Supporting Information

A Concise Synthesis of the Pennsylvania Green Fluorophore and Labeling of Intracellular Targets with O^6-Benzylguanine Derivatives

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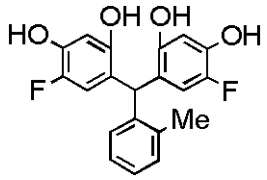
Experimental section

General. Chemical reagents were obtained from Acros, Aldrich, or TCI America. Solvents were from EM Science. Tissue culture reagents were obtained from Valley Biomedical Inc., Mediatech, BD Biosciences, and Gibco. Commercial grade reagents were used without further purification unless otherwise noted. Anhydrous solvents were obtained after passage through a drying column of a solvent purification system from GlassContour (Laguna Beach, CA). All reactions were performed under an atmosphere of dry argon or nitrogen. Reactions were monitored by analytical thin-layer chromatography on plates coated with 0.25 mm silica gel 60 F_{254} (EM Science). TLC plates were visualized by UV irradiation (254 nm and 365 nm) or stained with a solution of phosphomolybdic acid and sulfuric acid in ethanol (1:1:20). Flash column chromatography employed ICN SiliTech Silica Gel (32-63 μm). Purification by preparative reverse-phase HPLC employed an Agilent 1100 preparative pump / gradient extension instrument equipped with a Hamilton PRP-1 (polystyrene-divinylbenzene) column (7 μm particle size, 21.5 mm x 25 cm). The HPLC flow rate was increased from 15 mL/min (t = 0 min) to 25 mL/min (t = 2 min) and maintained at 25 mL/min for the remainder of the run unless otherwise noted. Melting points were measured with a Thomas Hoover capillary melting point apparatus and are uncorrected. Infrared spectra were obtained with a Perkin Elmer 1600 Series FTIR. NMR spectra were obtained with Bruker CDPX-300, DPX-300, AMX-360, or DRX-400 instruments with chemical shifts reported in parts per million (ppm, δ) referenced to either $CDCl_3$ (^{1}H 7.27 ppm; ^{13}C 77.23 ppm) or CD_3OD (^{1}H, 4.78 ppm; ^{13}C, 49.0 ppm). High-resolution mass spectra were obtained from the Pennsylvania State University Mass Spectrometry Facility (ESI and CI). Peaks are reported as *m*/*z*.

Labeling of AGT fusion proteins expressed in Chinese hamster ovary (CHO) cells. The CHO-K1 cell line (ATCC# CCL-61) was cultivated in F-12K medium supplemented with 10% FBS, 100 units/mL penicillin and 0.1 mg/mL streptomycin. Cells were propagated in a humidified 5% CO_2 incubator at 37 °C. The day before transient transfection, exponentially growing cells (1 x 10^5 cells/well) were seeded on collagen-coated glass coverslips in a 6-well plate. The cells were transfected using FuGene (Roche), according to the manufacturer's instructions, using a plasmid / FuGene ratio of 1:3 for pSEMXT-26M-CaaX and 2:3 for pSEMXT-H2B-26M (AGT expression vectors provided by Covalys Biosciences). The cells were incubated for an additional 24 h to allow expression of AGT fusion proteins, cells were washed twice with growth media (2 mL) and compounds **4** or **5** (5 μM) were added for 1 h at 37 °C. Cells were rinsed twice with fresh growth media (2 mL) followed by incubation for an additional 30 min in the presence of fresh media (2 mL) to allow diffusion of any unbound fluorophores from cells. Cells were rinsed again with media (2 mL) before imaging of live cells by confocal laser scanning and differential interference contrast (DIC) microscopy.

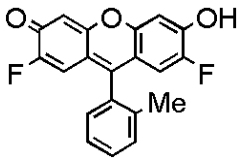
Microscopy. An inverted Zeiss LSM 5 Pascal confocal laser-scanning microscope fitted with a Plan Apochromat oil-immersion objective (63 X) was employed. Fluorophores were excited with the 488 spectral line of an argon ion laser (25 mW, 1% laser power) and emitted photons were collected through a 505 nm LP filter. For cellular imaging, coverslips bearing living cells were carefully removed from the 6-well plate and mounted on microscope slides bearing a press-to-seal silicone isolator filled with cell culture medium to preserve viability during the course of the experiment.

Synthetic procedures and compound characterization data



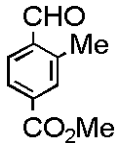
8

4,4'-[(2-Methylphenyl)methylene]bis(6-fluorobenzene-1,3-diol) (8)
4-Fluororesorcinol (**7**, 100 mg, 0.78 mmol, prepared as described in Yang, J. J. et al. *Heteroatom Chem*. **1998**, *9*, 229-239) and 2-methylbenzaldehyde (**6**, 43 μL, 0.37 mmol) were dissolved in CH_2Cl_2 / ether (1:1, 4 mL). Methanesulfonic acid (400 μL) was added and the reaction was stirred at 23 °C for 16 h. The solution was diluted with ether (5 mL), poured into saturated aqueous $NaHCO_3$ (10 mL) and extracted with ethyl acetate (2 x 20 mL). The combined organic extracts were rinsed with saturated aqueous sodium chloride (50 mL) and dried over anhydrous sodium sulfate. Flash column chromatography (CH_2Cl_2 / MeOH, 9:1) afforded **8** as a tan solid (109 mg, 83%). m.p. 130-132 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.90 (bs, 4H), 7.10-7.03 (m, 3H), 6.70 (m, 1H), 6.45 (d, J = 7.9 Hz, 2H), 6.21 (d, J = 12.4 Hz, 2H), 5.28 (s, 1H), 2.08 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) 150.8, 145.7, 143.1, 142.9, 142.77, 142.74, 135.9, 130.0, 127.6, 125.8, 125.4, 120.1, 120.0, 116.2, 115.9, 104.5, 19.0; IR (film) ν_{max} 3320, 2925 cm^{-1}; HRMS (TOF ES-) m/z 357.0932 (MH^-, $C_{20}H_{16}O_4F_2$ requires 357.0938).



