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Characteristics of key intermediates generated in uncatalyzed bis(2,4-dinitrophenyl) oxalate (DNPO) chemiluminescence reactions

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The characteristics of two distinct perylene chemiluminescence (CL) decay curves generated by the reaction between bis(2,4-dinitrophenyl) oxalate (DNPO) and H_2O_2 in the absence of base catalysts were investigated. When the intensity of peroxyoxalate chemiluminescence (PO-CL) *vs.* time was measured under relatively low H_2O_2 and high DNPO concentrations in ethyl acetate, a slowly decaying curve appeared. However, upon increasing the H_2O_2 concentration under the same DNPO-CL reaction conditions, two distinct emission maxima were observed: a fast decaying CL curve and a slowly decaying CL curve. The fast decaying CL curve appeared alone when the H_2O_2 concentration greatly exceeded the DNPO concentration in ethyl acetate. To learn more about the properties of both CL decay curves, we further investigated the effects of adding H_2O or phenols having different nucleophilicity [2,4-dinitrophenol (DNP), 2,4-dichlorophenol (DCP), pentachlorophenol (PCP), and 2,4,6-trichlorophenol (TCP)] and the stability of DNPO used in the CL reaction. The slowly decaying curve was predominant with the addition of H_2O and relatively strong nucleophile. Based on the observed results, we propose that the likely high-energy intermediates generated in DNPO-CL reactions without added base catalysts are hydroperoxyoxalate ester and a six-membered cyclic peroxide.

Introduction

Although various high pressure liquid chromatography (HPLC) systems with a peroxyoxalate chemiluminescence (PO-CL) detector have been successfully used for quantifying fluorescent compounds at trace levels,[1–3] the identity of the high-energy intermediate(s) generated during PO-CL reactions continues to be debated by several research groups.[4–15]

Alvarez *et al.*[8] first proposed the existence of multiple intermediates capable of generating light during PO-CL reactions, including two high-energy intermediates (1,2-dioxetanedione and 1,2-dioxetanone). Chokshi *et al.*[9] proposed the hydroperoxyoxalate ester as a possible high-energy intermediate capable of interacting with fluorescent compounds by studying the bis(2,6-difluorophenyl) oxalate (DFPO) CL reaction using ^{19}F-NMR. Based on the kinetics of photoinitiated CL reactions, Milofsky and Birks[10] proposed a six-membered cyclic peroxide as an intermediate capable of generating singlet-excited states of fluorescent molecules. Lee *et al.*[11] proposed that bis(2,4-dinitrophenyl) oxalate (DNPO) CL reactions exhibit complex pathways (*e.g.*, direct CL reaction, base-catalyzed CL reaction, and acid–base CL reaction) that involve many of the highly energetic intermediates previously suggested by other research groups.[4–10] According to Lee *et al.*, formation of various key intermediates in DNPO-CL reactions is strongly dependent on the concentrations of DNPO and H_2O_2, and is influenced by the nature of bases such as imidazole, triethylamine or sodium salicylate, and the solvent property.[11] In particular, Lee *et al.* obtained two different decay curves when they measured CL intensities generated by the reaction of DNPO and excess H_2O_2 in the presence of imidazole (ImH) and 1-aminoanthracene ($[H_2O_2] > [DNPO] > [ImH]$).[11] Lee *et al.* proposed that the relatively rapidly decaying curve might be generated by the interaction between fluorescent molecules having low oxidative half-wave potentials and an intermediate (*e.g.*, hydroxyperoxyoxalate ester) formed from the reaction (*e.g.*, direct CL reaction) between DNPO and H_2O_2 with a base in low viscosity solvents (*e.g.*, acetonitrile, ethyl acetate). For example, they were not able to obtain the fast decaying curve in DNPO-CL reactions with fluorescent compounds having relatively high oxidative half-wave potentials (*e.g.*, anthracene, perylene).

Based on their results, Lee *et al.* proposed that the high-energy intermediate producing the fast decaying curve was the hydroperoxyoxalate ester.[11] Also, they proposed that the slowly decaying curve generated in the base-catalyzed CL reaction is always predominant in DNPO-CL reactions in non-aqueous solvents because the intermediate is more reactive with fluorescent molecules than the hydroperoxyoxalate ester.

