Synthesis of a far-red fluorophore and its use as an esterase probe in living cells
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Materials and Methods
Unless otherwise stated, reactions were magnetically stirred in flame-dried glassware under a nitrogen atmosphere. Anhydrous solvents were purchased in septum-sealed bottles and kept under nitrogen. All chemicals were purchased from Sigma-Aldrich, Fisher Scientific, or VWR Scientific and used as received. Reactions were monitored by thin-layer chromatography (TLC) on glass-backed silica gel plates (Silicycle 60 Å, 250 µM). Flash column chromatography was performed with the indicated solvents on Silicycle SiliaFlash P60.

Mass spectra were acquired at Portland State University's bioanalytical mass-spectrometry facility on a ThermoElectron LTQ-Orbitrap Discovery high resolution mass spectrometer with electrospray ionization (ESI).

NMR spectra were taken at ambient temperature at Portland State University's NMR facility. $^1$H-NMR data was obtained in the specified solvent on either a Bruker Avance ARX-400 at 400 MHz or a Bruker Avance ARX-600 at 600 MHz as indicated. $^{13}$C-NMR data was obtained in the specified solvent on either a Bruker Avance ARX-400 at 101 MHz or a Bruker Avance ARX-600 at 151 MHz as indicated. Spectra were calibrated to the residual solvent peak. Chemical shifts are reported in ppm. Coupling constants ($J$) are reported in Hertz (Hz) and rounded to the nearest 0.1 Hz. Multiplicities are defined as: s = singlet, d = doublet, dd = doublet of doublets, t = triplet, m = multiplet, br s = broad singlet.

1,3-dichloro-7-hydroxy-2H-spiro[acridine-9,1'-cyclohexane]-2',5'-diene-2,4'-dione (DSACO)$^{1,2}$

In a 125 mL Erlenmeyer flask open to the atmosphere, NaNO$_2$ was stirred with 0.5 mL H$_2$O. A solution of 23.75 mL concentrated H$_2$SO$_4$ and 0.75 mL H$_2$O was then added slowly to the flask. After gas evolution was complete and the solution had cooled to ambient temperature, ~50 glass beads (4 mm dia.) were added to the flask, followed by chloroindophenol (0.5818 g, 2.01 mmol, 1 equiv) to give a dark purple solution. The flask was then placed in a water bath and heated to 35 °C. Phenol (0.7638 g, 8.12 mmol, 4 equiv) was added in four portions over 35 min. After an additional 25 min, the reaction mixture was cooled, the glass beads removed, and the reaction mixture poured onto ice. The flask was rinsed with H$_2$O and MeOH. After the ice had melted, the solution was transferred to a separatory funnel and extracted with EtO (6 x 25 mL). The combined organic layers were washed with H$_2$O and brine before extraction with 3% Na$_2$CO$_3$ (aq) (6 x 25 mL). The combined aqueous extracts gave a deep blue solution, which was placed under a stream of air for 30 min. In order to decompose any remaining starting material and undesired side products, K$_3$Fe$_5$(CN)$_6$ (3.5 g) was then added to the flask and the resulting blue-green
solution protected from light and allowed to stir for 1.5 days. CaCl$_2$•H$_2$O (10 g) was added to the solution, which was then cooled to 0 °C and slowly quenched with concentrated HCl. When gas evolution had ceased, the resulting red suspension was extracted with Et$_2$O (5 x 30 mL). The combined organic layers were washed twice with brine, dried over MgSO$_4$, and concentrated in vacuo to give a dark red-brown solid (0.3206 g, 45% yield).

**DSACO:**
$^1$H-NMR (600 MHz; DMSO-d$_6$): δ 10.88 (br s, 1H), 7.93 (s, 1H), 7.62 (d, $J = 8.6$ Hz, 1H), 7.03-7.01 (m, 2H), 6.90 (dd, $J = 8.6, 2.6$ Hz, 1H), 6.54-6.52 (m, 2H).
$^{13}$C-NMR (151 MHz, DMSO-d$_6$): δ 185.14, 172.06, 161.85, 146.91, 146.11, 139.54, 137.15, 135.58, 135.20, 133.47, 131.84, 129.06, 125.92, 117.33, 115.45, 47.44.
ESI-HRMS: m/z calculated for C$_{18}$H$_8$Cl$_2$NO$_3$ ([M-H]$: 355.9876; found 355.9877.

