SUPPORTING INFORMATION

Zwitterionic Near-Infrared Fluorophores and their *In Vivo* Fate

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All chemicals and solvents were of American Chemical Society grade or HPLC purity and were used as received. HPLC grade acetonitrile (CH_3CN) and water were purchased from VWR International (West Chester, PA) and American Bioanalytic (Natick, MA), respectively. All other chemicals were purchased from Fisher Scientific (Pittsburgh, PA, USA) and Sigma-Aldrich (Saint Louis, MO). Melting points (mp, open Pyrex capillary) were measured on a Thomas Hoover apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on Bruker Avance (400 MHz) spectrometer or Varian Unity INOVA (600 MHz) vertical bore spectrometer using 5 mm high-resolution probes. Vis/NIR absorption spectra were recorded on a Perkin Elmer Lambda 20 spectrophotometer. High-resolution mass spectra (HRMS) were recorded on a VG Analytical 70-SE spectrometer and elemental analysis was performed using a Perkin-Elmer 2400 Series II CHN analyzer.

2,3,3-Trimethyl-3*H*-indole-5-sulfonic acid (3). A mixture of 4-hydrazinobenzenesulfonic acid (20 g, 97.5 mmol), sodium acetate (16 g, 195 mmol), and 3-methyl-2-butanone (14.9 mL, 139 mmol) in glacial acetic acid (97 mL) was heated at 110˚C under a nitrogen atmosphere in a sealed tube. The crude product was filtered, washed with methyl *tert*-butyl ether (MTBE), and collected after precipitation as a brown solid (19.4 g, 83%); mp 292 to 293˚C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.25 (s, 6*H)*, 2.22 (s, 3*H)*, 7.10

to 7.30 (br. s, 1*H)*, 7.36 (d, *J* = 8.0 Hz, 1*H)*, 7.58 (d, *J* = 8.0 Hz, 1*H)*, 7.65 (s, 1*H)*. ^{13}C NMR (100 MHz, DMSO-d_6): δ 15.0, 22.4, 53.1, 118.5, 125.0, 145.0, 153.5, 171.5, 173.4, 188.8.

2,3,3-Trimethyl-1-[3-(trimethylammonio)propyl]-3*H*-indolium-5-sulfonic acid dibromide (5). A mixture of 2,3,3-trimethyl-3*H*-indole-5-sulfonic acid **3** (7.17 g, 36.4 mmol) and (3-bromopropyl)trimethyl ammonium bromide (10.5 g, 40 mmol) in toluene (60 mL) was heated at 130˚C for 72 h under a nitrogen atmosphere. The mixture was cooled to room temperature and the solvent was decanted. The crude product was crystallized from methanol and MTBE to afford pink crystals, which was used in the next step without further purification (**5**, 11 g, 81%); mp 232 to 235˚C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.56 (s, 6*H)*, 2.51 (s, 3*H)*, 3.07 (m, 2*H)*, 3.12 (s, 9*H)*, 3.62 (t, *J* = 7.2 Hz, 2*H)*, 4.50 (t, *J* = 7.2 Hz, 2*H)*, 7.71 (d, *J* = 8.0 Hz, 1*H)*, 7.79 (d, *J* = 8.0 Hz, 1*H)*, 8.01 (s, 1*H)*. ^{13}C NMR (100 MHz, DMSO-d_6): δ 15.0, 21.6, 22.3, 45.2, 53.1, 55.0, 62.4, 115.4, 121.2, 126.8, 141.3, 142.0, 149.9, 199.1. HRMS (ESI) calculated for $C_{17}H_{27}N_2O_3S$ $[M]^+$ *m/z* 339.1740, found *m/z* 339.1742.

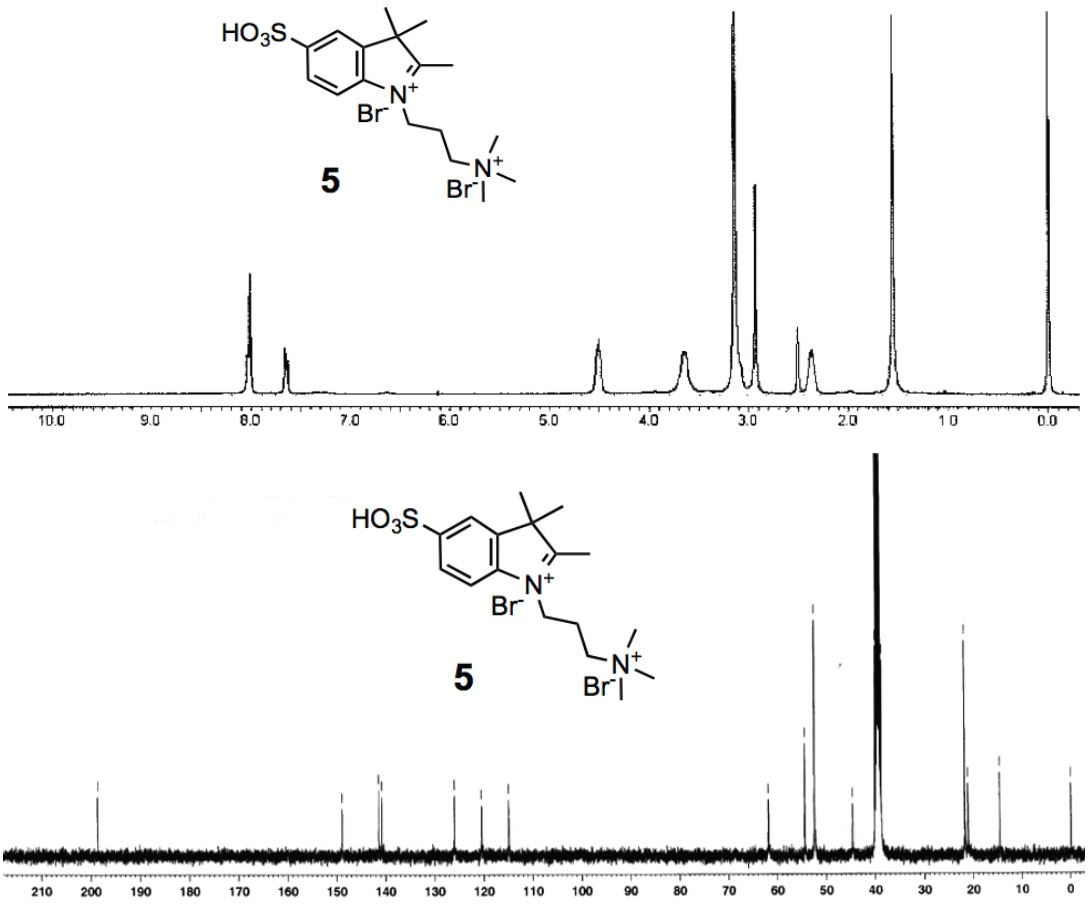

Figure S1a. ^{1}H NMR (top) and ^{13}C NMR (bottom) of compound #**5** in DMSO-d_6.

2,3,3-Trimethyl-1-[3-(trimethylammonio)propyl]-3*H*-indolium bromide (6). A mixture of 2,3,3-trimethyl-3*H*-indole **4** (1.59 g, 10 mmol) and (3-bromopropyl)trimethylammonium bromide (2.87 g, 11 mmol) in toluene (50 mL) was reflux for 72 h under a nitrogen atmosphere in a sealed tube. The reaction mixture was cooled to room temperature, and then concentrated under reduced pressure to furnish a red residue. The crude product was crystallized from acetone/methanol (5:1) to afford a pink solid **6** (2.52 g, 61%); mp 197 to 199˚C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.58 (s, 6*H)*, 2.41 (m, 2*H)*, 2.99 (s, 3*H)*, 3.17 (s, 9*H)*, 3.98 (t, J = 7.6 Hz, 2*H)*, 4.57 (t, J = 7.6 Hz, 2*H)*, 7.45 (m, 2*H)*, 7.86 (d, J = 5.2 Hz, 1*H)*, 8.19 (d, J = 5.2 Hz, 1*H)*. ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.3, 22.0, 25.4, 44.6, 52.5, 61.9, 64.9, 115.6, 123.5, 129.0, 129.4, 140.9, 141.8, 197.8. HRMS (ESI) calculated for $C_{17}H_{27}N_2$ $[M]^+$ *m/z* 259.2174, found *m/z* 259.2177.

