Supporting Information

Evaluation of Enzyme Substrate Radiotracers as PET/MRS Hybrid Imaging Agents

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Table of Contents

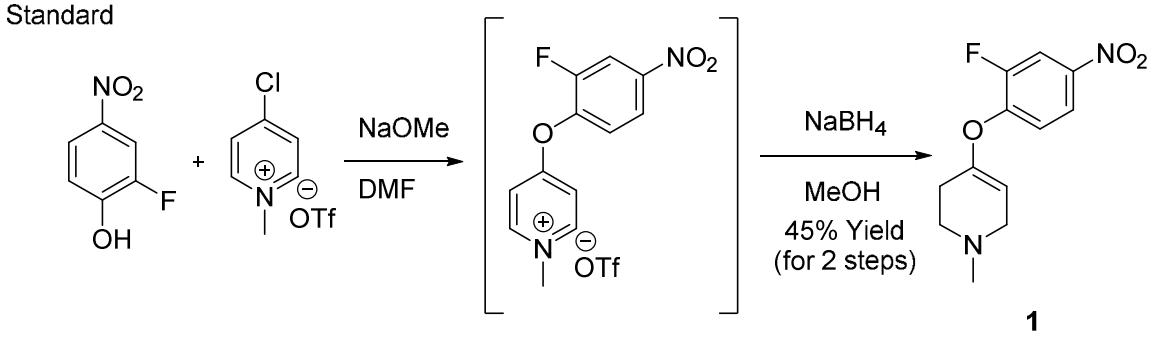
1. General Experimental

All solvents and reagents were commercially available and used without further purification unless otherwise stated. 2-Fluoro-4-nitrophenol, 4-hydroxypyridine, and 4-chloropyridine hydrochloride were obtained from Sigma-Aldrich. NMR spectra were recorded with a Varian 400 MHz instrument at room temperature with tetramethylsilane (TMS) as an internal standard. Mass spectra were performed on a Micromass LCT time-of-flight mass spectrometer or an Agilent Q-TOF HPLC-MS employing the electrospray ionization (ESI) method. High-performance liquid chromatography (HPLC) was performed using a Shimadzu LC-2010A HT system equipped with a Bioscan B-FC-1000 radiation detector. The reference standard and precursor were prepared as described previously (Brooks et al., 2015).

2. Synthesis and Characterization of Standards and Precursors

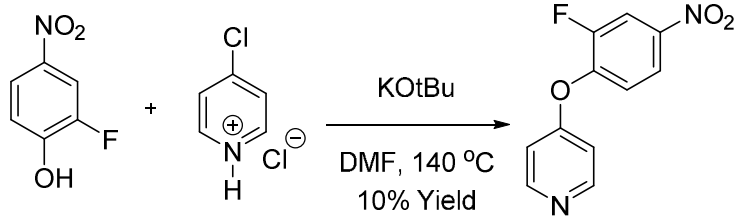
Reaction Schemes:

Standard



1

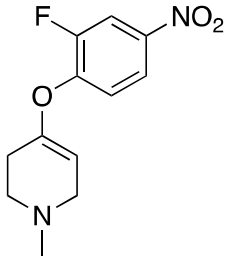
Radiolabeling Precursor



2

Scheme S1: Synthesis of standard (**1**) and precursor were completed consistent with procedures in previous literature(Brooks et al., 2015a)(Kalgutkar, Castagnoli, Hall, & Castagnoli, 1994).

Preparation of 4-(2-fluoro-4-nitrophenoxy)-1-methyl-1,2,3,6-tetrahydropyridine (1)

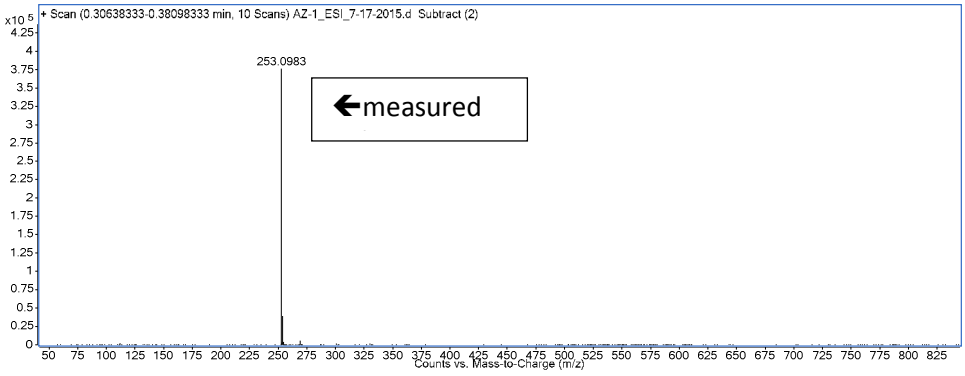


(**1**)

