SUPPORTING MATERIAL FOR:

Structure Based Design of Irreversible KAT II Inhibitors: Discovery of New Potency Enhancing Interactions

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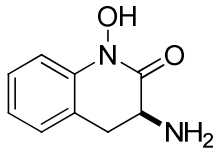
Supporting Information

General Experimental Details.

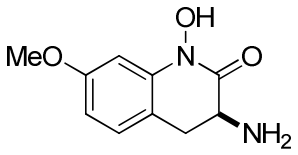
Experiments were generally carried out under inert atmosphere (nitrogen or argon), particularly in cases where oxygen- or moisture-sensitive reagents or intermediates were employed. Commercial solvents and reagents were generally used without further purification, including anhydrous solvents where appropriate (generally Sure-Seal™ products from the Aldrich Chemical Company, Milwaukee, Wisconsin). Mass spectrometry data is reported from either liquid chromatography-mass spectrometry (LCMS), atmospheric pressure chemical ionization (APCI), or gas chromatography-mass spectrometry (GCMS). Chemical shifts for nuclear magnetic resonance (NMR) data are expressed in parts per million (ppm, d) referenced to residual peaks from the deuterated solvents employed.

General Methods for the Preparation and Analytical Data for Compounds Illustrated in Tables 1, 2 and 3:



1

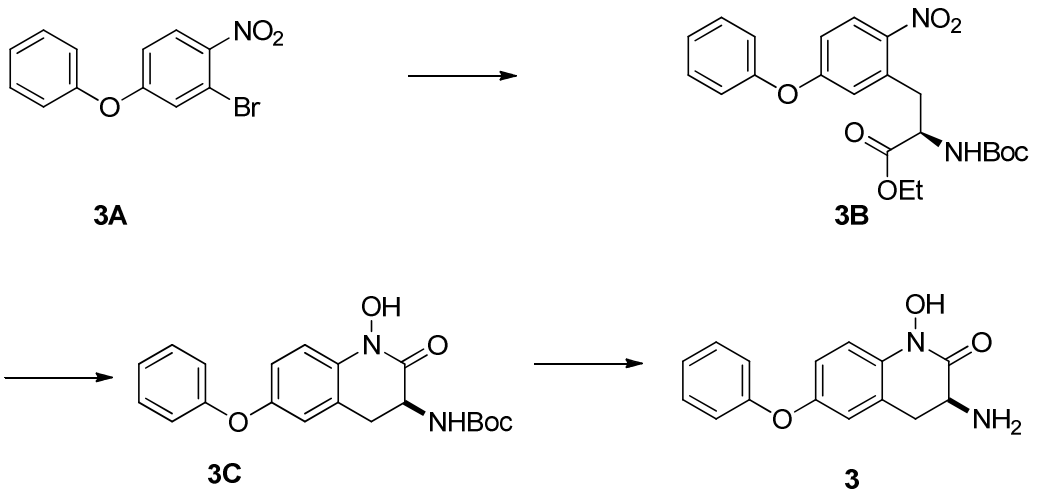
(3*S*)-3-amino-1-hydroxy-3,4-dihydroquinolin-2(1*H*)-one (1) Prepared as previously reported and the physical data were identical in all respects.[1]



2

(3*S*)-2-Amino-1-hydroxy-7-methoxy-3,4-dihydroquinolin-2(1*H*)-one (2): Prepared as previously reported and the physical data were identical in all respects.[2]

$[\alpha]_D^{20}$ -65 (C = 0.20, CH_3OH) HRMS (m/z): $[M+H]^+$ calcd for $C_{10}H_{13}N_2O_3$, 209.0921; found 209.0918.



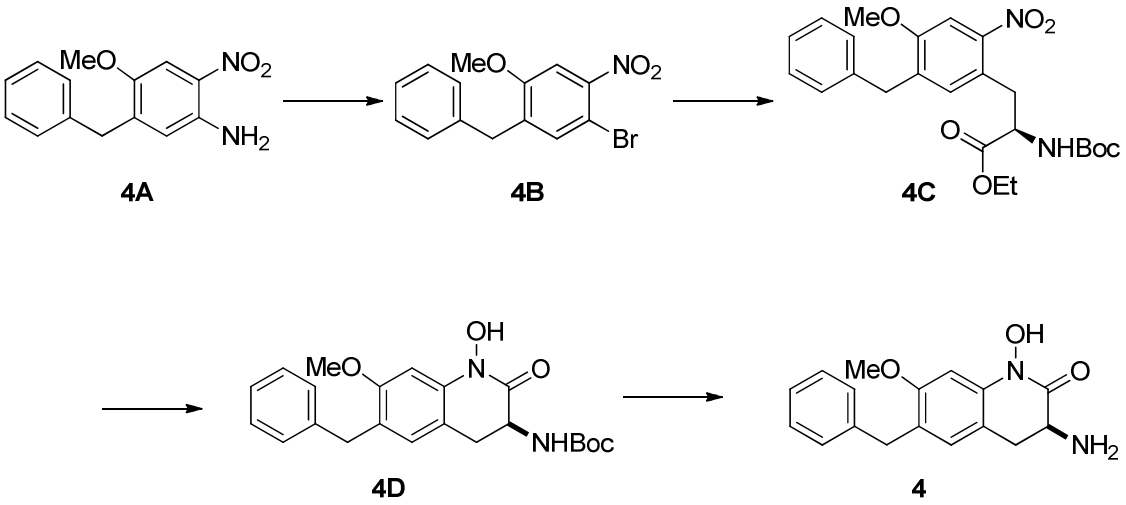
2-bromo-1-nitro-4-penoxybenzene (3A) Phenol (11.1 g, 118 mmol) was added to a suspension of CS_2CO_3 (46.2 g, 142 mmol) in MeCN (295 ml). The resulting solution was stirred at RT for 10 min., then 2-bromo-4-fluoronitrobenzene (26.0 g, 118 mmol) was added, and the reaction mixture was heated to 50 °C for 65 h. The reaction mixture was cooled to RT and filtered to remove CS_2CO_3. The filtrate was concentrated in vacuo, and the resulting residue was dissolved in EtOAc (150 ml) and washed with aqueous sodium hydroxide solution (1 N, 250 ml), water (2 x 250 ml), and saturated aqueous sodium chloride solution (250 ml). The separated organic phase was dried over Na_2SO_4, filtered and concentrated in vacuo. Purification by silica gel chromatography (100% heptanes) provided the title compound as a pale yellow oil. (32.7 g, 94% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.95 (d, J = 9.1 Hz, 1 H), 7.43-7.49 (m, 2H), 7.26-7.31 (m, 2H), 7.08-7.12 (m, 2H), 6.97 (dd, J = 9.1, 2.6 Hz, 1H).

