Electronic Supplementary Material (ESI) for Chemical Communications

Supporting Information to Accompany "**A Two-Photon Fluorescent Probe for Amyloid-β Plaque in Living Mice**"

Cheol Ho Heo,[a] ‡ Kyung Ho Kim,[b] ‡ Hyung Joong Kim,[a] Sung Hoon Baik,[b] Hyundong Song,[b] Yong Soo Kim,[c] Jeewoo Lee,[c] Inhee Mook-jung, *[b] and Hwan Myung Kim*[a]

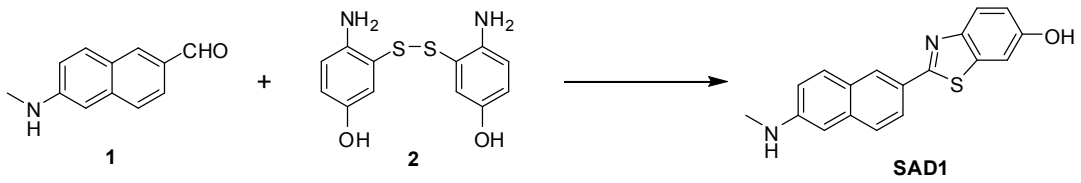
[a]*Division of Energy Systems Research, Ajou University, Suwon 443-749, Korea.*

[b]*Department of Biochemistry and Biological sciences, Seoul National University College of Medicine, 103 Daehak-ro Jongno-gu, Seoul 110-799, Korea.*

[c]*College of pharmacy, Seoul National University, 1 Gwanak-ro, Gwanak-gu, Seoul, 151-742. Korea.*

Table of Contents

Synthesis of SAD1. MeO-X04, PIB, **1**, and **2** were prepared by the literature methods.[1-4] Synthesis of SAD1 is described below.



SAD1. Compound **1** (0.50 g, 2.69 mmol), **2** (0.76 g, 2.69 mmol) and *p*-toluenesulfonic acid (0.05 g, 0.27 mmol) were dissolved in dry DMF (30 mL) and the reaction mixture was stirred at 90 °C under nitrogen atmosphere for 8 h. After cooling to room temperature, the solvent was evaporated in vacuo and the crude product was purified by column chromatography using 5 % EtOAc in $CHCl_3$ as the eluent to give SAD1 as a dark green solid. Yield: 0.38 g (46 %); m.p. 243-245 °C; ^{1}H NMR (400 MHz, $CDCl_3$+DMSO-d_6): δ 9.17 (s, 1H), 8.27 (d, J = 2.0 Hz, 1H), 7.99 (dd, J = 8.8, 2.0 Hz, 1H), 7.82 (d, J = 8.8 Hz, 1H), 7.69 (d, J = 8.8 Hz, 1H), 7.67 (d, J = 8.8 Hz, 1H), 7.32 (d, J = 2.4 Hz, 1H), 7.01 (dd, J = 8.8, 2.4 Hz, 1H), 6.97 (dd, J = 8.8, 2.4 Hz, 1H), 6.75 (d, J = 2.4 Hz, 1H), 4.74 (br s, 1H), 2.95 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$+DMSO-d_6): 164.7, 155.9, 149.6, 147.9, 137.1, 136.2, 129.8, 127.1, 126.6, 126.4, 126.3, 124.6, 123.4, 119.6, 116.4, 107.3, 102.1, 30.3; HRMS (FAB^{+}): m/z calcd for [$C_{18}H_{14}N_2OS$+H^{+}]: 307.0905, found: 307.0905.

Spectroscopic measurements. Absorption spectra were recorded on a S-3100 UV-Vis spectrophotometer and fluorescence spectra were obtained with FluoroMate FS-2 fluorescence spectrophotometer with a 1 cm standard quartz cell. The fluorescence quantum yield was determined by using coumarin 307 (Φ = 0.95 in MeOH) as the reference by the literature method.[5] The one-photon absorption and emission spectra of PIB, MeO-X04, and SAD1 are depicted in Figure S1. The results are summarized in Table S1.

