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Electronic Supplementary Information

De Novo Formation of Citrate-based Fluorophores on *N*-Termini of Peptides and Proteins in Cells and Tissues

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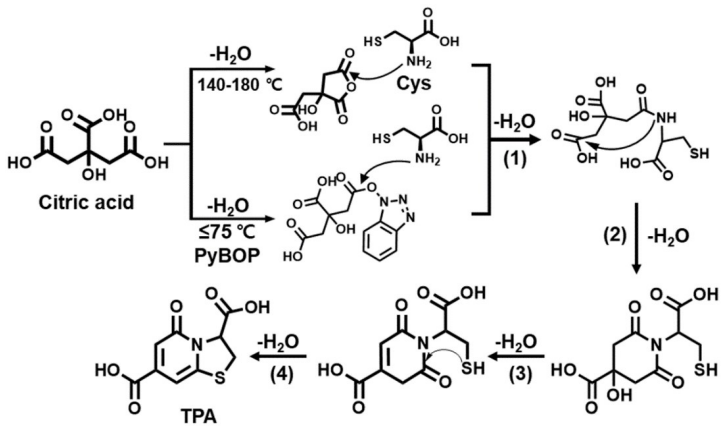
1. Materials

N-α-Fmoc protected L-amino acids, Rink Amide MBHA resin (0.45 mmol/g loading), and benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) were purchased from BeadTech, Korea. Citric acid monohydrate was purchased from DAEJUNG, Korea. L-cysteine hydrochloide monohydrate (L-Cys·HCl·H_2O) was purchased from BIOSESANG INC., Korea. Glycine was purchased from Merck Millipore, USA. *N*,*N*-Diisopropylethylamine (DIPEA), triisopropylsilane (TIS), glutaraldehyde (GA), and 1,2-ethanedithiol (EDT) were purchased from Tokyo Chemical Industry (TCI), Japan. Dimethyl sulfoxide (DMSO), N-butylamine, *N*,*N'*-diisopropylcarbodiimide (DIC), L-serine, L-methionine, 1-hydroxybenzotriazole hydrate (HOBt), DL-dithiothreitol (DTT), trifluoroacetic acid (TFA), triethylsilane (TES), paraformaldehyde (PFA), gelatin, sodium borohydride and piperidine were purchased from Sigma-Aldrich, USA. *N*,*N*-Dimethyl formamide (DMF), 1,2-dichloromethane (DCM), acetonitrile (ACN), ethyl acetate (EtOAc), *n*-hexane (*n*-Hex) and diethyl ether were purchased from Samchun Chemical, Korea. *HeLa* (human cervical cancer), *THP-1* (human monocyte cell) and *NIH3T3* (mouse fibroblast) cells were purchased from Korean Cell Line Bank. *HEK293T* (human embryonic kidney) cells were purchased from American Type Culture Collection (ATCC). SYTO 59 red fluorescent nucleic acid stain (5 mM Solution in DMSO) and concanavalin A, Alexa Fluor 594 conjugate were purchased from Thermo Fisher Scientific, USA. Anti-TOMM20 antibody [EPR15581-54] - Mitochondrial Marker (Alexa Fluor® 647), Anti-NUP133 antibody [EPR10808(B)] and Donkey Anti-Rabbit IgG H&L (Alexa Fluor® 594) were purchased from abcam. Dulbecco's modified eagle's medium (DMEM), Dulbecco's phosphate buffered saline (DPBS) and fetal bovine serum (FBS) were purchased from WELGENE, Korea. 0.05% Trypsin-EDTA (1×) was purchased from Gibco, USA.

2. Instruments

All ^{1}H NMR-spectra were analyzed with a Varian NMR (500 MHz) from USA and a Bruker Avance DPX-300 (300 MHz) from Germany. Microwave irradiation for synthesis and modification of peptides was provided by a CEM Discover microwave from USA. Photoluminescence excitation and emission were measured with a JASCO FP-8300 spectrofluorometer from Japan. High-performance liquid chromatography (HPLC) analyses and separation were carried out on a LC-20 series SHIMADZU system (Japan) equipped with a C18 Zorbax (5 µm, 9.4 × 250 mm) at 30^{o}C. 0.1% TFA in water and acetonitrile were used a as eluents at a flow rate of 4 mL/min. Quality of peptides was monitored with a MALDI-TOF mass anlyzer (Bruker, Germany, DE/microflex LT). LC-ESI-MS measurements were performed on an Agilent 6100 series system equipped with an Agilent 6120 quadrupole MS. Confocal laser scanning microscopic (CLSM) images were acquired using a Zeiss DE/LSM 510 NLO (Carl Zeiss, Germany) with a 500× objective lens (C-Apochromat, Carl Zeiss).

3. A Proposed Mechanism of TPA Formation



Scheme S1 A proposed mechanism of TPA formation with/without coupling reagents. (1) amide formation, (2) imide formation, (3) dehydration, (4) intramolecular condensation.[1-3]

4. Methods

Synthesis of 5-oxo-2,3-dihydro-5*H*-[1,3]thiazolo[3,2-*a*]pyridine-3,7-dicarboxylic acid (TPA)

Citric acid monohydrate (0.100 g, 0.476 mmol) was dissolved in DMF (0.5 mL) and DIPEA (81.0 µL, 0.476 mmol) was added to the solution. PyBOP (0.248 g, 0.476 mmol) in DMF (1 mL) was added to the citric acid solution. L-Cys·HCl·H_2O (0.085 g, 0.48 mmol) in DMF (0.5 mL) was added to the yellowish solution. Then, the solution was heated by microwave at 75°C (25 W) or stirred at room temperature (25°C). After the reaction, the product with a strong absorption band at 365 nm was purified using HPLC (Fig. S1). The fraction was collected and freeze-dried to obtain the pale yellowish solid. ^{1}H NMR (500 MHz, DMSO-d_6): δ 6.64-6.60 (s, 1H), 6.55-6.51 (s, 1H), 5.51-5.42(d, 1H), 3.97-3.85(m, 1H), 3.66-3.56(m, 1H). LC/MS (m/z): $[M+H]^+$ calcd. for $C_9H_7NO_5S$; 241.0 found. 242.2 $[M+H]^+$.

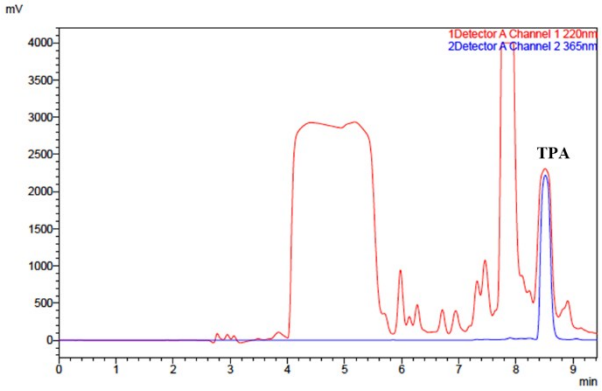
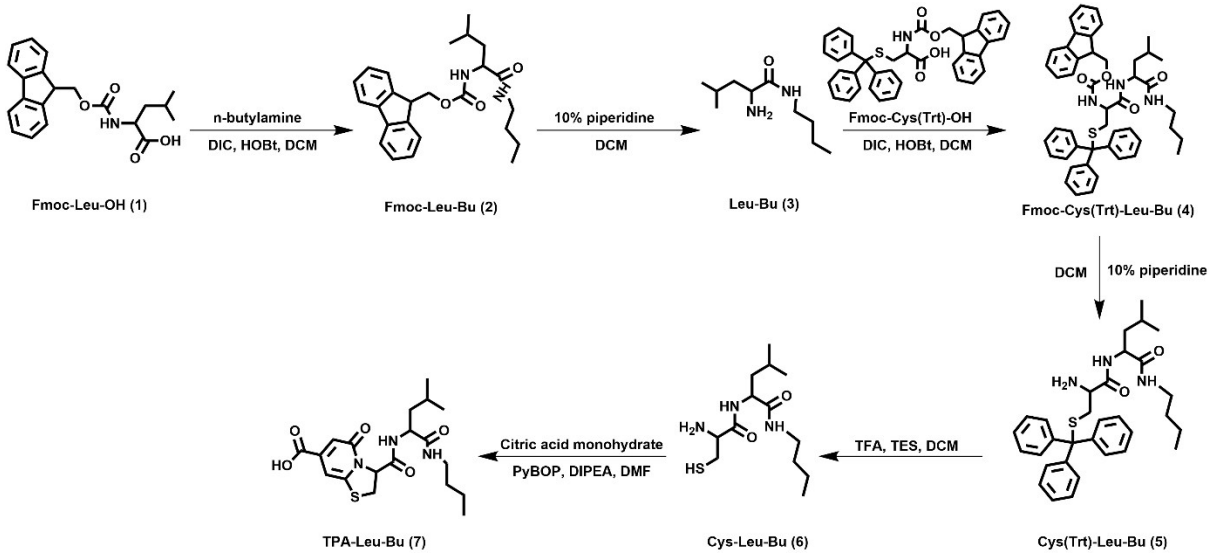
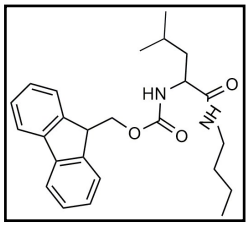


Fig. S1 Chromatograms of a mixture containing citric acid and L-cysteine heated by microwave.



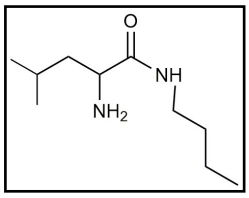
Scheme S2 Synthesis of TPA-Leu-Bu (**7**); model peptide in liquid phase.

Synthesis of Fmoc-Leu-Bu (2)



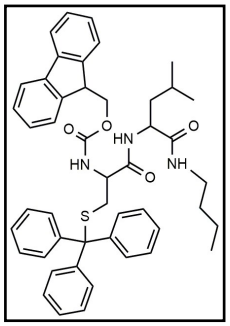
Fmoc-Leu-OH **(1)** (1.00 g, 2.83 mmol) and DIC (0.438 mL, 2.83 mmol) were dissolved in DCM (10 mL). Then, HOBt (0.382 g, 2.83 mmol) dissolved in DCM (10 mL) and n-butylamine (0.336 mL, 3.34 mmol) were added to the **(1)** solution. The solution was stirred at room temperature for 3 h. The reaction solution was concentrated via rotary evaporation. The mixture was purified by silica gel chromatography (*n*-Hex:EtOAc=1:1) to obtain a white solid (yield 87%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.78-7.28 (m, 8H), 4.43-4.41 (d, 2H), 4.23-4.19 (t, 1H), 4.12 (s, 1H), 3.25-3.23 (d, 2H), 1.61-1.25 (m, 6H), 0.93-0.88 (m, 9H).

Synthesis of Leu-Bu (3)



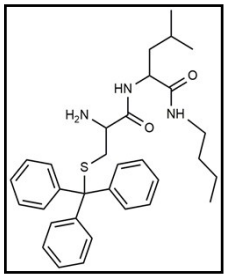
Compound **(2)** (0.100 g, 0.245 mmol) was dissolved in 10% piperidine in DCM (10 mL). The solution was stirred at room temperature for 1 h. The reaction solution was concentrated via rotary evaporation. The product was obtained as a white solid (yield 71%) by silica gel chromatography (DCM:MeOH=10:1). ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.10-3.04 (m, 3H),1.74-1.61 (m, 1H), 1.42-1.14 (m, 6H), 0.89-0.82 (m, 9H).

