Supplementary Material (ESI) for Chemical Communications

Supporting Information

Photoactivable Bioluminescent Probes for Imaging Luciferase Activity

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Material and General methods:

Materials. All reagents were purchased from Aldrich or Fluka. Commercially available reagents were used without further purification, unless noted otherwise. Anhydrous solvents for organic syntheses were purchased from Aldrich and stored over activated molecular sieves (4 Å). Thin-layer chromatography (TLC) was performed on precoated silica gel 60F-254 glass plates.

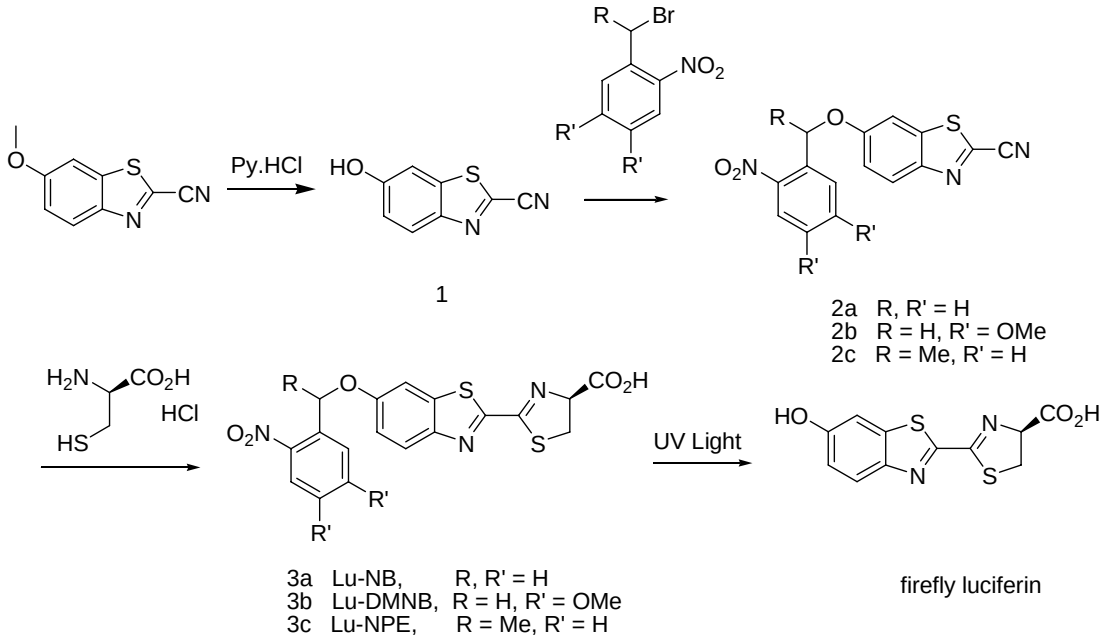
Instruments. NMR spectra were recorded on JEOL 400 MHz spectrometer. Chemical shifts (δ, ppm) were reported against TMS (0 ppm). Mass spectra (MS) were measured with a Thermo Polaris Q for EI and Thermo LCQ Deca XP MAX for ESI. UV-vis spectra were recorded in a 5-mm path quartz cell on a Beckman coulter DU800 spectrometer. Fluorescence spectroscopic studies were performed on a Varian Cary eclipse Fluorescence spectrophotometer. Photoirradiation experiments were carried out with UV lamp (Blak-Ray, B-100AP/R, 100 w/365 nm, Intensity: 8.9 mW/cm^2). Bioluminescence studies were carried out on a Turner Biosystems 20/20^n Luminometer. HPLC analysis was performed on a reverse-phase column with a Shimadzu HPLC system. Analytical reverse-phase high performance liquid chromatography (HPLC) was performed on Alltima C-18 column (250×3.0mm) at a flow rate of 1.0 mL/min and semi-preparative HPLC was performed on the similar C-18 column (250×10mm) at a flow rate of 3 mL/min.

Synthesis and characterization of the "caged" substrates.

2-cyano-6-hydroxybenzothiazole (1)

In a three-necked flask, pyridine hydrochloride (4 g) was heated to 180°C and 2-cyano-6-methoxybenzothiazole (0.200 g, 1.05 mmol) was added slowly. The mixture was stirred at this temperature for 1h and cooled in ice bath. Then 10% sodium bicarbonate solution was added and the mixture was extracted with EA (15 ml × 3). The combined organic layer was washed with brine, dried over Na_2SO_4 and concentrated. The residue was purified by column chromatography on silica gel using MeOH and DCM (1:20) as eluent to give 0.115 g of white solid. Yield: 62.2%. ^{1}HNMR (DMSO-d6) δ

(ppm): 10.59 (br, 1H), 8.06 (d, J = 9.1 Hz, 1H), 7.58 (d, J = 2.3 Hz, 1H), 7.17 (dd, J = 9.1, 2.3 Hz, 1H); ^{13}CNMR (MeOH-d4) δ (ppm): 160.6, 147.5, 139.2, 134.1, 126.8, 119.8, 114.5, 107.2; MS (EI): m/z calcd for $C_8H_4N_2OS$ 176.00, found 176.11 $[M]^+$



Scheme S1. Scheme of synthesis of caged luciferin derivatives

2-cyano-6-(2-nitrobenzyloxy)benzothiazole (2a)

2-cyano-6-hydroxybenzothiazole (**1**, 0.053 g, 0.30 mmol), 2-nitrobenzylbromide (0.077 g, 0.36 mmol) and potassium carbonate (0.117 g, 0.85 mmol) was added in 5 ml of acetone. The mixture was refluxed for 1h. After cooled, the solid was filtered off and the filtrate was concentrated and purified by column chromatography on silica gel with DCM and Hexane (1:3) as eluent to give 0.085 g white solid. Yield: 90.0%. ^{1}HNMR ($CDCl_3$) δ (ppm): 8.22 (d, J = 8.2 Hz, 1H), 8.14 (d, J = 9.2 Hz, 1H), 7.88 (d, J = 7.8 Hz, 1H), 7.72 (d, J = 7.8 Hz, 1H), 7.54 (d, J = 8.2 Hz, 1H), 7.46 (d, J = 2.3 Hz, 1H), 7.36 (dd, J = 2.3, 9.2 Hz, 1H), 5.60 (s, 2H), ^{13}CNMR ($CDCl_3$) δ (ppm): 158.9, 147.5, 147.0, 137.5, 134.3, 134.2, 132.6, 129.0, 128.5, 126.3, 125.4, 118.9, 113.2, 104.6, 67.7; MS (EI): m/z calcd for $C_{15}H_9N_3O_3S$ 311.04, found 311.13 $[M]^+$

Compounds **2b** and **2c** were synthesized similarly as **2a** from **1**

2-cyano-6-(4,5-dimethoxy-2-nitrobenzyloxy)benzothiazole(2b) Yield: 88.6%**.** ^{1}HNMR ($CDCl_3$) δ (ppm): 8.14 (d, J = 9.2 Hz, 1H), 7.80 (s, 1H), 7.48 (d, J = 2.3 Hz, 1H), 7.376-7.39 (m, 1H), 7.31 (s, 1H), 5.60 (s, 2H), 3.97 (s, 3H), 3.98 (s, 3H)**,** ^{13}CNMR ($CDCl_3$) δ (ppm): 158.8, 154.2, 148.3, 147.5, 139.3, 137.5, 134.2, 127.9, 126.3, 118.9, 113.1, 109.3, 108.3, 104.7, 67.9, 56.6, 31.0**;** MS (EI): m/z calcd for $C_{17}H_{13}N_3O_5S$ 371.06, found 371.15 $[M]^+$

2-cyano-6-(1-(2-nitrophenyl)ethoxy)benzothiazole (2c) Yield: 94.5%, ^{1}HNMR ($CDCl_3$) δ (ppm): 8.02-8.06 (m, 2H), 7.73 (m, 1H), 7.60 (m, 1H), 7.44 (m, 1H), 7.19-7.25 (m, 2H), 6.15 (q, J = 6.4 Hz, 1H), 1.75 (d, J = 6.4 Hz, 3H), ^{13}CNMR ($CDCl_3$) δ (ppm): 158.1, 147.6, 147.2, 138.0, 137.4, 134.5, 134.0, 129.0, 127.4, 126.2, 125.2, 119.3, 113.2, 105.5, 72.5, 23.7; MS (EI): m/z calcd for $C_{16}H_{11}N_3O_3S$ 325.05, found 325.08 $[M]^+$

(S)-2-(6'-(2-nitrobenzyloxy)-2'-benzothiazolyl)-Δ^2-thiazoline-4-carboxylic acid (3a)

