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Dimethyl- and Monomethyloxyluciferins as Analogs of the Product of the Bioluminescence Reaction Catalyzed by Firefly Luciferase

O. V. Leont'eva*, T. N. Vlasova, and N. N. Ugarova

Faculty of Chemistry, Lomonosov Moscow State University, 119992 Moscow, Russia; fax: (7-495) 939-2660; E-mail: ovl@enz.chem.msu.ru

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Abstract—The absorption and fluorescence spectra of dimethyloxyluciferin (DMOL) and monomethyloxyluciferin (MMOL) were studied at pH 3.0-12.0. In the range of pH 3.0-8.0, the fluorescence spectrum of DMOL exhibits a maximum at λ_{em} = 639 nm. At higher pH values an additional emission maximum appears at λ_{em} = 500 nm (wavelength of excitation maximum λ_{ex} = 350 nm), which intensity increases with time. It is shown that this peak corresponds to the product of DMOL decomposition at pH > 8.0. The absorption spectra of MMOL were studied in the range of pH 6.0-9.0. At pH 8.0-9.0, the absorption spectrum of MMOL exhibits one peak at λ_{abs} = 440 nm. At pH 7.3-7.7, an additional band appears with maximum at λ_{abs} = 390 nm. At pH 6.0-7.0 two maxima are observed, at λ_{abs} = 375 and 440 nm. The fluorescence spectra of MMOL (pH 6.0-9.7, λ_{ex} = 440 or 375 nm) exhibit one maximum. It is shown that decomposition of DMOL and MMOL in aqueous solutions results in products of similar structure. DMOL and MMOL are rather stable at the pH optimum of luciferase. It is suggested that they can be used as fluorescent markers for investigation of the active site of the enzyme.

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Key words: bioluminescence, fluorescence, firefly luciferase, luciferin, oxyluciferin, monomethyloxyluciferin, dimethyloxyluciferin

Firefly luciferase catalyses the oxidation of luciferin by oxygen in the presence of Mg-ATP. The first step of the reaction is the interaction of luciferin with ATP yielding luciferyladenylate, which is oxidized by oxygen yielding oxyluciferin in the singlet excited state. The transition of the reaction product into the ground state is accompanied by the emission of visible light. The quantum yield of the reaction is close to unity [1].

Luciferin (the substrate) and oxyluciferin (the product of the reaction) are molecules with clearly pronounced fluorescence properties [2]. For this reason, fluorescent methods are widely used for investigating the interaction of luciferase with the substrate, reaction product, and their analogs. Oxyluciferin is unstable in aqueous solutions, but we expect that its analogs dimethyloxyluciferin (DMOL) and monomethyloxyluciferin (MMOL) are more stable (Fig. 1). In contrast to the oxyluciferin, the MMOL molecule contains a methyl group at the α-position with respect to the carbonyl group. MMOL, like oxyluciferin, can exist in ketone, enol, and enolate forms. DMOL contains two methyl groups, which prevents the transition of the ketone into the enol (Fig. 1). The spectral and physicochemical properties of these analogs have been little studied.

The goal of the present study was to investigate the spectral and fluorescence properties of MMOL and DMOL in buffer solutions over a wide range of pH.

MATERIALS AND METHODS

We used DMOL and MMOL synthesized by Weiss [3] and luciferin synthesized in our laboratory as described in [4]. All solutions were prepared using distilled deionized water obtained on a Milli Q unit (USA).

The absorption spectra were recorded using a Shimadzu UV-1202 spectrophotometer (Japan) in the

Abbreviations: DMOL) dimethyloxyluciferin; MMOL) monomethyloxyluciferin; λ_{em}, λ_{ex}, and λ_{abs}) the wavelengths of the maxima of emission, excitation, and absorption spectra, respectively.

* To whom correspondence should be addressed.

