Supporting information

In Situ Imaging of Aminopeptidase N Activity in Hepatocellular Carcinoma: A Migration Model for Tumour Using an Activatable Two-Photon NIR Fluorescent Probe

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General information and materials

All reagents used were obtained from commercial suppliers and were used without further purification unless otherwise stated. Solvents used were purified *via* standard methods. Twice-distilled purified water used in all experiments was from Milli-Q systems (18 MΩ·cm). ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance II 400 MHz spectrometer. Chemical shifts (δ) were reported as ppm (in MeOD or DMSO, with TMS as the internal standard). Fluorescence spectra were performed on a VAEIAN CARY Eclipse fluorescence spectrophotometer (Serial No.MY15210003) in 10 × 10 mm quartz cell. Excitation and emission slit widths were modified to adjust the fluorescence intensity to a suitable range. Absorption spectra were measured on Agilent Technologies CARY 60 UV-Vis spectrophotometer (Serial No.MY1523004) in 10×10 mm quartz cell. Mass spectrometric data were achieved with HP1100LC/MSD MS and an LC/Q-TOF-MS instruments. Mito-Tracker Green, Lyso-Tracker Green and Hoechst 33342 were purchased from Life Technologies Co. (USA). Nitroreductase, transglutaminase, γ-Glutamyltranspeptidase and bestatin were purchased from Sigma-Aldrich. Recombinant human aminopeptidase (APN) was purchased from R&D Systems China. 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). Flash column chromatography was performed using silica gel (100-200 mesh) purchased from Qingdao Ocean Chemicals. Tumor tissue slices were prepared from freezing microtome (LEICA CM1860 UV). All the interferential reagents were prepared based on published literatures. [1]

Determination of the detection limit

The detection limit (DL) was calculated based on the fluorescence titration of probe **DCM-APN** (10 μM) in the presence of APN (0-100 ng/mL). The fluorescence intensity of probe **DCM-APN** was measured and standard deviation of the blank measurement was achieved. The detection limit was calculated by using detection limit with 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 ($F_{664\ nm}$) versus various APN concentrations.

Determination of the quantum yield

The fluorescence quantum yields for compounds are used by Absolute PL Quantum Yield Spectrometer (HAMAMATSU C11347). Operating this system is simple. Load a sample and press the start button to measure the photoluminescence quantum yields, excitation wavelength dependence, PL excitation spectrum and other properties in a short time. The PL Quantum Yield (Φ) is expressed as the ratio of the number of photons emitted from molecules (PN_{em}) to that absorbed by molecules (PN_{abs}).

$$\Phi = PN_{em} / PN_{abs}$$

Cell incubation

Hepatoma carcinoma cells (HepG-2 cells), mouse melanoma (B16/BL6 cells) and normal liver cells (LO2 cells) were purchased from Institute of Basic Medical Sciences (IBMS) of the Chinese Academy of Medical Sciences. Except for HepG-2 cells treated with Dulbecco's modified Eagle's medium (DMEM, Invitrogen), others 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 ºC under a humidified atmosphere containing 5% CO_2.

Cytotoxicity 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. HepG-2 and LO2 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, 1, 2, 5 and 10 μM of probe **DCM-APN** for 24 h. Cells in culture

medium without probe **DCM-APN** were used as the control. Six replicate wells were used for each control and test concentration. 10 μL of MTT (5 mg/mL) prepared in PBS 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:

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

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 ºC in a 5% CO_2 humidified incubator. For the detection of endogenous APN, HepG-2, LO2 and B16/BL6 cells were treated with probe **DCM-APN** (2.5 μM) at 37 °C, followed by washing thrice using DMEM without FBS. Under the confocal fluorescence microscope (Olympus FV1000-IX81) with a 60 × objective lens, probe **DCM-APN** was excited at 488 nm (one-photon) and 800 (two-photon), next, fluorescence emission at 610-660 nm channel and 575-630 nm channel were gathered, respectively.

Cells motility assays

Cellualr morphology of living HepG-2 was immediately collected after wounding with about 350 micrometers. After 24 hours of cultivation at 37 °C, horizontal migration of cell pretreated with or without 100 μM bestatin was measured by Olympus viewer. Quantitative distance analysis of migration determined from 11 regions of interest (ROIs).

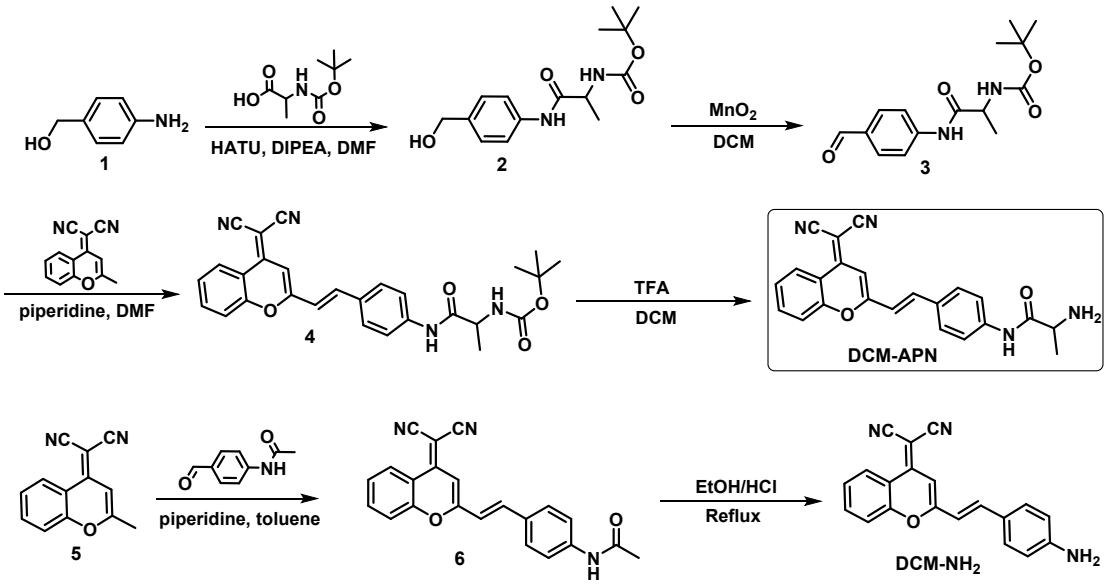
Visualization of APN activity in tissue

Tumor tissue slices of 20 μm or 200 μm were prepared from hepatoma tumour using freezing microtome (LEICA CM1860 UV). As a comparison, Normal slices were chosen from normal liver tissue. Next, these tissues were incubated with probe **DCM-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 **DCM-APN** was excited at 488 nm (one-photon) and 800 (two-photon), next, fluorescence emission at 610-660 nm (one-photon) and 575-630 nm (two-photon) of red channel were

gathered. In tissue depth imaging, the 3D images were constructed *via* z-scan mode under the same condition.

Visualization of APN activity in mice xenograft tumor model

All procedures were carried out in compliance with the guide for the care and use of laboratory animal resources and Dalian Medical University animal care and use committee, and were approved by the institutional animal care and use committee of the Dalian Medical University. For establishing a mouse tumor model, the HepG-2 cells were chosen 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 100 μM 150 μL probe **DCM-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.



