

## SUPPORTING INFORMATION

### Prolonged bioluminescence imaging in living cells and mice using novel pro-substrates for *Renilla* luciferase

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## 1. Synthesis

### General Procedure 1

Under argon the compound **1** (1 equiv.) was dissolved in anhydrous pyridine. The corresponding acid anhydride or acyl chloride was added (3-10 equiv.) and the reaction mixture stirred at 30 °C until starting material was consumed. The reaction mixture was firstly diluted with ethylacetate, and then washed three times with cold saturated copper sulfate solution. The extract was washed successively with brine solution and concentrated in vacuo. The crude product was purified by recrystallization from methanol.

### General Procedure 2

Under argon, to a solution of a mixture containing compound **1** (1 equiv.), potassium carbonate (1 equiv.) and potassium iodide (1 equiv.) in anhydrous DMF was added chloromethylpivalate or other (5-10 equiv.). The mixture stirred at 30 °C until complete. Water was added and product was extracted with ethyl acetate. The organic layer was washed with brine, dried and concentrated in vacuo. The residue was purified by recrystallization from methanol to afford the pure product.

**Compound 2:** The title compound **2** was synthesized from **1** (123 mg, 0.3 mmol, 1 equiv.) and acetic anhydride (85  $\mu$ L, 0.9 mmol, 3 equiv.) according to General Procedure 1. Pale pink solid (76.5 mg, 56.5%); Mp.171-173 °C.  $^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  8.69 (s, 1H), 7.85 – 7.66 (m, 4H), 7.62 – 7.53 (m, 3H), 7.40 – 7.14 (m, 8H), 4.08 (s, 2H), 2.40 (s, 3H).  $^{13}\text{C}$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  168.64 (1C), 150.62 (1C), 138.93 (1C), 137.36 (1C), 136.27 (2C), 136.10 (1C), 134.96 (1C), 131.30 (1C), 130.02 (1C), 129.68 (2C), 129.63 (2C), 129.32 (1C), 129.01 (2C), 128.96 (2C), 128.85 (1C), 127.41 (1C), 126.74 (2C), 126.09 (1C), 109.72 (1C), 32.87 (1C), 20.83 (1C). ESI-HRMS calcd.: 451.1354, found: 452.1433(M+H<sup>+</sup>), 474.1243 (M+Na<sup>+</sup>).

**Compound 3:** The title compound **3** was synthesized from **1** (123 mg, 0.3 mmol, 1 equiv.) and isobutyryl chloride (155  $\mu$ L, 1.5 mmol, 5 equiv.) according to General Procedure 1. Pale pink solid (64.8 mg, 45.1%); Mp.104-106 °C.  $^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  8.57 (s, 1H), 7.73 (td,  $J$  = 7.9, 3.0 Hz, 4H), 7.61 – 7.54 (m, 3H), 7.39 – 7.28 (m, 5H), 7.23 (dd,  $J$  = 15.7, 7.2 Hz, 3H), 4.04 (s, 2H), 3.09 – 3.00 (m, 1H), 1.27 (d,  $J$  = 7.0 Hz, 6H).  $^{13}\text{C}$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  175.28 (1C), 139.71 (1C), 138.26 (1C), 137.13 (2C), 136.92 (1C), 135.62 (1C), 132.09 (1C), 130.85 (1C), 130.49 (2C), 130.45 (1C), 129.92 (2C), 129.84 (1C), 129.79 (2C), 129.69 (3C), 128.14 (1C), 127.57 (1C), 126.93 (2C), 110.25 (1C), 33.69 (1C), 33.14 (1C), 18.89 (2C). ESI-HRMS calcd.: 479.1667, found: 480.1743(M+H<sup>+</sup>).

**Compound 4:** The title compound **4** was synthesized from **1** (123 mg, 0.3 mmol, 1 equiv.) and cyclohexanecarboxylic acid chloride (120  $\mu$ L, 0.9 mmol, 3 equiv) according to General Procedure 1. Pale pink solid (106.1 mg, 68.1%); Mp.95-97 °C.  $^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  8.53 (s, 1H), 7.81 – 7.67 (m, 4H), 7.61 – 7.51 (m, 3H),

7.41 – 7.29 (m, 5H), 7.29 – 7.14 (m, 3H), 4.03 (s, 2H), 2.85 (tt,  $J = 10.8, 3.6$  Hz, 1H), 2.11 – 1.95 (m, 2H), 1.79 – 1.70 (m, 2H), 1.64 (dd,  $J = 8.3, 3.8$  Hz, 1H), 1.56 – 1.44 (m, 2H), 1.42 – 1.31 (m, 2H), 1.22 (dd,  $J = 16.3, 6.9$  Hz, 1H).  $^{13}\text{C}$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  173.01 (1C), 150.70 (1C), 138.85 (1C), 137.45 (1C), 136.27 (2C), 136.10 (1C), 134.76 (1C), 131.33 (1C), 130.03 (1C), 129.68 (1C), 129.65 (1C), 129.13 (2C), 129.03 (2C), 128.99 (2C), 128.89 (2C), 127.41 (1C), 126.78 (1C), 126.16 (2C), 109.54 (1C), 41.99 (1C), 32.91 (1C), 28.57 (2C), 25.63 (1C), 25.16 (2C). ESI-HRMS calcd.: 519.1980, found: 520.2063 ( $\text{M}+\text{H}^+$ ).

**Compound 5:** The title compound **5** was synthesized from **1** (150 mg, 0.367 mmol, 1 equiv.) and trimethylacetyl chloride (225  $\mu\text{L}$ , 1.83 mmol, 5 equiv.) according to General Procedure 1. Pale pink solid (143 mg, 79.0%); Mp. 155–157  $^{\circ}\text{C}$ .  $^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  8.26 (s, 1H), 7.81 – 7.68 (m, 4H), 7.63 – 7.53 (m, 3H), 7.40 – 7.28 (m, 5H), 7.23 (dd,  $J = 14.6, 7.2$  Hz, 3H), 4.04 (s, 2H), 1.37 (s, 8H).  $^{13}\text{C}$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  176.82 (1C), 151.75 (1C), 139.65 (1C), 138.36 (1C), 137.12 (2C), 136.86 (1C), 135.63 (1C), 132.14 (1C), 130.85 (1C), 130.49 (2C), 130.45 (1C), 129.87 (4C), 129.83 (1C), 129.70 (2C), 128.12 (1C), 127.59 (1C), 127.04 (2C), 109.77 (1C), 39.69 (1C), 33.18 (1C), 27.20 (3C). ESI-HRMS calcd.: 493.1824, found: 494.1897 ( $\text{M}+\text{H}^+$ ).

**Compound 6:** The title compound **6** was synthesized from **1** (123 mg, 0.3 mmol, 1 equiv.) and 1-adamantanecarbonyl chloride (357.6 mg, 1.8 mmol, 6 equiv.) according to General Procedure 1. Pale pink solid (103.4 mg, 60.3%); Mp. 148–150  $^{\circ}\text{C}$ .  $^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  8.20 (s, 1H), 7.68 (dd,  $J = 6.7, 1.5$  Hz, 4H), 7.54 – 7.49 (m, 3H), 7.34 – 7.24 (m, 5H), 7.19 (dd,  $J = 11.8, 7.1$  Hz, 3H), 3.98 (s, 2H), 2.02 (s, 9H), 1.70 (s, 6H).  $^{13}\text{C}$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  173.33 (1C), 150.83 (1C), 138.79 (1C), 137.53 (1C), 136.27 (2C), 136.03 (1C), 134.78 (1C), 131.38 (1C), 130.04 (1C), 129.68 (2C), 129.64 (1C), 129.13 (2C), 129.06 (1C), 129.03 (2C), 128.91 (2C), 127.37 (1C), 126.80 (1C), 126.27 (2C), 109.03 (1C), 49.07 (1C), 42.06 (1C), 41.25 (1C), 38.11 (2C), 36.11 (2C), 32.94 (1C), 27.49 (2C). ESI-HRMS calcd.: 571.2293, found: 572.2275 ( $\text{M}+\text{H}^+$ ).

