Discovery and Optimisation of wt-RET/KDR-selective Inhibitors of RET^{V804M} Kinase

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Analytical Data Summary for all Compounds

COMPOUND	MF	MW	pH 4				pH 10				NMR Purity
			RT	Obs. MW	Purity	Adduct	RT	Obs. MW	Purity	Adduct	
5	C19H16N6O	344.37	0.62	343.2	>95	[M-H]-	0.73	343.2	>95	[M-H]-	>95
11	C21H20N6O	372.42	0.69	371.5	>95	[M-H]-	0.81	No ion	>95	No ion	90-95
12	C19H16N6O	344.37	0.64	343.2	>95	[M-H]-	0.75	343.2	>95	[M-H]-	>95
13	C19H16N6O	344.37	0.62	343.2	>95	[M-H]-	0.76	343.2	>95	[M-H]-	>95
14	C17H20N6O	324.38	0.58	323.2	>95	[M-H]-	0.75	323.2	90-95	[M-H]-	>95
15	C18H15N5	301.35	0.8	302.5	>95	[M+H]+	0.98	302.5	>95	[M+H]+	90-95
16	C19H16N6O	344.37	0.63	343.2	>95	[M-H]-	0.75	343.2	>95	[M-H]-	>95
17	C18H16N6O2S	380.42	0.64	379.1	>95	[M-H]-	0.77	379.2	>95	[M-H]-	>95
18	C19H15N5O2	345.36	0.68	346.3	>95	[M+H]+	0.57	346.3	>95	[M+H]+	>95
19	C20H18N6O	358.4	0.65	359.6	90-95	[M+H]+	0.76	359.6	>95	[M+H]+	>95
20	C20H18N6O	358.4	0.66	359.5	>95	[M+H]+	0.79	359.5	>95	[M+H]+	>95
21	C19H18N6O2S	394.45	0.69	393.2	>95	[M-H]-	0.81	393.2	<85	[M-H]-	>95
22	C19H15N7	341.37	0.69	342.6	85-90	[M+H]+	0.83	342.6	85-90	[M+H]+	85-90
23	C19H15N5O2	345.36	0.79	346.5	>95	[M+H]+	0.95	346.5	>95	[M+H]+	>95
27	C19H15N5O2	345.36	0.88	346.5	90-95	[M+H]+	1.03	346.5	90-95	[M+H]+	90-95
28	C20H16N4O2	344.37	1.20	345.5	>95	[M+H]+	1.19	345.5	>95	[M+H]+	>95
29	C18H13N5O2	331.33	0.77	332.5	>95	[M+H]+	0.83	332.5	>95	[M+H]+	>95
30	C19H21N5O2	351.4	0.85	352.2	>95	[M+H]+	0.90	352.2	>95	[M+H]+	>95
31	C18H19N5O2	337.38	0.83	338.2	>95	[M+H]+	0.88	336.3	>95	[M-H]-	>95
32	C17H19N5O2	325.37	0.73	326.6	>95	[M+H]+	1.01	326.6	>95	[M+H]+	>95
33	C20H23N5O2	365.43	0.81	364.4	90-95	[M-H]-	1.21	364.2	90-95	[M-H]-	90-95
34	C18H21N5O2	339.39	0.75	338.3	>95	[M-H]-	1.06	338.3	>95	[M-H]-	>95
35	C15H14N4O3	298.3	0.88	299.5	>95	[M+H]+	0.89	299.5	90-95	[M+H]+	>95

Experimental Procedures

Flash chromatography was performed using pre-packed silica gel cartridges (KP-Sil SNAP, Biotage, Hengoed UK or RediSep Rf, Isco). Thin layer chromatography was conducted with 5 × 10 cm plates coated with Merck Type 60 F_{254} silica gel to a thickness of 0.25 mm. All reagents obtained from commercial sources were used without further purification. Anhydrous solvents were obtained from the Sigma-Aldrich Chemical Company Ltd. or Fisher Chemicals Ltd., and used without further drying. HPLC grade solvents were obtained from Fisher Chemicals Ltd.

All compounds were > 90% purity as determined by examination of both the LC-MS and ^{1}H NMR spectra unless otherwise indicated. Where Cl or Br was present, expected isotopic distribution patterns were observed.

^{1}H NMR

Proton (^{1}H) and carbon (^{13}C) NMR spectra were recorded on a 300 MHz Bruker spectrometer. Solutions were typically prepared in either deuterochloroform ($CDCl_3$), deuteromethanol (CD_3OD) or deuterated dimethylsulfoxide (d_6-DMSO) with chemical shifts referenced to tetramethylsilane (TMS) or deuterated solvent as an internal standard. ^{1}H NMR data are reported indicating the chemical shift (δ), the integration (e.g. 1H), the multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad; dd, doublet of doublets etc.) and the coupling constant (J) in Hz (app implies apparent coupling on broadened signals). Deuterated solvents were obtained from the Sigma-Aldrich Chemical Company, Goss or Fluorochem.

Analytical LC-MS

LC-MS analyses were performed on a Waters Acquity UPLC system fitted with BEH C18 1.7 µM columns (2.1 × 50 mm) and with UV diode array detection (210–400 nm). Positive and negative mass ion detection was performed using a Waters SQD detector. Analyses were performed with either buffered acidic or basic solvents and gradients as detailed below:

Low pH:

Solvent A – Water + 10 mM ammonium formate + 0.1% formic acid

Solvent B – Acetonitrile + 5% water + 0.1% formic acid

High pH:

Solvent A – Water + 10 mM ammonium hydrogen carbonate + 0.1% ammonia solution

Solvent B – Acetonitrile + 0.1% ammonia solution

Gradient:

Time	Flow rate (mL/min)	% Solvent A	% Solvent B
0	0.6	95	5
1.2	0.6	5	95
1.7	0.6	5	95
1.8	0.6	95	5

Preparative HPLC

Some compounds were purified by preparative HPLC on a Waters FractionLynx MS autopurification system, with a Waters XBridge 5 μm C18, 100 mm × 19 mm i.d. column, running at a flow rate of 20 mL/min with UV diode array detection (210–400 nm) and mass-directed collection using both positive and negative mass ion detection.

Purifications were performed using buffered acidic or basic solvent systems as appropriate. Compound retention times on the system were routinely assessed using a 30-50 μL test injection and a standard gradient, and then purified using an appropriately chosen focussed gradient as detailed below, based upon observed retention time.