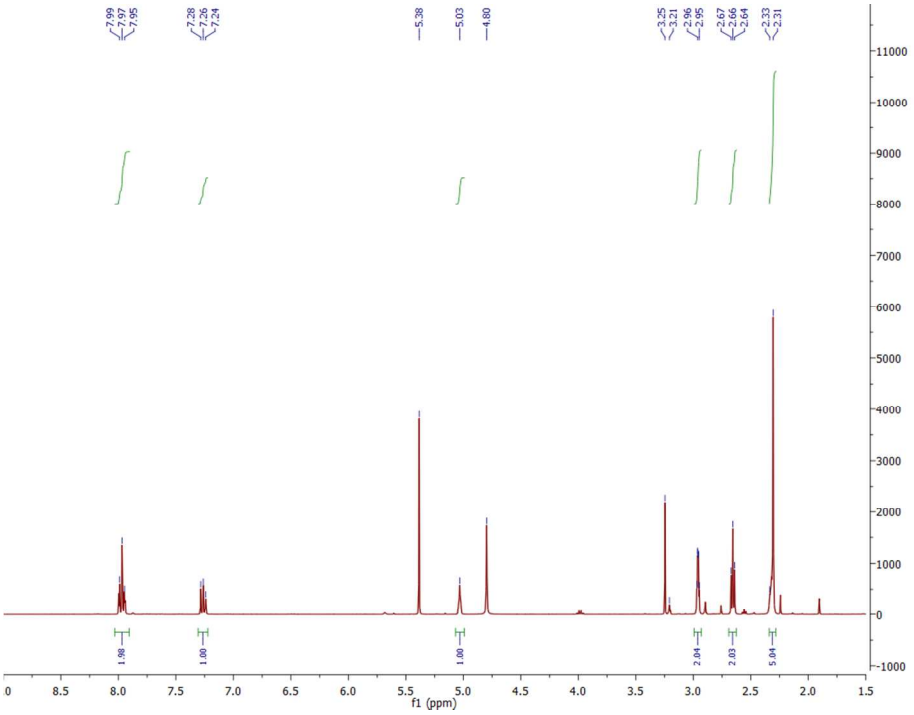
2-Fluoro-4-nitrophenol (0.11 g; 0.72 mmol) was added to sodium methoxide (0.047 g; 0.87 mmol) dissolved in DMF (3 mL), and the mixture was stirred for 10 min. 4-Chloro-1-methylpyridin-1-ium triflate (0.20g; 0.72 mmol) was added, and the reaction was stirred for 18 h. The solvent was removed *in vacuo*, and the resulting intermediate was suspended in methanol (3 mL). The reaction mixture was cooled to 0 °C in a water ice bath, and $NaBH_4$ (0.069 g; 1.81 mmol) was added slowly. After 1 h, the solvent was removed *in vacuo*. Water and ethyl acetate were added to the residue, and the mixture was transfer to a separatory funnel. The product was extracted with ethyl acetate (3×), dried over sodium sulfate, filtered, and concentrated *in vacuo*. The product was purified by flash silica gel chromatography (dichloromethane, methanol gradient; 0%MeOH/100% DCM → 10%MeOH/90%DCM). The product was collected as a yellow semi-solid in 45% yield (0.082 g). ^{1}H NMR (400 MHz; MeOH-d_4)/δ (ppm): 7.99-7.95 (2H, m), 7.26 (1H, t, *J* = 8.7), 5.03 (1H, br), 2.96 (2H, dd, *J* = 6.0, 2.6), 2.66 (2H, t, J = 6.0), 2.33-2.31 (5H, m); ^{19}F NMR (376 MHz; MeOH-d_4)/δ (ppm): -130.0; HRMS calcd for $[M + H]^+$ (M = $C_{12}H_{13}FN_2O_3$), 253.0983; found, 253.0983.

4-(2-fluoro-4-nitrophenoxy)-1-methyl-1,2,3,6-tetrahydropyridine (1) Data:

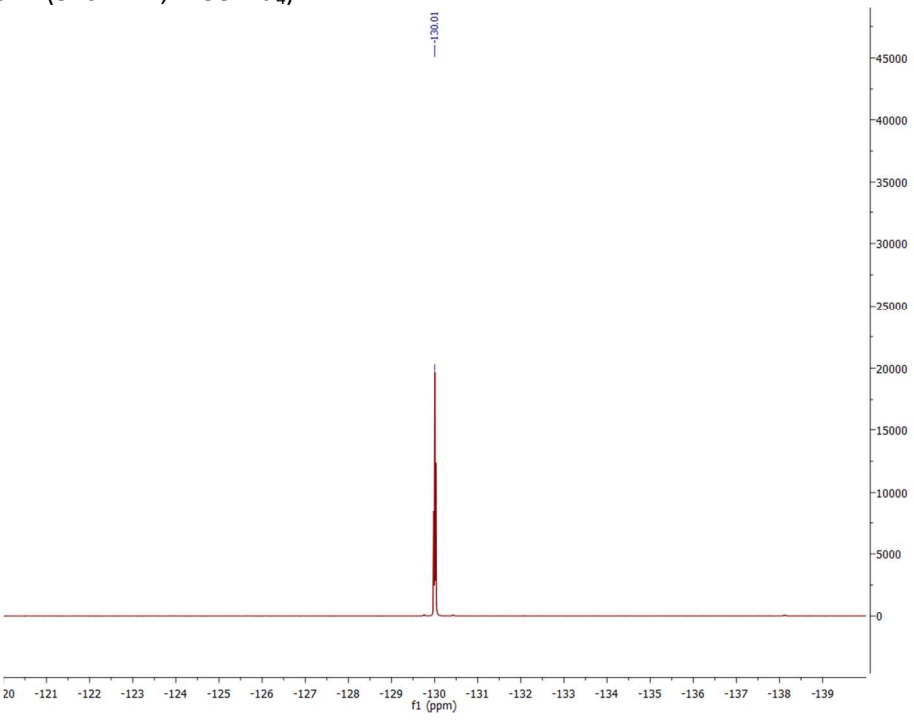
HR-MS of **1**



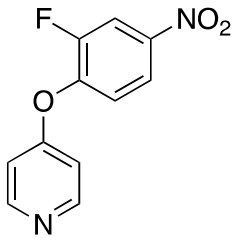
^{1}H NMR of **1** (400 MHz; MeOH-d_4)