Scheme S1. Synthetic procedures of probe **DCM-APN**

Synthesis of probe DCM-APN

Synthesis of compound 2

Compound **1** (189 mg, 1 mM), (tert-butoxycarbonyl)-L-alanine (378 mg, 2 mM), HATU (760 mg, 2 mM) and DIPEA (259 mg, 2 mM) were dissolved in 5 mL dry DMF. The mixture was stirred at room temperature for overnight under N_2 protection. After removing the solvent

by reduced pressure distillation, compound **2** was obtained measured through mass spectrometry. ESI-MS: m/z calcd for $C_{15}H_{22}N_2NaO_4^+$ $[M+Na]^+$: 317.14, found: 317.10.

Synthesis of compound 3

Compound **2** (294 mg, 1mM) and MnO_2 (870 mg, 10 mM) were dissolved in 5 mL dry DCM. This mixture was stirred at 30 °C for overnight and vacuum filter. The filtrate was obtained and further through silica gel column chromatography to obtain compound **3** measured by mass spectrometry. ESI-MS: m/z calcd for $C_{15}H_{20}KN_2O_4^+$ $[M+Na]^+$: 331.11, found: 331.09.

Synthesis of compound 4

Compound **3** (292 mg, 1 mM), compound 5 (312 mg, 1.5 mM) and a drop piperidine were dissolved in 5 mL dry DMF. This mixture was stirred at 120 °C for 4 h and vacuum filter. Crude red solid of compound **4** was obtained finally without further purification for the next step. ESI-MS: m/z calcd for $C_{28}H_{26}N_4NaO_4^+$ $[M+Na]^+$: 505.1846, found: 505.1850.

Synthesis of compound 5, 6 and DCM-NH_2

Compound **5, 6** and **DCM-NH_2** were synthesized according to the previous literature reported. [2]

DCM-NH_2:^{1}H NMR (400 MHz, DMSO-d_6) δ 8.73 (d, J = 8.3 Hz, 1H), 7.89 (t, J = 7.4 Hz, 1H), 7.78 (d, J = 8.3 Hz, 1H), 7.62-7.48 (m, 2H), 7.49 (d, J = 8.4 Hz, 1H), 7.09 (d, J = 15.8 Hz, 1H), 6.87 (s, 1H), 6.62 (d, J = 8.5 Hz, 2H), 6.02 (s, 2H).

ESI-HRMS: *m/z* $C_{20}H_{14}N_3O^+$ $[M+H]^+$: 312.1131, found 312.1127.

Synthesis of probe DCM-APN

Compound **4** (48 mg, 0.1 mM) was dissolved in 2 mL dry CH_2Cl_2 and stirred at room temperature for 20 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. After that, the crude product was purified through silica gel column chromatography to obtain 12 mg red probe **DCM-APN** (Yield 31%). ^{1}H NMR (400 MHz, MeOD)

δ 8.86 (d, *J* = 8.4 Hz, 1H), 7.84 (t, *J* = 7.6 Hz, 1H), 7.70 (m, 6H), 7.52 (t, *J* = 7.6 Hz, 1H), 7.08 (d, *J* = 16.0 Hz, 1H), 6.93 (s, 1H), 3.68 (q, *J* = 6.9 Hz, 1H), 1.42 (d, *J* = 6.9 Hz, 3H). ESI-HRMS: m/z calcd for $C_{23}H_{19}N_4O_2^+$ $[M+H]^+$: 383.1503, found: 383.1508.

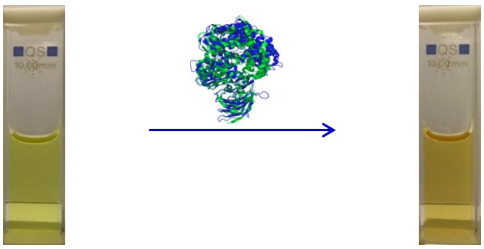


Fig. S1 The color change of the solution of probe **DCM-APN** (10 μM) in present of 200 ng/mL APN in aqueous solution.

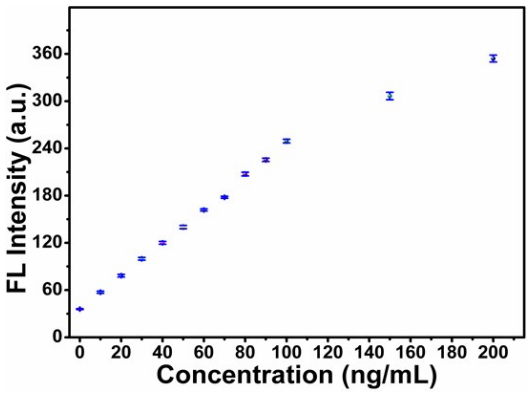


Fig. S2 Fluorescence intensity of probe **DCM-APN** (10 μM) approximately 664 nm towards various APN concentration (0-200 ng/mL) for 120 min in aqueous solution (PBS/DMSO=7:3, v/v, 0.01 M, pH=7.4).

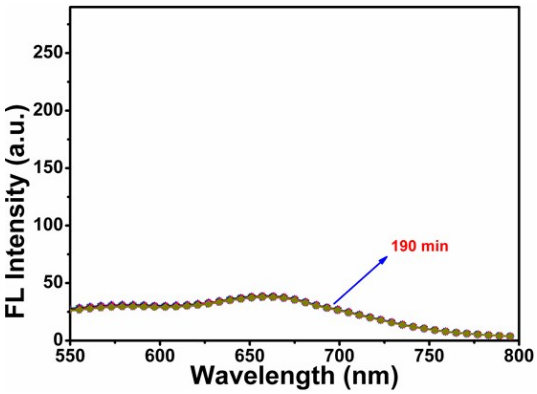


Fig. S3 Fluorescence emission spectra of probe **DCM-APN** (10 μM) were recorded in aqueous solution as a function of time (0-190 min).

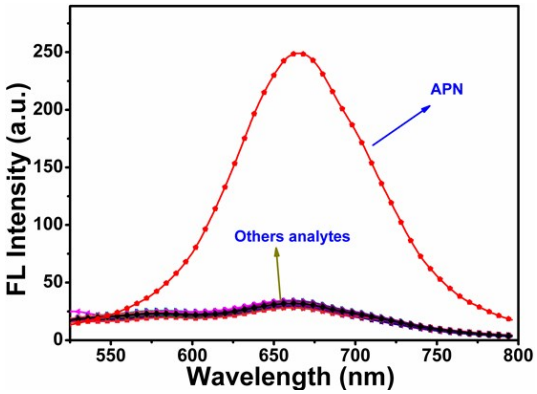


Fig. S4 Fluorescence emission spectra of probe **DCM-APN** (10 μM) towards various analytes were recorded in aqueous solution.

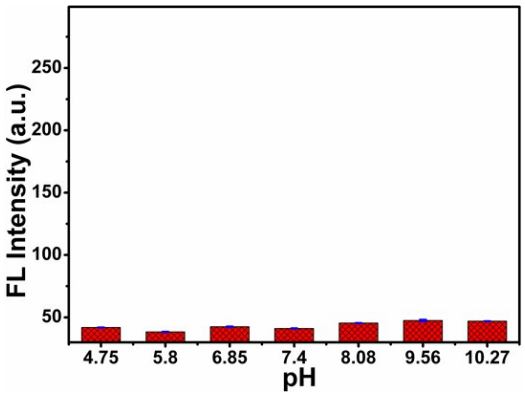


Fig. S5 The stability of probe **DCM-APN** (10 μM) in different pH value.