2

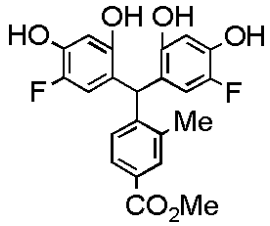
2,7-Difluoro-6-hydroxy-9-(2-methylphenyl)-3*H*-xanthen-3-one (2, Pennsylvania Green)
4,4'-[(2-Methylphenyl)methylene]bis(6-fluorobenzene-1,3-diol) (**8**, 50 mg, 0.14 mmol) and *p*-TsOH (212 mg, 1.12 mmol) were dissolved in dry toluene (14 mL, 0.01 M) and heated to reflux (111 °C) in a Dean Stark apparatus for 24 h. After cooling to 23 °C, the solution was neutralized with aqueous KOH (2 M) to pH = 7. The solution was acidified by dropwise addition of concentrated HCl to pH = 3. The precipitate was extracted into ethyl acetate (3 x 25 mL) and washed with cold aqueous HCl (1 M, 25 mL) followed by saturated aqueous sodium chloride (25 mL). The combined organic extracts were dried over anhydrous sodium sulfate and solvent was removed *in vacuo*. Flash column chromatography (AcOH / MeOH / CH_2Cl_2, 0.1:1:8.9) afforded **2** (18 mg, 37% yield) as an orange solid. Spectral data was identical to that reported in Mottram, L. F. et al. *Org. Lett*. **2006**, *8*, 581-584.



10

Methyl 4-formyl-3-methylbenzoate (10)

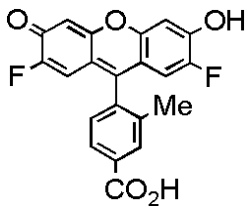
Methyl 4-iodo-3-methylbenzoate (**9**, 4.0 g, 14.6 mmol, TCI America) was dissolved in dry THF (100 mL) and purged with dry argon. The solution was cooled to -15 °C, *i*-PrMgCl (29.1 mL of 2.0 M in hexanes, 58.2 mmol) was added, and the reaction was stirred at -15 °C for 2 h. Dry DMF (5.65 mL, 72.8 mmol) was added and the reaction was allowed to warm to 23 °C over 1 h. The reaction was quenched with aqueous HCl (1 M, 100 mL) and extracted with ethyl acetate (3 x 100 mL). The combined organic extracts were rinsed with saturated aqueous sodium chloride (100 mL) and dried over anhydrous sodium sulfate. Flash column chromatography (hexane / ethyl acetate, 9:1) afforded **10** (2.44 g, 95% yield) as an oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.10 (s, 1H), 7.72 (d, *J* = 8.1 Hz, 1H), 7.66 (s 1H), 7.72 (d, *J* = 7.9 Hz, 1H), 3.74 (s, 3H), 2.47 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) 191.4, 165.4, 139.9, 136.4, 133.4, 132.2, 130.9, 126.7, 51.9, 18.7; IR (film) ν_{max} 2927, 2848, 2740, 1724, 1690 cm^{-1}; HRMS (TOF ESI+) *m/z* 179.0716 (MH^+, $C_9H_{10}O_3$ requires 179.0708).



11

Methyl 4-[bis(5-fluoro-2,4-dihydroxyphenyl)methyl]-3-methylbenzoate (11)

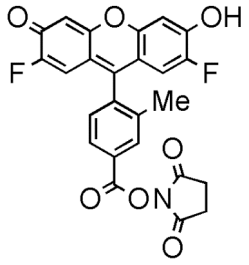
4-Fluororesorcinol (1.0 g, 7.8 mmol, prepared as described in Yang, J. J. et al. *Heteroatom Chem*. **1998**, *9*, 229-239) and methyl 4-formyl-3-methylbenzoate (**10**, 658 mg, 3.7 mmol) were dissolved in dry CH_2Cl_2 / dry Et_2O (1:1, 40 mL). Distilled methanesulfonic acid (4.0 mL) was added and the reaction was stirred at 23 °C for 6 h. The solution was diluted with ether (20 mL) and poured into saturated aqueous $NaHCO_3$ (100 mL). Concentrated aqueous HCl was added dropwise until pH = 3. The solution was extracted with ethyl acetate (3 x 20 mL) and dried over anhydrous sodium sulfate. Flash column chromatography (MeOH / CH_2Cl_2, 1:9) afforded **11** (1.34 g, 88% yield) as a tan solid. m.p. 132-136 °C; ^{1}H NMR (300 MHz, CD_3OD) δ 8.18 (s, 1H), 8.12 (d, *J* = 7.9 Hz, 1H), 7.30 (d, *J* = 8.0 Hz, 1H), 6.85 (d, *J* = 7.6 Hz, 2H), 6.71 (d, *J* = 11.2 Hz, 2H), 6.37 (s, 1H), 4.25 (s, 3H), 2.55 (s, 3H); ^{13}C NMR (75 MHz, CD_3OD) 168.4, 150.7, 148.6, 147.1, 144.0, 143.2, 143.0, 137.5, 131.5, 128.2, 127.7, 127.0, 120.6, 120.5, 116.8, 116.5, 104.9, 39.8, 19.2; IR (film) ν_{max} 3412, 2960, 1689 cm^{-1}; HRMS (TOF AP+) *m/z* 417.1156 (MH^+, $C_{22}H_{18}O_6F_2$ requires 417.1150).



