Synthesis and quantitative characterization of coumarin-caged D-luciferin

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Abstract

Caged luciferin compounds of firefly luciferins have recently drawn much attention since firefly bioluminescence, in which D-luciferin acts as a substrate, is widely used in noninvasive gene-expression imaging, studies of *in vivo* cell trafficking, and the detection of enzyme activity. The objectives of this study are the development of new caged luciferins and the quantitative determination of the photophysical parameters of their photo-decomposition. We synthesized 7-(diethylaminocoumarin)-4-(yl)methyl caged D-luciferin (DEACM-caged D-luciferin) and quantitatively characterized its absorption spectrum, bioluminescence, and photoproducts using chiral HPLC chromatography, as a function of light-irradiation time. We observed that 4 min of UV irradiation generated maximum D-luciferin concentrations, which corresponds to 16.2% of the original DEACM-caged-D-luciferin concentration. Moreover, we evaluated not only the rate of photocleavage (0.20 /min) from DEACM-caged D-luciferin to luciferin but also the rate of caged-luciferin degradation that did not produce luciferin (0.28 /min) and the rate of luciferin decomposition (0.20 /min) after exposure to irradiation with a 70 mW/cm^2 high-pressure mercury lamp (254 – 600 nm). The formation rate of L-luciferin via DEACM-

caged–D-luciferin photocleavage was smaller by a factor of 1/10 compared with that of D-luciferin. These quantitative measurements and simultaneous evaluations of photocleavage, degradation, and decomposition are the most important and original methodology presented in this study.

Keywords

DEACM-caged D-luciferin; Firefly Bioluminescence; Quantum Yield; Rate of photocleavage; Chiral HPLC chromatography

INTRODUCTION

The so-called caged compounds [1–9] remain inactive due to cage-forming groups, until activated by an external trigger, such as light, pH, or a specific chemical. Firefly bioluminescence, where D-luciferin is the substrate, is widely utilized as a noninvasive gene-expression imaging technique to image *in vivo* cellular mechanisms and to detect enzyme activity [10–12]. The caged compounds of D-luciferin are often used to image cell–cell contacts, to monitor gene silencing, and to perform real-time imaging of cell–cell coupling dynamics [2,7–8].

Tracking dynamic properties onto the desired time scales and/or at a targeted cell location in cells, tissues, or live animals require photolyzable caged luciferins [5–6]. To date, only a few photolyzable caged luciferins are available, such as 1-(2-nitrophenyl)ethyl-luciferin (NPE-luciferin) [7]. Only 1-(4,5-dimethoxy-2- nitrophenyl)-ethyl luciferin (DMNPE-luciferin) [2] is commercially available. However, this compound suffers from serious drawbacks, such as spontaneous hydrolysis and the occurrence of slow dark reactions after photolysis [9]. Hence, an urgent need exists for the development of new caged luciferins. Moreover, in order to design appropriate parameters for caged-luciferin

photolysis, quantitative data is required, such as the time and fluence of light irradiation necessary to cleave the caged groups, the rate of luciferins generation, and the rate of luciferins damage/decomposition, as well as the formation of side products. Such quantitative parameters are currently unavailable.

The objectives of this study follow the development of new caged luciferins and the quantitative determination of their photophysical light-induced decomposition parameters. Specifically, we prepared and characterized 7-(diethylaminocoumarin)-4-(yl)methyl-caged D-luciferin (DEACM-caged D-luciferin). We determined its photochemical decomposition products and assessed its bioluminescence as a function of irradiation time.

MATERIALS AND METHODS

Materials. All reagents were purchased from the Sigma-Aldrich Chemical Co. and were used without further modifications unless otherwise specified. Dichloromethane (DCM), methanol (MeOH), tetrahydrofuran (THF) were purified using a two column solvent purification system packed with activated alumina provided by Innovative Technology Inc. Water was deionized using a Millipore MilliQ system. The GTA buffer is composed of 3,3-dimethylglutaric acid (0.05

M, Wako), tris(hydroxymethyl)aminomethane (0.05 M, Wako) and 2-amino-2-methyl-1,3-propanediol (0.05 M, Wako) dissolved in water. Both $MgSO_4$ and adenosine-5'-triphosphate disodium salt trihydrate (ATP) were purchased from Wako Pure Chemical Industries, Ltd. The firefly luciferase, derived from *Photinus pyralis*, was bought from Sigma-Aldrich Co. LLC. Merck silica gel 60 with particle sizes ranging from 40–63 mm was used in Flash chromatography (FC).

Instrumentation. ^{1}H-NMR spectra were recorded on a Bruker AMX-400 (400 MHz) instrument. Sample solutions were prepared by dissolving 10 mg of compound in 1 mL of solvent ($CDCl_3$). UV-Vis spectra were measured on an Agilent 8453 UV-visible spectrophotometer equipped with a Peltier temperature control accessory. A 1 cm-path length quartz cuvette was used in all measurements. Mass spectra were recorded on a Micromass Autospec TOF instrument equipped with a LSIM source (Centre Regional de Spectrometrie de Masse, Universite de Montreal, Montreal, QC, Canada).

Synthesis. 7-Diethylamino-4-hydroxymethylcoumarin (a) was synthesized from 4-methyl-7-diethylaminocoumarin according to procedures described in Schönleber et al. [3]. Figure 2 shows the ^{1}H-NMR spectrum of compound **a**. d ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 7.31 (d, J = 9.0

92 Hz, 1H), 6.56 (dd, J = 9.0, 2.4 Hz, 1H), 6.50 (d, J = 2.4 Hz, 1H), 6.26 (s, 1H), 4.82 (s, 2H), 3.39

93 (q, J = 7.1, 4H), 2.30 (s, 1H), 1.20 (t, J = 7.1, 6H).

94 2-Cyano-6-hydroxybenzothiazole (b) was synthesized from 2-cyano-6- methoxybenzothiazole

95 according to procedures described in Shao et al. [7]. ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 9.80

96 (br, 1H), 7.86 (d, J = 9.0 Hz, 1H), 7.23(d, J = 2.3 Hz, 1H), 7.06 (dd, J = 9.0, 2.3 Hz, 1H) (Figure

97 3).

