Chemiluminescence of *Cypridina* Luciferin Analogues. Part 1. Effect of pH on Rates of Spontaneous Autoxidation of CLA in Aqueous Buffer Solutions

Ken Fujimori,*[,a] Hiromitsu Nakajima,[a] Katsuhisa Akutsu,[a] Motohiro Mitani,[b] Hideo Sawada[b] and Masaharu Nakayama[b]
[a] *Department of Chemistry, University of Tsukuba, Tsukuba, Ibaraki, 305 Japan*
[b] *Tsukuba Research Laboratory, NOF Corporation, Tsukuba, Ibaraki, 300–26 Japan*

From the pH–rate profile of autoxidation of the *Cypridina* luciferin analogue {2-methyl-6-phenylimidazo[1,2-*a*]pyrazin-3(7*H*)-one, CLA}, CLA was found not to react with ground-state molecular oxygen at a measurable rate, while the conjugate base of CLA was found to react with molecular oxygen with a second-order rate constant of 0.434 ± 0.001 $dm^3\ mol^{-1}\ s^{-1}$ at 25 °C. No chain reaction process contributed in the autoxidation of CLA. Superoxide dismutase (SOD) was observed to reduce the rate of the autoxidation to a small extent in the reaction with low-pH buffers, while SOD did not affect the reaction in buffers of pH higher than 7. The intimate radical pair ($CLA^{\bullet}O_2^{\bullet -}$) is proposed to be the primary product of the reaction.

In connection with the mechanism of bioluminescence of the *Cypridina* luciferin/*Cypridina* luciferase system,[1,2] the chemiluminescence of various *Cypridina* luciferin analogues has been intensively investigated.[1–3] The actual light emitters in the chemiluminescence of the *Cypridina* luciferin analogues have been identified as singlet excited *Cypridina* oxyluciferin analogues. As shown in Scheme 1, the singlet excited states of the anionic forms of the oxyluciferin analogues are formed primarily and these then disappear *via* two pathways: light emission or protonation to give the singlet excited oxyluciferin analogues of the neutral forms which also emit light.[1,3]

Pioneering work on the chemiluminescent reactions of *Cypridina* luciferin analogues with ground-state molecular oxygen was carried out in aprotic solvents containing a strong base or in a mixture of diglyme and aqueous acetate buffer;[1,3] however, no quantitative kinetic investigation on the autoxidation of *Cypridina* luciferin analogues at various pH has been reported. In order to clarify the mechanism of the autoxidation of 2-methyl-6-phenylimidazo[1,2-*a*]pyrazin-3(7*H*)-one (CLA), we determined the second-order rate constants of the reactions between ground-state molecular oxygen and either CLA or its conjugate base (CLA^-). The effect of superoxide dismutase on the rate of the autoxidation of CLA was also investigated at different pH to characterize the CLA radical–superoxide anion radical pair which is proposed to be a primary product in this reaction.

Experimental

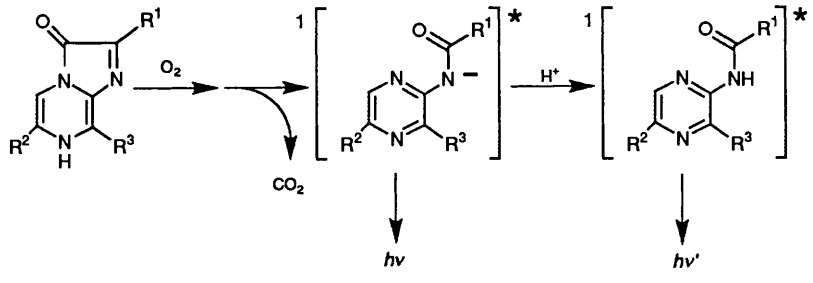
Equipment.—Chemiluminescence and fluorescence spectra were recorded on a Hitachi fluorescence spectrophotometer F4500. Emission of photons was counted by means of an Aloka luminescence reader. Electronic spectra and decay kinetics of CLA were measured with JASCO UV–VIS spectrophotometers Ubest-50 and Ubest-35. These spectrophotometers were equipped with a thermostatted water circulator NESLAB RTE-210. All reactions were carried out at 25.0 ± 0.1 °C. The pH was measured with a Horiba pH meter M-11.

