Electronic Supplementary Information

An Allylated Firefly Luciferin Analogue with Luciferase Specific Response in Living Cells

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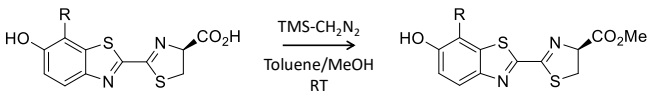
1. Materials and Methods

General Experimental Procedures

Reagents were purchased from Wako Pure Chemical, Tokyo Chemical Industries and Aldrich Chemical Company and were used without further purification. ^{1}H-NMR and ^{13}C-NMR spectra were obtained on JEOL JNM AL-400, JEOL JNM ECX-400, and JEOL JNM-α400 spectrometers in CD_3OD solution using tetramethylsilane as an internal standard. Reference values for residual solvents were taken as $\delta = 3.30$ (CD_3OD) ppm for ^{1}H-NMR and $\delta = 49.00$ (CD_3OD) ppm for ^{13}C-NMR. Coupling constants (*J*) are given in Hz and are uncorrected. High-resolution mass spectra were obtained on a Waters LCT Premier XE (ESI) instrument. For preparative and analytical TLC, silica gel plates (Kieselgel 60 F254, E. Merck AG, Germany) were used, with UV light (254 nm) and ninhydrin visualization of spots. For column chromatography, Kanto Chemical Silica 60N (spherical, neutral, 63-210 μm) was used. All reactions were carried out under an inert (Ar or N_2) atmosphere unless stated otherwise.

Chemiluminescence Spectra

The chemiluminescence emission from the luciferin methyl esters with t-BuOK in DMSO was measured on an AB-2270 luminometer. A solution of the luciferin methyl ester (2.5 mM) in DMSO (200 μL) was placed in a polystyrene tube. This solution was treated with t-BuOK (250 mM) in DMSO (40 μL), which was injected with a syringe, to initiate the chemiluminescence reaction. The emission spectra were measured on an AB-1850 spectrophotometer for 180 s (slit width: 1.0 mm; exposure time: 180 s) with final concentrations of 1.25 mM (substrate) and 125 mM (t-BuOK). The luciferin methyl esters were prepared as previously reported.[1]



D-luciferin: R = H
7`-AllylLuc: R = allyl

D-luciferin methyl ester: R = H
7`-AllylLuc methyl ester: R = allyl

Absorbance and Fluorescence Spectra

D-luciferin and 7`-AllylLuc (5 μM) were dissolved in 0.1 M GTA buffer (pH 8.0) and absorbance and fluorescence spectra were obtained using a VARIOSKAN FLASH (Thermo Scientific, USA) microplate reader with the excitation light and excitation/emission slit widths set to 330 nm and 1 nm, respectively. Spectral data were recorded at 25 °C in a 96-well microplate over the wavelength range of 300-800 nm.

The composition of GTA buffer is 3,3-dimethylglutaric acid, tris(hydroxymethyl)aminomethane, 2-amino-2-methyl-1,3-propanediol, and pH was adjusted with 1M HCl or 1M NaOH solution.

Bioluminescence Emission Spectra

All bioluminescence emission spectra with luciferases were recorded on an integrating sphere-based multichannel spectrometer equipped with a liquid N_2-cooled CCD detector as described previously.[2] The absolute spectral sensitivity was calibrated with a spectral irradiance standard lamp. Bioluminescence reactions were carried out at ambient temperature in 10 μL of reaction cocktail containing substrates and luciferase. Reactions were initiated by injection of 90 μL of ATP-Mg^{2+} solution (3 mM ATP-2Na, 8 mM $MgSO_4 \cdot 7H_2O$ in 0.1 M GTA buffer).

Bioluminescence intensities and Kinetic Reaction Analysis

Kinetic reaction analysis was performed with a custom-built luminometer equipped with a Hamamatsu H11890-01 photomultiplier tube (PMT). Bioluminescence reactions were carried out at ambient temperature in reaction cocktails containing 10 μL of 10-200 μM luciferin and 10 μL of 10 μg/mL luciferase. The reaction was initiated by injection of 80 μL of ATP-Mg^{2+} solution (3 mM ATP-2Na, 8 mM $MgSO_4 \cdot 7H_2O$ in 0.1 M GTA buffer, pH 8.0). The relative light intensity was collected immediately and integrated over 10 s. The values of Michaelis-Menten constants K_m and maximum velocities V_{max} were determined by Lineweaver-Burk plots.

Determination of Quantum Yields[2, 3]

The custom-built luminometer equipped with the Hamamatsu H11890-01 PMT was used to determine the bioluminescence quantum yield values. The absolute responsivity of the luminometer was calibrated with an integrating sphere-based multichannel spectrometer equipped with a liquid N_2-cooled CCD detector and was determined from linear fittings of the plots of the relative count value measured by the luminometer and the absolute value measure by the integrating sphere. The time-integrated total number of photons emitted in the bioluminescence

reaction was measured using the calibrated luminometer. The reaction was initiated by injection of 200 μL of ATP-Mg^{2+} solution (3 mM ATP-2Na, 8 mM $MgSO_4 \cdot 7H_2O$ in 0.1 M GTA buffer, pH 8.0) to the mixed solution of 10 μL of 10 nM luciferin solution and 10 μL of 1.0 mg/mL luciferase solution in a test tube, which was placed in advance into the luminometer. The reaction was monitored until the light-emitting reaction was complete. The quantum yield values were calculated from the obtained total number of photons and that of the luciferin molecules.

Plasmid Construction

For expression in mammalian cells, the genes of Fluc, Eluc, SLG, SLO, and SLR were cloned into pcDNA 3.1/V5-His A vector (Invitrogen, USA) using standard molecular biology techniques as follows. The gene of Fluc was amplified by polymerase chain reaction (PCR) from pGL4.13 (Promega, USA) using primers 5'- ATGCAAGCTTGCCACCATGGAAGATGCCAAAAAC-3' and 5'- GCATCTCGAGCACGGCGATCTTGCC-3'. The gene of Eluc was amplified by PCR from pEluc-test (Toyobo, Japan) using primers 5'- ATGCAAGCTTGCCACCATGGAGAGAGAGAAG-3' and 5'- GCATCTCGAGCAGCTTAGAAGCCTT-3'. The gene of SLG was amplified by PCR from pSLG-SV40 (Toyobo, Japan) using primers 5'- ATGCAAGCTTGCCACCATGGCTAACGAGATCATC-3' and 5'- GCATCTCGAGCAGCTTGGACTTCTT-3'. The gene of SLO was amplified by PCR from pSLO-SV40 (Toyobo, Japan) using primers 5'- ATGCAAGCTTGCCACCATGGCTAACGAGATCATC-3' and 5'- GCATCTCGAGCAGCTTGGACTTCTT-3'. The gene of SLR was amplified by PCR from pSLR-SV40 (Toyobo, Japan) using primers 5'- ATGCAAGCTTGCCACCATGGAAGAAGAGAACATC-3' and 5'- GCATCTCGAGCAGCTTGGACTTGGC-3'. All the PCR-amplified fragments were digested with Hind III and Xho I and subsequently ligated into pcDNA 3.1/V5-His A. The sequences of the amplified regions were confirmed by DNA sequencing (Eurofins Genomics, Japan). All the expression plasmids were purified using a QIAGEN plasmid kit (Qiagen, USA).

Cell Culture and Plasmid Transfection

COS-7 cells were cultured in Dulbecco's modified Eagle's medium (Sigma, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco, USA) at 37°C in 5% CO_2. The day before plasmid transfection, cells were plated onto

black 96-well plates (Greiner, Austria) at a density of 10,000 cells per well. Plasmid transfection was performed using Lipofectamine 3000 (Life Technologies, USA) according to the manufacturer's instructions. Forty-eight hours after plasmid transfection, live cell bioluminescence assays were performed.

Live Cell Bioluminescence Assays

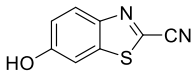
Transfected COS-7 cells were washed with HBSS once. For live cell assays, the cells in 96-well plates were incubated with 100 μL of luciferin in HBSS. Live cell bioluminescence assays were performed using a microplate luminometer (Centro XS3 LB960; Berthold Technologies, Germany) without filter. Imaging of cells was performed by using an *in vivo* imaging system (Lumazone FA, SHOSIN EM CORPORATION, Japan) equipped with EM-CCD (Electron Multiplying Charge Coupled Device, Nippon Roper) as a detector without emission filter. For this purpose, the cells were plated in 24-well plates. The light emission 5 min after incubation of luciferin (500 μM) was integrated. Exposure time = 10 s.

Cytotoxicity Assay

The cytotoxicity of 7`-AllylLuc on COS-7 cells was evaluated by the MTT assay. The cells were seeded in 96-well plates at a density of 10,000 cells per well and cultured for 48 h in Dulbecco's modified Eagle's medium (Sigma, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco, USA) at 37°C in 5% CO_2. The cultured cells were then exposed to 7`-AllylLuc in DMEM with 10% FBS at 37°C for 24 h in a 5% CO_2 incubator. Subsequently, MTT in PBS (-) was added to each well (finally diluted to 500 μg/mL). After an additional 4 h incubation, the medium was removed and washed with 200 μL of PBS (-). Then, 200 μL of acidified isopropanol (0.04 mol/L HCl) was added to each well. The culture plates were shaken for 10 min on an oscillator. The absorbance at 570 nm was measured with a hybrid multi-mode microplate reader. The relative percentage of cell survival was calculated based on the 100% arbitrary absorbance obtained for the blank control. Reported values represent the mean s.e.m. of 4 replicates on the same plate.

