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Investigation of the Superoxide Anion-Triggered Chemiluminescence of Coelenterazine Analogs

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Abstract: Reactive oxygen species (ROS), including superoxide anion, are involved in regulating various signaling pathways and are also responsible for oxidative stress. Sensing superoxide anion is of particular importance due to its biological significance. One potential approach is to use Coelenterazine as a chemiluminescent probe for the dynamic sensing of this ROS. In this study, we investigated the superoxide anion-triggered chemiluminescence of native Coelenterazine and two halogenated analogs and found that they showed a ~100-fold enhancement of light emission in aqueous solution, which was significantly reduced in methanol and nonexistent in aprotic solvents. In fact, Coelenterazine showed more intense light emission in aprotic solvents and, interestingly, although the light emission of the analogs seemed relatively unaffected by the solvents, their chemiluminescence was significantly quenched in water compared to methanol and, especially, to aprotic media. This suggests that the quenching effect observed for Coelenterazine is responsible for the differences in aqueous media, rather than an intrinsic enhanced emission by the analogs. In summary, we present Coelenterazine analogs that could serve as a basis for enhanced sensing of superoxide anion, providing information that could further our understanding of this chemiluminescent system.

Keywords: chemiluminescence; Coelenterazine; superoxide anion; luminescence; reactive oxygen species; imidazopyrazinones

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1. Introduction

Reactive oxygen species (ROS) are crucial in the regulation of several signal transduction pathways essential for biological development. They can directly react or modify the structure of transcription factors, genes, and proteins [1–4]. Examples of ROS include singlet oxygen, hydrogen peroxide, hydroxyl radical, and superoxide anion. Superoxide anion is a reduced form of molecular oxygen that consists of 2 oxygen atoms with a negative electrical charge and 17 electrons [3].

Superoxide anion is primarily produced in the respiratory chain and results from the one-electron reduction of molecular oxygen [2,3]. It is also one of the first species generated by various cellular systems [3]. Its production can lead to the formation of different ROS, which can be both damaging and involved in cell signaling [2,3]. Superoxide anion can react with reducing compounds or biomacromolecules, allowing for the control of the expression of target substances and the regulation of associated redox reactions [1]. However, its imbalance can lead to deleterious effects [5]. So, superoxide anion is involved in inter- and intracellular signaling pathways associated with different cellular events [6] and is one of the most important ROS responsible for oxidative stress [3], leading to detrimental health effects such as inflammation and cancer [7–10].

The sensing of superoxide anion is particularly important but its determination is challenging due to its high reactivity and low short-term survival rate [1]. Direct determination can be achieved using electron paramagnetic resonance (EPR) spectroscopy [11,12], which is not suitable for routine analysis [1]. Indirect determination can be achieved through different approaches, including the use of electrochemical and fluorescent (bio)sensors and surface-enhanced Raman spectroscopy (SERS) [1]. Among the possible indirect approaches, the use of chemiluminescent probes has significant potential [13–15].

Chemiluminescence involves the emission of radiation that results from chemiexcitation due to a chemical reaction [16–18]. Chemiluminescent systems do not require photoexcitation for generating the light-emitting excited state, resulting in a reduced probability of autofluorescence from the background signal [19]. Consequently, luminescent signals can be generated with high sensitivity and almost no background noise [18]. This remarkable feature makes chemiluminescent systems ideal for the sensitive, dynamic, real-time, and non-invasive determination of target analytes in intact biological systems [13,20,21].

Marine Coelenterazine (Figure 1) and related imidazopyrazinone-based analogs are considered the most promising chemiluminescent systems for detecting superoxide anion (Scheme 1). They are capable of both chemi- and bioluminescence (in the absence/presence of either luciferase enzymes or photoproteins, respectively) [22–25] and are present in many bioluminescent organisms present in the sea [26]. One of the attractive features of Coelenterazine is that its chemiluminescent reaction can be triggered selectively by superoxide anion, leading to light emission from the chemiexcited chemiluminophore, Coelenteramide (Scheme 1) [13–15,27–30]. Moreover, unlike other chemiluminescent probes such as lucigenin, [31] Coelenterazine and its analogs do not enhance superoxide production, which is an important advantage for the sensing of this ROS species. As a result, researchers have been using them as sensitive and dynamic probes for the detection of superoxide anion [13–15,27–30].