Low pH:

Solvent A – Water + 10 mM ammonium formate + 0.1% formic acid

Solvent B – Acetonitrile + 5% water +0.1% formic acid

High pH:

Solvent A – Water + 10 mM ammonium formate + 0.1% ammonia solution

Solvent B – Acetonitrile + 5% water + 0.1% ammonia solution

Standard Gradient:

Time	Flow rate (mL/min)	% Solvent A	% Solvent B
0	20	90	10
0.3	20	90	10
8.5	20	2	98
12	20	2	98
12.5	0	2	98

Focussed Gradients:

		% Solvent B				
		Retention time on standard gradient (min.)				
Time	Flow rate (mL/min)	0 – 5.2	4.9 – 6.6	6.3 – 7.5	7.3 – 9.5	9.3 - 12
0	20	10	10	10	10	10
0.25	20	10	10	10	10	10
0.35	20	10	20	35	45	60
10	20	45	55	65	75	98
12	20	98	98	98	98	98
12.5	0	98	98	98	98	98

Representative synthetic procedures

Preparation of intermediates 7-10 (Scheme 1)

*3-Bromo-N-(3-pyridylmethyl)pyrazolo[1,5-a]pyrimidin-5-amine (**7**)*

A mixture of 3-bromo-5-chloro-pyrazolo[1,5-a]pyrimidine **6** (1.0 g, 4.3 mmol), DIPEA (1.42 mL, 8.6 mmol) and 3-(aminomethyl)pyridine (0.53 mL, 5.16 mmol) in IPA (6 mL) was heated at 80 ˚C for 16 h. The reaction was cooled to RT, diluted with water (20 mL) and extracted with EtOAc (3 × 20 mL). The combined organics were washed with water and brine, dried over $MgSO_4$ and concentrated to give a sticky solid which was purified by flash chromatography eluting with EtOAc to give the title compound (680 mg, 2.2 mmol, 52%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.65 (dd, *J* = 2.3, 0.8 Hz, 1H), 8.55 – 8.43 (m, 2H), 8.21 (t, *J* = 5.7 Hz, 1H), 7.90 (s, 1H), 7.87 – 7.76 (m, 1H), 7.37 (ddd, *J* = 7.8, 4.8, 0.9 Hz, 1H), 6.35 (d, *J* = 7.6 Hz, 1H), 4.59 (d, *J* = 5.7 Hz, 2H).

*3-Bromo-N-(4-pyridylmethyl)pyrazolo[1,5-a]pyrimidin-5-amine (**8**)*

A mixture of 3-bromo-5-chloro-pyrazolo[1,5-a]pyrimidine **6** (1.0 g, 4.3 mmol), 4-pyridylmethanamine (1.31 mL, 12.9 mmol), DIPEA (2.13 mL, 12.9 mmol) and DMF (2 mL) was heated to 100 °C for 5 h. The reaction was cooled to RT, diluted with water (50 mL) and extracted with EtOAc (3 × 50 ml). The combined organics were washed with water and brine, dried over $MgSO_4$ and concentrated to return an orange solid which was purified by flash chromatography eluting with 0 to 5% MeOH in DCM to give the title compound (733 mg, 2.41 mmol, 56%) as a yellow solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.58 – 8.47 (m, 3H), 8.26 (t, *J* = 5.9 Hz, 1H), 7.90 (s, 1H), 7.41 – 7.33 (m, 2H), 6.39 (d, *J* = 7.6 Hz, 1H), 4.62 (d, *J* = 5.9 Hz, 2H).

*3-Bromo-N-(2-pyridylmethyl)pyrazolo[1,5-a]pyrimidin-5-amine (**9**)*

A mixture of 3-bromo-5-chloro-pyrazolo[1,5-a]pyrimidine **6** (1.0 g, 4.3 mmol), DIPEA (1.42 mL, 8.6 mmol) and 2-(aminomethyl)pyridine (0.53 mL, 5.16 mmol) in IPA (6 mL) was heated to 80 °C for 16 h. The reaction was cooled to RT, diluted with water (20 mL) and extracted with EtOAc (3 × 20 ml). The combined organics were washed with water and brine, dried over $MgSO_4$ and concentrated to return a sticky solid which was purified by flash chromatography eluting EtOAc to give the title compound (760 mg, 2.50 mmol, 58%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.59 – 8.47 (m, 2H), 8.24 (t, *J* = 5.8 Hz, 1H), 7.89 (s, 1H), 7.77 (td, *J* = 7.7, 1.8 Hz, 1H), 7.43 (dt, *J* = 7.8, 1.1 Hz, 1H), 7.29 (ddd, *J* = 7.6, 4.8, 1.2 Hz, 1H), 6.46 (d, *J* = 7.6 Hz, 1H), 4.69 (d, *J* = 5.7 Hz, 2H).

N-(3-Bromopyrazolo[1,5-a]pyrimidin-5-yl)-N',N'-dimethyl-ethane-1,2-diamine (**10**)

A mixture of 3-bromo-5-chloro-pyrazolo[1,5-a]pyrimidine **6** (2.0 g, 8.6 mmol), *N,N*-dimethylethylenediamine (2.82 mL, 25.8 mmol) and DIPEA (4.27 mL, 25.8 mmol) in DMF (10 mL) was heated to 120 °C for 30 min under

microwave irradiation. The reaction was cooled to RT, diluted with EtOAc (50 mL) and washed with water (2 × 25 mL). The combined aqueous phases were back-extracted with EtOAc (25 mL) then the combined organics were washed with brine (25 ml), dried over $MgSO_4$, filtered and concentrated to give the title compound (2.54 g, 8.9 mmol, quantitative yield) as an orange solid containing residual DMF which was used without further purification. ^{1}H NMR (300 MHz, Chloroform-*d*) δ 8.05 (d, *J* = 7.5 Hz, 1H), 7.72 (s, 1H), 5.99 (d, *J* = 7.6 Hz, 1H), 5.63 (br s, 1H), 3.48 (q, *J* = 5.4 Hz, 2H), 2.48 (dd, *J* = 6.3, 5.4 Hz, 2H), 2.20 (s, 6H).

Suzuki couplings (Scheme 1)

*4-[5-(4-Pyridylmethylamino)pyrazolo[1,5-a]pyrimidin-3-yl]benzamide (**5**)*

A mixture of **8** (100 mg, 0.33 mmol), aq K_3PO_4 (1M, 1.0 mL, 0.99 mmol), (4-carbamoylphenyl)boronic acid (71 mg, 0.43 mmol), Pd-118 (11 mg, 0.02 mmol) and 1,4-dioxane (3 mL) was heated to 120 °C for 30 min in the microwave. The upper organic layer was purified by preparative HPLC (high pH) to return the title compound (31 mg, 0.09 mmol, 27%) as a pale yellow solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.62 – 8.51 (m, 3H), 8.45 – 8.35 (m, 2H), 7.94 (d, *J* = 8.3 Hz, 2H), 7.88 (s, 1H), 7.85 – 7.76 (m, 2H), 7.46 – 7.38 (m, 2H), 7.22 (s, 1H), 6.44 (d, *J* = 7.6 Hz, 1H), 4.65 (d, *J* = 5.7 Hz, 2H). HRMS (ESI) *m/z* $[M + H]^+$ calcd for $C_{19}H_{17}N_6O$ 345.1458, found 345.1448.

*N,N-Dimethyl-4-[5-(4-pyridylmethylamino)pyrazolo[1,5-a]pyrimidin-3-yl]benzamide (**11**)*

A mixture of **8** (152 mg, 0.50 mmol), $Pd(PPh_3)_4$ (58 mg, 0.05 mmol) and K_2CO_3 (207 mg, 1.5 mmol) in degassed 1,4-dioxane (3.5 mL) and water (1.5 mL) was heated at 150 °C for 30 min in the microwave. The reaction mixture was diluted with water (25 mL) and extracted with DCM. The combined organic extracts were filtered through a hydrophobic frit, concentrated then purified by preparative HPLC (high pH) to give the title compound (13 mg, 0.035 mmol, 7%) as an off-white powder. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.57 (d, *J* = 7.6 Hz, 1H), 8.55 – 8.48 (m, 2H), 8.43 – 8.32 (m, 2H), 7.91 (d, *J* = 8.3 Hz, 2H), 7.44 – 7.40 (m, 2H), 7.36 – 7.30 (m, 2H), 6.44 (d, *J* = 7.6 Hz, 1H), 4.64 (d, *J* = 5.7 Hz, 2H), 3.32 (s, 3H), 2.98 (s, 3H - obscured by water peak).

