Supporting Information

Near-Infrared Fluorescent Probe for Evaluating Acetylcholinesterase Effect in Aging Process and Dietary Restriction via Fluorescence Imaging

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1. General Experimental Section

Instruments. Mice imaging was performed on Perkinelmer IVIS Lumina XRMS Series III In Vivo Imaging System. Fluorescence spectra were determined using a HORIBA Scientific Fluoromax-4 spectro fluorometer with a Xenon lamp and 1.0-cm quartz cells. Absorption spectra were measured on Thermo Scientific NanoDrop 2000/2000C spectrophotometer. All pH measurements were performed with a basic pH-Meter PH-3C digital pH-meter (Lei Ci Device Works, Shanghai) with a combined glass-calomel electrode. Mass spectra were taken on LCQ Fleet LC-MS System (Thermo Fisher Scientific). ^{1}H NMR, ^{13}C NMR spectra were recorded on a Bruker spectrometer. MTT assay was carried out by a microplate reader (Tecan, Austria). The fluorescence images of cells and tissue sections were taken using a confocal laser scanning microscope (Japan Olympus Co., Ltd) with an objective lens (×60). Intracellular fluorescence detection was carried out on flow cytometry (Aria, BD) with excitation at 680 nm and emission at 720-780 nm.

Materials. All reactions were performed under argon protection and dark, monitored by TLC (Hailang, Yantai). Flash chromatography was carried out using silica gel (300-400 mesh). The purity of BD-AChE was separated on a Shimadzu LC-20AT HPLC system equipped with fluorescence and UV-vis absorption detectors. When it was used for imaging, the purity of BD-AChE was greater than 95%. All chemicals used in synthesis were analytical reagent grade, and were used as received. Ultrapure water was used throughout. Trifluoroacetic acid (TFA), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich. N,N-diisopropylethylamine (DIPEA) were purchased from Beijing InnoChem Science & Technology Co., Ltd. HEPES was obtained from Aladdin. Rat adrenal chromaffin tumor cells (PC12 cells) were obtained from the Committee on Type Culture Collection of the Chinese Academy of Sciences.

Spectrophotometric experiments. Absorption spectra were obtained with 1.0-cm cuvette cells. The probes BD-AChE was added to a 10.0-mL color comparison tube. After dilution to 10 μM with 5 mM HEPES buffer, different concentrations of AChE were added. The mixture was incubated at 37 ^{o}C for 15 min before measurement. Fluorescence spectra were obtained with a 1.0-cm quartz cells by Xenon lamp. The probe BD-AChE was added to a 10.0-mL color comparison tube, respectively. After diluted to 10 μM BD-AChE with 5 mM HEPES buffer, different concentrations of AChE were added. The mixture was incubated at 37 ^{o}C for 15 min before measurement.

Preparation of analytes. BD-AChE (1 mM) was prepared in DMSO and stored at 4°C in darkness. S-nitrosoglutathione (GSNO) was synthesized from GSH. Peroxynitrite ($ONOO^-$) solution was synthesized. NO was generated in form of 3-(Aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5, 100 μM/ml). NO_2^- was

generated from $NaNO_2$. O_2^- was created by the enzymatic reaction of xanthine/xanthineoxidase (XA/XO; 6.0 μM/ 3 mU) at 25 °C for 5 min. Methyl linoleate (MeLH) and 2,2'-azobis-(2,4-dimethyl) valeronitrile (AMVN) were used to produce MeLOOH. ClO^- was generated from NaClO. All other reagents and chemicals were all from commercial sources and used without further purification. Water used in all experiments was doubly distilled and purified by a Milli-Q system (Millipore, Bedford, MA, USA).

Cell cultures. PC12 cells were obtained from the Committee on Type Culture Collection of the Chinese Academy of Sciences. PC12 cells were cultured in RPMI 1640 supplemented with 10 % fetal bovine serum, 1% penicillin and 1% streptomycin at 37 °C (w/v) in an incubator in 5 % CO_2 / 95 % air. One day before imaging, the cells were detached and placed in glass-bottomed dishes.

Cell transfection: PC12 cells were selected as the cell models of overexpression of AChE by Calcium Phosphate Cell Transfection Kit. The AChE genes were transiently overexpressed by a pcDNA3-AChE plasmid (Vigene Biosciences Ltd.). Identical empty vectors lacking a cDNA insert were employed as control.

