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Full Length Article

Quenching Effect of some Amino Acids on Peroxyoxalate Chemiluminescence of the Biphenylquinoxaline Derivative, Dibenzo [a,c] Phenazin-11amine

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ABSTRACT
The quenching effect of L-cysteine,L-methionine, L-lysine, L-arginine and aspartic acid on strong chemiluminescence of bis-(2,4,6-trichlorophenyl)oxalate–H_2O_2 system in the presence of 2,3-biphenylquinoxalinewas studied. The chemiluminescence parameters were evaluated from computer fitting ofthe resulting intensity-time plots. These systems resulted in Stern–Volmer plots in the quencher concentration range of 1.67×10^{-5} to 1.67×10^{-4} M with $k_Q$values of 44758, 24976, 13648, 11206 and 7116 for Met, Cys, Asp, Arg and Lys respectively.
Keywords: Chemiluminescence, peroxyoxalate, quinoxaline, quenching, amino acid

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INTRODUCTION

A peroxyoxalate chemiluminescence (PO-CL) method has been widely utilized in environmental, pharmaceutical and biomedical analysis owing to its high sensitivity and a need of simple instrumentation without a light source [1,2]. It has been clearly shown that there are at least two characteristics necessary in the design of useful reagents for the PO-CL. First, the existence of some electron-withdrawing groups around the central peroxyoxalate moiety to facilitate the generation of reactive intermediates responsible for the excitation energy transfer to a fluorophore compound [3] and second, sufficient solubility of the leaving group in the solvent used.

Quinoxalines are well known fluorescent compounds with high quantum yields and have attracted much attention due to their potential functions for high technology applications [4, 5]. These compounds have been utilized as fluorescence probes in some of elaboratedchemosensors.Quinoxalines have shown broad biological activities such as in vitro antitumor activity [6], antiviral [7], herbicidal [8] and anti-inflammatory activity [9], hence they are an important class of nitrogen-containing heterocycles and useful intermediates in organic synthesis [10, 11].

Quinoxalinederivative ofbiphenylquinoxaline (dibenzo[a,c]phenazin-11amine)was used (Fig. 1). Quinoxaline derivative is found intense and useful fluoropher compound containing aromatic functional group with lowenergy $\pi \rightarrow \pi^*$ transition level (green light emission).

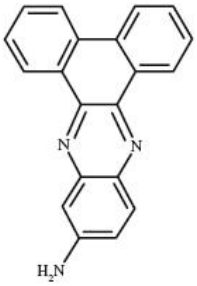


Fig. 1 Molecular structure of the biphenylquinoxaline derivative.

Amino acids are important biologically active compounds in biological tissues and fluids, and foods. In living organism, they play an important role in protein synthesis, lymphocyte growth and metabolism [12]. Analytical methods, including HPLC [13], capillary electrophoresis [14, 15], fluorescence [16], and spectrophotometry [17], have been developed for the determination of amino acids.
There are a small number of reports describing chemiluminescence reactions of individual amino acids which have mainly been applied to the analysis of pharmaceutical formulations [18-25].

Experimental

Reagents

All chemical compounds were reagent-grade and purchased from Fluka chemical company (CH-9470, Buchs, Switzerland) and used as received without further purification. Quinoxaline derivative was prepared from the reaction of 4-Nitro-O-phenylen diamine with benzyl and 9,10-phenanthroquinone in glacial acetic acid as a solvent and then were reduced by hydrazine monohydrate as described in the reference [26, 27].

Apparatus

Chemiluminescence detection was carried out with a homemade apparatus equipped with a model BPY47 photocell (Leybold, Huerth, Germany). The apparatus was connected to a personal computer via a suitable interface (Micropars, Tehran, Iran). Experiments were carried out with magnetic stirring (500 rpm) in a light-tight flattened bottom glass cell of 15 mm diameter at room temperature.
Steady-state fluorescence spectra were recorded on a Perkin Elmer, LS-3B spectrofluorimeter instrument. The excitation monochromator was set at 430 nm for the biphenylquinoxalinecompound. Spectral bandwidth of 3 nm was used.

