Supplementary Information

FRET-based imaging of transbilayer movement of pepducin in living cells by novel intracellular bioreductively activatable fluorescent probes

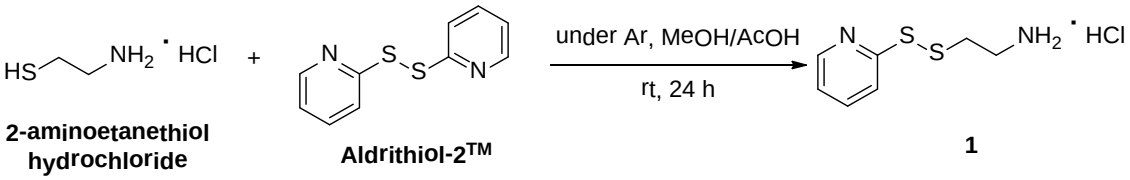
Mieko Tsuji, Satoshi Ueda, Tasuku Hirayama, Kensuke Okuda, Yoshiaki Sakaguchi, Aoi Isono, Hideko Nagasawa*

Laboratory of Pharmaceutical and Medicinal Chemistry, Gifu Pharmaceutical University, 1-25-4 Daigaku-nishi, Gifu, 501-1196, Japan

Contents:

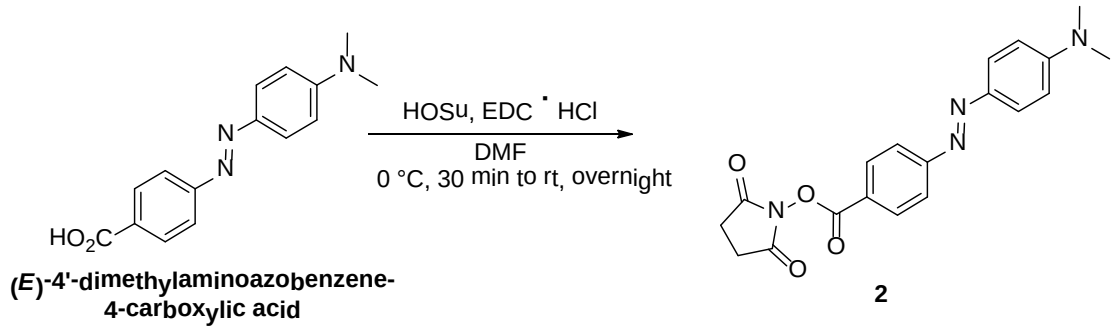
General: All chemicals used in this study were commercial products and were further purified by the standard methods, if necessary. Electron impact (EI) or fast atom bombardment (FAB) mass spectra were recorded on a JEOL JMS-SX102A mass spectrometer. Low-resolution mass spectra using electron spray ionization (ESI) were obtained on HP1100 series (Hewlett-Packard). High-resolution mass spectra (HRMS) were measured on JEOL JMS-SX102A or on LCMS-IT-TOF (Shimadzu). ^{1}H NMR spectra were obtained on a JEOL ECA-500 spectrometer at 500 MHz or JEOL JNM AL-400 spectrometer at 400 MHz. ^{13}C-NMR spectra were obtained on a JEOL ECA -500 spectrometer at 125 MHz or JEOL JNM AL-400 spectrometer at 100 MHz. Spectra were obtained in $CDCl_3$, d_6-DMSO or CD_3OD. Chemical shifts of ^{1}H-NMR are referenced to tetramethylsilane (0.00 ppm). Chemical shifts of ^{13}C-NMR are referenced to $CDCl_3$ (77.0 ppm), d_6-DMSO (39.5 ppm) and CD_3OD (49.0 ppm). Unless otherwise stated, products were purified on a silica gel column chromatography (Taiko-shoji AP-300S). RP-HPLC analyses were performed on 20-AD series (Shimadzu) or HP1100 series (Hewlett-Packard) equipped with Waters Symmetry C18 analytical column (Waters, 4.6 × 75 mm) or Inertsil C4 analytical column (GL sciences, 3 × 150 mm). For analysis, a solvent system consisting of 0.05% formic acid in ultra-pure water (v/v, solvent A) and 0.05% formic acid in MeCN (v/v solvent B) or a solvent system consisting of 0.1% TFA in ultra-pure water (v/v, solvent C) and 0.1% TFA in MeCN (v/v solvent D) was used and the eluting products were detected by light absorption at 220 nm and 496 nm. MPLC separation was performed on C18 Ultrapack column (YAMAZEN) with YFLC W-Prep 2XY (YAMAZEN) using a solvent system consisting of 0.1% TFA in ultra-pure water (v/v) and 0.1% TFA in MeCN (v/v) and the eluting products were detected by UV light absorption at 220 nm. Confocal fluorescence images were acquired with a Zeiss LSM 700 laser-scanning microscope system. Experiments were performed with a 20× or 40× oil-immersion objective lens. Fluorescence spectra were measured by FP-6600 (JASCO).

Synthesis



2-(2-Pyridinyldisulfanyl)-ethanamine hydrochloride (1)[1]

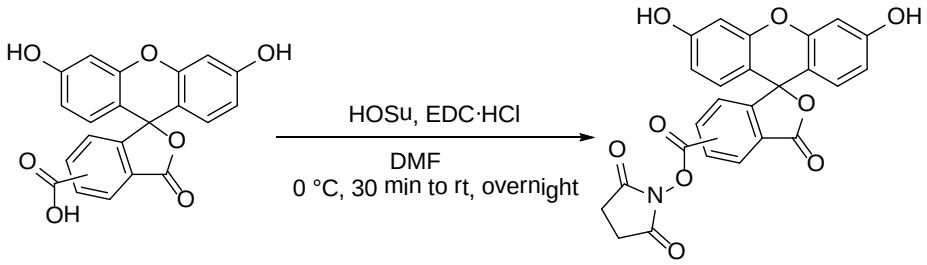
Aldrithiol-2™ (2.0 g, 10 mmol) was dissolved in MeOH (20 mL) and AcOH (0.80 mL). To the solution was added a solution of 2-aminoethanethiol hydrochloride (570 mg, 5.0 mmol) in MeOH (10 mL) within 30 min. The reaction was stirred under argon for 24 h at room temperature. After evaporation of the solvent, the residual oil was washed with Et_2O (2 times). The crude compound was dissolved in MeOH, and the product was precipitated out by addition of excess Et_2O (2 times) to afford **1** (780 mg, 70% crude yield) as a colorless solid. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 3.14 (t, J = 6.2 Hz, 2H), 3.30 (t, J = 6.2 Hz, 2H), 7.22–7.27 (m, 1H), 7.37–7.39 (m, 1H), 7.60–7.64 (m, 1H), 8.69–8.70 (m, 1H), 9.35 (br s, 3H); LRMS (FAB) m/z: $[M+H]^+$ 187.



