Supporting Information

SAR Development of Lysine-Based Irreversible Inhibitors of Transglutaminase 2 for Huntington's Disease

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Contents

Included is a table of inhibitors (Table 1) resynthesized from Marrano, et al., *Bioorg. Med. Chem.* **2001**, *9*, 1923-1928, and tested in-house. The data provides a rank ordering of compound potency that is in close agreement with that published. A general method for preparation of the carbamates in Table 1 is exemplified by the synthesis of **3e**. This is followed by a disclosure of general experimental details, experimental methods for the synthesis of key compound **4l**, and methods for the synthesis of compounds **5**, **6**, and **7**. The synthesis of **4l** is representative of the methods used to prepare the compounds listed in Table 2. In addition, experimental details are provided for the solubility, microsomal stability, MDCK-MDR1, and plasma stability assays.

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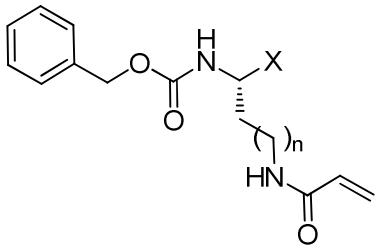
Table 1. TG2 SAR of Cbz-Protected Diamino Acid Acrylamides[a]



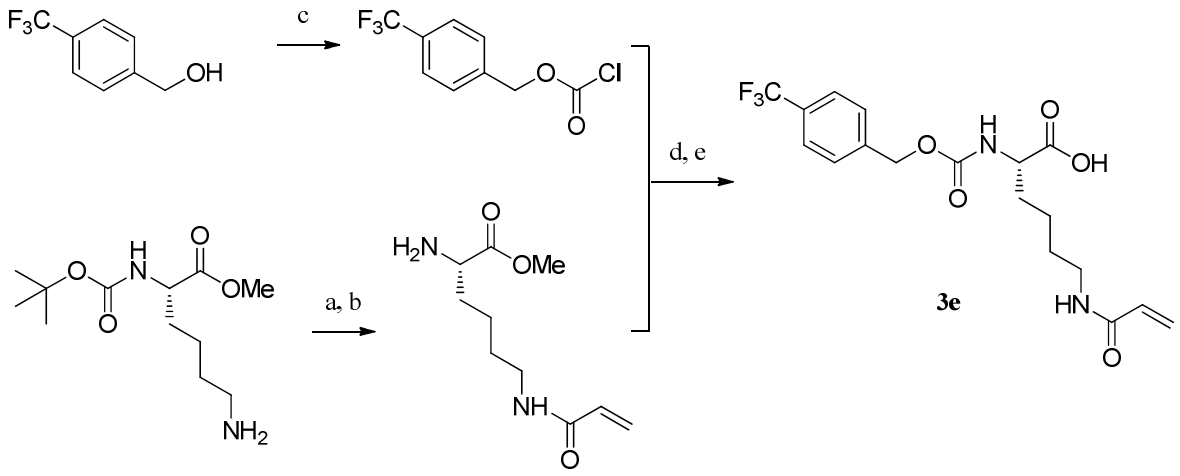
Cmpd No.	X	n	TG2 IC_{50} ± SD (μM)
	$CONHCH_2CO_2H$	3	1.5 ± 0.020
1a	CO_2H	3	2.7 ± 0.32
(*R*)-**1a**	CO_2H	3	36
	CO_2H	2	25
	CO_2H	1	> 80
	CO_2H	0	> 80

[a]Values accompanied by standard deviation were averaged from at least two independent experiments; they were otherwise obtained in a single determination.

General Experimental details

Commercially available reagents and solvents (HPLC grade) were used without further purification. ^1H and ^{13}C NMR spectra were recorded on a Bruker DRX 500MHz spectrometer or Bruker DPX 250MHz spectrometer in deuterated solvents. Chemical shifts (δ) are in parts per million. Thin-layer chromatography (TLC) analysis was performed with Kieselgel 60 F_{254} (Merck) plates and visualized using UV light.

Analytical HPLC-MS was performed on Shimadzu LCMS-2010EV systems using reverse phase Atlantis dC18 columns (3 μm, 2.1 X 50 mm), gradient 5-100% B (A = water/ 0.1% formic acid, B = acetonitrile/ 0.1% formic acid) over 3 min, injection volume 3 μL, flow = 1.0 mL/min. UV spectra were recorded at 215 nm using a Waters 2788 dual wavelength UV detector. Mass spectra were obtained over the range m/z 150 to 850 at a sampling rate of 2 scans per second using Waters LCT or analytical HPLC-MS on Shimadzu LCMS-2010EV systems using reverse phase Water Atlantis dC18 columns (3μm, 2.1 X 100 mm), gradient 5-100% B (A = water/ 0.1% formic acid, B = acetonitrile/ 0.1% formic acid) over 7 min, injection volume 3 μL, flow = 0.6 mL/min. UV spectra were recorded at 215 nm using a Waters 2996 photo diode array. Data were integrated and reported using Shimadzu psiport software. All compounds display purity of >95% as determined by this method, unless stated otherwise.



[a]Reagents and Conditions: (a) Acyloyl chloride, 1N NaOH, THF (aq), 0 °C, 30 min; (b) HCl, MeOH, r.t., 20 h; (c) Phosgene, toluene, r.t., 10 mins; (d) DIPEA, DMF, r.t., 18 h; (e) 2N NaOH, THF (aq), r.t., 18 h

Scheme 1. Description of the synthesis of compound **3e**

(2*S*)-6-(Prop-2-enamido)-2-[({[4-(trifluoromethyl)phenyl]methoxy}carbonyl)amino]hexanoic acid (3e)

(2*S*)-2-{[(tert-Butoxy)carbonyl]amino}-6-(prop-2-enamido)hexanoic acid

Acryloyl chloride (1.6 mL, 20.0 mmol) as a solution in THF (10 mL) and 1M NaOH (3 mL) were added drop wise concurrently over 20 minutes to a cooled (0 °C), stirred solution of Boc-Lysine (5.0 g, 20.0 mmol) in 1M NaOH (3 mL) and the mixture was stirred for a further 5 minutes. After this time the reaction mixture was quenched by the addition of saturated NaCl solution (5 mL) and acidified to pH 1 with concentrated HCl before being extracted with ethyl acetate (3 x 10 mL). The combined organic extracts were washed sequentially with saturated sodium bicarbonate (10 mL) and brine (10 mL) before being

dried ($MgSO_4$), filtered, and concentrated. The resulting residue was purified using flash column chromatography (elution; 5% Methanol/EtOAc to 10% Methanol/EtOAc) to give the title compound (2.0 g, 33% yield) as a colorless oil. δ_H (500 MHz, DMSO) *m/z* (ES^+) $(M+Na)^+$ 323.

