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Supporting Information

Two-photon fluorescence imaging of the cerebral peroxynitrite stress in Alzheimer's disease

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1. Materials and instruments

Phosphate buffer solutions were prepared using distilled water. Deferiprone (DFP) was obtained from Macklin. Ferrostatin-1 (Fer-1) was bought from MedChemExpress. 3-Morpholinosydnonimine hydrochloride (SIN-1) was acquired from Sigma-Aldrich. Human $A\beta_{42}$ peptide was obtained from GL Biochem (Shanghai) Ltd. DMEM medium, fetal bovine serum, penicillin, and streptomycin were obtained from Gibco Invitrogen. Natural products and other chemical reagents were acquired from commercial sources and directly used without further purification.

Peroxynitrite ($ONOO^-$) solution was prepared as previous method. Briefly, a mixture of sodium nitrite (0.6 M) and hydrogen peroxide (0.7 M) was acidified with hydrochloric acid (0.6 M), and sodium hydroxide (1.5 M) was added within 1−2 s to make the solution alkaline. The $ONOO^-$ concentration was estimated by using an extinction coefficient of 1670 $M^{-1}cm^{-1}$ at 302 nm. $C_{ONOO^-} = Abs_{302\ nm}/1.67$ (mM). Hydrogen peroxide (H_2O_2), hypochlorite (ClO^-) and *tert*-butyl hydroperoxide (*t*-BuOOH) were delivered from commercial aqueous solutions respectively. Superoxide solution ($O_2^{\bullet-}$) was prepared by adding KO_2 to dry dimethylsulfoxide and stirring vigorously for 10 min. Hydroxyl radicals ($^{\bullet}OH$) was generated *in situ* by reaction of Fe^{2+} with H_2O_2. Singlet oxygen ($^1O_2$) was generated *in situ* by addition of the H_2O_2 stock solution into a solution containing 10 equivalents of HClO. Nitric oxide (NO) was used from a stock solution prepared by sodium nitroprusside. Hydrogen sulfide (H_2S) was prepared from NaHS solid, and SO_3^{2-} was prepared from Na_2SO_3 solid. Additionally, the rest of reactive species (Cys, Hcy, and GSH), anions (NO_3^-, NO_2^-, CH_3COO^-, SO_4^{2-}, CO_3^{2-}, and PO_4^{3-}), and metal ions (Na^+, K^+, Ca^{2+}, Zn^{2+}, Mg^{2+}, Cu^{2+}, Fe^{2+}, and Fe^{3+}) were prepared by dissolving the corresponding solid into ultrapure water.

NMR spectroscopic characterization was taken on a Bruker Advance 400 MHz spectrometer. HRMS spectra were obtained by a Bruker MaXis UHR-TOF

instrument. Fluorescence measurements were done using a FLS-980 fluorospectrometer from Edinburgh Instruments or Hitachi F-4700 fluorescence spectrophotometer. The response mechanism of probe was monitored by HPLC-MS on an Ultimate 3000 LC system coupled with a Bruker MaXis UHR-TOF mass spectrometer. Two-photon fluorescence imaging of live cells and mice was performed on a Zeiss LSM 880 confocal laser scanning microscope.

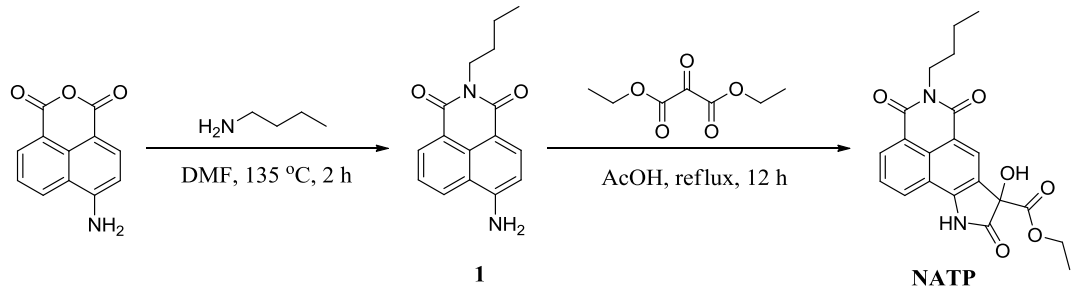
2. Photophysical property characterization

The probe was dissolved in DMSO to produce 1 mM stock solution, which was diluted to 2 μM as the testing solutions with phosphate buffer (50 mM, pH 7.4).

To test the fluorescence responses of the probe towards $ONOO^-$ or other analytes, aliquots of probe stock solutions were diluted with phosphate buffer (50 mM, pH 7.4) and treated with analytes to make sure both the probe and analytes were kept at desired final concentrations. The testing solutions were quickly and vigorously shaken. The fluorescence spectra were recorded immediately or after incubation at 37 ℃ for 10 min with excitation at 445 nm.

To test the fluorescence responses of the probe towards $ONOO^-$ at different pH values, aliquots of probe stock solutions were diluted with universal buffer solution (0.1 M citric acid, 0.1 M KH_2PO_4, 0.1 M $Na_2B_4O_7$, 0.1 M Tris, and 0.1 M KCl) of specified pH value and treated with $ONOO^-$ to make sure both the probe and $ONOO^-$ were kept at desired final concentrations. After quick and vigorous shaking, the fluorescence intensity was measured after incubation at 37 ℃ for 10 min with excitation at 445 nm.

3. Synthetic procedures



Scheme S1. Synthetic pathway of NATP

NATP was readily prepared through a cyclization reaction from 4-amino-1,8-naphthalimide derivative outlined in Scheme S1. All chemical reagents were acquired from commercial sources and directly used without further purification. The solvents were distilled or purified before use if necessary.

Synthesis of Compound 1. To a solution of 4-amino-1,8-naphthalic anhydride (CAS 6492-86-0, 426 mg, 2.0 mmol) in 10 mL DMF was added 1-butylamine (CAS 109-73-9, 1.96 mL, 20.0 mmol). The resulting mixture was stirred at 135 ℃ for 2 h and then concentrated. The residue was purified by flash column chromatography on silica gel with ethyl acetate and petroleum ether (1/3, V/V) to afford a solid (429 mg, 80%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.60 (d, J = 8.3 Hz, 1H), 8.41 (d, J = 7.9 Hz, 1H), 8.18 (d, J = 8.4 Hz, 1H), 7.69−7.58 (m, 1H), 7.42 (s, 2H), 6.83 (d, J = 8.4 Hz, 1H), 4.01 (t, J = 7.2 Hz, 2H), 1.65−1.50 (m, 2H), 1.41−1.25 (m, 2H), 0.91 (t, J = 7.3 Hz, 3H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6): δ 163.72, 162.87, 152.64, 133.87, 130.91, 129.64, 129.21, 123.90, 121.77, 119.35, 108.13, 107.59, 29.84, 19.86, 13.75 ppm.

