Supporting Information for:

Disassembly-Driven Turn-On Fluorescent Nanoprobes for Selective Protein Detection

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Supplementary Figures

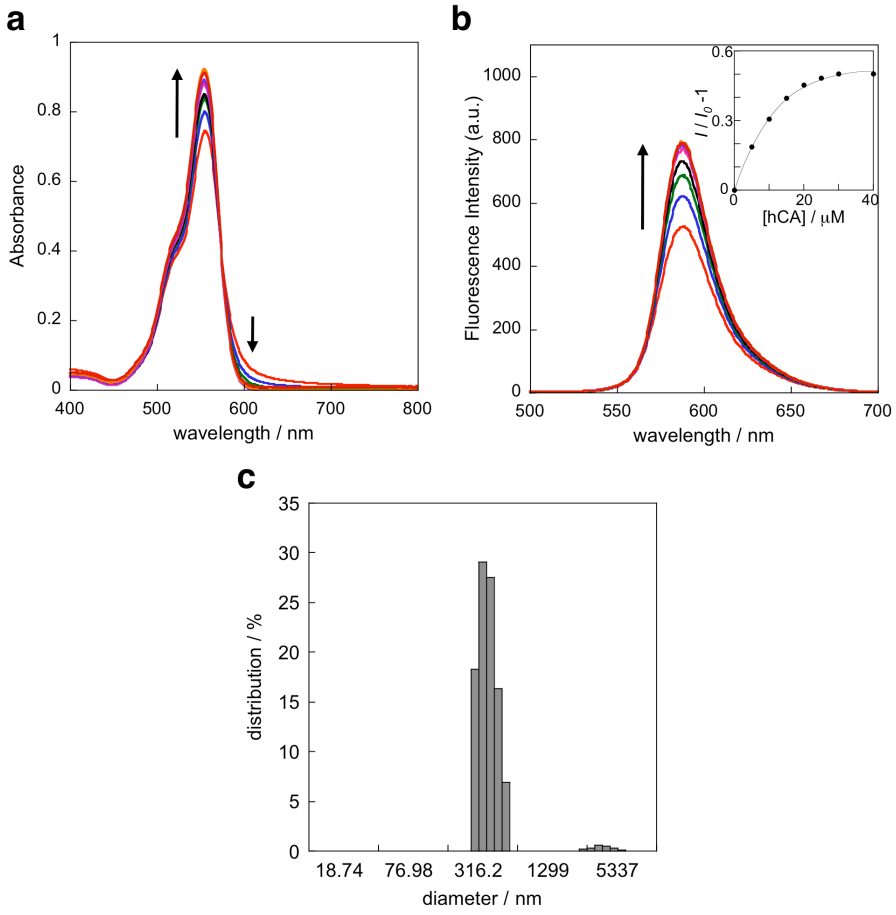


Figure S1. Detection and spectroscopic properties of probe **2**. (a) UV-visible absorption spectral change of probe **2** (15 μM) upon the addition of hCA (0–40 μM). (b) Fluorescence spectral change (λ_{ex} = 480 nm) of probe **2** (15 μM) upon the addition of hCA (0–40 μM). (Inset) Fluorescence titration curve (λ_{em} = 586 nm). (c) DLS analysis of particle-size distribution of probe **2** (15 μM). The DLS measurements showed that probe **2** formed submicron-sized aggregates in buffer solution, which indicates that the TMR fluorophore is less sensitive to the aggregation state in its quenching in this system. All experiments were performed in 50 mM HEPES buffer, 0.5 M NaCl (pH 7.2).

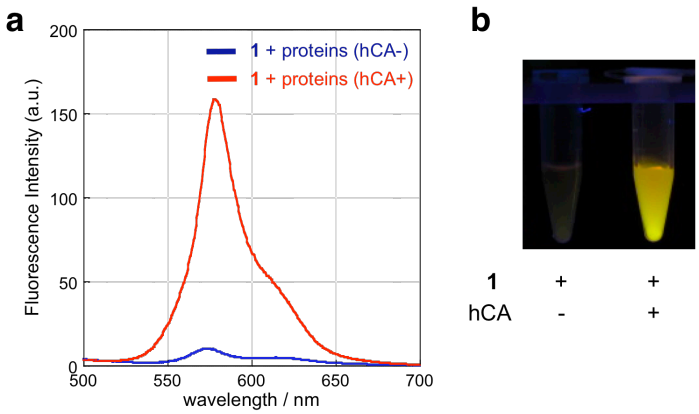


Figure S2. Detection of hCA in protein mixtures using probe **1**. (a) Fluorescence spectra (λ_{ex} = 468 nm) of probe **1** (25 μM) in a protein mixture solution (hemoglobin, bovine serum albmin, concanavalin A and chymotrypsin, each 1.5 mg/ml) containing or non-containing hCA (25 μM) in 50 mM HEPES buffer (pH = 7.2). (b) Photograph of probe **1** (25 μM) in the protein mixture solution containing or non-containing hCA (25 μM). The image was obtained with UV excitation (λ_{ex} = 365 nm).

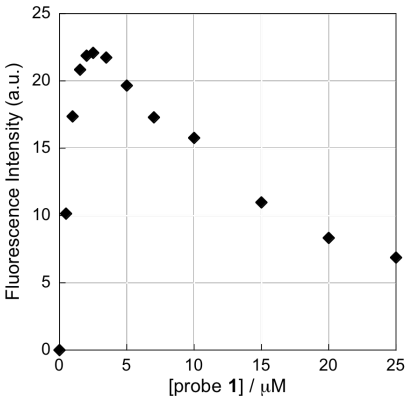


Figure S3. Plots of the fluorescence intensity (λ_{em} = 573 nm) in 50 mM HEPES buffer (pH 7.2) versus probe **1** concentration.

