Real-Time Bioluminescence Imaging of Nitroreductase in Breast cancer bone metastasis

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Table of Contents

1. Experimental Details……S3
2. Reaction Mechanism between QTZ-NTR and NTR……S4
3. Cells Experiments……S5
4. NTR Expression and Purification……S6
5. Synthesis, NMR, HRMS and HPLC Data……S8
6. Reference……S17

1. Experimental Details

1.1 General Procedures

All reagents were used without further purification and purchased from commercial suppliers (unless otherwise specified). Ultrapure water was purified by the Milli-Q system with a resistivity of 18.25 MΩ·cm. The ^{1}H NMR (600 MHz) and ^{13}C NMR (151 MHz) spectra recorded on at 25°C using Bruker AV 600 MHz spectrometers were reported as parts per million (ppm) from tetramethylsilane as the internal standard. High-resolution mass spectra (HRMS) were determined by a Bruker MicroToF ESI LC-MS System in positive-ion mode. High-performance liquid chromatography (HPLC) (Agilent Technologies, USA) was conducted. Purities of new compounds 4, 5 reached at least >95% pure by HPLC (Figure S14 and S18). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), and other cell culture reagents were purchased from Gibco (Thermo Fisher Scientific, CA). LB agar and 2YT were ordered from BD Difco™. Isopropyl-β-D-thiogalactoside (IPTG) was purchased from Anatrace. 4-12% Bis-Tris gels for SDS-PAGE were purchased from Invitrogen. QuickChange Lightning Multi Site-Directed Mutagenesis Kit was purchased from Agilent Technologies (Cat. 210515). Oligonucleotide primers were purchased from Integrated DNA Technologies and Eurofins Genomics (Table S3 lists the oligonucleotides used in this report). Plasmid DNA preparation was carried out with the GenCatch™ Plus Plasmid DNA Miniprep Kit and GenCatch™ Advanced Gel Extraction Kit. FastBreak™ Cell Lysis Reagent 10x was purchased from Promega (Cat. V8572). Ni-NTA Agarose was obtained from Qiagen (Cat. 30230).

1.2 Preparation of Stock Solutions

The stock solutions (2 mM) of QTZ-NTR and QTZ-control were prepared in DMSO. The test probes solution was prepared by dilution of stock solution in PBS buffer (pH 7.4, 10 mM) or ultrapure water. All aqueous solution was prepared in ultrapure water with a resistivity of 18.25 MW•cm (purified by Millipore, USA). For typical luminescence measurements, a solution of QTZ-NTR was incubated with a certain amount of analytes (Nitroreductase (NTR) or interference) at 37°C in 5% DMSO (final concentration of probe: 10 μM). Then luminescent intensity was measured in a microplate reader (Infinite 200 PRO, Tecan). For animal study, we used a nanodrug carrier - Pluronic F127 [1] to improve the biocompatibility of QTZ, QTZ-NTR or QTZ-Control. The preparation process was as follows: First, 0.43 mg QTZ, 0.56 mg QTZ-NTR or 0.52 mg QTZ-Control was dissolved in 100 μL of THF, then 10.00 mg F127 was added. After thorough stirring, the mixture was slowly added to 1.5 mL PBS. The excess THF was removed using a rotary evaporator, and the final volume was adjusted to 1 mL with PBS.

1.3 Preparation of various analysis

Various analytes were prepared according to the reported literature.[2] The testing solutions of $CaCl_2$, KCl, $ZnSO_4$, $HgCl_2$, $FeSO_4$, and $MgCl_2$, $Na_2S_2O_3$, Na_2SO_3, NaSH, L-Cysteine, Vitamine C, L-arginine, NADPH were prepared by dissolving or diluting each of them in ultrapure water. Nitric Oxide (NO) was produced by slowly adding 4 M sulfuric acid to sodium nitrite solids, and the NO gas was purified before use by passing it through a NaOH solution to eliminate NO_2. All test solutions in the presence Nanonluciferase (Nluc) (0.5 μg/mL) were waited for 20 minutes for measurement after treating with NTR and other analytes.

1.4 Detection of Limit (LOD) of QTZ-NTR towards NTR

The detection limit (LOD) was calculated to be 0.051 μM based on the luminescence titration experiments of QTZ-NTR (10 μM) with and without NTR. To obtain the slope (K), a curve of bioluminescence intensity versus increasing NTR concentration was plotted, as shown in the figure 1E. The LOD was derived using the formula LOD = 3σ/K, where σ represents the standard deviation of 10 blank measurements, and K is the slope of the bioluminescence intensity in response to NTR

concentration.

1.5 Cell culture

The 4T1.2-Nluc cells were maintained in DMEM with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin streptomycin) at 37°C in a humidified incubator with 5% CO_2. The medium was changed every 24 ~ 48 h. The cells were subcultured at 90% confluence with 0.25% trypsin (w/v) every 2 ~ 3 days. Luciferase activities were determined using a multimode microplate reader (Infinite 200 PRO, Tecan). Total bioluminescent intensity counts are integrated over wavelengths from 400 to 800nm, and the exposure time is 1000 ms.

1.6 Mice Model

Balb/c female mice, 6 weeks of age, were purchased from Jackson Laboratory. All mouse protocols were in accordance with NIH guidelines and were approved by the Institutional Animal Care and Use Committee of Rice University. Animals were imaged using IVIS Lumina II (Advanced Molecular Vision), following the recommended procedures and manufacturer's settings. The breast cancer bone metastasis model was established in Balb/c mice by injecting 4T1.2-Nluc cells (5 X 10^4 cells) into the left tibia, while simultaneously injecting 4T1.2-Nluc cells (1 X 10^6) into the right mammary fat pad to create an orthotopic breast cancer primary tumor.

1.7 Imaging of NTR in Breast Cancer Bone Metastasis Model

During the experiment, the mice were anesthetized with isoflurane. For mornitoring the tumor growth, On 11st, 17th, 22nd and 27th day, QTZ (1mM, 100 μL) was intravenously (retro-orbital (R.O.)) injected into three mice. For NTR detection, QTZ-NTR (1mM, 100 μL) was intravenously (R.O.) injected into three mice on 12th, 18th, 23rd and 28th day. On 19th and 29th day, QTZ-Control (1mM, 100 μL) was intravenously (R.O.) injected into mice. As the inhibitors experiments, on 28th day, dicoumarol (5 mM and 10 mM) was intraperitoneally injected into the mice. Bioluminescence intensity was then measured for 60 s using the IVIS Imager.

2. Reaction Mechanism between QTZ-NTR and NTR

2.1 HR-MS Verification

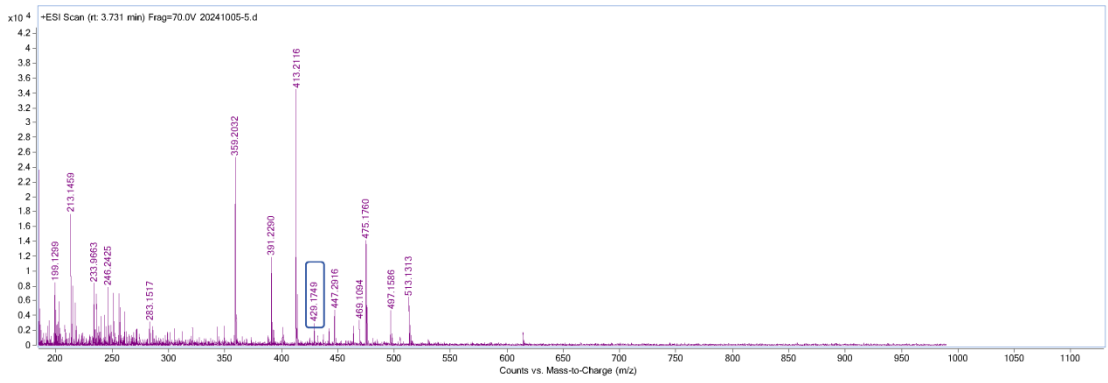
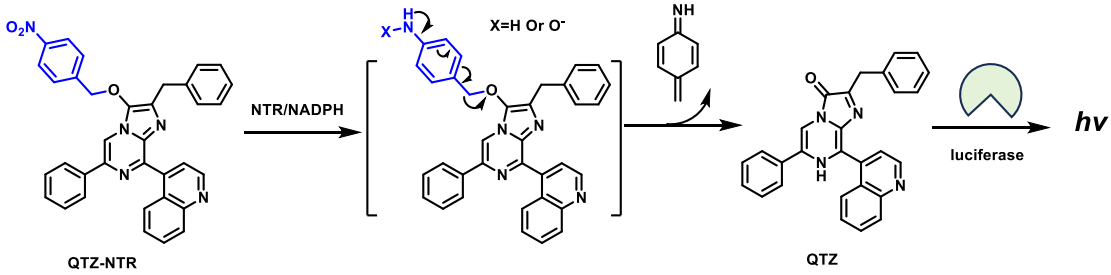


Figure S1. HR-MS analysis of the products in the reaction of QTZ-NTR (10 μM) with NTR (1 μg/mL) and NADPH (250 μg/mL) in PBS (pH = 7.4) after incubation for 5 min at 37.4°C

2.2 Proposed Mechanism



Scheme S1. Suggested mechanism for QTZ-NTR and NTR.

