Tetrahedron 69 (2013) 3847–3856

Contents lists available at SciVerse ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

Development of simple firefly luciferin analogs emitting blue, green, red, and near-infrared biological window light

Satoshi Iwano, Rika Obata, Chihiro Miura, Masahiro Kiyama, Kazutoshi Hama, Mitsuhiro Nakamura, Yoshiharu Amano, Satoshi Kojima, Takashi Hirano, Shojiro Maki *, Haruki Niwa *

Department of Engineering Science, The University of Electro-Communications, Chofu, Tokyo 182-8585, Japan

ARTICLE INFO

Article history:
Received 22 January 2013
Received in revised form 11 March 2013
Accepted 12 March 2013
Available online 18 March 2013

Keywords:
Bioluminescence
Firefly
Luciferin analog
Near-infrared
Biological window

ABSTRACT

Simple firefly luciferin analogs emitting blue, green, and red light were developed. The longest emission maximum was observed at 675 nm, which belongs to the NIR biological window (650–900 nm), useful for deep site bioimaging of living animals. The analogs showed a slow rise of emission intensity compared with the rapid emission of natural luciferin. The light emission of the adenylated analogs was strongly enhanced compared with those of analogs themselves.

1. Introduction

Firefly luciferase is well known for its highly efficient light emission by catalyzing the oxidation of substrate D-luciferin (D-LH_2). The accepted catalytic mechanism of firefly bioluminescence is shown in Fig. 1.[1–3] This enzymatic light emission process does not require an external light source to fluorescence, thereby an extremely high signal-to-noise ratio is provided by this process. Because of its substrate specificity and high sensitivity, firefly luciferase and luciferin combination is widely used in the detection of ATP, and biological studies, with luciferase as a reporter gene in cell culture systems,[4] and recently in noninvasive whole-body bioimaging.[5] For the bioimaging technique, emissions of light with various colors are useful. In particular, red, or more desirably, the light around the 650–900 nm region termed the near-infrared (NIR) biological window,[6] is suitable for noninvasive whole-body imaging. Since the light of the NIR window is not strongly absorbed by oxygenated hemoglobin and melanin in animal tissues, the light is expected to be useful for deeper site imaging.

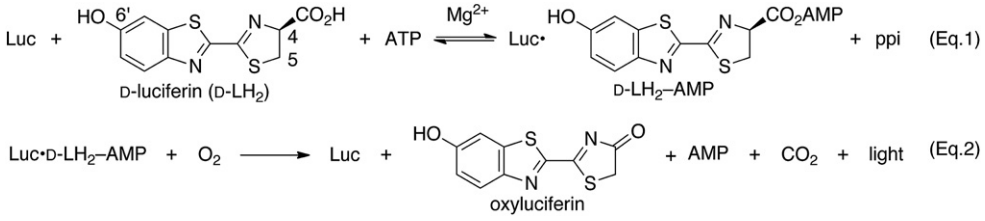


Fig. 1. Proposed reaction catalyzed by firefly luciferase. In the first step of the reaction, luciferase (Luc) catalyzes adenylation of D-LH_2 with ATP in the presence of Mg^{2+} to generate the intermediate luciferyl–AMP (D-LH_2–AMP) accompanied by pyrophosphate (ppi) (Eq. 1). Then the oxidative decarboxylation of the intermediate gives excited-state oxyluciferin, which then releases visible light in the course of relaxation to the ground state (Eq. 2).

In addition to Lampyridae (firefly) luciferases, several different isozymes are known to emit light in different colors using the same

* Corresponding authors. Tel.: +81 42 443 5484; fax: +81 42 486 1966 (H.N.); tel.: +81 42 443 5493; fax: +81 42 486 1966 (S.M.); e-mail addresses: maki@pc.uec.ac.jp (S. Maki), niwa@pc.uec.ac.jp (H. Niwa).

0040-4020/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.tet.2013.03.050

substrate D-LH_2.[7] Based on a bioengineering study, several mutant luciferases emitting various colors were developed.[8,9] Among various luciferases including their mutants, the shortest emission maximum wavelength was 534 nm and the longest was 623 nm.[7] Theoretical study of the light emitter oxyluciferin indicated an expected emission range from natural D-LH_2 to be 421–626 nm.[10] This study suggested that a new substrate scaffold, other than the natural luciferin chromophore, is required in order to obtain a wider emission range and longer maximum emission wavelength with Lampyridae luciferases including their mutants.

After the structure of luciferin was elucidated and synthesized in the 1960s, some modified luciferin analogs, such as 6′-aminoluciferin,[11] were synthesized and a few of them functioned as light-emitting substrates.[3] More recently, *N*-alkylated 6′-aminoluciferins were found to act as substrates[12] and cyclic alkylaminoluciferin was reported to show red light emission (607 nm) by using the mutant of *Photuris pennsylvanica* firefly luciferase (Ultra-Glo).[13] Some modified luciferins were newly developed for color change and specific purposes.[14–22] However, most synthetic bioluminescent luciferin analogs consist of benzothiazole and 4-carboxythiazolin-2-yl rings unchanged from natural luciferin, except for quinolyl-, coumaryl-, naphthylluciferins,[17,23a] and very recently published[23b] heterocyclic luciferin analogs.

In this study, we have substituted a benzothiazole moiety with a simplified aromatic structure to investigate the effect of π conjugation to the emission wavelength (Fig. 2).

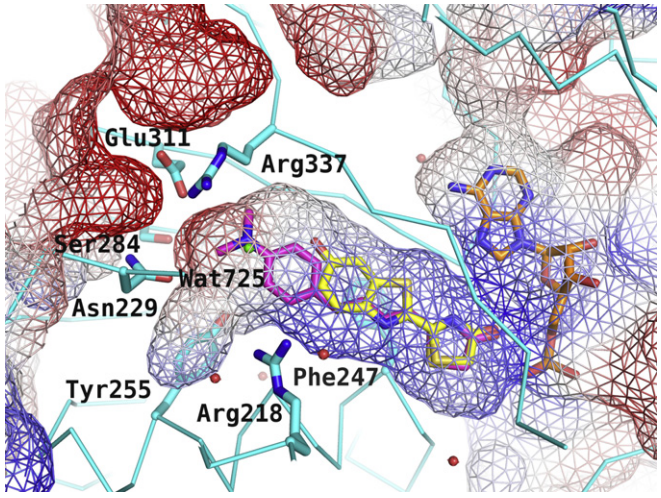


Fig. 3. Structure around plausible ligand-binding site of *P. pyralis* luciferase and overlay model of oxy-**3b**, oxyluciferin and AMP. The backbone structure of luciferase (4G36) is shown in cyan, and the electrostatic surface is presented as a wire mesh with negative and positive charges in red and blue, respectively. The original ligand DLSA is omitted from the figure for clarity. Side chains of Arg218, Ans229, Phe247, Tyr255, Ser284, Glu311, and Arg337 are shown in stick form. The view was sliced to show the binding pocket and some residues were omitted from the figure for clarity. Water molecules are shown as red spheres, except Wat725 is colored green. The ligand atoms are displayed in stick form with the carbon atom of oxy-**3b** in magenta, oxyluciferin in lemon yellow and AMP in orange. Nitrogen is colored blue, oxygen is colored red, sulfur is colored yellow, and phosphorus is colored deep orange.

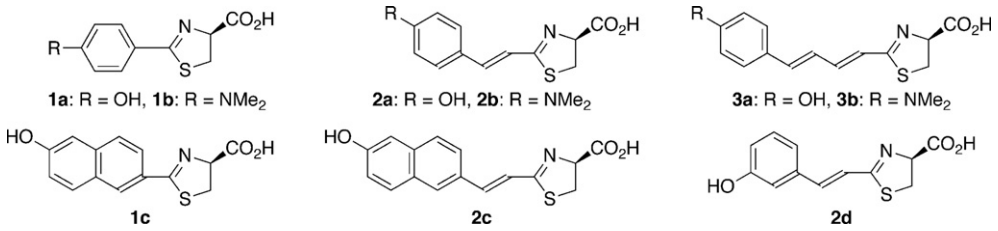


Fig. 2. Chemical structure of synthetic substrates (**1**–**3**).

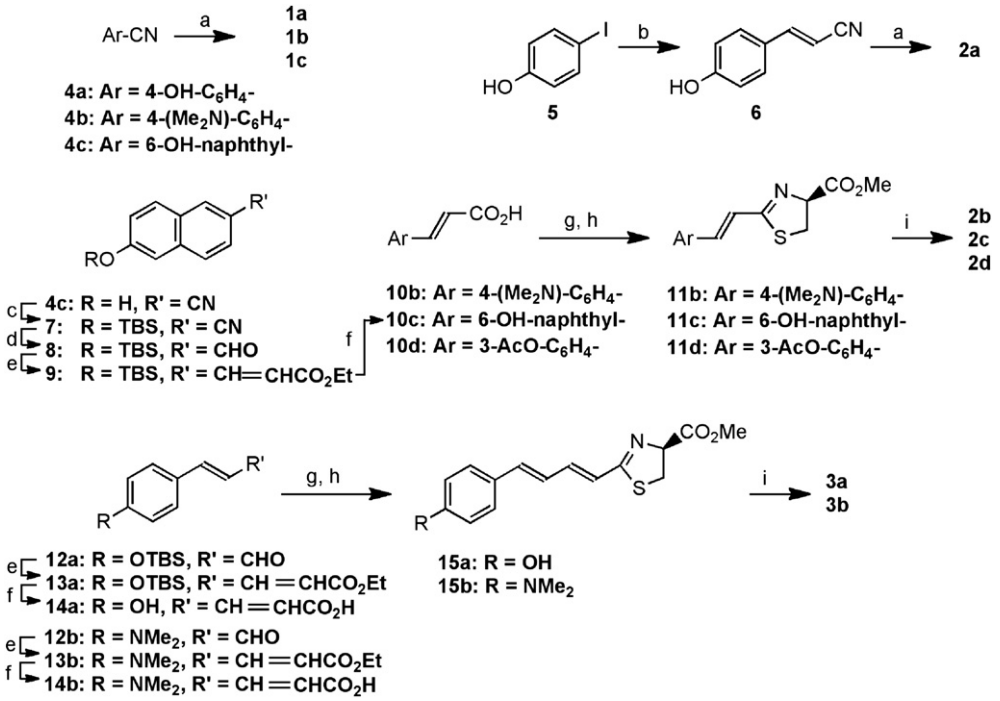
2. Results and discussion

2.1. Molecular design and synthesis of luciferin analogs

Because the scaffolds of these analogs are different from those of existing substrates, we have used molecular modeling to predict the interaction between intermediate species of one of the longest analogs, **3b**, and luciferase. Fig. 3 shows a plausible location of the model compound oxy-**3b** and AMP at the active site of the *Photinus pyralis* luciferase. The crystal structures used (PDB accession number 4G36[23c] and 2D1R[32]) were downloaded from the RSCB protein data bank. Structural alignment, generation of electrostatic surface potential and construction of the figure were carried out using PyMOL (DeLano Scientific; http://www.pymol.org). The coordinates of the DLSA-bound *P. pyralis* luciferase structure (chain B of 3G36) superimposed closely on the oxyluciferin and AMP-bound *Luciola cruciata* luciferase structure (2D1R), giving a root-mean-square deviation of 1.1 Å based on 3227 atoms. The local minimum conformation model of oxy-**3b** was obtained using Spartan '04 (Wavefunction; http://www.wavefun.com) by Hartree–Fock 3-21G calculation. The 4,5-dihydro-4-oxothiazol-2-yl moiety in oxy-**3b** was manually aligned onto both a 4,5-dihydro-4-oxothiazol-2-yl ring of the oxyluciferin and a thiazole ring of DLSA. As shown in Fig. 3, the extended *E*,*E*-form of oxy-**3b** fitted into the narrow and deep substrate-binding site. Several polar amino acid side chains (Arg218, Ans229, Tyr255, Ser284, Glu311, and Arg337) faced the bottom of the binding pocket and provided the rather negative electrostatic surface expected in a cation-stabilizing environment. Notably, the nitrogen atom of oxy-**3b** closely overlapped the water molecule (Wat725) of the 4G36 crystal structure. This preliminary evaluation indicated that it is structurally possible to locate the designed analogs on the native luciferin binding site.

