Supporting Information

Evaluation of 5-(Trifluoromethyl)-1,2,4-oxadiazole-Based Class IIa HDAC Inhibitors for Huntington's Disease

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[§]The authors dedicate this manuscript to the memory of Dr. Alexander H. Borchers who passed away on August 3rd, 2019.

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1) In vitro assay descriptions

a) Biological assays

i) Biochemical and cellular potency

To assess the biochemical and cellular potency, we utilized a two-step fluorogenic assay which measures HDAC-mediated catalytic conversion of synthetic acetylated lysine substrates. Detailed biological assay methods have previously been reported by our group [1, 2], and assay-substrate combinations for biochemical and cellular assays are shown in **SI Table 4**. In addition to the methods previously outlined, biochemical assays for HDAC6 were performed using an overexpressed lysate, due to the instability of purified HDAC6. Briefly, HDAC6-Halotag expressing plasmid (Promega; 50 µg) was mixed with a three-fold excess of Lipofectamine LTX (Invitrogen) and an equivalent amount of PLUS reagent (Invitrogen) for 20 min before transfection into HEK-Freestyle cells (50 x 10^6; Invitrogen). Twenty-four h post-transfection, cells were lysed using NP40 lysis buffer (25 mM Tris-HCL pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP40, 10% glycerol containing HALT protease/phosphatase inhibitors) and normalized to 5 mg/mL using assay buffer (50 mM Tris-HCl, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, pH 8.0). Assays were performed as HDAC4 biochemical assays previously described, with 50 µg/well lysate replacing the purified HDAC4 enzyme. Activity was normalized to vehicle and inhibition mediated by a selective HDAC6 inhibitor.

In addition to the use of Jurkat E6.1 cells, class I and Class IIb cellular assays were performed using HEK Freestyle cells (Invitrogen). Cells were plated at 40,000 cells/well in a tissue-culture treated 384-well V-bottom plate and treated with compounds for 4 h at 37°C, 5% CO_2. Following treatment, cells were lysed (0.8 % Tween 20, 0.2% SDS, 4 mM EDTA, 4 mM PMSF, HALT protease/phosphatase inhibitors), acidified by addition of HCl to a final concentration of 0.04 M and incubated overnight at 4°C. Following incubation, 1 µl/well was transferred onto a nitrocellulose membrane, dried at RT, and blocked for 1 h at RT in LI-COR Odyssey blocking buffer. Paired primary antibodies (Anti-acetyl-Histone H4 (Lys12) Antibody, Millipore (04-119) and Anti-Histone H4 antibody, Abcam (ab17036) or Acetylated α Tubulin (6-11B-1), Santa Cruz (sc-2390) and α-Tubulin (11H10), Cell Signalling Technology (2125) were incubated at recommended concentrations for 2 h at RT followed by washing in T-TBS and incubation with the appropriate secondary antibodies (LI-COR) for 2 h at RT before washing and detection on a LI-COR Odyssey imager. Activity was normalized to the activity of known selective pharmacological inhibitors.

ii) Binding of compounds to histone deacetylase 4 (HDAC4) catalytic domain using surface plasmon resonance (SPR) technology

This assay was carried out with a Biacore T200 platform. HDAC4 catalytic domain, which was produced in-house, was immobilized by direct amine coupling on the sensor surface. Compounds were passed over the immobilized HDAC4 surface and changes in refractive index caused by binding were measured. Each compound was tested in a 10-point concentration-response curve on three separate days including a known compound as a reference standard (**SI Table 1**). Steady state affinity and kinetic data were analyzed using Biacore T200 software with a 1:1 binding model. The quality of kinetic fits was assessed from statistical parameters (Chi squared, SE and plots of residuals).

Biacore T200 affinity run programming

The Biacore T200 was programmed for the affinity titration run with the appropriate compounds, molecular weight and concentrations using the following parameters, all with a flow rate of 30 μL/min throughout:

1) 10 start-up (running buffer) injections
2) 180 s injections of each compound dilution, from lowest to highest concentration, beginning and ending with zero controls for each compound (columns 1 and 12, respectively).
3) 180 s dissociation time following each injection
4) 30 s regeneration with 1 mM NaOH following dissociation
5) 30 s stabilization period following regeneration
6) Control injections after every 12 cycles (cycle = steps 2 through 5) that comprised reference compound (1 μM) calibrator injection and a blank (run buffer) injection
7) Solvent correction sequence every 48 cycles, including at the beginning and end of the run

Preparation and conditioning of Biacore CM5 sensor chip

With HBS-N as the run buffer, a new CM5 sensor chip was inserted into the Biacore T200 and the instrument was primed. The normalization protocol was run with Bianormalization solution. The chip was then conditioned with the following sequence of injections in a manual run with a flow rate of 100 μL/min across all four flow cells:

1) 2 x 6 second injections of 100 mM HCl
2) 2 x 6 second injections of 50 mM NaOH
3) 2 x 6 second injections of 0.5% SDS

HDAC4 immobilization

An immobilization wizard was employed consisting of the following sequence of steps, using HBS-N as the immobilization run buffer and a flow rate of 10 µL/min. Flow cell 1 (Fc1) was not derivatized but used for reference subtraction. One of the other flow cells (Fc2 to 4) was used for HDAC4 immobilization:

1) To activate the surface, an EDC (N-ethyl-N′-(3-diethylaminopropyl)carbodiimide) solution and NHS (N-hydroxysuccinimide) solution were mixed and injected for 600 s (104 µL each required, each in a separate 7 mm Biacore vial along with an empty vial for on board mixing)
2) 300 s injection of protected HDAC4
3) 420 s injection of ethanolamine to block the surface

A final immobilization level of 8,000 to 13,000 RU of HDAC4 was expected from this procedure.

Zn conditioning

Immediately following HDAC4 immobilization, the buffer was changed to HDAC4 run buffer and the instrument was re-primed. The HDAC4 surface was conditioned with an injection of 1 mM $ZnCl_2$ in HDAC4 run buffer for 5 min in a manual run with a flow rate of 5 µL/min, followed by equilibration for an additional 5 min.

iii) Cerep off-target liability assessment

Radioligand displacement assays of compound **12** (10 µM) was conducted against a diverse panel of CNS and other peripheral targets (receptors, ion channels, enzyme and transporters) to assess off-target liability (Diversity panel, Item# P9, Cerep, France, now available through Eurofins Discovery Services). Experimental conditions for each assay can be found on the provider webpage.
https://www.eurofinsdiscoveryservices.com/catalogmanagement/viewitem/Diversity-Panel/P9

iv) Dot Blots

Cell lysate or tissue was homogenized by mechanical disruption in lysis buffer (0.8 % Tween 20, 0.2% SDS, 4 mM EDTA, 4 mM PMSF, HALT protease/phosphatase inhibitors), acidified by addition of HCl to a final concentration of 0.04 M (where required for histone extraction) and incubated overnight at 4°C. Following incubation, 1 µL/well was transferred onto a nitrocellulose membrane, dried at RT, and blocked for 1 h at RT in LI-COR Odyssey blocking buffer. Paired primary antibodies (Anti-acetyl-Histone H4 (Lys12) Antibody, Millipore (04-119) and Anti-Histone H4 antibody, Abcam (ab17036) or Acetylated α Tubulin (6-11B-1), Santa Cruz (sc-2390) and α-Tubulin (11H10), Cell Signalling Technology (2125) were incubated at recommended concentrations for 2 h at RT followed by washing in T-TBS and incubation with

the appropriate secondary antibodies (LI-COR) for 2 h at RT before washing and detection on a LI-COR Odyssey imager.

v) Western Blots

Tissue or cellular samples were lysed and treated as per dot blots. Ten micrograms of protein per sample was loaded following denaturation in LDS loading buffer (Invitrogen; 95°C, 10 min), separation via electrophoresis 4-12% Bis-Tris gels at 200V for 35 min using MES running buffer, before blocking in 5% BSA in TSB-T for 1 h at RT, washed, incubated with primary antibody (diluted in TBS-T) at 4°C overnight, before being washed and incubated with appropriate secondary antibody at RT for 1 h before washing and detection on a LI-COR Odyssey imager.

b) ADME assays

For full details on mouse liver microsomal stability, permeability and brain tissue binding assays see previous publication [2].

i) Cytochrome P450 inhibition

CYP450 inhibition incubations were performed as described in Discovery UK Standard Operating Procedure, ADME-SOP-66 (version 3). The test compound was prepared in 0.1 M Tris buffer, pH 7.4 to a final top concentration of 50 µM. Test and control compounds were serially diluted (1 in 3) and incubated at six concentrations (0.3% DMSO final) at 37°C, with pooled human liver microsomes, in the presence of NADPH (1 mM final concentration). Conditions of incubation were optimised to maintain first order reaction kinetics and to minimise the potential for non-specific binding of the seven probe or study compounds.

At the specified times, shown below, reactions were terminated with acetonitrile containing an analytical internal standard, samples centrifuged and the supernatant fractions analysed for probe substrate metabolites by mass spectrometry (LC-MS/MS). The instrument responses were normalised to internal standard and compared to the appropriate solvent controls to determine the amount of metabolite formed from the probe substrates relative to these “uninhibited” controls.

The CYP450 isoforms studied, and their respective probe substrates and incubation conditions, are shown below.

CYP450 isoform	Microsome conc. (mg/mL)	Substrate	Substrate conc. (µM)	Metabolite	Incubation time (min)
CYP1A2	1	Phenacetin	100	Acetaminophen	30
CYP2C8	0.25	Amodiaquine	10	Desethylamodiaquine	15
CYP2C9	0.25	Diclofenac	15	1'-OH-diclofenac	15
CYP2C19	1	(*S*)-(+)-Mephenytoin	100	4'-OH-mephenytoin	30
CYP2D6	0.25	Dextromethorphan	25	Dextrorphan	15
CYP3A4	0.25	Midazolam	7	1'-OH-midazolam	15
CYP3A4	0.5	Testosterone	100	6'-OH-testosterone	30

CYP3A4 is known for atypical inhibition kinetics where the extent of inhibition observed depends on the probe substrate used, therefore two substrates are routinely tested for this isoform. Variation in probe turnover between plate wells means that inhibition values recorded up to 20% may not be significant.

Reference competitive inhibitors and control (non-inhibitor) compounds were included with each CYP450 isoform assayed to demonstrate the potential for observing specific and potent interactions under the conditions used. The reference and control inhibitor conditions for each isoform are shown below:

CYP450 isoform	Reference (inhibitor)	Concentration (µM)	Control (non-inhibitor)	Concentration (µM)
CYP1A2	2-Aminofluorine	50	Sulfaphenazole	100
CYP2C8	Felodipine	10	Quinidine	30
CYP2C9	Sulfaphenazole	2.5	Furafylline	100
CYP2C19	Tranylcypromine	125	Phenacetin	100
CYP2D6	Quinidine	2.5	Furafylline	100
CYP3A4	Ketoconazole	3	Sulfaphenazole	100

The results are reported as percentage inhibition and IC_{50} values (concentration resulting in a 50% reduction in probe metabolite formation) were calculated using a non-linear sigmoidal dose response equation (IDBS Biobook software):

% inhibition = 100-((metabolite formation at selected inhibitor concentration)/(metabolite formation in the DMSO control))*100

IC_{50} (µM) = Bottom + ((Top-Bottom)/(1+10^((LogIC_{50} - X)*Hill Slope)

ii) Plasma stability

Test and control samples (simvastatin, eucatropine), prepared in DMSO, were incubated at an initial concentration of 5 µM (1% DMSO final, n=2) with C57BL/6 mouse plasma or bovine serum albumin at 37°C. Aliquots were removed at 0, 5, 15, 30 and 60 minutes for termination of reactions and compound extraction with acetonitrile containing an analytical internal standard. Samples were centrifuged and the supernatent fractions analysed for parent compound by mass sprectrometry (LC-MS/MS).

iii) Stability in simulated gastric fluid

Compounds in DMSO stock solution were diluted (n=2) in simulated gastric fluid (stock solution comprising NaCl (1.0 g), pepsin (1.6 g) and HCl (1 N, 3.5 mL) in H_2O (750 mL)) to a concentration of 10 µM (2% DMSO final) and mixed at 37°C on an orbital shaker for 4 h, with aliquots removed at 2 and 4 h. DMSO containing an internal analytical standard was added to the aliquots, vortex-mixed and analyzed immediately by mass spectrometry (LC-MS/MS). Equivalent t=0 samples were also included, with sample preparation staggered to allow sequential injections of timed aliquots and the t=0 samples. The amount of compound remaining was determined from the MS response in each sample relative to that in the t=0 samples (normalised for internal standard). Ln plots of the % remaining were used to determine the half-life of compound dissapearance from the relationship $t_{1/2}$(min) = -0.693/λ, where λ is the slope of the Ln concentration vs time curve.

2) Live animal experiments and statement of approval and guidelines

a) Methods

i) Pharmacokinetic evaluation

The in-life phase was conducted at Xenometrics. Bioanalysis and PK analysis were conducted at Charles River Laboratories.

Administration of Compound 12 in C57/BL6 mice

Concentrations of Compound 12 were determined following a single intravenous bolus (iv, 3.3 mg /kg) or oral bolus gavage (po, 11.5 mg/kg , 10 mg /kg, 30 mg/kg and 100 mg/kg) administration to male C57BI/6J

mice (Hilltop Laboratories (Scottsdale, PA). Animals used in the study were acclimated to the study room environment for 8 days prior to IV dose administration, and 14 days prior to PO dose administration. Mice were individually housed in suspended wire caging, and were kept on a 12/12 hour light/dark cycle (6AM/6PM; lights on) except when interrupted for study procedures. Temperature and humidity in the animal room were regulated and continuously monitored to maintain an average room temperature in the range of 18 to 26°C, an average relative humidity of 30% to 70%, and an average daily airflow of at least 10 fresh air changes per hour. Animals were fed LabDiet™ Certified Rodent Diet 5002 Meal food ad libitum and had access to water *ad libitum*.

