Supporting Information for:

Site-specific covalent labeling of His-tag fused proteins with *N*-acyl-*N*-alkyl sulfonamide reagent

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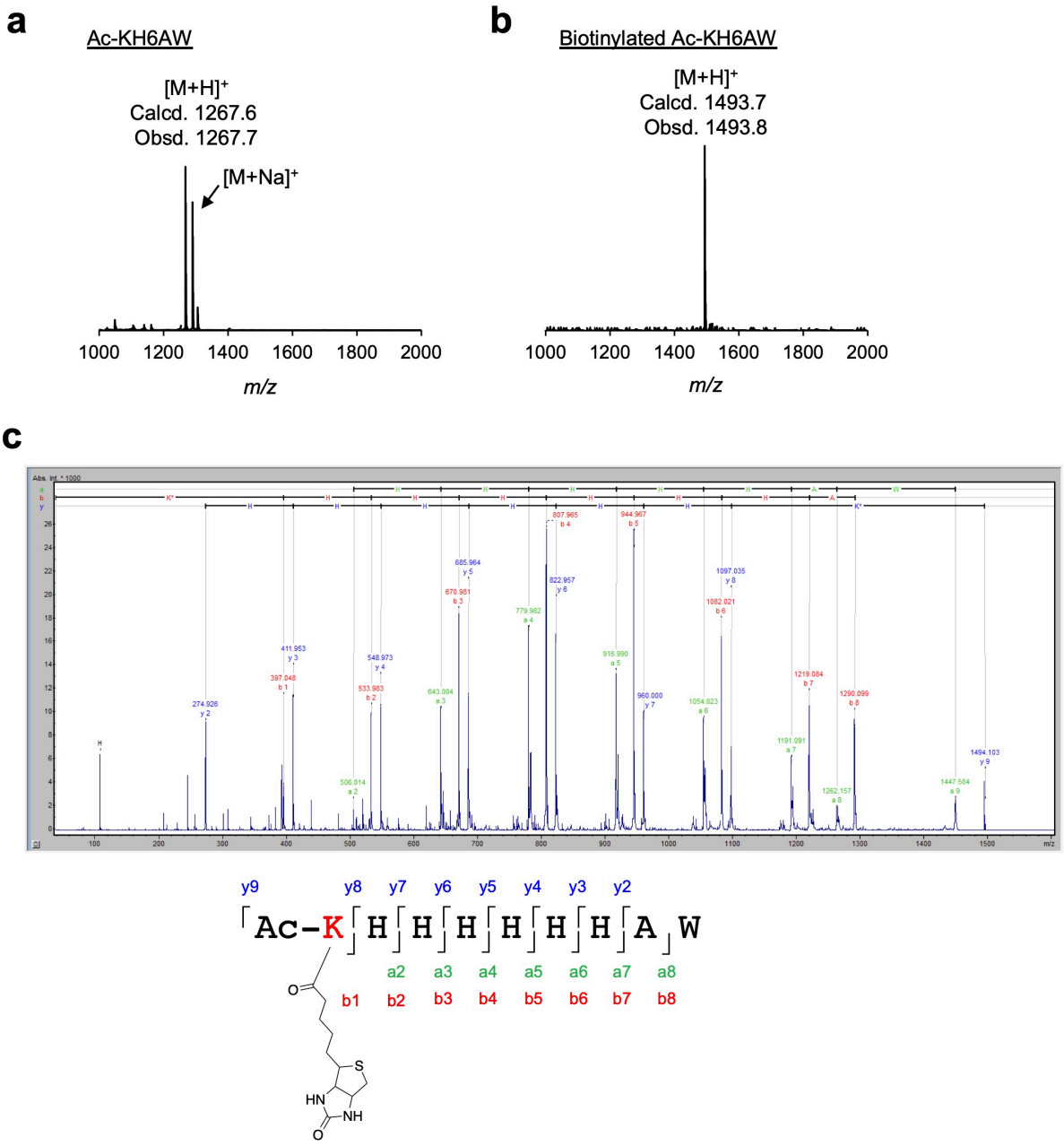

Figure S1 Mass spectral analysis of modification of Ac-KH6AW peptide with compound **1**. (a, b) MALDI-TOF MS spectrum of the peptide (a) before and (b) after labeling. Reaction condition: 1 μM peptide, 4 μM **1**, 8 μM $NiCl_2$, 37 °C, PBS, pH 7.4, 1 h. (c) MALDI-TOF/TOF MS analysis of the peptide labeled with **1**. This data clearly indicated that the biotin moiety is introduced at the ε-amino group of lysine residue in Ac-KH6AW peptide.

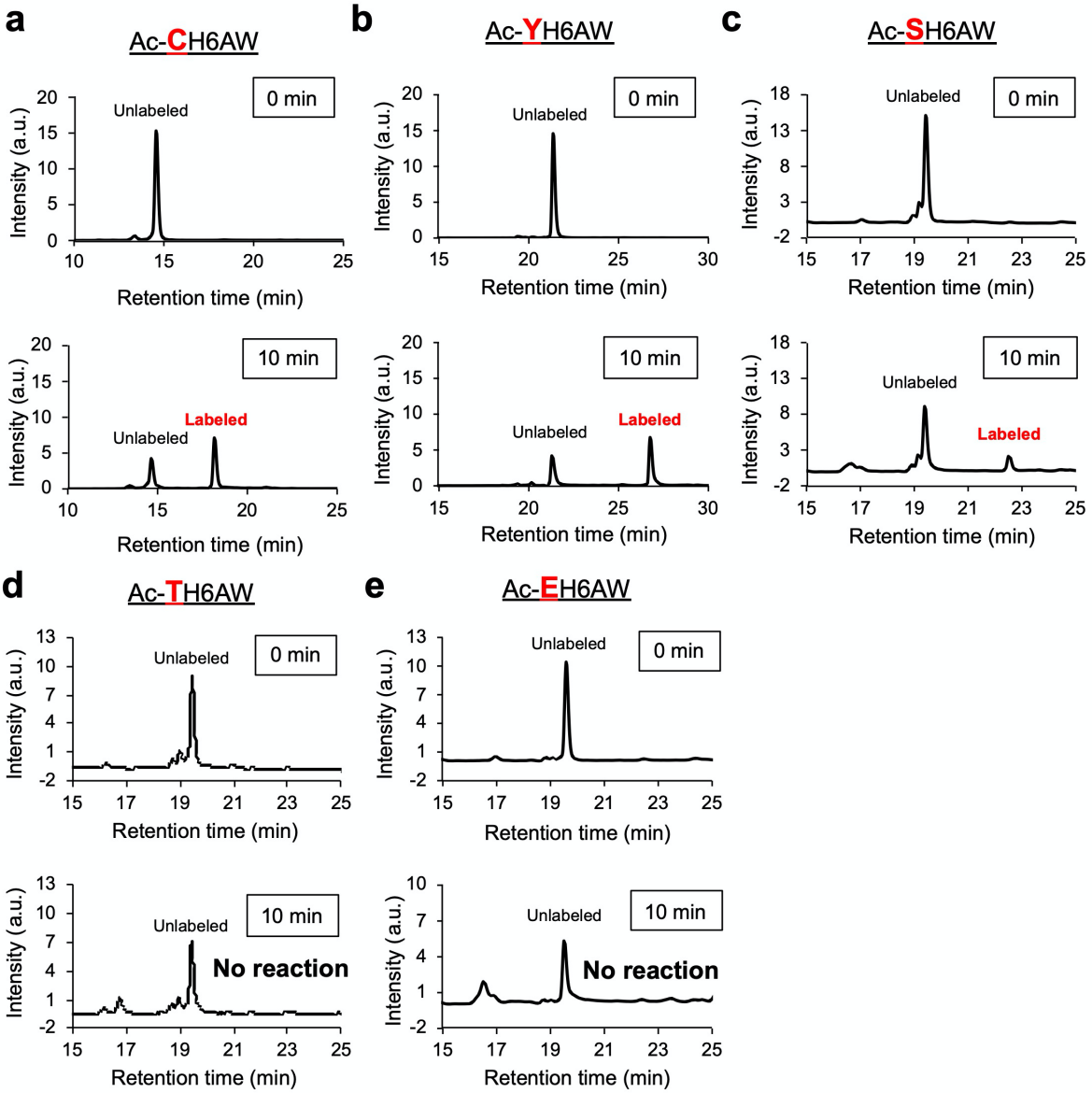

Figure S2 HPLC analysis of the reaction between Ac-KXH6AW and **1** at 10 min. X= (a) C, (b) Y, (c) S, (d) T, (e) E. The chromatograms were recorded with UV absorption at 280 nm for (c, d, e) and with fluorescence of tryptophan for (a, b). Gradients; A (CH_3CN containing 0.1% TFA) : B (H_2O containing 0.1% TFA) = 5 : 95 (0 min) to 35 : 65 (30 min), or 10 : 90 (0 min) to 35 : 65 (25 min). The yields were shown in Figure 3c.