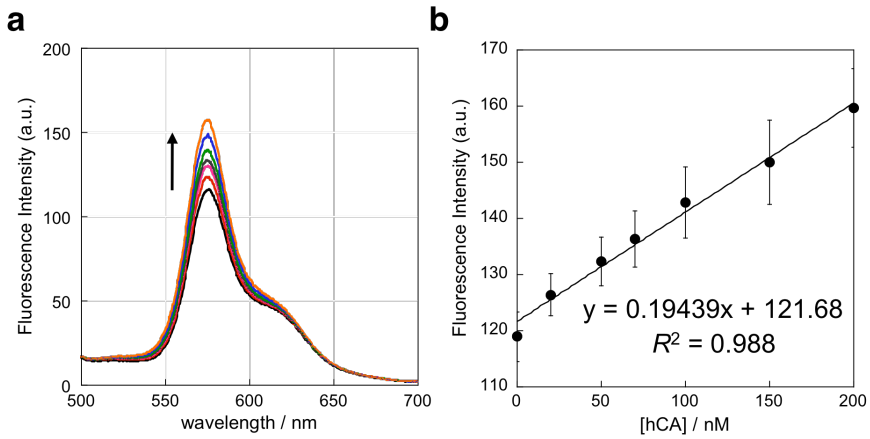


Figure S4. Estimation of the detection limit of probe **1** for hCA. (a) Fluorescence spectral change (λ_{ex} = 468 nm) of probe **1** (25 μM) upon the addition of hCA (0–200 nM) in 50 mM HEPES buffer (pH 7.2). (b) Plots of the fluorescence intensity (λ_{em} = 575 nm) versus hCA concentration. Error bars represent standard deviations of three experiments. Note that for this experiment, an emission slit width of 15 nm was used for more sensitive detection (an emission slit width of 5 nm was used for data shown in Figure 1b and 2c).

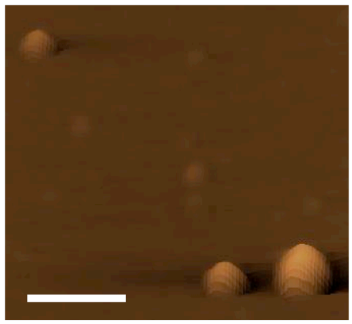


Figure S5. AFM image of the self-assembled probe **1** (25 μM) spin-coated onto a mica surface (scale bar, 500 nm).

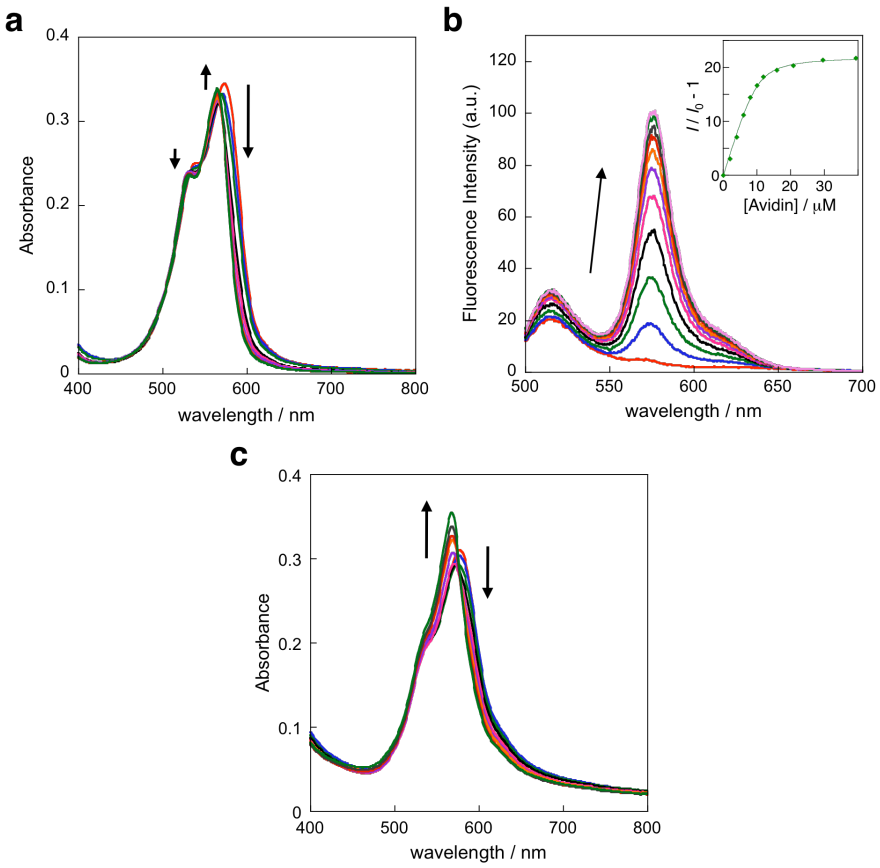


Figure S6. Detection and spectroscopic properties of probes **3** and **4**. (a) UV-visible absorption spectral change of probe **3** (10 μM) upon the addition of avidin (0–40 μM) in 50 mM HEPES buffer (pH 7.2). (b) Fluorescence spectral change of probe **3** (10 μM) upon the addition of avidin (0−40 μM) in 50 mM HEPES buffer (pH 7.2) (λ_{ex} = 480 nm). (Inset) Fluorescence titration curve (λ_{em} = 576 nm). (c) UV-visible absorption spectral change of probe **4** (10 μM) upon the addition of trypsin (0–100 μM) in 50 mM HEPES buffer, 0.5 M NaCl (pH 7.2).