Materials.—Distilled water was passed through ion-exchange resin before use. All buffer solutions were treated with Millipore Chelex 100 before use to eliminate any traces of iron and other heavy-metal ions. CLA was prepared by a known method.[4] CLA hydrochloride was prepared by mixing equimolar amounts of CLA and hydrochloric acid and was recrystallized from MeOH, m.p. 205 °C (decomp.) (in an evacuated tube) (Found: C, 59.7; H, 4.7; N, 16.0. $C_{13}H_{12}ClN_3$ requires C, 59.66; H, 4.62; N, 16.06%). OCLA was prepared by acetylation of 3-amino-6-phenylpyrazine with acetyl chloride in the presence of pyridine, m.p. 148 °C (EtOH). Superoxide dismutase (Cu–Zn type), SOD, from bovine erythrocyte was purchased from Wako.

Method.—Photometric titration was performed by measuring electronic spectra in 0.02 mol dm^{-3} Britton-Robinson buffers of various pH. The emission kinetics of the autoxidation of CLA were determined by counting the photons emitted from the reaction mixture. The reaction was started by addition of an aqueous solution of CLA hydrochloride to 2 cm^3 of buffer in a reaction tube mounted in the thermostatted luminescence reader. The rate of disappearance of CLA was measured by following the decreasing absorbance of CLA in a quartz UV cuvette equipped with water jacket.

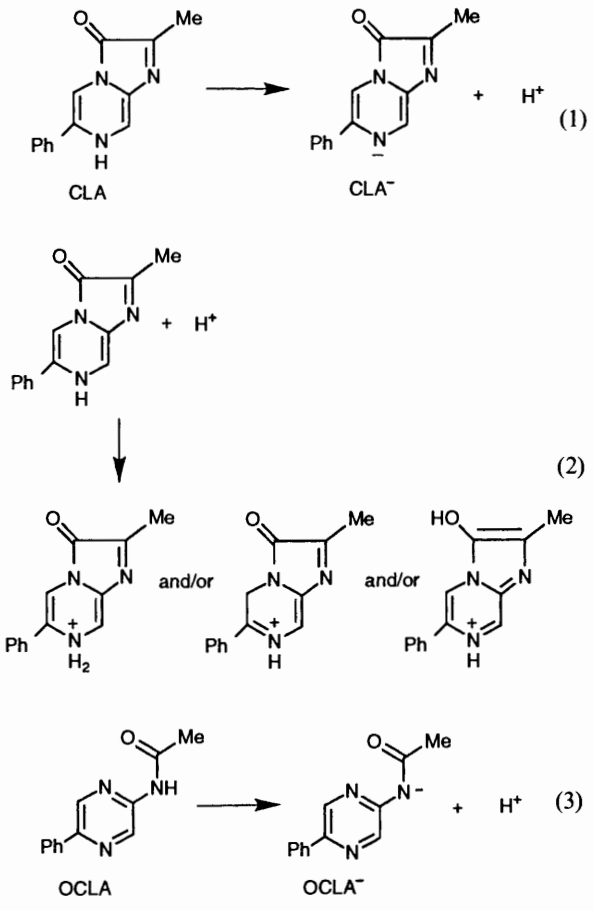
Results and Discussion

Dissociation Constants of CLA.—Both CLA and the corresponding oxyluciferin analogue (OCLA), 3-acetamido-6-phenyl-



Scheme 1

pyrazine, of which the singlet excited state is the actual emitter in the chemiluminescence of CLA, may behave as both nitrogen acids and a base as shown in eqns. (1)–(3). In fact the UV–VIS spectra of both compounds changed as a function of pH. Only spectra for CLA are shown in Fig. 1(*a*).