(2,5-Dioxo-1-pyrrolidinyl) (*E*)-4'-dimethylaminoazobenzene-4-carboxylate (2)[2]

To a solution of (*E*)-4'-dimethylaminoazobenzene-4-carboxylic acid (200 mg, 0.70 mmol) and *N*-hydroxysuccinimide (HOSu, 110 mg, 0.97 mmol) in DMF (10 mL) was added EDC·HCl (200 mg, 1.0 mmol) at 0 °C. The reaction mixture was stirred at 0 °C for 30 min and then at room temperature overnight. After evaporation *in vacuo*, the residue was partitioned between

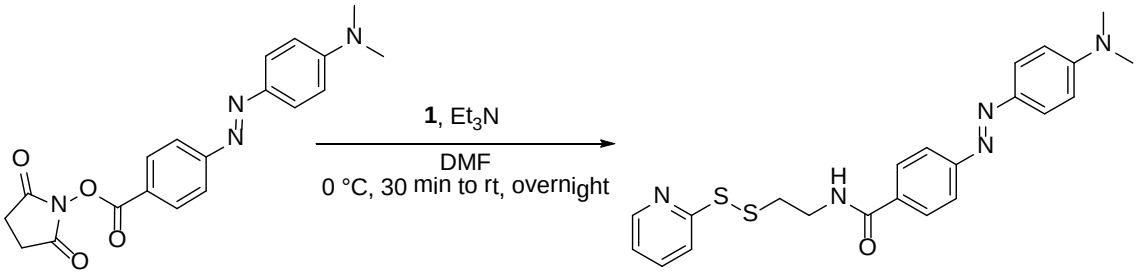
CH_2Cl_2 (120 mL) and H_2O (80 mL × 3). The organic layer was dried over anhydrous $MgSO_4$, and the solvent was removed *in vacuo*. The residue was purified by silica gel chromatography (hexane : ethyl acetate = 3:1, AcOH 0.5% → hexane : ethyl acetate = 1:1, AcOH 0.5% → CH_2Cl_2) to give **2** (157 mg, 58% yield) as an orange powder. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 2.91 (s, 4H), 3.12 (s, 6H), 6.76 (d, J = 9.2 Hz, 2H), 7.92 (d, J = 8.7 Hz, 4H), 8.23 (d, J = 8.7 Hz, 2H); ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 25.7, 40.3, 111.5, 122.4, 124.6, 125.8, 131.7, 143.7, 153.2, 157.2, 161.7, 169.2; LRMS (EI) m/z: $[M]^+$ 366.



5(6)-carboxyfluorescein **3**

2,5-Dioxo-1-pyrrolidinyl 3',6'-dihydroxy-3-oxospiro [isobenzofuran-1(3*H*),9'-[9*H*]xanthene]-5(6)-carboxylate (3)[3]

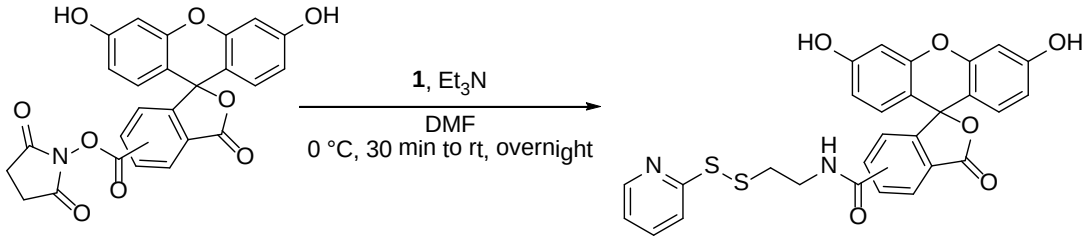
To a solution of 5(6)-carboxyfluorescein (1.0 g, 2.6 mmol) in DMF (10 mL) was added EDC·HCl (767 mg, 4.0 mmol) followed by HOSu (460 mg, 4.0 mmol). The reaction mixture was stirred at 0 °C for 30 min and then at room temperature overnight under dark condition. After evaporation *in vacuo*, the residue was dissolved in ethyl acetate (120 mL) and washed with 0.5% citric acid (80 mL × 3) and brine (80 mL × 3). The organic extracts were dried over anhydrous $MgSO_4$, and the solvent was removed in *vacuo* to afford the crude compound **3** (0.78 g, 60% yield) as a yellow powder. This material was used for the next step without further purification. Analytical HPLC condition: linear gradient of solvent D into C, 10 to 90% D over 15 min. r.t. = 10.8 min; LRMS (ESI) m/z: $[M+H]^+$ 474.



2 **4**

(*E*)-*N*-[2-(2-pyridinyldithio)ethyl]-4'-dimethylaminoazobenzene-4-carboxamide (4)[2]

To a solution of **2** (760 mg, 2.0 mmol) in DMF (10 mL) was added Et_3N (2.6 mL, 18.6 mmol) and **1** (600 mg, 2.7 mmol). The mixture was stirred at 0 °C for 30 min and then at room temperature overnight. The reaction mixture was evaporated *in vacuo*. The residue was dissolved in CH_2Cl_2 (120 mL) then washed with brine (80 mL × 3), 0.5% citric acid (80 mL × 3) successively. The organic layer was dried over anhydrous $MgSO_4$, and the solvent was removed *in vacuo*. The residue was purified by silica-gel chromatography (CH_2Cl_2) to give **4** (800 mg, 90% yield) as an orange powder. Analytical HPLC condition: linear gradient of solvent D in solvent C, 30 to 95% over 15 min. r.t. = 10.3 min; LRMS (ESI) m/z: $[M+H]^+$ 438.

