Bioluminescence

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Novel Mechanism of Bioluminescence: Oxidative Decarboxylation of a Moiety Adjacent to the Light Emitter of *Fridericia* Luciferin**

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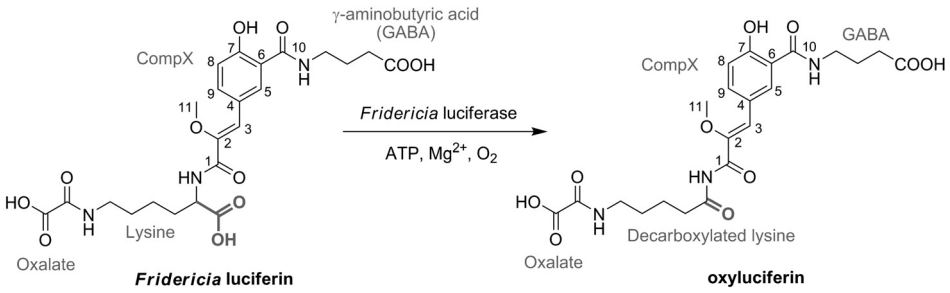
Abstract: *A novel luciferin from a bioluminescent Siberian earthworm Fridericia heliota was recently described. In this study, the Fridericia oxyluciferin was isolated and its structure elucidated. The results provide insight into a novel bioluminescence mechanism in nature. Oxidative decarboxylation of a lysine fragment of the luciferin supplies energy for light generation, while a fluorescent CompX moiety remains intact and serves as the light emitter.*

Bioluminescence (BL)—the production of "cold light" by a living organism—is generally mediated by the oxidation of a small organic molecule called luciferin by molecular oxygen in a reaction catalyzed by a specific luciferase enzyme.[1] We recently reported the structure of a novel luciferin from the Siberian bioluminescent earthworm *Fridericia heliota*.[2] We found this luciferin to be a key component of a novel ATP-dependent bioluminescent system, along with a specific luciferase, ATP, Mg^{2+} ions, and molecular oxygen[3] The *Fridericia* luciferin turned out to be an unusual peptide formed from oxalic acid, L-lysine, modified tyrosine (CompX[4]), and γ-aminobutyric acid residues. Herein, we report the structural elucidation of the *Fridericia* luciferin oxidation product, i.e., the oxyluciferin. The results provide a structural basis for understanding the novel light generation mechanism underlying the bioluminescence of *Fridericia heliota*.

Oxyluciferin was prepared by mixing 0.18 mg of synthetic *Fridericia* luciferin[2] with excess ATP and crude *Fridericia* luciferase obtained through partial purification of protein extract from 40 g of the worm biomass[5] (Scheme 1, see the Supporting Information for details). The reaction mixture produced constant eye-visible luminescence and the reaction course was monitored by HPLC analysis of aliquots (Figure 1), which showed formation of a major product of luciferin oxidation with a retention time of 18.2 min. After 25 h, approximately 62 % of the luciferin was consumed. At this point, the reaction was stopped by adding formic acid and the mixture was subjected to solid-phase extraction with subsequent HPLC to give 0.8 optical density units of oxyluciferin (absorption at 310 nm).



Scheme 1. The bioluminescence reaction of *Fridericia heliota*, including structures of the luciferin and oxyluciferin.

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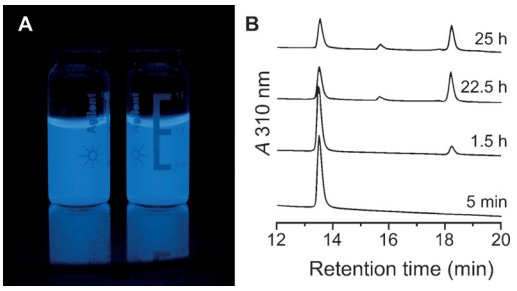
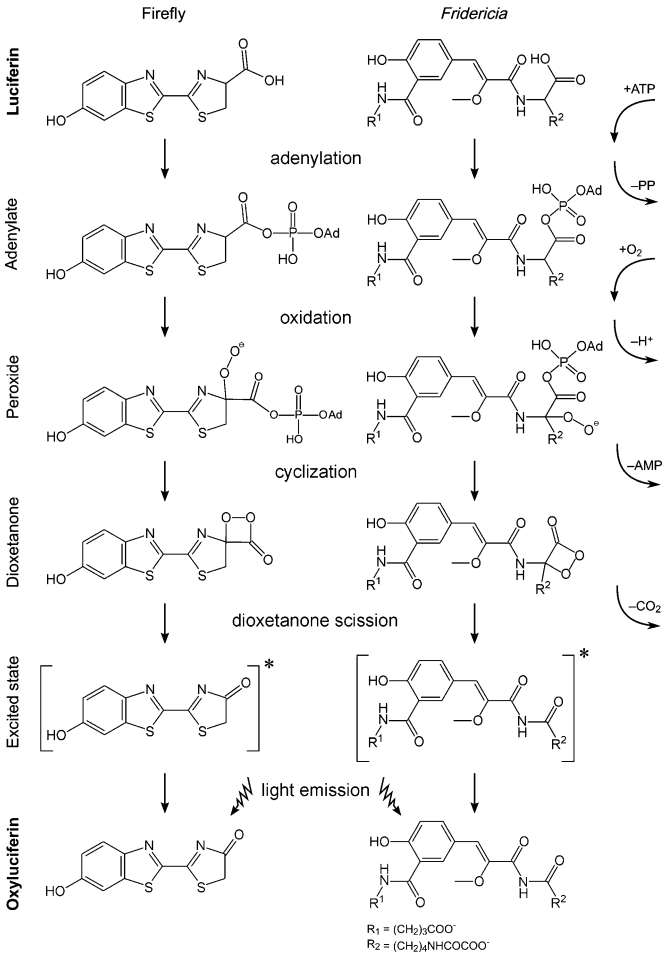


