Electronic Supplementary Material (ESI) for ChemComm.

Electronic Supplementary Information

Intracellular synthesis of $_{D}$-aminoluciferin for bioluminescence generation

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Contents:

1. General methods

All the starting materials were obtained from Adamas or BaoMan Inc. (Shanghai). Commercially available reagents were used without further purification, unless noted otherwise. 2-cyano-6-aminobenzothiazole (CBT) was obtained from Shanghai Chemical Pharm-Intermediate Tech. Co.. All chemicals were reagent grade or better. The electrospray ionization time-of-flight mass (ESI-TOF-MS) spectra were obtained on an Exactive Plus (Thermo Fisher Scientific, CA, USA) mass spectrometer. MALDI-TOF/TOF mass spectra were obtained on a time-of-flight Ultrflex II mass spectrometer (Bruker Daltonics). The spectra of electrospray ionization-mass spectrometry (ESI-MS) were recorded on a LCQ Advantage MAX ion trap mass spectrometer (Thermo Fisher). HPLC analyses were performed on an Agilent 1200 HPLC system equipped with a G1322A pump and in-line diode array UV detector using an Agilent Zorbax 300SB - C18 RP column with CH_3CN (0.1% of trifluoroacetic acid (TFA)) and water (0.1% of TFA) as the eluent. ^1H NMR spectra were obtained on a Bruker AV-300 MHz spectrometer. UV-vis absorption spectra were recorded on a Perkin-Elmer lambda 25 spectrophotometer. Dynamic light scattering (DLS) was measured on a Zeta Sizer Nano Series (Malvern Instruments). Fluorescence microscopic images were taken under a fluorescence microscope OLMPUS IX71.

Cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM, Hycolon) supplemented with 10 % fetal bovine serum at 37 °C, 5% CO_2, and humid atmosphere.

Five-week-old (weighting 20 g) BALB/c nude mice were used for animal experiments. All animals received care in compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals. The procedures were approved by the University of Science

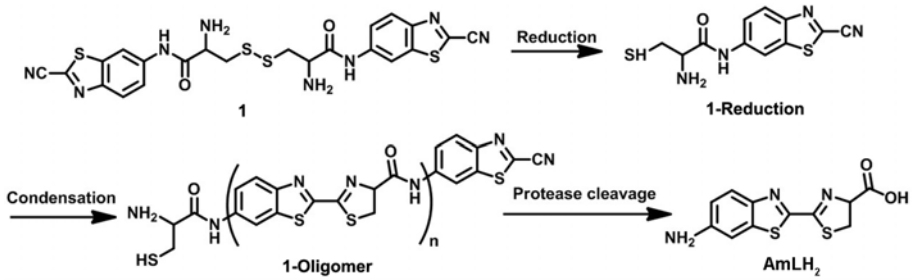
and Technology of China Animal Care and Use Committee.

MTT assay

The cytotoxicity was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay with MDA-MB-231 cells. Cells growing in log phase were seeded into 96-well cell culture plate at 3×10^3/well. The cells were incubated for 24 h at 37 °C under 5% CO_2. The solutions of $_D$-**1** or $_L$-**1** (100 μL/well) at concentrations of 12.5, 25, 50, or 100 μM in 100 μL of medium were added to the wells, respectively. The cells were incubated for 1, 2, or 3 day at 37 °C under 5% CO_2. Ten μL solution of 5 mg/mL MTT dissolved in PBS buffer (pH 7.4) was added to each well of the 96-well plate. Then 100 μL DMSO was added to each well to dissolve the formazan after an additional 4 h of incubation. The data were obtained using an enzyme-linked immunosorbent assay (ELISA) reader (VARIOSKAN FLASH) to detect its absorption at 490 nm. The following formula was used to calculate the viability of cell growth: viability (%) = (mean of absorbance value of treatment group/mean of absorbance value of control) × 100.

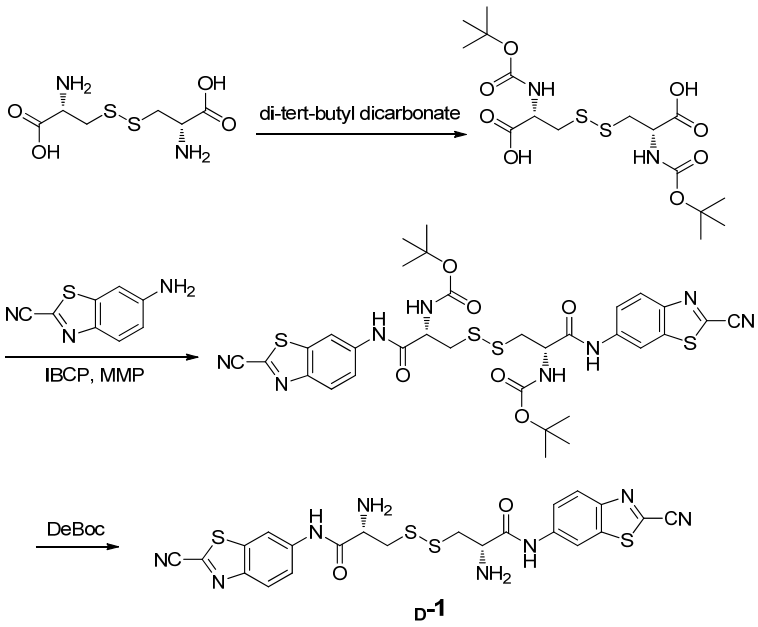
2. Syntheses and Characterizations of $_D$-1 or $_L$-1

Scheme S1. Schematic illustration of reduction-controlled condensation to form linear oligomer and subsequent proteolysis of the oligomer to yield aminoluciferin.



