Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Supporting Information

Rhodol-based Thallium Sensors of Cellular Imaging of Potassium Channels

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Contents

I. Chemical synthesis

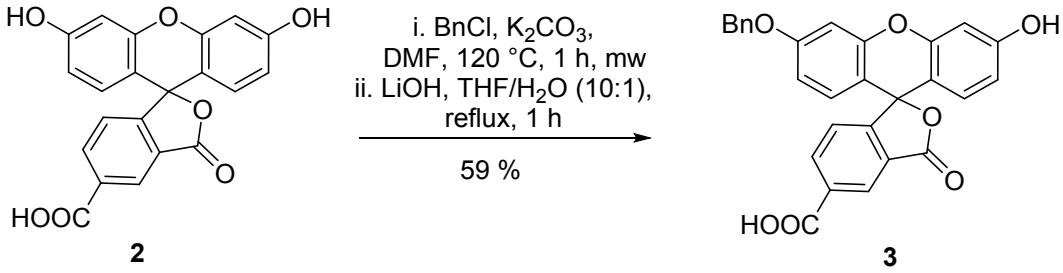
1. General Methods: All non-aqueous reactions were performed in flame-dried flasks under an atmosphere of argon. Stainless steel syringes were used to transfer air- and moisture-sensitive liquids. Reaction temperatures were controlled using a thermocouple thermometer and analog hotplate stirrer. Reactions were conducted at room temperature (rt, approximately 23 °C) unless otherwise noted. Flash column chromatography was conducted using silica gel 230-400 mesh. Analytical thin-layer chromatography (TLC) was performed on E. Merck silica gel 60 F254 plates and visualized using UV and iodine stain.

2. Materials: Acetic anhydride, trifluoromethanesulfonic anhydride, and HATU were purchased from Oakwood Products (Estill, SC). Diisopropylamine, azetidine hydrochloride, 3,3-di-fluoroazetidine hydrochloride, and cesium carbonate were purchased from Combi-Blocks (San Diego, CA). Pyrrolidine was purchase from Acros. 5-carboxyfluorescein[1] and *O*-*tert*-butyl-di-isopropylisourea[2] were prepared according to literature procedures. All other solvents and chemicals were purchased from Sigma-Aldrich. *N*,*N*-dimethylformamide (DMF), dichloromethane, pyridine, toluene (PhMe), triethylamine (Et_3N), and dimethylsulfoxide (DMSO) were used as received in a bottle with a Sure/Seal. Trifluoromethanesulfonic anhydride was distilled from P_2O_5 prior to use. Deuterated solvents were purchased from Cambridge Isotope Laboratories.

3. Instrumentation: ^{1}H NMR spectra were recorded on Bruker 400 or 600 MHz spectrometers and are reported relative to deuterated solvent signals. Data for ^{1}H NMR spectra are reported as follows: chemical shift (δ ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet, br = broad, app = apparent), coupling constants (Hz), and integration. ^{13}C NMR spectra were recorded on Bruker 100 or 150 MHz spectrometers and are reported relative to deuterated solvent signals. Low resolution mass spectrometry (LRMS) was conducted and recorded on an Agilent Technologies 6130 Quadrupole instrument. Preparative scale HPLC was

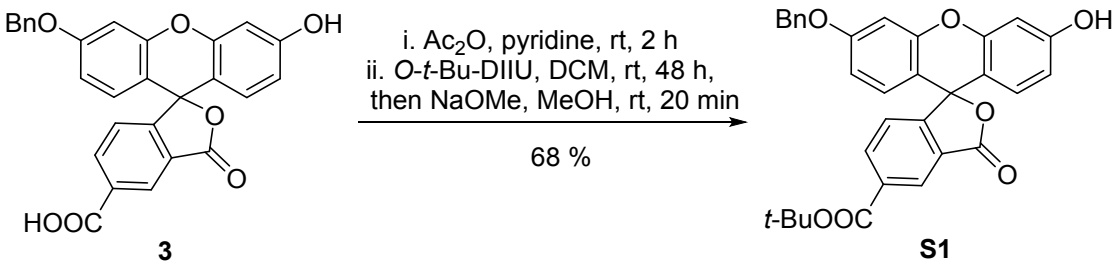
conducted on a Gilson HPLC machine. Automated flash chromatography was performed on a Teledyne Isco purification system.

4. Synthetic procedures and compound characterization



3'-(benzyloxy)-6'-hydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-5-carboxylic acid (**3**). To a stirred solution of 1.35 g (3.59 mmol, 1.0 eq) of **2** in 10 mL of DMF in a 20 mL microwave vial was added 2.48 g (18.0 mmol, 5.0 eq) of K_2CO_3 and 1.66 mL (14.4 mmol, 4.0 eq) of benzyl chloride. The vial was capped, flushed with Ar, and maintained at 120 °C under microwave irradiation for 1 h. The reaction was poured into 1 N HCl, the mixture extracted with EtOAc (3x), the organic layers combined and washed with brine, and concentrated. The residue was dissolved in 120 mL of THF and 12 mL of H_2O. To this solution was added 431 mg (18.0 mmol, 5.0 eq) of LiOH. The solution was refluxed for 1 h when the reaction was deemed complete by LCMS. The solution was acidifed with 1 N HCl and THF removed *in vacuo*. The resulting mixture was extracted with ethyl acetate (3x), the organic layers were combined, washed with brine, and passed through a phase separator (Biotage). The solution was concentrated and the residue was purified by flash chromatography with hexane and ethyl acetate plus 1% acetic acid to provide 0.99 g (59 %) of **3**. ^{1}H NMR (400 MHz, acetone-d6) δ 8.56 – 8.55 (app q, 1H), 8.40 (dd, J = 8.02 Hz, J = 1.50 Hz, 1H), 7.51 – 7.47 (m, 2H), 7.43 – 7.36 (m, 3H), 7.35 – 7.30 (m, 1H), 6.96 – 6.95 (app d, 1H), 6.83 – 6.77 (m, 3H), 6.74 (d, J = 8.64 Hz, 1H), 6.65 (dd, J = 8.68 Hz, J = 2.40 Hz, 1H), 5.20 (s, 2H); ^{13}C NMR (100 MHz, acetone-d6) δ 168.6, 166.3, 161.6, 160.5, 157.6, 153.2, 137.7, 137.0, 133.5, 130.2, 130.1, 129.4, 128.8, 128.5, 128.3, 126.8, 125.4, 113.6, 113.4, 112.0,

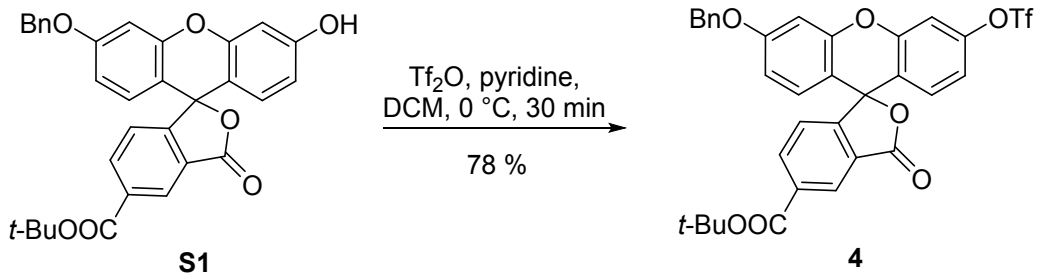
110.8, 103.4, 102.6, 83.8, 70.8; LRMS calculated for $C_{28}H_{18}O_7$ $[M+H]^+$ m/z: 467.1, measured 467.0.



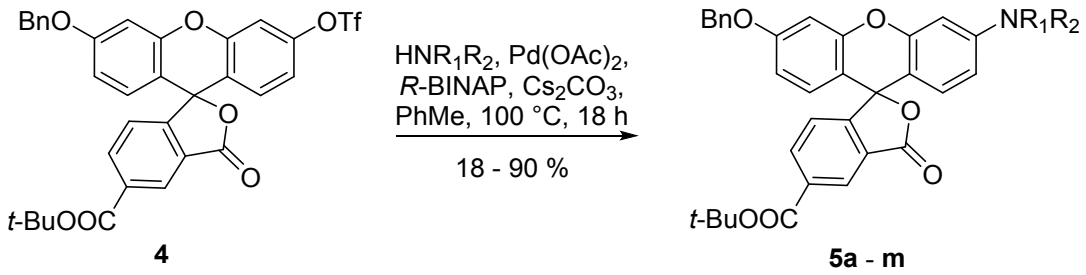
3 **S1**

tert-butyl 3'-(benzyloxy)-6'-hydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-5-carboxylate (**S1**). To a stirred solution of 0.49 g (1.05 mmol, 1.0 eq) of **3** in pyridine (5 mL) was added 149 µL (1.58 mmol, 1.5 eq) of acetic anhydride. The solution was stirred at room temperature for 2 h until judged complete by LCMS. The solution was concentrated, the resulting residue acidified with 1 N HCl, extracted with ethyl acetate (3x), washed with brine, and passed through a phase separator (Biotage). The solution was concentrated *in vacuo* and the residue was dissolved in dichloromethane (5 mL). To this solution was added 630 mg (3.15 mmol, 3.0 eq) of *O*-*t*-Bu-DIIU. The solution was stirred for 48 h at room temperature. The resulting suspension was filtered, the solids washed with dichloromethane, the organics combined and concentrated, and the residue dissolved in methanol (5 mL). To this solution was added 1.1 mL (5.25 mmol, 5.0 eq) of 25 % sodium methoxide in methanol. The mixture was stirred for 20 min at room temperature, quenched with 1 N HCl, concentrated and extracted with ethyl acetate (3x). The organic layers were combined, washed with brine, passed through a phase separator (Biotage), and concentrated *in vacuo*. The residue was purified by flash chromatography to provide 0.37 g (68 %) of **S1**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.62 (s, 1H), 8.29 (dd, J = 8.02 Hz, J = 1.34 Hz, 1H), 7.44 – 7.30 (m, 5H), 7.21 (d, J = 8.04 Hz, 1H), 6.83 (d, J = 2.08 Hz, 1H), 6.74 (d, J = 2.00 Hz, 1H), 6.70 - 6.62 (m, 2H), 6.57 – 6.50 (m, 2H), 5.07 (s, 2H), 1.64 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.5, 164.4, 160.8, 158.6, 156.6, 152.6, 136.3, 136.2, 134.2, 129.2, 129.1, 128.8,

127.6, 127.1, 126.6, 124.3, 112.7, 112.6, 110.7, 110.3, 103.4, 102.1, 84.7, 82.7, 70.4, 28.3; LRMS calculated for $C_{32}H_{26}O_7$ $[M+H]^+$ m/z: 523.2, measured 523.6.

