Electronic Supplementary Information

A novel near-infrared fluorescent probe for detection of early-stage Aβ protofibrils in Alzheimer's disease

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Contents

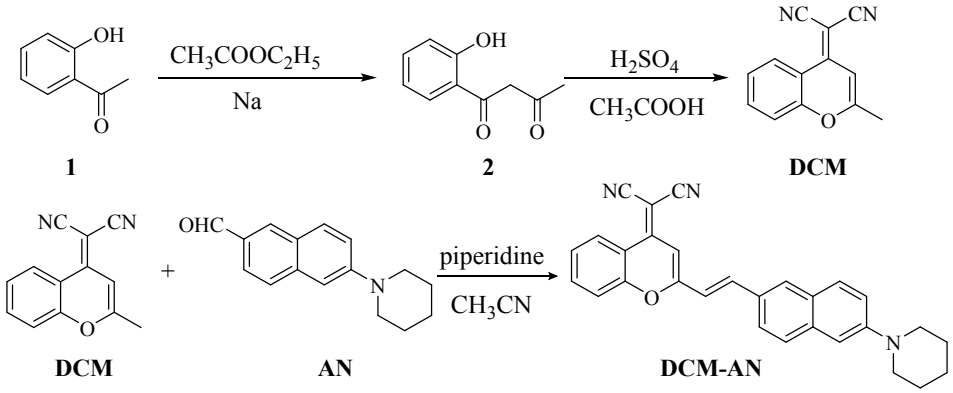
1. Materials and general methods

All of the starting materials were obtained from commercial suppliers and used as received. Moisture sensitive reactions were performed under an atmosphere of dry argon. 1-(2-Hydroxyphenyl)ethan-1-one and other chemicals were supplied from J&K Scientific Ltd. Aβ (1-42) monomer was purchased from GL Biochem (Shanghai) Ltd. Column chromatography was carried out on silica gel (200–300 mesh).

^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Mercury plus-Varian instrument. Proton chemical shifts are reported in parts per million downfield from tetramethylsilane (TMS). HR-MS was obtained on an LTQ-Orbitrap mass spectrometer (Thermo Fisher, San Jose, CA). UV-visible spectra were recorded on a Shimadzu UV-2550 spectrometer. Steady-state emission experiments at room temperature were measured on an Edinburgh instruments spectrometer (FS-920).

All the in *vivo* experiments were performed in compliance with the relevant laws and institutional guidelines, and the institutional committees of both Fudan University and Shanghai University of Medicine & Health Sciences have approved the experiments.

2. Synthesis details of DCM-AN



Scheme S1 The synthetic route of **DCM-AN**

AN was synthesized according to our previous report.[1]

Synthesis of DCM. Compound **1** (5.0 g, 36.5 mmol) was dissolved in 100 mL ethyl acetate (EA), and then sodium (4.0 g, 18.0 mmol) was added in the solution. The grayish-green solid was filtered after violently stirring for 4h at ambient temperature.

The solid was dissolved in 100 mL deionized water, followed by the adjustment of pH of the solution to neutral. The aqueous solution was extracted with 100 mL EA and the organic layer was dried over $MgSO_4$, filtered and concentrated to yield the final crude product **2** as a brown solid which was directly used in the next reaction without further purification.

Sulfuric acid (4.6 mL) was slowly added to an AcOH solution (70 mL) containing compound **2**. The mixture was refluxed for about 30 min and then was poured into 800 mL ice water, followed by the adjustment of pH of the solution to neutral with Na_2CO_3. The aqueous solution was extracted with methylene dichloride and the organic layer was dried over $MgSO_4$, filtered and concentrated to yield the final crude product DCM as an acicular gray solid (85% yield) via flash column chromatography (EtOAc: petroleum ether = 1: 5). ^{1}H NMR (400 MHz, CD_3CN) δ = 8.89 (d, J = 8.4 Hz, 1H), 7.72 (t, J = 7.6 Hz, 1H), 7.47 – 7.42 (m, 2H), 6.70 (s, 1H), 2.44 (s, 3H).

Synthesis of DCM-AN. To the mixture of DCM (43.7 mg, 0.21 mmol) and AN (54.9 mg, 0.23 mmol) in CH_3CN, piperidine (2.0 μL, 0.02 mmol) was added and the mixture was stirred at 50 ^{o}C under nitrogen for 12 hours. After completion, the crude mixture was concentrated under reduced pressure and the product was obtained via flash column chromatography (EtOAc: petroleum ether = 1: 5) as a dark red solid (83% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.87 (d, J = 8.3 Hz, 1H), 7.79 (s, 1H), 7.75 – 7.51 (m, 6H), 7.42 (t, J = 7.7 Hz, 1H), 7.29 (s, 1H), 6.75-6.72 (m, 2H), 5.30 (s, 1H), 3.34 (s, 4H), 1.77-1.66 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ = 157.9, 152.7, 152.3, 139.5, 136.0, 134.4, 130.0, 129.5, 127.5, 125.7, 123.6, 119.7, 118.5, 117.8, 117.1, 116.6, 116.0, 114.9, 106.3, 61.7, 50.00 (s), 29.7, 25.5, 24.2. HR-MS (ESI, m/z): calcd for $C_{29}H_{23}N_3O$ $[M+H]^+$, 430.1919, found 430.1924. FT-IR (cm^{-1}): 3352, 2954, 2922, 2852, 2207, 1603.