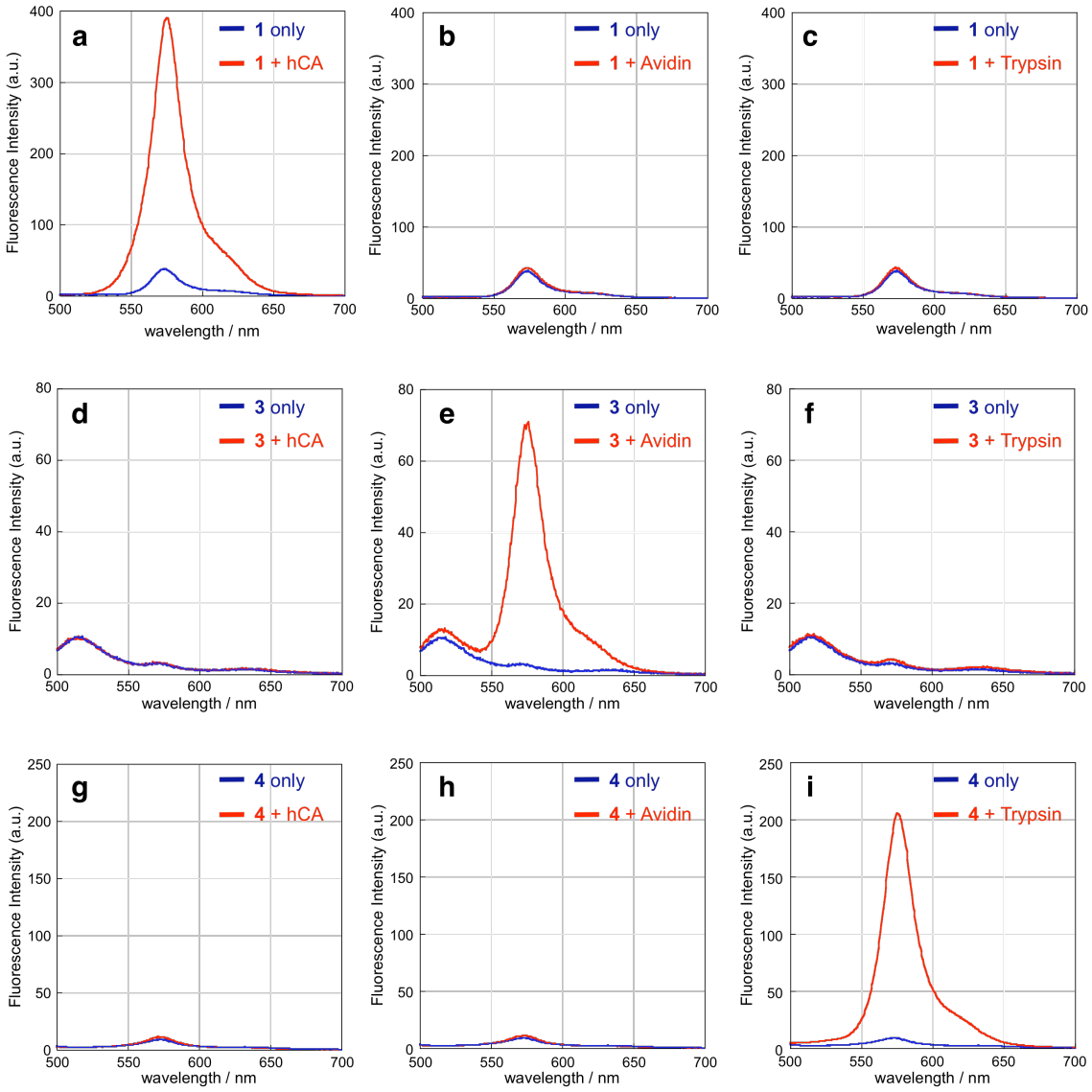


Figure S7. Fluorescence spectra of probes **1**, **3** and **4** without or with hCA, avidin or trypsin. (a–c) Probe **1** (10 μM) in 50 mM HEPES buffer (pH = 7.2) (λ_{ex} = 468 nm). (d–f) Probe **3** (10 μM) in 50 mM HEPES buffer (pH = 7.2) (λ_{ex} = 480 nm). (g–i) Probe **4** (10 μM) in 50 mM HEPES buffer, 0.5 M NaCl (pH = 7.2) (λ_{ex} = 480 nm). In all experiments, hCA (30 μM), avidin (30 μM) or trypsin (100 μM).

Supplementary Methods

General materials and methods.

All proteins were purchased from commercial suppliers (Sigma, Wako Pure Chemical Industries or Funakoshi). BODIPY 558/568 SE (compound **1-3**) was purchased from Invitrogen/Molecular Probes. Other chemical reagents and solvents were purchased from commercial chemical suppliers and used without further purification. UV-visible absorption spectra were recorded on a Shimadzu UV-visible 2550 spectrometer. Fluorescence spectra were measured by a Perkin-Elmer LS55 fluorescent spectrometer. ^{1}H NMR spectra were acquired with a Varian Mercury-400 (400 MHz) spectrometer. FAB mass spectra were recorded on a JEOL JMS-HX110A mass spectrometer.

All probes were dissolved in dimethylsulfoxide (DMSO) as a stock solution. The concentrations of probes **1**, **3** and **4** were determined by the absorbance at 559 nm in MeOH using the molar extinction coefficient of 97,000 $M^{-1}cm^{-1}$.[S2] The concentration of probe **2** was determined by the absorbance at 543 nm in MeOH using the molar extinction coefficient of 92,000 $M^{-1}cm^{-1}$.[S2] hCA and avidin were dissolved in 50 mM HEPES buffer (pH 7.2), and trypsin was dissolved in 50 mM HEPES buffer containing 0.5 M NaCl (pH 7.2). The concentrations of these proteins were determined by the absorbance at 280 nm using the molar extinction coefficient of 49,000 $M^{-1}cm^{-1}$ for hCA,[S3] 35,700 $M^{-1}cm^{-1}$ for avidin,[S4] and 36,700 $M^{-1}cm^{-1}$ for trypsin[S5].

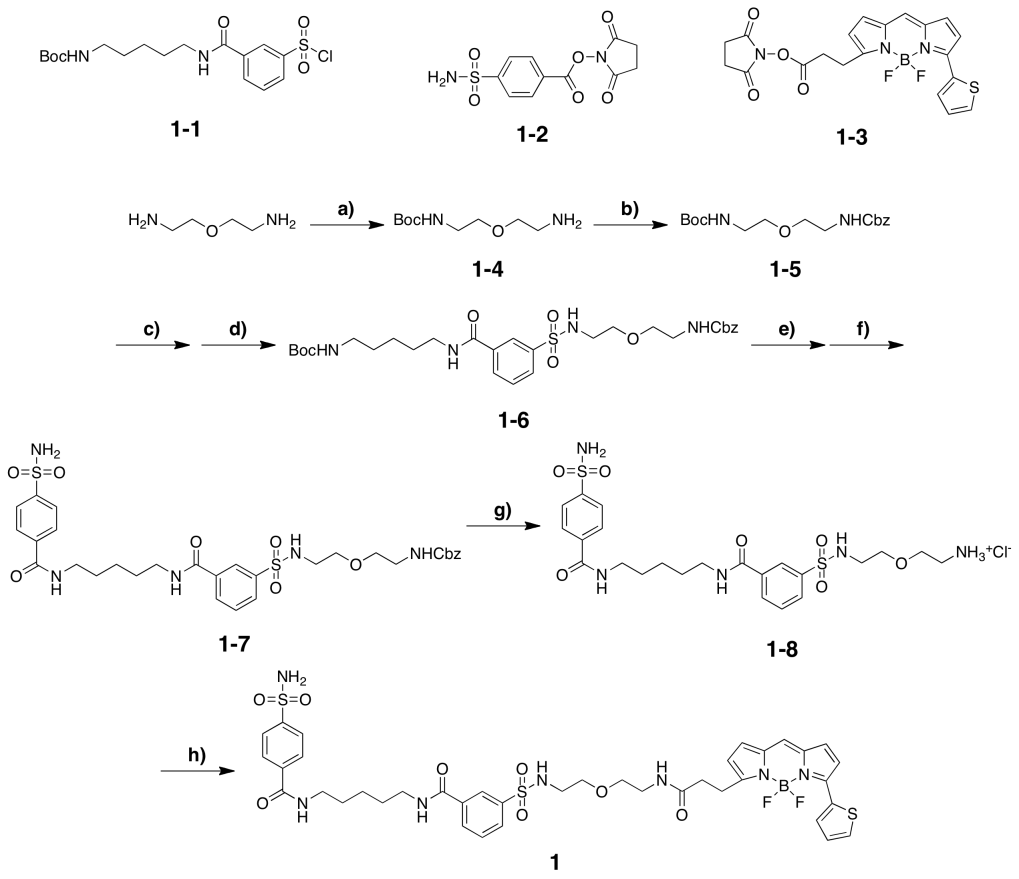
Dynamic light scattering (DLS) and atomic force microscopy (AFM) measurements.

