Supporting Information

Sequential ordering among multicolor fluorophores for protein labeling facility via aggregation–elimination based β–lactam probes

Kalyan K. Sadhu, Shin Mizukami, Shuji Watanabe and Kazuya Kikuchi*

Division of Advanced Science and Biotechnology, Graduate School of Engineering, Immunology Frontier Research Center Osaka University,
2-1 Yamadaoka, Suita, Osaka 565-0871, Japan

* To whom correspondence should be addressed: kkikuchi@mls.eng.osaka-u.ac.jp

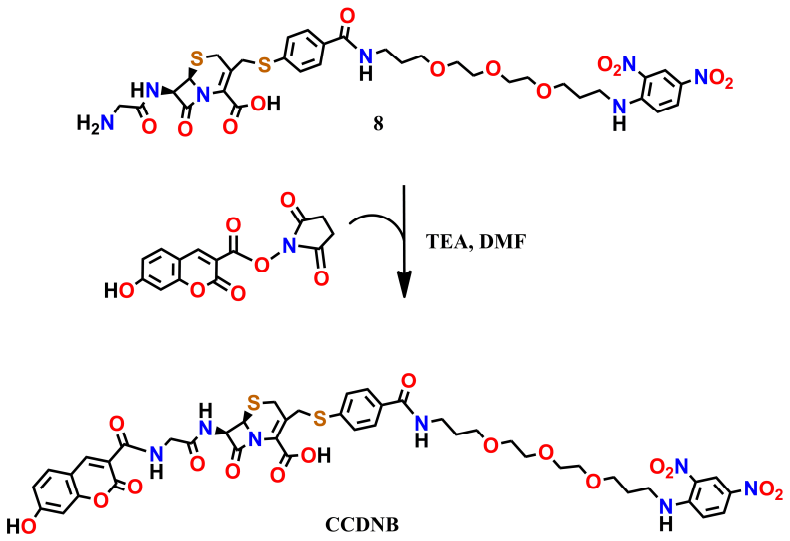
CONTENTS:

1. Materials and Instruments

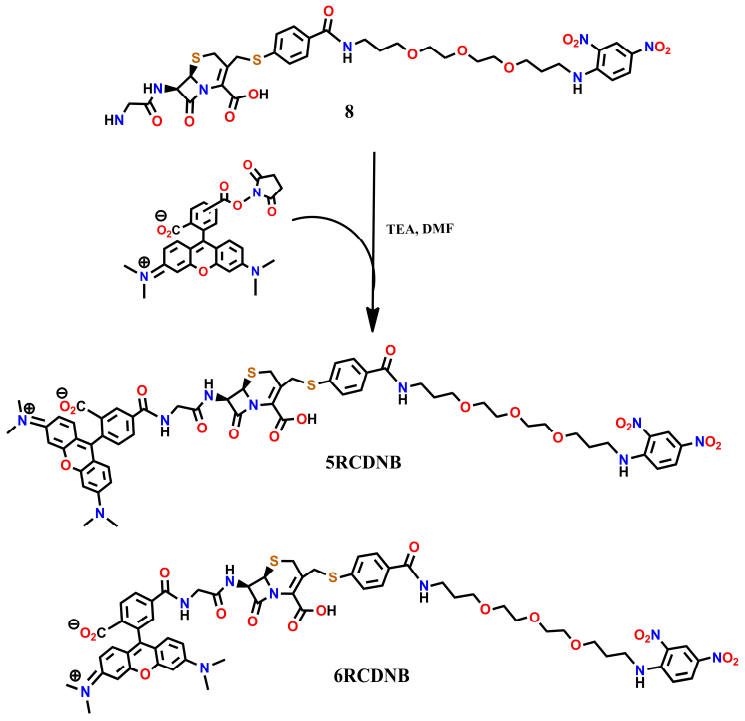
General Chemicals and biological reagent were similar to our previous report.[S1] 7-Amino-3-chloromethyl-3-cephem-4-carboxylic acid *p*-methoxybenzyl ester hydrochloride (ACLE·HCl) was obtained from Otsuka Chemical Co. Ltd.