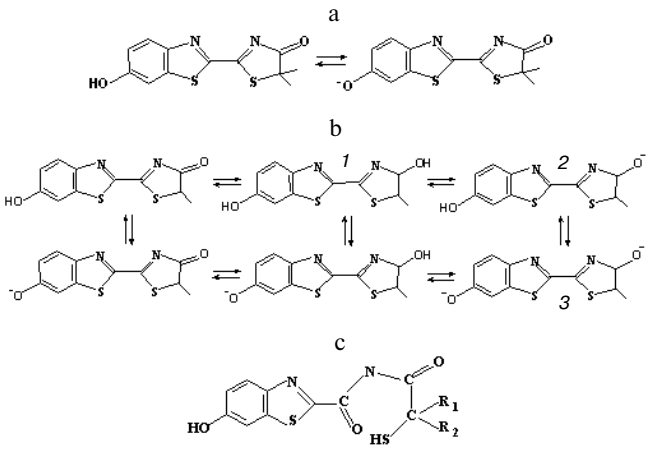

Fig. 1. Structures of dimethyloxyluciferin (a), monomethyloxyluciferin (b), and the products of their decomposition (c).

range 250-600 nm. DMOL or MMOL (20 μl of 10 mM solution in dioxane) were added into 2 ml of a buffer solution with fixed pH value.

The fluorescence spectra were recorded on a Perkin Elmer LS50B spectrofluorimeter (USA) in the range 450-750 nm. DMOL or MMOL (2 μl of 1 mM solution in dioxane) were added into 2 ml of a buffer solution of fixed pH value. To obtain the constant of the decomposition of the compounds, the cuvette with the investigated preparation was incubated at constant temperature and the fluorescence spectrum was taken after different time intervals.

The product of DMOL decomposition was obtained by incubation of 1 mM DMOL solution in 0.05 M Tris-acetate buffer containing 2 mM EDTA and 10 mM $MgSO_4$, pH 12, for 15 min. The product of MMOL decomposition was obtained by incubation of 1 mM MMOL solution in 0.05 M Tris-acetate buffer containing 2 mM EDTA and 10 mM $MgSO_4$, pH 12, for 72 h.

Thin layer chromatography assay of the decomposition products of DMOL, MMOL, and luciferin was performed using UV 254 silica gel plates (Silufor, Czechia) in the system butanol–acetic acid–water (7 : 1 : 1 v/v). A solution of the investigated compound (10 μl) was applied to a plate. The chromatogram was developed under ultraviolet light (λ = 254 nm).

RESULTS AND DISCUSSION

Absorption and fluorescence spectra of DMOL. In aqueous solutions (pH 6.0-9.6) the absorption spectra of DMOL (Fig. 2) indicate the existence of equilibrium between the phenolic (λ_{abs} = 383 nm) and phenolate (λ_{abs} = 485 nm) forms of DMOL with p*K* value of 7.8, this differing from the p*K* value of the phenol group of oxyluciferin (7.4) [2]. However, the absorption spectra of DMOL at pH > 8.0 exhibit significant absorption in the region 300-450 nm, whereas the content of the phenolic form under these conditions does not exceed 2%. These data suggest that at high pH values DMOL decomposes yielding a new product. Incubation of DMOL at pH 9.0 for 15-20 min resulted in the appearance of a new peak with λ_{abs} = 350 nm. Thus, the absorption spectrum of DMOL in alkaline solutions is the sum of the spectra of the phenolate form of DMOL and its decomposition product.

It is known that the excited phenol molecule exhibits decreased p*K* value. The approximate p*K** value of the excited molecule estimated using the absorption spectra by Ferster's method [5] is –3.91. Consequently, the DMOL molecule in the excited state is a strong acid existing in the phenolate form within the investigated range of pH. Thus, only one peak corresponding to the fluorescence of the phenolate ion must be observed in the fluorescence spectra. Actually, at λ_{ex} = 485 nm (that is λ_{abs} for the phenolate form of DMOL), one peak was observed (λ_{em} = 639 nm) at all studied pH values. The fluorescence intensity decreased with decrease in pH, this being explained by the decrease in the concentration of the original phenolate ion. At λ_{ex} = 383 nm (λ_{abs} of the phenolic form of DMOL) and pH < 8.0 we also observed one emission maximum (λ_{em} = 639 nm). At pH > 8.0, two peaks were observed: yellow-green and red fluores-

