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Supplementary Information

for

Unusual fluorescence of o-phenylazonaphthol derivatives with aggregation-induced emission and their use in two-photon cell imaging

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1. General Information

1.1 Materials and characterization

Aniline (1a), p-bromoaniline (1b), 2-naphthol (2a), 6-bromo-2-naphthol (2b), sodium nitrite, triethylamine (TEA), and tetrahydrofuran (THF) were purchased from Sigma-Aldrich (U.S.A.) and used without further purification. Enzymes including esterase (from porcine liver), glucose oxidase (GOx, from Aspergillus niger), trypsin (from porcine pancreas), and pepsin (from porcine gastric mucosa) and phosphate-buffered saline (PBS) solution (pH 7.2) were also purchased from Sigma-Aldrich. Acetic anhydride was purchased from Duksan Chemical (Korea) and dimethyl sulfoxide (DMSO), hydrochloric acid, sodium chloride, and sodium hydroxide were purchased from Samchun Chemicals (Korea). All chemicals and solvents were used without further purification. 1H-NMR spectra were obtained from a Bruker Fourier-300 spectrometer (Korea Basic Science Institute). UV-vis absorption spectra were recorded on a PerkinElmer Lambda 35 spectrometer. The photoluminescence spectra were taken using a Varian Cary Eclipse spectrometer and a Jasco spectrofluorometer (FP-8600). The absolute fluorescence quantum yields (QYs) were obtained using a Fluorolog-3 with time-correlated single photon counting (TCSPC). The X-ray diffraction (XRD) pattern was obtained using Bruker AXS (APEX 2 model).

2. Experimental Procedures

2.1 Synthesis

Synthesis of 1-(phenylazo)- 2-naphthol (AN-OH)

Compound 1a (1.583 g, 17.4 mmol) was added to 36% hydrochloric acid solution (4 mL) in water (6 mL) and the mixture was cooled using an ice bath containing sodium chloride with rapid stirring. A cold solution of sodium nitrite (1.20 g, 17.4 mmol) in water (10 mL) was added to the mixture rapidly, followed by stirring at 5 ^{o}C. During the diazotization with continuous stirring, 2a (2.451 g, 17.4 mmol) was dissolved in water (300 mL) containing sodium hydroxide (1.40 g) and the mixture was cooled to 5 ^{o}C. After 3-4 min, the diazonium salt solution was added to the solution containing 2a. Then, hydrochloric acid solution (10 mL) was poured into the mixture and warmed to room temperature. After stirring for 1 h, the solid was isolated by filtration. The precipitate was washed with water for neutralization of the acidic solution and then with ethanol and finally dried under vacuum. The product was obtained as an orange powder (yield: 3.62 g, 86%). 1H NMR (300 MHz, CDCl3): 16.25 (s, 1H), 8.57 (d, 1H), 7.76-7.28 (m, 9H), 6.87 ppm (d, 1H).

Synthesis of 1-(p-bromophenylazo)-6-bromo-2-naphthol (AN-Br-OH)

The synthesis and workup procedures were the same as used for AN-OH. For AN-Br-OH. 1b (2.9 g, 17.4 mmol) and 2b (3.9 g, 17.4 mmol) were used. The product was obtained as a red powder (yield: 6.56 g, 94%). 1H NMR (300 MHz, CDCl3): 15.93 (s, 1H), 8.42 (d, 1H), 7.77 (d, 1H), 7.66-7.61 ppm (m, 6H).

Synthesis of 1-(phenyldiazenyl)naphthalen-2-yl acetate (AN-acetate)

In a three-neck round-bottom flask, AN-OH (1.0 g, 4 mmol) was dissolved in anhydrous THF (35 mL).

TEA (16.5 mL) was added to the solution and stirred at 0 °C. Acetic anhydride (7.56 mL, 80 mmol) was added to the solution. The mixture was stirred at 0 °C for 2 h and then allowed to warm to room temperature and stirred overnight. The reaction mixture was poured into water and the mixture was extracted with chloroform. The organic layer was washed with brine, 0.5 N HCl, brine, and saturated sodium bicarbonate solution, and dried over magnesium sulfate. The organic solvent was removed by evaporation. The product was purified by column chromatography with silica gel (eluent: hexane/methylene chloride (2:1)). A pale-orange solid was obtained after recrystallization from ethanol (yield: 0.72 g, 62%). 1H NMR (300 MHz, CDCl3): 8.63 (d, 1H), 7.97-7.89 (m, 4H), 7.62-7.53 (m, 5H), 7.31 (d, 1H), 2.31 ppm (s, 3H).

