

SUPPORTING INFORMATION

A Coelenterazine-type Bioluminescent Probe for Nitroreductase Imaging

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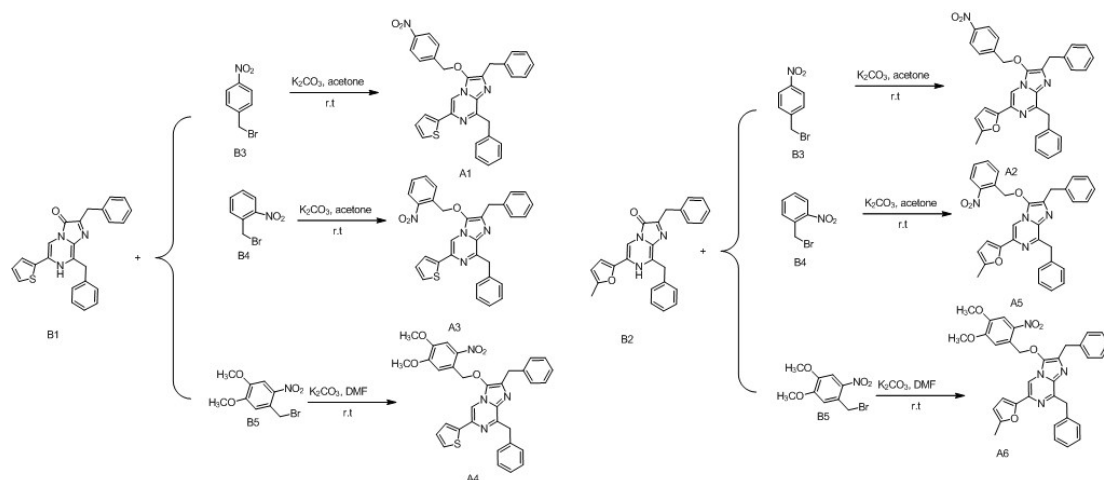
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1. Synthesis



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%. ¹H NMR (500 MHz, DMSO-*d*₆) δ 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). ¹³C NMR (500 MHz, DMSO-*d*₆) δ 152.23, 147.90, 143.94, 142.20, 139.52, 138.26, 137.26, 133.98, 132.10, 130.02, 129.69, 129.01, 128.74, 128.72, 127.53, 126.87, 126.60, 124.04, 123.91, 109.10, 75.36, 38.82, 32.87. HRMS *m/z* calcd. for C₃₁H₂₄N₄O₃S [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%. ¹H NMR (500 MHz, DMSO-*d*₆) δ 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 (s, 2H), 2.34 (s, 3H). ¹³C NMR (500 MHz, DMSO-*d*₆) δ 152.79, 152.76, 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 C₃₂H₂₆N₄O₄ [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%. ¹H NMR (500 MHz, DMSO-*d*₆) δ 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). ¹³C NMR (500 MHz, DMSO-*d*₆) δ 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 C₃₂H₂₆N₄O₄ [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%. ¹H NMR (500 MHz, DMSO-*d*₆) δ 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). ¹³C NMR (500 MHz, DMSO-*d*₆) δ 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 C₃₂H₂₆N₄O₄ [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%. ¹H NMR (500 MHz, DMSO-*d*₆) δ 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). ¹³C NMR (500 MHz, DMSO-*d*₆) δ 152.77, 150.03, 147.86, 139.53, 138.33, 137.25, 134.68, 133.70, 131.94, 131.16, 130.46, 130.25, 129.66, 129.07, 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 C₃₂H₂₆N₄O₄ [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%. ¹H NMR (500 MHz, DMSO-*d*₆) δ 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). ¹³C NMR (500 MHz, DMSO-*d*₆) δ 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, 126.31, 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 C₃₂H₂₆N₄O₄ [M+H]⁺ 591.2165, found 591.2230.

2. Selectivity and sensitivity assay *in vitro*

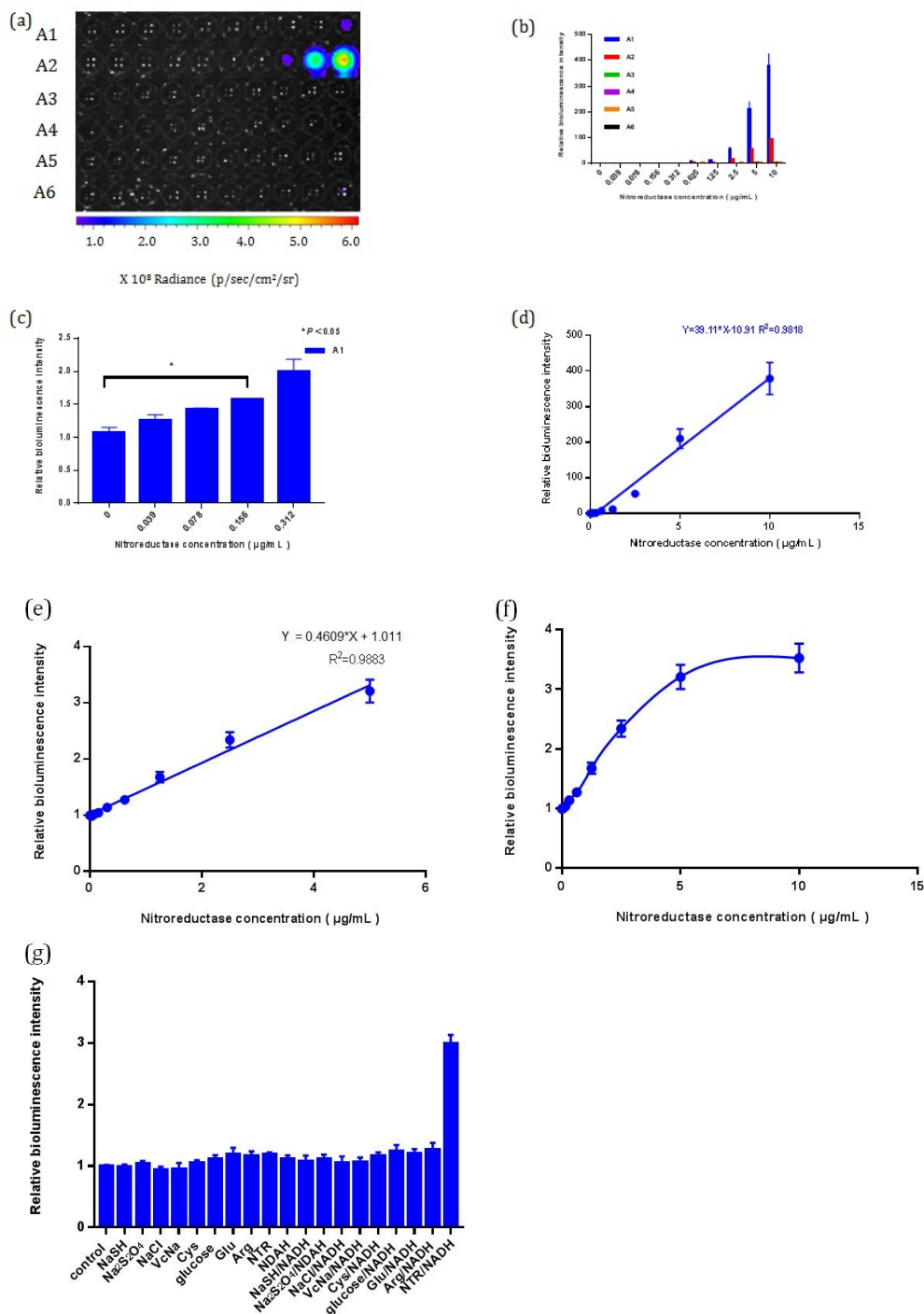


Figure S1. The bioluminescence intensity of probes with different concentrations of NTR. (a) bioluminescence imaging of probes with NTR in a concentration dependent manner (0, 0.039, 0.078, 0.156, 0.312, 0.625, 1.25, 2.5, 5, 10 μ L); (b) bioluminescent signals measured from (a); (c) bioluminescence signals at low concentration off nitroreductase (0, 0.039, 0.078, 0.156, 0.312 μ g/mL). * $P < 0.05$; (d) linear relationship between the bioluminescence intensity of the probe A1 and the nitroreductase concentrations (0-10 μ g/mL, $R^2 = 0.9818$); (e) linear relationship between the bioluminescence intensity of probe A5 and nitroreductase concentrations (0-5 μ g/mL, $R^2 =$