Methyl (2*S*)-2-amino-6-(prop-2-enamido)hexanoate

Concentrated HCl (3 mL) was added dropwise to a stirred solution of (2*S*)-2-{[(tert-butoxy)carbonyl]amino}-6-(prop-2-enamido)hexanoic acid (2.0 g, 6.7 mmol) in methanol (30 mL) and the resulting solution was stirred at room temperature for 18 hours. The resulting mixture was concentrated to give the title compound (1.3 g, 90% yield) as a colourless oil. *m/z* (ES^+) $(M+H)^+$ 215.

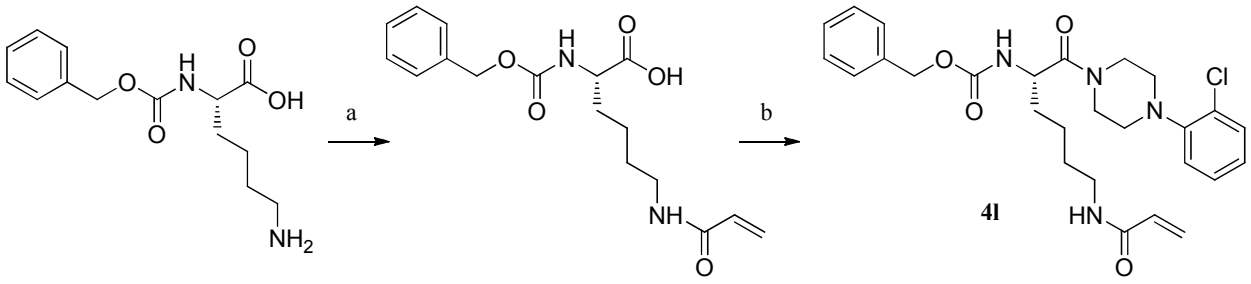
Methyl (2*S*)-6-(prop-2-enamido)-2-[({[4-(trifluoromethyl)phenyl]methoxy}carbonyl)amino]hexanoate

Carbonyl dichloride (0.5 mL of a 20% solution in toluene) was added dropwise over 5 minutes to a stirred solution of 4-trifluoromethylbenzyl alcohol (0.2 g, 1.2 mmol) in toluene (1 mL) and the resulting solution was stirred at room temperature for 30 minutes. After this time, the reaction mixture was concentrated, re-dissolved in DMF (2 mL) and added dropwise to a stirred solution of methyl (2*S*)-2-amino-6-(prop-2-enamido)hexanoate (0.2 g, 0.9 mmol) and diisopropylethyl amine (0.3 mL, 1.8 mmol) in DMF (3 mL) and the resulting mixture stirred at room temperature for 3 hours. After this time, the mixture was concentrated and the resulting residue purified using preparative HPLC to give the title compound (0.05 g, 13% yield) as a white powder. *m/z* (ES^+) $(M+Na)^+$ 439.

(2*S*)-6-(Prop-2-enamido)-2-[({[4-(trifluoromethyl)phenyl]methoxy}carbonyl)amino]hexanoic acid (3e)

2M sodium hydroxide (1mL, 2.0mmol) was added in one portion to a stirred solution of methyl (2*S*)-6-(prop-2-enamido)-2-[({[4-(trifluoromethyl)phenyl]methoxy}carbonyl)amino]hexanoate (0.05 g, 0.12 mmol) in THF and the resulting mixture stirred at room temperature for 18 hours. After this time, the reaction mixture was acidifed to pH 1 with 2M HCl solution, the mixture was extracted with ethyl acetate (3 x 10 mL) and the organic extracts combined. The organic was dried ($MgSO_4$), filtered and concentrated. The resulting residue was purified using flash column chromatography (elution; 5% Methanol/DCM to 10% Methanol/DCM) to give the title compound as a colourless oil. δ_H (250 MHz, DMSO) 12.57 (s, 1H), 8.08 (s, 1H), 7.89 – 7.41 (m, 5H), 6.33 – 5.89 (m, 2H), 5.55 (dd, *J* = 2.60, 9.73 Hz, 1H), 5.13 (s, 2H), 4.04 – 3.80 (m, 1H), 3.20 – 2.96 (m, 2H), 1.86 – 1.12 (m, 6H). δ_C (126 MHz, DMSO) 173.93, 164.47, 156.02, 142.04, 131.87, 127.91, 125.32, 125.20, 124.87, 64.49, 53.87, 39.52, 38.33,

30.43, 28.68, 23.16. *m/z* (ES^+) $(M+H)^+$ 403. HRMS (ES^+) *m/z* 403.1494 (403.1485 Calcd for $C_{18}H_{21}F_3N_2O_5$ M+H).



[a]Reagents and Conditions: (a) Acryloyl chloride, 1M NaOH, THF, H_2O, 0 °C, 30 mins; (b) DIPEA, HATU, HOBt, 1-(2-chlorophenyl)piperazine, r.t., 1h.

Scheme 2. Description of the synthesis of compound **4l**

Benzyl *N*-[(2*S*)-1-[4-(2-chlorophenyl)piperazin-1-yl]-1-oxo-6-(prop-2-enamido)hexan-2-yl]carbamate (4l).

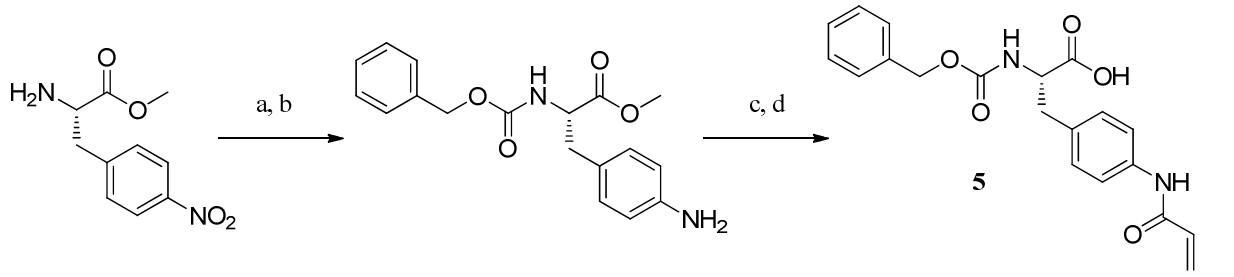
(2*S*)-2-{[(Benzyloxy)carbonyl]amino}-6-(prop-2-enamido)hexanoic acid

