Supporting Information

Intracellular Self-Assembly of Cyclic $_D$-Luciferin Nanoparticles for Persistent Bioluminescence Imaging of Fatty Acid Amide Hydrolase

Yue Yuan,[†,§] Fuqiang Wang,[‡,§] Wei Tang,[†] Zhanling Ding,[†] Lin Wang,[⊥] Lili Liang,[†] Zhen Zheng,[†] Huafeng Zhang,[⊥] and Gaolin Liang[†,*]

[†]CAS Key Laboratory of Soft Matter Chemistry, Hefei Science Center CAS, Department of Chemistry, University of Science and Technology of China, 96 Jinzhai Road, Hefei, Anhui 230026, China

[‡]State Key Laboratory of Reproductive Medicine, Analysis Center, Nanjing Medical University, Nanjing, Jiangsu 210093, China

[⊥]School of Life Sciences, University of Science and Technology of China, Hefei, Anhui 230027, China

[§]These authors contributed equally.

Correspondence and requests for materials should be addressed to e-mail: G. L. (e-mail: gliang@ustc.edu.cn).

Contents

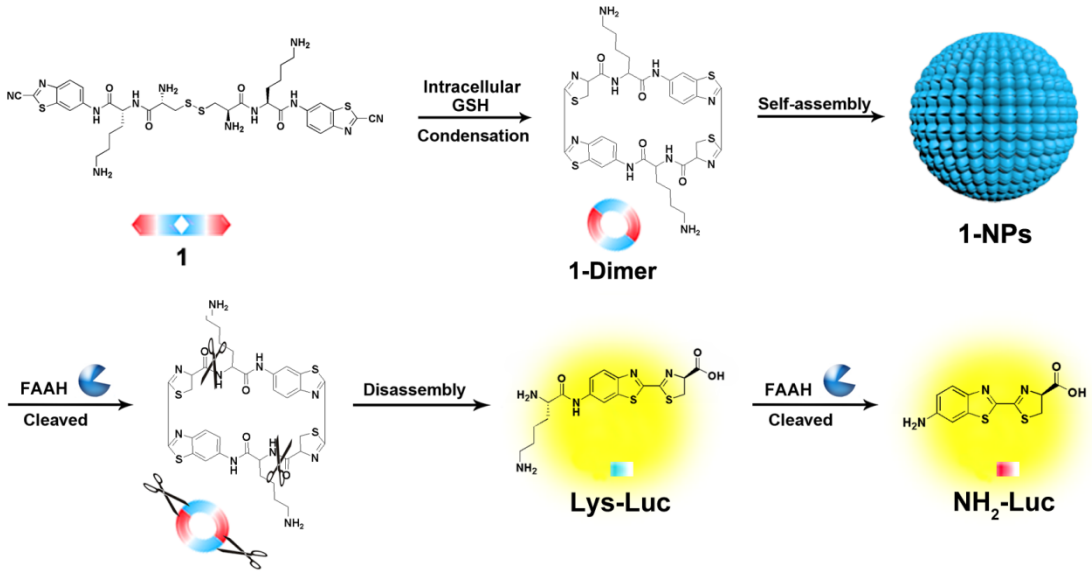
1. General methods

All the starting materials were obtained from Sigma-Aldrich, Adamas, or Sangon Biotech. Commercially available reagents were used without further purification, unless noted otherwise. All chemicals were reagent grade or better. Ultrapure water (18.2 MΩ. cm) was used throughout the experiment. Firefly luciferase plasmid was purchased from Promega, the expression and purification protocol of firefly luciferase was according to the literature method (Mofford, D. M., Adams, S. T., Reddy, G. S. K. K., Reddy, G. R., Miller, S. C. Luciferin amides enable *in vivo* bioluminescence detection of endogenous fatty acid amide hydrolase activity. *J. Am. Chem. Soc.* 2015, 137, 8684-8687). MALDI-TOF/TOF mass spectra were obtained on an UltrafleXtreme MALDI-TOF mass spectrometer from Bruker Daltonics. 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. UV-vis absorption spectra were recorded by a Pekin-Elmer lambda 25 spectrophotometer. Bioluminescence images were obtained from xenogen IVIS Lumina II small animal imaging system (Caliper Life Sciences). 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. 5 weeks 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.

2. Syntheses and Characterizations of 1, AMA, Lys-Luc, NH_2-Luc, and 1-Alexa 488

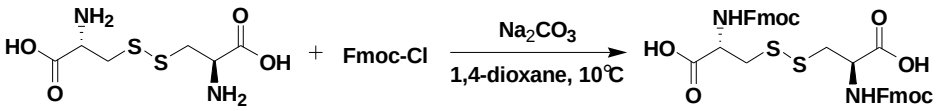
Scheme S1. Detailed schematic illustration of GSH-controlled self-assembly and FAAH-instructed disassembly of cyclic $_D$-luciferin-based **1-NPs** for persistent bioluminescence imaging of FAAH.



2-cyano-6-aminobenzothiazole (CBT) was synthesized following the literature method (White, E. H., Worther, H., Seliger, H. H., McElroy, W. D. Amino analogs of firefly luciferin and biological activity thereof. *J. Am. Chem. Soc.* 1966, 88, 2015-2019).

Preparation of Fmoc-$_D$-Cystine:

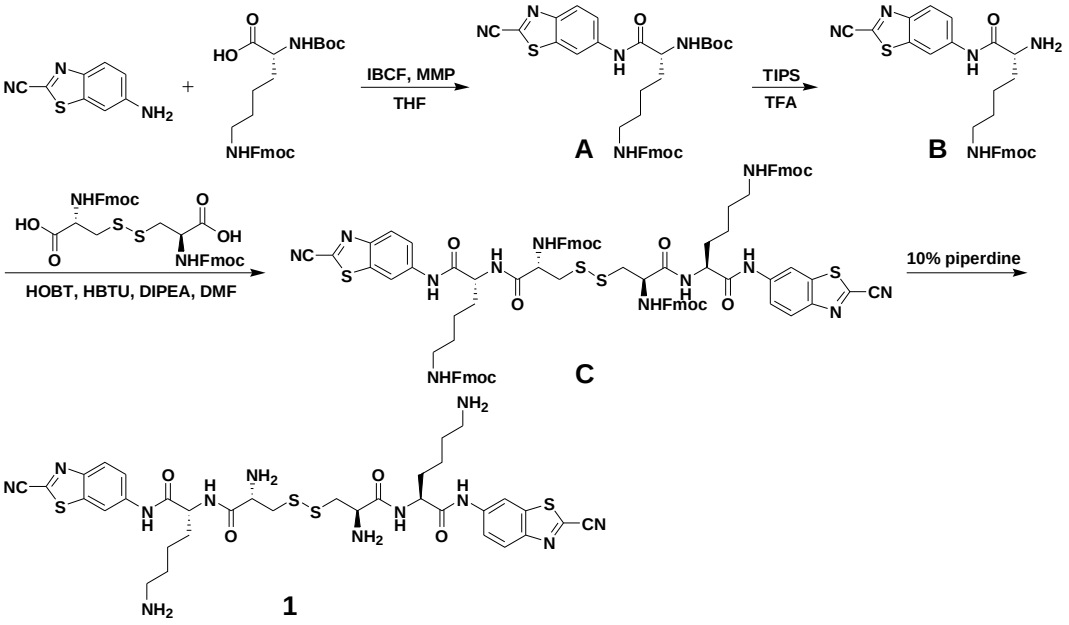
Scheme S2. The synthetic route for Fmoc-$_D$-Cystine.



$_D$-Cystine (500 mg, 2.08 mmol) and Na_2CO_3 (460 mg, 4.36 mmol) were dissolved in 30 mL H_2O at 10 °C, then Fmoc-Cl (1185 mg, 4.58 mmol, dissolved in 12 mL 1,4-dioxane) was added dropwise to the reaction mixture and further stirred for 2 h at 10 °C then overnight at room temperature. The pure product Fmoc-$_D$-Cystine (1026 mg, 72%) was obtained after the precipitates were washed with H_2O and ether for three times, respectively. MS: calc. $C_{36}H_{31}N_2O_8S_2$ $[M\text{-}H]^-$ = 683.15, obsvd. ESI-MS: *m*/*z* 683.06 (Figure S1).

Preparation of ($_D$-Cys-Lys-CBT)$_2$ (**1**):

Scheme S3. The synthetic route for **1**.