Compounds were formulated from solid at a concentration of 0.66 mg /mL in 10:40:50 ratio of DMSO:PEG400 and water (IV administration) or in 10% hydroxypropyl-β-cyclodextrin (1.15, 1, 3 or 10 mg/ml for oral gavage). The dose formulations were made up on the day of the study and kept at room temperature until dosing. Animals were dosed in a non-fasted state. Following IV or po administration (3 mg/kg and 11.5, 10, 30 and 100 mg/kg dose), at the allotted time-point after dosing, mice were bled as a terminal collection via cardiac puncture under Isoflurane inhalation anesthesia. The brain (halved) and muscle samples were then collected immediately. The whole blood samples were collected into Na Heparin tubes and were held on wet ice. Whole blood was diluted 1:1 with purified Aries water within 15 minutes of collection and mixed well. Brain and muscle tissues were rinsed with saline to remove any surface blood, snap frozen in liquid nitrogen, and were stored at -70°C ± 10°C until analysis.

Bioanalysis

Concentrations of compound **12** in mouse blood, brain, and muscle were determined using an LC-MS/MS assay developed at Charles River Laboratories. The lower limits of quantification (LLOQ) for this assay were 5.2 nM in blood, 6.5 nM in brain and 65 nM in muscle. Calibration standards in blood (2.0 – 20,000 ng eq./mL) were prepared by adding compound **12** to control C57BL/6 mouse blood. Calibration standards in brain and muscle (1 – 10,000 ng eq./g) were prepared by adding compound **12** to control C57BL/6 mouse brain or muscle. Reserpine was used as an internal standard in the LC-MS/MS assay.

Pharmacokinetic Analysis

Pharmacokinetic analyses of compound **12** concentrations in blood, brain and muscle (gastrocnemius) were performed using Phoenix® WinNonlin®, version 6.3 (Certera™ Pharsight, L.P., St. Louis, Missouri, USA).

ii) Tolerability studies

The study was conducted at Psychogenics, USA. 16 WT and 16 R62 mixed gender mice of the CBAxC57BL6/J strain background (8 weeks of age at start of dosing) were used in this study. Mice were singly housed and maintained on a 12/12 light/dark cycle. Testing was conducted during the light cycle and all assessments were carried out at consistent times throughout the study. The room temperature was maintained between 20 and 23°C with a relative humidity maintained between 30% and 70%. Chow and water was provided ad libitum.

Compound **12** was formulated in 10% (w/v) hydroxypropyl-β-cyclodextrin (HP-β-CD) in water to a final concentration of 1, 3, & 10 mg/mL giving doses of 10, 30 & 100 mg/kg in a 10 mL/kg dose volume. Compound **12** (10, 30 and 100mg/kg) and its vehicle was administered by oral gavage twice daily at a dose volume of 10 mL/kg.

The animals were evaluated before treatment start in order to detect any abnormalities that would suggest that an animal has to be removed from the experiment (baseline). Twice a week for 2 weeks, animals were observed ~30 min before injection and at 60 and 90 min after the injection. The observations include body temperature (at –30 min, 60 min, and 24 hr after injection), neurological, and motor evaluation as described in **SI Table 9**. A score of 0 indicates normal activity of each specific outcome evaluated while scores of 1 or 2 grade the level of the abnormalities observed, as moderate or severe, respectively. This rating system was used for all the parameters except for locomotor activity, provoked biting, touch escape and struggle by the tail. Body weights were measured on days 1, 3, 5, 8, 10, 12 and 15. These parameters receive either a 0 or 1 rating and were evaluated with notations as to the direction of an observed abnormality. In order to indicate the direction of the change, a letter A or B was added when those parameters were abnormal. 1A indicates an increase in the activity observed while 1B indicates a decrease.

Activity chambers (Med Associates Inc, St Albans, VT; 27 x 27 x 20.3 cm) are equipped with infrared (IR) beams. Mice were placed in the center of the chamber and their behavior recorded for 30 min. Quantitative analysis was performed on the following five dependent measures: total locomotion, locomotion in the center of the open field, rearing rate in the center, total rearing frequency and velocity. Open field testing was conducted once during each week of dosing during the MTD to determine whether compound treatment affects spontaneous locomotor activity. Open field testing was conducted 30 minutes following compound administration.

Passive signs and manipulation responses (**SI Table 9**) were summed over the course of a session and analyzed using the Kruskal-Wallis test for effects of treatment in WT and R62 mice, and the Mann-Whitney

test for effects of genotype. Open field, body temperature and body weight data was analyzed using repeated measures analysis of variance (ANOVA) with treatment and genotype as factors.

b) Statement identifying approving committee and certification that such experiments were performed in accordance with national regulations.

US: Studies were performed in a facility that is registered with the USDA as an animal research facility, fully accredited by AAALAC, and that maintains a PHS Animal Welfare Assurance. All animal experiments were conducted according to the National Institute of Health (NIH) *Guide for the Care and Use of Laboratory Animals (2011)*.

UK: Studies were performed under UK Home Office Guidelines.

3) Additional compound data

a) Compound preparation and characterization

i) General methods

All chemicals were purchased from commercial suppliers and used as received. All reactions involving air- or moisture-sensitive reagents were performed under a nitrogen atmosphere using dried solvents and glassware. Flash chromatography was carried out with prepacked SiO_2 SNAP cartridges (KP-SIL) from Biotage using a Biotage Isolera Four system using gradient elution. Analytical thin-layer chromatography (TLC) was performed on silica using Polygram®SIL G/UV254 with fluorescent indicator (200 µm thickness), and visualized under UV light. NMR spectra were recorded on a Bruker AV 400 (^{1}H = 400.13 MHz, ^{13}C = 100.60 MHz) instrument spectrometer and referenced to tetramethylsilane. The following abbreviations are used: br = broad signal, s = singlet, br s = broad singlet, d = doublet, dd = doublet of doublets, t = triplet, q = quartet, m = multiplet. Carbon spectra were proton decoupled and coupling quoted for coupling to fluorine. Preparative HPLC was performed on a Waters Sunfire OBD C18 10 µm column (150 mm × 19 mm), Phenomenex Luna phenylhexyl 10 µm column (150 mm × 21.2 mm) or a Waters Xbridge phenyl 5 µm column (100 mm × 19 mm), eluting with mixtures of water-acetonitrile or water-

methanol, optionally containing a modifier (0.1% v/v formic acid or 10 mM ammonium bicarbonate). Low-resolution mass spectra were recorded on a Waters ZQ single quadrapole LCMS in $ESCi^+$, $ESCi^-$ mode, or a Quattro Micro LC− MS−MS in $ESCi^+$, $ESCi^-$ mode). High-resolution mass spectra were recorded on a Waters Acquity UPLC system and Waters Xevo G2 TOF mass spectrometer. HPLC purity was assessed using one of three systems (See analytical methods table below). All final compounds were purified to >95% chemical and optical purity (analytical methods detailed in compound experimental). Supercritical Fluid Chromatography (SFC) used a Waters Thar Prep100 preparative SFC system (P200 CO_2 pump, 2545 modifier pump, 2998 UV/VIS detector, 2767 liquid handler with Stacked Injection Module). The Waters 2767 liquid handler acted as both auto-sampler and fraction collector. Chiral HPLC purification used either Diacel Chiralpak IA or IC columns. Each sample was run under both un-modified and basic conditions (5.0 μL injection, 5/95 gradient for 5 minutes) across ethanol, methanol and isopropanol. Compounds were named with the aid of the Cambridgesoft Chemistry Cartridge (v. 9.0.0.182) software. As used herein, the following abbreviations have the following meanings. If an abbreviation is not defined, it has its generally accepted meaning.

Abbreviations

DCM:	Dichloromethane
DIPE:	Diisopropyl ether
DIPEA:	Diisopropylethylamine
DMAP:	4-Dimethylaminopyridine
DMF:	Dimethylformamide
DMSO:	Dimethyl sulfoxide
EDC:	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
ES+:	Electrospray Positive Ionisation
ES-:	Electrospray Negative Ionisation

Et_2O:	Diethyl ether
EtOAc:	Ethyl acetate
h:	Hours
HATU:	(1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate)
HBTU:	*N,N,N',N'*-Tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate
HOPO:	2-Hydroxypyridine-*N*-oxide
HPLC:	High Performance Liquid Chromatography
i-hex:	iso-Hexane
IPA:	*iso*-Propyl alcohol
LCMS:	Liquid Chromatography Mass Spectrometry
M:	Mass
MeCN:	Acetonitrile
MeOH:	Methanol
NMR:	Nuclear Magnetic Resonance
RT:	Retention time
r.t.:	Room temperature
SFC:	Supercritical Fluid Chromatography
SCX:	Strong Cation Exchange
TFA:	Trifluoroacetic acid
TFAA:	Trifluoroacetic anhydride
THF:	Tetrahydrofuran

ii) Analytical Conditions

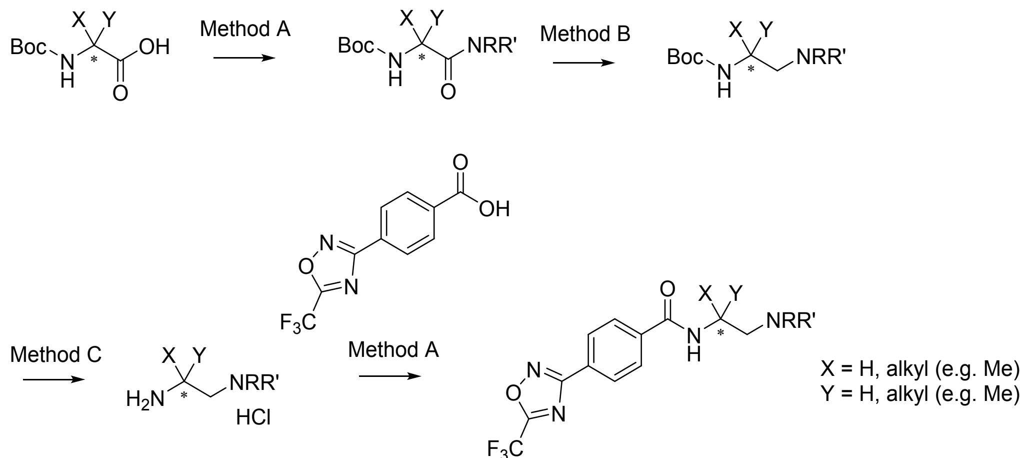
Analytical Method #	Description
Analytical method 1	Solvents: Acetonitrile (far UV grade) with 0.1% (v/v) formic acid. Water (high purity *via* PureLab Option unit) with 0.1% formic acid Column: Phenomenex Luna 5 µm C18 (2),100 x 4.6 mm (Plus guard cartridge) Flow Rate: 2 mL/min gradient: A: Water/formic acid B: MeCN/formic acid Time A% B% 0.00 95 5 3.50 5 95 5.50 5 95 5.60 95 5 6.50 95 5 Typical Injections 2-7 µL (concentration ~ 0.2-1.0 mg/mL)

Analytical method 2	Solvents: - Acetonitrile (Far UV grade) Water (High purity via PureLab Option unit) with 10mM ammonium bicarbonate (ammonium hydrogen carbonate) Column: - Waters Xterra MS 5μ C18, 100 x 4.6 mm (Plus guard cartridge) Flow Rate: - 2 mL/min Gradient: - A: Water / Bicarb B: MeCN Time A% B% 0.00 95 5 0.50 95 5 4.00 5 95 5.50 5 95 5.60 95 5 6.50 95 5 Typical Injections 2-7 μL (concentration ~ 0.2 -1 mg/mL)
Analytical method 3	Solvents: Acetonitrile (Far UV grade) with 0.1% (V/V) formic acid Water (High purity *via* PureLab Ultra unit) with 0.1% formic acid Column: Hichrom ACE 3 C18-AR mixed mode column 100x4.6 mm Flow Rate: 1 mL/min gradient: A: Water / formic B: MeCN/formic Time A% B% 0.00 98 2 3.00 98 2 12.00 0 100 15.4 0 100 15.5 98 2 17 98 2 Typical Injections 0.2-10 μL (concentration ~ 0.2-1.0 mg/mL)

Analytical method 4	Solvents: - Acetonitrile (Far UV grade) with 0.1% (V/V) formic acid Water (High purity via PureLab Ultra unit) with 0.1% formic acid Column: Supelco, Ascentis® Express C18 or Hichrom Halo C18, 2.7 μm C18, 150 x 4.6 mm. Both latest technology fused core columns Flow Rate: 1mL/min Gradient: A: Water / formic B: MeCN/formic Time A% B% 0.00 96 4 3.00 96 4 9.00 0 100 13.6 0 100 13.7 96 4 15 96 4 Typical Injections 0.2-10 μL (concentration ~ 0.2-1.0 mg/mL)

iii) General Synthetic Methods

Scheme 1 – Amide coupling route



Method A (amide coupling)

To a solution of carboxylic acid (1.50 mmol) in DCM (10 mL) at r.t. were added EDC (351 mg, 1.83 mmol) and HOPO (203 mg, 1.83 mmol). The mixture was stirred for 30 min to give a complete solution then amine (free base or hydrochloride salt) (1.65 mmol) and DIPEA (1.3 mL, 7.5 mmol) were added and the mixture stirred at r.t. for 18 h. The mixture was washed with water, passed through a phase separation cartridge and concentrated.

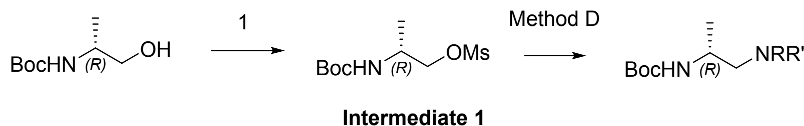
Method B (amide reduction)

To a solution of amide (1.30 mmol) in diethyl ether (50 mL) at -15 °C under nitrogen was added dropwise lithium aluminum hydride (1.30 mL, 2.6 mmol, 2.0 M in THF) over 20 min. The reaction was maintained at -10°C for 1.5 h then quenched with water (1.5 mL), 2 N NaOH (1 mL) and further water (2 mL). The reaction was warmed to r.t. and stirred for 30 min, then $MgSO_4$ was added and the reaction was filtered through Celite, washing well with EtOAc. The filtrate was concentrated and the residue purified by passage through an SCX cartridge (5g), eluting with 0-10% 7 M methanolic ammonia in DCM.

