

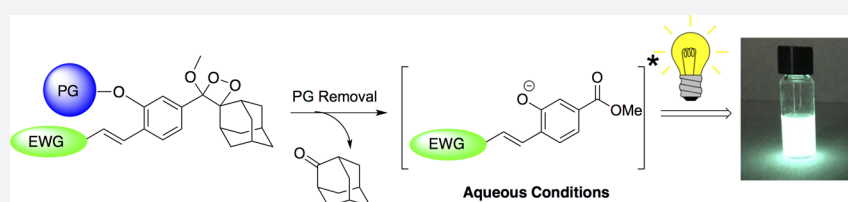
# Opening a Gateway for Chemiluminescence Cell Imaging: Distinctive Methodology for Design of Bright Chemiluminescent Dioxetane Probes

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## Supporting Information



**ABSTRACT:** Chemiluminescence probes are considered to be among the most sensitive diagnostic tools that provide high signal-to-noise ratio for various applications such as DNA detection and immunoassays. We have developed a new molecular methodology to design and foresee light-emission properties of turn-ON chemiluminescence dioxetane probes suitable for use under physiological conditions. The methodology is based on incorporation of a substituent on the benzoate species obtained during the chemiexcitation pathway of Schaap's adamantylidene–dioxetane probe. The substituent effect was initially evaluated on the fluorescence emission generated by the benzoate species and then on the chemiluminescence of the dioxetane luminophores. A striking substituent effect on the chemiluminescence efficiency of the probes was obtained when acrylate and acrylonitrile electron-withdrawing groups were installed. The chemiluminescence quantum yield of the best probe was more than 3 orders of magnitude higher than that of a standard, commercially available adamantylidene–dioxetane probe. These are the most powerful chemiluminescence dioxetane probes synthesized to date that are suitable for use under aqueous conditions. One of our probes was capable of providing high-quality chemiluminescence cell images based on endogenous activity of  $\beta$ -galactosidase. This is the first demonstration of cell imaging achieved by a non-luciferin small-molecule probe with direct chemiluminescence mode of emission. We anticipate that the strategy presented here will lead to development of efficient chemiluminescence probes for various applications in the field of sensing and imaging.

## INTRODUCTION

Chemiluminescence assays are among the most sensitive methods for determination of enzyme activity and analyte concentrations due to their high signal-to-noise ratio.<sup>1</sup> Hence, chemiluminescence probes are utilized in a broad range of analytical applications such as immunoassays and assays involving DNA.<sup>2</sup> Most chemiluminescence probes produce light emission following reaction with an oxidizing agent. Such probes usually undergo an oxidation step to form an unstable strained peroxide, which rapidly decomposes to generate an emissive species in its excited state that decays to its ground state through emission of light. The oxidation-based mechanism is utilized for activation of common chemiluminescence substrates such as luminol<sup>3</sup> and oxalate esters.<sup>4</sup> In addition, oxidation-activated chemiluminescence has been used to detect and image reactive oxygen species (ROS) *in vitro* and *in vivo*.<sup>5–16</sup>

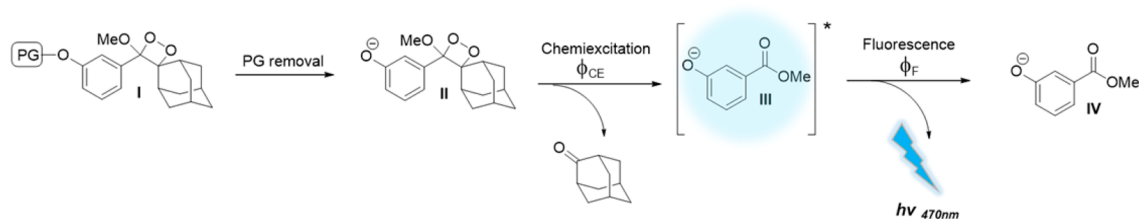
Schaap's adamantylidene–dioxetane (Figure 1, structure I) is the only known chemiluminescence probe that contains a stable dioxetane moiety.<sup>17–19</sup> Therefore, this probe does not require an oxidation step to trigger its chemiluminescence and, thus, can detect a wide range of chemical and biological activities. A

schematic diagram illustrating the adamantylidene–dioxetane chemiluminescence pathway is depicted in Figure 1. Schaap's dioxetane I is equipped with an analyte-responsive protecting group (PG), which is used to mask the phenol moiety of the probe. Removal of the protecting group by the analyte of interest generates an unstable phenolate–dioxetane species II, which decomposes through a chemiexcitation process to produce the excited intermediate benzoate ester III and adamantanone. The excited intermediate decays to its ground state (benzoate ester IV) through an emission of a blue photon.

In order to achieve bright chemiluminescence, the resulting excited species must be an efficient emitter (i.e., it must have high fluorescence quantum yield). Benzoate ester III emits strong light in organic solvents like DMSO, but it is a weakly emissive fluorophore under aqueous conditions.<sup>20</sup> Therefore, the direct chemiluminescence generated by emission of the corresponding dioxetane probe in water is very weak. The weak emission nature of benzoate ester III under aqueous conditions motivated us to

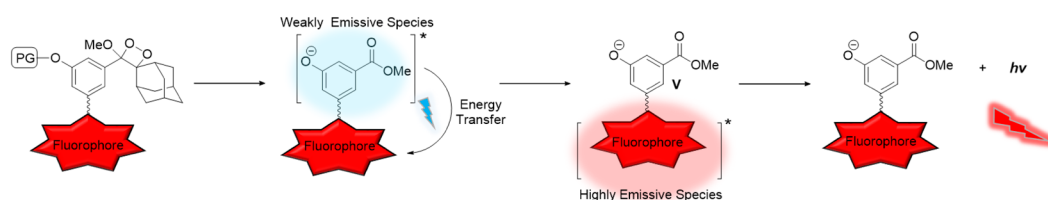
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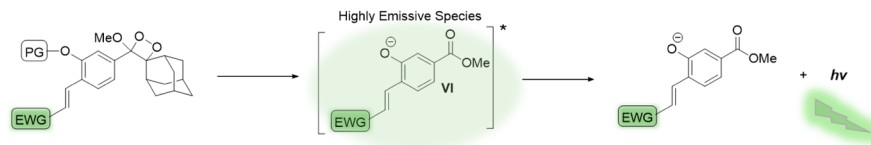


