Electronic Supplementary Material (ESI) for Medicinal Chemistry Communications

Supporting information for

Development of substituted 7-pheny-4-aminobenzothieno[3,2-*d*] pyrimidines as potent LIMK1 inhibitors

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Biological Experimental

To measure LIMK1 activity, 12 nM of LIMK1 enzyme (Upstate Biotechnology, Lake Placid, NY) was incubated with 10 μM cofilin-2 protein substrate and 10 μM ATP in reaction buffer containing 20 mM HEPES pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 0.25 mM EGTA, 0.01 % Triton X-100, 0.01 % (w/v) chicken ovalbumine, and 1 mM DTT in low volume 384 well assay plates (Greiner, Frickenhausen, Germany). Test compounds were diluted in DMSO to 100-fold of the final concentration; robotic pin tool transfer (V&P Scientific, San Diego, CA and MiniTrak IX, Perkin Elmer, Waltham, MA) was used to deliver the compound dilutions into assay buffer prior to addition of the kinase. After incubation for 60 minutes at room temperature the endpoint was detected using either of the two following methods: The remaining ATP was detected by addition of equal volumes of Kinase-Glo reagent (Promega, Madison, WI) and measurement of the luminescence on an EnVision 2103 plate reader (Perkin Elmer). Alternatively the ADP formed was quantified by adding Transceener^{TM} ADP FP tracer (4 nM final) and anti-ADP antibody (14.1 μg/ml final) in Stop & Detect buffer (BellBrook Labs, Madison, WI) and measurement of the fluorescence polarisation on an EnVision 2103 reader. The data was standardized according to positive and negative controls on the same plate (DMSO without inhibitor and no enzyme, respectively) and IC_{50} were calculated by non-linear regression based on a four-parameter logistic model. Both measurement methods yielded comparable IC_{50} for the compound classes described here.

Solubility Estimates using Nephelometry

Compound in DMSO was spiked into either pH 6.5 phosphate buffer or 0.01M HCl (approx pH 2.0) with the final DMSO concentration being 1%. Samples were then analysed via nephelometry to determine a solubility range. (See C. D. Bevan and R. S. Lloyd, Anal. Chem. 2000, 72, 1781-1787).

Chromatographic LogD Measurement

Partition coefficient values ($LogD_{7.4}$) of the test compounds were estimated by correlation of their chromatographic retention properties against the characteristics of a series of standard compounds with known partition coefficient values. The method employed is a gradient HPLC based derivation of the method developed by Lombardo (See F. Lombardo *et al*, J. Med. Chem. 2001, 44, 2490-2497).

Chromatographic Protein Binding Estimation

Protein binding values of the test compounds were estimated by correlation of their chromatographic retention properties on a human albumin column against the characteristics of a series of standard compounds with known protein binding values. The method employed is a gradient HPLC based derivation of the method developed by Valko (See K. Valko *et al*, Journal of Pharmaceutical Sciences (2003), 92(11), 2236-48).

Protein Binding in Human Plasma

Aliquots of human plasma spiked with test compound and equilibrated at 37°C were removed from a larger incubation of spiked plasma and centrifuged (42,000 rpm for 4.2 h at 37°C) after which the supernatant plasma water was sampled for analysis. The concentration of test compound in samples of plasma-water (C_{spun}) and the bulk unspun plasma (incubated at 37°C for the duration of the ultracentrifuge run; C_{unspun}) was determined by LC-MS and the percentage of test compound bound to plasma proteins (% bound) was calculated according to the equation:

$$\%\,\text{bound} = \frac{\left(C_{unspun} - C_{spun}\right)}{C_{unspun}} * 100$$

In Vitro Metabolism in Mouse and Human Liver Microsomes

Metabolic stability was assessed by incubating test compounds individually (1 µM) at 37°C with either mouse or human liver microsomes. The metabolic reaction was initiated by the addition of an NADPH-regenerating system and quenched at various time points over the incubation period by the addition of acetonitrile. The relative loss of parent compound and formation of metabolic products was monitored by LC-MS.