Acryloyl chloride (0.14 mL, 2.0 mmol) solution in THF (1 mL) and 1M NaOH (2 mL) were added drop wise concurrently over 20 minutes to a cooled (0 °C), stirred solution of Cbz-Lysine (0.5 g, 2.0 mmol) in 1M NaOH (2 mL) and the mixture was stirred for a further 5 minutes. After this time the reaction mixture was quenched by the addition of saturated NaCl solution (5 mL) and acidified to pH 1 with concentrated HCl before being extracted with ethyl acetate (3 x 10 mL). The combined organic extracts were washed sequentially with saturated sodium bicarbonate (10 mL) and brine (10 mL) before being dried ($MgSO_4$), filtered, and concentrated to give the title compound (0.55 g, 93% yield) as a colorless oil. δ_H (500 MHz, DMSO) 8.06 (s, 1H), 7.54 (d, *J* = 7.85 Hz, 1H), 7.37 – 7.34 (m, 4H), 6.26 – 6.14 (m, 1H), 6.11 – 5.99 (m, 1H), 5.60 – 5.45 (m, 1H), 5.03 (s, 2H), 4.03 (q, *J* = 7.11 Hz, 1H), 3.96 – 3.84 (m, 1H), 3.09 (q, *J* = 6.47 Hz, 2H), 1.76 – 1.51 (m, 2H), 1.42 – 1.28 (m, 5H). *m/z* (ES^+) $(M+H)^+$ 335.

Benzyl *N*-[(2*S*)-1-[4-(2-chlorophenyl)piperazin-1-yl]-1-oxo-6-(prop-2-enamido)hexan-2-yl]carbamate (4l)

Diisopropylethyl amine (0.91 mL, 5.4 mmol) was added portion wise over 5 minutes to a stirred solution of (2*S*)-2-{[(benzyloxy)carbonyl]amino}-6-(prop-2-enamido)hexanoic acid (0.45 g, 1.4 mmol), HATU (0.76

g, 2.0 mmol), HOBt (0.27 g, 2.0 mmol), and 1-(2-chlorophenyl)piperazine (0.26 g, 1.34 mmol) in DMF (5 mL). The resulting mixture was stirred at room temperature under a nitrogen atmosphere for 2 hours. After this time, the reaction mixture was concentrated and the resulting residue partitioned between DCM (50 mL) and 1M HCl (10 mL). The organic layer was separated and washed sequentially with saturated sodium bicarbonate (10 mL) and brine (10 mL) before being dried ($MgSO_4$), filtered, and concentrated. The crude residue was then purified using flash column chromatography (elution: 100% methylene chloride to 2% methanol, 98% methylene chloride) and the resulting oil suspended in heptanes (5 mL) and sonicated for 30 seconds. The resulting solid precipitate was collected by filtration and dried under vacuum to give the title compound (0.12 g, 18% yield) as an off-white solid. δ_H (500 MHz, DMSO) 8.06 (t, *J* = 5.50 Hz, 1H), 7.51 (d, *J* = 8.17 Hz, 1H), 7.43 (d, *J* = 7.84 Hz, 1H), 7.38 – 7.24 (m, 6H), 7.18 – 7.02 (m, 2H), 6.19 (dd, *J* = 10.13, 17.08 Hz, 1H), 6.05 (dd, *J* = 2.22, 17.09 Hz, 1H), 5.55 (dd, *J* = 2.19, 10.12 Hz, 1H), 5.09 – 4.98 (m, 2H), 4.50 – 4.38 (m, 1H), 3.74 – 3.49 (m, 4H), 3.11 (q, *J* = 6.62 Hz, 2H), 2.93 (d, *J* = 18.87 Hz, 4H), 1.57 (ddd, *J* = 7.82, 13.17, 27.79 Hz, 2H), 1.47 – 1.27 (m, 4H). δ_C (126 MHz, DMSO) 170.22, 164.45, 155.96, 148.55, 137.11, 131.89, 130.38, 128.36, 128.15, 127.83, 127.78, 124.87, 124.34, 121.16, 65.40, 51.25, 50.84, 50.52, 45.20, 41.78, 38.30, 31.01, 28.83, 22.80. *m/z* (ES^+) $(M+H)^+$ 513/515. HRMS (ES^+) *m/z* 513.2251 (513.2269 Calcd for $C_{27}H_{33}ClN_4O_4$ M+H).



[a]Reagents and Conditions: (a) N-(Benzyloxycarbonyloxy)succinimide, DIPEA, DMF, r.t., 2h; (b) Fe powder, EtOH/H_2O (5:1), sat NH_4Cl (aq), 75 °C, 2h; (c) Acryloyl chloride, DIPEA, THF, r.t., 2h; (d) LiOH, THF/H_2O (2:1), r.t., 2h.

Scheme 3. Synthetic route for the synthesis of compound **5**

(*S*)-3-(4-Acrylamidophenyl)-2-(((benzyloxy)methyl)amino)propanoic acid (5).

(*S*)-Methyl 3-(4-aminophenyl)-2-(((benzyloxy)carbonyl)amino)propanoate

Diisopropylethylamine (1.4 mL, 8.4 mmol) was added portion wise over 5 minutes to a stirred suspension of (*S*)-methyl 2-amino-3-(4-nitrophenyl)propanoate (2.0 g, 7.7 mmol) and N-(benzyloxycarbonyloxy)succinimide (2.1 g, 8.4 mmol) in DMF (15 mL) and the resulting mixture was

stirred at room temperature under a nitrogen atmosphere for 2 hours. After this time, the reaction mixture was concentrated and the resulting residue was purified using flash column chromatography (elution: 100% methylene chloride to 2% methanol, 98% methylene chloride) to give the title compound (2.7 g, 98% yield) as a white powder. *m/z* (ES^+) $(M+H)^+$ 359.

(*S*)-Methyl 3-(4-aminophenyl)-2-(((benzyloxy)carbonyl)amino)propanoate

Iron powder (0.3 g, 5.6 mmol) was added in one portion to a stirred solution of (*S*)-methyl 3-(4-aminophenyl)-2-(((benzyloxy)carbonyl)amino)propanoate (1.0 g, 2.8 mmol) and saturated ammonium chloride (1 mL) in an ethanol-water mixture (5:1, 15 mL). The resulting mixture was then heated to 75 ^oC and stirred at this temperature for 2 hours. After this time, the reaction mixture was cooled to room temperature and filtered through a pad of celite, the celite was then washed with DCM (50 mL) and the filtrate concentrated to give the title compound (0.91 g, 99% yield) as a white solid. *m/z* (ES^+) $(M+H)^+$ 329.