**Figure 1.** Activation pathway of Schaap's dioxetane (PG, protecting group).

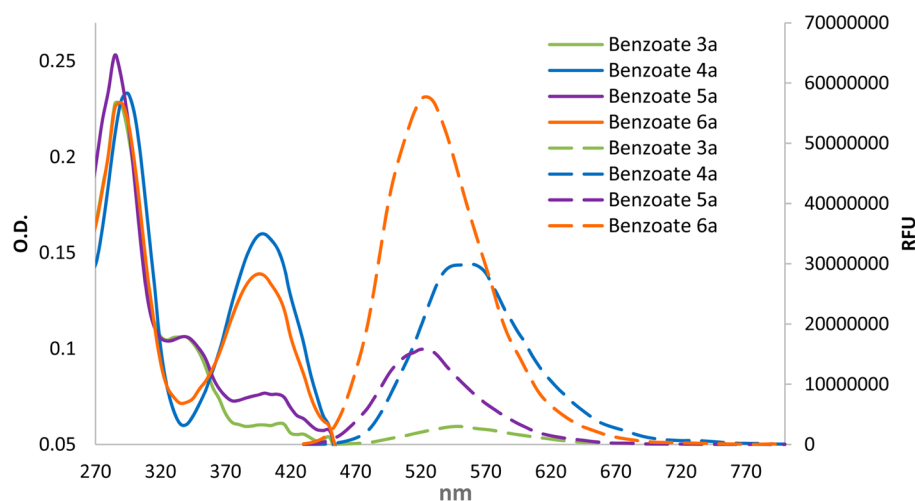
**(A) Previous Work – Indirect Chemiluminescence**



**(B) This Work – Direct Chemiluminescence**



**Figure 2.** (A) In previous work, indirect chemiluminescence amplification was obtained by energy transfer to a fluorogenic dye. (B) Here, direct chemiluminescence amplification was obtained by a substituent effect.



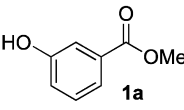
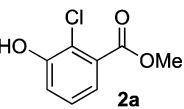
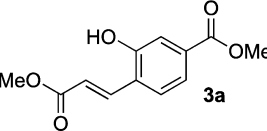
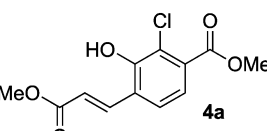
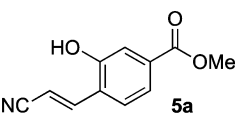
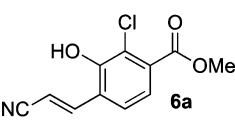
**Figure 3.** Absorbance (solid line) and fluorescence (dashed line) spectra of benzoates 3a, 4a, 5a, and 6a [ $50 \mu\text{M}$ ] in PBS [ $100 \text{ mM}$ ], pH 7.4, 5% DMSO, (excitation wavelength = 400 nm).

search for new methods to amplify its chemiluminescent emission that would enable biological use. We have recently reported a practical synthetic route to adamantylidene-dioxetane conjugated with fluorescent dyes.<sup>21</sup> The dioxetane-fluorophore conjugate decomposes upon its activation to generate the typical excited benzoate. The latter can transfer its energy to the attached fluorophore yielding a highly emissive intermediate (Figure 2A, compound V). The chemiluminescent emission of such conjugates was significantly amplified under physiological conditions through the energy transfer mechanism.

The conjugation to a fluorescent dye resulted in signal amplification through an indirect chemiluminescence pathway.

We reasoned that it should be possible to amplify the chemiluminescence emission through a direct mode of action. In order to achieve direct amplification, the emissive character of the originally formed benzoate had to be improved. We, therefore, sought to introduce an electron-withdrawing group (EWG) at a conjugated position to the phenolate donor of benzoate ester III (Figure 2B). Such a donor-acceptor pair design (VI) should increase the emissive nature the benzoate species.<sup>22</sup> To the best of our knowledge, the influence of a conjugated electron-withdrawing group on the aromatic moiety of Schaap's chemiluminescence probes has not been studied before under physiologically relevant pHs.<sup>23–27</sup> Here we report

Table 1. Spectroscopic Fluorescence Parameters Measured for Selected Phenol–Benzoate Derivatives in PBS, pH 7.4

Phenol-Benzoate	$\lambda_{\text{max}_{\text{ex}}}$ [nm]	$\lambda_{\text{max}_{\text{em}}}$ [nm]	$\epsilon_{400\text{nm}}$	$\Phi_{\text{Fluoresc.}}$	Relative $\Phi_{\text{Fluoresc}}$
 <b>1a</b>	290	470	ND	ND	negligible
 <b>2a</b>	290	470	ND	ND	negligible
 <b>3a</b>	400	540	1200	3.1%	1
 <b>4a</b>	400	540	7400	5.2%	1.7
 <b>5a</b>	400	525	2200	24.5%	7.9
 <b>6a</b>	400	525	7700	29.8%	9.6

an extraordinary enhancement effect of chemiluminescence emission under physiological conditions, which results from distinct substituents installed on Schaap's adamantylidene–dioxetane probe. In addition, by utilizing one of our extremely bright chemiluminescence probes, we were able to obtain chemiluminescence cell images with unprecedented quality.

