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Electronic Supplementary Information

Addressing the Autofluorescence Issue in Deep Tissue Imaging by Two-Photon Microscopy: Significance of Far-Red Emitting Dyes

Yong Woong Jun,[a] Hye Rim Kim,[a] Ye Jin Reo,[a] Mingchong Dai[a] and Kyo Han Ahn*[a]

[a]Department of Chemistry, Pohang University of Science and Technology (POSTECH), 77 Cheongam-Ro, Nam-Gu, Pohang, Gyungbuk, Republic of Korea 37673

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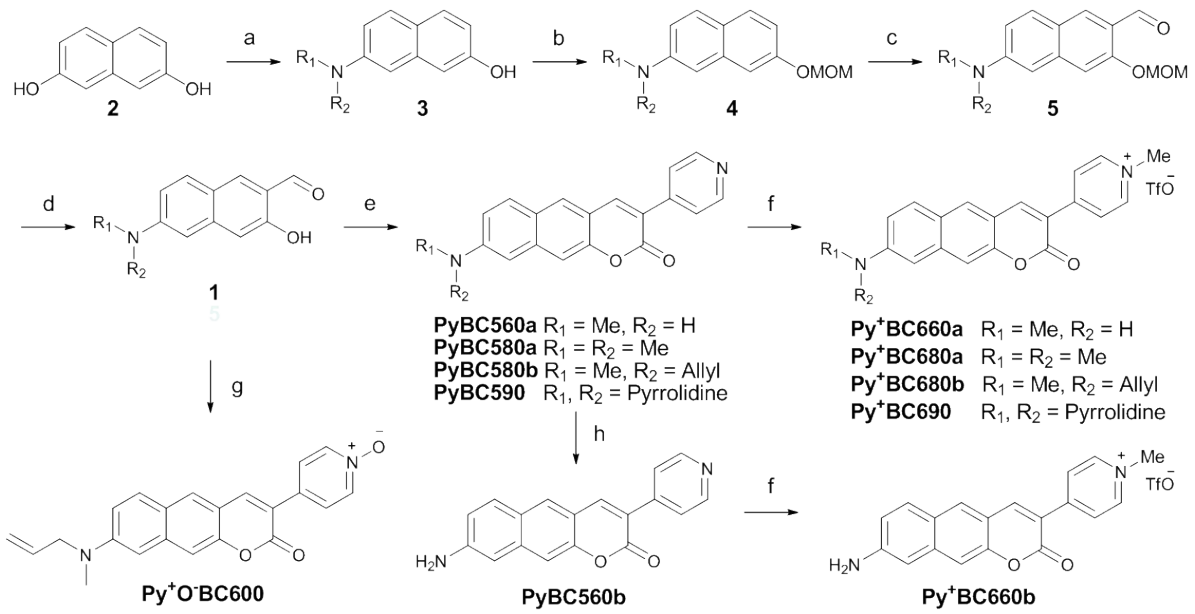
1. Scheme



Scheme S1. Reagents and conditions: a) $Na_2S_2O_5$, HNR_1R_2, H_2O, 150 °C 8 h. b) NaH, DMF, chloromethyl methyl ether, -15 °C, 7 h. c) *t*-BuLi, Et_2O; DMF, -15 °C, 2 h. d) HCl, *i*-PrOH, 60 °C, 3 h. e) 4-Pyridineacetic acid hydrochloride, EDC, HOBt, Et_3N, CH_2Cl_2, 25 °C, 24 h. f) $CF_3SO_3CH_3$, CH_2Cl_2, 25 °C 4 h. g) 4-(2-Ethoxy-2-oxoethyl)pyridine 1-oxide, piperidine, EtOH, 30 °C, 8 h. h) $Pd(PPh_3)_4$, NDMBA, 60 °C, 5 h.

Synthesis

6-(Dialkyl- or monoalkylamino)-3-hydroxy-2-naphthaldehydes (1). These compounds were synthesized by following the reported procedure[1] using the corresponding amines.

3-(Pyridin-4-yl)-8-(pyrrolidin-1-yl)-2H-benzo[*g*]chromen-2-one (PyBC590). A solution of 3-hydroxy-6-(pyrrolidin-1-yl)-2-naphthaldehyde (100 mg, 0.41 mmol)[1] and 4-pyridineacetic acid hydrochloride (108 mg, 0.62 mmol) in dichloromethane (2 mL) was treated with triethylamine (173 µL, 1.24 mmol). After being stirred for 10 min, the solution was treated with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC, 119 mg, 0.62 mmol) and 1-hydroxybenzotriazole hydrate (HOBt, 84 mg, 0.62 mmol). The resulting mixture, after being stirred for 24 h at room temperature, was subjected to extraction with dichloromethane. The organic layer was washed with brine, dried over anhydrous Na_2SO_4, and concentrated. The crude product was purified by silica gel column chromatography (eluent: MeOH/CH_2Cl_2 = 3/97) to afford **PyBC590** as an orange solid (120 mg, 84%). ^{1}H NMR (300 MHz, $CDCl_3$, 298 K): δ 8.68 (d, *J* = 4.5 Hz, 2H), 7.99 (s, 1H), 7.87 (s, 1H), 7.76 (d, *J* = 9.2 Hz, 1H), 7.69 (d, *J* = 4.5 Hz, 2H), 7.44 (s, 1H), 7.01 (dd, *J* = 9.2 2.4 Hz, 1H), 6.69 (d, *J* = 2.4 Hz, 1H), 3.47 (t, *J* = 6.6 Hz, 4H), 2.14–2.07 (m, 4H). ^{13}C NMR (600 MHz, $CDCl_3$, 298 K): δ 159.8, 150.6, 149.5, 147.2, 142.4, 141.5, 137.2, 129.4, 128.5, 122.9, 122.1, 121.6, 115.8, 114.5, 108.7, 102.7, 47.2 (2 carbons), 25.0 (2 carbons). HRMS (ESI) *m/z*: $[M + H]^+$ calcd for $C_{22}H_{18}N_2O_2$, 342.1368; found, 343.1447.

1-Methyl-4-(2-oxo-8-(pyrrolidin-1-yl)-2H-benzo[g]chromen-3-yl)pyridinium trifluoromethanesulfonate (Py$^+$BC690). To a solution of **PyBC590** (50 mg, 0.15 mmol) in dichloromethane (1 mL) was added methyl trifluoromethanesulfonate (MeOTf,

25 µL, 0.22 mmol) dropwise, and the resulting solution was stirred for 4 h at room temperature. The organic solvent was removed under reduced pressure, and the residue was washed with dichloromethane and hexane (1:9) several times, and then dried in vacuum to give **Py⁺BC690** as a red-violet solid (63 mg, 85%). mp 282 °C, ^{1}H NMR (600 MHz, DMSO-d_6, 298 K): δ 8.95 (d, *J* = 7.2 Hz, 2H), 8.88 (s, 1H), 8.54 (d, *J* = 7.2 Hz, 2H), 8.22 (s, 1H), 7.93 (d, *J* = 9.6 Hz, 1H), 7.52 (s, 1H), 7.12 (dd, *J* = 9.6 2.4 Hz, 1H), 6.82 (d, *J* = 2.4 Hz, 1H), 4.32 (s, 3H), 3.44 (t, *J* = 6.6 Hz, 4H), 2.03 (m, 4H). ^{13}C NMR (600 MHz, DMSO-d_6, 298 K): δ 159.5, 151.3, 150.8, 148.7, 147.2, 145.3, 138.8, 132.1, 131.3, 125.4, 123.6, 117.4, 116.9 114.8, 108.7, 103,6, 48.0, 47.6 (2 carbons), 25.4 (2 carbons). IR (KBr): ν = 3129, 3047, 2964, 2848, 1701, 1630, 1581, 1551, 1322, 1300, 1262, 1229, 1201, 1185, 1141 cm^{-1}. HRMS (ESI) *m/z*: $[M + H]^+$ calcd for $C_{23}H_{21}N_2O_2$, 357.1603; found, 357.1603.

8-(Dimethylamino)-3-(pyridin-4-yl)-2H-benzo[*g*]chromen-2-one (PyBC580a). Starting from 6-(dimethylamino)-3-hydroxy-2-naphthaldehyde (30 mg, 0.14 mmol), this compound was similarly synthesized as an orange solid (33.4 mg, 74%). ^{1}H NMR (500 MHz, $CDCl_3$, δ): 8.69 (d, *J* = 4.5 Hz, 2H), 8.01 (s, 1H), 7.90 (s, 1H), 7.77 (d, *J* = 9.3 Hz, 1H), 7.69 (d, *J* = 4.5 Hz, 2H), 7.48 (s, 1H), 7.16 (dd, *J* = 9.3 2.3 Hz, 1H), 6.84 (d, *J* = 2.3 Hz, 1H), 3.15 (s, 6H). ^{13}C NMR (600 MHz, $CDCl_3$+MeOD, δ): 160.4, 151.0, 150.3, 149.5, 143.3, 142.4, 137.5, 129.8, 129.0, 123.7, 122.9, 122.1, 116.2, 115.4, 109.7, 104.1, 40.2 (2 carbons). HRMS (ESI) *m/z*: $[M + H]^+$ calcd for $C_{20}H_{16}N_2O_2$, 316.1212; found, 317.1290.

4-(8-(Dimethylamino)-2-oxo-2H-benzo[*g*]chromen-3-yl)-1-methylpyridinium trifluoromethanesulfonate (Py⁺BC680a). Starting from **PyBC580a** (30 mg, 0.095 mmol), this compound was prepared as a red-violet solid (37 mg, 81%). mp 319°C, ^{1}H NMR (300 MHz, DMSO-d_6, 298 K): δ 8.97 (d, *J* = 7.2 Hz, 2H), 8.90 (s, 1H), 8.53 (d, *J* = 7.2 Hz, 2H), 8.25 (s, 1H), 7.96 (d, *J* = 9.3 Hz, 1H), 7.58 (s, 1H), 7.30 (dd, *J* = 9.3 2.4 Hz, 1H), 6.99 (d, *J* = 2.4 Hz, 1H), 4.33 (s, 3H), 3.13 (s, 6H). ^{13}C NMR (500 MHz, DMSO-d_6, 298 K): δ 159.0, 150.7, 150.7, 150.2, 146.7, 144.9, 138.1, 131.3, 130.6, 130.1, 125.0, 123.1, 117.0, 116.4, 114.7, 108.7, 103.6, 47.2. IR (KBr): ν = 3127, 3048, 1701, 1644, 1615, 1580, 1554, 1507, 1472, 1440, 1392, 1323, 1299, 1260, 1223, 1198, 1182, 1141, 1060, 1029 cm^{-1}. HRMS (ESI) *m/z*: $[M + H]^+$ calcd for $C_{21}H_{19}N_2O_2$, 331.1447; found, 331.1447.

8-(Allyl(methyl)amino)-3-(pyridin-4-yl)-2H-benzo[*g*]chromen-2-one (PyBC580b). Starting from 6-(allyl(methyl)amino)-3-hydroxy-2-naphthaldehyde (130 mg, 0.54 mmol), this compound was synthesized as an orange solid (144 mg, 78%). ^{1}H NMR (300 MHz, $CDCl_3$, 298 K): δ 8.69 (d, *J* = 5.7 Hz, 2H), 8.00 (s, 1H), 7.89 (s, 1H), 7.76 (d, *J* = 9.3 Hz, 1H), 7.69 (d, *J* = 5.7 Hz, 2H), 7.47 (s, 1H), 7.12 (dd, *J* = 9.3 2.4 Hz, 1H), 6.85 (d, *J* = 2.4 Hz, 1H), 5.96–5.84 (m, 1H), 5.24–5.16 (m, 2H), 4.10 (d, *J* = 4.8 Hz, 2H), 3.13 (s, 3H). ^{13}C NMR (300 MHz, $CDCl_3$ + MeOD, 298 K): δ 160.6, 150.9, 149.5, 149.2, 143.6, 142.7, 137.6, 132.7, 130.0, 129.1, 123.7, 123.0, 121.6, 116.5, 116.3, 115.3, 109.6, 103.9, 54.8, 38.2. HRMS (ESI) *m/z*: $[M + H]^+$ calcd for $C_{22}H_{18}N_2O_2$, 342.1368; found, 343.1447.

4-(8-(Allyl(methyl)amino)-2-oxo-2H-benzo[*g*]chromen-3-yl)-1-methylpyridinium trifluoromethanesulfonate (Py⁺BC680b). Starting from **PyBC580b** (44 mg, 0.13 mmol), this compound was prepared as a red-violet solid (58 mg, 88%). mp 246 °C, ^{1}H NMR (600 MHz, DMSO-d_6, 298 K): δ 8.98 (d, *J* = 6.6 Hz, 2H), 8.90 (s, 1H), 8.54 (d, *J* = 6.6 Hz, 2H), 8.25 (s, 1H), 7.94 (d, *J* = 9.0

Hz, 1H), 7.58 (s, 1H), 7.28 (dd, *J* = 9.0, 2.4 Hz, 1H), 7.02 (d, *J* = 2.4 Hz, 1H), 5.94–5.89 (m, 1H), 5.20–5.15 (m, 2H), 4.34 (s, 3H), 4.18 (d, *J* = 4.8 Hz, 2H), 3.12 (s, 3H). ^{13}C NMR (600 MHz, DMSO-d_6, 298 K): δ 159.5, 151.2, 150.7, 150.3, 147.2, 145.4, 138.7, 133.8, 131.7, 131.0, 125.6, 123.7, 117.6, 117.0, 116.7, 115.2, 109.2, 104.1, 54.4, 47.7, 38.6. IR (KBr): ν = 3125, 3047, 1701, 1640, 1623, 1579, 1550, 1494, 1436, 1395, 1325, 1305, 1260, 1224, 1202, 1185, 1140 cm^{-1}. HRMS (ESI) *m/z*: [M + H]$^+$ calcd for $C_{23}H_{21}N_2O_2$, 357.1603; found, 357.1603.