Methyl *N*-(*tert*-butoxycarbonyl)-2-nitro-5-phenoxy-*L*-phenylalaninate (3B) Freshly distilled DMF (45 ml) was added to Zn powder (20.0 g, 306 mmol) under N_2. Trimethylsilyl chloride (8.0 ml, ~0.2 eq.) was added at RT and the resulting suspension was stirred vigorously for 35 min. The resulting pale orange supernatant was removed via syringe. The activated Zn was washed with DMF (2 x 30 ml). After removal of the DMF, the activated zinc was dried under vacuum using a heat gun. Methyl *N*-(*tert*-butoxycarbonyl)-3-iodo-*L*-alaninate (37.0 g, 112 mmol) was freshly recrystallized from petroleum ether, dried in vacuo, and dissolved in freshly distilled DMF (93 ml) and the solution was added to the activated zinc at 0 °C. After 5 min. the cooling bath was removed. The reaction mixture was stirred for 20 min. in a RT water bath at which time TLC analysis indicated disappearance of the starting iodide. The grayish supernatant was transferred via syringe into a dry flask under N_2 and the remaining zinc metal was washed with DMF (20 ml) to the flask containing the combined DMF fractions was added

sequentially a solution of 2-bromo-1-nitro-4-phenoxybenzene (**3B**) (30.0 g, 102 mmol) in DMF (18 ml), $Pd(OAc)_2$ (1.1 g, 5.1 mmol), then XPhos (4.9 g, 10.2 mmol). The resulting brown solution was stirred at RT, and the solution turned red within 1 h. The reaction mixture stirred at RT for 16 h and then poured into EtOAc (400 ml). The resulting suspension was filtered through celite. The filtrate was washed with water (2 x 400ml) and brine (400 ml) and the separated aqueous phases were back extracted with EtOAc (2 x 150 ml). The combined organic extracts were dried over Na_2SO_4, filtered and concentrated in vacuo. The crude residue was purified by silica gel chromatography (0-25% EtOAc/heptane) to provide the product as a pale yellow solid (27.9 g, 66% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.03 (d, J = 8.8 Hz, 1H), 7.44 (dd, J 7.9, 7.9 Hz, 2H), 7.27-7.24 (m, 1H), 7.10-7.07 (m, 2H), 6.92-6.87 (m, 2H), 5.17 (br d, J = 8Hz, 1H), 4.72-4.65 (m, 1H), 3.73 (s, 3H), 3.57 (dd, J = 13.3, 5.2 Hz, 1H), 3.21 (dd, J = 13, 9 Hz, 1H), 1.38 (s, 9H). LCMS *m/z* 415.1 (M-1).

***Tert*-butyl [(3*S*)-1-hydroxy-2-oxo-6-phenoxy-1,2,3,4-tetrahydroquinolin-3-yl]carbamate (3C).** In three equal batches, methyl *N*-(*tert*-butoxycarbonyl)-2-nitro-5-phenoxy-*L*-phenylalaninate (**3B**) (9.33 g, 22.3 mmol) was dissolved in pyridine (250 ml) in a Parr bottle and Pt/C (5% w/w dry catalyst, 4.4 g, 1.1 mmol) was added. The combined reaction mixtures were filtered through Celite with EtOAc washing. The filtrate was concentrated in vacuo and the crude residue was purified by silica gel chromatography (20-50% EtOAc/heptanes) to provide the desired material as a solid (19.2 g, 77% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37-7.31 (m, 3H), 7.09 (tt, J = 7.4, 1.1 Hz, 1H), 6.99-6.89 (m, 4H), 4.39 (dd, J = 12, 8 Hz, 1H), 3.06-2.96 (m, 2H), 1.47 (s, 9H). LCMS *m/z* 369.1 (M-1).

(3*S*)-3-amino-1-hydroxy-6-phenoxy-3,4-dihydroquinolin-2(1*H*)-one, hydrochloride salt (3). In two equal batches, *tert*-butyl [(3*S*)-1-hydroxy-2-oxo-6-phenoxy-1,2,3,4-tetrahydroquinolin-3-yl]carbamate **(3C)** (8.6 g, 23.2 mmol) was added to a 0 °C solution of HCl in dioxane (4 N, 100 ml) with stirring. After 5 mins. the ice bath was removed and the reaction mixture was maintained at RT for 1 h. Diethyl ether (800 ml) was added, the batches were combined, and precipitate was collected by filtration. The precipitate was washed with diethyl ether and residual solvent was removed under vacuum. The resulting pale pink solid was slurried in cold MeOH (100 ml) and filtered. The resulting solid was further washed with diethyl ether and then dried in a vacuum oven at 45 °C for 45 h to yield the desired product as a white solid (13.1 g, 92% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40-7.33 (m, 3H), 7.13 (tt, J = 7.4, 1.1 Hz, 1H), 7.02-6.97 (m, 4H), 4.35 (dd, J = 14.2, 6.7 Hz, 1H), 3.23 (dd, J = 15, 6.8 Hz, 1H), 3.16 (br dd, J = 14.8, 14.4 Hz, 1h). ^{13}C NMR (400 MHz, DMSO-d6) δ 161.6, 157.5, 152.7, 135.5, 130.5, 123.7, 122.5, 119.4, 118.8, 118.6, 115.4, 48.4, 29.2 HRMS (m/z): $[M+H]^+$ calcd for $C_{15}H_{14}N_2O_3$, 271.1077; found 271.1086. $[\alpha]^{20}_D$ -8.15 (C = 0.65, CH_3OH)



5-Benzyl-4-methoxy-2-nitroaniline (4A) Benzylzinc chloride (0.5 M solution in THF, 10.1 ml, 5.05 mmol) was added to a suspension of 5-bromo-4-methoxy-2-nitroaniline (1.26 g, 5.10 mmol) (see L. A. Hasvold *et al., Bioorg. Med. Chem. Lett.* **2008,** *18,* 2311-2315), palladium (II) acetate (47.1 mg, 0.210 mmol) and 2-dicyclohexylphosphino-2',6'-dimethoxybiphenyl (172 mg, 0.420 mmol) in THF (4.2 ml) that had been stirred for 5 min. The resulting solution was stirred for 18 h at RT. After addition of saturated aqueous ammonium chloride solution (20 ml), the mixture was extracted with EtOAc (3 x 3 ml), and the combined organic layers were dried over sodium sulfate, filtered and concentrated *in vacuo*. Silica gel chromatography (Gradient: 0% - 50% EtOAc/Heptane) provided the product as an orange solid (940 mg, 71%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.52 (s, 1H), 7.32 (br dd, J = 7.6, 7.1 Hz, 2H), 7.23-7.27 (m, 1H), 7.20 (br d, J = 7.4 Hz, 2H), 6.42 (s, 1H), 5.84 (br s, 2H), 3.93 (s, 2H), 3.83 (s, 3H).