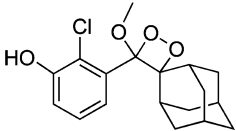
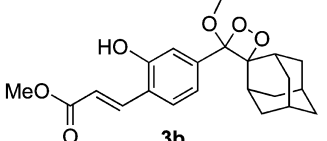
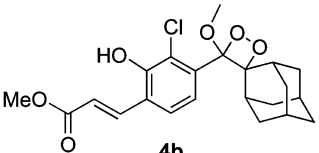
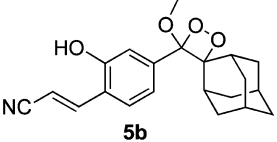
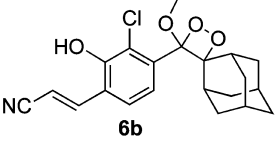
## RESULTS AND DISCUSSION

To evaluate the substituent effect, we synthesized numerous phenol–benzoate derivatives with acceptor substituents at *ortho* and *para* positions of the phenol and measured their fluorescence emission in PBS buffer at pH 7.4 (see the [Supporting Information](#) for synthetic procedures). The most significant effect was obtained when an acceptor was incorporated at the *ortho* position of the phenol. Following a screen of several electron-withdrawing groups, we chose to focus on methyl acrylate and acrylonitrile substituents. In addition, we also examined the effect of incorporation of chlorine substituent at the *ortho* position of the phenol. Chlorine substituent was previously used in Schaap's chemiluminescent probes to reduce the phenol's  $pK_a$ .<sup>28</sup> The reduced  $pK_a$  enriches the relative concentration of the phenolate, thus enhancing the rate of the chemiluminescent decomposition in physiological pH. The absorbance and fluorescence spectra of selected phenol–benzoate derivatives are shown in [Figure 3](#), and their molecular structures and spectroscopic parameters are summarized in [Table 1](#).

The emissive species generated by the chemiexcitation of commercially available adamantylidene–dioxetane probes are eventually the excited states of benzoates **1a** or **2a**. These benzoates do not present any measurable fluorescence under physiological conditions. However, incorporation of methyl acrylate or acrylonitrile substituents at the *ortho* position of the phenol (benzoates **3a** and **5a**) resulted in highly fluorogenic phenol–benzoate derivatives (quantum yields 3.1% and 24.5%, respectively) with maximum emission wavelengths of 540 and 525 nm, respectively. Insertion of an additional chlorine substituent at the other *ortho* position (benzoates **4a** and **6a**) resulted in an increase of the extinction coefficient ( $\lambda = 400$  nm) in comparison to parent benzoates (**3a** and **5a**), and thus enhanced the brightness of the fluorophores. This rise of the extinction coefficient is attributed to the increased concentration of the phenolate species under physiological conditions, produced by the electron-withdrawing effect of the chlorine substituent. However, it did not change the emission wavelength, and also had only minor effect on fluorescence quantum yield.

These results suggest that incorporation of the methyl acrylate and acrylonitrile substituents (with or without the chlorine) in the dioxetane chemiluminescent luminophores could strengthen the emissive nature of the released benzoate. Such a substituent effect would lead to a significant increase in chemiluminescence quantum yield of the dioxetane under physiological conditions. To test this hypothesis, we synthesized five different adamantylidene–dioxetane luminophores (see [Supporting](#)

**Table 2. Molecular Structure and Chemiluminescence Parameters of Adamantylidene–Dioxetane Luminophores with Different *Ortho* Substituents (Luminophores 2b–6b [1  $\mu$ M] in PBS [100 mM], pH 7.4, 5% DMSO, 37 °C)**

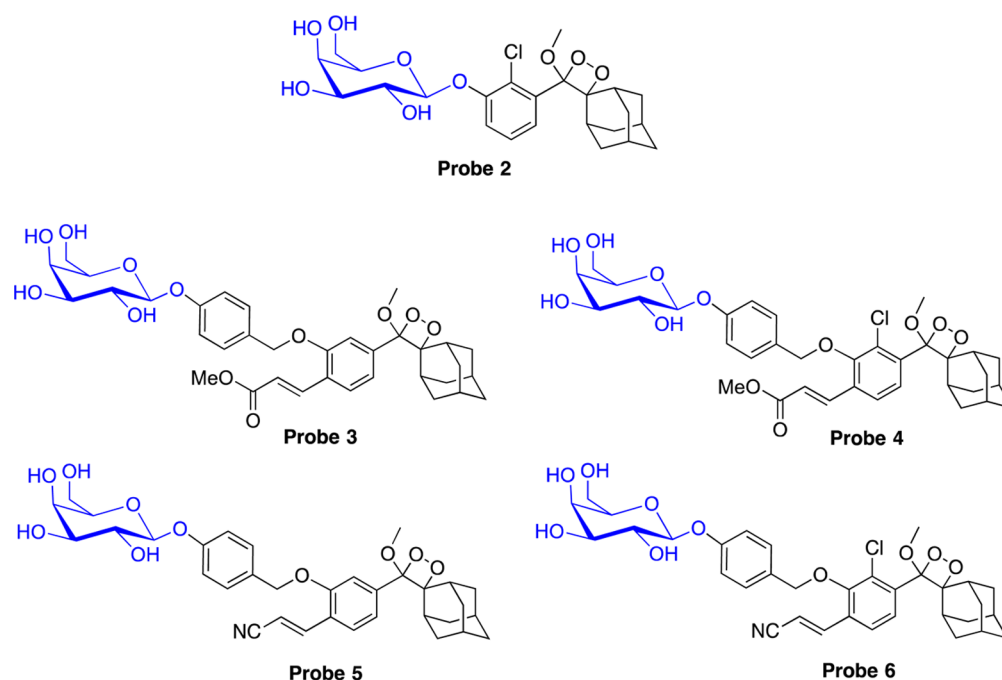
Probe	$\lambda_{\text{maxCL}}$ [nm]	$T_{1/2}$ (min)	Relative CL Emission	$\phi_{\text{CL(H}_2\text{O)}}$ %
 <b>2b</b>	470	17	1	$3.2 \times 10^{-3}$
 <b>3b</b>	540	23	724	2.3
 <b>4b</b>	540	7	780	2.5
 <b>5b</b>	525	22	2295	7.4
 <b>6b</b>	525	10	3043	9.8