As a simple bioluminescence chromophore for luciferase substrate, we chose a 4-hydroxyphenyl group as an aromatic part. This part was connected to a 4-carboxythiazolin-2-yl ring directly (analog **1a**), or was connected through one (analog **2a**) or two (analog **3a**) double bonds to study the effect of π conjugation on the emission wavelength (Fig. 2). A 4-(dimethylamino)phenyl group was also selected as an aromatic part (**1b**–**3b**) with an expectation of a red shift of light emission, through the electron donating effect of the alkylamino group. In addition, 6-hydroxynaphthalen-2-yl analogs **1c**[23a] and **2c** were also anticipated as shifting emission maxima toward red, because of longer π-conjugated systems than that of the corresponding 4-hydroxyphenyl analogs. The 3-hydroxystyryl-type luciferin analog **2d** was also prepared to evaluate the importance of the hydroxy group position for bioluminescence activity.

The synthesis of luciferin analogs was conducted as shown in Scheme 1. We utilized D-cysteine or (*S*)-trityl-D-cysteine methyl ester for constructing the chiral thiazoline ring. Thus, the analogs **1a**, **1b**, **1c**, and **2a** were synthesized directly by the coupling of D-cysteine with the corresponding nitriles, **4a**, **4b**, **4c**, and **6**,



Scheme 1. Synthesis of aromatic analogs **1**–**3**. Synthetic conditions: (a) D-Cys·HCl, NaOHaq, EtOH. (b) Acrylonitrile, $Pd(OAc)_2$, CH_3CO_2K, K_2CO_3, water, reflux. (c) TBDMSCl, imidazole. (d) DIBAL. (e) $Ph_3P{=}CHCO_2Et$, toluene, reflux. (f) NaOHaq *i*-PrOH. (g) D-Cys(*S*-Trt)-OMe, EDC, DMAP, DMF, rt. (h) Ph_3PO, Tf_2O, CH_2Cl_2. (i) Esterase, EtOH, 10 mM NH_4HCO_3, pH 7.8, 36 °C.

respectively. The analogs **2b**, **2c**, **2d**, **3a**, and **3b** were obtained by the enzymatic hydrolysis (porcine liver esterase) of the corresponding methyl esters **11b**, **11c**, **11d**, **15a**, and **15b**, which were constructed from the corresponding carboxylic acids **10b**, **10c**, **10d**, **14a**, and **14b** via coupling with (*S*)-trityl-D-cysteine methyl ester followed by the formation of the thiazoline rings.

2.2. Bioluminescence activity of luciferin analogs

Bioluminescence activity assays were performed with wild-type *P. pyralis* luciferase and ATP–Mg for each compound, and the emitted light count was integrated for a fixed time (180 s). The emission spectra were measured from 400 nm to 750 nm (Fig. 4).

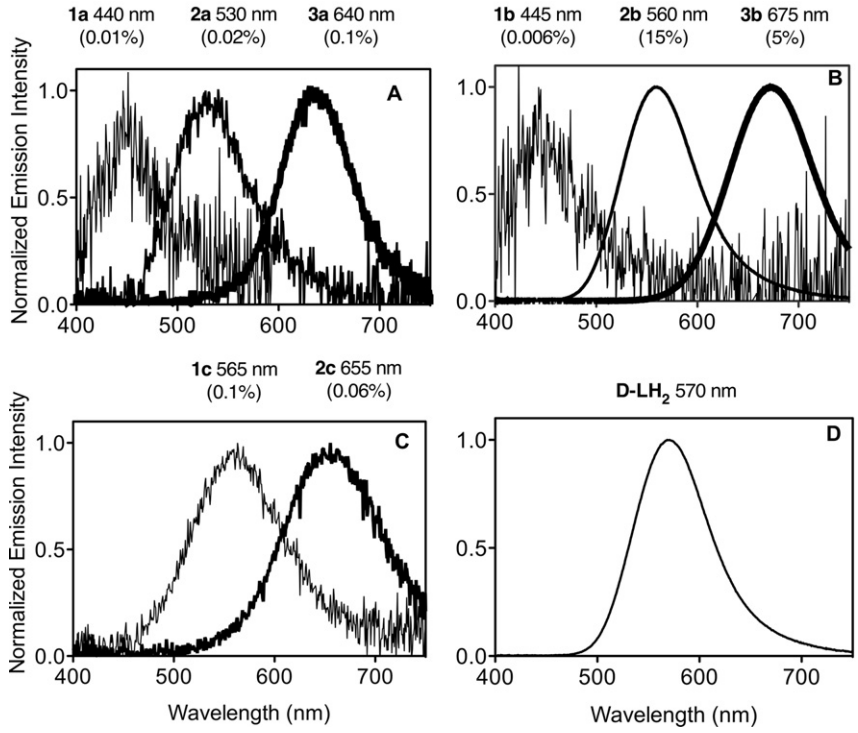


Fig. 4. Bioluminescence emission spectra of compounds of (A) 4-hydroxyphenyl, (B) 4-(dimethylamino)phenyl, (C) 6-hydroxynaphthalen-2-yl analogs, and (D) natural substrate D-LH_2. All spectra were normalized at the emission maxima, and relative light emission intensity compared with that of natural D-LH_2 is shown in parenthesis in %.

All the synthetic aromatic analogs except **2d** exhibited light-emitting activities. In preliminary measurements, we observed that the light emission intensity of 1:1 mixtures of D- and L-**2b** were approximately 1:3 those of pure D-**2b**. This means that the inhibition ability of L-**2b** and the optical purity of the analogs are not crucial in evaluating the light emission ability of the analogs when we use *P. pyralis* luciferase. Thus, we used the racemic mixtures without further separation. The light emission intensity increased proportionally with an increasing amount of luciferase and/or ATP–Mg (data not shown). Analogs **2b** and **3b** showed comparatively potent bioluminescence activity with 15% and 5% relative light intensities compared with that of natural D-LH_2, respectively. However, the light emission intensities of the analogs other than **2b** and **3b** were less than 1% of that of the natural substrate. Although the bioluminescent intensity of **3b** is only 5% of that of D-LH_2 in aqueous solution, the absorption coefficient of blood against 675 nm light (λ_{max} generated by bioluminescence of **3b**) is less than 1:30 of that for 560 nm light (λ_{max} generated by the bioluminescence of D-LH_2). This means that the light generated by the bioluminescence of **3b** passes through blood-containing tissue better than that of D-LH_2 under the same conditions. Observed emission maximum and light emission intensity for **1c** (λ_{max} 565 nm and 0.1% relative light yield compared with that of D-LH_2) were considerably different from the previously reported values for **1c** (λ_{max} 524 nm, 1.5% relative light yield compared with that of D-LH_2 at pH 9.3).[23a] This discrepancy may be because of the difference in the experimental conditions.

The inactive 3-hydroxystyryl-type luciferin analog **2d** compared with the active 4-hydroxystyryl-type luciferin analog **2a**, as in the case of phenolic hydroxy positional isomers of luciferin,[24] suggested that synthetic substrates bind and act in a similar manner to native D-LH_2, and that the position of the hydroxyl group is one of the important factors for light emission ability. However, the slow emission profile indicated a difference of environment at the catalytic site of the luciferase. The light emission from all the synthetic substrates showed a slow rise to maximum intensity (about a minute), and was sustained for about 1 min, as distinct from light emission from D-LH_2, which rapidly reached maximum intensity within a few seconds followed by a steep decay (will be discussed below).

Among the bioluminescently active compounds, obvious structure–emission wavelength relationships were found. The insertion of conjugated double bonds between an aromatic ring and a thiazoline ring showed an emission maximum wavelength with an approximately 100 nm shift to red per double bond. In addition, the emission maximum of 4-(dimethylamino)phenyl analogs (**1b**–**3b**) exhibited an approximately 30 nm shift to red, compared with those of corresponding 4-hydroxyphenyl analogs (**1a**–**3a**). Similarly, the emission maximum of 6-hydroxynaphthalen-2-yl analogs (**1c** and **2c**) exhibited an approximately 130 nm shift to red, compared with those of the corresponding 4-hydroxyphenyl analogs (**1a** and **2a**). Thus, we obtained the synthetic luciferase substrates emitting a variety of colors, such as blue/purple, blue, green/yellow-green, and red. The shortest blue emission (λ_{max}=440 nm) was observed for **1a**. Notably, the longest wavelength observed for **3b** (λ_{max}=675 nm) was in the NIR window region. Therefore, these results represent the capability of designing light-emitting substrates to obtain a wider range of emission wavelengths than those from native D-LH_2.

One of the reasons for these wavelength shifts was indicated to be that the length of the π conjugation controls the wavelength of the emission maximum. Theoretical calculation for the excited species and prediction of the spectroscopic properties are challenging fields,[25] especially the characterization of light emitters (excited oxyluciferins) interacting with various intramolecular factors, including hydrogen bonds and Coulomb interaction, and polarity in the luciferase active site. Commonly, a +30 nm shift per conjugated double bond was used to estimate the UV wavelength of the organic compounds.[26] Even from this rough estimate, a +100 nm shift per double bond seemed to be a bigger shift than we expected.