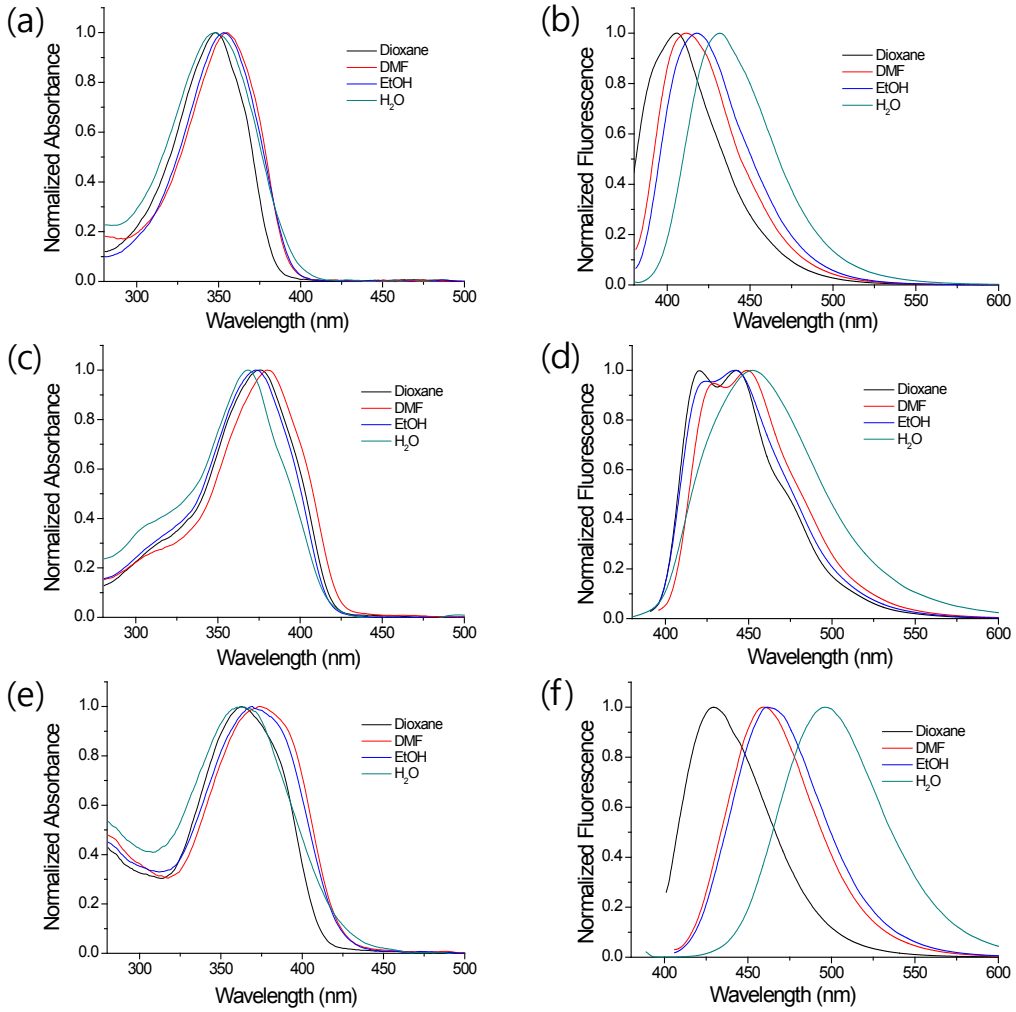


Figure S1. (a, c, e) Normalized absorption and (b, d, f) emission spectra of (a, b) PIB, (c, d) MeO-X04 and (e, f) SAD1 in 1,4-dioxane, DMF, EtOH, and PBS buffer (pH 7.4).

Table S1. Photophysical data for PIB, MeO-X04 and SAD1 in various solvents.

Compd	Solvent (E_T^N)[a]	$\lambda_{max}^{(1)}$ [b]	λ_{max}^{fl} [c]	Φ[d]	$\lambda_{max}^{(2)}$ [e]	Φ_{max} [f]
PIB	Dioxane (0.164)	348	405	1.00		
	DMF (0.386)	355	412	1.00		
	EtOH (0.654)	353	417	1.00	740	40
	PBS (1.00)[g]	348	431	1.00	740	45
MeO-X04	Dioxane (0.164)	376	443	1.00		
	DMF (0.386)	379	450	1.00		
	EtOH (0.654)	372	444	1.00	720	75
	PBS (1.00)[g]	370	452	0.10	720	10
SAD1	Dioxane (0.164)	363	429	1.00		
	DMF (0.386)	373	460	1.00		
	EtOH (0.654)	370	465	1.00	750	170
	PBS (1.00)[g]	362	497	0.24	750	10

a) The numbers in the parenthesis are normalized empirical parameter of solvent polarity.[6] b) λ_{max} of the one-photon absorption spectra in nm. c) λ_{max} of the one-photon emission spectra in nm. d) Fluorescence quantum yield. e) λ_{max} of the two-photon excitation spectra in nm. f) The peak two-photon action cross-section in 10^{-50} cm^4/photon (GM). g) PBS buffer (pH 7.4). The E_T^N value is for water.

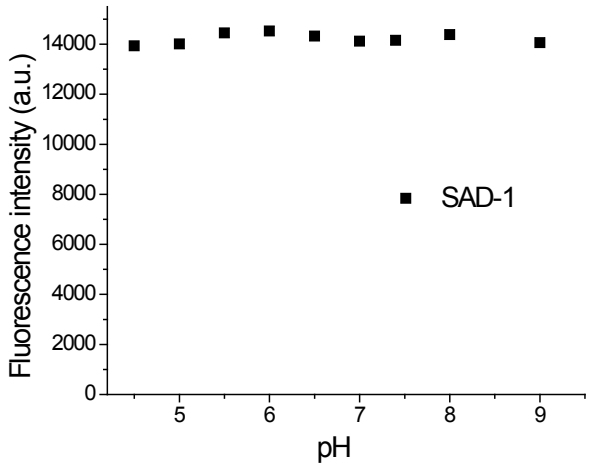


Figure S2. Effect of pH for SAD1 in universal buffer (0.1 M citric acid, 0.1 M KH_2PO_4, 0.1 M $Na_2B_4O_7$, 0.1 M Tris, 0.1 M KCl).

Solubility of SAD1, PIB, and Methoxy-X04 in PBS buffer. Small amount of dye was dissolved in DMSO to prepare the stock solutions (1.0×10^{-2} M). The solution was diluted to ($6.0 \times 10^{-3} \sim 6.0 \times 10^{-5}$) M and added to a cuvette containing 3.0 mL of PBS buffer (pH 7.4) by using a micro syringe. In all cases, the concentration of DMSO in H_2O was maintained to be 0.2 %.[7] The plots of absorption intensity against the total amount of the dye injected to the cuvette were linear at low dye content and showed downward curvature as more dye was added (Figure S3). The maximum point in the linear region was taken as the solubility. The solubility of SAD1, PIB, and MeO-X04 in PBS buffer were 2.0, 3.0, and 0.2 μM, respectively.