QTZ-NTR was catalyzed by NTR in the presence of NADPH, followed by 1,6-rearrangement-elimination reaction (p-azaquinone methide elimination), resulting in the release of QTZ[3]. The oxidative mechanism of QTZ under Nluc is detailed in the references.[4]

3. Cells Experiments

3.1 Lentivirus Packing and Establishment of Nanoluc-Expressing Stable Cell Lines

The Nanoluc gene was amplified from gBlock - DNA sequence of Nanoluc (Table S1) from twist bioscience using oligos RL43 and RL44 (Table S2). pLV-EF1a vector was amplified using oligos RL45-RL48 (Table S2). pLV-EF1a vector was a gift from Dr. Yu J. Cao's lab at Peking University. Nanoluc gene was inserted into pLV-EF1a vector via Gibson assembly and confirmed by sequencing oligos RL49 and RL50 (Table S2). pLV-EF1a-Nanoluc vector was co-transfected with pMDLg/pRRE (Plasmid #12251), pRSV-Rev (Plasmid #12253) and pCMV-VSV-G (Plasmid #8454) using Lipofectamine™ 2000 Transfection Reagent (Invitrogen™). HEK-293T was cultured in DEME without P/S, the cell culture media containing lentiviruses were collected at 48 h, filtered through 0.45 μm PES syringe filters.

4T1.2 cells were cultured in DMEM. Half of DMEM was replaced by the cell culture media containing lentiviruses when it reaches 50% confluency, the final medium contains 10 ug/ml hexadimethrine bromide. The cells were cultured in the media containing lentiviruses for 48 h, the medium was replaced by fresh DMEM after 48 h, and the 4T1.2-Nluc cells were allowed to recover for 24 h. The cell was treated with trypsin, single-cell clonal was obtained by serial dilution. Morphology and nanoluciferase expression were checked for each single cell clone. Various 4T1.2-Nluc clones were collected for future use.

Table S1. DNA and protein sequences of the Nanoluciferase.

DNA sequence of Nanoluc	atggtcttcacactcgaagatttcgttggggactggcgacagacagccggctacaacctggaccaagtccttgaacag ggaggtgtgtccagtttgtttcagaatctcggggtgtccgtaactccgatccaaaggattgtcctgagcggtgaaaatgg gctgaagatcgacatccatgtcatcatcccgtatgaaggtctgagcggcgaccaaatgggccagatcgaaaaaatttt taaggtggtgtaccctgtggatgatcatcactttaaggtgatcctgcactatggcacactggtaatcgacggggttacgc cgaacatgatcgactatttcggacggccgtatgaaggcatcgccgtgttcgacggcaaaaagatcactgtaacaggg accctgtggaacggcaacaaaattatcgacgagcgcctgatcaaccccgacggctccctgctgttccgagtaaccat caacggagtgaccggctggcggctgtgcgaacgcattctggcgtaa
Protein sequence of Nanoluc	MVFTLEDFVGDWRQTAGYNLDQVLEQGGVSSLFQNLGVSVTPIQRIVLSGENGLKI DIHVIIPYEGLSGDQMGQIEKIFKVVYPVDDHHFKVILHYGTLVIDGVTPNMIDYFGRP YEGIAVFDGKKITVTGTLWNGNKIIDERLINPDGSLLFRVTINGVTGWRLCERILA*

Table S2. Oligonucleotides used in this study

RL43	ccgcggccgcccggggatccgccaccatggtcttcacact
RL44	gtaatccagaggttgattgtcgacttacgccagaatgcgttcgc
RL45	gtcgacaatcaacctctggattac
RL46	ggatccccgggcggccgc

RL47	cgcctggtatctttatagtcctgtcg
RL48	cgacaggactataaagataccaggcg
RL49	GGAATTTGCCCTTTTTGAGTTTGG
RL50	gaagcaatagcatgatacaaaggcattaaag

3.2 Nanoluciferase Expression and Purification

Plasmid pET28a-Nanoluc-3F10H was transformed into BL21 (DE3) and the plate was grown overnight at 37 °C. The next day, a colony was picked into 2YT medium, growing overnight. Then the starter culture was used to inoculate Luria-Bertani (LB) medium at 37 °C. After the OD600 of the bacteria culture reached 0.6, 1mM Isopropyl β-d-1-thiogalactopyranoside (IPTG) was added to induce protein expression. After growth at 30 °C overnight, cells were harvested by centrifugation at 4000 rpm for 10 min and lysed using sonication. Proteins were purified from supernatant on Ni-NTA resin (Qiagen) following the manufacturer's instructions. The purified protein was used for SDS-PAGE and ESI-MS analysis.

3.3 Cytotoxicity Assay

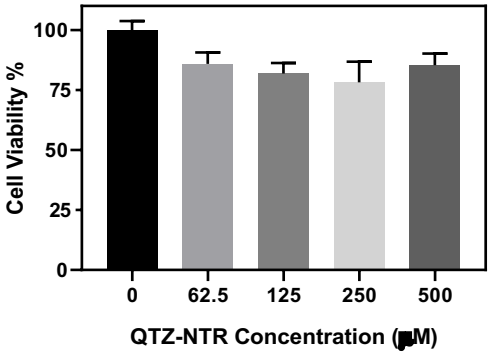


Figure S2. Cell viability of QTZ-NTR on 4T1.2-Nluc cells. The cells were incubated for 24 h with various concentrations of QTZ-NTR (0–500 μM) through the standard CCK-8 assay. The cell viability is directly related to the absorbance at 450 nm. The error bars indicate ± SD.

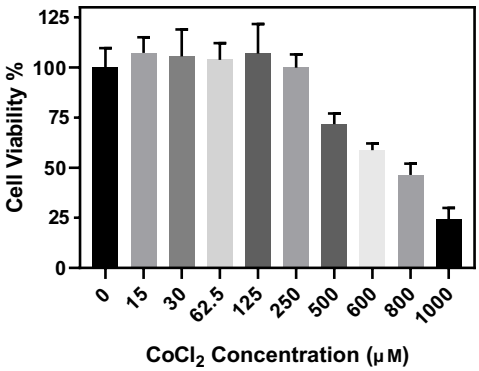


Figure S3. Cell viability of $CoCl_2$ on 4T1.2-Nluc cells. The cells were incubated for 24 h with various concentrations of $CoCl_2$ (0–1000 μM) through the standard CCK-8 assay. The cell viability is directly related to the absorbance at 450 nm. The error bars indicate ± SD.

4. NTR Expression and Purification

4.1 Plasmid Construction

For plasmid pET22b-T5-NTR-His_6, vector pET22b-T5-His6 was amplified by PCR from plasmid pET22b-Ts-sfGFP-His_6 in our previous work[5] (by primer mz377, mz378). Insertion NTR (*E. coli*

nitroreductase) was subcloned from *E. coli* genome (by primer mz375, mz376). The two PCR products listed above then were assembled via Gibson assembly. DNA sequence of NTR is listed in Table S4.

Table S3. Oligonucleotide information in this study.

Oligonucleotide	Sequence (5'-3')
mz375	GGAGAAATTACATATGGATATCATTTCTGTCGCCTTAAAGCGT
mz376	CACTTCGGTTAAGGTGATGTTTTGCG
mz377	TCACCTTAACCGAAGTGCACCACCACCACCACCACTAAAGCT
mz378	GAAATGATATCCATATGTAATTTCTCCTCTTTAATGAATTCTGTGTGAAATTG

Table S4. DNA sequence in this study.