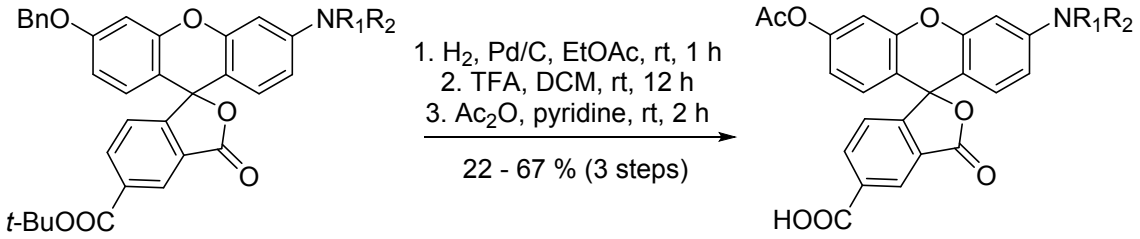


tert-butyl 3'-(benzyloxy)-3-oxo-6'-(((trifluoromethyl)sulfonyl)oxy)-3H-spiro[isobenzofuran-1,9'-xanthene]-5-carboxylate (**4**). To a stirred solution of 0.37 g (0.709 mmol, 1.0 eq) of **S1** and 228 µL (2.84 mmol, 4.0 eq) of pyridine in dichloromethane (5 mL) at 0 °C was added 238 µL (1.42 mmol, 2.0 eq) of trifluoromethanesulfonic anhydride. The solution was stirred at 0 °C for 30 min at which point the reaction was judged complete by TLC. The solution was concentrated and the resulting residue purified by flash chromatography to provide 0.36 g (78 %) of **4** as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.63 (d, J = 0.60 Hz, 1H), 8.32 (dd, J = 8.02 Hz, J = 1.42 Hz, 1H), 7.44 – 7.32 (m, 5H), 7.27 – 7.25 (m, 1H), 7.22 (d, J = 7.88 Hz, 1H), 6.96 (dd, J = 8.80 Hz, J = 2.40 Hz, 1H), 6.90 – 6.86 (m, 2H), 6.74 (dd, J = 8.84 Hz, J = 2.36 Hz, 1H), 6.69 (d, J = 8.80 Hz, 1H), 5.10 (s, 2H), 1.64 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.3, 164.0, 161.0, 156.0, 152.1, 151.9, 150.2, 136.5, 134.7, 130.1, 129.1, 128.9, 128.4, 127.6, 126.8, 126.7, 124.1, 119.3, 116.9, 113.4, 110.8, 110.3, 102.2, 82.7, 81.8, 70.5, 28.3; ^{19}F NMR (376 MHz, $CDCl_3$) δ 75.7; LRMS calculated for $C_{33}H_{25}F_3O_9S$ $[M+H]^+$ m/z: 655.1, measured 655.5.



General procedure for Buchwald-Hartwig coupling

To a stirred solution of triflate **4** (1.0 eq) dissolved in toluene (0.08 M) in a microwave vial was added $Pd(OAc)_2$ (0.1 eq), *R*-BINAP (0.15 eq), Cs_2CO_3 (4 eq), and amine (1.2 eq). The tube was sealed and maintained at 100 °C on a heating block for 18 h. The mixture was cooled, filtered through celite, and concentrated *in vacuo*. The crude residue was purified by flash chromatography to provide products **5a** – **m** (18 – 90 %).



5a - **m** **6a** - **m**

General hydrogenolysis procedure

To a stirred solution of benzyl ether (1.0 eq) in ethyl acetate (1 mL) was added 10 % palladium on carbon (0.05 mol % of palladium). The solution was flushed with Ar and then H_2. The reaction was vigorously stirred at room temperature under an atmosphere of H_2 until it was judged complete by LCMS (~1 h). The reaction was flushed with Ar, filtered through celite, and concentrated to provide crude phenol. The material was carried on crude to the next step.

General procedure for *t*-butyl ester deprotection

To a suspension of phenol (1.0 eq) in DCM (2.0 mL) was added TFA (400 µL). The resulting solution was stirred overnight at room temperature. The reaction was judged complete by LCMS and concentrated. The product was concentrated and carried on crude to the next step.

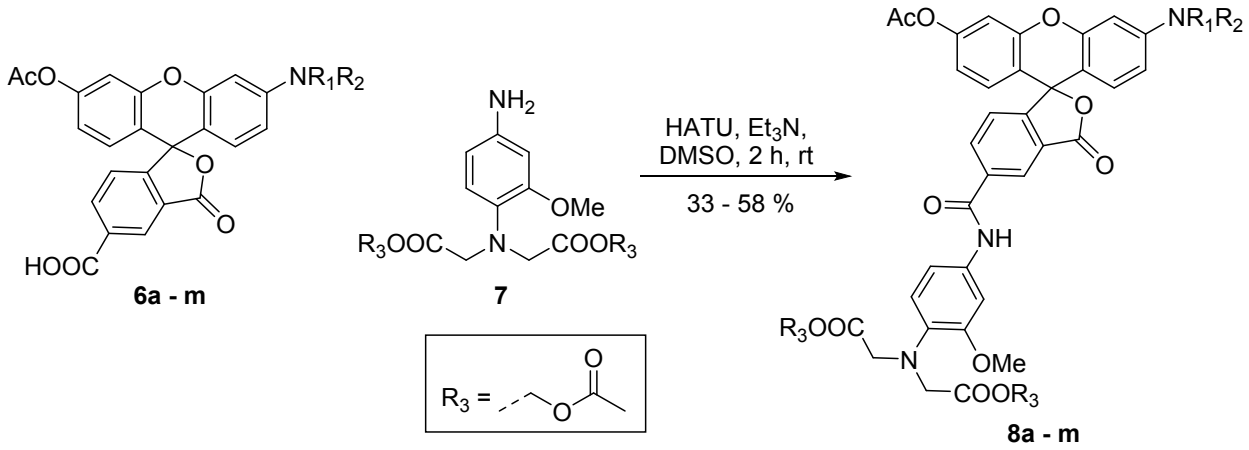
General *O*-acetylation procedure

To a solution of phenol in pyridine (1.0 mL) was added acetic anhydride (1.5 eq). The solution was stirred until judged complete by LCMS. The solution was concentrated, dissolved in DMSO,

and purified by preparative scale reverse phase HPLC using an acetonitrile and water with 0.1 % TFA gradient. Fractions containing product were lyophilized to provide **6a** – **m** (22 – 67 %).

Synthesis of **6a** and **6b**

The common intermediate for the synthesis of **6a** and **6b** was prepared by Buchwald-Hartwig coupling of **4** with *N*-*tert*-butylcarbamate followed by hydrogenolysis of the benzyl ether as described above. **6a** was prepared by *O*-acetylation followed by ester and *N*-boc deprotection under the *t*-butyl ester deprotection conditions described. **6b** was prepared first by ester and *N*-boc deprotection followed by bis-acetylation accomplished by increasing the amount of acetic anhydride in the above *O*-acetylation conditions to 3 equivalents.



General coupling procedure

To a stirred solution of **6a** - **m** in DMSO was added HATU (1.5 eq), triethylamine (2 eq), and **7**[3] (1.1 eq). The solution was stirred until judged complete by LCMS (1 – 2 h). The product was purified by preparative reverse phase HPLC using an acetonitrile and water with 0.1 % TFA gradient. Fractions containing product were lyophilized to provide **8a** – **m** (33 – 58 %).

Compound storage

Compounds **8a** - **m** were dissolved in DMSO and stored as 10 mM stock solutions at -20 °C. These conditions were generally well tolerated with the exception of **8a** which appeared to

undergo transacetylation and equilibrated to a mixture of **8a**, **8b**, and mono-deacetylated species upon storage. To prevent this, dry aliquots of **8a** were prepared and dissolved in DMSO immediately before use.

bis(acetoxymethyl) 2,2'-((4-(3'-acetoxy-6'-amino-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-5-ylcarboxamido)-2-methoxyphenyl)azanediyl)diacetate (**8a**). ^{1}H NMR (600 MHz, CD_3CN) δ 8.93 (s, 1H), 8.60 (s, 1H), 8.29 (d, J = 7.80 Hz, 1H), 7.44 (s, 1H), 7.40 (d, J = 7.86 Hz, 1H), 7.25 (s, 1H), 7.20 (d, J = 8.04 Hz, 1H), 7.01 (d, J = 8.64 Hz, 1H), 6.95 (d, J = 8.16 Hz, 1H), 6.83 (d, J = 8.58 Hz, 1H), 6.78 (d, J = 8.58 Hz, 1H), 6.65 (s, 1H), 6.59 (d, J = 8.34 Hz, 1H), 5.71 (s, 4H), 4.13 (s, 4H), 3.80 (s, 3H), 2.28 (s, 3H), 2.07 (s, 6H); ^{13}C NMR (150 MHz, CD_3CN) δ 171.1, 170.5, 170.0, 168.5, 164.8, 155.5, 154.9, 153.6, 152.2, 138.2, 135.9, 134.9, 134.4, 131.2, 130.5, 129.0, 127.1, 126.6, 119.9, 119.8, 115.6, 113.9, 111.5, 106.7, 100.6, 80.1, 56.3, 54.4, 21.3, 20.9; LRMS calculated for $C_{40}H_{35}N_3O_{15}$ $[M+H]^+$ m/z: 798.2; measured 798.4.