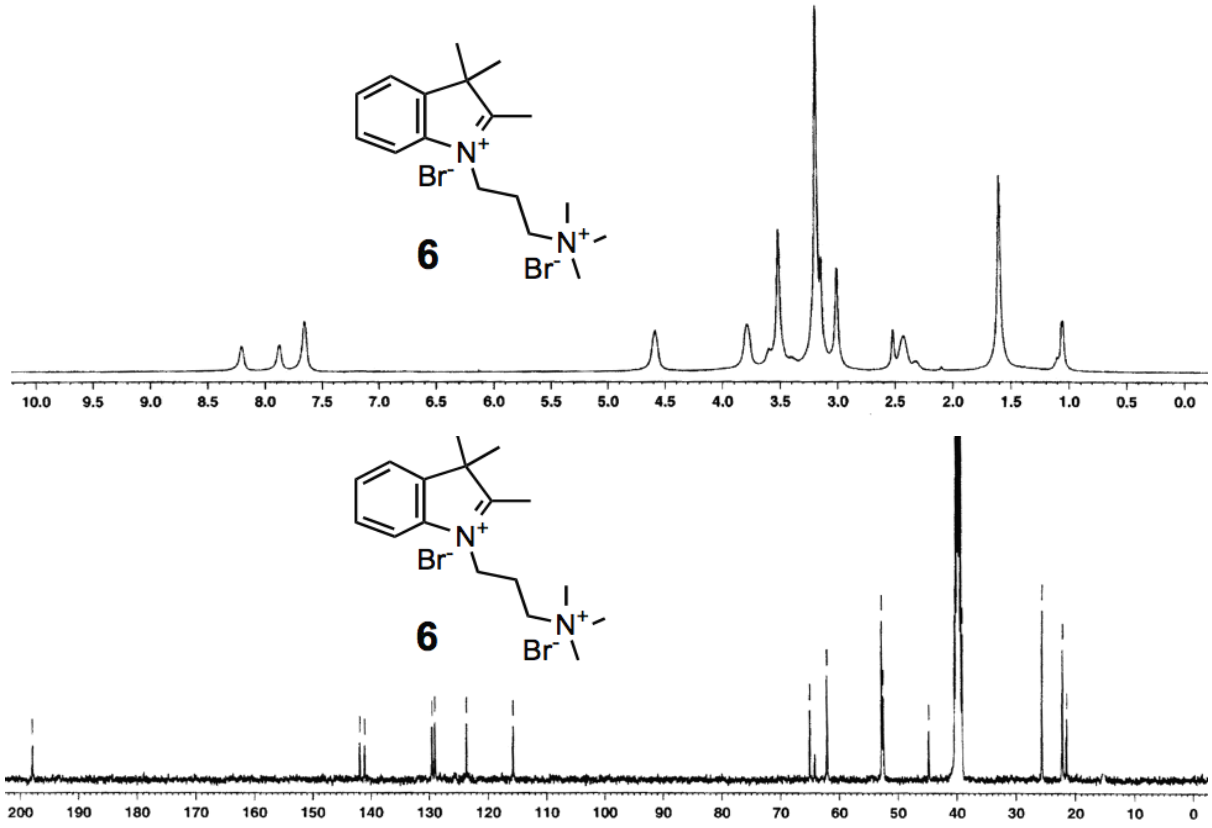

Figure S1b. ^{1}H NMR (top) and ^{13}C NMR (bottom) of compound #**6** in DMSO-d_6.

2-((*E*)-2-((*E*)-2-chloro-3-((*E*)-2-(3,3-dimethyl-5-sulfonato-1-(3-(trimethylammonio)propyl) indolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-3,3-dimethyl-1-(3-(trimethyl-ammonio)propyl)-3*H*-indol-1-ium-5-sulfonate disodium bromide (8). A mixture of bromide salt **5** (1.26 g, 2.41 mmol), Vilsmeier-Haack reagent **7** (0.433 g, 1.5 mmol), and anhydrous sodium acetate (0.37 g, 4.5 mmol) in absolute ethanol (50 mL) was heated under reflux for 6 h under a nitrogen atmosphere. The reaction

mixture was cooled to room temperature, and then concentrated under reduced pressure to yield a brown residue. The crude product was washed with dichloromethane to furnish a brownish-green solid **8**, which was collected, suspended in methanol (10 mL), filtered and dried *in vacuo* to yield a golden-green solid (1.2 g, 1.1 mmol, 73%); mp 274 to 277°C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.72 (s, 12*H)*, 1.88 (m, 2*H)*, 2.18 (m, 4*H)*, 2.76 (m, 4*H)*, 3.08 (s, 18*H)*, 3.49 (m, 4*H)*, 4.18 (m, 4*H)*, 6.36 (d, *J* = 14 Hz, 2*H)*, 7.45 (d, *J* = 8.0 Hz, 2*H)*, 7.70 (d, *J* = 8.0 Hz, 2*H)*, 7.85 (s, 2*H)*, 8.31 (d, *J* = 14 Hz, 2*H)*. ^{13}C NMR (150 MHz, D_2O + DMSO-d_6): δ 17.74, 20.72, 23.58, 30.46, 55.10, 55.68, 59.45, 65.58, 68.22, 104.97, 113.62, 122.92, 129.66, 131.19, 143.94, 145.55, 145.67, 147.38, 176.09. HRMS (ESI) calculated for $C_{42}H_{58}ClN_4O_6S_2$ $[M]^+$ *m/z* 813.3481, found *m/z* 813.3486. Vis/NIR in MeOH; λ_{max} = 780 nm.

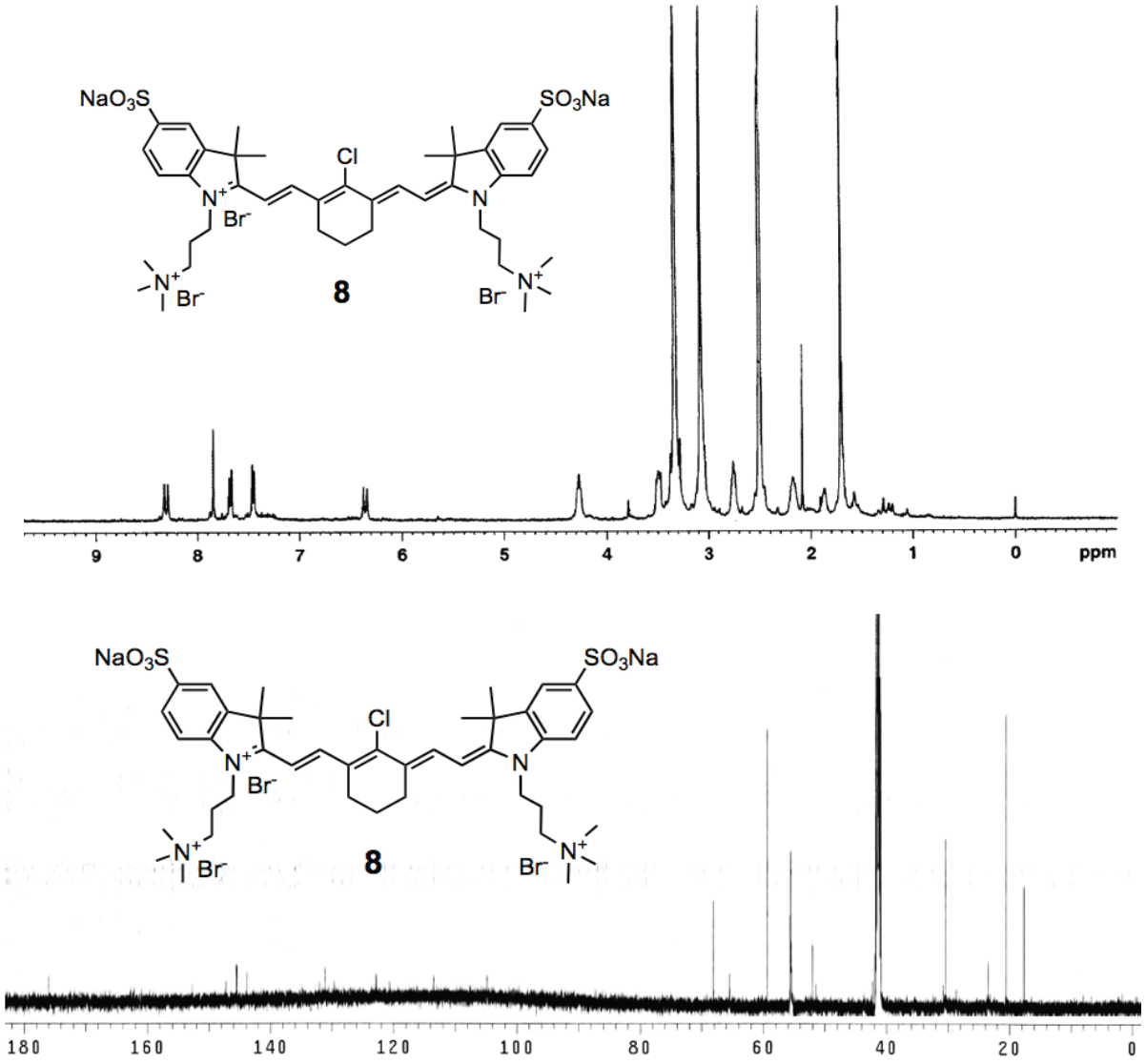

Figure S1c. ^{1}H NMR (top) and ^{13}C NMR (bottom) of compound #**8** in D_2O + DMSO-d_6.

