Electronic Supplementary Information

Practical fluorescence detection of acrolein in human plasma via a two-step tethering approach

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Materials and instruments. General reagents were purchased from Sigma-Aldrich, Wako Pure Chemical Industries, and Tokyo Chemical Industry, and used without further purification. TentaGel S NH-NH-Boc microbeads were purchased from RAPP Polymere GmbH (#30137, particle size: 130 μm, capacity 0.23 mmol/g). ^{1}H NMR and ^{13}C NMR spectra were recorded on a JEOL JNM-LA300. Mass spectra were recorded on a JEOL JMS-T100LC (ESI+, ESI-) mass spectrometer. Preparative HPLC purification was performed on a reversed-phase ODS column (GL Sciences, Inertsil Prep-ODS 30 mm x 250 mm) fitted on a JASCO PU-1587 HPLC system. UV-visible spectra were obtained on a Shimadzu UV-1600. Fluorescence spectroscopic studies were performed with a Hitachi F4500.

Fluorescence measurements. Measurements were performed in 1 cm quartz cells at 25 °C. The excitation and emission slit widths were 2.5 nm. The photomultiplier voltage was 400 V. Relative fluorescence quantum yield was obtained by comparing the area under the emission spectrum of the test sample excited at 554 nm with that of a solution of rhodamine B in ethanol, which has a quantum yield of 0.65.[S1]

Deprotection of TentaGel-NH-NH-Boc microbeads. TentaGel-NH-NH-Boc microbeads (200 mg) were added to a reaction vessel and swollen in DMF for 1 hr, then deprotection was performed with 95% trifluoroacetic acid, 2.5% triethylsilane, and 2.5% H_2O (2 mL total volume) for 2 hr at 25 °C by gently agitating the vessel. The microbeads were washed with dichloromethane (x 8). Next, 90% DMF and 10% N,N-diisopropylethylamine (2 mL total volume) were added to the vessel. The vessel was agitated for 5 min at 25 °C, and the microbeads were washed with dichloromethane (x 8) and dried to yield TentaGel-NH-NH_2 microbeads.

Two-step tethering assay using confocal fluorescence microscopy (in sodium phosphate buffer). First, acrolein (10 μM final) was added to 50 mM sodium phosphate buffer (pH 7.4) in micro test tubes. Then, 3.0 μL of 100 μM TAMRA-C2-SH in DMF was added to 57.8 μL of the above acrolein solution. A blank sample was similarly prepared without acrolein. The microtubes were incubated for 1 hr at 40 °C, then 19.3 μL of 200 mM sodium phosphate buffer (pH 1.44) and 0.4 mg of TentaGel-NH-NH_2 microbeads were added. Incubation was performed for 3 hr at 40 °C, then the microbeads were washed with DMF for 1 min and with 50 mM sodium phosphate buffer for 1 min. The microbeads were placed on a slide glass and the fluorescence intensity was measured with a confocal laser scanning microscope TCS SP5X AOBS (Leica Microsystems) equipped with a 10/0.4 objective lens. The excitation wavelength was 554 nm (white light laser), and the emission was filtered using a 570-700 nm barrier filter. For investigation of selectivity, 4-hydroxy-2-nonenal (HNE), 4-oxo-2-nonenal, crotonaldehyde, propionaldehyde, N-acetylcysteine, or glutathione (5 μM each) was added in place of acrolein, and the assay was performed as described above.

Detection in human plasma. Human plasma (PLA013016, obtained from BIOPREDIC International) was centrifuged in an Ultracel YM-10 centrifuge tube (Millipore) before use (10,000 rpm, 4 °C, 15 min) to remove proteins. Acrolein diluted with 50 mM sodium phosphate buffer (pH 7.4) was added to the filtrate to give final concentrations of 0, 1, 2, and 3 μM (sodium phosphate buffer/plasma = 1/9). Then, 3.0 μL of 100 μM TAMRA-C2-SH in DMF was added to 57.8 μL of plasma containing acrolein in micro test tubes. The microtubes were incubated for 1 hr at 40 °C, and 12.2 μL of 200 mM sodium phosphate buffer (pH 1.44) and 0.4 mg of TentaGel-NH-NH_2 microbeads were added. Incubation, washing, and measurement steps were performed as described above. A blank sample was prepared as follows: 3.0 μL of 100 μM TAMRA-C2-SH in DMF solution was added to 57.8 μL of plasma (containing no additional acrolein) in micro test tubes. The microtubes were incubated for 1 hr at 40 °C, then 12.2 μL of 200 mM sodium phosphate buffer (pH 1.44) and 0.4 mg of TentaGel-NH-Boc microbeads were added to the reaction mixture.