98 Alcohol **a** (0.42 g, 2.2 mmol), compound **b** (0.32 g, 1.8 mmol), and triphenylphosphine (TPP,

99 0.58 g, 2.2 mmol) were dissolved in dry THF (20 mL) in a round bottom flask. Under magnetic

100 stirring, diisopropylazodicarboxylate (DIAD, 0.45 g, 2.2 mmol) was added dropwise. After

101 complete addition, the mixture was stirred for 24 hours at room temperature. The orange

102 precipitate that formed was separated by filtration, dried, and purified by recrystallization from a

103 DCM/MeOH (2:1) mixture. The final title compound **c** formed as flaky, orange crystals. Yield

104 0.37 g, 60%. ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm) of **c**: 8.16 (d, J = 9.1, 1H), 7.46 (d, J = 2.5 Hz,

105 1H), 7.40(d, J = 9.1 Hz, 1H), 7.36 (dd, J = 9.1, 2.5 Hz, 1H), 6.63 (dd, J = 9.1, 2.5 Hz, 1H), 6.58

106 (d, J = 2.5 Hz, 1H), 6.28 (s, 1H), 5.29 (s, 2H), 3.46 (q, J = 7.1, 7.1 Hz, 4H), 1.25 (t, J = 7.1,7.1

107 Hz, 6H) (Figure 4).

Compound **c** (0.11 g, 0.32 mmol) was dispersed in 20 mL of a DCM/MeOH (1:1) mixture, and D-cysteine hydrochloride monohydrate (0.078 g, 0.45 mmol) was added. The suspension was stirred until the solid was completely dissolved. Then, 0.64 mL of a 10% $NaHCO_3$ aqueous solution was added and the mixture was stirred for 15 min. Afterwards, the mixture was acidified with 1 N HCl to a pH of 2–3 and extracted with DCM (30 mL ´ 3). The combined organic phase was washed with brine, dried over anhydrous Na_2SO_4, and concentrated. The product was purified by FC using a DCM/Hexanes/MeOH (7:2:1) solution as the eluent. Yield 0.12 g, 74%. Figure 5 shows the ^{1}H-NMR spectrum of this chemical species whose peaks were as follows: 8.10 (d, J = 9.0, 1H), 7.97 (d, J = 2.4 Hz, 1H), 7.61 (d, J = 9.0 Hz, 1H), 7.38 (dd, J = 9.0, 2.5 Hz, 1H), 6.74 (dd, J = 9.1, 2.4 Hz, 1H), 6.57 (d, J = 2.4 Hz, 1H), 6.19 (s, 1H), 5.44 (s, 2H), 5.27 (t, J = 8.9 Hz, 1H) 3.71 (q, J = 9.1, 2.3 Hz, 2H), 3.42 (q, J = 6.9, 6.9 Hz, 4H), 1.14 (t, J = 6.9, 6.9 Hz, 6H). ^{13}C-NMR (400 MHz, DMSO-d_6), δ (ppm): 161.4, 160.1, 158.1, 156.5, 152.0, 151.3, 148.5, 137.8, 126.8, 126.4, 118.5, 109.5, 107.1, 106.3, 106.0, 97.8, 86.8, 44.5, 13.2. MS (ESI): m/z calculated for C25H23N3O5S2, 509.61, was 510.16 $[M+H]^+$.

The 7-(diethylaminocoumarin)-4-(yl)methyl-caged D-luciferin (DEACM-caged D-luciferin) (d) solution was prepared by dissolving 5.1 mg of **d** in several drops of DMSO. After complete

dissolution, DI water was added to dilute the DMSO solution to a volume of 200 mL. The concentration of **d** in this solution was 0.05 mM. The melting point of **d** was 200ºC (± 10 ºC).

HPLC analysis. The sample (40 mL) containing the analyte in the water/DMSO (8:2) solution was injected into an analytical HPLC system consisting of a Waters 1500 HPLC System (Nihon Waters, Tokyo, Japan), a Waters 2489 UV/Visible Detector, a Waters 1525 Binary HPLC Pump, and a chiral column Chi ralcel CHIRALPAK IG (4.6 mm φ × 150 mm, Daicel Chemical Industries, Tokyo, Ja- pan) (See Figure S4). Compounds were eluted by increasing the MeCN concentration, in water, from 10 to 90% over a period of 20 min at a flow rate of 1.0 mL/min, simultaneously measuring absorbance (λ = 330 nm) (See Figure S5).

Quantitative characterization For UV/Vis measurement, D-luciferin was dissolved in a water/DMSO 95/5 v/v solution and DEACM-caged D-luciferin was dissolved in a water/DMSO 4/1 v/v solution (2.0×10^{-4} M, 0.5 mL). The solutions were poured into borosilicate glass tubes (12×75 mm) for UV/Vis spectram mesurements before and after UV-light irradiation (70 mW/cm^2, 254–600 nm). The UV irradiation device was a multipurpose exposure unit (Multilight; Ushio Inc.) equipped with standard irradiation optics (ML-251A/B, PM-25C-100) and a UV lamps (USH-250BY).

Results and Discussion

DEACM-caged-D-luciferin synthetic routes, photocleavage, and bioluminescence processes are presented in Figure 1. The DEACM-caged D-luciferin precursor, i.e., 2-cyano-6-((7-diethylaminocoumarin-4-yl)methoxy)benzothiazole (compound **c**), was prepared by coupling 2-cyano-6-hydroxybenzothiazole (compound **b**) to 7-diethylamino-4-hydroxymethylcoumarin (compound a) via a Mitsunobu coupling using THF as a solvent and the acidic 2-cyano-6-hydroxybenzothiazole phenol as a nucleophile. The reaction product (compound **c**) was insoluble in THF, which allowed its purification simply by recrystallization from THF. Subsequently, we reacted compound **c** with D-cysteine to obtain the target product (compound **d**) with a high yield [13]. In Fig. 5, we observed the successful condensation of D-cysteine to precursor **c** based on the appearance of two new resonances at 5.27 and 3.71 ppm, which belong to the methine (9) and methylene (10) groups, respectively, from the incorporated D-cysteine [7]. In addition, the integration of these resonances have a similar ratio with the other proton resonances in this compound.

Figure 6 presents the UV/Vis spectra of the DEACM-caged-D-luciferin solution before (black curve) and after UV-light irradiation for 4 and 20 min (red and blue curves, respectively). The

UV/VIS spectrum of the D-luciferin solution is also shown in Figure 6 as a reference (black dotted curve). Solution pH ranged from 5.5 to 6.8. The DEACM-caged-D-luciferin spectrum prior to UV irradiation has absorption bands at 384, 339, and <250 nm, while the main adsorption band for D-luciferin is centered at 327 nm. A simple linear interpolation of the two absorption spectra does not match the DEACM-caged-D-luciferin solution spectra after 4- and 20-min UV irradiation, which suggests that UV-light irradiation generates side products and/or products formed by damage/decomposition in addition to D-luciferin.

In order to understand D-luciferin formation during UV irradiation of DEACM-caged D-luciferin and to quantitatively determine the D-luciferin yield, we measured the *in-vitro* bioluminescence and quantum emission yield using a calibrated total-photon-flux charge coupled device (CCD)-spectrometer system [14]. Table 1 lists the bioluminescence parameters and experimental conditions.

The normalized bioluminescence spectra that we recorded for the DEACM-caged-D-luciferin solution after UV-light irradiation and for a D-luciferin solution with a pH of 8 are presented in Figure 7(a). The position and shape of the emission band for both spectra are nearly identical.