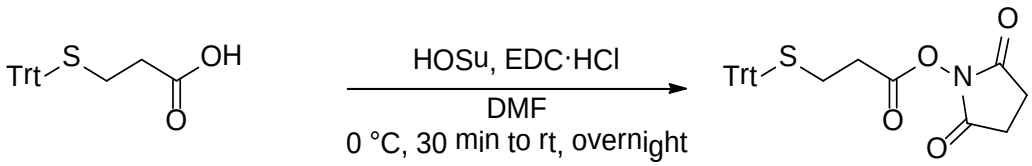


3 **5**

***N*-[2-(2-pyridinyldithio)ethyl]-3',6'-dihydroxy-3-oxospiro[isobenzofuran-1(3*H*),9'-[9*H*]xanthene]-5(6)-carboxamide (5)**[2]

To a solution of **3** (470 mg, 1.0 mmol) in DMF (10 mL) was added Et_3N (0.70 mL, 5.0mmol) and **1** (330 mg, 1.5 mmol). The mixture was stirred at 0 °C for 30 min and then at the room temperature overnight. The reaction mixture was evaporated *in vacuo*. The residue was purified by MPLC followed by lyophilization to give **5** (250 mg, 47% yield) as a yellow powder. ^{1}H-NMR (CD_3OD, 500MHz): δ 3.00 (t, J = 6.3 Hz, 0.8H), 3.12 (t, J = 6.6 Hz, 1.2H), 3.64 (t, J

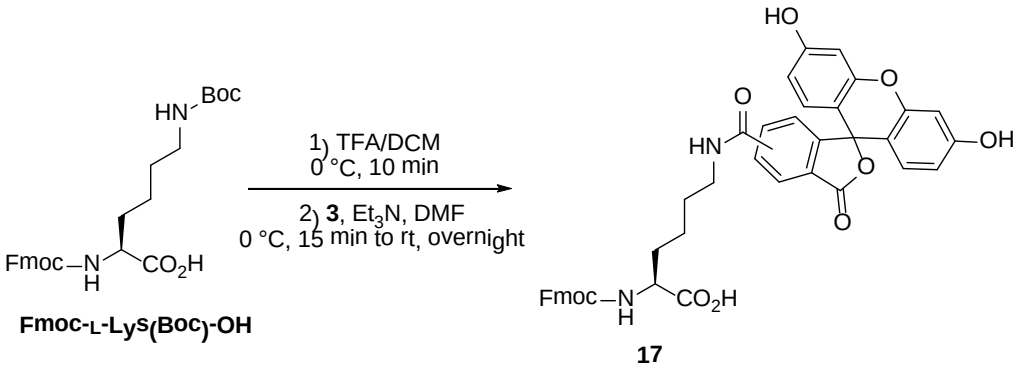
= 6.3 Hz, 0.8H), 3.76 (t, *J* = 6.6 Hz, 1.2H), 6.72–6.78 (m, 2.4H), 6.87–6.93 (m, 3.6H), 7.17–7.19 (m, 0.4H), 7.25–7.27 (m, 0.6H), 7.39 (d, *J* = 8.2 Hz, 0.6H), 7.68 (s, 0.4H), 7.70–7.78 (m, 0.8H), 7.82–7.90 (m, 1.2H), 8.15 (d, *J* = 8.2 Hz, 0.4H), 8.21–8.23 (m, 1H), 8.30 (d, *J* = 5.0 Hz, 0.4H), 8.42 (d, *J* = 5.0 Hz, 0.6H), 8.55 (s, 0.6H); ^{13}C-NMR (CD_3OD, 125 MHz): δ 38.6, 40.0, 103.5, 113.0, 113.2, 116.1, 116.2, 121.9, 122.7, 125.8, 127.1, 127.7, 130.0, 130.5, 131.5, 134.5, 137.8, 139.7, 139.9, 141.3, 149.9, 150.0; HRMS (ESI) m/z: calcd for $C_{28}H_{21}N_2O_6S_2^+$ $[M+H]^+$ 545.0836, found 545.0862.



3-(Tritylthio)propionic acid **16**

2,5-Dioxo-1-pyrrolidinyl 3-[(triphenylmethyl)thio]propanoate (16)[4]

3-(Tritylthio)propionic acid (200 mg, 0.57 mmol) was dissolved in DMF (2.0 mL), to which HOSu (99 mg, 0.86 mmol) and EDC·HCl (170 mg, 0.86 mmol) were added at 0 °C. The mixture was stirred at 0 °C for 30 min and then at room temperature overnight. The reaction mixture was diluted with ethyl acetate (80 mL), and the solution was washed with 10% citric acid (20 mL × 3), sat. $NaHCO_3$ (20 mL × 3) and water (20 mL × 3), and then the organic layer was dried over anhydrous $MgSO_4$. Removal of solvent *in vacuo* to give compound **16** (crude, 180 mg, 59%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.40 (t, *J* = 7.3 Hz, 2 H), 2.55 (t, *J* = 7.3 Hz, 2 H), 2.80 (s, 4 H), 7.28–7.31 (m, 9 H), 7.43–7.45 (m, 6H).