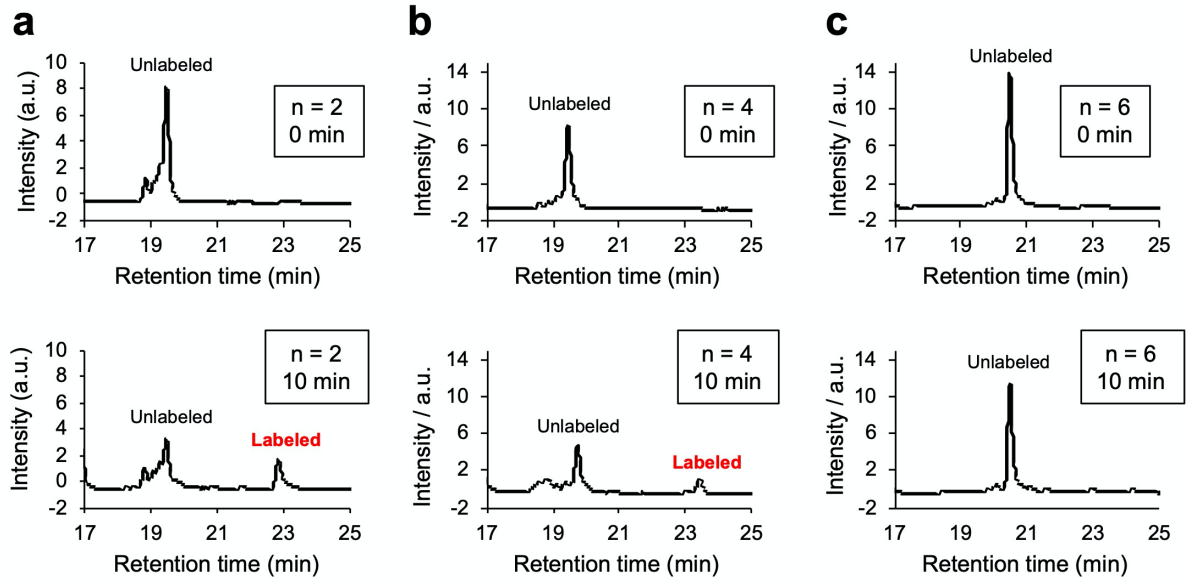

Figure S3 HPLC analysis of the reaction between Ac-KA$_n$H6AW and **1** at 10 min. (a) n = 2, (b) n = 4, (c) n = 6. The chromatograms were recorded with UV absorption at 280 nm. Gradients; A (CH_3CN containing 0.1% TFA) : B (H_2O containing 0.1% TFA) = 5 : 95 (0 min) to 35 : 65 (30 min). The yields were shown in Figure 3d.

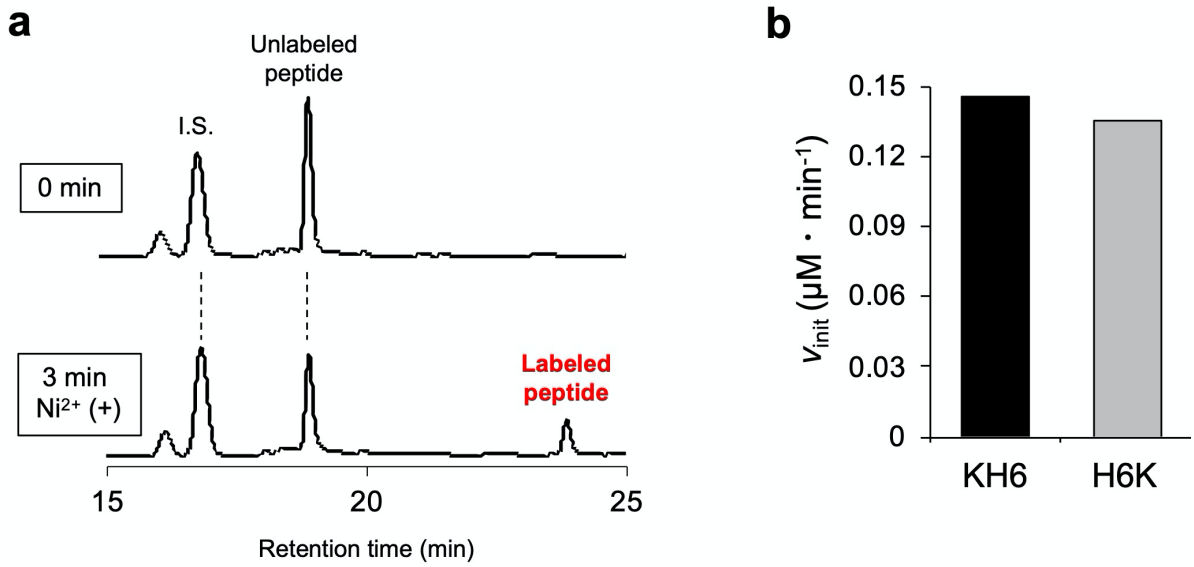

Figure S4 Kinetic analysis of the reaction between Ac-H6KAW and **1**. (a) HPLC analysis of the labeling reaction between Ac-H6KAW peptide (1 μM) and **1** (4 μM) in the presence of Ni^{2+} (8 μM) at 3 min. (b) Initial rates ($\mu M \cdot min^{-1}$) of labeling with Ac-KH6AW peptide (KH6, black) and Ac-H6KAW peptide (H6K, grey). This data shows that the reaction kinetics does not significantly alter between these sequences.

a

b

MGSKHHHHHH SSGLVPRGSG MMVSKGEELF TGVVPILVEL
DGDVNGHKFS VSGEGEGDAT YGKLTLKFIC TTGKLPVPWP
TLVTTLTYGV QCFSRYPDHM KQHDFFKSAM PEGYVQERTI
FFKDDGNYKT RAEVKFEGDT LVNRIELKGI DFKEDGNILG
HKLEYNYNSH NVYIMADKQK NGIKVNFKIR HNIEDGSVQL
ADHYQQNTPI GDGPVLLPDN HYLSTQSALS KDPNEKRDHM
VLLEFVTAAG ITLGMDELYK EFELRRQAGSG

Figure S5 (a) Primary structure and (b) sequence of KH6-tagged EGFP used in this work. Red, KH6 tag; Magenta, Thrombin site; Green, EGFP.

