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CYPRIDINA BIOLUMINESCENCE V. STRUCTURE OF EMITTING SPECIES IN THE LUMINESCENCE OF CYPRIDINA LUCIFERIN AND ITS RELATED COMPOUNDS

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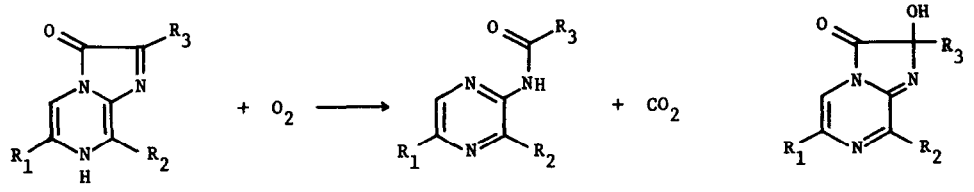
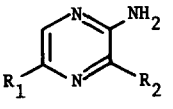
2-Methyl-3,7-dihydroimidazo[1,2-a]pyrazin-3-one (I) chemiluminesces with consumption of one mole of oxygen and produces acetamidopyrazine (II); anion of which being considered to be the emitter of this luminescence reaction, since the fluorescence spectrum of the pyrazine in diethylene glycol dimethyl ether (diglyme) containing potassium t-butoxide is in agreement with the luminescence spectrum (1). This result suggested that the product of luminescence of Cypridina luciferin (III) could be the acylaminopyrazine IV. Reinvestigation of the



(I, III, VII) (II, IV, VI, VIII) (IV')

(I) and (II): R_1=R_2= H, R_3= CH_3; (VI): R_1= 3-indolyl, R_2=H, R_3=CH_3
(III), (IV), (IV') and (V): R_1= 3-indolyl, R_3= $-CH(CH_3)CH_2CH_3$
R_2= $-CH_2CH_2CH_2NHC(=NH)NH_2$
(VII) and (VIII): R_1= phenyl, R_2= H, R_3= CH_3



(V)

structure of Cypridina oxyluciferin, which is considered as a primary product of Cypridina bioluminescence (2), led to the conclusion that oxyluciferin has the structure IV (3) and not the structure IV' previously assigned (2). Thus, the oxyluciferin produced during the bio-

luminescence and the oxyluciferin synthesized from etioluciferin (V) and α-methylbutyric acid anhydride in pyridine show the same characteristic spectra in strongly acidic solutions (Fig. 1), as well as they give the same Rf values on t.l.c. A synthetic model compound, 2-acetamido-5-(indol-3-yl)pyrazine (VI), m.p. 263° (dec.), gives spectra of the similar type. Comparison of fluorescence spectra of these substances in neutral and in basic solutions also supports the above conclusion (Fig. 2). NMR spectrum of IV previously reported (2) does not contradict this new formula. Furthermore, luciferin (III) shows, after chemiluminescence ceased, the same characteristic fluorescence spectra.

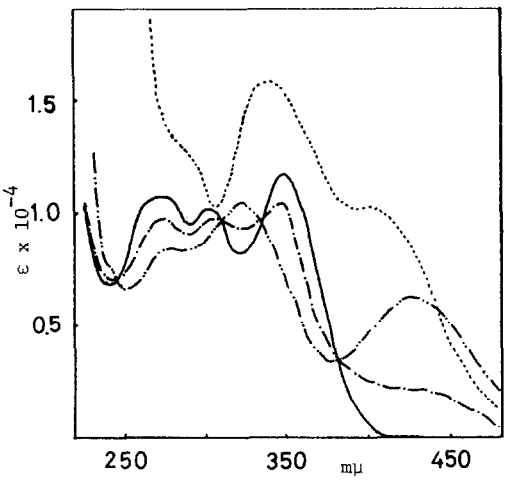


FIG. 1

Absorption spectra of:
——— IV in MeOH
—·— IV in MeOH contg. 0.07N HCl
—··— IV in MeOH contg. 1N HCl
····· VI in DMSO + t-BuOK

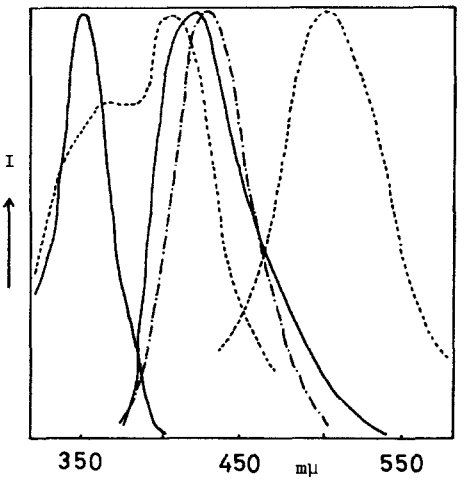


FIG. 2

Fluorescence spectra of:
——— IV in diglyme
····· IV in diglyme + t-BuOK
Left: excitation spectra
Right: emission spectra
Luminescence spectrum:
—·— III in diglyme + acetate buffer