Procedures

Chemiluminescence and fluorescence spectra
Solution Iwas made with mixing 1.0 ml of TCPO (0.01 M), 0.5 mL of the quinoxaline derivative (0.001 in ethyl acetate) and 1.5 ml EtOAc. Solution II contained 2 mlof hydrogen peroxide (3.2 M) and 1.0 ml of sodium salicylate(0.1 M) in methanol. Solution I was transferred into glasscell using polypropene syringes. Then 100 µL of solution IIwas injected in the glass cell, chemiluminescence spectrum was recorded soon after mixing of solutions. The fluorescence spectra were carried out using freshly prepared solutions containing 5×10^{-4} M of fluorophores in ethylacetate, using a 3-cm quartz cuvette.
Effect of aminoacids concentration on PO–CL of thequinoxaline derivative
The cell was charged by 1.5 ml EtOAC, 0.5 mL of quinoxaline derivative (0.001M inEtOAc), 1.0ml TCPO (0.01M in EtOAc), and the various concentration of an aminoacids (0.001M in MeOH).The light intensity decay curves were them obtained after introduction of100 µL of solution contained 2 ml of hydrogen peroxide (3.2 M) and 1.0 ml of sodium salicylate(0.1 M) in methanol was injected into the cell.

RESULTS AND DISCUSSION

Peroxyoxalatechemiluminescence (PO–CL) is wellknownas one of the most efficient non-biological light producingsystems [28-34]. The possible mechanistic pathway forthe PO–CL process involves the following steps [35-37]:

$$TCPO + H_2O_2 \xrightarrow{K_1} C_2O_4 + 2TCP \quad (1)$$

$$C_2O_4 + F \xrightarrow{K_2} [C_2O_4 \cdot F^+] \quad (2)$$

$$[C_2O_4^- \cdot F^+] \xrightarrow{K_3} F^+ + 2CO_2 \quad (3)$$

$$F^* \xrightarrow{K_4} F + h\nu_1 \quad (4)$$

$$F^* \xrightarrow{K_5} F + \text{heat} \quad (5)$$

where TCP is 2,4,6-trichlorophenol and F is fluorescer.

The crucial step in luminescence quenching is assumed to be the reaction of the quencher Q with the highly energetic intermediate C_2O_4 to give non chemiluminescent products, in competition with the reaction of fluorescer, as follows:

$$C_2O_4 + Q \xrightarrow{K_q} \text{non-chemiluminescent products} \quad (2a)$$

The florescence spectra of compound areshown in Fig. 2.

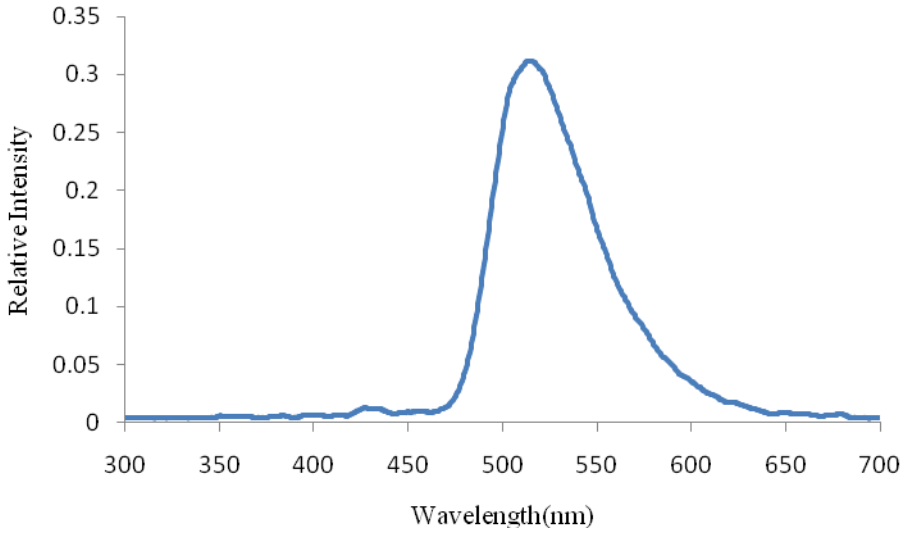
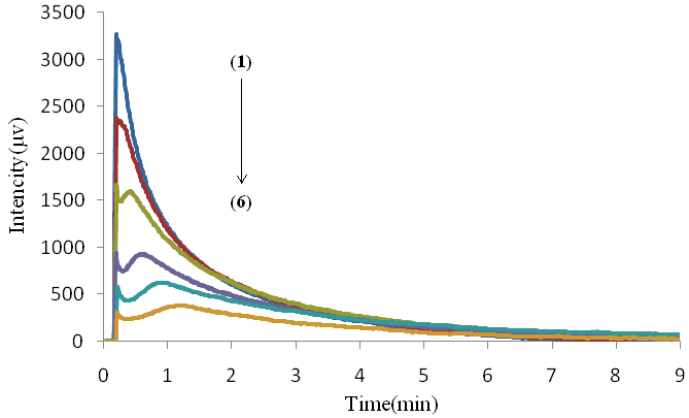
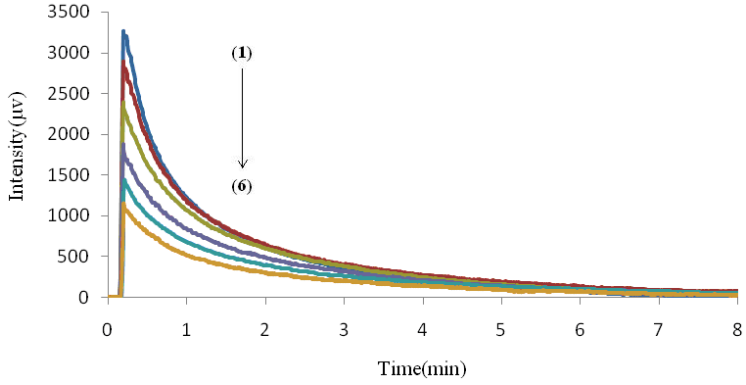