In general, trace levels of fluorescent compounds can be detected by the reaction of DNPO and H_2O_2 without a catalyst, which is the most fundamental PO-CL reaction. This is because DNPO is more sensitive and selective than bis(2,4,6-trichlorophenyl) oxalate (TCPO) and bis(pentachlorophenyl) oxalate (PCPO). To date, however, key intermediate(s) formed under the simplest PO-CL reaction conditions have never been proposed. In the present paper, we investigate the properties of two decay curves generated by the reaction of DNPO and H_2O_2

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without added catalyst in the presence of perylene. Based on results observed for this simplest PO-CL reaction condition, we propose the identity of key intermediates capable of interacting with perylene to produce light emission.

Experimental

Chemicals

DNPO, TCPO, PCPO, 2,4-dinitrophenol (DNP), 2,4-dichlorophenol (DCP), pentachlorophenol (PCP), 2,4,6-trichlorophenol (TCP), H_2O_2 (50%), 1-aminoanthracene, anthracene, and perylene were all purchased from Aldrich. H_2O_2 was stored in a refrigerator at -10 °C. It was titrated with $KMnO_4$ solution (Aldrich) to determine the exact concentration before use. Spectrophotometric grade ethyl acetate and acetonitrile purchased from Baker were used as the solvents. Deionized water of resistivity greater than 17.8 MΩ cm^{-1} was obtained from a Millipore Super Q system.

Procedures

A typical CL reaction was conducted at room temperature (22.0 ± 1.0 °C) in a 1-cm fluorescence cell placed in the sample compartment of a spectrofluorometer (PTI, Inc.). H_2O_2 solution (0.5 ml) was added to the cell followed by 0.5 ml of the perylene solution. The reaction was initiated by injecting 1.0 ml of oxalate solution. Each experiment was repeated three times. Fresh solutions were prepared daily and kept in the dark. DNPO was used within 4 hours after being prepared. The CL intensity *vs.* time was monitored by the spectrofluorometer at the maximum emission wavelength (468 nm) of perylene. DNP formed from the DNPO-CL reaction was observed by absorbance at 420 nm using the kinetics function of an 8452A diode array spectrophotometer (Hewlett Packard).

Results and discussion

Decay curves in uncatalyzed DNPO-CL reactions

Fig. 1 shows typical perylene CL intensity *vs.* time profiles for the reaction of H_2O_2 and DNPO in the absence of catalysts. With a low H_2O_2 concentration, only the slow decay curve was observed. Upon increasing the H_2O_2 concentration, both a fast decay curve and a slow decay curve appeared. With the highest H_2O_2 concentration, the fast decay curve was predominant.

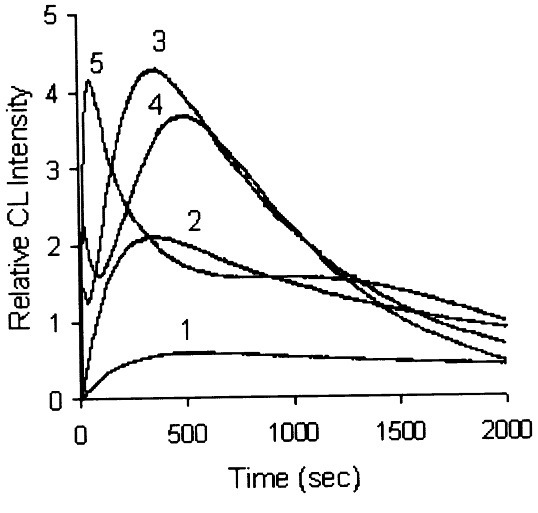


Fig. 1 Effect of H_2O_2 concentration in the uncatalyzed DNPO-CL reactions in ethyl acetate. $[H_2O_2]$ (mM) = 4.5 (1), 14.0 (2), 42.0 (3), 84.0 (4), and 168.0 (5), [DNPO] = 0.5 mM, [perylene] = 0.05 mM.

