h at 37 °C under a humidified atmosphere containing 5% CO_2.

MTT assays

Measurement of cell viability was tested by reducing of MTT (3-(4, 5)-dimethylthiahiazo (-2-yl)-3, 5-diphenytetrazoliumromide) to formazan crystals using mitochondrial dehydrogenases. Cells were seeded in 96-well microplates (Nunc, Denmark) at a density of 1 × 10^5 cells/mL in 100 μL medium containing 10 % FBS. After 24 h of cell attachment, the plates were then washed with 100 μL / well PBS. The cells were then cultured in medium with 0, 2, 5, 10 and 15 μM of YH-APN for 24 h. Cells in culture medium without YH-APN were used as the control. Six replicate wells were used for each control and test concentration. 100 μL of MTT (0.5 mg/mL) prepared in DMEM solution was added to each well and the plates were incubated at 37 °C for another 4 h in a 5% CO_2 humidified incubator. The medium was then carefully removed, and the purple crystals were lysed in 200 μL DMSO. Optical density of solutions was determined on a microplate reader (Thermo Fisher Scientific) at 490 nm. Cell viability was expressed as a percent of the control culture value, and it was calculated using the following equation:

$$\text{Cells viability (\%)} = (\text{OD dye} - \text{OD blank}) / (\text{OD control} - \text{OD blank}) \times 100$$

Imaging endogenous APN activity of living cells

HepG-2, LO2 and B16/BL6 cells were seeded in glass-bottom culture dishes at approximately concentration of 2 × 10^4 cells/mL and allowed to culture for 24 h at 37 °C in a 5% CO_2 humidified incubator. For the detection of endogenous APN enzyme, HepG-2, LO2 and B16/BL6 cells were treated with probe YH-APN (5 μM) 37 °C, followed by washing thrice with free DMEM for three times. Under the confocal fluorescence microscope (Olympus FV1000-IX81) with a 60 × objective lens, probe YH-APN was excited at 488 nm, next, fluorescence emission at 655-755 nm channel was gathered.

Mixed cell cultivation

The detailed operation method is referred to the published literature.[3]

Cells motility assays

Cellualr morphology of living HepG-2 was immediately collected after wounding with 419 ± 20, 427 ± 18 μm, respectively. After 12 and 24 hours of cultivation at 37 °C, horizontal migration of cell pretreated with or without 100 μM Ube was measured by Olympus viewer. Quantitative distance analysis of migration determined from 11 regions of interest (ROIs).

Visualization of APN activity in tissue

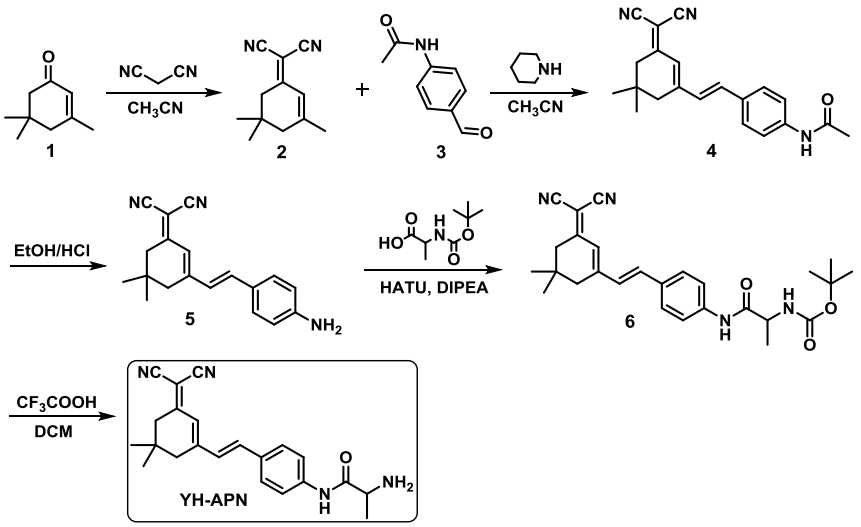
Tumor tissue slices of 20 μm were prepared from hepatoma tumor using freezing microtome (LEICA CM1860 UV). As a comparison, Normal slices were chose from normal liver tissue. Next, these tissues were incubated with YH-APN at 37 °C for 30 min, followed by washing thrice with phosphate buffer saline (0.01 M, pH 7.4). Under the confocal fluorescence microscope (Olympus FV1000-IX81) with a 60 × objective lens, probe YH-APN was excited at 488 nm, next, fluorescence emission at 655-755 nm of red channel was gathered. In tissue depth imaging, the 3D images were constructed via z-scan mode under two-photon platform.

Visualization of APN activity in mice xenograft tumor model

All the animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Dalian Medical University and experiments were approved by Dalian Medical University Animal Care and Use Committee. For establishing a mouse tumor model, the HepG-2 cells (1×10^6 cells) were chose to transplant under the armpit of approximately 15-20 g female nude BABL/c mice. After 15 days inoculation, the xenograft tumor mice were given with YH-APN through tumor injection within the period of mice anesthesia. After that, the imaging of mice was carried out on a NightOWL II LB983 small animal in vivo imaging system with a 475 nm (fwhm 20 nm) excitation and a 665 nm (fwhm 20 nm) emission filter.

Tumor model with metastasis

4T1 cells (0.3×10^6) were subcutaneously into the mammary gland of BALB/c mice.[4] Mice were euthanized after about 5 weeks, and then spleen and liver samples were discreetly taken out from body.



Scheme S1. Synthetic procedures of YH-APN

Synthesis of YH-APN

Synthesis of compound 2

The compound 2 was synthesized according to the previous the previous literature.[5]

Synthesis of compound 4

A solution of compound 2 (500 mg, 2.69 mM), compound 3 (456 mg, 2.80 mM) and 1-3 drop piperidine in 5 mL dry toluene was refluxed with stirring for 3 h under N_2 protection. The solid which separated upon cooling to room temperature was washed by dry CH_3CN (3 × 5 mL) to obtain 507.5 mg light-red compound 4 (Yield 57%) without further purification. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.12 (s, 1H), 7.64 (s, 4H), 7.26 (q, J = 16.1 Hz, 2H), 6.83 (s, 1H), 2.59 (s, 2H), 2.52 (s, 2H), 2.07 (s, 3H), 1.01 (s, 6H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 170.68, 168.99, 156.63, 141.17, 137.92, 131.11, 129.15, 128.36, 122.61, 119.40, 114.45, 113.64, 76.10, 42.76, 38.62, 32.12, 27.90, 24.59. ESI-MS: m/z calcd for $C_{21}H_{22}N_3O^+$ $[M+H]^+$ 332.18, found 332.17.

Synthesis of compound 5

Compound 4 (66.2 mg, 0.2 mM) was dissolved in 12 mL the mix solution of EtOH/HCl (v/v 1:2) under N_2 protection. This mixture was stirred at 105 °C for 8 h and cooled down to room temperature. After neutralization with 5% $NaHCO_3$, the solution was extracted by EtOAc and washed with brine, dried with anhydrous Na_2SO_4. After removal of the solvent under reduced pressure by evaporation, the crude product was purified by silica column

chromatography using hexane/EtOAc (v/v 5:1) as eluent, obtaining 24.9 mg red solid compound 5 (Yield 43%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.42 (d, J = 8.4 Hz, 2H), 7.19 (d, J = 15.9 Hz, 1H), 7.06 (d, J = 15.9 Hz, 1H), 6.72 (s, 1H), 6.57 (d, J = 8.4 Hz, 2H), 5.87 (s, 2H), 2.58 (s, 2H), 2.51 (s, 2H), 1.01 (s, 6H). ESI-HRMS: m/z calcd for $C_{19}H_{20}N_3^+$ $[M+H]^+$ 290.1652, found 290.1649.

Synthesis of compound 6

A solution of compound 5 (58.9 mg, 0.10 mM), N-(tert-Butoxycarbonyl)-L-alanine (28.4 mg, 0.15 mM), HATU (114 mg, 0.30 mM) and DIPEA (38.8 mg, 0.30 mM) in 5 mL dry DMF was stirred for 8 h under room temperature. After adding 15 mL EtOAc into the solution, it was washed by water for three times. The combined organic phase was dried with Na_2SO_4, and crude product 6 was obtained without further purification to the next step. ESI-MS: m/z calcd for $C_{27}H_{32}N_4NaO_3^+$ $[M+Na]^+$ 483.24, found 483.31.