2-cyano-6-(2-nitrobenzyloxy)benzothiazole (**2a**, 0.040 g, 0.13 mmol) and D-cysteine hydrochloride monohydrate (0.040 g, 0.23 mmol) was added to the mixed solvent of 2 ml of DCM and 2 ml of methanol. The mixture was stirred till the solid dissolved. Then 0.32 ml of 10% $NaHCO_3$ aqueous solution was added and the mixture was stirred for 15min. The mixture was acidified with 1N HCl to pH 2-3 and extracted with DCM (20 ml × 3). The combined organic layer was washed with brine, dried over Na_2SO_4 and concentrated. The product was obtained as yellow solid. Yield: 83.4%. ^{1}HNMR (DMSO-d6) δ (ppm): 8.16 (d, J = 7.8 Hz, 1H), 8.08 (d, J = 9.2 Hz, 1H), 7.87 (d, J = 2.3 Hz, 1H), 7.80 (m, 2H), 7.62-7.65 (m, 1H), 7.31 (dd, J = 9.2, 2.3 Hz, 1H), 5.85 (s, 2H), 5.42 (t, J = 9.2 Hz, 1H), 3.66-3.80 (m, 2H); ^{13}CNMR(DMSO-d6) δ (ppm): 171.7, 164.9, 158.9, 157.9, 148.0, 147.9, 137.6, 134.7, 132.7, 129.8, 129.7, 125.5, 125.4, 118.0, 106.5, 78.6, 67.5, 35.3; MS (ESI): m/z calcd for $C_{18}H_{13}N_3O_5S_2$ 415.03, found 416.64 $[M+H]^+$

Compounds **3b** and **3c** were synthesized similarly as **3a** from **2b** and **2c**

(S)-2-(6'-(4,5-dimethoxy-2-nitrobenzyloxy)-2'-benzothiazolyl)-Δ^2-thiazoline-4-carboxylic acid (3b)
Yield: 87.6%. ^{1}HNMR (DMSO-d6) δ (ppm): 8.08 (d, J = 9.2 Hz, 1H), 7.91 (d, J = 2.8 Hz, 1H), 7.74 (s, 1H), 7.38 (s, 1H), 7.34 (dd, J = 9.2, 2.8 Hz, 1H), 5.52 (s, 2H), 5.39-5.43 (m, 1H), 3.87 (s, 3H), 3.88 (s, 3H), 3.65-3.77 (m, 2H); ^{13}CNMR (DMSO-d6) δ (ppm): 171.7, 164.8, 158.9, 158.0, 153.8, 148.5, 148.0, 140.2, 137.6, 127.3, 125.4, 118.0, 111.8, 108.8, 106.6, 78.8, 67.8, 56.8, 56.6, 35.3; MS (ESI): m/z calcd for $C_{20}H_{17}N_3O_7S_2$ 475.05, found 476.31 $[M+H]^+$

(S)-2-(6'-(1-(2-nitrophenyl)ethoxy)-2'-benzothiazolyl)-Δ^2-thiazoline-4-carboxylic acid (3c) Yield: 75.8%. ^{1}HNMR($CDCl_3$) δ(ppm): 9.43 (br, 1H), 8.00 (d, J = 8.2 Hz, 1H), 7.92 (d, J = 9.2Hz, 1H), 7.73 (d, J = 7.4 Hz, 1H), 7.56 (t, J = 7.4 Hz, 1H), 7.38(t, J = 7.8 Hz, 1H), 7.18 (s, 1H), 7.07 (d, J = 9.2 Hz, 1H), 6.11 (q, J = 6.0 Hz, 1H), 5.37-5.42 (m, 1H), 3.67-3.77 (m, 2H), 1.70 (d, J = 6.0 Hz, 3H); ^{13}CNMR ($CDCl_3$) δ (ppm): 173.8, 167.5, 158.0, 156.9, 148.0, 147.4, 138.4, 137.8, 134.4, 128.7, 127.4, 125.5, 125.0, 117.7, 106.3, 72.2, 53.6, 35.1, 23.6; MS (ESI): m/z calcd for $C_{19}H_{15}N_3O_5S_2$ 429.05, found 430.57 $[M+H]^+$

Photoirradiation Experiments in Cuvette and photoconversion efficiency determination

A solution (600 μL, 1 μM, in 10 mM PBS containing 0.05% DMSO as cosolvent) of each compound was transferred to a 5-mm path quartz cell. The cell containing the solution was illuminated at 365 nm for a specified period. After each irradiation, the fluorescence spectrum was recorded in the range from 425 nm to 670 nm, with 350nm excitation. The integrated fluorescence intensity was plotted against irradiation time.

The photoconversion efficiency was determined as the ratio of the integrated fluorescence intensity of the luciferin derivatives after each episode of UV illumination against that of luciferin(600 μL, 1 μM, in 10 mM PBS containing 0.05% DMSO as cosolvent). Among them, compound 3c reached 70% conversion faster than the other two compounds.

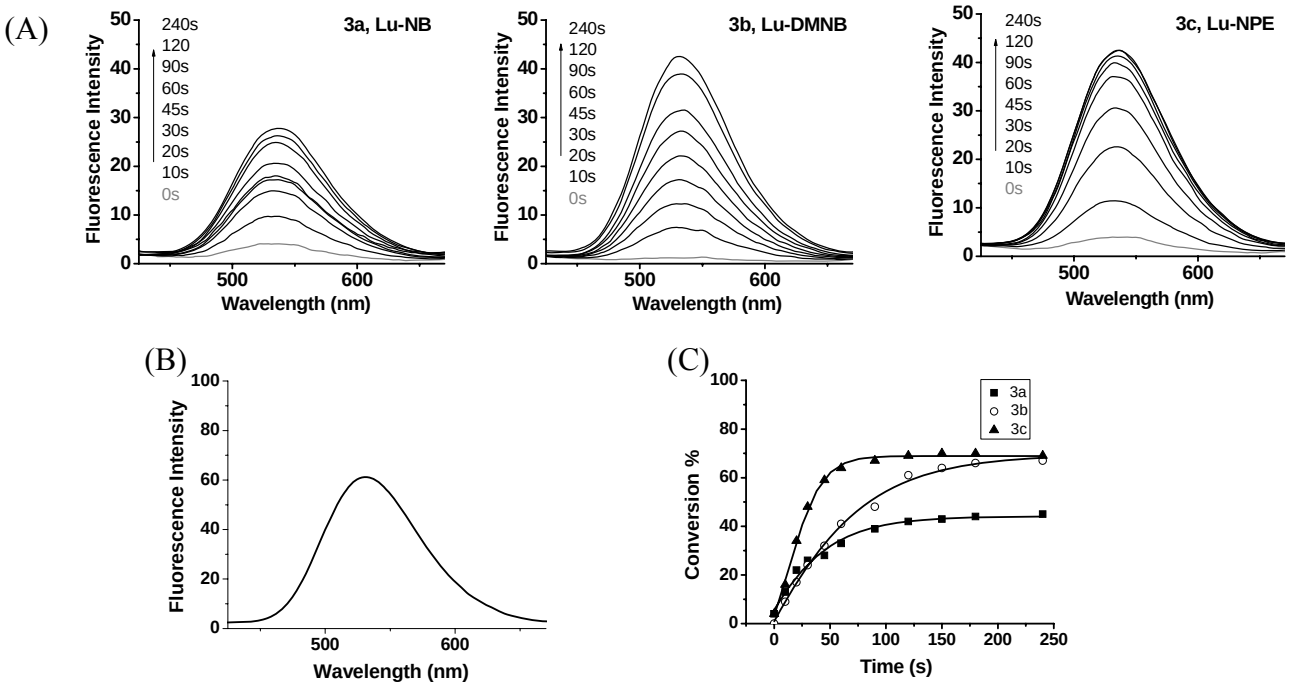


Figure S1. (A) Change of fluorescence spectra of "caged" Lucifeirn (sample concentration: 1 μM, in 10 mM PBS containing 0.05% DMSO as co-solvent) upon UV irradiation (365 nm). (B) Fluorescence spectrum of D-Luciferin (1 μM, in 10 mM PBS containing 0.05% DMSO as co-solvent). (C) Time course of the photoconversion of "caged" luciferins to D-luciferin.

