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β-Deuterium Isotope Effects on Firefly Luciferase Bioluminescence

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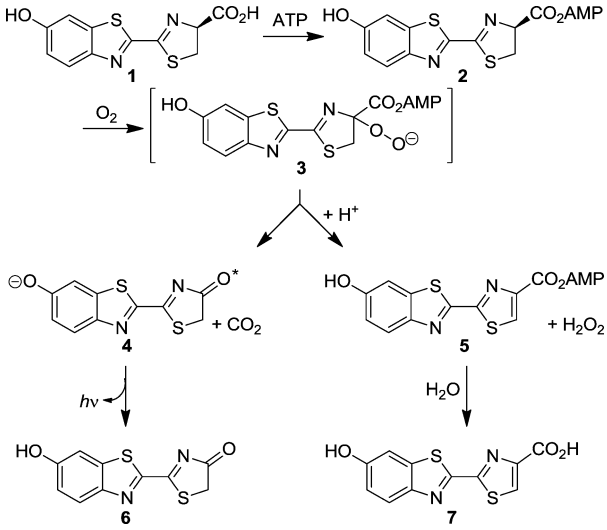
A 5,5-d_2-luciferin was prepared to measure isotope effects on reactions of two intermediates in firefly bioluminescence: emission by oxyluciferin and elimination of a putative luciferyl adenylate hydroperoxide to dehydroluciferin. A negligible isotope effect on bioluminescence provides further support for the belief that the emitting species is the keto-phenolate of oxyluciferin and rules out its excited-state tautomerization, one potential contribution to a bioluminescence quantum yield less than unity. A small isotope effect on dehydroluciferin formation supports a single-electron-transfer mechanism for reaction of the luciferyl adenylate enolate with oxygen to form the hydroperoxide or dehydroluciferin. Partitioning between the dioxetanone intermediate (en route to oxyluciferin) and dehydroluciferin is determined, not by the fate of the hydroperoxide, but by that of the radical formed from luciferyl adenylate, and the kinetic isotope effect (KIE) reflects H-atom abstraction by superoxide.



Scheme 1. Accepted pathway of firefly bioluminescence.

The reaction pathway of firefly bioluminescence is well established (Scheme 1).[1] The stoichiometric substrate luciferin (**1**) is converted to acyl adenylate (**2**) that reacts with oxygen to form presumably the hydroperoxy-adenylate (**3**),[2,3] an intermediate that has never been directly observed. Two paths can be envisioned for **3**. The first involves conversion via the dioxetanone to carbon dioxide and the excited state of oxyluciferin (**4**), which emits. A side reaction diverts some of **3** to hydrogen peroxide plus dehydroluciferin adenylate (**5**), which hydrolyzes to dehydroluciferin (**7**). Past studies have determined a **6**/**7** ratio of 4:1.[4]

Many mechanistic details of this process have been worked out previously. Deep investigations of **4** strongly suggest that, despite a large number of possible ionic states and tautomeric forms, the emitting species is the keto-phenolate shown.[5] For many years, luciferase had been considered to be highly efficient, but careful modern measurements have shown instead a quantum yield (Φ) of 0.42.[6] The basis of this quantum yield of less than unity is not understood, but possibilities for non-radiative loss of the chemically generated excited state **4** include internal conversion or a reaction such as excited-state ionization or tautomerization.[7] The oxyluciferin product **6** had been believed to be highly unstable, but recent work showed it simply requires careful handling.[8] Isotopically labeled luciferins have been used to show the expected primary α-deuterium kinetic isotope effect of approximately 2 on formation of the enolate of **2** for oxygenation.[9]

We became interested in the β-deuterium isotope effect (β-related to the reaction site at C4 of the thiazoline ring) on the firefly luciferase reaction, because of its potential to yield insight into multiple features of this classical mechanism. Once the β-deuterated luciferin enters into this reaction sequence, it can exert primary isotope effects on two consecutive mechanistic steps. It could also show a β-secondary isotope effect on the formation of **3**, but its magnitude would be small compared to the two primary isotope effects. They are worthy of study, because they could impact bioluminescent efficiency and provide a deeper understanding of mechanism.

For example, the strong similarity between the bioluminescence spectrum with the native substrate luciferin and a synthetic substrate analog, 5,5-dimethylluciferin, provides experimental evidence that the keto form is the only emitter.[10] As 5,5-dimethylluciferin gives an oxyluciferin excited state that