The other possible reason apart from π conjugation was supposed to be a solvent effect. Previously, we have investigated the light-color modulation mechanism using 5,5-dimethyloxyluciferin and aminoluciferin analogs as model compounds, and showed that it depended on the base/solvent combination.[27,28] When the emission species is exposed into the polar water from a generally hydrophobic binding site, the emission color may shift to red. Therefore, we presumed the environment of the emission species of the synthetic compounds was more polar than that for natural D-LH_2. The domain alteration hypothesis of firefly luciferase[29,30] seems to be supportive of this assumption. Firefly luciferase is a family of adenylating enzymes containing acyl- and aryl-CoA synthetases, and nonribosomal peptide synthetases.[31] Many of them are proposed for the domain movement during the catalytic reaction. According to their crystal structural studies,[23c,32] the putative substrate-binding site of luciferase is within a cavity of the N-terminal domain, and the AMP moiety is located in-between the N- and C-terminal domains. When this domain movement occurred after the adenylation of D-LH_2 and the C-terminal domain covered the substrate inside the enzyme, the emission species was supposed to exist in the hydrophobic environment. If we assumed that, like native D-LH_2, adenylated synthetic substrates did not induce domain alteration, then the emission species might have been exposed to water, which may have resulted in an emission shift toward red. Study of the whole C-domain-lacking mutant of firefly luciferase showed the importance of the C-terminal domain for bioluminescence.[33] Interestingly, this luciferase mutant, with an altered N-domain only, retains its luminescence activity and showed red emission around 620 nm irrespective of pH with D-LH_2–AMP. We assumed this result also supports the presumption that unnatural conformation of the C-domain causes a polar active site environment for the synthetic compounds.

2.3. Bioluminescence activity of adenylated derivatives of luciferin analogs

Next, we compared the bioluminescence activity of D-LH_2 and analogs to the corresponding adenylated derivatives. The use of the adenylated derivatives allows us to skip the adenylation step in bioluminescence reaction (Fig. 1, Eq. 1), and allows the luciferase to only catalyze the oxidation step of the adenylated substrates (Fig. 1, Eq. 2). If the adenylation step is the rate-determining step in light emission, the speed of light emission should be increased to enhance light emission intensity. The adenylated derivatives were prepared by reported procedures,[34] purified by HPLC just prior to use, and subjected to bioluminescence reaction. The adenylated substrate derivatives were rather stable under acidic conditions and no epimerization or hydrolysis was observed in pH 4 buffer solution or in 0.05% TFA-containing HPLC eluent over 1 h at room temperature. However, they were somewhat unstable under basic conditions measuring bioluminescence activity: the half-life for hydrolysis and epimerization in the pH 8 buffer was ca. 1 h and 10 min, respectively, at room temperature. Thus, the adenylated material was prepared and purified by HPLC under acidic conditions just prior to use. The bioluminescence emission profiles of the most active anaolg **2b** and its adenylated **2b**–**AMP** are shown in Fig. 5 as representative results. The emission maxima were not changed by adenylation, though the emission intensity was enhanced 10 to several hundred times with shorter rise times (8 s), as for D-LH_2 (6 s). Thus, the slow rise of light emission intensity of the analog itself was suggested to be responsible for the slow reaction of the adenylation step. The emission intensity of adenylated analogs decayed more slowly than that of adenylated D-LH_2. Compared

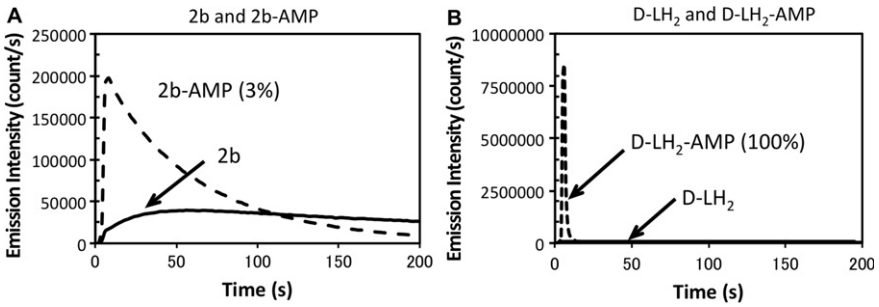


Fig. 5. Comparison of changes in bioluminescence over time of **2b**/ATP with **2b**–AMP, and of D-LH_2/ATP with D-LH_2–AMP. The most active **2b** was selected as a delegate for synthesized compounds. (A) **2b**/ATP (thick line) and **2b**–AMP (dashed line). (B) D-LH_2/ATP (thick line) and D-LH_2–AMP (dashed line). Relative light emission intensity of **2b**–AMP compared with D-LH_2–AMP is shown in parenthesis in %.

with D-LH_2, D-LH_2–AMP showed a burst of light emission followed by drastic decay. If this quick decay of D-LH_2–AMP is because of product inhibition,[1] synthetic analogs seem to have fewer product inhibition properties compared with those of oxyluciferin from natural D-LH_2. The synthetic analogs for *P. pyralis* luciferase may be useful for in vivo whole-body imaging, because of the duration of substantial maximum light production suitable for detecting more stable signals, coupled with availability of vectors for luciferase gene to introduce it into living organisms.

Recently, bioluminescence resonance energy transfer (BRET) systems utilizing chemically modified luciferin[18,19] and firefly luciferase[17,35] were successfully developed to obtain light emission in the NIR window region,[18,19] and the longest wavelength was recorded at 783 nm.[35] This is a far longer wavelength than we observed from **3b**, though our compounds do not require the injection of modified luciferase into organisms to obtain red emission. Nevertheless, our compounds may also have the potential to be used as BRET donors. It should be noted that according to the functional specification of the photomultiplier tube (Hamamatsu R4220) of the luminometer used, sensitivity at 700 nm is more than 10 times less than at 600 nm. We will therefore be required to prepare more suitable apparatus for further development of luciferase substrates with NIR range emission.

3. Conclusion

We developed simple luciferin analogs with various light emission colors ranging from blue to the NIR window region. From study of the structure–emission wavelength relationship, we found that the introduction of conjugated double bonds between aromatic parts and the thiazoline ring was very effective for elongation of wavelengths of emission maxima, producing an approximately 100 nm longer wavelength shift per conjugated double bond. The shortest emission wavelength was observed with **1a** (λ_{max} 440 nm), and the longest with **3b** (λ_{max} 675 nm), reached within the NIR window region of light suitable for bioimaging of deep sites in living animals. The light emission from analogs showed slow increases to maximum intensity (about a minute), and was sustained for about 1 min, being different from that of D-LH_2 light emission, which rapidly reaches maximum intensity within a few seconds followed by a steep decay. The rather weak bioluminescence intensity of analogs could be enhanced 10 to several hundred times by their derivatization to corresponding adenylated substrates.

4. Experimental section

4.1. General

4.1.1. Materials and general method for synthesis. Starting materials and reagents were obtained from commercial suppliers and used without further purification. Porcine liver esterase was purchased from Sigma (E3019, lyophilized powder). Solvents used for anhydrous conditions were distilled, or dried over 4 Å molecular sieves. Cation exchange resins (Organo, Amberlite IR-120B NA, and IRA400OH AG) were used to remove ions. Merck precoated Kieselgel 60 F_{254} plates with 0.25 mm (Art. 5715) and 0.5 mm (Art. 5744) thickness were used for analytical and preparative thin layer chromatography (TLC). Visualization of TLC was accomplished with UV-light and by treatment with suitable staining reagents. Merck Kieselgel 60 (Art. 7734) was used for column chromatography. Solvents were removed with a rotary evaporator under reduced pressure at a temperature below 40 °C. Melting points were measured using a Yamato MP-2 instrument and are uncorrected. Optical purity of synthesized luciferin analogs was analyzed by HPLC (Agilent 1100 series) using a chiral column (Daicel Chemical Industries, OD-RH or OZ-RH, 5 μm, 4.6×150 mm) with linear gradient of 10%–90% acetonitrile in H_2O over 30 min (flow rate 0.5 mL/min) as eluent. Unless otherwise stated, chiral analyses were performed using the OD-RH column. A UV detector set at 330 nm was used for peak detection. The retention times of L-LH_2 and D-LH_2 were 17.2 and 18.2 min, respectively. Synthesized compounds were used for the luminescence assay without further purification. IR spectra were measured using a Horiba FT 730 spectrometer. ^1H and ^{13}C NMR spectra were recorded on JEOL Lambda 270 [270 MHz (^1H) and 67.8 MHz (^{13}C)] and JEOL ECA 500 [500 MHz (^1H) and 125 MHz (^{13}C)] instruments. Chemical shifts are reported in parts per million (δ) downfield from internal tetramethylsilane (δ=0) and coupling constants in hertz. Fast atom bombardment mass spectra (FAB-MS) were measured with a Finnigan MAT TSQ-700 instrument (glycerol matrix). Electron ionization mass spectra (EI-MS) were measured with a JEOL JMS 600H instrument. High-resolution electrospray ionization mass spectra (HR-ESI-MS) were measured with JEOL JMS T1000LC mass spectrometers, using tuned conditions of needle voltage: 2000 V, orifice 1 voltage: 85 V, orifice 2 voltage: 5 V, ring lens voltage: 10 V, desolvating gas: 250 °C, orifice 1 temperature: 80 °C, delivery of sample: infusion method, flow speed: 10–30 μL/min (according to the sample).

4.1.2. Materials and general method for luminescence assay. A 1 mg/mL stock solution of commercial luciferase purchased from Promega (QuantiLum recombinant *P. pyralis* luciferase, E1701) and Sigma (L9506) was prepared by dissolving the luciferase in 50 mM Tris–HCl buffer (pH 8.0) containing 10% glycerol and was stored at −80 °C. Just prior to use, the luciferase stock solutions were diluted 100-fold with 50 mM potassium phosphate buffer (pH 6.0) containing 35% glycerol. The diluted luciferase solution was ice-cooled until use. ATP–Mg was purchased from Nacalai Tesque, and the buffer chemicals from Wako Chemicals or Kanto Chemicals. Stock solution of substrates was prepared by dissolving 5 mM of the substrates in 50 mM potassium phosphate buffer (pH 6.0), and was

stored at −80 °C. Deionized water (Millipore, Milli-RX-12α) was used for aqueous assay solutions. pH was monitored with a Horiba F-23 pH meter. Bioluminescence intensity was measured using an ATTO AB-2200 or AB-2270 luminometer (Hamamatsu, R4220 photomultiplier tube) and bioluminescence spectra were recorded using an ATTO AB-1850 spectrophotometer. Adenylated derivatives were prepared according to the literature,[34] and purified using an HPLC system (Agilent 1100 series) equipped with a Mightysil C_{18} reverse phase column (5 μm, 4.6×250 mm) just prior to use. The flow rate of the mobile phase consisting of a mixture of acetonitrile and 0.05% TFA aqueous solution was 0.5 mL/min, and the column temperature was maintained at 20 °C. A linear gradient of 10%–90% acetonitrile over 30 min was applied, and a UV detector set at 330 nm was used for peak detection. D-LH_2–AMP was eluted at 13.4 min, followed by L-LH_2–AMP (13.6 min), and D-LH_2 (20.9 min). D-**2b**–AMP was eluted at 22.2 min, followed by L-**2b**–AMP (22.8 min), and **2b** (26.8 min). The volatiles of the eluted fraction were removed under reduced pressure and the residual aqueous solution containing adenylated product was promptly subjected to bioluminescence activity. The purity and concentration of the synthesized adenylates were determined to be more than 95% using a calibration curve prepared with the HPLC system.