**Compound 7:** The title compound **7** was synthesized from **1** (150 mg, 0.367 mmol, 1 equiv.) and chloromethyl butyrate (382  $\mu\text{L}$ , 3 mmol, 8 equiv.) according to General Procedure 2. Yellow viscous powder (86.4 mg, 46.2%); Mp. 97–99  $^{\circ}\text{C}$ .  $^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  8.62 (s, 1H), 7.77 (dd,  $J = 8.0, 1.5$  Hz, 2H), 7.72 – 7.68 (m, 2H), 7.56 (dd,  $J = 5.0, 1.8$  Hz, 3H), 7.36 – 7.30 (m, 7H), 7.26 – 7.20 (m, 1H), 5.79 (s, 2H), 4.11 (s, 2H), 2.26 (t,  $J = 7.3$  Hz, 2H), 1.52 – 1.38 (m, 2H), 0.76 (t,  $J = 7.4$  Hz, 3H).  $^{13}\text{C}$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  173.12 (1C), 151.08 (1C), 140.39 (1C), 138.00 (1C), 137.06 (2C), 136.75 (1C), 134.45 (1C), 131.28 (1C), 130.76 (1C), 130.45 (2C), 130.06 (1C), 129.87 (2C), 129.80 (2C), 129.70 (1C), 129.62 (2C), 128.32 (1C), 127.44 (1C), 126.74 (2C), 110.12 (1C), 90.37 (1C), 35.66 (1C), 32.58 (1C), 17.98 (1C), 13.73 (1C). ESI-HRMS calcd.: 509.1773, found: 510.1854 ( $\text{M}+\text{H}^+$ ), 532.1666 ( $\text{M}+\text{Na}^+$ ).

**Compound 8:** The title compound **8** was synthesized from **1** (123 mg, 0.3 mmol, 1 equiv.) and chloromethyl pivalate (432  $\mu\text{L}$ , 3 mmol, 10 equiv.) according to General

Procedure 2. Pale pink solid (76.5 mg, 48.7%); Mp.93-95 °C. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 8.57 (s, 1H), 7.76 (dd, *J* = 8.0, 1.5 Hz, 2H), 7.73 – 7.66 (m, 2H), 7.59 – 7.51 (m, 3H), 7.38 – 7.28 (m, 7H), 7.26 – 7.18 (m, 1H), 5.83 (s, 2H), 4.15 (s, 2H), 1.08 (s, 9H). <sup>13</sup>C NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 178.11 (1C), 151.49 (1C), 140.34 (1C), 137.95 (1C), 137.04 (2C), 137.01 (1C), 136.89 (1C), 134.20 (1C), 131.21 (1C), 130.75 (1C), 130.44 (2C), 129.88 (2C), 129.80 (2C), 129.71 (1C), 129.65 (2C), 128.30 (1C), 127.47 (1C), 126.70 (2C), 110.08 (1C), 91.25 (1C), 38.90 (1C), 32.72 (1C), 27.11 (3C). ESI-HRMS calcd.: 523.1930, found: 524.2000 (M+H<sup>+</sup>)

Compound **9**: The title compound **9** was synthesized from **1** (123 mg, 0.3 mmol, 1 equiv.) and diethyl pyrocarbonate (434 μL, 3 mmol, 10 equiv.) according to General Procedure 1. Pale pink solid (53.4 mg, 37.0%); Mp.150-152 °C. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 8.83 (s, 1H), 7.78 (dd, *J* = 6.4, 2.9 Hz, 2H), 7.72 (dd, *J* = 6.4, 2.9 Hz, 2H), 7.58-7.54 (m, 3H), 7.38 – 7.19 (m, 8H), 4.29 (q, *J* = 7.1 Hz, 2H), 4.10 (s, 1H), 1.31 (t, *J* = 7.1 Hz, 3H). <sup>13</sup>C NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 151.35 (1C), 150.69 (1C), 138.65 (1C), 137.51 (1C), 136.28 (2C), 136.07 (1C), 134.75 (1C), 131.04 (1C), 130.06 (1C), 129.94 (1C), 129.71 (2C), 129.22 (2C), 129.04 (1C), 128.97 (2C), 128.89 (2C), 127.36 (1C), 126.81 (1C), 126.17 (2C), 109.95 (1C), 66.83 (1C), 32.98 (1C), 14.25 (1C). ESI-HRMS calcd.: 481.1640, found: 482.1588 (M+H<sup>+</sup>).

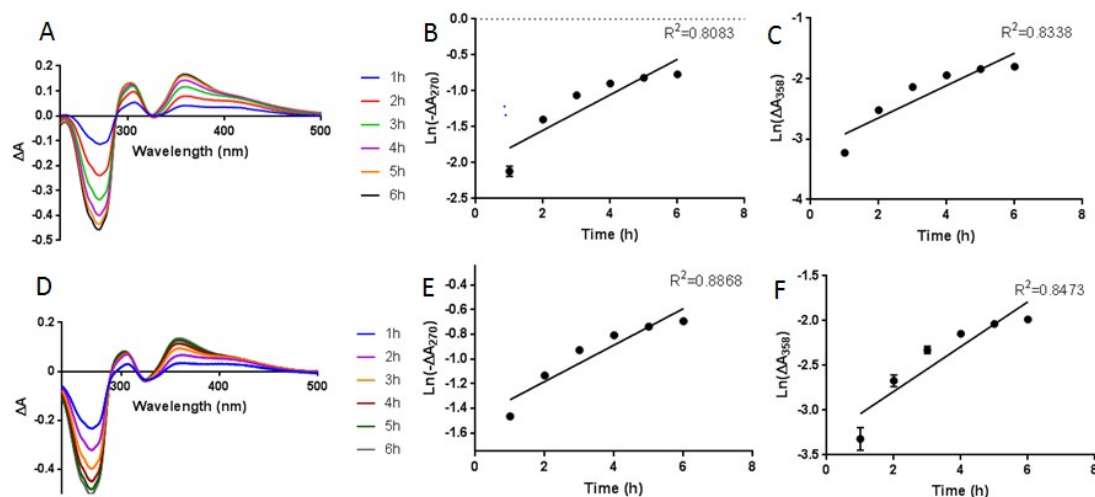
Compound **10**: The title compound **10** was synthesized from **1** (123 mg, 0.3 mmol, 1 equiv.) and di-tert-butyl dicarbonate (740 μL, 3 mmol, 10 equiv.) according to General Procedure 1. Pale pink solid (50.4 mg, 33.0%); Mp:135-137 °C. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 8.74 (s, 1H), 7.79 (d, *J* = 7.8 Hz, 2H), 7.72 (dd, *J* = 5.8, 2.4 Hz, 2H), 7.57 (d, *J* = 5.0 Hz, 3H), 7.39 – 7.20 (m, 8H), 4.08 (s, 2H), 1.49 (s, 9H). <sup>13</sup>C NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 151.58 (1C), 150.33 (1C), 139.61 (1C), 138.29 (1C), 137.08 (2C), 136.91 (1C), 135.55 (1C), 131.82 (1C), 130.82 (1C), 130.78 (1C), 130.49 (2C), 129.90 (2C), 129.82 (1C), 129.79 (2C), 129.69 (2C), 128.18 (1C), 127.58 (1C), 126.94 (2C), 110.34 (1C), 86.95 (1C), 33.06 (1C), 27.40 (3C). ESI-HRMS calcd.: 509.1773, found: 510.1842 (M+H<sup>+</sup>)

Compound **11**: The title compound **11** was synthesized from **1** (123 mg, 0.3 mmol, 1 equiv.) and chloromethyl isopropyl carbonate (398.8 μL, 3 mmol, 10 equiv.) according to General Procedure 2. Pale pink solid (77.7 mg, 49.3%); Mp.105-107 °C. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 8.58 (s, 1H), 7.75 (d, *J* = 6.5 Hz, 2H), 7.70 (dd, *J* = 6.4, 2.8 Hz, 2H), 7.58 – 7.54 (m, 3H), 7.35-7.31 (m, 7H), 7.22 (td, *J* = 8.3, 4.0 Hz, 1H), 5.78 (s, 2H), 4.81-4.75 (m, 1H), 4.09 (s, 2H), 3.17 (d, *J* = 5.2 Hz, 2H), 1.15 (d, *J* = 6.2 Hz, 6H). <sup>13</sup>C NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 154.34 (1C), 151.51 (1C), 140.19 (1C), 138.06 (1C), 137.07 (2C), 136.47 (1C), 134.73 (1C), 131.27 (1C), 130.80 (1C), 130.47 (2C), 129.94 (2C), 129.81 (2C), 129.75 (1C), 129.64 (2C), 128.24 (1C), 127.48 (1C), 126.76 (2C), 110.14 (1C), 93.30 (1C), 73.80 (1C), 49.37 (1C), 32.64 (1C), 21.80 (2C). ESI-HRMS calcd.: 525.1722, found: 526.1799 (M+H<sup>+</sup>).

