Discrimination between Streptavidin and Avidin with Fluorescent Affinity-Based Probes

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Supporting Information

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Experimental

Materials and Reagents

All chemicals were purchased from Aladdin Corporation and used without further purification. Ultra-pure water was prepared through Sartorius Arium 611DI system.

Spectral measurement

Stock solutions of the dyes (3×10^{-4} M) were prepared in DMF, while those of biotin (14.4×10^{-3} M), SA and AV (1.0 mg/mL) were prepared in PBS (10 mM, pH 7.0). The stock solutions of the dyes were diluted with corresponding solvents to acquired 10 μM dye solution. Absorption spectra were measured with an Evolution 220 UV-Visible spectrophotometer (Thermo Scientific). Fluorescent spectra were carried out on a Lumina Fluorescence Spectrometer (Thermo Scientific). NMR spectra were performed with a Bruker AV-400 spectrometer (400M Hz). Mass spectra were recorded on a MA 1212 Instrument using standard condition (ESI, 70 ev).

Titration of SA/AV by the probe

20 μL of the dye stock solution were added to 2 mL of the phosphate above buffer solution (10 mM, pH 7.0) to keep the dye concentration being 3 μM. 0 - 200 μL of 1.0 mg/mL SA or AV PBS solutions were added into 2 mL of the dye solution (3 μM).

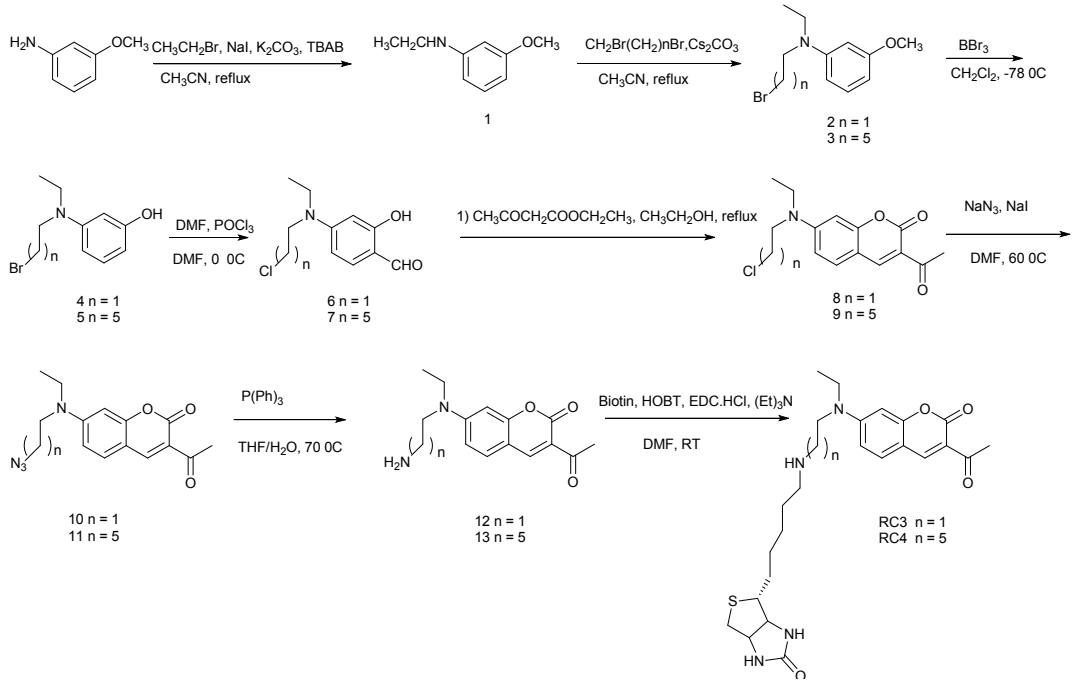
Titration of probe by SA/AV

200 μL of 1mg/mL SA or AV stock solution were added to 1.8 mL of the PBS to keep the protein concentration being 100 μg/mL. 0-72 μL of the dye stock solutions were added into 2 mL of 100 μg/mL protein solution.

Competitive fluorescence assay of biotin

To 2 mL of the dye solution (3 μM) added 60 μL of 1.0 mg/mL SA - PBS solution followed by adding 20 μL of the biotin stock solution after 10 min.

Synthesis



Scheme S1 Synthesis procedures of RC3 and RC4

Synthesis of compound 1: Commercially available 3-methoxyaniline (1.67 g, 13.6 mmol), K_2CO_3 (2.82 g, 20.4 mmol), NaI (203.9 mg, 1.36 mmol) and TBAB (438 mg, 1.36 mmol) were added to acetonitrile (80 mL) in a 250 mL flask. 1.48 g (13.6 mmol) of bromoethane were added slowly to the above solution, and the mixed solution was refluxed for 8 h. After filtration, the solvent was removed under reduced pressure, and the brown residue was purified by column chromatography to give colourless oil (1.14 g, yield: 55.4%). ^{1}H NMR (400MHz, $CDCl_3$) δ (ppm): 7.08 (t, J = 8.1 Hz, 1H), 6.26 (dd, J_1 = 1.9 Hz, J_2 = 8.1 Hz, 1H), 6.22 (dd, J_1 = 1.5 Hz, J_2 = 8.10 Hz, 1H), 6.16 (s, 1H), 3.77 (s, 3H), 3.14 (q, J = 7.2 Hz, 2H), 1.25 (t, J = 7.2 Hz, 3H).

Synthesis of compound 2 and compound 3: 12.42 g (66.1 mmol) of 1, 2-dibromoethane were added slowly to acetonitrile (80 mL) with compound **1** (1.00 g, 6.61 mmol) and Cs_2CO_3 (3.23 g, 9.92 mmol). The above solution was refluxed for 18 h. After filtration, the solvent was removed under reduced pressure, and the brown residue was purified by column chromatography to give compound **2** as colourless oil (1.43 g, yield: 88.7%). ^{1}H NMR (400MHz, $CDCl_3$) δ (ppm): 7.08 (t, J = 8.1 Hz, 1H), 6.26 (dd, J_1 = 1.9 Hz, J_2 = 8.1 Hz, 1H), 6.22 (dd, J_1 = 1.5 Hz, J_2 = 8.1 Hz, 1H), 6.16 (s, 1H), 3.77 (s, 3H), 3.66 (t, J = 8.3 Hz, 2H), 3.46 (t, J = 8.3 Hz, 2H), 3.40 (q, J = 7.0 Hz, 2H), 1.18 (t, J = 7.0 Hz, 3H). Compound **3** was obtained by the same procedures with 1, 6-dibromohexane (16.13 g) instead of 1, 2-dibromoethane (1.64 g, 78.9%). ^{1}H NMR (400MHz, $CDCl_3$) δ (ppm): 7.08 (t, J = 8.1 Hz, 1H), 6.26 (dd, J_1 = 1.9 Hz, J_2 = 8.1 Hz, 1H), 6.22 (dd, J_1 = 1.5 Hz, J_2 = 8.1 Hz, 1H), 6.16 (s, 1H), 3.55 (t, J = 6.4 Hz, 2H), 3.41 (t, J = 6.5 Hz, 2H), 3.31 (q, J = 7.1 Hz, 2H), 1.83 – 1.76 (m, 2H), 1.68 – 1.60 (m, 2H), 1.54 -1.46 (m, 2H), 1.40 – 1.33 (m, 2H), 1.18 (t, J = 7.0 Hz, 3H).

Synthesis of compound 4 and compound 5: Under nitrogen atmosphere and at -78 °C, 1 mL (10.6 mmol) BBr_3 was added slowly to 40 mL of compound **2** (1.05 g, 4.30 mmol) in dichloromethane. The solution was stirred at -78 °C for 30 min and at room temperature for another 3 h. Then the above solution was poured into 100 mL iced water and stirred for 10 minutes. The crude product was extracted with dichloromethane. The organic layer was washed with saturated $NaHCO_3$ (100 mL × 2) and brine (100 mL × 2), followed by concentrated under vacuo and purified by column chromatography to give compound **4** as colourless oil (610 mg, yield: 61.6%). ^{1}H NMR (400MHz, DMSO-d_6) δ (ppm): 9.17 (s, 1H), 6.96 (m, 1H), 6.15 - 6.05 (m, 3H), 3.71 (t, J = 7.3 Hz, 2H), 3.59 - 3.53 (m, 4H), 1.07 (t, J = 7.0 Hz, 3H). Compound **5** was obtained by the same procedures with compound **3** (1.05 g) instead of compound **2** (680 mg, 67.8%). ^{1}H NMR (400MHz, $CDCl_3$) δ (ppm): 9.17 (s, 1H), 6.96 (m, 1H), 6.15 - 6.05 (m, 3H), 3.55 (t, J = 6.4 Hz, 2H), 3.41 (t, J = 6.5 Hz, 2H), 3.31 (q, J = 7.1 Hz, 2H), 1.83 – 1.76 (m, 2H), 1.68 – 1.60 (m, 2H), 1.54 -1.46 (m, 2H), 1.40 – 1.33 (m, 2H), 1.18 (t, J = 7.0 Hz, 3H).