3

4-(2,7-Difluoro-6-hydroxy-3-oxo-3H-xanthen-9-yl)-3-methyl-benzoic acid (**3**, 4-carboxy-Pennsylvania Green)

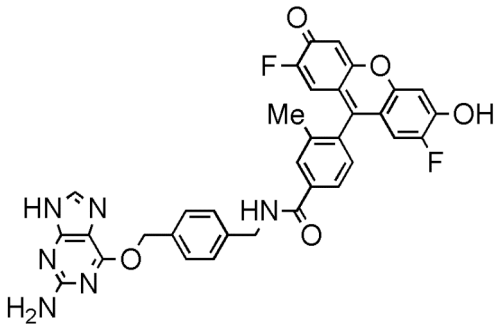
Methyl 4-[bis(5-fluoro-2,4-dihydroxyphenyl)methyl]-3-methylbenzoate (**11**) (100 mg, 0.24 mmol) and *p*-TsOH (0.91 g, 4.8 mmol) were dissolved in dry toluene (24 mL) and heated to reflux (111 °C) in a Dean Stark apparatus for 24 h. The reaction was cooled to 23 °C, aqueous KOH (2 M) was added until pH = 10, and the reaction was stirred at 23 °C for 1 h. After hydrolysis of the methyl ester was complete as evidenced by TLC, concentrated aqueous HCl was added dropwise until pH = 3. This solution was extracted with ethyl acetate (3 x 25 mL), and the organic layers were washed with ice-cold HCl (1 M, 25 mL) followed by saturated aqueous sodium chloride (25 mL). The combined organic extracts were dried over anhydrous sodium sulfate and solvent was removed *in vacuo*. Flash column chromatography (AcOH / MeOH / CH_2Cl_2, 0.1:1:8.9) afforded **3** (34.8 mg, 38% yield) as an orange solid. Spectral data was identical to that reported in Mottram, L. F. et al. *Org. Lett.* **2006**, *8*, 581-584.



12

1-{[4-(2,7-Difluoro-6-hydroxy-3-oxo-3*H*-xanthen-9-yl)-3-methylbenzoyl]oxy}pyrrolidine-2,5-dione (12, 4-carboxy-Pennsylvania Green NHS ester**)**

4-Carboxy-Pennsylvania Green (**3**, 49 mg, 0.13 mmol), DCC (32 mg, 0.15 mmol) and *N*-hydroxysuccinimide (30 mg, 0.26 mmol) were dissolved in dry THF (20 mL). After stirring for 4 h at 23 °C, the urea byproduct was removed by filtration. The solution was concentrated *in vacuo*. Flash column chromatography (MeOH / CH_2Cl_2, 1:9) afforded **12** (55 mg, 89% yield) as an orange solid. m.p. 225-230 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.25 (s, 1H), 8.13 (d, J = 7.7 Hz, 1H), 7.55 (d, J = 7.9 Hz, 1H), 6.79 (d, J = 6.8 Hz, 2H), 6.73 (d, J = 11.1 Hz, 2H), 2.92 (s, 4H), 2.12 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) 172.8, 170.3, 161.6, 1504.1, 153.5, 150.7, 148.2, 138.9, 137.7, 132.1, 130.2, 127.9, 125.7, 113.5, 113.4, 111.4, 111.1, 105.1, 26.6, 18.9; IR (film) ν_{max} 3413, 2923, 1735, 1647, 1192 cm^{-1}; HRMS (TOF ES-) 478.0758 *m/z* (MH^-, $C_{25}H_{14}O_7F_2N$ requires 478.0738). The ^{1}H NMR spectrum (400 MHz) of **12** in CD_3OD / D_2O / NaOD (99:0.7:0.3) is shown in Figure S3.



4

***N*-(4-{[(2-Amino-9*H*-purin-6-yl)oxy]methyl}benzyl)-4-(2,7-difluoro-3,6-dihydroxy-9*H*-xanthen-9-yl)-3-methylbenzamide (4)**

A solution of 1-{[4-(2,7-difluoro-6-hydroxy-3-oxo-3H-xanthen-9-yl)-3-methylbenzoyl]oxy}pyrrolidine-2,5-dione (**12**, 10 mg, 0.02 mmol) in DMF (1 mL) was added to O^6-[4-(aminomethyl)benzyl]guanine (**14**, 6.5 mg, 0.024 mmol, Covalys Biosciences) and triethylamine (14 μL, 0.1 mmol) in DMF (500 μL). The reaction was stirred at 23 °C for 16 h. The solvent was removed *in vacuo*. Purification by preparative reverse-phase HPLC employed a linear gradient of water / acetonitrile (99:1 to 1:99 over 30 min, 0.1% TFA) to afford **4** as a orange solid (10.0 mg, 83%). m.p. > 280 °C; ^{1}H NMR (300 MHz, CD_3OD) δ 8.25 (bs, 1H), 7.85 (s, 1H), 7.80 (d, J = 7.6 Hz, 1H), 7.45 (d, J = 7.8 Hz, 2H), 7.53 (d, J = 8.1 Hz, 2H), 7.27 (d, J = 7.9 Hz, 1H), 6.83 (d, J = 7.1 Hz, 2H), 6.64 (d, J = 10.8 Hz, 2H), 5.47 (s, 2H), 4.47 (s, 2H), 2.02 (s, 3H); IR (film) ν_{max} 3518, 3280, 2954, 1650 cm^{-1}; HRMS (TOF ESI+) m/z 657.1675 (MNa^+, $C_{34}H_{24}O_5F_2N_6$ requires 657.1644). Analysis of **4** by reverse phase HPLC is shown in Figure S1. The ^{1}H NMR spectrum (300 MHz) of **4** in CD_3OD is shown in Figure S4.