Synthesis of Fmoc-Cys(Trt)-Leu-Bu (4)



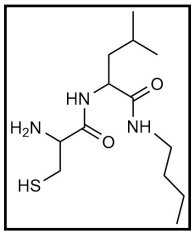
Compound **(3)** (0.100 g, 0.537 mmol), HOBt (72.6 mg, 0.537 mmol), and Fmoc-Cys(Trt)-OH(315 mg, 0.537 mmol) were dissolved in DCM (2 mL). Then, DIC (83.2 μL, 0.537 mmol) was added to the **(3)** solution. The solution was stirred at room temperature for 3 h. The reaction solution was concentrated via rotary evaporation. The product was obtained as a white solid (yield 24%) by silica gel chromatography (*n*-Hex:EtOAc=3:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.18-7.19 (m, 23H), 4.89-4.87 (d, 1H), 4.41-4.29 (m, 3H), 4.20-4.15 (t, 3H), 3.13-3.11 (d, 2H), 2.73-2.59 (m, 2H), 1.78-1.75 (m, 1H), 1.58-1.20 (m, 6H), 0.89-0.82 (m, 9H).

Synthesis of Cys(Trt)-Leu-Bu (5)



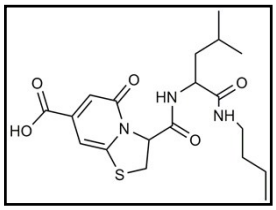
Compound **(4)** (0.100 g, 0.133 mmol) was dissolved in 10% piperidine in DCM (1 mL). The solution was stirred at room temperature for 1 h. The reaction solution was concentrated via rotary evaporation. The product was obtained as a pale yellowish solid (yield 36%) by silica gel chromatography (*n*-Hex:EtOAc=5:1 and then, EtOAc only). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.45-7.19 (m, 15H), 4.28-4.21 (m, 1H), 3.26-3.08 (m, 2H), 3.03-2.99 (q, 1H), 2.69-2.50 (m, 2H), 1.69-1.32 (m, 7H), 0.91-0.85 (m, 9H).

Synthesis of Cys-Leu-Bu (6)



Compound **(5)** (0.100 g, 0.188 mmol) was dissolved in DCM (1 mL). TES (0.300 mL, 1.88 mmol) and TFA (3 mL) were added to the (5) solution. The solution was stirred at room temperature for 2 h. After the reaction, the solution was concentrated by a nitrogen gas blowing and rotary evaporation. Then, a white solid was obtained by precipitaion in diethyl ether. The precipitate was collected by centrifugation at 4,000 rpm for 10 min and washed with diethyl ether (×4). The white solid product was dried by high vacuum (yield 31%). ^{1}H NMR (300 MHz, D_2O): δ 4.34-4.23 (m, 2H), 3.33-3.02 (m, 4H), 1.68-1.44 (m, 5H),1.36-1.24 (m, 2H), 0.95-0.86 (m, 9H).

Synthesis of TPA-Leu-Bu (7)



Citric acid monohydrate (5.0 mg, 0.024 mmol) was dissolved in DMSO (50 μL) and DIPEA (4.1 μL, 0.024 mmol) was added to the solution. PyBOP (12.4 mg, 0.0240 mmol) in DMSO (100 μL) was added to the citric acid solution. Compound **(6)** (6.9 mg, 0.024 mmol) was dissolved in DMSO (50 μL) and added to the citric acid solution. Then, the solution was heated by microwave at 75°C (25 W) for 30 min. After the reaction, the product with a strong absorption at 365 nm was purified using HPLC (Fig. S2). The purified fraction was freeze-dried to obtain a pale yellowish solid (yield 13%). ^{1}H NMR (500 MHz, D_2O): δ 6.75 (s, 1H), 6.59 (s, 1H), 5.61-5.60(d, 1H), 4.33-4.32(m, 1H), 4.00-3.96(m, 1H), 3.57-3.53(m, 1H), 3.25-3.15(m, 2H), 1.68-1.29(m, 7H), 0.96-0.88(m, 9H). LC/MS (m/z): calcd. for $C_{19}H_{27}N_3O_5S$; 409.2 found. 410.2 $[M+H]^+$.

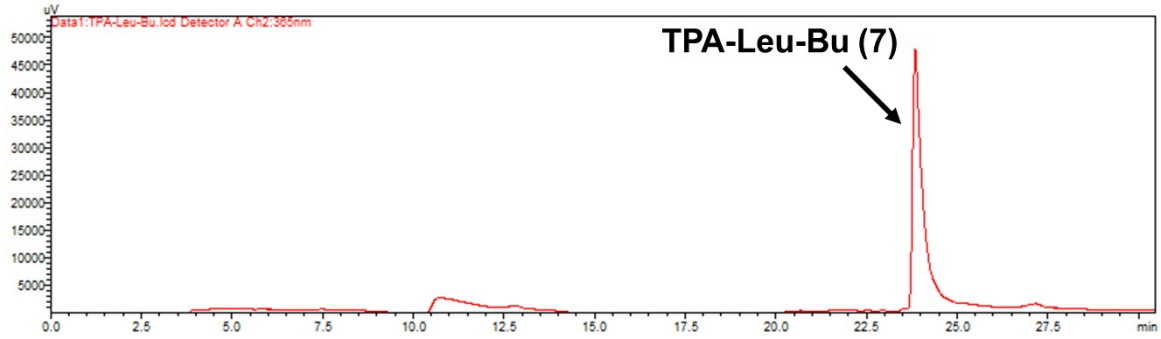
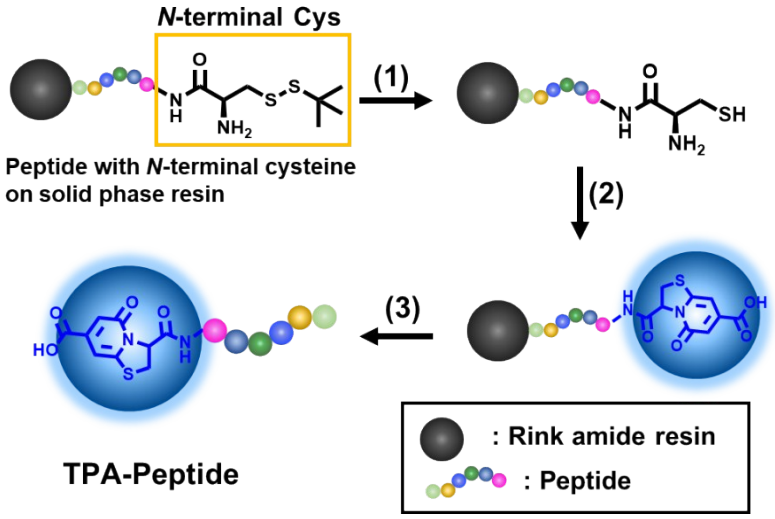


Fig. S2 A HPLC chromatogram of a TPA-Leu-Bu reaction mixture.



Scheme S3 Synthetic scheme of TPA-peptides on SPPS resin. (1): deprotection of S-(*tert*-butylthio) group, (2): formation of TPA on the peptide, (3): cleavage of the TPA-peptide from the resin.

Synthesis of TAT and LK peptides with an *N*-terminal cysteine on a solid phase resin

TAT and LK peptides with an *N*-terminal cysteine (sequence: CYGRKKRRQRRR and CLKKLLKLLKKLLKLAG) was synthesized by Fmoc-based solid-phase peptide synthesis chemistry using a Rink Amide MBHA resin. The peptide was synthesized in a 90 μmol scale. The typical conditions for the microwave-assisted peptide synthesis were used[4,5]: Fmoc-deprotection with 20% piperidine at 75°C (50 W) for 20 min and peptide coupling with PyBOP and DIPEA at 75°C (25 W) for

20 min.

First of all, Rink Amide resins (200 mg, 0.090 mmol, 0.45 mmol/g loading) were deprotected with 20% peperidine in DMF (2 mL) under N_2 bubbling, and then the first amino acid (350 mg, 0.540 mmol), PyBOP (281 mg, 0.540 mmol) and DIPEA (200 μL, 1.18 mmol) were added to the suspension. With microwave irradiation, the suspension was stirred under N_2 atmosphere for 20 min. Reaction byproducts were removed with DMF and DCM several times. After the coupling step, Fmoc-protected amino acid was deprotected with 20% piperidine in DMF and the reaction byproducts were removed with DMF and DCM several times. The coupling and deprotection steps were repeated with different Fmoc-protected amino acids sequentially until the deprotection of the last Fmoc-protected amino acid (Fmoc-Cys) was complete.

Deprotection of S-(*tert*-butylthio) in TAT and LK peptides on the solid phase peptide resin

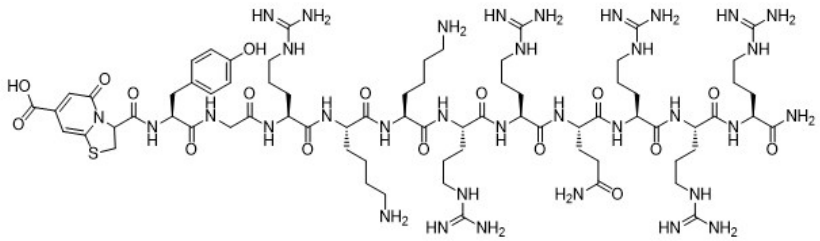
For deprotection of S-(*tert*-butylthio) protecting group with an *N*-terminal cysteine amino acid in the prepared peptides on the resin, the swollen resin (100 mg, 0.045 mmol) was reacted with DTT (250 mg, 1.62 mmol) in DMF (2 mL) for 1 h at 75°C (50 W) by using microwave heating. Reaction byproducts were removed with DMF and DCM several times. LRMS (MALDI) *m/z*: Anal. calcd. for $[M+H]^+$ $C_{67}H_{123}N_{33}O_{15}S$ (Cys-TAT): 1662.97; found: 1662.50. Anal. calcd. for $[M+H]^+$ and $[M+Na]^+$ $C_{92}H_{176}N_{24}O_{17}S$ (Cys-LK): 1923.35, 1945.33; found: 1923.27, 1945.17.

Formation of TPA on the peptides conjugated to the solid phase peptide resin

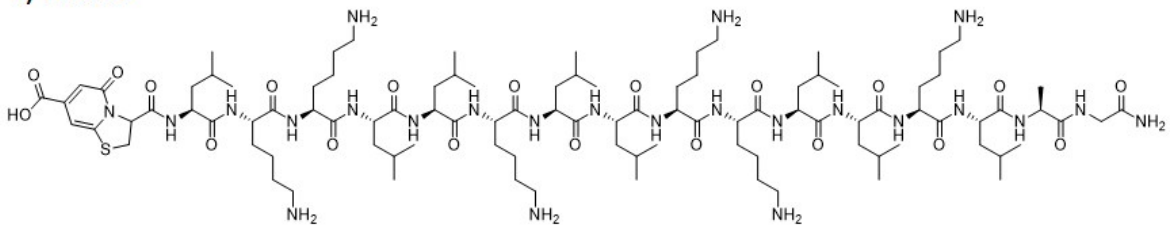
Citric acid monohydrate (189 mg, 0.900 mmol) was dissolved in DMF (1 mL). Then, DIPEA (154.4 μL, 0.90 mmol) and PyBOP (468 mg, 0.90 mmol) dissolved in DMF (1 mL) was added to the citric acid solution. The swollen resin (100 mg, 0.045 mmol) in DMF (2 mL) were reacted with the prepared solution and EDT (50 μL) for 1 h at 75°C (25 W) by using microwave heating.