Synthesis of compound 6 and compound 7: Under nitrogen atmosphere and at 0 °C, 1.5 mL $POCl_3$ (16.51 mmol) were injected slowly into 2 mL freshly distilled DMF and the mixed solution was stirred for 30 min. Then compound **4** (1.90 g, 8.26 mmol) in 3 mL freshly distilled DMF were injected slowly to the above mixture. After stirred at 0 °C for 30 min and at 60 °C for 4 h, the reaction mixture was poured into 100 mL ice-water and extracted with dichloromethane. The organic layer was washed with saturated $NaHCO_3$ (100 mL × 2) and brine (100 mL × 2), followed by concentrated under vacuo and purified by column chromatography to give compound **6** as white solid (1.25 g, yield: 70.9%). ^{1}H NMR (400MHz, DMSO-d_6) δ (ppm): 11.15 (s, 1H), 9.69 (s, 1H), 7.47 (d, J = 8.9 Hz, 1H), 6.41 (dd, J_1 = 8.9 Hz, J_2 = 2.3 Hz, 1H), 6.13 (d, $_1$ = 2.3 Hz, 1H), 3.75 (t, J = 5.3 Hz, 2H), 3.72 (t, J = 5.6 Hz, 2H), 3.49 (q, J = 7.1 Hz, 2H), 1.23 (t, J = 7.1 Hz, 3H).

Compound **7** was obtained by the same procedures with compound **5** instead of compound **4** (1.45 g, 69.8%). ^{1}H NMR (400MHz, DMSO-d_6) δ (ppm): 11.15 (s, 1H), 9.69 (s, 1H), 7.47 (d, J = 8.9 Hz, 1H), 6.41 (dd, J_1 = 8.9 Hz, J_2 = 2.3 Hz, 1H), 6.13 (d, $_1$ = 2.3 Hz, 1H), 3.55 (t, J = 6.4 Hz, 2H), 3.41 (t, J = 6.5 Hz, 2H), 3.31 (q, J = 7.1 Hz, 2H), 1.83 – 1.76 (m, 2H), 1.68 – 1.60 (m, 2H), 1.54 -1.46 (m, 2H), 1.40 – 1.33 (m, 2H), 1.18 (t, J = 7.0 Hz, 3H).

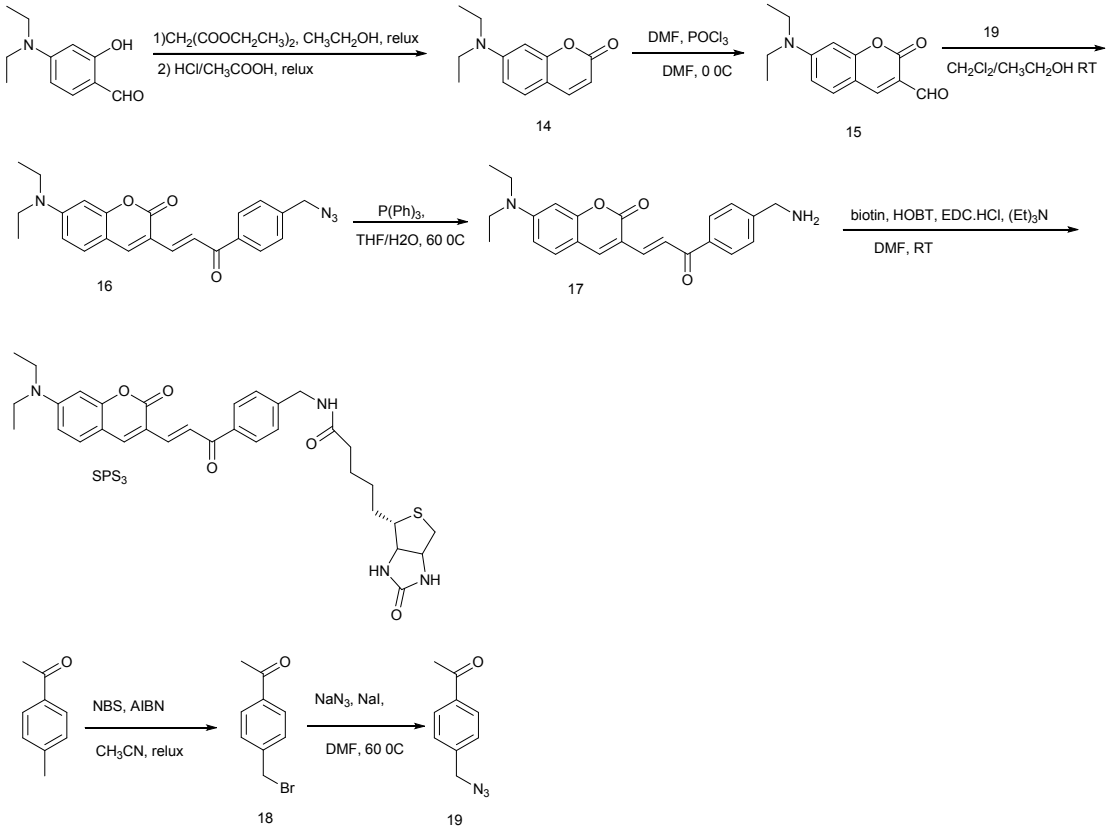
Synthesis of compound 8 and compound 9: Compound **6** (500 mg, 2.20 mmol), ethyl acetoacetate (428 mg, 3.29 mmol) and 0.5 mL piperidine were dissolved in 100 mL absolute ethanol. After refluxed for 8 h, the solvent was removed under the reduced pressure, and purified with column chromatography to give compound **8** as a yellow solid (523 mg, yield: 81.1%). ^{1}H NMR (400MHz, $CDCl_3$) δ (ppm): 8.45 (s, 1H), 7.45 (d, J = 8.9 Hz, 1H), 6.66 (dd, J_1 = 2.4 Hz, J_2 = 8.9 Hz, 1H), 6.52 (d, J = 2.2 Hz, 1H), 3.75 (t, J = 6.4 Hz, 2H), 3.66 (t, J = 6.5 Hz, 2H), 3.55 (q, J = 7.1 Hz, 2H), 2.69 (s, 3H), 1.26 (t, J = 7.1 Hz, 3H). Compound **9** was obtained (510 mg, 84.9%) by the same procedures with compound **7** (500 mg) instead of compound **6**. ^{1}H NMR (400MHz, $CDCl_3$) δ (ppm): 8.45 (s, 1H), 7.45 (d, J = 8.9 Hz, 1H), 6.66 (dd, J_1 = 2.4 Hz, J_2 = 8.9 Hz, 1H), 6.52 (d, J = 2.2 Hz, 1H), 3.55 (t, J = 6.4 Hz, 2H), 3.47 (t, J = 6.5 Hz, 2H), 3.36 (q, J = 7.1 Hz, 2H), 2.69 (s, 3H), 1.83 – 1.76 (m, 2H), 1.68 – 1.60 (m, 2H), 1.54 -1.46 (m, 2H), 1.40 – 1.33 (m, 2H), 1.26 (t, J = 7.1 Hz, 3H).