171 These observations confirm that DEACM-caged D-luciferin via UV-light irradiation produced
172 D-luciferin.

173 In Figure 7(b), we present the bioluminescence quantum yields (QY) of each solution as a
174 function of UV-light irradiation time. Bioluminescence QY is defined as the ratio of the number
175 of photons produced to the number of DEACM-caged-D-luciferin molecules. Note that the
176 bioluminescence QY of D-luciferin is 41% at a pH of 8 [14]. As shown in Figure 7(b), the
177 experimental QY increases from 0 to 6.0% as UV-irradiation time increases from 0 to 4 min.
178 Experimental QY decreases with further increases in the UV-irradiation time, which implies that
179 UV irradiation not only cleaves but also damages the caged compound and D-luciferin.

180 Figure S5 shows the HPLC chromatograms (normalized absorbance at 330 nm *vs*. Time) of
181 DEACM-caged D-luciferin, L-luciferin, and D-luciferin (See Supporting Information). DEACM-
182 caged D-luciferin elutes with retention times of 23.6 and 24.6 min, while L-luciferin and D-
183 luciferin elute, respectively, at 9.7 and 10.2 min.

184 Figure 8 presents DEACM-caged-D-luciferin solution chromatograms, monitored at 330 nm,
185 after UV irradiation. In the chromatograms, signal intensity from DEACM-caged D-luciferin
186 (23.6 and 24.6 mins) decreased with irradiation, while signals from L-luciferin and D-luciferin

187 (9.7 and 10.2 mins) increased in intensity, implying that DEACM-caged D-luciferin was
188 consumed and L-luciferin and D-luciferin were produced by DEACM-caged-D-luciferin
189 photolysis.

190 Based on these chromatograms and calibration curves established for D- and L-luciferins, we
191 evaluated the molar concentrations of DEACM-caged D-luciferin (c_{CL}), D-luciferin(c_{DL}), L-
192 luciferin (c_{LL}), and luciferin ($c_L=c_{DL}+c_{LL}$), as a function of the UV-irradiation time, t. Using our
193 data, we derived the relative fractions, i.e., R_{CL}, R_{DL}, R_{LL}, and $R_L=R_{DL}+R_{LL}$, of the concentrations
194 to the initial concentrations, c^0_{CL}, for DEACM-caged D-luciferins based on the following
195 equations:

196 $$R_{CL}=c_{CL}/c^0_{CL}, \quad (1)$$
197 $$R_L=c_L/c^0_{CL}. \quad (2)$$

198 Changes in R_{CL} (black dots) and R_L (green dots) as a function of the UV-irradiation time are
199 presented in Figure 9(a). The fraction, $R_{L(broken)}$, of lost caged luciferins that did not produce
200 luciferin plus lost luciferins due to light-induced decomposition is also shown in Figure 9(a) (open
201 circles). As shown in Fig. 1, 1 mole of luciferin should be produced upon irradiation of 1 mole of
202 DEACM-caged D-luciferin.

203 $$R_{L(broken)} + R_L = 1 - R_{CL} \quad (3)$$

After 4 min of UV irradiation, R_L reaches a maximum value of 17.8%. DEACM-caged D-luciferin cleavage reactions end after 7 min, since the DEACM-caged D-luciferin fraction remains constant after this time. We defined three rates (inversed constants): 1) DEACM-caged D-luciferin cleavage to luciferin (γ_1), 2) caged-luciferin degradation that does not produce luciferin (γ_2), and 3) luciferin decomposition (γ_3). Via simple rate-equation calculations, we express the time dependence of R_{CL} and R_L, as:

$$R_{CL} = \exp[-(\gamma_1+\gamma_2)t], \quad (4)$$

$$R_L = [\gamma_1/(\gamma_1+\gamma_2-\gamma_3)]x\{ \exp(-\gamma_3 t) -\exp[-(\gamma_1+\gamma_2)t] \}. \quad (5)$$

Three fitting curves are drawn in Fig. 9(a) using Eqs. (3–5) where:

$$\gamma_1=0.20 \text{ /min.} \quad (6)$$

$$\gamma_2=0.28 \text{ /min.} \quad (7)$$

$$\gamma_3=0.20 \text{ /min.} \quad (8)$$

In other words, the rates γ_1 and γ_2 of caged-luciferin photocleavage are 0.20/min and 0.28/min, and the rate γ_3 of luciferin damage or decomposition is 0.20/min, during irradiation produced from a 70 mW/cm^2 light (254–600 nm) high-pressure mercury UV lamp.

Figure 9(b) shows the proportions of D-luciferin R_{DL} (blue dots) and L-luciferin R_{LL} (red dots) as a function of the UV-irradiation time, t. The three rates, i.e., R_L, R_{DL} and R_{LL}, are at a maximum at t = 4 min, with intensities of 17.8, 16.2, and 1.6%, respectively.

From the bioluminescence QY data presented in Fig. 7(b), we evaluated the relative D-luciferin fraction, R'_{DL}:

$$R'_{DL} = QY / 41\%, \quad (9)$$

which is denoted by pink triangles in Fig. 9(b). The R_{DL} (blue dots) and R'_{DL} (pink triangles) data agree well, especially for t < 7 min.

Using the R_L fitting curve shown in Fig. 9(a), we drew fitting curves for both R_{LL} and R_{DL} in Fig. 9(b), based on the following equations:

$$R_{DL} = (1-p)\, R_L \quad (10)$$

$$R_{LL} = p\, R_L, \quad (11)$$

where p = 0.09. These results indicate that L-luciferin formation is lower than D-luciferin formation by approximately 1/10.

Finally, we note that agreement between R_{DL} with R'_{DL}, and the measured data for R_{CL}, R_L, R_{DL}, and R_{LL} with the fitting curves is quite good for irradiation times from t = 0 to 7

min. At longer times, discrepancies between the experimental and the fit data become much larger. Side products produced via photolysis may cause errors during the quantification of weak HPLC signals for solution chromatograms irradiated during periods greater than 7 min.

Conclusions

In summary, we synthesized and quantitatively characterized DEACM-caged D-luciferin by measuring absorption spectra, HPLC, and bioluminescence. We confirmed that 4-min UV irradiation generates the maximum D-luciferin concentration corresponding to a photochemical reaction quantum yield of 16.2%. Moreover, we evaluated not only the rate of photocleavage (0.20/min) from DEACM-caged D-luciferin to luciferin, but also the rate of caged-luciferin degradation that did not produce luciferin (0.28/min) as well as the rate of luciferin decomposition (0.20/min) during irradiation with a 70 mW/cm^2 high-pressure mercury lamp (254–600 nm).

