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Photoinduced Dynamics of Oxyluciferin Analogues: Unusual Enol "Super"photoacidity and Evidence for Keto–Enol Isomerization

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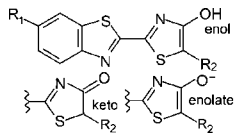
Supporting Information

ABSTRACT: The first systematic pico-nanosecond time-resolved spectroscopic study of the firefly emitter oxyluciferin and two of its chemically modified analogues revealed that in the excited state the enol group is more acidic than the phenol group. The 6′-dehydroxylated derivative, in which only the 4-enolic hydroxyl proton is acidic, has an experimentally determined pK_a* of 0.9 in dimethyl sulfoxide and an estimated pK_a* of −0.3 in water. Moreover, this compound provided direct evidence that in a nonpolar, basic environment the keto form in the excited state can tautomerize into the enol, which subsequently undergoes excited-state proton transfer (ESPT) to produce enolate ion. This observation presents the first experimental evidence of excited-state keto–enol tautomerization of a firefly fluorophore, and it could be important in resolving the enol–keto conundrum related to the color-tuning mechanism of firefly bioluminescence. The 6′-dehydroxylated form of oxyluciferin adds a very rare case of a stable enol to the family of "super"photoacids.

The bioluminescence (BL) reaction catalyzed by firefly luciferase (**Luc**)[1] holds the record for the emission quantum yield[2] of BL reactions with exogenous substrates, which from all natural BL reactions is second only to the yield of Green Fluorescent Protein.[2] The utility of this distinctive property for rapid trace detection of adenosine triphosphate (ATP) in bacterial contamination assays has long been realized.[3] Current bioanalytical applications include gene regulation and expression,[4,5] multicolor in vivo bioluminescence imaging,[6,7] BL microbiological analysis,[8] and BL enzyme immunoassays.[9] Future prospects that are being exploited, such as bioluminogenic substrate conjugates,[10] bioluminescence resonance energy transfer (BRET) in luciferase–protein dyads,[11] fluorescence resonance energy transfer (FRET)-based imaging with luciferase–quantum dot conjugates,[12,13] and sequential BRET–FRET,[14] have triggered ample research efforts, both experimental and theoretical,[15] aimed at better understanding and optimizing the firefly BL reaction. A four-step reaction sequence is commonly accepted as the mechanism of firefly BL,[1] initiated by adenylation of the substrate, D-(−)-luciferin (**LH_2**), with ATP in the **Luc** active pocket, followed by oxidation and chemiexcitation[16] to generate the first excited state of the emitter, oxyluciferin (**$OxyLH_2$**, Scheme 1), which then deexcites, giving the impressive bursts of green-yellow light communicated by fireflies.

Scheme 1. Structures of the Enol, Keto, and Enolate Forms of Firefly Oxyluciferin ($OxyLH_2$) and Its Synthetic Analogues



	R_1	R_2
HOxyLH	H	H
$OxyLH_2$	OH	H
$MOxyLH_2$	OH	CH_3

Despite being of critical relevance for enhancing its bioanalytical performance, two key aspects of this BL system have long remained a subject of speculation and dispute.[17] First, the exact chemical identity of **Luc**-complexed **$OxyLH_2$**,[18] which at various stages of the BL reaction sequence and under various conditions can exist in one of six chemical forms [partially listed in Table 1 and fully presented in Chart S1 in the Supporting Information (SI)], has not been determined yet. A second thoroughly debated yet unresolved issue is the molecular origin of natural or point-mutation-induced energy modulation of the **Luc** emission. Including some natural orange-emitting species of

Table 1. Ground-State Chemical Species of the Three Fluorophores in Various Solvents[a]

	solvent		
	DMSO	H_2O	toluene/DBU
HOxyLH	enol	enol	keto
			enolate
$OxyLH_2$	phenol–enol	phenol–enol	phenolate–keto
		phenolate–enol	phenolate–enol
			phenolate–enolate[b]
$MOxyLH_2$	phenol–enol	phenol–enol	phenolate–keto
			phenolate–enol
			phenolate–enolate[b]

[a]Based on ^{1}H NMR and UV–vis data. [b]Minor contributor.

