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Sulfonamides are an overlooked class of electron donors in luminogenic luciferins and fluorescent dyes

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Abstract

Many fluorophores, and all bright light-emitting substrates for firefly luciferase, contain hydroxyl or amine electron donors. Sulfonamides were found to be capable of serving as replacements for these canonical groups. Unlike “caged” carboxamides, sulfonamide donors enable bioluminescence, and sulfonamidyl luciferins, coumarins, rhodols, and rhodamines are fluorescent in water.

Graphical Abstract

There is only one natural substrate for firefly luciferase: D-luciferin. The light-emitting chemistry of this molecule is not unique, as a broad range of synthetic substrates have now been shown capable of supporting bioluminescence.^{1–3} However, most luminogenic examples are push-pull chromophores that contain hydroxyl, amine, or alkylamine electron donors (Figure 1). Here we show that a new class of sulfonamidyl luciferin analogues are fluorescent molecules that are luminogenic substrates for firefly luciferase. Furthermore, we find that sulfonamidyl analogues of coumarin and xanthene dyes are also bright fluorophores. Sulfonamides are thus an overlooked class of electron donors that can be incorporated into both fluorescent and bioluminescent probes.

In recent work to explore and expand the range of accessible luciferin analogues, we found that Buchwald–Hartwig amination of 6-bromo-2-cyanobenzothiazole **1** with Xantphos and

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ASSOCIATED CONTENT

Supporting Information

Experimental details and spectra (PDF). The Supporting Information is available free of charge on the ACS Publications website.

Cs_2CO_3 allowed ready access to a wide variety of 6-substituted luciferin precursors.⁴ When these reaction conditions were applied to methanesulfonamide, attack at the electrophilic nitrile occurred instead, resulting in the imide **2** (Scheme 1). Fortunately, simply replacing the ligand and base with tBuXPhos ⁵ and K_3PO_4 allowed the synthesis of **3** to proceed in 67% yield. Condensation with D-cysteine yielded the 6'-methanesulfonamide (mesyl) luciferin analogue **3a**.

In sharp contrast to 6'-acylamino luciferin analogues – which are “caged” derivatives with little-to-no bioluminescent emission⁴ – we found that **3a** is luminogenic (Figure 2). This marked difference could be due in part to the lower pKa of sulfonamides. Consistent with this hypothesis, **3a** exhibits pH-dependent fluorescence (pKa of 7.8), suggesting that light emission occurs from the sulfonamide anion. Furthermore, the N-methylated sulfonamide analogue **4a** exhibits negligible bioluminescence, and its weakened fluorescence is blue-shifted by ~80 nm and lacks pH dependence (Figure 2, Table S1).

To further probe the scope of sulfonamide luminescence behavior, we synthesized a large panel of ~40 sulfonamidyl luciferin analogues (Schemes S1–S5). Since firefly luciferase also has fatty acyl-CoA synthetase activity,^{6–8} sulfonamides with aliphatic chains were expected to extend into the elongated binding pocket (**3a–15a**). Bulky analogues were synthesized to probe the steric scope of accepted substrates (**16a–34a**), and electronic modifications were made to further probe the importance of the sulfonamide pKa on its emissive properties (**35a–44a**).

The modest light emission of **3a** could be greatly improved upon (Figure 3). Generally, more lipophilic derivatives performed better as luminogenic substrates, but interestingly, there is a substantial drop-off in luminescent activity for the hexylsulfonamide **14a** compared to the butyl, pentyl, and octyl analogues (**9a**, **12a**, **15a**). Bulky phenethyl, 2,6-dimethylphenyl and mesityl analogues (**25a**, **31a**, **32a**) are also bright emitters. Intriguingly, the maximal emission from **31a** is ~10% of that achieved with D-luciferin (Figure S1). Furthermore, the decay in luminescence from the sulfonamides one minute after substrate injection is only ~3-fold from the initial rate, similar to D-luciferin, rather than ~10–100-fold as observed for many aminoluciferin analogues (Figures 2 and S1).^{9,10} Surprised by the brightness of many sulfonamides, we performed LC/MS to verify that there is no contaminating D-luciferin or aminoluciferin (SI).