DLS experiments were performed at 25 ℃ in 50 mM HEPES buffer (pH 7.2) using a plastic cuvette (3 ml volume). The measurements were performed on a NICOMP 380zls. The scattering angle was 90^{o}, and the laser wavelength was 785 nm.

In AFM experiments, a solution of probe **1** (25 μM) was spin-coated onto a freshly cleaved mica surface and dried *in vacuo*. Images of the sample were obtained with a tapping-mode AFM on a SHIMADSU SP-9600 microscope.

Synthesis.

Synthesis of probe 1



Scheme S1. Synthetic scheme of probe **1**. Reaction conditions: a) Boc_2O and Na_2CO_3 in dry dioxane; b) Z-Cl and DIEA in dry CH_2Cl_2; c) TFA in CH_2Cl_2; d) **1-1**,[S1] DMAP and DIEA in dry CH_2Cl_2; e) TFA in CH_2Cl_2; f) **1-2** [S1] and DIEA in CH_2Cl_2; g) H_2 and Pd/C in dry MeOH, HCl; h) **1-3** and DIEA in dry MeOH.

Compound 1-4

To a solution of 2,2'-oxybis(ethylamine) (2.60 g, 25.0 mmol) in dry dioxane (100 ml) was added Na_2CO_3 (2.65 g, 25.0 mmol), and subsequently a dioxane solution (40 ml) of Boc_2O (2.18 g, 10.0 mmol) dropwisely. The reaction mixture was stirred at room temperature for 1.5 h. After the precipitate was removed by filtration, the filtrate was

evaporated. The crude residue was dissolved in 5% aqueous citric acid (300 ml) and washed with diethyl ether (300 ml, 2 times). The pH of the aqueous layer was adjusted to ca. 12 with 1 N KOH, and the solution was extracted with chloroform (200 ml, 2 times). The organic layer was dried over anhydrous $MgSO_4$, evaporated and dried *in vacuo* to yield compound **1-4** (1.07 g, 5.24 mmol, 52%) as a colorless oil. ^{1}H NMR (400 MHz; $CDCl_3$): δ 4.98 (br, 1H), 3.50 (t, 2H, J = 5.2 Hz), 3.46 (t, 2H, J = 5.2 Hz), 3.31 (m, 2H), 2.85 (t, 2H, J = 5.2 Hz), 1.43 (s, 9H).

Compound 1-5

To a solution of compound **1-4** (1.02 g, 4.99 mmol) in dry CH_2Cl_2 (10 ml) was added triethylamine (TEA) (2.05 ml, 14.7 mmol) and benzyloxycarbonyl chloride (Z-Cl) (769 μl, 14.7 mmol). The reaction mixture was stirred at room temperature for 10 h. After diluting the reaction mixture with CH_2Cl_2 (100 ml), the solution was washed with 5% aqueous citric acid (200 ml, 2 times) and brine (100 ml). The organic layer was dried over anhydrous $MgSO_4$, filtered and evaporated. The crude product was purified by column chromatography on silica gel ($CHCl_3$/MeOH, 30:1) to yield compound **1-5** (650 mg, 1.92 mmol, 38%) as a colorless oil. ^{1}H NMR (400 MHz; $CDCl_3$): δ 7.36−7.30 (m, 5H), 5.15 (br, 1H), 5.11 (s, 2H), 4.86 (br, 1H), 3.53−3.48 (m, 4H), 3.38 (m, 2H), 3.29 (m, 2H), 1.43 (s, 9H).

Compound 1-6

To a solution of compound **1-5** (600 mg, 1.77 mmol) in CH_2Cl_2 (15 ml) was added trifluoroacetic acid (TFA) (6 ml) at room temperature. The reaction mixture was stirred for 30 min. After co-evaporation with toluene (8 ml, 2 times), the residue was dried *in vacuo* to yield a deprotected form of compound **1-5**. To a solution of a part of the above obtained compound (109 mg, 0.309 mmol) in dry CH_2Cl_2 (5.0 ml) was added compound **1-1**[S1] (150 mg, 0.370 mmol), 4-dimethylaminopyridine (DMAP) (11.3 mg, 92.5 μmol) and *N*,*N*'-diisopropylethylamine (DIEA) (156 μl, 0.896 mmol). The reaction mixture was stirred at room temperature for 5 h. After evaporation, the crude residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH, 25:1) to yield compound **1-6** (155 mg, 0.255 mmol, 83%) as a colorless oil. ^{1}H NMR (400 MHz;

$CDCl_3$): δ 8.29 (s, 1H), 8.03 (d, 1H, *J* = 8.0 Hz), 7.95 (d, 1H, *J* = 8.0 Hz), 7.53 (t, 1H, *J* = 8.0 Hz), 7.32-7.30 (m, 5H), 6.85 (br, 1H), 5.75 (br, 1H), 5.45 (br, 1H), 5.09 (s, 2H), 4.62 (br, 1H), 3.43−3.39 (m, 6H), 3.29 (m, 2H), 3.12-3.06 (m, 4H), 1.59−1.33 (m, 15H).