These quantitative measurements and simultaneous evaluations of photocleavage, degradation, and decomposition, are the important and original results of this study, which

has never been performed in previous studies [7]. Our quantitative procedures proposed in this study will be a standard methodology for caged-luciferin photolysis to trigger luciferin.

Based on the results of this work, we will elucidate the electronic excited states of caged luciferin using quantum-chemistry calculations. This will lead to theoretical predictions of suitable wavelengths for caged-luciferin photolysis. We will then perform quantitative experiments with the light source of these wavelength in the near future.

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References

[1] G. C. R. Ellis-Davies, Caged compounds: photorelease technology for control of cellular chemistry and physiology, Nature Methods 4 (2007) 619-628..

[2] J. I. W. E. I. Yang, D. B. Thomason, An easily synthesized, photolyzable luciferase substrate for in vivo luciferase activity measurement, Biotechniques 15 (1993) 848-850.

[3] R. O. Schönleber, J. Bendig, V. Hagen, B. Giese, Rapid photolytic release of cytidine 5′-diphosphate from a coumarin derivative: a new tool for the investigation of ribonucleotide reductases, Bioorganic & Medicinal Chemistry 10 (2002) 97-101.

[4] T. Eckardt, V. Hagen, B. Schade, R. Schmidt, C. Schweitzer, J. Deactivation Behavior and Excited-State Properties of (Coumarin-4-yl)methyl Derivatives. 2. Photocleavage of Selected (Coumarin-4-yl)methyl-Caged Adenosine Cyclic 3′,5′-Monophosphates with Fluorescence Enhancement, J. Org. Chem. 67 (2002) 703-710.

[5] D.M. Rothman, M. D. Shults, B. Imperiali, Chemical approaches for investigating phosphorylation in signal transduction networks, Trends Cell Biol. 15 (2005) 502-510.

[6] D. S. Laurence, The preparation and in vivo applications of caged peptides and proteins, Curr. Opin. Chem. Biol. 9 (2005) 570-575.

[7] Q. Shao, T. Jiang, G. Ren, Z. Cheng, B. Xing, Photoactivable bioluminescent probes for imaging luciferase activity, Chem. Commun. 27 (2009) 4028-4030.

[8] W. B. Porterfield, K. A. Jones, D. C. McCutcheon, J. A. Prescher, A “Caged” Luciferin for Imaging Cell–Cell Contacts, Journal of the American Chemical Society 137 (2015) 8656-8659.

[9] T. Kageyama, M. Tanaka, T. Sekiya, S. Y. Ohno, N. Wada, The Reaction Process of Firefly Bioluminescence Triggered by Photolysis of Caged-ATP, Photochemistry and Photobiology 87 (2011) 653-658.

[10] F. McCapra, Chemical generation of excited states: The basis of chemiluminescence and bioluminescence, Methods Enzymol. 305 (2000) 3-47.

[11] O. Shimomura, Bioluminescence: Chemical Principles and Methods. World Scientific, 2006, p1-29.

[12] T. S. Bailey, N. T. Donor, S. P. Naughton, M. D. Pluth, A simple bioluminescent method for measuring D-amino acid oxidase activity, Chem. Commun. 51 (2015) 5425-5428.

[13] K. C. K. Swamy, N. N. B. Kumar, E. Balaraman, K. V. P. P. Kumar, Mitsunobu and Related Reactions: Advances and Applications, Chemical Reviews 109 (2009) 2551-2651.

[14] Y. Ando, K. Niwa, N. Yamada, T. Enomoto, T. Irie, H. Kubota, Y. Ohmiya, H. Akiyama. Firefly bioluminescence quantum yield and colour change by pH-sensitive green emission, Nature Photonics 2 (2008) 44-47.

Table 1. Parameters for firefly bioluminescence measurements.

Reagents	Concentration (M)	Volume (μL)	Final concentration (M)
GTA (pH 8)	0.15	35	
Mg^{2+}	0.1	5	5.0×10^{-3}
coumarin caged D-luciferin	1.0×10^{-6}	5	5.0×10^{-8}
Luciferase	2.0×10^{-5}	5	1.0×10^{-6}
ATP	1.0×10^{-3}	50	5.0×10^{-4}