*Synthesis of **A***: The isobutyl chloroformate (IBCF, 136 mg, 1.0 mmol) was added to a mixture of Boc-Lys(Fmoc)-OH (468 mg, 1.0 mmol) and 4-methylmorpholine (MMP, 101 mg, 1.0 mmol) in THF (8.0 mL) at 0 °C and the reaction mixture was stirred for 30 min. 2-cyano-6-aminobenzothiazole (CBT, 175 mg, 1.0 mmol) was added to the

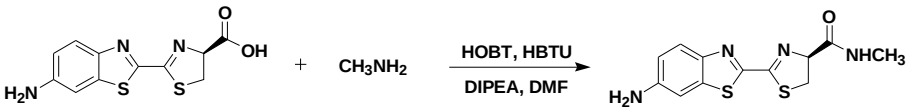
reaction mixture and further stirred for 2 h at 0 °C then overnight at room temperature. The pure product **A** (531 mg, 85%) was obtained after HPLC purification. MS: calc. $C_{34}H_{36}N_5O_5S$ $[M+H]^+$ = 626.24, obsvd. ESI-MS: *m*/*z* 625.96.

*Synthesis of **B***: The Boc protecting group of **A** was 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 compound **B** (420 mg, 92%) after HPLC purification. MS: calc. $C_{29}H_{28}N_5O_3S$ $[M+H]^+$ = 526.19, obsvd. ESI-MS: *m*/*z* 526.10.

*Synthesis of **1***: Compound **B** (420 mg, 0.8 mmol), Fmoc-$_D$-Cystine (274 mg, 0.4 mmol), O-benzotriazol-1-yl-N,N,N,N-tetramethyluronium hexafluorophosphate (HBTU, 303 mg, 0.8 mmol), and N-Hydroxybenzotriazole (HOBT, 108 mg, 0.8 mmol) in 16 mL DMF were stirred overnight in the presence of N,N-Diisopropylethylamine (DIPEA, 103 mg, 0.8 mmol). Then 50 mL CH_3CN/H_2O (50/50, V/V) was added into the mixture, the precipitate was centrifuged and freeze-dried to yield compound **C** in good yield. The Fmoc protecting group of **C** was cleaved with 15 % piperidine in DMF for 5 min at 0 °C to yield compound **1** (374 mg) after HPLC purification. MS: calculated for **1** $C_{34}H_{43}N_{12}O_4S_4$ $[(M+H)^+]$: 811.24130; obsvd. HR-MALDI-TOF/MS: *m*/*z* 811.24130 (Figure S2). ^{1}H NMR of **1** (300 MHz, CD_3OD) δ (ppm): 8.64 (d, 2 H), 8.12 (d, 2 H), 7.75 (dd, 2 H), 4.52 (dd, 2 H), 4.35 (dd, 2 H), 3.50 (dd, 2 H), 3.18 (dd, 2 H), 2.92 (t, 4 H), 1.92 (m, 4 H), 1.70 (m, 4 H), 1.52 (m, 4 H) (Figure S3). ^{13}C NMR of **1** (75 MHz, CD_3OD) δ (ppm): 170.66, 166.98, 148.06, 138.60, 136.20, 135.22, 124.18, 120.58, 112.17, 111.20, 54.43, 51.36, 38.59, 37.54, 30.56, 26.50, 22.15 (Figure S4).

Preparation of aminoluciferin methyl amide (**AMA**):

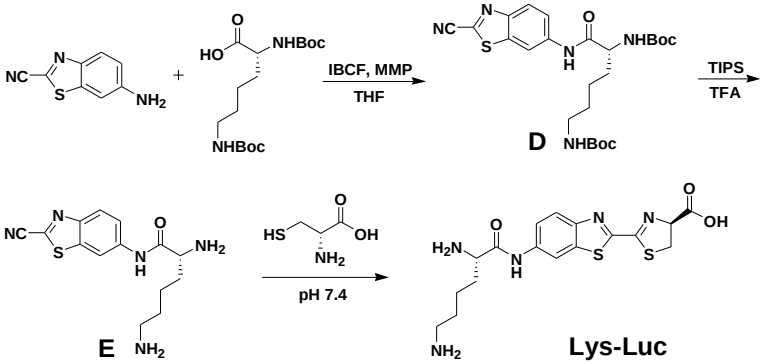
Scheme S4. The synthetic route for **AMA**.



*Synthesis of **AMA***: Aminoluciferin (112 mg, 0.4 mmol), CH_3NH_2 (16 mg, 0.5 mmol), O-benzotriazol-1-yl-N,N,N,N-tetramethyluronium hexafluorophosphate (HBTU, 152 mg, 0.4 mmol), and N-Hydroxybenzotriazole (HOBT, 54 mg, 0.4 mmol) in 5 mL DMF were stirred overnight in the presence of N,N-Diisopropylethylamine (DIPEA, 52 mg, 0.4 mmol) to yield compound **B** (104 mg, 89%) after HPLC purification. MS: calc. $C_{12}H_{13}N_4OS_2$ $[M+H]^+$ = 293.05308, obsvd. ESI-MS: *m*/*z* 293.05308 (Figure S9). ^{1}H NMR of **AMA** (300 MHz, CD_3OD) δ (ppm): 7.80 (d, 1 H), 7.20 (d, 1 H), 6.98 (dd, 1 H), 5.25 (t, 1 H), 3.71 (m, 2 H), 2.84 (s, 3 H) (Figure S10).

Preparation of Lys-Aminoluciferin (**Lys-Luc**):

Scheme S5. The synthetic route for **Lys-Luc**.



*Synthesis of **D***: The IBCF (140 mg, 1.0 mmol) was added to a mixture of Boc-Lys(Boc)-OH•DCHA (528 mg, 1.0 mmol) and MMP (208 mg, 2.0 mmol) in THF (8.0 mL) at 0 °C and the reaction mixture was stirred for 30 min. CBT (175 mg, 1.0 mmol) was added to the reaction mixture and further stirred for 1 h at 0 °C then overnight at room temperature. The pure product **D** (401.6 mg, yield: 80%) was

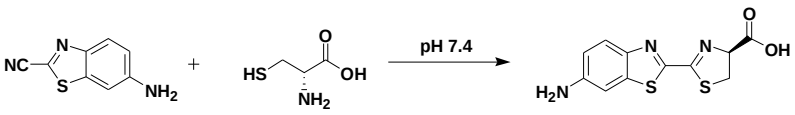
obtained after HPLC purification.

*Synthesis of **E**:* The Boc protecting groups of compound **D** were removed with 95 % TFA in CH_2Cl_2 for 3 h in presence of TIPS (1.6 mg, 10 μmol). Compound **E** (222.6 mg, yield: 92 %) was obtained after HPLC purification using water-methanol added with 0.1% TFA as the eluent (from 7:3 to 1:9).

*Synthesis of **Lys-Luc***: Compound **E** (172.4 mg, 0.52 mmol) was added to the mixture of $_D$-Cys (124.4 mg, 0.52 mmol), TCEP (148.5 mg, 0.52 mmol), H_2O (2 mL) and DMSO (2 mL). Subsequently, the pH of reaction mixture was adjusted to 7.0 and the mixture was stirred for 3 h at room temperature. Compound **Lys-Luc** (180.7 mg, yield: 78%) was purified with HPLC using water-acetonitrile added with 0.1% TFA as the eluent. MS: calculated for **Lys-Luc** $C_{17}H_{22}N_5O_3S_2$ $[(M+H)^+]$: 408.11641; obsvd. HR-MALDI-TOF/MS: *m*/*z* 408.11641 (Figure S11). ^{1}H NMR of **Lys-Luc** (300 MHz, CD_3OD) δ (ppm): 8.63 (d, 1 H), 8.11 (dd, 1 H), 7.70 (dd, 1 H), 5.43 (t, 1 H), 4.10 (q, 1 H), 3.79 (dd, 1 H), 3.66 (m, 1 H), 2.95 (t, 2 H), 2.04 (m, 2 H),1.74 (m, 2 H), 1.57 (m, 2 H) (Figure S12).

