Applications of Azo-Based Probes for Imaging Retinal Hypoxia

Md. Imam Uddin,[1] Stephanie M. Evans,[1] Jason R. Craft,[1] Lawrence J. Marnett,[3] Md. Jashim Uddin,*[3] and Ashwath Jayagopal,*[1,2]

[1] Department of Ophthalmology and Visual Sciences, Vanderbilt Eye Institute, Vanderbilt University Medical Center, Nashville, TN

[2] Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, TN

[3] A. B. Hancock, Jr., Memorial Laboratory for Cancer Research, Department of Biochemistry, Chemistry and Pharmacology, Vanderbilt Institute of Chemical Biology, Center for Molecular Toxicology and Vanderbilt-Ingram Cancer Center, Vanderbilt University School of Medicine, Nashville, TN

*__Corresponding authors:__

Ashwath Jayagopal, PhD

Vanderbilt Eye Institute

8010 Medical Center East

Nashville, Tennessee 37232

Phone: 615-343-7851

E-mail: ash.jayagopal@vanderbilt.edu

Md. Jashim Uddin, PhD

Department of Biochemistry

Vanderbilt University School of Medicine

Nashville, Tennessee 37232

E-mail: jashim.uddin@Vanderbilt.Edu

Supplementary Information

General Techniques

All chemicals were purchased and used as received unless otherwise indicated. Moisture sensitive reactions were performed in oven-dried glassware under a positive pressure of nitrogen or argon. Air and moisture-sensitive compounds were introduced via syringe or cannula through a rubber septum. HPLC grade solvents were obtained from Fisher Scientific (Pittsburgh, PA). All reagents, NIR fuorescent dyes and deuterated solvents were purchased from the Aldrich Chemical Company (Milwaukee, WI) and used without further purification. The dark quencher (BHQ-3 amine) from Biosearch Technologies (Petaluma, CA), and the Oregon Green 488 carboxylic acid, succinimidyl ester, 5-isomer was purchased from Life Technologies (grand Island, NY) and used without further purification.

Chromatography

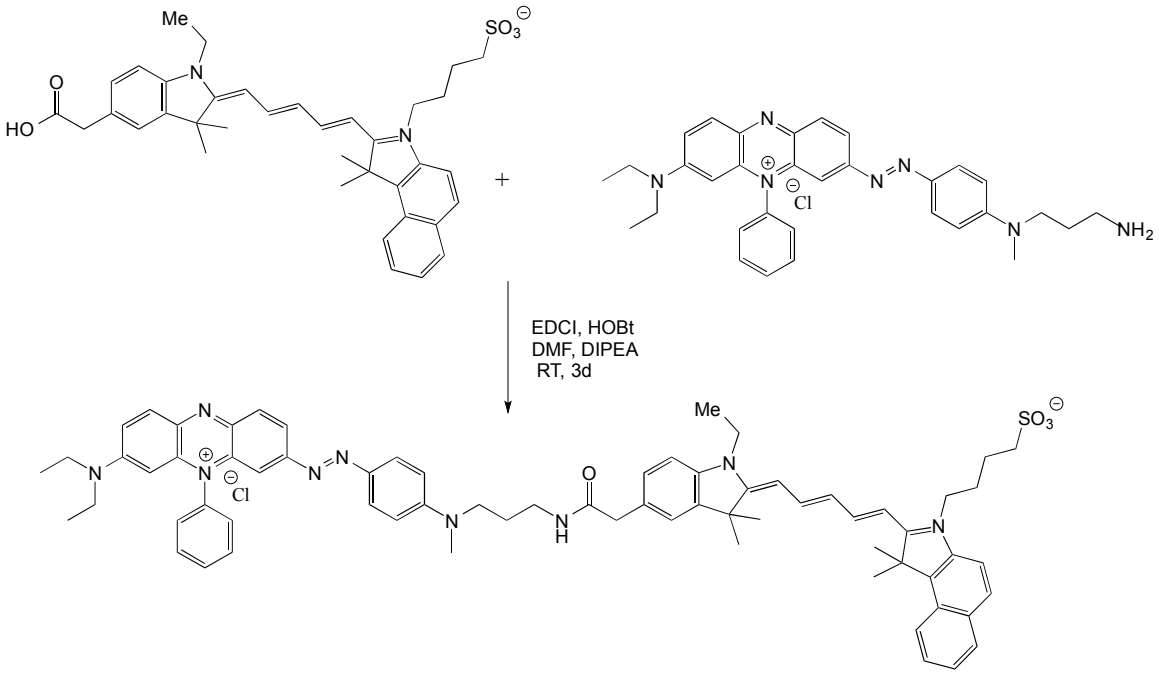
Silica gel column chromatography was performed using Sorbent silica gel standard grad, porosity 60 Å, particle size 32-63 (μm) (230 x 450 mesh), surface area 500-600 m^2/g, bulk density 0.4 g/mL, pH range 6.5-7.5, purchased from Sorbent Technologies (Atlanta, GA). The analytical HPLC of the fluorescence compounds were performed on a Waters 2996 HPLC system with a UV or fluorescence detector using C18 reverse-phase columns.

