Supporting Information

A Two-Photon Fluorescent Probe for Thiols in Live Cells and Tissues

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Synthesis of ASS and ACC.

1, **3**, and **5** were prepared by the literature methods.^[1] Synthesis of **ASS** and **ACC** is described below.

Compound 4. To a solution of crude **3** (500 mg, 1.6 mmol), pyridine (0.64 mL, 7.9 mmol), **1** (630 mg, 3.2 mmol) in DMF (5 mL), a catalytic amount of *N,N*-dimethylaminopyridine (DMAP) were added and stirred for 14 h at RT. The solvent was removed *in vacuo* and the resulting brown residue was dissolved in CH_2Cl_2 and washed twice with 1% HCl (aq). The crude product was purified by silica-gel column chromatography using hexane/ethyl acetate (4:1) as the eluent. Yield, 310 mg (41 %); IR (KBr): 3361, 1714, 1681 cm^{-1} ; ^1H NMR (CDCl_3 , 300 MHz): δ 8.45 (d, J = 1.9 Hz, 1H) 8.06 (dd, J = 1.9, 8.8 Hz, 1H), 7.96 (d, J = 8.8 Hz, 1H), 7.86 (d, J = 8.8 Hz, 1H), 7.72 (d, J = 1.9 Hz, 1H), 7.55 (d, J = 1.9 Hz, 8.8 Hz, 1H), 4.41 (t, J = 6.6 Hz, 2H), 3.44 (s, 3H), 3.41 (t, J = 6.6 Hz, 2H), 2.92 (t, J = 6.6 Hz, 2H), 2.75 (t, J = 6.6 Hz, 2H), 2.73 (s, 3H), 1.43 (s, 9H). ^{13}C (CDCl_3 , 100 MHz): δ 198.2, 155.3, 136.8, 136.0, 134.7, 130.7, 130.4, 130.0, 128.4, 125.7, 124.8, 122.9, 120.8, 63.9, 39.3, 38.8, 38.0, 37.4, 28.6, 26.9, 14.4. Anal. Calcd for $\text{C}_{23}\text{H}_{30}\text{N}_2\text{O}_5\text{S}_2$: C, 57.72; H, 6.32; N, 5.85; S, 13.40. Found: C, 57.64; H, 6.47; N, 5.75; S, 13.30

ASS. 4 (300 mg, 6.8 mmol) was dissolved in $\text{CF}_3\text{CO}_2\text{H}/\text{CH}_2\text{Cl}_2$ (1/1, 10 mL) at 0 $^\circ\text{C}$ under darkness. The solution was stirred for 1 h at 0 $^\circ\text{C}$, warmed to RT, stirred for 2h, and the solvent was removed *in vacuo*. The residue was dissolved in MeOH and filtered. Using prep-HPLC, ASS was purified with a mixture of 10 % eluent A (HPLC-grade MeCN containing 0.1% TFA) and 90 % eluent B (HPLC-grade water containing 0.1% TFA) for 10 min, followed by a linear gradient to 80 % eluent A and 20 % eluent B over the period of 120 min, with a flow rate of 10 mL/min. The pure ASS was collected as brown oil. Yield, 140 mg (46 %); IR (KBr): 3442, 1712, 1680 cm^{-1} ; ^1H NMR (CD_3OD , 300 MHz): δ

8.60 (d, $J = 2.0$ Hz, 1H), 8.06 (d, $J = 8.9$ Hz, 1H), 8.04 (dd, $J = 2.0$ Hz, 8.9 Hz, 1H), 7.93 (d, $J = 8.9$ Hz, 1H), 7.84 (d, $J = 2.0$ Hz, 1H), 7.60 (dd, $J = 2.0$ Hz, 8.9 Hz 1H), 4.42 (t, $J = 6.5$ Hz, 2H) 3.43 (s, 3H), 3.22 (t, $J = 6.5$ Hz, 2H), 3.00 (t, $J = 6.5$ Hz, 2H), 2.90 (t, $J = 6.5$ Hz, 2H), 2.72 (s, 3H). ^{13}C NMR (CD_3OD , 100 MHz): δ 199.0, 155.7, 147.8, 143.2, 136.1, 134.6, 130.9, 130.2, 130.1, 128.1, 125.6, 124.1, 123.0, 63.7, 37.8, 36.9, 36.6, 34.2, 29.9, 25.5. Anal. Calcd for $\text{C}_{20}\text{H}_{23}\text{F}_3\text{N}_2\text{O}_5\text{S}_2$: C, 48.77; H, 4.71; N, 5.69; S, 13.02. Found: C, 48.69; H, 4.82; N, 5.72; S, 13.10.