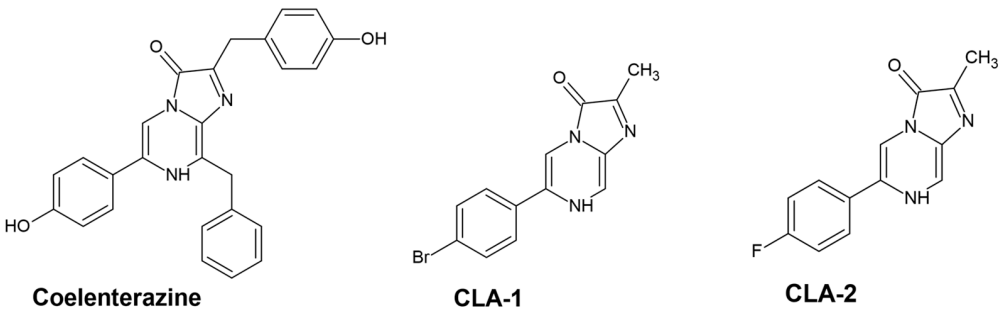
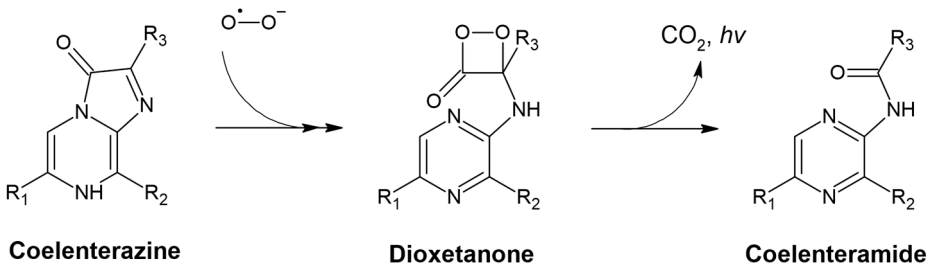


Figure 1. Molecular structures for native Coelenterazine, CLA-1, and CLA-2.



Scheme 1. Schematic representation of the overall superoxide anion-triggered chemiluminescent reaction of Coelenterazine [13–15,27–30]. The interaction between Coelenterazine and superoxide anion ultimately leads to the formation of a high-energy peroxide intermediate, dioxetanone. Its subsequent decomposition yields the light emitter, Coelenteramide, in a singlet excited state that decays to the ground state with the emission of visible light.

In recent years, our group has focused on developing novel Coelenterazine Analogs (CLA) with both new and enhanced properties [32–37]. Among the newly obtained compounds, we discovered that certain mono- and dibrominated CLA species exhibited significantly enhanced superoxide anion-triggered chemiluminescent emission in solution compared to native Coelenterazine and commercial analogs [35–37]. This improved emission is expected to be highly advantageous for the development of novel Coelenterazine-based probes for superoxide anion. However, some doubts regarding these novel compounds must be addressed. For instance, it is essential to understand why this enhancement occurs. The previously reported CLA species all share the common feature of incorporating bromine heteroatoms into their structures [35–37], which are not present in native Coelenterazine. Nevertheless, there is still not enough evidence to guarantee that bromine heteroatoms are a prerequisite for enhanced emission [35–37]. Therefore, assessing how, or if, the chemiluminescence of these compounds is affected by the medium in which they are studied is of critical importance. This can be especially relevant considering recent evidence indicating that the fluorescent quantum yields of the Coelenteramide versions of some CLA species are medium-dependent [38].

In this study, we compare the superoxide anion-triggered chemiluminescence of native Coelenterazine with that of two CLA species (Figure 1). CLA-1 contains an imidazopyrazinone core, with the phenol, benzyl, and *p*-cresol moieties replaced with a bromophenyl moiety, a hydrogen atom, and a methyl group, respectively. CLA-2 is similar to CLA-1 but the phenol is replaced with a fluorophenyl moiety. By comparing these different compounds, we aim to determine the potential effect of bromine heteroatoms on their superoxide anion-triggered emissions and assess any potential influences of their structure on their response to superoxide anion.

2. Materials and Methods

Native Coelenterazine was purchased from NanoLight Technology, dissolved in methanol, and stored at −20 °C. After their synthesis, as described below, stock solutions of CLA-1 and CLA-2 were also prepared in methanol and stored at −20 °C.