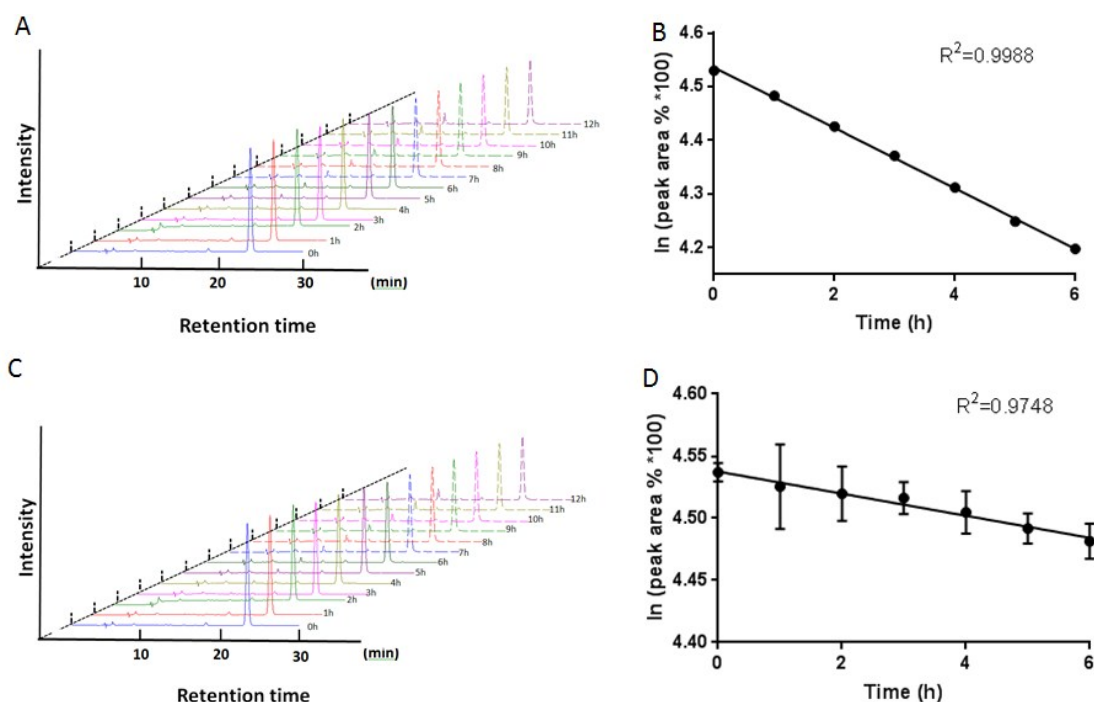
acetyl-BDC: The title compound acetyl-BDC was synthesized from **1** (50mg, 0.11 mmol, equiv) acetic anhydride (118mg, 1.1 mmol, 10 equiv.) according to General

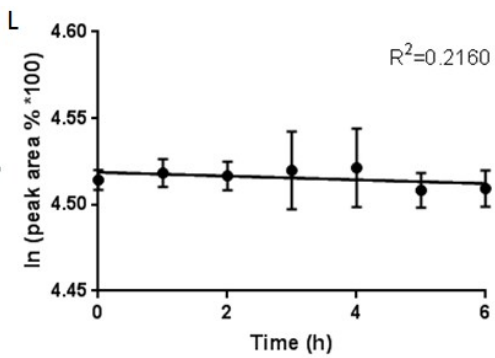
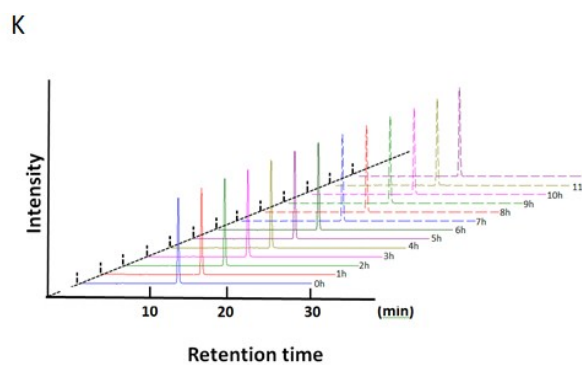
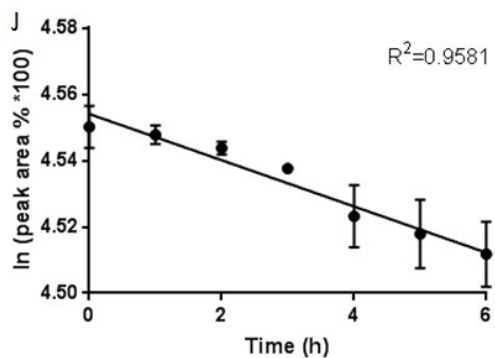
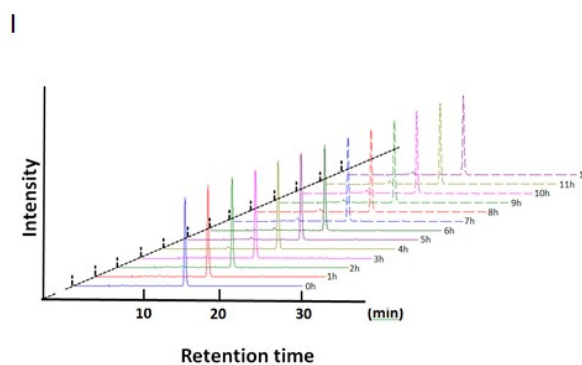
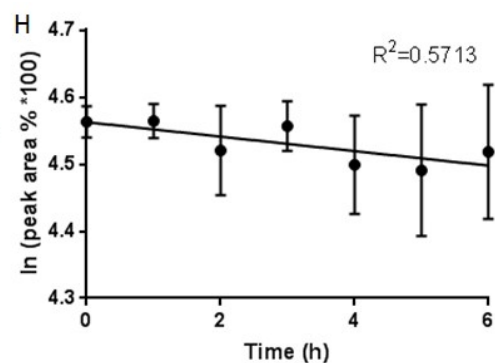
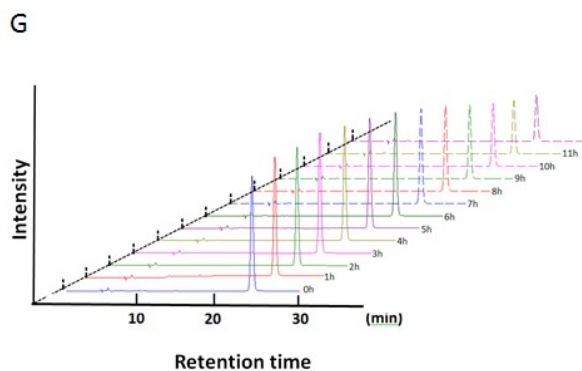
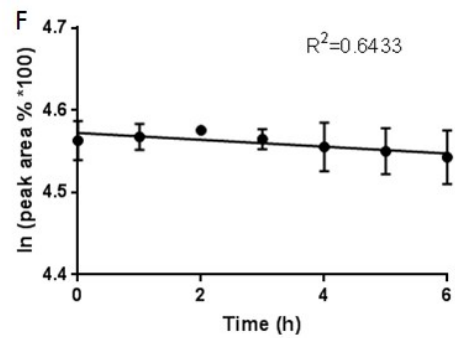
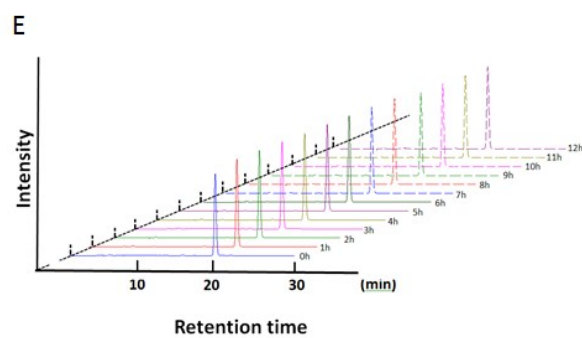
Procedure 1. Yellow oil (15 mg, 27.3%); Purity 95.27%;  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  7.87 (d,  $J = 7.4$  Hz, 2H), 7.77 (s, 1H), 7.61 (d,  $J = 7.4$  Hz, 2H), 7.51 – 7.18 (m, 11H), 4.61 (s, 2H), 4.20 (s, 2H), 2.15 (s, 3H). ESI-HRMS calcd.: 433.1790, found:  $[\text{M}+\text{H}]^+$  434.1862.