Flow cytometry. FCM assay was applied to detect the intramolecular generation of AChE with probe BD-AChE. The PC12 cells were cultivated at 2.0×10^5 cells/well in 6-well plates, the PC12 cells with different treated, and then treated with 5 μM BD-AChE at 37 °C for 30 min. After harvest, PC12 cells were washed, and resuspended with PBS and then analyzed by flow cytometry.

Cytotoxicity of Cy-AChE. The cytotoxicity of BD-AChE was measured by the MTT assay. PC12 cells were seeded into 96-well cell culture plate at a final density of 8×10^3 cells/well. And then different concentrations of BD-AChE (0 μM, 20 μM, 40 μM, 60 μM, 80 μM, 100 μM) were supplemented to the wells. The PC12 cells were then treated at 37 °C for 24 h under 5% CO_2. Subsequently, MTT was supplemented to every well (final concentration 5 mg/mL) for an additional at 37 °C 4 h under 5% CO_2, then formazan crystals which were dissolved in 150 μL DMSO formed. We qualified the amount of MTT formazan with the absorbance (OD) at 570 nm by a microplate reader (Tecan, Austria). Calculation of IC_{50} values were performed according to Koella and Huber. These results are the mean standard deviation of six separate measurements.

Confocal Imaging. The fluorescence images of PC12 cells were performed by a confocal laser scanning microscope (Japan Olympus Co., Ltd) with an objective lens (× 60). Excitation wavelength was 680 nm, and the emission was collected from 720 nm to 780 nm.

***In vivo* imaging in living mice.** All the Balb/C mice were administered an intracranial injection of BD-AChE

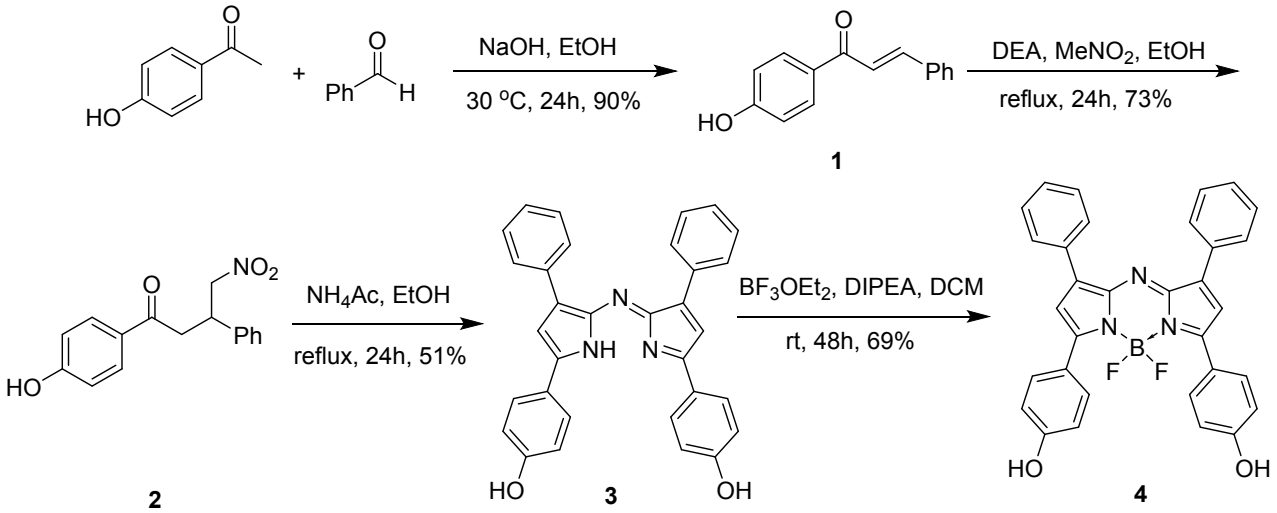
(100 μM, 50 μL in 1:99 DMSO/saline v/v). Eventually, all groups of Balb/C mice were anesthetized by i.p. injections of 4% chloral hydrate (0.25 ml). Mice images were taken by a Perkinelmer IVIS Lumina XRMS Series III In Vivo Imaging System, with an excitation filter of 680 nm and an emission of 720 - 780 nm. The results were the mean standard deviation of five separate measurements. Besides, we merged the fluorescence image with the bright field image to clearly show the reaction site of the Balb/C mice. All mice experimental procedures were performed in conformity with institutional regulations and guidelines for the use and care of laboratory animals, and the related protocols were approved by the Institutional Animal Care and Use Committee in Binzhou Medical University, Yantai, China. Approval Number: No. BZ2014-102R.

