

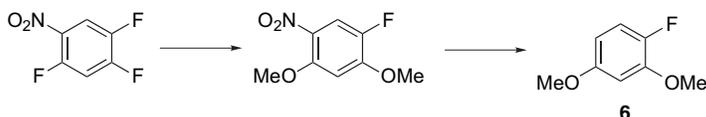
Supporting Information

Experimental

General Procedures. For all compounds, ^1H NMR, ^{13}C NMR and ^{19}F NMR spectra were recorded on a Bruker AC-300 (300 MHz) NMR spectrometer. Proton and carbon chemical shifts are reported relative to TMS using either TMS or the residue solvent signal as internal standard. Carbon NMRs are proton-decoupled, and coupling constants to fluorine are not reported. Fluorine chemical shifts are reported relative to CF_3COOH (-78.5 ppm) as an external standard. Electrospray mass spectra (ESI) data were obtained from the Laboratory for Macromolecular Analysis and Proteomics of the Albert Einstein College of Medicine.

All moisture and air sensitive experiments were performed under a positive pressure of N_2 or Ar in oven dried glassware. Reagent and anhydrous solvents were bought from Aldrich Chemical Company and used without further purification.

1,3-Dimethoxy-4-fluorobenzene (6). See Wei-Chuan Sun, Kyle R. Gee, Dieter H. Klaubert and Richard P. Haugland; *J. Org. Chem.* 1997, 62, 6469-6475.

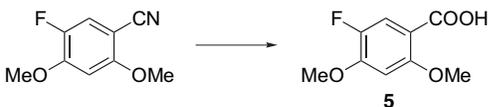


5-Fluoro-2,4-dimethoxy-benzonitrile. Sodium methoxide (25% wt in MeOH, 32 mL) was added dropwise to a solution of 2,4,5-trifluorobenzonitrile (10g, 63.7 mmol) in MeOH (160 mL) under nitrogen at 0 °C. The resulting reaction mixture was stirred at reflux for 48 h. The reaction was then quenched with 1 M citric acid and the MeOH was removed *in vacuo*. The residue was taken up in EtOAc, washed with brine, dried over Na_2SO_4 , concentrated *in vacuo* and recrystallized from EtOAc/hexane to furnish the desired product in 95% yield (11 g). ^1H NMR (300 MHz, CDCl_3) δ 7.25 (d, 1 H, $J = 10.2$ Hz), 6.52 (d, 1 H, $J = 6.8$ Hz), 3.97 (s, 3 H), 3.93 (s, 3 H); ^{13}C NMR (75 MHz, CDCl_3) δ 159.3, 159.2, 152.8, 152.6, 147.6, 144.4, 119.8, 119.5, 115.8, 115.7, 97.4, 97.3, 92.1, 92.0, 56.6, 56.5; ^{19}F NMR (282 MHz, CDCl_3) δ -143.4 (dd, 1 F); mass spectrum, calculated for $\text{C}_9\text{H}_8\text{FNO}_2$ (MH^+) 182.1, Found 182.1.

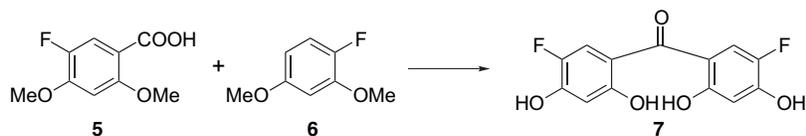


5-Fluoro-2,4-dimethoxy-benzoic acid (5). A solution of 3.1 g (17.2 mmol) of 2,4-dimethoxy-5-fluorobenzonitrile and 100 mL of 6N aqueous sodium hydroxide in 200 mL of methanol was stirred at reflux overnight, cooled to 0 °C, acidified to pH 1 - 2 with 6N aqueous hydrochloric acid, and partitioned between ethyl acetate and brine. The organic extract was dried over

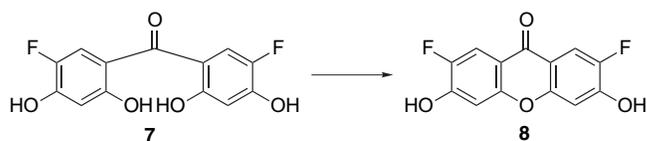
Na₂SO₄, and the solvents were removed *in vacuo* to give crude 2,4-dimethoxy-5-fluorobenzoic acid as a light-yellow solid. Recrystallization from ethyl acetate and hexane afforded 3.2 g (93%) of **5** as a white solid. ¹H NMR (300 MHz, DMSO-d₆) δ 12.4 (s, 1 H), 7.49 (d, 1 H, *J* = 12 Hz), 6.83 (d, 1 H, *J* = 7.2 Hz), 3.92 (s, 3 H), 3.84 (s, 3 H); ¹³C NMR (75 MHz, DMSO-d₆) δ 165.5, 165.5, 156.7, 151.1, 151.0, 146.3, 143.2, 117.9, 117.6, 111.2, 111.1, 99.3, 56.6, 56.4; ¹⁹F NMR (282 MHz, DMSO-d₆) δ -146.2 (dd, 1F); mass spectrum, calculated for C₉H₉FO₄ (MH⁺) 201.1, Found 200.9.