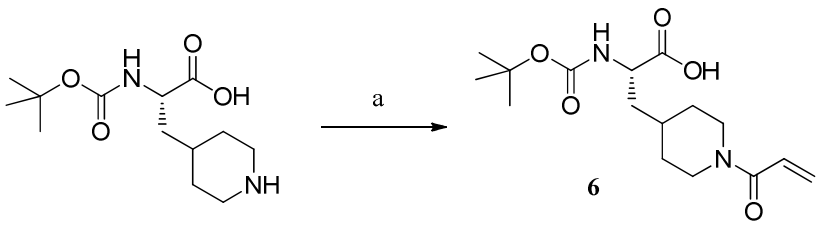
(*S*)-Methyl 3-(4-acrylamidophenyl)-2-(((benzyloxy)methyl)amino)propanoate

Acryloyl chloride (0.14 mL, 1.68 mmol) was added drop wise over 5 minutes to a stirred solution of (*S*)-methyl 3-(4-aminophenyl)-2-(((benzyloxy)carbonyl)amino)propanoate (0.5 g, 1.5 mmol) and diisopropylethylamine (0.22 mL, 1.68 mmol) in THF (7 mL). The resulting mixture was then stirred at room temperature under a nitrogen atmosphere for 2 hours. After this time, the reaction mixture was concentrated and the resulting residue purified using flash column chromatography (elution: 100% methylene chloride to 2% methanol, 98% methylene chloride) to give the title compound (0.32 g, 55% yield) as a white solid. *m/z* (ES^+) $(M+H)^+$ 383.

(*S*)-3-(4-Acrylamidophenyl)-2-(((benzyloxy)methyl)amino)propanoic acid (5)

Lithium hydroxide (0.01 g, 0.43 mmol) was added in one portion to a stirred solution of (*S*)-methyl 3-(4-acrylamidophenyl)-2-(((benzyloxy)methyl)amino)propanoate (0.15 g, 0.39 mmol) in a solution of THF-water (2:1, 3 mL) and the resulting mixture was then stirred at room temperature for 2 hours. After this time, the reaction mixture was diluted with saturated ammonium chloride solution (5 mL) and extracted with methylene chloride (3 x 20 mL). The combined organic extracts were dried ($MgSO_4$), filtered, and concentrated to give the title compound (0.03 g, 24% yield) as a white solid. δ_H (500 MHz, DMSO) 12.75 (s, 1H), 10.11 (s, 1H), 7.65 (d, *J* = 8.42 Hz, 1H), 7.57 (d, *J* = 8.38 Hz, 2H), 7.37 – 7.24 (m, 5H), 7.20 (d, *J* = 8.41 Hz, 2H), 6.43 (dd, *J* = 10.14, 16.95 Hz, 1H), 6.25 (dd, *J* = 1.92, 16.98 Hz, 1H), 5.75 (dd, *J* = 1.91, 10.12

Hz, 1H), 4.97 (s, 2H), 4.20 – 4.10 (m, 1H), 3.02 (dd, *J* = 4.37, 13.82 Hz, 1H), 2.79 (dd, *J* = 10.67, 13.67 Hz, 1H). δ_C (126 MHz, DMSO) 173.56, 163.19, 156.19, 137.61, 137.20, 133.15, 132.10, 129.63, 128.49, 127.91, 127.70, 126.94, 119.33, 65.43, 55.80, 36.18. *m/z* (ES^+) $(M+H)^+$ 369. HRMS (ES^+) *m/z* 369.1443 (369.145 Calcd for $C_{20}H_{20}N_2O_5$ M+H).

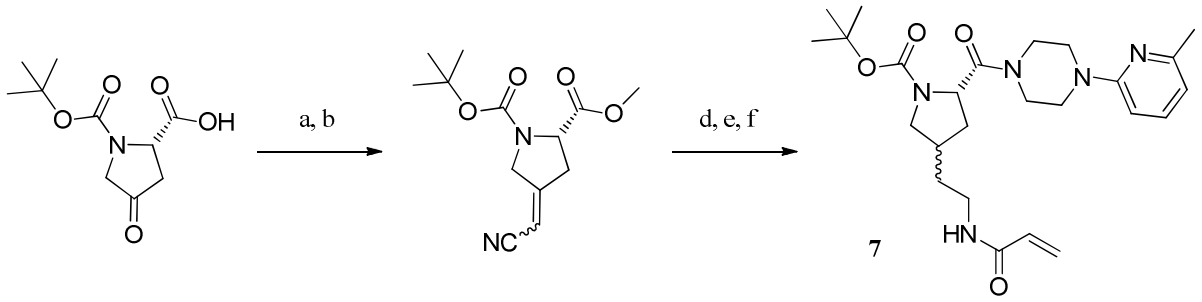


[a]Reagents and Conditions: (a) Acryloyl chloride, 1M NaOH, THF, H_2O, 0 °C, 30 mins.

Scheme 4. Description of the synthesis of piperidine **6**

(*S*)-3-(1-acryloylpiperidin-4-yl)-2-((*tert*-butoxycarbonyl)amino)propanoic acid (6).

Acryloyl chloride (0.06 mL, 0.74 mmol) solution in THF (1 mL) and 1M NaOH (2 mL) were added drop wise concurrently over 20 minutes to a cooled (0 °C), stirred solution of (*S*)-2-((*tert*-butoxycarbonyl)amino)-3-(piperidin-4-yl)propanoic acid (0.2 g, 0.74 mmol) in water (2 mL) and the mixture was stirred for a further 1 hour. After this time the reaction mixture was quenched by the addition of saturated NaCl solution (5 mL) and acidified to pH 1 with concentrated HCl before being extracted with ethyl acetate (3 x 10 mL). The combined organic extracts were washed sequentially with saturated sodium bicarbonate (10 mL) and brine (10 mL) before being dried ($MgSO_4$), filtered, and concentrated. The resulting residue was purified using preparative HPLC to give the title compound (0.01 g, 4% yield) as a white powder. δ_H (500 MHz, $CDCl_3$) 6.56 (dd, *J* = 10.62, 16.84 Hz, 1H), 6.26 (d, *J* = 16.85 Hz, 1H), 5.76 – 5.60 (m, 1H), 4.99 (s, 1H), 4.65 (s, 1H), 4.38 (s, 1H), 3.98 (s, 1H), 3.04 (s, 1H), 2.63 (s, 1H), 1.91 (s, 1H), 1.75 (d, *J* = 19.73 Hz, 3H), 1.58 (s, 1H), 1.45 (s, 9H), 1.29 – 1.08 (m, 1H). *m/z* (ES^+) $(M+Na)^+$ 349.



