Aminonaphthalene 2-Cyanoacrylate (ANCA) Probes Fluorescently Discriminate between Amyloid-β and Prion Plaques in Brain

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The syntheses of molecules 1-3 were published previously. For convenience, a description of their syntheses and characterization are given here:

**General Notes for Chemical Synthesis**

All reagents were purchased at highest commercial quality and used without further purification except where noted. Air- and moisture-sensitive liquids and solutions were transferred via syringe or stainless steel cannula. Organic solutions were concentrated by rotary evaporation below 40 °C at approximately 20 mmHg. All non-aqueous reactions were carried out under anhydrous conditions. Yields refer to chromatographically and spectroscopically (¹H NMR, ¹³C NMR) homogeneous materials, unless otherwise stated. Reactions were monitored by thin-layer chromatography (TLC) carried out on 0.25 mm Dynamic Adsorbents, Inc. silica gel plates (60F-254) and visualized under UV light and/or developed by dipping in solutions of 10% ethanolic phosphomolybdic acid (PMA) and applying heat. Dynamic Adsorbents, Inc. silica gel (60, particle size 0.040-0.063 mm) was used for flash chromatography. NMR spectra were recorded on the Varian Mercury 400, 300 and/or Unity 500 MHz instruments and calibrated using the residual non-deuterated solvent as an internal reference. The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, b = broad. High resolution mass spectra (HRMS) were recorded on a VG 7070 HS mass spectrometer under electron spray ionization (ESI) or electron impact (EI) conditions.

![Chemical Reaction](image)

**6-bromo-2-naphtaldehyde (S1)**

To a solution of DIBAL-H (1.0 M in heptane, 34 mL, 34 mmol) at 0 °C under argon, a solution of 6-bromonaphthalene-2-carboxylate (3.0 g, 11 mmol) in anhydrous THF was added dropwise. The reaction mixture was allowed to warm up to room temperature and left stirring overnight. Upon completion, MeOH was added, followed by a saturated sodium potassium tartrate solution and ethylacetate. After the two phases were separated, the organic phase was washed with saturated solution of ammonium chloride and brine, dried over MgSO₄ and concentrated under reduced pressure to yield 6-bromo-2-(hydroxymethyl)naphthalene. Rᵢ = 0.33 (EtOAc:Hexanes 3:7); ¹H NMR (400 MHz, CDCl₃): δ 7.99 (bs, 1H), 7.77 (bs, 1H), 7.74 (d, J= 8.5 Hz,
1H), 7.69 (d, J= 8.7 Hz, 1H), 7.55 (dd, J= 1.7, 8.7 Hz, 1H), 7.49 (dd, J= 1.7, 8.5 Hz, 1H), 4.84 (bs, 2H); 
\(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 138.8, 133.9, 131.7, 129.7, 129.5, 127.4, 126.1, 125.2, 119.8, 65.2.

To a suspension of pyridinium chlorochromate (2.4 g, 11 mmol) in anhydrous CH\(_2\)Cl\(_2\) (60 mL) was added a solution of the above alcohol in anh. CH\(_2\)Cl\(_2\), and the reaction was heated under reflux for 5 hours. Upon completion, it was cooled to room temperature and poured into diethyl ether. The solution was then filtered through a pad of silica and concentrated under reduced pressure to yield S1 (2.4 g, 95%). S1: white solid; \(R_\text{f} = 0.67\) (EtOAc:Hexanes 3:7); \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 10.15 (s, 1H), 8.31 (bs, 1H), 8.08 (bs, 1H), 7.98 (dd, \(J= 1.5, 8.5\) Hz, 1H), 7.86 (m, 2H), 7.67 (dd, \(J= 1.5, 8.5\) Hz, 1H); 
\(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 191.8, 137.3, 134.3, 134.1, 131.0, 131.0, 130.6, 130.2, 128.2, 124.0, 123.6.