5,5'-difluoro-2,2',4,4'-tetrahydroxybenzophenone (7). 2,4-dimethoxy-5-fluorobenzoic acid **5** (5.4 g, 27 mmol) in dry CH₂Cl₂ (100 mL) was treated with 10.6 mL of oxalyl chloride under an Ar atmosphere and stirred at room temperature overnight. The excess reagent and solvent were removed under reduced pressure. The residue, 2,4-dimethoxy-5-fluorobenzoyl chloride, was redissolved in anhydrous CH₂Cl₂ (125 mL). 1,3-dimethoxy-4-fluorobenzene **6** (3.29 mL, 26 mmol) and AlCl₃ (10.8 g, 81 mmol) were added at 0 °C. After stirring at 0 °C for 1 h, the reaction mixture was allowed to warm to room temperature for 67 h, the mixture was then hydrolyzed with 1M HCl (300 mL) at 0 °C and extracted with EtOAc. The combined organic layers were washed with brine, dried over Na₂SO₄ and concentrated *in vacuo*. The crude product was purified by flash column chromatography, eluting with 30% - 40% EtOAc in hexane to yield **7** (3.3 g, 44%) as a yellow solid. ¹H NMR (300 MHz, MeOH-d₄) 7.14 (d, 2 H, *J* = 11.5 Hz), 6.46 (d, 2 H, *J* = 7.4 Hz); ¹³C NMR (75 MHz, MeOH-d₄) δ 199.1, 159.0, 152.9, 152.7, 147.7, 144.5, 119.2, 118.9, 114.2, 114.1, 106.1, 106.0; ¹⁹F NMR (282 MHz, MeOH-d₄) δ -149.8 (dd, 2F).

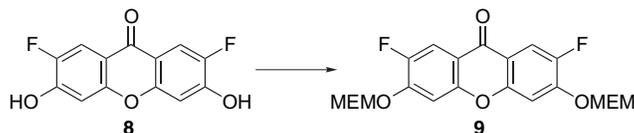


2,7-Difluoro-3,6-dihydroxy-xanthen-9-one (8). Compound **7** (0.75 g, 2.66 mmol) was heated in a sealed tube with H₂O (30 mL) at 200 - 220 °C for 2 - 3 h. On cooling, 2,7-difluoro-3,6-dihydroxyxanthone **8** precipitated out. The product was collected by filtration and washed with H₂O to give **8** (0.58 g, 83%) as a light yellow solid. ¹H NMR (300 MHz, DMSO-d₆) δ 11.4 (s, 2 H), 7.67 (d, 2 H, *J* = 10.9 Hz), 6.96 (d, 2 H, *J* = 7.1 Hz); ¹³C NMR (75 MHz, DMSO-d₆) δ 173.1, 153.1, 152.0, 151.8, 150.4, 147.2, 112.6, 112.5, 111.1, 110.8, 104.7, 104.6; ¹⁹F NMR (282 MHz, DMSO-d₆) δ -139.0 (dd, 2F); mass spectrum, calculated for C₁₃H₆F₂O₄ (MH⁺) 265.0, Found 264.9.

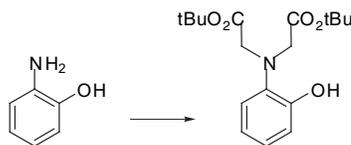


2,7-Difluoro-3,6-bis-(2-methoxyethoxymethoxy)-xanthen-9-one (9). A cloudy solution of **8** (2.4 g, 9.1 mmol) in dry THF (50 mL) was treated with sodium hydride (1.1 g, 45.8 mmol) at

0°C under an Ar atmosphere. The mixture was stirred at 0°C for 30 min and then 2-methoxyethoxymethyl chloride (5.2 mL, 45.8 mmol) was added. The mixture was stirred at 0°C for an additional 30 min and then warmed to room temperature overnight. The reaction mixture was cooled to 0°C, quenched with 1M citric acid (50mL), and then extracted with EtOAc. The combined organic layers were washed with brine, dried over Na₂SO₄ and concentrated *in vacuo*. The crude product was purified by flash column chromatography, eluting with CH₂Cl₂/hexane/EtOAc = 1:1:0.4 to yield 2.6 g (65%) of **9** as a white solid. R_f = 0.47 (in CH₂Cl₂/hexane/EtOAc = 1:1:0.4); ¹H NMR (300 MHz, CDCl₃) δ 7.91 (d, 2 H, J = 10.6 Hz), 7.28 (d, 2 H, J = 6.5 Hz), 5.46 (s, 4 H), 3.91 (m, 4 H), 3.60 (m, 4 H), 3.39 (s, 6 H); ¹³C NMR (75 MHz, CDCl₃) δ 174.4, 153.3, 153.2, 151.4, 151.0, 150.9, 148.2, 115.2, 115.1, 111.8, 111.6, 104.8, 94.2, 71.3, 68.6, 59.0; ¹⁹F NMR (282 MHz, CDCl₃) δ -137.0 (dd, 2 F); mass spectrum, calculated for C₂₁H₂₂F₂O₈ (MH⁺) 441.1, Found 441.0.