1-Benzyl-5-bromo-2-methoxy-4-nitrobenzene (4B) *tert*-butyl nitrite (557 mg, 5.40 mmol) was added to a solution of copper (II) bromide (1.77 g, 7.92 mmol) in MeCN (8 ml) and the mixture was heated to 60 °C. A solution of 5-benzyl-4-methoxy-2-nitroaniline (**4A**) (930 mg, 3.60 mmol) in MeCN (12 ml) was added dropwise and the reaction was stirred for 10 min. It was then poured into aqueous hydrochloric acid (2 N, 100 ml) and extracted with EtOAc (2 x 100 ml). The combined organic layers were washed with saturated aqueous sodium chloride solution (200 ml), dried over sodium sulfate, filtered and concentrated *in vacuo*. Purification via silica gel chromatography (100% hexanes) provided the product as a white solid (1.06 g of of roughly 60% purity as assessed by 1H NMR, estimated yield 55%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.41 (s, 1H), 7.37 (s, 1H), 7.32 (br dd, J = 7.5, 7.5 Hz, 2H), 7.23-7.27 (m, 1H), 7.19 (br d, J=8 Hz), 3.98 (s, 2H), 3.90 (s, 3H).

Methyl 3-benzyl-N-(*tert*-butoxycarbonyl)-*O*-methyl-6-nitro-L-tyrosinate (4C) Trimethylsilyl chloride (240 ul, 1.82 mmol) was added to a stirring suspension of Zn powder (609 mg, 9.31 mmol) in dry DMF (1.4 ml) and the mixture was stirred for 30 min. The stirring was stopped and the solids were allowed to settle for 10 min. at which time the supernatant was removed via syringe. The activated zinc was washed with DMF and the solvent was again removed with a syringe; the zinc was then dried under vacuum using a heat gun. A solution of methyl N-(*tert*-butoxycarbonyl)-3-iodo-L-alaninate (prepared according to S. Van Zutphan *et al.*, *Tet. Lett.* 2007, 48, 2857-2859) (1.33 g, 4.04 mmol) in DMF (1.0 M) was added to the dry activated zinc, and the resulting solution was transferred via syringe into a dry flask under nitrogen. To this was sequentially added 1-Benzyl-5-bromo-2-methoxy-4-nitrobenzene (**4B**) (1 g, 3.104 mmol), palladium(II)acetate (34.8 mg, 0.155 mmol) and then XPhos (dicyclohexyl(2',4',6'-triisopropylbiphenyl-2-yl)phosphine (148 mg, 0.1 mmol). The resulting solution was stirred at RT for 18 h. The reaction mixture was diluted with EtOAc (5 ml), washed with water (3 x 10 ml) and brine (1 x 10 ml) then dried over Mg_2SO_4, filtered and concentrated *in vacuo*. Purification via silica gel chromatography (0-30% EtOAc/Heptane) obtained the desired material as a gum (368 mg, 44% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.49 (br s, 1H), 7.29 (br dd, J=7.6, 7.2 Hz, 2H), 7.20-7.24 (m, 1H), 7.18 (br d, J=8 Hz, 2H), 7.02 (br s, 1H), 5.12 (br d, J=8.2 Hz, 1H), 4.59-4.65 (m, 1H), 3.98 (br s, 2H), 3.88 (br s, 3H), 3.65 (s, 3H), 3.43 (dd, J=13.7, 5.6 Hz, 1H), 3.18 (dd, J=13.5, 8.0 Hz), 1.38 (br s, 9H) LCMS m/z (M + 1) 445

***Tert*-Butyl [(3*S*)-6-benzyl-1-hydroxy-7-methoxy-2-oxo-1,2,3,4-tetrahydroquinolin-3-yl]carbamate (4D)** Ammonium formate (255 mg, 4.05 mmol, 5 eq.) was added to a solution of Methyl 3-benzyl-N-(*tert*-butoxycarbonyl)-*O*-methyl-6-nitro-L-tyrosinate (**4C**) (360 mg, 0.81 mmol) in pyridine (8.1 ml), followed by platinum on carbon (5%, 36 mg, 10% w/w). The black suspension was stirred at 60 °C for 18 h, then allowed to cool to RT and filtered though and Acrodisc® syringe filter (Pall Live Sciences). The filtrate was concentrated and the residue was purified via chromatography on silica gel (0-3% MeOH/CH_2Cl_2) to obtain the product as a light purple solid (86 mg, 27%) ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.79 (br s, 1H), 7.27-7.31 (m, 2H), 7.18-7.22 (m, 3H), 6.91 (s, 1H), 6.85 (s, 1H), 5.42 (br s 1H), 4.42-4.51 (m, 1H), 3.92 (quartet J=15.3, 10.6 Hz, 2H), 3.87 (s, 3H), 3.21-3.29 (m, 1H), 2.75 (br dd, J=15, 14 Hz, 1H), 1.45 (s, 9H).

(3*s*)-3-Amino-6-benzyl-1-hydroxy-7-methoxy-3,4-dihydroquinolin-2(1*H*)-one, hydrochloride salt (4)

Preparation of the hydrochloride salt was carried out by mixing the free base of the product with dichloromethane (2ml) and adding MeOH (2 drops). To this solution was added a solution of hydrogen

chloride (2 N in diethyl ether, 3 ml); solvents were removed under reduced pressure to yield the title product as a solid (60 mg, 85%). Characterization data was obtained on the neutral compound. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.22-7.26 (m, 2H), 7.13-7.20 (m, 3H), 6.68-6.81 (br m, 2H), 3.82 (br s, 3H), 3.40-3.69 (br m, 1H), 3.45 (br s , 2H), 2.58-2.91 (br m, 2H) ^{13}C NMR (400 MHz, MeOD) 165.4, 161.4, 144.6, 141.9, 133.4, 132.3, 131.8, 129.8, 129.5, 114.5, 100.8, 58.7, 52.9, 38.56, 32.24 HRMS (m/z): $[M+H]^+$ calcd for $C_{17}H_{18}N_2O_3$, 299.139; found 299.1391. $[\alpha]^{20}_D$ -8.17 (C = 0.57, CH_3OH)

Protocols for Inhibition Assays Using Recombinant Kynurenine Aminotransferase II

Human KAT II inhibition spectra assay

Formation of kynurenic acid (KYNA) is indirectly assessed by a decrease in light absorbance at 370 nm (OD370) as the L-kynurenine (KYN) substrate is converted by the human KAT II (hKAT II) enzyme into KYNA. An inhibitor would therefore inhibit the decrease in OD370.

The protocol was performed by placing the following reagents into a Costar 384 well black plate (30 µL total assay volume/well):

- 10 µL of 3x concentrated compound;
- 10 µL of 3x concentrated substrate mix (BGG (Sigma G-5009); 3 mM L-Kynurenine in 150 mM Tris Acetate (Sigma K3750); 3 mM α-ketoglutaric acid in 150 mM Tris Acetate (Sigma K2010); and 210 µM pyridoxal 5-phosphate (PLP) in 150 mM Tris Acetate (Sigma 9255)); and
- 10 µL of 3x concentrated enzyme (90 nM enzyme in 150 mM Tris Acetate with 0.3% bovine serum).

