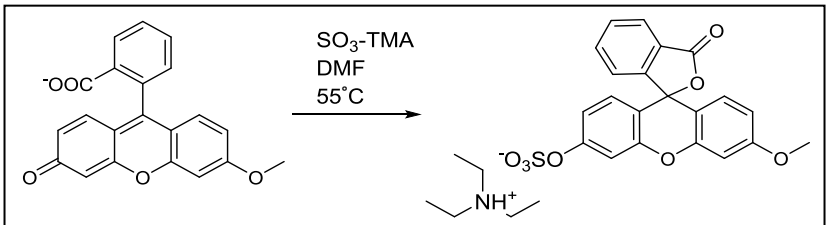
SUPPORTING INFORMATION

Materials and Methods

Synthesis and purification of 3-O-methylfluorescein sulfate (MFS)

All chemicals were purchased from Sigma-Aldrich unless otherwise indicated. 3-O-methylfluorescein (**MF**; MP Biomedicals, 81.6 mg, 236 μmol) was dissolved in anhydrous DMF (3 mL) in a round-bottom flask on ice under inert atmosphere (nitrogen gas). Sodium hydride (60% dispersion in mineral oil, 25.9 mg, 648 μmol) was added to the stirring solution of **MF**. The flask was removed from the ice bath and allowed to warm to room temperature for 15 min. Sulfur trioxide trimethylamine (166 mg, 1.19 mmol) was added, and the reaction was heated to 55 °C overnight. The reaction was then cooled and quenched with methanol. The solvent was evaporated, and the resulting yellow oil was purified by silica column chromatography using SiliaFlash P60 (SiliCycle) in methylene chloride and methanol (0% to 15% methanol) with 0.05% triethylamine to give a yellow oil (119 mg, 96% yield of **MFS** with a triethylammonium counterion). The compound was further purified by reversed phase HPLC using a Varian Pro Star system on a Dynamax 100 Å C18 preparative column. A linear gradient of water and methanol was used (95% water to 0% water). A peak corresponding to **MFS** was collected, and the solvent was evaporated to give **MFS** as a pale yellow oil (90.4 mg, 73% isolated yield). Stock solutions of **MFS** (100 mM in methanol) were stored at -20 °C.

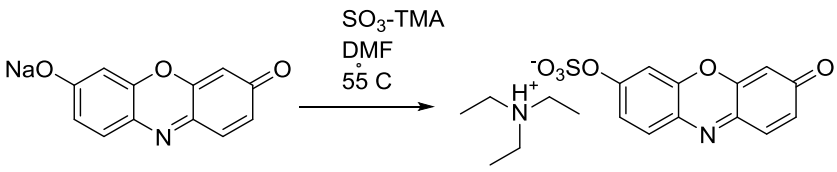


Scheme S1. Synthesis of MFS from MF.

^{1}H NMR (CD_3OD, 400 MHz): δ 7.93 (1H, d, *J* = 7.6 Hz), 7.69 (1H, dt, *J* = 6.6), 7.63 (1H, dt, *J* = 7.7), 7.24 (1H, d, *J* = 2.4), 7.1 (1H, d, *J* = 7.6), 6.9 (dd, *J* = 2.2, 7.2), 6.81 (1H, s), 6.7, (1H, d, 8.8), 6.6 (2H, s), 3.76 (3H, s). Spectrum also contained the triethylammonium counterion: 3.1 (4.9H, q, *J* = 7.4), 1.2 (7.4H, t, *J* = 7.2) (**Figure S1**). ^{13}C NMR (CD_3OD, 160 MHz): δ 171.41, 163.37, 155.95, 154.56, 153.94, 153.31, 136.92, 131.45, 130.15, 129.83, 127.88, 126.03, 125.37, 118.50, 116.57, 113.20, 112.23, 110.37, 102.04, 84.68, 56.32. Spectrum also contained the triethylammonium counterion: 48.09, 9.37 (**Figure S2**). High-resolution mass spectrometry (ESI, negative mode) was performed at the UC Berkeley Mass Spectrometry Laboratory. A peak corresponding to **MFS** ($C_{21}H_{13}O_8S^-$) was observed at 425.0333 m/z (calculated: 425.0336 m/z). A peak corresponding to the loss of the sulfate, to give **MF** ($C_{21}H_{13}O_5^-$), was observed at 345.0767 m/z (calculated: 345.07685 m/z).

Synthesis and purification of resorufin-sulfate (RS)

Resorufin sodium salt (104 mg, 442 μmol) was dissolved in anhydrous DMF (3 mL) in a round-bottom flask on ice under inert atmosphere (nitrogen gas). Sodium hydride (60% dispersion in mineral oil, 60 mg, 1.5 mmol) was added to the stirring solution. The flask was removed from the ice bath and allowed to warm to room temperature for 10 min. Sulfur trioxide trimethylamine (492 mg, 3.54 mmol) was added, and the reaction was heated to 55 °C overnight. The reaction was then cooled and quenched with methanol. The solvent was evaporated, and the resulting solid was purified by silica column chromatography using SiliaFlash P60 (SiliCycle) in methylene chloride and methanol (0% to 10% methanol) with 0.05% triethylamine to give a red solid (155 mg, 89% yield of **RS** with a triethylammonium counterion). The compound was then purified by reversed phase HPLC using a Varian Pro Star system on a Dynamax 100 Å C18 semi-preparative column. A linear gradient of water and methanol was used (100% water to 0% water). A single peak corresponding to **RS** was collected, and solvent was evaporated to give a pure yellow-orange solid (0.016 g, 9% isolated yield). The isolated yield was diminished by some decomposition of **RS** to form resorufin during the purification. Stock solutions of **RS** (20 mM in methanol) were stored at -20 °C under an inert atmosphere with desiccant.



Scheme S2. Synthesis of RS from resorufin.

^{1}H NMR (CD_3OD, 400 MHz): δ 7.73 (1H, d, *J* = 8.8), 7.46 (1H, d, *J* = 9.6), 7.35 (1H, d, *J* = 2.4), 7.26 (1H, dd, *J* = 2.2, 9), 6.76 (1H, d, 9.6), 6.25 (1H, d, *J* = 2). Spectrum also contains the triethylammonium counterion: 3.0 (5H, q, *J* = 7.1), 1.2 (8H, t, *J* = 7.4) (**Figure S3**). ^{13}C NMR (CD_3OD, 151 MHz): δ 214.84, 188.86, 158.54, 152.14, 148.38, 136.89, 135.38, 132.56, 131.80, 120.13, 109.02, 107.14. Spectrum also contains the triethylammonium counterion: 47.83, 9.79 (**Figure S4**). A peak corresponding to **RS** ($C_{12}H_6NO_6S^-$) was observed at 291.9918 m/z (calculated: 291.99213 m/z). A peak corresponding to the loss of the sulfate, to give resorufin ($C_{12}H_6NO_3^-$), was observed at 212.0352 m/z (calculated: 212.03532 m/z).

Characterization of fluorophores

Fluorescence properties of compounds were measured on a QuantaMaster spectrofluorimeter (PTI). Sample solutions were analyzed in fluorescence grade quartz cuvettes. Excitation and emission spectra for 1 μM **MF** and **MFS** were measured in 100 mM Tris buffer pH 7.9 (**Figure S5**). Excitation

and emission spectra for 1 μM resorufin and **RS** were measured in 100 mM Tris buffer pH 7.9 (**Figure S6**). All measurements were taken at room temperature.