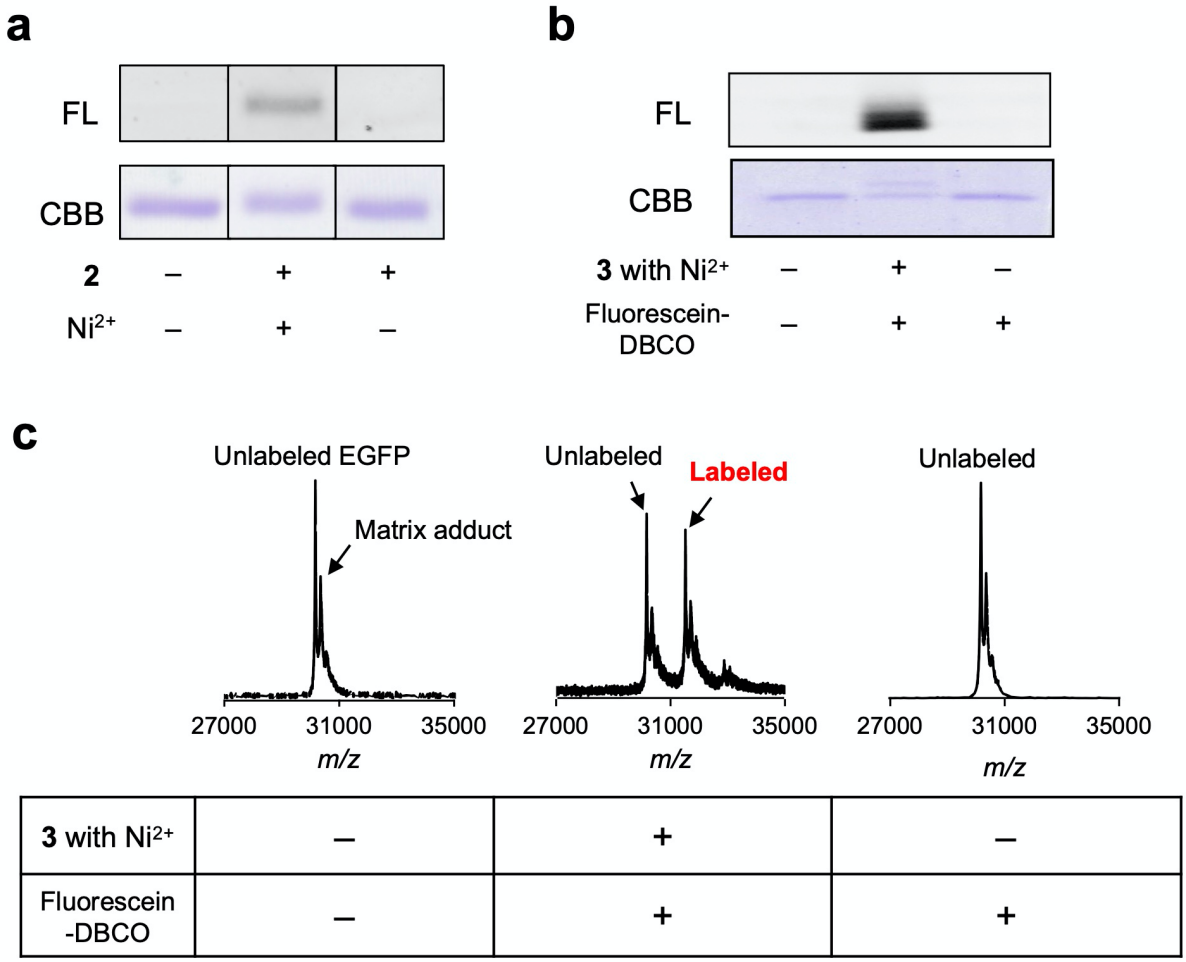

Figure S6 KH6-EGFP labeling with **2** and **3**. (a, b) SDS-PAGE analysis of the labeling reaction with (a) **2** and (b) **3**. FL, in-gel fluorescence; CBB, Coomassie Brilliant Blue. Reaction conditions: In the case of the Dc labeling, 1 μM KH6-EGFP, 3 μM **2**, 6 μM $NiCl_2$, 37 °C, PBS, pH 7.4, 30 min. In the case of the azide labeling, 5 μM KH6-EGFP, 10 μM **3** reagent, 20 μM $NiCl_2$, 37 °C, PBS, pH 7.4, 30 min, followed by treatment with 50 μM Fluorescein-DBCO for 1 h. (c) MALDI-TOF MS analysis of the reaction in (b). Unlabeled KH6-EGFP, obsd *m/z*: 30171; Single-labeled KH6-EGFP, obsd *m/z*: 31519.

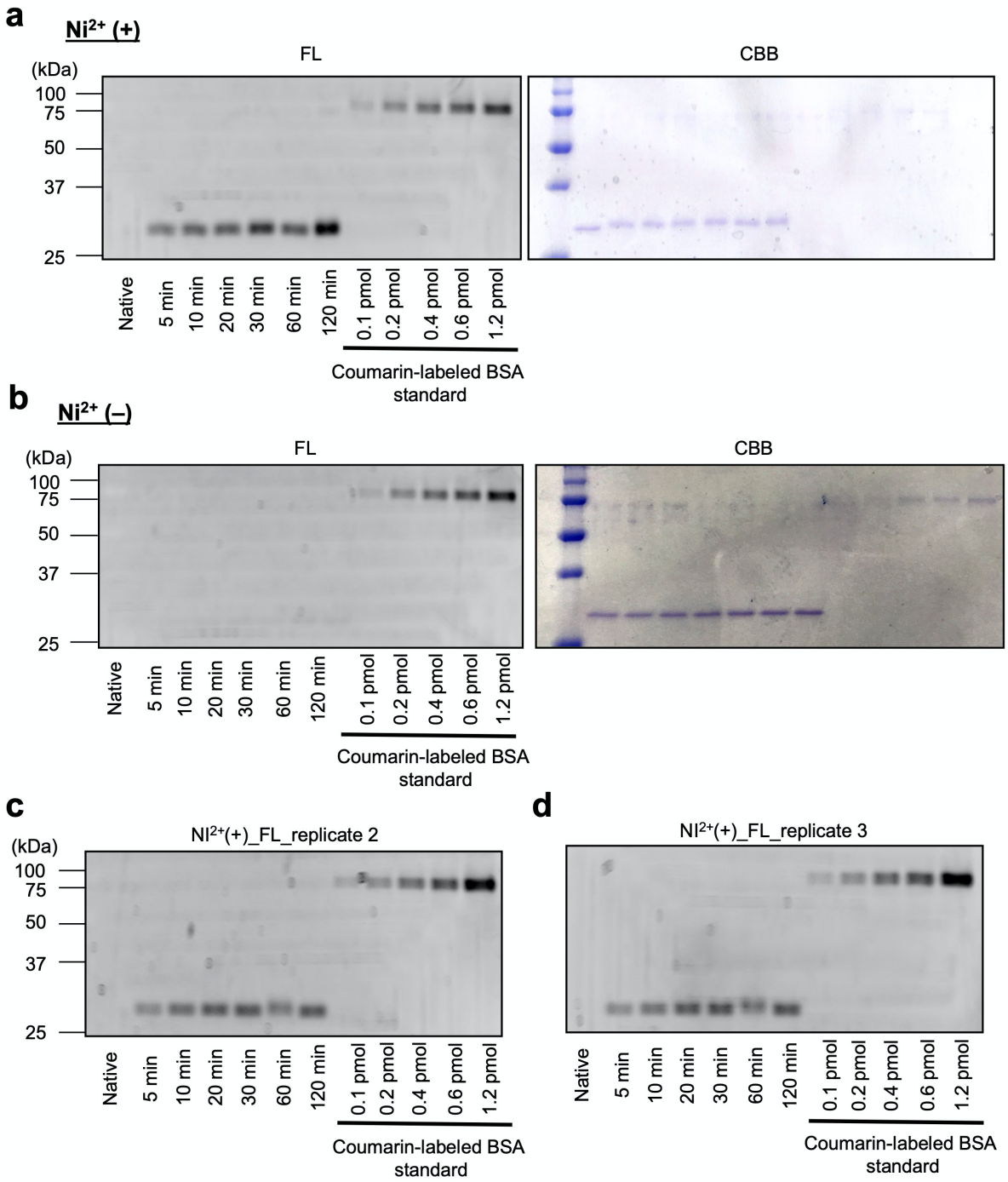

Figure S7 Time course analysis of the labeling of KH6-EGFP with **2**. (a, b) SDS-PAGE and in-gel fluorescence analysis of the labeling in the (a) presence and (b) absence of Ni^{2+} with a coumarin-modified bovine serum albumin (BSA) standard to determine the labeling yield. Reaction conditions: 1 µM KH6-EGFP, 3 µM **2**, 6 µM $NiCl_2$, 37 °C, PBS, pH 7.4, 0 – 120 min. Preparation of coumarin-modified BSA and calculation of labeling yields of samples are described in the method section. (c, d) Other two replicates of the time-course analysis.