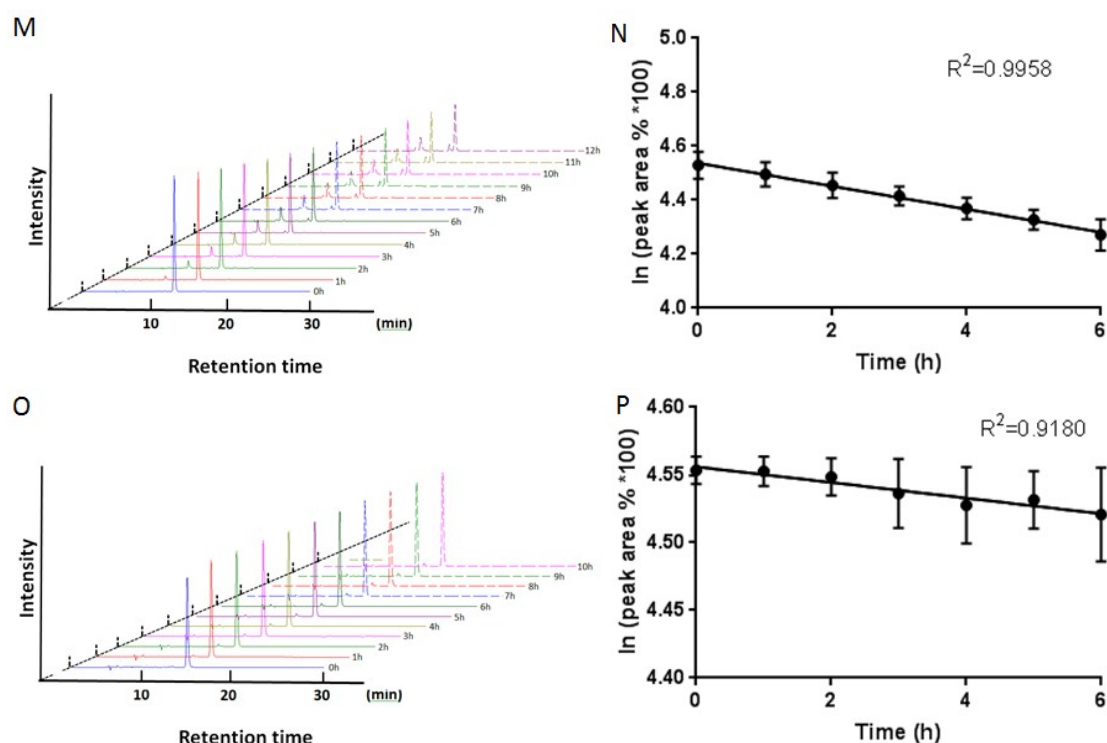
## 2. Stability evaluation in aqueous solution



**Figure S1.** *In vitro* stability study of the luciferin esters (UV): (A) differential absorption spectroscopy (compound **2**); (B) absorbance data analysis at 270 nm (compound **2**); (C) absorbance data analysis at 358 nm (compound **2**); (D) differential absorption spectroscopy (compound **10**); (E) absorbance data analysis at 270 nm (compound **10**); (F) absorbance data analysis at 358 nm (compound **10**).







**Figure S2.** *In vitro* stability study of the luciferin esters (HPLC): (A) HPLC analysis over time (compound **3**); (B) HPLC dynamics data analysis for the first 6 hours (compound **3**); (C) HPLC analysis over time (compound **4**); (D) HPLC dynamics data analysis for the first 6 hours (compound **4**); (E) HPLC analysis over time (compound **5**); (F) HPLC dynamics data analysis for the first 6 hours (compound **5**); (G) HPLC analysis over time (compound **6**); (H) HPLC dynamics data analysis for the first 6 hours (compound **6**); (I) HPLC analysis over time (compound **7**); (J) HPLC dynamics data analysis for the first 6 hours (compound **7**); (K) HPLC analysis over time (compound **8**); (L) HPLC dynamics data analysis for the first 6 hours (compound **8**); (M) HPLC analysis over time (compound **9**); (N) HPLC dynamics data analysis for the first 6 hours (compound **9**); (O) HPLC analysis over time (compound **11**); (P) HPLC dynamics data analysis for the first 6 hours (compound **11**);

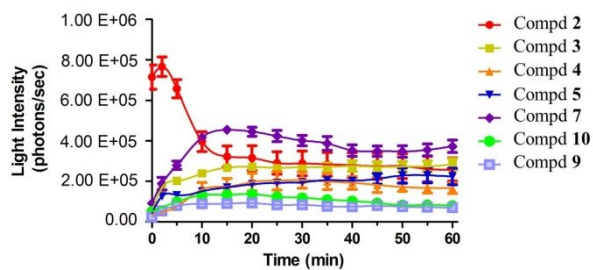
### 3. Cytotoxicity Tests

**Table S1.** Cytotoxicity of the luciferin esters.

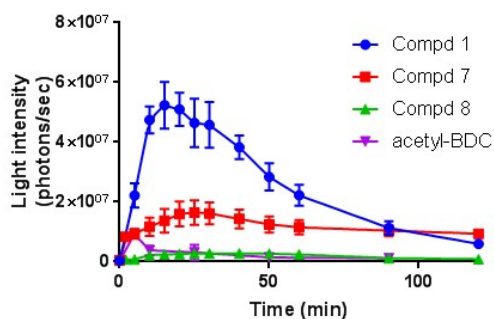
| Compound  | IC <sub>50</sub> ( $\mu\text{M}$ ) |
|-----------|------------------------------------|
| <b>2</b>  | 190.5 $\pm$ 6.12                   |
| <b>3</b>  | 542.4 $\pm$ 11.50                  |
| <b>4</b>  | > 1000                             |
| <b>5</b>  | > 1000                             |
| <b>6</b>  | 782.6 $\pm$ 16.67                  |
| <b>7</b>  | 381.6 $\pm$ 9.36                   |
| <b>8</b>  | > 1000                             |
| <b>9</b>  | 579.6 $\pm$ 13.12                  |
| <b>10</b> | 516.1 $\pm$ 9.57                   |

|    |        |
|----|--------|
| 11 | > 1000 |
|----|--------|

#### 4. Real-time imaging *in cellulo* and *in vivo*



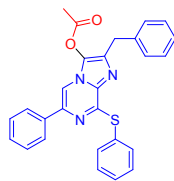
**Figure S3.** ES-2-Rluc cell imaging: the bioluminescence intensity of the luciferin esters in the cells over time.



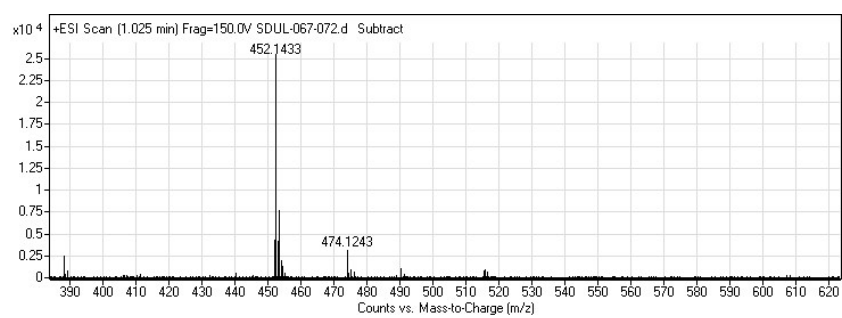
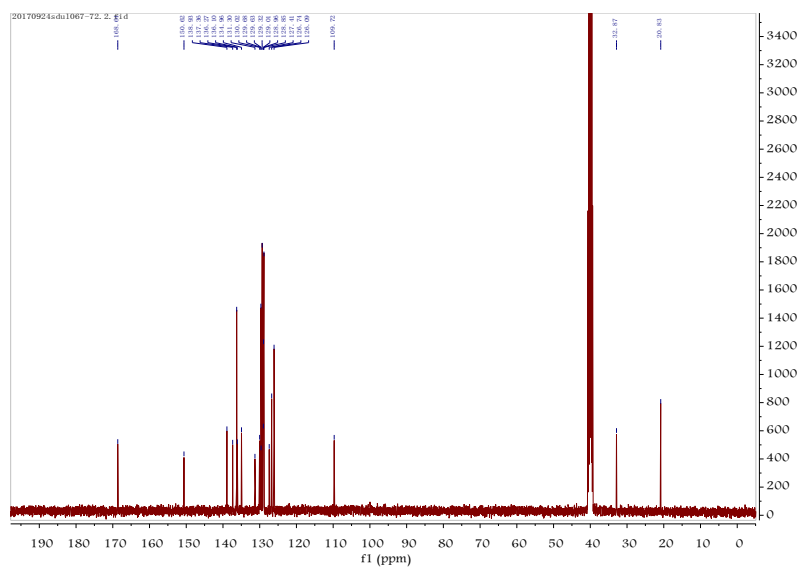
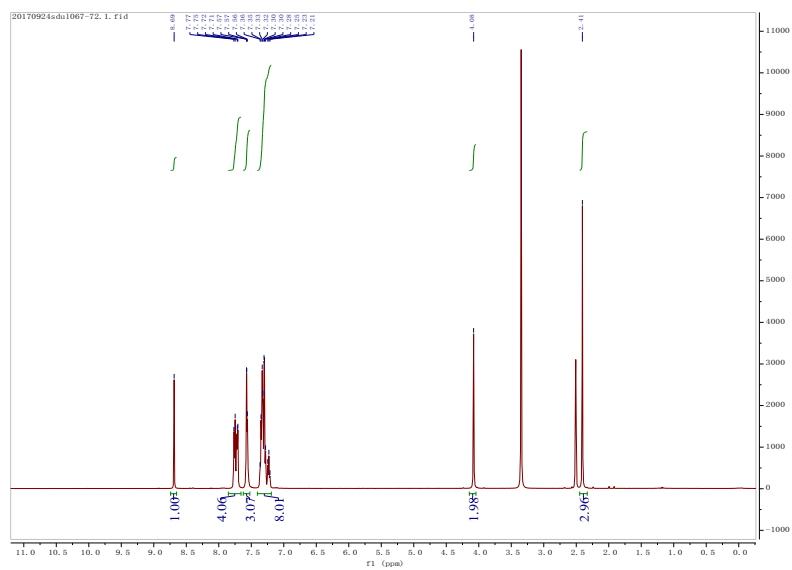
**Figure S4.** The bioluminescence intensity of the free luciferin and the luciferin esters *in vivo* over time.

