### Discovery of coumarin derivatives as fluorescence acceptors for intrinsic fluorescence resonance energy transfer of proteins

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### **Supplementary Information**

#### Experimental procedures, materials, and instrumentation

All chemicals were purchased from Sigma-Aldrich Chemical Co. or TCI and used without further purification, unless indicated otherwise. Thin-layer chromatography was performed using a Merck 60 F254 pre-coated silica gel plates which were visualized under ultraviolet light (254 nm), or by staining with KMnO<sub>4</sub> or Ninhydrin solutions followed by heating on a hot plate. Flash chromatography was performed with Merck silica gel (60 Å, 230–400 Mesh). Melting points, measured in capillary tubes, were uncorrected. IR spectra were recorded on a Bruker alpha-P FT-IR spectrometer. Optical rotations were determined at 30 °C using Autopol IV from Rudolph research analytical. Proton (<sup>1</sup>H) and carbon (<sup>13</sup>C) NMR spectra were recorded by a Varian Inova 400NB- and Inova 600 spectrometer in DMSO-*d*<sub>6</sub> unless otherwise stated. Chemical shifts ( $\delta$ ) are reported in the unit of parts per million (ppm) with reference to CDCl<sub>3</sub> (<sup>1</sup>H, 7.27; <sup>13</sup>C, 77.00), CD<sub>3</sub>OD (<sup>1</sup>H, 3.31; <sup>13</sup>C, 49.00), D<sub>2</sub>O (<sup>1</sup>H, 4.79) or DMSO-*d*<sub>6</sub> (<sup>1</sup>H, 2.50; <sup>13</sup>C, 39.51). The following abbreviations are used for the proton spectra multiplicities: s, singlet; d, doublet; t, triplet; q, quartet; qu, quintuplet; m, multiplet; br, broad. Coupling constants (*J*) reported in Hertz (Hz). Mass spectra were obtained using electrospray ionization and a time of flight analyzer (ESI-MS) mass spectrophotometer, from Agilent Technologies, Inc 1100 + G1958.

#### S1 Experimantal

#### Synthesis of Probe I



#### S.1.1 Methyl 3-(7-hydroxy-2-oxo-2H-chromen-3-yl)acrylate (1)

2,4-Dihydroxybenzaldehyde (1.5 g, 10.7 mmol) was dissolved in EtOH (30 mL). Dimethyl glutaconate (1.7 mL, 11.3 mmol) was added, followed by 6 drops of piperidine, and the reaction mixture was refluxed for 24 h. The reaction mixture was allowed to slowly cool to room temperature and was subsequently cooled to -20 °C. The yellow crystals were collected and dried to give the title compound in 86% yield (2 g). <sup>1</sup>H NMR (600 MHz, DMSO-d<sub>6</sub>)  $\delta$  10.92 (s, 1H), 8.40 (s, 1H), 7.56 (d, *J* = 8.6 Hz, 1H), 7.51 (d, *J* = 15.9 Hz, 1H), 6.88 – 6.82 (m, 2H), 6.74 (d, *J* = 1.6 Hz, 1H), 3.73 (s, 3H); <sup>13</sup>C NMR (600 MHz, DMSO-d<sub>6</sub>)  $\delta$  167.37, 163.55, 159.79, 156.01, 146.18, 139.73, 131.47, 119.94, 116.87, 114.65, 112.14, 102.59, 52.15; MS (ESI) *m*/*z* 269.1 [M + Na]<sup>+</sup>; IR v<sub>max</sub> (thin film)/cm<sup>-1</sup> 3253, 3045, 1713, 1692, 1594.

#### S.1.2 3-(7-hydroxy-2-oxo-2H-chromen-3-yl)acrylic acid (2)

Acrylate **1** (0.5 g, 2 mmol) was dissolved in a 33% HBr solution (5 mL) and the reaction mixture was stirred under reflux for 14 h. After completion of the reaction, as indicated by TLC analysis, the solvent was evaporated. The reaction mixture was diluted with H<sub>2</sub>O and subsequently extracted with EtOAc. The crude product was subjected to flash column chromatography purification (MeOH:CHCl<sub>3</sub>, 1:10) to give the title product as yellow solid in 54% yield (0.25 g). Mp 274-275 °C; <sup>1</sup>H NMR (600 MHz, DMSO-d<sub>6</sub>)  $\delta$  8.39 (s, 1H), 7.57 (d, *J* = 8.6 Hz, 1H), 7.47 (d, *J* = 15.9 Hz, 1H), 6.85 (dd, *J* = 8.5, 2.3 Hz, 1H), 6.81 (d, *J* = 15.9 Hz, 1H), 6.75 (d, *J* = 2.2 Hz, 1H); <sup>13</sup>C NMR (600 MHz, DMSO-d<sub>6</sub>)  $\delta$  168.26, 163.33, 159.89, 155.93, 145.62, 139.13, 131.39, 121.58, 117.16, 114.59, 112.20, 102.58; IR v<sub>max</sub> (thin film)/cm<sup>-1</sup> 3340, 1716, 1667, 1598. Anal. Calc. for C<sub>12</sub>H<sub>8</sub>O<sub>5</sub>(H<sub>2</sub>O)<sup>1</sup>/<sub>3</sub>; C, 60.51; H, 3.67.