bis(acetoxymethyl) 2,2'-((4-(3'-acetamido-6'-acetoxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-5-ylcarboxamido)-2-methoxyphenyl)azanediyl)diacetate (**8b**). ^{1}H NMR (600 MHz, CD_3CN) δ 8.88 (s, 1H), 8.55 – 8.51 (app d, 2H), 8.27 (dd, J = 8.01 Hz, J = 1.17 Hz, 1H), 7.82 (d, J = 1.62 Hz, 1H), 7.45 (d, J = 1.74 Hz, 1H), 7.37 (d, J = 8.04 Hz, 1H), 7.19 (dd, J = 8.58 Hz, J = 2.04 Hz, 1H), 7.15 (d, J = 2.16 Hz, 1H), 7.11 (dd, J = 8.67 Hz, J = 2.01 Hz, 1H), 6.91 (d, J = 8.70 Hz, 1H), 6.86 (dd, J = 8.64 Hz, J = 2.16 Hz, 1H), 6.83 (d, J = 8.58 Hz, 1H), 6.80 (d, J = 8.64 Hz, 1H), 5.71 (s, 4H), 4.13 (s, 4H), 3.81 (s, 3H), 2.27 (s, 3H), 2.08 (s, 3H), 2.06 (s, 6H); ^{13}C NMR (150 MHz, CD_3CN) δ 171.0, 170.5, 170.1, 169.2, 164.8, 156.3, 153.6, 152.7, 152.3, 152.2, 142.6, 138.4, 135.9, 134.5, 130.1, 129.5, 127.6, 125.3, 124.9, 119.9, 119.1, 117.2, 116.2, 113.84, 113.77, 111.5, 83.0, 80.1, 56.3, 54.4, 24.5, 21.2, 20.9; LRMS calculated for $C_{42}H_{37}N_3O_{16}$ $[M+H]^+$ m/z: 840.2, measured 840.3.

bis(acetoxymethyl) 2,2'-((4-(3'-acetoxy-6'-(dimethylamino)-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-5-carboxamido)-2-methoxyphenyl)azanediyl)diacetate (**8c**). ^{1}H NMR (600 MHz, acetone-d6) δ 9.76 (s, 1H), 8.55 (s, 1H), 8.40 (d, J = 7.98 Hz, 1H), 7.64 – 7.61 (m, 1H), 7.46 (d, J = 8.04 Hz, 1H), 7.38 – 7.34 (m, 1H), 7.16 (d, J = 1.80 Hz, 1H), 6.94 – 6.88 (m, 2H), 6.86 (d, J = 8.58 Hz, 1H), 6.67 (d, J = 8.94 Hz, 1H), 6.60 – 6.55 (m, 2H), 5.78 (s, 4H), 4.19 (s, 4H), 3.83 (s, 3H), 3.02 (s, 6H), 2.29 (s, 3H), 2.07 (s, 6H); ^{13}C NMR (150 MHz, acetone-d6) δ 170.8, 169.9, 169.4, 168.8, 164.4, 164.3, 156.1, 153.5, 153.4, 153.2, 153.0, 152.2, 138.3, 136.0, 135.5, 134.9, 134.8, 129.9, 129.5, 128.2, 125.3, 124.3, 119.8, 118.6, 117.7, 113.5, 113.4, 111.2, 110.4, 106.5, 106.4, 106.3, 99.0, 79.9, 56.2, 54.4, 40.3, 21.0, 20.6; LRMS calculated for $C_{42}H_{39}N_3O_{15}$ $[M+H]^+$ m/z: 826.3, measured 826.5.

bis(acetoxymethyl) 2,2'-((4-(3'-acetoxy-6'-(diethylamino)-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-5-ylcarboxamido)-2-methoxyphenyl)azanediyl)diacetate (**8d**) ^{1}H NMR (600 MHz, acetone-d6) δ 9.76 (s, 1H), 8.56 (s, 1H), 8.41 (dd, J = 7.95 Hz, J = 0.81 Hz, 1H), 7.63 (d, J = 1.98 Hz, 1H), 7.49 (d, J = 8.04 Hz, 1H), 7.36 (dd, J = 8.58 Hz, J = 2.10 Hz, 1H), 7.18 (d, J = 1.50 Hz, 1H), 6.95 – 6.89 (m, 2H), 6.86 (d, J = 8.58 Hz, 1H), 6.68 (d, J = 8.58 Hz, 1H), 6.61 – 6.57 (m, 2H), 5.78 (s, 4H), 4.20 (s, 4H), 3.83 (s, 3H), 3.48 (q, J = 7.02 Hz, 4H), 2.29 (s, 3H), 2.07 (s, 6H), 1.19 (t, J = 7.02 Hz, 6H); ^{13}C NMR (150 MHz, acetone-d6) δ 170.8, 169.9, 169.3, 168.8, 164.4, 155.9, 153.6, 153.4, 153.0, 152.2, 150.9, 138.3, 136.0, 135.5, 134.9, 129.9, 129.8, 128.4, 125.4, 124.4, 119.8, 118.5, 117.8, 113.5, 111.2, 110.0, 106.5, 105.6, 87.3, 79.9, 56.2, 54.4, 45.1, 21.0, 20.6, 12.7; LRMS calculated for $C_{44}H_{43}N_3O_{15}$ $[M+H]^+$ m/z ; 854.3, measured 854.5.

bis(acetoxymethyl) 2,2'-((4-(3'-acetoxy-6'-(dipropylamino)-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-5-ylcarboxamido)-2-methoxyphenyl)azanediyl)diacetate (**8e**). ^{1}H NMR (600 MHz, acetone-d6) δ 9.75 (s, 1H), 8.54 (s, 1H), 8.40 (dd, J = 7.98 Hz, J = 1.14 Hz, 1H), 7.63 (d, J = 2.16 Hz, 1H), 7.47 (d, J = 7.98 Hz, 1H), 7.36 (dd, J = 8.58 Hz, J = 2.22 Hz, 1H), 7.16 (d, J = 1.74 Hz, 1H), 6.93 – 6.88 (m, 2H), 6.86 (d, J = 8.58 Hz, 1H), 6.63 (d, J = 8.58 Hz, 1H), 6.54 – 6.51

(m, 2H), 5.78 (s, 4H), 4.20 (s, 4H), 3.83 (s, 3H), 3.36 (t, J = 7.68 Hz, 4H), 2.28 (s, 3H), 2.07 (s, 6H), 1.64 (sext, J = 7.48 Hz, 4H), 0.94 (t, J = 7.38 Hz, 6H); ^{13}C NMR (150 MHz, acetone-d6) δ 170.8, 170.0, 169.3, 168.8, 164.4, 153.5, 153.4, 153.0, 152.2, 151.3, 138.3, 136.0, 135.5, 134.9, 129.9, 129.6, 128.4, 125.4, 124.3, 119.8, 118.5, 117.8, 113.5, 111.2, 110.1, 106.5, 105.5, 98.3, 79.9, 56.2, 54.4, 53.2, 30.3, 21.0, 20.6, 11.5; LRMS calculated for $C_{46}H_{47}N_3O_{15}$ $[M+H]^+$ m/z; 882.3, measured 882.5.

bis(acetoxymethyl) 2,2'-((4-(3'-acetoxy-6'-(dibutylamino)-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-5-ylcarboxamido)-2-methoxyphenyl)azanediyl)diacetate (**8f**). ^{1}H NMR (600 MHz, acetone-d6) δ 9.77 (s, 1H), 8.54 (s, 1H), 8.40 (d, J = 7.98 Hz, 1H), 7.63 (d, J = 2.10 Hz, 1H), 7.47 (d, J = 7.98 Hz, 1H), 7.38 – 7.35 (m, 1H), 7.16 (d, J = 1.50 Hz, 1H), 6.92 – 6.87 (m, 2H), 6.86 (d, J = 8.64 Hz, 1H), 6.62 (d, J = 8.64 Hz, 1H), 6.54 – 6.50 (m, 2H), 5.78 (s, 4H), 4.20 (s, 4H), 3.83 (s, 3H), 3.38 (t, J = 7.68 Hz, 4H), 2.28 (s, 3H), 2.07 (s, 6H), 1.61 (p, J = 7.64 Hz, 4H), 1.38 (sext, J = 7.51 Hz, 4H), 0.95 (t, J = 7.38 Hz, 6H); ^{13}C NMR (150 MHz, acetone-d6) δ 170.8, 169.9, 169.3, 156.1, 153.5, 153.3, 153.0, 152.2, 151.2, 138.3, 136.0, 135.5, 134.9, 129.9, 129.6, 128.3, 125.4, 124.3, 119.8, 118.5, 117.8, 113.5, 111.2, 110.0, 106.5, 105.3, 98.3, 79.9, 56.2, 54.4, 51.2, 21.0, 20.8, 20.6, 14.2; LRMS calculated for $C_{48}H_{51}N_3O_{15}$ $[M+H]^+$ m/z: 910.3, measured 910.6.

bis(acetoxymethyl) 2,2'-((4-(3'-acetoxy-6'-(cyclohexylamino)-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-5-ylcarboxamido)-2-methoxyphenyl)azanediyl)diacetate (**8g**). ^{1}H NMR (600 MHz, acetone-d6) δ 9.76 (s, 1H), 8.54 (s, 1H), 8.40 (d, J = 7.26 Hz, 1H), 7.62 (d, J = 2.10 Hz, 1H), 7.47 (d, J = 8.04 Hz, 1H), 7.36 (dd, J = 8.58 Hz, J = 2.22 Hz, 1H), 7.15 (s, 1H), 6,89 (s, 2H), 6.85 (d, J = 8.64 Hz, 1H), 6.55 (d, J = 8.70 Hz, 1H), 6.50 (d, J = 2.04 Hz, 1H), 6.46 (dd, J = 8.73 Hz, J = 2.19 Hz, 1H), 5.78 (s, 4H), 4.19 (s, 4H), 3.83 (s, 3H), 3.40 – 3.43 (m, 2H), 2.28 (s, 3H), 2.07 (s, 6H), 1.80 – 1.74 (s, 2H), 1.67 – 1.62 (m, 1H), 1.48 – 1.39 (m, 2H), 1.31 – 1.19 (m, 3H); ^{13}C NMR (150 MHz, acetone-d6) δ 170.8,

169.9, 169.3, 168.8, 153.7, 153.4, 153.0, 152.2, 151.6, 136.0, 135.4, 129.9, 129.6, 128.4, 125.4, 124.4, 119.8, 118.5, 117.8, 113.5, 112.0, 111.2, 106.5, 98.3, 79.9, 56.2, 54.4, 51.9, 33.6, 30.3, 26.6, 25.6, 21.0, 20.5; LRMS calculated for $C_{46}H_{45}N_3O_{15}$ $[M+H]^+$ m/z: 880.3, measured 880.5.