Synthesis of 6-bromo-1-((4-bromophenyl)diazenyl)naphthalen-2-yl acetate (AN-Br-acetate)

AN-Br-OH (1.0 g, 2.46 mmol) was dissolved in anhydrous THF (90 mL) in a three-neck round-bottom flask. TEA (10 mL) was added to the solution and stirred at 0 °C. Acetic anhydride (6.9 mL, 67.9 mmol) was added to the stirred solution. Subsequent procedures were the same as used for the synthesis of AN-acetate. A red solid was obtained after recrystallization from ethanol (yield: 0.53 g, 48%). ^{1}H NMR (300 MHz, CDCl3): 8.51 (d, 1H), 8.08 (d, 1H), 7.87-7.81 (m, 3H), 7.74-7.68 (m, 3H), 7.34 (d, 1H), 2.32 ppm (s, 3H).

2.2 Esterase sensing

Stock solutions (1.0×10^{-2} M) of AN-acetate and AN-Br-acetate were prepared in DMSO, respectively. Stock solutions of esterase (100 U/mL) and other proteases (GOx, trypsin, and pepsin) were prepared in distilled deionized water, respectively. For esterase sensing, the stock solution of AN-acetate or AN-Br-acetate (30 μL) was added to a test tube, and then diluted to 3 mL with PBS buffer (10 mM, pH 7.2). The changes in fluorescence spectra of AN-acetate or AN-Br-acetate were recorded before and after the addition of esterase.

2.3 One-photon and two-photon fluorescence microscopy

One-photon and two-photon fluorescence images were obtained with spectral confocal and multiphoton microscopes (Leica TCS SP8 MP) with ×40 objective magnification. For two-photon excitation, probes were excited at 900 nm by a mode-locked femtosecond Ti:sapphire laser source (Mai Tai HP) with an output power of 2.04 W, which corresponded to about 9.88 × 105 W cm^{-2} average power in the focal plane.

2.4 Cell imaging

HepG2 cells and HeLa cells were cultured on 20 mm glass-bottomed dishes (NEST) using Dulbecco's Modified Eagle Medium (DMEM; WelGene), for HepG2 cells and Minimum Essential Media Eagle (MEM; WelGene), for HeLa cells containing 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL) for 48 h under 5% CO_2, 37 °C atmosphere. Before two-photon microscopic (TPM) imaging, the cultured medium was replaced with serum-free medium, then treated with probes including AN-acetate and AN-Br-acetate (10 μM) and incubated for 30 min. To investigate the photostability of the probes, the changes in the two-photon excited intensity of probe-labeled (10 μM) HeLa cells were measured at intervals of 2.0 s for 1 h.

2.5 Cell viability

An MTT kit (AbCareBio CL) assay was performed to assess the cytotoxicity of the probes. HeLa cells were cultured in 96-well-plate for 24 h, and then each different concentration of probes was added. After incubation for 2 h, the cultured medium was replaced with a serum-free medium containing 10% MTT, and further incubated for 2 h. MTT-containing medium was removed and DMSO was added to dissolve the formed precipitate. The changes in the absorbance were measured at 570 nm.

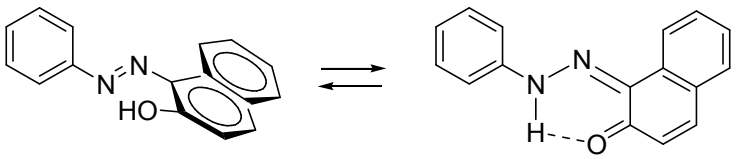
2.6 Measurement of two-photon cross section

The probes (1.0×10^{-5} M) were dissolved in PBS buffer (10 mM, pH = 7.4) and the two-photon excited fluorescence intensity was measured at 760−1040 nm. The intensity of the fluorescence spectra of the sample and reference emitted at the same excitation wavelength was determined, with rhodamine 6G as a reference. The two-photon action (TPA) cross section was calculated using the following equation:

$$\delta = \frac{\delta_r (S_s \Phi_r \varphi_r c_r)}{S_r \Phi_s \varphi_s c_s}$$

where the subscripts s and r stand for the sample and the reference molecules, respectively. The intensity of the signal collected by a CCD detector was denoted as S. Φ is the QY; and 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.

3. Supplementary Scheme S1 and Fig. S1-S9



Azo **Hydrazo**

Scheme S1. Tautomerism of freely-rotated, nonplanar azo tautomer and planar hydrazo tautomer. The restriction of intramolecular rotation in the hydrazo form occurred by intramolecular hydrogen bond.