NMR spectra were recorded on a JEOL JNM-AL400 instrument at 400 MHz for ^{1}H and at 100.4 MHz for ^{13}C NMR, using tetramethylsilane as an internal standard. Mass spectra were measured on a JEOL JMS-700 for FAB. UV-Visible absorbance spectra were measured using a Shimadzu UV1650PC spectrometer. Fluorescence spectra were measured using a Hitachi F4500 spectrometer. Slit width was 2.5 nm for both excitation and emission, and the photomultiplier voltage was 700 V. Fluorescence microscopic images were recorded using a confocal laser scanning microscope (Olympus, FLUOVIEW FV10i) equipped with a ×60 lens and the appropiate emission filters for coumarin and fluorescein probes. The excitation wavelengths were 405 nm for coumarin derivative and 473 nm for fluorescein derivative. The emission filter sets used were 420-460 nm for coumarin derivative and 490-540 nm for fluorescein derivative. FV10-ASW2.1 imaging software (Olympus) was used for imaging and data analysis. Silica gel column chromatography was performed using BW-300 (Fuji Silysia Chemical Ltd.). Fluorescence images of SDS-PAGE were visualized using an AE-6935B VISIRAYS-B (ATTO).

2. Syntheses of Compounds (Scheme S1 and S2)



Scheme S1. Synthetic route to **CCDNB**.



Scheme S2. Synthetic route to **5RCDNB** and **6RCDNB**.

3. Preparation of Proteins

All procedures were similar to our earlier experiments.[S1]

4. Labeling Experimental Procedures

HPLC Analysis. Preparative HPLC was performed with an Inertsil ODS-3 (10.0 mm × 250 mm) column (GL Sciences Inc.) using an HPLC system that comprised a pump (PU-2087, JASCO) and a detector (UV-2075, JASCO).

Detection of protein labeling by SDS-PAGE. WT TEM-1 or BL (20 μM) was added to a solution of each probe (30 μM) in 100 mM HEPES buffer (pH 7.4) at 25 °C. After 30 min, labeled protein was solubilized in 2 × SDS gel loading buffer (100 mM Tris-HCl buffer (pH 6.8), 2.5% SDS, 20% glycerol, and 10% mercaptoethanol). Fluorescence images of the gels were then captured using a digital camera (Nikon COOLPIX P6000). The gels were stained with Coomassie Brilliant Blue (CBB), and images of the stained gels were captured (Figure 1, Figure 5 and Figure S7-S9).

Fluorescence quantum yield. The fluorescent probes were dissolved in DMSO to obtain 10 mM stock solution; this solution was then diluted to the desired final concentrations by using buffer solutions. The fluorescence quantum yield of the probe was estimated in 100 mM HEPES buffer (pH 7.4) using different fluorescence standard for different probes, quinine bisulfate in 50 mM H_2SO_4 (ϕ = 0.55)[S2] and rhodamine B in ethanol (ϕ = 0.49)[S3] as references for comparison.

Fluorometric assay. Enzyme assays for the quantification of WT TEM-1 and BL were performed in 100 mM HEPES buffer (pH 7.4) at 25 ˚C. A total of 1.5 µL purified enzyme (20 µM) was added to 300 µL of buffer containing probes (1 µM for **CCDNB** and 0.5 µM for **5RCDNB** and **6RCDNB**). For labeling efficiency competition studies the concentration of the probes were 1 µM in each case. The samples were excited at 407 nm for coumarin and 558 nm for rhodamine, and the fluorescence intensity enhancement were monitored at 450 nm for coumarin and 575 nm for rhodamine (Figure 4, S10–S11).

Labeling of cell surface protein (BL-EGFR) with probes. HEK293T cells maintained in 10% FBS in DMEM (Invitrogen) at 37 ºC under 5% CO_2 were transfected with the pcDNA3.1(+)-BL-EGFR plasmids using Lipofectamine 2000 (Invitrogen). After 5–6 h, the culture medium was replaced with DMEM (without phenol red), and the cells were incubated at 37 ºC for 24 h. Then, the cells were washed three times with DMEM and incubated with 5 µM of the probes for 30 min in a CO_2 incubator. After the culture medium was replaced, microscopic images were acquired. FV10-ASW2.1 imaging software (Olympus) was used for imaging and data analysis.

