Supporting Information

Discovery, Synthesis, SAR Development of a Series of *N*-4-(2,5-dioxopyrrolidin-1-yl)-phenylpicolinamides: Characterization of VU0400195 (ML182) as a Positive Allosteric Modulator of Metabotropic Glutamate Receptor 4 ($mGlu_4$) with Oral Efficacy in an anti-Parkinsonian Animal Model

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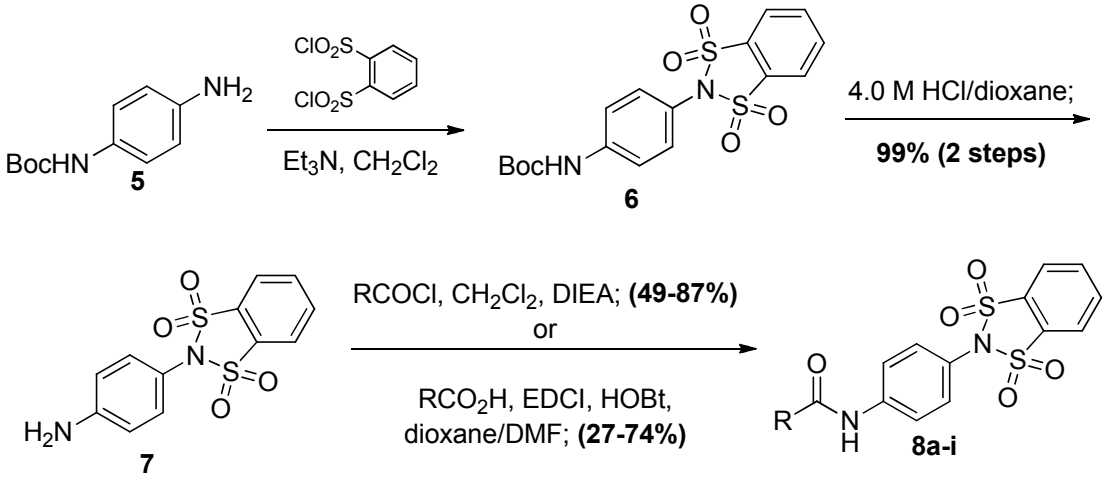
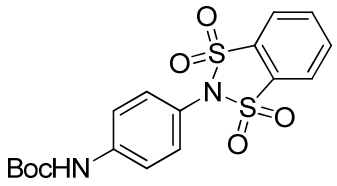
General Scheme



Supporting Information

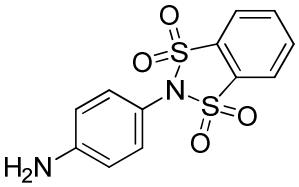
General.

General Procedures (Scheme 1):



***tert*-Butyl (4-(1,1,3,3-tetraoxidobenzo[*d*][1,3,2]dithiazol-2-yl)phenyl)carbamate** (**6**). A solution of *N*-Boc-*p*-phenylenediamine, **5**, (0.68 g, 3.26 mmol, 1.0 equivalents) and Et_3N (0.51 mL, 7.19 mmol, 2.2 equivalents) in dry DCM (29 mL) was added via syringe pump (8 mL/ h) to a refluxing solution of 1,2-benzenedisulfonyl dichloride (0.90 g, 3.27 mmol, 1.005 equivalents) in DCM (135 mL). After the complete addition, the reaction solution was maintained at reflux for an additional 2 h. The heat was removed and after 16 h at rt, the solvent was removed under reduced pressure. The residue of the crude reaction mixture was redissolved in DCM (75 mL) and washed with saturated $NaHCO_3$ (aq, 75 mL), brine (75 mL), and dried ($MgSO_4$). After the filtration and concentration under reduced pressure, analytical LCMS confirms product (**6**). The material was taken through to the deprotection step.

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.11-8.08 (m, 2 H), 7.99-7.96 (m, 2 H), 7.59-7.55 (m, 4 H), 6.66 (br s, 1 H), 1.54 (s, 9 H).

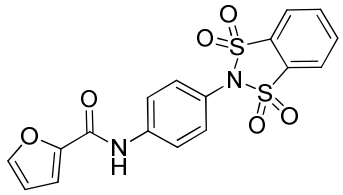


2-(4-Aminophenyl)benzo[d][1,3,2]dithiazole 1,1,3,3-tetraoxide (7). To a solution of **6** (3.26 mmol, 1.0 equivalents) in DCM (100 mL) at 0 °C was added dropwise 4N HCl in dioxane (20 mL). After 15 min, the ice bath was removed. Once the starting material was no longer evident by TLC, the solvent was removed affording the HCl salt (**7**), which was taken through to the amide formation step.

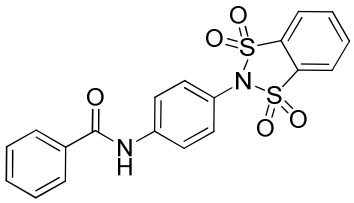
LCMS: R_T = 3.319 min, *m/z* = 311.0 $[M + H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.55-8.51 (m, 2 H), 8.21-8.17 (m, 2 H), 7.17-7.15 (m, 2 H), 6.75-6.73 (m, 2 H).

Amide formation (Method A) (8a-i). To each of the reaction test tubes was added a solution of the HCl salt (**7**) (1.0 equivalent) in DCM:DIEA (4:1) (1 mL, 0.1 M). The appropriate acid chloride (1.0 equivalent) was added and after 12 h at rt, the desired analogs were directly purified by mass-directed preparative HPLC.

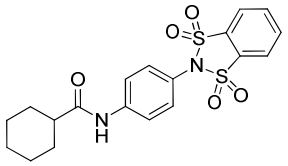
Amide Formation (Method B) (8a-i). To each of the reaction test tubes with the appropriate carboxylic acid (1.0 equivalent) in dry 1,4-dioxane:DMF:DIEA (2:2:1, 0.3M) at room temperature was charged with HOBt (1.0 equivalent) and EDCI (1.5 equivalents). After 15 min, the HCl salt (**7**, 1.0 equivalent) was added and the heterogeneous mixture was heated to 50 °C. After 12 h, the reaction mixture was added to EtOAc:water (1:1). The organic layer was separated and sequentially washed with water (2 x), brine and dried ($MgSO_4$). After filtration and concentration under reduced pressure, analytical LCMS confirms product (**8a-i**). The residue was purified by mass-directed preparative HPLC.



***N*-(4-(1,1,3,3-Tetraoxidobenzo[*d*][1,3,2]dithiazol-2-yl)phenyl)furan-2-carboxamide (8a):** Following the general procedure above (Method A), *N*-(4-(1,1,3,3-tetraoxidobenzo[*d*][1,3,2]dithiazol-2-yl)phenyl)furan-2-carboxamide was obtained as an off-white solid (87%).
LCMS: R_T = 2.88 min, >98% (214 nm), *m/z* = 405.1 $[M + H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.57 (br s, 1 H), 8.58-8.56 (m, 2 H), 8.21-8.19 (m, 2 H), 8.04 (d, 2 H, *J* = 8.8 Hz), 7.98 (d, 1 H, *J* = 1.6 Hz), 7.55 (d, 2 H, *J* = 8.8 Hz), 7.40 (d, 1 H, *J* = 3.6 Hz), 6.73 (dd, 1 H, *J* = 3.6, 1.6 Hz). HRMS, calc'd for $C_{17}H_{13}N_2O_6S_2$, 405.0215 $[M + H]^+$, found 405.0205.

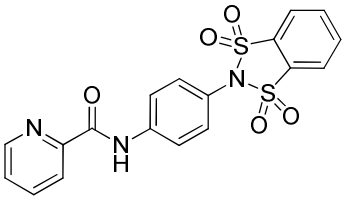


***N*-(4-(1,1,3,3-Tetraoxidobenzo[*d*][1,3,2]dithiazol-2-yl)phenyl)benzamide (8b):** Following the general procedure above (Method A), *N*-(4-(1,1,3,3-tetraoxidobenzo[d][1,3,2]dithiazol-2-yl)phenyl)benzamide was obtained as an tan solid.
LCMS: R_T = 3.08 min, >98% (214 nm), *m/z* = 415.1 $[M + H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.65 (br s, 1 H), 8.61-8.58 (m, 2 H), 8.25-8.22 (m, 2 H), 8.10 (d, 2 H, *J* = 8.8 Hz), 8.00 (d, 2 H, *J* = 8.4 Hz), 7.66-7.56 (m, 5 H). HRMS, calc'd for $C_{19}H_{14}N_2O_5NaS_2$ 437.0242 $[M + Na]^+$, found 437.0240.



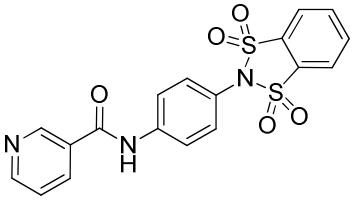
***N*-(4-(1,1,3,3-Tetraoxidobenzo[*d*][1,3,2]dithiazol-2-yl)phenyl)cyclohexanecarboxamide (8c):** Following the general procedure above (Method A), *N*-(4-(1,1,3,3-tetraoxidobenzo[*d*][1,3,2]dithiazol-2-yl)phenyl)cyclohexanecarboxamide was obtained as an off-white solid.

LCMS: R_T = 3.237 min, >98% (214 nm), *m/z* = 421.1 $[M + H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.24 (br s, 1 H), 8.59-8.57 (m, 2 H), 8.23-8.20 (m, 2 H), 7.89 (d, 1 H, *J* = 8.8 Hz), 7.50 (d, 1 H, *J* = 8.8 Hz), 2.38-2.33 (m, 1 H), 1.85-1.76 (m, 4 H), 1.70-1.62 (m, 1 H), 1.44-1.38 (m, 2 H), 1.30-1.20 (m, 3 H). HRMS, calc'd for $C_{19}H_{21}N_2O_5S_2$ 421.0892 $[M + H]^+$, found 421.0895.