Synthesis of compound 10 and compound 11: Compound **8** (400 mg, 1.36 mmol), NaI (20.4 mg, 0.13 mmol) and NaN_3 (442 mg, 6.81 mmol) were added to 5 mL freshly distilled DMF. After stirred under nitrogen at 80 °C for 6 h, the above solution was poured into 100 mL ice water and stirred for 10 minutes. The precipitate was filtered to give compound **10** as a yellow solid (360 mg, yield: 88.0%). ^{1}H NMR (400MHz, $CDCl_3$) δ (ppm): 8.45 (s, 1H), 7.44 (d, J = 8.9 Hz, 1H), 6.62 (dd, J_1 = 2.3 Hz, J_2 = 8.9 Hz, 1H), 6.53 (d, J = 2.0 Hz, 1H), 3.60 – 3.52 (m, 6H), 2.69 (s, 3H), 1.26 (t, J = 7.1 Hz, 3H). Compound **11** was obtained (345 mg, 95.4%) by the same procedures with compound **9** (400 mg) instead of compound **8**. ^{1}H NMR (400MHz, $CDCl_3$) δ (ppm): 8.45 (s, 1H), 7.44 (d, J = 8.9 Hz, 1H), 6.62 (dd, J_1 = 2.3 Hz, J_2 = 8.9 Hz, 1H), 6.53 (d, J = 2.0 Hz, 1H), 3.46 (q, J = 7.1 Hz, 2H), 3.36 (t, J = 6.4 Hz, 2H), 3.30 (t, J = 6.5 Hz, 2H), 2.69 (s, 3H), 1.83 – 1.76 (m, 2H), 1.68 – 1.60 (m, 2H), 1.54 -1.46 (m, 2H), 1.40 – 1.33 (m, 2H), 1.26 (t, J = 7.1 Hz, 3H).

Synthesis of compound 12 and compound 13: Compound **10** (360 mg, 1.20 mmol) and PPh_3 (472 mg, 1.80 mmol) were added to 20 mL of the mixed solvent [THF/H_2O = 4:1 (v/v)] under nitrogen atmosphere. After stirred for 24 h at 60 °C, the solvent was removed under vacuum. The residue was purified by column chromatography to give compound **12** as a yellow solid (160 mg, 48.7%). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.45 (s, 1H), 7.66 (d, J = 9.0 Hz, 1H), 6.84 (dd, J_1 = 2.2 Hz, J_2 = 9.1 Hz, 1H), 6.52 (d, J = 2.0 Hz, 1H), 3.53 – 3.45 (m, 4H), 2.74(t, J = 7.3 Hz, 2H), 2.53 (s, 3H), 1.13 (t, J = 7.0 Hz, 3H). Compound **13** was obtained (130 mg, 38.9%) by the same procedures with compound **11** (360 mg) instead of compound **10**. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.45 (s, 1H), 7.66 (d, J = 9.0 Hz, 1H), 6.84 (dd, J_1 = 2.2 Hz, J_2 = 9.1 Hz, 1H), 6.52 (d, J = 2.0 Hz, 1H), 3.53 – 3.45 (m, 4H), 2.74(t, J = 7.3 Hz, 2H), 2.53 (s, 3H), 1.83 – 1.76 (m, 2H), 1.68 – 1.60 (m, 2H), 1.54 -1.46 (m, 2H), 1.40 – 1.33 (m, 2H), 1.26 (t, J = 7.1 Hz, 3H).

Synthesis of RC3 and RC4: Biotin (89 mg, 0.36 mmol), EDC·HCl (90 mg, 0.42 mmol), HOBt (70 mg, 0.42 mmol) and Et_3N (90 μL, 0.65 mmol) were dissolved in freshly distilled DMF (5 mL) and stirred at room temperature for 50 min. Then 100 mg of compounds **12** (0.26 mmol) were added to the above solution. After stirred for another 5 h, the mixture was poured into 100 mL of ice water. The precipitate was washed in turn with saturated NH_4Cl, saturated $NaHCO_3$, brine and

then purified with column chromatography to give RC3 as a bright yellow solid (80 mg, 43.8%). ^{1}H NMR (400MHz, DMSO-d_6) δ (ppm): 8.50 (s, 1H), 7.99 (t, J = 5.7 Hz, 1H), 7.68 (d, J = 9.0 Hz, 1H), 6.87 (dd, J_1 = 2.2 Hz, J_2 = 9.0 Hz, 1H), 6.69 (d, J = 2.0 Hz, 1H), 6.41 (s, 1H), 6.35 (s, 1H), 4.32 – 4.29 (m, 1H), 4.13 – 4.09 (m, 1H), 3,52 – 3.45 (m, 4H), 3.26 (q, J = 6.4 Hz, 2H), 3.09 – 3.04 (m, 1H), 2.81 (dd, J_1 = 5.1 Hz, J_2 = 12.4 Hz, 1H), 2.58 (d, J = 12.4 Hz, 1H), 2.52 (s, 3H), 2.06 (t, J = 7.3 Hz, 2H), 1.62 – 1.41 (m, 4H), 1.31 – 1.23 (m, 2H), 1.13 (t, J = 6.9 Hz, 3H). ^{13}C NMR (400MHz, DMSO-d_6) δ: 194.2, 172.6, 162.7, 159.8, 158.1, 153.4, 147.6, 132.3, 115.3, 110.2, 107.7, 96.1, 61.0, 59.2, 55.4, 48.8, 45.7, 45.0, 36.1, 35.2, 30.1, 28.2, 28.0, 25.1, 12.0. HR-MS *m/z*: 501.2169 $(M+H)^+$; calculated molecular weight of $C_{25}H_{33}N_4O_5S$: 501.2172 for $(M+H)^+$. **RC4** was obtained (46 mg, 27.3%) by the same procedures with compound **13** (100 mg) instead of compound **12**. ^{1}H NMR (400MHz, DMSO-d_6) δ (ppm): 8.50 (s, 1H), 7.75 (t, J = 5.4 Hz, 1H), 7.66 (d, J = 9.0 Hz, 1H), 6.79 (dd, J_1 = 2.3 Hz, J_2 = 9.0 Hz, 1H), 6.56 (d, J = 2.1 Hz, 1H), 6.42 (s, 1H), 6.36 (s, 1H), 4.30 – 4.27 (m, 1H), 4.13 – 4.09 (m, 1H), 3.50 (q, J = 7.0 Hz, 2H), 3.42 – 3.37 (m, 2H), 3.10 – 3.00 (m, 3H), 2.80 (dd, J_1 = 5.1 Hz, J_2 = 12.4 Hz, 1H), 2.56 (d, J = 12.4 Hz, 1H), 2.51 (s, 3H), 2.04 (t, J = 7.3 Hz, 2H), 1.63 – 1.36 (m, 10H), 1.25 – 1.21 (m, 2H), 1.13 (t, J = 7.0 Hz, 3H). ^{13}C NMR (400MHz, DMSO-d_6) δ: 194.6, 172.2, 163.1, 160.3, 158.6, 153.6, 148.0, 132.8, 115.4, 110.6, 107.9, 96.3, 61.5, 59.6, 55.9, 52.4, 50.3, 45.3, 40.3, 38.7, 30.6, 29.6, 28.6, 28.5, 27.4, 26.7, 26.4, 25.8, 12.6. HR-MS *m/z*: 579.2617 $(M+Na)^+$; calculated molecular weight of $C_{29}H_{40}N_4O_5S$: 579.2617 for $(M+Na)^+$.



Scheme S2 synthesis procedures of SPS3

Synthesis of compound 14: Commercially available 4-(diethylamino) salicylaldehyde (1.93 g, 9.99 mmol), diethyl malonate (1.92 g, 11.98 mmol) and 1 mL piperidine were dissolved in 100

mL absolute alcohol. After refluxed for 8 h, the solvent was removed under the reduced pressure. Then 40 ml of mixed solution [concentrated HCl:glacial acetic acid＝1:1 (*V*:*V*)] was added to the crude product and refluxed for another 7 h. The reaction solution was poured into 300 mL of ice water after cooling to room temperature. The solution's pH was adjusted to ~5 with NaOH solution. The solid was filtered and purified over column chromatography to give a yellow solid of compound **14** (1.60 g, yield: 73.7%). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 10.12 (s, 1H), 8.26 (s, 1H), 7.42 (d, J＝9.0 Hz, 1H), 6.64 (dd, J＝2.5, 9.0 Hz, 1H), 6.49 (d, J＝2.3 Hz, 1H), 3.48 (q, J＝7.1 Hz, 4H), 1.26 (t, J＝7.1 Hz, 6H).