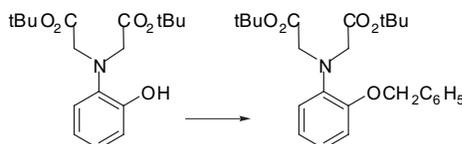


[*tert*-Butoxycarbonylmethyl-(2-hydroxy-phenyl)-amino]-acetic acid *tert*-butyl ester. A solution of 2-aminophenol (5.0 g, 45.8 mmol) and NaI (3.44 g, 23 mmol) in dry CH₃CN (150 mL) was treated with 1,8-bis(dimethylamino) naphthalene (21.6 g, 100.8 mmol) followed by *t*-butyl bromoacetate (14.2 mL, 96.2 mmol) under an Ar atmosphere and stirred at reflux overnight. After the mixture was cooled to room temperature, the precipitated salts were filtered off and washed with additional EtOAc. The combined organic layers were washed with brine, dried over Na₂SO₄ and concentrated *in vacuo*. The crude product was purified by flash column chromatography, eluting with CH₂Cl₂/hexane/EtOAc = 1:4:0.2 to yield 12.3 g (80%) of the desired product as a light yellow oil. R_f = 0.3 (in CH₂Cl₂/hexane/EtOAc = 1:4:0.2); ¹H NMR (300 MHz, CDCl₃) δ 7.29 (dd, 7.9 and 1.5 Hz, 1 H), 7.07 (m, 1 H), 6.94 (dd, 8.1 and 1.5 Hz, 1 H), 6.79 (m, 1 H), 5.30 (s, 1 H), 3.74 (s, 4 H), 1.46 (s, 18 H); ¹³C NMR (75 MHz, CDCl₃) δ 171.2, 153.6, 137.4, 127.3, 126.0, 119.9, 115.6, 81.9, 57.0, 28.0; mass spectrum, calculated for C₁₈H₂₇NO₅ (MH⁺) 338.2, Found 337.9.

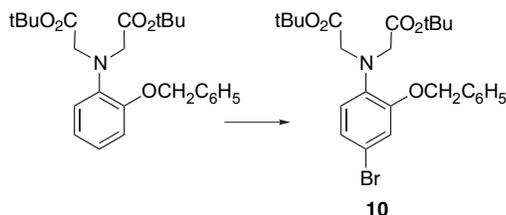


[(2-Benzyloxy-phenyl)-*tert*-butoxycarbonylmethyl-amino]-acetic acid *tert*-butyl ester. A solution of [*tert*-Butoxycarbonylmethyl-(2-hydroxy-phenyl)-amino]-acetic acid *tert*-butyl ester (7.4 g, 21.9 mmol) in dry THF (50 mL) was treated with sodium hydride (0.81 g, 33.8 mmol) at 0°C under an Ar atmosphere. After stirring at 0°C for 30 min, benzyl bromide (3.9 mL, 32.7 mmol) was added. The mixture was stirred at 0°C for another 30 min and then warmed to room temperature overnight. The reaction mixture was quenched with H₂O and extracted with EtOAc. The combined organic layers were washed with brine, dried over Na₂SO₄ and concentrated *in vacuo*. The crude product was purified by flash column chromatography, eluting with CH₂Cl₂/hexane/EtOAc = 1:4:0.2 to yield 6.3 g (67%) of the desired product as a colorless oil. R_f = 0.33 (in CH₂Cl₂/hexane/EtOAc = 1:4:0.2); ¹H NMR (300 MHz, CDCl₃) δ 7.45 - 7.28 (m, 5 H),

6.84 (m, 4 H), 5.12 (s, 2 H), 4.08 (s, 4 H), 1.40 (s, 18 H); ^{13}C NMR (75 MHz, CDCl_3) δ 170.5, 150.5, 139.6, 137.5, 128.4, 127.6, 127.2, 121.9, 121.4, 119.4, 114.7, 81.0, 70.9, 54.6, 28.0; mass spectrum, calculated for $\text{C}_{25}\text{H}_{33}\text{NO}_5$ (MH^+) 428.2, Found 427.9.