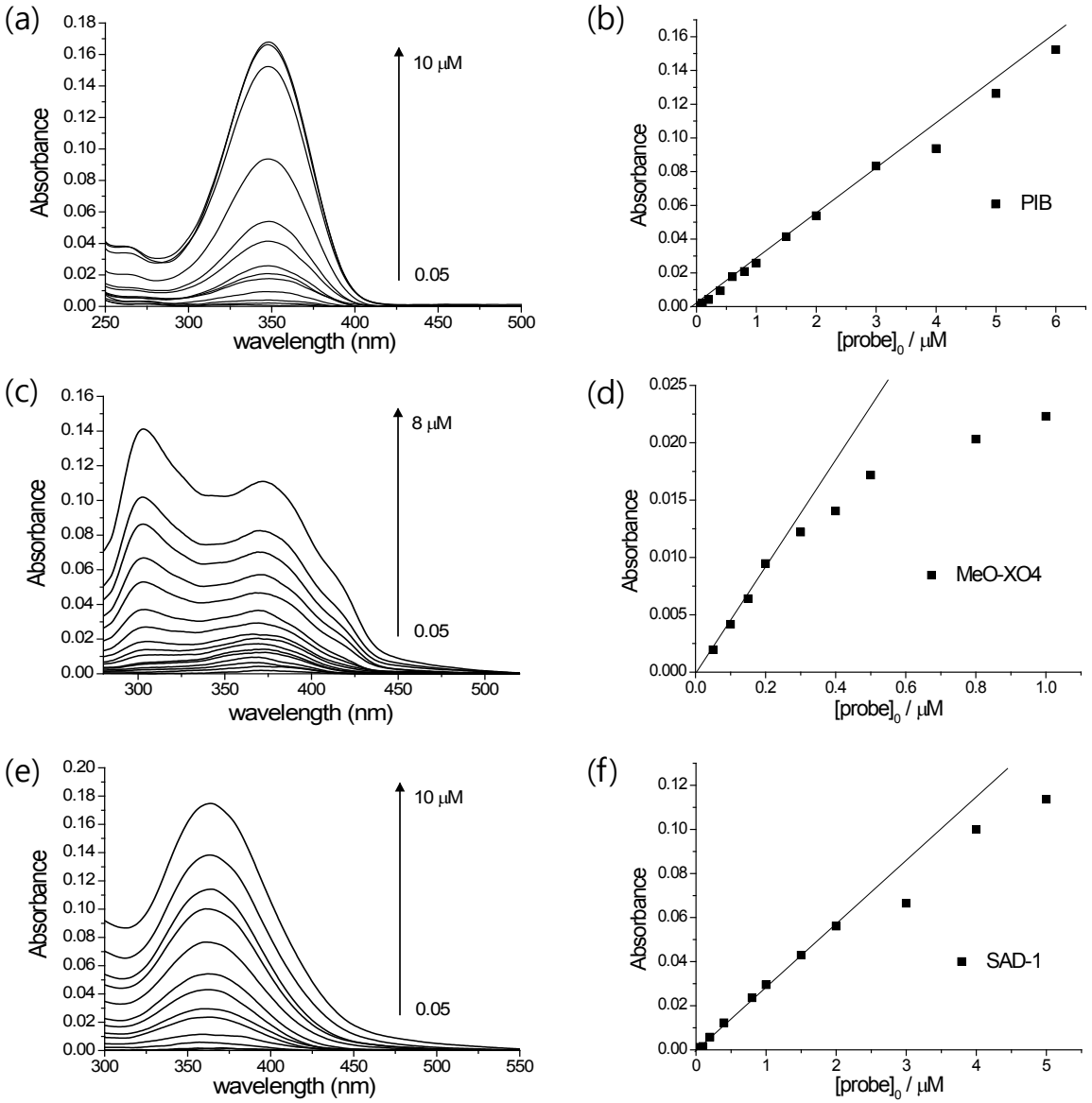


Figure S3. (a, c, e) One-photon absorption spectra and (b, d, f) plot of intensity against probe concentration for (a, b) PIB, (c, d) MeO-X04, and (e, f) SAD1 in PBS buffer (pH 7.4).

Octanol-water partition coefficient (log P_{oct}). Small aliquot (2 μL) of 20 mM SAD1 solution in DMSO was added to a vial containing 1 mL *n*-octanol by using a micro syringe. To this solution, 1 mL of PBS buffer was added. The resulting mixture was stirred vigorously and kept in dark for 1 day. The concentrations of probe in each layer were determined by the UV-Vis absorbance with their molar extinction coefficients as shown in Table S2. The log P_{oct} value was calculated by using log P_{oct} = log $[probe]_{oct}$ – log $[probe]_{PBS}$: where the $[probe]_{oct}$ and $[probe]_{PBS}$ are the concentrations of the probe in *n*-octanol and PBS, respectively. The log P_{oct} values for PIB, MeO-X04, and SAD1 are 1.2, 2.6, and 1.9, respectively.

Table S2. Molar extinction coefficients for PIB, MeO-X04 and SAD1 in *n*-octanol and PBS.

