Supporting Information to Accompany

"A small molecule two-photon fluorescent probe for intracellular sodium ions"

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Synthesis of ANa2. (S)-1-(6-acetylnaphthalen-2-yl)pyrrolidine-2-carboxylic acid¹ (A) and N-(2-methoxyphenyl)-N'-(4-amino-2-methoxyphenyl)-1,7-diaza-15-crown-5² (B) were prepared by the literature methods. Synthesis of ANa2 is described below.



ANa2. Compound A (0. 07 g, 0.25 mmol), B (0.11 g, 0.25 mmol) and hydroxybenzotriazole (HOBt, 0.01 g, 0.074 mmol) were dissolved in dry DCM (30 mL). To this mixture N,Ndiisopropylethylamine (DIEA, 0.07 g, 0.09 mL, 0.54 mmol) was added and the reaction mass was stirred under nitrogen atmosphere for 10 min. After stirring for 10 min, benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP, 0.39 g, 0.75 mmol) was added to the reaction mass and the reaction mass was allowed to stirring for 36 h under nitrogen atmosphere. The solvent was then removed in vacuo and the resulting residue dissolved in CHCl₃ (15 mL) and washed with water (3 x 10 mL). The organic layer dried over MgSO₄, and removed in vacuo to obtained the crude. The crude was purified by SiO₂ coloumn chromatography using chloroform/methanol (10:1 to 4:1) as the eluent to give ANa2 as a brown solid. Yield: 0.093 g (53 %); m.p. 145-148 °C; ¹H NMR (400 MHz, CDCl₃): δ 8.36 (s, 1H), 8.11 (s, 1H), 7.97 (dd, J = 2.0, 2.0 Hz, 1H), 7.87 (d, J = 9.2 Hz, 1H), 7.69 (d, J = 8.4 Hz, 1H), 7.34 (d, J = 2.8 Hz, 1H), 7.09 (dd, J = 2.8 Hz, 1H 2.8, 2.8 Hz, 1H), 7.02 (dd, J = 1.2, 2.0 Hz, 1H), 6.98 (d, J = 2.0 Hz, 1H), 6.94 – 6.90 (m, 2H), 6.88 – 6.80 (m, 2H), 6.70 (dd, J = 2.4, 2.4 Hz, 1H), 4.23 (m, 1H), 3.93 - 3.88 (m, 1H), 3.81 (s, 3H), 3.80 (s, 3H), 3.69 – 3.56 (m, 11H), 3.46 – 3.34 (m, 10H), 2.69 (s, 3H), 2.45 – 2.39 (m, 2H), 2.18 – 2.17 (m, 1H). ¹³C NMR (100 MHz, CDCl₃): 196.8, 172.5, 154.6, 154.3, 146.3, 149.9, 138.6, 136.9, 130.7, 129.9, 129.6, 129.2, 127.2, 125.3, 124.2, 123.1, 122.0, 119.9, 119.8, 116.2, 113.7, 111.1, 104.8, 103.9, 79.3, 74.6, 68.7, 67.0, 65.9, 65.6, 61.9, 56.2, 55.4, 55.1, 50.5, 48.6, 31.3, 28.4, 25.7, 23.3, 21.3; HRMS (FAB⁺): m/z calcd for $[C_{41}H_{50}N_4O_7 + Na^+]$: 733.3577, found: 733.3578.

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 ($\Phi = 0.95$ in MeOH) as the reference by the literature method.³



Figure S1. Normalized absorption (a) and emission (b) spectra of ANa2 in 1,4-dioxane, DMF, EtOH and buffer (10 mM MOPS, pH 7.0).

Compound	Solvent $(E_{T}^{N})^{(a)}$	$\lambda^{(1)}_{max}$, nm ^(b)	$\lambda_{\max}^{\mathrm{fl}}, \mathrm{nm}^{(\mathrm{c})}$
	1,4-Dioxane (0.164)	345	418
ANa2	DMF (0.386)	358	450
	EtOH (0.654)	365	477
	buffer (1.000) ^(d)	366	500

Table S1. Absorption and emission maxima of ANa2 in various solvent

a) The numbers in the parenthesis are normalized empirical parameter of solvent polarity.⁴ b,c) λ_{max} of the one-photon absorption and emission spectra in nm. d) 10 mM MOPS buffer (pH 7.0). The E_T^N value is for water.