The fluorescence quantum yields for **MF** and **RS** were determined by comparing the integrated emission of each fluorophore to the integrated emission of a known fluorophore[1]. Fluorescein in 0.1 N NaOH (quantum yield: 0.92) was used as a standard to find the quantum yield of **MF** in 0.1 N NaOH and **RS** in methanol. The quantum yield of **MF** was calculated to be 0.45, which is close to the reported value of 0.37[2], and the quantum yield of **RS** was calculated to be 0.011. **MFS** is non-fluorescent; therefore, we did not attempt to determine the quantum yield.

Probe stability in aqueous buffer

Stability studies were performed by monitoring the production of **MF** from **MFS** or the production of resorufin from **RS (Figure S7)**. Reactions were set-up in triplicate at 37 ˚C in Lysate Reaction Buffer [LR Buffer: 50 mM Tris (pH 7.5 at 37 °C), 100 mM NaCl, and 250 μM each $MnCl_2$, $MgCl_2$, and $CaCl_2$]. Each reaction contained 100 μM sulfated probe in 100 μL LR Buffer. Fluorescence of hydrolyzed **MF** and resorufin were measured every 15 min for 40 h on a fluorescence microplate reader (Molecular Devices Spectramax M3). For reactions with **MFS**, fluorescence of **MF** was measured (excite 475 nm, emit 525 nm, bp 515 nm). For reactions with **RS**, fluorescence of resorufin was measured (excite 550 nm, emit 600 nm, bp 590 nm). A standard curve was created by measuring the fluorescence of **MF** or resorufin at varying concentrations (50 nM to 500 nM). The standard curve was used to convert the measured fluorescence signal from arbitrary units to pmoles. The amount of hydrolyzed dye was determined by the change in **MF** or resorufin fluorescence over time.

Enzyme activation of probes with *Aerobacter aerogenes*

A. aerogenes sulfatase (Sigma-Aldrich, S1629) was evaluated with **MFS** and **RS** (**Figure S8**). This enzyme was used at a final concentration of 0.047 U/mL (enzyme units “U” are as defined by Sigma-Aldrich). Reactions were performed in triplicate in LR buffer. Kinetic parameters were determined using the method of initial rates[3] and plotted using GraphPad Prism. A general equation for Michaelis-Menten kinetics, y = (m1 * x) / (m2 + x), was used to fit data. Reactions were monitored by measuring the increase in **MF** or resorufin fluorescence on a fluorescence microplate reader (Molecular Devices Spectramax M3). A standard curve was created by measuring the fluorescence of **MF** (excite 475, emit 525 nm, bp 515 nm) at varying concentrations (10 nM to 5 μM). **MFS** concentrations ranged from 25 μM to 700 μM. Alternatively, a standard curve was created by measuring the fluorescence of resorufin (excite 550, emit 600 nm, bp 590 nm) at varying concentrations (100 nm to 1 μM). **RS**

concentrations ranged from 12.5 μM to 750 μM. *A. aerogenes* sulfatase was evaluated with DDAO-sulfate[4] and the commercial substrates 4-MUS and *p*-NPS as described[5].

Enzyme activation of probes with *Helix pomatia* sulfatase

H. pomatia sulfatase (Type H-1, Sigma, S9226) was evaluated with **MFS** and **RS** (**Figure S9**). The enzyme was used at a final concentration of 0.248 U/mL. Reactions were performed in triplicate in acidic buffer (100 mM potassium acetate, 100 mM NaCl, 250 μM $MgCl_2$, $CaCl_2$, and $MnCl_2$, pH 5) at 37 ˚C. Kinetic parameters were determined using the method of initial rates[3] and plotted using GraphPad Prism. A general equation for Michaelis-Menten kinetics, y = (m1 * x) / (m2 + x), was used to fit data. Reactions were monitored by measuring the increase in **MF** or resorufin fluorescence on a fluorescence microplate reader. A standard curve was created by measuring the fluorescence of **MF** (excite 475, emit 525 nm, bp 515 nm) at varying concentrations (125 nM to 5 μM). **MFS** concentrations ranged from 5 μM to 250 μM. A standard curve was created by measuring the fluorescence of resorufin (excite 550, emit 600 nm, bp 590 nm) at varying concentrations (25 nM to 0.5 μM). **RS** concentrations ranged from 3 μM to 500 μM. *H. pomatia* was evaluated with DDAO-sulfate as described[4].

Enzyme activation of probes with abalone entrails sulfatase type VIII

Sulfatase from abalone entrails (type VIII, Sigma-Aldrich, S9754) was evaluated with **MFS** and **RS** (**Figure S10**). The enzyme was used at a final concentration of 0.19 U/mL. Reactions were performed in triplicate in acidic buffer (100 mM potassium acetate, 100 mM NaCl, 250 μM $MgCl_2$, $CaCl_2$, and $MnCl_2$, pH 5) at 37 ˚C. Kinetic parameters were determined using the method of initial rates[3] and plotted using GraphPad Prism. Reactions were monitored by measuring the increase in **MF** or resorufin fluorescence on a fluorescence microplate reader (Molecular Devices). A standard curve was created by measuring the fluorescence of **MF** (excite 475, emit 525 nm, bp 515 nm) at varying concentrations (125 nM to 5 μM). **MFS** concentrations ranged from 70 μM to 1500 μM. Due to the substrate inhibition observed, a general equation for substrate inhibition, y = (m1 * x) / {m2 + [x * **(**1 + (x / m3)**)**]}, was used to fit the kinetic data. A standard curve was created by measuring the fluorescence of resorufin (excite 550, emit 600 nm) at varying concentrations (25 nM to 0.5 μM). **RS** concentrations ranged from 3 μM to 500 μM. A general equation for Michaelis-Menten kinetics, y = (m1 * x) / (m2 + x), was used to fit data since substrate inhibition was not observed. Abalone entrails was evaluated with DDAO-sulfate as described[4].

Detection Limit for *A. aerogenes* measured in a 96-well plate

The detection limit of **MFS**, **RS**, DDAO-sulfate, 4-MUS, *p*-NPS, and 4-nitrocatechol sulfate were evaluated with *A. aerogenes* sulfatase (**Table S1**). Each probe was used at a saturating concentration ($2*K_M$: 1.2 mM **MFS**; 324 μM **RS**; 328 μM DDAO-sulfate; 1.4 mM 4-MUS; 3.6 mM p-NPS; 3.6 mM 4-nitrocatechol sulfate). Reactions were performed in quadruplicate in LR Buffer. The amount of *A. aerogenes* ranged from 0.0315 to 1580 ng. The *A. aerogenes* sulfatase stock concentration was determined by the manufacturer and the sulfatase was used as received without further purification. Reactions were initiated by the addition of sulfatase probe and were monitored at 37 ˚C. Fluorescence of hydrolyzed **MF**, resorufin, DDAO, and 4-MU were measured at various time points, including 10 min (**Figure S11**) and 3 h (**Figure S12**). For reactions with **MFS**, fluorescence of **MF** was measured (excite 475, emit 525 nm, bp 515 nm). For reactions with **RS**, fluorescence of resorufin was measured (excite 550 nm, emit 600 nm, bp 590 nm). For reactions with DDAO-sulfate, fluorescence of DDAO was measured (excite 635 nm, emit 675 nm, bp 665 nm). For reactions with 4-MUS, fluorescence of 4-MU was measured (excite 360 nm, emit 450 nm, bp 435 nm). Although 4-MU fluorescence is enhanced by basification (> pH 7.8), fluorescence was directly monitored at pH 7.5. Absorbance of hydrolyzed *p*-NP and 4-nitrocatechol were measured at various time points, including 10 min (**Figure S11**) and 3 h (**Figure S12**). For reactions with *p*-NPS, absorbance of *p*-NP was measured at 405 nm. For reactions with 4-nitrocatechol sulfate, the absorbance of 4-nitrocatechol was measured at 535 nm. Significant signal ($p < 0.01$ or $p < 0.001$) over background was determined using an unpaired t-test in Microsoft Excel.