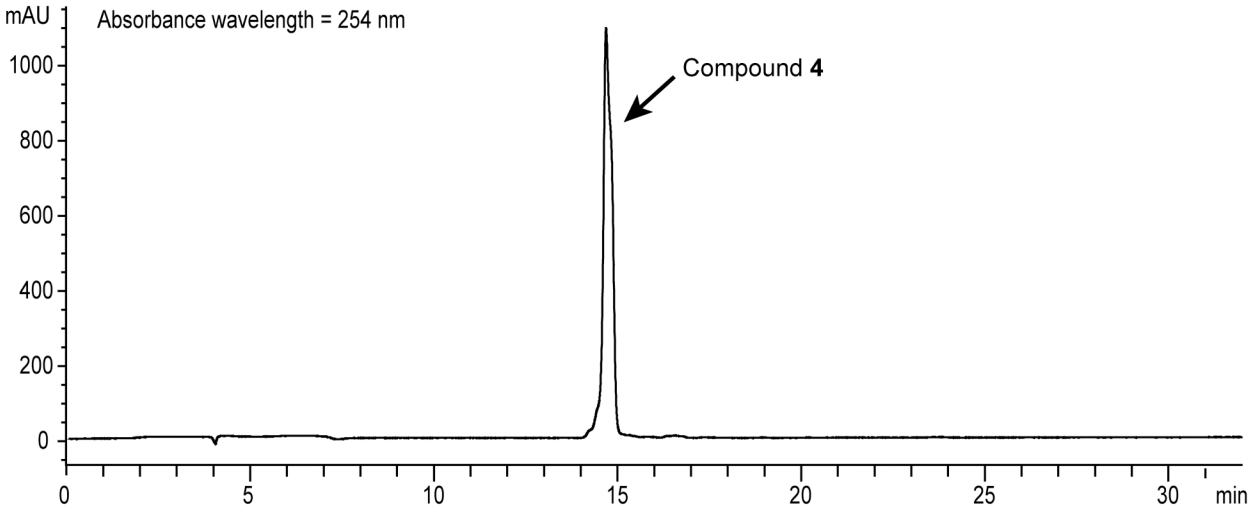
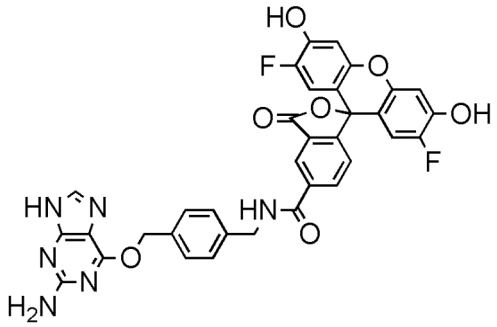


Figure S1. Analytical reverse-phase HPLC profile of compound **4** after purification by preparative reverse-phase HPLC (retention time = 14.7 min).



5

5-{[(4-{[(2-Amino-9*H*-purin-6-yl)oxy]methyl}benzyl)amino]carbonyl}-2-(2,7-difluor o-3,6-dihydroxy-9*H*-xanthen-9-yl)benzoate (5)

A solution of Oregon Green 488 carboxylic acid succinimidyl ester (**13**, 5 mg, 0.01 mmol, Invitrogen) in DMF (1 mL) was added to O^6-[4-(aminomethyl)benzyl]guanine (**14**, 3.2 mg, 0.012 mmol, Covalys Biosciences) and triethylamine (7 μL, 0.05 mmol) in DMF (500 μL). The reaction was stirred at 23 °C for 16 h. The solvent was removed *in vacuo*. Purification by preparative reverse-phase HPLC employed a linear gradient of water / acetonitrile (99:1 to 1:99 in 30 min, 0.1% TFA) to afford **5** as a orange solid (6.3 mg, 95%). m.p. > 280 °C; ^{1}H NMR (300 MHz, CD_3OD) δ 9.24 (t, J = 6.0 Hz, 1H), 8.37 (s, 1H), 8.14 (d, J = 8.0 Hz, 1H), 7.46 (d, J = 8.1 Hz, 2H), 7.36 (d, J = 7.9 Hz, 2H), 7.26 (d, J = 7.9 Hz, 1H), 6.74 (d, J = 7.5 Hz, 2H), 6.35 (d, J = 11.0 Hz, 2H), 5.55 (s, 2H), 4.51 (bs, 2H); HRMS (TOF ESI+) m/z 665.1596 (MNa^+, $C_{34}H_{22}O_7F_2N_6$ requires 665.1577). Analysis of **5** by reverse phase HPLC is shown in Figure S2. The ^{1}H NMR spectrum (300 MHz) of **5** in CD_3OD is shown in Figure S5.