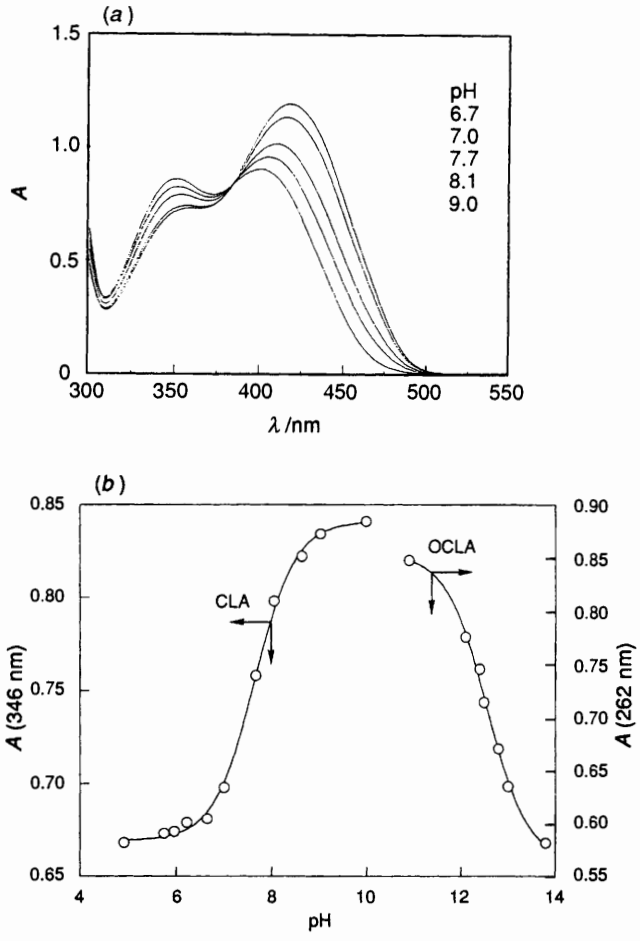


Fig. 1(*a*) Selected UV–VIS spectra of CLA; (*b*) plots of $A_{346\ nm}$ for CLA and $A_{262\ nm}$ for OCLA against pH. [CLA] = 120 μmol dm^{-3}; [OCLA] = 56.0 μmol dm^{-3}. The lines are theoretical curves based on pK_a = 7.64 for CLA and pK_a = 12.58 for OCLA.

Values of pK_{BH^+} and pK_a for CLA were determined to be 2.12 ± 0.36 and 7.64 ± 0.03, respectively, while pK_a for OCLA was found to be 12.56 ± 0.04 from the photometric titration curves shown in Fig. 1(*b*). The theoretical titration curves based on the pK_a values obtained above are shown by solid lines together with experimental results in Fig. 1(*b*). The pK_a value observed for CLA is a little smaller than that for the naturally occurring *Cypridina* luciferin (pK_a = 8.4).[1]

Chemiluminescence of CLA and Fluorescence of OCLA.—Since Goto reported that CLA emits light at the rate first order with respect to the concentration of CLA,[1] the initial rates of the chemiluminescence by the autoxidation of CLA were measured at various pH to evaluate the quantitative reactivities of CLA and CLA$^-$ toward molecular oxygen. The results are shown in Figs. 2 and 3. Unfortunately, however, the attempt was unsuccessful, since the chemiluminescence was found to be a complex function of pH as mentioned below.

Chemiluminescence spectra of the autoxidation of CLA are shown in Fig. 2(*a*). The chemiluminescence spectrum obtained at pH 11.1 differs from the fluorescence spectrum [Fig. 2(*b*)] of OCLA recorded under the same conditions, but is identical with the fluorescence spectrum of OCLA$^-$ obtained in 1 mol dm^{-3} aqueous NaOH. The chemiluminescence spectrum obtained for a solution at pH 7 can be superimposed on the fluorescence spectrum of OCLA recorded in buffer solution at pH 11.1. These observations clearly indicate that the autoxidation of CLA$^-$ affords primarily the singlet excited anionic form of the emitter, ^{1}(OCLA$^-$)*, regardless of the pH of the solution. This conclusion is essentially the same result reported by McCapra[3] and Goto.[1] ^{1}(OCLA$^-$)* decays by two competitive paths, *i.e.*, transition to the ground state of OCLA$^-$ emitting light (λ_{em} = 455 nm) and the protonation of ^{1}(OCLA$^-$)* to give the acid form of the emitter, ^{1}(OCLA)*, which emits light at λ_{em} = 380 nm. The quantum yield of the fluorescence of the latter is greater than that of the former.[5] The pH–rate profile for the chemiluminescence shown in Fig. 3 was found not to be suitable for the determination of the rate of the second-order reaction of CLA with superoxide. Thus, the rate of the autoxidation was measured by following the decay of CLA.

Kinetics for Decay of CLA.—In order to determine the mechanism for the initial stage of autoxidation of CLA, kinetic experiments measuring the disappearance of CLA in aqueous buffers of various pH were carried out under three different conditions: under air, under air with SOD, and under an argon atmosphere. Under all these conditions, the disappearance of CLA followed first-order kinetics [eqn. (4)]. One example is shown in Fig. 4(*a*).

$$-\mathrm{d[CLA]}/\mathrm{d}t = k_{obs}[\mathrm{CLA}] \quad (4)$$

Apparent first-order rate constants obtained under these three conditions are denoted k_{air}, k_{SOD} and k_{Ar}, respectively. The results are listed in Table 1. Since k_{Ar} is independent of autoxidation, the apparent rate constant for the autoxidation of CLA (k_{auto}) is obtained by subtracting k_{Ar} from k_{air} [eqn. (5)].