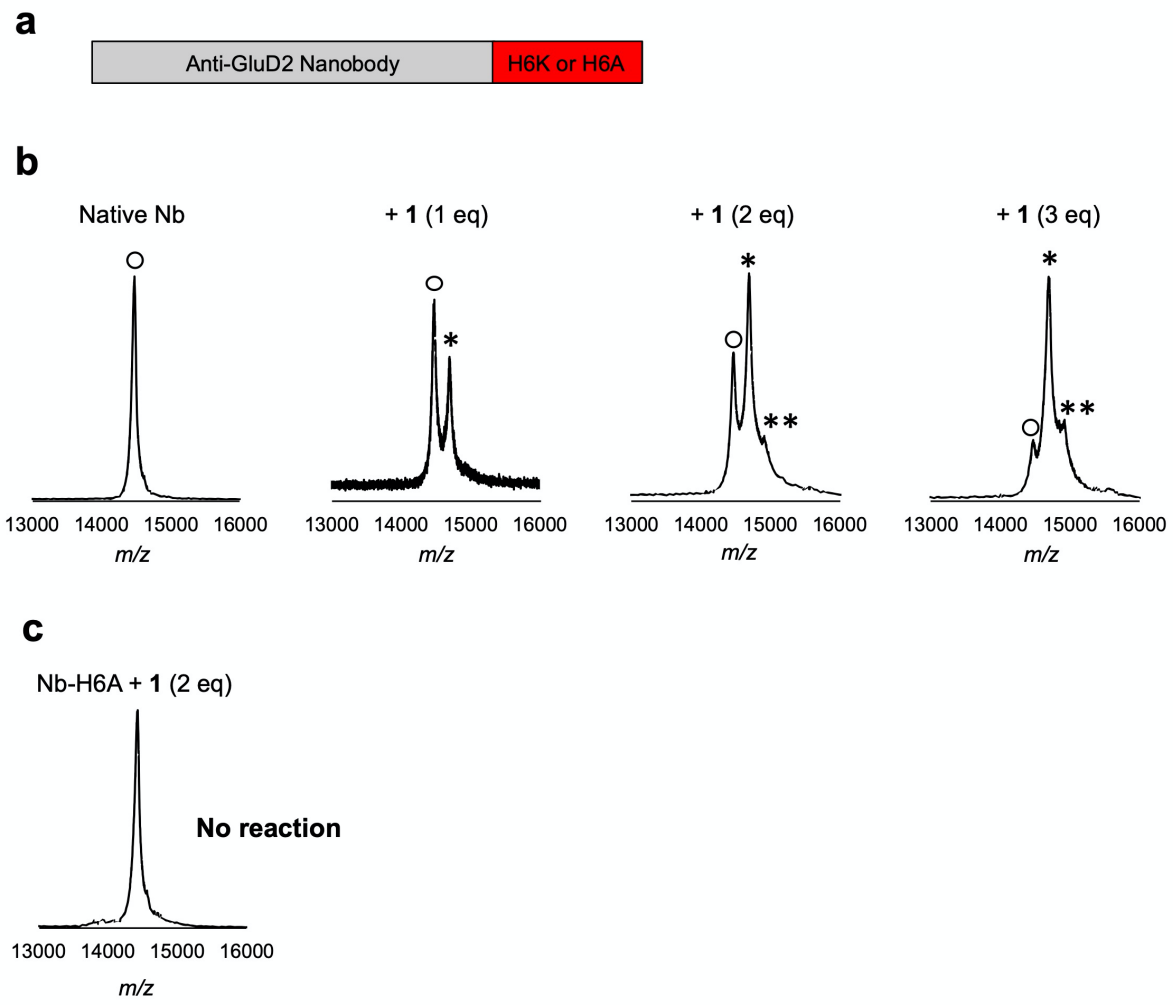

Figure S8 (a) Primary structure of anti-GluD2 nanobody tagged with H6K or H6A sequence at the C-terminus. (b, c) MALDI-TOF MS analysis of biotinylation of the (b) H6K- and (c) H6A-nanobody and with **1**. Reaction condition: 1 μM nanobody, 1–3 μM **1**, 2–6 μM $NiCl_2$, 37 °C, PBS, pH 7.4, 1 h. ○, native nanobody (obsd *m/z*: 14476); *, single-labeled nanobody (obsd *m/z*: 14702), **, double-labeled nanobody (obsd *m/z*: 14928).

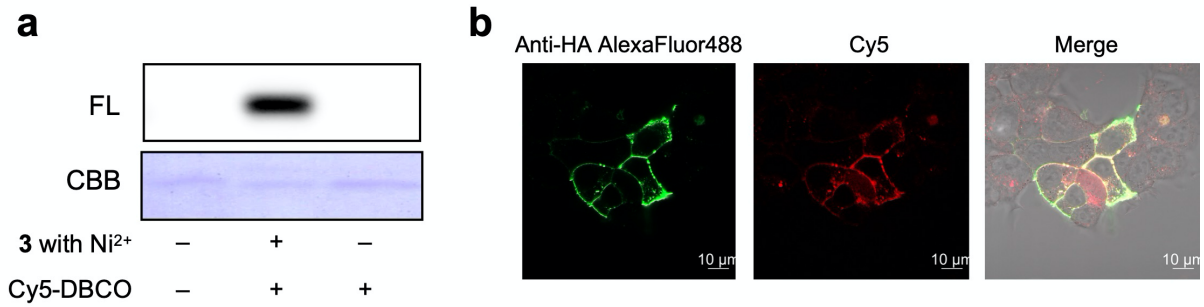

Figure S9 (a) SDS-PAGE analysis of the incorporation of Cy5 into the nanobody by **3** and Cy5-DBCO. FL, in-gel fluorescence; CBB, Coomassie Brilliant Blue. Reaction condition: the H6K-tagged nanobody (1 μM) was treated with **3** (1 μM) and $NiCl_2$ (2 μM) at 37 °C for 1 h, followed by incubation with Cy5-DBCO (10 μM) for 1 h. (b) Membrane surface GluD2 was visualized by treatment of an anti-HA antibody conjugated with AlexaFluor488 (green) and the Cy5-modified nanobody (red).

Experimental Section

General materials and methods for the biochemical/biological experiments

Unless otherwise noted, all proteins/enzymes and reagents were obtained from commercial suppliers (Sigma-Aldrich, Tokyo Chemical Industry (TCI), Fujifilm Wako Pure Chemical Corporation, Sasaki Chemical, Bio-Rad, Thermo Fisher Scientific, or Watanabe Chemical Industries) and used without further purification. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) spectra were recorded on an Autoflex III or UltrafleXtreme (Bruker Daltonics) using α-cyano-4-hydroxycinnamic acid (CHCA) or sinapinic acid (SA) as the matrix. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting were carried out using a Bio-Rad Mini-Protean III electrophoresis apparatus. Fluorescence gel images and chemical luminescent signals using Chemi-Lumi one (Nacalai Tesque) or ECL Prime (GE Healthcare) were acquired with a FUSION FX (Vilber). Reversed-phase HPLC (RP-HPLC) was carried out on a Hitachi Chromaster system equipped with a Chromaster 5430 diode array detector, a Chromaster 5440 fluorescence detector, and a YMC-Pack ODS-A column (5 μm, 250 × 4.6 mm) at a flow rate of 1.0 mL/min. UV detection was at 220 nm. All runs used linear gradients of acetonitrile containing 0.1% TFA (solvent A) and 0.1% aqueous TFA (solvent B).

