Chemiluminescence of 5-(azo-*para*-phenylene-*N*-aza-15-crown-5)-phthalhydrazide

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Abstract

5-(Azo-*para*-phenylene-*N*-aza-15-crown-5)-phthalhydrazide presents weak fluorescence and (in the presence of aqueous phosphate buffer at pH = 10.1 with H_2O_2, with or without Fe^{3+} or Cu^{2+} ions) a significant chemiluminescence (CL) at λ_{CL} = 425 nm. Linear correlations exist between the hydrogen peroxide concentration and the intensity of the CL. For similarly intense chemiluminiscence, in the presence of cupric ions, the H_2O_2 concentration range is ten times lower than in their absence. This observation allows the determination of low concentrations of H_2O_2 and of Cu^{2+} ions.

Keywords: Chemiluminescence bioanalytical reagent, 5-(azo-*para*-phenylene-*N*-aza-15-crown-5)-phthalhydrazide

Introduction

The chemiluminescence (CL) of organic compounds[1] has both theoretical and practical interest. Phthalhydrazide derivatives have been prominent; especially luminol, (5-amino-2,3-dihydrophthalazine-1,4-dione) has many uses owing to its fluorescence (FL) and chemiluminescence.[1-7] In alkaline medium, in the presence of oxygen,[2-4,8] hydrogen peroxide, and transitional metal ions such as Fe^{3+}, its CL intensity (I_{CL}) is enhanced.[9-11] Based on its CL, luminol is being used for the bioanalytical determination of enzymatically-formed oxidizing species such as the superoxide anion and the hydroxyl radical.[12-13] It is also possible to determine cholesterol via the H_2O_2 concentration formed by enzymatic oxidation.[14] Electro-

chemiluminescence determinations have recently been reported using luminol or its chemical modifications such as deposition on gold[15,16] or silver nanoparticles,[17] on graphite-methacrylate[18] or glass-carbon composite electrodes[19] on platinum electrodes[20] or as a film formed by copolymerization with aniline.[21] Derivatives of luminol with azo-calixarenes[22] have also been reported to present weak FL and significant CL. Also phthalhydrazide-ionophores such as styrylphthalhydrazides with crown ethers[23] and aza-crowned isoluminol[24] were obtained.

5-(Azo-*para*-phenylene-*N*-aza-15-crown-5)-phthalhydrazide **1** was designed and synthesized for its pH-dependent chromogenic (indicator) properties[25] due to its azo group, and for its chromoionophoric behavior in the presence of lithium and sodium cations[26] due to its crown ether moiety. The tunable CL is caused by the luminol moiety, and the azo bridge between it and the crown ether moiety allows electronic communication between these two moieties. In the present paper we report a study of the CL when **1** is oxidized in alkaline medium by hydrogen peroxide, with or without Fe^{3+} or Cu^{2+} ions.