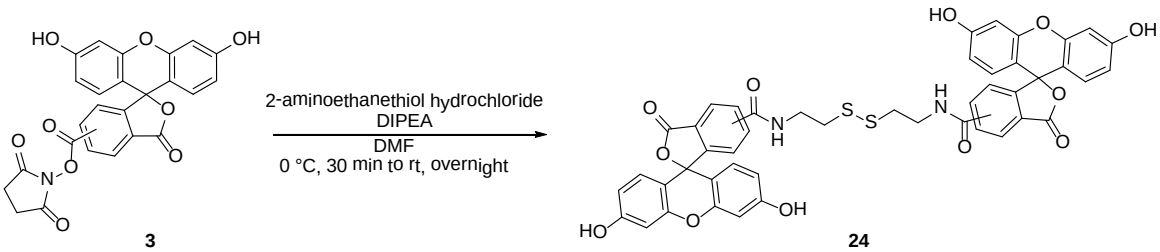


Fmoc-L-Lys(Boc)-OH **17**

***N*^{6}-[(3',6'-dihydroxy-3-oxospiro[isobenzofuran-1(3*H*),9'-[9*H*]xanthene)-5(6)-carbonyl]-*N*^{2}-[(9*H*-fluoren-9-ylmethoxy)carbonyl]-L-lysine (17)**

CAS: 1158088-23-3

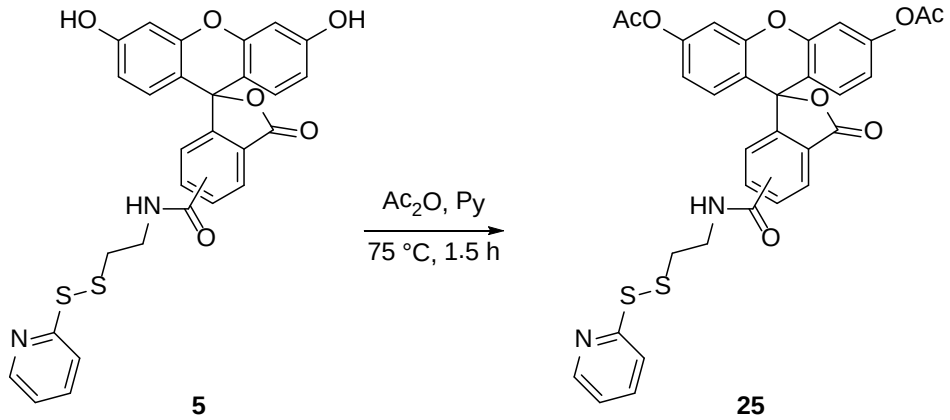
To a solution of Fmoc-L-Lys(Boc)-OH (470 mg, 1.0 mmol) in CH_2Cl_2/TFA (1:1) (12 mL) which was stirred at 0 °C for 10 min, and the solvent was removed *in vacuo*. To a solution of the residue in DMF (4 mL) was added Et_3N (0.14 mL, 1.0 mmol) and **3** (570 mg, 1.2 mmol), and the reaction mixture was stirred at 0 °C for 15 min and then at room temperature overnight under dark condition. After the additional Et_3N (0.11 mL, 0.75 mmol) was added, the reaction mixture was stirred at room temperature overnight. The solvent was removed *in vacuo*. The residue was dissolved in ethyl acetate (160 mL), and the mixture was washed with brine (40 mL × 3) and 0.5% citric acid (40 mL × 3). The organic layer was dried over anhydrous $MgSO_4$, and the solvent was removed *in vacuo* to afford **17** (1.0 g, 69% crude yield for two steps) as a yellow powder. Analytical HPLC condition: linear gradient of solvent D in solvent C, 10 to 90% over 30 min. r.t. = 20.0 min; LRMS (ESI) m/z: $[M+H]^+$ 727.



3 **24**

***N,N'*-(dithiodi-2,1-ethanediyl)bis[3',6'-dihydroxy-3-oxospiro[isobenzofuran-1(3*H*),9'-[9*H*]xanthene]-5(6)-carboxamide] (24)**[5]

To a solution of **3** (300 mg, 0.63 mmol) in DMF (3.0 mL) was added DIPEA (0.54 mL, 5.0 mmol) and 2-aminoethanethiol hydrochloride (116 mg, 0.76 mmol). The mixture was stirred at 0 °C for 30 min and then at the room temperature overnight. The reaction mixture was filtered, and the filtrate was evaporated *in vacuo*. The residue was purified by MPLC followed by lyophilization to give **24** (27.8 mg, 10% yield) as a yellow powder. ^{1}H-NMR (d_6-DMSO, 500 MHz): δ 2.81–3.00 (m, 4H), 3.45–3.64 (m, 4H), 6.53–6.60 (m, 8H), 6.69 (s, 4H), 7.38 (s, 1H), 7.68 (d, J = 14.3 Hz, 1H), 8.07 (s, 1H), 8.17–8.26 (m, 2H), 8.46 (d, J = 14.3 Hz, 1H), 8.86 (d, J = 23.1 Hz, 1H), 9.01 (d, J = 23.1 Hz, 1H), 10.17 (br s, 4H); HRMS (ESI) m/z: calcd for $C_{46}H_{31}N_2O_{12}S_2^-$ [M-H]$^-$ 867.1324, found 867.1329.



5 **25**

3-Oxo-5(6)-[2-(pyridin-2-yldisulfanyl)ethylcarbamoyl]-3*H*-spiro[isobenzofuran-1,9'-[9*H*]xanthene]-3',6'-diyl diacetate (25)