Test compound concentration versus time data was fitted to an exponential decay function to determine the first-order rate constant for substrate depletion. In cases where clear deviation from first-order kinetics was evident, only the initial linear portion of the profile was utilised to determine the degradation rate constant (k). Each substrate depletion rate constant was then used to calculate an *in vitro* intrinsic clearance value ($CL_{int,in\ vitro}$) according to the equation:

$$CL_{int,\ in\ vitro} = \frac{k}{\text{microsomal protein content (0.4 mg protein/mL)}}$$

CYP450 isoform inhibition using a specific substrate approach

A single concentration (20 μM) of the test compound was incubated at 37°C concomitantly with a specific substrate for an individual CYP450 isoform at ≤ K_m (i.e. phenacetin 50 μM, diclofenac 6 μM, (*S*)-mephenytoin 50 μM, dextromethorphan 3 μM, midazolam 2.5 μM and testosterone 50 μM) in human liver microsomes at a protein concentration of either 0.4 mg/mL (CYP1A2, CYP2C9, CYP3A4 and CYP2D6) or 1.0 mg/mL (CYP2C19). The reaction was initiated by the addition of an NADPH-regenerating system and was quenched by the addition of acetonitrile prior to determining the concentration (and apparent rate of formation) of the specific metabolite by LC-MS. The CYP450 inhibitory effect (i.e. % inhibition) of the test compound at a concentration of 20 μM was assessed according to the percent reduction in the apparent rate of formation of the specific metabolite, noting that the maximal metabolite formation occurs in the absence of inhibitor.

Chemistry Experimental

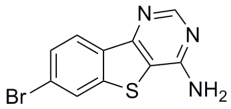
General

All non-aqueous reactions were performed in oven-dried glassware under an atmosphere of dry nitrogen, unless otherwise specified. Tetrahydrofuran was freshly distilled from sodium/benzophenone under N_2. Dichloromethane was freshly distilled from CaH_2 under N_2. All other solvents were reagent grade. Petroleum ether describes a mixture of hexanes in the bp range 40-60 °C. Analytical thin-layer chromatography was performed on Merck silica gel $60F_{254}$ aluminium-backed plates and were visualised by fluorescence quenching under UV light. Flash chromatography was performed with silica gel 60 (particle size 0.040-0.063mm). All NMR spectra were recorded on a Bruker Avance DRX 300 with the solvents indicated (^{1}H NMR at 300 MHz). Chemical shifts are reported in ppm on the δ scale, referenced to the appropriate solvent peak. LCMS were recorded on a Finnigan LCQ Advantage using a Finnigan Surveyor PDA Detector. LCMS conditions used to assess purity of compounds were as follows, column:

Gemini 3µ C18 20x4.0mm 110A; injection volume: 10 µL; flow rate 1.5 mL/min; gradient: 10-100% of B over 10 min, (solvent A: water; solvent B AcCN, 0.1% formic acid).

Experimental Procedures

7-Bromo-4-aminobenzothieno[3,2-d]pyrimidine **8**

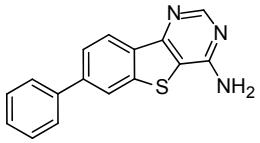


7-bromo-4-aminobenzothieno[3,2-*d*]pyrimidine **8** was synthesised from 4-bromo-2-fluorobenzonitrile following the protocol previously described.[1] LCMS- rt 5.50, M+H 280. ^{1}H-NMR (DMSO) δ 8.51 (1H, s), 8.47 (1H, d, *J* 1.7 Hz), 8.18 (1H, d, *J* 8.4 Hz), 7.70 (1H, dd, *J* 8.5 and 1.8 Hz), 7.60 (1H, bs).

Substituted 7-phenyl 4-aminobenzothieno[3,2-*d*]pyrimidines

Representative Example

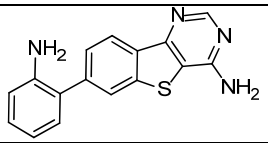
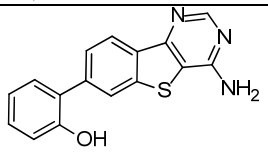
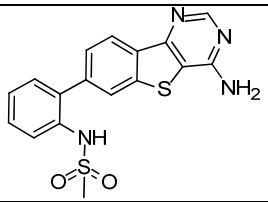
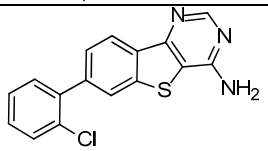
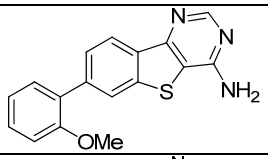
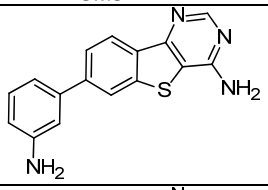
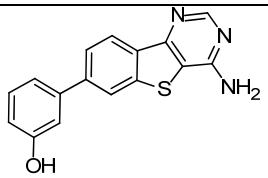
7-Phenyl-aminobenzothieno[3,2-*d*]pyrimidine **6**