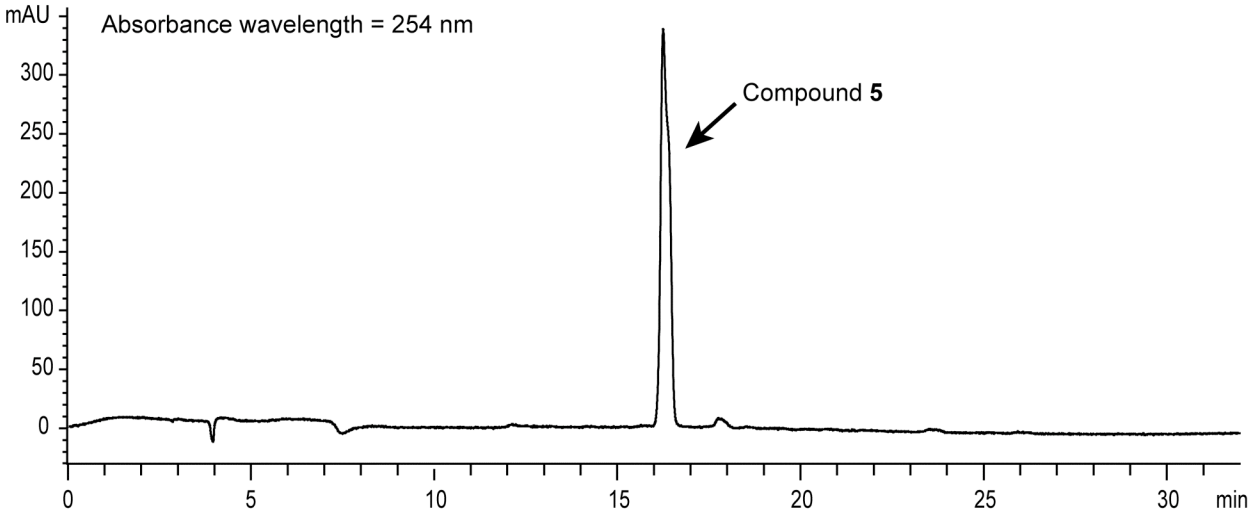


Figure S2. Analytical reverse-phase HPLC profile of compound **5** after purification by preparative reverse-phase HPLC (retention time = 16.3 min).

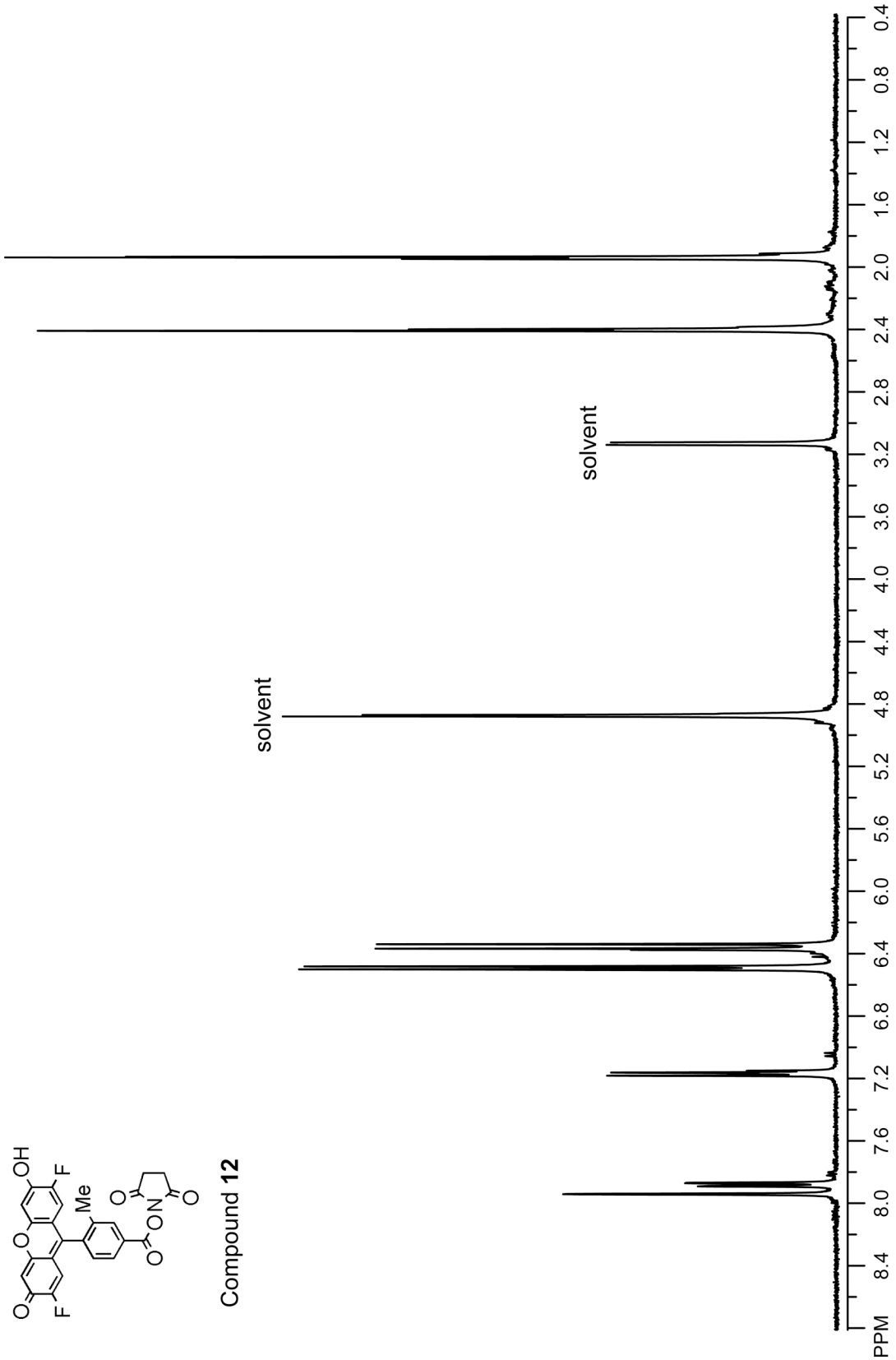


Figure S3. Proton NMR spectrum (400 MHz) of compound **12** in CD_3OD / D_2O / NaOD (99:0.7:0.3).