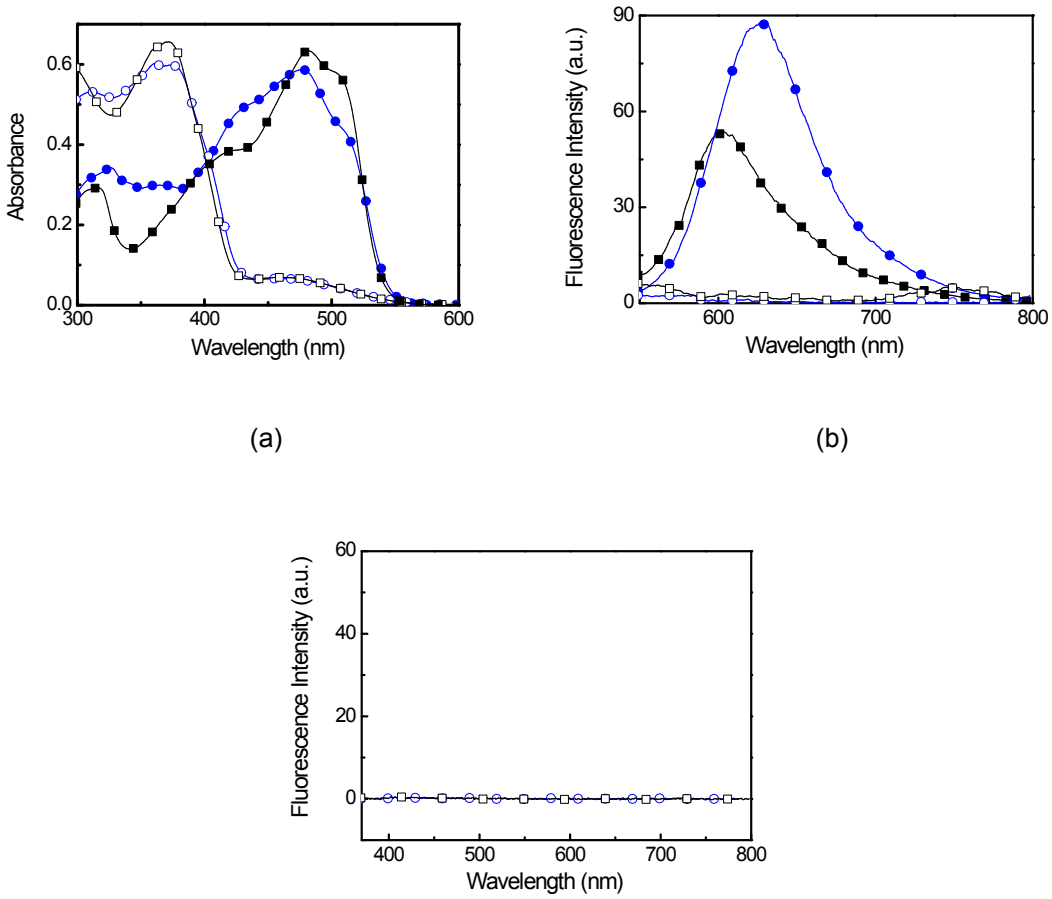
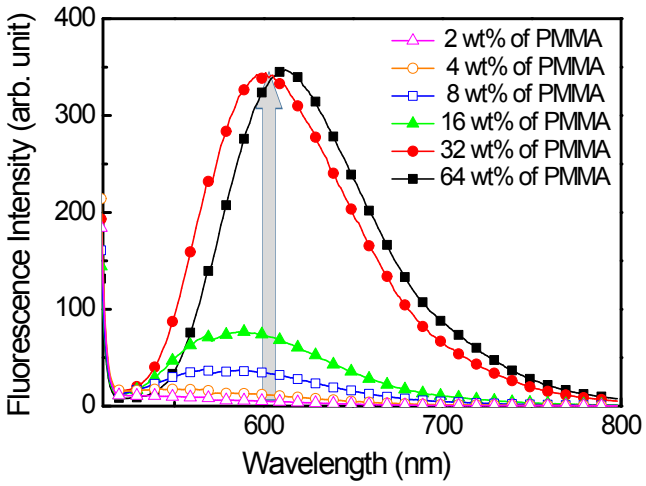
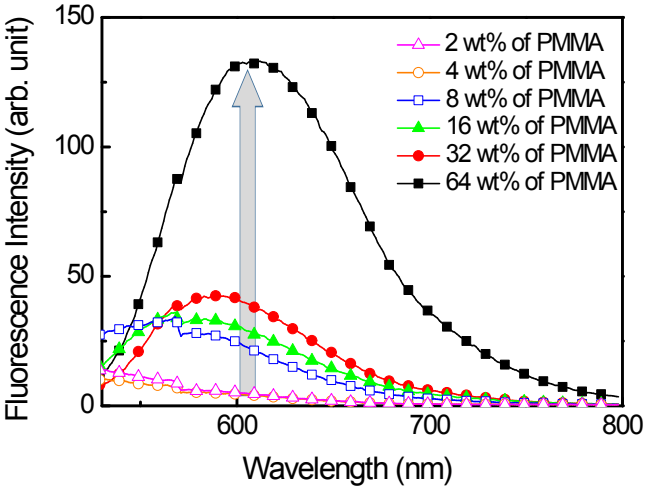