4.2. Preparation of luciferin analogs

*4.2.1. (S)-2-(4-Hydroxyphenyl)-4,5-dihydrothiazole-4-carboxylic acid (**1a**).* To a solution of 4-cyanophenol (**4a**) (120 mg, 1.01 mmol) and D-cysteine hydrochloride monohydrate (D-Cys·HCl) (211 mg, 1.20 mmol) in EtOH (5 mL) was added 1 M NaOH (5 mL). After the reaction mixture was stirred at 80 °C for 6 h, additional D-Cys·HCl (422 mg, 2.40 mmol) and 1 M NaOH (1 mL) were added, and the mixture was stirred at room temperature for 4 days. The reaction mixture was filtered and the filtration residue was washed with 50% aqueous EtOH (20 mL). The filtrate and washings were combined and the pH of the mixture was adjusted to pH 2 with 2 M HCl. The precipitates were collected by suction filtration, washed by distilled water, and dried under reduced pressure to give anaolg **1a** (111 mg, 50%) as a white powder. Mp 200–204 °C dec; 90% ee from chiral HPLC (retention time of L-isomer: 8.0 min, D-isomer: 8.7 min); ^{1}H NMR (270 MHz, CD_3OD) δ 3.70 (dd, J=7.9, 11.5 Hz, 1H, ABX system), 3.76 (dd, J=8.9, 11.5 Hz, 1H, ABX system), 5.23 (dd, J=7.9, 8.9 Hz, 1H, ABX system), 6.85 (d, J=8.9 Hz, 2H, AA′BB′ system), 7.74 (d, J=8.9 Hz, 2H, AA′BB′ system); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 36.0 (t), 78.0 (d), 116.5 (d)×2, 124.5 (s), 131.8 (d)×2, 163.0 (s), 173.9 (s), 174.6 (s); FT-IR ν_{max} (cm^{-1}): 2680, 1610, 1580, 1510; EI-MS m/z: 223 (M^+, 44%), 178 (100). HR-ESI-MS: m/z: $[M+Na]^+$ calcd for $C_{10}H_9NNaO_3S$, 246.0201; found, 246.0157.

*4.2.2. (S)-2-(4-(Dimethylamino)phenyl)-4,5-dihydrothiazole-4-carboxylic acid (**1b**).* To a solution of 4-(dimethylamino)benzonitrile (**4b**) (103 mg, 0.71 mmol) and D-Cys·HCl (371 mg, 2.12 mmol) in EtOH (4 mL) was added 1 M NaOH (5 mL). After the reaction mixture was stirred at 80 °C for 5 h, 1 M HCl (5 mL) was added, and the mixture was concentrated under reduced pressure. The residue was washed with distilled water to give analog **1b** (50.9 mg, 29%) as a yellow solid. 92% ee from chiral HPLC (retention time of L-isomer: 11.1 min, D-isomer: 11.5 min); ^{1}H NMR (270 MHz, $CDCl_3$) δ 3.01 (s, 6H), 3.50 (dd, J=9.2, 11 Hz, 1H, ABX system), 3.61 (dd, J=9.2, 11 Hz, 1H, ABX system), 5.00 (dd, J=9.2, 9.2 Hz, 1H, ABX system), 6.71 (d, J=7.0 Hz, 2H, AA′BB′ system), 7.71 (d, J=7.0 Hz, 2H, AA′BB′ system); FT-IR ν_{max} (cm^{-1}): 3392, 1608; ESI-MS m/z: 251 [$(M+H)^+$]. HR-ESI-MS: m/z: $[M+Na]^+$ calcd for $C_{12}H_{14}N_2NaO_2S$, 273.0674; found, 273.0649.

*4.2.3. (S)-2-(6-Hydroxynaphthalen-2-yl)-4,5-dihydrothiazole-4-carboxylic acid (**1c**).* In a similar manner to that used for the preparation of **1b**, 6-cyano-2-naphthol (**4c**) (102 mg, 0.60 mmol) was treated with D-Cys·HCl (290 mg, 1.65 mmol) and 1 M NaOH (2.5 mL) in EtOH (5 mL) to afford anaolg **1c** quantitatively as a yellow solid. Mp 197 °C dec (lit.[23a] 201.5–203.5 °C dec); 90% ee from chiral HPLC (retention time of L-isomer: 10.8 min, D-isomer: 11.2 min); ^{1}H NMR (270 MHz, CD_3OD) δ 3.71 (dd, J=8.9, 11.2 Hz, 1H, ABX system), 3.76 (dd, J=8.9, 11.2 Hz, 1H, ABX system), 5.33 (dd, J=8.9, 8.9 Hz, 1H, ABX system), 7.12–7.16 (complex, 2H), 7.68 (d, J=8.9 Hz, 1H), 7.83 (d, J=9.6 Hz, 1H), 7.90 (dd, J=1.6, 8.9 Hz, 1H), 8.21 (br d, J=1.6 Hz, 1H); ^{13}C NMR (67.8 MHz, CD_3OD) δ 37.1 (t), 83.0 (d), 110.0 (d), 120.3 (d), 126.2 (d), 127.4 (d), 128.8 (s), 128.9 (s), 130.4 (d), 131.7 (d), 138.1 (s)×2, 158.4 (s), 170.0 (s), 178.5 (s); FT-IR ν_{max} (cm^{-1}): 3022, 1589, 1483; HR-ESI-MS m/z: $[M+H]^+$ calcd for $C_{14}H_{12}NO_3S$, 274.0538; found, 247.0533, and $[M+H-CO_2]^+$ calcd for $C_{13}H_{12}NOS$, 230.0640; found, 230.0644.

*4.2.4. (S,E)-2-(4-Hydroxystyryl)-4,5-dihydrothiazole-4-carboxylic acid (**2a**).* Following the literature,[36] to a solution of 4-iodophenol (**5**) (1100 mg, 5.0 mmol) in water (10 mL) were added CH_3CO_2K (491 mg, 5.0 mmol), K_2CO_3 (865 mg, 6.3 mmol), acrylonitrile (495 μL, 7.5 mmol), and $Pd(OAc)_2$ (11 mg, 0.05 mmol), and the mixture was heated under reflux for 30 min. After the reaction mixture was cooled to room temperature, precipitates were filtered off through a pad of Celite and the filter cake was washed with water (50 mL). The filtrate and washings were combined, adjusted to pH 7 with 2 M HCl, and the products were extracted with EtOAc (40 mL). The organic layers were combined, dried over Na_2SO_4, and concentrated to leave a white solid. Recrystallization from EtOAc/*i*-Pr_2O gave nitrile **6** (417 mg, 58%) as colorless needles being a mixture of stereoisomers (cis/trans=1:3 by ^{1}H NMR). ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.30 (d, J=12.5 Hz, 0.25H cis), 5.85 (d, J=16 Hz, 0.75H trans), 6.75 (d, J=8 Hz, 1.5H trans, AA′BB′ system), 6.77 (d, J=9.0 Hz, 0.5H cis, AA′BB′ system), 7.08 (d, J=12.5 Hz, 0.25H cis), 7.33 (d, J=16 Hz, 0.75H trans), 7.35 (d, J=8 Hz, 1.5H trans, AA′BB′ system), 7.66 (d, J=9.0 Hz, 0.5H cis, AA′BB′ system). To a solution of D-Cys·HCl (106 mg, 0.60 mmol) and nitrile **6** (65.0 mg, 0.45 mmol) in MeOH (2 mL) was added 1 M NaOH (2 mL) and the mixture was stirred at 80 °C for 6 h. The reaction mixture was neutralized with 1 M HCl and was directly purified using a Sep-Pak cartridge (Waters, C_{18}, water to 60% MeOH stepwise gradient) to give anaolg **2a** (9.2 mg, 8%) as a pale-yellow solid. Mp 138–140 °C dec; 98% ee; from chiral HPLC (retention time of L-isomer: 12.3 min, D-isomer: 12.9 min); ^{1}H NMR (270 MHz, CD_3OD) δ 3.52 (dd, J=8.9, 10.9 Hz, 1H, ABX system), 3.61 (dd, J=8.9, 10.9 Hz, 1H, ABX system), 5.01 (dd, J=8.9, 8.9 Hz, 1H, ABX system), 6.80 (d, J=8.9 Hz, 2H, AA′BB′ system), 6.91 (d, J=16.0 Hz, 1H), 7.10 (d, J=16.0 Hz, 1H), 7.42 (d, J=8.9 Hz, 2H, AA′BB′ system); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 36.5 (t), 81.2 (d), 116.9 (d)×2, 119.7 (d), 128.0 (s), 130.5 (d)×2, 143.7 (d), 160.7 (s), 172.0 (s), 177.5 (s); FT-IR ν_{max} (cm^{-1}): 3151, 1626, 1568; ESI-MS m/z: 250 [$(M+H)^+$]. HR-ESI-MS: m/z: $[M+H]^+$ calcd for $C_{12}H_{12}NO_3S$, 250.0538; found, 250.0516.

4.2.5. S-Trityl-D-cysteine methyl ester [D-Cys(S-Trt)-OMe]. To a solution of *S*-trityl-D-cysteine (504 mg, 1.39 mmol) in MeOH (100 mL) was added 4 M HCl (5.4 mL in 1,4-dioxane), and the mixture was stirred at ambient temperature for 17 days. The reaction mixture was neutralized by adding ion exchange resin IRA400OH AG. The resin was filtered off and washed with MeOH. The filtrate and washings were combined and concentrated in vacuo. The residue was purified by silica gel column chromatography (Hex/EtOAc=1:1) to give D-Cys(*S*-Trt)-OMe 455 mg (86%) as a pale-yellow oil. ^{1}H NMR (270 MHz, $CDCl_3$) δ 2.47 (dd, J=7.7, 12.4 Hz, 1H, ABX system), 2.60 (dd, J=4.8, 12.4 Hz, 1H, ABX system), 3.20 (br dd, J=4.8, 7.7 Hz, 1H, ABX system), 3.65 (s, 3H), 7.18–7.31 (complex, 9H, 3× C_6H_3), 7.40–7.45 (complex, 6H, 3× C_6H_2); ^{13}C

NMR (67.8 MHz, $CDCl_3$) δ 36.9 (t), 52.1 (q), 53.8 (d), 66.8 (s), 126.8 (d)×3, 127.9 (d)×6, 129.6 (d)×6, 144.5 (s)×3, 174.2 (s); FT-IR ν_{max} (cm^{-1}): 3381, 3315, 1739, 1595; FAB-MS *m*/*z*: 378 ($M+H^+$, 10%), 243 (100).