Preparation of Aminoluciferin (**NH_2-Luc**):

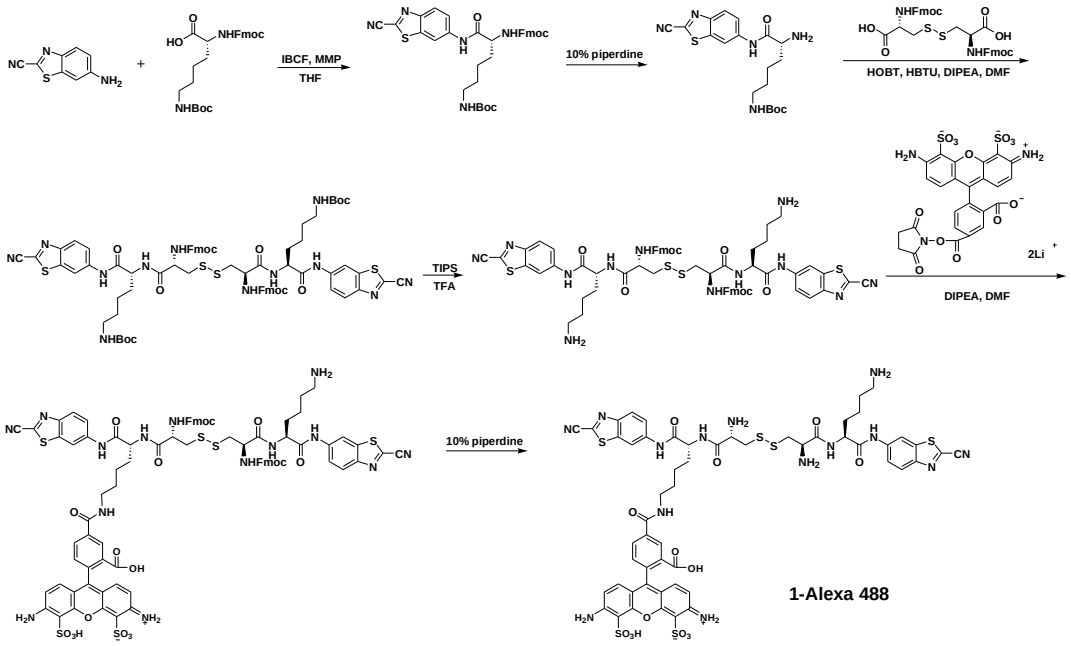
Scheme S6. The synthetic route for **NH_2-Luc**.



Cysteine (121 mg, 1.00 mmol) was dissolved in water and the pH value was adjusted to 7.4 with sodium carbonate and then CBT (140 mg, 0.800 mmol) dissolved in CH_3OH was added. CBT was consumed evidenced by thin-layer chromatography (TLC). The methanol was removed under reduced pressure, and the remaining

aqueous solution was acidified to pH 3 with 1 M HCl. With adjustment of the pH, yellow solid precipitated continuously. The precipitation was filtered and washed three times with water (2 mL × 3) to yield aminoluciferin (159 mg, 71.4%) dried under a freeze-dryer. MS: calculated for **NH_2-Luc** $C_{11}H_{10}N_3O_2S_2$ $[(M+H)^+]$: 280.02144; obsvd. HR-MALDI-TOF/MS: *m*/*z* 280.02144 (Figure S13). ^{1}H NMR of **NH_2-Luc** (300 MHz, CD_3OD) δ (ppm): 7.79 (d, 1 H), 7.16 (d, 1 H), 6.96 (dd, 1 H), 5.25 (t, 1 H), 3.75 (dd, 2 H), 2.84 (s, 3 H) (Figure S14).

Scheme S7. The synthetic route for **1-Alexa 488**.



3. Supporting Figures and Tables

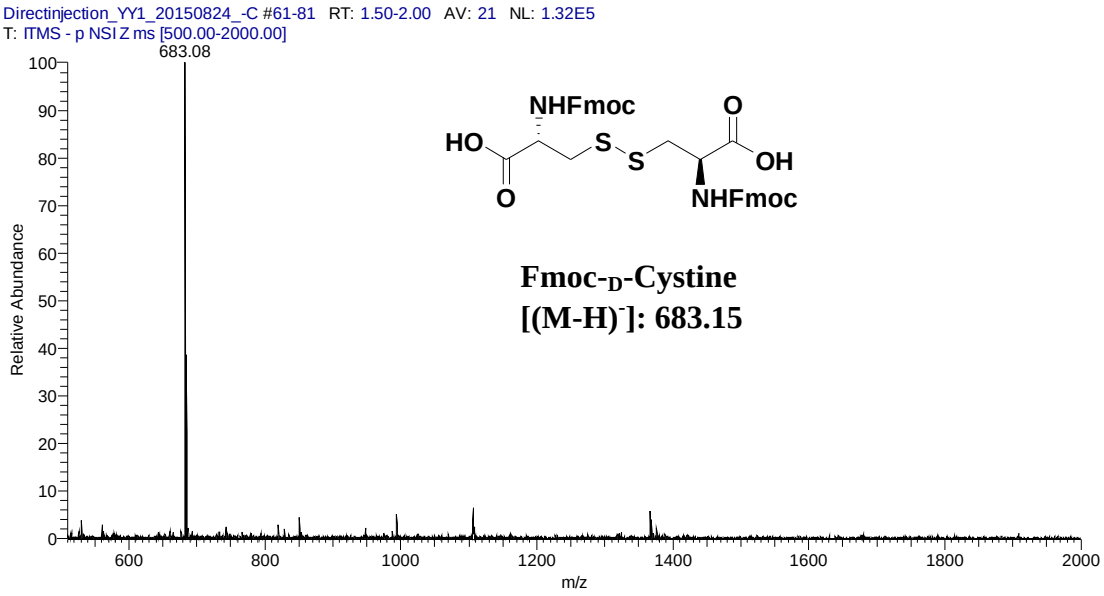


Figure S1. ESI-MS spectrum of Fmoc-$_D$-Cystine.

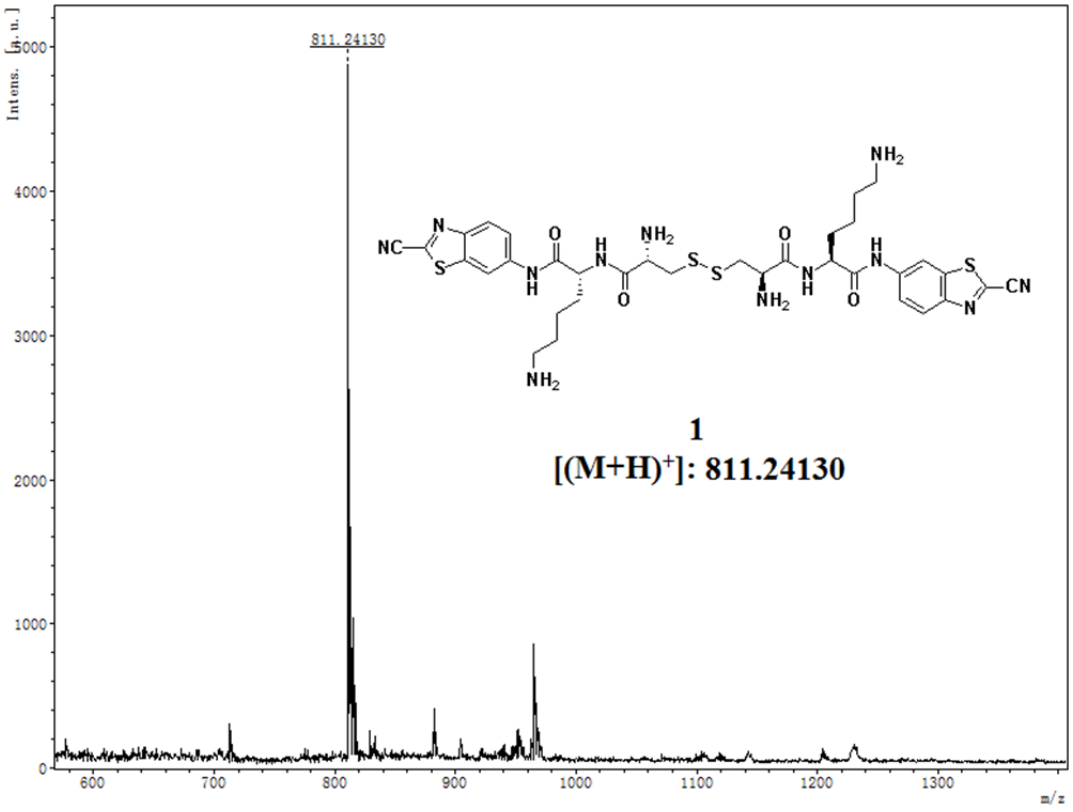


Figure S2. HR-MALDI-TOF/MS spectrum of **1**.