Method C (Boc removal)

To a solution of Boc-amine (0.76 mmol) in DCM (5 mL) at 0 °C under nitrogen was added dropwise 4 N HCl in dioxane (0.8 mL, 3.0 mmol). The solution was warmed to r.t. and stirred for 1 h, then concentrated.

Scheme 2 – Mesylate displacement route



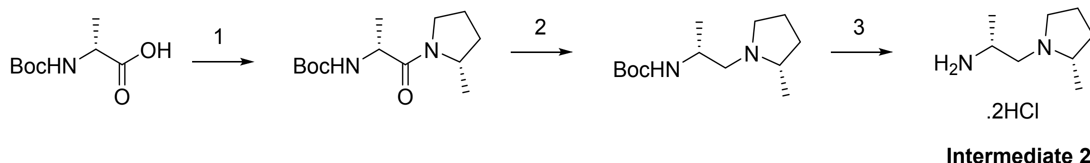
Step 1: (*R*)-2-((*tert*-butoxycarbonyl)amino)propyl methanesulfonate (**Intermediate 1**)

A solution of methanesulfonyl chloride (7.29 mL, 94.2 mmol) in dry DCM (140 mL) was added dropwise, over a period of 1.5 h, to a solution of (*R*)-*tert*-butyl (1-hydroxypropan-2-yl)carbamate (15 g, 85.6 mmol) and triethylamine (17.9 mL, 128.4 mmol) in DCM (300 mL) at 0°C. The reaction was stirred at 0 °C for 30 min, then 1 h at r.t, before washing with water (300 mL), $NaHCO_3$ (300 mL) and brine (200 mL). The organics were passed through a phase separator, and concentrated under reduced pressure to give the *title compound* as a salmon pink solid (21.5 g, 99%).

Method D

Amine (7.12 mmol) was added to **intermediate 1** (900 mg, 3.56 mmol) and cesium carbonate (3.48 g, 10.7 mmol) in DMF (8 mL) and the mixture stirred at 65°C for 24 h. The reaction mixture was cooled to r.t and filtered through celite, washing with MeOH. The filtrate was concentrated and the residue purified by passage through an SCX cartridge (5 g), eluting with 0-10% 7 M methanolic ammonia in DCM.

(*R*)-1-((*S*)-2-Methylpyrrolidin-1-yl)propan-2-amine dihydrochloride (**Intermediate 2**)



Step 1: *tert*-Butyl ((*R*)-1-((*S*)-2-methylpyrrolidin-1-yl)-1-oxopropan-2-yl)carbamate

Following **method A** from (*R*)-2-((*tert*-butoxycarbonyl)amino)propanoic acid (1.14 g, 6.04 mmol) and(*S*)-2-methylpyrrolidine (565 mg, 6.65 mmol) gave the *title compound* as a yellow oil which was progressed to the next step as crude.

Step 2: *tert*-Butyl ((*R*)-1-((*S*)-2-methylpyrrolidin-1-yl)propan-2-yl)carbamate

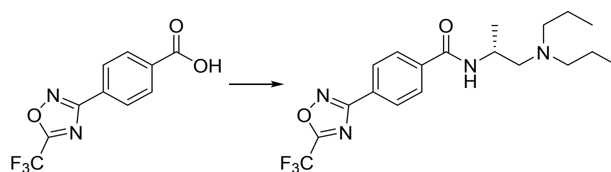
Following **method B** from *tert*-butyl ((*R*)-1-((*S*)-2-methylpyrrolidin-1-yl)-1-oxopropan-2-yl)carbamate (1.55 g, 6.04 mmol) gave the *title compound* as a colorless oil (1.43g, 97%).

Step 3: (*R*)-1-((*S*)-2-Methylpyrrolidin-1-yl)propan-2-amine dihydrochloride (**Intermediate 2**)

Following **method C** from *tert*-butyl ((*R*)-1-((*S*)-2-methylpyrrolidin-1-yl)propan-2-yl)carbamate (1.43 g, 5.88 mmol) gave the *title compound* as a colorless oil (850 mg, 81%).

Compound 2

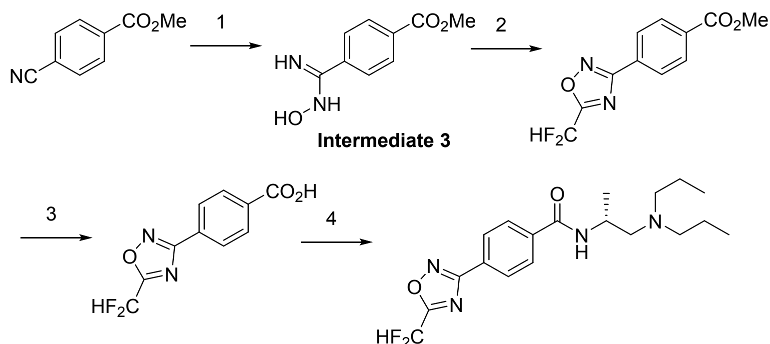
(R)-N-(1-(Dipropylamino)propan-2-yl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide



4-(5-(Trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzoic acid (200 mg, 0.775 mmol), HBTU (379 mg, 1 mmol), DMF (2.5 mL), (*R*)-*N*1,*N*1-dipropylpropane-1,2-diamine dihydrochloride (230 mg, 1 mmol) and triethylamine (0.5 mL) were combined and stirred at r.t. for 16 h. Reaction mixture was then purified by preparative-HPLC to give the *title compound* as an off-white solid (32 mg). LCMS (ES+) calc'd for $C_{19}H_{25}F_3N_4O_2$ $(M+H)^+$: 399, found: 399; RT 4.28 min (Analytical method 2); ^1H NMR δ (ppm) (400 MHz, DMSO-d_6) d 8.38 (d, J=7.4 Hz, 1H), 8.17 (d, J=8.5 Hz, 2H), 8.06 (d, J=8.5 Hz, 2H), 4.17 (dd, J=6.5, 13.5 Hz, 1H), 2.44 - 2.43 (m, 6H), 1.43 (dd, J=7.2, 14.4 Hz, 4H), 1.18 (d, J=6.7 Hz, 3H), 0.84 (dd, J=7.3, 7.3 Hz, 6H); ^{13}C NMR δ (ppm) (100 MHz, DMSO-d_6) 168.00, 165.21 (q, J=29 Hz), 164.63, 138.23, 128.38, 127.33, 126.59, 115.77 (q, J=272 Hz), 59.40, 55.86, 43.56, 19.72, 18.73, 11.61; HRMS calc'd for $C_{19}H_{25}F_3N_4O_2$ $(M+H)^+$: 399.2002, found 399.2014.

Compound 3

(*R*)-4-(5-(Difluoromethyl)-1,2,4-oxadiazol-3-yl)-N-(1-(dipropylamino)propan-2-yl)benzamide



Step1: Methyl 4-(*N'*-hydroxycarbamimidoyl)benzoate (**Intermediate 3**)

Methyl 4-cyanobenzoate (540 mg, 3.4 mmol), hydroxylamine hydrochloride (306 mg, 4.4 mmol), sodium hydrogen carbonate (428 mg, 5.1 mmol) and EtOH (30 mL) were combined and heated to reflux for 2.5 h. Reaction mixture was cooled to r.t.. The solid was removed by filtration and the filtrate evaporated to

dryness to give the *title compound* (550 mg, 64%) which was used in the next step without further purification.

Step 2: Methyl 4-(5-(difluoromethyl)-1,2,4-oxadiazol-3-yl)benzoate

Methyl 4-(*N*-hydroxycarbamimidoyl)benzoate (1.4 g, 7.2 mmol), THF (50 mL) and 1,1-difluoroacetic anhydride (1.9 g, 10.8 mmol) were combined and heated to 75°C under a nitrogen atmosphere for 18 h. Reaction mixture was then diluted with EtOAc, washed with 10% aq. Na_2CO_3 soln. and evaporated to dryness to give the *title compound* (1.35 g, 73%) which was used directly in the next step without further purification.

Step 3: 4-(5-(Difluoromethyl)-1,2,4-oxadiazol-3-yl)benzoic acid

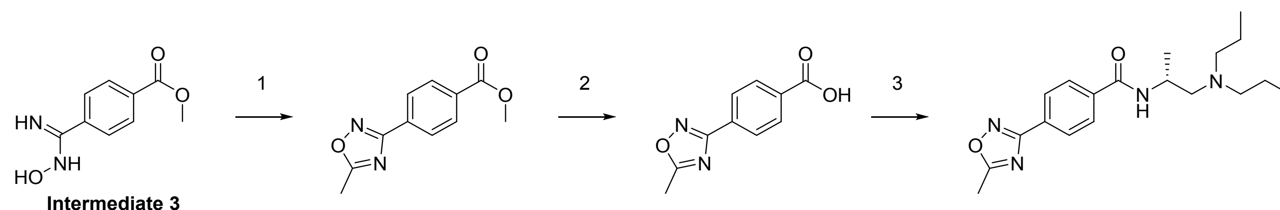
Methyl 4-(5-(difluoromethyl)-1,2,4-oxadiazol-3-yl)benzoate (1.4 g, 5.5 mmol), THF (20 mL), and 5 N HCl (20 mL) were combined and heated to 65°C for 20 h. The reaction mixture was then evaporated to dryness to give the *title compound* (5.5 mmol) which was used directly in the next step.

Step 4: (*R*)-4-(5-(Difluoromethyl)-1,2,4-oxadiazol-3-yl)-N-(1-(dipropylamino)propan-2-yl)benzamide

4-(5-(Difluoromethyl)-1,2,4-oxadiazol-3-yl)benzoic acid (280 mg, 1.04 mmol), HBTU (432 mg, 1.14 mmol), triethylamine (0.78 mL, 5.2 mmol), (*R*)-*N*1,*N*1-dipropylpropane-1,2-diamine (180 mg, 1.14 mmol) and DMF (10 mL) were combined and stirred at r.t. for 1.5 h. The reaction mixture was evaporated to dryness and purified by preparative-HPLC to give the *title compound* (29 mg) as an off white solid. LCMS (ES+) calc'd for $C_{19}H_{26}F_2N_4O_2$ $(M+H)^+$: 381, found: 381; RT 9.32 min (Analytical method 3); ^{1}H NMR δ (ppm) (400 MHz, DMSO-d_6) d 8.32 (d, J=8.4 Hz, 1H), 8.15 (d, J=8.5 Hz, 2H), 8.04 (d, J=8.5 Hz, 2H), 7.57 (t, J=51.6 Hz, 1H), 4.16 - 4.09 (m, 1H), 2.55 - 2.32 (m, 6H), 1.40 (dd, J=7.3, 14.6 Hz, 4H), 1.17 (d, J=6.7 Hz, 3H), 0.84 (dd, J=7.3, 7.3 Hz, 6H); ^{13}C NMR δ (ppm) (100 MHz, DMSO-d_6) 170.92 (t, J=30 Hz), 168.10, 165.03, 138.56, 128.73, 127.67, 127.52, 107.27 (t, J=240 Hz), 60.06, 56.65, 44.36, 20.48, 19.15, 12.24; HRMS calc'd for $C_{19}H_{26}F_2N_4O_2$ $(M+H)^+$: 381.2096, found 381.2092.

Compound 4

(*R*)-*N*-(1-(dipropylamino)propan-2-yl)-4-(5-methyl-1,2,4-oxadiazol-3-yl)benzamide formate salt



Step 1: Methyl 4-(5-methyl-1,2,4-oxadiazol-3-yl)benzoate

To a stirred solution of methyl methyl 4-(*N*'-hydroxycarbamimidoyl)benzoate (**Intermediate 3**) (770 mg, 3.96 mmol) in THF (50 mL) was added acetic anhydride (608 mg, 5.94 mL) and the mixture refluxed for 3 h. The reaction mixture was cooled to r.t., and solvent was removed under reduced pressure. The residue was partitioned between EtOAc (20 mL) and water (20 mL), and the organic layer was collected, dried ($MgSO_4$), filtered and concentrated to dryness to give the *title compound* (594 mg, 69%). LCMS (ES+) calc'd for $C_{11}H_{10}N_2O_3$ $(M+H)^+$: 219, found: 219.

Step 2: 4-(5-Methyl-1,2,4-oxadiazol-3-yl)benzoic acid

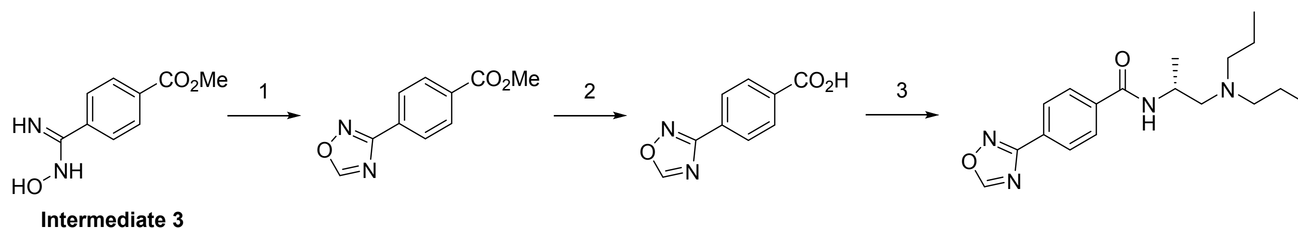
To a stirred solution of methyl 4-(5-methyl-1,2,4-oxadiazol-3-yl)benzoate (594 mg, 27.2 mmol) in THF (10 mL) was added 4 M HCl in dioxane (1 mL, 4 mmol) and the mixture was refluxed for 18 h. Conc. HCl (0.2 mL) was added and the mixture refluxed for a further 4 h. The reaction mixture was cooled to r.t. and the volatiles removed under reduced pressure to give the *title compound* (120 mg, 26%) which was used directly in the next step. LCMS (ES+) calc'd for $C_{10}H_8N_2O_3$ $(M+H)^+$: 205, found: 205.