Spectroscopic Analysis

The NMR spectra were collected on a Bruker AV-II console operating at 600.13 MHz. Experimental conditions included 2048 x 512 data matrix, 13 ppm sweep width, recycle delay of 1.5 seconds and 4 scans per increment. The data were processed using squared sinebell window function, symmetrized, and displayed in magnitude mode. Chemical shifts were reported in ppm using the residual of DMSO-d_6 as the internal standard (2.49 ppm for ^{1}H and 39.52 ppm for ^{13}C while DMSO-d_6 was used). The following abbreviations were used: s = singlet, d = doublet, t = triplet, q = quartet and m = multiplet. High-resolution mass spectra were acquired with a Waters Synapt hybrid quadrupole/oa-Q-ToF mass spectrometer equipped with a dual channel ES-CI source. All compounds used for biological assays were ≥ 95% purity based on analytical HPLC monitored at 650 nm.

Synthetis of HYPOX-3

To a stirred solution of NIR-667-carboxylic acid (8.0 μmol) in dimethyl formamide (2 mL) was added BHQ3-amine (9.6 μmol), 1-hydroxybenzotriazole hydrate (9.6 μmol), N,N-diisopropylethylamine (9.6 μmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (9.6 μmol) at 25 ^{o}C. The resultant mixture was stirred for 3 days at 25 ^{o}C. Removal of the solvent *in vacuo* afforded a residue, which was purified by silica gel column chromatography using $CHCl_3$:MeOH:NH_4OH (35:7:1).



Scheme 1. Synthesis of Conjugate HYPOX-3

Deep blue solid (65%). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 9.62 (bs, 1H), 8.42 (d, *J* = 8.8 Hz, 1H), 8.40 (t, *J* = 13.3 Hz, 1H), 8.30 (t, *J* = 13.3 Hz, 1H), 8.2-8.22 (m, 2H), 8.17-8.15 (m, 2H), 8.08 (t, *J* = 9.4 Hz, 2H), 7.98-7.90 (m, 5H), 7.80-7.77 (m, 4H), 7.66 (t, *J* = 7.6 Hz, 1H), 7.61 (d, *J* = 8.8 Hz, 2H), 7.53-7.50 (m, 2H), 7.35 (d, *J* = 8.1 Hz, 1H), 7.29 (d, *J* = 8.1 Hz, 1H), 7.19 (s, 1H), 6.70 (d, *J* = 9.1 Hz, 2H), 6.53 (m, 2H), 6.40 (d, *J* = 13.7 Hz, 1H), 6.21 (d, *J* = 13.7 Hz, 1H), 5.69 (s, 1H), 4.25-4.22 (m, 2H), 4.09-4.06 (m, 2H), 3.79 (m, 2H), 3.49 (s, 3H), 3.14-3.13 (m, 2H), 3.08-3.05 (m, 8H), 1.92 (s, 6H), 1.85 (m, 2H), 1.77 (m, 2H), 1.72 (m, 2H), 1.67 (s, 6H), 1.19 (t, *J* = 7.2 Hz, 9H); Mass (ESI-) calcd for $C_{69}H_{76}N_9O_4S$ [M-Cl]: 1126.57; found: 1126.87.