Plates were sealed and incubated at 37 °C for 15-20 h before reading OD370 on a SpectraMax Plus plate reader. IC_{50} values were generated by comparing the efficacy of compounds across a concentration range to inhibit a reduction in the OD370 value relative to assay wells with DMSO added in place of concentrated compound.

KAT II kinetic assay:

Test compounds were dissolved in 100% DMSO and diluted to the required concentrations in 100% DMSO. An additional aqueous dilution was made so that the compound at 3x final concentration was 1.0% DMSO in the assay specific buffer. Compounds were tested at 11 concentrations. Final DMSO concentrations in the assay plate were equal to 0.33%.

Assay Methodology

KATII enzyme activity was followed by measuring the loss of absorbance of the L-KYN substrate at an absorbance wavelength of 370 nm. The KAT II assays were run in a 384 well format at a final volume of 30 µL using 150 mM Tris Acetate buffer (pH7.0), 1 mM L-KYN,1 mM α-ketoglutaric acid, 70 µM PLP, 0.1% BGG and either 30 nM human KATII enzyme or 5 nM rat KAT II enzyme.

Compound was diluted in 100% DMSO and spotted prior to the addition of the other reagents. Enzyme was always added last. Assay plates were sealed around the edges with tape and immediately read on a SpectraMax plate reader at an absorbance wavelength of 370 nm. The SpectraMax plate reader was set up to read every 5 min for 16 hours.

The following steps are taken to ensure consistent production of kinetic read data:

1. A 10 µL aliquot of the compound dilutions (described above in compound preparation) was added to the assay plate by hand followed by a quick spin to ensure compound was collected at bottom of well.
2. A 10 µL aliquot of substrate mix containing the L-KYN, α-ketoglutaric acid and PLP was then added to the assay plate via a Multidrop instrument.
3. Finally, a 10 µL aliquot of a 3x concentration of enzyme stock soluton was added last to initiate the reaction via a Multidrop instrument.
4. The microplate lid was placed onto the assay plate and taped to seal in humidity, and the assay plate was put into the SpectraMax reader. A quick vibration on the plate platform was done to ensure mixing, and the absorbance was read (wavelength of 370 nm) every 5 min over 16 h at room temperature.

hKATI assay

The hKATI selectivity assay was run in a 384 well format using 200mM Potassium Phosphate buffer (pH7.4), 1mM L-kynurenine (Sigma K3750), 1mM pyruvate (Sigma P8574), 70uM pyridoxal 5-phosphate (Sigma 9255), 0.1% BGG (Sigma G5009) and 50nM hKATI enzyme. Compounds were diluted in 100% DMSO and spotted prior to the addition of the other reagents. Plates were sealed and incubated in a humidified incubator at 37 ^{o}C for 16 hrs and read on a SpectraMax at OD370.

Human KATIII Assay

The hKATIII selectivity assay was run in a 384 well format using 100 mM Potassium Phosphate dibasic/100 mM boric acid buffer (pH 9.0), 1 mM L-kynurenine (Sigma K3750), 1 mM phenylpyruvate (Sigma P8001), 70 mM pyridoxal 5-phosphate (Sigma 9255), 0.1% BGG (Sigma G5009) and 200 nM hKATIII enzyme. Compounds were diluted in 100% DMSO and spotted prior to the addition of the other reagents. Plates were sealed and incubated in a humidified incubator at 42 ^{o}C for 24 hrs and read on a SpectraMax at OD370.

Determination of Potencies (k_{inact}/K_I values)

The direct substrate absorbance loss assay described above was performed for the determination of potencies (k_{inact}/K_I values). The overall potency, k_{inact}/K_I values, were determined using the general approaches previously reported.[3,4] Reaction progress curves (decrease in OD 370 nm with time) were obtained in the presence of eleven concentrations of inhibitor with top dose at 1 μM and diluted by 2 fold to 1 nM. Data analysis was performed using GraphPad Prism version 5.01 for Windows, GraphPad Software, San Diego, California USA. Each progress curve was fit to a one phase exponential decay model to determine $k_{observed}$ (k_{obs}) values at each inhibitor concentration. For the human KATII enzyme, a 6 hour time window (5 minutes to 360 minutes) was used to derive the *kobs* value across all inhibitor concentrations. The inhibitor dissociation constant (K_I) and the first-order rate constant of enzyme inactivation at infinite inhibitor concentration (k_{inact}) were then obtained by fitting the *kobs vs*. [I] curves as described in the references. [3,4]

Experimental Protocols for Table 3

Nonspecific Binding to Brain Tissue

Brain free fraction measurement for compounds 1 and 3 was determined through the use of a 96-well equilibrium dialysis apparatus (Banker et al., 2003).[5] Spectra-Por 2 membranes with molecular cutoff of 12 to 14 kDa used for the dialysis were obtained from Spectrum Laboratories Inc. (Rancho Dominguez, CA). The membranes were conditioned in deionized water for 20 min followed by 30% ethanol for 20 min. Following a thorough rinse with deionized water, the membranes were subsequently stored in 0.10 M sodium phosphate buffer (pH 7.4) until needed. Previously frozen rat brains (Pel-Freez Biologicals, Rogers, AR) were thawed the day of the experiment. Brain samples were diluted with 2 volumes of 0.10 M sodium phosphate buffer and homogenized via a Kinematica Polytron homogenizer (Kinematica, Inc., Bohemia, NY). Brain homogenate (1/3 total dilution) was pH adjusted to 7.4 and then spiked with the test drug (1 uM) and 150 μL aliquots were loaded into the 96-well equilibrium dialysis plate and dialyzed versus 150 μL of 0.10 M sodium phosphate buffer. The apparatus was covered with a CO_2 permeable membrane and equilibrium was achieved following a 6 h incubation in a cell culture incubator (37 °C, 5% CO_2). After reaching equilibrium, 100 μL aliquots of each matrix were removed from the 96-well equilibrium dialysis apparatus and added to equal amount of blank matrix in 1.2 mL polypropylene tubes to employ a mixed matrix analysis technique. Mixed matrix analysis allows for direct comparison of analytical data when all samples are composed of an identical matrix, thus buffer

samples are added to control brain homogenate and brain homogenate samples are added to an equal volume of control buffer. The samples were analyzed identically to the *in vivo* samples (via LC-MS/MS) with the output of analyte area/internal standard ratios in place of concentration data. As described by Kalvass and Maurer (2002),[6] the unbound fraction in total (undiluted) brain was calculated via the below equation, where D and fu,measured represent the fold dilution of brain tissue and the free fraction determined as the ratio of area counts in buffer versus diluted brain tissue, respectively (Kalvass and Maurer, 2002).[6]

$$Total\ (undiluted)\ fu = \frac{1/\mathrm{D}}{\left(\left(\frac{1}{\mathrm{fu,measured}}\right) - 1\right) + 1/\mathrm{D}}$$

Animals

Male Sprague-Dawley rats weighing 200 to 250 g were obtained from Charles River Laboratories International Inc. (Wilmington, MA). Upon arrival, the rats were maintained for at least 2 days on a 12-h light/dark cycle in a temperature- and humidity-controlled environment with free access to food and water. The rats were housed in clear polycarbonate boxes containing sawdust. The study was conducted in accordance with approved Pfizer Animal Care and Use Procedures.

