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Chemiluminescence

The Light Emitter of the 2-Coumaranone Chemiluminescence: Theoretical and Experimental Elucidation of a Possible Model for Bioluminescent Systems

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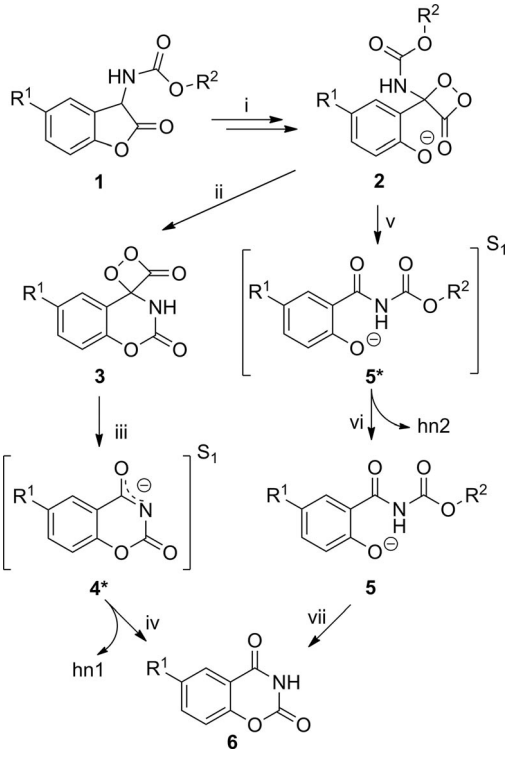
Abstract: The bioluminescence reaction of many biological subspecies, most notably fireflies, forms dioxetanone derivatives as high-energy intermediates. The thermal instability of dioxetanones complicates understanding of the transition from the ground state to the first excited state that leads to light emission. Herein, we report the reaction mechanism of 2-coumaranones, synthetically accessible strongly chemiluminescent materials that mimic the bioluminescence reaction. The pathways of chemiexcitation and photorelaxation are clarified on the basis of synthetic evidence and spectrochemical as well as computational mechanistic analysis.

Introduction

Chemiluminescence (CL) and bioluminescence (BL) are photochemical phenomena that lead to emission of light in the visible region of the electromagnetic spectrum by following a sequence of chemical reactions. In the case of BL, this excitation by chemical transformation occurs inside enzymes (luciferases). In CL, the reaction normally takes place in solution. The formation of a 1,2-dioxetanone moiety is common with many of the known CL and BL reactions.[1,2] This chemical group is formed if molecular oxygen reacts with the subtract, which lead to the formation of a peroxide bond (see compound **2** in Scheme 1). Decomposition of this intermediate leads to a luminophore (oxyluciferin in BL or light emitter) in its first singlet excited state (S_1) (chemiexcitation), which relaxes by emission of light (photorelaxation).[3–5] Despite the increase in the use of BL and CL as probes, the comprehension of these systems remains incomplete. Indeed, the difficulty of getting RX structures from biological systems limits the number of available BL luminophores, whereas many of them are deemed very unstable in pure form, and their structures remain uncertain or are unknown. For instance, although the BL of the Siberian glowworm (*Fridericia heliota*) and some fungal species have been known for centu-



Scheme 1. Two suggested pathways for the decomposition of 1,2-dioxetanone intermediate **2** in the chemiluminescence of 2-coumaranone derivatives **1**. R^1 is a halogen and R^2 is an alkyl or aryl group (examples are described in ref.[10]). Both closed-ring lactone **4*** and salicylamide-like structure **5*** are viable candidates for the excited-state structure of the light-emitting species. In one path (ii,iii,iv) the cyclization occurs before dioxetanone decomposition, and in the other path (v,vi,vii) the cyclization occurs after dioxetanone decomposition and light emission.