Step 3: (*R*)-*N*-(1-(dipropylamino)propan-2-yl)-4-(5-methyl-1,2,4-oxadiazol-3-yl)benzamide

4-(5-Methyl-1,2,4-oxadiazol-3-yl)benzoic acid (120 mg, 0.62 mmol), HBTU (258 mg, 0.68 mmol), DMF (10 mL) and (*R*)-*N*1,*N*1-dipropylpropane-1,2-diamine (107 mg, 0.68 mmol) were combined and stirred at r.t. for 72 h. The solvent was removed under reduced pressure, before partitioning the residue between EtOAc (20 mL) and water (20 mL). The organic layer was collected, dried ($MgSO_4$) filtered and concentrated to dryness. Purification by preparative-HPLC gave the *title compound* as a white solid (10 mg) as a formate salt. LCMS (ES+) calc'd for $C_{19}H_{28}N_4O_2$ $(M+H)^+$: 345, found: 345; RT 2.79 min (Analytical method 1) ^{1}H NMR δ (ppm) (400 MHz, $CDCl_3$) d 9.46 (s, 1H), 8.17 (d, J=8.7 Hz, 2H), 8.00 (d, J=8.3 Hz, 2H), 7.45 (s, 1H), 4.45 (dd, J=6.7, 15.2 Hz, 1H), 3.56 (dd, J=10.1, 13.7 Hz, 1H), 3.39 - 3.04 (m, 6H), 2.67 (s, 3H), 1.93 - 1.73 (m, 3H), 1.50 (d, J=7.0 Hz, 3H), 1.12 (dd, J=7.3, 7.3 Hz, 3H), 0.95 (dd, J=7.3, 7.3 Hz, 3H); HRMS calc'd for $C_{19}H_{28}N_4O_2$ $(M+H)^+$: 344.2284, found 344.2216.

Compound 5

(*R*)-*N*-(1-(Dipropylamino)propan-2-yl)-4-(1,2,4-oxadiazol-3-yl)benzamide



Step 1: Methyl 4-(1,2,4-oxadiazol-3-yl)benzoate

Methyl 4-(*N*'-hydroxycarbamimidoyl)benzoate (**Intermediate 3**) (1.55 g, 7.99 mmol), trimethyl orthoformate (20 mL) and $BF_3.OEt_2$ (2 drops) were combined in a sealed tube and microwave heated to 110°C for 1 h. Reaction mixture was diluted with EtOAc, washed with sat. aq. $NaHCO_3$, and evaporated to dryness to give the *title compound* (1.2 g, 74%) as a yellow oil which was used crude in next step.

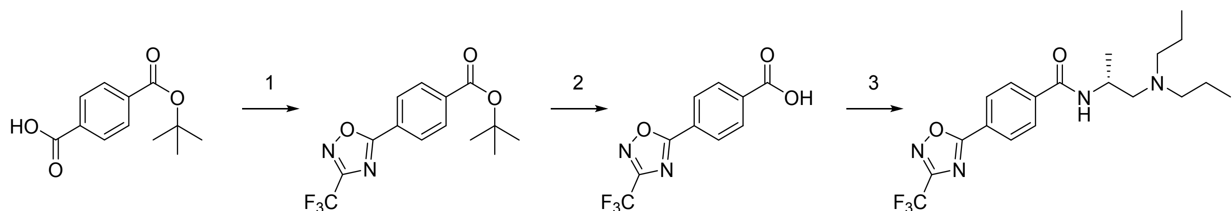
Step 2: 4-(1,2,4-Oxadiazol-3-yl)benzoic acid

Methyl 4-(1,2,4-oxadiazol-3-yl)benzoate (1.2 g, 5.88 mmol), methanol (25 mL) and NaOH (15% aq. soln.) (5 mL, 18.8 mmol) were combined and heated to 80°C for 16 h. Methanol was removed *in vacuo* and water (20 mL) added followed by acidification with conc. HCl. The resulting precipitate was filtered off and dried to give the *title compound* (1.0 g, 90%) as a beige solid which was used crude in next step.

Step 3: (*R*)-*N*-(1-(Dipropylamino)propan-2-yl)-4-(1,2,4-oxadiazol-3-yl)benzamide
4-(1,2,4-Oxadiazol-3-yl)benzoic acid (190 mg, 1 mmol), DCM (10 mL) and THF (5 mL) were combined under a nitrogen atmosphere. Oxalyl chloride (0.2 mL) was added followed by DMF (1 drop). The reaction mixture was stirred for 1 h then evaporated to dryness. Crude material was combined with THF (10 mL), triethylamine (101 mg, 1 mmol) and (*R*)-*N*1,*N*1-dipropylpropane-1,2-diamine (160 mg, 1 mmol) and stirred for 18 h. Reaction mixture was evaporated to dryness and purified by preparative-HPLC to give the *title compound* as a white sticky solid (30 mg). LCMS (ES+) calc'd for $C_{18}H_{26}N_4O_2$ $(M+H)^+$: 331, found: 331; RT 3.14 min (Analytical method 2); ^{1}H NMR δ (ppm) (400 MHz, DMSO-d_6) d 9.77 (s, 1H), 8.28 (d, J=8.3 Hz, 1H), 8.13 (d, J=8.5 Hz, 2H), 8.02 (d, J=8.5 Hz, 2H), 4.17 - 4.08 (m, 1H), 2.49 - 2.32 (m, 6H), 1.40 (dd, J=7.3, 14.6 Hz, 4H), 1.17 (d, J=6.7 Hz, 3H), 0.84 (dd, J=7.3, 7.3 Hz, 6H); ^{13}C NMR δ (ppm) (100 MHz, DMSO-d_6) 167.67, 166.37, 164.69, 163.74, 137.58, 128.12, 128.00, 127.40, 127.02, 59.55, 56.14, 43.83, 19.96, 18.69, 11.75; HRMS calc'd for $C_{18}H_{26}N_4O_2$ $(M+H)^+$: 331.2128, found 331.2168.

Compound 6

(*R*)-*N*-(1-(dipropylamino)propan-2-yl)-4-(3-(trifluoromethyl)-1,2,4-oxadiazol-5-yl)benzamide



Step 1: *tert*-Butyl 4-(3-(trifluoromethyl)-1,2,4-oxadiazol-5-yl)benzoate

To a stirred solution of 4-(*tert*-butoxycarbonyl)benzoic acid (440 mg, 2 mmol) in dry DCM (4 mL) was added 2,2,2-trifluoro-N'-hydroxyacetimidamide (350 mg, 2.7 mmol). The mixture was stirred at r.t. for 1 h, then concentrated to dryness. Toluene (4 mL) was added and the reaction mixture was heated at reflux for 18 h, before removing solvent under reduced pressure, and partitioning the residue between EtOAc (20 mL) and water (20 mL). The organic layer was collected and dried ($MgSO_4$), before filtering and concentrating to dryness. Purification by flash silica chromatography (gradient elution 0 to 100% EtOAc in *i*-hex) gave the *title compound* (140 mg, 25%).

Step 2: 4-(3-(Trifluoromethyl)-1,2,4-oxadiazol-5-yl)benzoic acid

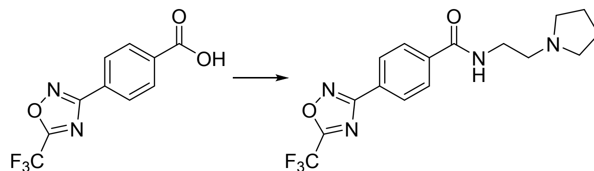
To a stirred solution of *tert*-butyl 4-(3-(trifluoromethyl)-1,2,4-oxadiazol-5-yl)benzoate (140 mg, 0.44 mmol) in DCM (1 mL) was added TFA (0.5 mL, 6.5 mmol) and triethylsilane (0.2 mL, 12 mmol). The mixture was stirred at r.t. for 1 h, then concentrated to dryness to give the *title compound* (110 mg, 96%) which was used crude in next step.

Step 3: (*R*)-*N*-(1-(dipropylamino)propan-2-yl)-4-(3-(trifluoromethyl)-1,2,4-oxadiazol-5-yl)benzamide

4-(3-(Trifluoromethyl)-1,2,4-oxadiazol-5-yl)benzoic acid (110 mg, 0.43 mmol), HATU (245 mg, 0.60 mmol), DIPEA (0.46 mL, 2.58 mmol), DMAP (20 mg, 0.16 mmol), (*R*)-*N*1,*N*1-dipropylpropane-1,2-diamine bis-TFA salt (193 mg, 0.50 mmol) and DMF (1.5 mL) were combined and stirred at r.t. for 2 h. The reaction mixture was evaporated to dryness and purified by preparative-HPLC to give the *title compound* (50 mg) as a formate salt, which was removed by passage through SCX-cartridge eluting with 0-10% 7 M methanolic ammonia in DCM to give the *title compound* as a colourless solid (12 mg). LCMS (ES+) calc'd for $C_{19}H_{25}F_3N_4O_2$ $(M+H)^+$: 399, found: 399; RT 2.79 min (Analytical method 1); ^{1}H NMR δ (ppm) (400 MHz, $CDCl_3$) d 8.26 (d, J=8.6 Hz, 2H), 7.97 (d, J=8.1 Hz, 2H), 7.03 - 6.98 (m, 1H), 4.01 (dd, J=12.9, 12.9 Hz, 1H), 2.57 - 2.31 (m, 6H), 1.55 - 1.37 (m, 4H), 1.34 (d, J=6.3 Hz, 3H), 0.86 (dd, J=7.3, 7.3 Hz, 6H); ^{13}C NMR δ (ppm) (100 MHz, DMSO-d_6) 177.48, 164.24, 161.06 (q, J=39 Hz), 139.70, 128.43, 128.38, 124.15, 118.02 (q, J=270 Hz), 59.57, 56.15, 43.98, 20.00, 18.65, 11.75; HRMS calc'd for $C_{19}H_{25}F_3N_4O_2$ $(M+H)^+$: 399.2002, found 399.2014.

Compound 7

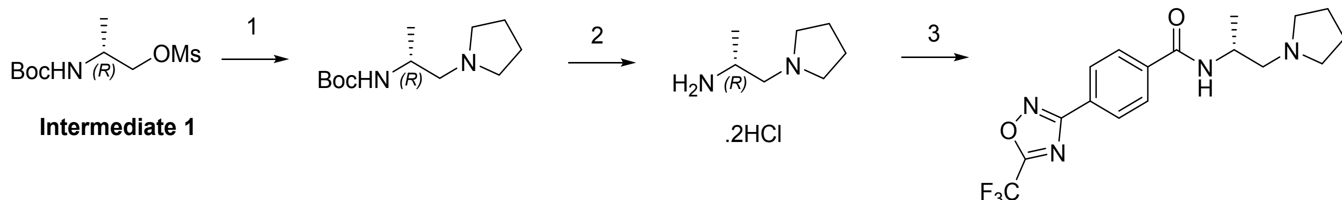
N-(2-(Pyrrolidin-1-yl)ethyl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide



4-(5-(Trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzoic acid (200 mg, 0.78 mmol), HBTU (379 mg, 1 mmol), DMF (2.5 mL) and 2-(pyrrolidin-1-yl)ethan-1-amine (0.5 mL) were combined and stirred at r.t. for 18 h. Reaction mixture was then purified by preparative-HPLC to give the *title compound* as a white solid (25 mg) as a formate salt. LCMS (ES+) calc'd for $C_{16}H_{17}F_3N_4O_2$ $(M+H)^+$: 355, found: 355; RT 2.56 min (Analytical method 1); ^{1}H NMR δ (ppm) (400 MHz, DMSO-d_6) d 8.71 (dd, J=5.6, 5.6 Hz, 1H), 8.21 - 8.16 (m, 2H), 8.07 (d, J=8.5 Hz, 2H), 3.43 (dd, J=6.8, 12.7 Hz, 4H), 2.64 (dd, J=6.9, 6.9 Hz, 2H), 2.60 (s, 2H), 1.74 - 1.67 (m, 4H); ^{13}C NMR δ (ppm) (100 MHz, DMSO-d_6) 167.99, 165.21 (q, J=43 Hz), 165.14, 137.98, 128.35, 127.39, 126.63, 115.77 (q, J=271 Hz), 54.71, 53.61, 35.57, 23.13; HRMS calc'd for $C_{16}H_{17}F_3N_4O_2$ $(M+H)^+$: calc. 355.1378, found 355.1396.

Compound 8

(*R*)-*N*-(1-(Pyrrolidin-1-yl)propan-2-yl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide



Step 1: *tert*-Butyl (*R*)-(1-(pyrrolidin-1-yl)propan-2-yl)carbamate

Following **method D** from **Intermediate 1** (6 g, 23.7 mmol) and pyrrolidine (4.14 mL, 47.4 mmol) gave the *title compound* (3.2 g, 59%).

Step 2: (*R*)-1-(Pyrrolidin-1-yl)propan-2-amine dihydrochloride

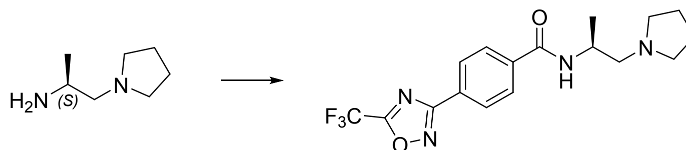
Following **method C** from *tert*-butyl (*R*)-(1-(pyrrolidin-1-yl)propan-2-yl)carbamate (3.2 g, 14 mmol) gave the *title compound* (2.33 g, 83%).

Step 3: (*R*)-*N*-(1-(Pyrrolidin-1-yl)propan-2-yl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide

Following **method A** from (*R*)-1-(pyrrolidin-1-yl)propan-2-amine dihydrochloride (2.29 g, 14 mmol) and 4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzoic acid (3.6 g, 14 mmol). Purification by silica gel column chromatography (gradient elution, DCM to 10% MeOH in DCM) followed by recrystallization from DIPE afforded *the title compound* as a colourless solid (2.15 g). LCMS (ES+) calc'd for $C_{17}H_{19}F_3N_4O_2$ $(M+H)^+$: 369, found: 369; RT 2.57 min (Analytical method 1); LCMS (ES+) 369 $(M+H)^+$, RT 8.99 min (*Analytical Method 3*); ^{1}H NMR δ (ppm) (400 MHz, $CDCl_3$) δ 8.19 (d, J=8.2 Hz, 2H), 7.92 (d, J=8.3 Hz, 2H), 6.77 (d, J=3.3 Hz, 1H), 4.15 - 4.07 (m, 1H), 2.71 (dd, J=9.0, 12.2 Hz, 1H), 2.62 - 2.47 (m, 5H), 1.80 - 1.75 (m, 4H), 1.34 (d, J=6.4 Hz, 3H); ^{13}C NMR δ (ppm) (100 MHz, DMSO-d_6) 168.00, 165.22 (q, J=44 Hz), 164.68, 138.16, 128.44, 127.30, 126.59, 115.76 (q, J=272 Hz), 60.28, 53.71, 44.04, 23.06, 18.97; HRMS calc'd for $C_{17}H_{19}F_3N_4O_2$ $(M+H)^+$: 369.1534, found 369.1566.

