Development of near-infrared firefly luciferin analogue reacted with wild-type and mutant luciferases

Nobuo Kitada¹,² | Ryohei Saito¹,³ | Rika Obata¹ | Satoshi Iwano⁴ | Kazuma Karube¹ | Atsushi Miyawaki⁴ | Takashi Hirano¹ | Shojiro A. Maki¹,²

¹Department of Engineering Science, Graduate School of Informatics and Engineering, The University of Electro-Communications, Chofu, Japan
²Center for Neuroscience and Biomedical Engineering, The University of Electro-Communications, Chofu, Japan
³School of Pharmacy, Tokyo University of Pharmacy and Life Science, Tokyo, Japan
⁴Laboratory for Cell Function and Dynamics, Center for Brain Science, Saitama, Japan

Abstract
Interestingly, only the D-form of firefly luciferin produces light by luciferin–luciferase (L–L) reaction. Certain firefly luciferin analogues with modified structures maintain bioluminescence (BL) activity; however, all L-form luciferin analogues show no BL activity. To this date, our group has developed luciferin analogues with moderate BL activity that produce light of various wavelengths. For in vivo bioluminescence imaging, one of the important factors for detection sensitivity is tissue permeability of the number of photons emitted by L–L reaction, and the wavelengths of light in the near-infrared (NIR) range (700–900 nm) are most appropriate for the purpose. Some NIR luciferin analogues by us had performance for in vivo experiments to make it possible to detect photons from deep target tissues in mice with high sensitivity, whereas only a few of them can produce NIR light by the L–L reactions with wild-type luciferase and/or mutant luciferase. Based on the structure–activity relationships, we designed and synthesized here a luciferin analogue with the 5-allyl-6-dimethylamino-2-naphthylethenyl moiety. This analogue exhibited NIR BL emissions with wild-type luciferase (λmax = 705 nm) and mutant luciferase AlaLuc (λmax = 655 nm).

KEYWORDS
Akaluc, luciferin analogues, luciferin-luciferase reaction, mutant luciferase, near-infrared bioluminescence, Photinus pyralis luciferase, TokeOni

1 | INTRODUCTION

Firefly bioluminescence (BL) showed light emission caused by the reaction of firefly luciferin (1, Figure 1) catalyzed with firefly luciferase in the presence of Mg²⁺, in
which 1 is first adenylated with ATP followed by the oxidative reaction with O₂ to generate oxyluciferin with a yellow-green light (λ_max = 560 nm). This reaction is termed as the luciferin–luciferase (L–L) reaction. Firefly luciferin and luciferase are biosynthesized in the body of firefly, and 1 has a chiral center at C3 with the same stereochemistry as unnatural D-cysteine (D-form). Interestingly, despite the fact that the L-form of firefly luciferin has significantly low BL activity of L–L reaction; however, we reported that the L-form of firefly luciferin is able to produce light by conversion to D-form through the luciferyl-CoA under the action of luciferase.

The L–L reaction is applied to optical imaging techniques in the fundamental research fields of medical and biological sciences. One of the solutions to improve optical in vivo imaging technique is an increase in the permeability of light from deep site of biological tissue. Because the permeability of near-infrared (NIR) light is higher than that of visible light (450–600 nm) for biological tissue, researchers have been engaged in developing luciferin analogues and mutant luciferases producing NIR light by the L–L reactions. These luciferin analogues and mutant luciferases successfully enabled high-resolution optical in vivo imaging compared with the use of the wild-type luciferin 1 and luciferase. Our group developed luciferin analogues producing light with various wavelengths and some of the analogues were tested for in vivo experiments. Then, we confirmed that the analogues enabled to detect light emission from the deep target tissue of mice with high sensitively. In addition, Aka-BLI, which is the combination of a NIR luciferin analogue, TokeOni (2, Figure 1) with a mutant luciferase, Akaluc, produced NIR light and made it possible to detect the BL emission from the brain in a marmoset.