The synthesis of CLA compounds followed a general synthetic pathway [32–37], which included an initial Suzuki-Miyaura cross-coupling between 5-bromopyrazin-2-amine and the appropriated organoboranes. This resulted in the formation of an aminopyrazine-based intermediate, which is generally called Coelenteramine. The target CLA was then obtained through condensation of the resulting Coelenteramine with methyl glyoxal.

CLA-1 was then prepared according to the procedures described in [32]. CLA-2 was also synthesized following the same general pathway with some modifications. In short, a solution of methyl glyoxal (1.396 mmol, 1.5 equiv.) and 3-bromo-5-(4-fluorophenyl)pyrazin-2-amine (a fluorinated Coelenteramine analog) (0.931 mmol, 1 equiv) in ethanol (9 mL) was deoxygenated with N_2. It should be noted that the Coelenteramine analog was obtained as described in [36]. The resulting mixture was then cooled to 0 °C, HCl (37%, 3.35 mmol, 3.6 equiv) was added, and the solution was stirred until it reached room temperature. Then, the solution was stirred at 70 °C for 2.5 h and at room temperature overnight. The resulting solution was concentrated under reduced pressure to yield a brown oil, which was redissolved in the minimum amount of ethyl acetate. The solution was then precipitated with diethyl ether and vacuum-dried, resulting in the formation of CLA-2 as an ochre solid with a yield of 94%.

Ice-water and silicon baths were used for reactions at low and high temperatures, respectively, with all reaction temperatures referring to the external bath. Organic extracts were dried over anhydrous Na_2SO_4, filtered, and concentrated using a rotary evaporator (Büchi® Rotavapor® R-210, Büchi® B-491 Heating Bath 120V, KNF Neuberger D-79112 Vacuum Pump N 035.1.2 AN.18). Reactions were monitored by thin-layer chromatography (TLC) using aluminum-backed Merck 60 F254 silica gel plates and *n*-hexanes-ethyl acetate solvent systems. After visualization under ultraviolet light at 254 nm and 365 nm, the plates were developed by immersing them in a solution consisting of a mixture of *p*-anisaldehyde

(2.5%), acetic acid (1%), and sulfuric acid (3.4%) in 95% ethanol, followed by heating. More details regarding the synthesis processes can be found in the Supplementary Materials.

The structure of CLA-2 was confirmed by NMR and FT-MS analysis. NMR spectra were recorded in both acetone-d_6 or methanol-d_4 solutions on a Bruker NMR spectrometer (Bruker Advance III 400 MHz Ascend, 9.4 Tesla). The chemical shifts are reported on the δ scale (ppm) using the residual solvent signals (δ = 2.050 ppm (^{1}H, qu, acetone-d_6), 2.840 (^{1}H, s, acetone-d_6)) or (δ = 3.31 ppm (^{1}H, qu, methanol-d_4), 4.78 ppm (^{1}H, s, methanol-d_4)) as internal standards. The coupling constants (J) are reported in Hz. FT-MS analysis was performed on an LTQ OrbitrapTM XL hybrid mass spectrometer (Thermo Fischer Scientific, Bremen, Germany) controlled by LTQ Tune Plus and Xcalibur 2.1.0. The ^{1}H-NMR and high-resolution mass spectra for CLA-2 are available in the Supplementary Materials.

Chemiluminescent kinetic measurements were performed using a homemade luminometer equipped with a Hamamatsu HC135-01 photomultiplier tube. This setup also included an automatic burette, a sample holder, and a PC for data acquisition. The reactions were performed at least in sextuplicate at room temperature in various media, including methanol, deionized water, and *n*,*n*-dimethylformamide (DMF). Reaction mixtures in water and DMF also contained 1% of sodium acetate buffer pH 5.2. Final volumes of 500 µL and compound concentrations of 5 µM were used for all compounds. Light was integrated and recorded at 0.1 s intervals. In the protic solvent, chemiluminescence was triggered by mixing superoxide anion and either Coelenterazine, CLA-1, or CLA-2. Potassium superoxide was used as the source for superoxide anion, following its previous use in similar applications [35–37,39,40]. The amount of potassium superoxide used was 5 mg. In the aprotic solvents, chemiluminescence was triggered spontaneously by the addition of protic solvent to Coelenterazine/CLA-1/2.