#### S.1.3 tert-butyl (2-(3-(7-hydroxy-2-oxo-2H-chromen-3-yl)acrylamido)ethyl)carbamate (3)

Acrylic acid 2 (0.05 g, 0.22 mmol) was dissolved in N,N-dimethyl formamide (DMF, 0.5 mL) followed by the addition of a 0.1M solution of mono-N-Boc-ethylenediamine (0.04 g, 0.26 mmol) in dichloromethane. The reaction mixture was cooled to 0  $^{\circ}$ C, N-(3-dimethylaminopropyl)-N'-

ethylcarbodiimide hydrochloride (EDC, 0.05 g, 0.26 mmol) and 4-(dimethylamino)pyridine (DMAP, 0.002 g, 0.01 mmol), dissolve in dichloromethane (1 mL), were added. The reaction mixture was allowed to warm-up to room temperature over a period of 1h and stirring continued for 12h. The reaction mixture was diluted with sat. aq. NaHCO<sub>3</sub> (3 mL) and subsequently extracted with EtOAc (3 times). The combined organic layer was washed with 10% aq. citric acid. The organic extracts were washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, and concentrated *in vacuo*. The crude product was purified by flash chromatography (MeOH : DCM, 1:20) to yield the title compound in 48% yield (0.04 g). Mp 218-219 °C; <sup>1</sup>H NMR (600 MHz, DMSO-d<sub>6</sub>)  $\delta$  8.24 (s, 2H), 7.58 (d, *J* = 8.6 Hz, 1H), 7.30 (d, *J* = 15.5 Hz, 1H), 7.04 (d, *J* = 15.6 Hz, 1H), 6.84 (dd, *J* = 8.5, 2.2 Hz, 2H), 6.75 (d, *J* = 2.1 Hz, 1H), 3.20 (dd, *J* = 12.4, 6.3 Hz, 2H), 3.04 (dd, *J* = 12.3, 6.2 Hz, 2H), 1.38 (s, 9H); <sup>13</sup>C NMR (600 MHz, DMSO-d<sub>6</sub>)  $\delta$  165.98, 162.93, 159.86, 156.25, 155.56, 145.09, 134.40, 131.07, 125.12, 117.81, 114.47, 112.29, 102.49, 78.33, 40.39, 39.59, 28.89; MS (ESI) *m/z* 397.2 [M + Na]<sup>+</sup>; IR v<sub>max</sub> (thin film)/cm<sup>-1</sup> 3326, 2974, 1702, 1603, 1529. Anal. Calc. for C<sub>19</sub>H<sub>22</sub>N<sub>2</sub>O<sub>6</sub>(H<sub>2</sub>O)<sup>3</sup>/<sub>2</sub>; C, 56.85; H, 6.28; N, 6.98. Found: C, 57.25; H, 6.34; N, 6.96.

#### S.1.4 N-(2-aminoethyl)-3-(7-hydroxy-2-oxo-2H-chromen-3-yl)acrylamide (4)

Amide **3** (0.09 g, 0.24 mmol) was dissolved in a 1:1(v/v) mixture of TFA and DCM (2 mL) at 0°C. The reaction was stirred at room temperature for 0.5 h. The reaction mixture was concentrated to provide the crude title compound as its TFA salt in a yield of 97% (0.09 g).

mp 222-223 °C; <sup>1</sup>H NMR (600 MHz, DMSO-d<sub>6</sub>)  $\delta$  8.44 (t, J = 5.6 Hz, 1H), 8.27 (s, 1H), 7.58 (d, J = 8.6 Hz, 1H), 7.33 (d, J = 15.6 Hz, 1H), 7.07 (d, J = 15.6 Hz, 1H), 6.86 (dd, J = 8.5, 2.2 Hz, 1H), 6.77 (d, J = 2.1 Hz, 1H), 3.41 (d, J = 5.9 Hz, 2H), 3.10 (dt, J = 17.3, 8.6 Hz, 1H), 2.93 (d, J = 6.3 Hz, 2H), 1.18 (t, J = 7.3 Hz, 1H); <sup>13</sup>C NMR (600 MHz, DMSO-d<sub>6</sub>)  $\delta$  166.65, 163.11, 159.87, 155.62, 145.47, 134.94, 131.14, 124.62, 117.60, 114.54, 112.25, 102.51, 37.40, 9.23 ; IR v<sub>max</sub> (thin film)/cm<sup>-1</sup> 3288, 2920, 1667, 1591, 1557, 1124. Anal. Calc. for C<sub>14</sub>H<sub>14</sub>N<sub>2</sub>O<sub>4</sub>TFA<sub>2</sub>(H<sub>2</sub>O)<sup>2</sup>/<sub>3</sub>; C, 42.03; H, 3.4; N, 5.45. Found: C, 42.04; H, 3.18; N, 5.22.