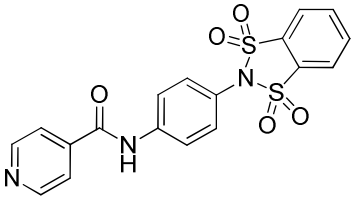
N-(4-(1,1,3,3-Tetraoxidobenzo[d][1,3,2]dithiazol-2-yl)phenyl)picolinamide (8d): Following the general procedure above (Method A), N-(4-(1,1,3,3-tetraoxidobenzo[d][1,3,2]dithiazol-2-yl)phenyl)picolinamide was obtained as an tan solid (49%).

Analytical LCMS: R_T = 3.074 min, >98% (214 nm), *m/z* = 416.1 $[M + H]^+$; ^{1}H NMR (400 MHz, d_6-DMSO): δ 11.08 (br s, 1 H), 8.79 (d, 1 H, *J* = 4.0 Hz), 8.60-8.59 (m, 2 H), 8.24-8.21 (m, 5 H), 8.11 (t, 1 H, *J* = 8.0 Hz), 7.73 (t, 1 H, *J* = 4.8 Hz), 7.59 (d, 2 H, *J* = 8.4 Hz); ^{13}C NMR (125 MHz, *d*-DMSO-d_6) δ 163.5, 149.8, 148.9, 142.1, 138.7, 136.8, 134.0, 133.0, 127.7, 123.8, 123.1, 122.4, 119.1. HRMS, calc'd for $C_{18}H_{13}N_3O_5NaS_2$ $[M + Na^+]$, 438.0194; found 438.0194.



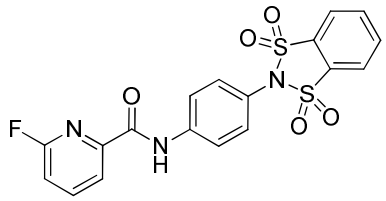
***N*-(4-(1,1,3,3-Tetraoxidobenzo[*d*][1,3,2]dithiazol-2-yl)phenyl)nicotinamide (8e):** Following the general procedure above (Method B), N-(4-(1,1,3,3-tetraoxidobenzo[d][1,3,2]dithiazol-2-yl)phenyl)nicotinamide was obtained as an tan solid.

Analytical LCMS: R_T = 0.698 min, >98% (220 nm), *m/z* = 417.0 $[M + H]^+$.

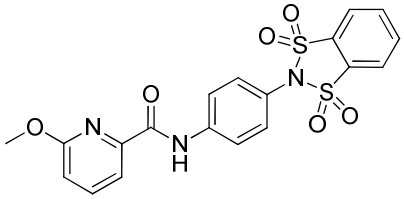


***N*-(4-(1,1,3,3-Tetraoxidobenzo[*d*][1,3,2]dithiazol-2-yl)phenyl)isonicotinamide (8f):** Following the general procedure above (Method B), *N*-(4-(1,1,3,3-tetraoxidobenzo[*d*][1,3,2]dithiazol-2-yl)phenyl)isonicotinamide was obtained as an tan solid.

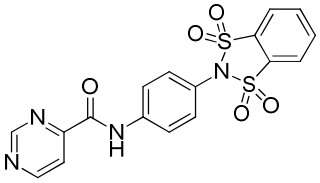
Analytical LCMS: R_T = 0.708 min, >98% (220 nm), *m/z* = 417.0 $[M + H]^+$.



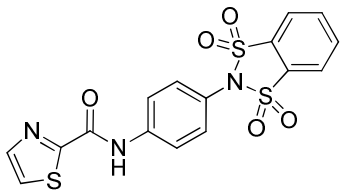
6-Fluoro-*N*-(4-(1,1,3,3-tetraoxidobenzo[*d*][1,3,2]dithiazol-2-yl)phenyl)picolinamide (8g): Following the general procedure above (Method B), 6-fluoro-*N*-(4-(1,1,3,3-tetraoxidobenzo[*d*][1,3,2]dithiazol-2-yl)phenyl)picolinamide was obtained as an white solid (88%).
LCMS: R_T = 3.18 min, >98% (214 nm), *m/z* = 434.0 $[M + H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.90 (br s, 1 H), 8.61-8.59 (m, 2 H), 8.29 (dd, 1 H, *J* = 15.6, 8.0 Hz), 8.24-8.22 (m, 2 H), 8.20 (d, 2 H, *J* = 8.8 Hz), 8.14 (d, 1 H, *J* = 6.4 Hz), 7.60 (d, 2 H, *J* = 8.4 Hz), 7.54 (d, 1 H, *J* = 7.6 Hz). HRMS, calc'd for $C_{18}H_{13}N_3O_5FS_2$ 434.0281 $[M + H]^+$, found 434.0281.



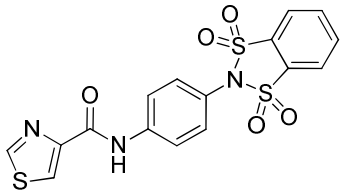
6-Methoxy-*N*-(4-(1,1,3,3-tetraoxidobenzo[*d*][1,3,2]dithiazol-2-yl)phenyl)picolinamide (8h): Following the general procedure above (Method B), 6-methoxy-*N*-(4-(1,1,3,3-tetraoxidobenzo[*d*][1,3,2]dithiazol-2-yl)phenyl)picolinamide was obtained.
LCMS: R_T = 2.414 min., >98% (214 nm), *m/z* = 446.1 $[M + H]^+$.



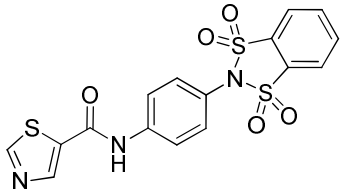
***N*-(4-(1,1,3,3-Tetraoxidobenzo[*d*][1,3,2]dithiazol-2-yl)phenyl)pyrimidine-4-carboxamide (8i):** Following the general procedure above (Method B), *N*-(4-(1,1,3,3-tetraoxidobenzo[*d*][1,3,2]dithiazol-2-yl)phenyl)pyrimidine-4-carboxamide was obtained.
LCMS: R_T = 2.80 min, >98% (214 nm), *m/z* = 417.1 $[M + H]^+$.



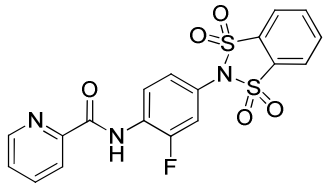
***N*-(4-(1,1,3,3-Tetraoxidobenzo[*d*][1,3,2]dithiazol-2-yl)phenyl)thiazole-2-carboxamide (8j):** Following the general procedure (Method A), *N*-(4-(1,1,3,3-tetraoxidobenzo[*d*][1,3,2]dithiazol-2-yl)phenyl)thiazole-2-carboxamide was obtained.
LCMS: R_T = 3.008 min., >98% (214 nm), *m/z* = 422.0 $[M + H]^+$.



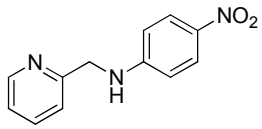
***N*-(4-(1,1,3,3-Tetraoxidobenzo[*d*][1,3,2]dithiazol-2-yl)phenyl)thiazole-4-carboxamide (8k):** Following the general procedure above (Method B), *N*-(4-(1,1,3,3-tetraoxidobenzo[*d*][1,3,2]dithiazol-2-yl)phenyl)thiazole-4-carboxamide was obtained.
LCMS: R_T = 2.97 min, >98% (214 nm), *m/z* = 422.0 $[M + H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.82 (s, 1 H), 9.34 (d, 1 H, *J* = 2.1 Hz), 8.63 (m, 3 H), 8.25 (m, 2 H), 8.24, (d, 2 H, *J* = 8.9 Hz), 7.60 (d, 2 H, *J* = 8.9 Hz). HRMS, calc'd for $C_{16}H_{11}N_3O_5S_3$ 421.9939 $[M]^+$, found 421.9937.



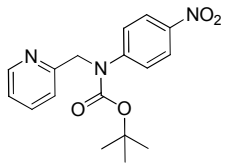
***N*-(4-(1,1,3,3-Tetraoxidobenzo[*d*][1,3,2]dithiazol-2-yl)phenyl)thiazole-5-carboxamide (8l):** Following the general procedure (Method A), *N*-(4-(1,1,3,3-tetraoxidobenzo[*d*][1,3,2]dithiazol-2-yl)phenyl)thiazole-5-carboxamide was obtained.
LCMS: R_T = 0.761 min., >98% (220 nm), *m/z* = 422.0 $[M + H]^+$.



***N*-(2-Fluoro-4-(1,1,3,3-tetraoxidobenzo[*d*][1,3,2]dithiazol-2-yl)phenyl)picolinamide (9a).**
LCMS: R_T = 2.24 min, 87% (214 nm), *m/z* = 434 $[M + H]^+$.

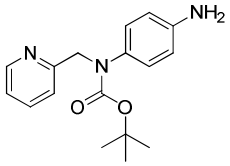


4-Nitro-*N*-(pyridin-2-ylmethyl)aniline: To a solution of 1-fluoro-4-nitrobenzene (1.06 mL, 10.0 mmol) and DIEA (3.10 mL, 22.0 mmol) in DMF (10 mL) was added 2-(aminomethyl)pyridine (1.14 mL, 11.0 mmol). The rxn was heated to 70-75 °C. After 12 h, the brown solution was added to ethyl acetate:H_2O (1:1, 50 mL). The organic layer was separated and washed with H_2O (2 x 25 mL) and Brine (25 mL). After drying over $MgSO_4$, the organic layer was filtered and concentrated. The residue was purified by flash column chromatography (10-100% ethyl acetate:hexanes) to provide a yellow solid (1.40g, 61% yield):
R_f = 0.25 (50% EtOAc/hexanes). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.61 (d, 1 H, *J* = 4.8 Hz), 8.11 (d, 2 H, *J* = 8.8 Hz), 7.75 (t, 1 H, *J* = 6.8 Hz), 7.33 (d, 2 H, *J* = 11.8 Hz), 7.31 (d, 1 H, *J* = 10.8 Hz), 6.64 (d, 2 H, *J* = 9.0 Hz), 5.90 (br s, 1 H), 4.56 (s, 2 H).