Figure 1. A) Luminescence of the reaction mixture. B) HPLC profiles of the bioluminescence reaction at 5 min, 1.5 h, 22.5 h, and 25 h. The peaks at 13.7 and 18.2 min correspond to luciferin and oxyluciferin, respectively.

The ESI-HRMS spectra of pure oxyluciferin showed a molecular ion with $m/z = 494.1769$, which perfectly matches the molecular formula $C_{22}H_{28}N_3O_{10}^+$. This formula corresponds to the loss of two hydrogen atoms, one carbon atom, and one oxygen atom compared to *Fridericia* luciferin. NMR analysis of the oxyluciferin (1H, COSY and HSQC spectra) showed disappearance of the lysine α-H and essential changes to the chemical shift and multiplicity of the lysine β-CH_2 protons compared to luciferin (two multiplets at 1.91 and 1.78 ppm vs a triplet at 2.62 ppm; Figure 2 and Table S1 in the Supporting Information). A downfield shift of the CompX H-3 proton by approximately 0.2 ppm was also observed, while all other protons remained intact and showed unchanged NMR signals. Taken together, these data provide unambiguous evidence that *Fridericia* oxyluciferin is produced through oxidative decarboxylation of the lysine moiety, contrary to our initial hypothesis that the oxalate fragment is oxidized during bioluminescence.[2]

The mechanism of light production by the bioluminescence system of *Fridericia heliota* seems to be highly similar to that of the well-studied firefly bioluminescence (Scheme 2). The reaction probably starts with the formation of a luciferin adenylate at the lysine carboxy group, followed by deprotonation of the neighboring lysine α-CH, addition of an oxygen molecule to the carbanion to give a peroxide, nucleophilic attack of the peroxy group at the adenylated carboxy group with the release of AMP and formation of the dioxetanone



Scheme 2. Proposed mechanism of Fridericia bioluminescence compared to the well-studied firefly bioluminescence mechanism. The acid–base interactions (such as protonation state of the phenolic groups, excited-state proton transfer, etc) are not shown for simplicity.

cycle, and finally, electrocyclic scission of the dioxetanone ring to release a CO_2 molecule and form oxyluciferin, in a manner analogous to the firefly system.[1,6] However, in the firefly bioluminescence mechanism, the newly formed carbonyl group becomes part of the conjugated π system of the light emitter, while in the *Fridericia* mechanism, the π system of the light emitter (CompX fragment) remains unchanged during oxidation.

The role of the CompX moiety as the light emitter is supported by the close similarity of the luciferin fluorescence emission spectrum to its bioluminescence emission spectrum (λ_{max} 466 and 480 nm, respectively)[2] and also to the fluorescence emission spectrum of oxyluciferin (Figure S3). The fluorescence quantum yield of oxyluciferin in water at pH 5.7 was determined to be as low as 0.16%. Taking into account the bright luminescence of the live worms, which is visible with the naked eye, the bioluminescence quantum yield of *Fridericia* luciferin is likely much higher than 0.16%. This seeming contradiction is probably explained by the dramatic increase in the