[a]Reagents and Conditions: (a) $TMSCHN_2$, DCM, MeOH, r.t., 18h; (b) $(EtO)_2P(O)CH_2CN$, LHMDS, THF, 0 °C to r.t., 1h; (c) PtO_2, H_2, EtOH, $CHCl_3$, 45mins; (d) Acryloyl chloride, DIPEA, THF, r.t., 18h; (e) LiOH, THF, H_2O, r.t., 18h; (f) 1-(6-Methylpyridin-2-yl)piperazine, HATU, HOBt, DIPEA, DMF, r.t., 18h.

Scheme 5. Synthetic route for the preparation of pyrrolidine **7**

(2*S*)-*tert*-Butyl 4-(2-acrylamidoethyl)-2-(4-(6-methylpyridin-2-yl)piperazine-1-carbonyl)pyrrolidine-1-carboxylate (7).

(*S*)-1-*tert*-Butyl 2-methyl 4-oxopyrrolidine-1,2-dicarboxylate

Trimethylsilane diazomethane (2M solution in heptane, 3.1 mL, 6.2 mmol) was added portion wise over 5 minutes to a cooled (0 °C), stirred solution of (*S*)-1-(*tert*-butoxycarbonyl)-4-oxopyrrolidine-2-carboxylic acid (0.95 g, 4.1 mmol) in a methylene chloride-methanol mixture (10:3, 10 mL) and the mixture was stirred under a nitrogen atmosphere for 10 minutes before being allowed to warm to room temperature and stirred at this temperature for 18 hours. After this time, the reaction was quenched by the addition of acetic acid (1 mL) and the resulting mixture was concentrated to give the title compound (1.0 g, 99% yield) as a yellow oil which was taken on directly without further purification.

(*S*)-1-*tert*-Butyl 2-methyl 4-(cyanomethylene)pyrrolidine-1,2-dicarboxylate

Lithium hexamethyldisilazide(LHMDS, 1 M solution in THF, 4.8 mL, 4.8 mmol) was added drop wise over 5 minutes to a stirred solution of diethyl (cyanomethyl)phosphonate (0.78 mL, 4.8 mmol) in THF (10 mL) and the resulting mixture was stirred under a nitrogen atmosphere for 10 minutes. After this time, the reaction mixture was cannulated into a cold (0 °C), stirred solution of (*S*)-1-*tert*-butyl 2-methyl 4-oxopyrrolidine-1,2-dicarboxylate (1.0 g, 4.4 mmol) in THF (5 mL) and the resulting mixture was stirred for 10 minutes under a nitrogen atmosphere. After this time, the mixture was warmed to room temperature and stirred for a further 45 minutes before being quenched by the addition of saturated ammonium chloride (10 mL). The resulting mixture was extracted using ethyl acetate (4 x 30 mL) and the

combined organic extracts were dried (Na_2SO_4), filtered, and concentrated to give the title compound (1.13 g, 99% yield) as a colorless oil. *m/z* (ES^+) $(M+Na)^+$ 289.

(2*S*)-1-*tert*-Butyl 2-methyl 4-(2-aminoethyl)pyrrolidine-1,2-dicarboxylate

Platinum oxide (0.12 g, 0.43 mmol) was added in one portion to a stirred solution of (*S*)-1-*tert*-butyl 2-methyl 4-(cyanomethylene)pyrrolidine-1,2-dicarboxylate (1.16 g, 4.3 mmol) in an ethanol-chloroform mixture (10:2, 15 mL). The resulting mixture was flushed with nitrogen via a vacuum manifold before being evacuated, flushed with hydrogen, and stirred at room temperature for 3 hours. After this time, the mixture was flushed with nitrogen before being filtered through celite. The bed of celite washed with ethanol (20 mL) and the combined filtrate was concentrated to give the title compound (1.0 g, 83% yield) as a brown oil which was taken on directly without further purification.

(2*S*)-1-*tert*-Butyl 2-methyl 4-(2-acrylamidoethyl)pyrrolidine-1,2-dicarboxylate

Acryloyl chloride (0.43 mL, 5.24 mmol) was added drop wise over 5 minutes to a stirred solution of (2*S*)-1-*tert*-butyl 2-methyl 4-(2-aminoethyl)pyrrolidine-1,2-dicarboxylate (1.0 g, 4.4 mmol) and diisopropylethylamine (2.2 mL, 13.1 mmol) in THF (25 mL). The resulting mixture was then stirred at room temperature under a nitrogen atmosphere for 2 hours. After this time, the reaction mixture was concentrated and the resulting residue purified using flash column chromatography (elution: 100% methylene chloride to 6% methanol, 94% methylene chloride) to give the title compound (0.53 g, 37% yield) as a colorless oil. δ_H (500 MHz, $CDCl_3$) 7.06 (s, 1H), 6.38 – 5.98 (m, 2H), 5.71 (d, *J* = 10.16 Hz, 1H), 4.23 (dt, *J* = 8.09, 16.69 Hz, 1H), 3.86 – 3.64 (m, 4H), 3.36 (q, *J* = 6.94 Hz, 2H), 3.05 (t, *J* = 10.13 Hz, 1H), 2.48 (dt, *J* = 6.77, 12.94 Hz, 1H), 2.15 (dd, *J* = 5.84, 20.74 Hz, 1H), 1.68 (tt, *J* = 8.03, 15.59 Hz, 3H), 1.39 (s, 9H). *m/z* (ES^+) $(M+H)^+$ 349.

(2*S*)-4-(2-Acrylamidoethyl)-1-(*tert*-butoxycarbonyl)pyrrolidine-2-carboxylic acid

Lithium hydroxide (2M solution, 3.7 mL, 7.35 mmol) was added in one portion to a stirred solution of (2*S*)-1-*tert*-butyl 2-methyl 4-(2-acrylamidoethyl)pyrrolidine-1,2-dicarboxylate (0.48 g, 1.47 mmol) in a solution of THF-water (2:1, 3 mL) and the resulting mixture was then stirred at room temperature for 2 hours. After this time, the reaction mixture was diluted with saturated ammonium chloride solution (5 mL) and extracted with methylene chloride (3 x 20 mL). The combined organic extracts were dried ($MgSO_4$), filtered, and concentrated to give the title compound (0.48 g, 98% yield) as a white solid. *m/z* (ES^+) $(M+H)^+$ 335.

