Supplementary Information for

A 2-Aza-Cope Reactivity-Based Platform for Ratiometric Fluorescence Imaging of Formaldehyde in Living Cells

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General Methods. All reactions utilizing air- or moisture-sensitive reagents were performed in dried glassware under an atmosphere of dry N_2. When dry solvent was used, the solvent was passed over activated alumina. Other reagents were used without further purification. Silica gel P60 (SiliCycle) was used for column chromatography and SiliCycle 60 F254 silica gel (precoated sheets, 0.25 mm thick) was used for analytical thin layer chromatography and visualized by fluorescence quenching under UV light. Coumarins **1**, **2**, and RFAP-1-Ald were synthesized according to literature procedures.[1] 2-(2-((6-chlorohexyl)oxy)ethoxy)ethan-1-amine was synthesized according to literature procedures.[2] Prenylboronic acid solution was prepared using a slightly modified literature procedure.[3] 3-methyl-2-buten-1-ol and tetrahydroxydiboron were purchased from AK Scientific (Union City, CA); ethyl 4-pentenoate was purchased from TCI America (Portland, OR); 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate was purchased from ChemPep Inc. (Wellington, FL); 4-hydroxynonenal solution was purchased from Cayman Chemical (Ann Arbor, MI); glucosone was purchased from Santa Cruz Biotech (Dallas, TX); and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO). ^{1}H NMR and ^{13}C NMR spectra were collected in $CDCl_3$ or C_6D_6 (Cambridge Isotope Laboratories, Cambridge, MA) at 25 °C on Bruker AVB-400, AVQ-400, AV-500, and AV-600 with ^{13}C operating frequencies of 101 MHz, 126 MHz, and 150 MHz, respectively, at the College of Chemistry NMR Facility at the University of California, Berkeley. All chemical shifts are reported in the standard δ notation of parts per million relative to residual solvent peak at 7.26 ($CDCl_3$) or 7.16 (C_6D_6) for ^{1}H and 77.16 ($CDCl_3$) or 128.06 (C_6D_6) for ^{13}C as an internal reference. Splitting patterns are indicated as follows: br, broad; s, singlet; d, doublet; t, triplet; m, multiplet; dd, doublet of doublets. Low-resolution electrospray mass spectral analyses were carried out using a LC-MS (Agilent Technology 6130, Quadrupole LC/MS). High resolution mass spectral analyses (ESI-MS) were carried out at the College of Chemistry Mass Spectrometry Facility at the University of California, Berkeley.

Probe Synthesis and New Compound Characterization.

Preparation of RFAP-0. *Synthesis of Compound **2***. Compound **2** was prepared through alkyl cuprate addition into compound **1** according to published procedures.[1]

*Synthesis of compound **3***. To a solution of compound **2** (100 mg, 0.23 mmol) in 5 mL of 4:1 MeOH:DCM at 0 °C was added 0.33 mL of NH_3 solution (7 N in MeOH, 2.3 mmol). The reaction mixture was stirred at 0 °C for 30 min, then allylboronic acid pinacol ester (51 µL, 0.27 mmol) was added, and the reaction mixture was warmed to ambient temperature and stirred for 10 h. The solvent was removed under reduced pressure, and purification by silica column chromatography (0→3% MeOH /DCM) afforded compound **3** as a pale yellow solid (105 mg, 96%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.09 (s, 1H), 5.74 (ddt, *J* = 17.1, 10.0, 7.2 Hz, 1H), 5.08 (dd, *J* = 17.1, 2.0 Hz, 1H), 4.99 (dd, *J* = 10.0, 2.1 Hz, 1H), 4.04 (br s, 1H), 3.72 (t, *J* = 5.6 Hz, 2H), 3.23 (dt, *J* = 10.9, 6.0 Hz, 4H), 2.87 (t, *J* = 6.5 Hz, 2H), 2.84 – 2.72 (m, 4H), 2.72 – 2.59 (m, 2H), 2.34 (br s, 2H), 2.08 – 1.85 (m, 4H), 1.80 – 1.70 (m, 2H), 0.94 (s, 9H), 0.09 (s, 6H). LRMS calcd. for $C_{28}H_{43}N_2O_3Si$ (M+H) 483.3, found 483.5.

Synthesis of RFAP-0. Compound **3** (20 mg, 40 μmol) was dissolved in 1.5 mL of 3:1 AcOH:H_2O, and the reaction mixture was stirred for 10 hours. The reaction mixture was carefully basified with 10% K_2CO_3/H_2O to pH >11, then extracted with DCM (4 x 25 mL). The combined organic layers were washed with brine (1 x 100 mL). The organic layer was dried over anhydrous Na_2SO_4, and the solvent was removed under reduced pressure. Purification by silica column chromatography (gradient from DCM to 10% MeOH/DCM) afforded RFAP-0 as a yellow powder (11 mg, 72% yield). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.06 (s, 1H), 5.69 (dt, *J* = 17.0, 8.1 Hz, 1H), 5.12 (d, *J* = 17.0 Hz, 1H), 5.05 (d, *J* = 9.9 Hz, 1H), 4.60 (br s, 1H), 3.66 – 3.35 (m, 7H), 3.26 (dt, *J* = 11.6, 5.5 Hz, 4H), 3.08 – 2.98 (m, 1H), 2.90 – 2.82 (m, 2H), 2.81 – 2.71 (m, 3H), 2.03 – 1.92 (m, 4H), 1.82 (br s, 1H). HRMS calcd. for $C_{22}H_{29}N_2O_3$ (M+H) 369.2173, found 369.2175.

Preparation of prenylboronic acid solution. A 0.3 M solution of H_2PdCl_4 was prepared by dissolving $PdCl_2$ (54 mg, 0.3 mmol) in 1 mL 0.9 M aqueous HCl and stirring for 8 h. Prenol (0.61 mL, 6 mmol) was dissolved in 12 mL of 4:1 DMSO:H_2O, followed by addition of 0.3 M H_2PdCl_4 (0.98 mL, 0.3 mmol) and $B_2(OH)_4$ (0.65 g, 7.2 mmol). The reaction mixture was stirred under N_2 for 10 h, then diluted with 22 mL $CHCl_3$ and stirred for an additional 10 minutes. The reaction mixture was filtered through a cotton plug, then 20 mL brine was added. The biphasic mixture was vigorously agitated and the layers were allowed to separate. The organic layer was collected, followed by additional brine washes (2 x 20 mL). The organic layer was dried over Na_2SO_4, the volume was measured, and the solution was used directly in further reactions. The yield was assumed to be 50%,[3] resulting in ca. 0.13 M prenylboronic acid solution.

Preparation of RFAP-1. *Synthesis of Compound **5***. Commercial 7N NH_3 solution (0.33 mL, 2.3 mmol) was added to coumarin aldehyde **2** (100 mg, 230 μmol) dissolved in 5 mL of 4:1 MeOH:DCM and stirred under N_2 for 30 minutes. Freshly prepared (see preparation above) 0.13 M prenylboronic acid solution (2.3 mL, 300 μmol) was added, and a white precipitate immediately formed. After stirring for 1 hour, the reaction mixture became homogeneous. The reaction mixture was stirred for an additional 9 h, and TLC (5% MeOH/DCM) showed complete consumption of starting material. The solvent was removed under reduced pressure. Purification by silica column chromatography (gradient from 1% MeOH/DCM with 0.2% NH_4OH to 3% MeOH/DCM with 0.2% NH_4OH) afforded **5** as a yellow powder (110 mg, 95% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.08 (s, 1H), 5.93 (dd, J = 17.3, 10.8 Hz, 1H), 5.01 – 4.91 (m, 2H), 3.87 (s, 1H), 3.75 – 3.64 (m, 2H), 3.26 – 3.15 (m, 4H), 2.95 – 2.76 (m, 4H), 2.75 (t, J = 6.4 Hz, 2H), 2.33 (br s, 2H), 2.01 – 1.88 (m, 4H), 1.80 – 1.65 (m, 2H), 1.08 (d, J = 12.5 Hz, 6H), 0.93 (s, 9H), 0.08 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 161.9, 153.3, 150.4, 147.0, 145.1, 122.4, 118.3, 118.1, 111.6, 108.2, 106.4, 62.5, 58.5, 49.9, 49.5, 43.5, 32.7, 27.9, 26.1, 25.5, 25.3, 24.0, 21.8, 20.8, 20.4, 18.4, -5.2; LRMS calcd. for $C_{30}H_{47}N_2O_3Si$ (M+H) 511.34, found 511.5.

Synthesis of RFAP-1. Compound **5** (53 mg, 100 μmol) was dissolved in 4 mL of 3:1 AcOH:H_2O, and the reaction mixture was stirred for 10 hours. The reaction mixture was carefully basified with 10% K_2CO_3/H_2O to pH >11, then extracted with DCM (4 x 25 mL). The combined organic layers were washed with brine (1 x 100 mL). The organic layer was dried over anhydrous Na_2SO_4, and the solvent was removed under reduced pressure. Purification by silica column chromatography (gradient from 4% MeOH/DCM to 10% MeOH/DCM) afforded RFAP-1 as a yellow powder (28 mg, 68% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.06 (s, 1H), 5.97 – 5.84 (m, 1H), 5.05 – 4.95 (m, 2H), 4.09 (s, 1H), 3.63 – 3.56 (m, 1H), 3.52 – 3.43 (m, 1H), 3.40 – 3.19 (m, 7H), 3.17 – 3.06 (m, 1H), 2.95 – 2.81 (m, 3H), 2.81 – 2.71 (m, 2H), 2.03 – 1.90 (m, 5H), 1.79 – 1.68

(m, 1H), 1.08 (s, 3H), 1.04 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 162.2, 154.7, 150.6, 146.1, 145.6, 122.7, 118.5, 115.5, 113.1, 108.0, 106.5, 60.1, 58.2, 50.0, 49.6, 43.5, 32.2, 28.0, 25.7, 25.0, 23.4, 21.8, 20.8, 20.5. HRMS calcd. for $C_{24}H_{33}N_2O_3$ (M+H) 397.2486, found 397.2484.

Preparation of RFAP-2. *Synthesis of Compound **7***. To prepare a solution of the alkylborane coupling partner **6**, a round-bottom flask was charged with ethyl 4-pentenoate (0.56 mL, 3.9 mmol) and commercial 0.5 M 9-BBN solution (8.6 mL, 4.3 mmol). The reaction mixture was stirred overnight and used directly for Suzuki coupling.

A 2-neck round-bottom flask was charged with Compound **1** (0.66 g, 2.2 mmol), potassium phosphate tribasic monohydrate (0.81 g, 3.5 mmol), and PEPPSI-IPr catalyst (60 mg, 90 µmol). The atmosphere was replaced with N_2 by 5 evacuation/refill cycles, then a freshly-prepared 0.4 M solution of compound **6** (8.8 mL, 3.5 mmol) was added. The reaction mixture was stirred for 10 h, and TLC (EtOAc) showed complete consumption of starting material. The reaction mixture was diluted with 50 mL EtOAc, run through a plug of silica gel (with EtOAc washes), and concentrated under reduced pressure. Purification by silica column chromatography (gradient from 25% EtOAc/hexanes to 35% EtOAc/hexanes) afforded **7** as an orange oil (0.5 g, 58% yield). ^{1}H NMR (400 MHz, C_6D_6) δ 10.85 (s, 1H), 7.02 (s, 1H), 3.97 (q, J = 7.1 Hz, 2H), 3.22 – 3.11 (m, 2H), 2.61 (t, J = 6.5 Hz, 2H), 2.57 – 2.47 (m, 4H), 2.35 (t, J = 6.1 Hz, 2H), 2.21 (t, J = 7.2 Hz, 2H), 1.81 (p, J = 7.3 Hz, 2H), 1.64 – 1.51 (m, 2H), 1.41 – 1.34 (m, 4H), 0.97 (t, J = 7.1 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 190.9, 173.6, 163.6, 163.1, 152.6, 148.6, 124.3, 119.6, 110.4, 108.2, 106.1, 60.3, 50.3, 49.8, 34.0, 29.9, 27.8, 27.3, 25.3, 21.2, 20.2, 20.2, 14.3. LRMS calcd. for $C_{23}H_{28}NO_5$ (M+H) 398.20, found 398.4.