Gene Name	Sequence (5'-3')
NTR	ATGGATATCATTTCTGTCGCCTTAAAGCGTCATTCCACTAAGGCATTTGATGCCAGCA AAAAACTTACCCCGGAACAGGCCGAGCAGATCAAAACGCTACTGCAATACAGCCCA TCCAGCACCAACTCCCAGCCGTGGCATTTTATTGTTGCCAGCACGGAAGAAGGTAA AGCGCGTGTTGCCAAATCCGCTGCCGGTAATTACGTGTTCAACGAGCGTAAAATGC TTGATGCCTCGCACGTCGTGGTGTTCTGTGCAAAAACCGCGATGGACGATGTCTGG CTGAAGCTGGTTGTTGACCAGGAAGATGCCGATGGCCGCTTTGCCACGCCGGAAG CGAAAGCCGCGAACGATAAAGGTCGCAAGTTCTTCGCTGATATGCACCGTAAAGATC TGCATGATGATGCAGAGTGGATGGCAAAACAGGTTTATCTCAACGTCGGTAACTTCC TGCTCGGCGTGGCGGCTCTGGGTCTGGACGCGGTACCCATCGAAGGTTTTGACGC CGCCATCCTCGATGCAGAATTTGGTCTGAAAGAGAAAGGCTACACCAGTCTGGTGG TTGTTCCGGTAGGTCATCACAGCGTTGAAGATTTTAACGCTACGCTGCCGAAATCTC GTCTGCCGCAAAACATCACCTTAACCGAAGTG

4.2 NTR Protein Expression and Purification from *E. coli*

E. coli. BL21(DE3) cells were transformed with plasmid (pET22b-T5-NTR-His_6) and grown in 2YT medium at 37°C as starter culture. The protein expression was carried out in Luria-Bertani (LB) medium with 100 x starter culture. Expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactoside (IPTG) at OD 0.6 and cells were grown for an additional 18 h at 20°C. Cells were harvested by centrifugation at 4000 rpm for 10 min. The cell pellets were suspended in wash buffer (1x phosphate buffered saline (PBS) with 20 mM imidazole) and lysed by sonication. The resulting cell lysate was clarified by centrifugation at 10000 rpm for 30 min. Protein was purified on Ni-NTA resin (Qiagen) following the manufacturer's instructions. The purified protein was used for SDS-PAGE (Figure S4). Protein yield of NTR was ~ 5 mg/L LB.

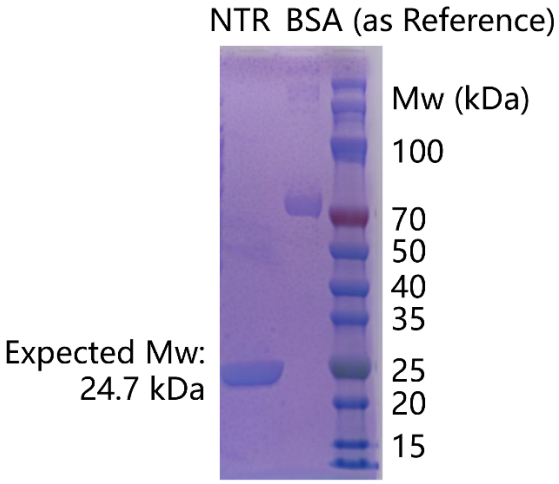
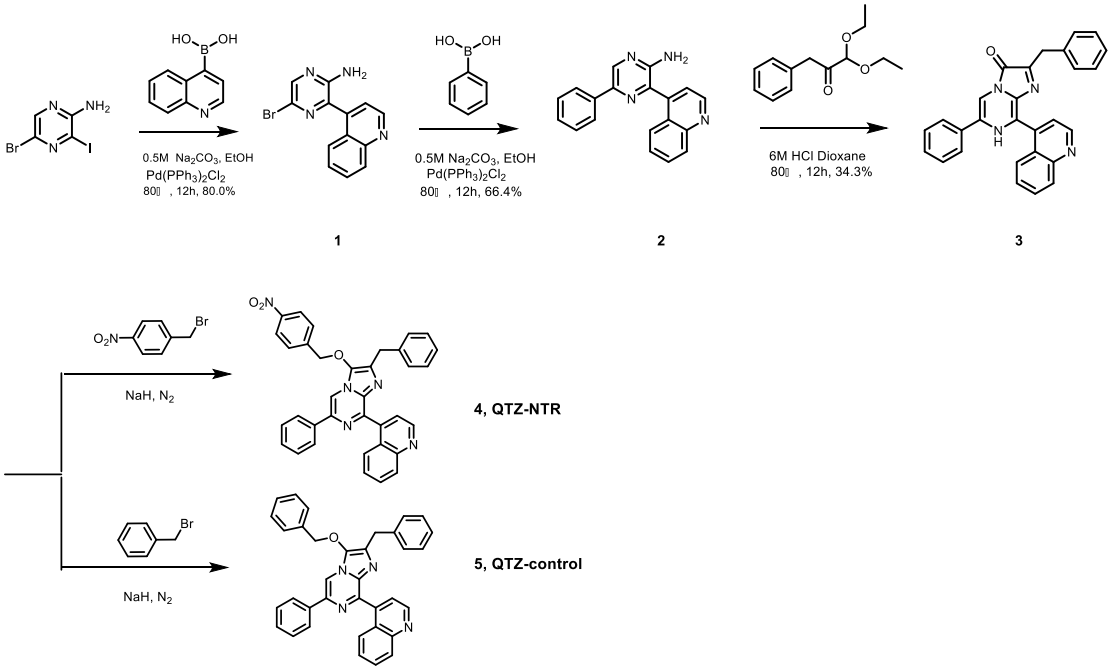


Figure S4. SDS-PAGE analysis of NTR.

5. Synthesis, NMR, HRMS and HPLC Data



Scheme S2. Synthetic routes for QTZ-NTR and QTZ-control

5.1 Synthesis and Characterization

Synthesis of compounds 1, 2, and 3 were prepared by literature-reported methods with a minor modification.[4]

5-bromo-3-(quinolin-4-yl)pyrazin-2-amine (1)

In a 100 mL round-bottom flask, $Pd(PPh_3)_2Cl_2$ (140.4 mg, 0.2 mmol, 0.1 eq) and 5-Bromo-3-iodopyrazin-2-amine (597.7 mg, 2 mmol, 1 eq) were dissolved in 50 mL of ethanol. Quinoline-4-boronic acid (346.1 mg, 2 mmol, 1 eq) and 0.5 M Na_2CO_3 solution (4 mL, 4 mmol, 2 eq) were subsequently added. The resulting mixture was stirred overnight at 80°C under nitrogen. The solvent was removed under vacuum, and the residue was extracted with ethyl acetate (3 × 100 mL). The combined organic layers were dried over

anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography eluting with EA/Hex (1:1) to afford Compound 1 as a pale yellow powder (yield: 480.0 mg, 80.0%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.94 (d, *J* = 4.2 Hz, 1H), 8.26 (s, 1H), 8.15 (d, *J* = 8.2 Hz, 1H), 7.76 (t, *J* = 7.6 Hz, 1H), 7.68 (t, *J* = 8.7 Hz, 1H), 7.57 (dd, *J* = 17.5, 9.7 Hz, 1H), 7.48 (dd, *J* = 11.0, 3.1 Hz, 1H), 4.71 (s, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 151.63, 150.34, 148.87, 144.86, 140.95, 137.23, 130.38, 130.22, 127.77, 126.83, 125.07, 124.81, 121.54.

