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Spectroscopic studies of the color modulation mechanism of firefly (beetle) bioluminescence with amino-analogs of luciferin and oxyluciferin†

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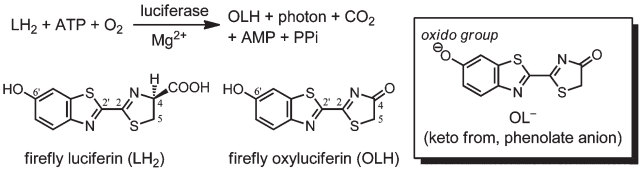
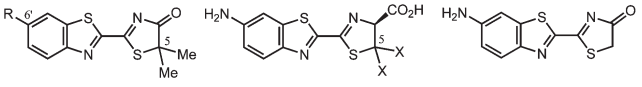
Spectroscopic properties of amino-analogs of luciferin and oxyluciferin were investigated to confirm the color modulation mechanism of firefly (beetle) bioluminescence. Fluorescence solvatochromic character of aminooxyluciferin analogs indicates that the bioluminescence of aminoluciferin is useful for evaluating the polarity of a luciferase active site.

Fireflies and luminous beetles such as click beetles and railroad worms show bioluminescence with the same luciferin–luciferase (L–L) reaction.[1] An enzyme, luciferase catalyzes the luminescence reaction of firefly luciferin (LH_2), ATP and O_2, to give oxyluciferin (OLH) and a photon of visible light (Scheme 1). One of the important characteristics of the bioluminescent system is photon generation in a wide range of colors from green to red (λ_{max} 530–640 nm)[2] using the common LH_2. To better use the bioluminescent system for practical applications, we have to clarify the fundamental chemical subjects including the mechanism of color modulation.

Color variation of the bioluminescence is caused by the changes in the structure and properties of the excited OLH inside the active site of a luciferase. The most plausible candidate structure of the light-emitter is the keto-form of the phenolate anion (OL^-, Scheme 1) which is supported by the mechanistic evidence reported by Branchini *et al.*,[3] while other possible structures were proposed.[4] We recently supported this conclusion by clarifying the spectroscopic properties of the phenolate anion of 5,5-dimethyl OLH analog (**1**-O^-), whose methyl groups make the π-system fix the keto form.[5] In fact, the fluorescence maximum of **1**-O^- varied in the range of green to red color depending on two factors: (i) the polarity of the molecular environment and (ii) the bonding interaction between the oxido group and a counter cation. Therefore, these two factors will play essential roles in the color modulation mechanism of the bioluminescence. To establish the mechanism, we need to understand the property of the excited state of OL^- having the oxido group at C6′ as a strong electron-donating group. To reveal the role of the oxido group of OL^-, we compared the spectroscopic properties of OLH analogs (**1**-NMe_2, **1**-NH_2 and **1**-H) having a substituent R (NMe_2, NH_2 and H) at C6′ with those of **1**-OH, **1**-O^- and methoxy analog **1**-OMe.[5] We now report how the spectroscopic property of **1** is affected by the electron-donating ability of a series of R, explaining the color variation of firefly (beetle) bioluminescence. In addition, the data of amino analog **1**-NH_2 are useful for evaluating the bioluminescence property of aminoluciferin (ALH), whose fundamentals have been well studied.[6] Because ALH is a useful analog as a red-emitting substrate,[7] it is valuable to decipher bioluminescence color modulation behavior with ALH. For this purpose, we reinvestigated the bioluminescence of ALH together with that of the 5,5-dimethyl analog (Me_2ALH), that is the luciferin analog corresponding to **1**-NH_2. We also report here the color modulation mechanism of the ALH bioluminescence.