Measurement of Fluorescence Quantum Efficiency

A stock solution (50 μM, in 10 mM PBS) of each compound was prepared. Absorption spectra were obtained with a 10 mM PBS (pH 7.4) solution of each compound at the desired concentration, adjusted by appropriate addition of the 50 μM stock solution. For determination of the quantum efficiency of fluorescence (Φ_f), dapoxyl sulfonic acid was used as a fluorescence standard.[1] The quantum efficiency

of fluorescence was obtained with the following equation (F denotes integrated fluorescence intensity at each wavelength)

$$\Phi_f/\Phi_{f0} = (1\text{-}10^{-A0})F/(1\text{-}10^{-A})F_0$$

A, F and Φ_f: absorbance, integrated fluorescence intensity and fluorescence quantum efficiency of sample; A_0, F_0 and Φ_{f0}: absorbance, integrated fluorescence intensity and fluorescence quantum efficiency of reference compound.

The quantum efficiencies of the different substrates are listed as following:
3a: 0.011±0.001; 3b: 0.0017±0.0003; 3c: 0.0043±0.0003; Luciferin: 0.11 and Reference compound: dapoxyl sulfonic acid: 0.04

HPLC analysis and Determination of Uncaging Cross Section

The uncaging products were confirmed by HPLC analysis. All the samples were eluted with an isocratic mixture of 50% acetonitrile and 50% water containing 0.1% TFA at a flow rate of 1 ml/min. The chromatograms were displayed by monitoring the absorbance at 325 nm to show the "photocaged" substrates and two photolyzed products. The HPLC chromatogram of D-luciferin was applied as a reference (trace c in Fig. S2A, S2B and S2C). The different peaks before and after UV illumination were collected and confirmed by mass spectrum.

As the data shown in HPLC analysis, the peak at retention time of 1.6 min in trace c (Fig. S2A, S2B or S2C) was D-luciferin, which was confirmed by Mass Spectrum. (280.80, [M+H]+; calcd for $C_{11}H_8N_2O_3S_2$, 280.00). For the substrate 3a, besides the peak at 9.2 min (3a), another two peaks at 8.2 min and 1.6 min were identified upon 1 min UV illumination. But the photolytic efficiency is low. For the substrate 3b, besides the product of D-luciferin at 1.6 min, another new peak at 6.7 min was the photolysis product 4, 5-dimethoxy-2-nitrosobenzaldehyde. (196.28, [M+H]+; calcd for $C_9H_9NO_4$, 195.05). Substrate 3c contained two chiral centers in the structure and thus indicated two peaks in HPLC spectrum. Both of the two diastereomers NPE-luciferin derivatives exhibited the same properties toward the UV illumination. After 1 min UV illumination, only little amount of substrates can be monitored, demonstrating the good photolactivity of NPE-luciferin substrate. (Fig. S2C).

The uncaging cross section measures the efficiency of photolysis. It equals the product of the quantum efficiency of uncaging (Φ_{365}) and the molecular extinction coefficient (ε_{365}) at the wavelength of photolysis. The quantum yields for luciferin release from NB- (3a), DMNB- (3b) and NPE- (3c) caged substrates were determined by competition with the 2-nitrobenzyl alcohol standard compound(Φ_{365} =

0.45).[2] The disappearance quantum yield of 0.69, 0.35 and 0.63 could be determined from this experiment by HPLC analysis.

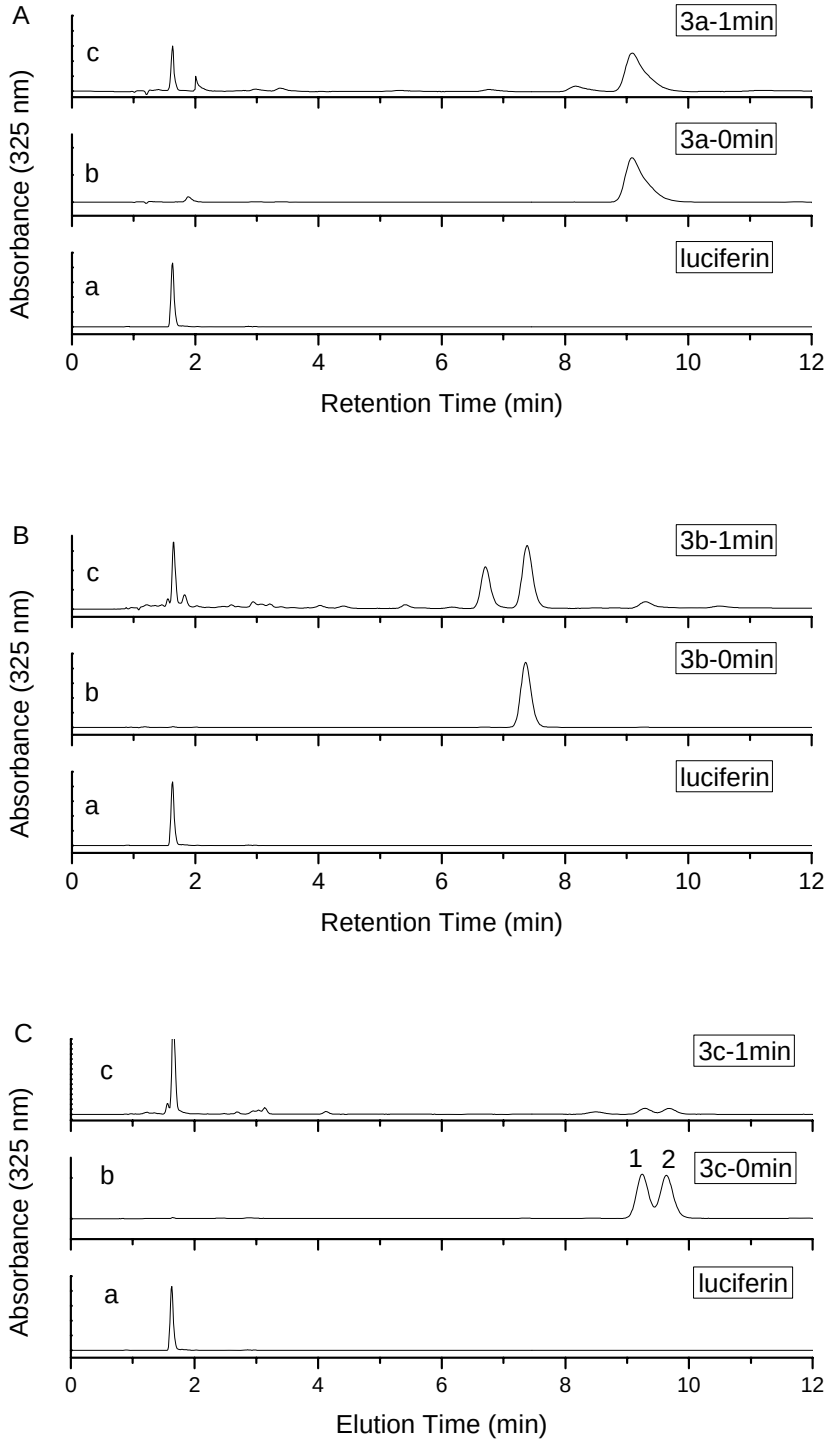


Figure S2. HPLC analysis of the photolysis products of 3a(A), 3b(B) and 3c(C). Trace a is the chromatogram of Luciferin (50 μM, in 10 mM PBS, pH 7.4). Trace b and trace c are the chromatograms of caged compounds before and after illumination respectively. Illumination time: 1min.

A solution (50 μM, in 10 mM PBS, pH 7.4, containing 2.5% DMSO as cosolvent) of each compound was prepared and illuminated at 365 nm for the specified period. After each irradiation, a 20 μL of the irradiated solution was subjected to HPLC analysis. Disappearance of caged substrate was monitored in terms of peak area at 325 nm absorbance. Remaining caged substrate [%] was plotted versus irradiation time [sec]. Each plot was fitted to a linear equation and the slope (k^{sample}, k^{standard}) was determined. Uncaging quantum efficiency was calculated according to the following equation:

$$\Phi_{365}^{\text{sample}}/\Phi_{365}^{\text{standard}} = \varepsilon_{365}^{\text{standard}} k^{\text{sample}} / (\varepsilon_{365}^{\text{sample}} k^{\text{standard}})$$