5-phenyl-3-(quinolin-4-yl)pyrazin-2-amine (2)

In a 100 mL round-bottom flask, $Pd(PPh_3)_2Cl_2$ (56.2 mg, 0.08 mmol, 0.1 eq) and Compound 1 (240.0 mg, 0.8 mmol, 1 eq) were dissolved in 50 mL of ethanol. Phenyl boronic acid (195.3 mg, 1.6 mmol, 2 equiv.) and 0.5 M Na_2CO_3 solution (1.6 mL, 1.6 mmol, 2 equiv.) were subsequently added. The resulting mixture was stirred overnight at 80°C under nitrogen. The solvent was removed under vacuum, and the residue was extracted with ethyl acetate (3 × 100 mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography eluting with EA/Hex (1:1) to afford Compound 2 as a light yellow powder (yield: 158.4mg, 66.4%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.03 (d, *J* = 4.3 Hz, 1H), 8.74 (s, 1H), 8.14 (d, *J* = 8.4 Hz, 1H), 7.94 (d, *J* = 7.5 Hz, 2H), 7.82 – 7.77 (m, 1H), 7.73 (d, *J* = 8.3 Hz, 1H), 7.65 (t, *J* = 6.9 Hz, 1H), 7.63 – 7.55 (m, 1H), 7.41 (t, *J* = 7.7 Hz, 2H), 7.36 – 7.28 (m, 1H), 6.34 (s, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 170.84, 153.27, 151.00, 148.88, 143.11, 139.93, 139.81, 137.20, 135.94, 134.56, 133.49, 132.81, 132.53, 132.00, 131.93, 130.50, 130.06, 129.98, 129.27, 129.22, 128.17, 127.83, 127.41, 126.44, 125.83, 125.44, 125.22, 122.25.

2-benzyl-6-phenyl-8-(quinolin-4-yl)imidazo[1,2-*a*]pyrazin-3(7*H*)-one (3)

In a 50 mL round-bottom flask, Compound 2 (29.8 mg, 0.1 mmol, 1 eq) and 1,1-diethoxy-3-phenylpropan-2-one (88.9 mg, 0.4 mmol, 4 eq) were dissolved in 5 mL of degassed 1,4-dioxane. 0.5 mL of 6 M hydrochloric acid (30 eq) was added dropwise. The resulting mixture was stirred overnight at 80°C under nitrogen. The solvent was removed under vacuum, and the residue was extracted with ethyl acetate (3 × 50 mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography under nitrogen pressure eluting with EA/Hex (5:1) to afford Compound 3 as an orange powder, sealed under nitrogen (yield: 14.7 mg, 34.3%). ^{1}H NMR (600 MHz, MeOD) δ 9.02 (t, *J* = 7.7 Hz, 1H), 8.25 (s, 1H), 8.16 (t, *J* = 15.5 Hz, 1H), 7.99 (t, *J* = 10.4 Hz, 1H), 7.94 – 7.87 (m, 2H), 7.87 – 7.80 (m, 2H), 7.62 (t, *J* = 7.5 Hz, 1H), 7.48 (t, *J* = 7.5 Hz, 2H), 7.45 – 7.38 (m, 2H), 7.24 (t, *J* = 9.0 Hz, 2H), 7.20 (t, *J* = 7.6 Hz, 2H), 7.16 – 7.08 (m, 1H), 4.07 (s, 2H). ^{13}C NMR (151 MHz, MeOD) δ 149.48, 147.89, 138.99, 131.64, 130.08, 128.66, 128.61, 128.54, 128.44, 128.39, 128.27, 127.97, 127.41, 127.16, 126.66, 126.26, 125.81, 125.42, 125.22, 122.53, 109.39, 31.73.

General Synthetic Procedure for Compounds 4 and 5

In a 25 mL round-bottom flask, NaH (1.7 mg, 0.069 mmol, 3eq) and Compound 3 (10.0 mg,

0.023 mmol, 1eq) were dissolved in 1 mL of THF and stirred under nitrogen at room temperature for 15 minutes, during which the solution color changed from orange to black. Bromide (0.069 mmol, 3eq) was then added dropwise. The resulting mixture was stirred overnight at 80°C under nitrogen. The solvent was removed under vacuum, and the residue was extracted with ethyl acetate (3 × 10 mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography eluting with Acetone/Hex (2:1) to afford Compounds 4 and 5.

4-(2-benzyl-3-((4-nitrobenzyl)oxy)-6-phenylimidazo[1,2-*a*]pyrazin-8-yl)quinolone (4)

Yield: 22.1%. ^{1}H NMR (600 MHz, Acetone-d_6) δ 8.97 (d, *J* = 4.1 Hz, 1H), 8.63 (s, 1H), 8.21 (d, *J* = 8.5 Hz, 1H), 8.14 (d, *J* = 8.3 Hz, 2H), 8.07 (t, *J* = 7.6 Hz, 1H), 7.97 (t, *J* = 5.7 Hz, 3H), 7.68 (t, *J* = 7.2 Hz, 3H), 7.45 (dd, *J* = 16.1, 8.2 Hz, 1H), 7.37 – 7.32 (m, 2H), 7.28 (t, *J* = 7.4 Hz, 1H), 7.14 (d, *J* = 7.7 Hz, 2H), 7.09 (t, *J* = 7.5 Hz, 2H), 7.02 (t, *J* = 7.3 Hz, 1H), 5.35 (s, 2H), 3.97 (s, 2H). ^{13}C NMR (151 MHz, Acetone-d_6) δ 150.17, 149.54, 148.49, 147.91, 143.95, 141.48, 139.62, 138.47, 137.64, 137.09, 135.27, 132.85, 130.36, 130.25, 129.80, 129.59, 129.13, 129.04, 128.91, 128.69, 127.08, 126.65, 126.60, 126.56, 125.74, 124.01, 123.81, 123.33, 111.59, 75.72. ESI-MS(m/z): calcd $C_{35}H_{26}N_5O_3$ for $[M + H]^+$ = 564.2036, found: 564.2046.

4-(2-benzyl-3-(benzyloxy)-6-phenylimidazo[1,2-*a*]pyrazin-8-yl)quinoline (5)

Yield: 27.2%. ^{1}H NMR (600 MHz, Acetone-d_6) δ 9.10 (d, *J* = 4.3 Hz, 1H), 8.52 (s, 1H), 8.32 (dd, *J* = 8.5, 0.8 Hz, 1H), 8.18 (t, *J* = 7.1 Hz, 1H), 8.09 (d, *J* = 4.3 Hz, 1H), 8.06 (m, 2H), 7.83 – 7.78 (m, 1H), 7.61 – 7.56 (m, 1H), 7.53 – 7.46 (m, 4H), 7.45 – 7.37 (m, 4H), 7.31 (d, *J* = 7.6 Hz, 2H), 7.27 – 7.22 (m, 2H), 7.17 (t, *J* = 7.3 Hz, 1H), 5.30 (s, 2H), 4.06 (s, 2H). ^{13}C NMR (151 MHz, Acetone-d_6) δ 149.78, 149.13, 147.34, 141.15, 139.41, 137.81, 137.38, 136.72, 136.27, 135.20, 132.34, 129.83, 129.24, 129.18, 129.00, 128.73, 128.72, 128.69, 128.44, 128.31, 126.67, 126.28, 126.23, 126.14, 122.92, 111.03, 77.19, 32.87. ESI-MS(m/z): calcd $C_{35}H_{27}N_4O$ for $[M + H]^+$ = 519.2185, found: 519.2208.