3. The measurement of K_d

The apparent binding constant (K_d) of DCM-AN to Aβ protofibril (5 μM) was measured from the double reciprocal of the fluorescence intensity maximum (F_{max}) and

the concentration of the probe.[2] The plot is shown in Fig. S1. The K_d corresponds to the -1/(x-intercept) of the linear regression.

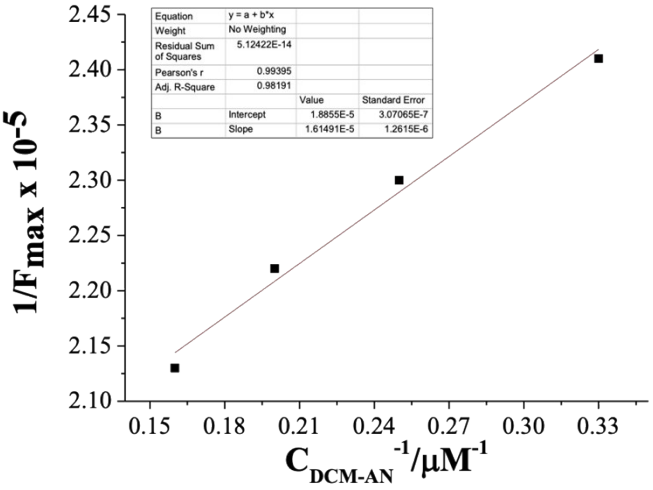


Fig. S1 Determination of the apparent binding constant (K_d) of probe ($R^2 = 0.98$) to Aβ protofibrils, λ_{ex}=500 nm.

4. Preparation of Aβ aggregates, oligomer and monomer

The preparation of Aβ species was depicted in our previous work.[1] Aβ monomer was purchased from GL Biochem (Shanghai) Ltd and further purified using HPLC. Purified monomer was stored in hexafluoroisopropanol (HFIP). 10 μL of Aβ monomer in HFIP were dried with argon gas and then reconstituted in 1.0 mL of distilled water.[4] This procedure is important for the following measurements.

Preparation of Aβ oligomers: the method was referred to Kayed's procedure reported in science (*Science*, 2003, **300**, 486). Briefly, Soluble oligomers were prepared by dissolving 1.0 mg Aβ in 400 μL hexafluoroisopropanol (HFIP) for 15 min at room temperature. 100 μL of the resulting seedless Aβ solution was added to 900 μL dd H_2O in a siliconized Eppendorf tube. After 15 min incubation at room temperature, the samples were centrifuged for 15 min. at 10,000 rpm and the supernatant fraction (pH 2.8-3.5) was transferred to a new siliconized tube and subjected to a gentle stream of N_2 for 5-10 min to evaporate the HFIP. The samples were then stirred at 500 rpm using a Teflon coated micro stir bar for 24 h at 22 °C.

Preparation of Aβ aggregates: Briefly, Aβ (1-42) monomer was dissolved in phosphate buffered saline (PBS, pH = 7.31). This solution was magnetically stirred at

1200 rpm for three days at room temperature. Then pre-aggregated Aβ solution was obtained.

Preparation of Aβ protofibrils: This was performed according to Lashuel's reported procedure.[3] Briefly, 1.0 mg Aβ monomer was dissolved in 50 μL anhydrous DMSO, and then 800 μL ultrapure water was carefully added. After that, 10 μL 2M Tris-base solution (pH=7.5) was immediately added and this solution was incubated at room temperature for 5 minutes. The obtained Aβ protofibrils were confirmed by dynamic light scattering (DLS) and TEM (Figure S2).

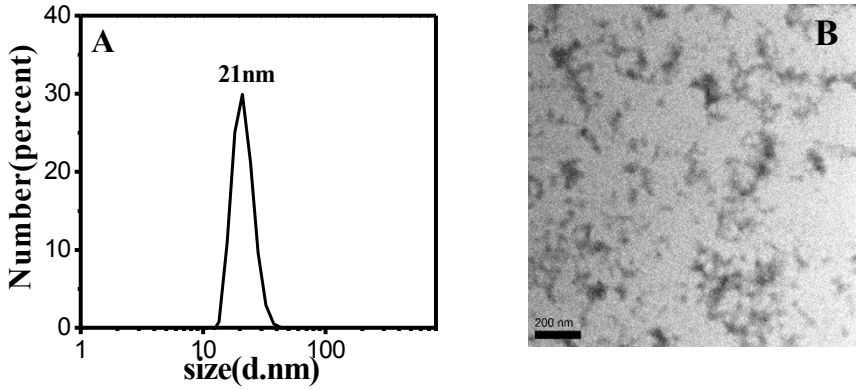


Fig. S2 Aβ protofibril was measured by DLS (A) and TEM (B, negative staining with PTA).