2-((*E*)-2-((*E*)-2-chloro-3-((*E*)-2-(3,3-dimethyl-1-(3-(trimethylammonio)propyl)indolin-2-ylidene) ethylidene)cyclohex-1-en-1-yl)vinyl)-3,3-dimethyl-1-(3-(trimethylammonio)propyl)-3*H*-indol-1-ium bromide (9). A mixture of bromide salt **6** (840 mg, 2 mmol), Vilsmeier-Haack reagent **7** (359 mg, 1 mmol), and anhydrous sodium acetate (492 mg, 6 mmol) in absolute ethanol (50 mL) was heated under reflux at 100˚C for 5 h. The mixture was cooled to room temperature, and then concentrated under reduced pressure to yield a brown residue. The reaction mixture was concentrated to dryness and the brown residue was washed with dichloromethane and MTBE (1:1 ratio) to yield dye **9** (260 mg, 29%); ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.80 (s, 12*H)*, 2.40 (m, 4*H)*, 2.87 (m, 4*H)*, 3.24 (s, 18*H)*, 3.33 (m, 2*H)*, 3.75 (m, 4*H)*, 4.36 (t, *J* = 7.2 Hz, 4*H)*, 6.44 (d, *J* = 14.0 Hz, 2*H)*, 7.34 (t, *J* = 7.2 Hz, 2*H)*, 7.50 (m, 4*H)*, 7.58 (d, *J* = 7.2 Hz, 2*H)*, 8.49 (d, *J* = 14.0 Hz, 2*H)*. ^{13}C NMR (100 MHz, DMSO-d_6): δ 22.27, 22.51, 27.93, 28.62, 42.2,0 50.87, 54.01, 64.53, 102.93, 112.55, 123.77, 126.82, 129.32, 130.21, 142.68, 143.38, 146.23, 151.74, 174.45. MS-MALDI calculated for $C_{42}H_{60}ClN_4$ $[M]^+$ *m/z* 655.4561, found *m/z* 655.4564. Vis/NIR in MeOH; λ_{max} = 780 nm.

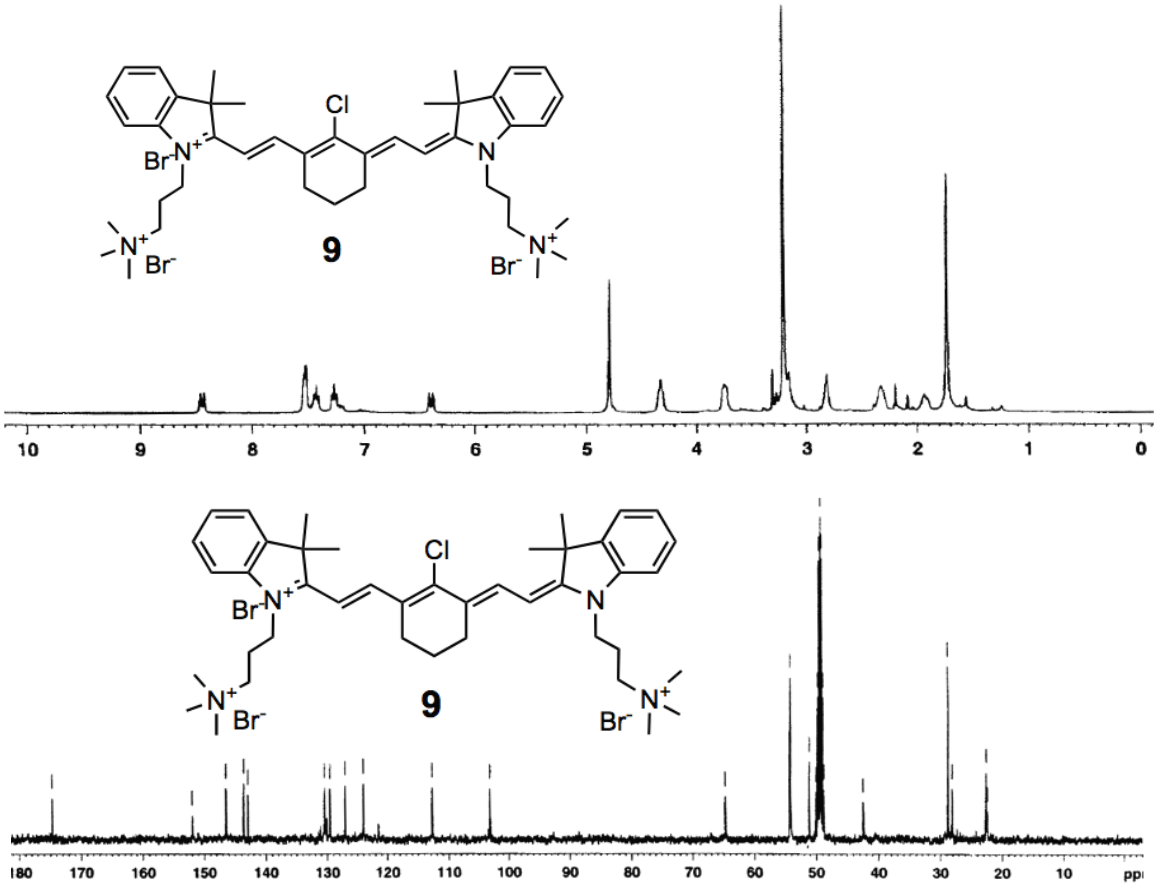

Figure S1d. ^{1}H NMR (top) and ^{13}C NMR (bottom) of compound #**9** in DMSO-d_6.

2-((*E*)-2-((*E*)-2-(4-(2-carboxyethyl)phenoxy)-3-((*E*)-2-(3,3-dimethyl-5-sulfonato-1-(3-(tri-methyl ammonio)-propyl)indolin-2-ylidene)ethylidene)cyclohex-1-enyl)vinyl)-3,3-dimethyl-1-(3-(trimethyl ammonio)-propyl)-3*H*-indolium-5-sulfonate disodium bromide (10; ZW800-1). We present 3 analytical-scale substitution routes for ZW800-1, and validated preparative-scale routes for ZW800-1 and ZW800-3a.

Preparation of 10 (ZW800-1) using sodium hydride (NaH) – Analytical scale method #1: A solution of 3-(4-hydroxyphenyl)propionic acid (33.1 mg, 0.2 mmol) and sodium hydride (8 mg of 60% NaH oil dispersion 0.2 mmol) in DMSO (2 mL) was stirred for 30 min under nitrogen atmosphere to form the phenolate. Chloro dye **8** (110 mg, 0.1 mmol or 22 mg, 0.02 mmol) was added, and the reaction mixture was heated under microwave conditions depicted in **Table S1**. The crude product was washed with MTBE 3 times and precipitated with methanol and MTBE (20 mL, 1:4) to yield **10** (ZW800-1) as a dark green solid. The conversion ratio from the chloro atom to phenoxypropionic acid and the final yield were calculated by LC-MS spectroscopy (80% conversion yield, 69% yield).

Preparation of 10 (ZW800-1) using sodium carbonate (Na_2CO_3) – Analytical scale method #2: 3-(4-hydroxyphenyl)propionic acid (33.1 mg, 0.2 mmol) and anhydrous sodium carbonate (21.2 mg, 0.2 mmol) were suspended in DMSO (2 mL) and stirred at room temperature for 30 min under nitrogen atmosphere. Chloro dye **8** (110 mg, 0.1 mmol or 22 mg, 0.02 mmol) was added and the reaction mixture was heated under microwave conditions depicted in **Table S1**. The crude product was washed with MTBE 3 times and precipitated with methanol and MTBE (20 mL, 1:4) to yield **10** (ZW800-1) as a dark green solid (75% conversion yield, 53% yield).

Preparation of 10 (ZW800-1) using sodium hydroxide (NaOH) – Analytical scale method #3: 3-(4-hydroxyphenyl)propionic acid (33.1 mg, 0.2 mmol) and sodium hydroxide (8 mg, 0.2 mmol) were suspended in DMSO (2 mL) and stirred at room temperature for 30 min under nitrogen atmosphere. Chloro dye **8** (110 mg, 0.1 mmol or 22 mg, 0.02 mmol) was added, and the mixture was heated under microwave conditions depicted in **Table S1**. The crude product was washed with MTBE 3 times and precipitated with methanol and MTBE (20 mL, 1:4) to yield **10** (ZW800-1) as a dark green solid (82% conversion yield, 41% yield).