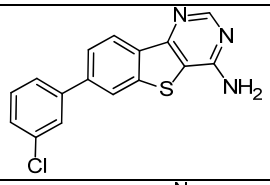
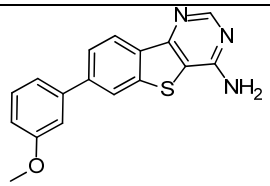
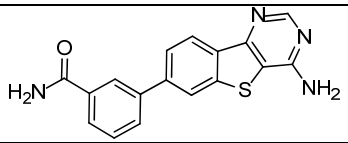
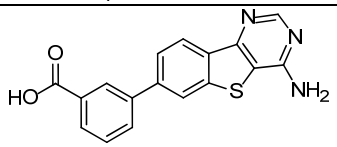
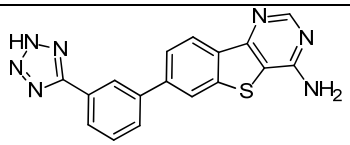
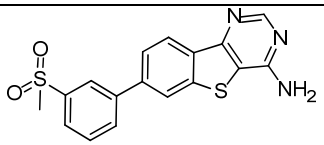
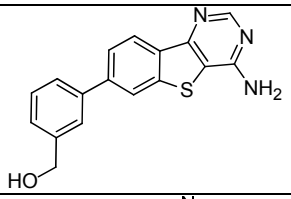
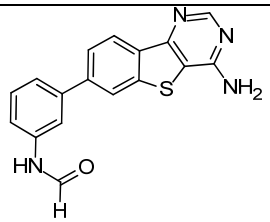
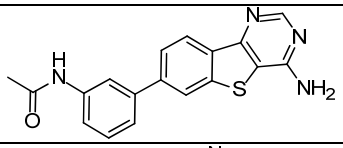
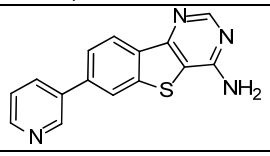


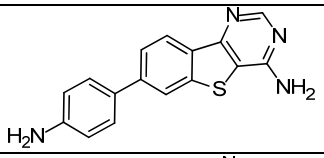
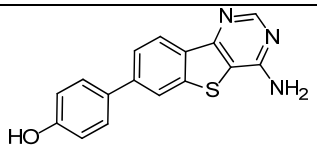
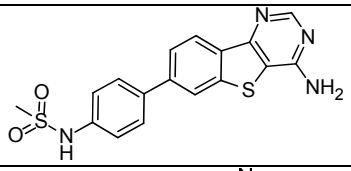
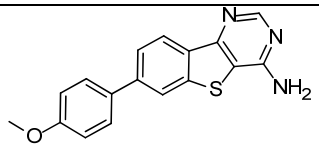
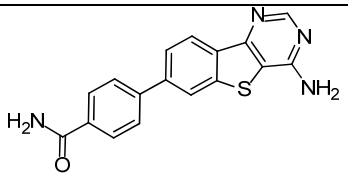
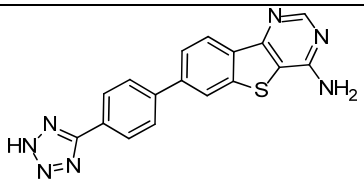
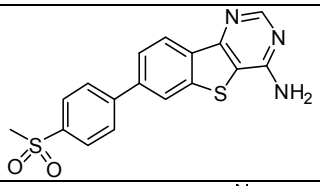
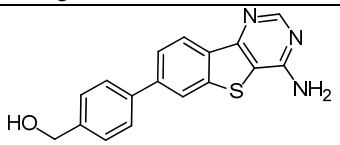
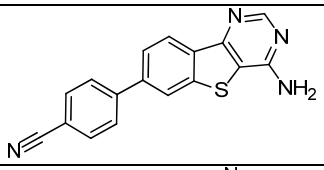
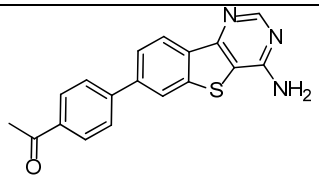
A mixture of 7-bromo-aminobenzothieno[3,2-*d*]pyrimidine **8** (0.36 mmol), potassium carbonate (0.89 mmol), phenylboronic acid (0.4 mmol), tetrabutylammonium bromide (0.036 mmol) and $PdCl_2(PPh_3)_2$ (0.036 mmol) in a solution of dioxane (4 mL) and water (1 mL) were heated for 120 ^{o}C under microwave irradiation for 2h. 10% Citric acid solution (10 mL) was added and the precipitate that resulted was filtered off, washed with water, and dried in a vacuum oven. The solid was dissolved in a dilute methanol/tetrahydrofuran mixture with warming, and filtered. The filtrate was concentrated to dryness *in vacuo*. The resulting residue is triturated with diethyl ether, and filtered off to give the pyrimidine **6** as a tanned solid (70%). Alternatively, the crude residue is applied to column chromatography gradient eluting with 100% dichloromethane to 15% methanol dichloromethane. LCMS – rt 7.53, M+H 278. ^{1}H-NMR (DMSO) δ 8.52 (1H, s), 8.46-8.45 (1H, m), 8.33 (1H, d, *J* 8.8 Hz), 7.88-7.81 (3H, m), 7.54-7.41 (5H, m).