(Φ_{365}) Uncaging Quantum Efficiency at 365nm

(ε_{365}) Molar Extinction Coefficient

($\Phi^*\varepsilon$) Uncaging Cross Section

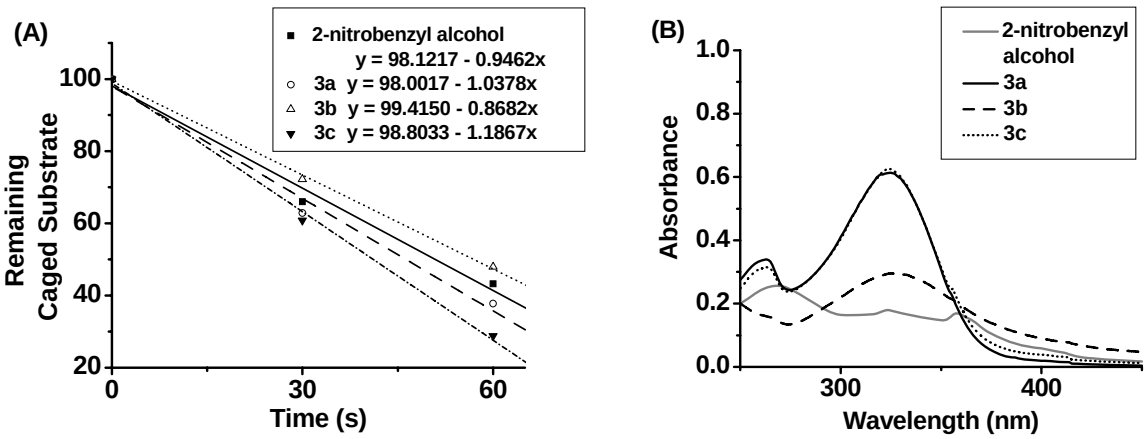


Figure S3. (A) Remaining caged substrates versus irradiation time plot. (B) Absorption spectra (250-450 nm) of caged luciferin derivatives and 2-nitrobenzyl alcohol (50 μM, in 10mM PBS)

	3a	**3b**	**3c**
Φ_f	0.011±0.001	0.0017±0.0003	0.0043±0.0003
Φ_{365}	0.69	0.35	0.63
ε_{365}	4304	6864	5280
$\Phi^*\varepsilon$	2970	2402	3326

Table S1. Fluorescent and photochemical properties of caged luciferin derivatives.

***In vitro* Luciferase Assay:**

A 2.5 µl aliquot of 1 mM DMSO stock solution of each conjugate (A, D, E) was diluted by adding 197.5 µl PBS buffer solution, pH 7.4 in a quartz cuvette. The cuvette containing the prepared solution was placed in a UV lamp (365nm). After irradiation(1min UV exposure treatment), firefly luciferase, ATP, $MgCl_2$ were added into each solution, with final concentration of 75 µg/ml, 1 mM and 2.5 mM, respectively. Then, luminescence intensity was recorded through a luminometer (Turner BioSystems, 20/20n Luminometer; Turner BioSystems, Sunnyvale, CA, USA). As a comparison, luminescence of all three samples before UV irradiation was also measured.

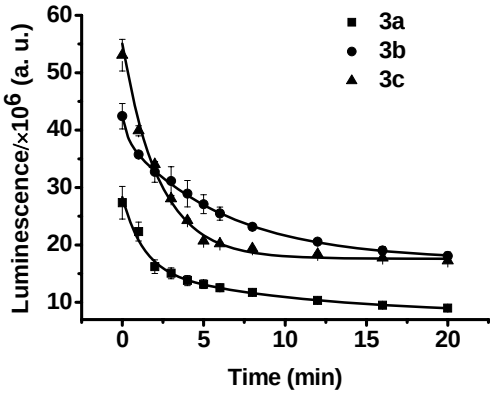


Figure S4. Bioluminescence of three substrates (12.5 µ M) after uncaging with 1 min UV irradiation and treatment with 75 µg/ml of firefly luciferase in PBS buffer, pH 7.4.

Cell Lysis Assay:

C6 glioma cell line was bought from American Type Culture Collection (ATCC Cat No.: CCL-107, Manassas, VA) and maintained in F-12K medium (ATCC, Manassas, VA) containing 10% FBS (Invitrogen, Burlington, Canada). PGL3 control plasmid was purchased from promega (Madison, WI) containing SV40 promoter, enhancer and firefly luciferase reporter gene. C6 glioma cell line was transfected with PGL3 control plasmid using Lipofectamine 2000 (Invitrogen, Burlington, Canada). After that, Lysis of various cell numbers were added into 12.5 µM compounds solution including 1 mM ATP and 2.5 mM $MgCl_2$ and the resultant light measured.

Cell and animal imaging assays:

Cell imaging assays:

fLuc transfected C6 glioma cell were plated in a 35-mm-diameter glass-bottomed dish (MatTek Corp., Ashland, MA) and cultured overnight in F-12K medium with 10% FBS. The medium was removed and the cells were washed twice with 2 ml PBS buffer. Then F-12K medium (2ml) solution containing 25 µM

compounds 3a, 3b or 3c was added and the cells were incubated for 1hr in an incubator with 37 ℃ and 5% CO_2. Then, the cells were washed twice with PBS (2 ml) and illuminated with the UV lamp. After exposure, the fluorescence imaging was acquired with a confocal fluorescence microscope (Nikon，Eclipse TE2000-E), using a super high pressure mercury lamp (Nikon, TE2-PS100W) with an excitation filter: 460/40nm and emission filter: 535/50nm.

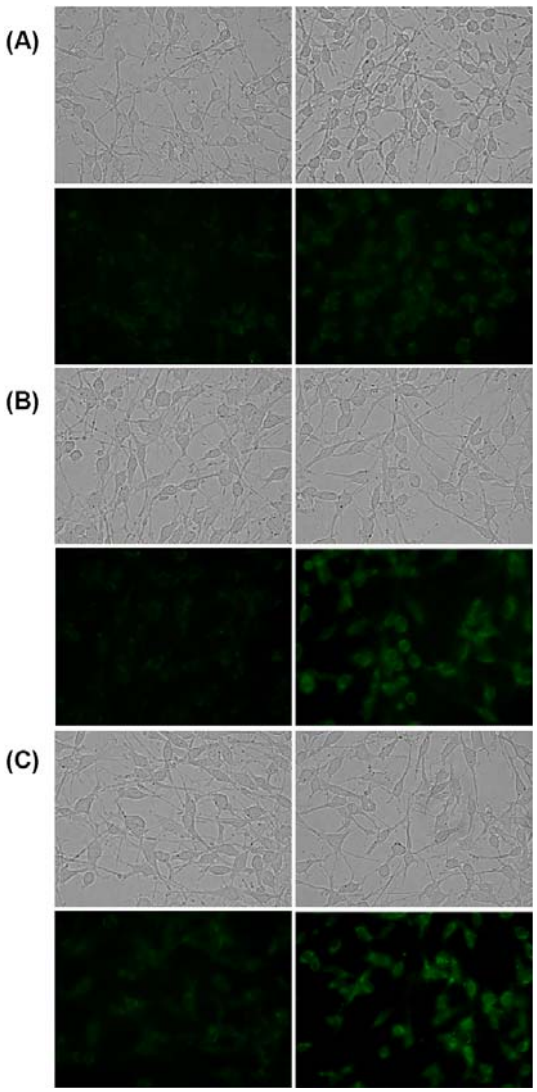


Figure S5. Cell application of compounds 3a (A), 3b (B) and 3c (C) (25 μM). Differential interference contrast and fluorescence imaging before (left) and after 1 min irradiation (right).

Cytotoxicity assays:

According to the procedures in cell imaging assays, fLuc transfected C6 glioma cells were treated with 25 μM of D-luciferin, "caged" luciferin derivatives 3a, 3b and 3c. After 1hr incubation and 1min UV irradiation to the "caged" substrates, the cytotoxicity activity was evaluated by an MTT assay as

previously described.[3]

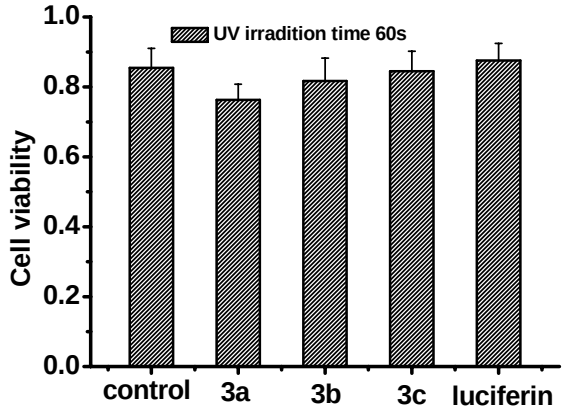


Figure S6 Cell viability of compound 3a, 3b, 3c and luciferin. As a control, cells without compound incubation were also treated with 1min UV irradiation.