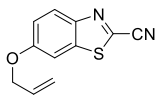
2. Synthesis Procedure

2-Cyano-6-hydroxybenzothiazole (2)



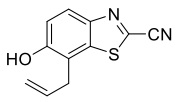
Pyridine hydrochloride (excess) and **1** (1.18 g, 6.22 mmol) were combined in a flask and stirred at 180 °C for 2h. The resulting brown residue was suspended in ethyl acetate and washed with saturated $NaHCO_3$ aq., H_2O, and brine. The organic layers were combined, dried over Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (ethyl acetate/*n*-hexane = 1/3) to give **2** (839.3 mg, 77%) as a pale yellow solid. ^{1}H-NMR (400 MHz, CD_3OD): δ 7.98 (d, J = 8.8 Hz, 1H), 7.39 (d, J = 2.4 Hz, 1H), 7.16 (dd, J = 2.4, 8.8 Hz, 1H) ppm; ^{13}C-NMR (100 MHz, CD_3OD): δ 160.3, 147.3, 139.0, 133.9, 126.6, 119.6, 114.3, 107.0 ppm; HRMS (ESI^-) Calcd for $C_8H_3N_2OS$ $[M+H]^+$: 174.9966, Found: 174.9971.

6-Allyloxy-2-cyanobenzothiazole (3)



A solution of **2** (429.3 mg, 2.44 mmol) and allyl bromide (0.60 mL, 7.09 mmol) in DMF (5 mL) was treated with K_2CO_3 (368.1 mg, 2.66 mmol) and stirred at r.t. for 20 h. The reaction mixture was then added into water and extracted with ethyl acetate. The organic layers were combined, dried over Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (ethyl acetate/*n*-hexane = 1/5) to give **3** (504.9 mg, 96%) as a white solid. ^{1}H-NMR (400 MHz, CD_3OD): δ 8.05 (d, J = 9.3 Hz, 1H), 7.63 (d, J = 2.4 Hz, 1H), 7.30 (dd, J = 2.4, 9.3 Hz, 1H), 6.10 (tdd, 1H), 5.45 (ddd, J = 9.3 Hz, 1H), 5.31 (ddd, J = 9.3 Hz, 1H), 4.67 (td, J = 9.3 Hz, 2H) ppm; ^{13}C-NMR (100 MHz, CD_3OD): δ 160.9, 148.1, 138.9, 135.0, 134.0, 126.4, 120.1, 118.3, 114.2, 105.6, 70.5 ppm; HRMS (ESI^+) Calcd for $C_{11}H_9N_2OS$ $[M+H]^+$: 217.0436, Found: 217.0458.

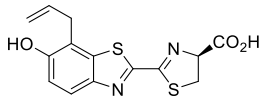
7-Allyl-2-cyano-6-hydroxybenzothiazole (4)



3 (504.9 mg, 2.34 mmol) was heated at 180 °C under an Ar atmosphere for 1h. The residue was dissolved and extracted with ethyl acetate. The organic layers were combined, dried over Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (ethyl acetate/*n*-hexane = 1/10) to give **4** (304.0 mg, 95% (b.r.s.m.)) as a white solid. The remaining starting material (**3**) (183.96 mg, 36%) was recovered as a white solid. ^{1}H-NMR (400 MHz, CD_3OD): δ 8.05 (d, J = 9.3 Hz, 1H), 7.63 (d, J = 2.4 Hz, 1H), 7.30 (dd, J = 2.4, 9.3 Hz, 1H), 6.10 (tdd, 1H), 5.45 (ddd, J = 9.3 Hz, 1H), 5.31 (ddd, J = 9.3 Hz, 1H), 4.67 (td, J = 9.3 Hz, 2H) ppm; ^{13}C-NMR (100 MHz, CD_3OD): δ 156.9, 147.2,

139.2, 134.7, 133.8, 124.6, 119.2, 119.0, 117.2, 114.3, 34.7 ppm; HRMS (ESI^-) Calcd for $C_{11}H_7N_2OS$ $[M-H]^-$: 215.0279, Found: 215.0266.

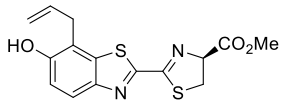
(*S*)-2-(7-allyl-6-hydroxybenzo[*d*]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid (7`-AllylLuc)



4 (66.2 mg, 0.307 mmol) was dissolved in methanol (2 mL), and then D-cysteine hydrochloride monohydrate (56.6 mg, 0.322 mmol) in aqueous K_2CO_3 solution (1.5 mL, pH7.8) was added and stirred at r.t. for 1 h. After complete reaction, the mixture was diluted with saturated $NaHCO_3$ solution and washed with ethyl acetate. Then, the aqueous layer was acidified with 1 M HCl aq. and extracted with ethyl acetate. The combined organic layers were dried over Na_2SO_4, filtered and concentrated *in vacuo* to give **7`-AllylLuc** (95.6 mg, 97%) as a yellow solid. ^{1}H-NMR (400 MHz, CD_3OD): δ 7.78 (d, J = 8.8 Hz, 1H), 7.09 (d, J = 8.8 Hz, 1H), 5.92 (m, 1H), 5.39 (t, J = 9.2 Hz, 1H), 5.08 (ddd, J = 2.0, 10.4, 17.2 Hz, 2H), 3.75 (ddd, J = 9.3 Hz, 2H), 3.59 (m, 2H) ppm; ^{13}C-NMR (100 MHz, CD_3OD): δ 173.4, 167.7, 158.5, 155.7, 148.1, 139.5, 123.9, 119.5, 117.7, 116.7, 79.5, 79.4, 35.9, 34.8 ppm; HRMS (ESI^+) Calcd for $C_{14}H_{13}N_2O_3S_2$ $[M+H]^+$: 321.0368, Found: 321.0378.

The optical purity was determined by CSP HPLC analysis (Chiracel OZ-RH, eluent: 10-90% H_2O/CH_3CN, flow 0.3 mL/min) > 99 % ee [t_R (minor) = 16.4 min, t_R (major) = 19.1 min].

methyl (*S*)-2-(7-allyl-6-hydroxybenzo[*d*]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylate (7`-AllylLuc methyl ester)



To a solution of **7`-AllylLuc** (22.8 mg, 71.1 μmol) in Toluene/MeOH (7.0 mL, v/v=1:7) was added $TMSCH_2N_2$ (130 μL, *ca*. 0.6 M solution in hexane) at r.t., and the reaction mixture was stirred for 2 h. After concentration *in vacuo*, the crude product was purified by silica gel column chromatography (methanol/chloroform = 1/9) to give **7`-AllylLuc methyl ester** (23.4 mg, 99%) as a pale yellow solid. ^{1}H-NMR (400 MHz, CD_3OD): δ 7.77 (d, J = 8.8 Hz, 1H), 7.09 (d, J = 8.8 Hz, 1H), 5.97-5.87 (m, 1H), 5.41 (t, J = 9.2 Hz, 1H), 5.08 (ddd, J = 2.0, 10.4, 17.2 Hz, 2H), 3.96 (s, 3H), 3.78 (dd, J = 1.6, 6.4 Hz, 2H), 3.31 (m, 2H) ppm; ^{13}C-NMR (100 MHz, CD_3OD): δ 172.2, 168.0, 158.3, 155.7, 148.1, 139.5, 135.1, 123.9, 119.5, 117.7, 116.7, 79.3, 53.2, 35.7, 34.8 ppm; HRMS (ESI^+) Calcd for $C_{15}H_{15}N_2O_3S_2$ $[M+H]^+$: 335.0519, Found: 335.0524.

The optical purity was not determined.

Supplemental Figures

3-1. Supplemental Figure 1

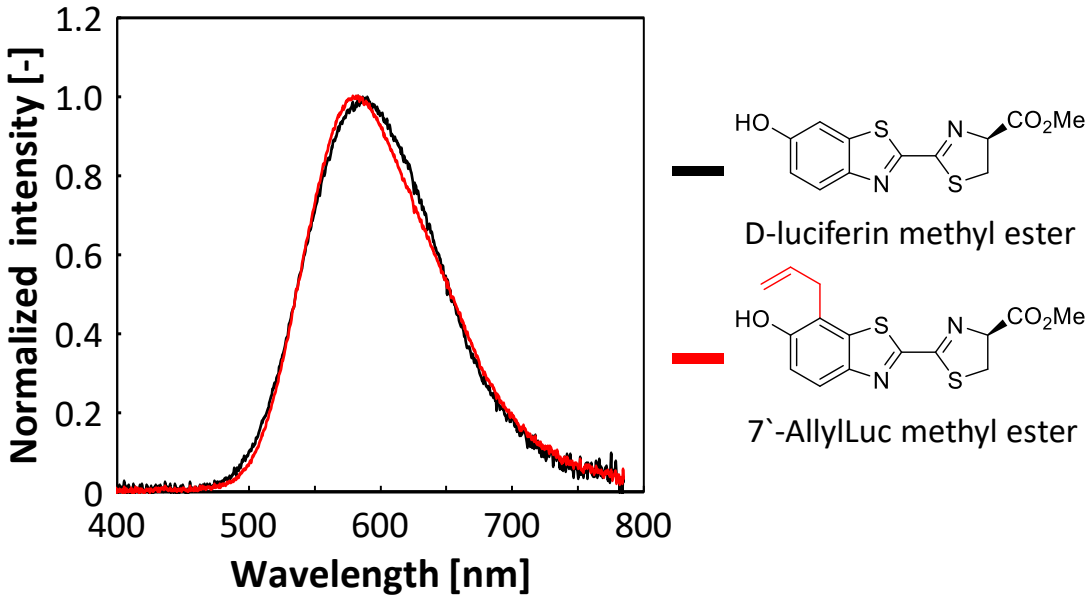


Figure S1. Chemiluminescence spectra of D-luciferin methyl ester (black) and 7`-AllylLuc methyl ester (red) with t-BuOK in DMSO.