Western blot assay. All the pre-treated cells were lysed in cell lysis buffer containing the protease inhibitor PMSF. The cell lysates were centrifuged at 15,000 rpm for 10 min at 4 °C. Protein concentrations were measured using the BCA Protein Assay Reagent with BSA to establish a standard curve. Equal proteins from PC12 cells were lysed in SDS sample buffer (62.5 mM Tris-HCl pH 6.8, 2 % SDS, 6 % glycerol, 0.005 % bromophenol blue, and 2.5 % 2-mercaptoethanol) and then boiled for 5 min at 95 °C. Proteins were separated by using SDS-polyacrylamide gel electrophoresis and were then transferred to polyvinylidene difluoride membranes (Merck Millipore, Darmstadt, Germany) at 100 V for 1 h. Membranes were blocked with 5 % non-fat milk in Tris-buffered saline (TBS) containing 0.1 % Tween-20 (TBS-T) (20 mM Tris pH 7.5, 137 mM NaCl, and 0.1 % Tween 20) for 60 min and then incubated for 1 h at room temperature or overnight at 4°C with the anti-Acetylcholinesterase antibody (ab183591, Abcam). After membranes were washed with TBS-T, they were incubated with horseradish peroxidase (HRP)- conjugated anti-rabbit secondary antibodies (Abcam) at room temperature for 1 h. After samples washed with TBS-T, protein bands were detected by using Immobilon Western Chemiluminescent HRP Substrate with the luminescent image analyzer.

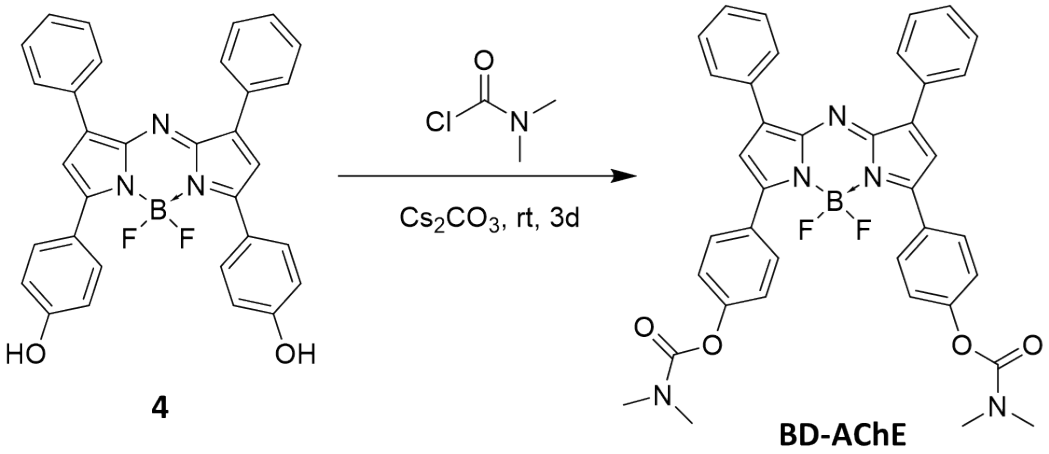
Tissue preparation. Mice were sacrificed by cervical dislocation at a fixed time of the day (13:00 h) and their brains were excised out immediately, washed in chilled physiological saline and blotted dry. A 10% (w/v) homogenate of these tissue was prepared in ice-cold 0.32 M sucrose solution. The homogenate was centrifuged for 30 min at 4 °C. The supernatant thus obtained were used for the assay of AChE protein estimation.

2. Synthesis of Fluorophore

Scheme S1. Synthesis of BODIPY fluorophore (Compound 4)



Scheme S2. Synthesis of **BD-AChE**



Synthesis of 1: 4'-Hydroxyacetophenone (13.6 g,100 mmol) in ethanol (140 mL) was added to the solution of sodium hydroxide (8.4 g, 210 mmol) in 60 mL water under ice bath，then benzaldehyde（10.13 mL, 100 mmol）diluted with ethanol (140 mL) was dropwised to the cold solution. The resulting solution was stirred at room temperature for 15 h, and then the HCl was added to the solution. Finally, the solution was cold in ice water, filtered, and crystallized with ethanol to yield light yellow crystal chalcone **1** (20.16 g, 90%), m.p.180-182 ºC. ^{1}H NMR (500 MHz, $CDCl_3$-D_1) δ (ppm): 10.41 (s, 1H), 8.08-8.05 (m, 2H), 7.92-7.85 (m, 3H), 7.69-7.66 (m, 1H), 7.47-7.43 (m, 3H), 6.91-6.88 (m, 2H). LC-MS (ESI^+): m/z $C_{15}H_{12}O_2$ calcd. 224.0837, found $[M-H]^-$ 223.0758.