Preparation of 10 (ZW800-1) using disodium 3-(4-oxidophenyl)propanoate (SOPP) – Preparative scale method: SOPP ($C_9H_8Na_2O_3$, MW 210.14) was prepared by adding 3-(4-hydroxyphenyl)propionic acid (16.6 g, 100 mmol) into a solution of sodium hydroxide (8 g, 200 mmol) in water. The mixture was stirred at room temperature for 2 h, followed by lyophilization. The pale yellow solid was dried under vacuum for 24 h and used for the next step without further purification. In the

following step, chloro dye **8** (110 mg, 0.1 mmol) and 2 or 10 equiv of SOPP (42 mg or 210 mg) were dispersed in DMSO or DMF (2 mL) under nitrogen atmosphere. The mixture was heated under microwave conditions depicted in **Table S1**. As a control, conventional substitution reactions for ZW800-1 were prepared by adding chloro dye **8** (5.5 g, 5 mmol) and 2 or 10 equiv of SOPP (2.1 g or 10.5 g) in DMSO, followed by heating the mixture in a 65°C oil bath under nitrogen atmosphere for 6 to 24 h.

Based on this systematic analysis, the optimal condition for preparative synthesis of ZW800-1 included DMSO as solvent, 2 equiv of SOPP, and 30 min of microwave irradiation at 65˚C. The crude product was washed with MTBE 3 times and precipitated with methanol and MTBE (20 mL, 1:4) to yield **10** (ZW800-1) as a dark green solid (98% conversion yield, 85% yield); ^{1}H NMR (600 MHz, D_2O): δ 0.943 (s, 12*H)*, 1.71 (m, 2*H)*, 2.06 (m, 4*H)*, 2.35 (m, 2*H)*, 2.45 (m, 2*H)*, 2.63 (m, 2*H)*, 2.97 (s, 18 *H)*, 3.38 (m, 4*H)*, 3.83 (m, 4*H)*, 5.97 (d, *J* = 13.2 Hz, 2*H)*, 6.81 (d, *J* = 7.8 Hz, 2*H)*, 7.03 (d, *J* = 7.2 Hz, 4*H)*, 7.45 (s, 2*H)*, 7.56 (d, *J* = 6.6 Hz, 2*H)*, 7.66 (d, *J* = 13.2 Hz, 2*H)*. ^{13}C NMR (150 MHz, D_2O): δ 19.82, 22.61, 23.36, 29.92, 39.90, 41.62, 42.79, 42.90, 47.52, 51.18, 51.41, 51.65, 55.54, 55.71, 60.02, 65.59, 66.23, 66.94, 103.32, 108.48, 113.42, 117.44, 118.36, 122.49, 125.39, 126.09, 127.12, 129.08, 129.63, 132.22, 132.96, 135.82, 137.66, 138.29, 139.45, 140.03, 141.47, 142.94, 143.70, 145.87, 148.44, 150.51, 152.08, 157.56, 159.09, 159.44, 160.80, 167.82, 175.04, 183.81, 184.48. MS-MALDI calculated for $C_{51}H_{66}N_4O_9S_2$ $[M-H]^-$ *m/z* 941.43, found *m/z* 941.48. Vis/NIR in MeOH; λ_{max} = 770 nm.

Table S1. Optimization of microwave-assisted substitution of phenoxypropionic acid on the central *meso*-position of ZW800-1 (**8** --> **10**; see **Figure 1a**).

#	Solvent	Base		Temp	Time	Conversion	Yield
		Type	Equiv	(˚C)	(min)	Yield (%)*	(%)
1	DMF	SOPP	2	65	5	3	trace
2	DMF	SOPP	2	65	10	7	trace
3	DMF	SOPP	2	65	30	20	10
4	DMF	SOPP	10	65	5	10	trace
5	DMF	SOPP	10	65	10	9	trace
6	DMF	SOPP	10	65	30	23	11
7	DMF	SOPP	2	100	5	42	30
8	DMF	SOPP	2	100	10	56	44
9	DMF	SOPP	2	100	30	82	68
10	DMF	SOPP	10	100	5	53	46
11	DMF	SOPP	10	100	10	70	52
12	DMF	SOPP	10	100	30	86	74
13	DMF	SOPP	2	135	5	100	trace
14	DMF	SOPP	2	135	10	100	trace
15	DMF	SOPP	2	135	30	100	trace
16	DMF	SOPP	10	135	5	100	trace
17	DMF	SOPP	10	135	10	100	trace
18	DMF	SOPP	10	135	30	100	trace
19	DMSO	SOPP	2	65	2	55	42
20	DMSO	SOPP	2	65	5	56	40
21	DMSO	SOPP	2	65	10	60	43
22	DMSO	SOPP	2	65	20	75	55
23	DMSO	SOPP	2	65	30	98	85
24	DMSO	SOPP	10	65	30	98	85
25	DMSO	SOPP	2	130	2	100	10
26	DMSO	SOPP	2	130	5	100	12
27	DMSO	SOPP	2	130	10	100	15
28	DMSO	SOPP	2	130	20	100	10
29	DMSO	SOPP	2	130	30	100	trace
30	DMSO	SOPP	10	100	30	100	trace
31	DMSO	NaH	2	65	30	76	59
32	DMSO	NaH	10	65	30	80	69
33	DMSO	Na_2CO_3	2	65	30	64	35
34	DMSO	Na_2CO_3	10	65	30	75	53
35	DMSO	NaOH	2	65	30	65	42
36	DMSO	NaOH	10	65	30	82	41

SOPP, disodium 3-(4-oxidophenyl)propanoate; NaH, sodium hydride; Na_2CO_3, sodium carbonate; NaOH, sodium hydroxide. Conversion yield (%) and final yield (%) were analyzed by LC-MS. Dark gray: preparative scale synthesis and purification; White: analytical scale synthesis and purification.

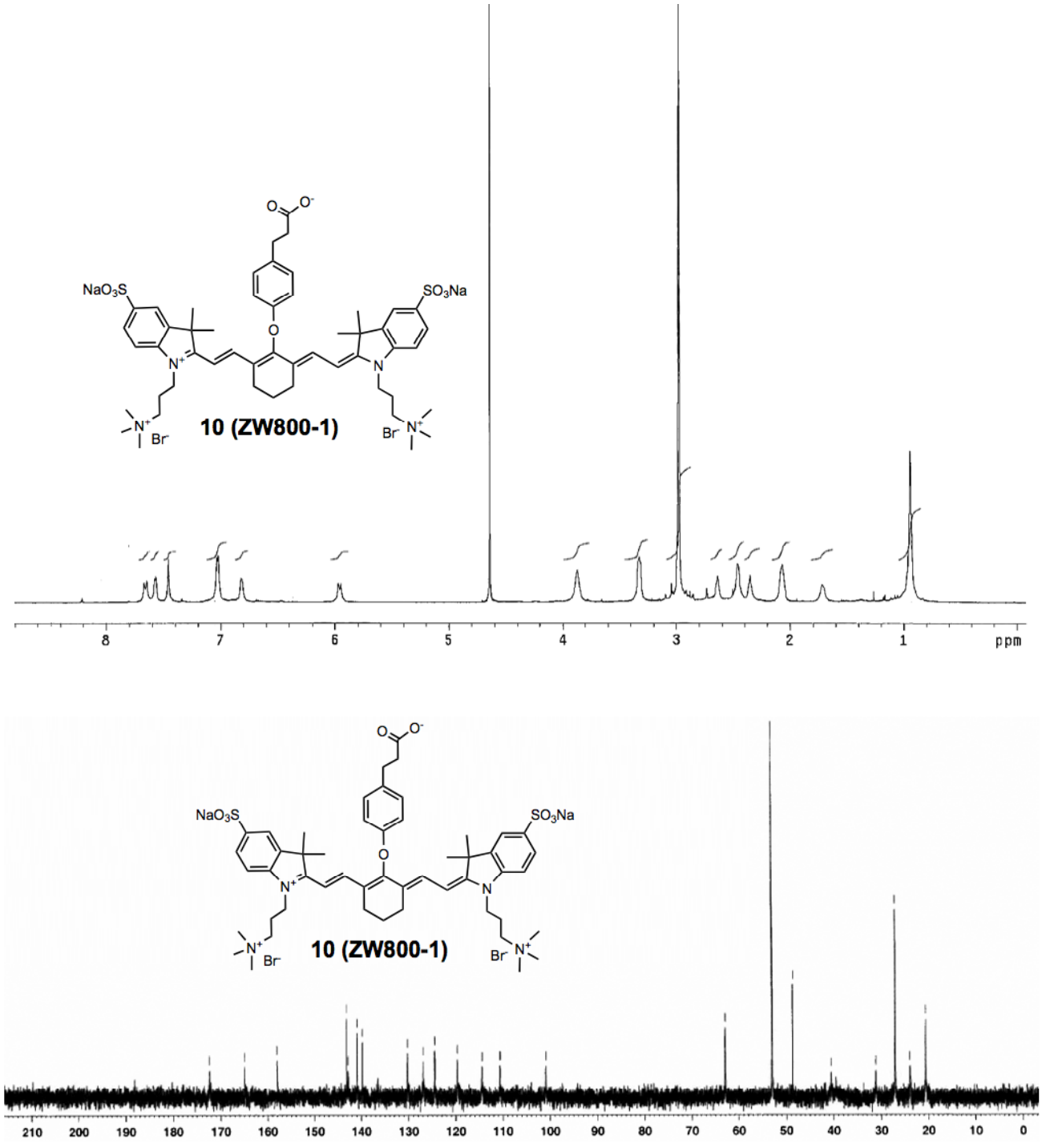

Figure S1e. ^{1}H NMR (top) and ^{13}C NMR (bottom) of compound **10** (ZW800-1) in D_2O.