**DSACO-2-AME and DSACO-7-AME**

DSACO (0.1195 g, 0.319 mmol, 1 equiv) and tetrabutylammonium hydrogensulfate (0.0109 g, 0.032 mmol, 0.1 equiv) were combined in CH$_2$Cl$_2$ (3 mL) in a 20 mL scintillation vial. Potassium carbonate (0.1323 g, 0.957 mmol, 3 equiv) in water (1 mL) was added to the vial, followed by the remaining water (1 mL, 0.16 M total). The biphasic mixture was stirred for 10 min, after which the bromomethyl acetate (0.125 mL, 1.28 mmol, 4 equiv) was added dropwise via syringe. The remaining CH$_2$Cl$_2$ (3 mL, 0.05 M total) was added, then the vial was sealed and wrapped in foil. The reaction mixture was stirred at room temperature for 3 d. The contents of the vial were transferred to a separatory funnel with additional CH$_2$Cl$_2$ and H$_2$O, the layers were separated and the aqueous layer was extracted with CH$_2$Cl$_2$ (3 x 15 mL). The combined organic layers were washed with brine, dried over MgSO$_4$, filtered, and concentrated in vacuo. Purification via column chromatography (SiO$_2$, 10 → 20% EtOAc/45 → 35% hexanes/45% CH$_2$Cl$_2$) afforded two products. DSACO-7-AME (R$_f$ = 0.49, 25% EtOAc/30%hexanes/45% CH$_2$Cl$_2$) was an orange solid (0.0210 g, 15%). DSACO-2-AME (R$_f$ = 0.37, 25% EtOAc/30%hexanes/45% CH$_2$Cl$_2$) was a yellow solid (0.0589 g, 43%).

**DSACO-7-AME:**
$^1$H-NMR (400 MHz; CDCl$_3$): δ 7.70 (d, $J = 8.9$ Hz, 1H), 7.69 (s, 1H), 7.10 (dd, $J = 8.7, 2.7$ Hz, 1H), 6.86 (d, $J = 2.7$ Hz, 1H), 6.68-6.65 (m, 2H), 6.61-6.57 (m, 2H), 5.74 (s, 2H), 2.11 (s, 3H).
$^{13}$C-NMR (101 MHz; CDCl$_3$): δ 185.00, 172.48, 169.54, 159.77, 148.04, 144.90, 140.43, 139.38, 137.81, 136.22, 135.03, 130.48, 125.43, 116.91, 116.84, 84.62, 47.63, 20.91.
ESI-HRMS: m/z calculated for C$_{21}$H$_{13}$Cl$_2$NO$_3$ ([M+][+]$: 429.0176; found 429.0169.

**DSACO-2-AME:**
$^1$H-NMR (400 MHz; CDCl$_3$): δ 7.81 (s, 1H), 7.40 (d, $J = 9.8$ Hz, 1H), 6.67 (dd, $J = 9.6, 2.0$ Hz, 2H), 6.67-6.63 (m, 2H), 6.53-6.49 (m, 2H), 6.48 (d, $J = 2.0$ Hz, 1H), 5.71 (s, 2H), 2.08 (s, 3H).
$^{13}$C-NMR (101 MHz, CDCl$_3$): $\delta$ 186.00, 184.89, 170.05, 153.04, 152.13, 146.65, 141.03, 140.42, 134.41, 132.97, 132.94, 132.67, 131.29, 129.85, 122.96, 88.02, 46.40, 20.90.

ESI-HRMS: m/z calculated for C$_{21}$H$_{13}$Cl$_2$NO$_5$ ([M+e]$: 429.0176; found 429.0176.