# S.1.5 N-(2-(3-(7-hydroxy-2-oxo-2H-chromen-3-yl)acrylamido) ethyl)-5-(2-oxohexahydro-1H-thieno[3,4-d] imidazol-4-yl) pentanamide (I)

Crude TFA salt **4** (0.1 g, 0.26 mmol) and biotin N-hydroxysuccinimide ester (0.089 g, 0.26 mmol) were dissolved in DMF (2 mL) and the reaction mixture was cooled to 0  $^{\circ}$ C. Triethylamine (Et<sub>3</sub>N) (0.06 mL, 0.42 mmol) was added to the reaction mixture and stirred for 5 h followed by the addition of H<sub>2</sub>O. The reaction mixture was extracted with EtOAc. The solvent was evaporated and the title product was obtained by recrystallization from MeOH and Et<sub>2</sub>O in a yield of 46%. (0.06 g)

 $[\alpha]_D{}^{30} = + 27.7^{\circ}$  (c= 0.5, DMSO); Mp 220-221 °C; <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>)  $\delta$  8.41 – 8.13 (m, 2H), 7.87 (t, J = 5.4 Hz, 1H), 7.57 (d, J = 8.6 Hz, 1H), 7.30 (d, J = 15.6 Hz, 1H), 7.03 (d, J = 15.6 Hz, 1H), 6.84 (dd, J = 8.5, 1.9 Hz, 1H), 6.75 (d, J = 2.0 Hz, 1H), 6.38 (d, J = 28.7 Hz, 2H), 4.46 – 4.15 (m, 1H), 4.16 – 4.02 (m, 1H), 3.16 (ddd, J = 20.9, 13.2, 6.4 Hz, 4H), 2.80 (dd, J = 12.4, 5.0 Hz, 1H), 2.56 (d, J = 12.4 Hz, 1H), 2.06 (t, J = 7.4 Hz, 2H), 1.43 (m, J = 38.7, 31.1, 18.5, 10.2 Hz, 8H).; <sup>13</sup>C NMR (400 MHz, DMSO-d<sub>6</sub>)  $\delta$  172.60, 165.73, 163.09, 162.80, 159.63, 155.31, 144.84, 134.17, 130.81, 124.81, 117.48, 114.27, 111.99, 109.99, 102.25, 61.40, 59.60, 55.78, 35.64, 28.57, 28.44, 25.62 ; IR v<sub>max</sub> (thin film)/cm<sup>-1</sup> 3372, 2921, 1730, 1655, 1598, 1567.



S.1.6 tert-butyl (2-(2-(7-hydroxy-2-oxo-2H-chromen-4-yl)acetamido)ethyl)carbamate (5)

7-Hydroxycoumarin-4-acetic acid (2.0 g, 9.1 mmol) was dissolved in DMF (20 mL) and a 1.6M solution of mono-N-Boc-ethylenediamine (1.6 g, 10.0 mmol) in DCM was added. The resulting mixture was cooled to 0 °C and a solution of EDC (2.6 g, 13.6 mmol) and DMAP (54 mg, 0.1 mmol) in DCM (40 mL) was added. The reaction mixture was allowed to warm-up to room temperature over a period of 1h and stirring continued for 12h. The reaction mixture was diluted with sat. aq. NaHCO<sub>3</sub> (60 mL) and extracted with EtOAc (3 times). The combined organic layer was washed with 10% aq. citric acid and the organic extract was washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated *in vacuo*. The residue was purified by flash column chromatography (MeOH: DCM, 1:10) to yield the title compound in 79% yield (2.6 g). Mp 111-113 °C; <sup>1</sup>H NMR (600 MHz, DMSO-d<sub>6</sub>)  $\delta$  10.54 (s, 1H), 8.20 (d, *J* = 5.2 Hz, 1H), 7.59 (d, *J* = 8.7 Hz, 1H), 6.79 (dd, *J* = 8.7, 2.2 Hz, 2H), 6.71 (d, *J* = 2.3 Hz, 1H), 6.16 (s, 1H), 3.63 (s, 2H), 3.09 (dd, *J* = 12.1, 6.1 Hz, 2H), 2.98 (dd, *J* = 12.2, 6.1 Hz, 2H), 1.38 (s, 9H); <sup>13</sup>C NMR (600 MHz, DMSO-d<sub>6</sub>)  $\delta$  168.45, 161.78, 160.88, 156.27, 155.65, 151.73, 127.39, 113.55, 112.47, 112.16, 104.99, 102.90, 78.35, 28.88; IR v<sub>max</sub> (thin film)/cm<sup>-1</sup> 3357, 3331, 3195, 2990, 1693, 1685, 1654.