We interpret the results shown in Fig. 1 as supporting our hypothesis of at least two high-energy intermediates capable of generating an excited singlet state of perylene in DNPO-CL reactions without an added catalyst. The two decay profiles shown in Fig. 1 are similar to the two peaks observed by Lee *et al.* who measured 1-aminoanthracene CL intensity generated by the reaction of DNPO and H_2O_2 in ImH-catalyzed CL reactions ($[H_2O_2] > [DNPO] > [ImH]$).[11] However, when the highest H_2O_2 concentration reacted with DNPO in the presence of perylene as shown in Fig. 1, the fast decaying curve dominated. These results are contrary to the observations of Lee *et al.* that the slowly decaying curve always dominated.[11]

The fast decay curve shown in Fig. 1 is generated from the interaction between perylene (0.85 V) and a high-energy intermediate formed from the reaction between H_2O_2 and DNPO. We also observed the fast decay curve using anthracene (1.09 V) and 1-aminoanthracene (0.40 V) as the fluorescent compounds under the same reaction conditions as curve 4 in Fig. 1 ($[H_2O_2]$ = 84 mM, [DNPO] = 0.5 mM, and [fluorophor] = 0.5 mM) (data not shown). The intensity ratio of the fast decay CL curve to the slow decay CL curve in the presence of each of the three fluorescent compounds is dependent on the oxidative half-wave potential of each fluorescent compound (1-aminoanthracene > perylene > anthracene). These results are consistent with the results obtained by Lee *et al.*[11] They suggested that the possible high energy intermediate capable of generating the fast CL decay curve is hydroperoxyoxalate ester. They called this reaction a direct CL reaction.[11]

Milofsky and Birks[10] suggested that ImH acts as a base-catalyst in PO-CL reactions, and that the decay curve observed in the absence of an added base such as ImH may result because TCP produced from the reaction between TCPO and H_2O_2 acts as a nucleophile in this reaction. Based on this reference, we propose that the slow decay curve obtained in Fig. 1 might be generated by the base-catalyzed DNPO-CL reactions like the decay curves observed by Milofsky and Birks[10] and Lee *et al.*[11,15] In other words, DNP produced from the reaction between DNPO and H_2O_2 becomes the nucleophile in this reaction. They suggested that the possible high energy intermediate capable of generating the slow CL decay curve is a six-membered cyclic peroxide.[10,11,15]

Fig. 1 shows that the appearance of the two decay curves generated in the DNPO-CL reaction depends on the concentration of reagents (DNPO *vs.* H_2O_2) in the presence of perylene. Therefore, after isolating each CL curve by changing the concentrations of DNPO and H_2O_2 in the presence of perylene, we were able to further investigate various properties of the decay curves.

Effects of other oxalates

We conducted experiments using TCPO and PCPO instead of DNPO for the same experimental conditions as Fig. 1 (data not shown). When PCPO reacted with excess H_2O_2 (540 mM), a weak fast decay CL curve appeared even though the slow decay CL curve predominated. However, even at H_2O_2 concentrations higher than 540 mM, the fast decay CL curve was not present for TCPO. These results suggest that a high-energy intermediate capable of interacting with fluorescent molecules to generate a fast decay CL curve must have a strong electron withdrawing group in its structure. This is consistent with our belief that the CL efficiency of the fast decaying curve depends on the strength of electron withdrawing group (DNP > PCP > TCP) substituted in oxalate esters.

Direct CL reaction

Fast decay curves generated in DNPO-CL reactions with the highest excesses of H_2O_2 in ethyl acetate exhibited a characteristic light intensity *vs.* time profile. For these conditions, the reaction kinetics were well fit by the simple reaction scheme shown in eqn. (1) where A and C are the initial reactant(s) and final products respectively.

$$A \xrightarrow{k_r} B \xrightarrow{k_f} C$$

The CL intensity is presumed to be proportional to the concentration of intermediate species, B. Both reaction steps are assumed to be irreversible pseudo-first-order reactions with rate constants k_r and k_f corresponding to the rise and fall, respectively in the CL intensity *vs.* time profiles. Based on these simplifying assumptions, an integrated rate equation can then be obtained for the CL intensity *vs.* time.[7,16]

$$\mathrm{CL} \propto [\mathrm{B}] = \frac{I_{\max} k_r}{k_f - k_r}[\exp(-k_r t) - \exp(-k_f t)]$$

Using this simple model for the reaction kinetics, k_r and k_f were obtained from a nonlinear least-squares fit of eqn. (2) to the experimental data using the optimization toolbox in Matlab 5.2. The reported rate constant is an average of at least three independent measurements.

To determine if a reactive intermediate leading to the fast decay curve is consistent with the hydroperoxyoxalate ester proposed by Lee *et al.*,[11] we calculated the rate constants of the fast decay curves obtained in the DNPO-CL reactions having H_2O_2 in large excess over DNPO as shown in Fig. 2.