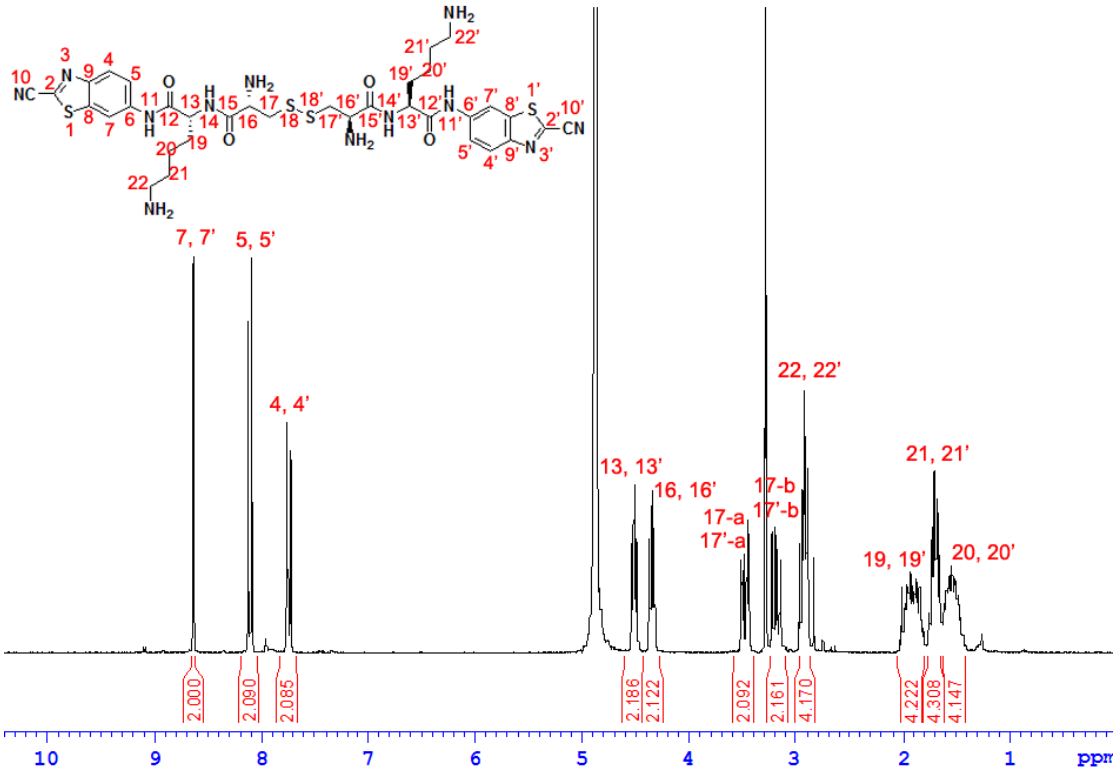


Figure S3. ^{1}H NMR spectrum of **1**.

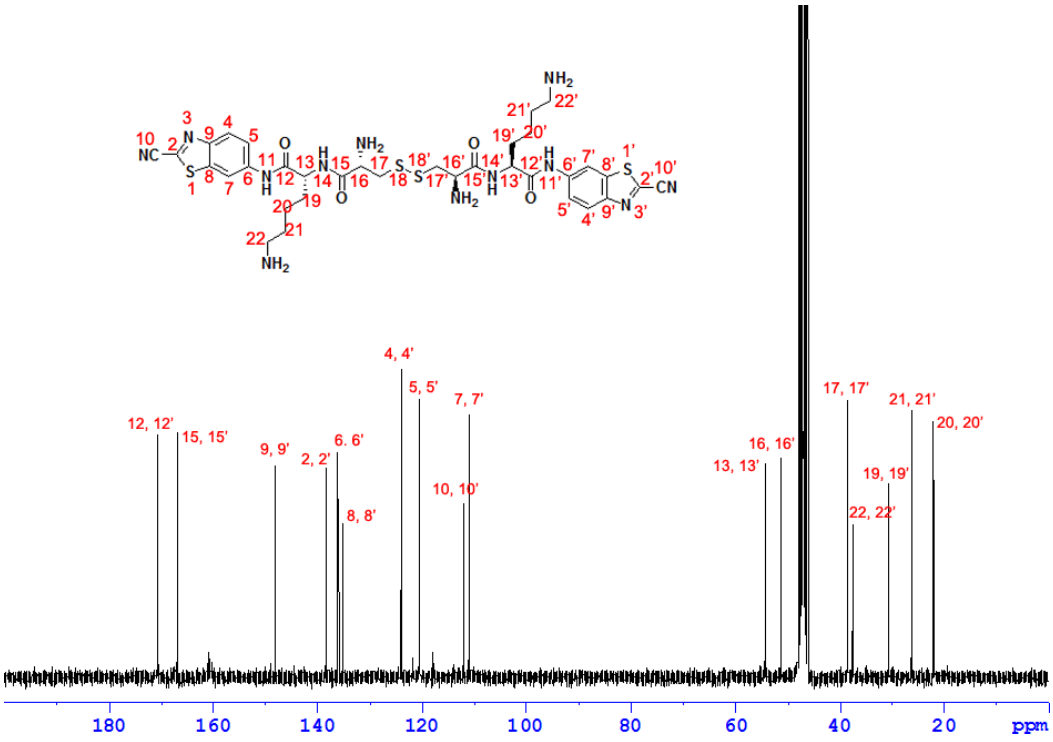


Figure S4. ^{13}C NMR spectrum of **1**.

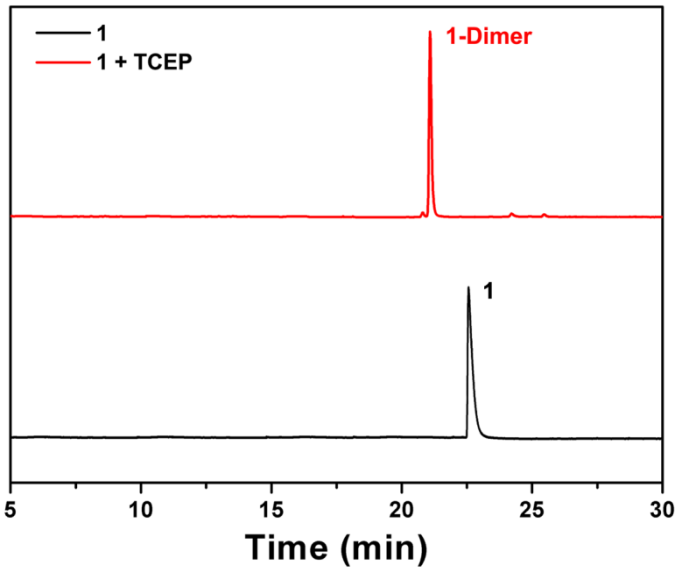


Figure S5. HPLC traces of 500 μM **1** in FAAH buffer with (red) or without (black) 1 mM TCEP for 30 min at 37 °C.

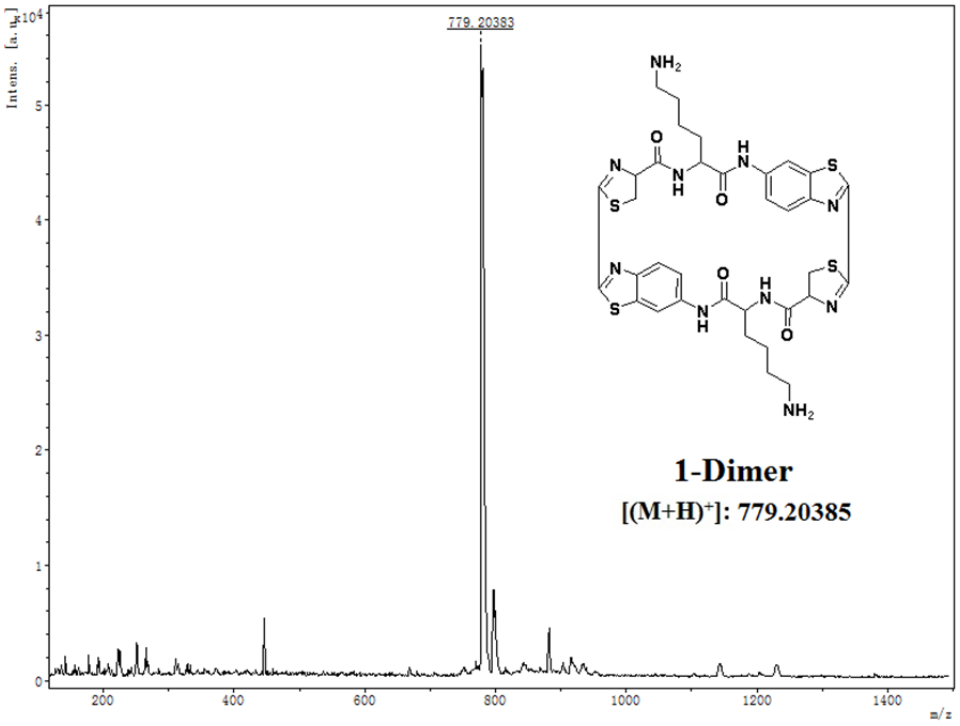


Figure S6. HR-MALDI-TOF/MS spectrum of HPLC peak at 21.1 min in Figure S5.