Cleavage of the TPA-peptides from the solid phase peptide resin

1) TPA-TAT



2) TPA-LK



Cleavage of the peptide from the resin was carried out by treatment with TFA/TIS/water (total volume 4.00 mL, v/v=95:2.5:2.5) for 4 h at room temperature. The resin was then separated by filtration and further washed with TFA. The filtrate was concentrated by a nitrogen gas blowing. The synthesized peptide was precipitated in a mixture of *n*-hexane and diethyl ether (v/v=50:50). The resulting suspension was centrifuged at 4,000 rpm for 15 min. After the supernatant was decanted, the pellet was dissolved in DMF and was purified with HPLC. The peptide was lyophilized affording a white powder. LRMS (MALDI) *m/z*: Anal. calcd. for $[M+H]^+$ $C_{73}H_{124}N_{33}O_{18}S$ (TPA-TAT): 1782.94; found: 1782.87 and $C_{98}H_{177}N_{24}O_{20}S$ (TPA-LK): 2043.30; found: 2044.87.

Formation of fluorophores on amino acids or tripeptides

For the formation of fluorophores on free amino acids, citric acid monohydrate (10 mg, 48 μmol) was dissolved in DMSO (0.4 mL) and DIPEA (8.1 μL, 48 μmol) was added to the solution. And then, an amino acid (Cys: 5.8 mg, 48 μmol or Ser: 5.0 mg, 48 μmol) in H_2O (0.6 mL) was added to the citrate solution. Finally, PyBOP (25 mg, 48 μmol) in DMSO (1.0 mL) was added to the solution. The solution was incubated at 25°C for the time-dependent analysis of the fluorescnce generation. The

fluorescence intensity at the maximum emission wavelength of each mixture was measured with a JASCO FP-8300 spectrofluorometer coninuously by an excitation at the maximum absorption wavelength.

For the formation of fluorophores on tripeptides, citric acid monohydrate (2.5 mg, 12 μmol) was dissolved in DMSO (100 μL) and DIPEA (2.0 μL, 12 μmol) was added to the solution. And then, a tripeptide (CGG: 2.8 mg, 12 μmol, SGG: 2.6 mg, 12 μmol or MGG: 3.1 mg, 12 μmol) in H_2O (150 μL) was added to the citrate solution. Finally, PyBOP (6.2 mg, 11.9 μmol) in DMSO (250 μL) was added to the solution. The fluorescence intensity of each mixture was measured in the same manner as above.

Cell culture

HeLa (human cervical cancer), *HEK293T* (human embryonic kidney) and *NIH3T3* (mouse fibroblast) cells were cultured in DMEM supplemented 10% FBS. *THP-1* (human monocyte cell) cells were cultured in RPMI 16409 supplemented 5% FBS. The *THP-1* mococytes were differentiated into macrophages (*THP-1M*) by treatment of with phorbol 12-myristate 13-acetate (PMA). *THP-1* cells (50,000 cells/well) were seeded on gelatin coated coverslips and incubated with PMA (10 ng/mL) for 72 h. All the cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C in a incubator.

CLSM observation of *HeLa* cells treated with TPA-labeled peptides

HeLa cells (10,000 cells/well) were cultured in 180 μL of DMEM supplemented with 10% FBS in a confocal dish (SPL Lifesciences, South Korea) at 37°C in a 5% CO_2 incubator. After 24 h, the cells were washed with PBS (×3), the medium was exchanged with fresh DMEM supplemented with 2% FBS. Then, TPA-TAT peptide (final concentration: 5 μM) or TPA-LK peptide (final concentration: 500 nM) in deionized water was added to the dish and the cells were further incubated for 12 h. Cells were washed 4 times with fresh medium for the removal of peptides outside the cells, and fresh medium was added. Nucleus was stained with a SYTO 59 solution (final concentration: 5 μM) for 30 min prior to the CLSM imaging. After further washing with fresh medium (×3), CLSM images were acquired.

Red channel (560 nm long-pass filter) images were obtained by excitation with a 1 mW laser at 543 nm and blue channel (390-465 nm) images were done by two-photon excitation with 2 mW lasers at 780 nm (TPA-TAT) or 720 nm (TPA-LK), respectively. The line profiles of each fluorescence in the CLSM image are shown in Fig. S19. The Imaris software was used to analyze the fluorescence colocalization of more than 500 intracellular fluorescent pixels in a CLSM image. The colcalization degree of blue fluorescence with red fluorescence was caluclated as below.

$$Degree\ of\ fluorescence\ colocalization\ (\%) = \frac{Number\ of\ colocalized\ pixels\ with\ blue\ and\ red\ fluorescence}{Total\ number\ of\ pixels\ with\ blue\ fluorescence}$$

Cell fixation

14-mm^2 glass coverslips were coated with 0.1% gelatin in ultrapure water. Coverslips were placed in a 6-well plate and seeded with 50,000 cells overnight. For ER and mitochondria staining, cells were washed and fixed with 3% PFA + 0.1% GA in PBS for 10 min, followed by reduction with 0.1% sodium borohydride for 7 min at RT and quenching in 100 mM glycine for 10 min at RT. Finally, cells were washed with PBS three times for 5 min each. For nuclear pore complex (NPC) staining, cells were rinsed with 2.4% PFA in PBS and extracted with 0.4% Triton X-100 in PBS for 3 min. And the cells were fixed with 2.4% PFA in PBS for 30 min, quenched for 5 min with 50 mM NH_4Cl solution, and washed with PBS three times for 5 min each.

Staining of fixed cells by the DNFC method

First, Citric acid monohydrate (0.010 g, 0.0476 mmol) was dissolved in DMSO/H_2O (v/v=70/30, 0.5 mL) and DIPEA (8.1 μL, 0.0476 mmol) was added to the solution. Then PyBOP (0.0248 g, 0.0476 mmol) was dissolved in DMSO/H_2O (v/v=70/30, 1.0 mL) and the solution was added to the citric acid solution. Fixed cells in glass chamber were stained with the prepared solution at room temperature. Generally, the staining was performed under the dark and shaking for 30 min. After the staining, the cells were washed by DMSO and DPBS 4 times, respectively.

CLSM observation of the DNFC-stained cells

After staining of fixed cells, CLSM images were acquired by using LSM880 (Carl Zeiss). Blue (410-500 nm) and green (500-600 nm) channel images were obtained under excitation at 405 nm with a laser power of 5 mW. Red channel (600-700 nm) images of ConA, TOMM20, and NPC were obtained under excitation at 543 nm with a laser power of 10 mW, 633 nm with a laser power of 5 mW and 543 nm with a laser power of 10 mW, respectively.

Colocalization of DNFC fluorescence with organelles

For ER staining, Alexa Fluor 594-conjugated concanavalin A (ConA) in DPBS (10 μg/mL) was added to the DNFC-stained cells. After 30 min-incubation under the dark and with shaking, the cells were washed with DPBS 4 times. For the staining of mitochondria and NPC, the DNFC-stained cells were incubated with a blocking buffer (3% BSA in DPBS) for 30 min and added with anti-TOMM20 antibody and anti-NPC antibody (2 μg/mL) in the blocking buffer, respectively. After 3 h, the cells were washed three times with the blocking buffer and further incubated with secondary antibodies (4 μg/mL) in the blocking buffer for 1 h. The secondary antibody for TOMM20 visualization was labeled with Alexa Fluor 647 and that for NPC visualization was labeled with Alexa Fluor 594, respectively. Finally, the cells were washed with DPBS extensively.

After obtaining the CLSM images, the Imaris software was used to analyze the fluorescence colocalization of more than 500 intracellular fluorescent pixels in a CLSM image. The colcalization degree of blue/green fluorescence from the DNFC staining with red fluorescence from each organell-targeting reagent was caluclated as below.

$$Degree\ of\ blue\ (or\ green)\ fluorescence\ colocalization\ (\%) = \frac{Number\ of\ colocalized\ pixels\ with\ blue\ (or\ green)\ and\ red\ fluorescence}{Total\ number\ of\ pixels\ with\ blue\ (or\ green)\ fluorescence}$$

Human tissue sample acquisition

Human tissue samples were collected between February and April in 2018 at the department of plastic and reconstructive surgery in the Boramae Medical Center of Seoul National University. A

keloid tissue of the earlobe, a basal cell carcinoma sample of the facial skin, and a dermatofibrosarcoma protuberance sample of the back were offered for this study. The institutional review board of the Boramae Medical Center reviewed and approved the study protocol (IRB No. 30-2017-11 and 26-2017-20) and all patients provided informed consent agreements. Subject identification was blinded at tissue sampling. Briefly, tissue specimens used surplus tissues remaining after wide excision of skin cancer or keloid resection surgery.

Production of formalin fixed paraffin-embedded tissues and microsection

On gross, tissues were dissected to about 3 mm in thickness, then tissues were soaked into 4% formalin solution for 1 day. The formalin-fixed tissues were placed into the automatic tissue processor instrument (EFTP) (Intelsint: Villarbasse. Turin, Italy), in which the tissues were submerged in each reagent and permeated through a series of reagents overnight according to the standard protocol.[6] Next day, the tissues were embedded in paraffin wax blocks. Tissue-paraffin blocks (after cooling) were serially microsectioned to 3 μm in the thickness using a rotary microtome apparatus. Each tissue slice was put on a glass slide for the DNFC staining or the conventional histochemical staining.

H&E- and MT-staining of human tissues for observation with optical microscopy

Hematoxylin and eosin (H&E) stain was performed using the autostainer (Leica autostainer XL) (Leica Biosystems: Newcastle Upon Tyne, UK). The staining procedures were as follows; tissue slices were dewaxed, rehydrated through descending grades of alcohol to water, stain in hematoxylin, washed well in running tap water, differentiated in acid alcohol, washed well, stained in eosin Y, washed in running tap water, dehydrated through alcohols, and cleared with xylene. As for Masson trichrome (MT) stain, tissues were deparaffinized and rehydrated, then submerged in Boulin's solution overnight. After rinsed with distilled water, tissues were stained by Weigert's iron hematoxylin, washed wall in tap water, treated with phosphomolybdic acid solution, stained with aniline blue solution, washed lightly, treated with acetic acid solution, dehydrated, and cleared in xylene. Finally, each stained-tissue on the glass slide was mounted in permanent mounting medium, and microscopic features were interpreted using an optical microscope.

Staining of human tissues by the DNFC method

Citric acid monohydrate (0.200 g, 0.952 mmol) was dissolved in DMSO (1.0 mL) and DIPEA (162 μL, 0.952 mmol) was added to the solution. PyBOP (0.496 g, 0.952 mmol) was dissolved in DMSO (2.0 mL) and the solution was added to the citric acid solutions. Human tissue samples in a glass chamber were incubated in the prepared solution for 30 min at room temperature under the dark condition and with shaking. After the staining, the samples were washed with DMSO and DPBS 4 times, respectively.

CLSM observation of the DNFC-stained human tissues

After staining of human tissues, CLSM images were acquired by using LSM880 (Carl Zeiss). Blue (410-500 nm) and green (500-600 nm) channel images were obtained under excitation at 405 nm with a laser power of 5 mW.