3. Results and Discussion

The first step in this study involved measuring the chemiluminescent kinetic profiles of Coelenterazine, CLA-1, and CLA-2 in a protic solvent. Specifically, the measurements were first performed in methanol using potassium superoxide, which is a commonly used source of superoxide anion, to trigger the reaction [35–37,39,40]. The resulting kinetic profiles, which represent light emission as a function of time, can be seen in Figure 2 (when normalized).

We can see that the chemiluminescent reaction proceeded with a flash-type profile, with a quick burst of light, followed by a rapid decay to basal levels. In fact, all of the studied compounds displayed qualitatively similar chemiluminescent profiles, with the emission of light finishing within the first minute. Nevertheless, the emission lifetime appeared to be shorter for CLA-1/2 than for native Coelenterazine. We attempted to measure chemiluminescence in deionized water but native Coelenterazine had too low a light emission to be measured. It should be mentioned that chemiluminescent reactions typically exhibit lower light-emission intensities in aqueous solution due to energy loss to water molecules [41,42]. Indeed, previous studies have demonstrated that the chemiluminescence of Coelenterazine can be completely quenched in solvent mixtures with water contents as low as 20% [22]. However, it should be noted that this particular study was performed without the addition of superoxide anion as the triggering agent [22].

Studies have shown that the addition of acidic buffer can enhance the chemiluminescence of Coelenterazine and its analogs [43–46]. To investigate this further, we attempted to measure the chemiluminescent emission in deionized water with the addition of acetate buffer pH 5.2 (1%). Remarkably, light emissions were then detected for all the compounds in this aqueous solution (Figure 2), and the results were similar to those observed in methanol. Interestingly, the peaks appeared to be more sharply defined in deionized water/acetate buffer. However, it should be noted that some of the observed features of the chemiluminescent kinetic profiles may not solely result from the intrinsic properties of the studied compounds. Instead, they may be attributed to the behavior of the superoxide

anion, which can be quite unstable in protic solvents, particularly in aqueous solution due to its tendency for spontaneous disproportionation and strong solvation [47,48].