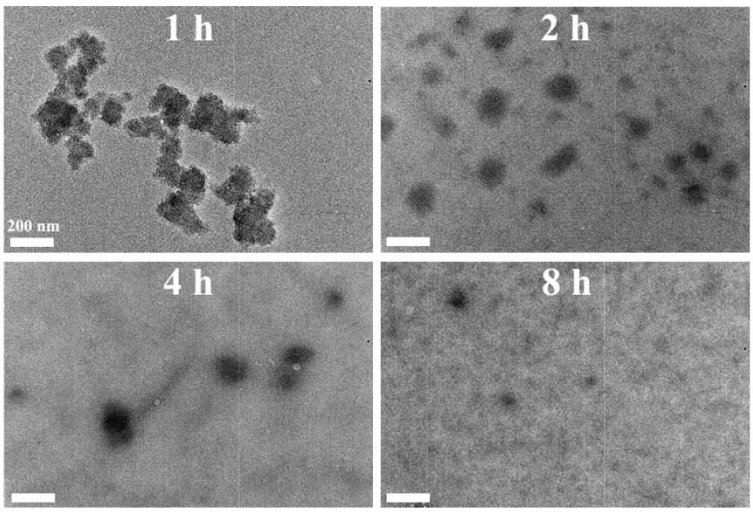


Figure S7. TEM images of 500 μM **1-NPs** after incubation with 10 μM FAAH at 37 °C for 1 h, 2 h, 4 h, and 8 h, respectively.

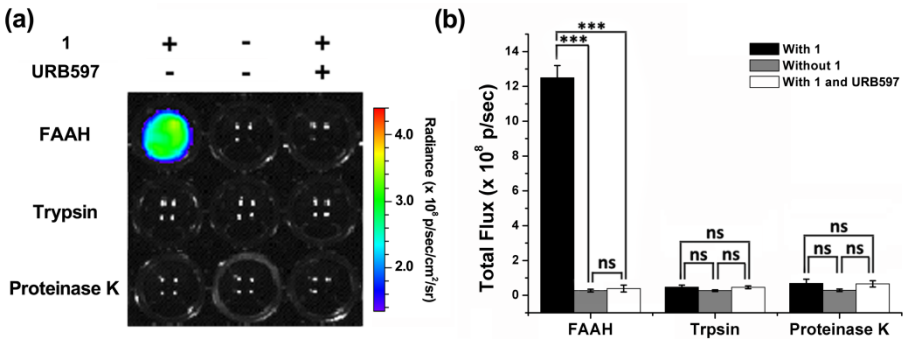


Figure S8. (a) Bioluminescence images of 2 μM FAAH, 2 μM trpsin, and 2 μM proteinase K in 1 mM TCEP incubated with (left) or without (middle) 500 μM **1**, or co-incubated with **1** and UBR597 (right). (b) Quantified total photon output in a. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns = not significant by t-test.

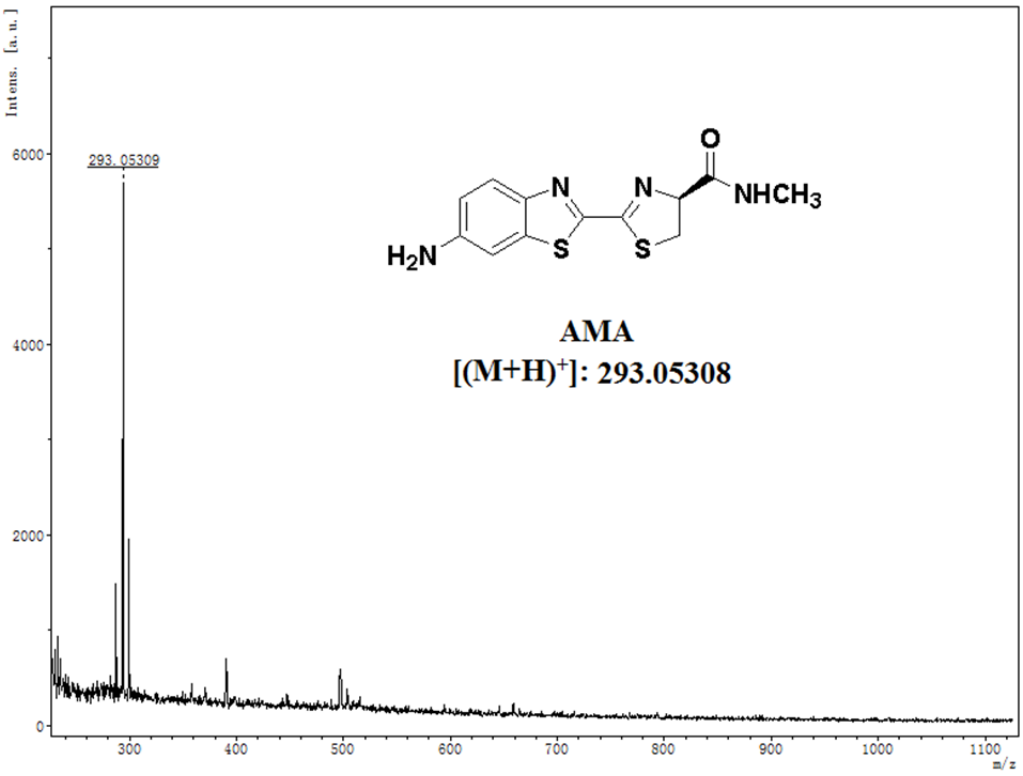


Figure S9. HR-MALDI-TOF/MS spectrum of **AMA**.

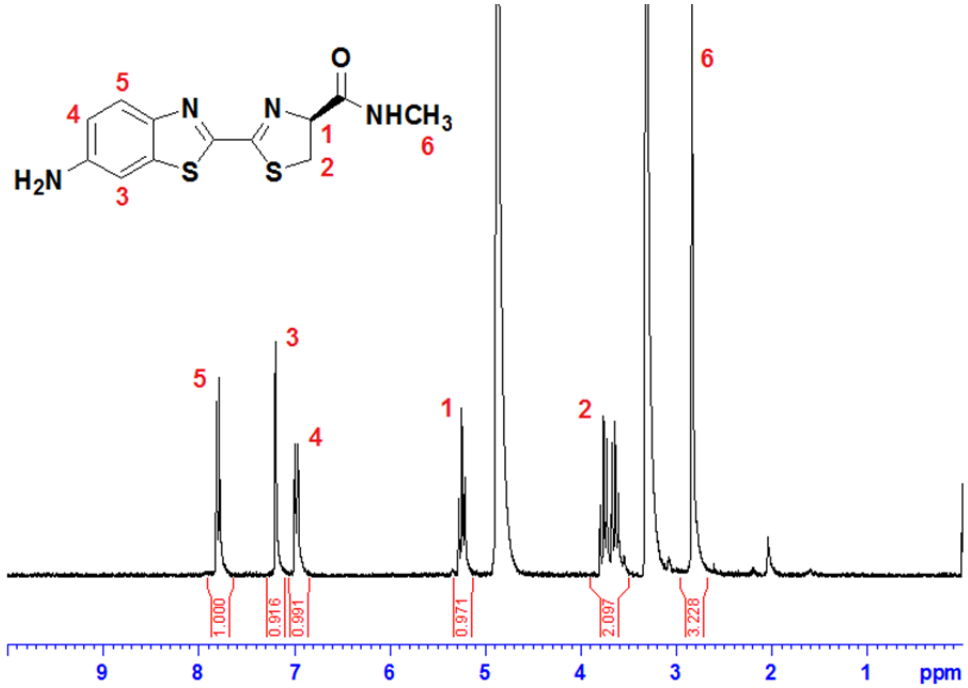


Figure S10. ^{1}H NMR spectrum of **AMA**.