Scheme 1



1-OH: R = OH
1-O^-: R = O^-
1-OMe: R = OMe
1-NMe_2: R = NMe_2
1-NH_2: R = NH_2
1-H: R = H

ALH: X = H
Me_2ALH: X = Me

AOL

OLH analogs **1**-NMe_2, **1**-NH_2 and **1**-H were prepared by the method for preparing **1**-OH.[5] The Hammett σ_p constants of R for **1**-NMe_2, **1**-NH_2 and **1**-H are −0.83, −0.66 and 0.00, respectively, and those of hydroxyl, oxido and methoxy groups for **1**-OH, **1**-O^- and **1**-OMe are −0.37, −0.81 and −0.27,

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†Electronic supplementary information (ESI) available: General methods, synthesis and NMR spectra of luciferin and oxyluciferin analogs, measurements of bioluminescence activities and spectra, spectroscopic data of oxyluciferin analogs, and DFT and TDDFT calculation data. See DOI: 10.1039/c2pp25106j

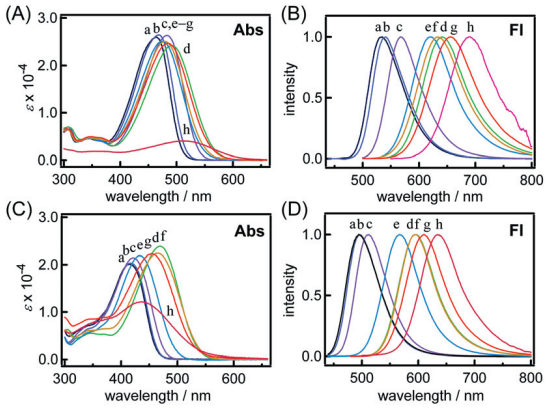


Fig. 1 UV/visible absorption (Abs) and fluorescence (Fl) spectra of **1**-NMe_2 (A and B) and **1**-NH_2 (C and D) in *p*-xylene (a), benzene (b), chloroform (c), DMSO (d), acetonitrile (e), 2-propanol (f), methanol (g), and H_2O (h) at 25 °C.

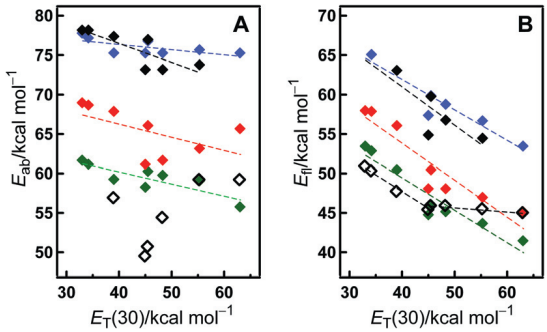


Fig. 2 E_{ab} and E_{fl} values for **1**-NMe_2 (♦) and **1**-NH_2 (♦) together with those for **1**-OH (♦), **1**-O^- (◊) and **1**-OMe (♦) plotted as a function of $E_T(30)$.

