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Spectroscopic properties of amine-substituted analogues of firefly luciferin and oxyluciferin†

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ABSTRACT

Spectroscopic and photophysical properties of firefly luciferin and oxyluciferin analogues with an amine substituent (NH_2, NHMe and NMe_2) at the C6' position were studied based on absorption and fluorescence measurements. Their π-electronic properties were investigated by DFT and TD-DFT calculations. These compounds showed fluorescence solvatochromism with good quantum yields. An increase in the electron donating strength of the substituent led to the bathochromic shift of the fluorescence maximum. The fluorescence maxima of the luciferin analogues and the corresponding oxyluciferin analogues in a solvent were well correlated to each other. Based on the obtained data, the polarity of a luciferase active site was explained. As a result, the maximum wavelength of bioluminescence for a luciferin analogue was readily predicted by measuring the photoluminescence of the luciferin analogue in place of that of the corresponding oxyluciferin analogue.

INTRODUCTION

Firefly bioluminescence produces photons by a luciferin–luciferase (L–L) reaction, in which firefly luciferin (LH_2) reacts with ATP and O_2 under the enzymatic action of luciferase, to give oxyluciferin (OLH), CO_2 and pyrophosphate (PPi) (Schemes 1A and B) (1,2). It is one of the characteristics of firefly bioluminescence to generate light that varies from green to red colors. The emission colors are dependent on different varieties of luciferases and conditions surrounding the reaction site, such as pH, coexisting metal ions, and temperature (3–13). It has been revealed that the light emitter is OLH. The luminescence properties of OLH and its analogues have been investigated to understand the color

modulation mechanism (14–34). Breakthrough evidence for the mechanism was reported using a 5,5-dimethylluciferin adenylate, that is, the light emitter in the excited singlet (S_1) state generated by the L–L reaction has a structure of the keto-phenolate anion (OL^-, Scheme 1B) (35,36). Further studies on the spectroscopic properties of the phenolate anion of 5,5-dimethyl analogue of OLH supported the proposed mechanism (37). It was clarified that the physical properties of OL^- in the S_1 state were modulated both by the strength of the bonding interaction with a counter cation in the luciferase active site and the polarity of the active site environment.

<Scheme 1>

To confirm the color modulation mechanism, we have been paying attention to aminoluciferin (**1a**), which is bioluminescence active and shows red emission by the L–L reaction with *Photinus pyralis* (Ppy) luciferase in a pH-independent manner (38). Our previous studies of the 5,5-dimethyl analogue of **1a** (Scheme 1C) led to the conclusion that the S_1 state of aminooxyluciferin (**2a**) in the keto form is the light emitting center of the L–L reaction of **1a** (39,40). Because **2a** will mainly exist as the enol form in solutions like OLH (31), 5,5-dimethyl analogue **3a** is useful for predicting the spectroscopic property of the keto form of **2a** (39). Based on the spectroscopic property of **3a**, it was suggested that the amino (NH_2) group of **2a** works as an electron donating substituent toward the π-electronic system for the polarized property of the S_1 state showing fluorescence solvatochromism. Because the NH_2 group is basic contrast to the acidic hydroxy (OH) group, the physical properties of the S_1 state of **2a** will be modulated only by the polarity of the luciferase active site (41). To support the suggested mechanism, it is required to understand the accurate photophysical property of the keto form of **2a** by using **3a**.

Furthermore, a variety of amine-substituted luciferin analogues such as amine-functionalized analogues (42,43), fluorophore conjugated analogues (44–46), π-modified analogues (47–50) and cyclic amine-fused analogues (51,52) were recently prepared, and their luminescence properties were investigated for imaging applications. For these studies, luciferin analogues **1b** and **1c** with the methylamino (NHMe) and dimethylamino (NMe_2) groups, respectively, were investigated as standard substrates for the L–L reactions (44,45,51,52). Interestingly, the emission maxima (λ_{bl}) of the L–L