*4-[5-(2-Pyridylmethylamino)pyrazolo[1,5-a]pyrimidin-3-yl]benzamide (**12**)*

Prepared in the same manner as **5** but using **9** (100 mg, 0.33 mmol) to give the title compound (13 mg, 0.039 mmol, 12%) as an off-white powder. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.68 (dd, *J* = 2.3, 0.9 Hz, 1H), 8.56 (d, *J* = 7.6 Hz, 1H), 8.47 (dd, *J* = 4.8, 1.7 Hz, 1H), 8.42 (s, 1H), 8.35 (t, *J* = 5.7 Hz, 1H), 8.08 – 7.99 (m, 2H), 7.95 – 7.79 (m, 4H), 7.39 (ddd, *J* = 7.9, 4.7, 0.9 Hz, 1H), 7.23 (s, 1H), 6.40 (d, *J* = 7.6 Hz, 1H), 4.66 (d, *J* = 5.6 Hz, 2H).

*4-[5-(3-Pyridylmethylamino)pyrazolo[1,5-a]pyrimidin-3-yl]benzamide (**13**)*

A mixture of **7** (50 mg, 0.16 mmol), (4-carbamoylphenyl)boronic acid (54 mg, 0.33 mmol), Pd-118 (5 mg, 0.01 mmol), aq K_3PO_4 (1M, 0.49 mL, 0.49 mmol) and 1,4-dioxane (3 mL) was heated to 120 °C for 30 min in the microwave. The reaction was diluted with EtOAc and the organic layer separated. The aqueous was washed with 3:1 DCM:IPA and the organic layers combined and evaporated *in vacuo*. The residue was purified by preparative HPLC to return the title compound (12 mg, 0.03 mmol, 22%) as an off-white powder. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.68 (dd, J = 2.2, 0.9 Hz, 1H), 8.56 (d, J = 7.6 Hz, 1H), 8.46 (dd, J = 4.8, 1.7 Hz, 1H), 8.42 (s, 1H), 8.35 (t, J = 5.7 Hz, 1H), 8.09 – 8.00 (m, 2H), 7.90 – 7.78 (m, 4H), 7.39 (ddd, J = 7.8, 4.8, 0.9 Hz, 1H), 7.23 (s, 1H), 6.40 (d, J = 7.5 Hz, 1H), 4.66 (d, J = 5.6 Hz, 2H). HRMS (ESI) *m/z* $[M + H]^+$ calcd for $C_{19}H_{17}N_6O$ 345.1458, found 345.1454.

*4-[5-[2-(Dimethylamino)ethylamino]pyrazolo[1,5-a]pyrimidin-3-yl]benzamide (**14**)*

Prepared in the same manner as **13** but using **10** (100 mg, 0.35 mmol) to give the title compound (22 mg, 0.07 mmol, 19%) as a yellow powder. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.50 (d, J = 7.6 Hz, 1H), 8.42 (s, 1H), 8.16 (d, J = 8.1 Hz, 3H), 7.92 – 7.81 (m, 3H), 7.71 (s, 1H), 7.22 (s, 1H), 6.38 (d, J = 7.6 Hz, 1H), 3.56 (q, J = 6.3 Hz, 2H), 2.58 (t, J = 6.7 Hz, 2H), 2.29 (s, 6H).

*3-Phenyl-N-(4-pyridylmethyl)pyrazolo[1,5-a]pyrimidin-5-amine (**15**)*

Prepared in the same manner as **13** to give the title compound (26 mg, 0.09 mmol, 26%) as a pale yellow solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.60 – 8.49 (m, 3H), 8.32 (s, 2H), 7.91 – 7.81 (m, 2H), 7.45 – 7.37 (m, 2H), 7.35 – 7.22 (m, 2H), 7.08 (ddt, J = 7.8, 6.9, 1.3 Hz, 1H), 6.42 (d, J = 7.5 Hz, 1H), 4.63 (d, J = 5.7 Hz, 2H).

*3-[5-(4-Pyridylmethylamino)pyrazolo[1,5-a]pyrimidin-3-yl]benzamide (**16**)*

Prepared in the same manner as **13** to give the title compound (21 mg, 0.06 mmol, 19%) as a pale yellow solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.56 (d, J = 7.6 Hz, 1H), 8.55 – 8.47 (m, 2H), 8.45 (t, J = 1.7 Hz, 1H), 8.41 – 8.31 (m, 2H), 8.06 (d, J = 7.8 Hz, 1H), 7.93 (s, 1H), 7.58 (dt, J = 7.8, 1.4 Hz, 1H), 7.49 – 7.41 (m, 2H), 7.41 – 7.30 (m, 2H), 6.42 (d, J = 7.6 Hz, 1H), 4.67 (d, J = 5.8 Hz, 2H).

*4-[5-(4-Pyridylmethylamino)pyrazolo[1,5-a]pyrimidin-3-yl]benzenesulfonamide (**17**)*

Prepared in the same manner as **13** to give the title compound (13 mg, 0.03 mmol, 10%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.59 (d, J = 7.5 Hz, 1H), 8.56 – 8.50 (m, 2H), 8.50 – 8.39 (m, 2H), 8.02 (d, J = 8.3 Hz, 2H), 7.75 – 7.67 (m, 2H), 7.47 – 7.39 (m, 2H), 7.24 (s, 2H), 6.47 (d, J = 7.6 Hz, 1H), 4.66 (d, J = 5.6 Hz, 2H).

*4-[5-(4-Pyridylmethylamino)pyrazolo[1,5-a]pyrimidin-3-yl]benzoic acid (**18**)*

A mixture of **7** (100 mg, 0.33 mmol), 4-ethoxycarbonylphenyl boronic acid (128 mg, 0.66 mmol), Pd-118 (11 mg, 0.02 mmol), aq K_3PO_4 (1M, 0.49 mL, 0.49 mmol) and 1,4-dioxane (3 mL) was heated to 120 °C for 30 min in the microwave. The reaction was diluted with EtOAc (6 mL), separated, dried over $MgSO_4$ and concentrated. The crude product was purified by flash chromatography eluting with MeOH (0-10%) in EtOAc to give ethyl 4-[5-(4-pyridylmethylamino)pyrazolo[1,5-a]pyrimidin-3-yl]benzoate (111 mg, 0.29 mmol, 90%). To a solution of this intermediate ester in THF (3 mL) was added LiOH (21 mg, 0.88 mmol) and water and the mixture heated to 60 °C for 6 h. The solution was treated with NH_4Cl (100 mg) and stirred for 30 min. The mixture was evaporated to dryness and then the residue triturated with $CHCl_3$. The resultant solid was triturated with methanol followed by water, then dried to give the title compound (16 mg, 0.05 mmol, 16%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.65 (s, 1H), 8.59 (d, J = 7.5 Hz, 1H), 8.52 (d, J = 5.1 Hz, 2H), 8.49 – 8.39 (m, 2H), 7.98 (d, J = 8.1 Hz, 2H), 7.84 (d, J = 8.1 Hz, 2H), 7.43 (d, J = 5.0 Hz, 2H), 6.46 (d, J = 7.6 Hz, 1H), 4.66 (d, J = 5.6 Hz, 2H).