respectively.[8] Thus, the electron-donating abilities of the dimethylamino and oxido groups are similar to each other. UV/visible absorption and fluorescence spectra of **1**-NMe_2, **1**-NH_2 and **1**-H were measured in various solvents (*p*-xylene, benzene, chloroform, DMSO, acetonitrile, 2-propanol, methanol and H_2O). Analogs **1**-NMe_2 and **1**-NH_2 showed intense fluorescence ($\Phi_F > 0.1$) similar to **1**-O^- (Fig. 1), while fluorescence of **1**-H was not observed. The spectroscopic data of **1** are summarized in Tables S1 and S2† in the ESI with the solvent polarity scale $E_T(30)$ (in kcal mol^{-1}) that has been used for evaluating dipolar character of a molecule.[9,10] Analogs **1**-NMe_2 and **1**-NH_2 exhibited solvatochromism of the absorption and fluorescence bands, and their absorption maxima (λ_{ab}) and fluorescence maxima (λ_{fl}) show a characteristic dependency on the solvent polarity. Variation widths of the fluorescent state energies (E_{fl}) estimated from λ_{fl} of **1**-NMe_2 and **1**-NH_2 were larger than those of the absorption energies (E_{ab}) estimated from their λ_{ab}. The spectral changes of **1**-NMe_2 and **1**-NH_2 were evaluated by correlating the E_{fl} and E_{ab} values (in kcal mol^{-1}) with $E_T(30)$ (Fig. 2). Fig. 2 also shows the plots of the E_{ab} and E_{fl} values for **1**-OH, **1**-OMe, and **1**-O^- generated by using 1,1,3,3-tetramethylguanidine. The E_{ab}–$E_T(30)$ and E_{fl}–$E_T(30)$ plots for **1**-NMe_2 and **1**-NH_2 show linear correlations similar to those for **1**-OH and **1**-OMe, while the deviations of E_{ab} are larger than those of E_{fl}. The correlations were estimated to be $E_{ab} = -0.15E_T(30) + 66$ ($r = -0.85$) and $E_{fl} = -0.41E_T(30) + 66$ ($r = -0.95$) for **1**-NMe_2 and $E_{ab} = -0.17E_T(30) + 73$ ($r = -0.55$) and $E_{fl} = -0.47E_T(30) + 73$ ($r = -0.93$) for **1**-NH_2. In the case of **1**-O^-, the E_{ab}–$E_T(30)$ plot shows a low correlation and the E_{fl}–$E_T(30)$ plot shows two correlation lines, because the electronic absorption and fluorescence of **1**-O^- are affected by the structural character of the ion pair of **1**-O^- and a counter cation as reported previously.[5] The negative slopes of the E_{ab}–$E_T(30)$ and E_{fl}–$E_T(30)$ correlations for **1**-NMe_2 and **1**-NH_2 indicate that both their ground (S_0) and excited singlet (S_1) states have dipolar character. The slopes of the E_{fl}–$E_T(30)$ plots are steeper than those of the corresponding E_{ab}–$E_T(30)$ plots, indicating that the dipolar character of the S_1 states of **1**-NMe_2 and **1**-NH_2 is stronger than that of the corresponding S_0 states because their electronic excitations have intramolecular charge transfer (ICT) character. The E_{ab} and E_{fl} are in the order **1**-OH ≈ **1**-OMe > **1**-NH_2 > **1**-NMe_2 in a common solvent, indicating that the increase of the electron-donating ability of R causes a red shift of the λ_{ab} and λ_{fl}. The E_{fl} of **1**-O^- is smaller than that of **1**-NMe_2 in a less polar solvent and is varied between those of **1**-NMe_2 and **1**-NH_2 in a polar protic solvent. The result indicates that the oxido group at C6′ of OL^- plays a role as an electron-donating group stronger than the dimethylamino group in less polar environments, while its electron-donating ability is depressed by hydrogen bonding interactions in a protic polar solvent. Analogs **1**-NMe_2 and **1**-NH_2 showed high Φ_F in the range from 0.1 to 0.9 similar to **1**-O^-, while **1**-OH and **1**-OMe showed low Φ_F below 0.2 and the fluorescence of **1**-H disappeared. Therefore, we can conclude that the electron-donating ability of R at C6′ in the OLH structure is essential for fluorescence solvatochromism and increase of the fluorescence efficiency.

To understand the electronic effect of the substituent R at C6′ on the spectroscopic properties of **1**, we carried out DFT and TDDFT calculations of **1**-NMe_2, **1**-NH_2 and **1**-H using the B3LYP/6-31+G(d) method.[11] Calculation data are summarized in Table S3† together with the reported data of **1**-OH and **1**-OCH_3.[5,12] The electric dipole allowed transitions to the excited singlet states with the lowest excitation energies are $S_0 \rightarrow S_1$ for **1**-NMe_2 and **1**-NH_2 and $S_0 \rightarrow S_3$ for **1**-H. The former $S_0 \rightarrow S_1$ transitions are mainly contributed by the HOMO to LUMO excitation and the latter $S_0 \rightarrow S_3$ transition is mainly contributed by the HOMO − 2 to LUMO excitation. The Kohn–Sham frontier orbitals of **1**-NMe_2, **1**-NH_2 and **1**-H (Fig. S6† in the ESI) indicate that these allowed transitions are of π–π* type. Excitation wavelengths (λ_{ex}) for the allowed transitions of **1**-NMe_2 (438 nm), **1**-NH_2 (401 nm) and **1**-H (340 nm) correspond to the observed λ_{ab} (**1**-NMe_2, 468 nm; **1**-NH_2, 417 nm; **1**-H, 328 nm) in benzene. The tendency of the red shift of λ_{ex} with increase of the electron-donating ability of R matches the spectroscopic observations for **1**. This red shift of λ_{ex} originates from a change of the frontier orbital levels; an increase of the electron-donating ability of R causes a rise of the HOMO level steeper than that of the LUMO level. Oscillator strengths (*f*) of **1**-NMe_2 and **1**-NH_2 are similar to those of OL^- and **1**-O^-, and larger than those of the remaining analogs. Because a fluorescence rate constant is theoretically related to *f*,[13] introduction of a strong electron-donating R such as the amino group will accelerate the