Compound **5** (100 mg, 0.18 mmol) was dissolved in 5 mL of acetic anhydride and 75 μL of pyridine. The solution was heated at 75 °C for 1.5 h and then poured into ice-water. The crude precipitate was dissolved in $CHCl_3$ (60 mL) and the solution was washed sequentially with 5% acetic acid (40 mL × 3) and brine (40 mL × 3), dried over anhydrous $MgSO_4$, and concentrated under vacuum. The residue was purified by chromatography on silica gel (hexane : ethyl acetate = 1:3) to afford a mixture of diacetate as a white solid (30 mg, 38 %). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 2.24 (s, 6H), 2.94 (m, 2H), 3.69 (m, 2H), 6.81–6.72 (m, 4H), 6.94–6.91 (m, 0.5H), 7.04 (dd, J = 7.5, 1.2 Hz, 2H), 7.10–7.13 (m, 0.5H), 7.32 (d, J = 8.2 Hz, 0.5H), 7.40 (d, J = 8.2 Hz, 0.5H), 7.44–7.49 (m, 0.5H), 7.53–7.59 (m, 0.5H), 7.60 (s, 0.5H), 7.91–7.95 (m, 0.5H), 8.05 (d, J = 8.2 Hz, 0.5H), 8.14 (d, J = 8.2 Hz, 0.5H), 8.19–8.23 (m, 0.5H), 8.46 (m, 1.5H), 8.63 (t, J = 6.0 Hz, 0.5H), 8.59 (t, J = 6.0 Hz, 0.5H); ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 168.49, 168.41, 167.90, 165.13, 151.90, 151.20, 151.16, 149.60, 149.03, 136.72, 128.60, 128.54, 128.02, 121.64, 121.58, 121.52, 117.59, 117.57, 115.47, 110.23, 81.90, 60.37, 38.47, 37.09; HRMS (ESI) m/z: calcd for $C_{32}H_{25}N_2O_8S_2^+$ $[M+H]^+$ 629.1052, found 629.1079.

HPLC analyses of 11-15, and 20

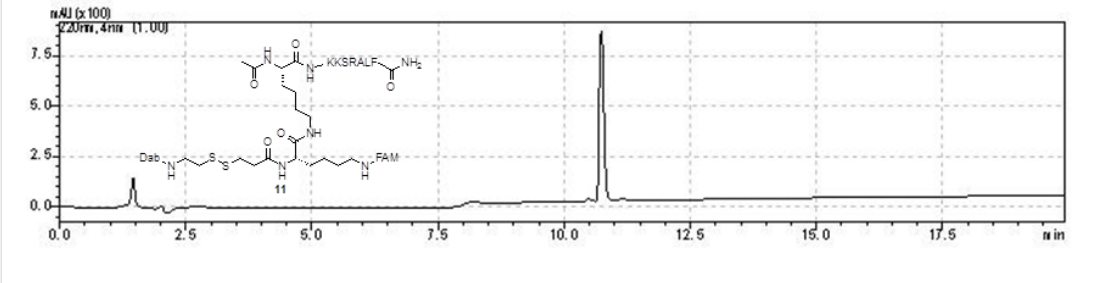


Figure S1. HPLC chart of **11**. Analytical HPLC condition: column; a Waters Symmetry C18 analytical column (Waters, 4.6 × 75 mm), flow rate 0.5 mL/min, linear gradient of solvent B in solvent A; 10% for 5min then 10 to 90% over 15 min.

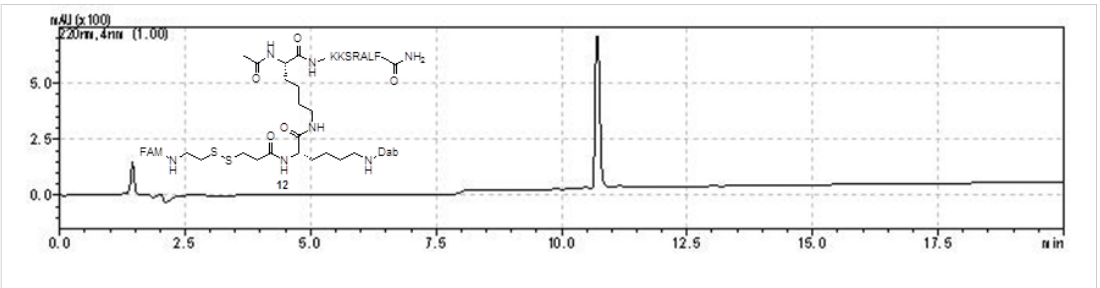


Figure S2. HPLC chart of **12**. Analytical HPLC condition: column; a Waters Symmetry C18 analytical column (Waters, 4.6 × 75 mm), flow rate 0.5 mL/min, linear gradient of solvent B in solvent A; 10% for 5min, 10 to 95% over 15 min.

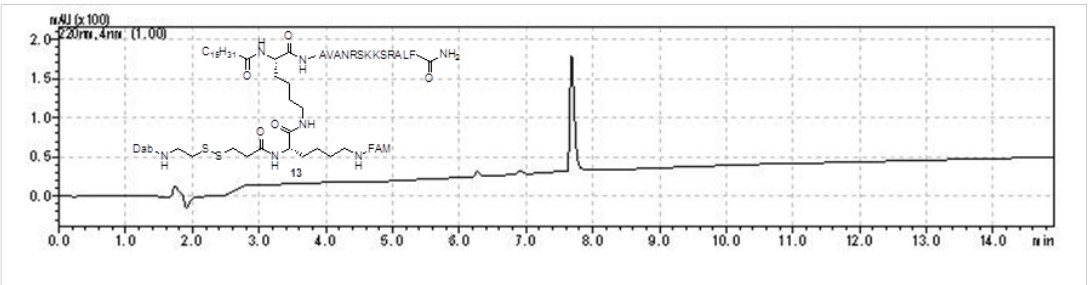


Figure S3. HPLC chart of **13**. Analytical HPLC condition: column; a Waters Symmetry C18 analytical column (Waters, 4.6 × 75 mm), flow rate 0.5 mL/min, linear gradient of solvent B in solvent A; 10 to 90% over 15 min.