2-((*E*)-2-((*E*)-2-(4-(2-carboxyethyl)phenoxy)-3-((*E*)-2-(3,3-dimethyl-1-(3-(trimethyl ammonio)propyl)indolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-3,3-dimethyl-1-(3-(trimethylammonio)propyl)-3*H*-indol-1-ium bromide (11; ZW800-3a) – *Preparative scale method*: Chloro dye **9** (89.6 mg, 0.1 mmol) and 2 equiv of SOPP (42 mg, 0.2 mmol) were dissolved in DMSO (2 mL) under nitrogen atmosphere. The mixture was heated at 65°C for 30 min under a microwave. The crude product was washed with MTBE 3 times to yield **11** (ZW800-3a) as a dark green solid (99% conversion yield, 92% yield); ^{1}H NMR (600 MHz, DMSO-d_6): δ 1.27 (s, 12*H)*, 2.42 (m, 4*H)*, 2.10 (m, 4*H)*, 2.68 (t, *J* = 7.8 Hz, 2*H)*, 2.74 (m, 4*H)*, 3.07 (s, 18*H)*, 3.58 (m, 4*H)*, 4.16 (m, 4*H)*, 6.21 (d, *J* = 13.8 Hz, 2*H)*, 6.64 (d, *J* = 7.8 Hz, 2*H)*, 6.99 (d, *J* = 8.4 Hz, 2*H)*, 7.05 (d, *J* = 8.4 Hz, 2*H)*, 7.23 (m, 2*H)*, 7.40 (t, *J* = 7.2 Hz, 2*H)*, 7.44 (d, *J* = 7.8 Hz, 2*H)*, 7.51 (d, *J* = 7.2 Hz, 2*H)*, 7.86 (d, *J* = 13.8 Hz, 2*H)*. ^{13}C NMR (400 MHz, DMSO-d_6): 20.74, 23.91, 27.27, 28.24, 30.20, 37.09, 38.89, 48.62, 52.44, 62.32, 100.38, 111.20, 114.14, 115.05, 122.10, 122.49, 128.95, 130.08, 131.43, 140.92, 141.41, 141.78, 155.55, 171.63, 174.55. MS-MALDI calculated for $C_{51}H_{68}N_4O_3$ $[M]^+$ *m/z* 784.54, found *m/z* 784.35. Vis/NIR in MeOH; λ_{max} = 765 nm.

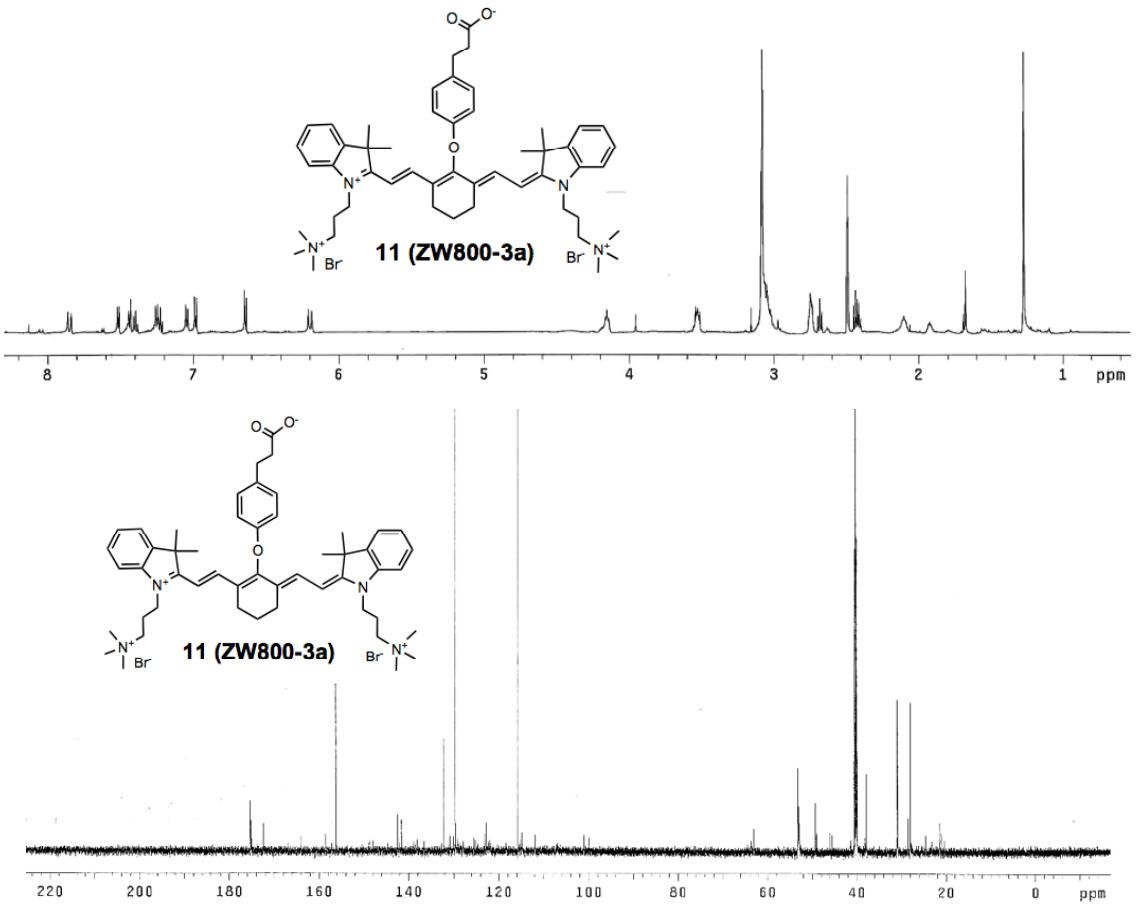

Figure S1f. ^{1}H NMR (top) and ^{13}C NMR (bottom) of compound **11** (ZW800-3a) in DMSO-d_6.

MALDI-TOF Mass Spectroscopy (Figure S2): Chemical structures of the series of NIR fluorophores in PBS, FBS, MeOH, DMSO, or urine were analyzed using a μFocus Plate (HST, Inc., Newark, NJ) by UltraFlex III MALDI-TOF mass spectroscopy (Bruker Daltonics, Inc., Billerica, MA). Desalting microcolumns, packed with Poros R2 resin (Applied Biosystems, Framingham, MA), were used to remove excess salts and contaminants from serum and urine samples. Positive and negative spectra were collected using a solid state Smartbeam™ laser (Bruker Daltonics) operating at 100 Hz in reflectron mode (mass range 50-2,500 Da).

Optical and Physicochemical Property Analyses (Figures S3 and S4): All optical measurements were performed at 37˚C in 100% FBS buffered with 50 mM HEPES, pH 7.4. For fluorescence quantum yield (QY) measurements, ICG in DMSO (QY 13%) was used as a calibration standard under conditions of matched absorbance at 770 nm. For *in vitro* optical property measurements, online fiberoptic HR2000 absorbance (200-1100 nm) and USB2000FL fluorescence (350-1,000 nm) spectrometers (Ocean Optics, Dunedin, FL) were used. NIR excitation was provided by a 770 nm NIR laser diode light source (Electro Optical Components, Santa Rosa, CA) set to 8 mW and coupled through a 300-μm core diameter, NA 0.22 fiber (Fiberguide Industries, Stirling, NJ).