**Spectral Characterization**

The spectral properties of DDAO, DSACO, DSACO-2-AME, and DSACO-7-AME are summarized in Table S1. Absorbance and emission spectra were acquired on a Tecan Infinite M200 Pro microplate reader in Starna Cell quartz cuvettes (1 nm step size) and in black 96-well microplates (10 reads per well, 1 nm step size) respectively. In order to remain in the linear range of the instrument, compounds were diluted to 10 $\mu$M for absorbance reads and 1 $\mu$M for fluorescence reads. Excitation wavelengths were 618 nm (DSACO), 602 nm (DDAO), 454 nm (DSACO-7-AME), and 388 nm (DSACO-2-AME).

The extinction coefficients were determined for DDAO, DSACO, DSACO-2-AME, and DSACO-7-AME in 10 mM HEPES (pH 7.3). Four independent stocks of each compound in DMSO were prepared from purified solid (20 mM for DSACO or 5 mM for DDAO, DSACO-2-AME, and DSACO-7-AME). 1 mM solutions in DMSO were prepared from the stock solutions. Two independent 10 $\mu$M solutions of each compound in HEPES were prepared from the 1 mM solutions. Absorbance was measured with a Tecan Infinite M200 Pro microplate reader in quartz cuvettes at the wavelength of maximum absorption. The molar extinction coefficients were calculated using Beer's law ($\varepsilon = \frac{A}{c \cdot l}$; $\varepsilon$ = extinction coefficient, $A$ = absorbance, $c$ = concentration, $l$ = pathlength).

The relative quantum yields of DDAO and DSACO were determined using oxazine 1 (Anaspec, $\phi = 0.15$ in EtOH) as the reference. A 10 $\mu$M solution of each dye was prepared from a 1 mM stock in DMSO and its absorbance measured with a Tecan Infinite M200 Pro microplate reader in quartz cuvettes at 520 nm. For each compound, three independent dilutions at a calculated absorbance of 0.01 were then prepared from the 10 $\mu$M solution. Three samples from each dilution (for a total of $n = 9$) were then excited at 520 nm in a 96-well black plate and the fluorescence emission curves recorded by a Tecan Infinite M200 Pro microplate reader. The total fluorescence was calculated by numerical integration of the area under the curve in GraphPad Prism 6. DDAO and DSACO were evaluated in both HEPES (pH 7.3) and EtOH.

The relative quantum yield of DSACO-7-AME was determined using fluorescein (Sigma-Aldrich, $\phi = 0.89$ in 0.1 M NaOH) as the reference. A 10 $\mu$M solution of each probe was prepared from a 1 mM stock in DMSO and its absorbance measured with a Tecan Infinite M200 Pro microplate reader in quartz cuvettes at 400 nm. For each compound, three independent dilutions at a calculated absorbance of 0.01 were then prepared from the 10 $\mu$M solution. Three samples from each dilution (for a total of $n = 9$) were then excited at 400 nm in a 96-well black plate and the fluorescence emission curves recorded by a Tecan Infinite M200 Pro microplate reader. The total fluorescence was calculated by numerical integration of the area under the curve in GraphPad Prism 6. DSACO-7-AME was evaluated in EtOH. DSACO-2-AME was not evaluated, as it displayed no detectable fluorescence.
**Determination of pKₐ**
The pKₐ of DSACO was determined by measuring the fluorescence of the dye in citric acid buffer (pH = 2.6 to 7.5). DSACO (1 mM in DMSO) was diluted to 5 µM at each pH ($n = 3$). Fluorescence measurements were performed on a Tecan Infinite M200 Pro microplate reader with $\lambda_{ex} = 618$ nm and $\lambda_{em} = 678$ nm. Data were fit to a sigmoidal curve in GraphPad Prism (Figure S2) and the inflection point used to determine the pKₐ.