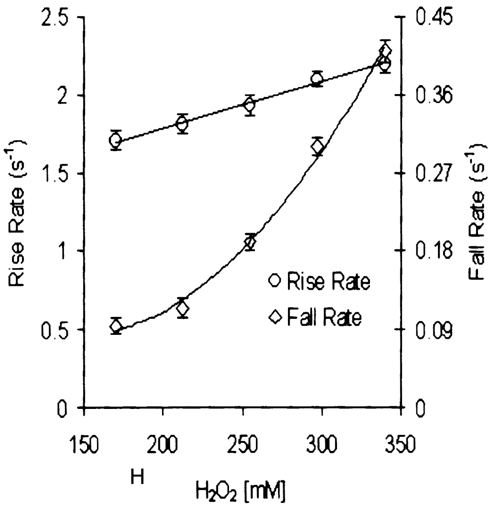


Fig. 2 Effect of H_2O_2 concentration on the rise rate constants (k_r) and the fall rate constants (k_f) for the direct CL reactions in ethyl acetate. [DNPO] = 0.001 mM, [perylene] = 0.02 mM.

Fig. 2 illustrates the dependence of k_r and k_f on H_2O_2 concentration. The rise rate constants (k_r) were linearly dependent on H_2O_2 concentration, whereas the fall rate constants (k_f) appeared to show a second-order dependence on H_2O_2 concentration. However, this latter observation most likely indicates that the fall rate coefficients determined under these conditions are also being affected slightly by the slow decay CL curve generated from a different CL reaction pathway.

The effect of varying the DNPO concentration (0.003 to 0.05 mM) on k_r and k_f was investigated using constant perylene (0.05 mM) and H_2O_2 (340 mM) concentrations in ethyl acetate (data not shown). There was no significant change, suggesting that the pseudo-first-order rate constants k_r and k_f are independent of DNPO concentration (k_r = 1.90 ± 0.03 s^{-1}, k_f = 0.095 ± 0.004 s^{-1}). In other words, when the H_2O_2 concentration greatly exceeded the DNPO concentration in ethyl acetate, the effect of DNPO concentration was not observed in the reaction.

The effect of varying the perylene concentration (0.006 to 0.25 mM) on k_r and k_f was investigated using constant DNPO (0.05 mM) and H_2O_2 (340 mM) concentrations in ethyl acetate (data not shown). Both kinetic parameters were independent of the concentration of perylene (k_r = 1.85 ± 0.02 s^{-1}, k_f = 0.099 ± 0.004 s^{-1}) because perylene does not react with the other reagents (DNPO and H_2O_2) to generate high-energy intermediates.

DNP is one of the final products generated in the DNPO-CL reaction, and Fig. 3 shows the effect of varying its concentration when DNPO, H_2O_2, and perylene concentrations are constant in ethyl acetate. Both the CL intensity and area under the fast decay curves decreased upon increasing the DNP concentration. DNP appears to interrupt the interaction between perylene and the high-energy intermediate used to generate the fast decay curve.

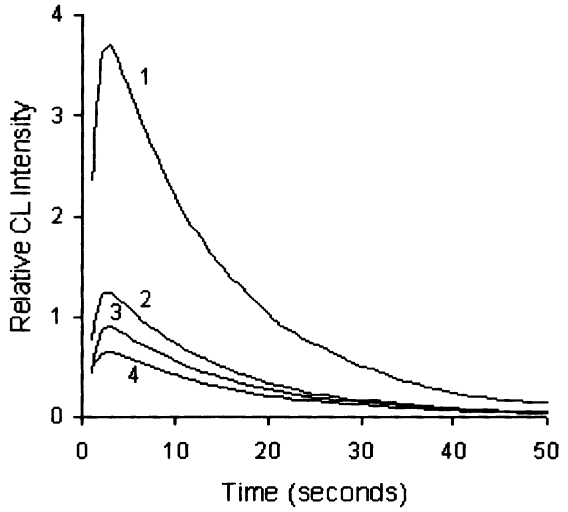


Fig. 3 Effect of DNP concentration in the direct CL reaction in ethyl acetate. [DNP] (mM) = 0 (1), 0.006 (2), 0.032 (3), and 0.160 (4), [perylene] = 0.025 mM, [DNPO] = 0.005 mM, [H_2O_2] = 34.0 mM.