^{19}F NMR of **1** (376 MHz; MeOH-d_4)



Preparation of 4-(2-fluoro-4-nitrophenoxy)pyridine (2)

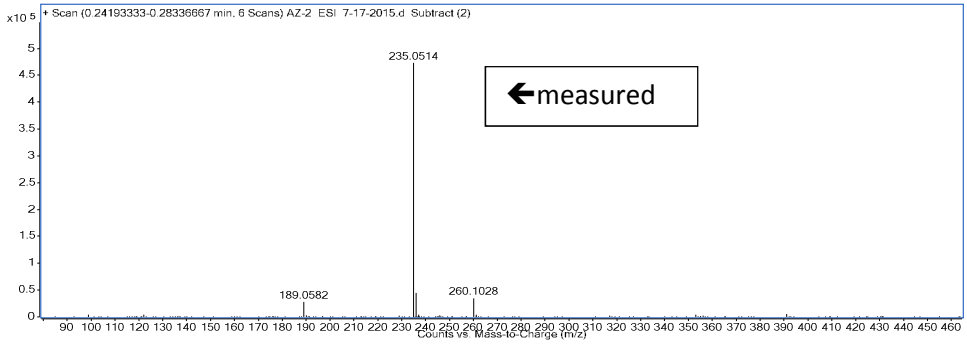


(**2**)

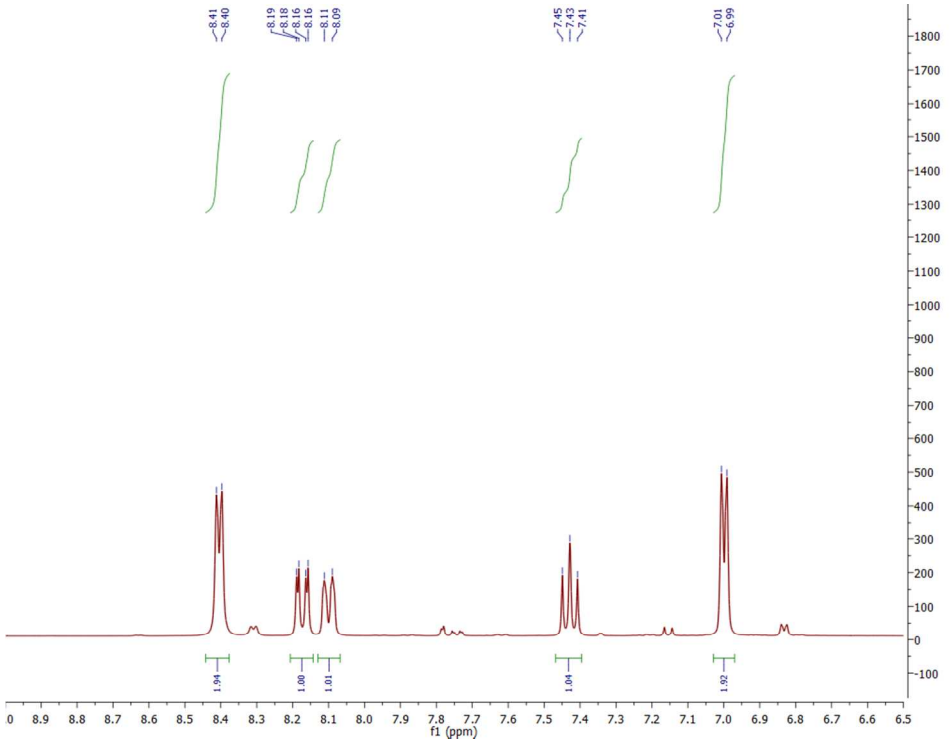
Phenol starting material (0.25 g; 1.6 mmol) was added to potassium *tert*-butoxide (0.33 g; 2.9 mmol) dissolved in DMF (7 mL). The reaction mixture was heated at 140 °C, 4-chloropyridine hydrochloride (0.20 g; 1.33 mmol) was added, and the reaction was stirred for 18 h. The reaction was cooled to room temperature and quenched with aqueous saturated sodium bicarbonate. The product was extracted with ethyl acetate (3×), dried over sodium sulfate, filtered, and concentrated *in vacuo*. The product was purified by flash silica gel chromatography (hexanes, ethyl acetate gradient; 0%EtOAc/100% hexanes → 50% EtOAc/50% hexanes). The product was collected as a red solid in 10% yield (0.031 g). ^{1}H NMR (400 MHz; MeOH-d_4)/δ (ppm): 8.41 (2H, d, *J* = 6.0), 8.17 (1H, dd, *J* = 10.3, 2.5), 8.10 (1H, d, *J* = 9.0), 7.43 (1H, t, *J* = 8.5), 7.0 (2H, d, *J* = 6.0); ^{19}F NMR (376 MHz; MeOH-d_4)/δ (ppm): -126.9; HRMS calcd for $[M + H]^+$ (M = $C_{11}H_7FN_2O_3$), 235.0513; found, 235.0514.