Peptide labeling and HPLC analysis

2 μL of reagent (1.5 mM in DMSO) and 2 μL of $NiCl_2$ aq. (3 mM) was mixed in a tube and incubated at room temperature for 4 min. The mixture was added to a solution of peptide (1 μM) and benzene sulfonamide (8 μM, internal standard) in PBS (pH 7.4) and incubated at 37 °C. At each time point, the reaction was quenched by adding EDTA (final conc. 0.6 mM), and the solution was mixed with 100 μL of DMSO and injected to HPLC for analysis. The chromatograms were recorded with UV absorption at 220 or 280 nm, and tryptophan fluorescence (Ex: 280 nm/Em: 350 nm). Gradients; A (CH_3CN containing 0.1% TFA) : B (H_2O containing 0.1% TFA) = 5 : 95 (0 min) to 35 : 65 (30 min), or 10 : 90 (0 min) to 35 : 65 (25 min). The reaction yield was calculated by comparing the peak area corresponding to the unmodified and modified peptide, or internal standard.

MALDI-TOF/TOF analysis of the labeled peptide

The labeled peptide fractionated by HPLC was mixed with CHCA solution (10 mg/mL, CH_3CN:H_2O = 1:1 with 0.1% TFA), spotted on the MALDI-TOF MS plate and analyzed by MALDI-TOF/TOF MS (Bruker Daltonics, UltrafleXtreme). The raw MS data files were analyzed by FlexAnalysis 3.4 (Bruker) to create peak lists based on the recorded spectra. The

mass peak assigned as the **1**-modified peptide was selected and subjected to MSMS analysis with the LIFT mode with a precursor mass tolerance and a fragment ion mass tolerance of 0.5 Da. The MSMS spectra were analyzed by Biotools and Sequence editor (Bruker).

Preparation of KH6-tagged EGFP

The DNA fragments coding KH6 and thrombin site (MGSKHHHHHHSSGLVPRGS) was inserted into NcoI-NdeI site of pET28a-EGFP[S1] with DNA ligation mix (TAKARA). The sequences of the 5'-phosphorylated DNA fragments were as follows: (forward) 5'-catgggcagcaagcatcatcatcatcacagcagcggcctggtgccgcgcggcagcgg-3' and (backward) 5'-taccgctgccgcgcggcaccaggccgctgctgtgatgatgatgatgcttgctgcc-3' (purchased from FASMAC). pET28a-KH6-EGFP plasmid was transformed into *E. coli* BL21(DE3) pLysS. The cells were grown in 1 L of Terrific Broth (TB) at 37 °C until an optical density (OD) at 600 nm increased to 0.6 ~ 0.8, and further grown at 16 °C for 24 h with IPTG induction (0.25 mM). The cell suspension was centrifuged for 20 min at 4700 rpm at 4 °C. The cells were re-suspended in 40 mL of Lysis buffer (50 mM HEPES, 100 mM NaCl, 10% glycerol, pH 7.4) and lysed by sonication (10 shots x 10 sec, Branson Sonifier 450). Insoluble materials were removed by centrifugation for 10 min at 12,000 rpm to collect the soluble fraction containing KH6-EGFP. The EGFP purification was performed with TALON resin (Clontech). The soluble fraction (40 mL) was adsorbed on 1 mL of TALON resin pre-filled with washing buffer (50 mM HEPES, 100 mM NaCl, 30 mM imidazole, pH 7.2) in a plastic column. The resin was washed with washing buffer and then the resin-bound protein was eluted from the column with elution buffer (50mM HEPES, 100 mM NaCl, pH 7.2, 150 mM imidazole). The fractions containing the purified EGFP was collected and dialyzed three times with HEPES buffer (50 mM HEPES, 100 mM NaCl, pH 7.4) to remove the excess imidazole. The concentration of KH6-EGFP was determined by UV absorbance at 488 nm based on the reported extinction coefficient of EGFP (ε = 55,000 $M^{-1}cm^{-1}$). The solution of the EGFP was stored at 4 °C.

Labeling of KH6- or H6K-tagged protein

The tagged protein (1 μM) was incubated with reagent (3 μM) in the presence (6 μM) or absence of Ni^{2+} in PBS buffer (pH 7.4) at 37 °C. Aliquots at different time points were taken and then quenched using EDTA (final 0.6 mM), and the reaction was analyzed by SDS-PAGE or MALDI-TOF MS (matrix: SA). For the azide labeled protein, DBCO-dPEG12-carboxyfluorescein (10 equivalent for protein, Quanta BioDesign) or DBCO-Cy5 (10 equivalent for protein, Sigma-Aldrich) was used.

Thrombin digestion

The biotin-labeled KH6-EGFP was treated with thrombin (1unit per 25 pmol of KH6-EGFP in PBS, pH 7.4, 22°C, 16 hr) to cleave the tag site. The cleavage was evaluated by SDS-PAGE.

Determination of labeling yields with coumarin-modified BSA standard

The labeling yields of KH6-EGFP were determined according to a standard coumarin-modified BSA which was prepared as follows: 500 μM of 7-(diethylamino)coumarin-3-carboxylic acid *N*-succinimidyl ester was added to a PBS buffer solution containing 50 μM of BSA. After incubation at room temperature overnight, the reaction mixture was purified by gel filtration (TOYOPEARL HW-40F) with PBS as the eluent. The labeling yield of coumarin-BSA was determined to be 500% according to absorbance data and extinction coefficients of BSA ($\varepsilon_{BSA,280nm}$ = 43824 $M^{-1}cm^{-1}$) and coumarin ($\varepsilon_{Dc,280nm}$ = 5000 $M^{-1}cm^{-1}$, $\varepsilon_{Dc,416nm}$ = 45000 $M^{-1}cm^{-1}$).[S2] The labeling yields of KH6-EGFP were calculated by in-gel fluorescence according to the following equation with coumarin-BSA as the standard.

$$Y_x = Y_s \times \frac{I_x}{I_s} \times \frac{A_s}{A_x}$$

where *Y* is labeling yield, *I* is in-gel fluorescence intensity, *A* is protein amount (mol) in the well, *x* and *s* represent sample and standard, respectively.

Preparation of H6K-tagged anti-GluD2 nanobody

A lysine residue was inserted at the C-terminus of anti-GluD2 nanobody using site-directed mutagenesis with a plasmid encoding anti-GluD2 nanobody and the following primers: (Forward) 5'- caccatcaccatcacaaataatacccgtacgacgttccg-3', (backward) 5'-gtgcggtggtggcggtggcgggacggtgacctgggtccc-3' (FASMAC). The obtained plasmid was transformed into *E. coli* BL21(DE3) Singles (Novagen). The cells were grown in 1 L of Terrific Broth (TB) at 37 °C until an optical density (OD) at 600 nm increased to ~ 1.0, and further grown at 37 °C for 5 h with IPTG induction (1 mM). The nanobody was extracted and purified according to the reported procedure.[S3] The concentration of nanobody was determined by BCA assay (Pierce). The purified nanobody was stored at 4 °C.

Visualization of GluD2 transiently expressing in HEK293T cells

HEK293T cells (2×10^5 cells) were seeded on a 3.5 cm dish (Falcon) and incubated in Dulbecco's Modified Eagle's Medium (DMEM, High glucose, Sigma Aldrich) supplemented with 10% fetal bovine serum (FBS, Gibco), penicillin (100 units/ml), streptomycin (100 mg/ml), and amphotericin B (250 ng/ ml) for 24 h at 37 °C under 5% CO_2. The cells were transfected with 2 μg pCAGGS2(HA-GluD2) and Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's instruction. The cells were trypsinized and transferred to the poly-L-lysine coated glass bottomed dish (Matsunami). After incubation 18 h, the cells were incubated in PBS (–) containing 1 μM the nanobody-biotin conjugate on ice for 3 min. The cells were washed one time using DMEM-HEPES (Gibco) followed by incubation in DMEM HEPES containing Streptavidin-HiLyte647 (5 μg/mL)/ Anti-HA alexa488 (5 μg/mL) on ice for 3 min. After washing twice with DMEM-HEPES, the cells were analyzed with a confocal microscope (LSM800, Carl Zeiss).