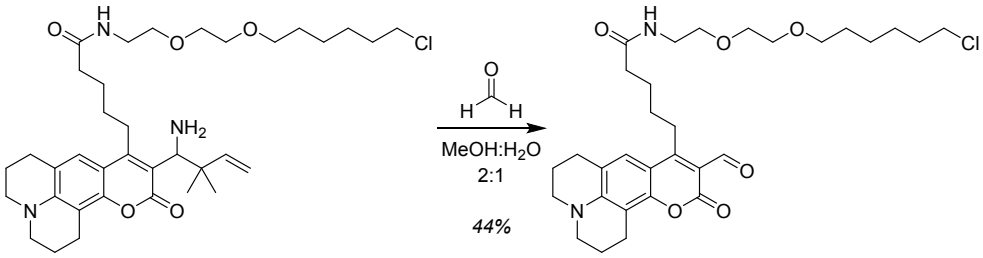
*Synthesis of Compound **8***. Commercial 7N NH_3 solution (3.2 mL, 23 mmol) was added to coumarin aldehyde **7** (0.9 g, 2.3 mmol) dissolved in 25 mL of 3:2 MeOH:DCM and stirred under N_2 for 30 minutes. Freshly prepared (see preparation above) 0.13 M prenylboronic acid solution (35 mL, 4.6 mmol) was added, and the reaction mixture was stirred for 10 h. The solvent was removed under reduced pressure. The residue was dissolved in 100 mL DCM and washed sequentially with 1% K_2CO_3 (2 x 100 mL) and brine (1 x 100 mL). The organic layer was dried over anhydrous Na_2SO_4, and the solvent was removed under reduced pressure. Purification by silica column chromatography (gradient from DCM to 3% MeOH/DCM) afforded **8** as a yellow oil (0.66 g, 62% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.98 (s, 1H), 5.93 (dd, J = 17.3, 10.8 Hz, 1H), 5.03 – 4.93 (m, 2H), 4.12 (q, J = 7.2 Hz, 2H), 3.82 (s, 1H), 3.28 – 3.17 (m, 4H), 2.89 – 2.65 (m, 6H), 2.56 – 2.26 (m, 4H), 2.02 – 1.90 (m, 4H), 1.83 – 1.73 (m, 2H), 1.68 –1.50 (m, 2H), 1.24 (t, J = 7.1 Hz, 3H), 1.10 (s, 3H), 1.07 (s, 3H); ^{13}C NMR (101 MHz, C_6D_6) δ 172.7, 161.4, 151.6, 151.1, 147.9, 145.0, 122.6, 120.3, 117.9, 111.2, 108.7, 107.0, 60.2, 59.5, 49.8, 49.4, 43.8, 33.9, 29.2, 28.6, 28.1, 25.4, 25.2, 25.1, 22.1, 21.0, 20.8, 14.4. LRMS calcd. for $C_{28}H_{39}N_2O_4$ (M+H) 467.29, found 467.4.

*Synthesis of Compound **9***. Compound **8** (0.28 g, 0.6 mmol) was dissolved in 10 mL of 3:1:1 MeOH:THF:H_2O and LiOH•H_2O (33 mg, 0.78 mmol) was added. The reaction was stirred for 10 h, and TLC (20% MeOH/DCM) showed complete consumption of starting material. The solvent was removed under reduced pressure. Purification by silica column chromatography (gradient from 5% MeOH/DCM to 20% MeOH/DCM) afforded **9** as a yellow powder (0.25 g, 90% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.19 (br s, 3H), 7.01 (s, 1H), 5.91 (dd, J = 17.4, 10.7 Hz, 1H), 5.11 – 5.01 (m, 2H), 4.43 (s, 1H), 3.27 – 3.12 (m, 4H), 2.86 – 2.68 (m, 5H), 2.62 (br s, 1H), 2.20 (br s, 2H), 2.02 – 1.83 (m, 4H), 1.83 – 1.69 (br s, 1H), 1.68 – 1.50 (br s,

3H), 1.16 (s, 3H), 1.13 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 179.3, 162.5, 156.8, 150.6, 145.8, 144.6, 122.6, 118.7, 113.8, 111.5, 107.9, 106.2, 56.3, 49.9, 49.5, 42.6, 35.6, 28.4, 28.0, 27.9, 25.8, 25.0, 24.1, 21.6, 20.6, 20.3. LRMS calcd. for $C_{26}H_{35}N_2O_4$ (M+H) 439.26, found 439.4.

Synthesis of RFAP-2. A flame-dried round bottom flask was charged with compound **9** (5 mg, 11.4 µmol), 2-(2-((6-chlorohexyl)oxy)ethoxy)ethan-1-amine (8 mg, 34.2 µmol), and HATU (13 mg, 34.2 µmol), followed by addition of 1 mL dry DMF and triethylamine (10 µL, 57 µmol). The reaction mixture as stirred under N_2 for 8 h. The solvent was removed under reduced pressure. Purification by silica column chromatography (gradient from 6% MeOH/DCM to 8% MeOH/DCM) afforded RFAP-2 as a yellow powder (1.6 mg, 22% yield). ^{1}H NMR (400 MHz, C_6D_6) δ 7.80 (br s, 2H), 7.29 (s, 1H), 6.84 (s, 1H), 6.15 (dd, J = 17.3, 10.8 Hz, 1H), 5.26 (d, J = 10.8 Hz, 1H), 5.15 (d, J = 17.3 Hz, 1H), 4.77 (s, 1H), 3.52 – 3.39 (m, 6H), 3.38 – 3.29 (m, 3H), 3.22 (t, J = 6.7 Hz, 2H), 2.96 – 2.84 (m, 2H), 2.84 – 2.54 (m, 8H), 2.40 – 2.29 (m, 2H), 2.01 – 1.89 (m, 2), 1.88 – 1.76 (m, 2H), 1.70 – 1.40 (m, 9H), 1.29 – 1.11 (m, 10H); ^{13}C NMR (101 MHz, C_6D_6) δ 174.7, 163.4, 159.6, 151.2, 146.9, 143.0, 123.7, 120.1, 116.4, 108.0, 107.1, 106.1, 71.2, 70.3, 70.2, 69.9, 57.5, 49.9, 49.3, 45.2, 43.0, 39.7, 36.3, 32.9, 29.8, 29.7, 28.9, 27.9, 27.0, 26.1, 25.7, 25.0, 22.4, 21.6, 20.6, 20.5. HRMS calcd. for $C_{36}H_{55}ClN_3O_5$ (M+H) 644.3825, found 644.3823.

Preparation of RFAP-2-Ald.



RFAP-2 **RFAP-2-Ald**

Synthesis of RFAP-2-Ald. RFAP-2 (1 mg, 1.5 µmol) was dissolved in 1 mL 2:1 MeOH:H_2O and 15 µL of a 1 M solution of formaldehyde (15 µmol) were added. The reaction mixture was stirred for 10 h, then the solvent was removed under reduced pressure. Purification by silica column chromatography (gradient from DCM to 2.5% MeOH/DCM) afforded RFAP-2-Ald as an orange film (0.4 mg, 44% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.34 (s, 1H), 7.21 (s, 1H), 6.21 (s, 1H), 3.61 – 3.51 (m, 5H), 3.48 – 3.43 (m, 3H), 3.35 (dt, *J* = 9.3, 5.8 Hz, 2H), 3.23 (t, *J* = 8.2 Hz, 2H), 2.88 (t, *J* = 6.4 Hz, 2H), 2.80 (t, *J* = 6.2 Hz, 2H), 2.31 (t, *J* = 7.4 Hz, 2H), 2.03 – 1.93 (m, 3H), 1.86 (p, *J* = 7.3 Hz, 2H), 1.77 (p, *J* = 6.8 Hz, 2H), 1.64 – 1.51 (m, 15H*), 1.47 – 1.33 (m, 4H). *Note: multiplet at 1.64 – 1.51 overlaps with H_2O peak. HRMS calcd. For $C_{31}H_{43}ClN_2O_6Na$ (M+Na) 597.2702, found 597.2707.

Spectroscopic Materials and Methods. Milli-Q water was used to prepare all aqueous solutions. All spectroscopic measurements were performed in 20 mM PBS, pH 7.4. Absorption spectra were recorded using a Varian Cary 50 spectrophotometer, and fluorescence spectra were recorded using a Photon Technology International Quanta Master 4 L-format scan spectrofluorometer equipped with an LPS-220B 75-W xenon lamp and power supply, A-1010B lamp housing with integrated igniter, switchable 814 photocounting/analog photomultiplier detection unit, and MD5020 motor driver. Samples for absorption

and emission measurements were contained in 1-cm × 1-cm quartz cuvettes (1.4-mL volume, Starna). All excitation spectra were corrected for the emission profile of the xenon lamp.

Quantum Yield. Quantum yield was determined using coumarin 153 (λ_{ex} = 420 nm) and fluorescein (λ_{ex} = 470 nm) as standards according to a published method.[4] For RFAP-1-Ald, RFAP-1, RFAP-2, coumarin 153, and fluorescein, the absorbance spectra were measured within an absorbance range below 0.1. The quantum yield was calculated according to the equation:

$$\phi_{sample} = \phi_{standard} (Grad_{sample}/Grad_{standard})(\eta_{sample}^2/\eta_{standard}^2)$$

where ϕ is the quantum yield, $\phi_{coumarin\ 153}$ = 0.38 in EtOH,[4] $\phi_{fluorescein}$ = 0.91 in 0.1 M aqueous NaOH,[4] Grad is the slope of the plot of absorbance versus integrated emission intensity, and η is the refractive index of the solvent.

RFAP-0, RFAP-1, and RFAP-2 Fluorescence Response to FA. 995 μL of a 10.05 μM solution of RFAP-0, RFAP-1, or RFAP-2 in 20 mM PBS (pH 7.4) was prepared by diluting a 5 mM DMSO stock solution of RFAP-0, RFAP-1, or RFAP-2 into pre-warmed PBS (37 °C) in a 1-cm × 1-cm quartz cuvette, followed by a 15 minute incubation. 5 μL of 20 mM stock solution of FA (freshly prepared by diluting commercial 37 wt. % in H_2O FA solution) was added (for a final concentration of 100 μM), and the mixture was mixed by vigorous pipetting for 5 s, followed by acquisition of the t = 0 spectrum. Excitation spectra (λ_{ex} = 400–500 nm, λ_{em} = 510 nm) were collected at 0, 30, 60, 90, and 120 min. Temperature was maintained at 37 °C throughout the experiment by immersing the cuvette in a heated water bath between measurements.

RFAP-0 and RFAP-1 Bimolecular Rate Constant Determination. 995 μL of a 10.05 μM solution of RFAP-0 or RFAP-1 in 20 mM PBS (pH 7.4) was prepared by diluting a 5 mM DMSO stock solution of RFAP-0 or RFAP-1 into pre-warmed PBS (37 °C) in a 1-cm × 1-cm quartz cuvette, followed by a 15 minute incubation. 5 μL of 20 mM stock solution of FA (freshly prepared by diluting commercial 37 wt. % in H_2O FA solution) was added (for a final concentration of 100 μM), and the mixture was mixed by vigorous pipetting for 5 s, followed by acquisition of the t = 0 spectrum. Excitation spectra (λ_{ex} = 400–500 nm, λ_{em} = 510 nm) were collected at timepoints until saturation was observed. Temperature was maintained at 37 °C throughout the experiment by immersing the cuvette in a heated water bath between measurements. The reaction was assumed to be pseudo-first order under these conditions, and the slope of a best-fit line to the plot of $\ln((R_{max}-R)/(R_{max}-R_0))$ vs time (where R_{max} indicates saturated excitation ratio, R_0 indicates initial excitation ratio, and R indicates excitation ratio at the current timepoint) was divided by the concentration of FA (100 μM) to give the bimolecular rate constant.

Selectivity Tests. 995 μL of a 10.05 μM solution of RFAP-1 or RFAP-2 (unless otherwise specified) in 20 mM PBS (pH 7.4) was prepared by diluting a 5 mM DMSO stock solution of RFAP-1 or RFAP-2 into pre-warmed PBS (37 °C) in a 1-cm × 1-cm quartz cuvette, followed by a 15-minute incubation. The analyte of interest was added to the cuvette to bring the concentration of analyte to 100 μM (unless otherwise specified) and the concentration of RFAP-1 or RFAP-2 to 10 μM, followed by mixing by vigorous pipetting

for 5 seconds, and a t = 0 spectrum was acquired. The cuvette was placed in a 37 °C water bath. Excitation spectra were recorded by quickly removing the cuvette from the water bath, obtaining the spectrum, and returning the cuvette to the bath. Spectra were taken at t = 0, 30, 60, 90, and 120 min. Stock solutions and addition volumes were as follows.

FA: 5 μL of a 20 mM stock solution of FA in Milli-Q water (freshly prepared by diluting commercial 37 wt. % FA solution with Milli-Q water).

Acetaldehyde: 5 μL of a 20 mM stock solution of acetaldehyde in Milli-Q water (freshly prepared by diluting neat acetaldehyde with Milli-Q water).

Glucose: 5 μL of a 200 mM stock solution of glucose in Milli-Q water (freshly prepared by dissolving glucose in Milli-Q water); final concentration 1 mM glucose.

4-hydroxynonenal (4-HNE): 5 μL of 20 mM stock solution of 4-HNE in 7:1 EtOH:Milli-Q water (diluted from commercial 64 mM EtOH stock with Milli-Q water).

Dehydroascorbate: 5 μL of a 20 mM stock solution of dehydroascorbic acid in 1:1 DMSO:Milli-Q water (freshly prepared by dissolving dehydroascorbic acid in 1:1 DMSO:Milli-Q water).

Glucosone: 5 μL of a 20 mM stock solution of glucosone in Milli-Q water (freshly prepared by dissolving gluocosone in Milli-Q water).