reactions of **1b** and **1c** with Ppy luciferase are red-shifted compared to that of **1a**. While the emitting site on the L–L reaction of a luciferin analogue was the S_1 state of the corresponding oxyluciferin analogue, the λ_{bl} value was evaluated based on the fluorescence property of the luciferin analogue in place of the oxyluciferin analogue (44, 52) considering the similarity of LH_2 and OLH in the spectroscopic behaviors (16, 53). In fact, it was found that the fluorescence emission maxima (λ_{fl}) of the cyclic amine-fused luciferin analogues are linearly correlated with their λ_{bl} values (52). To confirm the relationship between the λ_{bl} and λ_{fl} values of a luciferin analogue, we require deep understanding of the spectroscopic properties of a luciferin analogue and the corresponding oxyluciferin analogue. In the present work, we investigated the spectroscopic and photophysical properties of luciferin analogues **1a**-**c** and 5,5-dimethyloxyluciferin analogues **3a**-**c** having the amine substituent. The Hammett σ_p values of the NH_2, NHMe, and NMe_2 groups are −0.66, −0.70, and −0.83, respectively, indicating the order of the electron donating strength of the substituent is NH_2<NHMe<NMe_2 (54). We were, therefore, able to systematically evaluate the spectroscopic and photophysical data for **1a**-**c** and **3a**-**c** based on the amine substituent effect on the electronic properties of their S_1 states. The results were applicable to analyzing the λ_{bl} values of the L–L reactions of **1a**-**c** with Ppy luciferase.

MATERIALS AND METHODS

General. Melting points were determined with a Yamato MP-21 apparatus. IR spectra were obtained with a Nicolet 6700 spectrometer with an ATR attachment. High-resolution electro-spray ionization (ESI) mass spectra were recorded on a JEOL JMS-T100LC mass spectrometer. ^{1}H NMR spectra were obtained with a JEOL ECA-500 instrument (500 MHz). UV/visible absorption spectra were measured with Agilent Technologies Cary 60 spectrophotometer (scan speed, 600 nm/min; data interval, 1 nm). Fluorescence spectra and fluorescence quantum yields were measured with a Hamamatsu Photonics Quantaurus-QY absolute PL quantum yields measurement system. Fluorescence lifetimes (τ_f) were measured with a Hamamatsu Photonics Tau time-correlated single-photon counting fluorimeter system. Spectroscopic measurements were performed with sample solutions (1.0×10^{-5} mol/L) in a quartz cuvette (1 cm path length) at 25 ± 1 °C. Spectral-grade solvents were used for the

measurements of UV/visible absorption and fluorescence. Bioluminescence spectra were measured with an ATTO AB-1850 spectrophotometer (data interval, 1 nm). Density functional theory (DFT) calculations were performed with the Gaussian 09 program (Rev. D.01) (55). DFT includes the B3LYP functional with the 6-31+G(d) basis set (56−58). Molecular graphics were made with GaussView, Version 5 (59).

*Preparation of luciferin analogues **1a-c** and oxyluciferin analogues **3a-c***. Luciferin analogues **1a-c** and oxyluciferin analogues **3a** and **3c** were prepare according to the literatures (38,39,44,51). Oxyluciferin analogue **3b** was synthesized from 2-cyano-6-methylaminobenzothiazole as followed.

*Oxyluciferin analogue **3b***. Ethyl 2-(ethoxycarbonothioylthio)-2-methylpropanoate (187 mg, 0.55 mmol) was dissolved in ethylenediamine (2.8 ml) under Ar, and the reaction mixture was stirred at room temperature for 2 h. The reaction was quenched by adding saturated NH_4Cl aqueous solution, and ethyl 2-mercapto-2-methylpropanoate was extracted with dichloromethane (50 mL × 2). The organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The yellow oily residue containing ethyl 2-mercapto-2-methylpropanoate (141 mg) was dissolved in dehydrated ethanol (4 mL) under Ar, and 2-cyano-6-methyaminobenzothiazole (50 mg, 0.27 mmol) and triethylamine (120 μL, 8.8 mmol) were added to the solution. After heating at reflux for 3 h, the cooled reaction mixture was concentrated in vacuo. The residue was purified by silica gel TLC twice [hexane/chloroform (1:2) and chloroform/ethyl acetate (2:1)], to give **3b** (14 mg, 18%) as reddish brown crystals: mp 257−260 °C. δ_H ($CDCl_3$) 1.72 (6 H, s), 2.95 (3 H, d, *J* 4.6 Hz), 4.36 (1 H, br s), 6.86 (1 H, dd, *J* 2.3 Hz, *J* 8.9 Hz), 6.97 (1 H, d, *J* 2.3 Hz), and 7.94 (1 H, d, *J* 9.2 Hz). ν /cm^{-1} 3384, 2965, 2917, 1712, 1614, and 1552. *m/z* (ESI) Found: 292.0577 ($[M+H]^+$). $C_{13}H_{14}N_3OS_2$ requires 292.0578; Found: 314.0408 ($[M+Na]^+$). $C_{13}H_{13}N_3NaOS_2$ requires 314.0398.