Synthesis of compound 15: 1.0 mL $POCl_3$ (11.0 mmol) was injected in freshly distilled DMF slowly under nitrogen atmosphere at 0 °C and stirred for 30 min. Then compound **1** (1.20 g, 5.52 mmol) in 5 mL distilled DMF was injected to the reaction mixture at 0 °C slowly. After stirred for another 30 min, the solution was slowly warmed to 60 ℃ and stirred for 4h. Then the reaction solution was poured into 100 mL ice water and its pH was adjusted to ~5 with NaOH solution. The solid was filtered and was purified by column chromatography to give a orange-brown solid (850 mg, yield: 62.7 %). ^{1}H NMR (400MHz, DMSO-d_6) δ: 8.49 (s, 1H), 8.06 (d, J = 8.2 Hz, 2H), 8.03 (d, J = 15.5 Hz, 1H), 7.67 (d, J = 15.4 Hz, 1H), 7.57 (d, J = 8.2 Hz, 2H), 7.50 (d, J = 9.0 Hz, 1H), 6.81 (dd, J_1 = 1.5 Hz, J_2 = 9.0 Hz, 1H), 6.61 (s, 1H), 6.48 (s, 1H), 6.38 (s, 1H),4.60 (s, 2H), 3.48 (q, J = 7.0 Hz, 4H), 1.15 (t, J = 7.0 Hz, 6H)

Synthesis of compound 18: Commercially available p-methylacetophenone (3.0 g, 22.36 mmol), NBS (6.4 g, 36.1 mmol) and AIBN (367 mg, 2.24 mmol) were added to 100 mL acetonitrile in 250 mL flask. After refluxed for 6 h, the solvent was removed under reduced pressure and the residue was further purified by column chromatography to give compound **18** as a colorless oil (3.8 g, yield: 79.8%). ^{1}H NMR (400MHz, $CDCl_3$) δ: 7.93 (d, J = 8.3 Hz, 1H), 7.48 (d, J = 8.2 Hz, 1H), 4.50 (s, 2H), 2.60 (s, 3H)

Synthesis of compound 19: Compound **18** (1.10 g, 5.16 mmol), NaI (77 mg, 0.52mmol) and NaN_3 (1.01 g, 15.5 mmol) were added in 5 mL distilled DMF, the solution was stirred under nitrogen at 80 °C for 8 h. Then the reaction solution was poured into 100 mL ice water and extracted with dichloromethane and water. The organic layer was washed with sat. $NaHCO_3$ (100 mL × 2) and brine (100 mL × 2), concentrated in vacuo and purified by column chromatography to give compound **19** as a colorless oil (600 mg, yield: 66.3%). ^{1}H NMR (400MHz, $CDCl_3$) δ: 7.93 (d, J = 8.3 Hz, 1H), 7.48 (d, J = 8.2 Hz, 1H), 4.43 (s, 2H), 2.61 (s, 3H)

Synthesis of compound 16: Compound **15** (300 mg, 1.22 mmol) and compound **19** (386 mg, 2.20mmol) were added to 20 mL of the mixed solvent [CH_2Cl_2/anhydrous CH_3CH_2OH＝1:1 (*V*:*V*)], then 10 drops of pyrrolidine were droped into the above solution. The mixture was stirred at room temperature for 1 d, and the solvent was removed under reduced pressure. The residue was purified by column chromatography to give compound **16** as a bright red solid (210 mg, 42.1%). ^{1}H NMR (400MHz, DMSO-d_6) δ: 8.49 (s, 1H), 8.06 (d, J = 8.2 Hz, 2H), 8.03 (d, J = 15.4 Hz, 1H), 7.67 (d, J = 15.4 Hz, 1H), 7.57 (d, J = 8.2 Hz, 2H), 7.50 (d, J = 9.0 Hz, 1H), 6.80 (dd, J_1 = 9.0 Hz, J_2 = 2.2 Hz, 1H), 6.60 (d, J = 2.1 Hz, 1H), 4.60 (s, 2H), 3.48 (q, J = 7.0 Hz, 4H), 1.15 (t, J = 7.0 Hz, 6H).

Synthesis of compound 17: Compound **16** (210 mg, 0.52 mmol) and PPh_3 (205 mg, 0.78 mmol) were added to 20 mL of the mixed solvent [THF/H_2O＝4:1 (*V*:*V*)] under nitrogen atmosphere. After stirring for 24 h at 60 °C, the solvent was removed under vacuum. The residue was purified by column chromatography to give compound **17** as a bright red solid (150 mg, 71.3%). ^{1}H NMR

(400MHz, DMSO-d_6) δ: 8.49 (s, 1H), 8.06-8.00 (m, 3H), 7.66 (d, J = 15.4 Hz, 1H), 7.56 (d, J = 8.1 Hz, 2H), 7.51 (d, J = 9.0 Hz, 1H), 6.80 (dd, J_1 = 1.5 Hz, J_2 = 8.9 Hz, 1H), 6.6 (s, 1H), 3.86 (s, 2H), 3.49 (q, J = 7.0 Hz, 4H), 1.15 (t, J = 7.0 Hz, 6H)

Synthesis of SPS3: Biotin (65 mg, 0.26 mmol), EDC·HCl (65 mg, 0.31 mmol), HOBt (54 mg, 0.31 mmol) and Et_3N (65 μL, 0.47 mmol) in DMF (5 mL) at room temperature. After stirred for 50 minutes, compounds **4** (105 mg, 0.26 mmol) was added to the above solution and stirred for another 5 h. Then the reaction solution was poured into 100 mL ice water, and the solid was filtered. The solid was further washed with sat. NH_4Cl, sat. $NaHCO_3$ and brine and purified by column chromatography to give **SPS3** as a bright red solid (82 mg, yield: 51.2%). ^{1}H NMR (400MHz, DMSO-d_6) δ: 8.48 (s, 1H), 8.04-8.00 (m, 3H), 7.66 (d, J = 15.3 Hz, 1H), 7.51 (d, J = 9.0 Hz, 1H), 7.43 (d, J = 8.0 Hz, 2H), 6.80 (dd, J_1 = 1.5 Hz, J_2 = 8.9 Hz, 1H), 6.61 (s, 1H), 4.35 (d, J = 5.6 Hz, 2H), 4.32 (t, J = 6.3 Hz, 1H), 4.12 (t, J = 5.0 Hz, 1H), 3.49 (q, J = 6.9 Hz, 4H), 3.07 (m, 1H), 2.83 (dd, J_1 = 7.4 Hz, J_2 = 12.4 Hz, 4H), 2.59 (d, J = 12.4 Hz, 1H), 2.18 (t, J = 7.2 Hz, 2H), 1.64-1.43 (m, 4H), 1.38-1.29 (m, 2H), 1.15 (t, J = 7.0 Hz, 6H). ^{13}C NMR (400MHz, DMSO-d_6) δ: 188.9, 172.7, 163.2, 160.4, 156.9, 152.4, 146.1, 145.6, 139.8, 136.9, 131.1, 128.8,127.7, 127.3, 113.7, 110.4, 108.3, 96.7, 61.5, 59.7, 55.9, 45.9, 44.8, 42.3, 35.7, 28.7, 28.5, 25.8, 12.8. HR-MS *m*/*z*: 603.2643 $(M+H)^+$; calculated molecular weight of $C_{33}H_{38}N_4O_5S$: 603.2596 for $(M+H)^+$.