(2*S*)-*tert*-Butyl 4-(2-acrylamidoethyl)-2-(4-(6-methylpyridin-2-yl)piperazine-1-carbonyl)pyrrolidine-1-carboxylate (7)

Diisopropylethylamine (1.3 mL, 7.8 mmol) was added portion wise over 5 minutes to a stirred solution of (2*S*)-4-(2-acrylamidoethyl)-1-(*tert*-butoxycarbonyl)pyrrolidine-2-carboxylic acid (0.48 g, 1.57 mmol), HATU (0.66 g, 1.73 mmol), HOBt (0.23 g, 1.77 mmol) and 1-(6-methylpyridin-2-yl)piperazine (0.28 g, 1.57 mmol) in DMF (5 mL) and the resulting mixture was stirred at room temperature under a nitrogen atmosphere for 2 hours. After this time, the reaction mixture was concentrated and the resulting residue partitioned between methylene chloride (50 mL) and 1M HCl (10 mL). The organic layer was separated and washed sequentially with saturated sodium bicarbonate (10 mL) and brine (10 mL) before being dried ($MgSO_4$), filtered, and concentrated. The crude residue was then purified using flash column chromatography (elution: 100% methylene chloride to 6% methanol, 94% methylene chloride) to give the title compound as a mixture of diastereoisomers (0.04 g, 5% yield) as a pale yellow oil. δ_H (500 MHz, DMSO) 7.42 (s, 1H), 6.55 (dd, *J* = 7.36, 10.78 Hz, 1H), 6.45 (d, *J* = 5.41 Hz, 1H), 6.32 – 6.23 (m, 1H), 6.13 – 5.99 (m, 1H), 5.75 – 5.59 (m, 2H), 4.62 (dt, *J* = 8.20, 45.29 Hz, 1H), 3.89 – 3.56 (m, 8H), 3.50 (t, *J* = 5.06 Hz, 1H), 3.35 (ddd, *J* = 6.99, 12.36, 23.56 Hz, 2H), 3.16 – 3.01 (m, 1H), 2.52 – 2.37 (m, 4H), 2.28 – 2.09 (m, 1H), 1.66 (dt, *J* = 7.06, 14.03 Hz, 3H), 1.54 – 1.34 (m, 9H). *m/z* (ES^+) $(M+H)^+$ 472.

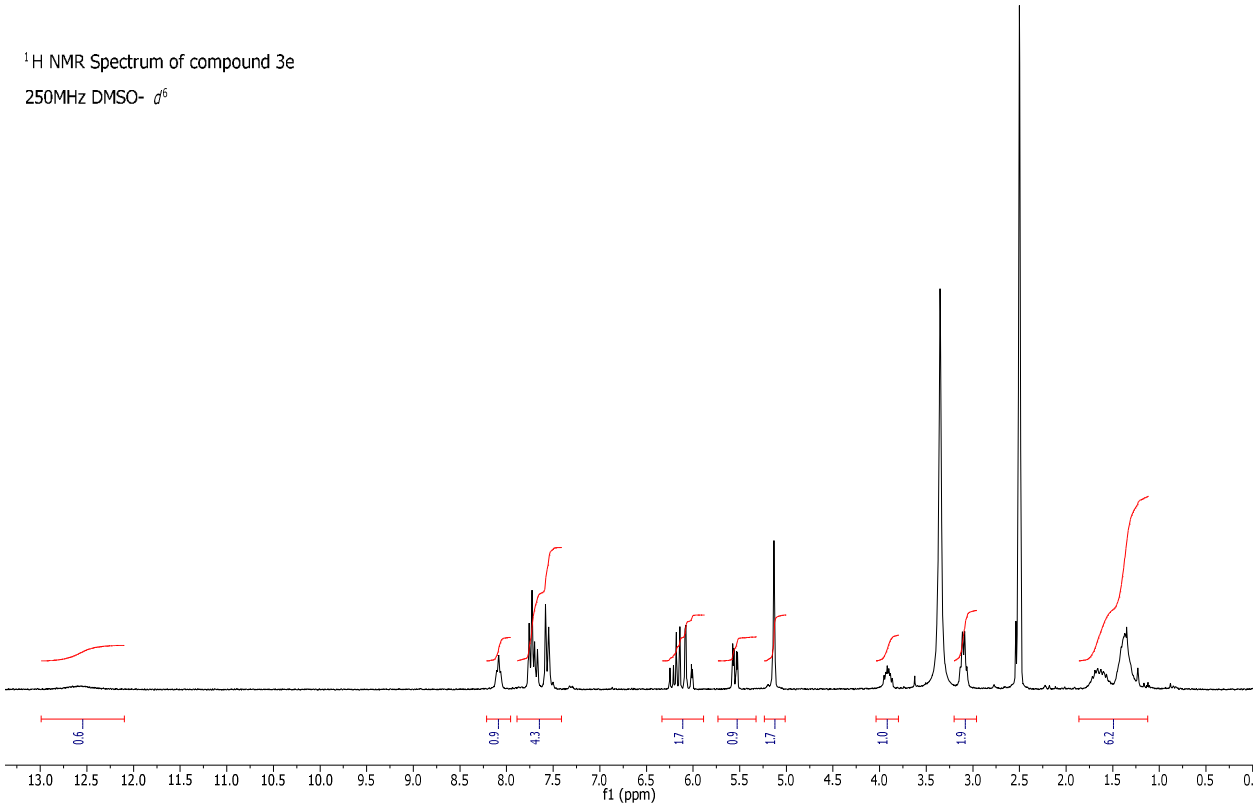


Figure 1. 250 MHz ^{1}H NMR spectrum of **3e**.