Synthesis of 2: A solution of chalcone **1** (2.02 g, 9 mmol) in EtOH (15 mL) was treated with diethylamine (4.6 mL, 45 mmol) and nitromethane (4.8 mL, 90 mmol), then heated under reflux for 24 h. The solution was cooled and then neutralized with HCl, partitioned between EtOAc and H_2O (1:1). The organic layer was separated, dried over sodium sulfate and evaporated. The residue was stirred in cold Et_2O for 10 min and filtered to give the

product as yellowish crystalline compound 2 (1.87 g, 73%), m.p. 112-113 ºC. ^{1}H NMR (500 MHz, DMSO-D_6) δ (ppm): 10.20 (s, 1H), 7.77 (d, 2H), 7.28-7.20 (m, 5H), 6.78 (m, 2H), 4.86 (m, 2H), 3.93 (m, 1H), 3.81 (m, 2H). LC-MS (ESI$^+$): m/z $C_{16}H_{15}NO_4$ calcd. 285.1001, found [M-H]$^-$ 284.0928.

Synthesis of 3: Compound 2 (2.0 g, 7.0 mmol) and ammonium acetate (18.9 g, 245 mmol) in EtOH (50 mL) were heated under reflux for 24 h. The reaction was cooled to room temperature. Then, the product was spin steamed and extracted in EtOAc and H_2O (1:1).The organic layer was evaporated to dry and yielded **3** as blue-black solid (1.72 g, 51%), mp 245-246 °C. ^{1}H NMR (500 MHz, DMSO-D_6) δ (ppm): 10.26-10.21 (s, 2H), 7.53-7.24 (m, 13H), 7.10-7.07 (m, 3H), 6.49-6.47 (d, 4H), 5.26 (s, 1H). LC-MS (ESI$^+$): m/z $C_{32}H_{23}N_3O_2$ calcd. 481.1790, found [M-H]$^-$ 480.1717.

Synthesis of 4: Compound 3 (0.15 g, 0.31 mmol) was dissolved in anhydrous CH_2Cl_2, treated with diisopropylethylamine (0.54 mL, 3.11 mmol) and BF_3diethyletherate (0.55 mL, 4.35 mmol), and stirred under argon for 48 h. The colour of solution changed from blue to green. The course of the reaction is confirmed by thin-layer chromatography (TLC).The product was extracted in CH_2Cl_2/H_2O (1:1), and the organic layer evaporated to dryness, and then purified by column chromatography on silica eluting with CH_2Cl_2/EtOAc (4:1) gave the Aza-BODIPY as atropurpureus solid (0.11 g, 69%), m.p 253-254 °C. ^{1}H NMR (500 MHz, DMSO-D_6) δ (ppm): 10.46 (s, 2H), 8.16-7.80 (m, 8H), 7.56-7.48 (m, 6H), 7.48-7.45 (m, 2H), 6.96-6.94 (4H). LC-MS (ESI$^+$): m/z $C_{32}H_{22}BF_2N_3O_2$ calcd. 529.1773, found [M-H]$^-$ 528.1702.

Synthesis of BD-AChE: Compound 4 (0.529 g, 1 mmol) and cesium carbonate (0.326 g, 1 mmol) were dissolved in dichloromethane, and the mixture was stirred under argon at room temperature. After 30 min, dimethylcarbamoyl chloride was added to the mixture (400 μL). Then, the mixture was stirred for 3 days, and additional dimethylcarbamoyl chloride was added twice each day (200 μL). The mixture was purified by column chromatography on silica eluting with CH_2Cl_2/CH_3OH (10:1) gave a yellow product (0.395 g, 59%). ^{1}H NMR (500 MHz, CD_3OD-D_4) δ (ppm): 8.16-8.14 (m,1H), 8.06-8.04 (m, 2H), 7.88-7.80 (m, 2H), 7.51-6.92 (m, 13H), 4.85 (s, 12H), 2.97-2.84 (m, 2H). ^{13}C NMR (125 MHz, CD_3OD-D_4) δ (ppm): 166.1, 163.5, 158.6, 157.7, 157.4, 150.3, 139.5, 138.7, 130.8, 129.5, 129.0, 128.9, 128.8, 128.7, 128.6, 128.3, 128.2, 128.1, 127.9, 127.6, 126.8, 121.7, 115.8, 115.3, 115.1, 37.5. LC-MS (ESI$^+$): m/z $C_{38}H_{32}BF_2N_5O_4$, calcd 671.2515, [M+H]$^+$ 672.1768.