Results and Discussion

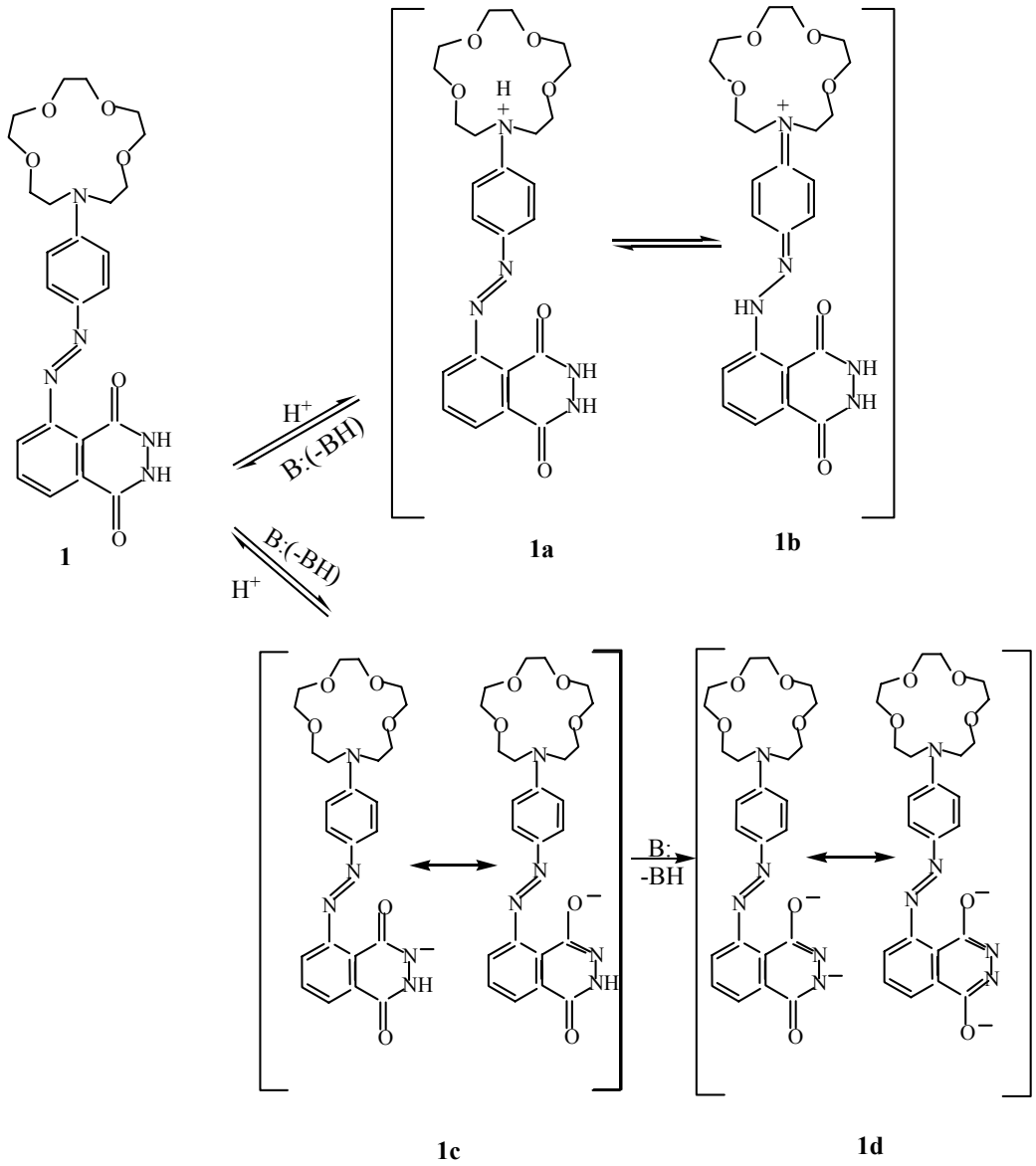
Chromogenic, solubility, and fluorescence properties of coumpound 1

In Scheme 1 one can see the various chemical species formed from **1** in acidic media (only two lactam tautomers are shown, **1a** and **1b** although lactim forms are also posible) and in alkaline media; the resonance forms of the monoanion **1c** and of the dianion **1d** influence chromogenic properties.[25] Acidification of **1** is accompanied by a bathochromic shift due probably to the *para*-quinone-diimidic tautomer **1b**, whereas in basic medium a hypsochromic effect takes place, with phthalhydrazide resonance structures of **1c** and **1d**.

The amphoteric nature of **1** determines its solubility. Therefore neutral **1** is soluble in organic solvents, whereas its alkaline salts **1c** and **1d** are soluble in water as in the case of luminol,[7,9] and the ratio between mono- and dianion (Scheme 1) is strongly dependent on the pH. In sodium phosphate buffer at pH = 10.1, under photoexcitation with λ_{ex} = 420 nm, the FL intensity of **1** was 30 times lower than that of luminol. This effect may be attributable to electron-attracting effect of the azo group, similarly to the congeneric azo-calixarene derivatives.[22]

Chemiluminescence

First, we performed qualitative investigations for finding optimal reaction conditions, then experiments for confirming the putative reaction mechanism, and finally we collected quantitative data. These results are presented below sequentially.