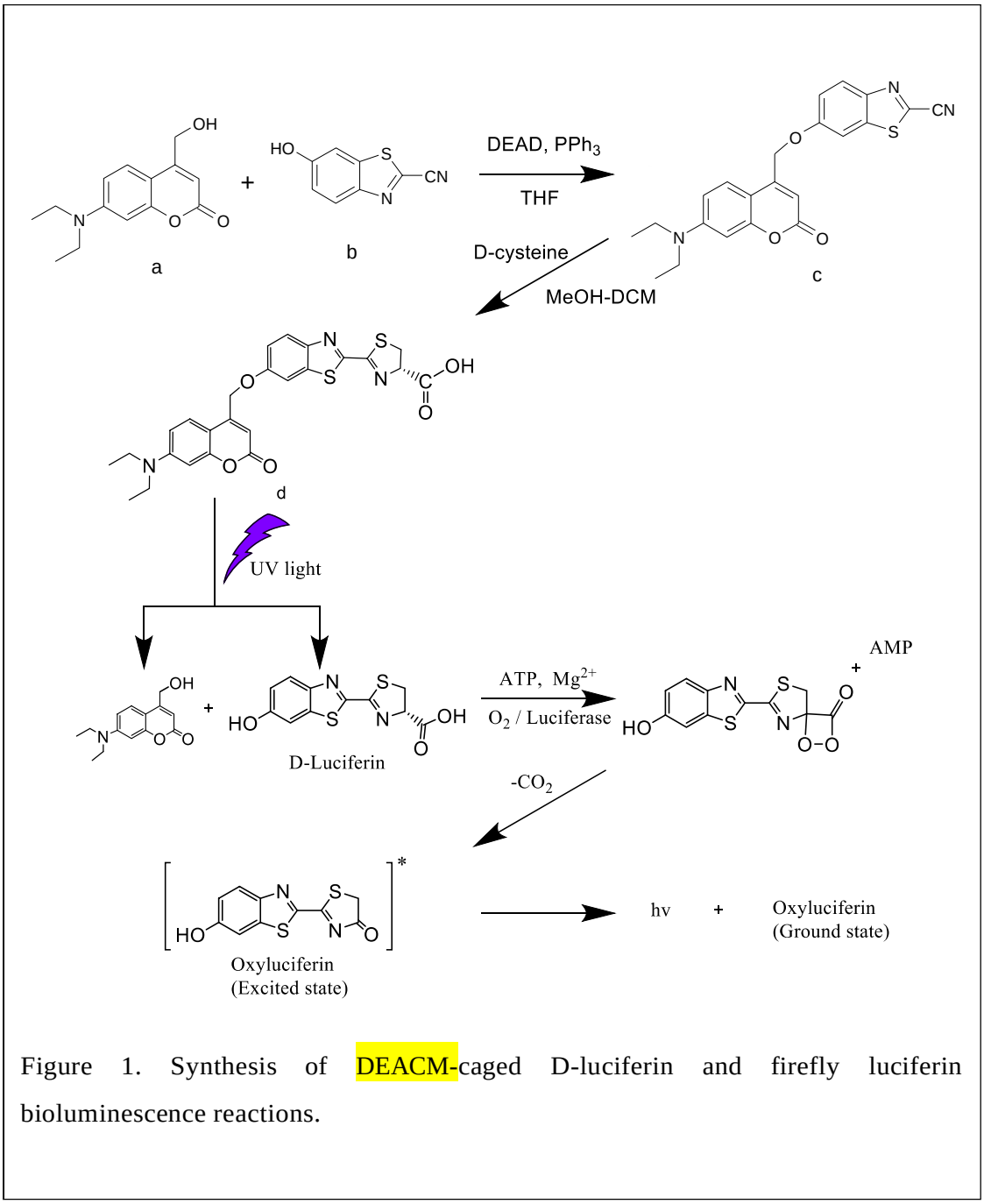

Figure 1. Synthesis of DEACM-caged D-luciferin and firefly luciferin bioluminescence reactions.

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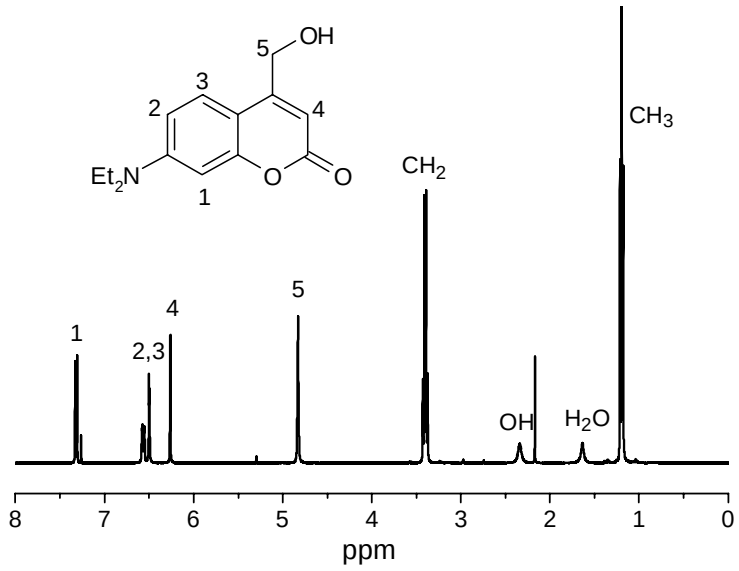

Figure 2. ^{1}H-NMR spectrum of 7-Diethylamino-4-hydroxymethylcoumarin (a).

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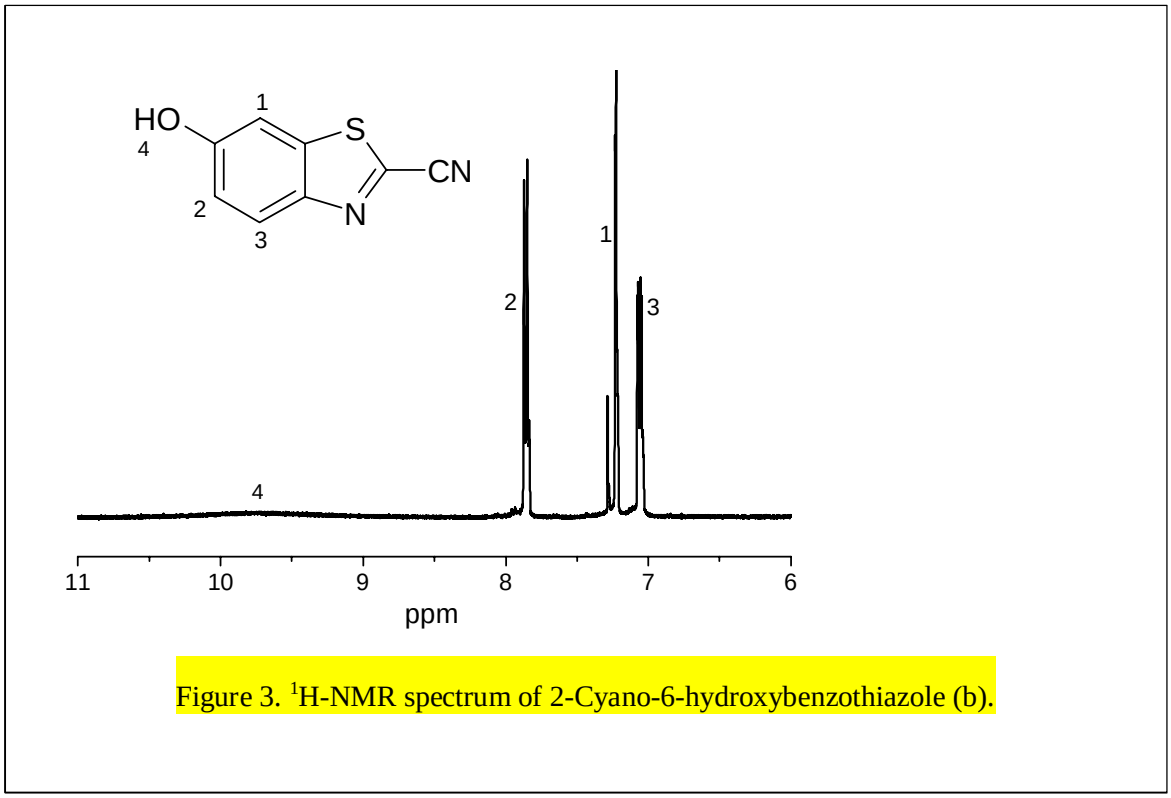

Figure 3. ^{1}H-NMR spectrum of 2-Cyano-6-hydroxybenzothiazole (b).