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ries, the structures of their substrates were characterized only recently.[6–8] The protocols for cases in which synthetic procedures are available[9,10] are laborious and cost ineffective, which greatly limits prospects for systematic studies of the related CL and BL mechanisms. The recent interest in new fluorescent probes[11–13] calls for the design and convenient access to diverse, chemically robust, stable, bright luminophores with tunable emission energies. All properties are crucial for their utility as labels in bioanalytical applications, typically with microscopic imaging techniques.

Results and Discussion

To fulfil the demand for chemically variable luminophores, we recently prepared new derivatives of 2-coumaranones. This class of chemiluminescent compounds (**1** Scheme 1) first synthesized in 1979[14–18] are easily accessible and are strongly chemiluminescent.[19–21] The structures of the ethyl and methyl derivatives were confirmed by X-ray diffraction analysis (Figure 1; for details see the Supporting Information, Figures S6–S9). Upon treatment with base [i.e., 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU)] in polar aprotic solvents (e.g., MeCN, DMF, or acetone), these compounds emit bright blue light that is readily observable even with the naked eye in daylight (Figure 2, a). The CL also occurs after oxidation with a minimum amount of horse radish peroxidase (Figure 2, b). The energy released in this reaction can be further transferred to a fluorescence dye; the color of the secondary emission can be tuned across a wide range of the visible spectrum by selecting the dye (Figure 2, c–n). The 2-coumaranones own a 1,2-dioxetanone structure as an intermediate (see compound **2** in Scheme 1)[21] in common with many of the known CL and BL reactions.[1,2,22] Thus, they can be convenient analogues of BL precursors that provide instant access to this unstable high-energy intermediate, the structure of which has not yet been directly observed. Herein, the CL mechanism and the structure of the light emitter of 2-coumaranones were delineated to obtain fundamental insight into the molecular mechanism of their light emission. This required a combination of quantum chemical calculations and spectrochemical and structural analysis. The reaction pathways that have been advanced for CL are shown in Scheme 1. Until now, studies on these compounds have not distinguished between these two pathways.[14,19,20] Reaction of **1** with molecular oxygen (path i) initially leads to high-energy 1,2-dioxetanone intermediate **2**. Decomposition of **2** can occur by cyclization (path ii), whereby spirocyclic 1,2-dioxetanone **3** is generated. Spirocycle **3** decomposes by releasing CO_2 (path iii) to afford product lactone **4** in its excited state (**4***). After light emission (path iv), the lactone relaxes to its ground state (GS) **6**. As an alternative pathway, 1,2-dioxetanone **2** can decompose directly by releasing CO_2 (path v) to afford the excited state (i.e., **5***) of compound **5**, which subsequently relaxes and cyclizes (path vii) to product **6**.

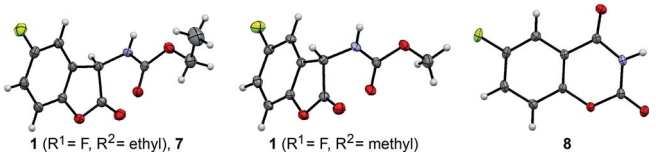


Figure 1. Crystal structures of compound **7** in Scheme 2 (**1** with R^1 = F and R^2 = Et in Scheme 1), **1** (with R^1 = F and R^2 = Me in Scheme 1) and **8**.