*N-[4-[5-(4-Pyridylmethylamino)pyrazolo[1,5-a]pyrimidin-3-yl]phenyl]acetamide (**19**)*

Prepared in the same manner as **11** to give the title compound (42 mg, 0.12 mmol, 23%) as an off-white powder. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.84 (s, 1H), 8.58 – 8.47 (m, 3H), 8.28 – 8.22 (m, 1H), 7.76 (d, J = 8.6 Hz, 2H), 7.52 – 7.44 (m, 2H), 7.44 – 7.36 (m, 2H), 6.39 (d, J = 7.6 Hz, 1H), 4.63 (d, J = 5.7 Hz, 2H), 2.03 (s, 2H).

*N-[3-[5-(4-Pyridylmethylamino)pyrazolo[1,5-a]pyrimidin-3-yl]phenyl]acetamide (**20**)*

Prepared in the same manner as **11** to give the title compound (16 mg, 0.04 mmol, 12%) as an off-white powder. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.83 (s, 1H), 8.54 (d, J = 7.6 Hz, 1H), 8.52 – 8.45 (m, 2H), 8.38 – 8.17 (m, 3H), 7.57 – 7.51 (m, 1H), 7.45 – 7.37 (m, 2H), 7.24 – 7.17 (m, 2H), 6.40 (d, J = 7.5 Hz, 1H), 4.75 (d, J = 5.8 Hz, 2H), 2.06 (s, 3H).

*N-[4-[5-(4-Pyridylmethylamino)pyrazolo[1,5-a]pyrimidin-3-yl]phenyl]methanesulfonamide (**21**)*

Prepared in the same manner as **13** to give the title compound (45 mg, 0.11 mmol, 34%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.54 (s, 1H), 8.58 – 8.47 (m, 3H), 8.28 (s, 2H), 7.88 – 7.78 (m, 2H), 7.45 – 7.37 (m, 2H), 7.20 – 7.10 (m, 2H), 6.41 (d, J = 7.6 Hz, 1H), 4.64 (d, J = 5.6 Hz, 2H), 2.96 (s, 3H).

*3-(1H-Indazol-6-yl)-N-(4-pyridylmethyl)pyrazolo[1,5-a]pyrimidin-5-amine (**22**)*

Prepared in the same manner as **11** to give the title compound (10 mg, 0.03 mmol, 6%) as an off-white powder. ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.91 (s, 1H), 8.57 (d, J = 7.6 Hz, 1H), 8.53 – 8.49 (m, 2H), 8.39 (d, J = 17.0 Hz, 2H),

8.21 (q, *J* = 1.1 Hz, 1H), 7.98 (s, 1H), 7.66 (t, *J* = 1.3 Hz, 2H), 7.57 – 7.42 (m, 2H), 7.41 – 7.29 (m, 1H), 6.42 (d, *J* = 7.5 Hz, 1H), 4.71 (d, *J* = 5.8 Hz, 2H).

*3-(1,3-Benzodioxol-5-yl)-N-(4-pyridylmethyl)pyrazolo[1,5-a]pyrimidin-5-amine (**23**)*

Prepared in the same manner as **13** to give the title compound (16 mg, 0.05 mmol, 13%) as a tan solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.63 – 8.42 (m, 3H), 8.34 – 8.23 (m, 2H), 7.52 (d, *J* = 1.7 Hz, 1H), 7.43 – 7.33 (m, 3H), 6.86 (d, *J* = 8.1 Hz, 1H), 6.38 (d, *J* = 7.6 Hz, 1H), 5.98 (s, 2H), 4.61 (d, *J* = 5.7 Hz, 2H).

Synthesis of intermediates 24, 25 and 26 (Scheme 2)

*4-(1,3-Benzodioxol-5-yl)-1H-pyrazol-3-amine (**24**)*

Prepared an a manner similar to that previously described.[1]

To a stirred suspension of sodium methoxide (1.31 g, 24.2 mmol) in toluene (50 mL) at 0 ˚C was added a mixture of peperacetonitrile (3.0 g, 18.62 mmol) and ethyl formate (1.95 mL, 24.2 mmol) in toluene (25 mL). After 20 minutes the ice bath was removed and the mixture stirred at room temperature for 2 h producing a pink suspension. Ice water was added and stirred until the suspension dissolved, upon which the aqueous and organic phases were separated. The organic phase was washed with 0.5 M sodium hydroxide (2 × 100 mL). The aqueous phases were combined, the pH adjusted to ~4, ice water added and the mixture stirred overnight. The resulting beige precipitate was isolated by filtration and washed with water to give the intermediate 2-(benzo[d][1,3]dioxol-5-yl)-3-oxopropanenitrile. MS (ES–) m/z 188 (M–H). To this solid was added methanol (25 mL) and water (5 mL). The mixture was cooled to 0 °C before addition of semicarbazide (2.1 g, 27.92 mmol). The mixture was stirred at 0 °C for 10 min, then for a further hour at room temperature keeping the pH at 9-10 with 5N sodium hydroxide. The reaction mixture was then diluted with water (200 mL), cooled in an ice-bath and allowed to stir overnight. The resulting beige precipitate was isolated by filtration. To this solid was added methanol (150 mL) and 5N sodium hydroxide solution (15 mL) and the mixture was heated at reflux for 30 minutes. After cooling, water (300 mL) was added and the mixture was cooled in an ice-bath and allowed to stir overnight. The resulting precipitate was filtered and dried *in vacuo* to return the title compound (2.33 g, 10.32 mmol, 55%) as a beige solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.64 (s, 1H), 7.62 (s, 1H), 7.10 – 7.03 (m, 1H), 6.95 (d, *J* = 8.2 Hz, 1H), 6.87 (d, *J* = 8.0 Hz, 1H), 5.97 (s, 2H), 4.52 (s, 2H).

*3-(1,3-Benzodioxol-5-yl)-4H-pyrazolo[1,5-a]pyrimidin-5-one (**25**)*

Prepared in a manner similar to that previously described.[2]

To a mixture of **24** (2.33 g, 11.49 mmol) and 1,3-dimethyluracil (1.77 g, 12.63 mmol) in EtOH (50 mL) was added NaOEt solution (21% w/w in EtOH, 5.57 mL, 14.93 mmol). The reaction mixture heated at reflux for 3.5 h. After

cooling, the mixture was concentrated in *vacuo*, the residue added to ice and neutralised with glacial acetic acid. The resulting mixture was stirred overnight and the resulting precipitate filtered, washed with water and dried to return the title compound (2.56 g, 9.53 mmol, 83%) as a yellow solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.05 (s, 1H), 8.56 (d, *J* = 7.7 Hz, 1H), 8.13 (s, 1H), 7.27 (d, *J* = 1.7 Hz, 1H), 7.15 (dd, *J* = 8.1, 1.8 Hz, 1H), 6.93 (d, *J* = 8.1 Hz, 1H), 6.08 (d, *J* = 7.8 Hz, 1H), 6.02 (s, 2H).