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

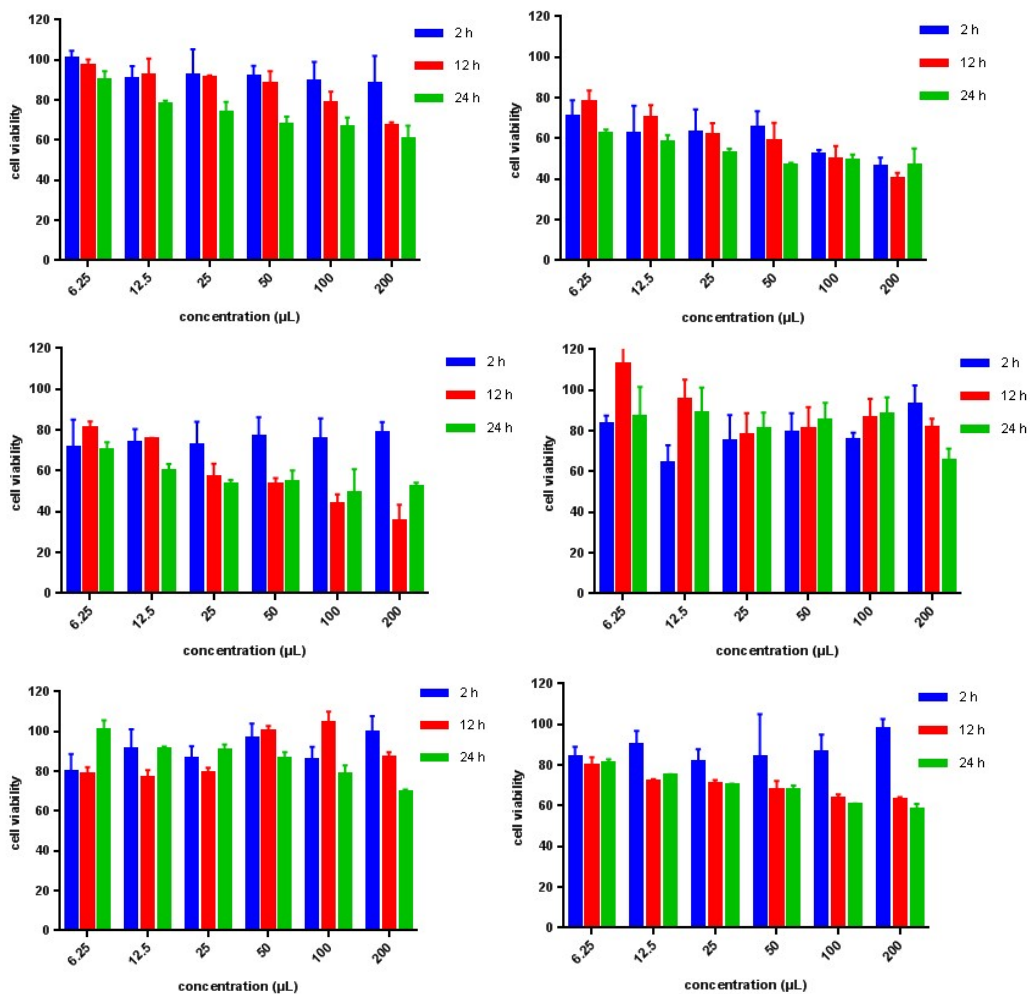


Figure S2. The cell viability of ES-2-Rluc cells after incubation with different concentrations of probes and time points.

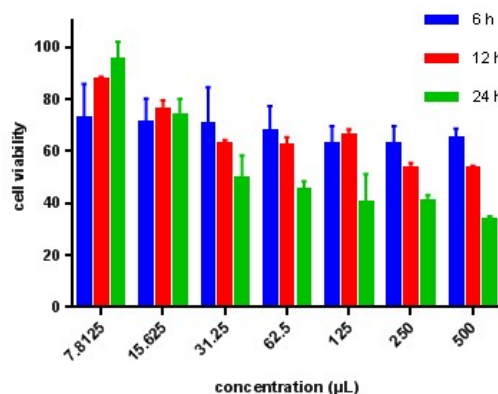
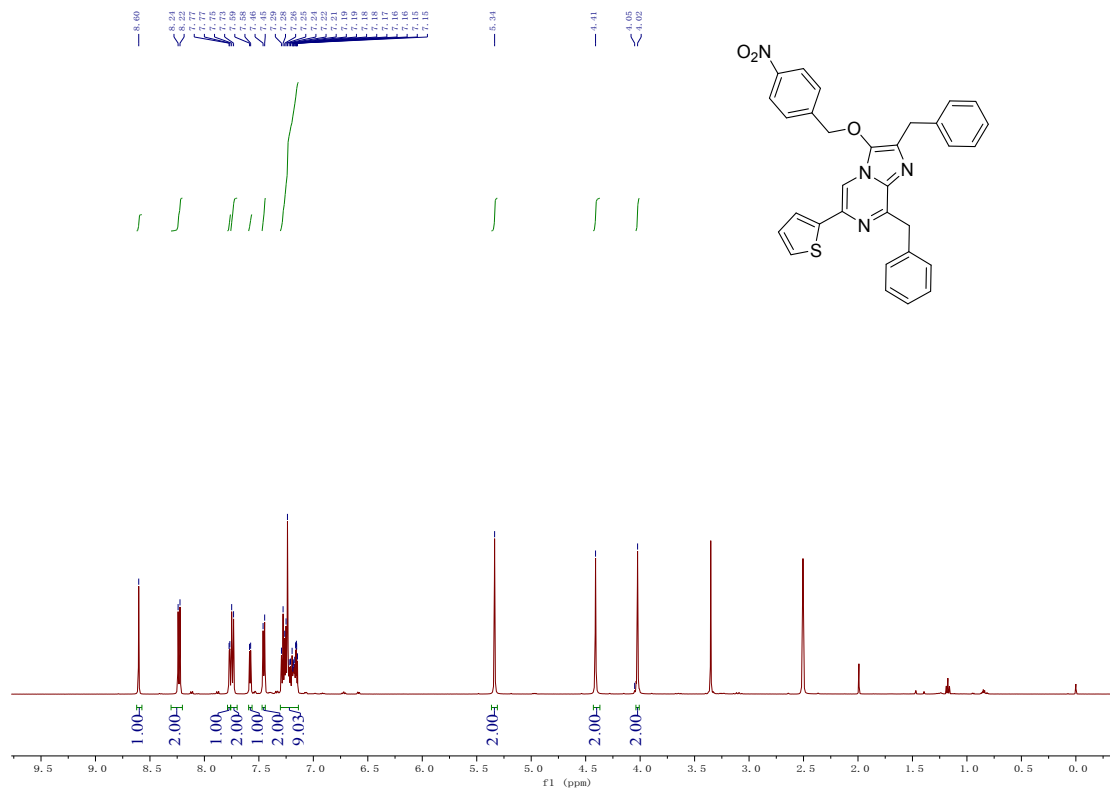


