Supplementary Information For
Aminoluciferin 4-Hydroxyphenyl Amide Enables Bioluminescence Detection of Endogenous Tyrosinase

Chunchao Tang,‡a Lei Jin,b Yuxing Lin,a Jing Su,c Yingai Sun,a Pan Liu,a Qi Li,a Guankui Wang,a Zheng Zhang,a Lupeu Du*a and Minyong Li,a,d*

‡ Department of Medicinal Chemistry, Key Laboratory of Chemical Biology (MOE), School of Pharmacy, Shandong University, Jinan, Shandong 250012, China. Tel./fax: +86-531-8838-2076; E-mail: ml@shu.edu.cn
b DNA Laboratory of Criminal Investigation Squad, Yongkang Municipal Public Security Bureau, Yongkang, Zhejiang 321300, China
c State Key Laboratory of Biobased Material and Green Papermaking, Qilu University of Technology, Shandong Academy of Sciences, Jinan, 250353.
d State Key Laboratory of Microbial Technology, Shandong University, Jinan, Shandong 250100, China
* These authors contributed equally.

1. Materials and Instruments

Unless otherwise noted, all solvents and reagents available were purchased from commercial sources. 0.9% N.S represent 0.9% sodium chloride solution. Tyrosinase was obtained from Shuyanye company. B16F10-Fluc cells expressing firefly luciferase were employed from Beijing Biocytogen Co., Ltd. ES-2-Fluc cells (human ovarian cancer cell lines) expressing firefly luciferase, which were supplied with Cellcyto. NMR data was obtained for H NMR (400 MHz) and 13C NMR (100 MHz) on a Bruker AV-400 spectrometer. Mass spectral analyses were obtained on an API 4000 spectrometer (ESI-HRMS). Tris-HCl buffer (50 mM, pH 7.4, 10 mM MgCl2) was used in the bioluminescent measurement. HPLC analysis was undertaken with a 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, CA). The IVIS Lumina (Xenogen) imaging system (Caliper Life Sciences, USA) with a cooled CCD camera was used to capture bioluminescence signals.

2. Synthetic Procedures

![Scheme S1 Synthesis of Lumityr, TyrBP-1, TyrBP-2, TyrBP-3 and TyrBP-4.]

The 3-(bromomethyl)phenol, aminoluciferin and cybLuc were synthesised according to the reported literature.1-2

Synthesis of Intermediate 1

2-Cyano-6-aminobenzothiazole (50 mg, 0.285 mmol) and 140 μL DIPEA were dissolved in 3 mL THF, and to this solution was added the BTC (85 mg, 0.285 mmol) in THF, stirred at 0 °C for 30 min, then stirred at r.t for 2 h. Finally, the p-aminophenol (93 mg, 0.855 mmol) was added and stirred at r.t overnight. The organic solvent was removed in vacuo then diluted with ethylacetate (50 mL) and washed with H2O (50 mL × 3). The organic layer was then dried over MgSO4, filtered, and concentrated in vacuo. The product was purified via column chromatography with hexane-ethyl acetate (8:1) to provide 2a as a pale yellow solid (15 mg, 17%), m.p.: 244-248 °C. 

1H NMR (400 MHz, DMSO) δ 9.26 – 9.07 (m, 2H), 8.51 (dd, J = 24.8, 23.4 Hz, 2H), 8.16 (dd, J = 19.6, 9.0 Hz, 1H), 7.65 (dd, J = 9.0, 1.7 Hz, 1H), 7.25 (d, J = 8.7 Hz, 2H), 6.70 (t, J = 9.0 Hz, 2H).

13C NMR (100 MHz, DMSO) δ 153.35, 152.98, 147.05, 141.48, 137.54, 133.96, 131.13, 125.25, 121.13, 120.44, 115.72, 114.21, 109.65. For C15H12N2O2S (M+H)+, 310.0524, found 311.0597.