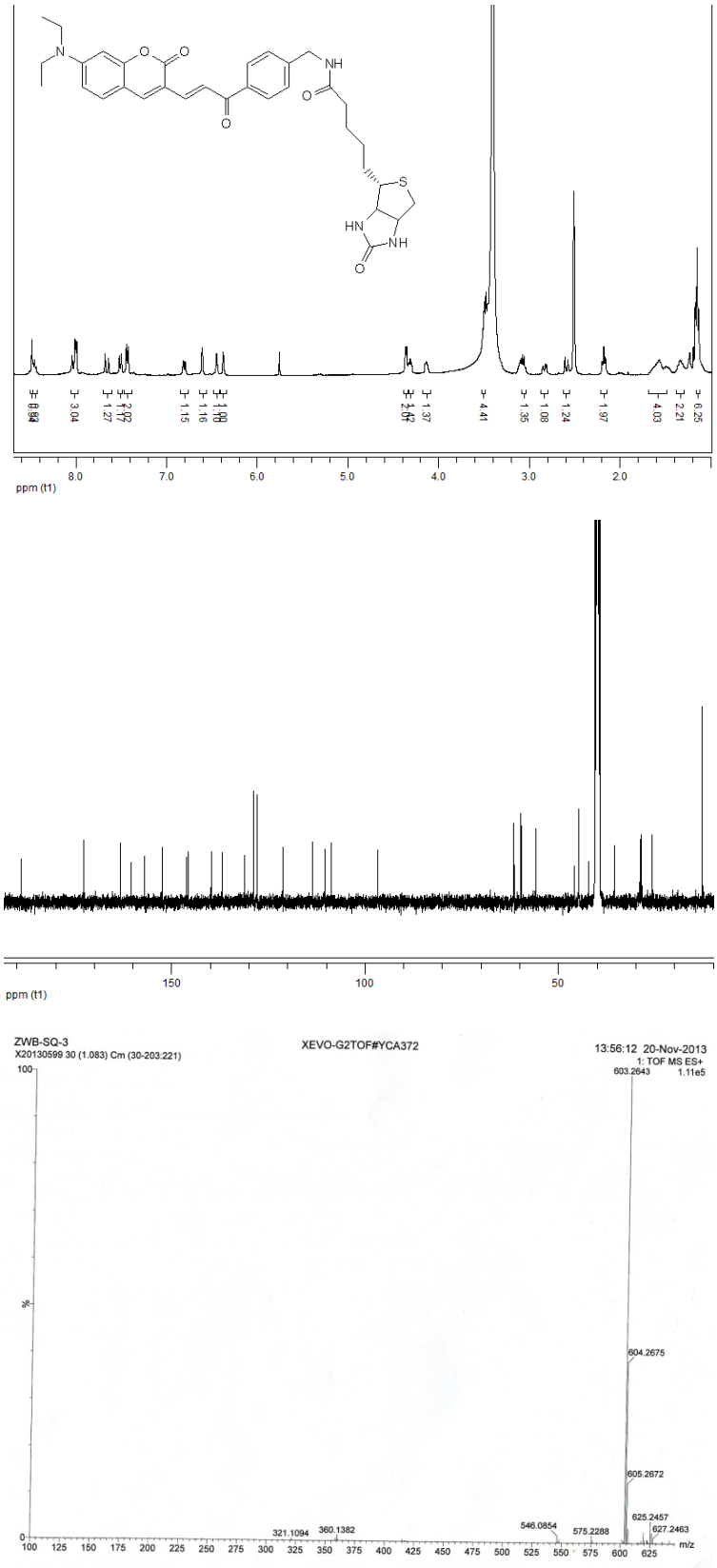


Fig. S1 ^{1}H-NMR, ^{13}C-NMR and HRMS spectra of SPS3.

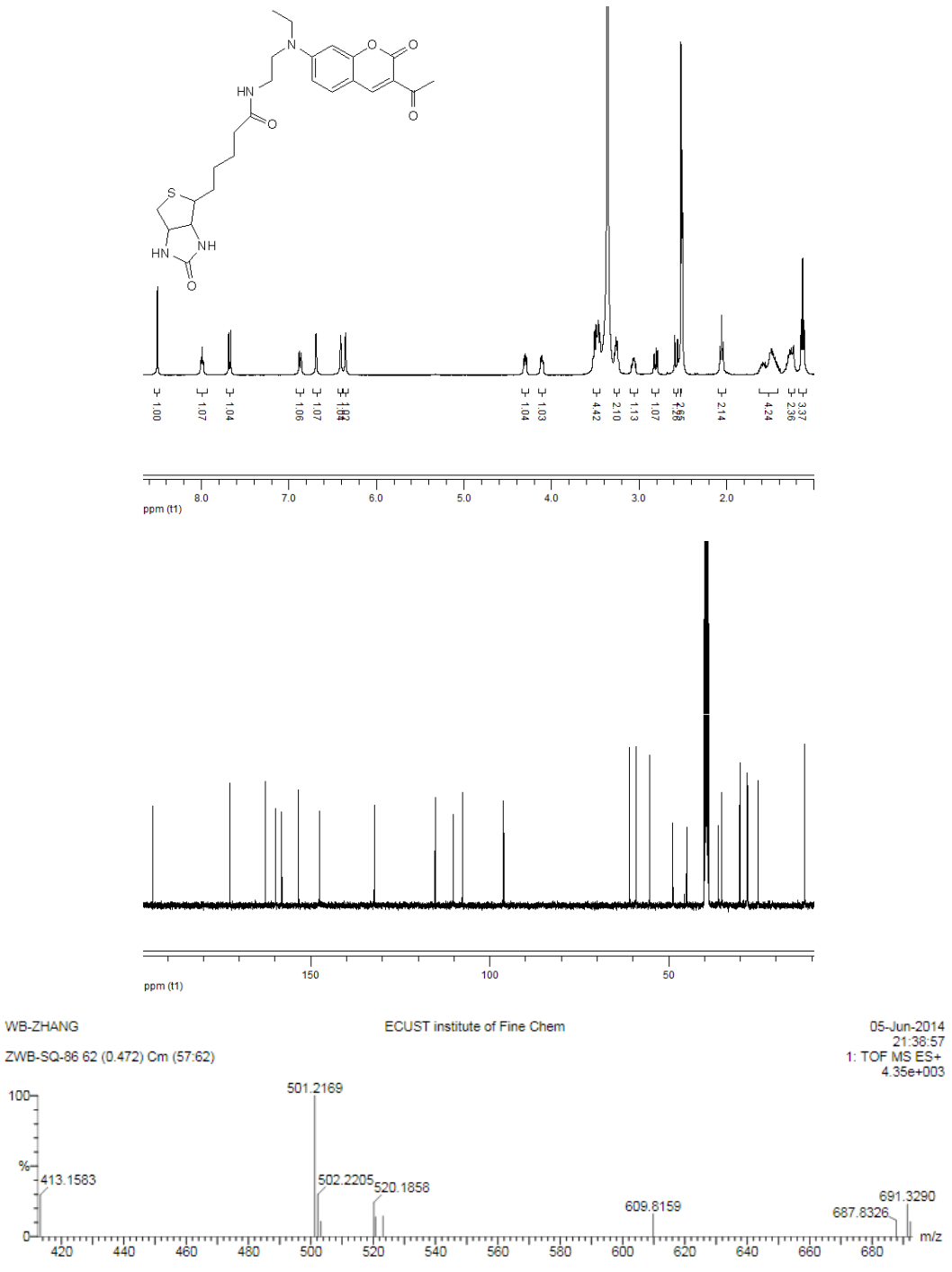
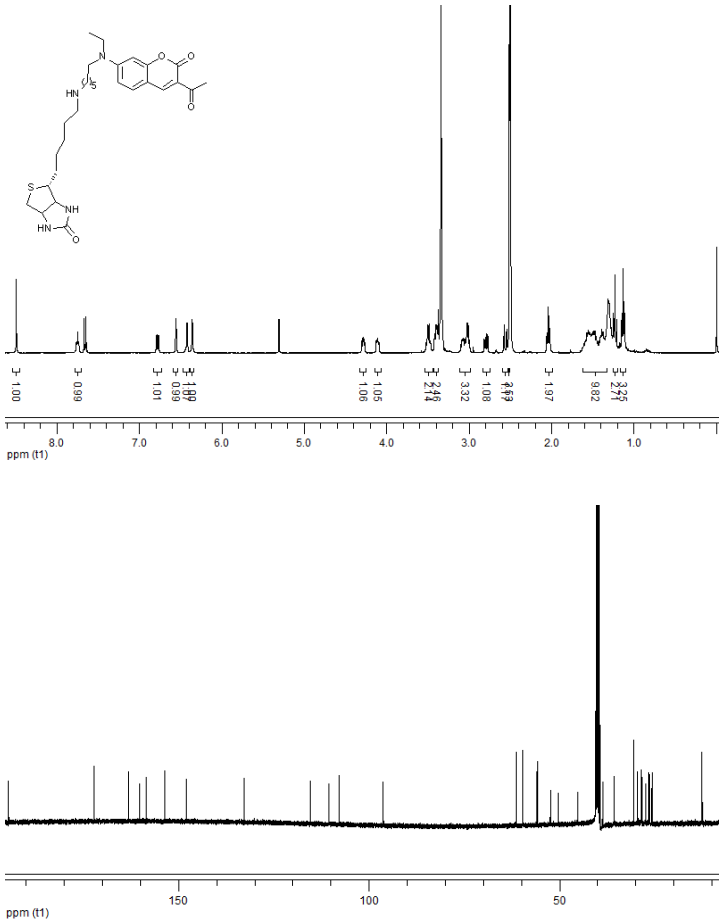
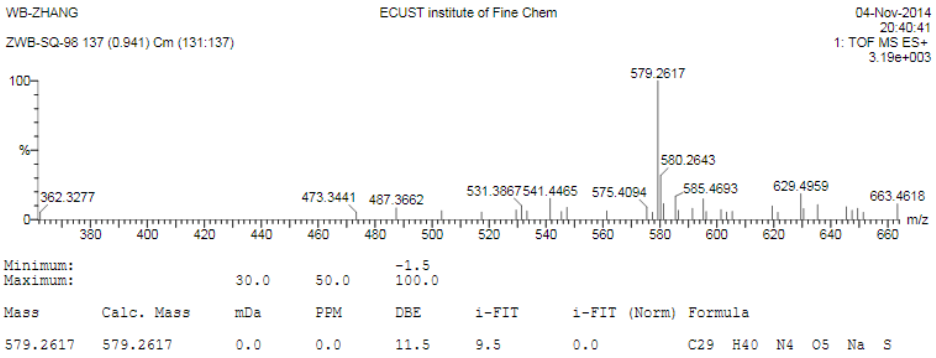


Fig. S2 ^{1}H-NMR, ^{13}C-NMR and HRMS spectra of RC3.