Sodium pyruvate: 5 μL of a 20 mM stock solution of sodium pyruvate in Milli-Q water (freshly prepared by dissolving sodium pyruvate in Milli-Q water).

Oxaloacetate: 5 μL of a 20 mM stock solution of oxaloacetate in Milli-Q water (freshly prepared by dissolving oxaloacetic acid in Milli-Q water).

H_2O_2: 5 μL of a 20 mM stock solution of H_2O_2 in Milli-Q water (freshly prepared by diluting commercial 9.8 M H_2O_2 with Milli-Q water).

Glutathione: All buffers and stocks were deoxygenated by bubbling a stream of nitrogen gas for 30 minutes. 25 μL of a 200 mM stock solution of glutathione in PBS (freshly prepared by dissolving glutathione in deoxygenated PBS; pH was brought to 7.4 with deoxygenated 1 M NaOH) was added to 975 μL of a 10.25 μM solution of RFAP-2 in deoxygenated PBS. The screw-top cuvette was capped for the duration of the timecourse. Final concentration 5 mM glutathione.

Methylglyoxal: 5 μL of a 20 mM stock solution of methylglyoxal in Milli-Q water or 5 μL of 2 mM stock solution of methylglyoxal in Milli-Q water (freshly prepared by diluting neat methylglyoxal with Milli-Q water); final concentration 100 μM or 10 μM methylglyoxal.

Sodium Bisulfite: 5 μL of a 40 mM stock solution of sodium bisulfite in Milli-Q water (freshly prepared by dissolving sodium bisulfite in Milli-Q water); final concentration 200 μM.

Caffeine: 14.1 μL of a 71 mM stock solution of caffeine in Milli-Q water (freshly prepared by dissolving caffeine in Milli-Q water) was added to 986 μL of a 10.14 μM solution of RFAP-2 in PBS. Final concentration 1 mM caffeine.

RFAP-2 *In Vitro* Detection Limit. 995 μL of a 10.05 μM solution of RFAP-2 in 20 mM PBS (pH 7.4) was prepared by diluting a 5 mM DMSO stock solution of RFAP-2 into pre-warmed PBS (37 °C) in a 1-cm × 1-cm quartz cuvette, followed by a 15-minute incubation. 5 μL of a FA stock solution (8 mM, 6 mM, 4 mM, 2 mM, or 1 mM, freshly prepared by diluting commercial 37 wt. % FA solution with Milli-Q water) or 5 μL of PBS was added (for a final concentration of 40 μM, 30 μM, 20 μM, 10 μM, 5 μM, or 0 μM FA), and the mixture was mixed by vigorous pipetting for 5 s and placed in a 37 °C water bath. Excitation spectra were obtained after 120 min. The standard deviation (σ) of three replicates of blank (0 μM FA) samples as well as the slope of the linear regression curve fitted to the 470/420 nm excitation ratio in the range of [FA] from 0 to 40 μM were used to determine the detection limit (3σ/slope) of 0.3 μM according to published methods.[5]

Cell Culture Procedures. Cells were maintained by the UC Berkeley Tissue Culture Facility. HEK293T cells were maintained as a monolayer in exponential growth at 37 °C in a 5% CO_2 atmosphere in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Seradigm), 1% glutamax (Gibco), and 1% non-essential amino acids (NEAA, Gibco). HeLa, RKO, and U-2OS were maintained as a monolayer in exponential growth at 37 °C in a 5% CO_2 atmosphere in DMEM supplemented with 10% FBS and 1% glutamax. MCF-7 and SH-SY5Y cells were maintained as a monolayer in exponential growth at 37 °C in a 5% CO_2 atmosphere in DMEM supplemented with 10% FBS, 1% glutamax, 1% NEAA, and sodium pyruvate. MCF-10A were maintained as a monolayer in exponential growth at 37 °C in a 5% CO_2 atmosphere in DMEM/F-12 supplemented with 5% horse serum (Hyclone), 10 mM HEPES, 0.5 mg/mL hydrocortizone, 100 ng/mL cholera toxin, 20 ng/mL EGF, 10 μg/ml insulin. ADH5 -/- and genetically matched WT HAP1 cells were maintained in exponential growth at 37 °C in a 5% CO_2 atmosphere as a monolayer in Iscove's Modified Dulbecco's Medium, high glucose, (IMDM, Invitrogen) supplemented with 10% FBS. One day before imaging, HEK293T, MCF-7, SH-SY5Y, HAP1 ADH5 -/-, and HAP1 WT cells were passaged and plated on poly-L-lysine-coated 4-well Lab Tek borosilicate chambered coverglass slides (Nunc) at 1.8 × 105 per well and allowed to grow to between 70 and 80% confluence before imaging. One day before imaging, HeLa, MCF-10A, RKO, and U- 2OS cells were passaged and plated on 4-well Lab Tek borosilicate chambered coverglass slides and allowed to grow to between 70 and 80% confluence before imaging.

Confocal Fluorescence Imaging Experiments. Confocal fluorescence imaging studies were performed with a Zeiss laser scanning microscope 710 with a 20x or 63x oil immersion objective lens using Zen 2009 software (Carl Zeiss). RFAP-1 and RFAP-2 were excited using 405 nm diode laser and 488 nm argon laser, and emission was collected using a META detector between 410 to 550 nm (405 nm excitation) and 493 to 550 nm (488 nm excitation). BSS (136.9 mM NaCl, 5.37 mM KCl, 1.26 mM $CaCl_2$, 0.81 mM $MgSO_4$, 0.44 mM KH_2PO_4, 0.335 mM Na_2HPO_4, 10 mM PIPES; pH to 7.2 with NaOH) was used as the imaging buffer for all confocal experiments. The cells were imaged at 37 °C throughout the course of the experiment. Image analysis and quantification was performed using ImageJ (National Institutes of Health). For quantification of excitation ratio, three fields of cells within the same well were imaged. A region of interest (ROI) was

created around each cell in each image. The mean fluorescence intensity of each cell was measured (using "Measure" function) for each excitation wavelength and averaged across the three fields imaged. For each condition, multiple wells were analyzed using this process, and the 488/405 excitation ratios were averaged across independent experiments for statistical analysis. Statistical analyses for multiple comparisons were performed using one-way ANOVA with the Bonferroni correction in the statistical analysis software, R.

Exogenous FA Addition Experiments in Various Cell Types. One day before imaging, HEK293T, MCF-7, and SH-SY5Y cells were passaged and plated on poly-L-lysine-coated 4-well Lab Tek borosilicate chambered coverglass slides and allowed to grow to between 70 and 80% confluence before imaging. One day before imaging, HeLa, MCF-10A, RKO, and U- 2OS cells were passaged and plated on 4-well Lab Tek borosilicate chambered coverglass slides and allowed to grow to between 70 and 80% confluence before imaging. The DMEM media was aspirated from the chambers containing cells and replaced with 500 μL DMEM (supplemented with 10% FBS and glutamax) containing 10 μM RFAP-1 or RFAP-2 (diluted from 5 mM stock in DMSO) and incubated at 37 °C for 30 minutes. The buffer was then replaced with 500 μL fresh DMEM (supplemented with 10% FBS and glutamax) containing no probe, and the cells were incubated at 37 °C for 10 minutes to remove any excess probe. The buffer was then replaced with 500 μL BSS, and cells were imaged to provide the t = 0 timepoint. 200 μL of the buffer was removed from each well and mixed with vehicle control (5 μL H_2O) or FA (5 μL of 20.2 mM FA for 200 μM final concentration upon re-addition to well, 5 μL of 10.1 mM FA for 100 μM final concentration upon re-addition to well, 5 μL of 5.05 mM FA for 50 μM final concentration upon re-addition to well; 5 μL of 25.025 mM FA for 25 μM final concentration upon re-addition to well; all FA stocks freshly prepared by diluting 37 wt. % commercial FA). The cells were then incubated at 37 °C for 30 or 60 min prior to imaging for the t = 30 or t = 60 timepoint.

HAP1 ADH5 -/- and WT Confocal Imaging and Flow Cytometry Experiments.

Confocal Imaging Experiments. One day before imaging, ADH5-/- and WT HAP1 cells were passaged and plated on poly-L-lysine-coated 4-well Lab Tek borosilicate chambered coverglass slides and allowed to grow to between 70 and 80% confluence before imaging. The media was aspirated from the chambers containing cells and replaced with 500 μL BSS containing 500 nM RFAP-2 (diluted from 500 μM stock in DMSO) and incubated at 37 °C for 30 minutes. The buffer was then replaced with 500 μL BSS supplemented with either vehicle control (5 μL H_2O) or 100 μM FA (5 μL of a 10.1 mM FA stock). The cells were then incubated at 37 °C for 60 min prior to imaging for the t = 60 timepoint.

Flow Cytometry Experiments. One day before imaging, ADH5 -/- and WT HAP1 cells were passaged and plated on 12-well polystyrene culture plates (Corning) and allowed to grow to 80% confluence. The media was aspirated from the wells containing cells and replaced with 500 μL BSS containing 500 nM RFAP-2 (diluted from 500 μM stock in DMSO) and incubated at 37 °C for 30 minutes. The buffer was then replaced with 500 μL BSS supplemented with either vehicle control (5 μL H_2O) or 100 μM FA (5 μL of a 10.1 mM FA stock). The cells were then incubated at 37 °C for 60 min, then exchanged into PBS containing 5 nM Sytox Red (Invitrogen) and incubated for 5 min. Cells were dislodged from wells by gentle agitation and filtered

through 35 µm nylon mesh cap into a 12 x 75 mm polystyrene tube (Corning) for flow cytometry. Cells were analyzed using a BD LSRFortessa X-20 cell analyzer equipped with 405 nm, 488 nm, and 640 nm lasers; fluorescence emission was collected using standard AmCyan filter (525/50 nm bandpass filter with 475 nm longpass filter) for 405 nm excitation, standard FITC filter (530/30 nm bandpass filter with 505 nm longpass filter) for 488 nm excitation, and standard APC filter (670/14 nm bandpass filter) for 640 nm excitation. Quantification was performed using FlowJo software (FlowJo, LLC); live cells were selected and median 488/405 nm excitation ratios were averaged across 3 technical replicates per biological replicate. Median 488/405 nm excitation ratios were then averaged across independent biological replicates for statistical analysis. Statistical analyses for multiple comparisons were performed using one-way ANOVA with the Bonferroni correction in the statistical analysis software, R.

Supplementary Figures.

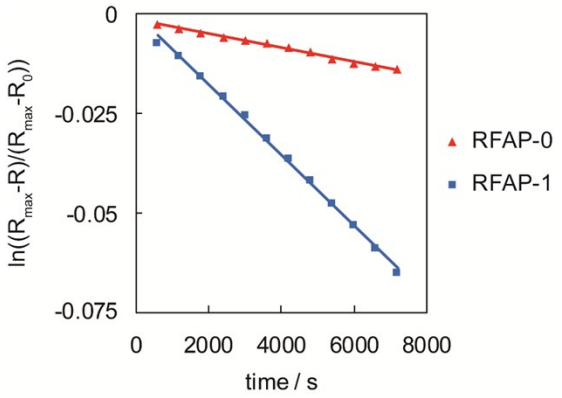


Figure S1. Linearized integrated rate law of reaction between 100 μM FA and 10 μM RFAP-0 (red triangles) or RFAP-1 (blue squares) assuming pseudo-first order kinetics.

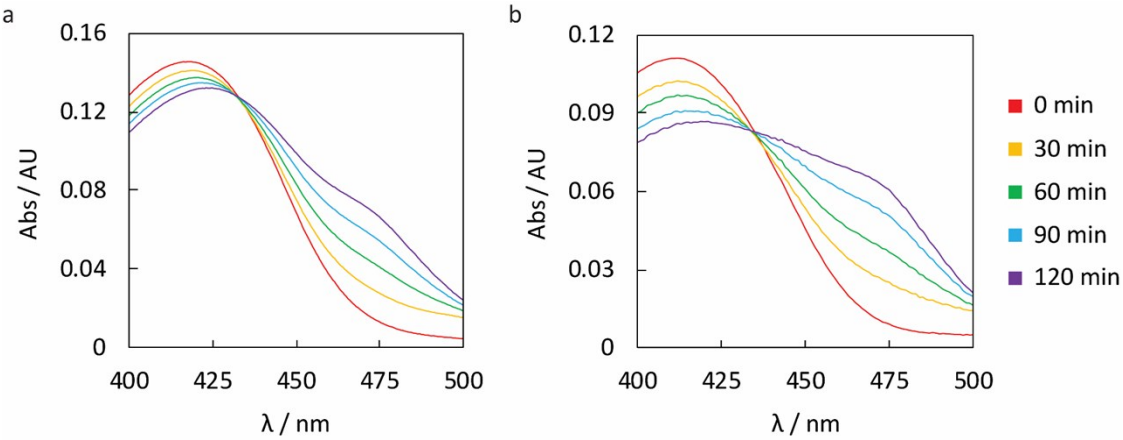


Figure S2. UV-visible FA response for RFAP-1 and RFAP-2. Data were acquired in 20 mM PBS (pH 7.4). (a) UV-visible response of 10 μM RFAP-1 to 100 μM FA. (b) UV-visible response of 10 μM RFAP-2 to 100 μM FA. Time points represent 0, 30, 60, 90, and 120 minutes after addition of 100 μM FA; saturation was not reached during this time.

