Supporting Information

“Near-infrared Fluorescence Probes for Enzymes Based on Binding Affinity Modulation of Squarylium Dye Scaffold”

(Analytical Chemistry)

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Experimental Procedure

1. Synthesis and Characterization

Materials

General chemicals were of the best grade available, supplied by Tokyo Chemical Industries, Wako Pure Chemical, Aldrich Chemical Co., Alfa Aesar, or Acros Organics, and were used without further purification.

Instruments

$^1$H NMR and $^{13}$C NMR spectra were recorded on a JEOL JNM-LA300 instrument (300 MHz for $^1$H NMR and 75 MHz for $^{13}$C NMR) or a JEOL JNM-LA400 instrument (400 MHz for $^1$H NMR and 100 MHz for $^{13}$C NMR); $\delta$ values are in ppm relative to TMS. Mass spectra (MS) were measured with a JEOL JMS-T100LC AccuTOF (ESI). HPLC purification and analyses were performed on a reverse-phase column (GL Sciences (Tokyo, Japan) Inertsil ODS-3 30 mm × 250 mm or 10 mm × 250 mm for purification and Inertsil ODS-3 4.6 mm × 250 mm for analyses) fitted on a Jasco PU-1587 system or a Jasco PU-2080 system for purification and a Jasco PU-980 system for analyses, using eluent A (H$_2$O with 0.1% TFA) and eluent B (CH$_3$CN with 20% H$_2$O and 0.1% TFA).
Synthetic procedures

Cy5F, Cy5MeO, Cy5Bn, Cy5PH, and Cy5SO₃H were prepared according to the previous reports.¹ SR1, SR2

Scheme 1. Synthesis of Cy5CF₃.

5-(Trifluoromethyl)-2,3,3-trimethylindolenine (2)

4-(Trifluoromethyl)phenylhydrazine (1) (4.8 g, 27 mmol) and 3-methyl-2-butanone (3 mL, 28 mmol) were dissolved in methanol (30 mL) and concentrated hydrochloric acid (1 mL). The reaction mixture was refluxed for 10 hours, then allowed to cool to room temperature, and the solvent was removed under reduced pressure. To the resulting residue was added dichloromethane, and the whole was washed with a 2 N aqueous solution of sodium hydroxide. The organic extracts were dried over Na₂SO₄, filtered, and evaporated to afford 2 as a brown oil (6.2 g, yield quant).¹ H NMR (300 MHz, DMSO-d₆): δ 7.83 (s, 1H), 7.63 (dd, 1H, J = 8.3, 1.5 Hz), 7.59 (d, 1H, J = 8.3 Hz), 2.24 (s, 3H), 1.27 (s, 6H). ¹³C NMR (75 MHz, DMSO-d₆): δ 191.7, 156.7, 146.9, 130.1, 126.5, 126.0, 125.6, 125.2, 125.1, 125.0, 124.9, 124.7, 122.9, 119.6, 119.3, 118.9, 118.8, 118.7, 53.8, 22.0, 15.2. HRMS (ESI⁺): Calcd for [M+H]⁺, 228.1000, Found, 228.0966 (−3.4 mmu).
1-(β-Carboxyethyl)-5-(trifluoromethyl)-2,3,3-trimethylindolenium iodide (3)

Compound 2 (6.2 g, 27 mmol) and 3-iodopropionic acid (5.5 g 28 mmol) were dissolved in acetonitrile (20 mL). The reaction mixture was refluxed for 12 hours, then allowed to cool to room temperature, and the solvent was removed under reduced pressure. To the resulting residue was added dichloromethane, and the whole was washed with a saturated aqueous solution of sodium bicarbonate. The organic extracts were dried over Na₂SO₄, filtered, and evaporated to afford 3 as a brown solid (1.1 g, yield 10% crude). The crude 3 was used without further purification in the next reaction. HRMS (ESI⁺): Calcd for [M–I]⁺, 300.1211, Found, 300.1216 (+0.5 mmu).

1,1'-Bis-(β-carboxyethyl)-5,5'-bis-(trifluoromethyl)-3,3,3',3'-tetramethylindodicarbocyanine iodide (Cy5CF₃)

Malonaldehyde dianilide hydrochloride (0.3 g, 1.2 mmol) was dissolved in a mixture of dichloromethane (5 mL) and DIEA (0.3 mL). To this mixture was added dropwise a mixture of acetic anhydride (0.2 mL) and dichloromethane (2 mL). The reaction mixture was stirred at room temperature for 4 hours, then added dropwise to refluxing acetonitrile solution (15 mL) containing 3 (0.74 g, 1.7 mmol) and sodium acetate (0.4 g, 4.9 mmol). The resulting mixture was refluxed for 10 hours, then allowed to cool to room temperature, and the solvent was removed under reduced pressure. To the residue was added dichloromethane, and the whole was washed with saturated aqueous solution of sodium bicarbonate. The organic extracts were dried over Na₂SO₄, filtered, and evaporated. The crude product was purified by semi-preparative HPLC until a single peak of the product was obtained, affording Cy5CF₃ as a dark gold powder (11 mg, yield 2%). ¹H NMR (300 MHz, CD₃OD): δ 8.34 (t, 2H, J = 13.1 Hz), 7.77-7.52 (m, 6H), 6.74 (t, 1H, J = 13.1 Hz), 6.74 (d, 2H, J = 13.1 Hz), 4.39 (t, 4H, J = 7.2 Hz), 2.64 (t, 4H, J = 7.2 Hz), 1.76 (s, 12H). ¹³C NMR (100 MHz, CD₃OD): δ 176.9, 157.1, 146.5, 143.4, 128.7, 128.0, 127.7, 127.4, 127.2, 124.5, 120.5, 112.8, 106.0, 50.7, 42.9, 35.6, 27.7. HRMS (ESI⁺): Calcd for [M–I]⁺, 635.2345, Found, 635.2377 (+3.2 mmu).
Scheme 2. Synthesis of Sq.

1-(β-Carboxyethyl)-2,3,3-trimethylindolenium iodide (5)
3-Iodopropionic acid (32.0 g, 0.16 mol) was suspended in 2,3,3-trimethylindolenine (4) (25 mL, 0.15 mol). The suspension was stirred at 100 °C for 15 hours under an argon atmosphere, then allowed to cool to room temperature, and the resulting solid was collected by filtration. The solid was washed with chloroform and dried under reduced pressure to afford 5 as a pink solid (19.9 g, yield 37%). ^1H NMR (300 MHz, DMSO-d$_6$): δ 7.97-7.59 (m, 4H), 4.64 (t, 2H, J = 7.0 Hz), 2.97 (t, 2H, J = 7.0 Hz), 2.85 (s, 3H), 1.51 (s, 6H). ^13C NMR (75 MHz, DMSO-d$_6$): δ 198.0, 171.6, 141.8, 140.8, 129.4, 129.0, 123.6, 115.6, 54.4, 43.7, 31.2, 22.0, 14.7. HRMS (ESI$^+$): Calcd for [M–I]$^+$, 232.1338, Found, 232.1322 (+1.6 mmu).