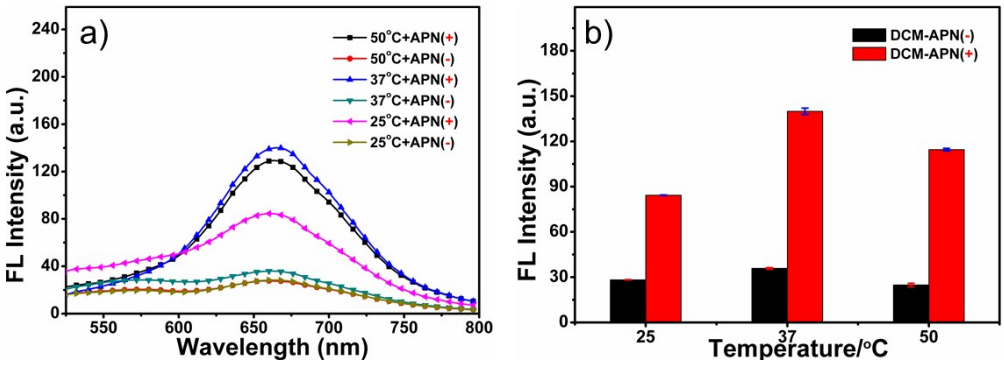


Fig. S6 The temperature effect of probe **DCM-APN** (10 μM) and its activity toward 50 mg/mL APN in aqueous solution.

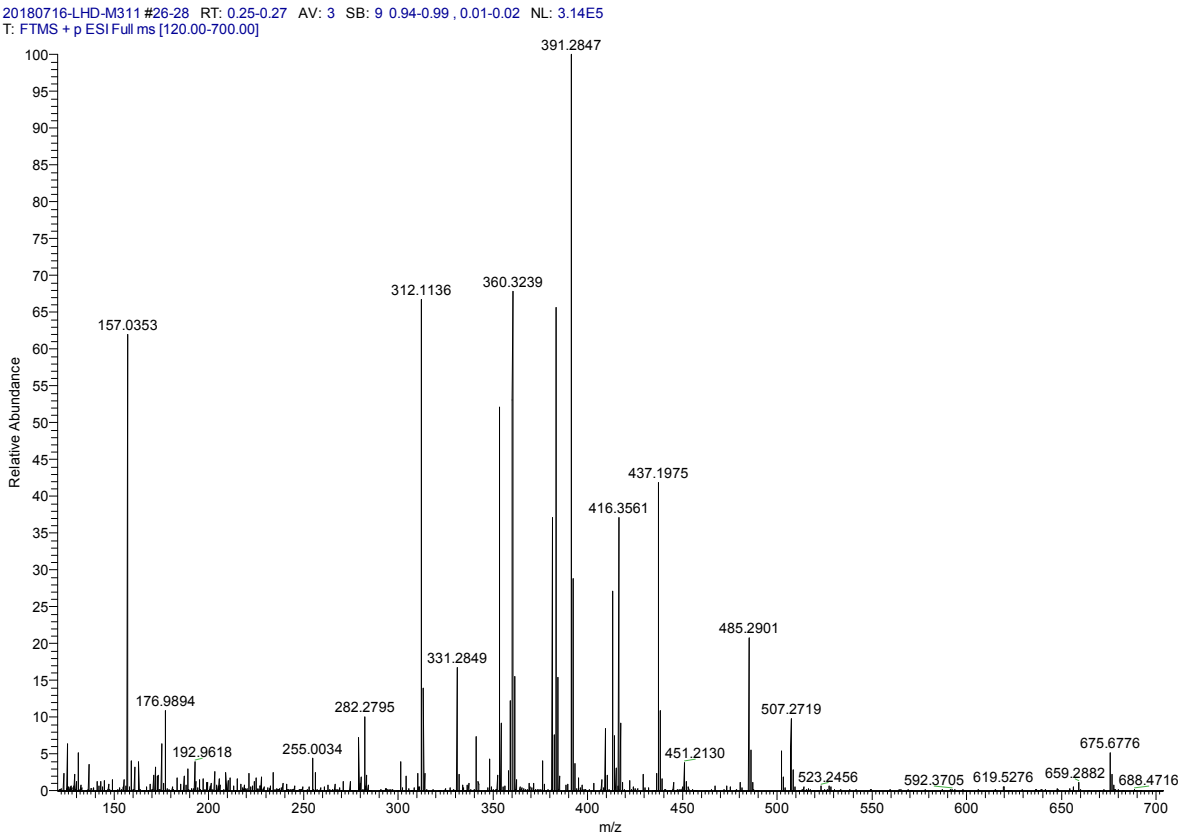


Fig. S7. ESI-HRMS verify mechanism.

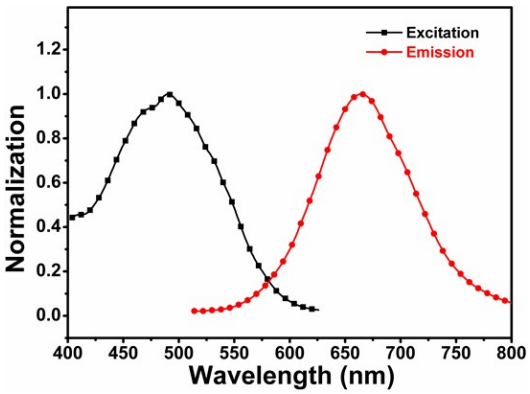


Fig. S8. Normalization excitation and emission spectra of probe **DCM-NH_2** (5 μM) were shown in aqueous solution.

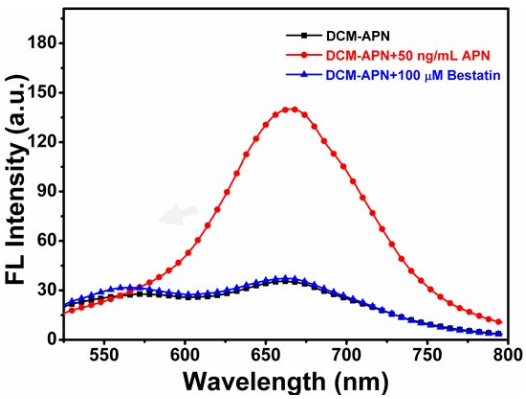


Fig. S9. Inhibition experiments of probe **DCM-APN** towards APN in aqueous buffer. Insert black line: only 10 μM **DCM-APN**; red line: 10 μM **DCM-APN** + 50 ng/mL APN; blue line: 10 μM **DCM-APN** + 100 μM Bestatin + 50 ng/mL.