Synthesis of probe NATP. To a solution of Compound 1 (268 mg, 1.0 mmol) in 3 mL acetic acid was added diethyl ketomalonate (348 mg, 2.0 mmol). The resulting mixture was stirred at reflux for 12 h. The solvent was evaporated, and the residue was purified using flash column chromatography on silica gel using dichloromethane and methanol (35/1, V/V) as eluent to obtain a solid (344 mg, 86%). HRMS (ESI):

calculated for $C_{21}H_{19}N_2O_6^-$ $(M-H)^-$ 395.1249, found 395.1308. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.13 (s, 1H), 8.56 (d, J = 8.4 Hz, 1H), 8.52 (d, J = 7.1 Hz, 1H), 8.29 (s, 1H), 7.89 (t, J = 7.8 Hz, 1H), 7.53 (s, 1H), 4.19 (dq, J = 11.2, 7.1 Hz, 1H), 4.12−4.04 (m, 1H), 4.01 (t, J = 7.2 Hz, 2H), 1.68−1.47 (m, 2H), 1.38−1.28 (m, 2H), 1.07 (t, J = 7.1 Hz, 3H), 0.91 (t, J = 7.3 Hz, 3H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6): δ 175.68, 168.46, 163.19, 162.99, 146.47, 132.19, 129.23, 129.10, 127.09, 125.81, 124.79, 122.64, 117.11, 115.83, 77.67, 61.94, 29.68, 19.85, 13.91, 13.78 ppm.

4. Absorption spectra

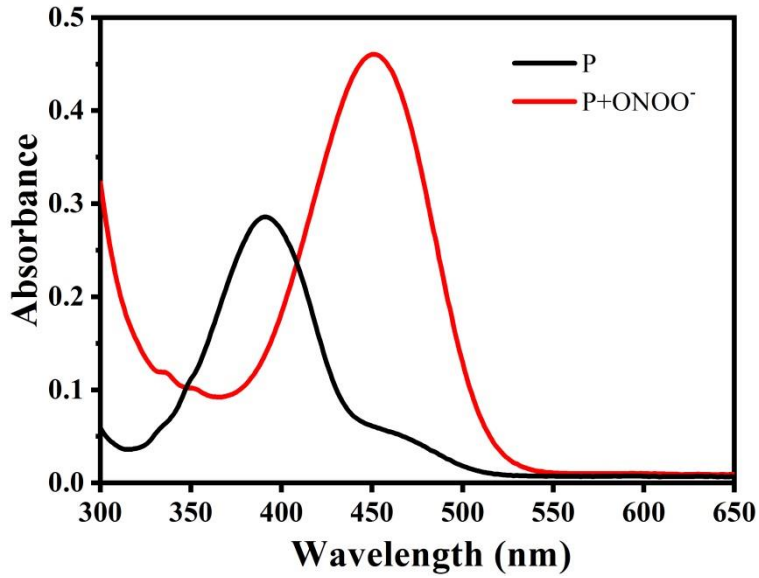


Fig. S1 Absorption spectra of NATP in the absence (black line) and presence (red line) of $ONOO^-$. The final concentration of NATP was 5 μM, and $ONOO^-$ concentration was 30 μM. Data were acquired in phosphate buffer (50 mM, pH 7.4) after incubation at 37 ℃ for 10 min.

5. Specificity evaluation

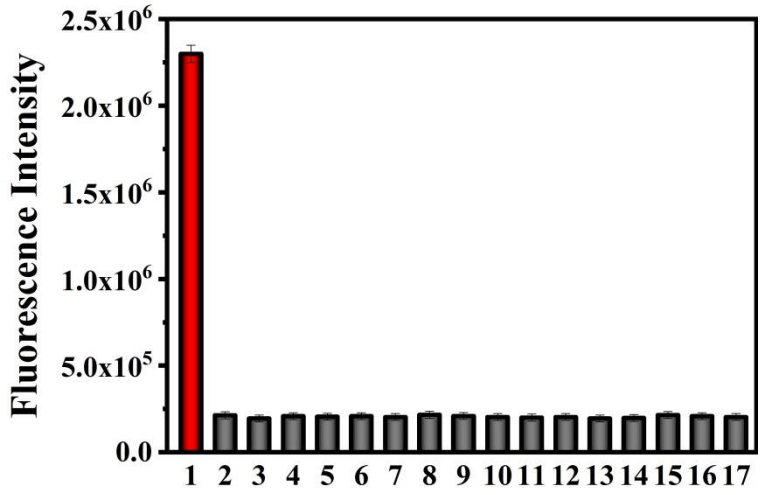


Fig. S2 Fluorescence intensities of NATP at 565 nm with 100 μM analytes unless otherwise noted: (1) 18 μM $ONOO^-$, (2) blank, (3) NO_3^-, (4) NO_2^-, (5) CH_3COO^-, (6) SO_4^{2-}, (7) SO_3^{2-}, (8) CO_3^{2-}, (9) PO_4^{3-}, (10) Na^+, (11) K^+, (12) Ca^{2+}, (13) Mg^{2+}, (14) Zn^{2+}, (15) Cu^{2+}, (16) Fe^{2+}, and (17) Fe^{3+}. The final concentration of NATP was 2 μM. All tests were done in 50 mM phosphate buffer (pH 7.4, 2‰ DMSO) at 37 ℃ with excitation at 445 nm.

6. Effect of pH value

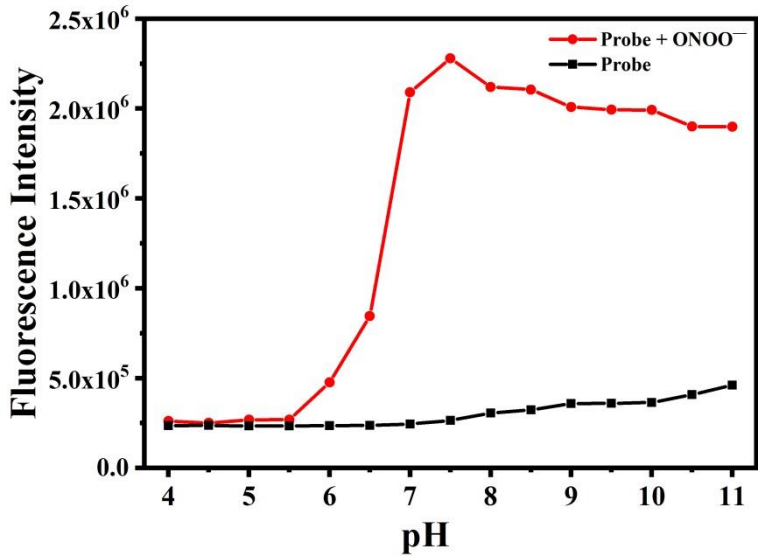


Fig. S3 Fluorescence responses of NATP in the absence and presence of $ONOO^-$ in the pH range 4.0−11.0. The final concentration of NATP was 2 μM. The final

$ONOO^-$ concentration was 18 μM. Fluorescence intensity at 565 nm was acquired in universal buffer solution (0.1 M citric acid, 0.1 M KH_2PO_4, 0.1 M $Na_2B_4O_7$, 0.1 M Tris, and 0.1 M KCl) containing 2‰ DMSO after incubation at 37 ℃ for 10 min, with excitation at 445 nm.