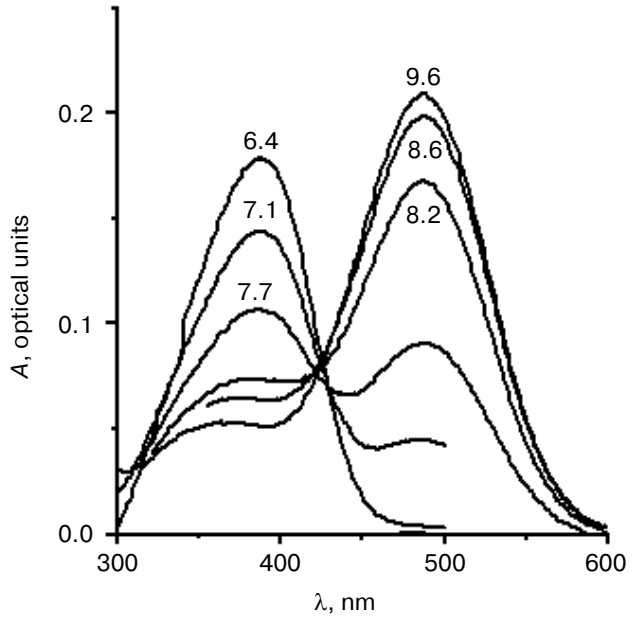

Fig. 2. Absorption spectra of dimethyl oxyluciferin at different pH values (indicated by the numbers near the curves). The spectra were taken in 0.05 M Tris-acetate buffer containing 2 mM EDTA and 10 mM $MgSO_4$. DMOL concentration was $2 \cdot 10^{-5}$ M.

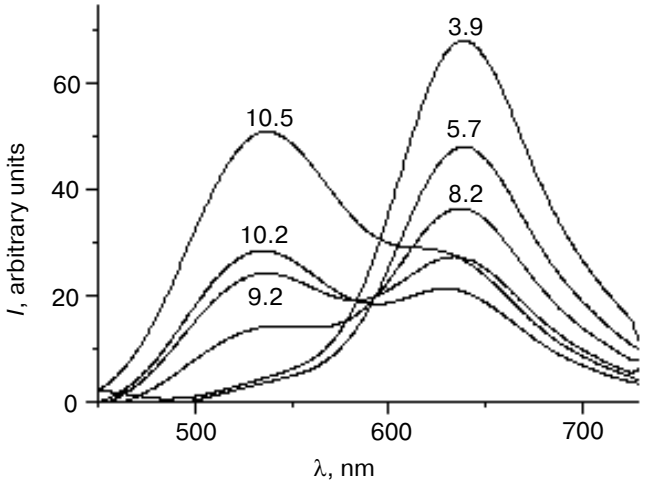

Fig. 3. Fluorescence spectra of dimethyloxyluciferin at different pH values (indicated by the numbers near the curves). The conditions are described in the legend to Fig. 2. The concentration of DMOL was 10^{-6} M, λ_{ex} = 383 nm.

cence (λ_{em} = 500 and 639 nm, respectively) (Fig. 3). Similar results were obtained previously [3], but they were not explained. The authors observed red fluorescence at pH 6.0, and the yellow-green fluorescence at pH > 8.0. The intensity of the yellow-green fluorescence increased with increasing pH, while the intensity of the red fluorescence decreased. Also, the intensity of the yellow-green fluorescence increased with time and did not disappear on acidification of the solution. The yellow-green fluorescence (λ_{em} = 500 nm) is presumably due to the decomposition of DMOL at high pH values, since the rate of DMOL decomposition significantly depends on pH.