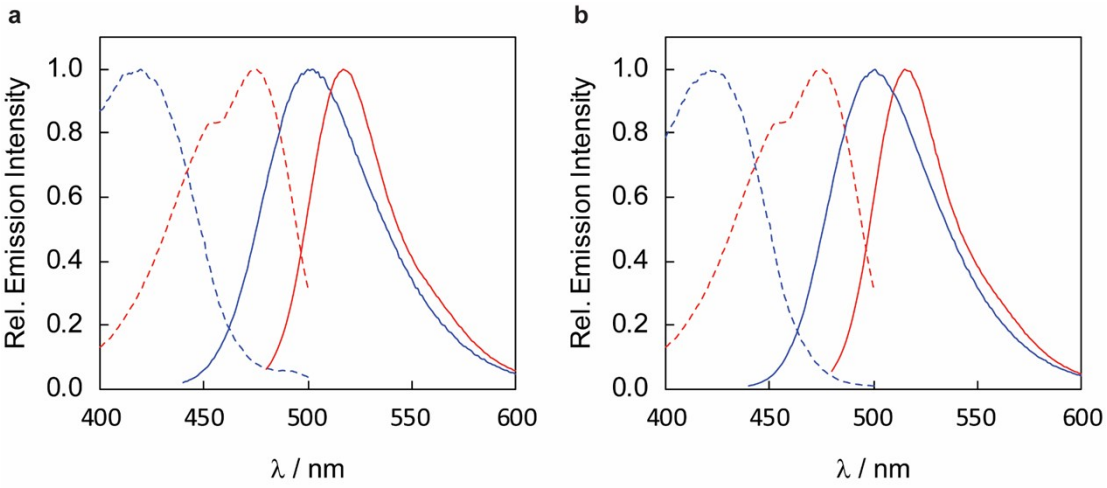


Figure S3. Normalized excitation and emission spectra for RFAP-1, RFAP-1-Ald, RFAP-2, and RFAP-2-Ald (all at 10 µM). Data were acquired in 20 mM PBS (pH 7.4) at 37 °C. (a) Spectra for RFAP-1 (blue traces) and RFAP-1-Ald (red traces). RFAP-1 excitation spectrum (blue, dashed trace) acquired with λ_{em} = 510 nm and emission spectrum (blue, solid trace) with λ_{ex} = 420 nm; RFAP-1-Ald excitation spectrum (red, dashed trace) acquired with λ_{em} = 510 nm and emission spectrum (red, solid trace) with λ_{ex} = 470 nm. (b) Spectra for RFAP-2 (blue traces) and RFAP-2-Ald (red traces). RFAP-2 excitation spectrum (blue, dashed trace) acquired with λ_{em} = 510 nm and emission spectrum (blue, solid trace) with λ_{ex} = 420 nm; RFAP-2-Ald excitation spectrum (red, dashed trace) acquired with λ_{em} = 510 nm and emission spectrum (red, solid trace) with λ_{ex} = 470 nm.

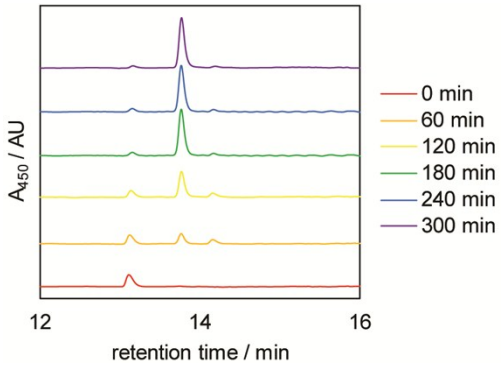


Figure S4. LC-MS timecourse of reaction between 100 µM RFAP-1 and 1 mM FA at 25 °C in 20 mM PBS (pH 7.4). HPLC runs used a linear gradient from 5 % MeCN / 95 % H_2O / 0.05 % formic acid to 95 % MeCN / 5 % H_2O / 0.05 % formic acid over 16 min using an Agilent 300extend-C18, 3.5 µm, 4.6 × 100mm column. Traces represent reverse-phase HPLC profile at 450 nm at different timepoints during reaction.

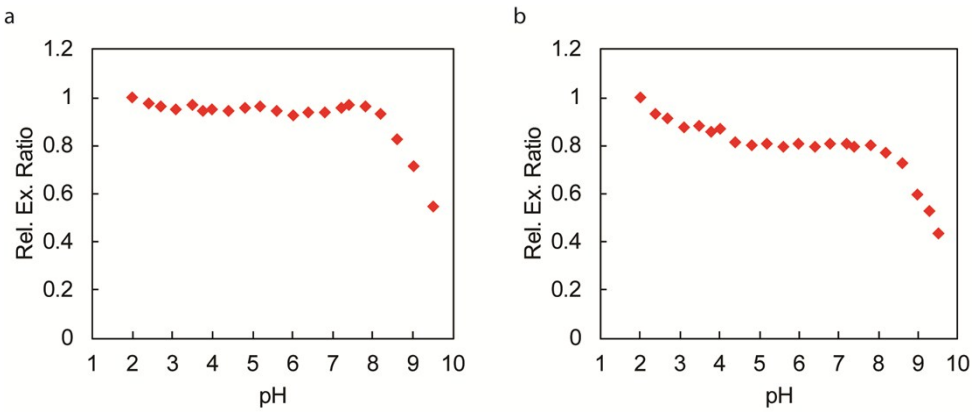


Figure S5. Excitation ratio pH profiles for (a) RFAP-1 and (b) RFAP-2. Data were acquired at 37 °C with 10 μM probe in 50 mM glycine (pH 2–3.8), 50 mM NaOAc (pH 4–5.6), 50 mM MOPS (pH 6–6.8), 50 mM HEPES (pH 7.2–8.2), and 50 mM bicine (pH 8.6–9.5).

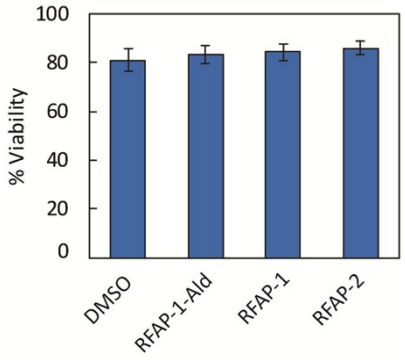


Figure S6. Flow cytometric analysis of HEK293T cell viability using Sytox Red staining. Cells were plated in 12-well polystyrene culture plates (Corning). After the designated treatments below, cells were dislodged from wells by gentle agitation and filtered through 35 μm nylon mesh cap into a 12 x 75 mm polystyrene tube (Corning) for flow cytometry. Cell viability was calculated as the percentage of Sytox Red-negative cells. HEK293T cells were incubated with vehicle (DMSO), 10 μM RFAP-1, 10 μM RFAP-2, or 10 μM RFAP-1-Ald in BSS at 37 °C for 60 min, then exchanged into PBS containing 5 nM Sytox Red and incubated for 10 min before flow cytometry. Error bars denote SEM, n=3.

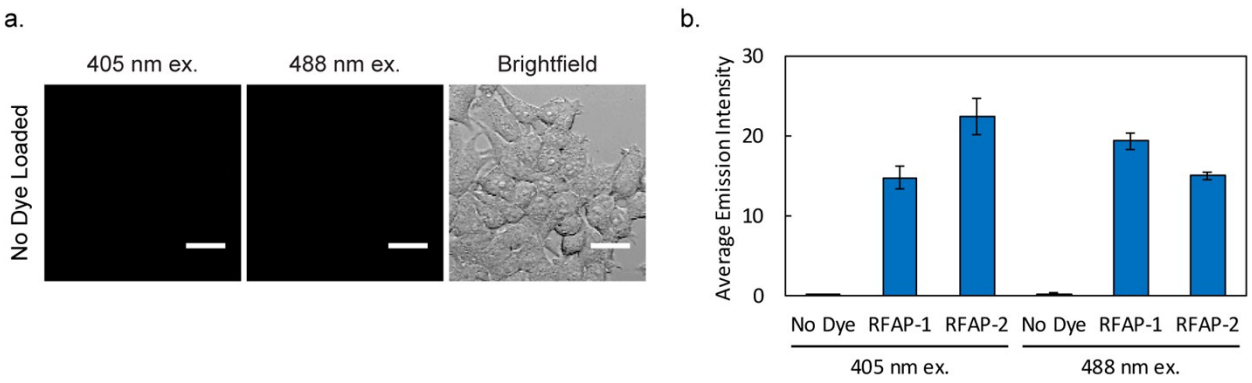


Figure S7. No-dye control has negligible fluorescence background using the same laser settings used for imaging RFAP-1 and RFAP-2. (a) Images of HEK293T cells treated with BSS for 30 min at 37 °C, washed with fresh BSS, then imaged. Scale bar represents 25 μm. (b) Quantification of fluorescence intensity in no-dye control compared to average fluorescence intensities of HEK93T cells loaded with 10 μM RFAP-1 or RFAP-2 under the same laser settings. Error bars represent SEM, n = 3 replicates with 3 fields of cells per replicate.

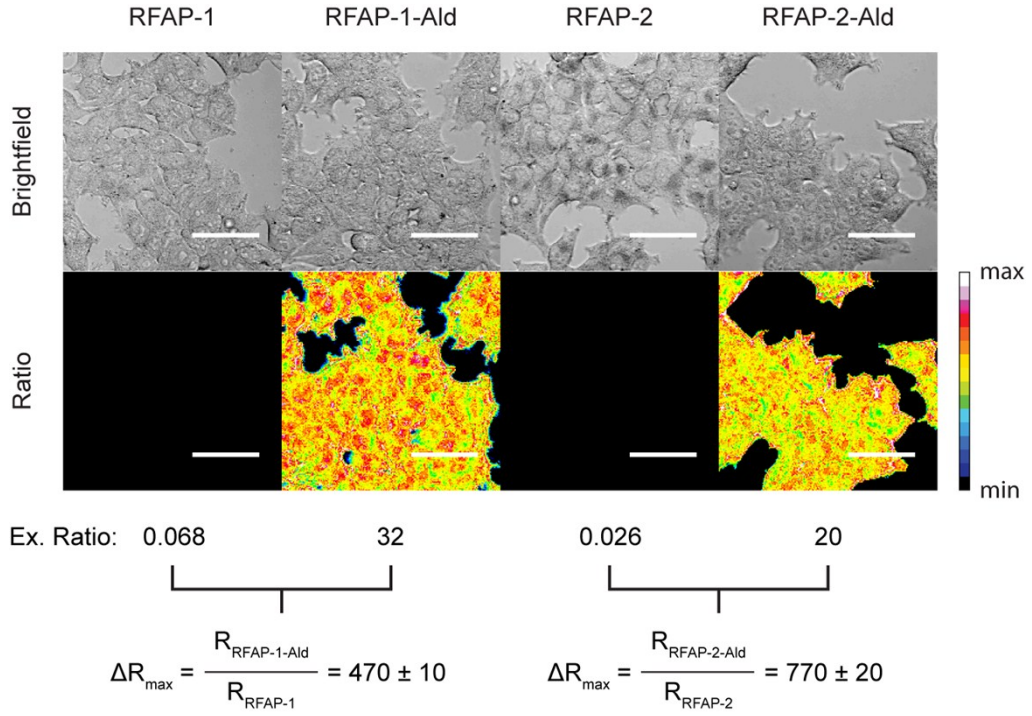


Figure S8. Maximum ratio change *in cellulo*. HEK293T cells were treated with 10 μM RFAP-1, RFAP-1-Ald, RFAP-2, or RFAP-2-Ald in BSS for 30 min at 37 °C, washed with fresh BSS, then imaged. Note that laser settings were altered so that RFAP-1-Ald and RFAP-2-Ald would not have saturated pixels for this analysis. Excitation ratios are an average across 2 replicates with 3 fields of cells per replicate; error represents SEM. Scale bar represents 50 μm.