4-(2-fluoro-4-nitrophenoxy)pyridine (2) Data:

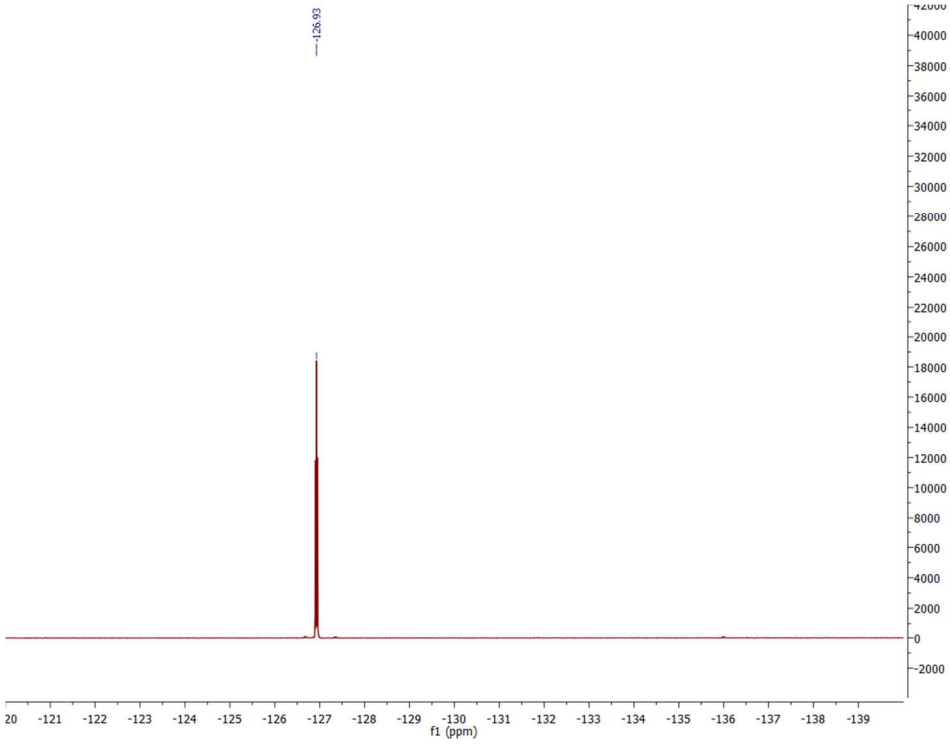
HR-MS of **2**



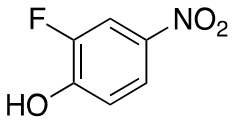
^{1}H NMR of **2** (400 MHz; MeOH-d_4)



^{19}F NMR of **2** (376 MHz; MeOH-d_4)



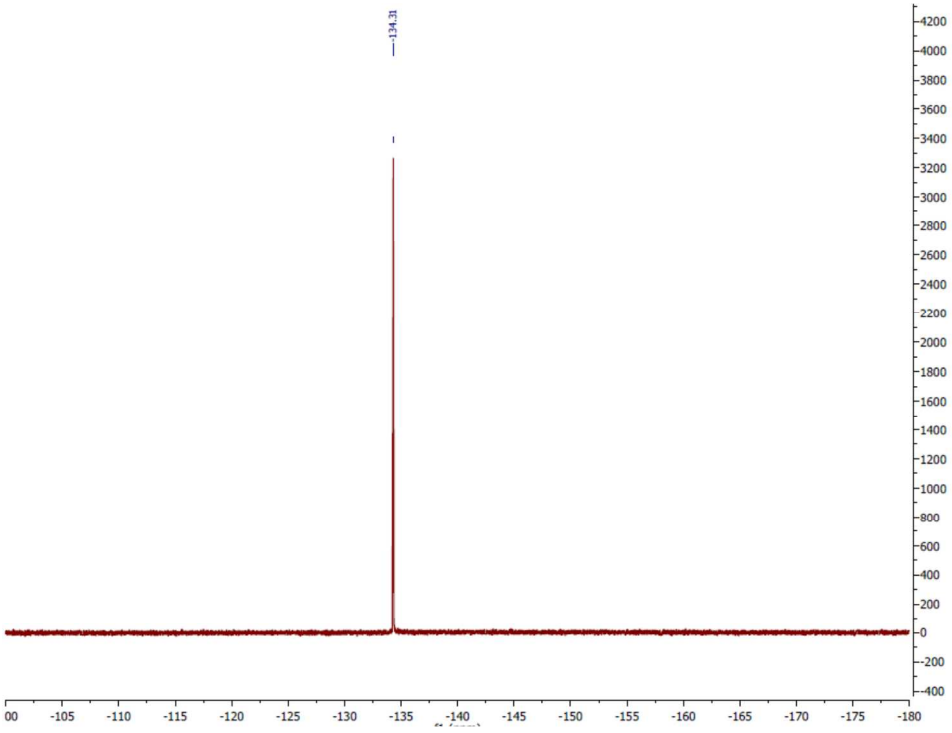
2-Fluoro-4-nitrophenol (4)