**General procedure for the synthesis of 6-amino-substituted napthaldehydes (S2-S4)**

In dry and degassed toluene (0.8 mL), were added Pd(OAc)\(_2\) (0.022 mmol) and P(tBu)\(_3\) (0.078 mmol) After stirring for 20 min, S1 (0.207 mmol), the appropriate amine (0.249 mmol) and Cs\(_2\)CO\(_3\) (0.280 mmol) were added and the reaction left stirring for three days under reflux. After three days, the reaction was cooled at room temperature, diluted with CH\(_2\)Cl\(_2\), filtered, concentrated under reduced pressure and purified via silica gel flash chromatography (hexanes/EtOAc 0-10%).

**6-(piperidin-1-yl)naphthalene-2-carbaldehyde (S2).** 70% yield, yellow solid; \(R_\text{f} = 0.61\) (EtOAc:Hexanes 3:7); \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 10.03 (s, 1H), 8.15 (s, 1H), 7.83 (m, 2H), 7.68 (d, \(J= 8.6\) Hz, 1H) 7.32 (dd, \(J= 2.4, 9.1\) Hz, 1H), 7.08 (d, \(J= 2.4\) Hz, 1H), 3.38 (m, 4H), 1.78-1.63 (m, 6H); 
\(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 191.9, 151.9, 138.5, 134.4, 131.3, 130.4, 127.2, 126.3, 123.4, 119.5, 108.8, 49.6, 25.5, 24.3; HRMS Calc for C\(_{16}\)H\(_{18}\)NO (M+H)* 240.1383 found 240.1381.

**6-morpholinonaphthalene-2-carbaldehyde (S3).** 79% yield, yellow solid; \(R_\text{f} = 0.56\) (EtOAc:Hexanes 3:7); \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 10.06 (s, 1H), 8.20 (s, 1H), 7.88 (m, 2H), 7.73 (d, \(J= 8.4\) Hz, 1H), 7.32 (m, 1H), 7.11 (d, \(J= 1.2\) Hz, 1H), 3.92 (m, 4H), 3.36 (m, 4H); 
\(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 191.9, 151.3, 138.1, 134.2, 131.8, 130.6, 127.5, 127.0, 123.6, 118.7, 109.0, 66.7, 48.5; HRMS Calc for C\(_{15}\)H\(_{15}\)NO\(_2\)Na (M+Na)* 264.0995 found 264.0996.
6-(4-methylpiperazin-1-yl)naphthalene-2-carbaldehyde (S4). 77% yield, yellow solid; Rf = 0.36 (EtOAc:Hexanes 3:7); 1H NMR (300 MHz, CDCl3) δ 10.00 (s, 1H), 8.13 (s, 1H), 7.80 (m, 2H), 7.66 (d, J = 8.6 Hz, 1H), 7.28 (dd, J = 2.1, 9.2 Hz, 1H), 7.06 (d, J = 2.1 Hz, 1H), 3.36 (m, 4H), 2.57 (m, 4H), 2.33 (s, 3H); 13C NMR (100 MHz, CDCl3) δ 191.5, 151.0, 138.0, 134.0, 131.3, 130.2, 127.1, 126.4, 123.1, 118.7, 108.7, 54.5, 47.8, 45.7; HRMS Calc for C16H19N2O (M+H)+ 255.1492 found 255.1491.

2-(2-(2-methoxyethoxy)ethoxy)ethyl-2-cyanoacetate (S5). To a solution of 2-cyanoacetic acid (230 mg, 2.72 mmol), triethylene glycol monomethyl ether (0.4 mL, 2.27 mmol) in CH2Cl2 (2.5 mL) and DMAP (2 mg, 0.013 mmol) was added dropwise at 0 °C. Finally, DCC (560 mg, 2.72 mmol) was added and the reaction mixture was stirred at 0 °C for 6 hours. The reaction was diluted with CH2Cl2 and the formed DCU was filtered off. The filtrate was dried over MgSO4 and concentrated under reduced pressure. The residue was purified by silica gel flash chromatography to yield S5 (450 mg, 86% yield). S5: colorless liquid; Rf = 0.45 (100% ether); 1H NMR (400 MHz, CDCl3) δ 4.29 (m, 2H), 3.67, (m, 2H), 3.59 (m, 6H), 3.50 (m, 2H), 3.49 (s, 2H), 3.32 (s, 3H); 13C NMR (100 MHz, CDCl3) δ 163.0, 113.0, 71.7, 70.4, 70.3, 68.3, 65.5, 58.8, 24.5; HRMS: calcd. for C10H17NO5: (M+H)+ 232.1185, found 232.1199.