7. Mechanism investigation

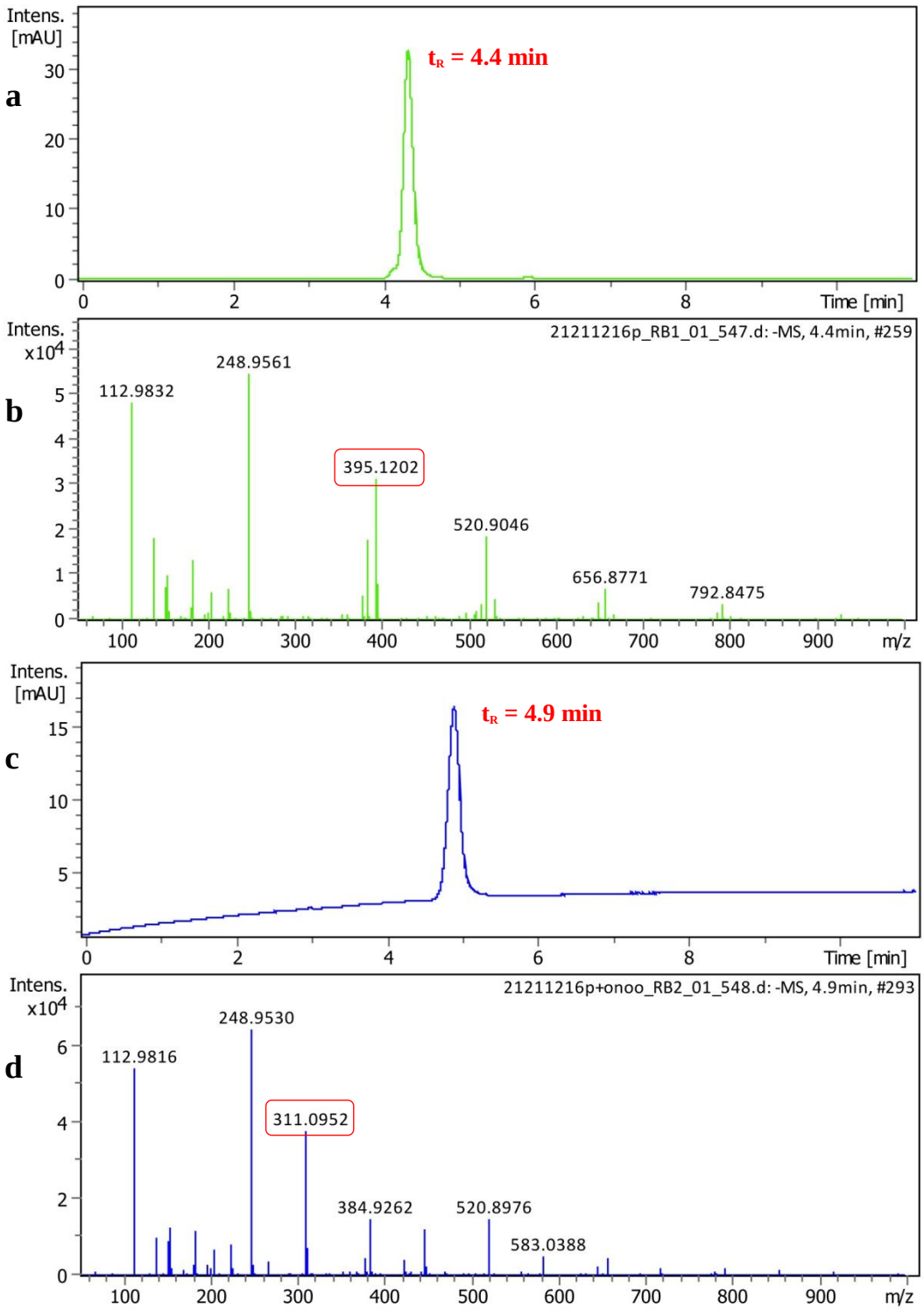


Fig. S4 HPLC-MS analysis of the reaction product of NATP with $ONOO^-$. NATP (100 μM) was incubated with 10 equivalents of $ONOO^-$ at 37 ℃, and then characterized by HPLC-MS spectrometry. (a) HPLC chromatogram of NATP alone.

(b) The corresponding MS data of the chromatographic peak at 4.4 min in Fig. S4a. (c) HPLC chromatogram of NATP incubated with $ONOO^-$. (d) The corresponding MS data of the chromatographic peak at 4.9 min in Fig. S4c.

To further confirm its chemical structure, the reaction product of NATP and $ONOO^-$ was extracted, separated, and subjected to ^1H NMR spectroscopy analysis. ^1H NMR (400 MHz, DMSO-d_6): δ 10.96 (br s, 1H), 9.01 (s, 1H), 8.76 (d, J = 8.4 Hz, 1H), 8.39 (d, J = 7.1 Hz, 1H), 8.20 (br s, 2H), 7.61 (t, J = 7.8 Hz, 1H), 4.01 (t, J = 7.2 Hz, 2H), 1.68−1.47 (m, 2H), 1.38−1.28 (m, 2H), 0.91 (t, J = 7.3 Hz, 3H) ppm. The ^1H NMR spectra of NATP and its product were compared in Fig. S5. The *n*-butyl protons locate at δ 4.01, 1.68−1.47, 1.38−1.28, and 0.91 ppm respectively, and display no obvious chemical shift after reaction (marked with red). The aromatic protons appear at δ 8.56, 8.52, 8.29, and 7.89 ppm, and shift to δ 8.76, 8.39, 9.01, and 7.61 ppm respectively after reaction (marked with pink). The protons of amide (CONH), hydroxyl (OH), and ethoxyl (OCH_2CH_3) of NATP locate at δ 12.13, 7.53, 4.19, 4.12−4.04, and 1.07 ppm, and completely disappear after reaction (marked with blue). Additionally, after reaction, the resonances at δ 10.96 and 8.20 ppm emerge as broad singlets and are attributing to the active protons of carboxyl (COOH) and amino (NH_2) groups of the reaction product (marked with green). The singles and chemical shift variations are consistent with the chemical conversion shown in Scheme 1.