RESULTS AND DISCUSSION

Spectroscopic properties of luciferin analogues 1a-c and oxyluciferin analogues 3a-c.

UV/visible absorption and fluorescence spectra of luciferin analogues **1a-c** were measured in DMSO,

acetonitrile, 2-propanol, methanol, and water (Figure 1). Unfortunately, **1a**-**c** showed low solubility in chloroform and benzene. UV/visible absorption and fluorescence spectra of oxyluciferin analogues **3a**-**c** were measured in *p*-xylene, benzene, chloroform, DMSO, acetonitrile, 2-propanol, and methanol (Figure 2). The analogues **3a**-**c** were insoluble and unstable in water. The spectroscopic data of the absorption maxima (λ_{ab}) of the lowest energy bands of the absorption and the fluorescence emission maxima (λ_{fl}) for **1a**-**c** and **3a**-**c** are summarized in Tables 1 and 2, respectively. In the case of luciferin analogues, the ranges of the λ_{fl} values in the used solvents are 472−520 nm for **1a**, 486−534 nm for **1b**, and 500−562 nm for **1c**, and their Φ_f values are over 0.23. In the case of oxyluciferin analogues, the ranges of the λ_{fl} values in the used solvents are 494−607 nm for **3a**, 511−623 nm for **3b**, and 535−656 nm for **3c**, and their Φ_f values are over 0.16. Interestingly, the λ_{ab} and λ_{fl} values of **1a**-**c** and **3a**-**c** show small red shifts with increasing the electron donating strength of the amine substituent, indicating that the amine substituents play a role in modulating the energy levels of the frontier orbitals of **1a**-**c** and **3a**-**c**. The solvent dependent variations of the λ_{fl} values of **1a**-**c** and **3a**-**c** are larger than those of λ_{ab} values, indicating that the excited singlet (S_1) states of both **1a**-**c** and **3a**-**c** have polarized character more than the corresponding ground (S_0) states (39).

<Figure 1>

<Figure 2>

<Table 1>

<Table 2>

The energy values of E_{fl} (in kcal mol^{-1}) estimated from the λ_{fl} values of **1a**-**c** and **3a**-**c** were correlated with the $E_T(30)$ values (60) (Figure 3). The data for all compounds in DMSO show a significant deviation from the linear correlations, suggesting that strong solvating interactions between the solvent and the excited molecules affect the π-electronic stabilities of **1a**-**c** and **3a**-**c**. Thus, the correlations between the E_{fl} and $E_T(30)$ values were analyzed by linear regression without the data in DMSO (dotted lines in Figure 3) expressed with equation (1).

$$E_{fl} = a\, E_T(30) + b \qquad (1)$$

From the slope and the intercept of the line, the coefficients (a and b) were determined, respectively, and summarized in Table 3. The negative correlations ($a < 0$) indicate that the S_1 states of **1a**-**c** and **3a**-**c** have dipole character more than the corresponding S_0 states. The electron donating strengths of the amine substituents of **1a**-**c** and **3a**-**c** clearly determine the E_{fl} values and have an insignificant effect on the slopes of the linear correlations.