8-(Methylamino)-3-(pyridin-4-yl)-2H-benzo[*g*]chromen-2-one (PyBC560a). A solution of 3-hydroxy-6-(methylamino)-2-naphthaldehyde (50 mg, 0.25 mmol)[1] and ethyl 4-pyridylacetate (45.6 µL, 0.30 mmol) in ethanol (2.5 mL) was treated with 2 drops of piperidine, and the resulting solution was stirred for 8 h at 70 °C. After being cooled to room temperature, the reaction mixture concentrated under reduced pressure. The residue was treated with a mixture of methanol and hexane (1:9) to give **PyBC560a** as an orange precipitate, which was filtered and dried (57 mg, 76%). ^{1}H NMR (300 MHz, DMSO-d_6, 298 K): δ 8.65 (d, *J* = 6.2 Hz, 2H), 8.49 (s, 1H), 8.11 (s, 1H), 7.78 (d, *J* = 6.2 Hz, 2H), 7.51 (s, 1H), 6.99 (dd, *J* = 9.0 2.0 Hz, 1H), 6.70 (d, *J* = 2.0, 1H), 6.66 (dd, *J* = 9.0, 5.1, 1H), 2.82 (d, *J* = 5.1 Hz, 3H). ^{13}C NMR (600 MHz, DMSO-d_6, 298 K): δ 160.0, 151.3, 150.6, 150.1, 143.5, 143.0, 138.4, 130.1, 130.0, 124.0, 123.1, 121.2, 119.3, 115.0, 108.8, 100.6. 29.9. HRMS (ESI) *m/z*: [M + H]$^+$ calcd for $C_{19}H_{14}N_2O_2$, 302.1060; found, 303.1134.

1-Methyl-4-(8-(methylamino)-2-oxo-2H-benzo[*g*]chromen-3-yl)pyridinium trifluoromethanesul-fonate (Py$^+$BC660a). Starting from **PyBC560a** (40 mg, 0.13 mmol), this compound was prepared as a red solid (52 mg, 84%). mp 311 °C, ^{1}H NMR (300MHz, DMSO-d_6, 298 K): δ 8.94 (d, *J* = 7.0 Hz, 2H), 8.85 (s, 1H), 8.51 (d, *J* = 7.0 Hz, 2H), 8.13 (s, 1H), 7.79 (d, *J* = 9.1 Hz, 1H), 7.45 (s, 1H), 6.99 (dd, *J* = 9.1 2.0 Hz, 1H), 6.69 (d, *J* = 2.0 Hz, 1H), 4.32 (s. 3H), 2.83 (s, 3H). ^{13}C NMR (300 MHz, DMSO-d_6, 298 K): δ 159.1, 150.9, 150.9, 150.2, 146.8, 144.8, 139.1, 131.3, 130.3, 124.9, 123.6, 119.1, 116.4, 114.1, 108.3, 100.2, 47.1, 29.3. IR (KBr): ν = 3566, 3006, 2990, 1715, 1623, 1586, 1520, 1504, 1475, 1276, 1260, 1168, 1031 cm^{-1}. HRMS (ESI) *m/z*: [M + H]$^+$ calcd for $C_{20}H_{17}N_2O_2$, 317.1285; found, 317.1290.

8-Amino-3-(pyridin-4-yl)-2H-benzo[*g*]chromen-2-one (PyBC560b). Starting from 6-(allylamino)-3-hydroxy-2-naphthaldehyde (100 mg, 0.44 mmol), which was synthesized by following the reported procedure by us,[1] 8-(allylamino)-3-(pyridin-4-yl)-2*H*-benzo[g]chromen-2-one (***N*-allyl-PyBC**) was synthesized as an orange solid (120 mg, 84%). ^{1}H NMR (300 MHz, $CDCl_3$, 298 K): δ 8.55 (d, *J* = 5.9 Hz, 2H), 8.00 (s, 1H), 7.84 (s, 1H), 7.68 (d, *J* = 5.9 Hz, 2H), 7.64 (d, *J* = 9.0 Hz, 1H), 7.38 (s, 1H), 6.88 (dd, *J* = 9.0 1.8 Hz, 1H), 6.69 (d, *J* = 1.8 Hz, 1H), 5.98–5.90 (m, 1H), 5.31–5.17 (m, 2H), 3.86 (d, *J* = 5.0 Hz, 2H). ^{13}C NMR (500 MHz, $CDCl_3$ + MeOD, 298 K): δ 161.0, 151.1, 149.3, 148.5, 143.6, 142.8, 138.1, 134.3, 130.1, 129.2, 124.6, 123.1, 122.0, 118.9, 116.8, 115.3, 109.8, 102.6, 45.9. A solution of ***N*-allyl-PyBC** (50 mg, 0.15 mmol) in anhydrous methanol (2 mL) was added to a round bottom flask containing tetrakis(triphenylphosphine)palladium (17.6 mg, 0.015 mmol) and *N,N*′-dimethylbarbituric acid (71.2 mg, 0.46 mmol) under argon condition. The resulting solution was stirred for 5 h at 60 °C to afford the crude product as precipitates, which was purified by column chromatography on a short pad of silica gel (eluent:

MeOH/CH_2Cl_2 = 1/9) to give **PyBC560b** as an orange solid (28 mg, 64%). ^{1}H NMR (500 MHz, DMSO-d_6, 298 K): δ 8.66 (d, *J* = 3.6 Hz, 2H), 8.49 (s, 1H), 8.11 (s, 1H), 7.79 (m, 3H), 7.43 (s, 1H), 6.99 (dd, *J* = 8.7 2.2 Hz, 1H), 6.84 (d, *J* = 2.2 Hz, 1H), 6.04 (s. 2H). ^{13}C NMR (500 MHz, DMSO-d_6, 298 K): δ 159.5, 150.6, 149.7, 149.6, 143.0, 137.5, 130.1, 129.6, 123.3, 122.5, 118.8, 114.5, 107.8, 104.0. HRMS (ESI) *m/z*: $[M + H]^+$ calcd for $C_{18}H_{12}N_2O_2$, 288.0899; found, 289.0977.

4-(8-Amino-2-oxo-2H-benzo[*g*]chromen-3-yl)-1-methylpyridinium trifluoromethanesulfonate (Py^+BC660b). Starting from **PyBC560b** (10 mg, 0.023 mmol), this compound was prepared as a red solid (7 mg, 67%). ^{1}H NMR (300 MHz, DMSO-d_6, 298 K): δ 8.97 (d, *J* = 6.7 Hz, 2H), 8.90 (s, 1H), 8.54 (d, *J* = 6.7 Hz, 2H), 8.18 (s, 1H), 7.84 (d, *J* = 9.2 Hz, 1H), 7.46 (s, 1H), 7.01 (dd, *J* = 9.2 2.1 Hz, 1H), 6.84 (d, *J* = 2.1 Hz 1H), 6.33 (s, 2H), 4.32 (s, 3H). ^{13}C NMR (500 MHz, DMSO-d_6, 298 K): δ 159.1, 150.8, 150.8, 150.3, 146.9, 144.9, 138.7, 131.5, 131.0, 125.0, 123.5, 119.1, 116.7, 114.2, 107.9, 104.0, 47.2. IR (KBr): ν = 3406, 3347, 3240, 3127, 1703, 1623, 1586, 1459, 1332, 1317, 1253, 1225, 1153, 1028 cm^{-1}. HRMS (ESI) *m/z*: $[M + H]^+$ calcd for $C_{19}H_{15}N_2O_2$, 303.1133; found, 303.1134.

4-(8-(Allyl(methyl)amino)-2-oxo-2H-benzo[*g*]chromen-3-yl)pyridine 1-oxide (Py^+O^-BC600). A solution of ethyl 2-(pyridin-4-yl)acetate (200 mg, 1.21 mmol) and meta-chloroperoxybenzoic acid (*m*CPBA, 418 mg, 2.42 mmol) in THF (2 mL) was stirred at room temperature for 10 h. The reaction mixture was concentrated under reduced pressure, and the crude product was purified by silica gel column chromatography (eluent: MeOH/CH_2Cl_2 = 7/93) to afford 4-(2-ethoxy-2-oxoethyl)pyridine *N*-oxide (146 mg, 67%). A solution of 6-(allyl(methyl)amino)-3-hydroxy-2-naphthaldehyde (50 mg, 0.21 mmol) and 4-(2-ethoxy-2-oxoethyl)pyridine 1-oxide (45 mg, 0.25 mmol) in ethanol (1 mL) was treated with 2 drops of piperidine, and the resulting solution was stirred for 8 h at 30 °C. The reaction mixture was concentrated under reduced pressure, and the crude product was purified by silica gel column chromatography (eluent: MeOH/CH_2Cl_2 = 3/97) to give **Py^+O^-BC600** as an orange solid (54 mg, 73%). mp 198 °C , ^{1}H NMR (300 MHz, $CDCl_3$, 298 K): δ 8.24 (d, *J* = 7.2 Hz, 2H), 7.99 (s, 1H), 7.88 (s, 1H), 7.80–7.74(m, 3H), 7.45 (s, 1H), 7.12 (dd, *J* = 9.3 2.4 Hz, 1H), 6.84 (d, *J* = 2.4 Hz, 1H), 5.95–5.83 (m, 1H), 5.24–5.15 (m, 2H), 4.10 (d, *J* = 4.8 Hz, 2H), 3.13 (s, 3H). ^{13}C NMR (500 MHz, $CDCl_3$ + MeOD, 298 K): δ 160.4, 150.9, 149.7, 142.1, 138.8, 137.9, 135.2, 132.7, 130.2, 129.4, 125.3, 123.9, 119.7, 116.7, 116.5, 115.3, 109.8, 104.1, 54.9, 38.4. IR (KBr): ν = 3420, 3111, 3081, 2958, 2925, 2855, 1716, 1630, 1590, 1564, 1489, 1448, 1394, 1374, 1310, 1247 cm^{-1}. HRMS (ESI) *m/z*: $[M + H]^+$ calcd for $C_{22}H_{18}N_2O_3$, 358.1317; found, 359.1396.

7-(Diethylamino)-3-(pyridin-4-yl)-2H-chromen-2-one (PyC). A solution of 4-(diethylamino)salicylaldehyde (100 mg, 0.52 mmol) and ethyl 4-pyridylacetate (118 μL, 0.78 mmol) in ethanol (2 mL) was treated with 2 drops of piperidine, and the resulting mixture was stirred for 24 h at 70 °C. After being cooled to room temperature, the reaction mixture was concentrated under reduced pressure to give the crude product, which was purified by silica gel column chromatography (eluent: EtOAc/Hexane = 2/8) to afford **PyC** (72 mg, 47%) as an yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$, 298 K): δ 8.63 (d, *J* = 5.6 Hz, 2H), 7.85 (s, 1H), 7.68 (d, *J* = 5.6 Hz, 2H), 7.35 (d, *J* = 8.8 Hz, 1H), 6.62 (dd, *J* = 8.8 2.4 Hz, 1H), 6.52 (d, *J* = 2.4 Hz, 1H),

3.48–3.41 (q, *J* = 7.1 Hz, 4H), 1.23 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR (500 MHz, $CDCl_3$, 298 K): δ 161.0, 156.9, 151.6, 149.9, 143.7, 142.1, 129.8, 122.5, 117.3, 109.5, 108.8, 97.2, 45.2 (2 carbons), 12.6 (2 carbons).