Synthesis of YH-APN

Compound 6 prepared above was dissolved in 2 mL dry DCM and stirred at 0 °C for 30 min. Next, 2 mL CH_2Cl_2-TFA (v/v 1:1) was added into above mixture *via* drop by drop style. When add was completed, the mixture system continued to stir overnight under room temperature. After that, the crude product was purified through silica gel column chromatography to obtain 11.5 mg red YH-APN (Total yield 32%). ^{1}H NMR (400 MHz, MeOD) δ 7.63 (q, J = 8.9 Hz, 4H), 7.21 (d, J = 16.2 Hz, 1H), 7.12 (d, J = 16.1 Hz, 1H), 6.85 (s, 1H), 4.12 – 3.96 (m, 1H), 2.63 (s, 2H), 2.56 (s, 2H), 1.59 (d, J = 7.1 Hz, 3H), 1.08 (s, 6H). ^{13}C NMR (100 MHz, MeOD) δ 169.80, 167.84, 155.13, 139.00, 136.50, 132.47, 128.32, 128.24, 122.62, 119.75, 112.43, 77.21, 49.55, 42.54, 38.46, 31.50, 26.67, 16.15. ESI-HRMS: m/z calcd for $C_{22}H_{25}N_4O^+$ $[M+H]^+$ 361.2023, found 361.2025.

Results and discussion

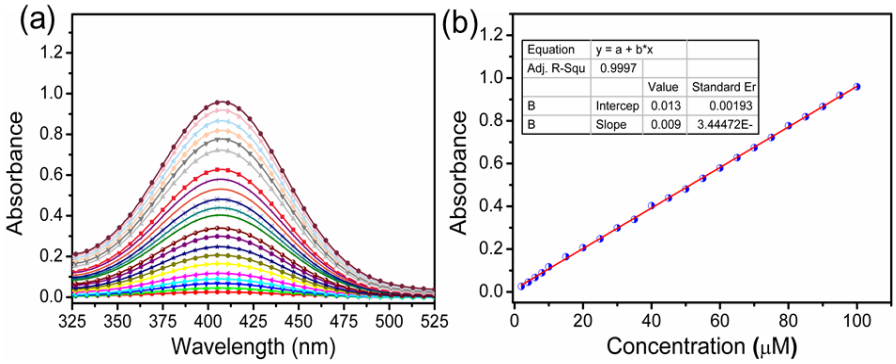


Figure S1. The solubility of YH-APN in PBS buffer solution (0.01 M, pH=7.4).

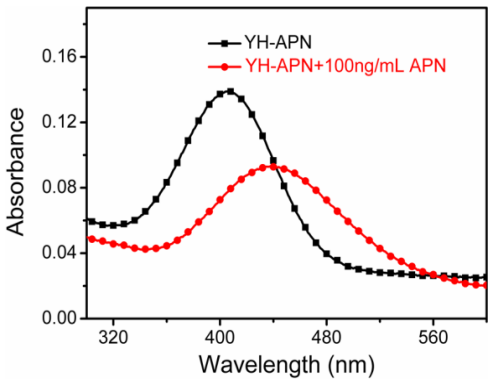


Figure S2. UV-Vis changes of YH-APN upon addition APN in PBS buffer solution (0.01 M, pH = 7.4).

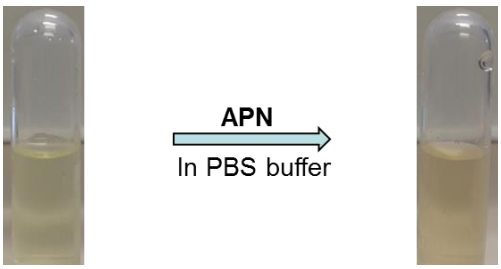


Figure S3. The color change of the solution of YH-APN (10 μM) in presence of (150 ng/mL) APN in PBS buffer solution (0.01 M, pH=7.4).

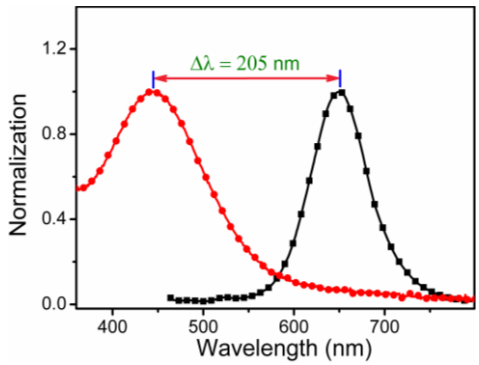


Figure S4. Stokes shift of YH-APN after adding APN in PBS buffer solution (0.01 M, pH = 7.4).

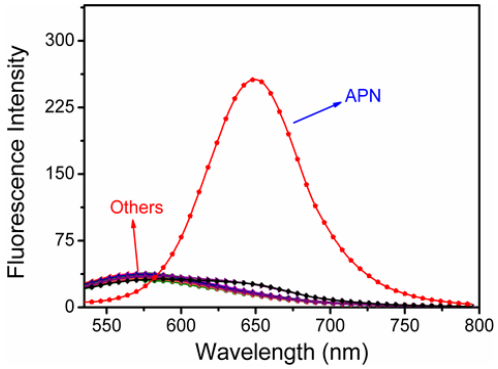


Figure S5. Fluorescence spectral of YH-APN (10 μM) towards various analytes (Except for special instructions, all other concentrations were 500 μM. including Na^+, K^+, Ca^{2+}, Ni^{2+}, Mg^{2+}, NH_4^+, F^-, Cl^-, Br^-, I^-, CH_3COO^-, HCO_3^-, CO_3^{2-}, S^{2-}, HPO_4^{2-}, NO_3^-, SO_4^{2-}, SCN^-, NO_2^-, Glutathione, Cysteine, Homocysteine, Ascorbic acid, NO, H_2O_2 (100 μM), nitroreductase (10 μg/mL), transglutaminase (60 mU/mL), γ-GGT (300 ng/mL), APN (100 ng/mL) were recorded in aqueous solution.

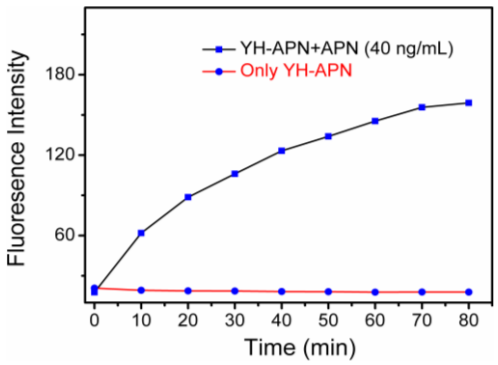


Figure S6. The time response of YH-APN (10 μM) in response to APN (40 ng/mL) in PBS buffer solution (0.01 M, pH=7.4).

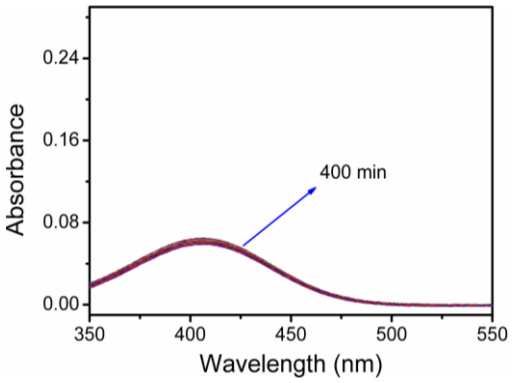


Figure S7. Absorption spectral of YH-APN (10 μM) were recorded in aqueous solution as a function of time.

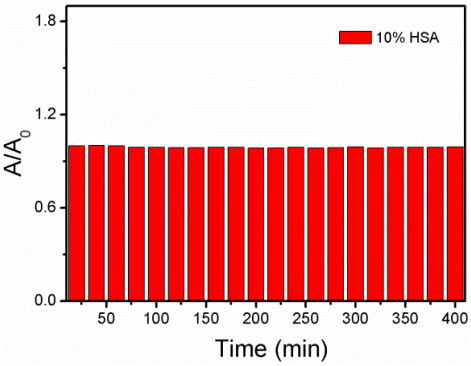


Figure S8. Normalization absorption intensity of YH-APN (10 μM) were recorded in 10% HSA solution as a function of time.

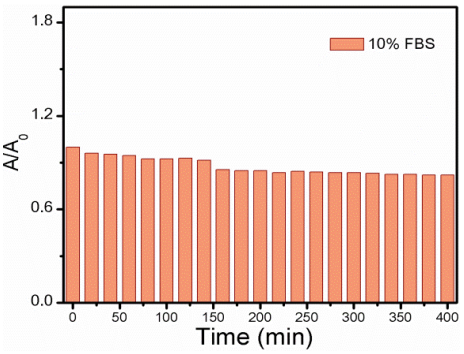


Figure S9. Normalization absorption intensity of YH-APN (10 μM) were recorded in 10% FBS solution as a function of time.