Fig. 2. The fluorescence emission spectra of 5×10^{-4} M of the biphenylquinoxaline with λ_{ex}=430 nm in ethyl acetate.

Fig. 3 (a and b) shows typical response curves (i.e., light intensityversus time) for the PO–CL system ofthe biphenylquinoxaline in the absenceand presence of methionine andarginine, respectively.Typically, in chemiluminescence system, soon after mixing of ingredients (e.g., the biphenylquinoxaline, bis-(2, 4, 6-trchlorophenyl) oxalate, H_2O_2 and sodium salycilate) the intensity of emitted light is risen rapidly and then exponential decay of light intensity follows. In the case of methionine there are two peaks.



3(a)



3(b)

Fig. 3 (a and b).Chemiluminescence emission intensity as a function of time for thebis-(2, 4, 6-trchlorophenyl) oxalate (TCPO), H_2O_2, 2,3-Biphenylquinoxaline and sodium salysilate system with constant concentration of TCPO (3.2×10^{-3} M), H_2O_2 (6.8×10^{-2} M), sodium salicylate (1.0×10^{-3} M),

biphenylquinoxaline (1.6×10^{-5}M) in the absence and presence of varying concentrations of Met (a)and Arg (b). The amino acid concentration (M) is: (1) 0.00,
(2) 1.67×10^{-5}, (3) 5×10^{-5}, (4) 8.33×10^{-5}, (5) 1.33×10^{-4}, (6) 1.67×10^{-4}.

However in the presence of amino acids, was found to quench the chemiluminscencesystem considerably. The resulting intensity decay curves inthe presence of increasing amino acids is also included in Fig. 3.Based on the proposed mechanism, and from steady-statekinetics calculations, the chemiluminescence intensity forfluorescer(F) in the absence of quenchers (Q) can be written as:

$I_0 = k\,[C_2O_4][F]$ (6)

where $k = k_2k_4/(k_4 + k_5)$.In the presence of quencher Q, the chemiluminscence intensity is reduced from I_0 to I, which is given by:

$$I = k\left(\frac{k_2[F]}{k_2[F] + k_{2a}[Q]}\right)[C_2O_4][F] \quad (7)$$

The ratio of I_0/I will thus result in a Stern–Volmer type expression as [38]:

$$\frac{I_0}{I} = 1 + \left(\frac{K_{2a}}{K_2[F]}\right)[Q] = 1 + K_Q[Q] \quad (8)$$

Thus, according to equation [35], the concentration of amino acids used (Q) can be determined by quenched chemiluminscence. For a measurement system based on quenching,$k_{2a}/k_2[F]$ should be as large as possible. This can be achievedby lowering the fluorescer concentration or by the choice ofa flourescer that has a low k_2 value. However, $k_2[F]$ cannotbe chosen too small because then I_0 will be very low and the
apparatus noise will largely affect the detection limit.The linear plots of I_0/I versus amino acid concentration for 5 amino acids are shown in Fig. 4.

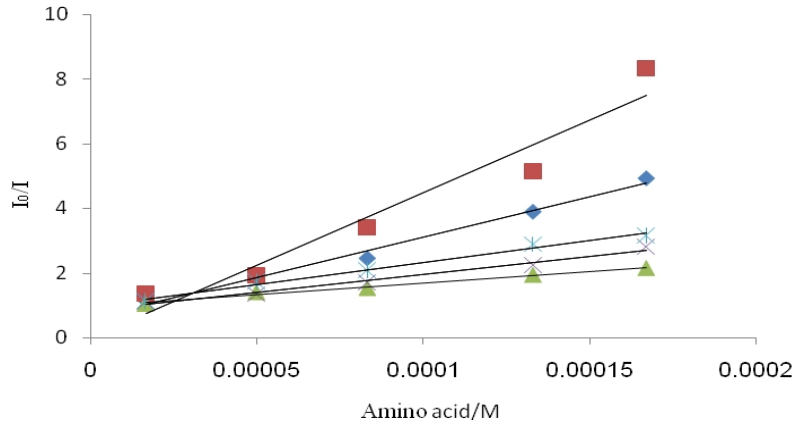


Fig. 4. Stern–Volmer plots for Met (■), Cys (◆), Asp (✳), Arg (✕) and Lys (▲).