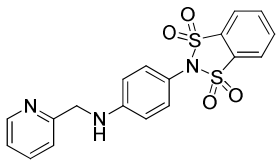
***tert*-Butyl (4-nitrophenyl)(pyridin-2-ylmethyl)carbamate:** To a solution of 4-nitro-*N*-(pyridin-2-ylmethyl)aniline (1.40 g; 6.11 mmol), DMAP (75 mg; 0.61 mmol), DIEA (1.28 mL; 9.17 mmol) in THF (60 mL) was added Boc_2O (2.0 g; 9.17 mmol). The rxn was heated to reflux. After 6h, TLC shows loss of starting material, so the heat was removed. The rxn was added to EtOAc:H2O (1:1, 100 mL) and the organic layer separated. The organic phase was washed with Brine (30 mL), dried ($MgSO_4$), filtered and concentrated. The crude residue was taken through to the next step without further purification.

R_f = 0.33 (33% EtOAc/hexanes); ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.58 (d, 1 H, *J* = 4.4 Hz), 8.18-8.14 (m, 2 H), 7.76 (t, 1 H, *J* = 8.0 Hz), 7.55-7.51 (m, 2 H), 7.34 (d, 1 H, *J* = 7.6 Hz), 7.29-7.26 (m, 1 H), 5.10 (s, 2 H), 1.42 (s, 9 H).



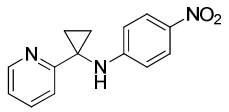
***tert*-Butyl (4-aminophenyl)(pyridin-2-ylmethyl)carbamate:** To a solution of *tert*-butyl (4-nitrophenyl)(pyridin-2-ylmethyl)carbamate (0.58 g, 1.76 mmol) in methanol (17 mL) was added Pd/C (50 mg) and an atmosphere of H_2 was applied. After 14 h, the rxn was filtered through a plug of Celite and concentrated. The residue was carried on without further purification.

LCMS: R_T = 0.79 min, >98% (214 nm), *m/z* = 300.0 $[M + H]^+$.



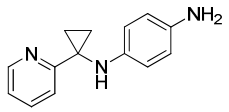
2-(4-((Pyridin-2-ylmethyl)amino)phenyl)benzo[*d*][1,3,2]dithiazole 1,1,3,3-tetraoxide (9b): To a solution of 1,2-benzenedisulfonyl dichloride (72 mg, 0.26 mmol) and DCE (3 mL) at 40 °C was added a solution of *tert*-butyl (4-aminophenyl)(pyridin-2-ylmethyl)carbamate (78 mg, 0.26 mmol) and DBU (0.09 mL, 0.57 mmol) in dry DCE (2 mL). After 48 h, the heat was removed and the rxn was added to DCM:H_2O (1:1, 5 mL) and the organic layer was separated. The aqueous layer was extracted with DCM (2 x 5 mL) and the collected organic layers were washed with Brine (5 mL), and dried over $MgSO_4$. After filtration and concentration under reduced pressure, the residue was purified by preparative HPLC. The pure material was dissolved in DCM (1 mL) and 4M HCl in dioxane (0.5 mL) was added. After 3h, the solvent was removed to provide 2-(4-((pyridin-2-ylmethyl)amino)phenyl)benzo[*d*][1,3,2]dithiazole 1,1,3,3-tetraoxide as an HCl Salt.

LCMS: R_T = 1.096 min, >98% (214 nm), *m/z* = 401.8 $[M + H]^+$.



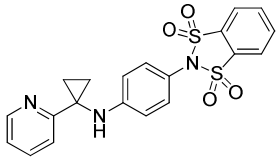
4-Nitro-*N*-(1-(pyridin-2-yl)cyclopropyl)aniline: To a solution of 1-fluoro-4-nitrobenzene (141 mg, 1.0 mmol) and DIEA (0.31 mL, 2.2 mmol) in DMF (1.5 mL) was added 1-(pyridin-2-yl)cyclopropanamine (141 mg, 1.05 mmol). The rxn was heated to 160 °C for 25 min in a microwave reactor. The rxn was added to ethyl acetate:H_2O (1:1, 50 mL). The organic layer was separated and washed with H_2O (3 x 15 mL). After drying over $MgSO_4$, the organic layer was filtered and concentrated. The crude material was taken through to the next step.

LCMS: R_T = 2.00 min, *m/z* = 256.1 $[M + H]^+$.



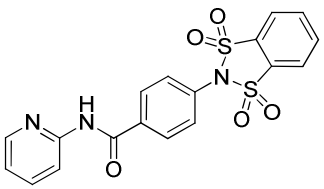
***N*-(1-(Pyridin-2-yl)cyclopropyl)benzene-1,4-diamine:** To a solution of 4-nitro-*N*-(1-(pyridin-2-yl)cyclopropyl)aniline in ethyl acetate (10 mL) was added Pd/C (50 mg) and an atmosphere of H_2 was applied. After 16 h, TLC confirmed the loss of starting material. The rxn was filtered through a plug of Celite and concentrated. The residue was carried on without further purification.

LCMS: R_T = 0.29 min,>98% (214 nm), *m/z* = 226.0 $[M + H]^+$.

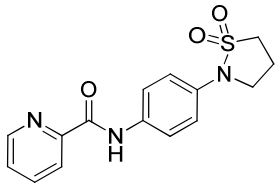


2-(4-((1-(Pyridin-2-yl)cyclopropyl)amino)phenyl)benzo[*d*][1,3,2]dithiazole 1,1,3,3-tetraoxide (9c): To a solution of 1,2-benzenedisulfonyl dichloride (0.23 g, 0.81 mmol) and DCE (2 mL) was added a solution of N1-(1-(pyridin-2-yl)cyclopropyl)benzene-1,4-diamine (0.18 g, 0.81 mmol) and DBU (0.27 mL, 1.78 mmol) in dry DCE (2 mL). After 16 h, the rxn was added to DCM:H_2O (1:1, 50 mL) and the organic layer was separated. The aqueous layer was extracted with DCM (2 x 10 mL) and the collected organic layers were washed with H_2O (20 mL), brine (25 mL), and dried over $MgSO_4$. After the filtration and concentration under reduced pressure, analytical LCMS confirms product (**9c**). The residue was purified by mass-directed preparative HPLC.

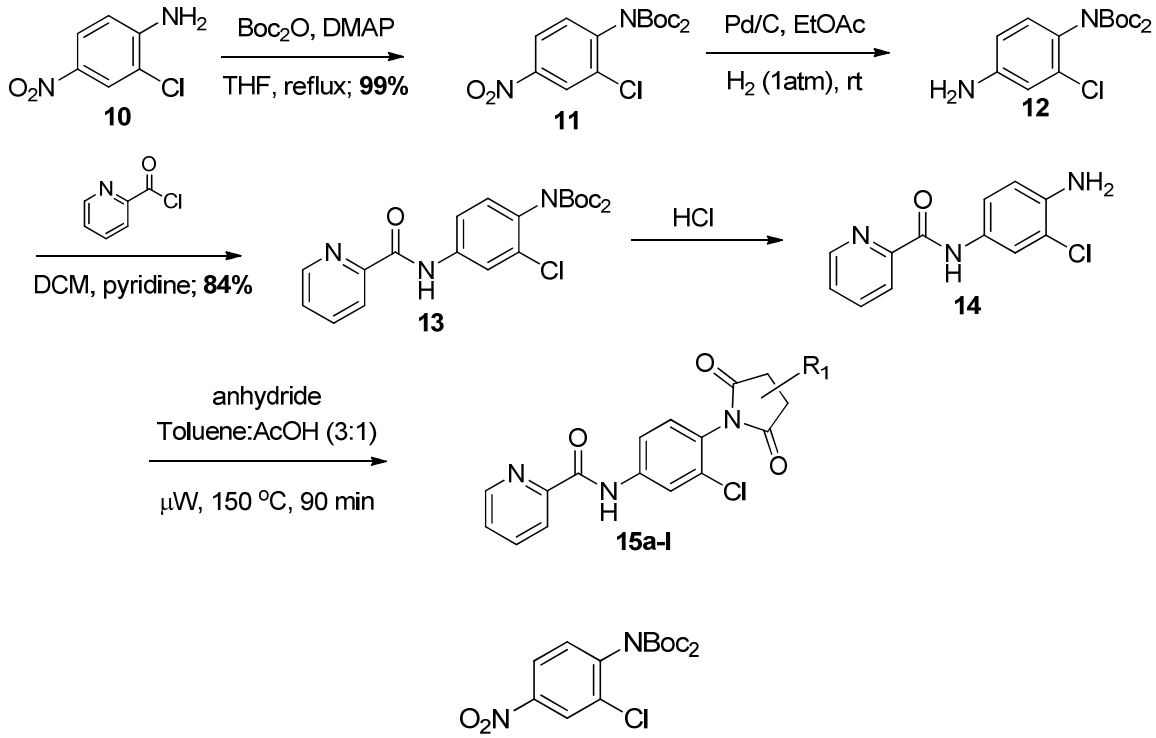
LCMS: R_T = 1.16 min, >90% (214 nm) and >98% (254 nm), *m/z* = 427.8 $[M + H]^+$.



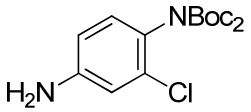
***N*-(Pyridin-2-yl)-4-(1,1,3,3-tetraoxidobenzo[*d*][1,3,2]dithiazol-2-yl)benzamide (9d).**
LCMS: R_T = 1.87 min, >98% (214 nm), *m/z* = 416 $[M + H]^+$.