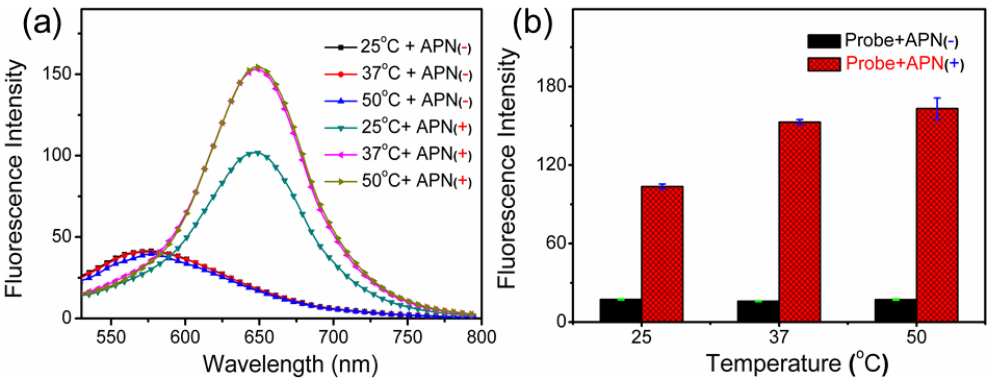


Figure S10. The temperature effect of YH-APN (10 μM) and its activity toward APN (50 ng/mL) in aqueous solution.

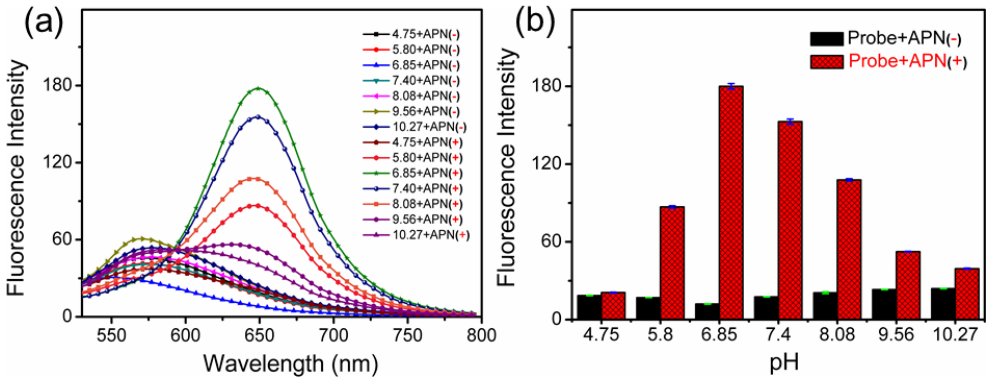


Figure S11. The stability of YH-APN (10 μM) in different pH value and its activity toward APN (50 ng/mL) in aqueous solution.

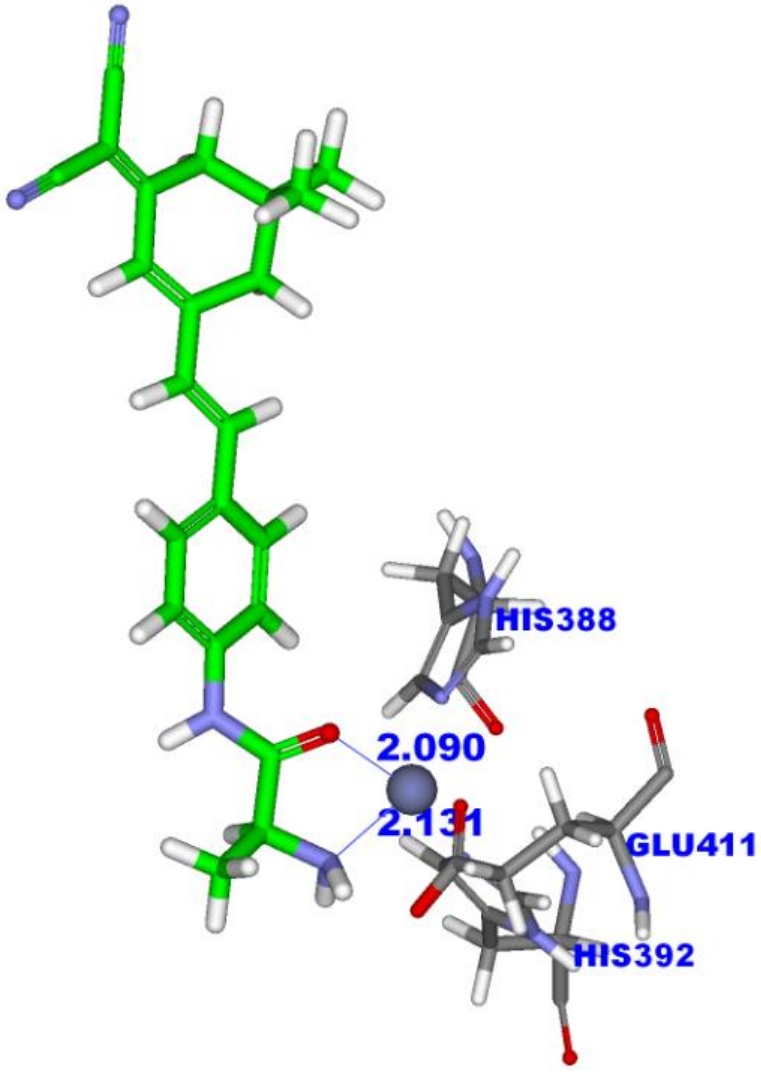


Figure S12. The coordination bond lengths of between YH-APN and zinc ion of APN via docking calculated.

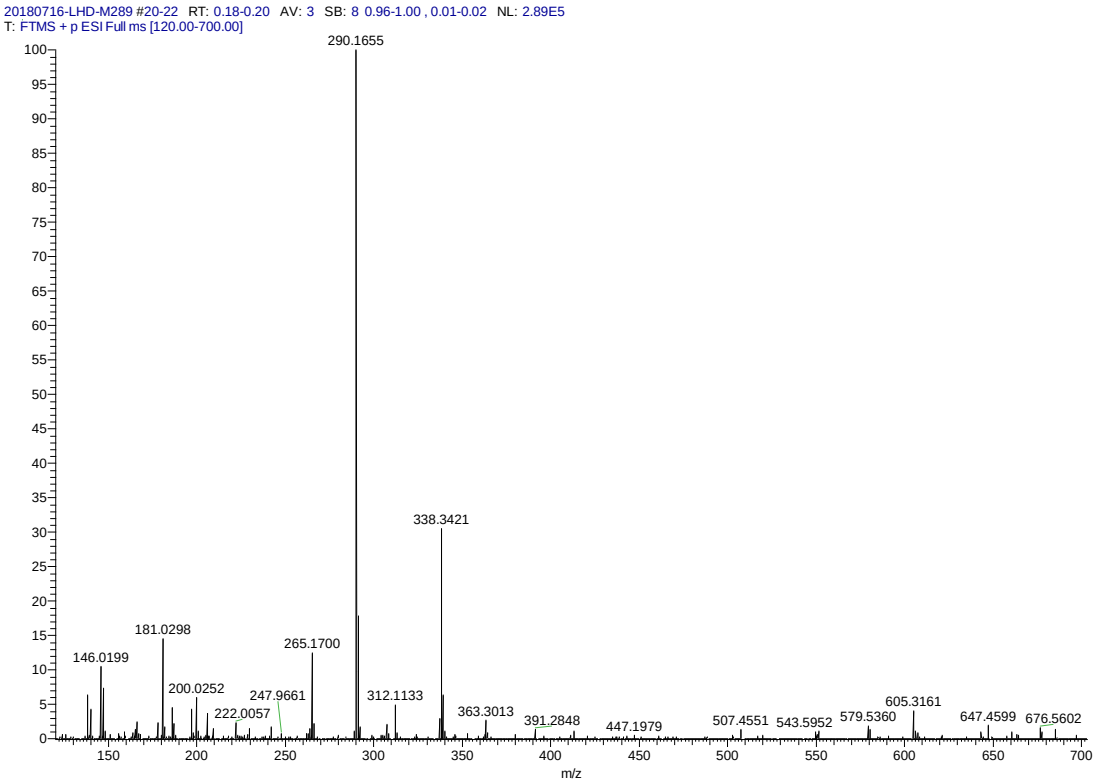


Figure S13. ESI-HRMS verify mechanism of YH-APN in response to APN.

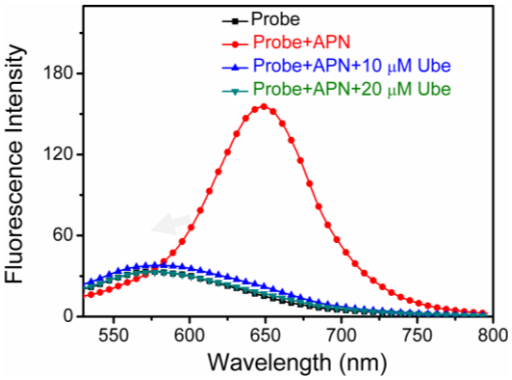


Figure S14. Inhibition experiments of YH-APN towards APN in PBS buffer solution (0.01 M, pH=7.4). Insert black line: Only 10 μM YH-APN; red line: 10 μM YH-APN + APN (50 ng/mL); blue line: 10 μM YH-APN + 10 μM Ube + APN (50 ng/mL); green line: 10 μM YH-APN + 20 μM Ube + APN (50 ng/mL).