Compound 9

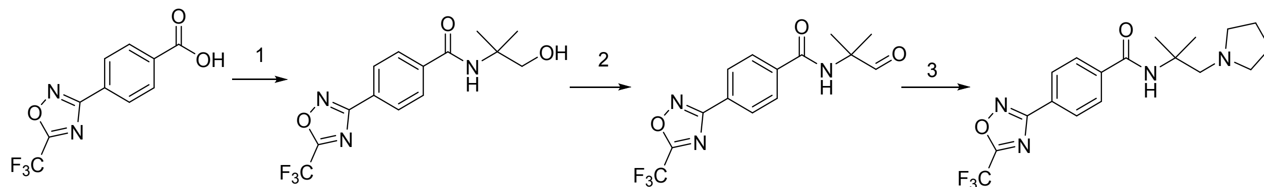
(*S*)-*N*-(1-(Pyrrolidin-1-yl)propan-2-yl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide



Following **method A** from (*S*)-1-(pyrrolidin-1-yl)propan-2-amine (167 mg, 1.30 mmol) and 4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzoic acid (335 mg, 1.30 mmol). Purification by preparative-HPLC gave the *title compound* as an off-white solid (319 mg). LCMS (ES+) calc'd for $C_{17}H_{19}F_3N_4O_2$ $(M+H)^+$: 369, found: 369; RT 2.56 min (Analytical method 1); ^{1}H NMR δ (ppm) (400 MHz, DMSO-d_6) 8.44 (d, J=8.2 Hz, 1H), 8.17 (d, J=8.2 Hz, 2H), 8.07 (d, J=8.5 Hz, 2H), 4.23 - 4.15 (m, 1H), 2.63 - 2.46 (m, 6H), 1.71 - 1.67 (m, 4H), 1.18 (d, J=6.7 Hz, 3H); ^{13}C NMR δ (ppm) (100 MHz, DMSO-d_6) 168.00, 165.22 (q, J=44 Hz), 164.68, 138.16, 128.44, 127.30, 126.59, 115.76 (q, J=272 Hz), 60.28, 53.71, 44.04, 23.06, 18.97; HRMS calc'd for $C_{19}H_{25}F_3N_4O_2$ $(M+H)^+$: 369.1534, found 369.1527.

Compound 10

N-(2-Methyl-1-(pyrrolidin-1-yl)propan-2-yl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide formate salt



Step 1: *N*-(1-Hydroxy-2-methylpropan-2-yl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide

4-(5-(Trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzoic acid (1 g, 3.88 mmol), HBTU (1.67 g, 4.4 mmol), 2-amino-2-methyl-1-propanol (400 mg, 4.5 mmol), DMF (10 mL) and triethylamine (1 mL) were combined and stirred at r.t. for 16 h. Reaction mixture was diluted with EtOAc, washed with water (4x) and evaporated to dryness onto silica. Purification by flash silica chromatography gave the *title compound* as a white solid (900 mg, 71%).

Step 2: *N*-(2-Methyl-1-oxopropan-2-yl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide

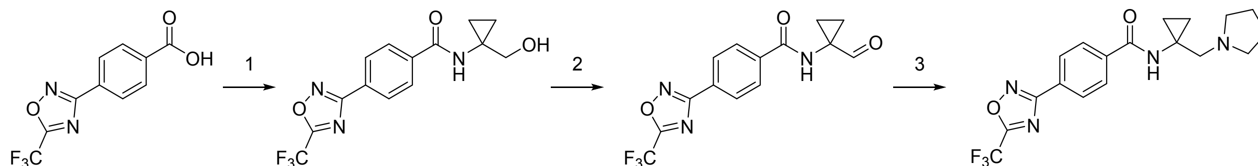
N-(1-Hydroxy-2-methylpropan-2-yl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide (850 mg, 2.58 mmol), DCM (70 mL) and Dess Martin periodinane (1.1 g, 2.6 mmol) were combined and stirred at r.t. for 16 h. Reaction mixture was then evaporated to dryness onto silica and purified by flash silica chromatography to give the *title compound* as a white solid (630 mg, 75%).

Step 3: *N*-(2-Methyl-1-(pyrrolidin-1-yl)propan-2-yl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide

N-(2-Methyl-1-oxopropan-2-yl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide (100 mg, 0.3 mmol), THF (9 mL), pyrrolidine (0.042 ml, 0.5 mmol), and $NaBH(OAc)_3$ (105 mg, 0.5 mmol) were combined and stirred at r.t. for 16 h. The reaction mixture was then evaporated to dryness and purified by preparative-HPLC to give the *title compound* as the formate salt as an off-white solid (54 mg). LCMS (ES+) calc'd for $C_{18}H_{21}F_3N_4O_2$ $(M+H)^+$: 395, found: 395; RT 2.68 min (Analytical method 1); ^{1}H NMR δ (ppm) (400 MHz, DMSO-d_6) 8.18 (1H, s), 8.14 (d, J=8.5 Hz, 2H), 7.99 (d, J=8.5 Hz, 2H), 7.90 (s, 1H), 2.86 (s, 2H), 2.64 (dd, J=5.8, 5.8 Hz, 4H), 1.69 - 1.64 (m, 4H), 1.38 (s, 6H); ^{13}C NMR δ (ppm) (100 MHz, DMSO-d_6) 168.47, 165.90, 165.24 (q, J=45 Hz), 164.19, 139.79, 128.90, 127.72, 126.82, 116.23 (q, J=272 Hz), 63.16, 56.07, 55.02, 26.29, 24.00; HRMS calc'd for for $C_{18}H_{21}F_3N_4O_2$ $(M+H)^+$: 383.1690, found 383.1727.

Compound 11

N-(1-(pyrrolidin-1-ylmethyl)cyclopropyl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide



Step 1: *N*-(1-(Hydroxymethyl)cyclopropyl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide

4-(5-(Trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzoic acid (1.6 g, 6.20 mmol), HATU (3.6 g, 9.47 mmol), DIPEA (4.1 mL, 23.4 mmol), DMAP (422 mg, 3.4 mmol), (1-aminocyclopropyl)methanol hydrochloride (960 mg, 9.30 mmol) and DMF (15 mL) were combined and stirred at r.t. for 4 h. Water (50 mL) was added to the reaction mixture and the resulting precipitate was collected by vacuum filtration to give the *title compound* (1.48 g, 69%).

Step 2: *N*-(1-Formylcyclopropyl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide

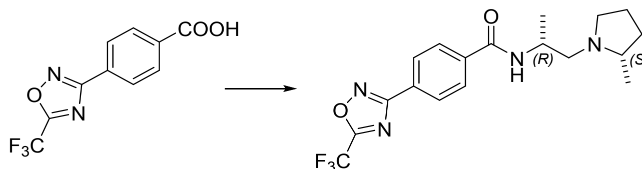
To a stirred solution of *N*-(1-(hydroxymethyl)cyclopropyl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide (1.48 g, 4.5 mmol) in DCM (105 mL) was added Dess-Martin periodinane (2.86 g, 6.8 mmol). The reaction mixture was stirred at r.t. for 4 h then filtered through a plug of Florisil, before concentrating the resulting solution under reduced pressure to give the *title compound* (2.2 g, >99%).

Step 3: *N*-(1-(Pyrrolidin-1-ylmethyl)cyclopropyl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide

To a stirred solution of *N*-(1-formylcyclopropyl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide (300 mg, 0.90 mmol) in THF (5 mL) was added pyrrolidine (0.085 mL, 1.0 mmol). This was stirred at r.t. for 2 h before addition of sodium cyanoborohydride (120 mg, 1.94 mmol) and the resulting solution was stirred at r.t. for 17 h. The reaction mixture was concentrated to dryness, partitioned between DCM (10 mL) and water (10 mL), the organic layer was collected by passage through a phase separator and concentrated to dryness. Purification by preparative-HPLC gave the *title compound* as an off-white solid (41 mg). LCMS (ES+) calc'd for $C_{18}H_{19}F_3N_4O_2$ (M+H)$^+$: 381, found: 381; RT 2.62 min (*Analytical Method 1*); ^{1}H NMR δ (ppm) (400 MHz, DMSO-d_6) d 8.87 (s, 1H), 8.20 (d, J=8.3 Hz, 2H), 8.10 (d, J=8.6 Hz, 2H), 2.71 (s, 2H), 2.64 - 2.58 (m, 4H), 1.72 (dd, J=5.1, 5.1 Hz, 4H), 0.85 (dd, J=5.8, 5.8 Hz, 2H), 0.79 - 0.73 (m, 2H). ^{13}C NMR δ (ppm) (100 MHz, DMSO-d_6) 168.02, 165.22 (q, J=45 Hz), 164.24, 138.68, 128.41, 127.33, 126.45, 116.33 (q, J=272 Hz), 60.04, 67.45, 54.96, 31.54, 23.34, 14.98; HRMS calc'd for $C_{18}H_{19}F_3N_4O_2$ (M+H)$^+$: 381.1534, found 381.1533.

Compound 12

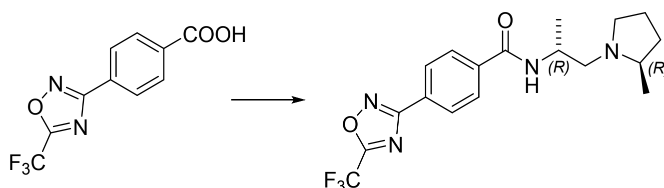
N-((*R*)-1-((*S*)-2-methylpyrrolidin-1-yl)propan-2-yl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide



A solution of 4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzoic acid (7.87 g, 30.5 mmol), intermediate 2 (6.55 g, 30.5 mmol), EDC (7.02 g, 36.6 mmol), HOPO (4.07 g, 36.6 mmol) and DIPEA (26 mL, 149 mmol) in DCM (175 mL) was stirred at r.t. for 67 h. NOTE: Addition of DIPEA is exothermic; a cold water bath was used to control the reaction temperature. The mixture was washed with water (2 x 50 mL) and concentrated. The residue was combined with other batches of the same reaction (using a total of 96.9 mmol 4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzoic acid). Purification by SFC gave the *title compound* as a white solid (11.3 g). LCMS (ES+) calc'd for $C_{18}H_{21}F_3N_4O_2$ $(M+H)^+$: 383, found: 383; RT 2.61 min (Analytical method 1); ^{1}H NMR δ (ppm) (400 MHz, DMSO-d_6) 8.44 (d, J=8.2 Hz, 1H), 8.16 (d, J=8.5 Hz, 2H), 8.06 (d, J=8.7 Hz, 2H), 4.18 - 4.09 (m, 1H), 3.14 - 3.07 (m, 1H), 2.70 (dd, J=9.7, 11.5 Hz, 1H), 2.34 - 2.09 (m, 3H), 1.91 - 1.81 (m, 1H), 1.71 - 1.61 (m, 2H), 1.34 - 1.23 (m, 1H), 1.18 (d, J=6.5 Hz, 3H), 1.03 (d, J=6.0 Hz, 3H); ^{13}C NMR δ (ppm) (100 MHz, DMSO-d_6) 168.00, 165.21, (q, J=43 Hz), 164.47, 138.25, 128.39, 127.35, 126.57, 115.77 (q, J=271 Hz), 59.38, 58.72, 53.61, 44.53, 32.33, 21.66, 19.17, 18.73; HRMS calc'd for $C_{18}H_{21}F_3N_4O_2$ $(M+H)^+$: 383.1690, found 383.1687.

Compound 13

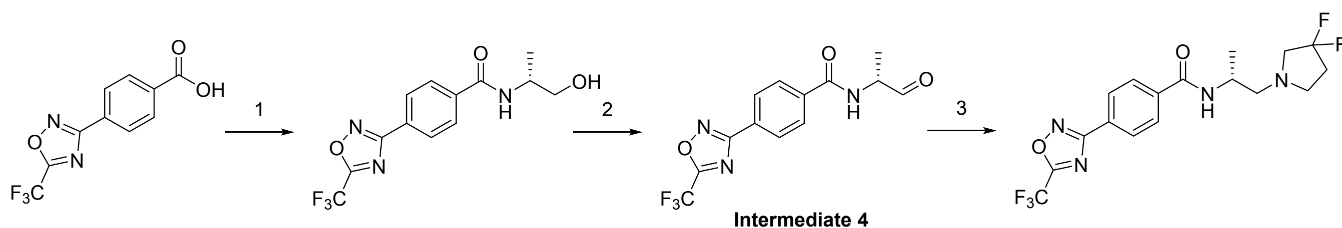
N-((*R*)-1-((*R*)-2-methylpyrrolidin-1-yl)propan-2-yl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide formate salt



4-(5-(Trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzoic acid (560 mg, 2.17 mmol), HATU (1.2 g, 3.20 mmol), DIPEA (1.55 mL, 8.68 mmol), DMAP (26 mg, 0.21 mmol), (R)-1-((R)-2-methylpyrrolidin-1-yl)propan-2-amine (340 mg, 2.30 mmol) and DMF (4 mL) were combined and stirred at r.t. for 3 h. The reaction mixture was evaporated to dryness and partitioned between DCM (10 mL) and water (10 mL). The organics were collected by passage through a phase separator and concentrated to dryness. Purification by preparative-HPLC gave the *title compound* (11 mg). LCMS (ES+) calc'd for $C_{18}H_{21}F_3N_4O_2$ $(M+H)^+$: 383, found: 383;

RT 2.65 min (Analytical method 1); ^{1}H NMR δ (ppm) (400 MHz, DMSO-d_6) 8.72 (d, J=8.8 Hz, 1H), 8.64 (s, 1H), 8.28 (d, J=8.3 Hz, 2H), 8.17 (d, J=8.6 Hz, 2H), 4.57 - 4.51 (m, 1H), 3.74 - 3.67 (m, 1H), 3.59 - 3.24 (m, 3H), 3.19 - 3.12 (m, 1H), 2.30 - 2.21 (m, 1H), 2.07 - 1.94 (m, 2H), 1.72 - 1.61 (m, 1H), 1.42 (d, J=6.3 Hz, 3H), 1.28 (d, J=6.8 Hz, 3H); ^{13}C NMR δ (ppm) (100 MHz, DMSO-d_6) 168.01, 165.21 (q, J=44 Hz), 164.80, 164.54, 138.24, 128.46, 127.27, 126.55, 115.76 (q, J=271 Hz), 61.09, 57.85, 53.51, 43.86, 31.85, 21.33, 19.07, 17.78; HRMS calc'd for $C_{18}H_{21}F_3N_4O_2$ $(M+H)^+$: 383.1690, found 383.1727.

