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Tetrahedron Letters
Tetrahedron Letters 47 (2006) 6057–6061

Chemiluminescence of 6-aryl-2-methylimidazo[1,2-*a*]pyrazin-3(7*H*)-ones in DMSO/TMG and in diglyme/acetate buffer: support for the chemiexcitation process to generate the singlet-excited state of neutral oxyluciferin in a high quantum yield in the *Cypridina* (*Vargula*) bioluminescence mechanism

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Received 25 May 2006; revised 19 June 2006; accepted 20 June 2006
Available online 10 July 2006

Abstract—The chemiluminescence of 6-aryl-2-methylimidazo[1,2-*a*]pyrazin-3(7*H*)-ones (Cypridina luciferin analogues) in DMSO/1,1,3,3-tetramethylguanidine and in diglyme/acetate buffer was investigated. The results indicate that the reaction mechanism that produces a high chemiluminescence quantum yield involves a chemiexcitation process from a neutral dioxetanone intermediate possessing an electron-donating aryl group ($\sigma_{Ar} < -0.6$) to the singlet-excited state of neutral acetamidopyrazine. This result may be applied to the reaction mechanism for *Cypridina* (*Vargula*) bioluminescence.

The luminous ostracod *Cypridina hilgendorfii* (presently *Vargula hilgendorfii*) produces blue light by a luciferin–luciferase reaction with a high bioluminescence quantum yield ($\Phi_{BL} = 0.3$).[1–3] In the luciferin–luciferase reaction, Cypridina luciferin is oxidized by triplet molecular oxygen (O_2) to give the singlet-excited state of the neutral amide form of Cypridina oxyluciferin (Scheme 1).[1b,2a] In Cypridina luciferin, the core structure for the bioluminescence reaction is an imidazo[1,2-*a*]pyrazin-3(7*H*)-one (imidazopyrazinone) ring.[4] To establish the reaction mechanism of *Cypridina* bioluminescence, we[5] and other groups[6–9] have studied the chemiluminescence reactions of imidazopyrazinones. These studies employed an aprotic solvent containing a base as the solution in which to run the chemiluminescence reactions. The initial study using DMSO containing $NaHCO_3$ or KOH for Cypridina luciferin produced low chemiluminescence quantum yields (Φ_{CL}).[10] Goto et al. subsequently obtained a high Φ_{CL} (ca. 10% of the observed Φ_{BL}) with diglyme containing an acetate buffer.[2a,7] This finding led them to predict that the chemiluminescence reaction in diglyme/acetate buffer proceeds via the same pathway as that of the bioluminescence reaction. However, the mechanism that produces this high Φ_{CL} has not been clarified.

To clarify the reason for the high Φ_{BL} in *Cypridina* bioluminescence, we compared the chemiluminescent properties of a series of substituted imidazopyrazinones in DMSO/base and in diglyme/acetate buffer, paying close attention to substituent effects. Specifically, we used 6-aryl-2-methylimidazopyrazinones **1H** as Cypridina luciferin analogues [**1Ha**, Ar = *p*-$(CH_3)_2NC_6H_4$; **1Hb**, Ar = *p*-$CH_3OC_6H_4$; **1Hc**, Ar = C_6H_5; **1Hd**, Ar = *p*-ClC_6H_4; **1He**, Ar = *p*-NCC_6H_4; **1Hf**, Ar = 3-indolyl; **1Hg**, Ar = 3-(*N*-methyl)indolyl; **1Hh**, Ar = 3-benzofuranyl; and **1Hi**, Ar = 3-benzothienyl (Scheme 1)]. In

Keywords: Chemiluminescence; *Cypridina* bioluminescence; Imidazopyrazinone; Substituent effects; Reaction mechanism.
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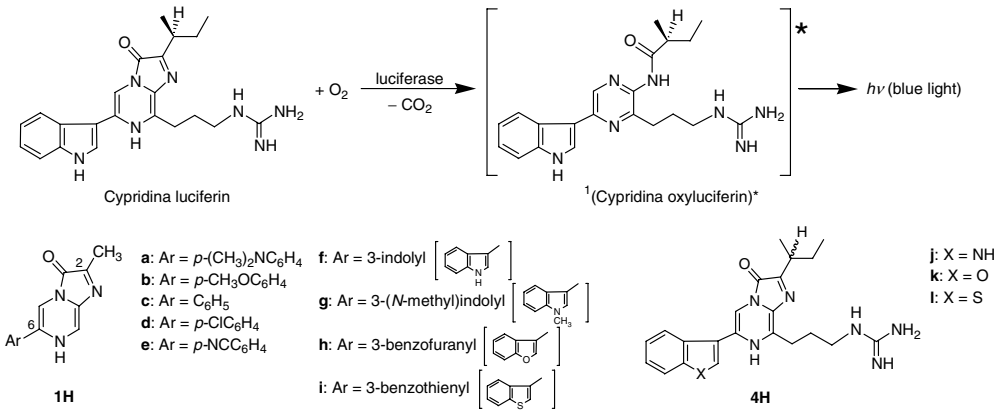
doi:10.1016/j.tetlet.2006.06.101