<Table 3>

<Figure 3>

Figure 4A shows the E_{fl} values of **1a**-**c** and **3a**-**c** in acetonitrile plotted as a function of the Hammett σ_p values. Since the plots showed straight lines with positive slopes, it is shown that the E_{fl} values of both **1a**-**c** and **3a**-**c** linearly decrease with an increase of the electron donating strength of the amine substituent. Therefore, the E_{fl} values of **1a**-**c** and **3a**-**c** in a solvent will have linear correlation. Figure 4B shows the E_{fl} values of **3a-c** in acetonitrile, 2-propanol and methanol plotted as the functions of the E_{fl} values of **1a-c** in the same solvents. The plots showed straight lines as expressed with equation (2).

$$E_{fl}(\mathbf{3}) = c\ E_{fl}(\mathbf{1}) + d \qquad (2)$$

From the slope and intercept of the line, the values of c and d were, respectively, determined to be 1.2 and −21 kcal mol^{-1} for acetonitrile, 1.4 and −35 kcal mol^{-1} for 2-propanol, and 1.1 and −18 kcal mol^{-1} for methanol. The obtained c values are similar among these solvents.

<Figure 4>

Fluorescence lifetimes of luciferin analogues 1a-c and oxyluciferin analogues 3a-c.

Fluorescence lifetimes (τ_f) of **1a**-**c** in acetonitrile and water and **3a**-**c** in benzene and acetonitrile are listed in Table 4. By using the τ_f and Φ_f values, the rate constants (k_f) of the fluorescence emission process were estimated using the equation, $k_f = \Phi_f \tau_f^{-1}$. The quantum yields (Φ_{nr}) and rate constants (k_{nr}) of the nonradiative decay processes were estimated with the equations, $\Phi_{nr} = 1-\Phi_f$ and $k_{nr} = \Phi_{nr}$

τ_f^{-1}, respectively. The k_f values of **1a**-**c** and **3a**-**c** are similar to each other (ca. $1–2 \times 10^8$ s^{-1}), while the k_{nr} values are slightly varied by the difference in the amine substituents and in the solvents. Because the k_f values of **3a**-**c** are slightly greater than the k_{nr} values, **3a**-**c** show great Φ_f values compared to **1a**-**c**.

<Table 4>

DFT calculations of luciferin analogues 1a-c and oxyluciferin analogues 2a-c and 3a-c.

To analyze the relationship between the spectroscopic and the π-electronic properties of **1a-c** and **3a-c**, DFT and TD-DFT calculations of **1–3** were carried out with the B3LYP/6-31+G(d) method (Table 5). The NHMe group at the C6' position of **1b**, **2b** and **3b** provide two conformations **I** and **II**. The example for **1b** is illustrated in Scheme 2. Thus, the data of the conformers **I** and **II** for **1b**, **2b** and **3b** were also shown in Table 5. The heats of formation of **I** and **II** for **1b**, **2b** and **3b** are similar to each other. The data are deposited in ESI as listed in Table S1.

<Table 5>

<Scheme 2>

The $S_0 \rightarrow S_1$ transitions of all the compounds are of π,π* for the main contribution of the HOMO→LUMO configurations. The energy differences ($\Delta E_{H–L}$) between HOMO and LUMO and the wavelengths (λ_{tr}) estimated from the calculated transition energies support the observations that the λ_{ab} and λ_{fl} values of **1a-c** and **3a-c** are red-shifted with an increase in the electron donating strength of the amine substituent. The increase of the electron donating strength of the substituent raises both the energy levels of HOMO and LUMO. In particular, the HOMO levels exhibit significant increases compared with the LUMO levels, resulting in decreases of the $\Delta E_{H–L}$ value.

The calculated $\Delta E_{H–L}$ values of **1a-c** are greater than those of **3a-c**. This result matches the observation that the λ_{ab} and λ_{fl} values of **1a-c** are blue-shifted compared to those of **3a-c**. The difference in the $\Delta E_{H–L}$ values is caused by the reason that the 4,5-dihydrothiazole ring of **1a-c** have a

π-electronic conjugation smaller than the 4(5*H*)-thiazolone ring of **3a-c**. To compare the π-electronic character of **1a-c** and **3a-c**, electronic distributions of the HOMOs and LUMOs of **1a-c** and **3a-c** are deposited in ESI (Figure S3). The HOMO and LUMO energy levels of **1a-c** are higher than those of **3a-c**. In particular, the difference in the LUMO energy levels of **1a-c** and **3a-c** is greater than that in the HOMO energy levels. This difference in the LUMO energy levels is caused by the reason that the π-electronic distributions in the LUMOs of **3a-c** are largely contributed from the thiazolone ring, whose π-conjugation is larger than the 4,5-dihydrothiazole ring of **1a-c**.