5. Computational Methods

Quantum mechanical calculations

The geometry optimization for **DCM-AN** molecule was performed by using density functional theory at the B3LYP/6-31G* level at the gas phase using Gaussian 09 program.[8]

Molecular docking search

DCM-AN docking searches with Aβ trimer and twelve Aβ monomers (named twelve polymer) were executed by using Autodock vina1.1.2 software package. The docking simulations were carried out with a box centered on the Aβ and employing 50 × 50 × 50 grid points. X-ray RCSB database (PDB ID: 4NTR[9] and 2MXU[10]) determined for trimer and twelve polymer of β-amyloid peptide were employed for structure models of Aβ oligomer and toxic Aβ protofibrils, respectively. In addition,

two other Aβ structures (PDB ID: 5KK3[11] and 2LMP[12]) which were more complicated than 4NTR and 2MXU, were chosen as working models for Aβ aggregates.

Molecular dynamics (MD) simulations

We performed all-atom, explicit-water MD simulations using NAMD soft package in Charmm force field.[13] Cl ions were added to keep system neutral and a TIP3P periodic water box was added, 9208 water molecules were added into the system. Then a 1000 steps energy minimization was carry out at 298 K, 1 BAR condition. Finally, MD simulation runs at the same temperature and pressure. Other parameters were the default value of the software.

6. AD transgenic mouse model

A triple transgenic model for Alzheimer's disease was used to examine the possible co-localization of **DCM-AN** probe staining with Aβ or tau immunoreactivity. The triple transgenic mice were generated by crossing 5XFAD transgenic mice with the tau P301S single transgenic mice. The 5XFAD transgenic mice [5] and P301S tau transgenic mice [6] were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). To maintain on a C57BL/6J background, the original 5XFAD mice were backcrossed to C57BL/6J mice for eight generations. These 5XFAD transgenic mice overexpress both mutant human APP (695) with the Swedish (K670N, M671L), Florida (I716V), and London (V717I) familial Alzheimer's disease (FAD) mutations and human PS1 harboring two FAD mutations, M146L and L286V. Expression of two transgenes is regulated by neural-specific elements of the mouse Thy1 promoter. Mutant tau transgenic mice express the P301S mutant form of human microtubule-associated protein tau with one N-terminal insert and four microtubule binding repeats, under control of the mouse prion promoter. Mice were genotyped by PCR as described previously. Male or female mice at age of 4-15 month-old were used in this study.[7] Animals were handled in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals.

7. Colocalization of probe labeling and AD pathology in the transgenic mouse brain

The brain tissue and immunofuorescent labeling were performed as previously described.[7] Mice were deeply anesthetized and transcardially perfused with PBS, followed by 8% formadehyde (Sigma-Aldrich) in PBS (pH = 7.4). The brains were post-fixed and frozen-sectioned (14 μm thickness) with a microtome (Leica Microsystems, GmbH, Wetzlar, Germany) for further analyses. Briefly, free-floating sections were blocked in a blocking solution containing 10% goat serum, 1% BSA and 0.4% Triton X-100. Incubation with primary antibodies took place overnight at 4 °C. Then, one of following antibodies was employed each for colocalization studies: β-Amyloid oligomer-specific monoclonal antibody (Agrisera, Sweden), β-Amyloid monoclonal antibody (Sigma-Aldrich), Aβ40 and Aβ42 polyclonal antibodies (Cell Signaling), or conformation-dependent tau antibody MC1 (Peter Davies), at dilutions from 1:400 to 1:100. After rinsing, sections were incubated in the solution of goat anti-mouse or goat anti-rabbit secondary antibody conjugated with Alexa 488 (Molecular Probes, Carlsbad, CA, USA) for 2 h at room temperature. The images for Aβ or tau immunofuorescent reactivity followed by **DCM-AN** probe (40 μM) for Aβ antibody were taken sequentially using a microscope (BX51, Olympus, Japan) equipped with a DP72 digital camera.

8. Cell viability

The cytotoxicity was performed by 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) assay with RAW cell lines. Cells growing in log phase were planted into a 96-well cell culture plate at 1×10^5/ well. The cells were incubated for 12 h at 37 ^oC under 5% CO_2 in an incubator. A solution of **DCM-AN** (100.0 μL/well) at concentrations of 1.0, 2.5, 5.0, 10.0, 25.0, 50.0, 100.0 μM in nutrient with 10‰ DMSO was added to the wells of the treatment group, respectively. The cells were subsequently incubated for 12 h and 36 h at 37 ^oC under 5% CO_2. Thereafter, MTT (0.5 mg/mL) was added to each well and the plate was incubated for an additional 4 h at 37 ^oC under 5% CO_2. The optical density OD490 value (Abs.) of each well was measured by means of a Tecan Infinite M200 monochromator-based multifunction microplate reader, which was used as cell viability.

9. Additional absorption and fluorescent spectra

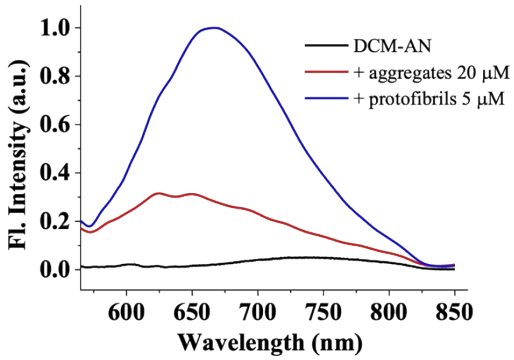


Fig. S3 Fluorescence spectrum of **DCM-AN** (2.0 μM) with Aβ protofibrils (5.0 μM) and Aβ aggregates (20.0 μM) , λ_{ex}=500 nm.