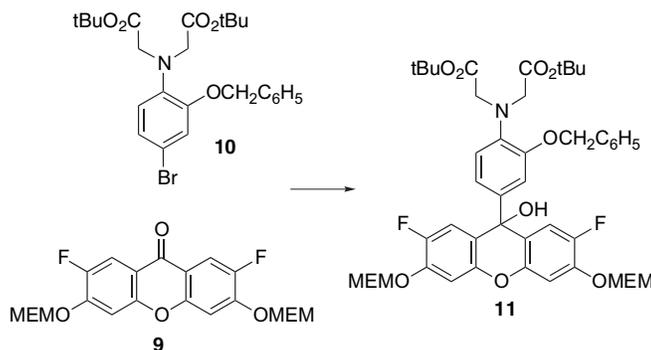


[(2-Benzyloxy-4-bromo-phenyl)-tert-butoxycarbonylmethyl-amino]-acetic acid tert-butyl ester (10). To a $-78\text{ }^\circ\text{C}$ solution of [(2-Benzyloxy-phenyl)-tert-butoxycarbonylmethyl-amino]-acetic acid tert-butyl ester (6.3 g, 14.7 mmol) in CH_2Cl_2 (100 mL) was added pyridine (1.8 mL, 22.1 mmol) followed by bromine (0.91 mL, 17.6 mmol) under an Ar atmosphere. After 30 min, the mixture was allowed to warm to room temperature and then washed with water, 5% sodium bicarbonate, and brine. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by flash column chromatography, eluting with CH_2Cl_2 /hexane/EtOAc = 1:4:0.2 to yield 5.9 g (79 %) of **10** as a white solid. R_f = 0.43 (in CH_2Cl_2 /hexane/EtOAc = 1:4:0.2); ^1H NMR (300 MHz, CDCl_3) δ 7.45 - 7.26 (m, 5 H), 6.99 (m, 2 H), 6.73 (m, 1 H), 5.09 (s, 2 H), 4.04 (s, 4 H), 1.41 (s, 18 H); ^{13}C NMR (75 MHz, CDCl_3) δ 170.1, 151.1, 138.7, 136.6, 128.6, 127.9, 127.4, 124.1, 120.5, 117.6, 113.7, 81.2, 71.2, 54.5, 28.0; mass spectrum, calculated for $\text{C}_{25}\text{H}_{32}\text{BrNO}_5$ (MH^+) 506.2, Found 505.8.

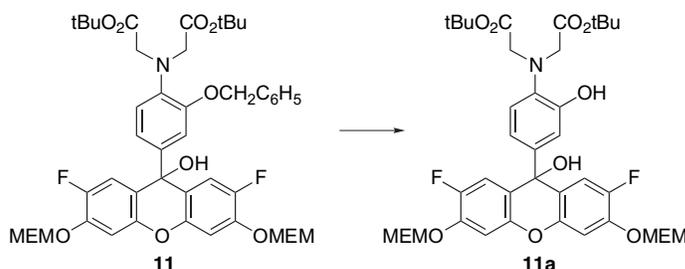


({2-Benzyloxy-4-[2,7-difluoro-9-hydroxy-3,6-bis-(2-methoxyethoxymethoxy)-9H xanthen-9-yl]-phenyl}-tert-butoxycarbonylmethyl-amino)-acetic acid tert-butyl ester (11). The bromide **10** (807 mg, 1.59 mmol) was dissolved in 20 mL THF/2-methyltetrahydrofuran (1:1) and cooled to $-105\text{ }^\circ\text{C}$ in a liquid N_2 /diethyl ether bath. After stirring at $-105\text{ }^\circ\text{C}$ for 10 min, 4.5 mL of tert-butyllithium (1.1 M in pentanes) was added dropwise. Stirring was continued for another 15 min. 2,7-difluoro-3,6-bis-(2-methoxy-ethoxymethoxy)-xanthen-9-one **9** (912 mg, 2.07 mmol) dissolved in THF (10 mL) was added dropwise to the reaction mixture. The mixture was stirred at $-105\text{ }^\circ\text{C}$ for 30 min, the cooling bath removed, and the reaction solution was then stirred for an additional 15 min. The resulting mixture was added to 150 mL NH_4Cl (sat) and extracted twice with EtOAc. The combined organic layers were washed with brine, dried over Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by flash column chromatography, eluting with 50% EtOAc in hexane (with 0.1% Et_3N) to yield 956 mg (69%) of **11** as a orange-red oil. R_f = 0.33 (in 50% EtOAc/hexane); ^1H NMR (300 MHz, CDCl_3) δ 7.32 - 7.27 (m, 5 H), 6.97 (d, 2 H, J = 6.98 Hz), 6.86 (m, 3 H), 6.72 (m, 2 H), 5.30 (s, 4 H), 5.05 (s, 2 H), 4.02 (s, 4 H), 3.85 (m, 4 H), 3.56 (m, 4 H), 3.36 (s, 6 H), 1.38 (s, 18 H); ^{13}C NMR (75 MHz, CDCl_3) δ 170.5, 150.8, 149.3, 147.6, 145.6, 145.5, 145.4, 145.2, 140.5, 138.4, 136.9, 128.3, 127.7, 127.5, 119.9, 119.8, 119.3, 118.4, 115.2, 114.9, 113.0, 104.9, 94.5, 81.0, 71.4, 70.6, 69.7,

68.1, 58.9, 54.6, 28.0; ^{19}F NMR (282 MHz, CDCl_3) δ -139.0 (dd, 2 F); mass spectrum, calculated for $\text{C}_{46}\text{H}_{55}\text{F}_2\text{NO}_{13}$ (MH^+) 868.4, Found 867.8.