Compound 1-7

To a solution of compound **1-6** (140 mg, 0.231 mmol) in CH_2Cl_2 (12 ml) was added TFA (6 ml). The reaction mixture was stirred at room temperature for 30 min. After co-evaporation with toluene (10 ml, 2 times), the residue was dried *in vacuo* to yield a deprotected form of compound **1-6**. To a solution of the deprotected compound in dry DMF (3.0 ml) was added compound **1-2** [S1] (89.8 mg, 0.301 mmol) and DIEA (117 μl, 0.672 mmol). The reaction mixture was stirred at room temperature for 1 h. After evaporation, the residue was dissolved in AcOEt (50 ml) and washed with saturated aqueous $NaHCO_3$ (50 ml, 3 times) and brine (50 ml). The organic layer was dried over anhydrous $MgSO_4$, filtered and evaporated. The crude product was purified by column chromatography on silica gel ($CHCl_3$/MeOH, 10:1) to yield compound **1-7** (135 mg, 0.196 mmol, 85%) as a white amorphous. ^{1}H NMR (400 MHz; CD_3OD): δ 8.29 (s, 1H), 8.00-7.90 (m, 6H), 7.61 (t, 1H, *J* = 8.0 Hz), 7.33−7.27 (m, 5H), 5.06 (s, 2H), 3.51−3.34 (m, 8H), 3.20 (t, 2H, *J* = 5.2 Hz), 3.06 (t, 2H, *J* = 5.2 Hz), 1.68 (m, 4H), 1.47 (m, 2H).

Compound 1-8

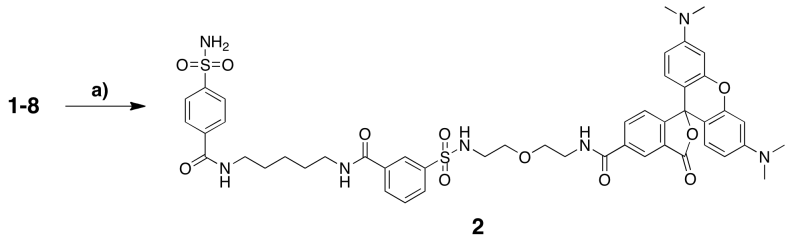
To a solution of compound **1-7** (85 mg, 0.123 mmol) in dry MeOH (5.0 ml) was added 10 wt.% Pd/C (40 mg). The reaction mixture was stirred at room temperature for 4 h under H_2 atmosphere. After the solution was filtered, the filtrate was acidified with conc. HCl (50 μl) and diluted with MeOH (5 ml) and water (25 ml). The resulting solution was lyophilized to yield compound **1-8** (50 mg, 0.084 mmol, 68%) as a white solid. ^{1}H NMR (400 MHz; CD_3OD): δ 8.30 (s, 1H), 8.03−7.99 (m, 2H), 7.96−7.90 (m, 4H), 7.65 (t, 1H, *J* = 8.0 Hz), 3.60 (t, 2H, *J* = 5.2 Hz), 3.51 (t, 2H, *J* = 5.2 Hz), 3.45-3.40 (m, 4H), 3.13−3.09 (m, 4H), 1.72-1.67 (m, 4H), 1.48 (m, 2H).

Compound 1

To a solution of compound **1-8** (5.0 mg, 8.44 μmol) in dry MeOH (0.5 ml) was

added compound **1-3** (3.40 mg, 7.67 μmol) and DIEA (2.96 μl, 17.0 μmol). The reaction mixture was stirred for 1 h at room temperature. After evaporation, the crude residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH, 20:1 to 10:1) to yield compound **1** (4.5 mg, 5.09 μmol, 66%) as a purple solid. ^{1}H NMR (400 MHz; CD_3OD): δ 8.29 (s, 1H), 8.09 (d, 1H, *J* = 4.0 Hz), 7.99−7.89 (m, 6H), 7.63−7.59 (m, 2H), 7.42 (s, 1H), 7.18−7.15 (m, 2H), 7.10 (d, 1H, *J* = 4.0 Hz), 6.85 (d, 1H, *J* = 4.0 Hz), 6.45 (d, 1H, *J* = 4.0 Hz), 3.42−3.36 (m, 12H), 3.06 (t, 2H, *J* = 5.6 Hz), 2.63 (t, 2H, *J* = 7.2 Hz), 1.66 (m, 4H), 1.48−1.42 (m, 2H). HR-FAB MS (NBA): calcd for $C_{39}H_{45}BF_2N_7O_8S_3$ $[M+H]^+$ = 884.2553; obsd 884.2556.

Synthesis of probe 2

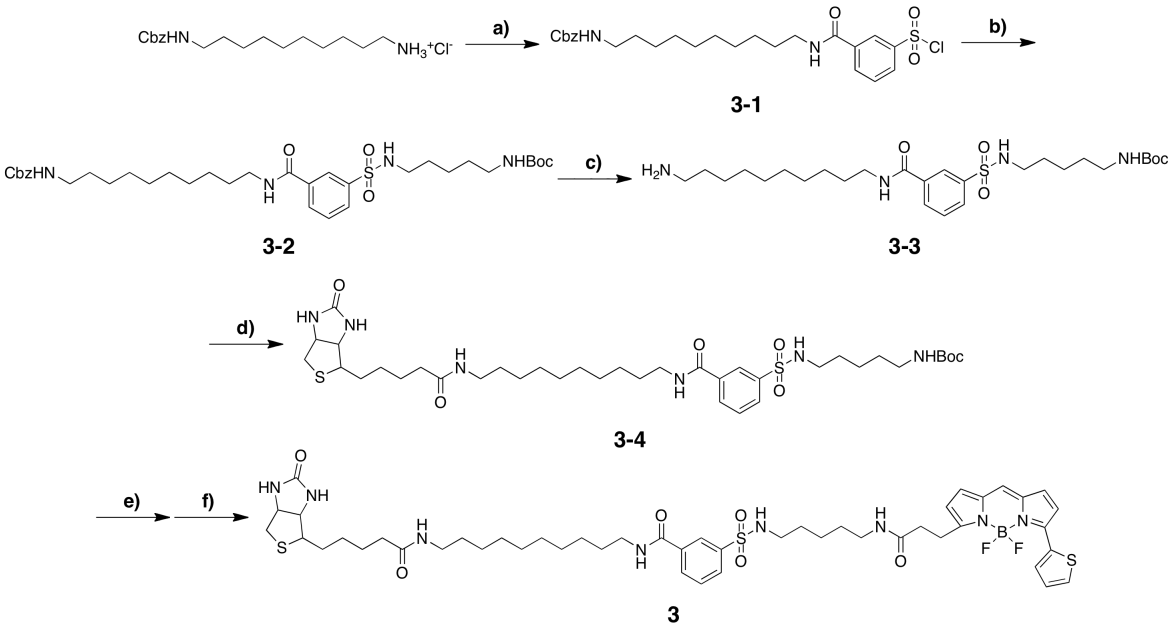


Scheme S2. Synthetic scheme of probe **2**. Reaction condition: a) 5(6)-carboxytetramethylrhodamine, WSC•HCl, HOBt•H_2O and DIEA in dry DMF.