Fig. S1. (a) UV-vis (in THF) and (b) fluorescence spectra (solid) of AN-OH (■), AN-Br-OH (●), AN-acetate (□), and AN-Br-acetate (○). [AN-OH] = 2.09 × 10^{-4} M; [AN-Br-OH] = 1.5 × 10^{-5} M; [AN-acetate] = 1.07 × 10^{-4} M; [AN-Br-acetate] = 3.4 × 10^{-5} M. (c) Fluorescence spectra of AN-acetate (□) and AN-Br-acetate (○) in THF solution. Excitation wavelengths (nm): AN-OH (482), AN-Br-OH (487), AN-acetate (370), and AN-Br-acetate (360).



(a)



(b)

Fig. S2. Change in the fluorescence spectra of PMMA films containing (a) AN-Br-OH and (b) AN-OH with various concentrations.

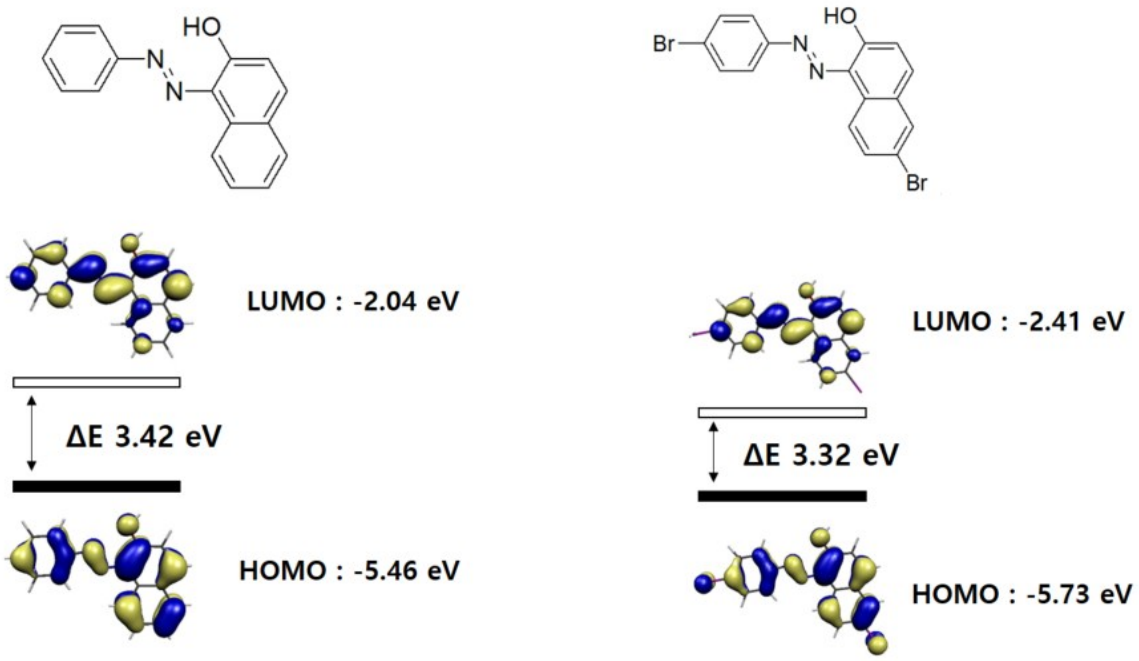


Fig. S3. HOMO and LUMO energy levels of AN-OH and AN-Br-OH calculated using B3LYP/6-31G(d).