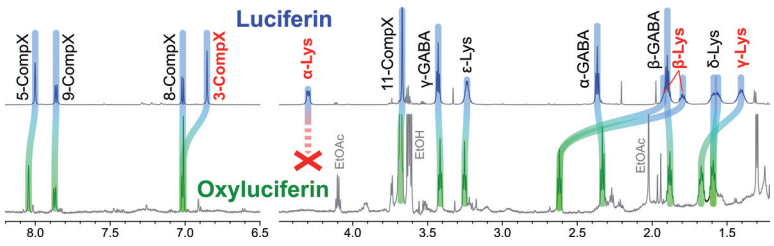


Figure 2. Comparison of the ^{1}H NMR spectra of *Fridericia* luciferin (blue, 800 MHz) and oxyluciferin (green, 700 MHz) in D_2O at pH 5.0. The disappearance of the signal for the lysine α-H and the changes in chemical shift and multiplicity of the signals for the lysine β- and γ-CH_2 protons are marked in red. Corresponding chemical shifts are linked by blue–green gradients. See Figure S23 for the 2D NMR spectra used for signal assignment and Table S1 for NMR chemical shifts. Impurities in the NMR spectrum are shown in gray. Assignments are shown for some impurities.

fluorescence quantum yield (FQY) of a fluorophore upon steric stabilization (including possible photoinduced *cis–trans* isomerization of the CompX double bond) when bound to the active site of luciferase. Such an increase in the FQY of a ligand through specific binding to a macromolecular host is well-described for the chromophore of green fluorescent protein (GFP).[7]

To demonstrate the role of the luciferin adenylate at the lysine carboxy group as an intermediate in *Fridericia* bioluminescence, we attempted its chemical synthesis. However, the activation of the lysine carboxy group by any known method (activated esters, mixed anhydrides, etc) led to decomposition of the substrate and produced complex mixtures. The CompX moiety probably facilitates the deprotonation of a nearby lysine α-H, thereby rendering the luciferin derivatives activated at the lysine carboxy group (including the adenylate itself) unstable. This conclusion is supported by the observed chemiluminescence of a model compound for luciferin adenylate, which contains a *tert*-butylated lysine carboxy group, upon the action of bases (Figure 3 and the Supporting Information).

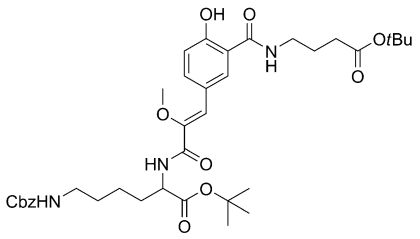


Figure 3. Structure of a chemiluminescent model compound for luciferin adenylate. Cbz = carboxybenzyl.

The characterization of the reactants and the products of the *Fridericia* bioluminescence system confirms the decarboxylation process that results in oxyluciferin formation. This reaction most likely proceeds through the enzymatic formation of highly reactive dioxetanone. Based on the literature, there are two possible mechanisms for the conversion of the dioxetanone chemical energy into luminescence of the chromophore (CompX).[8–10] The first mechanism is an indirect chemiluminescence of CompX by singlet–singlet energy transfer. Highly exergonic thermolysis of the dioxetanone may result in the formation of singlet and/or triplet carbonyl products. The fluorescence of the former may then be reabsorbed and emitted by CompX.[11] However, the efficiency of the formation of an excited singlet state in carbonyls is usually less than 1%, so the most probable mechanism of CompX chemiexcitation is the one proposed by Schuster (Scheme S1 in the Supporting Information).[12] This mechanism, known as chemically-initiated electron-exchange luminescence (CIEEL) involves two sequential electron transfer steps. First, the electron-rich chromophore donates an electron to the peroxide moiety of the dioxetanone. This leads to cleavage of the O–O bond, release of CO_2, and electron back-transfer resulting in the formation of the excited chromophore. Formally, a proposed intermediate in the reaction of *Friderica* luciferin belongs to the class of CIEEL-active dioxetanes[8,9] and thus may be responsible for the observed bioluminescence. It is important to note that the energy and electron-transfer bioluminescence mechanisms do not require conjugation between the aromatic residue of the CompX and the carboxylate group. Nevertheless, one should take into account the possibility of tautomerization of the newly formed carbonyl through the α-NH group to give N=C–OH, which would enable conjugation to the fluorophore. Similar keto–enol photoinduced tautomerization was recently proposed for firefly oxyluciferin.[13]

In conclusion, we report the isolation and structural elucidation of *Fridericia* oxyluciferin, which provides insight into a novel bioluminescence mechanism in nature. Oxidative decarboxylation of a lysine fragment of luciferin supplies the energy for light generation, while a fluorescent CompX moiety remains intact and serves as a light emitter. Further efforts in our group will be focused on the isolation and sequencing of *Fridericia* luciferase, as well as on creating functionally active synthetic analogues of *Fridericia* luciferin with red-shifted bioluminescence emission spectra.

Keywords: bioluminescence · bioorganic chemistry · luciferin · oxidative decarboxylation · peptides

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