Compound 2

To a solution of compound **1-8** (10 mg, 16.9 μmol) in dry DMF (0.5 ml) was added 5(6)-carboxytetramethylrhodamine (9.43 mg, 21.9 μmol), WSCI•HCl (5.46 mg, 28.5 μmol), HOBt•H_2O (4.36 mg, 28.5 μmol) and DIEA (11.8 μl, 67.7 μmol). The reaction mixture was stirred for 2 h at room temperature. The crude residue was purified by reversed phase HPLC (C18 (ODS) column; a linear gradient of solution A/solution B, 5:95 to 65:35 over 30 min; solution A, CH_3CN with 0.1% TFA; solution B, H_2O with 0.1% TFA). The collected fraction was lyophilized to yield compound **2** (5-isomer) (2.00 mg, 2.07 μmol, 12%) as a red solid. ^{1}H NMR (400 MHz; CD_3OD): δ 8.77 (s, 1H), 8.30 (s, 1H), 8.27 (d, 1H, J = 7.6 Hz), 8.01−7.86 (m, 6H), 7.63 (t, 1H, J = 8.0 Hz), 7.54 (d, 1H, J = 8.0 Hz), 7.15 (d, 2H, J = 9.2 Hz), 7.02 (dd, 2H, J = 9.2, 2.4 Hz), 6.95 (d, 2H, J = 2.4 Hz), 3.59−3.23 (m, 22H), 3.10 (t, 2H, J = 6.0 Hz), 1.69−1.61 (m, 4H), 1.45 (m, 2H). HR-FAB MS (NBA): calcd for $C_{48}H_{53}N_7O_{11}S_2$ $[M]^+$ = 967.3244; obsd 967.3259.

Synthesis of probe 3



Scheme S3. Synthetic scheme of probe **3**. Reaction conditions: a) 3-(chlorosulfonyl)benzoyl chloride and DIEA in dry CH_2Cl_2; b) *N*-(*tert*-butoxycarbonyl)-1,5-diaminopentane and DIEA in dry CH_2Cl_2; c) H_2 and Pd/C in MeOH/AcOEt; d) biotin-NHS ester [S1] in dry DMF; e) TFA in CH_2Cl_2; f) **1-3** and DIEA in dry DMF.

Compound 3-1

To a stirred solution of 3-(chlorosulfonyl)benzoyl chloride (3.6 g, 15.1 mmol) in dry CH_2Cl_2 (20 ml) was added dropwisely a solution of *N*-1-carbobenzoxy-1,10-diaminodecane hydrochloride (2.00 g, 5.83 mmol) and DIEA (9.0 ml, 51.7 mmol) in dry CH_2Cl_2 (180 ml) on ice. The reaction mixture was stirred for 1 h on ice, and for 2 h at room temperature. After evaporation, the crude residue was purified by column chromatography on silica gel (AcOEt/Hexane, 1:1) to give compound **3-1** (1.62 g, 3.18 mmol, 55%) as a colorless oil. ^{1}H NMR (400 MHz; $CDCl_3$): δ 8.36 (s, 1H), 8.20−8.15 (m, 2H), 7.72 (t, 1H, J = 8.0 Hz), 7.36−7.31 (m, 5H), 6.24 (br, 1H), 5.09 (s, 2H), 4.73 (br, 1H), 3.48 (m, 2H), 3.18 (m, 2H), 1.64 (m, 2H), 1.51−1.29 (m, 14H).

Compound 3-2

To a stirred solution of compound **3-1** (1.50 g, 2.95 mmol) in dry CH_2Cl_2 (70 ml) was added a solution of *N*-(*tert*-butoxycarbonyl)-1,5-diaminopentane (658 mg, 3.25 mmol) and DIEA (1.54 ml , 8.84 mmol) in dry CH_2Cl_2 (10 ml). The reaction mixture was stirred overnight at room temperature, and washed with 5% aqueous citric acid (100 ml) and brine (100 ml). The organic layer was dried over anhydrous $MgSO_4$, filtered and evaporated to give compound **3-2** (1.99 g, 2.95 mmol, quant.) as a white solid. ^{1}H NMR (400 MHz; $CDCl_3$): δ 8.27 (s, 1H), 8.04 (d, 1H, *J* = 7.6 Hz), 7.97 (d, 1H, *J* = 6.8 Hz), 7.58 (t, 1H, *J* = 8.0 Hz), 7.36−7.29 (m, 5H), 6.67 (br, 1H), 5.13 (br, 1H), 5.09 (s, 2H), 4.81 (br, 1H), 4.60 (br, 1H), 3.43 (m, 2H), 3.18 (m, 2H), 3.02 (m, 2H), 2.94 (m, 2H), 1.60 (m, 2H), 1.48−1.27 (m, 29H).

Compound 3-3

To a stirred solution of compound **3-2** (1.99 g, 2.95 mmol) in dry MeOH (70 ml) and AcOEt (20 ml) was added 10 wt.% Pd/C (1.00 g). The reaction mixture was stirred for 7 h at room temperature under H_2 atmosphere. After filtration, the filtrate was evaporated to give compound **3-3** (1.36 g, 2.51 mmol, 85%) as a colorless oil. ^{1}H NMR (400 MHz; CD_3OD): δ 8.27 (s, 1H), 8.03−7.97 (m, 2H), 7.66 (t, 1H, *J* = 8.0 Hz), 3.38 (t, 2H, *J* = 7.2 Hz), 2.96 (t, 2H, *J* = 7.2 Hz), 2.86 (t, 2H, *J* = 7.2 Hz), 2.66 (t, 2H, *J* = 7.2 Hz), 1.63 (m, 2H), 1.50−1.25 (m, 29H).

