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An Expanded Set of Fluorogenic Sulfatase Activity Probes

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Abstract

Fluorogenic probes that are activated by an enzymatic transformation are ideally suited for profiling enzyme activities in biological systems. Here, we describe two fluorogenic enzyme probes, 3-O-methylfluorescein-sulfate and resorufin-sulfate, that can be used to detect sulfatases in mycobacterial lysates. Both probes were validated with a set of commercial sulfatases and used to reveal species specific sulfatase banding patterns in a gel-resolved assay of mycobacterial lysates. The fluorogenic probes described here are suitable for various assays, and provide a starting point for creating new sulfatase probes with improved selectivity for mycobacterial sulfatases.

Keywords

sulfatase; fluorescent probe; *Mycobacterium tuberculosis*; protein imaging; hydrolase

Fluorogenic probes targeting distinct enzyme classes are powerful tools for applications ranging from enzyme assays to therapeutic screens.^[1] Enzyme probes can be used to assign function and substrate specificity to uncharacterized proteins, which is particularly useful for pathogens like *Mycobacterium tuberculosis* (*M. tuberculosis*) that have a high proportion of unannotated genes.^[2] Already, fluorogenic enzyme probes have made an impact in the development of diagnostics for detection of *M. tuberculosis*. For example, cephalosporin-based fluorogenic probes activated by the *M. tuberculosis* β -lactamase, BlaC, have been used to detect infections in mice^[3] and in sputum samples.^[4] Second-generation BlaC-activated probes^[4] appear to offer enhanced specificity for *M. tuberculosis* β -lactamase over the many other β -lactamases encoded by other gram negative bacteria.^[5]

Sulfatases are a class of enzyme that are genetically conserved across the different mycobacterial species that cause tuberculosis.^[6] We recently reported on the use of fluorogenic probes to examine mycobacterial sulfatase activity in a variety of species and strains.^[7] In that work, we developed a new fluorogenic probe, 7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one)(DDAO)-sulfate, which was used to detect sulfatase

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activity in protein gel-resolved mycobacterial lysates. The assay revealed that mycobacteria have distinct sulfatase activity patterns, or “fingerprints”. DDAO-sulfate, and the new fluorogenic sulfatase probes described here, could be used to further elucidate sulfatase function and regulation.

Beyond DDAO-sulfate, there is just one other type of fluorogenic probe that has been reported for detecting sulfatase activity, the coumarin derivatives [e.g., 4-methylumbelliferyl sulfate (4-MUS), 4-methylumbelliferyl sulfamate, and closely related analogues].^[8] This limited repertoire stands in contrast to the many fluorogenic probes that have been developed for detection of proteases,^[9] phosphatases,^[10] esterases,^[11] β -lactamases,^[3–4, 12] and glycosidases.^[13] The hydrolysis product of 4-MUS and DDAO-sulfate, 4-methylumbelliferone (4-MU; $\lambda_{\text{max}} = 360$, $\lambda_{\text{emit}} = 450$ nm) and DDAO ($\lambda_{\text{max}} = 600$, $\lambda_{\text{emit}} = 660$ nm), respectively, enable fluorescence detection at the extreme ends of the visible spectrum; these products cannot be detected on many low-cost instruments. Therefore, we sought to expand the spectrum of sulfatase-activated probes to allow greater flexibility for enzyme assays and multicolor imaging. We surveyed the existing fluorophore scaffolds and decided to target fluorescein and resorufin, two bright fluorescent probes. These fluorophore scaffolds excite and emit in the middle of the visible range, which broadens their applicability by enabling them to be detected on most plate readers, gel imagers, and standard fluorescence microscopes.