1-(β-Carboxyethyl)-2-[3'-[1''-(β-carboxyethyl)-1'',3''-dihydro-3'',3''-dimethyl-2H-indol-2''-ylidenemethyl]-2'-hydroxy-4'-oxo-2'-cyclobuten-1'-ylidenemethyl]-3,3-dimethylindolenium (Sq)
Compound 5 (0.72 g, 2.00 mmol) and squaric acid (104 mg, 0.91 mmol) were dissolved in toluene (10 mL), 1-butanol (10 mL), and pyridine (5 mL). The flask was fitted with a Dean-Stark trap, and the mixture was refluxed for 12 hours under an argon atmosphere, then allowed to cool to room temperature. The solvent was removed under reduced pressure. The resulting residue was dissolved in 2 N HCl aq. (10 mL) and MeOH (10 mL), and the solution was refluxed for 12 hours to cleave the n-butyl ester, then allowed to cool to room temperature. The resulting solid was collected by filtration and washed with acetone to afford Sq as a green powder (216 mg, yield 44%). ^1H NMR (400 MHz, DMSO-d$_6$): δ 12.59 (s, 1H), 7.52 (d, 2H, J = 7.3 Hz), 7.36-7.34 (m, 4H), 7.15-7.10 (m, 2H), 5.81 (s, 2H), 4.33 (t, 4H, J = 6.8 Hz), 2.70 (t, 4H, J = 6.8 Hz), 1.68 (s, 12H). HRMS (ESI$^+$): Calcd for [M+H]$^+$, 541.2339, Found, 541.2365 (+2.6 mmu).
Scheme 3. Synthesis of SqSO₃H.

1-(β-Carboxyethyl)-2-{3'-(1''-(β-carboxyethyl)-1'',3''-dihydro-3''-dimethyl-5''-sulfo-2H-indol-2''-ylidenemethyl] -2'-hydroxy-4'-oxo-2'-cyclobuten-1'-ylidenemethyl]-3,3-dimethylindolenium-5-sulfonic acid (SqSO₃H)

1-(β-Carboxyethyl)-2,3,3-trimethylindolenium-5-sulfonate (6) was prepared according to the literature. Compound 6 (0.65 g, 1.86 mmol) and squaric acid (107 mg, 0.94 mmol) were dissolved in toluene (15 mL), 1-butanol (15 mL), and pyridine (5 mL). The flask was fitted with a Dean-Stark trap, and the mixture was refluxed for 12 hours under an argon atmosphere, then allowed to cool to room temperature. The solvent was removed under reduced pressure. The crude product was purified by semi-preparative HPLC until a single peak of the product was obtained, affording SqSO₃H as a dark red powder (13 mg, yield 2%). ¹H NMR (300 MHz, D₂O): δ 7.60-7.57 (m, 5H), 7.12 (d, 2H, J = 8.1 Hz), 5.65 (s, 2H), 4.21 (t, 4H, J = 6.6 Hz), 2.67 (t, 4H, J = 6.6 Hz), 1.40 (s, 12H). ¹³C NMR (75 MHz, D₂O): δ 185.1, 175.4, 172.7, 150.1, 144.5, 142.7, 139.1, 126.7, 120.3, 111.2, 87.1, 40.2, 39.3, 31.9, 26.3. HRMS (ESI⁻): Calcd for [M⁻H]⁻, 699.1318, Found, 699.1286 (−3.2 mmu).
Scheme 4. Synthesis of SqNaphtho.

1-(β-Carboxyethyl)-2,3,3-trimethylbenzoindolenium iodide (8)

2,3,3-Trimethylbenzoindolenine (7) (7.0 g, 33 mmol) and 3-iodopropionic acid (6.8 g, 34 mmol) were dissolved in o-dichlorobenzene (80 mL). The reaction mixture was stirred at 100 °C for 20 hours, and then allowed to cool to room temperature. The resulting solid was collected by filtration, washed with diethyl ether, and dried under reduced pressure to afford 8 as a white solid (11.0 g, yield 81%). 1H NMR (300 MHz, DMSO-<sub>d6</sub>): δ 8.41 (d, 1H, J = 8.8 Hz), 8.32 (d, 1H, J = 8.8 Hz), 8.23 (t, 2H, J = 8.4 Hz), 7.80-7.76 (m, 2H), 4.83 (t, 2H, J = 6.6 Hz), 3.10 (t, 2H, J = 6.6 Hz), 3.03 (s, 3H), 1.79 (s, 6H). 13C NMR (75 MHz, DMSO-<sub>d6</sub>): δ 197.7, 171.5, 138.3, 136.7, 132.9, 130.6, 129.7, 128.4, 127.2, 127.1, 123.4, 113.4, 55.6, 43.9, 31.3, 21.4, 14.4. HRMS (ESI<sup>+</sup>): Calcd for [M—I]<sup>+</sup>, 282.1494, Found, 282.1499 (+0.5 mmu).

1-(β-Carboxyethyl)-2-{3'-[1''-((β-carboxyethyl)-1'',3''-dihydro-3'',3''-dimethyl-2H-benzoindol-2''-ylidenemethyl]-2'-hydroxy-4'-oxo-2'-cyclobuten-1'-ylidenemethyl]-3,3-dimethylbenzoindolenium (SqNaphtho)

Compound 8 (1.1 g, 2.7 mmol) and squaric acid (149 mg, 1.3 mmol) were dissolved in toluene (15 mL), 1-butanol (15 mL), and pyridine (5 mL). The flask was fitted with a Dean-Stark trap. The mixture was refluxed for 10 hours under an argon atmosphere, then allowed to cool to room temperature, and the solvent was removed under reduced pressure. The crude product was purified by semi-preparative HPLC until a single peak of the product was obtained, affording SqNaphtho as a green powder (25 mg, yield 3%). 1H NMR (300 MHz, DMSO-<sub>d6</sub>): δ 8.21 (d, 2H, J = 8.8 Hz), 8.01 (d, 4H, J = 8.8 Hz), 7.69 (d, 2H, J = 9.5 Hz), 7.61 (t, 2H, J = 7.3 Hz), 7.45 (t, 2H, J = 7.3 Hz), 5.91 (s, 2H), 4.47 (t, 4H, J = 7.0 Hz), 2.77 (t, 4H, J = 7.0 Hz), 1.95 (s, 12H). 13C NMR (100 MHz, DMSO-<sub>d6</sub>): δ 181.3, 177.8, 172.1, 170.7, 139.7, 133.3, 131.2, 129.9, 129.8, 128.1, 127.6, 124.5, 122.5, 111.7, 86.4, 58.4, 50.8, 31.6, 26.4. HRMS (ESI<sup>+</sup>): Calcd for [M+H]<sup>+</sup>, 641.2652, Found, 641.2685 (+3.3 mmu).
Scheme 5. Synthesis of SqSO₃H-Me.