bis(acetoxymethyl) 2,2'-((4-(3'-acetoxy-6'-(butylamino)-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-5-ylcarboxamido)-2-methoxyphenyl)azanediyl)diacetate (**8h**). ^{1}H NMR (600 MHz, acetone-d6) δ 9.76 (s, 1H), 8.54 (s, 1H), 8.40 (d, J = 8.04 Hz, 1H), 7.62 (d, J = 1.92 Hz, 1H), 7.47 (d, J = 8.04 Hz, 1H), 7.36 (dd, J = 8.55 Hz, J = 2.07 Hz, 1H), 7.16 (d, J = 0.72 Hz, 1H), 6.93 – 6.88 (m, 2H), 6.86 (d, J = 8.58 Hz, 1H), 6.58 (d, J = 8.52 Hz, 1H), 6.50 – 6.46 (m, 2H), 5.78 (s, 4H), 4.20 (s, 4H), 3.83 (s, 3H), 3.19 (t, J = 7.05 Hz, 2H), 2.29 (s, 3H), 2.07 (s, 6H), 1.64 (p, J = 7.60 Hz, 2H), 1.46 (sext, J = 8.28 Hz, 2H), 0.95 (t, J = 7.38 Hz, 3H); ^{13}C NMR (150 MHz, acetone-d6) δ 170.8, 170.0, 169.3, 168.8, 164.4, 153.7, 153.4, 153.0, 152.2, 138.3, 136.0, 135.4, 134.9, 129.9, 128.4, 125.5, 124.4, 119.8, 118.6, 117.8, 113.5, 111.7, 111.2, 106.5, 97.9, 79.9, 56.2, 54.4, 42.6, 31.9, 30.3, 21.0, 20.9, 20.6, 14.1; LRMS calculated for $C_{44}H_{43}N_3O_{15}$ $[M+H]^+$ m/z: 854.3, measured 854.1.

bis(acetoxymethyl) 2,2'-((4-(3'-acetoxy-3-oxo-6'-(phenylamino)-3H-spiro[isobenzofuran-1,9'-xanthen]-5-ylcarboxamido)-2-methoxyphenyl)azanediyl)diacetate (**8i**). ^{1}H NMR (600 MHz, CD_3CN) δ 8.89 (s, 1H), 8.54 (s, 1H), 8.29 (d, J = 7.92 Hz, 1H), 7.45 (s, 1H), 7.39 (d, J = 7.98 Hz, 1H), 7.35 (t, J = 7.83 Hz, 2H), 7.22 – 7.18 (m, 3H), 7.14 (d, J = 2.04 Hz, 1H), 7.06 (t, J = 7.35 Hz, 1H), 6.95 (d, J = 2.04 Hz, 1H), 6.93 (d, J = 8.70 Hz, 1H), 6.88 (dd, J = 8.67 Hz, J = 2.07 Hz, 1H), 6.83 (d, J = 8.58 Hz, 1H), 6.80 (dd, J = 8.70 Hz, J = 2.01 Hz, 1H), 6.74 (d, J = 8.76 Hz, 1H), 5.71 (s, 4H), 4.13 (s, 4H), 3.81 (s, 3H), 2.27 (s, 3H), 2.06 (s, 6H); ^{13}C NMR (150 MHz, CD_3CN) δ 170.5, 170.1, 169.0, 164.8, 153.9, 153.0, 152.2, 142.2, 138.3, 135.9, 135.6, 134.5, 130.5, 130.3, 130.2, 128.2, 125.8, 125.3, 123.8, 120.9, 119.9, 117.7, 114.5, 113.8, 111.5, 106.7, 102.0, 80.1, 56.3, 54.4, 21.3, 20.9; LRMS calculated for $C_{46}H_{39}N_3O_{15}$ $[M+H]^+$ m/z: 874.2, measured 874.4.

bis(acetoxymethyl) 2,2'-((4-(3'-acetoxy-6'-(azetidin-1-yl)-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-5-ylcarboxamido)-2-methoxyphenyl)azanediyl)diacetate (**8j**). ^{1}H NMR (600 MHz, CD_3CN) δ 8.90 (s, 1H), 8.57 (s, 1H), 8.29 (dd, J = 8.01 Hz, J = 1.47 Hz, 1H), 7.45 (d, J = 1.98 Hz, 1H), 7.39 (d, J = 7.92 Hz, 1H), 7.22 – 7.18 (m, 2H), 6.96 (d, J = 8.70 Hz, 1H), 6.90 (dd, J = 8.70 Hz, J = 2.16 Hz, 1H), 6.83 (d, J = 8.64 Hz, 1H), 6.76 (d, J = 9.36 Hz, 1H), 6.34 – 6.31 (m, 2H), 5.71 (s, 4H), 4.13 (s, 4H), 4.03 (t, J = 7.38 Hz, 4H), 3.81 (s, 3H), 2.41 (p, J = 7.38 Hz, 2H), 2.27 (s, 3H), 2.06 (s, 6H); ^{13}C NMR (150 MHz, CD_3CN) δ 171.1, 170.5, 170.1, 168.7, 164. 8, 153.3, 152.2, 138.2, 135.9, 135.2, 134.5, 130.5, 130.3, 128.8, 119.9, 119.4, 113.8, 111.5, 106.7, 97.5, 80.1, 56.3, 54.4, 53.2, 21.3, 20.9, 17.1; LRMS calculated for $C_{43}H_{39}N_3O_{15}$ $[M+H]^+$ m/z: 838.2, measured 838.5.

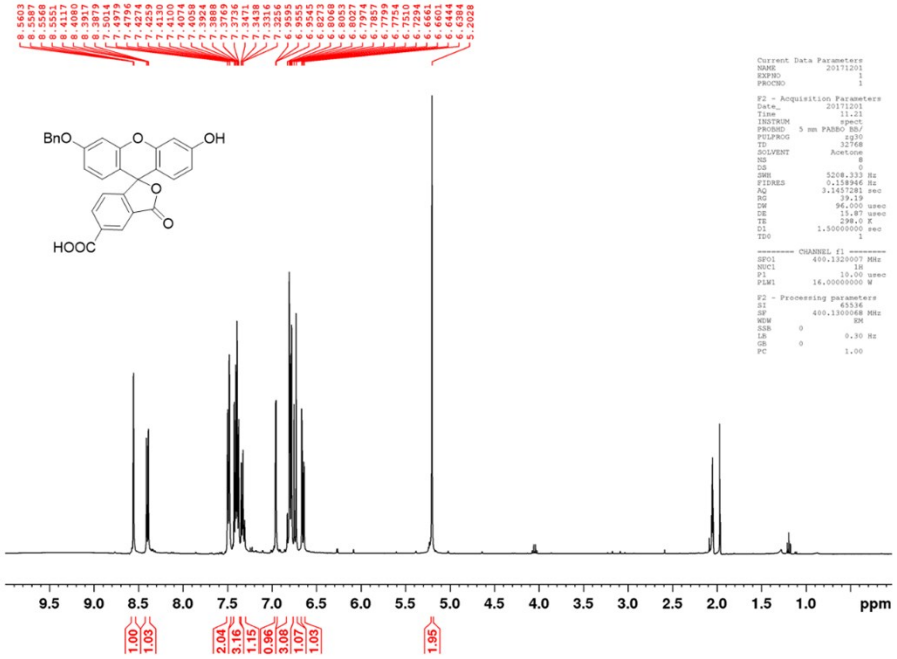
bis(acetoxymethyl) 2,2'-((4-(3'-acetoxy-6'-(3,3-difluoroazetidin-1-yl)-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-5-ylcarboxamido)-2-methoxyphenyl)azanediyl)diacetate (**8k**). ^{1}H NMR (600 MHz, CD_3CN) δ 8.88 (s, 1H), 8.51 (s, 1H), 8.27 (dd, J = 8.01 Hz, J = 1.17 Hz, 1H), 7.45 (d, J = 1.80 Hz, 1H), 7.35 (d, J = 8.04 Hz, 1H), 7.19 (dd, J = 8.58 Hz, J = 2.10 Hz, 1H), 7.12 (d, J = 2.16 Hz, 1H), 6.88 (d, J = 8.64 Hz, 1H), 6.86 – 6.82 (m, 2H), 6.72 (d, J = 8.64 Hz, 1H), 6.44 (d, J = 2.28 Hz, 1H), 6.33 (dd, J = 8.64 Hz, J = 2.34 Hz, 1H), 5.71 (s, 4H), 4.30 (t, J = 12.00 Hz, 4H), 4.13 (s, 4H), 3.81 (s, 3H), 2.26 (s, 3H), 2.06 (s, 6H); ^{13}C NMR (150 MHz, CD_3CN) δ 171.0, 170.5, 170.1, 169.2, 164.8, 156.2, 153.5, 153.0, 152.7, 152.2, 138.3, 135.9, 135.8, 134.5, 130.1, 130.0, 127.9, 125.2, 124.8, 119.9, 119.0, 117.5, 113.8, 111.5, 110.7, $_{109}$.1, 106.6, 100.2, 83.8, 80.1, 64.1, 63.9, 63.8, 56.3, 54.4, 21.2. 20.9; LRMS calculated for $C_{43}H_{37}F_2N_3O_{15}$ $[M+H]^+$ m/z: 874.2, measured 874.4.