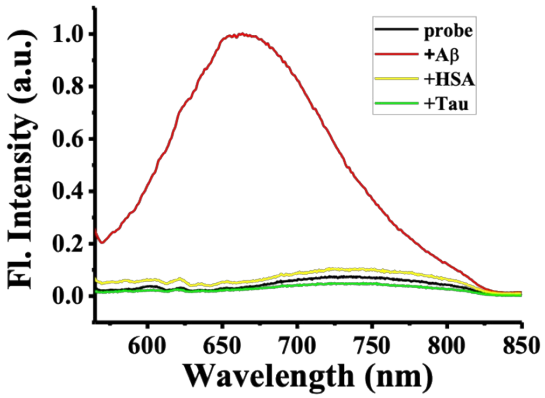


Fig. S4 Fluorescence spectra of **DCM-AN** (2.0 μM) with HSA (5.0 μM) and tau protein (5.0 μM), λ_{ex}=500 nm.

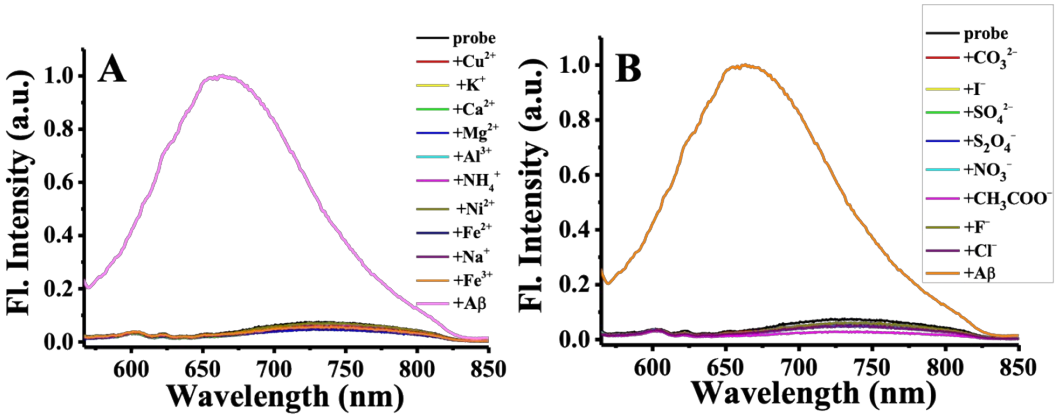


Fig. S5 Fluorescence spectra of **DCM-AN** (2.0 μM) with a series of cations (5.0 μM) including Cu^{2+}, K^{+}, Ca^{2+}, Mg^{2+}, Al^{3+}, NH_4^{+}, Ni^{2+}, Fe^{2+}, Na^{+}, and Fe^{3+}, and anions (5.0 μM) including CO_3^{2-}, I^{-}, SO_4^{2-}, $S_2O_4^{-}$, NO_3^{-}, CH_3COO^{-}, F^{-}, and Cl^{-}, λ_{ex}=500 nm.

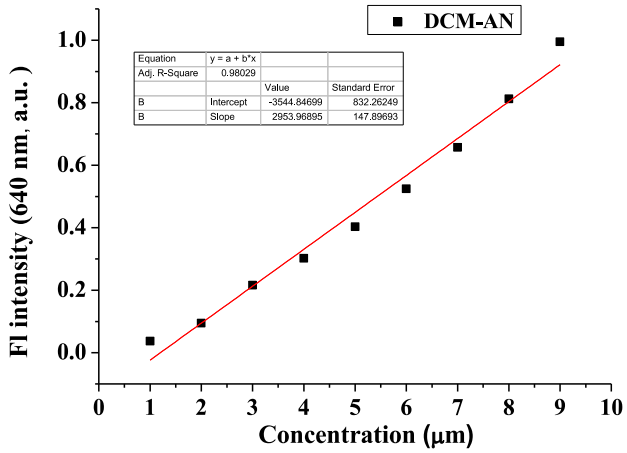


Fig. S6 The linear relationship of fluorescence intensity at 661 nm and the concentration of Aβ protofibrils. λ_{ex}=500 nm

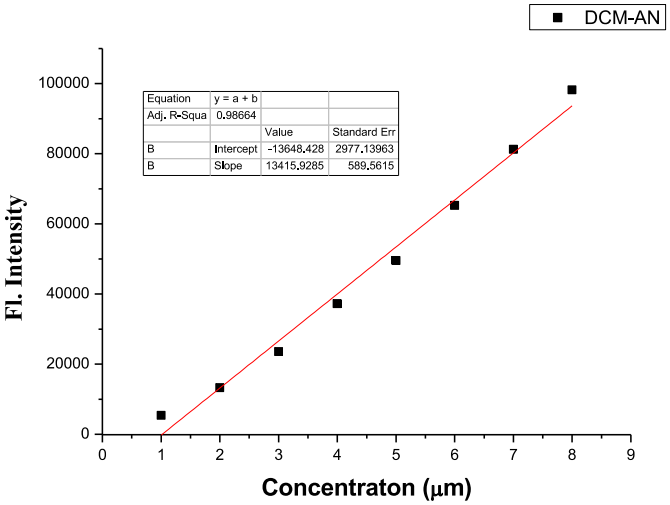


Fig. S7 The detection limit was calculated with 3σ/k， where σ is the standard deviation of blank measurement and k is the slope in this figure. λ_{ex}=500 nm