Most fluorogenic fluoresceins require two sequential hydrolysis steps to produce the bright fluorescent parent fluorophore.^[10b, 10c, 11a] In contrast, 3-O-methylfluorescein (**MF**; $\lambda_{\text{max}} = 472$ nm, $\lambda_{\text{emit}} = 510$ nm, $\Phi = 0.45$) is an enhanced xanthene that can be locked in a non-fluorescent lactone form through a single modification (Scheme 1). Fluorogenic **MF** derivatives have been used to assay proteases^[9b] and phospholipase,^[14] but not sulfatases. A few sulfated fluoresceins have been reported as fluorogenic substrates, but were only utilized as a phosphate isostere in phosphatase assays (e.g., to examine phosphatase inhibition).^[10b, 10c]

Resorufin ($\lambda_{\text{max}} = 574$ nm, $\lambda_{\text{emit}} = 581$ nm, $\Phi = 0.74$ ^[15]) is red-shifted compared to **MF** and has a high fluorescence quantum yield. Fluorogenic resorufin substrates have been developed for several hydrolases, including esterases^[11a, 16] and glycosidases.^[13b] Moreover, resorufin has been used to monitor sulfotransferase activity. Beckmann found that sulfation of resorufin by a phenol sulfotransferase resulted in a loss of fluorescence.^[17] The sulfation product was not isolated or characterized, but was proposed to be resorufin-sulfate (**RS**). Beckmann suggested that sulfatase activity could be monitored by the reverse reaction, namely starting with sulfated resorufin and measuring the sulfatase-catalyzed hydrolysis to form resorufin, but this was not subsequently explored.

Here, we report the synthesis of two fluorogenic substrates for detecting sulfatase activity: 3-O-methylfluorescein-sulfate (**MFS**) and **RS** (Scheme 1). We validated both **MFS** and **RS** with commercial sulfatases and mycobacterial lysates, and then applied these probes in a protein gel assay to examine sulfatase activity with a panel of mycobacterial pathogens.

We synthesized **MFS** from **MF** in one step by sulfation of the phenol with sulfur trioxide trimethylamine (see Supporting Information for experimental details). **MFS** was colorless and non-fluorescent, as expected for the spirolactonized compound.^[18] In contrast, **MF** had a fluorescence quantum yield of 0.45 and bright green fluorescence (see Scheme 1 and Figure S5). The large change in quantum yield upon hydrolysis makes **MFS** an exceptionally high contrast “turn-on” probe. The stability of **MFS** in the absence of enzyme was determined by monitoring the formation of **MF** over time in Tris buffer (pH 7.5) at 37 °C. After 40 hours, only 0.04% of **MFS** was hydrolyzed to form **MF** (see Figure S7).

We validated **MFS** as a sulfatase substrate using three commercial sulfatase sources: *Aerobacter aerogenes*, *Helix pomatia*, and abalone entrails (Table 1). Sulfatase activity from *A. aerogenes* was evaluated at 37 °C in Tris buffer (pH 7.5). The V_{\max} for **MFS** was determined to be 1.25 ± 0.08 pmoles/sec, and the K_M was 598 ± 67 μM . This was broadly comparable to this enzyme's K_M for other substrates, including DDAO-sulfate (164 ± 14 μM),^[7] *p*-nitrophenyl sulfate (*p*-NPS; 1800 ± 120 μM),^[19] and 4-MUS (700 ± 160 μM).^[19]

The enzymatic hydrolysis of **MFS** was further evaluated at an acidic pH, which is optimal for sulfatases from *H. pomatia* and abalone entrails. The formation of **MF** was monitored in real time at 37 °C in potassium acetate buffer (pH 5.0); hydrolysis of **MFS** in the absence of enzyme was negligible. With *H. pomatia*, the V_{\max} for **MFS** was determined to be 6.9 ± 0.2 pmoles/sec, and the K_M was 24.8 ± 2.2 μM . This is similar to the reported K_M value for DDAO-sulfate (13 ± 3 μM).^[7] With abalone entrails, the V_{\max} was 10.4 ± 3.3 pmoles/sec, and the K_M value was 1070 ± 420 μM . With this sulfatase source, substrate inhibition was observed ($K_i = 770 \pm 370$ μM). The K_M for DDAO-sulfate was 29 ± 3 μM for abalone entrails.^[7] From the *in vitro* kinetic assays, we conclude that **MFS** is a versatile substrate that can be used to monitor sulfatase activity in a range of conditions, including low pH.