2. Figures

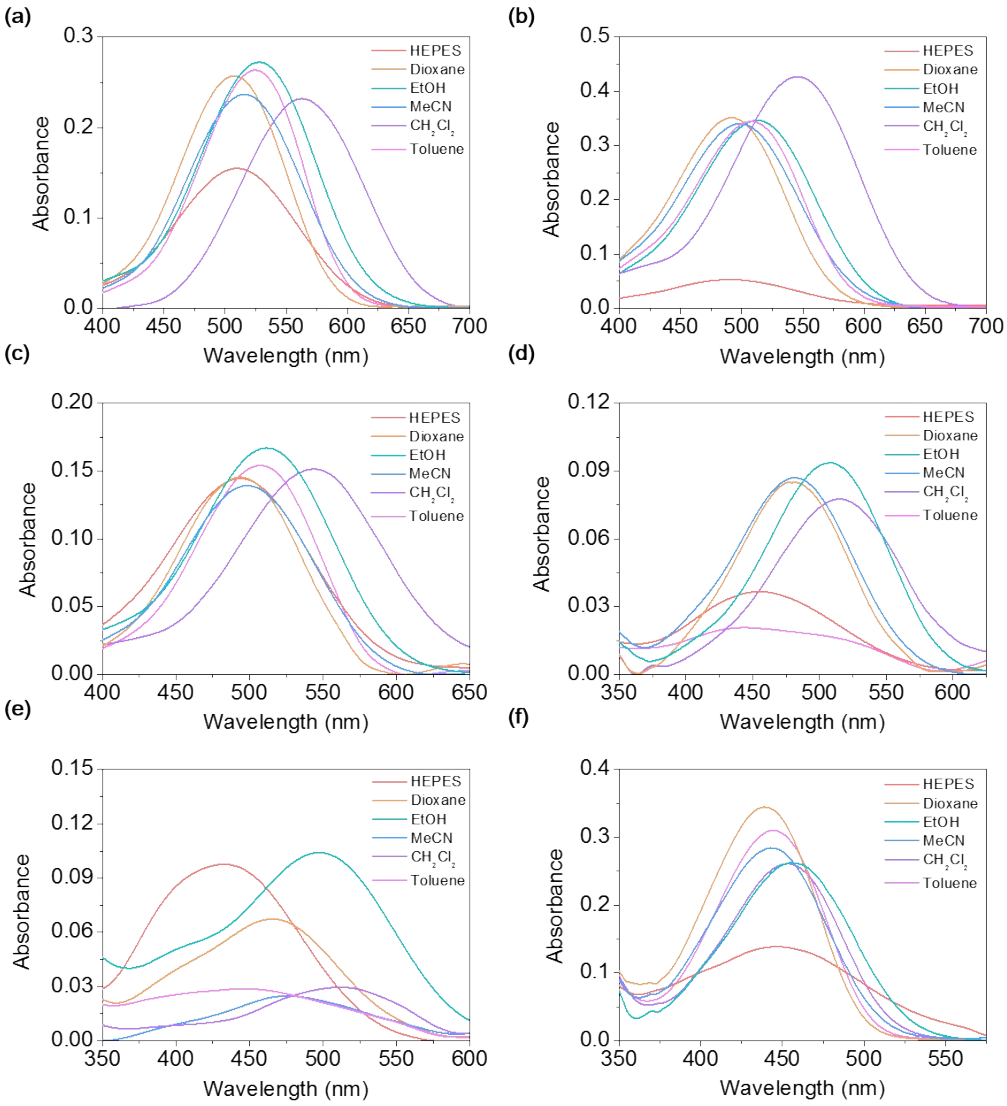


Fig. S1. UV/Vis absorption spectra of (a) **Py⁺BC690**, (b) **Py⁺BC680a**, (c) **Py⁺BC680b**, (d) **Py⁺BC660a**, (e) **Py⁺BC660b,** and (f) **Py⁺O⁻BC600,** measured in different solvents. All the measurements were conducted at 25 °C for each of the compounds (10 μM) dissolved in the given solvent.

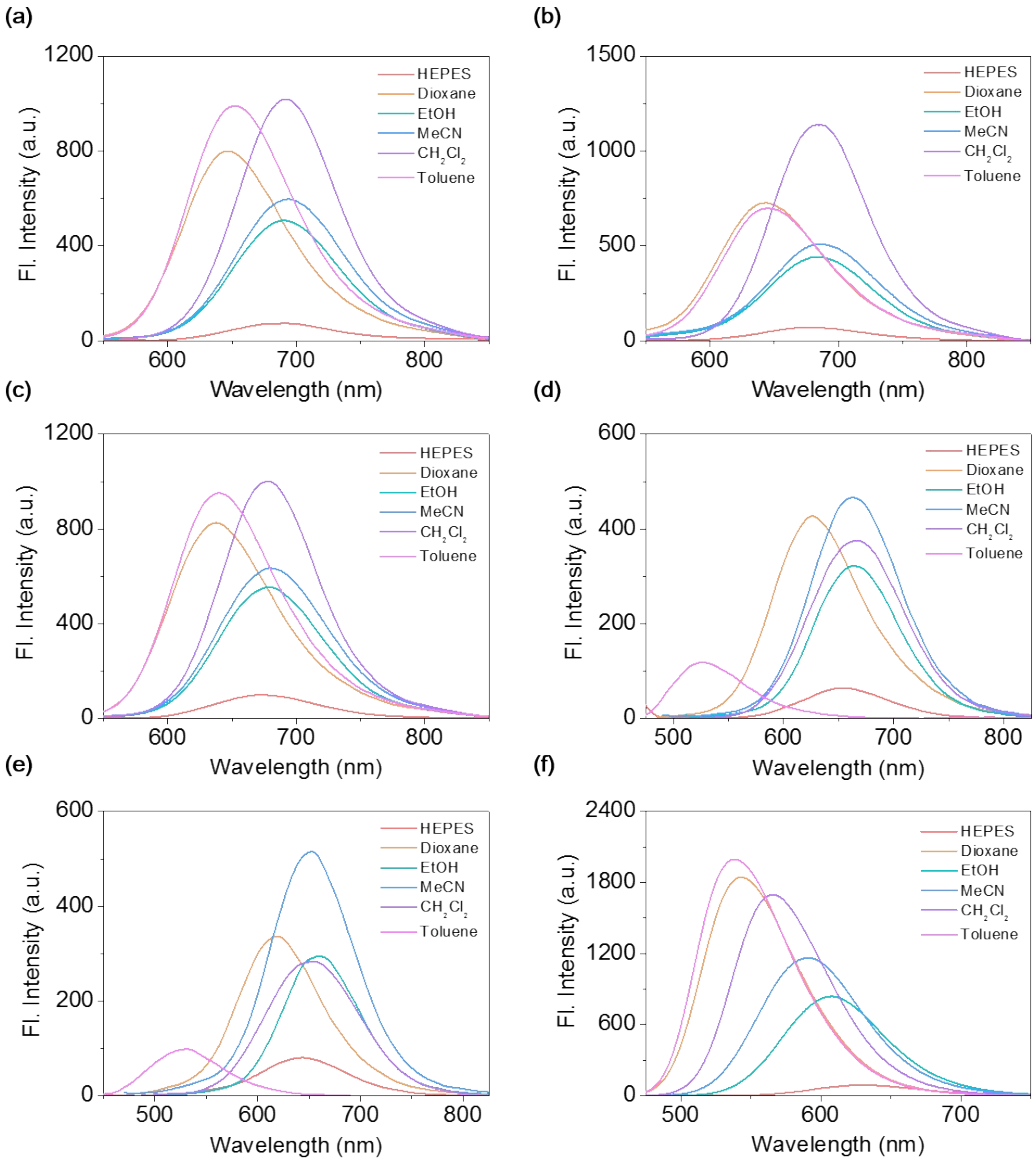


Fig. S2. Fluorescence emission spectra of (a) **Py$^+$BC690,** (b) **Py$^+$BC680a**, (c) **Py$^+$BC680b**, (d) **Py$^+$BC660a**, (e) **Py$^+$BC660b**, and (f) **Py$^+$O$^-$BC600**, measured in different solvents. All the measurements were conducted at 25 °C for each of the compounds (10 μM) dissolved in the given solvent. The fluorescence emission spectra were measured under excitation at the maximum absorption wavelength of each dye.

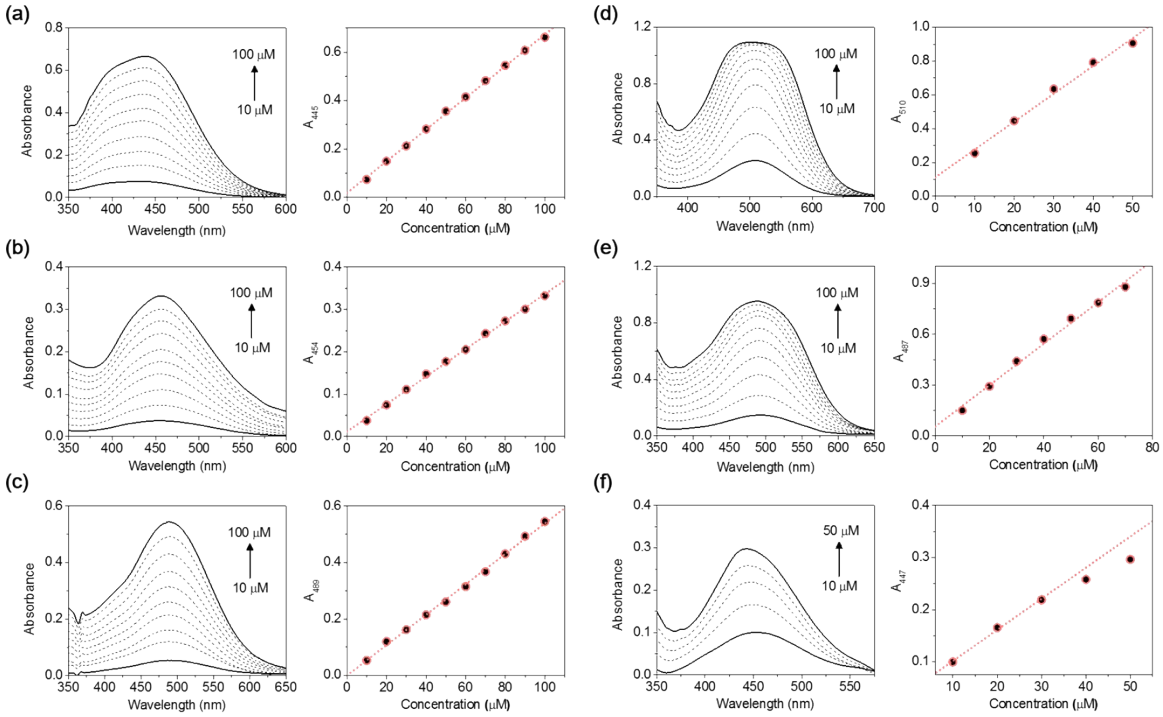


Fig. S3. UV/Vis absorption spectra of (a) **Py⁺BC690,** (b) **Py⁺BC680a**, (c) **Py⁺BC680b**, (d) **Py⁺BC660a**, (e) **Py⁺BC660b**, and (f) **Py⁺O⁻BC600**, at different concentrations (10–100 μM) in HEPES buffer (10 mM, pH 7.4, ≤ 1% DMSO). Note: We were able to measure the solubility of **Py⁺BC690** up to 50 μM where the absorbance was saturated.

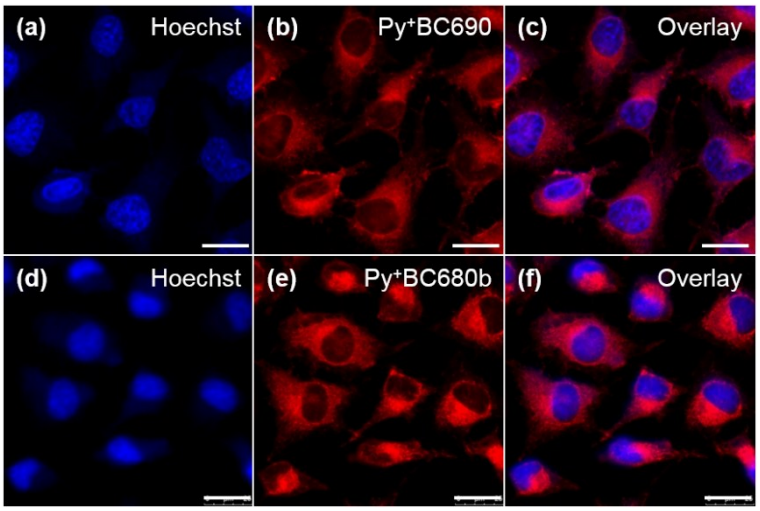


Fig. S4. 2PM imaging of HeLa cells incubated with **Py⁺BC** dyes. (a, d) Cell images stained with Hoechst 33342, a nucleus staining reference dye. (b, e) Cell images stained with **Py⁺BC690** and **Py⁺BC680b**, respectively. Concentration of dyes: 3 μg/mL for the Hoechst dye; 10 μM for **Py⁺BC** dyes. Excitation wavelengths: 405 nm for the Hoechst dye and 900 nm for the **Py⁺BC** dyes (under TPM). Emission wavelengths collected: 410–450 nm for the Hoechst dye; 565–675 nm for the **Py⁺BC** dyes.

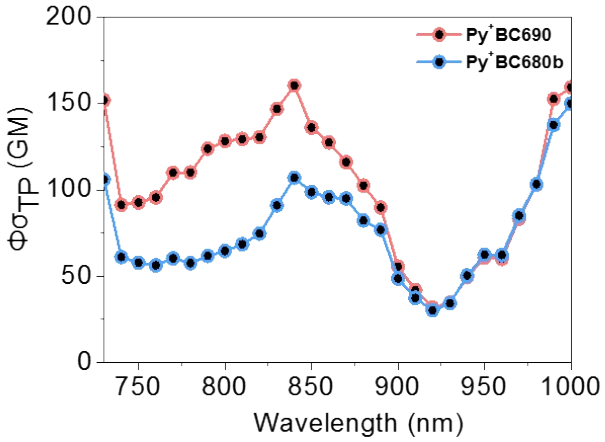


Fig. S5. Two-photon action spectra of **Py⁺BC690** and **Py⁺BC680b.** The two-photon action cross section (TPACS) values were measured for the **Py⁺BC** dyes at 100 µM in DMSO using Rhodamine B (100 µM) in MeOH as a reference dye.