General procedure for the synthesis of fluorescent probes (1-3)

To a round bottom flask containing a solution of aldehyde (0.21 mmol) and the appropriate 2-cyanoacetate (0.23 mmol) in THF (0.8 mL), piperidine (0.02 mmol) was added and the mixture left stirring at 50 °C. The reaction was monitored by TLC and was completed within 21 hours. The crude mixture was concentrated under reduced pressure and the product was purified via flash column chromatography (10-30% EtOAc in hexanes).
(E)-2-(2-(2-methoxyethoxy)ethoxy)ethyl-2-cyano-3-(2-(piperidin-1-yl)napthalen-6-yl) acrylate (1). 90% yield; red liquid; $R_f = 0.44$ (EtOAc:Hexanes 1:1); $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.31 (s, 1H), 8.22 (bs, 1H), 8.10 (d, $J= 8.8$ Hz, 1H), 7.76 (d, $J= 9.2$ Hz, 1H), 7.65 (d, $J= 8.8$ Hz, 1H), 7.30 (dd, $J= 2.1$, 9.2 Hz, 1H), 7.05 (d, $J= 2.1$ Hz, 1H), 4.47 (m, 2H), 3.83 (m, 2H), 3.74-3.66 (m, 6H), 3.56 (m, 2H), 3.42-3.38 (m, 4H), 3.37 (s, 3H), 1.74 (m, 6H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 163.3, 155.4, 151.9, 137.7, 134.7, 130.6, 127.2, 126.4, 125.9, 125.6, 119.2, 116.4, 108.3, 71.8, 70.7, 70.5, 70.5, 68.7, 65.3, 58.9, 49.3, 25.4, 24.3; HRMS Calc for C$_{26}$H$_{32}$N$_2$O$_5$Na (M+Na)$^+$ 475.2203 found 475.2197.

(E)-2-(2-(2-methoxyethoxy)ethoxy)ethyl-2-cyano-3-(2-morpholinonapthalen-6-yl) acrylate (2). 83% yield; red liquid; $R_f = 0.76$ (2% MeOH in CH$_2$Cl$_2$); $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 8.31 (s, 1H), 8.24 (s, 1H), 8.11 (dd, $J= 1.9$, 8.8 Hz, 1H), 7.80 (d, $J= 9.1$ Hz, 1H), 7.69 (d, $J= 8.8$ Hz, 1H), 7.28 (m, 1H), 7.06 (d, $J= 1.9$ Hz, 1H), 4.47 (m, 2H), 3.90 (m, 4H), 3.83 (m, 2H), 3.70 (m, 6H), 3.55 (m, 2H), 3.35 (m, 7H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 163.0, 155.2, 151.3, 137.2, 134.4, 130.6, 127.4, 127.0, 126.1, 126.0, 118.5, 116.1, 108.5, 99.4, 71.8, 70.7, 70.5, 70.4, 68.6, 66.5, 65.3, 58.9, 48.2; HRMS Calc for C$_{25}$H$_{30}$N$_2$O$_6$Na (M+Na)$^+$ 477.1996 found 477.1995.

(E)-2-(2-(2-methoxyethoxy)ethoxy)ethyl-2-cyano-3-(2-(4-methylpiperazin-1-yl) napthalen-6-yl)acrylate (3). 85% yield; red liquid; $R_f = 0.71$ (2% MeOH in CH$_2$Cl$_2$); $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.31 (s, 1H), 8.23 (s, 1H), 8.10 (d, $J= 8.6$ Hz, 1H), 7.78 (d, $J= 9.1$ Hz, 1H), 7.67 (d, $J= 8.6$ Hz, 1H), 7.29 (d, $J= 9.1$ Hz, 1H), 7.06 (s, 1H), 4.46 (m, 2H), 3.83 (m, 2H), 3.73 (m, 2H), 3.67 (m, 4H), 3.55 (m, 2H) 3.42 (bs, 4H), 3.36 (s, 3H), 2.61 (bs, 4H), 2.37 (s, 3H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 163.2, 155.4, 151.4, 137.5, 134.5, 130.6, 127.4, 126.9, 126.1, 126.1, 119.0, 116.2, 108.7, 99.3, 71.9, 70.8, 70.6, 70.5, 68.8, 65.4, 59.0, 54.8, 48.0, 46.1; HRMS Calc for C$_{26}$H$_{34}$N$_5$O$_5$ (M+H)$^+$ 468.2493 found 468.2494.
Staining of brain tissue sections