Although, there are a number of luciferin analogues, only limited analogues can produce NIR light (over 700 nm) reacted with wild-type luciferase. To design a new luciferin analogue, we have evaluated a structure–BL activity relationship of our luciferin analogues for the wavelength of L–L reaction with wild-type luciferase (Figure 2). One conclusion of the evaluations lead us to design analogue 3 based on the data of 2, 4, and 5. The BL emission maximum (λ_BL) of 2 with the dimethylamino group is 35-nm red shifted from that of 4 with the hydroxyl group, although 2 and 4 have the common phenyl-1,3-butadiene structure. When 4 and 5, both of which contain the hydroxyl group, are compared, the λ_BL of 5 is 50-nm red shifted from that of 4. Hence, we designed 3 to have the 5-allyl-2-naphthylethenyl moiety and a dimethylamino group at C6. The structure–BL activity relationship predicts that the λ_BL value of 3 will be 725 nm. In this report, we prepared 3 and investigated its BL activity with Photinus pyralis (Ppy) luciferase and Akaluc, comparing its properties to those of 1, 2, and 5.
2 | MATERIALS AND METHODS

2.1 | General

Commercially available reagents and solvents were used without further purification. For bioluminescence measurements, TokeOni (2) was provided by Kurogane Kasei Co., Ltd. and recombinant Ppy luciferase (Quantilum® recombinant luciferase, E1701, Promega) was used. Wako Silica gel 70 F254 thin-layer chromatography plates were used for analytical thin-layer chromatography, and Kanto Chemical Silica gel 60 N (spherical, neutral) was used for column chromatography. For preparative flash chromatography, an automated system (Smart Flash EPLC AI-580S, Yamazen Corp., Japan) was used with universal columns of silica gel. Melting points were measured with a Yanaco MP-500P. IR spectra were obtained with a Nicolet 6700 spectrometer with an attenuated total reflection attachment. 1H and 13C nuclear magnetic resonance (NMR) spectra were recorded on a JEOL ECA-500 instrument (500 MHz for 1H and 126 MHz for 13C). High-resolution electrospray ionization mass spectra were obtained with a JEOL JMS-T100LC mass spectrometer (500 MHz for 1H and 126 MHz for 13C) using a Daicel chiral column (Daicel Chemical Industries, OD-RH, 5 μm (HPLC; Agilent 1100 series) using a Daicel chiral column (Daicel Chemical Industries, OD-RH, 5 μm, 150 mm, flow rate 0.5 ml/min). Bioluminescence spectra were measured with an ATTO AB-1850 spectrophotometer. Bioluminescence intensities were monitored with a TokeOni (2) instrument (500 MHz for 1H and 126 MHz for 13C).

2.2 | Synthesis of NIR analogue 3

2.2.1 | Bromoamine 3b

A solution of 6-amino-2-naphthoic acid methyl ester (3a) (5.35 g, 26.6 mmol) in dimethyl sulphoxide (DMSO; 50 ml) and N-bromosuccinimide (4.84 g, 27.2 mmol) was added, and the mixture was stirred for 10 min at r.t. The reaction mixture was diluted with water and extracted with ethyl acetate (3 × 150 ml). The combined organic layers were dried over Na2SO4, filtered, and the solvent was removed under reduced pressure. The obtained residue was purified by silica gel column chromatography (hexane/ethyl acetate = 4/1) to yield bromoamine 3b (6.97 g, 24.9 mmol, 93%) as a light brown solid: 1H NMR (500 MHz, CDCl3) δ 8.43 (d, J = 1.7 Hz, 1H), 8.06 (dd, J = 8.6, 1.7 Hz, 1H), 8.03 (d, J = 8.6 Hz, 1H), 7.70 (d, J = 8.6 Hz, 1H), 7.03 (d, J = 8.6 Hz, 1H), 3.96 (s, 3H); 13C NMR (126 MHz, CDCl3) δ 167.29, 144.31, 135.62, 131.30, 130.16, 127.46, 127.34, 125.09, 124.36, 118.24, 103.40, 77.38, 77.12, 76.86, 52.20; HR-ESI-MS: m/z: [M + H]+ calcd for C12H11BrNO2, 279.9973, 281.9953; found, 279.9931, 281.9910.401.