(**4**)

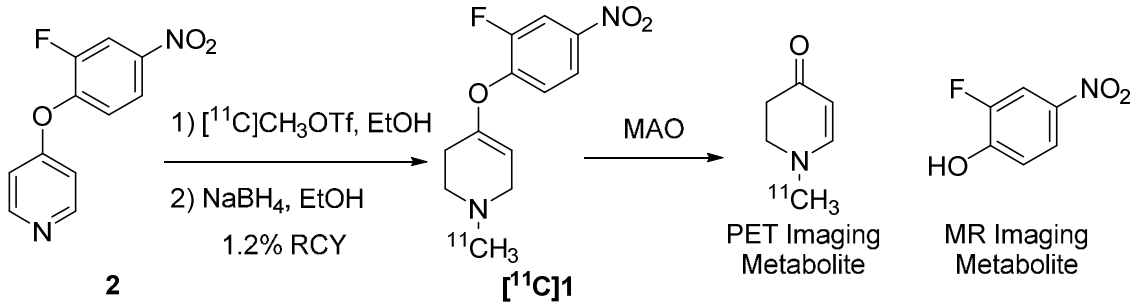
2-Fluoro-4-nitrophenol was obtained from Sigma-Aldrich and used as received.

^{19}F NMR of MR Metabolite 2-Fluoro-4-nitrophenol **4** (376 MHz; MeOH-d_4)



3. Radiosynthesis and Analysis

Radiochemistry Scheme



Scheme S2: Radiosynthesis of **[^{11}C]1** and its MAO metabolites.

Radiochemistry General Considerations

Reagents and solvents were commercially available and used without further purification, unless otherwise noted. Sodium chloride (0.9% USP) and sterile water for injection (USP) were purchased from Hospira; dehydrated alcohol for injection (USP) was obtained from Akorn Inc. Shimalite-Nickle was purchased from Shimadzu, iodine was obtained from EMD, phosphorus pentoxide was acquired from Fluka, molecular sieves were purchased from Alltech, and HPLC columns were acquired from Phenomenex. Other synthesis components were obtained as follows: sterile filters were acquired from Millipore, C18-light Sep-Paks and Porapak Q were purchased from Waters Corporation, and 10 cc sterile vials were obtained from HollisterStier. Sep-Paks were flushed with 10 mL of ethanol followed by 10 mL of sterile water prior to use.

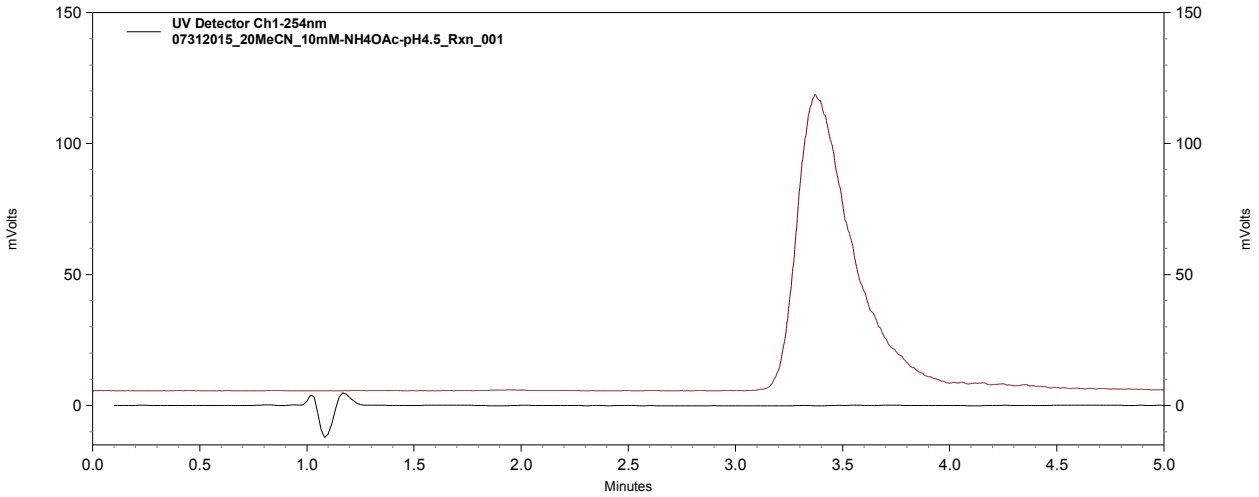
Radiochemical Synthesis of 4-(2-fluoro-4-nitrophenoxy)-1-[^{11}C]methyl-1,2,3,6-tetrahydropyridine ([^{11}C]1)