Absorption and fluorescence spectra of MMOL. Absorption spectra of MMOL were studied in buffer solutions in the range of pH 6-9. In the range of pH 8-9, a single peak was observed at λ_{abs} = 440 nm (Fig. 4a). At pH 7.3-7.7, an additional peak appeared at λ_{abs} = 390 nm (Fig. 4b). At pH 6-7, a peak at λ_{abs} = 375 nm and a shoulder at λ_{abs} = 440 nm were revealed (Fig. 4c). The changes in the absorption spectra of MMOL at different pH values are probably due to the fact that, depending on pH, MMOL can exist in different forms (Fig. 1). At pH > 8.0 the MMOL molecule exists as the dianion (form *3*). At pH < 8.0 the MMOL molecule can be protonated at two positions (the hydroxythiazole and phenol groups). As demonstrated in [6], the hydroxythiazole group of MMOL is more acidic than the phenolic group, and thus the phenolic group will be protonated first. Consequently, the peak with λ_{abs} = 390 nm corresponds to the monoanion with the protonated phenolic group (Fig. 1, form *2*). The peak at λ_{abs} = 375 nm must correspond to the neutral MMOL molecule (Fig. 1, form *1*). The transition dianion ↔ monoanion is characterized by the p*K* value of 7.8. Thus, the absorption spectra of MMOL suggest that different forms of the molecule exist in a rather narrow pH interval, which prevented determination of the accurate p*K* value for their transition.

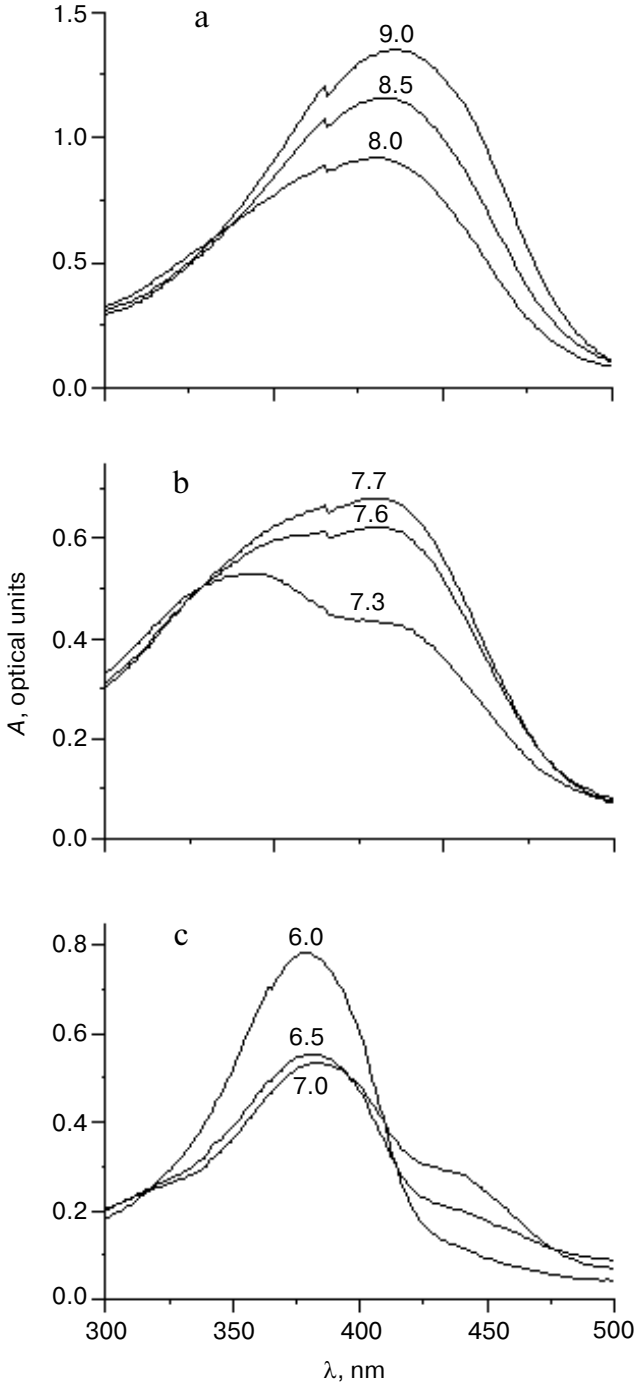

Fig. 4. Absorption spectra of monomethyloxyluciferin at different pH values (indicated by the numbers near the curves). The conditions are described in the legend to Fig. 2. The concentration of MMOL was 10^{-5} M.