3-(1,3-Benzodioxol-5-yl)-5-chloro-pyrazolo[1,5-a]pyrimidine (***26***)

To a mixture of **25** (1.0 g, 3.92 mmol) and $POCl_3$ (20 mL) was added 2 drops DMF and the mixture was heated at reflux for 1.5 h. After cooling to room temperature the reaction mixture was concentrated to yield a dark green residue which was partitioned between sat. aq. $NaHCO_3$ and DCM. The aqueous layer was separated and further extracted with DCM and the combined organic layers were passed through a hydrophobic frit. Concentration in *vacuo* returned the title compound (935 mg, 3.07 mmol, 78 %) as a dark green solid which was used without further purification. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.17 (d, *J* = 7.2 Hz, 1H), 8.74 (s, 1H), 7.60 (d, *J* = 1.7 Hz, 1H), 7.56 (dd, *J* = 8.1, 1.7 Hz, 1H), 7.15 (d, *J* = 7.3 Hz, 1H), 7.02 (d, *J* = 8.1 Hz, 1H), 6.05 (s, 2H).

Synthesis of 27-35 (Scheme 2)

*3-(1,3-Benzodioxol-5-yl)-N-(2-pyridylmethyl)pyrazolo[1,5-a]pyrimidin-5-amine (**27**)*

A mixture of **26** (100 mg, 0.37 mmol), 2-picolylamine (75 µL, 0.73 mmol), DIPEA (120 µL, 0.73 mmol) in IPA (1 mL) was heated at 120 ˚C for 30 minutes in the microwave. The mixture was concentrated *in vacuo* and purified by preparative HPLC (high pH) to give the title compound (15 mg, 0.04 mmol, 11%) as a white powder. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.59 – 8.46 (m, 2H), 8.30 – 8.22 (m, 2H), 7.75 (t, *J* = 7.9 Hz, 1H), 7.55 (s, 1H), 7.50 – 7.35 (m, 2H), 7.25 (t, *J* = 6.2 Hz, 1H), 6.85 (d, *J* = 8.2 Hz, 1H), 6.41 (d, *J* = 7.7 Hz, 1H), 5.98 (s, 2H), 4.68 (d, *J* = 5.6 Hz, 2H).

*3-(1,3-Benzodioxol-5-yl)-N-benzyl-pyrazolo[1,5-a]pyrimidin-5-amine (**28**)*

Prepared in the same manner as **27** to give the title compound (5 mg, 0.02 mmol, 4%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.48 (d, *J* = 7.6 Hz, 1H), 8.26 (s, 1H), 8.17 (t, *J* = 5.8 Hz, 1H), 7.65 (d, *J* = 1.7 Hz, 1H), 7.48 (dd, *J* = 8.1, 1.7 Hz, 1H), 7.46 – 7.39 (m, 2H), 7.39 – 7.30 (m, 2H), 7.30 – 7.19 (m, 1H), 6.89 (d, *J* = 8.2 Hz, 1H), 6.33 (d, *J* = 7.6 Hz, 1H), 5.98 (s, 2H), 4.59 (d, *J* = 5.7 Hz, 2H).

*3-(1,3-Benzodioxol-5-yl)-N-(4-pyridyl)pyrazolo[1,5-a]pyrimidin-5-amine (**29**)*

Prepared in the same manner as **27** to give the title compound (104 mg, 0.31 mmol, 86%) as an orange solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.51 (d, *J* = 7.6 Hz, 1H), 9.12 (s, 2H), 8.99 (d, *J* = 7.4 Hz, 2H), 8.84 (s, 1H), 7.70 – 7.58 (m, 3H), 7.11 (d, *J* = 7.3 Hz, 2H), 7.07 – 6.99 (m, 1H), 6.06 (s, 2H).

*3-(1,3-Benzodioxol-5-yl)-N-(4-piperidylmethyl)pyrazolo[1,5-a]pyrimidin-5-amine (**30**)*

To a solution of **26** (40 mg, 0.15 mmol) in DMF (0.50 mL) was added 1-Boc-4-(aminomethyl)piperidine (125 mg, 0.58 mmol). The reaction was stirred at 100 °C for 60 min then diluted to 1 mL with 1:1 acetonitrile:water and purified by preparative HPLC (high pH) to give the title compound (6 mg, 0.02 mmol, 12%), presumably via thermal deprotection under the reaction conditions. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.46 (d, J = 7.5 Hz, 1H), 8.39 (s, 1H), 8.27 (s, 1H), 7.80 – 7.70 (m, 2H), 7.54 (dd, J = 8.2, 1.7 Hz, 1H), 6.92 (d, J = 8.2 Hz, 1H), 6.29 (d, J = 7.6 Hz, 1H), 5.99 (s, 2H), 3.31 (t, J = 6.1 Hz, 2H), 3.20 – 3.10 (m, 2H), 2.75 – 2.60 (m, 2H), 1.94 – 1.86 (m, 1H), 1.85 – 1.75 (m, 2H), 1.39 – 1.21 (m, 2H).

*3-(1,3-Benzodioxol-5-yl)-N-(4-piperidyl)pyrazolo[1,5-a]pyrimidin-5-amine (**31**)*

To a solution of **26** (40 mg, 0.15 mmol) in DMF (0.50 mL) was added 4-amino-1-Boc-piperidine (0.13 mL, 0.58 mmol). The reaction was stirred at 100 °C for 60 min then diluted to 1 mL with 1:1 acetonitrile:water and purified by preparative HPLC (high pH) to give the title compound (13 mg, 0.04 mmol, 26%), presumably via thermal deprotection under the reaction conditions. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.48 (d, J = 7.6 Hz, 1H), 8.36 (s, 1H), 8.28 (s, 1H), 7.73 (d, J = 1.7 Hz, 1H), 7.69 (d, J = 6.6 Hz, 1H), 7.53 (dd, J = 8.2, 1.7 Hz, 1H), 6.91 (d, J = 8.1 Hz, 1H), 6.27 (d, J = 7.6 Hz, 1H), 5.99 (s, 2H), 4.06 – 4.00 (m, 1H), 3.27 – 3.10 (m, 2H), 2.96 – 2.81 (m, 2H), 2.17 – 2.07 (m, 2H), 1.68 – 1.50 (m, 2H).

*N-[3-(1,3-Benzodioxol-5-yl)pyrazolo[1,5-a]pyrimidin-5-yl]-N',N'-dimethyl-ethane-1,2-diamine (**32**)*

Prepared in the same manner as **27** to give the title compound (31 mg, 0.10 mmol, 26%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.45 (d, J = 7.6 Hz, 1H), 8.27 (s, 1H), 7.74 (d, J = 1.7 Hz, 1H), 7.67 – 7.44 (m, 2H), 6.90 (d, J = 8.1 Hz, 1H), 6.33 (d, J = 7.6 Hz, 1H), 5.99 (s, 2H), 3.52 (q, J = 6.3 Hz, 2H), 2.56 (t, J = 6.7 Hz, 2H), 2.26 (s, 6H).