Compound 6. Prepared by the same procedure as described for the synthesis of **4** except that **5** (140 mg 0.50 mmol) was used in place of **3**. Purification by silica-gel column chromatography using hexane/ethyl acetate (4:1) as the eluent afforded **5**. Yield, 90 mg (41 %); IR (KBr): 3365, 1710, 1680 cm^{-1} ; ^1H (CDCl_3 , 300 MHz): δ 8.43 (d, $J = 1.6$ Hz, 1H), 8.03 (dd, $J = 1.6$ Hz, 8.7 Hz 1H), 7.93 (d, $J = 8.7$ Hz, 1H), 7.83 (d, $J = 8.7$ Hz, 1H), 7.68 (d, $J = 1.6$ Hz, 1H), 7.53 (dd, $J = 1.6$ Hz, 8.7 Hz, 1H), 4.13 (t, $J = 6.6$ Hz, 2H) 3.41 (s, 3H), 3.06 (t, $J = 6.6$ Hz 2H), 2.71 (s, 3H), 1.61 (m 4H), 1.42 (s, 9H), 1.30 (m, 4H). ^{13}C (CDCl_3 , 100MHz): δ 198.2, 155.8, 143.5, 136.0, 134.5, 130.5, 130.2, 130.1, 128.3, 125.8, 125.1, 124.7, 122.5, 66.2, 40.7, 37.8, 30.2, 29.0, 28.6, 28.3, 27.0, 26.6, 25.8. Anal. Calcd for $\text{C}_{25}\text{H}_{34}\text{N}_2\text{O}_5$: C, 67.85; H, 7.74; N, 6.33. Found: C, 67.52; H, 7.81; N, 6.20

ACC. Prepared by the same procedure as described for the synthesis of ASS except that **6** (50 mg 0.11 mmol) was used in place of **4**. **ACC** was purified by prep-HPLC using the same protocol as described above. Yield, 32 mg (62 %); IR (KBr): 3423, 1685, 1677 cm^{-1} ; ^1H NMR (CD_3OD , 300 MHz): δ 8.60 (d, $J = 1.9$ Hz, 1H), 8.06 (d, $J = 8.8$ Hz, 1H),, 8.05 (dd, $J = 1.9$ Hz, 8.8 Hz, 1H), 7.93 (d, $J = 8.8$ Hz, 1H), 7.82 (d, $J = 1.9$ Hz, 1H), 7.58 (dd, $J = 1.9$ Hz, 8.8 Hz, 1H), 4.15 (t, $J = 6.9$ Hz, 2H), 3.41 (s, 3H), 2.86 (t, $J = 6.9$ Hz, 2H), 2.72 (s, 3H) 1.63 (m, 4H), 1.37 (m, 4H). ^{13}C (CD_3OD , 100 MHz): δ 199.0, 156.2, 143.4, 136.2, 134.5, 130.8, 130.1, 128.8, 128.0, 127.5, 125.7, 124.8, 124.1, 65.9, 39.4, 36.8, 28.5, 27.3, 25.8, 25.5, 25.3, 25.1. Anal. Calcd for $\text{C}_{22}\text{H}_{27}\text{F}_3\text{N}_2\text{O}_5$: C, 57.89; H, 5.96; N, 6.14. Found: C, 57.86; H, 5.97; N, 6.19.