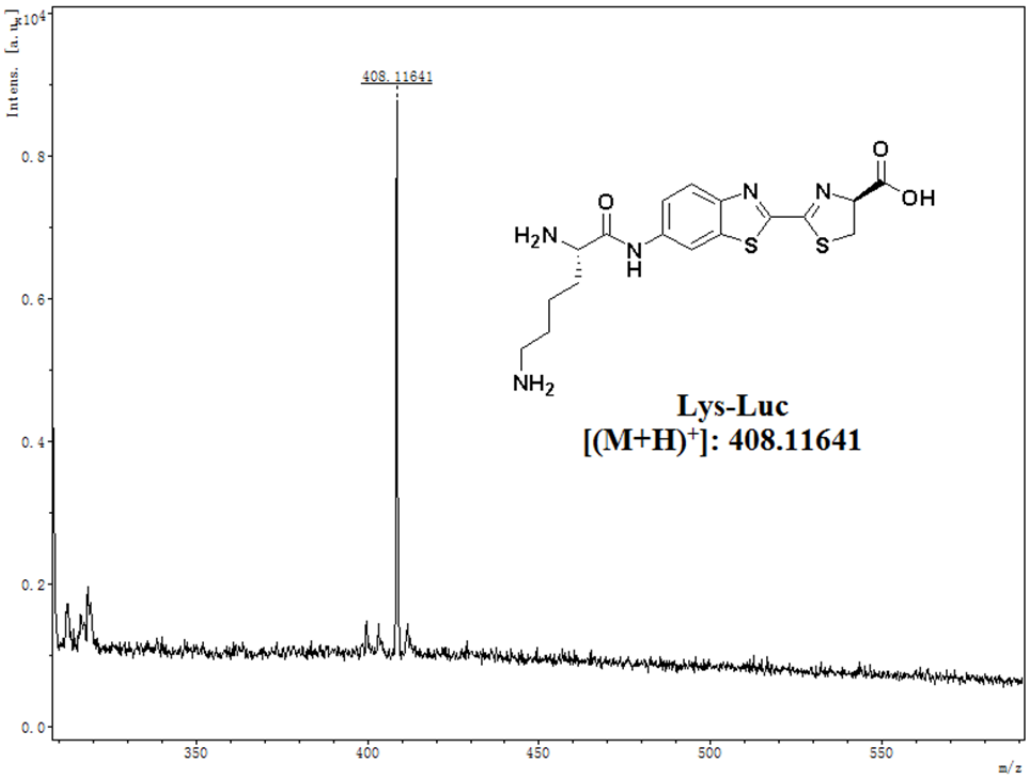


Figure S11. HR-MALDI-TOF/MS spectrum of **Lys-Luc**.

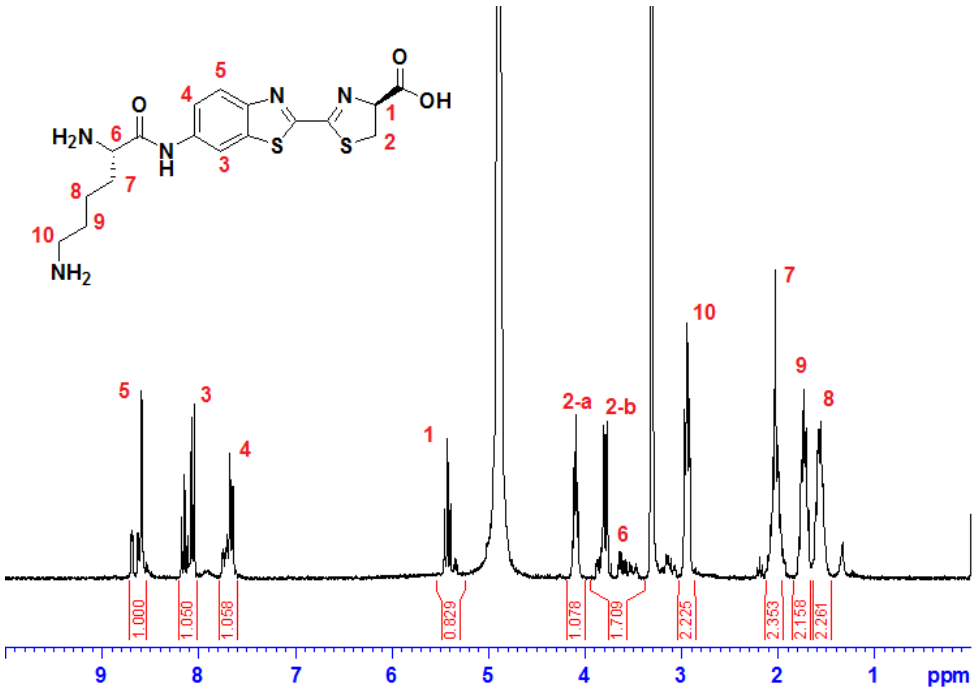


Figure S12. ^{1}H NMR spectrum of **Lys-Luc**.

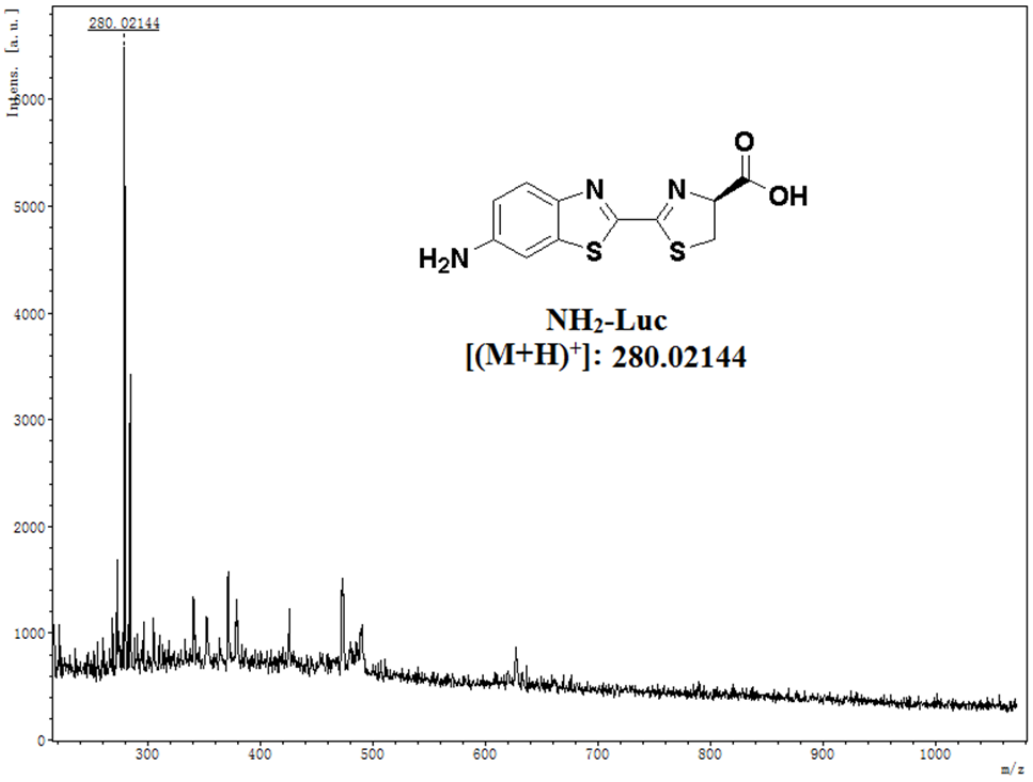


Figure S13. HR-MALDI-TOF/MS spectrum of **NH_2-Luc**.

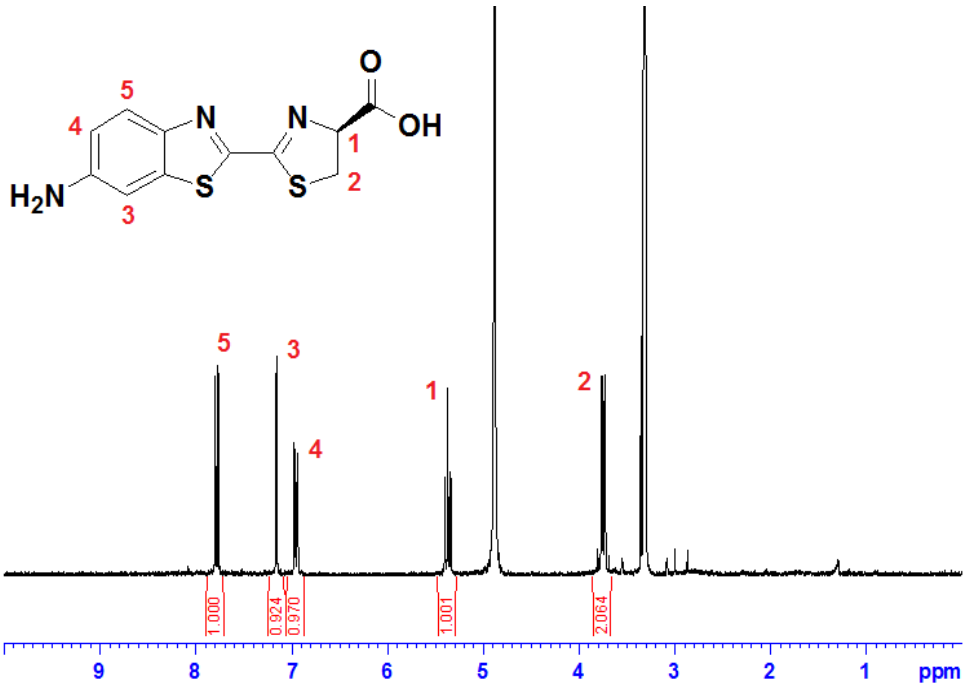


Figure S14. ^{1}H NMR spectrum of **NH_2-Luc**.