5.2 ^{1}H, ^{13}C NMR and HRMS and HPLC Data

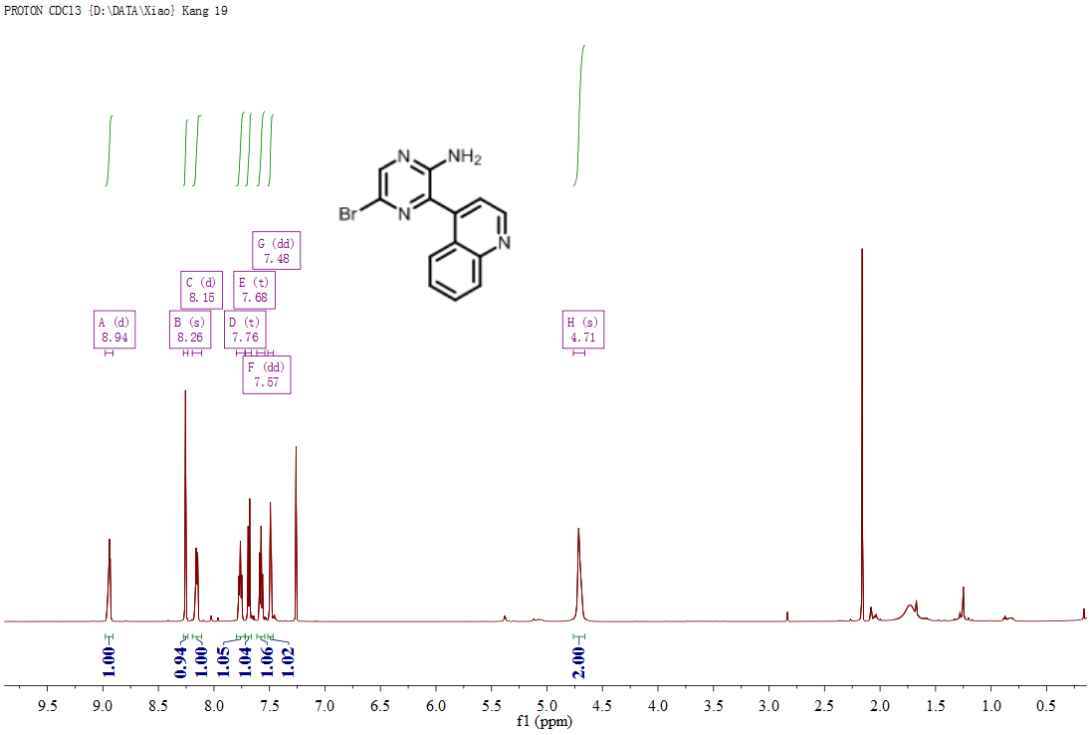


Figure S5. The ^{1}H-NMR spectrum of compound 1 ($CDCl_3$, 298 K, 600 MHz)

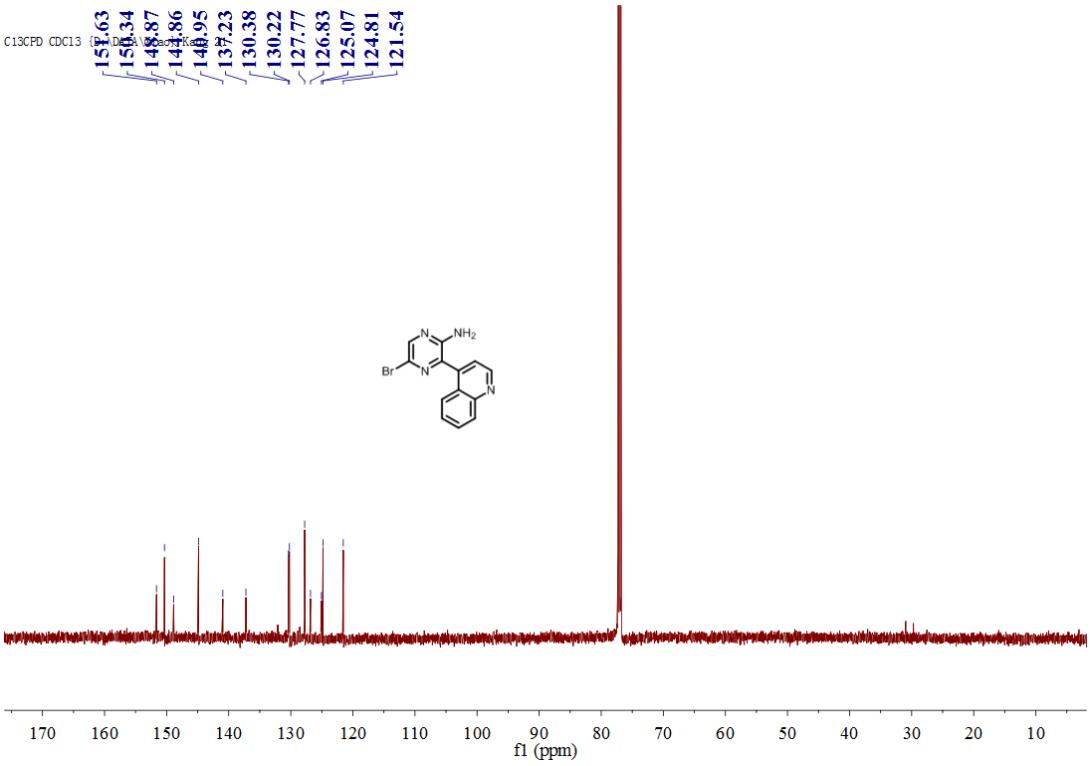


Figure S6. The ^{13}C-NMR spectrum of compound 1 ($CDCl_3$, 298 K, 151 MHz)

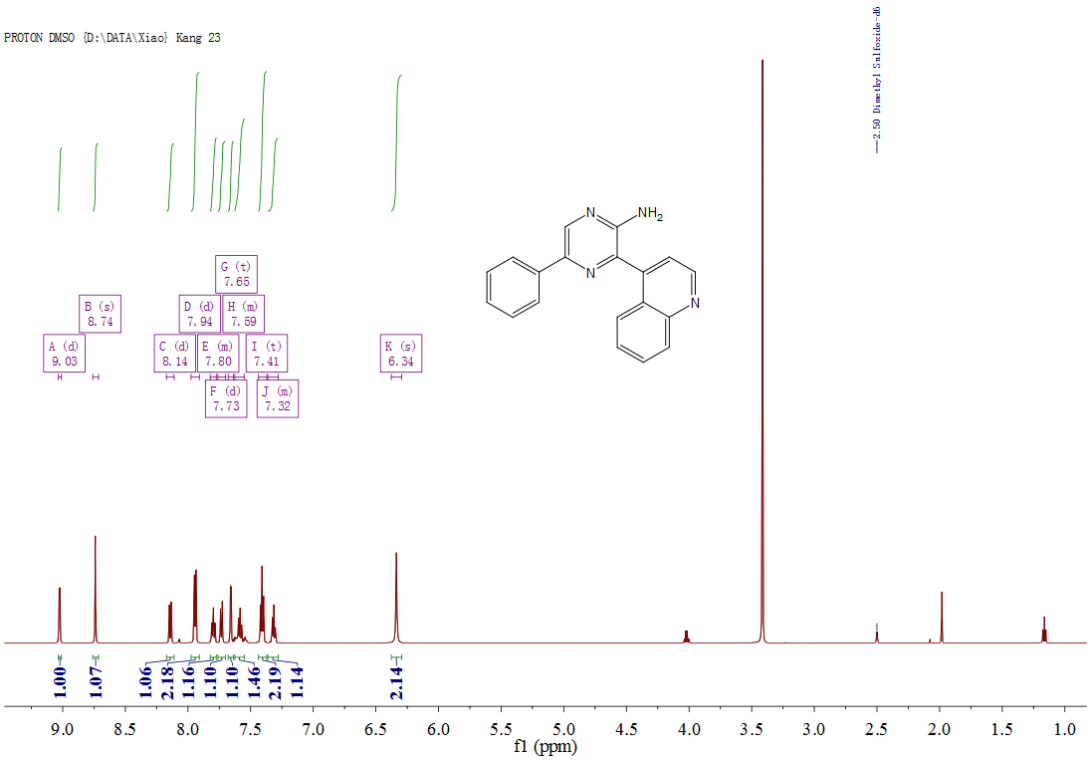


Figure S7. The ^{1}H-NMR spectrum of compound 2 (DMSO-d_6, 298 K, 600 MHz)