Dose Administration and Sample Collection

Rats were administered a single subcutaneous dose of either compound 1 or compound 3 at 10 mg/kg (n = 3 per dose per time point). Dosing solution was prepared as a solution in sterile water and administered in a 2 mL/kg volume in the mid-back region. Rats were euthanized in a CO_2 chamber at 0.25, 0.5, 1, and 3 h post-dose for compound 1 and at 0.5, 1, 2, 4, and 7 h post-dose following administration of compound 3. CSF was collected via puncture of the cisterna magna using a 23 gauge needle attached to polyethylene tubing and a syringe. Whole blood was collected by cardiac puncture into Vacutainer tubes containing heparin and stored on ice until centrifuged for the preparation of plasma. Whole brains were collected by decapitation, rinsed with phosphate-buffered saline, and weighed. CSF and whole brains were immediately frozen on dry ice upon collection.

Sample Analysis

All samples were quantified via HPLC-MS/MS. Whole brain samples were thawed, diluted 1:4 (w/v) with water, and homogenized using a Kinematica Polytron homogenizer (Kinematica, Inc., Bohemia, NY). Standards and controls were prepared in a similar manner using a brain homogenate prepared from untreated animals. Plasma, csf, and brain homogenate samples were prepared for analysis by deproteination with acetonitrile containing a structurally similar internal standard. For compound 1, a 50 µL aliquot of sample was precipitated with 100 µl of acetonitrile, vortexed, and centrifuged. Following centrifugation, the supernatant was transferred to a clean 96-well injection block and evaporated under a flow of nitrogen at 37 ºC and reconstituted in 75 µl of 50/50 methanol/water. 10 µl was subsequently injected on the LC-MS/MS for analysis. For analysis of compound 3, a 50 µL aliquot of sample was precipitated with 300 µl of acetonitrile containing internal standard, vortexed and centrifuged. The supernatant was dried down under nitrogen and reconstituted in 80 µl of 95/5 water/acetonitrile. 5 µl was then injected on the LC/MS for analysis.

LC-MS/MS analysis was carried out for using a high-performance liquid chromatography system consisting of a Shimadzu LC-20 binary pump (Shimadzu Scientific Instruments, Columbia, MD) with CTC PAL autosampler (Leap Technologies, Carrboro, NC) interfaced to a Micromass Quattro Ultima mass spectrometer (Waters, Milford, MA) for compound 1. The mass spectrometer was fitted with an electrospray ionization Z-spray interface that was operated in a positive ion mode. Multiple reaction monitoring was performed with the transition m/z 178.9 to 116.1. In regards to the analysis of compound 3, the interface of the LC system was to an API 4000 LC/MS/MS quadrupole tandem mass spectrometer (Applied Biosystems/ MDS Sciex Inc., Ontario, Canada). The mass spectrometer was operated in positive ion ESI mode for the detection of compound 3 and the internal standard. Multiple reaction monitoring was performed with the transitions m/z 271 to 160.

Compound 1 and the internal standard were separated on a Phenomenex Synergi Hydro-RP column (30 x 2.0 mm, 2.5 µm) by gradient elution. Mobile phase A consisted of 20 mM ammonium acetate with 0.1% formic acid and 0.1% isopropanol and mobile phase B consisted of acetonitrile. During the first half-minute of the chromatographic run, the mobile phase solvents were held at a constant ratio 100:0 (A:B) that was followed by a 1.0 minute linear gradient to a mobile phase solvent ratio of 10:90 (A:B). The intermediate condition was held for 0.5 minute followed by an immediate return to the starting conditions. The starting conditions were then maintained for a one-minute period of re-equilibration. A flow rate of 0.3 ml/min was used. All raw data was processed using MassLynx Software ver. 4.0

(Waters, Milford, MA). The lower limit of quantification (LLOQ) was 1.0 ng/ml for plasma, csf, and brain homogenate (conversion to 5 ng/g) samples. The upper limit of quantification (ULOQ) was 1000 ng/mL.

Compound 3 and the internal standard were separated on an Analytical Sales and Service Advantage Echelon C18 column (50 x 2.1 mm, 4 µm) by gradient elution. Mobile phase A consisted of 5 mM ammonium formate with 0.1% Formic acid. Mobile phase B consisted of acetonitrile. The gradient began at a hold of 0% B for 0.3 min and was then ramped up to 40% B over 2.2 minutes, immediately ramped to 90% B, held there for 0.4 minutes, and then returned back to 0% B for the remaining 0.6 minutes of the method for re-equilibration. A flow rate of 0.45 ml/min was used. All raw data was processed using Analyst Software ver. 1.4.2 (Applied Biosystems/ MDS Sciex Inc., Ontario, Canada). The lower limit of quantification (LLOQ) for compound 3 was 1.22 ng/mL. The upper limit of quantification (ULOQ) was 2500 ng/mL. Pharmacokinetic parameters were determined by non-compartmental analysis for AUC calculation using WinNonlin (v5.2, Pharsight, Cary, NC).

Experimental Methods for Figure 6

Animal care and handling. All animals used in these studies were housed two per cage, in environmentally controlled animal quarters (light/dark-4:00 am/4:00 pm) with free access to food and water for not less than 7 days prior to testing except as noted in method. All procedures were in accordance with the Pfizer Laboratory Animal Care Program and were approved by the Institutional Animal Care and use Committee of Pfizer Global Research and Development, Groton CT.

Microdialysis in freely moving rats. Male Sprague-Dawley rats (280 – 340 g) underwent surgery to implant unilateral microdialysis guide cannulae at Charles River (Raleigh, NC). Guide cannulae (Bioanalytical Systems, Inc., West Lafayette, ID) targeting the PFC (bregma: AP +3.7 mm, ML -0.7 mm, DV -2.0 mm; Paxinos and Watson 1997) were implanted under isoflurane anesthesia and fixed to the skull using bone screws and dental acrylic. Approximately 18 h before testing, 4 mm microdialysis probes (BR-4; Bioanalytical Systems, Inc.) with a polyacrylonitrile membrane (OD 340 nm, MW cut-off 30,000 Daltons) were perfused with artificial CSF (147 mM NaCl, 2.7 mM KCl, 1.0 mM $MgCl_2$ hexahydrate, 1.3 mM $CaCl_2$ dihydrate; 1.5 µl/min). Microdialysate samples (30 µl) were continuously collected and automatically injected every 20 or 60 min. (overnight) for on-line HPLC/Fluorometric analysis.

Rats (n = 4) were dosed subcutaneously (SC) with vehicle (2 ml/kg) or test compound dissolved in vehicle. Vehicle consisted of 1% acetic acid in distilled water (v/v). Test compound or vehicle was administered 2 hours after basal KYNA levels, see below, had stabilized and the effects on KYNA levels were monitored for 4 hours. Data are shown as mean ± standard error of the mean.