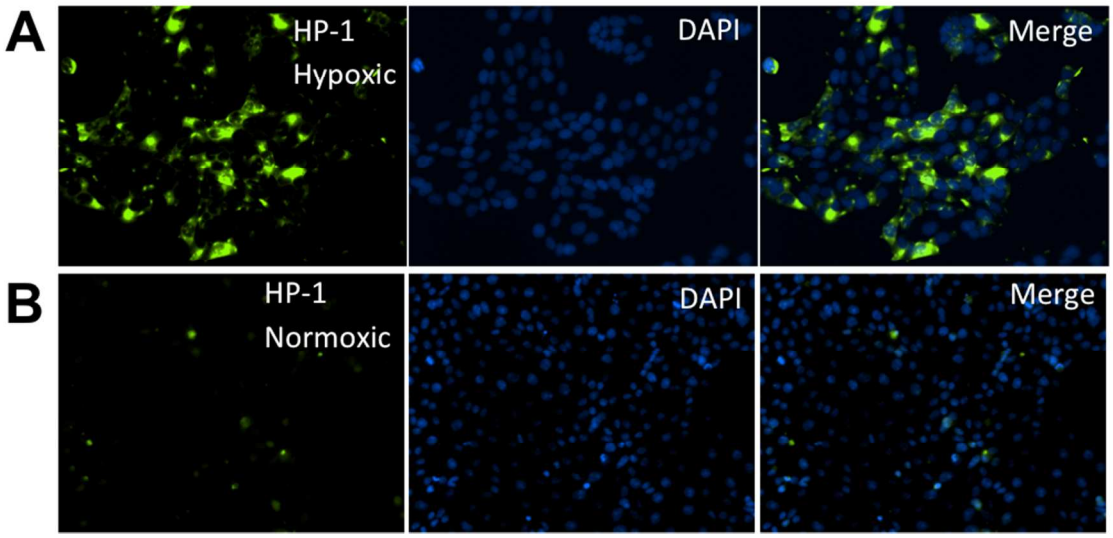


Figure S1. Detection of hypoxia in R28 cells using immunostaining of hypoxyprobe adducts with anti-rabbit AF488 secondary antibody (Hypoxyprobe Inc.). (A) Significant enhancement of fluorescence signal was observed in hypoxic cells incubated with pimonidazole (100 μM) for 4hours. (B) Normoxic cells showed minimal fluorescence signal after incubated with pimonidazole (100 μM) for 4hours.