#### S.1.7 N-(2-aminoethyl)-2-(7-hydroxy-2-oxo-2H-chromen-4-yl)acetamide (6)

Amide **5** (0.05 g, 0.138 mmol) was dissolved in a 1:1 (v/v) mixture of trifluoroacetic acid (TFA) and DCM (2 mL) at 0  $^{\circ}$ C. The reaction mixture was stirred at room temperature for 0.5 h. After removal of the solvents and trice coevaporation with DCM, the crude title compound was obtained as its TFA salt. The crude product was crystallized from MeOH and Et<sub>2</sub>O to give the title compound in a yield of 81% (0.04 g). Mp 111-113  $^{\circ}$ C; <sup>1</sup>H NMR (600 MHz, DMSO-d<sub>6</sub>)  $\delta$  8.42 (t, *J* = 5.5 Hz, 1H), 7.88 (s, 2H), 7.60 (d, *J* = 8.7 Hz, 1H), 6.80 (dd, *J* = 8.7, 2.3 Hz, 1H), 6.73 (d, *J* = 2.3 Hz, 1H), 6.18 (s, 1H), 3.68 (s, 2H), 3.35 (s, 1H), 3.31 (d, *J* = 6.1 Hz, 2H), 2.88 (t, *J* = 6.5 Hz, 2H); <sup>13</sup>C NMR (600 MHz, DMSO-d<sub>6</sub>)  $\delta$  169.21, 161.91, 160.89, 155.67, 151.46, 127.42, 113.59, 112.54, 112.12, 102.94, 39.28, 39.07, 37.36; IR v<sub>max</sub> (thin film)/cm<sup>-1</sup> 3609, 1663, 1603, 1561.

# S.1.8 N-(2-(2-(7-hydroxy-2-oxo-2H-chromen-4-yl)acetamido)ethyl)-5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide (II)

TFA salt **6** (0.05 g, 0.13 mmol) and biotin N-hydroxysuccinimide ester (0.55 g, 0.16 mmol) were dissolved in DMF (1 mL) and cooled to 0  $^{\circ}$ C. Next, Et<sub>3</sub>N (0.036 mL, 0.26 mmol) was added to the reaction mixture and stirred for 5h. The solvent was removed *in vacuo* and the residue was washed with H<sub>2</sub>O. The residue was purified by flash column chromatography (MeOH: DCM, 1:10) to yield

the title compound in a yield of 63% (0.04 g).  $[\alpha]_D{}^{30} = +96.5^\circ$  (c= 0.5, DMSO); Mp 191-192 °C; <sup>1</sup>H NMR (600 MHz, DMSO-d<sub>6</sub>) 8.19 (s, 1H), 7.79 (s, 1H), 7.55 (d, J = 8.8 Hz, 1H), 6.77 (dd, J = 8.7, 2.4 Hz, 1H), 6.69 (d, J = 2.3 Hz, 1H), 6.41 (s, 1H), 6.35 (s, 1H), 6.13 (s, 1H), 4.27 (dd, J = 7.5, 5.3 Hz, 1H), 4.15 – 4.04 (m, 1H), 3.60 (s, 2H), 3.07 (t, J = 7.6 Hz, 5H), 2.78 (dd, J = 12.4, 5.2 Hz, 1H), 2.54 (d, J = 12.4 Hz, 1H), 2.00 (t, J = 7.5 Hz, 2H), 1.63 – 1.18 (m, 7H); <sup>13</sup>C NMR (600 MHz, CDCl<sub>3</sub>)  $\delta$  28.7, 35.9, 105.0, 108.3, 108.6, 114.0 (t, J=21 Hz), 118.0, 122.5, 130.5, 136.2, 137.6 (d, J=243 Hz), 142.1 (d, J=257 Hz), 143.9 (d, J=251 Hz), 151.1, 171.5; IR v<sub>max</sub> (thin film)/cm<sup>-1</sup> 3274, 2928, 1697, 1683, 1638, 1607, 1558, 1543. Anal. Calc. for C<sub>23</sub>H<sub>28</sub>N<sub>4</sub>O<sub>6</sub>S(H<sub>2</sub>O) $\frac{3}{2}$ ; C, 53.58; H, 6.06; N, 10.87. Found: C, 53.26; H, 6.02; N, 10.69.

#### Synthesis of probe III



#### S.1.9 ethyl 2-(7-(dimethylamino)-2-oxo-2H-chromen-4-yl)acetate (7)

To a dry solution of dimethylaminophenol (6.86 g, 0.05 mol) and ethyl acetonedicarboxylate (10 mL, 0.06 mol) in anhydrous ethanol (30 mL), was added anhydrous ZnCl<sub>2</sub> (8.2 g, 0.06 mol), and the reaction mixture was stirred under reflux for 15 h. The reaction mixture was coold to room temperature and poured into an ice-water mixture while vigorously stirring. The resulting dark oil slowly solidified on contact with cold ethanol. The crude product was crystallized from ethanol to yield the title compound in a yield of 25% (3.4 g). <sup>1</sup>H NMR (600 MHz, DMSO-d<sub>6</sub>)  $\delta$  7.40 (d, *J* = 9.0 Hz, 1H), 6.68 (dd, *J* = 9.0, 2.6 Hz, 1H), 6.51 (d, *J* = 2.5 Hz, 1H), 6.01 (s, 1H), 4.07 (q, *J* = 7.1 Hz, 2H), 3.83 (s, 2H), 2.98 (s, 6H), 1.14 (t, *J* = 7.1 Hz, 3H); <sup>13</sup>C NMR (600 MHz, DMSO-d<sub>6</sub>  $\delta$  169.94, 161.24, 156.07, 153.50, 150.29, 126.57, 110.30, 109.77, 108.56, 98.13, 61.46, 40.36, 37.56, 14.66; IR v<sub>max</sub> (thin film)/cm<sup>-1</sup> 2979, 1717, 1595.