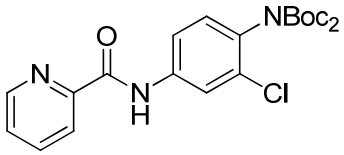
***N*-(4-(1,1-Dioxidoisothiazolidin-2-yl)phenyl)picolinamide (16):** To a suspension of NaH (60% in mineral oil, 96 mg, 2.4 mmol) in DMF (6 mL) at 0 °C was added 1,3-propane sultam (0.29 g, (2.4 mmol), dropwise. After 15 min, the ice bath was removed. After an additional 30 min at rt, a solution of 1-Fluoro-4-nitrobenzene (0.21 mL, 2.0 mmol) in DMF (2 mL) was added. After 12 h, the rxn was added to EtOAc:NH_4Cl (aq) (1:1, 60 mL). The organic layer was washed with water (2 x 30 mL), Brine (30 mL) and dried ($MgSO_4$). Filtered and concentrated to provide a bright yellow solid. The material was dissolved in EtOAc (50 mL) and after 5% Pd/C (~50 mg) was added, an H_2 atmosphere was applied to the rxn. After 24 h, rxn mixture was filtered through a pad of Celite and concentrated to afford 2-(4-aminophenyl)isothiazolidine 1,1-dioxide in >90% purity. Following the general Amide Formation procedure above (Method A), *N*-(4-(1,1-dioxidoisothiazolidin-2-yl)phenyl)picolinamide was obtained.
LCMS: R_T = 2.539 min, >98% (214 nm), *m/z* = 318.1 $[M + H]^+$.



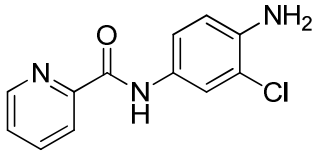
Bis(*tert*-butyl-2-chloro-4-nitrophenyl)carbamate (11). To a solution of 2-chloro-4-nitroaniline (5.0 g, 29 mmol) and DMAP (50 mg, 0.41 mmol) in dry THF (250 mL) was added Boc_2O (16.0 g, 73.3 mmol). The rxn mixture was heated to reflux. After 1h, the solvent was removed. The residue was redissolved in EtOAc:0.5 N HCl (aqueous) and the organic layer was separated. The aqueous layer was extracted with EtOAc (3 x 50 mL) and the collected organic layers were washed with Brine (100 mL). The solution was dried ($MgSO_4$), filtered and concentrated to afford bis(*tert*-butyl-2-chloro-4-nitrophenyl)carbamate.
LCMS: R_T = 3.74 min., >98% @ 254 nM, *m/z* = 767.2 $[2M + Na]^+$.



Bis(*tert*-butyl-4-amino-2-chlorophenyl)carbamate (12). To a solution of bis(*tert*-butyl-2-chloro-4-nitrophenyl)carbamate. (29.0 mmol) in EtOAc (120 mL) was added 5% Pd/C (150 mg) and an H_2 atmosphere was applied. After 12 h, TLC confirmed loss of starting material. The reaction mixture was filtered through Celite and concentrated to afford bis(*tert*-butyl-4-amino-2-chlorophenyl)carbamate which was used without further purification.
^{1}H NMR (400 MHz, $CDCl_3$) δ 6.94 (d, 1 H, *J* = 8.4 Hz), 6.72 (d, 1 H, *J* = 2.8 Hz), 6.53 (dd, 1 H, *J* = 8.4, 2.8 Hz), 3.75 (br s, 2 H), 1.41 (s, 18 H).

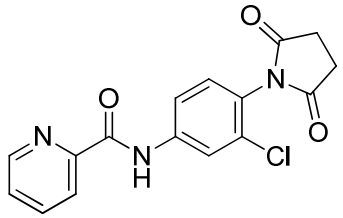


Bis(*tert*-butyl 2-chloro-4-(picolinamido)phenyl)carbamate (13). A solution of bis(*tert*-butyl-4-amino-2-chlorophenyl)carbamate (29.0 mmol) in DCM (25 mL) at 0 °C was subjected to DIEA (10.2 mL, 72.5 mmol) followed by picolinoyl chloride hydrochloride (5.68 g, 31.9 mmol). After 12 h, the reaction was added to EtOAc:H_2O (1:1, 120 mL). The organic layer was washed with water (2 x 50 mL), brine (50 mL) and dried (MgSO4). The mixture was filtered and concentrated to provide bis(*tert*-butyl 2-chloro-4-(picolinamido)phenyl)carbamate.
^{1}H NMR (400 MHz, $CDCl_3$) δ 10.17 (br s, 1 H), 8.63 (d, 1 H, *J* = 4.0 Hz), 8.32 (d, 1 H, *J* = 8.0), 8.05 (d, 1 H, *J* = 2.4 Hz), 7.96 (ddd, 1 H, *J* = 8.0, 8.0, 1.2), 7.66 (dd, 1 H, *J* = 8.4, 2.4), 7.55-7.52 (m, 1 H), 7.20 (d, 1 H, *J* = 8.8 Hz), 1.40 (s, 18 H).

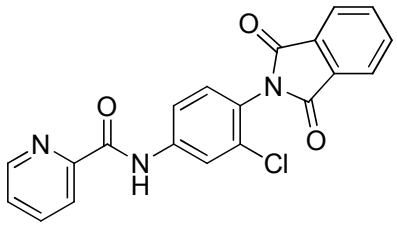


***N*-(4-Amino-3-chlorophenyl)picolinamide (14).** To a solution of bis(*tert*-butyl 2-chloro-4-(picolinamido)phenyl)carbamate (29.0 mmol) in DCM (300 mL) at 0 °C was added dropwise 4N HCl in dioxane (50 mL). After 15 min, the ice bath was removed. Once the starting material was no longer evident by TLC, the solvent was removed affording the HCl salt. The crude material was redissolved with EtOAc (100 mL) and washed with $NaHCO_3$ (aqueous)**.** The organic layer was dried (MgSO4), filtered and concentrated to afford *N*-(4-amino-3-chlorophenyl)picolinamide. LCMS: R_T = 1.15 min., >98% @ 254 nM, *m/z* = 248.0 $[M + H]^+$.

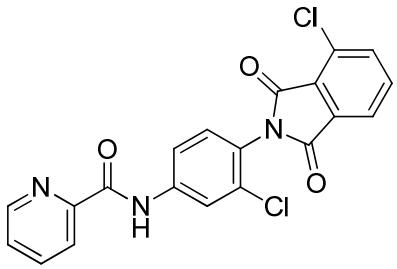
General Cyclic Imide formation. To a solution of *N*-(4-amino-3-chlorophenyl)picolinamide (1.0 equivalent) in toluene:acetic acid (3:1) was added desired anhydride (1.25 equivalents) and the mixture was heated to 150 °C in the microwave for 90 min. After LCMS confirmed the product, the rxn was added to EtOAc:$NaHCO_3$ (sat'd) (1:1) and the organic layer was separated, washed with Brine and dried ($MgSO_4$). After the organic layer was filtered and concentrated, the material was purified by LC purification (Gilson) or crystallization.



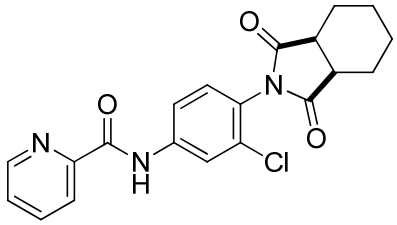
***N*-(3-Chloro-4-(2,5-dioxopyrrolidin-1-yl)phenyl)picolinamide (15a).** Following the general procedure, *N*-(3-chloro-4-(2,5-dioxopyrrolidin-1-yl)phenyl)picolinamide was obtained.
LCMS: R_T = 2.52 min., >98% @ 214 nM, *m/z* = 330.1 $[M + H]^+$.



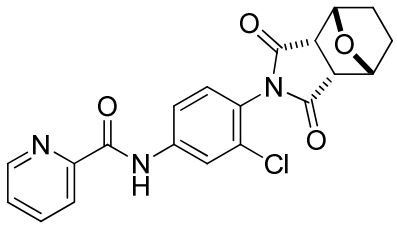
***N*-(3-Chloro-4-(1,3-dioxoisoindolin-2-yl)phenyl)picolinamide (15b).** Following the general procedure, *N*-(3-chloro-4-(1,3-dioxoisoindolin-2-yl)phenyl)picolinamide was obtained.
LCMS: R_T = 1.85 min., >98% @ 214 nM, *m/z* = 378.0 $[M + H]^+$.



***N*-(3-Chloro-4-(4-chloro-1,3-dioxoisoindolin-2-yl)phenyl)picolinamide (15c).** Following the general procedure, *N*-(3-chloro-4-(4-chloro-1,3-dioxoisoindolin-2-yl)phenyl)picolinamide was obtained.
LCMS: R_T = 2.07 min., >98% @ 214 nM, *m/z* = 412.0 $[M + H]^+$.

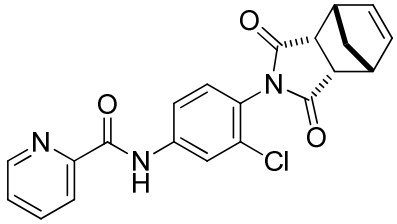


***N*-(3-Chloro-4-((3a*R*,7a*S*)-1,3-dioxohexahydro-1*H*-isoindol-2(3*H*)-yl)phenyl)picolinamide (15d).** Following the general procedure, *N*-(3-chloro-4-((3aR,7aS)-1,3-dioxohexahydro-1*H*-isoindol-2(3*H*)-yl)phenyl)picolinamide was obtained.
LCMS: R_T = 1.43 min., >98% @ 214 nM, *m/z* = 384.1 $[M + H]^+$.