For compound **1**, mean basal KYNA levels = 0.71 ± 0.16 nM (n = 4)

For compound **3**, mean basal KYNA levels = 1.38 ± 0.35 nM (n = 4)

KAT II Protein Isolation and X-ray Crystallography Experimental Protocols

Expression and Purification:

Based on our first crystal structure of a human KAT II complex with PF-4859989[1] we systematically tested the effect of surface mutations in the protein, with the goal of forcing the protein molecules to pack differently in the crystal so as to improve diffraction resolution. Of many different mutants examined, one (K240S/F241G), consistently gave better crystals, and was the one picked. Full length human KAT II (residues 1-425, Uniprot Q8N5Z0, K240S/F241G), with a C-terminal thrombin cleavage site (-LVPRGSLE-) followed by a hexa-histidine tag was expressed in Sf21 insect cells. The protein was purified as described before.[1] Its specific activity and Ki for standard inhibitors were no different from the WT protein (data not shown), but replacement of the surface lysine and phenylalanine by smaller side chains led to different packing interactions that gave crystals that diffracted to higher resolution than the original 3.2 Å structure.

Crystallization:

Crystals were grown by vapour diffusion as hanging drops. Protein at 10 mg/ml was incubated at room temperature for 2 h with compound (1 mM) and pyridoxal phosphate (2 mM). Interestingly, each compound crystallized in more than one set of crystallization conditions. The best crystals for each compound, which were picked for data collection, were obtained in conditions described below.

Compound 2: 2 µl drops, containing equal volumes of protein solution and reservoir solution (200 mM MgAcetate, 0.1M NaCacodylate pH 6.5, 20% PEG8K), were equilibrated against 1mL of reservoir solution.

Compound 3: 2 µl drops, containing equal volumes of protein solution and reservoir solution (250 mM NaSCN, 0.1M NaCitrate pH 5.6, 26% PEG4K), were equilibrated against 1mL of reservoir solution.

Compound 4: 2 µl drops, containing equal volumes of protein solution and reservoir solution (0.1M Hepes pH 7.5, 10% propanol, 20% PEG4K), were equilibrated against 1mL of reservoir solution.

Structure determination and refinement:

For all three structures, diffraction data were collected from a single crystal flash frozen in a stream of dry nitrogen at 100 K, using 25% glycerol as a cryoprotectant. Data were collected at the IMCA-CAT beamline 17-ID at the Advanced Photon Source. Use of the IMCA-CAT beamline 17-ID at the Advanced Photon Source was supported by the companies of the Industrial Macromolecular

Crystallography Association through a contract with Hauptman-Woodward Medical Research Institute. Use of the Advanced Photon Source was supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract No. DE-AC02-06CH11357.

The structures were solved by molecular replacement, using the previously reported structure of human KAT II as a starting model.[7] Refinement was carried out using autoBUSTER[8], using NCS restraints implemented as Local Structure Similarity Restraints[9], while fitting was done in COOT as distributed with the CCP4 package[10] Complete data collection and refinement statistics are given in Table S1,S2 and S3.

Table S3. Data Collection and Refinement Statistics – Compound 2

Data Collection	
Space Group	$P322_1$
Unit cell: *a,b,c*, Å	111.94, 111.94, 137.51
Unit cell: $\alpha,\beta,\gamma(°)$	90.0, 90.0, 120.0
Resolution (Å)	2.4
Total measurements	427757(63123)*
Unique reflections	39279(5657)
R_{sym} (%)	6.9 (49.7)
<I>/<SigI>	23.7 (5.2)
Completeness	100.0 (100.0)
Redundancy	10.9 (11.2)

*Numbers in parentheses reflect the values in the highest resolution shell (2.54 - 2.40 Å).

Model Refinement	
No. of molecules in A.U.	2
No. of non-H atoms in refinement:	
Protein	6640
Solvent	265
Ligands	60
Maximum resolution (Å)	2.41
R_{work}[a] (%)	18.7
R_{free} (%)	21.3
Mean B value (Å^2)	52.2
Rms deviations from ideal geometry	
Bonds (Å)	0.009
Angles (°)	1.18
Peptide Omega Torsion Angles (°)	3.19
Number of TLS Groups	2

[a]$R_{work} = \Sigma||F_{obs}| - |F_{calc}|/ \Sigma|F_{obs}|$. R_{free} is equivalent to R_{work}, except that it is calculated for a randomly chosen 5% of reflections omitted from the refinement process.

Table S1. Data Collection and Refinement Statistics – Compound 3

Data Collection	
Space Group	$P2_12_12_1$
Unit cell: *a,b,c*, Å	85.74, 114.97, 116.55
Unit cell: $\alpha,\beta,\gamma(°)$	90.0, 90.0, 90.0
Resolution (Å)	2.1
Total measurements	286621(9319)*
Unique reflections	60372(2273)
R_{sym} (%)	8.4 (45.1)
<I>/<SigI>	17.54 (2.63)
Completeness	89.1 (68.1)
Redundancy	4.75 (4.1)

*Numbers in parentheses reflect the values in the highest resolution shell (2.14 - 2.1 Å).

Model Refinement	
No. of molecules in A.U.	2
No. of non-H atoms in refinement:	
Protein	6676
Solvent	630
Ligands	84
Maximum resolution (Å)	2.1
R_{work}[a] (%)	17.8
R_{free} (%)	20.2
Mean B value (Å^2)	35.6
Rms deviations from ideal geometry	
Bonds (Å)	0.009
Angles (°)	1.11
Peptide Omega Torsion Angles (°)	3.07
Number of TLS Groups	2

[a]$R_{work} = \Sigma||F_{obs}| - |F_{calc}|/ \Sigma|F_{obs}|$. R_{free} is equivalent to R_{work}, except that it is calculated for a randomly chosen 5% of reflections omitted from the refinement process.