*Preparation of CBT-$_D$-Cystine-CBT ($_D$-**1**) and CBT-$_L$-Cystine-CBT ($_L$-**1**) (CBT = 2-cyano-6-aminobenzothiazole).*

Scheme S2. Synthetic route for $_D$-**1**.



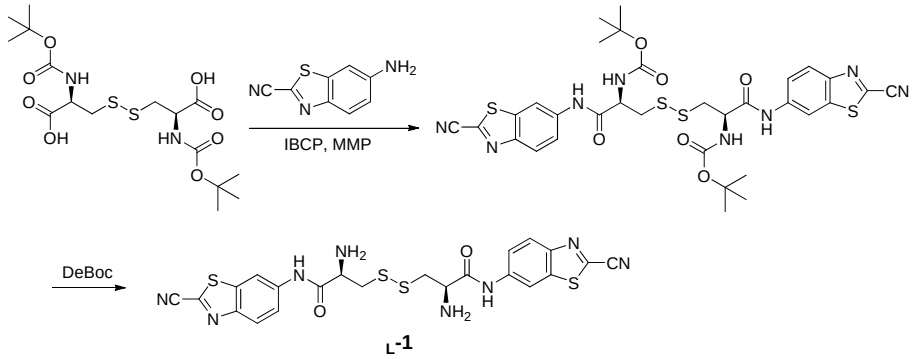
*Synthesis of $_D$-**1***:

$_D$-Cystine (1 g, 4.16 mmol) was dissolve in 9:1 water: tetrahydrofuran (10 mL). 6 M NaOH in water was added drop wise until pH 10 was reached. Then di-tert-butyl dicarbonate (2.4 g, 11.2 mmol) was added drop wise into that solution. The reaction mixture was stirred for 24 hrs. Then the reaction mixture acidified by drop wise addition of 2 (N) HCl with stirring until

the solution reached pH 2. The solids were extracted with ethyl acetate (3 × 20 mL) and the combined organic layer washed with pH 2 water (2 × 10 mL) followed by brine solution and dried over sodium sulphate. Solution was filtered and concentrated under reduced pressure to get a white solid compound. The white solids were washed with hexane for several times to get completely pure N-Boc-$_D$-Cystine (1.6 g, yield 89%).

The isobutyl chloroformate (IBCF, 27.8 mg, 0.2 mmol) was added to a mixture of N-Boc-$_D$-Cystine (44 mg, 0.1 mmol) and 4-methylmorpholine (MMP, 20.2 mg, 0.2 mmol) in THF (4.0 mL) at 0 °C and the reaction mixture was stirred for 30 min. 2-cyano-6-aminobenzothiazole (CBT, 35.0 mg, 0.2 mmol) was added to the reaction mixture and further stirred for 2 h at 0 °C then overnight at room temperature. The pure product was obtained after HPLC purification. The Boc protecting groups were then cleaved with 95% TFA in CH_2Cl_2 for 3 h at room temperature in presence of Triisopropylsilane (TIPS, 1.6 mg, 10 μmol) to yield the final product $_D$-**1** (15 mg, total yield 27.3%) which was purified with HPLC. ^{1}H NMR of $_D$-**1** (d_6-DMSO, 300 MHz) δ (ppm): 8.59 (d, J = 1.8 Hz, 2H), 8.23 (d, J = 9.0 Hz, 2H), 7.77 (dd, J_1 = 9.0 Hz, J_2 = 9.0 Hz, 2H), 4.32 (s, 2H), 3.32 (m, 4H) (Figure S1). ^{13}C NMR of $_D$-**1** (75 MHz, DMSO) δ (ppm): 166.2, 148.1, 138.2, 136.6, 135.8, 124.9, 120.9, 113.4, 112.1, 52.1, 38.0 (Figure S2). MS: calculated for $_D$-**1** ($C_{22}H_{19}N_8O_2S_4$) [$(M+H)^+$]: 555.0514; obsvd. HR-ESI-TOF/MS: *m/z* 555.0516 (Figure S3).

Scheme S2. Synthetic route for $_L$-**1**.



$_L$-**1**

*Synthesis of $_L$-**1***:

The isobutyl chloroformate (IBCF, 27.8 mg, 0.2 mmol) was added to a mixture of N-Boc-$_L$-Cystine (44 mg, 0.1 mmol) and 4-methylmorpholine (MMP, 20.2 mg, 0.2 mmol) in THF (4.0 mL) at 0 °C and the reaction mixture was stirred for 30 min. 2-cyano-6-aminobenzothiazole (CBT, 35.0 mg, 0.2 mmol) was added to the reaction mixture and further stirred for 2 h at 0 °C then overnight at room temperature. The pure product was obtained after HPLC purification. The Boc protecting groups were then cleaved with 95% TFA in CH_2Cl_2 for 3 h at room temperature in presence of Triisopropylsilane (TIPS, 1.6 mg, 10 μmol) to yield the final product $_L$-**1** (20 mg, total yield 36.4%) which was purified by HPLC. ^{1}H NMR of $_L$-**1** (d_6-DMSO, 300 MHz) δ (ppm): 8.59 (d, J = 1.8 Hz, 2H), 8.23 (d, J = 9.0 Hz, 2H), 7.77 (dd, J_1 = 9.0 Hz, J_2 = 9.0 Hz, 2H), 4.32 (s, 2H), 3.32 (m, 4H) (Figure S4). ^{13}C NMR of $_L$-**1** (75 MHz, DMSO) δ (ppm): 166.2, 148.1, 138.2, 136.6, 135.9, 125.0, 120.9, 113.4, 112.2, 52.1, 38.0 (Figure S5). MS: calculated for $_L$-**1** ($C_{22}H_{19}N_8O_2S_4$) [$(M+H)^+$]: 555.0514; obsvd. HR-ESI-TOF/MS: *m/z* 555.0517 (Figure S6).

Synthesis of $_D$-aminoluciferin:

$_D$-cysteine hydrochloride monohydrate (30.0 mg, 0.171 mM) and CBT (28.7 mg, 0.16 mM) were suspended in MeOH : H_2O (2: 1, v/v, 10 mL) in a 20 mL vial. Potassium carbonate (22.7

mg, 0.16 mM) was then added to the mixture, and the resulting bright yellow-green solution was stirred under N_2 for 20 min. Upon consumption of CBT as evidenced by thin layer chromatograph analysis, the methanol was removed in vacuo and the remaining aqueous solution was acidified to pH 3 with 1 M HCl, and then D-aminoluciferin precipitated. Pure product was obtained after filtration and washing with water in high yield. ^{1}H NMR of D-aminoluciferin (300 MHz, CD_3OD) δ (ppm): 7.87 (d, J = 8.9 Hz, 1 H), 7.24 (d, J = 2.0 Hz, 1 H), 7.06 (dd, J_1 = 8.9 Hz, J_2 = 2.0 Hz, 1 H), 5.47 (t, J_1 = 9.0 Hz, 1 H), 3.82 (m, 2 H) (Figure S6).

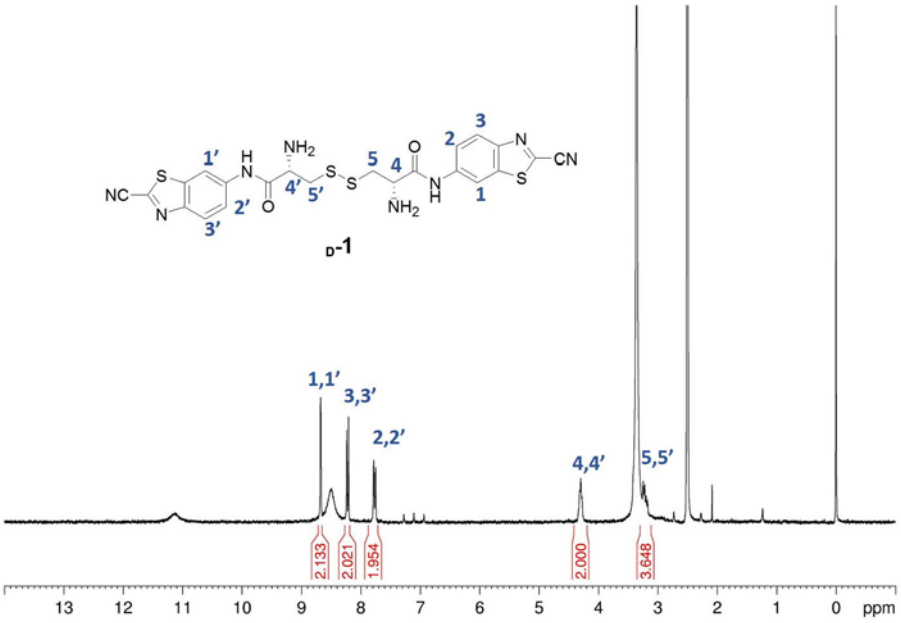


Figure S1. ^{1}H NMR spectrum of D-**1** in d_6-DMSO.

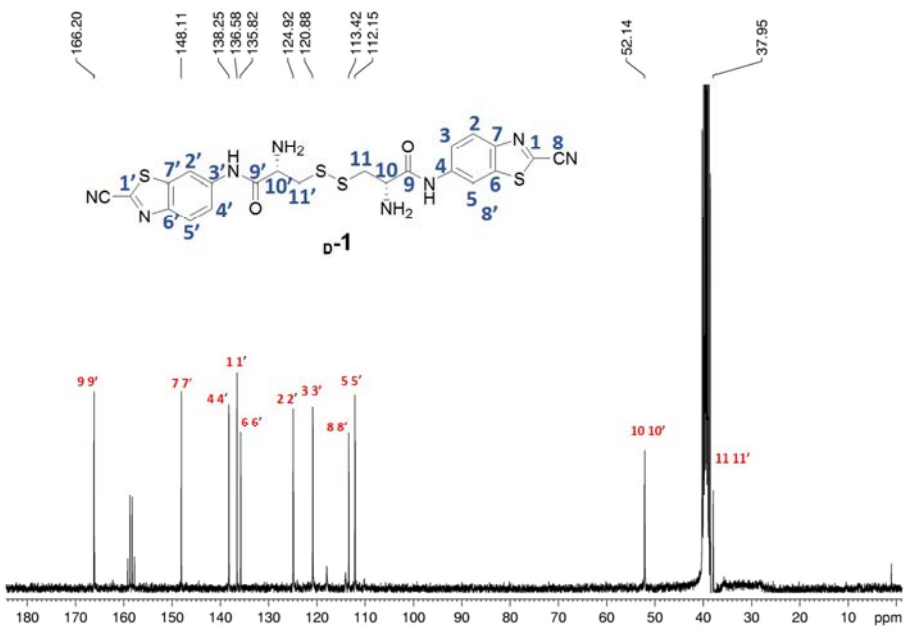


Figure S2. ^{13}C NMR spectrum of $_{D}$-**1** in d_6-DMSO.