Information for synthetic procedures) containing unmasked phenol groups (Table 2). Upon deprotonation of the phenol, the luminophores underwent chemiexcitation decomposition to release the benzoates (Table 1) in their excited state. Next, we measured the chemiluminescence emission spectra and total light emission of the luminophores under physiological conditions. The molecular structure of the dioxetane luminophores and their chemiluminescence parameters are summarized in Table 2. Predictably, the chemiluminescence emission spectra of the dioxetane luminophores overlapped with the fluorescence emission spectra of their corresponding benzoates (Figure 3).

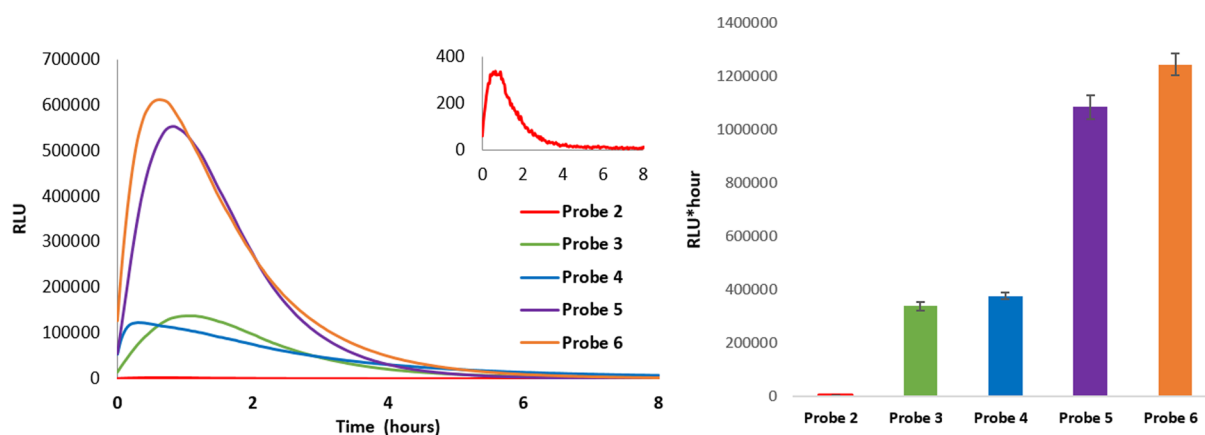
The dioxetane luminophores exhibited chemiluminescent exponential decay kinetic profiles with varied half-lives ( $T_{1/2}$ ; see the Supporting Information for kinetic plots). Dioxetane **2b** was used as a reference compound since its chemiluminescence quantum yield under aqueous conditions is known ( $3.2 \times 10^{-3}\%$ ).<sup>29,30</sup> The chemiluminescence emission of dioxetane **2a** in water was extremely weak; however, dioxetane luminophores **3b**, **4b**, **5b**, and **6b** exhibited remarkably strong chemiluminescence

emission signals upon their deprotonation in PBS, pH 7.4. Luminophore **3b** (with the methyl acrylate substituent) had an emission signal about 700-fold stronger than that of dioxetane **2b**. The chemiluminescence quantum yield of **3b** was 2.3%. Luminophore **4b** (with the methyl acrylate substituent and an additional chlorine substituent) showed a similar signal enhancement with a shorter  $T_{1/2}$  (7 min) relative to luminophore **3b** ( $T_{1/2}$  of 23 min). Luminophore **6b** (with acrylonitrile and an additional chlorine) had a chemiluminescence quantum yield of 9.8% and showed the highest enhancement of chemiluminescence emission, about 3000-fold higher than that of dioxetane **2a**. A similar faster kinetic profile was observed when the chlorine substituent was present on the luminophore ( $T_{1/2}$  of 10 min for probe **6b** vs 22 min for **5b**).

Turn-ON chemiluminescence probes can be simply prepared by masking the phenol functional group of the dioxetane luminophores with an enzyme-responsive substrate. To evaluate this option, we synthesized five different adamantylidene–dioxetane probes (**2–6**) based on dioxetane luminophores **2b–**



**Figure 4.** Molecular structures of chemiluminescence probes for detection of  $\beta$ -galactosidase.



**Figure 5.** (Left) Chemiluminescence kinetic profiles of probes 2, 3, 4, 5, and 6 [ $1 \mu\text{M}$ ] in PBS [ $100 \text{ mM}$ ], pH 7.4, 10% DMSO in the presence of 1.5 units/mL  $\beta$ -galactosidase at room temperature. The inset focuses on the kinetic profile of probe 2. (Right) Total light emitted from each probe.