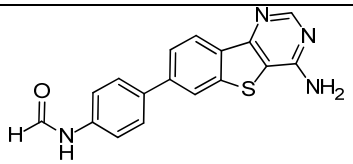
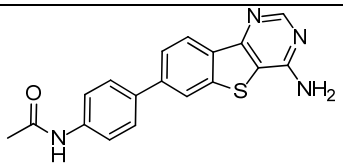
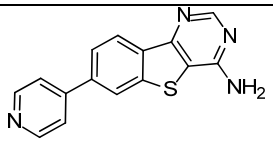
Table 1. Substituted 7-phenyl 4-aminobenzothieno[3,2-*d*]pyrimidines

Substituted 7-phenyl-4-aminobenzothieno[3,2-*d*]pyrimidine examples were synthesised from the corresponding boronic acids in the same manner as 7-phenyl-aminobenzothieno[3,2-*d*]pyrimidine **6**.

#	Structure/name	Reagent used	LCMS data
9		2-Amino-phenylboronic acid	rt- 6.70, M+H 293
10		2-Hydroxyphenylboronic acid	rt- 6.84, M+H 294
11		[(2-Methylsulfonyl) aminophenyl] boronic acid, pinacol ester	rt 6.17, M+H 371
12		2-Chlorophenylboronic acid	rt- 7.79, M+H 312
13		2-Methoxy-phenylboronic acid	rt- 7.45, M+H 308
14		3-Aminophenylboronic acid	rt – 4.42, M+H 293
15		3-Hydroxyphenylboronic acid	rt- 6.78, M+H 294

16		3-Chlorophenylboronic acid	rt- 6.91, M+H 312
17		3-Methoxy-phenylboronic acid	rt- 7.55, M+H 308
18		3-Aminocarbonylphenyl boronic acid	rt- 6.39, M+H 321
19		3-Carboxyphenylboronic acid	rt – 6.96, M+H 322
20		3-(Tetrazol-5-yl)phenyl boronic acid	rt – 6.99, M+H 346
21		3-(Methylsulfonyl)phenyl boronic acid	rt – 6.92, M+H 356
22		3-Hydroxymethyl-phenylboronic acid	rt- 6.63, M+H 308
23		3-Formylaminophenyl boronic acid, pinacol ester	rt- 6.67, M+H 321
24		3-Acetamidophenylboronic acid	rt- 5.64, M+H 335
25		Pyridin-3-ylboronic acid	rt – 0.90, M+H 279