Synthesis of Intermediate 2

The preparation method was described as 1, provide a pale yellow solid (19 mg, 14%), m.p.: 184-189 °C.
Synthesis of LumiTyr

To the solution of compound 1 (14 mg, 0.045 mmol) in 4 mL DCM/MeOH (1:1) was added the mixture of D-cysteine hydrochloride (13 mg, 0.09 mmol) and potassium carbonate (16 mg, 0.09 mmol) dissolved in 2 mL H2O/MeOH (1:1). The reaction was stirred for 30 min at r.t. DCM and MeOH were then removed from the flask under low pressure prior to the addition of 0.1 M aqueous HCl solution until pH 5-6 was reached. Precipitate formed immediately. The precipitate was filtered off and washed with ice water. The precipitate was dried under reduced pressure to provide a yellow solid (17 mg, 90%). m.p.: 186-190 °C Analytical RP HPLC (phenomenex, C8, 250 4.6 mm column) 60% Methanol with 0.1% trifluoroacetic acid 0.6 mL/min at 330 nm, Rt: 14.40 min.

1H NMR (400 MHz, DMSO) δ 13.20 (s, 1H), 9.11 (d, J = 12.8 Hz, 2H), 8.58 (s, 1H), 8.42 (d, J = 1.7 Hz, 1H), 8.04 (d, J = 8.9 Hz, 1H), 7.50 (dd, J = 8.9, 1.9 Hz, 1H), 7.25 (d, J = 8.8 Hz, 2H), 6.70 (d, J = 8.7 Hz, 2H), 5.43 (t, J = 9.0 Hz, 1H), 3.92 – 3.56 (m, 2H).

13C NMR (100 MHz, DMSO) δ 171.66, 164.85, 158.29, 153.23, 153.11, 148.00, 140.22, 137.14, 131.28, 124.70, 121.00, 119.27, 115.70, 109.96, 78.59, 35.17.

For C18 H14 N2O12S2 (M+H)+, 415.0456 , found 415.0529

Synthesis of TyrBP-1

The preparation method was described as LumiTyr, provide a red solid (13 mg, 81%). m.p.: 188-192 °C. Analytical RP HPLC (phenomenex, C8, 250 4.6 mm column) 70% Methanol with 0.1% trifluoroacetic acid 0.8 mL/min at 350 nm, Rt: 3.61 min

1H NMR (400 MHz, DMSO) δ 9.39 (s, 2H), 9.01 (s, 1H), 8.43 (s, 1H), 8.06 (d, J = 8.9 Hz, 1H), 7.53 (d, J = 8.8 Hz, 1H), 7.06 (t, J = 7.9 Hz, 2H), 6.85 (d, J = 8.0 Hz, 1H), 6.39 (d, J = 7.9 Hz, 1H), 5.43 (t, J = 9.0 Hz, 1H), 3.74 (dt, J = 19.4, 10.9 Hz, 2H).

13C NMR (100 MHz, DMSO) δ 171.70, 164.78, 158.46, 152.83, 148.12, 141.03, 140.00, 137.13, 129.94, 124.72, 119.29, 110.08, 109.67, 109.44, 105.77, 78.69, 35.21.

For C18 H12 N2O12S2 (M-H)−, 415.0456 , found 415.0531

Synthesis of Intermediate 3

3-(bromomethyl)phenol (106 mg, 0.56 mmol), 2-Cyano-6-hydroxybenzothiazole (100 mg, 0.56 mmol) and K2CO3 (74 mg, 0.56 mmol) were dissolved in 2 mL DMF, then stir at 50 °C overnight, the reaction was diluted with 10 mL ethylacetate and H2O (50 mL X 3), then the organic layer was dried over Na2SO4, filtered, and recrystallized from ethanol for purifying. White solid (45 mg, 28%), m.p.: 160-164 °C.

1H NMR (400 MHz, DMSO) δ 9.48 (s, 1H), 8.17 (d, J = 9.1 Hz, 1H), 7.96 (d, J = 2.5 Hz, 1H), 7.40 (dd, J = 9.1, 2.6 Hz, 1H), 7.20 (t, J = 7.8 Hz, 1H), 6.88 (dd, J = 9.0, 4.8 Hz, 2H), 6.74 (dd, J = 8.1, 1.6 Hz, 1H).