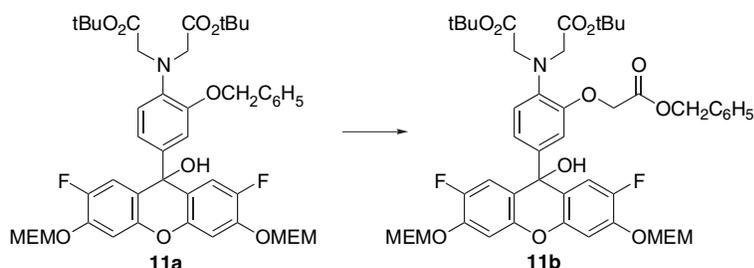


(tert-Butoxycarbonylmethyl-4-[2,7-difluoro-9-hydroxy-3,6-bis-(2-methoxyethoxymethoxy)-9H-xanthen-9-yl]-2-hydroxy-phenyl}-amino)-acetic acid tert-butyl ester (11a). To a solution of **11** (956 mg, 1.1 mmol) in 95% ethanol (25 mL) and EtOAc (25 mL) was added 10% Pd/C (130 mg). The resulting mixture was stirred at room temperature overnight under H_2 (1 atm). The catalyst was then removed by filtration and, after evaporation to dryness, the crude product was purified by flash column chromatography, eluting with 40% EtOAc in hexane (with 0.1% Et_3N) to yield 671 mg (78%) of **11a** as a light yellow oil. R_f = 0.3 (in 40% EtOAc/hexane); ^1H NMR (300 MHz, CDCl_3) δ 8.1 (s, 1 H), 7.18 (d, 1 H, J = 8.0 Hz), 6.95 (d, 2 H, J = 7.2 Hz), 6.69 (m, 3 H), 6.57 (m, 1 H), 5.28 (s, 4 H), 4.94 (s, 1 H), 3.85 (m, 4 H), 3.69 (s, 4 H), 3.57 (m, 4 H), 3.38 (s, 6 H), 1.41 (s, 18 H); ^{13}C NMR (75 MHz, CDCl_3) δ 171.2, 153.8, 150.6, 147.4, 146.5, 144.8, 144.4, 144.2, 136.3, 126.3, 119.6, 116.7, 116.6, 116.1, 115.8, 115.5, 105.9, 94.6, 81.9, 71.4, 68.0, 59.0, 56.9, 43.1, 27.9; ^{19}F NMR (282 MHz, CDCl_3) δ -140.4 (dd, 2 F); mass spectrum, calculated for $\text{C}_{46}\text{H}_{55}\text{F}_2\text{NO}_{13}$ (M-17) 761.3, Found 761.7.

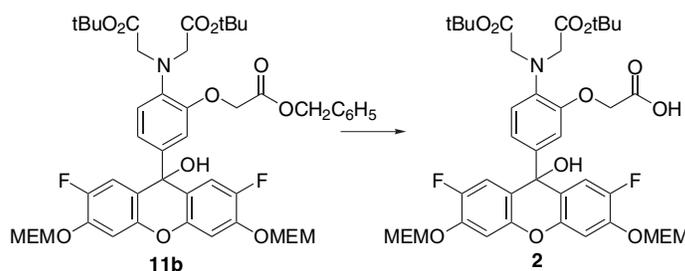


(2-Benzyloxycarbonylmethoxy-4-[2,7-difluoro-9-hydroxy-3,6-bis-(2-methoxyethoxymethoxy)-9H-xanthen-9-yl]-phenyl)-tert-butoxycarbonylmethyl-amino)-acetic acid tert-butyl ester (11b). A solution of **11a** (497 mg, 0.64 mmol) in DMF (10 mL) was treated with sodium hydride (18.5 mg, 0.77 mmol) at 0°C under an Ar atmosphere. After stirring at 0°C for 30 min, benzyl 2-bromoacetate (0.15 mL, 0.96 mmol) was added. The mixture was stirred at 0°C for another 30 min and then warmed to room temperature overnight. The solvent was evaporated to dryness and extracted with EtOAc/ H_2O . The combined organic layers were washed with brine, dried over Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by flash column chromatography, eluting with 40% EtOAc in Hexane (with 0.1% Et_3N) to yield 453 mg (76%) of **11b** as a light yellow oil. R_f = 0.28 (in 40% EtOAc/hexane); ^1H NMR

(300 MHz, CDCl₃) δ 7.32 (m, 5 H), 6.96 (d, 2 H, *J* = 7.2 Hz), 6.86 (d, 1 H, *J* = 8.4 Hz), 6.72 (dd, 1 H, *J* = 7.8 and 1.5 Hz), 6.59 (m, 3 H), 5.28 (s, 4 H), 5.15 (s, 2 H), 4.89 (s, 1 H), 4.64 (s, 2 H), 4.01 (s, 4 H), 3.86 (m, 4 H), 3.58 (m, 4 H), 3.39 (s, 6 H), 1.39 (s, 18 H); ¹³C NMR (75 MHz, CDCl₃) δ 170.3, 168.8, 150.7, 149.9, 147.5, 146.6, 146.5, 144.4, 144.3, 139.1, 138.9, 135.3, 128.6, 128.4, 128.3, 122.9, 120.0, 117.0, 116.9, 116.2, 116.1, 115.8, 105.9, 94.7, 81.2, 71.5, 68.1, 66.7, 66.6, 59.0, 54.5, 42.6, 28.0; ¹⁹F NMR (282 MHz, CDCl₃) δ -140.2 (dd, 2 F); mass spectrum, calculated for C₄₆H₅₅F₂NO₁₃ (M-17) 909.4, Found 910.1.