Probe	Solvent	ε (10^{-4}·$M^{-1}cm^{-1}$)
PIB	*n*-octanol	3.48
	PBS	2.71
MeO-X04	*n*-octanol	5.44
	PBS	4.46
SAD-1	*n*-octanol	3.55
	PBS	3.09

Surface plasmon resonance spectroscopy (SPR). The protocol of surface plasmon resonance spectroscopy (SPR) in this study of real-time direct binding of Aβ Oligomer (AβO) and probe was performed as described by Maezawa et al. 2006 using a Biacore X-100 (GE Healthcare, Piscataway, NJ, USA).[8] The Sensor chip SA with pre-immobilized streptavidin (GE Healthcare, Piscataway, NJ, USA) in one flow cell was first saturated with biotinylated AβO, prepared by mixing a 1 : 10 ratio of biotinylated (at *N*-terminus) and unbiotinylated-$Aβ_{42}$ peptide. 140 μL of biotinylated AβO (10 μM) was injected to a flow cell for 15 min at a 10 μL/min rate to allow saturation of the streptavidin chip by AβO via the tight biotin–streptavidin binding. To analyze the binding kinetics, various concentrations of probe diluted in HBS-EP buffer (consisting of 0.01 mol/L HEPES, 0.15 mol/L NaCl, 3 mmol/L EDTA, 0.005% surfactant P20, pH 7.6) were injected onto the sensor chip for 180 sec at 20 μL/min, and the response unit (RU) was then recorded.[9] After injection of the analyte was stopped, HBS-EP buffer contaning 0.5% vehicle (DMSO) was poured over the chip for 300 sec at a 20 μL/min rate to allow the bound analytes to dissociate from the immobilized AβO, and dissociation curves were obtained. The RU elicited by injecting 0.5% of DMSO included HBS-EP buffer was used as the vehicle control. Biacore X-100 control software was used to measure the changes in RU and to plot the binding curve. The curves obtained from the SPR experiments were analyzed and the dissociation constant (K_d) of probe to immobilized AβO was calculated using kinetic evaluation software (Biacore X-100). The K_d was derived from the equation, $K_d = k_d\,(s^{-1})/k_a\,(M^{-1}s^{-1})$, where k_d and k_a are dissociation- and association-rate constants, respectively.

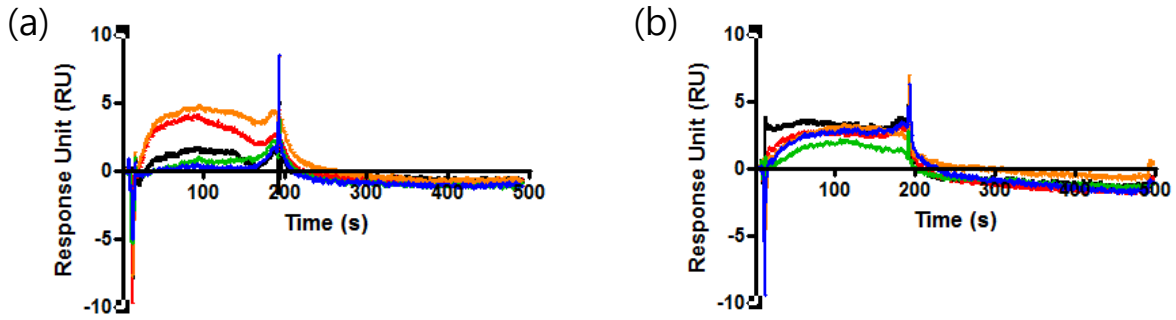


Figure S4. SPR analysis with binding kinetics following the interaction of (a) SAD1 and (b) PIB to Aβ Oligomer.

Measurement of Two-Photon Cross Section. The two-photon cross section (δ) was determined by using femto second (fs) fluorescence measurement technique as described.[10] Probes (1.0×10^{-6} M) were dissolved in EtOH or PBS buffer (pH 7.4) and the two-photon induced fluorescence intensity was measured at 720−880 nm by using rhodamine 6G as the reference, whose two-photon property has been well characterized in the literature.[11] The intensities of the two-photon induced fluorescence spectra of the reference and sample emitted at the same excitation wavelength were determined. The TPA cross section was calculated by using $\delta = \delta_r(S_s\Phi_r\phi_r c_r)/(S_r\Phi_s\phi_s c_s)$: where the subscripts *s* and *r* stand for the sample and reference molecules. The intensity of the signal collected by a CCD detector was denoted as *S*. Φ is the fluorescence quantum yield. ϕ is the overall fluorescence collection efficiency of the experimental apparatus. The number density of the molecules in solution was denoted as *c*. δ_r is the TPA cross section of the reference molecule.

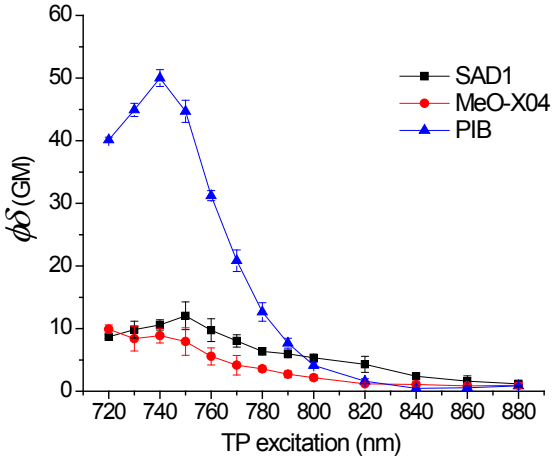


Figure S5. Two-photon action spectra of PIB, MeO-X04 and SAD1 in PBS buffer (pH 7.4).