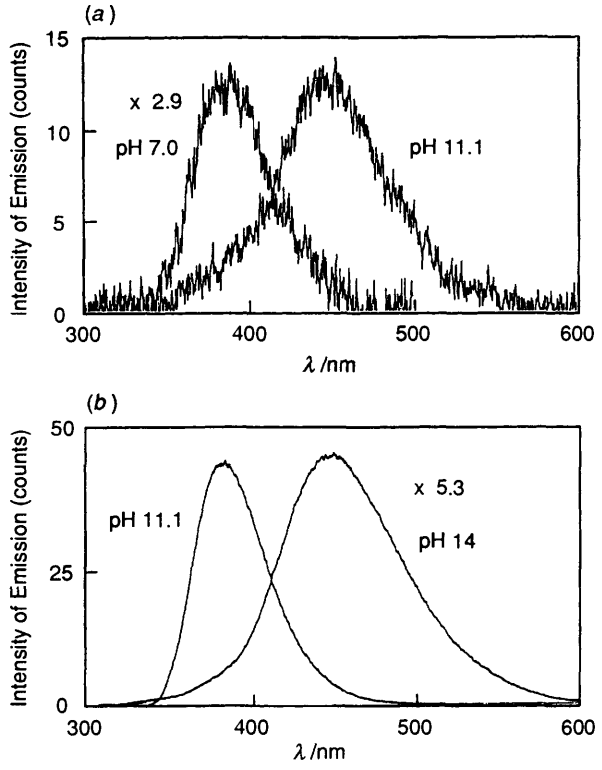


Fig. 2 (*a*) Chemiluminescence spectra of CLA taken in buffer at pH 7.0 and 11.1, $[CLA]_0 = 14.6\ \mu mol\ dm^{-3}$; (*b*) fluorescence spectra of OCLA (excitation light 313 nm) recorded at pH 11.1 and 14

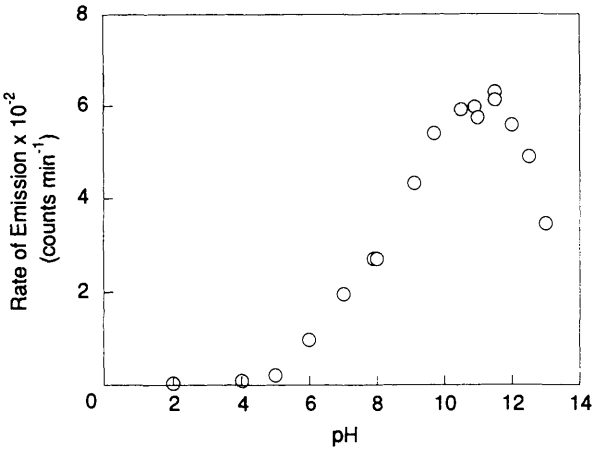


Fig. 3 Plot of initial rate of emission of the chemiluminescence reaction against pH; under air, $[CLA] = 6.84\ \mu mol\ dm^{-3}$

$$\text{Rate of autoxidation} = k_{auto}[CLA] = (k_{air} - k_{Ar})[CLA] \quad (5)$$

As shown in Fig. 4(*b*), the rate of the autoxidation was found to be first order with respect to the concentration of oxygen, and hence the rate equation is given by eqn. (6). These kinetic results

$$\text{Rate of autoxidation} = k_o[CLA][O_2] \quad (6)$$

suggest that the autoxidation of CLA involves no chain-reaction path, but is a clear bimolecular reaction between CLA and dioxygen. This is also supported by the kinetic observation that the presence of SOD changed the rate of the autoxidation hardly at all as will be discussed later.