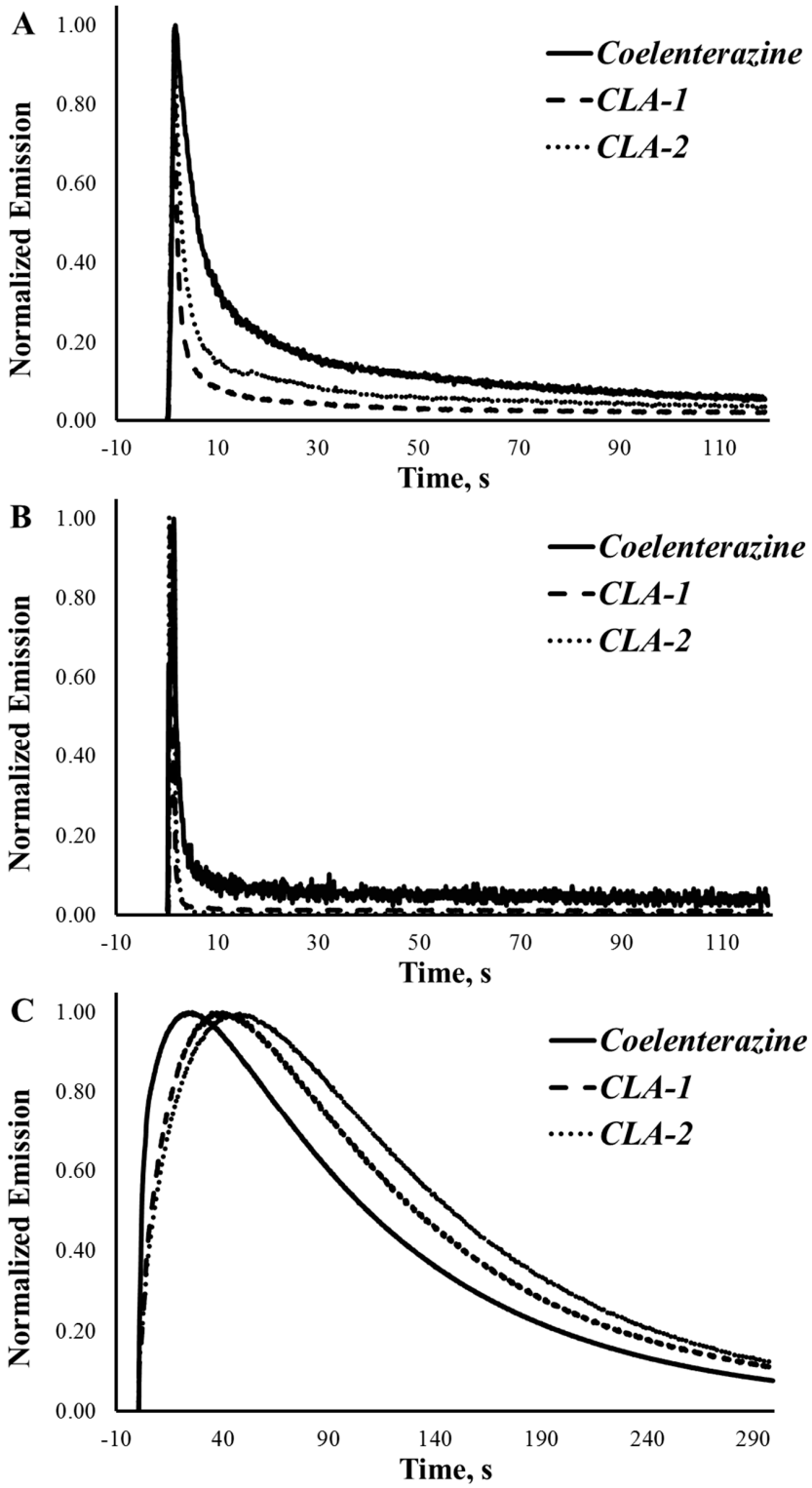


Figure 2. Representative and normalized chemiluminescent emissions as a function of time for Coelenterazine, CLA-1, and CLA-2. The reactions were initiated by adding 5 mg of potassium superoxide to either methanol (**A**) or deionized water/1% acetate buffer pH 5.2 (**B**). Chemiluminescence measured in DMF/1% acetate buffer pH 5.2 (**C**). Final volume was 500 µL, with a concentration of 5 µM for all compounds.

Finally, we measured the chemiluminescence of these compounds in DMF with the addition of acetate buffer pH 5.2 (1%) [35,36,43]. Previous studies have demonstrated that besides being triggered directly by superoxide anion, Coelenterazine and its derivatives can also emit light spontaneously in certain aprotic solvents such as DMF and dimethyl sulfoxide (DMSO) [43–46,49,50]. In these reactions, the chemiluminescent signal is typically more stable than when triggered by superoxide anion in protic media [35–37]. Thus, by studying the chemiluminescence of these compounds in aprotic media, we can better determine their intrinsic properties, without potential interference from the instability of superoxide anion in protic media.

Figure 2 presents the chemiluminescent kinetic profiles of Coelenterazine, CLA-1, and CLA-2 in DMF/acetate buffer pH 5.2 (1%). Although the chemiluminescence still exhibited a somewhat flash-type profile, the light emission persisted significantly longer compared to the reactions in both methanol and deionized water (Figure 2). Specifically, the maximum light emission was only achieved between the first 30 and 60 s of the reaction, and the decay was notably slower, with basal levels only being reached after approximately 5 min. Among the studied compounds, native Coelenterazine exhibited the fastest kinetics, reaching the light-emission maximum plateau faster and showing a faster decay. It was followed by CLA-1 and then CLA-2.

We performed a quantitative analysis by calculating the chemiluminescence enhancement ratio between CLA-1/CLA-2 and native Coelenterazine in the three aforementioned media [35,36] using Equation (1). The results are presented in Table 1.

$$\textit{Chemiluminescence Enhancement Ratio} = \frac{\textit{Light Emission Maxima of either CLA}-1 \textit{ or CLA}-2}{\textit{Light Emission Maxima of Coelenterazine}} \quad (1)$$

Table 1. Measured chemiluminescence enhancement ratios between the chemiluminescent reactions of CLA-1 or CLA-2, and native Coelenterazine. These ratios were obtained using Equation (1) while considering the light-emission maxima in the different solvents. Measurements were performed in either methanol, deionized water/1% acetate buffer pH 5.2, or DMF/1% acetate buffer pH 5.2. In protic solvents, the reactions were triggered by the addition of 5 mg of potassium superoxide. Final volume was 500 µL, with a compound concentration of 5 µM.