10. Working models for theoretical calculations

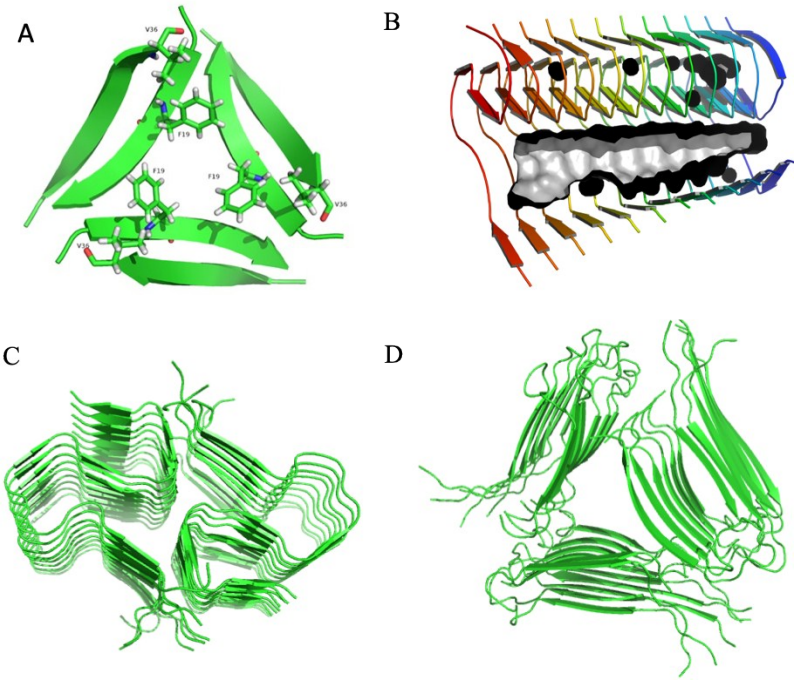


Fig. S8 Working models for (A) Aβ trimer, (B) Aβ twelve polymer, (C and D) Aβ aggregates.

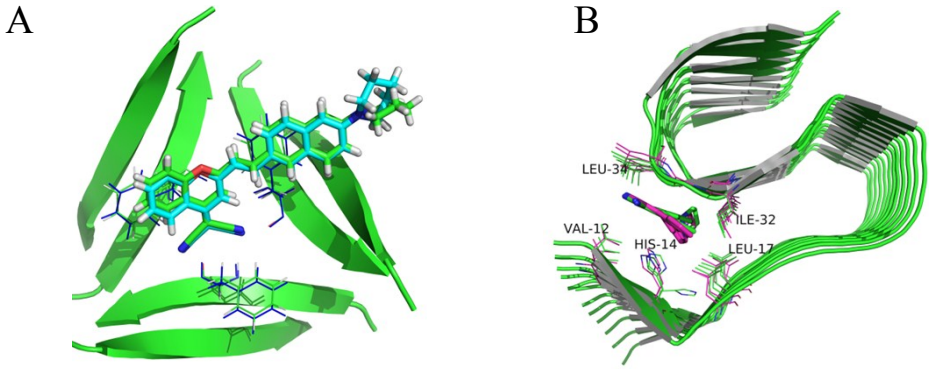


Fig. S9 The Molecular dynamics simulations of **DCM-AN** with (A) Aβ trimer and (B) Aβ twelve polymer.

Table S1 SASA shrinks before and after binding with two working models

	Before binding	After binding	Shrink (ΔSASA)
Twelve-polymer	15300.857 Å^2	15159.366 Å^2	141.491 Å^2
Trimer	24703.236 Å^2	24572.652 Å^2	130.584 Å^2
Aggregates Fig. S4 C	18634.187 Å^2	18628.119 Å^2	6.068 Å^2
Aggregates Fig. S4 D	28305.437 Å^2	28288.730 Å^2	16.707 ^{2}

11. Toxicity of DCM-AN and additional brain section images

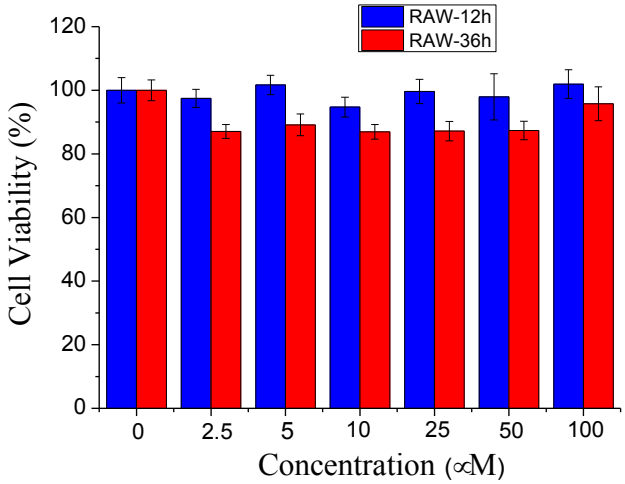


Fig. S10 Cell viability values (%) estimated by MTT assay RAW cells, which were cultured in the presence of 0-100 μM **DCM-AN** for 12 h and 36 h at 37 °C.