Two-Photon Fluorescence Microscopy. To visualize SAD1, PIB or MeO-X04 amyloid plaques staining, 20X water immersion-objective lens (W Plan-Apochromat 20x/1.0 DIC M27 70 mm, Carl Zeiss Inc. Germany) of two photon laser scanning microscope (LSM 7 MP, Carl Zeiss Inc, Goettingen, Germany) was used for imaging acquisition. SAD1, PIB or MeO-X04 was excited by a Titanium-Sapphire femtosecond laser (Chameleon Ultra®, Coherent, Santa Clara, CA) at 750 nm and the emission was collected at 420-480 nm. *z*-stacks of 0~400 μm were taken for 3D image of amyloid plaques with 0.77 μm *z*-resolution and 512×512 pixels per image frame (0.22 μm/pixel dwell time). 2D image of maximum intensity and 3D imaging was processed with Zen 2011 software (Carl Zeiss Inc, Goettingen, Germany).

Hippocampal slice preparation for ex vivo two photon imaging. 5XFAD mice was sacrificed with cervical dislocation and whole brain was rapidly removed from the cranium and placed for 30 sec in ice cold artificial cerebrospinal fluid (aCSF) containing (in mM) NaCl (124), KCl (3), NaH_2PO_4 (1.25), $MgCl_2$ (1), $NaHCO_3$ (36), D-glucose (10), $CaCl_2$ (2) and bubbled with 95% O_2/5% CO_2. The brain was trimmed to expose the hippocampus, affixed to the stage of a vibrating blade microtome (Model VT1000S; Leica, Nussloch, Germany) and cut horizontally at 350 μm and maintained in cold aCSF. The slice includes hippocampus and surrounding cortical area. Three to four slices from the medial hippocampus were transferred to a recovery chamber supplied with oxygenated 95% O_2/5% CO_2 aCSF at 37 °C temperature at least 30 min. After recovery, 10 μM of SAD1, PIB or MeO-X04 containing aCSF was applied to hippocampal slices for 45 min for amyloid plaque staining in 5% of CO_2 incubator. For two photon imaging experiment, SAD1, PIB or MeO-X04 stained hippocampal slice was transferred to recording chamber and stabilized with slice anchor (Warner instrument, Hamden, CT, USA). Temperature (32 °C) controlled by fluidic inline heater (Live cell instrument, Seoul, Korea) and oxygenated aCSF was perfused to hippocampal slice with peristaltic pump (Gilson Inc, Middleton, WI, USA) during entire two photon imaging.

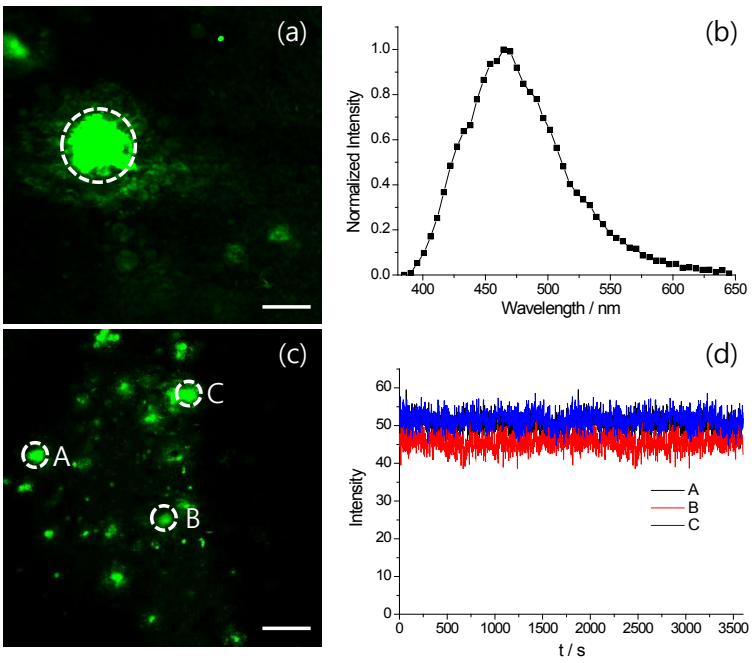


Figure S6. (a,c) TPM images of a hippocampal slice of brain from transgenic 5XFAD mice stained with 10 μM SAD1 for 45 min at 37 °C. (b) Two-photon excited fluorescence spectrum from the white circle in (a). (d) The relative TPEF intensity from the A-C in Figure (c) as a function of time. The digitized intensity was recorded with 1.63 sec intervals for the duration of one hour using *xyt* mode. The TPEF intensities were collected upon excitation at 750 nm with fs pulse. Scale bars, (a) 20 and (c) 50 μm.

Cranial window surgery and preparation for *in vivo* two photon imaging. We used 5XFAD mice (Tg6799; B6SJL-Tg [APPSwFlLon, PSEN*M146L*L286V] 6799Vas/J, stock no. 006554, The Jackson laboratory, Bar Harbor, USA) aged 4~10 months, which overexpress three mutations (Swedish, Florida, and London) of human APP 695 and two mutations (M146L and L286V) of human PS1.[12] A cranial window with 3~4 mm diameter over the right frontal cortex (approximately 0.5~3.5 mm rostral from bregma and 0.5~3.5 mm from midline) was surgically implanted as described by Mostany and Porter-Cailliau, 2008.[13] 5XFAD mice was anesthetized with isoflurane (4% for induction, 1.5~2% for surgery) and secured in a custom-built stereotaxic frame installed with warming pad. Before surgery, dexamethasone (0.2 mg/kg) and carprofen (5 mg/kg) was subcutaneously administrated to prevent the swelling of brain and/or inflammatory response, respectively. After removing skin and periosteum, lidocaine and epinephrine mixture was also topically applied to entire surface of brain to prevent excessive bleeding or pain during the surgical processes. Lines forming a circle about 3~4 mm diameter were a gently drilled onto the skull surface with a dental drill. Using an angled forceps, the skull was gently removed and care was given not to disturb or punctuate the dura mater (Gelfoam was also applied to surface to stop any small bleeding which is occasionally occurred when skull is removed). 5 mm round coverslips was laid on top of the dura mater and glued to the skull using dental acryl to close the craniotomy.