Minimum:				-1.5			
Maximum:		30.0	50.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
579.2617	579.2617	0.0	0.0	11.5	9.5	0.0	C29 H40 N4 O5 Na S

Fig. S3 ^{1}H-NMR, ^{13}C-NMR and HRMS spectra of RC4.

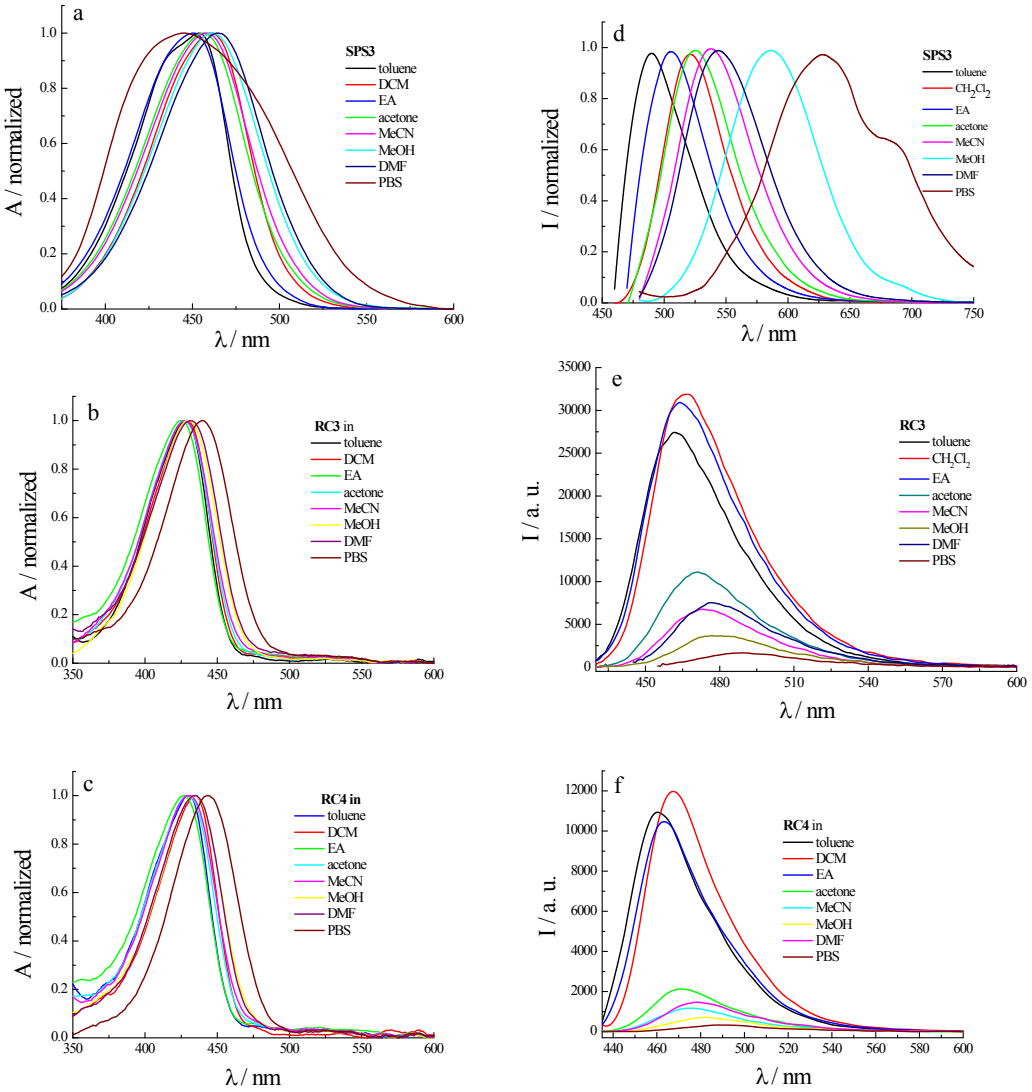


Fig. S4 The normalized absorption (a-c) and emission (d-f) spectra of three dyes in various solvents, [SPS3] = [RC3] =[RC4] = 10 μM.

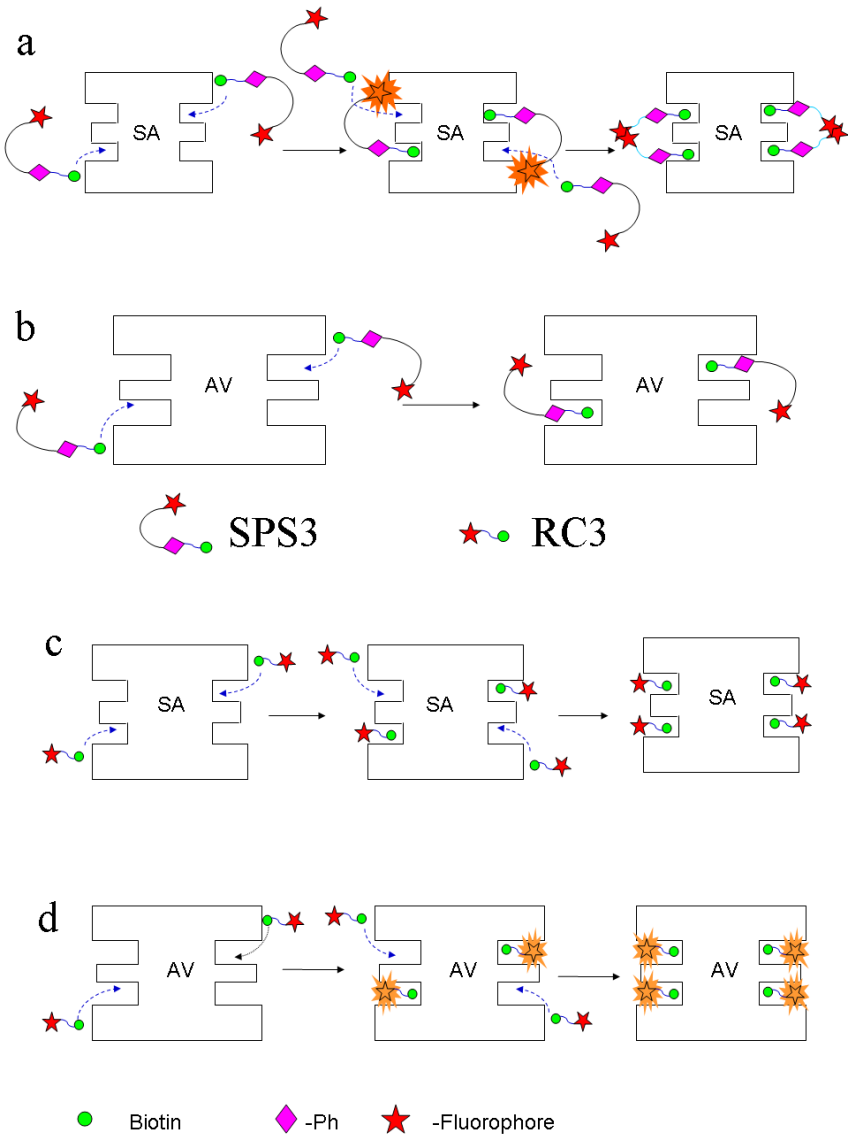


Fig. S5 The proposed binding models of SPS3 (a-b)/RC3 (c-d) to AV/SA.