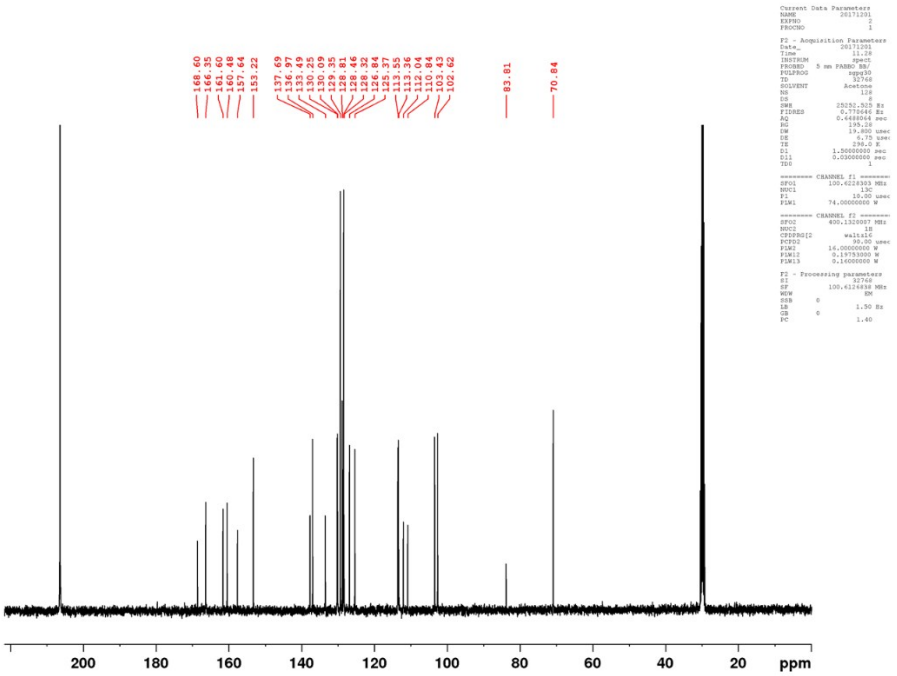
bis(acetoxymethyl) 2,2'-((4-(3'-acetoxy-3-oxo-6'-(pyrrolidin-1-yl)-3H-spiro[isobenzofuran-1,9'-xanthen]-5-ylcarboxamido)-2-methoxyphenyl)azanediyl)diacetate (**8l**). ^{1}H NMR (600 MHz, CD_3CN) δ 8.92 (s, 1H), 8.53 (s, 1H), 8.27 (dd, J = 8.01 Hz, J = 1.41 Hz, 1H),7.45 (dd, J = 1.86 Hz, 1H), 7.36 (d, J = 7.98 Hz, 1H), 7.20 (dd, J = 8.58 Hz, J = 2.10 Hz, 1H), 7.14 (d, J = 2.16

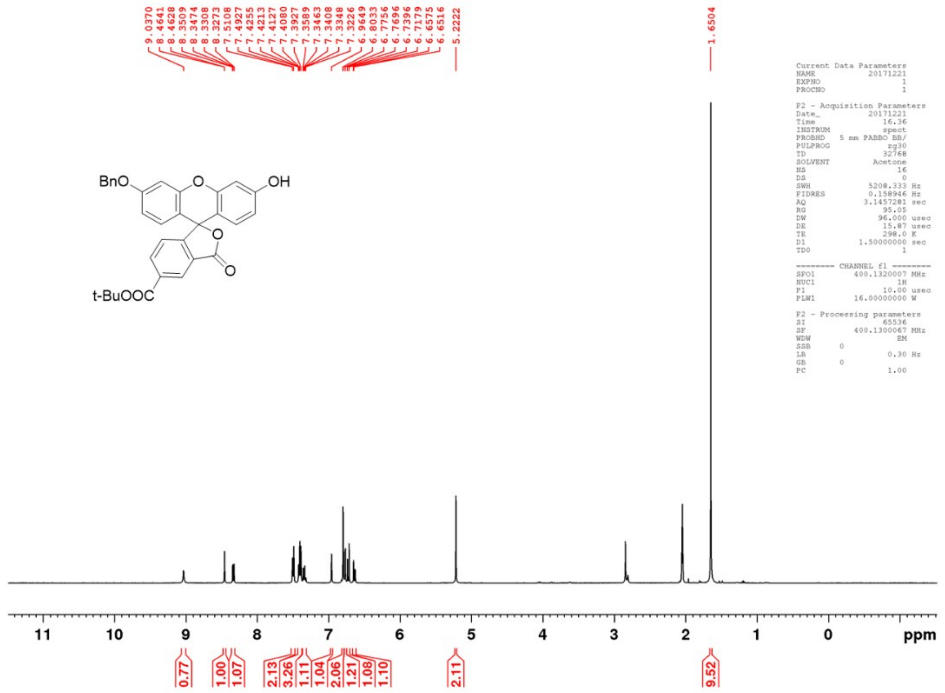
Hz, 1H), 6.90 (d, J = 8.64 Hz, 1H), 6.85 (dd, J = 8.67 Hz, J = 2.25 Hz, 1H), 6.83 (d, J = 8.58 Hz, 1H), 6.68 (d, J = 9.42 Hz, 1H), 6.45 – 6.41 (m, 2H), 5.71 (s, 4H), 4.13 (s, 4H), 3.81 (s, 3H), 3.36 – 3.34 (m, 4H), 2.27 (s, 3H), 2.06 (s, 6H), 2.03 – 1.99 (m, 4H); ^{13}C NMR (150 MHz, CD_3CN) δ 169.2, 164.9, 153.8, 153.2, 152.2, 138.2, 135.8, 135.4, 134.6, 130.2, 130.0, 128.5, 125.8, 125.2, 119.9, 118.9, 118.0, 113.8, 111.4, 111.3, 106.7, 98.4, 80.1, 56.3, 54.4, 48.7, 41.3, 26.1, 21.3, 20.9; LRMS calculated for $C_{44}H_{41}N_3O_{15}$ $[M+H]^+$ m/z: 852.3, measured 852.4.

bis(acetoxymethyl) 2,2'-((4-(3'-acetoxy-3-oxo-6'-(piperidin-1-yl)-3H-spiro[isobenzofuran-1,9'-xanthen]-5-ylcarboxamido)-2-methoxyphenyl)azanediyl)diacetate (**8m**). ^{1}H NMR (600 MHz, acetone-d6) δ 9.76 (s, 1H), 8.55 (s, 1H), 8.40 (d, J = 7.86 Hz, 1H), 7.65 (d, J = 2.28 Hz, 1H), 7.49 – 7.46 (m, 1H), 7.38 – 7.34 (m, 1H), 7.16 (d, J = 1.98 Hz, 1H), 6.94 – 6.88 (m, 2H), 6.87 – 6.84 (m, 1H), 6.80 – 6.76 (m, 2H), 6.71 – 6.67 (m, 1H), 5.78 (app t, 4H), 4.19 (s, 4H), 3.82 (s, 3H), 3.32 (s, 4H), 2.29 (app t, 3H), 2.07 (app t, 6H), 1.69 – 1.60 (m, 6H); ^{13}C NMR (150 MHz, acetone-d6) δ 170.8, 169.9, 169.3, 168.8, 164.3, 156.1, 154.6, 153.4, 153.2, 152.9, 152.2, 138.4, 136.0, 135.6, 134.9, 129.9, 129.4, 128.1, 125.3, 124.4, 119.8, 118.6, 117.6, 113.5, 113.3, 111.2, 108.1, 106.5, 106.4, 102.1, 79.9, 56.2, 54.4, 49.7, 30.3, 26.1, 25.0, 21.0, 20.6; LRMS calculated for $C_{45}H_{43}N_3O_{15}$ $[M+H]^+$ m/z: 866.3, measured 866.4.