## 5. $^1\text{H}$ NMR, $^{13}\text{C}$ NMR and HR-MS spectra

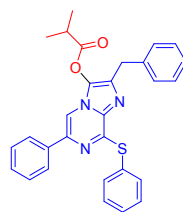
Compound 2:



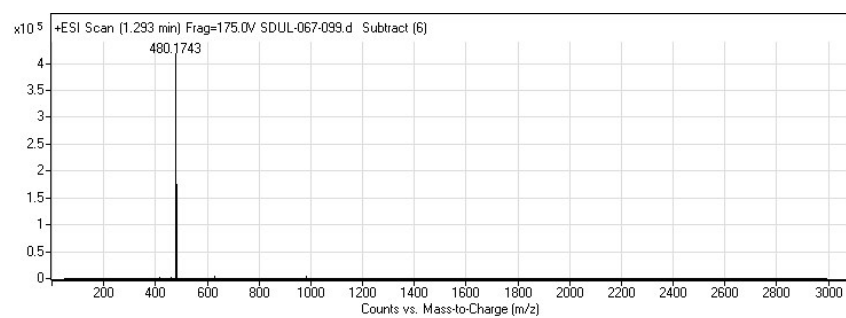
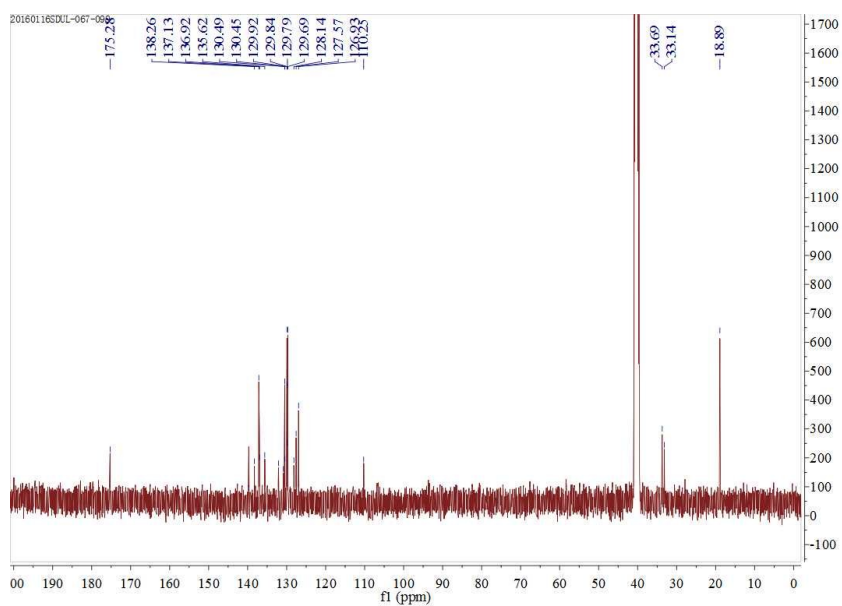
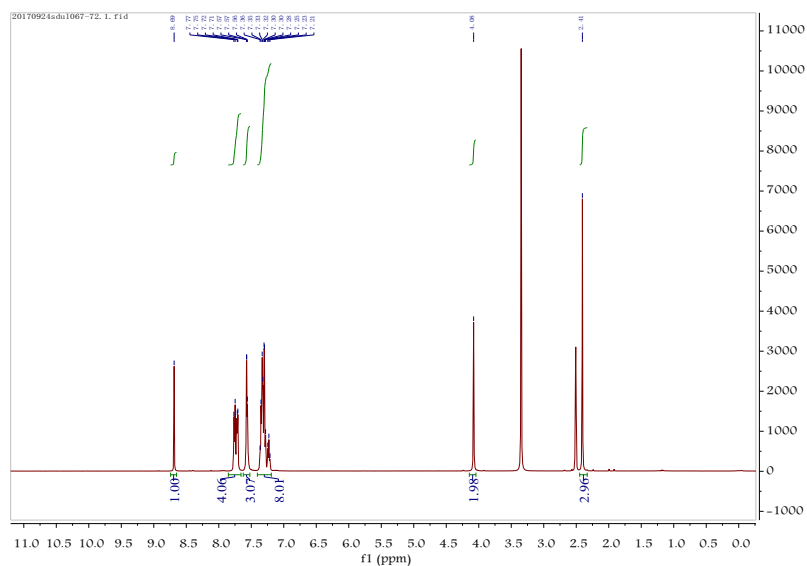
2



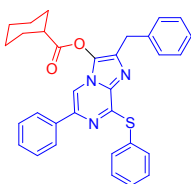
Compound **3**:



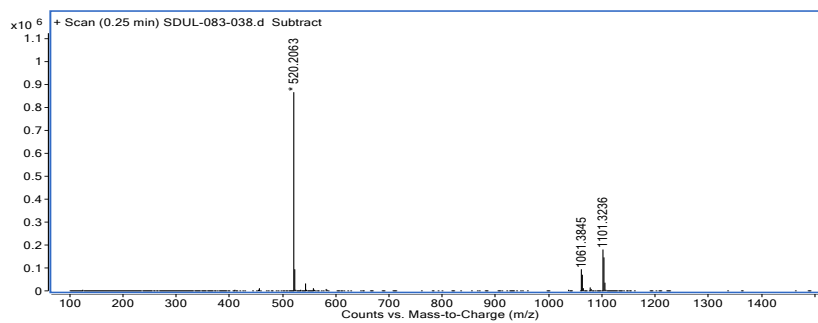
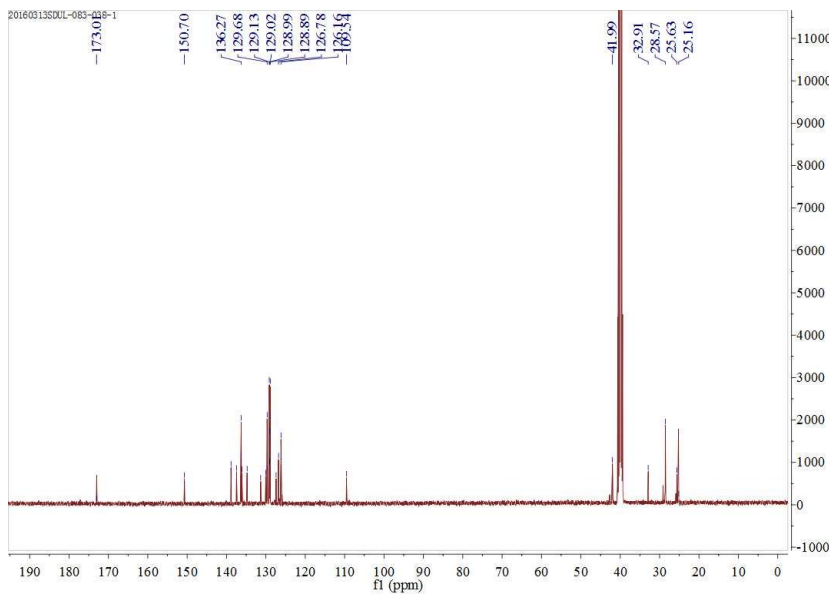
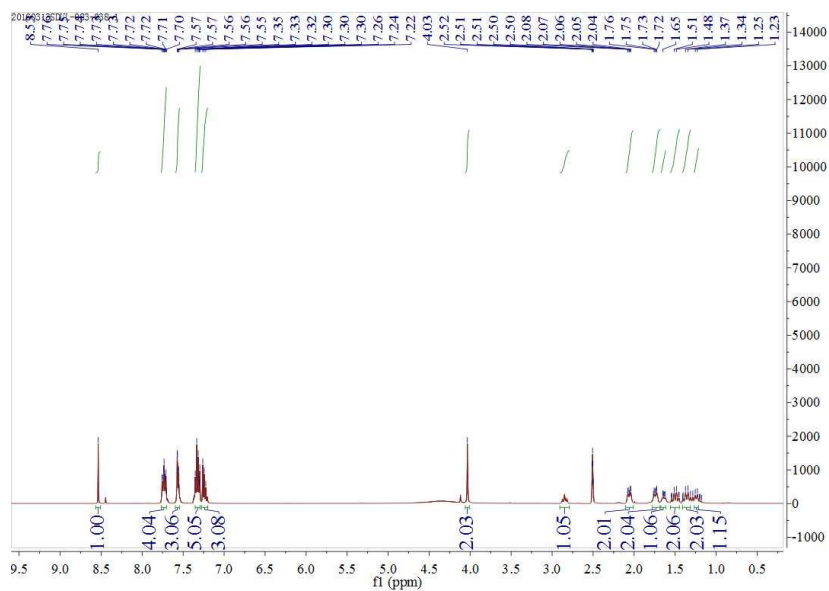
**3**