Spectroscopic measurements. Absorption spectra were recorded on a Hewlett-Packard 8453 diode array spectrophotometer, and fluorescence spectra were obtained with Amico-Bowman series 2 luminescence spectrometer with a 1-cm standard quartz cell. The fluorescence quantum yield was determined by using Coumarin 307 and Rhodamine B as the reference by the literature method.^[2] The spectral data obtained under various conditions are summarized in Figure S1 and Table S1.

Figure S1. (a, b) Normalized absorption and (c, d) emission spectra of ASS (a, c) and **1** (b, d) in 1,4-dioxane, DMF, EtOH, and H₂O.

Table S1. Photophysical properties of ASS and **1** in various solvents

Solvent (E_T^N) ^[a]	$\lambda_{\max}^{(1)}$ [b]		$\lambda_{\max}^{\text{fl}}$ [b]		Φ ^[c]	
	ASS	1	ASS	1	ASS	1
Dioxane (0.164)	293	370	448	427	2.0×10^{-3}	0.21
DMF (0.386)	305	389	444	450	3.0×10^{-3}	0.49
EtOH (0.654)	307	396	441	477	6.0×10^{-3}	0.44
H ₂ O (1.00)	321	382	457	503	2.2×10^{-1}	0.17

[a] The numbers in the parenthesis are normalized empirical parameter of solvent polarity.^[3] [b] $\lambda_{\max}$ of the one-photon absorption and emission spectra in nm. [c] Fluorescence quantum yield. The uncertainty is $\pm 15\%$.

Figure S2. (a) The change of two-photon fluorescence intensity with time for the reaction of ASS (5 μM) with 2-aminoethanethiol (10 mM) in MOPS buffer. (b) Plots of $\ln(F_\infty - F)$ vs time for the reaction of ASS (μM) with 2-aminoethanethiol (10 mM) in MOPS buffer for the one- and two-photon processes. (c) Plots of $\ln(F_\infty - F)$ vs time for the reaction of ASS (μM) with various 2-aminoethanethiol concentration (10–100 mM) in MOPS buffer.

Product analysis. The reaction of ASS (5 μM) with 2-aminoethanethiol (10 mM) was carried out for 2 hr at RT in MOPS buffer (3 mL, pH = 7.4). The HPLC traces of ASS, **1**, and the reaction are shown below (Figure S3). The result shows that **1** is the only product.

Figure S3. HPLC traces of (a) ASS, (b) **1**, (c) the reaction product between ASS and 2-aminoethanthiol. Assigned peaks were confirmed with ESI⁺. HPLC Conditions: 1.0 mL/min flow rate, 5% A to 90% A over 20 min, detected at 307 nm. Solvent A is acetonitrile with 0.1% TFA and solvent B is water with 0.1% TFA.

Figure S4. Fluorescence responses of ASS toward GSH and other analytes (30 eq, 1, Ca^{2+} ; 2, K^{+} ; 3, Na^{+} ; 4, Fe^{3+} ; 5, Zn^{2+} ; 6, Mg^{2+} ; 7, Glu; 8, Ser; 9, Val; 10, Met; 11, Ala; 12, Ile; 13, H_2O_2 ; 14, GSH only) in MOPS buffer. White and black bars represent the fluorescence intensity of ASS ($5\mu\text{M}$) in the presence of various analytes before and 3 hr after addition of GSH, respectively.

Figure S5. Effect of the pH on the one-photon fluorescence intensity of ASS (■) and the reaction

product between ASS and 2-aminoethanethiol (○) in MOPS buffer. The reaction of ASS (5 μM) with 2-aminoethanethiol (10 mM) was carried out for 2 hr at RT in MOPS buffer (3 mL). The excitation wavelength was 365 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.^[4] ASS and **1** were dissolved in 30 mM PBC buffer (137 mM NaCl, 3 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4) at concentrations of 5.0×10^{-6} M and then the two-photon induced fluorescence intensity was measured at 740–940 nm by using fluorescein (8.0×10^{-5} M, pH = 11) as the reference, whose two-photon property has been well characterized in the literature.^[5] To measure the two-photon cross section (δ) of the reaction product between ASS (5.0×10^{-6} M) and 2-aminoethanethiol (10 mM), the reaction mixture was kept at RT for 2 hrs before the measurement was conducted. 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.