3-2. Supplemental Figure 2

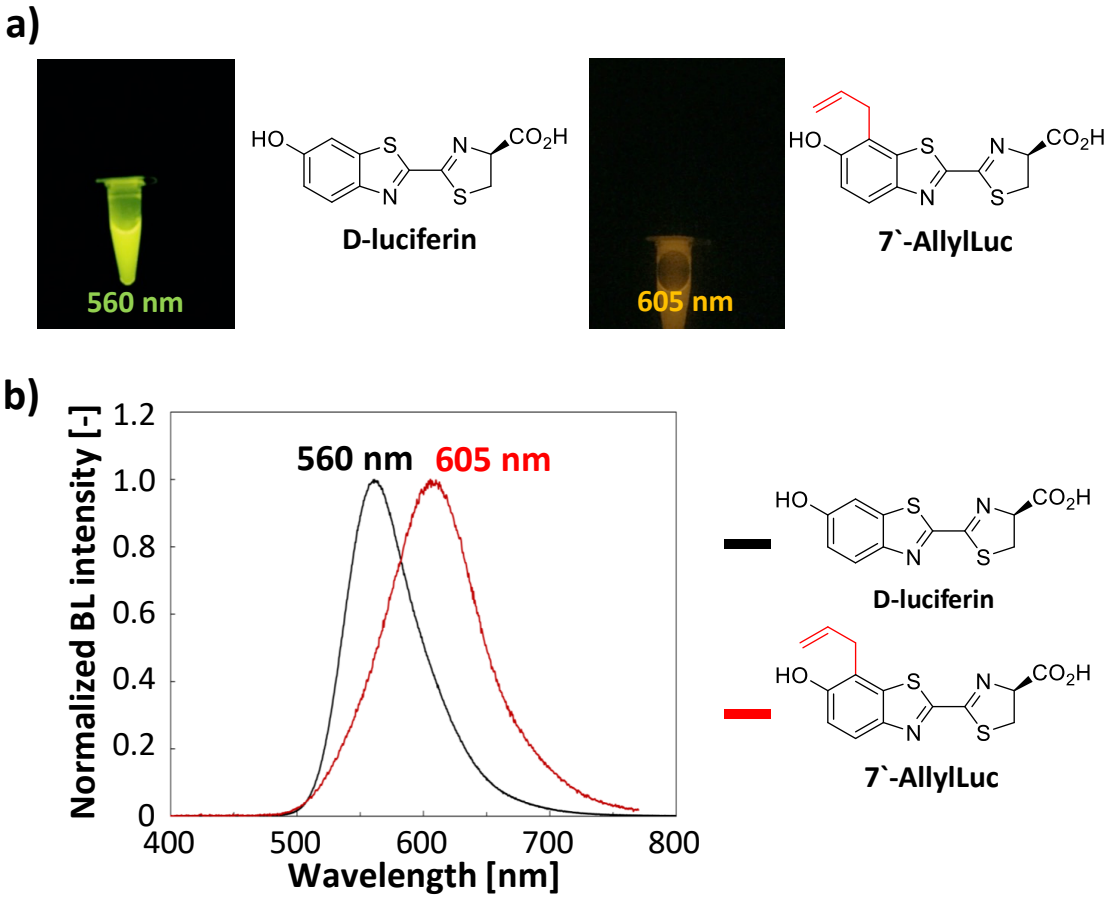


Figure S2. Fluc-catalyzed bioluminescence emission of D-luciferin and 7`-AllylLuc: a) bioluminescence images of D-luciferin and 7`-AllylLuc incubated with high concentrations of Fluc (1.0 µg/mL), b) bioluminescence emission spectra for D-luciferin and 7`-AllylLuc.

3-3. Supplemental Figure 3

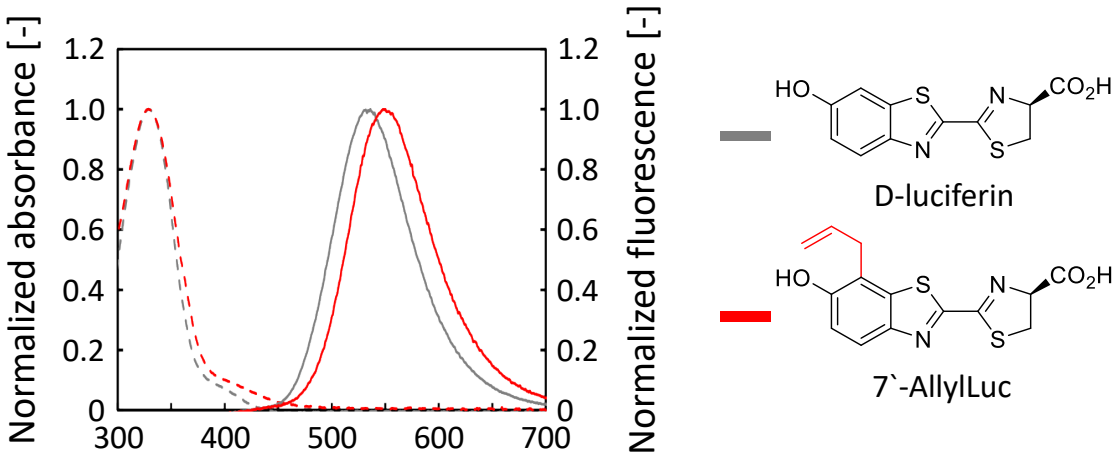


Figure S3. Absorbance (dotted line) and fluorescence (solid line) spectra of D-luciferin (black) and 7`-AllylLuc (red) recorded at pH 8.0 in 0.1M GTA buffer; excited at 330 nm.

3-4. Supplemental Figure 4

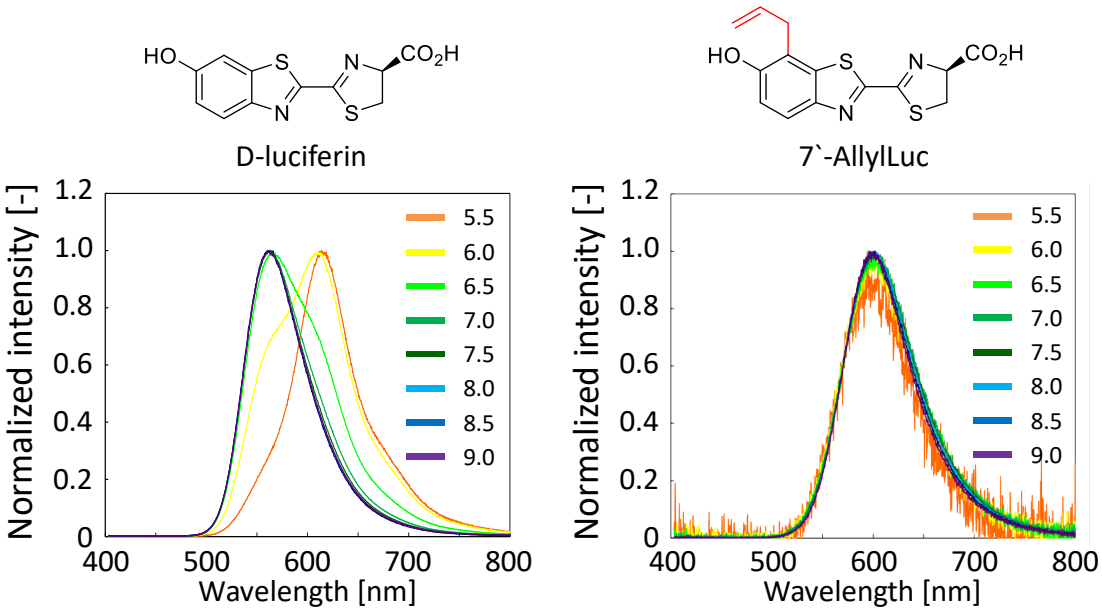


Figure S4. Bioluminescence spectra of D-luciferin and 7`-AllylLuc with Fluc at various pH values.

It is widely known that the luminescence color of D-luciferin in combination with Fluc changes from green to red with increasing pH values.[4] In contrast, the luminescence color of 7`-AllylLuc remains unaffected by pH changes.