Potassium salt of 1,2,3,3-tetramethyl-5-sulfoindolenium iodide (10)

Potassium salt of 2,3,3-trimethylindolenine-5-sulfonate (9) was prepared according to the literature. Compound 9 (1.0 g, 3.6 mmol) was dissolved in acetonitrile (10 mL) and methyl iodide (40 mL), and the mixture was refluxed for 10 hours, then allowed to cool to room temperature, and the resulting solid was collected by filtration. The solid was washed with n-hexane and dried under reduced pressure to afford 10 as a brown solid (0.9 g, yield 60%). ¹H NMR (300 MHz, DMSO-δ₆): δ 7.95 (s, 1H), 7.83 (d, 1H, J = 8.8 Hz), 7.74 (d, 1H, J = 8.8 Hz), 3.97 (s, 3H), 2.78 (s, 3H), 1.51 (s, 6H). ¹³C NMR (75 MHz, DMSO-δ₆): δ 196.9, 149.0, 142.0, 141.2, 126.1, 120.4, 114.6, 54.0, 34.9, 21.6, 14.4. HRMS (ESI⁺): Calcd for [M–K]+H⁺, 254.0851, Found, 254.0836 (–1.5 mmu).

2-[3’-(1”,3”-Dihydro-1”,3”,3”-trimethyl-5”-sulfo-2H-indol-2”-ylidenemethyl)-2’-hydroxy-4’-oxo-2’-cyclobuten-1’-ylidenemethyl]-1,3,3-trimethylindolenium-5-sulfonic acid (SqSO₃H-Me)

Compound 10 (153 mg, 0.36 mmol) and squaric acid (37 mg, 0.32 mmol) were dissolved in toluene (7.5 mL), 1-butanol (7.5 mL), and pyridine (5 mL). The flask was fitted with a Dean-Stark trap, and the mixture was refluxed for 2 hours under an argon atmosphere, then allowed to cool to room temperature. The resulting solid was collected by filtration, washed with acetone, and purified by semi-preparative HPLC until a single peak of the product was obtained, affording SqSO₃H-Me as a dark red powder (11 mg, yield 10%). ¹H NMR (300 MHz, D₂O): δ 7.59-7.56 (m, 4H), 7.05 (d, 2H, J = 8.8 Hz), 5.59 (s, 2H), 3.46 (s, 6H), 1.36 (s, 12H). ¹³C NMR (100 MHz, D₂O): δ 216.1, 173.7, 172.9, 145.2, 142.4, 138.6, 126.4, 119.8, 110.7, 86.6, 49.2, 31.2, 26.0. HRMS (ESI⁻): Calcd for [M–2H+Na]⁻, 605.1028, Found, 605.1031 (+0.3 mmu).
Scheme 6. Synthesis of 5SqOH.

5-Hydroxy-2,3,3-trimethylindolenine (13)

5-Methoxy-2,3,3-trimethylindolenine (12) was prepared from 4-methoxyphenyl hydrazine hydrochloride (11) (10.5 g, 60 mmol) and 3-methyl-2-butanone (6.6 mL, 61 mmol) according to the previous report. To a solution of compound 12 in CH₂Cl₂ (30 mL) at 0 °C was added dropwise 1 M BBr₃ in CH₂Cl₂ (70 mL, 70 mmol), and the mixture was stirred at room temperature for 12 hours. Then, a saturated aqueous solution of sodium bicarbonate was added to it, and the whole was extracted with CH₂Cl₂. The organic extracts were dried over Na₂SO₄, filtered, and evaporated to afford 13 as a brown solid (8.3 g, yield 79% 2 steps). H NMR (300 MHz, CDCl₃): δ 10.35 (s, 1H), 7.30 (d, 1H, J = 8.1 Hz), 6.87 (d, 1H, J = 2.2 Hz), 6.80 (dd, 1H, J = 8.1, 2.2 Hz), 2.26 (s, 3H), 1.28 (s, 6H). 13C NMR (75 MHz, CDCl₃): δ 185.8, 155.9, 147.1, 144.6, 119.8, 114.1, 109.8, 53.6, 23.1, 14.8. HRMS (ESI⁺): Calcd for [M+H]⁺, 176.1075, Found, 176.1034 (−4.1 mmu).
**5-Hydroxy-1,2,3,3-tetramethylindolenium iodide (14)**

Compound 13 (1.6 g, 9.1 mmol) was dissolved in methyl iodide (50 mL). The solution was refluxed for 12 hours, then allowed to cool to room temperature, and the resulting solid was collected by filtration. The solid was washed with n-hexane and dried under reduced pressure to afford 14 as a brown solid (2.5 g, yield 87%). $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 10.24 (s, 1H), 7.69 (d, 1H, $J = 8.8$ Hz), 7.13 (d, 1H, $J = 2.2$ Hz), 6.94 (dd, 1H, $J = 8.8, 2.2$ Hz), 3.91 (s, 3H), 2.68 (s, 3H), 1.47 (s, 6H). $^{13}$C NMR (75 MHz, DMSO-$d_6$): $\delta$ 191.9, 159.0, 143.7, 143.1, 116.0, 115.0, 110.3, 53.4, 34.5, 21.8, 13.6. HRMS (ESI$^+$): Calcd for [M−I]$^+$, 190.1232, Found, 190.1227 (−0.5 mmu).

**2-[3''-(1'',3''-Dihydro-5''-hydroxy-1'',3'',3''-trimethyl -2H-indol-2''-ylidenemethyl)-2'-hydroxy-4'-oxo-2'-cyclobuten-1'-ylidenemethyl]-5-hydroxy-1,3,3-trimethylindolenium (5SqOH)**

Compound 14 (56 mg, 0.18 mmol) and squaric acid (12 mg, 0.11 mmol) were dissolved in toluene (5 mL), 1-butanol (5 mL), and pyridine (1 mL). The flask was fitted with a Dean-Stark trap. The reaction mixture was refluxed for 12 hours, then allowed to cool to room temperature, and the whole was washed with 2 N HCl aq. The organic extracts were dried over Na$_2$SO$_4$, filtered, and evaporated. The crude product was purified by semi-preparative HPLC until a single peak of the product was obtained, affording 5SqOH as a dark green powder (1.2 mg, yield 3%). $^1$H NMR (300 MHz, DMF-$d_7$): $\delta$ 9.54 (s, 2H), 7.05 (d, 2H, $J = 8.1$ Hz), 6.88 (s, 2H), 6.72 (dd, 2H, $J = 8.1, 2.2$ Hz), 5.62 (s, 2H), 3.48 (s, 6H), 1.61 (s, 12H). HRMS (ESI$^+$): Calcd for [M+Na]$^+$, 479.1947, Found, 479.1940 (−0.7 mmu).
Scheme 7. Synthesis of 6SqOH.