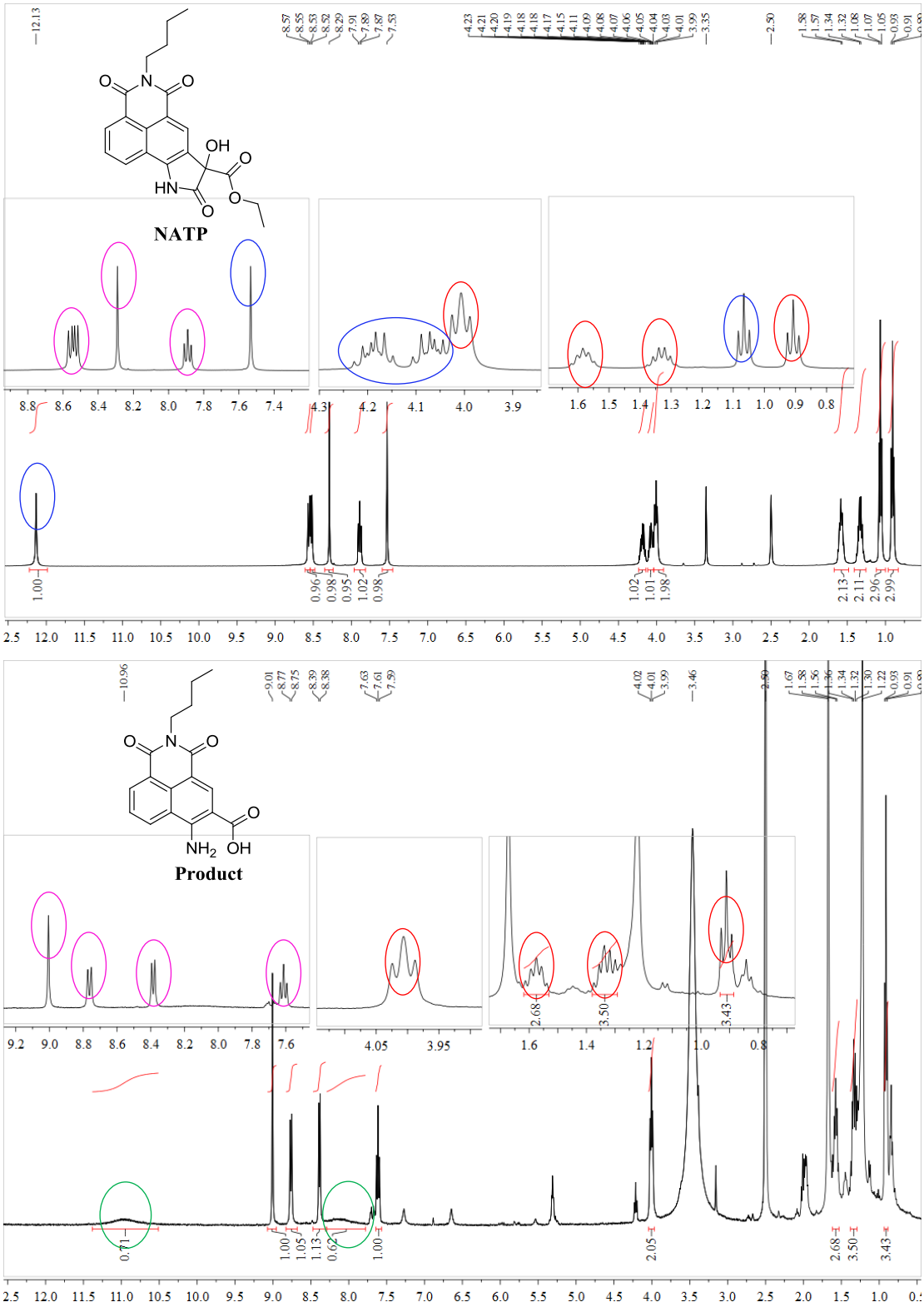
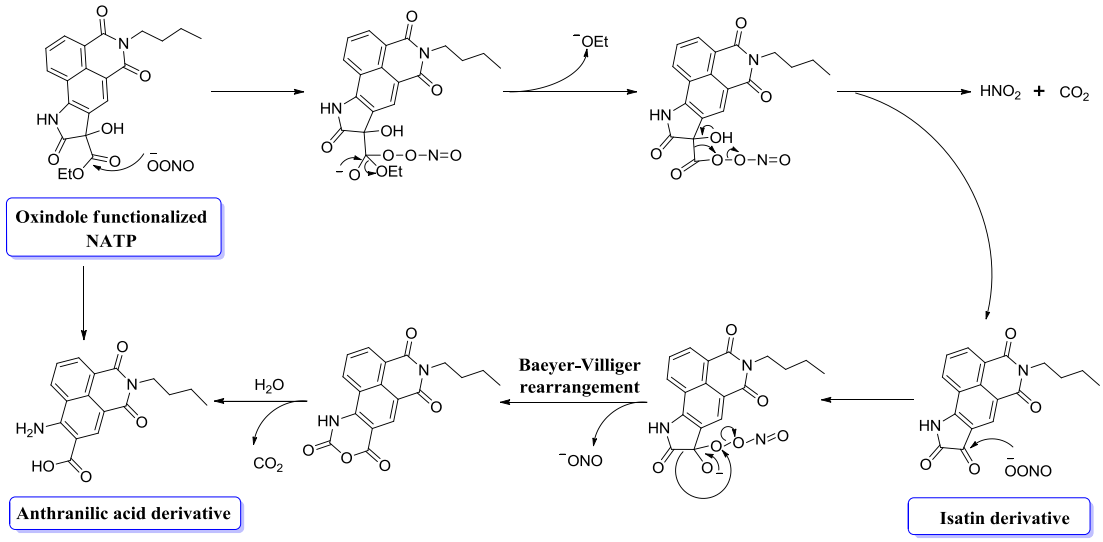


Fig. S5 ^{1}H NMR spectra of NATP and its reaction product with $ONOO^{-}$.



Scheme S2. Proposed reaction mechanism of $ONOO^-$ and oxindole functionalized probe NATP.

8. MTT assay

PC12 cells were seeded into a 96-well microtiter plate at 37 ℃ in a 5% CO_2/95% air incubator for 24 h. The cells were incubated for an additional 12 h with different concentrations of tested probe (0, 1, 10, 20, 50, and 100 μM), respectively. Then the cells were washed with PBS three times. Subsequently, MTT solution (200 μL, 0.5 mg/mL) was added to each well and the cells were incubated at 37 ℃. After 4 h, the remaining MTT was removed, and the formazan crystals were dissolved in 200 μL of DMSO with gentle agitation for 5 min. The absorbance at 490 nm was measured using a TRITURUS microplate reader.

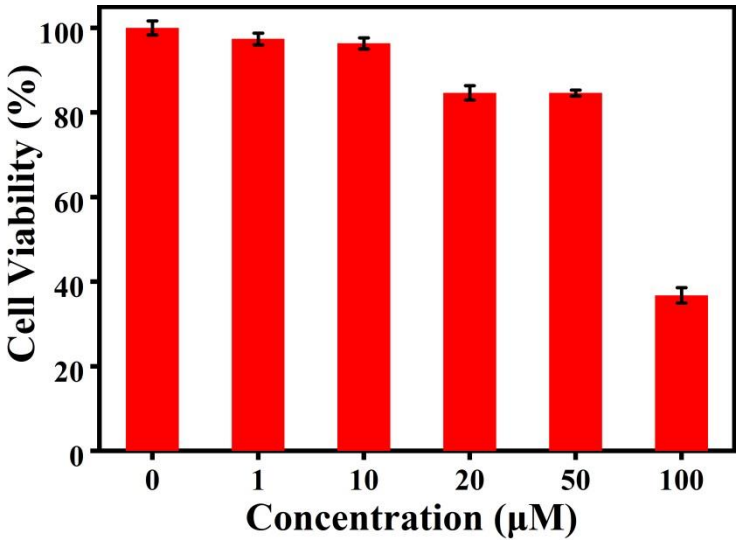


Fig. S6 MTT assay of PC12 cells with different concentrations of NATP. The IC_{50} value was calculated to be 73 μM.

9. Cell culture and imaging

PC12 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin, and 1% streptomycin at 37 ℃ in a 5% CO_2/95% air incubator (MCO-5AC, Sanyo). One day before imaging, the cells were detached and replanted on glass-bottomed dishes. Cell imaging was performed on a Zeiss LSM 880 confocal microscope. The emission of NATP was collected at 535−595 nm with two-photon excitation at 800 nm.

For monitoring of $ONOO^-$ released from SIN-1 in live cells, PC12 cells were pretreated with 500 μM SIN-1 for 30 min before being washed three times with PBS; then, they were incubated with 5 μM NATP for 20 min before being rinsed with PBS. For the negative control group, the cells were pretreated with PBS instead of SIN-1. For the scavenging assay, the cells were treated with 100 μM uric acid for 3 h in advance. All cells were washed three times with PBS before cell imaging.

For monitoring of $ONOO^-$ variations induced by $A\beta_{42}$ peptide with varied concentrations, PC12 cells were divided into six parallel groups and pretreated with 0, 5, 10, 15, 20, or 30 μM $A\beta_{42}$ peptide for 24 h respectively. Then they were incubated with 5 μM NATP for 20 min before being rinsed with PBS.

For monitoring of $ONOO^-$ variations induced by $A\beta_{42}$ peptide with different incubation time, PC12 cells were divided into six parallel groups and pretreated with 30 μM $A\beta_{42}$ peptide for 0, 6, 8, 12, 16, or 24 h respectively. Then they were incubated with 5 μM NATP for 20 min before being rinsed with PBS.