5. ^{1}H NMR Spectra of Compounds

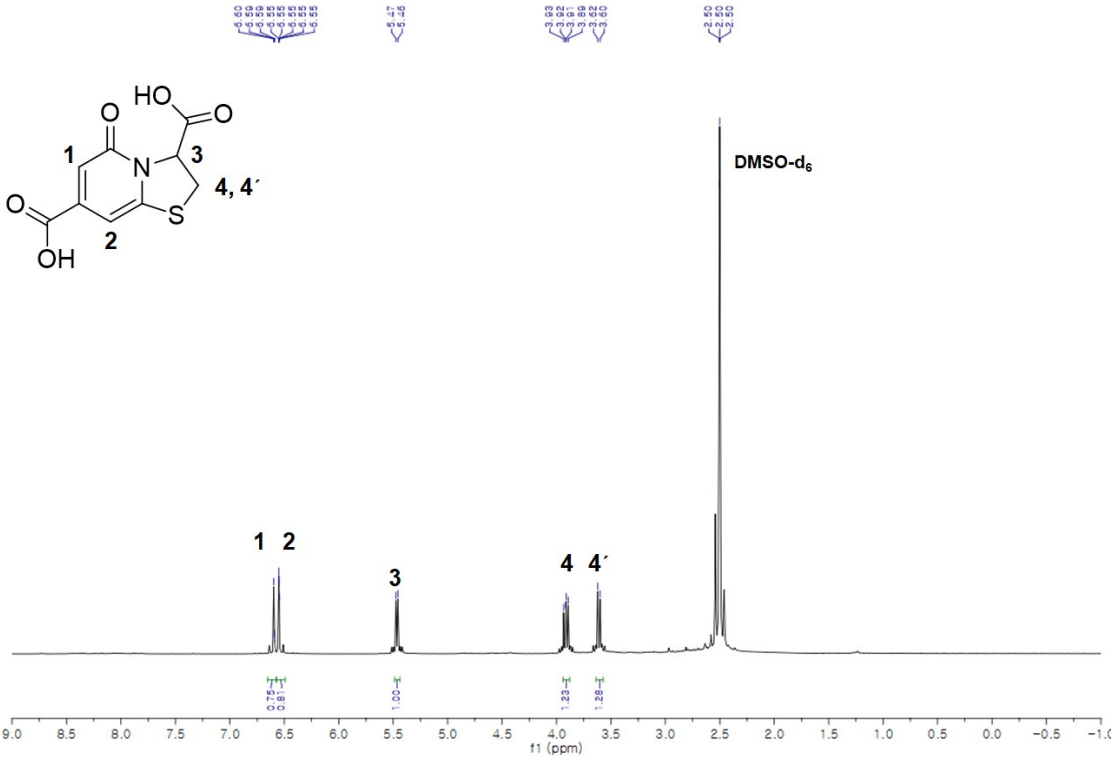


Fig. S3 ^{1}H NMR spectrum of TPA in DMSO-d_6.

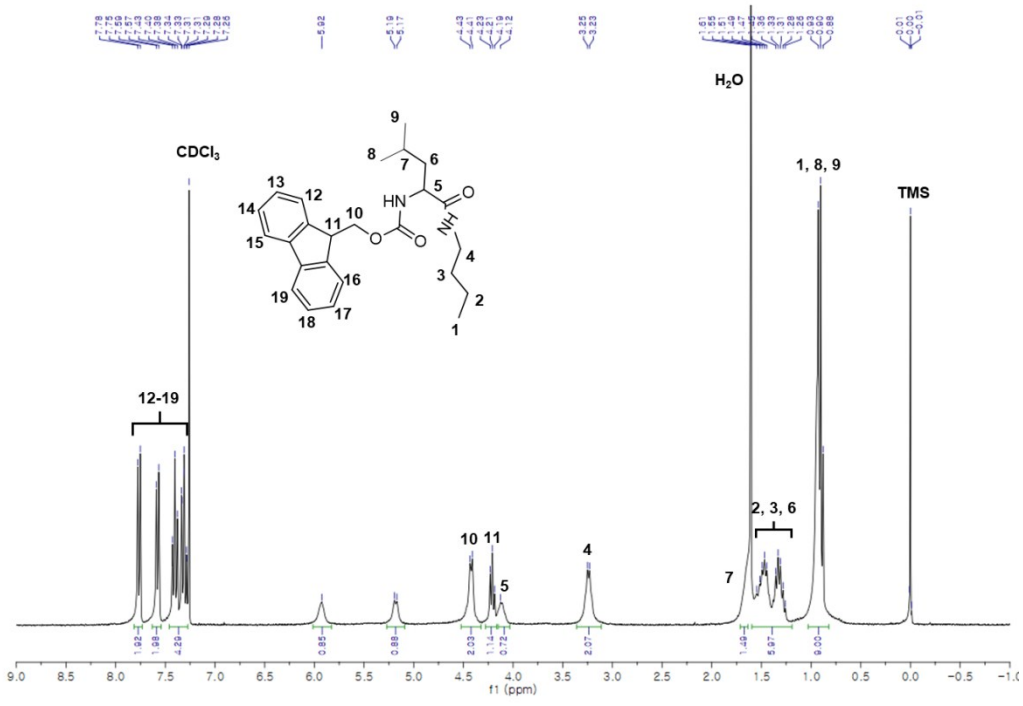


Fig. S4 ^{1}H NMR spectrum of compound **2** in $CDCl_3$.

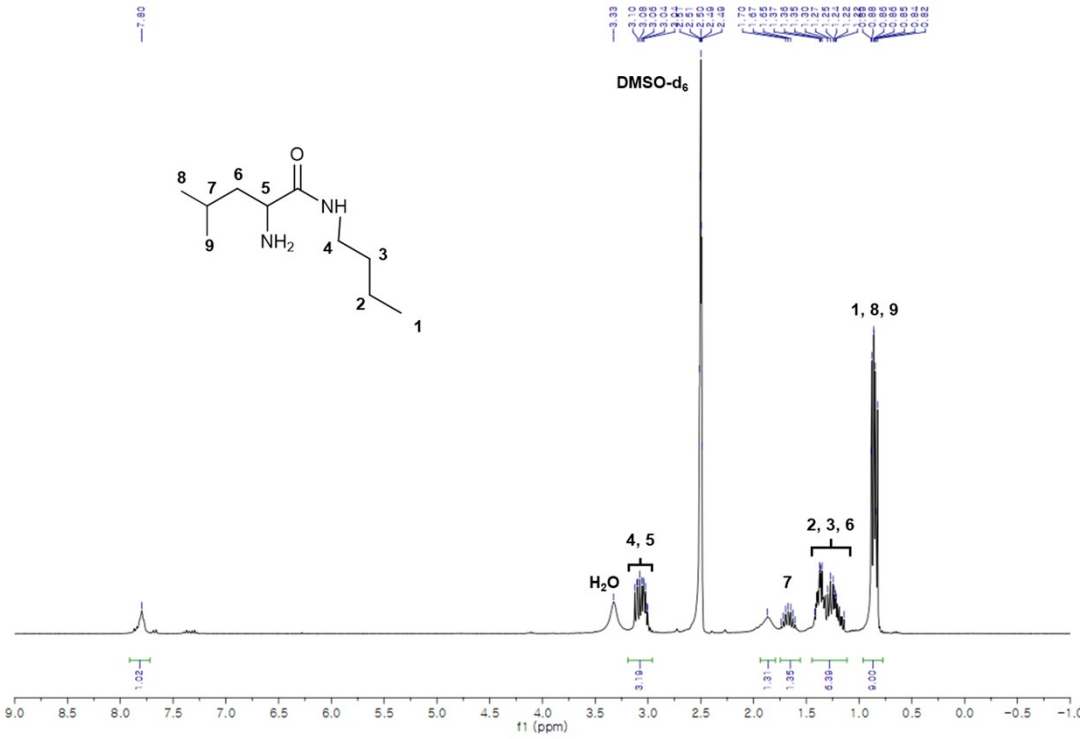


Fig. S5 ^{1}H NMR spectrum of compound **3** in $CDCl_3$.

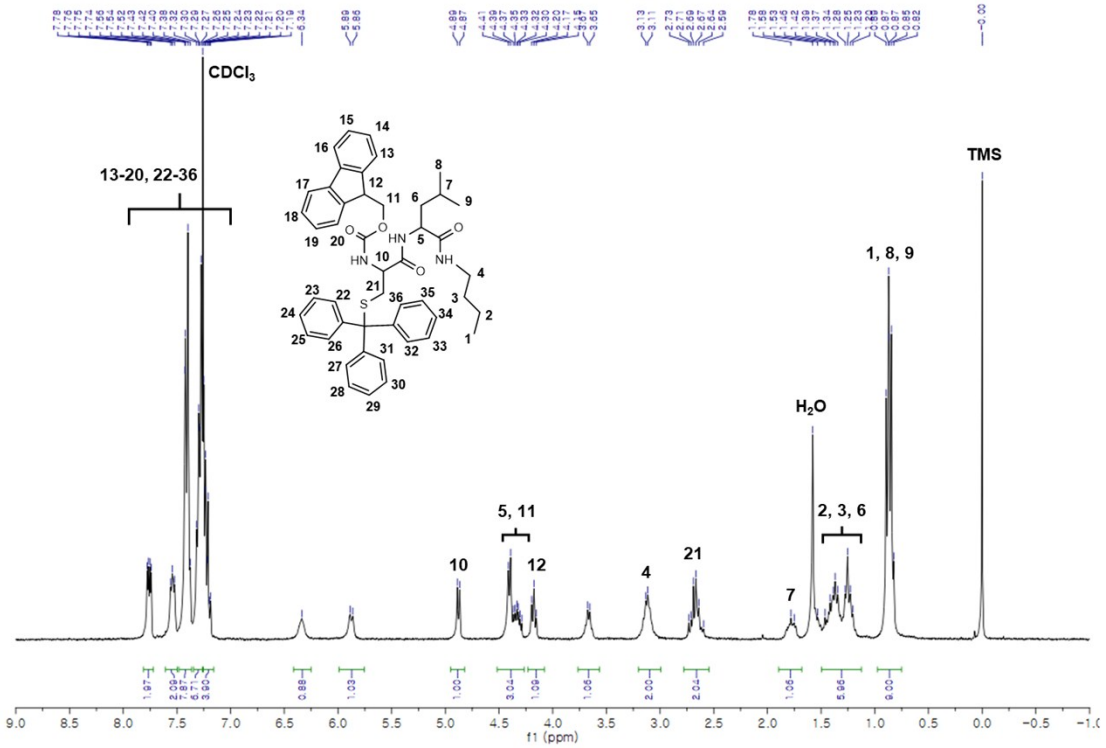


Fig. S6 ^{1}H NMR spectrum of compound **4** in $CDCl_3$.