The analogues **2a-c** and **3a-c** show similar values in each term of the calculated data (Table 5), indicating that the introduction of the two methyl groups at the C5 position of **2a-c** has little effect on the π-electronic character of **2a-c** as explained in the previous report on the 5,5-dimethyl analogue of OLH (37). In fact, the difference in the λ_{tr} values of **2a-c** and **3a-c** are less than 2 nm, supporting that the 5,5-dimethyl analogues **3a-c** are useful compounds for predicting the spectroscopic properties of the keto form of **2a-c**.

For the NHMe analogues **1b**, **2b**, and **3b**, the difference in the π-electronic character of the conformers **I** and **II** were investigated. We regard the differences in the ΔE_{H-L} and λ_{tr} values between **I** and **II** as being small. For instance, the conformers **I** and **II** of **1b** have the λ_{tr} values being 368 and 373 nm, respectively.

Bioluminescence properties of luciferin analogues 1a-c.

Bioluminescence properties of **1a-c** with Ppy luciferase have been reported by several groups, showing that the emission maxima (λ_{bl}) of the L–L reactions of **1a-c** are red-shifted with increasing the electron donating ability of the amine substituent (44,45,51,52). We reinvestigated the L–L reactions of **1a-c** with Ppy luciferase in GTA buffer at pH 8.0 and 6.0, and obtained the reproduced result deposited in ESI (Figure S4). The λ_{bl} values are 595, 610, and 620 nm for **1a**, **1b**, and **1c**, respectively, at pH 8.0 and 6.0. Because the excited light emitters generated by the reactions of **1a-c** are the keto form of **2a-c** (39,40), the λ_{bl} values of **1a-c** are reasonably evaluated by the λ_{fl} values of **3a-c** in place of **2a-c**. The λ_{bl} values are similar to the λ_{fl} values of **3a** in 2-propanol (593 nm), **3b** in 2-

propanol (604 nm), and **3c** in acetonitrile (621 nm), respectively. This result indicates that the S_1 states of **2a-c** generated from **1a-c** are stabilized by the microenvironments of the luciferase active site as they are by the solvation in 2-propanol and acetonitrile. Thus, the E_{fl}–E_T (30) plots in Figure 3 are useful as rulers for evaluating the polarity of a luciferase active site as proposed in the previous reports (39–41).

Figure 5 shows the energy values of E_{bl} (in kcal mol^{-1}) estimated from the λ_{bl} values on the L–L reactions of **1a-c** plotted as a function of the E_{fl} value for **1a-c** and **3a-c** in acetonitrile. The plots showed straight lines as expressed with equation (3).

$$E_{bl} = e\ E_{fl}(\mathbf{1}) + f \qquad (3)$$

From the slope and intercept of the line, the values of e and f were, respectively, determined to be 0.52 and 17 kcal mol^{-1} for **1a-c**, and 0.43 and 26 kcal mol^{-1} for **3a-c**. Because the E_{fl} values of **1a-c** and **3a-c** in a solvent are linearly correlated to each other (Figure 4B), it is reasonable for the E_{bl}–E_{fl} plots in Figure 5 to show good linear correlations. This result indicates that the S_1 states of **2a-c** generated from **1a-c** locate at the similar position in the active site of Ppy luciferase, whose microenvironments provide a polarity equally stabilizing the π-electron systems of the excited molecules. Similar result was obtained for the cyclic amine-fused luciferin analogues (52). The results obtained in this work are adoptable for predicting the λ_{bl} value of bioluminescence from a luciferin analogue with the λ_{fl} of the photoluminescence in a particular solvent based on the similarity in the spectroscopic behaviors of luciferin analogues and the corresponding oxyluciferin analogues.