The resulting regression equations in the concentration range 1.67×10^{-5} to 1.67×10^{-4} M are as follows:

Met : $I_0/I = 0.009 + 44758$ [Met]; r = 0.9685
Cys : $I_0/I = 0.610 + 24976$ [Cys]; r = 0.9945
Asp : $I_0/I = 0.965 + 13648$ [Asp]; r = 0.9945
Arg : $I_0/I = 0.837 + 11206$ [Arg]; r = 0.9920
Lyz: $I_0/I = 0.977 + 7116$ [Lys]; r = 0.9935

Therefore the measured k_Q values are 44758, 24976, 13648, 11206 and 7116 for Met, Cys, Asp, Arg and Lys respectively.

It is noteworthy that detection of amino acids by quenched chemiluminescence is based on the decrease in the resulting signal. Hence, the noise of this luminescence signal should be reduced as much as possible to achieve a favorable signal–noise ratio for quenched chemilumenescence. This can be reached most easily at high I_0, since a greater dynamic range is then obtained. From these considerations, it can be concluded that the biphenylquinoxaline with an inherently high PO–CL intensity is very appropriate for the quenched chemiluminescence mode.

In order to evaluate the kinetic data for the PO-CL system, a pooled-intermediate model was used [39-41].According to this model, the CL reaction is simplified as:

$$A \xrightarrow{K_r} B \xrightarrow{K_f} C \quad (9)$$

where A, B and C represent pools of reactants, intermediatesand products, respectively, and both reaction stepsdesignated by the rate constants k_r and k_f are irreversible first order reactions. The integrated rate equation for the CLintensity versus time is:

$$I_t = \left[\frac{Mk_r}{k_f - k_r}\right]\left[\exp(-k_r t) - \exp(-k_f t)\right] \quad (10)$$

Where, I_t is the CL intensity at time t, M is a theoretical maximum level of intensity if the reactants were entirelyconverted to a CL-generating material, k_r and k_f are, respectively, the first order rate constants for the rise andfall of the burst of CL. A further advantage of this model is that it not only allows the determination of parameters M, $k_r$and k_f, but also it permits an estimate of the intensity at maximum level (J), the time of maximum intensity (τ_{max})and the total light yield (Y), as follows:

$$J = M\left(\frac{k_f}{k_r}\right)^{[k_f/(k_r - k_f)]} \quad (11)$$

$$\tau_{max} = \frac{\ln(K_f/K_r)}{K_f - K_r} \quad (12)$$

$$Y = \int_0^\infty I_t\, dt = \frac{M}{K_f} \quad (13)$$

In this work, a non-linear least-squares curve fitting program KINFIT [42] was used to evaluate the M, k_r and k_f values from the corresponding CL intensity-time plots. Atypical computer fitting of the CL intensity time plots is shown in Fig. 5. The theoretical values of maximum intensity (J), and corresponding time (τ_{max}) and Y can be calculated by Eqs. 11-13using parameters of k_f, k_r and M which were determined from the resulting CL intensity-time plots.