Compound 14



Step 1: (*R*)-*N*-(1-hydroxypropan-2-yl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide

Following **method A** from (*R*)-2-aminopropan-1-ol (1.51 mL, 19.37 mmol) and 4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzoic acid (5.0 g, 19.37 mmol). Purification by silica gel column chromatography (gradient elution, 100% *i*-hex to 100% ethyl acetate) followed by trituration with diethyl ether gave the *title compound* as an off-white solid. The filtrate was concentrated and the resultant solid triturated with DCM and diethyl ether to afford the *title compound*. The two batches were combined and used without further purification (4.03 g, 66%). ^{1}H NMR (400 MHz, MeOD) δ 8.23 (d, J=8.7 Hz, 2H), 8.04 (d, J=8.7 Hz, 2H), 4.28 - 4.19 (m, 1H), 3.69 - 3.59 (m, 2H), 1.29 (d, J=6.8 Hz, 3H).

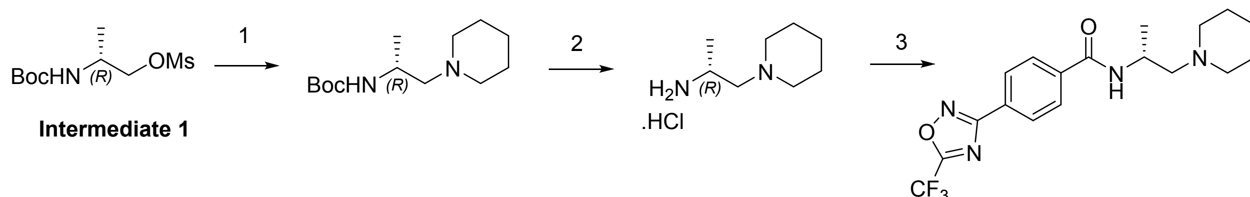
Step 2: (*R*)-*N*-(1-Oxopropan-2-yl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide (**Intermediate 4**)

Dess Martin Periodinane (6.06 g, 14.28 mmol) was added to (*R*)-*N*-(1-hydroxypropan-2-yl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide (3.0 g, 9.52 mmol) in DCM (150 mL). After 3 h the reaction mixture was quenched with methanol (50 mL). The reaction mixture was stirred for 30 minutes before concentrating under reduced pressure. Purification by silica gel column chromatography (gradient elution; 0-40% ethyl acetate / *i*-hex) afforded the *title compound* as a white solid (2.46 g, 82%). ^{1}H NMR δ (ppm) (400 MHz, $CDCl_3$) 9.68 (s, 1H), 8.22 (d, J=8.2 Hz, 2H), 7.98 (d, J=8.2 Hz, 2H), 6.92 (d, J=5.6 Hz, 1H), 4.83 - 4.75 (m, 1H), 1.57 (d, J=7.8 Hz, 3H).

Step 3: (*R*)-*N*-(1-(3,3-Difluoropyrrolidin-1-yl)propan-2-yl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide

To a stirred solution of (*R*)-*N*-(1-oxopropan-2-yl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide (97 mg, 0.31 mmol) and 3,3'-difluoropyrrolidine (44.5 mg, 0.31 mmol) in THF (3 mL) was added sodium triacetoxyborohydride (197 mg, 0.93 mmol). The reaction was stirred for 18 h then 2 mL methanol was added. After 3 h the reaction was quenched with brine aqnd extracted with ethyl acetate (x3). The combined organic solvents were dried (magnesium sulfate), filtered and concentrated under reduced pressure. Purification by preparative-HPLC gave the *title compound* as a colourless solid (18 mg). LCMS (ES+) calc'd for $C_{17}H_{17}F_5N_4O_2$ $(M+H)^+$: 405, found: 405; RT 3.57 min (*Analytical Method 2*); ^{1}H NMR δ (ppm) (400 MHz, DMSO-d_6) 8.47 (d, J=8.3 Hz, 1H), 8.17 (d, J=8.5 Hz, 2H), 8.06 (d, J=8.5 Hz, 2H), 4.22 - 4.14 (m, 1H), 3.03 - 2.85 (m, 2H), 2.75 (dd, J=7.0, 7.0 Hz, 2H), 2.64 - 2.52 (m, 2H), 2.28 - 2.15 (m, 2H), 1.18 (d, J=6.7 Hz, 3H); ^{13}C NMR δ (ppm) (100 MHz, DMSO-d_6) 167.99, 165.22 (q, J=44 Hz), 164.68, 138.20, 130.59 (t, J= 245 Hz), 128.40, 127.38, 126.61, 115.77 (q, J=271 Hz), 61.45 (t, J=28 Hz), 60.01, 51.92, 43.79, 35.21 (t, J=24 Hz), 18.88; HRMS calc'd for $C_{17}H_{17}F_5N_4O_2$ $(M+H)^+$: 405.1346, found 405.1331.

Compound 15



Step 1: *tert*-Butyl (*R*)-(1-(piperidin-1-yl)propan-2-yl)carbamate

Following **method D** from **intermediate 1** (500 mg, 1.97 mmol) and piperidine (392 µL, 3.95 mmol) gave the *title compound* as a yellow oil (60 mg, 13%).

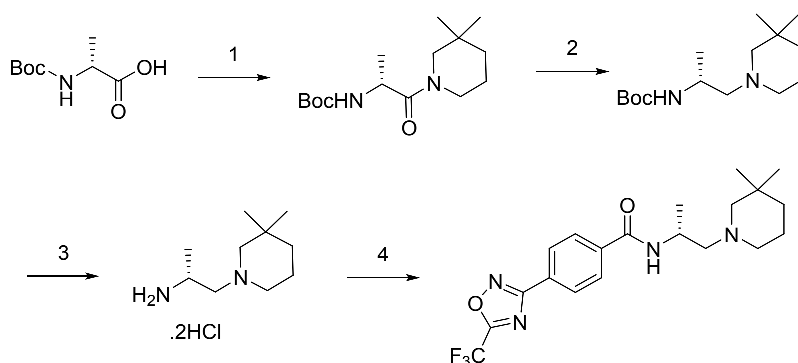
Step 2: (*R*)-1-(Piperidin-1-yl)propan-2-amine hydrochloride

To a solution of *tert*-butyl (*R*)-(1-(piperidin-1-yl)propan-2-yl)carbamate (60 mg, 0.25 mmol) in anhydrous DCM (1 mL) was added 4 M HCl in dioxane (0.25 mL). The reaction mixture was stirred at r.t for 3 h. The mixture was concentrated under reduced pressure to give the *title compound* (0.25 mmol).

Step 3: (*R*)-*N*-(1-(Piperidin-1-yl)propan-2-yl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide

Following **method A** from 4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzoic acid (65 mg, 0.25 mmol) and (*R*)-1-(piperidin-1-yl)propan-2-amine hydrochloride (0.25 mmol). Purification by preparative-HPLC and SFC gave the *title compound* as a white solid (24 mg). LCMS (ES+) calc'd for $C_{18}H_{21}F_3N_4O_2$ $(M+H)^+$: 383, found: 383; RT 3.49 min (*Analytical Method 2*); ^1H NMR δ (ppm) (400 MHz, DMSO-d_6) 8.41 (d, J = 8.2 Hz, 1H), 8.21 (d, J = 8.4 Hz, 2H), 8.10 (d, J = 8 Hz, 2H), 4.30 - 4.23 (m, 1H), 2.50 - 2.36 (m, 5H), 2.35-2.25 (m, 1H), 1.58 - 1.45 (m, 4H), 1.45-1.34 (m, 2H), 1.20 (d, J = 6.8 Hz, 3H).

Compound 16



Step 1: *tert*-Butyl (*R*)-(1-(3,3-dimethylpiperidin-1-yl)-1-oxopropan-2-yl)carbamate

Following **method A** from (*R*)-2-((*tert*-butoxycarbonyl)amino)propanoic acid (250 mg, 1.32 mmol) and 3,3-dimethylpiperidine (164 mg, 1.45 mmol). The *title compound* was obtained as a pale yellow gum (376 mg, >99%) which was used crude in the next step.

Step 2: *tert*-Butyl (*R*)-(1-(3,3-dimethylpiperidin-1-yl)propan-2-yl)carbamate

Following **method B** from *tert*-butyl (*R*)-(1-(3,3-dimethylpiperidin-1-yl)-1-oxopropan-2-yl)carbamate (376 mg, 1.25 mmol) gave the *title compound* as a tan oil (355 mg) which was used without further purification in the next step.

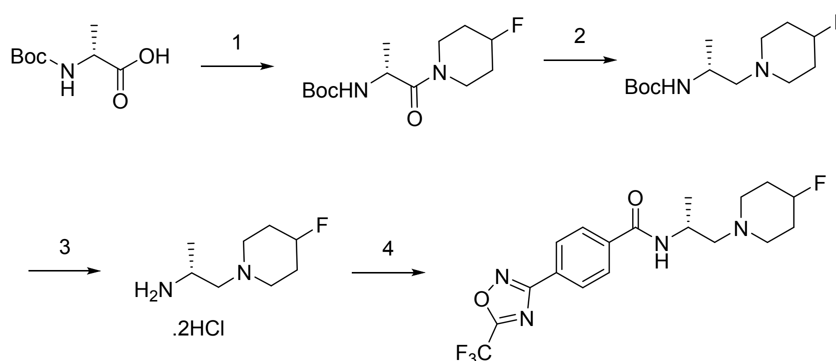
Step 3: (*R*)-1-(3,3-Dimethylpiperidin-1-yl)propan-2-amine dihydrodrochloride

Following **method C** from *tert*-butyl (*R*)-(1-(3,3-dimethylpiperidin-1-yl)propan-2-yl)carbamate (355 mg) afforded the *title compound* as a tan oil (222 mg, 66%) which was used without further purification in the next step.

Step 4: (*R*)-*N*-(1-(3,3-dimethylpiperidin-1-yl)propan-2-yl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide

Following **method A** from (*R*)-1-(3,3-Dimethylpiperidin-1-yl)propan-2-amine dihydrodrochloride (222 mg, 0.82 mmol) and 4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzoic acid (194 mg, 0.75 mmol). Purification by preparative-HPLC gave the *title compound* as a tan solid (15 mg). LCMS (ES+) calc'd for $C_{20}H_{25}F_3N_4O_2$ $(M+H)^+$: 411, found: 411; RT 2.84 min (*Analytical Method 1*); ^1H NMR δ (ppm) (400 MHz, DMSO-d_6) d 8.35 (d, J=8.3 Hz, 1H), 8.16 (d, J=8.5 Hz, 2H), 8.04 (d, J=8.5 Hz, 2H), 4.26 - 4.17 (m, 1H), 2.41 - 2.33 (m, 3H), 2.23 (dd, J=7.4, 12.2 Hz, 1H), 2.05 (s, 2H), 1.53 - 1.45 (m, 2H), 1.16 (d, J=6.5 Hz, 5H), 0.87 (d, J=7.5 Hz, 6H); ^{13}C NMR δ (ppm) (100 MHz, DMSO-d_6) 168.01, 165.19 (q, J=44 Hz), 164.58, 138.48, 128.33, 127.31, 126.47, 115.77 (q, J=272 Hz), 65.64, 63.36, 54.58, 42.71, 37.18, 30.53, 27.07, 22.33, 18.77; HRMS calc'd for $C_{20}H_{25}F_3N_4O_2$ $(M+H)^+$: 411.2002, found 411.2040.

Compound 17



Step 1: *tert*-Butyl (*R*)-(1-(4-Fluoropiperidin-1-yl)-1-oxopropan-2-yl)carbamate

Following **method A** from (*tert*-butoxycarbonyl)-D-alanine (2.20 g, 11.6 mmol) and 4-fluoropiperidine hydrochloride (1.32 g, 12.8 mmol) gave the *title compound* as a yellow oil (2.86 g, 90%).

Step 2: *tet*-butyl (*R*)-(1-(4-Fluoropiperidin-1-yl)propan-2-yl)carbamate

Following **method B** from *tert*-butyl (*R*)-(1-(4-fluoropiperidin-1-yl)-1-oxopropan-2-yl)carbamate (1.5 g, 5.47 mmol) gave the *title compound* as a yellow oil (652 mg, 46%).

Step 3: (*R*)-1-(4-Fluoropiperidin-1-yl)propan-2-amine dihydrochloride

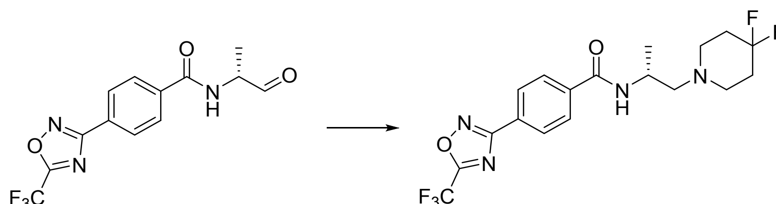
Following **method C** from *tert*-butyl (*R*)-(1-(4-fluoropiperidin-1-yl)propan-2-yl)carbamate (260 mg, 1.00 mmL). Trituration of the crude product with diethyl ether afforded a yellow solid (172 mg, 74%).

