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Bioluminescent Probes of Sulfatase Activity

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Sulfation of glycans, proteins, and small molecules is a biological regulatory modification that plays important roles in normal and disease processes.[1] The enzymes that install and hydrolyze sulfate esters are the sulfotransferases and sulfatases, respectively. The sulfatases in particular have been associated with a number of congenital and acquired disease states. [2] Steroid sulfatase inhibitors are under evaluation as anticancer drugs,[3] and the association of heparan sulfatases with cancer[2b,4] suggests future opportunities for therapeutic approaches that target this enzyme class. Moreover, sulfatases are predicted from the genome sequences of many pathogenic microbes, including *Mycobacterium tuberculosis*,[5] although their biological functions are not well defined.

Fundamental studies of sulfatase function, as well as in vitro screens and in vivo diagnostics, would benefit tremendously from chemical probes that report on sulfatase activity. The current handful of commercial reagents (e.g., the fluorogenic substrate 4-methylumbelliferyl sulfate (4-MUS) and the chromogenic substrates *p*-nitrophenyl sulfate (*p*NPS) and *p*-nitrocatechol sulfate) reflect a limited conceptual framework in which a sulfated reporter dye must be directly hydrolyzed by the enzyme. This dual requirement of optical activity and sulfatase substrate activity cannot be met by many desirable reporters; this restricts the modalities that can be used to investigate sulfatases. Furthermore, reliance on an optical reporter limits applications involving screening/selection or common clinical imaging modalities (e.g., MRI and PET).

We sought to develop a versatile strategy for probing sulfatase activity that separates the structural elements associated with substrate and reporter functions. We envisioned a sulfated self-immolating caging compound as a broad-spectrum sulfatase substrate.[6] Hydrolysis of the sulfate ester leads to decomposition of the linker and uncaging of a reporter group (Scheme 1A). Other hydrolytic enzymes, such as esterases, glycosidases, and phosphatases, have been targeted with caged dyes or prodrugs by using a similar strategy.[7] A major advantage of this approach is that the required aromatic substrate portion of the heterobifunctional molecule can be structurally modulated to optimize specificity for a target enzyme without perturbing the reporter moiety.

Herein, we describe an initial application of this strategy to the development of a panel of bioluminescent sulfatase reporters (compounds **1–3**, Scheme 1B). The compounds comprise variously fluorinated aryl sulfates conjugated to aminoluciferin, a substrate for firefly

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luciferase.[8] Acylation of aminoluciferin's amino group is known to extinguish bioluminescence.[8] Thus, sulfatase-mediated uncaging was expected to activate the reporter and enable detection of enzyme activity with the high sensitivity characteristic of bioluminescence.[9] The fluorine substituents of compounds **2** and **3** were installed to improve the sulfatase reaction rates, which are known to correlate positively with leaving group ability of the desulfated product.[10] We employed alternative substitution patterns to investigate potential differences in selectivity exhibited by sulfatases from different sources. Here we demonstrate that compounds **1–3** can report on sulfatase activities from both human and pro-karyotic cell samples.

Compounds **4–6** were key intermediates in the syntheses of **1–3**; their preparation is described in the Supporting Information. The neopentyl sulfate protecting group reported by Simpson and Widlanski[11] facilitated the synthesis by masking the sulfate's charge, and could be removed at the end of the synthesis by mild nucleophilic displacement. We integrated the protected aryl sulfate moieties into the construction of the luciferin scaffold, as shown in Scheme 2. Known heterocycle **7**[8] was converted into isocyanate **8**, which was then condensed with **4**, **5**, or **6** in a one-pot procedure. Treatment of the products **9**, **10**, and **11** with D-cysteine, an established transformation in the synthesis of luciferin, formed intermediates **12**, **13**, and **14**, respectively. A final deprotection with NaN₃ in warm dimethyl sulfoxide (DMSO) yielded the caged luciferins **1–3**.

We evaluated the kinetic parameters of compounds **1–3** with a commercially available sulfatase from *Aerobacter aerogenes* (Table 1). Analysis of the $V_{\max}$ values revealed that addition of fluorine substituents did indeed increase the enzymatic reaction rates, as predicted. Similar to other arylsulfatases, we observed substrate inhibition at higher concentrations (see the Supporting Information).[12] It is likely that an optimal concentration will need to be determined when initiating a new application. To confirm that the cages masked bioluminescence, we incubated compounds **1–3** with or without *A. aerogenes* sulfatase and then, after a period of time, added commercially available luciferase and ATP to initiate bioluminescence. As shown in Figure 1, luminescence was observed only in the presence of sulfatase. During the time course of the experiment, enzyme-mediated hydrolysis released 10–20 % of the potential aminoluciferin; nonenzymatic hydrolysis was minimal (<5 %).

To compare our probes with existing sulfatase reporters, we also determined the kinetic parameters of *p*NPS and 4-MUS. The K_m values, 1800 ± 120 and 700 ± 160 μ M, respectively, were found to be somewhat higher than those determined for **1–3**.

We next sought to evaluate compounds **1–3** as probes of sulfatase activity from cell lysates and conditioned media. Although the *E. coli* genome possesses several putative sulfatases,

laboratory strains of *E. coli* do not exhibit sulfatase activity.[13] To generate a sulfatase-active strain, we therefore cloned the putative *E. coli* sulfatase YidJ (Supporting Information) into an inducible expression plasmid, and used this to transform *E. coli* BL21(DE3) cells. As shown in Figure 2A, in the presence of compound **1**, **2**, or **3**, lysate from *E. coli* expressing YidJ produced bioluminescence up to 22 times greater than that produced by heat-inactivated lysate.[14] Under similar incubation conditions, 4-MUS and *p*NPS displayed signals 25 and 24 times above background, respectively, although their greater K_m values necessitated the use of higher concentrations (1 and 2 mM, respectively, see the Supporting Information). We performed similar comparisons for the sulfatase samples below, and observed comparable results. These results are detailed in the Supporting Information.