<Figure 5>

CONCLUSION

We investigated the spectroscopic properties of luciferin analogues **1a**-**c** and oxyluciferin analogues **3a**-**c** having the amine substituent (NH_2, NHMe and NMe_2) with the different electron donating strength. Their photophysical properties were analyzed by fluorescence lifetime measurements. All of **1a**-**c** and **3a**-**c** show fluorescence solvatochromism with appreciably large Φ_f values. The fluorescence

solvatochromism is originated from strongly polarized character of the S_1 state. The increase in the electron donating strength of the amine substituent for **1a**-**c** and **3a**-**c** leads to the bathochromic shift in the λ_{fl} value. The E_{fl} values estimated from the λ_{fl} values of **1a**-**c** and **3a**-**c** are linearly correlated to the Hammett σ_p values. In addition, it was confirmed that the E_{fl} values of **1a**-**c** are linearly correlated to those of **3a**-**c**. The substituent effect of the amine family on the π-electronic properties of the luciferin and oxyluciferin skeletons were reasonably explained by the DFT and TD-DFT calculation data. Based on the obtained spectroscopic data of **1a**-**c** and **3a**-**c**, we confirmed that the E_{bl} values estimated from the λ_{bl} values on the L–L reactions of **1a-c** with Ppy luciferase are shown to be expressed with equation (3) as a function of the E_{fl} value for **1a**-**c** and **3a**-**c** (Figure 5). The result supports the usefulness of the method to predict the λ_{bl} values of luciferin analogues with the λ_{fl} values of the luciferin analogues themselves in a particular solvent, when the S_1 states of oxyluciferin analogues produced by the L–L reactions locate at a similar position in the luciferase active site. The conclusion of this study provides a theoretical guarantee to design a novel luciferin analogue for a desired purpose based on the fluorescence property of the luciferin analogue in place of the corresponding oxyluciferin analogue.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Fluorescence decay curves of **1a** (a and b), **1b** (c and d) and **1c** (e and f) in acetonitrile and water at 295 K.

Figure S2. Fluorescence decay curves of **3a** (a and b), **3b** (c and d) and **3c** (e and f) in benzene and acetonitrile 295 K.

Figure S3. HOMOs and LUMOs of **1a-c** and **3a-c**. The data of **1b** and **3b** are for the conformers **I**.

Figure S4. Bioluminescence spectra for the L–L reactions of **1a**-**c** with Ppy luciferase at (A) pH 8.0 and (B) pH 6.0 at room temperature.

Table S1. Heats of formation of **1**–**3** with DFT using B3LYP/6-31+G(d).

Table S2. Cartesian Coordinates (in Å) of luciferin analogue **1a** (R = NH_2).

Table S3. Cartesian Coordinates (in Å) of luciferin analogue **1b**-**I** (R = NHMe).

Table S4. Cartesian Coordinates (in Å) of luciferin analogue **1b**-**II** (R = NHMe).

Table S5. Cartesian Coordinates (in Å) of luciferin analogue **1c** (R = NMe_2).

Table S6. Cartesian Coordinates (in Å) of oxyluciferin analogue **2b**-**I** (R = NHMe).

Table S7. Cartesian Coordinates (in Å) of oxyluciferin analogue **2b**-**II** (R = NHMe).

Table S8. Cartesian Coordinates (in Å) of oxyluciferin analogue **3b**-**I** (R = NHMe).

Table S9. Cartesian Coordinates (in Å) of oxyluciferin analogue **3b**-**II** (R = NHMe).

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Tables

Table 1. Electronic absorption properties of luciferin analogues **1a**-**c** and oxyluciferin analogues **3a**-**c** in various solvents at 298 K.

Solvent [$E_T(30)$[a]]	λ_{ab} / nm (ε / 10^4 dm^3 mol^{-1} cm^{-1})[b]					
	1a (NH_2)	**1b** (NHMe)	**1c** (NMe_2)	**3a** (NH_2)	**3b** (NHMe)	**3c** (NMe_2)
p-Xylene [33.1]	---	---	---	415 (1.8)	443 (1.9)	465 (2.6)
Benzene [34.3]	---	---	---	417 (1.7)	446 (2.0)	469 (2.6)
Chloroform [39.1]	---	---	---	423 (1.5)	455 (1.8)	483 (2.7)
DMSO [45.1]	375 (1.6)	386 (1.3)	388 (1.8)	470 (2.2)	483 (2.0)	492 (2.4)
Acetonitrile [45.6]	359 (1.3)	378 (1.1)	386 (1.5)	434 (2.0)	458 (2.0)	476 (2.5)
2-Propanol [48.4]	360 (1.3)	381 (1.3)	379 (1.7)	467 (1.9)	479 (2.1)	480 (2.5)
Methanol [55.4]	362 (1.4)	382 (1.2)	387 (1.7)	455 (1.9)	473 (2.0)	486 (2.5)
Water [63.1]	351 (1.4)	373 (0.98)	383 (1.2)	---	---	---