Logarithms of k_{auto} are plotted in Fig. 5 against the pH of the medium. Although log k_{auto} is constant in the pH range greater than the pK_a of CLA, it decreases with the pH in the region pH < pK_a of CLA. This observation clearly reveals that CLA is inert to molecular oxygen and that CLA^- is actually the active species that reacts with molecular oxygen. Thus, k_o may be expressed by eqn. (7), where k_{oa} and k_{ob} are second-order rate

$$k_o = (k_{oa}[H^+] + k_{ob}K_a)/([H^+] + K_a) \quad (7)$$

constants for the reactions of CLA and CLA^- with O_2, respectively. Upon treatment of the experimental k_{auto} data and $[O_2] = 2.58 \times 10^{-4}$ mol dm^{-3} with eqn. (7) by using a non-linear least-squares method, one obtains k_{ob} and pK_a values of $0.434 \pm 0.001\ dm^3\ mol^{-1}\ s^{-1}$ and 7.41 ± 0.09, respectively, while k_{oa} is almost zero ($1.9 \pm 2.6 \times 10^{-3}\ dm^3\ mol^{-1}\ s^{-1}$). The kinetic p$K_a$ value of CLA thus obtained is well in accordance with the pK_a determined by the photometric titration of CLA.

Interestingly, values of k_{Ar} in Table 1 were found to obey eqn. (7), *i.e.*, the pH–log k_{Ar} prophile is the same shape as that for k_{auto} shown in Fig. 5. Eqn. (8) was obtained by treating the data

$$k_{Ar} = (4.35 \pm 0.19) \times 10^{-5}/([H^+] + 10^{7.6 \pm 0.15}) \quad (8)$$

by a non-linear least-squares method. The kinetic pK value obtained, 7.60 ± 0.15, is identical with the thermodynamic pK_a of CLA. These observations suggest that the major reaction path of the decay of CLA under argon is an oxidation process rather than by hydrolysis, which was first considered to be responsible for the decay of CLA under argon. Although all reagents and water were carefully treated with ion-exchange resin to remove transition-metal ions as mentioned in the Experimental section, an unknown oxidant might be involved in the system to promote the oxidation of CLA^-. In other words, CLA^- is very suspectible to oxidants.

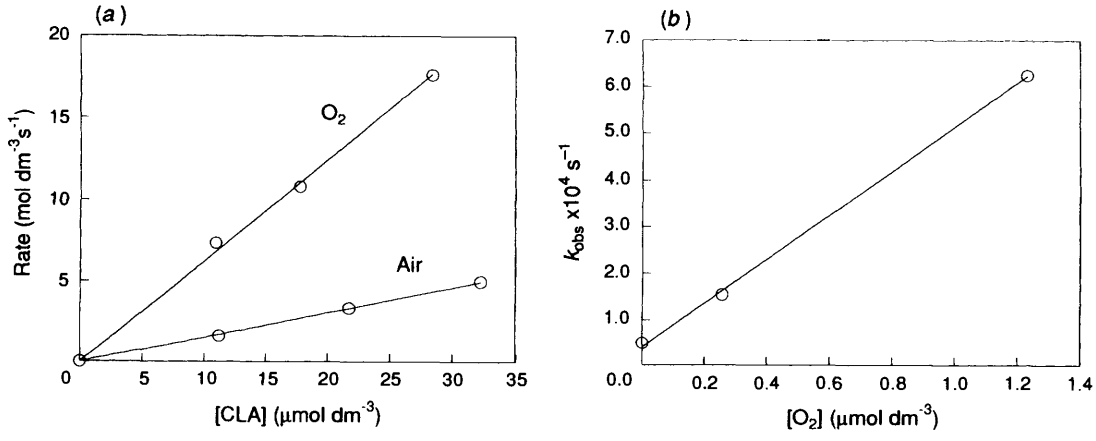


Fig. 4 The rate of the autoxidation of CLA at pH 11.1 as a function of the concentrations of (*a*) CLA with $[O_2] = 0.258$ mmol dm^{-3}, under air and 1.22 mmol dm^{-3} under oxygen, and (*b*) O_2 with $[CLA]_0 = 22.4\ \mu mol\ dm^{-3}$

Table 1 First-order rate constants of autoxidation of CLA at 25 °C

pH	$k_{air}/10^{-6}$ s^{-1}	$k_{SOD}/10^{-6}$ s^{-1} [a]	$k_{Ar}/10^{-6}$ s^{-1}
5.5	2.27 ± 0.10	1.88 ± 0.10	0.434 ± 0.015
6.0	5.43 ± 0.14	4.61 ± 0.15	2.94 ± 0.00
6.5	17.5 ± 0.8	13.6 ± 0.7	4.74 ± 0.16
7.0	44.5 ± 1.1	44.7 ± 0.8	8.89 ± 0.16
7.0	34.6 ± 0.9	40.1 ± 1.3	21.6 ± 1.6
8.0	96.0 ± 2.4	97.3 ± 1.2	43.6 ± 1.5
9.0	155 ± 1	146 ± 1	35.7 ± 1.0
10.0	161 ± 2	160 ± 3	40.4 ± 0.7
11.1	154 ± 1	144 ± 2	49.2 ± 1.1
12.0	145 ± 1	143 ± 1	46.3 ± 0.5

[a] [SOD] = 1.12 μg cm^{-3}.