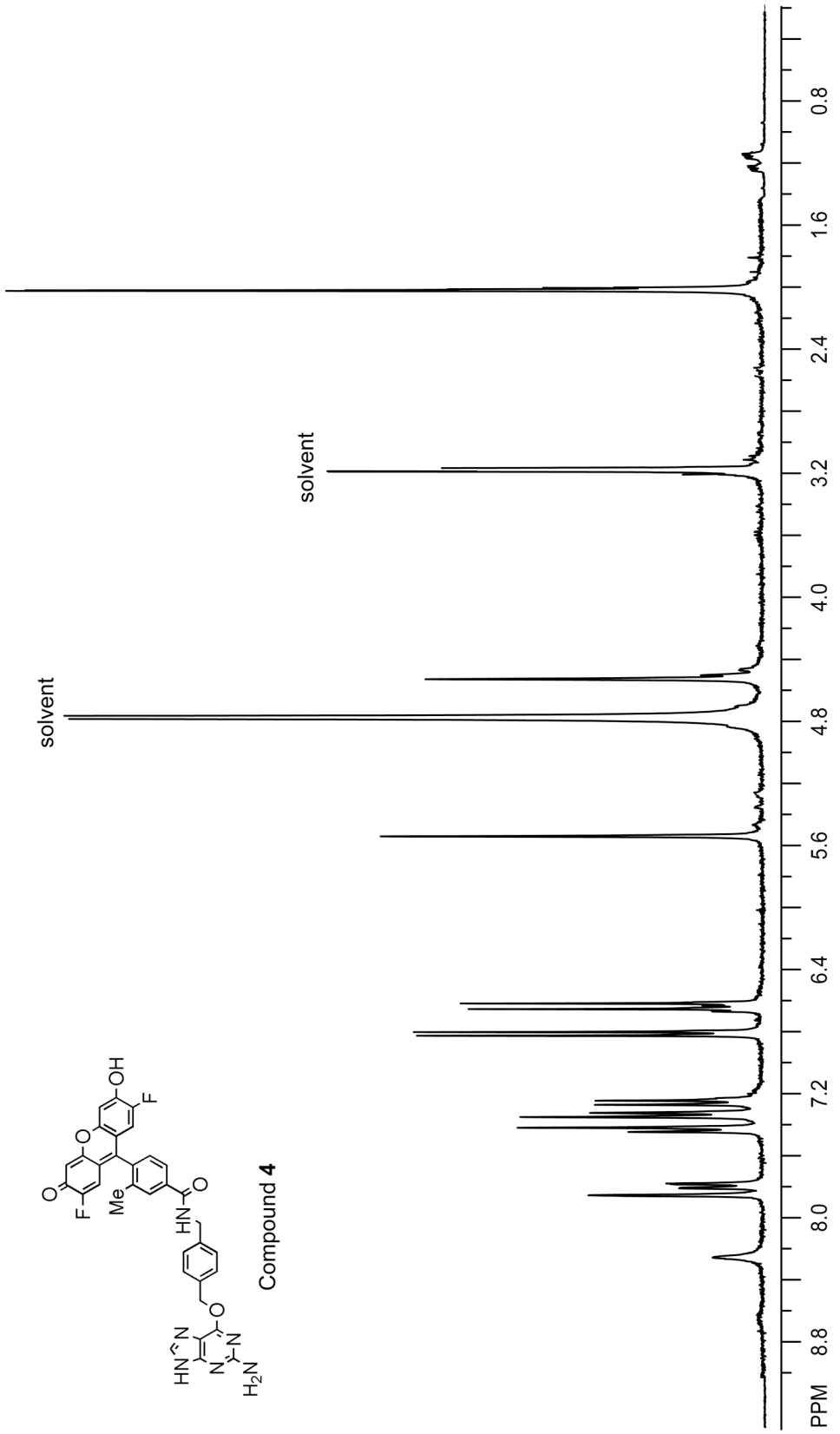


Figure S4. Proton NMR spectrum (300 MHz) of compound **4** in CD_3OD.