We next sought to synthesize a fluorogenic substrate based on resorufin. We synthesized **RS**, which is blue-shifted and dark ($\lambda_{\max} = 455$, $\lambda_{\text{emit}} = 570$ nm, $\Phi = 0.01$) compared to resorufin (see Scheme 1 and Figure S6). Similar to **MFS**, **RS** is a favorable turn-on probe due to its large change in quantum yield upon hydrolysis to form resorufin. The conversion of **RS** to form resorufin in the absence of enzyme was negligible in Tris buffer (pH 7.5) at 37 °C. After 40 hours, only 0.05% of **RS** was hydrolyzed (see Figure S7). Thus, **RS** offers a similarly high hydrolytic stability as **MFS**.

We determined the kinetic parameters of **RS** with three sulfatases (Table 2). Hydrolysis by *A. aerogenes* ($K_M = 162 \pm 10$ μM , $V_{\max} = 10.1 \pm 0.2$ pmoles/sec) was monitored at pH 7.5, while conversion by *H. pomatia* ($K_M = 17.5 \pm 0.9$ μM , $V_{\max} = 5.00 \pm 0.06$ pmoles/sec) and abalone entrails ($K_M = 131 \pm 11$ μM , $V_{\max} = 2.72 \pm 0.09$ pmoles/sec) was monitored at pH 5.0. At either pH value, the background hydrolysis was negligible in the absence of sulfatase.

We used *A. aerogenes* sulfatase to determine the detection limit for various fluorogenic (**MFS**, **RS**, DDAO-sulfate, 4-MUS) or colorimetric (*p*-NPS and 4-nitrocatechol sulfate) probes. The detection limit was evaluated at saturating probe concentration (i.e., at a concentration twice the K_M). Each probe was incubated in Tris buffer (pH 7.5) at 37 °C with

varying amounts of sulfatase (0.315 to 1580 ng), and the signal from hydrolyzed probe was measured at two time points: 10 min and 3 h (Table S1). At 10 min, **RS**, 4-MUS, and *p*-NPS were the most sensitive probes and detected 15.8 ng of sulfatase (Figure S11). **MFS** and 4-nitrocatechol sulfate required 158 ng for detection, and DDAO-sulfate had the highest detection limit (315 ng).

After a 3 h incubation with *A. aerogenes* sulfatase, the detection limits had improved 5- to 20-fold for all probes: **RS** (1.58 ng), 4-MUS (3.15 ng), *p*-NPS (1.58 ng), **MFS** (31.5 ng), 4-nitrocatechol sulfate (31.5 ng) and DDAO-sulfate (15.8 ng) (Figure S12). Although all of the probes could detect low nanogram quantities of *A. aerogenes* sulfatase, these results do highlight the high sensitivity offered by **RS**. It is worth noting that the high pK_a of 4-MU ($pK_a = 7.8$), *p*-nitrophenol ($pK_a = 7.2$), and 4-nitrocatechol ($pK_a = 6.9$) precludes their use in continuous assays at low pH. In contrast, the low pK_a values for resorufin ($pK_a = 5.8$), **MF** ($pK_a = 4.8$) and DDAO ($pK_a = 5.0$) improve their versatility because many sulfatases are most active at acidic pH, including lysosomal sulfatases^[20] and sulfatases from *H. pomatia* and abalone entrails.