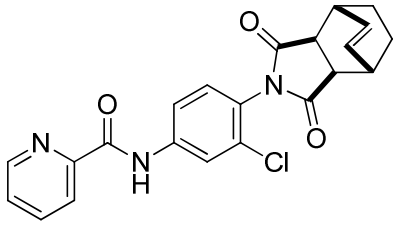
***N*-(3-Chloro-4-((3a*R*,4*R*,7*S*,7a*S*)-1,3-dioxohexahydro-1*H*-4,7-epoxyisoindol-2(3*H*)-yl)phenyl)picolinamide (15e).** Following the general procedure, *N*-(3-chloro-4-((3aR,4R,7S,7aS)-1,3-dioxohexahydro-1*H*-4,7-epoxyisoindol-2(3*H*)-yl)phenyl)picolinamide was obtained.

LCMS: R_T = 0.79 min., >98% @ 214 nM, *m/z* = 420.0 $[M + Na]^+$.



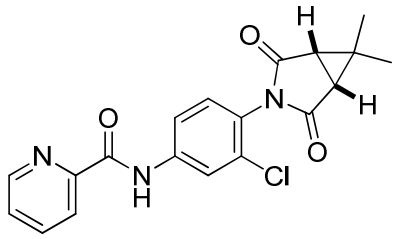
***N*-(3-Chloro-4-((1*R*,2*S*,3*R*,4*S*-bicyclo[2.2.1]hept-5-ene-1,3-dioxo-1*H*-isoindol-1-yl) phenyl)picolinamide (15f).** Following the general procedure, *N*-(3-Chloro-4-((1R,2S,3R,4S-bicyclo[2.2.1]hept-5-ene-1,3-dioxo-1*H*-isoindol-1-yl) phenyl)picolinamide was obtained (mixture of diastereomers).

LCMS: R_T = 1.367 min, >98% @ 254 nM, *m/z* = 394.0 $[M + H]^+$. ^{1}H NMR (400 MHz, MeOD) δ 8.81 (d, 1 H, *J* = 4.4 Hz), 8.41 (d, 1 H, *J* = 7.6 Hz), 8.31-8.28 (m, 1 H), 8.20 (d, 1 H, *J* = 2.4 Hz), 7.84 (m, 1 H), 7.80 (d, 1 H, *J* = 2.4 Hz), 7.05 (d, 1 H, *J* = 8.4 Hz), 6.31 (s, 2 H), 3.61-3.56 (m, 2 H), 3.44-3.42 (m, 2 H), 1.79-1.76 (m, 1 H), 1.71-1.68 (m, 1 H). ^{13}C NMR (125 MHz, *d*-DMSO-d_6, mixture of diastereomers) δ 176.5, 176.4, 163.44, 163.42, 149.8, 149.7, 148.9, 140.7, 140.4, 138.7, 135.3, 135.0, 131.7, 131.2, 130.6, 127.7, 126.0, 125.6, 123.1, 121.34, 121.31, 120.2, 119.9, 52.4, 52.1, 46.9, 45.7, 45.3, 44.9. HRMS, calc'd for $C_{21}H_{17}N_3O_3Cl$ $[M + H]^+$, 394.0958; found 394.0959.

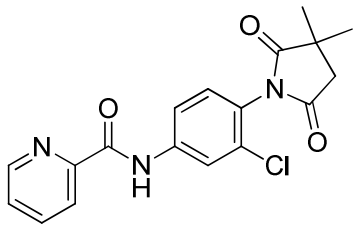


***N*-(3-Chloro-4-((1*R*,2*S*,3*R*,4*S*-bicyclo[2.2.2]oct-5-ene-1,3-dioxo-1*H*-isoindol-1-yl) phenyl)picolinamide (15g).** Following the general procedure, *N*-(3-Chloro-4-((1R,2S,3R,4S-bicyclo[2.2.2]oct-5-ene-1,3-dioxo-1*H*-isoindol-1-yl) phenyl)picolinamide was obtained.

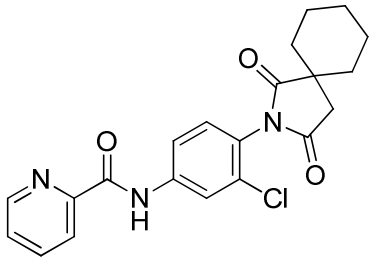
LCMS: R_T = 0.850 min, >98% @ 254 nM, *m/z* = 430.0 $[M + Na]^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.15 (br s, 1 H), 8.64 (d, 1 H, *J* = 4.8 Hz), 8.38 (d, 1 H, *J* = 10.0 Hz), 8.11 (d, 1 H, *J* = 2.0 Hz), 7.95 (ddd, 1 H, *J* = 13.2, 13.2, 1.6 Hz), 7.76 (dd, 1 H, *J* = 8.4, 2.0 Hz), 7.55-7.46 (m, 1 H), 7.21 (d, 1 H, *J* = 12.4 Hz), 6.38-6.30 (m, 1 H), 3.30 (br s, 2 H), 3.03 (s, 2 H), 1.68-1.62 (m, 2 H), 1.49-1.41 (m, 2 H). HRMS, calc'd for $C_{22}H_{19}N_3O_3Cl$ $[M + H]^+$, 408.1115; found 408.1115.



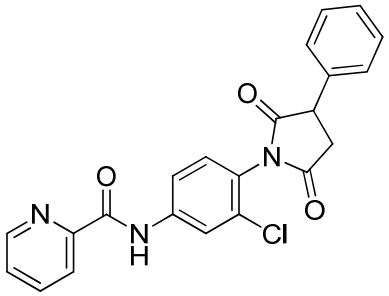
***N*-(3-Chloro-4-((1*R*,5*S*)-6,6-dimethyl-2,4-dioxo-3-azabicyclo[3.1.0]hexan-3-yl)phenyl)picolinamide (15h).** Following the general procedure, *N*-(3-chloro-4-((1*R*,5*S*)-6,6-dimethyl-2,4-dioxo-3-azabicyclo[3.1.0]hexan-3-yl)phenyl)picolinamide was obtained.
LCMS: R_T = 1.32 min., >98% @ 254 nM, *m/z* = 370.2 $[M + H]^+$.



***N*-(3-Chloro-4-(3,3-dimethyl-2,5-dioxopyrrolidin-1-yl)phenyl)picolinamide (15i).** Following the general procedure, *N*-(3-chloro-4-(3,3-dimethyl-2,5-dioxopyrrolidin-1-yl)phenyl)picolinamide was obtained.
LCMS: R_T = 1.35 min., >98% @ 254 nM, *m/z* = 358.1 $[M + H]^+$.



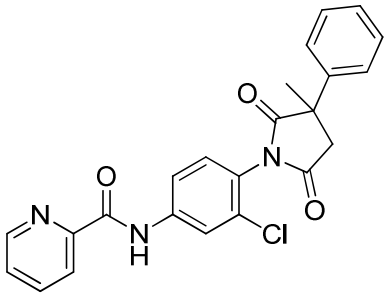
***N*-(3-Chloro-4-(1,3-dioxo-2-azaspiro[4.5]decan-2-yl)phenyl)picolinamide (15j).** Following the general procedure, *N*-(3-chloro-4-(1,3-dioxo-2-azaspiro[4.5]decan-2-yl)phenyl)picolinamide was obtained.
LCMS: R_T = 1.439 min, >98% @ 254 nM, *m/z* = 398.2 $[M + H]^+$. ^{1}H NMR (400 MHz, *d*-DMSO-d_6) δ 11.01 (br s, 1 H), 8.76 (d, 1 H, *J* = 4.4 Hz), 8.24 (d, 1 H, *J* = 2.0 Hz), 8.17 (d, 1 H, *J* = 8.0 Hz), 8.08 (ddd, 1 H, *J* = 8.0, 8.0, 2.0 Hz), 7.99 (dd, 1 H, *J* = 8.4, 2.0 Hz), 7.70 (ddd, 1 H, *J* = 7.6, 4.8, 1.2 Hz), 7.39 (d, 1 H, *J* = 8.4 Hz), 2.86 (d, 1 H, *J* = 18.0 Hz), 2.78 (d, 1 H, *J* = 18.4 Hz), 1.77-1.60 (m, 7 H), 1.46-1.24 (m, 3 H). ^{13}C NMR (125 MHz, *d*-DMSO-d_6) δ 181.6, 175.1, 163.4, 149.7, 148.9, 140.7, 138.7, 131.7, 131.7, 131.1, 127.7, 125.8, 123.1, 121.3, 120.1, 45.3, 39.3, 34.0, 32.5, 25.1, 22.0, 21.9. HRMS, calc'd for $C_{21}H_{21}N_3O_3Cl$ $[M + H]^+$, 398.1271; found 398.1271.



***N*-(3-Chloro-4-(2,5-dioxo-3-phenylpyrrolidin-1-yl)phenyl)picolinamide (15k).** Following the general procedure, *N*-(3-chloro-4-(2,5-dioxo-3-phenylpyrrolidin-1-yl)phenyl)picolinamide was obtained as a mixture of diastereomers (dr 2:1).

Major diastereomer

LCMS: R_T = 1.370 min, >98% @ 254 nM, *m/z* = 406.2 $[M + H]^+$. ^{1}H NMR (400 MHz, *d*-DMSO-d_6) δ 11.03 (br s, 1 H), 8.76 (d, 1 H, *J* = 4.8 Hz), 8.28 (d, 1 H, *J* = 2.4 Hz), 8.17 (d, 1 H, *J* = 8.0 Hz), 8.08 (ddd, 1 H, *J* = 7.6, 7.6, 1.6 Hz), 8.01 (dd, 1 H, *J* = 8.8, 2.4 Hz), 7.72-7.68 (m, 1 H), 7.52-7.30 (m, 6 H), 4.36 (dd, 1 H, *J* = 9.6, 5.2 Hz), 3.42 (dd, 1 H, *J* = 18.4, 9.6 Hz), 2.97 (dd, 1 H, *J* = 18.4, 5.2 Hz). ^{13}C NMR (125 MHz, *d*-DMSO-d_6, mixture of diasteromers) δ 177.1, 175.4, 175.1, 163.3, 149.5, 148.7, 140.8, 140.7, 138.9, 138.5, 137.9, 131.8, 131.7, 131.2, 130.9, 129.3, 129.1, 128.7, 128.4, 127.9, 127.7, 126.0, 125.9, 123.2, 121.4, 121.3, 120.24, 120.15, 46.5, 46.2, 37.7, 37.5. HRMS, calc'd for $C_{22}H_{17}N_3O_3Cl$ $[M + H]^+$, 406.0958; found 406.0958.