2.2.2 | Dimethylamine 3c

To a solution of bromoamine 3b (2.39 g, 8.54 mmol) in tetrahydrofuran (30 ml), sodium cyanoborohydride (2.63 g, 41.9 mmol) and formaldehyde (35% in H2O, 15 ml, 195 mmol) were added, and the mixture was stirred in an ice bath. The mixture was slowly added to acetic acid (4 ml, 70 mmol) and stirred for 14 h. To the reaction mixture, saturated NaHCO3 aqueous solution (100 ml) was added to quench the reaction. Further, the reaction mixture was diluted with water and extracted with ethyl acetate (3 × 100 ml). The combined organic layers were dried over Na2SO4, filtered, and the solvent was removed under reduced pressure. The obtained residue was purified by silica gel column chromatography (hexane only to hexane/ethyl acetate = 3/1) to yield dimethylamine 3c (912 mg, 2.96 mmol, 35%) as a white solid: 1H NMR (500 MHz, CDCl3) δ 8.50 (s, 1H), 8.31 (d, J = 8.6 Hz, 1H), 8.08 (dd, J = 9.2, 1.7 Hz, 1H), 7.86 (d, J = 8.6 Hz, 1H), 7.41 (d, J = 8.6 Hz, 1H), 3.97 (s, 3H); 1H NMR (126 MHz, CDCl3) δ 167.11, 152.03, 135.86, 131.09, 130.02, 129.85, 127.15, 126.82, 126.28, 120.87, 116.27, 52.32, 44.40; HR-ESI-MS: m/z: [M + H]+ calcd for C12H12BrNO2, 308.0286, 310.0266; found, 308.0295, 310.0274.

2.2.3 | Allyl dimethylamine 3d

To a solution of dimethylamine 3c (2.61 g, 8.46 mmol) in dimethylformamide (40 ml), allyltributyltin (3.4 ml, 11 mmol), LiCl (1.14 g, 28.3 mmol), and Pd (PPh3)2Cl2 (584 mg, 0.832 mmol) were added, and the mixture was stirred for 10 h at 90°C. The reaction mixture was purified by silica gel column chromatography with 10 wt% K2CO3 (hexane/ethyl acetate = 1/1). The obtained crude compound was purified by silica gel column chromatography (hexane/ethyl acetate = 4/1) to yield allyl dimethylamine 3d (2.15 g, 7.99 mmol, 94%) as a colorless oil: 1H NMR (500 MHz, CDCl3) δ 8.54 (d, J = 1.7 Hz, 1H), 8.02 (dd, J = 8.9, 2.0 Hz, 1H), 7.95 (d, J = 8.6 Hz,
2.2.4 | Allyl alcohol 3e

A solution of allyl dimethyamine 3d (2.15 mg, 7.99 mmol) in dry toluene (30 ml) under Ar at 0°C was slowly added 1.0-M diisobutylaluminium hydride (DIBAL-H) in toluene (16.0 ml, 16 mmol), and the mixture was stirred for 1 h at r.t. Then to the reaction mixture was added 1-M hydrochloric acid (10 ml). The mixed solution was extracted with ethyl acetate (3 × 100 ml). The combined organic layers were dried over Na2SO4, filtered, and the solvent was removed under reduced pressure. The obtained residue was purified by silica gel column chromatography (hexane/ethyl acetate = 3/1) to yield alcohol 3e (1.65 mg, 6.83 mmol, 85%) as a colorless oil: 1H NMR (500 MHz, CDCl3) δ 7.92 (d, J = 9.2 Hz, 1H), 7.69 (d, J = 9.2 Hz, 2H), 7.41 (d, J = 8.6 Hz, 2H), 6.12 (qd, J = 11.0, 5.3 Hz, 1H), 5.03 (d, J = 10.3 Hz, 1H), 4.88 (d, J = 17.2 Hz, 1H), 4.78 (d, J = 6.9 Hz, 2H), 3.99 (q, J = 7.2 Hz, 2H), 2.75 (s, 6H); 13C NMR (126 MHz, CDCl3) δ 150.19, 137.84, 136.63, 132.92, 130.89, 128.76, 127.75, 126.04, 125.50, 125.31, 120.16, 115.60, 65.46, 45.77, 31.15; HR-ESI-MS: m/z: [M + H]+ calcd for C18H20NO2, 310.1807; found, 310.1801.