For monitoring of $ONOO^-$ during Aβ induced ferroptosis, PC12 cells were divided into six parallel groups. Cells in groups (b) and (f) were pretreated with 30 μM $A\beta_{42}$ peptide for 16 h before being washed three times with PBS; then, they were incubated with 5 μM NATP for 20 min before being rinsed with PBS. For the negative control groups (a) and (e), the cells were pretreated with PBS instead of $A\beta_{42}$ peptide. For the inhibiting group (c), the cells were treated with 10 μM Fer-1 for 2 h in advance. For the inhibiting group (g), the cells were treated with 50 μM DFP for 2 h in advance. All

cells were washed three times with PBS before cell imaging.

For monitoring of $ONOO^-$ upon treatment with natural products, PC12 cells were divided into twelve parallel groups. The cells were pretreated with 20 μM natural products for 12 h before being washed three times with PBS: (a) blank, (b) blank, (c) curcumin, (d) α-tocopherol succinate, (e) L-ascorbic acid, (f) UP302, (g) caffeic acid, (h) (S)-naringenin, (i) lipoic acid, (j) resveratrol, (k) propyl gallate, and (l) uric acid. Then all the cells except group (a) were treated with 30 μM $A\beta_{42}$ peptide for 16 h being washed three times with PBS. These treatments were followed by incubation with 5 μM NATP for 20 min. All cells were washed three times with PBS before cell imaging.

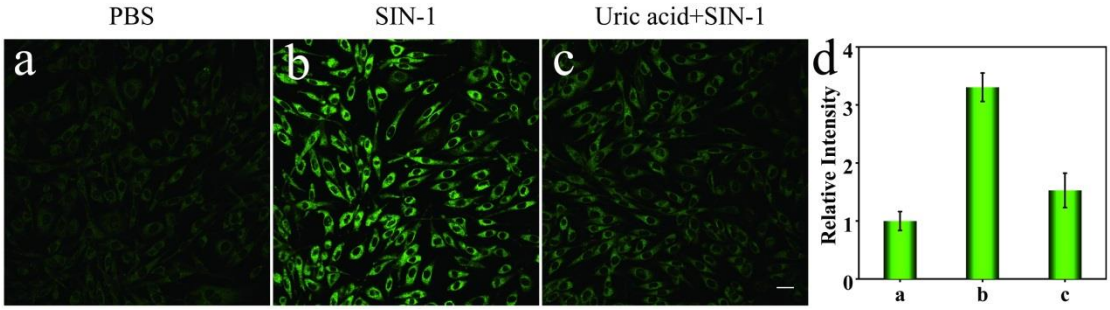


Fig. S7 Two-photon fluorescence imaging of $ONOO^-$ in live cells. PC12 cells were treated with (a) PBS, (b) SIN-1 (500 μM, 30 min), and (c) uric acid (100 μM, 3 h) + SIN-1 (500 μM, 30 min), followed by incubation with 5 μM NATP for 20 min. (d) Relative mean fluorescence intensities of (a−c). Images were obtained by collecting the emissions at 535−595 nm upon two-photon excitation at 800 nm. Scale bar represents 20 μm.

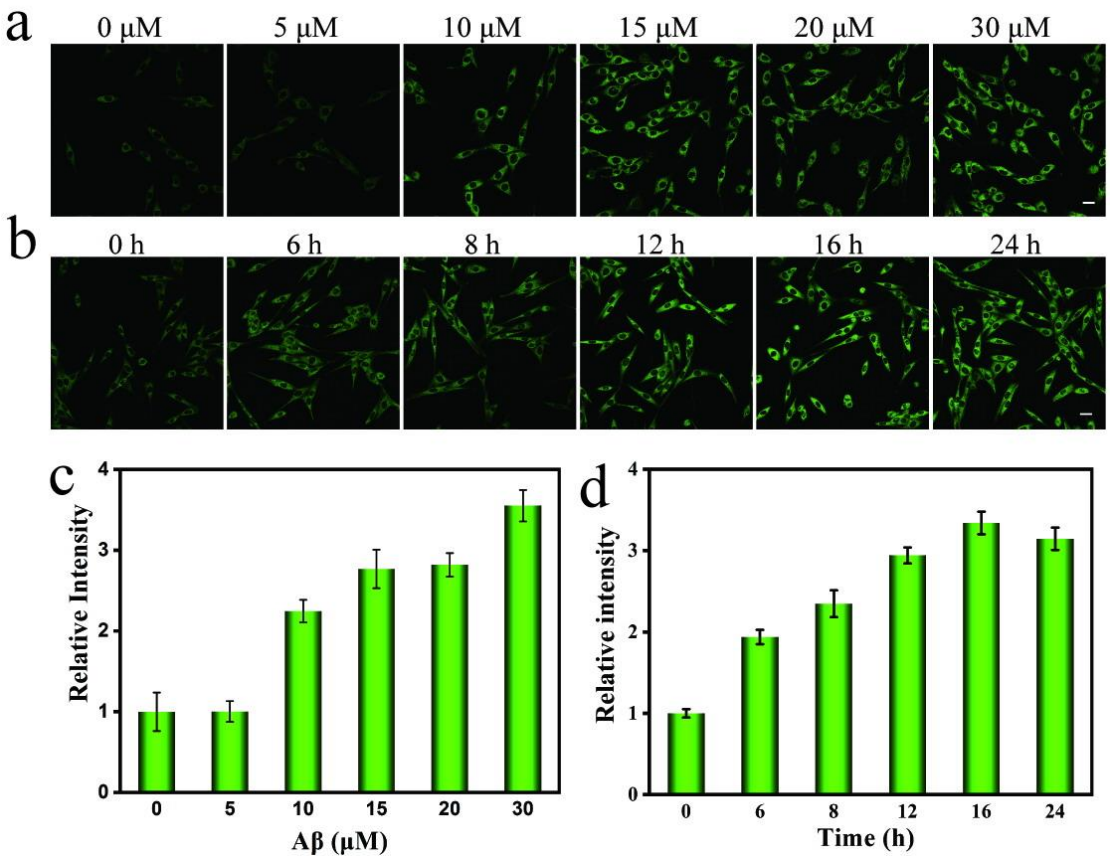


Fig. S8 Two-photon fluorescence imaging of $ONOO^-$ in PC12 cells induced by Aβ stimulation. (a) Cells were treated with 0, 5, 10, 15, 20, or 30 μM $A\beta_{42}$ peptide for 24 h, and then incubated with 5 μM NATP for 20 min. (b) Cells were treated with 30 μM $A\beta_{42}$ peptide for 0, 6, 8, 12, 16, or 24 h, and then incubated with 5 μM NATP for 20 min. Images were obtained by collecting the emissions at 535−595 nm upon two-photon excitation at 800 nm. Scale bar represents 20 μm. (c, d) Relative mean fluorescence intensities of cells in (a) and (b) were quantified.