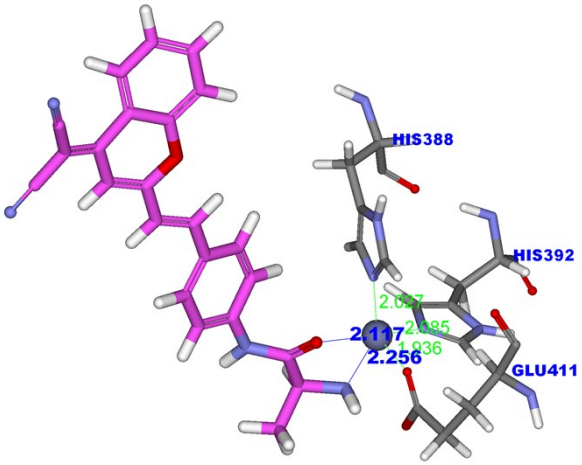


Fig. S10. The bonding mode between probe **DCM-APN** and APN. The gray sections represent three residues of APN; the pink part on behalf of probe **DCM-APN**; The gray point represents zinc ion.

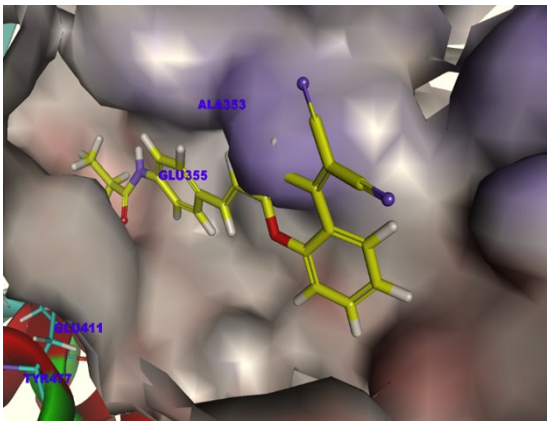


Fig. S11. Probe **DCM-APN** reached coordination center of zinc ion through wide-opening hydrophobic cavity.

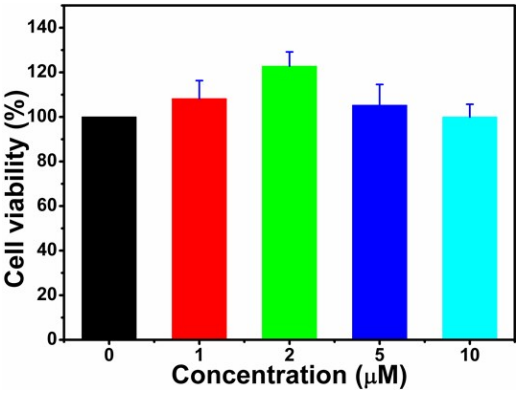


Fig. S12. Cytotoxicity of probe **DCM-APN** in HepG-2 cells.

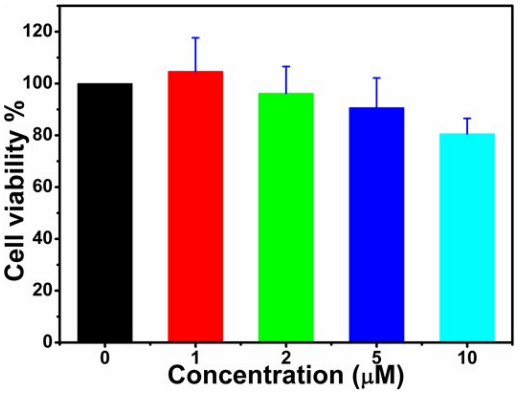


Fig. S13. Cytotoxicity of probe **DCM-APN** in LO2 cells.

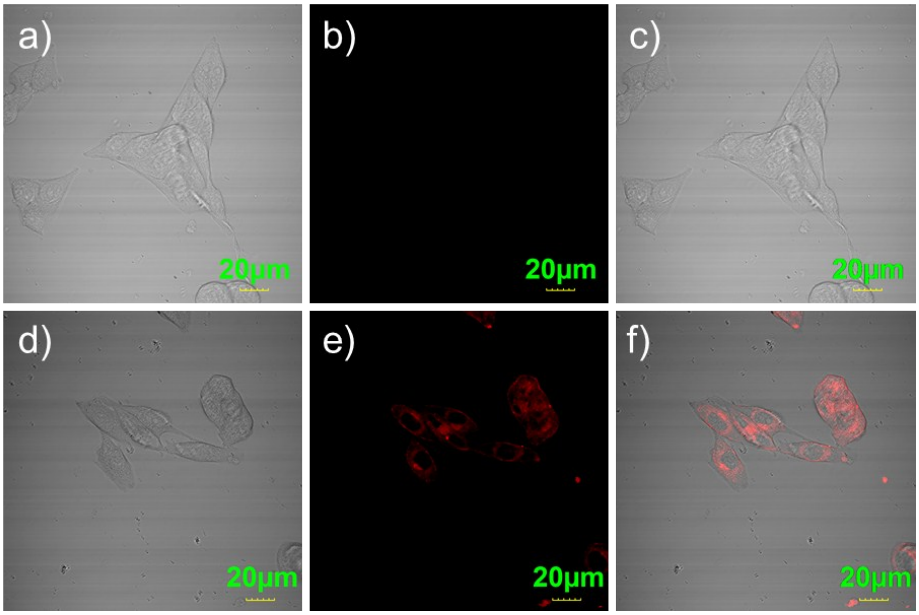


Fig. S14. Fluorescence imaging of probe **DCM-APN** in B16/BL6 cells. a, b and c) control group; d, e and f) treated with 2.5 μM probe **DCM-APN**. λex = 488 nm and λem = 610-660 nm. Scale bar = 20 μm.

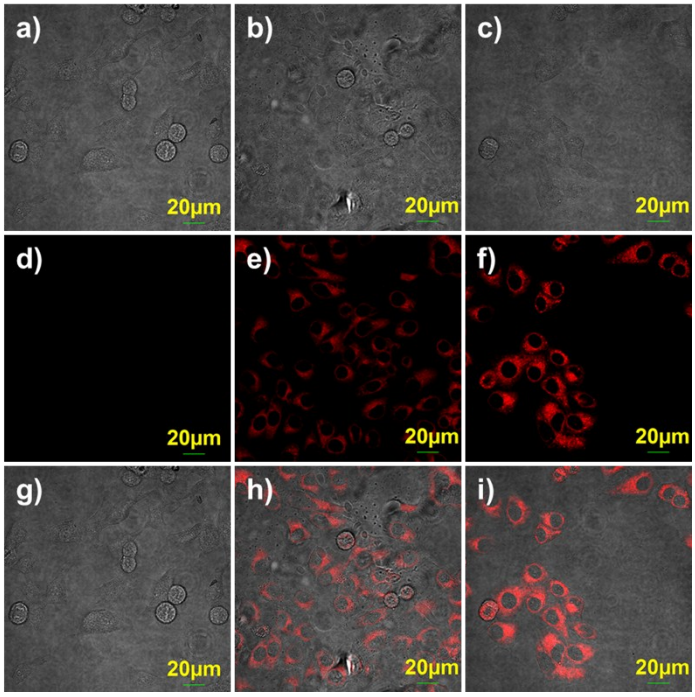


Fig. S15. Fluorescence imaging of probe **DCM-APN** in living HepG-2 cells as a function of time. a, d and g) control group; b, e and h) treated with 2. 5 μM probe **DCM-APN** for 10 min; c, f and i) treated with 2. 5 μM probe **DCM-APN** for 30 min. λex = 488 nm and λem = 610-660 nm. Scale bar = 20 μm.