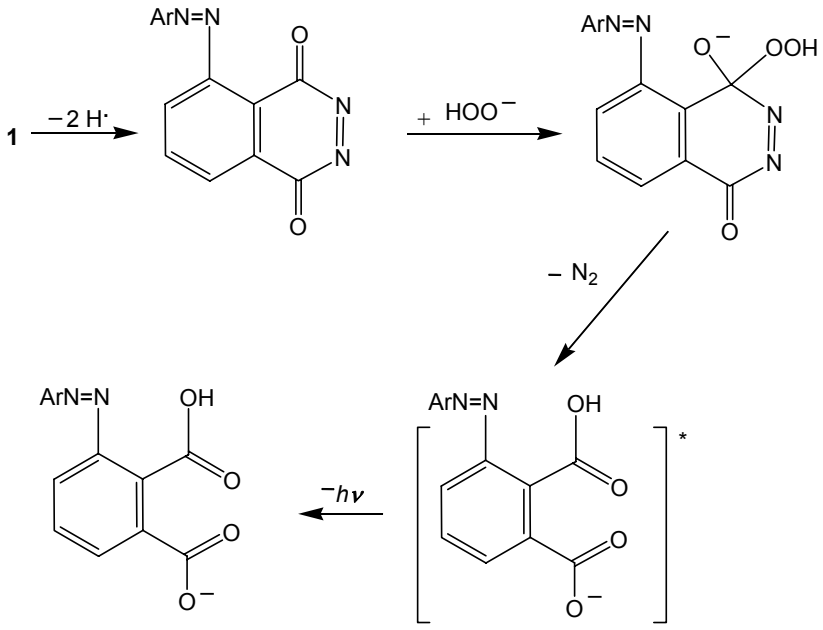
Scheme 1. Behavior of compound **1** (for neutral solutions, λ_{max} = 509 nm) in acidic (**1 a**, **b**, λ_{max} = 534 nm) and in alkaline media (**1 c**, **1d**, λ_{max} = 429 nm).

Qualitative experiments and reaction mechanism

The pH of the buffer (Na_2HPO_4 + NaOH) was selected to be 10.1, by analogy with literature values: pH = 10.15 for luminol,[10,23] and pH = 10.5 for the phthalhydrazide-azo-calixarene derivative.[22] Experiments were carried out at room temperature (25ºC). Higher pH values than 10.1 cause precipitation when using Fe^{3+} and Cu^{2+} salts.

At an optimal concentration of 10.3 μM with or without Fe^{3+} or Cu^{2+} ions, compound **1** with the phosphate buffer presented CL on treatment with H_2O_2, after 5 s at λ_{CL} = 425 nm, like luminol[7-9] but differing slightly from the azo-calixarene congener which had λ_{CL} = 420 nm.[22]

Following Merényi and coworkers,[13,27,28] it is generally accepted[29-31] that the CL of luminol starts with electron transfer processes between redox couples.[32,33] By analogy with luminol, we assume that **1** is first dehydrogenated, allowing it then to add a nucleophilic hydroperoxide anion, and then to release a dinitrogen molecule leaving an excited phthalic acid derivative responsible for emitting a photon.[3,4,8-10] (Scheme 2).



Scheme 2. The probable process for CL of compound **1**.