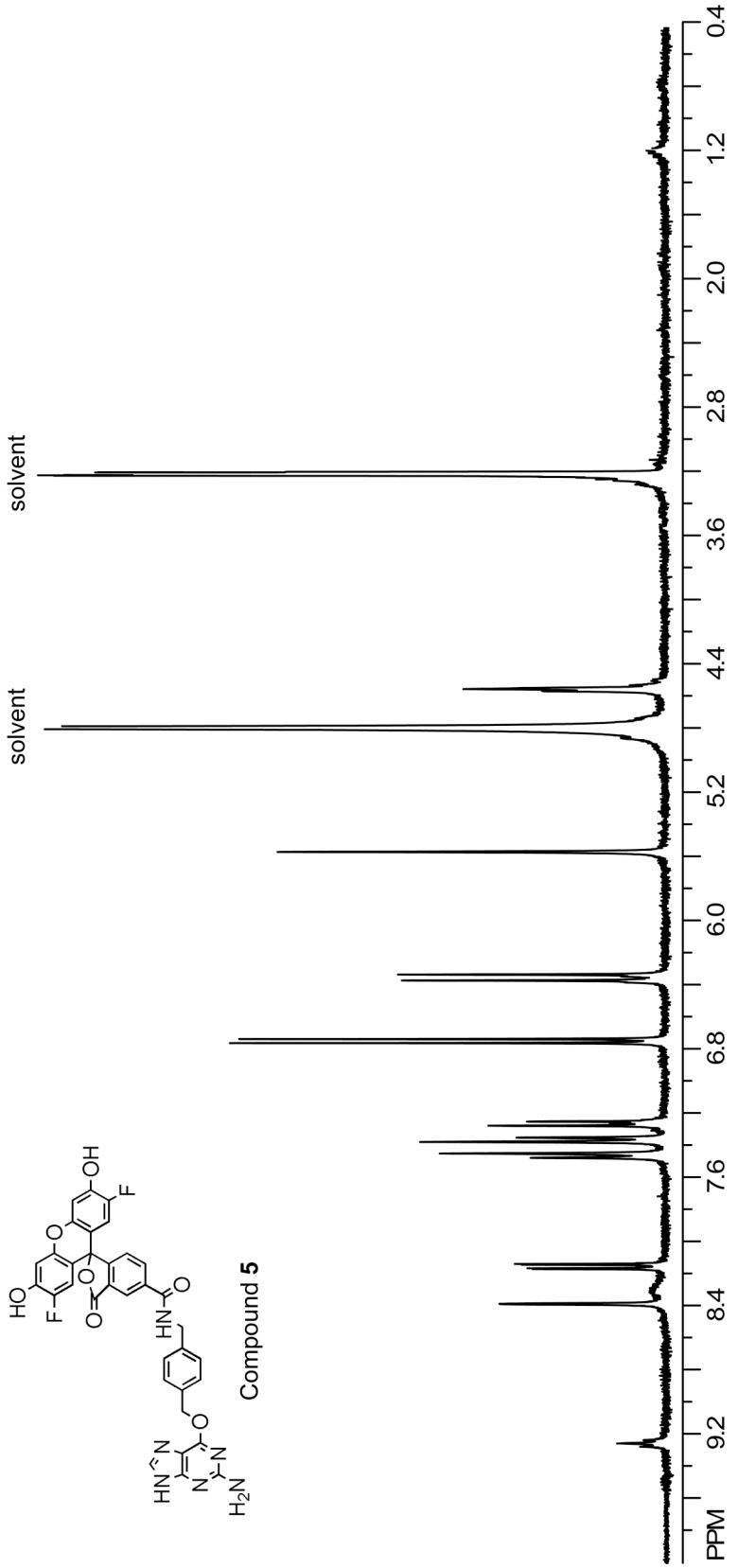


Figure S5. Proton NMR spectrum (300 MHz) of compound **5** in CD_3OD.

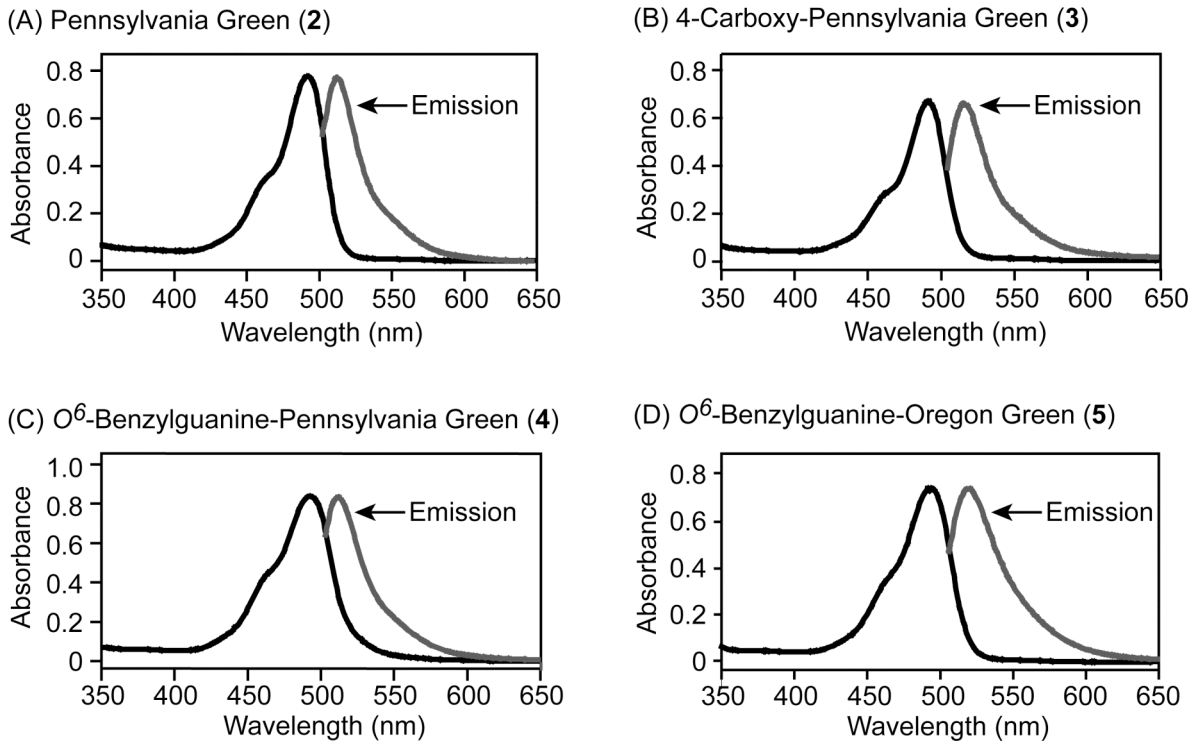


Figure S6. Absorbance and emission spectra of compounds **2**-**5** (10 μM (abs.) and 10 nM (em.)) in PBS (pH 7.4). Emission spectra are in arbitrary intensity units.