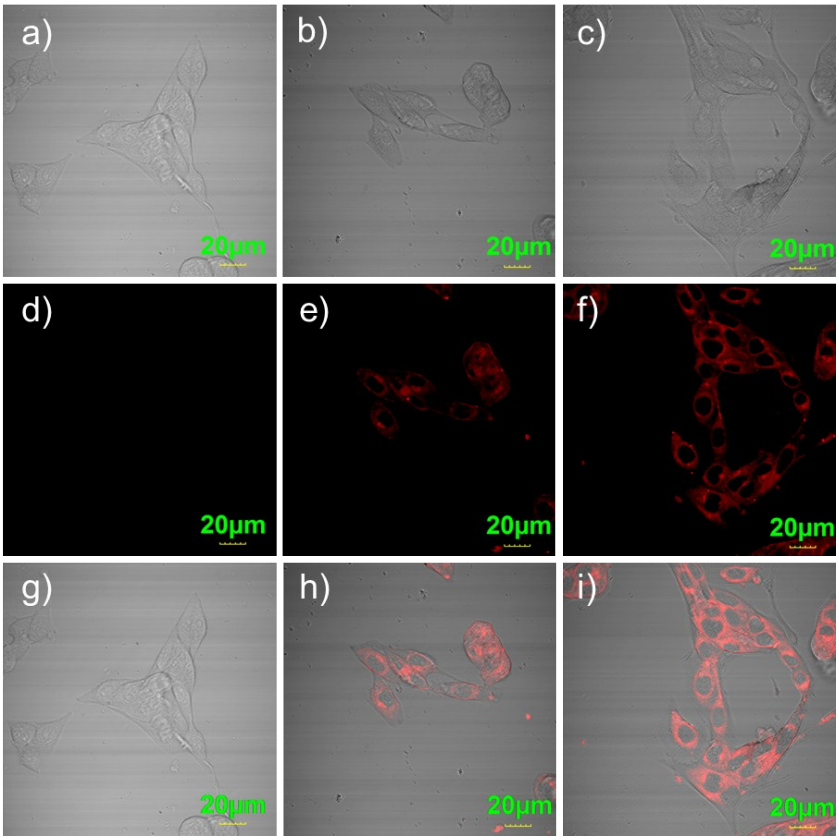


Fig. S16. Fluorescence imaging of probe **DCM-APN** in living B16/BL6 cells as a function of time. a, d and g) control group; b, e and h) treated with 2.5 μM probe **DCM-APN** for 10 min; c, f and i) treated with 2.5 μM probe **DCM-APN** for 30 min. λex = 488 nm and λem = 610-660 nm. Scale bar = 20 μm.

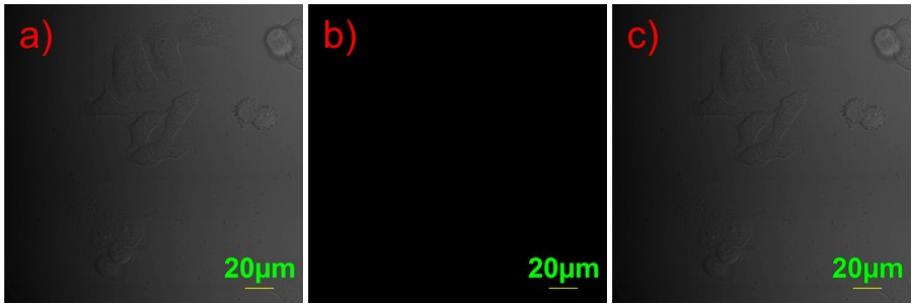


Fig. S17. Two-photon imaging of living HepG-2 cells, λex= 800 nm and λem = 575-630 nm. Scale bar = 20 μm.

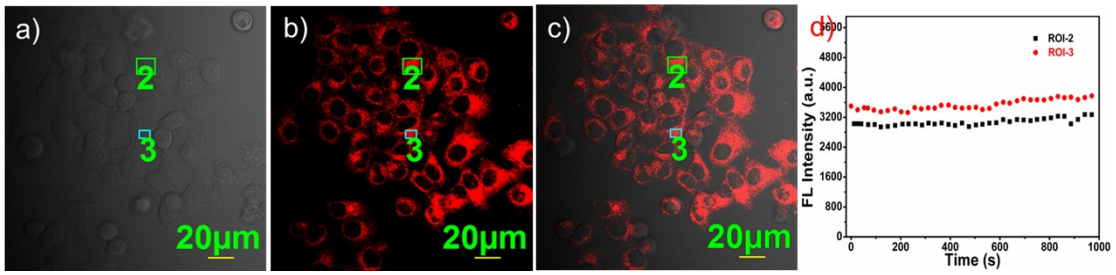


Fig. S18. Two-photon photo-stability evaluation in living HepG-2 cells. a) bright imaging; b) fluorescence imaging; c) merged imaging; d) fluorescence intensities of regions of interests (ROIs, 2 and 3) as a function of time. λex = 800 nm and λem = 575-630 nm. Scale bar = 20 μm.

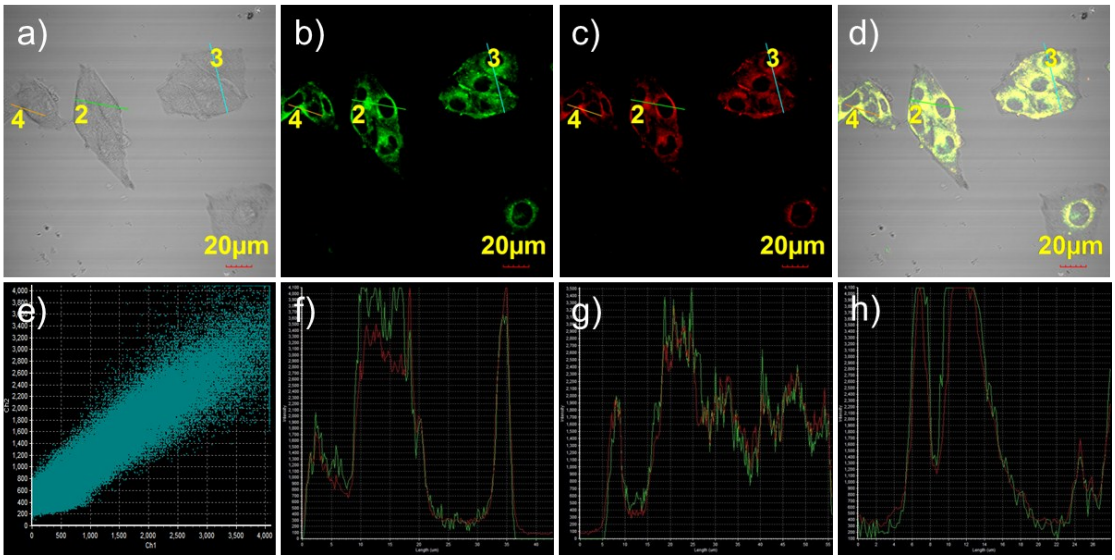


Fig. S19. Fluorescence imaging of subcellular localization in living B16/BL6 cells. a) bright imaging; b) commercial Lyso-Tracker (λex = 488 nm, λem = 500-560 nm) dye channel; c) NIR channel of probe **DCM-APN** (λex = 488 nm, λem = 610-660 nm); d) merged channel; e) Pearson's correlation (P = 0.97); f, g and h) intensity profiles with ROI (regions of interest, 2, 3, and 4, respectively). Scale bar = 20 μm.