Compound	Methanol	Water/Acetate Buffer	DMF/Acetate Buffer
CLA-1	$4.01 \times 10^{0} \pm 5.41 \times 10^{-1}$	$1.09 \times 10^{2} \pm 6.48 \times 10^{1}$	$1.32 \times 10^{-1} \pm 2.97 \times 10^{-2}$
CLA-2	$1.98 \times 10^{0} \pm 1.02 \times 10^{-1}$	$1.05 \times 10^{2} \pm 5.80 \times 10^{1}$	$1.60 \times 10^{-1} \pm 4.33 \times 10^{-2}$

Both CLA compounds presented higher light-emission intensities than native Coelenterazine in methanol, with intensities 2 to 4 times higher. Both CLA compounds showed more enhanced light-emission intensities in aqueous solution that were about 100 times higher than that of Coelenterazine. These results suggest that CLA-1 and CLA-2 have the potential to be used as probes for superoxide anion in biological media, given their significantly enhanced chemiluminescence regarding native Coelenterazine, and following the previous validation of this latter molecule as a probe for this ROS [14,15]. These findings are also consistent with the performance of other CLA compounds we have developed [35–37]. Nevertheless, it should be noted that other CLA compounds have shown even higher emission enhancements than Coelenterazine, with ratios of up to 1.11×10^{4} [35,36]. Moreover, since CLA-1 and CLA-2 have exhibited similar relative light-emission intensities to Coelenterazine, it appears that the introduction of bromine heteroatoms is not a prerequisite for the enhanced emission of these compounds in protic solvents, especially in aqueous solution [35–37]. However, it should be noted that it was previously determined that the Coelenteramide versions of CLA-1 and CLA-2 presented blue-shifted emissions compared to native Coelenteramide [38]. This may limit the suitability of these compounds for certain

biological applications, meaning that further optimization and tuning of their luminescent properties would be beneficial.

Contrary to the observations in protic solvents, the chemiluminescence of CLA-1/2 appeared to be significantly reduced compared to that of native Coelenterazine (Table 1). In other words, the light-emission intensities of these compounds were not enhanced but were instead substantially decreased relative to Coelenterazine. Thus, the enhanced superoxide anion-triggered chemiluminescence of CLA compounds is not an intrinsic feature of these compounds [35–37], as it appears to be dependent on the reaction media.

Given these findings, it is important to investigate the factors that affect the chemiluminescence of these compounds in different media and explain the observed differences in light emission. To this end, we assessed the relative light-emission efficiency of the three compounds in the three reaction media. We then calculated the ratios between the light-emission maxima of the chemiluminescent reaction of each compound in different media using water/acetate buffer as the reference ($\frac{Methanol\ or\ DMF/Acetate\ Buffer}{Water/Acetate\ Bufffer}$). The results are presented in Table 2.

Table 2. Measured ratios between the obtained light-emission maxima of the chemiluminescent reactions of Coelenterazine, CLA-1, and CLA-2 in different media with deionized water/1% acetate buffer pH 5.2 as a reference. Measurements were performed in either methanol, deionized water/1% acetate buffer pH 5.2, or DMF/1% acetate buffer pH 5.2. When in protic solvents, reactions were triggered by the addition of 5 mg of potassium superoxide. Final volume was 500 µL, with a concentration of 5 µM for all compounds.

Compound	$\frac{Methanol}{Water \mid Acetate\ Buffer}$	$\frac{DMF \mid Acetate\ Buffer}{Water \mid Acetate\ Buffer}$
Coelenterazine	$8.98 \times 10^{0} \pm 3.56 \times 10^{0}$	$1.31 \times 10^{3} \pm 3.16 \times 10^{2}$
CLA-1	$6.19 \times 10^{-1} \pm 2.44 \times 10^{-1}$	$2.50 \times 10^{0} \pm 5.07 \times 10^{-1}$
CLA-2	$1.93 \times 10^{-1} \pm 8.22 \times 10^{-2}$	$2.00 \times 10^{0} \pm 4.36 \times 10^{-1}$

CLA-1 and CLA-2 exhibited similar behavior, with higher light-emission intensities observed in water/acetate buffer than in methanol. This could be partly attributed to the addition of acidic buffer in the former scenario [43–46]. In contrast, when switching from protic to aprotic media, the light-emission intensity increased by approximately 2–2.5 times.