We recently described a new assay for mycobacterial strain assignment based on patterns of sulfatase activity revealed by hydrolysis of DDAO-sulfate.^[7] In the current work, we used this assay to detect sulfatase activities in a set of mycobacterial species using **MFS**, **RS**, and DDAO-sulfate. We examined lysates from *Mycobacterium marinum*, *Mycobacterium intracellulare*, *Mycobacterium flavescens*, *Mycobacterium peregrinum*, *Mycobacterium nonchromogenicum*, *Mycobacterium bovis* (bacillus Calmette-Guérin, BCG), *Mycobacterium avium*, and three strains of *M. tuberculosis* (H37Rv, CDC1551, and Erdman). Three identically prepared native protein gels were used to resolve lysates before incubation for less than 10 min with each sulfatase probe. Each gel was imaged on a fluorescence scanner to reveal fluorescent banding patterns dependent on species- and strain-specific sulfatase activity (Figure 1). Application of DDAO-sulfate demonstrated that all of the examined mycobacterial species had active sulfatases, although the observed activity in *M. peregrinum* was low. Hydrolyzed **MFS** was not detected in lanes containing resolved *M. tuberculosis* lysates (Figure 1A, lanes 8-10), but the other seven species produced detectable **MF** bands. For **RS**, every species except *M. peregrinum* had sulfatase activity that could be detected. However, **RS** produced fewer bands than DDAO-sulfate. For example, in the resolved *M. tuberculosis* (Erdman) lysate (lane 10), DDAO-sulfate is hydrolyzed by a Type III sulfatase,^[21] ZnSulf-B,^[7] to produce a distinctive band at a low apparent molecular weight, but this activity could not be detected by incubation with **RS** or **MFS**. The lowest sulfatase band produced by *M. flavescens* (lane 3) and *M. nonchromogenicum* (lane 5) was also not visible for gels exposed to **RS** or **MFS**. It is interesting to note that the banding pattern for *M. avium* sulfatases (lane 7) was altered for the gel exposed to **RS** compared to the other two sulfated probes. The intensity of the lower sulfatase band was greater than the higher band following incubation with **RS**, while the opposite pattern was observed for **MFS** and DDAO-sulfate. For this species, DDAO-sulfate produced three total bands while **RS** and **MFS** each produced two.

Probes that are broadly hydrolyzed by different mycobacterial sulfatases are useful for examining patterns of activity among related pathogens, while more specific probes would be valuable for developing a simple enzyme-specific diagnostic assay. The differences in the sulfatase fingerprints observed with **MFS**, **RS**, and DDAO-sulfate might reflect variations in substrate suitability for different mycobacterial sulfatases. The gel-resolved *M. tuberculosis* sulfatases did not hydrolyze **MFS**, but could hydrolyze both **RS** and DDAO-sulfate. It is tempting to speculate that **MFS** is the least optimal mycobacterial sulfatase substrate due to the added bulk of the benzofuranone moiety on **MFS**, which is absent in both **RS** and DDAO-sulfate. Although natural substrates have yet to be identified for the mycobacterial sulfatases, the structural differences among fluorogenic probes could be used to evaluate the specificity of sulfatases in mycobacterial species and strains. For example, our results indicate that the *M. tuberculosis* sulfatase AtsB^[7] can hydrolyze both **RS** and DDAO-sulfate, but not **MFS**. The variation in sulfatase activity patterns observed for **MFS** versus **RS** could form the basis for developing new fluorogenic probes with improved specificity for a particular sulfatase enzyme.

Mammalian cells express sulfatases^[20], which might accept **MFS**, **RS**, or DDAO-sulfate as substrates. Using this in-gel assay, we analyzed mammalian cell lysates from five sources (BT549, HEK293, CHO, Rat-1, and Cos-7). Notably, none of the three probes were hydrolyzed by gel-resolved mammalian lysates (see Figure S13). It is unclear whether the mammalian sulfatase activity was lost during sample preparation or if the assay conditions disfavoured the catalysis by mammalian enzymes. The assay was performed in Tris buffer pH 7.5, and many of the mammalian sulfatases have acidic pH optima. Either way, the lack of detectable mammalian sulfatase activity suggests that our assay might be useful for evaluating mycobacterial sulfatase activity in infected macrophages^[7] or human sputum samples.