Based on the experimental results, the property of the high-energy intermediate generating the fast decay curve in the reaction is consistent with that of the intermediate generating the fast decay curve observed from the direct CL reaction when a low catalyst concentration is added.[11] When ImH (0.01 mM) was used as a base catalyst in the DNPO-CL reaction under otherwise similar reactive conditions as Fig. 1, we observed both a fast decay curve and a slow decay curve (data not shown). From the results obtained here, we suggest that the direct CL reaction to generate a fast decaying CL curve is predominant when a low concentration of DNPO reacts with excess H_2O_2 in the absence of a base catalyst. In addition, DNP as a final product in the reaction acts as an inhibitor (quencher) for the direct CL reaction.

DNP-catalyzed CL reaction

Milofsky and Birks[10] first postulated that the possible reactive intermediate in their photoinitiated CL reaction was a six-membered cyclic peroxide. They also suggested that the decay curve obtained in the absence of added bases might be due to reactions with phenols, such as TCP, generated as a final product in the photoinitiated PO-CL reaction.[10] To determine if the reactive intermediate of the slow decay curve observed in Fig. 1 is a six-membered cyclic peroxide, we added DNP from 0 to 0.04 mM for conditions similar to curve 1 in Fig. 1. As shown in Fig. 4, with the gradual increase of DNP concentration, the relative CL intensity of the slow decay curves increased and then at a certain point began to decrease whereas the opposite trend was observed for the time required to reach the maximum emission, $\tau_{\max}$. In this reaction, DNP acts as a nucleophile to generate the reactive intermediate (six-membered cyclic peroxide) up to a certain point and then acts as an inhibitor. When DCP, PCP or TCP was used in the experiment instead of DNP under the same reaction condition as Fig. 4 they also acted as nucleophiles as shown in Fig. 5. Based on these results, we

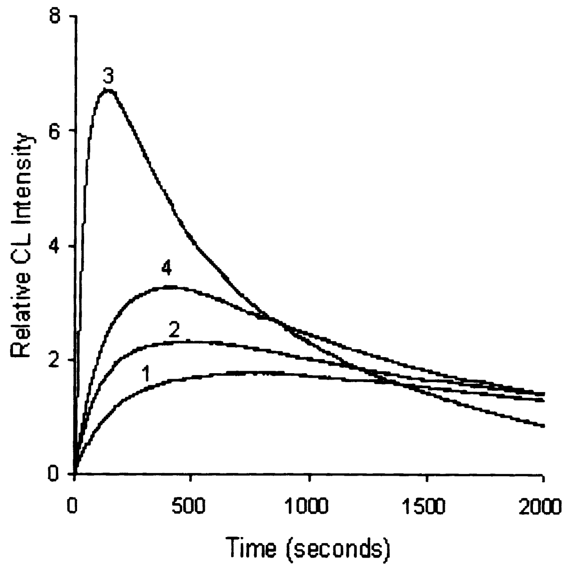


Fig. 4 Effect of DNP concentration on the DNP-catalyzed nucleophilic CL reaction in ethyl acetate. [DNP] (mM) = 0 (1), 0.006 (2), 0.032 (3), and 0.160 (4), [perylene] = 0.025 mM, [DNPO] = 2.0 mM, $[H_2O_2]$ = 10.0 mM.

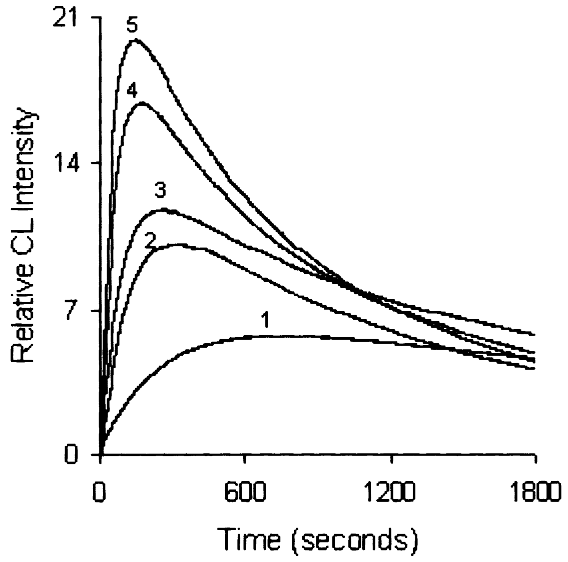


Fig. 5 Effect of phenols (0.01 mM) added in the four types of PO-CL reactions; 1. no addition, 2. DCP, 3. TCP, 4. PCP, 5. DNP. $[H_2O_2]$ = 5.6 mM, [DNPO] = 3.0 mM, [perylene] = 0.1 mM.

believe that CL observed from the reaction between TCPO or PCPO and H_2O_2 in the absence of a weak base comes from TCP- or PCP-catalyzed nucleophilic CL reactions.