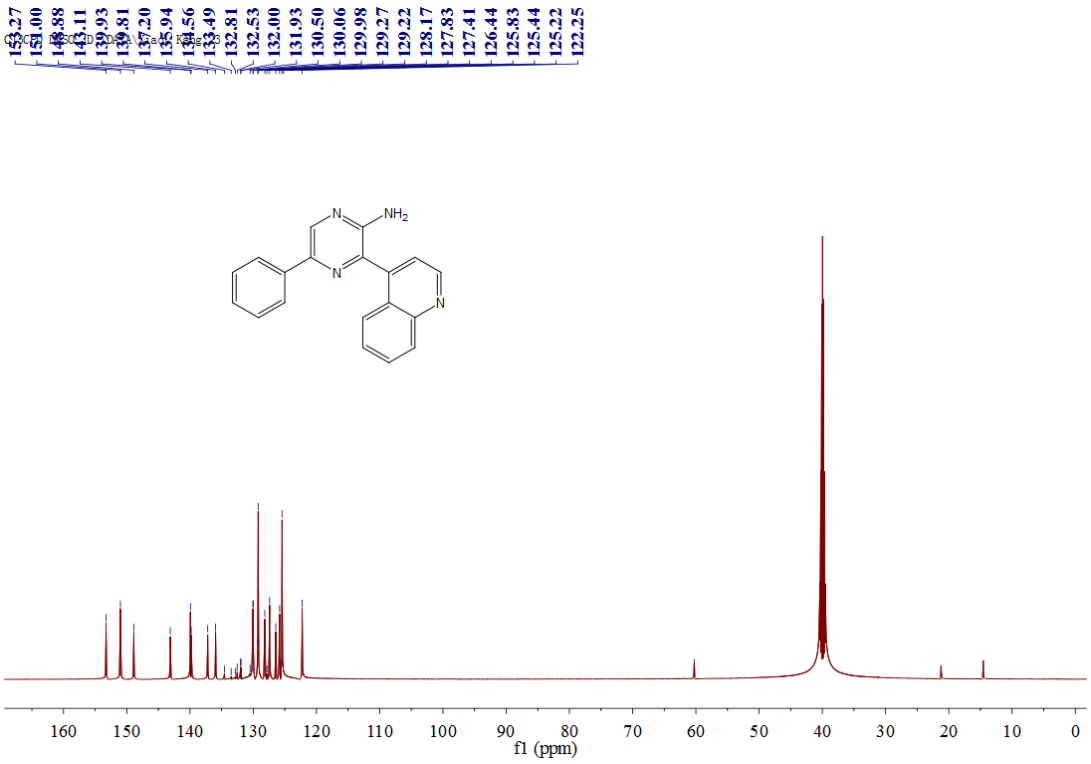


Figure S8. The ^{13}C-NMR spectrum of compound 2 (DMSO-d_6, 298 K, 151 MHz)

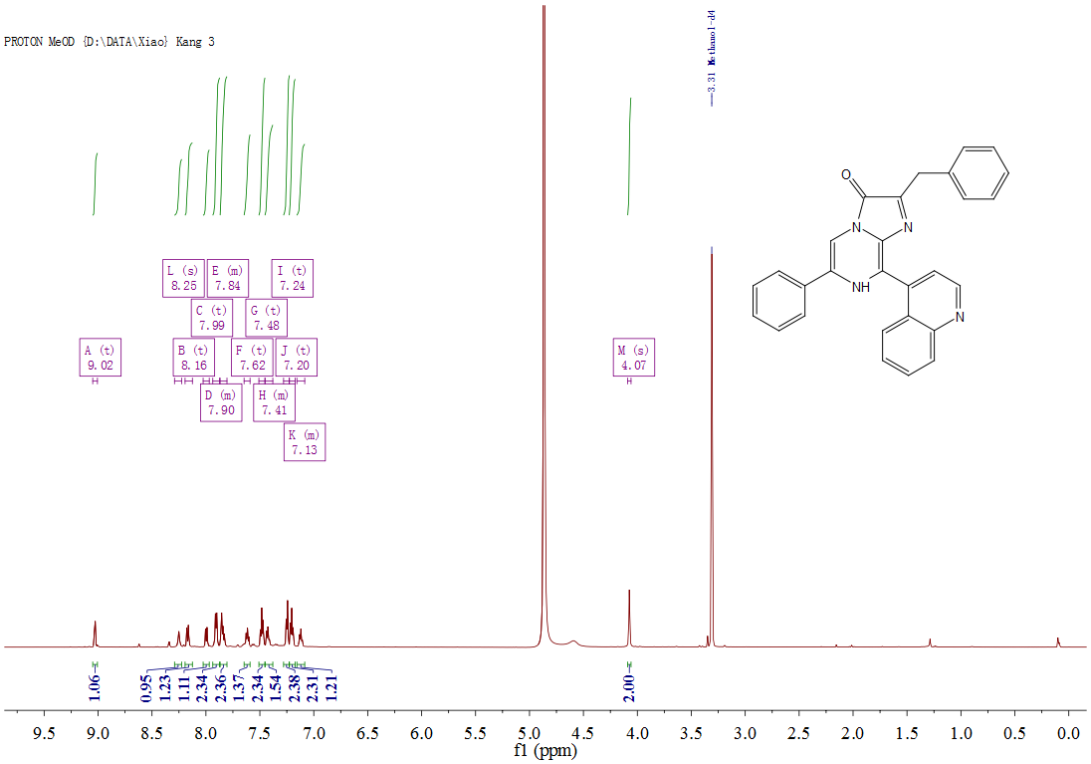


Figure S9. The ^{1}H-NMR spectrum of compound 3 (MeOD, 298 K, 600 MHz)

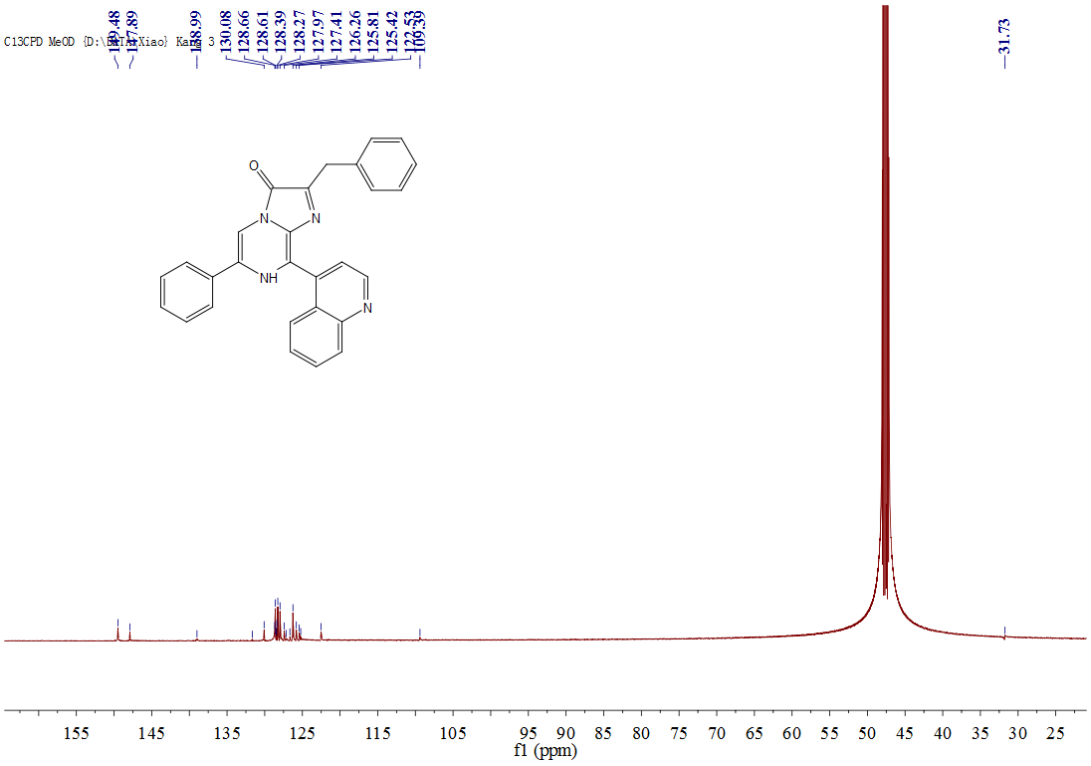


Figure S10. The ^{13}C-NMR spectrum of compound 3 (MeOD, 298 K, 151 MHz)