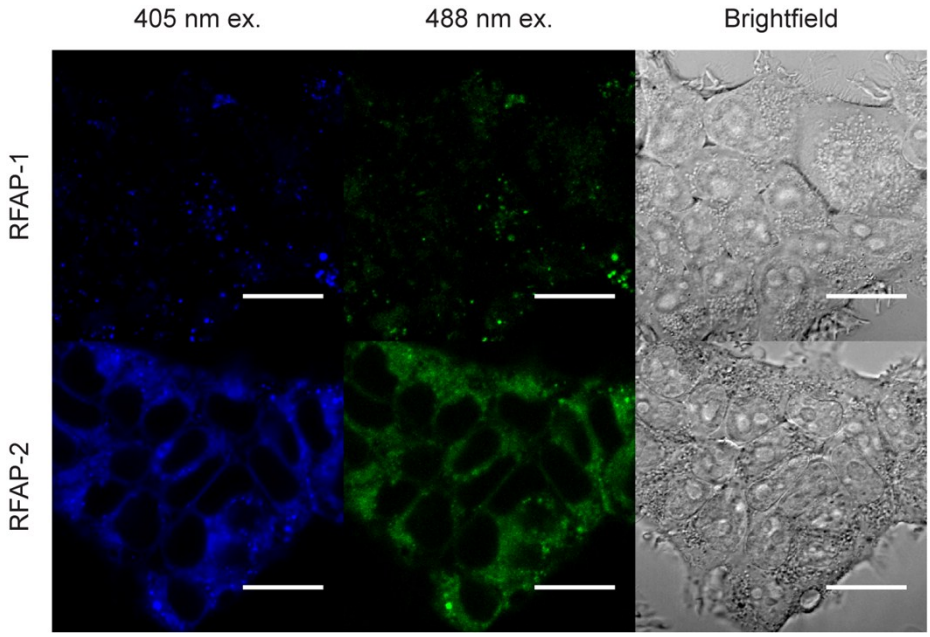


Figure S9. RFAP-1 displays punctate staining in HEK293T cells. HEK293T cells were treated with 10 μM RFAP-1 or RFAP-2 in BSS for 30 min at 37 °C, washed with fresh BSS, then imaged. Scale bar represents 20 μm.

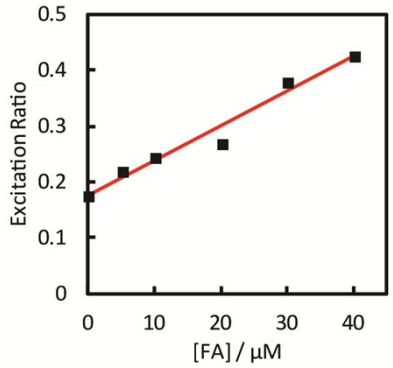


Figure S10. *In vitro* detection limit determination for RFAP-2. Excitation spectra were acquired in 20 mM PBS (pH 7.4) at 37 °C with emission at λ_{em} = 510 nm. Bars represent 470/420 nm excitation ratio 2 hours after FA addition. The standard deviation (σ) of three replicates of blank (0 μM FA) samples as well as the slope of the linear regression curve fitted to the 470/420 nm excitation ratio in the range of [FA] from 0 to 40 μM were used to determine the detection limit (3σ/slope) of 0.3 μM.

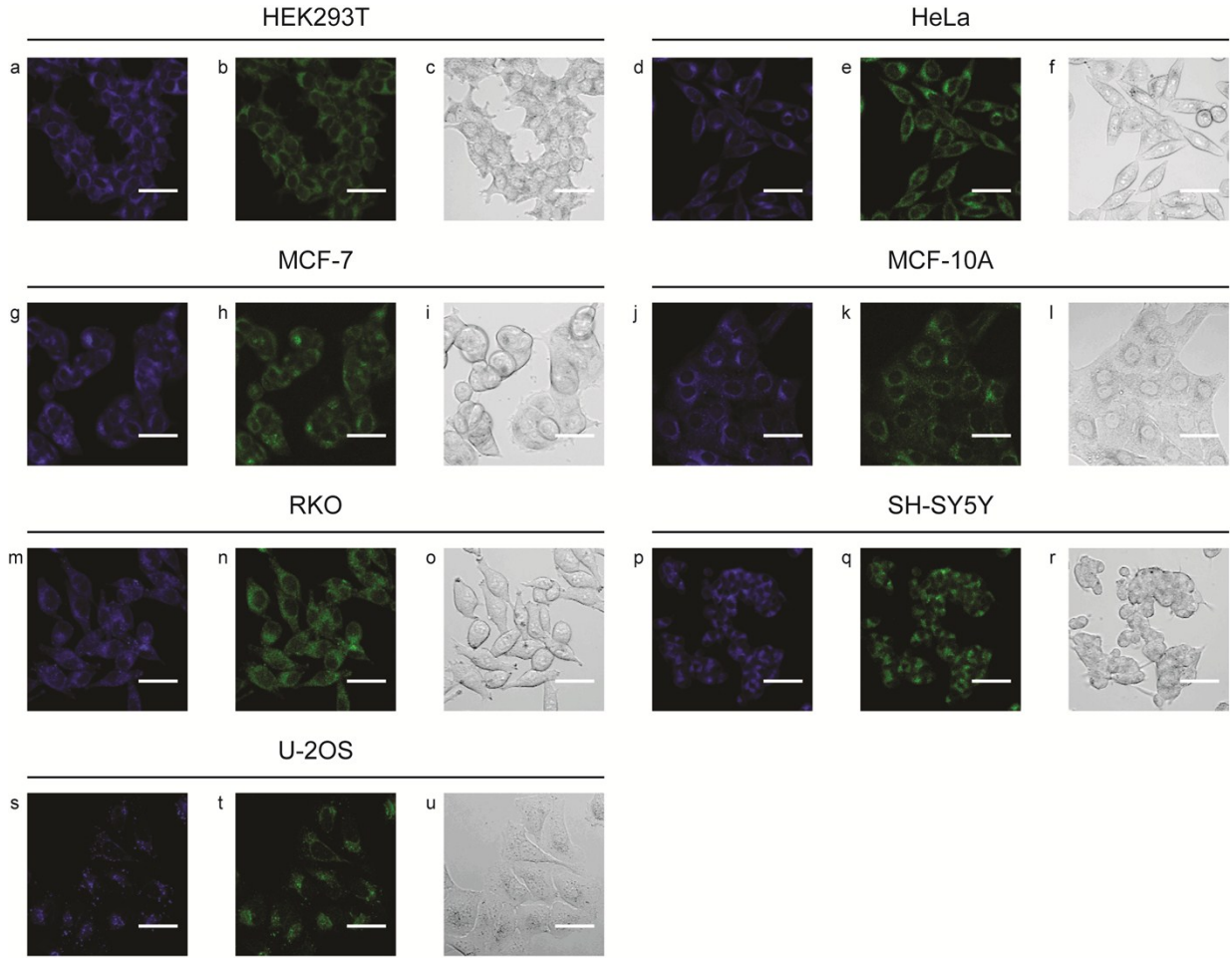


Figure S11. RFAP-2 staining patterns in various cell lines. Cells were treated with 10 μM RFAP-2 in BSS for 30 min at 37 °C, washed with fresh BSS, then imaged. Scale bar represents 40 μm in all images. HEK293T cells: (a) 405 nm excitation, (b) 488 nm excitation, (c) bright-field; HeLa cells: (d) 405 nm excitation, (e) 488 nm excitation, (f) bright-field; MCF-7 cells: (g) 405 nm excitation, (h) 488 nm excitation, (i) bright-field; MCF-10A cells: (j) 405 nm excitation, (k) 488 nm excitation, (l) bright-field; RKO cells: (m) 405 nm excitation, (n) 488 nm excitation, (o) bright-field; SH-SY5Y cells: (p) 405 nm excitation, (q) 488 nm excitation, (r) bright-field; U-2OS cells: (s) 405 nm excitation, (t) 488 nm excitation, (u) bright-field.

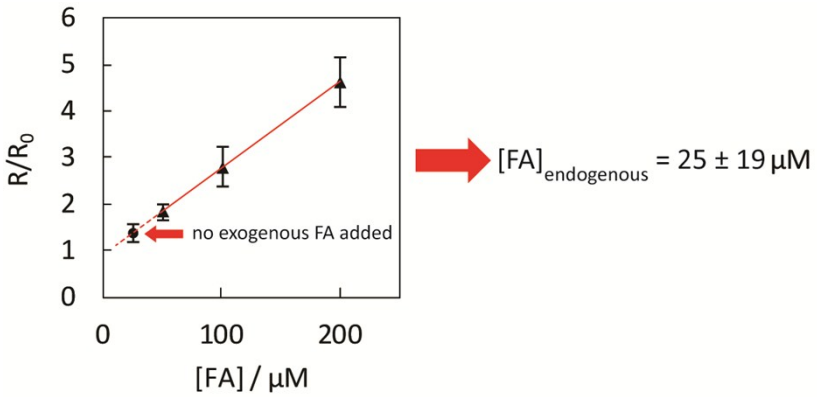


Figure S12. Calibration curve for [FA] in cells using RFAP-2. Intracellular [FA] was assumed to be equivalent to exogenous [FA] (except in case of no exogenous FA) to construct calibration curve. Least-squares fit was extrapolated to observed R/R_0 for $[FA]_{exogenous}$ = 0 µM to give predicted $[FA]_{endogenous}$ = 25 ± 19 µM, where ± 19 µM is the 95% confidence interval.

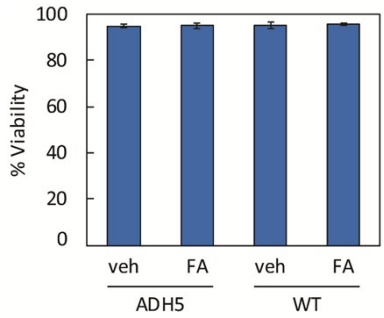


Figure S13. Flow cytometric analysis of ADH5 -/- and WT cell viability using Sytox Red staining. Cells were plated in 12-well polystyrene culture plates (Corning). After the designated treatments below, cells were dislodged from wells by gentle agitation and filtered through 35 µm nylon mesh cap into a 12 x 75 mm polystyrene tube (Corning) for flow cytometry. Cell viability was calculated as the percentage of Sytox Red-negative cells. ADH5 -/- and WT HAP1 cells were incubated with 500 nM RFAP-1 in BSS at 37 °C for 30 min, then exchanged into BSS containing either vehicle control or 100 µM FA and incubated at 37 °C for a further 60 min. Cells were then exchanged into PBS containing 5 nM Sytox Red and incubated for 10 min before flow cytometry. Error bars denote SEM, n=3.

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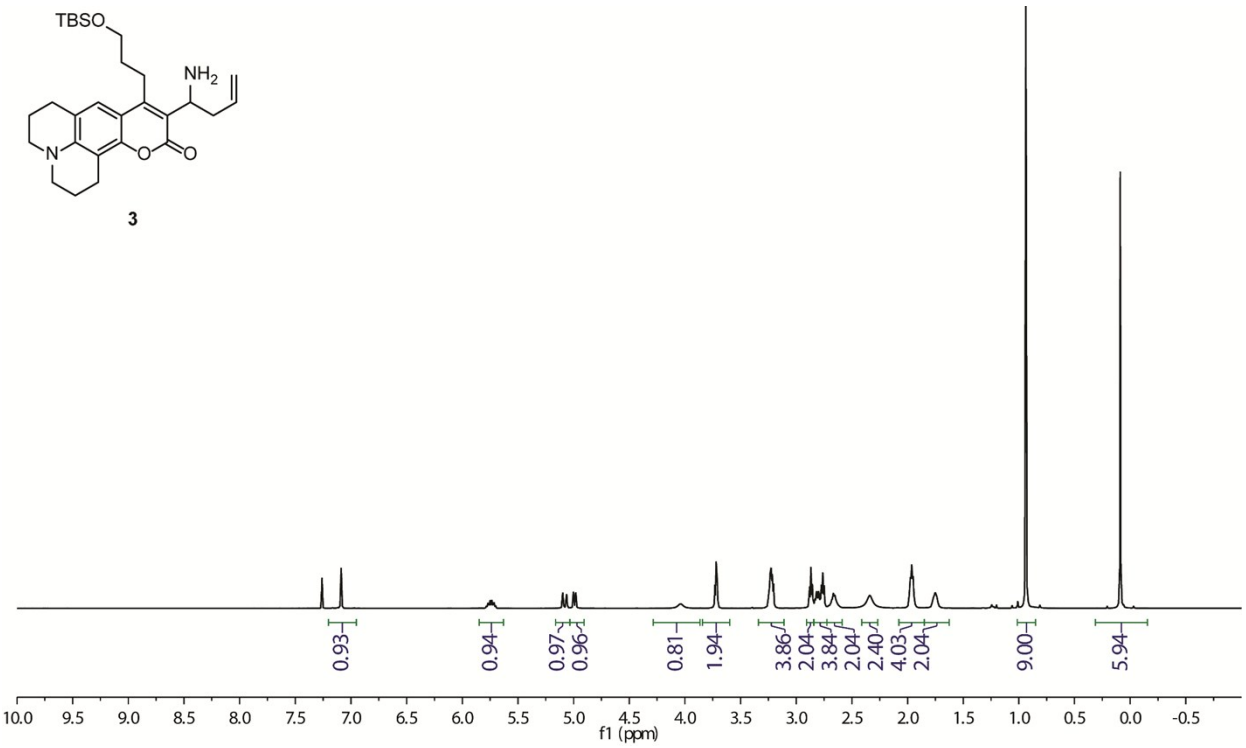
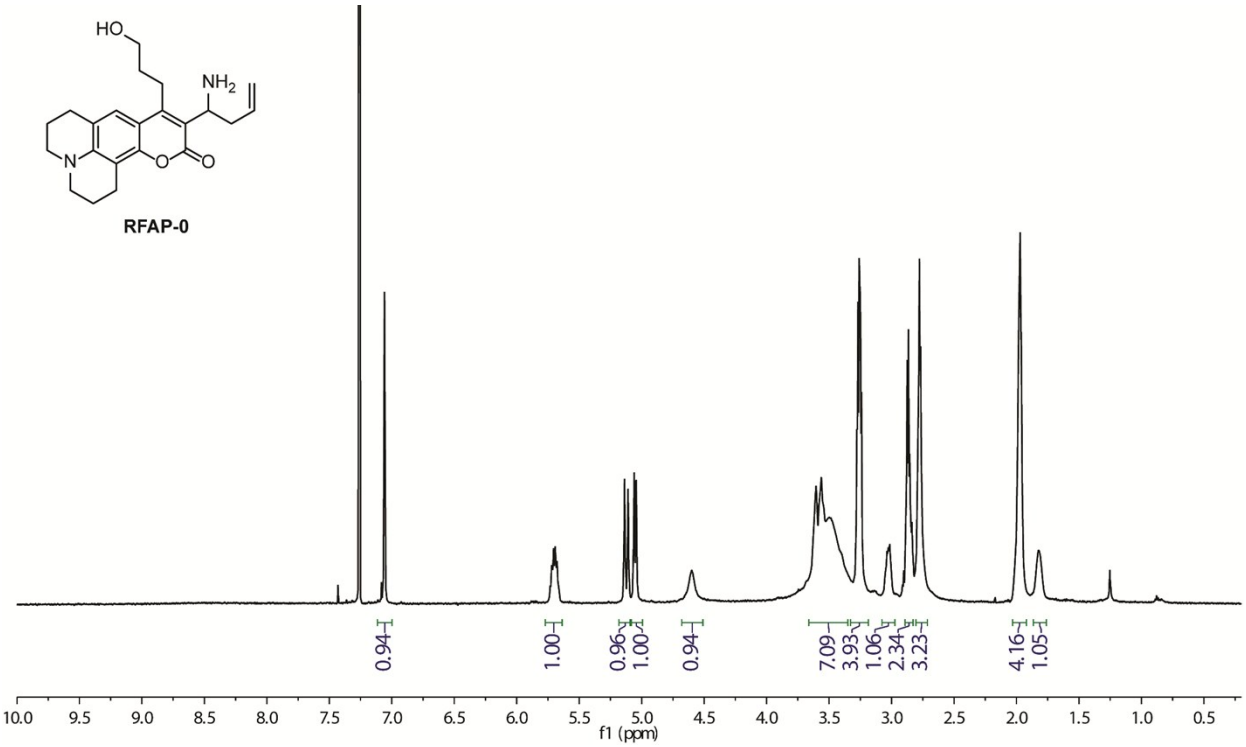
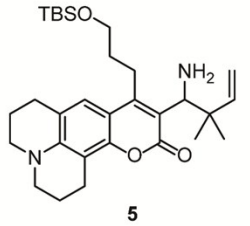
Copies of NMR Spectra.