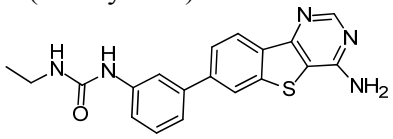
26		2-Aminophenylboronic acid	rt- 7.04, M+H 293
27		4-Hydroxyphenylboronic acid	rt- 5.61, M+H 294
28		[(4-Methylsulfonyl) aminophenyl] boronic acid, pinacol ester	rt 6.72, M+H 371
29		4-Methoxy-phenylboronic acid	rt-7.41, M+H 308
30		4-aminocarbonylphenyl boronic acid	rt- 6.33, M+H 321
31		4-(Tetrazol-5-yl)phenyl boronic acid	rt – 6.87, M+H 346
32		4-(Methylsulfonyl)phenyl boronic acid	rt – 6.92, M+H 356
33		4-Hydroxymethyl-phenylboronic acid	rt- 6.58, M+H 308
34		4-Cyanophenylboronic acid	rt- 7.38, M+H 303
35		4-Acetylphenylboronic acid	rt- 7.19, M+H 320

36		4-Formylaminophenyl boronic acid, pinacol ester	rt – 6.52, M+H 321
37		4-Acetamidophenylboronic acid	rt- 6.63, M+H 335
38		Pyridin-4-ylboronic acid	rt- 0.90, M+H 279

Substituted ureido 7-phenyl 4-aminobenzothieno[3,2-*d*]pyrimidines

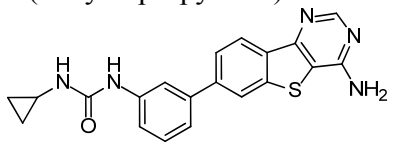
Representative Example

7-(3-Ethylurea)-4-aminobenzothieno[3,2-*d*]pyrimidine **40**



A solution of 7-(3-amino)-4-aminobenzothieno[3,2-*d*]pyrimidine **14** (0.07 mmol) and ethyl isocyanate (1.09 mmol) in anhydrous THF (1 mL) was stirred at 50 ^{o}C for 16 h. After this time, the solvent was removed *in vacuo*. The resulting residue was triturated with diethyl ether, and filtered to give the urea **40** as a tanned solid (80%). LCMS- rt 4.86, M+H 363; ^{1}H-NMR (DMSO) δ 8.55 (1H, s), 8.53 (1H, s), 8.36 (1H, s), 8.33 (1H,s), 7.90 (1H, s), 7.81-7.77 (1H, m), 7.54 (2H, bs), 7.38-7.34 (3H, m), 6.18-6.14 (1H, bs), 3.40-3.20 (2H, m), 1.07 (3H, t *J* 7.2 Hz).

7-(3-Cyclopropylurea)-4-aminobenzothieno[3,2-*d*]pyrimidine



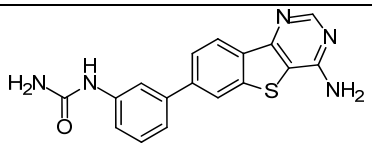
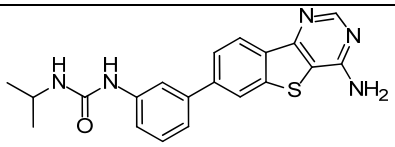
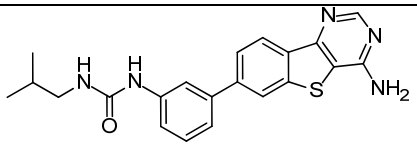
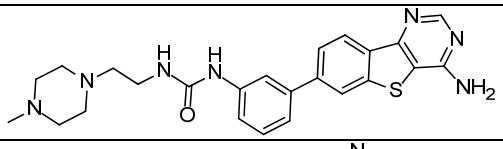
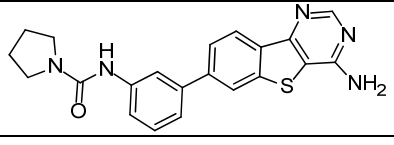
A solution of 7-(3-amino)-4-aminobenzothieno[3,2-*d*]pyrimidine **14** (0.51 mmol), pyridine (1.54 mmol) and phenyl chloroformate (0.59 mmol) in *N*,*N*-dimethylformamide (3 mL) containing under nitrogen was stirred at 0 ^{o}C for 45 min and then warmed to 25 ^{o}C for 2 h. After this time, water was added and an precipitate formed. This was filtered, washed with water and then oven

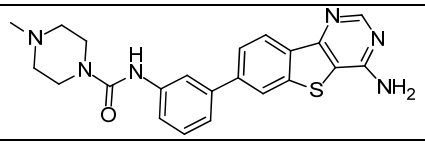
dried to give the 7-(3-phenylcarbamate)-4-aminobenzothieno[3,2-*d*]pyrimidine as an off-white solid (80%).