3-5. Supplemental Figure 5

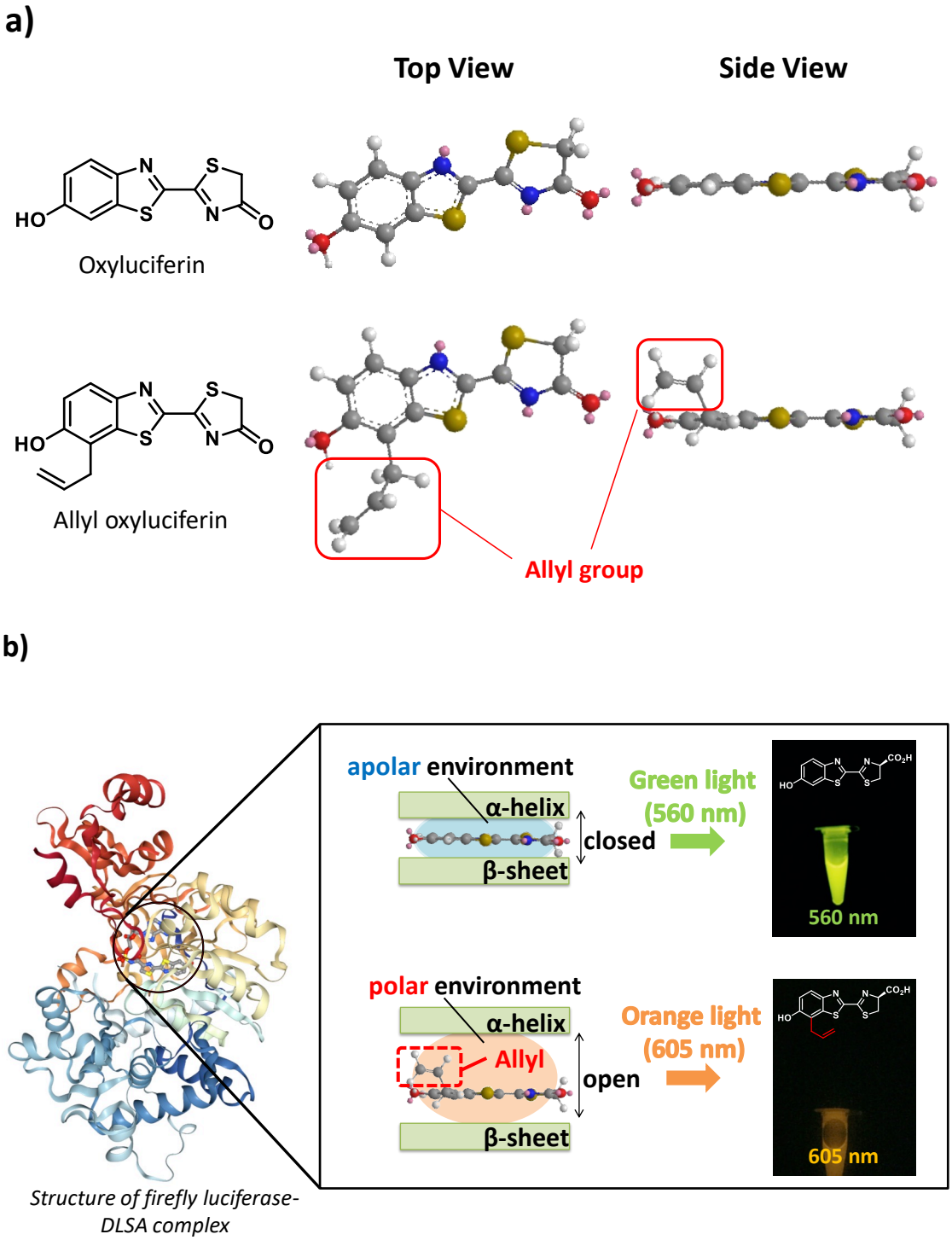


Figure S5. a) Molecular structures of oxyluciferin and ally-oxyluciferin calculated by MM2. The allyl group is not part of the planar luciferin structure. Color scheme: grey = carbon; white = hydrogen; red = oxygen; blue = nitrogen; yellow = sulfur; pink = lone pair. b) Schematic diagram of luciferase active site with 7`-AllylLuc.

3-6. Supplemental Figure 6

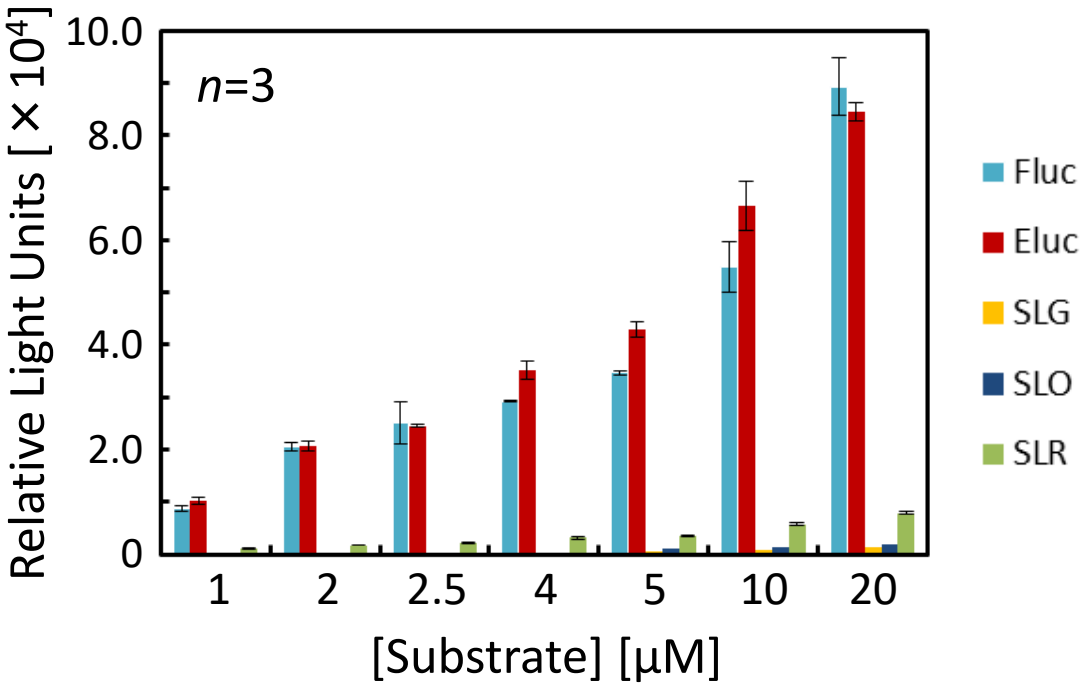


Figure S6. Initial bioluminescence intensity from 7`-AllylLuc with purified luciferases (1 μg/mL) at various luciferin concentrations (1-20 μM). Data were recorded immediately after addition of ATP-Mg^{2+} solution. The initial luminescence intensity is defined as the peak intensity within the first 10 s of ATP-Mg^{2+} solution addition. Error bars represent the standard deviation of 3 experiments.

3-7. Supplemental Figure 7

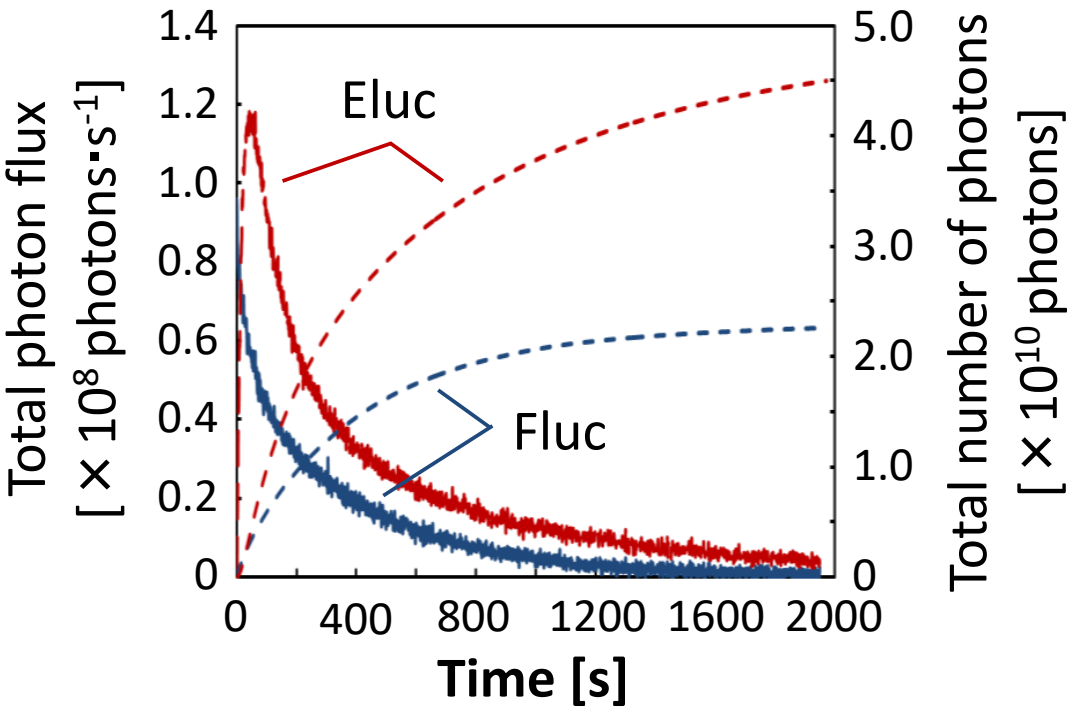


Figure S7. Temporal behavior of total photon flux (solid line, left-hand axis) and time-integrated total number of photons (dotted line, right-hand axis) for 7`-AllylLuc with Fluc or Eluc.