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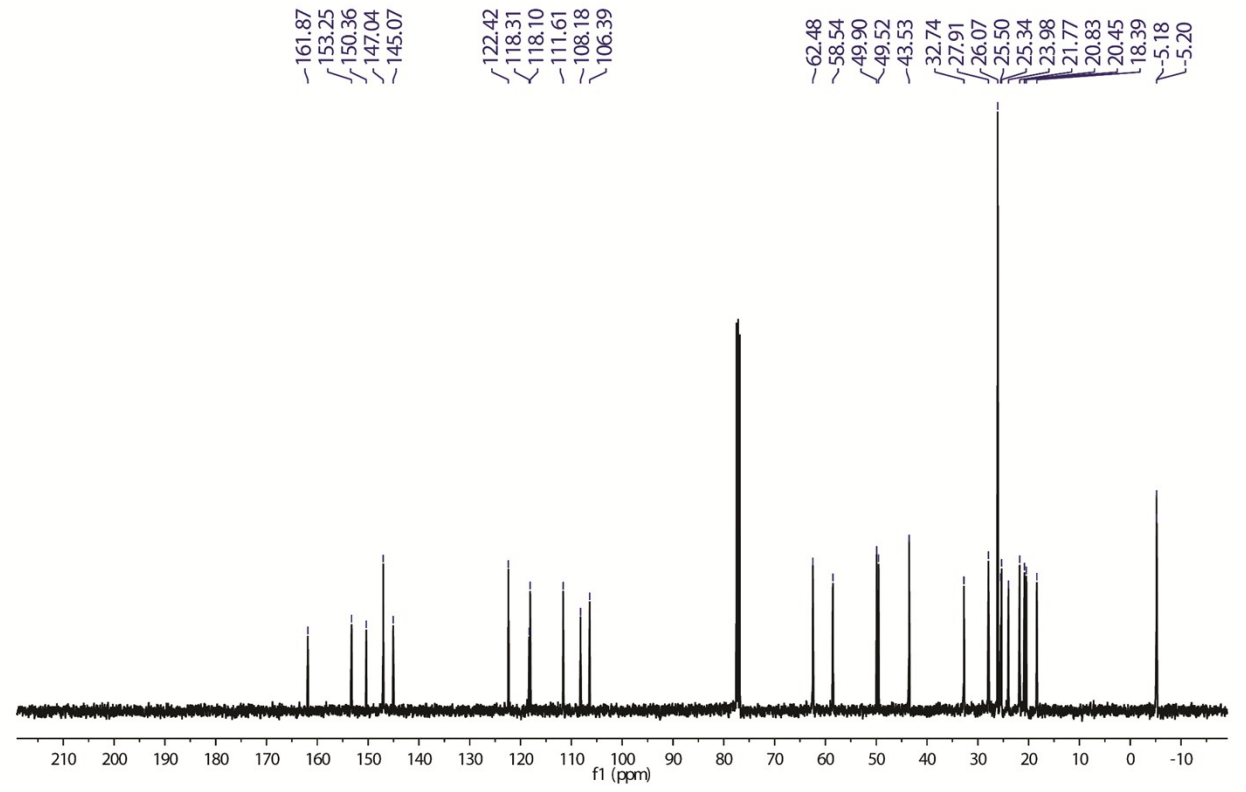
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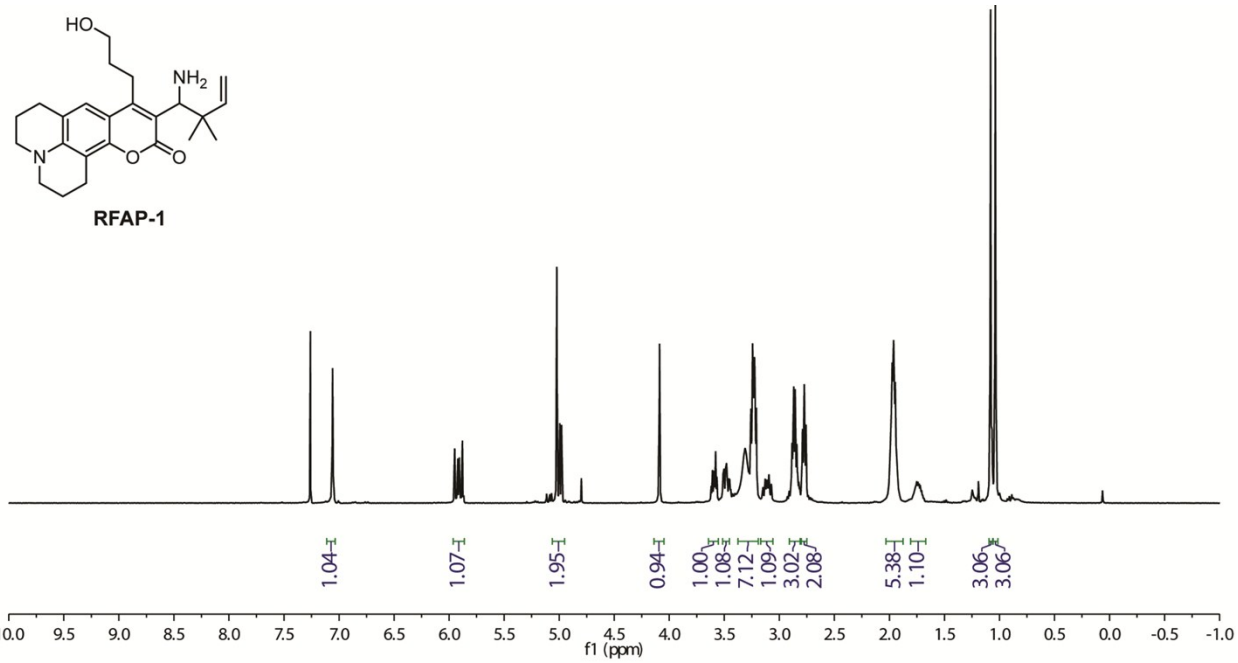
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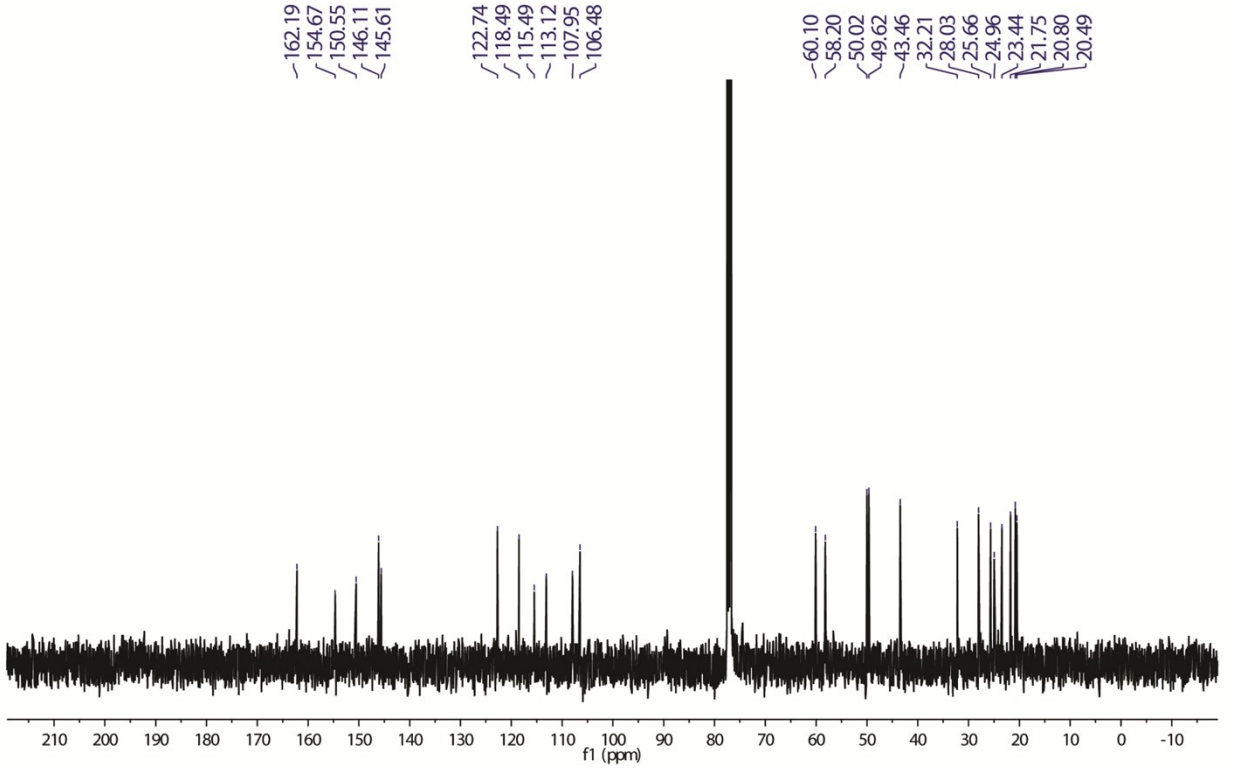
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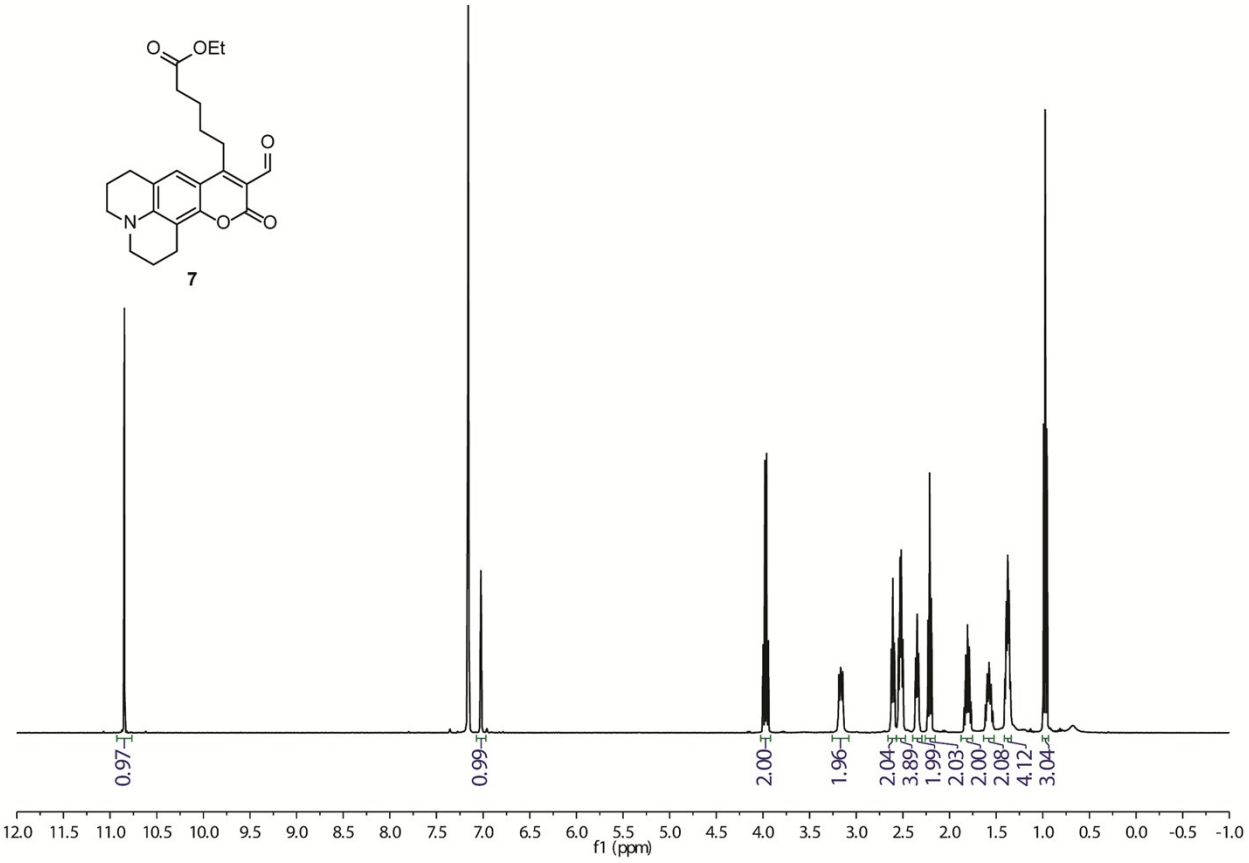
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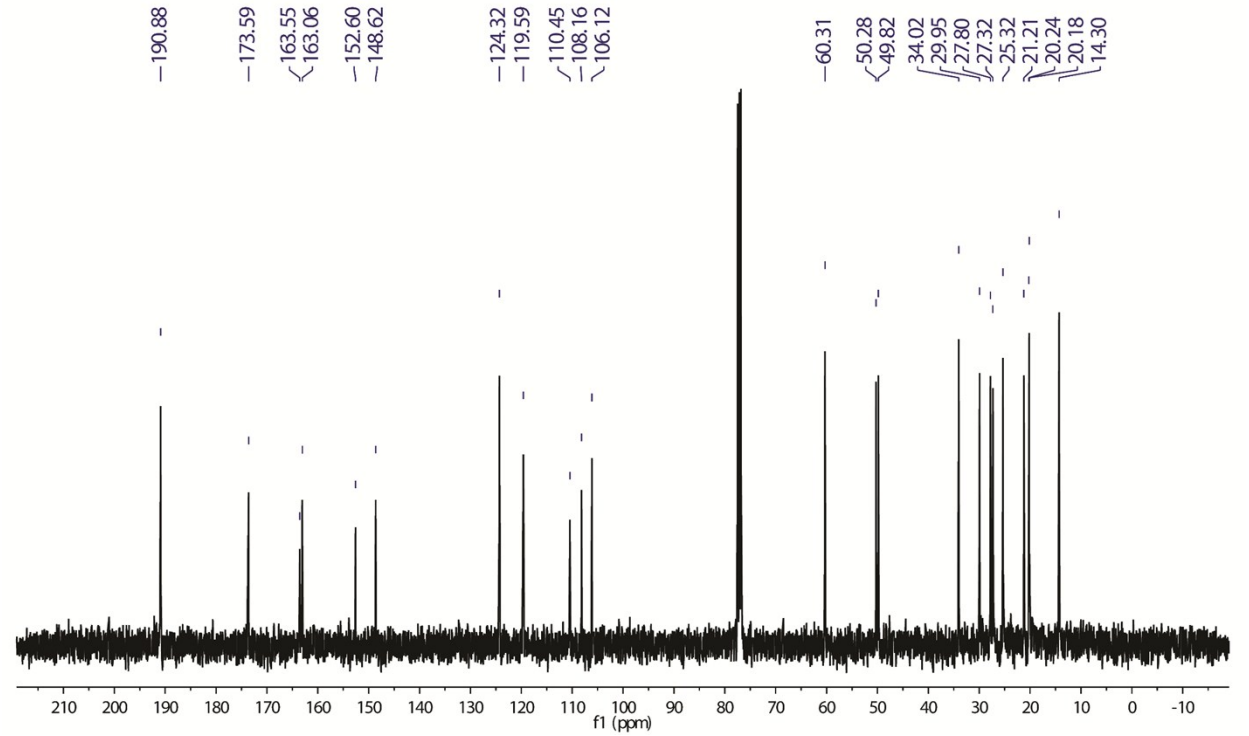
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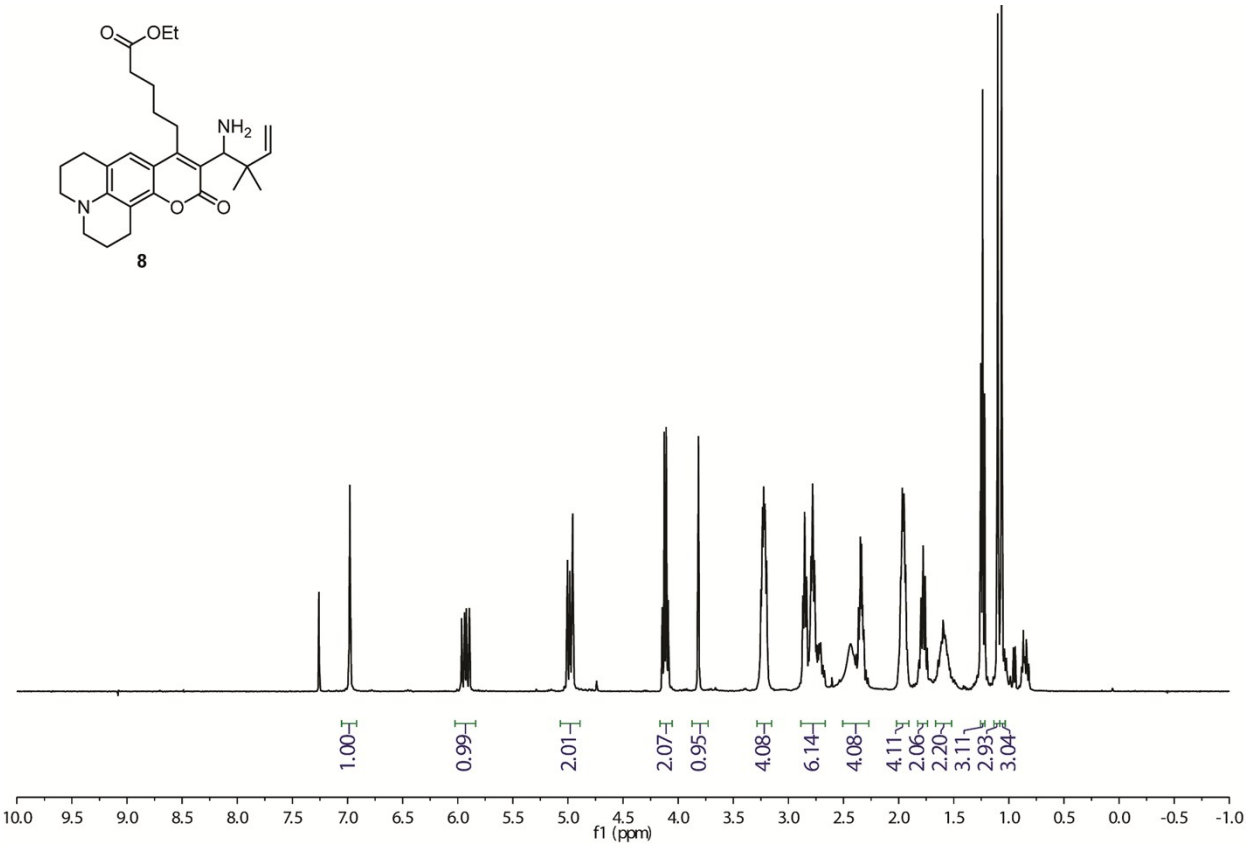