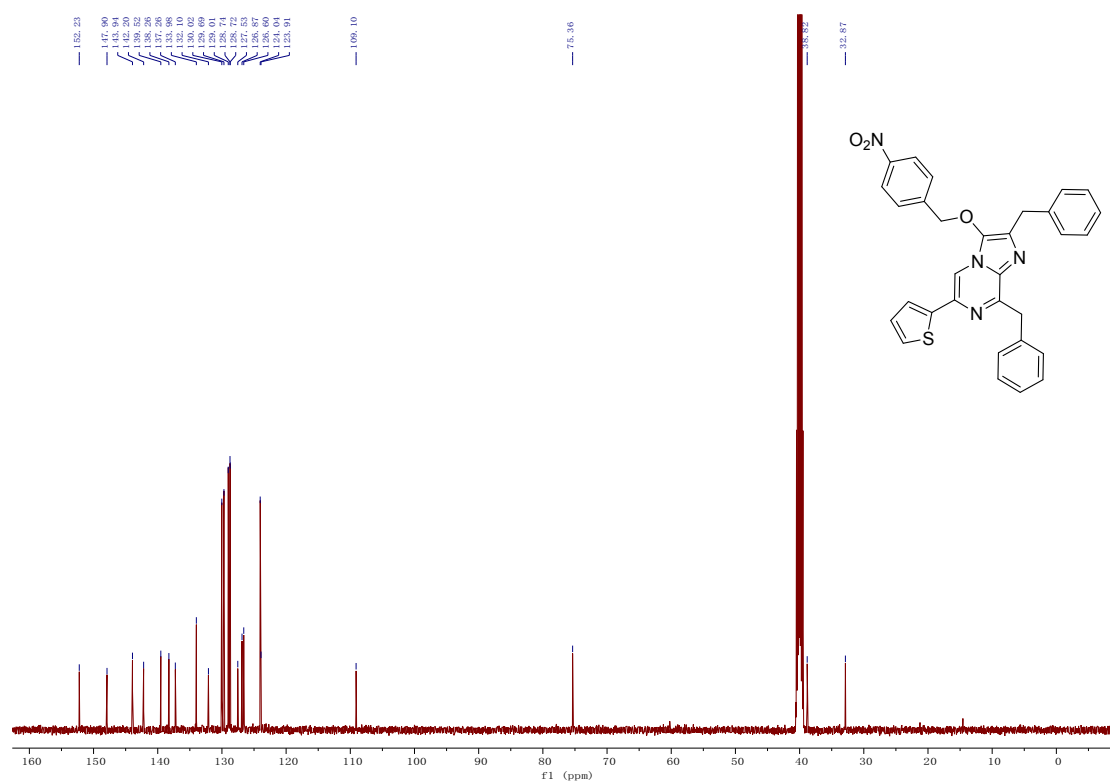
Figure S3. The cell viability of ES-2-Rluc cells after incubation with various concentrations of CoCl₂ and time points.