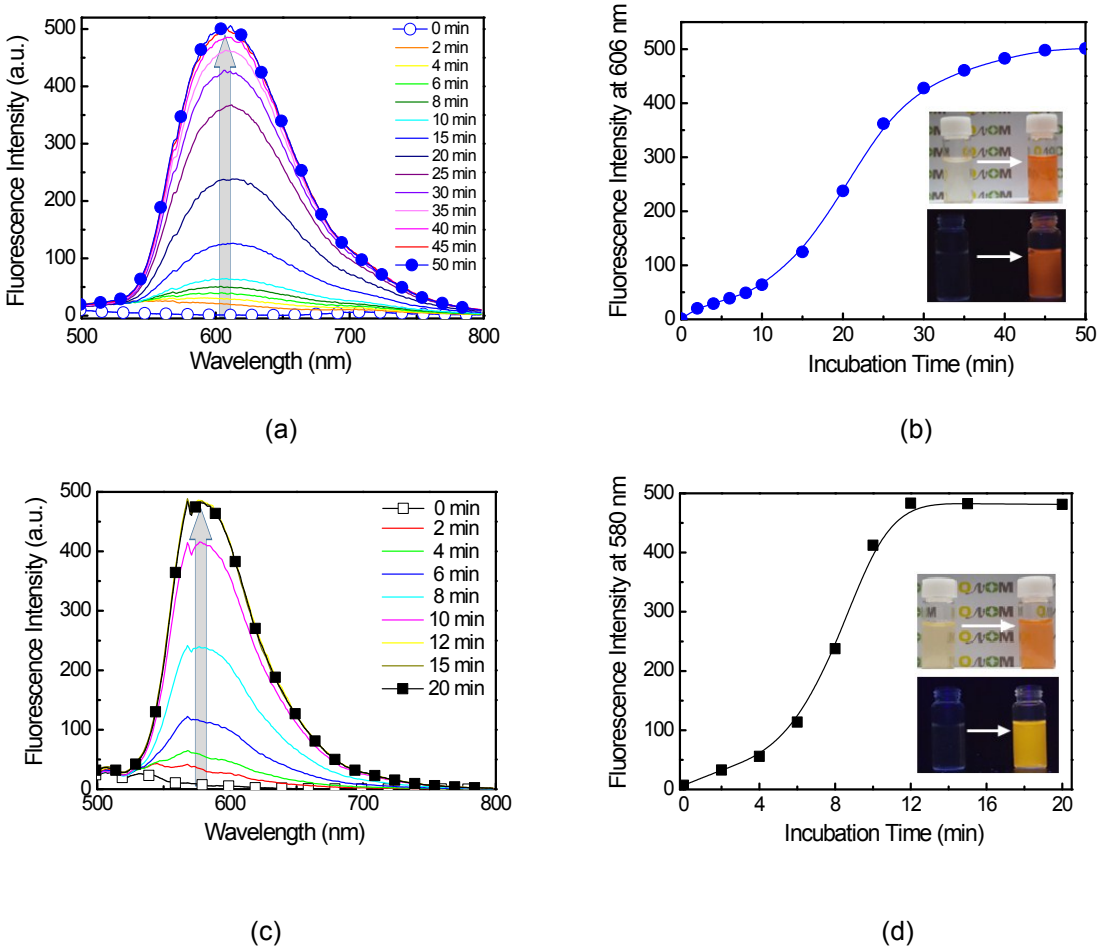


Fig. S4. Changes in fluorescence spectra of (a) AN-Br-acetate and (c) AN-acetate in DMSO (10 mM) according to incubation time of esterase (0.1 U/mL) in 10 mM PBS buffer (PBS buffer:DMSO = 33:1; pH 7.2; 37 °C). Relationship between the fluorescence intensity of (b) AN-Br-acetate at 606 nm and (d) AN-acetate at 580 nm and incubation time. Inset photographs represent AN-Br-acetate (b) and AN-acetate solutions (c) at incubation times of 0 min and 50 min (AN-Br-acetate) and 20 min (AN-acetate) taken under ambient light (upper) and UV light (365 nm, bottom).