4- & 6-Methoxy-2,3,3-trimethylindolenine, isomer mixture (16)

3-Methoxyphenylhydrazine hydrochloride (15) (561 mg, 3.2 mmol) and 3-methyl-2-butanone (350 μL, 3.3 mmol) were dissolved in methanol (10 mL) and concentrated hydrochloric acid (1 mL). The reaction mixture was refluxed for 12 hours, then allowed to cool to room temperature, and the solvent was removed under reduced pressure. To the resulting residue was added CH₂Cl₂, and the whole was washed with a saturated aqueous solution of sodium bicarbonate. The organic extracts were dried over Na₂SO₄, filtered, and evaporated to afford 16 as a brown oil (580 mg, yield 96%). ¹H NMR (300 MHz, CDCl₃): δ 7.25 (t, 1H, J = 8.1 Hz), 7.17 (d, 1H, J = 8.1 Hz), 7.15 (d, 2H, J = 8.1 Hz), 7.12 (d, 2H, J = 2.2 Hz), 6.75 (dd, 2H, J = 8.1, 2.2 Hz), 6.71 (d, 1H, J = 8.1 Hz), 3.86 (s, 3H), 3.83 (s, 6H), 2.27 (s, 6H), 2.25 (s, 3H), 1.38 (s, 6H), 1.28 (s, 12H). ¹³C NMR (100 MHz, CDCl₃): δ 189.0, 188.6, 159.4, 155.2, 154.6, 154.5, 137.4, 129.7, 128.3, 121.1, 112.5, 110.5, 107.4, 105.4, 55.1, 54.9, 54.6, 54.1, 22.9, 20.3, 15.1, 14.7. HRMS (ESI⁺): Calcd for [M+H]⁺, 190.1232, Found, 190.1241 (+0.9 mmu).
4- & 6-Hydroxy-2,3,3-trimethylindolenine, isomer mixture (17)
To a solution of compound 16 (580 mg, 3.1 mmol) in CH₂Cl₂ (5 mL) at 0 °C was added dropwise 1 M BBr₃ in CH₂Cl₂ (5 mL, 5 mmol). The reaction mixture was stirred at room temperature for 10 hours, then a saturated aqueous solution of sodium bicarbonate was added, and the whole was extracted with CH₂Cl₂. The organic extracts were dried over Na₂SO₄, filtered, and evaporated to afford 17 as a purple solid (542 mg, yield quant). ¹H NMR (300 MHz, CDCl₃): δ 7.13-7.09 (m, 4H), 7.08 (d, 2H, J = 2.2 Hz), 6.70 (dd, 2H, J = 8.1, 2.2 Hz), 6.57 (dt, 1H, J = 8.8, 3.7 Hz), 2.29 (s, 3H), 2.27 (s, 6H), 1.47 (s, 6H), 1.28 (s, 12H). ¹³C NMR (100 MHz, CDCl₃): δ 189.8, 189.5, 157.2, 153.4, 153.1, 153.0, 135.8, 129.5, 128.3, 121.5, 113.2, 112.7, 110.7, 107.1, 53.9, 52.8, 23.1, 20.3, 14.8, 14.4. HRMS (ESI⁺): Calcd for [M+H]⁺, 176.1075, Found, 176.1069 (~0.6 mmu).

4- & 6-Hydroxy-1,2,3,3-tetramethylindolenium iodide, isomer mixture (18)
Compound 17 (49 mg, 0.28 mmol) was dissolved in methyl iodide (5 mL). The solution was refluxed for 8 hours, then allowed to cool to room temperature, and the solvent was removed under reduced pressure. The resulting residue was washed with diethyl ether. The crude 18 was used without further purification in the next reaction. HRMS (ESI⁺): Calcd for [M–I]⁺, 190.1232, Found, 190.1187 (~4.5 mmu).

2-(3''-(1'',3''-Dihydro-6''-hydroxy-1'',3'',3''-trimethyl-2H-indol-2''-ylidenemethyl)-2'-hydroxy-4'-oxo-2'-cyclobuten-1'-ylidenemethyl)-6-hydroxy-1,3,3-trimethylindolenium (6SqOH)
Compound 18 (60 mg, 0.19 mmol) and squaric acid (10 mg, 0.09 mmol) were dissolved in toluene (5 mL), 1-butanol (5 mL), and pyridine (1 mL). The flask was fitted with a Dean-Stark trap. The mixture was refluxed for 6 hours under an argon atmosphere, then allowed to cool to room temperature, and the whole was washed with an aqueous solution of ammonium chloride. The organic extracts were dried over Na₂SO₄, filtered, and evaporated. The crude product was purified by semi-preparative HPLC until a single peak of the product was obtained, affording 6SqOH as a dark green powder (0.9 mg, yield 2%). ¹H NMR (300 MHz, DMSO-d₆): δ 9.61 (s, 2H), 7.24 (d, 2H, J = 8.1 Hz), 6.68 (d, 2H, J = 2.2 Hz), 6.55 (dd, 2H, J = 8.1, 2.2 Hz), 5.71 (s, 2H), 3.49 (s, 6H), 1.62 (s, 12H). HRMS (ESI⁺): Calcd for [M+H]⁺, 457.2127, Found, 457.2129 (+0.2 mmu).

S11
**Scheme 8.** Synthesis of 5SqPhos and 5SqmonoPhos.

**2,3,3-Trimethylindolenine-5-phosphoric acid diethyl ester (19)**

To a mixture of compound 13 (0.78 g, 4.5 mmol) and sodium hydride (0.15 g, 6.3 mmol) in chloroform (40 mL) was added diethylchlorophosphate (0.65 mL, 4.5 mmol), and the mixture was stirred at room temperature under an argon atmosphere. After 10 hours, the solvent was removed under reduced pressure. To the resulting residue was added CH₂Cl₂, and the whole was washed with H₂O. The organic extracts were dried over Na₂SO₄, filtered, and evaporated. The crude product was purified by silica gel chromatography (3% methanol / CH₂Cl₂) to afford 19 as a purple solid (0.64 g, yield 46%). ¹H NMR (300 MHz, CDCl₃): δ 7.46 (d, 1H, J = 8.1 Hz), 7.17 (s, 1H), 7.13 (dd, 1H, J = 8.1, 1.5 Hz), 4.23 (tt, 4H, J = 14.7, 7.3 Hz), 2.27 (s, 3H), 1.35 (td, 6H, J = 7.3, 1.5 Hz), 1.30 (s, 6H).