#### S.1.10 2-(7-(dimethylamino)-2-oxo-2H-chromen-4-yl)acetic acid (8)

Ethyl 7-dimethylaminocoumarin 7 (1 g, 3.6 mmol) was dissolved in a mixture of THF-H<sub>2</sub>O (3:1, v/v, 6 mL) and cooled to 0 °C. Next, 2 M aq. LiOH solution (3.6 mL, 7.26 mmol) was added dropwise to the reaction mixture and stirring continued for 0.5 h at room temperature. The mixture was diluted with water and subsequently washed with Et<sub>2</sub>O. The aqueous layer was acidified to pH 2 with a 2 M aq. HCl solution. The formed precipitate was collected by filtration and air dried to give the title compound as a yellow solid in 85% yield (0.76 g). <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>)  $\delta$  7.46 (d, *J* = 9.0 Hz, 1H), 6.73 (dd, *J* = 9.0, 2.5 Hz, 1H), 6.55 (d, *J* = 2.5 Hz, 1H), 6.05 (s, 1H), 3.78 (s, 2H), 3.02 (s, 6H); <sup>13</sup>C NMR (400 MHz, DMSO-d<sub>6</sub>)  $\delta$  171.44, 161.32, 156.04, 153.44, 150.87, 126.63, 110.23, 109.75, 108.74, 98.09, 40.35, 37.91; MS (ESI) *m*/*z* 248.1 [M + H]<sup>+</sup>; IR v<sub>max</sub> (thin film)/cm<sup>-1</sup> 1698, 1602.

S.1.11 tert-butyl (2-(2-(7-(dimethylamino)-2-oxo-2H-chromen-4-yl)acetamido)ethyl)carbamate (9) 7-Dimethylaminocoumarin-4-acetic acid 8 (0.5 g, 2 mmol) was dissolved in DMF (5 mL) and a solution of mono-N-Boc-ethylenediamine (0.4 g, 2.2 mmol) in DCM (15 mL) was added. Next, the mixture was cooled to 0  $^{\circ}$ C and a solution of EDC (0.57 g, 3 mmol) and DMAP (0.013 g, 0.1 mmol) in DCM (10 mL) was added. The reaction mixture was allowed to warm-up to room temperature over a period of 1h and stirring continued for 12 h. The reaction mixture was diluted with sat. aq. NaHCO<sub>3</sub> (15 mL) and subsequently extracted with EtOAc. The organic layer was washed with 10% aq. citric acid. The organic layer was washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated *in vacuo*. The residue was washed with Et<sub>2</sub>O and crystallized from EtOH and Et<sub>2</sub>O to yield the title compound in 81% yield (0.63 g). <sup>1</sup>H NMR (600 MHz, DMSO-d<sub>6</sub>)  $\delta$  8.19 (t, *J* = 5.4 Hz, 1H), 7.53 (d, *J* = 9.0 Hz, 1H), 6.80 (t, *J* = 5.5 Hz, 1H), 6.72 (dd, *J* = 9.0, 2.6 Hz, 1H), 6.55 (d, *J* = 2.5 Hz, 1H), 5.99 (s, 1H), 3.59 (s, 2H), 3.09 (dd, *J* = 12.4, 6.3 Hz, 2H), 3.02 (s, 6H), 2.98 (dd, *J* = 12.4, 6.2 Hz, 2H), 1.38 (s, 9H); <sup>13</sup>C NMR (600 MHz, DMSO-d<sub>6</sub>)  $\delta$  168.61, 161.33, 156.27, 156.03, 153.44, 151.80, 126.68, 110.12, 109.68, 108.90, 104.99, 98.13, 78.36, 40.38, 39.62, 39.46, 28.88; MS (ESI) *m/z* 412.2 [M + Na]<sup>+</sup>; IR v<sub>max</sub> (thin film)/cm<sup>-1</sup> 3334, 3272, 2930, 1735, 1682, 1643, 1617.