NMR spectra



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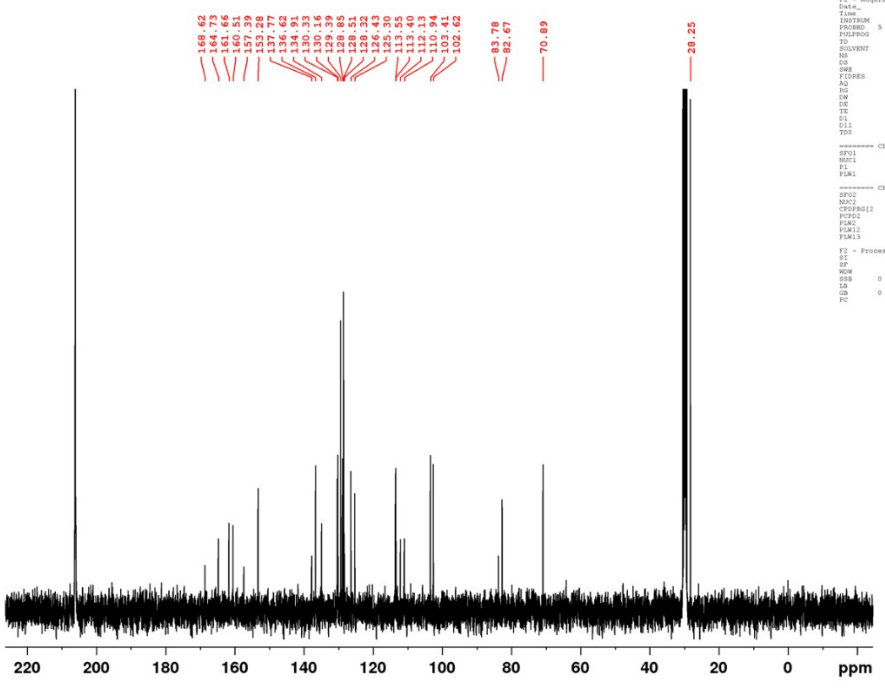
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128.51
128.32
126.43
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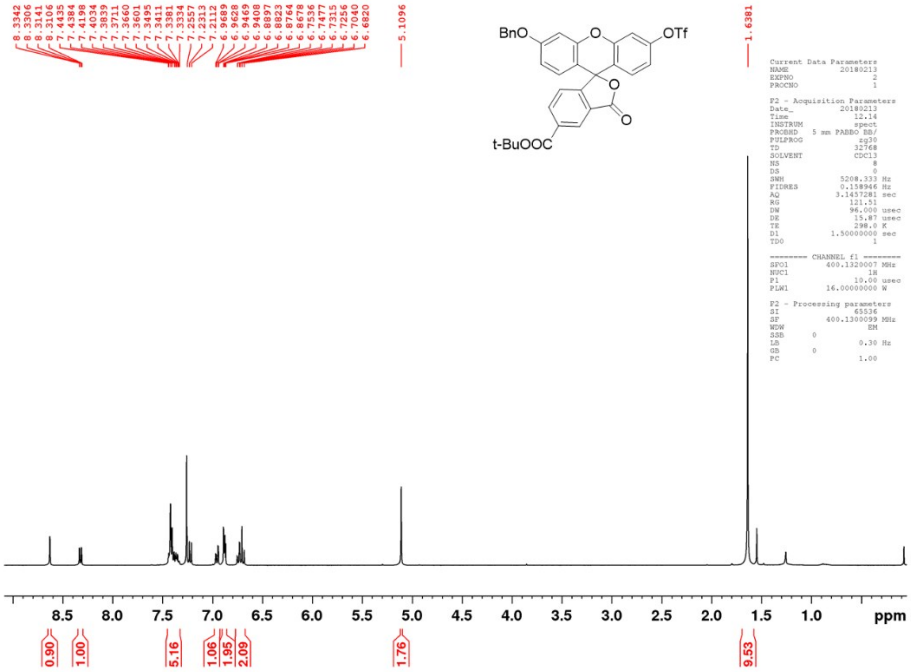
ppm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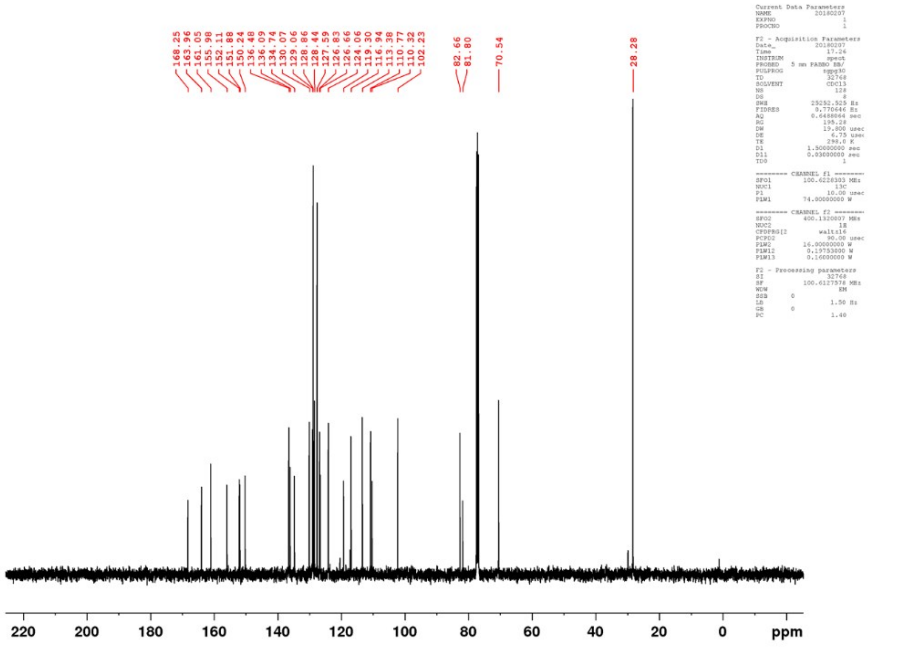
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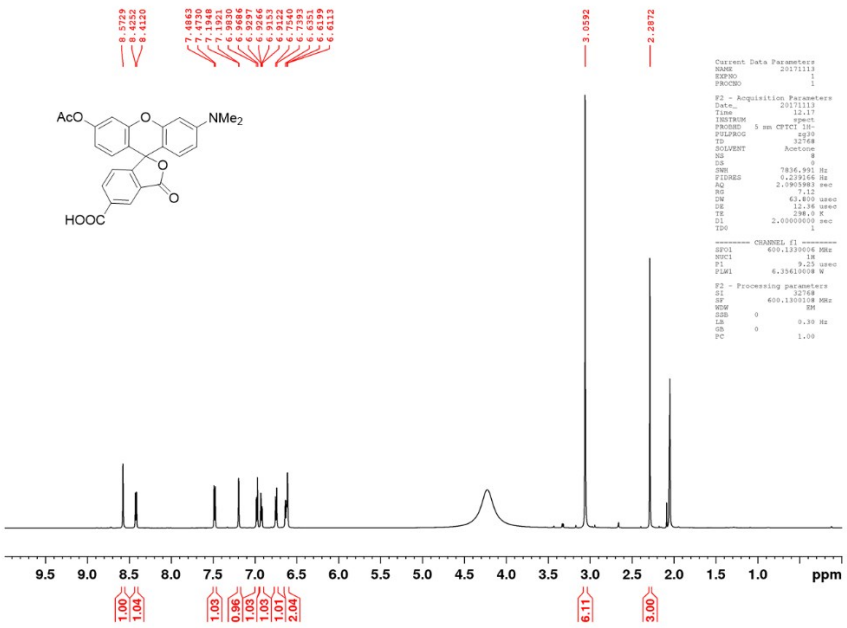
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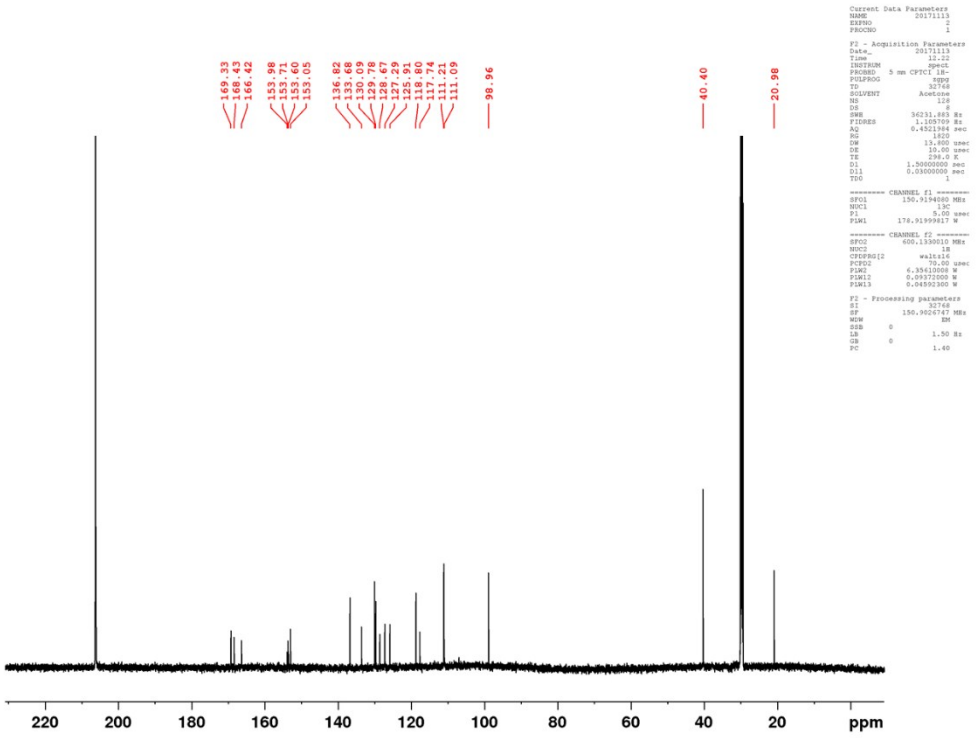
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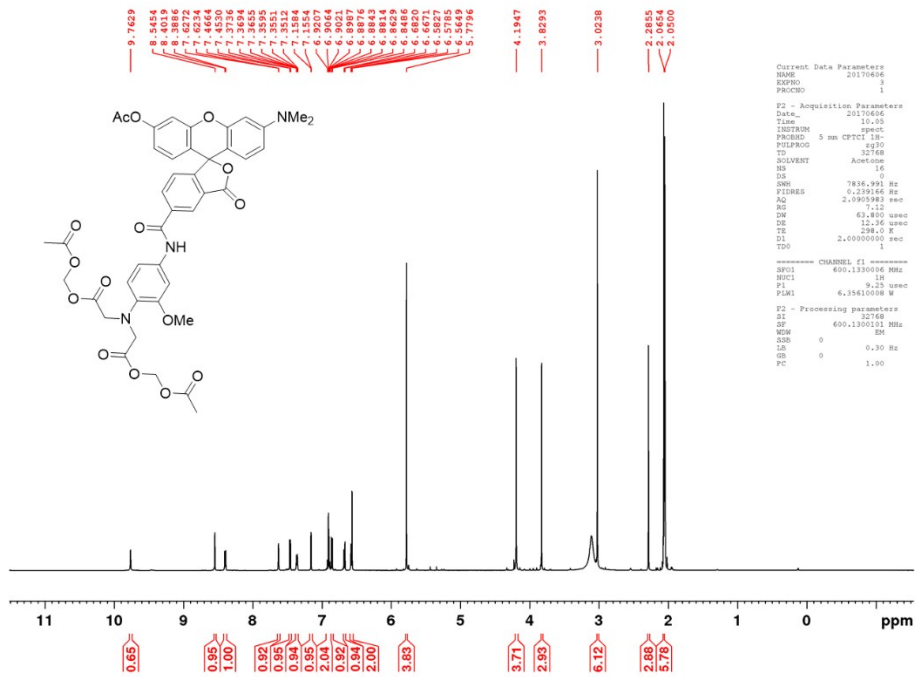
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0.95
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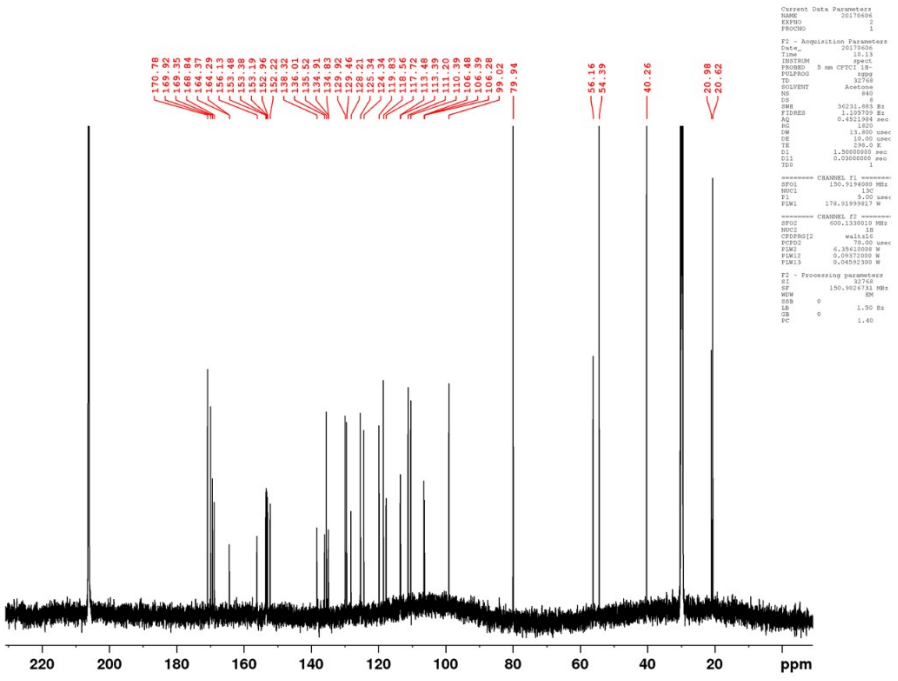
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20
ppm

II. Determination of Photochemical Properties

Evaluation of the photochemical properties of the active Thallium sensing species was accomplished by saponifying 10 µL of each pro-dye stock solution by incubating with 90 µL of 0.1 M KOH for 20 min at 37 °C with the exception of **8b** which was treated with 1% NH_4OH for 1 h at 37 °C. The saponified pro-dyes were diluted into 150 mM KCl buffered with 10 mM HEPES, pH = 7.22 with 50 µM of EDTA. All photochemical measurements were taken in this buffer. Absorbance and fluorescence emission spectra were recorded on a Molecular Devices Spectromax M5 using polystyrene cuvettes with path length = 1 cm at 23 °C. Molar absorptivities are reported for λ_{max}. The emission wavelengths monitored for recording excitation spectra, the excitation wavelengths used for recording emission spectra, and the corresponding emission cutoff filters used are listed below. Spectra for **8b** and **8i** were not recorded because they exhibit very weak fluorescence.