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click beetles, red-emitting railroad worms, and fireflies,[19,20] wild-type and mutant luciferases can utilize identical BL reactions and fluorophores to produce light within the 530−640 nm range. In the absence of direct experimental evidence on the excited-state structure of the emitter, hypotheses regarding both the energy variation and the chemical form of the fluorophore have been advanced and disputed on the basis of the ground-state structures, model spectrochemistry, and computational results.

Some of us have recently pointed out[17a] that *dynamic* aspects of the excited state of the emitter could be critically important in explaining the key events in firefly BL emission, but this assumption has yet to be experimentally supported. Previously, on the basis of steady-state emission results, differences in the excited-state photoacidities of **OxyLH$_2$** and its derivatives were noted,[17] although a mechanism for that observation could not be proposed. Intrigued by this result, here we employed the natural **Luc** fluorophore (**OxyLH$_2$**) together with two unnatural synthetic derivatives (Scheme 1) in a systematic study of the effects of the medium on their dynamic photochemical behavior. A comparative pico-nanosecond time-resolved photophysical study of three molecules—the parent molecule **OxyLH$_2$**; **HOxyLH**, a fluorophore that lacks one of the two proton-generating hydroxyl groups; and **MOxyLH$_2$**, which is supplemented with a methyl group for increased stability and could shed light on substitution effects[17a]—provided direct evidence of the fate of the excited emitter. The selection of the time window in our study (40 ps−50 ns) was based on the relevance of this time scale for the natural bioluminescence (the emitter in the luciferase pocket deexcites on the nanosecond scale). This first systematic and comparative study of the time-resolved dynamics revealed an unexpected excited-state acid−base behavior of **OxyLH$_2$** and its derivatives and a previously unnoticed photoacidity of the enol group. These phenomena indicate new viable routes in the deexcitation of the emitter and are likely to become of critical importance for complete understanding of the mechanisms underlying firefly BL.

In the natural BL, deexcitation from the two possible charge-neutral forms of **OxyLH$_2$**, phenol−keto and phenol−enol (see the structure in Scheme 1) has been discredited as main emission event on the basis of the prohibitively high energy gap, which would result in blue emission; such blue emission has not yet been observed with natural firefly **Luc**.[17] The natural emitter, **OxyLH$_2$**, is a photoacid with an estimated pK_a* of −0.5.[21] The lack of excited-state proton transfer (ESPT) in methanol[17,21] as a good test for "super"photoacidity (pK_a* < 0),[22] however, indicates that its excited-state acidity was overestimated. Nevertheless, ESPT from doubly protonated (neutral) (**OxyLH$_2$**)* in aqueous solvents produced by photoexcitation in model solutions would normally be ascribed to deprotonation of the phenol group.[21] The recent results on the model photochemistry of the chemical precursor **LH$_2$** were treated in a similar manner.[23] Thus, removal of the phenol hydroxyl functionality should suppress the ESPT and stimulate emission from the neutral molecule.

To our surprise, instead of complete suppression of the ESPT relative to (**OxyLH$_2$**)*, with (**HOxyLH**)* we observed strong emission at 612 nm from (**HOxyL$^-$**)* as clear evidence of ESPT from the *enol* hydroxyl group in dimethyl sulfoxide (DMSO) (Figure 1). Moreover, (**HOxyLH**)* is a *stronger* photoacid than both (**OxyLH$_2$**)* and (**MOxyLH$_2$**)*, providing evidence that the ESPT reactivity of (**OxyLH$_2$**)*, which was previously associated with phenol photodissociation, has been misinterpreted. Further support for our hypothesis of the stronger photoacidity of the