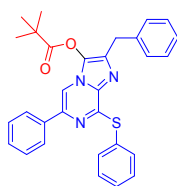
Compound 4:



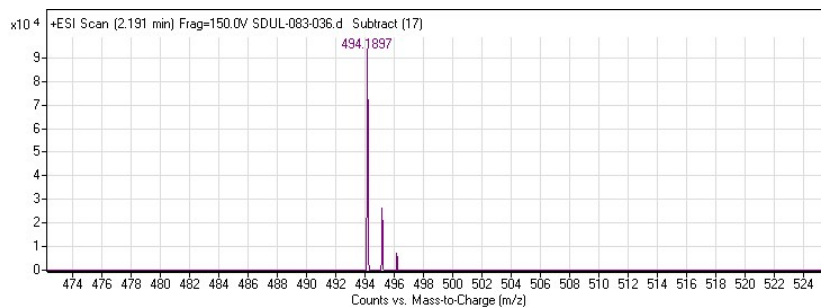
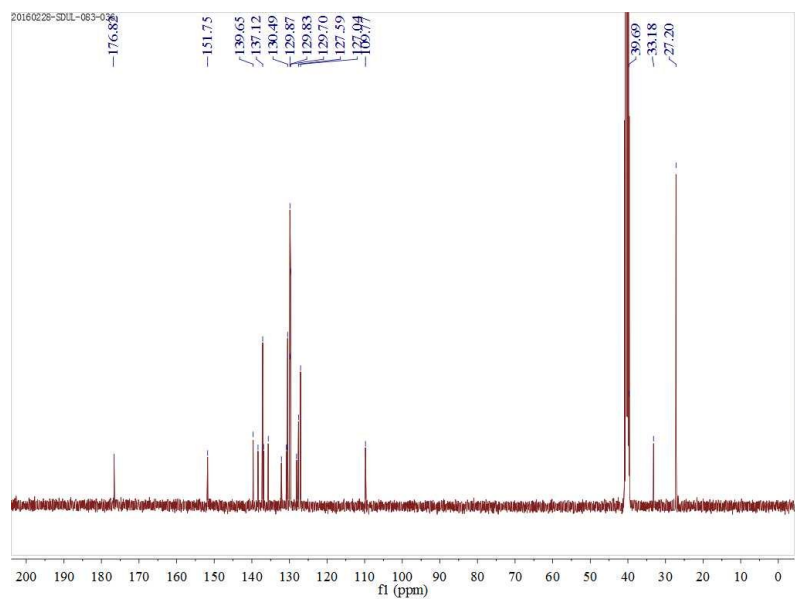
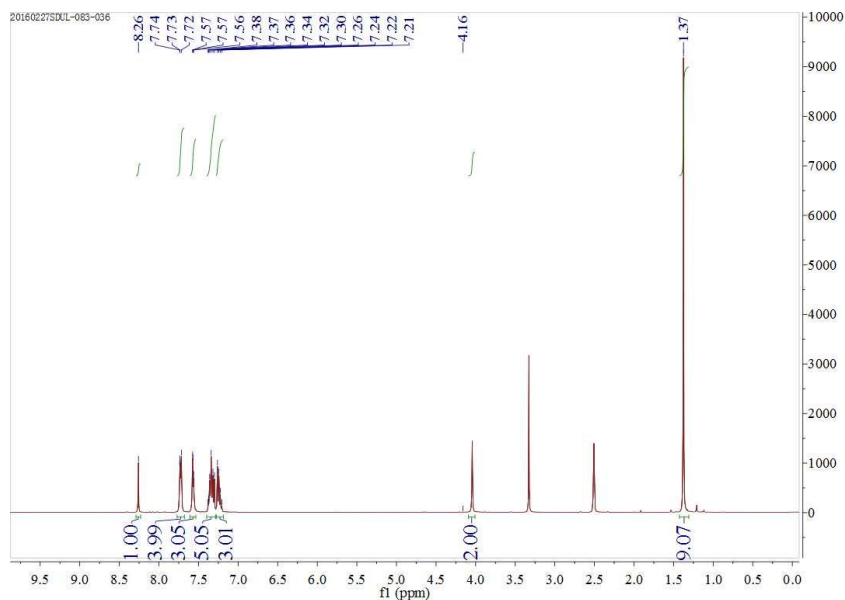
4



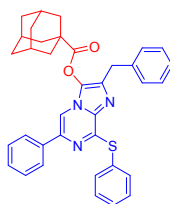
Compound 5:



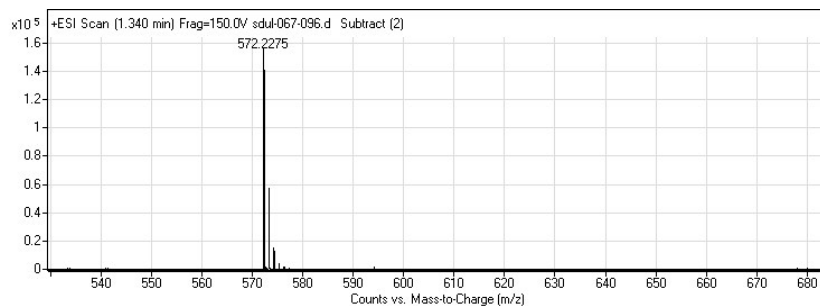
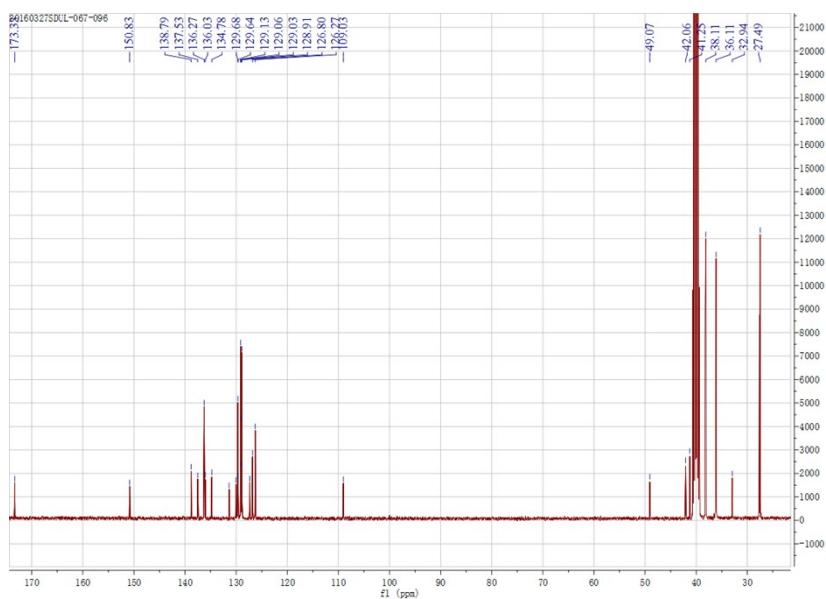
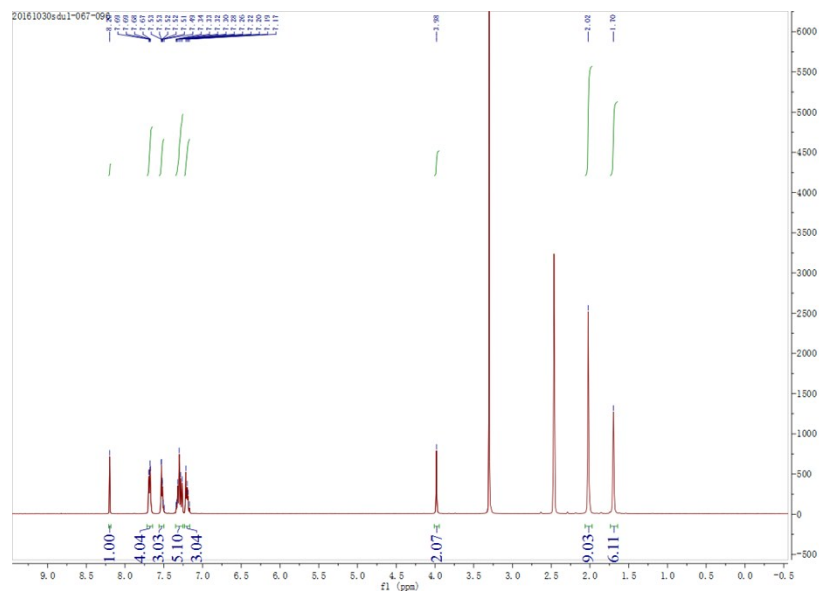
5



