SUPPORTING INFORMATION

A Coelenterazine-type Bioluminescent Probe for Nitroreductase Imaging

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CONTENT

1. Synthesis ................................................................................................................S-2
2. Selectivity and sensitivity assay in vitro .................................................................S-4
3. Cytotoxicity evaluation of probes and cobalt chloride...........................................S-4
4. NMR, ESI-HRMS and HPLC spectra .....................................................................S-5
1. Synthesis

**Scheme S1:** The synthesis route of NTR probes

### 2,8-dibenzyl-3-((4-nitrobenzyl)oxy)-6-(thiophen-2-yl)imidazo[1,2-a]pyrazine (A1)

Yield: 94.88%. Melting point: 118.3-120.0 °C, Purity: 99.83%. $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 8.60 (s, 1H), 8.23 (d, $J = 8.7$ Hz, 2H), 7.77 (d, $J = 3.5$ Hz, 1H), 7.74 (d, $J = 8.6$ Hz, 2H), 7.58 (d, $J = 5.0$ Hz, 1H), 7.45 (d, $J = 7.3$ Hz, 2H), 7.35 - 6.44 (m, 9H), 5.34 (s, 2H), 4.41 (s, 2H), 4.04 (d, $J = 13.5$ Hz, 2H). $^{13}$C NMR (500 MHz, DMSO-$d_6$) $\delta$ 152.23, 147.90, 143.94, 142.20, 139.52, 138.26, 137.26, 133.98, 131.00, 129.67, 129.04, 128.75, 128.71, 126.85, 126.60, 124.04, 123.91, 109.10, 75.36, 38.82, 32.87. HRMS m/z calcd. for C$_{31}$H$_{24}$N$_4$O$_3$S [M+H]$^+$ 533.1647, found 533.1634.

### 2,8-dibenzyl-6-(5-methylfuran-2-yl)-3-((4-nitrobenzyl)oxy)imidazo[1,2-a]pyrazine (A2)

Yield: 29.6%. Melting point: 117.0-119.7 °C, Purity: 99.33%. $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 8.24 (d, $J = 8.3$ Hz, 2H), 8.02 (s, 1H), 7.72 (d, $J = 8.2$ Hz, 2H), 7.44 (d, $J = 6.9$ Hz, 2H), 7.30-7.15 (m, 8H), 6.82 (d, $J = 2.3$ Hz, 1H), 6.21 (d, $J = 13.6$ Hz, 1H), 5.33 (s, 2H), 4.40 (s, 2H), 4.04 (d, 2H), 2.34 (s, 3H). $^{13}$C NMR (500 MHz, DMSO-$d_6$) $\delta$ 152.79, 152.76, 152.57, 149.98, 147.95, 144.03, 139.53, 138.30, 137.44, 133.62, 131.90, 131.11, 130.03, 129.67, 129.04, 128.75, 128.71, 126.85, 126.60, 124.15, 109.65, 108.69, 107.92, 75.63, 38.99, 32.86, 13.91. HRMS m/z calcd for C$_{32}$H$_{26}$N$_4$O$_4$ [M+H]$^+$ 531.2032, found 531.2032.

### 2,8-dibenzyl-3-((2-nitrobenzyl)oxy)-6-(thiophen-2-yl)imidazo[1,2-a]pyrazine (A3)

Yield: 33.6%. Melting point: 133.4-135.8 °C, Purity: 99.79%. $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 8.58 (s, 1H), 8.17 (d, $J = 7.3$ Hz, 1H), 8.00 -7.79 (m, 2H), 7.70 (d, $J = 10.1$ Hz, 2H), 7.58 (s, 1H), 7.46 (s, 2H), 7.24 (dd, $J = 44.5$, 23.8 Hz, 9H), 5.51 (s, 2H), 4.43 (s, 2H), 4.03 (s, 2H). $^{13}$C NMR (500 MHz, DMSO-$d_6$) $\delta$ 152.26, 147.83, 142.21, 139.52, 138.26, 137.10, 134.66, 134.06, 133.98, 132.15, 131.90, 130.56, 130.20, 129.68, 129.04, 128.72, 127.51, 126.86, 126.60, 125.25, 123.80, 109.05, 73.27, 38.82, 32.91. HRMS m/z calcd for C$_{32}$H$_{26}$N$_4$O$_4$ [M+H]$^+$ 533.1569, found 533.1650.
2,8-dibenzyl-3-((4,5-dimethoxy-2-nitrobenzyl)oxy)-6-(thiophen-2-yl)imidazo[1,2-a]pyrazine (A4)
Yield: 67.0%. Melting point: 126.8-130.43 °C, Purity: 99.54%. $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 8.56 (s, 1H), 7.75-7.67 (m, 2H), 7.58 (d, $J = 4.8$ Hz, 1H), 7.46 (d, $J = 7.6$ Hz, 2H), 7.33-7.25 (m, 3H), 7.21 (dd, $J = 14.9$, 5.8 Hz, 5H), 7.17-7.13 (m, 2H), 5.48 (s, 2H), 4.42 (s, 2H), 4.01 (s, 2H), 3.87 (s, 3H), 3.81 (s, 3H). $^{13}$C NMR (500 MHz, DMSO-$d_6$) $\delta$ 153.52, 152.22, 148.79, 142.22, 140.40, 139.51, 138.32, 137.13, 134.11, 133.93, 132.15, 129.65, 128.93, 128.72, 128.69, 127.47, 126.86, 126.56, 126.31, 123.76, 112.68, 109.08, 108.64, 73.46, 56.72, 56.59, 38.83, 32.86. HRMS m/z calcd for C$_{32}$H$_{26}$N$_4$O$_4$ [M+H]$^+$ 593.1780, found 593.1851.