After surgery, mouse was allowed to recover for 1 week for two photon imaging. To stain amyloid plaque in mice brain 10 mg/kg of SAD1, PIB or MeO-X04 (5 mg/ml in 10% DMSO, 45% propylene glycol, and 45% saline) was injected into mice intraperitoneally (*i.p.*) 24 hr prior to two photon imaging (Klunk et al., 2002).[1] For *in vivo* imaging, SAD1, PIB or MeO-X04 injected mouse was anesthetized with isoflurane (2% for induction, 1~1.5 % for imaging). Mouse was transferred to stereotaxic device equipped with heating plate (Model: US-R-20, Live cell instrument, Seoul, Korea) and secured under the two photon laser scanning microscope. All animal procedures were performed under guidance of the Principle of Laboratory Animal Care (NIH publication No. 85–23, revised 1985) and the Animal Care and Use Guidelines of Seoul National University, Seoul, Korea.

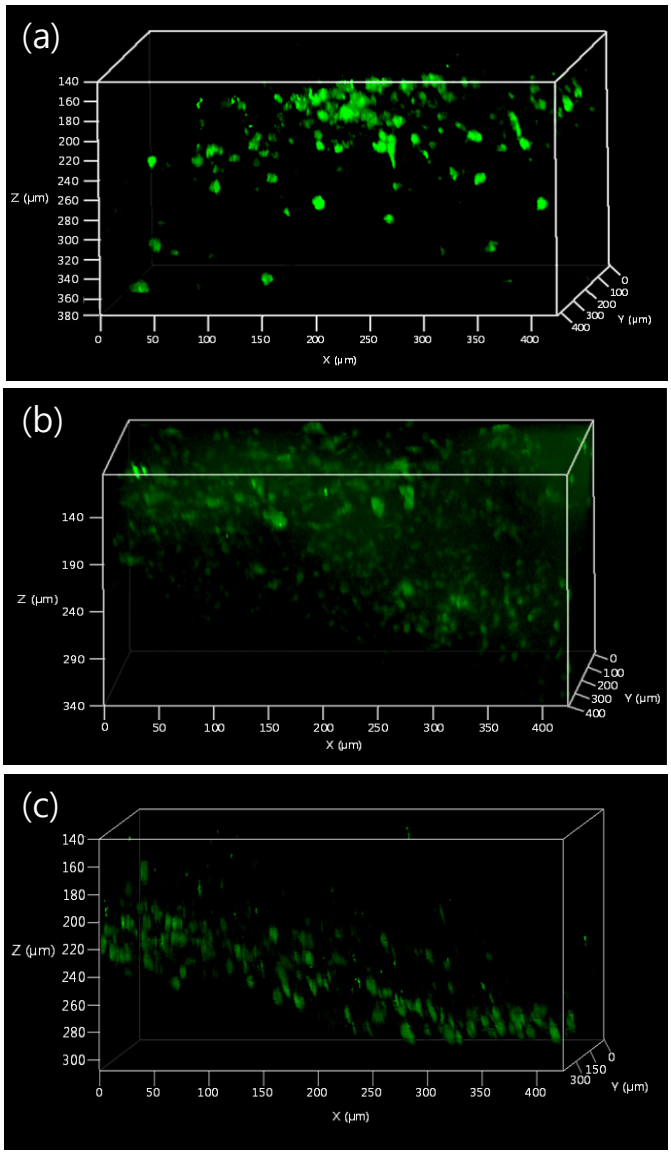


Figure S7. 3D reconstructed TP images of the frontal cortex of transgenic 5XFAD mice after i.p. injection of 10 mg/kg (a) SAD1, (b) PIB, and (c) MeO-X04. 300 TPM images acquired with 20x magnification along the z direction at the depth up to (a) 380, (b) 340 and (c) 300 μm, respectively, from the surface of the cortex. The TPEF were collected at 420-480 nm upon excitation at 750 nm with fs pulse. The images shown are representative of the images obtained in the repeat experiments (n = 5).