Table S1: Lowest detectable amount of *A. aerogenes* sulfatase with each probe.

	Detection Limit[a] (ng)	
Probe (concentration)	**10 min**	**3 h**
MFS (1.2 mM)	158	31.5
RS (324 μM)	15.8	1.58
DDAO-sulfate (328 μM)	315	15.8
4-MUS (1.4 mM)	15.8	3.15
p-NPS (3.6 mM)	15.8	1.58
4-nitrocatechol sulfate (3.6 mM)	158	31.5

[a]The detection limit is the minimal amount of *A. aerogenes* sulfatase required to give a significant signal ($p < 0.01$) above the enzyme-free control.

Strains and Culture

A variety of mycobacterial species were purchased from American Type Tissue Culture Collection (ATCC), including *M. marinum* (ATCC BAA-535), *M. flavescens* (ATCC 14474), *M. intracellulare*

(ATCC 35847), *M. nonchromogenicum* (ATCC 19530), and *M. peregrinum* (ATCC 14467). These species were all treated as BioSafety Level 2 pathogens. *M. bovis* (BCG), *M. avium*, *M. kansasii*, and *M. tuberculosis* (H37Rv, CDC1551, Erdman) were obtained from Prof. Lee Riley (UC Berkeley). These species were all treated as BioSafety Level 3 pathogens.

M. tuberculosis strains, *M. avium*, *M. bovis*, and *M. kansasii* were grown at 37 °C in Middlebrook 7H9 broth or on 7H11 agar supplemented with 0.5% glycerol, 0.05% Tween-80, and 10% OADC. Other species were grown at 30 °C (*M. marinum* and *M. intracellulare*) or 37 °C (*M. flavescens*, *M. nonchromogenicum*, and *M. peregrinum*) in 7H9 broth or 7H10 agar supplemented with 0.5% glycerol, 0.05% Tween-80, and 10% OADC. Cultures were grown to log phase ($0D_{600}$ = 0.5 to 1.5) before harvesting by centrifugation at 4 °C. The supernatant was discarded, and the pellet was frozen (-20 °C) prior to lysis.

Preparation of Clarified Lysates

Mycobacterial cell pellets were put in Lysis Buffer [50 mM Tris (pH 7 at 4 °C), 100 mM NaCl, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, plus an EDTA-free Protease Inhibitor Tablet (Roche)]. Cells were lysed by mechanical disruption using 0.1 mm zirconia/silica beads (BioSpec Products) on a FastPrep (MP Biomedical) bead-beating instrument. Crude lysates were clarified by centrifugation (10 min, 16,000*g*, 4 °C). Mammalian cell lysates were processed using a previously described procedure[4]. Mycobacterial cell pellets from BioSafety Level 3 pathogens were lysed in Lysis Buffer supplemented with 0.1% Triton X-100 and sterilized by filtration twice through a 0.2 μm filter (Acrodisc PVDF 13 mm syringe filters). Total protein concentrations of clarified lysates were determined using a Pierce BCA protein assay kit (Thermo Scientific). Lysates were stored in Lysis Buffer supplemented with 5% glycerol at -80 °C until use.

In-gel Activity Assay

Mycobacterial lysates (10 ng to 10 μg) were resolved by native gel electrophoresis (4-15% Tris-Cl Criterion gel, Bio-Rad) in 1X Tris-Glycine buffer (Bio-Rad). Protein loading dye did not contain DTT or SDS. Gels were run at 180 V for 55 min in a Criterion electrophoresis cell on top of ice before soaking in LR Buffer with 10 μM fluorogenic probe (**MFS**, **RS**, or DDAO-sulfate). Gels were imaged on a fluorescence scanner (Typhoon 9410 Variable Mode Imager, GE Healthcare). **MF** was detected following excitation at 488 nm, with emission collected after passage through a 526 nm sp filter. Resorufin was detected following excitation at 532 nm, with emission collected at 580 nm (bp 30). DDAO was detected following excitation at 633 nm, with emission collected at 670 nm (bp 30). For the experiment shown in **Figure 1**, we also analyzed mammalian cell lysates (e.g., BT549, HEK293, CHO,

Rat-1 fibroblasts and COS-7). The expanded view, which shows the resolved mammalian cell lysates, is shown in **Figure S13**. Gel images were analyzed in ImageJ[6].

In-Gel Sulfatase Detection Limit Assay Methods

Native gels (4-15% Tris-Cl) were used to resolve mycobacterial lysates from *M. avium*, *M. bovis* (BCG), *M. kansasii*, *M. tuberculosis* (H37Rv), *M. tuberculosis* (Erdman), and *M. marinum*. Total amounts of lysate, including 10 µg, 1 µg, 100ng, and 10ng, were resolved by electrophoresis (180V, 55 min, on ice). Then each gel was soaked in LR buffer containing 50 µM **MFS** or 25 µM **RS**; probe concentrations were optimized to give a high signal to noise. Gels were imaged after 15 min incubation with probes on a fluorescence gel scanner. Gel images were analyzed in ImageJ[6].