4. NMR, ESI-HRMS and HPLC spectra

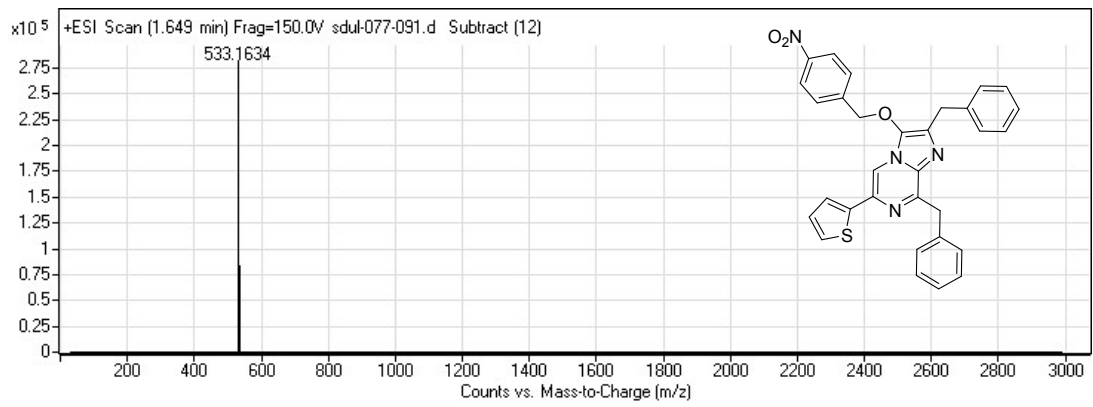
¹HNMR spectra of A1



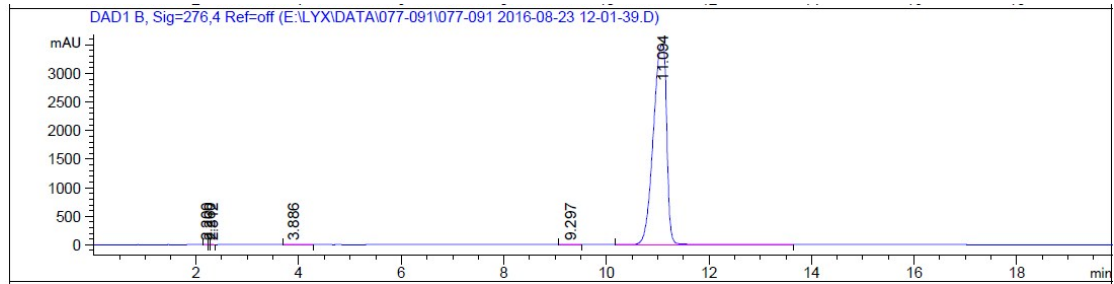
¹³CNMR spectra of A1



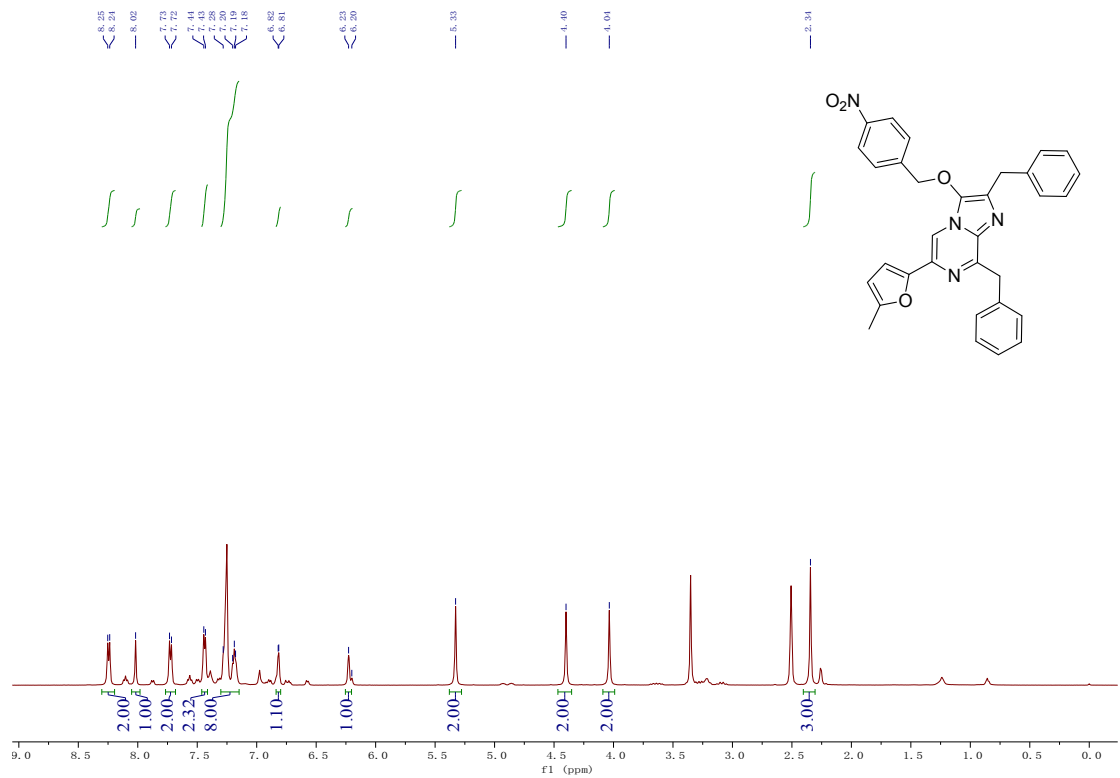
HRMS spectra of A1



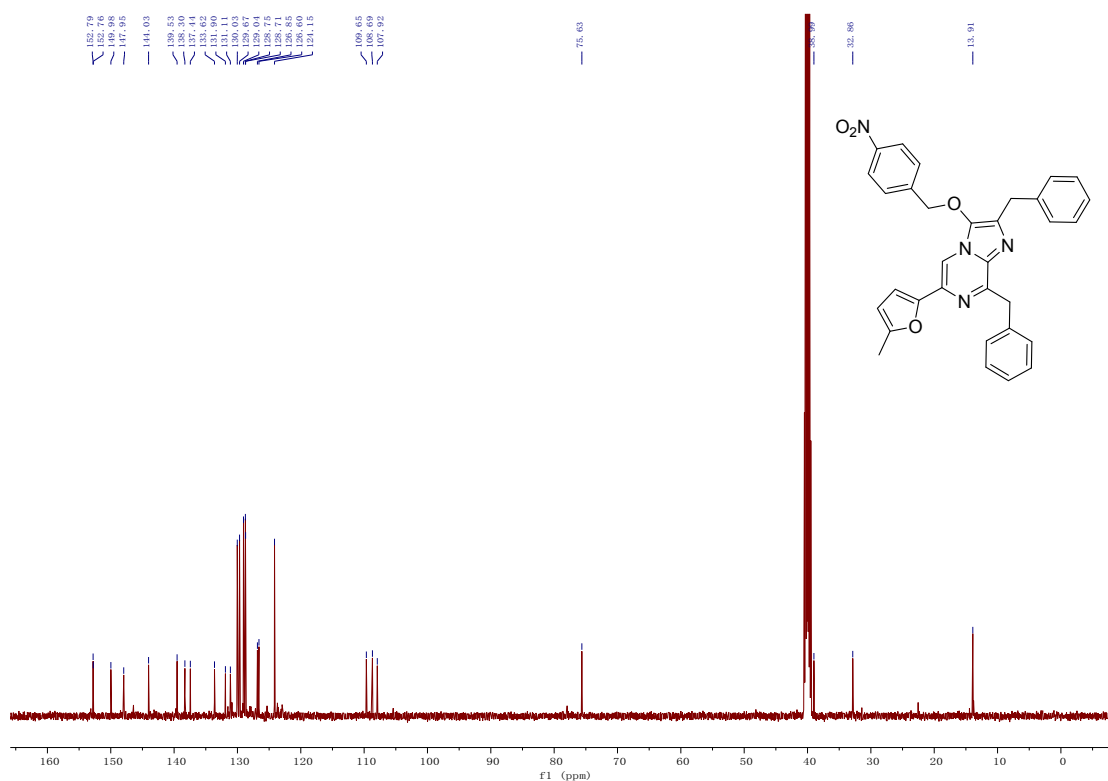
HPLC spectra of A1