In vivo Bioluminescent Imaging:

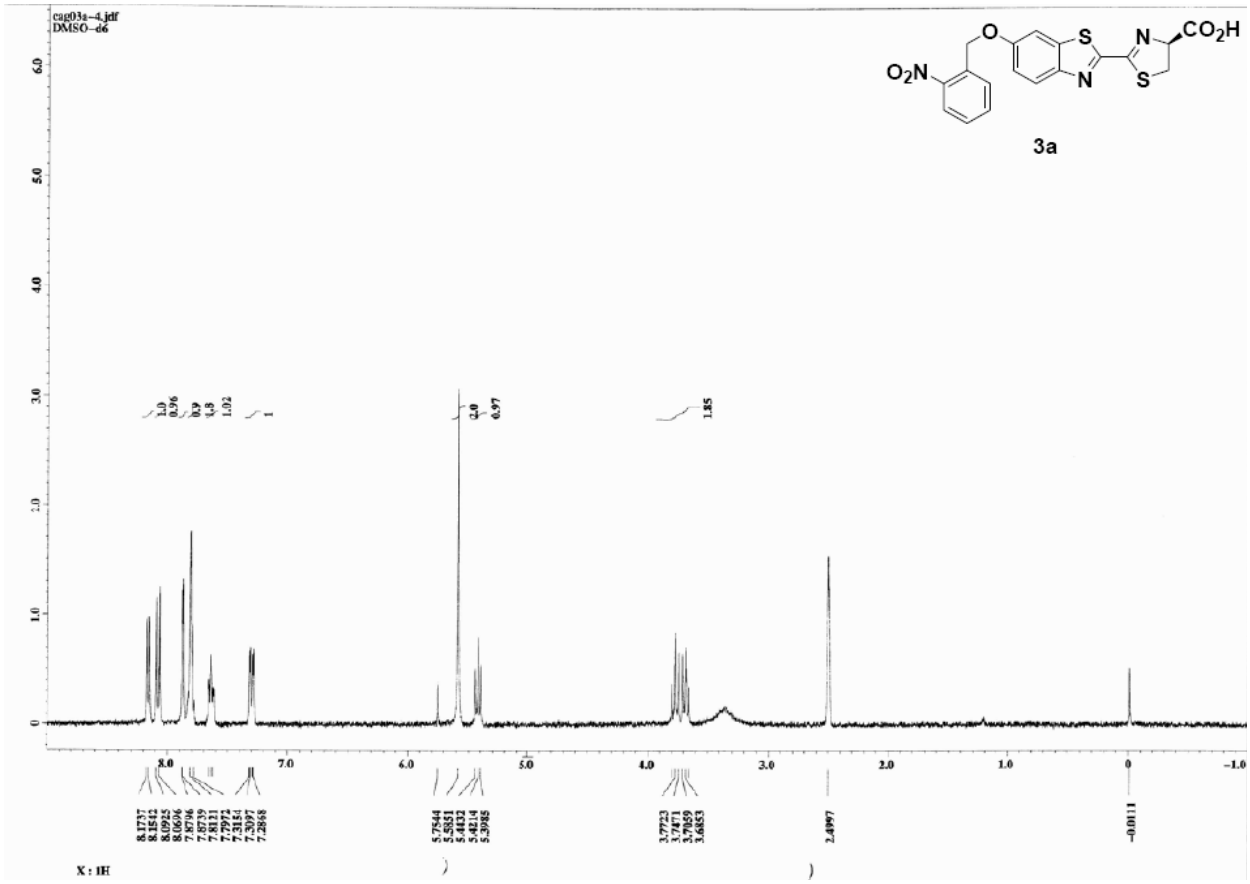
fLuc transfected C6 cells were injected subcutaneously into the left ear and right shoulder of a nude mice. After ten days implantation, the mice were subsequently anesthesized in a chamber filled with 2% isofluorane in oxygen, and then transferred to the light-tight chamber of IVIS 200 (Xenogen, Alameda, CA). After 10 min, "caged" luciferin derivatives were injected via tail vein or intraperitoneally and the images were acquired sequentially for indicated periods of time (about 30 mins). In the typical experiments, the mice were injected with substrates and followed by 4 minutes UV irradiation. Then the bioluminescent scanning experiments were conducted at the different time intervals. As control, the similar imaging experiments were also carried out for the mice which were administrated by intraperitoneal injection of the caged substrates but without UV illumination. In comparison, the same amount of D-luciferin was also injected to each mouse to identify the fLuc activity.

Reference:

(1) Z. Diwu, C. L. Zhang, D. H. Klaubert, R. P. Haugland, *J. Photochem. Photobiol. A*: 2000, **131**, 95-100.

(2) R. T. Cummings, G. A. Krafft, *Tetrahedron Lett.*, 1988, **29(1)**, 65-68.