Synthesis

General materials and methods for organic synthesis

All chemical reagents and solvents were obtained from commercial suppliers (Tokyo Chemical Industry (TCI), Sigma-Aldrich, Fujifilm-Wako Pure Chemical Corporation, Watanabe Chemical Industries, or Kanto Chemical Co., Inc.) and used without further purification. Thin layer chromatography (TLC) was performed on silica gel 60 F254 precoated aluminium sheets (Merck) and visualized by fluorescence quenching, fluorescence by 365 nm excitation, I_2 staining and ninhydrin staining. Chromatographic purification was accomplished using flash column chromatography on silica gel 60 N (neutral, 40–50 μm, Kanto Chemical). ^{1}H NMR spectra were recorded in deuterated solvents on a Varian Mercury 400 (400 MHz) spectrometer and calibrated to tetramethylsilane (= 0 ppm) or residual solvent peak. Multiplicities are abbreviated as follows: s = singlet, brs = broad singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = double doublet. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) spectra were recorded on an Autoflex III instrument (Bruker Daltonics) using α-cyano-4-hydroxycinnamic acid (CHCA) as the matrix. High-resolution mass spectra were measured on an Exactive (Thermo Scientific) equipped with electron spray ionization (ESI). Reversed-phase HPLC (RP-HPLC) was carried out on a Hitachi LaChrom L-7100 system equipped with a LaChrom L-7400 UV detector, and a YMC-Pack ODS-A column (5 μm, 250 × 20 mm) at a flow rate of 9.9 mL/min. UV/Vis detection was at 220 nm and 418 nm. All runs used linear gradients of acetonitrile containing 0.1% TFA (solvent A) and 0.1% aqueous TFA (solvent B).

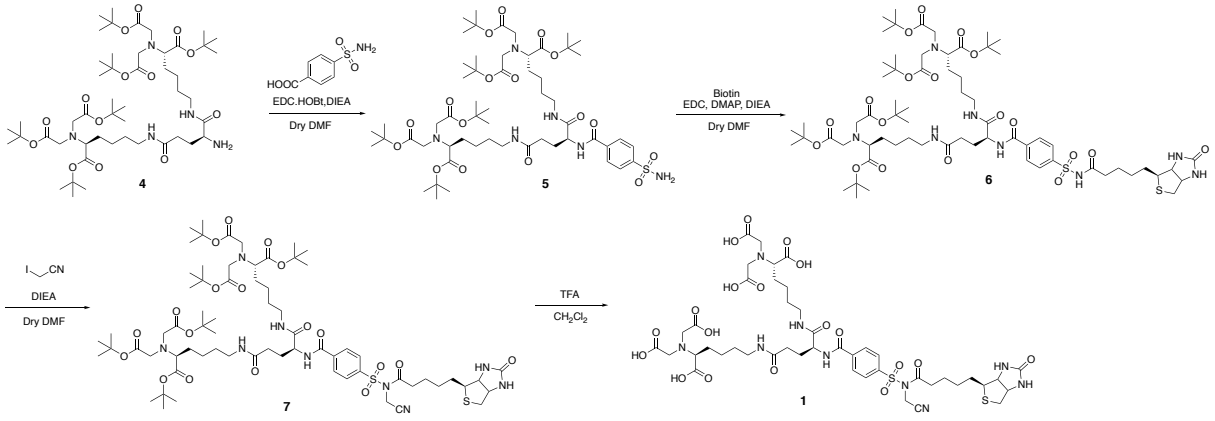

Figure S10 Synthetic scheme of **1**.

*Compound **5***

To a solution of **4**[S4] (84.3 mg, 0.08 mmol) in dry DMF (2 mL) was added 1-Hydroxybenzotriazole Monohydrate (HOBt) (20 mg, 0.13 mmol), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide Hydrochloride (EDC) (25 mg, 0.13 mmol), 4-Sulfamoylbenzoic acid (21 mg, 0.1 mmol) and *N,N,*-diisopropylethylamine (DIEA) (45 µL, 0.24 mmol), and the resulting solution was stirred at room temperature for 4 h. The mixture was concentated under reduced pressure and chromatographed over silica gel with $CHCl_3$/MeOH (30:1) as the mobile phase to yield **5** as an oily liquid. Yield: 90 mg, 90%; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.40 (d, 1H, *J* = 4.0 Hz), 7.95 (d, 2H, *J* = 8.0 Hz), 7.90 (d, 2H, *J* = 8.0 Hz), 7.05 (t, 1H, *J* = 4.0 Hz), 6.50 (t, 1H, *J* = 4.0 Hz), 6.03 (s, 2H,), 4.57 (m, 1H), 3.50-2.85 (m, 14H), 2.60- 2.20 (m, 4H), 1.65-1.55 (m, 12H), 1.45 (s, 18H), 1.43 (s, 36H); ESI-MS: Calcd. for $(M+Na)^+$:1177.6288 , found:1177.6289

*Compound **6***

To a solution of compound **5** (44.4 mg, 0.038 mmol) in dry DMF (2 mL) was added biotin (45 mg, 0.039 mmol), EDC (11.2 mg, 0.057 mmol), 4-dimethylaminopyridine (DMAP) (5.7 mg, 0.046 mmol) and DIEA (20.3 µL, 0.11 mmol). The reaction mixture was stirred overnight at room temperature. The solvent was evaporated, and the residue was chromatographed over silica gel with $CHCl_3$/MeOH (15:1) as mobile phase to yield **6** as an oily liquid. Yield: 43.6 mg, 82%; ^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD): δ 8.13 (d, 2H, J = 8.0 Hz), 8.06 (d, 2H, *J* = 8.0 Hz), 7.91 (t, 1H, *J* = 4.0 Hz), 7.69 (t, 1H, *J* = 4.0 Hz), 4.66 (m, 2H), 4.24 (m, 1H), 3.46-3.05 (m, 17H) 2.95-1.5 (m, 24H), 1.48 (s, 18H), 1.46 (s, 36H); ESI-MS: Calcd. for $(M+Na)^+$:1403.7064 , found:1403.7075

*Compound **7***

Compound **6** (43.6 mg, 0.03 mmol) was dissolved in dry DMF (2 mL). To this solution, Iodoacetonitrile (22.7 µL, 0.3 mmol) and DIEA (54.9 µL, 0.3 mmol) were added and the solution was stirred for 3 h at room temperature. The reaction mixture was concentrated and the residue was column chromatographed over silica gel with $CHCl_3$:MeOH (30:1) as the moving phase to yield **7** colorless sticky oil. Yield: 26.8 mg, 60%; ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.09 (d, 1H, *J* = 8.0 Hz), 8.22 (d, 2H, *J* = 8.0 Hz), 8.07 (d, 2H, *J* = 8.0 Hz), 7.10 (t, 1H, *J* = 4.0 Hz), 6.71 (t, 1H, *J* = 4.0 Hz), 6.26 (brs, 1H), 5.3 (s, 1H), 4.79 (s, 2H), 4.58 (m, 2H), 4.29 (m, 1H), 3.50-3.34 (m, 8H), 3.33-3.0 (m, 7H), 2.98-2.78 (m, 4H), 2.70-2.08 (m, 6H), 1.80-1.37 (m, 70H). ESI-MS: Calcd. for $(M+Na)^+$:1442.7173, found:1442.7165.

*Compound **1***

To a solution of **7** (10 mg, 7 µmol) in dichloromethane (500 µL), was added TFA (500 µL) and stirred the reaction mixture for 6 hours at room temperature. The solvent was coevaporated with Toluene (0.5 mL × 2). The residue was purified by RP-HPLC (A:B) 0 min = 5:95, 35 min = 40:60, 40 min = 100:0, 45 min = 5 : 95; to yield compound **1** as a colorless oily liquid. Yield: 3.8 mg, 52%; H NMR (400 MHz, CD_3OD): δ 8.13 (m, 4H), 4.9 (s, 2H), 4.46 (m, 2H), 4.25 (m, 1H), 3.76-3.40 (m, 13H), 3.26-3.04 (4H), 2.91 (m, 1H), 2.70 (m, 3H), 2.43-1.45 (m, 20H); ESI-MS: Calcd. for $(M+H)^+$:1084.3598, found:1084.3596.