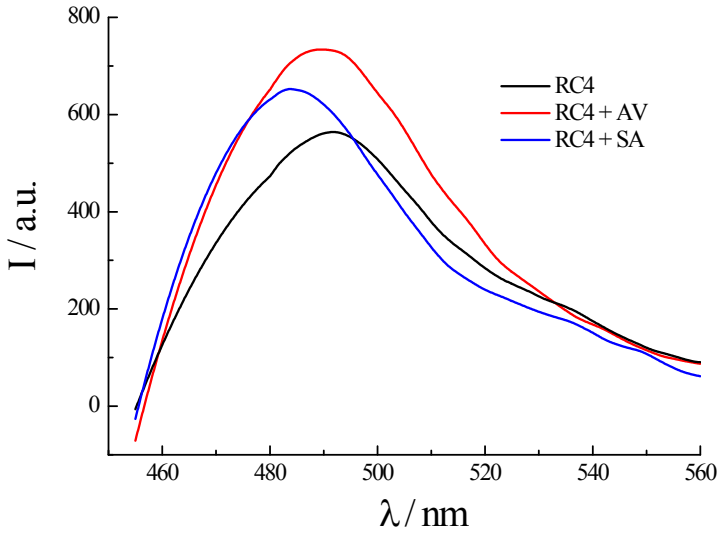


Fig. S6 The emission spectra of RC4 in the absence and presence of SA/AV; 10 mM PBS, pH 7.0, [RC4] = 3 μM, [SA] = [AV] = 1.5 μM, λ_{ex} = 435 nm.

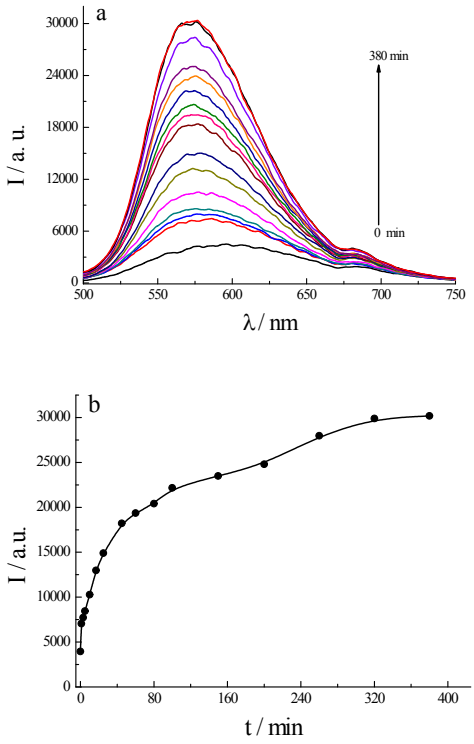


Fig. S7 Time-dependent emission spectra (a) and time-dependent fluorescence intensity at λ = 560 nm (b) of SPS3-SA-biotin system. [SPS3] = 3 μM, [SA] = 0.75 μM, [biotin] = 144 μM, 10 mM PBS, pH 7.0, λ_{ex} = 465 nm.

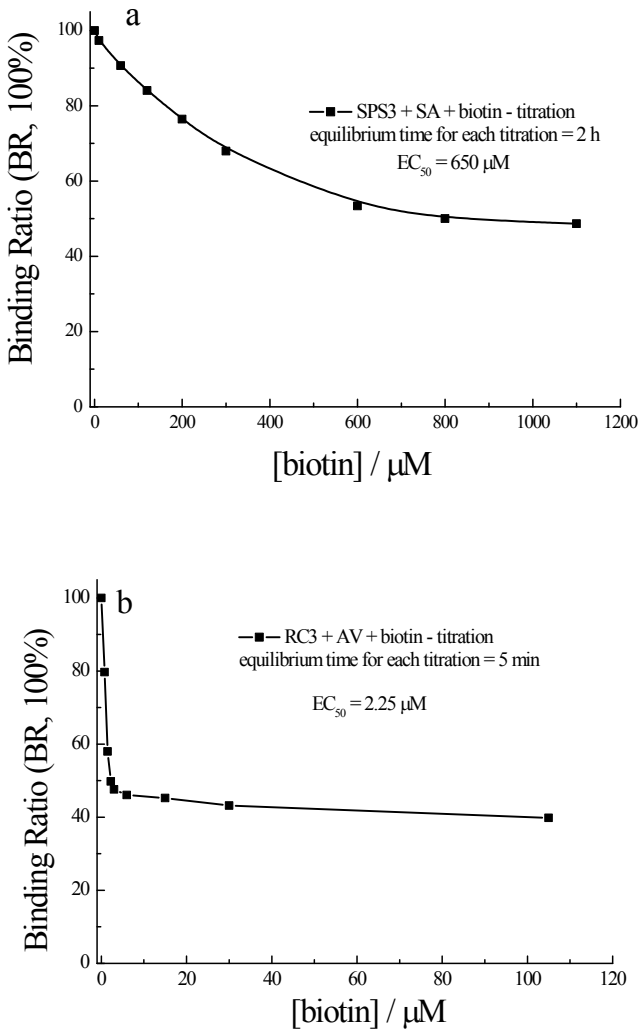


Fig. S8 Competition titration of biotin to SPS3-SA (a) and RC3-AV (b) systems, [SPS3] = [RC3] = 6 μM, [SA] = [AV] = 1.5 μM.

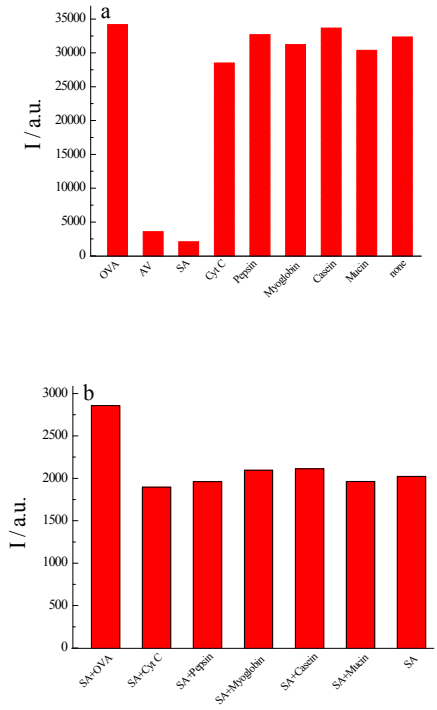


Fig. S9 The fluorescence intensity of SPS3-BSA system in the presence of 0.1 mg/mL of various proteins (a), and the competition of SPS3-BSA system toward SA over other proteins (b). [SA] = [AV] = 0.45 μM (0.03 mg/mL), [BSA] = 3.0 mg/mL, [SPS3] = 3.0 μM, 10 mM PBS, pH 7.0, λ_{ex} = 465 nm, λ_{em} = 550 nm.

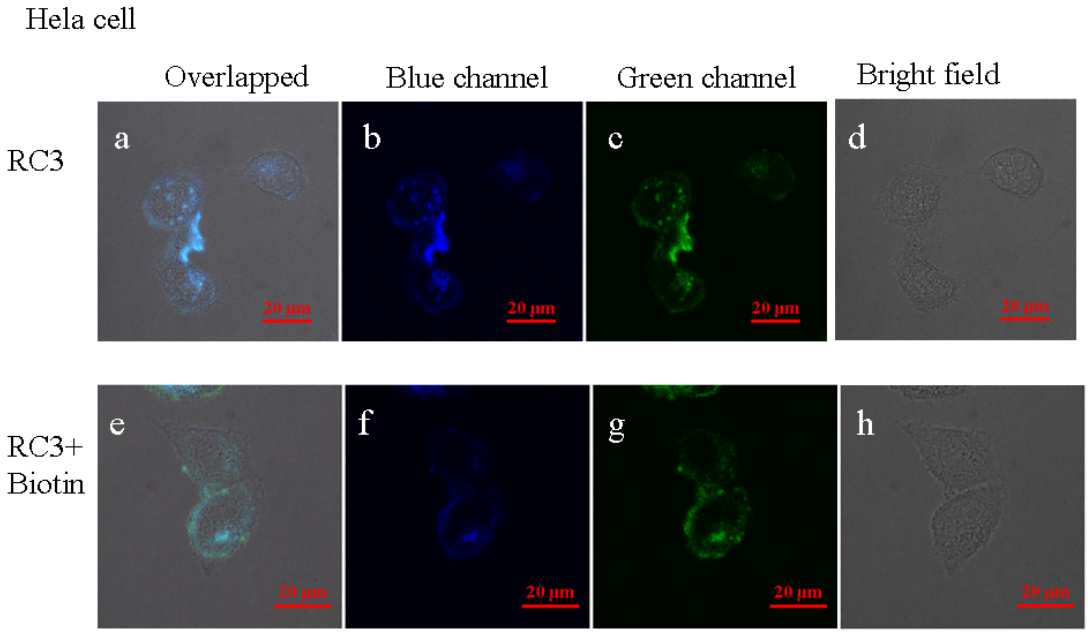


Fig. S10 Confocal fluorescence (b-c, f-g), bright-field (d, h) and the merged fluorescence (a, e) images of living HeLa cells incubated with RC3 (10 μM) for 60 min (a-d) and pretreated with 10 μM biotin for 1h (e-h) followed by incubated with RC3 for another 1h. Excited at 404 nm; (b, f): blue channel; (c, g): green channel.