We explored the sensitivity of the in-gel assay for detecting sulfatases using **MFS** and **RS** (Figure 2). We imaged fluorescence produced by sulfatases from lysates (10 ng to 10 µg total protein) obtained from six different mycobacterial sources: *M. avium*, *M. bovis* (BCG), *Mycobacterium kansasii*, *M. tuberculosis* (H37Rv and Erdman), and *M. marinum*. Following 15 min incubation with 50 µM **MFS**, sulfatase bands were produced by lysates (10 or 1 µg) from *M. avium*, *M. bovis* (BCG), *M. kansasii*, and *M. marinum*. No bands were observed for either *M. tuberculosis* strain at any amount of lysate examined. **MFS** detected sulfatase activity in as little as 100 ng of *M. kansasii* or *M. marinum* lysate. Sulfatase activity could not be detected with **MFS** in lanes containing 10 ng of lysate (data not shown). Incubation with 25 µM **RS** for 15 min produced sulfatase bands in 10 µg of *M. tuberculosis* (Erdman) lysate. **RS** could detect activity in 10 or 1 µg of lysate from *M. avium*, *M. bovis* (BCG), *M. kansasii*, *M. tuberculosis* (H37Rv), and *M. marinum*. It should be noted that as little as 100 ng of lysate produced detectable sulfatase bands from *M. avium* and *M. kansasii*, although this low amount of activity could only be observed by adjusting the contrast of the image. **RS** could not detect sulfatase activity from 10 ng of resolved proteins for any lysates (data not shown). With either **MFS** or **RS**, the in-gel assay format was most informative when a larger amount of lysate was resolved, but sulfatase activity could be detected in a small amount of lysate for some species.

In conclusion, we synthesized and characterized two new fluorogenic probes, **MFS** and **RS**, which were used to detect sulfatase activity. **MFS** is non-fluorescent and **RS** is effectively non-fluorescent ($\Phi = 0.01$). Following sulfatase hydrolysis, both probes display a dramatic increase in quantum yield, making these two excellent turn-on probes for applications requiring very low background fluorescence. The kinetic parameters for both probes were determined using sulfatases from *A. aerogenes*, *H. pomatia*, and abalone entrails. We used *A. aerogenes* sulfatase to compare the detection limit for hydrolysis of **MFS** and **RS** to other known sulfatase substrates in a 96-well plate format. We found that **RS** was particularly sensitive and could detect at least as little *A. aerogenes* enzyme as the pre-existing fluorogenic substrate 4-MUS. **RS** could detect 10- to 20-fold less enzyme than DDAO-sulfate. The detection limit for **MFS** was higher than **RS**, and more comparable to the colorimetric substrate 4-nitrocatechol sulfate. Furthermore, we used an in-gel assay format to examine mycobacterial sulfatase activity in gel-resolved lysates and found that each probe could reveal a variety of sulfatases. **MFS** and **RS** expand the set of available substrates for evaluating sulfatase activity in a variety of formats.