Water Solubility of ANa2 in MOPS buffer. Small amount of dye was dissolved in DMSO to prepare the stock solutions $(1.0 \times 10^{-2} \text{ M})$. 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 MOPS buffer (10 mM, pH 7.0) by using a micro syringe. In all cases, the concentration of DMSO in H₂O was maintained to be 0.2 %.⁵ The plots of fluorescence intensity against the total amount of the dye injected to the cuvette were linear at low dye concentration and showed downward curvature as more dye was added (Figure S2). The maximum point in the linear region was taken as the solubility. The solubility of ANa2 in MOPS buffer was 1.5 μ M.



Figure S2. (a) One-photon fluorescence spectra and (b) plot of fluorescence intensity against the concentration of ANa2 in 3.0 mL MOPS buffer (pH 7.0). The excitation wavelength was 360 nm.

Compound	$\lambda_{\max}^{(1)/\lambda_{\max}^{fl(b)}}$	$\Phi^{(c)}$	$K_{d}^{\text{ Na/}}\!K_{d}^{\text{ Na/}K(d)}$	$K_d^{K(e)}$	FEF ^(f)	$\lambda_{\max}^{(2)(g)}$	$\delta\Phi^{(h)}$
ANa2	366/500	0.023				750	3
ANa2/ Na ⁺	366/500	0.35	18(19.6)/21.6(22)	296	15(16)	750	83

Table S2. Photophysical data for ANa2.^(a)

a) All data measured in 10 mM MOPS buffer in the absence and presence of 500 mM of free Na⁺. b) λ_{max} of the one-photon absorption and emission spectra in nm. c) Fluorescence quantum yield. The uncertainity is $\pm 10\%$. d) Dissociation constants for Na⁺ in mM measured by one-photon processes in the absence (K_d^{Na}) and presence $(K_d^{\text{Na/K}})$ of K (135 mM), the uncertainty is $\pm 10\%$. The number in the parentheses is measured by two-photon process. e) Dissociation constants for K⁺ in mM measured by one-photon processes. The uncertainty is $\pm 10\%$. f) Fluorescence enhancement factor, $(F-F_{\text{min}})/F_{\text{min}}$ by one-photon excitation spectra in nm. h) Two-photon action cross section in 10⁻⁵⁰ cm⁴ s photon⁻¹ (GM). The uncertainty is $\pm 15\%$.

Determination of Apparent Dissociation Constant: A series of calibration solutions containing various $[Na^+]$ was prepared by mixing two solutions (*solution A* containing 135 mM KCl and *solution B* containing 500 mM NaCl) in various ratios.⁶ Both solutions contained 1 μ M ANa2, 10 mM MOPS, and they were adjusted to pH 7.0.

To determine the K_d for ANa2-Na⁺, the fluorescence spectrum was recorded with 3.0 mL of *solution A* (0 µM free Na⁺) at 20 °C. Then 8 µL of this solution was discarded and replaced by 8 µL of *solution B*, and the spectrum was recorded. This brings the [Na⁺] to 1.35 mM with no change in the [probe]. Further iterations attained 2.70, 8.10, 10.8, 16.2, 27.0, 35.1, 43.2, 54.0, 94.5, 135.0, 250.0, 350.0, 450.0 and 500.0 mM free Na⁺ by successively discarding 0.016, 0.048, 0.064, 0.097, 0.162, 0.216, 0.259, 0.324, 0.567, 0.810, 1.5, 2.1, 2.7 and 3.0 mL of *solution A* and replacing each with an equal volume of *solution B*.

When a 1:1 metal-ligand complex is formed between probe and Na^+ , one can describe the equilibrium as follows, where L and M represent probe and Na^+ , respectively.

The total probe and metal ion concentration are defined as $[L]_0 = [L] + [LM]$ and $[M]_0 = [M] + [LM]$, respectively. With $[L]_0$ and $[M]_0$, the value of K_d is given by:

$$[LM]^{2} - ([L]_{0} + [M]_{0} + K_{d})[LM] + [L]_{0}[M]_{0} = 0,$$

or
$$(F-F_{min}) = \left[\left\{ ([L]_0 + [M]_0 + K_d) - (([L]_0 + [M]_0 + K_d)^2 - 4[L]_0[M]_0)^{1/2} \right\} / 2[L]_0 \right] (F_{max} - F_{min})..(2)$$

where *F* is the observed fluorescence intensity, F_{min} is the minimum fluorescence intensity, and F_{max} is the maximum fluorescence intensity. The K_d value that best fits the titration curve (Figures 1c, S3 and S4) with Eq 2 was calculated by using the Excel program as reported.⁷