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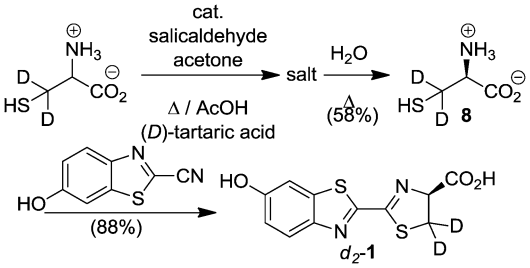
cannot tautomerize, there is no possibility of an enol contributing to its emission. A weakness of that study is the necessity to pre-synthesize the adenylate of 5,5-dimethylluciferin, because its adenylate cannot be generated by luciferase. However, firefly luciferase accepts the adenylate as a substrate. Dideuteration of luciferin in lieu of dimethylation should impede tautomerization in d_2-**4**, but, unlike 5,5-dimethylluciferin, both half-reactions of bioluminescence can be studied with d_2-**1**. A change in bioluminescence with this dideuterated substrate would signal something other than the keto form being involved in the photochemistry of **4**. Kinetic isotope effects on simple ketone enolizations are 4–6,[11] and enzyme-catalyzed enolizations have values of similar magnitude.[12] Tautomerization of a ketone triplet excited state via tunneling has a very large isotope effect of 700,[13] but the excited singlet is the species that is relevant to bioluminescence.

Another reason to examine the isotope effect with d_2-luciferin is the dehydroluciferin side products. Both **5** and **7** are inhibitors of firefly luciferase bioluminescence,[14] with inhibition constant (K_I) values of approximately 5 nM and 0.15 μM, respectively; the former being a multi-substrate adduct inhibitor.[15] These compare to a 0.5 μM K_I for oxyluciferin[12,16] and a low μM Michaelis constant (K_M) for luciferin. As the dehydroluciferins are produced to the extent of 20% in each turnover, their formation contributes substantially to the loss of free, active enzyme. Their production formally entails β-elimination of hydrogen peroxide from **3**, as proposed by White et al.[2a] This process could exhibit a substantial isotope effect that would affect the **4**/**5** partitioning. Reducing the amount of **3** diverted to **5** and **7** would affect the bioluminescence efficiency and could affect enzyme activity during turnover. Although we could identify no good precedents for hydrogen peroxide β-eliminations, conventional β-eliminations often exhibit near-maximal deuterium isotope effects of approximately 7. Reducing the formation of dehydroluciferin should increase the uninhibited enzyme following each turnover, which should result in greater integrated bioluminescence and a gentler slope of its decay. For example, if the isotope effect on dehydroluciferin formation were 4, d_2-**1** would cut its production per turnover from 20% to 6% and double the amount of active enzyme remaining after five turnovers.

The D-d_2-luciferin required for this study was prepared as shown in Scheme 2. Commercial d_2-cysteine was deracemized by chiral conversion using the method of Shiraiwa et al.[17] This process converts Cys to the thiazolidine with acetone. The salicaldehyde catalyst forms a Schiff base with the cyclic amine, labilizing the α-hydrogen and establishing a racemizing equilibrium. Tartaric acid forms an insoluble salt with one enantiomer, removing it from the equilibrium. The salt is hydrolyzed to give D-(−)-d_2-cysteine, which has $[\alpha]_D$ −6.5° (*c* 4.00, 5 N HCl) ($[\alpha]_D$ +6.5° (*c* 4.00, 5 N HCl) for L-cysteine). The conventional luciferin synthesis was then applied to provide d_2-**1**.



Scheme 2. Synthesis of d_2-luciferin.

Isotope effects on enzymatic reactions are well known and heavily studied.[18] They are more complex to consider than those involved in elementary chemical processes. How they are measured determines whether they are catalytic rate constant (k_{cat}) isotope effects or k_{cat}/K_M isotope effects and what mechanistic information they provide. However, for both steps in the bioluminescence mechanism that could be affected by 5,5-d_2-luciferin, tautomerization of **4** and formation of **5**, the isotope effect influences the partitioning of an intermediate between competing pathways, so k_H/k_D (kinetic isotope effect) can be considered on this basis.

The first question addressed with d_2-luciferin was the isotope effect on bioluminescence. Under identical incubation conditions with recombinant wild-type *P. pyralis* luciferase, we were unable to detect any difference between **1** and d_2-**1** in the bioluminescence emission spectrum or intensity.

A second study examined the isotope effect on dehydroluciferin production. The formation of **6** and **7** was determined in end-point assays. The HPLC analytical method of Silva and coworkers[4] was used to measure the absolute amounts of oxyluciferin and dehydroluciferin formed from **1** and d_2-**1**, calibrated with an authentic sample of dehydroluciferin, and their ratio was used to calculate the isotope effect. Results are summarized in Table 1. From these data, the isotope effect based on the organic products is 2.13 ± 0.14.

Table 1. Production of **6** and **7** from **1** and d_2-**1**.

	1	d_2-**1**
6[a]	45.06 ± 0.15	38.97 ± 0.49
7[a]	11.26 ± 0.04	4.57 ± 0.30
6/**7**	4.002 ± 0.01	8.53 ± 0.56

[a] [nmol].

A third study was also related to dehydroluciferin production, but analyzed the other product. A fluorescence method based on Amplex Red and horseradish peroxidase[19] was used to measure hydrogen peroxide formation in triplicate reactions using **1** or d_2-**1**. The kinetic isotope effect (KIE) determined from hydrogen peroxide formation is 1.12 ± 0.02, which here is a k_{cat} isotope effect. Reasons for the divergence between this value and that determined by using dehydroluciferin are not obvious. However, the data in Table 1 are based on a ratio of products in the same reaction, which should be more reliable. The isotope effect on hydrogen peroxide arises from separate reactions, where consistency between trials is more difficult to attain.