Experimental Section

Please see the online Supporting Information for detailed experimental protocols for the synthesis and evaluation of **MFS** and **RS** and additional data (Table S1 and Figures S1–13).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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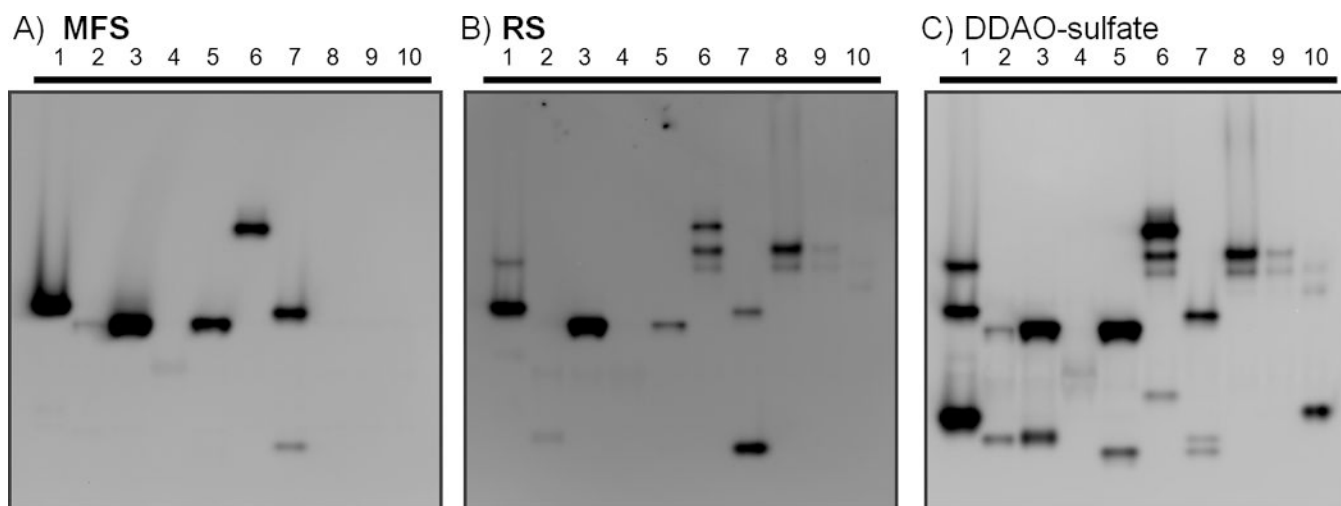


Figure 1. Sulfatase-activated fluorophores reveal different patterns of activity. Mycobacterial lysates (10 μ g of total protein per lane) from a variety of species were resolved by native protein gel electrophoresis on a gradient gel (4-15%). Each gel was incubated for 7-8 min with 10 μ M **MFS (A)**, **RS (B)**, or **DDAO-sulfate (C)**. 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 were also evaluated in this assay; see Figure S13 in the *Supporting Information*.