#### S.1.12 N-(2-aminoethyl)-2-(7-(dimethylamino)-2-oxo-2H-chromen-4-yl)acetamide (10)

Amide **9** (0.08 g, 0.205 mmol) was dissolved in a 1:1 (v/v) mixture of TFA and DCM (2 mL) at 0  $^{\circ}$ C. The reaction was stirred at room temperature for 0.5 h. The TFA salt of the title compound was isolated after removal of the solvent and coevaporation with DCM (x3). The crude product was crystallized from MeOH and Et<sub>2</sub>O to give the title compound in 67% yield (0.05 g). <sup>1</sup>H NMR (600 MHz, DMSO-d<sub>6</sub>)  $\delta$  8.63 (t, J = 5.4 Hz, 1H), 8.10 (s, 3H), 7.59 (d, J = 8.9 Hz, 1H), 6.72 (dd, J = 8.9, 2.1 Hz, 1H), 6.55 (d, J = 2.1 Hz, 1H), 6.03 (s, 1H), 3.66 (s, 2H), 3.33 (dd, J = 12.1, 6.1 Hz, 2H), 3.02 (s, 6H), 2.87 (dd, J = 11.5, 5.7 Hz, 2H).; <sup>13</sup>C NMR (600 MHz, DMSO-d<sub>6</sub>)  $\delta$  169.18, 161.33, 156.04, 153.45, 151.61, 126.85, 110.20, 109.69, 108.89, 98.11, 40.39, 39.30, 39.04, 37.34; MS (ESI) *m/z* 290.2 [M + H]<sup>+</sup>; IR v<sub>max</sub> (thin film)/cm<sup>-1</sup> 3213, 2837, 1677, 1608, 1520.

# *S.1.13 N-(2-(2-(7-(dimethylamino)-2-oxo-2H-chromen-4-yl)acetamido)ethyl)-5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide* (**III**)

TFA salt 10 (0.04 g, 0.1 mmol) and biotin N-hydroxysuccinimide ester (0.41 g, 0.12 mmol) were dissolved in DMF (1 mL) and cooled to 0 °C. Next, Et<sub>3</sub>N (0.03 mL, 0.2 mmol) was added to the reaction mixture and stirred for 5h. The solvents were removed *in vacuo* and the residue washed with H<sub>2</sub>O. The residue was purified by flash column chromatography (MeOH: DCM, 1:10) to yield the title compound in 58% yield (0.03 g).  $[\alpha]_D^{30} = + 88.6^{\circ}$  (c= 0.5, DMSO); Mp 255-256°C; <sup>1</sup>H NMR (600 MHz, DMSO-d<sub>6</sub>)  $\delta$  8.23 (s, 1H), 7.83 (s, 1H), 7.53 (d, 1H), 6.74 (d, 1H), 6.56 (s, 1H), 6.53 (d, 2H), 5.99 (s, 1H), 4.32 (t, 1H), 4.13 (t, 1H), 3.58 (s, 1H), 3.09 (s, 5H), 3.02 (s, 5H), 2.83 (dd, 1H), 2.55 (d, 1H), 2.04 (t, 2H), 1.48 – 1.27 (s, 6H); <sup>13</sup>C NMR (600 MHz, CDCl<sub>3</sub>)  $\delta$  28.7, 35.9, 105.0, 108.3, 108.6, 114.0 (t, J=21 Hz), 118.0, 122.5, 130.5, 136.2, 137.6 (d, J=243 Hz), 142.1 (d, J=257 Hz), 143.9 (d, J=251 Hz), 151.1, 171.5; IR v<sub>max</sub> (thin film)/cm<sup>-1</sup> 3378, 3285, 2936, 1712, 1670, 1634, 1628, 1550. Anal. Calc. for C<sub>25</sub>H<sub>33</sub>N<sub>5</sub>O<sub>5</sub>S(H<sub>2</sub>O)<sup>2</sup>/<sub>3</sub>; C, 56.91; H, 6.56; N, 13.27. Found: C, 57.46; H, 6.61; N, 13.13.



### *S.1.14 N-(2-(naphthalen-1-ylamino)ethyl)-5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide* (*IV*)

N-(1-naphthyl)-ethylenediamine (0.25 g, 1.1 mmol) and biotin N-hydroxysuccinimide ester (0.38 g, 1.1 mmol) were dissolved in DMF (5 mL). At 0 °C, Et<sub>3</sub>N (0.58 mL, 4.1 mmol) was added to the reaction mixture and stirred for 5h. The solvents were removed *in vacuo* and the residue washed with H<sub>2</sub>O. The precipitate was purified by flash column chromatography (MeOH: DCM, 1: 10) to give the title compound in a yield of 83% yield (0.38 g).  $[\alpha]_D{}^{30} = + 88.3^{\circ}$  (c= 0.5, DMSO); <sup>1</sup>H NMR (600 MHz, DMSO-d<sub>6</sub>)  $\delta$  8.07 (dd, *J* = 15.5, 7.1 Hz, 2H), 7.77 – 7.73 (m, 1H), 7.46 – 7.37 (m, 2H), 7.28 (t, *J* = 7.9 Hz, 1H), 7.10 (d, *J* = 8.1 Hz, 1H), 6.55 (d, *J* = 7.6 Hz, 1H), 6.37 (d, *J* = 34.7 Hz, 2H), 6.20 (t, *J* = 5.1 Hz, 1H), 4.25 (dd, *J* = 7.5, 5.2 Hz, 1H), 4.06 – 3.99 (m, 1H), 3.40 (dd, *J* = 12.3, 6.0 Hz, 2H), 3.26 (dd, *J* = 11.8, 6.2 Hz, 2H), 2.99 – 2.92 (m, 1H), 2.75 (dd, *J* = 12.4, 5.1 Hz, 1H), 2.55 (d, *J* = 12.4 Hz, 1H), 2.11 (t, *J* = 7.4 Hz, 2H), 1.68 – 1.48 (m, 3H), 1.48 – 1.37 (m, 1H), 1.34 – 1.24 (m, 2H); <sup>13</sup>C NMR (151 MHz, DMSO-d<sub>6</sub>)  $\delta$  173.59, 163.34, 144.56, 134.68, 128.63, 127.50, 126.26, 124.64, 123.50, 121.98, 116.05, 103.28, 61.62, 59.81, 56.03, 44.30, 38.31, 35.91, 28.78, 28.65, 25.96; MS (ESI) *m/z* 413.3 [M + H]<sup>+</sup>; IR v<sub>max</sub> (thin film)/cm<sup>-1</sup> 3306, 3201, 2939, 1708, 1696, 1633.