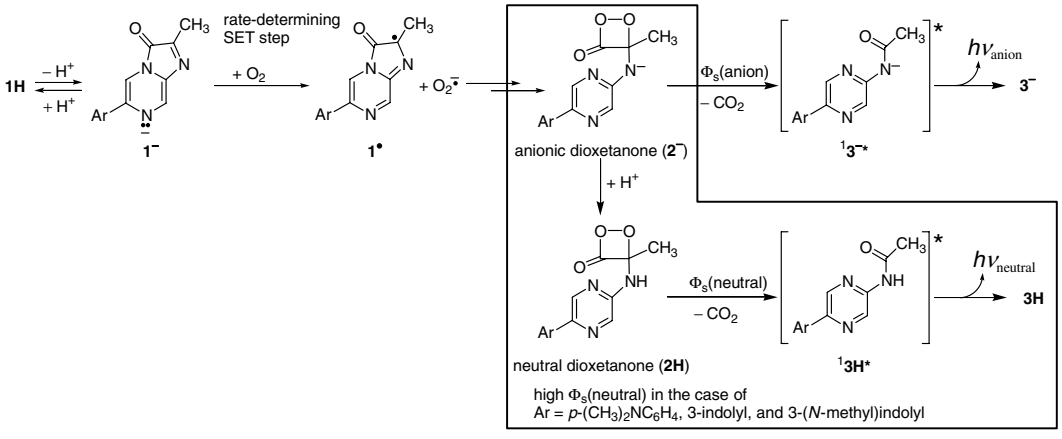


Scheme 1. Top: the *Cypridina* bioluminescence reaction. Bottom: Cypridina luciferin analogues **1H** and **4H**.

particular, the 3-indolyl derivative **1Hf** was chosen as a prototype for Cypridina luciferin.[11,12] Here report the chemiluminescent properties of the series **1H** in these two solvents. They indicate two chemiexcitation processes in the chemiluminescence reactions of **1H**, one of which generates the singlet-excited state of neutral acetamidopyrazine (an oxyluciferin analogue) in a high quantum yield when Ar is an electron-donating group.

We first examined the chemiluminescence reactions of **1H** in aerated DMSO containing 1,1,3,3-tetramethylguanidine (TMG) at 25 ± 1 °C.[13] TMG, a moderate base, generated anion $\mathbf{1}^-$ (Scheme 2).[5c] The reactions of **1H** were traced by monitoring the intensity (I) of the total emitted light, and the observed kinetics indicated a pseudo-first-order reaction similar to that observed for the reactions of **1H** in acetonitrile.[5c,14] The rate constants (k_{obsd}) of the pseudo-first-order kinetics for **1H** are summarized in Table 1, along with the corresponding emission wavelengths (λ_{em}) and values for Φ_{CL}.[15] It can be seen in Figure 1 that the $\log[k_{obsd}(\mathbf{1H})/k_{obsd}(\mathbf{1Hc})]$ values for **1Ha–e** are linearly correlated to the Hammett constant σ_p of the *para*-substituent,[17] indicating that the reaction rate is determined by the single electron transfer (SET) from $\mathbf{1}^-$ to O_2 in the overall reaction process (Scheme 2).[5c] The linear relationship $\log[k_{obsd}(\mathbf{1H})/k_{obsd}(\mathbf{1Hc})] = -0.37\sigma_p - 0.03$ ($r = 0.994$) is used to estimate the electron-donating ability (σ_{Ar}) of the Ar groups. We define $\sigma_{Ar} = \sigma_p$ for **1Ha–e** with phenyl and *p*-substituted phenyl groups. The σ_{Ar} values for 3-indolyl, 3-(*N*-methyl)indolyl, 3-benzofuranyl, and 3-benzothienyl groups in **1Hf–i** were estimated as −0.92, −0.67, +0.45, and +0.29, respectively, by substituting their k_{obsd} values into the above equation. These values indicate that 3-indolyl and 3-(*N*-methyl)indolyl groups have electron-donating abilities similar to that of the *p*-(dimethylamino)phenyl group, while 3-benzofuranyl and 3-benzothienyl groups have electron-donating abilities that fall between those of the *p*-cyanophenyl and *p*-chlorophenyl groups.