2.2.5 | Allyl aldehyde 3f

To a solution of alcohol 3e (1.46 mg, 6.07 mmol) in dichloromethane (50 ml), Dess–Martin periodinane (2.71 g, 6.39 mmol) and pyridine (1.0 ml, 12 mmol) were added, and the mixture was stirred for 5 h at r.t. The reaction mixture was diluted with water and extracted with chloroform (3 × 100 ml). The combined organic layer was dried over Na2SO4, filtered, and the solvent was removed under reduced pressure. The obtained residue was purified by silica gel column chromatography (hexane/ethyl acetate = 5/1) to yield allyl aldehyde 3f (477 mg, 2.00 mmol, 33%) as a yellow oil: 1H NMR (500 MHz, CDCl3) δ 7.91–7.95 (m, 3H), 7.77 (d, J = 8.6 Hz, 1H), 7.66 (dd, J = 8.6, 1.7 Hz, 1H), 7.45 (d, J = 8.6 Hz, 1H), 7.26 (s, 2H), 6.53 (d, J = 16.0 Hz, 1H), 6.11–6.19 (m, 1H), 5.06–5.09 (m, 1H), 4.88–4.92 (m, 1H), 3.98 (t, J = 2.6 Hz, 2H), 2.78 (d, J = 7.4 Hz, 6H); 13C NMR (126 MHz, CDCl3) δ 172.26, 151.76, 147.09, 137.44, 134.59, 130.91, 130.39, 129.70, 128.50, 128.27, 125.73, 125.49, 120.53, 116.54, 115.77, 45.39, 31.16; HR-ESI-MS: m/z: [M + H]+ calcd for C19H20NO2, 282.1494; found, 282.1469.

2.2.6 | Allyl ethyl ester 3g

A solution of allyl aldehyde 3f (474 mg, 1.98 mmol) in toluene (15 ml) and (carbethoxymethylene) triphenylphosphorane (2.03 g, 5.82 mmol) was added, and the mixture was stirred for 7 h at r.t. The reaction mixture was purified by silica gel column chromatography (hexane/ethyl acetate = 7/1) to yield allyl ethyl ester 3g (584 mg, 1.89 mmol, 95%) as a green-yellow oil: 1H NMR (500 MHz, CDCl3) δ 7.91 (d, J = 9.2 Hz, 1H), 7.86 (d, J = 1.1 Hz, 1H), 7.82 (d, J = 16.0 Hz, 1H), 7.74 (d, J = 8.6 Hz, 1H), 7.63 (dd, J = 9.2, 1.7 Hz, 1H), 7.43 (d, J = 9.2 Hz, 1H), 6.50 (d, J = 16.0 Hz, 1H), 6.10–6.18 (m, 1H), 5.06 (dd, J = 10.3, 1.7 Hz, 1H), 4.89 (dd, J = 17.2, 1.7 Hz, 1H), 4.28 (q, J = 7.1 Hz, 2H), 3.98 (t, J = 2.6 Hz, 2H), 2.77 (s, 6H), 1.35 (t, J = 7.2 Hz, 3H); 13C NMR (126 MHz, CDCl3) δ 167.35, 151.61, 144.85, 137.58, 134.40, 130.55, 130.43, 130.17, 128.44, 125.71, 125.32, 120.56, 117.57, 115.80, 60.54, 45.51, 31.22, 14.46; HR-ESI-MS: m/z: [M + H]+ calcd for C20H24NO2, 310.1807; found, 310.1801.

2.2.7 | Carboxylic acid 3h

A solution of allyl ethyl ester 3g (198 mg, 0.642 mmol) in 2-propanol (4 ml) was added 1-M NaOH aq. (2 ml), and the mixture was heated at reflux for 3 h. After cooling, the reaction mixture was neutralized by adding 1-M HCl aq. The mixed solution was extracted with ethyl acetate (3 × 100 ml). The combined organic layers were dried over Na2SO4, filtered, and the solvent was removed under reduced pressure to give carboxylic acid 3h (148 mg, 0.525 mmol, 82%) as a green-yellow solid: 1H NMR (500 MHz, CDCl3) δ 7.89–7.95 (m, 3H), 7.77 (d, J = 8.6 Hz, 1H), 7.66 (dd, J = 8.6, 1.7 Hz, 1H), 7.45 (d, J = 8.6 Hz, 1H), 7.26 (s, 2H), 6.53 (d, J = 16.0 Hz, 1H), 6.11–6.19 (m, 1H), 5.06–5.09 (m, 1H), 4.88–4.92 (m, 1H), 3.98 (t, J = 2.6 Hz, 2H), 2.78 (d, J = 7.4 Hz, 6H); 13C NMR (126 MHz, CDCl3) δ 172.26, 151.76, 147.09, 137.44, 134.59, 130.91, 130.39, 129.70, 128.50, 128.27, 125.73, 125.49, 120.53, 116.54, 115.77, 45.39, 31.16; HR-ESI-MS: m/z: [M + H]+ calcd for C18H20NO2, 282.1494; found, 282.1469.
2.2.8 | Amide 3i