(3) Y. H. Choi, F. Liu, J. S. Kim, Y. K. Choi, J. S. Park, S. W. Kim, *J. Control. Rel.*, 1998, **54**, 39-48.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3a
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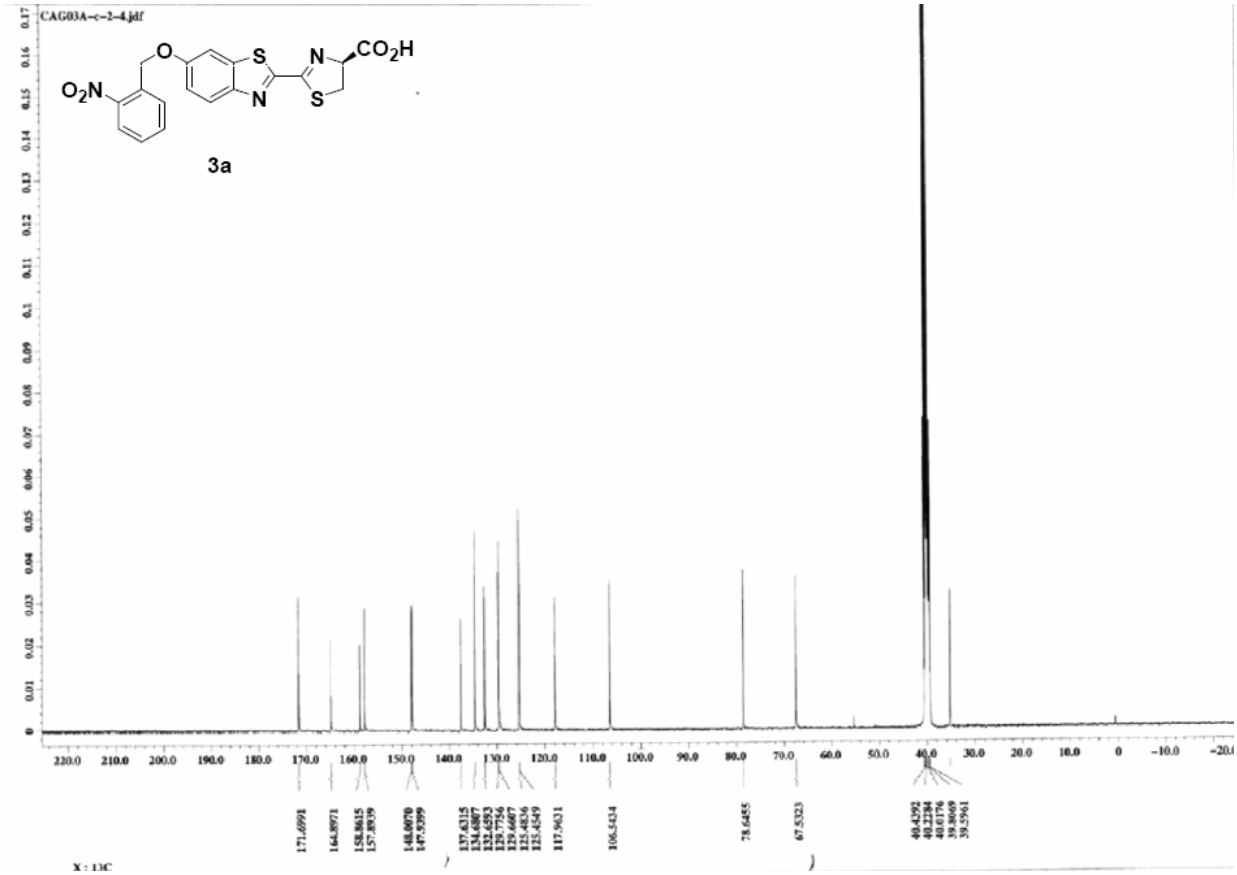
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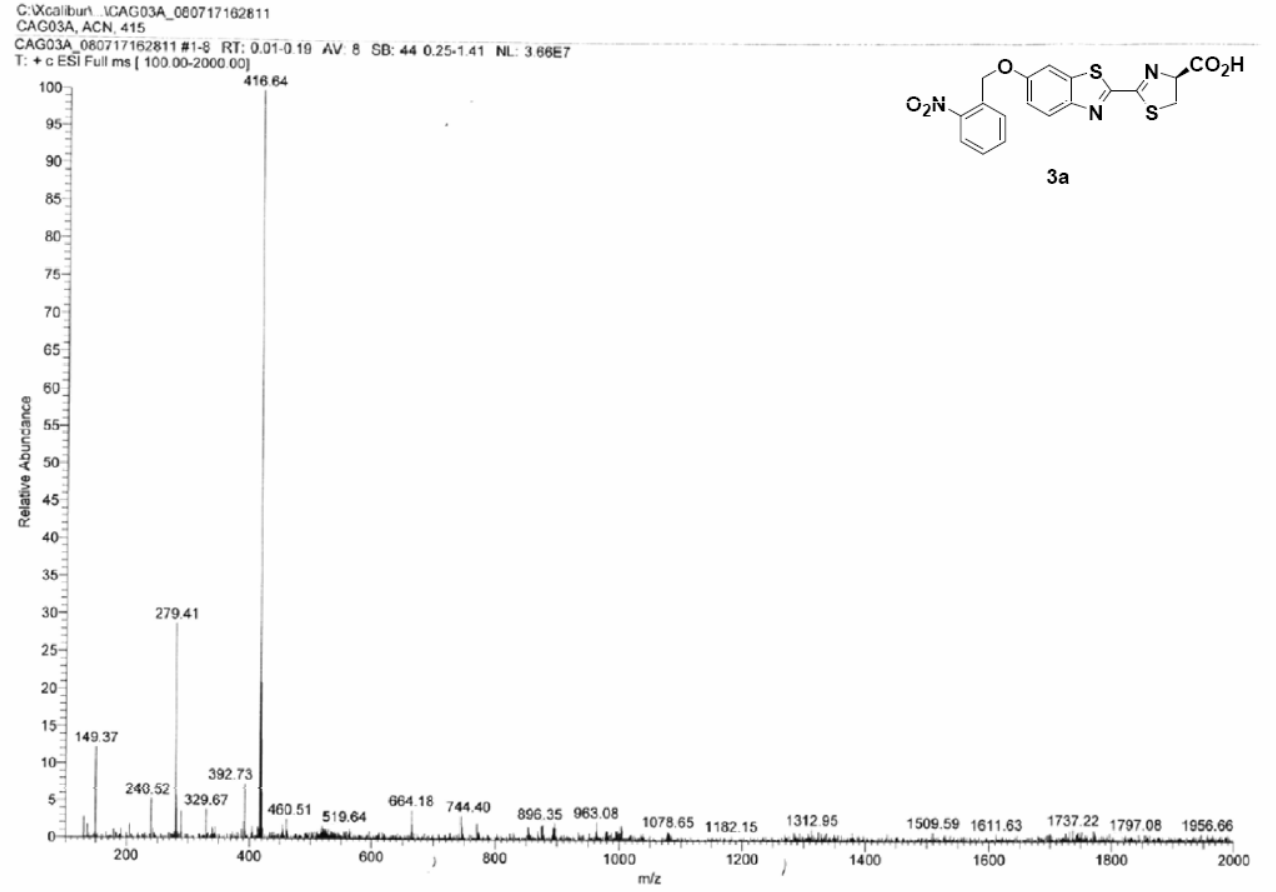
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Relative Abundance
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240.52
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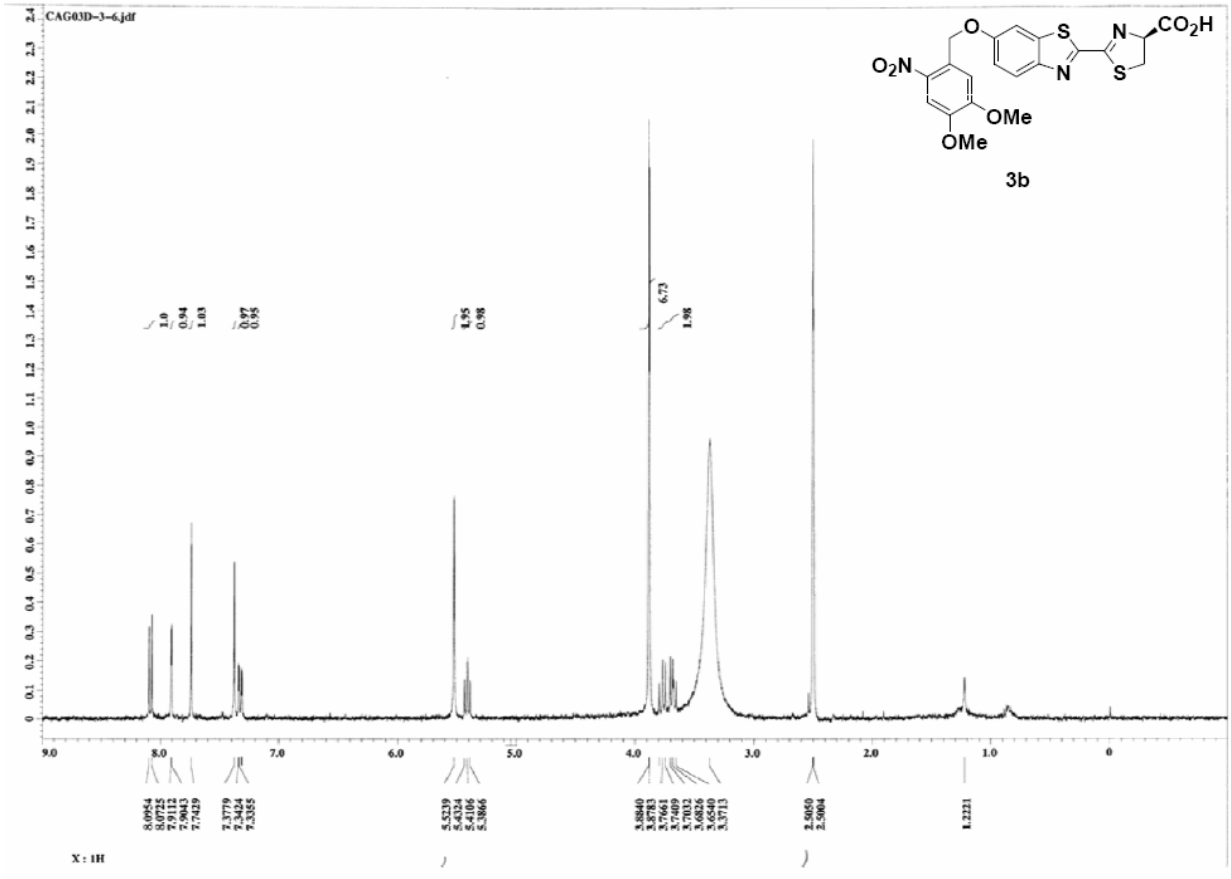