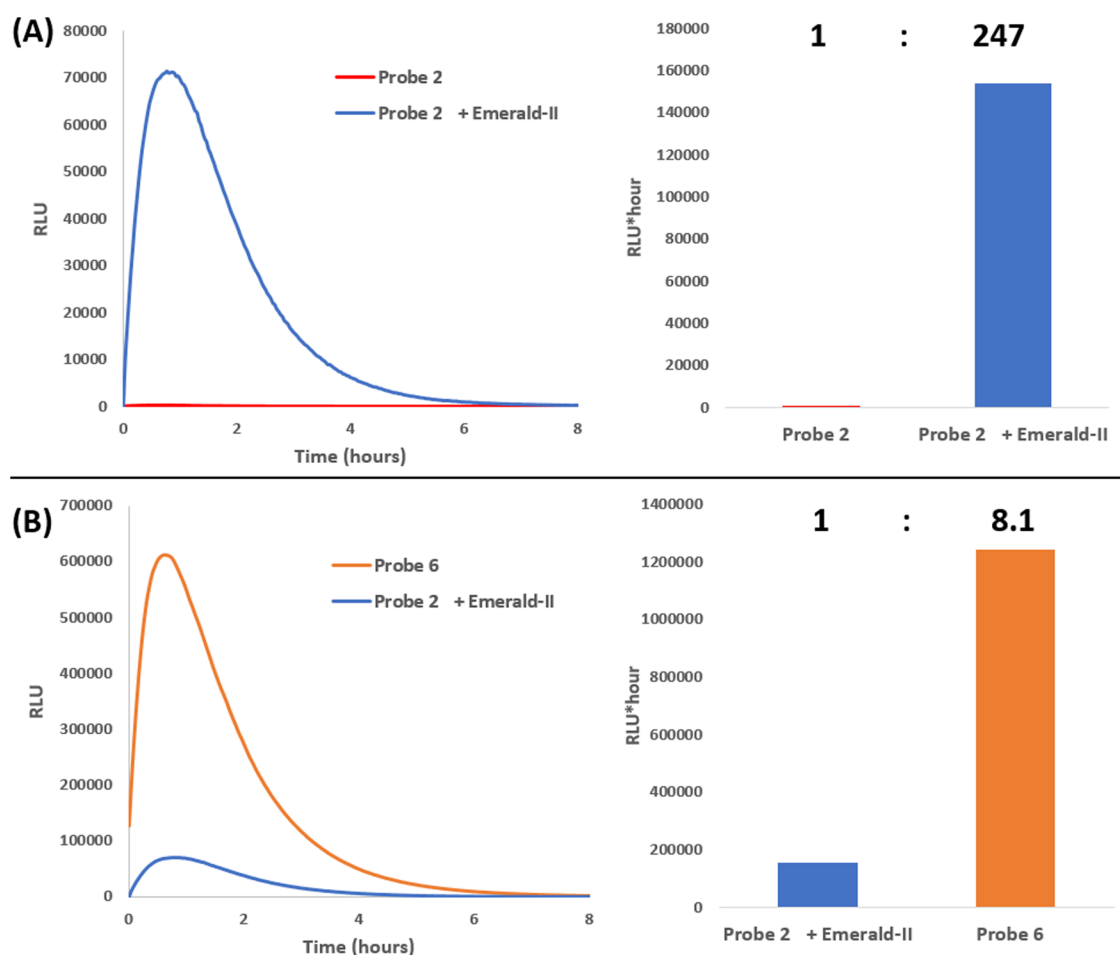
**6b**, where the phenols were masked with a triggering substrate suitable for activation by  $\beta$ -galactosidase (Figure 4; see the Supporting Information for synthetic procedures). To avoid steric interference (as a result of the *ortho* substituent) at the enzyme cleavage site, a short self-immolative spacer was installed between the phenolic oxygen and the galactose substrate as previously described.<sup>31–33</sup> Next, we measured the chemiluminescence emission of the probes, as a function of time, in the presence and in the absence of  $\beta$ -galactosidase. The kinetic profiles of the chemiluminescence signals and their relative emission intensities are shown in Figure 5.

The probes exhibited a typical chemiluminescent kinetic profile in the presence of  $\beta$ -galactosidase with an initial signal increase to a maximum followed by a slow decrease to zero. Probes 3, 4, 5, and 6 exhibited remarkably strong chemiluminescence emission signal under aqueous conditions in the presence of  $\beta$ -galactosidase; however, probe 2 produced extremely weak emission (Figure 5, inset). Probe 3 showed an emission signal about 500-fold stronger than that of probe 2.

Probe 4 (with the chlorine substituent) showed similar signal enhancement with a faster kinetic profile than probe 3 (the deviation in the kinetic profile observed for probe 4 might be attributed to low solubility at room temperature). Probe 6 showed the highest enhancement of chemiluminescence emission: about 1800-fold higher than that of probe 2. No light emission was observed from the probes in the absence of  $\beta$ -galactosidase.

The striking enhancement of chemiluminescence emission obtained by the new dioxetane luminophores encouraged us to compare the signal intensity of probe 6 to that of commercial chemiluminescence assays. There are several commercially available chemiluminescence probes based on the adamantylidene–dioxetane. Since the chemiluminescence emission of these probes is very weak under aqueous conditions, a surfactant–dye adduct (enhancer) is usually added in order to amplify the signal of the assay.<sup>34</sup> The surfactant reduces water-induced quenching by providing a hydrophobic environment for the chemiluminescent reaction that transfers the emitted light to excite the nearby