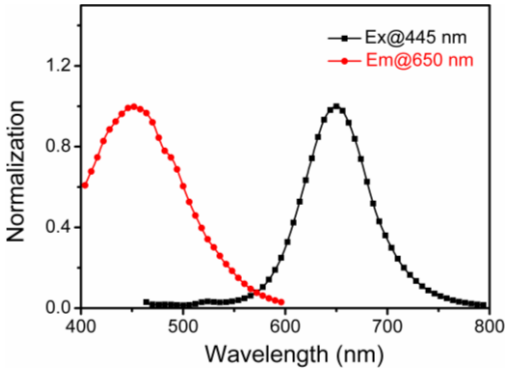


Figure S15. Normalization excitation and emission spectra of YH-APN (10 μM) with 150 ng/mL APN for 80 min in PBS buffer solution (0.01 M, pH=7.4).

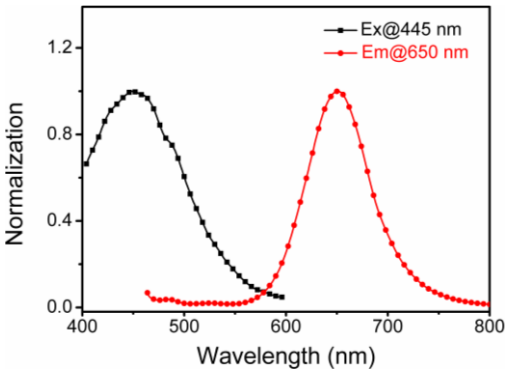


Figure S16. Normalization excitation and emission spectra of intermediate 5 (10 μM) in PBS buffer solution (0.01 M, pH=7.4).

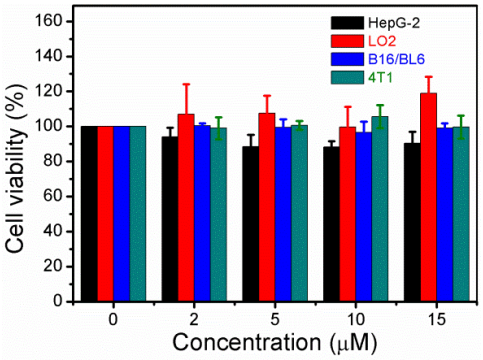


Figure S17. MTT assays of YH-APN in various cells.

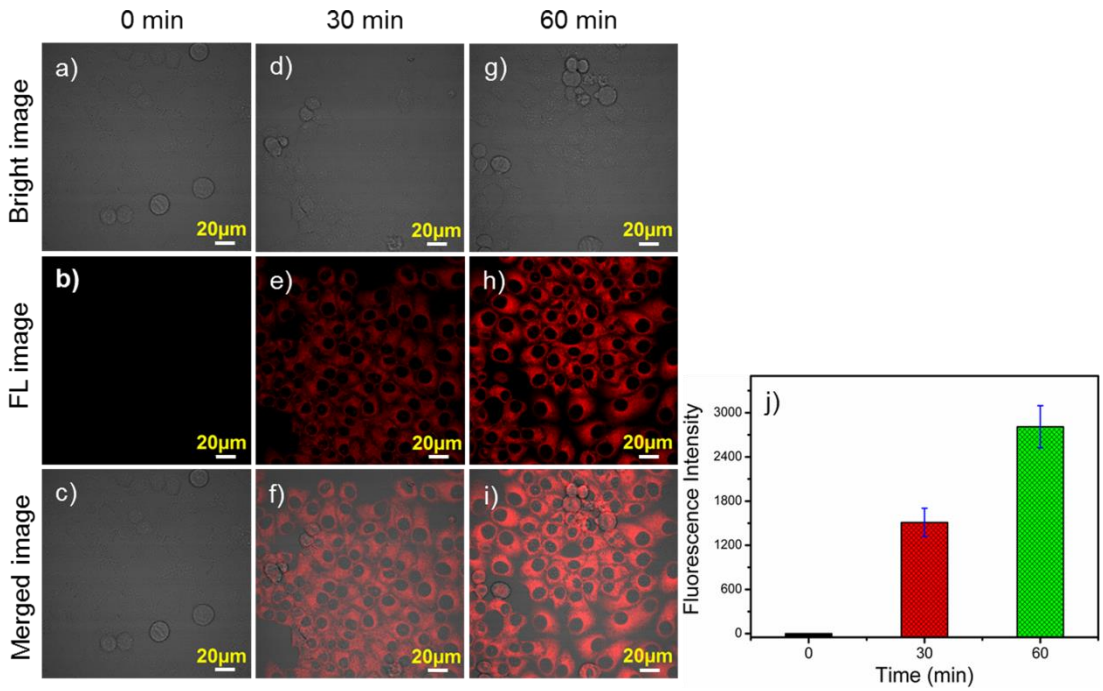


Figure S18. Fluorescence imaging of 5 μM YH-APN in living HepG-2 cells as a function of time. a-c) 0 min, d-f) 30 min, g-i) 60 min, j) fluorescence intensities were gathered from cell. Error bar = RSD (n = 9). λex = 488 nm and λem = 655-755 nm. Scale bar = 20 μm.

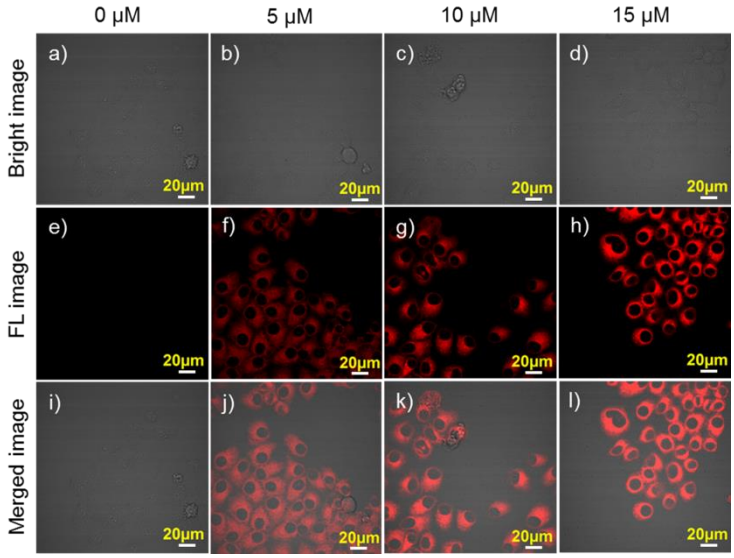


Figure S19. Dose-dependent of YH-APN in living HepG-2 cells for 30 min. a, e and f) 0 μM; b, f and j) 5 μM; c, g, and k) 10 μM; d, h, and l) 15 μM. λex = 488 nm and λem = 655-755 nm. Scale bar = 20 μm.

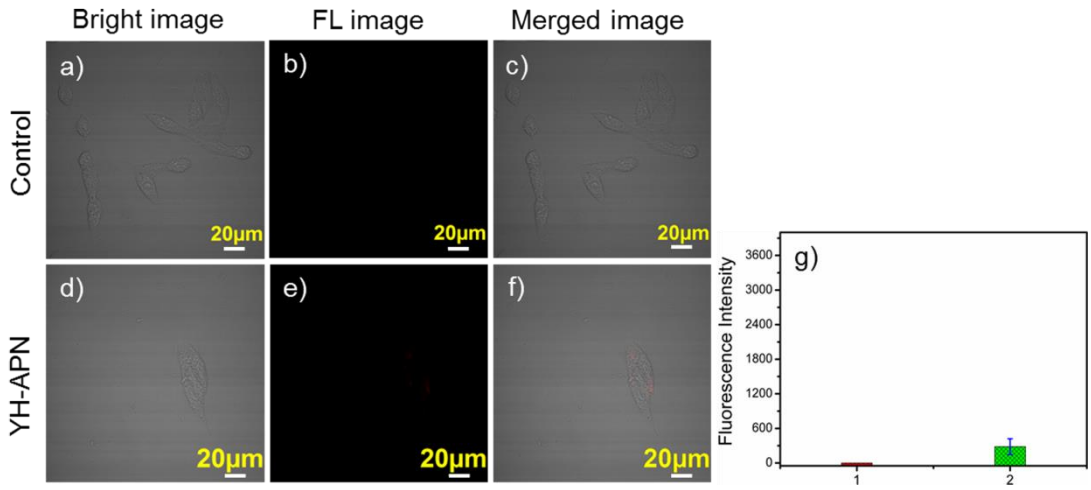


Figure S20. Fluorescence imaging in living COS-7 cells for 30 min. a-c) control group; d-f) treated with 5 μM probe YH-APN; g) fluorescence intensities were gathered from cell. Error bar = RSD (n = 9). λex = 488 nm and λem = 655-755 nm. Scale bar = 20 μm.