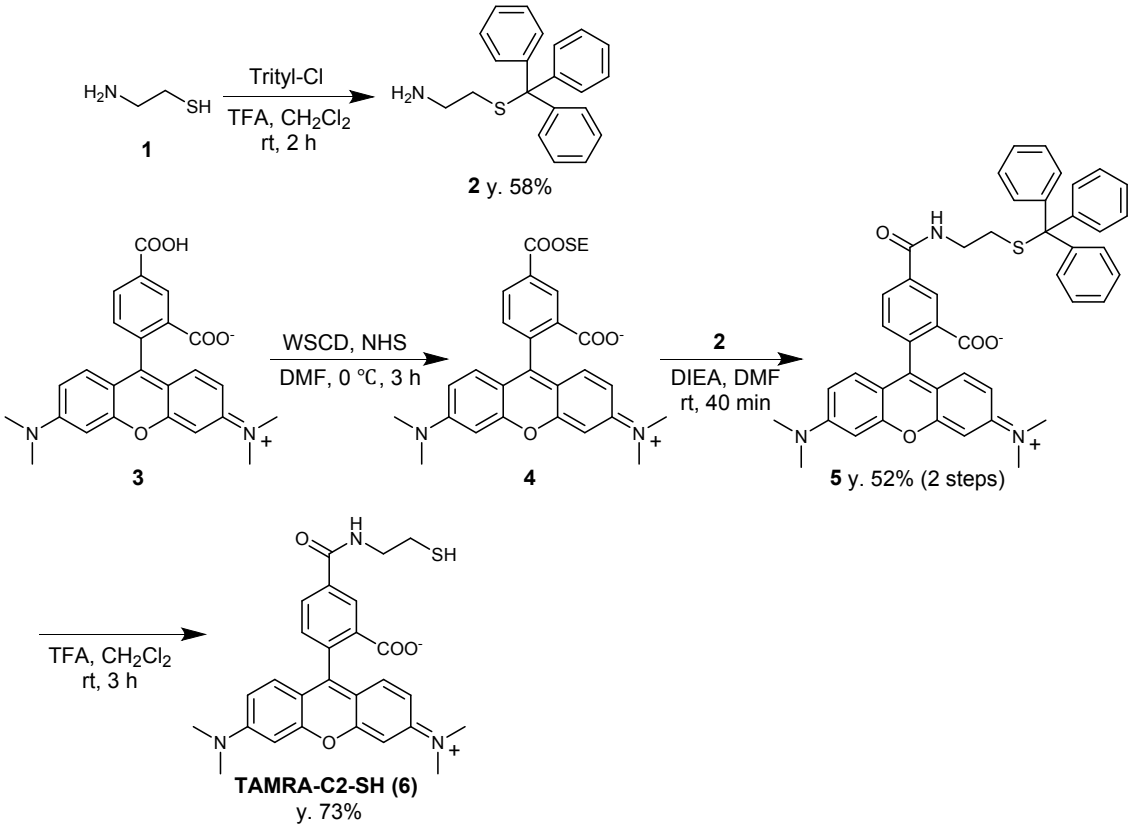
Detection in human plasma (conventional method). Plasma containing acrolein was prepared as described above. Assays were performed according to the method reported by Alarlcon.[S2] After the

reaction, centrifugation was performed (12,000 rpm, 4 °C, 10 min) and the fluorescence intensity of the supernatants was obtained with a Hitachi F4500 spectrofluorometer (Ex./Em. = 358/505 nm).