Gel-Filtration Chromatography (GFC; Figure S5): Gel-filtration analysis was performed with a Waters 996 PDA Detector (200-800 nm) and a Waters 2475 Multi-Wavelength Fluorescence Detector (λ_1 Exc = 770 nm, Em = 790 nm; λ_2 Exc = 800 nm, Em = 820 nm). For measurement of the effect of serum protein adsorption, 100 μM NIR fluorophores were incubated in PBS (pH 7.4) and 100% FBS supplemented with 50 mM HEPES (pH 7.4) at 37˚C for 4 h prior to loading 20 μL on a 5 μm, 8 × 300 mm, 60 Å Diol size-exclusion column (Diol-60, YMC, Japan). For the mobile phase, 0.1 M PBS (pH 6.8) containing 0.2 M Na_2SO_4, 0.5% Triton X-100 (Dow Chemicals, Midland, MI) and 0.5% CHAPS (Fisher) was used with a flow rate of 1 mL/min. Calibration of hydrodynamic diameter (HD) was performed by injecting 20 μL of protein standards containing thyroglobulin (M1, 669 kDa, 18.8 nm HD), γ-globulin (M2, 158 kDa, 11.1 nm HD), ovalbumin (M3, 44 kDa, 6.1 nm HD), myoglobin (M4, 17 kDa, 3.8 nm HD), and vitamin B_{12} (M5, 1.3 kDa, 1.5 nm HD). All HD measurements were performed 3 times in independent experiments.

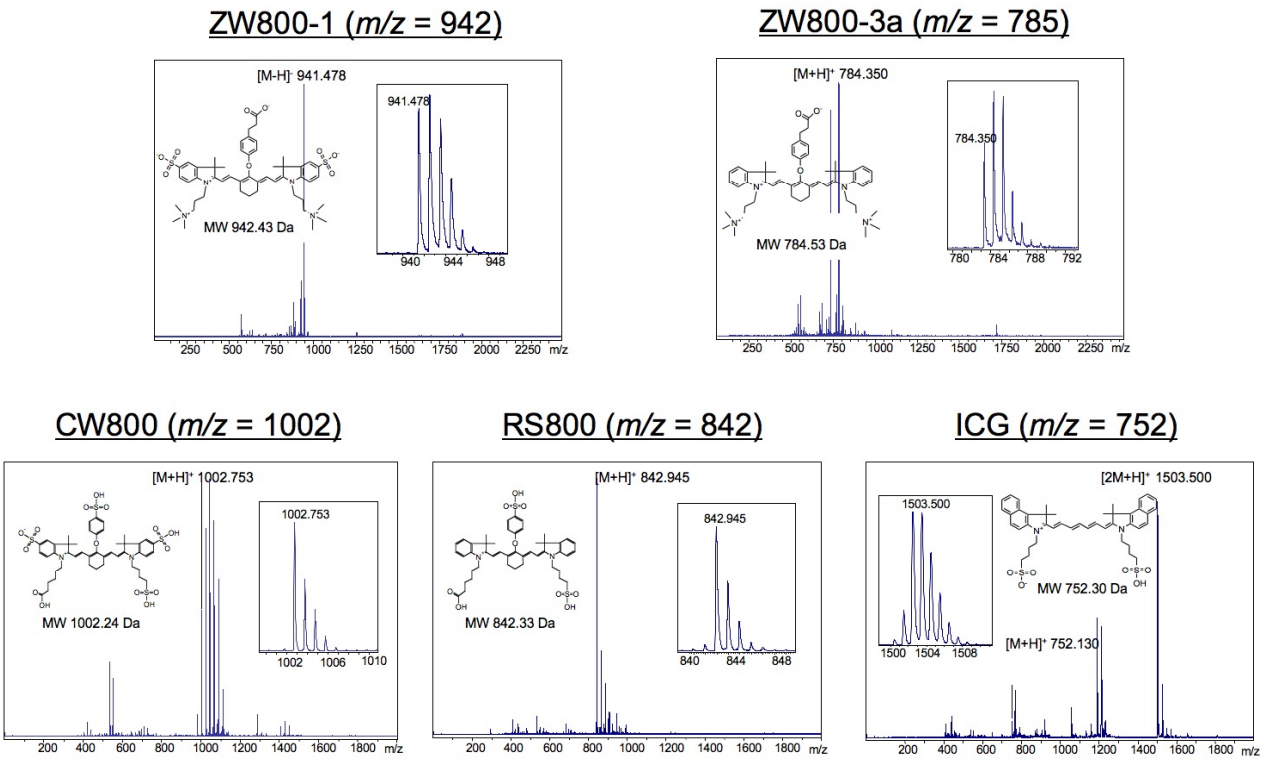

Figure S2. MALDI-TOF MS of ZW800-1 (*m/z* = 942), ZW800-3a (*m/z* = 785), CW800 (*m/z* = 1002), RS800 (*m/z* = 842), and ICG (*m/z* = 752) in 100% serum. Samples were incubated in FBS supplemented with 50 mM HEPES (pH 7.4) at 37˚C for 4 h, and filtered by a desalting microcolumn, packed with porous R2 resin prior to MALDI-TOF injection.

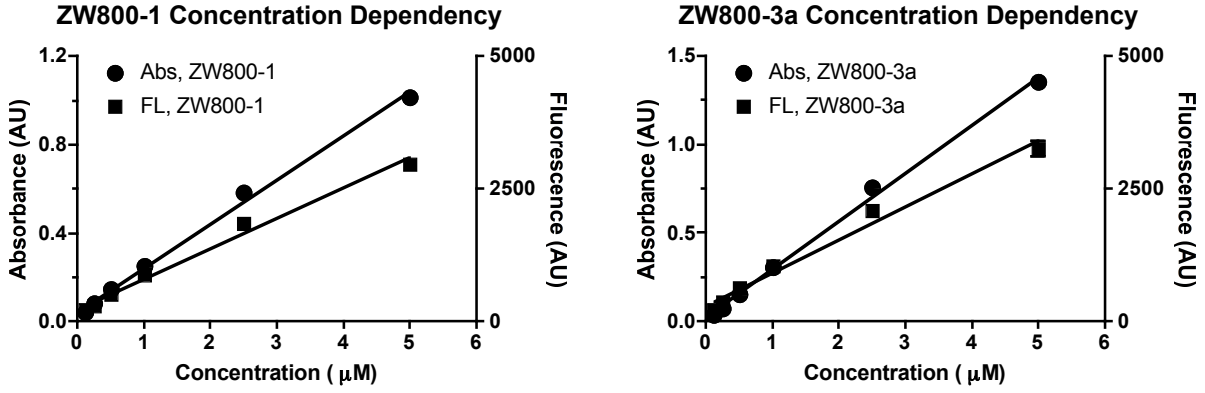

Figure S3. Concentration-dependent absorption and fluorescence emission spectra of ZW800-1 and ZW800-3a in 100% FBS. R^2 values for absorbance (Abs) and fluorescence emission (FL) for ZW800-1 are 0.9954 and 0.9884, respectively, while those for ZW800-3a are 0.9967and 0.9824, respectively.

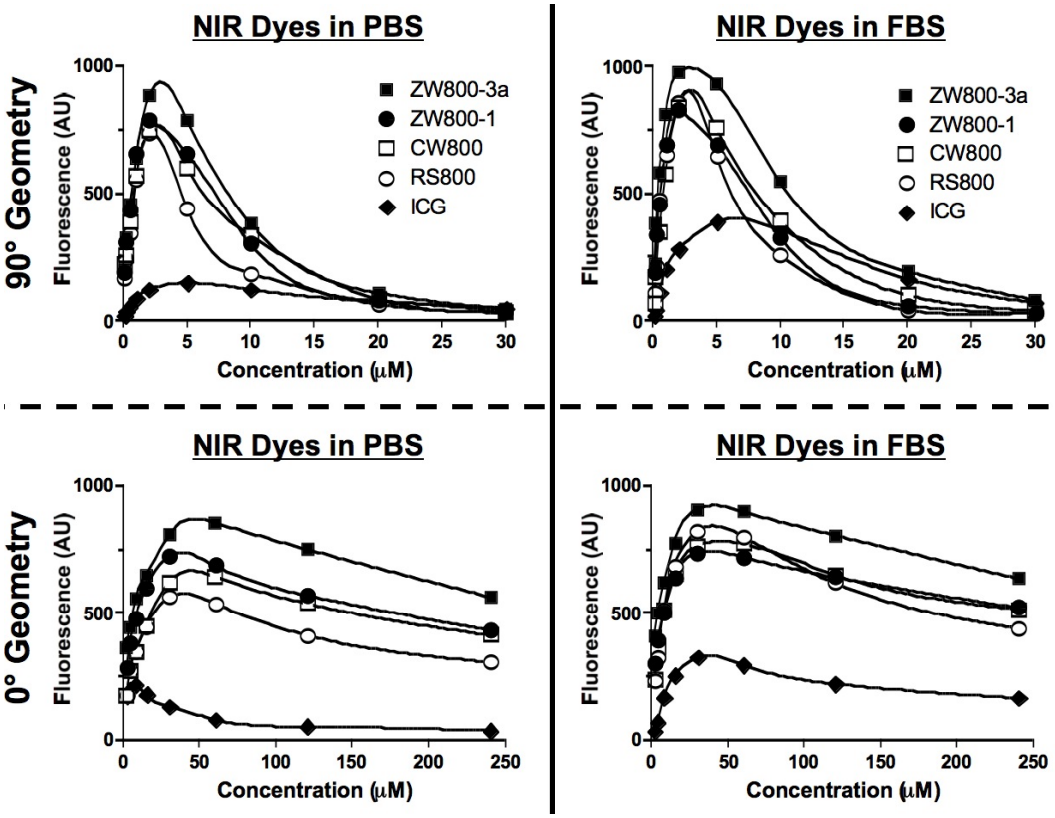

Figure S4. The effect of fluorophore concentration on total fluorescence yield (i.e., quenching) measured in the 90˚ geometry using a 770-nm NIR laser diode light source or in the 0˚ geometry using the FLARE™ imaging system after incubating fluorophores in PBS and in 100% FBS supplemented with 50 mM HEPES (pH 7.4) at 37˚C for 4 h.