¹³C NMR (75 MHz, CDCl₃): δ 188.0, 150.5, 148.5, 148.4, 147.1, 120.2, 119.0, 118.9, 113.7, 113.6, 64.5, 64.4, 54.0, 22.9, 16.1, 15.9, 15.3. HRMS (ESI⁺): Caled for [M+H]⁺, 312.1365, Found, 312.1340 (~2.5 mnu).

**5-Diethylphosphonooxy-1,2,3,3-tetramethyl-indolenium iodide (20)**

Compound 19 (0.64 g, 2.1 mmol) was dissolved in methyl iodide (10 mL). The solution was refluxed for 24 hours, then allowed to cool to room temperature, and the resulting solid was collected by filtration. The solid was washed with diethyl ether and dried under reduced pressure to afford 20 as a purple solid (0.65 g, yield 68%). ¹H NMR (300 MHz, CDCl₃): δ 7.72 (d, 1H, J = 8.8 Hz), 7.42-7.39 (m, 2H), 4.28-4.18 (m, 7H), 3.06 (s, 3H), 1.64 (s,
6H), 1.36 (t, 6H, J = 7.0 Hz). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 195.6, 152.1, 152.0, 143.1, 138.4, 120.9, 120.8, 116.6, 115.1, 115.0, 65.1, 65.0, 54.6, 37.5, 22.9, 17.3, 16.0, 15.9. HRMS (ESI$^+$): Calcd for [M–I]$^+$, 326.1521, Found, 326.1559 (+3.8 mmu).

1,2,3,3-Tetramethyl-5-phosphonooxindolenium iodide (21)

To a solution of compound 20 (32 mg, 70 $\mu$mol) in CH$_2$Cl$_2$ (10 mL) was added bromotrimethylsilane (0.1 mL, 0.75 mmol), and the mixture was stirred at room temperature under an argon atmosphere. The resulting solid was collected by filtration, washed with diethyl ether, and dried under reduced pressure to afford 21 as a yellow powder (23 mg, yield 83%). $^1$H NMR (300 MHz, D$_2$O): $\delta$ 7.56 (d, 1H, J = 8.1 Hz), 7.38 (s, 1H), 7.24 (d, 1H, J = 8.1 Hz), 3.84 (s, 3H), 2.61 (s, 3H), 1.42 (s, 6H). $^{13}$C NMR (100 MHz, D$_2$O): $\delta$ 198.0, 155.5, 155.4, 145.8, 140.5, 123.4, 123.3, 118.4, 118.0, 117.9, 56.7, 36.8, 23.8, 15.5. HRMS (ESI$^-$): Calcd for [M–I–2H]$^-$, 268.0739, Found, 268.0730 (–0.9 mmu).

2-[3'-(1'',3'',3''-Dihydro-1'',3'',3''-trimethyl-5''-phosphonooxy-2H-indol-2''-ylidenemethyl)-2'-hydroxy-4'-oxo-2'-cyclobuten-1'-ylidenemethyl]-5-phosphonooxy-1,3,3-trimethylindolenium (5SqPhos)

2-[3'-(1'',3'',3''-Dihydro-5''-hydroxy-1'',3'',3''-trimethyl-2H-indol-2''-ylidenemethyl)-2'-hydroxy-4'-oxo-2'-cyclobuten-1'-ylidenemethyl]-5-phosphonooxy-1,3,3-trimethylindolenium (5SqmonoPhos)

Compound 21 (34 mg, 86 $\mu$mol) and squaric acid (5 mg, 44 $\mu$mol) were dissolved in toluene (5 mL), 1-butanol (5 mL), and pyridine (1 mL). The flask was fitted with a Dean-Stark trap, and the reaction mixture was refluxed for 12 hours, then allowed to cool to room temperature. The solvent was removed under reduced pressure, and the crude product was purified by semi-preparative HPLC until a single peak of each product was obtained, affording 5SqPhos as a dark green powder (7.7 mg, yield 29%) and 5SqmonoPhos as a dark green powder (5.6 mg, yield 24%). 5SqPhos: $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 7.25–7.14 (m, 6H), 5.68 (s, 2H), 3.56 (s, 6H), 1.57 (s, 12H). HRMS (ESI$^-$): Calcd for [M–H]$^-$, 615.1297, Found, 615.1272 (–2.5 mmu). 5SqmonoPhos: $^1$H NMR (300 MHz, DMF-$d_7$): $\delta$ 7.36 (s, 1H), 7.26 (m, 3H), 7.04 (d, 1H, J = 2.2 Hz), 6.87 (dd, 1H, J = 8.1, 2.2 Hz), 5.84 (s, 1H), 5.76 (s, 1H), 3.67 (s, 3H), 3.58 (s, 3H), 1.74 (s, 12H). HRMS (ESI$^-$): Calcd for [M–H]$^-$, 535.1634, Found, 535.1610 (–2.4 mmu).

5-Ethylphosphonooxy-2-[(5''-ethylphosphonooxy-1''''-dihydro-1''''-3''''-trimethyl-2H-indol-2''''-ylidenemethyl)-2'-hydroxy-4'-oxo-2'-cyclobuten-1'-ylidenemethyl]-1,3,3-trimethylindolenium (5SqPhos-diEt)

Compound 20 (147 mg, 0.32 mmol) and squaric acid (20 mg, 0.18 mmol) were dissolved in toluene (5 mL), 1-butanol (5 mL), and pyridine (2 mL). The flask was fitted with a Dean-Stark trap, and the reaction mixture was refluxed for 12 hours, then allowed to cool to room temperature. The whole was neutralized with 2 N HCl aq., and extracted with CH₂Cl₂. The organic extracts were dried over Na₂SO₄, filtered, and evaporated. The crude product was purified by semi-preparative HPLC until a single peak of the product was obtained, affording 5SqPhos-diEt as a dark red powder (64 mg, yield 59%). ¹H NMR (300 MHz, DMSO-d₆): δ 7.33-7.30 (m, 4H), 7.17 (d, 2H, J = 8.1 Hz), 5.72 (s, 2H), 4.11-4.06 (m, 4H), 3.56 (s, 6H), 1.68 (s, 12H), 1.23 (t, 6H, J = 7.0 Hz). ¹³C NMR (75 MHz, DMSO-d₆): δ 180.6, 178.3, 169.5, 147.0, 142.6, 139.3, 119.3, 114.7, 110.6, 86.4, 63.0, 62.9, 48.7, 30.7, 26.4, 16.1, 16.0. HRMS (ESI⁻): Calcd for [M–H]⁻, 671.1923, Found, 671.1918 (–0.5 mmu).
Scheme 10. Synthesis of 6SqPhos and 6SqmonoPhos.