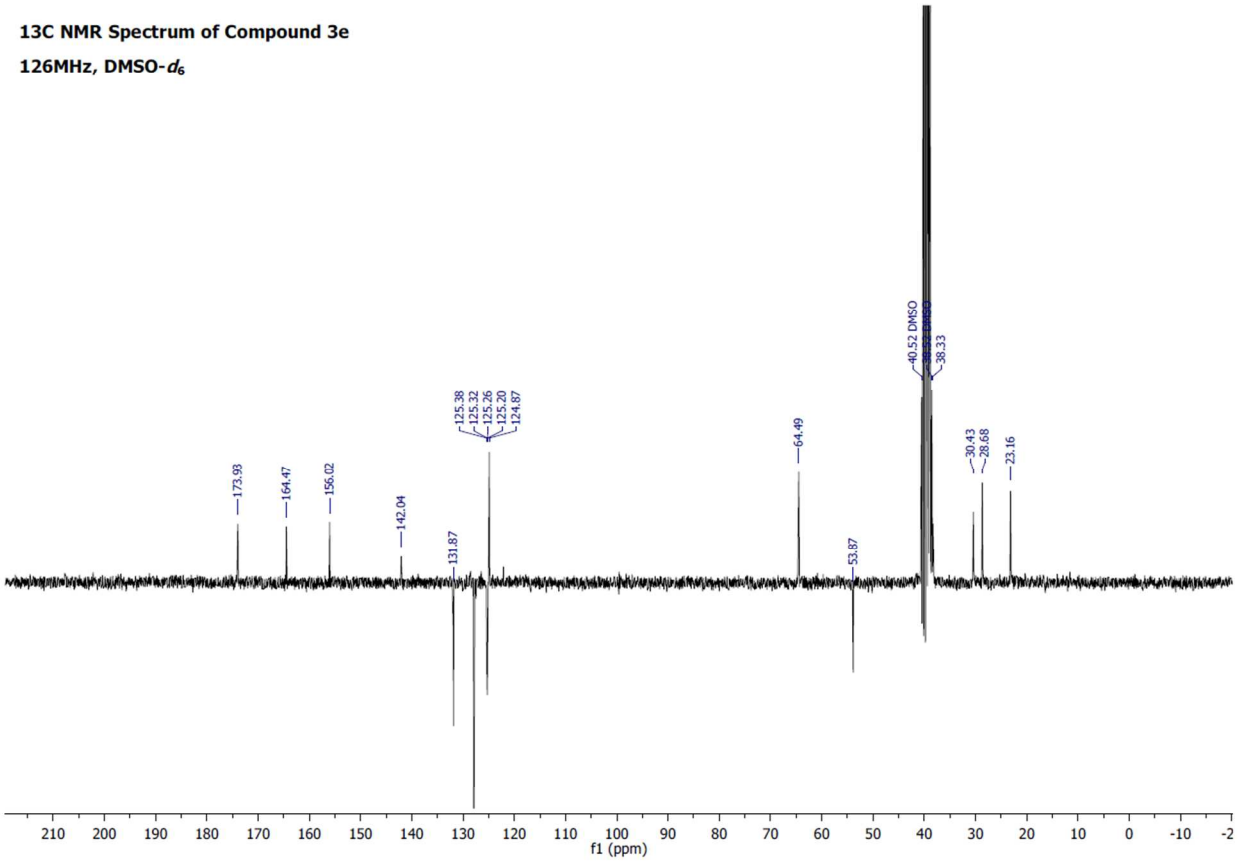


Figure 2. 126 MHz ^{13}C NMR spectrum of **3e**.

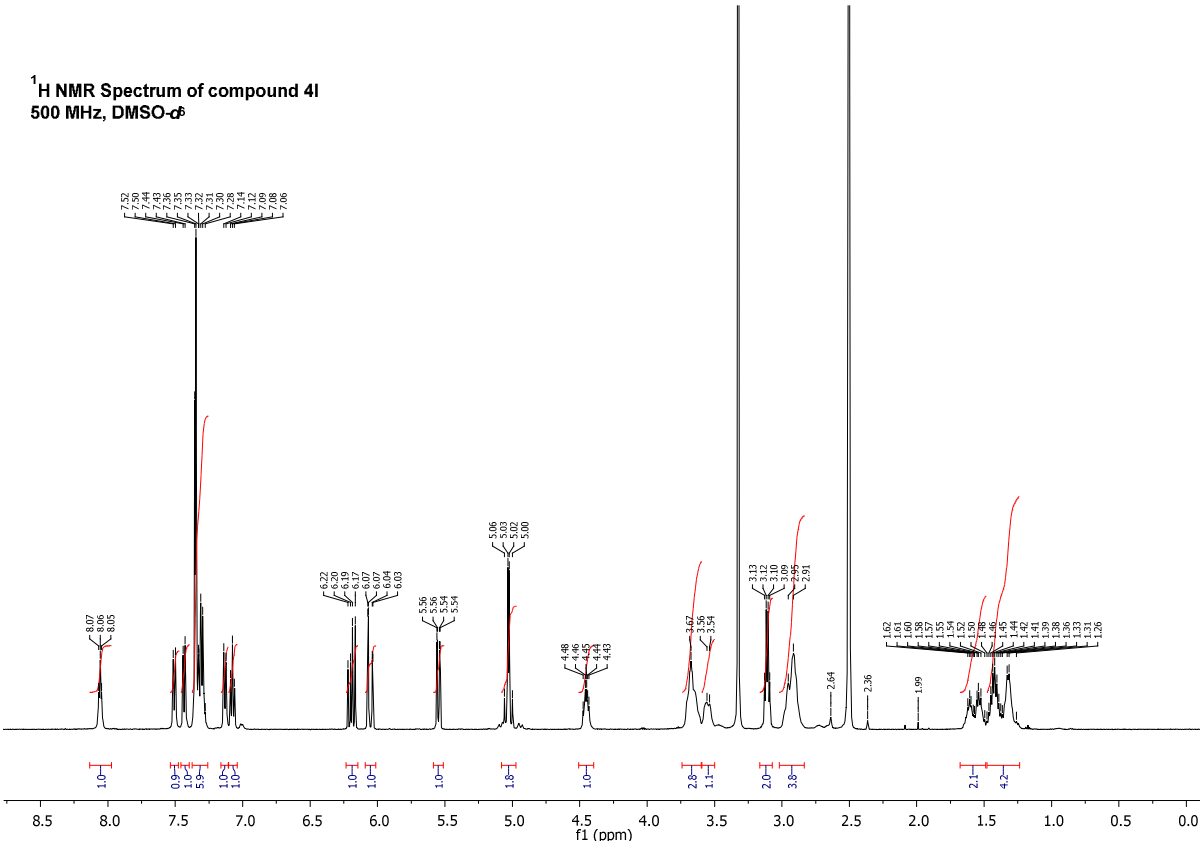


Figure 3. 500 MHz ^{1}H NMR spectrum of **4l**.