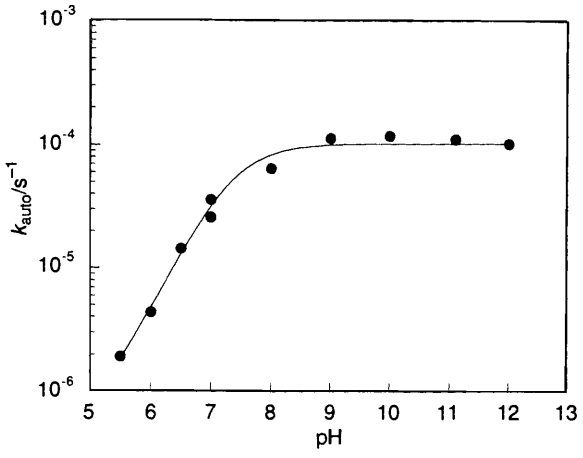


Fig. 5 Plots of k_{auto} (under air) against pH; the solid line is the theoretical curve based on k_{oa} = 0.0019 dm^3 mol^{-1} s^{-1}, k_{ob} = 0.434 dm^3 mol^{-1} s^{-1} and pK_a = 7.41 for the autoxidation of CLA under air

According to the spin correlation rule, reaction of a singlet anion with triplet molecular oxygen generates a triplet species at first, which is eventually converted into singlet product(s). Russell *et al.* discussed this point in their pioneering works on the autoxidation of carbanions.[6] Later, Jefford argued this problem in detail and suggested the following two paths for the reaction.[7] One is initiated by a single electron transfer from an anion to triplet molecular oxygen as shown in mechanism (9).

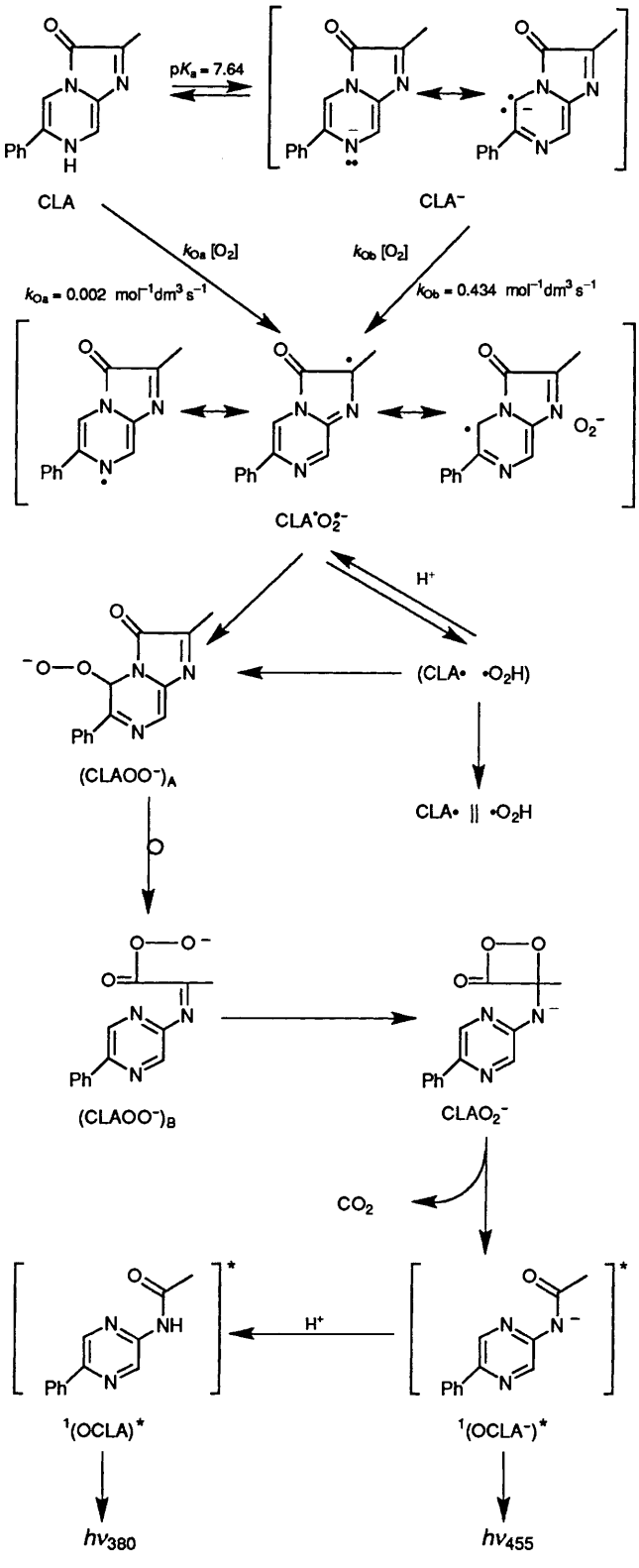
$$R^- + O_2 \longrightarrow {}^3[R^\bullet + O_2^{\bullet -}] \longrightarrow {}^1[R^\bullet + O_2^{\bullet -}] \longrightarrow RO_2^- \quad (9)$$