Production was carried out as previously described using a TracerLab $FX_{C\text{-}Pro}$ automated radiochemistry synthesis module (General Electric, GE)(Brooks et al., 2015b). [^{11}C]Carbon dioxide was produced using a GE PETTrace cyclotron (40 μA beam for 30 min) and converted by standard procedures into carbon-11-labeled methyl triflate ([^{11}C]CH_3OTf). The [^{11}C]CH_3OTf in helium carrier gas was bubbled into a vial containing a solution of 4-(2-fluoro-4-nitrophenoxy)pyridine (1 mg) dissolved in ethanol (0.2 mL). At the completion of transfer of radioactivity into the reaction vial, the ethanol solution was then transferred to a second conical vial containing sodium borohydride (2 mg) in ethanol (0.3 mL). The resulting mixture was stirred for 5 min at room temperature, and then the reaction was quenched by addition of HPLC buffer (30% MeCN, 10 mM NH_4OAc, pH 4.5) . The crude product was loaded onto a semi-preparative HPLC loop. The product was purified by reverse-phase chromatography (Prodigy ODS prep, 250 × 10 mm, 10 μ, 4 mL/min), collected (retention time of 5.7 min), diluted into dilution flask containing H_2O (40

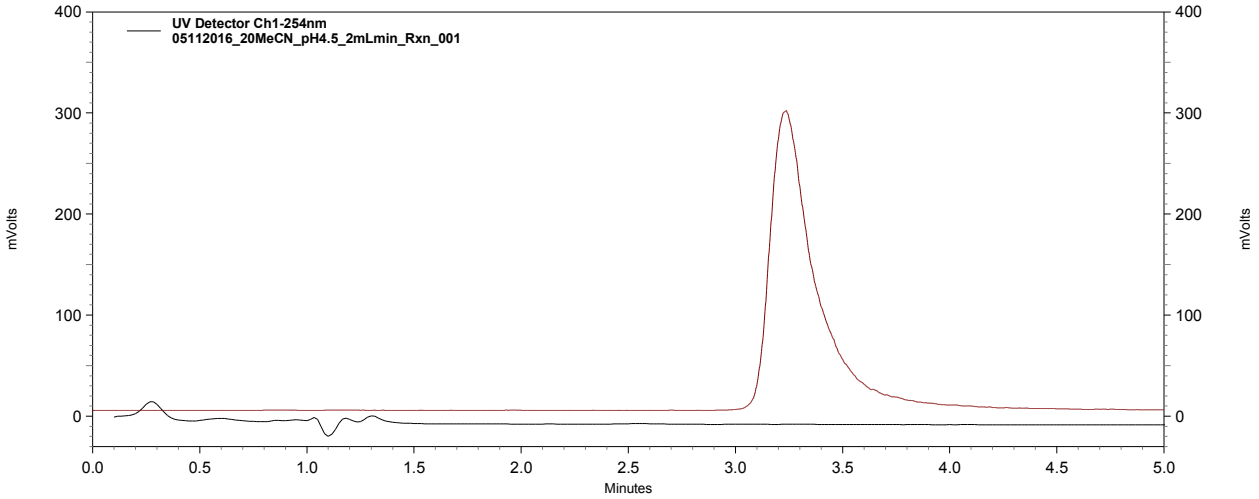
mL) and 1% NH_4OH (2 mL), and reformulated using a C-18 extraction disk into a final 5 mL total volume of 10% ethanol in saline. The doses produced (10.6 ± 5.6 mCi; non-decay corrected Radiochemical Yield = 1.2% based on 900 mCi of starting [^{11}C]CH_3OTf; Radiochemical Purity greater than 99%) were assessed via standard quality control techniques and were appropriate for rodent and nonhuman primate studies. Overall synthesis times were 30 min from end-of-bombardment.

[^{11}C]1 Molar Activity Studies (UV-254 nm Black; RAD Red)