Table S2. Data Collection and Refinement Statistics – Compound 4

Data Collection	
Space Group	$P2_1$
Unit cell: *a,b,c*, Å	69.15, 107.05, 116.60
Unit cell: $\alpha,\beta,\gamma(°)$	90.0, 94.5, 90.0
Resolution (Å)	2.4
Total measurements	226058(31721)*
Unique reflections	62628(3193)
R_{sym} (%)	11.4 (48.9)
<I>/<SigI>	8.0 (3.0)
Completeness	98.1 (97.7)
Redundancy	3.6 (3.5)

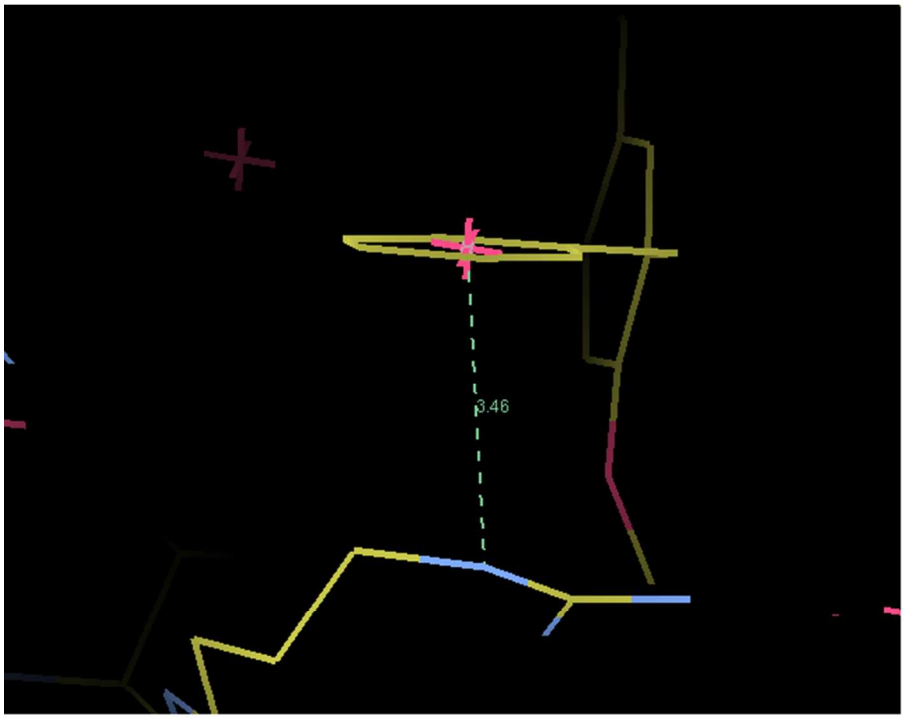
*Numbers in parentheses reflect the values in the highest resolution shell (2.56 - 2.42 Å).

Model Refinement	
No. of molecules in A.U.	4
No. of non-H atoms in refinement:	
Protein	13376
Solvent	745
Ligands	148
Maximum resolution (Å)	2.43
R_{work}[a] (%)	17.5
R_{free} (%)	22.9
Mean B value ($Å^2$)	52.4
Rms deviations from ideal geometry	
Bonds (Å)	0.010
Angles (°)	1.24
Peptide Omega Torsion Angles (°)	2.92
Number of TLS Groups	4

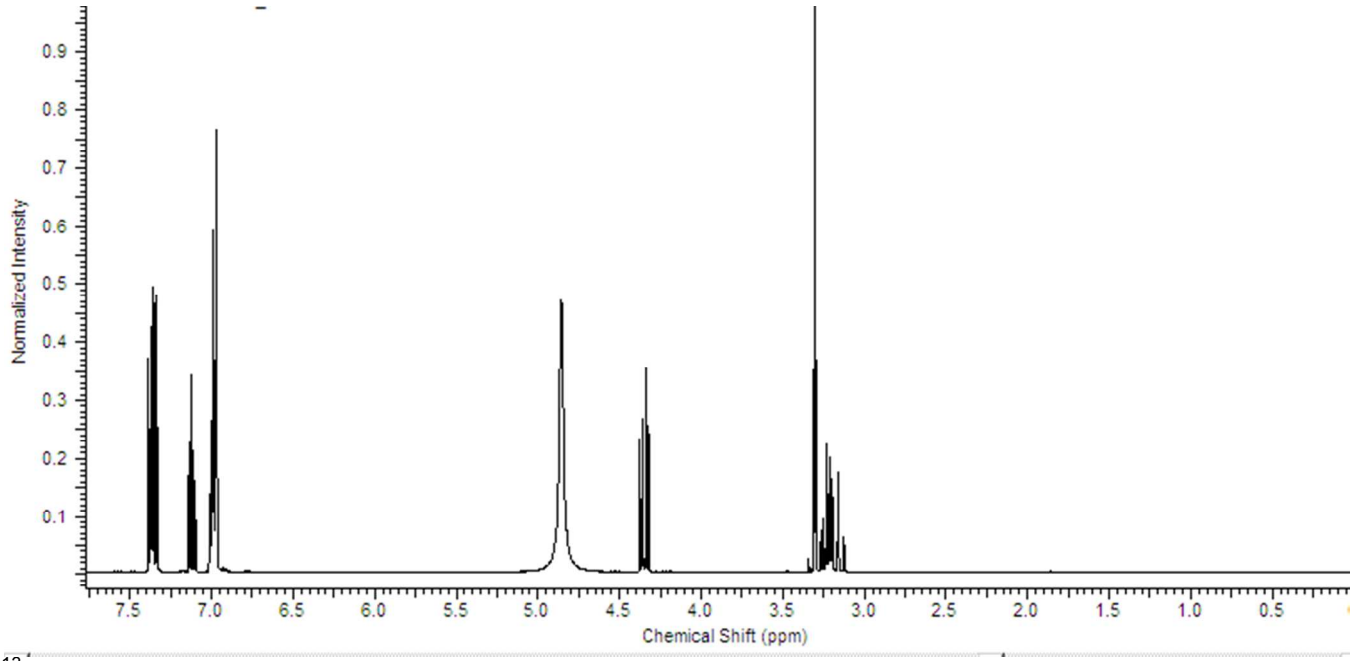
[a]$R_{work} = \Sigma||F_{obs}| - |F_{calc}|/ \Sigma|F_{obs}|$. R_{free} is equivalent to R_{work}, except that it is calculated for a randomly chosen 5% of reflections omitted from the refinement process.

Distance Calculation Between the Phenyl Ring of Compound 4 and Arg-20 in Figure 4