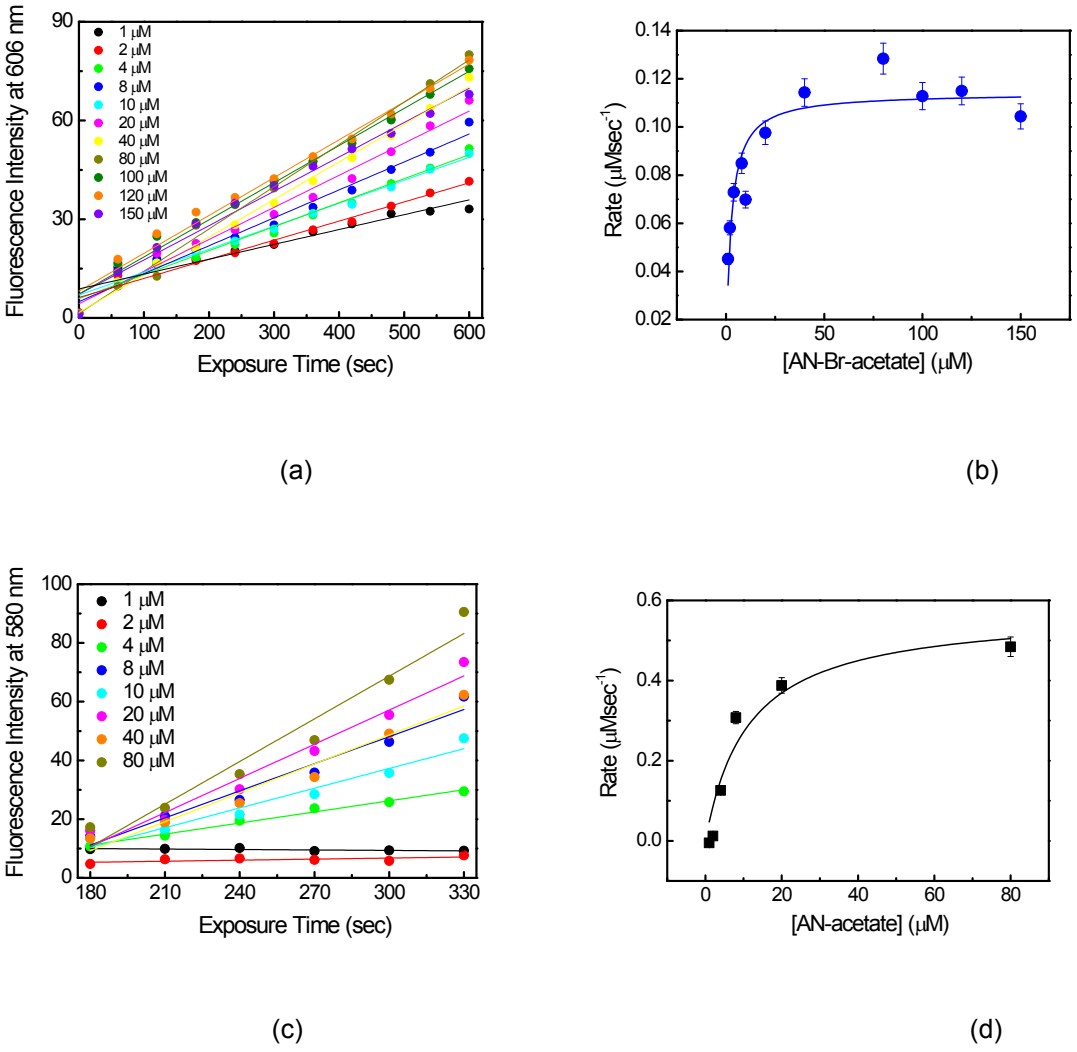


Fig. S5. Hydrolysis plots of (a) AN-Br-acetate and (c) AN-acetate with various concentrations upon exposure to esterase (0.1 U/mL) in PBS buffer solution (10 mM, pH 7.2, 37 ˚C). Relationship between the hydrolysis rate and the concentrations of (b) AN-Br-acetate and (d) AN-acetate.

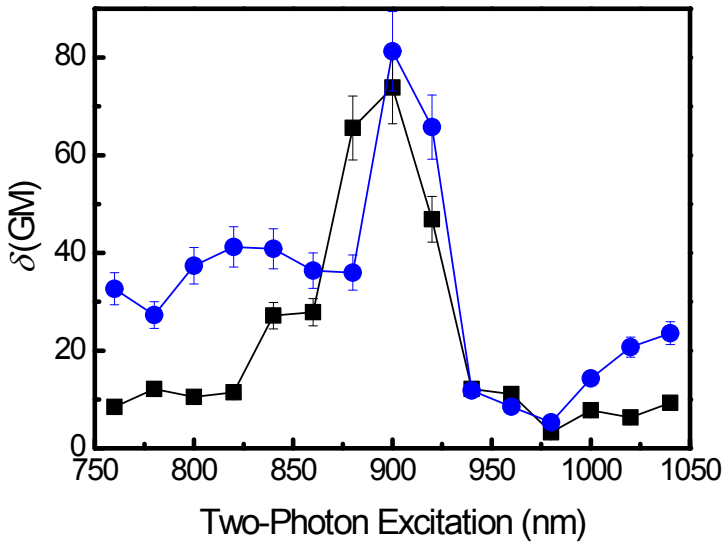


Fig. S6. Two-photon excitation spectra of AN-OH (●) and AN-Br-OH (■) in PBS buffer (10 mM, pH 7.4).