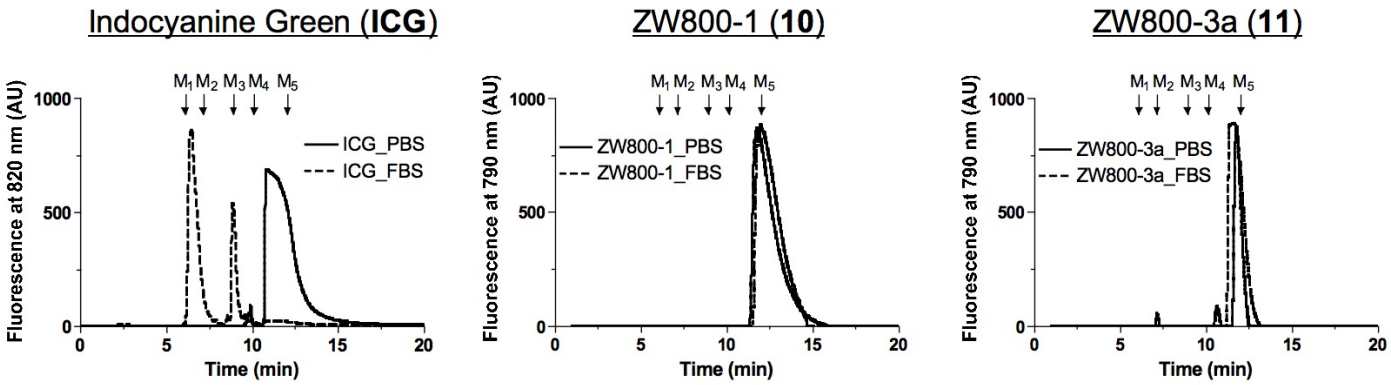

Figure S5. Gel-filtration analyses of ICG, ZW800-1 (**10**), and ZW800-3a (**11**) in PBS (solid line) or 100% FBS (dotted line). HD of NIR fluorophores after incubating at 37˚C for 4 h as measured using a fluorescence detector. Molecular weight markers M1 to M5 are indicated by arrows.

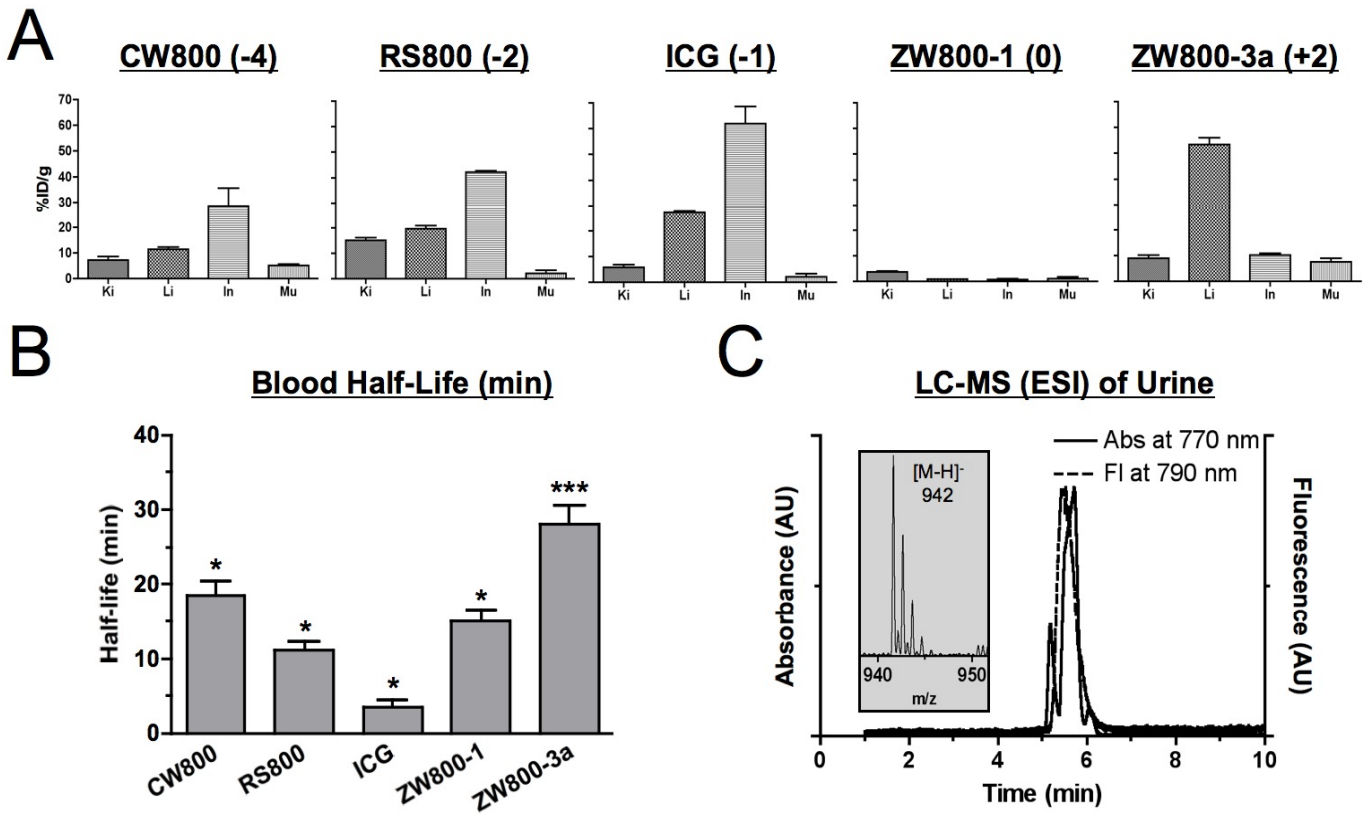

Figure S6. *In vivo* biodistribution and clearance of NIR fluorophores having systematically varying net charge. 40 pmol/g of NIR fluorophores were injected intravenously into CD-1 mice and their biodistribution (**A**) and blood half-life (**B**) were measured over 4 h. **P* < 0.05 and ****P* < 0.001. **C**) Renal excretion of ZW800-1 (right bottom) was confirmed by analyzing mouse urine 4 h postinjection. Each data point is the mean ± S.D. from N = 5 animals.