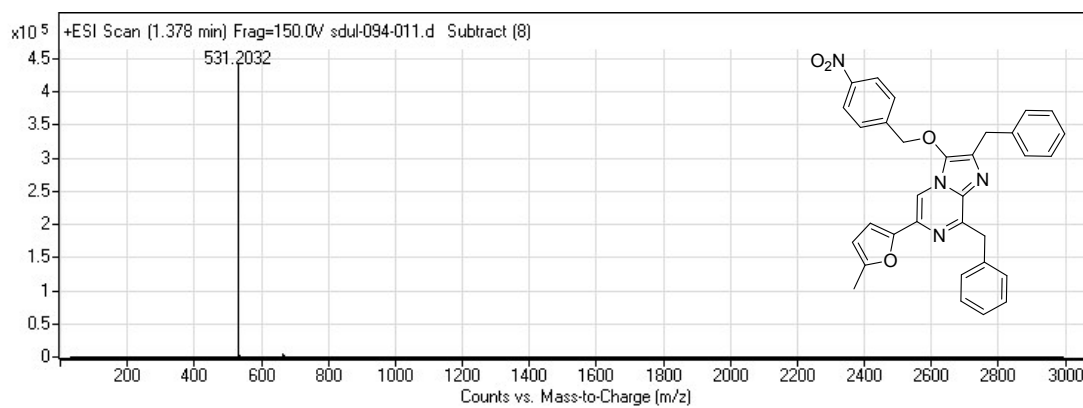
¹HNMR spectra of A2



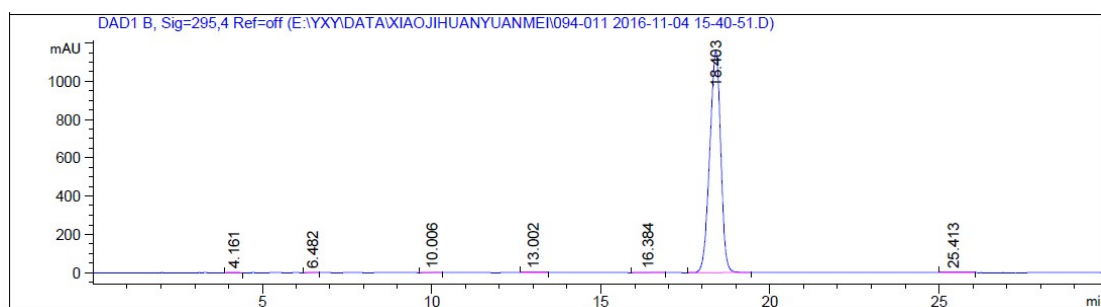
¹³CNMR spectra of A2



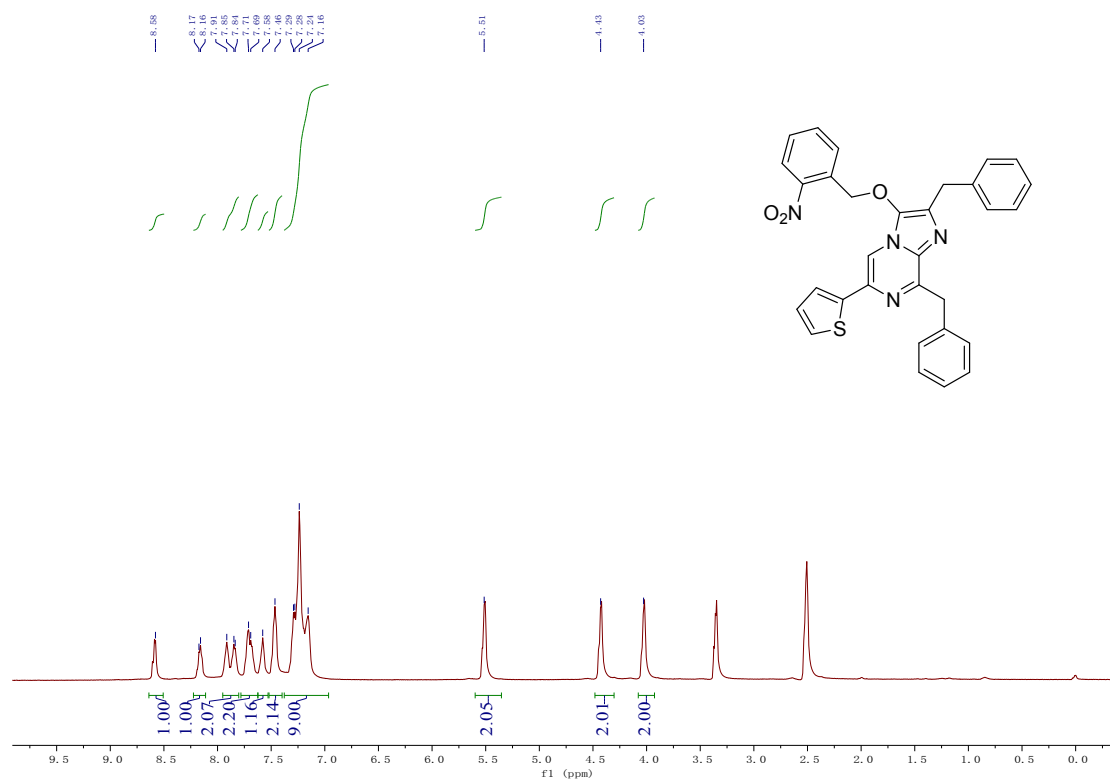
HRMS spectra of A2



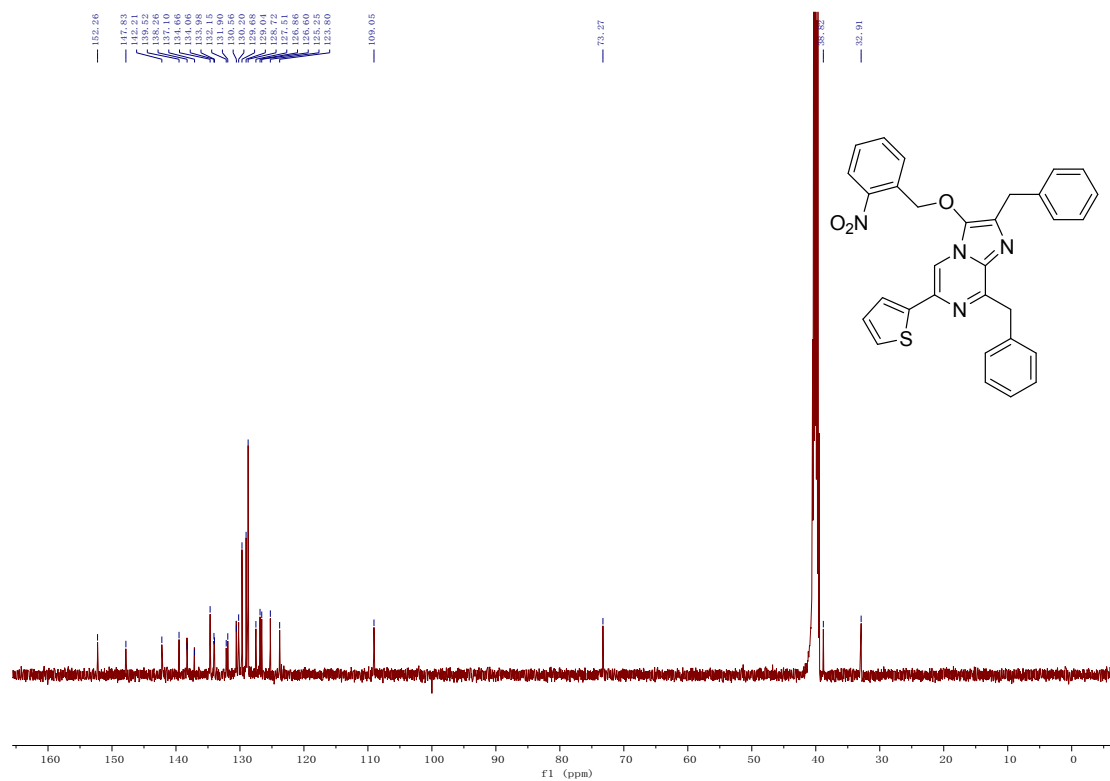
HPLC spectra of A2



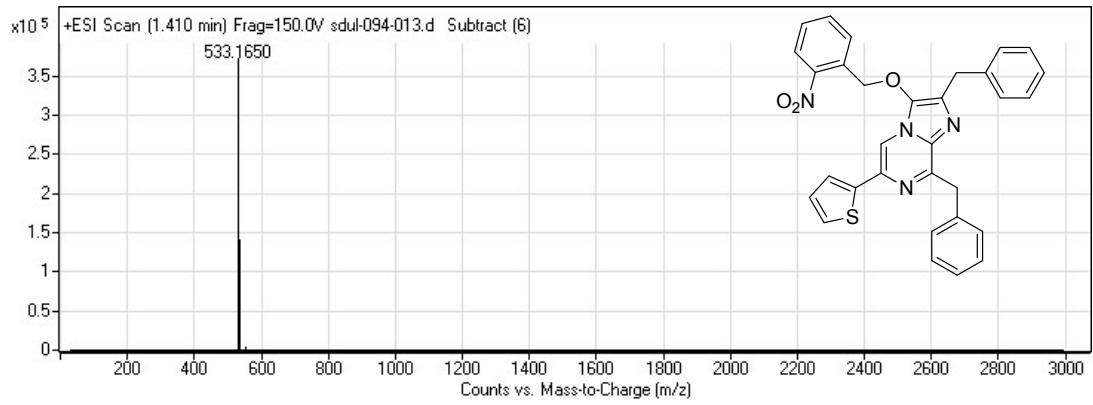