We measured the pH-sensitivity of fluorescence for a subset of these new analogues (Table S1). Compared to **3a**, the pKa values for benzenesulfonamide **27a** and trifluoropropyl (TFP) sulfonamide **41a** are lowered from 7.8 to 7.4, and these analogues exhibited the highest quantum yields. On the other hand, the pKa values for **9a**, **25a**, and **32a** were raised to 8.6, 8.2, and 8.6, respectively, yet they remain good luminogenic luciferase substrates. This suggests that deprotonation can occur within the active site for all of these analogues. For reference, the pKa of D-luciferin is 8.7.¹¹

Bioluminescence from most of the sulfonamides occurred at a wavelength similar to that of D-luciferin (547–555 nm). However, **3a** and **9a** emit at 636 and 642 nm, respectively (Figure S2). This difference appears to derive from a subtle orientation and/or protonation effect

within the enzyme pocket, and not the pKa value *per se*, as this red-shift is not observed for similar analogues **12a** and **15a**. Furthermore, some of the sulfonamides, such as **29a** and **41a**, give bimodal emission. D-luciferin is known to exhibit similarly environmentally-sensitive emission, including examples of bimodal emission.^{12,13}

In live cells, the brightest substrates include the mesityl and *n*-butyl sulfonamides **32a** and **9a** (Figures 4, S3). Surprisingly, *n*-octyl sulfonamide **15a** gave weaker than expected emission based on the *in vitro* results (Figure 3), suggesting that its ability to access the intracellular luciferase is restricted, perhaps through sequestration in cell membranes and/or engagement with other cellular targets. In cell lysates, the relative performance of **15a** was substantially improved. Notably, sulfonamidyl luciferin analogues do not suffer from the product inhibition normally associated with alkylated aminoluciferins in cell lysates (Figure 4),¹² consistent with their behavior using the purified luciferase (Figure 2, S1).

Because all of the sulfonamidyl luciferins are fluorescent, it was surprising to find few documented examples of fluorophores with sulfonamide donors. Shibata et al. have reported two rhodamines containing 2,4-dinitrobenzenesulfonamide donors, designed as “caged” sensors for thiols, neither of which exhibited appreciable fluorescence.¹⁴ Nitrobenzenesulfonamide coumarin and cresyl violet dyes are similarly nonfluorescent, presumably due to electron-transfer quenching.¹⁵ Toluene-sulfonamide-substituted coumarin, cresyl violet, and rhodamine dyes have been reported, but their fluorescence properties have scarcely been investigated.^{15–18}

The lack of reported sulfonamidyl dyes may be due to the perception that they will not be fluorescent, coupled with the need for deprotonation. To determine whether sulfonamide donors can be utilized more broadly in fluorescent scaffolds other than luciferin analogues, we synthesized TFP sulfonamidyl coumarin **45**, which should have a low pKa and no electron-transfer quenching (Figure 5). In water at physiological pH, **45** is 89% as bright as the aminocoumarin, with a similar extinction coefficient and emission that is red-shifted by 15 nm (Table S2). Next, we synthesized sulfonamidyl rhodol **46** and rhodamine **47**. The bis-sulfonamide rhodamine **47** is colorless in ethanol, and the mono-sulfonamide rhodol **46** nearly so, suggesting they exist predominantly as the spirolactone (Figure 1). However, in water, we expected they would deprotonate to a ring-opened, anionic form. Indeed, the pKa of rhodol **46** is 5.9, slightly lower than that of fluorescein (Figure S4). TFP-rhodol **46** has an extinction coefficient of 56,400 M⁻¹cm⁻¹ at 499 nm, and fluoresces at 526 nm with a quantum yield of 0.78 (Table S3). Impressively, this is brighter than most rhodols with dialkylamine donors.^{19,20} Moreover, the bis-TFP rhodamine **47** is also fluorescent, with a quantum yield of 0.40 and extinction coefficient of 51,000 M⁻¹cm⁻¹, suggesting that sulfonamides could also be incorporated into related red-shifted scaffolds such as oxazines²¹ and azasilines,²² as well as sulfone,²³ phosphorus,^{24–26} and Si-substituted rhodamines²⁷ and rhodols.²⁸

In summary, sulfonamide electron donors do not behave like “caged” carboxamides, but instead expand the scope of luminogenic molecules beyond the canonical hydroxyl and amino-substituted analogues. The relative brightness of sulfonamide-substituted fluorophores suggests that further improvements in bioluminescence from sulfonamidyl

luciferins can be achieved through mutation, and the ability to easily tune the molecular properties of sulfonamidyl luciferins can aid in the construction of selective new luciferin-luciferase pairs^{9,12,29} as well as specific bioluminescent sensors that can take advantage of this new functionality.^{30,31} Furthermore, sulfonamide donors have previously overlooked potential for incorporation into fluorescent probes, opening up new possibilities in fluorophore design.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Examples of luciferins and xanthenes.

Figure 2.

Bioluminescence and fluorescence of **3a–4a**. Top: Rapid injection of 0.5 nM luciferase into 250 μ M luciferin. Bottom: fluorescence properties of **3a** and **4a**.

Figure 3. Sulfonamidyl luciferins (top) and their relative light emission after rapid injection into 0.5 nM purified firefly luciferase (bottom).

Figure 4. Bioluminescence of sulfonamidyl luciferins in live (top) and lysed mammalian cells (bottom).

Figure 5. Properties of fluorophores with sulfonamide donors (depicted in ring-opened, protonated form).

Scheme 1.
Synthesis of sulfonamidyl luciferins.