	Excitation Spectra		Emission Spectra	
Compounds	Wavelength (nm)	Cutoff (nm)	Wavelength (nm)	Cutoff (nm)
8a	535	530	480	495
8g, **8h**, **8k**	540	530	505	515
8c-f, **8j**, **8l**, **8m**	560	550	520	530

III. Cell Culture

HEK-293 cells co-expressing GIRK1 and GIRK2 were cultured to 80 % confluence in a T75 flask (Techno Plastic Products, Trasadingen, CH) at 37 °C and 5% CO_2 in medium consisting of Minimal Essential Medium (Cellgro, Manassas, VA) containing 1× Glutagro (Cellgro, Manassas, VA) and 10% (v/v) heat-inactivated fetal bovine serum (Life Technologies, Carlsbad, CA). The cells were dislodged by treatment with TrypLE (Gibco/Life Technologies, Carlsbad, CA), diluted in medium, and plated on clear bottomed, black-walled, amine coated 384 well plates (BD Biosciences, Billerica, MA) at a density ~20,000 cells per well. The plated cells were incubated overnight at 37 °C and 5% CO_2 and used for the assay the next day.

IV. Confocal microscopy

HEK cells co-expressing GIRK 1 and GIRK2 were imaged by confocal microscopy using a Zeiss LSM880 AiryScan and a 40x/1.30 C Plan-Apochromat Oil objective on a 8-well IbiTreat μ-Slide (Ibidi, Madison, WI). Thallos was imaged using a 488 nm laser and emission collected 493 – 576 nm, **8c** and Rhodamine 123 were imaged using a 514 nm laser and emission collected 519 – 683 nm, and Hoechst was imaged using a 405 nm laser and emission collected 462 – 680 nm. Cells were plated on the slide the previous day and culture medium was replaced by 20 μL of assay buffer (1X HBSS, 4 mM $NaHCO_3$, and 20 mM HEPES, pH = 7.3) containing either 1 μM of Thallos, 5 μM of **8c**, or 1 μM of rhodamine 123 (Sigma-Aldrich, St. Louis, MO). Cells were incubated in the dark for 1 h at room temperature. The dye loading buffer was then replaced with assay buffer with 1 μg/mL of Hoechst 33342 (Life Technologies, Carlsbad, CA) and incubated for 15 min at room temperature. The second dye loading buffer was then replaced with 20 μL of assay buffer and the cells were imaged.

V. Tl^+ flux assays

General procedure:

Dye loading: Pro-dyes **8a** – **m** were diluted to a concentration of 5 μM in assay buffer (1X HBSS, 4 mM $NaHCO_3$, and 20 mM HEPES, pH = 7.3). Dye loading solutions of Thallos were prepared by dissolving a 25 μg aliquot in 30 μL of 6.7 % Pluoronic F-127 (w/v) in DMSO and diluting the resulting solution 1:1000 into assay buffer. Culture medium was removed and replaced with 20 μL of dye loading buffer and incubated in the dark at room temperature for 1 h.

Assay: Assays were conducted on a WaveFront Biosciences Panoptic 1 or 2 (WaveFront Biosciences, Franklin, TN) as indicated. Dye loading solutions were removed and replaced with assay buffer immediately before imaging unless otherwise noted. 10 s following commencement of plate imaging, 20 μL of compound at 2.5x concentration in assay buffer was added and incubated for 120 s at which point 10 μL of Tl^+ at 5x concentration in stimulus buffer (125 mM

$NaHCO_3$, 1.8 mM $CaSO_4$, 1 mM $MgSO_4$, 5 mM glucose, 10 mM HEPES, pH 7.4, and Tl_2SO_4), and images taken for the following 120 s.

On/off ratio of **8a** – **m** determined in HEK-293 cells co-expressing GIRK1 and GIRK2

The general Tl^+ flux assay procedure outlined above was conducted on a WaveFront Biosciences Panoptic 1 using a checkerboard of 10 μM of ML297 (final concentration) and vehicle. 1 mM of Tl_2SO_4 (final concentration) was used for stimulus. Assay and stimulus buffers were supplemented with 2 mM of Allura Red AC. **8c** – **m** were imaged using 517/20 nm excitation and 562/40 nm emission filters and **8a** and **8b** were imaged using 480/40 nm excitation and 538/40 nm emission filters (Semrock, Rochester, NY). On/off ratios were calculated by dividing the fluorescence intensity after saturation following Tl^+ addition by the fluorescence intensity immediately after compound addition (n = 6).

ML297 concentration-response curves

General: All concentration-response data were collected on a WaveFront Biosciences Panoptic 2. Thallos treated cells were imaged using 482/35 nm excitation and 536/40 nm emission filters. **8c** treated cells were imaged using 529/24 nm excitation and 565/24 nm emission filters (unless otherwise noted). FluxOR Red treated cells were imaged using 544/24 nm excitation and 593/40 nm emission filters (all filters purchased from Semrock, Rochester, NY). FluxOR Red reagent was prepared according to the manufacturer's procedure. 5 μM of **8c** was used for dye loading for all conditions. Dye loading solutions of Thallos were prepared by following the reagent preparation steps listed above and diluting this 1:1000 into the corresponding dye loading buffer.

FluxOR Red procedure with background suppressor: The manufacturer's procedure was followed with the exceptions that the dye loading solution was replaced with 20 μL of assay buffer, solutions of ML297 were prepared in assay buffer plus background suppressor, 20 μL of compound solution was added and 10 μL stimulus buffer (basal) was added according to the general assay protocol described above.

FluxOR Red procedure without background suppressor: The FluxOR Red manufacturer's procedure was followed except the background suppressor was replaced by H_2O in all buffers.

Bicarbonate buffer with Allura Red AC. The assay and stimulus buffers described in the general Tl^+ flux procedure were used for dye loading, assay buffer with ML297, and stimulus buffer. All buffers were supplemented with 1 mM of Allura Red AC. The wash step to remove dye loading buffer was omitted.

Bicarbonate buffer without Allura Red AC: The general Tl^+ flux procedure was followed without any solutions supplemented with Allura Red AC.

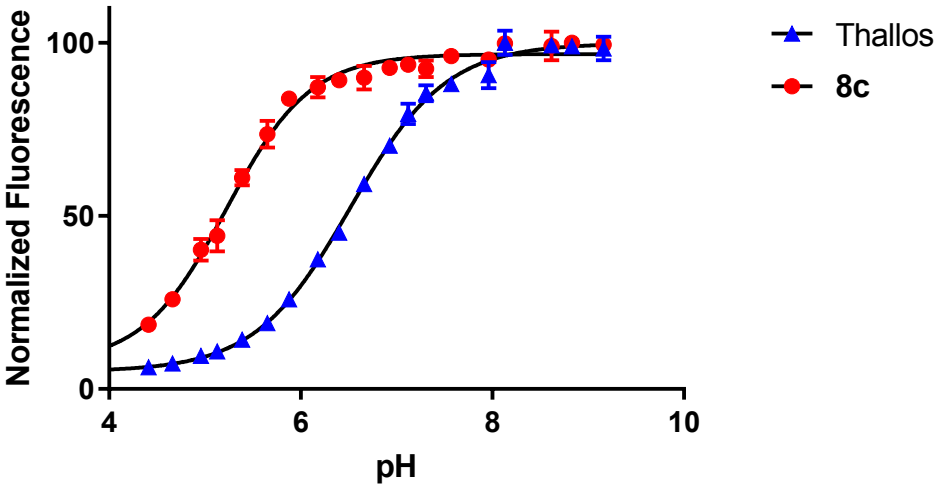
Data from all experiments were processed as follows: The fluorescence intensity values of the first 6 time points for each well were averaged and the fluorescence intensity for all time points in the well was divided by this value to provide F/F_o for each time point. The average F/F_o of the vehicle control was then subtracted from each curve and the normalized, control subtracted fluorescence intensity value was sampled at a time point several seconds after Tl^+ addition. These data were averaged (n = 6 – 12 for each concentration of ML297) and fit to a curve using GraphPad Prism. Fold increases in fluorescence reported in the main paper were calculated by averaging F/F_o between 100 and 110 s after Tl^+ addition. Data presented in Figure 3 and Supplemental Figure 3 were obtained using **8c** with the FluxOR Red kit as described above under the FluxOR Red with background suppressor procedure.