Chemiluminescence spectrum of Cypridina luciferin (III) in diglyme containing acetate buffer (pH 5.6) (1) is similar to the fluorescence spectrum of oxyluciferin (IV) in the same solvent and different from that in a basic solution (Fig. 2), indicating that the emitting species is un-ionized, rather than ionized, molecule of oxyluciferin (IV). Interestingly, 6-phenyl derivative of I (VII), m.p. ca. 259° (dec.), in a favorable conditions gives chemiluminescence, whose spectrum suggests that the emission comes from un-ionized and ionized 2-acetamido-5-phenylpyrazine (VIII), m.p. 166.5-168°, in excited states (Fig. 3). In this

case, proton-transfer reactions are involved in the molecules in electronically excited states. Since the spent solutions of this luminescence show fluorescence spectra same as that of the neutral molecules of VIII (the reverse reaction, k'_{H_2O}, is negligible), it is apparent that anion of the acylaminopyrazine VIII is formed first in excited states and then protonated to produce the excited neutral molecules (Fig. 3). Deuterium isotope effect also

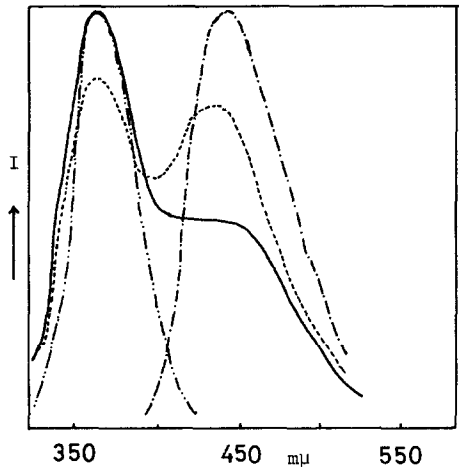


FIG. 3

Luminescence spectra of:
——— VII in 3 ml. diglyme + 20 μl. 0.1M acetate buffer (pH 5.6)
····· VII in 3 ml. diglyme + 20 μl. 0.1M acetate buffer in D_2O (pH 5.6)
Fluorescence spectra of:
—·—·— VIII in diglyme + acetate buffer
—··— VIII in diglyme + t-BuOK

supports this mechanism. Thus, when an ionized form is produced first and then protonated to give neutral molecules in excited states, luminescence intensity corresponding to the ionized form becomes stronger in D_2O than in H_2O, since rates of proton-transfer reactions are usually faster in H_2O than in D_2O (Fig. 3)#. The effect same to that in D_2O is also observed when the concentration of water in the reaction mixture is decreased (4).

$$\text{(VII)} \xrightarrow[\text{slow}]{O_2} R^{-*} + H_3O^+ \underset{k'_{H_2O}}{\overset{k_{H_2O}}{\rightleftharpoons}} RH^* + H_2O$$

$$R^{-*} \longrightarrow R^- + h\nu_1 \qquad RH^* \longrightarrow RH + h\nu_2$$

RH = (VIII) $\qquad k_{H_2O} > k_{D_2O}$

* = singlet excited state

If pKa value of the excited state of VIII in D_2O were higher than that in H_2O, this conclusion is not altered.

In the case of bioluminescence of Cypridina luciferin (III) in phosphate buffer (pH 7.0) in the presence of Cypridina luciferase, the luminescence spectrum ($\lambda_{max.}$ 450 mμ) is not in agreement with the fluorescence spectrum of oxyluciferin (IV) ($\lambda_{max.}$ 480 mμ). While the fluorescence intensity of IV in organic solvents such as DMSO ($\lambda_{max.}$ 427 mμ) or diglyme ($\lambda_{max.}$ 417 mμ) is strong, in aqueous solutions it is very weak (ca. 1/200 of that in the org. solvents).# If we assume that the emitter of Cypridina bioluminescence is oxyluciferin (IV), the fluorescence quantum yield of oxyluciferin in aqueous solutions must be high, since the bioluminescence quantum yield is high (5), and the luminescence spectrum must be identical with the fluorescence spectrum of oxyluciferin. These contradictions may be explained by assuming the presence of an enzyme-substrate complex, in which the emitter, oxyluciferin in excited states, is in environment similar to that in the aprotic solvents.

Satisfactory elemental analyses were obtained for all new compounds whose m.p. are given.

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If the enzyme (crude) is present, however, the fluorescence intensity at 480 mμ is rapidly increased and the spectrum is finally agreed with that of etioluciferin (V); hydrolysis of the acyl group occuring rapidly in the presence of the enzyme (or a contaminated enzyme). Thus, spent solutions for bioluminescence of luciferin show fluorescence spectrum identical with that of etioluciferin (V).