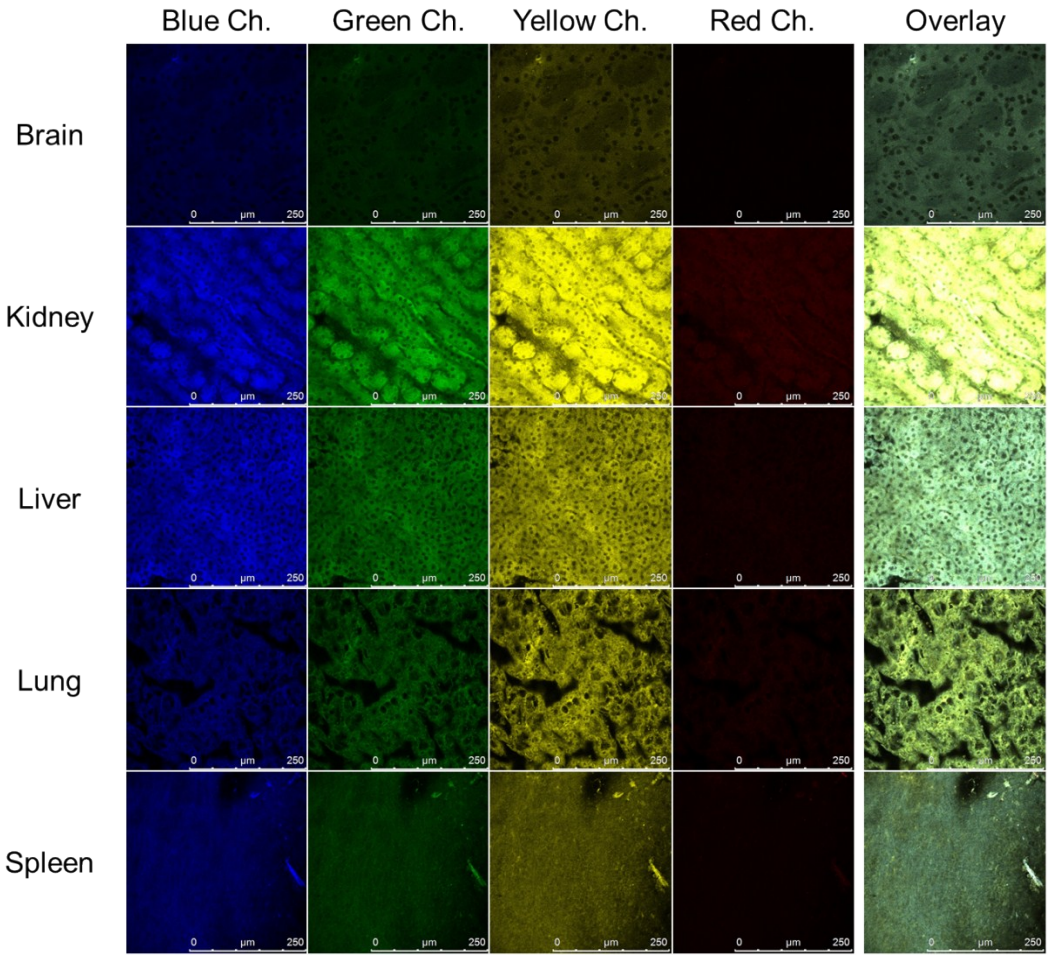


Fig. S6. Autofluorescence analysis for different tissues depending on the emission channels and kinds of tissues, measured under two-photon excitation at 850 nm.

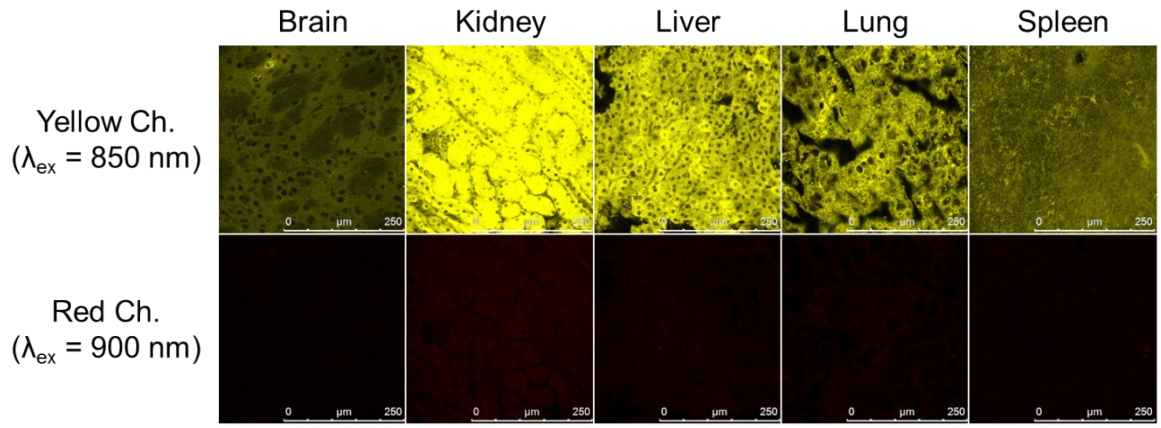


Fig. S7. Autofluorescence in tissue imaging dependent on the emission channels: between the yellow and red channels. The emission from the yellow channel was obtained under excitation at 850 nm and that from the red channel under excitation at 900 nm (at the doubled wavelength of the maximum absorption wavelength of the yellow or the red emitting dye).

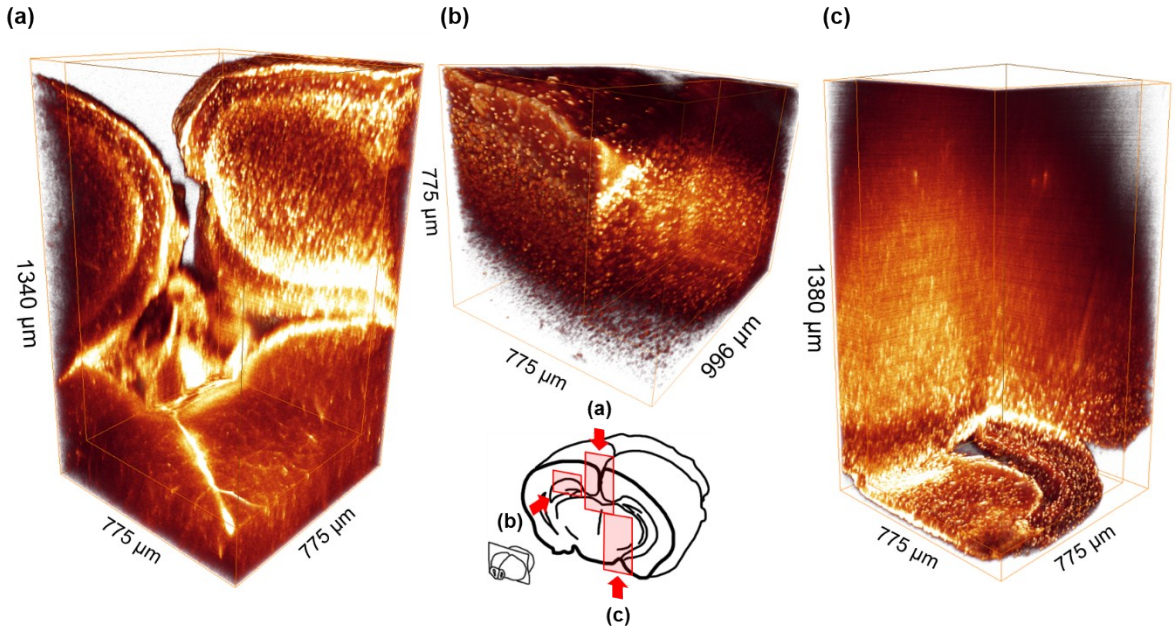


Fig. S8. 3D 2PM images of mouse brain tissue stained with **Py⁺BC690** after the BABB clearance procedure. Corner cup images from the (a) top, (b) side, and (c) bottom, which were collected through the red channel (625–675 nm) at every 2 μm of depth while excited at 900 nm. Laser power was gradually increased from 5 mW to 50 mW (compensation excitation).

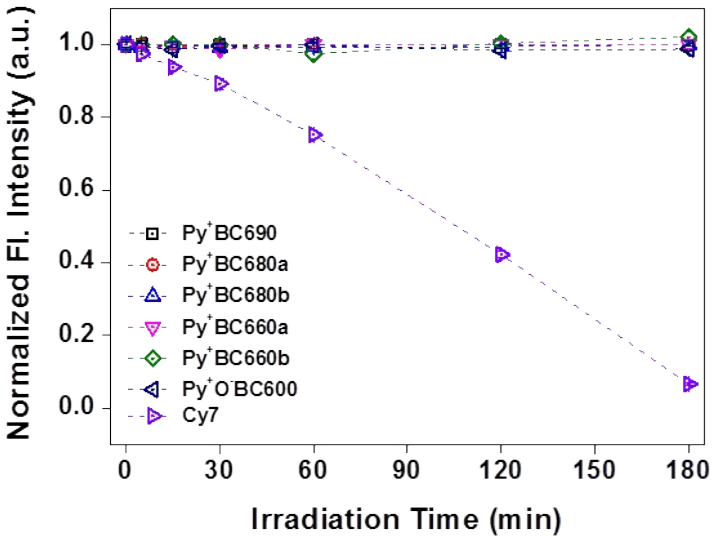


Fig. S9. Photostability spectra in time under UV irradiation (at 365 nm) with 10 μM of dyes in EtOH. The fluorescence emission was measured under excitation at the maximum absorption wavelength of each dye.

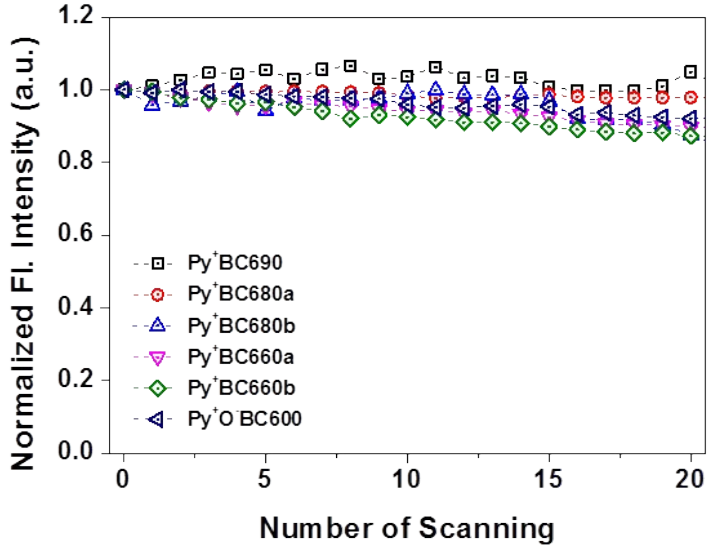


Fig. S10. Photostability spectra under two-photon excitation (900 nm) in HeLa cells incubated with 10 μM of dyes. The fluorescence emission was collected in the range from 410 to 675 nm.

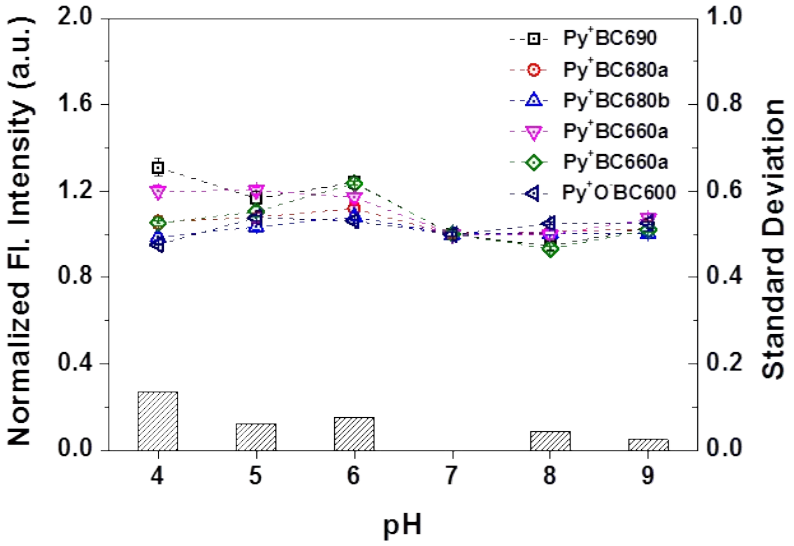


Fig. S11. Variation of fluorescent intensity depending on pH. Line and symbol indicate normalized fluorescent intensity (based on pH 7) on each pH and bar graph indicate the standard deviation of normalized fluorescent intensity. Measured in 1X universal buffer with 10 μM of **Py⁺BC** dyes.