**Esterase and Lipase Screen**
Esterases and lipases (Table S2) were purchased from Sigma-Aldrich. Lipases were purchased as part of the Lipase Basic Kit (product 62327). Enzymes were prepared as 10 mg/mL stocks and diluted to 5 µg/mL in 10 mM HEPES (pH 7.3). Heat-killed PLE was prepared by incubating PLE at 90 °C for 20 min. Probe concentrations were 5 µM. After incubation at 37 °C for 10 min, DSACO fluorescence ($\lambda_{ex} = 618$ nm, $\lambda_{em} = 678$ nm) was measured on a Tecan Infinite M200 Pro microplate reader.

**PLE Detection Limit**
Solutions of DSACO-2-AME, DSACO-7-AME, and fluorescein diacetate were prepared at 10 µM in 10 mM HEPES (pH 7.3). PLE was diluted in 10 mM HEPES (pH 7.3) such that the final amount of enzyme in each reaction ranged from 0 to 100 pg for fluorescein diacetate or 0 to 300 pg for DSACO-2-AME and DSACO-7-AME. After incubation at 37 °C for 20 min, the fluorescence (DSACO: $\lambda_{ex} = 618$ nm, $\lambda_{em} = 678$ nm; fluorescein: $\lambda_{ex} = 490$ nm, $\lambda_{em} = 525$ nm) was measured by a Tecan Infinite M200 Pro microplate reader (Figure S3). The detection limit was calculated as the lowest amount of PLE where fluorescence was statistically significant ($p < 0.01$) compared to a no-enzyme control. Significance was calculated in GraphPad Prism using an unpaired, two-tailed t-test with Welch's correction.

**Kinetic Characterization with PLE**
Probes were evaluated in triplicate with PLE at a final concentration of 50 ng/mL in 10 mM HEPES (pH 7.3) with 20% MeCN, to improve substrate solubility. The amount of enzyme was chosen to allow an accurate measurement of the initial rate. Probe and enzyme solutions were incubated separately at 37 °C for 5 min prior to the addition of the enzyme to the probe. Fluorescence (DSACO: $\lambda_{ex} = 618$ nm, $\lambda_{em} = 678$ nm) was then recorded every 14 s by a Tecan Infinite M200 Pro microplate reader. Kinetic parameters were calculated via the initial rates method using GraphPad Prism. The data fit the Michaelis-Menten model of enzyme kinetics ($V = V_{max}\frac{[S]}{K_M+[S]}$), where V = reaction rate, S = substrate concentration, and K_M = Michaelis constant) (Figure S6). Due to solubility limits, the K_M was extrapolated.

**Hydrolitic Stability**
DSACO-2-AME and DSACO-7-AME were diluted to 5 µM in PBS (pH 7.4), DMEM [High Glucose Dulbecco's Modified Eagle Medium (HyClone)], or DMEM-FBS [DMEM supplemented with 10% fetal bovine serum (HyClone)]. The samples were incubated at 37 °C in a 96-well plate, and the fluorescence ($\lambda_{ex} = 618$ nm, $\lambda_{em} = 678$ nm) was recorded every 1 min for 2h and then every 10 min for an additional 19 h on a Tecan Infinite M200 Pro microplate reader. The stability curves are shown in Figure S7. Samples containing serum were completely hydrolyzed during the course of the experiment and GraphPad Prism was used to fit the data to a
one phase association model \( (Y = Y_0 + (\text{Plateau} - Y_0) \ast (1 - e^{-Kx})) \), where \( Y \) = fluorescence, \( K \) = the rate constant, and \( x \) = time. The half-life was calculated as \( t_{1/2} = \frac{\ln(2)}{K} \). Since the probes in PBS did not completely hydrolyze, the plateau was calculated by treating 5 \( \mu \)M of each probe with 20 \( \mu \)g/mL of PLE. The one-phase association model was constrained by this value in order to calculate \( t_{1/2} \). Calculated half-lives are listed in Table S3.