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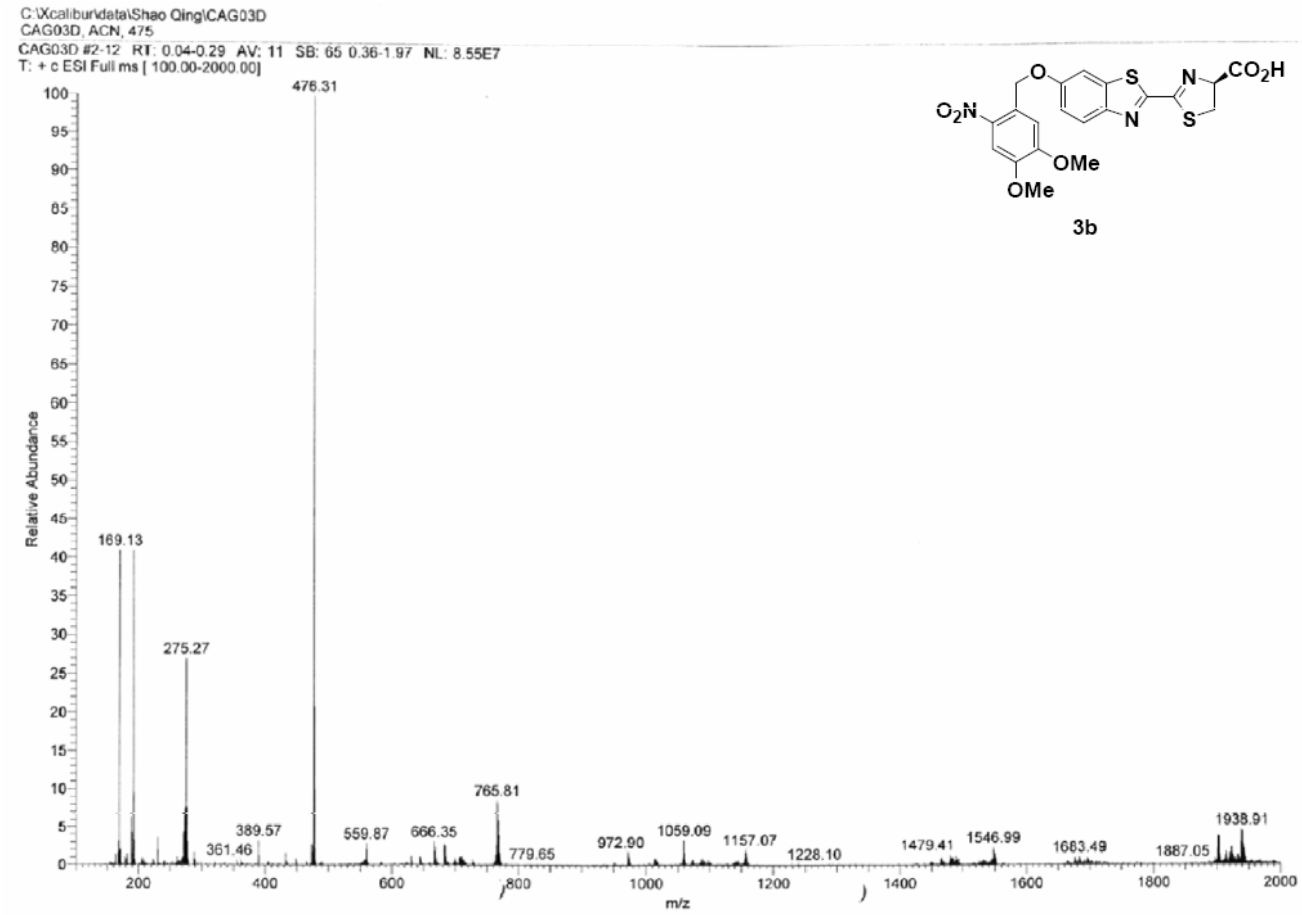
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Relative Abundance
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OMe
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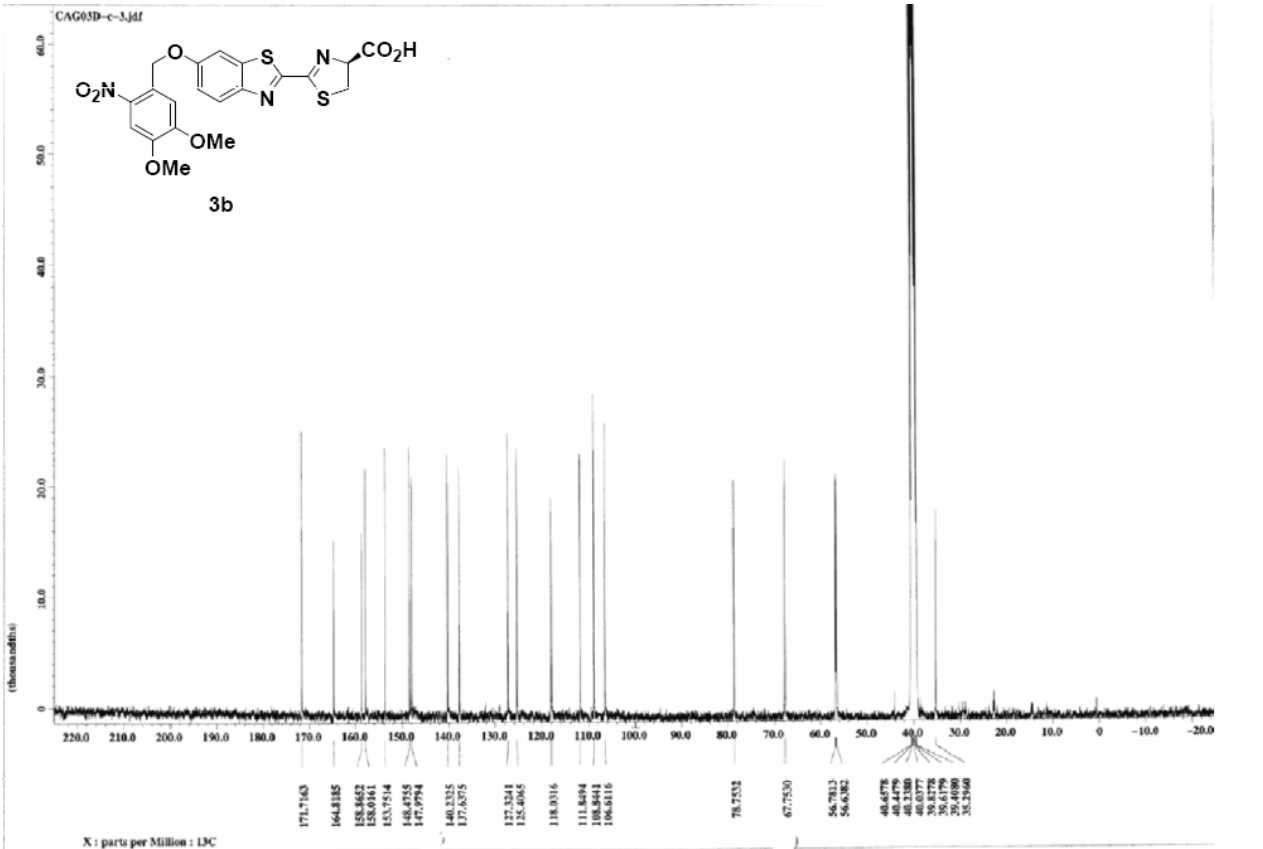
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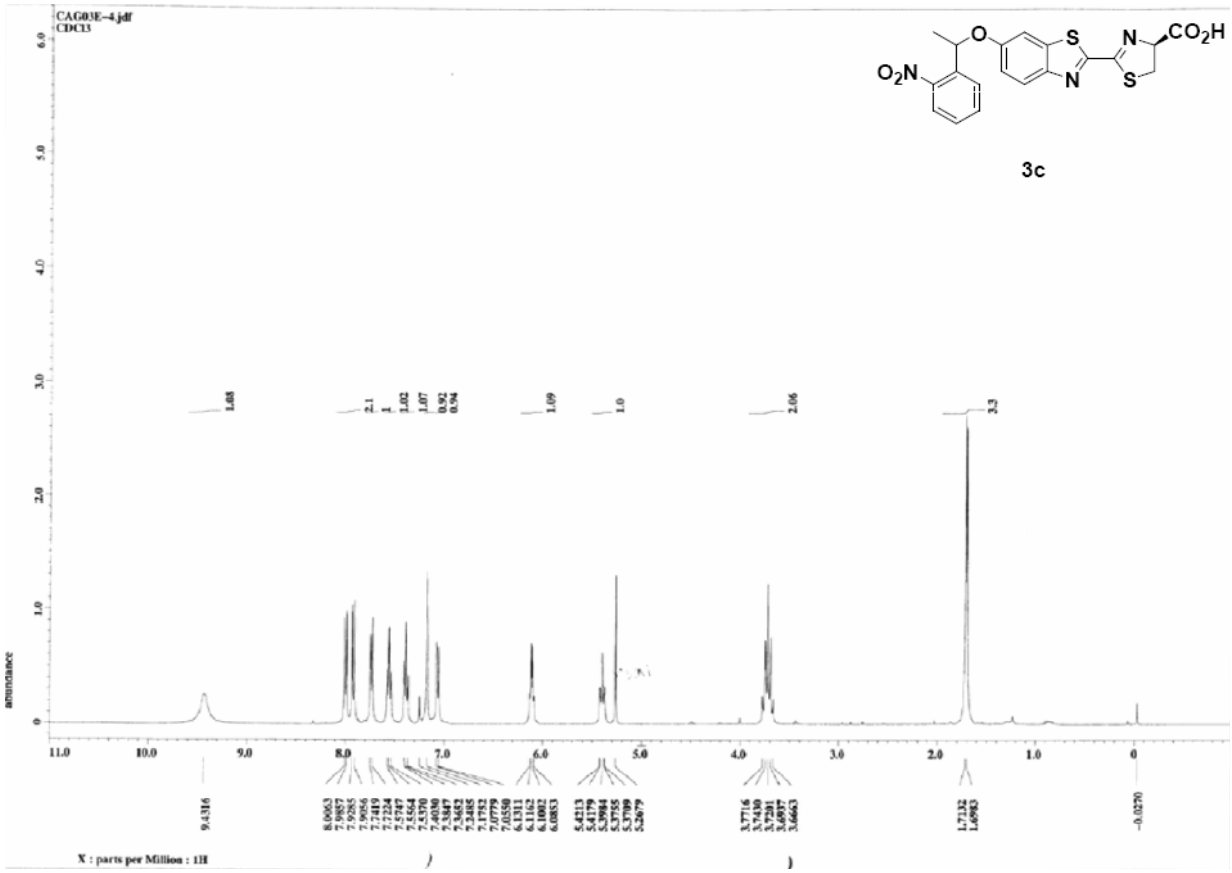
O2N
OMe
OMe
CO2H
3b
X : parts per Million : 13C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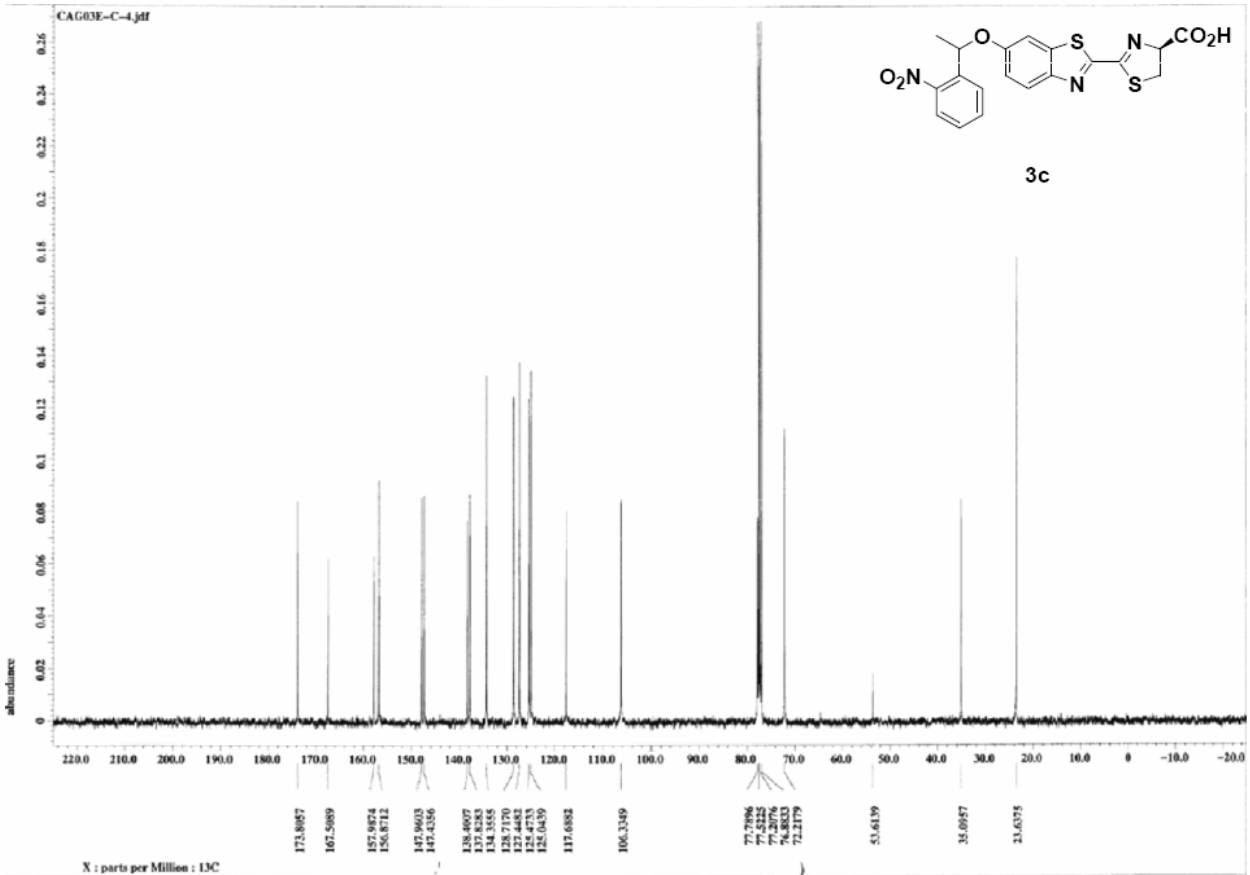