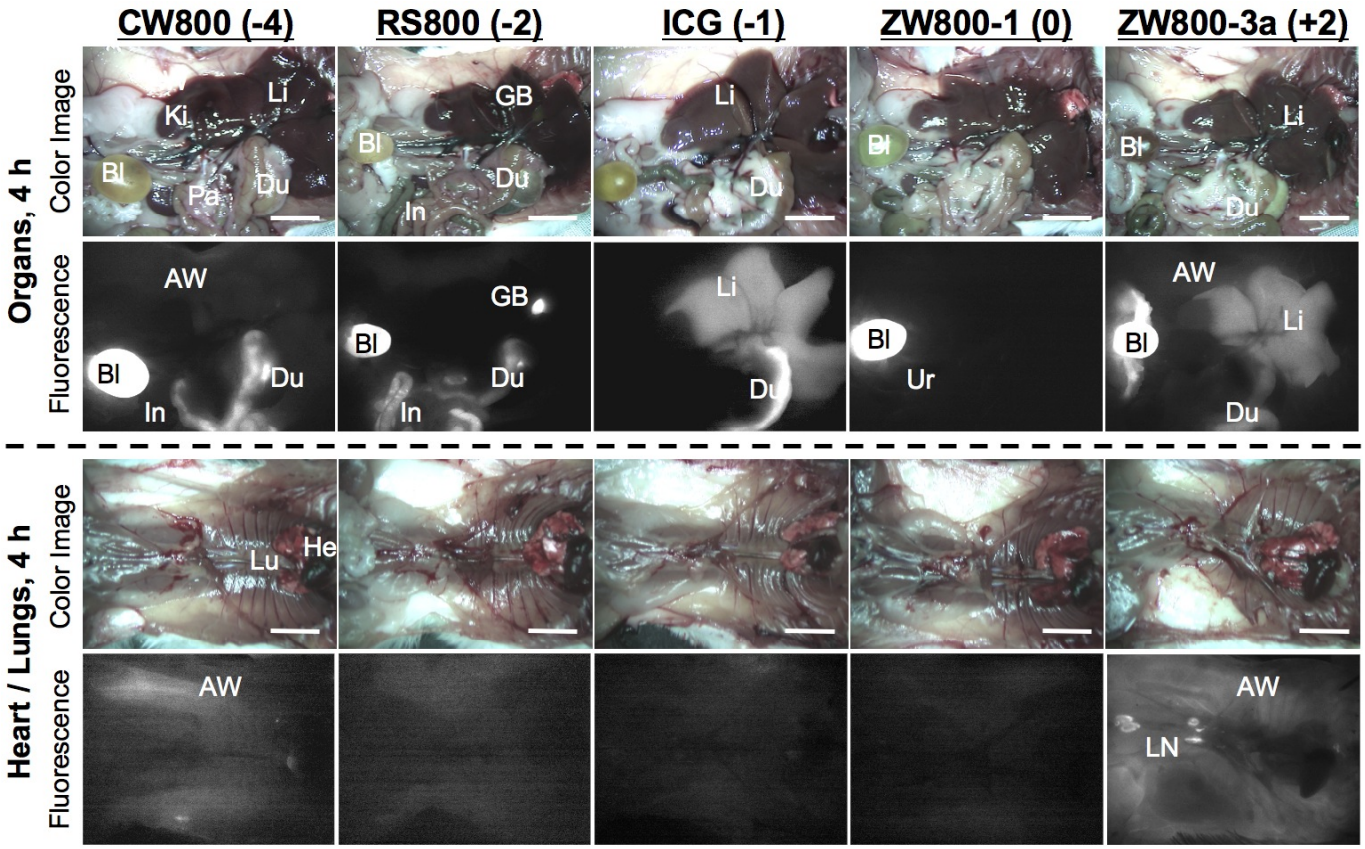

Figure S7. Biodistribution and clearance of NIR fluorophores into CD-1 mice. 40 pmol/g of each fluorophore was intravenously injected and imaged 4 h postinjection. Shown are color and 800 nm NIR fluorescence images of surgically exposed *in situ* organs (top 2 panels) and heart/lungs (bottom 2 panels). Abbreviations used are: AW, abdominal wall; Bl, bladder; BD, bile duct; Du, duodenum; GB, gall bladder; He, heart; In, intestine; Ki, kidneys; Li, liver; LN, lymph node; Lu, lungs; Mu, muscle; Pa, pancreas; and Ur, ureter. Each set of NIR fluorescence images has an identical camera exposure time and normalization. Scale bars = 1 cm.

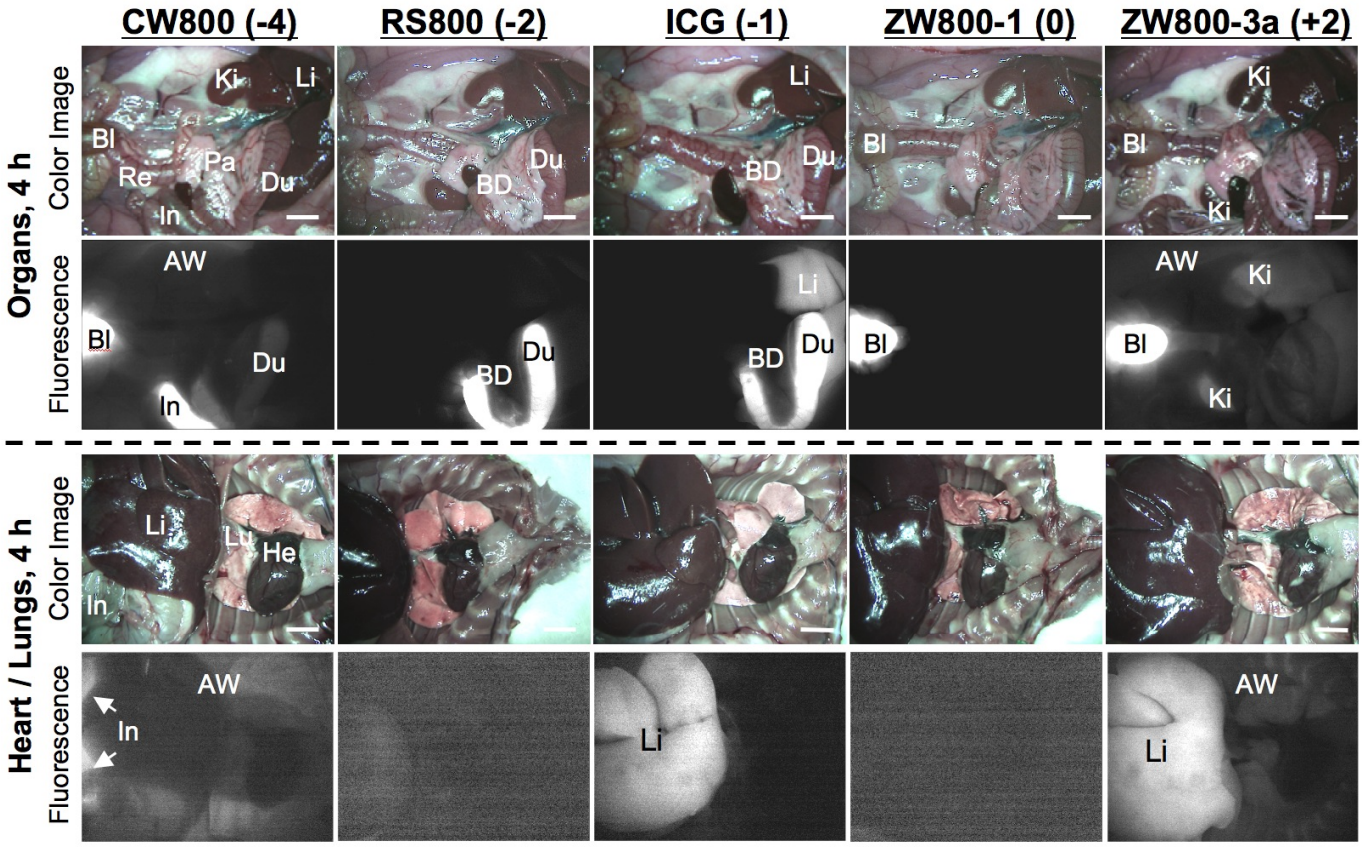

Figure S8. Biodistribution and clearance of NIR fluorophores into SD rats. 40 pmol/g of each fluorophore was intravenously injected and imaged 4 h postinjection. Shown are color and 800 nm NIR fluorescence images of surgically exposed *in situ* organs (top 2 panels) and heart/lungs (bottom 2 panels). Abbreviations used are: AW, abdominal wall; BD, bile duct; Bl, bladder; Du, duodenum; In, intestine; Ki, kidneys; Li, liver; Lu, lungs; LN, lymph node; Lu, lungs; Pa, pancreas; and Re, rectum. NIR fluorescence images have an identical camera exposure time and normalization. Scale bars = 1 cm.

SUPPORTING VIDEOS

Video S1 Real-time biodistribution and excretion of ZW800-1 over a 4 h time lapse. 40 pmol/g of ZW800-1 was injected intravenously into a 250-g SD rat. Shown are color video (left) and NIR fluorescence video (right). λ_{Exc} = 760 ± 20 nm and λ_{Em} = 795 nm longpass. Arrows mark the position of the ureters.

Video S2 Real-time biodistribution and excretion of ZW800-3a over a 4 h time lapse. 40 pmol/g of ZW800-3a was injected intravenously into a 250-g SD rat. Shown are color video (left) and NIR fluorescence video (right). λ_{Exc} = 760 ± 20 nm and λ_{Em} = 795 nm longpass. Arrows mark the position of the ureters and arrowheads mark the position of the common bile duct.

Video S3 Real-time biodistribution and elimination of CW800 over a 4 h time lapse. 40 pmol/g of CW800 was injected intravenously into a 250-g SD rat. Shown are color video (left) and NIR fluorescence video (right). λ_{Exc} = 760 ± 20 nm and λ_{Em} = 795 nm longpass. Arrows mark the position of the ureters and arrowheads mark the position of the common bile duct.