Stability of DNPO

Although DNPO is less stable in organic solution and has a higher background intensity than other oxalates such as TCPO and PCPO, it exhibits faster reaction kinetics and the greatest CL intensity in PO-CL reactions.[17] Several research groups have measured the decomposition of TCPO in acetonitrile.[18–20] For example, when Hanaoka[19] used Pyrex borosilicate and sodium borosilicate glass bottles as reservoirs for TCPO solution, no deactivation of TCPO was observed for at least 6 hours in acetonitrile. In the present study, we stored DNPO solutions (0.4 mM) in a Pyrex borosilicate glass bottle for 10 hours and every hour measured CL intensity with H_2O_2 (9.0 mM) and perylene (0.13 mM) (data not shown). There were negligible changes in either the CL intensity or τ_{max} over a 4-hour storage period. However, after 6 hours of storage the CL intensity of the slow decay curve increased sharply (data not shown). After 10 hours of storage in a Pyrex borosilicate glass bottle the presence of DNP formed by the decomposition of DNPO (0.4 mM) in acetonitrile was observed by absorbance at 420 nm. Thus, we always prepared fresh DNPO solutions in organic solvent every 4 hours because DNPO dissolved in organic solvents is unstable in Pyrex borosilicate glass bottles for longer storage periods.

Effects of H_2O

Fig. 6 shows the effect of varying H_2O concentration with constant DNPO, H_2O_2, and perylene concentrations in acetonitrile. Curve 1 in Fig. 6, obtained without H_2O, shows that the slow decay curve predominates. However, the fast decay curve becomes dominant with increasing H_2O concentration. In particular, curve 4, obtained by adding a large excess of H_2O, shows that the slow decay curve disappears and the CL intensity of the fast decay curve increases.

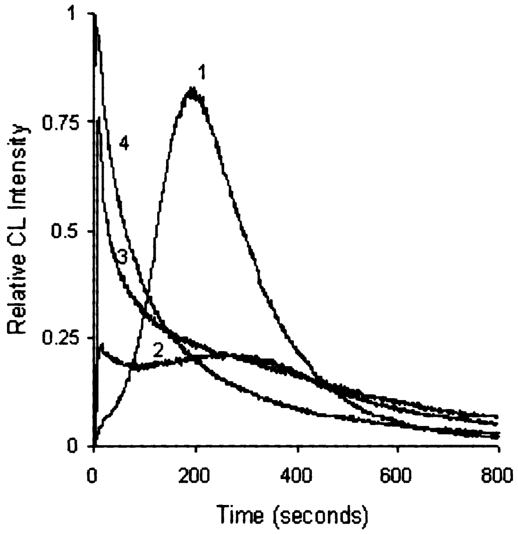


Fig. 6 Effect of H_2O concentration in the DNPO-CL reaction without an added catalyst in acetonitrile. $[H_2O]$ (M) = 0 (1), 1.11 (2), 1.67 (3) and 2.22 (4), [DNPO] = 0.2 mM, $[H_2O_2]$ = 20.0 mM, [perylene] = 0.02 mM.

To understand the mechanistic details of the reaction of aryl oxalates with H_2O, several research groups have studied the hydrolysis kinetics of DNPO in aqueous solution using UV[20,21] and IR spectroscopies.[22] These researchers have suggested that DNPO is hydrolyzed in aqueous solution (such as acetonitrile–water mixtures) with low water content, forming 2 moles of the corresponding DNP from 1 mole of DNPO. An overall hydrolysis reaction of DNPO in aqueous acetonitrile solution proposed by Neuvonen[21] is consistent with the concept of the occurrence of aryl hydroperoxyoxalate ester as an intermediate generated in the reaction between aryl oxalate and H_2O_2.[6,7,9,11] Based on references,[20–22] we surmise that the CL decay curves shown in Fig. 6 were obtained from three competitively reactive pathways which are 1) the direct CL reaction, 2) the DNP-catalyzed nucleophile CL reaction, and 3) the hydrolysis reaction of DNPO. Therefore, the fast decay curve predominates with increasing H_2O concentration under the reaction conditions in Fig. 6 because the excess DNP produced from the hydrolysis reaction of DNPO in aqueous solvent is an inhibitor (quencher) rather than a nucleophile in DNP-catalyzed CL reaction. In other words, the slow decay curve disappears when a large excess of H_2O is present (curve 4 in Fig. 6) because DNPO is hydrolyzed to DNP which acts as a quencher. Therefore, the decay curves of Fig. 6 were obtained from the three competitive reaction pathways (direct CL reaction, DNP-catalyzed CL reaction and hydrolysis reaction of DNPO).