3. Absorption Spectra of BD-AChE

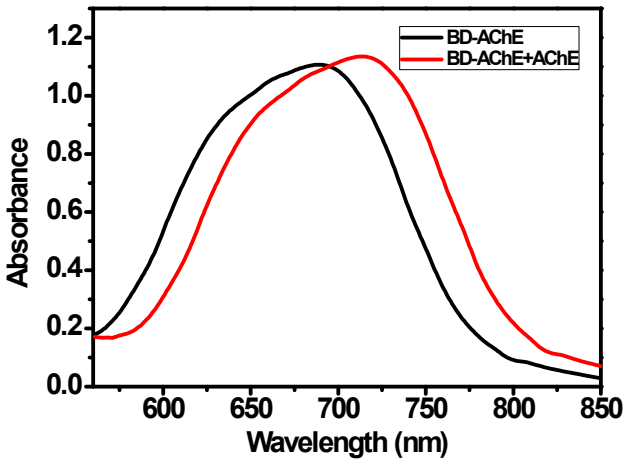


Figure S1. Absorption spectra of BD-AChE and BD-AChE + AChE. The black curve was the absorption spectrum of BD-AChE. The red curve was recorded after treatment with AChE.

4. MTT Assay for BD-AChE

PC12 cells were cultured in RPMI 1640 supplemented with 10% FBS in an atmosphere of 5% CO_2 and 95% air at 37 ^oC. The cells (8000/cell) were plated into 96-well plates and allowed to adhere for 24 hours. Subsequently, the cells were incubated with 0, 20, 40, 60, 80 and 100 μM (final concentration) of BD-AChE at 37 ^oC in an atmosphere of 5% CO_2 and 95% air for 24 h. An untreated assay was also performed under the same conditions. MTT solution (5.0 mg/mL in PBS, 20 μL) was added to each well, and 4 h later, the remaining MTT solution was carefully removed. In addition, DMSO (150 μL) was added to each well to dissolve the formazan crystals. The plate was shaken for 10 min and the absorbance was measured at 570 nm and 630 nm using a microplate reader (TECAN infinite M200pro).