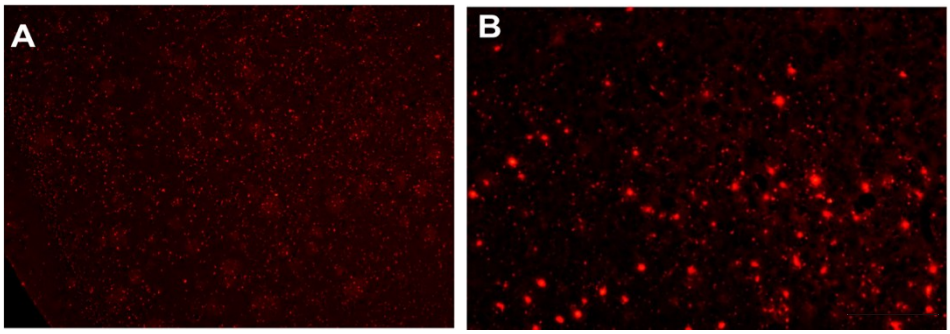


Fig. S11 The fluorescent images of **DCM-AN** *in vitro* labeling on brain sections from an AD transgenic mouse model. A: negative control without **DCM-AN** labeling; B: **DCM-AN** (0.1 μM) *in vitro* labeling. Scale bar: 200 μm.

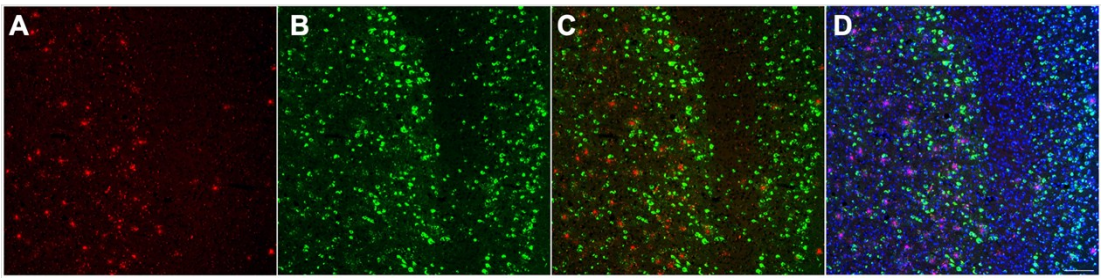


Fig. S12 Colocalization of **DCM-AN** labeling with pathological tau antibody immunostaining was examined in the brain sections of triple transgenic mice for AD. (A) **DCM-AN** staining; (B) MC1 immunostaining for pathological conformation of tau; (C) the merged images; (D) the merged image counter-stained with DAPI. Scale bar: 100 μm.

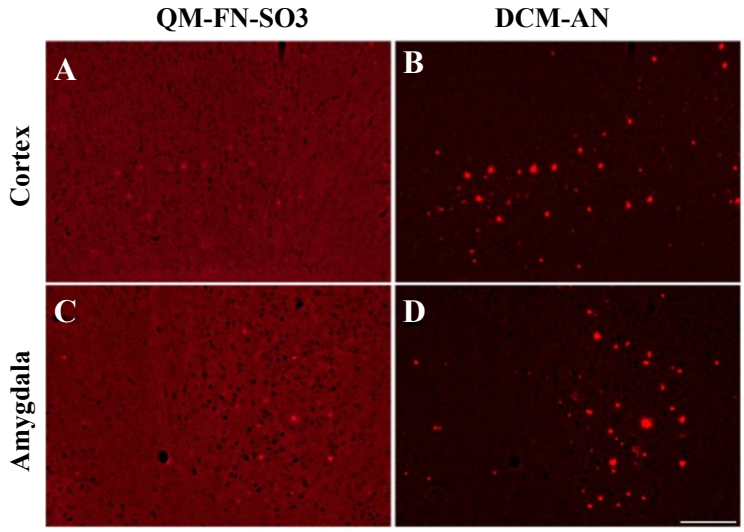


Fig. S13 Capability of **DCM-AN** in detecting early Aβ pathology as compared with that of a Aβ aggregate probe QM-FN-SO3. A and C were stained with QM-FN-SO3; B and D were stained with DCM-AN. A and B were from cortex; C and D were from amygdala. The brain sections from 4-month-old APP/PS1 transgenic mice were used for this comparative study. Scale bar: 200 μm.