The other involves the initial formation of an anion–molecular oxygen CT-complex in the triplet state which changes to a singlet CT-complex eventually affording a hydroperoxide anion as shown in mechanism (10).

$$R^- + O_2 \longrightarrow {}^3[R^{\delta -} \cdots O_2^{\delta -}] \longrightarrow {}^1[R^{\delta -} \cdots O_2^{\delta -}] \longrightarrow RO_2^- \quad (10)$$

To clarify the mechanism of the reaction of CLA$^-$ with molecular oxygen, the rates of the reaction were measured in the presence of superoxide dismutase (SOD), which is known to be sufficiently active to quench superoxide in the pH range from 5–12.[8] If the reaction proceeds by the mechanism (9), to some extent the radical pair may give a separated free CLA radical and superoxide anion radical which can be quenched by SOD. Since superoxide anion radical consumes CLA,[1,5] quenching of superoxide anion radicals should reduce the apparent rate of the autoxidation of CLA. This was not the case in buffer solutions at pH greater than 8, while in the lower pH region the presence of SOD was found to reduce the rate a little. This trend became slightly more apparent as the pH decreased. Although the effect is very small, the fact that SOD affects the kinetics suggests that the reaction between CLA$^-$ and triplet molecular oxygen proceeds *via* the single electron transfer mechanism (9).

All the experimental results may be best explained by the mechanism shown in Scheme 2. The single electron oxidation of CLA$^-$ by O_2 generates an intimate pair of the CLA radical and superoxide anion radical, (CLA$^\bullet$ $O_2^{\bullet -}$), the combination of which takes place faster than diffusion. On the other hand,

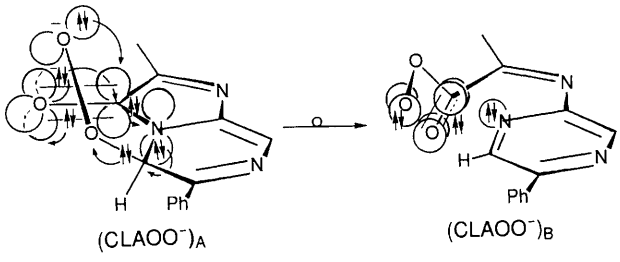


Scheme 2 Autoxidation of CLA (25 °C)

the combination of the radical pair competes with protonation of $O_2^{\bullet -}$ to form the protonated radical pair, (CLA$^{\bullet}$ $^{\bullet}O_2H$). Since the hydroperoxyl radical may move faster than the highly solvated superoxide anion radical in water, (CLA$^{\bullet}$ $^{\bullet}O_2H$) may have a much greater chance of separation than (CLA$^{\bullet}$ $O_2^{\bullet -}$). Then, in low-pH buffer, very small amounts of (CLA$^{\bullet}$ $O_2^{\bullet -}$) are converted into separated pairs *via* the protonated pair (CLA$^{\bullet}$ $^{\bullet}O_2H$).