To a solution of carboxylic acid 3h (255 mg, 0.905 mmol) and S-trityl-d-cysteine methyl ester (521 mg, 1.38 mmol) in dimethylformamide (10 ml), 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) was added, and the reaction mixture was stirred for 7 h at r.t. The reaction was quenched by adding water (50 ml), and the product was extracted with ethyl acetate (3 × 100 ml). The combined organic layer was dried over Na2SO4, filtered, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (hexane/ethyl acetate = 2/1) to yield amide 3i (551 mg, 0.859 mmol, 95%) as a green-yellow solid. 1H NMR (500 MHz, CDCl3) δ 7.93 (d, J = 8.6 Hz, 1H), 7.85 (d, J = 1.7 Hz, 1H), 7.73 (d, J = 6.9 Hz, 1H), 7.62 (dd, J = 9.2, 1.7 Hz, 1H), 7.44 (d, J = 9.2 Hz, 1H), 7.39–7.41 (m, 6H), 7.27–7.30 (m, 6H), 7.19–7.24 (m, 3H), 6.44 (d, J = 15.5 Hz, 1H), 6.11–6.19 (m, 2H), 5.06–5.09 (m, 1H), 4.90–4.94 (m, 1H), 4.78–4.82 (m, 1H), 3.99 (q, J = 1.9 Hz, 2H), 3.74 (s, 3H), 2.78 (d, J = 4.0 Hz, 6H); 13C NMR (126 MHz, CDCl3) δ 171.01, 165.45, 151.34, 144.31, 141.98, 137.51, 134.13, 130.56, 130.24, 129.50, 128.40, 128.30, 128.03, 126.92, 125.57, 123.43, 120.43, 119.20, 115.72, 66.98, 52.71, 51.20, 45.45, 34.02, 31.11; HR-ESI-MS: m/z: [M + Na]+ calc'd for C41H40N2KO3S, 679.2397; found, 679.2415.

2.2.9 | Thiazolidine ester 3j

To a solution of trifluoromethanesulfonic anhydride (Tf2O) (0.30 ml, 1.8 mmol) in dichloromethane (5 ml), a solution of amide 3i (540 mg, 0.843 mmol) in dichloromethane (5 ml) was added under Ar at 0°C, and the mixture was stirred for 10 min. Saturated NaHCO3 aq. was added to the reaction mixture for neutralization. The product was extracted with chloroform (3 × 50 ml). The combined organic layer was dried over Na2SO4, filtered, and concentrated under reduced pressure. The crude products were purified by silica gel column chromatography (hexane/ethyl acetate = 5/1) to yield thiazolidine ester 3j (8.7 mg, 0.023 mmol, 28%) as an orange solid: 1H NMR (500 MHz, CDCl3) δ 7.91 (t, J = 9.5 Hz, 2H), 7.77 (d, J = 9.2 Hz, 1H), 7.67 (dd, J = 8.9, 1.4 Hz, 1H), 7.47 (d, J = 8.6 Hz, 1H), 7.36 (d, J = 16.0 Hz, 1H), 7.15 (d, J = 16.0 Hz, 1H), 6.08–6.15 (m, 1H), 5.17 (t, J = 8.9 Hz, 1H), 5.02 (dd, J = 10.3, 1.7 Hz, 1H), 4.80–4.84 (m, 1H), 3.96 (t, J = 2.6 Hz, 2H), 3.59–3.69 (m, 2H), 2.75 (s, 6H); 13C NMR (126 MHz, CD2OD) δ 173.25, 171.83, 151.38, 143.03, 137.56, 134.08, 130.89, 130.58, 129.50, 128.40, 128.30, 128.03, 126.92, 125.57, 123.43, 120.43, 119.20, 115.70, 66.98, 52.71, 51.20, 45.45, 34.02, 31.11; HR-ESI-MS: m/z: [M + H]+ calc'd for C41H40N2KO3S, 679.2397; found, 679.2415.