Detection in mouse plasma treated with CPA. The experimental procedures in the present study were performed in compliance with Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan), and were approved by the Animal Care and Use Committee at the Graduate School of Pharmaceutical Sciences, the University of Tokyo. A mouse was anesthetized and CPA (20 mg/kg, in 100 μL saline) was injected intravenously. Saline (100 μL) was injected into a control mouse. After 30 min, the mice were sacrificed and blood was collected from the heart (0.8-1.0 mL). Centrifugation was performed (4,000 rpm, 4 °C, 10 min) after addition of 1 mg EDTA-2Na to the blood. Subsequently, plasma was centrifuged in a YM-10 centrifuge tube (10,000 rpm, 4 °C, 15 min). Then, 3.0 μL of 100 μM TAMRA-C2-SH in DMF was added to 57.8 μL of plasma, and the mixture was incubated for 1 hr at 40 °C. Next, 15.2 μL of 200 mM sodium phosphate buffer (pH 1.44) and 0.4 mg of TentaGel-NH-NH_2 microbeads were added to the reaction mixture. Incubation, washing, and measurement steps were performed as described above. A blank sample was prepared using 0.4 mg of TentaGel-NH-NH-Boc microbeads.

Calibration curve. A calibration curve was obtained using human plasma with added acrolein. Plasma was centrifuged in an Ultracel YM-10 centrifuge tube before use (10,000 rpm, 4 °C, 15 min). Acrolein diluted with 50 mM sodium phosphate buffer (pH 7.4) was added to the filtrate to give final concentrations of 0, 1, 2, 3 and 4 μM (sodium phosphate buffer/plasma = 1/9). Reactions and measurement procedures were as described above (see “Detection in human plasma”). The Y intercept was not corrected to zero, because some acrolein and other unsaturated carbonyl compounds would have been present originally in the plasma.

Synthesis of TAMRA-C2-SH



Scheme S1 Synthesis of TAMRA-C2-SH (**6**)

Synthesis of 2. Cysteamine (**1**, 261 mg, 2.3 mmol) was added to a solution of dry CH_2Cl_2 (5 mL) containing trityl chloride (558 mg, 2.0 mmol), and trifluoroacetic acid (0.4 mL) was added dropwise to the mixture. After incubation for 2 hr at 25 °C under an Ar atmosphere, 1 N NaOH (3 mL) was added to the mixture. The product was extracted with ethyl acetate, and the organic solution was washed with brine, dried with anhydrous Na_2SO_4, and evaporated. The crude product was purified by column chromatography over silica gel (AcOEt/methanol = 9/1) to afford **2** as a white solid (374 mg, 1.2 mmol, 58%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.33 (t, 2H, J = 6.6 Hz), 2.39 (br, 2H), 2.53 (t, 2H, J = 6.6 Hz), 7.18 – 7.30 (m, 9H), 7.42 (d, 6H, J = 7.3 Hz). ^{13}C NMR (75 MHz, DMSO-d_6) δ 35.2, 40.6, 66.6, 126.7, 127.9, 129.5, 144.7. HRMS (ESI$^+$): *m/z* calcd for $[M + H]^+$, 320.1473; Found, 320.1478.

Synthesis of 5. *N*-Hydroxysuccinimide (42.8 mg, 0.37 mmol) and water-soluble carbodiimide (75.2 mg, 0.37 mmol) dissolved in DMF (2 mL) were added dropwise to a solution of 5-carboxytetramethylrhodamine (**3**, 120 mg, 0.28 mmol) in DMF (2 mL) at 0 °C. The mixture was stirred at 0 °C with a $CaCl_2$ tube for 3 hr, and acetone (5 mL) and 100 mM sodium phosphate buffer (pH 6.0, 15 mL) were added to it. The product was extracted with ethyl acetate, and the organic solution was washed