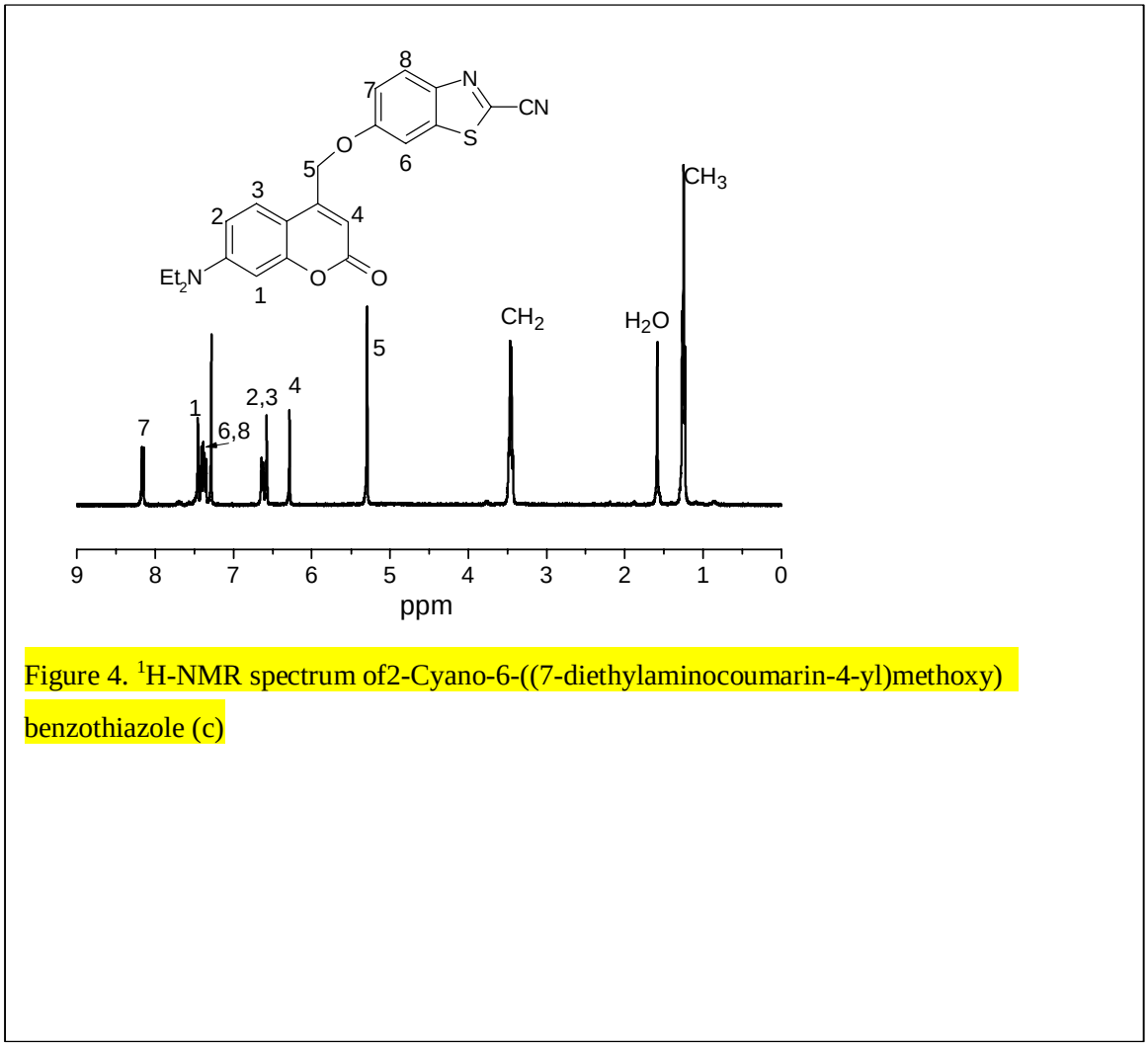

Figure 4. ^{1}H-NMR spectrum of2-Cyano-6-((7-diethylaminocoumarin-4-yl)methoxy) benzothiazole (c)

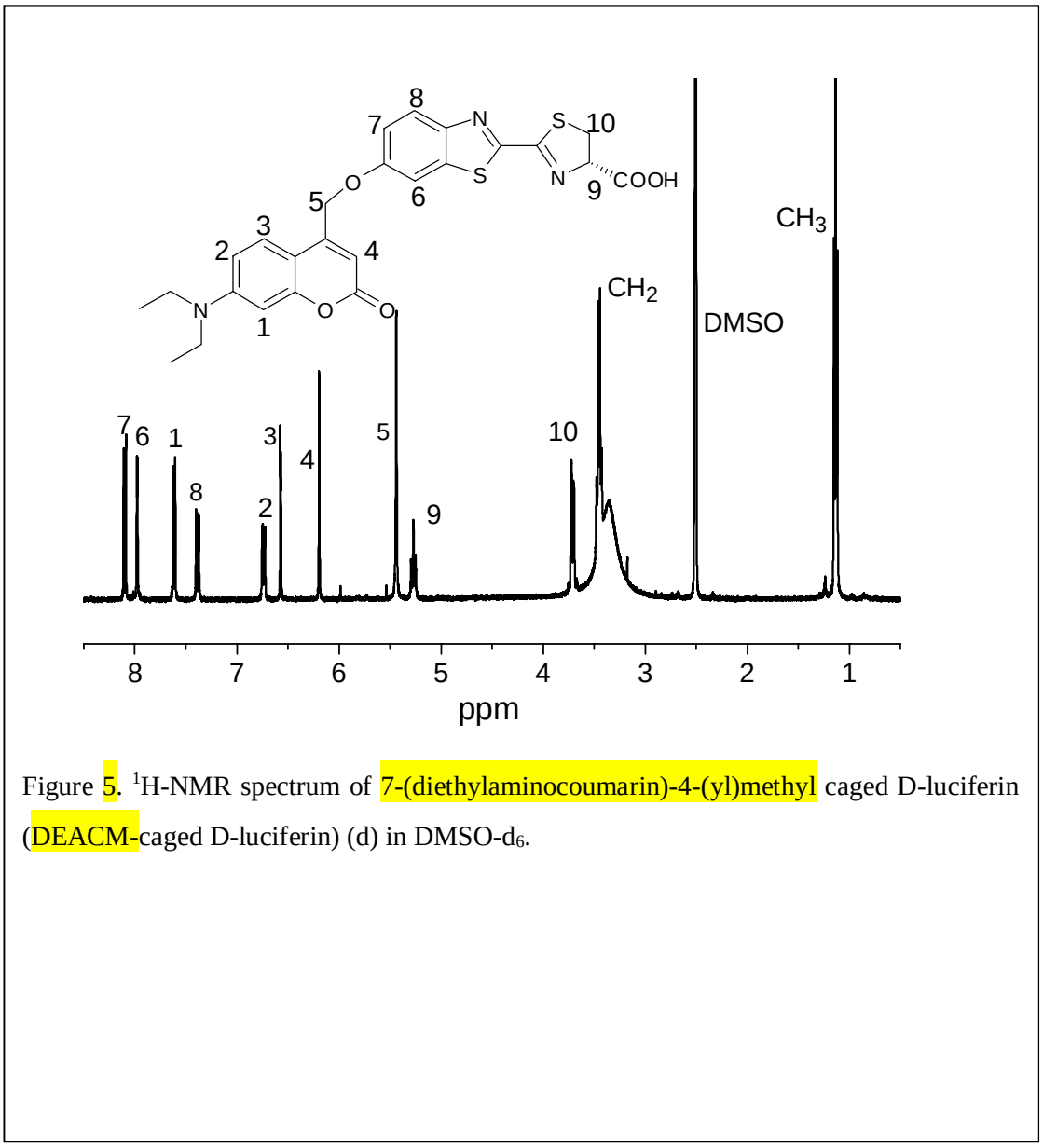

Figure 5. ^{1}H-NMR spectrum of 7-(diethylaminocoumarin)-4-(yl)methyl caged D-luciferin (DEACM-caged D-luciferin) (d) in DMSO-d_6.