VI. pK_a determination

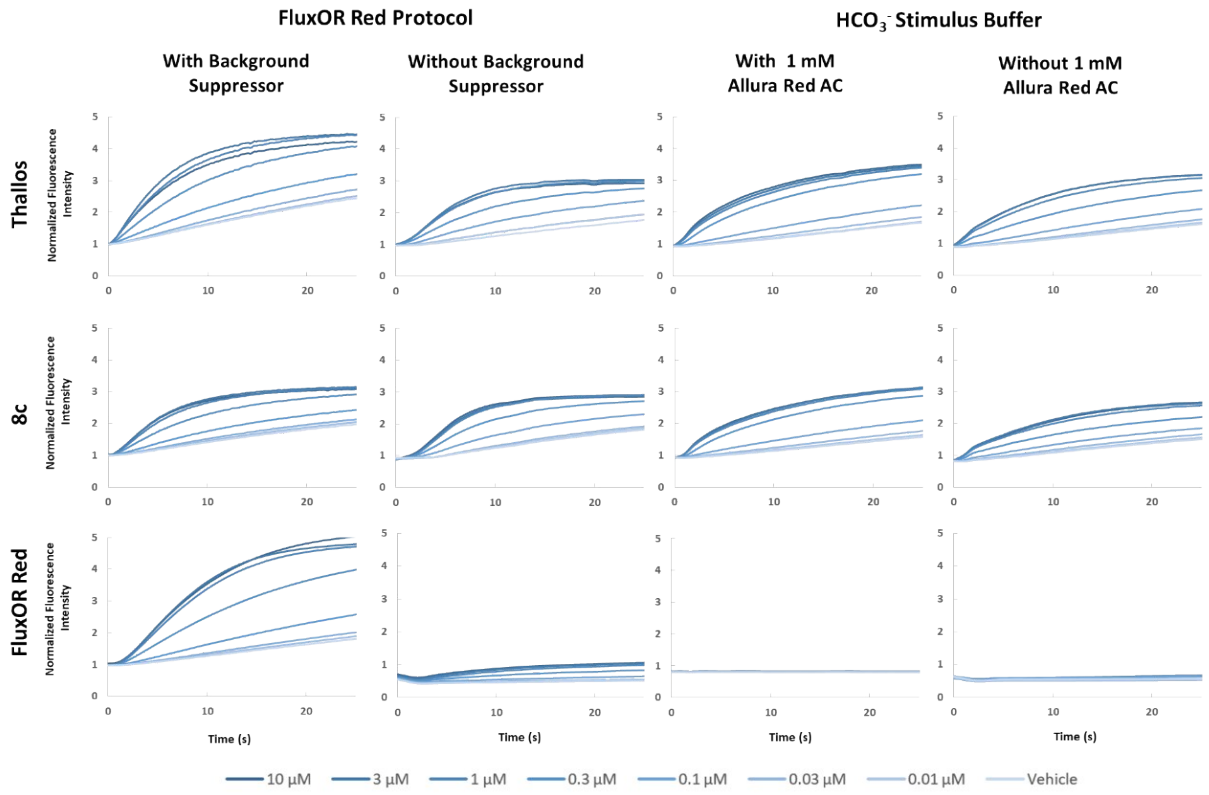
Saponified **8c** and Thallos were prepared and stored as 10 mM solutions in DMSO. These were diluted to 200 μM in DMSO. 5 μL of these solutions were diluted into 195 μL of buffers consisting of 150 mM KCl, 50 μM EDTA, and 10 mM of either potassium citrate (pH = 4 – 6), potassium phosphate (pH = 6.25 – 8), or Tris-HCl (pH = 8 – 9.5) on a 96-well, black walled, clear bottom plate (Greiner Bio-one, Monroe, NC). Fluorescence measurements were recorded on a Biotek Neo Synergy plate reader using excitation and emission wavelengths of 485 nm and 528

nm for Thallos and 500 nm and 565 nm for **8c**. Fluorescence intensity data were average for each pH (n = 3) and the data fit to a curve using GraphPad Prism.

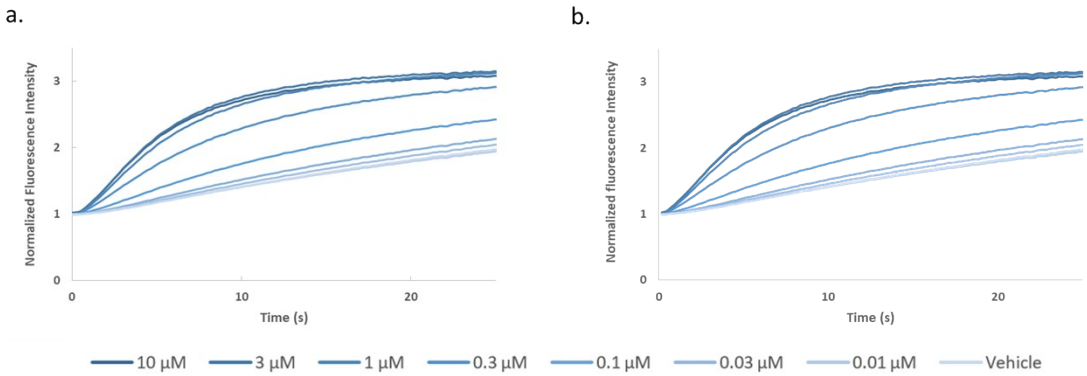
VI. Supplemental Figures and Tables



Supplemental Figure 1: Comparison of pH sensitivity between Thallos and **8c**.



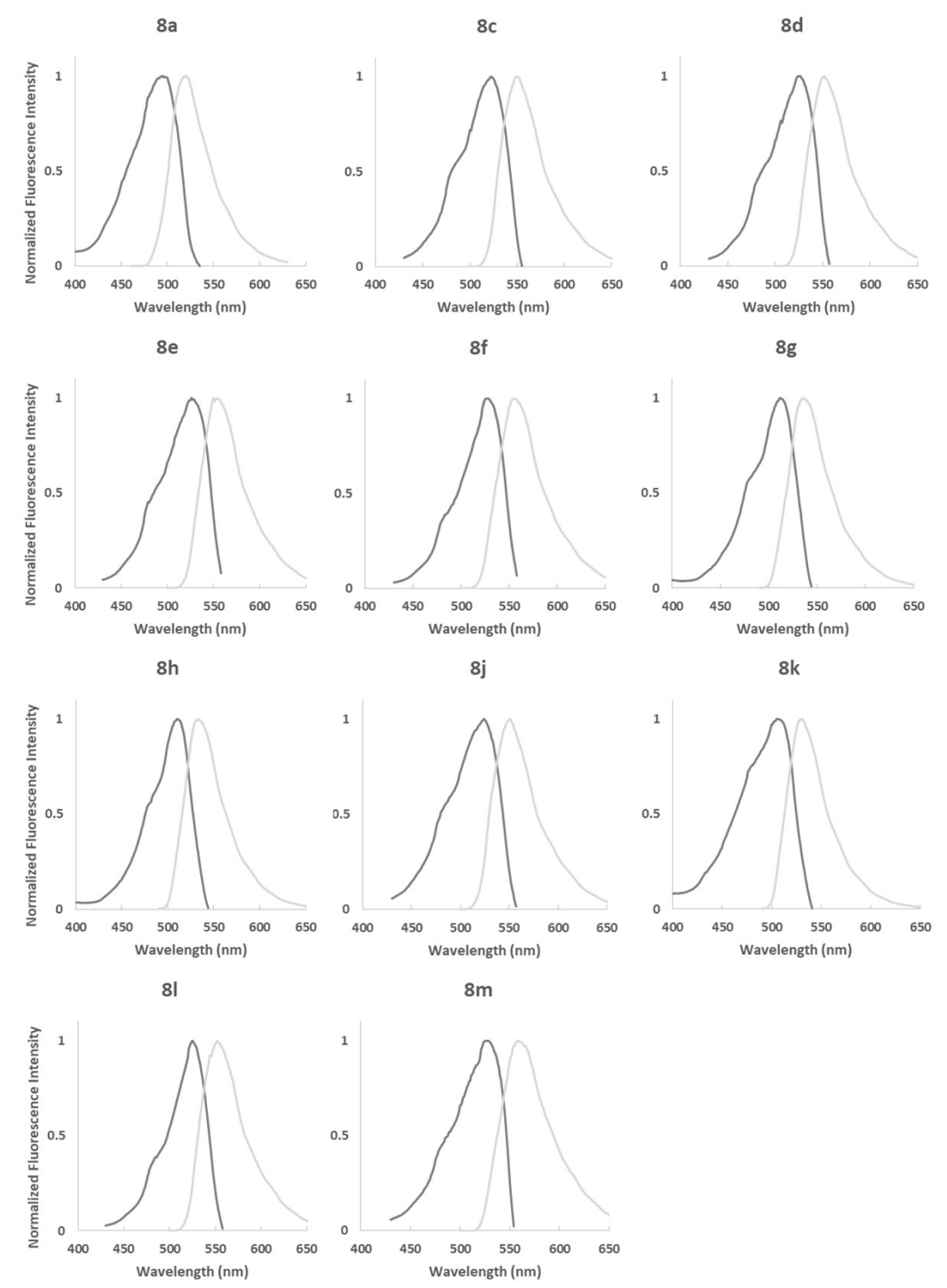
Supplemental Figure 2. Comparison of dynamic range between Thallos, **8c**, and FluxOR Red under the indicated conditions. Tl^+ flux assays were conducted in HEK-293 cells co-expressing GIRK1 and GIRK2 and treated with varying concentrations of the GIRK activator ML297. Fluorescence intensity data from each well were normalized to the averaged fluorescence intensity of the first 6 time points of the experiment and the normalized fluorescence data for each replicate time point were averaged (n = 6 – 12). The plots show normalized fluorescence intensity beginning at the moment of Tl^+ stimulus addition.



Supplemental Figure 3. Comparison of data obtained during a Tl^+ flux assay to generate a ML297 concentration response curve using **8c** as the indicator and (a) 529/24 nm excitation and 565/24 nm emission filters and (b) 544/24 nm excitation and 593/40 nm emission filters. Fluorescence intensity data from each well were normalized to the averaged fluorescence intensity of the first 6 time points of the experiment and the normalized fluorescence data for each replicate time point were averaged (n = 6 – 12). The plots show normalized fluorescence intensity after addition of Tl^+ stimulus.

	FluxOR Red Kit		HCO_3^- Stimulus Buffer	
	Background Suppressor		1 mM Allura Red AC	
Dye	**+**	**-**	**+**	**-**
Thallos	**6.57 ± 0.04**	**6.65 ± 0.02**	**6.66 ± 0.01**	**6.61 ± 0.01**
FluxOR Red	**6.64 ± 0.01**	**6.41 ± 0.03**	**ND**	**7.02 ± 0.1**
8c	**6.59 ± 0.01**	**6.61 ± 0.02**	**6.69 ± 0.01**	**6.59 ± 0.03**
8c (544x/593m)	**6.63 ± 0.01**	**6.66 ± 0.04**		

Supplemental Table 1: pEC_{50} and standard error of ML297 activation of GIRK1 and GIRK2 co-expressing HEK-293 cells determined from concentration response curves fit using data obtained under the corresponding conditions.



Supplemental Figure 4: Excitation and emission spectra. Spectra were recorded as described and normalized to the maximum value for each condition.

VII: References

[1] P. Hammershøj, E. K. P. Kumar, P. Harris, T. L. Andresen, M. H. Clausen, *European J. Org. Chem.* **2015**, *2015*, 7301–7309.

[2] K. R. West, K. D. Bake, S. Otto, *Org. Lett.* **2005**, *7*, 2615–2618.

[3] C. D. Weaver, *Thallium Fluorescent Ion Indicator and Assay*, **2015**, US9,103,791 B1.