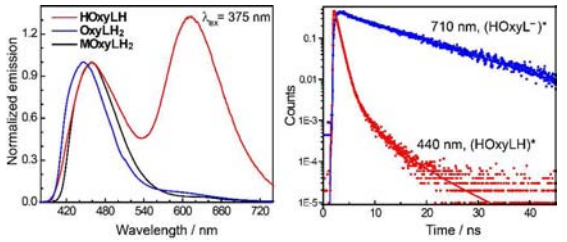

Figure 1. Left: Normalized emission spectra of oxyluciferin and its synthetic analogues in DMSO. Right: Time-resolved fluorescence decays of (**HOxyLH**)* and (**HOxyL$^-$**)* in DMSO (λ_{ex} = 372 nm) and the respective fits obtained using the SSDP approach.[26−28]

enol group relative to the phenol group was obtained using two selectively O-methylated derivatives of **OxyLH$_2$**: whereas methylation of the phenol group resulted in emission from the enolate ion (540 nm), methylation of the enol resulted in emission from the neutral species (455 nm).[24] The decreasing photoacidity in the series **HOxyLH** > **OxyLH$_2$** > **MOxyLH$_2$** can be explained as a cumulative effect from electron-donating groups (hydroxyl and methyl) to the chromophore, reducing its overall acidity for enolic protolytic photodissociation. Although the rise times in water could not be determined with the resolution of our setup, the time scale in DMSO (and other solvents) is evident from Figure 1. It is noteworthy that hydroxyaromatic photoacids are usually based on phenolic compounds, and similar examples of nontraditional photoacids are extremely rare. **HOxyLH** presents a very rare example of photoacidity of a stable enol group in a five-membered ring, which is even more surprising considering that, although not unknown, stable enols are rather exceptional.[25]

Previously, some of us successfully described ESPT of various "super"photoacids[26,27] within the spherically symmetric diffusion problem (SSDP) approach[28] by fitting the nonexponential fluorescence decays of the conjugate acids and bases to the numerical solution of the coupled Debye−Smoluchowski equations. Being devoid of the phenol group, **HOxyLH** is an excellent model for studying the enol photochemistry of **OxyLH$_2$**. Figure 1 shows the fluorescence decay curves of **HOxyLH** in DMSO monitored at wavelengths selected for the emission of the neutral form [(**HOxyLH**)*, λ_{em} = 440 nm] and the ion [(**HOxyL$^-$**)*, λ_{em} = 710 nm], and Table 2 contains the

Table 2. Lifetimes of (HOxyLH)* from Separate Analyses without ESPT (Acetonitrile, Chloroform, Toluene) and with ESPT and/or Tautomerization (Water, Toluene/DBU)

solvent	λ_{em}^{max}/nm[a]	τ_1/ns	τ_2/ns
acetonitrile	440	2.7	4.7
chloroform	440	0.64	1.79
toluene	430	1.17	1.77
water	550	0.37	7.9
toluene/DBU	590, 605	2.4 (590 nm)	7.9 (605 nm)

[a]Approximate maximum of the decay surface measured with a spectral step of 15 nm.

relevant photophysical parameters. The fluorescence decay at 440 nm was nonexponential and consistent with the geminate recombination mechanism (Chart 1).[26−29] The fitted rate constants of the protolytic dissociation and geminate recombination were obtained as k_d = 1.44 ns^{-1} and k_r = 5.9 ns^{-1}, respectively, and the quenching rate constant was found to be k_q

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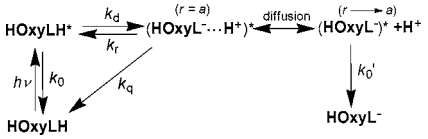
Chart 1. Excited-State Reaction Pathways of the Enol Form of HOxyLH as a Model of $OxyLH_2$ Enol Deexcitation



= 9.5 ns^{-1}. The k_d/k_r ratio results in[26−28] pK_a^* = 0.9 for **HOxyLH*** in DMSO. Accounting for the known pK_a^* difference between water and DMSO for another "super"photoacid (5-cyano-2-naphthol),[26] we estimated pK_a^* = −0.3 in water, which places **HOxyLH** among the "super"photoacids. An analogous analysis of the fluorescence decay was not applicable to **$OxyLH_2$** and **$MOxyLH_2$** because their ESPT in DMSO was extremely weak (Figure 1). The decay of the neutral forms of these fluorophores was monoexponential over more than 3 orders of magnitude, thus reflecting the overall protolytic photodissociation rate.