***N*-(3-Chloro-4-(3-methyl-2,5-dioxo-3-phenylpyrrolidin-1-yl)phenyl)picolinamide (15l).** Following the general procedure, *N*-(3-chloro-4-(3-methyl-2,5-dioxo-3-phenylpyrrolidin-1-yl)phenyl)picolinamide was obtained as a mixture of diastereomers (dr 1:1).

Major diastereomer

LCMS: R_T = 1.425 min, >98% @ 254 nM, *m/z* = 442.0 $[M + Na]^+$. ^{1}H NMR (400 MHz, *d*-DMSO-d_6) δ 11.03 (br s, 1 H), 8.76 (d, 1 H, *J* = 5.6 Hz), 8.26 (d, 1 H, *J* = 2.4 Hz), 8.18-8.16 (m, 1 H), 8.10-8.07 (m, 1 H), 8.00 (dd, 1 H, *J* = 4.4, 2.4 Hz), 7.72-7.69 (m, 1 H), 7.56-7.47 (m, 3 H), 7.41 (dd, 2 H, *J* = 8.0, 7.2 Hz), 7.34-7.29 (m, 1 H), 3.26 (d, 1 H, *J* = 18.4 Hz), 3.19 (d, 1 H, *J* = 18.4 Hz), 1.77 (s, 3 H). HRMS, calc'd for $C_{23}H_{19}N_3O_3Cl$ $[M + H]^+$, 420.1115; found 420.1115.

In Vitro Pharmacology:

Cell culture. Human $mGlu_4$ ($hmGlu_4$)/CHO cells stably transfected with the chimeric G protein Gqi5 in pIRESneo3 (Invitrogen, Carlsbad, CA) were cultured in 90% Dulbecco's Modified Eagle Media (DMEM), 10% dialyzed fetal bovine serum (FBS), 100 units/ml penicillin/streptomycin, 20 mM HEPES (pH 7.3), 1 mM sodium pyruvate, 2 mM glutamine, 400 μg/ml G418 sufate (Mediatech, Inc., Herndon, VA) and 5 nM methotrexate (Calbiochem, EMD Chemicals, Gibbstown, NJ). Human Embryonic Kidney (HEK-293) cell lines co-expressing rat mGluR 2, 3, 4, 7 or 8 and GIRK potassium channels were grown in growth medium containing 45% DMEM, 45% F-12, 10% FBS, 20 mM HEPES, 2 mM L-glutamine, antibiotic/antimycotic non-essential amino acids, 700 μg/ml G418, and 0.6 μg/ml puromycin. All cells were maintained at 37ºC in the presence of 5% CO2. Rat mGluR1 and 5 cells were cultured as described in Hempstapat et al., 2007. All cell culture reagents were purchased from Invitrogen Corp. (Carlsbad, CA) unless otherwise noted.

Calcium mobilization assays. Calcium assays were used to assess activity of compounds at mGluRs 1, 4, and 5. Assays were performed within Vanderbilt University's High-Throughput Screening Center and the primary mGluR4 HTS has been described in detail. Human mGluR4/Gqi5/CHO cells (30,000 cells/20 μl/well) were plated in blackwalled, clear-bottomed, TC treated, 384 well plates (Greiner Bio-One, Monroe, North Carolina) in DMEM containing 10% dialyzed FBS, 20 mM HEPES, and 1 mM sodium pyruvate (Plating Medium). The cells were grown overnight at 37 °C in the presence of 5% CO_2. The following day, the medium was replaced with 20 μL of 1 μM Fluo-4, AM dye (Invitrogen, Carlsbad, CA); the dye was prepared by mixing a 2.3 mM stock in DMSO in a 1:1 ratio with 10% (w/v) pluronic acid F-127 and then diluting in Assay Buffer (Hank's balanced salt solution, 20 mM HEPES and 2.5 mM Probenecid (Sigma-Aldrich, St. Louis, MO)). Cells were dye loaded for 45 minutes at 37 °C. Dye was removed and replaced with 20 μL of Assay Buffer; the cells were equilibrated with Assay Buffer for 10 minutes at room temperature prior to the start of the assay. In addition, on the day of assay, test compounds were transferred to daughter plates using an Echo acoustic plate reformatter (Labcyte, Sunnyvale, CA) and then diluted into Assay Buffer. Ca2+ flux was measured using the Functional Drug Screening System 7000 (FDSS7000, Hamamatsu, Japan). Baseline readings were taken (10 images at 1 Hz, excitation, 470 ± 20 nm, emission, 540 ± 30 nm) and then 20 μl/well test compounds at 2X their final concentration were added using the FDSS's integrated pipettor. Cells were incubated with compounds for approximately 2.5 minutes and then an EC_{20} concentration of glutamate was applied; 2 minutes later an EC_{80} concentration of glutamate was added. Data was collected at ½ Hz until 10 seconds prior to agonist addition, when the rate was increased to 1 Hz for the remainder of the assay. For concentration-response curve experiments, compounds were serially diluted 1:3 into 10 point concentration response curves and were transferred to daughter plates using the Echo. Test compounds were applied and followed by EC_{20} and EC_{80} concentrations of glutamate as described above. For fold shift experiments, compounds were added at 2X their final concentration and then increasing concentrations of glutamate were added in the presence of vehicle or the appropriate concentration of test compound. Curves were fitted using a four point logistical equation using Microsoft XLfit (IDBS, Bridgewater, NJ). Subsequent confirmations of concentration response parameters were performed using independent serial dilutions of source compounds and data from multiple days experiments were integrated and fit using a four point logistical equation in GraphPad Prism (GraphPad Software, Inc., La Jolla, CA).

Thallium flux assays. Compound activity at the rat group II and group III mGluRs was assessed using thallium flux through GIRK channels, a method that has been described in detail.3 Briefly, cells were plated into 384 well, black-walled, clear-bottom poly-D-lysine coated plates at a

density of 15,000 cells/20 μl/well in Plating Medium and incubated overnight at 37°C in the presence of 5% CO2. The following day, the medium from the cells and 20 μl/well of 1.7 μM FlouZin-2 dye (Invitrogen, Carlsbad, CA); the dye was prepared by mixing a mM stock in DMSO in a 1:1 ratio with 10% (w/v) pluronic acid F-127 and then diluting in Assay Buffer. Cells were incubated for 1 h at room temperature and the dye was replaced with 20 μl/well of Assay Buffer. For these assays, compounds were added at 2x final concentration and then 2.5 min later the appropriate concentration of agonist was added using the FDSS 7000. Agonists were diluted in thallium buffer (125 mM sodium bicarbonate, 1 mM magnesium sulfate, 1.8 mM calcium sulfate, 5 mM glucose, 12 mM thallium sulfate, 10 mM HEPES) at 5x the final concentration to be assayed. Five frames of data were collected (excitation, 470 ± 20 nm emission, 540 ± 30 nm) at ½ Hz prior to compound addition. Data collection continued at ½ Hz until 10 seconds prior to agonist addition, when the rate was increased to 1 Hz for 2 min after agonist addition. Data were analyzed as described. For the concentration-response curve experiments, compounds were serially diluted 1:3 into 10 point concentration response curves and were transferred to daughter plates using the Echo. Test compounds were applied at 2X the final concentrations and followed by EC_{20} concentrations of glutamate 2.5 minutes later. For fold shift experiments, compounds were added at 2X their final concentration and then increasing concentrations of glutamate were added in the presence of vehicle or the appropriate concentration of test compound. For selectivity experiments, full concentration-response curves of glutamate or L-AP4 (for mGluR7) were performed in the presence of a 30 μM concentration of compound, and compounds that affected the concentration-response by less than 2 fold in terms of potency or efficacy were designated as inactive. For mGluR5, full concentration-response curves were performed for selected compounds in the presence of either appropriate EC_{20} or EC_{80} concentrations of glutamate.