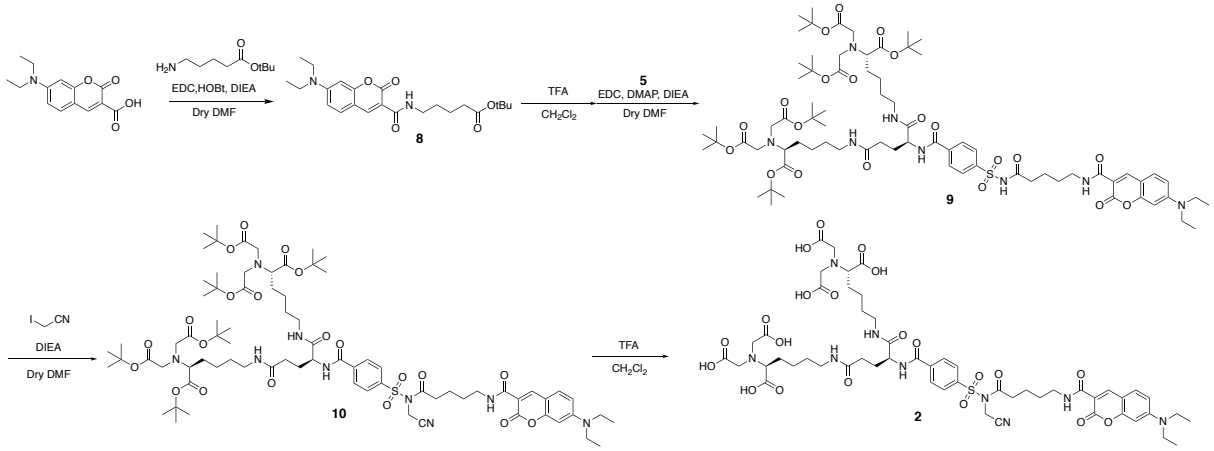

Figure S11 Synthetic scheme of **2**.

*Compound **8***

To 7-(Diethylamino)coumarin-3-carboxylic acid (200 mg, 0.76 mmol), H-Ape(5)-OtBu.HCl (176.2 mg, 0.84 mmol), EDC (293.7 mg, 1.53 mmol), HOBt (234.4 mg, 1.53 mmol) were added in a 50 mL clean eggplant flask. To this mixture was added dry DMF (3.5 mL), DIEA (533.1 µL, 3.06 mmol) and stirred for 5 h at room temperature. After the reaction completion, the reaction mixture was subjected to liquid-liquid extraction using DCM/sat. $NaHCO_3$. Organic layer was collected, and aqueous layer was extracted with DCM (2 times). Organic layers were mixed together and washed using brine solution (1 time). Collected organic layer was dried over Na_2SO_4 and filtered. Solvent was evaporated under vacuum. The obtained residue was purified by column chromatography on silica gel (60N) using CHCl3/MeOH 80:1 to yield target compound as a yellow solid. Yield: (290 mg, 94 %). ^1H NMR (400 MHz, $CDCl_3$): δ 8.80 (t, 1 H, *J* = 4.0 Hz), 8.70 (s, 1 H), 7.42 (d, 1 H, *J* = 8.0 Hz), 6.62 (d, 1 H, *J* = 8.0 Hz), 6.50 (s, 1 H), 3.45 (m, 6 H) 2.26 (t, 2 H, *J* = 8.0 Hz), 1.66 (br, 4 H), 1.44 (s, 9 H), 1.24 (t, 6 H, *J* = 8.0 Hz); ESI-MS: Calcd. for $(M+Na)^+$:439.2203, found:439.2209.

*Compound **9***

Compound **8** (11.5 mg, 0.028 mmol) was dissolved in TFA/DCM (1:1, 1 mL) and stirred at room temperature for 2 h. The solvent was coevaporated with toluene (0.5 mL*2). Carried to next step without purification. To 10 mg of this intermediate compound, was added EDC (8 mg, 0.041 mmol), DMAP (5 mg, 0.40 mmol), compound **5** (28 mg, 0.0.024 mmol), DIEA (21.1 µL, 0.12 mmol) and dry DMF (1.5 mL), and stirred at room temperature for overnight. Solvent was evaporated under vacuum, and the crude mixture was purified by column chromatography over silica gel using $CHCl_3$:MeOH = 40:1 to 30:1. Yield:33 mg, 82 % over two steps. ^{1}HNMR ($CDCl_3$, 400 MHz): δ 10.71 (brs, 1H), 9.14 (t, 1H, *J* = 4.0 Hz) 9.00 (s, 1H), 8.75 (d, 1H, *J* = 4.0 Hz), 8.09 (d, 2H, *J* = 8.0 Hz), 8.03 (d, 2H, *J* = 8.0 Hz), 7.54 (d, 1H, *J* = 8.0 Hz), 7.10 (t, 1 H), 6.66 (dd, 1H, *J* = 8.0 Hz), 6.51 (s, 1H), 4.54 (m, 1H), 3.54-3.14 (m, 16H), 2.59 (m, 1H), 2.37 (m, 4H), 2.27-2.03 (m, 2H), 1.77-1.40 (m, 71H), 1.24 (t, 6H, *J* = 8.0 Hz). ESI-MS: Calcd. for $(M+Na)^+$:1519.7868, found:1519.7845.

*Compound ***10***

To a solution of **9** (33 mg, 0.022 mmol) in dry DMF (0.5 mL) was added Iodoacetonitrile (15.9 µL, 0.22 mmol) and DIEA (38.3 µL, 0.22 mmol), and the mixture was stirred at room temperature for 3 h. Solvent was evaporated in vacuo and the residue was purified by flash chromatography over silica gel using $CHCl_3$/MeOH = (80:1) to yield the target compound as yellow oily liquid (23.1 mg, 70 %).^{1}HNMR ($CDCl_3$, 400 MHz): δ 9.11 (d, 1 H, *J* = 4.0 Hz), 8.80 (t, 1 H, *J* = 4.0 Hz), 8.71 (s, 1 H), 8.20 (d, 2H, *J* = 8.0 Hz), 8.06 (d, 2H, *J* = 8.0 Hz), 7.46 (d, 1H, *J* = 8.0 Hz), 7.08 (t, 1H, *J* = 4.0 Hz),6.73 (t, 1 H, *J* = 4.0 Hz), 6.65 (dd, 1 H J_1 = 8.0 Hz, J_2 = 4.0 Hz), 6.50 (s, 1 H), 4.79 (s, 2 H), 4.54 (m, 1H), 3.54-3.0 (m, 18H), 2.71 (m, 2H), 2.58 (m, 1H), 2.38 (m,2H), 2.3-2.07(m, 2H), 1.77-1.37 (m, 71 H), 1.24 (t, , 6H, *J* = 8.0 Hz). ESI-MS: Calcd. for $(M+Na)^+$:1558.7977, found:1558.7973.

*Compound **2***

Compound **10** (10 mg, 6.5 µmol) was dissolved in TFA/DCM (2 mL, 1:1) and stirred at room temperature for 2 h. The solvent was coevaporated with toluene (0.5 mL x 2) and the crude compound was purified by RP-HPLC (A:B) = 20:80 (0 min), 60:40 (40 min), 100:0 (45 min), 20:80 (50 min) to obtain the target product **2** as a stickly yellow oil (4.1 mg, 51 %) . ^{1}HNMR (CD_3OD, 400 MHz): δ 8.62 (s, 1 H), 8.15 (s, 4 H), 7.56 (d, 1 H, *J* = 4.0 Hz), 6.82 (d, 1 H, *J* = 8.0 Hz), 6.58 (d, 1 H, *J* = 4.0 Hz), 4.90 (s, 2 H), 4.5 (m, 1 H), 3.70-3.42 (m, 16H), 3.24-3.05 (m, 4H), 2.76 (t, 2H, *J* = 8.0 Hz), 2.40-1.40 (m, 20H), 1.23 (t, 6H, *J* = 8.0 Hz).

ESI-MS: Calcd. for $(M-H)^-$:1198.4256, found:1198.4273.