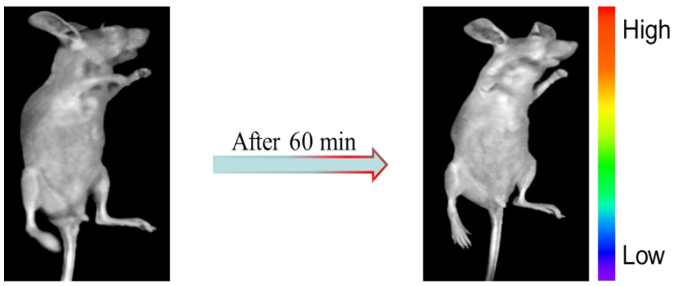


Fig. S20. Fluorescence imaging of APN activity in the healthy bare BABL/c mice after subcutaneous injection with probe **DCM-APN** (100 μM, 150 μL) under excitation at 475 nm (fwhm 20 nm) and emission at 655 nm (fwhm 20 nm).

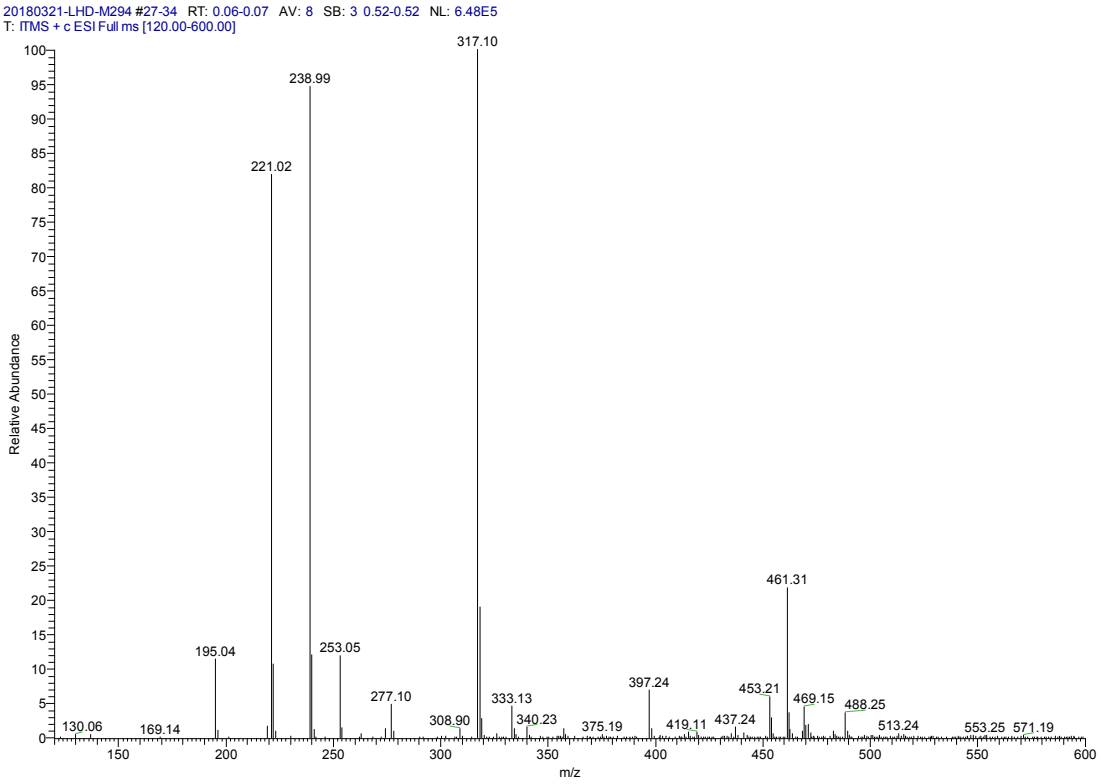


Fig. S21. ESI-MS of compound **2**.

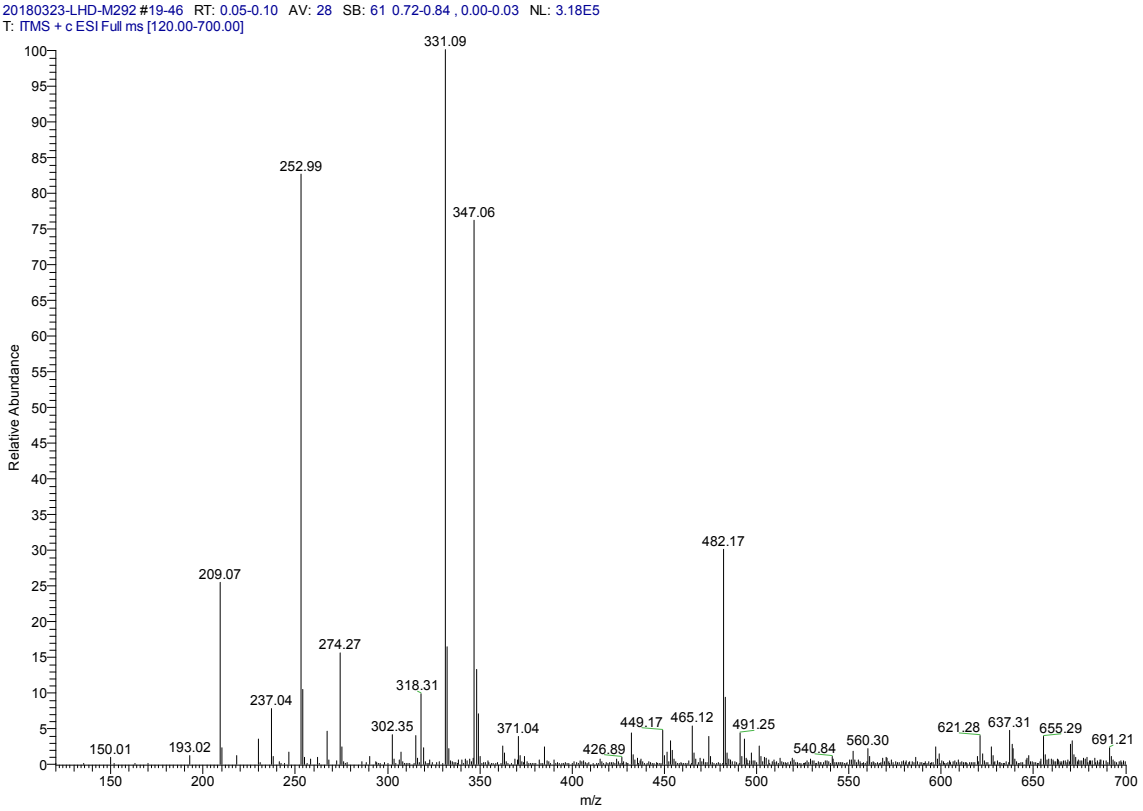


Fig. S22. ESI-MS of compound **3**.