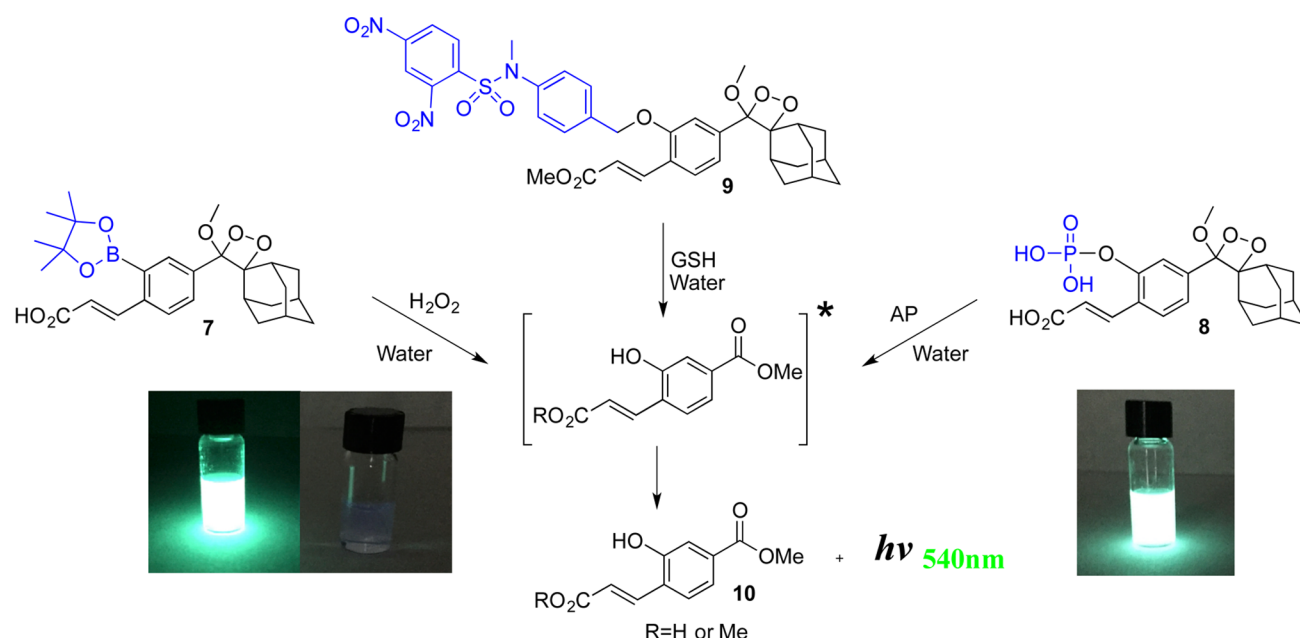
**Figure 6.** (A) Chemiluminescence kinetic profiles of probe 2 [ $1 \mu\text{M}$ ] in the presence of 1.5 units/mL  $\beta$ -galactosidase with and without Emerald-II enhancer (10%) in PBS [100 mM], pH 7.4, 10% DMSO, and count of total light emitted. (B) Chemiluminescence kinetic profiles of probe 2 [ $1 \mu\text{M}$ ] with Emerald-II enhancer [10%] and probe 6 [ $1 \mu\text{M}$ ] in PBS [100 mM], pH 7.4, 10% DMSO, in the presence of 1.5 units/mL  $\beta$ -galactosidase, and count of total light emitted.

fluorogenic dye. Consequently, the low-efficiency luminescence process is amplified significantly in aqueous medium in the presence of surfactant.<sup>35</sup> Commercially available Emerald-II enhancer (10%) was added to probe 2 in the presence of  $\beta$ -galactosidase (in PBS, pH 7.4), and its chemiluminescence emission was compared to that of probe 6 without enhancer. The obtained results are presented in Figure 6. Emerald-II enhancer amplified the chemiluminescence emission of probe 2 by 247-fold (Figure 6A). Remarkably, the chemiluminescence emission signal from probe 6 without enhancer was more than 8-fold stronger than that of probe 2 with the Emerald-II enhancer (Figure 6B). This unprecedented result suggests that a simple small-molecule dioxetane compound like probe 6 can produce chemiluminescence emission that is about an order of magnitude stronger than the signal produced by a two-component system (dioxetane 2 and Emerald-II enhancer). Since our probes produce relatively highly emissive benzoate species under aqueous conditions, addition of the Emerald-II enhancer had only a slight effect on their chemiluminescence emission (see Supporting Information).

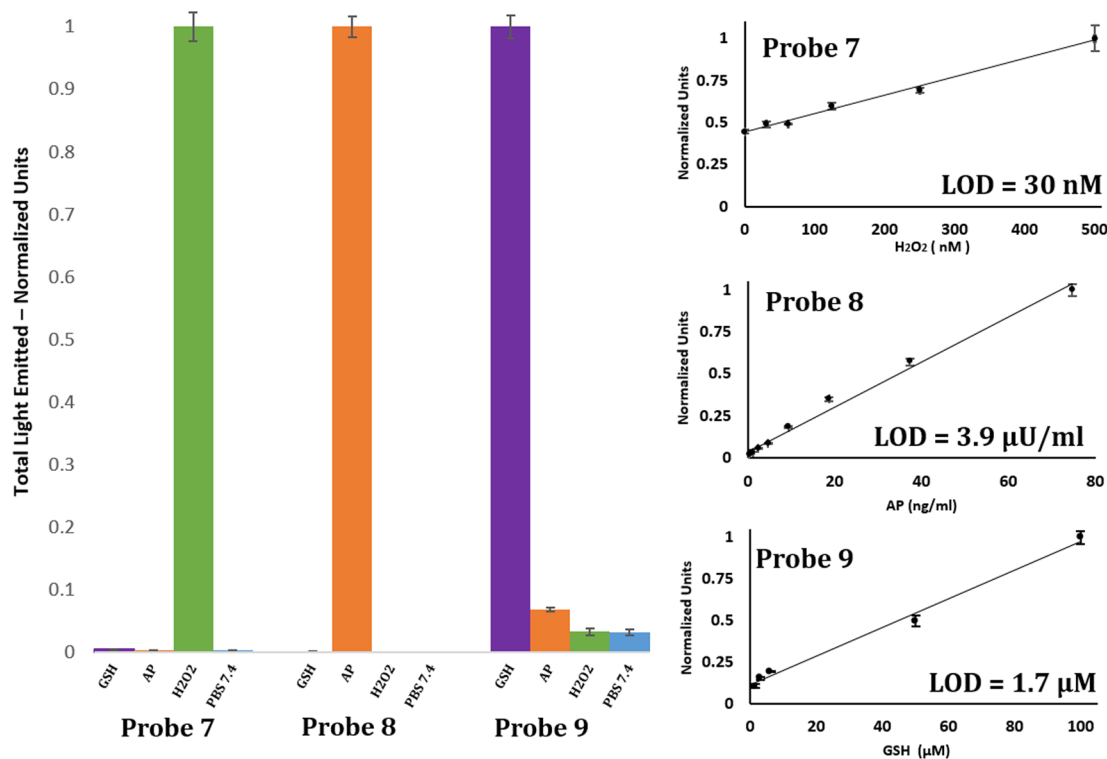
The activation of our chemiluminescence probes is based on removal of a protecting group from the phenolic moiety. Therefore, different phenol protecting groups could be incorporated as triggering substrates for various analytes or enzymes.<sup>36</sup> To demonstrate this modular feature, we synthesized