Supplemental Figures

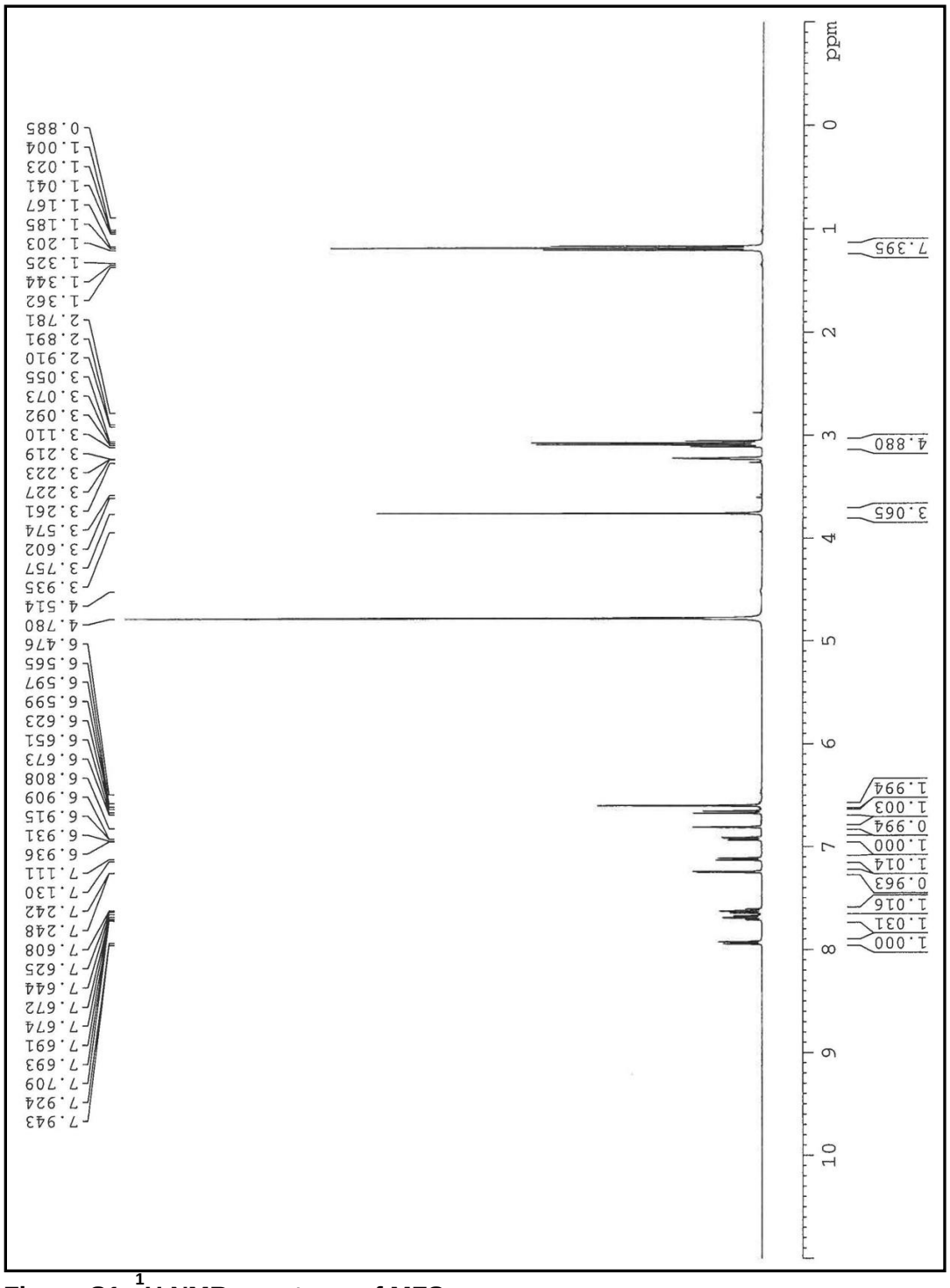


Figure S1. ^{1}H NMR spectrum of MFS.