The results shown in Fig. 6 are inconsistent with those obtained in Figs. 3 and 4. To determine the different spectral trends shown in the figures, we separated the fast decay curve

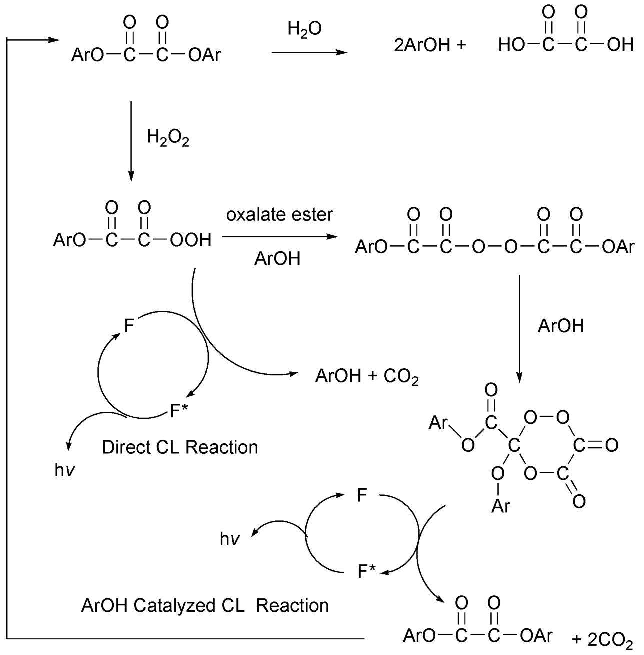
Table 1 Comparison between the direct CL reaction and DNP-catalyzed nucleophilic CL reaction in the absence of base catalysts

	Direct	DNP-catalyzed nucleophilic
τ_{max}	Fast	Slow
H_2O_2	Dominates in excess concentration	Dominates in relatively low concentration
DNPO	Dominates in low concentration	Dominates in excess concentration
H_2O	CL intensity decreases with its addition	CL intensity increases with its addition
DNP	CL intensity decreases with its addition	CL intensity increases with its addition

(direct CL reaction) and the slow decay curve (DNP-catalyzed CL reaction) as shown previously in Figs. 3 and 4. The effect of H_2O for each reactive condition (direct CL reaction: [DNPO] = 0.01 mM, [H_2O_2] = 34.0 mM, [perylene] = 0.025 mM; DNP-catalyzed CL reaction: [DNPO] = 3.0 mM, [H_2O_2] = 10.0 mM, [perylene] = 0.025 mM) in acetonitrile was then investigated (data not shown). The results obtained under both reaction conditions were consistent with those shown in Figs. 3 and 4. In other words, the intensity obtained from the direct CL reaction in the presence of H_2O decreased, whereas the intensity shown in the DNP-catalyzed CL reaction with H_2O increased. The decay curve observed under either the direct CL reaction or the DNP-catalyzed CL reaction in aqueous solution is obtained from two competitive reaction pathways (the direct CL reaction and the hydrolysis reaction of DNPO, or the DNP-catalyzed nucleophilic CL reaction and the hydrolysis reaction of DNPO). Therefore, DNP generated from the hydrolysis reaction of DNPO with low water content acts as a quencher in the direct CL reaction and acts as a nucleophile in the DNP-catalyzed CL reaction.

Conclusions

Based on the present results observed under the most fundamental PO-CL reaction conditions, we propose possible high energy intermediates formed in DNPO-CL reactions in the absence of catalysts (Scheme 1). The CL effects observed from DNPO molecules formed in the DNP-catalyzed CL reaction might not be significant because large DNP concentrations produced from both the direct CL reaction and DNP-catalyzed CL reaction act to inhibit (quench) the two DNPO-CL reaction pathways. Table 1 summarizes the major differences between the direct CL reaction and the DNP-catalyzed CL reaction.



Scheme 1

Acknowledgements

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