{2-(Bis-tert-butoxycarbonylmethyl-amino)-5-[2,7-difluoro-9-hydroxy 3,6-bis-(2-methoxyethoxymethoxy)-9H-xanthen-9-yl]-phenoxy}-acetic acid (2). To a solution of **11b** (743 mg, 0.80 mmol) in 95% ethanol (25 mL) and EtOAc (25 mL) was added 10% Pd/C (135 mg). The resulting mixture was stirred at room temperature overnight under H₂ (1 atm). The catalyst was then removed by filtration and, after evaporation to dryness, the crude product was purified by flash column chromatography, eluting with 10% MeOH in CH₂Cl₂ to yield 595 mg (89%) of **2** as a red foaming solid. *R_f* = 0.43 (in 10% MeOH/CH₂Cl₂); ¹H NMR (300 MHz, CDCl₃) δ 10.0 (br, 1 H), 6.93 (d, 2 H, *J* = 7.2 Hz), 6.86 (d, 1 H, *J* = 8.2 Hz), 6.71 (dd, 1 H, *J* = 8.2 and 1.3 Hz), 6.62 (m, 3 H), 5.24 (s, 4 H), 4.93 (s, 1 H), 4.53 (s, 2 H), 3.91 (s, 4 H), 3.83 (m, 4 H), 3.56 (m, 4 H), 3.35 (s, 6 H), 1.34 (s, 18 H); ¹³C NMR (75 MHz, CDCl₃) δ 171.0, 170.5, 150.6, 150.5, 147.4, 146.4, 146.3, 144.4, 144.2, 140.8, 138.7, 122.3, 120.7, 116.6, 116.5, 115.9, 115.6, 115.4, 105.9, 94.6, 81.7, 71.3, 67.9, 67.3, 58.8, 55.0, 42.7, 27.8; ¹⁹F NMR (282 MHz, CDCl₃) δ -140.0 (dd, 2 F); mass spectrum, calculated for C₄₆H₅₅F₂NO₁₃ (M-17) 819.3, Found 819.8.

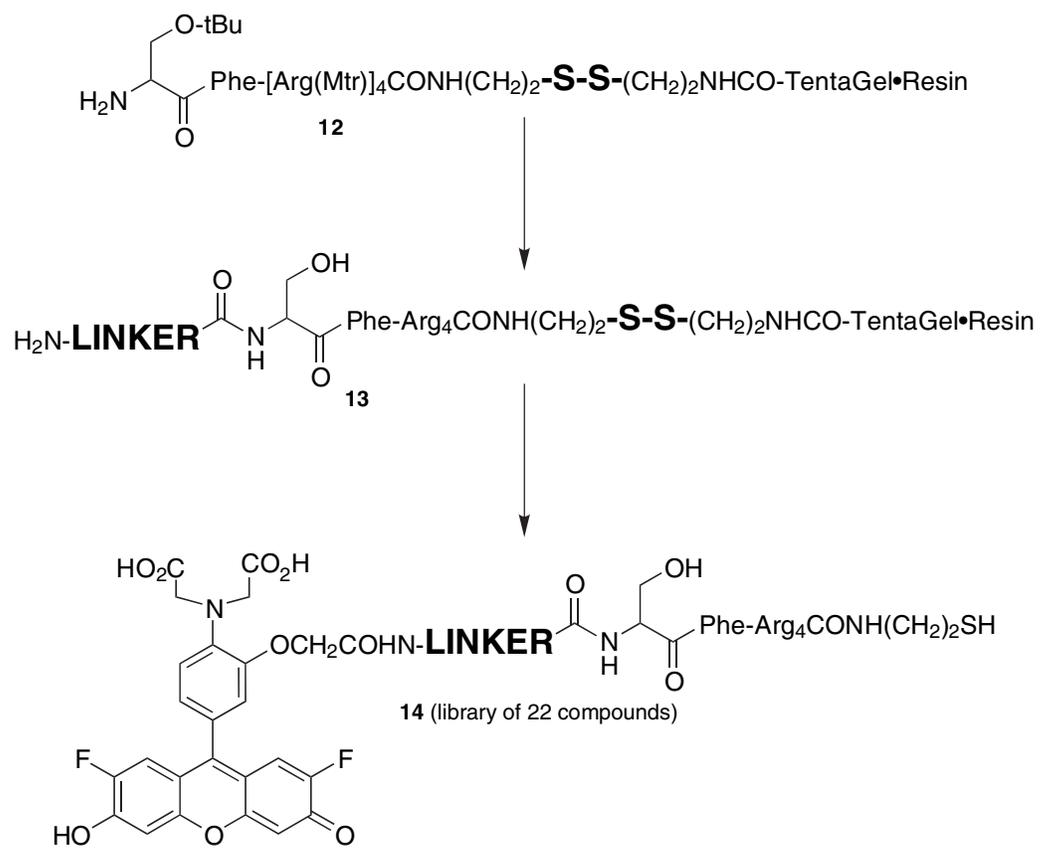


H₂N-Ser(tBu)-Phe-[Arg(Mtr)]₄-cystamine-Tentagel Resin (12). Cystamine dihydrochloride (10 eq, 2.25 g) was added to a mixture of TentaGel S COOH resin (90 μm, 5 g, 0.2 mmol/g), BOP (1.2 eq, 0.53 g), HOBT (1.2 eq, 0.184 g), N-methylmorpholine (NMM) (30 eq, 3.3 mL) in 20 mL DMF and subsequently shaken overnight at ambient temperature. The free amine substitution level was determined as 0.01 mmol/g. This low substitution level is ideal for our purposes since this not only ensures a higher coupling yield but, in addition, larger quantities of resin (with greater weight accuracy) can be subsequently introduced into the 96-well plates. The