Figure S3: <sup>1</sup>H-NMR spectrum of compound 2



Figure S4: <sup>13</sup>C-NMR spectrum of compound 2





Figure S5: <sup>1</sup>H-NMR spectrum of compound 3

Figure S6: <sup>13</sup>C-NMR spectrum of compound 3



Figure S7: <sup>1</sup>H-NMR spectrum of compound 4



Figure S8: <sup>13</sup>C-NMR spectrum of compound 4



Figure S9: <sup>1</sup>H-NMR spectrum of compound I



Figure S10: <sup>13</sup>C-NMR spectrum of compound I



Figure S11: <sup>1</sup>H-NMR spectrum of compound 5





Figure S12: <sup>13</sup>C-NMR spectrum of compound 5

Figure S13: <sup>1</sup>H-NMR spectrum of compound 6





Figure S14: <sup>13</sup>C-NMR spectrum of compound 6

Figure S15: <sup>1</sup>H-NMR spectrum of compound II



Figure S16: <sup>13</sup>C-NMR spectrum of compound II



Figure S17: <sup>1</sup>H-NMR spectrum of compound 7



Figure S18: <sup>13</sup>C-NMR spectrum of compound 7



Figure S19: <sup>1</sup>H-NMR spectrum of compound 8



Figure S20: <sup>13</sup>C-NMR spectrum of compound 8



Figure S21: <sup>1</sup>H-NMR spectrum of compound 9



Figure S22: <sup>13</sup>C-NMR spectrum of compound 9



Figure S23: <sup>1</sup>H-NMR spectrum of compound 10





Figure S25: <sup>1</sup>H-NMR spectrum of compound III



Figure S26: <sup>13</sup>C-NMR spectrum of compound III



Figure S27: <sup>1</sup>H-NMR spectrum of compound IV



Figure S28: <sup>13</sup>C-NMR spectrum of compound IV

#### S2 Calculation of R0 and iFRET efficiency

iFRET efficiencies and Förster distances between the donor and the acceptor fluorophores were calculated according to previously described methods.[11c] The overlap integral ( $J(\lambda)$ ) expresses the degree of spectral overlap between the donor emission and the acceptor absorption. From various spectral overlap calculation methods, a method was chosen in which  $J(\lambda)$  is expressed in  $M^{-1}$ cm<sup>-1</sup>nm<sup>4</sup>, expressed in the following equation's:

$$A = \varepsilon_A(\lambda) c l \tag{1}$$

$$F_{\rm D}(\lambda) = \frac{f_{\rm D}(\lambda)}{\int_0^\infty f_{\rm D}(\lambda) d\lambda}$$
(2)

$$J(\lambda) = \int_0^\infty F_{\rm D}(\lambda) \,\varepsilon_{\rm A}(\lambda) \,\lambda^4 \,\mathrm{d}\lambda \tag{3}$$

The first equation gives the relationship of the absorption A in which  $\varepsilon_A(\lambda)$  represents the molar extinction coefficient of the acceptor, at each wavelength  $(M^{-1}\text{cm}^{-1})$ , c is the micro-molar concentration and l is the cell diameter (1 cm). The molar concentration of each probe was measured with a UV spectrophotometer.

In case of probe **II** for example:

$$\epsilon_{A(300 \sim 420 \text{ nm})} = A_{(300 \sim 420 \text{ nm})} / (0.0000144 \text{ M})*(1 \text{ cm})$$

In the second equation,  $F_D(\lambda)$  represents the normalized donor emission spectrum, in which the total area is set to 1. In this equation,  $f_D(\lambda)$ , the specific wavelength intensity, is divided by the total fluorescence intensity,  $\int_0^{\infty} f_D(\lambda) d\lambda$ , and the wavelength interval at which each probe is measured is depicted in ESI-S5 (spectra overlap area) e.g. the interval at which probe **II** was measured is from 300 nm to 420 nm.

In case of probe **II** for example:

 $F_{D(340 \text{ nm})} = f_{D(340 \text{ nm})} / \int_{300}^{420} f_{D}(\lambda) d\lambda$ 

The third equation, describes the calculation of the overlap integral ( $J(\lambda)$ ), of the intervals described in ESI-S5 (spectra-overlap area). For example, the interval of probe **II** is 300 nm to 420 nm. The parameter for the integration was decided at 1 nm intervals. To this end,  $F_D(\lambda) \varepsilon_A(\lambda) \lambda^4 d\lambda$  was calculated for all the wavelengths in this interval, followed by their integration.