**Cell Culture, Fluorescence Microscopy, and Image Processing**

Rat-1 fibroblasts were obtained from ATCC. Cells were grown in DMEM (HyClone) supplemented with 10% FBS (HyClone) and 100 units/mL penicillin and 100 \( \mu \)g/mL streptomycin in a humidified incubator at 37 \( ^\circ \)C (5% CO\(_2\)). Cultured cells were plated in 35-mm dishes with 20 mm glass microwells (Cellvis) and grown to 90% confluency. Cells were counterstained with 5 \( \mu \)g/mL Hoechst 33342 (Invitrogen) and washed once with warm PBS. Cells were then incubated with 10 \( \mu \)M of probe for 20 min at 37 \( ^\circ \)C in serum-free DMEM. The cells were washed once with warm PBS and imaged in serum-free DMEM. Fluorescence was imaged on a confocal microscope (Yokogawa CSU-W1 spinning disk confocal mounted on a Nikon TiE) using a Nikon 40x/1.3 Plan Fluor oil objective at OHSU’s Advanced Light Microscopy Core. Hoechst 33342 fluorescence was obtained by excitation at 405 nm, and DSACO fluorescence was obtained by excitation at 640 nm.

The brightness and contrast settings were manually adjusted for each image using ImageJ software. Standardized minimum and maximum pixel values were applied to images in the DSACO channel (Figure 4) in order to compare the two probes to background. The settings were readjusted for Figure S4 in order to compare cells treated with DSACO-7-AME to cells treated with DMSO in order to highlight the fluorescent labeling observed for that probe.

**Cell viability assay**

The affect of probe exposure on cell viability was evaluated by measuring the amount of ATP produced by treated cells, which is a direct indicator of metabolically active cells. Rat-1 fibroblasts were seeded into a 96-well tissue culture plate (Costar #3610) and grown overnight to 70-80% confluence. Cells were washed once with warm PBS, and then treated with each compound in media at 37 \( ^\circ \)C for 1 h or 24 h. For the short-exposure (1 h) experiment, cells were treated with probe in serum-free DMEM in order to mimic our live-cell imaging experiments. For the long-exposure (24 h) experiment, cells were incubated with probe in complete medium (DMEM + 10% FBS). Each probe (DSACO, DSACO-2-AME, DSACO-7-AME, and FDA) was evaluated at 10 \( \mu \)M final concentration (0.2% DMSO in media), and mock-treated cells (0.2% DMSO in media) were used as a negative control. Cells treated with hygromycin B (HygB; 2 mM), a known cytotoxic agent, served as a positive control. After incubation with the compounds, plates were cooled to room temperature. ATP levels were measured using the CellTiter-Glo 2.0 assay (Promega), following the manufacturer’s instructions. Luminescence was recorded on a GloMax Multimodal microplate reader after a 25 min room temperature incubation with the CellTiter-Glo 2.0 reagent (Figure S5).

Exposure to our probes for 1 h or 24 h did not affect the viability of Rat-1 fibroblasts. None of the compounds, including HygB, decreased viability after 1 h. However, the cytotoxic treatment
were effective at decreasing cell viability after a 24 h exposure while DSACO, DSACO-2-AME, DSACO-7-AME, and FDA all maintained at least 95\% of the viability of the mock-treated cells.
Supplementary Figures and Tables

**Figure S1.** Absorbance and emission spectra of the carbazine dyes DSACO and DDAO. Samples were prepared in 10 mM HEPES (pH 7.4). Absorbance is indicated by solid lines, emission by dashed lines.

**Figure S2.** Determination of the pKₐ of DSACO.
Figure S3. The PLE detection limit of 10 µM probe after a 20 min incubation. a) DSACO-2-AME. b) DSACO-7-AME. c) Fluorescein diacetate. Labeled responses were statistically significant (p < 0.01) compared with the no-enzyme control. Error bars represent one standard deviation; n = 4.
Figure S4. Live-cell imaging of esterase activity in Rat1-fibroblast cells via confocal fluorescence microscopy. The cells were incubated with 10 μM DMSO or 10 μM DSACO-7-AME for 20 min, then washed and imaged to detect DSACO fluorescence ($\lambda_{ex} = 640$ nm). Cells were counterstained with Hoechst 33342 ($\lambda_{ex} = 405$ nm). Scale bars represent 50 μm.
Figure S5. Luminescence of Rat-1 fibroblasts treated with 10 µM DSACO, DSACO-2-AME, DSACO-7-AME, FDA, or HygB. Luminescence was measured after 1 h in serum-free DMEM (a) or after 24 h in DMEM supplemented with 10% serum (b).