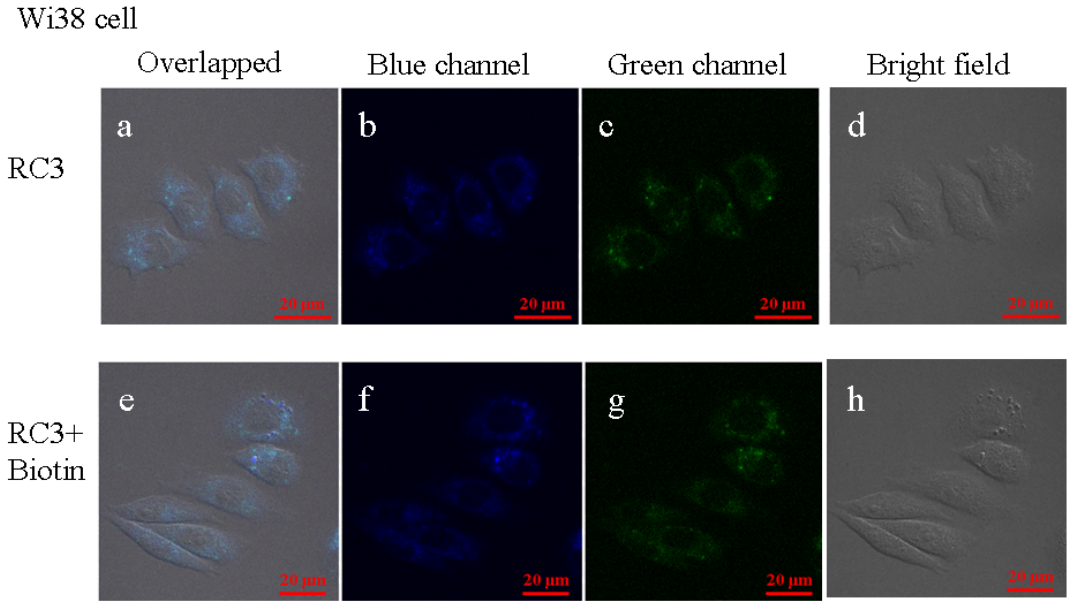


Fig. S11 Confocal fluorescence (b-c, f-g), bright-field (d, h) and the merged fluorescence (a, e) images of living normal Lung cells Wi38 incubated with RC3 (10 μM) for 60 min (a-d) and pretreated with 10 μM biotin for 1h (e-h) followed by incubated with RC3 for another 1h. Excited at 404 nm; (b, f): blue channel; (c, g): green channel.

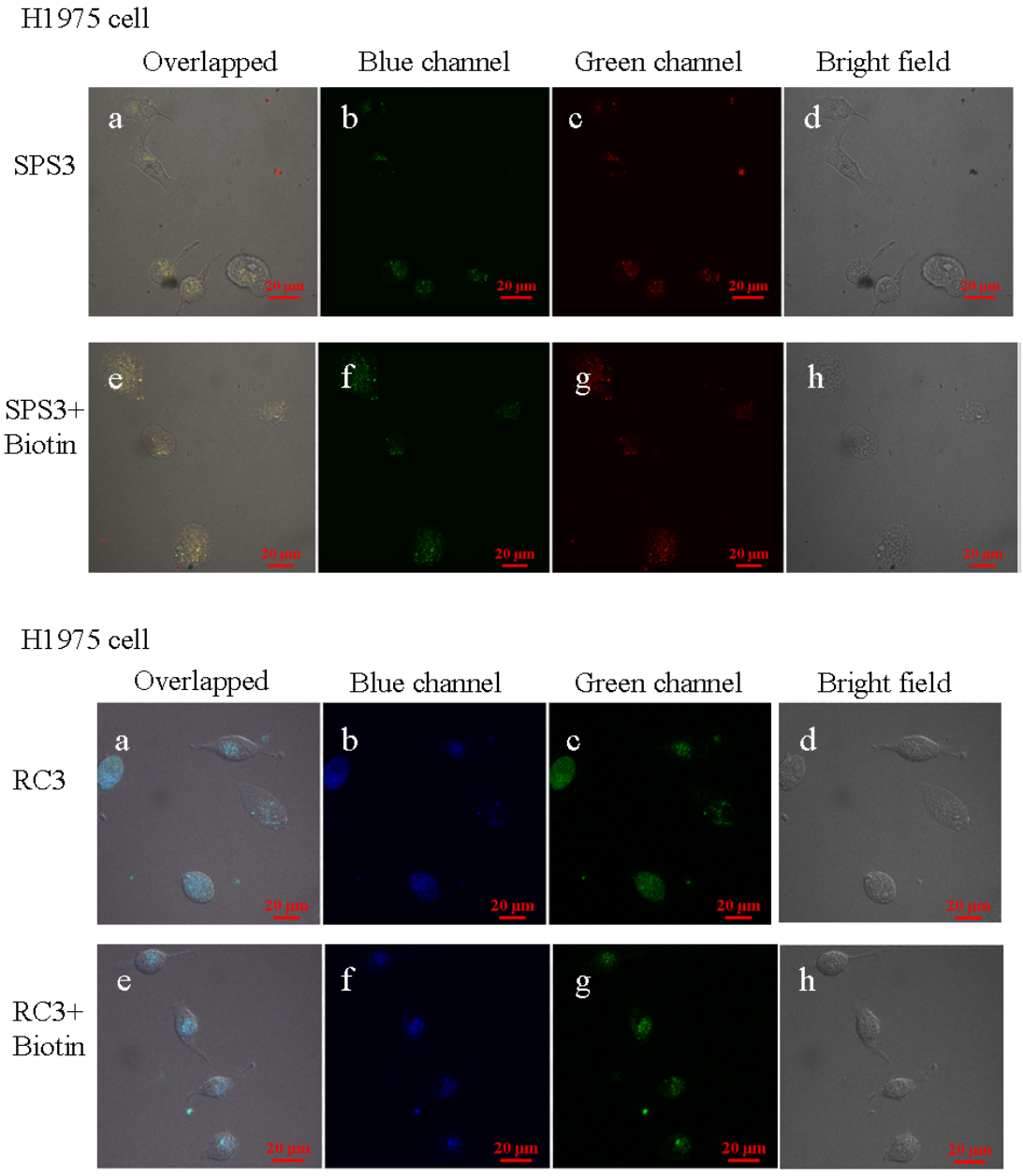


Fig. S12 Confocal fluorescence (b-c, f-g), bright-field (d, h) and the merged fluorescence (a, e) images of living Lung cancer cells H1975 incubated with SPS3/RC3 (10 μM) for 60 min (a-d) and pretreated with 10 μM biotin for 1h (e-h) followed by incubated with SPS3/RC3 for another 1h. For SPS3 excited at 488 nm; (b, f): green channel; (c, g): red channel; for RC3 excited at 404 nm; (b, f): blue channel; (c, g): green channel.

Table S1 The photophysical properties of three compounds in various solvents

Solvent[b]	Probe	λ_{ab} / nm	λ_{em} / nm	Stokes shift / nm	[a]Φ_{fl}
toluene	SPS3	454	490	36	0.63
	RC3	428	460	32	1.04
	RC4	429	460	31	0.99
dichloromethane	SPS3	461	521	60	0.45
	RC3	429	466	37	0.97
	RC4	435	468	33	0.88
ethylacetate	SPS3	451	506	55	0.57
	RC3	425	463	38	0.85
	RC4	427	463	36	0.67
acetone	SPS3	457	525	68	0.49
	RC3	427	471	44	0.36
	RC4	429	471	42	0.15
acetonitrile	SPS3	459	537	78	0.51
	RC3	428	473	45	0.23
	RC4	429	475	46	0.08
Methanol	SPS3	462	586	124	0.14
	RC3	429	480	51	0.11
	RC4	435	482	47	0.05
DMF	SPS3	465	543	78	0.47
	RC3	429	478	49	0.24
	RC4	435	479	44	0.10
PBS	SPS3	445	628	183	0.004
	RC3	440	489	49	0.053
	RC4	443	488	45	0.027

[a] Coumarin 153 (Φ_f = 0.38 in ethanol) was used as the reference; [b] containing 1% DMF.

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