*4.2.6. (S,E)-2-(4-(Dimethylamino)styryl)-4,5-dihydrothiazole-4-carboxylic acid (**2b**).* To a solution of 4-(dimethylamino)cinnamic acid (**10b**) (92.1 mg, 0.48 mmol) in DMF (5 mL) were added D-Cys(*S*-Trt)-OMe (101 mg, 0.53 mmol), 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDC) (311 mg, 1.62 mmol), and 4-(dimethylamino)pyridine (DMAP) (151 mg, 1.23 mmol) under Ar. After the reaction mixture was stirred at room temperature for 24 h, water (100 mL) was added. The products were extracted with EtOAc (3×100 mL). The combined organic layers were dried over Na_2SO_4, filtered, and concentrated. The residue obtained was purified by silica gel column chromatography (Hex/EtOAc=1:1) to give an *N*-acyl-*S*-trityl D-cysteine methyl ester derivative, (*S*)-methyl 2-((*E*)-3-(4-(dimethylamino)phenyl)acrylamido)-3-(tritylthio)propanoate (259 mg, 89%) as a yellow oil (step g in Scheme 1). ^1H NMR (500 MHz, $CDCl_3$) δ 2.72 (m, 2H, ABX system), 3.01 (s, 6H), 3.72 (s, 3H), 4.78 (m, 1H, ABX system), 6.15 (d, *J*=15.5 Hz, 1H), 6.20 (d, *J*=5.9 Hz, 1H, *NH*), 6.68 (d, *J*=8.6 Hz, 2H, AA′BB′ system), 7.20–7.42 (complex, 17H), 7.52 (d, *J*=15.5 Hz, 1H); ESI-MS *m*/*z*: 573 [$(M+Na)^+$]. To a solution of the amide obtained above (118 mg, 0.21 mmol) in dry CH_2Cl_2 (10 mL) were added triphenylphosphine oxide (Ph_3PO) (124 mg, 0.45 mmol) and trifluoromethanesulfonic anhydride (Tf_2O) (360 μL, 2.14 mmol) under Ar.[37] After the reaction mixture was stirred at room temperature for 40 min, the reaction was quenched by adding water (50 mL), and the products were extracted with $CHCl_3$ (50 mL), and then EtOAc (2×50 mL). The combined organic layers were dried over Na_2SO_4, filtered, and concentrated. The residue was purified by silica gel column chromatography (Hex/EtOAc=1:2) to give thiazoline ester **11b** (44.2 mg, **7**1%) as a yellow solid (step h in Scheme 1). ^1H NMR (500 MHz, $CDCl_3$) δ 2.96 (s, 6H), 3.53 (m, 2H, ABX system), 3.79 (s, 3H), 5.14 (dd, *J*=8.6, 8.6 Hz, 1H, ABX system), 6.63 (d, *J*=7.5 Hz, 2H, AA′BB′ system), 6.88 (d, *J*=15.5 Hz, 1H), 7.04 (d, *J*=15.5 Hz, 1H), 7.34 (d, *J*=7.5 Hz, 2H, AA′BB′ system); FT-IR ν_{max} (cm^{-1}): 1724; ESI-MS *m*/*z*: 291 [$(M+H)^+$]. To a mixture of thiazoline ester **11b** (21.1 mg, 0.07 mmol) in EtOH (2 mL) and 10 mM NH_4HCO_3 (6 mL) was added porcine liver esterase (9.2 mg), and the reaction mixture was stirred at 37 °C under Ar for 19 h. After evaporation of the reaction mixture, the residue obtained was suspended in a mixture of MeOH/$CHCl_3$. The precipitate was filtered off, and the filtrate and washings of the residue were combined and concentrated to give anaolg **2b** (14.1 mg, 71%) as an orange solid (step i in Scheme 1). 26% ee from chiral HPLC (retention time of L-isomer: 13.2 min, D-isomer: 12.9 min); ^1H NMR (500 MHz, CD_3OD) δ 3.00 (s, 6H), 3.62 (m, 2H, ABX system), 5.01 (dd, *J*=8.6, 8.6 Hz, 1H, ABX system), 6.72 (d, *J*=9.0 Hz, 2H, AA′BB′ system), 6.86 (d, *J*=16 Hz, 1H) 7.21 (d, *J*=16 Hz, 1H), 7.44 (d, *J*=9.0 Hz, 2H, AA′BB′ system); ^{13}C NMR (125 MHz, CD_3OD, δ): 31.2 (t), 34.5 (q)×2, 79.7 (d), 112.5 (d)×2, 115.9 (d), 122.5 (s), 130.1 (d)×2, 144.1 (d), 152.1 (s), 170.4 (s), 172.2 (s); FT-IR ν_{max} (cm^{-1}): 3392, 1602; HR-ESI-MS *m*/*z*: $[M+H]^+$ calcd for $C_{14}H_{17}N_2O_2S$, 277.1011; found, 277.0989.

*4.2.7. (S,E)-2-(2-(6-Hydroxynaphthalen-2-yl)vinyl)-4,5-dihydrothiazole-4-carboxylic acid (**2c**).* To a solution of 6-cyano-2-naphthol (**4c**) (50.2 mg, 0.30 mmol) in DMF (0.5 mL) were added *tert*-butyldimethylsilyl chloride (TBDMSCl, 143 mg, 0.95 mmol) and imidazole (160.7 mg, 2.40 mmol). After the mixture was stirred at room temperature for 1 h, water (40 mL) was added. The products were extracted from the diluted mixture with EtOAc (3×60 mL). The combined organic layers were dried over Na_2SO_4, filtered, and concentrated. The residue was purified by silica gel column chromatography (Hex/EtOAc=8:1) to yield 6-(*tert*-butyldimethylsilyloxy)naphthalene-2-carbonitrile (**7**) (69.9 mg, 83%) as a colorless oil. ^1H NMR (270 MHz, CD_3OD) δ 0.23 (s, 6H), 0.97 (s, 9H), 7.12 (complex, 2H), 7.48 (dd, *J*=1.6, 8.6 Hz, 1H), 7.69 (d, *J*=8.9 Hz, 1H), 7.73 (d, *J*=8.9 Hz, 1H), 8.08 (d, *J*=0.5 Hz, 1H); FT-IR ν_{max} (cm^{-1}): 3689, 2225, 1274; ESI-MS *m*/*z*: 284 [$(M+H)^+$], 306[$(M+Na)^+$]. Nitrile **7** (99.3 mg, 0.35 mmol) was dissolved in toluene (10 mL) under Ar. To the mixture was added 1 M solution of diisobutylaluminium hydride (DIBAL) in toluene solution (0.5 mL). After the reaction mixture was stirred at room temperature for 1 h, acetone (10 mL) was added to the ice-water-cooled reaction mixture for decomposition of the excess reagent. To the mixture were added satd potassium sodium tartrate (20 mL) and water (30 mL). The products were extracted from the suspension with EtOAc (3×50 mL). The combined organic layers were dried over Na_2SO_4, and evaporated. The residue was purified by PTLC (Hex/EtOAc=10:1) to afford aldehyde **8** (74.4 mg, 74%) as a yellow oil. ^1H NMR (270 MHz, $CDCl_3$) δ 0.28 (s, 6H), 1.03 (s, 9H), 7.17 (dd, *J*=2.3, 8.6 Hz, 1H), 7.23 (br d, *J*=2.3 Hz, 1H), 7.76 (d, *J*=8.6 Hz, 1H), 7.89 (d, *J*=8.6 Hz, 1H), 7.90 (dd, *J*=1.6, 8.6 Hz, 1H), 8.26 (s, 1H), 10.09 (s, 1H); FT-IR ν_{max} (cm^{-1}): 1716, 1274; ESI-MS *m*/*z*: 287 [$(M+H)^+$]. To a solution of aldehyde **8** (63.9 mg, 0.22 mmol) in toluene (2 mL) was added ethoxycarbonylmethylene-triphenylphosphoran ($Ph_3P{=}CHCO_2Et$) (121 mg, 0.349 mmol) and the mixture was stirred at room temperature for 5 h. After dilution of the reaction mixture with water (50 mL), the products were extracted with EtOAc (3×50 mL). The combined organic layers were dried over Na_2SO_4 and evaporated. The residue was purified by PTLC (Hex/EtOAc=25:1) to give ester **9** (76.6 mg, 97%) as a yellow oil (step e in Scheme 1). ^1H NMR (270 MHz, $CDCl_3$) δ 0.25 (s, 6H), 1.00 (s, 9H), 1.34 (t, *J*=7.0 Hz, 3H), 4.27 (q, *J*=7.0 Hz, 2H), 6.47 (d, *J*=16.1 Hz, 1H), 7.11 (dd, *J*=2.4, 8.9 Hz, 1H), 7.19 (d, *J*=2.4 Hz, 1H), 7.60–7.79, complex, 4H), 7.85 (br s, 1H); FT-IR ν_{max} (cm^{-1}): 1623, 1274; ESI-MS *m*/*z*: 357 [$(M+H)^+$]. To a solution of ester **9** (90.8 mg, 0.253 mmol) in *i*-PrOH (3 mL) was added 1 M NaOH (5 mL) and the mixture was stirred at room temperature for 5 h. The reaction mixture was neutralized by adding ion exchange resin IR-120B NA. The resin was removed by filtering. The filtrate and washings were combined, and evaporated to give acid **10c** quantitatively as a pale-yellow oil (step f in Scheme 1). ^1H NMR (270 MHz, CD_3OD) δ 6.50 (d, *J*=15.7 Hz, 1H), 7.10 (dd, *J*=2.2, 7.6 Hz, 1H), 7.11 (br s, 1H), 7.65 (m, 2H), 7.77 (dd, *J*=1.6, 8.1 Hz, 1H), 7.80 (d, *J*=15.7 Hz, 1H), 7.90 (s 1H); FT-IR ν_{max} (cm^{-1}): 3689, 1670; ESI-MS *m*/*z*: 237 [$(M+Na)^+$]. In a similar manner to that used in step g in the synthesis of analog **2b**, acid **10c** (54.9 mg, 0.25 mmol) was coupled with D-Cys(*S*-Trt)-OMe to give an *N*-acyl-*S*-trityl D-cysteine methyl ester derivative, (*S*)-methyl 2-((*E*)-3-(2-hydroxynaphthalen-6-yl)acrylamido)-3-(tritylthio)propanoate (58.4 mg, 40%) as a pale-yellow oil. ^1H NMR (270 MHz, $CDCl_3$) δ 2.75 (m, 2H, ABX system), 3.75 (s, 3H), 4.77 (dd, *J*=2.7, 7.9 Hz, 1H, ABX system), 6.35 (d, *J*=16.1 Hz, 1H), 6.90–7.80 (complex, 23H); FT-IR ν_{max} (cm^{-1}): 3689, 3565, 1868; ESI-MS *m*/*z*: 574 [$(M+H)^+$]. In a similar manner to that used in step h in the synthesis of analog **2b**, the amide obtained above (60.3 mg, 0.11 mmol) was cyclized to yield thiazoline ester **11c** (17.4 mg, 55%) as a yellow solid. ^1H NMR (270 MHz, $CDCl_3$) δ 3.63 (m, 2H, ABX system), 3.81 (s, 3H), 5.27 (dd, *J*=8.9, 8.9 Hz, 1H, ABX system), 7.07–7.13 (complex, 3H), 7.33 (d, *J*=16.1 Hz, 1H), 7.65 (complex, 2H), 7.76 (m, 1H), 7.88 (br s, 1H); FT-IR ν_{max} (cm^{-1}): 3689, 1733; ESI-MS *m*/*z*: 314 [$(M+H)^+$], 336 [$(M+Na)^+$]. In a similar manner to that used in step i in the synthesis of analog **2b**, thiazoline ester **11c** (6.3 mg, 0.02 mmol) was hydrolyzed with porcine liver esterase to give anaolg **2c** quantitatively as a pale-yellow solid. 22% ee from chiral HPLC (OZ-RH column, retention time of L-isomer: 17.4 min, D-isomer: 18.2 min; ^1H NMR (270 MHz, CD_3OD) δ 3.61 (m, 2H, ABX system), 5.09 (dd, *J*=8.9, 8.9 Hz, 1H, ABX system), 7.06–7.17 (complex, 3H), 7.30 (dd, *J*=16.1 Hz, 1H), 7.65–7.87 (complex, 4H); FT-IR ν_{max} (cm^{-1}): 3689,