2,3,3-Trimethylindolenine-4- & 6-phosphoric acid diethyl ester, isomer mixture (22)
To a mixture of compound 17 (108 mg, 0.62 mmol) and sodium hydride (60 mg, 2.5 mmol) in DMF (5 mL) was added diethylchlorophosphate (0.15 mL, 1.0 mmol), and the mixture was stirred at room temperature under an argon atmosphere. After 8 hours, the solvent was removed under reduced pressure. To the resulting residue was added CH₂Cl₂, and the whole was washed with H₂O. The organic extracts were dried over Na₂SO₄, filtered, and evaporated. The crude product was purified by silica gel chromatography (5% methanol / CH₂Cl₂) to afford 22 as a brown solid (37 mg, yield 19%). ¹H NMR (300 MHz, CDCl₃): δ 7.35-7.34 (m, 3H), 7.25-7.20 (m, 4H), 7.09 (dd, 2H, J = 8.1, 2.2 Hz), 4.31-4.22 (m, 12H), 2.28 (s, 6H), 2.27 (s, 3H), 1.43-1.34 (m, 24H), 1.29 (s, 12H). HRMS (ESI⁺): Calcd for [M+H]⁺, 312.1364, Found, 312.1358 (−0.6 mmu).

4- & 6-Diethylphosphonooxy-1,2,3,3-tetramethylindolenium iodide, isomer mixture (23)
Compound 22 (68 mg, 0.22 mmol) was dissolved in methyl iodide (10 mL). The solution was refluxed for 10 hours, then allowed to cool to room temperature, and the solvent was removed under reduced pressure to afford...
23 as a brown solid (98 mg, yield 98% crude). The crude 23 was used without further purification in the next reaction. HRMS (ESI⁻): Calcd for [M–I]⁻, 326.1521, Found, 326.1502 (−1.9 mmu).

1,2,3,3-Tetramethyl-6-phosphonooxyindolenium iodide (24)

To a solution of compound 23 (98 mg, 0.22 mmol) in CH₂Cl₂ (5 mL) was added bromotrimethylsilane (0.3 mL, 2.3 mmol), and the mixture was stirred at room temperature under an argon atmosphere. The resulting solid was collected by filtration, washed with diethyl ether, and dried under reduced pressure to afford 24 as a purple powder (40 mg, yield 46%). ¹H NMR (400 MHz, CD₃OD): δ 7.68 (d, 1H, J = 8.2 Hz), 7.61 (d, 1H, J = 1.7 Hz), 7.41 (dd, 1H, J = 8.2, 1.7 Hz), 4.01 (s, 3H), 1.56 (s, 6H), 3H of the 2-methyl group were not observed because of H/D exchange. ¹³C NMR (100 MHz, CD₃OD): δ 199.0, 153.6, 153.5, 143.7, 138.1, 124.9, 123.0, 122.9, 108.6, 108.5, 55.2, 35.4, 22.5, 20.5. HRMS (ESI⁻): Calcd for [M–I–2H]⁻, 268.0738, Found, 268.0699 (−3.9 mmu).

2-[3′-(1′′,3′′-Dihydro-1′′,3′′,3′′-trimethyl-6′′-phosphonooxy-2H-indol-2′′-ylidenemethyl)-2′-hydroxy-4′-oxo-2′-cyclobuten-1′-ylidenemethyl]-6-phosphonooxy-1,3,3-trimethylindolenium (6SqPhos)

2-[3′-(1′′,3′′-Dihydro-6′′-hydroxy-1′′,3′′,3′′-trimethyl-2H-indol-2′′-ylidenemethyl)-2′-hydroxy-4′-oxo-2′-cyclobuten-1′-ylidenemethyl]-6-phosphonooxy-1,3,3-trimethylindolenium (6SqmonoPhos)

Compound 24 (24 mg, 60 µmol) and squaric acid (4 mg, 35 µmol) were dissolved in toluene (5 mL), 1-butanol (5 mL), acetonitrile (5 mL), and pyridine (1 mL). The flask was fitted with a Dean-Stark trap, and the mixture was refluxed for 6 hours under an argon atmosphere, then allowed to cool to room temperature. The solvent was removed under reduced pressure. The crude product was purified by semi-preparative HPLC until a single peak of each product was obtained, affording 6SqPhos as a dark green powder (0.9 mg, yield 5%) and 6SqmonoPhos as a dark green powder (1.2 mg, yield 7%). 6SqPhos: ¹H NMR (400 MHz, DMSO-d₆): δ 7.51 (d, 2H, J = 7.8 Hz), 7.19 (s, 2H), 7.06 (dd, 2H, J = 7.8, 1.5 Hz), 5.68 (s, 2H), 3.63 (s, 6H), 1.71 (s, 12H). HRMS (ESI⁻): Calcd for [M–H]⁻, 615.1297, Found, 615.1332 (+3.5 mmu). 6SqmonoPhos: ¹H NMR (400 MHz, DMSO-d₆): δ 7.42 (d, 1H, J = 7.8 Hz), 7.27 (d, 1H, J = 7.8 Hz), 7.06 (s, 1H), 6.93 (d, 1H, J = 7.8 Hz), 6.71 (d, 1H, J = 2.0 Hz), 6.58 (dd, 1H, J = 7.8, 2.0 Hz), 5.77 (s, 1H), 5.72 (s, 1H), 3.53 (s, 3H), 3.51 (s, 3H), 1.66 (s, 6H), 1.63 (s, 6H). HRMS (ESI⁻): Calcd for [M–H]⁻, 535.1634, Found, 535.1654 (+2.0 mmu).

6-(2',3',4',6'-Tetra-O-acetyl-β-D-galactosyloxy)-2,3,3-trimethylindolenine (26)

2,3,4,6-Tetra-O-acetyl-α-D-galactopyranosyl bromide (25) was prepared from β-D-galactose pentaacetate, bismuth (III) bromide, and bromotrimethylsilane in CH₂Cl₂ according to the literature. To a mixture of compound 17 (120 mg, 0.68 mmol) and cesium carbonate (1.0 g, 3.1 mmol) in DMF (5 mL) was added a 1 M solution of 25 in DMF (2 mL, 2 mmol), and the whole was stirred at room temperature under an argon atmosphere. After 21 hours, the solvent was removed under reduced pressure. To the resulting residue was added CH₂Cl₂ and the whole was washed with an aqueous solution of ammonium chloride. The organic extracts were dried over Na₂SO₄, filtered, and evaporated. The crude product was purified by silica gel chromatography (ethyl acetate / n-hexane, 1:1) to afford 26 as a dark red powder (29 mg, yield 8%).