Table S1. Bioluminescence quantum yields (n = 3)

Substrate	***Bioluminescence quantum yield*** ϕ_{BL}	
	Fluc	**Eluc**
D-luciferin	0.41±0.07	0.61±0.08
7`-AllylLuc	0.043±0.007	0.056±0.013

3-8. Supplemental Figure 8

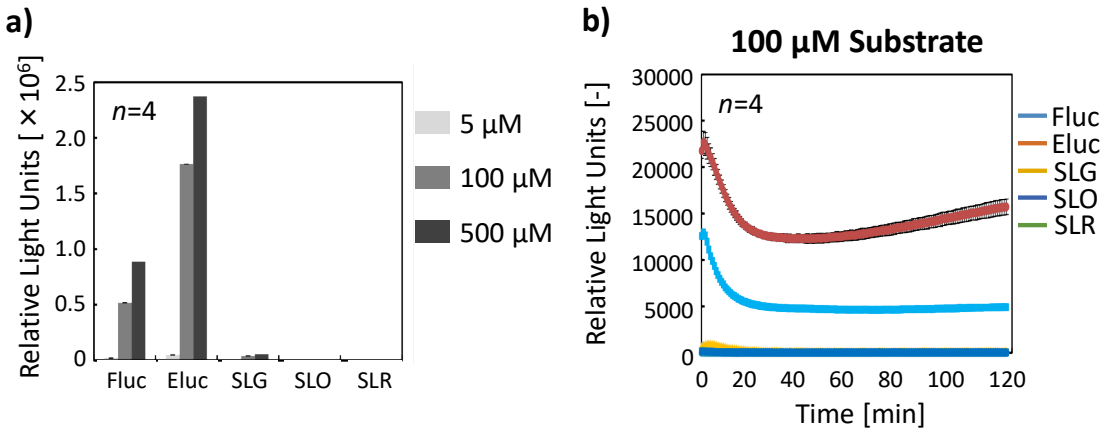


Figure S8. Light emission from luciferase-expressing COS-7 cells treated with D-luciferin: a) total intensities of dose-response emission, b) time courses at 100 µM substrate. Error bars represent the standard deviation of 4 experiments.

3-9. Supplemental Figure 9

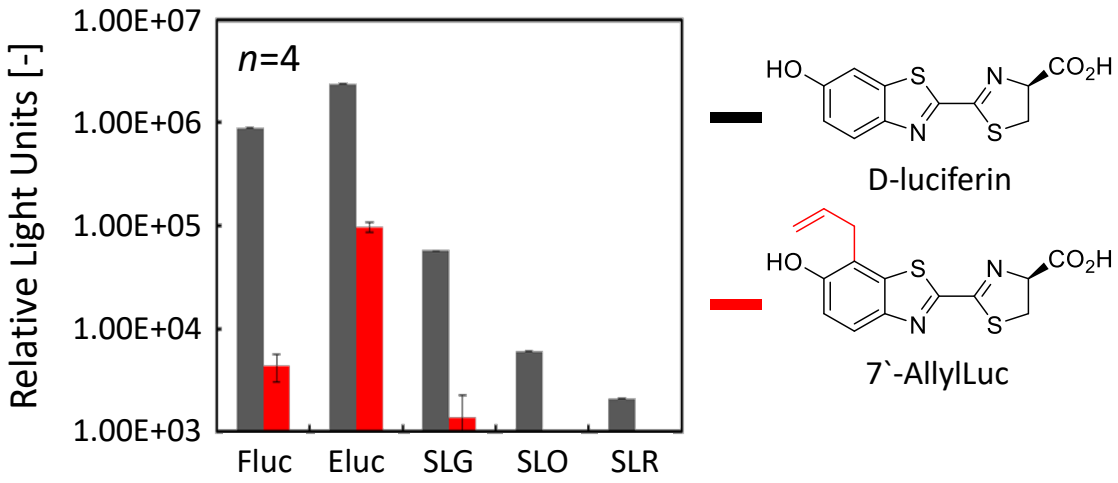


Figure S9. Light emission intensities from luciferase-expressing COS-7 cells treated with D-luciferin and 7`-AllylLuc at 500 μM substrate. Error bars represent the standard deviation of 4 experiments.

3-10. Supplemental Figure 10

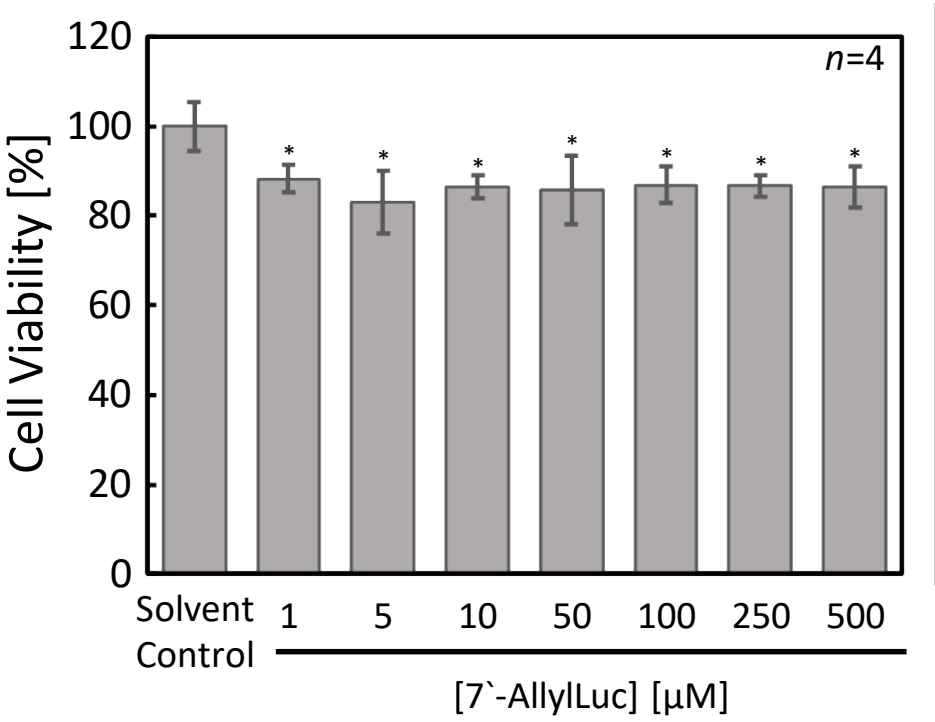


Figure S10. Cell viability evaluated by MTT assay. Cell viabilities were measured after 24 h treatment with 7`-AllylLuc at the indicated concentration. Error bars represent means ± SEM (*n*=4). *Not Significant (*t*-test)

Supplementary References

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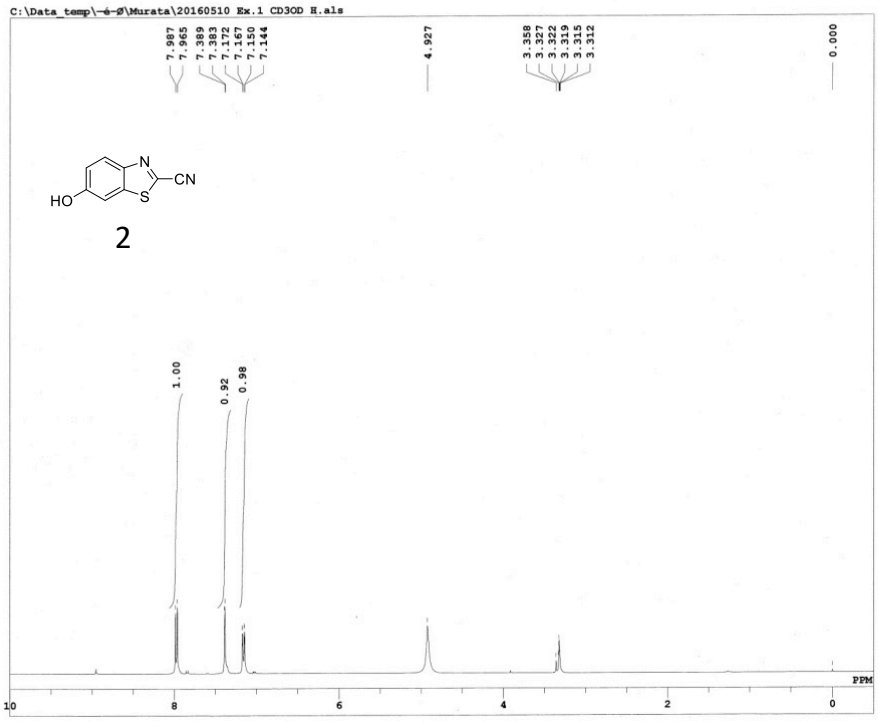
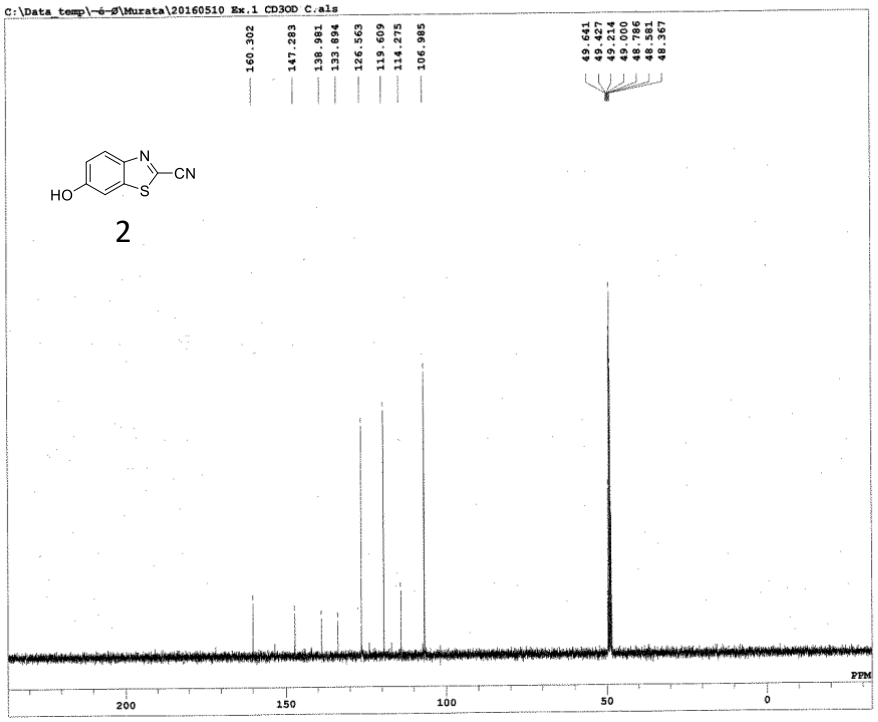
3. K. Niwa, Y. Ichino and Y. Ohmiya, Quantum yield measurements of firefly bioluminescence reactions using commercial luminometer, *Chem. Lett.*, **2010**, *39*, 291-293.
4. Y. Ando, K. Niwa, N. Yamada, T. Enomoto, T. Irie, H. Kubota, Y. Ohmiya and H. Akiyama, Firefly bioluminescence quantum yield and colour change by pH-sensitive green emission, *Nat. Photonics*, **2008**, *2*, 44-47.