Further quantification of the excited-state dynamics was accomplished by global and target analysis of the ESPT from the three compounds to solvents of varying polarity. Since the dynamics of these amphotheric compounds depends on their ground-state populations, it was extremely important to know the species existing prior to excitation. By a combination of ^1H NMR and UV/vis-absorption spectroscopies it has been previously established[17] that in all polar solvents or in the presence of even small amounts of polar solvents or hydrogen-bond acceptors, all of these compounds exist in solution as enols that are hydrogen-bonded to the solvent or dimerized at higher concentrations (Table 1). Only in nonpolar solvents in the presence of base could we detect a significant fraction of the ionic keto tautomer, supposedly identical with the BL-emitting form, in equilibrium with the neutral phenol−keto form. Thus, water, DMSO, and a mixture of toluene and 1,8-diazabicyclo[5.4.0]-undec-7-ene (DBU) (0.3 M) were selected for profiling the excited-state dynamics across various polarities. Global analysis was applied to the data in two ways: First, the data were fitted to a parallel model in which n_{comp} excited species decay mono-exponentially in parallel, with n_{comp} rate constants k_i (e.g., see **$OxyLH_2$** in water in Figures S1 and S2 in the SI). In this procedure, the spectral shapes were left unconstrained, yielding decay-associated spectra (DAS) (Figure S2). Second, the data were fitted to a full compartmental scheme (the target model) including all possible branching routes and equilibria between compartments. This allowed estimation of the spectra of each of the excited species, that is, species-associated spectra (SAS).[30,31]

The steady-state emission showed[17] that in nonbasic solvents (acetonitrile, chloroform, toluene), **HOxyLH** does not undergo ESPT. Nevertheless, the fluorescence decay in each of these solvents was nonexponential, indicating conformational processes of the excited fluorophore (Table 2). Global analysis revealed two spectrally identical components, confirming the absence of chemical transformations of the photoexcited **HOxyLH** in these solvents.

On the other hand, excitation in aqueous solution at neutral pH, where only the enol form of **HOxyLH** exists, resulted in an ESPT scenario typical of a number of strong photoacids (Chart 1).[26] The emission spectrum consisted of an extremely weak band at 440 nm from the neutral species and a strong band from the ESPT product (enolate) centered at 550 nm (Figure S3). The decay kinetics at 440 nm was below the time resolution of our time-correlated single-photon counting setup (40 ps). The decay at 550 nm was biexponential with lifetimes of 0.37 and 7.9 ns (Table 2). As with other photoacids, the nonexponential behavior of the ESPT product is prescribed to efficient diabatic geminate proton recombination.[27] Importantly, the target analysis could not reveal the time evolution of each decaying species, thus confirming our hypothesis of only one emitter undergoing competitive diabatic and adiabatic geminate recombination.

In contrast to aqueous solutions, in the toluene/DBU mixture the equilibrium between the enolate and keto forms of **HOxyLH** exists in the ground state (Table 1). The emission bands of the enolate and keto forms are very close and overlap with a common maximum at λ_{em} = 601 nm, the keto form having a higher emission energy.[17b] Indeed, the current target analysis of the time-resolved data (SAS in Figure 2) confirmed two closely lying

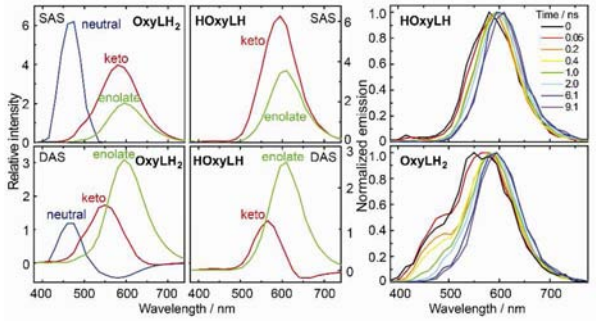


Figure 2. Left: SAS and DAS derived from the target analysis of the fluorescence decay of **$OxyLH_2$** and **HOxyLH** in toluene/DBU. Right: Selected time-dependent emission spectra in toluene/DBU.