In order to determine the K_d^{TP} for the two-photon process, the TPEF intensity were recorded in the range of 450-550 nm with a DM IRE2 Microscope (Leica) excited by a mode-locked titanium-sapphire laser source (Mai Tai HP; Spectra Physics, 80 MHz pulse frequency, 100 fs pulse width) set at wavelength 750 nm and output power 2510 mW, which corresponded to approximately 10 mW average power in the focal plane. The TPEF titration curves (Figures 1c and S3) were obtained and fitted Eq. 2.



Figure S3. (a) One-photon absorption. and (b,c) one-photon and two-photon emission spectra of 1 μ M ANa2 (10 mM MOPS, pH 7.0) in the presence of free Na⁺ (0–500 mM). (d) One- (\bigcirc) and two-photon (\bigcirc) fluorescence titration curves for the complexation of ANa2 with free Na⁺ (0–500 mM). (e) Benesi-Hildebrand plots for the complexation of ANa2 with free Na⁺ (0–500 mM). The excitation wavelengths for one- and two-photon processes were 360 and 750 nm, respectively. The solid lines in (d) and (e) are the calculated values (Eq. 2 and Ref. 7).



Figure S4. (a) One-photon emission spectra for 1 μ M ANa2 (10 mM MOPS, pH 7.0) with free K⁺ (0–1000 mM). (b) Titration curve for the complexation of 1 μ M ANa2 (10 mM MOPS buffer, pH 7.0) with free K⁺ (0-1000 mM). (c) Benesi-Hildebrand plots for the complexation of ANa2 with free K⁺. The excitation wavelength was 360 nm. The solid lines in (b) and (c) are the calculated values (Eq 2 and Ref. 7).



Figure S5. Plot of the fluorescence intensity for ANa2 versus [Na⁺] in MOPS buffer (10 mM MOPS, pH 7.0). The detection limit (102 \pm 3 μ M) was calculated with 3 σ /k; where σ is the standard deviation of blank measurements and k is the slop in Figure S5.⁸ The excitation wavelength was 360 nm.



Figure S6. (a) The relative fluorescence intensity of ANa2 (10 mM MOPS, pH 7.0) in the presence of 200 mM for K⁺, 5 mM Li⁺, Ca²⁺, Mg²⁺, 100 μ M for Zn²⁺, Fe²⁺, Cu²⁺, Cu⁺, Mn²⁺, Co²⁺, Ni²⁺ (gray bars) and subsequent addition of 100 mM of Na⁺ (black bars). (b) Effect of the pH on the one-photon fluorescence intensity of 1 μ M ANa2 in the presence of 0 (\Box) and 500 mM (\blacksquare) of NaCl in 50 mM MOPS buffer. The excitation wavelengths were 360 nm.

Measurement of Two-Photon Cross Section. The two-photon cross section (δ) was determined by using femto second (fs) fluorescence measurement technique as described.⁹ ANa2 was dissolved in 10 mM MOPS buffer at concentration 1.0×10^{-6} M in the absence or presence of 500 mM Na⁺ 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.¹⁰ 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 Eq. 3.

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.

Cell Culture. HeLa human cervical carcinoma cells (ATCC, Manassas, VA, USA) were cultured in DMEM (WelGene Inc, Seoul, Korea) supplemented with 10 % FBS (WelGene), penicillin (100 units/ml), and streptomycin (100 μ g/mL). Two days before imaging, the cells were passed and plated on glass-bottomed dishes (NEST). All the cells were maintained in a humidified atmosphere of 5/95 (v/v) of CO₂/air at 37 °C. For labeling, the growth medium was removed and replaced with serum-free DMEM. The cells were treated and incubated with 1.0 μ L of 1 mM ANa2 in DMSO stock solution (1.0 μ M ANa2) at 37 °C under 5 % CO₂ for 30 min.