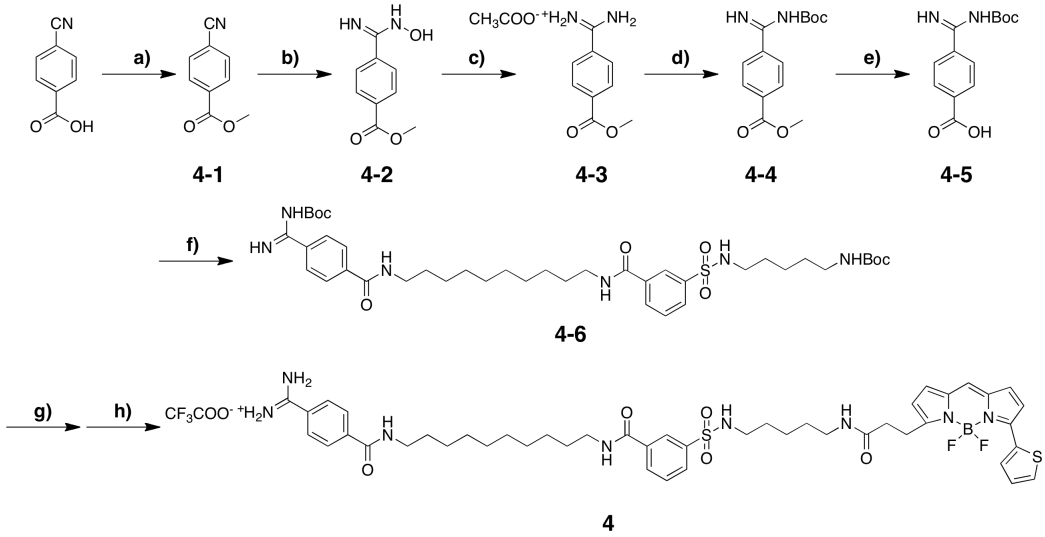
Compound 3-4

To a stirred solution of compound **3-3** (53 mg, 0.098 mmol) in dry DMF (2 ml) was added biotin-NHS ester[S1] (32 mg, 0.094 mmol). The reaction mixture was stirred overnight at room temperature. After evaporation, the crude residue was purified by reprecipitation with MeOH and water (1:5). The precipitate was collected by filtration and dried *in vacuo* to give compound **3-4** (63 mg, 0.082 mmol, 87%) as a white solid. ^{1}H NMR (400 MHz; CD_3OD): δ 8.28 (s, 1H), 8.02 (d, 1H, *J* = 8.0 Hz), 7.98 (d, 1H, *J* = 8.0 Hz), 7.66 (t, 1H, *J* = 8.0 Hz), 4.50−4.46 (m, 1H), 4.31−4.28 (m, 1H), 3.38 (t, 2H, *J* = 6.8 Hz), 3.22−3.13 (m, 3H), 2.99−2.89 (m, 3H), 2.86 (t, 2H, *J* = 7.2 Hz), 2.70 (d, 1H, *J* = 12.4 Hz), 2.18 (t, 2H, *J* = 7.2 Hz), 1.42 (s, 9H), 1.74−1.24 (m, 28H).

Compound 3

To a stirred solution of compound **3-4** (6.5 mg, 8.5 μmol) in CH_2Cl_2 (4 ml) was added TFA (1 ml). The reaction mixture was stirred for 1 h at room temperature. After co-evaporation with toluene (10 ml), the residue was dried *in vacuo* to yield a deprotected form of compound **3-4**. To a solution of the deprotected compound **3-4** in dry DMF (1.0 ml) was added compound **1-3** (2.5 mg, 5.6 μmol) and DIEA (8 μl, 46 μmol). The reaction mixture was stirred for 1 h at room temperature. After evaporation, the crude residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH, 10:1) to give compound **3** (5.2 mg, 5.2 μmol, 93%) as a purple solid. ^{1}H NMR (400 MHz; CD_3OD): δ 8.28 (s, 1H), 8.11 (d, 1H, *J* = 4.0 Hz), 8.02 (d, 1H, *J* = 7.6 Hz), 7.98 (d, 1H, *J* = 8.4 Hz), 7.67−7.63 (m, 2H), 7.44 (s, 1H), 7.20−7.16 (m, 2H), 7.13 (d, 1H, *J* = 4.4 Hz). 6.87 (d, 1H, *J* = 4.0 Hz), 6.45 (d, 1H, *J* = 4.0 Hz), 4.48−4.45 (m, 1H), 4.29−4.26 (m, 1H), 3.37 (t, 2H, *J* = 7.2 Hz), 3.20−3.09 (m, 5H), 2.90 (dd, 1H, *J* = 12.8, 5.2 Hz), 2.86 (t, 2H, *J* = 7.2 Hz), 2.69 (d, 1H, *J* = 12.8 Hz), 2.64−2.60 (m, 2H), 2.17 (t, 2H, *J* = 6.8 Hz), 1.74−1.24 (m, 30H). HR-FAB MS (NBA): calcd for $C_{48}H_{66}BF_2N_8O_6S_3$ $[M+H]^+$ = 995.4329; obsd 995.4335.

Synthesis of probe 4



Scheme S4. Synthetic scheme of probe **4**. Reaction conditions: a) HCl in MeOH; b) NH_2OH/HCl and K_2CO_3 in MeOH/H_2O; c) Ac_2O, H_2 and Pd/C in AcOH; d) Boc_2O and TEA in dry MeOH; e) 1 N NaOHaq./MeOH; f) **3-3**, WSC•HCl, HOBt•H_2O and DIEA in dry DMF; g) TFA in CH_2Cl_2; h) **1-3** and DIEA in dry DMF.

Compound 4-1

To a stirred solution of 4-cyanobenzoic acid (2.6 g, 17.7 mmol) in MeOH (40 ml) was added conc. HCl aq (5 ml). The reaction mixture was stirred overnight at room temperature. After evaporation, the residue was dissolved in AcOEt (400 ml) and washed with saturated aqueous $NaHCO_3$ (200 ml) and brine (200 ml). The organic layer was dried over anhydrous $MgSO_4$, filtered and evaporated to give compound **4-1** (2.5 g, 15.5 mmol, 88%) as a white solid. ^{1}H NMR (400 MHz; $CDCl_3$): δ 8.14 (d, 2H, J = 8.0 Hz), 7.75 (d, 2H, J = 8.0 Hz), 3.97 (s, 3H).