¹H NMR spectra of A3



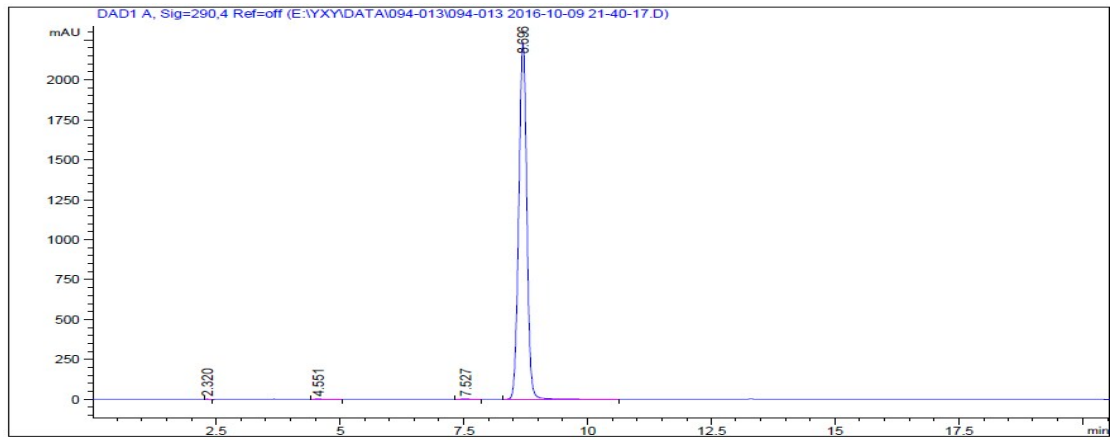
¹³C NMR spectra of A3



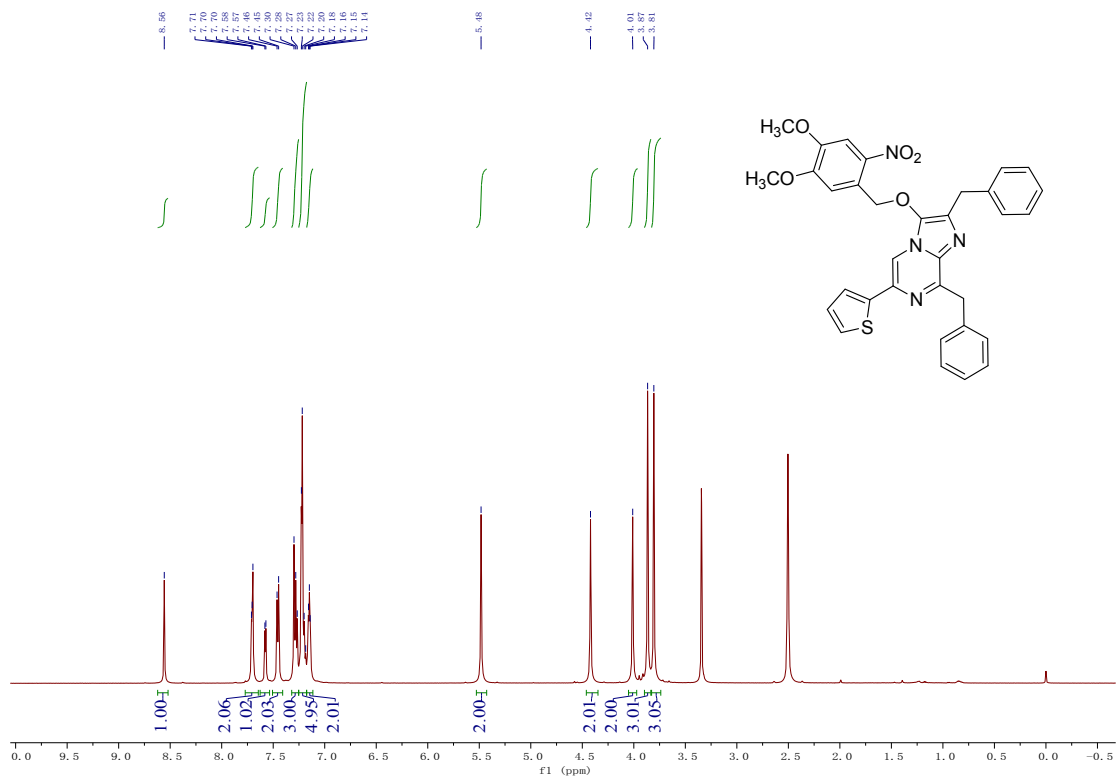
HRMS spectra of A3



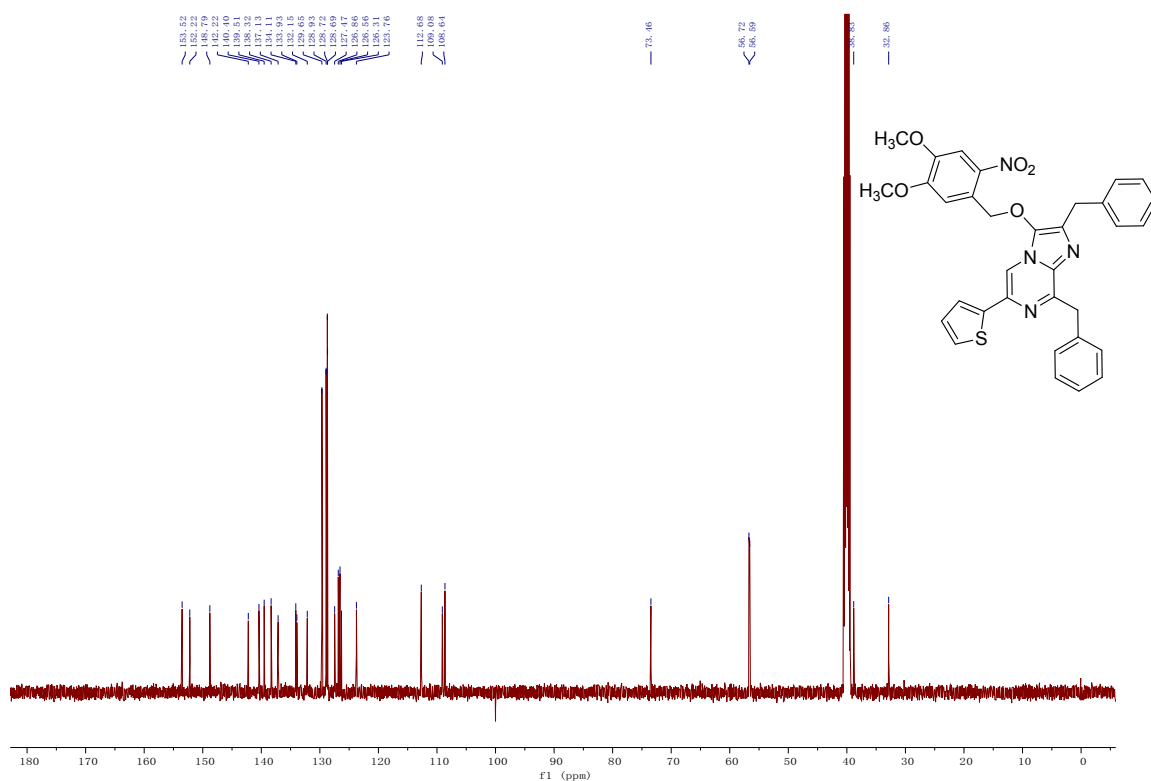
HPLC spectra of A3



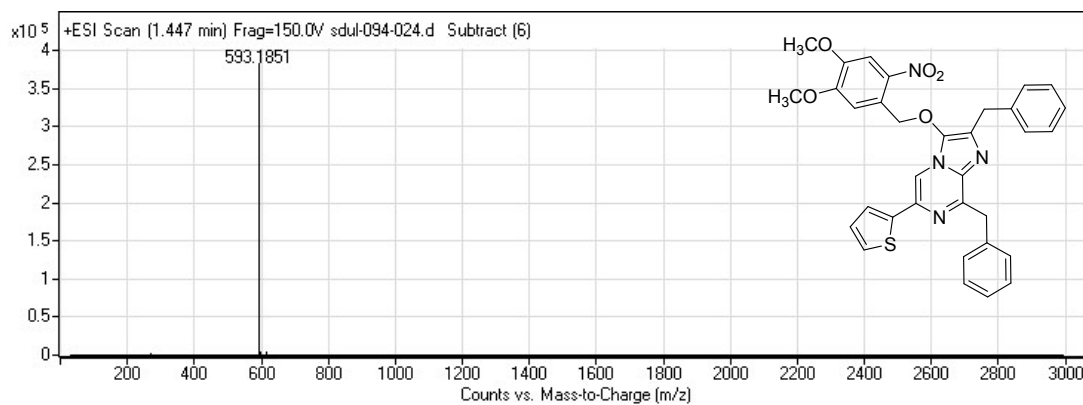
¹HNMR spectra of A4