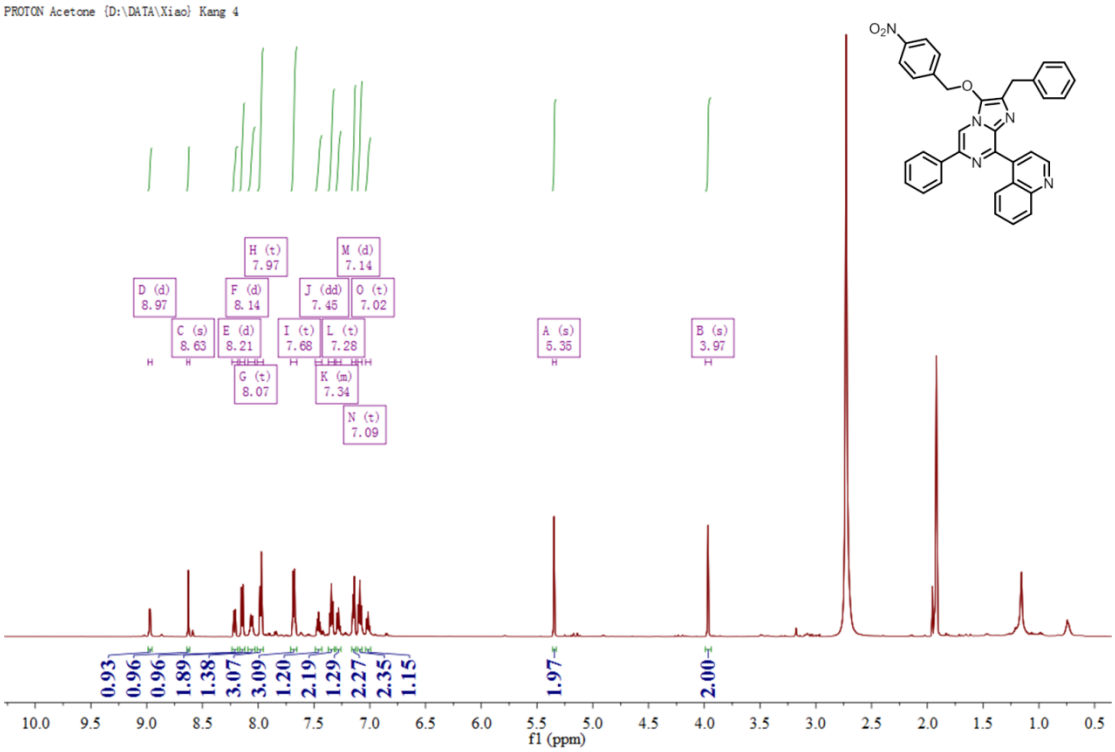


Figure S11. The ^{1}H-NMR spectrum of compound 4 (Acetone-d_6, 298 K, 600 MHz)

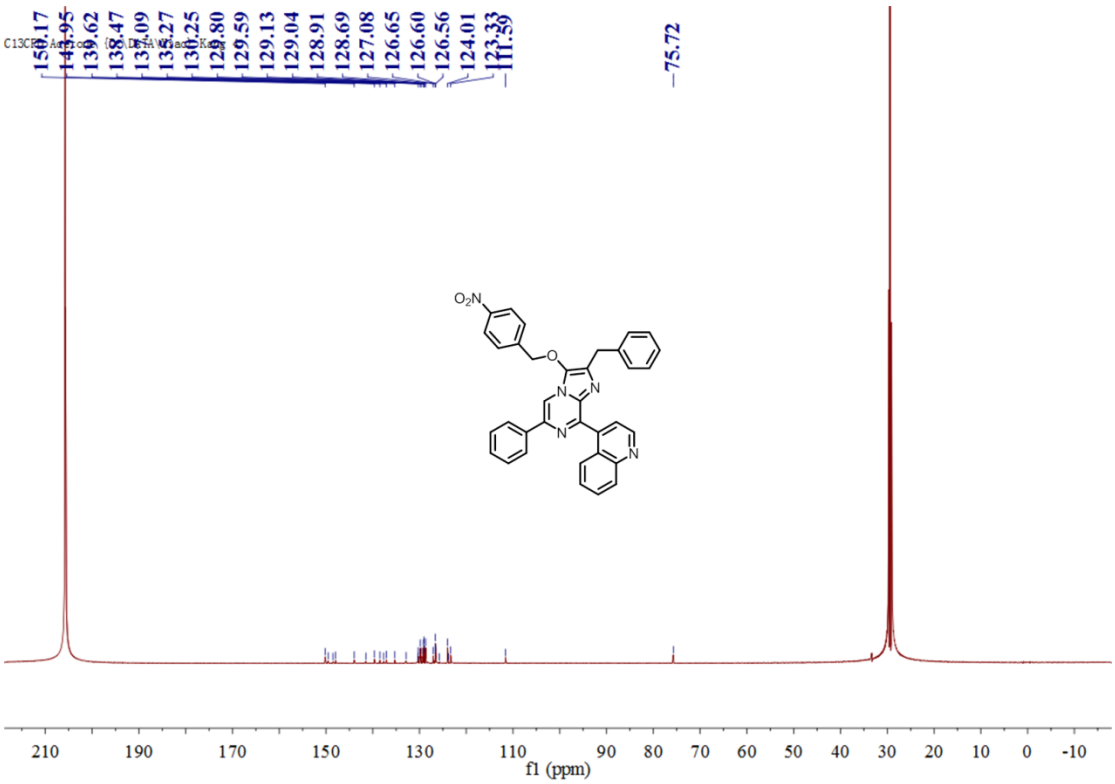


Figure S12. The ^{13}C-NMR spectrum of compound 4 (Acetone-d_6, 298 K, 151 MHz)

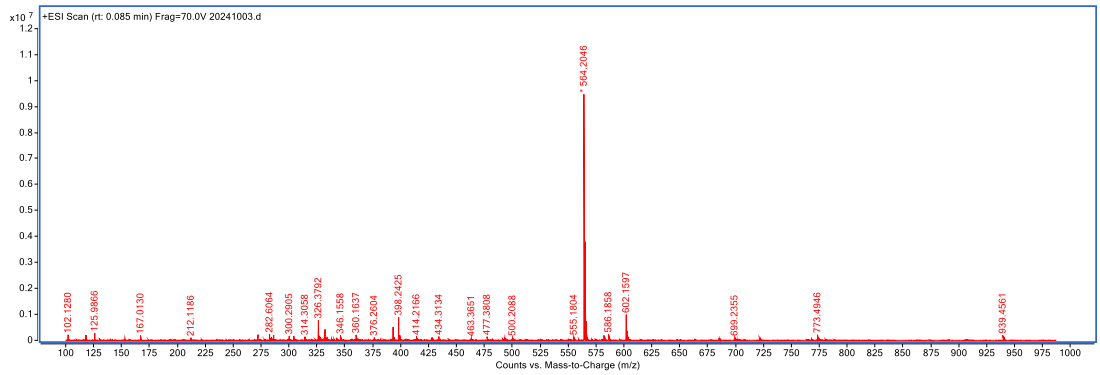


Figure S13. The HRMS spectrum of compound 4

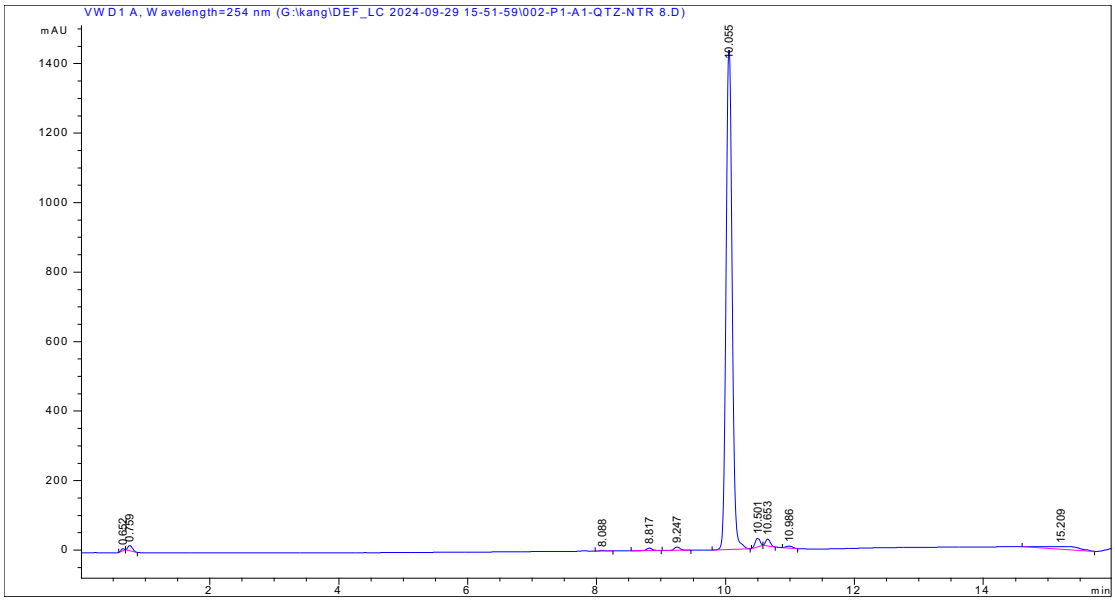


Figure S14. HPLC Analysis of Compound 4: t_R = 10.055 min over 16 min of 0.5 mL min^{-1} mobile phase (10-100% MeCN aqueous solution), purity 98.6%.