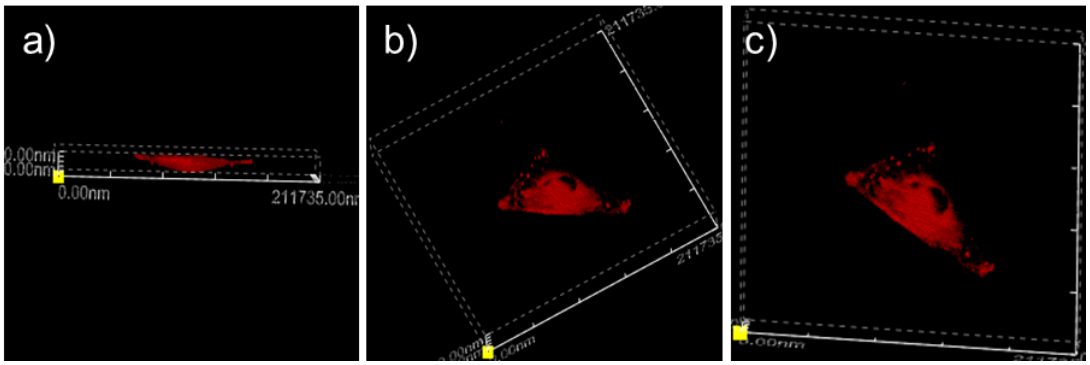


Figure S21. 3D imaging of YH-APN in B16/BL6 cells. λex = 488 nm and λem = 655-755 nm.

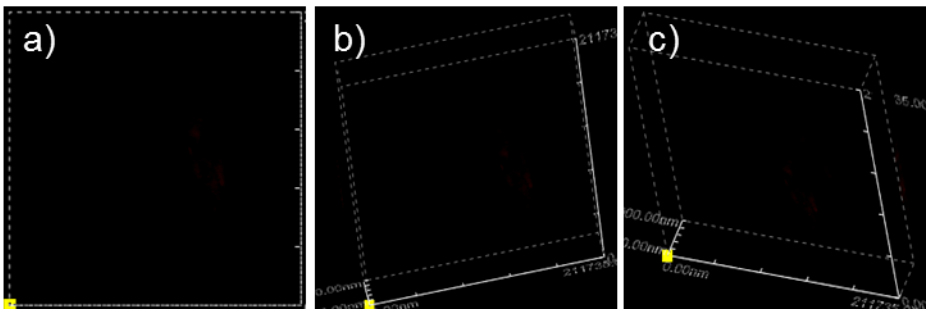


Figure S22. 3D imaging of YH-APN in COS-7 cells. λex = 488 nm and λem = 655-755 nm.

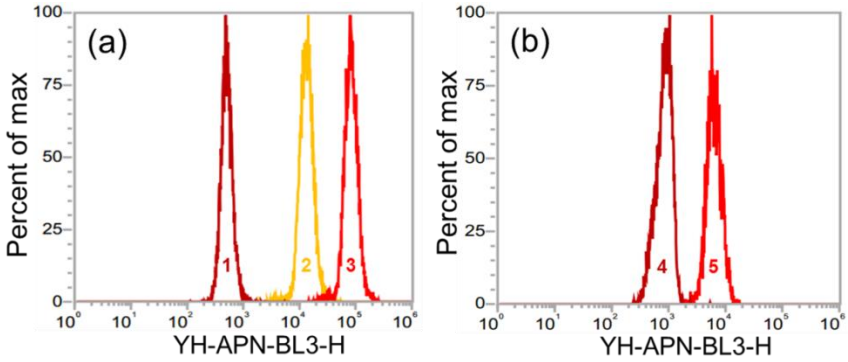


Figure S23. Flow cytometry analysis of YH-APN in living HepG-2 and LO2 cells. HepG-2 cells: 1) control group; 2) pre-treated with Ube, then added YH-APN; 3) incubated with YH-APN. LO2 cells: 4) control group; 5) incubated with YH-APN. λex = 488 nm and λem = 675-715 nm.

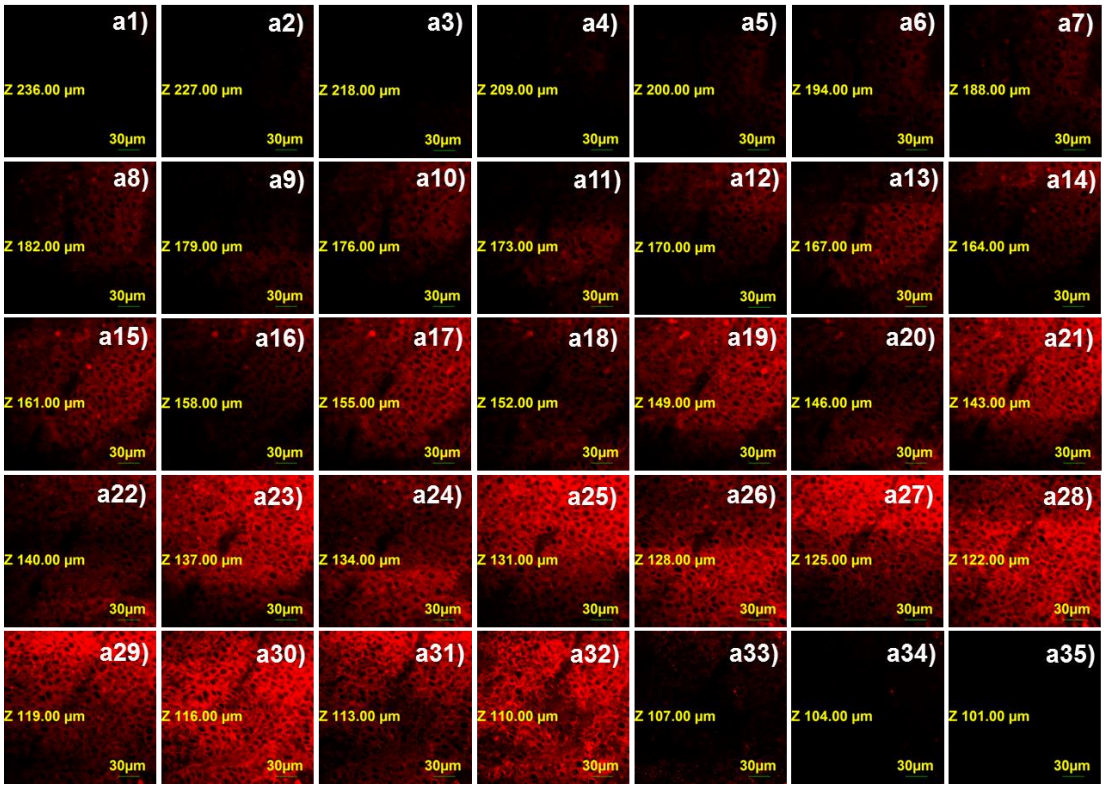


Figure S24. The depth imaging of hepatocellular carcinoma tissue under two-photon excitation. Scale bar = 30 μm.

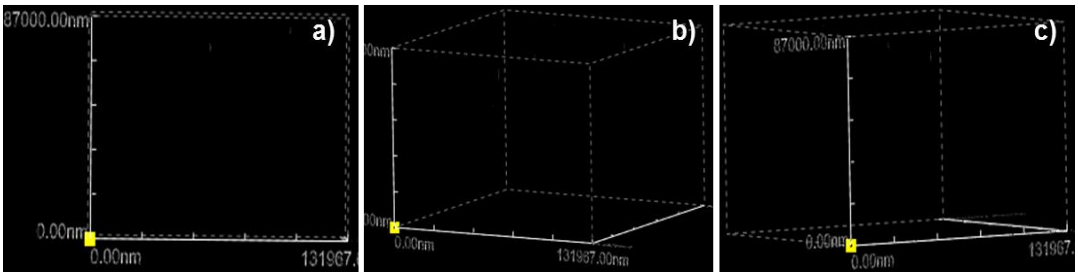


Figure S25. 3D imaging of normal liver tissue treated with 5 μM probe YH-APN at 37 °C for 30 min. λex = 488 nm and λem = 655-755 nm. Scale bar = 20 μm.