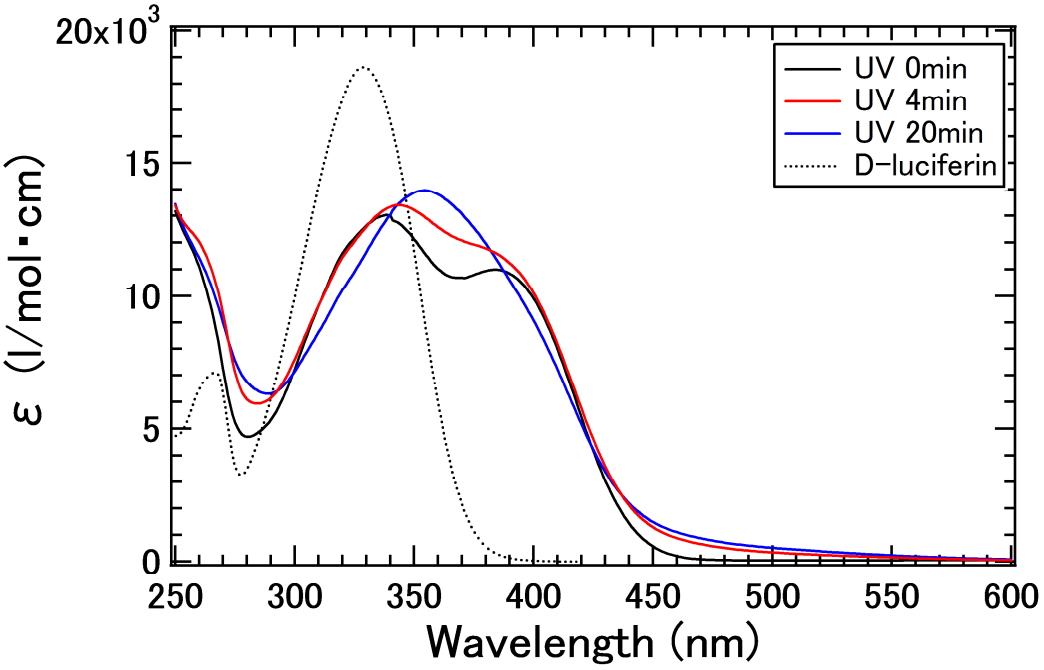

Figure 6. . UV/Vis spectra in mixed solvents (95% pure water, 5% DMSO) composed of DEACM-caged D-luciferin, which was irradiated with UV light for 0 (black curve), 4 (red curve), and 20 (blue curve) minutes. We also include the D-luciferin (black dotted line) UV/Vis spectrum for comparison.

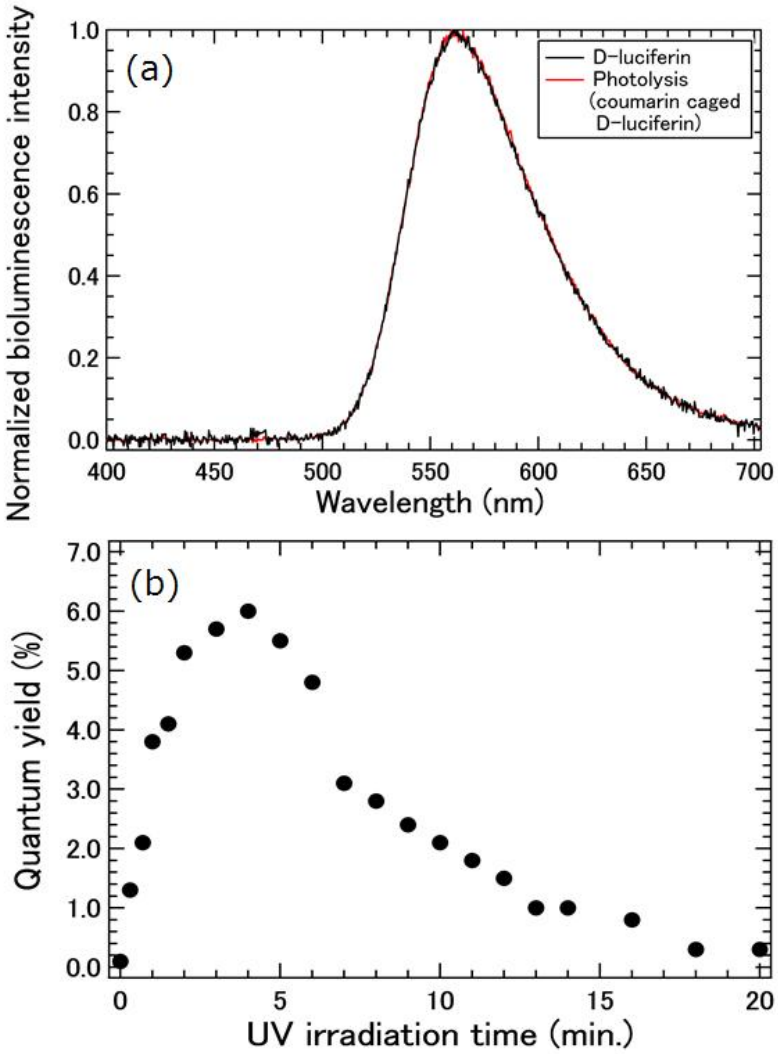

Figure 7. (a) Bioluminescence spectrum in a GTA buffer solution (pH 8) composed of DEACM-caged D-luciferin (red line), which was irradiated with UV light (D-luciferin, black line, is shown for comparison). (b) The bioluminescence quantum yield when DEACM-caged D-luciferin was irradiated with UV light.