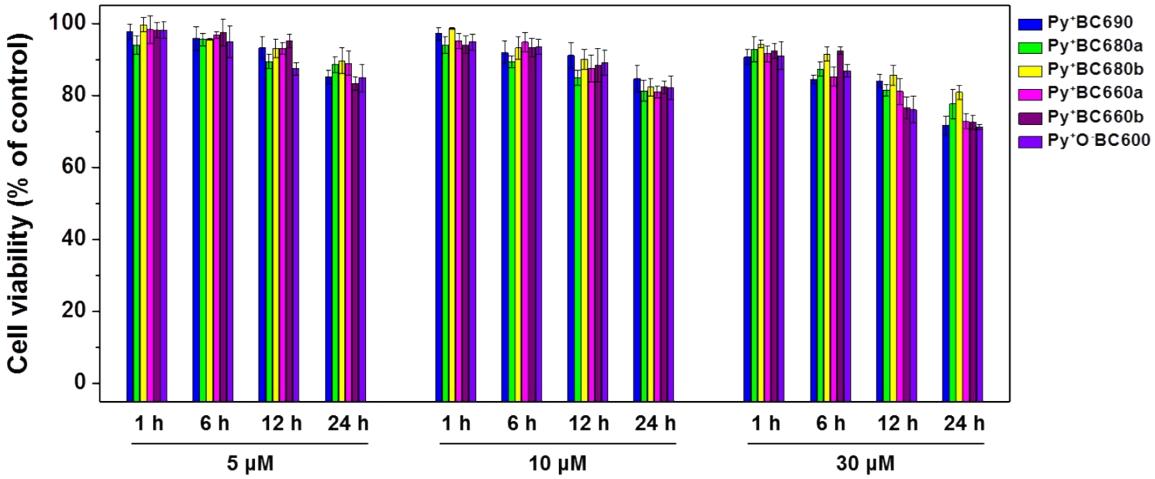


Fig. S12. Cell viability evaluation by CCK-8 assay with HeLa cells treated with **Py⁺BC** dyes at various concentrations (5, 10, and 30 μM) within 24 h.

3. Tables

Table S1. Maximum absorbance wavelengths [λ_{abs} (nm)] of **Py$^+$BC** derivatives in different solvents[a]

	Py$^+$BC690	Py$^+$BC680a	Py$^+$BC680b	Py$^+$BC660a	Py$^+$BC660b	Py$^+$O$^-$BC600
HEPES buffer	510	489	487	454	445	447
Dioxane	507	492	493	475	465	440
EtOH	527	513	515	506	499	456
MeCN	517	500	499	483	464	445
CH_2Cl_2	562	546	542	517	492	456
DMSO	513	498	499	492	490	454
Toluene	525	506	506	435	425	447

[a]All the measurements were conducted at 25 °C for each of the compounds (10 μM) dissolved in the given solvent.

Table S2. Maximum emission wavelengths [λ_{em} (nm)) of **Py$^+$BC** derivatives in different solvents[a]

	Py$^+$BC690	Py$^+$BC680a	Py$^+$BC680b	Py$^+$BC660a	Py$^+$BC660b	Py$^+$O$^-$BC600
HEPES buffer	689	678	675	657	645	630
Dioxane	647	643	637	626	619	543
EtOH	691	681	680	663	660	606
MeCN	694	685	680	663	651	591
CH_2Cl_2	694	685	678	666	656	565
DMSO	700	664	686	673	664	605
Toluene	652	645	640	526	530	538

[a]All the measurements were conducted at 25 °C for each of the compounds (10 μM) dissolved in the given solvent. The fluorescence emission spectra were measured under the excitation at the maximum absorption wavelength of each dye.

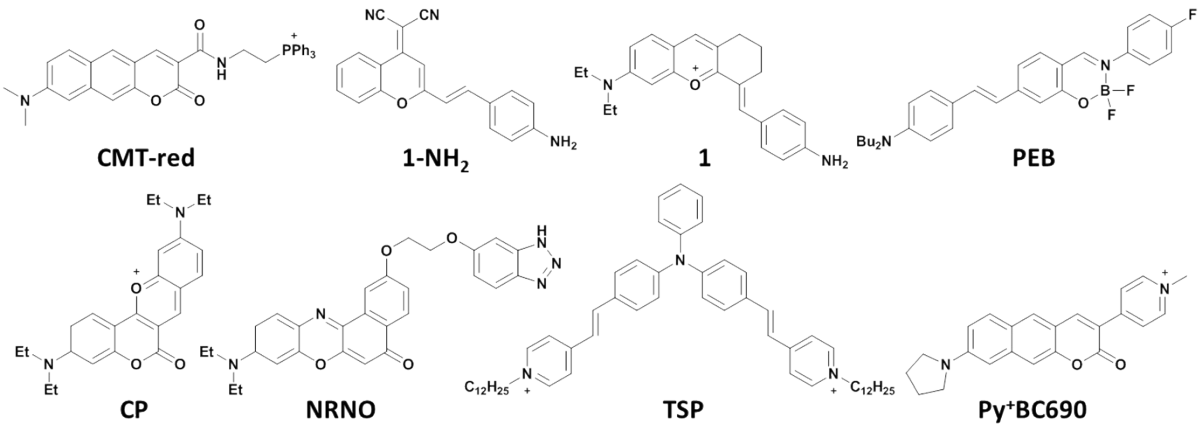
Table S3. Molar extinction coefficients [ε ($Lmol^{-1}cm^{-1}$)] of **Py$^+$BC** derivatives in different solvents

	Py$^+$BC690	Py$^+$BC680a	Py$^+$BC680b	Py$^+$BC660a	Py$^+$BC660b	Py$^+$O$^-$BC600
HEPES buffer	15991	5307	14761	3755	10477	14085
Dioxane	26033	35259	17094	11566	7070	25332
EtOH	27577	34818	17258	12915	11593	35464
MeCN	24167	34174	15618	10380	9773	21073
CH_2Cl_2	22311	42870	17441	7803	6594	31209
DMSO	23402	32800	15234	8972	4938	33535
Toluene	26554	34615	16971	1531	1686	41244

Table S4. Quantum yields (Φ_F) of **Py⁺BC** derivatives in different solvents

	Py⁺BC690	Py⁺BC680a	Py⁺BC680b	Py⁺BC660a	Py⁺BC660b	Py⁺O⁻BC600
Water	0.007	0.009	0.013	0.013	0.013	0.085
EtOH	0.029	0.060	0.068	0.058	0.044	0.097
MeCN	0.048	0.070	0.063	0.070	0.063	0.398
CH_2Cl_2	0.037	0.082	0.072	0.073	0.054	0.489
DMSO	0.022	0.034	0.047	0.041	0.035	0.243

Table S5. Photophysical properties of two-photon absorbing red and far-red emitting dyes.



Name	λ_{em} (nm)	δΦ (GM)	M.W.	Photo-stability ($t_{1/2}$)	Water Solubility
CMT-red	626[a]	50[b]	650.1	1200 sec[g]	6 μM
1-NH_2	670[f]	50[e]	311.1	Stable[j]	n.d.
1	725[a]	180[a]	359.2	Stable[i]	n.d.
PEB	617[a]	290[d]	492.3	n.d.	n.d.
CP	645[a]	n.d.	393.2	n.d.	n.d.
NRNO	650[a]	38[a]	497.2	n.d.	n.d.
TSP	610[a]	0.03[c]	789.6	900 sec[g]	n.d.
Py⁺BC690	700[e]	150[e]	357.2	Stable[g,h]	>100 μM

Measured in [a]aqueous buffer, [b]Dioxane, [c]MeOH, [d]toluene, [e]DMSO, and [f]aqueous/organic mixture (50:50). Irradiated under [g]two-photon excitation, [h]UV (365 nm), [i]mercury lamp (100 W), and [j]tungsten lamp (500 W). n.d.; not determined.

4. Notes

Fluorescence quantum yield measurement.

The fluorescence quantum yields were measured by using rhodamine 6G in ethanol as a reference. The sample solutions were excited by a laser light at the wavelengths tuned to 500, 510, and 520 nm, and fluorescence was detected by a spectrograph equipped with a CCD detector. The full emission spectra were measured and integrated to give the quantum yields. After confirming no uncertainty in the calculated quantum yield of rhodamine 6G compared with the literature values (literature quantum yield is 0.91[2]), we measured the quantum yields of all the **Py⁺BC** derivatives using rhodamine 6G as a reference. The quantum yields of the compounds in various solvents were calculated according to **Equation S1** as below. Where Φ is the quantum yield, r represents references, I is the measured integrated emission intensity, n is the refractive index, and A is the optical density

$$\Phi = \Phi_r \times \frac{A_r}{I_r} \times \frac{I}{A} \times \frac{n^2}{n_r^2} \quad \text{(S1)}$$

Determination of two-photon action cross-section value. TPACS values were measured following the known method.[3,4] Two equations are referred from the references as below.

$$\frac{< F(t) >_{cal}}{< F(t) >_{new}} = \frac{\Phi_{cal}\eta_{2cal}\sigma_{2cal}C_{cal} < P_{cal}(t) >^2 n_{cal}}{\Phi_{new}\eta_{2new}\sigma_{2new}C_{new} < P_{new}(t) >^2 n_{new}} \quad \text{(S2)}$$

The Equation S2 is the main equation that calculates TPACS using a reference dye and Equation S3 could be extracted from Equation S2.

$$\sigma_{2new}(\lambda)\eta_{2new} = \frac{\Phi_{cal}\eta_{2cal}\sigma_{2cal}(\lambda)C_{cal} < P_{cal}(t) >^2 < F(t) >_{new} n_{cal}}{\Phi_{new}C_{new} \quad < P_{new}(t) >^2 < F(t) >_{cal} n_{new}} \quad \text{(S3)}$$

(σ_2 = two-photon absorption cross section; η = quantum efficiency; σ_{TPE} (two photon action cross section = ση; <F(t)> = time averaged fluorescence emission; C =fluorophore concentration; <P(t)> = time averaged laser power; n=refractive index of sample; Φ = fluorescence collection efficiency)

Φ_{cal} and Φ_{new} are identical in the same experimental setup, and $<P_{cal}(t)>$, $<P_{new}(t)>$ are also identical when same laser is applied. TPACS values of samples could be calculated by putting values of known TPACS (two-photon action cross section) (ση), concentration (C), detected emission (<F(t)>), and known refractive index (n) into either of the two equations.

Rhodamine B in methanol (100 μM) was used as a reference, and 100 μM of **Py⁺BC690** or **Py⁺BC680b** in DMSO was used for the measurements. Each refractive index of a given solvent was applied (assuming that the refractive index of sample is almost the same as that of pure solvent). 100 μL of a sample was loaded in the well slide and covered with cover glass. The edge of cover glass was coated with transparent manicure to prevent the evaporation of solvent and then mounted on a vibration isolation table. Two-photon excitation was performed with a Ti-sapphire laser (Chameleon Vision II, Coherent) at 140 fs pulse width and 80 MHz pulse repetition rate. The emission intensity was collected through an HCX APO 10× objective lens (Leica,

Germany) of a two-photon microscopy (TCS SP5 II, Leica, Germany) equipped with HyD detector (Leica, Germany).

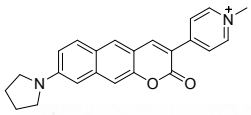
Cell viability evaluation. Cell viability was assesed by measuring their ability to metabolize CCK-8 (Cell Counting Kit-8, Dojindo molecular technologies, Inc.) in HeLa cell line. Cells were seeded into 96-well plates at a density of 5×10^3 cells per well in Dulbecco's Modified Eagle's Medium (DMEM) and incubated at 37 °C for 24 h in a humidified atmosphere of 5% CO_2 in the air. The **Py$^+$BC** dyes at various concentrations (5, 10 and 30 μM) were added into the culture media in the plate and control group was treated with PBS buffer (10 mM, pH 7.4). The plates were incubated for 1, 6, 12 or 24 h and 10 μL of CCK-8 solution was added to each well of the plate. After incubation for 1 h, absorbance at 450 nm was measured using a microplate reader (Multiskan EX, Thermo Eletron). Results were expressed as a percentage in comparison to the absorbance of non-treated cells (Fig S12).