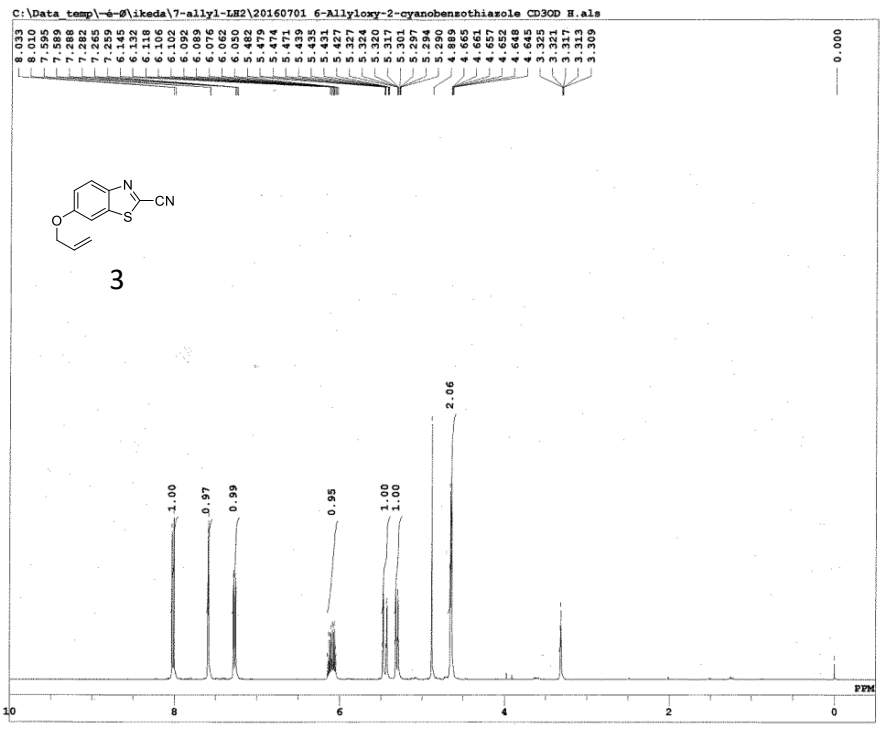
3. NMR spectra



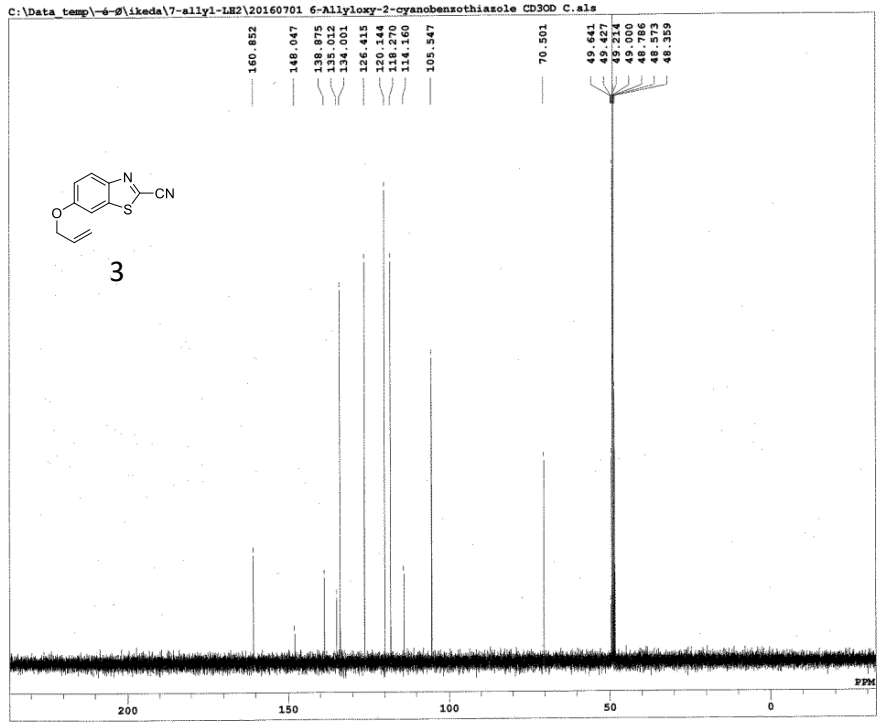

```
EXMOD   SINGL
IRMOD   NON
OBNUC   1H
OBFRQ        400.05 MHz
OBSET          0.00 KHz
PW1            5.30 usec
POINT         16384
SAMPO         16384
TIMES             8
SCANS             8
DUMMY             1
FREQU        8000.00 Hz
FILTR          4000 Hz
ACQTM        2.0480 sec
DEADT         72.35 usec
DELAY         50.00 usec
PD           2.0000 sec
ADBIT            16
RGAIN            19
IRNUC   1H
IRFRQ        400.05 MHz
IRSET          0.00 KHz
IRRPW            45 usec
IRATN           511
IRRNS             0
IRBP1            30
IRBP2             6
BF             1.20 Hz
T1             0.00
T2             0.00
T3            90.00
T4           100.00
XE          4202.64 Hz
XS           104.98 Hz
REFVL
DFILE   20160510 Ex.1 CD3OD
MENUF   non
SEMFL   TH5
LKSET          0.00 KHz
LKLEV           180
LGAIN            23
LKPHS           318
LKSIG           626
CTEMP          22.5 c
CSPED            17 Hz
FIELD           916
```



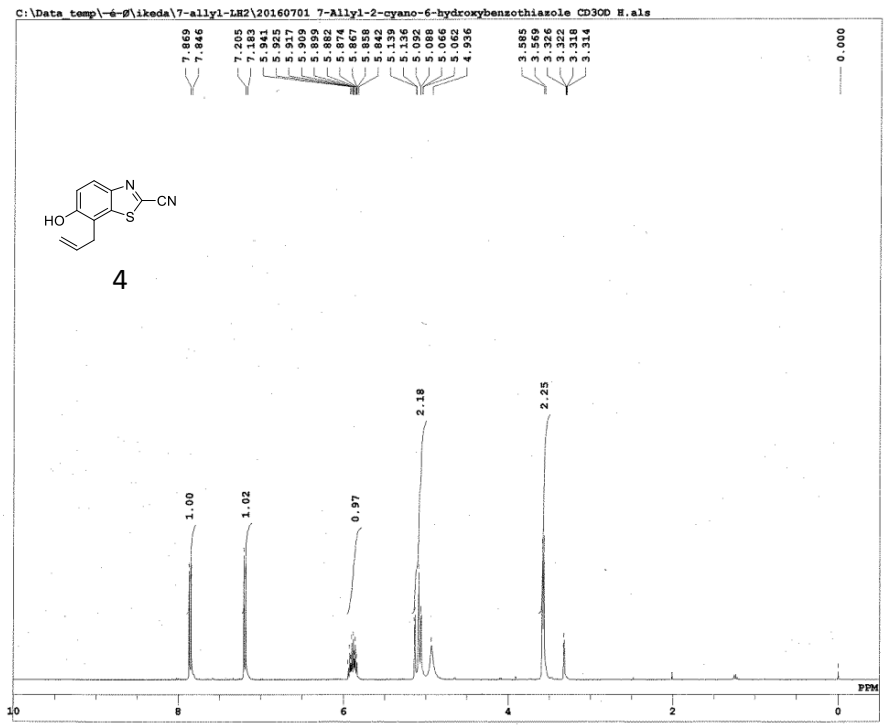

```
EXMOD   SINGL
IRMOD   BCM
OBNUC   13C
OBFRQ        100.50 MHz
OBSET          0.00 KHz
PW1            4.80 usec
POINT         32768
SAMPO         32768
TIMES           500
SCANS           235
DUMMY             1
FREQU       27100.27 Hz
FILTR         13550 Hz
ACQTM        1.2091 sec
DEADT         19.74 usec
DELAY         14.76 usec
PD           1.0000 sec
ADBIT            16
RGAIN            33
IRNUC   1H
IRFRQ        400.05 MHz
IRSET          0.00 KHz
IRRPW            45 usec
IRATN           511
IRRNS             0
IRBP1            30
IRBP2             6
BF             1.20 Hz
T1             0.00
T2             0.00
T3            90.00
T4           100.00
XE          27100.27 Hz
XS              0.00 Hz
REFVL
DFILE   20160510 Ex.1 CD3OD
MENUF   bcm
SEMFL   TH5
LKSET          0.00 KHz
LKLEV           180
LGAIN            16
LKPHS           318
LKSIG           685
CTEMP          23.2 c
CSPED            16 Hz
FIELD           764
```