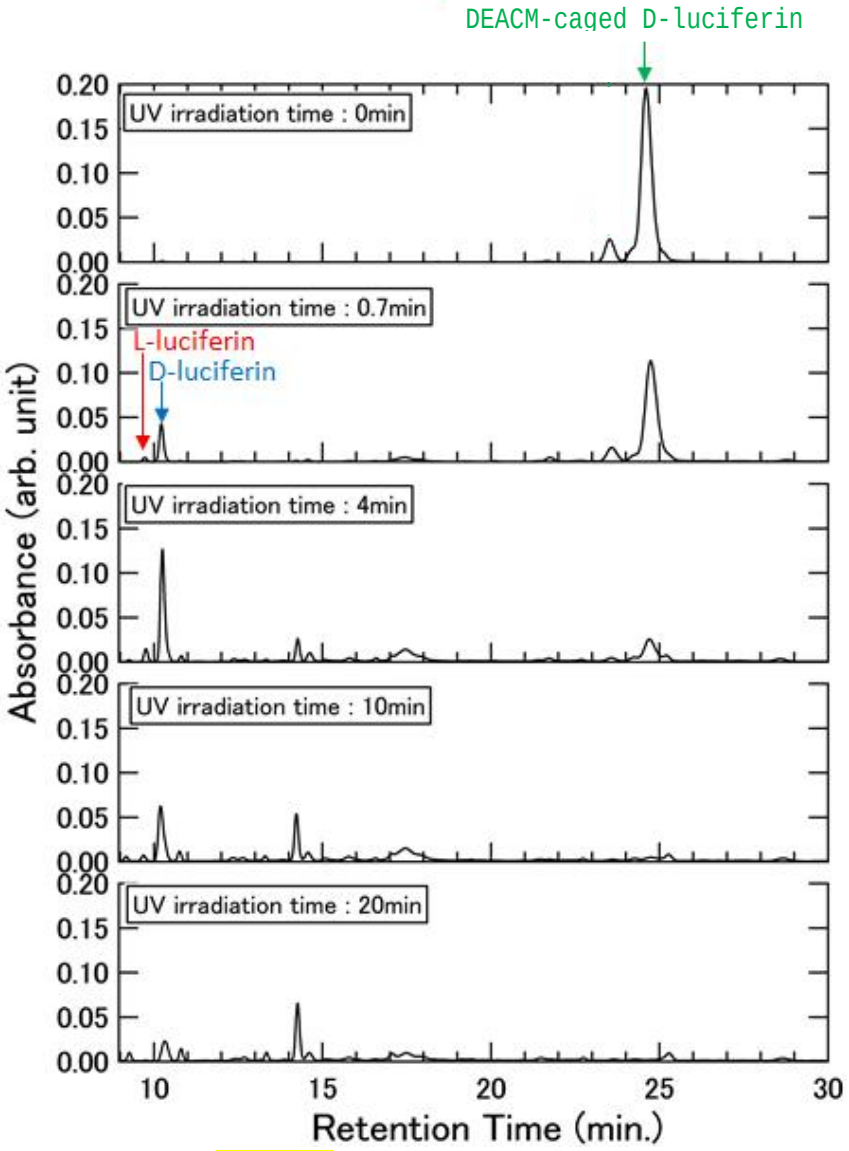

Figure 8. HPLC analysis of DEACM-caged D-luciferin irradiated with UV light.

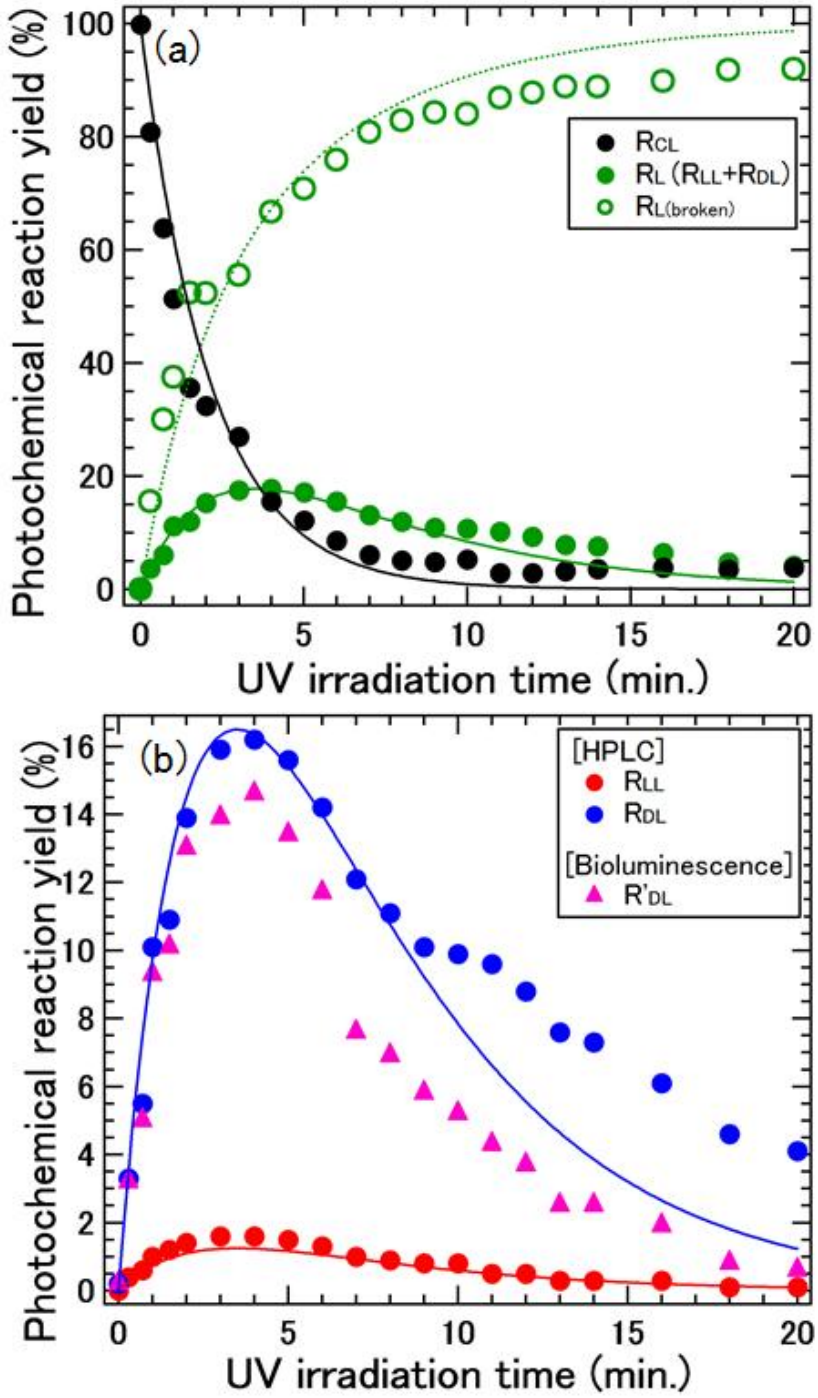

Figure 9. (a) Concentration ratios of DEACM-caged D-luciferin (R_{CL}) and the luciferin (R_L) *vs.* UV irradiation times, which were characterized by HPLC measurements. The ratio of the broken caged luciferin plus broken luciferin by UV irradiation ($R_{L(broken)}$) is also protted. (b) Concentration ratios of L-luciferin (R_{LL}) and D-luciferin (R_{DL}) characterized by HPLC measurements. The concentration ratio of DEACM-caged D-luciferin (R'_{DL}) characterized by bioluminescence is also plotted.