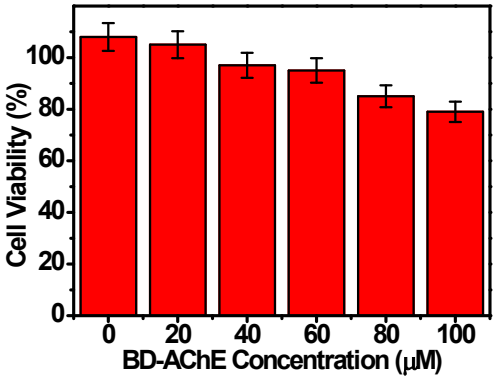


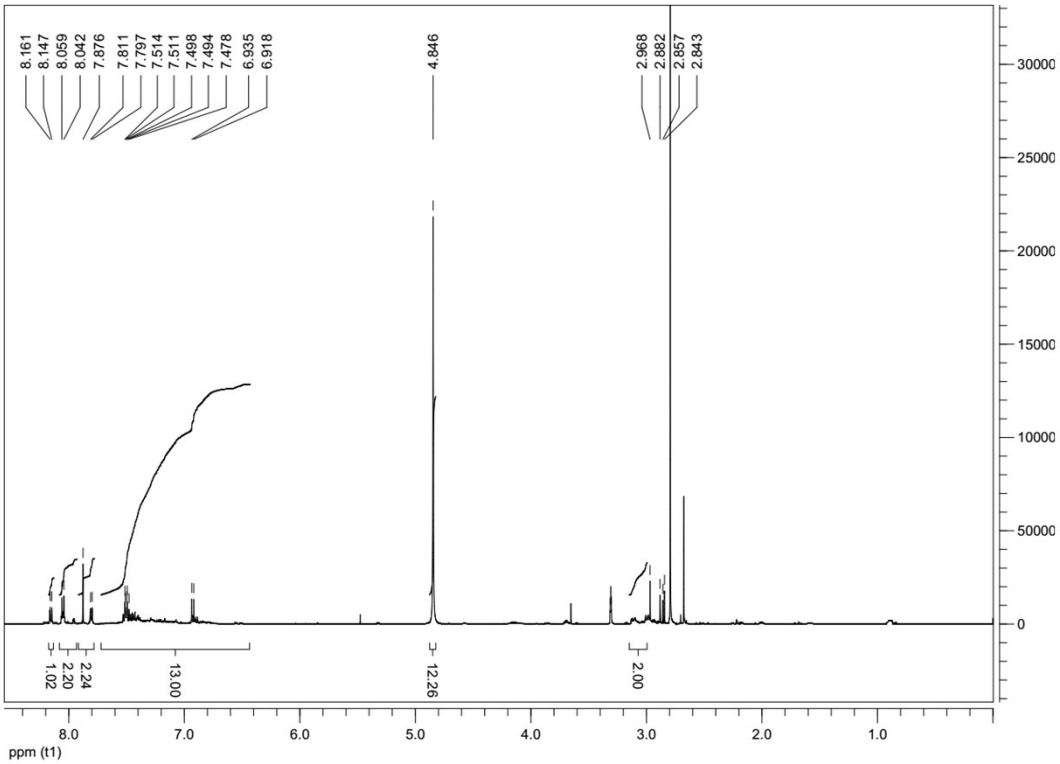
Figure S2. The 24 h cell viability of PC12 cells for BD-AChE, the concentration of BD-AChE was 0, 20, 40, 60, 80 and 100 μM.

5. Bright-field cell images of those shown in Figure 2 (in manuscript)

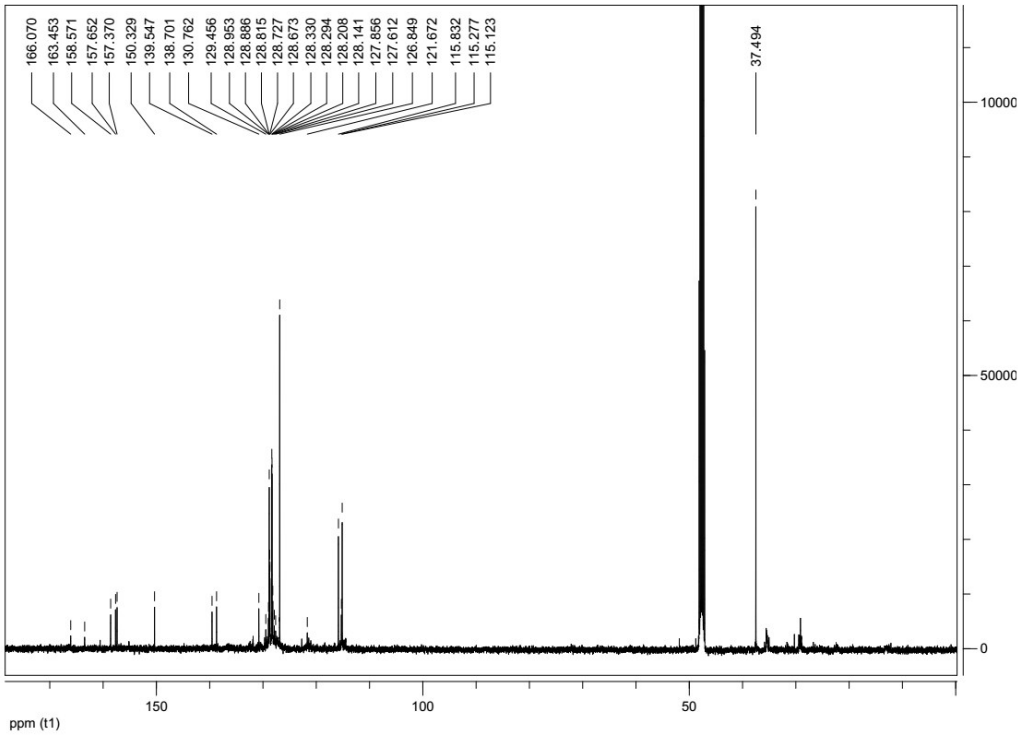
Figure S3. Bright-field images of Figure 2 (in manuscript). Scale bars: 20 µm.

6. Characterization (^{1}H NMR and ^{13}C NMR) of synthesized BD-AChE

^{1}H NMR of BD-AChE



^{13}C NMR of BD-AChE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