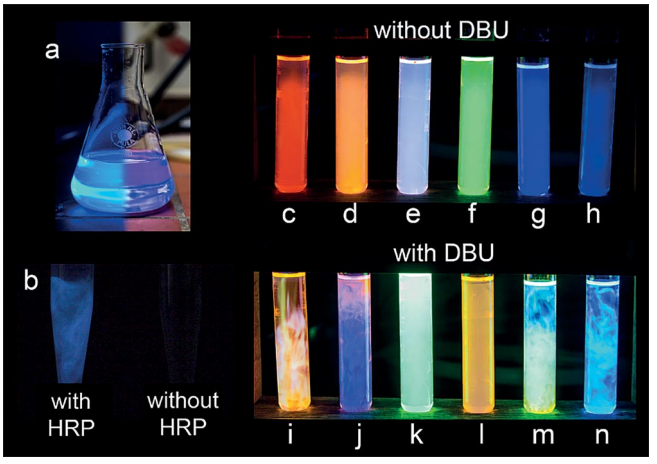
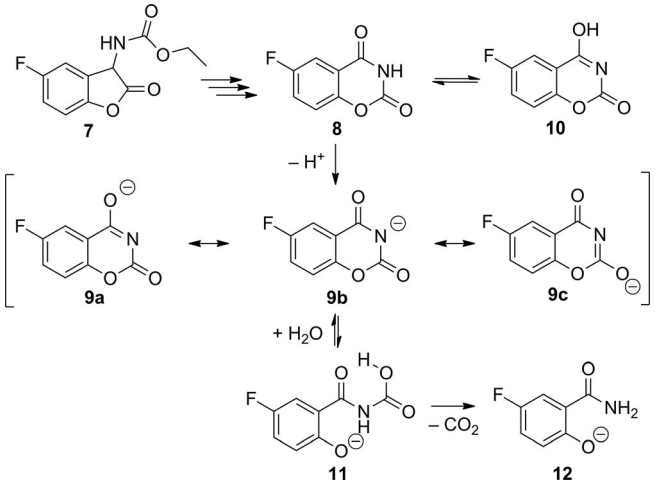


Figure 2. Chemiluminescence and fluorescence of 2-coumaranones. (a) The chemiluminescence is visible in daylight. (b) Chemiluminescence is induced by horseradish peroxidase (HRP). (c–n) Fluorescence of organic dyes induced by chemiluminescent light from the 2-coumaranones in DMF before (c–h) and after (i–n) the addition of DBU as the base. Organic dyes: (c,i) a thiazole dye (for the structure of the dye, see the Supporting Information), (d,j) Rhodamine 6G, (e,k) sodium fluoresceinate, (f,l) 9,10-bis(phenylethynyl)anthracene, (g,m) pyranine. Panels h and n show control experiments (no dye added).

To decipher the reaction pathways for the CL of 2-coumaranones and to identify the light emitter in the CL reaction, we focused on compound **7** (Scheme 2, or **1** with R^1 = F and R^2 = Et in Scheme 1). The chemical product of the CL of **7**, compound **8**, was isolated from the reaction mixture and its structure was confirmed by X-ray diffraction (for details of the structure analysis, see the Supporting Information). This result confirmed that **8** was indeed the product of CL, at least after isolation as described earlier.[15] The maximum wavelength of the CL emission of **7** was $\lambda_{em,CL}$ = 433 nm with a quantum yield of $(4.5 \pm 0.2) \times 10^2$ E mol^{-1}. The fluorescence maximum ($\lambda_{em,fl}$ = 434 nm) of the reaction mixture after any observable CL had ceased ("spent solution"), excited at λ = 366 nm, was (within spectral resolution) identical to that of CL emission. However, if product **8** was excited in MeCN at the maximum absorption in the absence of base, isolated N-protonated product **8** showed only very weak, hardly detectable fluorescence with $\lambda_{em,fl}$ = 328 nm. This observation indicated that the protonated form of **8** is neither the emitter in the CL reaction nor emitting in the spent solution. This prompted us to consider other protonated states as candidates, including tautomeric form **10** and its conjugate base **9**, the resonance structures of which, **9a**, **9b**, and **9c**, could be generated by deprotonation of **8** with a strong base such as DBU (Scheme 2). α-Azacoumarinolate anion **9** could hydrolyze in strong basic solution to open-chain carbaminic acid **11**. Indeed, free carbaminic acids such as **11** are well known to be thermally labile, even at room temperature;[23] decomposition by release of CO_2 would lead to the phenolate of 4-fluorosalicylamide **12**. All of these reactions are viable pathways and, thus, were examined as possible routes for the

ring opening before (light emitter) and after the emission (in the spent solution). We first focused our attention on the simplest product, 4-fluorosalicylamide **12**. Salicylamides are known to exhibit intense blue fluorescence.[24] We prepared **12** and analyzed its absorption and fluorescence spectra (Figure 3; for details, see the Supporting Information and Table S1).