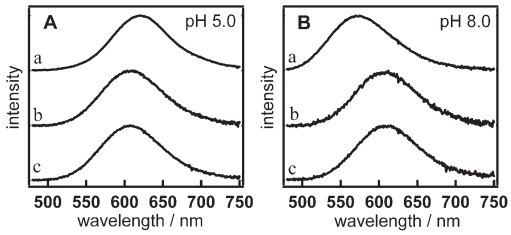
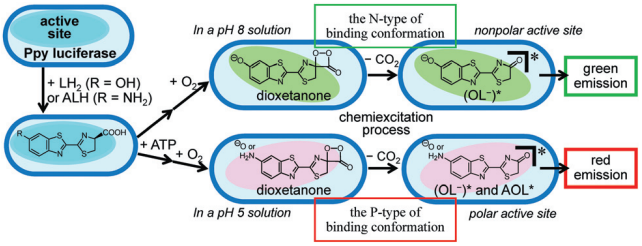


Fig. 3 Bioluminescence spectra of LH_2 (a), ALH (b), and Me_2ALH (c) with a recombinant *Photinus pyralis* (Ppy) luciferase in 0.10 M potassium phosphate buffers at pH 5.0 (A) and pH 8.0 (B).



Scheme 2

fluorescence emission process to lead to a high Φ_F. In the cases of **1**-NMe_2 and **1**-NH_2, electrons of their HOMOs and LUMOs are distributed around the benzothiazole and thiazolylthiazole moieties, respectively, in a manner similar to those of OL^- and **1**-O^-. Thus, the HOMO to LUMO excitation has ICT character to enlarge the dipole moments of the S_1 states of **1**-NMe_2 and **1**-NH_2. This conclusion matches that obtained from the evaluation of the solvent-dependent fluorescence spectral change described above.

To evaluate the bioluminescent property of ALH, we have to confirm the S_1 state structure of aminooxyluciferin (AOL) during the bioluminescence reaction. For this purpose, we investigated L–L reactions of Me_2ALH with a recombinant *Photinus pyralis* (Ppy) luciferase and the luminescence properties of Me_2ALH were compared with those of LH_2 and ALH.[3,6] L–L reactions with low concentrations of substrates [luciferins (20 μM), Mg·ATP (80 μM), and PPy luciferase (30 nM)] showed steady light emission,[14] and the relative intensity ratios for the reactions with LH_2, ALH and Me_2ALH were 1.0 : 0.13 : 0.001 at pH 8.0 and 1.0 : 0.90 : 0.003 at pH 5.0. Bioluminescence spectra (Fig. 3) of Me_2ALH at pH 5.0 and 8.0 coincided with each other and showed the emission maxima (λ_{bl}) at 608 nm. Observed spectra of LH_2 and ALH at pH 5.0 and 8.0 (Fig. 3) showed λ_{bl} reproducing the reported values: 619 and 571 nm at pH 5.0 and 8.0 for LH_2, respectively, and 609 nm at pH 5.0 and 8.0 for ALH.[6] Bioluminescence spectra of ALH and Me_2ALH coincided with each other, indicating that the S_1 state of AOL generated by the L–L reactions has the keto form. This result also indicates that the two methyl groups at C5 of Me_2ALH influence to a lesser extent the spectroscopic properties. This is supported by DFT calculations of AOL (Table S4†), which showed that the π-electronic properties and excitation character of **1**-NH_2 and AOL were similar to each other. Me_2ALH showed pH-independent bioluminescence spectra similar to those of ALH.[6] This result confirms that the amino groups at C6′ of ALH and Me_2ALH play a role to induce their pH-independent luminescence property of the L–L reactions.