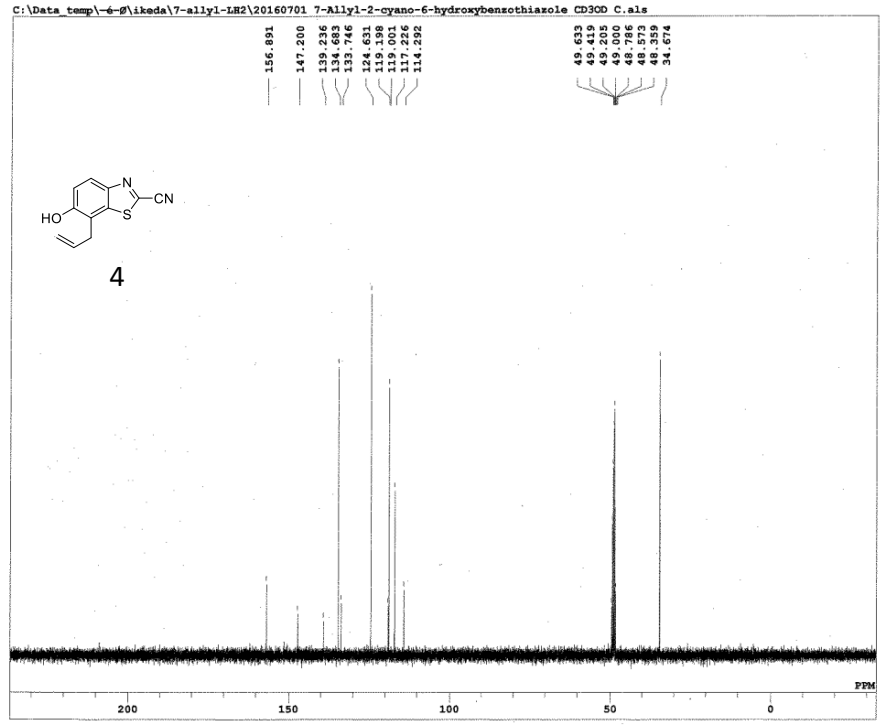

```
EXMOD   SINGL
IRMOD   NON
OBNUC   1H
OBFRQ         400.05 MHz
OBSET           0.00 KHz
PW1             5.30 usec
POINT          32768
SAMPO          32768
TIMES              8
SCANS              8
DUMMY              1
FREQU         8000.00 Hz
FILTR            4000 Hz
ACQTM          4.0960 sec
DEADT           72.35 usec
DELAY           50.00 usec
PD             2.0000 sec
ADBIT              16
RGAIN              17
IRNUC   1H
IRFRQ         400.05 MHz
IRSET           0.00 KHz
IRRFW             45 usec
IRATN             511
IRRNS               0
IRBP1              30
IRBP2               6
BF              0.10 Hz
T1              0.00
T2              0.00
T3             90.00
T4            100.00
XE           4202.15 Hz
XS            104.00 Hz
REFVL
DFILE   20160701 6-Allyloxy
MENUF   non
SHMFL   th5_201601
LKSET           0.00 KHz
LKLEV            180
LGAIN             25
LKPHS            318
LKSIG            707
CTEMP          21.4 c
CSPED            18 Hz
FIELD            959
```



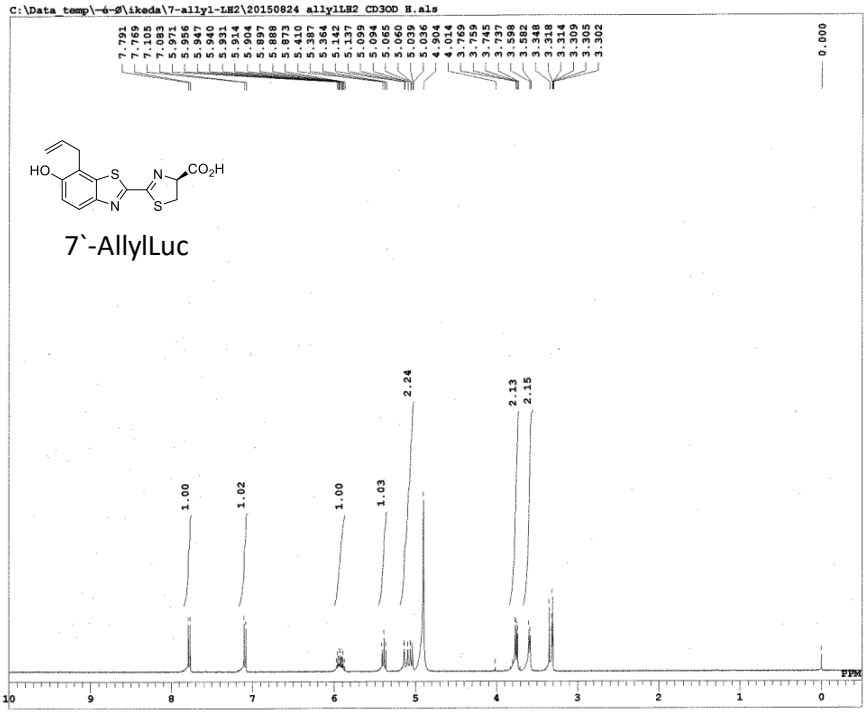

```
EXMOD   SINGL
IRMOD   BCM
OBNUC   13C
OBFRQ         100.50 MHz
OBSET           0.00 KHz
PW1             4.80 usec
POINT          32768
SAMPO          32768
TIMES            500
SCANS            151
DUMMY              1
FREQU        27100.27 Hz
FILTR           13550 Hz
ACQTM          1.2091 sec
DEADT           19.74 usec
DELAY           14.76 usec
PD             1.0000 sec
ADBIT              16
RGAIN              33
IRNUC   1H
IRFRQ         400.05 MHz
IRSET           0.00 KHz
IRRFW             45 usec
IRATN             511
IRRNS               0
IRBP1              30
IRBP2               6
BF              0.10 Hz
T1              0.00
T2              0.00
T3             90.00
T4            100.00
XE           27100.27 Hz
XS               0.00 Hz
REFVL
DFILE   20160701 6-Allyloxy
MENUF   bcm
SHMFL   th5_201601
LKSET           0.00 KHz
LKLEV            180
LGAIN             17
LKPHS            318
LKSIG            915
CTEMP          22.3 c
CSPED            13 Hz
FIELD            796
```



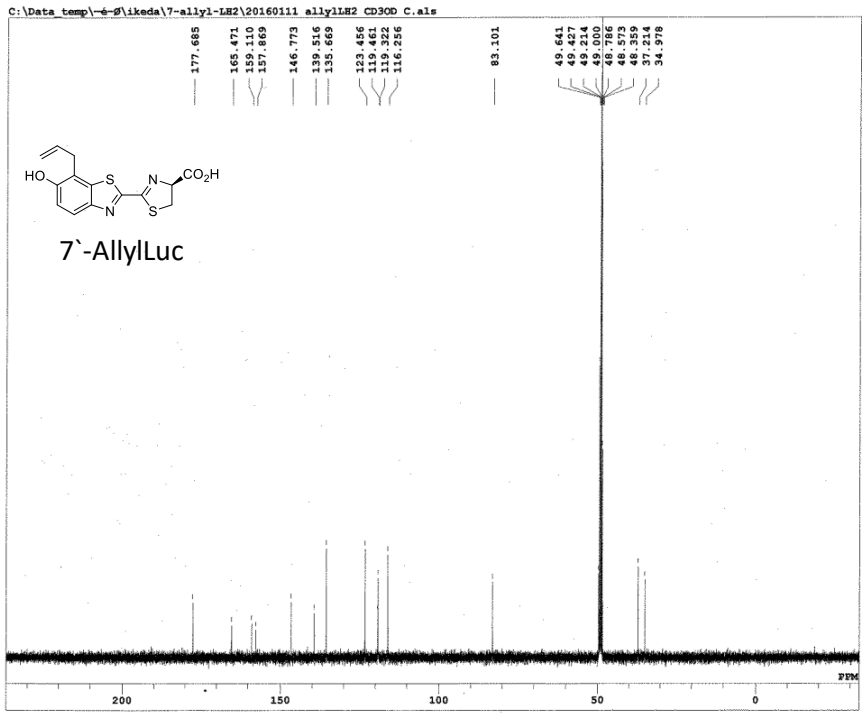

```
EXMOD   SINGL
IRMOD   NON
OBNUC   1H
OBFRQ         400.05 MHz
OBSET           0.00 KHz
PW1             5.30 usec
POINT          16384
SAMPO          16384
TIMES              8
SCANS              8
DUMMY              1
FREQU         8000.00 Hz
FILTR            4000 Hz
ACQTM          2.0480 sec
DEADT           72.35 usec
DELAY           50.00 usec
PD             2.0000 sec
ADBIT              16
RGAIN              18
IRNUC   1H
IRFRQ         400.05 MHz
IRSET           0.00 KHz
IRRPW             45 usec
IRATN            511
IRRNS              0
IRBP1             30
IRBP2              6
BF              0.10 Hz
T1              0.00
T2              0.00
T3             90.00
T4            100.00
XE           4202.64 Hz
XS            105.96 Hz
REFVL
DFILE   20160701 7-Allyl-2-
MENUF   non
SHMFL   th5_201601
LKSET           0.00 KHz
LKLEV            180
LGAIN             17
LKPHS            318
LKSIG            451
CTEMP          22.1 c
CSPED            21 Hz
FIELD            796
```