three additional probes for detection of the analytes hydrogen peroxide<sup>37</sup> and glutathione (GSH) and the enzyme alkaline phosphatase (AP)<sup>35</sup> (see Supporting Information for synthetic procedures). Probe 7 was equipped with a boronic ester substrate for hydrogen peroxide, probe 8 with a phosphate group as a substrate for AP, and probe 9 with dinitro-benzene-sulfonyl group as a substrate for GSH (Figure 7). The probes were prepared with an acrylic acid or methyl acrylate substituent at the *ortho* position of the phenolic oxygen. The presence of an ionizable carboxylic acid group significantly increased the aqueous solubility of probes 7 and 8 and enabled us to conduct evaluations at relatively high concentrations. At a concentration of 1 mM (pH 10), probes 7 and 8 produced bright green luminescence upon reaction with their analyte/enzyme. As described above, the probes decomposed upon activation to release the excited state of the corresponding benzoate. The acrylate substituent efficiently increased the emissive nature of the released benzoate to produce strong light emission, clearly visible to the naked eye (Figure 7). Probe 9 has relatively moderate aqueous solubility with an applicable concentration range between 1 and 10  $\mu\text{M}$ .

To evaluate the sensitivity and selectivity of probes 7, 8, and 9 to detect analyte/enzyme, we determined limits of detection (LOD). The probes exhibited very good selectivity for their analytes of choice under physiological conditions (Figure 8).



**Figure 7.** Water-soluble chemiluminescence probes for detection of hydrogen peroxide (probe 7) and alkaline phosphatase (probe 8) produce visible bright green luminescence under aqueous conditions. Left photograph: Comparison between light emission observed by 1 mM probe 7 (left vial) to that of 1 mM luminol (right vial) upon incubation with hydrogen peroxide under aqueous conditions at pH 10. Probe 9 is a chemiluminescence probe for detection of GSH.



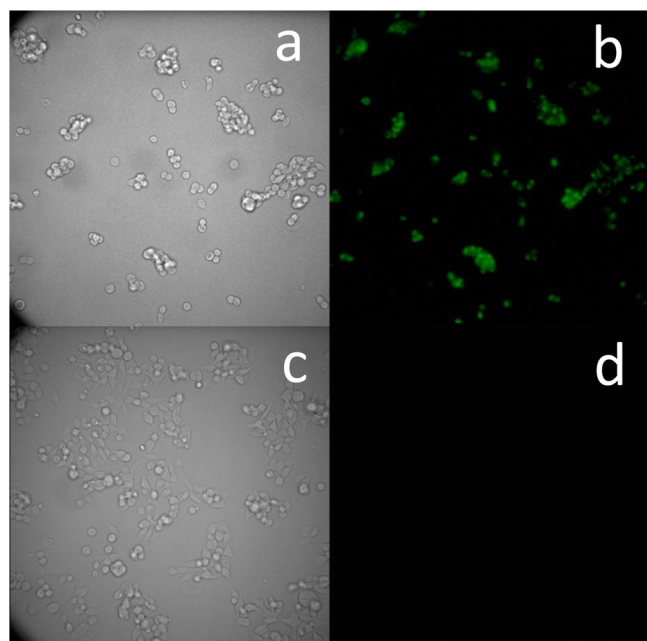
**Figure 8.** (Left) Total light emitted from probe 7 [100  $\mu\text{M}$ ], probe 8 [10  $\mu\text{M}$ ], and probe 9 [10  $\mu\text{M}$ ] in the presence of hydrogen peroxide [1 mM, green], alkaline phosphatase [1.5 EU/mL, orange], and glutathione [1 mM, purple]. Measurements were conducted in PBS [100 mM], pH 7.4, with 10% DMSO at room temperature. (Right) Total light emitted from probe 7 [500  $\mu\text{M}$ ], probe 8 [500  $\mu\text{M}$ ], and probe 9 [10  $\mu\text{M}$ ] in PBS [100 mM], pH 7.4 with 10% DMSO, over a period of 1 h over a range of stimulus concentrations. We determined a detection limit (blank + 3 SD) for each probe.

Probe 7 detected hydrogen peroxide with an LOD value of 30 nM. Probe 8 detected alkaline phosphatase with an LOD of 3.9  $\mu\text{U}/\text{ml}$ , and probe 9 detected GSH with an LOD of 1.7  $\mu\text{M}$ .