2.2.10 | NIR analogue 3

A solution of thiazolidine ester 3j (32.8 mg, 0.0862 mmol) in 4 M HCl aq. (1 ml) and tetrahydrofuran (1 ml) was stirred at r.t. for 18 h. After neutralization of the reaction mixture by adding NaHCO3, the mixture was then concentrated under reduced pressure. The crude products were purified by automated flash chromatography (Smart Flash EPCLC AI-580S, ULTRAPACK COLUMNS C18, H2O/methanol = 9/1 to 1/9) to yield NIR analogue 3 (8.7 mg, 0.023 mmol, 28%) as an orange solid: m.p. 160–164 °C; IR (attenuated total reflection, cm−1): 1590, 1369, 1195, 1143, 983, 955, 816; 1H NMR (500 MHz, CD3OD) δ 7.91 (t, J = 9.5 Hz, 2H), 7.77 (d, J = 9.2 Hz, 1H), 7.67 (dd, J = 8.9, 1.4 Hz, 1H), 7.47 (d, J = 8.6 Hz, 1H), 7.36 (d, J = 16.0 Hz, 1H), 7.15 (d, J = 16.0 Hz, 1H), 6.08–6.15 (m, 1H), 5.17 (t, J = 8.9 Hz, 1H), 5.02 (dd, J = 10.3, 1.7 Hz, 1H), 4.80–4.84 (m, 1H), 3.96 (t, J = 2.6 Hz, 2H), 3.59–3.69 (m, 2H), 2.75 (s, 6H); 13C NMR (126 MHz, CD3OD) δ 173.25, 171.83, 151.38, 143.03, 137.56, 134.08, 130.89, 130.58, 129.50, 128.40, 128.30, 128.03, 126.92, 125.57, 123.43, 120.43, 119.20, 115.70, 66.98, 52.71, 51.20, 45.45, 34.02, 31.11; HR-ESI-MS: m/z: [M + H]+ calc'd for C22H25N2O2S, 381.1637; found, 381.1623.

2.3 | Luminescence measurements

Bioluminescence activities of 3 together with those of wild-type luciferin 1 and TokeOni (2) were investigated using Ppy luciferase and Akaluc. The substrates were dissolved in 50-mM potassium phosphate buffer (KPB, pH 6.0), Ppy luciferase and Akaluc were dissolved in 50-mM KPB (pH 8.0) containing 35% glycerol, and Mg-ATP was dissolved in ultrapure water. An L-L reaction was initiated by injection of 10 μl of Mg-ATP (200 μM) into a mixture of 5 μl of a substrate solution (100 μM), 5 μl of luciferase solution (1 mg/ml), and 5 μl of KPB (500 mM, pH 8.0). Emission spectra were measured on the AB-1850 spectrophotometer in the range of 400–790 nm (slit width: 1.0 mm; exposure time: 10 min [3 and 5] or 15 s [1 and 2]). Light emission intensity by
Ppy luciferase was monitored on an AB-1850 spectrophotometer to provide emission spectra (slit width: 1.0 mm; exposure time: 1 s; scan: 600), and light intensity was determined as the intensity at the \( \lambda_{BL} \) value of the emission spectrum.

Chemiluminescence emission spectra for the reactions of the luciferin methyl esters of 1–2, 5, and 3j with \( t\)-BuOK in DMSO under air were measured on an AB-1850 spectrophotometer (slit width: 1.0 mm; exposure time: 180 s). A solution of the luciferin methyl ester (2.5 mm) in DMSO (200 \( \mu l \)) was placed in a polystyrene tube. This solution was mixed with \( t\)-BuOK (250 mm) in DMSO (200 \( \mu l \)), which was injected with a syringe, to initiate the chemiluminescence reaction with final concentrations of the substrate (1.25 mm) and \( t\)-BuOK (125 mm).

### RESULTS AND DISCUSSION

#### 3.1 Synthesis of luciferin analogue 3

Analogue 3 was prepared according the procedure as shown in Scheme 1. The synthesis of 3 was started from bromination of commercially available methyl ester 3a to obtain 3b. Dimethylation of 3b followed by allylation yielded 3d. Alcohol 3e was prepared from 3d via diisobutylaluminium hydride (DIBAL-H) reduction. Allylaldehyde 3f was prepared via oxidation of 3e,
followed by Dess–Martin periodinane. Wittig reaction of $3f$ with (carbethoxymethylene)triphenylphosphorane ($\text{Ph}_3\text{PCHCOOEt}$) afforded ethyl ester $3g$, which was hydrolyzed to give carboxylic acid $3h$. The condensation of $3h$ with $S$-trityl-$d$-cysteine methyl ester gave amide $3i$, and the following thiazoline ring formation with trifluoromethanesulfonic anhydride ($\text{Tf}_2\text{O}$) and triphenylphosphine oxide ($\text{Ph}_3\text{PO}$) afforded ester $3j$.