However, the results for native Coelenterazine showed a markedly different trend (Table 2). Chemiluminescence was more intense in methanol than in water/acetate buffer by a factor of about 9, which was opposite to the behavior observed for CLA-1 and CLA-2. More importantly, the light-emission intensity maximum of Coelenterazine in DMF/acetate buffer was about 1.31×10^{3} times higher than that in water/acetate buffer, whereas for the two CLA analogs, the increase was only of $2.00–2.50 \times 10^{0}$.

So, the results suggest that although the chemiluminescence of CLA-1/CLA-2 is relatively unaffected by the reaction media, the light emission of Coelenterazine is significantly reduced in water compared to other solvents. Therefore, while the observed outcome is indeed an enhanced emission by CLA-1/CLA-2, it appears to be less of an enhancement and more of an inhibition of the chemiluminescence of Coelenterazine in aqueous solution, which barely affects CLA compounds. As mentioned earlier, chemiluminescent systems typically exhibit lower light-emission intensities in aqueous solution due to the energy loss to water molecules [41,42]. Hence, the reduction in emission intensity for Coelenterazine in water is not unexpected. What may be surprising is that CLA-1/2, as with other similar CLA derivatives [35–37], are relatively unaffected by this quenching effect. Thus, our findings point out the relative insensitivity of CLA-1/CLA-2 to changes in media as the reason behind the enhanced emission in water, in contrast to the sensitivity of Coelenterazine to solvent effects.

It is worth noting that we recently conducted a study on the Coelenteramide versions of both CLA-1 and CLA-2 [38]. Unlike their chemiluminescent reactions, the fluorescence

quantum yields of their Coelenteramide versions were indeed relevantly affected by the solvent. For the CLA-1-based Coelenteramide, the quantum yield ranged from 8 to 17%, with a higher value observed in methanol. On the other hand, the CLA-2-based compound showed a quantum yield range of 12–26%, with a higher value observed in water. These yields were both significantly influenced by the solvent in opposite ways [38], contrary to what was observed for the chemiluminescence emission (Tables 1 and 2). Thus, we can hypothesize that the relative insensitivity of the chemiluminescence emission intensities of these compounds to the reaction media is not related to the fluorescence quantum yield of the resulting chemiluminophore. We expect that these variations in the quantum yield of the Coelenteramide-based species are compensated for by changes in the chemiexcitation yield and/or ground-state chemical reaction for the CLA compounds.

Finally, we measured the chemiluminescence half-life (in seconds, s) of the three compounds in the three reaction conditions, as shown in Table 3. We observed significant differences between the solvents, particularly between the protic and aprotic media. Specifically, the measured half-life values in the aprotic solvents were higher than 1 (for Coelenterazine) and 2 min (for CLA-1 and CLA-2), whereas in the protic solvents, the obtained values ranged from ~1 to ~6 s. The observed longer emission half-life values in the aprotic solvents could be partially explained by the instability of superoxide anion in protic media, especially in aqueous solution [47,48]. In fact, for all three compounds, the half-life values were higher in methanol than in water/acetate buffer. With respect to the differences between the compounds, there was no noticeable variation in the half-life values for reactions in water/acetate buffer. However, in methanol, the emission half-life of Coelenterazine was longer than that of CLA-1 and CLA-2. Conversely, in DMF/acetate buffer, CLA-1/CLA-2 presented longer half-life values than Coelenterazine. Notably, there were no relevant differences between CLA-1 and CLA-2 in the three solvents. Therefore, replacing the bromine heteroatom in CLA-1 with fluorine in CLA-2 did not seem to lead to significant changes in the studied properties of these compounds.

Table 3. Light-emission half-life (in seconds, s) for chemiluminescent reactions of native Coelenterazine, CLA-1, and CLA-2 in different media: methanol, deionized water/1% acetate buffer pH 5.2, or DMF/1% acetate buffer pH 5.2. Reactions were initiated by adding 5 mg of potassium superoxide to the protic solvents. Final volume was 500 μL, with a compound concentration of 5 μM.