Step 4: (*R*)-*N*-(1-(4-fluoropiperidin-1-yl)propan-2-yl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide

Following **method A** from (*R*)-1-(4-fluoropiperidin-1-yl)propan-2-amine dihydrochloride (116 mg, 0.50 mmol) and 4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzoic acid (129 mg, 0.50 mmol). Purification by preparative-HPLC gave the *title compound* as a white solid (100 mg). LCMS (ES+) calc'd for $C_{18}H_{20}F_4N_4O_2$ $(M+H)^+$: 401, found: 401; RT 9.24 min (*Analytical Method 3*); ^{1}H NMR δ (ppm) (400 MHz, $CDCl_3$) 8.21 (d, J=8.6 Hz, 2H), 7.92 (d, J=8.6 Hz, 2H), 6.69 (d, J=3.9 Hz, 1H), 4.75 - 4.59 (m, 1H), 4.19 - 4.11 (m, 1H), 2.72 (s, 1H), 2.57 - 2.43 (m, 4H), 2.36 - 2.33 (m, 1H), 1.93 - 1.83 (m, 4H), 1.33 (d, J=6.4 Hz, 3H); ^{13}C NMR δ (ppm) (100 MHz, DMSO-d_6) 168.00, 165.21 (q, J=44 Hz), 164.69, 138.39, 128.38, 127.36, 126.54, 115.77 (q, J=272 Hz), 88.62 (d, J=168 Hz), 62.71, 49.55 (t, J=6 Hz), 42.96, 31.32 (d, J=19 Hz), 18.92; HRMS calc'd for $C_{18}H_{20}F_4N_4O_2$ $(M+H)^+$: 401.1596, found 401.1615.

Compound 18

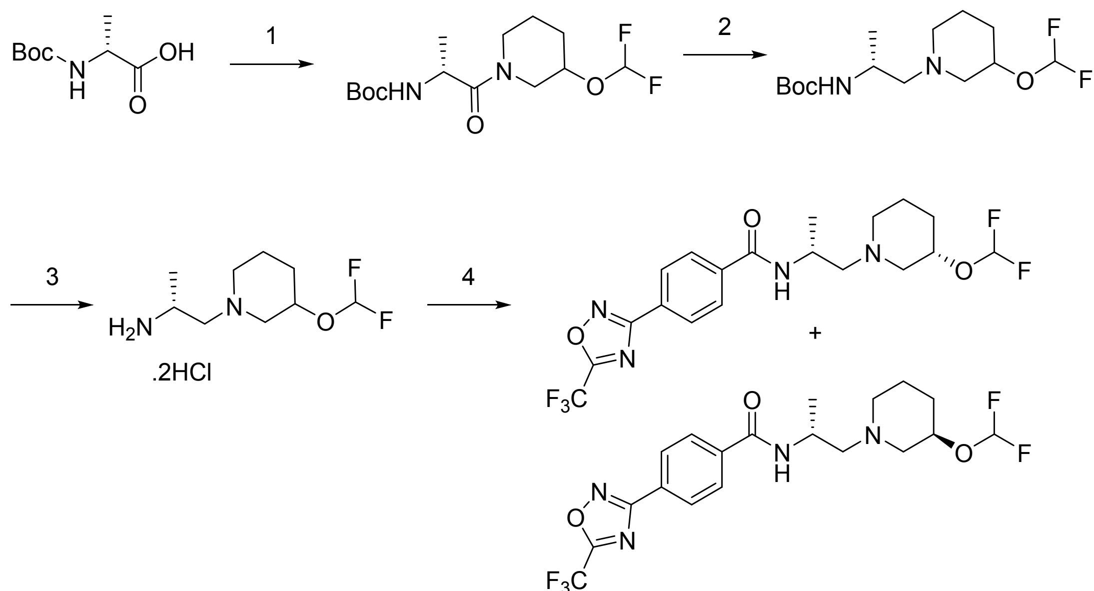
(*R*)-*N*-(1-(4,4-Difluoropiperidin-1-yl)propan-2-yl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide



To a stirred solution of (*R*)-*N*-(1-oxopropan-2-yl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide (91 mg, 0.29 mmol) in DCM:AcOH (10:1, 3 mL) was added 4,4'-difluoropiperidine hydrochloride (55 mg, 0.35 mmol) and PS-triethylammonium cyanoborohydride (145 mg, 0.58 mmol, 4.0 mmol / g loading). The reaction was shaken for 5 h before filtering and concentrating under reduced pressure. Purification by preparative-HPLC gave the *title compound* as a white solid (48 mg). LCMS (ES+) calc'd for $C_{18}H_{19}F_5N_4O_2$ $(M+H)^+$: 419, found: 419; RT 2.71 min (*Analytical Method 1*); ^{1}H NMR δ (ppm) (400 MHz, DMSO-d_6) 8.41 (d, J=8.2 Hz, 1H), 8.17 (d, J=8.4 Hz, 2H), 8.06 (d, J=8.3 Hz, 2H), 4.26 - 4.18 (m, 1H), 2.57 - 2.54 (m, 5H), 2.42 - 2.33 (m, 1H), 1.98 - 1.87 (m, 4H), 1.18 (d, J=6.6 Hz, 3H); ^{13}C NMR δ (ppm) (100 MHz, DMSO-d_6) 168.00, 165.21(q, J=43 Hz), 164.75, 138.35, 128.38, 127.37, 126.55, 122.79 (t, J=239 Hz), 115.77 (q, J=272 Hz), 61.74, 49.76 (t, J=5 Hz), 43.06, 33.55 (t, J=22 Hz), 18.84; HRMS calc'd for $C_{18}H_{19}F_5N_4O_2$ $(M+H)^+$: 419.1502, found 419.1523.

Compounds 19 and 20

N-((*R*)-1-((*R*)-3-(difluoromethoxy)piperidin-1-yl)propan-2-yl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide and *N*-((*R*)-1-((*S*)-3-(difluoromethoxy)piperidin-1-yl)propan-2-yl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide.



Step 1: *tert*-butyl ((2*R*)-1-(3-(difluoromethoxy)piperidin-1-yl)-1-oxopropan-2-yl)carbamate

Following **method A** from (*R*)-2-((*tert*-butoxycarbonyl)amino)propanoic acid (284 mg, 1.50 mmol) and (±)-3-(difluoromethoxy)piperidine (250 mg, 1.65 mmol). The *title compound* was obtained as a yellow gum (424 mg, 87%).

Step 2: *tert*-butyl ((2*R*)-1-(3-(difluoromethoxy)piperidin-1-yl)propan-2-yl)carbamate

Following **method B** from *tert*-butyl ((2*R*)-1-(3-(difluoromethoxy)piperidin-1-yl)-1-oxopropan-2-yl)carbamate (420 mg, 1.30 mmol). The *title compound* was obtained as a pale yellow gum (234 mg, 58%).

Step 3: (2*R*)-1-(3-(difluoromethoxy)piperidin-1-yl)propan-2-amine dihydrochloride

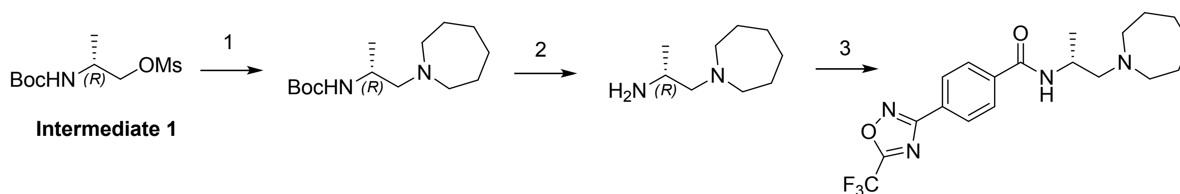
Following **method C** from *tert*-butyl ((2*R*)-1-(3-(difluoromethoxy)piperidin-1-yl)propan-2-yl)carbamate (234 mg, 0.76 mmol). The *title compound* was obtained as a pale yellow foam (210 mg, 98%).

Step 4: *N*-((*R*)-1-((*R*)-3-(difluoromethoxy)piperidin-1-yl)propan-2-yl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide and *N*-((*R*)-1-((*S*)-3-(difluoromethoxy)piperidin-1-yl)propan-2-yl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide.

Following **method A** from 4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzoic acid (178 mg, 0.69 mmol) and (2*R*)-1-(3-(difluoromethoxy)piperidin-1-yl)propan-2-amine dihydrochloride (210 mg, 0.76 mmol). The crude material was purified by preparative HPLC and chiral preparative SFC to give the *title compounds* as white solids. *N*-((*R*)-1-((*abs*)-3-(difluoromethoxy)piperidin-1-yl)propan-2-yl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide (50 mg): LCMS (ES+) calc'd for $C_{19}H_{21}F_5N_4O_3$ $(M+H)^+$: 449, found: 449; RT 2.74 min (*Analytical Method 1*); ^{1}H NMR δ (ppm) (400 MHz, DMSO-d_6) 8.40 (d, J = 8.3 Hz, 1H), 8.17 (d, J = 8.5 Hz, 2H), 8.05 (d, J = 8.5 Hz, 2H), 6.72 (t, J = 76.3 Hz, 1H), 4.27 - 4.16 (m, 1H), 4.10 - 4.01 (m, 1H), 2.91 (dd, J = 3.0, 10.4 Hz, 1H), 2.68 - 2.60 (m, 1H), 2.35 (dd, J = 7.0, 12.4 Hz, 1H), 2.19 - 2.05 (m, 2H), 1.91 - 1.82 (m, 1H), 1.71 - 1.63 (m, 1H), 1.47 - 1.27 (m, 2H), 1.16 (d, J = 6.5 Hz, 3H), 1H obscured by DMSO peak. *N*-((*R*)-1-((*abs*)-3-(difluoromethoxy)piperidin-1-yl)propan-2-yl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide (53 mg): LCMS (ES+) calc'd for $C_{19}H_{21}F_5N_4O_3$ $(M+H)^+$: 449, found: 449; RT 2.72 min (*Analytical Method 1*); ^{1}H NMR δ (ppm) (400 MHz, DMSO-d_6) 8.40 (1d, J = 8.2 Hz, 1H), 8.17 (d, J = 8.5 Hz, 2H), 8.05 (d, J = 8.5 Hz, 2H), 6.73 (t, J = 76.4 Hz, 1H), 4.27 - 4.16 (m, 1H), 4.11 - 4.02 (m, 1H), 2.92 (dd, J = 3.3, 10.6 Hz, 1H), 2.72 - 2.67 (m, 1H), 2.35 (dd, J = 6.7, 12.5 Hz, 1H), 2.15 - 2.02 (m, 2H), 1.92 - 1.83 (m, 1H), 1.70 - 1.62 (m, 1H), 1.46 - 1.25 (m, 2H), 1.16 (d, J = 6.7 Hz, 3H), 1 proton obscured by DMSO peak; ^{13}C NMR δ (ppm) (100 MHz, DMSO-d_6) 168.46, 165.68 (q, J=44 Hz), 165.16, 138.83, 128.84, 127.82, 127.01, 117.62 (t, J=253 Hz), 116.24 (q, J=272 Hz), 72.31, 63.14, 58.54, 53.08, 43.26, 30.90, 23.18, 19.39; HRMS calc'd for $C_{19}H_{21}F_5N_4O_3$ $(M+H)^+$: 449.1607, found 449.1624.

Compound 21

(*R*)-*N*-(1-(Azepan-1-yl)propan-2-yl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide



Step 1: (*R*)-*tert*-Butyl (1-(azepan-1-yl)propan-2-yl)carbamate

Following **method D** from **intermediate 1** (1.0 g, 3.95 mmol) and azepane (890 µL, 7.91 mmol) gave the *title compound* as a yellow oil (489 mg, 48%).

Step 2: (*R*)-1-(Azepan-1-yl)propan-2-amine

To a solution of (*R*)-*tert*-butyl (1-(azepan-1-yl)propan-2-yl)carbamate (489 mg, 1.91 mmol) in anhydrous DCM (40 mL) was added TFA (0.8 mL). The reaction mixture was stirred at r.t for 2 h. The mixture was concentrated under reduced pressure and passed through an SCX cartridge eluting with 0-10% 7 M methanolic ammonia in DCM. The basic fractions were concentrated to give the *title compound* as a yellow oil (310 mg, >99%).

Step 3: (*R*)-*N*-(1-(Azepan-1-yl)propan-2-yl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide

Following **method A** from 4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzoic acid (427 mg, 1.65 mmol) and (*R*)-1-(azepan-1-yl)propan-2-amine (310 mg, 1.91 mmol). Purification by preparative-HPLC and SFC gave the *title compound* as a white solid (45 mg). LCMS (ES+) calc'd for $C_{19}H_{23}F_3N_4O_2$ $(M+H)^+$: 397, found: 397; RT 2.70 min (*Analytical Method 1*); ^1H NMR δ (ppm) (400 MHz, DMSO-d_6) 8.35 (d, J = 8.2 Hz, 1H), 8.17 (d, J = 8.7 Hz, 2H), 8.05 (d, J = 8.5 Hz, 2H), 4.17 - 4.09 (m, 1H), 2.68 - 2.59 (m, 5H), 2.45 (dd, J = 7.0, 12.4 Hz, 1H), 1.58 - 1.51 (m, 8H), 1.16 (d, J = 6.7 Hz, 3H); ^{13}C NMR δ (ppm) (100 MHz, DMSO-d_6) 168.47, 165.67 (q, J=44 Hz), 165.09, 138.91, 128.82, 127.82, 126.97, 116.24 (q, J=272 Hz), 62.96, 55.49, 44.35, 28.63, 27.11, 19.19; HRMS calc'd for $C_{19}H_{23}F_3N_4O_2$ $(M+H)^+$: 397.1846, found 397.1849.