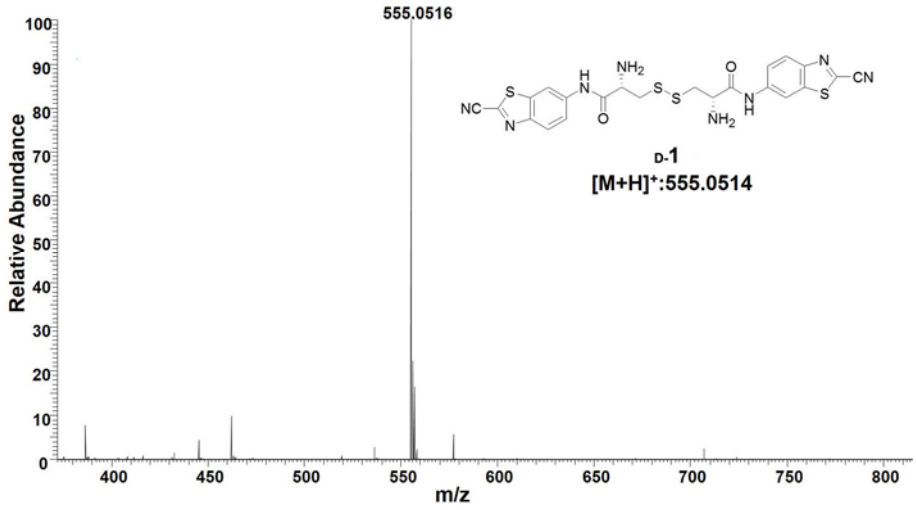


Figure S3. ESI-TOF/MS spectrum of $_{D}$-**1**.

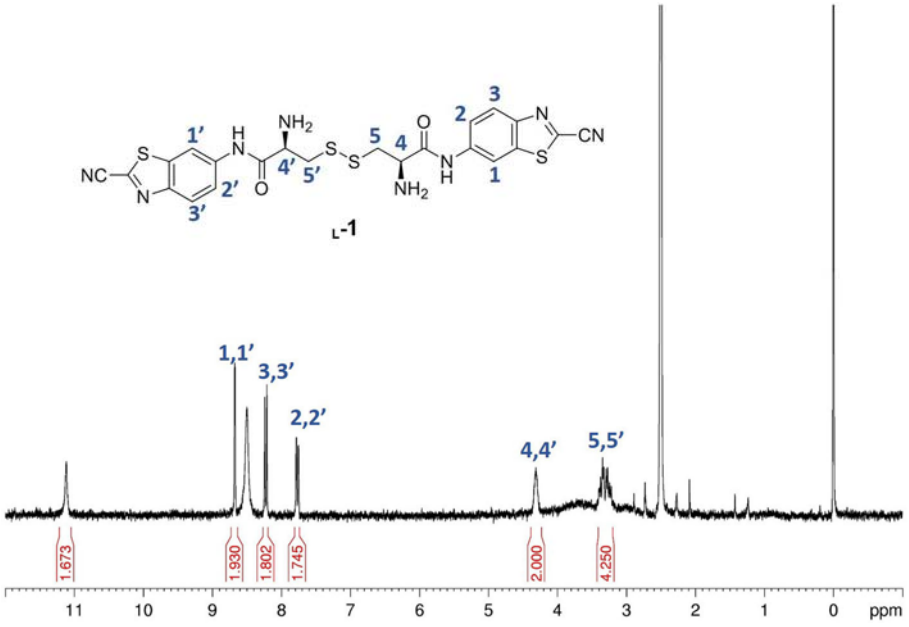


Figure S4. ^{1}H NMR spectrum of $_{L}$-**1** in d_6-DMSO.