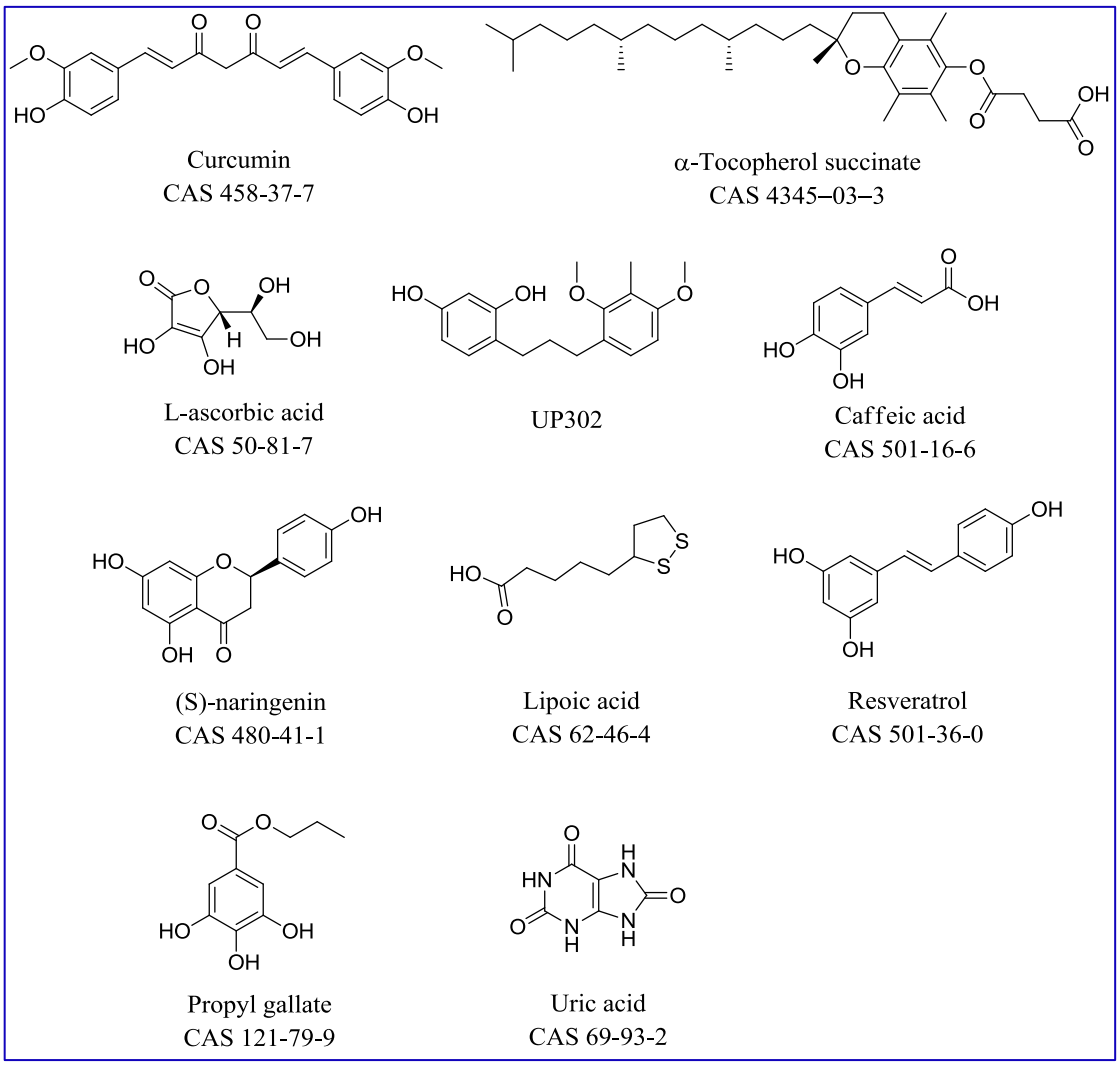


Fig. S9 The chemical structures of the screened natural products.

10. Western blotting

For Western blots experiment, PC12 cells were divided into five parallel groups named blank group, Aβ group, 1 + Aβ group, 2 + Aβ group, and 3 + Aβ group, and given varied treatment. For blank group, the cells were given no treatment. For Aβ group, the cells were treated with 30 μM $A\beta_{42}$ peptide for 16 h. For 1 + Aβ group, the cells were pretreated with 20 μM ascorbic acid (1) for 12 h, and then treated with 30 μM $A\beta_{42}$ peptide for 16 h. For 2 + Aβ group, the cells were pretreated with 20 μM caffeic acid (2) for 12 h, and then treated with 30 μM $A\beta_{42}$ peptide for 16 h. For 3 + Aβ group, the cells were pretreated with 20 μM uric acid (3) for 12 h, and then treated with 30 μM $A\beta_{42}$ peptide for 16 h. Cells samples were collected by trypsinization and

pelleted by centrifugation at 3 000 rpm for 3 min at 25 ℃, followed by washing with PBS (1 mL) three times and the cell pellets were flash frozen in liquid nitrogen and ultrasonicated followed by centrifugation for 30 min with 20 000 g at 4 ℃. The resulting supernatant (soluble cell lysate) was collected and protein concentration was determined via BCA assay (Pierce, Thermo Scientific). Protein concentration was adjusted to 3 mg/mL with PBS before use. Protein samples were mixed with the loading buffer, boiled for 5 min and set on ice immediately. Then samples and protein ladder were loaded onto the gel along with positive and negative controls. Gel was ran at 80 V for 30 min and followed by increasing to 100 V for a period of time depending on the position of the prey proteins on the gel. Next, proteins were transferred onto the membrane. Traditional sandwich method was used to transfer proteins from gel to membrane. Then, the membrane was sealed with QuickBlock™ Blocking Buffer for 15 min under room temperature, washed with the PBST buffer for three times, incubated with primary antibody (GPX4 Antibody, 1: 1000) for 12 h, washed with the TBST buffer for three times and incubated with a secondary antibody (Goat Anti-Rabbit IgG (H+L), 1: 5000) for 2 h at room temperature. After incubation, the samples were washed with the TBST buffer for three times and rinsed with PBS buffer. Finally, chemiluminescent detection of the bound bait protein was assayed by an ECL kit, according to the manufacturer's instruction. Blotting was imaged using a ChemiDoc™ Touch Imaging System (Bio-Rad).

11. Mouse models

All animal experiments were approved by the Animal Care and Use Committee of Shandong Normal University, and were performed in compliance with the Animal Management Rules of the Ministry of Health of the People's Republic of China and the Guidelines for the Care and Use of Laboratory Animals of Shandong Normal University.

The injection solution of NATP was freshly prepared in using DMSO. NATP (20 mg/kg, 100 μL) was injected intraperitoneally into Kunming mice (ca. 28 g). As a

comparison, another group of mice were injected with PBS without NATP. Thirty minutes later, after anesthesia, the mice were sacrificed, and the brains were isolated and then homogenized in methanol. After centrifugation at 7 830 rpm for 5 min, the resulting supernatants were concentrated and then filtered. Fluorescence spectra of the extractions were measured using a Hitachi F4700 fluorospectrometer.

The mice were fasted for 12 h to avoid possible food fluorescence interference to the dye fluorescence before imaging. The injection solution of NATP was freshly prepared in a mixture of 20% DMSO and 80% PBS. NATP (10 mg/kg, 200 μL) was injected intraperitoneally into healthy (4 months old) and APP/PS1 double transgenic mice (4 months old). Thirty minutes later, after anesthesia, the mouse scalp was carefully removed and the skull maintained intact, and then the brain of live mice was imaged on a Zeiss LSM 880 confocal laser scanning microscope. Images were obtained by collecting the emissions at 495−595 nm upon two-photon excitation at 800 nm.