Table 1. Rate constants of the decomposition of mono- and dimethyloxyluciferin at different pH values

pH	k_{eff}, min^{-1}	$t_{1/2}$, min	pH	k_{eff}, min^{-1}	$t_{1/2}$, min
	MMOL			DMOL	
6.0	0.02	70	7.8	0.001	521
7.8	0.02	65	9.0	0.002	385
9.0	0.002	475	10.4	0.02	46

The fluorescence spectra of MMOL at pH 6.0-9.7 (λ_{ex} = 440 or 375 nm) have a single maximum. The pK^* value of the excited MMOL molecule is approximately 0.7. This explains the existence of one maximum in the fluorescence spectra at all studied pH values. The maximum at λ_{em} = 550 nm corresponds to the fluorescence of the dianion of MMOL. Excitation at 440 nm (the absorption band of the dianion) does not affect the position of λ_{em} = 550 nm in the range of pH 6.0-9.7. For excitation at 375 nm (the absorption band of the neutral molecule) in the range of pH 6.0-9.7, the λ_{em} position of MMOL is shifted to shorter wavelength with increasing pH. This is presumably connected to the fact that the λ_{em} and λ_{abs} of the product of MMOL decomposition are rather close to those at the alkaline pH values, and in this case the fluorescence spectrum of MMOL is the sum of the fluorescence spectra of MMOL and the product of its decomposition.

Stability of dimethyl- and monomethyloxyluciferin in aqueous solutions. Stability of DMOL and MMOL was investigated at different pH values. The decomposition of MMOL was monitored by the decrease in the optical density at λ_{abs} (440 nm at pH 9.0 and 7.8, and 375 nm at pH 6.0) with time. The dependence of the optical density on time was used to calculate the rate constants of decomposition of MMOL (Table 1). The decomposition of DMOL was monitored by the changes in the intensity of the yellow-green fluorescence (λ_{em} = 500 nm) with time. At pH 10.5, the increase in the intensity of the yellow-green fluorescence is described by a first order kinetic curve with the constant k = 0.02 min^{-1}. The data presented in Table 1 show that MMOL is more stable at alkaline pH values compared to DMOL, and DMOL is more stable at acid pH values compared to MMOL. Nevertheless, at pH 7.8 corresponding to the maximum of firefly luciferase catalytic activity, the half-life time values ($t_{1/2}$) of MMOL and DMOL are 65 min and ~6 h, respectively (Table 1). This allows using MMOL and DMOL as fluorescent markers for investigation of the active site of luciferase in buffer solutions.

Table 2. Spectral properties of the decomposition products of DMOL and MMOL in aqueous solutions

pH	λ_{abs}, nm	λ_{em}, nm
< 8.8	310	520
> 8.8	350	500

Thin layer chromatography assay of the reaction mixture demonstrated that decomposition of MMOL and DMOL yielded a product, whose spectral properties in aqueous solutions (Table 2) are similar to those of the product of oxyluciferin decomposition (λ_{em} = 498 nm) described in [7]. The authors identified this product as the derivative of 6-hydroxybenzthiazol-2-carboxamide, i.e., a product resulting from breaking of the C–S bond. By analogy of the results of work [7], the structure of the decomposition products of DMOL and MMOL in aqueous solutions can be presented in the following way (Fig. 1, form *3*).

The suggested amide structure is supported by the experimental data. The fluorescence spectrum of an amide shifts to shorter wavelength, this indicating a decrease in the system of conjugated bonds. This is possible only on breaking of the thiazole ring. The λ_{em} value of the amide coincides with the λ_{em} of 2-CN-6-OH-benzthiazole obtained in [8]. According to the amide structure, the products of decomposition of DMOL and MMOL must differ in the number of the methyl groups at the α-position with respect to the carbonyl group. To test this assumption, we used thin layer chromatography assay. The distribution coefficients of the decomposition products of DMOL, MMOL, and luciferin were determined. The value R_m was calculated using the equation: $R_m = \log(1/R_f - 1)$, where R_f is the distribution coefficient of the investigated substance. As expected according to [9], R_m depends linearly on the number of the methyl groups in the α-position with respect to the carbonyl group.

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