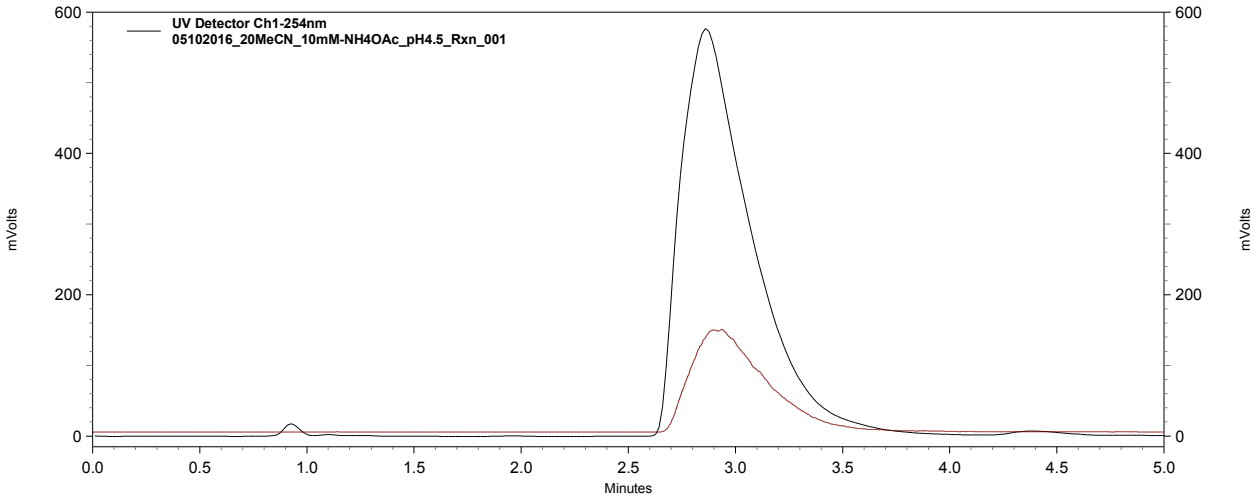
High Molar Activity Trace - 1



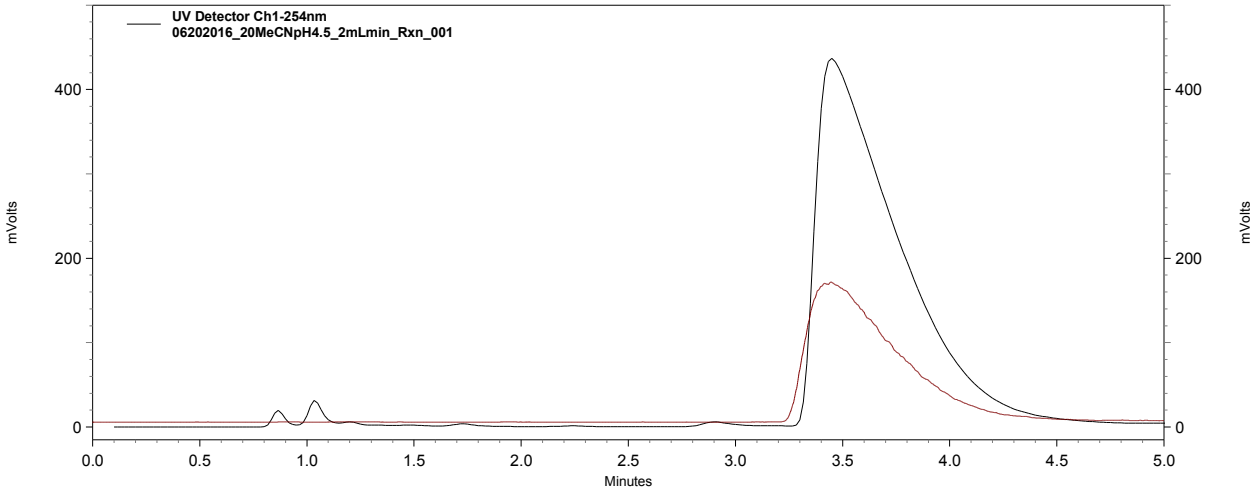
High Molar Activity - 2



Low Molar Activity - 1



Low Molar Activity - 2

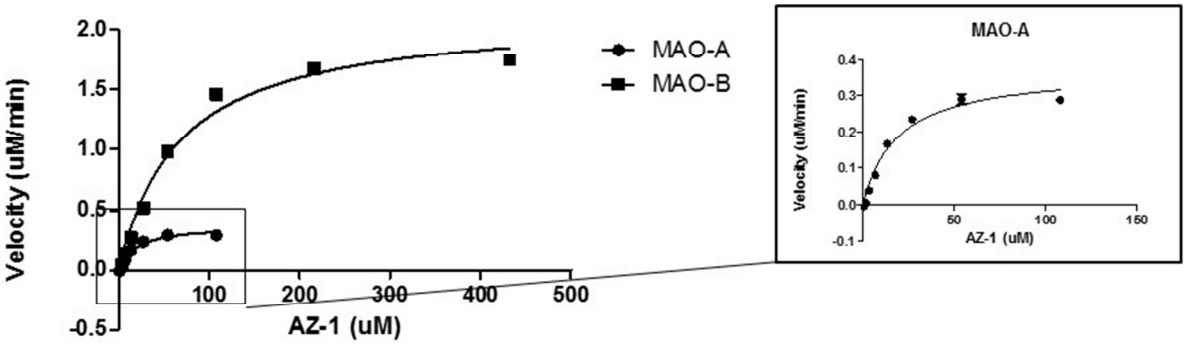


4. MAO Enzyme In Vitro Analysis

Absorbance Assay Experimental

Michaelis-Menten kinetic parameters were calculated for the compounds using hMAO-A and –B Supersome (Creative Biomart). In brief, assays were performed using Supersomes (mitochondrial membrane preps of insect cells overexpressing human MAO-A or –B) at a concentration of 80 ug/ml. Fresh stocks of substrate were dissolved in DMSO and diluted to various concentrations ranging from 200 uM to 1 nM. Reaction progress was measured by appearance of absorbant product, 2-fluoro-4-nitrophenol. Reactions were monitored in real time using a Biotek Hybrid plate reader (absorbance 410 nm) at 37 °C for 60 min. The resultant absorbance units were converted to concentration of product using a reference standard curve. Michaelis-Menten curves were plotted and parameters computed in GraphPad Prism. Assays were done in duplicate, in three independent experiments using fresh stock of enzyme, test compound, and reference standard.

Michaelis-Menten Curves and Parameters



MAO-A	K_M (uM)	19 +/- 2.3
	k_{cat} (min^{-1})	0.0416 +/- 0.0016
MAO-B	K_M (uM)	67 +/- 4.5
	k_{cat} (min^{-1})	0.240 +/- 0.048

Figure S1: Data for MAO–A and –B experiments graphed and kinetic parameters computed.