Monitoring the intensity of CL in time, the following results were obtained:
(i) when only H_2O_2 was employed, the I_{CL} value decreased for 50 s reaching a plateau (Figure 1A); even after 5 minutes I_{CL} did not decrease to a half;
(ii) with a high ratio $[H_2O_2]/[M^{n+}]$ ($M^{n+} = Fe^{3+}$ or Cu^{2+}, the I_{CL} value decreased for 50 s and then starts to increase (Figure 1B, 1C); even after 5 minutes I_{CL} did not decrease to a half;
(iii) inversely, with a low ratio $[H_2O_2]/[M^{n+}]$ ($M^{n+} = Fe^{3+}$ or Cu^{2+}), I_{CL} started higher (Figure 1D) but decreased for 50 s, and had reached half of its value after 15 s.

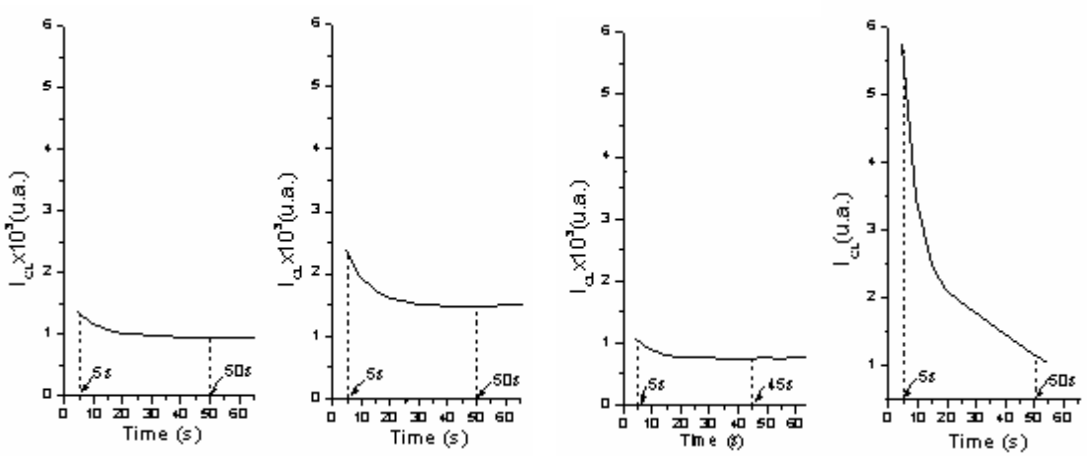


Figure 1. I_{CL} *vs.* time of the solutions of **1** ($1.03 \cdot 10^{-5}$M) in sodium phosphate buffer (pH=10.10) at 425 nm with: A) H_2O_2 (60 μg·mL^{-1}); B) H_2O_2 (60 μg·mL^{-1}) + Fe(III) ($44 \cdot 10^{-3}$ μg·mL^{-1}); C) H_2O_2 (60 μg·mL^{-1}) + Cu(II) ($2.3 \cdot 10^{-3}$ μg·mL^{-1}); D) H_2O_2 (3.0 μg·mL^{-1}) + Cu(II) ($5.9 \cdot 10^{-3}$ μg·mL^{-1}).

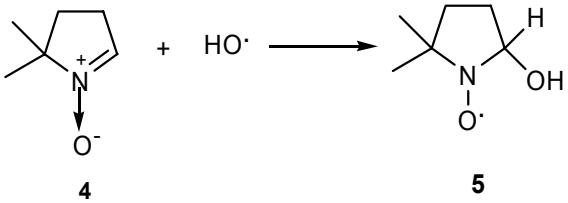
After monitoring at λ_{CL} = 425 nm, it was not posible to evidence by TLC the reaction products because less than 5% of the compound **1** had reacted, as determined from the UV-Vis spectra. This situation is similar to that observed for the CL of luminol.[9]

When transition metal cations were also present, the processes responsible for oxidizing **1** are represented by equations (1) and (2) (where: $M^{n+} = Fe^{3+}$ or Cu^{2+} and $M^{(n-1)+} = Fe^{2+}$ or Cu^{1+}), which afford hydroxyl radicals via reaction (2).