bands at 590 nm (keto) and 605 nm (enolate) that decayed with lifetimes of 2.4 and 7.9 ns, respectively. More importantly, the analysis demonstrated slow *photoinduced base-catalyzed enolization*,[32] and the DAS confirmed this spectral interconversion. This result represents the first direct evidence of the enolization of the excited keto form in the **$OxyLH_2$** family. This process must involve keto−enol tautomerization and subsequent ESPT from the enol to 0.3 M DBU; since deprotonation of the enol is ultrafast, we believe that the rate-limiting factor is the tautomerization step with a characteristic time of 2.4 ns. Such enolization of the keto form should be considered as a possible reaction pathway for the native emitter in the scarcely polar active pocket of the luciferase, where a basic residue (adenosine monophosphate) approaches the keto/enol group of the emitter.[18]

As mentioned above, **$OxyLH_2$** is much weaker photoacid than **HOxyLH**, an immediate reason being electron donation from the additional hydroxyl group to the conjugated π system. In toluene/DBU, **$OxyLH_2$** behaves similarly to **HOxyLH**, although the weaker photoacidity results in stronger emission from the neutral species (or the contact ion pair) at 460 nm (Chart 2). The latter decayed with τ = 0.59 ns, undergoing ESPT with a rate constant $k'_{ESPT} = (0.59\ ns)^{-1} - (1.77\ ns)^{-1} = 1.1\ ns^{-1}$ (Table 1) and being transformed into the red-shifted band. In the dominating emission band at 550−600 nm, a conversion of the component at 580 nm (τ = 3.0 ns, keto) to a species emitting at 595 nm (τ = 8.5 ns, enolate) was detected (Figure 2 and Figure S5). The ESPT in water was also similar to that of **HOxyLH**, and likewise, it was retarded (Figures S1, S2, and S5−S8). A pronounced kinetic isotope effect (2.3) was detected for the ESPT from the neutral form (Figure S8). In addition to the

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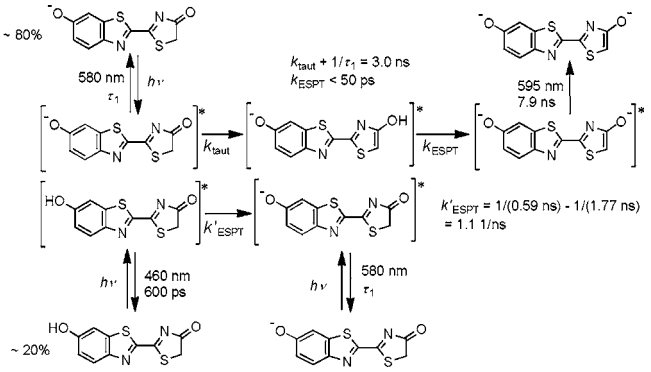
Chart 2. Reaction Pathways of Oxyluciferin in Toluene/DBU



dominating emission from the long-lived phenol−enolate form at 560 nm, a short-lived species (0.53 ns) emitting at 630 nm was observed. We tentatively attribute this emission to the formation of phenolate−enolate species.

In conclusion, using target analysis of the time-resolved pico-nanosecond-scale emission data of firefly oxyluciferin and its synthetic analogues, we have observed a number of unexpected photoinduced phenomena. All of them are associated with the previously ignored prototropic behavior of the enolic moiety of the chromophores, which was found to have a much smaller pK_a* than the phenol group. First, for the first time we have demonstrated an unusual enhancement of the photoacidity of the stable enolic group in a synthetic oxyluciferin derivative. Our results indicate that the protolytic photodissociation of the native emitter, oxyluciferin, is related to the enol rather than the phenol reactivity. Second, we have demonstrated relatively slow photoinduced keto−enol tautomerization in nonpolar solvents in presence of an external base. In terms of the BL mechanism, this could imply that although the emitter is allegedly produced as the keto tautomer, it can undergo excited-state tautomerization and subsequent deprotonation to emit from its enol or enolate forms. A detailed study of the related photophysical processes is now underway.

■ ASSOCIATED CONTENT

Supporting Information

Full scheme of equilibria in **OxyLH$_2$** and details of global and target analysis performed with Glotaran. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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