^{19}F NMR Experimental

Turnover was observed for compounds using MAO-A Supersome (Creative Biomart). In brief, assays were performed using Supersomes (mitochondrial membrane preps of insect cells overexpressing human MAO-A) at a concentration of 12 unit/ml (unit of activity defined by manufacturer and varied stock to stock). Fresh stocks of substrate were dissolved in DMSO and diluted to 100 uM in HEPES (100mM, pH 7.4). Reaction progressed in glass vials at 37 °C until desired stop time. Reaction was stopped at 0, 15, 45, 75, and 120 minutes by addition of clorgyline in excess (1.6mM). Entire reaction solutions were transferred to a 5 mm NMR tube for ^{19}F-NMR spectroscopy. NMR spec details: ^{19}F NMR experiments were performed on a Varian 400 MHz instrument (376 MHz for ^{19}F-NMR) at room temperature, 4096 scans were performed for each sample with a 1 second relaxation delay between scans, and data was analyzed with Mnova software from Mestrelab Research.

5. In Vivo Imaging Studies with [^{11}C]1

General Considerations for Animal Imaging

All animal studies were performed in accordance with the standards set by the University Committee on Use and Care of Animals (UCUCA) at the University of Michigan.

Rodent MicroPET Imaging

Rodent imaging studies were done with female Sprague Dawley rats. The animals were anesthetized (isoflurane), intubated, and positioned in a Concorde MicroPET R4 scanner. *Normal molar activity* ***[^{11}C]1*** *Scan* (n = 2, animal weight = 270 ± 124 g): Following a transmission scan, the animal was injected i.v. (via tail vein catheter as a bolus over 1 min) with **[^{11}C]1** (0.44 ± 0.008 mCi; >7000 Ci/mmol) and the head imaged for 60 min (5 x 1 min frames – 2 x 2.5 min frames – 2 x 5 min frames – 4 x 10 min frames). *Low molar activity* ***[^{11}C]1*** *Scan* (n = 2, animal weight = 297 ± 38 g): Following a transmission scan, the animal was injected i.v. (via tail vein catheter as a bolus over 1 min) with **[^{11}C]1** (0.51±0.06 mCi; 0.50 Ci/mmol) and the head imaged for 60 min (5 x 1 min frames – 2 x 2.5 min frames – 2 x 5 min frames – 4 x 10 min frames). In each case, emission data were corrected for attenuation and scatter, and reconstructed using the 3D maximum a priori (3D MAP) method. By using a summed image, regions of interest (ROI) were drawn over the whole brain on multiple planes, and the volumetric ROIs were then applied to the full dynamic data set to generate time-radioactivity curves.

[^{11}C]1-Rodent Data

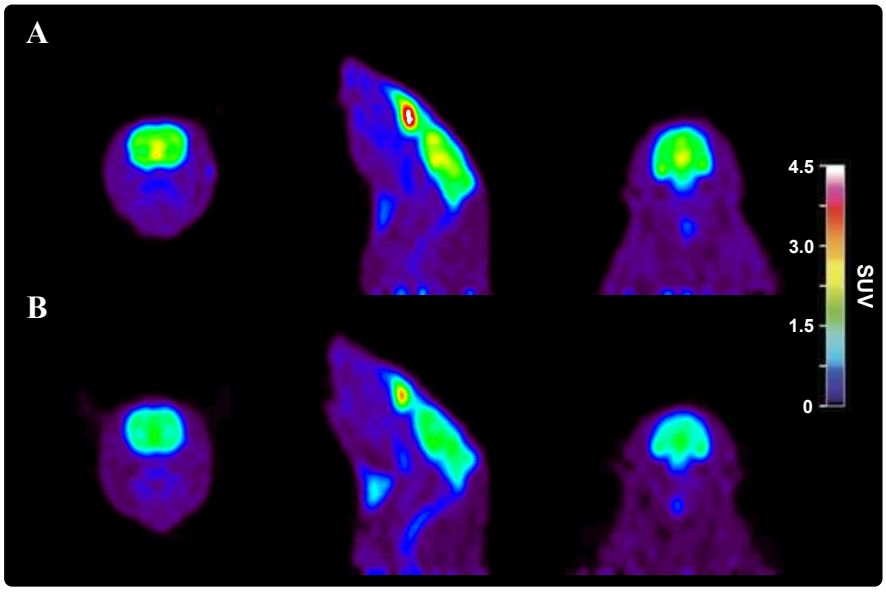


Figure S3: [^{11}C]1 at low and high molar activity; transverse, coronal and sagittal planes are presented for comparison. A) Summed frames of Sprague-Dawley rat injected with low molar activity (20.7 MBq, 0.025 GBq/µmol, 270 g rat) dose compared to B) summed frames of Sprague-Dawley rat injected with high molar activity (16.3 MBq, >259 GBq/µmol, 358 g rat) dose. Images are normalized to rat body weight and injected dose.

6. References

Brooks, A. F., Shao, X., Quesada, C. A., Sherman, P., Scott, P. J. H., & Kilbourn, M. R. (2015). In Vivo Metabolic Trapping Radiotracers for Imaging Monoamine Oxidase-A and -B Enzymatic Activity. *ACS Chemical Neuroscience*, *6*(12), 1965–1971. https://doi.org/10.1021/acschemneuro.5b00223

Kalgutkar, A. S., Castagnoli, K., Hall, A., & Castagnoli, N. (1994). Novel 4-(Aryloxy)tetrahydropyridine Analogs of MPTP as Monoamine Oxidase A and B Substrates. *Journal of Medicinal Chemistry*, *37*(7), 944–949. https://doi.org/10.1021/jm00033a012