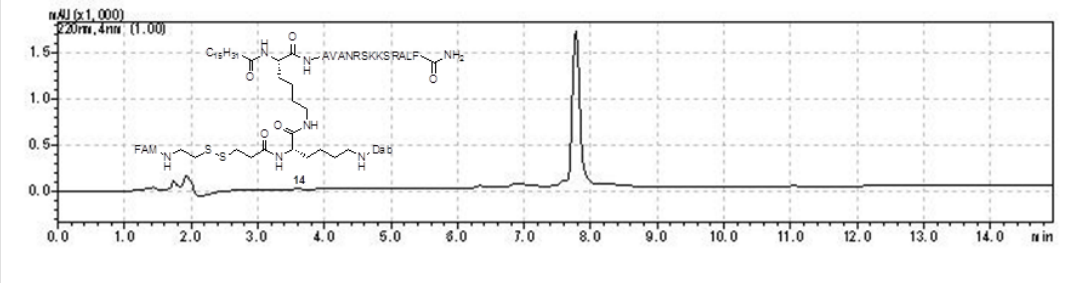


Figure S4. HPLC chart of **14**. Analytical HPLC condition: column; an Inertsil C4 analytical column (GL Science, 3 × 150 mm), flow rate 0.5 mL/min, linear gradient of solvent B in solvent A; 10 to 90% over 15 min.

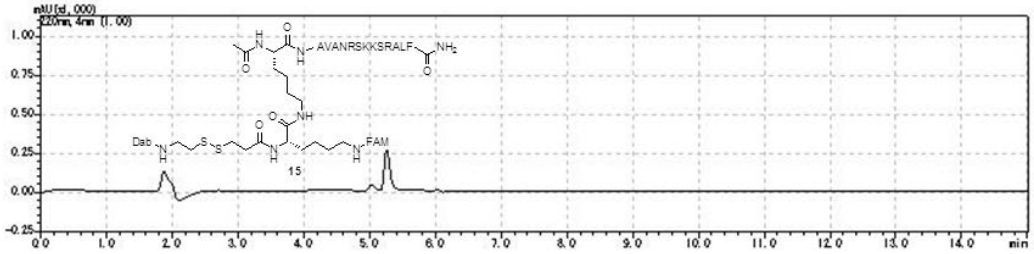


Figure S5. HPLC chart of **15**. Analytical HPLC condition: column; a Waters Symmetry C18 analytical column (Waters, 4.6 × 75 mm), flow rate 0.5 mL/min, linear gradient of solvent B in solvent A; 10 to 90% over 15 min.

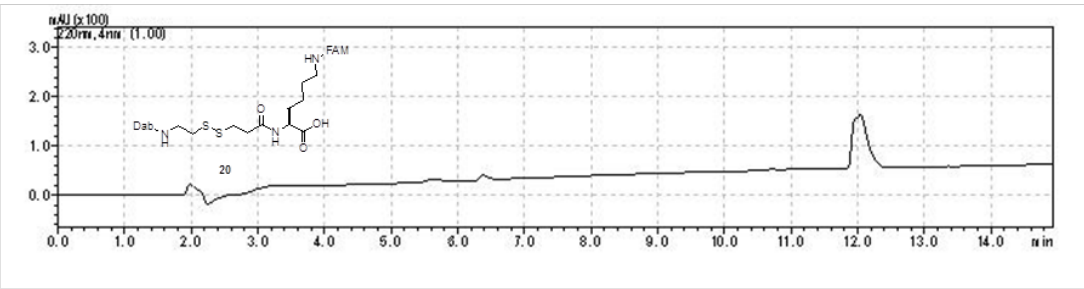
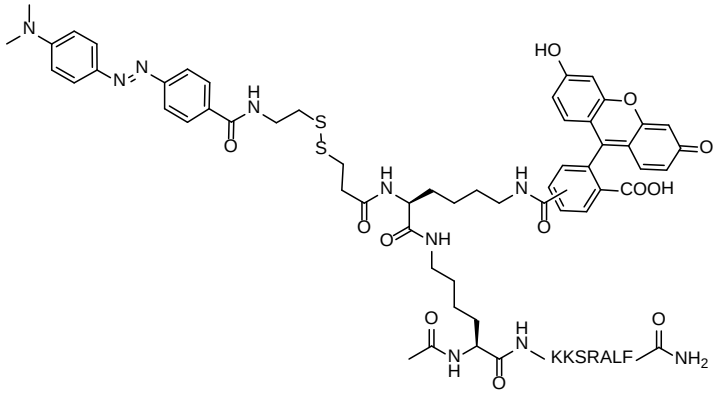


Figure S6. HPLC chart of **20**. Analytical HPLC condition: column; a Waters Symmetry C18 analytical column (Waters, 4.6 × 75 mm), flow rate 0.5 mL/min, linear gradient of solvent B in solvent A; 10 to 90% over 15 min.

GSH-concentration-dependency of fluorescence intensity derived from model peptide 11



11

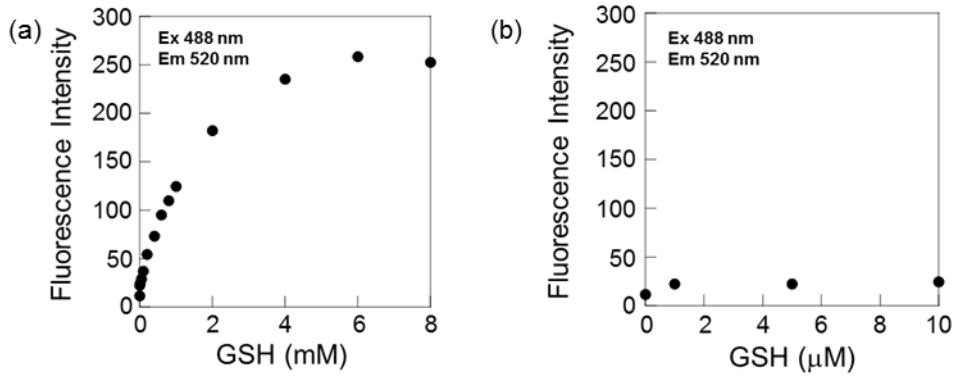
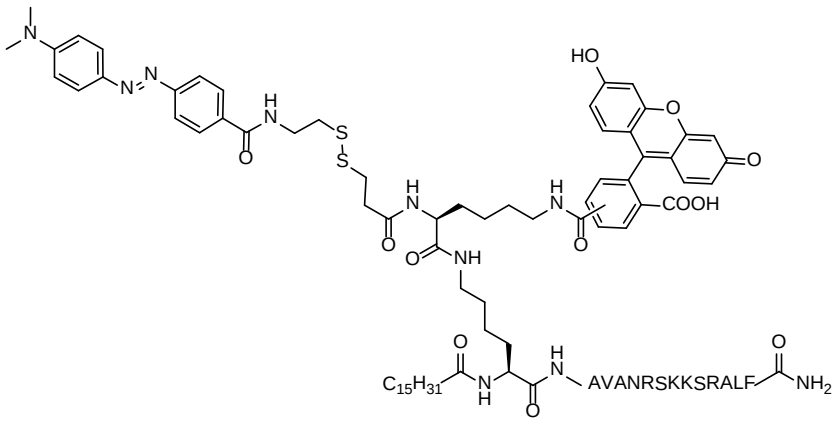