1H NMR (300 MHz, CDCl₃): δ 7.20 (d, 1H, J = 2.2 Hz), 7.16 (d, 1H, J = 8.1 Hz), 6.86 (dd, 1H, J = 8.1, 2.2 Hz), 5.48 (m, 2H), 5.11 (dd, 1H, J = 10.6, 3.3 Hz), 5.05 (d, 1H, J = 8.1 Hz), 4.26-4.03 (m, 3H), 2.27 (s, 3H), 2.19 (s, 3H), 2.07 (s, 3H), 2.02 (s, 3H), 1.28 (s, 6H, a).

13C NMR (75 MHz, CDCl₃): δ 189.7, 170.4, 170.2, 170.1, 169.4, 157.0, 154.9, 140.5, 121.5, 114.1, 108.9, 100.2, 71.0, 70.9, 68.7, 66.9, 61.2, 53.3, 23.1, 21.0, 20.7, 20.6, 20.5, 15.5. HRMS (ESI⁺): Calcd for [M+Na]⁺, 528.1846, Found, 528.1804 (–4.2 mmu).
6-(2',3',4',6'-Tetra-O-acetyl-\(\beta\)-D-galactosyloxy)-1,2,3,3-tetramethylindolenium iodide (27)

Compound 26 (29 mg, 57 \(\mu\)mol) was dissolved in methyl iodide (10 mL). The reaction mixture was refluxed for 12 hours, then allowed to cool to room temperature, and the solvent was removed under reduced pressure. The resulting residue was washed with diethyl ether, affording 27 as a dark yellow solid (32 mg, yield 87%). 1H NMR (300 MHz, CDCl\(_3\)): \(\delta\) 7.57 (d, 1H, \(J = 2.2\) Hz), 7.45 (d, 1H, \(J = 8.1\) Hz), 7.20 (dd, 1H, \(J = 8.1, 2.2\) Hz), 5.62 (d, 1H, \(J = 8.1\) Hz), 5.52-5.49 (m, 2H), 5.23 (dd, 1H, \(J = 10.3, 2.9\) Hz), 4.59 (t, 1H, \(J = 6.2\) Hz), 4.33 (s, 3H), 4.19 (d, 2H, \(J = 6.2\) Hz), 3.09 (s, 3H), 2.18 (s, 3H), 2.10 (s, 3H), 2.06 (s, 3H), 2.01 (s, 3H), 1.65 (s, 6H). 13C NMR (75 MHz, CDCl\(_3\)): \(\delta\) 197.1, 170.3, 170.1, 169.8, 169.5, 157.6, 142.8, 135.3, 123.5, 119.0, 104.4, 98.5, 71.2, 70.6, 68.5, 67.0, 61.3, 54.2, 37.8, 23.1, 20.8, 20.6, 20.5, 17.2. HRMS (ESI\(^+\)): Calcd for [M–I]\(^+\), 520.2183, Found, 520.2144 (–3.9 mmu).

6-(2''',3'''',4'''',6''''-Tetra-O-acetyl-\(\beta\)-D-galactosyloxy)-2-{3'-[6''-(2'''',3'''',4'''',6''''-tetra-O-acetyl-\(\beta\)-D-galactosyloxy)-1'',3''-dihydro-1'',3'',3''-trimethyl-2H-indol-2''-ylidenemethyl]-2'-hydroxy-4'-oxo-2'-cyclobuten-1'-ylidenemethyl}-1,3,3-trimethylindolenium (6SqGal-Ac)

Compound 27 (32 mg, 49 \(\mu\)mol) and squaric acid (4 mg, 35 \(\mu\)mol) were dissolved in toluene (5 mL), 1-butanol (5 mL) and pyridine (1 mL). The flask was fitted with a Dean-Stark trap, and the reaction mixture was refluxed for 8 hours, then allowed to cool to room temperature, and washed with an aqueous solution of ammonium chloride. The organic extracts were dried over Na\(_2\)SO\(_4\), filtered, and evaporated. The crude 6SqGal-Ac was used without further purification in the next reaction. HRMS (ESI\(^+\)): Calcd for [M+H]\(^+\), 1117.4029, Found, 1117.4003 (–2.6 mmu).
To a solution of 6SqGal-Ac in methanol (10 mL) was added 0.1 M sodium methoxide in methanol (2.5 mL, 250 μmol), and the mixture was stirred at room temperature under an argon atmosphere. After 3 hours, the reaction mixture was neutralized with Amberlite IR-120 (H⁺). The Amberlite IR-120 was filtered off, and the filtrate was evaporated under reduced pressure. The resulting residue was purified by semi-preparative HPLC until a single peak of the product was obtained, affording 6SqGal as a dark red powder (7.7 mg, yield 40% 2 steps). ¹H NMR (300 MHz, DMSO-d₆): δ 7.38 (d, 2H, J = 8.8 Hz), 7.02 (s, 2H), 6.81 (d, 2H, J = 8.8 Hz), 5.75 (s, 2H), 4.87 (d, 2H, J = 7.3 Hz), 3.71 (d, 2H, J = 2.9 Hz), 3.63-3.48 (m, 16H), 1.66 (s, 12H). HRMS (ESI⁺): Calcd for [M+H]⁺, 781.3184, Found, 781.3198 (+1.4 mmu).
2. Fluorescence Detection on Western Blots

Dot blot assay for estimation of washing efficiency

Polyvinylidene fluoride (PVDF) membrane was first blocked with membrane blocking agent for 60 minutes, and then various amounts of 1 mM stock solutions of dyes (5 µL, 3 µL, 1 µL) were directly dropped onto the membrane. The membrane was air-dried for a few minutes, and white light and fluorescence images were acquired with MAESTRO. Then, the membrane was washed with Tris-buffered saline containing 0.1% Tween 20 (T-TBS) for 15 minutes, and white light and fluorescence images were acquired again.

Dot blot assay for comparison of signal intensity

Various amounts (1 units, 0.5 units, and 0.25 units) of ALP were directly dropped onto PVDF membrane, and the membrane was air-dried for 30 minutes and blocked with membrane blocking agent for 60 minutes. The membrane was then incubated in a 1 µM solution of 6SqmonoPhos or DDAO-Phosphate in 2 M Tris buffer (pH 8.0) with gentle agitation at room temperature. After 10 minutes, the membrane was rinsed with water and the fluorescence image was acquired. For estimation of washing efficiency, the membrane was washed with T-TBS for 15 minutes and the fluorescence image was acquired again.
3. *In Vivo Imaging*

**X-Gal staining**

After imaging experiments, the liver was perfused with saline, isolated, and placed in 6-well chamber slides on ice. X-Gal staining was performed using X-Gal Staining Assay Kit (Genlantis, Inc., San Diego, CA) according to the manufacturer’s instructions.
4. Supporting References


Supporting Figures

[Cy5 structure image]

[Cy6F structure image]

[Cy9H8O structure image]

[Cy6PH structure image]

[Cy6CF3 structure image]
Figure S1. Absorption (left) and fluorescence (right) spectra of cyanine dyes in 0.1 M sodium phosphate buffer (black) or fetal bovine serum (FBS, blue).
**Figure S2.** Absorption (left) and fluorescence (right) spectra of squarylium dyes in 0.1 M sodium phosphate buffer (black) or FBS (blue).