Figure S6. Kinetic evaluation of DSACO-2-AME (7.5 to 80 µM) with PLE.
Figure S7. Hydrolytic stability of DSACO-2-AME and DSACO-7-AME. a) 10% FBS in DMEM, b) DMEM without FBS, c) PBS (pH 7.4).
Table S1. Summary of spectral data for DDAO, DSACO, DSACO-2-AME, and DSACO-7-AME.

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<th>compound</th>
<th>solvent</th>
<th>$\lambda_{abs}$ (nm)</th>
<th>$\lambda_{em}$ (nm)</th>
<th>$\varepsilon$ (M$^{-1}$ cm$^{-1}$)</th>
<th>$\phi$</th>
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<tr>
<td>DDAO</td>
<td>HEPES (pH 7.3)</td>
<td>602</td>
<td>659</td>
<td>36,000 ± 4,000</td>
<td>0.39 ± 0.04</td>
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<tr>
<td>DSACO</td>
<td>HEPES (pH 7.3)</td>
<td>618</td>
<td>678</td>
<td>34,000 ± 4,000</td>
<td>0.22 ± 0.01</td>
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<td>DSACO</td>
<td>EtOH</td>
<td>668</td>
<td>682</td>
<td>32,000 ± 2,000</td>
<td>0.29 ± 0.02</td>
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<td>DSACO-2-AME</td>
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<td>388</td>
<td>not fluorescent</td>
<td>15,600 ± 300</td>
<td>n/a</td>
</tr>
<tr>
<td>DSACO-7-AME</td>
<td>EtOH</td>
<td>462</td>
<td>622</td>
<td>13,800 ± 700</td>
<td>0.012 ± 0.001</td>
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Table S2. Enzymes used for probe characterization.

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<td>porcine liver esterase</td>
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<td>Saccharomyces cerevisiae esterase</td>
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<td>Bacillus subtilis esterase</td>
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Table S3. Half-life (h) for the hydrolysis of DSACO-2-AME and DSACO-7-AME under different conditions.

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<th>DMEM-FBS (h)</th>
<th>DMEM (h)</th>
<th>PBS (h)</th>
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<tr>
<td>DSACO-2-AME</td>
<td>0.4</td>
<td>25</td>
<td>27</td>
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<tr>
<td>DSACO-7-AME</td>
<td>1.5</td>
<td>64</td>
<td>83</td>
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Figure S8a. $^1$H-NMR spectrum of DSACO (600 MHz; DMSO-d$_6$).
**Figure S8b.** $^{13}$C-NMR spectrum of DSACO (151 MHz; DMSO-$d_6$).
Figure S9a. $^1$H-NMR spectrum of DSACO-7-AME (400 MHz; CDCl$_3$).
Figure S9b. $^{13}$C-NMR spectrum of DSACO-2-AME (101 MHz, CDCl$_3$).
Figure S9c. HSQC spectrum of DSACO-7-AME (CDCl₃).
Figure S9d. HMBC spectrum of DSACO-7-AME (CDCl$_3$).
Figure S10a. $^1$H-NMR spectrum of DSACO-2-AME (400 MHz; CDCl$_3$).
Figure S10b. $^{13}$C-NMR spectrum of DSACO-2-AME (101 MHz; CDCl$_3$).
Figure S10c. HSQC spectrum of DSACO-2-AME (CDCl₃).
Figure S10d. HMBC spectrum of DSACO-2-AME (CDCl₃).
References and Notes


2 Reference 1 reports the absorbance maximum at 620 nm and the pKₐ at 5.0. The only other data provided for this compound is the potential (Eₘ₈ = 190 mV) and the molar (linear) absorption coefficient (log₁₀ε = 4.67).