Selectivity Studies for 15f:

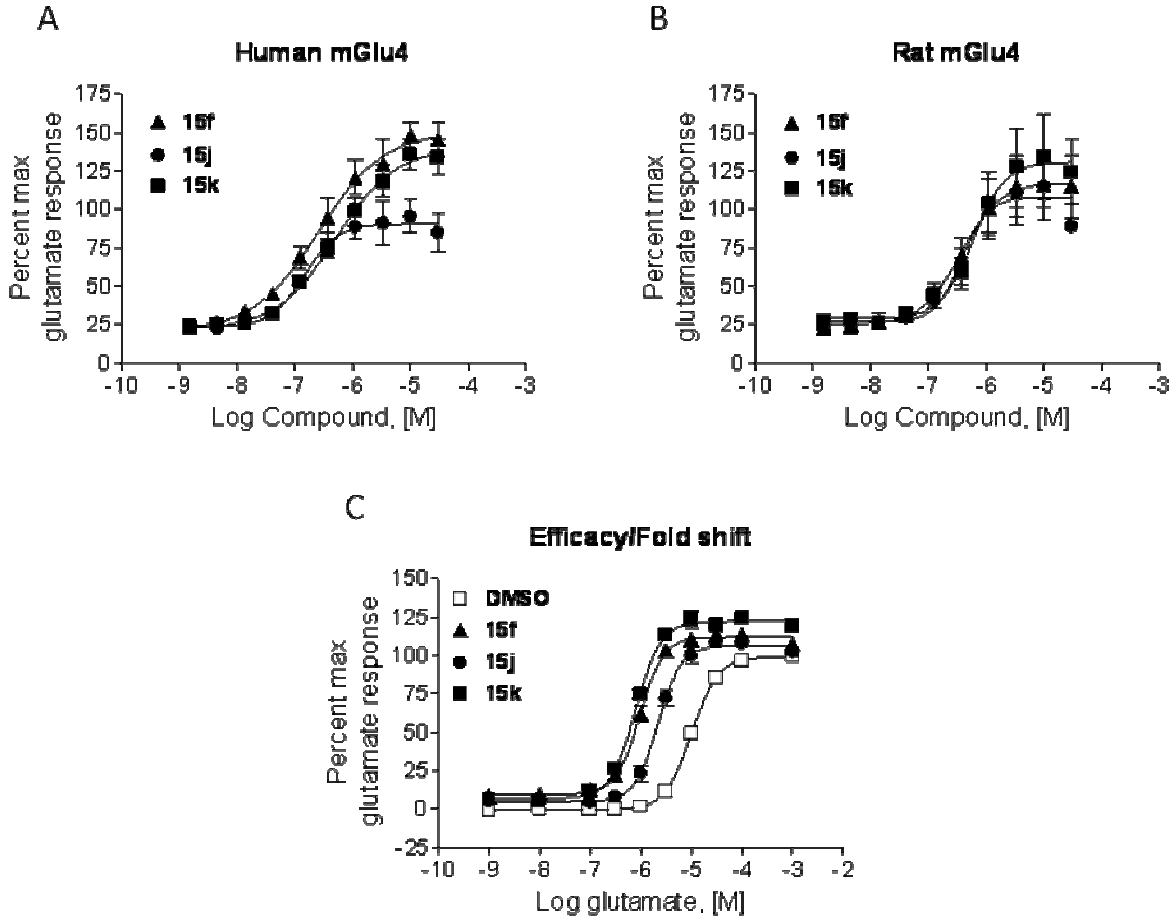
Rat $mGlu_1$ and $mGlu_5$. HEK cells expressing rat $mGlu_1$ or rat $mGlu_5$ were used for selectivity screening and were plated at a density of 15,000 cells/20 µl/well in Assay Media (DMEM containing 10% dialyzed FBS, 20 mM HEPES, and 100 units/mL penicillin/streptomycin). Effects of VU0400195 on rat $mGlu_1$ and $mGlu_5$ were assessed using calcium mobilization and by measuring the glutamate concentration-response relationship in the presence and absence of 10 µM VU0400195. Using a double-addition protocol, VU0400195 was added to the cells, followed 2.5 minutes later by a full concentration-response of glutamate. Shifts of the concentration-response relationship were used to assess potential potentiator (left shift of more than 2 fold) or antagonist (right shift of more than 2 fold or depression of the max response by at least 75%) activity of VU0400195. In these studies, VU0400195 was shown to weakly potentiate mGlu5 responses. Compounds were further assessed for potency at the group I mGlus activity by performing a full concentration-response curve of VU0400195, starting at 30 µM and serially diluted using 1:3 dilutions, in the presence of an EC_{20} concentration of glutamate.

Rat $mGlu_2$, $mGlu_3$, $mGlu_7$, $mGlu_8$ and human $mGlu_6$. Compound activity at the rat group II and III mGlus, was assessed using thallium flux through G-protein-coupled inwardly-rectifying potassium (GIRK) channels as described above for $mGlu_4$ "fold shift" experiments. Cells were plated at a density of 15,000 cells/20 µl/well in Assay Media. Effects of VU0400195 were assessed by measuring the glutamate concentration-response relationship in the presence and absence of 10 µM VU0400195. Using a double-addition protocol, VU0400195 was added to the cells, followed 2.5 minutes later by a full concentration-response of glutamate or, in the case of $mGlu_7$, L-AP4. As above, shifts of the concentration-response relationship were used to assess potential potentiator (left shift of more than 2 fold) or antagonist (right shift of more than 2 fold or depression of the max response by at least 75%) activity of VU0400195. In these studies, VU0400195 showed weak PAM activity at mGlus 6 and 7. Compounds were further assessed for potency at the group II and III mGlus activity by performing a full concentration-response curve of VU0400195, starting at 30 µM and serially diluted using 1:3 dilutions, in the presence of an EC_{20} concentration of glutamate.

Supplemental Table 1. Selectivity profile of **15f** across the mGlu subtypes

Receptor	**mGlu1**	**mGlu2**	**mGlu3**	**mGlu4**	**mGlu5**	**mGlu6**	**mGlu7**	**mGlu8**
Category	Inactive	Inactive	Inactive	PAM	PAM	PAM	PAM	Inactive
EC50 (μM) ± SEM	N/A	N/A	N/A	.33 ± 0.05	1.7 ± 0.07	>10	2.9 ± 0.6	N/A
% Glu Max at 30 μM	4.4 ± 0.4*	29.1 ± 1.2	30.5 ± 2.3	103.1 ± 2.9	64.3 ± 0.8	45.8 ± 3.0	64.0 ± 1.4	34.6 ± 0.8

15f, **15j** and **15k** were initially assessed for their ability to shift a glutamate concentration-response curve (CRC) (Table 7). This strategy allows the determination of PAM (left shift of the glutamate CRC) or antagonist (right shift or decrease in the maximal response) activity of the compound at a 10 μM concentration. In these studies, **15f** showed weak PAM activity at mGlu5, mGlu6, and mGlu7. **15f** was then examined for concentration-dependent activity using a 10 point CRC of compound, starting at 30 μM and serially diluting by 3, pre-applied prior to an EC_{20} concentration of glutamate. For all compounds, the % Glu max at 30 μM is reported and, for active compounds, the potency as a PAM is reported as well. *Compound **15f** exhibited weak inhibition of an EC_{20} concentration of glutamate and was further examined for antagonist activity using an EC_{80} glutamate concentration. In these antagonist experiments, **15f** was determined to be inactive (EC_{80} response in the presence of 30 μM **15f** was 80.6±2.3%). Data represent the mean ± SEM of three experiments performed in singlicate.



Supplemental Figure 2. ***N*-4-(2,5-dioxopyrrolidin-1-yl)-phenylpicolinamide $mGlu_4$ positive allosteric modulators are potent at both human and rat mGlu4 in vitro and exhibit robust efficacy in shifting a glutamate concentration-response curve to the left.** A. CHO cells expressing the human mGlu4 receptor wand the chimeric G protein G_{qi5} were incubated with increasing concentrations of PAMs and an EC_{20} concentration of glutamate and calcium responses were measured. The potencies of these PAMs were: **15f**, 290 ± 55 nM; **15j**, 158 ± 20 nM; **15k**, 488 ± 35 nM. B. HEK cells expressing the rat mGlu4 receptor and G protein inwardly rectifying potassium channels were incubated with increasing concentrations of PAMs and an EC_{20} concentration of glutamate and thallium flux responses were measured. The potencies of these PAMs were: **15f**, 376 ± 23 nM; **15j**, 398 ± 38 nM; **15k**,VU0400071, 590 ± 15 nM. C. A 30 μM concentration of each PAM was incubated with increasing concentrations of glutamate and the leftward shift of the glutamate concentration-response curve was calculated: **15f**, 11.2 ± 0.8 nM fold; **15j**, 4.8±0.2 fold; **15k**, 12.1 ± 1.0 fold.

In vitro PK Analysis:

Microsomal stability: The metabolic stability of each compound was investigated in rat hepatic microsomes (BD Biosciences, Billerica, MA) using substrate depletion methodology (% parent compound remaining). In separate 96-well plates for each time point, a mixture of 0.1M potassium phosphate-buffered (pH 7.4), 1µM test compound, 0.5 mg/mL microsomes, and 1mM NADPH (t=3, 7, 15, 25, or 45min) or buffer (t=0) were continually incubated at 37°C under ambient oxygenation. At the respective time, each plate's reaction was precipitated by the addition of 2 volumes of ice-cold acetonitrile containing glyburide as an internal standard (50 ng/mL). The plates were centrifuged at 3000 rpm (4°C) for 10 min. The resulting supernatants were transferred and diluted 1:1 (supernatant: water) into new 96-well plates in preparation for LC/MS/MS analysis. Each compound was assayed in triplicate within the same 96-well plate. The in vitro half-life ($t_{1/2}$, min, Eq. 1), intrinsic clearance (*CLint*, mL/min/kg, Eq. 2) and subsequent predicted hepatic clearance (*CLhep*, mL/min/kg, Eq. 3) was determined employing the following equations:

1) $t_{1/2}$ = Ln(2) / k ; where k represents the slope from linear regression analysis (% test compound remaining)

2) *CLint* = (0.693 / $t_{1/2}$) (rxn volume / mg of microsomes) (45 mg microsomes / gram of liver) (20[a] gm of liver / kg body weight); [a]scale-up factors of 20 (human) and 45 (rat)

3) $$CLhep = \frac{Q \cdot CL\text{int}}{Q + CL\text{int}}$$

Plasma Protein Binding. The protein binding of each compound was determined in rat plasma via equilibrium dialysis employing Single-Use RED Plates with inserts (ThermoFisher Scientific, Rochester, NY). Plasma (220 µL) was added to the 96 well plate containing test compound (5 µL) and mixed thoroughly. Subsequently, 200 µL of the plasma-compound mixture was transferred to the *cis* chamber (red) of the RED plate, with an accompanying 350 µL of phosphate buffer (25 mM, pH 7.4) in the *trans* chamber. The RED plate was sealed and incubated for 4 hours at 37°C with shaking. At completion, 50 µL aliquots from each chamber were diluted 1:1 (50 µL) with either plasma (*cis*) or buffer (*trans*) and transferred to a new 96 well plate, at which time ice-cold acetonitrile (2 volumes) was added to extract the matrices. The plate was centrifuged (3000 rpm, 10 min) and supernatants transferred and diluted 1:1 (supernatant: water) into a new 96 well plate, which was then sealed in preparation for LC/MS/MS analysis. Each compound was assayed in triplicate within the same 96-well plate.