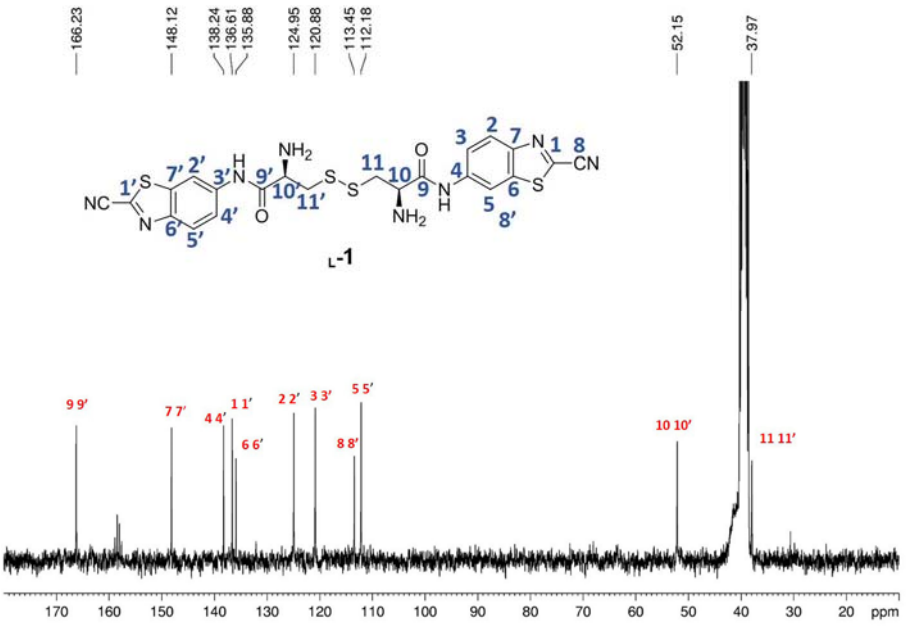


Figure S5. ^{13}C NMR spectrum of $_{L}$-**1** in d_6-DMSO.

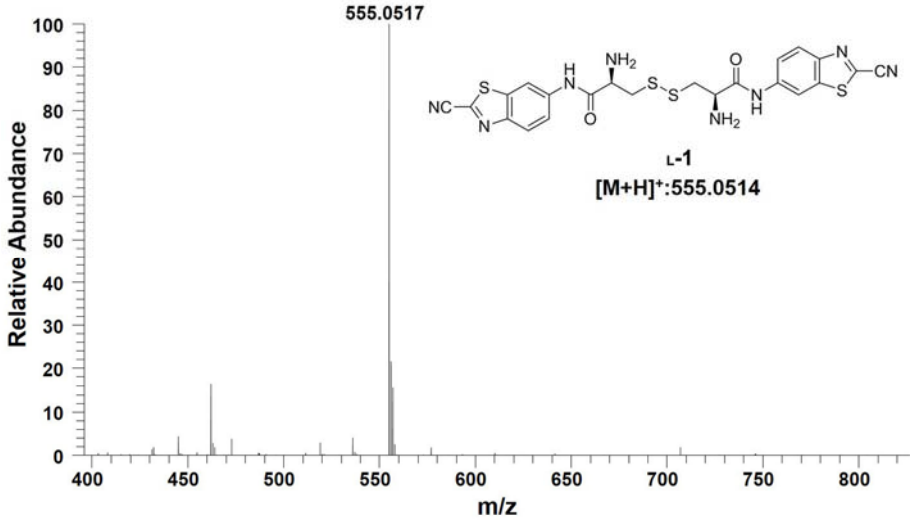


Figure S6. ESI-TOF/MS spectrum of $_{L}$-**1**.

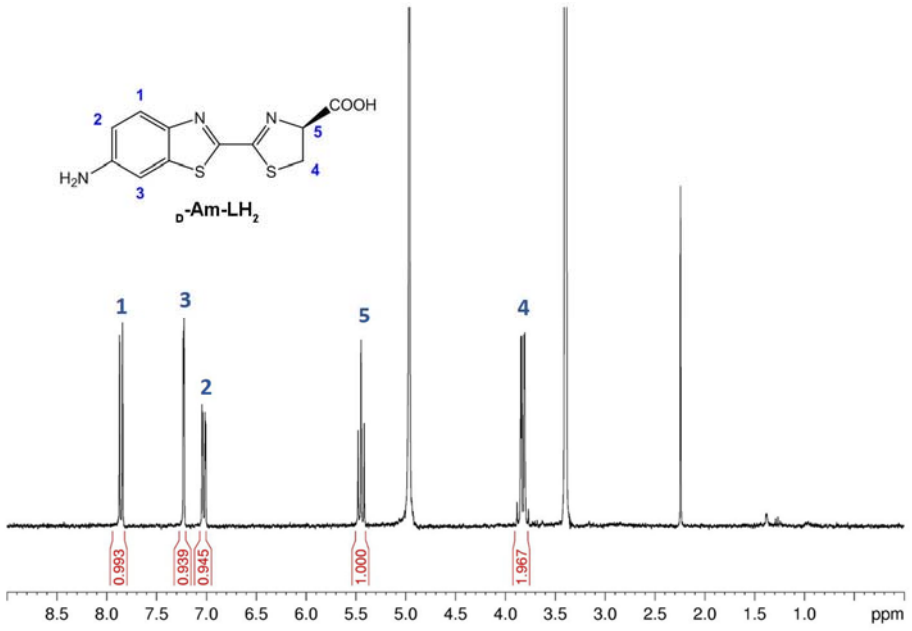


Figure S7. ^{1}H NMR spectrum of $_{D}$-aminoluciferin in CD_3OD.