A solution of the carbamate (0.05 mmol) in 1,4-dioxane (0.5 mL) containing excess cyclopropylamine (6.91 mmol) was stirred at 70 ^{o}C for 4 h. After this time the solvent and excess amine was removed *in vacuo*. Diethyl ether was added and the precipitate that resulted was filtered, washing with diethylether, to give the urea as a light brown solid (80%). LCMS- rt 6.86, M+H 376; ^{1}H-NMR (DMSO) δ 8.34 (1H, 8.53), 8.43 (1H, s), 8.37-8.33 (3H, m), 7.93 (1H, s), 7.82-7.78 (1H, m), 7.54 (2H, bs), 7.41-7.35 (3H, m), 6.46 (1H, bs), 2.57-2.54 (1H, m), 0.66-0.64 (2H, m), 0.44-0.42 (2H, m).

Table 2. Substituted ureido 7-phenyl 4-aminobenzothieno[3,2-*d*]pyrimidines

Substituted ureido 7-phenyl-4-aminobenzothieno[3,2-*d*]pyrimidine examples were synthesised from the corresponding isocyanate in the same manner as 7-(3-ethylurea)-4-aminobenzothieno[3,2-*d*]pyrimidine **40**, or from the corresponding amine in the same manner as 7-(3-cyclopropylurea)-4-aminobenzothieno[3,2-*d*]pyrimidine.

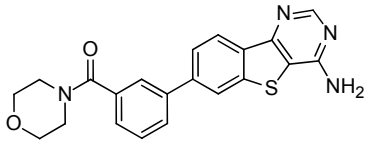
#	Structure/name	Reagent used	LCMS data
39		Potassium isocyanate	rt- 6.45, M+H 336
41		Isopropylamine	rt- 6.86, M+H 376
42		2-Methylpropyl-amine	rt-6.86, M+H 376
43		2-(4-Methylpiperazin-1-yl)ethyl amine	rt- 6.17, M+H 462
44		Pyrrolidine	rt- 7.06, M+H 390

45		*N*-Methyl piperazine	rt- 0.20, M+H 419

Substituted amido 7-phenyl 4-aminobenzothieno[3,2-*d*]pyrimidines

Representative Example

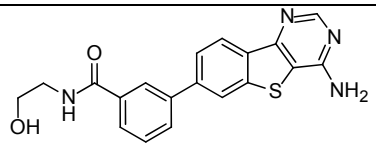
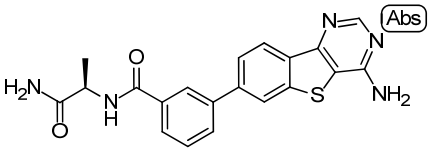
7-(3-*N*,*N*-Morpholinylbenzamide) 4-aminobenzothieno[3,2-*d*] pyrimidine **49**

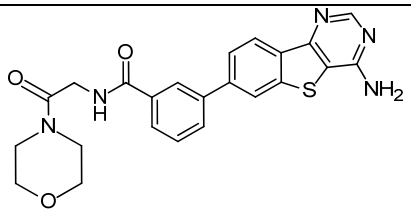
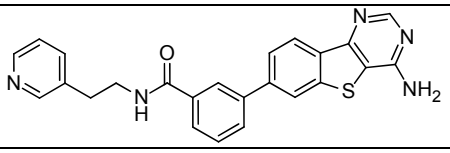


A mixture of 7-(3-carboxy)-4-aminobenzothieno[3,2-*d*]pyrimidine **19** (0.09 mmol), diisopropylethylamine (0.42 mmol) and HBTU (0.13 mmol) in *N*,*N*-dimethylformamide (0.3 mL), was allowed to stir for 5 min. Morpholine (0.18 mmol) was added and the solution was allowed to stir for 16 h. Ice-water was added and the precipitate that formed was filtered off, washing with water and dried in a vacuum oven to give the amide **49** as a tanned solid (90%). LCMS- rt 6.66, M+H 391; ^{1}H-NMR (DMSO) δ 8.58 (1H, s), 8.55 (1H, s), 8.37, (1H, s), 7.94-7.42 (7H, m), 3.62 (4H, bs), 2.97 (4H, bs).