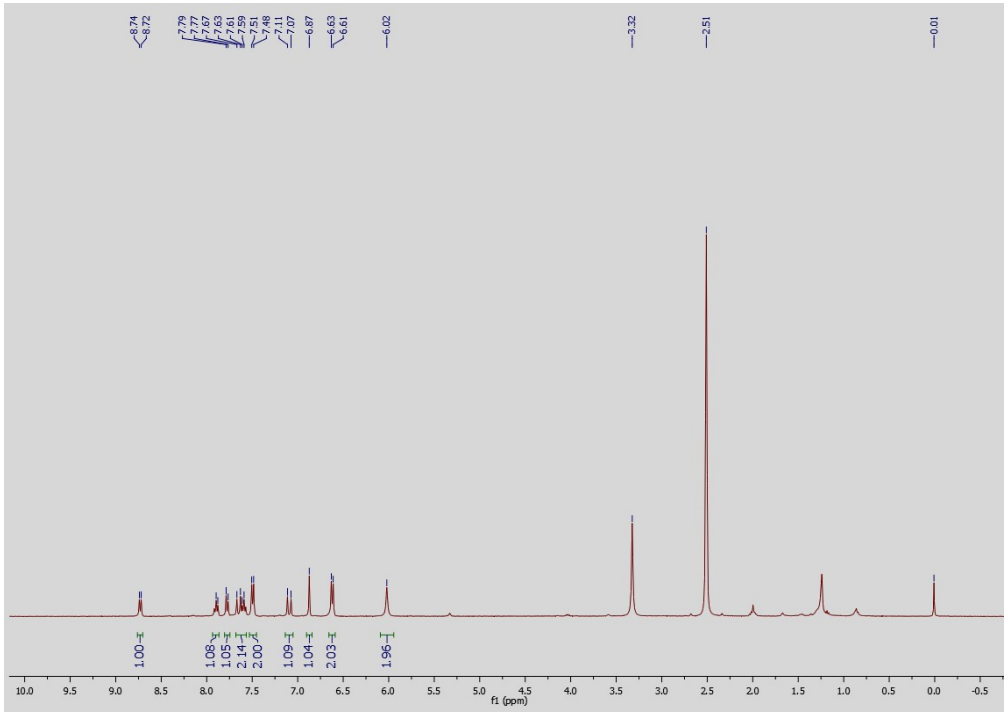


Fig. S23. ^{1}H NMR of **DCM-NH$_2$** in DMSO-d_6.

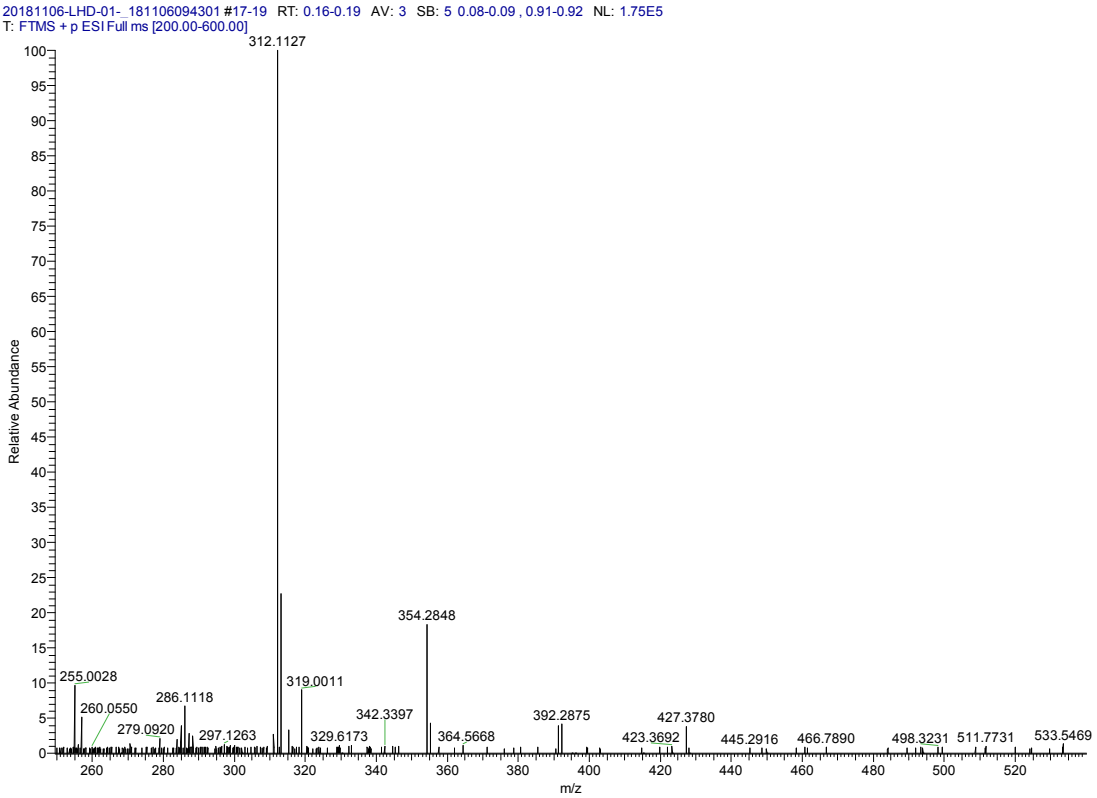


Fig. S24. ESI-HRMS of **DCM-NH$_2$**.