Based on previous studies,[5a,b,6,7,11,18] the observed λ_{em} values (467–474 nm) for **1H** in DMSO/TMG indicate that light emission occurs from the singlet-excited state of the acetamidopyrazine anion $^1\mathbf{3}^{-*}$ (Scheme 2). This indicates that $^1\mathbf{3}^{-*}$ is generated by the thermal decomposition of the anionic dioxetanone $\mathbf{2}^-$.[19] The small change observed in Φ_{CL} (0.2×10^{-3}–1.2×10^{-3}) on varying the Ar group indicates that the chemiexcitation process from $\mathbf{2}^-$ to $^1\mathbf{3}^{-*}$ is not greatly affected by a change in the electronic properties of $\mathbf{2}^-$. This conclusion is consistent with findings in our previous report.[5b]



Scheme 2. Chemiluminescence reaction mechanism of **1H** involving the two chemiexcitation processes.

Table 1. Hammett constants (σ_p) and chemiluminescence data (k_{obsd}, λ_{em}, and Φ_{CL}) for **1H** in aerated DMSO/TMG and in aerated diglyme/acetate buffer at 25 °C

Substrate [Ar]	σ_p or (σ_{Ar})	DMSO/TMG[a]			Diglyme/acetate buffer[b]	
		k_{obsd}[c] (mol^{-1} L s^{-1})	λ_{em}[d] (nm)	Φ_{CL}[e] (10^{-3})	λ_{em}[d] (nm)	Φ_{CL}[e] (10^{-3})
1Ha [*p*-$(CH_3)_2NC_6H_4$]	−0.83	0.156	474	1.2	491	15
1Hb [*p*-$CH_3OC_6H_4$]	−0.27	0.099	471	1.1	394	3.1
1Hc [C_6H_5]	+0.00	0.085	467	0.74	390 sh, 445	0.75
1Hd [*p*-ClC_6H_4]	+0.23	0.067	467	0.34	390 sh, 453	0.55
1He [*p*-NCC_6H_4]	+0.66	0.042	474	0.31	453	0.86
1Hf [3-indolyl]	(−0.92)	0.174	474	0.48	432	9.0
1Hg [3-(*N*-methyl)indolyl]	(−0.67)	0.140	473	0.43	437	7.2
1Hh [3-benzofuranyl]	(+0.45)	0.054	470	0.50	389, 450 sh	1.1
1Hi [3-benzothienyl]	(+0.29)	0.062	467	0.24	401, 450 sh	0.55

[a] The concentration of TMG in DMSO was 0.10 mol L^{-1}.
[b] 0.10 mol L^{-1} of acetate buffer (pH 5.6, 0.66% v/v) was mixed in diglyme.
[c] Pseudo-first-order rate constants.
[d] Emission maxima of the chemiluminescence spectra.
[e] Chemiluminescence quantum yields.

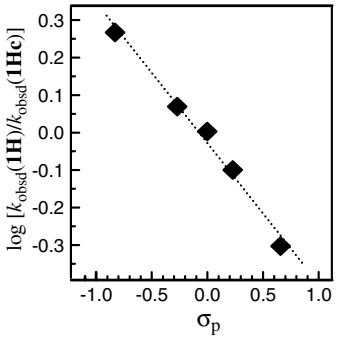


Figure 1. Hammett plot of the log[k_{obsd}(**1H**)/k_{obsd}(**1Hc**)] values for **1Ha–e** against the σ_p constant.