with brine, dried with anhydrous Na_2SO_4, and evaporated to give oily, crude **4**. Then, **2** (88.8 mg, 0.28 mmol) dissolved in DMF (1.5 mL) was added dropwise to the crude **4**, and the mixture was stirred for 40 min at 25 °C. The solvent was evaporated and the residue was purified by preparative reverse-phase HPLC with a linear gradient from 40% to 100% solvent B (solvent A: H_2O containing 0.1% trifluoroacetic acid; solvent B: acetonitrile/H_2O = 80/20 containing 0.1% trifluoroacetic acid) in 20 min. **5** (103 mg, 0.14 mmol, 52%) was obtained as a red solid. ^{1}H NMR (300 MHz, CD_3OD) δ 2.52 (t, 2H, J = 7.3 Hz), 3.27 (s, 12H), 3.37 – 3.41 (m, 2H), 6.93 – 7.42 (m, 21H), 7.48 (d, 1H, J = 8.1 Hz), 8.21 (dd, 1H, J = 2.2, 8.1 Hz), 8.75 (d, 1H, J = 2.2 Hz). ^{13}C NMR (75 MHz, CD_3OD) δ 32.8, 40.1, 40.9, 67.8, 97.4, 114.7, 115.5, 127.9, 129.0, 130.7, 131.4, 131.9, 132.3, 132.8, 137.5, 138.1, 146.2, 158.9, 159.0, 160.6, 167.3, 167.9. HRMS (ESI$^+$): *m/z* calcd for $[M + H]^+$, 732.2896; Found, 732.2858.

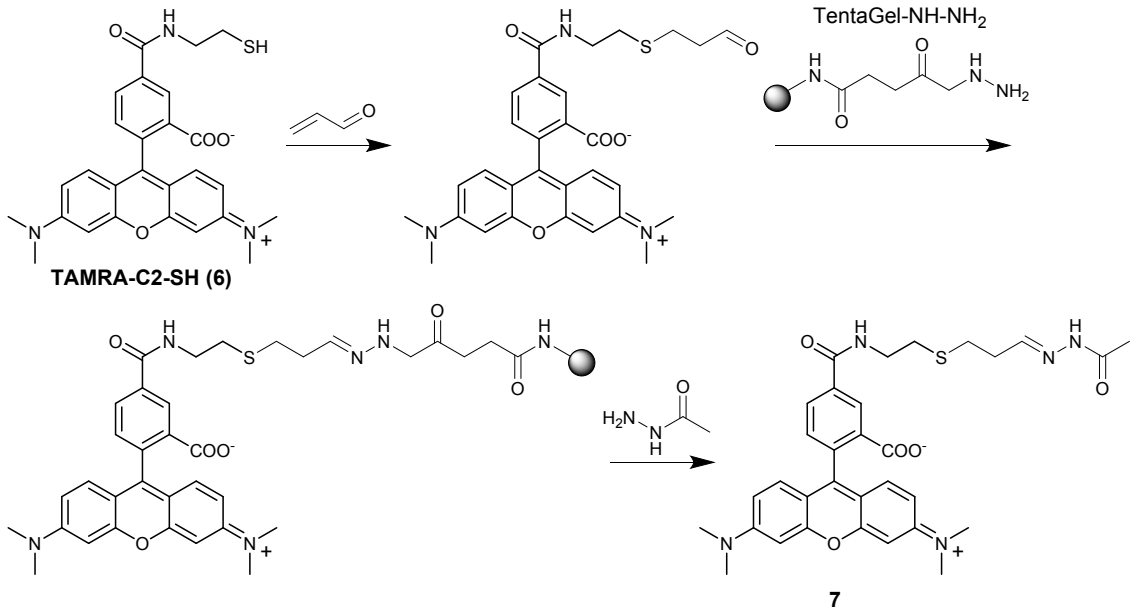
Synthesis of TAMRA-C2-SH (6). Trifluoroacetic acid (3 mL) and triethylsilane (20 μL) were added to **5** (7.9 mg, 0.011 mmol) dissolved in CH_2Cl_2 (0.2 mL) at 0 °C. The mixture was stirred for 3 hr at 0 °C and then evaporated to dryness. The crude product was purified by preparative reverse-phase HPLC with a linear gradient from 40% to 100% solvent B (solvent A: H_2O containing 0.1% trifluoroacetic acid; solvent B: acetonitrile/H_2O = 80/20 containing 0.1% trifluoroacetic acid) in 20 min. **6** (3.9 mg, 0.008 mmol, 73%) was obtained as a red solid. ^{1}H NMR (300 MHz, CD_3OD) δ 2.80 (t, 2H, J = 7.4 Hz), 3.30 (s, 12H), 3.61 – 3.66 (m, 2H), 6.98 – 7.16 (m, 6H), 7.54 (d, 1H, J = 8.0 Hz), 8.27 (dd, 1H, J = 1.7, 8.0 Hz), 8.79 (d, 1H, J = 1.7 Hz). ^{13}C NMR (75 MHz, CD_3OD) δ 24.4, 40.9, 44.6, 97.5, 114.7, 115.6, 131.3, 131.9, 132.0, 132.3, 132.9, 137.5, 138.2, 159.0, 159.0, 160.6, 167.3, 168.2. HRMS (ESI$^+$): *m/z* calcd for $[M + H]^+$, 490.1801; Found, 490.1806. The photophysical properties of this compound are summarized in Table S1.