There are a number of possible ways to combine CLA$^{\bullet}$ with $O_2^{\bullet -}$ as exemplified by the canonical forms of CLA$^{\bullet}$ in Scheme 2. Among them, the pioneering workers predicted the coupling of CLA$^{\bullet}$ at the 2- and/or 5-positions. Scheme 2 illustrates the most plausible mechanism, *i.e.*, the coupling with $O_2^{\bullet -}$ takes place at the 5-position. This is based on the observation by Goto *et al.* that CLA$^{\bullet}$ combines reversibly at the 4-position affording the dimer under anaerobic conditions[7] and a study of coelenterazine by Teranishi *et al.*[10]

$(CLAOO^-)_A$ may afford $^1(OCLA^-)^*$ *via* the rearranged peroxy acid $(CLAOO^-)_B$ and the dioxetanone intermediate, $(CLAO_2)$. The concerted rearangement of $(CLAOO^-)_A$ to $(CLAOO^-)_B$ may take place in the same manner as the concerted S_N2 reaction on the nitroso-nitrogen[11] and the intramolecular acyl migration in the acid anhydride.[12] The stereoelectronic course is shown in Scheme 3. Observations



Scheme 3

leading to this conclusion are (*a*) the orientation of the entering group and the leaving group is quasi-perpendicular and (*b*) the trigonal plane of the reaction centre rotates 90° during the reaction.

As was mentioned earlier, $^1(OCLA^-)^*$ is formed initially by decarboxylation of intermediate dioxetanone and, if the concentration of H^+ is high enough to compete, protonation with the light-emitting process, the emission from $^1(OCLA)^*$, becomes observable. Unfortunately the intensity of the light is so weak in this autoxidation of CLA that we could not undertake a quantitative treatment of the competitive pathways of $^1(OCLA^-)^*$.

Acknowledgements

This work is partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (No. 02804037).

References

1 T. Goto, *Pure Appl. Chem.*, 1968, **17**, 421; *The Role of Oxygen in Chemistry and Biochemistry*, eds. W. Ando and Y. Moro-oka, Elsevier, Amsterdam, 1989, pp. 367–382.
2 Y. Kishi, T. Goto, Y. Hirata, O. Shimomura and F. H. Johnson, *Tetrahedron Lett.*, 1966, 3427; O. Shimomura and F. H. Johnson, *Photochem. Photobiol.*, 1970, **12**, 291; O. Shimomura and F. H. Johnson, *Biochem, Biophys. Res. Commun.*, 1971, **44**, 340.
3 F. McCapra and Y. O. Chang, *J. Chem. Soc., Chem. Commun.*, 1967, 1011; F. McCapra, Y. O. Chang and V. P. Francois, *J. Chem. Soc., Chem. Commun.*, 1968, 22.
4 S. Inoue, S. Sugiura, H. Kakoi and T. Goto, *Tetrahedron Lett.*, 1969, 1606.
5 K. Akutsu, H. Nakajima and K. Fujimori, to be published.
6 G. A. Russell, A. G. Bemis, E. J. Geels, E. G. Janzen and A. J. Moye, 'Oxidation of Organic Compounds 1', *Advances in Chemistry*, Series No. 75, 1968, 174, Am. Chem. Soc., Washington DC; J. A. Howard, *Free Radicals*, ed. J. K. Kochi, 1973, Vol. 2, pp. 37–41, Wiley, New York.
7 C. W. Jefford, *Molecular Mechanism of Enzyme-Catalyzed Dioxygenation*, ed. C. W. Jefford and P. A. Cadby, Springer, New York, 1981, pp. 191–265.
8 B. H. Bielski, D. E. Cabelli, R. L. Arudi and A. B. Ross, *J. Phys. Chem. Ref. Data*, 1985, **14**, 1083; D. E. Cabelli, D. Allen, B. H. J. Bielski, *J. Biol. Chem.*, 1989, **264**, 9967.
9 Y. Toya, S. Nakatsuka and T. Goto, *Tetrahedron Lett.*, 1985, **26**, 239.
10 K. Teranishi, M. Isobe, T. Yamada and T. Goto, *Tetrahedron Lett.*, 1992, **33**, 1303.
11 S. Oae, N. Asai and K. Fujimori, *J. Chem. Soc., Perkin Trans. 2*, 1978, 571, 1124.
12 K. Fujimori and S. Oae, *J. Chem. Soc., Perkin Trans. 2*, 1989, 1335.

Paper 3/02951D
Received 24*th May* 1993
Accepted 9*th August* 1993