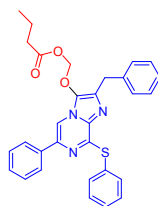
Compound **6**:



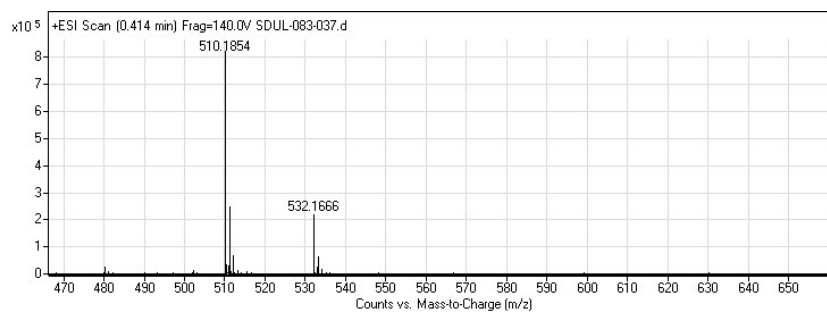
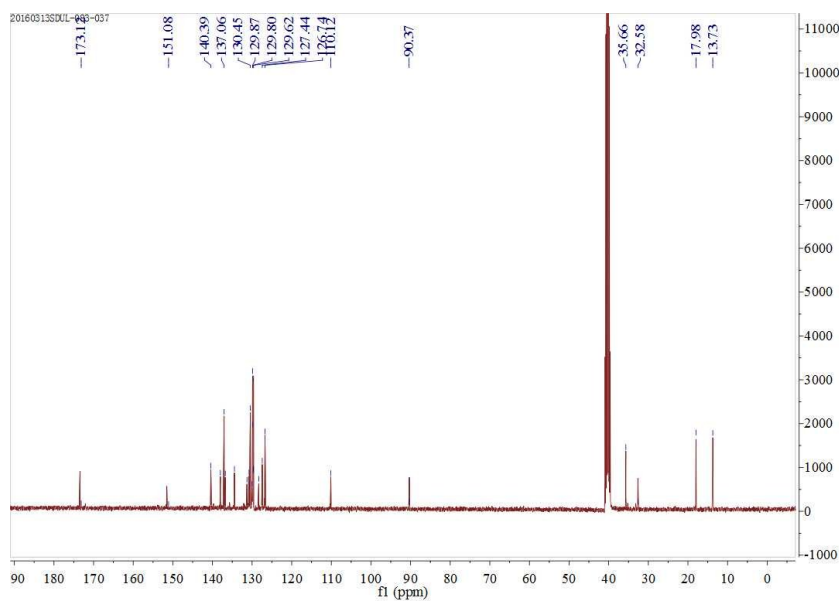
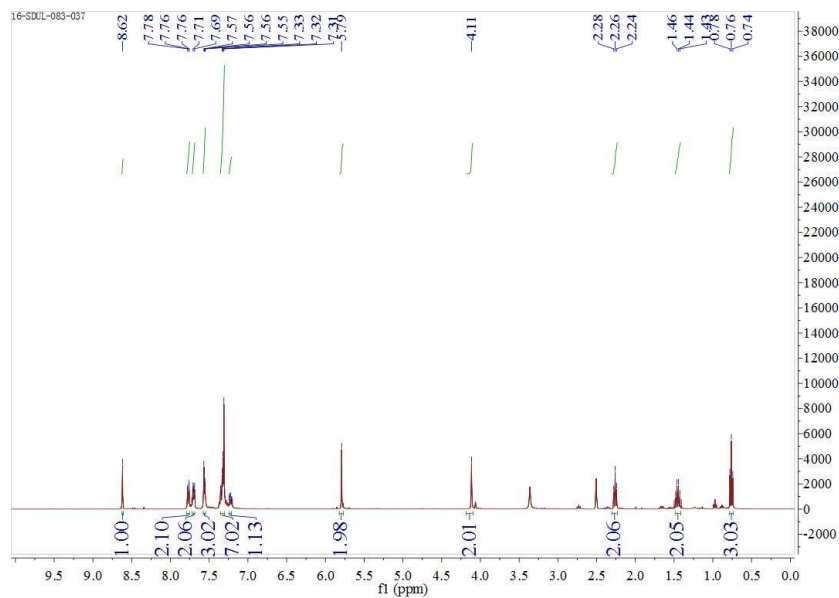
**6**



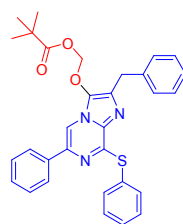
Compound **7**:



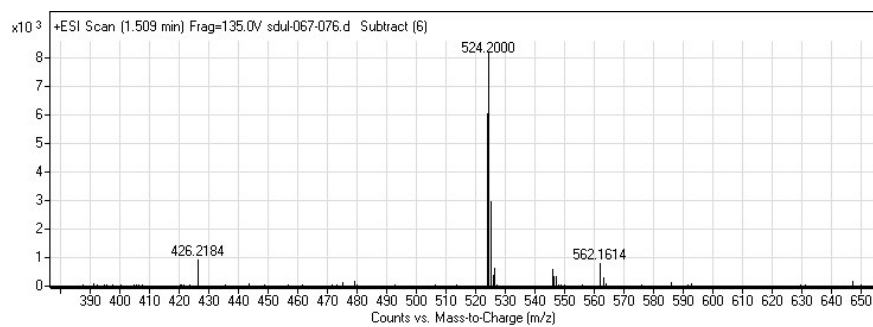
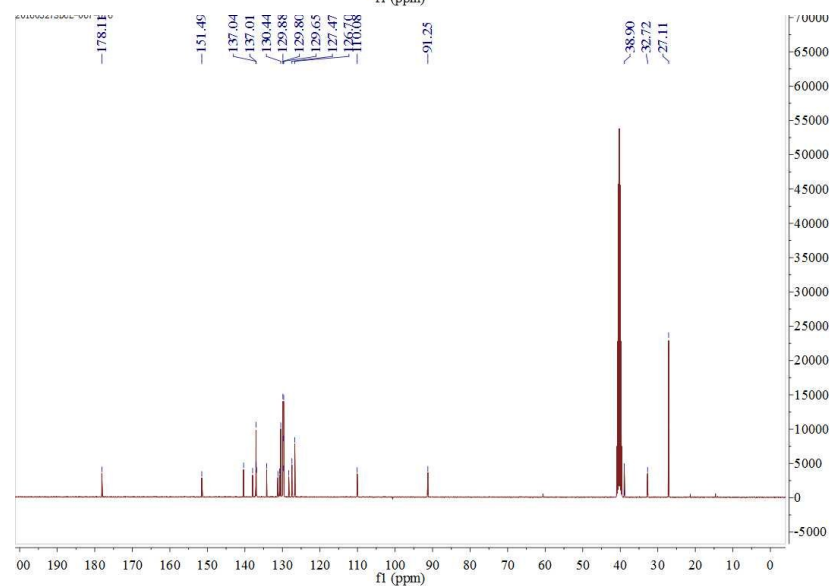
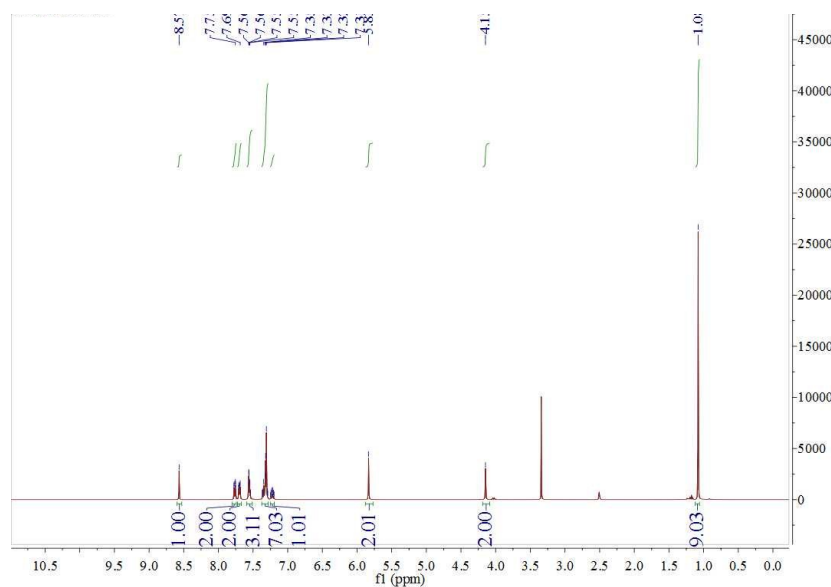
**7**