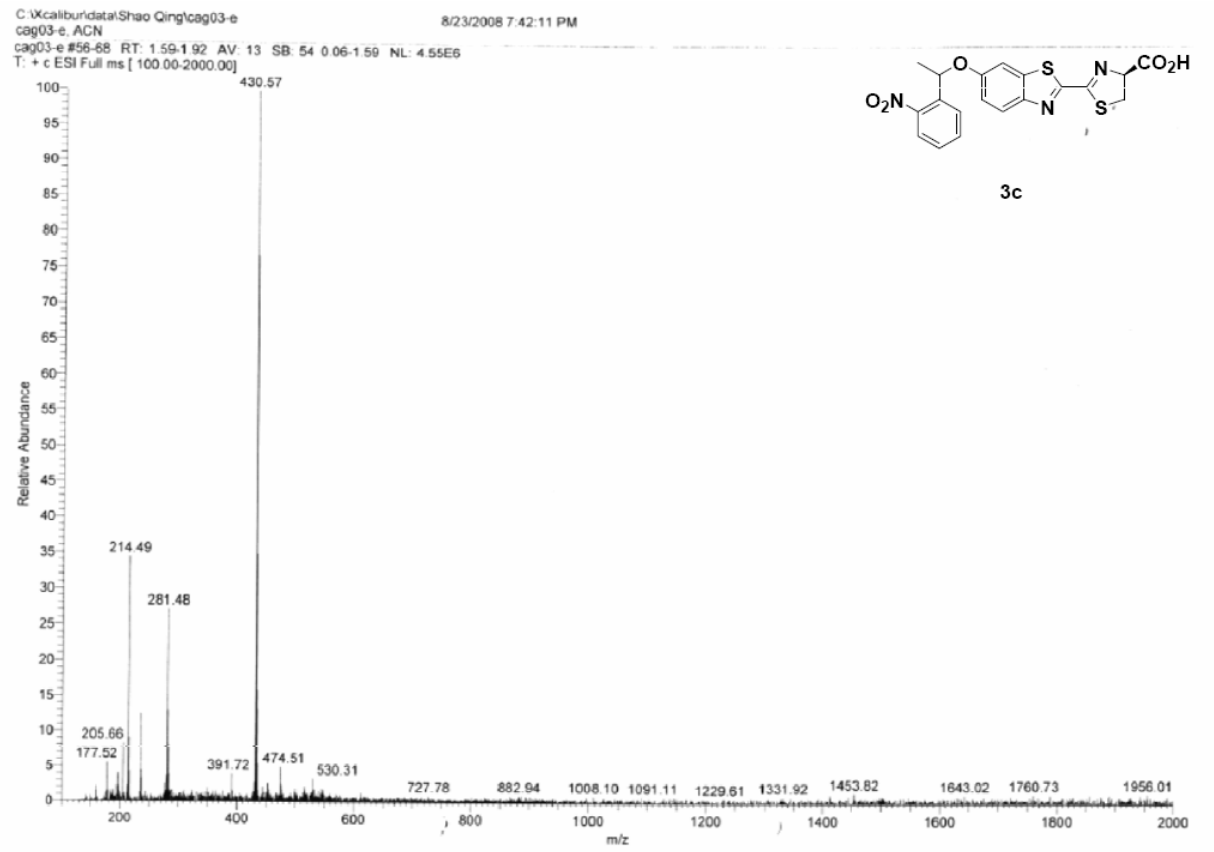
CDCl3
3c
abundance
X : parts per Million : 1H


CAG03E-C-4.jdf
3c
abundance
X : parts per Million : 13C


C:\Xcalibur\data\Shao Qing\cag03-e
cag03-e, ACN
8/23/2008 7:42:11 PM
cag03-e #56-68 RT: 1.59-1.92 AV: 13 SB: 54 0.06-1.59 NL: 4.55E6
T: + c ESI Full ms [100.00-2000.00]
Relative Abundance
m/z
430.57
214.49
281.48
205.66
177.52
391.72
474.51
530.31
727.78
882.94
1008.10
1091.11
1229.61
1331.92
1453.82
1643.02
1760.73
1956.01
O2N
O
S
N
CO2H
3c