5. Supporting Figures

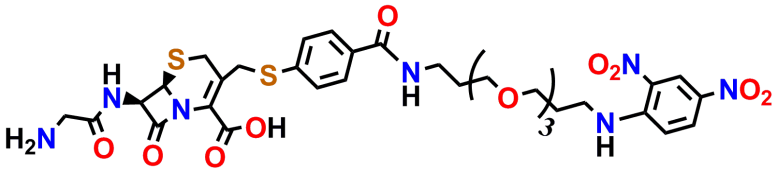


Figure S1. Molecular structure of compound **8**

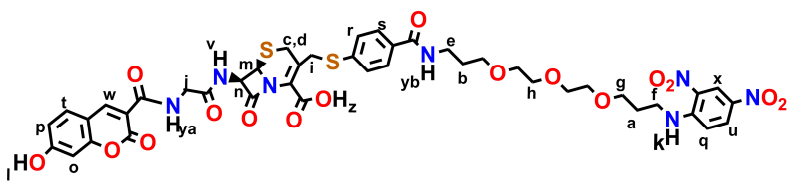


Figure S2. Chemical structure of **CCDNB** for NMR analysis.

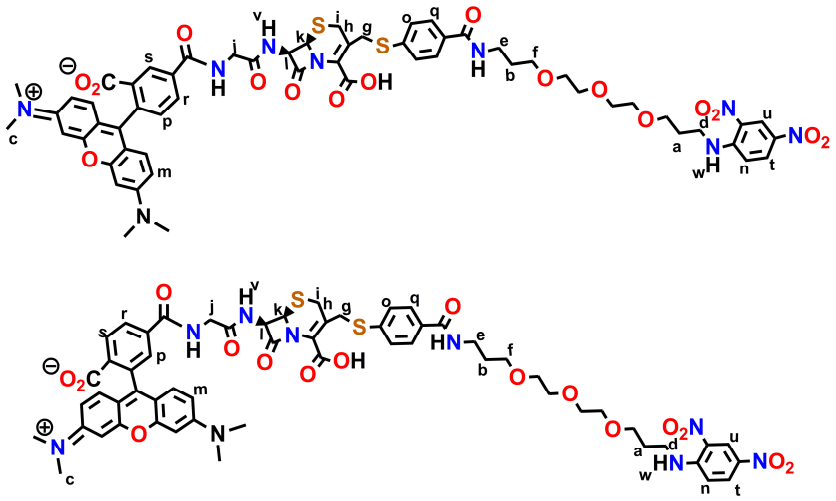


Figure S3. Chemical structure of **5RCDNB** (above) and **6RCDNB** (bottom) for NMR analysis.

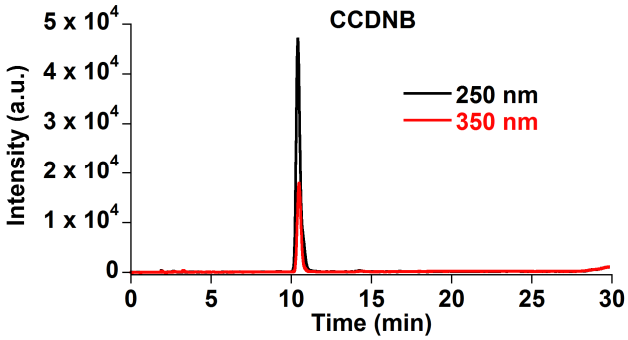


Figure S4. Analytical HPLC data of **CCDNB** for purity assay with the following eluent condition

Time (min)	Percentage of eluent A	Percentage of eluent B
0	70	30
25	60	40
30	10	90

eluent A: 0.1% formic acid in water
eluent B: 0.1% formic acid in acetonitrile

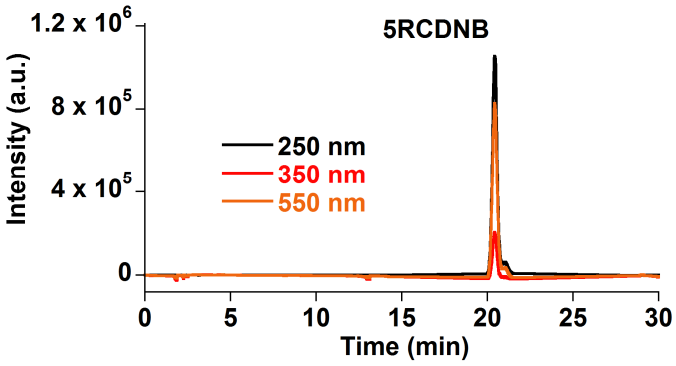


Figure S5. Analytical HPLC data of **5RCDNB** for purity assay with the following eluent condition