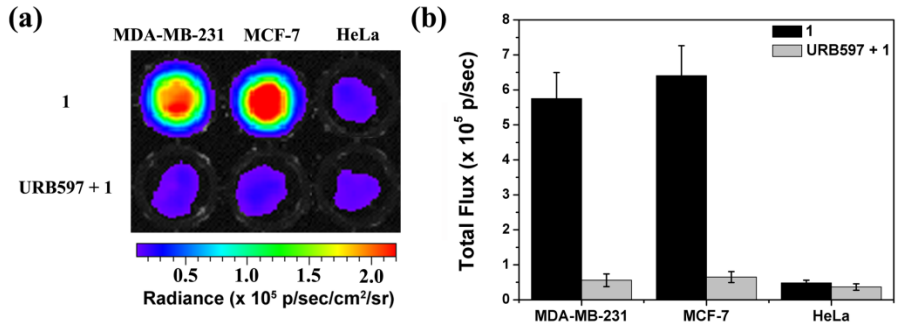


Figure S15. (a) Bioluminescence images of lysates of MDA-MB-231 cells, MCF-7 cells, and HeLa cells incubated with 250 μM **1** (top) or 100 μM URB597 with 250 μM **1** (bottom) in the presence of fLuc for 6 h respectively. (b) Quantified total photon output in a. Each error bar represents the standard deviation of three independent experiments.

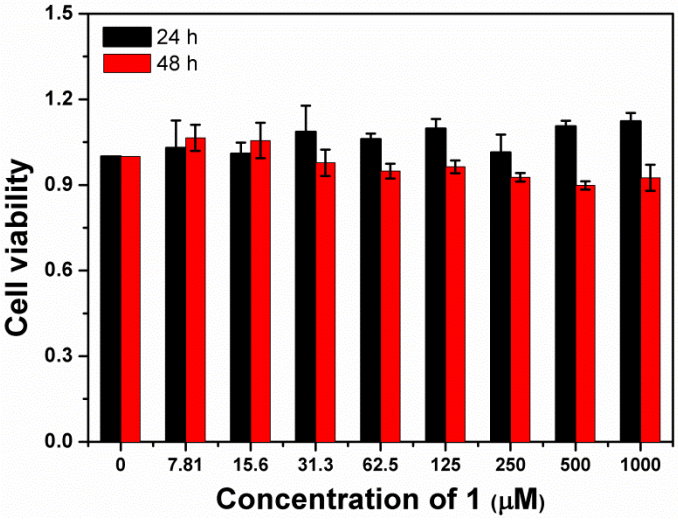


Figure S16. Cell viability of MDA-MB-231 cells treated with **1** at different concentrations for 24 h (black) or 48 h (red).

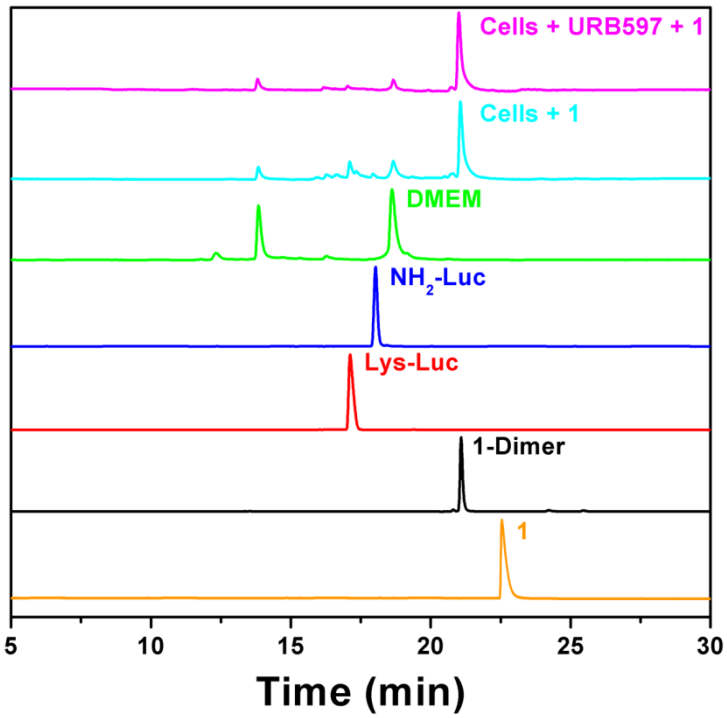


Figure S17. HPLC traces of **1** (orange); **1-Dimer** (black); **Lys-Luc** (red); **NH_2-Luc** (blue); High glucose DMEM (green); 2 mM **1** incubated with parental MDA-MB-231 cells (cyan) or 100 μM URB597-pretreated parental MDA-MB-231 cells (magenta) in serum-free culture medium at 37 °C for 2 h.

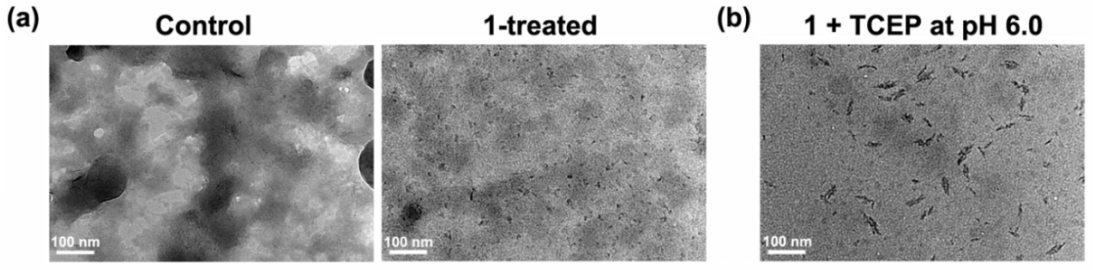


Figure S18. (a) TEM images of lysates of 100 μM URB597-treated parental MDA-MB-231 cells (left) and 100 μM URB597-pretreated parental MDA-MB-231 cells incubated with 2 mM **1** at 37 °C for 2 h (right). (b) TEM image of **1-NPs** formed by 500 μM **1** treated with 1 mM TCEP for 30 min at 37 °C and pH 6.0.

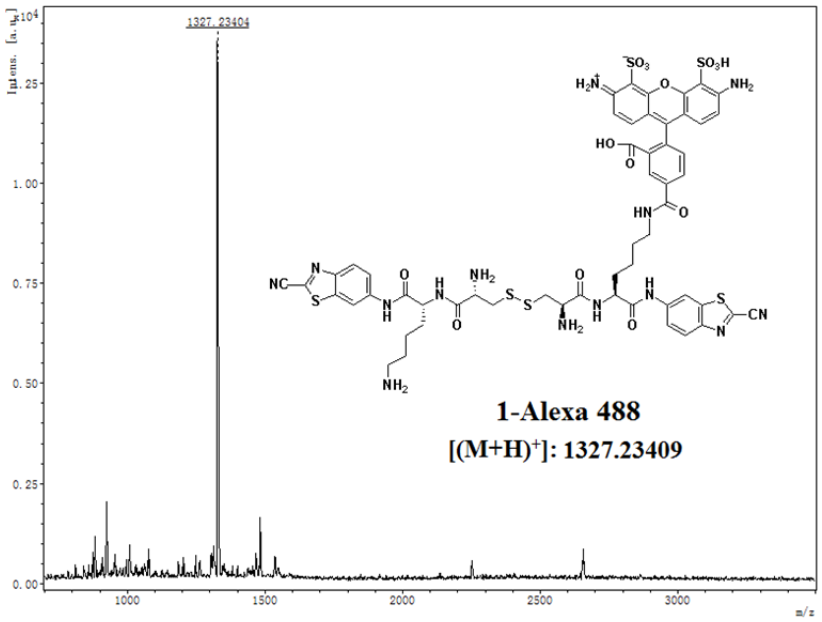


Figure S19. HR-MALDI-TOF/MS spectrum of **1-Alexa 488**.