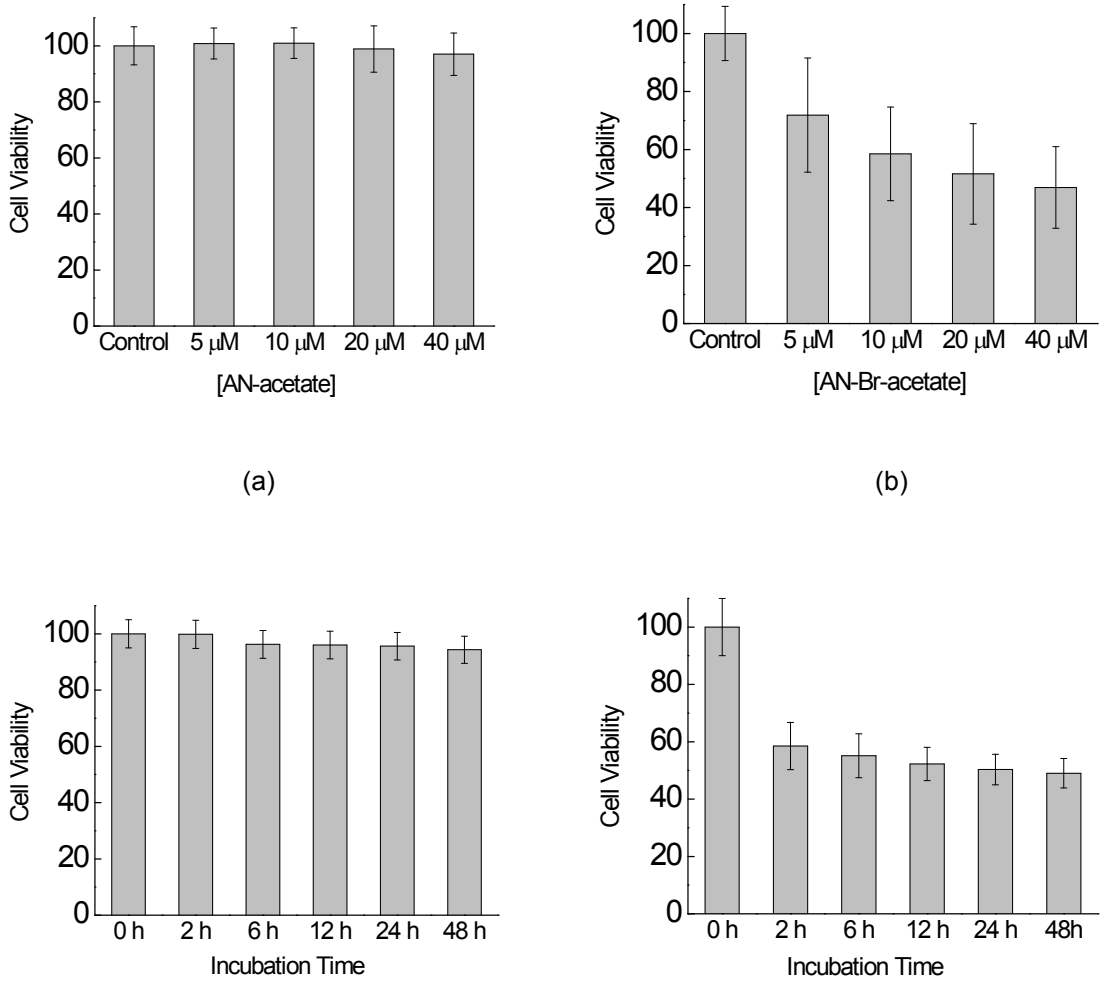


Fig. S7. Cytotoxicity test using MTT assays. HeLa cells were incubated at 37 °C for 2 h with various concentrations of (a) AN-acetate and (b) AN-Br-acetate and incubated at 37 °C for various times at the concentrations (10 μM) of (c) AN-acetate and (d) AN-Br-acetate.