Compound 4-2

To a stirred solution of compound **4-1** (2.5 g, 15.5 mmol) in MeOH (240 ml) and water (120 ml) was added NH_2OH/HCl (1.3 g, 19 mmol) and K_2CO_3 (1.1 g, 7.96 mmol). The reaction mixture was refluxed overnight at 90 ℃. After evaporation, the residue

was dissolved in CH_2Cl_2 (500 ml) and washed with water (300 ml, 2 times). The organic layer was dried over anhydrous $MgSO_4$, filtered and evaporated to give compound **4-2** (1.4 g, 7.21 mmol, 47%) as a white solid. ^{1}H NMR (400 MHz; $CDCl_3$): δ 8.03 (d, 2H, *J* = 8.8 Hz), 7.76 (d, 2H, *J* = 8.8 Hz), 3.92 (s, 3H).

Compound 4-3

To a stirred solution of compound **4-2** (0.76 g, 3.91 mmol) in AcOH (20 ml) was added Ac_2O (530 μl, 5.61 mmol). After stirring at room temperature for 5 min, 10 wt.% Pd/C (200 mg) was added to the above solution. The reaction mixture was stirred for 2 h at room temperature under H_2 atmosphere. The solution was filtered and evaporated to give compound **4-3** (0.80 g, 3.36 mmol, 86%) as a white solid. ^{1}H NMR (400 MHz; CD_3OD): δ 8.22 (dd, 2H, *J* = 8.8 Hz), 7.90 (d, 2H, *J* = 8.8 Hz), 3.97 (s, 3H).

Compound 4-4

To a stirred solution of compound **4-3** (0.51 g, 2.14 mmol) and TEA (150 μl, 1.08 mmol) in dry MeOH (50 ml) was added Boc_2O (0.71 g, 3.25 mmol). The reaction mixture was stirred for 4 h at 40 ℃. After evaporation, the crude residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH = 5:1) to give compound **4-4** (0.46 g, 1.65 mmol, 77%) as a yellow oil. ^{1}H NMR (400 MHz; CD_3OD): δ 8.10 (d, 2H, *J* = 8.4 Hz), 7.89 (d, 2H, *J* = 8.4 Hz), 3.94 (s, 3H), 1.53 (s, 9H).

Compound 4-5

To a stirred solution of compound **4-4** (100 mg, 0.36 mmol) in MeOH (10 ml) was added 1 N NaOH (5 ml). The reaction mixture was stirred for 2 h at room temperature. After evaporation, the residue was dissolved in water. The solution was acidified to pH 4 with 1 N HCl, and extracted with AcOEt (200 ml, 2 times). The organic layer was dried over anhydrous $MgSO_4$, evaporated and dried *in vacuo* to give compound **4-5** (83 mg, 0.31 mmol, 86%) as a white solid. ^{1}H NMR (400 MHz; CD_3OD): δ 8.11 (d, 2H, *J* = 8.4 Hz), 7.83 (d, 2H, *J* = 8.4 Hz), 1.57 (s, 9H).

Compound 4-6

To a stirred solution of compound **3-3** (27 mg, 0.050 mmol) in dry DMF (1 ml) was added compound **4-5** (13 mg, 0.049 mmol), WSCI•HCl (9 mg, 0.047 mmol), HOBt•H_2O (7 mg, 0.046 mmol) and DIEA (17 μl, 0.098 mmol). The reaction mixture was stirred overnight at room temperature. After evaporation, the resude was dissolved in AcOEt (20 ml) and washed with saturated aqueous $NaHCO_3$ (30 ml) and brine (30 ml, 2 times). The organic layer was dried over anhydrous Na_2SO_4, filtered, and evaporated to give compound **4-6** (46 mg, 0.058 mmol, quant) as a yellow oil. ^{1}H NMR (400 MHz; $CDCl_3$): δ 8.25 (s, 1H), 8.00 (d, 1H, *J* = 8.0 Hz), 7.92 (d, 1H, *J* = 8.0 Hz), 7.79 (d, 2H, *J* = 8.8 Hz), 7.69 (d, 2H, *J* = 8.8 Hz), 7.52 (t, 1H, *J* = 7.6 Hz), 7.16 (br, 1H), 6.89 (br, 1H), 5.58 (br, 1H), 4.68 (br, 1H), 3.39 (m, 4H), 2.97 (dd, 2H, *J* = 12.8, 6.8 Hz), 2.89 (m, 2H), 1.53 (s, 9H), 1.42 (s, 9H), 1.62-1.23 (m, 22H).

Compound 4

To a stirred solution of compound **4-6** (8.5 mg, 10.8 μmol) in CH_2Cl_2 (4.5 ml) was added TFA (0.5 ml). The reaction mixture was stirred for 1 h at room temperature. After co-evaporation with toluene (10 ml), the residue was dried *in vacuo* to yield a deprotected form of compound **4-6**. To a solution of the deprotected compound **4-6** in dry DMF (1.0 ml) was added compound **1-3** (2.5 mg, 5.6 μmol) and DIEA (1.8 μl, 10.3 μmol). The reaction mixture was stirred at room temperature for 1 h. After evaporation, the crude residue was purified by reversed phase HPLC (C18 (ODS) column; a linear gradient of solution A/solution B, 10:90 to 70:30 over 40 min; solution A, CH_3CN with 0.1% TFA; solution B, H_2O with 0.1% TFA) to give compound **4** (1.2 mg, 1.17 μmol, 21%) as a purple solid. ^{1}H NMR (400 MHz; CD_3OD): δ 8.27 (s, 1H), 8.11 (d, 1H, *J* = 4.0 Hz), 8.01−7.97 (m, 4H), 7.86 (d, 2H, *J* = 8.8 Hz), 7.67−7.63 (m, 2H), 7.44 (s, 1H), 7.20−7.16 (m, 2H), 7.13 (d, 1H, *J* = 4.0 Hz), 6.87 (d, 1H, *J* = 3.6 Hz), 6.44 (d, 1H, *J* = 4.0 Hz), 3.34 (m, 4H), 3.10 (t, 2H, *J* = 6.8 Hz), 2.85 (t, 2H, *J* = 6.8 Hz), 2.62 (t, 2H, *J* = 8.0 Hz), 1.61−1.24 (m, 24H). HR-FAB MS (NBA): calcd for $C_{46}H_{58}BF_2N_8O_5S_2$ $[M+H]^+$ = 915.4033; obsd 915.4030.

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