1716; ESI-MS *m/z*: 300 [(M+H)$^+$]. HR-ESI-MS *m/z*: [M+H]$^+$ calcd for $C_{16}H_{14}NO_3S$, 300.0694; found, 300.0689.

*4.2.8. (S,E)-2-(3-Hydroystyryl)-4,5-dihydrothiazole-4-carboxylic acid (**2d**).* To a solution of 3-hydroxycinnamic acid (1.80 g, 11.0 mmol) in CH_2Cl_2 (200 mL) were added acetic anhydride (4.0 mL, 42 mmol) and DMAP (6.70 g, 54.8 mmol), and the mixture was stirred at room temperature for 4 h. Water (150 mL) was added to the reaction mixture and the products were extracted from the diluted mixture with CH_2Cl_2 (100 mL) and EtOAc (2×80 mL) successively. The organic layers were combined, dried over $MgSO_4$, and concentrated. The residue was purified by silica gel column chromatography (Hex/EtOAc=2:1) to yield 3-acetoxycinnamic acid (**10d**) (1.28 g, 71%) as colorless needles. Mp 140–142 °C; ^{1}H NMR (270 MHz, CD_3OD) δ 2.28 (s, 3H), 6.49 (d, *J*=16.0 Hz, 1H), 7.12 (d, *J*=7.4 Hz, 1H, AA′BB′ system), 7.34–7.46 (complex, 3H), 7.63 (d, *J*=16.0 Hz, 1H); ^{13}C NMR (67.8 MHz, CD_3OD) δ 20.9 (q), 121.0 (d), 122.1 (d), 124.6 (d), 126.6 (d), 130.9 (d), 137.5 (s), 144.8 (s), 152.7 (s), 170.5 (s), 171.0 (s); FT-IR ν_{max} (cm^{-1}): 3037, 1761, 1687, 1631; EI-MS *m/z*: 206 (M$^+$, 26%), 164 (100). In a similar manner to that used in step g in the synthesis of analog **2b**, acid **10d** (133 mg, 0.65 mmol) was coupled with D-Cys(*S*-Trt)-OMe to give an *N*-acyl-*S*-trityl D-cysteine methyl ester derivative, (*S*)-methyl 2-((*E*)-3-(3-acetoxyphenyl)acrylamido)-3-(tritylthio)propanoate (159 mg, 89%) as a pale-yellow oil. ^{1}H NMR (270 MHz, $CDCl_3$) δ 2.31 (s, 3H), 2.70 (dd, *J*=4.8, 12.5 Hz, 1H, ABX system), 2.78 (dd, *J*=5.4, 12.5 Hz, 1H, ABX system), 3.72 (s, 3H), 4.75 (ddd, *J*=4.8, 5.4, 7.9 Hz, 1H, ABX–XY system), 6.18 (br d, *J*=7.9 Hz, 1H, *NH*, *XY system*), 6.33 (d, *J*=15.6 Hz, 1H), 7.09 (m, 1H of AA′BB′ system), 7.17–7.41 (complex, 18H), 7.55 (d, *J*=15.6 Hz, 1H); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 21.2 (q), 33.9 (t), 51.2 (d), 52.7 (q), 67.0 (s), 120.6 (d), 121.0 (d), 122.9 (d), 125.6 (d), 126.9 (d)×3, 128.0 (d)×6, 129.5 (d)×6, 129.8 (d), 136.3 (s), 140.7 (d), 144.3 (s)×3, 151.0 (s), 164.9 (s), 169.3 (s), 170.9 (s); FT-IR ν_{max} (cm^{-1}): 3283, 1764, 1739, 1663, 1624; FAB-MS *m/z*: 566 (M+H$^+$, 1%), 243 (100). In a similar manner to that used in step h in the synthesis of analog **2b**, the amide obtained above (508 mg, 0.90 mmol) was cyclized to yield thiazoline ester **11d** (93.6 mg, 36%) as a colorless oil. ^{1}H NMR (270 MHz, $CDCl_3$) δ 2.32 (s, 3H), 3.58 (dd, *J*=9.2, 11.2 Hz, 1H, ABX system), 3.65 (dd, *J*=9.2, 11.2 Hz, 1H, ABX system), 3.84 (s, 3H), 5.22 (dd, *J*=9.2, 9.2 Hz, 1H, ABX system), 7.06–7.10 (complex, 3H), 7.21 (m, 1H), 7.34–7.42 (complex, 2H); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 21.1 (q), 34.7 (t), 52.9 (q), 78.0 (d), 120.6 (d), 122.9 (d), 123.2 (d), 124.9 (d), 129.9 (d), 136.6 (s), 141.1 (d), 151.1 (s), 169.3 (s), 169.8 (s), 171.1 (s); FT-IR ν_{max} (cm^{-1}): 1768, 1743, 1633; EI-MS *m/z*: 305 (M$^+$, 31%), 246 (100). In a similar manner to that used in step i in the synthesis of analog **2b**, thiazoline ester **11d** (38.8 mg, 0.13 mmol) was hydrolyzed with porcine liver esterase to give analog **2d** quantitatively as a yellow powder. Mp 163–165 °C dec; 5% ee from chiral HPLC (retention time of L-isomer: 14.5 min, D-isomer: 16.6 min); ^{1}H NMR (270 MHz, CD_3OD) δ 3.54 (dd, *J*=8.9, 10.9 Hz, 1H, ABX system), 3.63 (dd, *J*=9.2, 10.9 Hz, 1H, ABX system), 5.05 (dd, *J*=8.9. 9.2 Hz, 1H, ABX system), 6.79 (ddd, *J*=1.0, 2.3, 7.9 Hz, 1H), 6. 79–7.23 (complex, 5H); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 36.5 (t), 81.2 (d), 114.7 (d), 118.1 (d), 120.4 (d), 122.9 (d), 131.0 (d), 137.8 (s), 143.5 (d), 159.1 (s), 171.5 (s), 177.1 (s); FT-IR ν_{max} (cm^{-1}): 3180, 1583, 1628; EI-MS *m/z*: 249 (M$^+$, 15%), 204 (98), 145 (100). HR-ESI-MS: *m/z*: [M+H]$^+$ calcd for $C_{12}H_{12}NO_3S$, 250.0538; found, 250.0493.