FIGURE 3  The bioluminescence spectra of $1$–$3$ and $5$ reacted with $Ppy$ luciferase A, and Akaluc B, respectively

FIGURE 4  The most stable optimized structures of the luciferin forms $3$ and $5$ and optimized structures of the oxyluciferin forms $\text{oxy-}3$ and $\text{oxy-}5$ (phenolate) having the conformations corresponding to the structures of $3$ and $5$
Finally, acid hydrolysis of 3j produced target analogue 3.

3.2 | Bioluminescence activity of analogues 3 and 5

BL activity and emission spectrum of 3 together with those of 1, 2, and 5 were investigated with wild-type recombinant Ppy luciferase and a mutant luciferase, Akaluc (Table 1 and Figure 3).

Before investigating BL properties, the d- and l-forms of 3 were separated by HPLC with a chiral octadecysilane column, and their fractions were screened for BL measurements. The d-form of 3 showed sufficient luminescence with Ppy luciferase, whereas the l-form of 3 showed negligible luminescence similar to the background (Table S1). Similar to wild-type luciferin 1, NIR luciferin analogue produces light only in the ground (Table S1). Similar to wild-type luciferin, showed negligible luminescence similar to the back-

3

eed experiments. The light intensity (Rel. Int.) obtained through the L−L reaction with Ppy luciferase during the initial 600 s for 3 was 1.3% as a relative value compared with that for 1 (Table 1), and the Rel. Int. value was similar to that of 5 (0.8%). The light intensity of 3 with Akaluc was weaker than that with Ppy luciferase and could not be determined relative intensity. These results indicate that 3 and 5 have weak BL activities compared with 2. The \( \lambda_{BL} \) value of 3 was recorded at 705 nm with Ppy luciferase (Table 1 and Figure 3), which red shifted from that of 5 (690 nm). The \( \lambda_{BL} \) values of 3 and 5 are 155 and 120 nm longer than that of 1, respectively, and even 30 and 15 nm longer than that of 2, respectively. On the other hand, the emission spectra of 3 measured with Akaluc showed the \( \lambda_{BL} \) value at 665 nm (Table 1 and Figure 3), which is red shifted by 15 nm compared with that of 2. Also, the \( \lambda_{BL} \) value of 5 reacted with Akaluc was observed at 660 nm that is same as that of 2. To investigate the cause of the variation in \( \lambda_{BL} \) values for 3 and 5, chemiluminescence reaction of the methyl esters of 1–3 and 5 were performed in DMSO containing t-BuOK under air. The chemiluminescence emission maxima (\( \lambda_{CL} \)) of 1–3 and 5 were observed at 595, 685, 685, and 620 nm, respectively (Table 1 and Figure S1). The \( \lambda_{CL} \) value of 3 is same as that of 2, and the \( \lambda_{CL} \) value of 5 is blue shifted by 65 nm compared with that of 2.

4 | DFT AND TIME-DEPENDENT DFT CALCULATIONS FOR OXY-2, OXY-3, AND OXY-5

To further evaluate the observed \( \lambda_{BL} \) and \( \lambda_{CL} \) values for 3, the electronic properties of the oxyluciferin form of 3 (\( \text{oxy} \)-3) together with that of the oxyluciferin form of 5 (\( \text{oxy} \)-5) were investigated using DFT and time-dependent DFT (TD-DFT) calculations with the B3LYP/6–31 + G(d) method. Prior performing a search for the most stable optimized structures of \( \text{oxy} \)-3 and \( \text{oxy} \)-5, we found the most stable optimized structures of the luciferin forms 3 and 5. We then used the structures of 3 and 5 shown in Figure 4 as the basis for starting conformations of \( \text{oxy} \)-3 and \( \text{oxy} \)-5 for further calculations because the structures of 3 and \( \text{oxy} \)-3 have steric hindrance between the allyl and dimethylamino groups, and their dimethylamino groups are twisted and pyramidal. Next, we analyzed the electronic transition properties of the oxyluciferin forms (Table 2). In the case of \( \text{oxy} \)-5, the phenolate anion and its sodium salt model were calculated in the manner similar to the previous literature.

Table 2 summarizes vertical excitation energies (\( E_{ex} \)), excitation wavelengths (\( \lambda_{ex} \)), oscillator strengths (f), and configurations of the allowed transitions to the excited singlet states with the lowest energies for \( \text{oxy} \)-3, \( \text{oxy} \)-5 (phenolate), and \( \text{oxy} \)-5(ONa) together with those for \( \text{oxy} \)-2.16 The \( S_0 \) → \( S_1 \) transitions of \( \text{oxy} \)-3 and \( \text{oxy} \)-5(phenolate) are \( \pi \), \( \pi^* \) transitions corresponding to the highest occupied molecular orbital (HOMO) → lowest unoccupied molecular orbital (LUMO) → lowest unoccupied molecular orbital (LUMO+1, respectively.