4) Supplemental Tables

SPR				
Compound	$K_{D(SS)}$	K_{on}	K_{off}	t_R
2	0.044	5.0 x 10^5	0.007	2.5
Positive control[a]	0.11	1.5 x 10^5	0.016	1.0

SI Table 1. SPR data for compound **2** and positive control. The K_D (μM; steady state), K_{on} ($M^{-1}s^{-1}$) and K_{off} (s^{-1}) values are shown as geometric mean (standard deviation <50% of the mean) and are derived from at least two independent binding sensorgrams; t_{R} = residence time (minutes; $1/K_{off}$). [a](1*S*,2*R*,3*R*)-2-(8-Chloro-2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-3-(2-fluorophenyl)-N-hydroxycyclopropanecarboxamide (Compound **25f** from patent WO2012103008)

Target	M1	M2	M3	M4	M5	σ_1	L-Ca^{2+} (diltiazem)	Na^+ channel
Percent of specific binding (10 μM)	91	87	86	79	84	77	70	63

SI Table 2. Cerep data for compound **12**. Hits are shown that displayed >50% of control specific binding (screened at 10 μM).

Target	M1	M2	M3	M4	hERG IonWorks™
K_d (μM)	5.2	13	19	31	3.9

SI Table 3. Compound **12**: K_d data for muscarinic receptors M1-M4 (Cerep), and hERG (IonWorks™). Geometric mean of three experiments; standard deviations are <25% of the mean.

Assay	**Substrate**
HDAC1	Ac-Arg-Gly-Lys(Ac)

HDAC2	Ac-Arg-Gly-Lys(Ac)
HDAC3	Boc Lys(Ac)
HDAC8	Boc Lys(TFA)
HDAC6	Boc Lys(Ac)
HDAC4cat	Boc Lys(TFA)
HDAC5cat	Boc Lys(TFA)
HDAC7	Boc Lys(TFA)
HDAC9	Boc Lys(TFA)
Class I / IIb cellular (Jurkat)	Boc Lys(Ac)
Class I cellular (HEK293)	H4K12
Class IIb cellular (HEK293)	a-Tubulin
Class IIa cellular (Jurkat)	Boc Lys(TFA)
Class IIa cellular (HEK293)	Boc Lys(TFA)

SI Table 4. Assay-substrate combinations for biochemical and cellular assays.

PK PARAMETER	UNITS	BLOOD		BRAIN	MUSCLE
		IV[a]	PO[b]	PO[b]	PO[b]
$AUC_{(0\text{-last})}$	nM h	2000	8400	24000	6800
$AUC_{(0\text{-infi})}$	nM h	2000	8400	24000	7000
AUC_{norm}	nM h kg/mg	620	730	2100	600
Oral Bioavailability (F)	%	NA	≥100	NA	NA
Observed C_{max}	nM	NA	5100	13000	4100
Observed C_{max}Norm	nM kg/mg	NA	440	1100	350
Observed T_{max}	h	NA	0.5	1	0.5
Clearance (CL)	L/h/kg	4.2	NA	NA	NA
Volume of Distribution at Steady State (Vd_{ss})	L/kg	3.8	NA	NA	NA
Mean Residency Time (MRT)	h	0.90	NA	NA	NA
Half-life ($t_{½}$)	h	0.72	0.93	0.88	0.72
Half-life Regression Time Points	h	1, 2, 4	1, 2, 4, 8	2, 4, 8	1, 2, 4

SI Table 5. Summary of pharmacokinetic parameters following a single IV and PO dose of **12** to fed, male, C57BL/6 mice. NA: not applicable PK parameters due to route and/or matrix. [a] Compound **12** dosed at 3.3 mg/kg, 0.66 mg eq./mL in DMSO:PEG400:water (10:50:40 v/v/v). [b] Compound **12** dosed at 11.5 mg/kg, 1.15 mg/mL in 10% hydroxypropyl-β-cyclodextrin in water (solution).

Time-point (h)	Brain: Blood ratio	Muscle: Blood ratio
	Mean ± SD	Mean ± SD
0.083	0.25 ±ND	ND
0.25	1.5 ± 0.14	0.54 ± 0.11
0.5	2.4 ± 0.5	0.8 ± 0.13
1	3.4 ± 0.17	0.93 ± 0.18
2	3.4 ± 0.39	0.94 ± 0.1
4	3.1 ± 0.45	0.94 ± 0.2
8	2 ± 0.32	ND
16	1.2	ND

SI Table 6. Tissue-to-blood concentration ratios following PO (11.5 mg/kg) administration of compound **12** to fed, male, C57BL/6 mice. ND: Not determined as levels in one or both matrices were below the lower limit of quantification. Mean was calculated when at least two concentration ratios were available. SD was calculated when at least three concentration ratios were available.

PK PARAMETER	UNITS	Blood			Brain			Muscle		
		PO	PO	PO	PO	PO	PO	PO	PO	PO
		10 mg/kg	30 mg/kg	100 mg/kg	10 mg/kg	30 mg/kg	100 mg/kg	10 mg/kg	30 mg/kg	100 mg/kg
AUC(0-last)	nM h	4700	21000	72000	10000	75000	470000	5300	27000	140000
AUC(0-inf)	nM h	4900	21000	79000	10000	75000	470000	5300	27000	140000
AUCnorm	nM h kg/mg	490	720	790	1000	2500	4700	530	900	1400
Observed Cmax	nM	2500	7400	18000	6300	41000	150000	2600	11000	50000
Observed CmaxNorm	nM kg/mg	250	250	180	630	1400	1500	260	380	500
Observed Tmax	h	0.5	0.5	0.5	1.0	0.5	0.5	1.0	0.5	0.5
Half-life (t½)	h	0.73	1.4	3.2	0.8	1.1	1.8	0.91	1.1	2.2
Half-life Regression Time Points	h	1,2,4	1,2,4,8	1,2,4,8,12	1,2,4,8	1,2,4,8,12	1,2,4,8,12	2,4,8	1,2,4,8,12	1,2,4,8,12

SI Table 7. Summary of pharmacokinetic parameters following a single PO doses of **12** at 10, 30 or 100 mg eq./kg to fed male C57Bl/6 mice.

Formulation	Duration (days)	Target concentration (mg/mL)	Concentration in unfiltered formulation (mg/mL)	Concentration in filtered formulation (mg/mL)	% in solution (filtered concentration vs unfiltered)	Stability (% intact in unfiltered formulation vs day0)	Macroscopic analysis
10% (w/v) hydroxypropyl-β-cyclodextrin (HP-β-CD) in citrate buffer pH 5.0	0	2.5	2.47	2.44	98.8	100	Clear solution
	1	2.5	2.45	2.51	102.6	99.1	Clear solution
	7	2.5	2.50	2.58	103.4	101.0	Clear solution
	15	2.5	2.43	2.41	99.6	98.1	Clear solution
	30	2.5	2.36	2.38	101.1	95.4	Clear solution

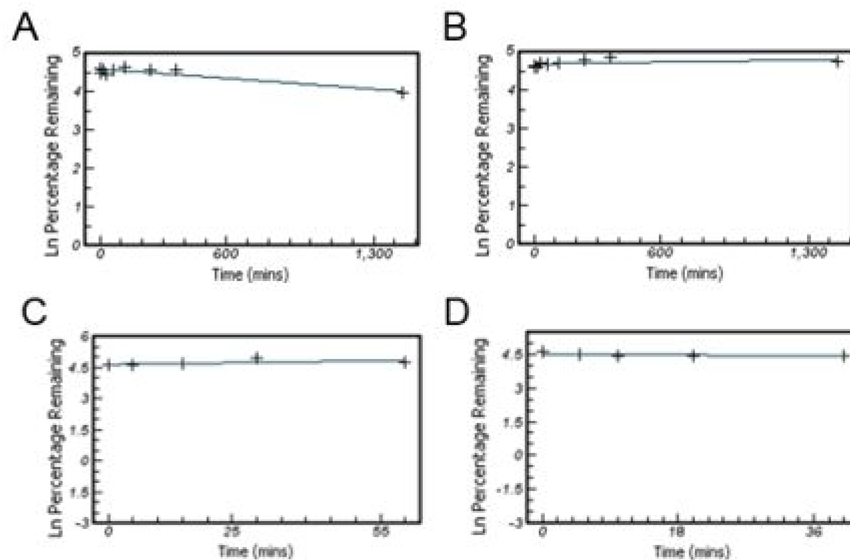
SI Table 8. Vehicle stability studies of compound **12**.

Passive signs
Locomotor activity (0-1A- 1B)
Shallow respiration (0-2)
Ataxia (0-2)
Head tremor (0-2)
Body tremor (0-2)
Tail tremor (0-2)
Straub tail (0-2)
Seizure (0-2)
Stereotypy (0-2)
Flat body posture (0-2)
Retropulsion (0-2)
Shivering (0-2)

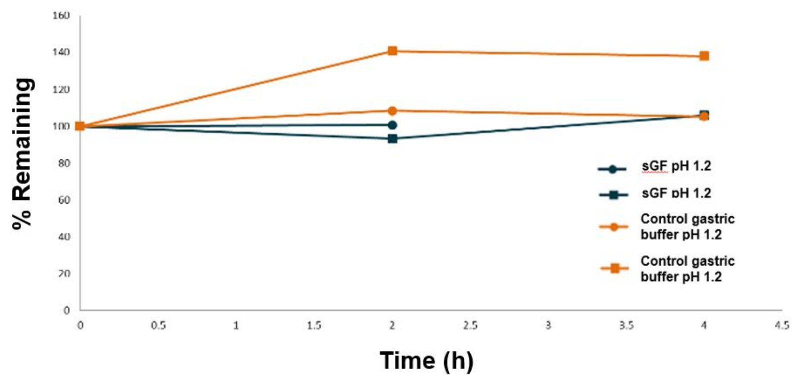
Manipulations
Flaccidity (0-2)
Struggle by tail (0-1A-1B)
Body temperature (deg C)
Irritability (0-2)
Provoked biting (0-1A-1B)
Loss of paw pull (0-2)
Seizure by tail (0-2)
Touch escape (0-1A-1B)

SI Table 9. Neurological, and motor evaluation scoring system for tolerability study

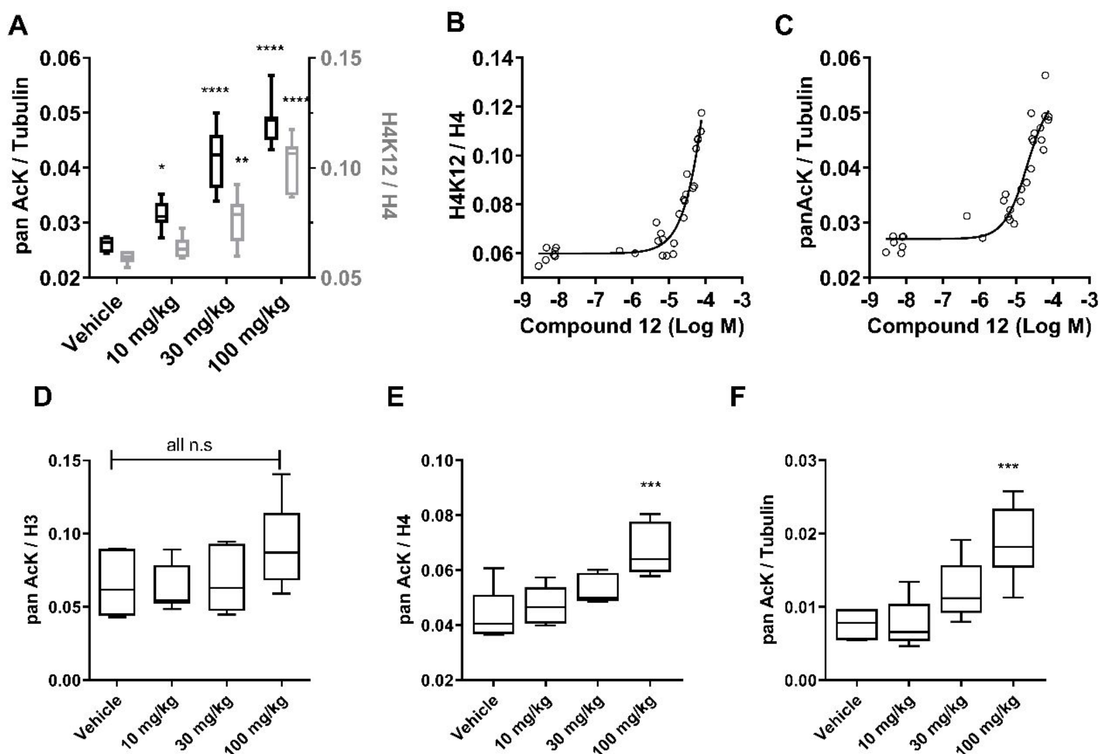
5) Supplemental Figures



SI Figure 1. Stability studies of compound **12** in mouse plasma (**A**), bovine serum albumin (**B**), mouse blood (**C**) and mouse liver microsomes without cofactor (**D**).



SI Figure 2. Stability studies of compound **12** in simulated gastric fluid (sGF)



SI Figure 3. Terminal Class I/IIb biomarker analysis from pooled WT and R6/2 mouse brain tissue dosed *p.o.* with 12 *b.i.d.* for 14.5 days. Brain tissue collected 2 h post final dose. H4K12 acetylation was normalized to H4 histone while the pan-AcK signal was normalized to tubulin levels. **A**. Dose-dependent increase in both pan-AcK (black boxes) and specific H4K12 acetylation (grey boxes) compared to vehicle-treated mice (dot blot technique). No difference in acetylation levels were detected between WT and R6/2 mice (data is pooled by genotype). Boxes indicate median and interquartile range, whiskers indicate minimum-maximum values. **B-C**. Correlation of total brain exposure of 12 to increase in H4K12 acetylation (**B**) or global acetylation (**C**) at 2h post last dose from all drug treated mice. Data in B-C are fit by a non-linear sigmoidal dose response fit (variable 4 parameter fit) to estimate EC_{50}. **D-E** Western blot quantitation of pan AcK band corresponding to H3 (**D**) and H4 (**E**) normalized to H3 and H4 total protein band intensity respectively. **F** Western blot quantitation of pan AcK band corresponding to tubulin molecular weight normalized to total tubulin protein band intensity. Statistics performed by ordinary One-way ANOVA with Dunnett's multiple comparison test: $p < 0.05$*, $p < 0.001$***.

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