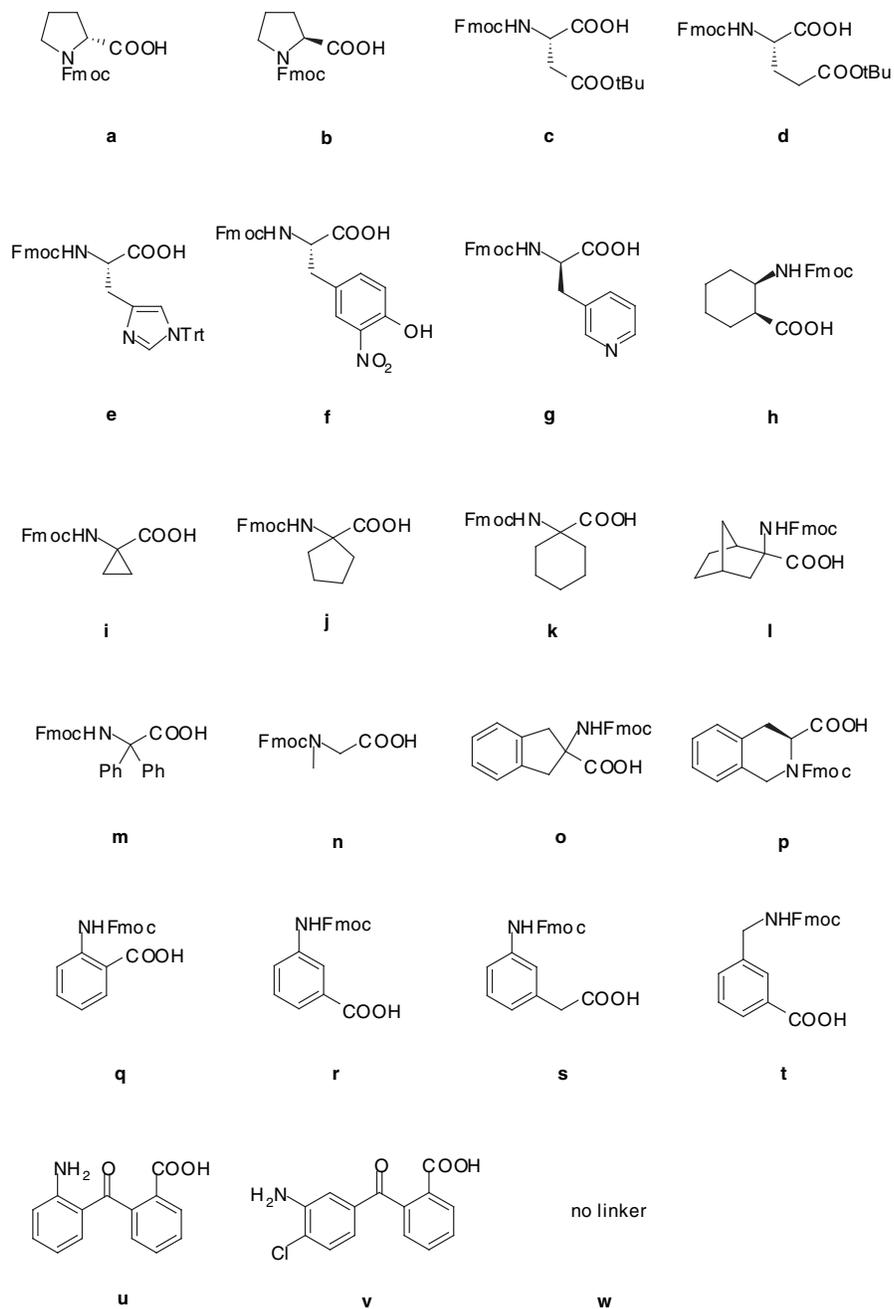
peptide NH₂-Ser(tBu)-Phe-[Arg(Mtr)]₄ was synthesized on the cystamine-substituted TentaGel resin using an Fmoc solid phase peptide synthesis protocol.