Compound	Methanol	Water/Acetate Buffer	DMF/Acetate Buffer
Coelenterazine	6.4 ± 1.3	1.2 ± 0.6	89.2 ± 3.0
CLA-1	2.3 ± 0.2	1.5 ± 0.3	134.0 ± 5.3
CLA-2	2.7 ± 0.2	1.5 ± 0.6	141.9 ± 4.0

4. Conclusions

Reactive oxygen species, or ROS, play an important role in the regulation of different signal transduction pathways required for biological development. Among the most relevant ROS species is superoxide anion, a reduced form of molecular oxygen and a primary product in the respiratory chain. Due to its involvement in inter- and intracellular signaling pathways that are associated with several cellular events, superoxide anion attracts significant biological interest. However, it can also be responsible for oxidative stress and deleterious health effects such as inflammation and cancer.

Given this, the ability to sense superoxide anion is highly relevant but its high reactivity and low short-term survival rate make this task challenging. One promising sensing strategy is the use of chemiluminescent probes such as marine Coelenterazine. Coelenterazine exhibits selectivity toward superoxide anion, which, when added to the high signal-to-noise ratio of chemiluminescent systems, enables dynamic, sensitive, and selective sensing of this ROS.

Here, we performed a comparative study of the superoxide anion-triggered chemiluminescent reaction of native Coelenterazine and two halogenated analogs (bearing either

a bromine or a fluorine heteroatom). Both analogs showed a significant enhancement of superoxide anion-triggered enhanced emission in aqueous solution, with an increase of up to 100 times compared to native Coelenterazine. Therefore, these compounds hold great potential as the foundation for developing novel Coelenterazine-based chemiluminescent probes for sensing superoxide anion in biological media.

However, the difference in emission between the analogs and Coelenterazine varied with the used solvent. In methanol, the analogs only enhanced the superoxide anion-triggered emission by 2 to 4 times. Interestingly, in *n*,*n*-dimethylformamide, in the absence of superoxide anion, the intensity of light emitted by Coelenterazine was significantly higher than that of the analogs. Furthermore, the light-emission intensities of the analogs remained relatively constant across protic and aprotic media, whereas that of Coelenterazine was significantly decreased in aqueous solution. This indicates that the analogs did not show a superoxide anion-triggered enhanced emission, but rather that the chemiluminescent reaction of native Coelenterazine suffered a relevant quenching effect in aqueous solution, which barely affected the analogs. Finally, we also did not observe any significant differences in the studied properties of the analogs that could be attributed to a specific halogen element. Overall, these findings advance our understanding of this important type of chemiluminescent system.

5. Patents

WO20219211808—Chemiluminescent Imidazopyrazinone-Based Photosensitizers with Available Singlet and Triplet Excited States.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app13116617/s1. Figure S1: ^{1}H NMR spectrum (400 MHz, Acetone) for 6-(4-fluorophenyl)-2-methylimidazo [1,2-a]pyrazin-3(7H)-one (CLA-2). δ = 9.31–9.30 (d, J = 1.3 Hz, 1H), 9.26–9.24 (d, J = 1.4 Hz, 1H), 8.24–8.19 (m, 2H), 7.33–7.28 (m, 2H), 2.65–2.53 (s, 3H). Figure S2: ^{1}H NMR spectrum (400 MHz, MeOD) for 6-(4-fluorophenyl)-2-methylimidazo[1,2-a]pyrazin-3(7H)-one (CLA-2). δ = 9.05–9.04 (d, J = 1.4 Hz, 1H), 8.61–8.58 (d, J = 1.4 Hz, 1H), 7.96–7.92 (m, 2H), 7.18–7.11 (m, 2H), 2.94–2.30 (s, 3H). Figure S3: FTMS-ESI (+) spectrum for 6-(4-fluorophenyl)-2-methylimidazo [1,2-a]pyrazin-3(7H)-one (CLA-2), *m*/*z*: calcd for [C13H11FN3O]+: 244.0886 [M + H]$^{+}$; found 244.0886 [C13H11FN3O]$^{+}$. Figure S4: FTMS-ESI (−) spectrum for 6-(4-fluorophenyl)-2-methylimidazo [1,2-a]pyrazin-3(7H)-one (CLA-2), *m*/*z*: calcd for [C13H9FN3O]$^{-}$: 242.0730 [M + H]$^{+}$; found 242.0765 [C13H9FN3O]$^{-}$.

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