$$H_2O_2 + M^{(n-1)+} \rightarrow HO\cdot + M^{n+} + HO^- \text{ (Fenton reaction)} \quad (1)$$

$$M^{n+} + e^- \rightleftharpoons M^{(n-1)+} \quad (2)$$

For checking that Cu^{2+} behaves like Fe^{3+} in the presence of H_2O_2, we used EPR spectroscopy for spin-trapping with 5,5-dimethyl-1-pyrroline N-oxide[34-36] (DMPO, **4**) any short-lived free radicals such as HO· (Scheme 4). We did obtain EPR quartets with 1:2:2:1 intensities only in the presence of Fe^{3+} or Cu^{2+} cations, and we ascribe these EPR spectra (Fig. 2) to the persistent free radical **5**, formed from **4** and a hydroxyl free radical.



4 **5**

Scheme 4. Reaction of diamagnetic DMPO **4** with HO· radical yielding the paramagnetic nitroxide **5** with practically equal hyperfine splitting constants a_H and a_N.

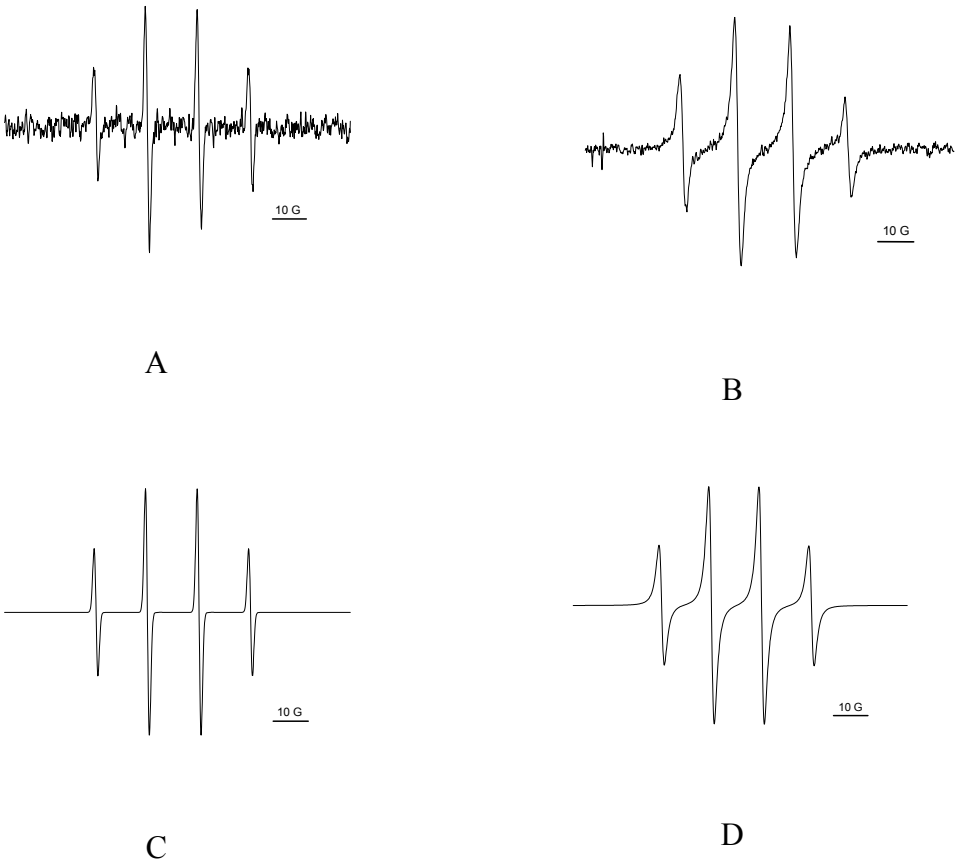


Figure 2. EPR experimental (A, B) and simulated (C, D) spectra (see experimental part), of compound **1** in phosphate buffer in the presence of DMPO and H_2O_2: (A, C) with Fe^{3+} (a_N=14.95 G and a_H= 14.75 G); (B, D) with Cu^{2+} (a_N=15.00 G and a_H=14.80 G).