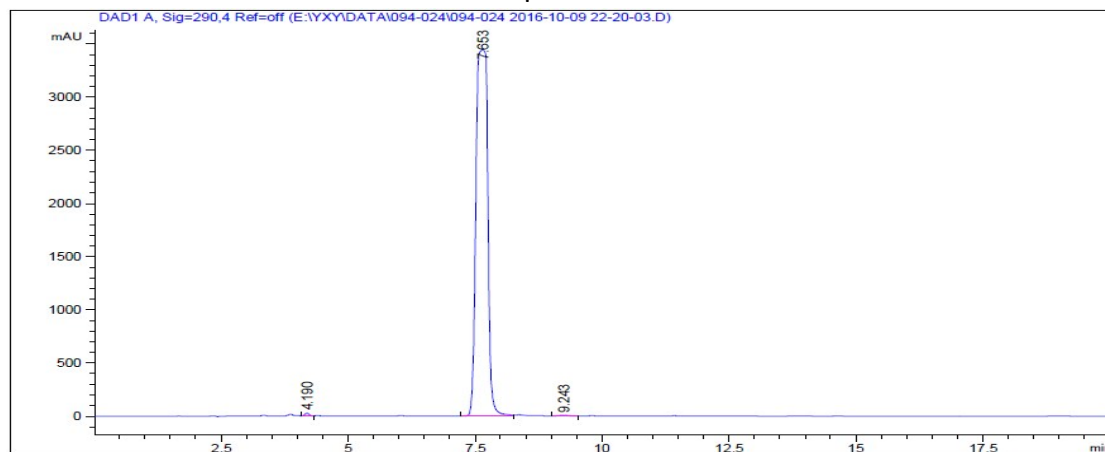
¹³CNMR spectra of A4



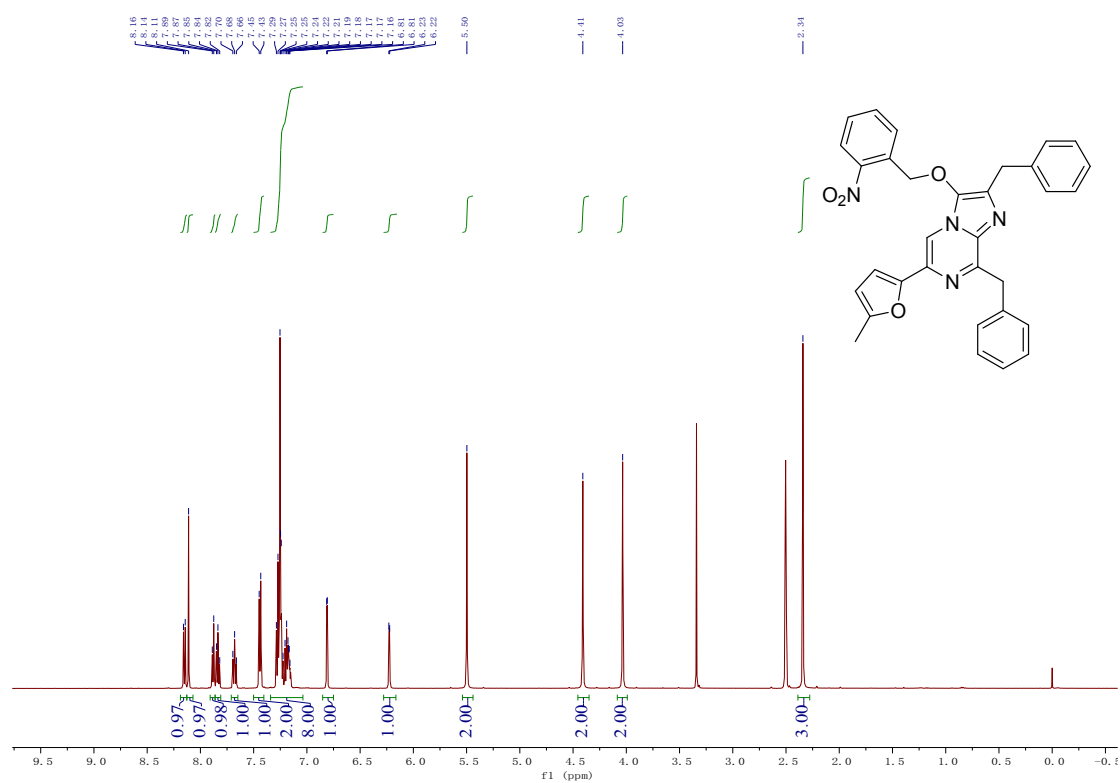
HRMS spectra of A4



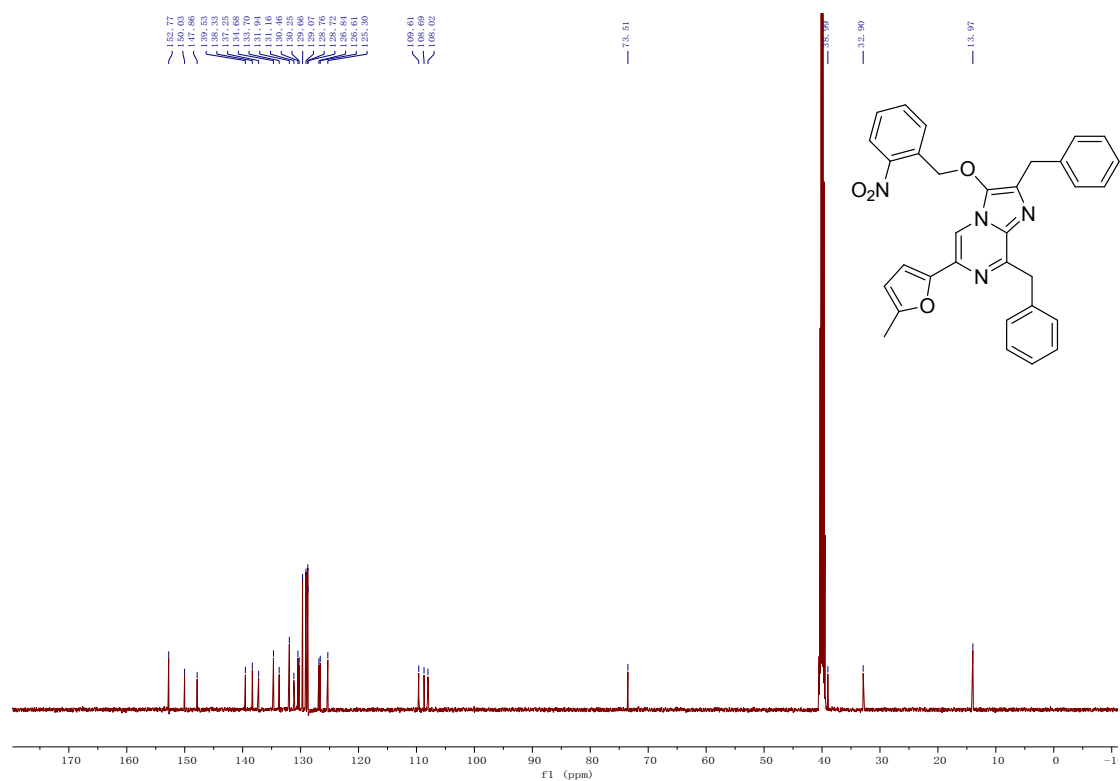
HPLC spectra of A4



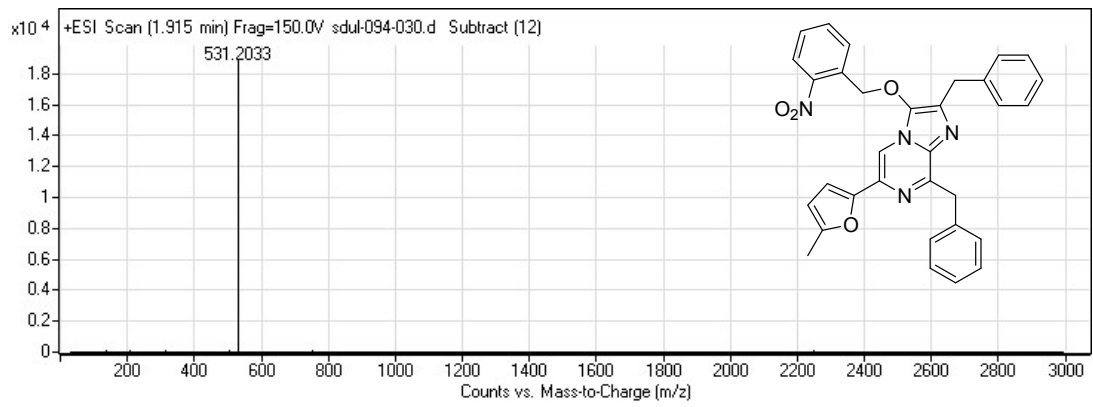
¹H NMR spectra of A5



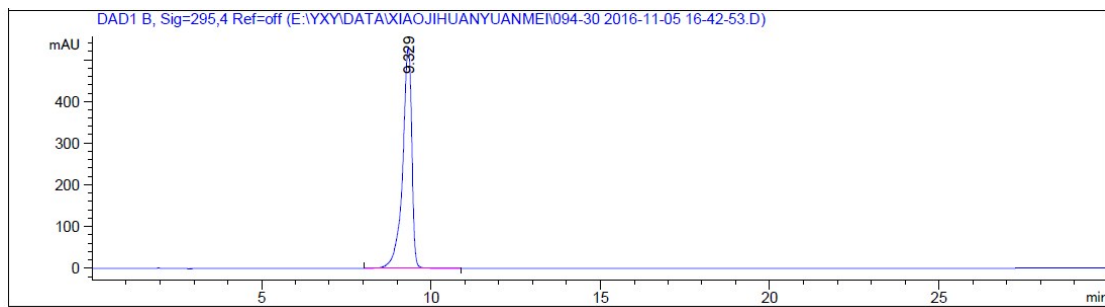