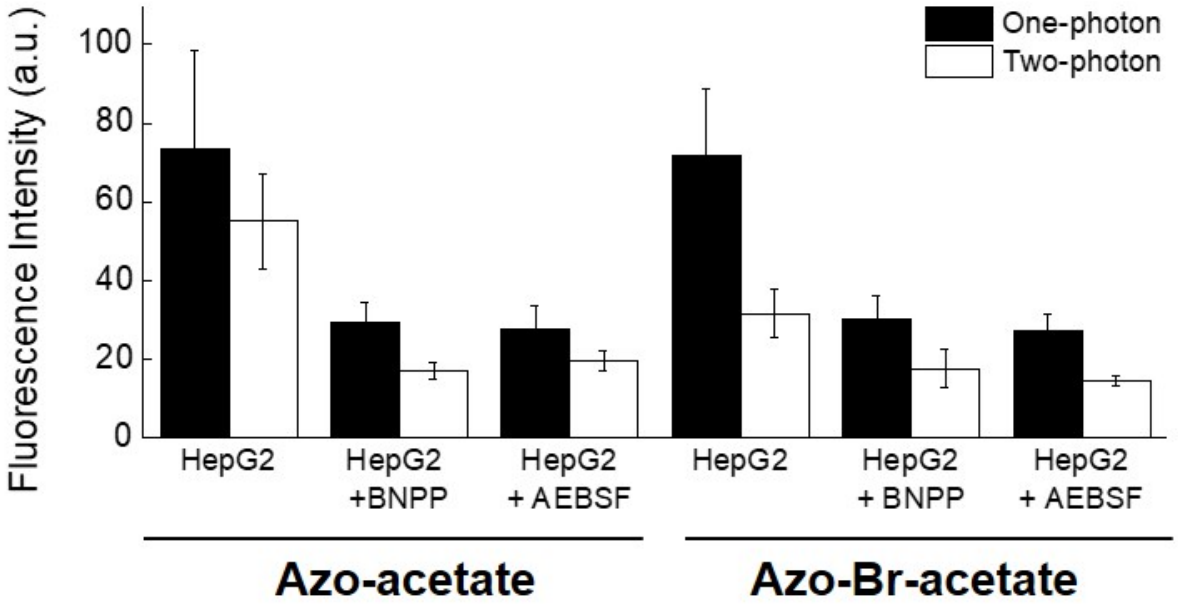


Fig. S8. Relative fluorescence intensity of the corresponding images (Excitation wavelengths 488 nm for OP and 900 nm for TP).

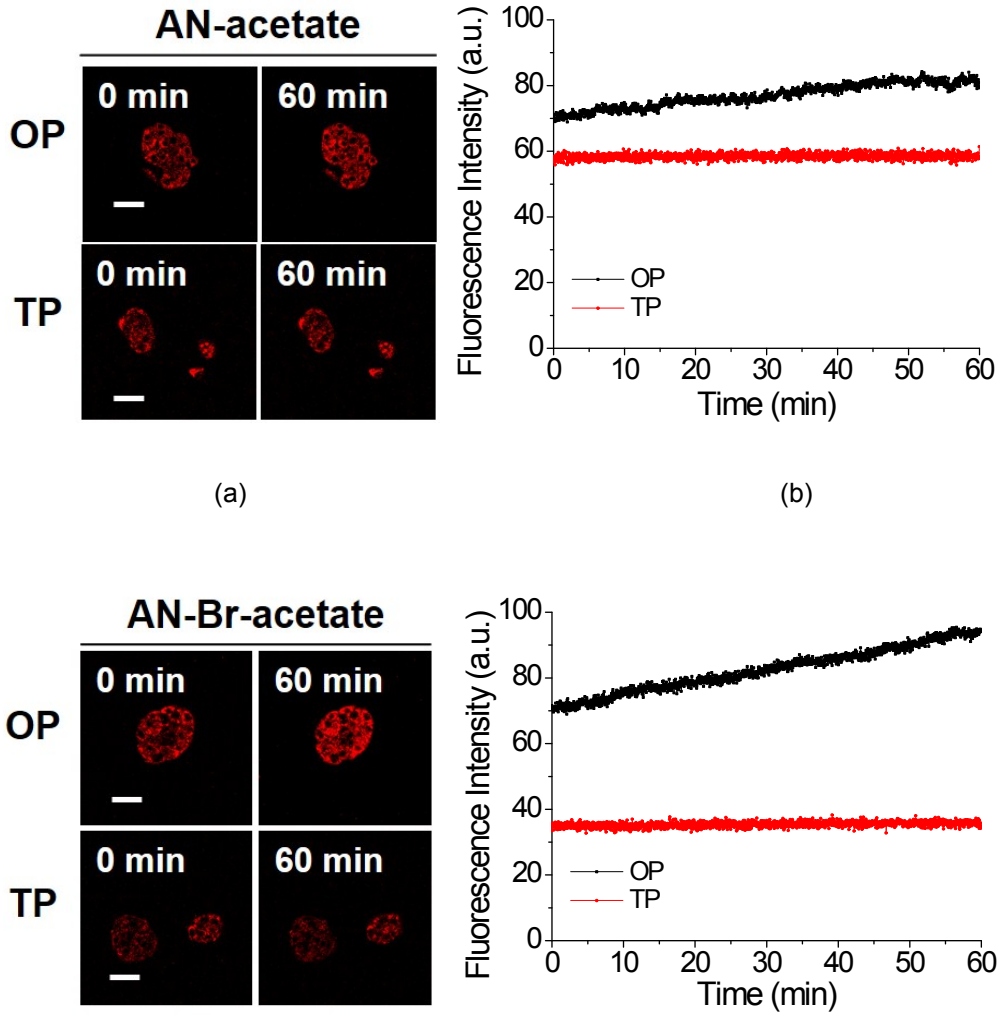


Fig. S9. OPM and TPM images of HepG2 cells stained with (a) AN-acetate (10 μM) and (c) AN-Br-acetate (10 μM) at 0 and 60 min irradiation of 488 nm (OP) and 900 nm (TP). The changes in the intensity of (b) AN-acetate and (d) AN-Br-acetate as a function of time.

Table S1. Crystallographic parameters of AN-Br-OH

Symmetry	Monoclinic
a (Å)	4.58
b (Å)	15.70
c (Å)	20.55
α (°)	103.44
β (°)	89.88
γ (°)	98.52