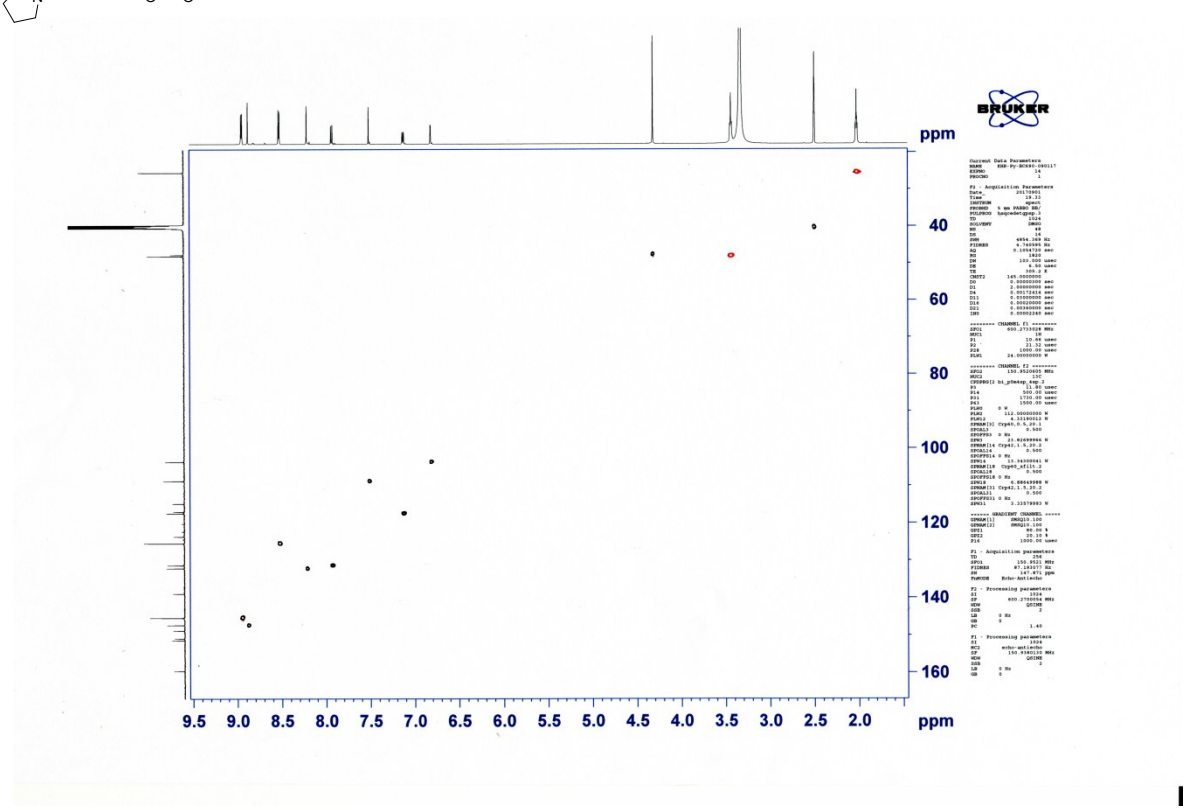
5. Reference

1. I. Kim, D. Kim, S. Sambasivan and K. H. Ahn, *Asian. J. Org. Chem.,* 2012, **1**, 60–64.
2. C. Würth, M. Grabolle, J. Pauli, M. Spieles and U. Resch-Genger, *Nat. Protoc.,* 2013, **8**, 1535–1550.
3. M. A. Albota, C. Xu and W. W. Webb, *Appl. Opt.,* 1998, **37**, 7352–7356.
4. C. Xu and W. W. Webb, *J. Opt. Soc. Am. B,* 1996, **13**, 481–491.

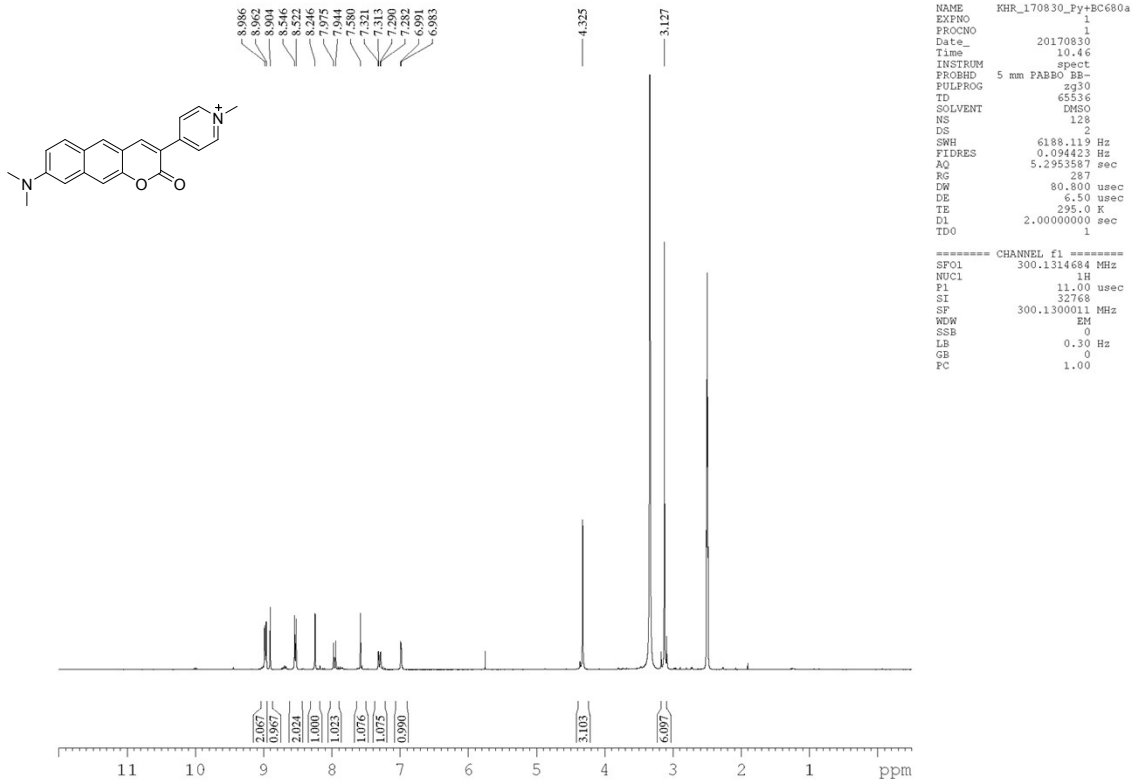
6. ^{1}H and ^{13}C NMR Spectra

$Py^{+}BC690$





Py⁺BC680a

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SOLVENT                      DMSO
NS                            128
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FIDRES                   0.094423 Hz
AQ                       5.2953587 sec
RG                            287
DW                         80.800 usec
DE                          6.50 usec
TE                         295.0 K
D1                    2.00000000 sec
TD0                              1

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GB                            0
PC                         1.00
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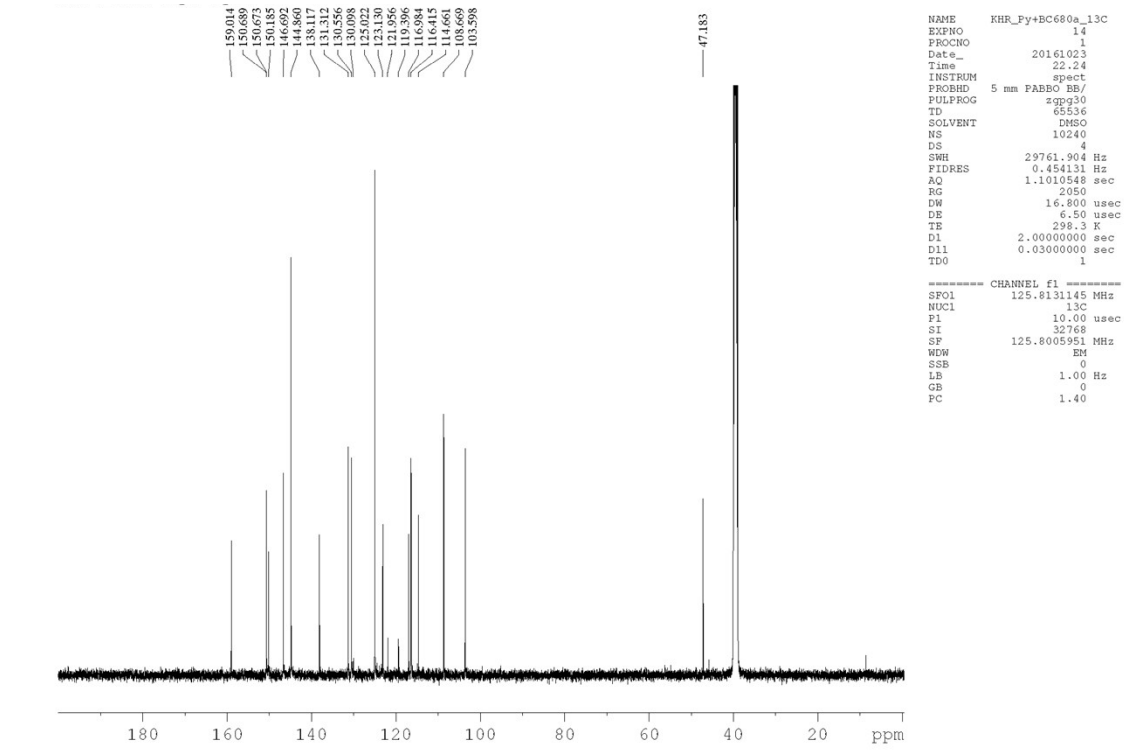


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SOLVENT                      DMSO
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DS                               4
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FIDRES                   0.454131 Hz
AQ                       1.1010548 sec
RG                            2050
DW                         16.800 usec
DE                          6.50 usec
TE                         298.3 K
D1                    2.00000000 sec
D11                   0.03000000 sec
TD0                              1

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P1                      10.00 usec
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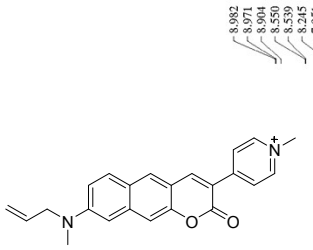
Py$^+$BC680b

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SOLVENT                DMSO
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RG                      101
DW                 40.533 usec
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TE                  306.2 K
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PC                     1.00
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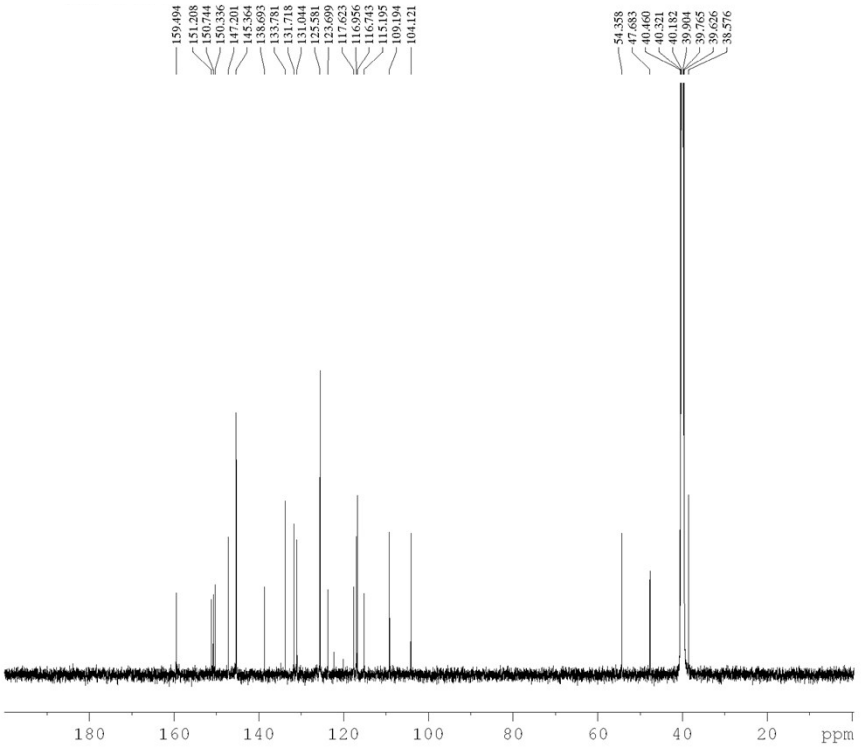


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TE                  305.1 K
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TD0                       1

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GB                        0
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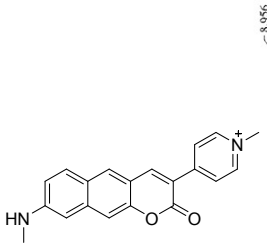
Py$^+$BC660a

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TD                    65536
SOLVENT                DMSO
NS                       64
DS                        2
SWH                6188.119 Hz
FIDRES                0.094423 Hz
AQ                 5.2953587 sec
RG                      287
DW                    80.800 usec
DE                     6.50 usec
TE                    295.6 K
D1                 2.00000000 sec
TD0                       1

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NUC1                     1H
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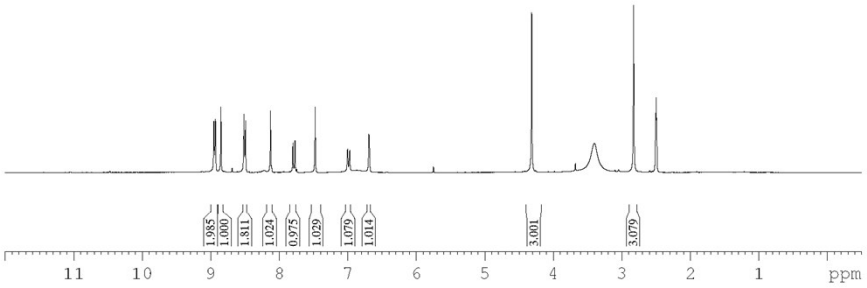