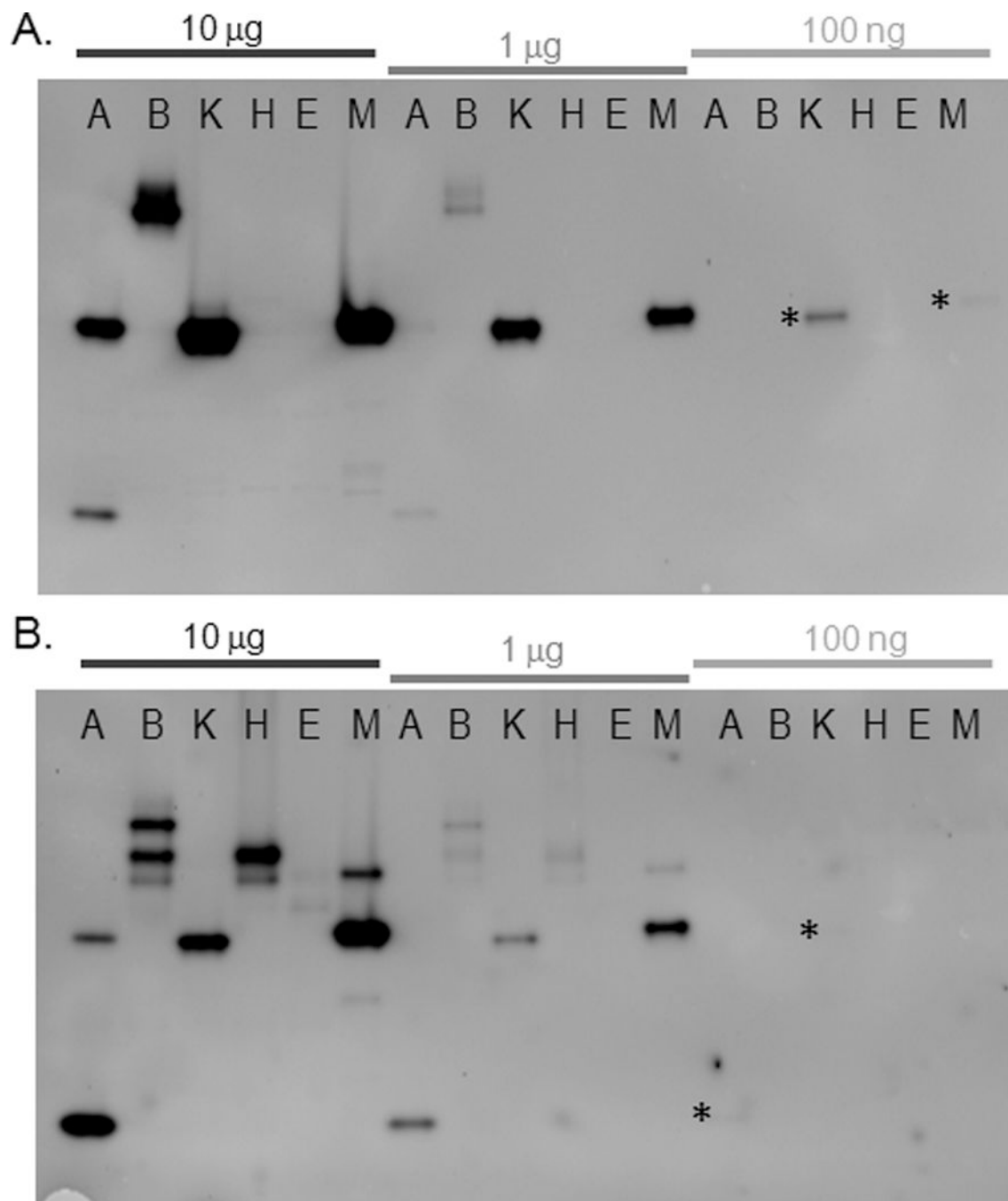
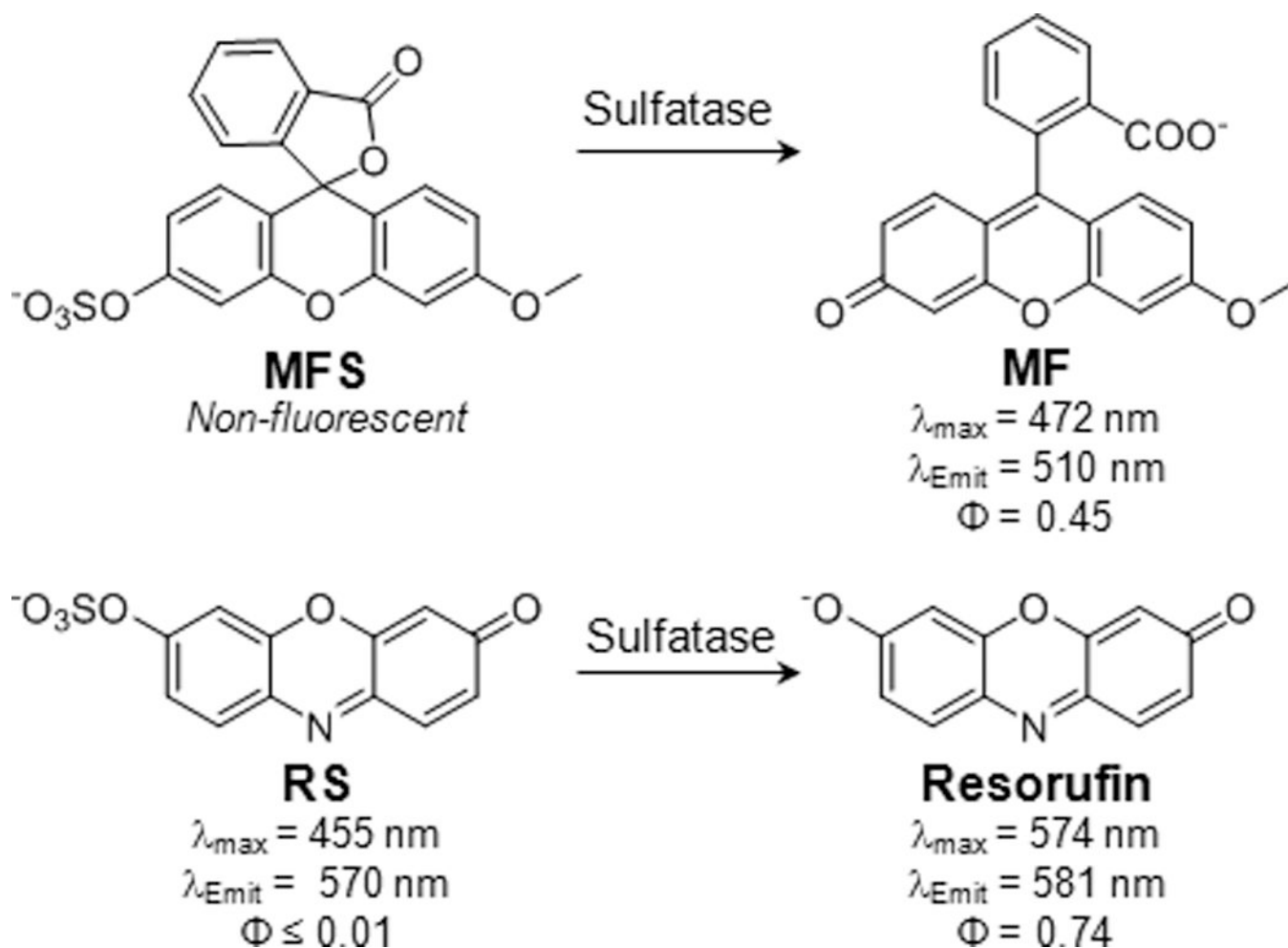