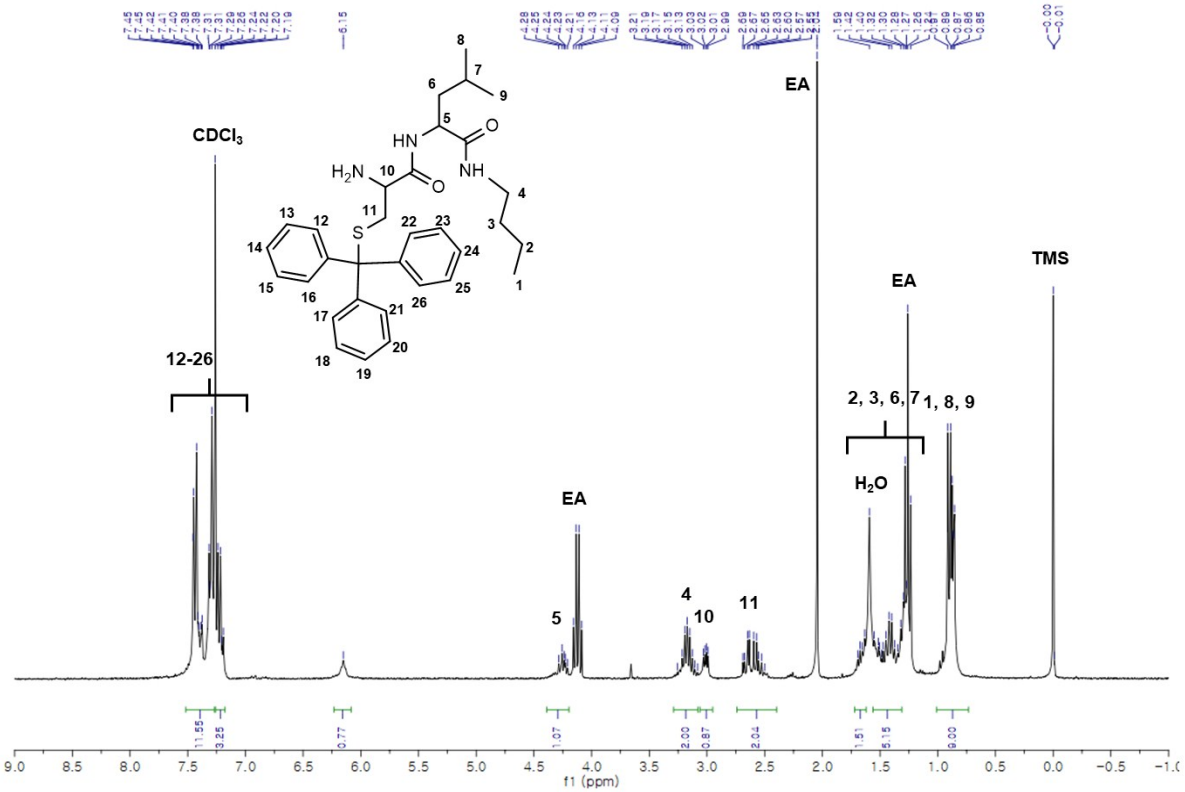


Fig. S7 ^{1}H NMR spectrum of compound **5** in $CDCl_3$.

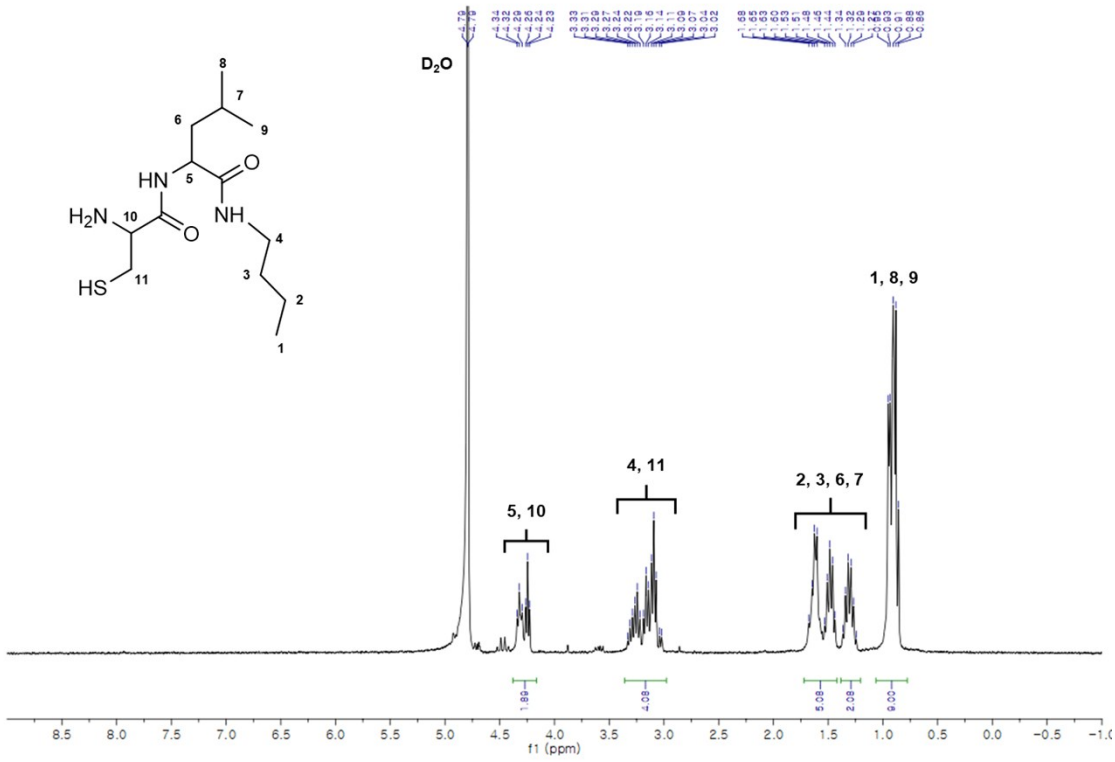


Fig. S8 ^{1}H NMR spectrum of compound **6** in D_2O.

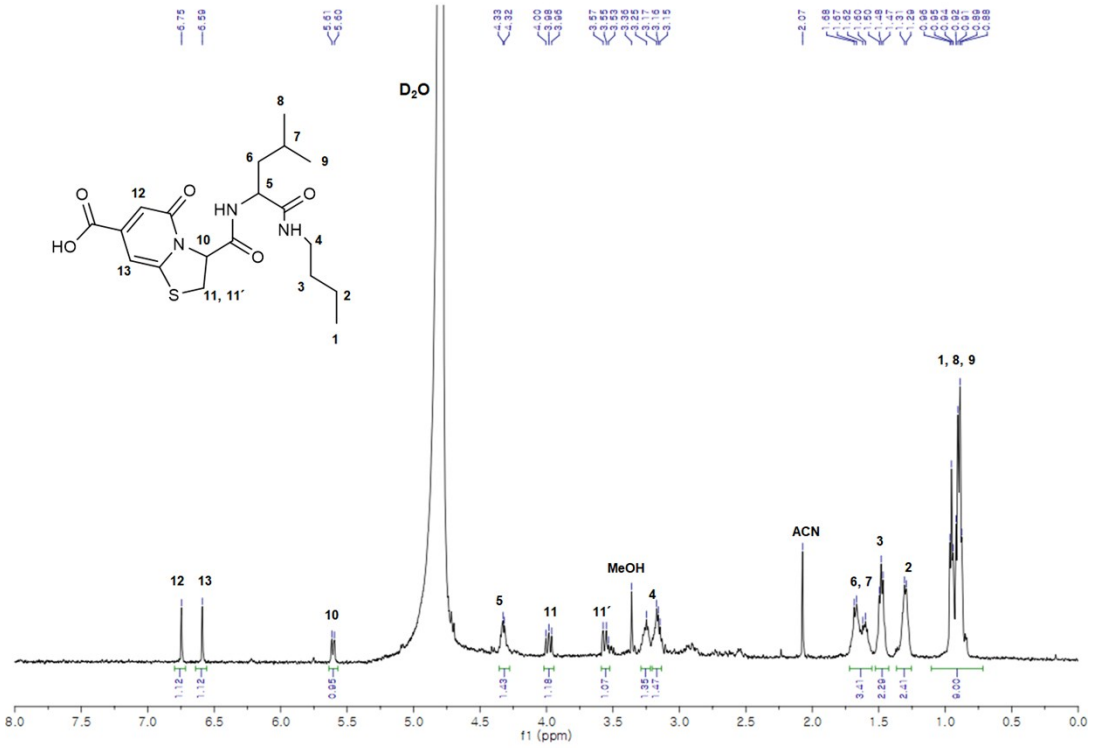


Fig. S9 ^{1}H NMR spectrum of compound **7** in D_2O.

6. MS Spectra of TPA and Compound 7

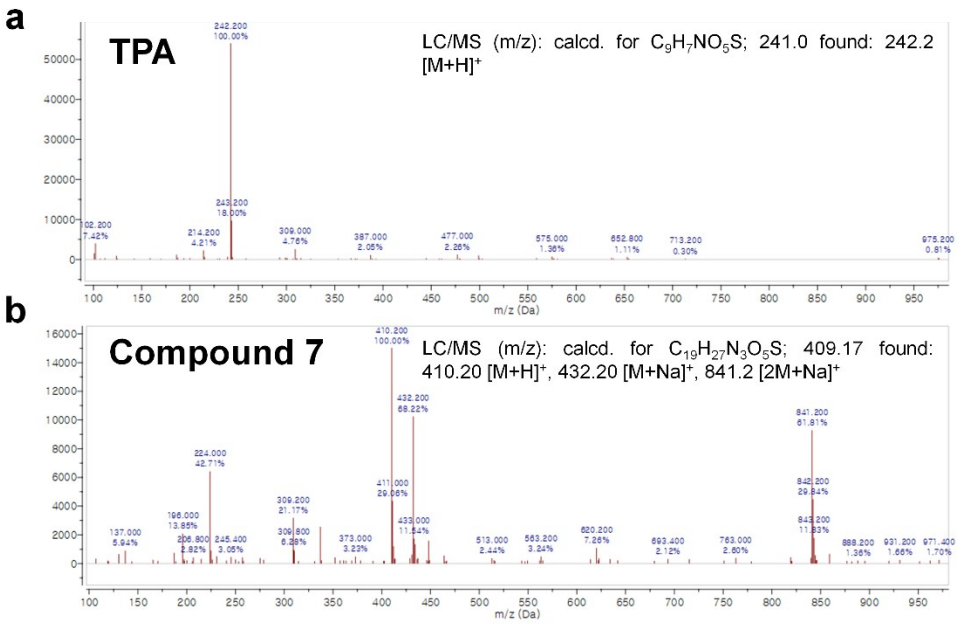


Fig. S10 LC/MS spectra of (a) TPA and (b) compound **7**.