In case of probe **II** for example:

$$J_{(340 \text{ nm})} = \int_{300}^{420} F_{\rm D}(\lambda) \, \varepsilon_{\rm A}(\lambda) \, \lambda^4 \, \mathrm{d}\lambda$$

The Förster distances ( $R_0$ ), in nm, were calculated by substituting  $J(\lambda)$  into the following equation:

$$R_0 = 0.0211 [\kappa^2 n^{-4} Q_{\rm D} J(\lambda)]^{1/6}$$
(5)

The factor 0.0211 is a constant originating. Kappa<sup>2</sup> ( $\kappa^2$ ) is a geometrical factor that relates the orientation of the donor and acceptor transition moments and  $\kappa^2$  varies from 0 to 4. Assuming fluorescence oriental randomization of the donor and the acceptor, unhinded and independent rotation of dipoles, a value of 2/3 is obtained for  $\kappa^2$  (natural state). In this "Förster distance equation", *n* is the refraction index of the solvent (*n* phosphate buffer (pH 7.4)) = 1.333). In this equation *Q*<sub>D</sub> is the quantum yield of the donor (tryptophan = 0.2).<sup>Reference 20 in the main paper</sup> The Förster distance (*R*<sub>0</sub>) depends on the overlap integral of the donor emission with the acceptor absorption (*J*( $\lambda$ )) (equation 5)

In case of probe **II** for example:

$$R_0 = 0.0211[(2/3)(1.333)^{-4}(0.2) (J_{300\sim420 \text{ nm}})]^{1/6}$$

Energy transfer efficiency is measured by a decrease in the steady-state fluorescence emission intensity of the donor, in the absence  $(I_d)$  and presence  $(I_{da})$  of the acceptor, given by the following equation:

$$\mathbf{E} = 1 - \mathbf{I}_{da} / \mathbf{I}_d \tag{6}$$

 $I_{da}$  or  $I_d$  is area of the total wavelength intensity and the interval of each probe is depicted in Table 2(main paper). In the fixed interval, Energy transfer efficiency is calculated by the sum of  $I_{da}$  or  $I_d$ .



S3 Emission spectra of probes I, II, III and IV versus BSA and SAV by excitation at 280 nm

**Figure S28**. The fluorescence was measured after binding reactions by excitation at 280 nm. The slits of excitation and emission were at 10 nm. (The concentration of all molecules is 150 nM in (a),(c) and is 50 nM in (b),(d))

- (a) A, BSA alone; B, SAV alone; C, Probe I alone; D, BSA + Probe I; E, SAV + Probe I
  (b) A, BSA alone; B, SAV alone; C, Probe II alone; D, BSA + Probe II; E, SAV + Probe II
  (c) A, BSA alone; B, SAV alone; C, Probe III alone; D, BSA + Probe III; E, SAV + Probe III
- (d) A, BSA alone; B, SAV alone; C, Probe IV alone; D, BSA + Probe IV; E, SAV + Probe IV



S4 Emission spectra of probes II versus SAV and denatured SAV by excitation at 280 nm

**Figure S29.** The fluorescence was measured after binding reactions by excitation at 280 nm. The slits of excitation and emission were at 10 nm, 6 nm. (The concentration of all molecules is 50 nM in (a),(b))

(a) A, Denatured SAV alone; B, SAV alone; C, Probe II alone; D, Denatured SAV + Probe II; E, SAV + Probe II ( in pH 7.4 1x PBS buffer)

(**b**) A, Denatured SAV alone; B, SAV alone; C, Probe II alone; D, Denatured SAV + Probe II; E, SAV + Probe II ( in 0.3 % SDS contained pH 7.4 1x PBS buffer)

Streptavidin denaturation was performed by heating a solution of SAV to 100  $\degree$ C for 10 min. When the SAV was denatured in the absence of sodium dodecyl sulfate (SDS) solution, the iFRET effect was observed due to refolding of SAV. To prevent refolding, the experiment was repeated in the presence of 0.3 % SDS, resulting in the disappearance of the iFRET effect, as shown in spectra **b**.



### S5 Absorption spectra of probes I ~ IV and emission spectrum of SAV by excitation at 280 nm

**Figure S30.** The figure showed the emission spectrum of donor (SAV) and the absorption spectra of acceptors (probes). A, emission spectrum of SAV; B, absorption spectrum of probe I; C, absorption spectrum of probe II; D, absorption spectrum of probe III; E, absorption spectrum of probe IV.

As shown in the above figure, probe III showed weak absorption, which results from lower extinction coefficient than others. The following table showed the extinction coefficients of the probes.

Probe	<b>E</b> A <sup><i>a</i></sup>	$\mathbf{\Phi}_{\mathbf{f}^b}$
Ι	389000	0.26
II	613000	0.47
III	254000	0.06
IV	727000	0.50

Table S1. Extinction coefficients and quantum yields of the probes  $I \sim IV$ .

<sup>a</sup>Extinction coefficient. <sup>b</sup>Quantum yield