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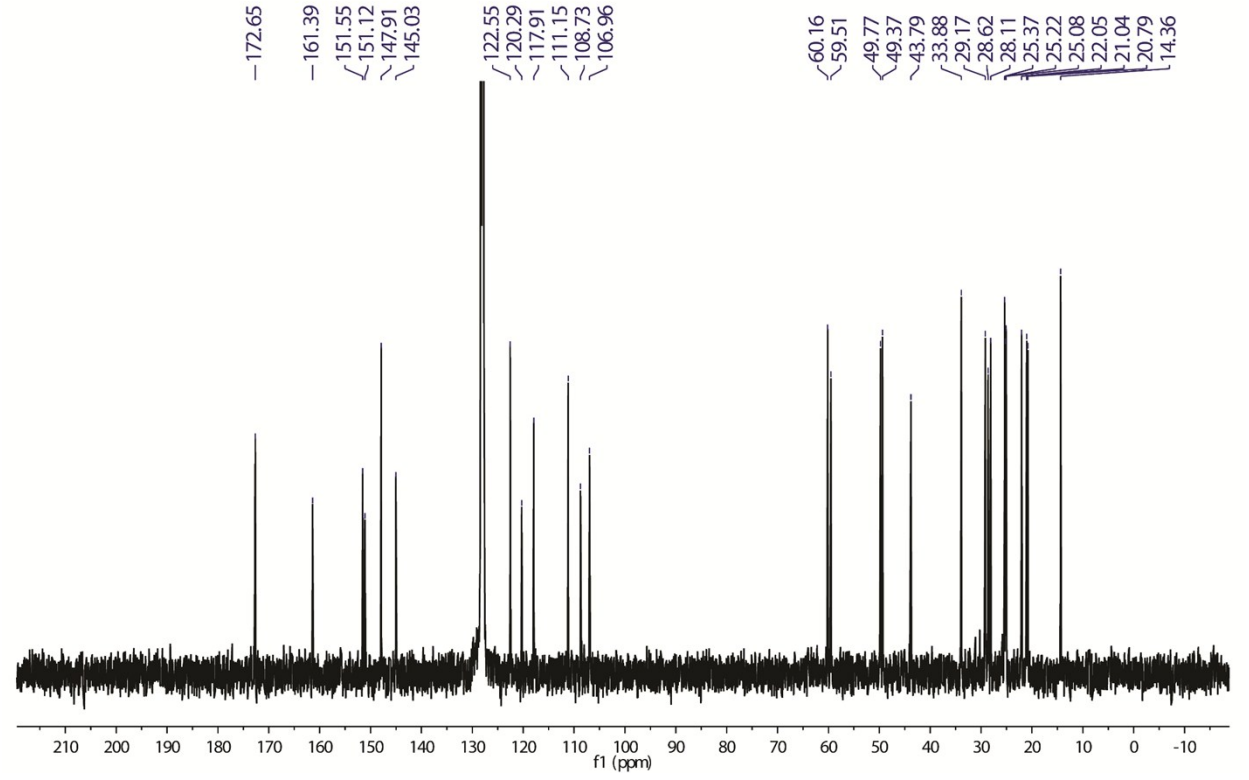
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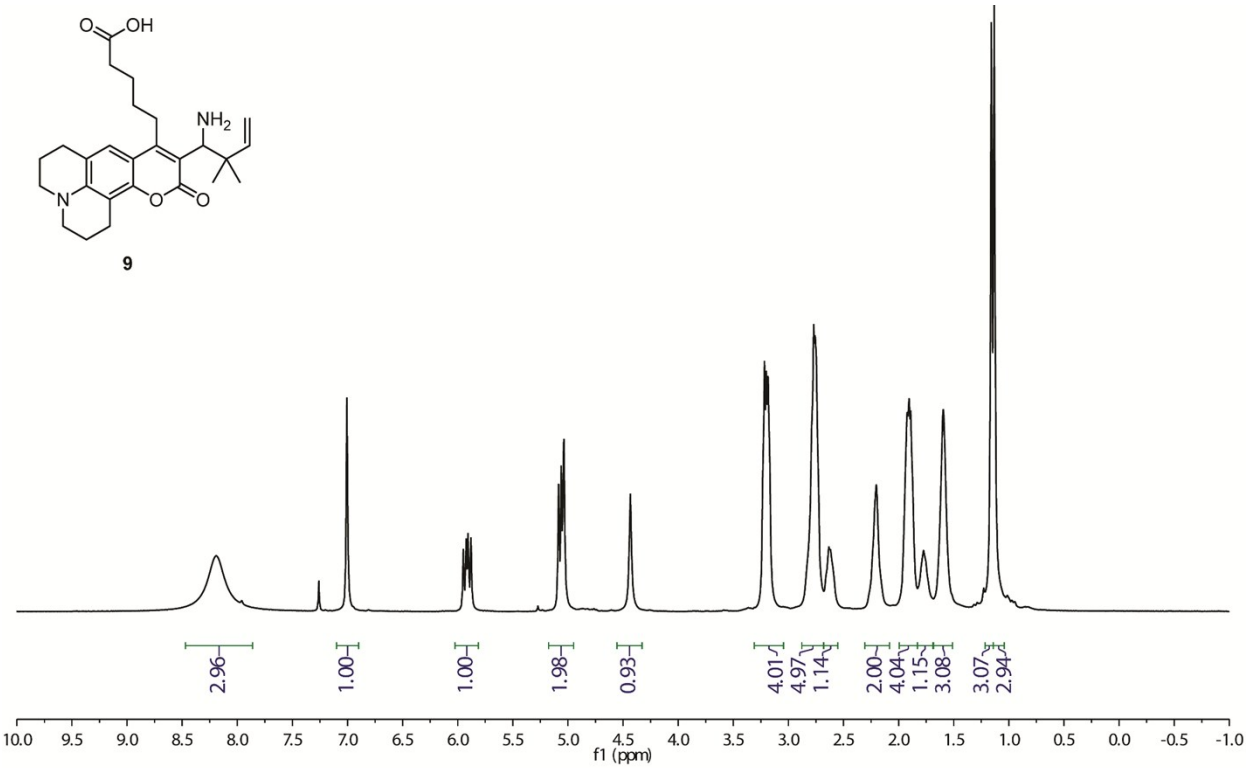
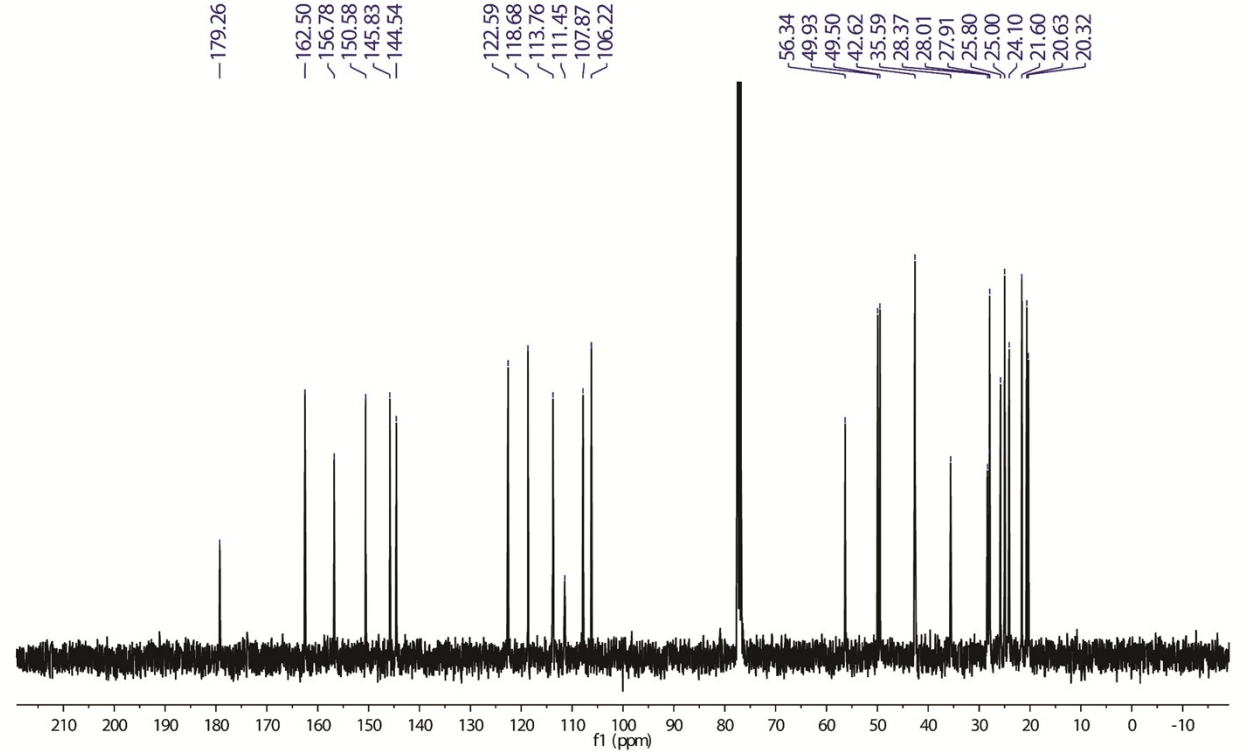
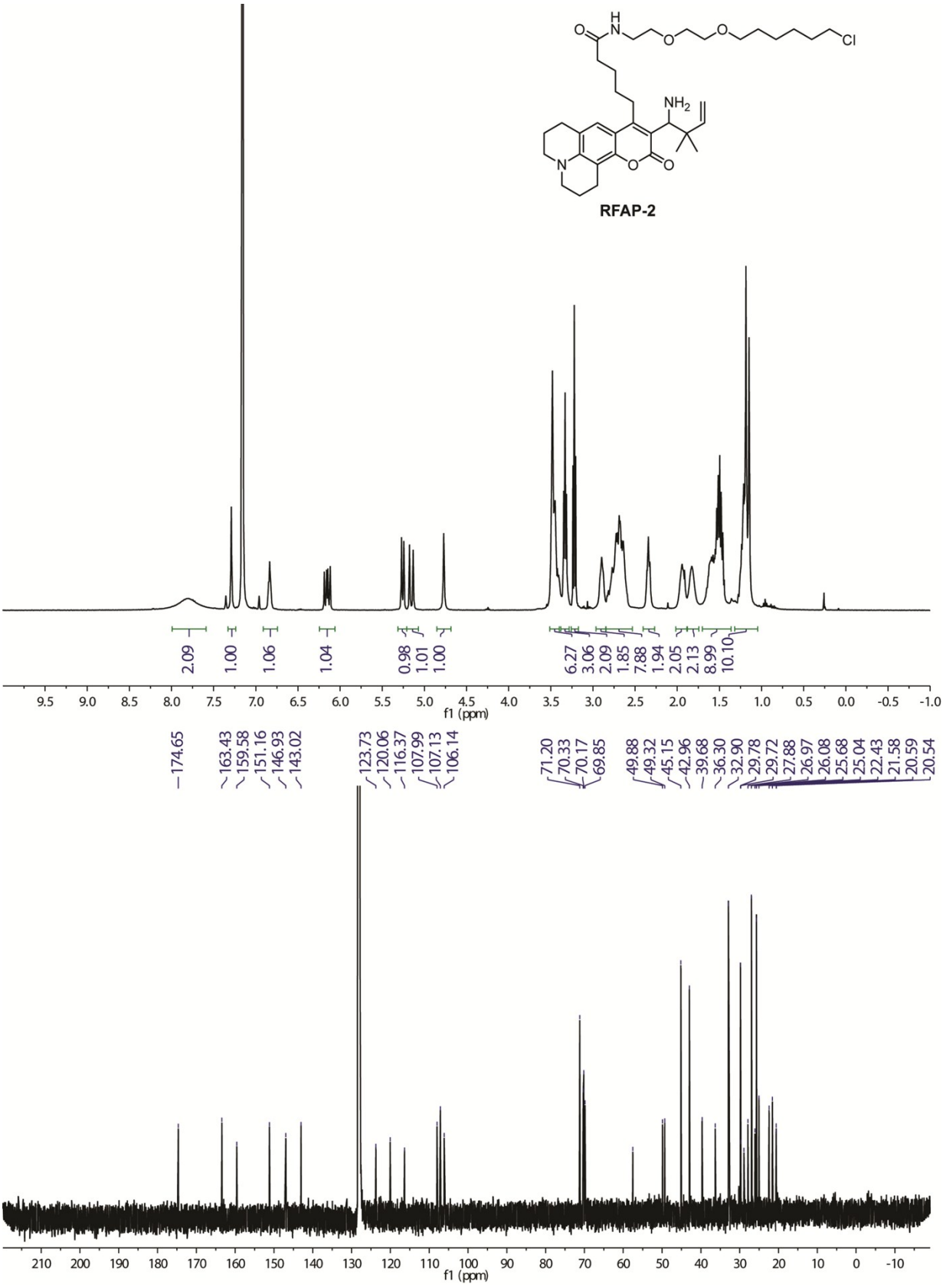
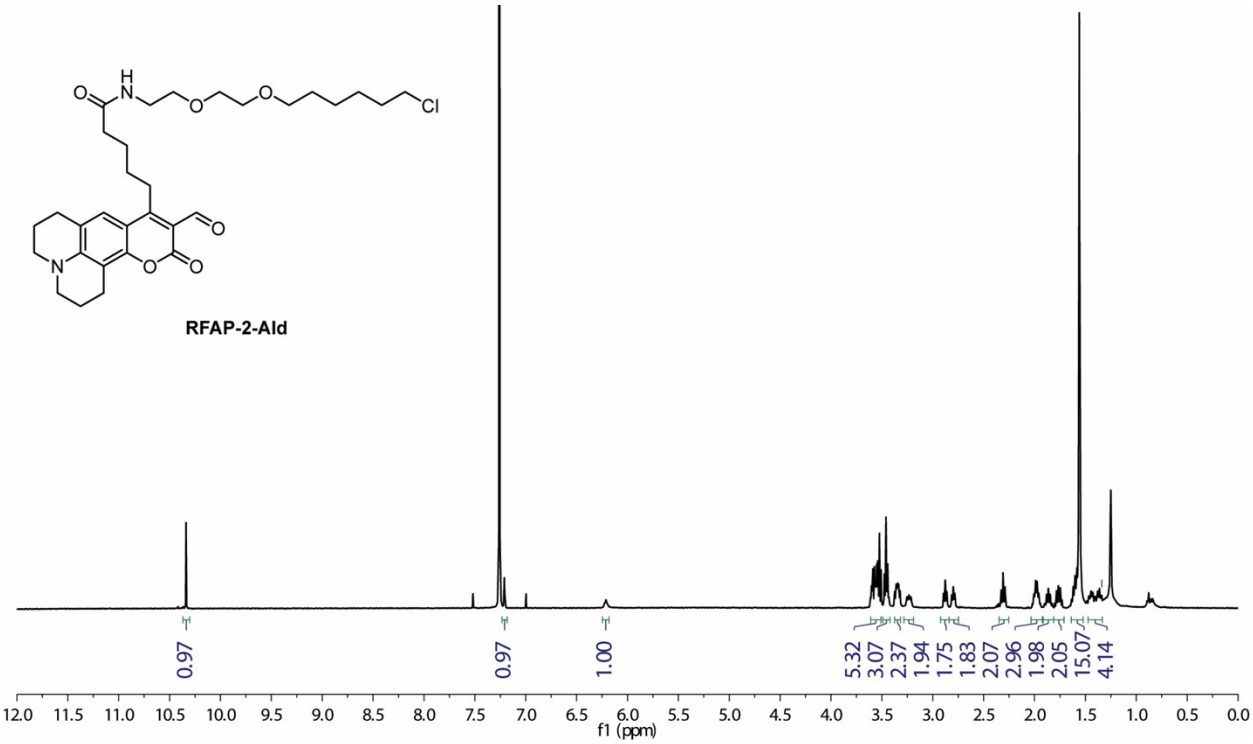
106.96
60.16
59.51
49.77
49.37
43.79
33.88
29.17
28.62
28.11
25.37
25.22
25.08
22.05
21.04
20.79
14.36
f1 (ppm)


O
OH
NH2
N
O
O
9
2.96
1.00
1.00
1.98
0.93
4.01
4.97
1.14
2.00
4.04
1.15
3.08
3.07
2.94
10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5 -1.0
f1 (ppm)


179.26
162.50
156.78
150.58
145.83
144.54
122.59
118.68
113.76
111.45
107.87
106.22
56.34
49.93
49.50
42.62
35.59
28.37
28.01
27.91
25.80
25.00
24.10
21.60
20.63
20.32
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)


RFAP-2
2.09
1.00
1.06
1.04
0.98
1.01
1.00
6.27
3.06
2.09
1.85
7.88
1.94
2.05
2.13
8.99
10.10
f1 (ppm)
174.65
163.43
159.58
151.16
146.93
143.02
123.73
120.06
116.37
107.99
107.13
106.14
71.20
70.33
70.17
69.85
49.88
49.32
45.15
42.96
39.68
36.30
32.90
29.78
29.72
27.88
26.97
26.08
25.68
25.04
22.43
21.58
20.59
20.54
f1 (ppm)


RFAP-2-Ald
0.97
0.97
1.00
5.32
3.07
2.37
1.94
1.75
1.83
2.07
2.96
1.98
2.05
15.07
4.14
f1 (ppm)