3. Supporting figures and tables

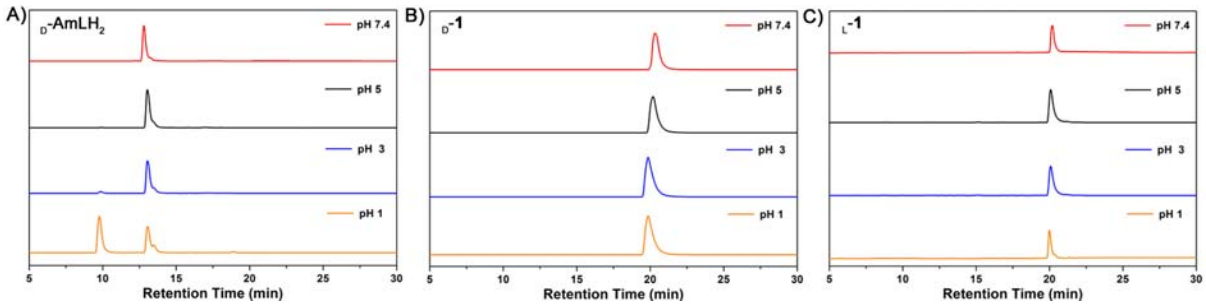


Figure S8. HPLC traces of $_{D}$-AmLH$_2$ (A), $_{D}$-**1** (B), or $_{L}$-**1** (C) in phosphate buffer at 37 °C for 1 h at pH 7.4 (red), pH 5 (black), pH 3 (blue), or pH 1 (orange). Wavelength for detection: 320 nm.

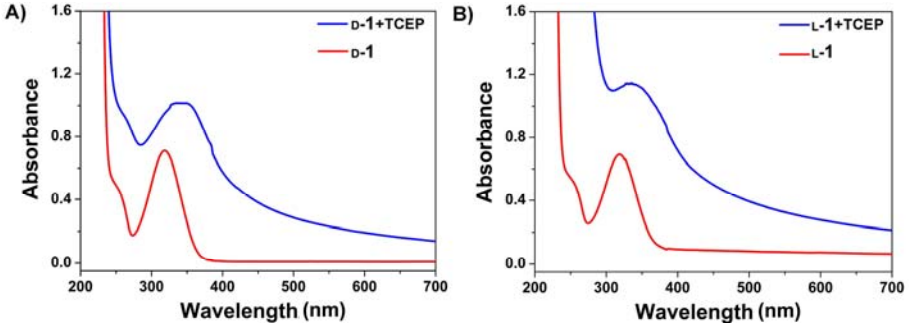


Figure S9. A) UV-Vis absorption spectra of 50 μM $_{D}$-**1** before (red) and after (blue) incubation with 1 mM TCEP at 37 °C for 0.5 h. B) UV-Vis absorption spectra of 50 μM $_{L}$-**1** before (red) and after (blue) incubation with 1 mM TCEP at 37 °C for 0.5 h. Note that the same phosphate buffer (0.2 M phosphate buffer at pH 7.4) to dissolve $_{L}$-**1** and $_{D}$-**1** was used as the reagent blank.

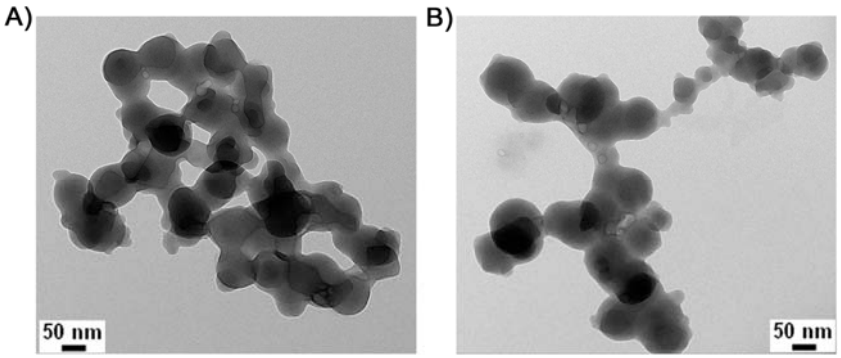


Figure S10. TEM images for 50 μM $_{D}$-**1** (A) or $_{L}$-**1** (B) after incubation with 1 mM TCEP at 37 °C for 0.5 h.

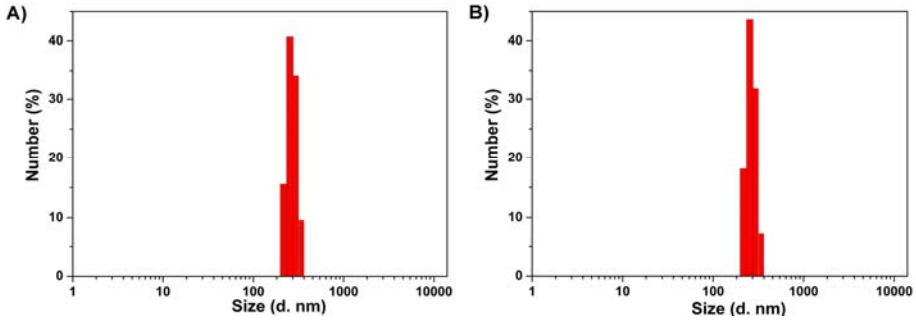


Figure S11. Dynamic light scattering measurements for 50 μM $_{D}$-**1** (A) or $_{L}$-**1** (B) after incubation with 1 mM TCEP at 37 °C for 0.5 h.