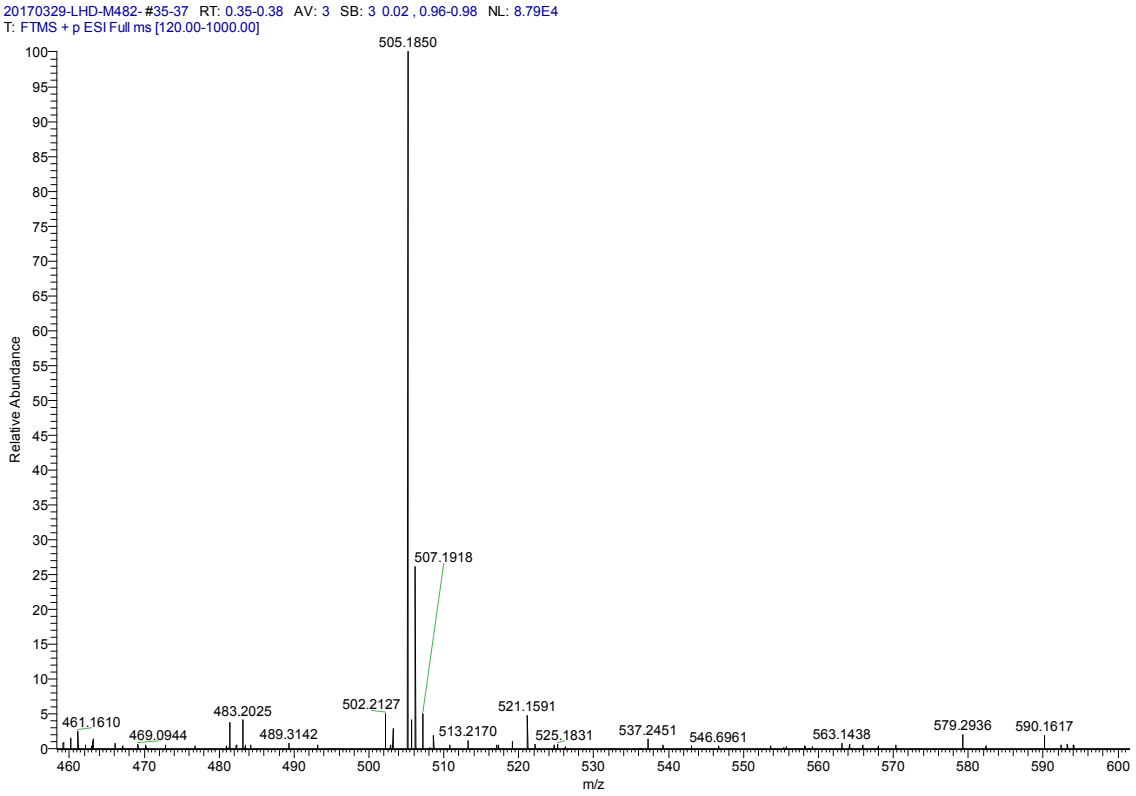


Fig. S25. ESI-HRMS of compound **4**.

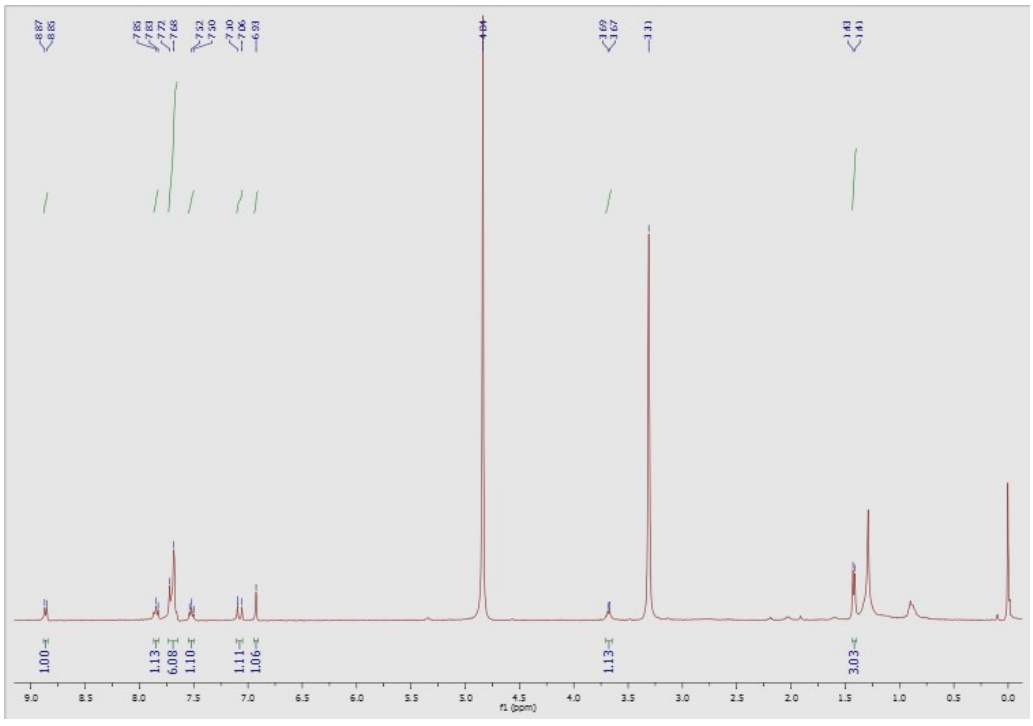


Fig. S26. ^{1}H NMR of **DCM-APN** in MeOD.

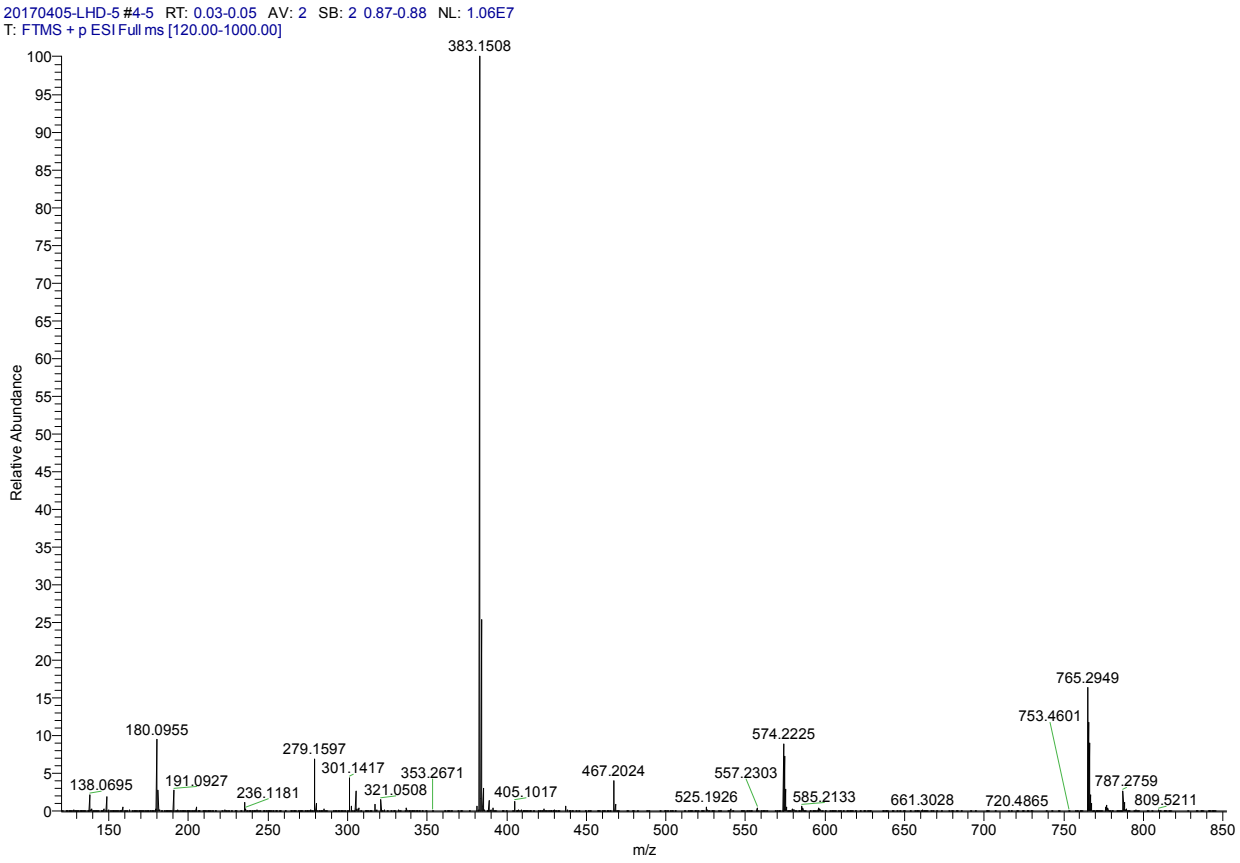


Fig. S27. ESI-HRMS of compound **DCM-APN**.

References

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