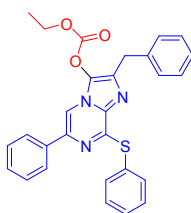
Compound **8**:



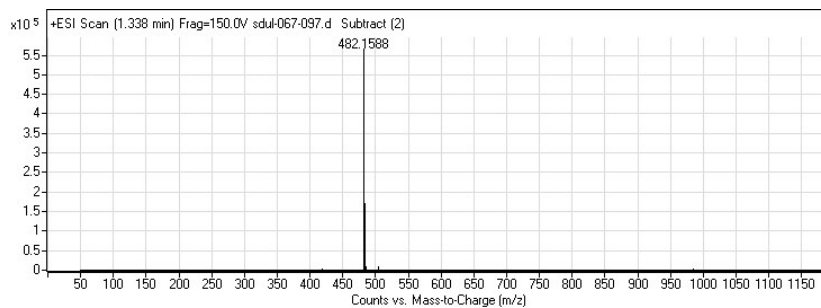
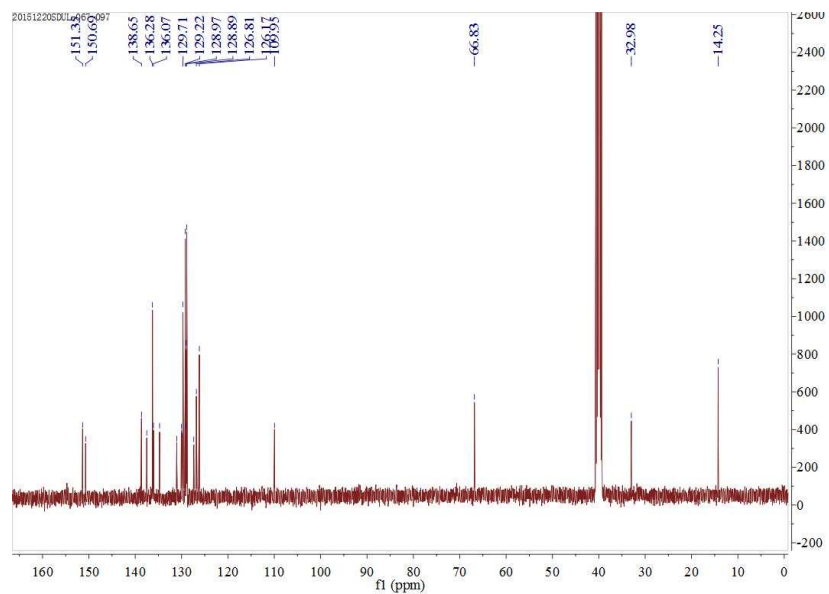
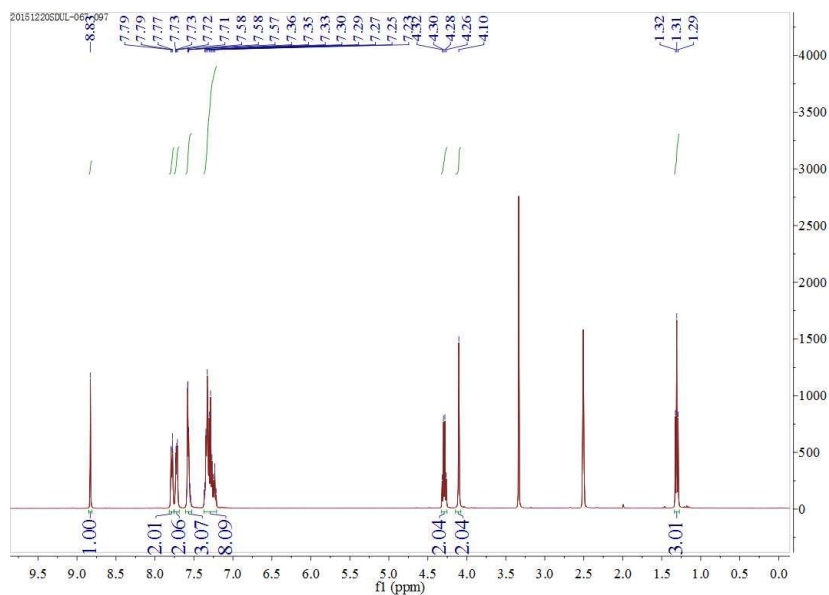
**8**



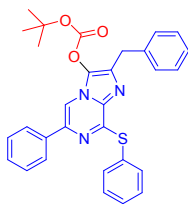
Compound **9**:



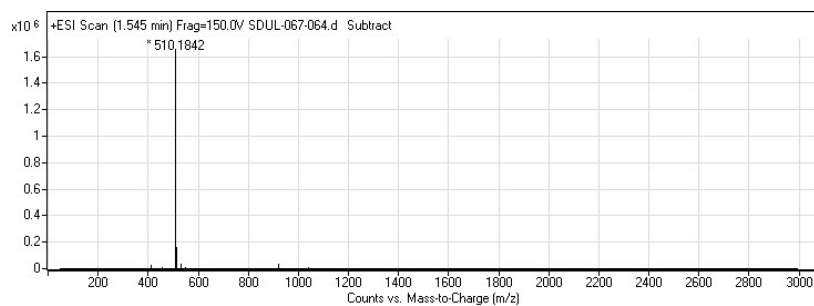
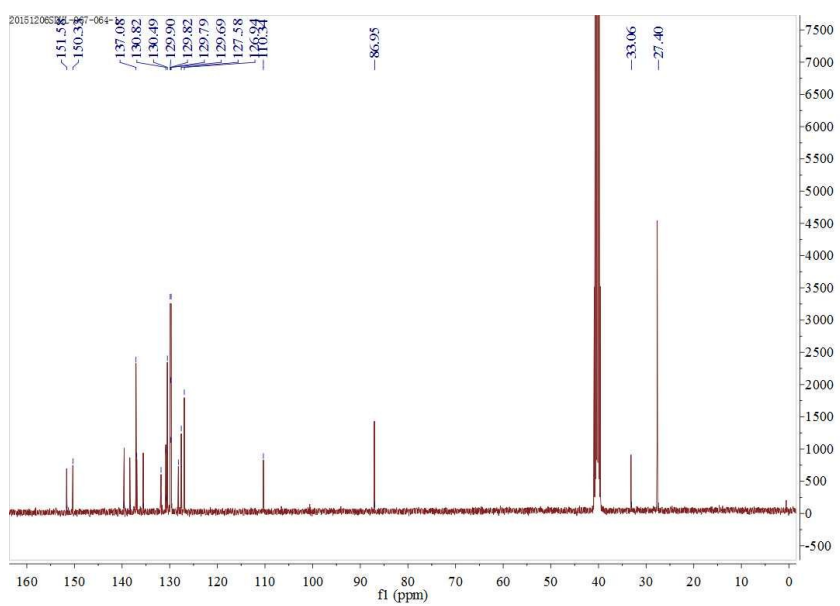
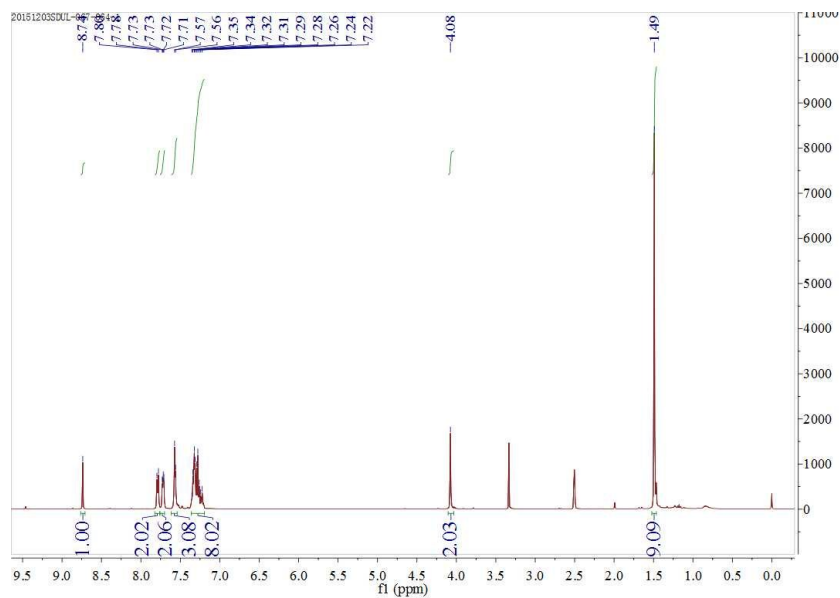
**9**



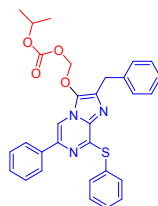
Compound **10**:



**10**



Compound **11**:



**11**