Time (min)	Percentage of eluent A	Percentage of eluent B
0	70	30
25	60	40
30	10	90

eluent A: 0.1% formic acid in water
eluent B: 0.1% formic acid in acetonitrile

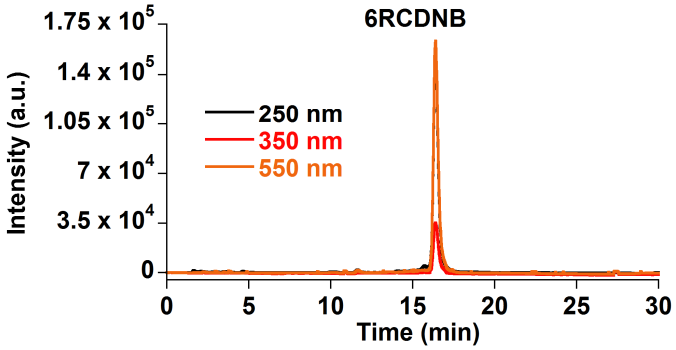


Figure S6. Analytical HPLC data of **6RCDNB** for purity assay with the following eluent condition

Time (min)	Percentage of eluent A	Percentage of eluent B
0	70	30
25	60	40
30	10	90

eluent A: 0.1% formic acid in water
eluent B: 0.1% formic acid in acetonitrile

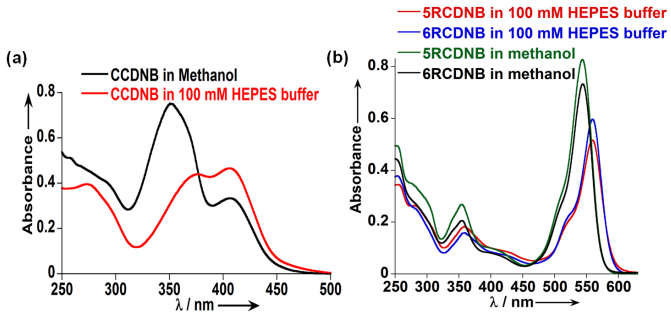


Figure S7. Absorption spectra of the probes (a) **CCNB** (conc. of **CCDNB** 10 μM) and (b) **5RCDNB** and **6RCDNB** (conc. of each solution 5 μM) in methanol and 100 mM HEPES buffer.

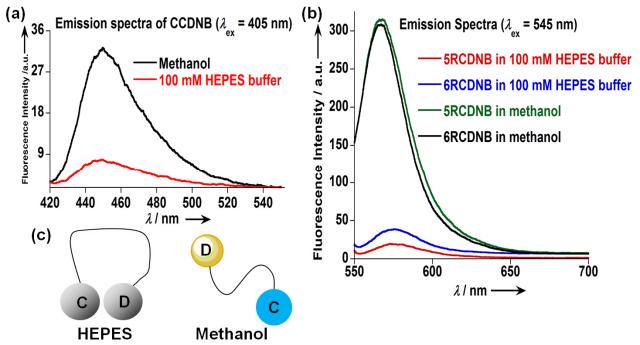


Figure S8. Emission spectra of the probes (a) **CCNB** (conc. of **CCDNB** 1.0 μM), (b) **5RCDNB** and **6RCDNB** (conc. of each solution 0.5 μM) in methanol and 100 mM HEPES buffer and (c) cartoon diagram representing the favorable aggregation phenomenon between fluorophore and quencher in different medium.

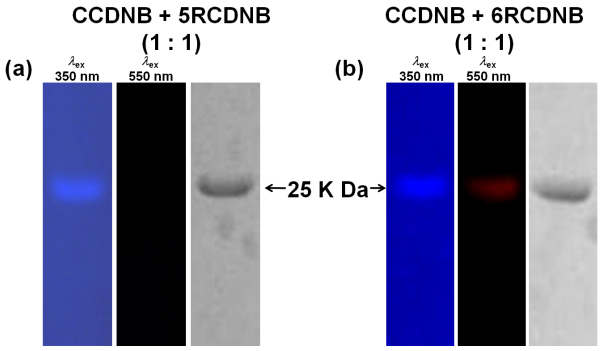


Figure S9. Fluorescence (left and middle) and CBB-stained (right) gel images of BL-tag incubated with (a) equimolar mixture of **CCDNB** and **5RCDNB** and (b) equimolar mixture of **CCDNB** and **6RCDNB**.