12. Characteristic of the compounds

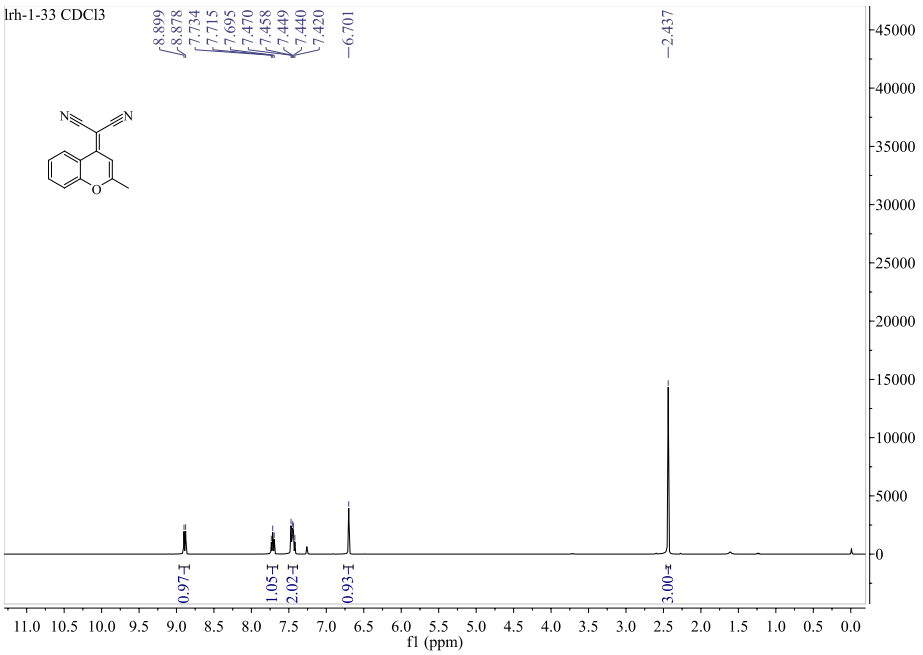


Fig. S14 The ^{1}H NMR spectrum of DCM (400 MHz, CD_3Cl).

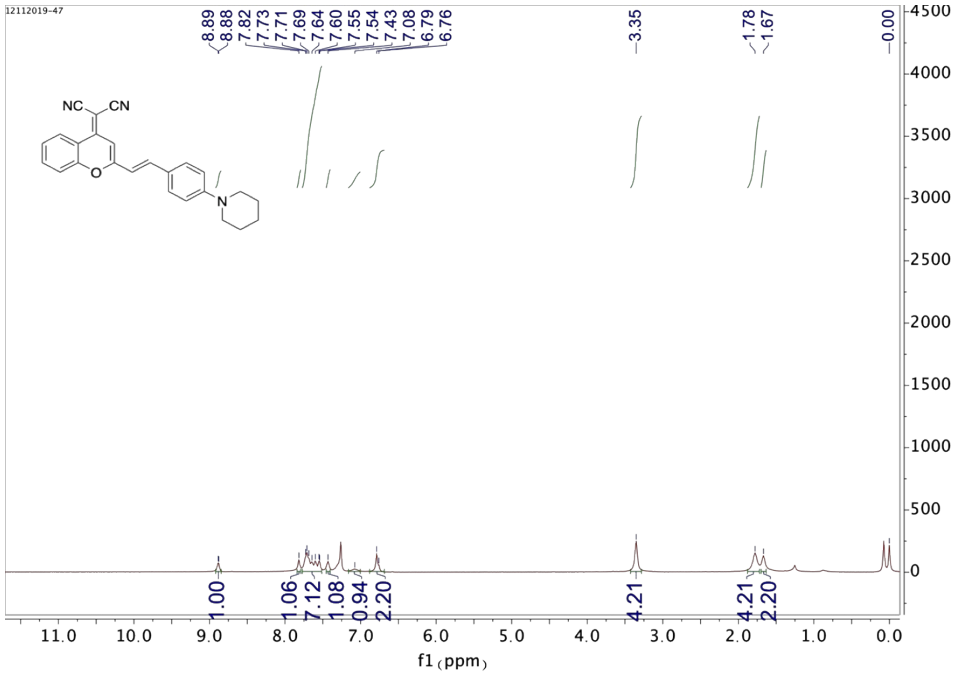


Fig. S15 The ^{1}H NMR spectrum of **DCM-AN** (400 MHz, CD_3Cl).

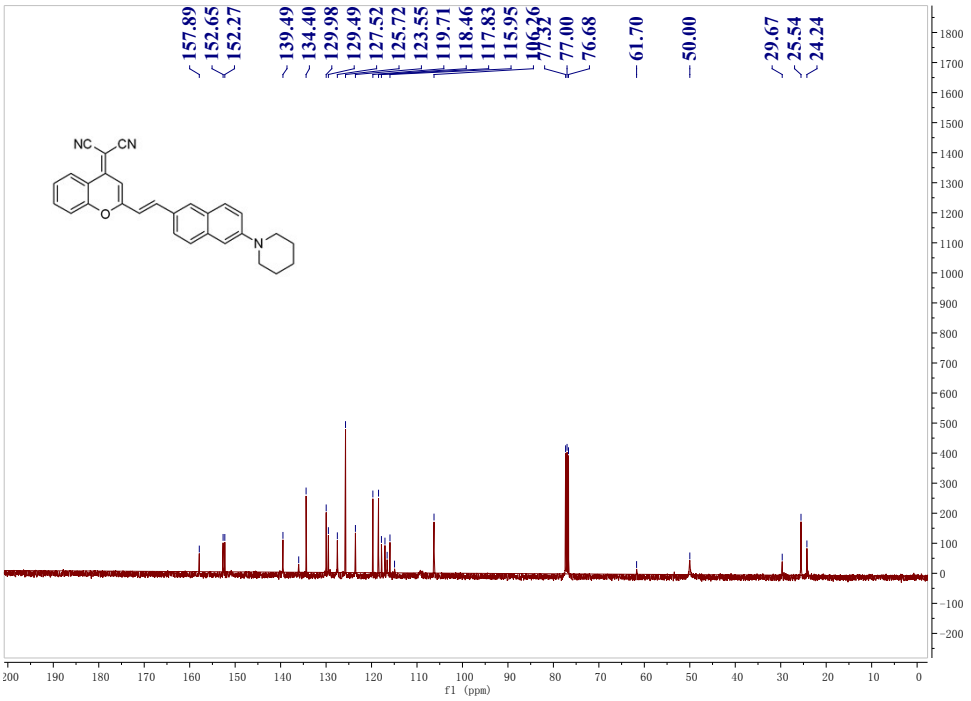


Fig. S16 The ^{13}C NMR spectrum of **DCM-AN** (100MHz, $CDCl_3$).