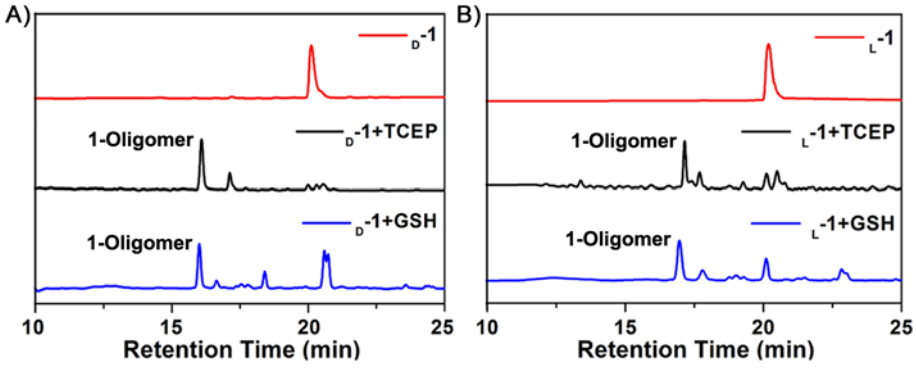


Figure S12. A) HPLC traces of $_{D}$-**1** (red), 50 μM $_{D}$-**1** incubated with 1 mM TCEP at 37 °C for 0.5 h (black), 50 μM $_{D}$-**1** incubated with 1 mM GSH at 37 °C for 0.5 h (blue). Wavelength for detection: 320 nm. B) HPLC traces of $_{L}$-**1** (red), 50 μM $_{L}$-**1** incubated with 1 mM TCEP at

37 °C for 0.5 h (black), 50 μM $_{L}$-**1** incubated with 1 mM GSH at 37 °C for 0.5 h (blue). Wavelength for detection: 320 nm.

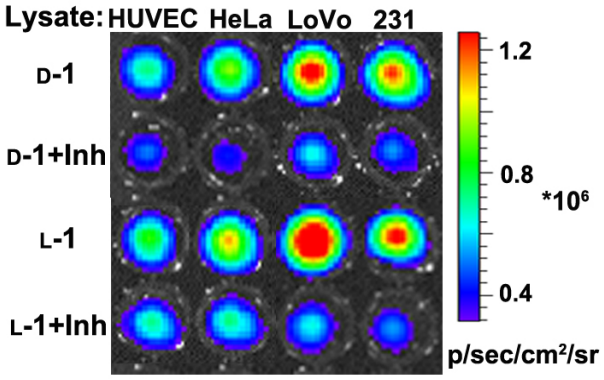


Figure S13. Bioluminescence images of the $_{D}$-**1-Oligomer** (top row) or $_{L}$-**1-Oligomer** (third row) incubated with the lysates of different cell lines, and $_{D}$-**1-Oligomer** incubated with protease inhibitor phenylmethanesulfonyl fluoride (PMSF) and cell lysate (second row) or $_{L}$-**1-Oligomer** co-incubated with protease inhibitor PMSF and cell lysate (bottom row) at 37 °C for 6 h, followed by the addition of 1 μM fLuc into the mixtures.

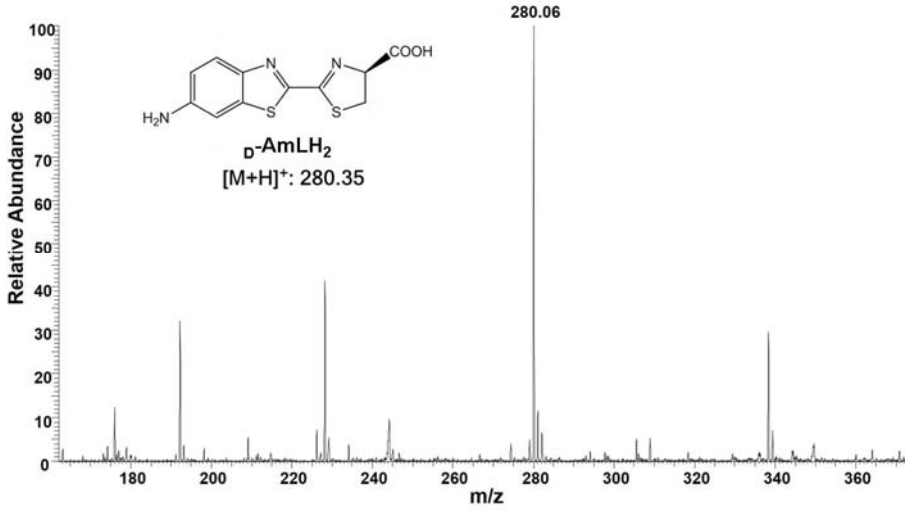


Figure S14. ESI-MS spectrum of the HPLC peak at retention time of 12.1 min in the blue trace in Figure 2B, confirming the formation of $_{D}$-aminoluciferin ($_{D}$-$AmLH_2$).

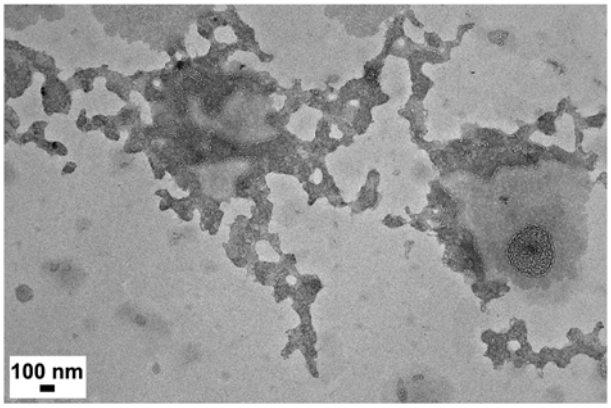


Figure S15. TEM image of the lysate of MDA-MB-231 cells after incubation with 50 μM $_{D}$-**1** at 37 °C for 0.5 h.