Table 3. Substituted amido 7-phenyl 4-aminobenzothieno[3,2-*d*]pyrimidines

Substituted amido 7-phenyl-4-aminobenzothieno[3,2-*d*]pyrimidine examples were synthesised from the corresponding amines in the same manner as 7-(3-*N*,*N*-morpholinylbenzamide) 4-aminobenzothieno[3,2-*d*] pyrimidine **49**.

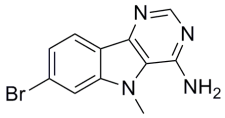
#	Structure/name	Reagent used	LCMS data
46		2-Aminoethanol	rt-0.20, M+H 365
47		L-Alaninamide hydrochloride	rt-4.50, M+H 392

48		2-Amino-1-morpholinoethanone hydrochloride	rt- 6.50, M+H 448
50		3-(2-Aminoethyl) pyridine	rt-0.22, M+H 426

Substituted 7-phenyl-4-amino-5-methyl-5*H*-pyrimido[5,4-*b*]indoles

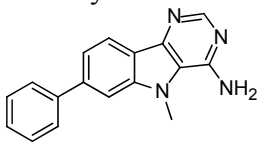
Representative Example

7-Bromo-4-amino-5-methyl-5*H*-pyrimido[5,4-*b*]indole **51**



7-Bromo-4-amino-5-methyl-5*H*-pyrimido[5,4-*b*]indole **51** was synthesised from 4-bromo-2-fluorobenzonitrile following the protocol of that previously described.[1] LCMS- rt 4.58, M+H 277. ^{1}H-NMR (DMSO) δ 8.27 (1H, s), 7.98-7.97 (2H, m), 7.34 (1H, dd, *J* 8.10 and 1.8 Hz), 6.96 (2H, bs), 4.05 (3H, s).

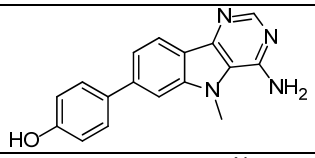
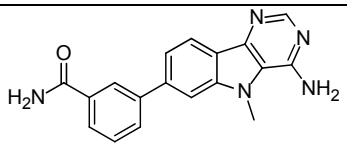
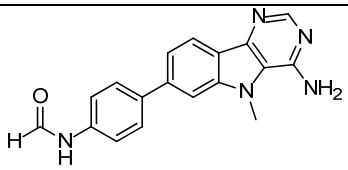
7-Phenyl-4-amino-5-methyl-5*H*-pyrimido[5,4-*b*]indole **7**



7-Phenyl-4-amino-5-methyl-5*H*-pyrimido[5,4-*b*]indole **7** was synthesised from 7-bromo-4-amino-5-methyl-5*H*-pyrimido[5,4-*b*]indole **51** in the same manner as 7-phenyl-aminobenzothieno[3,2-*d*]pyrimidine **6**. LCMS- rt 6.21, M+H 275. ^{1}H-NMR (DMSO) δ 8.28 (1H, s), 8.12 (1H, d, *J* 8.1 Hz), 7.95 (1H, s), 7.84-7.81 (2H, m), 7.55-7.39 (4H, m), 6.87 (2H, bs).

Table 4. Substituted 7-phenyl-4-amino-5-methyl-5*H*-pyrimido[5,4-*b*]indoles

Substituted 7-phenyl-4-amino-5-methyl-5*H*-pyrimido[5,4-*b*]indole examples were synthesised from the corresponding boronic acids in the same manner as 7-phenyl-4-amino-5-methyl-5*H*-pyrimido[5,4-*b*]indole **51**.

#	Structure/name	Reagent used	LCMS data
52		4-Hydroxyphenyl boronic acid	rt- 6.05, M+H 291
53		3-Aminocarbonyl phenylboronic acid	rt- 5.97, M+H 318
54		3-Formylaminophenyl boronic acid, pinacol ester	rt- 6.05, M+H 318

References

1. Sleebs, B. E.; Levit, A.; Street, I. P.; Falk, H.; Hammonds, T.; A.-C., W.; Charles, M. D.; Baell, J. B. *MedChemComm* **2011**, *xx*, xx.