Determination of quantum yields. Absorbance and emission spectra of compounds **2**-**5** were obtained with a Cary 500 Scan UV-Vis-NIR spectrophotometer and PTi MD-5020 fluorimeter in phosphate-buffered saline (PBS, pH 7.4, Figure S6). The quantum yields of compounds **2**-**5** were determined by the method of Williams, A. T. et al. (*Analyst* **1983**, *108*, 1067-1071, Figure S7). Samples were excited at 494 nm and the integrated fluorescence emission (504 nm to 650 nm) was quantified (a 1 cm path length quartz cuvette was used). This was repeated with increasing concentrations of sample (2 nM to 20 nM). Fluorescein (Φ = 0.92) and 5-carboxyfluorescein (Φ = 0.92) at pH = 9.0 were used as standards. The integrated fluorescence emission at a given concentration was plotted against the maximum absorbance of the sample at that concentration as extrapolated from absorbance measurements at higher concentrations. Linear least squares fitting of the data (including a zero intercept) was used to calculate the slope, which is proportional to the quantum yield of the samples. Quantum yields were calculated with the following equation using the average of the values measured for fluorescein and 5-carboxyfluorescein as standards:

$$\Phi_x = \Phi_{st}(Grad_x/Grad_{st})(\eta^2_x/\eta^2_{st})$$

Φ_{st} represents the quantum yield of the standard, Φ_x represents the quantum yield of the unknown, *Grad* is the slope of the best linear fit, and η is the refractive index of the solvent used (the refractive index ratio of standard and sample was assumed to be unity in these measurements because similar buffers were used for each measurement).

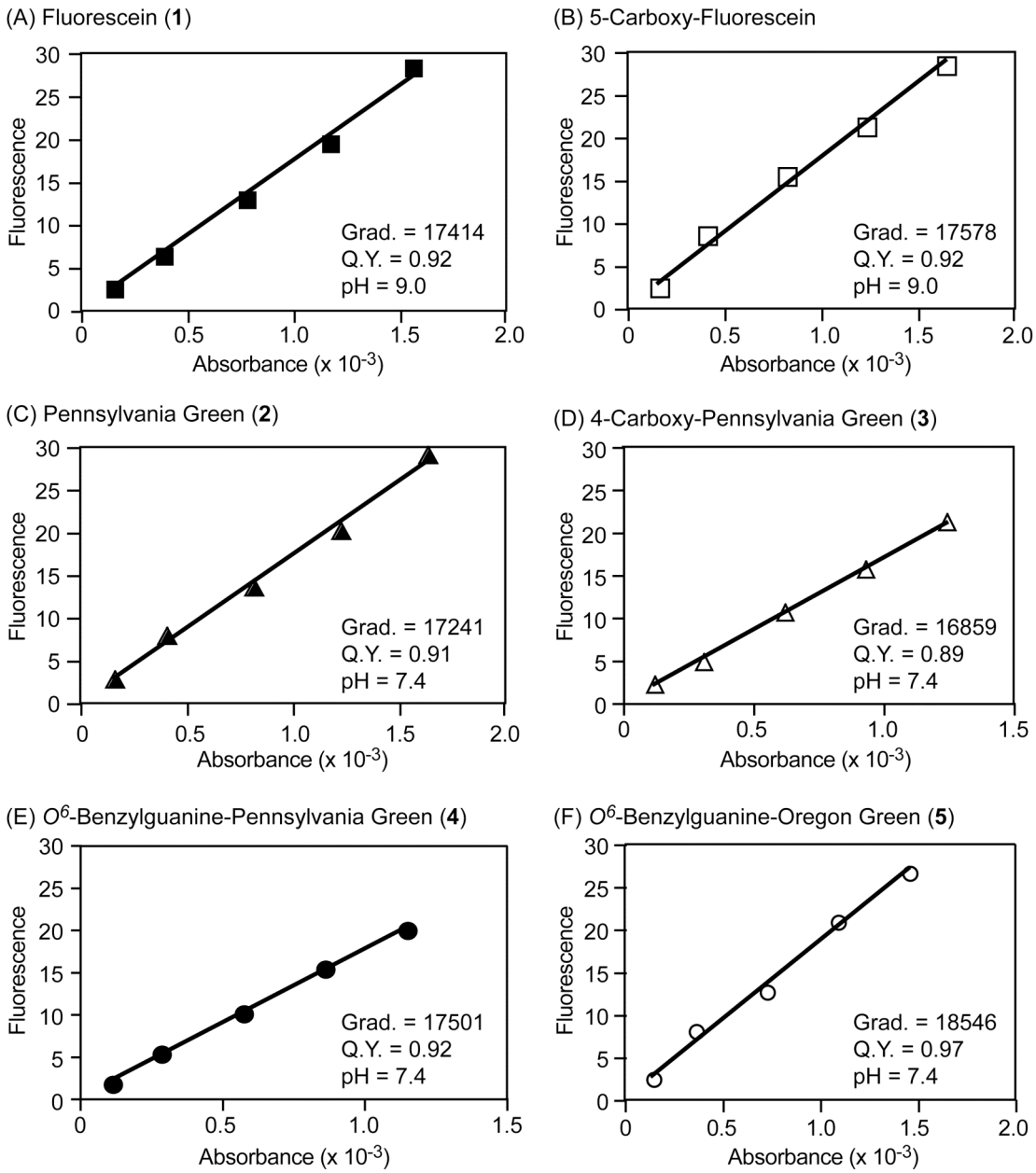


Figure S7. Fluorescence vs. absorbance plots used to determine the relative quantum yields of compounds **2**-**5** compared with fluorescein (**1**) and 5-carboxyfluorescein.

Determination of molar extinction coefficients (ε). Beer's Law plots of absorbance versus concentration were measured for compounds **2**-**5** in PBS (pH 7.4) with increasing concentrations of sample (0.1 μM to 2.0 μM). Absorbance values at 494 nm were determined for all samples. Linear least squares fitting of the data (including a zero intercept) was used to determine the slope, which corresponds to the extinction coefficient. Molar absorptivities (M^{-1} cm^{-1}) were calculated using the following equation:

Absorbance = ε [concentration (M)] L, where L = path length = 1 cm