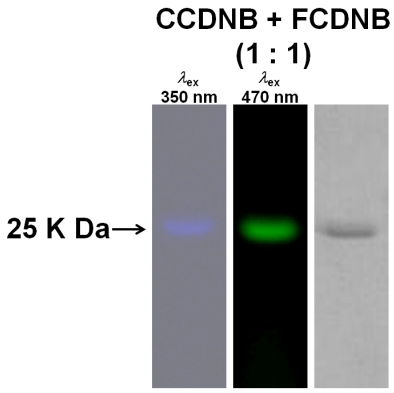


Figure S10. Fluorescence (left and middle) and CBB-stained (right) gel images of BL-tag incubated with equimolar mixture of **CCDNB** and **FCDNB**.

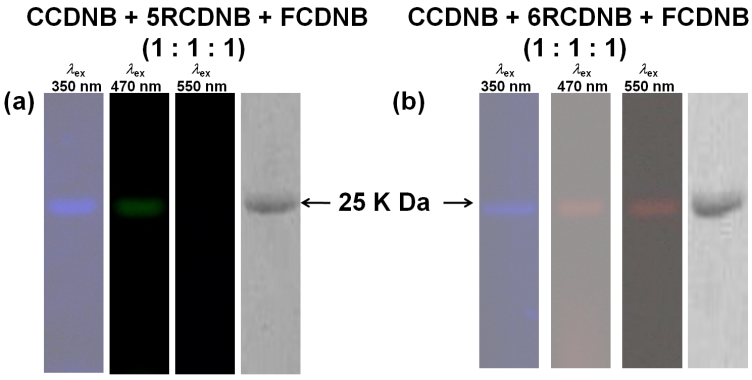


Figure S11. Fluorescence (left and middle) and CBB-stained (right) gel images of BL-tag incubated (a) equimolar mixture of **CCDNB, 5RCDNB** and **FCDNB** and (b) equimolar mixture of **CCDNB, 6RCDNB** and **FCDNB**.

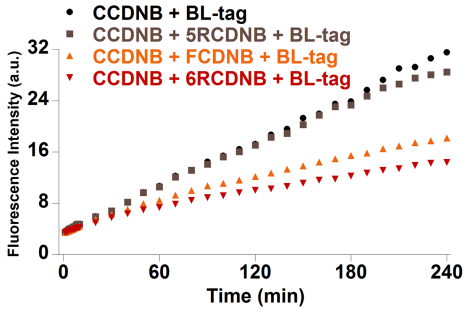


Figure S12. Time-dependent emission enhancement (λ_{ex} = 407 nm and λ_{em} = 450 nm) of **CCDNB** (conc. of **CCDNB** 1.0 μM) in presence of BL-tag with equimolar amount of other probes in 100 mM HEPES buffer (pH 7.4) containing 0.1% DMSO at 25 °C.

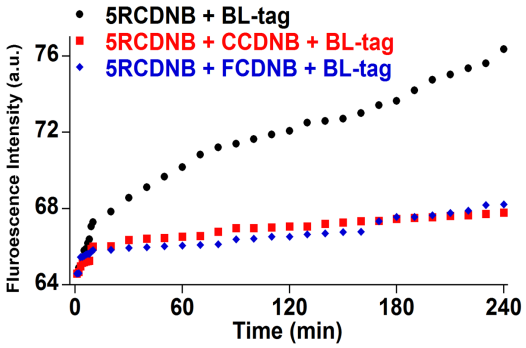


Figure S13. Time-dependent emission enhancement (λ_{ex} = 558 nm and λ_{em} = 575 nm) of **5RCDNB** (conc. of **5RCDNB** 1.0 μM) in presence of BL-tag with equimolar amount of fluorescein and coumarin probes in 100 mM HEPES buffer (pH 7.4) containing 0.1% DMSO at 25 °C.

6. Supporting References

S1. S. Mizukami, S. Watanabe, Y. Hori and K. Kikuchi, *J. Am. Chem. Soc.,* **2009**, *131*, 5016-5017.
S2. R. W. Dawson, W. M. Windsor, *J. Phys. Chem.* **1968**, *72*, 3251–3260.
S3. K. G. Casey, E. L. Quitevis, *J. Phys. Chem.* **1988**, *92*, 6590–6594.