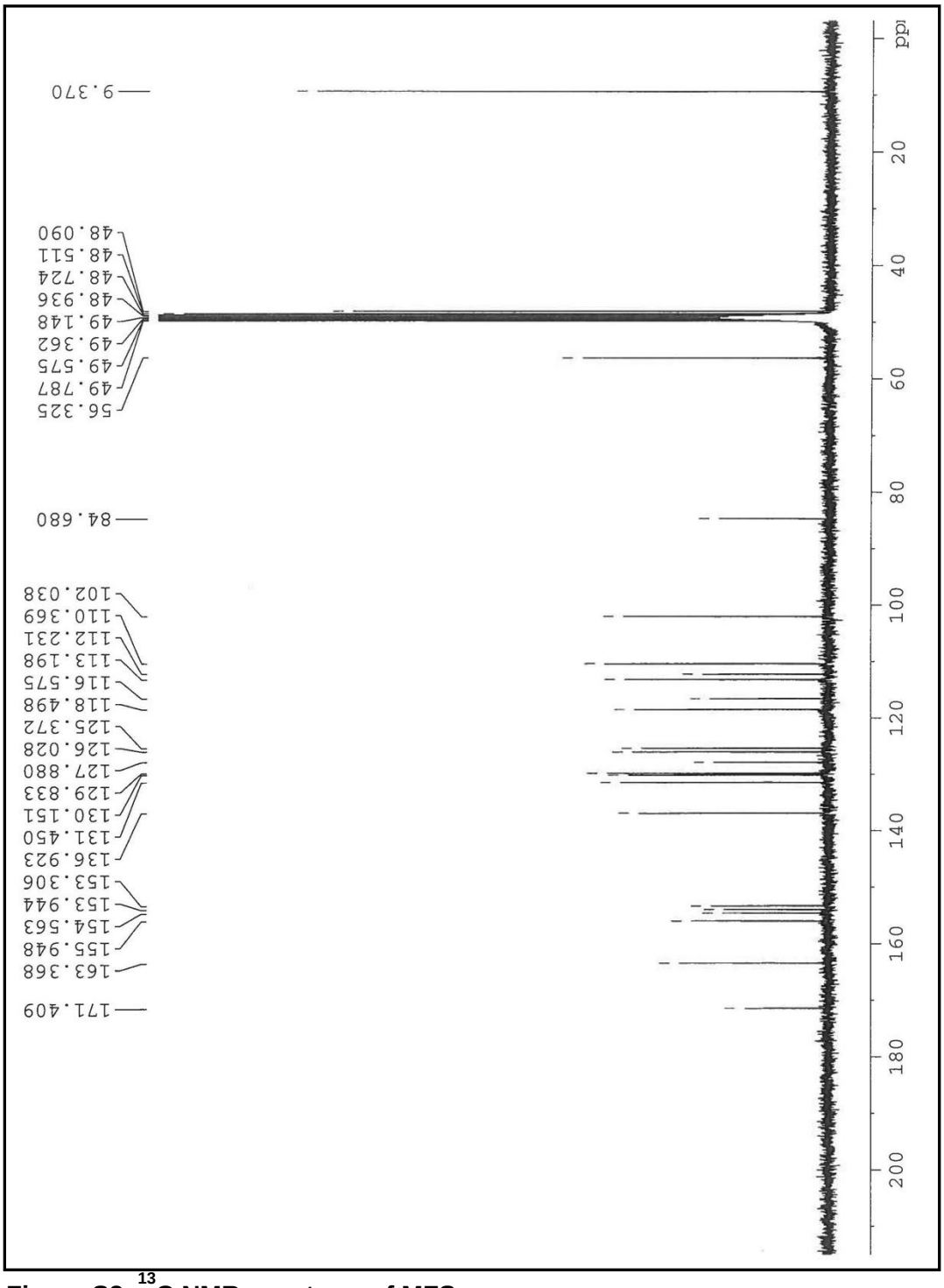


Figure S2. ^{13}**C NMR spectrum of MFS.**

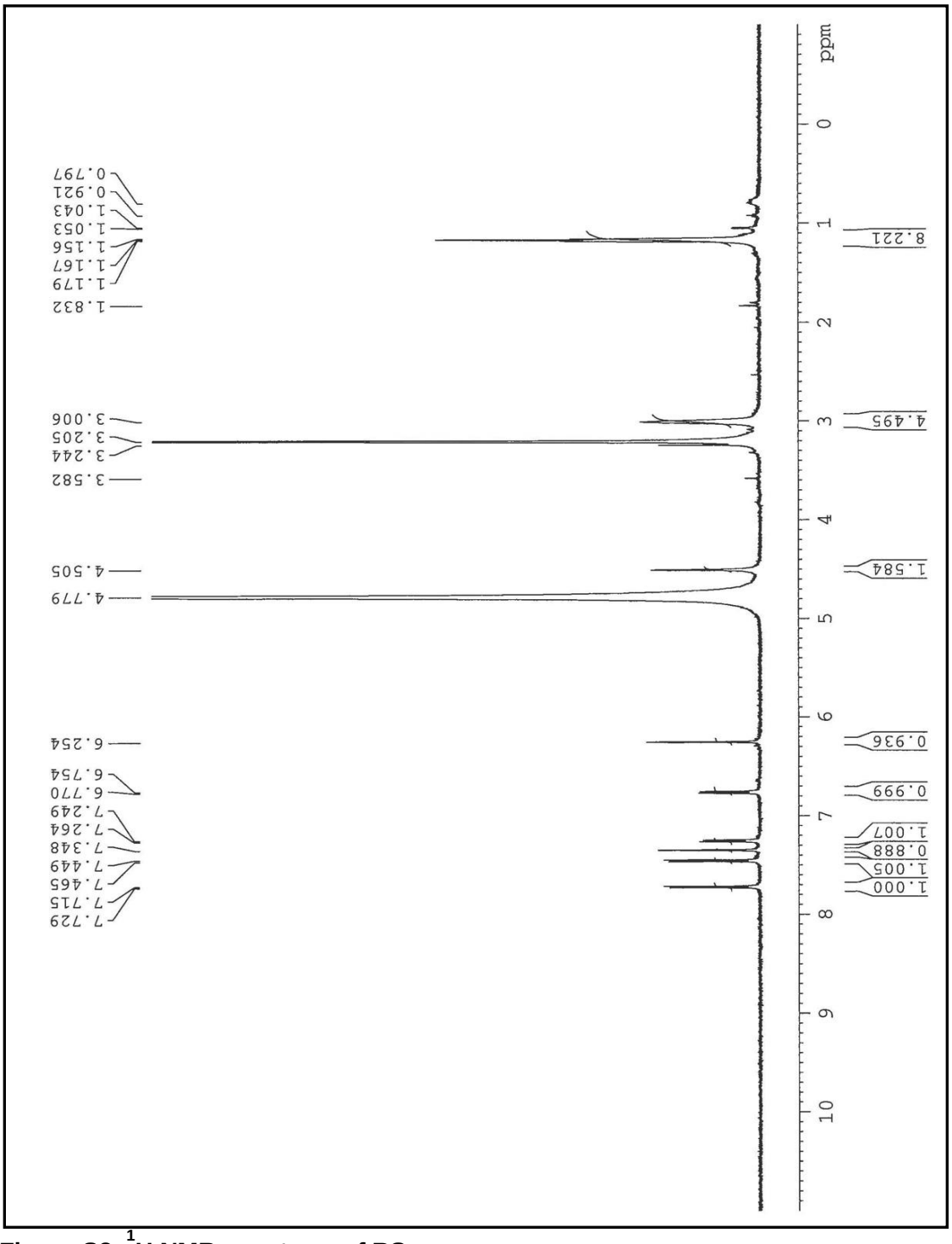


Figure S3. ^{1}H NMR spectrum of RS.

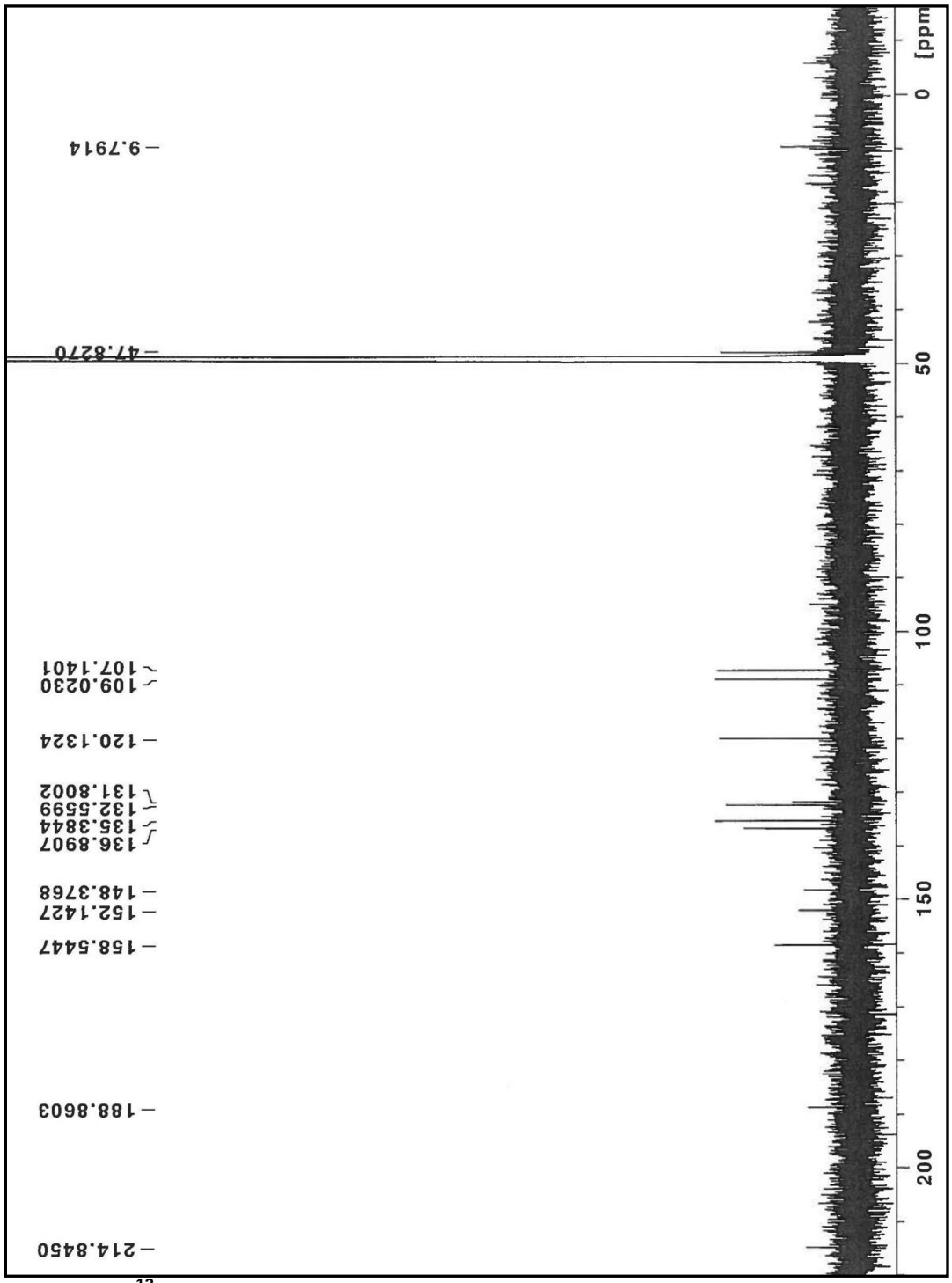


Figure S4. ^{13}C NMR spectrum of RS.