^{1}H-NMR, ^{13}C-NMR, and MS spectra

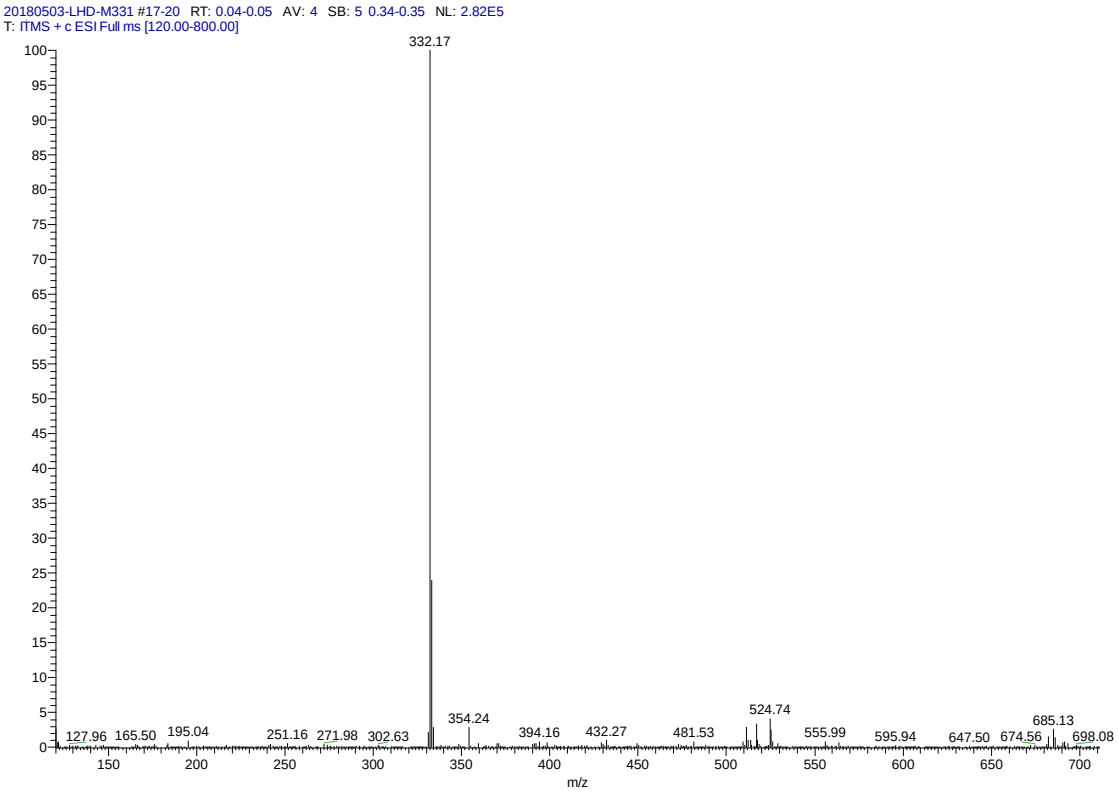


Figure S26. ESI-MS of compound 4.

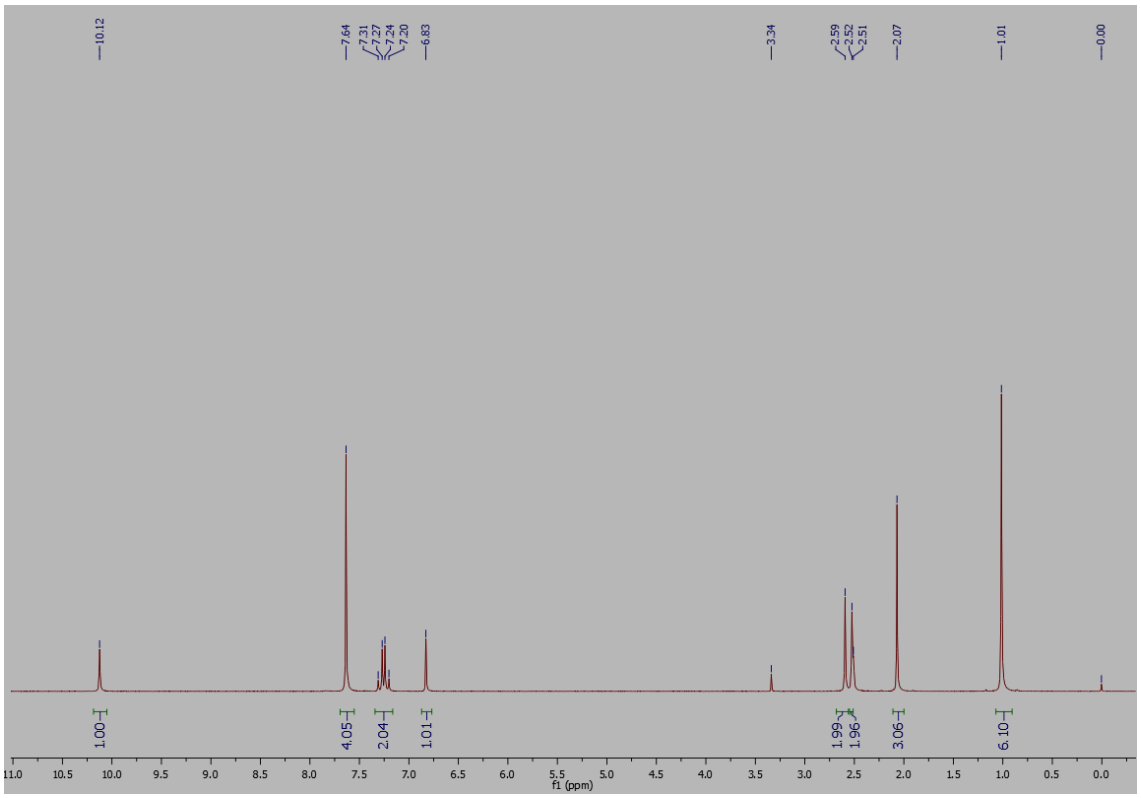


Figure S27. ^{1}H NMR of compound 4 in DMSO-d_6.

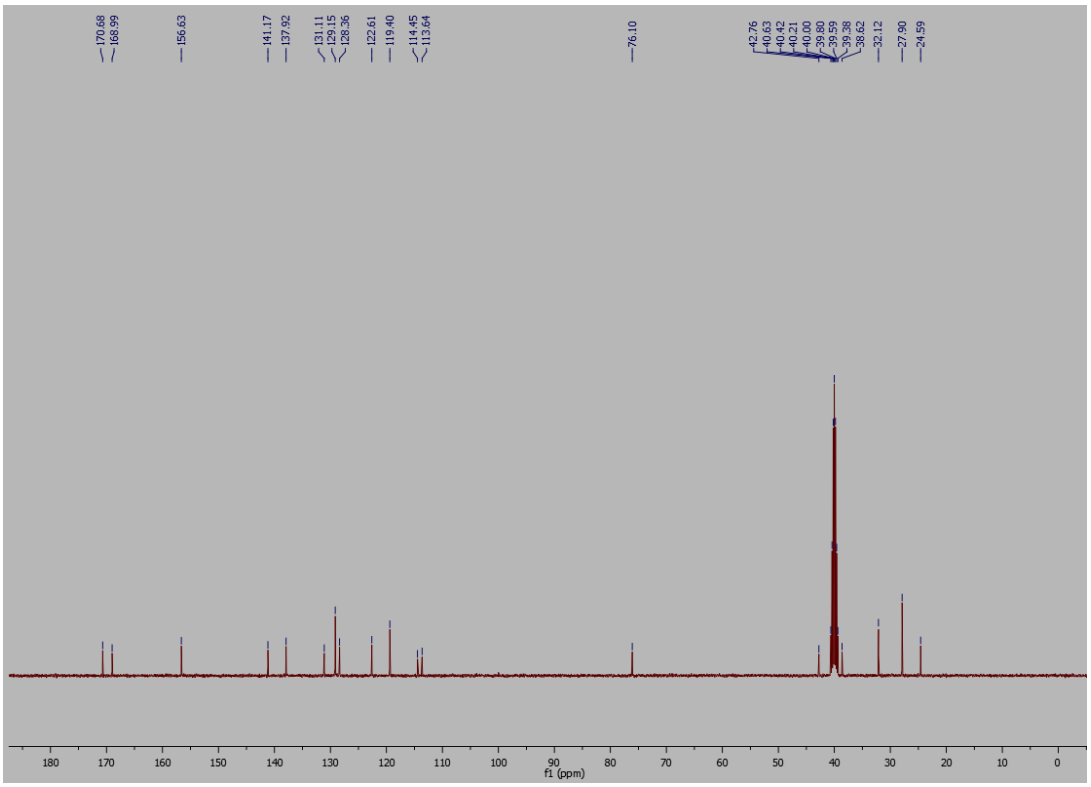


Figure S28. ^{13}C NMR of compound 4 in DMSO-d_6.