*4.2.9. (S)-2-((1E,3E)-4-(4-Hydroxyphenyl)buta-1,3-dienyl)-4,5-dihydrothiazole-4-carboxylic acid (**3a**).* To a solution of ethyl 4-(*tert*-butyldimethylsilyloxy)cinnamate[38] (197 mg, 0.64 mmol) in toluene (2 mL) was added DIBAL (1 M solution in toluene, 3.0 mL, 3 mmol) and the mixture was stirred at room temperature for 3 h. The reaction was quenched by adding water (100 mL), and the products were extracted from the quenched mixture with EtOAc (3×10 mL). The organic layers were dried over Na_2SO_4 and evaporated. The residue was purified by PTLC (Hex/EtOAc=2:1) to obtain 4-(*tert*-butyldimethylsilyloxy)cinnamyl alcohol (141 mg, 83%) as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.21 (s, 6H), 0.98 (s, 9H), 4.30 (br s, 2H), 6.25 (d, *J*=16 Hz, 1H), 6.56 (d, *J*=16 Hz, 1H), 6.80 (d, *J*=8.6 Hz, 2H, AA′BB′ system), 7.28 (d, *J*=8.6 Hz, 2H, AA′BB′ system); FT-IR ν_{max} (cm^{-1}): 3388; EI-MS *m/z*: 264 (M$^+$, 54%), 207 (100). To the 4-(*tert*-butyldimethylsilyloxy)cinnamyl alcohol obtained above (233 mg, 0.88 mmol) in CH_2Cl_2 (80 mL) was added MnO_2 (1.40 g, 16.1 mmol) and the mixture was vigorously stirred at room temperature for 4 h. The reaction mixture was filtered through a pad of Celite and the filtration cake was washed thoroughly with CH_2Cl_2. The filtrate and washings were combined and evaporated to give 4-(*tert*-butyldimethylsilyloxy)-cinnamaldehyde (**12a**) (229 mg, 99%) as a pale-yellow oil. A small portion was purified by PTLC (Hex/EtOAc=2:1) for analysis, and the rest was used for the next reaction step without further purification. ^{1}H NMR (270 MHz, $CDCl_3$) δ 0.22 (s, 6H), 0.98 (s, 9H), 6.58 (dd, *J*=7.8, 16 Hz, 1H), 6.87 (d, *J*=8.6 Hz, 2H, AA′BB′ system), 7.41 (d, *J*=16 Hz, 1H), 7.46 (d, *J*=8.6 Hz, 2H, AA′BB′ system), 9.64 (d, *J*=7.8 Hz, 1H); FT-IR ν_{max} (cm^{-1}): 1675; EI-MS *m/z*: 262 (M$^+$, 47%), 207 (25), 206 (100). In a similar manner to that used in step e in the synthesis of analog **2c**, aldehyde **12a** (47.6 mg, 0.18 mmol) was subjected to Wittig olefination to give ester **13a** (45.4 mg, 76%) as a yellow oil. ^{1}H NMR (270 MHz, $CDCl_3$) δ 0.22 (s, 6H), 0.99 (s, 9H), 1.32 (t, *J*=7.3 Hz, 3H), 4.25 (q, *J*=7.3 Hz, 2H), 6.00 (dd, *J*=7.8, 15 Hz, 1H), 6.76–6.89 (complex, 4H), 7.36 (d, *J*=7 Hz, 2H), 7.46 (d, *J*=15 Hz, 1H); FT-IR ν_{max} (cm^{-1}): 1705; EI-MS *m/z*: 332 (M$^+$, 41%), 275 (22), 218 (100). In a similar manner to that used in step f in the synthesis of analog **2c**, ester **13a** (9.7 mg, 0.03 mmol) was hydrolyzed with 1 M NaOH to give acid **14a** quantitatively as a yellow solid. ^{1}H NMR (270 MHz, CD_3OD) δ 5.91 (d, *J*=15 Hz, 1H), 6.79–6.92 (complex, 4H), 7.38 (d, *J*=8.9 Hz, 2H, AA′BB′ system), 7.45 (d, *J*=15 Hz, 1H); FT-IR ν_{max} (cm^{-1}): 3311, 1670; EI-MS *m/z*: 190 (M$^+$, 9%), 183 (100). In a similar manner to that used in step g in the synthesis of analog **2b**, acid **14a** (32.3 mg, 0.170 mmol) was condensed with D-Cys(*S-Trt*)-OMe to afford an *N*-acyl-*S*-trityl D-cysteine methyl ester derivative, (*S*)-methyl 2-((2E,4E)-5-(4-hydroxyphenyl)penta-2,4-dienamido)-3-(tritylthio)propanoate (44.5 mg, 48%) as a pale-yellow oil. ^{1}H NMR (270 MHz, $CDCl_3$) δ 2.71 (m, 2H, ABX system), 3.71 (s, 3H), 4.72 (m, 1H, ABX–XY system), 5.85 (d, *J*=15 Hz, 1H), 6.09 (d, *J*=8.0 Hz, 1H, *NH*, *XY system*), 6.67–7.46 (complex, 22H); FT-IR ν_{max} (cm^{-1}): 3290, 1739, 1652. In a similar manner to that used in step h in the synthesis of analog **2b**, the amide obtained above (44.5 mg, 0.08 mmol) was cyclized to give thiazoline ester **15a** (4.6 mg, 20%) as a yellow solid. ^{1}H NMR (270 MHz, CD_3OD) δ 3.60 (m, 2H, ABX system), 3.79 (s, 3H), 5.21 (dd, *J*=8.9, 8.9 Hz, 1H, ABX system), 6.52 (d, *J*=15 Hz, 1H), 6.76 (d, *J*=8.9 Hz, 2H, AA′BB′ system), 6.77–7.18 (m, 3H), 7.38 (d, *J*=8.6 Hz, 2H, AA′BB′ system). In a similar manner to that used in step i in the synthesis of analog **2b**, thiazoline ester **15a** (4.6 mg, 0.02 mmol) was hydrolyzed with porcine liver esterase to give analog **3a** quantitatively as a yellow solid. 40% ee from chiral HPLC (retention time of L-isomer: 16.2 min, D-isomer: 15.8 min); ^{1}H NMR (270 MHz, CD_3OD) δ 3.60 (m, 2H, ABX system), 5.21 (dd, *J*=7.3, 7.3 Hz, 1H, ABX system), 6.52 (d, *J*=15 Hz, 1H), 6.75–7.56 (complex, 7H), 7.37 (d, *J*=8.9 Hz, 2H, AA′BB′ system); ^{1}FT-IR ν_{max} (cm^{-1}): 3396, 1596; HR-ESI-MS *m/z*: [M+H]$^+$ calcd for $C_{14}H_{14}NO_3S$, 276.0694; found, 276.0694, [M+K]$^+$ calcd for $C_{14}H_{13}NKO_3S$, 314.0253; found, 314.0284.

*4.2.10. (S)-4,5-Dihydro-2-[(1E,3E)-4-(4-dimethylaminophenyl)buta-1,3-dienyl]thiazole-4-carboxylic acid (**3b**).* In a similar manner to that used in step e in the synthesis of analog **2c**, 4-(dimethylamino) cinnamaldehyde (**12b**) (504 mg, 2.88 mmol) was subjected to Wittig olefination to give ester **13b** (705 mg, 99%) as a yellow solid. Mp 115–120 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.30 (t, *J*=7.5 Hz, 3H), 3.00 (s, 6H), 4.20 (q, *J*=7.5 Hz, 2H), 5.88 (d, *J*=15.5 Hz, 1H),

6.66–6.71 (m, 3H), 6.82 (d, *J*=15.5 Hz, 1H), 7.35 (d, *J*=8 Hz, 2H, AA′BB′ system), 7.44 (dd, *J*=12, 15.5 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 14.5 (q), 40.3 (q)×2, 60.2 (t), 112.1 (d)×2, 118.3 (d), 121.9 (d), 124.2 (s), 128.7 (d)×2, 141.2 (d), 145.9 (d), 151.0 (s), 167.7 (s); FT-IR ν_{max} (cm^{-1}): 1701; ESI-MS *m*/*z*: 246 [$(M+H)^+$]. In a similar manner to that used in step f in the synthesis of analog **2c**, ester **13b** (411 mg, 1.68 mmol) was hydrolyzed with 1 M NaOH to give acid **14b** (353 mg, 96%) as a yellow solid. Mp 220–225 °C (lit.[39] 248 °C); ^{1}H NMR (500 MHz, CD_3OD) δ 2.97 (s, 6H), 5.84 (d, *J*=15 Hz, 1H), 6.70 (d, *J*=9.2 Hz, 2H, AA′BB′ system), 6.73–6.87 (m, 2H), 7.36 (d, *J*=9.2 Hz, 2H, AA′BB′ system), 7.40 (dd, *J*=11, 15 Hz, 1H); ^{13}C NMR (67.8 MHz, CD_3OD) δ 39.0 (q)×2, 111.9 (d)×2, 118.7 (d), 121.7 (s), 123.1 (d), 128. 6 (d), 130.4 (d), 137.1 (d), 140.6 (s), 145.0 (s), 167.8 (s); FT-IR ν_{max} (cm^{-1}): 2896, 1684; ESI-MS *m*/*z*: 218 [$(M+H)^+$]. In a similar manner to that used in step g in the synthesis of analog **2b**, acid **14b** (637 mg, 2.93 mmol) was coupled with D-Cys(*S*-Trt)-OMe to give an *N*-acyl-*S*-trityl D-cysteine methyl ester derivative, (*S*)-methyl 2-((2*E*,4*E*)-5-(4-(dimethylamino)phenyl)penta-2,4-dienamido)-3-(tritylthio)propanoate (1.2 g, 69%) as a yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.73 (m, 2H, ABX system), 2.99 (s, 6H), 3.71 (s, 3H), 4.77 (ddd, *J*=6.8, 6.8, 7.5 Hz, 1H, ABX–XY system), 5.86 (d, *J*=15 Hz, 1H), 6.18 (d, *J*=7.5 Hz, 1H, *NH*), 6.67 (d, *J*=8.0 Hz, 2H, AA′BB′ system), 6.70 (d, *J*=11.5 Hz, 1H), 6.81 (d, *J*=15.5 Hz, 1H), 7.22–7.42 (m, 18H); ^{13}C NMR (67.8 MHz, CD_3OD) δ 34.3 (t), 39.0 (q)×2, 53.0 (q), 53.2 (d), 68.3 (s), 98.7 (d)×2, 112.5 (d), 115.0 (d), 121.1 (s), 124.1 (d), 128.0 (d)×3, 129.0 (d)×8, 130.7 (d)×8, 145.9 (s), 148.0 (s), 158.0 (s), 159.6 (s), 161.0 (s), 172.0 (s); FT-IR ν_{max} (cm^{-1}): 1739, 1593; ESI-MS *m*/*z*: 599 [$(M+Na)^+$]. In a similar manner to that used in step h in the synthesis of analog **2b**, the amide obtained above (43.3 mg, 0.08 mmol) was cyclized to give thiazoline **15b** (17.6 mg, 74%) as a pale-yellow solid. Mp 145 °C dec; ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.99 (s, 6H), 3.55 (m, 2H, ABX system), 3.82 (s, 3H), 5.15 (dd, *J*=9.2, 9.2 Hz, 1H, ABX system), 6.53 (d, *J*=15 Hz, 1H), 6.66 (d, *J*=9.0 Hz, 2H, AA′BB′ system), 6.72 (m, 2H), 6.93 (dd, *J*=10, 15 Hz, 1H), 7.34 (d, *J*=9.0 Hz, 2H, AA′BB′ system); ^{13}C NMR (125 MHz, $CDCl_3$) δ 33.7 (t), 39.1 (q)×2, 51.7 (q), 76.8 (d), 112.0 (d)×2, 121.0 (d), 122.0 (d), 128.4 (d)×2, 140.5 (d), 144.8 (d), 151.3 (s), 171.3 (s), 171.3 (s); FT-IR ν_{max} (cm^{-1}): 1699; ESI-MS *m*/*z*: 317 [$(M+H)^+$]. In a similar manner to that used in step i in the synthesis of analog **2b**, thiazoline ester **15b** (39.9 mg, 0.13 mmol) was hydrolyzed with porcine liver esterase to give analog **3b** (37.9 mg, 99%) as a red solid. Mp 144 °C dec; 70% ee from chiral HPLC (OZ-RH column, retention time of L-isomer: 9.9 min, D-isomer: 12.0 min); ^{1}H NMR (500 MHz, CD_3OD) δ 2.97 (s, 6H), 3.58 (m, 2H, ABX system), 5.00 (t, *J*=8.9 Hz, 1H, ABX system), 6.49 (d, *J*=15 Hz, 1H), 6.70 (d, *J*=9.2 Hz, 2H, AA′BB′ system), 6.81 (m, 2H), 7.04 (dd, *J*=9.5, 15 Hz, 1H), 7.37 (d, *J*=9.2 Hz, 2H, AA′BB′ system); ^{13}C NMR (125 MHz, CD_3OD) δ 34.5 (t), 39.3 (q)×2, 78.0 (d), 112.0 (d)×2, 119.8 (d), 121.9 (d), 124.3 (s), 128.8 (d)×2, 141.9 (d), 146.2 (d), 151.5 (s), 166.0 (s), 172.0 (s); FT-IR ν_{max} (cm^{-1}): 3386, 1734, 989; ESI-MS *m*/*z*: 303 [$(M+H)^+$]. HR-ESI-MS *m*/*z*: $[M+H]^+$ calcd for $C_{16}H_{19}N_2O_2S$, 303.1167; found, 303.1145.