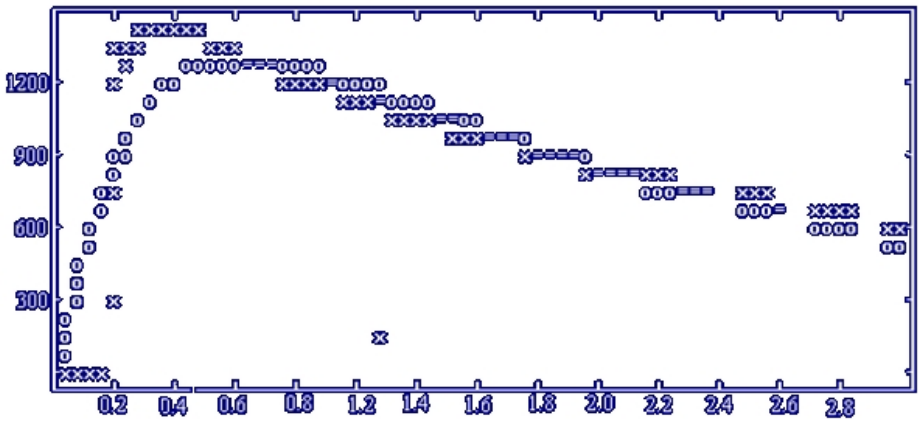


Fig. 5 A typical computer fit of the CL intensity–time plot for TCPO–H2O2–biphenylquinoxaline derivative–sodium salicylate system ([H_2O_2]=0.068 M, [sodium salicylate]=1.0×10^{-3}, [biphenylbiphenylquinoxaline]= 1.6×10^{-5} M, [TCPO]=3.2×10^{-3} M and [cystein]=1.67×10^{-4}M): (×) experimental point; (o) calculated point; (=) experimental and calculated points are the same within the resolution of the plot

All kinetic parameters are listed in Tables 1. The data given in Table 1 indicate that there is a satisfactory agreementbetween the calculated (J) and experimental (I) values of the intensity at the maximum CL for Met and Cys. The quenching effect of Cys and Met may be due to the presence of heavy atom sulfur that facility of the intersystem crossing of the fluorecer excited state.

Parameter changed	Concentration (M)	K_r (min^{-1})	K_f (min^{-1})	M	J	T_{exp}(min)	T_{max}(min)	Y	I
Arginine	1.67×10^{-5}	6.09±0.32	0.81±2.98	2658±65.8	1950	0.19	0.38	3269	2890
	5×10^{-5}	5.99±0.30	0.72±2.54	2172±49.5	1623	0.19	0.40	2985	2385
	8.33×10^{-5}	6.03±0.30	0.69±2.39	1647±36.6	1246	0.19	0.41	2401	1871
	1.33×10^{-4}	5.36±0.28	0.68±2.53	1308±31.2	970	0.21	0.44	1934	1437
	1.67×10^{-4}	5.72±0.29	0.694±2.51	1032±23.9	772	0.2	0.42	1488	1149
Lysine	1.67×10^{-5}	6.35±0.31	0.79±2.68	2820±64.1	2098	0.18	0.37	3573	3060
	5×10^{-5}	5.99±0.29	0.68±2.28	2114±45.1	1600	0.19	0.41	3101	2289
	8.33×10^{-5}	6.37±0.31	0.63±2.09	1833±37.5	1422	0.18	0.40	2908	2090
	1.33×10^{-4}	6.98±0.36	0.49±1.79	1226±24.2	1001	0.18	0.41	2458	1662
	1.67×10^{-4}	4.99±0.29	0.51±2.38	1157±30.0	890	0.24	0.51	2231	1501
Aspartic acid	1.67×10^{-5}	6.28±0.31	0.69±2.41	2582±57.3	1964	0.19	0.39	3713	2875
	5×10^{-5}	5.79±0.26	0.54±1.74	1790±33.2	1400	0.19	0.45	3285	1865
	8.33×10^{-5}	5.55±0.26	0.41±1.56	1291±24.2	1046	0.2	0.50	3097	1553
	1.33×10^{-4}	6.44±0.33	0.41±1.69	839±17.0	696	0.2	0.46	2040	1120
	1.67×10^{-4}	6.49±0.36	0.40±1.81	703±15.3	585	0.21	0.45	1726	1020
Cysteine	1.67×10^{-5}	5.47±0.28	0.81±2.96	2723±67.6	1954	0.2	0.41	3357	2729
	5×10^{-5}	5.65±0.25	0.58±1.81	1818±33.9	1403	0.19	0.45	3144	1752
	8.33×10^{-5}	5.07±0.22	0.46±1.54	1396±24.8	1097	0.26	0.52	3008	1309
	1.33×10^{-4}	5.19±0.21	0.39±1.29	892±14.3	724	0.31	0.54	2309	828
	1.67×10^{-4}	4.72±0.18	0.41±1.28	755±11.7	598	0.34	0.56	1826	656
Methionine	1.67×10^{-5}	5.26±0.26	0.79±2.81	2537±60.5	1811	0.2	0.42	3176	2375
	5×10^{-5}	4.44±0.19	0.64±2.02	1934±38.4	1394	0.2	0.51	2993	1663
	8.33×10^{-5}	3.54±0.13	0.52±0.01	1205±20.5	866	0.19	0.63	2314	943
	1.33×10^{-4}	2.06±9.25	0.45±2.11	895±24.2	586	0.83	0.94	1996	627
	1.67×10^{-4}	0.86±0.16	0.86±0.16	971±186.6	357	1.2	1.16	1127	387

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