Next, we examined the chemiluminescence reactions of **1H** in aerated diglyme containing acetate buffer (0.10 mol L^{-1} acetic acid–sodium acetate buffer, pH 5.6, 0.66% v/v) at 25 ± 1 °C. A plotting of the time course of *I* produced the growth and decay curve, shown in Figure 2, suggesting that reactions proceed via a stepwise process. The observed λ_{em} and Φ_{CL} values are summarized in Table 1. One particularly interesting result is that the Φ_{CL} values of **1Ha**, **1Hf**, and **1Hg**, whose Ar groups have electron-donating abilities (σ_{Ar} <−0.6), are over 0.007, while the Φ_{CL} values of the other derivatives (except **1Hb**) are in a range similar to those observed in DMSO/TMG. The Φ_{CL} value of the *p*-methoxyphenyl derivative **1Hb** falls between those of **1Ha** and **1Hc**. The Φ_{CL} value of the indolyl derivative **1Hf** is similar to that of Cypridina luciferin ($\Phi_{CL} = 10^{-2}$), as reported by Goto,[2a,7] indicating that the electron-donating indolyl group in Cypridina luciferin is an important contributor to the high Φ_{CL} value.

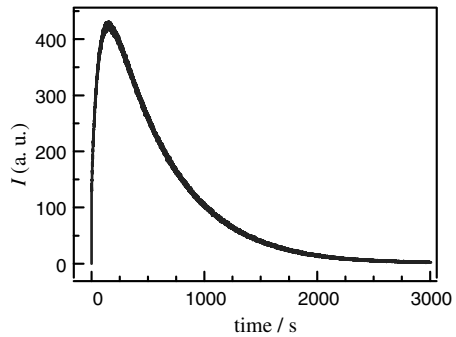
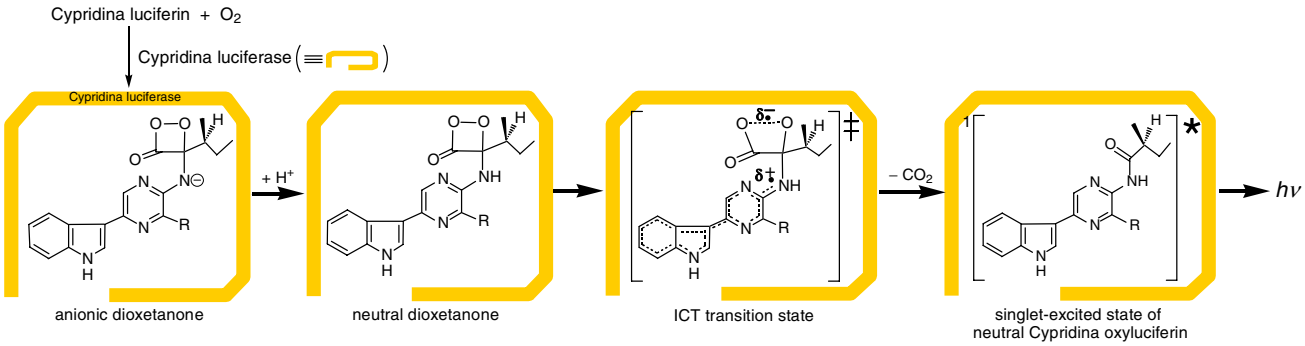


Figure 2. Time course of *I* for the chemiluminescence reaction of **1Hf** (1.0×10^{-6} mol L^{-1}) in aerated diglyme/acetate buffer at 25 ± 1 °C.

Emission spectra for the chemiluminescence of the high-Φ_{CL} derivatives **1Ha**, **1Hb**, **1Hf**, and **1Hg** indicate that light emission occurs from the singlet-excited state of neutral acetamidopyrazine (^{1}**3H***)[20] (Scheme 2); for **1Hc**, **1Hd**, **1Hh**, and **1Hi**, light emission is seen to arise from both ^{1}**3**$^{-*}$ and ^{1}**3H***; finally, the spectrum of **1He** shows light emission occurring only from ^{1}**3**$^{-*}$. These results indicate that an increase in the electron-donating ability of the Ar group favors the pathway that gives rise to the neutral species ^{1}**3H*** as the light emitting source.[21] In previous reports (including ours),[5a,11a] the generation of ^{1}**3H*** has been explained primarily by protonation of ^{1}**3**$^{-*}$, produced in the thermal decomposition of **2**$^-$ via a chemiexcitation process. However, the difference in sensitivity to the Ar group (as observed in the different Φ_{CL} values) depending upon whether the reaction is run in DMSO/TMG or diglyme/acetate buffer can most reasonably be explained by participation of a chemiexcitation process from **2H** to ^{1}**3H***, in addition to that from **2**$^-$ to ^{1}**3**$^{-*}$,[22] as chemiexcitations from **2H** to ^{1}**3H*** and from **2**$^-$ to ^{1}**3**$^{-*}$ would be expected to give different quantum yields for singlet-excited molecules (Φ_S).[25] The chemiluminescence reactions of **1Ha**, **1Hb**, **1Hf**, and **1Hg** in diglyme/acetate buffer give the corresponding **2H** by protonation of **2**$^-$, which have high basicities induced by an electron-donating Ar group, and chemiexcitations from **2H** to ^{1}**3H*** occur with a high Φ_S. Therefore, two factors are important in obtaining a high Φ_{CL}: the chemiexcitation process from **2H** to ^{1}**3H*** and the electron-donating ability of the Ar group in **2H** (σ_{Ar} <−0.6).