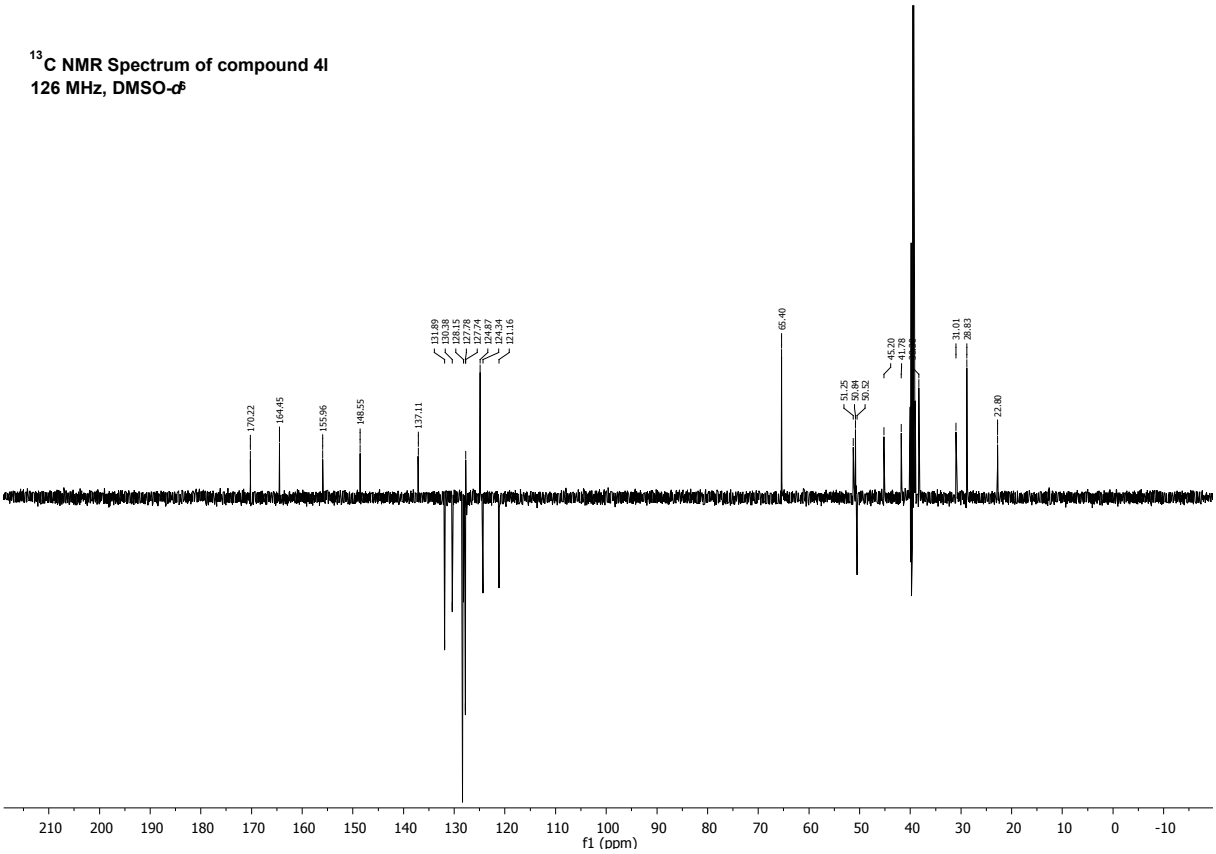


Figure 4. 126 MHz ^{13}C NMR spectrum of **4l**.

Kinetic Solubility (2% DMSO)

Using a 10 mM stock solution of each compound in 100% DMSO, dilutions are prepared to a theoretical concentration of 200 µM in both Phosphate Buffered Saline (PBS), pH 7.4 (2% DMSO final concentration) and in 100% DMSO. An aliquot of the 200 µM DMSO solution is then further diluted to 10 µM and all dilutions (n = 2 in 96-well plates) allowed to equilibrate at room temperature on an orbital shaker for two hours. The PBS dilutions are filtered using a Multiscreen HTS solubility filter plate (Millipore) and filtrates are analyzed by LC-UV and LC-MS. The concentration of compound in the PBS filtrate is determined by comparing the UV absorbance peak with that of the two DMSO dilutions which are used as calibration standards. Mass spectrometry is used to confirm the presence of the expected molecular ion in the UV peak measured. The effective range of the assay is 5 - 200 µM and compounds returning values close to the upper limit may have much higher solubility.

Plasma Stability

Incubations of test compound (1 µM initial concentration, n = 2) were carried out with pooled plasma or BSA (45 mg/mL in 0.1 M phosphate buffered saline pH 7.4). The incubations were performed at 37 °C. Samples (50 µL) were obtained from the incubation at 0, 10, 30, 120, 240, 360 and 1440 min, and added to 150 µL of acetonitrile containing carbamazepine as analytical internal standard to terminate the reaction. Samples were centrifuged and the supernatant fractions analyzed using LC-MS/MS.

Liver Microsomal Stability

Incubations of test compound (1 μM initial concentration, n = 2) were carried out with pooled hepatic liver microsomes (0.25 mg protein/mL in 0.1 M phosphate buffer pH7.4). NADPH (1 mM) was added to initiate the reactions. The incubations were performed at 37 °C. Samples (100 μL) were taken from the incubation at 0, 5, 10, 20 and 40 min and added to 100 μL of acetonitrile containing carbamazepine as analytical internal standard, to terminate the reaction. Samples were centrifuged and the supernatant fractions analyzed using LC-MS/MS.

Determinations of Analyte Stability in Plasma and Liver Microsomes

For all incubations, the instrument response (i.e. chromatographic peak area or peak height, normalized by internal standard), at each time-point were referenced to the zero time-point samples (as 100%) in order to determine the percentage of compound remaining at that time-point. Plots of the natural logarithm (Ln) of the percent of parent remaining for each compound, versus time, were used to determine the half-life in the incubation of interest.

Half-life values (t1/2) were calculated from the relationship: t1/2 (min) = -0.693/λ; where λ was the slope of the Ln concentration versus time curve.

For incubations in hepatic liver microsomes, the *in vitro* intrinsic clearance, Clint (μL/min/mg microsomal protein), was calculated using the following formula:

Clint = (0.693/t1/2 microsomal)*(ml incubation/mg microsomal protein)*(mg microsomal protein/g liver)*(g liver/kg body weight)

When quantification was required, calibration standards for parent compound and metabolites were prepared in control hepatic liver microsomes and extracted and analyzed as described for the study samples. Quantification of parent compound or metabolite was by extrapolation from the calibration line.

Permeability and Effective Efflux Ratio in MDCK-MDR1

The MDR1-MDCKII and wild type MDCKII cell lines were cultured in accordance with the guidelines provided by Solvo Biotechnology. Both wild-type MDCK and MDR1-MDCK cells were seeded at a cell density of 2.3 x 10^5 cells/well into 24-well Transwell plates and cultured for three days to form monolayers. Test compound was loaded into the donor compartments of the Transwell plate (24-well) bearing MDR1-MDCK or wild type MDCK monolayers. Test compound was added to either the apical or basolateral chambers of the Transwell plate assembly at a concentration of 10 μM in Hanks' Balanced Salt Solution containing 25 mM HEPES (pH 7.4). Lucifer Yellow was added to the apical buffer in all wells and its permeation monitored to assess integrity of the cell layer. As Lucifer Yellow (LY) cannot freely permeate lipophilic barriers, a high degree of LY transport indicates poor integrity of the cell layer and wells with LY permeability above 100 nm/s are rejected.