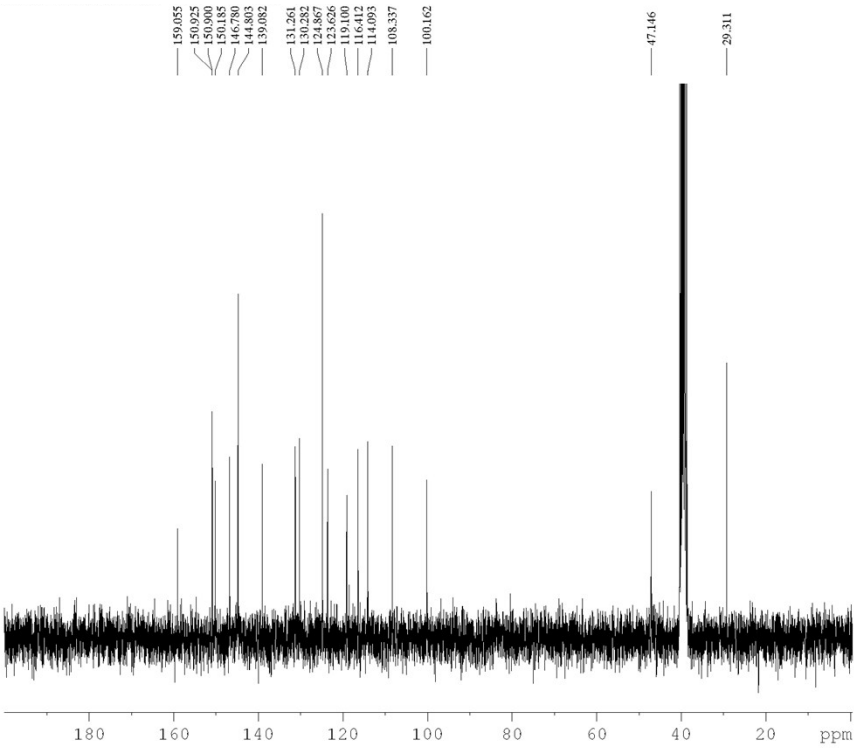


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TE                    295.8 K
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D11                0.03000000 sec
TD0                       1

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SSB                       0
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PC                     1.40
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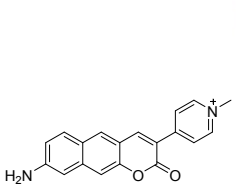
Py⁺BC660b

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TD                   65536
SOLVENT               DMSO
NS                      64
DS                       2
SWH               6188.119 Hz
FIDRES            0.094423 Hz
AQ                5.2953587 sec
RG                     406
DW                  80.800 usec
DE                   9.00 usec
TE                  295.2 K
D1                2.00000000 sec
TD0                      1

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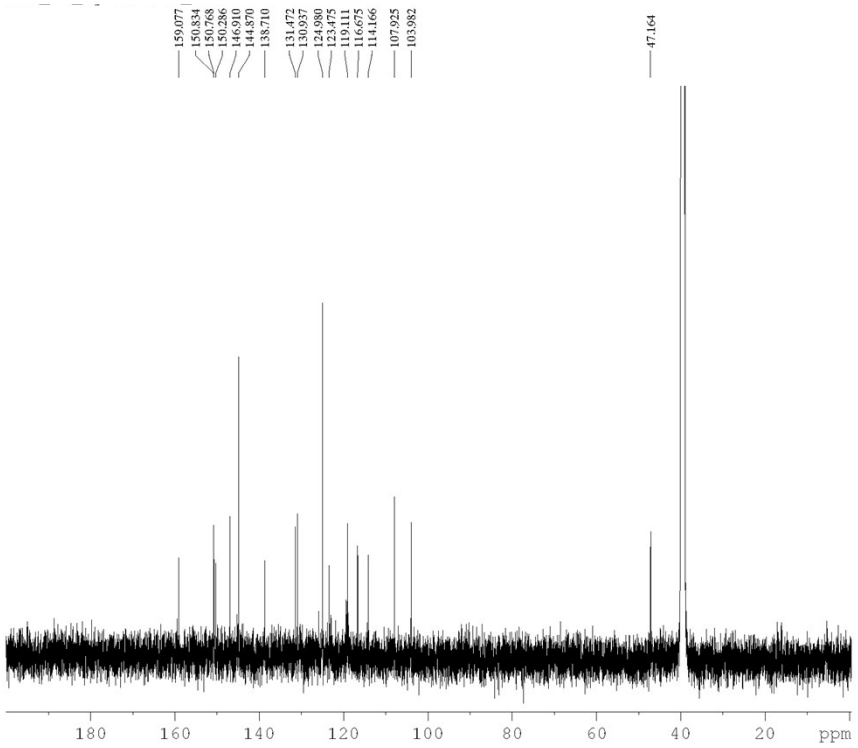


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TE                  298.2 K
D1                2.00000000 sec
D11               0.03000000 sec
TD0                      1

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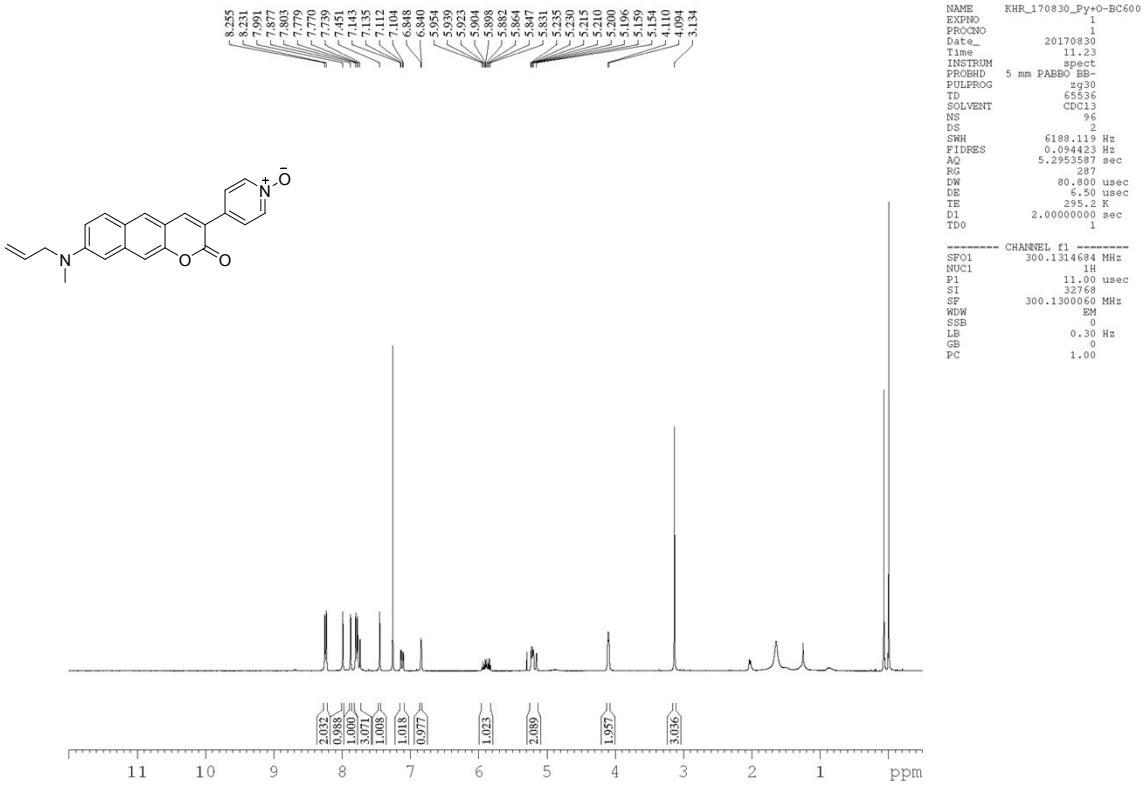
Py^{+}O^{-}BC600

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TD                           65536
SOLVENT                      CDCl3
NS                              96
DS                               2
SWH                     6188.119 Hz
FIDRES                  0.094423 Hz
AQ                      5.2953587 sec
RG                             287
DW                         80.800 usec
DE                          6.50 usec
TE                          295.2 K
D1                     2.00000000 sec
TD0                              1

======== CHANNEL f1 ========
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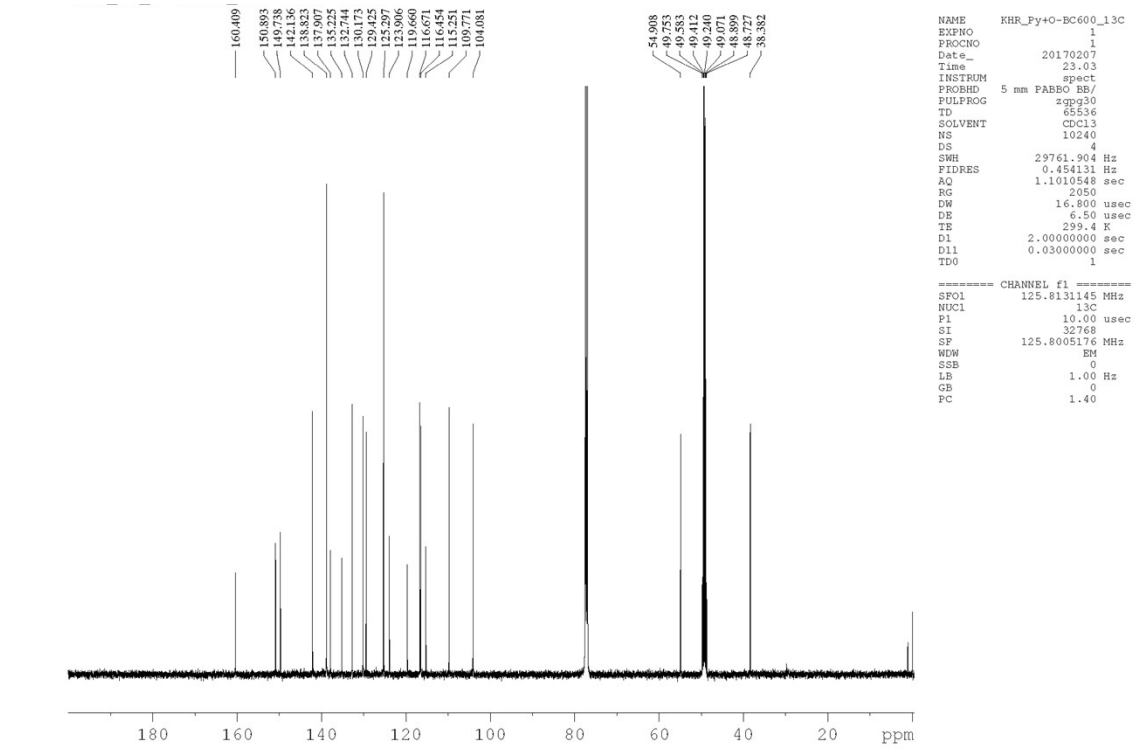
P1                         11.00 usec
SI                          32768
SP                  300.1300060 MHz
WDW                            EM
SSB                             0
LB                          0.30 Hz
GB                              0
PC                           1.00
```

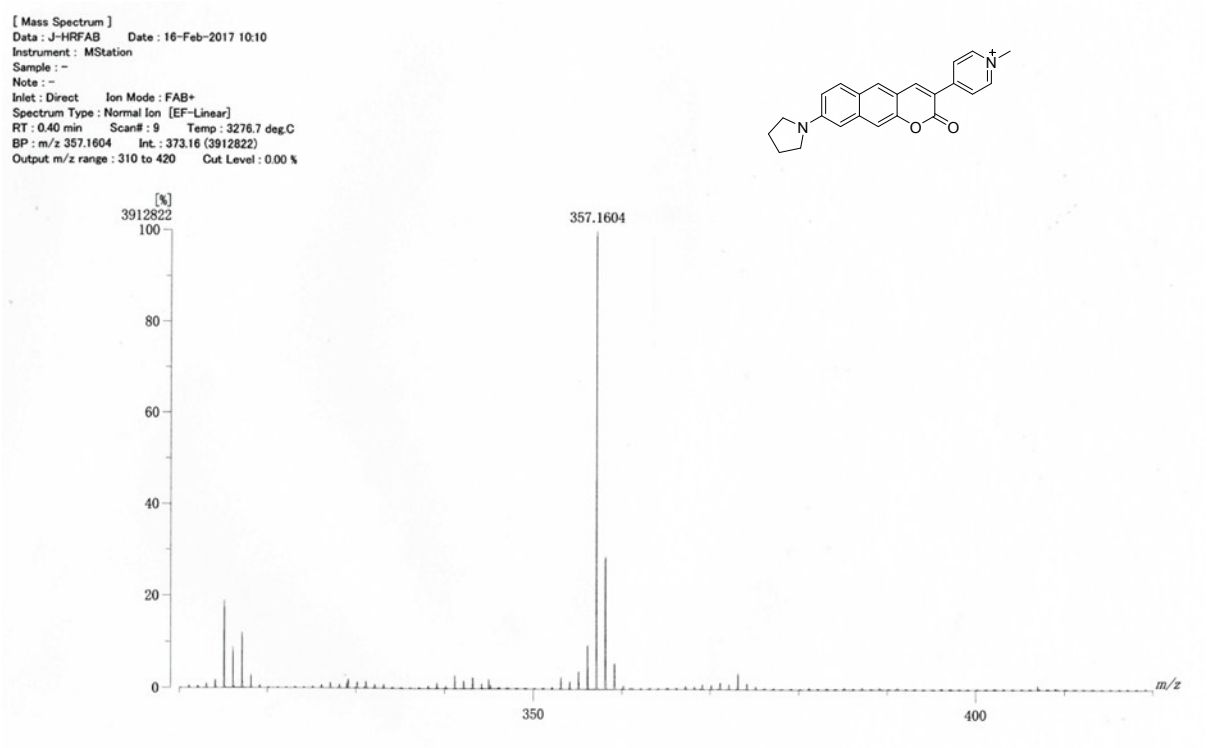