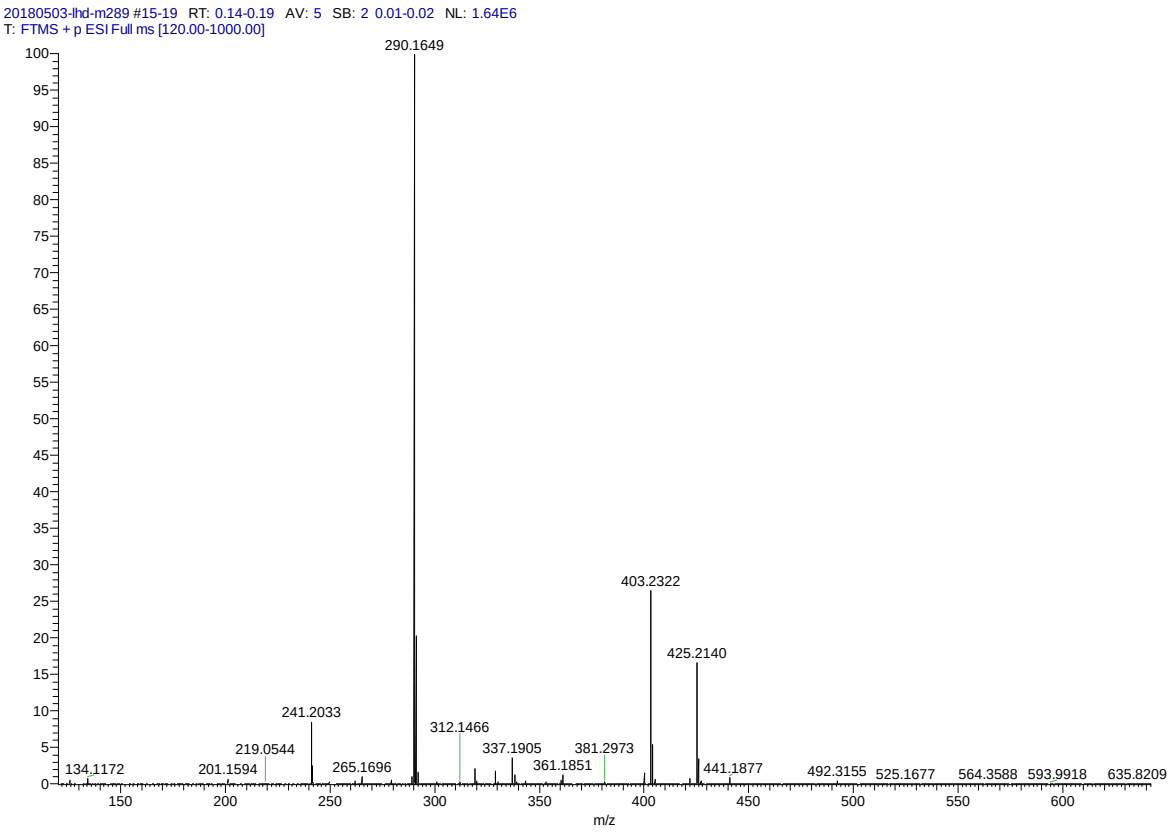


Figure S29. ESI-MS of compound 5.

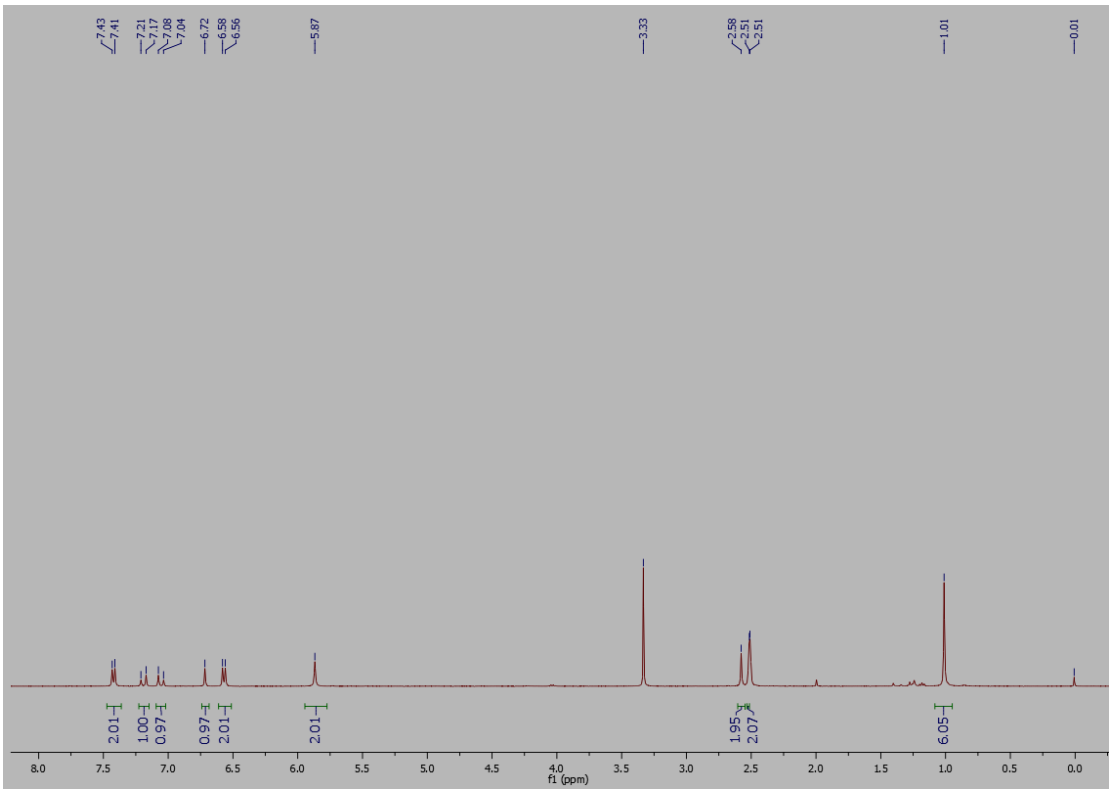


Figure S30. ^{1}H NMR of compound 5 in DMSO-d_6.

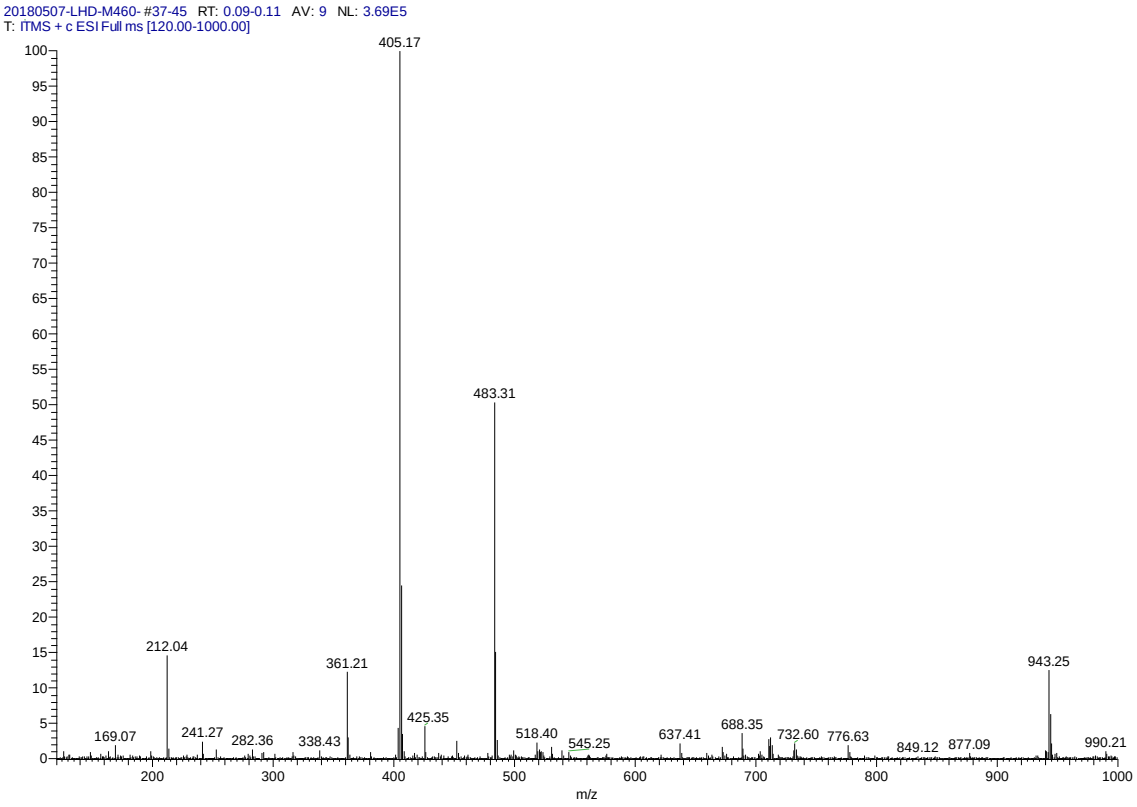


Figure S31. ESI-MS of compound 6.