Quantitative experiments

Determination of the hydrogen peroxide concentration with or without Cu^{2+}

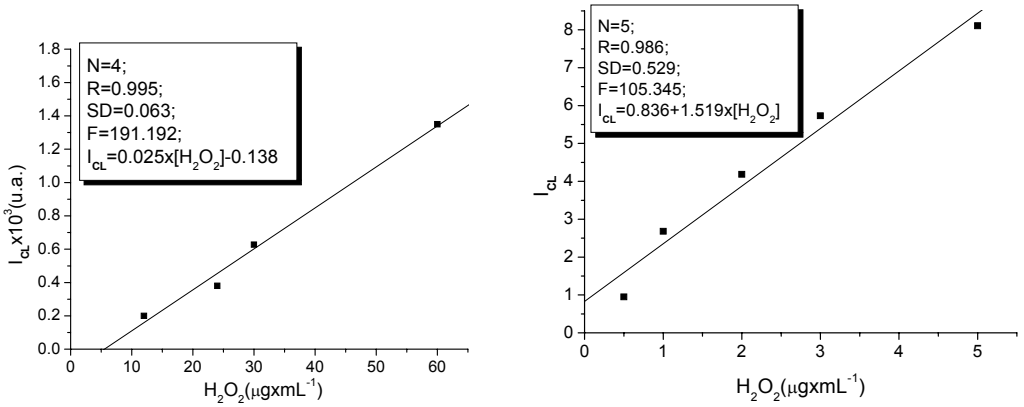
We investigated the possibility of determining the concentration of H_2O_2 in the absence or in the presence of Cu^{2+} ions using compound **1**, and we obtained the following results (Figure 3):

(i) In the absence of Cu^{2+} ions (Figure 3A), there is a linear correlation[37] (equation 3) for hydrogen peroxide concentrations ranging from 12 μg·mL^{-1} to 90 μg·mL^{-1};

$$I_{CL} = a + b\cdot[X] \quad (3)$$

where: I_{CL} = intensity of CL; [X] = concentration of H_2O_2, Fe^{3+} and Cu^{2+}.

(ii) In the presence of excess Cu^{2+} ions (Figure 3B), a similar linear correlation allows the determination of H_2O_2 in 20-times lower concentration range between 0.5 to 5 μg·mL^{-1}.



A B

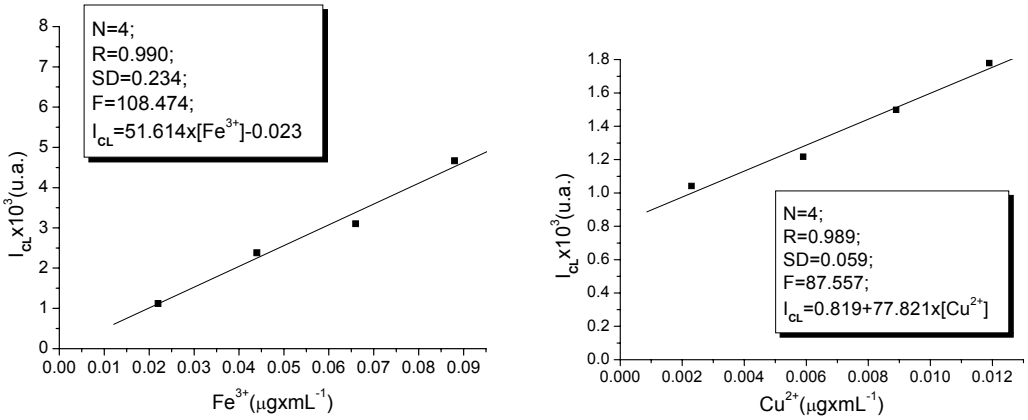
Figure 3. CL intensity of **1** ($1.03 \cdot 10^{-3}$M) at 425 nm (pH=10.10) after 5 s: (A) I_{CL} *vs* $[H_2O_2]$; (B) I_{CL} *vs* $[H_2O_2]$ in the presence of Cu^{2+} ($5.9 \cdot 10^{-3}$ μg·mL^{-1}).