**Figure S3.** Absorption (left) and fluorescence (right) spectra of Sq (top) and SqSO$_3$H (bottom) in 0.1 M sodium phosphate buffer containing various concentrations of BSA (0 to 13.5% w/v).

**Figure S4.** Relationship between concentration of BSA and the absorption wavelength change (left) or the fluorescence intensity (right). The values of the binding parameter $nK_a$ were estimated to be $\sim 3 \times 10^5$ M$^{-1}$ (1 $\mu$M Sq) and $\sim 5 \times 10^3$ M$^{-1}$ (1 $\mu$M SqSO$_3$H).
Figure S5. Absorption (left) and fluorescence (right) spectra of polymethine dyes in 0.1 M sodium phosphate buffer containing 1% w/v bovine γ-globulin (BGG).
Table S1. Photochemical properties of polymethine dyes in phosphate buffer containing 1% w/v BGG.

<table>
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<th>Emission max (nm)</th>
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$^a$ Excitation wavelength was 600 nm. $^b$ $\phi_{FL}$ is the relative fluorescence quantum yield estimated by using ca. 0.1 µM cresyl violet in MeOH (0.54) as a fluorescence standard. $\delta\lambda$ is the difference between the absorption or emission maxima in the buffer and in BGG solution.

Figure S6. Absorption (left) and fluorescence (right) spectra of Sq (top) or SqSO3H (bottom) in 20% FBS/0.1 M sodium phosphate buffer after addition of SDS.
Figure S7. Structures of squarylium dye derivatives bearing phosphate group(s).

Figure S8. Absorption (left) and fluorescence (right) spectra of 5SqPhos (top), 5SqmonoPhos (middle), and 5SqPhos-diEt (bottom) in 0.1 M sodium phosphate buffer (black) or FBS (blue).
**Table S2.** Photochemical properties of squarylium dyes bearing phosphate groups in phosphate buffer or FBS.

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<th>Absorption max (nm) in FBS</th>
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*a Excitation wavelength was 600 nm. b \( \phi_F \) is the relative fluorescence quantum yield estimated by using ca. 0.1 \( \mu \)M cresyl violet in MeOH (0.54) \(^19\) as a fluorescence standard. \( \delta \lambda \) is the difference between the absorption or emission maxima in the buffer and in FBS.

**Figure S9.** Absorption (left) and fluorescence (right) spectra of 6SqPhos (top) or 6SqOH (middle) in 0.1 M sodium phosphate buffer containing various concentrations of BSA (0 to 13.5% w/v). Bottom: relationship between concentration of BSA and the absorption wavelength change (left) or the fluorescence intensity (right). The values of the binding parameter \( nK_a \) were estimated to be \( \sim 2 \times 10^5 \) M\(^{-1}\) (1 \( \mu \)M 6SqOH) and \( \sim 2 \times 10^4 \) M\(^{-1}\) (1 \( \mu \)M 6SqPhos).
**Figure S10.** Absorption and fluorescence spectra of 6SqmonoPhos before and after addition of ALP (2.5 units) and time courses of fluorescence intensity observed with 6SqmonoPhos in the presence of various amounts of ALP. For details, see experimental procedures.

**Figure S11.** Relationship between the amount (units) of added ALP and the initial (from 75 sec to 85 sec) fluorescence increase rate.

**Figure S12.** HPLC chromatograms of 6SqPhos or 6SqmonoPhos, the reaction mixture of 6SqPhos or 6SqmonoPhos with ALP, and 6SqOH. Samples were analyzed by HPLC with linear gradient elution (eluent A/B = 80/20, 20 min, 0/100; flow rate = 1.0 mL/min). The monitored wavelength was 620 nm.
Figure S13. White light (left) and fluorescence (right) images of PVDF membrane before and after washing. Various amounts of 6SqOH (1st row), 6SqmonoPhos (2nd row, indicated as 6SqmPhos), and 6SqPhos (3rd row) were dropped onto the membrane.

Figure S14. Fluorescence intensity on PVDF membrane (Figure S13) and efficiency of washing estimated by dividing the fluorescence intensity after washing by the initial fluorescence intensity. Data are shown as mean ± standard deviation (3 membranes).
Figure S15. Correlation between total fluorescence intensity observed for each band (Figure 5) and amount of CYP3A2. Data are shown as mean ± standard deviation (6 gels in 3 independent experiments).

Figure S16. Comparison of signal intensity between 6SqmonoPhos (indicated as 6SqmPhos) and DDAO-Phos. White light and fluorescence images are representative data (left) and bars are shown as mean ± standard deviation (n = 3), *P < 0.05, n.s.; no significance.

Figure S17. Left: white light and fluorescence images of PVDF membrane after washing (Figure S16). Representative images are shown (n = 3). Right: washing efficiency estimated by dividing the fluorescence decrease after washing by the initial fluorescence intensity. Data are shown as mean ± standard deviation, *P < 0.05. For details, see experimental procedure.
Figure S18. Top: absorption (left) and fluorescence (right) spectra of 6SqGal in 0.1 M sodium phosphate buffer containing various concentrations of BSA (0 to 13.5% w/v). Bottom: relationship between concentration of BSA and the absorption wavelength change (left) or the fluorescence intensity (right). The values of the binding parameter $nK_a$ were estimated to be $\sim 2 \times 10^5$ M$^{-1}$ (1 $\mu$M 6SqOH) and $\sim 2 \times 10^3$ M$^{-1}$ (1 $\mu$M 6SqGal).

Figure S19. Relationship between the amount (units) of added $\beta$-galactosidase and the initial (from 75 sec to 85 sec) fluorescence increase rate.
Figure S20. HPLC chromatograms of 6SqGal, the reaction mixture of 6SqGal with β-galactosidase, and 6SqOH. Samples were analyzed by HPLC with linear gradient elution (eluent A/B = 80/20, 20 min, 0/100; flow rate = 1.0 mL/min). The monitored wavelengths were 620 nm for absorption and 650 nm for fluorescence, with excitation at 625 nm.

Figure S21. X-Gal staining of livers isolated from plasmid-transfected mice (top: pCMV-SPORT β-gal, bottom: pcDNA3.1, see Figure 9).

Figure S22. Comparison of average fluorescence intensity in the liver. Data are shown as mean ± standard deviation (n = 3).