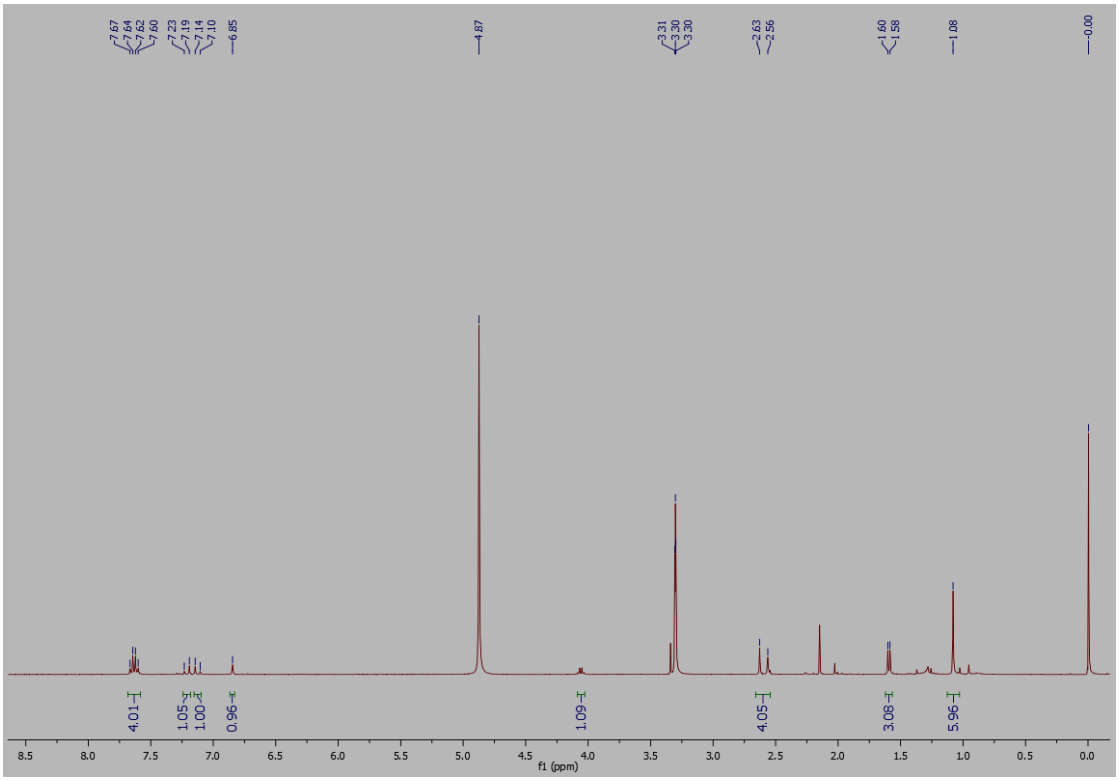


Figure S32. ^{1}H NMR of YH-APN in MeOD.

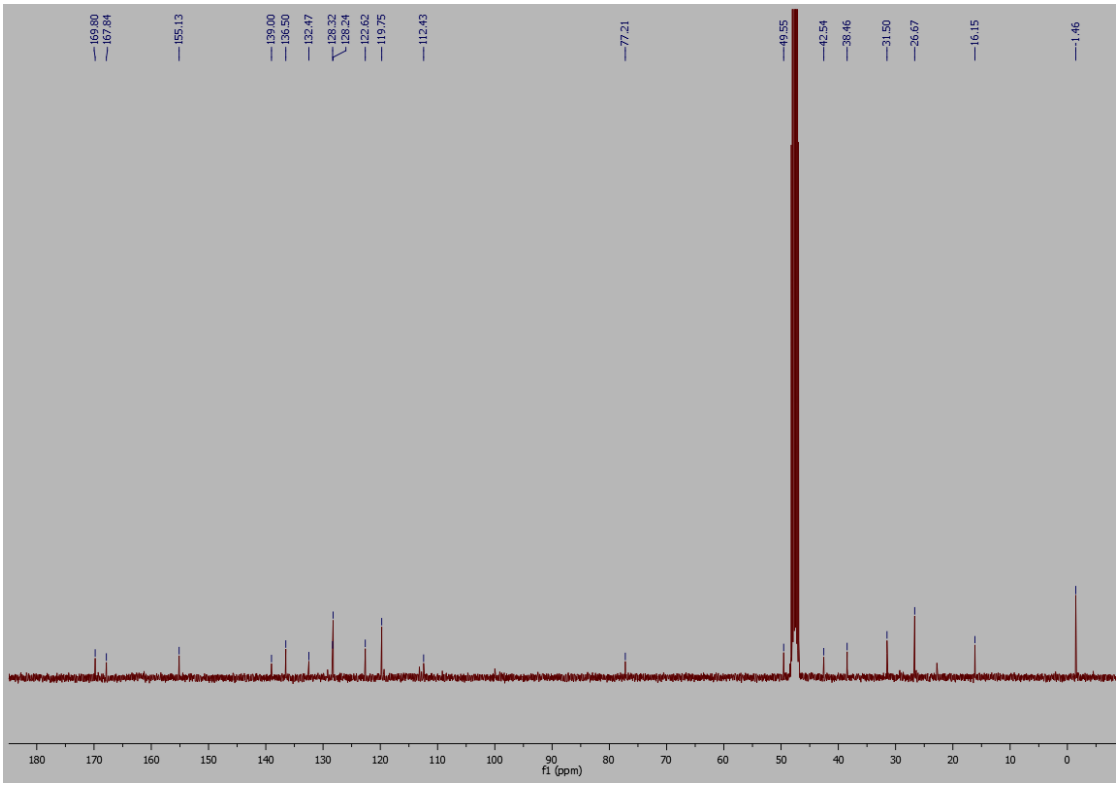


Figure S33. ^{13}C NMR of YH-APN in MeOD.

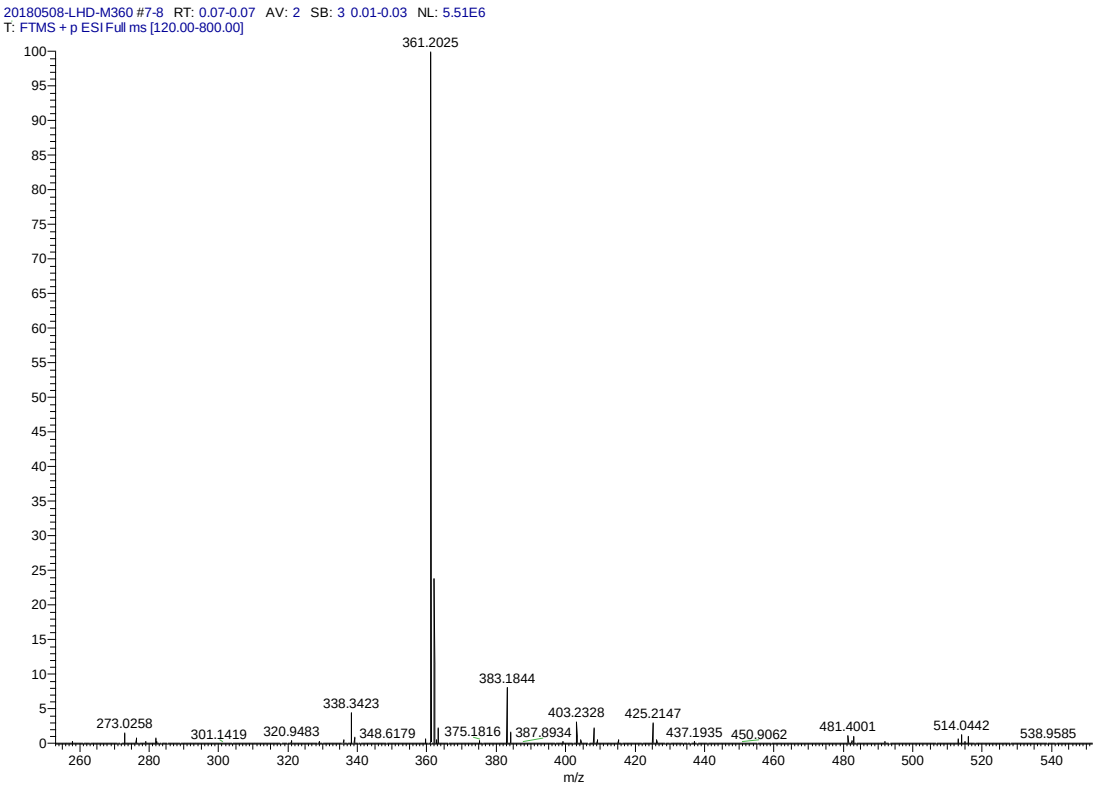


Figure S34. ESI-HRMS of YH-APN.

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