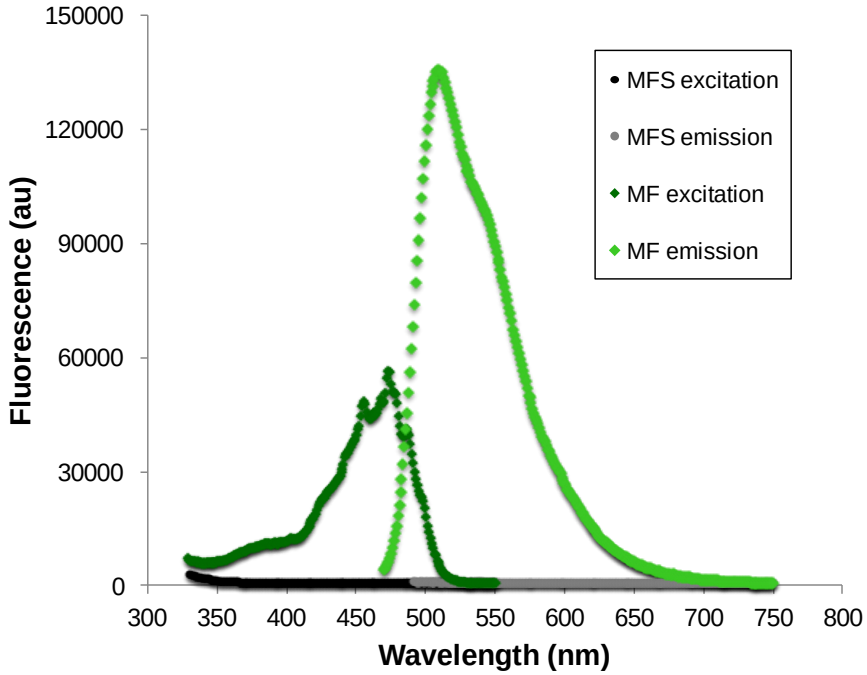


Figure S5. Excitation and emission spectra for 1 μM MF and MFS in Tris buffer, pH 7.9.

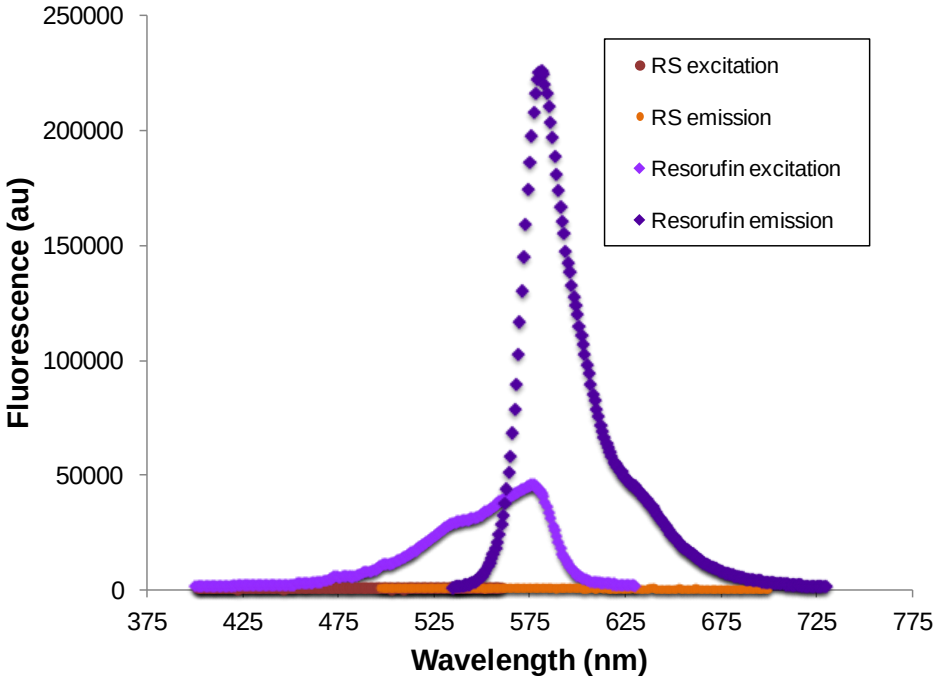


Figure S6. Excitation and emission spectra for 1 μM resorufin and RS in Tris buffer, pH 7.9.

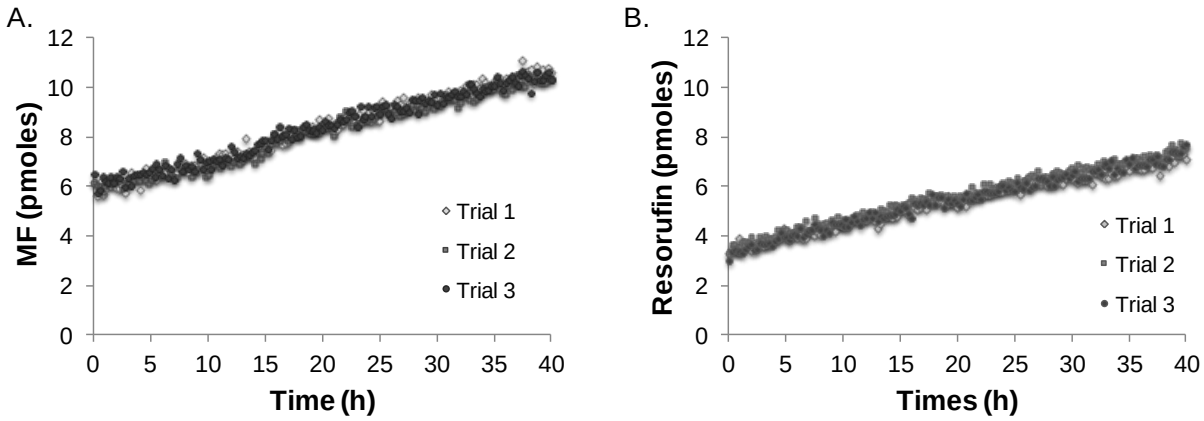


Figure S7. **Spontaneous hydrolysis of sulfated probes**. **A. MFS** (100 μM) was incubated in Tris buffer pH 7.5 and the formation of **MF** was monitored (excite 475 nm, emit 525 nm). **B. RS** was incubated in aqueous buffer pH 7.5 and the formation of resorufin was monitored (excite 550 nm, emit 600 nm).

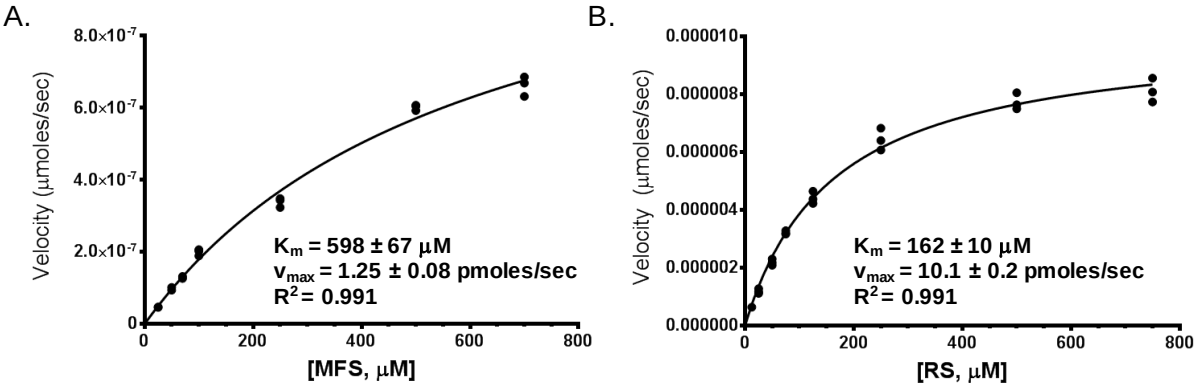


Figure S8. Kinetic data for MFS and RS hydrolysis with *A. aerogenes* sulfatase. Varying concentrations of A. **MFS** or B. **RS** were incubated with sulfatase, and the reaction progress was monitored by detecting formation of **MF** or resorufin. Each plot shows the initial rate as a function of substrate concentration with data fitting.