Scheme 2. Tautomerization of chemiluminescent product **8** to α-azacoumarin **10** and deprotonation of chemiluminescent end-product **8** to α-azacoumarinolate anion **9** (resonance structures **9a**, **9b**, and **9c**). Hydrolysis of lactone **9b** leads to open-chain carbaminic acid **11**, which decomposes to 4-fluorosalicylamide **12**. Structures are the most stable calculated ones (see Table S3).

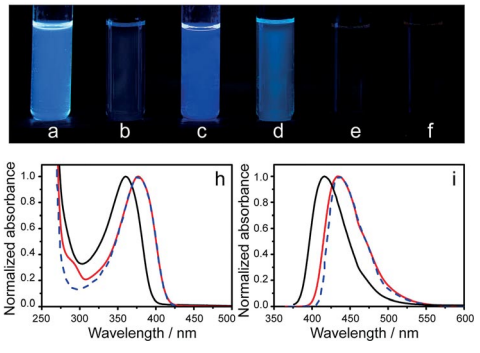


Figure 3. Fluorescence of potential emitters in DMF. (a) Compound **5** with DBU, (b) compound **5** without DBU, (c) fluorosalicylamide **12** with DBU, (d) fluorosalicylamide **12** without DBU, (e) isolated product **8** with DBU, and (f) isolated product **8** without DBU. (h) Absorption and (i) fluorescence spectra of 4-fluorosalicylamide **12** with DBU (black), compound **5** with DBU (blue), and the spent solution after decay of the chemiluminescence (red). The solutions were excited at their specific maximum absorption wavelength.

Except for a spectral shift, the absorption and fluorescence spectra of solution of **12** and the spent solution had similar appearance and identical Stokes shifts. These results were used to establish and calibrate a quantum calculation protocol for compound **12** (see Computations Section in the Supporting Information). The absorption and emission transitions of different isomers of **12** in MeCN were computed at the DFT B3LYP/6-31+G(d,p) level of theory. The strongly basic conditions in the experiments imply that the luminophore should exist as an anion. Besides, the theoretical results for the neutral conformers do not match the experimental data (Table S2). Calculations of the charged (–1) compound resulted in most stable anion form **12m** (Table S2), with λ_{abs} = 340 nm and λ_{em} = 405 nm, both values deviating by only 20 to 10 nm from the respective experimental values. The small magnitude of the blueshift (<0.1 eV) confirmed the reliability of the used DFT method for this system. The components in the solution after the CL decay were established by a combined experimental and theoretical approach. O-Protonated tautomer **10** was excluded on the basis of 2D-NOESY NMR spectroscopy (see the Supporting Information). The calculations were consistent with this result on the basis of the prohibitively high energy gap between **8** and **10** (ΔE = 73.4 kJ mol^{-1} in vacuo). Deprotonation of **8**, facilitated by base, results in **9**. Electronic calculations on deprotonated forms **9** showed that the negative charge is completely delocalized over the heteroatoms of the molecule (Figure S3). Three resonance structures are drawn in Scheme 2. Compound **10** and deprotonated basic form **9** are hetero-analogous to 4-hydroxycoumarin. These, in turn, are well known for their intense blue fluorescence, especially at basic pH values.[25] Therefore, **9** is a viable candidate as a fluorescent species. However, DFT calculations were consistent with the fact that the experimental fluorescence of anion **9** is nondetectable (Table S5, Figure 3, e).