General protocol for the preparation of the fluorophore-peptide conjugates (3 and 14). Both compound **3** and the corresponding linker-based library **14** (23 compounds) were prepared using the same protocol, with the exception that the library members were synthesized in a solvent resistant 96-well filter plate. 10 mg of the peptide-TentaGel resin **12** was introduced into 23 individual wells of a 96-well filter plate. In addition, each well contained a Fmoc-linker-COOH (10 eq), *O*-benzotriazol-1-yl-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (10 eq), 1-hydroxybenzotriazole (10 eq), and *N*-methylmorpholine (20 eq) in 100 μ L DMF. A total of 22 different Fmoc-linker-COOH linkers were employed. One additional well was reserved for the species that does not contain a linker. The plate was shaken overnight and then each well was subjected to a series of wash steps (3 \times 200 μ L DMF, 3 \times 200 μ L water, 3 \times 200 μ L DMF, 3 \times 200 μ L CH₂Cl₂, 2 \times 200 μ L MeOH, 2 \times 200 μ L 50 mM Tris pH 7.5). The *N*-terminal Fmoc protecting group was removed via double exposure to 30% piperidine in DMF for 30 min and then each well was subjected to a series of wash steps (3 \times 200 μ L DMF, 3 \times 200 μ L water, 3 \times 200 μ L DMF, 3 \times 200 μ L CH₂Cl₂, 2 \times 200 μ L MeOH, 2 \times 200 μ L 50 mM Tris pH 7.5). The side chain protecting groups on the peptide were removed via treatment with 95% trifluoroacetic acid/2.5% thioanisole/2.5% H₂O at room temperature overnight to furnish **13**. Each well was subsequently subjected to a series of wash steps (3 \times 200 μ L DMF, 3 \times 200 μ L water, 3 \times 200 μ L DMF, 3 \times 200 μ L CH₂Cl₂, 2 \times 200 μ L MeOH, 2 \times 200 μ L 50 mM Tris pH 7.5). {2-(Bis-tert-butoxycarbonylmethyl-amino)-5-[2,7-difluoro-9-hydroxy 3,6-bis-(2-methoxy-ethoxymethoxy)-9H-xanthen-9-yl]-phenoxy}-acetic acid (**4**) (5 eq) was coupled to the peptide-TentaGel resin **13** under standard conditions [*O*-benzotriazol-1-yl-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (5 eq), 1-hydroxybenzotriazole (5 eq), and *N*-methylmorpholine (10 eq) in 100 μ L DMF]. The attached xanthen was then deprotected and fully aromatized via exposure to 95% trifluoroacetic acid/5% H₂O. The fluorophore-peptide conjugates were cleaved from the disulfide-containing resin with 10 mM dithiothreitol (DTT) in Tris buffer (1 \times 200 μ L for 1 Hr; 2 \times 150 μ L for 1 hr each) and filtered into a receiving set of 96-well plates using a vacuum manifold to furnish the library **14**.

Mass spectrum, calculated for **14a** 1544.7, Found 1624.0 (M + 79.9); calculated for **14b** 1544.7, Found 1544.0; calculated for **14c** 1562.6, Found 1562.0; calculated for **14d** 1576.7, Found 1576.0; calculated for **14e** 1584.7.7, Found 1584.0; calculated for **14f** 1655.7, Found 1657.0 (M + 2); calculated for **14g** 1595.7, Found 1596.0; calculated for **14h** 1572.7, Found 1572.0; calculated for **14i** 1530.6, Found 1530.0; calculated for **14j** 1558.7, Found 1558.0; calculated for **14k** 1572.7, Found 1574.0; calculated for **14l** 1584.0, Found 1584.0 (M + 2); calculated for **14m** 1518.0, Found 1518.0; calculated for **14n** 1517.7, Found 1518.0; calculated for **14o** 1606.7, Found 1607.0; calculated for **14p** 1606.7, Found 1607.0 (M + 2); calculated for **14q** 1566.7, Found no identifiable mass ion; calculated for **14r** 1566.7, Found 1566.0; calculated for **14s** 1580.7, Found 1581.0; calculated for **14t** 1580.7, Found 1581.0; calculated for **14u** 1670.7, Found no identifiable mass ion; calculated for **14v** 1796.2, Found no identifiable mass ion; calculated for **14w** 1447.5, Found 1447.0;



The 22 linker monomers (plus compound w) employed in the preparation of library **14**:



Protein kinase C assay. The kinase-catalyzed reaction was performed in triplicate at 30 °C and initiated by addition of ATP to a solution of PKC α and fluorophore-appended peptide substrate **3** (except in the case of the library, where the assays were performed in a 96 well plate using a fluorescence plate reader). Final conditions: 62.5 mM HEPES, 3 mM MgCl₂, 0.3 mM CaCl₂, 0.1 mM EGTA, 1 mM DTT, 0.5 μ g/mL phosphatidylserine, 0.1 μ g/mL diacylglycerol, 1 mM ATP, and 13 nM PKC α (pH 7.4). After the addition of ATP, the solution was gently mixed and the time-dependent change in fluorescent intensity ($\lambda_{\text{excitation}} = 494 \text{ nm}$; $\lambda_{\text{emission}} = 521 \text{ nm}$) continuously monitored with a Photon Technology QM-1 spectrofluorimeter.

Ca²⁺ titration assay. The Ca²⁺ Calibration Kit #2 (purchased from Molecular Probes) was employed for these studies. The fluorescent peptide was dissolved in two buffers, one without (SOLUTION A: 10 mM MOPs, 100 mM KCl, 10 mM EGTA, pH 7.2) and one with (SOLUTION B: 10 mM MOPs, 100 mM KCl, 10 mM CaEGTA, pH 7.2) Ca²⁺. The final concentration of the peptide in both buffers was 1 μ M. Solution A was (200 μ L) added to the microcuvette and the fluorescence was recorded ($\lambda_{\text{excitation}} = 494 \text{ nm}$; $\lambda_{\text{emission}} = 521 \text{ nm}$). This was the fluorescence intensity with 0 Ca²⁺. A series of solutions containing the following free Ca²⁺ concentrations (based on the K_d of CaEGTA at pH 7.2 = 150.3 nM) was prepared: 0.038 μ M, 0.065 μ M, 0.1 μ M, 0.15 μ M, 0.225 μ M, 0.351 μ M, 0.602 μ M, 1.35 μ M, and 39.8 μ M.