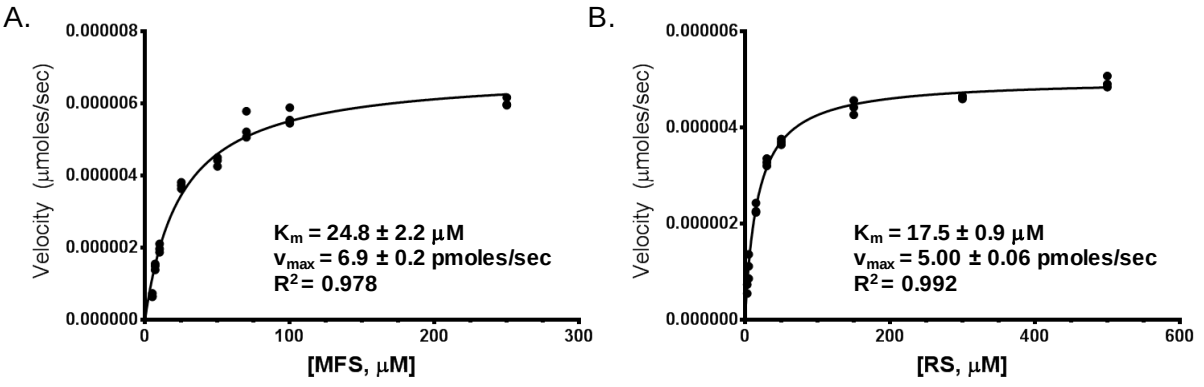


Figure S9. Kinetic data for MFS and RS hydrolysis with *Helix pomatia* sulfatase. Varying concentrations of A. **MFS** or B. **RS** were incubated with sulfatase, and the reaction progress was monitored by detecting formation of **MF** or resorufin. Each plot shows the initial rate as a function of substrate concentration with data fitting.

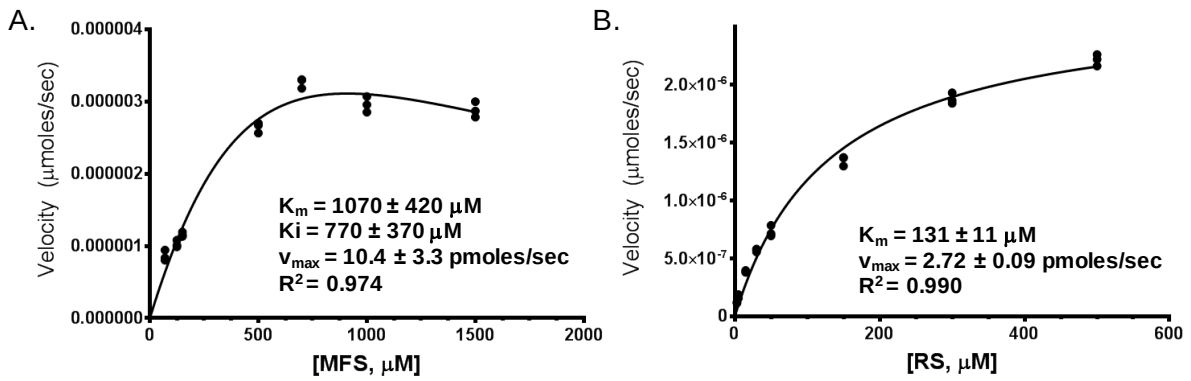


Figure S10. Kinetic data for MFS and RS hydrolysis with sulfatase from abalone entrails. Varying concentrations of A. **MFS** or B. **RS** were incubated with sulfatase, and the reaction progress was monitored by detecting formation of **MF** or resorufin. Each plot shows the initial rate as a function of substrate concentration with data fitting.

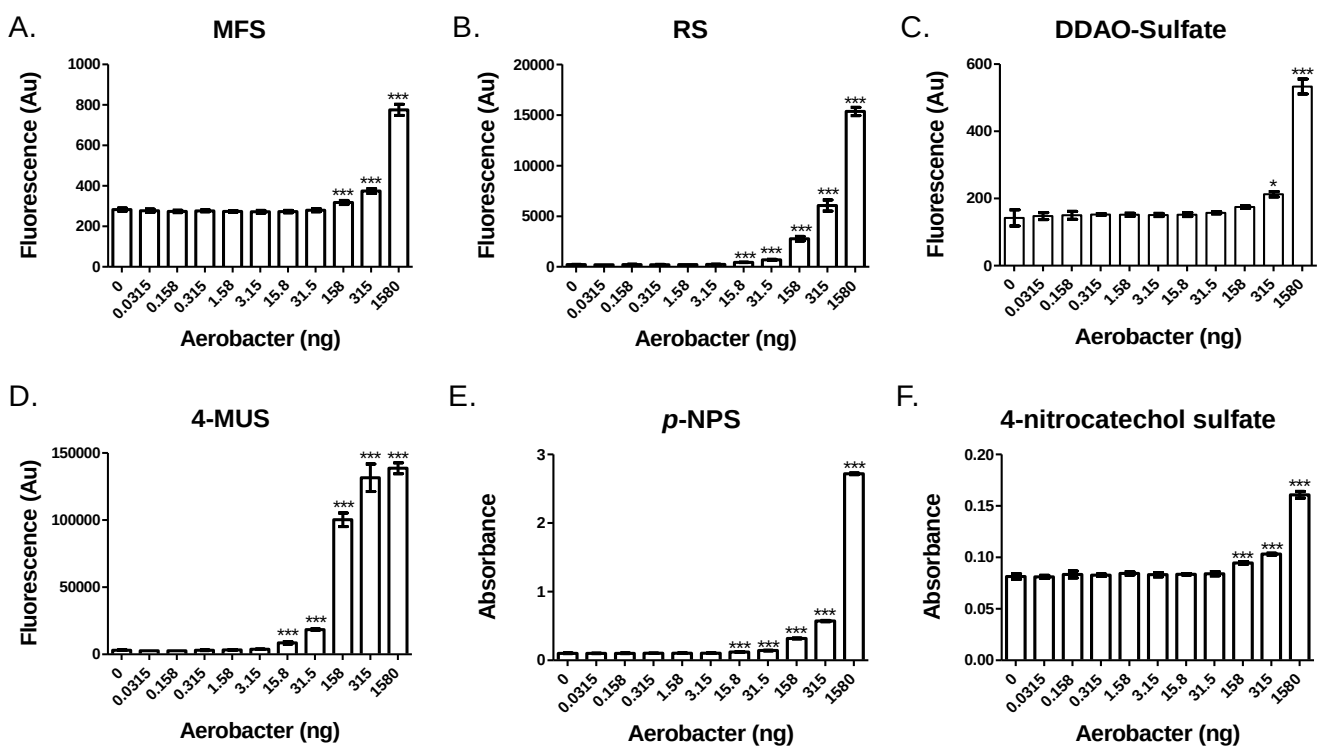


Figure S11. Detection limit for sulfatase probes measured with *A. aerogenes* sulfatase at 10 min. A. **MFS**, **B**. **RS**, **C**. DDAO-sulfate, **D**. 4-MUS, **E**. *p*-NPS, or **F**. 4-nitrocatechol sulfate. Measurements were made after 10 min incubation with enzyme in pH 7.5 Tris buffer at 37 °C. The fluorescence of **MF** (excite 475, emit 525), resorufin (excite 550, emit 600), DDAO (excite 635, emit 675), and 4-MU (excite 360, emit 450) was measured on a fluorescence plate reader. The absorbance of *p*-NP was measured at 405 nm and 4-nitrocatechol was measured at 535 nm. Error bars represent the standard deviation of four replicates. Detected signal that was significantly higher than the enzyme-free control are denoted by a * ($p < 0.01$) or a *** ($p < 0.001$).