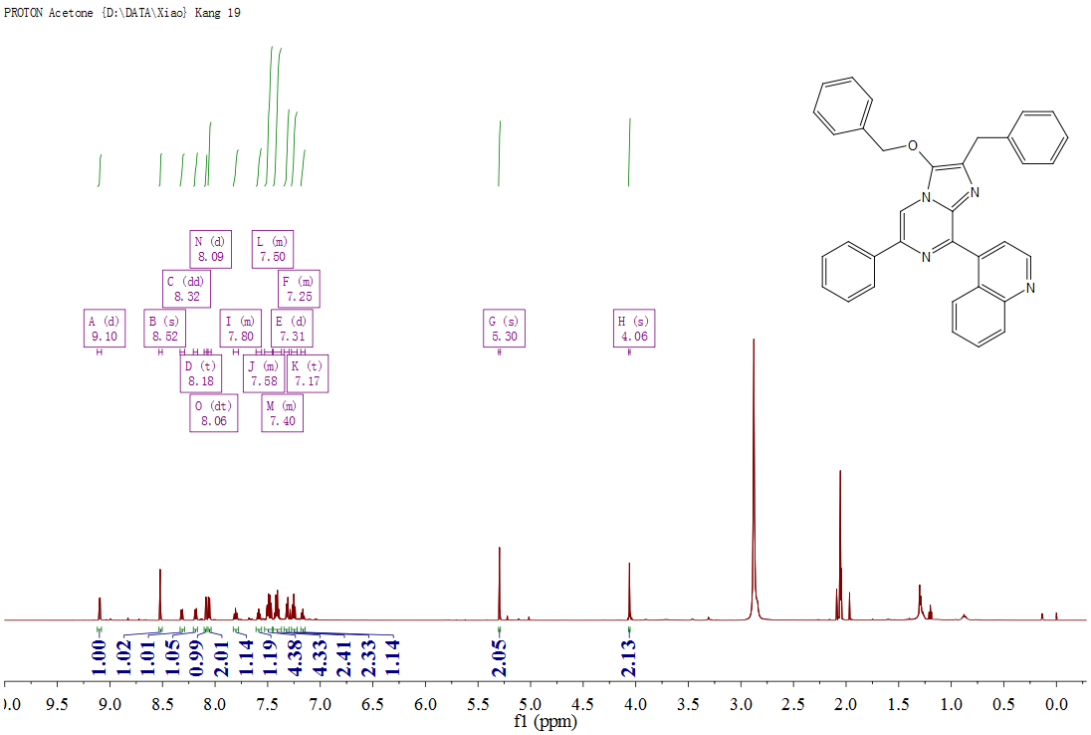


Figure S15. The ^{1}H-NMR spectrum of compound 5 (Acetone-d_6, 298 K, 600 MHz)

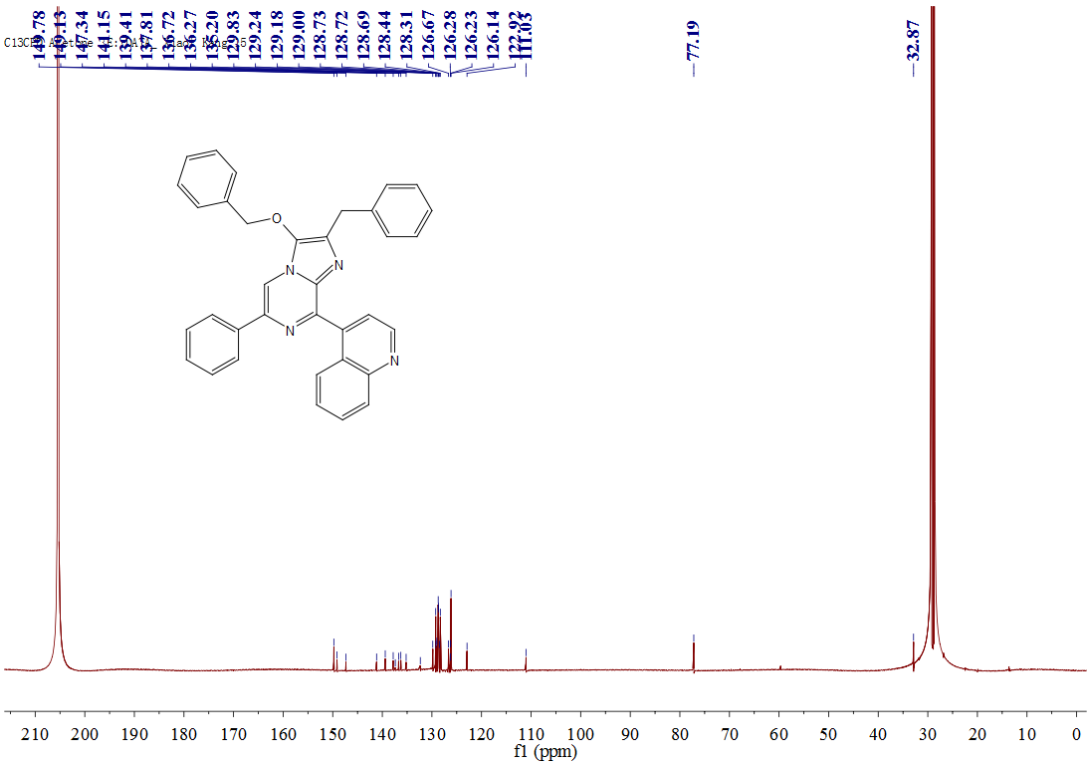


Figure S16. The ^{13}C-NMR spectrum of compound 5 (Acetone-d_6, 298 K, 151 MHz)

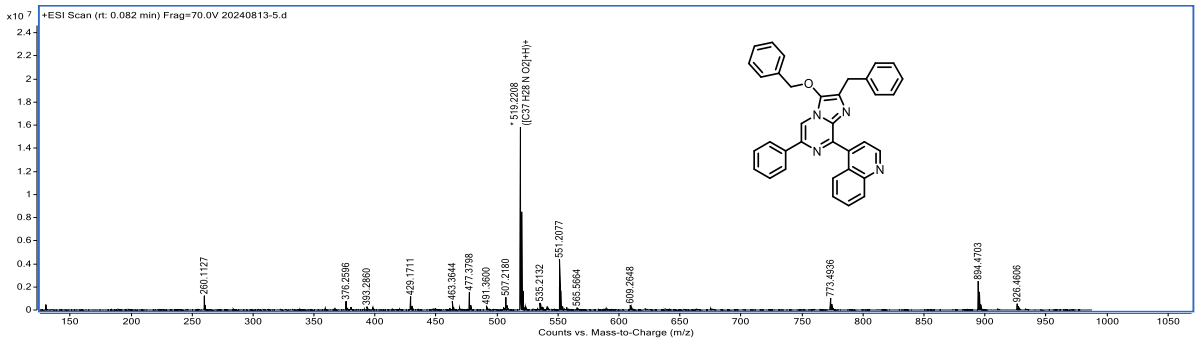


Figure S17. The HRMS spectrum of compound 5

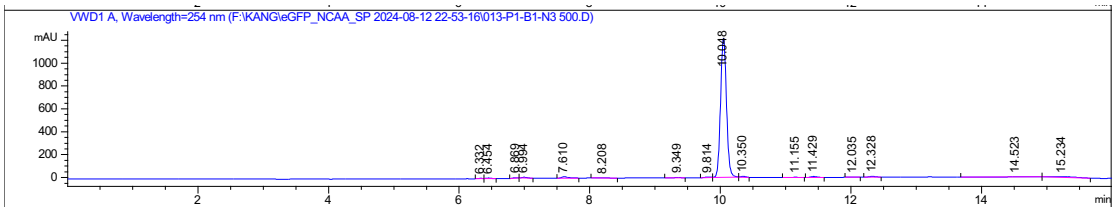


Figure S18. HPLC Analysis of Compound 5: t_R = 10.048 min over 16 min of 0.5 mL min^{-1} mobile phase (10-100% MeCN aqueous solution), purity 98.1%.

6. Reference

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