In the above correlations, slightly lower coefficients R would result if $a = 0$ in equation (3) for the cases presented in Figures 3A, 3B, and 4A, where the linear correlation is close to the origin of the Cartesian coordinates. However, in the case presented in Figure 4B, the origin is appreciably farther, so that *a* cannot be ignored.

We tested the effect of alkali metal cations, and found that in the presence of Li^+ salts $\lambda_{max} = 501$ nm and the CL has $\lambda_{max,CL}$ at 436 nm, whereas in the presence of Na^+ salts $\lambda_{max} = 492$ nm and the CL has $\lambda_{max,CL}$ at 437 nm.

Determination of concentrations for Cu^{2+} and Fe^{3+} cations

One can determine concentrations of Cu^{2+} salts by means of luminol CL.[38] We investigated the possibility of using similarly **1** for determining either Cu^{2+} or Fe^{3+} concentrations. It is known that luminol emits light in the presence of blood[11,39] or ferricyanide.[40] On applying the same equation (3) the results are displayed in Figure 4, where X = Fe^{3+} or Cu^{2+} in the concentration range from $22 \cdot 10^{-3}$ μg·mL^{-1} to $88 \cdot 10^{-3}$ μg·mL^{-1} for Fe^{3+} (Figure 4A), and from $2.3 \cdot 10^{-3}$ μg·mL^{-1} to $11.9 \cdot 10^{-3}$ μg·mL^{-1} for Cu^{2+} (Figure 4B).



A B

Figure 4. I_{CL} for **1** ($1.03 \cdot 10^{-3}$ M) at 425 nm (pH = 10.1) after 5 s in the presence of 60 μg·mL^{-1} H_2O_2 with different concentrations of Fe^{3+} (A) and of Cu^{2+} (B).

Conclusions

In alkaline solution (pH=10.1), compound **1** presents at 25°C a weak FL. In the same conditions, in the presence of H_2O_2, with or without Fe^{3+} or Cu^{2+} cations, compound **1** displays after 5 s a significant CL at 425 nm. After about 50 s secondary phenomena occur due to various reactive oxygen species. By spin trapping using DMPO and EPR spectrometry, it was possible to prove the formation of HO· in such conditions. The linear correlation between I_{CL} of **1** and oxidizing species (H_2O_2, Fe^{3+} or Cu^{2+}) encourages us to propose using **1** (with chromogenic properties depending on pH, and chromoionophoric properties depending on alkali metal cations) for analytical and bioanalytical assays (Figure 5).

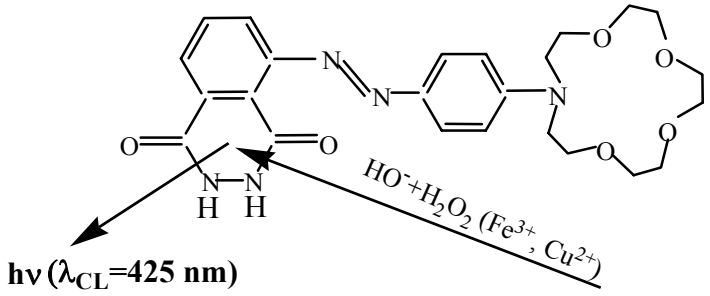


Figure 5. CL properties of the compound **1**.