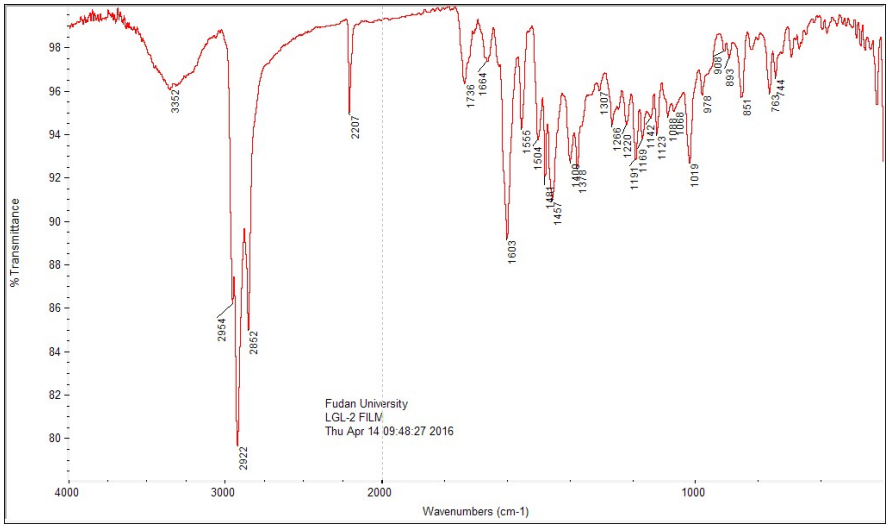


Fig. S17 The IR of **DCM-AN.** FT-IR (cm^{-1}): 2925 cm^{-1} (asymmetrical stretching vibration of CH_2, $\nu_{as\ CH2}$); 2850 cm^{-1} (symmetrical stretching vibration of CH_2, $\nu_{s\ CH2}$); 2207 cm^{-1} (stretching vibration of CN, ν_{CN}); 1603 cm^{-1} (stretching vibration of C=C, $\nu_{C=C}$); 1266 cm^{-1} (stretching vibration of C-N, ν_{C-N}); 1019 cm^{-1} (symmetrical stretching vibration of C-O-C, $\nu_{asC-O-C}$).

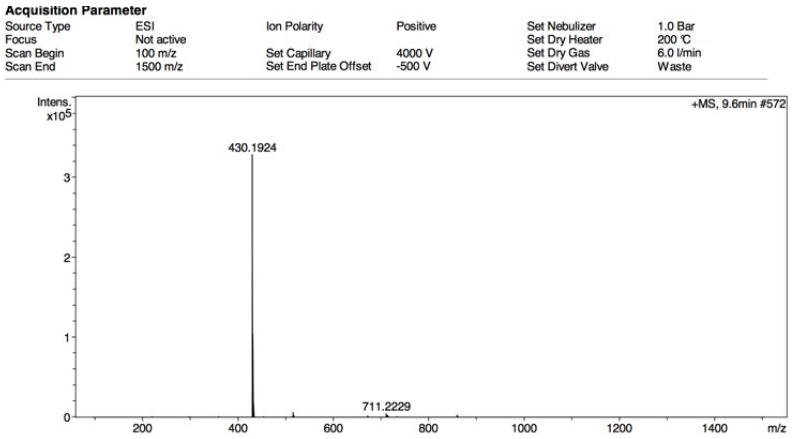


Fig. S18 The HRMS of **DCM-AN.**

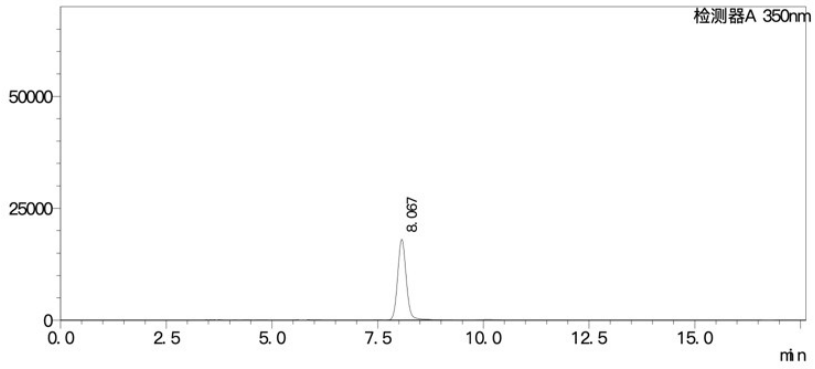


Fig. S19 The HPLC of **DCM-AN** (mobile phase: acetonitrile, flow rate: 1.0 mL/min, detection wavelength: 350 nm).

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