7. MS Spectra of Compounds of Peptides

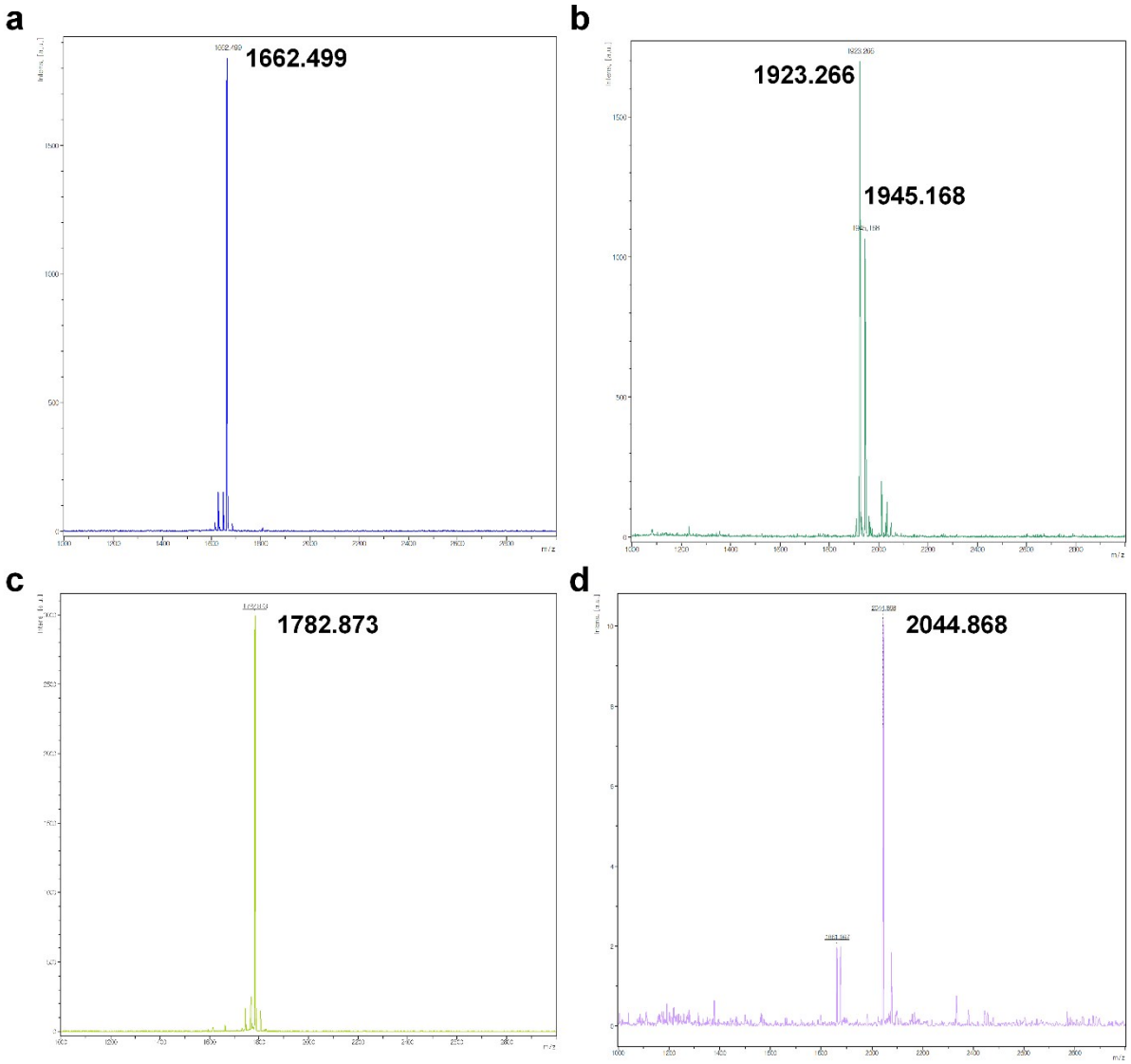


Fig. S11 MALDI-TOF spectra of (a) Cys-TAT peptide, (b) Cys-LK peptide, (c) TPA-TAT peptide and (d) TPA-LK peptide.

8. Photoluminescence (PL) Spectra

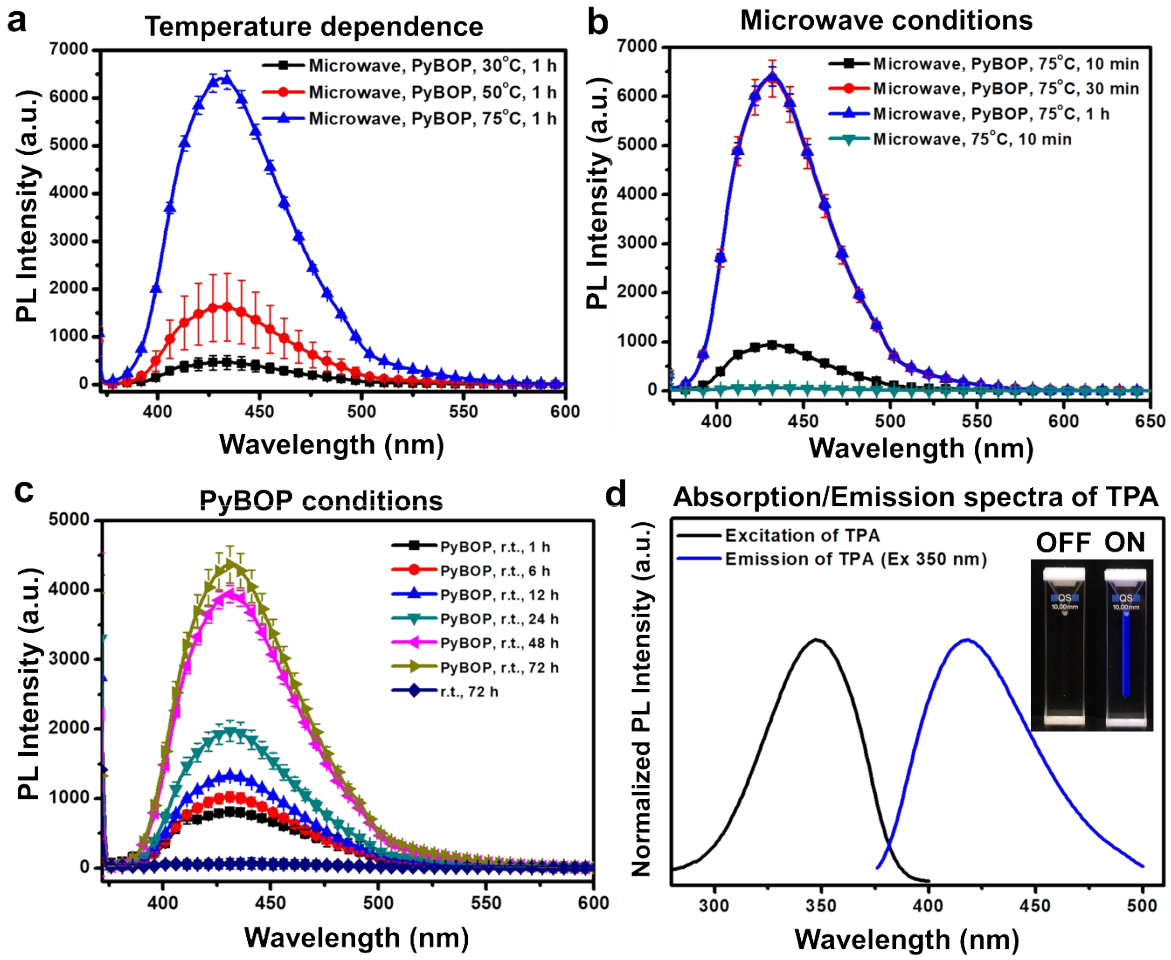


Fig. S12 Photoluminescence spectra of fluorophores and fluorophore-generated samples. Emission spectra (λ_{ex}= 365 nm) of mixtures in DMF containing the same amount of citric acid and L-cysteine which were treated with various reaction conditions: (a) with microwave irradiation at various reaction temperatures (black: 30°C, red: 50°C, blue: 75°C); (b) with microwave irradiation for various reaction time at 75°C (black: 10 min, red: 30 min, blue: 1 h, cyan: 10 min without PyBOP); (c) without microwave irradiation for various reaction time at 25°C. (black: 1 h, red: 6 h, blue: 12 h, cyan: 24 h, magenta: 48 h, brown: 72 h, navy: 72 h without PyBOP). (a-c) Each data point represents the average ± S.D. (n = 4). (d) Absorption and emission spectra of TPA prepared by the PyBOP-assisted synthesis in deionized water at 25°C. (solvent: deionized water, concentration: 0.2 mg/mL). For the emission spectrum, the excitation wavelength was 350 nm. Inset: Fluorescence of TPA illuminated by UV light (365 nm) (solvent: deionized water, concentration: 0.2 mg/mL).

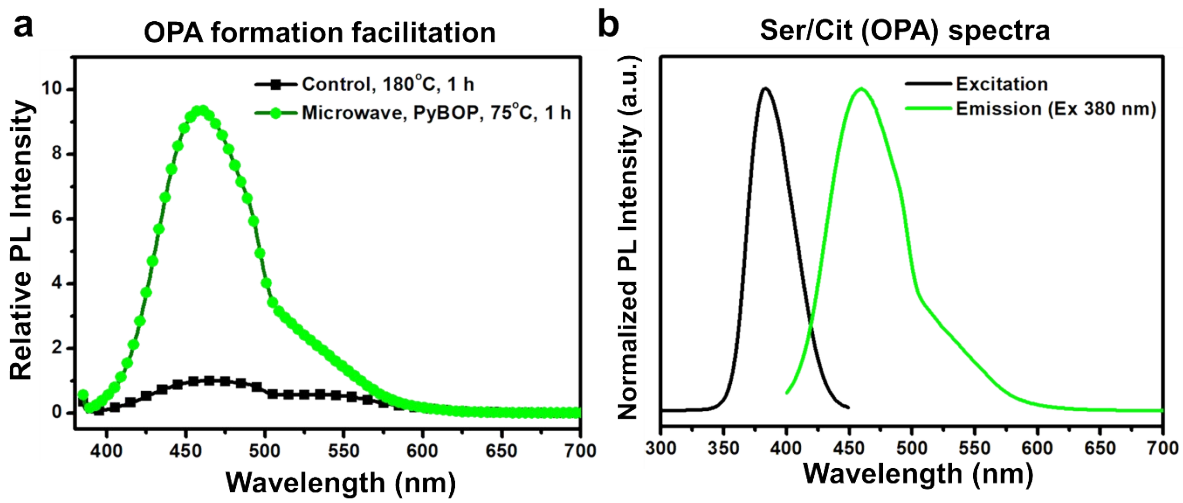


Fig. S13 (a) Emission spectra (λ_{ex} = 380 nm) of mixtures containing the same amount of citrate and L-serine in DMF after heating at 180°C (black) or the treatment in the same condition of (b). (b) Absorption and emission spectra (λ_{ex} = 380 nm) of a mixture of citrate (0.476 mmol) and L-serine (0.48 mmol) in DMF (2.0 mL) after incubation with PyBOP (0.476 mmol) and DIPEA (0.476 mmol) in presence of microwave irradiation for 1 h at 75°C.

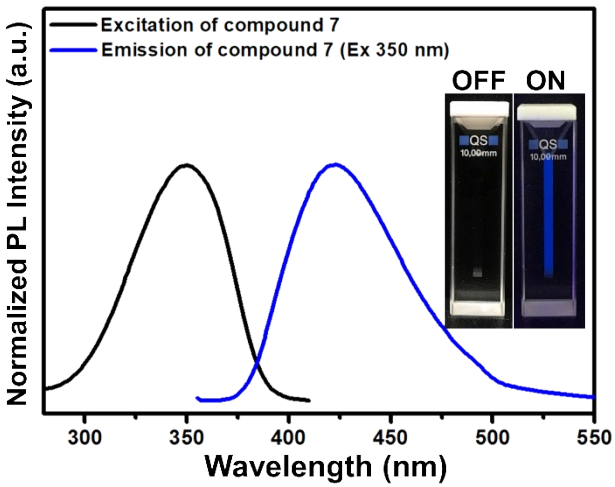


Fig. S14 Absorption and emission spectra of compound **7** (solvent: deionized water, concentration: 0.3 mg/mL) in deionized water. Inset: Fluorescence of compound **7** illuminated by UV light (365 nm) (solvent: deionized water, concentration: 0.3 mg/mL).

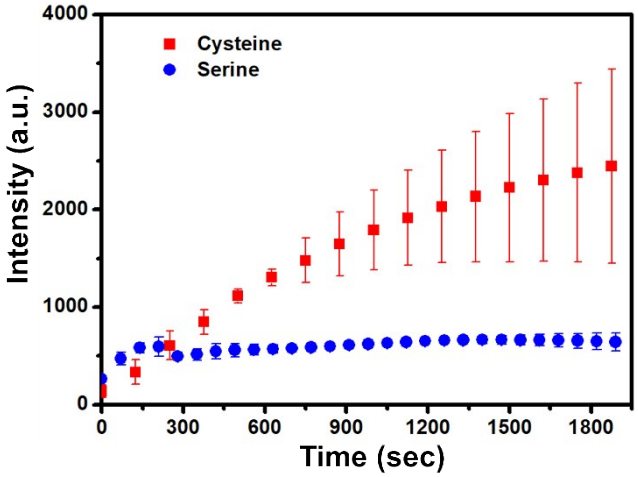


Fig. S15 Time-dependent fluorescence development of Cys/Cit and Ser/Cit mixtures after additon of PyBOP at 25°C in DMSO/H_2O (70/30). The each fluorescence intensity was measured at the maximum emission wavelength by excitation at the maximum absorption wavelength (maximum λ_{ex} of Cys/Cit= 370 nm and Ser/Cit= 400 nm). Each data points represent the average ± S.D. (*n* = 3).