Experimental Section

General. Hydrogen peroxide (30%), $CuCl_2 \cdot 2H_2O$, $Fe(NO_3)_3 \cdot 9H_2O$, NaOH, DMPO, and 5,5-dimethyl-1-pyrroline N-oxide **4** were from Aldrich and $Na_2HPO_4 \cdot 12H_2O$ from Sigma. The non-commercial compound 5-(azo-*para*-phenylene-N-aza-15-crown-5)-phthalhydrazide (**1**) was synthesized as described earlier.[25]

Solutions

The phosphate buffer was prepared from bidistilled water and $Na_2HPO_4 \cdot 12H_2O$ (17.956 g in 250 mL); into this solution under continuous stirring and pH measurement (with pH Meter 315 i/SET, WTW) a 0.2 M solution of NaOH was added till the pH reached 10.1. Buffer solutions were used immediately after preparation. In all experimental procedures fresh aqueous solutions were used. Compound **1** was dissolved at room temperature (25°C) in phosphate buffer (pH=10.1). The 0.3% (g/v) H_2O_2 solution was obtained by diluting 30%, H_2O_2 with bidistilled water at 25°C, and determining the concentration by titration with $KMnO_4$ and H_2SO_4. Solutions of transition metal salts (0.08% $Fe(NO_3)_3 \cdot 9H_2O$ and 0.008% $CuCl_2 \cdot 2H_2O$) were obtained with bidistilled water at 25°C.

Methods

CL measurements were performed with TD 20/20 Turner Design, USA (the points on the plot were obtained by integrating the light signal over periods of 5 s for five measurements, then average values were calculated, obtaining a maximum 10% relative scattering of the results from the mean value).

EPR spectra were recorded at room temperature after 8 minutes with compound **1** ($1.03 \cdot 10^{-4}$M) and H_2O_2 (60 $\mu g \cdot mL^{-1}$) in phosphate buffer (pH=10.1) in the presence of DMPO (10^{-1} M) with a JEOL FA 100 spectrometer with 100 kHz modulation frequency, at 0.998 mW microwave power, with 120 s sweep time, 0.7 G modulation amplitude, time constant 0.1 s The DMPO solution was freshly prepared; the simulated EPR spectra were performed with Winsim-program standard. Simulations yielded the hyperfine splitting constants with Fe^{3+} ($44 \cdot 10^{-3} \mu g \cdot mL^{-1}$) and with Cu^{2+} ($2.3 \cdot 10^{-3}$ $\mu g \cdot mL^{-1}$). The experimental spectra had sharper lines for Fe^{3+} than with Cu^{2+} (Figure 2).

Concentration determinations

(i) **Determination of H_2O_2 concentrations without Cu^{2+}.** An aqueous solution of **1** (100 μL) was admixed at 25 °C with a given volume of phosphate buffer (pH=10.1) and then rapidly with 4 to 20 μL of the 0.3 % H_2O_2 solution yielding a final volume of 1000 μL. After stirring for 5 s, I_{CL} was measured; the average of 5 measurements was obtained, as shown in Figure 3A.

(ii) **Determination of H_2O_2 concentrations in the presence of Cu^{2+}.** An aqueous solution of 1 (100 μL) was admixed at 25 °C with 20 μL of $CuCl_2 \cdot 2H_2O$ solution (0.596 $\mu g \cdot mL^{-1}$) and with a given volume of phosphate buffer so that on rapidly adding 0.5 to 5 $\mu g \cdot mL^{-1}$ of the 0.03 % H_2O_2

solution one obtains a final volume of 1000 μL. After stirring for 5 s, I_{CL} was measured; the average of 5 measurements was obtained, as shown in Figure 3B.

Determination of Cu^{2+} and Fe^{3+} concentrations. An aqueous solution of **1** (100 μL) was admixed at 25 °C with 2 to 8 μL of $Fe(NO_3)_3 \cdot 9H_2O$ solution or with 8 to 40 μL of $CuCl_2 \cdot 2H_2O$ solution; then a corresponding volume of the phosphate buffer was added so as to reach a total volume of 1000 μL after the rapid ddition of 20 μL (60 $\mu g \cdot ml^{-1}$) 0.3 % H_2O_2 solution. After stirring for 5 s, I_{CL} was measured; the average of 5 measurements was obtained, as shown in Fig. 4.

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