13C NMR (100 MHz, DMSO) δ 159.40, 157.97, 146.76, 138.07, 137.99, 134.24, 130.03, 125.81, 119.38, 118.73, 115.49, 114.96, 114.07, 110.61, 70.45.

For C18 H12 N2O12S2 (M+H)+, 282.0463 , found 283.0536

Synthesis of TyrBP-2

The preparation method was described as LumiTyr, provide a pale yellow solid (25 mg, 92%). m.p.: 80-86 °C. Analytical RP HPLC (phenomenex, C8, 250 4.6 mm column) 70% Methanol with 0.1% trifluoroacetic acid 0.8 mL/min at 350 nm, Rt: 5.50 min

1H NMR (400 MHz, DMSO) δ 8.05 (d, J = 9.0 Hz, 1H), 7.83 (d, J = 2.5 Hz, 1H), 7.26 (dd, J = 9.0, 2.6 Hz, 1H), 7.19 (t, J = 7.8 Hz, 1H), 6.88 (t, J = 5.3 Hz, 2H), 6.73 (dd, J = 8.1, 1.5 Hz, 1H), 5.45 – 5.33 (m, 1H), 5.14 (s, 2H), 3.82 – 3.63 (m, 2H).

13C NMR (100 MHz, DMSO) δ 171.56, 164.18, 158.62, 158.40, 157.97, 147.66, 138.38, 137.52, 129.98, 125.23, 118.68, 117.90, 115.40, 114.93, 106.33, 79.33, 70.34, 35.41.

For C18 H12 N2O12S2 (M-H)−, 385.0395 , found 385.0322.

Synthesis of TyrBP-3

Aminoluciferin (62 mg, 0.222 mmol) was dissolved in 6 mL MeOH, then added DMTMM (67.5 mg, 0.244 mmol) and p-aminophenol (26.5 mg, 0.244 mmol) in 2 mL MeOH, followed by stirring at room temperature overnight, the solvent was removed by evaporation, then was diluted with EA, washed with water ( 25 mL X 3), dried over anhydrous Na2SO4. The product was purified via column chromatography further recrystallized from EA for purifying, provided yellow solid (16 mg, 19.5%). m.p.: 184-188 °C. Analytical RP HPLC (phenomenex, C8, 250 4.6 mm column) 70% Methanol with 0.1% trifluoroacetic acid 0.8 mL/min at 380 nm, Rt: 8.95 min.

1H NMR (400 MHz, DMSO) δ 10.09 (s, 1H), 9.28 (s, 1H), 7.78 (d, J = 8.8 Hz, 1H), 7.45 (d, J = 8.8 Hz, 2H), 7.07 (d, J = 1.7 Hz, 1H), 6.86 (dd, J = 8.8, 1.9 Hz, 1H), 6.73 (d, J = 8.8 Hz, 2H), 5.84 (s, 2H), 5.38 (t, J = 9.3 Hz, 1H), 3.73 (t, J = 11.0 Hz, 2H).

13C NMR (100 MHz, DMSO) δ 167.46, 164.77, 154.17, 153.64, 149.74, 144.64, 138.34, 130.75, 125.05, 121.77, 116.44, 115.60, 103.48, 80.28, 34.47.

For C18 H12 N2O12S2(M+H)+, 371.0558 , found 371.0631.

Synthesis of TyrBP-4

The preparation method was described as TyrBP-3, provide pale yellow solid (11.2 mg, 22%). m.p.: 184-188 °C. Analytical RP HPLC (phenomenex, C8, 250 4.6 mm column) 70% Methanol with 0.1% trifluoroacetic acid 0.8 mL/min at 380 nm, Rt: 8.95 min.
9.0, 2.0 Hz, 1H), 6.79–6.66 (m, 3H), 5.35 (dt, \( J = 9.8, 7.0 \) Hz, 1H), 3.90 (dd, \( J = 16.3, 8.9 \) Hz, 2H), 3.72 (dd, \( J = 14.9, 7.3 \) Hz, 2H), 2.47–2.30 (m, 2H), 1.95–1.82 (m, 2H), 1.82–1.69 (m, 2H).