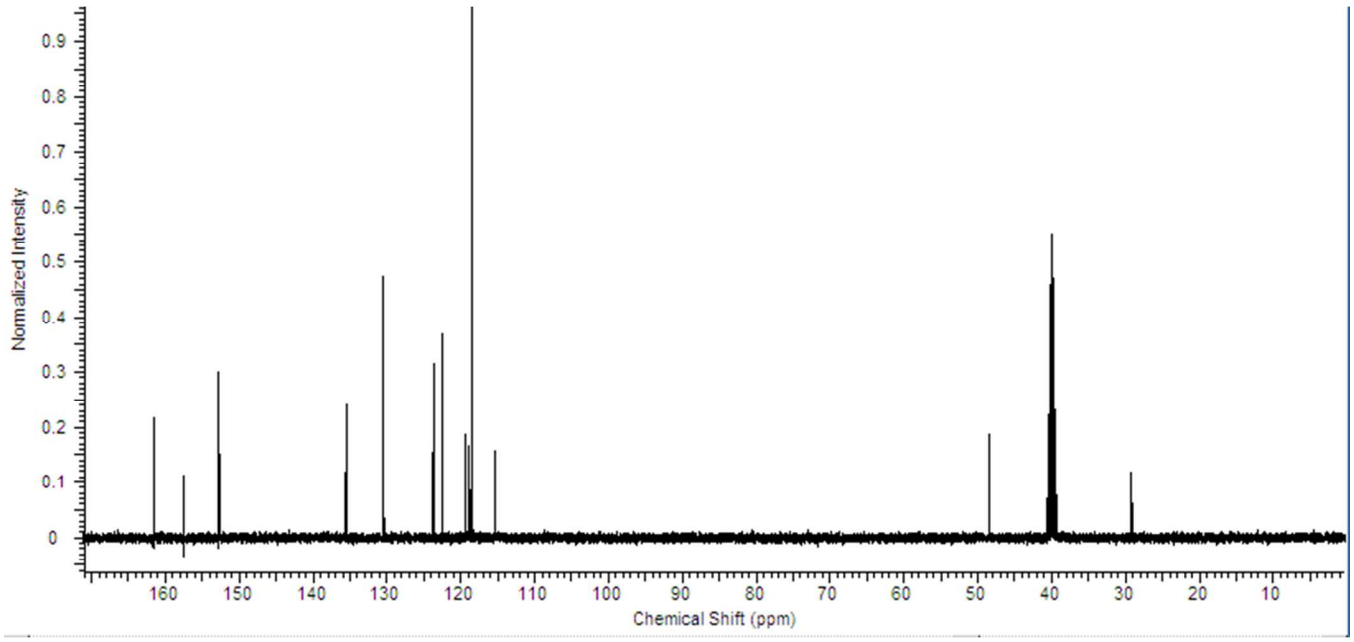
Distance measurement between the phenyl ring and Arg-20. A virtual atom was placed in the center of the phenyl ring. The distance measured from this virtual atom to the NH of Arg-20 was 3.5 angstroms.



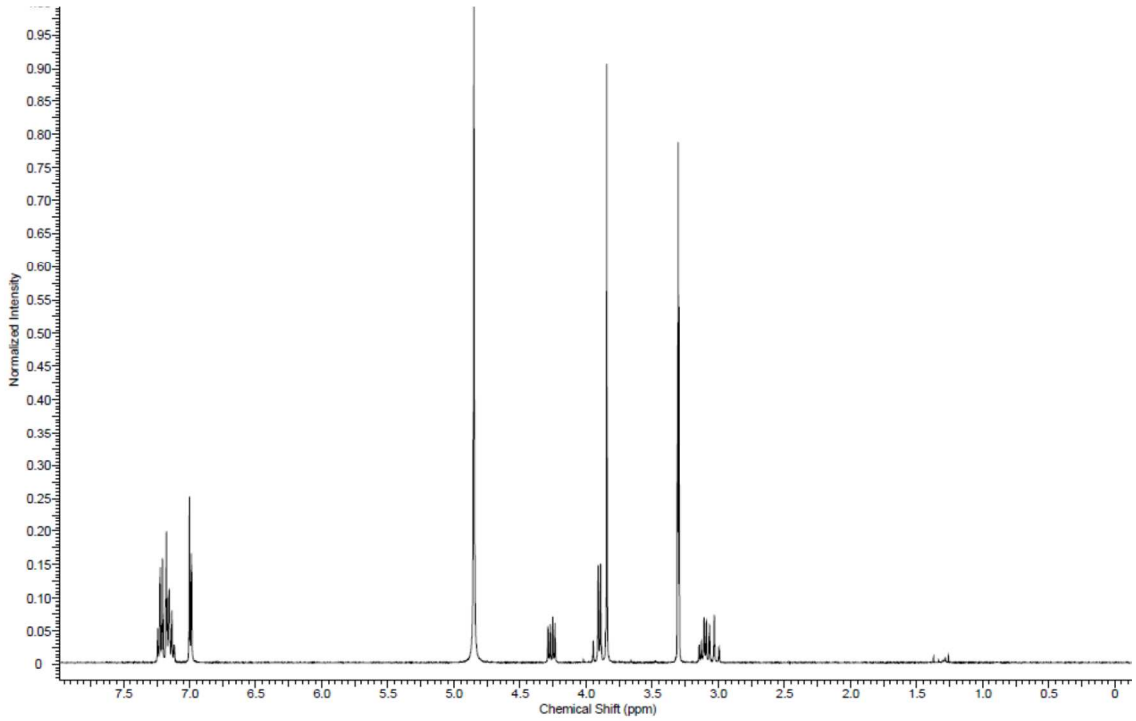
^{1}H NMR for compound **3**



^{13}C NMR for compound **3**



^{1}H NMR for compound **4**



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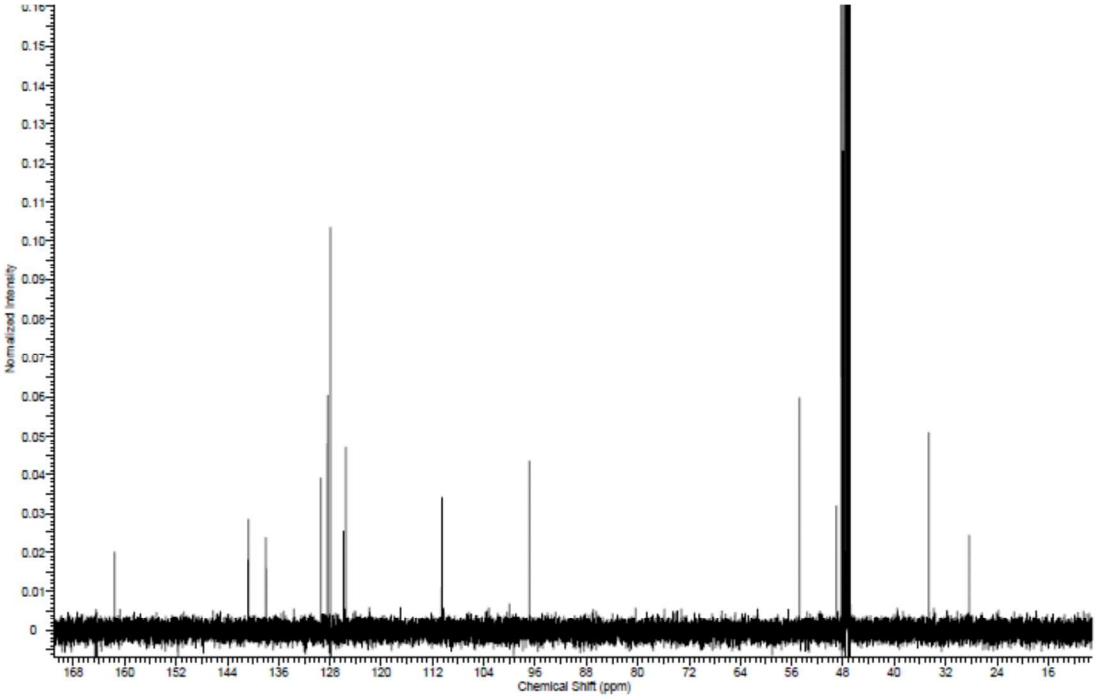
^{13}C NMR for compound **4**



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