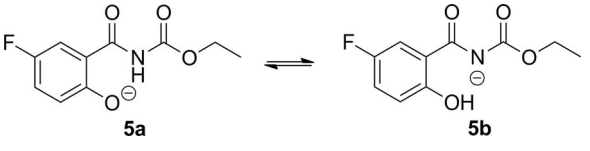
Furthermore, we considered the possibility that the fluorescence was generated by open-chain carbaminic acid **11**. As mentioned above, this compound is not very stable and decomposes rapidly to **12** or cyclizes to **9**. The computed Gibbs free energies of these two reactions are in favor of decomposition to **12** ($\Delta_r G$ = –50 kJ mol^{-1}) and **9** ($\Delta_r G$ = –25 kJ mol^{-1}). The DFT- and CASPT2-calculated absorption and emission wavelengths of **11** are remarkably close to the experimental results obtained on the spent solution (Table 1). Therefore, open-form **11**, which coexists with nondetectable fluorescent (Figure 2, e,f) cyclic form **9**, is capable of fluorescence.

Table 1. Experimental and calculated wavelength maxima for the most stable conformations of compounds **5** (conformer **5a**) and **11**.

	λ [nm]			
	Spent solution[a]	**5** (DFT)[b]	**5** (CASPT2)[c]	**11** (DFT)[b]
Absorption	378	367	374	369
Fluorescence	434	429	423	434

[a] For experimental details, see the Experimental Section in the Supporting Information. [b] B3LYP/6-31+G(d,p) with polarized continuum model (PCM) MeCN solvation. [c] CASPT2/ANO-RCC-VTZP in vacuo.

Compound **5*** (with R^1 = F and R^2 = Et in Scheme 1, Table S5, and excited ester form of **11**) appears as the most viable emitter in the CL reaction. The *O*-deprotonated form **5a** is stabilized by about 9.65 kJ mol^{-1} relative to *N*-deprotonated form **5b** (Scheme 3, Table S6, **5a** is the protonated structure drawn in Scheme 1). The spectral wavelength of this structure at the DFT and CASPT2 levels is reported in Table 1. We then focused on



Scheme 3. Different structures and isomers of compound **5**.

the synthesis of compound **5**. Initial attempts indicated that synthetic **5** is unstable in solution and decomposes quickly to final product **8**. Therefore, all subsequent procedures were performed below 5 °C and in the absence of light. The recorded absorption and emission spectra of deprotonated synthetic **5** correspond to those recorded from a spent solution (Figure 2) and are consistent with the predicted theoretical results. This finding is in agreement with the reaction path: v→vi→vii.

On the basis of these two arguments, we conclude that the emitter of the CL reaction of 2-coumaranone **7** is form **5a** of compound **5**.

How can this study be a model for bioluminescent systems? First, the present chemiluminescent system presents the formation and decomposition of the dioxetanone moiety, as in most bioluminescent systems, and all insight into such a decomposition can be relevant for bioluminescent systems. Second, emitter **5** may also be structurally related to the newly discovered *Fridericia heliota* oxyluciferin.[7] The latter has two moieties involved in bioluminescence: the lysine part, at which formation of dioxetanone and release of CO_2 occur, and a chromophore (CompX). The conjugated system of CompX is very similar to the herein-studied 2-coumaranone systems (see Figure S10). Thus, the present chemiluminescence system may be used to understand the role of the bioluminescent CompX moiety.

Conclusions

In summary, by using a combined experimental and computational approach, a fundamental step in the mechanism of a class of strongly chemiluminescent 2-coumaranones was herein clarified. The key intermediates were identified on the basis of direct comparison with their synthetic analogues, and conclusions were aided by computational assessment of the viable isomers. Both the experimental and computational results were consistent and showed that the open form was the light emitter, not the closed form, in the chemiluminescence of 2-coumaranones. The open form may be structurally related to the chromophore (CompX) of the *Fridericia heliota* bioluminescence system and provides a convenient model for the latter, which is difficult to access by experimental methods. The results presented herein highlight the successful utility of complementary approaches in resolving nontrivial mechanistic pathways. Further calculations on the formation and opening of the dioxetanone will provide additional information on this fundamental reaction in bio- and chemiluminescent systems.

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Keywords: Luminescence · Bioluminescence · Photochemistry · Reaction mechanisms · Oxygen heterocycles · Natural products

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