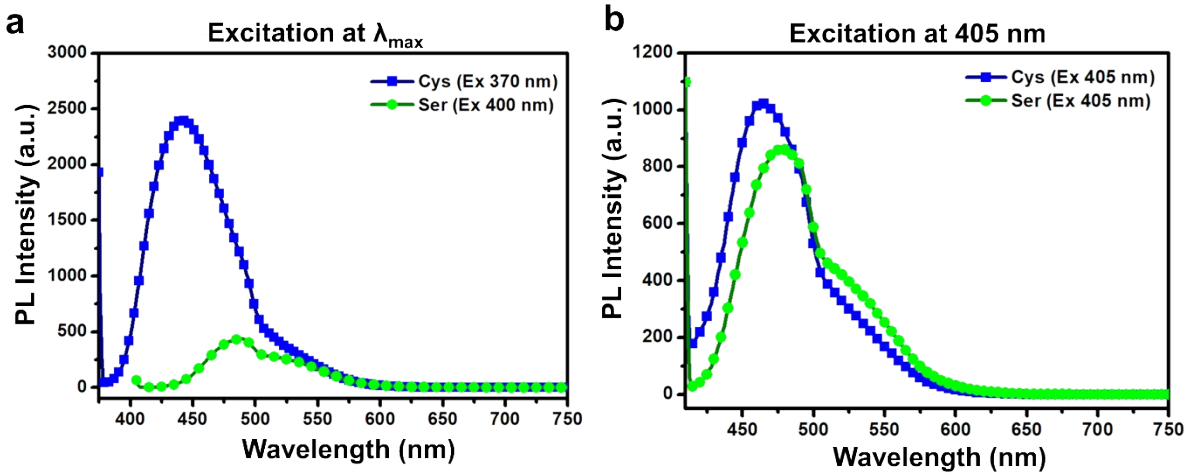


Fig. S16 (a) Comparison of fluorescence emission by excitation at the maximum absorption wavelength (maximum λ_{ex} of Cys/Cit= 370 nm and Ser/Cit= 400 nm) from a Cys/Cit mixture and a Ser/Cit mixture after incubation with PyBOP for 30 min at 25°C in DMSO/H_2O. (b) Comparison of fluorescence emission by excitation at 405 nm from a Cys/Cit mixture and a Ser/Cit mixture after incubation with PyBOP for 30 min at 25°C in DMSO/H_2O (70/30).

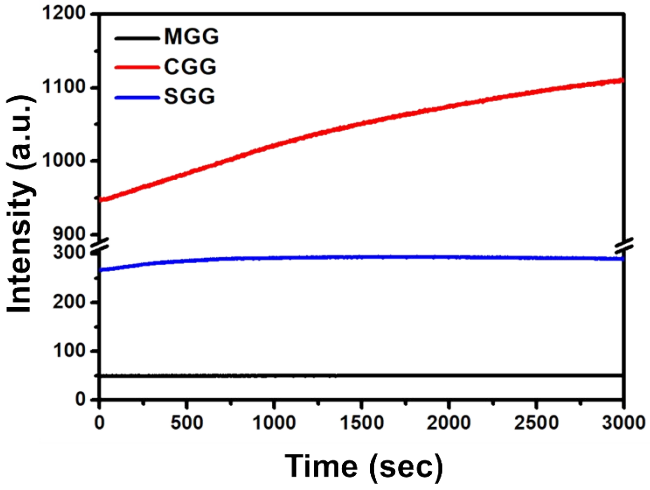


Fig. S17 Time-dependent fluorescence development of tripeptide/Cit mixtures after additon of PyBOP at 25°C in DMSO/H_2O (70/30). MGG, CGG and SGG represent L-methionine-glycine-glycine, L-cysteine-glycine-glycine and L-serine-glycine-glycine, respectively. Each fluorescence intensity was measured at the maximum emission wavelength by excitation at the maximum absorption wavelength.

9. Analysis of the CLSM Images of *HeLa* Cells Treated with TPA-labeled Peptides

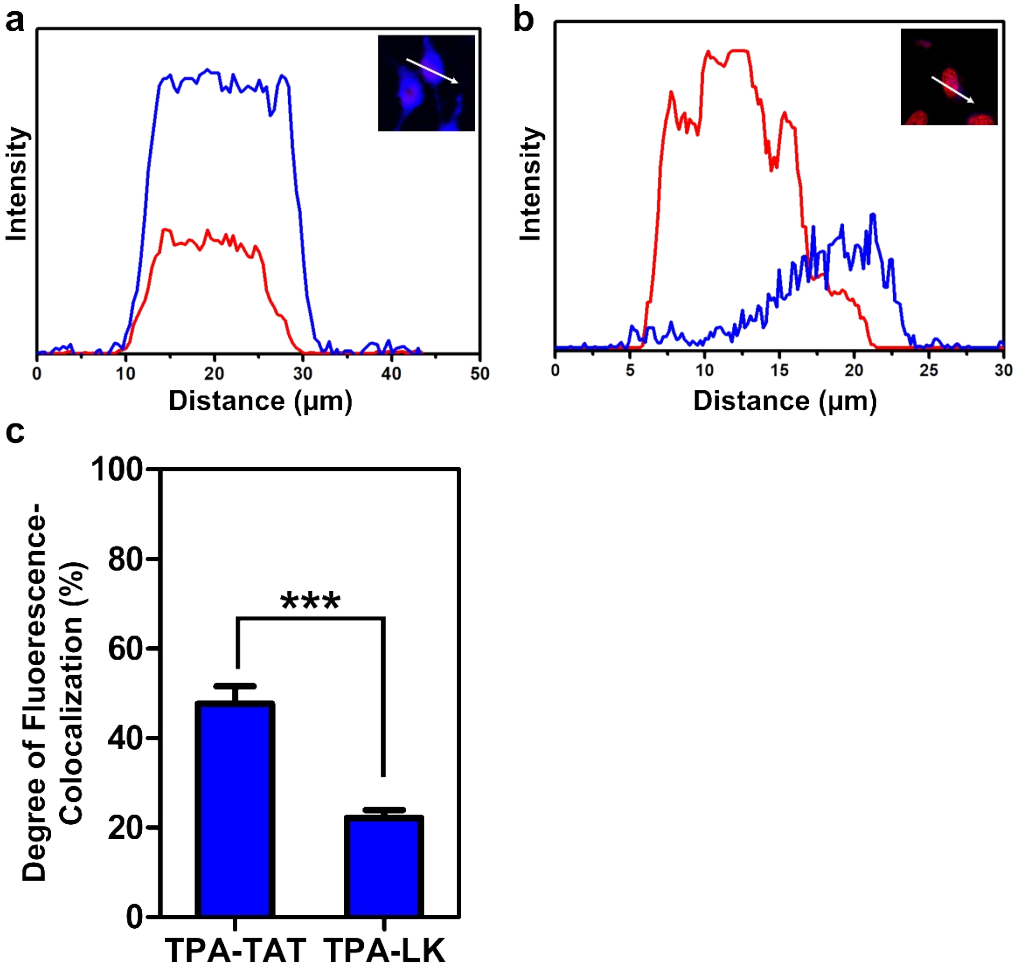


Fig. S18 Localization of TPA-labeled peptides in *HeLa* cells. Line profiles indicating the fluorescence distribution on *HeLa* cells treated with (a) TPA-TAT and (b) TPA-LK. The blue lines indicate the intensity profiles of blue fluorescence (390-465 nm) from TPA and the red lines indicate those of red fluorescnece (560 nm) from SYTO 59 through the white arrow lines in the CLSM images of cells. (c) Comparion of the colocalization ratio of blue fluorescence with red fluoresecence in *HeLa* cells. The colocalization ratio was calculated from over 500 points in a CLSM image of *HeLa* cells by the Imaris microscopy analysis software. Total four images (over 2,000 points) were analyzed and the values were averaged. Each bar represents the average ± S.D. (n = 4). The *** mark means $p < 0.0001$ through the Student's t-test.

10. CLSM Images of the DNFC-Stained Cells and Related Analyses

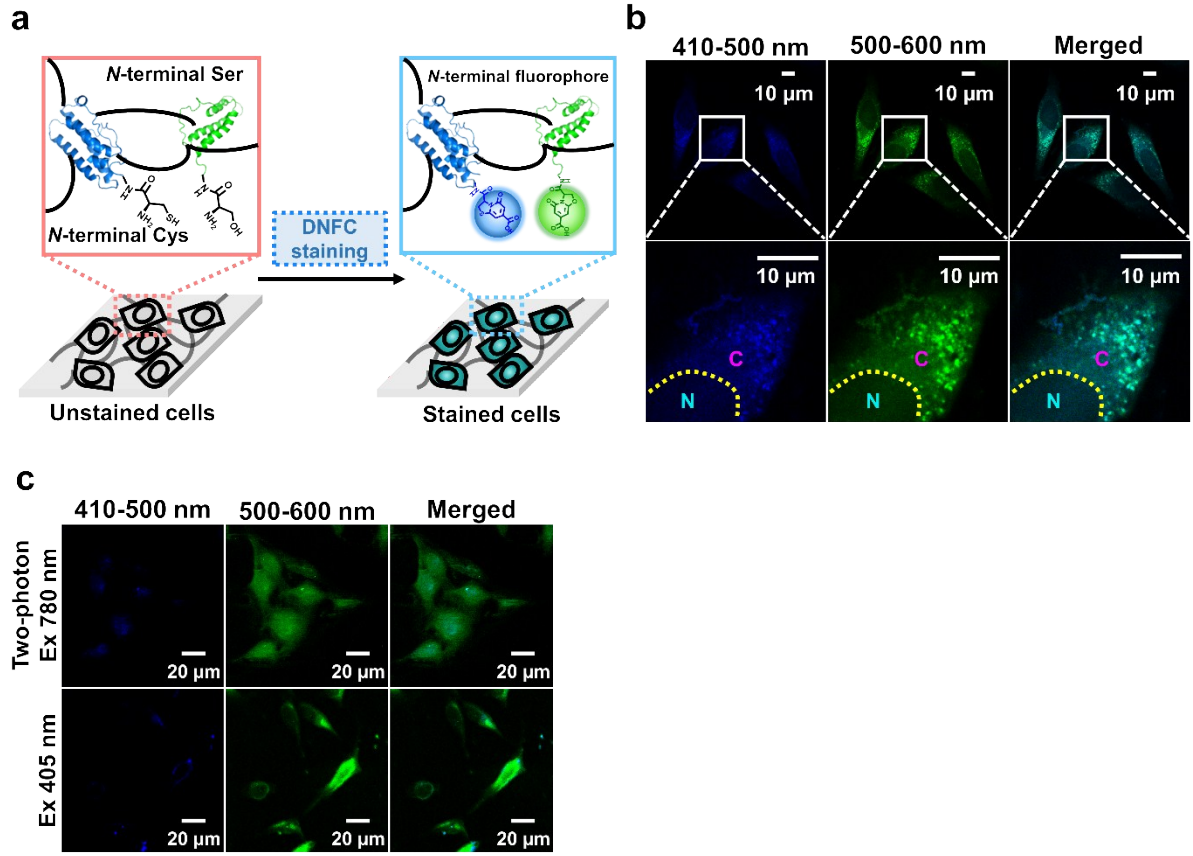


Fig. S19 Staining of fixed *HeLa* cells by the DNFC method. (a) Simplified scheme of DNFC staining on cells. (b) CLSM images of the DNFC-stained *HeLa* cells at the focal plane near the nucleus (upper panel) and the magnified CLSM images of the white squares (lower panel). (c) CLSM images of the DNFC-stained *HeLa* cells using different laser sources. The upper and lower panel images were obtained by excitation with a two-photon laser (2 mW) at 780 nm and a single-photon laser (5 mW) at 405 nm, respectively. Without indication, the DNFC staining was performed in DMSO/H_2O and the CLSM images were obtained with a 405 nm-laser (5 mW).