Figure S7. The fluorescence emissions (λ_{ex} = 488 nm, λ_{em} = 520 nm) were monitored, when the model peptide **11** (0.1 μM) was treated with GSH in the range of concentrations (a) from 0 to 8 mM or (b) from 1 to 10 μM in 100 mM HEPES buffer (pH 7.5) at 37 °C for 15 min.

Confocal microscopy images of MCF-7 cells treated with probe 13 with or without preincubation with bacitracin[6]



13

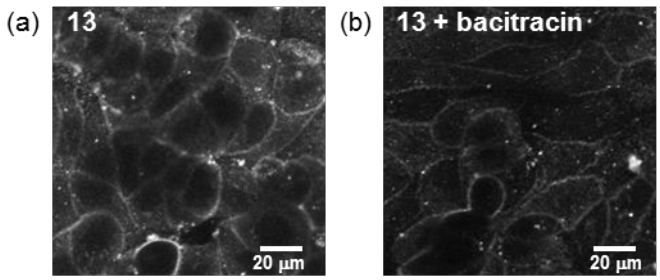
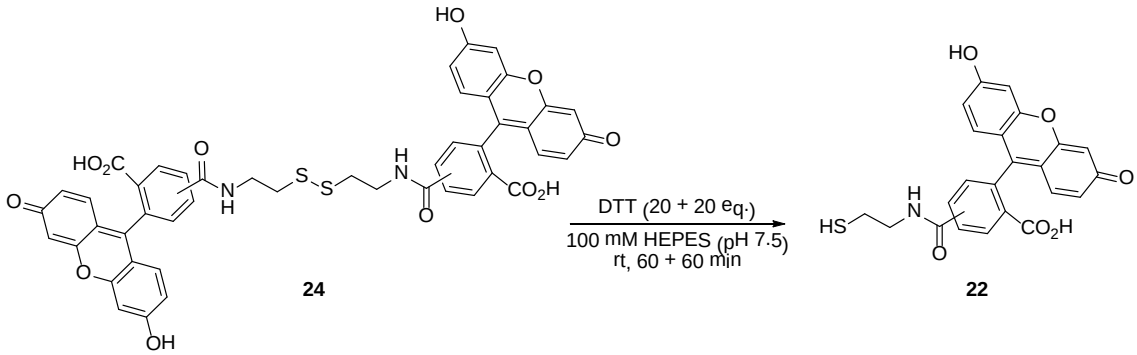


Figure S8. Confocal microscopy images of live MCF-7 cells. (a) 1 μM of **13** for 15 min. (b) Cells were incubated with 10 mM of bacitracin for 60 min prior to treatment with 1 μM of **13** for 15 min.

Reduction of the compound 24 with DTT



Compound **24** (5.0 mM in DMSO, 1.0 μL) was dissolved in 100 mM HEPES buffer (pH 7.5, 100 μL) at a concentration of 50 μM and reacted with DTT (20 eq.) at room temperature for 60 min. Additionally, to the reaction mixture was added DTT (20 eq.) and stirred at room temperature for 60 min .The reaction mixture was diluted to 12.5 μM with 0.05% formic acid in MeCN, and then was analyzed by LC-MS (Figure S9).

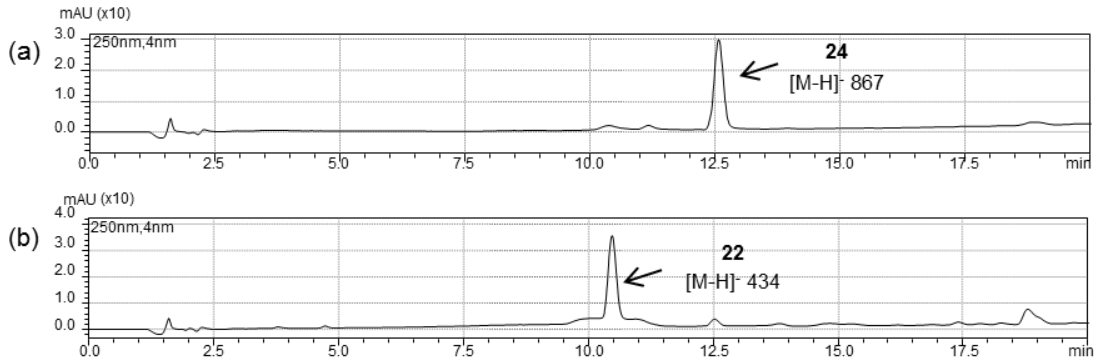
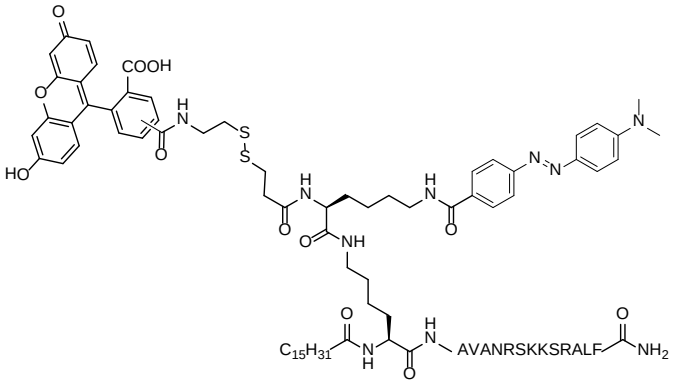


Figure S9. HPLC chart of monitoring the reaction of **24** and DTT. Analytical HPLC condition: column; a Waters Symmetry C18 analytical column (Waters, 4.6 × 75 mm), flow rate 0.5 mL/min, linear gradient of solvent B in solvent A; 10 to 90% over 20 min. (a) before and (b) after reduction with the above method.