A final study examined the effect of d_2-**1** on the time course of bioluminescence; deuteration was expected to enhance its

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persistence by reducing the formation of two powerful inhibitors. However, the low magnitude of the isotope effect mutes the effect compared to the potential benefits earlier discussed. As shown in Figure 1, there is but a slight advantage for d_2-**1**

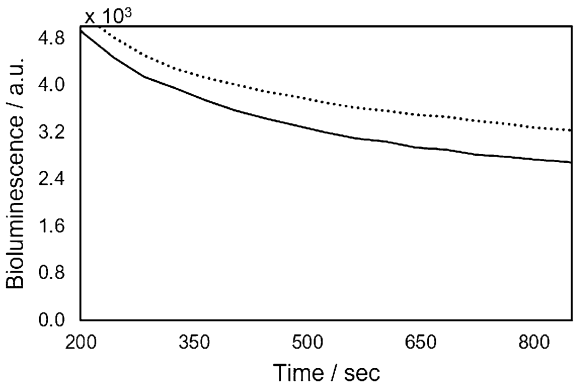
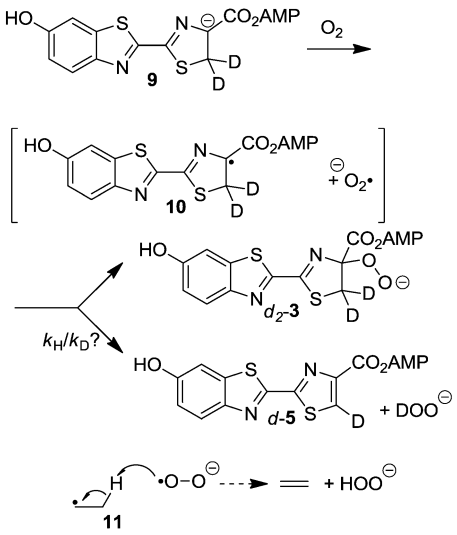


Figure 1. Time course of bioluminescence decay at late reaction times for **1** (solid) and d_2-**1** (dashed).

in bioluminescence decay at long reaction times. Although the reasons are unclear for the difference in isotope effects on dehydroluciferin formation measured via the two reaction products, the conclusion is apparent that the effect is small; this fact affects mechanistic possibilities for dehydroluciferin formation.

The lack of an isotope effect on oxyluciferin bioluminescence emission agrees with earlier beliefs that its keto form is the emitting species, further dispels outmoded notions that color modulation of the emission is based on keto-enol tautomerism,[20] and shows that CH ionization or tautomerization are not responsible for its excitation loss.

The small isotope effect on dehydroluciferin formation is difficult to reconcile with the idea that it is formed by β-elimination from **3**, as isotope effects on such reactions can be substantial. We, therefore, consider a modified mechanism for the reaction of **2** with oxygen, exemplified in Scheme 3 with the deuterated substrate. As proposed in 1981 by Kosower as well as more recently[2b,3,21] and then supported experimentally,[22] the enolate of the adenylate (**9**) transfers an electron to oxygen to form radical **10** and superoxide. Their recombination would produce hydroperoxy d_2-**3** that cyclizes to the dioxetanone. The proportion of bioluminescence and dehydroluciferin formation could be accounted for by the partitioning of **10** between recombination with superoxide (which should have only a very small β-secondary isotope effect) and H/D abstraction by superoxide. We postulate that the measured isotope effect reflects the latter reaction, a prototype of which is shown with a simple ethyl radical **11**. We have been unable to locate any precedent for such a process, and therefore have no frame of reference for its KIE. Other H-abstractions by superoxide are known,[23] but their KIEs are not. The idea that dehydroluciferin arises from elimination of **3** is thus supplanted—instead, it comes essentially from the direct, step-wise, radical-based oxidation of the enolate of **2** by oxygen. Other mechanisms may



Scheme 3. Alternative reaction pathways for the enolate of luciferyl adenylate.

also be conceived that are consistent with the small isotope effect on this step.

This view parallels work in bacterial bioluminescent systems, where a primary kinetic isotope effect of approximately 1.5 was interpreted to show rate-limiting electron transfer to the carbonyl of its bioluminescent substrate, a long-chain aldehyde.[24]

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: electron transfer · elimination · isotope effects · luminescence · tautomerization

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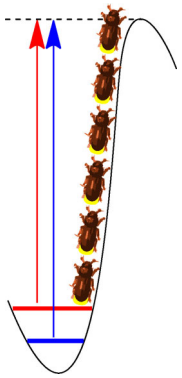
Product inhibition: Firefly flashing can be attributed to inhibitory products of the luciferase enzyme. A small isotope effect on the formation of one important product, dehydroluciferin, has mechanistic implications for bioluminescence production.



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