\(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \( \delta \) 168.84, 167.15, 154.35, 152.96, 147.02, 145.85, 138.86, 130.18, 125.35, 122.23, 115.76, 101.24, 79.20, 48.85, 35.29, 31.05, 29.71, 29.33, 15.37.

For \( \text{C}_{21}\text{H}_{20}\text{N}_{4}\text{O}_{2}\text{S}_{2} \) \( \text{(M-H)} \), found 423.0956.

3. Bioluminescent Assay

Figure S1 Tyrosinase concentration-dependent bioluminescence value changes from Figure 1. (A) The linear relationship of LumiTYR is described as \( Y = 2.272 \times 10^7 \times X + 9.504 \times 10^7 \) (\( R^2 = 0.9571 \)), and the linearity range of tyrosinase concentration is from 0 to 0–12.5 U·mL\(^{-1}\). (B) The linear relationship of TyrBP-2 is described as \( Y = 5.322 \times 10^6 \times X - 5.027 \times 10^7 \) (\( R^2 = 0.9733 \)), and the linearity range of tyrosinase concentration is from 0 to 5–100 U·mL\(^{-1}\). (C) The linear relationship of TyrBP-3 is described as \( Y = 3.528 \times 10^7 \times X + 1.171 \times 10^7 \) (\( R^2 = 0.9841 \)), and the linearity range of tyrosinase concentration is from 0 to 0–5 U·mL\(^{-1}\). (D) The linear relationship of TyrBP-4 is described as \( Y = 7.116 \times 10^7 \times X + 3.934 \times 10^7 \) (\( R^2 = 0.9925 \)), and the linearity range of tyrosinase concentration is from 0 to 0–5 U·mL\(^{-1}\).
<table>
<thead>
<tr>
<th>Number</th>
<th>TYR Probes</th>
<th>The Structures of Probes</th>
<th>LOD (U·mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LumiTYR</td>
<td><img src="image" alt="Structure of LumiTYR" /></td>
<td>8.4</td>
</tr>
<tr>
<td>2</td>
<td>TyrBP-1</td>
<td><img src="image" alt="Structure of TyrBP-1" /></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>TyrBP-2</td>
<td><img src="image" alt="Structure of TyrBP-2" /></td>
<td>12.5</td>
</tr>
<tr>
<td>4</td>
<td>TyrBP-3</td>
<td><img src="image" alt="Structure of TyrBP-3" /></td>
<td>0.65</td>
</tr>
<tr>
<td>5</td>
<td>TyrBP-4</td>
<td><img src="image" alt="Structure of TyrBP-4" /></td>
<td>1.1</td>
</tr>
</tbody>
</table>

**Table S1** The limit detection (LOD) of LumiTYR, TyrBP-1, TyrBP-2, TyrBP-3, and TyrBP-4 about tyrosianse

**Figure S2** The cytotoxic effect of four BL probes was determined by CCK8 assay. All assays were performed in triplicate and represented as the mean ± SEM.
Figure S3  Total flux of bioluminescence imaging of TyrBP-3 in a real-time manner in ES-2-Fluc cells. All assays were performed in quintuplicate and represented as the mean ± SEM.

Figure S4  The assay of tyrosinase activity in ES-2-Fluc cells and B16F10-Fluc cells in the presence of L-DOPA. The absorbance was read with a microplate reader at 475 nm. The results are the mean ± SEM (n=3)
4. $^1$H-NMR, $^{13}$C-NMR, HPLC and ESI-MS spectra of compounds

Intermediate 1
LumiTYR
Scan (0.17 min) 079-068.d Subtract

Counts vs. Mass-to-Charge (m/z)

DAD1 C, Sig=330.4 Ref-off (E:\CY3D\DATA079-067-TRR079-066-920-33-05.D)
Intermediate 2
Scan (0.216 min) L-2.d Subtract

Counts vs. Mass-to-Charge (m/z)
TyrBP-1

[Chemical structure image]

[Graphical data visualization]
TyrBP-2

\[
\begin{align*}
\text{OH} & \quad \text{O-} \\
\text{N} & \quad \text{S} \\
\text{N} & \quad \text{COOH}
\end{align*}
\]
Counts vs. Mass-to-Charge (m/z)