We are now able to evaluate the λ_{bl} values for the L–L reactions of ALH and Me_2ALH with Ppy luciferase based on the fluorescent property of **1**-NH_2. Because **1**-NH_2 exhibited fluorescence solvatochromism, the λ_{bl} values give information on the polarity of the active site of Ppy luciferase.[15] The λ_{bl} (608–9 nm) for ALH and Me_2ALH are similar to the λ_{fl} (610 nm) of **1**-NH_2 in methanol, indicating that the active site provides a polar environment like a methanol solution to the S_1 states of AOL and **1**-NH_2. Polar character of the active site for the excited AOL and **1**-NH_2 is not affected by the pH of solutions as explained below. On the other hand, the pH dependent bioluminescence of LH_2 indicates that the active site of Ppy luciferase provides two different polarity environments to the S_1 states of OL^-. Based on the fluorescent property of **1**-O^-,[5] it is predicted that the 571 nm emission occurs from the excited OL^- located in the active site with nonpolar character like a benzene solution and the 619 nm emission occurs in the active site with polar character like an alcoholic solution. It is also known that bioluminescence quantum yield (Φ_{BL}) with Ppy luciferase shows pH dependent character,[16] indicating that the nonpolar active site dominantly functions in a pH 8 solution to give a high Φ_{BL} and the polar active site dominantly functions in a pH 5 solution to give a low Φ_{BL}. These active sites are interconvertible depending on the pH of a solution. Thus, it is remarkable that the changes of λ_{bl} and Φ_{BL} are related to each other.[16,17] Because the Φ_{BL} value is mainly determined by the chemiexcitation efficiency,[18] a switch of the active sites with different polarities takes place before the chemiexcitation process (Scheme 2). LH_2 bonded in the active site of Ppy luciferase is adenylated and the following oxygenation gives the dioxetanone intermediate, which takes two types of possible binding conformations in the active site, termed N- and P-types (Scheme 2). Decompositions of the dioxetanones in two binding conformations in the active site generate the S_1 states of OL^- with the corresponding binding conformations with different chemiexcitation efficiencies. The excited OL^- in the N- and P-types of binding conformations are influenced by the nonpolar and polar character of the active site, respectively (Scheme 2). In the case of the ALH bioluminescence, the amino groups of the dioxetanone intermediate and the excited AOL lead to the P-type of binding conformations preferentially, showing red emission both in pH 5 and 8 solutions (Scheme 2). Thus, the hydroxyl group of LH_2 plays an important role for the interactions with the active site of Ppy luciferase to give the dioxetanone in the N-type of binding conformation in a pH 8 solution. Because a green emitting luciferase may have evolved from a proto-luciferase with the red emission ability,[19] the P-type of binding conformation will be a prototype for the dioxetanone in the active site and the active site has the ability to allow an analog structure of the dioxetanone to adopt the P-type binding conformation. The difference in the λ_{bl} for the red emission with ALH (609 nm) and LH_2 (619 nm) corresponds to the different electron-donating abilities of the amino group of AOL and the oxido group of OL^-. Therefore, the S_1 states of OL^- and AOL in the P-conformation are similarly affected by the polar character of the active site.

Conclusions

We have clarified the fluorescent properties of **1**-NH_2 and its related compounds and revealed that the amino group, a strong electron-donating group, plays important roles for a red-shift of λ_{fl}, fluorescence solvatochromism, and a high Φ_F value. This information leads to the conclusion that the strong electron-donating ability of the oxido group in OL^- is essential for the high efficiency and solvatochromic properties of OL^- as the light-emitter in the firefly (beetle) bioluminescence. It was also clarified that the ALH bioluminescence occurs by the light emission from the S_1 states of the keto form of AOL. The emission colors from the excited AOL are modulated only by the polarity of the active site, while those from the excited OL^- are modulated not only by the polarity but also by the covalent character of the O8′···H bonds between the excited OL^- and a counter cation.[5] Therefore, the λ_{bl} value for the bioluminescence with ALH is useful for evaluating the polarity of the active site of a luciferase by using the fluorescence data of **1**-NH_2 as a ruler for the polarity scale. Extended applications of the ALH bioluminescence to various luciferases are currently underway.

Acknowledgements

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