*3-(1,3-Benzodioxol-5-yl)-N-[2-(1-piperidyl)ethyl]pyrazolo[1,5-a]pyrimidin-5-amine (**33**)*

Prepared in the same manner as **30** to give the title compound (11 mg, 0.03 mmol, 16%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.45 (d, J = 7.6 Hz, 1H), 8.26 (s, 1H), 7.75 (d, J = 1.6 Hz, 1H), 7.62 – 7.48 (m, 2H), 6.88 (d, J = 8.1 Hz, 1H), 6.30 (d, J = 7.6 Hz, 1H), 5.99 (s, 2H), 3.51 (q, J = 6.5 Hz, 2H), 2.60 – 2.51 (m, 2H - overlapping with DMSO), 2.45 (s,4H - overlapping with DMSO), 1.52 (p, J = 5.7 Hz, 4H), 1.44 – 1.36 (m, 2H).

*N-[3-(1,3-Benzodioxol-5-yl)pyrazolo[1,5-a]pyrimidin-5-yl]-N',N'-dimethyl-propane-1,3-diamine **(34)***

Prepared in the same manner as **30** to give the title compound (21 mg, 0.062 mmol, 34%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.45 (d, J = 7.6 Hz, 1H), 8.27 (d, J = 3.6 Hz, 2H), 7.76 – 7.63 (m, 2H), 7.54 (dd, J = 8.2, 1.7 Hz, 1H), 6.91 (d, J = 8.1 Hz, 1H), 6.26 (d, J = 7.6 Hz, 1H), 5.99 (s, 2H), 3.42 (q, J = 6.6 Hz, 2H), 2.59 – 2.46 (m, 2H –

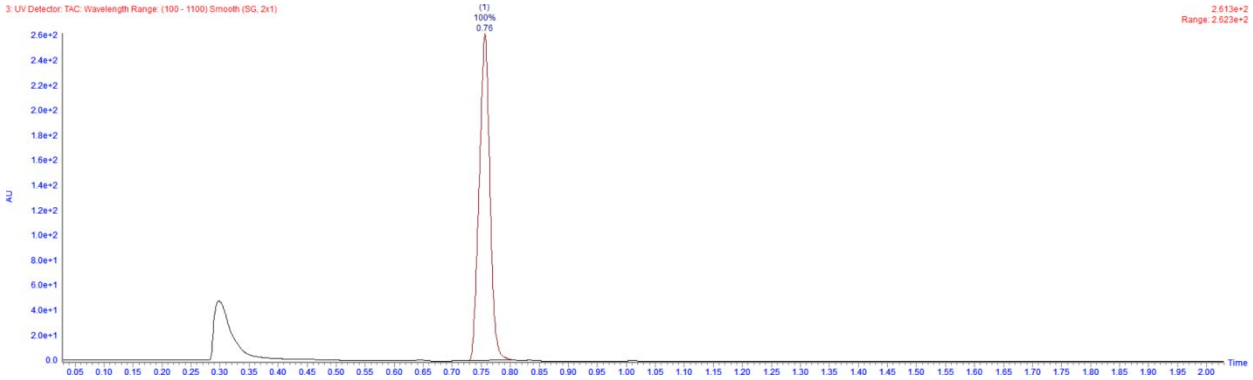
signal obscured by DMSO), 2.31 (s, 6H), 1.83 (p, J = 7.1 Hz, 2H). HRMS (ESI) *m/z* $[M + H]^+$ calcd for $C_{18}H_{22}N_5O_2$ 340.1768, found 340.1763.

*2-[[3-(1,3-Benzodioxol-5-yl)pyrazolo[1,5-a]pyrimidin-5-yl]amino]ethanol (**35**)*

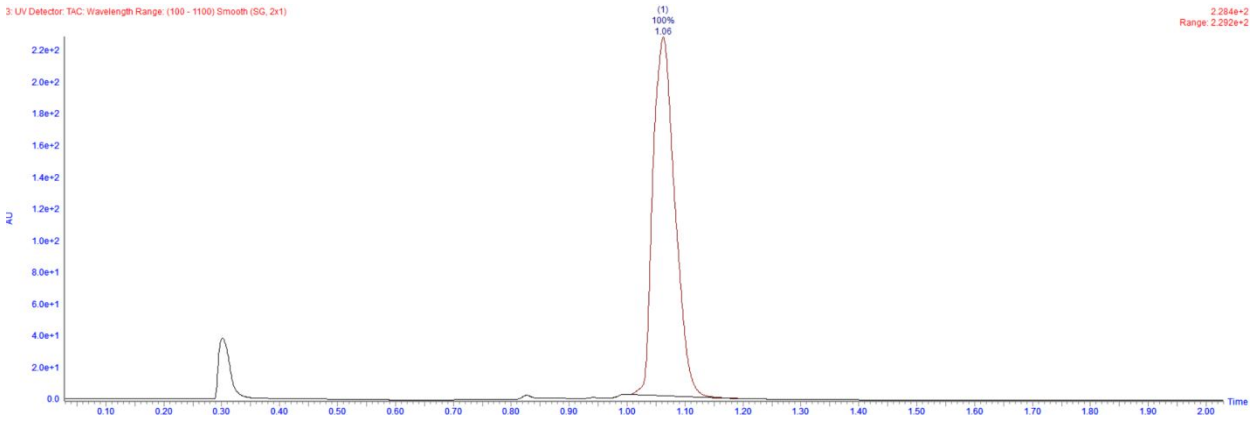
Prepared in the same manner as **27** to give the title compound (35 mg, 0.11 mmol, 31%) as a beige powder. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.44 (d, J = 7.5 Hz, 1H), 8.25 (s, 1H), 7.70 (d, J = 1.7 Hz, 1H), 7.63 (br s, 1H), 7.53 (dd, J = 8.1, 1.7 Hz, 1H), 6.91 (d, J = 8.1 Hz, 1H), 6.33 (d, J = 7.6 Hz, 1H), 5.98 (s, 2H), 4.81 (t, J = 5.2 Hz, 1H), 3.65 (q, J = 5.4 Hz, 2H), 3.48 (q, J = 5.6 Hz, 2H).