[a] $E_T(30)$ in kcal mol^{-1}. [b] Absorption maximum (λ_{ab}) and extinction coefficient (ε) in parenthesis.

Table 2. Fluorescence properties of luciferin analogues **1a**-**c** and oxyluciferin analogues **3a**-**c** in various solvents at 298 K.

Solvent [$E_T(30)$[a]]	λ_{fl} / nm (Φ_f)[b]					
	1a (NH_2)	**1b** (NHMe)	**1c** (NMe_2)	**2a** (NH_2)	**2b** (NHMe)	**2c** (NMe_2)
p-Xylene [33.1]	---	---	---	494 (0.44)	511 (0.81)	535 (0.85)
Benzene [34.3]	---	---	---	495 (0.54)	517 (0.85)	541 (0.87)
Chloroform [39.1]	---	---	---	511 (0.85)	534 (0.90)	567 (0.90)
DMSO [45.1]	498 (0.49)	513 (0.64)	514 (0.71)	593 (0.79)	611 (0.74)	640 (0.39)
Acetonitrile [45.6]	472 (0.33)	486 (0.50)	503 (0.57)	567 (0.84)	587 (0.81)	621 (0.50)
2-Propanol [48.4]	481 (0.54)	489 (0.63)	500 (0.70)	593 (0.69)	604 (0.67)	634 (0.46)
Methanol [55.4]	491 (0.58)	503 (0.67)	519 (0.67)	607 (0.42)	623 (0.39)	656 (0.16)
Water [63.1]	520 (0.57)	534 (0.57)	562 (0.23)	---	---	---

[a] $E_T(30)$ in kcal mol^{-1}. [b] Fluorescence maximum wavelengths (λ_{fl}) and quantum yields (Φ_f) in parenthesis.

Table 3. Coefficients (a and b) for equation (1).

Compounds	a	b / kcal mol^{-1}
1a (NH_2)	−0.32	76
1b (NHMe)	−0.31	74
1c (NMe_2)	−0.36	74
2a (NH_2)	−0.54	76
2b (NHMe)	−0.48	72
2c (NMe_2)	−0.46	68

Table 4. Photophysical properties of **1a-c** and **3a-c** at 295 K.

Compound	Solvent	Φ_f	τ_f / ns	k_f [a] /10^8 s^{-1}	Φ_{nr} [b]	k_{nr} [c] / 10^8 s^{-1}
1a (NH_2)	acetonitrile	0.33	3.8	0.87	0.67	1.8
	H_2O	0.57	4.8	1.2	0.43	0.9
1b (NHMe)	acetonitrile	0.5	3.9	1.3	0.5	1.3
	H_2O	0.57	4.5	1.3	0.43	0.96
1c (NMe_2)	acetonitrile	0.57	4.6	1.2	0.43	0.93
	H_2O	0.23	1.9	1.2	0.77	4.1
3a (NH_2)	benzene	0.41	2.3	1.8	0.59	2.6
	acetonitrile	0.84	4.6	1.8	0.16	0.35
3b (NHMe)	benzene	0.85	3.5	2.4	0.15	0.43
	acetonitrile	0.81	4.8	1.7	0.19	0.4
3c (NMe_2)	benzene	0.87	3.9	2.2	0.13	0.33
	acetonitrile	0.5	3.3	1.5	0.5	1.5

[a] Determined by $k_f = \Phi_f \tau_f^{-1}$. [b] Determined by $\Phi_{nr} = 1-\Phi_f$. [c] Determined by $k_{nr} = \Phi_{nr} \tau_f^{-1}$.