Nakamura et al.[12] reported substituent effects of the Ar group on the bioluminescent properties of Cypridina luciferin using racemic luciferin **4Hj** and its analogues **4Hk** and **4Hl** with 3-benzofuranyl and 3-benzothienyl



Scheme 3. Reaction mechanism for *Cypridina* bioluminescence via the neutral dioxetanone intermediate. R = 3-(1-guanidino)propyl.

groups, respectively. The Φ_{BL} ratio observed for **4Hj**, **4Hk**, and **4Hl** (100:6:11) is similar to their Φ_{CL} ratio (100:5:7) in diethyleneglycol monomethyl ether containing acetate buffer, a mixed solvent similar to diglyme/acetate buffer. In their study, Nakamura et al. concluded that these Φ_{BL} and Φ_{CL} ratios were determined by the same factor. The Φ_{CL} ratio observed for **1Hf**, **1Hh**, and **1Hi** in diglyme/acetate buffer (100:12:6) is also similar to the Φ_{BL} and Φ_{CL} ratios observed for **4Hj**, **4Hk**, and **4Hl**, indicating that Nakamura et al.'s findings are attributable to the electronic effects of the Ar group on the bioluminescent properties of Cypridina luciferin. Therefore, the factors that contribute to a high Φ_{CL} for the chemiluminescence of **1H** are applicable to the *Cypridina* bioluminescence mechanism. The singlet-excited state of neutral oxyluciferin is generated with a high Φ_S by the thermal decomposition of the neutral dioxetanone intermediate, as shown in Scheme 3, with the indolyl group acting as an electron-donating group. The 5-(3-indolyl)pyrazinamine moiety in the dioxetanone encourages the decomposition to go through an intramolecular charge transfer (ICT) transition state in the manner predicted by Yamaguchi et al.[26] in the charge transfer–induced luminescence (CTIL) mechanism for high Φ_S.

In conclusion, we have demonstrated that the differences in chemiluminescent properties observed in the series of **1H** are caused by a switch in the chemiexcitation process depending on the nature of the Ar group. In particular, when Ar is an electron-donating group the chemiexcitation process from **2H** to ^{1}**3H*** becomes dominant in diglyme/acetate buffer, and the Ar group in **2H** facilitates generation of a high Φ_S via the CTIL mechanism. This conclusion allows us to propose a reaction mechanism for *Cypridina* bioluminescence that includes chemiexcitation from a neutral dioxetanone intermediate to the singlet-excited state of neutral oxyluciferin. Further study to elucidate on the complete reaction mechanism for the bio- and chemiluminescence of imidazopyrazinones is now in progress.

Acknowledgments

We thank Mr. T. Irie and Mr. H. Kubota at ATTO Co. for their kind assistance in measuring chemiluminescence spectra. This work was supported by grants from the Japanese Ministry of Education, Culture, Sports, Science and Technology [No. 18510181 for S.M. and No. 14050008 (Priority Area No. 417) for H.I.]. H.I. gratefully acknowledges financial supports from the Izumi Foundation, the Shorai Foundation, and the Iketani Foundation for Science and Technology. We also thank Professor M. Ueda (Tohoku University) for his generous considerations.

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20. Fluorescence spectra of **3Ha**, **3Hb**, **3Hf**, and **3Hg** in diglyme/acetate buffer were identical with the corresponding chemiluminescence spectra for **1H**. As a preliminary result, fluorescence quantum yields for **3Ha**, **3Hb**, **3Hf**, and **3Hg** were ca. 0.3.
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