4.3. Measurement of bioluminescent activity

*4.3.1. Synthesis of **2b**–AMP and D-LH$_2$–AMP.* Following the literature,[34] adenylated **2b** and D-LH_2 were prepared and purified just prior to use. For **2b**-LH_2–AMP: under an argon atmosphere, a solution of *N*,*N*′-dicyclohexylcarbodiimide (20 mg, 0.097 mmol) in DMSO (0.8 mL) was added to a solution of **2b**-LH_2 (1 mg, 3.61 μmol) and (−)-adenosine-5′-monophosphoric acid (free acid, Oriental Yeast Co.) (10 mg, 0.029 mmol) in DMSO (0.5 mL). The reaction mixture was stirred vigorously for 10 min at room temperature, then acetone (1.5 mL) was added to quench the reaction. The white precipitates formed were deposited by centrifugation, and the supernatant was discarded. The precipitates were suspended in ice-cold acetone (1 mL) and centrifuged. This washing operation was repeated. The twice-washed precipitates were dissolved in distilled water containing 0.05% (v/v) trifluoroacetic acid (0.5 mL). The acetone dissolved in the solution was removed under reduced pressure. The resulting aqueous solution was promptly subjected to HPLC purification just prior to use as described in Section 4.1.2. D-LH_2–AMP was also prepared by essentially the same procedure and purified by HPLC just prior to use.

4.3.2. Measurements of bioluminescence intensities. Bioluminescence intensities of D-LH_2, synthesized analogs, and the adenylated derivatives were measured using an ATTO AB-2200 or AB-2270 luminometer (Hamamatsu, R4220 photomultiplier tube). A reaction mixture was prepared by mixing 20 μL of substrate (100 μM), 20 μL of luciferase solution (0.01 mg/mL), and 20 μL of potassium phosphate buffer (500 mM, pH 8.0). Luminescence reactions were initiated by injecting 40 μL of ATP-Mg (200 μM) into the reaction mixture at ambient temperature. To evaluate bioluminescence activity of the adenylated derivatives, 20 μL of the adenylated substrate (10 μM), 20 μL of potassium phosphate buffer (500 mM, pH 8.0), and water (40 μL) were mixed, and the luminescence reaction was initiated by adding 20 μL luciferase solution (1 μg/mL). In the both cases, light emission was monitored for 180 s with sampling intervals of 1 s. Emission intensities were expressed as the light count per second (cps).

4.3.3. Measurements of bioluminescence spectra. Bioluminescence spectra of synthesized analogs and the adenylated derivatives were recorded using an ATTO AB-1850 spectrophotometer. A reaction mixture was prepared by mixing 5 μL of a substrate (100 μM), 5 μL of luciferase solution (1 mg/mL), and 5 μL of potassium phosphate buffer (500 mM, pH 8.0). Luminescence reactions were initiated by injecting 10 μL of ATP–Mg (200 μM) into the reaction mixture. Emission spectra were measured in 1 nm increments from 400 nm to 750 nm. Bioluminescence emission wavelengths of the adenylated substrates were measured in a similar manner. Luminescence reactions were initiated by adding 5 μL of luciferase solution (1 mg/mL) to solutions of adenylated substrates (10 μM, 5 μL) in 5 μL of potassium phosphate buffer (500 mM, pH 8.0) and 10 μL water.

Acknowledgements

The authors are deeply grateful to Dr. Shigeru Nishiyama (Keio University, Yokohama, Japan) for enthusiastic support and critical review. This work was supported by Grants-in-Aid for Exploratory Research (No. 24650633) to S.M. from the Ministry of Education, Culture, Sports, Science and Technology, and Adaptable & Seamless Technology Transfer Program through Target-driven R&D (A-Step) (No. AS2321366E) to S.M. from the Japan Science and Technology Agency.

References and notes

1. Fraga, H. *Photochem. Photobiol. Sci.* **2008**, *7*, 146–158.
2. Marques, S. M.; Esteves de Silva, J. C. G. *IUBMB Life* **2009**, *61*, 6–17.
3. Inouye, S. *Cell. Mol. Life Sci.* **2010**, *67*, 387–404.
4. Thorne, N.; Ingles, J.; Auld, D. S. *Chem. Biol.* **2010**, *17*, 646–657.
5. Roda, A.; Guardigli, M.; Michelini, E.; Mairasoli, M. *Trends Anal. Chem.* **2009**, *28*, 307–322.
6. Weissleder, R. *Nat. Biotechnol.* **2001**, *19*, 316–317.
7. Viviani, V. R. *Cell. Mol. Life Sci.* **2002**, *59*, 1833–1850.
8. Kajiyama, N.; Nakano, E. *Protein Eng.* **1991**, *4*, 691–693.
9. Branchini, B. R.; Ablamsky, D. M.; Davis, A. L.; Southworth, T. L.; Bulter, B.; Fan, F.; Jathoul, A. P.; Pule, M. A. *Anal. Biochem.* **2010**, *396*, 290–297.
10. Naumov, P.; Ozawa, Y.; Ohkubo, K.; Fukuzumi, S. *J. Am. Chem. Soc.* **2009**, *131*, 11590–11605.
11. White, E. H.; Wörther, H.; Seliger, H. H.; McElroy, W. D. *J. Am. Chem. Soc.* **1966**, *88*, 2015–2019.
12. Woodroof, C. C.; Shultz, J. W.; Wood, M. G.; Osterman, J.; Cali, J. J.; Daily, W. J.; Meisenheimer, P. L.; Klaubert, D. H. *Biochemistry* **2008**, *47*, 10383–10393.

13. Reddy, G. R.; Thompson, W. C.; Miller, S. C. *J. Am. Chem. Soc.* **2010**, *132*, 13586–13587.
14. Zhou, W.; Shlts, J. W.; Murphy, N.; Hawkins, E. M.; Bernad, L.; Good, T.; Moothart, L.; Frackman, S.; Klaubert, D. H.; Wood, K. V. *Chem. Commun.* **2006**, 4620–4622.
15. Yang, W.; Liu, X.; Peng, X.; Li, P.; Wang, T.; Tai, G.; Li, X. J.; Zhou, Y. *Carbohydr. Res.* **2012**, *359*, 92–96.
16. Cohen, A. S.; Dubikovskaya, E. A.; Rush, J. S.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2010**, *132*, 8563–8565.
17. Takakura, H.; Sasakura, K.; Ueno, T.; Urano, Y.; Terai, T.; Hanaoka, K.; Tsuboi, T.; Nagano, T. *Chem.—Asian J.* **2010**, *5*, 2053–2061.
18. Takakura, H.; Kojima, R.; Urano, Y.; Terai, T.; Hanaoka, K.; Nagano, T. *Chem.—Asian J.* **2011**, *6*, 1800–1810.
19. Kojima, R.; Takakura, H.; Ozawa, T.; Tada, Y.; Nagano, T.; Urano, Y. *Angew. Chem., Int. Ed.* **2013**, *52*, 1175–1179.
20. Takakura, H.; Kojima, R.; Ozawa, T.; Nagano, T.; Urano, Y. *ChemBioChem* **2012**, *13*, 1424–1427.
21. Conley, N. R.; Dragulescu-Andrasi, A.; Rao, J.; Moerner, W. E. *Angew. Chem., Int. Ed.* **2012**, *51*, 3350–3353.
22. McCutcheon, D. C.; Paley, M. A.; Steinhardt, R. C.; Prescher, J. A. *J. Am. Chem. Soc.* **2012**, *134*, 7604–7607.
23. (a) Branchini, B. R.; Hayward, M. M.; Bamford, S.; Brennan, P. M.; Lajiness, E. J. *Photochem. Photobiol.* **1989**, *49*, 689–695; (b) Woodroofe, C. C.; Meisenheimer, P. L.; Klaubert, D. H.; Kovic, Y.; Rosenberg, J. C.; Behney, C. E.; Southworth, T. L.; Branchini, B. R. *Biochemistry* **2012**, *51*, 9807–9813; (c) Sundlow, J. A.; Fontaine, D. M.; Southworth, T. L.; Branchini, B. R.; Gulick, A. M. *Biochemistry* **2012**, *51*, 6493–6495.
24. White, E. H.; Wörther, H. *J. Org. Chem.* **1966**, *31*, 1484–1488.
25. Hosseinkhani, S. *Cell. Mol. Life Sci.* **2011**, *68*, 1167–1182.
26. Pretsch, E.; Buhlmann, C.; Affolter, C.; Amemiya, S. *Translation from the English Language Edition: Structure Determination of Organic Compounds*; Springer Tokyo: Tokyo, 2004; p 387 (in Japanese edition).
27. Hirano, T.; Hasumi, Y.; Ohtsuka, K.; Maki, S.; Niwa, H.; Yamaji, M.; Hashizume, D. *J. Am. Chem. Soc.* **2009**, *131*, 2385–2396.
28. Hirano, T.; Nagai, H.; Matsuhashi, T.; Hasumi, Y.; Iwano, S.; Ito, K.; Maki, S.; Niwa, H.; Viviani, V. R. *Photochem. Photobiol. Sci.* **2012**, *11*, 1281–1284.
29. DeLuca, M.; McElroy, W. D. *Biochemistry* **1974**, *13*, 921–925.
30. Branchini, B. R.; Rosenberg, J. C.; Fontaine, D. M.; Southworth, T. L.; Behny, C. E.; Uzasci, L. *J. Am. Chem. Soc.* **2011**, *133*, 11088–11091.
31. Gulick, A. M. *ACS Chem. Biol.* **2009**, *4*, 811–827.
32. Nakatsu, T.; Ichikawa, S.; Hiratake, J.; Saldanha, A.; Kobayashi, N.; Sakata, K.; Kato, H. *Nature* **2006**, *440*, 372–376.
33. Ayabe, K.; Zako, T.; Ueda, H. *FEBS Lett.* **2005**, *579*, 4389–4394.
34. Branchini, B. R.; Murtiashaw, M. H.; Magyar, R. A.; Portier, N. C.; Ruggiero, M. C.; Stroh, J. G. *J. Am. Chem. Soc.* **2002**, *124*, 2112–2113.
35. Branchini, B. R.; Ablamsky, D. M.; Rosenberg, J. C. *Bioconjugate Chem.* **2010**, *21*, 2023–2030.
36. Bumagin, N. A.; More, P. G.; Beletskaya, I. P. *J. Organomet. Chem.* **1989**, *371*, 397–401.
37. You, S.-L.; Razavi, H.; Kelly, J. W. *Angew. Chem., Int. Ed.* **2003**, *42*, 83–85.
38. Boschi, D.; Tron, G. C.; Lazzarato, L.; Chegaev, K.; Cena, C.; Stilo, A. D.; Giorgis, M.; Bertinaria, M.; Fruttero, R.; Gasco, A. *J. Med. Chem.* **2006**, *49*, 2886–2897.
39. Jana, S.; Ghosh, S.; Dalapati, S.; Kar, S.; Guchhait, N. *Spectrochim. Acta, Part A* **2011**, *78*, 463–468.