^{1}H-NMR, ^{13}C-NMR, and HRMS of SAD1

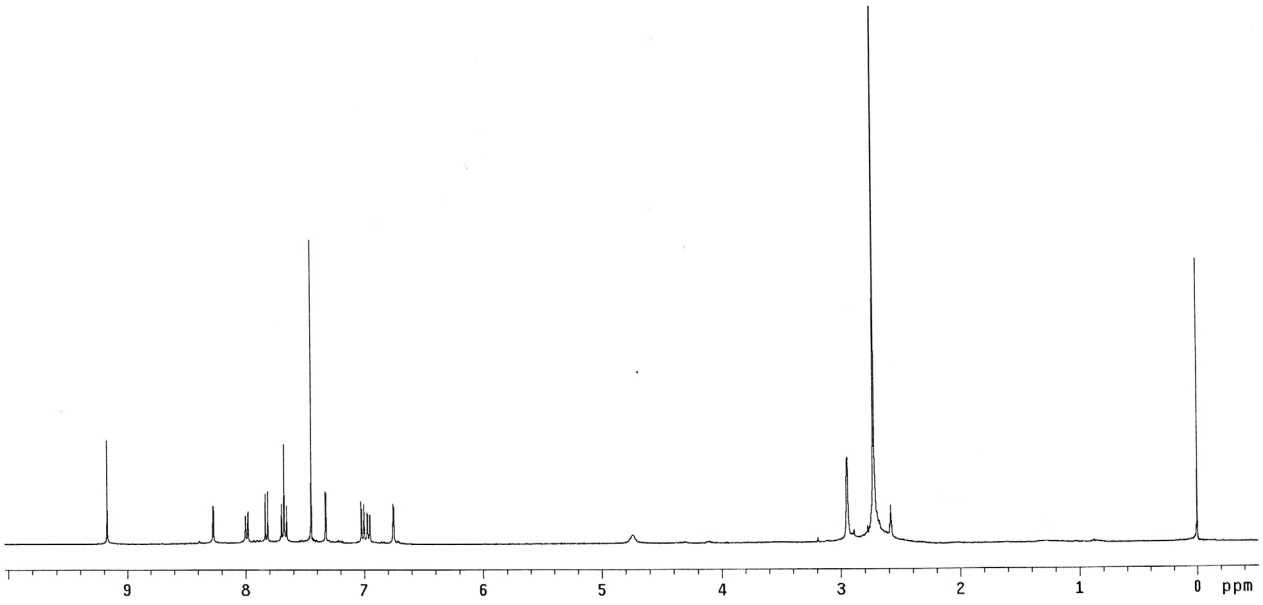


Figure S8. ^{1}H-NMR spectrum (400 MHz) of SAD1 in $CDCl_3$+DMSO-d_6.

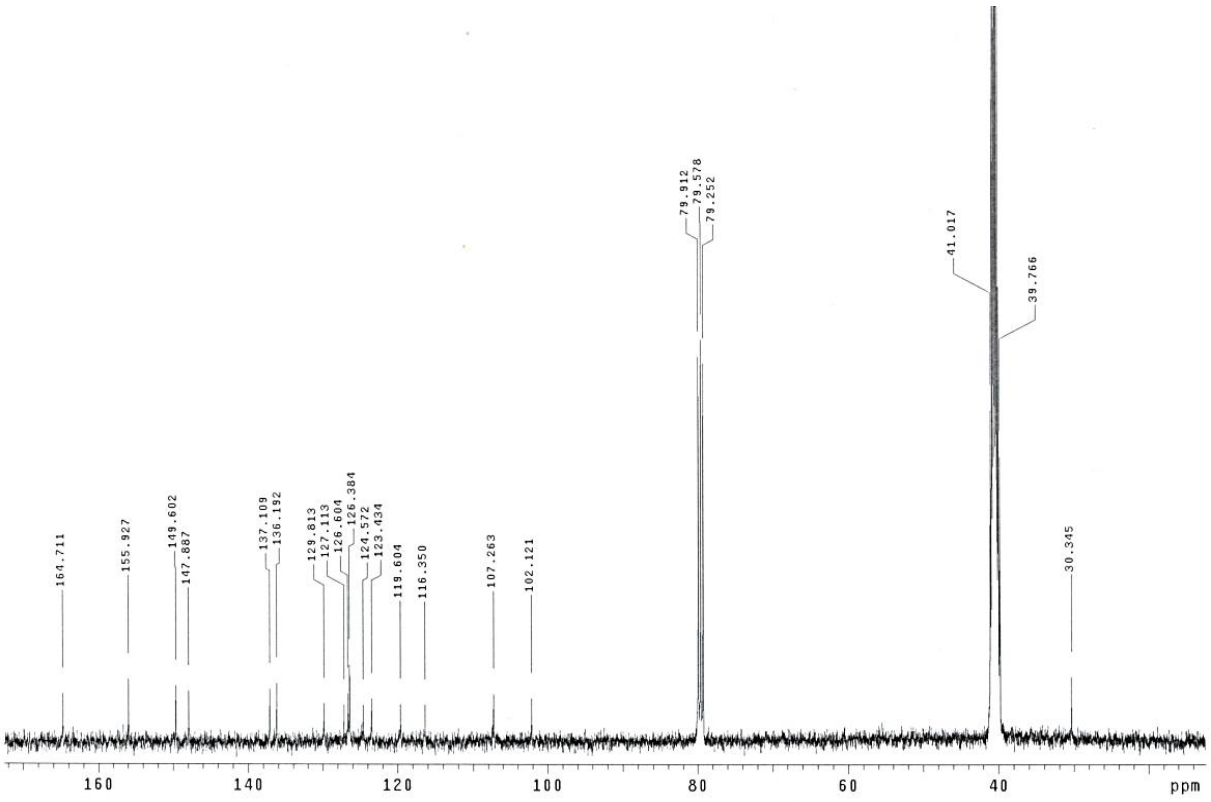


Figure S9. ^{13}C-NMR spectrum (100 MHz) of SAD1 in $CDCl_3$+DMSO-d_6.