Male and female transgenic mice overexpressing wild type mouse PrP (Tga20) were inoculated with the mouse-adapted prion strain mCWD and were euthanized upon developing terminal signs of prion disease. Transgenic mice (19959) harboring the Aβ plaques express the mutant human amyloid precursor protein 695 APP^{SweInd}, which bears both the Swedish (K670N/M671L) and the Indiana (V717F) mutations, under the control of the Syrian hamster prion protein promoter. Mice were euthanized between 9 - 13 months of age.

Frozen brain sections from transgenic mice producing Aβ and PrP^Sc protein were dried for 1 hr, treated with 100%, 95%, and 70% ethanol for 5 min each, and then rinsed with deionized water. Sections were then buffered with phosphate-buffered saline (1X PBS) for 30 min. Compounds 1, 2, and 3 were diluted 1:50 in 1X PBS (from stock solutions of 3mM) to a final concentration of 60μM, added to brain sections, incubated for 30 min at room temperature, washed with 1X PBS, and coverslipped using DAKO fluorescent mounting media.

Fluorescence microscopy

Brain tissue samples were excited with a 488 nm laser on an Olympus FluoView FV1000 spectral deconvolution confocal microscope. The emission spectra of compounds 1, 2, and 3 bound to Aβ, PrP^Sc, and background were collected in 1 nm increments from 450-650 nm. A minimum of 10 measurements were collected for each compound bound to plaque and non-plaque regions as a background control. The wavelength corresponding to the maximum relative fluorescence intensity was taken as the emission $\lambda_{max}$.

Spectroscopic studies of the ANCA probes versus pH

Each probe was dissolved in 95:5 deionized water:DMSO solution to a final concentration of 200 μM and titrated with 10 mM HCl and 1 M HCl with aliquots removed in approximately pH=0.5 increments starting from basic pH. Aliquots were evaluated on a SpectraMAX 190 absorbance microplate reader (Molecular Devices) in 1 nm increments from 250-550 nm. Fluorescence emission spectra for each aliquot were obtained using a PTI spectrofluorimeter in 1 nm increments from 400-650 nm. Each probe was excited with a wavelength corresponding to the spectral window in which both base probe and protonated probe showed good absorption. Maximum intensity wavelengths were plotted against pH and fit to a Boltzmann sigmoidal curve to estimate the pKₐ.
Fluorescence studies with aggregated Aβ peptides

Aggregated Aβ peptide was prepared as described previously.\textsuperscript{53} Briefly, we dissolved Aβ42 in PBS pH 7.4 to a final concentration of 100 µM. This solution was magnetically stirred at 1200 rpm for 3 days at room temperature. Aliquots of 15 µL of the pre-aggregated Aβ42 solution was added to 285 µL of the probe (5% DMSO in nanopure water) to attain a final concentration of 5 µM Aβ42 and 4 µM of the probe. The solution was transferred to a 300 µL cuvette and the fluorescence was measured.

**Figure S1.** Fluorescence emission spectra of ANCA probes 1-3 in PBS buffer in the presence or absence of aggregated Aβ peptide. Probe 1 was excited at $\lambda_{\text{max}} = 410$ nm. Probes 2 and 3 were excited at $\lambda_{\text{max}} = 400$ nm.
Determination of binding constant

Pre-aggregated Aβ42 (5 µM final concentration) was mixed with various concentrations of probes (10, 5, 2.5, 1.25 µM) in 5% DMSO in nanopure water and their K\(_d\)'s were determined as described previously\(^{S1}\).