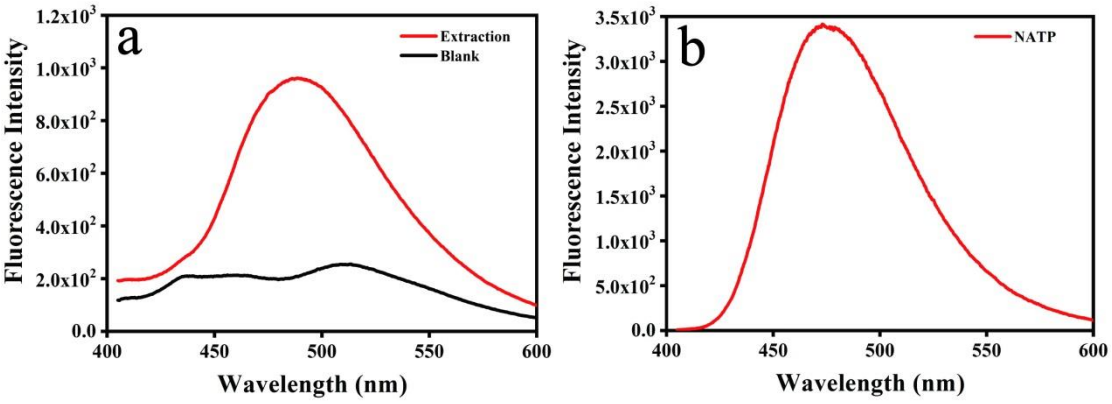


Fig. S10 (a) Fluorescence spectra of brain homogenates of mice in methanol. The mice were injected with NATP (red line) or PBS (black line), and then the brains were isolated, homogenized, and extracted in methanol. (b) Fluorescence spectrum of pure NATP in methanol.