Figure 2.

Sensitivity of sulfatase activity detection in mycobacterial lysates. Different amounts of mycobacterial lysates were separated by native gel, followed by incubation for 15 min with (A) MFS (50 μM) or (B) RS (25 μM). For the most faint bands, which were visible upon adjustment of the image contrast, an asterisk (*) is shown to the left of the band location. Lane designations are: A: *M. avium*; B: *M. bovis* (BCG); K: *M. kansasii*; H: *M. tuberculosis* (H37Rv); E: *M. tuberculosis* (Erdman); M: *M. marinum*.

**Scheme 1.**

Sulfatase-catalyzed hydrolysis of **MFS** and **RS** forms bright fluorescent products. **MFS** is non-fluorescent before hydrolysis to give **MF**, a product with a fluorescence quantum yield (Φ) of 0.45. **RS** is effectively non-fluorescent ($\Phi \leq 0.01$) before hydrolysis to produce resorufin ($\Phi = 0.74$).

Table 1Kinetic parameters for **MFS**.

Enzyme Source	<i>Aerobacter aerogenes</i> ^[a]	<i>Helix pomatia</i> ^[b]	<i>Abalone Entrails</i> ^[b,c]
K _M (μM)	598 ± 67	24.8 ± 2.2	1070 ± 420
V _{max} (pmoles/sec)	1.25 ± 0.08	6.9 ± 0.2	10.4 ± 3.3
V _{max} /K _M (nL/sec)	2.10	277	9.74

[a] Assay was performed in Tris buffer (pH 7.5) at 37 °C.

[b] Assay was performed in potassium acetate buffer (pH 5.0) at 37 °C.

[c] Inhibition was observed (K_i = 770 ± 370 μM).

Table 2Kinetic parameters for **RS**.

Enzyme Source	<i>Aerobacter aerogenes</i> ^[a]	<i>Helix pomatia</i> ^[b]	<i>Abalone Entrails</i> ^[b]
K _M (μM)	162 ± 10	17.5 ± 0.9	131 ± 11
V _{max} (pmoles/sec)	10.1 ± 0.2	5.00 ± 0.06	2.72 ± 0.09
V _{max} /K _M (nL/sec)	62.4	285	20.8

^[a] Assay was performed in Tris buffer (pH 7.5) at 37 °C.

^[b] Assay was performed in potassium acetate buffer (pH 5.0) at 37 °C.