*S1*

**Figure S2.** Plots of the fluorescence intensity versus concentration of probes 1-3 in the presence of aggregated Aβ peptide. The K\(_d\) was determined as described previously\(^{S1}\) by fitting the data to a one-site specific binding algorithm: \( Y = \frac{B_{\text{max}} \times X}{K_d + X} \), where X is the concentration of the probe, Y is specific binding fluorescence intensity, and \( B_{\text{max}} \) corresponds to the apparent maximal observable fluorescence upon binding of probes to aggregated Aβ42 peptide.
**Brightfield color imaging**

Brain tissue sections were imaged under an Olympus MVX10 Macroview equipped with a MWB2 (Japan) long-pass filter. Each sample was illuminated with epifluorescence and imaged with an exposure time of 0.2 sec. Congo red samples were imaged with an exposure time of 0.1 sec.

![Brightfield images](image)

**Figure S3.** Brightfield true color images of compounds 1, 2, and 3 bound to plaque deposits in brain tissue sections from mice. Scale bar = 100 μm. Aβ plaque deposits shown were located in the hippocampus; PrP^Sc deposits were located in the corpus callosum. Fluorescence emission maxima of Aβ and PrP^Sc stained plaques for compound 1 were measured at 535 ± 3 nm and 554 ± 2 nm, respectively. For compound 2, Aβ and PrP^Sc stained plaques had emission maxima at 525 ± 4 nm and 541 ± 4 nm, respectively. For compound 3, Aβ and PrP^Sc stained plaques had emission maxima at 515 ± 4 nm and 538 ± 3 nm, respectively.
Staining of frozen brain sections with Congo red

Frozen brain sections from transgenic mice with Aβ and PrPSc plaques were dried for 1 hr, treated with 100%, 95%, and 70% ethanol for 5 min each, and then rinsed with deionized water. Sections were then buffered with a working solution of sodium chloride in 1X PBS for 20 min. A 2% solution of Congo red was prepared in 1X PBS, added to brain sections, incubated for 1 hr at room temperature, washed with deionized water, and coverslipped using DAKO fluorescent mounting media.

Figure S4. Aβ and PrPSc deposits in brain tissue sections stained with Congo red. Scale bar = 100 μm. A) Image of Congo red staining in transgenic mice with deposits of Aβ. B) Real-color image of Congo red staining in prion-infected mice. C) Fluorescence emission spectra of stained plaques. The maximum fluorescence was measured at 593 ± 3 nm for Aβ deposits, and at 594 ± 8 nm for PrPSc deposits.
**Figure S5.** Plots of the fluorescence emission $\lambda_{\text{max}}$ versus Stokes shift for compounds 1-3 measured in different organic solvents. Each compound was dissolved in solvent to a final concentration of 1.5 $\mu$M. Error bars represent the standard deviation from at least two independent runs. Plots were fit with the linear function: $Y = m \cdot X + b$. The linear fits are shown in red, and the values for slope and intercept obtained from these fits are given within each graph.
**Solvent studies of probes**

Each probe was dissolved in dimethylsulfoxide (DMSO), methanol (MeOH), 2-propanol (iPrOH), dichloromethane (DCM), ethyl acetate, chloroform, and toluene to a final concentration of 1.5 μM. Excitation and fluorescence emission spectra of each solution was obtained on a PTI spectrofluorimeter in 1 nm increments from 350-520 nm and 400-650 nm, respectively. The wavelength corresponding to the maximum intensity was taken as $\lambda_{\text{max}}$.

*Figure S6.* Solvent studies on fluorescence emission of compounds 1-3. The values for $\lambda_{\text{max}}$ obtained from these experiments and the published values$^{34,35}$ for relative static permittivity and refractive indices of these solvents were used to generate the plots shown in Figure 4. N.F.I. = normalized fluorescence intensity.
Figure S7. Plots of the relationship of $1/\lambda_{\text{max}}$ for the emission of probes 1-3 versus either the static relative permittivity ($\varepsilon_0$) of the solvent alone (open circles, ○) or versus the static relative permittivity and the refractive index ($n$) of the solvent (open squares, □). Data were fit to the linear equation $y = m \cdot X + b$ with the best-fit line shown in red. The slopes ($m$) are in units of $\text{x}10^{-3}$ nm$^{-1}$. This analysis revealed that, with every probe, the linear correlation (as estimated by the $R^2$ values) between emission and $\varepsilon_0$ of the solvent was stronger when the refractive index of the solvent was not considered.
Supporting References