2,8-dibenzyl-6-(5-methylfuran-2-yl)-3-((2-nitrobenzyl)oxy)imidazo[1,2-a]pyrazine (A5)
Yield: 52.2%. Melting point: 133.1-136.5 °C, Purity: 100.00%. $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 8.15 (d, $J = 8.1$ Hz, 1H), 8.11 (s, 1H), 7.88 (d, $J = 6.8$ Hz, 1H), 7.83 (t, $J = 7.5$ Hz, 1H), 7.68 (t, $J = 8.4$ Hz, 1H), 7.44 (d, $J = 7.3$ Hz, 2H), 7.31-7.12 (m, 8H), 6.81 (d, $J = 3.2$ Hz, 1H), 6.23 (d, $J = 3.8$ Hz, 1H), 5.50 (s, 2H), 4.41 (s, 2H), 4.03 (s, 2H), 2.34 (s, 3H). $^{13}$C NMR (500 MHz, DMSO-$d_6$) $\delta$ 152.77, 150.03, 147.86, 138.50, 138.33, 137.25, 134.68, 133.70, 131.94, 131.16, 130.46, 130.25, 129.66, 128.76, 128.72, 126.84, 126.61, 125.30, 109.61, 108.69, 108.02, 73.51, 38.99, 32.90, 13.97. HRMS m/z calcd for C$_{32}$H$_{26}$N$_4$O$_4$ [M+H]$^+$ 531.1954, found 531.2033.

2,8-dibenzyl-3-((4,5-dimethoxy-2-nitrobenzyl)oxy)-6-(5-methylfuran-2-yl)imidazo[1,2-a]pyrazine (A6)
Yield: 51.4%. Melting point: 154.0-155.4 °C, Purity: 99.79%. $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 8.06 (s, 1H), 7.68 (s, 1H), 7.43 (d, $J = 7.4$ Hz, 2H), 7.32 (s, 1H), 7.30-7.12 (m, 8H), 6.80 (d, $J = 3.1$ Hz, 1H), 6.23 (d, $J = 2.8$ Hz, 1H), 5.47 (s, 2H), 4.41 (s, 2H), 4.04 (s, 2H), 3.85 (s, 3H), 3.82 (s, 3H), 2.34 (s, 3H). $^{13}$C NMR (500 MHz, DMSO-$d_6$) $\delta$ 153.56, 152.74, 150.02, 148.85, 140.48, 139.53, 138.37, 137.32, 133.73, 131.93, 131.08, 129.63, 129.00, 128.72, 126.84, 126.59, 126.59, 126.31, 112.64, 109.53, 108.68, 108.01, 73.70, 56.73, 56.58, 39.04, 32.88, 13.89. HRMS m/z calcd. for C$_{32}$H$_{26}$N$_4$O$_4$ [M+H]$^+$ 591.2165, found 591.2230.
2. Selectivity and sensitivity assay *in vitro*

**Figure S1.** The bioluminescence intensity of probes with different concentrations of NTR. (a) bioluminescence imaging of probes with NTR in a concentration dependent manner (0, 0.039, 0.078, 0.156, 0.312, 0.625, 1.25, 2.5, 5, 10 µL); (b) bioluminescent signals measured from (a); (c) bioluminescence signals at low concentration of nitroreductase (0, 0.039, 0.078, 0.156, 0.312 µg/mL). *P*<0.05; (d) linear relationship between the bioluminescence intensity of the probe A1 and the nitroreductase concentrations (0-10 µg/mL, R²= 0.9818); (e) linear relationship between the bioluminescence intensity of probe A5 and nitroreductase concentrations (0-5 µg/mL, R²=...
0.9883); (f) nitroreductase-independent relationship between the bioluminescence intensity of probe A5; (g) bioluminescence imaging of selectivity of A5 with various relevant reductants

3. Cytotoxicity evaluation of probes and cobalt chloride

Figure S2. The cell viability of ES-2-Rluc cells after incubation with different concentrations of probes and time points.

Figure S3. The cell viability of ES-2-Rluc cells after incubation with various concentrations of CoCl₂ and time points.
4. NMR, ESI-HRMS and HPLC spectra

$^1$HNMR spectra of A1

$^{13}$CNMR spectra of A1
HRMS spectra of A1

HPLC spectra of A1

$^1$HNMR spectra of A2
$^{13}$CNMR spectra of A2

HRMS spectra of A2

HPLC spectra of A2
$^1$HNMR spectra of A3

$^{13}$CNMR spectra of A3
HRMS spectra of A3

HPLC spectra of A3

$^1$HNMR spectra of A4
$^{13}$CNMR spectra of A4

![CNMR spectra](image)

HRMS spectra of A4

![HRMS spectra](image)

HPLC spectra of A4

![HPLC spectra](image)
$^1$HNMR spectra of A5

$^{13}$CNMR spectra of A5
$^{13}$CNMR spectra of A6

HRMS spectra of A6

HPLC spectra of A6