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Synthesis and bioluminescence of difluoroluciferin

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ABSTRACT

A new synthesis route to firefly luciferin analogs was developed via the synthesis of 5',7'-difluoroluciferin. As a luciferase substrate, it produces maximal bioluminescence at a much lower pH than is optimal for native luciferin, and at lower pH it gives much more of the red-shifted emission that is characteristic of the phenolate. These features are attributed to the enhanced acidity of the *o,o*-difluorophenol.

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Reporters and labels based on the bioluminescent reaction of D-luciferin with luciferases are pervasive in bioassays. Until the last few years, significant structural perturbations had not been made to the luciferin chromophore, but many modified luciferins are now known.¹ The pH optimum of firefly luciferase is 7.8, and the emission is yellow-green at this higher pH but more to the red at lower pH, reflecting (at least in part) different luciferin ionization states. This behavior recalls fluorescein, which requires a basic medium for maximum fluorescence. The acidity of its phenolic chromophore is increased by *o*-difluorination, creating the pH-insensitive (presumably because the phenol is always ionized) Oregon green dyes useful for live cell imaging, inter alia.² This fluorination strategy could be even more fruitful with the less acidic chromophore luciferin. 5'- and 7'-Fluoroluciferin are known,³ but our work was aimed at increasing the phenol acidity more acutely by *o*-difluorination, by analogy to Oregon green. There was little question if these compounds would be luciferase substrates, as fluorine is sterically only slightly larger than hydrogen. In this work, we applied a novel luciferin synthesis route to the preparation of 5',7'-difluoroluciferin (F₂-luc) and showed it has a useful pH-dependence of its bioluminescent properties.

The synthesis of F₂-luc (Scheme 1) begins with commercial 2,6-difluorophenol, which is transformed via known methods⁴ into difluoroanisidine (**1**). It is converted to the aminobenzothiazole by electrophilic aromatic substitution with BrSCN.⁵ The remainder

of the route follows White's precedent,⁶ using a modified Sandmeyer reaction⁷ to generate **2**, removing the methyl ether to give the phenol, with cyanide displacement yielding cyanobenzothiazole **3**. This nitrile is converted to F₂-luc (**4**) via the usual procedure.⁸ It is noteworthy that cyanobenzothiazoles much like **3** can be converted to D-luciferin analogs in vivo.⁹

Bioluminescence was examined with **4** as the substrate for wild-type *Photinus pyralis* firefly luciferase. As shown in Figure 1, at pH 8.0 its emission λ_{max} is 579 nm, somewhat different from native luciferin (560 nm; Fig. S1). Additionally, there is much greater emission farther to the red, with a second ~600 nm maximum. As the pH is reduced the 579 nm emission diminishes and the ~610 nm emission, which is comparable to that of luciferin, increases. Yet, the pH dependence of luciferase bioluminescence with **4** as substrate is much different from luciferin (Fig. 2); the greatest emission occurs at pH 6.0–6.5, whereas luciferin simply loses emission as the pH decreases from 8 (Fig. S2). There is presumably much greater ionization of the phenol in F₂-luc in the lower pH range compared to luciferin, as intended in our design. The pK_a of luciferin is 8.7,¹⁰ and the pK_a of oxyluciferin is 9.1.¹¹ The pK_a of F₂-oxyluciferin is estimated to be ca. 6.4, based on the 2.7 unit reduction in pK_a upon addition of fluorines at the 2- and 6-positions of phenol.¹² Comparing the pH dependence of the absorption spectra of **4** by theoretical methods proven for luciferin supports the idea that the phenol of F₂-luc is ionized at pH 6, whereas luciferin is not.¹³ It is interesting that F₂-luc and luciferin show their largest bioluminescence at pHs near their pK_as.

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Scheme 1. Reagents and conditions: (1) KSCN, AcOH, Br₂, 56%; (2) isoamyl-ONO, CuBr, MeCN, 68%; (3) BBr₃, CH₂Cl₂, –60 °C, 20 h, 90%; (4) NaCN, DMSO, ~100 °C, 4 h, 78%; (5) (R)-cysteine-HCl, H₂O, 86%.

Figure 1. Bioluminescence spectra of firefly luciferase with 5',7'-difluoroluciferin as substrate: pH dependence. Larger enzyme quantities were used at higher pH to obtain better emission data. The spectra are normalized to facilitate comparison. Reaction was initiated by adding 5 μ L of luciferase stock (0.2 pM–50 nM) to a solution of total volume of 0.245 mL, which included F₂-luc (5 μ M), ATP (1 mM), MgSO₄ (10 mM) and a 50 mM buffer of MOPS (for pH 7 and 8) or MES (for pH 5 and 6). Spectra were obtained using the instrument described in the experimental section.

We also examined the efficiency of **4** as a luciferase substrate. The concentration dependence of the emission was studied, which shows the expected saturation behavior with a K_m of 5 μ M at pH 7.8, compared to 10 μ M for luciferin under these conditions. Luciferase emission intensities were compared at pH 6.0 with **4** and luciferin each at 0.5 μ M. The bioluminescence from **4** was 12% that of luciferin. High emission intensity has generally not been observed with other luciferin analogs, a phenomenon that is not well-understood.

Since two other fluorinated luciferin analogs have been reported,^{1g,3} we compare F₂-luc to their reported pH sensitivity and bioluminescence properties. With 5'-fluoroluciferin (5F-luc), there is little variation in bioluminescence intensity at pHs from 8.3–6.9, and the efficiency is 20–25% of luciferin. Bioluminescence

Figure 2. Bioluminescence of firefly luciferase with F₂-luc as substrate: pH dependence. Readout was performed after manual injection of luciferase (to a final concentration of 1 nM) into a reaction mixture containing ATP, MgSO₄ buffer and F₂-luc (to a final concentration of 5 μ M). Assays were performed in triplicate and error bars represent standard deviations within the replicates.

of 5F-luc at pH 7.4 is blue-shifted by 25 nm compared to luciferin. With 7'-fluoroluciferin (7F-luc), its pH optimum is 7.5, and the pH sensitivity of its emission is essentially identical to luciferin. The efficiency with 7F-luc is 11% of luciferin. Its K_m is 4–5-fold lower than luciferin and its bioluminescence is red-shifted by either 11 or 21 nm, depending on the report. Neither pK_a is reported, but one predicts a 1.2 pH unit acidification of the phenol by an *o*-F.

It is intrinsic that the pH dependence of luciferase bioluminescence is a function not only of the chromophore ionization state but also the effects of pH on the enzyme. However, modern protein engineering methods can significantly influence the pH sensitivity of enzymatic activity, including luciferase activity.¹⁴ It has also been shown that luciferases of more diverse origin provide different pH sensitivities,¹⁵ in one case comparable to the pH optimum observed with **4**. We believe that difluoroluciferin (F₂-luc) will prove useful in circumstances where bioluminescence must be measured under varying pH regimes or in acidic cellular compartments, where the native luciferase/substrate pair perform poorly. Studying the basis for variance in pH dependence with substrate pK_a may also provide greater mechanistic insight into luciferase function.

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Supplementary data

Supplementary data (experimental procedures for the synthesis of F₂-luc and its assay as well as NMR and bioluminescence spectra) associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmcl.2014.08.048>.

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