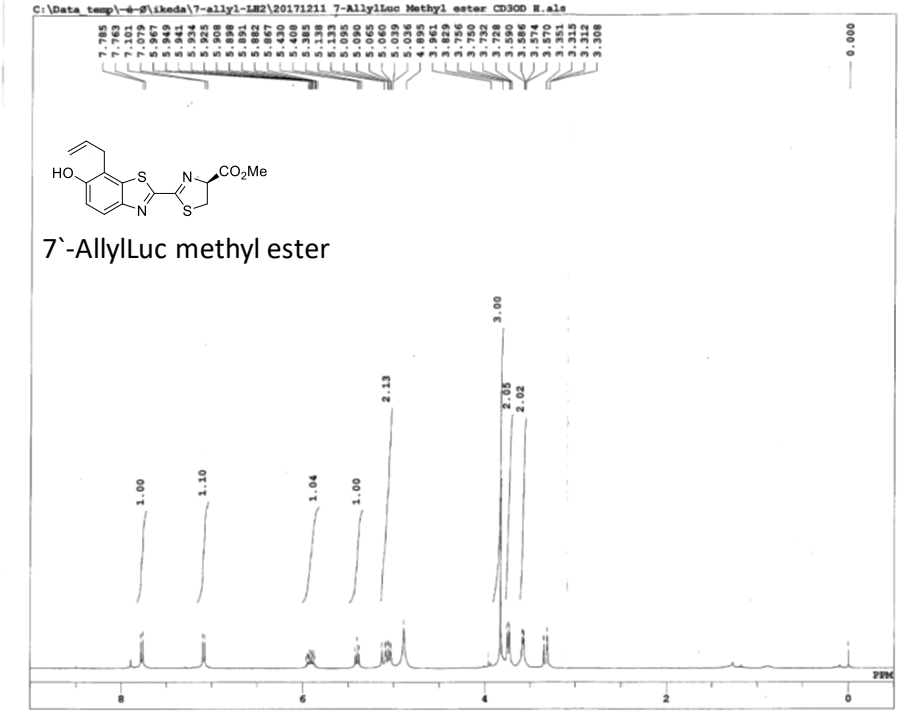

```
EXMOD   SINGL
IRMOD   BCM
OBNUC   13C
OBFRQ         100.50 MHz
OBSET           0.00 KHz
PW1             4.80 usec
POINT          32768
SAMPO          32768
TIMES            150
SCANS            150
DUMMY              1
FREQU        27100.27 Hz
FILTR           13550 Hz
ACQTM          1.2091 sec
DEADT           19.74 usec
DELAY           14.76 usec
PD             1.0000 sec
ADBIT              16
RGAIN              33
IRNUC   1H
IRFRQ         400.05 MHz
IRSET           0.00 KHz
IRRPW             45 usec
IRATN            511
IRRNS              0
IRBP1             30
IRBP2              6
BF              0.12 Hz
T1              0.00
T2              0.00
T3             90.00
T4            100.00
XE           27100.27 Hz
XS               0.00 Hz
REFVL
DFILE   20160701 7-Allyl-2-
MENUF   bcm
SHMFL   th5_201601
LKSET           0.00 KHz
LKLEV            180
LGAIN             17
LKPHS            318
LKSIG            792
CTEMP          22.5 c
CSPED            13 Hz
FIELD            800
```



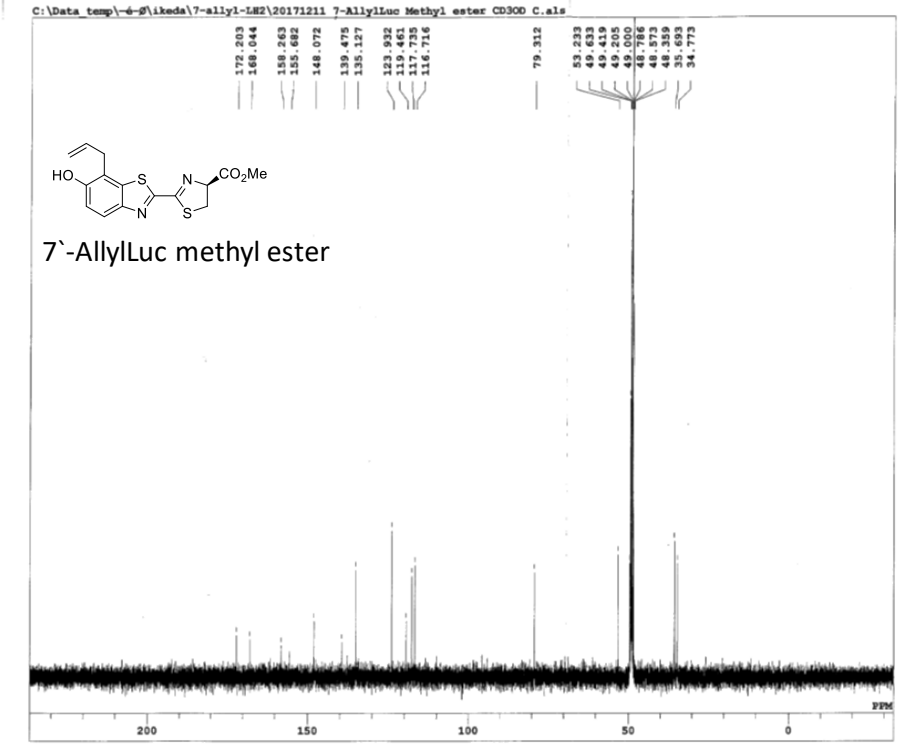

```
EXMOD   SINGL
IRMOD   NON
OBNUC   1H
OBFRQ         400.05 MHz
OBSET           0.00 KHz
PW1             5.30 usec
POINT          16384
SAMPO          16384
TIMES              8
SCANS              8
DUMMY              1
FREQU         8000.00 Hz
FILTR           4000 Hz
ACQTM          2.0480 sec
DEADT           72.35 usec
DELAY           50.00 usec
PD             2.0000 sec
ADBIT             16
RGAIN             25
IRNUC   1H
IRFRQ         400.05 MHz
IRSET           0.00 KHz
IRRFW             45 usec
IRATN            511
IRRNS              0
IRBP1             30
IRBP2              6
BF              0.12 Hz
T1              0.00
T2              0.00
T3             90.00
T4            100.00
XE           4202.64 Hz
XS            101.07 Hz
REFVL
DFILE   20150824 allylLH2 C
MENUF   non
SHMFL   TH5
LKSET           0.00 KHz
LKLEV            180
LGAIN             18
LKPHS            318
LKSIG            872
CTEMP          21.6 c
CSPED            14 Hz
FIELD            516
```




```
EXMOD   SINGL
IRMOD   BCM
OBNUC   13C
OBFRQ         100.50 MHz
OBSET           0.00 KHz
PW1             4.80 usec
POINT          32768
SAMPO          32768
TIMES           1200
SCANS           1200
DUMMY              1
FREQU        27100.27 Hz
FILTR          13550 Hz
ACQTM          1.2091 sec
DEADT           19.74 usec
DELAY           14.76 usec
PD             1.0000 sec
ADBIT             16
RGAIN             33
IRNUC   1H
IRFRQ         400.05 MHz
IRSET           0.00 KHz
IRRFW             45 usec
IRATN            511
IRRNS              0
IRBP1             30
IRBP2              6
BF              0.10 Hz
T1              0.00
T2              0.00
T3             90.00
T4            100.00
XE           27100.27 Hz
XS              0.00 Hz
REFVL
DFILE   20160111 allylLH2 C
MENUF   bcm
SHMFL   th5_201601
LKSET           0.00 KHz
LKLEV            180
LGAIN             23
LKPHS            318
LKSIG           2438
CTEMP          21.8 c
CSPED            11 Hz
FIELD            971
```




```
EXMOD   SINGL
IRMOD   NON
OBNUC   1H
OBFRQ        400.05 MHz
OBSET          0.00 KHz
PW1            5.30 usec
POINT         16384
SAMPO         16384
TIMES             8
SCANS             8
DUMMY             1
FREQU        8000.00 Hz
FILTR        4000 Hz
ACQTM          2.0480 sec
DEADT         72.35 usec
DELAY         50.00 usec
PD             2.0000 sec
ADBIT             16
RGAIN             19
IRNUC   1H
IRFRQ        400.05 MHz
IRSET          0.00 KHz
IRRPW            45 usec
IRATN           511
IRRNS             0
IRBP1            30
IRBP2             6
BF             0.12 Hz
T1             0.00
T2             0.00
T3            90.00
T4           100.00
XE          3802.25 Hz
XS           302.25 Hz
REFVL
DFILE   20171211 7-AllylLu
MENUF   non
SHMFL   th5_201703
LKSET          0.00 KHz
LKLEV           180
LGAIN            20
LKPHS           311
LKSIG           700
CTEMP         21.3 c
CSPED            14 Hz
FIELD          1214
```




```
EXMOD   SINGL
IRMOD   BCM
OBNUC   13C
OBFRQ        100.50 MHz
OBSET          0.00 KHz
PW1            4.80 usec
POINT         32768
SAMPO         32768
TIMES          1500
SCANS           339
DUMMY             1
FREQU       27100.27 Hz
FILTR         13550 Hz
ACQTM          1.2091 sec
DEADT         19.74 usec
DELAY         14.76 usec
PD             1.0000 sec
ADBIT             16
RGAIN             33
IRNUC   1H
IRFRQ        400.05 MHz
IRSET          0.00 KHz
IRRPW            45 usec
IRATN           511
IRRNS             0
IRBP1            30
IRBP2             6
BF             0.10 Hz
T1             0.00
T2             0.00
T3            90.00
T4           100.00
XE          27100.27 Hz
XS              0.00 Hz
REFVL
DFILE   20171211 7-AllylLuc
MENUF   bcm
SHMFL   th5_201703
LKSET          0.00 KHz
LKLEV           180
LGAIN            20
LKPHS           311
LKSIG          1048
CTEMP         22.3 c
CSPED            15 Hz
FIELD          1214
```