### Table 2: Time-dependent density functional theory calculation data for \( \text{oxy} \)-2, \( \text{oxy} \)-3, and \( \text{oxy} \)-5

| Compound          | Transition | \( E_{ex} \)/eV | \( \lambda_{ex} \)/nm (f) | Configuration |
|-------------------|------------|----------------|--------------------------|---------------|
| \( \text{oxy} \)-2 | \( S_0 \) → \( S_1 \) | 2.82           | 439 (1.38)               | H → L (0.70)  |
| \( \text{oxy} \)-3 | \( S_0 \) → \( S_1 \) | 2.85           | 435 (0.58)               | H → L (0.70)  |
| \( \text{oxy} \)-5(phenolate) | \( S_0 \) → \( S_1 \) | 2.40           | 516 (1.23)               | H → L (0.71) H → L (–0.14) |
| \( \text{oxy} \)-5(ONa) | \( S_0 \) → \( S_2 \) | 2.67           | 464 (0.87)               | H → L + 1 (0.70) |

*The allowed transition to the excited singlet state with the lowest excitation energy (\( S_0 \) → \( S_1 \) or \( S_0 \) → \( S_2 \)).

*Vertical excitation energy for the transition.

*Wavelength (\( \lambda_{ex} \)) estimated from the transition energy. Oscillator strength (f) is in the parenthesis.

*Configuration of excitation. Coefficient is in the parenthesis. H, L, and L + 1 denote highest occupied molecular orbital (HOMO), lowest unoccupied molecular orbital (LUMO), and LUMO+1, respectively.

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molecular orbital (LUMO) configuration and the $S_0 \rightarrow S_2$ transition of oxyl-5(ONa) is a $\pi$, $\pi^*$ transition corresponding to the HOMO $\rightarrow$ LUMO + 1 configuration. Although the $\lambda_{BL}$ value of 3 with Ppy luciferase is red shifted from that of 2, the $\lambda_{ex}$ values of oxyl-2 and oxyl-3 are similar. Results indicate that $\lambda_{BL}$ values were mainly determined by the effect of the active site of Ppy luciferase to stabilize the excited states of oxyl-2 and oxyl-3. Because the HOMO–LUMO transition of oxyl-2 has charge-transfer character, the $S_1$ state is more highly polarized than the ground state. $^{20}$ The HOMO and LUMO of oxyl-3 have primary electronic distributions at the (6-dimethylaminonaphtalenyl) and 2-ethenyl-1,3-thiazolone moieties, respectively (Figure 4), indicating that the HOMO has polarized character. The environment surrounding the excited oxyl-3 in Ppy luciferase will be more polar than that surrounding the excited oxyl-2, resulting in the red-shifted $\lambda_{BL}$ value of 3. The electronic distributions of the HOMO and LUMO of oxyl-3 indicate that the allyl group has no contribution to the $\pi$ electronic conjugation. The calculations showing that the $\lambda_{ex}$ values of oxyl-5(phenolate) and oxyl-5(ONa) are red shifted from that of oxyl-3 are opposite to the $\lambda_{BL}$ data with Ppy luciferase and Akaluc. Although the oxidized (O$^-$) group of oxyl-5(phenolate) and oxyl-5(ONa) has the potential to donate more electron density than that of the dimethylamino group of oxyl-3, the anionic property of the oxidized group in the luciferase active site may be weakened.

5 | CONCLUSION

We synthesized luciferin analogue 3 and investigated their luminescence properties. The $\lambda_{BL}$ values for 3 upon reaction with Ppy luciferase and mutant luciferase Akaluc were 705 and 665 nm, respectively. Furthermore, the results of BL and TD-DFT calculations suggest that the allyl group of 3 induced the excited oxyl-3 to be more stable in the active site of luciferase, thus increasing the $\lambda_{BL}$ value of 3 to over 700 nm. A $\lambda_{BL}$ value of over 700 nm is quite noteworthy; however, the intensity of 3 was very weak compared with those of 1 and 2. We should modify the new analogue design to produce a higher bioluminescence intensity for animal experiments.

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ORCID

Shojoiro A. Maki https://orcid.org/0000-0002-9741-0166

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