Since in chemiluminescence each molecule cannot emit more than one photon, signal intensity in absolute values is often

weaker than fluorescence signals. Therefore, to localize and quantify chemiluminescent and bioluminescent probes at single-cell resolution, a suitable microscope (like LV200 by Olympus) is required. So far, luminescence cell imaging was achieved solely by luciferin and its derivatives. We sought to evaluate the ability of

our probes to image cells overexpressing  $\beta$ -galactosidase using the LV200 microscope. After initial screening for cell permeability, probe 4 was selected for the imaging evaluation. HEK293 cells stably transfected with a vector that encodes *LacZ* gene and HEK293-WT (control) cells were incubated with probe 4 and then imaged using the LV200 (Figure 9). The



**Figure 9.** (a) Transmitted light image and (b) chemiluminescence microscopy image of HEK293-*LacZ* cells. (c) Transmitted light image and (d) chemiluminescence microscopy image of HEK293-WT cells. Images were obtained following 20 min incubation with cell culture medium containing probe 4 (5  $\mu$ M). Images were taken using the LV200 Olympus microscope using a 60 $\times$  objective and 40 s exposure time.

obtained images clearly show that the probe is activated by the expressed  $\beta$ -galactosidase. Probe 4 was able to produce high-quality chemiluminescence images of the HEK293-*LacZ* cells at a 40 s exposure time (Figure 9b). No chemiluminescence signal was observed in the HEK293-WT cells, which do not express  $\beta$ -galactosidase (Figure 9d). Almost 100% of the cell population was imaged by the probe. These are the first chemiluminescence microscopy cell images obtained by a small molecule probe with the direct emission mode that is other than luciferin.

Over the past 30 years or so, numerous examples of chemiluminescence probes based on Schaap's dioxetane have been reported in the literature.<sup>28,38–45</sup> In addition, Matsumoto has also explored other triggerable dioxetane probes with alternative groups in place of the phenols.<sup>46</sup> These probes were designed to release benzoate upon activation; such benzoate is a weakly emissive species under aqueous conditions. In this study, we aimed to redesign Schaap's dioxetane in order to develop chemiluminescence probes that are highly emissive in the biological environment. As explained in Figure 2, the chemiluminescence efficiency of Schaap's dioxetane essentially depends on the emissive nature of the obtained excited-state benzoate. Thus, we assumed that if a substituent increased the benzoate's fluorescent emission in water, it would similarly be able to intensify the chemiluminescence emission of the corresponding dioxetane probe under physiological conditions. Both acrylate and the acrylonitrile substituents at the *ortho*

position of the phenolic oxygen resulted in about 3 orders of magnitude enhancement under aqueous conditions relative to the unsubstituted probe. Interestingly, introduction of the acrylate substituent at the *para* position of the phenolic oxygen resulted in only moderate effects on the fluorescence of the benzoate and on the chemiluminescence of the adamantylidene-dioxetane (see Supporting Information). In our previous dioxetane-fluorophore conjugate probes, we used indirect chemiluminescence mode of action and were able to achieve up to a 100-fold signal amplification.<sup>21</sup> Remarkably, the direct chemiluminescence mode of action has produced probes with signal amplification greater than 1000-fold.

In commercial chemiluminescence assays, signal enhancement is achieved indirectly by energy transfer to a fluorescent dye (confined in micelles formed by an added surfactant). Due to high toxicity, such multicomponent probe systems are not suitable for living cell imaging.<sup>47</sup> Our new probes are composed of a single component, a small molecule with a direct mode of chemiluminescence emission and reasonable aqueous solubility. Such characteristics make these probes ideal for cell imaging applications. Probe 4 was able to provide excellent chemiluminescence cell images based on endogenous  $\beta$ -galactosidase activity. As explained above, in chemiluminescence, signal intensity in absolute values is often weaker than fluorescence signals since each molecule emits only one photon. Thus, only highly efficient bio- or chemiluminescence small molecule probes, like luciferin and its derivatives,<sup>48–50</sup> could be used for cell imaging.<sup>51</sup> As far as we know, in this work we present the first live cell images obtained using a non-luciferin small-molecule-based probe with a direct chemiluminescence mode of emission.

The modular synthetic strategy used enables installation of different triggering substrates on the dioxetane probe, allowing preparation of chemiluminescence probes triggered by various analytes or enzymes of interest. We have demonstrated this option by synthesis and evaluation of new chemiluminescence probes for detection of  $\beta$ -galactosidase, alkaline phosphatase, hydrogen peroxide, and ubiquitous thiols. The high chemiluminescence efficiency observed under aqueous conditions should make these probes ideal substrates for biochemical tests in the field of immunoassays.

## CONCLUSIONS

In summary, we have developed a new molecular methodology to obtain chemiluminescence probes with high emission efficiency under physiological conditions. The methodology is based on incorporation of a substituent on the benzoate species obtained during the chemiexcitation pathway of Schaap's adamantylidene-dioxetane probe. The substituent effect was initially evaluated on the fluorescence emission generated by the benzoate species and then on the chemiluminescence of the dioxetane luminophores. A striking substituent effect on the chemiluminescence efficiency of the probes was obtained when acrylate and acrylonitrile electron-withdrawing groups were installed. The chemiluminescence quantum yield of the best probe was more than 3 orders of magnitude higher than that of the standard commercially available adamantylidene-dioxetane probe. Currently, bio- and chemiluminescence cell imaging is limited to luciferin-related probes. One of our new probes was able to provide high-quality chemiluminescence cell images based on endogenous activity of  $\beta$ -galactosidase. To date, this is the first demonstration of cell-imaging achieved by a non-luciferin small-molecule probe with a direct chemiluminescence mode of emission. We anticipate that the notion presented in this



study will lead to a major transformation in the molecular design of small molecules that function as chemiluminescence-based probes.

## ■ ASSOCIATED CONTENT

### 📄 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscentsci.7b00058.

Synthetic procedures, NMR and MS characterization and spectra, CL kinetic measurements, spectral data, light-stability evaluation, and cell images (PDF)

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### Notes

The authors declare no competing financial interest.

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