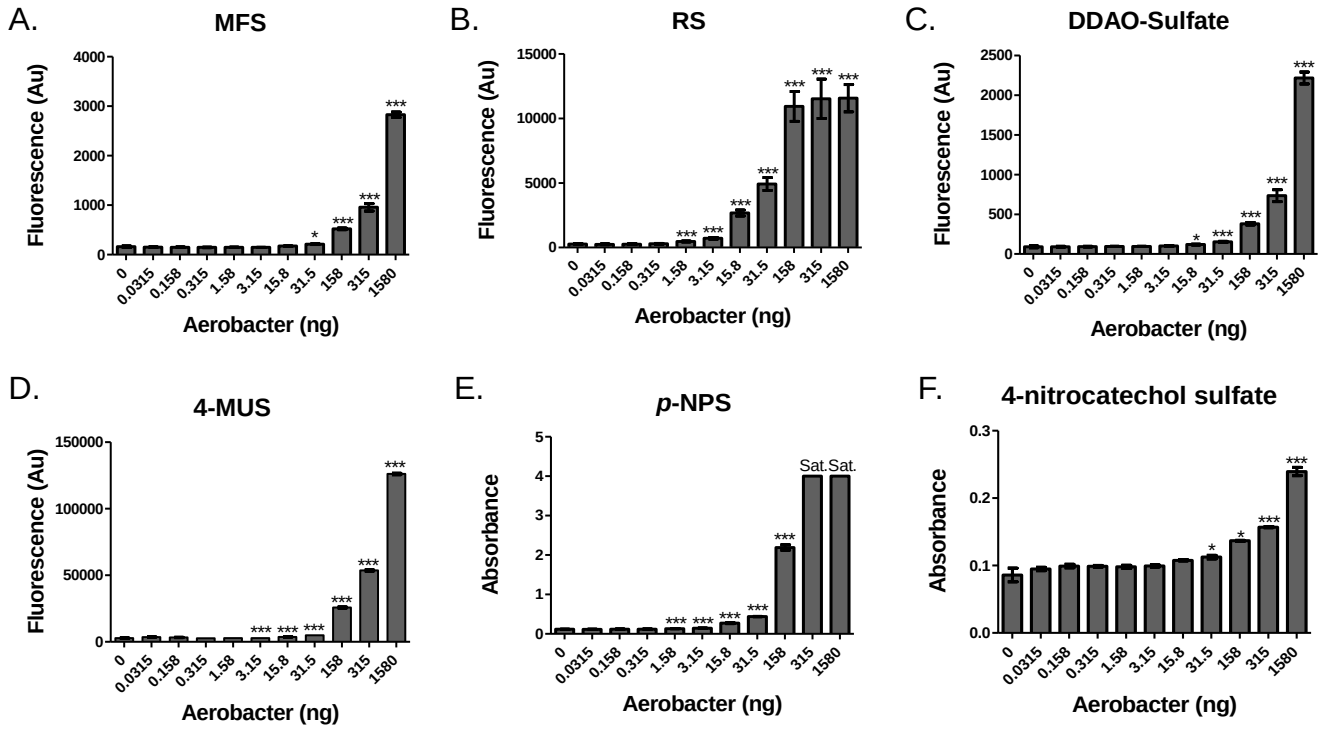


Figure S12. Detection limit for sulfatase probes measured with *A. aerogenes* sulfatase at 3 h. A. **MFS**, **B**. **RS**, **C**. DDAO-sulfate, **D**. 4-MUS, **E**. *p*-NPS, or **F**. 4-nitrocatechol sulfate. Measurements were made after 3 h incubation with enzyme in pH 7.5 Tris buffer at 37 °C. The fluorescence of **MF** (excite 475, emit 525), resorufin (excite 550, emit 600), DDAO (excite 635, emit 675), and 4-MU (excite 360, emit 450) was measured on a fluorescence plate reader. The absorbance of *p*-NP was measured at 405 nm and 4-nitrocatechol was measured at 535 nm. The absorbance of p-NP could not be measured at the two highest amounts of enzyme (315 and 1580 ng) due to saturation of the instrument detector (Sat.). Error bars represent the standard deviation of four replicates. Detected signal that was significantly higher than the enzyme-free control are denoted by a * ($p < 0.01$) or a *** ($p < 0.001$).

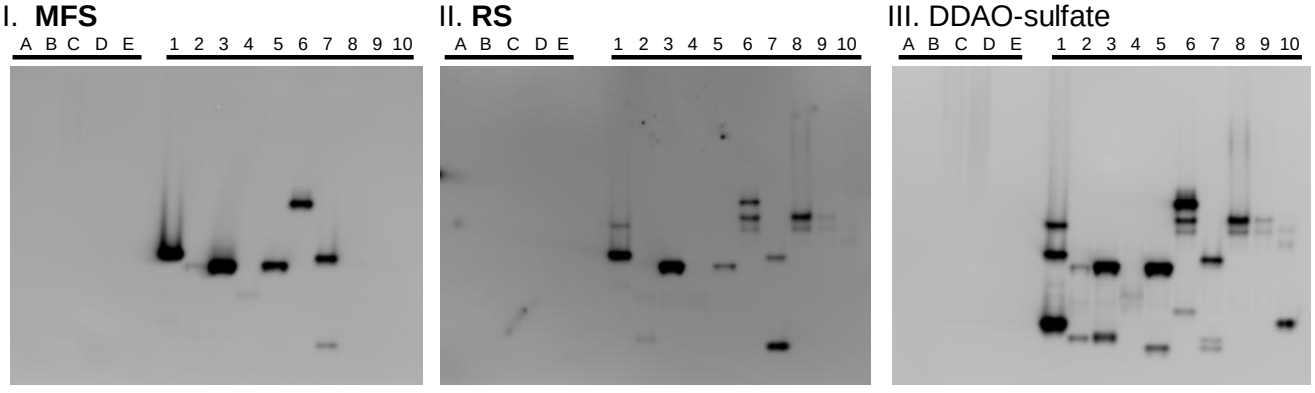


Figure S13. Sulfatase-activated fluorophores reveal different patterns of activity. Mycobacterial lysates (10 μg/lane) from a variety of species were resolved by native protein gel electrophoresis on a gradient gel (4-15%). Each gel was incubated for 7-8 m with 10 μM sulfatase probe [e.g., **MFS** (I), **RS** (II), or DDAO-sulfate (III)]. Gels were imaged to capture signal from each hydrolyzed probe. Lane 1: *M. marinum*, 2: *M. intracellulare*, 3: *M. flavescens*, 4: *M. peregrinum*, 5: *M. nonchromogenicum*, 6: *M. bovis* (BCG), 7: *M. avium*, 8: *M. tuberculosis* (H37Rv), 9: *M. tuberculosis* (CDC1551), 10: *M. tuberculosis* (Erdman). Select mammalian lysates from cell lines (100 μg/lane) were also evaluated in this assay (Lanes A-E). A: BT549, B: HEK293, C: CHO, D: Rat-1, E. COS-7.

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