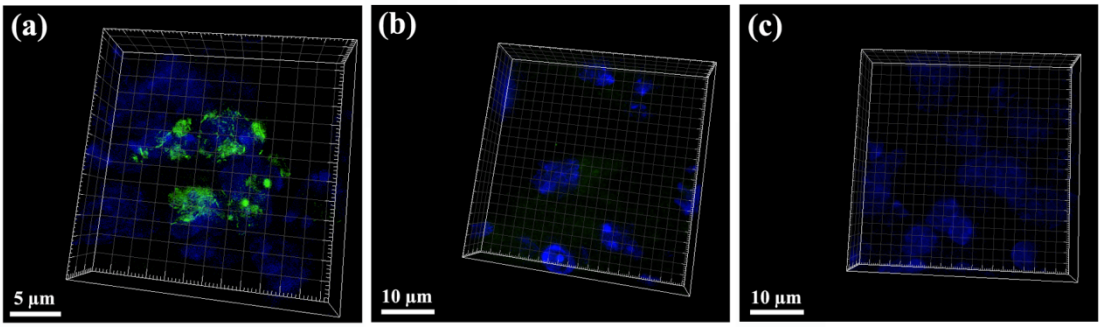


Figure S20. (a) 3D-SIM image of self-assembled fluorescent nanoparticles in MDA-MB-231 tumors of mice each i.p. injected with 45 nmol **1-Alexa 488**. Tumors were co-stained with DAPI. Green indicates the probe fluorescence, and blue indicates the nucleus. (b) 3D-SIM image of MDA-MB-231 tumor of mouse i.p. injected with 45 nmol **Alexa 488**. Tumors were co-stained with DAPI. (c) 3D-SIM image of blank MDA-MB-231 tumor.

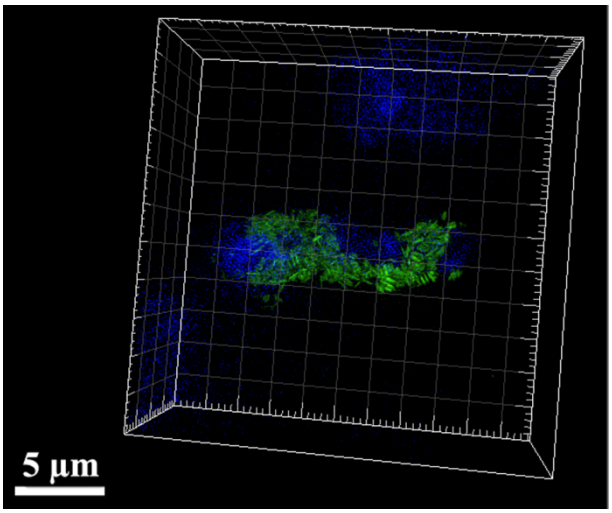


Figure S21. High magnification 3D-SIM image of self-assembled fluorescent nanoparticles in MDA-MB-231 tumor of mouse i.p. injected with 45 nmol **1-Alexa 488**.

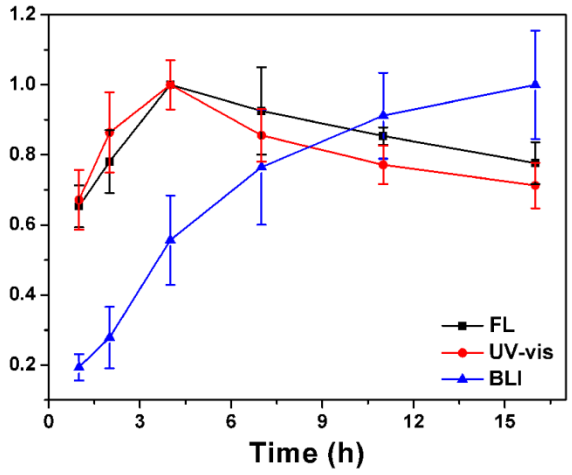


Figure S22. Normalized time-dependent fluorescence emission intensity at 525 nm (black line) or UV-vis absorbance difference of 510 nm to 700 nm (red line) of the tumor lysate supernatants of i.p. **1-Alexa 488**-injected mice at 1 h, 2 h, 4 h, 7 h, 11 h, and 16 h post injection, respectively. At indicated time points, the mice were euthanized and the tumors were resected and weighed. After the collected tumors were ground, freeze-dried and dissolved with DMSO at 0.25 g/mL, each of them was

sonicated in 4 °C for 30 min to prepare the organ homogenates. Then the organ homogenates were centrifuged at 16,000 rpm for 20 min to obtain the lysate supernatants. Blue line: Normalized BLI intensity of MDA-MB-231-fLuc tumor-bearing mice i.p. injected with 0.1 g/kg **1** (123 μmol/kg) at 1 h, 2 h, 4 h, 7 h, 11 h, and 16 h post injection, respectively.

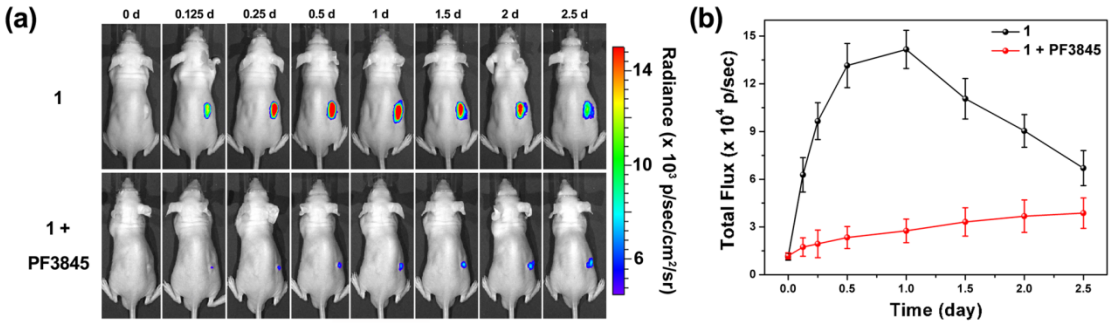


Figure S23. (a) Bioluminescence images of MDA-MB-231-fLuc tumor-bearing mice post intraperitoneally injected with 0.1 g/kg **1** (123 μmol/kg) (top), 1 mg/kg PF3845 (a more specific FAAH inhibitor than URB597) and 0.1 g/kg **1** (bottom) for 0.125 d, 0.25 d, 0.5 d, 1 d, 1.5 d, 2 d, and 2.5 d, respectively. (b) Quantified total photon output in a.

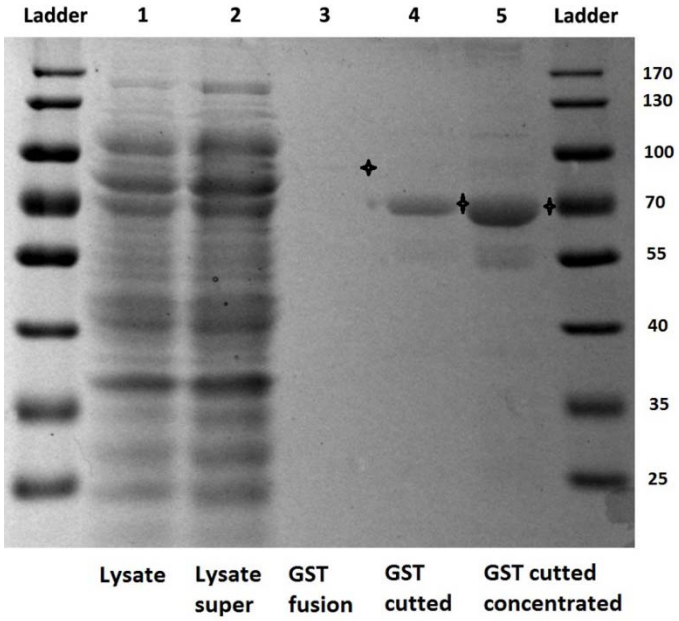


Figure S24. Lane 1, SDS-PAGE analysis of the recombinant GST tagged FAAH expressed in *E. Coli* lysate. Lane 2, recombinant GST tagged FAAH in *E. Coli* lysate supernatant. Lane 3, purified recombinant GST tagged FAAH. Lane 4, purified untagged recombinant FAAH. Lane 5, concentrated purified untagged recombinant FAAH.

Table S1. HPLC condition for Figure S5 and Figure S17.

Time (minute)	Flow (mL/min.)	H_2O % (0.1%TFA)	CH_3CN % (0.1%TFA)
0	1.0	99	1
3	1.0	99	1
35	1.0	35	65
37	1.0	35	65
38	1.0	99	1
40	1.0	99	1