12. NMR and HRMS spectra

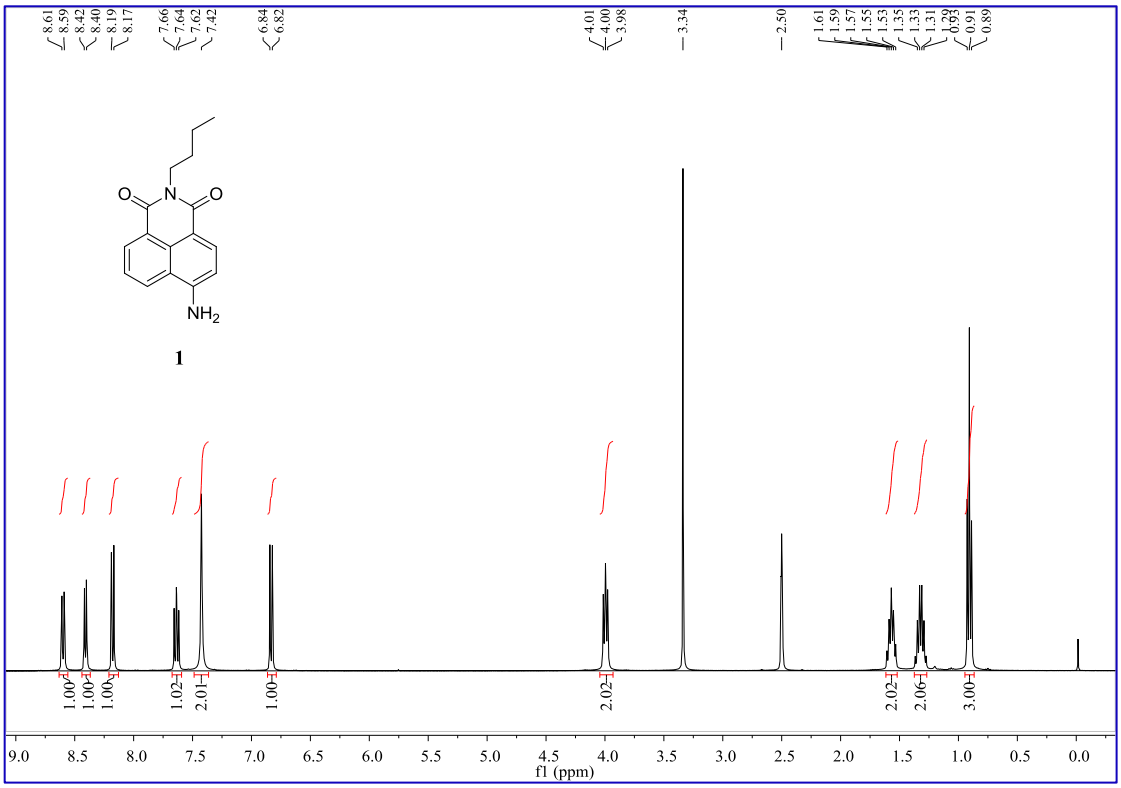


Fig. S11 ^{1}H NMR spectrum of Compound 1

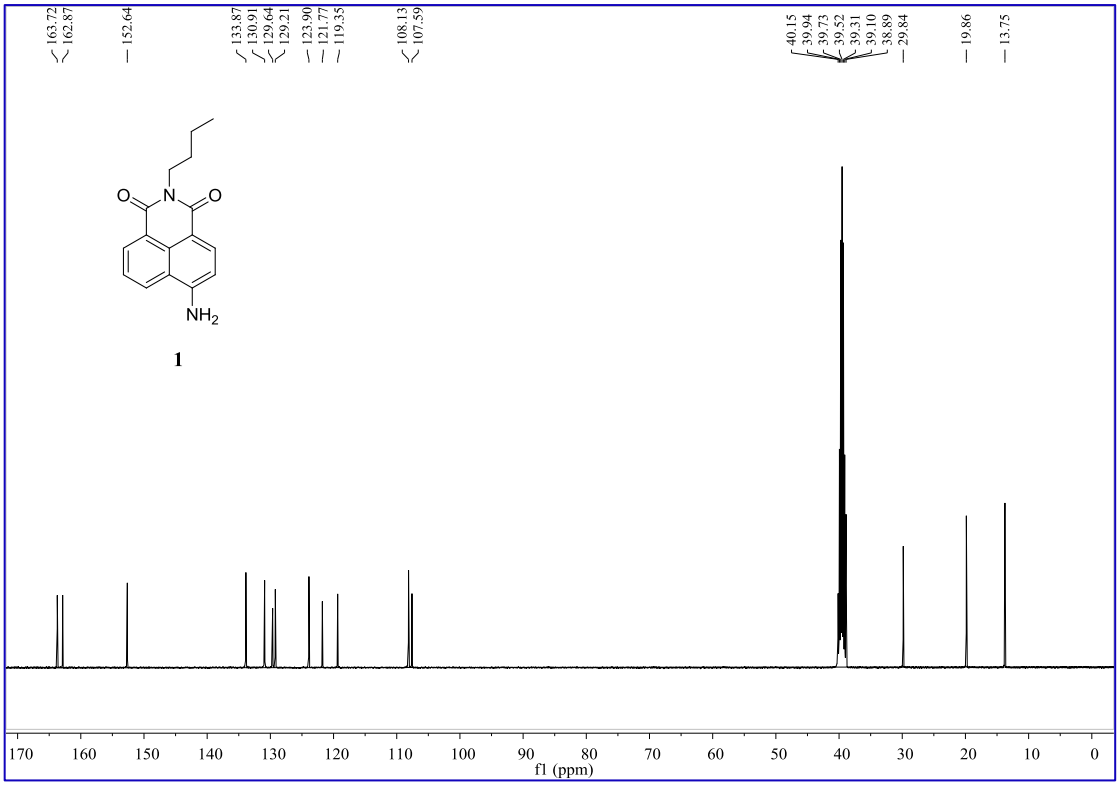


Fig. S12 ^{13}C NMR spectrum of Compound 1

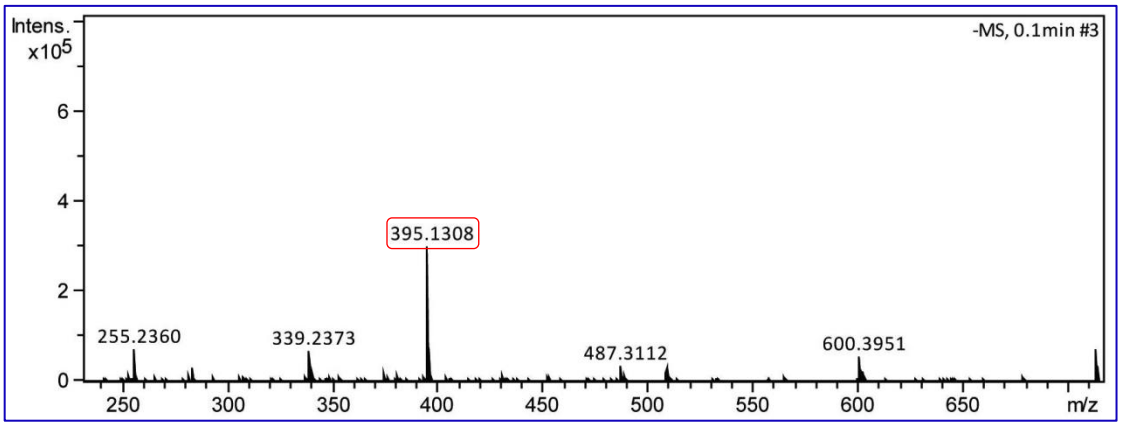


Fig. S13 HRMS spectrum of NATP

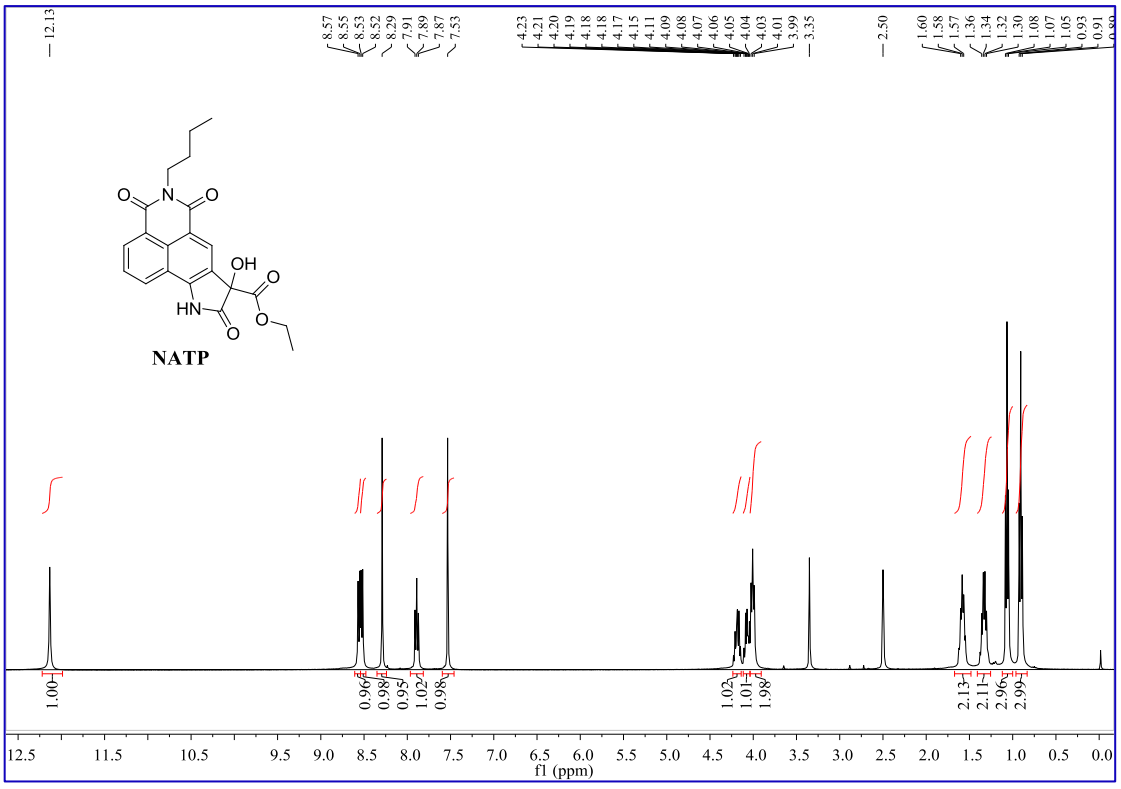


Fig. S14 ^{1}H NMR spectrum of NATP

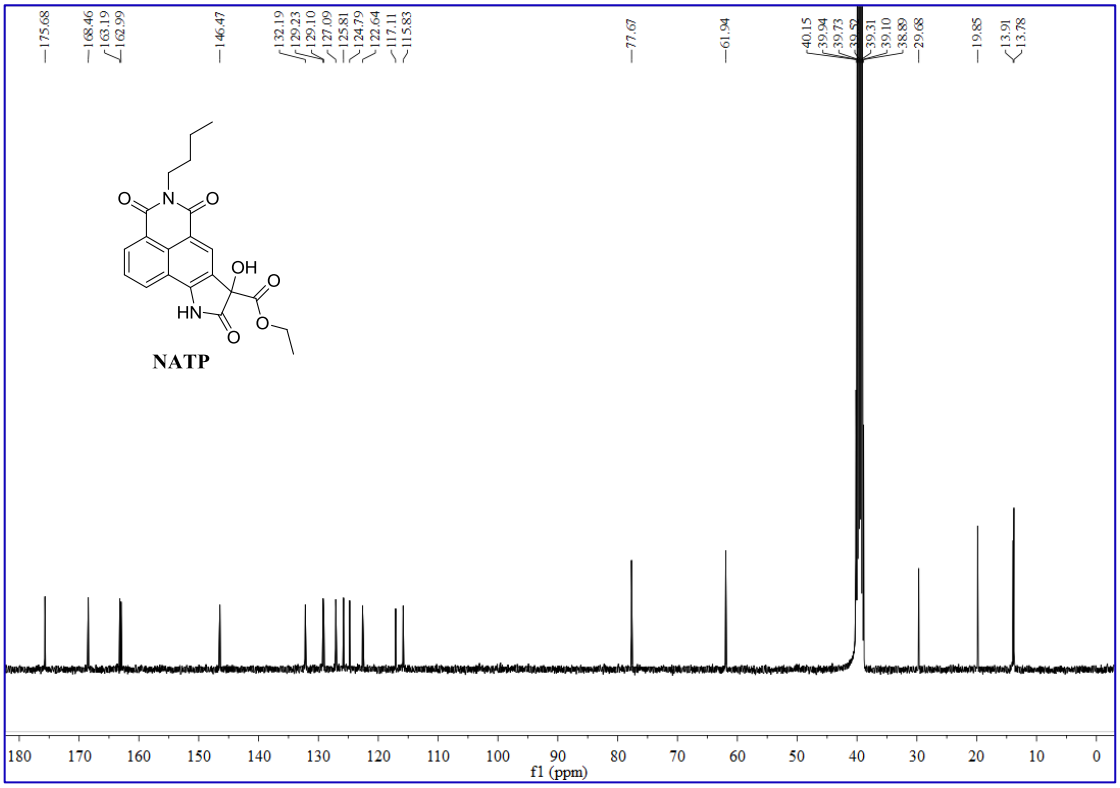


Fig. S15 ^{13}C NMR spectrum of NATP