Confocal microscopy images of MCF-7 cells treated with 14 without washing, 24 or 25



14

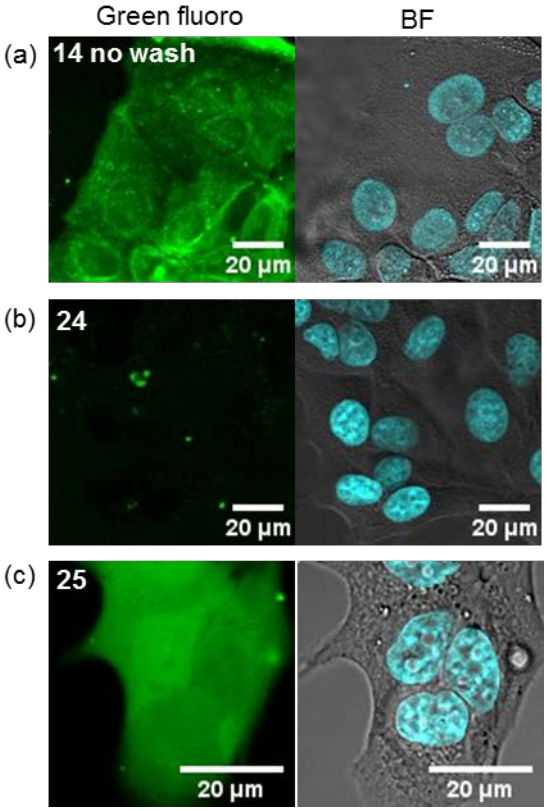
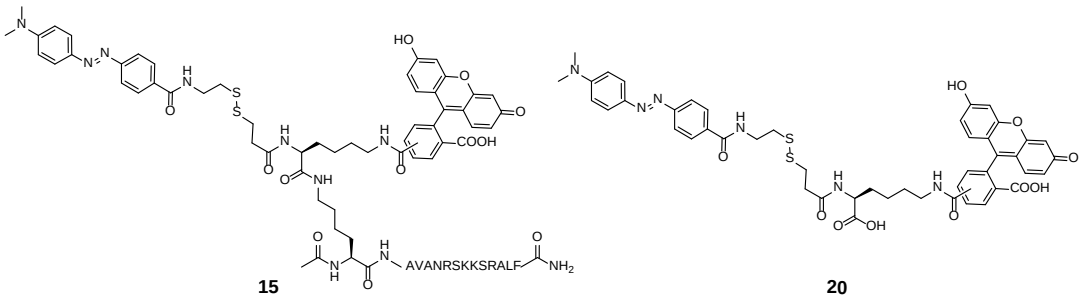


Figure S10. Confocal microscopy images and bright field images of live MCF-7 cells. (a) The cells were treated with 1 μM of **14** for 15 min, and then the image was acquired without washing. (b) The reduced **24** with DTT by the above method was diluted to 0.5 μM with MEM (–). Cells were treated with the solution of the reduced **24** for 15 min, (c) 1 μM of **25** for 15 min. BF images were overlaid by nuclear staining (Hoechst 33342). Excitation was provided with 488 nm laser.

Confocal microscopy images of MCF-7 cells treated with 15 or 20



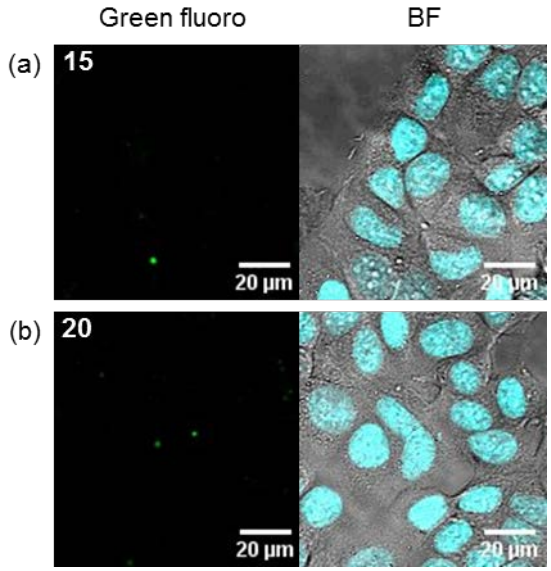


Figure S11. Confocal microscopy images and bright field images of live MCF-7 cells. Cells were treated with (a) 1 μM of **15** for 15 min, (b) 1 μM of **20** for 15 min. BF images were overlaid by nuclear staining (Hoechst 33342). Excitation was provided with 488 nm laser.

References:

1. C. Gnaccarini, S. Peter, U. Scheffer, S. Vonhoff, S. Klussmann and M. W. Göbel, *J. Am. Chem. Soc.*, 2006, **128**, 8063–8067.
2. Y.-J. Lee, S. Datta and J.-P. Pellois, *J. Am. Chem. Soc.*, 2008, **130**, 2398–2399.
3. M. Adamczyk, J. R. Fishpaugh and K. J. Heuser, *Bioconjugate Chem.*, 1997, **8**, 253–255.
4. M. Galibert, O. Renaudet, P. Dumy and D. Boturyn, *Angew. Chem. Int. Ed. Engl.*, 2011, **50**, 1901–1904.
5. J. Fernández-Carneado, M. Van Gool, V. Martos, S. Castel, P. Prados, J. de Mendoza and E. Giralt, *J. Am. Chem. Soc.*, 2005, **127**, 869–874.
6. J. Yang, H. Chen, I. R. Vlahov, J.-X. Cheng and P. S. Low, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 13872–13877.