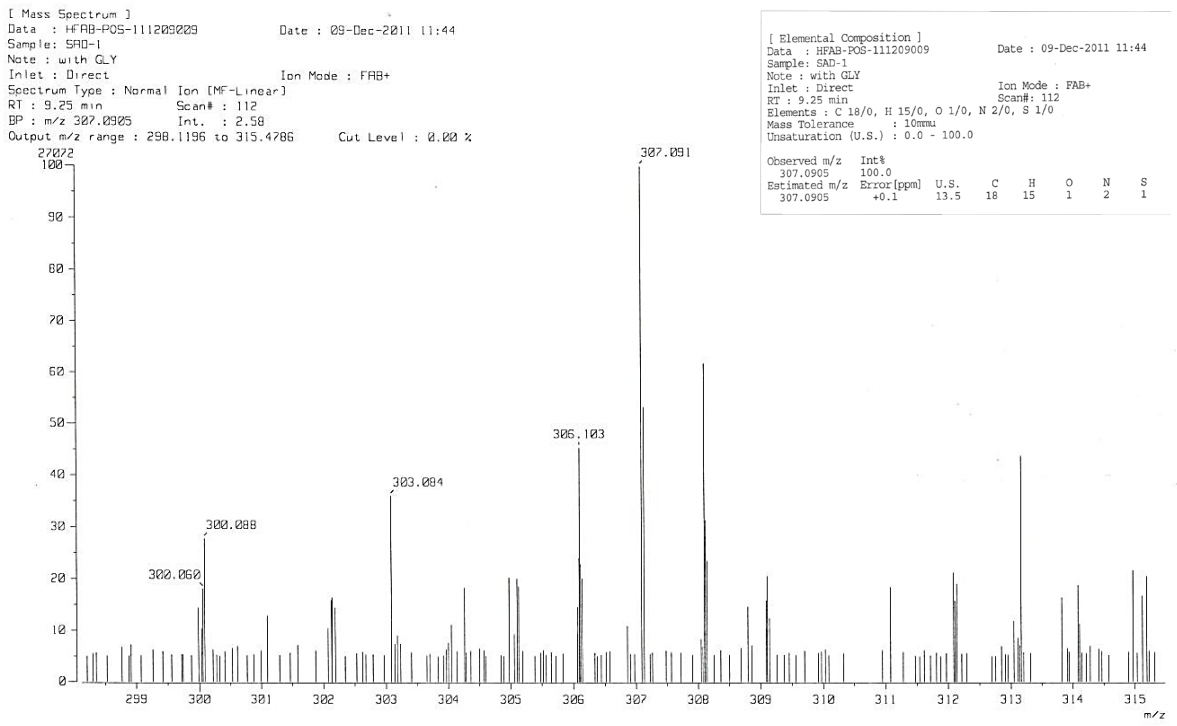


Figure S10. HRMS spectrum of SAD1.

References

(1) W. E. Klunk, B. J. Bacskai, C. A. Mathis, S. T. Kajdasz, M. E. McLellan, M. P. Frosch, M. L. Debnath, D. P. Holt, Y. Wang and B. T. Hyman, *J. Neuropathol. Exp. Neurol.*, 2002, **61**, 797.

(2) C. A. Mathis, Y. Wang, D. P. Holt, G. F. Huang, M. L. Debnath and W. E. Klunk, *J. Med. Chem.*, 2003, **46**, 2740.

(3) H. M. Kim, B. H. Jeong, J. Y. Hyon, M. J. An, M. S. Seo, J. H. Hong, K. J. Lee, C. H. Kim, T. Joo, S. C. Hong and B. R. Cho, *J. Am. Chem. Soc.*, 2008, **130**, 4246.

(4) B. E. Fink, A. V. Gavai, J. S. Tokarski, B. Goyal, R. Misra, H. Y. Xiao, S. D. Kimball, W. C. Han, D. Norris, T. E. Spires, D. You, M. M. Gottardis, M. V. Lorenzi and G. D. Vite, *Bioorg. Med. Chem. Lett.*, 2006, **16**, 1532.

(5) J. N. Demas and G. A. Crosby, *J. Phys. Chem.*, 1971, **75**, 991.

(6) C. Reichardt, *Chem. Rev.*, 1994, **94**, 2319.

(7) H. M. Kim, H. J. Choo, S. Y. Jung, Y. G. Ko, W. H. Park, S. J. Jeon, C. H. Kim, T. Joo and B. R. Cho, *ChemBioChem*, 2007, **8**, 553.

(8) I. Maezawa, H. S. Hong, H. C. Wu, S. K. Battina, S. Rana, T. Iwamoto, G. A. Radke, E. Pettersson, G. M. Martin, D. H. Hua and L. W. Jin, *J. Neurochem.*, 2006, **98**, 57.

(9) I. Maezawa, H. S. Hong, R. Liu, C. Y. Wu, R. H. Cheng, M. P. Kung, H. F. Kung, K. S. Lam, S. Oddo, F. M. Laferla and L. W. Jin, *J. Neurochem.*, 2008, **104**, 457.

(10) S. K. Lee, W. J. Yang, J. J. Choi, C. H. Kim, S. J. Jeon and B. R. Cho, *Org. Lett.*, 2005, **7**, 323.

(11) N. S. Makarov, M. Drobizhev and A. Rebane, *Opt. Express*, 2008, **6**, 4029.

(12) H. Oakley, S. L. Cole, S. Logan, E. Maus, P. Shao, J. Craft, A. Guillozet-Bongaarts, M. Ohno, J. Disterhoft, L. Van Eldik, R. Berry and R. Vassar, *J. Neurosci.*, 2006, **26**, 10129.

(13) R. Mostany and C. Portera-Cailliau, *J. Vis. Exp.*, 2008.