11. CLSM Images of DNFC-Stained Human Tissues and Related Analyses

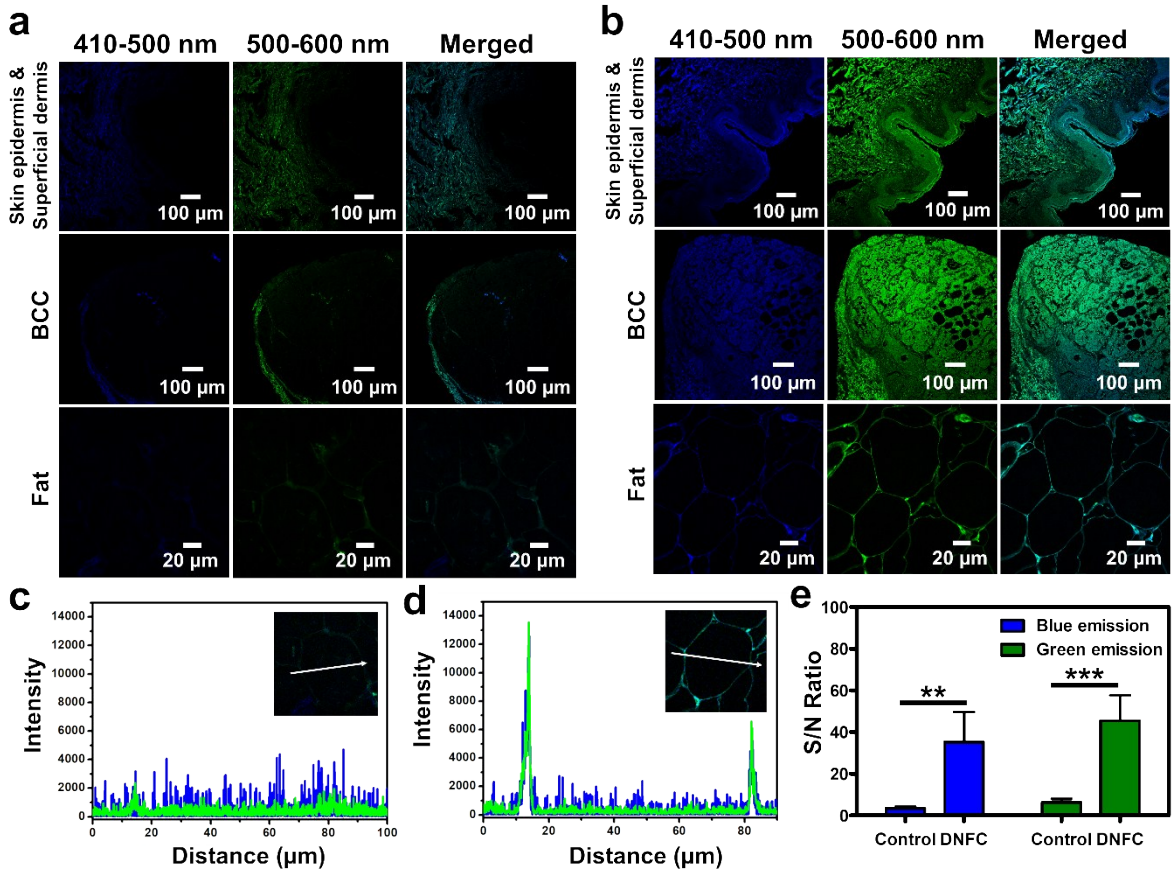


Fig. S20 Human tissues stained with the DNFC method. (a) CLSM images of a human skin/connective tissue and basal cell carcinoma (BCC) without the DNFC staining. The weak autofluorescence was observed in the image. (b) CLSM images of the DNFC-stained human skin tissue/connective tissue and BCC. Line profiles indicating the distribution of the blue (blue line) and green (green line) fluorescence in the fat tissue (c) without DNFC staining and (d) with DNFC staining through the white arrow line. (e) Comparison of the signal to noise (*S/N*) ratios of the fat tissues without DNFC staining (control; autofluorescence) and with DNFC staining. The fluorescence intensities of signals and noises were analyzed by using ImageJ software. Four different tissue samples (n = 4) were used for the comparison. Total 100 points were compared for blue fluorescence emission and green fluorescence emission, respectively. Each bar represents the average ± S.D. The ** and *** marks mean p < 0.01 and p< 0.001, and respectively (Student's *t*-test). All CLSM images were obtained under excitation at 405 nm. Blue (410-500 nm) and green (500-600 nm) channel images were obtained with laser power of 5 mW.

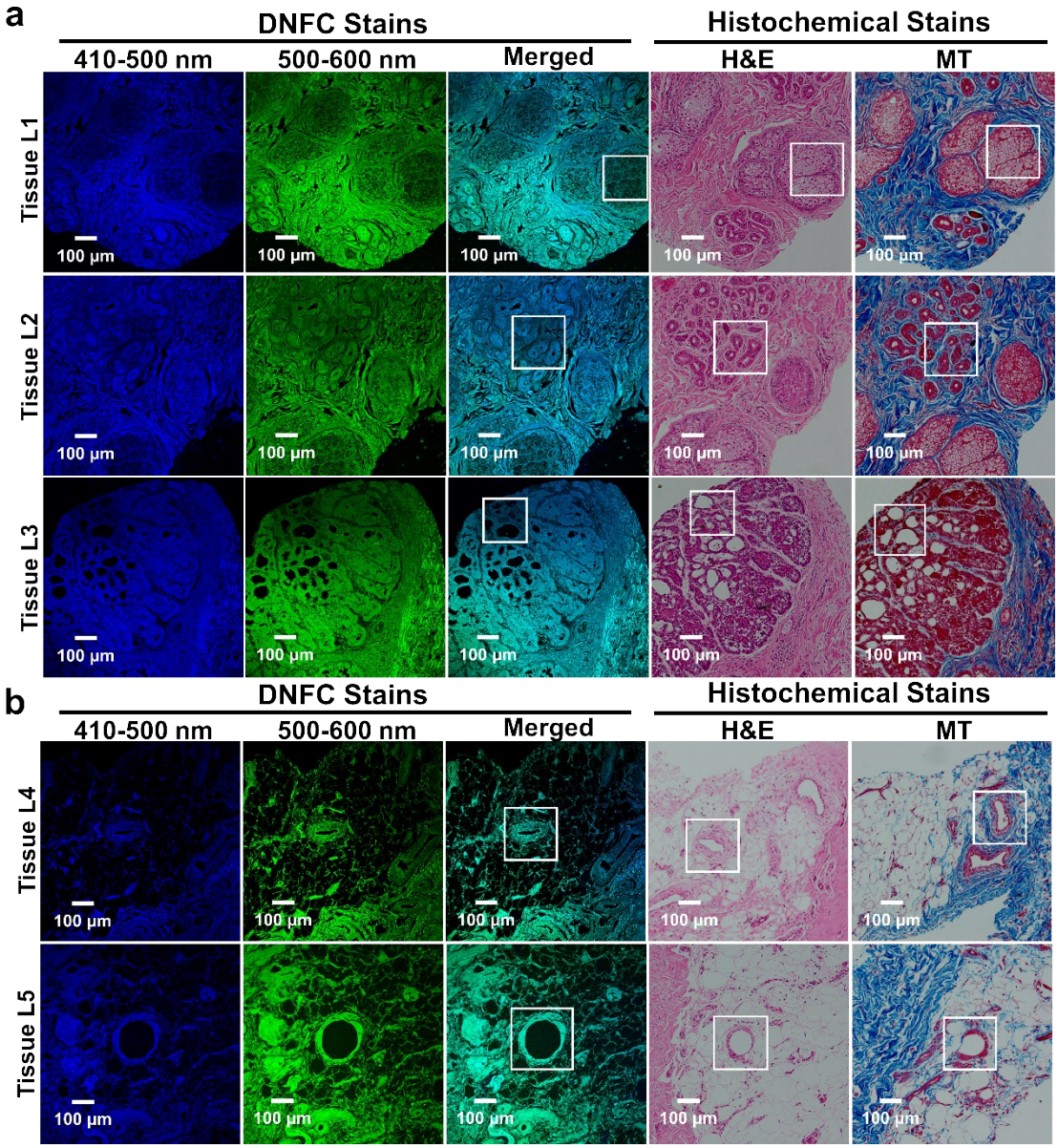


Fig. S21 Human tissues stained with the DNFC method. (a) CLSM images and light microscopic features of various locations substructures in human tissues from a patient with basal cell carcinoma stained with DNFC, H&E, and MT methods at a low magnification. White squares of Tissue L1, L2, L3 images indicate sebaceous glands, eccrine glands, an arteriole, basal cell carcinoma. (b) CLSM images of the DNFC-stained human skin/connective tissue samples. White squares of Tissue L4 and L5 images indicate an arteriole and histiocytes surrounding an adipocyte in fat necrosis. All CLSM images were obtained under excitation at 405 nm. Blue (410-500 nm) and green (500-600 nm) channel images were obtained with laser power of 5 mW.

Table S1 Summary of DNFC-staining characteristics of cells and tissues in this research.

	Name & Origin	Appearance	Staining characteristics
Cells (Fig. 2)	*HeLa* (human cervical cancer)	Polar	Fluorescence preferentially developed in the cytoplasm (ER) rather than in the nuclei green intensity > blue intensity
	HEK293T (human embryonic kidney)	Multipolar	Fluorescence preferentially developed in the cytoplasm rather than in the nuclei Lower fluorescence intensity than other cells green intensity > blue intensity
	THP-1M (human monocyte)	Irregular spherical	Much stronger blue fluorescence than other cells
	NIH3T3 (house fibroblast)	Multipolar	Fluorescence preferentially developed in the cytoplasm rather than in the nuclei green intensity > blue intensity
Human tissues (Fig.3)	Skin & subcutaneous fat (keloid of earlobe; Fig. 3ab)	Epidermis (stratum corneum and stratum spinosum layers) Dermis (collagen and elastic fibre bundle) Subcutaneous fat (adipocyte)	Keratin layers (stratum corneum), extracellular matrix of keratinocytes (stratum spinosum), fibre bundles (dermis) and extracellular matrix/cytoplasm of adipocytes (subcutaneous fat) strongly stained No staining in the lipid droplets
	Basal cell carcinoma (facial skin, Fig. 3c)	Subcutaneous substructures (sebaceous and eccrine glands) Tumor cells and stroma	Mesh-like structure (sebaceous gland), bright dot-like structures (eccrine gland) and tumor cells strongly stained No staining in the stroma
	Dermato-fibrosarcoma protuberance (back, Fig. 3d)	Arteriole (thick wall and lumen) Fat necrosis (histocytes and multinucleated giant cells)	Thick arteriole wall, histocytes and and giant cells strongly stained No staining in fat

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Author Contributions

D.J. carried out most experiments and analyses. D.C. performed analyses of DNFC-stained cells and tissues. C.S., Y.K., S.K. and D.K. contributed to the synthesis and analysis of the pyridone-based compounds. J.J. and S.H.N. performed cell experiments. M.S.C., J.U.P, and Y.L. designed and supervised most experiments and analyses. All authors discussed the results. D.J. prepared the first draft and all authors participated in the preparation of the final manuscript.