Analytical Data for Key Compounds

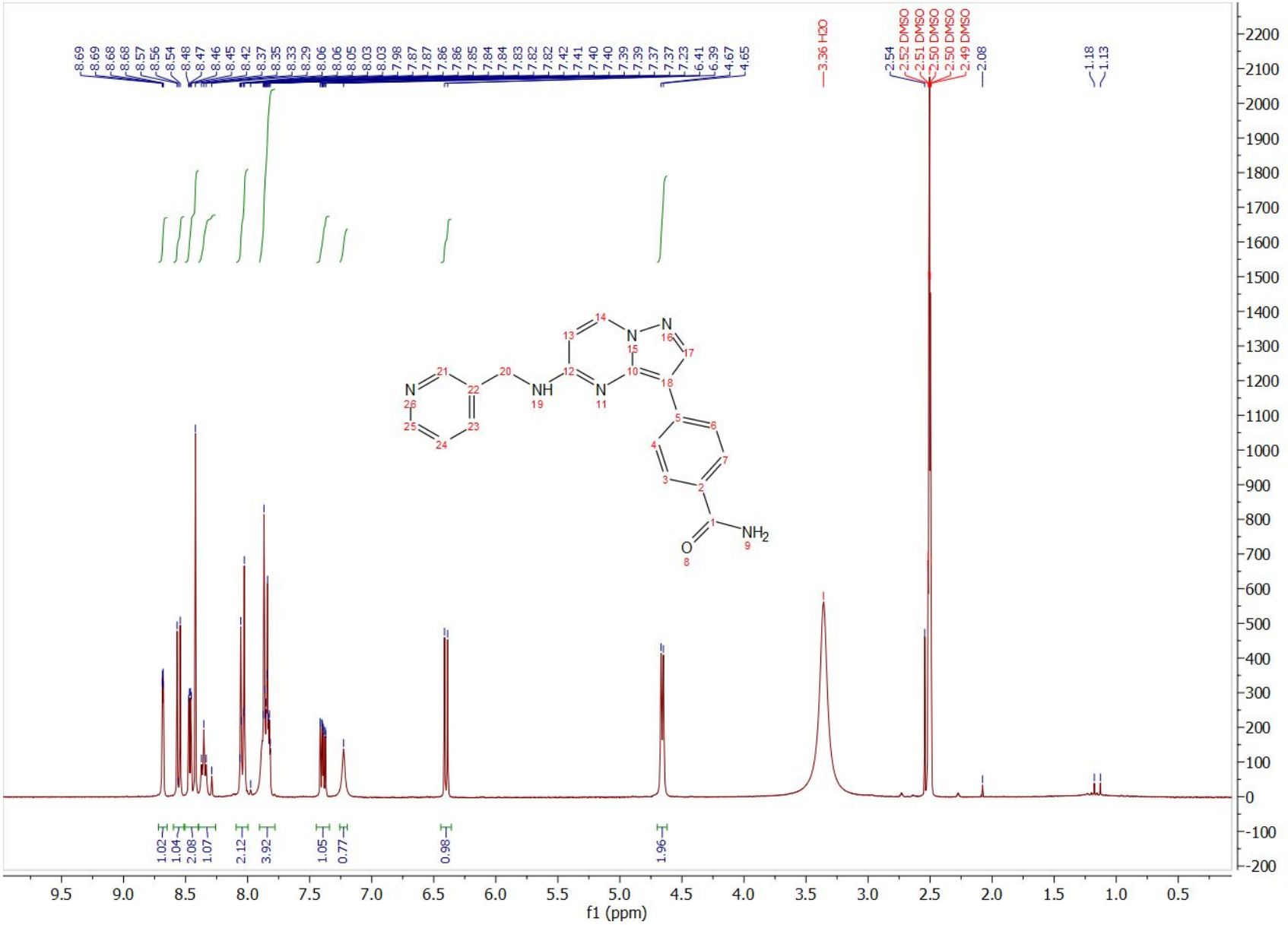
LC-MS Chromatogram for Compound **13**:



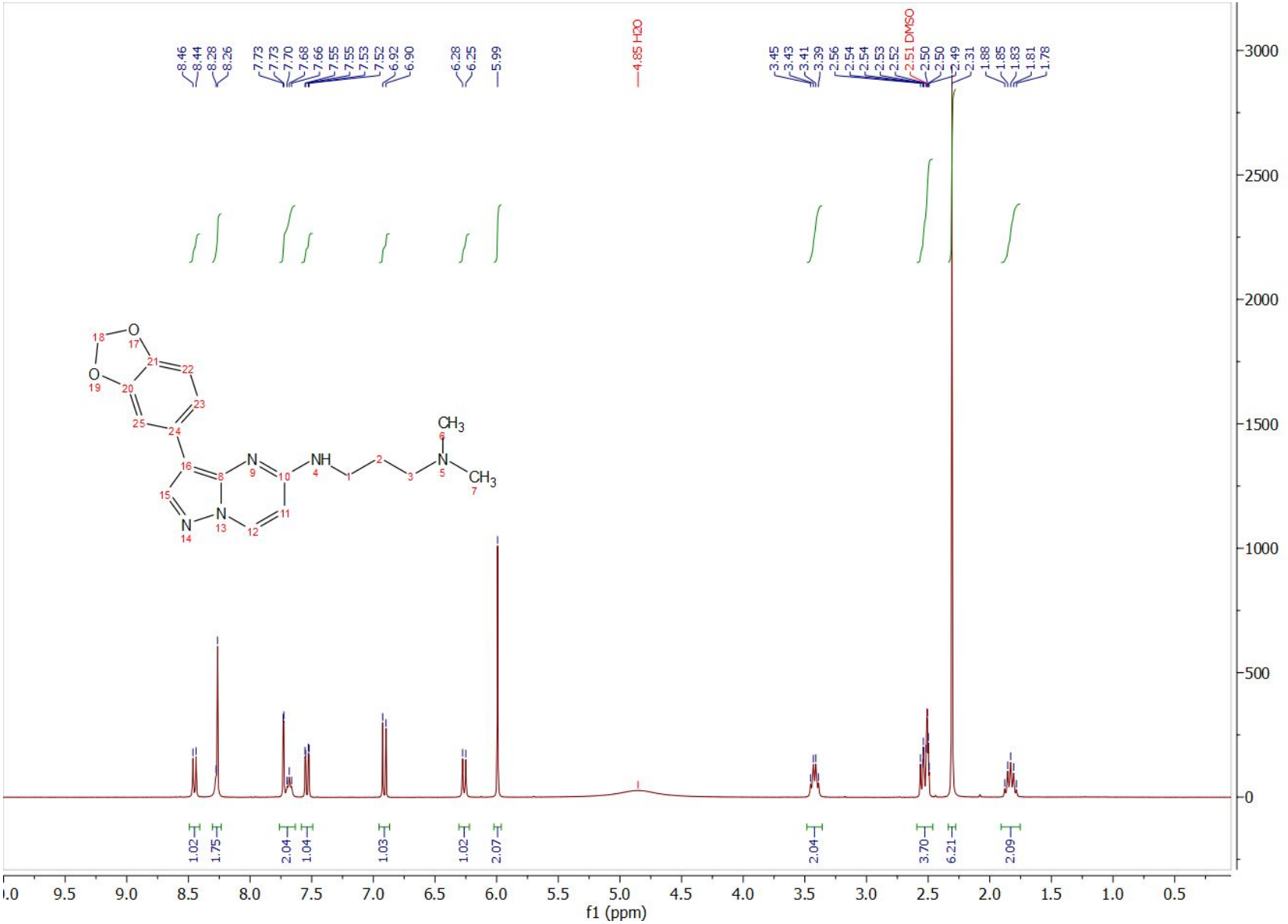
LC-MS Chromatogram for Compound **34**:



NMR Spectrum for Compound **13**:



NMR Spectrum for Compound **34**:



Biological Assay Protocols

Biochemical assay

Kinase activity was detected using CisBio HTRF kinEASE kit based on time-resolved fluorescence transfer (FRET). The assay was performed in 384-well white plates (Corning #3574) in a reaction volume of 10 µL containing 1× Cisbio enzymatic buffer supplemented with a final concentration of 5 mM $MgCl_2$, 1 mM DTT, 10 nM SEB and 0.01% Triton X100 for RET. The same buffer was used for the KDR biochemical assay with the addition of 2 mM $MnCl_2$. For RET^{V804M} the assay buffer was 1× Cisbio enzymatic buffer containing 1 mM DTT, 20 nM SEB, 2 mM $MgCl_2$ and 0.01% Triton X100.