Cell culture and imaging. HeLa human cervical carcinoma cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in DMEM (WelGene Inc, Seoul, Korea) supplemented with heat-inactivated 10% FBS (WelGene), penicillin (100 units/mL), and streptomycin (100 μg/mL). All the cell lines were maintained in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. Two days before imaging, the cells were detached and were replated on glass-bottomed dishes (MatTek). For labeling, the growth medium was removed and replaced with DMEN without FBS. The cells were incubated with ASS or ACC (3 μM), or pretreated with NEM (100 μM) for 30 min and incubated with ASS. The cells were pre-incubated with α-lipoic acid (500 μM) for a day, then washed and incubated with ASS. Following this incubation, the cells were washed three times with DMEM without FBS and imaged.

Two-photon fluorescence microscopy. Two-photon fluorescence microscopy images of probe-labeled cells and tissues were obtained with spectral confocal and multiphoton microscopes (Leica TCS SP2) with a ×100 (NA = 1.30 OIL) and ×20 (NA = 0.30 DRY) objective lens, respectively. The two-photon fluorescence microscopy images were obtained with a DM IRE2 Microscope (Leica) by exciting the probes with a mode-locked titanium-sapphire laser source (Coherent Chameleon, 90 MHz, 200 fs) set

at wavelength 780 nm and output power 1230 mW, which corresponded to approximately 10 mW average power in the focal plane. To obtain images at 360–460 nm and 520–620 nm range, internal PMTs were used to collect the signals in an 8 bit unsigned 512×512 pixels at 400 Hz scan speed.

The TPM images of HeLa cells labeled with 3- μ M ASS emitted very weak TPEF at 360-460 nm and strong TPEF at 500-620 nm, respectively (Figure S6 in the Supporting Information). For comparison, the TPM images of the cells labeled with acedan-derived TP probes for Mg^{2+} (AMg1)^[1] or Ca^{2+} (ACa1)^[6a] emitted TPEF in both regions, which had been attributed to the probes associated with membrane and cytosol, respectively, whereas those for Zn^{2+} (AZn1)^[6b] and pH (AH1 and AL1)^[6c] emitted TPEF only in the 500-620 nm range, probably because of the lower MW that may have facilitated the loading into the cytoplasm. Therefore, the observed result seems to indicate that ASS is predominantly located in the cytosol probably due to the low MW. Nevertheless, we have collected TPEF in the 500-620 nm range to absolutely rule out the possible interference from the membrane-bound probes.

Figure S6. Bright field (a) and TPM images (b-d) of HeLa cells labeled with 3 μ M ASS. The TPEF were collected at 360-460 nm (b) and 500-620 nm (c), respectively. (d) TPM image of HeLa cells labeled 3 μ M ACC. Two-photon excitation was provided at 780 nm with femto-second pulses. Cells shown are representative images from replicate experiments ($n = 5$). Scale bar, 30 μ m.

Preparation and staining of fresh rat Hippocampal slices. Slices were prepared from the hippocampi of 2-days-old rat (SD). Coronal slices were cut into 400 μ m-thick using a vibrating-blade microtome in artificial cerebrospinal fluid (ACSF; 124 mM NaCl, 3 mM KCl, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4 , 10 mM D-glucose, 2.4 mM $CaCl_2$, and 1.3 mM $MgSO_4$). Slices were incubated with 20 μ M ASS in ACSF bubbled with 95% O_2 and 5% CO_2 for 2 hr at 37 $^{\circ}C$. Slices were then washed three times with ACSF and transferred to glass-bottomed dishes (MatTek) and observed in a spectral confocal multiphoton microscope. To assess the effect of NEM, the slices were treated with 200 μ M of NEM for 30 min before ASS was added.

Figure S7. TPM images a fresh rat hippocampal slice stained with 20 μM ASS. Images were taken at depths of $\sim 90\text{--}180\text{ }\mu\text{m}$ with magnification at $10\times$. The TPEF were collected at 500–620 nm upon excitation at 780 nm with fs pulses. Scale bar, 300 μm .

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