```
NAME          KHR_Py+O-BC600_13C
EXPNO                             1
PROCNO                            1
Date_                      20170207
Time                          23.03
INSTRUM                       spect
PROBHD       5 mm PABBO BB/
PULPROG                    zgpg30
TD                           65536
SOLVENT                      CDCl3
NS                           10240
DS                               4
SWH                    29761.904 Hz
FIDRES                  0.454131 Hz
AQ                      1.1010548 sec
RG                            2050
DW                         16.800 usec
DE                          6.50 usec
TE                          299.4 K
D1                     2.00000000 sec
D11                    0.03000000 sec
TD0                              1

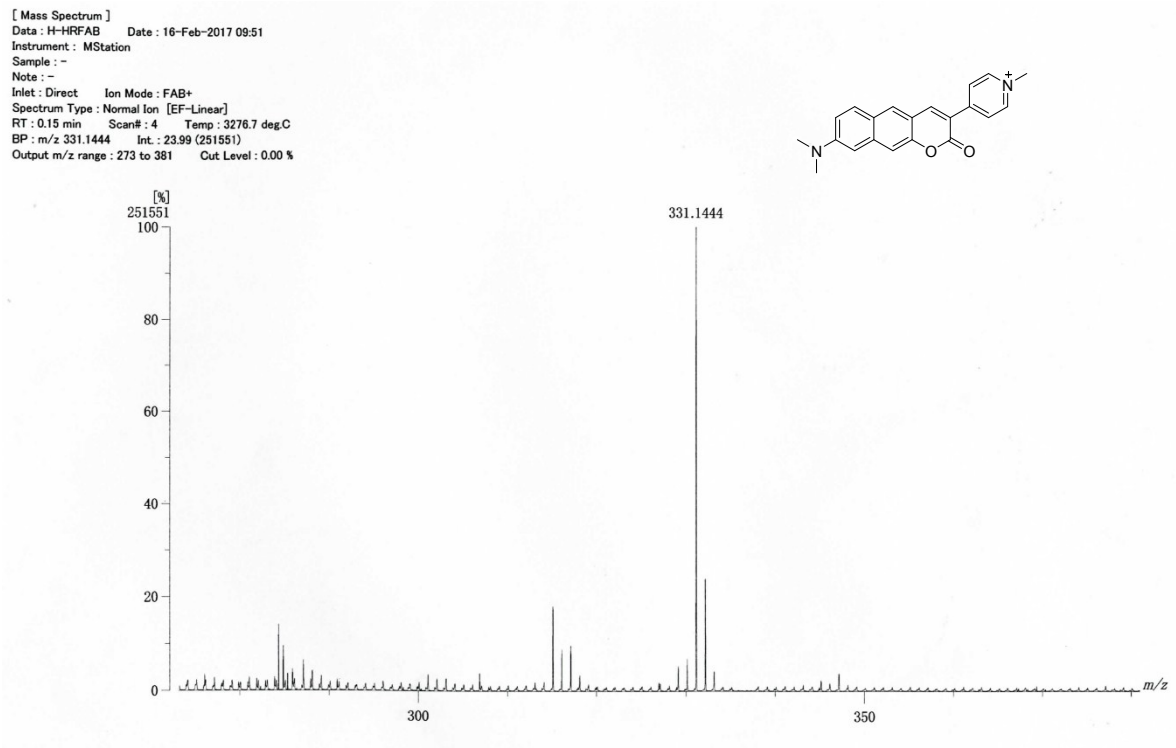
======== CHANNEL f1 ========
SFO1                125.8131145 MHz
NUC1                           13C
P1                         10.00 usec
SI                          32768
SF                  125.8005176 MHz
WDW                            EM
SSB                             0
LB                          1.00 Hz
GB                              0
PC                           1.40
```

7. HRMS Spectra

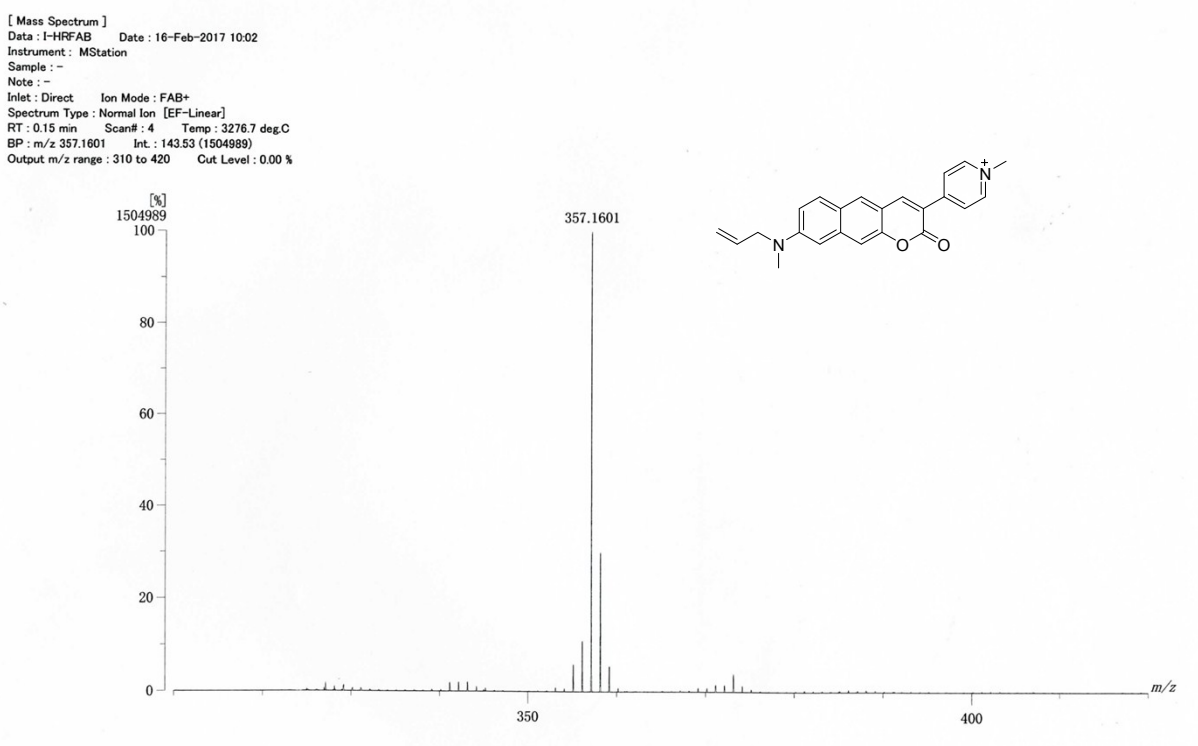
Py⁺BC690



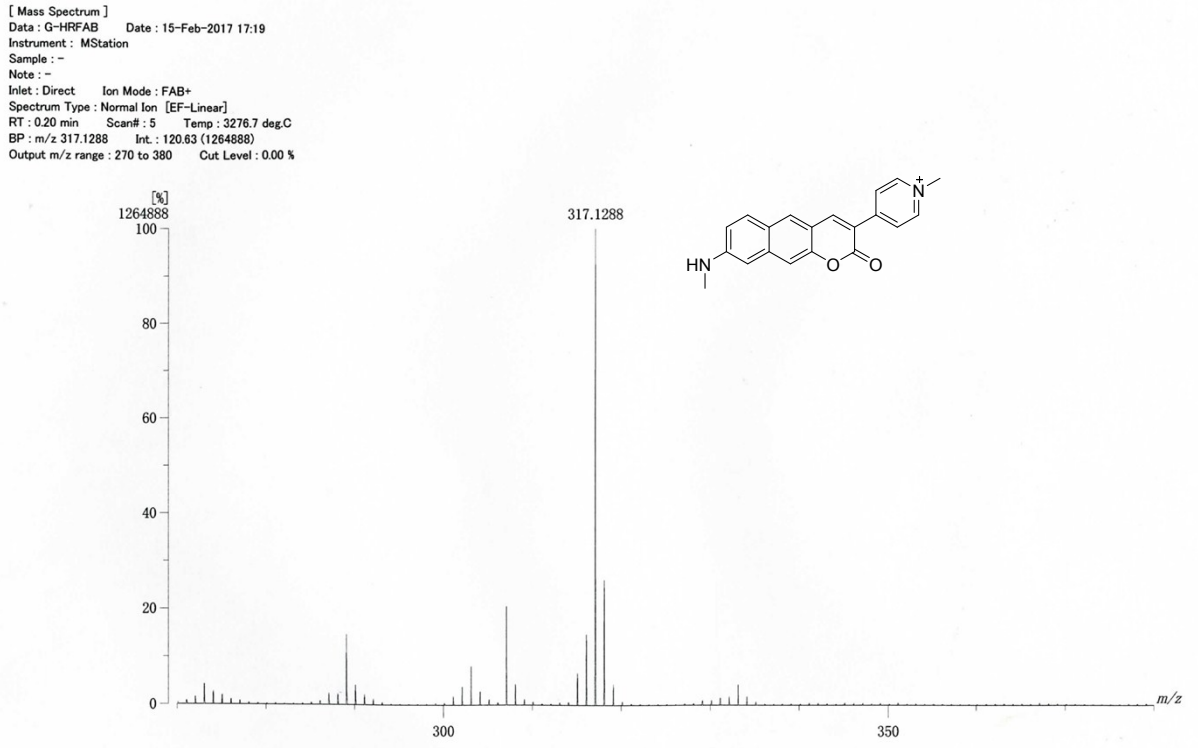
Py⁺BC680a



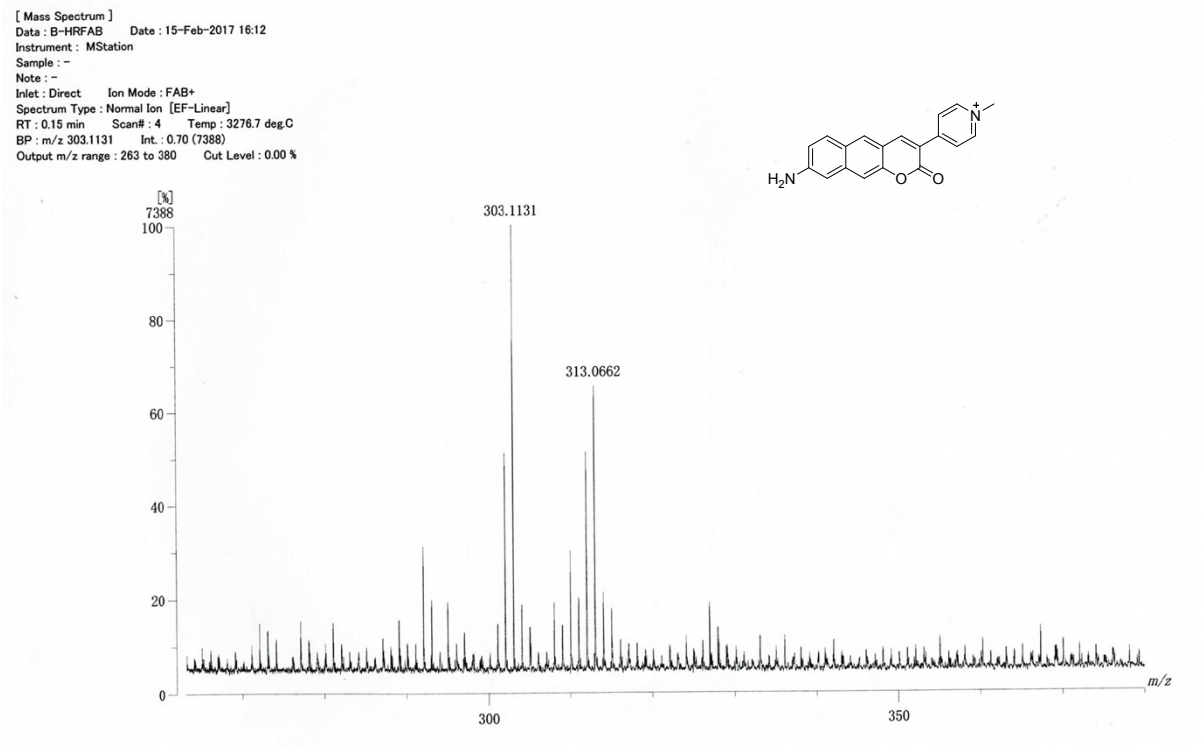
Py⁺BC680b



Py⁺BC660a



Py^{+}BC660b



Py^{+}O^{-}BC600