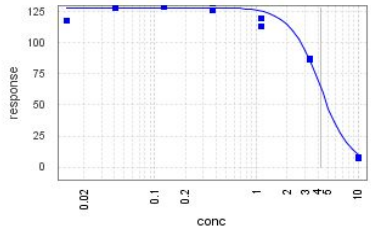
Inhibitors were pre-incubated in the plate for 15 minutes with 5 µL kinase and assay buffer at the following concentrations; 13 pM RET (Carna Biosciences; 08-159), 150 pM KDR (Millipore; 14-630) or 30 pM RET^{V804M} (Millipore 14-760). The reaction was initiated by the addition of 5 µL ATP and substrate at 2× final reaction concentrations. For RET, this was 18 µM and 2 µM; for KDR, this was 16 µM and 1 µM; for RET^{V804M}, this was 4 µM and 1.5 µM, respectively. Reactions were performed at ATP K_m for each target. The assay was allowed to proceed at room temperature for 30 minutes before terminating with the addition of 10 µL HTRF detection buffer containing EDTA supplemented with TK-antibody labelled with Eu^{3+}-Cryptate (1:100 dilution) and streptavidin-XL665 (128 nM). Following incubation at room temperature for 1 hour, FRET signal was measured using the Pherastar FS Microplate Reader.

BaF3 cellular assay

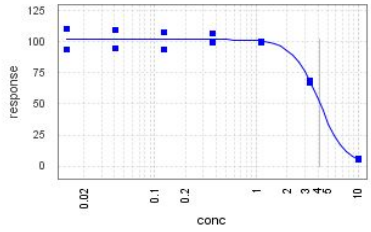
The system originally developed by Daley and Baltimore[3] was used, whereby IL3-dependent BaF3 cells are modified to express an activated recombinant kinase. Following removal of IL3, the modified cells are dependent on the activity of the recombinant kinase for survival and proliferation. BaF3 cell lines, harbouring KIF5B-RET (gift from Pasi Janne[4]), KDR (Advanced Cellular Dynamics, San Diego) and RET^{V804M} were maintained in RPMI-1640 media containing 10% FBS and appropriate antibiotics. Non-modified BaF3 cells (WT) were maintained in RPMI-1640 media containing 10% FBS and supplemented with 10 ng/mL recombinant mouse IL3 (R&D systems). For assessment of compound IC_{50}, cells were plated into 384-well plates at 1500 or 3000 cells per well in 30 µL culture medium and compounds dispensed using an acoustic liquid handling platform (Labcyte, Sunnyvale, CA). Following incubation of the cells for 48 hours at 37 °C in a humidified 5% CO_2 atmosphere, viability was determined by addition of 10 µL CellTiter-Glo reagent (Promega) and measurement of luminescence.

Representative IC_{50} curves from the BaF3 RET^{V804M} cellular assay

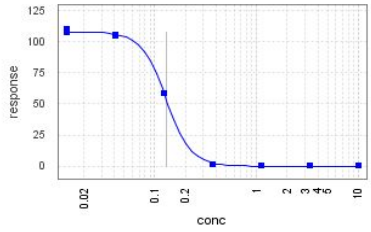
Compound 5



Compound 13



Compound 34



Crystallographic Details

Mammalian protein expression and purification

RET^{V804M} (residues 705-1013) was cloned into pcDNA.3 vector containing an amino-terminal Strep/$FLAG_2$ purification tag followed by a 3C cleavage site. Protein constructs were transiently transfected into suspension-grown HEK293F cells (Invitrogen) by adding 300 μg vector to 20 μg/ml polyethylenimine (PEI; Sigma) in 30 mL PBS, which was then incubated for 20 min at room temperature and added to 270 mL of cell culture. The cells were then harvested after 48 h as previously described.[5] Cell were lysed by sonication in 20 mM Tris-Cl pH 8.0, 300 mM NaCl, 1 mM DTT, and Roche c*O*mplete protease inhibitor (lysis buffer). The lysate was clarified by centrifugation at 30000 × *g* at 4 °C, then applied to Streptavidin resin and subsequently washed three times with 10 mL of lysis buffer. The protein was eluted with 20 mM Tris-Cl pH 8.0, 200 mM NaCl, 1 mM DTT, and 3 mM d-Desthiobiotin. The protein was treated with Precission protease overnight at 4 °C and further purified by gel filtration using a Superdex 75 16/60 column into 20 mM Tris-Cl pH 8.0, 100 mM NaCl, 1 mM DTT. The purified protein was concentrated to 3 mg/mL using centrifugal filter units with a molecular weight cut-off of 10 kDa.

Crystallisation, data collection and structure determination

Crystals of RET^{V804M} were grown at 18 °C by sitting-drop vapour diffusion against 0.1 M sodium citrate pH 4.5-5.5, 2.0 M sodium formate (mother liquor). For drop sizes of 0.5 μL concentrated protein + 0.5 μL mother liquor the crystals grew readily within one week. The crystals were soaked by adding 1 μl mother liquor containing an additional 30% ethylene glycol and 2 mM optimised lead compound **13** or **34** and incubating at 18 °C overnight. Crystals were cryo-cooled in liquid nitrogen and data collection was performed at Diamond Light Source beamline I04. For crystals soaked in compound **13** two datasets were collected from two separate crystals (1200 and 2980 images with 0.15° and 0.05° oscillation range, respectively) and merged for increased completeness and resolution of the data. For compound **34** 1800 images with 0.1° oscillation range were collected. The data were processed with XDS[6] and merged using AIMLESS.[7] The structure was solved by molecular replacement in PHASER[8] using a non-phosphorylated RET tyrosine kinase domain (PDB ID: 2IVS)[9] as a search model. Refinement was performed by alternatingly using REFMAC5[10] and COOT[11], with *B* factors being refined isotropically. Both final models show well-defined electron density for the respective compounds, which were modelled with full occupancy.

Data collection and refinement statistics of RET^{V804M} with compounds 13 and 34. Values in brackets refer to the highest-resolution shell.

Compound	**13**	**34**
PDB ID	6I83	6I82
Spacegroup	*C* 2	*P* 2_1
a (Å)	72.4	50.70
b (Å)	70.0	80.30
c (Å)	78.7	79.70
α (°)	90	90
β (°)	102.2	99.9
γ (°)	90	90
Resolution (Å)	49.77 - 1.88	45.84 - 2.05
Inner shell (Å)	(1.93 - 1.88)	(2.11 - 2.05)
R_{merge}	0.16 (>1)	0.08 (0.47)
Number of observations	174786 (5847)	120298 (4797)
Number unique	30797 (1646)	38208 (2324)
Mean (I) / σ(I)	4.9 (0.7)	7.5 (1.3)
Half-set correlation CC(1/2)	0.99 (0.38)	1.00 (0.73)
Completeness (%)	98.8 (82.6)	96.4 (75)
Multiplicity	5.7 (3.6)	3.1 (2.1)
R / R_{free}	0.21 / 0.24	0.18 / 0.23
Bond RMSD (Å)	0.004	0.010
Angle RMSD (°)	1.271	1.645
Average *B* factors (Å^2)		
Main chain	42.8	32.5
Side chain	48.2	38.7
Compound **13/34**	35.5	28.2
Water and solutes	48.9	37.8
Protein atoms	2345	4537
Compound **13/34** atoms	26	50
Water and solute atoms	134	278
Ramachandran plot (%)		
Favored	97.2	97.6
Allowed	2.1	1.8
Outliers	0.7	0.5

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