Table 5 Calculation data of **1–3** with DFT and TD-DFT using B3LYP/6-31+G(d).

Compound	HOMO	LUMO	ΔE_{H-L} [a]	Transitions [b]	λ_{tr} [c] / nm (*f*)	Configuration [d,e]
1a (NH_2)	−5.91	−2.12	3.80	$S_0 \rightarrow S_1$	354 (0.46)	H → L (0.68)
1b-I (NHMe)	−5.69	−2.04	3.65	$S_0 \rightarrow S_1$	368 (0.50)	H → L (0.69)
1b-II (NHMe)	−5.66	−2.06	3.61	$S_0 \rightarrow S_1$	373 (0.49)	H → L (0.69)
1c (NMe_2)	−5.54	−2.02	3.52	$S_0 \rightarrow S_1$	383 (0.51)	H → L (0.70)
2a (NH_2)	−6.19	−2.87	3.31	$S_0 \rightarrow S_1$	403 (0.46)	H → L (0.69)
2b-I (NHMe)	−5.95	−2.78	3.16	$S_0 \rightarrow S_1$	419 (0.53)	H → L (0.70)
2b-II (NHMe)	−5.92	−2.80	3.11	$S_0 \rightarrow S_1$	428 (0.49)	H → L (0.70)
2c (NMe_2)	−5.80	−2.76	3.03	$S_0 \rightarrow S_1$	439 (0.53)	H → L (0.70)

3a[f] (NH_2)	−6.13	−2.80	3.33	$S_0 \rightarrow S_1$	401 (0.49)	H → L (0.69)
3b-I (NHMe)	−5.90	−2.71	3.19	$S_0 \rightarrow S_1$	418 (0.55)	H → L (0.70)
3b-II (NHMe)	−5.87	−2.73	3.14	$S_0 \rightarrow S_1$	426 (0.52)	H → L (0.70)
3c[f] (NMe_2)	−5.75	−2.69	3.06	$S_0 \rightarrow S_1$	438 (0.55)	H → L (0.70)

[a] Energy difference between HOMO and LUMO. [b] The π,π^* transition to the excited singlet state with the lowest excitation energy. [c] Wavelengths estimated from transition energies. [d] Configuration of excitation. [e] H, H−1, and L denote the HOMO, HOMO−1, and LUMO, respectively. [f] Ref. 39.

Figure Captions

Scheme 1. (A) The luciferin−luciferase (L−L) reaction of firefly bioluminescence. (B) The molecular structures of luciferin (LH_2), oxyluciferin (OLH) and keto-phenolate anion of OLH (OL^-). (C) Amine-substituted luciferin analogues **1** and oxyluciferin analogues **2** and **3**.

Scheme 2. Conformers **I** and **II** of luciferin analogue **1b**.

Figure 1. UV/visible absorption (Abs) and fluorescence (Fl) spectra of **1a** (A), **1b** (B), and **1c** (C) in DMSO (a), acetonitrile (b), 2-propanol (c), methanol (d), and H_2O (e) at 298 K.

Figure 2. UV/visible absorption (Abs) and fluorescence (Fl) spectra of **3a** (A), **3b** (B), and **3c** (C) in *p*-xylene (a), benzene (b), chloroform (c), DMSO (d), acetonitrile (e), 2-propanol (f), and methanol (g) at 298 K.

Figure 3. E_{fl} values of **1a** (●), **1b** (○), **1c** (⊗), **3a** (■), **3b** (□), and **3c** (⊞) plotted as a function of $E_T(30)$. The data in DMSO are marked with an asterisk.

Figure 4. (A) E_{fl} values of **1a**-**c** (■) and **3a**-**c** (□) in acetonitrile plotted as a function of the Hammett σ_p values. (B) E_{fl} values (E_{fl}(**3**)) of **3a**, **3b**, and **3c** plotted as a function of E_{fl} values (E_{fl}(**1**)) of **1a**, **1b**, and **1c** in acetonitrile (■), 2-propanol (⊞) and methanol (□).

Figure 5. E_{bl} values of **1a-c** plotted as functions of E_{fl} values of **1a-c** (■) and **3a-c** (□) in acetonitrile.