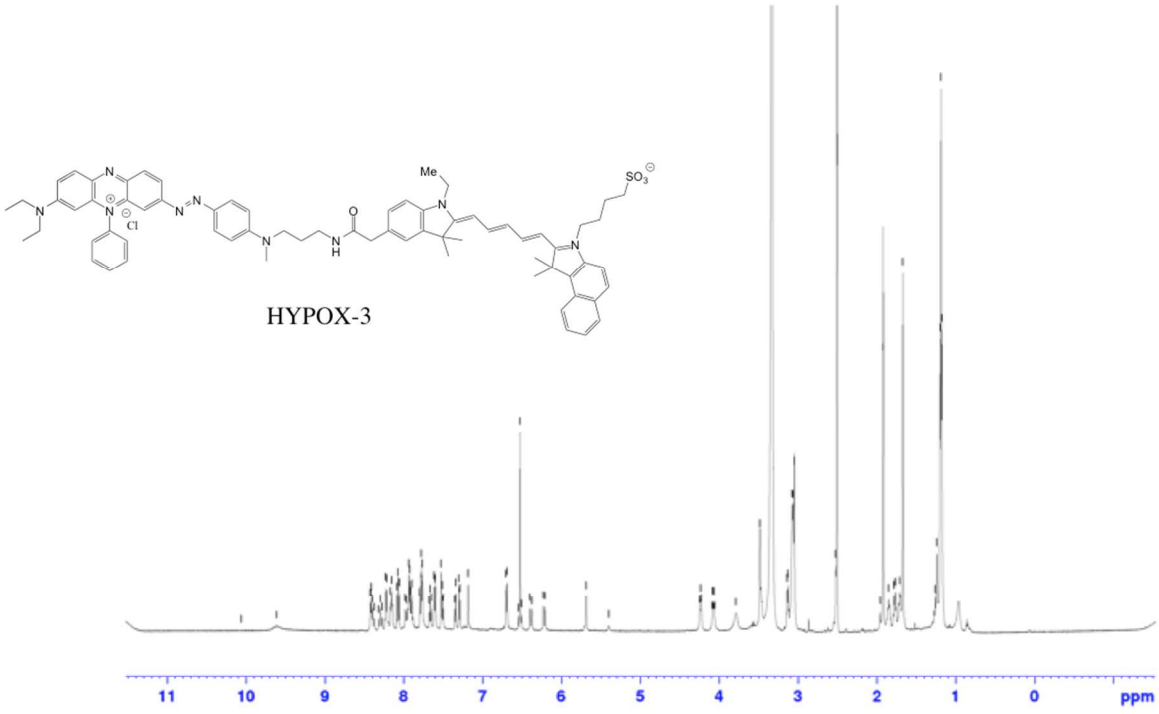


Figure S2. ^{1}H-NMR spectra for compound HYPOX-3.

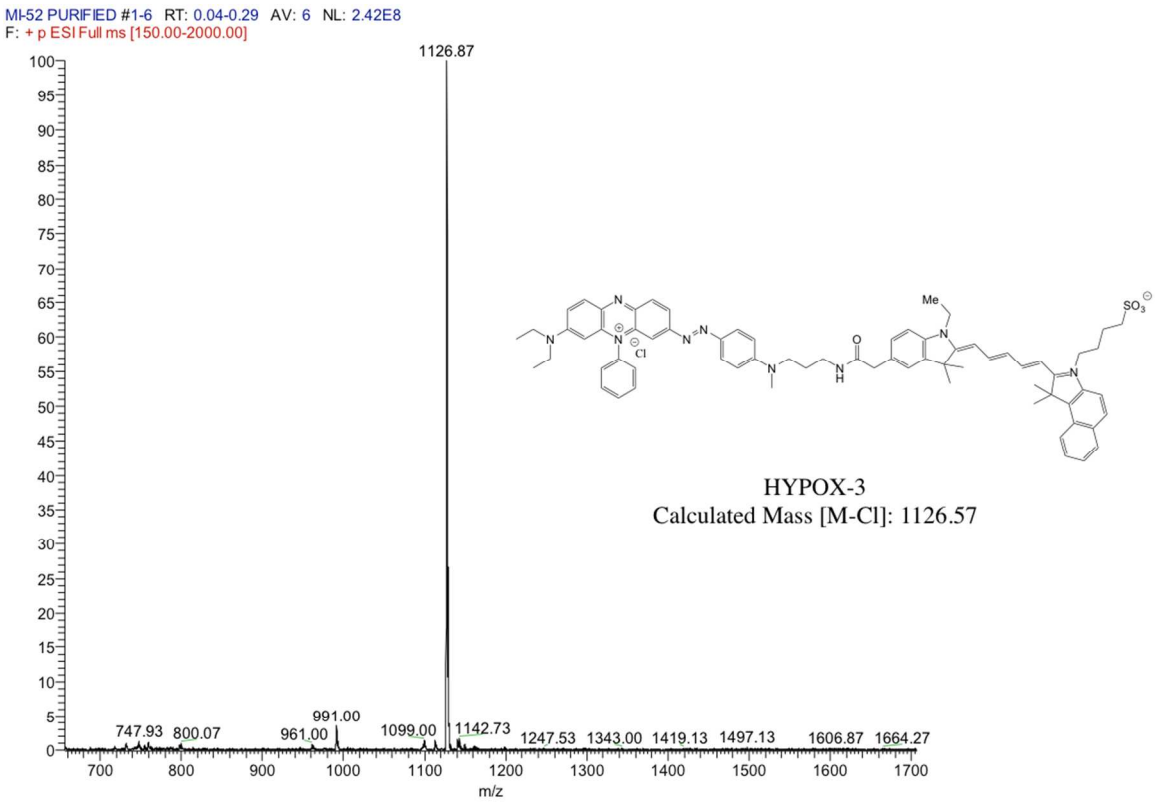


Figure S3. MS data for compound HYPOX-3