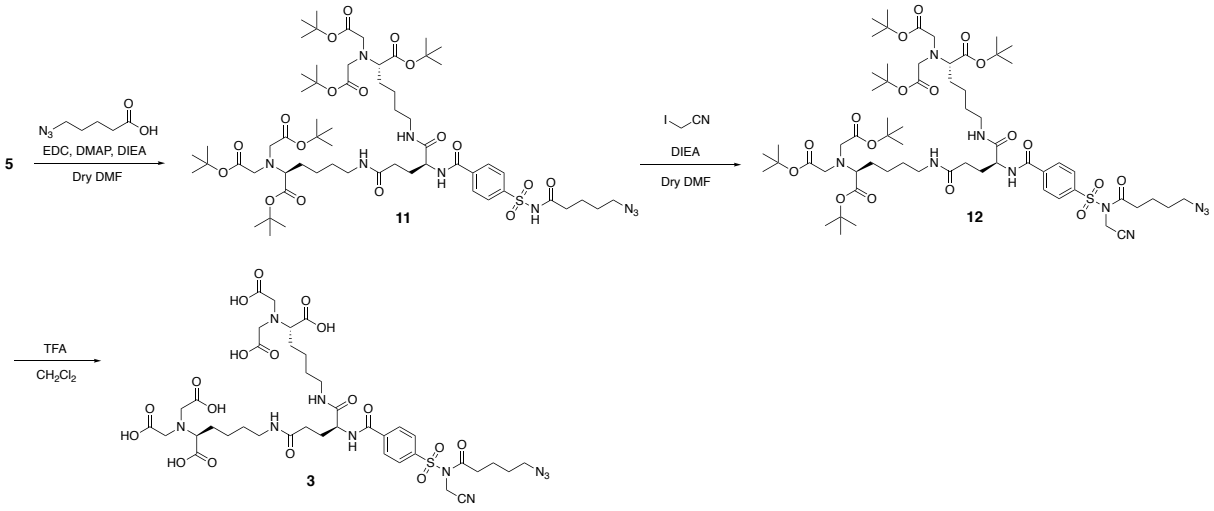

Figure S12 Synthetic scheme of **3**.

*Compound **11***

To a solution of **5** (14 mg, 0.012 mmol) in dry DMF (500 µL) was added 5-azido valeric acid (2 µL, 0.015 mmol), EDC (3.4 mg, 0.017 mmol), DMAP (1.77 mg, 0.014 mmol) and DIEA (6.32 µL, 0.048 mmol), and the mixture was stirred for 4 h at room temperature. Solvent was evaporated in vacuo and dried under vacuum, and the residue was purified by column chromatography over silica gel using $CHCl_3$/MeOH : 50:1 to yield the target compound as a colorless oily liquid (12.7 mg, 82 %). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.76 (d, 1H, *J* = 8.0 Hz), 8.06 (s, 4H), 7.03 (t, 1 H, *J* = 4.0 Hz), 6.64 (t, 1 H, *J* = 4.0 Hz), 4.56 (m, 1H), 3.52-3.12 (m, 16H), 2.62-2.07 (m, 6H) ,1.67-1.41 (m, 70H); ESI-MS: Calcd. for $(M+Na)^+$:1302.6877, found:1302.6883.

*Compound **12***

To a solution of compound **11** (12.7 mg, 9.9 µmol) in dry DMF (1 mL) was added Iodoacetonitrile (7.1 µL, 99µmol), DIEA (17.2 µL, 99 µmol) and stirred for 3 h at room temperature. Solvent was evaporated in vacuo. The resulting crude compound was purified by column chromatography on silica gel using $CHCl_3$/MeOH = 70:1 to obtain the target compound as colorless oily liquid. Yield : 6.5 mg, 50 %. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.14 (d, 1H, *J* = 8.0 Hz), 8.20 (d, 2H, *J* = 8.0 Hz), 8.04 (d, 2H, *J* = 8.0 Hz) 7.01 (t, 1 H, *J* = 4.0 Hz), 6.75 (t, 1H, *J* = 4.0 Hz), 4.76 (s, 2H), 4.52 (m, 1H), 3.49-3.36 (m, 8H), 3.36-3.17 (m,

8H), 2.72-2.59 (m, 3H) , 2.42 (m, 1H), 2.24 (m, 1H), 2.10 (m, 1H), 1.72-1.41 (m, 74H); ESI-MS: Calcd. for $(M+Na)^+$:1341.6986, found:1341.6988.

*Compound **3***

Compound **12** (6.5 mg, 4.92 μmol) was dissolved in TFA/DCM (1:1, 1.5 mL) and stirred at room temperature for 6 h. After the completion of the reaction, solvent was co-evaporated twice using Toluene (0.5 mL x 2). The residue was purified by RP-HPLC (A:B) 0 min = 5:95, 35 min = 40:60, 40 min = 100:0, 45 min = 5 : 95 to yield compound **3** as a colorless oily liquid. Yield: 2.2 mg, 46 %. ^{1}H NMR (CD_3OD, 400 MHz) δ 8.15 (s, 4H), 4.90 (s, 2H), 4.49 (m, 1H), 3.80-3.11 (m, 18H), 2.75 (t, 1H, *J* = 8.0 Hz), 2.37 (m, 2H), 2.22-2.02 (m, 2H), 1.88-1.40 (m, 15H); ESI-MS: Calcd. for $(M-H)^-$:981.3265, found: 981.3263.

Peptide synthesis

The peptides were synthesized manually on Rink Amide resin (Novabiochem) by standard Fmoc-based solid-phase peptide synthesis protocol. Fmoc-Cys(Trt)-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-His(Trt)-OH, Fmoc-Ala-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Tyr(*t*Bu)-OH and Fmoc-Trp(Boc)-OH were used as building blocks. Fmoc deprotection was performed with 20% piperidine in *N*-methylpyrrolidone (NMP), and coupling reactions were performed with a mixture of Fmoc-amino acid (3 eq.), HBTU (3 eq.), HOBt (3 eq.), and DIEA (6 eq.) in NMP. All coupling and Fmoc deprotection steps were monitored by the Kaiser test.For all peptides, N-terminus was acetylated using 25 % solution of acetic anhydride in dichloromethane. Following chain assembly, global deprotection and cleavage from the resin was performed with TFA containing 1% tri-isopropylsilane (TIS), 2.5% ethanedithiol, and 2.5% H_2O. The crude peptide products were precipitated by diisopropyl ether and purified by RP-HPLC (column; YMC-pack ODS-A, 250 x 20 mm, mobile phase; CH_3CN (containing 0.1% TFA) / H_2O (containing 0.1% TFA) = 5: 95 to 40:60 (linear gradient over 40 min), flow rate; 9.99 mL/min, detection; UV (220 nm). Molecular weight of the peptide was confirmed by MALDI-TOF mass spectrometry.

MALDI-TOF-MS (CHCA):

13 (Ac-KHHHHHHAW-NH_2) calcd for $[M+H]^+$ = 1267.6, obsd 1267.7;

14 (Ac- KAAHHHHHHAW -NH_2) calcd for $[M+H]^+$ = 1409.7, obsd 1409.7 ;

15 (Ac- KAAAAHHHHHHAW -NH_2) calcd for $[M+H]^+$ = 1551.7, obsd 1551.8;

16 (Ac- KAAAAAAHHHHHHAW -NH_2) calcd for $[M+H]^+$ = 1693.8, obsd 1693.8;

17 (Ac- CHHHHHHAW-NH_2) calcd for $[M+H]^+$ = 1242.5, obsd 1242.7 ;

18 (Ac- YHHHHHHAW-NH_2) calcd for $[M+H]^+$ = 1302.6, obsd 1303.3;

19 (Ac- SHHHHHHAW-NH_2) calcd for $[M+H]^+$ = 1226.5, obsd 1226.7;

20 (Ac- THHHHHHAW-NH_2) calcd for $[M+H]^+$ = 1240.5, obsd 1240.8;

21 (Ac- EHHHHHHAW-NH_2) calcd for $[M+H]^+$ = 1268.5, obsd 1268.7;

22 (Ac- HHHHHHKAW-NH_2) calcd for $[M+H]^+$ = 1267.6, obsd 1268.1.

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