¹³C NMR spectra of A5



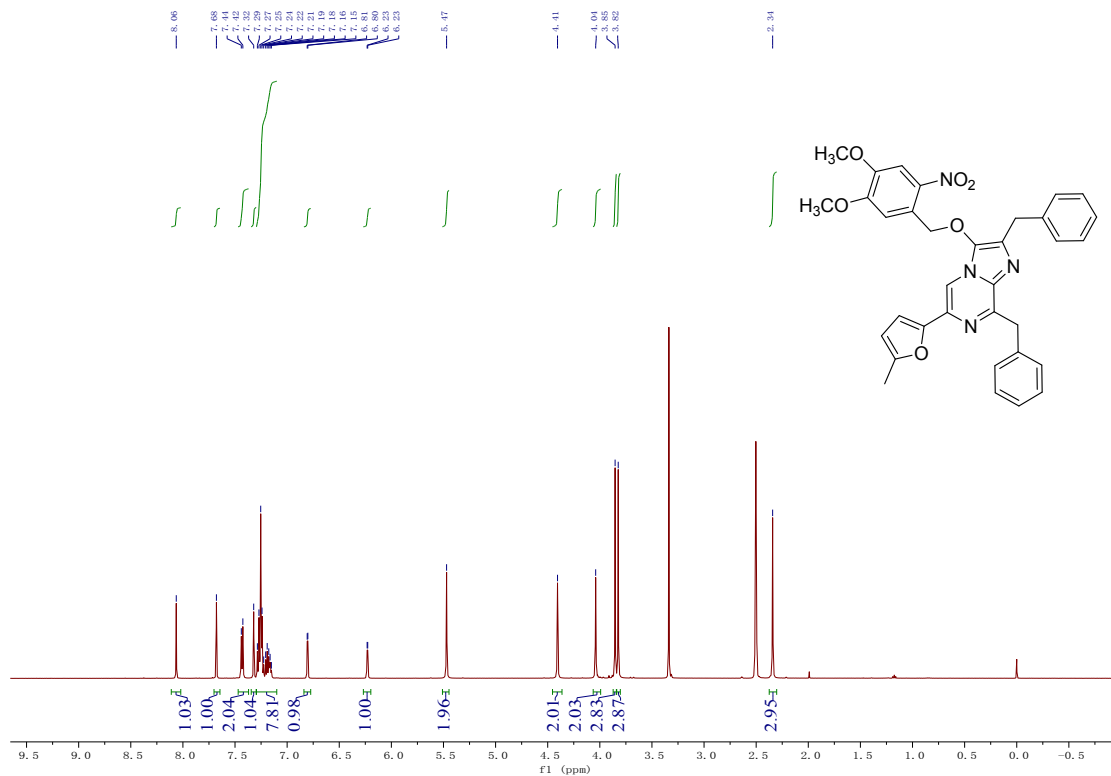
HRMS spectra of A5



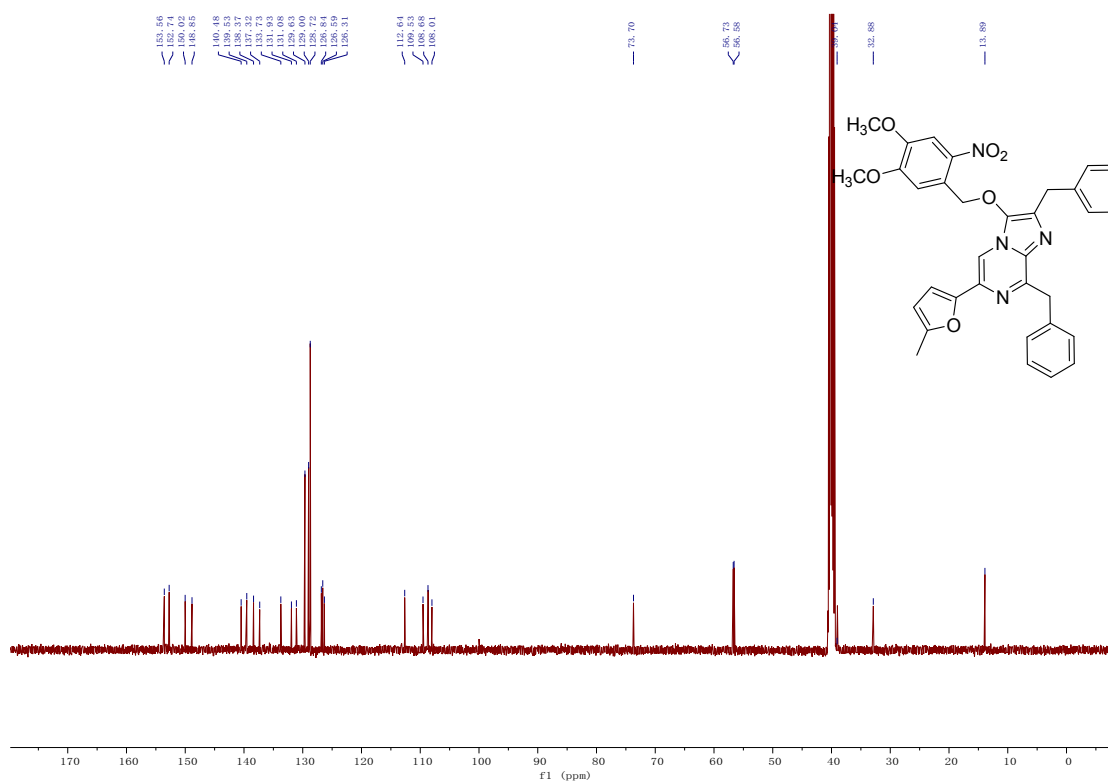
HPLC spectra of A5



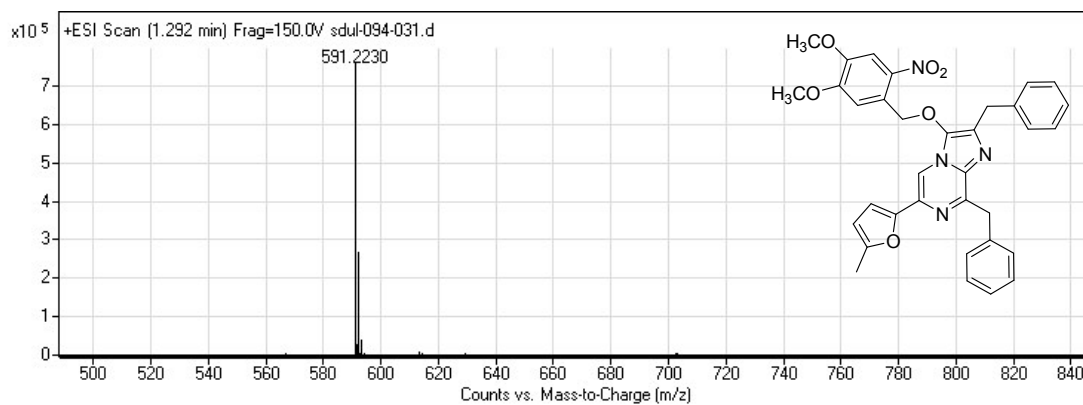
¹HNMR spectra of A6



¹³CNMR spectra of A6



HRMS spectra of A6



HPLC spectra of A6