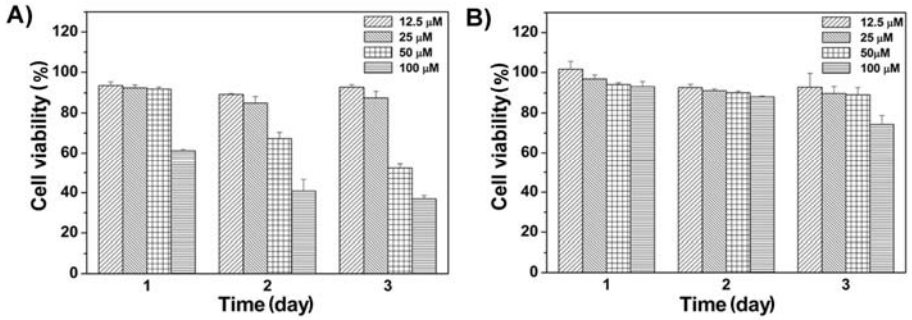


Figure S16. MTT assay of $_{D}$-**1** (A) and $_{L}$-**1** (B) on normal MDA-MB-231 cells (no luciferase-transfected). The experiments were performed in triplicate. Results are representative of three independent experiments. Error bars represent standard deviations.

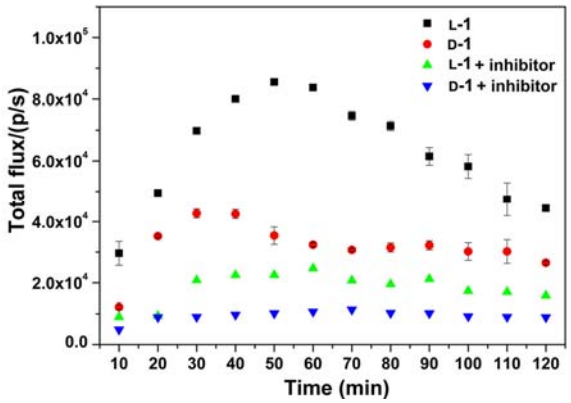


Figure S17. Quantified total photon output for cell image in Figure 3A. Each error bar represents the standard deviation of three independent experiments.

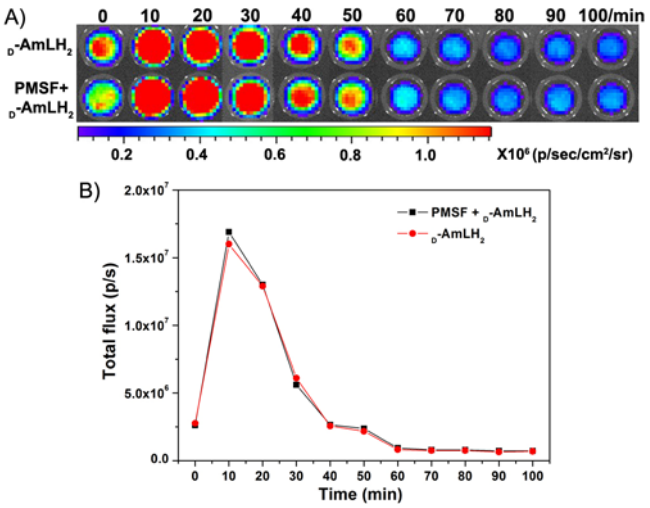


Figure 18. A) Time-course bioluminescence images of fLuc-transfected MDA-MB-231 cells incubated with 25 μM $_D$-AmLH$_2$ (top row), or pretreated with 500 μM protease inhibitor PMSF then 25 μM $_D$-AmLH$_2$ (bottom row) acquired at 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 min in serum-free culture medium at 37 °C. B) Quantified total photon output for the cell images in (A).

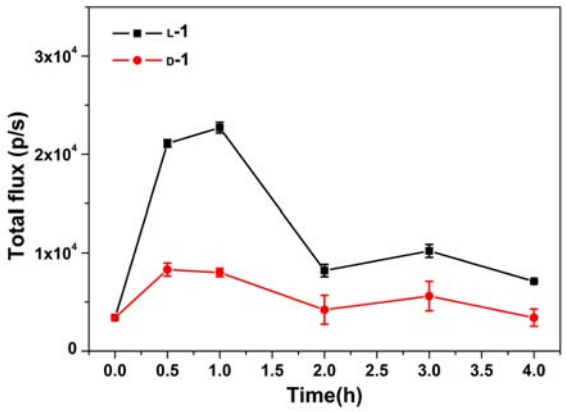


Figure S19. Quantified total photon output for mice image in Figure 3B. Each error bar represents the standard deviation of three independent experiments.

Table S1. HPLC condition for the HPLC traces in Figures 2, Figure S8 and Figure S12.

Time (min)	Flow (mL/min)	H_2O% (0.1% TFA)	CH_3CN% (0.1% TFA)
0	3.0	90	10
3	3.0	90	10
35	3.0	30	70
37	3.0	30	70
38	3.0	90	10
40	3.0	90	10