Primary astrocytes were cultured from the cortex of wild type mice brains. In brief, cortexes were removed and triturated in DMEM (Invitrogen, Carlsbad, CA, USA) containing 10 % FBS (HyClone, Logan, UT, USA), plated in 75 cm² T-flasks (0.5 hemisphere/flask), and incubated for 2~3 weeks. Microglia were detached from flasks by mild shaking, filtered through a nylon mesh to remove cell clumps, and cultured in DMEM containing 10% FBS.¹¹ Astrocytes remaining in the flask were harvested with 0.1% trypsin and cultured in DMEM containing 10% FBS. After 7-15 days in vitro, astrocytes were washed three times with serum-free media, and then incubated with 1.0 μ L of 1 mM ANa2 in DMSO stock solution (1.0 μ M ANa2) at 37 °C under 5 % CO₂ for 30 min.

Two-Photon Fluorescence Microscopy. Two-photon fluorescence microscopy images of ANa2 labeled cells and tissues were obtained with spectral confocal and multiphoton microscopes (Leica TCS SP8 MP) with $\times 10$ dry, $\times 40$ oil, $\times 63$ oil and $\times 100$ oil objectives, numerical aperture (NA) = 0.30, 1.30, 1.40, and 1.30, respectively. The two-photon fluorescence microscopy images were obtained with a DMI6000B Microscope (Leica) by exciting the probes with a mode-locked titanium-sapphire laser source (Mai Tai HP; Spectra Physics, 80 MHz pulse frequency, 100 fs pulse width) set at wavelength 750 nm and output power 2510 mW, which corresponded to approximately 10 mW average power in the focal plane. To obtain images at 450-550 nm range, internal PMTs were used to collect the signals in an 8 bit unsigned 512×512 and 1024×1024 pixels at 400 and 200 Hz scan speed, respectively.



Figure S7. (a,c) Pseudo colored TPM images of 1 μ M ANa2-labeled (a) HeLa cells and (c) astrocytes collected at 450-550 nm. (b,d) Two-photon excited fluorescence spectra for (b) HeLa cells and (d) astrocytes. The excitation wavelengths were 750 nm. Cells shown are representative images from replicate experiments (n = 5), Scale bar: (a,c) 47 μ m.

Photostability. Photostability of ANa2 was determined by monitoring the changes in TPEF intensity with time at two designated positions of 1 μ M ANa2 labeled HeLa cells (a) and astrocytes (b) chosen without bias (Figure S8a,c). The TPEF intensity remained nearly the same for 1 hr (Figures S8b,d), indicating high photostability.



Figure S8. TPM image of 1 μ M ANa2-labelled (a) HeLa cells and (c) astrocytes collected at 450-550 nm. The relative TPM intensity for (b) HeLa cells and (d) astrocytes as a function of time. The digitized intensity was recorded with 2.00 sec intervals for the dutation of one hour using *xyt* mode ($\lambda_{ex} = 750$ nm) with femto-second pulses. Cells shown are representative images from replicate experiments (n = 5), Scale bar: (a,c) 47 μ m.

Preparation and Staining of fresh rat Hippocampal slices. Slices were prepared from the hippocampi of 2-weeks-old rat (SD). Coronal slices were cut into 400 μ m-thick using a vibrating-blade microtome in artificial cerebrospinal fluid (ACSF; 138.6 mM NaCl, 3.5 mM KCl, 21 mM NaHCO₃, 0.6 mM NaH₂PO₄, 9.9 mM D-glucose, 1 mM CaCl₂, and 3 mM MgCl₂). Slices were incubated with 20 μ M ANa2 in ACSF bubbled with 95% O₂ and 5% CO₂ for 30 min at 37 °C. Slices were then washed three times with ACSF and transferred to glass-bottomed dishes (NEST) and observed in a spectral confocal multiphoton microscope. The TPM images of fresh rat Hippocampal slice labeled with 20 μ M ANa2 obtained at 90–180 μ m depth are shown in Figure S9.



Figure S9. Images of a rat hippocampal slice stained with 20 μ M ANa2. The TPEF were collected at 450-550 nm upon excitation at 750 nm with fs pulses. TPM images of the CA1-CA3 regions as well as dentate gyrus (DG) at 10x magnification. 15 TPM images along the z-direction at the depths of approximately 90–180 μ m were accumulated. Scale bar: 300 μ m.

¹H-NMR, ¹³C-NMR, and HRMS of **ANa2**



Figure S10. ¹H-NMR spectrum (400 MHz) of ANa2 in CDCl₃.



Figure S11. ¹³C-NMR spectrum (100 MHz) of ANa2 in CDCl₃.

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Figure S12. HRMS spectrum of ANa2.

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