Table S1 Photophysical properties of TAMRA-C2-SH.

Abs_{max} (nm)	Em_{max} (nm)	Φ_{fl}*
554	575	0.30

*Relative fluorescence quantum yield was obtained by comparing the area under the emission spectrum of the test sample excited at 554 nm with that of a solution of rhodamine B in ethanol, which has a quantum yield of 0.65.[S1]

Analytical HPLC. To confirm that the two reactions proceeded as expected, the product was cleaved from the microbeads (Scheme S2) and the cleaved material was analyzed by RP-HPLC (Fig. S1).



Scheme S2 Cleavage of product from microbeads after successive reactions of acrolein, TAMRA-C2-SH, and microbeads.

First, 4 μL of TAMRA-C2-SH (1 mM in DMF) was added to sodium phosphate buffer (pH 7.4, 36 μL) containing acrolein (100 μM TAMRA-C2-SH and 100 μM acrolein, final). The mixture was incubated for 1 hr at 40 °C, then 12 μL of 200 mM sodium phosphate buffer (pH 1.44) and 4 mg of TentaGel-NH-NH_2 microbeads were added to it. Incubation with microbeads was performed for 3 hr at 40 °C. The microbeads were then washed with DMF for 1 min and 50 mM sodium phosphate buffer (pH 7.4) for 1 min. Next, 2.5 M acetohydrazide (20 μL in DMF /HEPES-NaOH buffer (pH 5.8) = 1/1) was added to the microbeads. The mixture was incubated for 2 hr at 25 °C. The supernatant was taken and analyzed using an Inertsil 3 ODS column (4.6 mm x 250 mm, GL Sciences), fitted on a JASCO PU-980 HPLC system. Conditions: 20 min gradient from 40% to 100% solvent B (solvent A: 0.1 M triethylammonium acetate (pH 7.4); solvent B: 80% acetonitrile/20% 0.1 M triethylammonium acetate (pH 7.4)). HRMS (ESI^+): m/z calcd for $[M + H]^+$, 602.2437; Found, 602.2471.

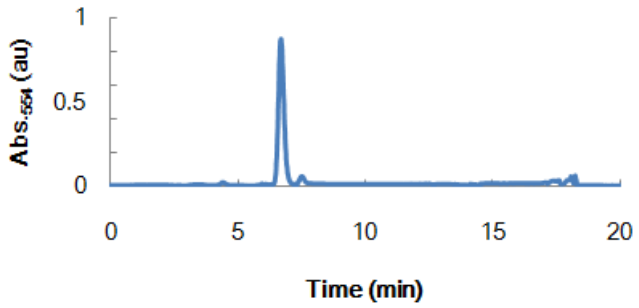


Fig. S1 HPLC chart of the reaction solution recorded with absorbance detection at 554 nm. *m/z* of the main peak matched the expected value for 7.

Supplementary References

S1. I. López Arbeloa and K. K. Rohatgi-Mukherjee, *Chem. Phys. Lett.,* 1986, **129,** 607.

S2. R. A. Alarcon, *Anal. Chem.,* 1968, **40,** 1704.