M. tuberculosis is known to possess abundant sulfated metabolites, some of which have been correlated with virulence.[5] Its genome encodes six putative sulfatases that could be relevant to survival or pathogenesis. To test compounds **1–3** as probes for *M. tuberculosis* sulfatases, we incubated them with cell lysates, and measured the resulting bioluminescence. As shown in Figure 2B, compounds **1–3** produced detectable bioluminescence, but at various levels. Compound **3**, the 3,5-di-fluoro analogue was the most active, while compound **1**, lacking fluorination, was the least active.

In addition to these two prokaryote-derived lysates, we examined cell lysate from the human-derived HEK293 cell line (Figure 2C). Conducting the sulfatase reactions at the slightly acidic pH of 5.6 produced enhanced bioluminescence relative to reactions performed at pH 7.5. This observation is consistent with the lysosomal residence of the majority of human sulfatases.[1] We also note that, in contrast to the results obtained with *M. tuberculosis* lysate, sulfatase activity from HEK293-derived lysate showed a preference for the 2,6-difluorinated analogue **2**. This qualitative difference in substrate preference suggests that compounds **2** and **3** might be used to probe species-specific sulfatase activities in a coculture infection model.

Finally, we tested two sources of conditioned media as a method of probing secreted sulfatase activities (Figure 3). Specifically, we used conditioned media from the above *M. tuberculosis* cultures and also from HEK293 cells stably expressing the heparan sulfatase HSulf2,[4] one of only two closely related sulfatases that are known to be secreted in humans. Unused mycobacterial culture media and conditioned media from parental HEK293 cells were used as negative controls, respectively. While the *M. tuberculosis*-conditioned medium exhibited activity with all caged luciferins, conditioned medium from the HSulf2-expressing HEK293 cells produced no more luminescence than conditioned medium from parental cells. Secretion of active HSulf2 was confirmed by analysis of the conditioned media by using the known substrate 4-MUS (Supporting Information).[4] We conclude from these studies that compounds **1–3** are not hydrolyzed to a significant extent by the secreted human sulfatases, whereas all three compounds are processed by the secreted *M. tuberculosis* sulfatase activity (although we cannot exclude intracellular sulfatases released from dead cells as a source of this activity). This observation suggests that these probes can be used for in vivo bioluminescence imaging of *M. tuberculosis* sulfatase activity with low background signal from secreted host enzymes.

We focused here on developing bioluminescent sulfatase probes whose substrate moieties can be modulated for specificity toward a particular enzyme activity. It should be noted, however, that the strategy can be generalized to other types of cargo, including chemotherapeutic agents or probes for clinical imaging (MRI or PET). Adapting the concept of sulfatase-activated cargo release to new diagnostic and therapeutic applications is a promising area of exploration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

The caging modules mask luciferin bioluminescence. *A. aerogenes* sulfatase was incubated with 6.25 μ M **1**, **2**, or **3** for 30 min (sufficient for ~10–20 % aminoluciferin release; see the Supporting Information), after which luminescence was initiated by the addition of luciferase and ATP (gray bars). The addition of H₂O instead of sulfatase served as a negative control (white bars). Error bars represent the standard deviation of three replicates. RLU=relative luminescence units.

Figure 2.

Sulfatase activity from cell lysates. Gray bars, untreated lysate; White bars, heat-treated lysate; 6.25 μ M **1**, **2**, or **3** was used in all reactions. A) Lysate from *E. coli* expressing the putative sulfatase YidJ. B) Lysate from *M. tuberculosis* strain H37Rv. C) Lysate from HEK293 cells at either pH 7.5 or pH 5.6. Luminescence was initiated by the addition of luciferase and ATP. Error bars represent the standard deviation of three replicates.

Figure 3.

Sulfatase activity in conditioned media from *M. tuberculosis* H37Rv and HEK293 cell cultures. Concentrated conditioned medium was incubated with 1 mM **1**, **2**, or **3** before luminescence was initiated by the addition of luciferase and ATP. For *M. tuberculosis*, gray bars represent the addition of conditioned medium and white bars represent the addition of fresh, unused medium. For HEK293, gray bars represent conditioned medium from a cell line stably transfected with HSulf2 and white bars represent conditioned medium from parental cells. Error bars represent the standard deviation of three replicates.

Scheme 1.

A) General strategy for the design of sulfatase activated probes. B) Bioluminescent sulfatase reporters.

Scheme 2.

Synthesis of caged aminoluciferin analogues **1–3**. a) Cl_2CO , dioxane; b) **4**, **5**, or **6**, DMAP, THF; c) D-Cys·HCl, K_2CO_3 , DCM/MeOH/ H_2O ; d) NaN_3 , DMSO.

Table 1Kinetic parameters for compounds **1–3** with *A. aerogenes* sulfatase.^[a]

Compound	1	2	3
K_m	156.8±8.1 μ M	29.8±1.0 μ M	90.1±38.7 μ M
Relative V_{max}	1	17.6	8.2

^[a]The reactions were monitored by the change in fluorescence maxima of the aminoluciferin analogue, after uncaging. See the Supporting Information for experimental details.