Microsomal stability and plasma protein binding samples were analyzed on a Thermo Electron TSQ Quantum Ultra triple quad detector via electrospray ionization (ESI) with two Themo Electron Accella pumps (San Jose, CA), and a Leap Technologies CTC PAL autosampler. Analytes were separated by gradient elution on a dual column system with two Waters Acquity BEH C18, 2.1x50mm, 1.7µm columns (Milford, MA) heated at 50°C. HPLC mobile phase A was 95:5:0.1 Water:Acetonitrile:Formic Acid, while mobile phase B was 95:5:0.1 Acetonitrile:Water:Formic Acid. Pump 1 runs the gradient: 95:5 (A:B) at 800µL/min hold 0 to 0.5min, linear ramp to 5:95 (A:B) 0.5 to 1.0min, 5:95 (A:B) hold 1.0 to 1.9min, return to 95:5 (A:B) at 1.9 min. While pump 1 runs the gradient method, pump 2 equilibrates the other column

isocratically with 95:5 (A:B). The total run time is 2.0 minutes per injection. All compounds are optimized using Thermo Electron's QuickQuan software.

Cytochrome P450 inhibition: A four-in-one, 96-well plate assay for determining IC_{50} values against human P450s 1A2, 2C9, 2D6 and 3A4 was developed based on previous reports (1,2). Human liver microsomes (final concentration of 0.1 mg/mL) and a substrate mixture containing the P450 probe substrates phenacetin (10μM), diclofenac (5μM), dextromethorphan (5μM) and midazolam (2μM) were added to a potassium phosphate buffered solution (0.1M, pH 7.4) and warmed to 37°C. The reaction mixture was divided evenly into the 96-well plate and various dilutions of each inhibitor/compound of interest (in duplicate) were then added to this reaction mixture such that the final concentration of each compound ranged from 100nM to 30μM. This mixture was allowed to pre-incubate for 15 minutes while shaking at 37°C. Buffer or NADPH (1mM) was added and the reaction mixture was incubated for an additional 8 minutes at 37°C prior to quenching with 2 volumes of ice-cold acetonitrile containing 50ng/mL of carbamazepine as internal standard. The plates were centrifuged at 4000 rpm (4°C) for 10 minutes and the supernatant was removed and diluted with water (1:4, v/v) in preparation for LC/MS/MS analysis. The IC_{50} values for each compound were obtained for the individual P450 enzymes by quantitating the inhibition of metabolite formation for each probe; acetaminophen (1A2), 4-hydroxydiclofenac (2C9), dextrorphan tartrate (2D6) and 1-hydroxymidazolam (3A4). Miconazole was included as a positive control broad spectrum P450 inhibiton (REF). For discrete 2C19 inhibition experiments, a similar assay design was employed with the following exceptions: the probe substrate was S-mephenytoin (40μM), the NADPH incubation with the reaction mixture went for 30 minutes, the supernatant was reconstituted 1:1 with water for analysis, and the metabolite used for quantitation was 4-hydroxymephenytoin.

Cytochrome P450 inhibition samples were analyzed via electrospray ionization (ESI) on an AB Sciex API-4000 (Foster City, CA) triple-quadrupole instrument that was coupled with Shimadzu LC-10AD pumps (Columbia, MD) and a Leap Technologies CTC PAL auto-sampler (Carrboro, NC). Analytes were separated by gradient elution using a Fortis C18 2.1 x 50 mm, 3 μm column (Fortis Technologies Ltd, Cheshire, UK) thermostated at 40°C. HPLC mobile phase A was 0.1% formic acid in water (pH unadjusted), mobile phase B was 0.1% formic acid in acetonitrile (pH unadjusted). The gradient started at 10% B after a 0.2 min hold and was linearly increased to 90% B over 1.3 min; returned to 10% B in 0.1 min followed by a re-equilibration (0.9 min). The total run time was 2.5 min and the HPLC flow rate was 0.5 mL/min. The source temperature was set at 500°C and mass spectral analyses were performed using multiple reaction monitoring (MRM), with transitions specific for each compound utilizing a Turbo-Ionspray® source in positive ionization mode (5.0 kV spray voltage). All data were analyzed using AB Sciex Analyst 1.5.1 software.

In vivo PK Analysis:

In vivo pharmacokinetics: Male Sprague-Dawley rats (n=2) weighing around 250-300g were purchased from Harlon laboratories (Indianapolis, IN) and implanted with catheters in the carotid artery and jugular vein. The cannulated animals were acclimated to their surroundings for approximately one week before dosing and provided food and water *ad libitum*. Parenteral administration of compounds to rats was achieved via a jugular vein catheter at a dose of 1 mg/kg (20% DMSO/80% saline) and a dose volume of 1 mL/kg. Blood collections via the carotid artery were performed at pre-dose, and at 2 min, 7 min, 15 min, 30 min, and 1, 2, 4, 7 and 24 hrs post dose. Samples were collected into chilled, EDTA-fortified tubes, centrifuged for 10 minutes at 3000 rpm (4°C), and resulting plasma aliquoted into 96-well plates for LC/MS/MS analysis. Pharmacokinetic parameters were obtained from noncompartmental analysis (WinNonLin, V5.3, Pharsight Corp., Mountain View, CA) of individual concentration-time profiles following the parenteral administration of a test article. For oral exposure studies, measuring both systemic plasma and CNS tissue exposure, compounds were administered (oral gavage) to fasted rats (n=2) as suspensions in 10% tween 80/0.5% methylcellulose at a dose of 10 mg/kg and in a dosing volume of 10 mL/kg; blood and whole brain samples were collected at 1.5 hours post-dose. Whole blood was collected into chilled, EDTA-fortified tubes, centrifuged for 10 minutes at 3000 rpm (4°C) and stored at -80°C until LC/MS/MS analysis. The brain samples were rinsed in PBS, snap frozen and stored at -80°C. Prior to LC/MS/MS analysis, brain samples were thawed to room temperature and subjected to mechanical homogenation employing a Mini-Beadbeater™ and 1.0 mm Zirconia/Silica Beads (BioSpec Products). All animal studies were approved by the Vanderbilt University Medical Center Institutional Animal Care and Use Committee. The animal care and use program is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

Three adult female rhesus monkeys (5-7 years old; 8-11 Kg) from the Yerkes National Primate Center breeding colony were used for blood collections in rhesus. In brief, animals were tranquilized with Ketamine (10 mg/kg, im) and Telazol (3 mg/kg, im), brought to an examination room, and received intravenous injections of compounds at a final concentration of 1 mg/kg. Blood collections (1 ml/time point) were then performed at the following time points post-injection: 5, 10, 20, 60, 120, 180 minutes, 6 hours, 8 hours, and 24 hours. Blood samples were then stored and processed the same way as those collected in rats.

Liquid chromatography/mass spectrometry analysis. In vivo samples were analyzed via electrospray ionization (ESI) on an AB Sciex API-4000 (Foster City, CA) triple-quadrupole instrument that was coupled with Shimadzu LC-10AD pumps (Columbia, MD) and a Leap Technologies CTC PAL auto-sampler (Carrboro, NC). Analytes were separated by gradient elution using a Fortis C18 2.1 x 50 mm, 3 µm column (Fortis Technologies Ltd, Cheshire, UK) thermostated at 40°C. HPLC mobile phase A was 0.1% formic acid in water (pH unadjusted), mobile phase B was 0.1% formic acid in acetonitrile (pH unadjusted). The gradient started at 10% B after a 0.2 min hold and was linearly increased to 90% B over 0.8 min; held at 90% B for 0.5 min and returned to 30% B in 0.1 min followed by a re-equilibration (0.9 min). The total run time was 2.5 min and the HPLC flow rate was 0.5 mL/min. The source temperature was set at 500°C and mass spectral analyses were performed using multiple reaction monitoring (MRM), with transitions specific for each compound utilizing a Turbo-Ionspray® source in positive ionization mode (5.0 kV spray voltage) (see Table 1). All data were analyzed using AB Sciex Analyst 1.5.1 software.

Table 1: Mass Spectrometer MRM Conditions

Compound	Q1 (m/z)	Q3 (m/z)	DP (volts)	EP (volts)	CE (volts)	CXP (volts)
15k	376.3	165.1	90	10	35	10

15j	398.3	106.0	90	10	33	5
15f	394.2	328.2	60	10	25	10

Catalepsy Methods:

Animals. Male Sprague-Dawley rats weighing between 275 and 300 g (Harlan Laboratories, Indianapolis, IN) were used for the behavioral studies and were maintained in accordance with American Association for the Accreditation of Laboratory Animal Care guidelines under a 12-h light/dark cycle (lights on at 6 AM, lights off at 6 PM) with free access to food and water. The experimental procedures, which were performed during the light cycle, were approved by the Institutional Animal Care and Use Committee of Vanderbilt University and conformed to the guidelines established by the National Research Council Guide for the Care and Use of Laboratory Animals.

Rats were injected with haloperidol (0.75mg/kg, i.p., dissolved in 8.5% lactic acid, pH = 6) 60 minutes prior to the oral administration of **15f** (VU0195 (0.1-56.6mg/kg) or vehicle (10% Tween 80). 30 minutes after compound administration all rats were tested for catalepsy. Catalepsy was assessed using a horizontal bar placed 6 cm from the testing surface. The forepaws of each rat were placed gently on the bar with the body positioned at an angle of ~45° to the testing surface. The latency in seconds required for the rat to remove one or both forepaws from the bar was manually measured with a cutoff of 30 seconds.